Genome-wide analysis of autophagy-related genes in *Medicago truncatula* highlights their roles in seed development and response to drought stress

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Autophagy is a highly conserved process of degradation of cytoplasmic constituents in eukaryotes. It is involved in the growth and development of plants, as well as in biotic and abiotic stress response. Although autophagy-related (ATG) genes have been identified and characterized in many plant species, little is known about this process in *Medicago truncatula*. In this study, 39 ATGs were identified, and their gene structures and conserved domains were systematically characterized in *M. truncatula*. Many cis-elements, related to hormone and stress responsiveness, were identified in the promoters of MtATGs. Phylogenetic and interaction network analyses suggested that the function of MtATGs is evolutionarily conserved in *Arabidopsis* and *M. truncatula*. The expression of MtATGs, at varied levels, was detected in all examined tissues. In addition, most of the MtATGs were highly induced during seed development and drought stress, which indicates that autophagy plays an important role in seed development and responses to drought stress in *M. truncatula*. In conclusion, this study gives a comprehensive overview of MtATGs and provides important clues for further functional analysis of autophagy in *M. truncatula*.

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

Previous studies have indicated that autophagy is broadly involved in the growth and development of plants. It has been reported that autophagy-defective mutants show accelerated leaf senescence in *Arabidopsis*. The *Osatg7* mutant showed complete sporophytic male sterility and reduced pollen germination activity, which suggests that autophagy plays critical roles in pollen development in rice. Increasing evidence highlights the crucial role of autophagy in starch and lipid metabolism in plants. 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 and heat, hypoxia, salt, and drought stresses.

*Medicago truncatula* is a model plant for genetic research on legumes that interact with rhizobia to develop nodules for nitrogen fixation. Despite its agronomical importance, the production of *M. truncatula* is threatened by abiotic stresses including high salt and drought stresses. To facilitate our understanding of the mechanism and function of autophagy in *M. truncatula*, it is necessary to first identify all the MtATGs. Based on the identification of MtATGs, further functional studies are required to elucidate the roles of autophagy in *M. truncatula*.
complete genome sequence of *M. truncatula* [23,24], herein, we provide a comprehensive description of *MtATGs*, including their genome-wide identification, characterization, and expression analysis. The results of this study lay the foundation for future research on the molecular mechanism of autophagy in *M. truncatula*.

**Materials and methods**

**Identification of MtATGs.** The identification of putative MtATGs was conducted using a bidirectional BLAST analytical strategy, and was performed using the BLASTP program that is integrated into the 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* in the second BLAST analysis were considered putative *MtATGs*. To further verify that the candidate genes are indeed *MtATGs*, the protein domain architectures were analyzed in the Pfam database ([http://pfam.xfam.org](http://pfam.xfam.org)) [25]. The chemical features of the *MtATG* proteins, including their molecular weights and theoretical isoelectric points, were obtained using the online tool ExPASy ([http://cdd.expasy.org/compute_pi/](http://cdd.expasy.org/compute_pi/)). Subcellular localization of *MtATGs* was predicted using the CELLO system ([http://cello.life.nctu.edu.tw](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 bioinformatic analysis toolkit Tbtools [26].

**Chromosomal location and gene duplication analysis.** *MtATGs* were mapped to the chromosomes based on their physical positions in the *M. truncatula* genome (MedtrA17.4.0). To investigate the synteny 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 [27]. The genome locations of *MtATGs* and the duplicated gene pairs were visualized using Tbtools [26].

**Protein sequence alignment and analysis of the phylogenetic relationship.** The phylogenetic analysis of *MtATGs* was performed using the MEGA7 software [28]. 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 are used as rates among sites, gaps/missing data are 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](http://bioinformatics.psb.ugent.be/webtools/plantcare/html)) [29].

**Construction of the protein–protein interaction (PPI) network.** The PPI networks were constructed using the STRING database ([http://www.string-db.org](http://www.string-db.org)). A total of 39 *MtATGs* were selected as input, and the PPI network of the *MtATGs* was constructed with a medium confidence (0.4).

**Analysis of the expression profiles using microarray data.** The *M. truncatula* microarray data were downloaded from the MtGEA v3 database ([https://mtgea.noble.org/v3/](https://mtgea.noble.org/v3/)) [30]. Expression values were normalized using the z-score method, and plotted using GraphPad Prism 8.

**Plant materials and growth conditions.** *Medicago truncatula* (cv. Jemalong A17) seeds were scarified with sulfuric acid, and vernalized on wetted filter paper at 4 °C for 7 days. Seedlings were grown in a greenhouse at 24 °C, 16-h light/8-h dark cycle, with humidity ranging from 60 to 80%. Different plant tissues (roots, stems, leaves, petioles, buds, flowers, and pods) were harvested from multiple plants. Material for the seed development was collected from pods at 5 different stages. For drought stress, 7-day-old seedlings were transferred by withholding watering for 2 days. For mannitol treatment, 2-weeks-old seedlings were transferred to liquid 1/2 MS medium supplemented with 300 mM mannitol for additional 2 days. All plant samples were frozen immediately in liquid nitrogen after harvest and stored at −80 °C until use. Plant material collections in this study complied with all institutional, national, and international guidelines and legislation.

**RNA isolation and quantitative PCR (qPCR) analysis.** Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The isolated RNA was reverse transcribed using ReverTra Ace qPCR RT Master Mix with gDNA Remover Kit (TOYOBO). qPCR was performed using the CFX Connect Real-Time PCR System (Bio-Rad) with the SYBR Premix ExTag Mix (Takara). *MtACTIN* (Medtr2g008050) was used as a reference gene. Three technical replicates were used for each reaction. The gene-specific primers for the qPCR analysis are listed in Supplementary Table S4.

**Protein blotting analysis.** Western blotting analysis of ATG8 lipidation was performed as previously described [26]. 2-weeks-old seedlings were ground in liquid nitrogen and homogenized in ice-cold RIPA buffer (50 mM Tris–HCl pH8.0, 150 mM NaCl, 1% NP-40, 0.5% Sodium Deoxycholate, 0.5% PVPP, 0.1% SDS). After centrifuged for 15 min at 12,000g, the supernatant fraction was transferred to a new microcentrifuge tube, and
eletrophoresis with 15% SDS-PAGE supplemented with 6 M Urea. Anti-ATG8a antibodies (ab77003, Abcam) were used in the immunoblotting analysis.

Monodansylcadaverine (MDC) staining and microscopy. MDC staining was performed as previously described. Briefly, lateral roots of M. truncatula were detached and stained with 0.75 mM MDC for 1 h. The root cells were observed using LSM 780 inverted microscope (Carl Zeiss) with a DAPI-specific filter.

Results

Genome-wide identification of ATGs in M. truncatula. To identify MtATGs, the BLASTP algorithm was employed in searches against M. truncatula proteome sequences (MedtrA17_4.0) using the amino acid sequences of A. thaliana ATGs (AtATGs) as queries. A total of 39 MtATGs were identified in M. truncatula (Table 1, Supplementary Tables S1, S2). The lengths of the MtATGs 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

| Gene name | Locus ID | Length (aa) | MW (kDa) | PI | Subcellular localization | Chromosome location |
|-----------|---------|-------------|----------|----|----------------------------|---------------------|
| MtATG1a   | Medtrg024100 | 696         | 77.38    | 5.69 | Nuclear                   | chr8:8817813.8824200 |
| MtATG1b   | Medtrg019410 | 737         | 82.03    | 7.13 | Nuclear                   | chr4:6057862.6065974 |
| MtATG1c   | Medtrg095620 | 290         | 32.87    | 7.09 | Extracellular             | chr3:4369826.43692334 |
| MtATG2    | Medtrg086370 | 1975        | 216.66   | 5.07 | Nuclear                   | chr4:33827078.33844760 |
| MtATG3    | Medtrg036265 | 310         | 35.27    | 4.5 | Cytoplasmic               | chr4:13052245.13057301 |
| MtATG4    | Medtrg018230 | 487         | 53.82    | 5.04 | Chloroplast               | chr7:3099369.30998401 |
| MtATG5    | Medtrg076920 | 361         | 41.14    | 4.31 | Nuclear                   | chr5:32086624.32813118 |
| MtATG6    | Medtrg018770 | 509         | 58.07    | 6.45 | Nuclear                   | chr3:5165817.5174556 |
| MtATG7    | Medtr0030540 | 698         | 76.88    | 5.38 | Plasma membrane           | scaffold003:305853.310747 |
| MtATG8a   | Medtrg023430 | 120         | 13.72    | 9.3 | Mitochondrial             | chr2:8277496.8280062 |
| MtATG8b   | Medtrg037225 | 120         | 14.13    | 7.82 | Nuclear                   | chr4:13715664.13717673 |
| MtATG8c   | Medtrg048510 | 120         | 13.89    | 9.29 | Cytoplasmic               | chr4:17207135.17210565 |
| MtATG8d   | Medtrg082830 | 108         | 12.37    | 7.51 | Cytoplasmic               | chr2:37163050.37165850 |
| MtATG8e   | Medtrg101090 | 122         | 14.06    | 8.76 | Cytoplasmic               | chr4:41752327.41755124 |
| MtATG8f   | Medtrg086310 | 121         | 14.10    | 8.18 | Cytoplasmic               | chr3:38625116.38626390 |
| MtATG8g   | Medtrg123760 | 118         | 13.82    | 9.74 | Nuclear                   | chr4:51007802.51010377 |
| MtATG8h   | Medtrg095640 | 62          | 7.09     | 9.1 | Extracellular             | chr7:38739885.38740615 |
| MtATG9a   | Medtrg096880 | 893         | 103.32   | 6.56 | Plasma membrane           | chr7:38799346.38805558 |
| MtATG9b   | Medtrg070160 | 866         | 99.95    | 6.7 | Plasma membrane           | chr1:30830518.30837261 |
| MtATG10   | Medtrg010140 | 235         | 27.01    | 4.77 | Extracellular             | chr8:2577226.2579513 |
| MtATG11   | Medtrg130370 | 1154        | 129.95   | 5.9 | Nuclear                   | chr4:54307709.54314660 |
| MtATG12   | Medtrg020500 | 124         | 10.59    | 9.07 | Plasma membrane           | chr8:7198866.7202464 |
| MtATG13a  | Medtrg068710 | 584         | 65.58    | 9.42 | Nuclear                   | chr5:29098184.29092600 |
| MtATG13b  | Medtrg095570 | 633         | 70.29    | 8.72 | Nuclear                   | chr3:43671041.43677624 |
| MtATG13c  | Medtrg093050 | 583         | 65.62    | 8.73 | Nuclear                   | chr3:38885014.38889871 |
| MtATG16a  | Medtrg075400 | 509         | 55.88    | 6.65 | Nuclear                   | chr3:34315943.34318708 |
| MtATG16b  | Medtrg104380 | 514         | 56.74    | 6.21 | Nuclear                   | chr4:43185561.43189052 |
| MtATG16c  | Medtrg060750 | 364         | 46.66    | 4.66 | Nuclear                   | chr4:1115999.1117649 |
| MtATG18a  | Medtrg083230 | 385         | 42.67    | 7.83 | Plasma membrane           | chr1:37037962.37041428 |
| MtATG18b  | Medtrg130190 | 372         | 40.38    | 7.64 | Nuclear                   | chr4:54209571.54215694 |
| MtATG18c  | Medtrg108520 | 418         | 45.73    | 7.44 | Plasma membrane           | chr7:44026127.44029925 |
| MtATG18d  | Medtrg088855 | 354         | 39.70    | 9.2 | Plasma membrane           | chr1:39776324.39778721 |
| MtATG18e  | Medtrg093590 | 415         | 46.14    | 7.95 | Plasma membrane           | chr3:42763022.42768303 |
| MtATG18f  | Medtrg082770 | 901         | 98.20    | 6.87 | Nuclear                   | chr2:34727900.34734357 |
| MtATG18g  | Medtrg089110 | 967         | 105.32   | 6.11 | Nuclear                   | chr1:40103141.40108943 |
| MtATG18h  | Medtrg082300 | 913         | 99.63    | 5.95 | Nuclear                   | chr1:36587909.36569198 |
| MtATG101  | Medtrg079240 | 218         | 25.48    | 6.43 | Cytoplasmic               | chr8:33765931.33771318 |
| MtVPS15   | Medtrg088835 | 1536        | 171.92   | 6.9 | Plasma membrane           | chr6:33989403.33999113 |
| MtVPS34   | Medtrg034120 | 808         | 92.65    | 6.47 | Cytoplasmic               | chr5:14747697.14758501 |

Table 1. Information related to ATGs and their encoded proteins in Medicago truncatula.
eight in different groups (three in the MtATG1 family, eight in the MtATG8 family, two in the MtATG9 family, three in the MtATG13 family, three in the MtATG16 family, and eight in the MtATG18 family) (Table 1).

The chromosomal distribution of MtATGs determined using the TBtools software is shown in Fig. 1. In total, 38 MtATGs were found to be distributed across all eight chromosomes except for MtATG7, which could not be 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) contains the maximum number (11) of MtATGs, whereas chromosome 6 has only one MtATG gene. Gene duplication is important for adaptation of plants to adverse and complex environments. In M. truncatula, 7 pairs of MtATGs were predicted to be segmentally duplicated. As shown in Fig. 1, these 7 pairs of duplicated MtATGs (MtATG8c and MtATG8d, MtATG8g and MtATG8f, MtATG9a and MtATG9b, MtATG13b and MtATG13c, MtATG16a and MtATG16b, MtATG18a and MtATG18c, MtATG18d and MtATG18e) are distributed across chromosomes 1, 2, 3, 4, 7, and 8. These duplications may have led to the expansion of MtATG families in M. truncatula.

The subcellular localization of the MtATGs was predicted using the CELLO system (http://cello.life.nctu.edu.tw). Most of the MtATGs were predicted to localize to the nucleus, plasma membrane, and cytoplasm, followed by extracellular space, chloroplast, and mitochondria (Table 1, Supplementary Figure S1). Furthermore, some MtATG families exhibited different subcellular localization. For example, MtATG8 proteins were predicted to be mainly cytoplasmic or nuclear, but were also found to localize to the mitochondria and extracellular space (Table 1). The prediction was the same for MtATG18 family members, which were localized to both the plasma membrane and nucleus (Table 1). Taken together, the diverse subcellular localization of MtATGs implies that they have distinct functions.

Figure 1. Chromosomal distribution and gene duplication of MtATGs. 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.
Phylogenetic analysis of MtATGs. To evaluate the evolutionary relationships of MtATGs, we conducted a phylogenetic analysis using the amino acid sequences of the multi-member subfamilies (MtATG1, MtATG8, MtATG9, MtATG13, MtATG16, and MtATG18) and their orthologs from Arabidopsis. As shown in Fig. 2, members of the MtATG1 and MtATG13 families were clustered in two branches (Fig. 2A, B). There are two ATG9s and three ATG16s in M. truncatula, whereas only one ATG9 and ATG16 in Arabidopsis (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 from the N-terminus were absent (Supplementary Figure S2). The C-terminal glycine residue in ATG8, which is exposed upon protease cleavage by ATG4, is essential for the conjugation of ATG8 to phosphatidylethanolamine. However, MtATG8h did not contain the C-terminal glycine residue. This result indicates that MtATG8h might function in other biological processes independent of autophagy. In addition, one MtATG8 member of clade II, MtATG8f, had a C-terminal extension after the Gly residue, whereas the MtATG8 members of clade II lack the C-terminal extension (Supplementary Figure S2). Eight MtATG18 members were also clustered in two branches like the MtATG8 family
members (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).

**Analyses of gene structures and distribution of conserved domains.** Gene structure is closely related to the expression pattern and function divergence of members of multigene families. Gene structure analysis revealed that all the MtATGs contain introns, with the number of exons 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 functional redundancy among these genes. However, differences in exon–intron patterns and exon numbers were also seen within some subfamilies, such as MtATG1t, MtATG8b/h, and MtATG18b/f (Fig. 3A).

The conserved domains of MtATGs were detected using the Pfam database\(^2^5\). In general, the composition of the conserved domains in MtATGs is comparable to that in Arabidopsis. Furthermore, members of the same MtATG family have similar domains. For example, all three MtATG1 proteins contain a protein kinase domain (Pkinase) at their N-terminus (Fig. 3B). In addition, almost all MtATG8 proteins (except MtATG8h) are similar in length and have identical ATG8 domains (Fig. 3). A similar phenomenon was also observed in the MtATG9 and MtATG13 subfamilies. However, exceptions were also found in the MtATG16 and MtATG18 subfamilies. All the MtATG16 family proteins have a C-terminal WD40 domain, but lack an N-terminal ATG16 domain in MtATG16c (Fig. 3B). MtATG18 proteins contain the WD40 domain except for MtATG18b and MtATG18h, but members of clade II (MtATG18f/g/h) have a C-terminal BCAS3 domain that is absent in members of clade I (Fig. 3B). The differences in the gene structure and conserved domains may be related to functional divergence among the different gene products within some MtATG families.

**Analysis of cis-elements 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\(^2^9\). Ninety-two putative cis-elements
were found among MtATG promoters (Supplementary Table S3). Among these, the TATA-box and CAAT-box are the most common cis-elements. Many of the identified cis-elements, such as ABRE (abscisic acid-related), TCA-element (salicylic acid-related), TCCACCT-motif and TGACG-motif (MeJA-related), TGA-element (auxin-related), TATC-box, and P-box and GARE-motif (gibberellin-related), are involved in hormone responsiveness (Fig. 4). Among these, cis-elements that respond to MeJA and ABA were found to be the most abundant. In addition, some stress-related elements are mainly related to anaerobic (ARE), defense (STRE and TC-rich repeats), drought (MBS), low temperature (LTR), and wound (WUN-motif) stresses (Fig. 4). The diversity of cis-elements in the promoter regions of MtATGs provided evidence for their potential biological functions in response to phytohormone, abiotic and biotic stresses.

Analysis of the protein–protein interaction network of MtATGs. To investigate the protein–protein interaction (PPI) between MtATGs, all the 39 MtATGs were submitted to the STRING (Search Tool for the Retrieval of Interacting Genes database) website. Twenty-two 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) interact with each other and function as the ATG1 kinase complex. The second module consists of two members of the PI3K complex, MtATG6 and MtVPS34, MtATG2 and six MtATG18 family members (MtATG18a, MtATG18b, MtATG18c, MtATG18f, MtATG18g, and MtATG18h), making up the third module, play a role in autophagic membrane recruitment. The last module, composed of MtATG4, MtATG5, MtATG12, and four MtATG8 members (MtATG8a, MtATG8d, MtATG8f, and MtATG8g), serves as the ubiquitin-like conjugation system. This interaction pattern of MtATGs is similar to that of Arabidopsis, suggesting that ATGs are possibly evolutionarily conserved in Arabidopsis and M. truncatula.
Expression patterns of MtATGs in different tissues and during seed development. To investigate the possible roles of MtATGs in the growth and development of plants, the expression patterns of their genes in different tissues and during different stages of seed development were determined. All the MtATGs were expressed in the tested tissues, indicating that autophagy is critical for growth and development of plants (Fig. 6A). However, MtATGs showed significantly distinct tissue-specific expression patterns in different tissues. Specifically, the expression levels of many MtATGs, 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 MtATGs (MtATG1a, MtATG11, MtATG2, MtATG7, MtATG9b, MtATG10, and MtATG11) were highly expressed in leaves, whereas others (MtATG3, MtATG8a, MtATG8c, MtATG8f, and MtATG11) were highly expressed in flowers (Fig. 6A). The results revealed that different MtATGs might function in different tissues. To validate the results of the microarray data, the expression profiles of several MtATGs (MtATG1a, MtATG2, MtATG4, MtATG5, MtATG8a, and

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**Figure 5.** Protein–protein interaction network of MtATGs. The associations among proteins are derived from various channels: textmining, experiments, databases, coexpression, neighborhood, gene fusion, and co-occurrence. The thickness of the lines indicates the strength of data support.
MtATG18b) were inspected by qPCR. Most of the selected genes were highly expressed in roots, which was very similar to those of microarray analysis (Fig. 6B).

Consistent with previous studies, most of the MtATGs were upregulated during seed development (Fig. 7A). In particular, MtATG2, MtATG3, MtATG4, MtATG5, MtATG6, MtATG13a, and MtATG18b, were highly expressed in the late stage of seed development (Fig. 7A). In contrast, a few MtATGs, including MtATG7 and MtATG8b, were downregulated after pollination (Fig. 7A). To validate the results of the microarray data, seeds were collected from pods at 5 different stages of seed developmental (Fig. 7B). As shown in Fig. 7C, the expression levels of five selected genes (MtATG2, MtATG4, MtATG5, MtATG8a, and MtATG18b) were considerably increased, only MtATG4 showed no gene expression change during seed development. These results were very similar to those of microarray analysis, and indicate that autophagy is essential for seed development in M. truncatula.

Expression of MtATGs in response to drought stress. To investigate the putative roles of autophagy in the response of M. truncatula to drought stress, the expression profiles of MtATGs were analyzed using microarray data from the MtGEA database\(^5,36\). Generally, most MtATGs were upregulated after drought treatment (Fig. 8A). Specifically, 26 of 34 MtATGs (e.g., MtATG11, MtATG8d, MtATG9a, and MtATG18b) were continuously upregulated when plants were subjected to drought stress by withholding watering, and the transcripts of MtATGs rapidly dropped to their basal levels after resuming the watering (Fig. 8A). Interestingly, MtATG8g showed an opposite trend: the expression level of MtATG8g dramatically decreased under drought stress compared with other MtATGs (Fig. 8A). To validate the results of the microarray data, six genes (MtATG1a, MtATG2, MtATG4, MtATG5, MtATG8a, and MtATG18b) were selected for independent validation by qPCR. The expression levels of most of the selected genes were significantly higher after 2 days of drought treatment (Fig. 8B). To examine autophagy activity under drought stress, antibodies against ATG8 protein...
by western blotting. ATG8 proteins are lipidated with phosphatidylethanolamine (PE) to promote autophagosome formation in response to drought treatment, whereas no changes in the level of ATG8-PE were detected under control condition (Fig. 8C). Furthermore, MDC staining showed that the number of autophagosomes was significantly increased after drought treatment (Fig. 8D). These results suggested that autophagy might play a crucial role in M. truncatula response to drought stress.

Discussion
In this study, 39 ATGs were identified in M. truncatula. These ATGs are similar to orthologous genes in Arabidopsis. For example, phylogenetic analysis revealed that ATG families in M. truncatula are very similar to those in Arabidopsis. In addition, the PPI network analysis shows that the interaction pattern of MtATGs is also similar to that of ATGs in Arabidopsis. These results indicate that the autophagy pathway is highly conserved across different plant species. However, the number of members in some ATG families differs among plant species. For example, the ATG8 family contains eight genes in M. truncatula, but nine in Arabidopsis, seven in rice, and thirteen in wheat. In addition, the gene structure and conserved domains of some MtATG families, such as MtATG16 and MtATG18 subfamilies, also differ from those of other plants. Furthermore, different types of
cis-elements were identified in the promoters of MtATGs in the same gene family. 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 future studies.

Autophagy has been shown to play crucial roles in the growth and development of plants. In this study, we found that all ATGs were expressed in the tested tissues of M. truncatula, but their expression levels varied among different tissues. The tissue-specific expression of MtATGs suggests that different functions are required in different tissues. Seed development consists of embryo morphogenesis and seed maturation. In rice, autophagy has been shown to be involved in the regulation of starch and sugar metabolism during seed maturation. In Norway spruce (Picea abies), autophagy is also involved in embryogenesis in which it regulates vacuolar cell death of the embryo suspensor. Furthermore, autophagy plays an important role in microspore embryogenesis in Brassica napus. The seed weight in autophagy-defective mutants of Arabidopsis and maize was reported to...
be lower than in the wild-type plants. 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, which indicates that autophagy is necessary for seed development in M. truncatula. Overall, autophagy plays crucial roles in the growth and development of plants through a pathway that is conserved across different species.

Autophagy has been demonstrated to promote plant survival by maintaining cellular homeostasis under drought stress. In A. thaliana, the transcriptional level of ATG18a was rapidly upregulated by mannitol treatment. In O. sativa, the expression levels of OsATG6 genes were also induced by drought stress. Moreover, ATG genes were upregulated by drought stress in many other plant species, such as barley, pepper, apple, and banana. Besides changes in gene expression, the Arabidopsis autophagy-defective mutants (atg5, atg7, and RNAi-ATG18a) showed more sensitivity to drought treatment than the wild type. Inhibition of autophagy by 3-MA or knockdown of ATG6 sensitized wheat seedlings to drought stress. Furthermore, virus-induced gene silencing of ATG8d or ATG18h significantly reduced drought tolerance in tomato. However, overexpression of MdATG5 or MdATG18a enhanced tolerance to drought stress in apple trees. In addition, overexpression of SiATG8a from foxtail millet improved drought tolerance in Arabidopsis. Recently, it was reported that autophagy improves drought tolerance in M. truncatula through degradation of the aquaporin MtiPIP2;7, which interacts with the cargo receptor MtCAS3.

Consistent with previous studies, our results reveal that the promoter of many MtATGs contain the drought-related MBS cis-element. Furthermore, the transcriptional levels of most of the MtATG genes, especially those of the MtATG8 family, significantly increased after drought treatment. The lipidation of ATG8 protein and accumulation of autophagosome are enhanced in M. truncatula with the cargo receptor MtCAS3. Consistent with previous studies, our results reveal that the promoter of many MtATGs contain the drought-related MBS cis-element. Furthermore, the transcriptional levels of most of the MtATG genes, especially those of the MtATG8 family, significantly increased after drought treatment. The lipidation of ATG8 protein and accumulation of autophagosome are enhanced in M. truncatula during drought stress. Our findings indicate that autophagy is largely induced by drought stress in M. truncatula, and can be considered an adaptive response under drought stress.

Conclusion

This study provided comprehensive analysis of ATGs in M. truncatula. In total, 39 ATGs were identified in M. truncatula. Members of the same ATG family showed similar gene structure and conserved domains. Analysis of cis-elements implied that MtATGs have potential biological functions in response to phytohormone, abiotic and biotic stresses. Phylogenetic and interaction network analyses suggested that the function of MtATGs is evolutionarily conserved in Arabidopsis and M. truncatula. The expression pattern of MtATGs indicates that autophagy possibly participates in seed development and plays an important role in plant responses to drought stress. In conclusion, this study gives a detailed overview of MtATGs and their expression patterns. The results obtained in this study provide useful information for further functional characterization of autophagy in M. truncatula.

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

Competing interests
The authors declare no competing interests.

Additional information
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