Genome-wide computational assay of the Mg transporter (MGT) gene family in rice (Oryza sativa L.)

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Research Article

Keywords: Biometals, Mg2+ ion, MGT/MRS family, Transmembrane transport, Protein structures, Oryza sativa L

Posted Date: June 22nd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1747664/v1

Abstract

One of the critical biometals in the cells, possessing important functions in multiple biochemical pathways, is Magnesium (Mg). The proper Mg homeostasis is regulated by the Mg transporters (MGTs) in the cells. Despite the identification of these genes in various plant species, such as rice, there is no systematic and computational assay of all the existed *MGT* genes in the rice genome. Here, we identified 23 non-redundant *MGTs* across the genome of *Oryza sativa* that were further analyzed according to the protein characteristics, duplication and synteny, protein structures, expression, and co-expression networks. Based on the conserved motifs, the distinguished OsMGTs were classified into three main clades, including MRS, NIPA, and CorA. The α/β patterns in the protein structures were highly similar in the NIPA and MRS members, with the conserved structures in the Mg^{2+}-binding and catalytic regions. The CorA clade-related proteins demonstrated the highest numbers of protein channels with Pro, Ser, Lys, Gly, and Tyr, as the critical binding residues. The expression analysis of *OsMGT* genes in various tissues showed that the MRS and NIPA groups *MGTs* may possess the critical functions during the rice development. Also, most of the up-regulated *OsMGTs* under stimuli exposure were from these clades of Mg transporters. Furthermore, 45 co-expressed genes were predicted for *OsMGTs* that were clustered into the three main groups involved in protein ubiquitination, amino acids/fatty acid degradations, and phenylpropanoid biosynthesis. Our in silico results can be informative for further evolutionary and functional genomics of the *MGT* genes in rice and other relatives.

Introduction

The magnesium (Mg) divalent cations are considered as the frequently found ions in nature that play important roles in the growth and development of plants and animals. The Mg cation has a regulatory function in plant growth, membrane stability, nucleic acid, and protein synthesis, photosynthesis, catalytic activities, and enzyme activation (Chen, et al. 2018; Li, et al. 2008; Mao, et al. 2014). Despite the important role of Mg as a vital nutrient, recent reports demonstrated a reduced content of Mg in the soil utilized in cereal crops cultivation (Senbayram, et al. 2015). The multiple types of silicates found in rock materials have been considered as the Mg source in soil that the substitution of Al^{3+} for Mg^{2+} may modify the soil Mg content (Gransee and Führs 2013). Furthermore, the extreme rainfall can lead to the saturation of acidic soils with some competing factors, such as Al^{3+}, H^{+}, Ca^{2+}, K^{+}, and Mn^{2+}, which can result in diminished Mg availability for the plant roots (Niu, et al. 2014). The Mg cation, as a cofactor necessary for the adjustment of kinase and polymerase enzymes activities (Li, et al. 2001), is important in proteins and nucleic acids production and regulates the ion equilibrium in the cell (Guo, et al. 2016; Hermans, et al. 2013). In addition, a wide range of adverse effects can be created on plant cells because of the Mg deficit that can eventually decrease photosynthesis and plant growth (Peng, et al. 2015). The Mg channels are involved in the adjustment of Mg concentrations in the tonoplast and a large amount of Mg ions accumulate in plant mesophyll tissues with the help of vacuoles (Chen, et al. 2018). Furthermore, the accumulation of Mg in the transmembrane regions of mitochondria and chloroplast had been recently reported (Chen, et al. 2018; Hermans, et al. 2013).

An effective transport network has been adjusted in plant cells to absorb and store Mg (Hermans, et al. 2013). The Mg transporters (MGTs) family comprises three NIPA, MRS, and CorA clades that have a fundamental function during the protection of Mg homeostasis in the plant cells (Li, et al. 2018; Saito, et al. 2013). The MGT-related proteins were extensively investigated and characterized. It was previously reported that MGT is an integral membrane protein with 1 acidic N-terminal periplasmic domain and 3 C-terminal transmembrane domains (Schock, et al. 2000; Smith, et al. 1995). A conserved GMN motif encompassing the Gly-Met-Asn residues can be found at

the hydrophobic region of these family-related proteins, which is predicted to be the catalytic section of the Mg transporters.

The MGT family genes have been distinguished in several plant species, including *Arabidopsis thaliana* (Schock, et al. 2000), *Zea mays* (Li, et al. 2016), *Solanum lycopersicum* (Regon, et al. 2019), *Pyrus* (Zhao, et al. 2018), *Poncirus trifoliate* (Liu, et al. 2019), and *Brassica napus* (Zhang, et al. 2019). The *MGT* genes are usually expressed in the plant's roots and engaged in the absorption, transferring, maintaining ionic homeostasis, and accumulation of Mg within the vacuole of the cell (Li, et al. 2008; Mao, et al. 2014). This gene family members are also important in pollen intine development (Chen, et al. 2009) and illustrated the high expression levels under aluminum toxicity exposure in acidic soils (Chen, et al. 2012). For example, in rice OsMRS2-5 is expressed in tissues other than flag leaves, and OsMRS2-8 is expressed in all tissues but rarely in leaves (Tong, et al. 2020). The Al-resistant genotypes in *Arabidopsis* and maize revealed a significant aptitude in Mg uptake and accumulation (Bose, et al. 2013; Li, et al. 2016; Li, et al. 2001). It was reported that the overexpression of an *Arabidopsis MGT1* in *Nicotiana benthamiana* led to an enhanced level of Mg uptake along with a diminished Al toxicity in transgenic plants (Deng, et al. 2006). It was also suggested that the silenced *MGT1* in rice can induce sensitivity to salt stress and raise the sodium ion concentration in the aerial parts (Chen, et al. 2017; Saito, et al. 2013). Therefore, a comprehensive study and characterizing the MGT family genes can prepare significant information regarding the gene structure, functions, and potential variation. Although only nine *MGT*-related genes have been experimentally studied in rice (Saito, et al. 2013), but there is no systematic assay of all MGT ion transporters in the rice genome. Li, et al. (2020) reported that an RNA-seq analysis showed the transcript abundance of all MGT members in rice was assessed. Also Among the nine MGTs, OsMGT3 showed an extremely high transcript level in shoots, and its transcript level was remarkably suppressed by Mg depletion. In the present study, we conducted a complete recognition and systematic analysis of this gene family members, their chromosomal locations, structural features, phylogenetic and syntenic relationships, and expression patterns in various tissues and under stimuli exposure for unraveling their function during the rice life cycle. Our findings can be helpful for further functional assay of Mg^{2+} transport-related genes in rice and ameliorate our knowledge regarding the activities of these transporters in plants.

Materials And Methods

Identification of Mg transporters in *Oryza sativa*

The HMM (hidden Markov models) profile of the Mg transporter domains, PF05653 and PF01544, was obtained from the Pfam database (http://pfam.xfam.org) (Finn, et al. 2013) and the HMM search (HMMER3.0), with an E^{-10}, was performed for identification of the putative Mg transporter proteins in the *Oryza sativa* genome. The identified Mg transporter proteins were manually checked for the specific domains by utilizing Pfam and SMART (http://smart.embl-heidelberg.de/webcite) (Schultz, et al. 2000) programs. The Ensembl (http://plants.ensembl.org) database have been used for identification of the corresponding DNA sequences and chromosomal location of genes (Bolser, et al. 2017). The physicochemical characteristics of MGT proteins were predicted via the ProtParam server (http://web.expasy.org/protparam) (Gasteiger, et al. 2005), and the possible transmembrane domains were detected through TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) (Krogh, et al. 2001).

Phylogenetic relationships, conserved motifs, and gene structures

The phylogenetic analysis was performed by creating a phylogenetic tree based on the maximum likelihood (ML) method under 1000 bootstrap replicates via MEGAX software (Kumar, et al. 2018) according to the protein

sequences of MGTs from rice, maize, and *Arabidopsis*. The MEME (Multiple Em for Motif Elicitation) program (http://meme-suite.org/tools/meme) (Bailey, et al. 2009) was also utilized for detection of the conserved motifs in MGT proteins. The *MGT* genes structures were predicted as the exon/intron patterns in the CDS sequences as compared with genomic regions through Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/) (Hu, et al. 2014).

Chromosomal map, gene duplication, and *Ka/Ks* substitution rates

The chromosomal localization of *MGT* genes was detected on the 12 chromosomes of rice by using the *O. sativa* genome info from the Ensembl and the MapChart software was eventually employed to generate a graphical chromosomal map (Voorrips 2002). The identified *OsMGT* genes were evaluated for the gene duplication events through an identity matrix between the aligned CDSs and the duplicated pairs were detected as the genes sharing ≥80% identity in their nucleotide sequences. The duplicated *OsMGT* gene pairs were subjected to a ClustalW codon alignment program in MEGAX software and the synonymous (*Ks*) and non-synonymous (*Ka*) substitution values were estimated utilizing the *Ka*/*Ks* Calculator tool (http://code.google.com/p/kaks calculator/wiki/KaKs_Calculator) (Zhang, et al. 2006). The time of duplication and divergence (million years ago) was also estimated through a synonymous mutation rate of λ substitutions per synonymous site per year as $T= [Ks/2\lambda\ (\lambda = 6.5 \times 10^{-9})] \times 10^{-6}$ (Yang, et al. 2008). The comparative synteny relationships of *MGT* genes among the orthologous pairs between rice-maize and rice-*Arabidopsis* were finally visualized at gene levels through Circos software (Krzywinski, et al. 2009).

Gene ontology, post-translational modifications, and promoter analysis

The gene ontology of MGT proteins was annotated by using the CELLO2GO (http://cello.life.nctu.edu.tw/cello2go/) program (Yu, et al. 2006). The post-translational modification rates in MGT proteins were estimated as the potential phosphorylated and N-glycosylated regions via NetPhos 3.1 server (Blom, et al. 2004) with potential value > 0.5 and NetNGlyc 1.0 server (Gupta, et al. 2002), respectively. The conserved *cis*-elements existing in the *OsMGT* promoter area were predicted by subjecting the 2000 bp upstream region of the start codon ATG in each putative *OsMGT* into the PlantCARE server (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot, et al. 2002).

Secondary structures of MGT proteins, 3D modeling, validation, and detection of the pocket sites

The secondary α/β structures and transmembrane helix of each MGT clades were predicted via the SOPMA program (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) (Geourjon and Deléage 1995) and Vadar server (http://vadar.wishartlab.com) (Willard, et al. 2003), respectively. The three-dimensional structures of the Mg transporter proteins from each cluster were predicted through Protein Homology/analogy Recognition Engine V 2.0 (Phyre2) server (http://www.sbg.bio.ic.ac.uk/*phyre2/Html/page.cgi?id=index) (Kelley, et al. 2015). The validation of the protein models performed via Ramachandran Plot Analysis (http://www.mordred.bioc.cam.ac.uk/*rapper/rampage.php) (Lovell, et al. 2003) and the BetaCavity Web server (http://voronoi.hanyang.ac.kr/betacavityweb/) had been utilized for estimation of the proteins molecular voids and pocket/channel regions (Kim, et al. 2015). The calculation of errors and plots in the protein's structure was done through the ProSA server (https://prosa.services.came.sbg.ac.at/prosa.php) (Wiederstein and Sippl 2007).

Expression profiling of *OsMGT* genes and prediction of co-expression networks based on RNA-seq data

The publicly available RNA-seq data related to the *Oryza sativa* cv Nipponbare genome in the Rice Genome Annotation Project database has been used for expression analysis of the Mg transporter family genes in multiple tissues (20 day-old leaves: SRX100757, post-emergence inflorescence: SRX100743, anther: SRX100746, pistil: SRX100747, seeds 5 days after pollination: SRX100749, embryo 25 days after pollination: SRX100753, endosperm 25 days after pollination: SRX100754, 35 days old callus: SRR074137, 90 days old immature panicle: SRR074145, and 2 weeks old roots: SRR074154); also for expression analysis under abiotic stimuli the data related to two weeks old seedlings treated with salinity (250 mM NaCl for 24 h: SRR074149), drought (lack of water for 5 days: SRR074142), and cold (4°C for 24 h: SRR074139) have been extracted from the database. The log2 transformed FPKM values of the expressed genes had been utilized to generate the heatmaps via the TBtools software (Chen, et al. 2020). Furthermore, the co-expression networks related to one candidate *OsMGT* gene from each clades were constructed using the ATTED-II ver 9.0 server (http://atted.jp/) (Obayashi, et al. 2018). The co-expressed genes with Mg transporters were more investigated according to the KEGG data.

Results

Identification of *OsMGT* genes and their characteristics

In the current study, 23 non-redundant putative MGT proteins, including 11 NIPA, 9 MRS, and 3 CorA members, were recognized in the genome of rice (Table 1). The predicted physicochemical characterizes in MGT proteins revealed high diversity. For example, the molecular weight (MW) ranged from 8.50 (in Os11g0197400) to 62.91 kDa (in Os01g0664100). Most of the identified MGT proteins in rice had acidic nature, because of the isoelectric point (pI) values lower than 7, and pI values ranged from 4.61 (in Os04g0430900) to 11.43 (in Os10g0545000). The predicted instability index (II) values suggested that ~48% of the identified MGTs can be classified as stable proteins, because of the values less than 40. Also, the grand average of hydropathicity (GRAVY) of rice MGT proteins ranged from -0.054 (in Os01g0601000 from CorA) to 0.789 (in Os06g0715700 from NIPA). Based on our results, three protein types including NIPA, MRS2, and CorA were recognized in MGT proteins based on the protein-specific domain distribution (Table 1).

Phylogenetic relationships and classification of OsMGTs

The evolutionary study of the MGT proteins classified them into the MRS, CorA, and NIPA clusters, which demonstrated similar motif patterns in each group (Fig. 1 a and b). As an instance, most of the proteins clustered in the MRS group possessed the motifs 5, 6, 7, 9, and 15 in their structures, while motifs 11 and 13 were detected in all CorA proteins, but a composition of motifs 1, 2, 3, 4, and 10 was predicted in the NIPA group members (Fig. 1 a and b) (Table S1). The conserved transmembrane domains and specific GMN motif at the C-terminal region of MGT proteins were detected in Fig. S1. In addition, the exon-intron structure of *OsMGT* genes was similar and specific in each cluster. For example, the NIPA group-related members could be suggested as the exon-rich genes with approximately 9-14 exons, while 3-6 coding exons have been predicted in the structure of the MRS-related genes (Fig. 1c). For further evolutionary assay of *MGT* genes, a phylogenetic tree was constructed using amino acid sequences of MGTs from *Oryza sativa*, *Arabidopsis thaliana*, and *Zea mays*. The analysis result demonstrated that MGT proteins could be clustered in six various groups (Fig. 2). The MRS-related MGTs of these three species were classified into group I, while groups II and III encompassed the CorA proteins; also the NIPA cluster-related members had been grouped into the IV, V, and VI sections. The groups I and VI, both with 32 members, were considered as the largest clades in the phylogenetic tree, while groups IV and III with 3 and 4 members, respectively, had been suggested as the smallest clusters. Moreover, the OsMGTs were present in all groups. According to the phylogeny

analysis, the proteins categorized in CorA and NIPA clades were sub-divided into more detailed sub-groups (Fig. 2). According to our findings, it can be suggested that some of the clustered genes may possess recent evolutionary origins, conferring similar functions in the cells.

Genomic distribution, duplication, and synteny analysis of *OsMGT* genes

All the *OsMGT* gene family members were successfully mapped onto the 9 out of 12 chromosomes of rice with an unequal distribution (Fig. 3). 7 *OsMGT* genes were mapped onto the chromosome 1, while only one *OsMGT* was predicted to be localized on each chromosome 10, 11, and 12. The gene duplication events can generate many genes in various species. Accordingly, 14 segmentally-duplicated/triplicated gene pairs classified in three main groups, including 2, 1, and 11 duplicated gene pairs in MRS, CorA, and NIPA clusters, respectively, were identified in the *OsMGT* gene family and each group has been demonstrated with colored lines, revealing the paralogous pairs (Fig. 4a). The NIPA group has endured the highest duplicated/triplicated events (Table 2). The paralogous duplicated pairs demonstrated the *Ka/Ks* ratios from 0.266 to 0.871, estimated to be occurred between 1.9 to 13.8 million years ago (MYA). The Ka/Ks ratios <1 in duplicated gene pairs from the *OsMGT* family in rice suggested that the genes have been impressed by purifying selection (Table 2). To identify the probable functions of the rice *MGT* genes, the synteny relationships between the rice, maize, and *Arabidopsis* genomes had been also investigated. According to the results, all the *OsMGT* genes showed synteny relationships with their orthologs in the *Arabidopsis* (~59%) and maize (~46%) genomes (Fig. 4b and c). These wide synteny relations at the gene level can demonstrate the close evolutionary relationships and wide rearrangement events of the rice chromosomes during the genome evolution process.

Gene ontology annotation, post-translational modifications, and promoter *cis*-elements

According to the subcellular localization analysis, most of the MGT proteins in rice were localized in the plasma membrane, vacuole, cytoplasm, and intracellular regions, with a fraction of them located into the nuclear, mitochondria, and plastid (Fig. S2). The assessment of the molecular function of *OsMGT* genes demonstrated that 73% of MGT proteins are engaged in Mg ion transmembrane transporter activity, and 27% of them have a function in transports of other metal ions (Fig. S2). Furthermore, the gene ontology annotation regarding the biological process illustrated that most of the OsMGT proteins are involved in ion transmembrane transport (55%), and the remaining can be engaged in the regulation of mRNA processing (20%), cell death (17%), and cellular nitrogen compound metabolic process (8%) (Fig. S2).

The post-transcriptional phosphorylation sites of OsMGT proteins illustrated a wide variety of phosphorylated serine (S) residues along with some changed threonine (T) and tyrosine (Y) sites (Fig. S3a). About 57% of OsMGTs, such as Os01g0601000 and Os01g0664100 from the CorA clade, Os01g0869200, Os04g0430900, Os01g0908500, and Os03g0137700 from the MRS group, and Os02g0518100, Os02g0498300, and Os05t0424800 belonging to the NIPA category, were predicted as the possible highly phosphorylated MGT proteins in rice. The proteins Os11g0197400 (NIPA), Os12g0566400 (CorA), Os05g0513400 (NIPA), and Os04g0501100 (MRS) were estimated to encompass limited phosphorylated residues in their structures after transcription. Also, most of the phosphorylation events have been occurred in the serine and threonine residues (Fig. S3a). As the important post-transcriptional modification regulating stimuli-induced responses, N-glycosylation sites of Mg transporter proteins were also predicted. Except for about 52% of OsMGTs, for example, Os12g0566400 from the CorA clade, Os01g0708300 in the NIPA group, and Os03g0742400 belonging to the MRS category, the other OsMGT proteins demonstrated some potential N-glycosylated residues at one to four sites (Fig. S3b). Based on the results,

Os03g0137700 and Os03g0684400 from the MRS clade were considered as the MGT proteins with many glycosylation sites (2-4 regions), while the other MGTs have been only subjected to one glycosylation during the modification process (Fig. S3b).

In the current assay, the *OsMGTs* promoter regions in the rice genome were investigated to find the putative *cis*-regulatory elements. The results demonstrated several kinds of *cis*-elements for dealing with various phytohormones and abiotic stimulus conditions (Table S2). The promoter common *cis*-elements, such as the core element TATA-box, and CAAT-box, were distinguished in all *OsMGT* genes. The ABRE (abscisic acid responsiveness), and TGACG-motif (Methyl jasmonate responsiveness) factors were predicted as the highly occurred hormone-responding *cis*-elements in approximately all the *OsMGT* promoters. The light-responsive G-Box and Box 4, anaerobic inducible ARE, WORKY binding site W-box-motif, and stress-responsive MYB elements were detected as the other regulatory *cis*-elements frequently distinguished in the *OsMGTs* promoter regions, revealing the significant potential engagement of these ion transporter genes in stimuli coping during the rice life cycle. The MBS (MYB binding site involved in drought-inducibility), LTR (low-temperature responsive), TC-rich repeats (regulating defensive reactions), TGA-element (auxin-responsive), TCA-element (salicylic acid-responsive), and WUN-motif (wounding responsiveness), were identified as the important abiotic/hormone stress-responsive elements significantly predicted in *OsMGT* genes, especially in the NIPA cluster (Table S2). Regarding our findings, various modulating *cis*-elements in dealing with phytohormones and environmental stresses were predicted upstream of the majority of *OsMGT* genes, suggesting the important potential of these ion transporters in rice growth and stress coping.

The secondary structures of the rice Mg transporter proteins

The secondary structures of three types of MGT proteins in rice were predicted using the SOPMA program. The secondary structures of these proteins in rice demonstrated similar patterns in each NIPA, MRS, and CorA cluster, with 23 (in MRS group) to 62% (in NIPA clade) α-helixes, 0 (in NIPA) to 17% (in MRS) β-sheets, 7-24% β-turns (in MRS), and 37 (in NIPA) to 67% (in MRS) coils, (Table 3). The secondary structure α/β patterns in approximately all the OsMGT proteins, especially in the NIPA and MRS clades, were highly similar, illustrating the same functions of these metal ion transporters in the cell. Thus, with significant transmembrane helices (3-9) in the NIPA group proteins as compared with the MRS and CorA clades (0-2), this clade may be effective in rice tolerance to stressful circumstances (Table 3).

The 3D structure modeling, validation, and pocket regions of OsMGT proteins

The MGT protein's three-dimensional structures were predicted under >90% confidence and their potential active sites were also illustrated. The 3D structures of MGT proteins in all MRS, CorA, and NIPA proteins demonstrated a typical frame comprising of various parallel β-turns and α-helixes. According to the results, it can be mentioned that these proteins possess a conserved structure. Despite some determined variations in protein sequences, a significant similarity, especially in the metal ion binding regions and catalytic sites, has been detected in all the MGT proteins. The predicted differences in some regions of these structures can refer to the various roles during transmembrane transport activity under stimuli exposure. Protein structures are exactly associated with the gene functions and also can reflect the phylogenetic relationships. Therefore, although diverse functional groups had been identified in MGTs, it can be proposed that the MGTs from multiple clades share a common catalytic mechanism in ion transmembrane transport and intracellular signaling pathway during stimulus conditions. For assessment of the accuracy and quality of the MGTs 3D models, the Ramachandran plot, as a common analysis

for comparison of the rotational angles of proteins, had been employed. According to the program parameters and models validation, the qualities of the MGTs model varied from 87% to 96%, proposing the reliability of the predicted 3D models and the worthy quality (Table 3). For further assessment of the probable errors within the protein models, the ProSA results demonstrated that in each protein model there were the regions with a significant rate of residues with lowest energy, confirming the modeling quality in various parts of these proteins.

The cavities and channel regions in the protein structure can be involved in the regulation of the protein functions in the cells. In this regard, the highest protein channels were estimated to be present in CorA proteins with 8-23 channel regions (Table 3 and Fig. 5). Also the MRS and NIPA group proteins with 4-13 and 3-15 channels, respectively, demonstrated an approximately similar pattern, proposing a similar function in the cells and during multiple stimuli exposure. Based on the results, it can be mentioned that the evolutionary divergence of OsMGTs can adjust the gene's function during several molecular pathways.

Expression analysis of *OsMGT* genes based on RNA-seq data

The log2-transformed FPKM values obtained from the rice RNA-seq data sets were utilized to comprehend the *OsMGT* genes functions and transcription patterns of them in various tissues/conditions. All of the *OsMGT* genes showed the expression level >3 at least in one of the rice tissues (Fig. 6a). Some *OsMGTs*, such as *Os03g0684400*, *Os03g0742400*, *Os10g0545000* belonging to the MRS clade, and *Os01g0873700*, and *Os01g0882300* from NIPA, demonstrated the remarkable transcription rates in all the rice tissues, illustrating the critical functions of these Mg transporters during the rice development, which may be adjusted through the regulatory *cis*-elements in the promoter of the genes (Table S2). Some of the *OsMGT* genes, such as *Os01g0601000* from the CorA group and *Os06g0715700* from the NIPA clade, revealed a tissue-specific expression pattern in the embryo and anther tissues, respectively. The Mg transporter genes were strongly expressed in the seed, inflorescence, anther, pistil, callus, and root tissues (Fig. 6b). It can be suggested that these *OsMGTs* are involved in multiple cellular functions during various developmental stages in rice.

The transcription magnitudes of the rice *OsMGT* family genes were also investigated in response to multiple abiotic stresses. Six out of 23 *OsMGTs*, including *Os02g0498300*, *Os06g0715700*, *Os01g0708300*, *Os05g0513400* from NIPA, and *Os03g0137700*, and *Os10g0545000* from MRS, were significantly up-regulated in response to all stimuli (Fig. 7a). Also, four Mg transporter genes, including *Os01g0869200*, *Os04g0501100* belonging to the MRS clade, and *Os05g0430700*, and *Os05t0424800* from NIPA, demonstrated the significant down-regulation under stimuli exposure (Fig. 7a). Between the abiotic stimulus conditions, drought stress has induced responses in ~39% of *OsMGTs*, while under salinity and chilling stresses exposure only ~26 and 9% of *OsMGT* genes had been induced, respectively (Fig. 8b). Based on our findings, two *OsMGTs*, namely *Os04g0373000* from NIPA and *Os12g0566400* from CorA, had been recognized as cold-responsive (Fig. 8b-c). Regarding the significant transcript magnitudes of the NIPA and MRS groups-related genes, they can be considered as the multiple stress dealing Mg transporters during the rice life cycle. These potentials in various *OsMGTs* may significantly result from the stress-responsive *cis*-elements present in their promoter regions (Table S2).

Co-expression network of Mg transporters in rice

The *OsMGT* genes' interactions with other rice genes and their roles in the cells have been investigated through a co-expression network. Based on our predictions, a total of 45 genes had been clustered into the five co-expression nodes in the group A to C in the co-expression network of each main cluster of *OsMGTs* (Fig. 8). According to the KEGG ontology, 13, 15, and 17 genes were predicted to play roles in the protein ubiquitination (osa04120), amino

acids (osa00280) and fatty acid (osa00071) degradations, and phenylpropanoid biosynthesis (osa00940) pathways in the co-expression networks, respectively (Table S3). Between the candidate Mg transporter genes, LOC4327682 (Os01g0708300) as a NIPA-related member in the clade A, demonstrated a significant correlation with an E3 ubiquitin-protein ligase RHB1A (LOC4348088), ras-related protein RABA1f (LOC4346744), and ubiquitin-conjugating enzyme E2 4 (LOC4348755), suggesting the important roles in ubiquitin-protein transferase activity and intracellular signal transduction (Table S3). As the other biological pathways modulated by NIPA and its co-expressed genes, protein polyubiquitination, protein phosphorylation, and plant organelle localization can be mentioned that manifest their probable roles in rice growth and stimuli coping. The *OsMGT* gene LOC4327867 (Os01g0664100), as a CorA candidate, had the functional neighbors, such as F-box protein SKIP28 (LOC4345622), acetyl-CoA acetyltransferase (LOC4326136), ABC transporter B family member 25 (LOC4339984), and disease resistance protein PIK6-NP-like (LOC4344770) in the clade B, which have the functions in developmental and stress-responsive pathways. In the co-expression network related to the candidate CorA, there were also the genes involved in transmembrane transport, amino acid catabolic process, and cellular metal ion homeostasis, which clarified the significant roles during various developmental stages. In clade C, the candidate MRS group-related gene LOC107275769 (Os04g0430900), along with the co-expressed genes MDIS1-interacting receptor-like kinase 1 (LOC4328049), peroxidase 27 (LOC4333262), and lipid-transfer protein 2 (LOC4325636) were predicted to be involved in cellular response to oxidative stress and defense response to abiotic stimuli (Fig. 8 and Table S3). Furthermore, the candidate MRS gene showed an interesting interaction with LOC4329975 and LOC4325035 genes, belonging to the AP2-like ethylene-responsive transcription factors, which have the potentials in the ethylene-activated signaling pathway and stress coping.

Discussion

The Mg ions balance in the cells is regulated through the Magnesium transporters (MGTs) that can interact with Ca^{2+} sensors, SnRK2 kinases, and CBLs in the modulation of the downstream genes engaged in coping with stimulus circumstances (Chen, et al. 2018; Manishankar, et al. 2018). This critical gene family has been investigated in various plant species such as *Arabidopsis thaliana* (Li, et al. 2001), *Brassica napus* (Zhang, et al. 2019), tomato (Regon, et al. 2019), *Saccharum spontaneummaize* (Wang, et al. 2019), maize (Li, et al. 2016), pear (Zhao, et al. 2018), and camelina and durum wheat (Faraji, et al. 2021). nine *MGT* genes were also identified in rice (Saito, et al. 2013), but there is no complete genome-wide systematic study of all MGTs in rice. In the current study, the *OsMGT* gene family has been widely investigated through genome data sets. We distinguished 23 non-redundant putative *Mg transporter* genes in the *O. sativa* genome. The conserved C-terminal GMN motif and TM domains, as the functional characteristics of MGT proteins (Smith, et al. 1995), were detected in all proteins. A large number of *MGT* genes, which may be affected stress resistance in plant species (Chen, et al. 2012), has been assumed to be related to ploidy level and genome size (Faraji, et al. 2020). About 61% of the identified OsMGT proteins were predicted to be acidophilic. In addition, the negative GRAVY values in OsMGTs can reveal their hydrophilic nature (Wilkins, et al. 1998). The OsMGTs distribution in various regions of the cell can also adjust the metal/Mg ions hemostasis in the plant cells (Wang, et al. 2019; Zhang, et al. 2019).

According to the evolutionary analysis, the MGTs from rice, maize, and *Arabidopsis* were classified into the six clades, including three NIPA, CorA, and MRS sub-families. The *MGT* family genes from *Arabidopsis* and rice had been previously grouped into the five clusters (Schock, et al. 2000). These genes predicted into the durum wheat and *Camelina sativa* had been also clustered into the eight groups (Faraji, et al. 2021), suggesting more varieties in MGT isoforms. The number of the NIPA clade-related proteins was significantly higher compared to the others,

demonstrating some duplication events that occurred in this clade of MGTs during the evolution process (Li, et al. 2016), which may be effective in stress adaptation of rice. According to the distribution of the conserved motifs, each clade of OsMGTs revealed similar motif patterns, which can confer similar functions and modulate the regulatory systems (Gebert, et al. 2009; Saito, et al. 2013).

The gene duplication events can generate multiple genes in some plant species such as rice, revealing a paleopolyploid origination for this crop (Magadum, et al. 2013). Moreover, the synonymous (*Ks*) and non-synonymous (*Ka*) substitution rates predicted for the duplicated gene pairs had been suggested as a way for estimation of the selection pressure and duplication times (Sheshadri, et al. 2016b). Based on the Ka/Ks ratios < 1 estimated for the duplicated *OsMGT* gene pairs, the genes have been affected by the purifying selection (Zhang, et al. 2006). It was revealed that the genes within a duplicated gene group have similar conserved motifs and may be functionally conserved. It can be mentioned that the conserved functions in genes may be adjusted through the purifying selection (Visser, et al. 2009). The *OsMGT* genes duplication in the rice genome may be considered as a significant ability for the evolutionary novelties. The wide synteny predicted between the genes from rice-maize and rice-*Arabidopsis* may suggest their close evolutionary relationships (Saito, et al. 2013), which may be the result of the chromosomal inversion rearrangement and duplication events (Magadum, et al. 2013). Our results suggested that most of the *OsMGT* genes had a common ancestor and functions with the counterparts from maize and *Arabidopsis*. The information obtained from the comparative synteny can offer an important background for understanding the crops' evolution (Zhao and Schranz 2017).

The biological processes regulated by the *OsMGT* family genes, based on the gene ontology annotation, demonstrated their significant involvement in ion transport, mRNA processing, cell death, and cellular nitrogen compound metabolic process. The enzyme activity in the cells can be significantly modulated by the Mg, as an essential cofactor (Chen, et al. 2018; Gransee and Führs 2013). It was previously reported that an induced mutation in the yeast *MRS2* gene significantly disarranged the RNA splicing, Mg uptake, and ion homeostasis in the cell organelles that eventually led to cell death (Bui, et al. 1999; Piskacek, et al. 2009). The nitrogen metabolisms can be regulated by some enzymes, such as glutamate dehydrogenase, nitrogen reductase, glutamate synthase, urease, and glutamic–oxaloacetic protease, which all act with the presence of Mg ion (Yin, et al. 2009). Therefore, the magnesium-related metabolisms can be significantly adjusted by MGTs through the regulation of the Mg distribution (Guo, et al. 2016; Hermans, et al. 2013).

In eukaryotic organisms, the potential post-translational phosphorylation and N-glycosylation of proteins contain important effects on protein functions (Blom, et al. 2004). According to our results, about 57% of OsMGTs, with most of them from the MRS category, were predicted as the highly phosphorylated MGT proteins especially in the serine and threonine residues. Also, the OsMGT proteins demonstrated some potential N-glycosylated residues at one to four sites, as an example, Os03g0137700 and Os03g0684400 from the MRS clade were considered as the MGT proteins with many glycosylation sites (2–4 regions). It was suggested that the post-translational modifications may alter the protein's stability and general features (Kia-Ki and Martinage 1992). Furthermore, protein phosphorylation can regulate the protein functions, and interactions, as well as modulate the signaling process in the cell (Blom, et al. 2004; Kazanecki, et al. 2007). The post-translational modifications, especially the phosphorylation and N-glycosylation, can importantly regulate the stress-coping aptitudes of genes (Hashiguchi and Komatsu 2016; Snider and Omary 2014), suggesting the stimuli coping ability for the OsMGTs with high rates of phosphorylation and N-glycosylation sites. The genes transcriptional adjustment under multiple stimulus conditions can be also controlled through the regulatory *cis*-elements in the genes promoter region (Sheshadri, et al. 2016a). Based on our predictions, multiple regulatory *cis*-elements responding to various phytohormones and

stresses were illustrated in most of *OsMGT* genes, which can reveal the significant potential of these genes during the various developmental and stimuli coping pathways (Zhang, et al. 2019). The presence of the light-responsive Box 4 and G-Box elements in the promoters, had been reported to be effective in genes regulation by the light signals that eventually induce the genes engaged in defensive pathways (Biłas, et al. 2016).

The protein structure can significantly demonstrate its activities and interactions in the cells (Zhang et al. 2016). According to our results, the secondary structures of OsMGT proteins revealed similar patterns in each NIPA, MRS, and CorA cluster, with 23–62% α-helixes, 0–17% β-sheets, 7–24% β-turns, and 37–67% coils. It was previously reported that in the protein's structure the β-turn and random coil can be engaged in conferring stress endurance (Braun, et al. 2013; Fukao 2012), suggesting the probable stimuli-coping aptitude of OsMGTs, especially in the MRS and NIPA clades. According to the reports, the proteins' structures in the stress enduring species contain the wide extended strands and transmembrane helix that may be effective in stability under stressful circumstances (Modarresi, et al. 2013). According to Faraji et al. (Faraji, et al. 2021), a highly similar secondary structure pattern had been predicted for the MGT proteins from the candidate species, which may demonstrate the similar functions of these proteins under multiple developmental processes and stimulus situations in various monocot and dicot plants. Furthermore, the 3D structure of OsMGT proteins demonstrated the conserved three-dimensional frame possessing multiple parallel α-helixes in each MRS, NIPA, and CorA clades. Similar to the previous reports (Faraji, et al. 2021), despite some diversities in protein sequences, an interesting similarity was found in the Mg-binding and catalytic regions of the OsMGT proteins. The variations in the proteins' structures can demonstrate the various roles during the regulation of cellular transport under stimuli exposure. Based on the 3D structure and GO annotation of MGT proteins and comparison of those with various species it can be suggested that they share a common catalytic mechanism in ion transport and mRNA processing. The cavities and channel regions in the protein structures have been reported to be involved in the adjustment of protein binding specificity and functions (Braun, et al. 2013; Fukao 2012). In accordance with the previous studies (Faraji, et al. 2021), the CorA-related proteins had the highest numbers of protein cavities. It has been reported that the similar structures in cavity and channel regions in MGT proteins can induce them to function similarly during different situations (Braun, et al. 2013; Fukao 2012)

The *OsMGT* family genes revealed different transcription patterns in multiple tissues and under various stimuli exposure. Some *OsMGTs*, belonging to the MRS (such as *Os03g0684400*) and NIPA (such as *Os01g0873700*) clades, demonstrated the remarkable transcription rates in all the rice tissues, illustrating the critical functions of these Mg transporters during the rice development (Chen, et al. 2009; Chen, et al. 2017; Saito, et al. 2013). Some of the *OsMGT* genes from the CorA (*Os01g0601000*) and NIPA (*Os06g0715700*) clades, also revealed a tissue-specific expression pattern in the embryo and anther tissues, suggesting their significant involvement in the development of tissues and transportation of Mg ion to these parts (Chen, et al. 2009; Gebert, et al. 2009). The Mg transporter genes were strongly expressed in the seed, inflorescence, anther, pistil, callus, and root tissues, thus it can be suggested that these *OsMGTs* are involved in multiple cellular functions during various developmental stages in rice (Saito, et al. 2013). It was previously reported that *MGT* genes can significantly regulate the ion balance under stimuli (Hermans, et al. 2013). In the current assay, six out of 23 *OsMGTs*, including four genes from NIPA (*Os11g0197400, Os06g0715700, Os01g0708300, and Os05g0513400*), and two from MRS (*Os03g0137700* and *Os10g0545000*), were significantly up-regulated in response to all stimuli. About 39% of *OsMGTs* demonstrated an induced expression in response to drought stress, while ~ 26 and 9% of *OsMGT* genes had been induced under salinity and cold stresses, respectively. Regarding the significant transcript magnitudes of the NIPA and MRS groups-related genes, they can be considered as the multiple stress dealing Mg transporters during the rice life cycle. These

potentials in various *OsMGTs* may be significantly adjusted by the stress-responsive *cis*-elements present in the promoter regions (Sheshadri, et al. 2016a). These genes can be offered as the candidates to enhance the stress endurance in rice. It was reported that the Mg deficiency can induce oxidative stress and stomata closing, which may decrease the transpiration of plant genes (Chen, et al. 2017; Kobayashi and Tanoi 2015). Moreover, it was reported that the *MGTs* can adjust the water use efficiency and drought resistance through the regulation of antioxidants enzymes, photosynthesis, and stomatal closure (Niu, et al. 2014; Tränkner, et al. 2016). It was previously suggested that the *OsMGT1* gene can significantly induce the *OsHKT1* activity that can reduce the excessive Na^+ in the rice tissues and improve endurance under salinity exposure (Chen, et al. 2017). Our RNA-seq-based expression analysis demonstrated that the *OsMGT* genes with various expression magnitudes in multiple tissues and under stimulus conditions can be engaged in Mg transporting system and multiple cellular pathways (Chen, et al. 2017; Saito, et al. 2013).

The co-expression networks predicted for various genes can prepare valuable insights regarding the potential interactions and various developmental and stress-responsive pathways in the cells (Hansen, et al. 2014). Based on our predictions, a total of 45 co-expressed genes clustered into the five co-expression nodes in the three main groups have been predicted for the candidate *OsMGTs*. According to the KEGG ontology, the co-expression network predicted for the rice NIPA gene plays a role in the protein transport, ubiquitination, and phosphorylation, which can be adjusted through the ras-related protein RABA1f, E3 ubiquitin-protein ligase, and serine/threonine-protein kinase SAPK9-like genes present in the cluster A (Dey, et al. 2016; Mazzucotelli, et al. 2006; Saito and Ueda 2009). The serine/threonine-protein kinase co-expressed with the rice NIPA-related *MGT* gene has also a role in intracellular signal transduction that can induce the stress responses under stimuli exposure (Dey, et al. 2016). The presence of the myosin-11 gene in the NIPA-related network may also illustrate the potential for organelle localization and vesicle transport (Ueda, et al. 2015). The predicted co-expression network of the rice CorA-related *MGT* gene demonstrated its correlation with the amino acids and fatty acid degradations. The co-expressed F-box protein SKIP28, acetyl-CoA acetyltransferase, and disease resistance protein PIK6-NP-like in the clade B suggested the critical activities of this co-expression network in the regulation of various developmental and stimuli coping pathways (Nazareno 2021; Risseeuw, et al. 2003). For example, it was reported that the PIK6-NP-like and aldehyde dehydrogenase 7 activities are important for the adjustment of the proline catabolic process and elimination of the reactive oxygen species under stress conditions (Nazareno 2021). According to the KEGG ontology, the co-expression network predicted for the rice MRS-related *MGT* gene plays an important function in the phenylpropanoid biosynthesis pathways. The candidate MRS group-related gene in the cluster C was co-expressed with the genes MDIS1-interacting receptor-like kinase 1, peroxidase, AP2-like ethylene-responsive transcription factors, and lipid-transfer protein 2 that were reported to be engaged in cellular response to oxidative stress, ethylene-activated signaling pathway, and defense response to various biotic and abiotic stimuli (Amako, et al. 1994; Faraji, et al. 2020; Trihemasava, et al. 2020). In the co-expression network related to the candidate *OsMGT* genes, there were also the genes involved in transmembrane transport, and regulation of the cellular metal ion homeostasis, which clarified the significant roles of these genes and their networks in the modulation of various developmental stages and stimuli coping during the rice life cycle.

Conclusion

In the current study, a complete assay has been performed to identify all the *OsMGT* family genes into the *O. sativa* genome and the diversities in the proteins characteristics, structures, and expression patterns have been systematically demonstrated. Our findings illustrated various aspects of the structure and function of *OsMGT*

genes in rice, which can be helpful for further insights into the signaling pathways and regulatory mechanisms adjusted by *MGTs* that may be utilized in the future functional genomics studies of the *MGT* members.

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests: The authors declare no competing interests

Funding :The authors did not receive support from any organization for the submitted work.

Author Contribution: Babaeian jelodar, Bagheri, Nemtzade Gharakhili, Hashemi-Petroudi and Mohahamdi designed the experiments. Mohamadi performed the experiments. Hashemi-Petroudi and Mohamadi analyzed the data. Mohamadi wrote the manuscript. All authors have read and approved the final manuscript.

Acknowledgements: The authors would like to thank the anonymous reviewers for their constructive comments and suggestions. We thank the patients and their families who participated in this study.

Authors' information: Not applicable

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Tables

Table 1 is available in the Supplementary Files section

Table 2

Duplication assay of the *MGT* gene family members in rice

Cluster	Gene 1	Gene 2	Ka	Ks	Ka/Ks	Divergence time (MYA)
MRS	*Os04g0501100*	*Os06g0650800*	0.054	0.062	0.871910807	4.7
	Os01g0869200	*Os01g0908500*	0.055	0.093	0.587627284	7.1
CorA	*Os01g0601000*	*Os01g0664100*	0.048	0.180	0.266199107	13.8
NIPA	*Os01g0873700*	*Os05g0513400*	0.051	0.090	0.568856024	6.8
	Os01g0873700	*Os05g0430700*	0.024	0.045	0.524462422	3.4
	Os01g0873700	*Os06g0715700*	0.050	0.063	0.795362257	4.8
	Os05g0513400	*Os05g0430700*	0.043	0.150	0.284059591	11.5
	Os05g0513400	*Os06g0715700*	0.050	0.119	0.421477465	9.1
	Os01g0882300	*Os05g0424800*	0.021	0.025	0.853886139	1.9
	Os01g0882300	*Os05g0513400*	0.051	0.127	0.398742168	9.7
	Os01g0882300	*Os04g0373000*	0.047	0.057	0.829117525	4.4
	Os05g0424800	*Os05g0513400*	0.050	0.128	0.387260509	9.8
	Os05g0424800	*Os04g0373000*	0.045	0.058	0.78902249	4.3
	Os04g0373000	*Os02g0498300*	0.022	0.048	0.451927773	3.7

Table 3

Properties of the secondary and tertiary structures of the rice Mg transporter proteins, their validation and channel numbers

Group	Protein ID	α-Helixes (%)	β-Sheets (%)	Coils (%)	Turns (%)	TM helix (%)	Channel number	Ramachandran plot (%)	z-values
NIPA	Os01g0873700	45	0	53	16	9	8	92	-4.33
	Os01g0882300	50	1	48	14	9	6	87	-3.66
	Os01g0708300	47	0	52	10	4	6	95	-0.85
	Os02g0518100	20	5	74	19	9	5	91	-5.77
	Os02g0498300	33	5	60	19	8	6	90	-2.02
	Os04g0373000	58	0	41	13	8	8	95	-2.79
	Os05g0430700	48	3	48	11	9	8	91	-3.21
	Os05t0424800	50	0	48	18	9	8	93	-5.03
	Os05g0513400	62	0	37	15	3	14	90	-3.09
	Os06g0715700	53	3	43	12	9	3	96	-3.19
	Os11g0197400	56	0	43	11	1	15	96	-2.79
MRS	Os01g0869200	31	5	62	20	2	8	89	-2.06
	Os01g0908500	40	9	50	21	2	6	92	-5.08
	Os03g0742400	48	2	48	24	2	4	96	-3.87
	Os03g0684400	33	11	54	7	2	7	90	-5.55
	Os03g0137700	23	14	62	20	2	9	88	-0.3
	Os04g0501100	31	17	51	5	0	7	94	-5.29
	Os04g0430900	24	8	67	14	2	5	92	-3.01
	Os06g0650800	27	7	65	11	2	9	92	-4.88
	Os10g0545000	48	9	41	8	0	13	90	-6.81
CorA	Os01g0601000	36	10	52	18	2	8	93	-3.25
	Os01g0664100	44	10	45	12	2	23	89	-3.21
	Os12g0566400	53	7	39	16	2	17	92	-4.25

Figures

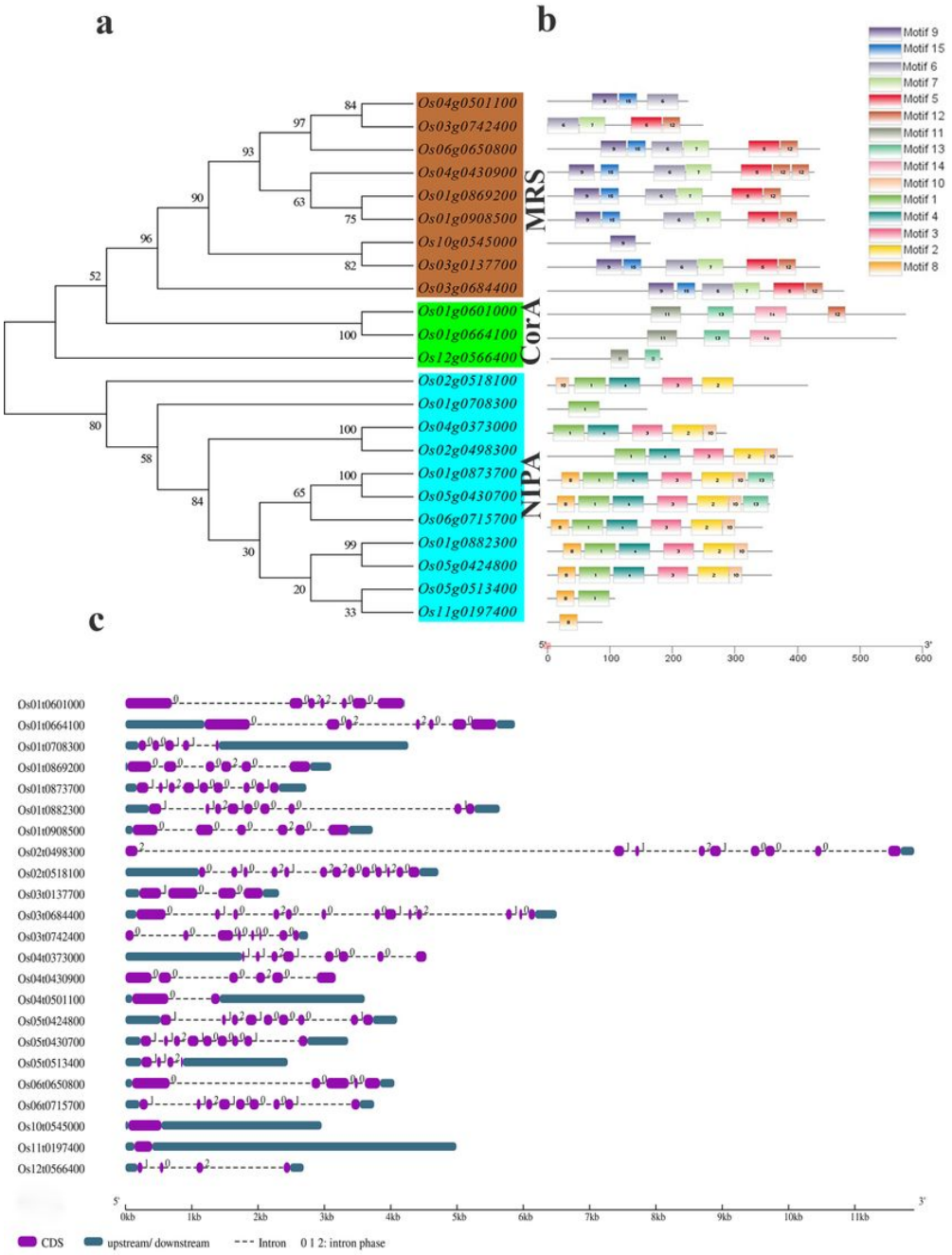

Figure 1

a: The phylogenetic relationships of the OsMGT proteins revealed three main clades of MRS, CorA, and NIPA. b: The conserved motifs, and c: gene structures predicted in the OsMGT members revealed a similar patterns in each clades.

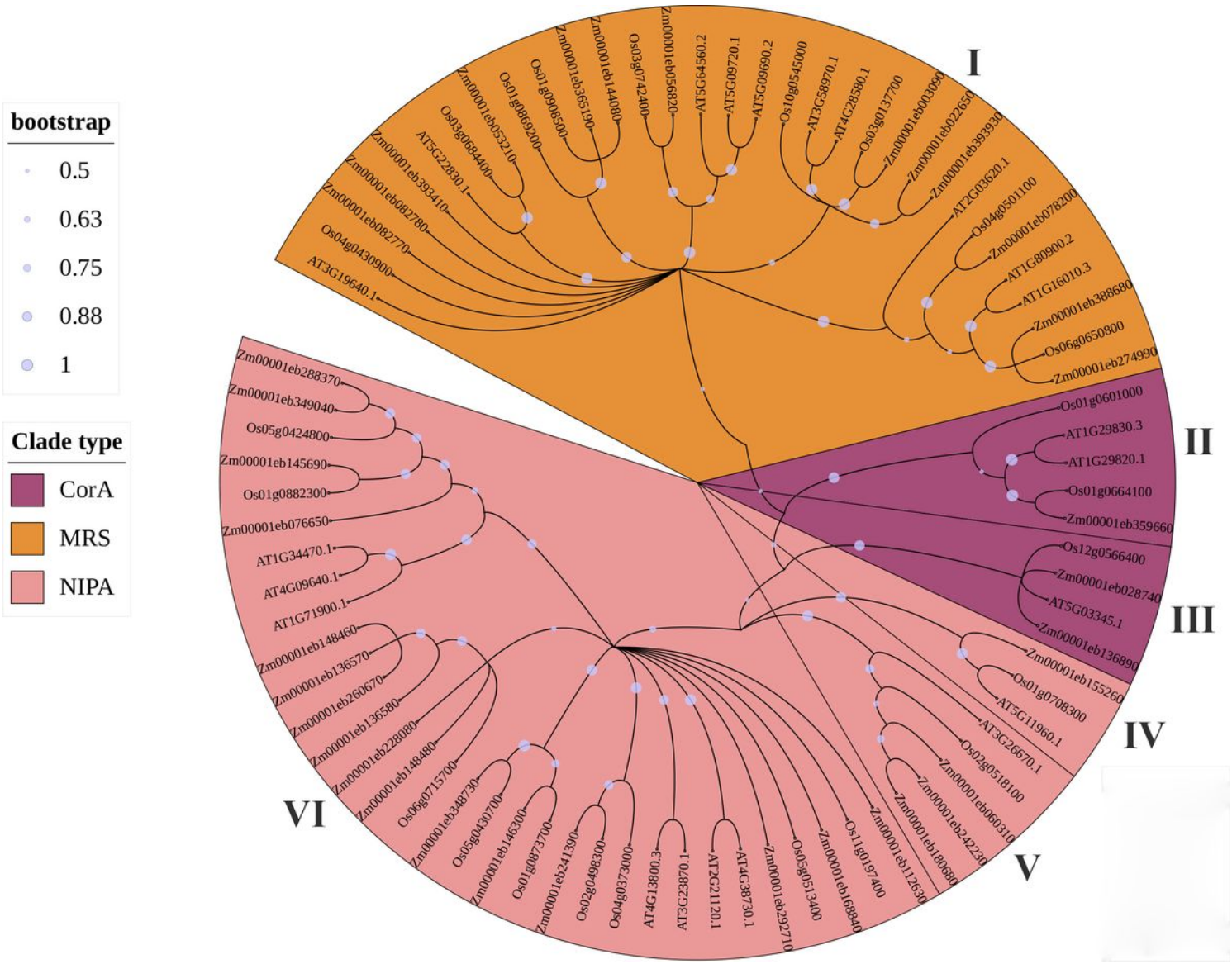

Figure 2

The phylogenetic relationships of MGT proteins from rice, maize, and *Arabidopsis* revealed three main clades of MRS, CorA, and NIPA that were further grouped into the six clusters.

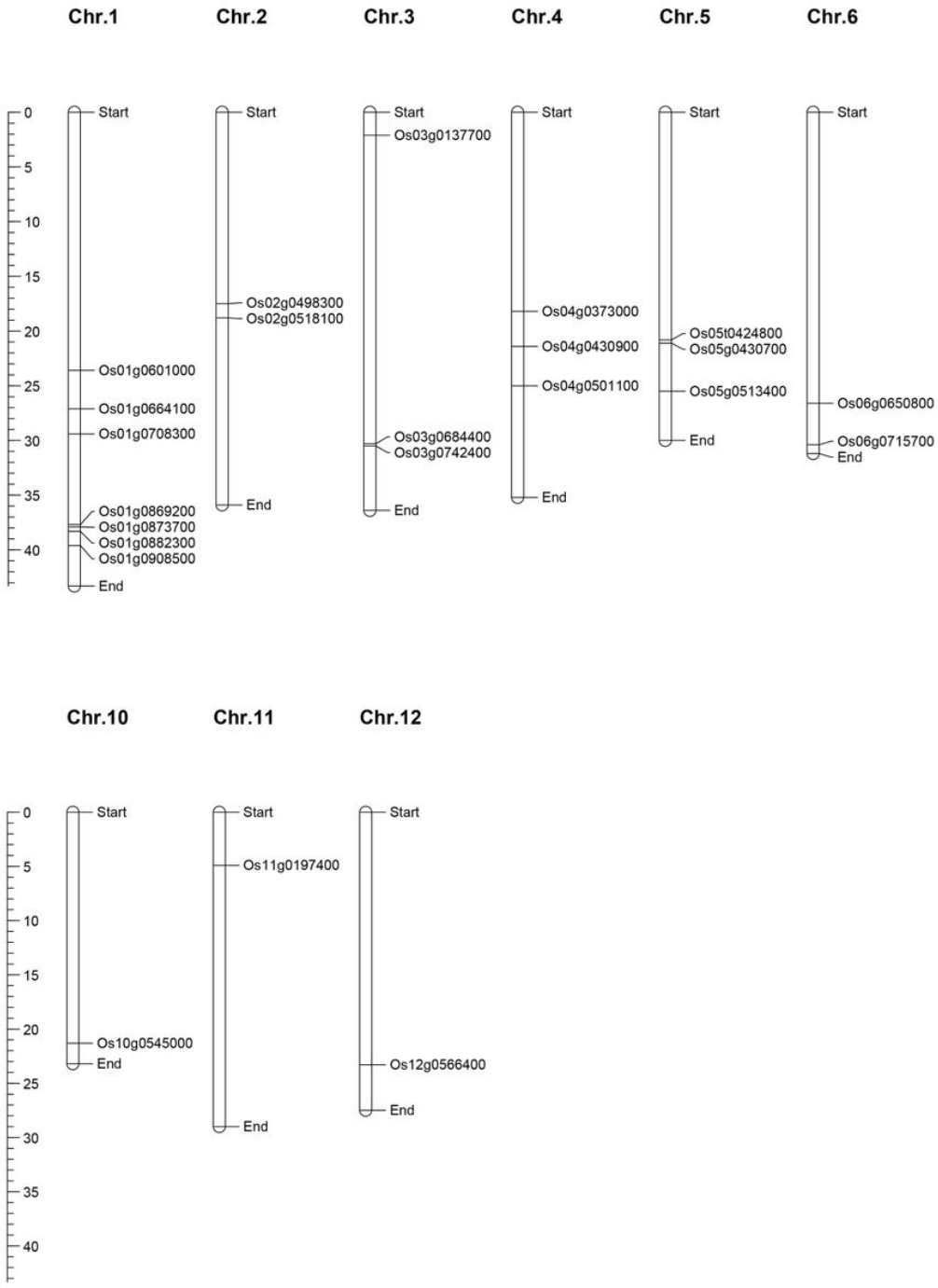

Figure 3

The chromosomal map of the *OsMGT* gene family members demonstrated the highest gene numbers on chromosomes 1, 4, and 5 with 7, 3, and 3 genes, respectively.

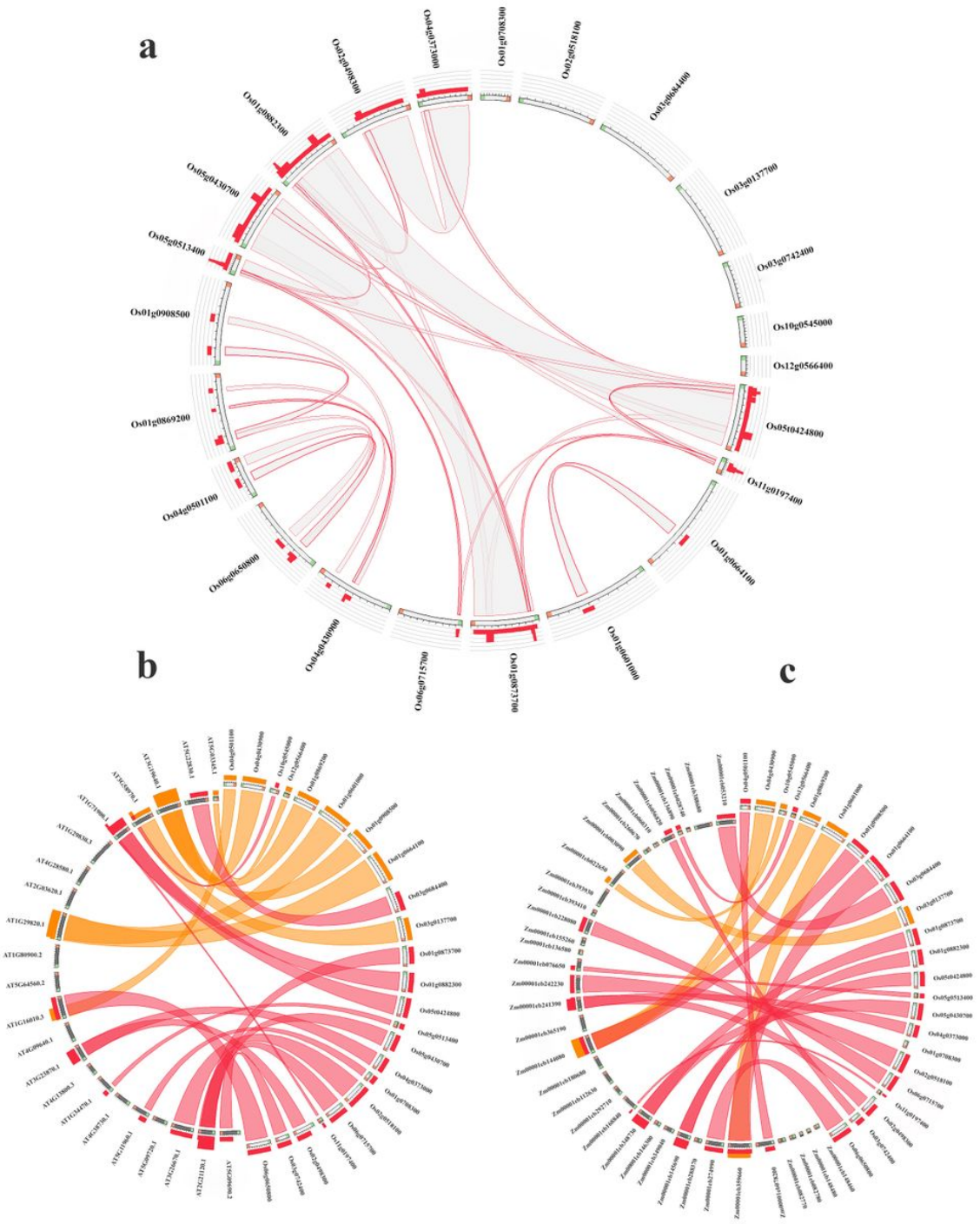

Figure 4

a: Duplication assay of the *OsMGT* genes in the rice genome revealed 14 segmentally-duplicated/triplicated gene pairs. b: The synteny relationships between the *MGT* genes from rice-*Arabidopsis* (~59%) and c: rice-maize (~46%) showed the high (in red) and low (in orange) strength syntenic blocks.

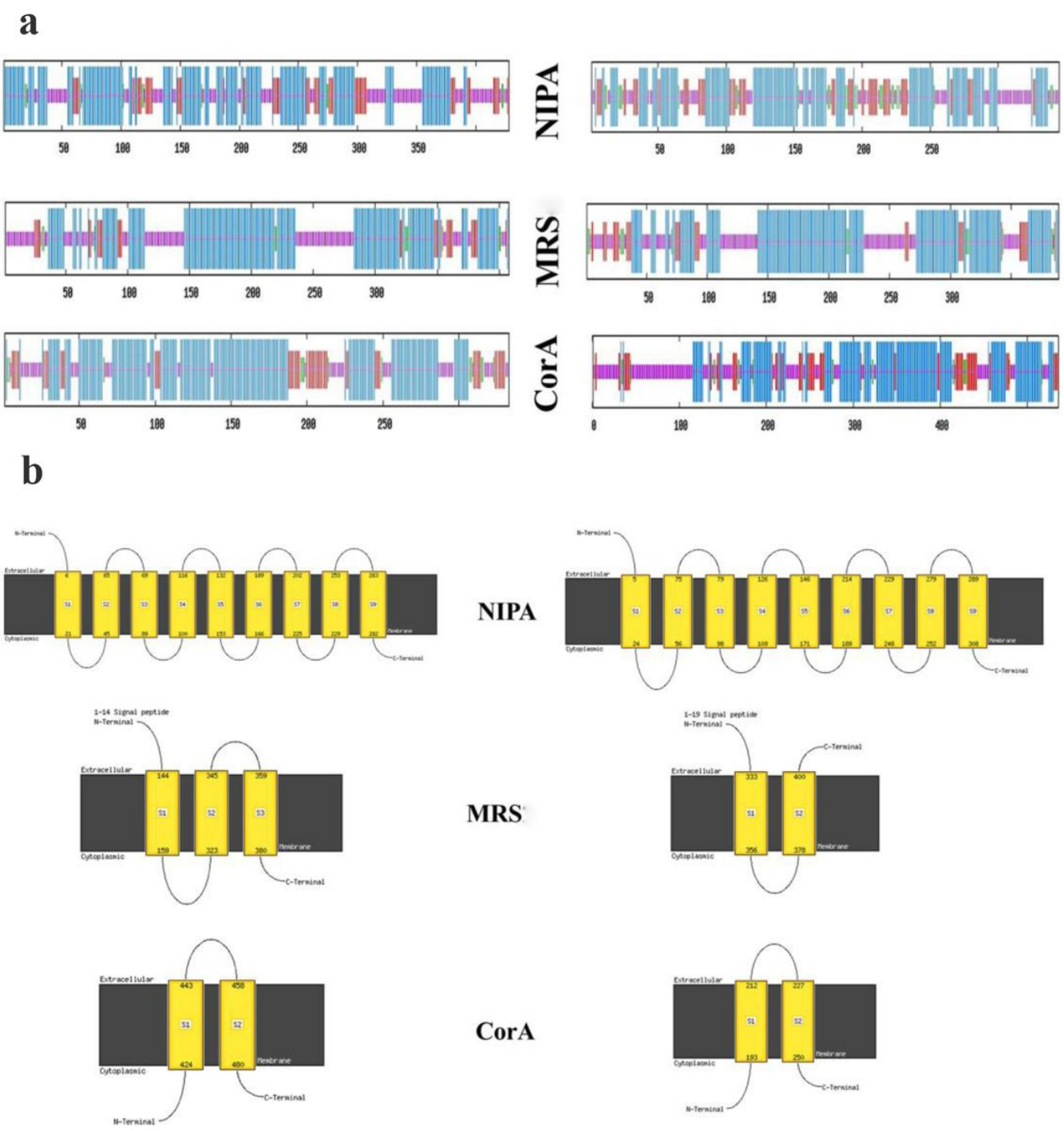

Figure 5

The protein channel regions predicted in the OsMGT proteins from various clades. The CorA proteins revealed the most channel regions that may clarify their significant functions during various conditions.

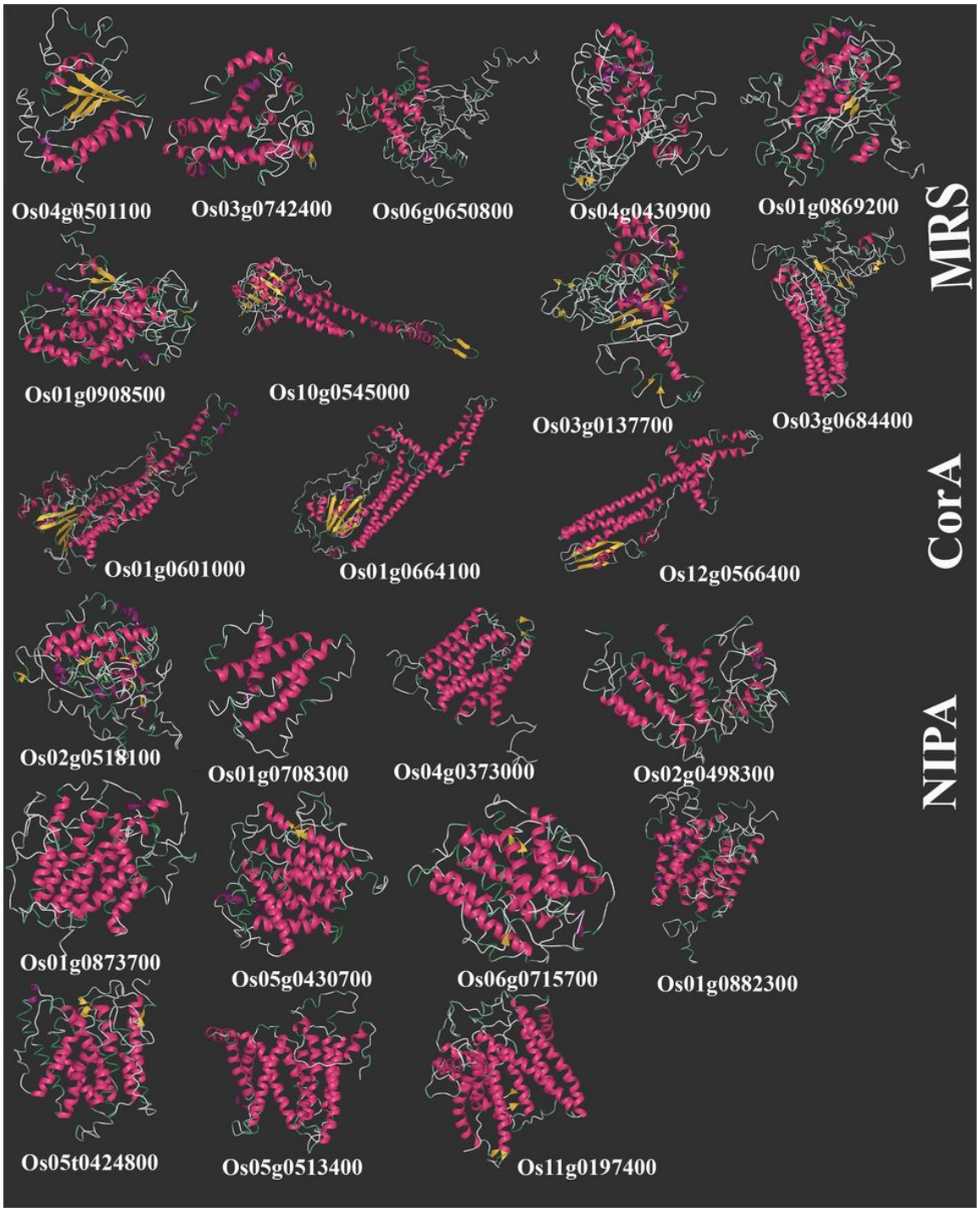

Figure 6

a: The tissue-specific expression patterns of the *OsMGT* genes and b: the number of expressed genes in each tissue. The highest number of *OsMGT* genes were expressed in seed, inflorescence, anther, pistil, and callus tissues.

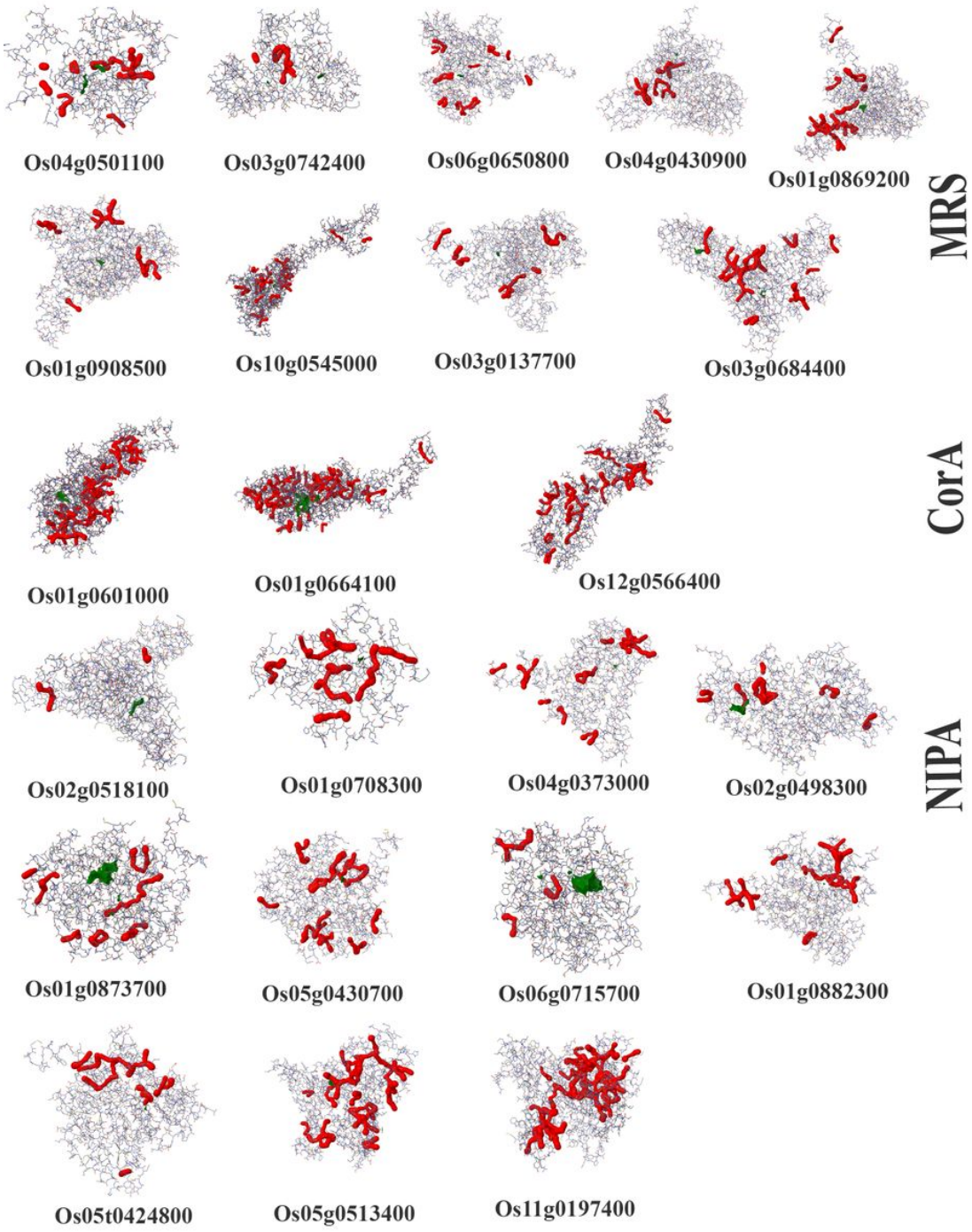

Figure 7

a: The transcription pattern of the *OsMGT* genes under stimulus conditions and b: the number of up and down-regulated genes under each abiotic stresses. c: The venn diagram of the expressed *OsMGTs* revealed six genes induced under multiple stresses. All expression magnitudes were extracted from the Rice Genome Annotation Project database.

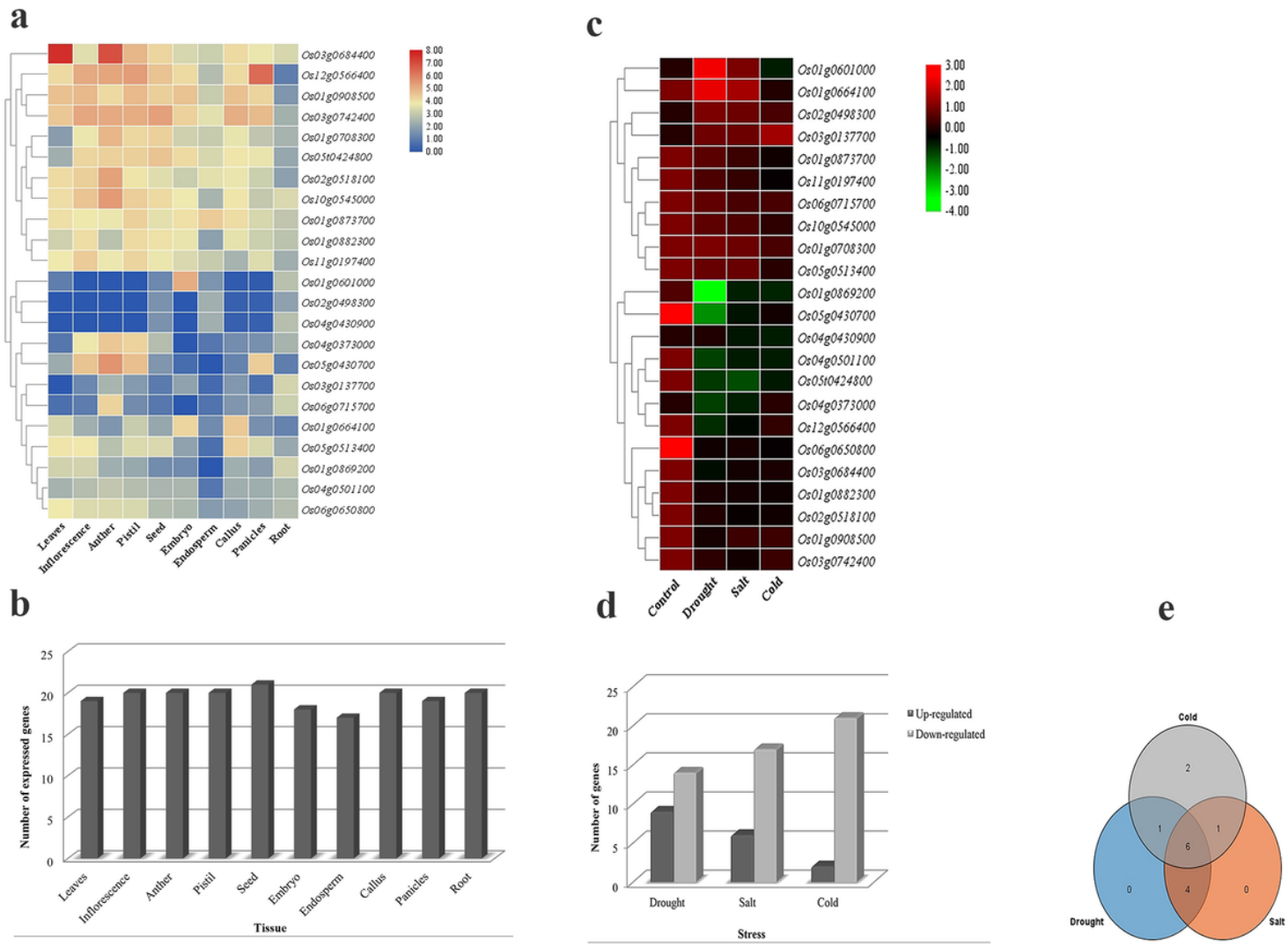

Figure 8

The co-expression networks related to the *OsMGT* genes from various clades demonstrated the important roles in ubiquitin-protein transferase activity for NIPA, the functions in developmental and stress-responsive pathways for CorA, and involvement in cellular response to oxidative stress for MRS group-related genes.

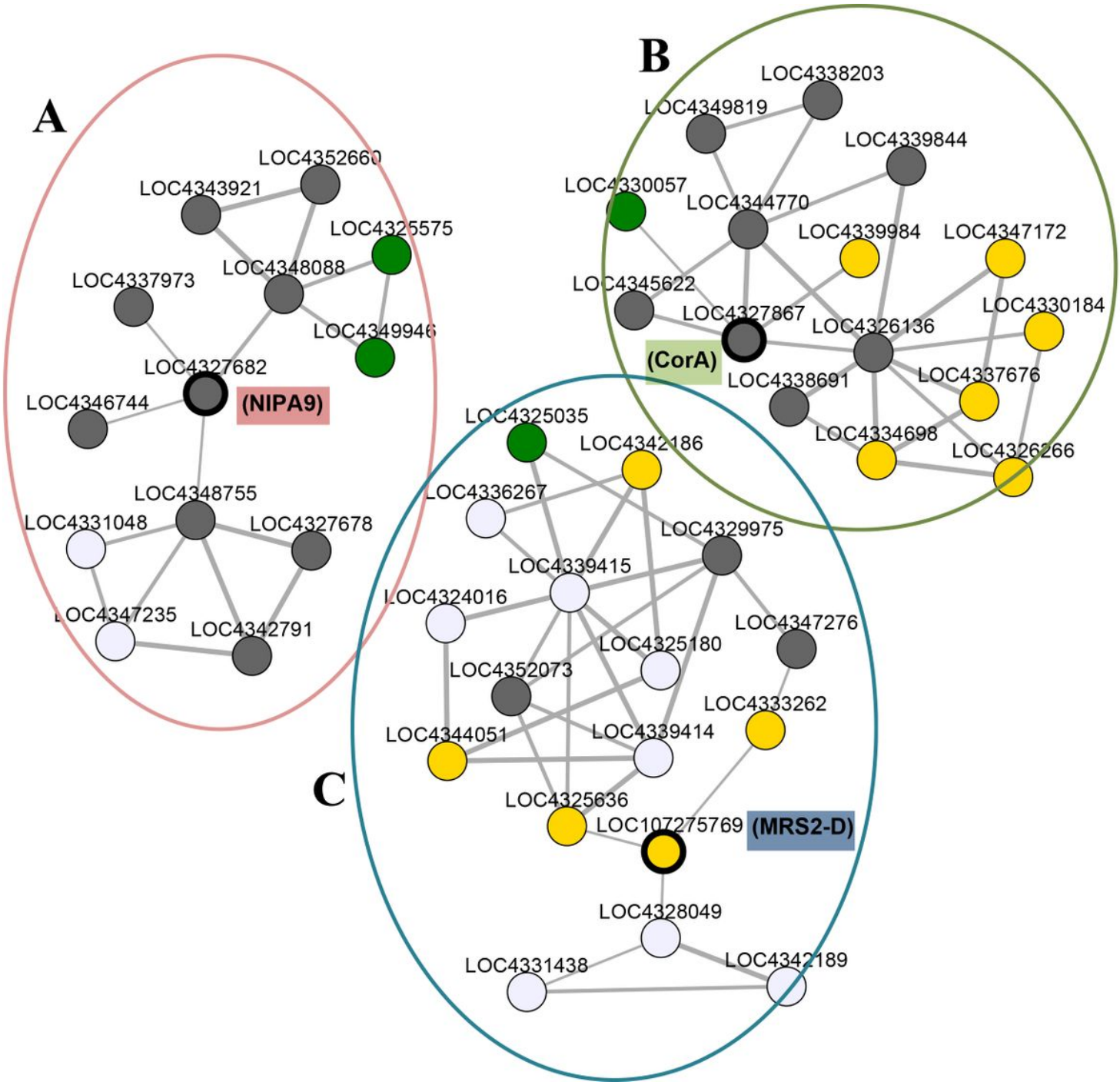

Figure 9

Legend not included with this version

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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- TableS2.xlsx

- TableS3.xlsx
- Fig.S1.jpg
- Fig.A.jpg
- Fig.B.jpg