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Genome-Wide Identification and Molecular Evolution of the Magnesium Transporter (MGT) Gene Family in *Citrullus lanatus* and *Cucumis sativus*

Parviz Heidari [1,*], Fatemeh Puresmaeli [1] and Freddy Mora-Poblete [2,*]

[1] Faculty of Agriculture, Shahrood University of Technology, Shahrood 3619995161, Iran
[2] Institute of Biological Sciences, University of Talca, Talca 3460000, Chile
* Correspondence: heidarip@shahroodut.ac.ir (P.H.); morapoblete@gmail.com (F.M.-P.)

Abstract: Magnesium transporters (MGTs) play a prominent role in the absorption, transportation, and storage of magnesium in plant cells. In the present study, MGT gene family members were identified and characterized into two species of Cucurbitaceae, including *Cucumis sativus* and *Citrullus lanatus*. Totals of 20 and 19 *MGT* genes were recognized in *Citrullus lanatus* and *Cucumis sativus*, respectively. According to their physicochemical properties, the members of each sub-class of MGTs in the species of Cucurbitaceae showed the close relationship. Proteins from NIPA class were identified as hydrophilic proteins with high stability. Based on phylogenetic analysis, MGT family members were classified into three groups, and NIPAs showed more diversity. Moreover, duplication events were not identified between the *MGT* genes in *C. lanatus* and *C. sativus*. According to pocket analysis, residues such as L, V, S, I, and A were frequently observed in the binding sites of MGT proteins in both studied species. The prediction of post-translation modifications revealed that MSR2 proteins have higher phosphorylation potentials than other sub-classes of MGT in both studied plants. The expression profile of *MGTs* showed that *MGTs* are more expressed in root tissues. In addition, *MGTs* showed differential expression in response to abiotic/biotic stresses as well as hormone application and *NIPAs* were more induced in response to stimuli in watermelon. The results of this study, as the primary work of MGT gene family, can be used in programs related to Cucurbitaceae breeding.

Keywords: magnesium; evolution analysis; plant gene families; Cucurbitaceae; gene sequence analysis; stresses

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1. Introduction

Magnesium (Mg) is a vital element for living cells involved in many critical cellular activities [1–3]. For instance, Mg as a cofactor is essential for the activity of many enzymes (>300 enzymes) such as kinase, polymerase, and H + -ATPase [4–6]. In addition, Mg, as the key atom of chlorophyll, affects the photosynthesis rate and plant growth [7]. Magnesium transporters (MGTs) are present in plants for Mg uptake, translocation, and cell storage. Based on sequence structure, MGT proteins have been classified into three groups, including MRS2, CorA, and NIPA [8]. The CorA protein, as a member of MGTs, was firstly identified in bacteria, *Salmonella typhimurium* [9], and in plants for the first time; MGT proteins were studied in the model plant, *Arabidopsis* [10]. The MRS2 and CorA proteins are recognized by a tripeptide conserved region, GMN (Glycine-Methionine-Asparagine), and two or three transmembrane (TM) domains in their C-terminal ends [11], while NIPAs contain several TMs in their structures [3,8]. However, our knowledge of the NIPA class is limited. Due to the important role of Mg in plants, members of the MGT gene family have been identified and studied in different plants such as *Arabidopsis* [12], *Triticum turgidum* and *Camelina sativa* [8], *Pyrus communis* [13],

Theobroma cacao [3], *Brassica napus* [14], *Zea mays* [15], *Poncirus trifoliata* [16], *Solanum lycopersicum* [17], *Oryza sativa* [11], and *Saccharum officinarum* [18]. Moreover, the function of recognized *MGT* genes was experimentally characterized in plant species.

MGT genes are distributed in various plant organs, including the root, flower, leaf, and stem, to balance magnesium concentrations [8]. Previous studies disclosed that some *MGTs* in root tissues are involved in up-taking Mg from the soil, including *OsMGT1* in *Oryza sativa*, and *AtMGT6* in *Arabidopsis thaliana* [19,20]. In addition, *AtMGT9* was identified as an Mg transporter translocating Mg from root tissues to shoot tissues in *Arabidopsis* [11]. Furthermore, *MGT* genes, such as *AtMGT5* and *AtMGT9*, have been recognized to be involved in pollen development in *Arabidopsis thaliana* [21–23]. Some MGT proteins are located in the membranes of cellular organelles and are involved in the distribution and accumulation of Mg within the cell. For instance, in *Arabidopsis thaliana*, AtMGT2 and AtMGT3 can accumulate Mg in the vacuole [24], while AtMGT10 maintains Mg homeostasis in chloroplasts [21]. It has also been reported that *MGTs* increase plants' tolerance to environmental stresses. For example, *OsMGT1* was identified as a gene related to the response to salt stress in rice [25]. In addition, a positive correlation has also been reported between aluminum (Al) stress tolerance in plants and the expression of *MGT* genes. Furthermore, *OsMGT1* in rice was recognized to be involved in the tolerance to Al stress [25]. Moreover, the transgenic lines for *AtMGT1* in *Nicotiana benthamiana* showed a reduction in Al toxicity [26]. It seems that increasing MGT activity and more Mg uptake play an important role in reducing the negative effects of some elements and ions.

Cucurbitaceae are the most diverse plant species, with more than 800 species known worldwide [27,28]. The important vegetable crops, including cucumber (*Cucumis sativus* L.), melon (*Cucumis melo* L.), watermelon (*Citrullus lanatus*), and squash (*Cucurbita L.*) belong to Cucurbitaceae. Due to the important role of the MGT gene family, no studies have been conducted to identify and structurally evaluate members of this gene family in species of Cucurbitaceae. In the current study, we focus on identifying and characterizing the members of MGT family in two species of Cucurbitaceae, *Citrullus lanatus* and *Cucumis sativus*. Moreover, phylogenetic relationships, protein structures, phosphorylation sites, expression patterns, and promoter region analyses of members of the MGT family were conducted in both species. The present results improve our understanding of the structure, regulatory systems, and function of the *MGTs* in two candidate species of Cucurbitaceae. As a primary study, it can be used in future studies related to functional analysis of *MGTs* in *Citrullus lanatus* and *Cucumis sativus*.

2. Results

2.1. Identification of MGT Gene Family in Watermelon, Cucumber, and Melon

By searching in three species of Cucurbitaceae, 20, 19, and 20 MGT genes were identified in *Citrullus lanatus* (*C. lanatus*), *Cucumis sativus* (*C. sativus*), and *Cucumis melo* (*C. melo*), respectively (Table 1, Table S1). In addition, subclasses of MGT, including MRS2, NIPA, and CorA, were identified based on their specific domain distributions (Table S1). Based on physicochemical properties, the MGTs in all three studied species of Cucurbitaceae were close to each other and almost similar. The protein lengths of MGTs in *C. lanatus* ranged from 323 amino acids (aa) to 548 aa, *C. sativus* from 305 to 567 aa, and *C. melo* from 175 to 546 aa. Moreover, the MW of MGTs in *C. lanatus* varied from 35.44 to 62.78 kDa, from 34.89 to 62.89 kDa in *C. sativus*, and from 20.17 to 63.23 kDa in *C. melo*. In addition, the pI of MGTs ranged from 4.86 to 8.32 in *C. lanatus*, from 4.87 to 9.63 in *C. sativus*, and from 4.60 to 9.47 in *C. melo*. The GRAVY (grand average of hydropathy) value of MGTs was between –0.35 and 0.77 in *C. lanatus*, between –0.37 and 0.87 in *C. sativus*, and between –0.45 and 1.07 in *C. melo*. Interestingly, proteins from subclasses MSR2 and CorA showed a positive GRAVY index, while most NIPAs showed a negative GRAVY. Moreover, according to the instability index, 45% of MGTs in *C. lanatus*, 53% in *C. sativus*, and 50% in *C. melo* were predicted as stable proteins. Moreover, most proteins of subclass NIPA were predicted as stable proteins than subclasses MSR2 and CorA proteins in all studied species. Based on

gene structure data, *MGT* genes have between 4 to 13 exons in *C. lanatus*, between 4 to 12 exons in *C. sativus*, and between 3 to 14 exons in *C. melo* (Table S1).

Table 1. Summary of MGTs properties in *C. lanatus*, *C. sativus*, and *C. melo*. Full details are provided in Table S1.

Attributes	*C. lanatus*	*C. sativus*	*C. melo*
Number of gene	20	19	20
Protein length (aa)	323–548	305–567	175–546
Exon number	4–13	4–12	3–14
pI	4.86–8.32	4.87–9.63	4.60–9.47
MW (KDa)	35.44–62.78	34.89–62.89	20.17–63.23
GRAVY	−0.35–0.77	−0.37–0.87	−0.45–1.07
Instability index	0.45% stable	0.53% stable	0.50% stable

2.2. Phylogenetic Analysis of the MGT Family

To better understand the evolutionary relationships of the MGT family, a phylogenetic tree was constructed for 85 members of this family in five different species, including *Arabidopsis thaliana*, *Oryza sativa*, *Citrullus lanatus*, *Cucumis sativus*, and *Cucumis melo* (Figure 1). According to the phylogenetic tree, MGT family members can be classified into three groups, including groups I, II, and III. Subclass NIPA proteins showed more diversity, and they were divided into two groups. Moreover, subclasses CorA and MSR2 were more closely related and were put together in group III.

2.3. Evolutionary Process in MGT Genes in Citrullus lanatus and Cucumis sativus

Duplication events were not identified between the *MGT* genes in *C. lanatus* and *C. sativus*. To investigate the duplication events between *MGTs* in *C. lanatus* and *C. sativus*, the Ks, Ka, and Ka/Ks for all gene pairs were calculated (Figure 2). The Ks value of *MGTs* in *C. lanatus* was detected between 1.8 and 2.8 (Figure 2a), and, in *C. sativus*, it was observed between 1.8 and 2.6 (Figure 2b). Moreover, the frequency of Ka/Ks of *MGTs* in *C. lanatus* was more observed between 0.5 and 0.6 (Figure 2c), while, in *C. sativus*, Ka/Ks ratio was frequently detected, ranging from 0.4 to 0.5 (Figure 2d). It seems that a similar evolutionary process has occurred in *C. lanatus* and *C. sativus* for the MGT gene family, and changes (mutations and duplication events) in members of this gene family may have occurred before the derivation of these two species.

2.4. Protein Structure of MGTs in C. lanatus and C. sativus

The three-dimensional structures of the MGT proteins, along with their binding sites in the two plants, *C. lanatus* and *C. sativus*, was predicted (Figure 3). Results showed that the members of each subclass, NIPA, CorA, and MSR2, have an almost similar structure in both studied plants. Moreover, various amino acids were predicted as ligand-binding residues in the MGT structures (Figure 4; Table S2). L, V, S, I, and A were also observed in the binding sites of MGT proteins in *C. lanatus* and *C. sativus*, indicating that these residues in molecular function of MGTs.

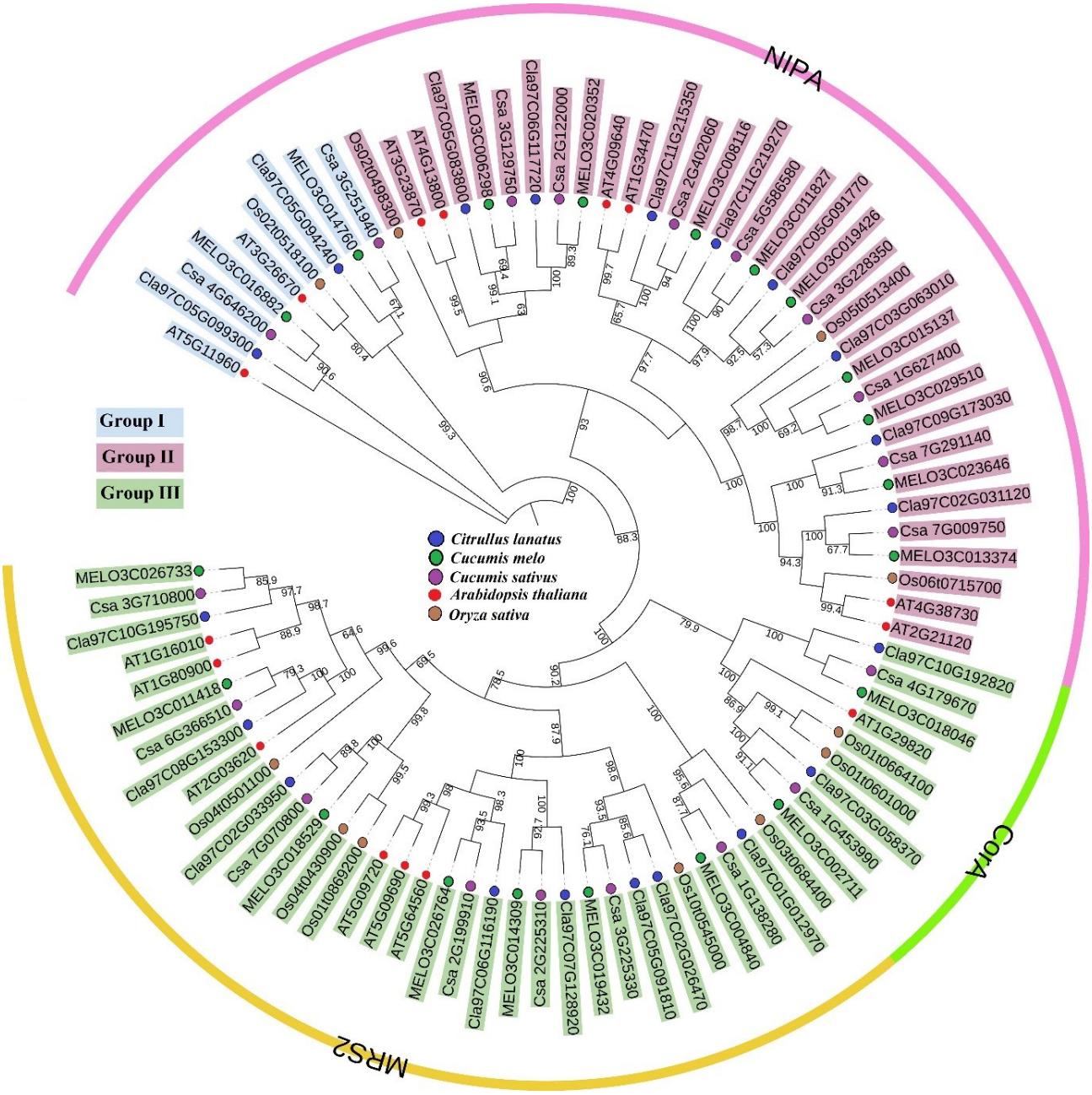

Figure 1. Phylogenetic analysis of MGT families in five different species, *Arabidopsis thaliana*, *Oryza sativa*, *Citrullus lanatus*, *Cucumis sativus*, and *Cucumis melo*. The phylogenetic tree was constructed by the maximum likelihood method. Different colored backgrounds indicate different groups.

2.5. Transmembrane Structure of MGTs

The transmembrane structures of three subclasses of MGTs were predicted in *C. lanatus* and *C. sativus* (Figure 5). The highest numbers of transmembrane helices were observed in NIPA proteins in both species with nine helices, while two helices in CorAs and between two and three helices in MSR2s were predicted. Both C- and N-terminal of the transmembrane structure of CorA proteins were observed in the cytoplasm, while C- and N-terminal of some MSR2 proteins were predicted to locate in extracellular. In addition, a signal peptide was expected in the N-terminal of MSR2 proteins. Overall, the results indicate that MGT proteins have an almost conserved transmembrane structure in both plants.

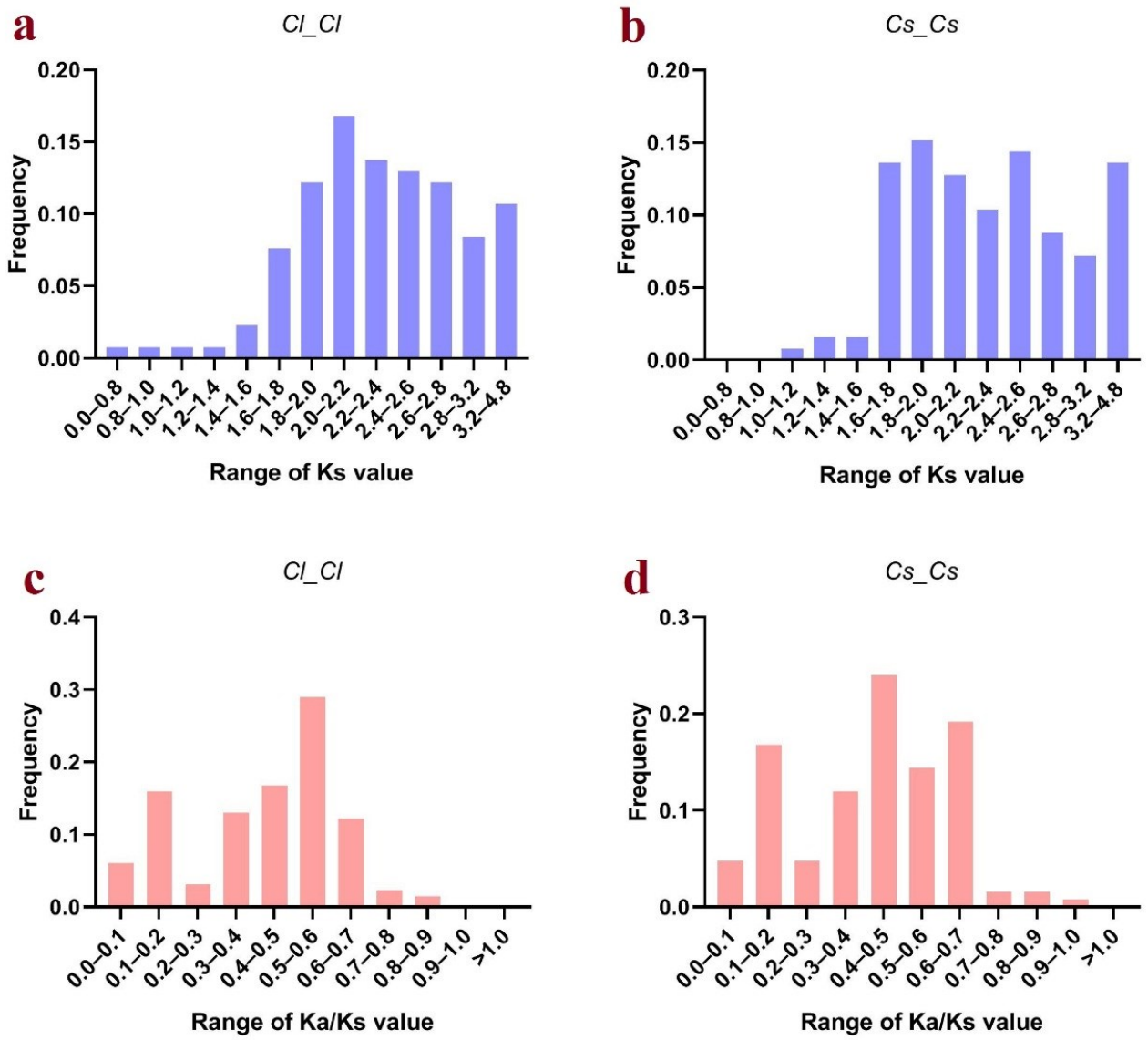

Figure 2. Ks value and Ka/Ks in *MGTs* in *C. lanatus* (Cl), and *C. sativus* (Cs). Frequency of Ks value between *MGTs* in *C. lanatus* (**a**) and *C. sativus* (**b**). Frequency of Ka/Ks ratio between *MGTs* in *C. lanatus* (**c**) and *C. sativus* (**d**).

2.6. Prediction of the Phosphorylation Site in MGT Proteins

The phosphorylation sites into MGTs of *C. lanatus* and *C. sativus* were predicted based on three amino acids, including serine (S), threonine (T), and tyrosine (Y) (Figure 6). The predicted phosphorylation sites in MGTs of *C. lanatus* varied from 4 to 18 sites and from 3 to 19 in *C. sativus*. MSR2 proteins showed higher phosphorylation potential than NIPAs in both studied plants. Moreover, Cla97C07G128920, as a MSR2 protein in *C. lanatus*, and Csa_1G453990, as a CorA protein, were identified as the MGT proteins with a high phosphorylation potential.

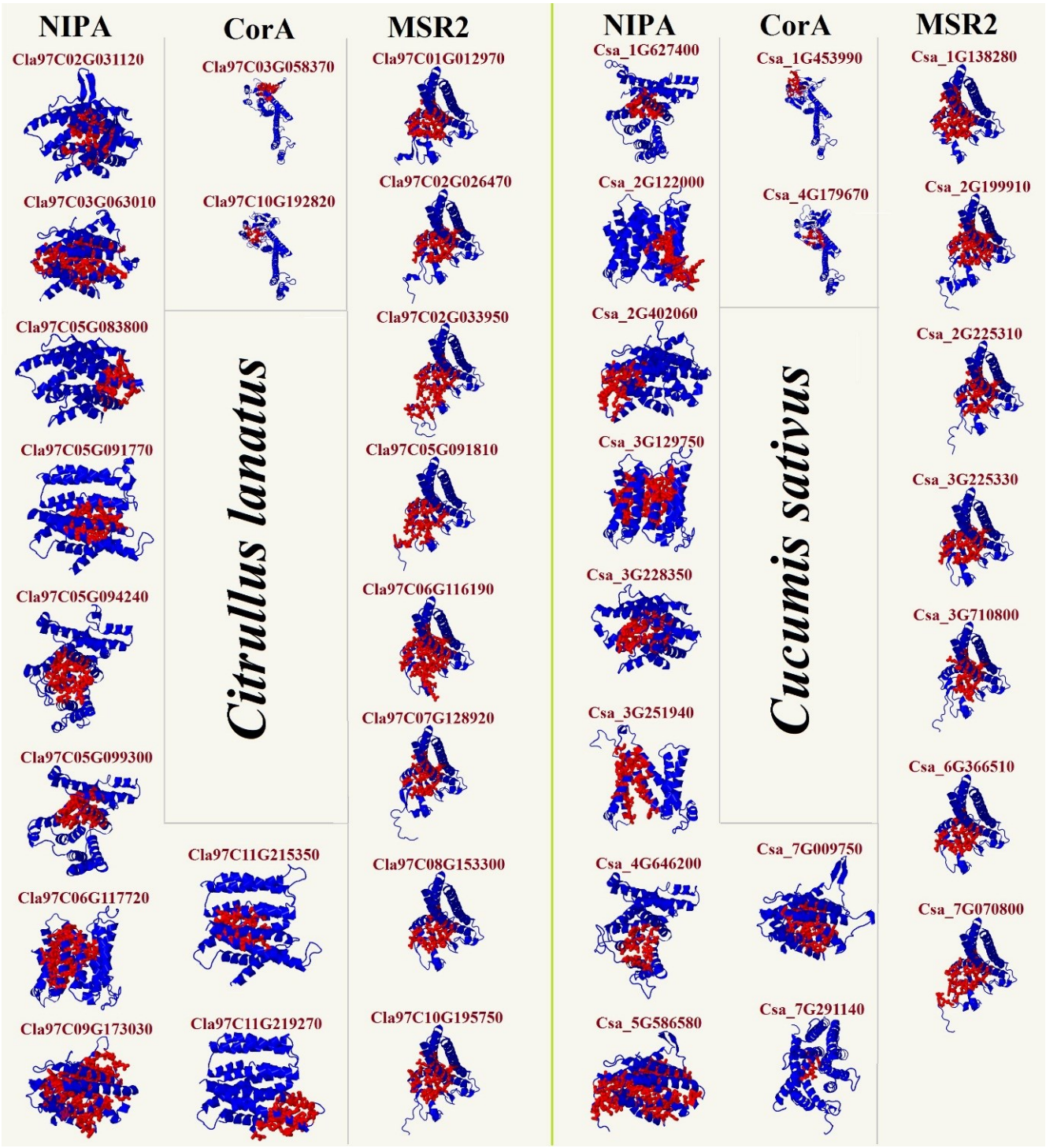

Figure 3. The predicted 3D structure of MGT proteins in *C. lanatus* and *C. sativus*. The region of ligand-binding sites in the predicted 3D structure of MGTs is highlighted by a red circle. More details are provided in Table S2.

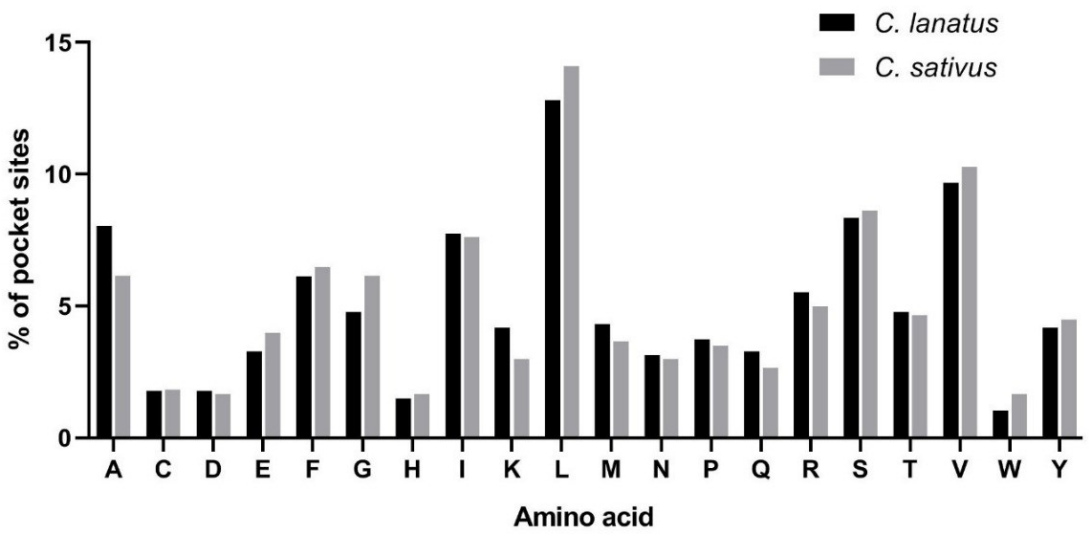

Figure 4. Percentage of amino acids in the predicted pocket sites of MGT proteins in *C. lanatus* and *C. sativus*.

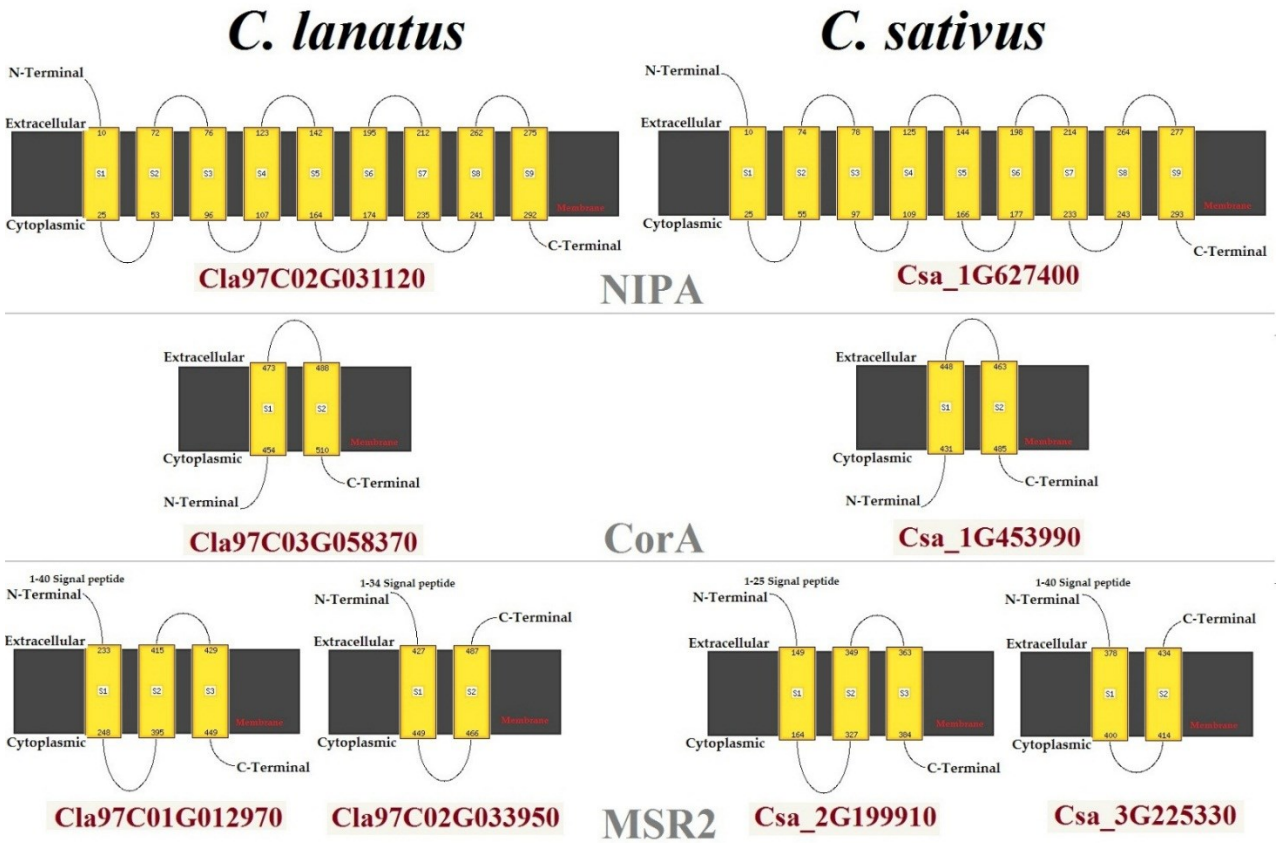

Figure 5. The distribution of transmembrane helices in various subclasses of MGTs in *C. lanatus* and *C. sativus*.

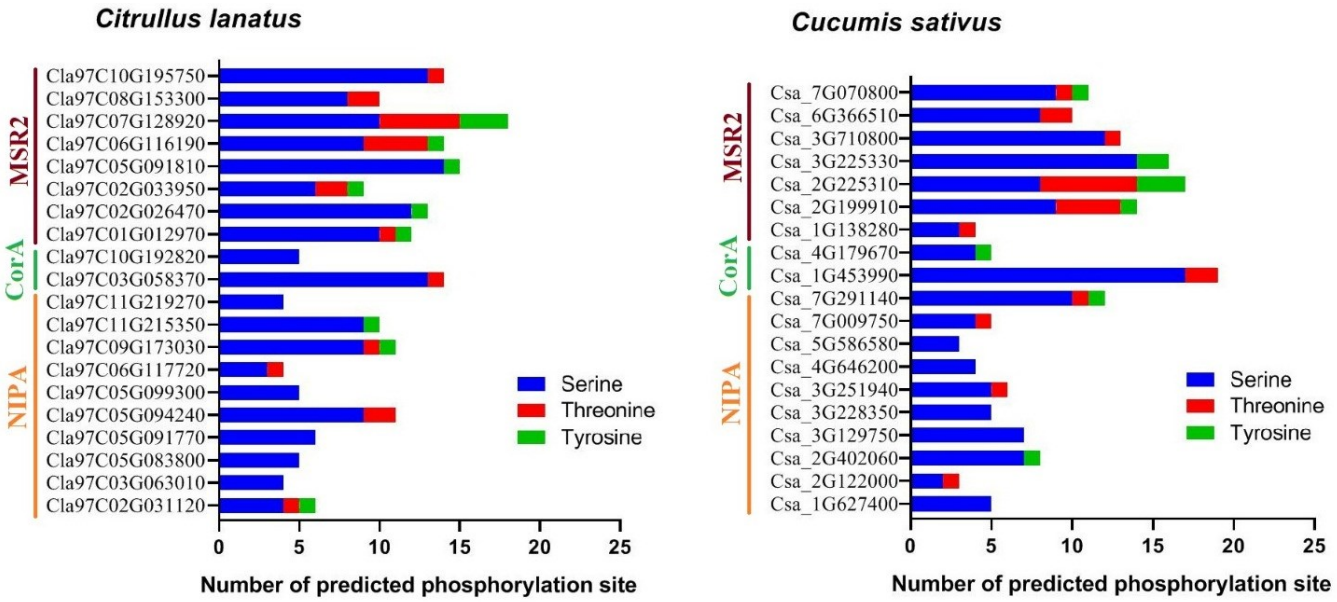

Figure 6. Prediction of phosphorylation site of MGT proteins in *C. lanatus* and *C. sativus*.

2.7. Co-Expression Network of MGTs in Cucumber

To understand the potential interaction of the MGT gene family members with other genes, as well as the involved pathways, an interaction network of MGT proteins was drawn in cucumber (Figure 7). The results show that NIPAs interacted highly with the protein phosphatase C (PP2C). In addition, some interactions were observed between NIPA and auxin-induced proteins. Moreover, MSR2 proteins showed strong interactions with acetyltransferase and CaCA antiporter proteins. Gene ontology (GO) analysis based on interaction network revealed molecular function terms including 8-amino-7-oxononanoate synthase activity, chlorophyllide a oxygenase activity, and transmembrane transporter activity were significantly involved in the MGT-interaction network. Moreover, biological processes, such as divalent metal ion transporter process, localization process, and cellular process, were linked with the MGT-interaction network.

2.8. Upstream Analysis of MGT Genes

The upstream region, 1500 bp, of the *MGT* genes *C. lanatus* and *C. sativus* was analyzed as the promoter region to identify *cis*-regulatory elements. All identified *cis*-elements were grouped according to their functions, including light-responsive elements (REs), hormone REs, stress REs, growth REs, and MYB binding site (Figure 8). The *cis*-regulatory elements related to stress REs were more observed in the promoter region of *MGTs* in *C. lanatus* (Figure 8a). In contrast, hormone REs were more observed in the upstream region of *MGTs* from *C. sativus* (Figure 8b). Moreover, abscisic acid (ABA) REs were frequently identified in the promoter region of *MGTs*, more in the cucumber *MGTs*, while auxin REs and methyl jasmonate (MeJA) REs were more frequently observed in the promoter region of *MGTs* from watermelon (Figure 8b,e). Stress REs were classified into four groups, including anaerobic, biotic, drought, and low-temperature stress (Figure 8c,f). Moreover, the regulatory elements of anaerobic were recognized with high frequency in the promoter region of *MGT* genes. Moreover, low-temperature REs were observed most often in the upstream region of *MGTs* from watermelon (Figure 8c), while regulatory elements that were responsive to drought stress were more frequently observed in promoter region of cucumber *MGTs* (Figure 8f).

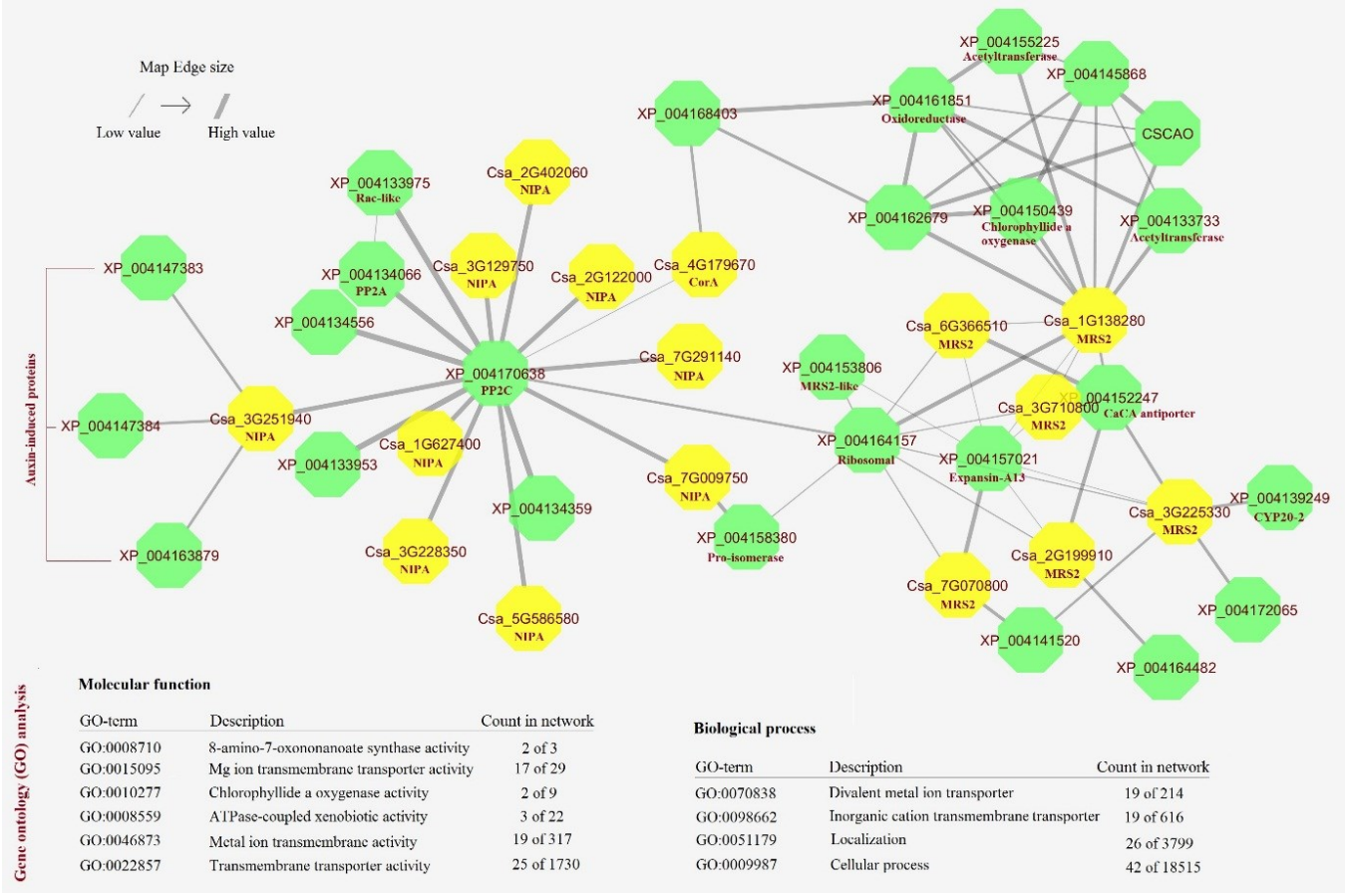

Figure 7. The interaction network of *MGTs* with other genes in *C. sativus*. The significant GO terms (FDR < 0.05) related to the network are provided based on molecular functions and biological process terms.

2.9. Expression Profile of MGTs in C. lanatus and C. sativus

The expression patterns of *MGT* genes in different tissues of *C. lanatus* and *C. sativus* were provided based on the RNA-Seq datasets (Figure 9). The *MGTs* in both studied plants showed low expressions in seeds, while high expression levels of *MGTs* were observed in root tissues. Three *MSR2* genes, including *Csa_2G225310*, *Csa_2G19910*, and *Csa_7G070800*, and three *NIPAs*, *Csa_7G291140*, *Csa_5G586580*, and *Csa_3G251940* showed high expression in shoot tissues of cucumber (Figure 9a). Moreover, an *MSR2* gene, *Cla97C02G033950*, showed substantial expression levels in all studied tissues in watermelon (Figure 9b), suggesting the important role of this gene during watermelon growth and expansion. The expression levels of *MGTs* were also investigated according to available RNA-seq datasets related to biotic and abiotic stresses and hormone treatments (Figure 10). The expression levels of a *NIPA*, *Csa_3G129750*, and an *MSR2* gene, *Csa_1G138280*, of cucumber were upregulated in response to NaCl stress (Figure 10a). Moreover, in response to the nematode, the expression pattern of *Csa_2G225310*, as an *MSR2* gene, was upregulated in cucumber, and *Csa_4G646200*, as a *NIPA* gene, showed an upregulation one day after infection (dai) by *Pseudoperonospora cubensis* (PC) (Figure 10a). In the watermelon, two *NIPA* genes, including *Cla97C05G083800* and *Cla97C03G063010*, were upregulated in response to drought stress and under mosaic virus stress, two *NIPAs*, *Cla97C05G083800* and *Cla97C02G031120*, also showed an upregulation (Figure 10b). Moreover, two *NIPAs* genes from watermelon were more induced in response to melatonin treatment and under low nitrogen content in the leaf (Figure 10b). It seems that *NIPAs* are more induced in response to stimuli in watermelon.

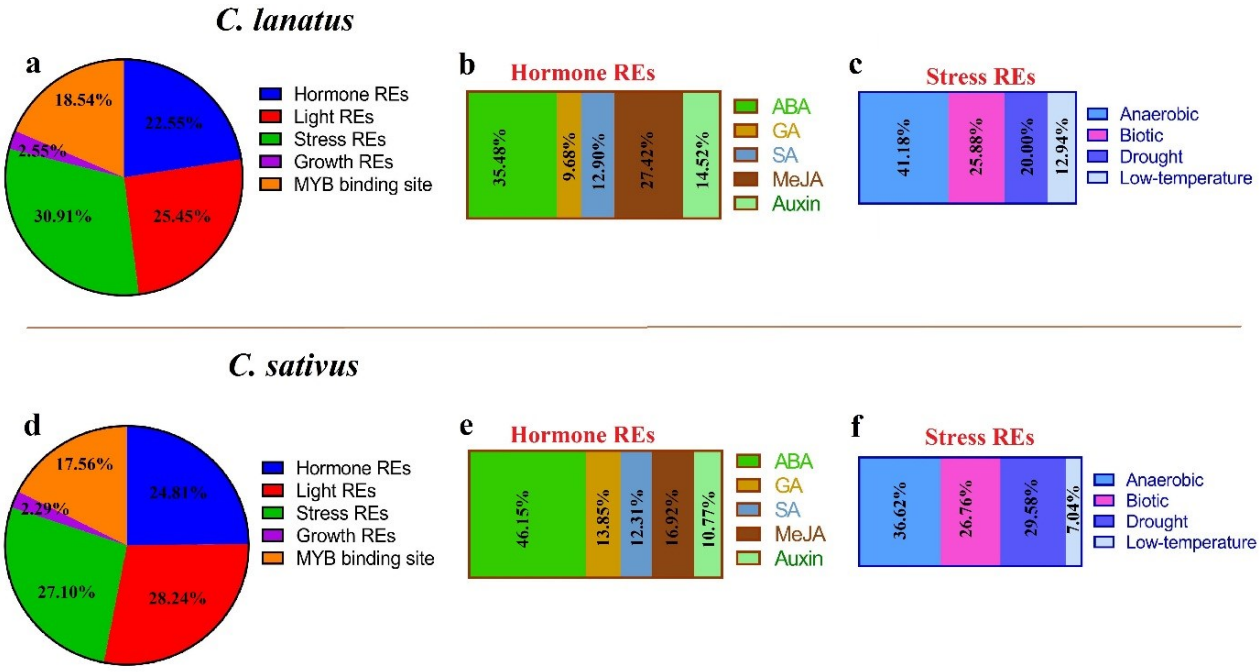

Figure 8. Proportion of *cis*-regulatory elements in upstream site (promoter regions) of *MGT* genes. The *cis*-regulatory elements were classified in hormone-responsive elements (REs), light REs, stress REs, growth REs, and MYB binding site in *C. lanatus* (**a**) and *C. sativus* (**d**). The percentage of *cis*-regulatory elements related to hormone REs in *C. lanatus* (**b**) and *C. sativus* (**e**), and stress REs in *C. lanatus* (**c**) and *C. sativus* (**f**). More details are provided in Table S3.

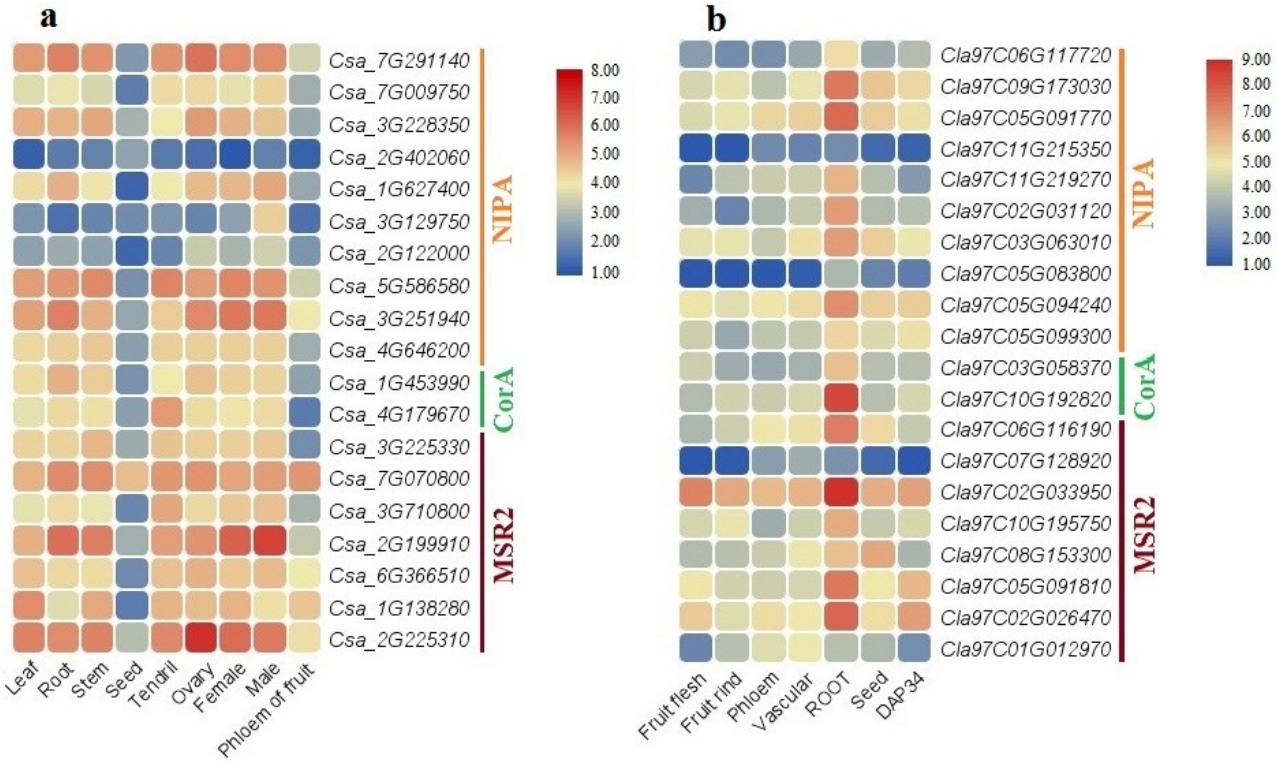

Figure 9. Heatmaps of expression of *MGT* genes in different tissues of *C. lanatus* (**a**) and *C. sativus* (**b**).

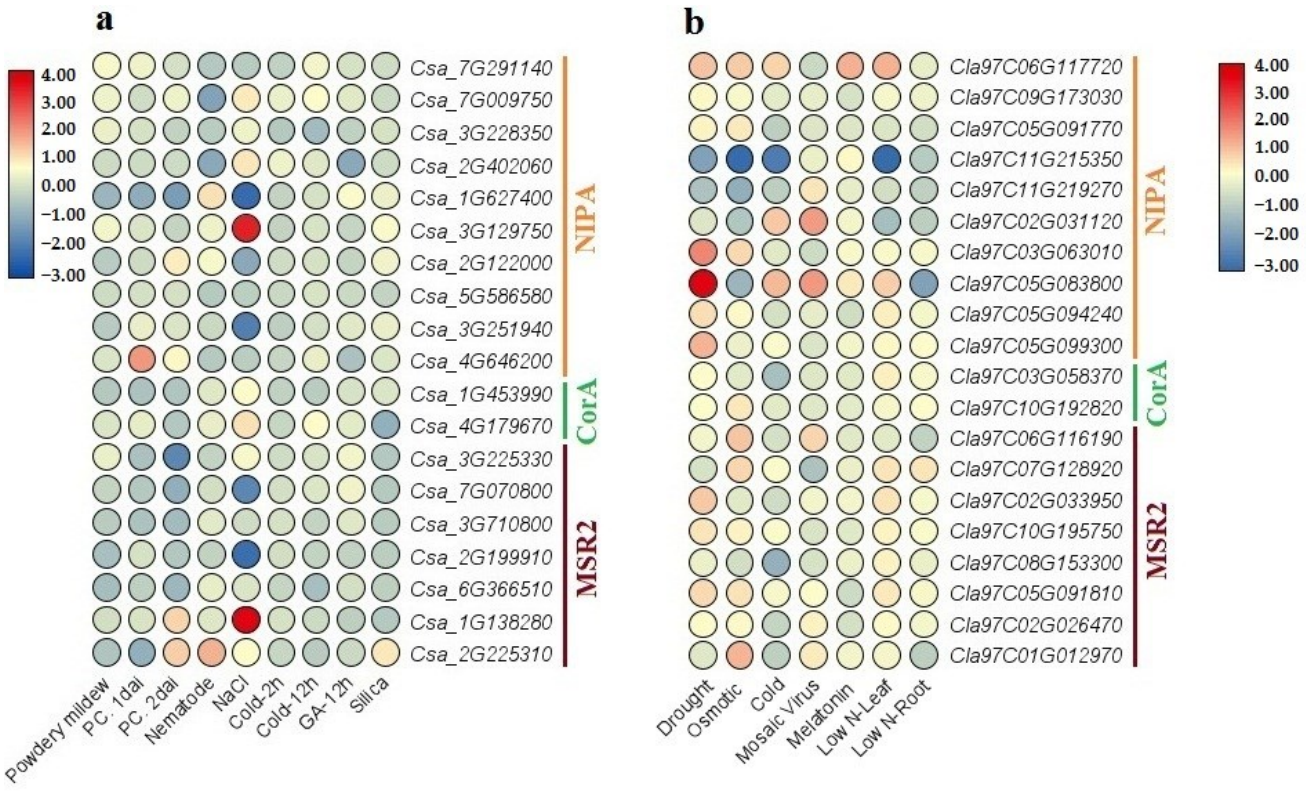

Figure 10. Heatmaps of expression of *MGT* genes respond to biotic and abiotic in *C. lanatus* (**a**) and *C. sativus* (**b**).

3. Discussion

Magnesium (Mg), in addition to being a basic element for plant growth as an essential cofactor, is also involved in the activity of enzymes and in metabolic and photosynthetic processes [29]. Magnesium transporters (MGTs) are fundamental in transmitting and maintaining Mg concentrations in various organelles and cell tissues. In the current study, as the first report, 20, 19, and 20 *MGT* genes were identified and characterized in three species of Cucurbitaceae, including *C. lanatus*, *C. sativus*, and *C. melo*, respectively. This number of genes is less than the number in *Gossypium hirsutum* (41 *MGTs*) [3], *Camelina sativa* (62 *MGTs*) [8], *Triticum turgidum* (41 *MGTs*) [8], and *Brassica napus* (36 *MGTs*) [14]; however, the number of identified genes is greater than that reported in the *Theobroma cacao* (18 *MGTs*) [3], *Corchorus capsularis* (16 *MGTs*) [3], *Pyrus bretschneideri* (16 *MGTs*) [13], *Zea mays* (12 *MGTs*) [15], *Poncirus trifoliata* (8 *MGTs*) [16], and *Fagaria vesca* (12 *MGTs*) [17]. The evolutionary events such as duplication and polyploidization have increased the number of *MGTs* in some plant species [30,31]. However, we did not find any duplication between the MGT family members in *C. lanatus* and *C. sativus*. We hypothesize that the duplication events probably did not occur after the derivation of these two species. Moreover, based on the structure of genes and physicochemical characteristics, an almost conserved state was observed between the studied species, further strengthening this hypothesis. Three subclasses of MGTs, including MSR2, CorA, and NIPA, were identified and compared, and the NIPAs showed a significant difference from the other two classes. For instance, the NIPAs were predicted as stable proteins. In addition, based on GRAVY as a solubility index [31,32], the NIPAs were predicted as hydrophilic proteins. There is limited information about the function of NIPA class in plants, and, due to the different structures, it is necessary to conduct more studies in the field of their functional analysis. Moreover, based on the analysis of gene structure, members of the MGT family had variations in the number of exons, especially in the *NIPAs*. The exon number can increase the diversity of the coding protein of a gene by affecting the post-transcriptional processes, such as alternative splicing [33,34]. Those with fewer

exons can activate rapidly in response to stress, and these genes play a stronger role in the process of adapting to adverse environmental conditions [35]. Evolutionary events such as duplication can affect the structure of genes. Our results reveal that the Ka/Ks ratios of all *MGTs* in both studied species, *C. lanatus* and *C. sativus*, were less than 1.0 indicating that purifying the (negative) selection was the most important force for motiving the evolution of *MGTs*. Moreover, it was stated that most *MGTs* emerged before the disclosure of angiosperms [18]. According to the phylogenetic analysis, MGT gene family members can be classified into three main groups; more diversity was observed among the orthologous NIPAs than CorA and MSR2. It seems that the evolutionary processes in the NIPAs were different from the other two groups, CorA and MSR2.

The prediction of the 3D structure of the MGT proteins showed that each subclass had an almost conserved structure in *C. lanatus* and *C. sativus*; however, differences in their binding sites were observed, indicating a parallel evolutionary trend in MGTs of both species. Moreover, leucine, valine, serine, isoleucine, and alanine were frequently predicted in pocket sites, suggesting that these residues were more related to the possible interactions of MGTs [36,37]. The interaction network of the MGTs in *C. sativus* showed that NIPAs interact more with PP2C and auxin-induced proteins. PP2C is a phosphatases involved in ABA signaling, and it was reported previously that Mg could induce Mg-dependent phosphatases PP2C heterodimer in response to heat stress [38]. In addition, hormone response elements were observed in the upstream regions of the *MGT* genes in both plants, indicating an interaction between Mg and phytohormones. Moreover, interactions between MSR2s and acetyltransferase and CaCA antiporter proteins were observed, and further studies are needed on how they interact in cucumber cells. The phosphorylation process is one of the key post-translational modifications that significantly affect the activity and stability of target proteins and also affect the regulation of cellular signaling pathways in response to adverse conditions [31,39,40]. Results reveal that MSR2 sub-class members have more potential sites for phosphorylation than NIPAs in both studied plant species, *C. lanatus* and *C. sativus*, suggesting that MSR2s have more potential to interact with kinases and other signaling components. Based on the expression profile, *MGTs* were expressed in various tissues in *C. lanatus* and *C. sativus*, indicating that they are involved in different biological processes. However, most *MGT* genes were expressed in the root tissues, indicating that *MGTs* are more involved in the uptake of Mg in the root and then the distribution of mg in other tissues. Moreover, *MGTs* showed various expression patterns in response to biotic and abiotic stresses, as well as hormone application. Interestingly, the results disclose that *NIPA* members are more frequently induced in response to stimuli in watermelon than *MSR2s* and *CorAs*. For instance, *Cla97C116215350* as a NIPA showed an upregulation in response to cold stress, melatonin application, and low nitrogen in leaf. Regarding the phylogenetic tree, two *MGT* genes of Arabidopsis, *AT4G09640* and *AT1G34470*, which were involved in antiviral defense [41], showed a closed relationship with *Cla97C116215350*. Findings suggest that this sub-class of *MGTs* may be an appropriate target group for further molecular breeding to release the watermelon-resistant lines. The specialized expression of *MGT* genes can also be related to their promoter region. These genes appear to be more heavily influenced and induced by the pathways dependent on the phytohormones such as ABA, auxin and MeJA. Moreover, it was revealed that the cell-signal transduction associated with hormone concentrations is induced in response to Mg toxicity/deficiency [42,43]. Moreover, previous studies disclosed that *MGTs* interact with Ca^{2+} sensors to induce the downstream signals correlated with plants' reaction to adverse environmental conditions [44].

4. Materials and Methods

4.1. Identification and Characterization of MGT Genes in C. lanatus, and C. sativus

To identify all sequences related to the MGT family, the MGT proteins of *Arabidopsis thaliana* were used as queries in the BLAST program in Ensembl Plants [45] against the genomes of *C. lanatus* and *C. sativus*. In addition, the orthologue of *MGTs* was identified in *Cucumis melo*,

and *Oryza sativa* was identified in the same way. The non-redundant sequences of MGTs were checked using CDD search [46] and Pfam database [47] to validate the presence of MGT domains. To predict the physicochemical properties, including the instability index, GRAVY, isoelectric points (pI), and molecular weight (MW) of MGTs, the ProtParam tool was applied [48].

4.2. Phylogenetic Analysis of MGTs

The amino acid sequences of MGTs from *C. lanatus, C. sativus, C. melo, Arabidopsis thaliana* (as a model plant from dicots), and *Oryza sativa* (as a model plant from monocots) were used to construct a phylogenetic tree. In the first step, all sequences were aligned using a multiple alignment tool, Clustal-Omega [49]. Then, the output of the Clustal-Omega was submitted to the IQ-TREE webserver [50] to estimate the phylogenetic relationships of MGTs using the Maximum likelihood (ML) method under 1000 bootstrap replicates. In the final step, the phylogenetic tree of MGT proteins was prepared by the interactive tree of life (iTOL version 5) tool [51].

4.3. Prediction of Ka and Ks

To recognize the duplicated genes, the cDNA sequences of *MGT* genes in *C. lanatus* and *C. sativus* were processed by the ClustalX v.21 program [52]. According to the identity matrix, the gene pairs with more than 90% identity were screened as a duplicated gene pairs [53]. In the present study, to understand mutations that affected protein sequencing during the evolutionary process, synonymous substitution (K_S) and nonsynonymous substitution (K_a) were investigated for all paired genes of MGT family in *C. lanatus* and *C. sativus*. Ks, Ka, and Ka/Ks were calculated using TBtools software [54].

4.4. Transmembrane Structure and Pocket Site Analysis of MGTs

To predict the 3D structure and transmembrane structure of MGTs in *C. lanatus* and *C. sativus*, the amino acid sequences were submitted to the Phyre2 server [55], and the predicted models with the highest similarity were selected. The pocket sites of each MGT were identified using the Phyre investigator tool of the Phyre2 server.

4.5. Prediction of Phosphorylation Sites into MGTs

The phosphorylation sites of each MGT protein in *C. lanatus* and *C. sativus* were predicted based on three amino acids, including serine (S), tyrosine (Y), and threonine (T), using the NetPhos 3.1 Server [56]. To predict the sites to a high percentage of confidence, the score was adjusted to scores of more than 0.90.

4.6. Protein-Protein Interaction Network

To construct the protein–protein interaction network between MGTs in *C. sativus*, the sequences of all MGTs were submitted to the STRING v11.5 database [57]. A maximum number of interactors was adjusted to no more than 5 interactors for the first shell and no more than 20 interactors for the second shell. Finally, the interaction networks were illustrated using Cytoscape v3.8.2 [58].

4.7. Promoter Analysis of MGT Genes

To identify the known *cis*-regulatory elements related to the response of hormones and stresses as well as those involved in growth, the upstream region (1500 bp before the start codon) of each *MGT* gene in *C. lanatus* and *C. sativus* was screened by the PlantCARE tool [59]. Finally, *cis*-regulatory elements were grouped according to their functions.

4.8. Gene Expression Profile of MGT Genes

To extract the expression patterns of *MGT* genes in *C. lanatus* and *C. sativus*, the available RNA-seq data from CuGenDBv1 (http://cucurbitgenomics.org/) (accessed on 1 August 2022) were used. Three RNA-seq datasets of different tissues of *C. sativus*, in-

cluding PRJNA80169 (leaf, stem, root, tendril, ovary, female, and male), PRJNA319011 (seed), and PRJNA263870 (phloem of fruit) were analyzed to extract the expression levels of *MGTs*. Moreover, the RNA-seq data of *C. lanatus* related to root tissue (PRJNA209092), 34 days after pollination (DAP) in fruit tissues (PRJNA221197), fruit flesh and fruit rind (SRP012849), seed (PRJNA319011), and phloem and vascular tissues (SRP012853) were used to find out the expression profile of *MGTs*. In addition to understanding the responses of *MGTs* to biotic/abiotic stresses and the exogenous application of hormones/elicitors, the RNA-seq datasets of *C. sativus* related to cold stress after 2 h and 12 h (PRJNA438923), NaCl (PRJNA437579), silica (PRJEB7612), GA at 12 h (PRJNA376073), nematode infection (PRJNA419665), powdery mildew infection (PRJNA321023), and one and two days after infection with *Pseudoperonospora cubensis* (PRJNA285071) and the RNA-seq datasets of *C. lanatus*, including PRJNA326331 (osmotic stress), PRJNA454040 (drought stress), PRJNA389184 (mosaic virus), PRJNA328189 (cold stress and melatonin application), and PRJNA422970 (low nitrogen (N) stress in leaf and root) were used and analyzed. The expression data of *MGTs* were extracted based on FPKM values. The expression profiles of the *MGTs* were illustrated in heatmaps based on log2 transformed method of FPKM + 1 for expression in tissues and the log2 fold change in response to stresses and hormone applications using TBtools software.

5. Conclusions

In the present study, MGT gene family members were identified and analyzed in two candidate species of Cucurbitaceae, *C. sativus* and *C. lanatus*, in a first report. The results reveal that a similar evolutionary process for the MGT gene family members has probably occurred in *C. lanatus* and *C. sativus*, and duplication events between *MGTs* may have occurred before the derivation of these two species. The *NIPAs* class showed great structural diversity and different expression patterns from the *MSR2* and *CorA* groups that should be considered more in future studies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12102253/s1, Table S1. List of the identified *MGT* genes and their characteristics in watermelon *(Citrullus lanatus),* cucumber (*Cucumis sativus),* and melon (*Cucumis melo).* Table S2. List of ligand-binding sites in the predicted 3D structure of MGTs in *C. lanatus*, and *C. sativus*. Table S3. Promoter important *cis* elements engaged in various developmental and stress responsive pathways in *MGT* genes of watermelon and cucumber.

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