

Genome-wide identification and expression analysis of autophagy genes in cucumber

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Abstract

Background

Autophagy is an evolutionarily conserved physiological and developmental process in eukaryotes. In this process, damaged proteins in cells are degraded and cytoplasmic materials recycled. When plants are exposed to stress conditions or their growth and development are blocked, autophagy is induced to maintain the cell homeostasis by degrading long-lived proteins in the cells and organelles that function abnormally due to aging or damage. Cell autophagy has multiple functions in plants, it is involved in growth and development, senescence, and responses to biotic and abiotic stress. So far, thirty three autophagy genes (ATG) have been found in rice, and more than 30 autophagy-related genes have been found in Arabidopsis, tobacco and corn, respectively. Four autophagy genes induced by salicylic acid were found in cucumber, but a little is still unknown about all of autophagy genes in the cucumber genome. Our experiment fully explored the ATG gene family of the cucumber genome based on bioinformatics methods and identified 20 CsATG genes. We systematically analyzed the structure, conserved motifs, expression and phylogenetic relationship of these ATG genes, which lays the foundation for exploring the function of the genes.

Results

A total of 20 putative ATG genes were identified in the cucumber genome. Gene duplication analysis showed that both fragmented and tandem duplication played vital roles in the amplification of the cucumber ATG gene family. Gene expression analysis showed that 16 CsATG genes were induced by the salicylic acid (SA) treatment, and 16 CsATG genes were down-regulated by Methyl jasmonate (MeJA) treatment. Under high salinity stress, 10 CsATG genes were induced in roots. Under drought stress, 16 CsATG genes were induced in roots. Under carbon starvation stress, all of 20 CsATG genes were induced to express in leaves, suggesting that cell autophagy has a potential role in nutritional starvation tolerance.

Conclusion

Our results clearly have deepened our understanding of the characteristics and functions of the cucumber ATG gene, and also found some new gene resources that can be used for the future development of cucumber and other crop varieties, which can resist stress.

Introduction

In early single-cell eukaryotes, in order to adapt to nutritional deficiencies or other forms of environmental stress, a mechanism for recovering and reusing nutrients has gradually evolved, which can protect the organism and regulate the abiotic stress response (Inoue, et al. 2006). In some nutrient-poor soils, there is often a lack of two nutrients, carbon and nitrogen. This will greatly affect the growth and development of plants. Therefore, the plant evolved an autophagy mechanism to adapt to the plant against nutrient

deficiency in the soil. Autophagy has the ability to degrade the damaged proteins and some organelles in the plant, thereby recycling and reusing its own nutrients to ensure normal growth and development of plants (Liu, et al. 2009). There are at least three types of autophagy in eukaryotes: macroautophagy, microautophagy, and chaperone-mediated autophagy. The most widely studied in plants is macroautophagy. The formation of autophagic vesicles (autophagosome) with a double membrane structure is biological feature of macroautophagy (Liu and Bassham 2012; Mizushima, et al. 2008; Mizushima, et al. 2011; Zhang, et al. 2016). Autophagic vesicles are formed by the expansion and growth of phagocytic vesicles (phagophore) (Yoshimoto 2012). The outer membrane of mature autophagic vesicles is fused with the vacuolar membrane and then releasing the autophagosomes that encapsulate the cytoplasmic components into the vacuole cavity and degrading them into amino acids and other small molecular substances by hydrolases. Cell autophagy is involved in many important biological processes such as plant growth, development, and response to biotic and abiotic stress. Also it is closely related to important agronomic traits of crops. Therefore, autophagy is considered to play a vital role in protecting plants against nutritional deficiency and various environmental stresses. Recently, more attention has been paid to the molecular and physiological mechanisms of autophagy.

To our knowledge of autophagy basically came from yeast. So far, 41 ATG genes have been founded in yeast. Many autophagy-defective mutants have been isolated and used to reveal the molecular mechanism of autophagy (Harding, et al. 1995; Kanki, et al. 2009; Klionsky, et al. 2003; Mizushima 2007; Noda, et al. 2002; Okamoto, et al. 2009; Suzuki, et al. 2010; Thumm, et al. 1994; Tsukada and Ohsumi 1993; Wang and Klionsky 2003; Yao, et al. 2015). According to the process of ATG proteins regulating autophagy pathways, these proteins can be divided into different functional groups (Shibutani and Yoshimori 2014; Xie and Klionsky 2007) (1) ATG1-ATG13 kinase complex is responsible for the induction of autophagy and it is negatively regulated by the TOR (target of rapamycin) kinase; (2) class III PI3K (phosphatidylinositol 3-kinase) complex contains ATG6 (called Beclin1 in mammals) and it plays an important role in the nucleation of vesicles; (3) ATG9-ATG2-ATG18 complex transports membrane lipids to phagocytic vesicles, thereby promoting the extension of phagocytic vesicles; (4) ATG12-ATG5 and ATG8-PE (ATG8-phosphatidylethanolamine) linkers generated by two ubiquitin-protein binding systems on the vesicle membrane extension and maturation of autophagic vesicles are essential.

With the development of genome-wide technology, a number of genes homologous to yeast ATG have been identified in plants and mammals. Sequence alignment results showed that the sequence homology between the Arabidopsis ATG protein and the corresponding yeast ATG protein is not high and only part of the protein can completely complement the phenotype of yeast mutant. The core autophagy mechanism in plant is similar to yeast. It was found that most of the important amino acid residues of yeast ATG protein were also conserved in Arabidopsis (Yoshimoto, et al. 2010). The discovery of these genes also can prove that the evolution of autophagy is highly conserved. In recent years, autophagy genes have been identified in Arabidopsis, rice, and maize genomes by genome sequencing and some of these genes have been functionally analyzed. There were 33 autophagy genes in rice, and over 30 autophagy-related genes were also found in tobacco and corn, respectively, and their functions were verified (Chung, et al. 2009; Li, et al. 2015; Su, et al. 2006; Xia, et al. 2011; Zhou, et al. 2015). The

autophagy of *Arabidopsis thaliana* is particularly well studied in plants. At present, 36 autophagy-related genes in *Arabidopsis* has been discovered (Avin-Wittenberg, et al. 2012). However, to our knowledge, there are very few studies on cucumber.

Cucumber (*Cucumis sativus* L.) is an important and popular vegetable crop of the family Cucurbitaceae and is widely cultivated in the temperate and tropical regions. Cucumbers are vulnerable to powdery mildew and downy mildew during their growth process, which affects the growth and development of cucumbers and causes serious economic damage. The aim of this study is to provide a clear role for the mechanism and a better understanding of cucumber autophagy gene for their potential roles in disease resistance. We conducted genome-wide analysis of cucumber ATG on the published cucumber genome, identified 20 putative autophagy genes, predicted the chromosomal positions and protein structures of these genes, then detected and analyzed their expression feature using qRT-PCR. Our results deepened our understanding of the characteristics and functions of the cucumber ATG gene, and also found new genetic resources for cucumber and other crop varieties.

Results

Identification of CsATG genes family members

A total of 20 putative CsATG genes were identified from the cucumber genome. ExPASy Proteomics Server (<http://expasy.org/>) was used to predict the molecular weight and the isoelectric point of the obtained 20 proteins (Table 1). It can be seen from the table 1 that the amino acid sequence length, predicted molecular weight, and predicted isoelectric point of the 20 CsATG proteins are quite different. The length of the CsATG protein sequence ranges from 87 amino acids (CsATG5a) to 1940 amino acids (CsATG2), and the molecular weight ranges from 9 to 215 kDa, and the predicted isoelectric point varies from 4.81 (CsATG10) to 9.77 (CsATG13a). Based on the name of cucumber ATG on NCBI GenBank, 20 putative CsATG genes were named respectively in reference to the results of homology alignment with *Arabidopsis thaliana*. Among the CsATGs, *CsATG27* and *CsATG10* have no corresponding names on NCBI GenBank, so they are named as new names.

Phylogenetic analysis of CsATG genes

To clarify the phylogenetic relationship of the autophagy protein family between cucumber and *Arabidopsis thaliana*, multiple alignments of ATG protein sequences from *C. sativus* and *A. thaliana* were performed using the Clustal X2.1 program, and a phylogenetic tree was constructed by MEGA (Ver. 5.0). As shown in Figure 1, the autophagy genes of the two species *C. sativus* and *A. thaliana* were divided into four subfamilies. GROUP1 contained *CsATG18b*, *CsATG5a*, *CsATG2*, *CsATG10* and *CsATG3*. GROUP2 contained *CsATG9* and *CsATG5b*. GROUP3 contained *CsATG16* and *CsATG27*. GROUP4 contained *CsATG5c*, *CsATG8a*, *CsATG8b*, *CsATG8c*, *CsATG8d*, *CsATG8e*, *CsATG8f*, *CsATG13a* and *CsATG13b*.

Gene structure analysis of CsATG genes

Exon-intron structural divergences within gene families have played a critical role in the evolution of multigene families (Zhang, et al. 2012). To further reveal the structural features of CsATG genes, their structures, including CDS and UTR, were analyzed (Figure 2). We found that their UTR number ranges from 0 to 3. *CsATG5a*, *CsATG5b*, *CsATG8a*, *CsATG11* and *CsATG27* do not contain UTR, and *CsATG18b* contains 3 UTR, other CsATGs contain 1 or 2 UTR. CDS number was from 1 to 13, *CsATG13a* contains only 1 CDS, *CsATG2* contains 13 CDS, *CsATG8b*, *CsATG8c*, *CsATG8d*, *CsATG8f* have the same number of CDS, The number of CDS members in other groups is different, and their number ranges from 2-11. Analysis of the CDS-UTR structure shows that some members of the ATG subfamily have the same or similar CDS-UTR structure. So these genes vary widely.

Synteny analysis of ATG genes between cucumber and Arabidopsis

Based on the analysis of the syntenic relationship between the cucumber *ATGs* and the *Arabidopsis thaliana ATGs* (Figure 3), the ATG homologous genes on chromosome 1 of cucumber can be found on chromosome 1, 2, 3, 4, 5 of *Arabidopsis*, and the ATG homologous genes on chromosome 2 of cucumber can correspond to chromosome 4 of *Arabidopsis*, and the ATG homologous genes on chromosome 3 of cucumber can correspond to chromosome 2 and 3 of *Arabidopsis*, and ATG homologous genes on chromosome 4 of cucumber can correspond to chromosome 5 of *Arabidopsis*, and the ATG of homologous genes on chromosome 5 of cucumber can correspond to chromosome 2, 3 and 4 of *Arabidopsis*, and the ATG homologous genes on chromosome 6 of cucumber can correspond to chromosome 1, 3, 4, and 5 of *Arabidopsis*. Therefore, the ATG genes of cucumber have a high colinearity with ATG genes of *Arabidopsis*.

Analysis of conserved motifs of ATG genes in cucumber

We used the MEME web server to search for conserved sequence motifs and to predict their composition and diversity of the motifs present in the CsATG proteins. A total of 20 different conserved motifs were found in these CsATG proteins. It is clear that some genes (*CsATG8a*, *CsATG8b*, *CsATG8c*, *CsATG8d*, *CsATG8e*, and *CsATG8f*) share the same motif (Figure 4), which shows that members of the same subfamily usually contain very similar or the same conserved structural motif. Some CsATGs, such as *CsATG5a*, *CsATG5b*, and *CsATG13a*, have no motif structure, but *CsATG9* contains a maximum of 11 motifs.

Chromosomal location of ATG genes in cucumber

The cucumber genome contains seven chromosomes. The physical location information of 20 cucumber autophagy genes was obtained from the PHYTOZOME (www.phytozome.net/) database. 20 CsATG genes were unevenly located on seven chromosomes of cucumber (Fig. 5). Chromosomes 3 and 6 each contained 6 autophagy genes, Chromosome 2 contained 3 autophagy genes, Chromosomes 1, 5, and 7 each contained 2 autophagy genes, and Chromosome 1 contained only 1 gene. The *CsATG8a*, *CsATG8e*, *CsATG8b*, and *CsATG18b* genes were segmental duplication, the *CsATG11*, *CsATG18b* genes were

tandem duplication, the *CsATG5a*, *CsATG5b*, and *CsATG5c* genes were tandem duplication, and the *CsATG13a* and *CsATG13b* genes were tandem duplication.

Selection pressure analysis of homologous gene pairs

Ks represents the background base replacement rate of the evolutionary process. Ks can be used to infer the time of the event, such as the whole genome doubling time which has an important role in exploring the origin of species. Through BLASTn sequence similarity analysis, we identified two putative homologous gene pairs (*CsATG8a/CsATG8e* and *CsATG8b/CsATG8e*) in the cucumber genome. To estimate the divergence time of the ATG gene, we calculated the Ka/Ks ratio of these two gene pairs. We also conducted a statistical analysis of the Ka/Ks ratio and Ks value. The Ks values of the *CsATG8a/CsATG8e*, and *CsATG8b/CsATG8e* paralogous pairs were 1.3233 and 2.0606. The Ka values of the *CsATG8a/CsATG8e*, and *CsATG8b/CsATG8e* paralogous pairs were 0.0956 and 0.1416. Then the large-scale duplication activity was about 108.46 and 168.9 MYA. The Ka/Ks distribution in the cucumber genome ranges from 0.068 to 0.072. When Ka/Ks <1, this indicates that a gene has undergone CED negative selection and purification selection. Therefore, in the cucumber genome, there is a strong purification selection in the ATG gene.

Expression profiles of ATG genes in cucumber under phytohormone, abiotic stress, and carbon starvation treatments

In order to investigate the CsATG genes response to salicylic acid (SA) and Methyl jasmonate (MeJA), qRT-PCR of 20 CsATG genes in leaves were performed. The result was showed in Figure 6. Under SA treatment, the expression of *CsATG16*, *CsATG11*, *CsATG18b*, and *CsATG5c* were down-regulated, while the other CsATG genes were up-regulated. The *CsATG2*, *CsATG3*, *CsATG9*, *CsATG101*, *CsATG27*, *CsATG8d*, *CsATG8f*, *CsATG13a*, and *CsATG13b* were up-regulated by more than 5 times. The time of the highest expression levels were at 6h for *CsATG8f* and *CsATG101*, at 9h for *CsATG8a*, *CsATG8b*, *CsATG8c*, *CsATG8d*, *CsATG8e*, *CsATG5a*, *CsATG5b*, and at 24h for *CsATG2*, *CsATG9*, *CsATG3*, *CsATG27* and *CsATG13a* after two hormones treatments. The ATG genes in the same subfamily exhibited the same expression patterns. Unlike SA treatment, under MeJA treatment (Figure 7) Most of ATG genes showed down-regulated expression (*CsATG5a*, *CsATG5b*, *CsATG5c*, *CsATG10*, *CsATG11*, *CsATG18b*, and *CsATG16*) or had no change in their expression level (*CsATG3*, *CsATG9*, *CsATG27*, *CsATG101*, *CsATG8d*, *CsATG13a*, and *CsATG13b*). While *CsATG8a*, *CsATG8b*, *CsATG8c*, *CsATG8e*, *CsATG8f*, and *CsATG2* were up-regulatory expression and their highest expression occurred at 9h. Interestingly, these up-regulated genes were also up-regulated under SA treatment. The genes *CsATG5c*, *CsATG11*, *CsATG18b*, and *CsATG16* were down-regulated genes under both SA and MeJA treatment. From the expression patterns of 20 CsATG genes, we can see that these ATG genes displayed different expression profiles after the treatment with the two hormones, which may be related to the two hormones participated different autophagy pathways.

Figure 7, Figure 8 and Figure 9 respectively showed the qRT-PCR results of 20 CsATG genes in the roots, stems and leaves of cucumber under drought, salt and carbon starvation.

In the roots (as shown in Fig. 7), under salt stress, the number of up-regulated CsATG genes (*CsATG101*, *CsATG11*, *CsATG10*, *CsATG10b*, *CsATG2*, *CsATG8a*, *CsATG8b*, *CsATG8d*, *CsATG8f*, *CsATG9* and *CsATG18b*) were about the same as the number of CsATG genes whose expression level did not change much (*CsATG3*, *CsATG27*, *CsATG5a*, *CsATG5b*, *CsATG8c*, *CsATG8e*, *CsATG13a*, *CsATG13b* and *CsATG16*). Under drought stress, only *CsATG10*, *CsATG101* and *CsATG8f* had no much change in the expression level. The other 17 CsATG genes were all up-regulated, in which the expression level of *CsATG2*, *CsATG3*, *CsATG5b*, *CsATG5c*, *CsATG8a*, *CsATG9* and *CsATG13a* were almost more than ten times that of the control group. Under the dark treatment, unlike salt and drought stress, all CsATG genes were up-regulated, in which nine CsATG genes (*CsATG3*, *CsATG27*, *CsATG5a*, *CsATG5b*, *CsATG5c*, *CsATG8f*, *CsATG13a*, *CsATG9* and *CsATG16*) had significant up-regulated expression, while these CsATG genes were interestingly not up- or down-regulated under salt stress. The expression levels of 20 CsATG genes after dark treatment were significantly higher than those under drought and salt stress.

In the stems (as shown in Fig. 8), under salt stress, there were only two ATG genes (*CsATG5c* and *CsATG8c*) whose expression were down-regulated. More ATG genes (*CsATG3*, *CsATG27*, *CsATG5a*, *CsATG5b*, *CsATG8a*, *CsATG8b*, *CsATG8e*, *CsATG13a*, and *CsATG16*) had no change in expression level. Moreover, significantly up-regulated CsATG genes were *CsATG101*, *CsATG10*, *CsATG2*, *CsATG9* and *CsATG13b*, whereas, others ATG genes (*CsATG8d*, *CsATG8f*, *CsATG11*, and *CsATG18b*) were slightly up-regulated. Under drought stress, *CsATG11*, *CsATG101* and *CsATG13b* had no much change in the expression level. These genes (*CsATG8c*, *CsATG8e*, and *CsATG8e*) were down-regulated, while other ATG genes were up-regulated; these three genes *CsATG3*, *CsATG5b* and *CsATG18b* were significantly up-regulated. Under the dark treatment, like salt stress, all CsATG genes were up-regulated, in which only five CsATG genes (*CsATG10*, *CsATG101*, *CsATG5b*, *CsATG5e*, and *CsATG13b*) had slightly up-regulated expression; other CsATG genes were significantly up-regulated.

In the leaves (as shown in Fig. 9), under salt stress, 13 CsATG genes *CsATG10*, *CsATG11*, *CsATG2*, *CsATG3*, *CsATG5c*, *CsATG8a*, *CsATG8b*, *CsATG8c*, *CsATG8d*, *CsATG8f*, *CsATG9*, *CsATG18b* and *CsATG13a* were slightly up-regulated. *CsATG16* was the only gene that was significantly up-regulated. The genes *CsATG27*, *CsATG5a* and *CsATG5b* had no change in expression level. Down-regulated genes were *CsATG13b*, *CsATG101* and *CsATG8e*. Under drought stress, there were seven ATG genes (*CsATG11*, *CsATG2*, *CsATG8a*, *CsATG8d*, *CsATG8f*, *CsATG18b* and *CsATG8e*) were slightly up-regulated, while only one ATG gene was significantly up-regulated, that was *CsATG16*. Most ATG genes had no change of expression level (*CsATG10*, *CsATG8b*, *CsATG9*, *CsATG8c*, *CsATG3*, *CsATG5b*, *CsATG5a* and *CsATG13a*) or were slightly down-regulated (*CsATG27*, *CsATG5c*, *CsATG13b*, and *CsATG101*). Under the dark treatment, all ATG genes were up-regulated, in which *CsATG101*, *CsATG2*, *CsATG27*, *CsATG5c*, *CsATG8a*, *CsATG8b*, *CsATG8c*, *CsATG8e*, and *CsATG18b* were slightly up-regulated, the other 11 ATG genes were significantly up-regulated more than 5 times. We found that most ATG genes had the highest expression level at 6h after treatment, then slightly or sharply decrease their expression until 24h.

Discussion

Identification of 20 CsATGs in the cucumber genome

In recent years, autophagy-related genes in multiple plant species have been identified. 41 ATG genes have been identified in yeast, and more than 30 ATG genes have been identified in the genomes of Arabidopsis, corn, rice and tobacco, respectively, which showed the evolutionary conservation of core autophagy mechanisms in different species. In our study, 20 ATG genes were found in the cucumber genome, which is much less than the number of autophagy genes in rice, tobacco and corn. Unlike yeast, which has only a single copy, autophagy genes present in other species have multiple copies, such as *ATG8*. There are 5, 9, and 11 copies of *ATG8* in tobacco, Arabidopsis and soybean, respectively (Chung, et al. 2009). In cucumber, the autophagy gene *ATG8* also has multiple copies, (*ATG8a-ATG8f*), which is similar to that in Arabidopsis, tobacco and so on.

Phylogenetic analysis of cucumber ATGs

The phylogenetic tree indicated that the ATG gene family of cucumber was divided into 4 groups (G1-G4), and each group contained ATG genes from both cucumber and Arabidopsis. Among these groups, CsATG family members are closely related to the corresponding members in Arabidopsis, such as: GROUP1 (*AtATG3* and *CsATG3*, *AtATG2* and *CsATG2*, *AtATG10* and *CsATG10*, *AtATG18b* and *CsATG18b*), GROUP2 (*AtATG9* and *CsATG9*), GROUP3 (*AtATG16* and *CsATG16*), GROUP4 (*AtATG8a* and *CsATG8a*, *AtATG8b* and *CsATG8b*, *AtATG8c* and *CsATG8c*, *AtATG8d* and *CsATG8d*, *AtATG8e* and *CsATG8e*, *AtATG8f* and *CsATG8f*, *AtATG11* and *CsATG11*, *AtATG101* and *CsATG101*, *AtATG13a* and *CsATG13a*, *AtATG13b* and *CsATG13b*). Each CsATG protein sequence is highly similar to a homologous protein in Arabidopsis, which indicating that their ATG genes are largely functionally similar, and these results are consistent with current understanding of plant evolutionary history (Yoshimoto, et al. 2004). So far, the expression or function of most autophagy genes in Arabidopsis has been reported, which lays the foundation for predicting the function of cucumber autophagy genes.

Analysis of ATG gene structure in cucumber

In general, the pattern of introns and exons in the genome provides important evidence for their evolutionary relationship. In this study, we comprehensively analyzed the exons and introns structure, distribution and length of ATG gene family members in cucumber. We found the conservation in the exon-intron structure of the ATG genes in cucumber, which was similar to that of Arabidopsis and our results revealed that they have a highly conserved conlinearity. It supports a close evolutionary relationship between the plants.

Analysis of conserved motifs of ATG genes in cucumber

This study found that *CsATG8a*, *CsATG8b*, *CsATG8c*, *CsATG8d*, *CsATG8e*, *CsATG8f* share the same motif. It can be seen that members of the same subfamily usually contain very similar or identical conservative structural motifs, which indicates that different members in the same group may have functional redundancy. However, the motif in *CsATG16*, *CsATG18b*, *CsATG5a*, and *CsATG5b* is different, which

indicates the different members of the same group have different functions and different responses to different environmental signals. Our results were similar to that of previous studies on tobacco and *Arabidopsis* (Xia, et al. 2011).

Chromosomal mapping of autophagy gene family in cucumber

Gene duplication (including tandem duplication, segmental duplication, and whole-genome duplication) plays an important role not only in the process of genome shuffling and genome expansion, but also in the diversification of gene functions and the large-scale generation of gene families (Vision, et al. 2000). In this study, it was found that the physical positions of 20 CsATG homologues were unevenly assigned to 7 chromosomes in cucumber. This is consistent with the uneven distribution of 33 OsATG genes on 12 chromosomes in rice (Xia, et al. 2011), and 37 SiATG genes on 9 chromosomes in millet (Liu and Bassham 2012). Both segmental duplication and tandem duplication of genes lead to multiple copies of the CsATG gene, which play an important role in the expansion of the CsATG gene family. In addition, some repetitive genes also play important roles in the evolution of new traits and in speciation. Some genes may play additional important role in long-term evolution.

Selection pressure analysis of homologous gene pairs

In genetics, the Ka/Ks ratio is an important parameter that determines whether positive Darwinian selection is related to gene differentiation (Juretic, et al. 2005). In this study, the Ka/Ks ratio of 2 pairs of duplicate genes in the CsATG gene family is <1 . Compared with both the Ka/Ks ratio of 4 pairs of duplicate genes in the OsATG gene family is <1 (Xia, et al. 2011) and the Ka/Ks ratio of 6 pairs of duplicate genes in the SiATG gene family is <1 (Li, et al. 2016), the CsATG gene family has less pressure for purification selection than the SiATG and OsATG gene families. In all organisms, most non-synonymous substitutions are deleterious mutations; only a few are neutral or advantageous mutations. The positive Darwinian selection will retain the advantages of non-synonymous mutations, and purification selection will gradually remove deleterious non-synonymous mutations (Juretic, et al. 2005).

Expression profiles of ATG genes in cucumber under phytohormone, abiotic stress, and carbon starvation treatments

Plants are often exposed multiple stresses, such as salt, cold and drought in nature, and have evolved a set of defense mechanisms to adapt to the environmental stresses. Plant hormones, such as auxin (IAA), gibberellin (GA), abscisic acid (ABA), salicylic acid (SA), and methyl jasmonate (MeJA), all participate in and regulate the stress response. Autophagy also plays an important role in plant stress response. In fact, plant hormones are closely related to cellular autophagy (Cao, et al. 2020; Porta and Jiménez-Nopala 2019). Although most of the *Arabidopsis* autophagy-deficient mutants may show signs of premature aging due to excessive accumulation of salicylic acid, these mutants can complete their life cycle under normal growth conditions. Tests on mutants of *Arabidopsis thaliana* with deficient in SA biosynthesis or its signaling have further demonstrated that the atg2 mutant requires the SA pathway for enhanced resistance to powdery mildew (Wang, et al. 2011). Some studies have shown that SA and its analogs can

activate autophagy in Arabidopsis cells, while autophagy negatively regulates SA synthesis and its signal transduction (Zhang, et al. 2017). Many studies have shown that there are complex networks of various hormones and their signaling pathways in plant growth and development and stress response (Koornneef, et al. 2008). In addition, a large number of gene chip data confirm that many genes are co-regulated by different hormones, which shows that hormone signals have a key function in coordinately regulating the same biological process in plants (De Paepe, et al. 2004).

In cucumber leaves after treatment with SA and MeJA, 20 autophagy genes showed different expression levels. Most ATG genes showed a downward trend under MeJA treatment, while most ATG genes showed an upward trend under SA treatment. There are six genes (*CsATG8a*, *CsATG8b*, *CsATG8c*, *CsATG8e*, *CsATG8f*, and *CsATG2*) that were up-regulated in both phytohormone treatment, and there are four genes (*CsATG5c*, *CsATG11*, *CsATG18b*, and *CsATG16*) that were down-regulated in both phytohormone treatments. This result is inconsistent with many previous studies. In millet, whether treated with SA or MeJA, only a few ATG genes were down-regulated, while the rest of the ATG genes were either up-regulated or remained unchanged (Li, et al. 2016). Of 30 ATG genes in tobacco, there were only 4 ATG genes and 11 ATG genes up-regulated by SA and JA, respectively, and there were only 1 ATG gene up-regulated by SA, while other ATG genes remained unchanged (Klionsky, et al. 2003). However, a recent study found that most of the ATG genes were up-regulated by LCSA when it is revealed that low concentration SA could inhibit MeJA-induced senescence in *Arabidopsis thaliana* leaves (Yin, et al. 2020). These results may indicate that there is a certain relationship among salicylic acid, methyl jasmonate and autophagy. Both the two hormones can induce the occurrence of autophagy, but the expression patterns of the genes caused by the two hormones are different, which can explain the different ways of SA and MeJA-induced autophagy in cucumber leaves.

High concentration of salts can cause ionic stress, and a large amount of oxidatively inactivated proteins will be produced in plant cells, which will cause greater damage to the plant. This is one of the reasons why stresses often induce plant autophagy. Studies have shown that some autophagy genes, such as *OsATG10b* in rice (Shin, et al. 2009), *AtATG18a* in Arabidopsis (Liu, et al. 2009) and *MdATG10* in apple (Huo, et al. 2020), were involved in salt stress. In this paper, we found that most of ATG genes response to salt stress, their expression decreased or increased under salt stress, except *CsATG16*, *CsATG5a*, *CsATG5b* and *CsATG27*. In general, when salt treatment of cucumber roots, stems and leaves, the highest expression levels of autophagy-related genes in response to salt stress were found in the leaves, followed by roots, and finally stems. This shows that autophagy responds to salt stress in different ways and in different degrees.

Drought stress can activate autophagy pathway in plants to favor their environmental adaptations. Many genes have been revealed to play an important role in drought resistance in plants and their overexpression can simultaneously improve plant resistance to drought stresses (Bao 2020; Kuzuoglu-Ozturk, et al. 2012; Li, et al. 2015; Luo, et al. 2020; Sun, et al. 2018; Tsugane, et al. 1999). In wheat, *ATG8* expression was strongly up-regulated under drought stress, especially in the roots when compared to leaves (Kuzuoglu-Ozturk, et al. 2012). In foxtail millet, *SiATG8a* expression was dramatically induced by

drought stress treatments (Li, et al. 2015). In this paper, we also found that most of ATG genes response to drought stress. In contrast to salt stress, the number of drought stresses responsive ATG genes in roots and stems was higher than in leaves, which imply that autophagy plays an important role in roots in response to drought stress.

Carbon is the highest content element in plants and plays an important role in the growth and development of plants. The roles of carbohydrates in plants include the energy consumed to maintain metabolism, the construction of carbon skeleton, the energy consumed during defense, and the maintenance of turgor pressure (Izumi, et al. 2013). Carbon starvation means that the organic carbon fixed by photosynthesis in plants cannot satisfy the consumption of respiration. This phenomenon often occurs during the growth and development of plants (McDowell and Sevanto 2010). In plants, in order to adapt to the nutrient deficiency, a mechanism for nutrient recovery and recycling has evolved. This mechanism is often called autophagy (Rodríguez, et al. 2014; Toyooka, et al. 2006). Studies have shown that autophagy-related genes will undergo a coordinated transient up-regulation during starvation, which provides a basis for plant autophagy to play an important role in the nutrient cycle (Chung, et al. 2009). The *Arabidopsis atg11* mutant appears more sensitive under nutrient-restricted conditions (Li, et al. 2014; Li and Vierstra 2014). In this paper, we found that the expression of 20 ATG genes in cucumber roots, stems, and leaves increased significantly under dark treatment compared to under other stress treatments (high salt and drought). Furthermore, the expression level of ATG genes in cucumber under dark treatment, like under drought stress, was higher in roots than in leaves, and the expression level was the lowest in stems. This indicates that there are differences in spatiotemporal expression in these three tissues, and autophagy responds to abiotic stress through different pathways.

Conclusion

In this paper, we identified a total of 20 cucumber ATG genes by genome-wide analysis, and analyzed the chromosomal distribution, intron-exon structures, duplication and divergence rates, et al. We explored the expression profile of the 20 CsATG genes in response to SA and MeJA, two hormones related with autophagy, especially in response to three kinds of abiotic stresses (salt stress, drought stress and carbon starvation stress). We found that 20 CsATG genes are specifically expressed in cucumber roots, stems and leaves, and can respond to high salt, drought and carbon starvation stresses to varying degrees. This study provides important clues for studying the function of cucumber ATG genes.

Methods

Plant materials and treatments

The experiment was conducted in the Key Laboratory of Plant Biology, Harbin Normal University from March to June, 2019.

Cucumber line 9930 was donated by Prof. Huang Sanwen (Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences), and was used for cloning of CsATG genes. Cucumber variety “Changchunmici” was used to expression analysis of CsATG genes. The seedlings were cultivated in a light incubator at a temperature of 28°C for 14 hours under light and a temperature of 24°C for 10 hours in the dark, and were used in following treatments when the seedlings grow to the fourth true leaf. For hormones treatment, 50 µL of 10 mM SA or 0.2% MeJA was added dropwise at 3 cm intervals between leaf veins. The seedlings were exposed to 200 mM NaCl solution for salt stress, or to 20% (w/v) polyethylene glycol (PEG6000) for drought stress. The seedlings were covered with black plastic bags for dark treatment (as carbon starvation stress). Samples from the roots, stems and leaves of the treated plants were harvested at 0 h, 6 h, 9 h and 24 h after the treatment, and frozen in liquid nitrogen and stored at -80 °C for RNA extraction.

Database searches for cucumber ATG genes

To identify the ATG gene members in cucumber, the Hidden Markov Model (HMM) profiles of all sequences containing an ATG domain (PF03987) were used to search the Cucurbit Genomics database (<http://www.cucurbitgenomics.org/organism/2>). All redundant sequences were removed using the ‘decrease redundancy’ tool at http://web.expasy.org/decrease_redundancy/. In addition, in order to verify the identity of these putative CsATGs, the non-redundant candidate ATG genes in cucumber were identified using the InterproScan program to confirm the existence of the conserved ATG domain, and all of the ATG genes without an ATG domain were discarded. Information for the CsATG genes and predicted proteins, including CDS lengths, the predicted number of amino acids, and physicochemical parameters were obtained from the CuGenDB (<http://cucurbitgenomics.org/>).

Phylogenetic analyses and intron-exon structure determination

To examine the domain organization of the predicted ATG proteins in cucumber in detail, multiple sequence alignments of ATG domain-containing sequences were performed using Clustal W software, and we constructed a phylogenetic tree based on the complete CsATG sequences using the N-J method as implemented in MEGA software (v5.1) with a bootstrap analysis of 1000 replicates. The combined phylogenetic tree of AtATG and CsATG protein sequences was generated using the same method. In addition, the predicted exon-intron structures of the CsATG genes were visualized using the online Gene Structures Display Server (<http://gsds.cbi.pku.edu.cn>) by comparing the cDNA with the corresponding genomic DNA sequences.

Chromosome location and

All genes were mapped to 7 cucumber chromosomes in ascending order of CsATG gene physical location (bp). From short arm telomeres to long arm telomeres, they were visualized using MapChart.

Identification of conserved protein sequence motifs

Conserved motifs present in the CsATG proteins were identified with the online MEME tool (<http://meme.sdsc.edu/meme/intro.html>) using the default parameter settings: maximum number of motifs = 20; optimum motif length range between 6 and 200. We also used the Pfam (<http://pfam.sanger.ac.uk/search>) and SMART (<http://smart.embl-heidelberg.de/>) databases to annotate the structural motifs.

Identification of paralogs and Arabidopsis orthologs in cucumber

We used BLASTn to perform all-against-all nucleotide sequence similarity searches of the transcribed ATG sequences to identify paralogous sequences as shown by Blanc and Wolfe. Sequences that showed at least 40% identity with aligned regions >300 bp were defined as paralogs. Putative Arabidopsis orthologs were identified by using each sequence as a query to search against all sequences from cucumber. If the ATG gene sequences gave the best hit, and >300 bp of the two sequences aligned, the two genes were then defined as being orthologous.

Calculation of Ka/Ks values

Pairwise alignments of the paralogous and orthologous ATG gene sequence pairs were performed with ClustalX 2.11, and the results were further analyzed using MEGA 6.0. A synonymous substitution (Ks) is defined as a mutation in which a nucleotide base is replaced by a different base in a protein-coding region of a gene that does not result in an amino acid change in the encoded protein, while a non-synonymous substitution (Ka) results in a change in the amino acid sequence of a protein. The non-synonymous and synonymous substitution rates were then calculated using DnaSP 5 to analyze gene duplication events. Ks can be used as a proxy for time when dating large-scale duplication events (Ina 1995). Therefore, the date of duplication events was subsequently converted into divergence time (T) using the formula $T = Ks / 2\lambda$ MYA for each gene pair ($\lambda = 6.1 \times 10^{-9}$).

Total RNA isolation and quantitative real-time PCR

The total RNA of the samples was extracted with the OminiPlant RNA Kit (DNase I) from Beijing CoWin Biotech Co., Ltd., and cDNA synthesis was finished using HiFiScript cDNA Synthesis Kit from Beijing CoWin Biotech Co., Ltd. Quantitative RT-PCR (qRT-PCR) was performed to detect the relative expression level of the CsATG genes using ABI 7500 Fast Real-Time PCR System.

We then designed 20 pairs of gene-specific primers using Primer Express 3.0. qRT-PCR amplifications were performed on an ABI 7300 Real-Time system (Applied Biosystems) in 20 μ L reactions containing 1 μ L of each gene-specific primer, 1 μ L of cDNA sample, 7 μ L dH₂O, and 10 μ L SYBR Green Master Mix reagent (Applied Biosystems). All primers for amplification of CsATG genes are given in Table 2. The qRT-PCR amplification conditions were: 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s, 55 °C for 15 s, and 72 °C for 10 s. A melting curve analysis was performed for each sample to verify the specificity of the reactions. There were three biological and three technical replicates performed for each sample. The relative expression levels were evaluated using the $\Delta\Delta$ CT method. It is worth noting that for the stress

treatments, relative gene expression [$2^{-\Delta\Delta CT}$, CK (0 h)] for each gene in the control plants was normalized to 1 as described previously (Schmittgen and Livak 2008).

Declarations

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Conflicts of interest/Competing interests

All authors declare that they have no competing interests.

Authors' contributions

GHD and NIE conceived and designed research. YY and YHH conducted experiments. YW conducted the bioinformatic work. AM and GR analyzed data. YY and GHD wrote the manuscript. All authors read and approved the manuscript.

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Tables

Table 1 Amino acid sequence length, predicted molecular weight and predicted isoelectric point of 20 CsATGs

Gene ID	Gene name	Gene Name in NCBI	Chromosome	Number of amino acids	Molecular weight	Isoelectric point
Csa1G002820	<i>CsATG16</i>	<i>CsATG16</i>	Chr1	511	56194.55	6.19
Csa1G062890	<i>CsATG8a</i>	<i>CsATG8c</i>	Chr1	118	13650.76	8.78
Csa2G036630	<i>CsATG11</i>	<i>CsATG11</i>	Chr2	1113	125745.37	5.71
Csa2G036700	<i>CsATG18b</i>	<i>CsATG18b</i>	Chr2	317	34676.75	7.68
Csa2G418350	<i>CsATG8b</i>	<i>CsATG8c</i>	Chr2	119	13731.96	8.62
Csa3G197920	<i>CsATG5a</i>	<i>CsATG5</i>	Chr3	87	9853.73	6.23
Csa3G197930	<i>CsATG5b</i>	<i>CsATG5</i>	Chr3	95	10927.65	5.82
Csa3G197940	<i>CsATG5c</i>	<i>CsATG5</i>	Chr3	247	27630.68	4.82
Csa3G207330	<i>CsATG2</i>	<i>CsATG2</i>	Chr3	1940	214677.03	5.43
Csa3G393920	<i>CsATG9</i>	<i>CsATG9</i>	Chr3	873	100515.82	6.24
Csa4G022890	<i>CsATG3</i>	<i>CsATG3</i>	Chr4	284	32432.02	6.42
Csa5G161950	<i>CsATG8c</i>	<i>CsATG8f</i>	Chr5	118	13689.82	8.71
Csa5G217160	<i>CsATG27</i>	□	Chr5	281	31272.59	8.95
Csa6G109660	<i>CsATG8d</i>	<i>CsATG8f</i>	Chr6	117	13504.43	6.74
Csa6G484550	<i>CsATG13a</i>	<i>CsATG13b</i>	Chr6	114	12382.08	9.77
Csa6G484560	<i>CsATG13b</i>	<i>CsATG13b</i>	Chr6	518	57544.66	9.16
Csa6G490120	<i>CsATG101</i>	<i>CsATG101</i>	Chr6	220	25842.86	6.31
Csa6G513730	<i>CsATG8e</i>	<i>CsATG8c</i>	Chr6	123	13721.89	9.33
Csa7G368220	<i>CsATG10</i>	□	Chr7	239	27350.99	4.81
Csa7G419640	<i>CsATG8f</i>	<i>CsATG8i</i>	Chr7	118	13644.74	7.74

Table 2 Primers for qRT-PCR analysis of the 20 CsATG genes.

Gene Name	Orientation	Primer Sequence (5'-3')
<i>CsATG16</i>	Forward	TACTAGTACAGGGGTTTGCTCTATC
	Reverse	GAAAGAGAAAGGTAAGGCTTGAGAC
<i>CsATG8a</i>	Forward	GTACCTTGTCCCTGCTGATCTGACT
	Reverse	AGCAGCAGTTGGAGGTAGTGTGTTT
<i>CsATG11</i>	Forward	TTCTGGCAGTGACAATGAAAATGAT
	Reverse	AAATCTTCTCCTTGTTTGCCTGCTT
<i>CsATG18b</i>	Forward	TCATATAGTTTTTCGCAGAGGATCAT
	Reverse	ACCTTCTGCTGCTTCTTTGGTTTAT
<i>CsATG8b</i>	Forward	ATATTTAGTTCCTGCTGATTTAACT
	Reverse	AGACATCATTGCAGCTGTAGGGGGC
<i>CsATG5a</i>	Forward	AATCTGAAGTCACCACCGTC
	Reverse	GTACCATTTAAGGGGCAAAC
<i>CsATG5b</i>	Forward	ATACCGCCTTGCAAATAC
	Reverse	AGGTTCCGACATGCTCAT
<i>CsATG5c</i>	Forward	TCGTCCTGTAGAGATCCGTG
	Reverse	TTTGTTGATTCCTCCTTGGT
<i>CsATG2</i>	Forward	TGTGGACCAGTTTTTTTGAATGCTTG
	Reverse	TGTGGACCAGTTTTTTTGAATGCTTG
<i>CsATG9</i>	Forward	ATTTTTCTTACTATTTGTTGATTGG
	Reverse	GACTGTTGTAATAGAAGTGACGGAT
<i>CsATG3</i>	Forward	ATGGTTCATTGGTTCGGAATTTTTC
	Reverse	ATTCCCAAGACCAAGAATCGGAATT
<i>CsATG8c</i>	Forward	ATACCTAGTTCCTGCAGACCTTACC
	Reverse	ATATTGCAGACATGATTGCTCCTGT
<i>CsATG27</i>	Forward	ATATAAAAATCCTGACAGGGGTGT
	Reverse	GGTCCCTGAATACCATCTGTCGTAC
<i>CsATG8d</i>	Forward	GTATCTAGTGCCTGCTGACCTGACT
	Reverse	TAGATGGCAGACATCAGTGATCCTG

<i>CsATG13a</i>	Forward	TCATCTTCTCTTGGACGAGATGAAA
	Reverse	CCTGCTACCTCTATCTGTCATCTCA
<i>CsATG13b</i>	Forward	GCGCCTGCCAAATGGTCTATTGCAG
	Reverse	GACTTTCCAATTGCAGAGCCAGCCG
<i>CsATG101</i>	Forward	ATATGTTTATCCTTTTATCAAGAGA
	Reverse	CTCTCTCCAGGATCAACTAGAATTT
<i>CsATG8e</i>	Forward	ATACCTCGTCCCCAAGGATTTAACT
	Reverse	AAATTGCAGACATCAATGCACCAGT
<i>CsATG10</i>	Forward	ATGGACAACCTCTGATGTTTGAAGA
	Reverse	GGATGCTCCTCCTGAGTAATAAATG
<i>CsATG8f</i>	Forward	ATGGGAAGAACCAAATCCTTCAAGG
	Reverse	GAAGGACTCCCCAAATCGTTGTTTCG

Figures

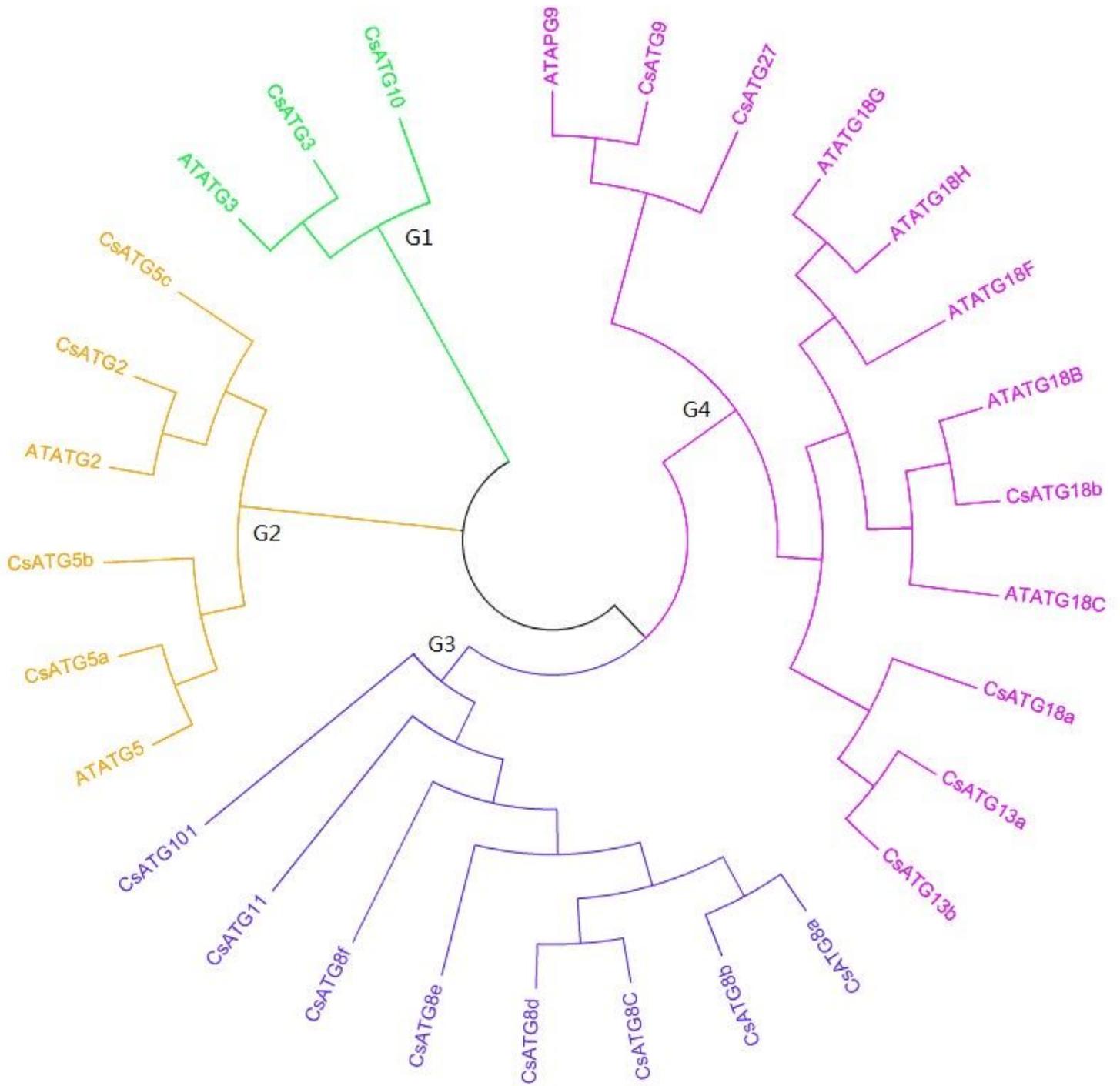


Figure 1

Phylogenetic tree of ATG proteins in *Arabidopsis* and *C. sativus*. The ATG genes of *C. sativus* are divided into four subfamilies (Groups 1-4, with blue, purple, green, and red colors, respectively). The tree was constructed from an analysis with MEGA 5.0 software using a maximum likelihood method.

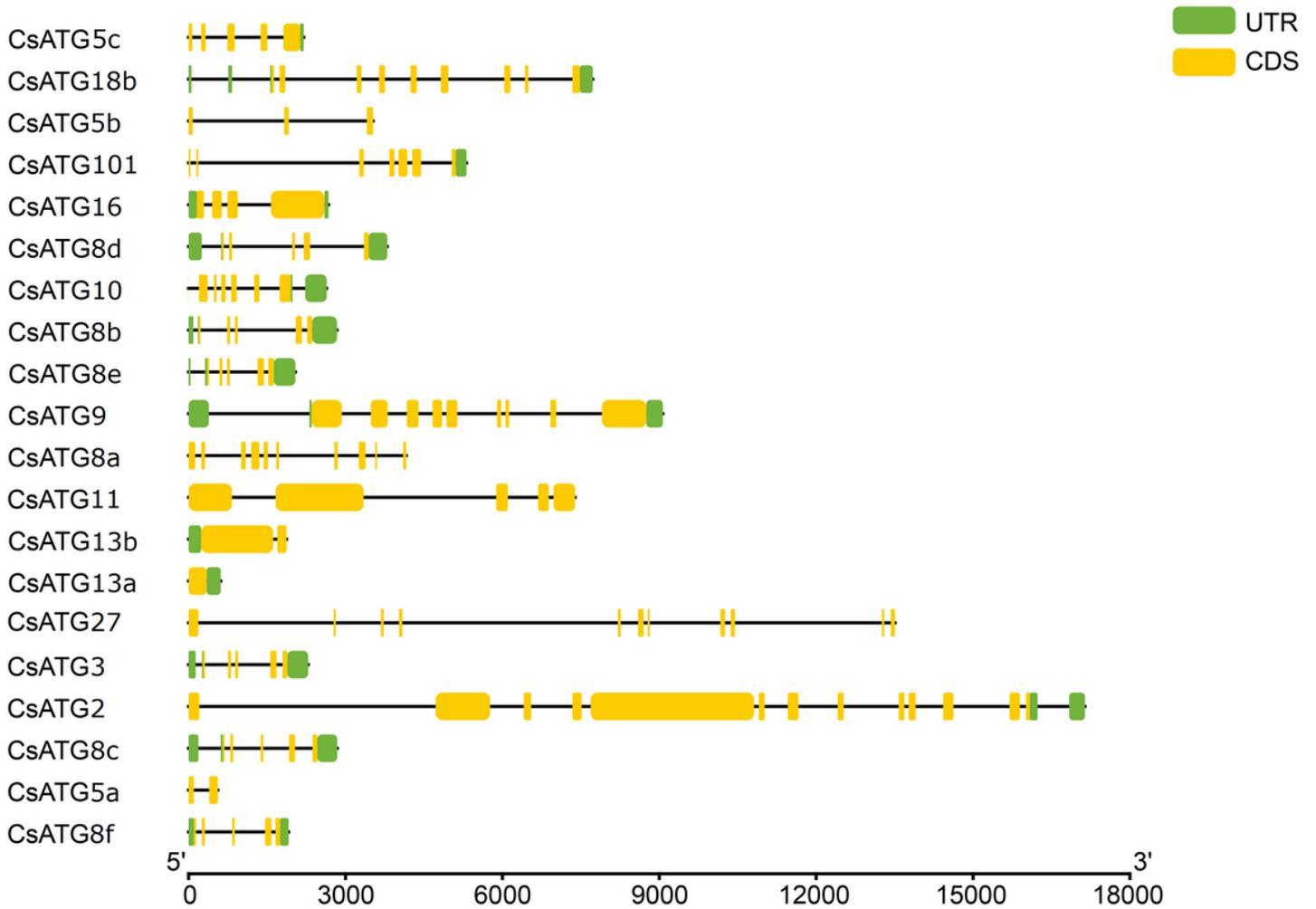


Figure 2

Exon-intron structure of the CsATG genes in *C. sativus*. The exon-intron structure coding sequence of the CsATG genes is compared with the corresponding genomic sequence using the GSDS program of the genetic structure. The green and yellow boxes represent untranslated upstream/downstream regions and coding region, respectively. Lines indicate introns.

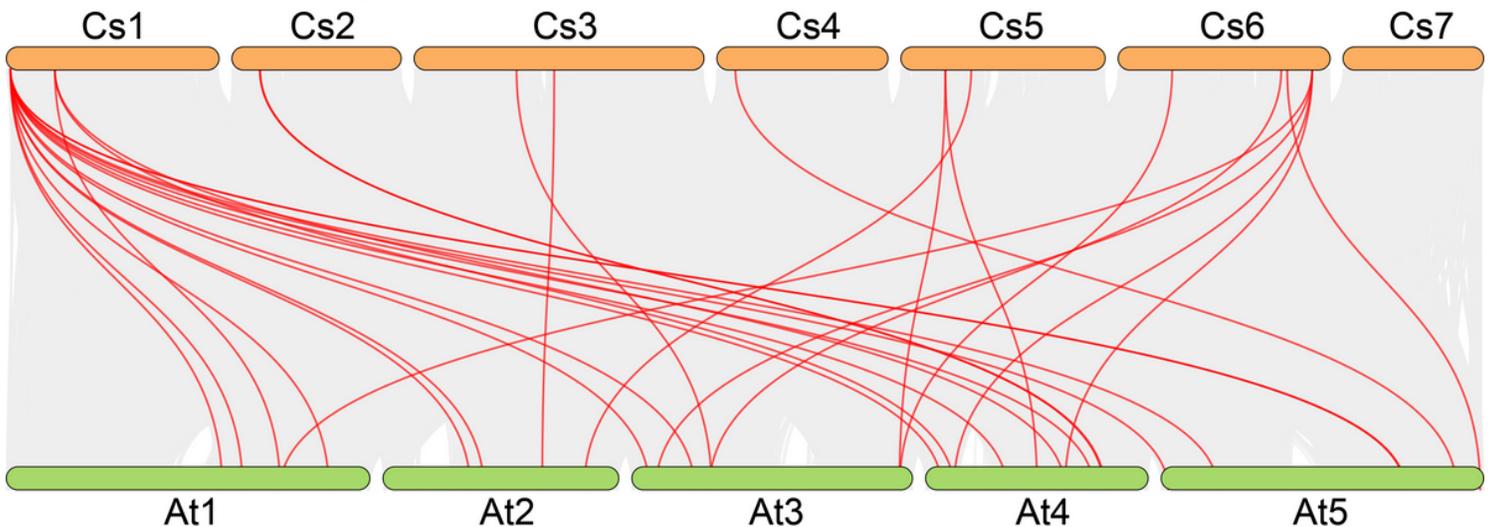


Figure 3

Synteny analysis of the ATG genes between *C. sativus* and Arabidopsis. The yellow and green bars represent the *C. sativus* and Arabidopsis chromosomes, respectively.

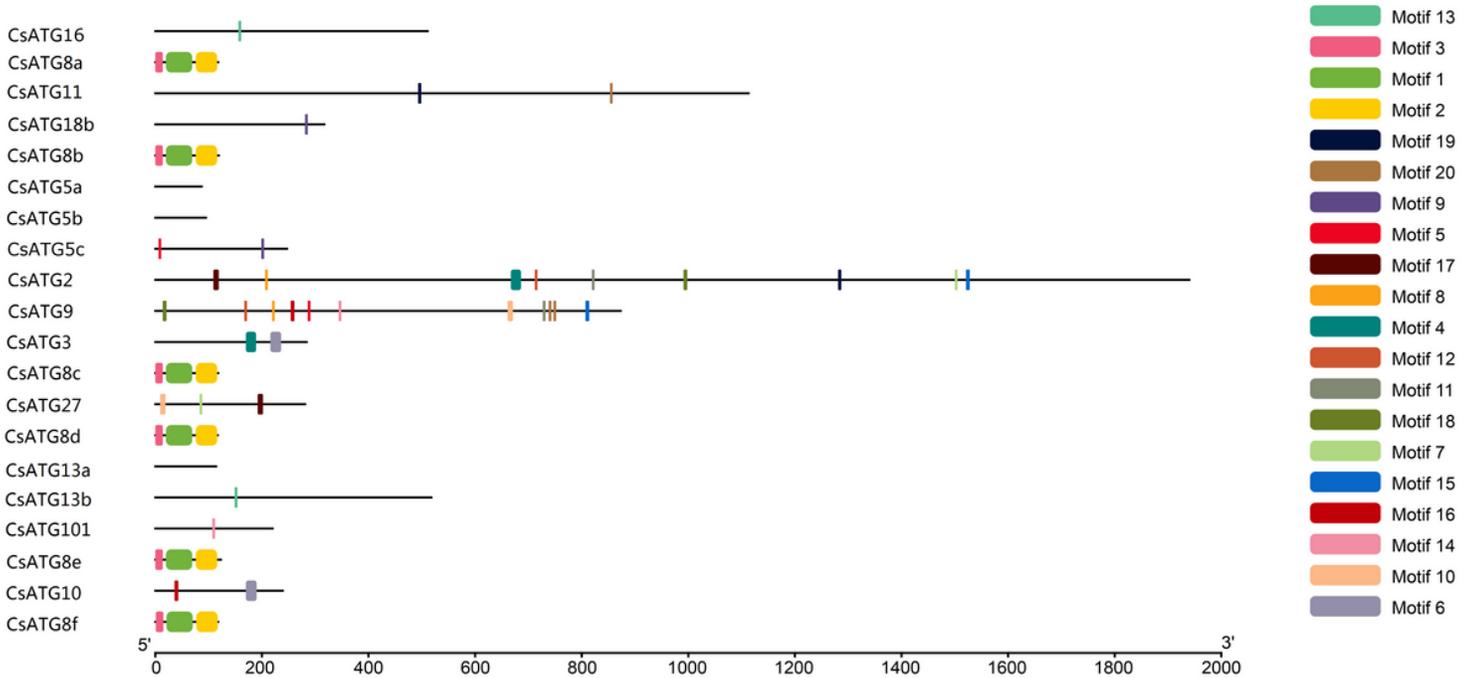


Figure 4

Conserved motifs of ATG proteins in *C. sativus*. Different color boxes represent different types of motifs, and the length of each box represents the actual motif size labeled by the number of nucleotides.

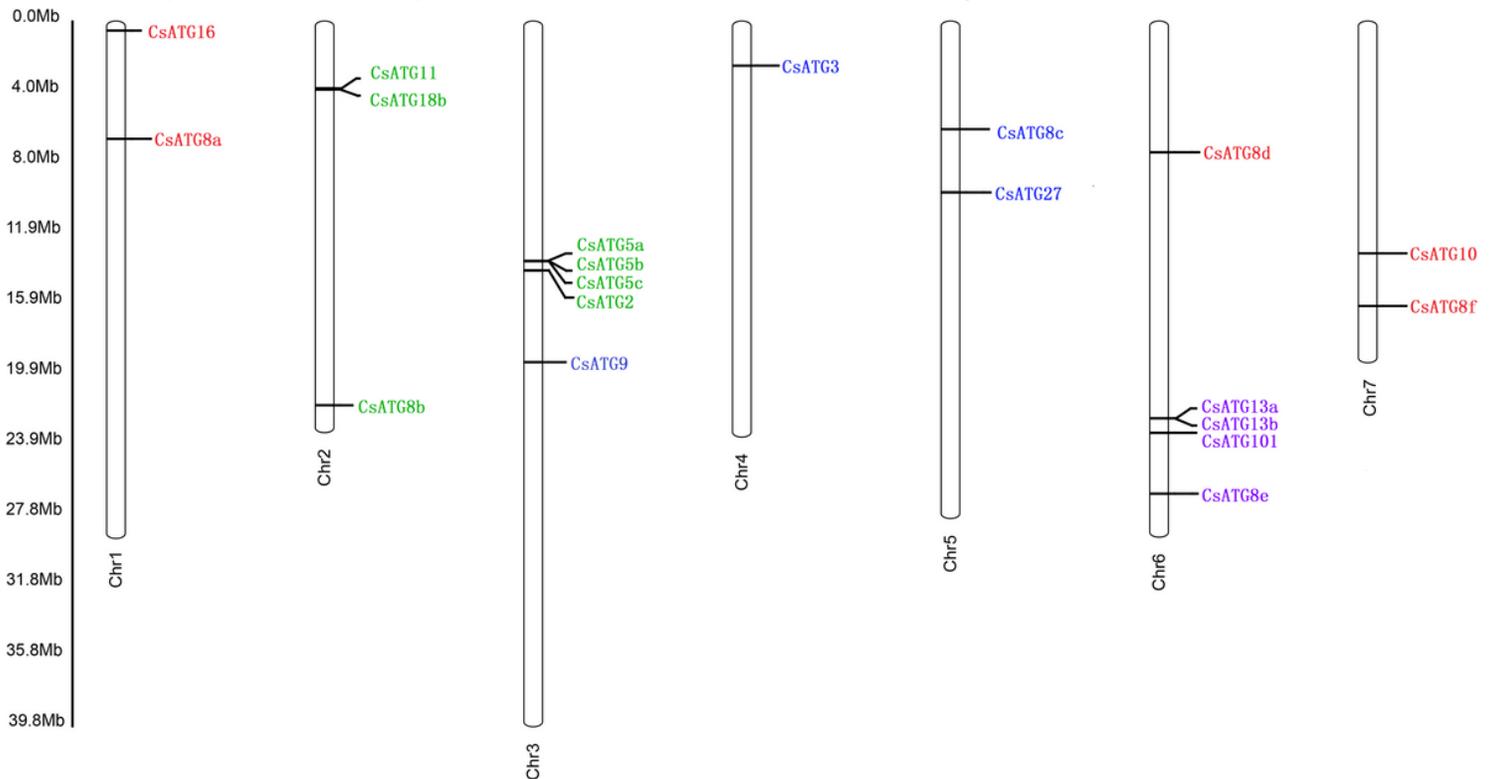


Figure 5

Distribution of 20 ATG genes on the seven chromosomes in *C. sativus*.

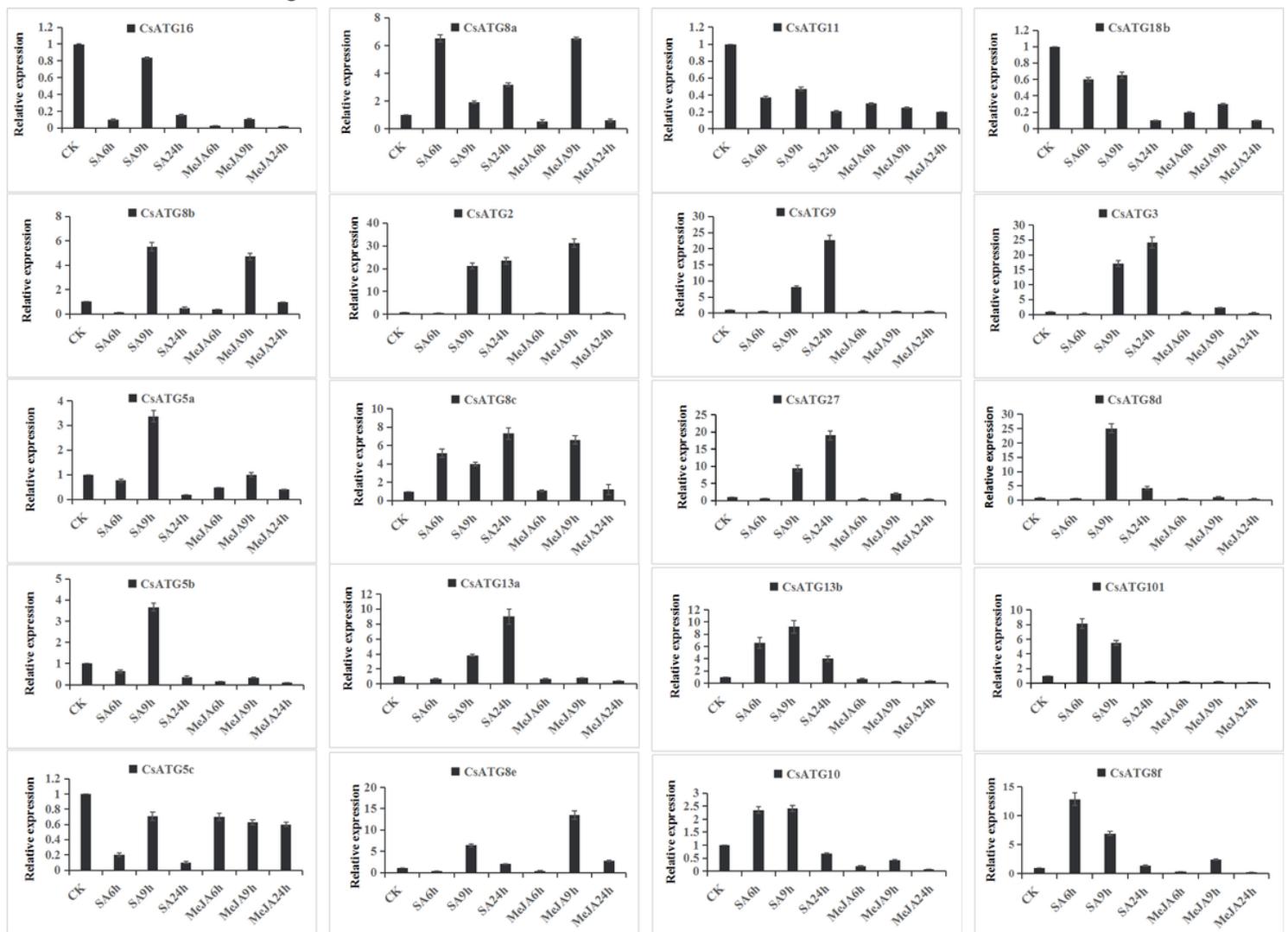


Figure 6

Expression of 20 CsATG genes in *C. sativus* under SA and MeJA treatment. The relative expression of each ATG in *C. sativus* seedlings under different hormone levels was normalized to Actin mRNA. 10 mM SA and 0.2% MeJA were added to the leaves for hormone induction treatment, and the expressions of each ATG were calculated at 6h, 9h, and 24h at the dropwise locations, and compared with the ATG of seedlings under normal growth conditions.

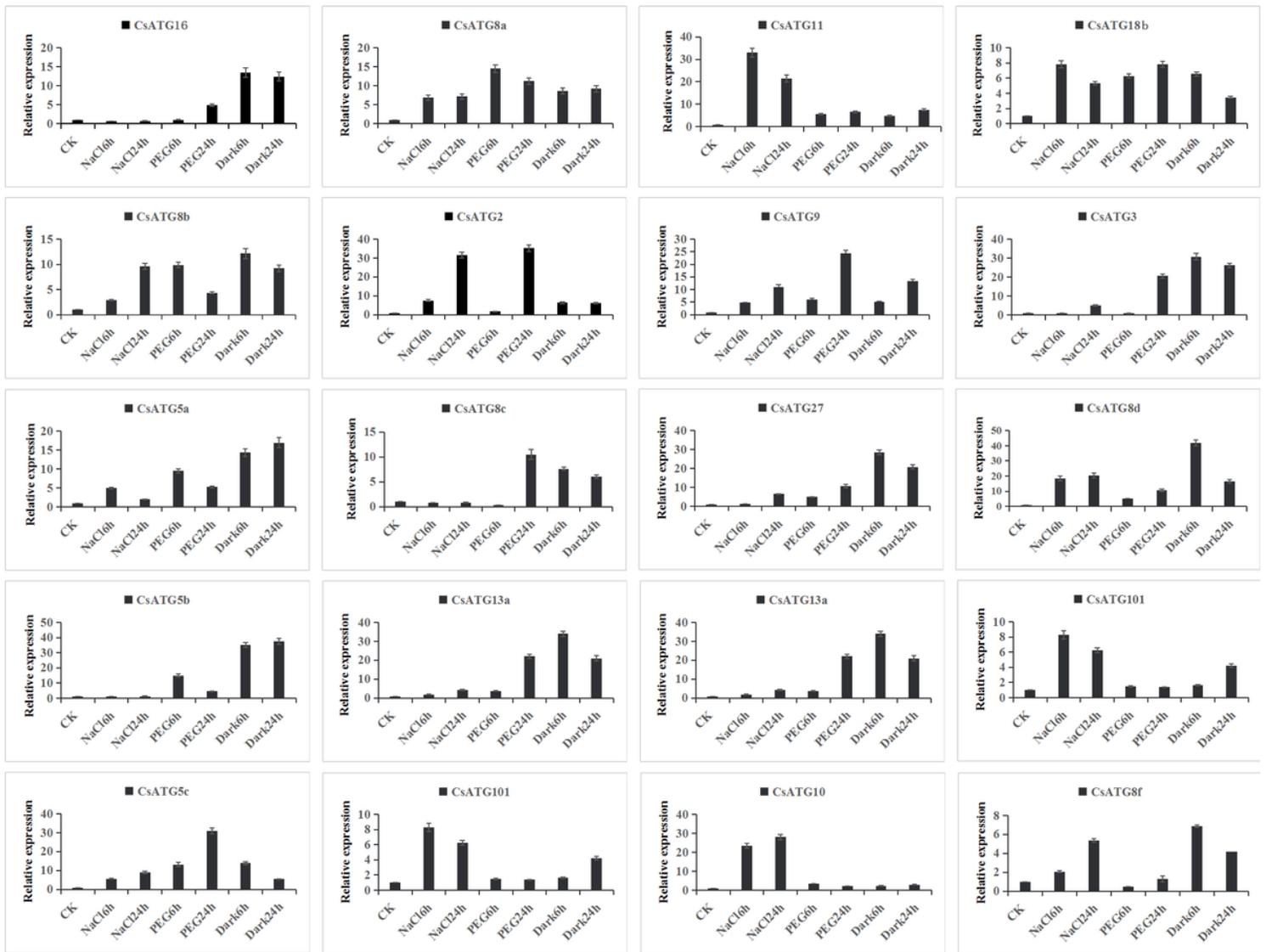


Figure 7

Expression of 20 CsATG genes in *Cucumis sativus* roots under three abiotic stresses. The relative expression of each ATG gene is normalized to Actin mRNA. The roots of the four-leaf stage seedlings were treated with 200 mM NaCl, 20% PEG6000, or in dark for 6h and 24h, respectively. The expression levels of each ATG were calculated and compared with the ATG expression levels of the seedlings under normal growth conditions.

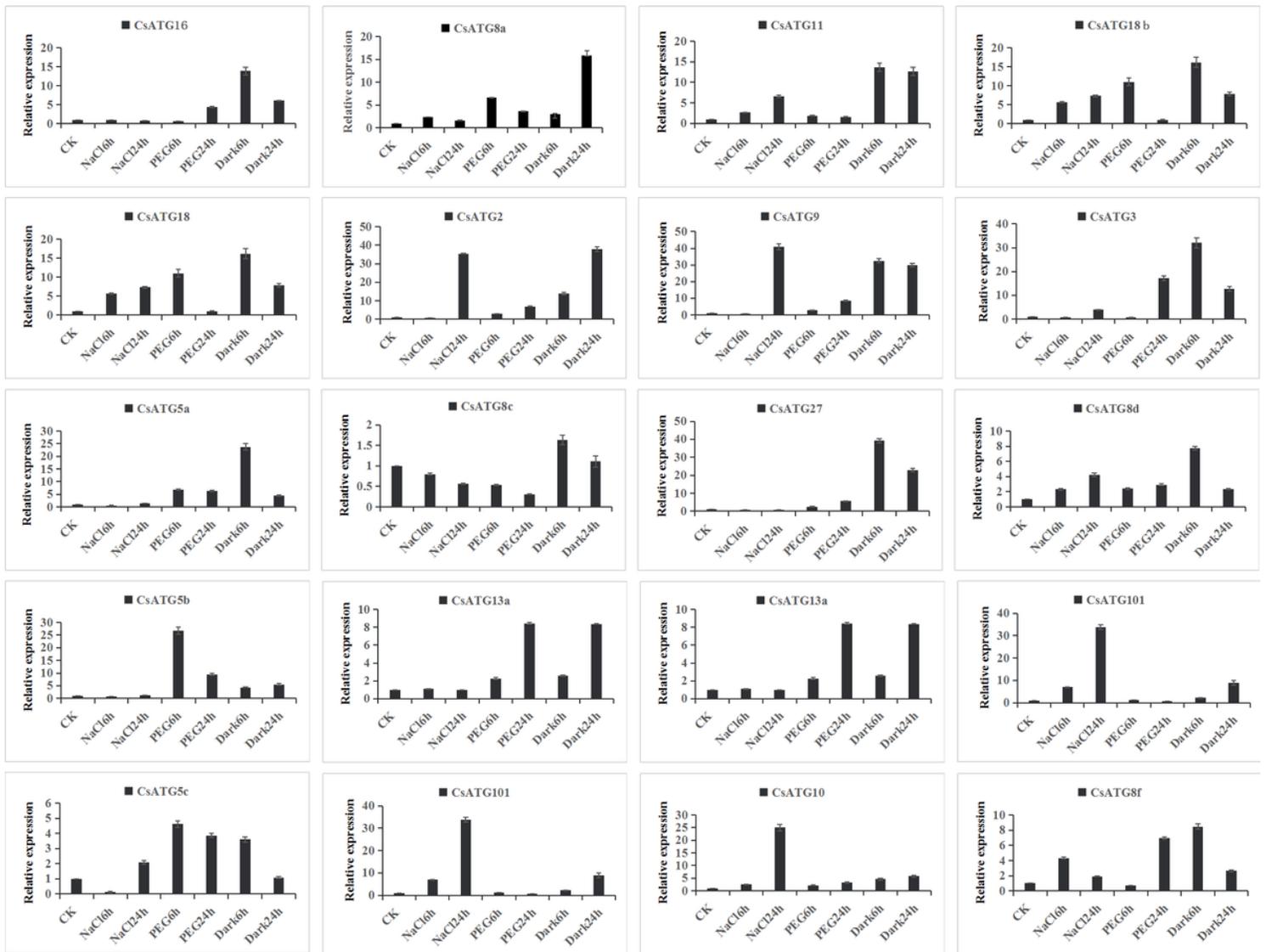


Figure 8

Expression of 20 CsATG genes in *C. sativus* stems under three abiotic stress. The relative expression of each ATG gene was normalized to Actin mRNA in stems of *C. sativus* seedlings under drought, high salt, and carbon starvation stress. Four-leaf stage *C. sativus* seedlings were treated with 200 mM NaCl, 20% PEG6000, or in dark for 6h and 24h, calculate the expression of each ATG, and compare with the ATG expression of seedlings under normal growth conditions.

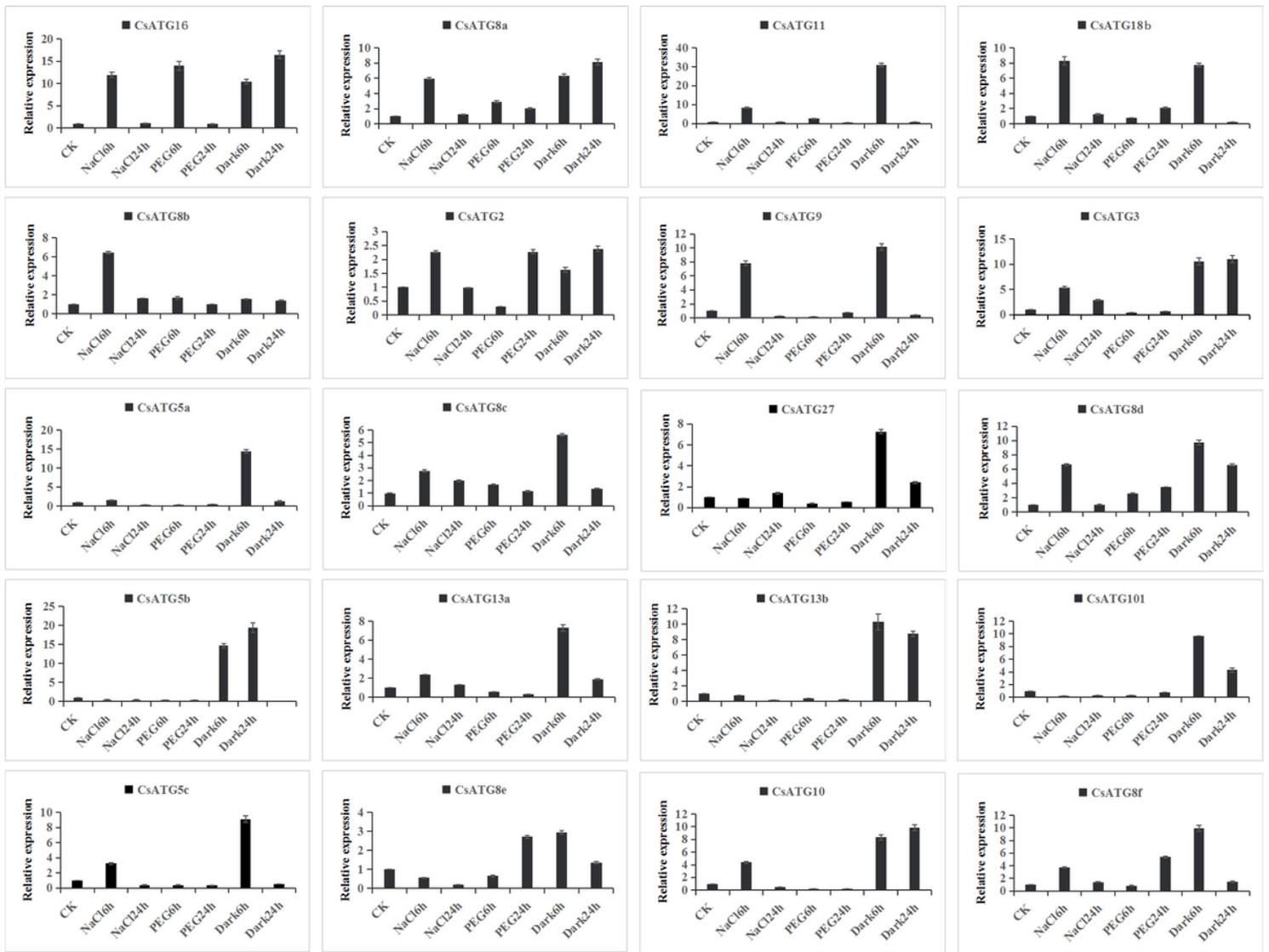


Figure 9

Expression of 20 CsATG genes in *C. sativus* leaves under three abiotic stresses. The relative expression of each ATG gene in leaves of *C. sativus* seedlings under drought, high salt and carbon starvation stress was normalized to Actin mRNA. The leaves of *C. sativus* seedlings were treated with 200 mM NaCl, 20% PEG6000, or in dark for 6h and 24h, calculate the expression of each ATG, and compare with the ATG expression of seedlings under normal growth conditions.