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Genome-wide sequence and expressional analysis of autophagy Gene family in bread wheat (*Triticum aestivum* L.)

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ABSTRACT

Autophagy, a highly conserved intracellular degradation system, is regarded to be responsible for self-defense and protect cells from abiotic stress. Extensive studies have demonstrated that autophagy plays a crucial role in regulating plant growth and development as well as in response to diverse stresses. However, little is known about autophagy-associated genes (ATGs) in wheat, especially those involved in the regulatory network of stress processes. In this study, a total of 108 putative wheat ATGs (TaATG) were obtained based on a genome-wide search approach. Phylogenetic analysis classified them into 13 subfamilies, of which the *TaAtg16* subfamily consisted of 29 members, ranking it the largest subfamily. The conserved motif compositions as well as their exon-intron structures were systematically analyzed and strongly supported the classification. The homoeologous genes tended to have similar gene features during wheat polyploidization. Furthermore, a total of 114 putative cis-elements were found, and those related to hormone, stress, and light responsiveness were abundantly presented in the promoter regions. Co-expression network analysis revealed that orthologous VAMP727 was the hub node of the whole network, and complex interactions were also found. Finally, the expression profiles of TaATGs among different tissues and under abiotic stresses were investigated to identify tissue-specific or stress-responsive candidates, and then 14 were validated by wet-lab analysis. Results showed that the *TaAtg8* subfamily played a crucial role in tissue autophagy and stress defense, which could be considered as processes that are candidates for further functional study. This was the first study to comprehensively investigate the ATG family in wheat, which ultimately provided important clues for further functional analysis and also took a step toward uncovering the evolutionary mechanism of ATG genes in wheat and beyond.

1. Introduction

In order to survive in adverse environmental conditions, plants evolve various mechanisms to resist or adjust to stresses. Autophagy, a major intracellular degradation system and evolutionarily conserved process in organisms, is one of such mechanisms that plants evolve for self-defense, and it can be induced by nutrient starvation and is essential for self-digestion to occur within the lysosome or vacuole in eukaryotic cells (Thompson and Vierstra, 2005; Yoshimoto et al., 2004). The macromolecules or organelles are sequestered into a double-membraned vesicle termed 'autophagosome'. Since it was first reported that vacuoles could induce autophagic degradation of cytosolic components under nutrient-deficient conditions in yeast (Takeshige et al., 1992; Yoshimoto et al., 2009), autophagy has increasingly attracted attention in biology as it is a highly interesting process, illustrating how cells break down and recycle damaged material and further function in abiotic stress resistance, such as under oxidative stress and starvation conditions (Chang et al., 2017; Shao et al., 2017; Shpilka et al., 2015; Yokota et al., 2017). Recent studies have revealed that the proteins of *ScAtg20* and *ScAtg24* can promote organelle autophagy in fission yeast (*Schizosaccharomyces pombe*) (Zhao et al., 2016). Under starvation conditions, fatty acid is delivered to the vacuole for degradation through autophagy induced by Vac8- and Atg24 (Shpilka et al., 2015). Most of the autophagy-associated genes (ATG) are

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involved not only in the process of degradation but also in the biosynthetic process. *ScAtg17-Atg13* complex formation plays an important role in normal autophagosome formation by binding and activating the *ScAtg1* kinase; this binding is generally enhanced under starvation conditions (Kabeya et al., 2005).

In plants, a large number of studies have demonstrated the essential roles ATG genes play in regulating plant growth and development as well as in stress responses (Guiboileau et al., 2012, 2013; Lv et al., 2014; Zhou et al., 2014a; Chen et al., 2015; Li et al., 2015; Luo et al., 2017). Autophagy mutants were initially isolated and characterized for functional analysis in *Arabidopsis* (Doelling et al., 2002). It was reported that disrupting the autophagy gene in *Arabidopsis* could accelerate leaf senescence and starvation-induced chlorosis (Hanaoka et al., 2002). KIN10, a plant ortholog of the mammalian AMPK, is involved in the positive regulation of autophagy, possibly by affecting the phosphorylation of *AtAtg1* (Chen et al., 2017). In rice (*Oryza sativa* L.), autophagy played an essential role in infection, pathogenesis, and development of the rice blast fungus (*Magnaporthe oryzae*) (Kershaw and Talbot, 2009; Zhou et al., 2017). In addition, autophagy-related protein *MoAtg14* was found to be involved in the differentiation, development, and pathogenicity of the rice blast fungus (Liu et al., 2017). Due to our improving knowledge on these species, the molecular mechanism and the role of ATG protein playing in autophagosome formation are gradually being understood. Increasing evidence shows that autophagy also plays a crucial role in diversifying cellular processes and maintaining cellular homeostasis under adverse environmental conditions (Masclaux-Daubresse, 2016). The repression of auxin-regulated TOR activity is necessary for autophagy activation in response to a subset of abiotic stress conditions (Pu et al., 2017), and overexpression of some autophagy-related genes can improve the resistance to low nitrogen, drought, hypoxia, and salt stress (Lv et al., 2014; Li et al., 2015; Luo et al., 2017). Generally, ATG4 and ATG8 subfamilies had more members than those of other subfamilies among the ATG genes family, and this phenomenon was conservatively found in yeast, animals as well as plants (Yoshimoto et al., 2004; Slobodkin and Elazar, 2013). *AtAtg8* belongs to a novel family of microtubule-binding proteins, which can operate both under favorable growth conditions and starvation stress (Yoshimoto et al., 2003; Ketelaar et al., 2004; Slavikova et al., 2005). *OsAtg8* interacting with the *OsAtg4* and *OsAtg8* conjugation pathway is conserved in rice and might play important roles in rice autophagy (Su et al., 2006). Comparative analysis of 28 ATG4 and 116 ATG8 genes from the available 18 different plant genomes reveals that ATG8 is evolutionarily conserved and that the cross-kingdom ATG8 processing is determined by the ATG8 sequence rather than that of ATG4 (Seo et al., 2016).

Bread wheat (*Triticum aestivum* L.) is one of the most widely grown crops worldwide, and serves as the staple food source for about 30% of the population around the world (International Wheat Genome Sequencing Consortium, 2014). By 2050, the world population is expected to increase to about 9 billion, and the production of wheat needs to increase by up to 70% to meet the future demands of 2050 (Tilman et al., 2002; Foley et al., 2011;). In addition to its agronomic importance, wheat is a newly formed allohexaploid species (2n = 6x = 42, AABBDD) with three (A, B, and D) homoeologous subgenomes, making it an ideal model for chromosome interaction and polyploidization studies in plants (Berkman et al., 2013). Recently, with the newly published genome draft sequence of hexaploid wheat genotype Chinese Spring (CS), a comprehensive investigation of the wheat gene family at the genome level discerning the homologous copies among the three sub-genomes has become accessible (International Wheat Genome Sequencing Consortium, 2014). The retention and dispersion of homologous genes will provide indispensable information about chromosome interaction during polyploidization. Up to now, genome-wide identification and characterization of ATG genes have been carried out in diverse plant species, such as rice (Xia et al., 2011), *Arabidopsis* (Xiong et al., 2005; Izumi et al., 2015), maize (Chung et al., 2009), tobacco (Zhou et al., 2015), and foxtail millet (Li et al., 2016). In light of its biological importance, the functions of some ATGs have been revealed in wheat. The *TaAtg4/Atg8*-associated autophagy process was found to play a negative role in the late stage of wheat immunity in response to Bgt, and was shown to be up-regulated by abiotic stresses and phytohormones (Pei et al., 2014). *TdATG8* is a positive regulator in the osmotic and drought stress response in wild emmer wheat (Kuzuoglu-Ozturk et al., 2012). In addition, three wheat ATG6s were identified to be essential for autophagy biogenesis and conveyed the immunity to powdery mildew (Yue et al., 2015). However, a systematical identification of the autophagy gene family in wheat has not been performed, especially of those involved in the regulatory networks of abiotic stress processes.

In this study, we searched the whole genome of bread wheat to identify autophagy gene family members using the bioinformatics analysis approach. Then, we examined the conserved motifs, gene structures, cis-regulatory elements, and conduced a phylogenetic relationship analysis to reveal the evolutionary and structural characteristics of wheat ATG genes (*TaATGs*). Furthermore, we analyzed the expression patterns in different tissues and under different abiotic stresses based on RNA-seq and further validated them by the qRT-PCR method, and then finally constructed their regulatory network. This study reports the genomic organization, gene structure, evolutionary features, and expressional patterns of wheat ATG genes, which may provide clues for further functional analysis and also contribute to better understanding the molecular mechanism of ATGs involved in regulating growth and development as well as stress processes in wheat.

2. Materials and methods

2.1. Identification of autophagy gene family in wheat

The protein sequences of the newly published wheat genome (TGAC_V1) were downloaded from the Ensemble database (http://plants.ensembl.org/Triticum_aestivum/Info/Index). In order to obtain a complete set of wheat ATG genes, an integrated method was employed in this study. First, a preliminary search for *TaAtg* homologs was performed using the key word 'autophagy' in the NCBI database (http://www.ncbi.nlm.nih.gov/) to collect autophagy-related genes from different species. Additionally, autophagy genes reported by previous studies were retrieved and downloaded as well (Xia et al., 2011; Li et al., 2016). The complete dataset of local ATG proteins was used to construct a HMM profile using the hmmbuild tool embedded in HMMER3.0 (http://hmmer.org/download.html), and the hmmsearch tool was further used to search for the HMM profile with an expected e-value of 0.01. Then, the wheat proteins were searched with the HMM profile and hits were extracted. A self-BLAST of hits was first carried out to remove the redundancy, and the alternative splices were manually excluded. The remaining proteins were considered as the putative wheat ATG genes. All the obtained *TaAtgs* were subsequently submitted to the PFAM database for the confirmation of the ATG domains (http://pfam.xfam.org/). The online ProtParam tool (http://web.expasy.org/compute_pi/) was used to compute the gravity, theoretical pI (isoelectric point), and Mw (molecular weight) of the putative *TaAtgs* in the ExPASy server. The subcellular localization of each *TaAtg* was predicted using the online tool CELLO v.2.5 (http://cello.life.nctu.edu.tw/).

2.2. Multiple sequence alignments and phylogenetic analysis

Multiple sequence alignment of all the obtained TaATG protein sequences was performed using ClustalX (version 2.0.9) with the default parameters (Thompson et al., 2002). An unrooted phylogenetic tree was constructed using MEGA 6.0 software based on the full-length TaATG protein sequences as well as those of ATG genes from yeast (*Saccharomyces cerevisiae,* Sc), foxtail millet (*Setaria italic*, Si), tobacco (*Nicotiana tabacum*, Nt), *Arabidopsis thaliana* (At), and rice (*Oryza Sativa*, Os). The parameters used for the phylogenetic tree were as follows: neighbor