Genome-wide Identification and Expression Analysis of Autophagy-related Genes (*ATGs*) in Medicago Truncatula

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Abstract

Background: Autophagy is a highly conserved degradation process of cytoplasmic constituents in eukaryotes. Autophagy is known to be involved in the regulation of plant growth and development, as well as biotic and abiotic stress response. Although autophagy-related genes (ATGs) have been identified and characterized in many plant species, little is known about the autophagy process in *Medicago truncatula*.

Results: In this study, 39 ATGs were identified in *M. truncatula* (MtATGs), and the gene structures and conserved domains of MtATGs were systematically characterized. In addition, many cis-elements which are related to hormone and stress responsiveness were identified in the promoters of MtATGs. Furthermore, phylogenetic analysis and interaction network analysis suggested that the function of MtATGs is evolutionarily conserved in *Arabidopsis* and *M. truncatula*. Gene expression analysis showed that most MtATGs were largely induced during seed development, but repressed by nodulation. Moreover, MtATGs were up-regulated in response to salt and drought stresses.

Conclusion: These results provide a comprehensive overview of the MtATGs, which provided important clues for further functional analysis of autophagy in *M. truncatula*.

Background

Autophagy is an evolutionarily conserved degradation process in eukaryotes, maintaining material and energy homeostasis through recycling damaged cytoplasmic constituents and unwanted cellular materials [1]. In *Arabidopsis*, more than 30 autophagy-related genes (ATGs) have been identified via homology-based cloning of yeast ATGs [2, 3]. They are involved in different stages of autophagosome formation, including phagophore induction, cargo capture, vesicle expansion and closure, and the delivery of the vesicles to the vacuole [4]. ATG proteins are functionally classified into four core functional groups, including the ATG1 kinase complex, PI3K complex, ATG9 recycling complex, and two ubiquitin-like conjugation systems [5]. To date, ATG genes have been characterized in many plant species, including *Arabidopsis thaliana*, rice (*Oryza sativa*), maize (*Zea mays*), tobacco (*Nicotiana tabacum*), and wheat (*Triticum aestivum*) [6-9].

Accumulating studies have indicated that autophagy is broadly involved in plant growth and development. It has been reported that autophagy-defective mutants show accelerated leaf senescence in Arabidopsis [10]. The *Osatg7* mutant showed complete sporophytic male sterility and reduced pollen germination activity, suggesting that autophagy plays critical roles in pollen development in rice [11]. Increasing evidence has highlighted the crucial role of autophagy in starch and lipid metabolism in plants [12, 13]. Moreover, autophagy, as a quality control mechanism, mediates the degradation of cellular components and contributes to cellular homeostasis, which is necessary for plants to survive various abiotic and biotic stresses, such as nutrient deficiencies, heat, hypoxia, salt, and drought stresses [14-18].

M. truncatula is a model plant for genetic research of legume plants that interact with rhizobia to develop nodules for nitrogen fixation [19-21]. Symbiotic nodulation is an adaptive strategy of legume plants for acclimatization to nitrogen-starved conditions [22, 23]. Autophagy is also an important mechanism in plants for surviving nitrogen deficiency in plants [24]. Our ability to study the crosstalk between autophagy and nodulation in legume plants, however, has thus far been limited by the lack of knowledge regarding autophagy mechanisms in *M. truncatula*. To facilitate our understanding of the mechanism and function of autophagy in *M. truncatula*, it is necessary to first identify all the *M. truncatula* ATGs (MtATGs). Based on the complete genome sequence of *M. truncatula* [25, 26], MtATGs were comprehensively described in this study, including their genome-wide identifications, and characterizations of their chromosomal locations, evolutionary relationships, cis-elements, gene and protein structures, and protein-protein interaction. In addition, the results of MtATG expression analysis suggested that autophagy plays vital roles in nodulation, seed development, and salt and drought stress responses.

Results

Genome-wide identification of ATGs in *M. truncatula*

To identify ATGs in *M. truncatula*, the BLASTP algorithm was employed in searching against *M. truncatula* proteome sequences (MedtrA17_4.0) using the amino acid sequences of *Arabidopsis thaliana* ATGs (AtATGs) as queries. A total of 39 MtATGs were identified in *M. truncatula* (Table S1). The lengths of the MtATG proteins ranged from 62 amino acids to 3768 amino acids. Most of the MtATGs (MtATG2, MtATG3, MtATG4, MtATG5, MtATG6, MtATG7, MtATG10, MtATG11, MtATG12, MtATG101, MtVPS15, and MtVPS34) contained a single member. A few of them (MtATG1, MtATG8, MtATG9, MtATG13, MtATG16, and MtATG18) contained multiple members, ranging from two to eight in different groups (three in MtATG1 family, eight in MtATG8 family, two in MtATG9 family, three in MtATG13 family, three in MtATG16 family, and eight in MtATG18 family) (Table S1).

The chromosomal distribution of *MtATGs* was displayed using TBtools software (Fig. 1). In total, 38 *MtATGs* were distributed across all eight chromosomes except for MtATG7, which did not mapped to any chromosome according to data from MedtrA17_4.0 (Fig. 1). The number of *MtATGs* located on each chromosome varies dramatically. Chromosome 4 (Chr4) contained the largest number of *MtATGs* with 11 genes, whereas the fewest was found on chromosome 6, which had only one *MtATG* gene. Gene duplication is important for plant adaptation to adverse and complex environments. In *M. truncatula*, 7 pairs of MtATG genes were predicted to be segmentally duplicated genes. As shown in Figure 1, these 7 pairs of duplicated MtATG genes (*MtATG8c* and *MtATG8d*, *MtATG8g* and *MtATG8f*, *MtATG9a* and *MtATG9b*, *MtATG13b* and *MtATG13c*, *MtATG16a* and *MtATG16b*, *MtATG18a* and *MtATG18c*, and *MtATG18d* and *MtATG18c*) are distributed across chromosomes 1, 2, 3, 4, 7, and 8. These duplications may have led to the expansion of MtATG gene families in *M. truncatula*.

Phylogenetic analysis of MtATGs

To evaluate the evolutionary relationships of MtATGs, we conducted phylogenetic analyses using the amino acid sequences of multi-member subfamilies (MtATG1s, MtATG8s, MtATG9s, MtATG13s, MtATG16s, and MtATG18s) and their orthologs from *Arabidopsis*. As shown in Figure 2, MtATG1 and MtATG13 family members were both clustered in two branches (Fig. 2a, b). *M. truncatula* consists of multiple members in ATG9 and ATG16 families, whereas *Arabidopsis* has only one member. In addition, members of MtATG9 and MtATG16 families were similar and clustered together, while AtATG9 and AtATG16 were clustered in different branches (Fig. 2c, d). ATG8 plays a central role in autophagy by promoting autophagosome formation and cargo recruitment. As in *Arabidopsis*, eight MtATG8 members were clustered into two distinct groups in *M. truncatula*; MtATG8a, MtATG8b, MtATG8c, MtATG8d, and MtATG8e were grouped into clade I, whereas MtATG8f, MtATG8g, and MtATG8h were clustered in clade II (Fig. 2e). MtATG8 proteins showed high identity with ATG8 proteins from *Arabidopsis*, except for MtATG8h, in which half of the amino acids were absent in the N-terminus (Fig. S2). The C-terminal glycine residue of ATG8, which is exposed by ATG4 protease cleavage, is essential for the conjugation of ATG8 to phosphatidylethanolamine (PE) [27]. However, MtATG8b did not contain the C-terminal glycine residue. This result indicated that MtATG8b might function in other biological processes independent of the autophagy pathway. In addition, one MtATG8 member of clade II, MtATG8f, had a C-terminal extension after the Gly residue, while the AtATG8 members of clade II lack the C-terminal extension (Fig. S2). Eight MtATG18 members were also clustered in two branches like MtATG8 family (Fig. 2f). Clade I of MtATG18 family consisted of MtATG18a, MtATG18b, MtATG18c MtATG18d, and MtATG18e, whereas clade II was made up of MtATG18f, MtATG18g, and MtATG18h (Fig. 2f).

Gene structure and conserved domain distribution analysis

Gene structure is closely related to the expression pattern and function divergence of members of the multigene family. Gene structure analysis revealed that all the MtATG genes contained introns, with the exon numbers ranging from 2 to 17 (Fig. 3a). In addition, similar exon-intron patterns and the same number of exons were observed in some ATG subfamilies, such as MtATG1a/b, MtATG8a/c/d/e/f/g, MtATG13a/b/c, MtATG18a/c/d/e, and MtATG18g/h (Fig. 3a). The similar gene structures suggest that functional redundancy exists among these genes. However, differences in exon-intron patterns and exon numbers also existed within some subfamilies, such as MtATG1t, MtATG8b/h, and MtATG18b/f (Fig. 3a).

The conserved domains of MtATGs were detected using the Pfam database [28]. In general, the composition of the conserved domain in MtATGs was comparable to that in *Arabidopsis*. Furthermore, the MtATG members of the same family have similar domains. For example, all three MtATG1 proteins contain a protein kinase domain (Pkinase) at the N-terminal (Fig. 3b). A similar phenomenon was also observed in the MtATG8, MtATG9, and MtATG13 subfamilies. However, exceptions were also found in the MtATG16 and MtATG18 subfamilies. All the MtATG16 family proteins had a C-terminal WD40 domain, but lacked an N-terminal ATG16 domain in MtATG16c (Fig. 3b). MtATG18 proteins contain WD40 domain except for MtATG18b and MtATG18h, but members of clade II (MtATG18f/g/h) had a C-terminal BCAS3 domain that was absent in members of clade I (Fig. 3b). The differences of gene structure and conserved

domains may be related to functional divergence among the different gene products within some MtATG gene families.

Cis-element analysis in the promoter regions of *MtATGs*

Cis-elements regulate genes through interactions with their corresponding transcription factors. To further understand the gene regulation network of MtATGs, cis-elements were identified using the online tool PlantCARE [29]. A total of 92 putative cis-elements were found among *MtATG* promoters (Table S2). Among these, the TATA-box and CAAT-box are the most common cis-elements. Many of the identified cis-elements were involved in hormone responsiveness, such as ABRE (abscisic acid-related), TCA-element (salicylic acid-related), TCCACCT-motif and TGACG-motif (MeJA-related), TGA-element (auxin-related), TATC-box, P-box and GARE-motif (gibberellin-related) (Fig. 4). Among these, cis-elements that respond to MeJA and ABA were the most abundant. In addition, some stress-related elements were mainly related to anaerobic (ARE), defense (STRE and TC-rich repeats), drought (MBS), low temperature (LTR), and wound (WUN-motif) stresses (Fig. 4).

Protein-protein interaction network analysis of MtATGs

To investigate the protein-protein interaction (PPI) between MtATGs, all 39 MtATGs were submitted to STRING (Search Tool for the Retrieval of Interacting Genes database). A total of 22 MtATGs were found to form a complex interaction network that can be divided into four major modules according to the functional classification in *Arabidopsis* (Fig. 5). In the first module, MtATG1a, MtATG11, MtATG101, and three MtATG13 members (MtATG13a, MtATG13b, MtATG13c) interacted with each other and function as the ATG1 kinase complex. The second module consisted of two members of the PI3K complex, MtATG6 and MtVPS34. MtATG2 and six MtATG18 family members (MtATG18a, MtATG18b, MtATG18c, MtATG18f, MtATG18g, and MtATG18h) made up the third module, which played a role in autophagic membrane recruitment. The last module served as a ubiquitin-like conjugation system and is composed of MtATG4, MtATG5, MtATG12, and four MtATG8 members (MtATG8a, MtATG8d, MtATG8f, and MtATG8g). This interaction pattern of MtATGs was similar to that of *Arabidopsis*, suggesting that ATG proteins are likely evolutionarily conserved in *Arabidopsis* and *M. truncatula*.

Gene expression patterns of *MtATGs* during growth and development

To investigate the possible roles of the MtATGs in growth and development, the expression patterns of these genes were determined among different tissues and different developmental stages in seeds and root nodules. *MtATG* microarray data were acquired from the MtGEA database (https://mtgea.noble.org/v3/). All of the MtATG genes were expressed in tested tissues, indicating that autophagy is critical for the growth and development of plants (Fig. 6a). However, MtATG genes showed significantly distinct tissue-specific expression patterns across tissues. Specifically, the expression levels of many MtATG genes, such as *MtATG4*, *MtATG8b*, *MtATG8g*, *MtATG9a*, *MtATG13a*, *MtATG13c*, *MtATG18b*, *MtATG18c*, *MtATG18e*, *MtATG18h*, *MtATG101*, *VPS15*, and *VPS34*, were significantly higher in roots than in other tissues (Fig. 6a). In addition, some MtATG genes (*MtATG1a*, *MtATG1t*, *MtATG2*,

MtATG7, *MtATG9b*, *MtATG10*, and *MtATG18f*) were highly expressed in leaves, whereas other MtATG genes (*MtATG3*, *MtATG8a*, *MtATG8e*, *MtATG8f*, and *MtATG11*) were highly expressed in flowers (Fig. 6a). The results revealed that different MtATG genes might function in different tissues.

Consistent with previous studies in maize and castor bean [7, 30], most MtATG genes were up-regulated during seed development (Fig. 6b). In particular, *MtATG1b*, *MtATG1t*, *MtATG2*, *MtATG3*, *MtATG4*, *MtATG5*, *MtATG6*, *MtATG13a*, and *MtATG18b*, were highly expressed in the late stage of seed development (36 dap) (Fig. 6b). In contrast, A few MtATG genes, including *MtATG7*, *MtATG8b*, *MtATG10*, and *MtATG13a*, were down-regulated after pollination (Fig. 6b). In addition, *MtATG1a* and *MtATG18h* were continuously expressed at low levels at all stages of seed development (Fig. 6b). These results revealed that autophagy is involved in seed development as a conserved pathway in plants.

M. truncatula can develop root nodules through its symbiotic association with rhizobia, which is an important strategy to enhance nitrogen acquisition in legumes [31]. To investigate the role of MtATGs in root nodule symbiosis, we analyzed their expression profiles during nodule development at different days post inoculation (dpi). The expression of most *MtATGs* rapidly decreased at the stages of early lumps (4 dpi), and showed a slight increase in immature nodules (10 dpi), before declining to relatively low expression levels in mature nodules (14 dpi) (Fig. 6c). In the stable nodule organs (28 dpi), the expression of *MtATGs* was even lower. However, some genes, including *MtATG9a*, *MtATG9b*, *MtATG13c* and *MtATG18g*, were found to be most highly expressed at 28 dpi (Fig. 6c). These results indicated that autophagy is inhibited during nodulation.

Gene expression of *MtATGs* in response to abiotic stresses

Previous studies indicated that autophagy is essential for plant response to abiotic stresses, including those of heat, hypoxia, salt and drought [32-35]. To investigate the putative roles of autophagy in response to various abiotic stresses in *M. truncatula*, the expression profiles of MtATGs under salt and drought stresses were analyzed using microarray data from the MtGEA database. As shown in Figure 7, *MtATGs* displayed similar expression patterns in response to salt and drought stresses. Most *MtATGs* were up-regulated after salt and drought treatments, which indicated that autophagy may play a positive role in response to salt/drought stresses. Specifically, 26 of 34 MtATG genes (e.g., *MtATG1t*, *MtATG8d*, *MtATG9a*, and *MtATG18b*, among others) were continuously up-regulated when plants were subjected to drought stress by withholding watering, and the transcripts of *MtATGs* rapidly dropped to their base levels after watering recovery (Fig. 7a). Interestingly, *MtATG8g* showed the opposite trend: the expression level of *MtATG8g* dramatically decreased under drought stress compared to other *MtATGs* (Fig. 7a). Furthermore, two members of the MtATG13 family, *MtATG13b* and *MtATG13c*, were also slightly down-regulated when watering was stopped (Fig. 7a). Similar to drought stress, the expression of most MtATGs was induced under high salinity conditions. Twenty-one of 34 MtATG genes were continuously induced by salt stress (Fig. 7b). However, the expression levels of several MtATG genes (*MtATG5*, *MtATG8c*, *MtATG18c*, and *MtATG18e*) were decreased after NaCl treatment (Fig. 7b). These results indicated that autophagy played an essential role in plant response to salt and drought stresses.

Discussion

In this study, 39 ATGs have been identified in *M. truncatula* (Fig. 1; Table S1). The ATG genes of *M. truncatula* are similar to orthologous genes in *Arabidopsis*. For example, most MtATG8s are similar in length and have identical ATG8 domains (Fig. 3). This result indicates that the autophagy pathway is highly conserved across different plant species. However, members of some MtATG families differ among plant species, such as ATG1, ATG4, ATG8, ATG9, ATG13, and ATG16 families (Fig. 2; Table S1). In addition, MtATG8h showed distinctive gene and protein structures compared with the homologue in *Arabidopsis*, in which half of N-terminal amino acids and UTRs are absent (Fig. 3a; Fig. S2). These results suggest that *M. truncatula* may have species-specific autophagy mechanism. Hence, it is necessary to illustrate the conserved and specific functions of MtATGs in further studies.

Autophagy has been shown to play crucial roles in plant growth and development [4]. In this study, we found that all ATG genes were expressed in the tested tissues of *M. truncatula*, but that their expression levels varied among different tissues, and especially differed over the course of the nodulation process (Fig. 6). Nodulation is a specific process for nitrogen fixation in leguminous plants [36]. A previous study reported that loss of PI3K function severely inhibited nodule formation in *M. truncatula* [37]. In addition, downregulation of ATG6 impaired the symbiosis between rhizobium and *P. vulgaris* [38]. These results revealed that autophagy is required for mycorrhization and nodulation in leguminous plants. On the contrary, silencing of TOR, a negative regulator of plant autophagy, caused a drastic reduction in nodule number in the common bean [39]. Consistent with this, we found that the transcript levels of most MtATGs decreased during nodule development (Fig. 6c). This result suggests a new role of autophagy in the nodulation of leguminous plants. Autophagy plays an important role in plant immunity. On one hand, autophagy protects plants from pathogen infection by limiting the spread of hypersensitive responses to programmed cell death (PCD) [40, 41]. On the other hand, autophagy mediates selective degradation of viral components to promote plant survival [41]. In turn, increasing evidence has shown that pathogens have developed diverse strategies to modulate host autophagy and evade autophagic clearance for their own survival [41, 42]. Therefore, we hypothesized that rhizobium represses gene expression of *MtATGs* to alleviate plant immune response and promote nodule formation, and then improve nitrogen fixation during nodulation.

Seed development consists of embryo morphogenesis and seed maturation [43]. In rice, autophagy has been shown to be involved in the regulation of starch and sugar metabolism during seed maturation [44]. In Norway spruce (*Picea abies*), autophagy is also involved in embryogenesis in which it regulates vacuolar cell death of the embryo suspensor [45]. Furthermore, autophagy plays an important role in microspore embryogenesis in *Brassica napus* [46]. Previous studies have reported that autophagy-defective mutants of *Arabidopsis* and maize exhibited lower seed weights [7, 47]. In the present study, we found that most of the *MtATGs* were induced during seed development and were highly expressed at the late stage of seed development (Fig. 6b), indicating that autophagy is necessary for seed development in *M. truncatula*. Overall, autophagy plays crucial roles in plant growth and development through a pathway that is conserved across different plant species.

Autophagy has been demonstrated to promote plant survival through maintaining cellular homeostasis under stress conditions, including under nutrient starvation, oxidative stress, and drought, salt, and other abiotic stresses [48]. An early study reported that autophagy could be induced by salt and drought stresses. In A. thaliana, the transcriptional level of *ATG18a* was rapidly unregulated by NaCl and mannitol treatments [49]. Furthermore, ATG8a and NBR1 proteins rapidly accumulated after NaCl treatment [50]. In *O. sativa*, the expression levels of OsATG6s were also induced by drought stress [51]. In addition to gene expression, the autophagy-defective plant, *RNAi-AtATG18a*, showed more sensitivity to drought and salt treatment than the wild type [52]. However, overexpression of *MdATG18a* enhances tolerance to drought stress in apple trees [53]. A recent study reported that autophagy improves drought tolerance in *M. truncatula* through degradation of the aquaporin MtPIP2;7, which interacts with the cargo receptor MtCAS31 [54]. Consistent with previous studies, our results revealed that the promoter of many MtATG genes contained the drought-related MBS cis-element (Fig. 4). Furthermore, the transcriptional levels of most of MtATG genes, especially those of the MtATG8 gene family, significantly increased after NaCl treatments (Fig. 7a). Our results suggest that autophagy is involved in the positive regulation of plant resistance to drought and salt stress in *M. truncatula*, and that autophagy plays a conserved role in drought and salt stress response across different plant species.

Conclusions

This study provides a comprehensive overview of the ATGs in *M. truncatula*. A total of 39 MtATGs were identified, and their chromosomal locations, evolutionary relationships, cis-elements, gene and protein structures, and protein-protein interaction were systematically characterized. Gene expression analysis showed that MtATGs played important roles in seed development, nodulation, and response to salt and drought stresses. Our work provides an important reference for further research on the functional analysis of autophagy in *M. truncatula*.

Materials And Methods

Identification of MtATGs

The identification of putative MtATGs was conducted using a bi-directional blast analytical strategy, and was performed using the BLASTP program that is integrated into BioEdit software. First, the protein sequences of published autophagy-related genes in *Arabidopsis* were used to search against *M. truncatula* proteome sequences (MedtrA17_4.0) with the e-value cutoff at 1×E-5. Then, all output *M. truncatula* protein sequences were aligned back to *Arabidopsis* proteome sequences. Only the *M. truncatula* genes that shared the highest similarities to the AtATGs from the second BLAST analysis were considered putative MtATGs. To further verify that the candidate MtATGs are indeed MtATGs, the protein domain architectures were analyzed in the Pfam database (http://pfam.xfam.org) [28]. The chemical features of the MtATG proteins, including their molecular weights and theoretical isoelectric points, were obtained using the online tool ExPASy (http://web.expasy.org/compute_pi/). Subcellular localization of MtATGs was predicted using the CELLO v2.5 system (http://cello.life.nctu.edu.tw). The gene and protein

structures of MtATGs were extracted from the annotation file of the *M. truncatula* genome (MedtrA17_4.0) and visualized with the integrating bioinformation analysis toolkit Tbtools [55].

Chromosomal location and gene duplication analysis

MtATGs were mapped to the *M. truncatula* chromosomes based on their physical positions in the the *M. truncatula* genome (MedtrA17_4.0). To investigate the synteny relationships of related genome regions in *M. truncatula*, putative orthologous genes were identified using the BLASTP program, and the results were used to generate a synteny map with the MCScanX program [56]. The genome locations of MtATGs and the duplicated gene pairs were visualized using Tbtools.

Protein sequence alignments and phylogenetic relationship analysis

Phylogenetic analysis of MtATGs was performed using MEGA7 software [57]. The amino acid sequences of MtATGs and AtATGs in different gene families were aligned independently using the ClustalW algorithm with the default parameters. An unrooted phylogenetic tree was constructed with the neighbor-joining statistical method, and the following parameters were used: uniform rates were used as rates among sites, gaps/missing data were treated as pairwise deletion, and the bootstrap analysis was performed with 1000 replicates to obtain a support value for each branch.

Identification of cis-elements

The 1.5 kb genomic DNA sequence upstream of the initiation codon of each *MtATG* was retrieved from the *M. truncatula* genome (MedtrA17_4.0). The assumed cis-elements of MtATGs were predicted using the PlantCARE web servers (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [29].

Protein-protein interaction (PPI) network Construction

PPI network were constructed using the STRING v11 database (http://www.string-db.org). A total of 39 MtATGs were selected as input, and the PPI network of the MtATGs were constructed with medium confidence (0.4).

Expression profile analysis using microarray data

The *M. truncatula* microarray data were downloaded from the MtGEA v3 database (https://mtgea.noble.org/v3/) [58] for datasets generated for different organs [59], drought stresses condition [60], and salt stresses condition [61]. Expression values were normalized using the z-score method or transformed to log2 fold change, and plotted using GraphPad Prism 8.

Abbreviations

ATGs: Autophagy-related genes; PI3K: Phosphatidylinositol-3-kinase; PE: Phosphatidylethanolamine; MeJA: Methyl jasmonate; ABA: Abscisic acid; VPS34: Vacuolar protein sorting 34; CAS31: Cold acclimation-specific 31; PIP2;7: Plasma membrane intrinsic protein 2;7; PPI: Protein-protein interaction;

DAP: Days after pollination; DPI: Days after being inoculated; PCD: Programmed cell death; RNAi: RNA Interference; MtGEA: Medicago truncatula Gene Expression Atlas; UTR: Untranslated region

Declarations

Author Contributions

L.C. conceived and designed the study. M.Y. and L.W. performed bioinformatics analysis. X. G. and C. L. prepared all of the figures and tables. M.Y., W.H., and L.C. wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

All data analyzed during this study are contained in the materials and methods section of the manuscript and its additional files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

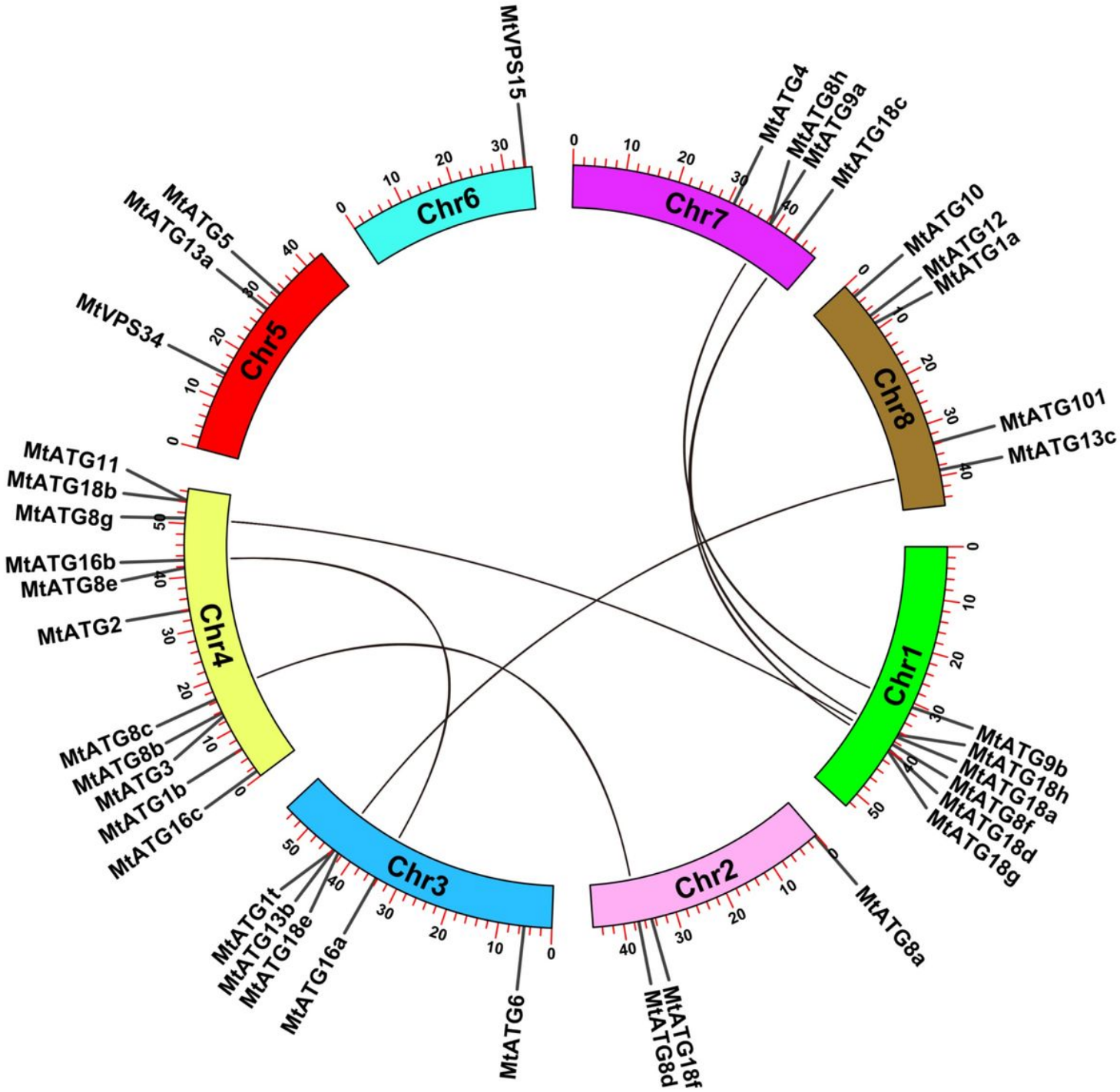
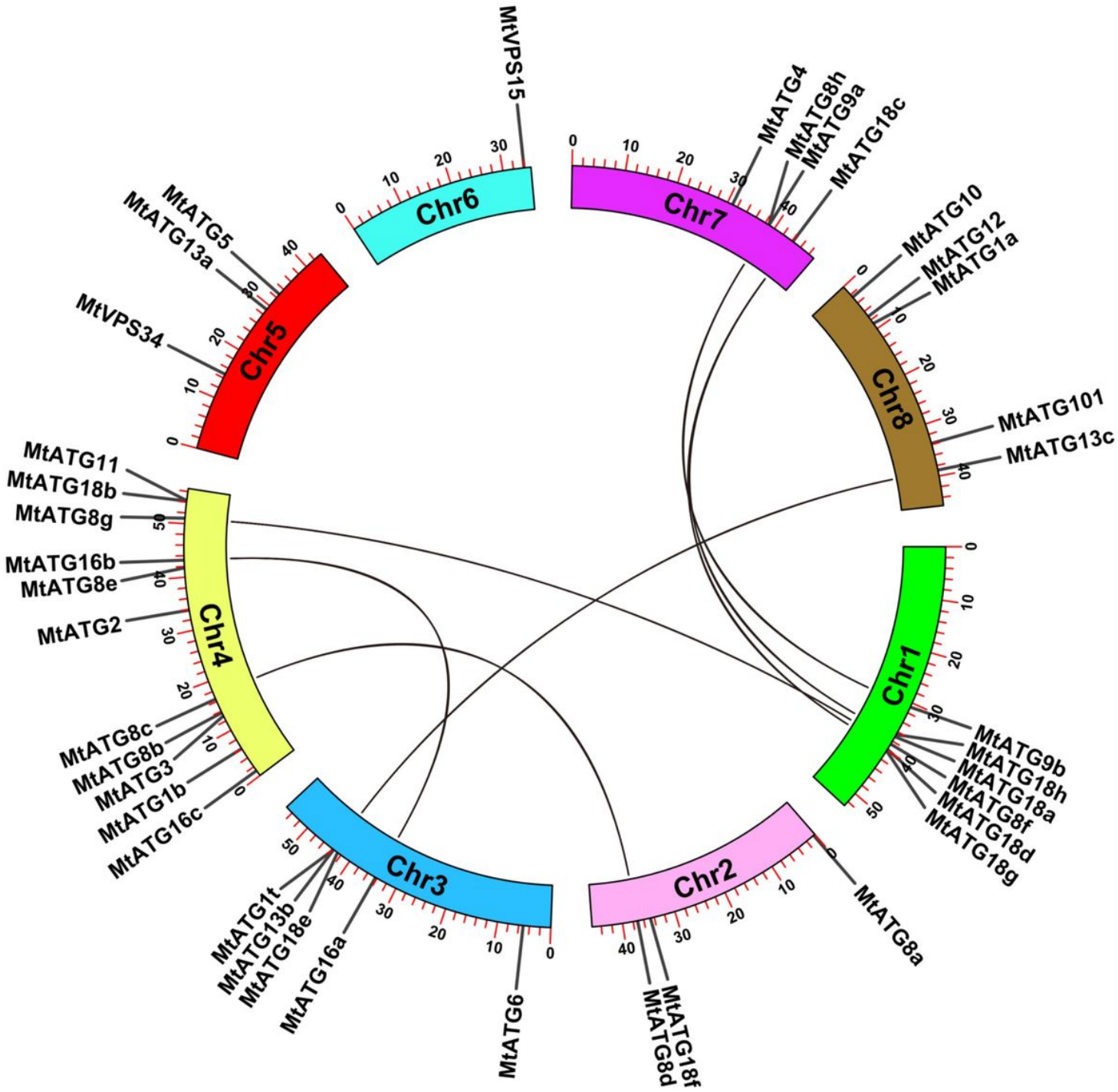


Figure 1

Chromosomal distribution and gene duplication of MtATG genes. The genome locations of MtATGs were retrieved from the M. truncatula genome annotation (MedtrA17_4.0) except for MtATG7. The duplications between MtATGs were analyzed by the MCScanX program and linked with black lines.



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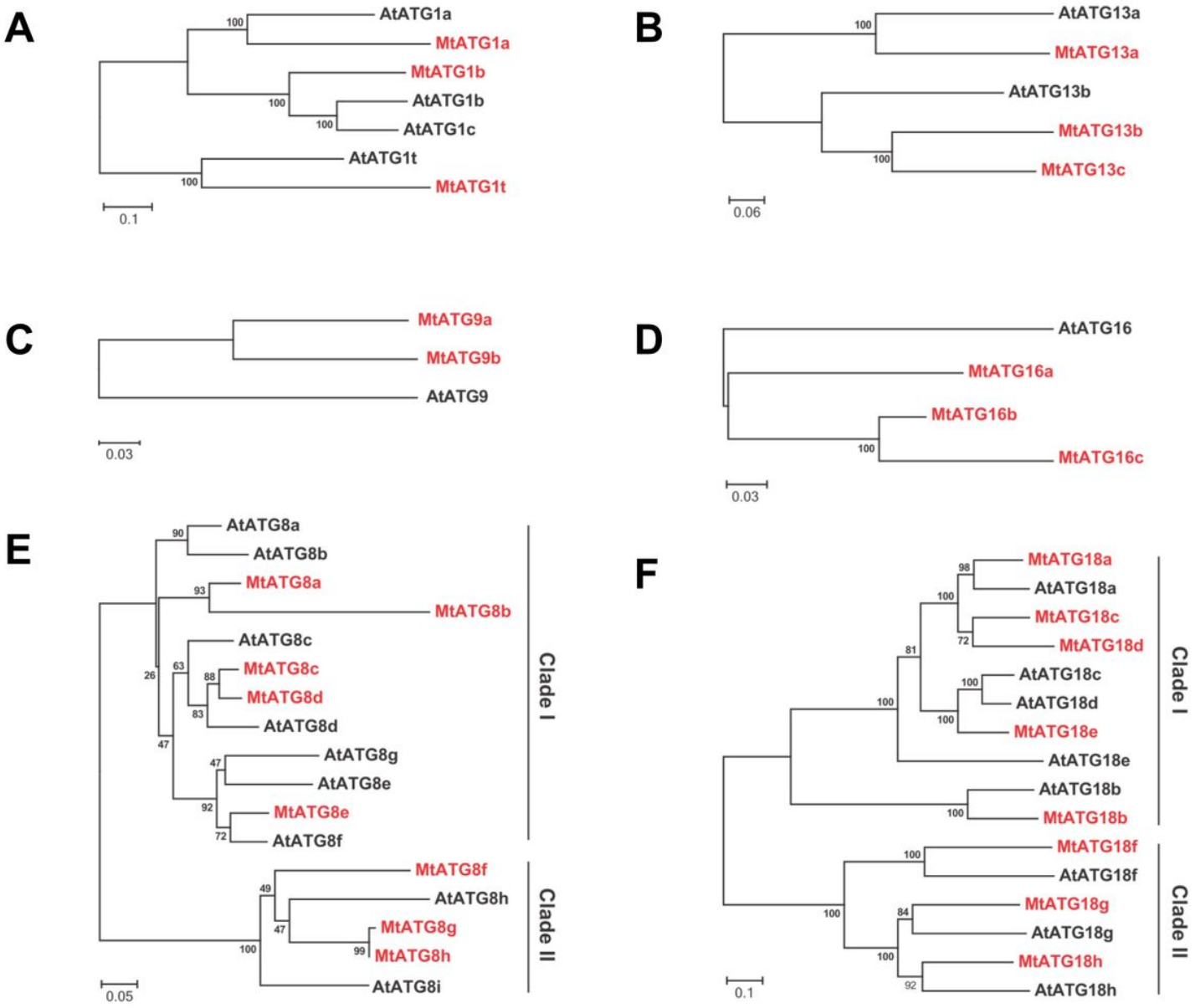
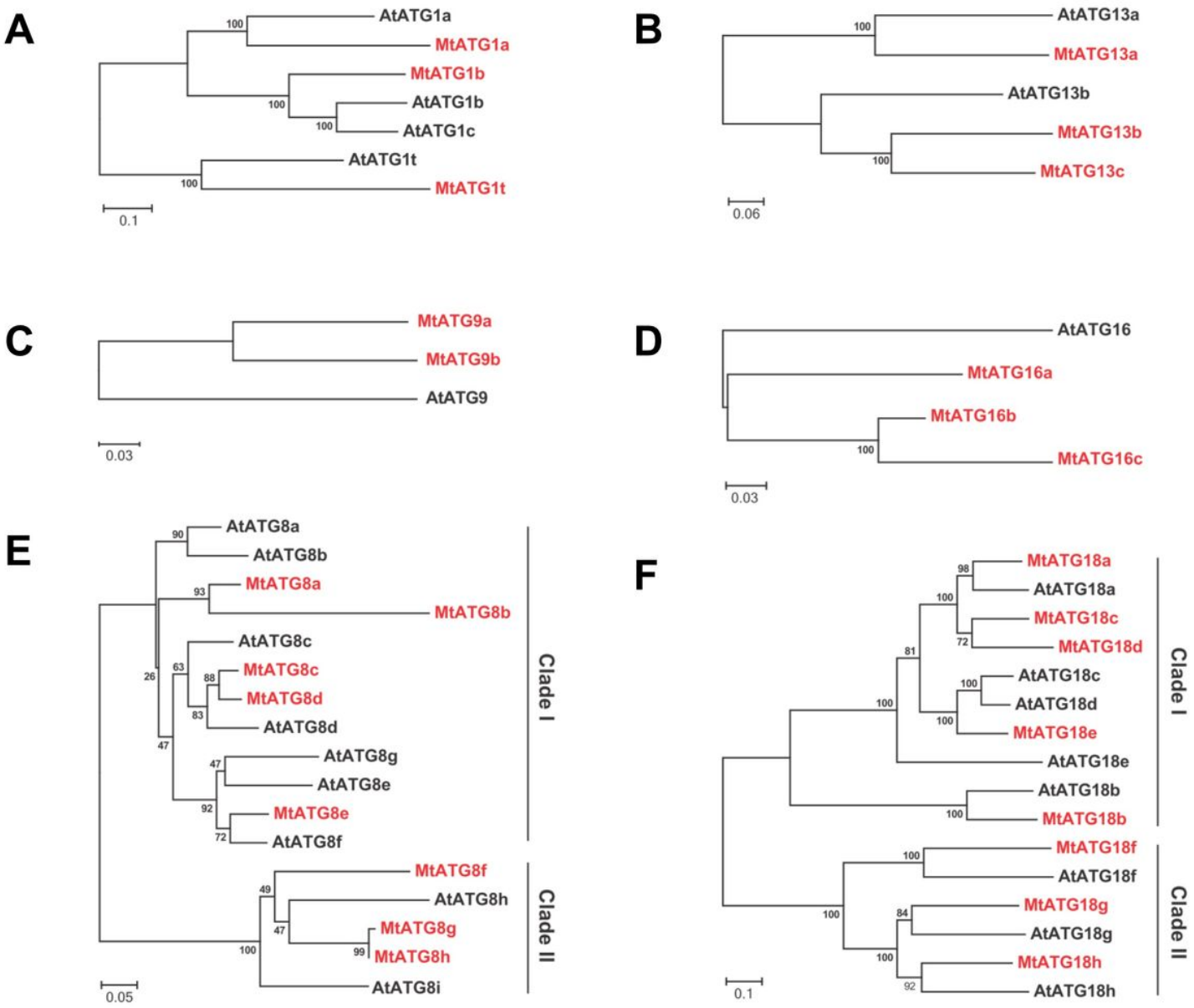


Figure 2

Phylogenetic analysis of ATGs from M. truncatula and A. thaliana. Phylogenetic tree of ATG1 (A), ATG13 (B), ATG9 (C), ATG16 (D), ATG8 (E), and ATG18 (F) families among M. truncatula and Arabidopsis. The unrooted tree was constructed using MEGA7 based on the multiple sequence alignment of the ATG protein sequences by the neighbor-joining (NJ) method. The number at each node represents the bootstrap value from 1000 replicates.



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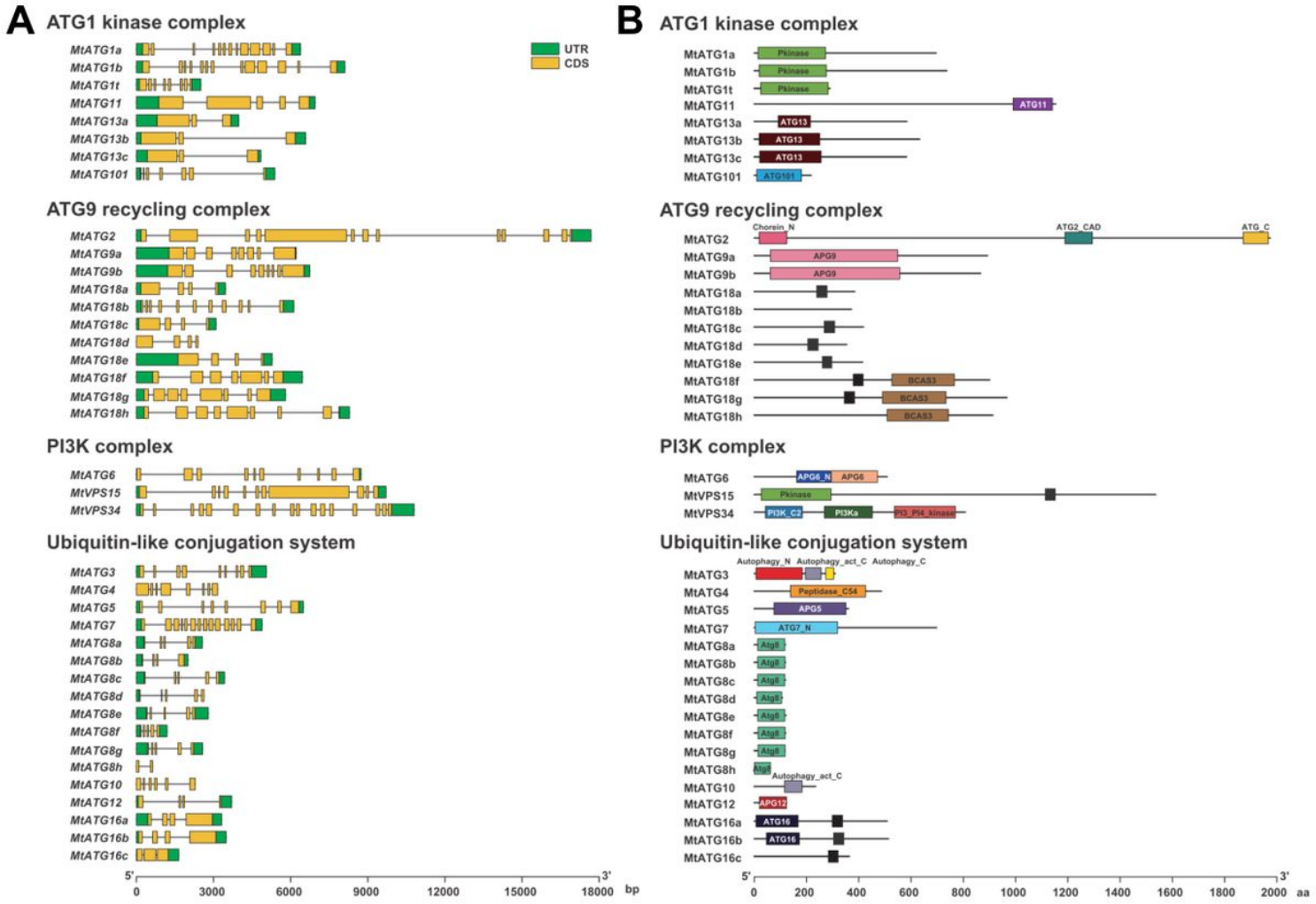
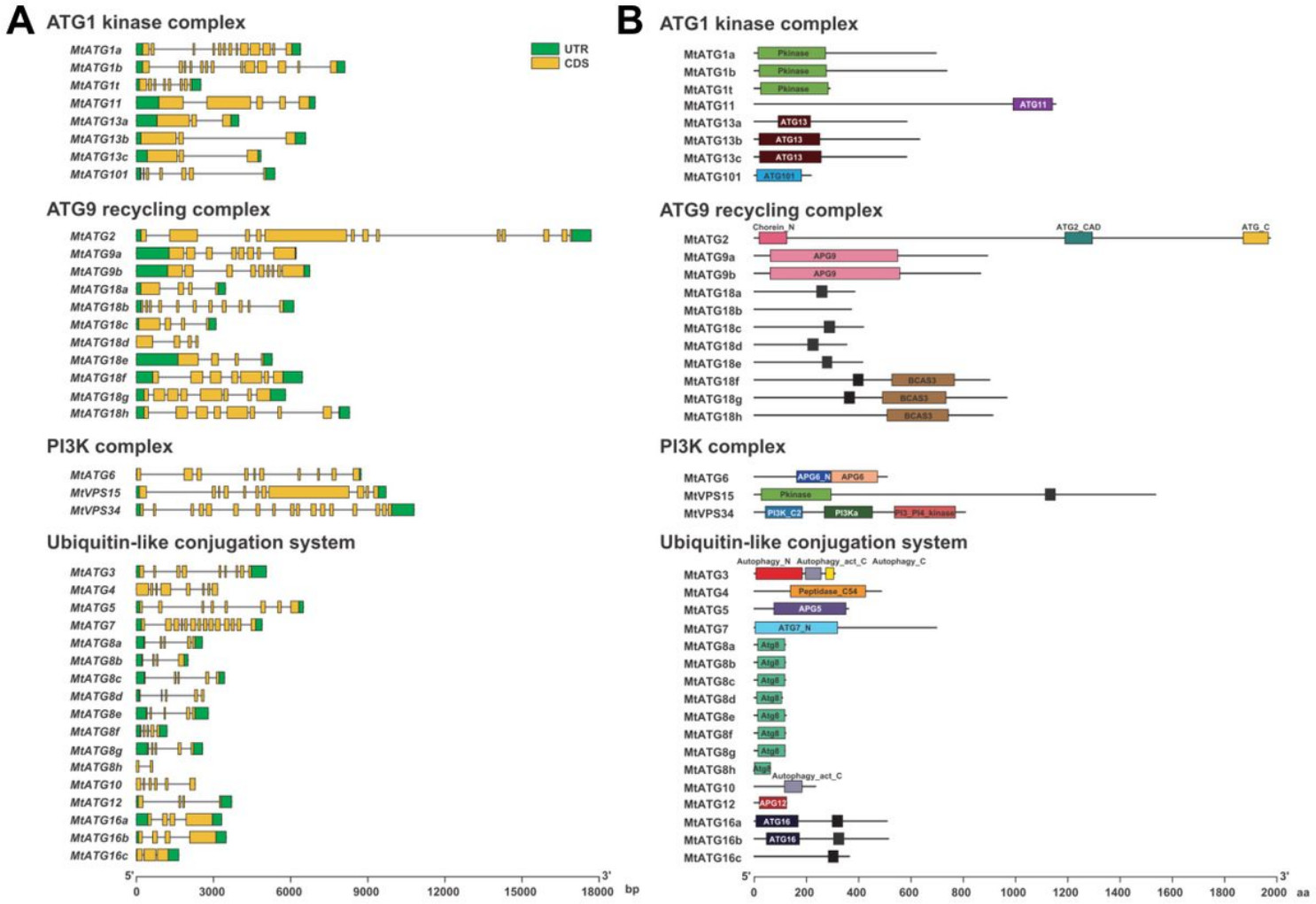


Figure 3

Gene structure and conserved domains of MtATGs. (A) Gene structure of MtATGs was illustrated according to M. truncatula genome annotation (MedtrA17_4.0), and the lengths of the exons and introns of each MtATG are exhibited proportionally. MtATGs are grouped based on their biological function in autophagy pathway. (B) The domain architectures were predicted by the Pfam database, and protein lengths of the MtATGs were acquired from the M. truncatula genome annotation (MedtrA17_4.0). The black box represents WD40 domain.



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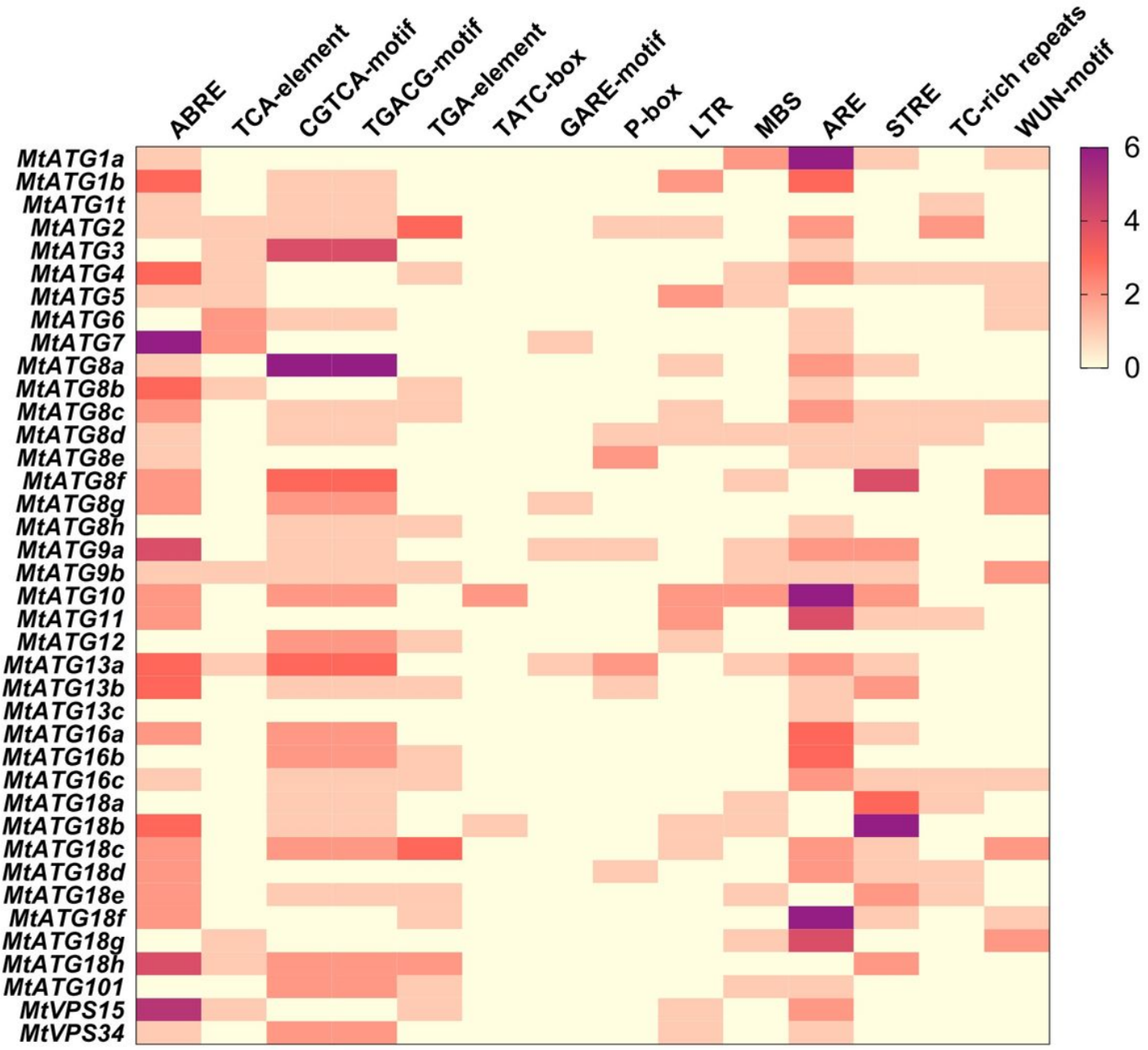
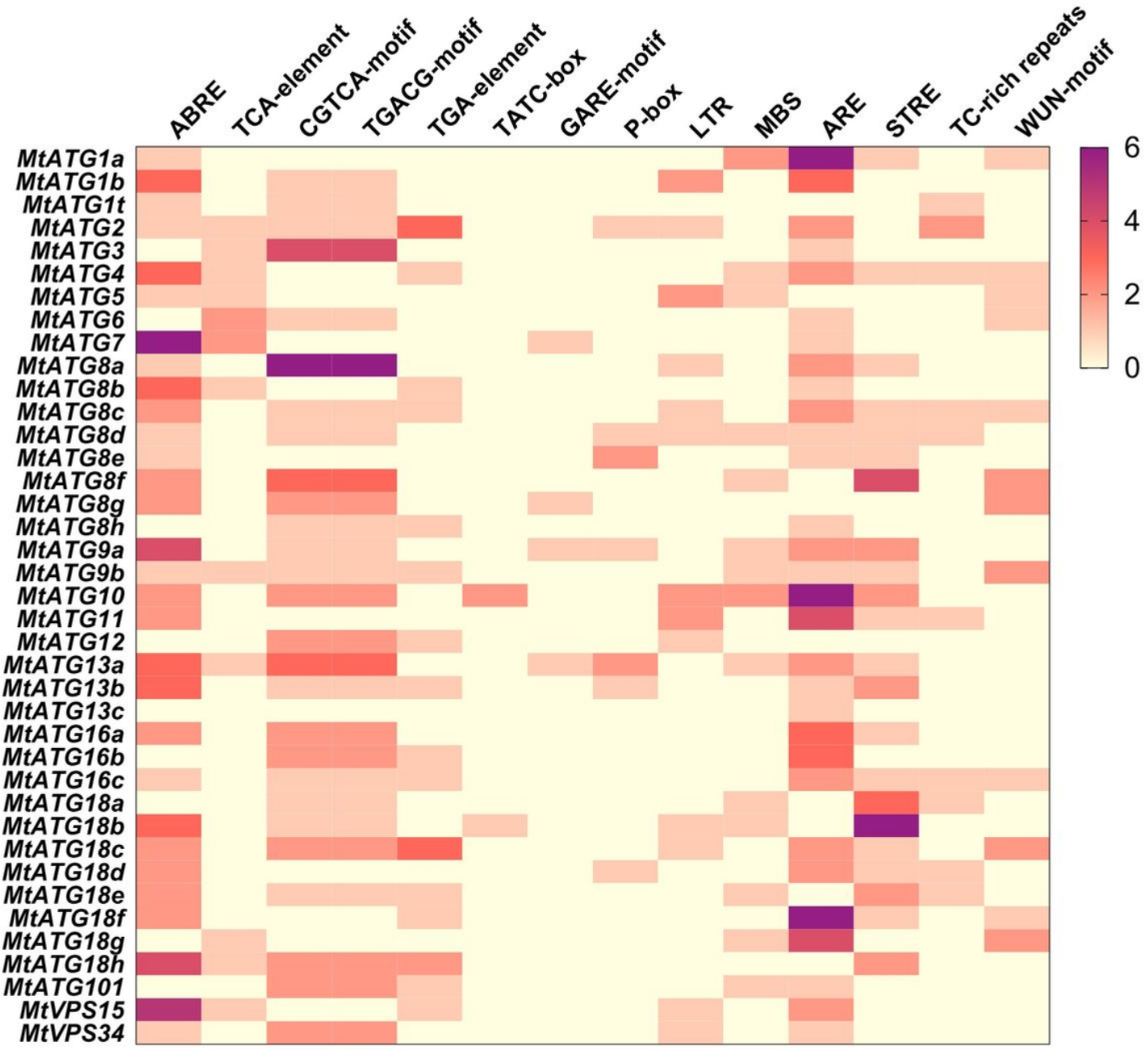


Figure 4

The number of cis-elements in promoter of MtATGs. The assumed cis-elements of MtATGs were predicted using the PlantCARE web servers, and the number of cis-elements in each promoter of MtATGs are visualized for heatmap using GraphPad Prism 8.



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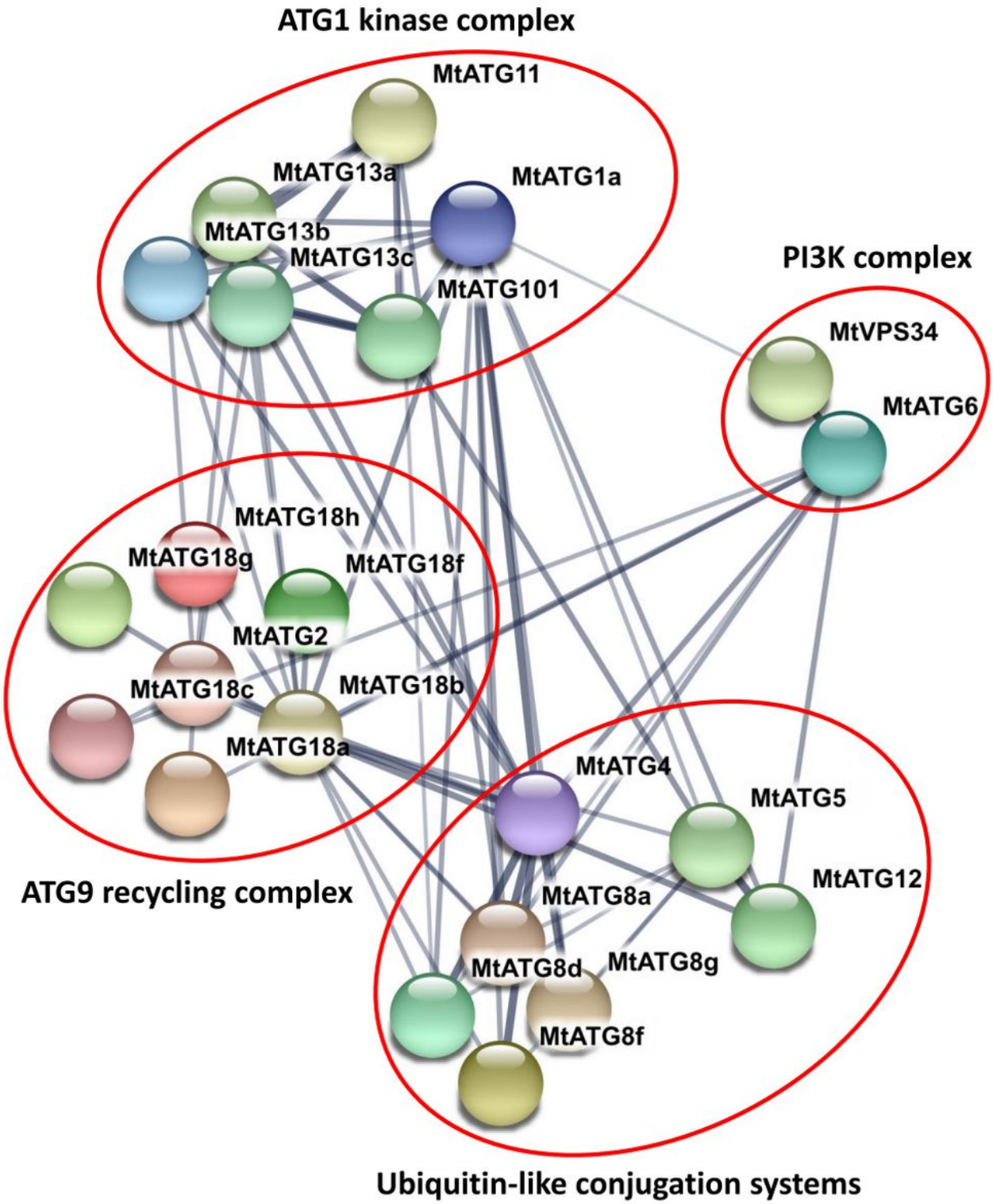
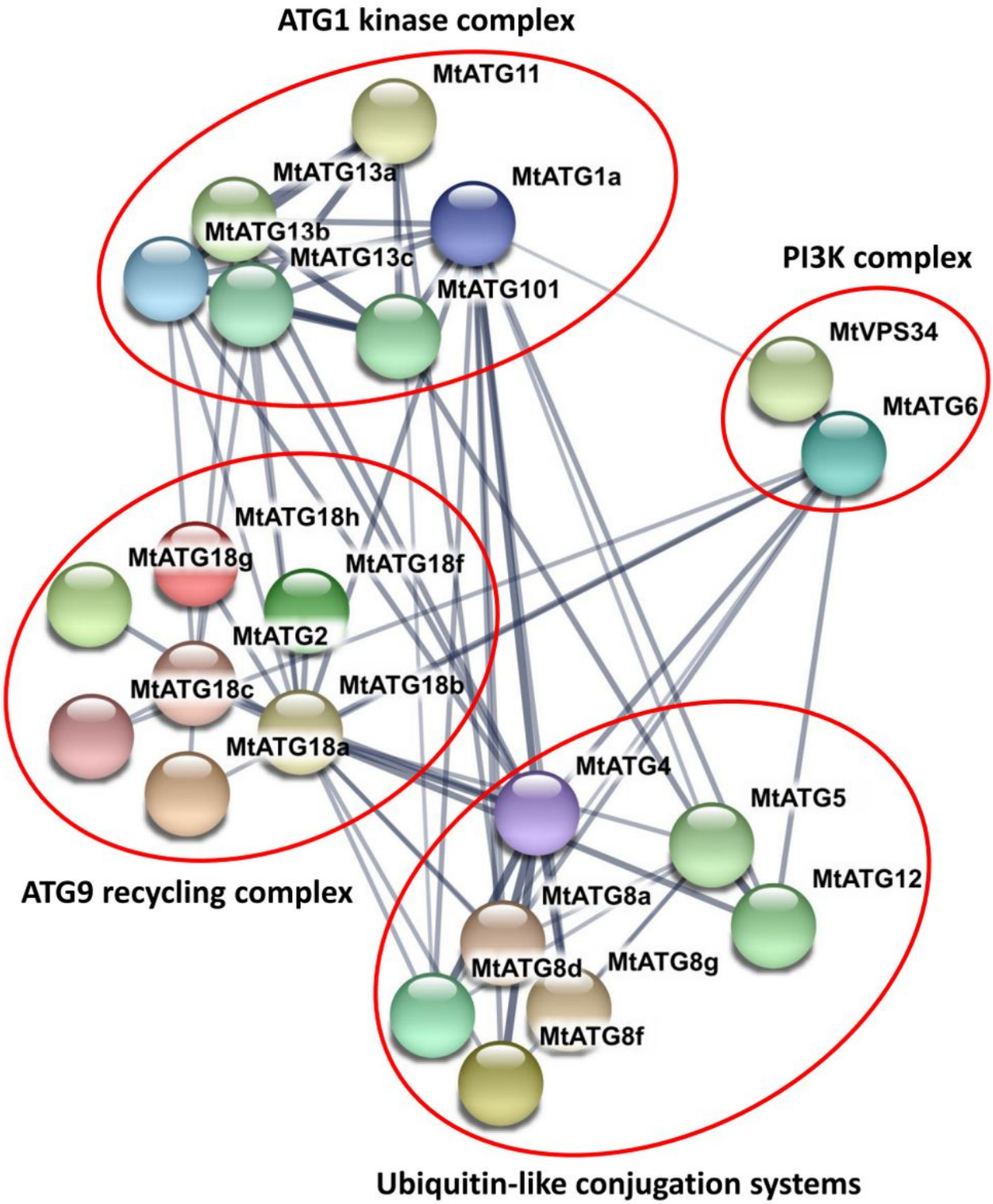


Figure 5

PPI network of MtATGs. The protein associations were derived from various channels: textmining, experiments, databases, co-expression, neighborhood, gene fusion, and co-occurrence. The line thickness indicates the strength of data support.



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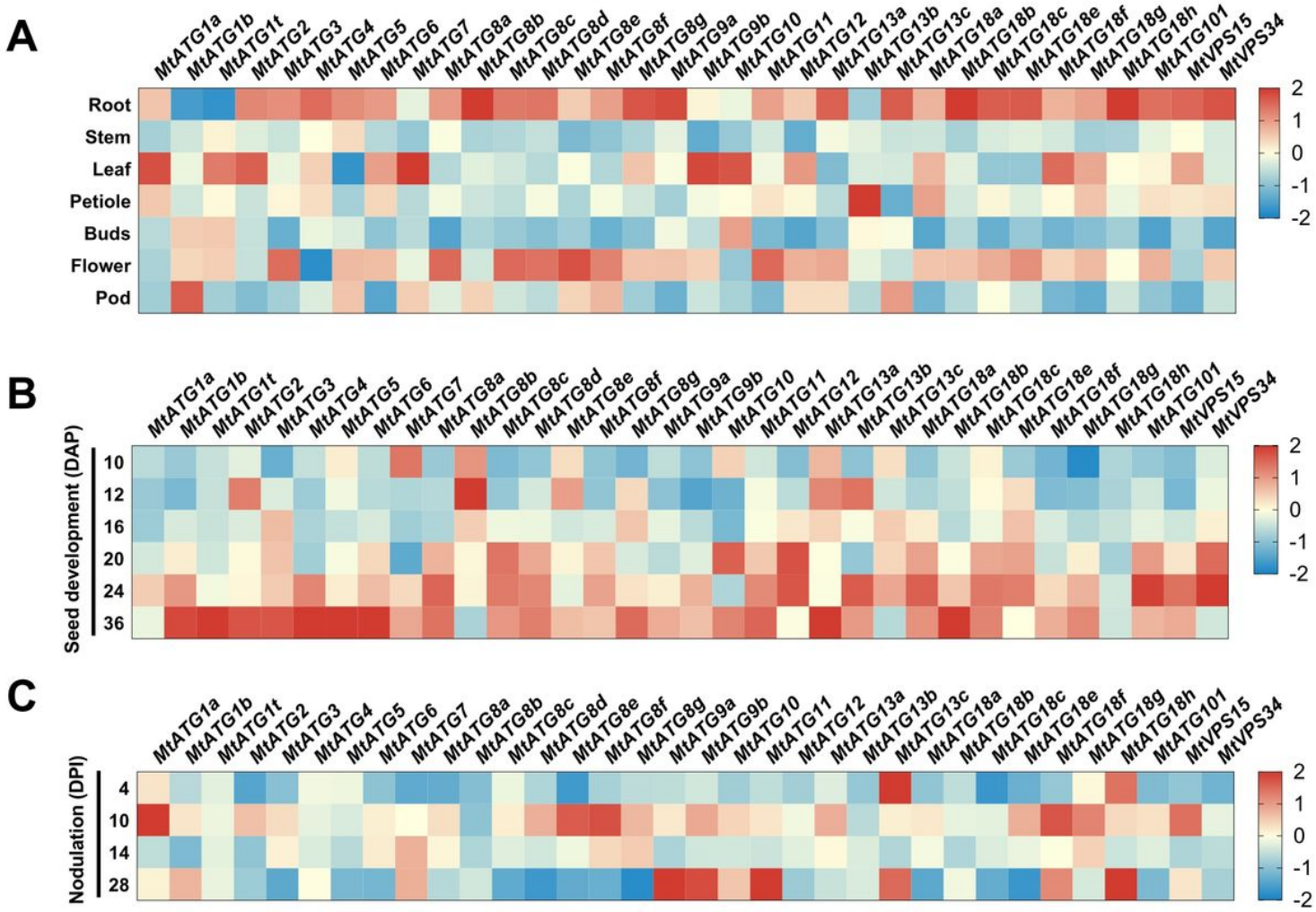
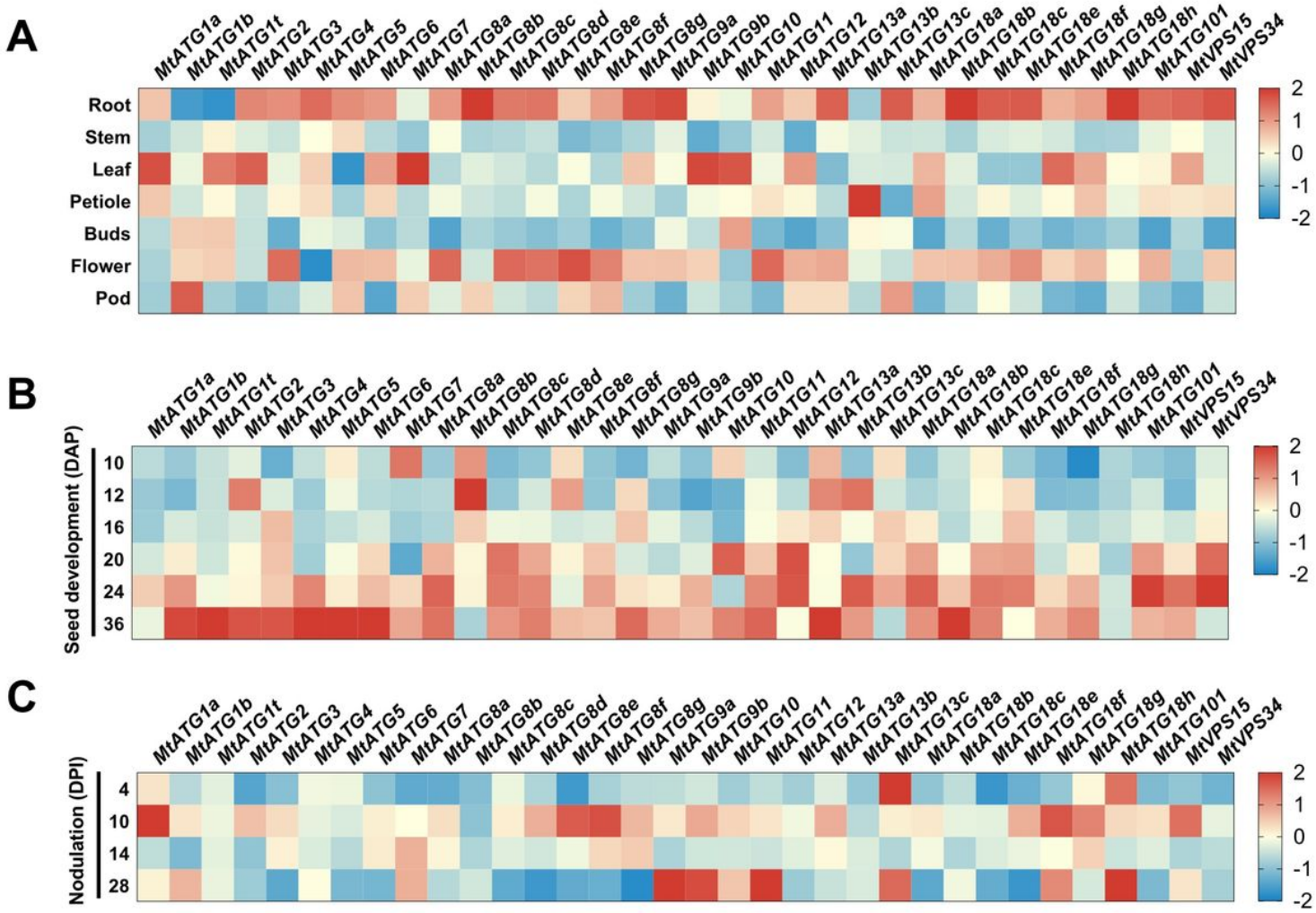


Figure 6

Expression patterns of MtATGs in different tissues, seed development and nodulation. (A) Expression Patterns of MtATGs in different tissues. Roots, stems, leaves, petioles, and shoot buds were harvested from multiple M. truncatula plants at 28 days after planting, besides flowers opened fully and pods ranged from 2.5 to 9.0 mm were collected. (B) Expression patterns of MtATGs in seed and nodulation development. Seeds were excised form pods at 10, 12, 16, 20, 24, and 36 days after pollination (DAP), and nodules were dissected from roots at indicated days after being inoculated (DPI) with S. meliloti. Scale bar represents the relative expression value after z-score normalization.



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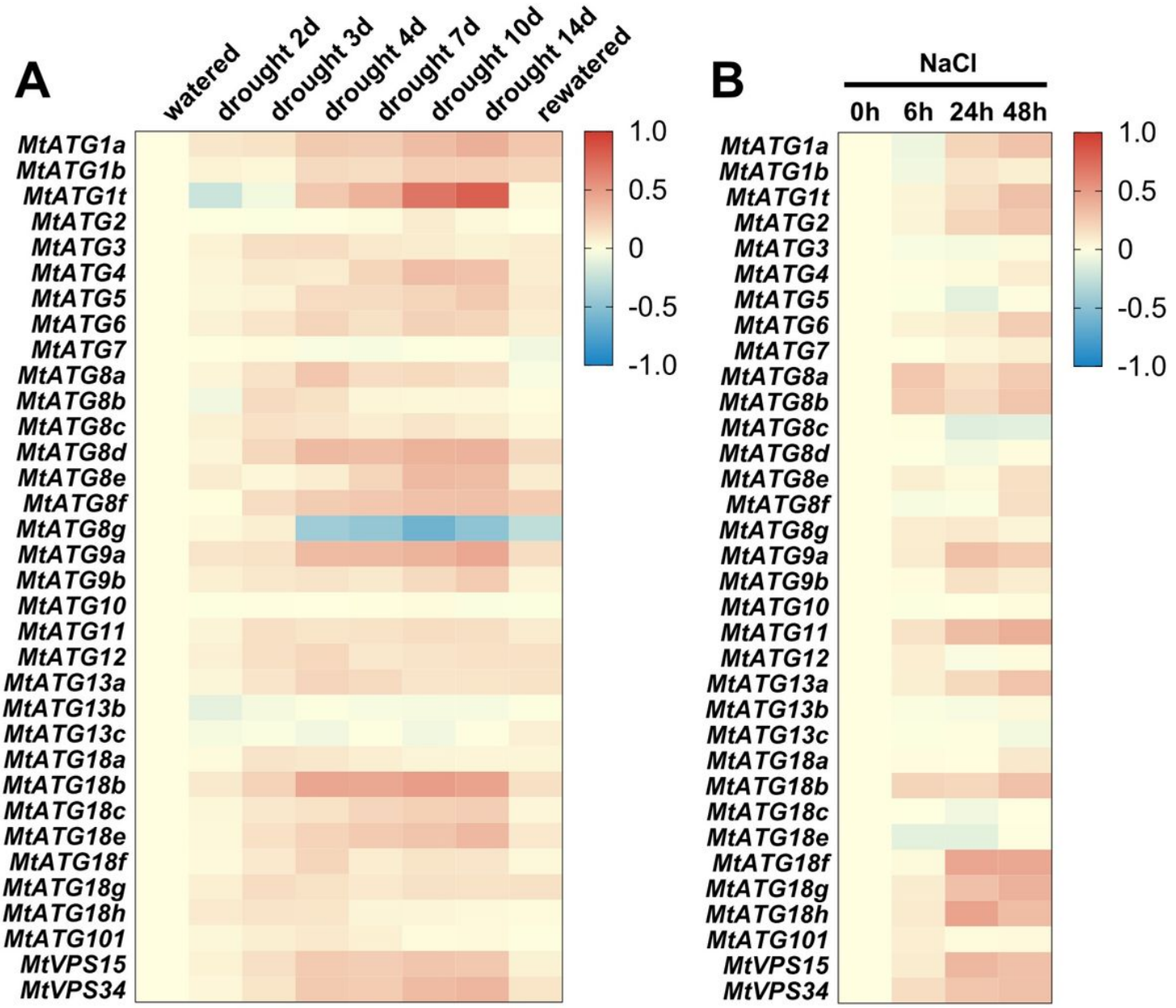
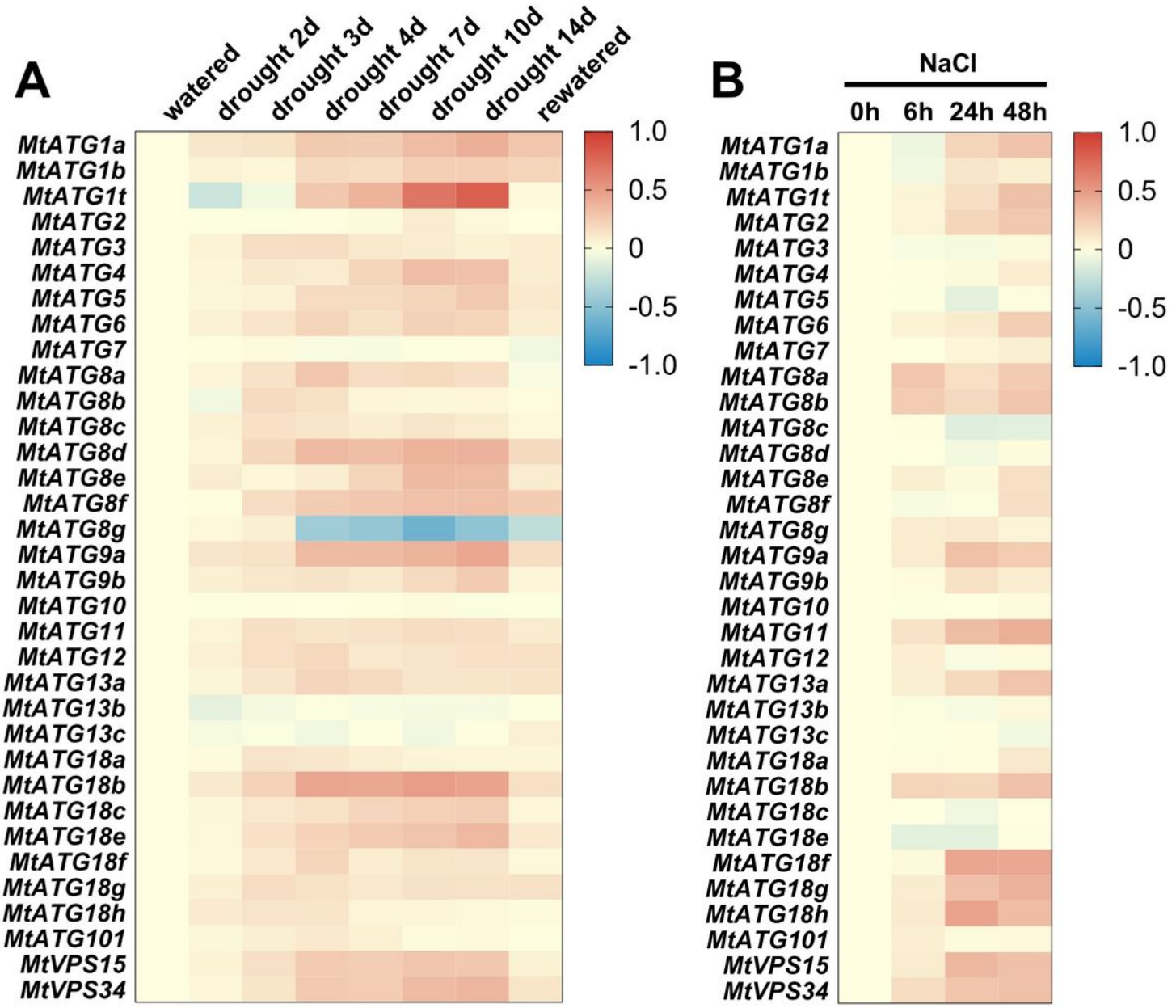


Figure 7

The expression levels of MtATGs under various abiotic stresses. (A) The expression levels of MtATGs under drought stress. Plants were watered from the soil top daily in the early morning until 24 d after planting. The seedlings were harvested under well-watered (controls), drought, and rewatered condition after a 14-day drought treatment. Samples were collected at the indicated time points. (B) The expression levels of MtATGs under salt stress. The seedlings were treated with 180 mM NaCl solution, and samples were collected at 0, 6, 24, and 48 hours after treatment. Scale bar represents fold the change (log2 value) relative to the corresponding control.



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