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Magnesium transporter Gene Family: Genome-Wide Identification and Characterization in *Theobroma cacao*, *Corchorus capsularis*, and *Gossypium hirsutum* of Family Malvaceae

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Abstract: Magnesium (Mg) is an element involved in various key cellular processes in plants. Mg transporter (MGT) genes play an important role in magnesium distribution and ionic balance maintenance. Here, MGT family members were identified and characterized in three species of the plant family Malvaceae, *Theobroma cacao*, *Corchorus capsularis*, and *Gossypium hirsutum*, to improve our understanding of their structure, regulatory systems, functions, and possible interactions. We identified 18, 41, and 16 putative non-redundant *MGT* genes from the genome of *T. cacao*, *G. hirsutum*, and *C. capsularis*, respectively, which clustered into three groups the maximum likelihood tree. Several segmental/tandem duplication events were determined between *MGT* genes. *MGTs* appear to have evolved slowly under a purifying selection. Analysis of gene promoter regions showed that *MGTs* have a high potential to respond to biotic/abiotic stresses and hormones. The expression patterns of *MGT* genes revealed a possible role in response to *P. megakarya* fungi in *T. cacao*, whereas *MGT* genes showed differential expression in various tissues and response to several abiotic stresses, including cold, salt, drought, and heat stress in *G. hirsutum*. The co-expression network of MGTs indicated that genes involved in auxin-responsive lipid metabolism, cell wall organization, and photoprotection can interact with MGTs.

Keywords: *magnesium transporter*; comparative analysis; Malvaceae; *Theobroma*; *Gossypium*; *Corchorus*; expression analysis; gene structure; phylogenetic analysis

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1. Introduction

Magnesium (Mg) is a critical bimetal that regulates biochemical processes and provides stability to membranes in plants [1,2]. Magnesium acts as a cofactor for polymerase, kinase, and H+-ATPase, which are necessary for synthesizing proteins and nucleic acid and for generating energy [3,4]. It is also required to maintain cation–anion homeostasis in the cell [5]. Various types of adverse effects have been reported in plants during Mg deficiency, including a reduction in photosynthesis, macromolecule synthesis, and plant growth and development [6–8]. Therefore, plants have developed an efficient transport system for absorption, storage, and Mg translocation [2]. The *Mg transporter* (*MGT*) gene family, also known as MRS2 or CorA, has an important role in the aforementioned essential functions [9,10]. Members of the MGT family are defined by two transmembrane domains in which a tripeptide motif GMN (glycine–methionine–asparagine) occurs at the C-terminal domain of the first transmembrane [11,12]. *MGTs* are expressed in root tissues

of plants that are more involved in up taking Mg (such as *MGT1* in rice and *MGT6* in *Arabidopsis*), transferring Mg from root to shoot (such as *MGT9* in *Arabidopsis)*, homeostasis by maintaining ionic balance (such as *MGT10* in *Arabidopsis*), and accumulation and translocation of Mg within, for instance, the vacuole of the cell (such as *MGT2* and *MGT3* in *Arabidopsis*) [11,13–16]. These genes are also crucial for pollen mitosis and pollen intine formation [15,17,18].

MGT genes also respond to changes in elemental concentration in soil, i.e., *MGT* genes showed high expression due to aluminum (Al) toxicity in acidic soil. In *Arabidopsis* and in maize, Al-tolerant genotypes were observed to have a high capacity for Mg uptake and accumulation [9,19]. The transformation and expression of *Arabidopsis MGT1* in *Nicotiana benthamiana* increased uptake of Mg and reduced toxicity of Al in transgenic lines [20]. In contrast, the knockout of *MGT1* in rice reduced tolerance to salt and was linked to a high content of sodium in shoot tissues [21]. The *MGT* genes are also important for plant adaptation to changing Mg status in soil [13]. Genes of the *MGT* family have been identified and characterized in several plant species, including *Arabidopsis* [22], rice [11], maize [9], pear [23], citrus [24], rapeseed [25], wild sugarcane [26], and tomato [27]. The plant family Malvaceae is one of the largest dicot families with 244 genera and 4225 species [28]. The family includes significant economic plant species, such as cotton (*Gossypium*) and jute (*Corchorus*) are important for fiber, whereas cacao (*Theobroma cacao* L.) is important for chocolate production [29,30]. Besides, the seed of *Theobroma cacao* contained 290 mg/100 g magnesium which is 4–5 time more than pea, corn, white wheat, and rice, and is included among the richest magnesium containing sources [31]. The cotton is considered as a white gold due to its industrial importance and in the world 25 million tons cotton are produce annually of worth of 600 billion dollars [32]. This important plant effected from Magnesium deficiency [33,34] whereas jute is the second most important natural fiber in terms of global consumption after cotton and specifically important in some countries such as Bangladesh [35]. Therefore, the comparative analysis and determining role of the MGT family can provide quality information about the gene structure, variation etc. To date, none of the studies, to the best of our knowledge, focuses on characterization of the *MGT* gene family despite the availability of nuclear genomes for the species of family Malvaceae [36,37] with the advancement of sequencing technologies. Here, we aim to: (i) identify and characterize *MGT* genes within three species of Malvaceae, including *T. cacao*, *C. capsularis*, and *G. hirsutum*, (ii) study evolutionary patterns and phylogenetic relationships, and (iii) determine roles of MGTs in growth and development of Malvaceae species.

2. Materials and Methods

2.1. Identification and Characterization of MGT Genes in T. cacao, C. capsularis, and G. hirsutum

The homologs of *Arabidopsis* MGT proteins, MGT7 (AT5G09690), CorA-like family protein (AT1G29820), MRS2-1 (AT1G16010), MRS2-10 (AT1G80900), NIPA7 (AT4G38730), NIPA1 (AT3G23870), and NIPA5 (AT4G09640), were BLAST with an expected value of E^{-10} in Ensembl Plants [38] for *T. cacao* and *Corchorus capsularis*, and in cotton genome database [39] for *Gossypium hirsutum* to identify *MGT* genes and retrieve protein sequences, coding sequences, genomic sequences, and promoter regions for various analyses following a previous approach [40]. The non-redundant protein sequences were selected based on CDD search (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml, accessed on 24 November 2020). The ProtParam program [41] was used to determine molecular weight (MW) and isoelectric points (*pI*), TMHMM Server version 2.0 [42] to predict transmembrane domains, and CELLO2GO [43] to determine the location of MGT proteins in the cell. PlantCARE [44] was used to analyze promoter regions.

2.2. Phylogenetic Inference, Conserved Protein Motifs, and Gene Structure

The protein sequences of *T. cacao*, *C. capsularis*, and *G. hirsutum* aligned in Geneious R8.1 [45] using ClustalW [46] and analyzed in IQ-tree for construction of a maximum likelihood phylogenetic tree under default parameters and 1000 bootstrap replications [47–49].

The Best-fit model JTT+G4 was chosen according to Bayesian Information Criterion using jModelTest 2 [50] and was employed to find relationship among genes. Finally, the iTOL, Integrative Tree of Life version 4 [51], was used for the improvement of tree representation. The MEME (Multiple Em for Motif Elicitation) server [52] was used to identify the conserved protein motifs in MGT. The gene structure of each *MGT* gene was constructed using the Gene Structure Display Server [53].

2.3. Gene Duplications and Synteny Analysis

The *MGT* genes with more than 85% identity in each species were selected as duplicated genes [54]. Then, the location diagram of duplicated genes in cacao and *G. hirsutum* were constructed using TBtools [55]. In addition, the synonymous (Ks) and non-synonymous (Ka) of each duplicated gene pair were calculated by DnaSP version 6 software [56]. Finally, the time of divergence of duplicated genes was determined using the following equation: $T = (Ks/2\lambda) \times 10^{-6}$ [57], where λ is substitutions per synonymous site per year and $\lambda = 6.5 \times 10^{-9}$. Moreover, the synteny relationship diagrams of *MGT* genes among the orthologous pairs of *T. cacao*–*G. hirsutum* and *T. cacao*–*C. capsularis* were created using Circos software [58].

2.4. Structure Analyses of MGT Proteins

The transmembrane and three-dimensional structures of the candidates of MGT subgroups MRS2, NIPA, and CorA proteins in *T. cacao*, *G. hirsutum*, and *C. capsularis* were predicted using the Phyre2 server [59], whereas docking analysis was performed to predict the ligand-binding regions (pocket sites) using DeepSite [60] and CASTp [61] tools and finally constructed in PyMOL [62].

2.5. Expression Analysis of TcMGTs and GhMGTs Using RNA-Seq Data

The publicly available RNA-seq data of cacao transcriptome with accession number GSE116041 [63] were used to find a possible differential expression of cacao *MGTs* concerning a fungi disease caused by *Phytophthora megakarya* after inoculation of the different time courses of 0 h, 6 h, 24 h, 48 h, and 72 h in two contrasting cultivars: Nanay (NA-32) as a susceptible cultivar and Scavina (SCA-6) as a fungal-resistant cultivar. Finally, the expression patterns of *TcMGTs* were illustrated in heatmaps based on log2 transformed using TBtools [55]. Furthermore, the expression profile of *GhMGTs* in various tissues (ovule, fiber, anther, bract, filament, leaf, pental, root, sepal, stem, and torus) and in response to various abiotic stresses (cold, heat, salt, and drought) was retrieved from available RNA-seq data of the cotton genome database (https://cottonfgd.org/, accessed on 9 March 2021) under project PRJNA490626 using the gene ID of each gene of the newly assembled genome as query [64]. The expression of *GhMGTs* in various tissues was analyzed and represented as a heatmap based on percentage expression of each gene using TBtools [55], while the data of abiotic stresses were analyzed and represented in heatmap after log2 transformation through TBtools [55].

3. Results

3.1. Sequence and Structure of MGT Genes

Altogether 18, 41, and 16 putative non-redundant MGT genes were identified from the genomes of *T. cacao*, *G. hirsutum*, and *C. capsularis*, respectively. All sequences (genomic, amino acids, coding sequences) of identified MGT genes are shown in Table S1. MGTs were characterized based on their sequences structure (Table S2) and three MGT sub-groups, including MRS2, NIPA, and CorA, were recognized according to the specific domain distribution (Table S2). Our findings revealed that MGTs in the three studied plant species are diverse in sequence length, molecular weight (MW), isoelectric point (pI), and exon number (Table S2). For instance, protein length varied from 321 amino acids (aa) to 632 aa in T. cacao, from 210 aa to 474 aa in *G. hirsutum*, and from 262 aa to 2417 aa in *C. capsularis* (Table 1). In addition, the predicted MW ranged from 32.75 kDa to 70.91 kDa in *T. cacao*,

from 32.66 kDa to 53.95 kDa in *G. hirsutum*, and from 29.82 kDa to 268.42 kDa in *C. capsularis* (Table 1). Moreover, the pI of MGTs was between 4.48 and 8.57 in *T. cacao*, between 4.76 and 9.57 in *G. hirsutum*, and between 4.79 and 8.60 in *C. capsularis* (Table 1). Based on pI value, 75% of MGTs in *C. capsularis*, 56% in *T. cacao*, and 49% in *G. hirsutum* were predicted to be acidophilic proteins (Table S2). In addition, the prediction of subcellular localization illustrated that most MGTs are located in the endomembrane or the plasma membrane (Table S2). The exon number of MGT genes varied between 4 and 15 in *T. cacao* and *G. hirsutum*, while the exon number varied between 4 and 21 in *C. capsularis* (Table 1).

Table 1. Summary of MGT properties in the three plant species *Theobroma cacao*, *Gossypium hirsutum*, and *Corchorus capsularis*.

Organism	Gene Number	Gene Length (bp)	Protein Length (aa)	MW (KDa)	pI	Exon Number
T. cacao	18	1212–2632	321–632	32.75–70.91	4.48–8.57	4–15
G. hirsutum	41	633–1425	210–474	32.66–53.95	4.76–9.57	4–15
C. capsularis	16	789–7254	262–2417	29.82–268.42	4.79–8.60	4–21

3.2. Phylogenetic Analysis and Classification of the MGT Gene Family

A phylogenetic tree of MGT proteins was constructed, comprising 18 TcMGT proteins from *T. cacao*, 41 GhMGTs from *G. hirsutum*, and 16 CcMGTs from *C. capsularis*. The MGT proteins clustered into three groups (groups I, II, and III) that group III included three sub-groups, 3a, b, and c (Figure 1). Six MGTs, including a CorA of Jute, namely CcMGT08, a MRS2 protein from cacao, namely TcMGT03, and four MRS2 proteins of cotton, namely GhMGT11, GhMGT12, GhMGT32, and GhMGT33, with similar structure contained 11 exons and were located in group I (Figure 1a,b). In addition, five MGT proteins were located in group II and four CorA proteins, namely TcMGT01, TcMGT16, CcMGT02, and CcMGT16, clustered in group III-a. In addition, 16 MRS2 proteins along with two CorA proteins were located in group III-b and all NIPA-type proteins were located in group III-c (Figure 1a,b). In addition, MGTs were analyzed based on distribution of conserved motifs in their protein sequence. Thirty-five conserved motifs were identified, and MGTs from group III showed more diversity than other groups (Figure 2). Motifs 12, 5, 18, 7, 10, and 2, frequently observed in MGTs and NIPA proteins, illustrated different patterns of conserved motif distribution. Moreover, CorA proteins also showed various conserved motifs (Figure 2).

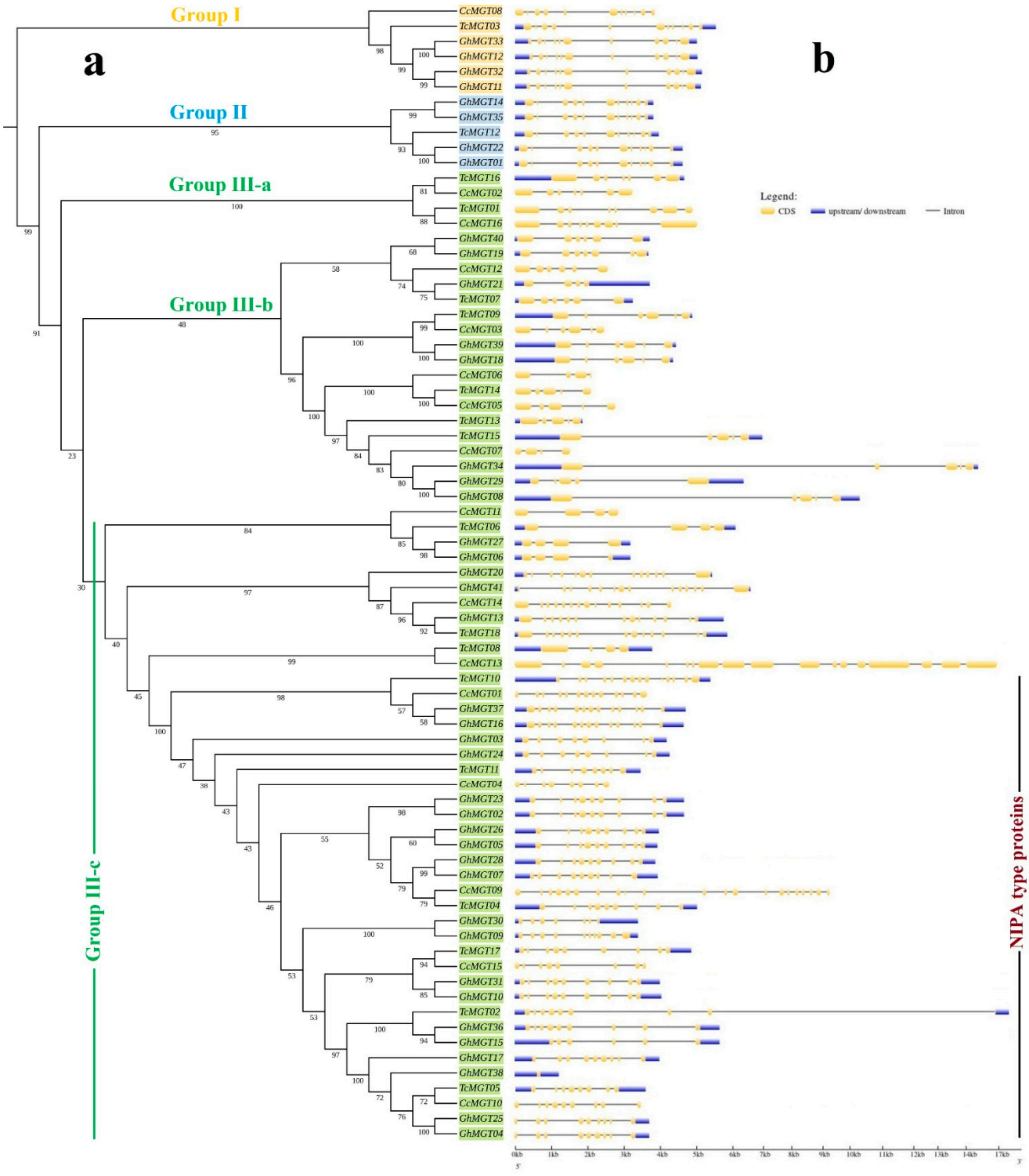

Figure 1. The phylogenetic tree of MGT proteins (**a**) and gene structure of *MGT* genes (**b**) of *Theobroma cacao*, *Gossypium hirsutum*, and *Corchorus capsularis*. The start of each gene name for the species is as follows: Tc: *Theobroma cacao*; Gh: *Gossypium hirsutum*; Cc; *Corchorus capsularis*.

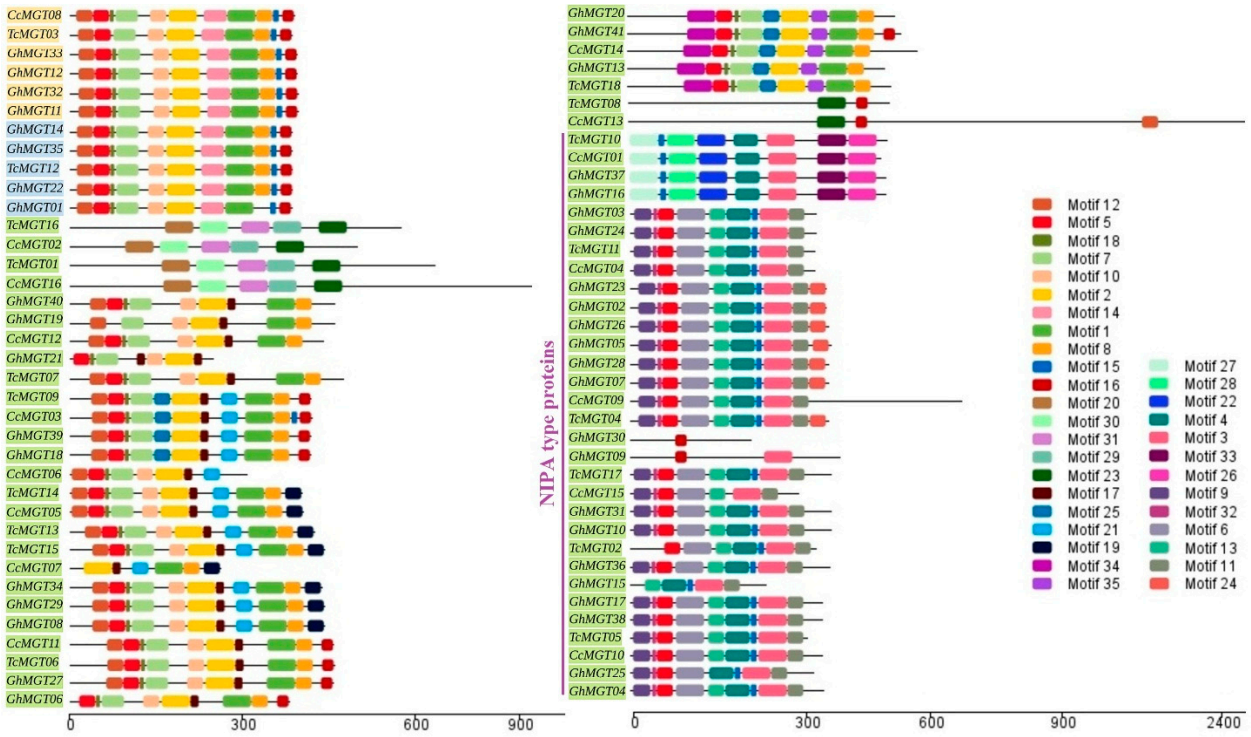

Figure 2. Conserved motif distribution in MGT proteins of *Theobroma cacao*, *Gossypium hirsutum*, and *Corchorus capsularis*.

3.3. Duplication Events and Synteny Analysis

The *TcMGT* genes were mapped onto 5 of 10 chromosomes in the cacao genome (Figure 3a), while *GhMGTs* were distributed over 22 of 26 chromosomes in the *G. hirsutum* genome (Figure 3b). Due to the incomplete physical map of the *C. capsularis* plant, the mapping of *CcMGT* genes is not provided. In the cacao genome, chromosome 2 encompassed the most significant number of *TcMGTs*, with five genes, while in *G. hirsutum* genome, the most significant number of *GhMGTs* were located on chromosomes A04 and D01 (Figure 3). In addition, the duplication events of *MGT* genes in selected plant species were investigated. Eight segmental duplication gene pairs were identified between 12 *TcMGT* genes of cacao (Figure 3a and Table S3). Tandem duplication events seemed to occur on chromosome 6 between three *TcMGT* genes of cacao, including, *TcMGT013*, *TcMGT14*, and *TcMGT15* (Figure 3a). In addition, four segmental duplication events were predicted for the *TcMGT03* gene (Table S3). Notably, a duplication event occurred around 10 MYA between two *CorA*- like genes in cacao, namely *TcMGT01* and *TcMGT16.* In addition, five segmental duplication gene pairs were recognized between *CcMGT* genes, and a triplication event was predicted between *CcMGT05* and *CcMGT06*, *CcMGT07*, and *CcMGT04* (Table S3). According to the *Ka/Ks* ratio, the first duplication was approximately 103 million years ago (MYA) between *CcMGT05* and *CcMGT04*. The most duplication events were observed between *GhMGT* genes in the *G. hirsutum* genome, with 22 segmental duplication gene pairs. Moreover, four *GhMGT* genes, namely *GhMGT02*, *GhMGT05*, *GhMGT23*, and *GhMGT26*, had a common ancestor and probably the first duplication event occurred approximately 68 MYA between *GhMGT23* and *GhMGT26* (Table S3). The intraspecies synteny of *MGT* genes was constructed between *T. cacao* and *G. hirsutum* and between *T. cacao* and *C. capsularis* (Figure 4). The 18 *TcMGT* genes in *T. cacao* illustrated 15 and 16 syntenic block relationships with *MGT* genes in *G. hirsutum* and *C. capsularis*, respectively (Figure 4a,b). Interestingly, TcMGT genes of cacao showed more syntenic relationships with *GhMGTs* of D-genome than A-genome.

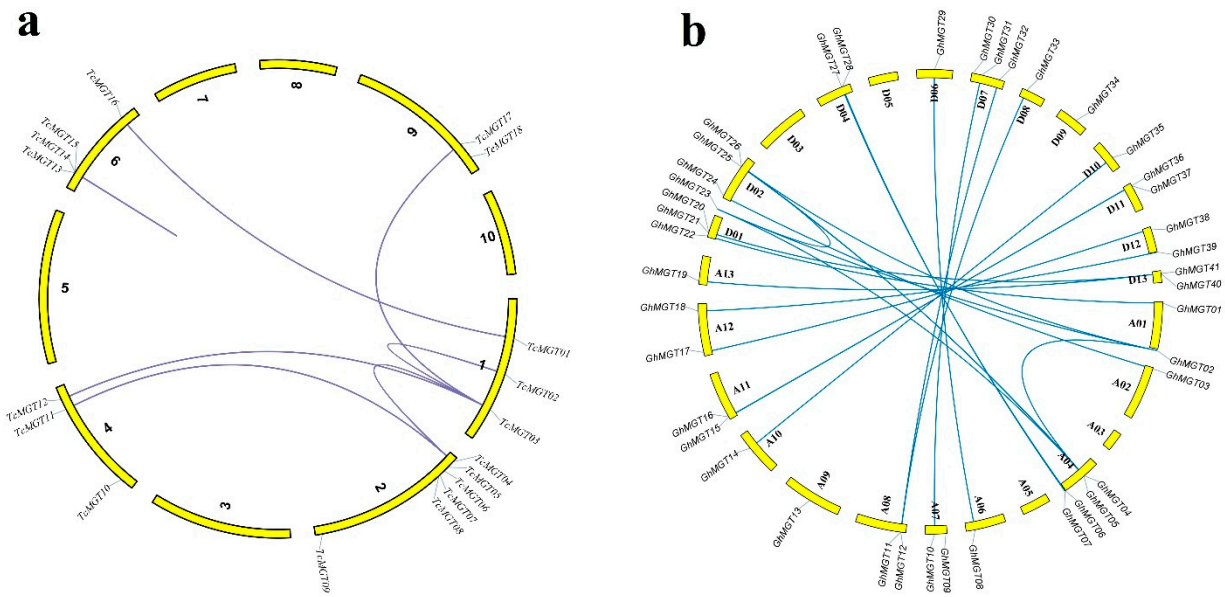

Figure 3. Location of *MGT* genes on the chromosome in *Theobroma cacao* (**a**), and *Gossypium hirsutum* (**b**). The duplicated genes are connected using blue lines.

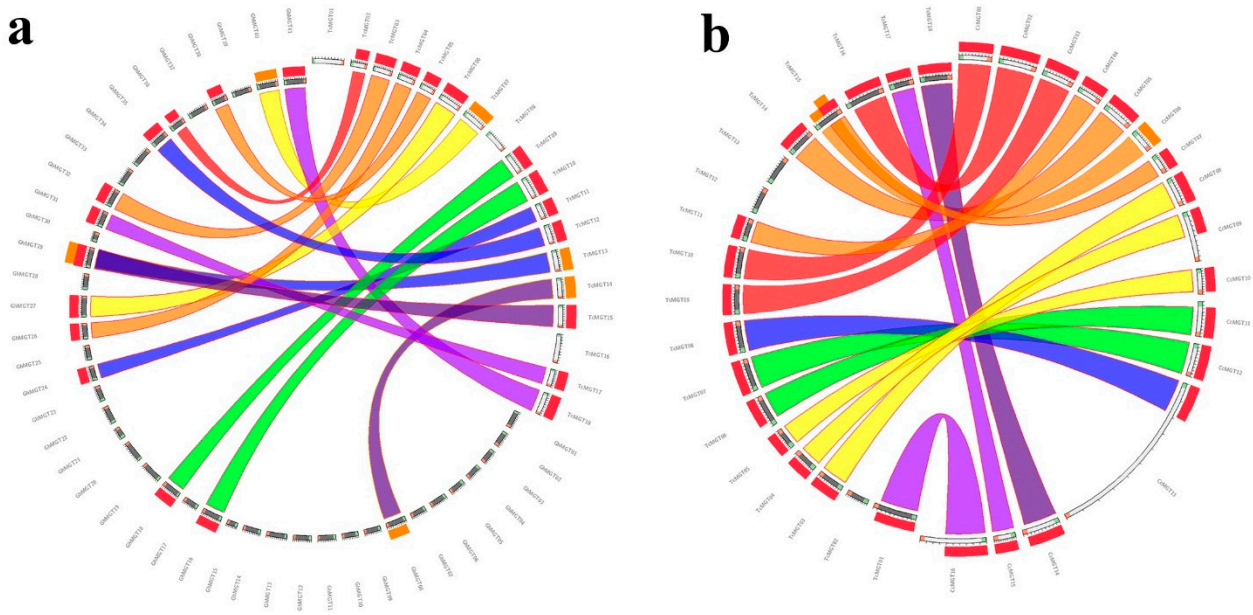

Figure 4. Synteny analysis of *MGT* genes. The syntenic blocks of cacao *MGTs* are constructed with *Gossypium hirsutum* (**a**), and *Corchorus capsularis* (**b**).

3.4. Protein Structure and Docking Analysis

The 3D structures of all candidates of three types of MGTs, namely NIPA, MRS2, and CorA, were predicted in *T. cacao*, *G. hirsutum*, and *C. capsularis* (Figure S1). Nine α-helices were observed in the predicted 3D structure of NIPAs in three studied plants, while fewer α-helices were predicted in the structure of MRS2 and CorA proteins (Figure S1). Furthermore, nine transmembrane helices with eight pores were predicted in the structure of NIPAs in three plants, while in candidate MRS2 proteins two transmembrane helices were observed in all studied plant species. However, both N-terminal and C-terminal of candidate MRS2 proteins from *T. cacao* and *C. capsularis* were predicted in the extracellular part, while in *G. hirsutum*, both N- and C-terminal were observed in the cytoplasmic part.

In the candidate CorA protein, three transmembrane helices were predicted in *G. hirsutum*, and two transmembrane helices were predicted in *T. cacao* and *C. capsularis* (Figure 5).

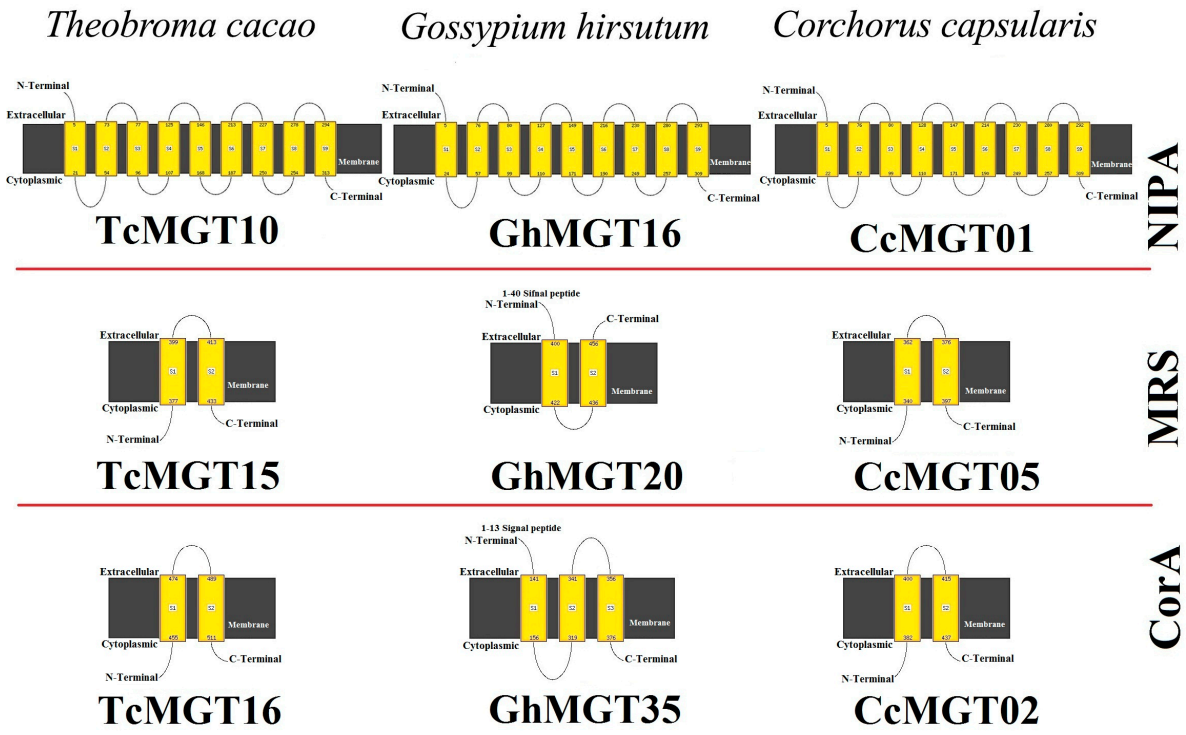

Figure 5. Predicted transmembrane helices in sub-groups of MGTs, namely NIPA, MSR2, and CorA, in *Theobroma cacao*, *Gossypium hirsutum*, and *Corchorus capsularis*.

Moreover, pocket sites of MGT proteins related to the active binding site were predicted in structures of candidate proteins. The results illustrated that sub-groups of MGT proteins are different based on the residues present in predicted pocket sites (Figure 6). Phenylalanine (PHE) amino acid was frequently observed in binding sites of NIPA proteins from *T. cacao* and *G. hirsutum*, while in *C. capsularis* isoleucine (ILE) and lysine (LYS) were more often observed in pocket sites. In candidate MRS2 proteins, proline (PRO), PHE, glutamine (GLN), asparagine (ASN), glutamic acid (GLU), and glycine (GLY) were frequently predicted in *T. cacao* as binding sites, while in *G. hirsutum* tyrosine (TYR), leucine (LEU), GLU, GLY, and GLN were more repeated in pocket sites. In addition, LEU residue was highly observed as a key binding site in candidate CcMGT of *C. capsularis*. In candidate CorA proteins, GLU amino acid was more often predicted in pocket sites of *T. cacao* and *G. hirsutum*, while PHE was frequently observed in pocket sites of candidate CorA protein in *C. capsularis*.

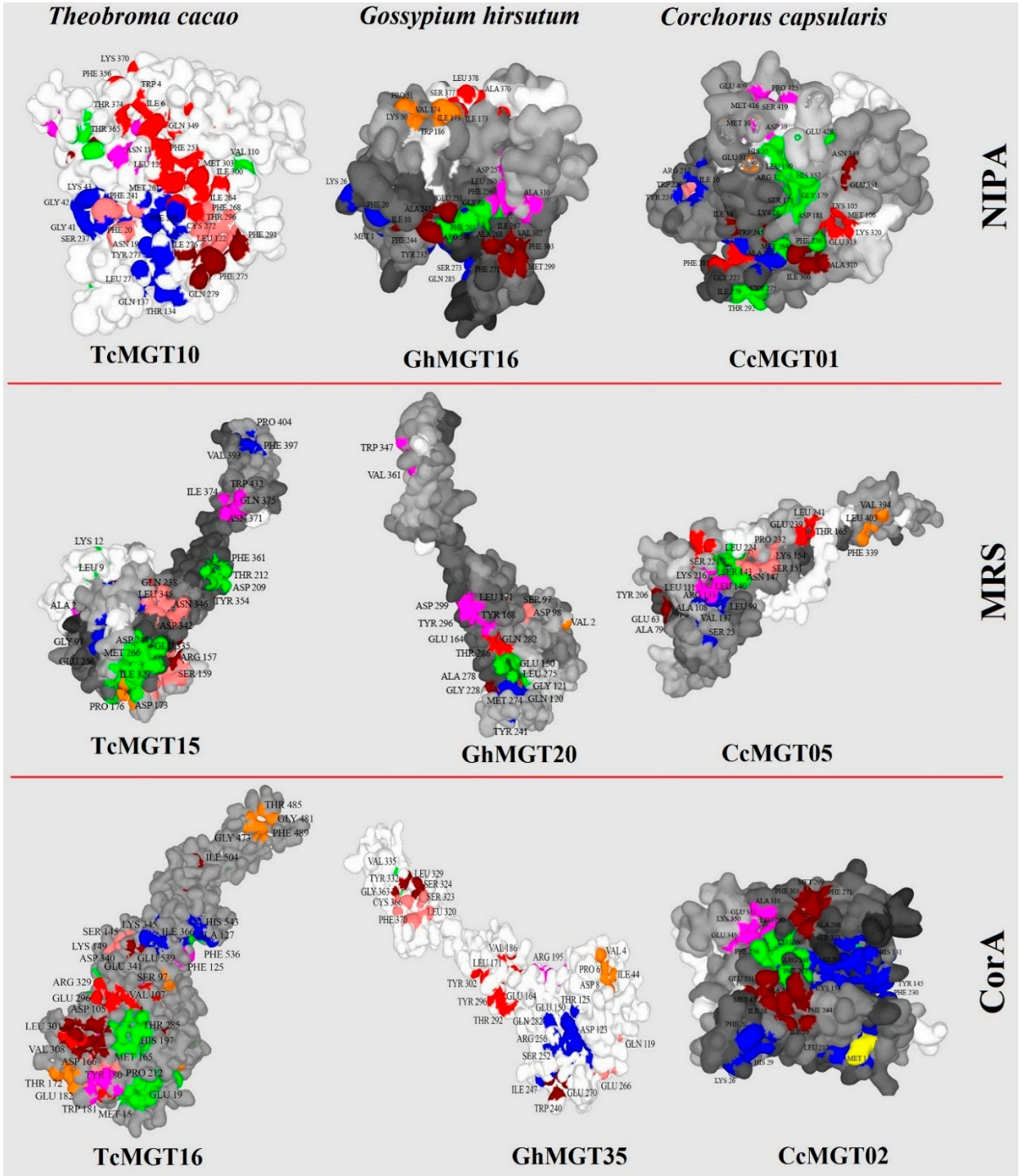

Figure 6. Docking analysis of candidates of sub-groups of MGTs, namely NIPA, MSR2, and CorA, in *Theobroma cacao*, *Gossypium hirsutum*, and *Corchorus capsularis*.

3.5. Distribution of Cis-Regulatory Elements in Promoter Region of MGT Genes

The promoter regions of *MGTs* in three plant species, comprising *T. cacao*, *G. hirsutum*, and *C. capsularis*, were analyzed and compared based on type and frequency of cis-regulatory elements. All recognized elements were classified into five groups: hormone-responsive elements, stress-responsive elements, light-responsive elements, growth-responsive elements, and binding sites of transcription factors. Our results revealed that *MGT*' promoters contain cis-regulatory elements related to stress response (Figure 7 and Table S4). In addition,

all stress-responsive elements were grouped in six classes related to drought, wounding, anaerobic, low temperature, biotic stress, and general stresses. However, cis-regulatory elements responsive to low temperature, anaerobic stresses, and biotic stresses were observed most often in the promoter region of *TcMGT* genes (Figure 7b). In addition, regulatory elements related to response to abscisic acid (ABA), salicylic acid (SA), auxin, gibberellin (GA), and methyl jasmonate (MeJA) were observed among hormone-responsive elements (Figure 7b,f,i). We found that *MGT* genes might be more frequently induced in response to ABA hormone.

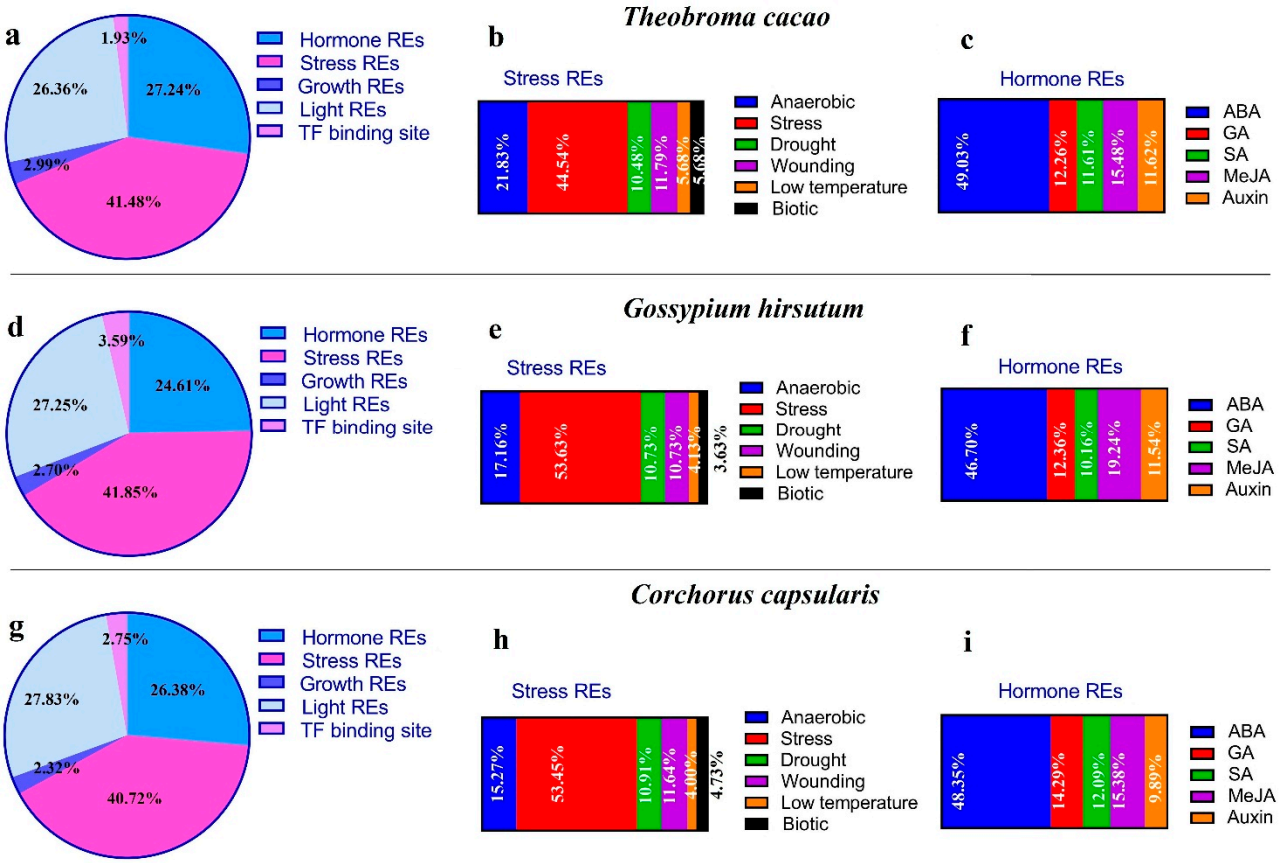

Figure 7. Proportion of cis-regulatory elements in promoter regions of *MGT* genes. Identified cis-regulatory elements were classified as hormone-responsive elements (REs), stress REs, growth REs, light REs, and transcription factor (TF) binding site in *Theobroma cacao* (**a**), *Gossypium hirsutum* (**d**), and *Corchorus capsularis* (**g**). Proportion of cis-regulatory elements related to stress responsiveness in *Theobroma cacao* (**b**), *Gossypium hirsutum* (**e**), and *Corchorus capsularis* (**h**). Proportion of different groups of hormone-related cis-regulatory elements in *Theobroma cacao* (**c**), *Gossypium hirsutum* (**f**), and *Corchorus capsularis* (**i**).

3.6. Expression Profile of TcMGT Genes

The expression levels of *TcMGTs* were also provided in response to *P. megakarya* after 0 h, 6 h, 24 h, and 72 h after infection using available RNA-seq data of two contrasting genotypes: *T. cacao* Nanay (fungal-susceptible cultivar) and Scavina (fungal-resistant cultivar) (Figure 8a,b). According to expression heatmaps, most *TcMGT* genes were less induced by a fungal infection, *P. megakarya*. A *NIPA* gene, *TcMGT02*, showed an upregulation after 48 h of fungal infection in both cultivars (Figure 8). In addition, two *MRS2* genes, *TcMGT18* and *TcMGT12*, were more expressed after 72 h in the fungal-resistant cultivar (Figure 8b).

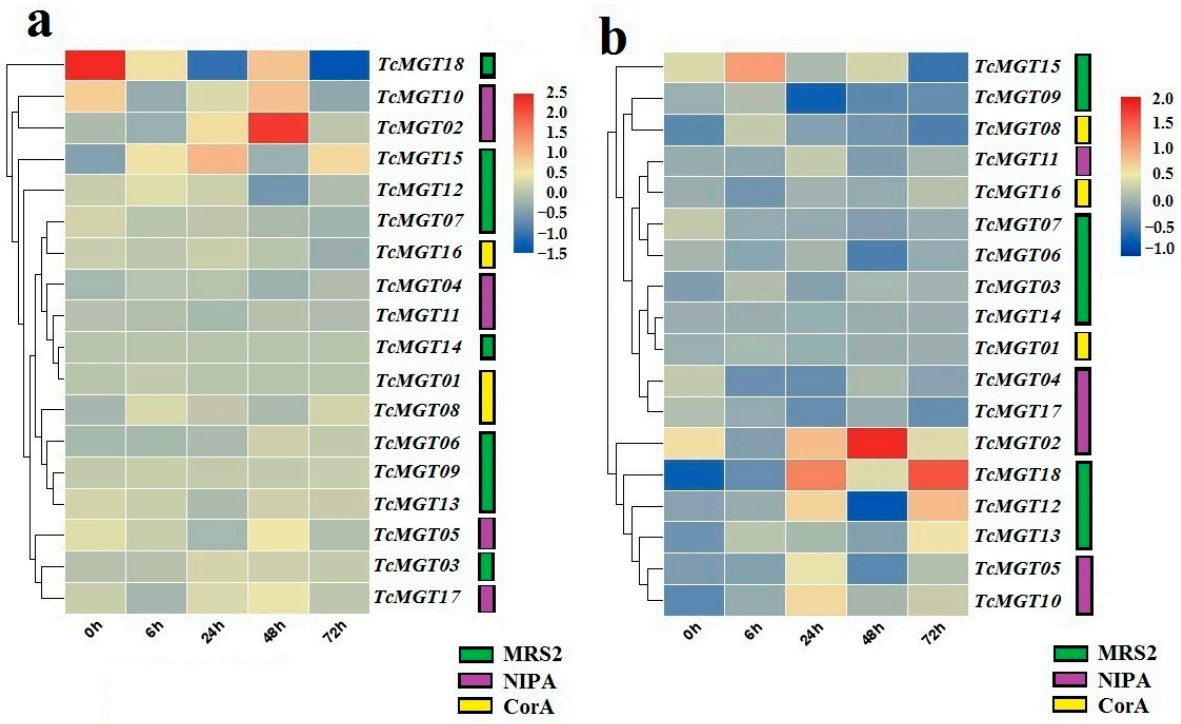

Figure 8. Expression profile of *TcMGT* genes in response *to P. megakarya* inoculation after 0 h, 6 h, 24 h, and 72 h. Nanay (NA-32) is the susceptible cultivar (**a**) and Scavina (SCA6) is the tolerant cultivar (**b**). The type of TcMGT protein sub-group is highlighted using different colors.

3.7. Expression Profile of GhMGT Genes

The expression profile of *GhMGT* genes was investigated in different tissues of *G. hirsutum* and under abiotic stresses, including cold, heat, drought, and salinity (Figure 9). The results illustrated that *GhMGTs* are involved in early and late responses to abiotic stresses (Figure 9a). For instance, a *MRS2* gene, *GhMGT32*, showed an upregulation in response to the temperature stresses of cold and heat after one hour (Figure 9a). Furthermore, *GhMGT24*, as a *NIPA* gene, was more upregulated in response to all studied abiotic stresses after 6 and 12 h. In addition, the expression profile of *GhMGT* genes showed that six *MRS2* genes, i.e., *GhMGT12*, *GhMGT33*, *GhMGT06*, *GhMGT41*, *GhMGT13*, and *GhMGT20*, are more expressed after 24 h of drought and salt stress. In the first hours of heat stress, *NIPA* genes are more expressed than *MRS2* genes in cotton. In addition, *CorA*- like genes of *GhMGT35* and *GhMGT14* showed more upregulation in response to drought and salt stress. Expression levels of *GhMGTs* were also evaluated in different tissues and organs of *G. hirsutum* (Figure 9b). The results show that *GhMGTs* were expressed in different organs for the proper distribution of magnesium throughout the cotton plant. In root tissues, *NIPA* genes are more expressed, while in leaf and torus tissues, *MRS2* genes are more expressed (Figure 9b). Furthermore, two *CorA*-like genes showed high expression in filament tissues. In addition, most *GhMGTs* are expressed in ovule tissues in 10 days post-anthesis (DPA) (Figure 9b).

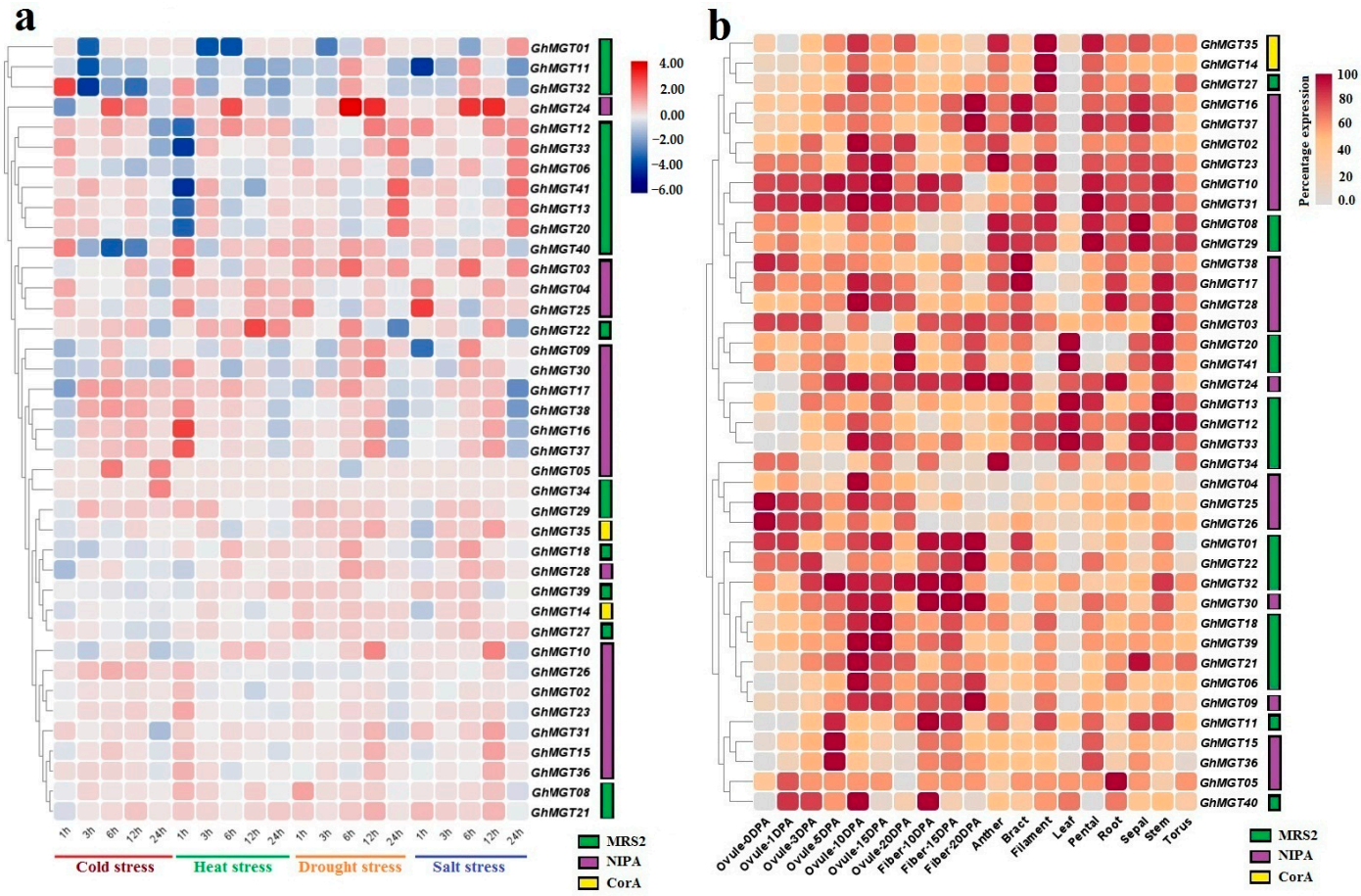

Figure 9. Expression profile of *GhMGT* genes in response to abiotic stresses, including cold, heat, drought, and salt stress (**a**), and in different tissues of cotton (**b**). The type of GhMGT protein sub-group is highlighted using different colors.

3.8. Co-Expression Network of MGT Genes

A co-expression network of *TcMGTs* was formed using their orthologs in the diploid model plant, *Arabidopsis* (Figure 10). All nodes in the co-expression network of *MGTs* were classified into four groups (I to IV) (Figure 10). In group I, three auxin-responsive genes, namely *SAUR4*, *7*, and *12*, two genes affecting lipid metabolism, *PLC* genes, two genes involving in disease resistance-responsive, namely *AT4G38700* and *AT2G21110*, and *CYP21-1* affecting protein folding showed high co-expression scores with *MGT* genes. In group II, a protein phosphatase 2C, *AT2G20050*, was found with high co-expression with three *MGT* genes. In group III, *MGT* genes showed high co-expression with *AT5G42070*, a hypothetical protein, *RPL15* (ribosomal protein), *AT2G23390* (acyl-CoA protein), and *CH1*. In group IV, high co-expression connections were observed between *MGT* genes and genes involving in cell wall organization/modification processes such as *S2LB*, *AGM1*, *AGM2*, *EXPA13*, *GXM2*, *GXM1*, and *QRT1*. In addition, *LrgB* involved in response to water deprivation and *MHX* encoding a mg/proton exchanger showed string co-expression with *MGTs*. Gene ontology (GO) analysis illustrated that the biological processes, including mg ion transporter, photoprotection, and cell wall pectin biosynthetic process, xylan biosynthetic process, and chloroplast organization, were significantly enriched based on all nodes of co-expression networks of *MGTs*.

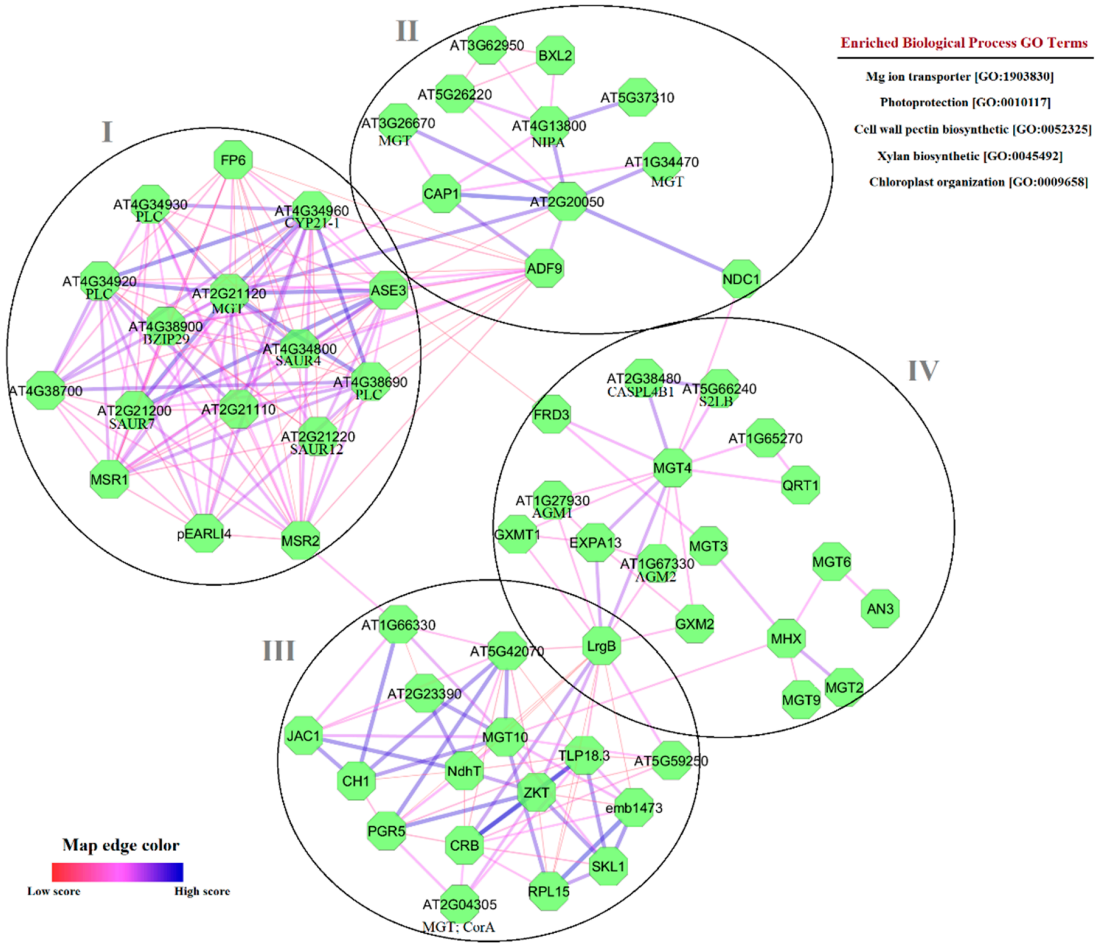

Figure 10. Co-expression networks of *MGT* genes based on the model plant *Arabidopsis thaliana*. Thickness and color intensity of each edge indicate the value of co-expression between two genes.

4. Discussion

Cocoa (*Theobroma cacao* L.) is an economical plant due to its wide use in producing chocolate, a popular commodity worldwide. Scientific research is underway to better understand the genome and metabolomes of this plant. In addition, several gene families, including *GASA* [40], *sucrose transporter* [65], *WRKY* [66], *NAC* [67], *desaturase* [68], and *sucrose synthase* [69], have been investigated and characterized in cacao. Due to the important role of magnesium ions in regulating plant growth and development, *magnesium transporters* (MGTs) have been investigated in cacao, upland cotton (*Gossypium hirsutum*), and white jute (*Corchorus capsularis*). We characterized 18 putative non-redundant *MGT* genes in cacao (*TcMGTs*) along with 41 *MGTs* in *G. hirsutum* (*GhMGTs*), and 16 *MGTs* from the genome of *C. capsularis* (*CcMGTs*). In previous studies, 62 *MGTs* in *Camelina sativa* [12], 41 *MGTs* in *Triticum turgidum* [12], 12 *MGTs* in *Zea mays* [70], 16 *MGTs* in *Pyrus bretschneideri* [23], 36 *MGTs* in *Brassica napus* [25], 12 *MGTs* in *Fagaria vesca* [27], and 8 *MGTs* in *Poncirus trifoliata* [24] were characterized. The number of *MGTs* is probably correlated with polyploidy events and genome size [12,71]. The prediction of the pI value of MGT proteins illustrated that CcMGTs are more acidophilic proteins than TcMGTs and GhMGTs, indicating that CcMGTs are mostly active under acidic conditions (pI < 6.50). This can be related to the optimal growing environment of white jute.

According to the phylogenetic analysis, MGT family members from *T. cacao*, *G. hirsutum*, and *C. capsularis* can be classified into three groups, and CcMGTs showed close relationships to TcMGTs. However, the MGT family proteins of *Arabidopsis* and rice were divided into five clusters based on phylogenetic analysis [11,72]. In the current study, MRS2

sub-group proteins showed more diversity than the other two sub-groups, indicating that *NIPA* and *CorA* genes may be derived from *MRS2* genes during the process of evolution. In addition, more *MGT* genes were identified in cotton, which is due to polyploidy in *G. hirsutum*. The analysis of gene structure showed that *TcMGTs* and *GhMGTs* contained 4 to 15 exons, while *CcMGTs* contained 4 to 21 exons. This suggests that under evolution events more insertions and deletions of introns occurred in *MGT* genes, especially in *CcMGTs*. In addition, the first duplication event was estimated to occur approximately 103 MYA between *CcMGT05*, as a *MRS2* gene, and *CcMGT04*, as a *NIPA* gene.

Several segmental/tandem duplication events were estimated to occur between *TcMGTs*. Notably, a gene cluster of three MRS2/*TcMGT* genes, namely *TcMGT013*, *TcMGT14*, and *TcMGT15*, was observed in chromosome 6, providing an avenue for further molecular investigations in cacao. In addition, the K_a/K_s ratios in most duplicated genes were less than one, suggesting that *MGTs* have evolved slowly under a purifying selection [54,71]. The comparisons between structures of MGT proteins revealed that NIPA proteins include the conserved structures with more transmembrane regions. More transmembrane regions could indicate a more important role for the NIPA protein group in transport of magnesium within the plant cell [12]. However, the transmembrane structure of MSR2 and CorA proteins in *T.* cacao and *C. capsularis* is highly conserved, while these proteins possess a different transmembrane structure in *G. hirsutum*, which may affect the ability of the magnesium transmembrane transport process [73,74]. Moreover, sub-groups of MGTs in the three studied plants showed diversity in the predicted pocket sites in the 3D structure. Overall, our findings suggest that PHE, GLU, LEU, GLY, and ILE, as the key binding sites, are associated with the function and interaction of MGTs in response to environmental stimuli and changes in ion/Mg concentration [54,75,76].

Magnesium transporters as well as other ion transporters are not only involved in response to Mg concentrations, but also their activity can be affected by changes in environmental conditions [10,12]. Analysis of gene promoter regions is one of the strategies to predict the response of target genes to various environmental factors [77,78]. *MGT* genes have a high potential to respond to stresses, both biotic and abiotic, as well as ABA based on the distribution of corresponding cis-regulatory elements in the promoter region. However, investigations that are more molecular are needed to confirm their functions. The black rod disease caused by the genus *Phytophthora* is a production limiting factor, reducing cacao production by around 20–25% [79]. On the other hand, expression studies of genes in beans of bulk cultivars, like disease resistance and fine flavor cocoa (disease susceptibility), may be of interest [80,81]. In the current study, the expression level of *TcMGT* genes was investigated using RNA sequencing data responses to *P. megakarya* in two contrasting cultivars of *T. cacao*, susceptible and fungal-resistant cultivars. Two TcMGTs, i.e., *TcMGT12* and *TcMGT18*, were identified as *P. megakarya* responsiveness genes by their upregulation specifically in the cacao-tolerant cultivar. These may be a good target for further molecular studies related to introducing the new cocao-resistant cultivars with high-quality, delicious chocolate. Furthermore, the expression profile of *GhMGTs* suggested that *MGT* genes are involved in response to abiotic stresses such as temperature stresses (cold and heat stress), drought, and salinity stress. Nevertheless, *GhMGTs* can be expressed in different plant tissues to regulate Mg homeostasis. Previous studies have indicated that *MGT* genes are associated with maintaining ion homeostasis in plant tissues during adverse conditions [20,21].

Mg transporter genes via affecting Na^+ transporters and K^+ transporters (HKTs) can improve salinity tolerance in plant species [82]. In addition, MGTs have been speculated to regulate the downstream pathways related to response to abiotic stresses by interacting with Ca^{2+} sensors [83]. Our findings revealed that the *MGT* duplicated gene pair could have diverse expression patterns, suggesting that these genes probably under some modifications or insertion/deletion in their sequence, CDS, or promoter regions have received novel functions [71,84]. Modifications, such as gains and losses of cis elements in promoters between duplicated gene pairs, e.g., parent and daughter genes, could occur

after duplication events, affecting the expression levels [85,86]. By constructing a network of co-expression genes, it is possible to identify other molecular pathways in which target genes are involved to gain a better understanding of the function of genes [54,87]. In this study, *MGT* genes showed diverse co-expressions with genes involved in auxin-responsive processes, lipid metabolism, cell wall organization, photoprotection, and chloroplast organization. Magnesium is a critical element of chlorophyll, affecting photosynthesis rate and biomass production [88,89]. Overall, *MGTs* appear to be involved in various pathways to control plant growth and development and response to adverse conditions.

5. Conclusions

A genome-wide analysis of *MGT* family genes was performed in the genomes of *Theobroma cacao, Corchorus capsularis, and Gossypium hirsutum*. Our findings provide insight into certain aspects of the sequence structure, evolutionary events, regulatory systems, and function of *MGT* genes in three species of Malvaceae. Furthermore, our results show that MGTs are involved in diverse cellular pathways, and they can interact with proteins associated with growth and development as well as with response to environmental stimuli. Further functional-molecular analyses are required to improve our understanding of the role of MGTs in cacao resistance to stress and to increase the quality of chocolate.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11081651/s1, Table S1. Details of each sequence of MGTs in *Theobroma cacao, Corchorus capsularis*, and *Gossypium hirsutum* analyzed in our study, including protein sequences, coding sequences, genomic sequences, and promoter regions. Table S2. A list and properties of the studied *MGT* genes in *Theobroma cacao, Corchorus capsularis*, and *Gossypium hirsutum.* Table S3. Predicted duplicated gene pairs in the MGT protein family in *Theobroma cacao, Corchorus capsularis*, and *Gossypium hirsutum.* Table S4. Cis-regulatory elements in promoter regions of the *MGT* gene family in *Theobroma cacao, Corchorus capsularis*, and *Gossypium hirsutum.* Figure S1. 3D structures of MGT proteins from various clades, namely NIPA, MRS2, and CorA, in *Theobroma cacao, Corchorus capsularis*, and *Gossypium hirsutum*.

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