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Regulation of ATG6/Beclin-1 homologs by abiotic stresses and hormones in rice (*Oryza sativa* L.)

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ABSTRACT. Autophagy, a complex and conserved mechanism, serving as a defense response in all eukaryotic organisms, is regulated by several proteins, among which ATG proteins are the most important due to their involvement in autophagosome formation. ATG6/Beclin-1 proteins, reported to be essential for autophagosome formation and assigned as a conserved domain, were subjected to database searches. We found three homologs in the rice (*Oryza sativa*) genome. A phylogeny tree was constructed to establish their across species relationship, which divided them into three distinct groups; two for plants, i.e., monocots and dicots, and one for animals. Evolutionary study of this family by critical amino acid conservation analysis revealed significant functional divergence. The finding of important stress-related *cis*-acting elements in the promoter region of rice *ATG6* genes demonstrated their involvement in

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abiotic stress responses. Furthermore, expression profiling of rice *ATG6* genes based on microarray data, as well as by semi-quantitative RT-PCR, showed differential expression when subjected to different stresses suggesting the involvement of *OsATG6* genes in abiotic stresses (heat, cold and drought) and hormone (abscisic acid) responses. Analysis of co-expressed genes showed that most of them annotated to DNA repair pathways and proteolysis, etc. Collectively, these results suggest the involvement of *OsATG6* genes in different stresses, and provide a basis for further functional studies to investigate the biological mechanism of action of these genes under abiotic stresses.

Key words: Autophagy; Abiotic stress; Phylogeny; Rice

INTRODUCTION

Autophagy, a conserved lysosomal pathway, is used by eukaryotes when they are affected by growth-limiting nutrient deficiency (Harrison-Lowe and Olsen, 2008). This pathway is intended to recycle cellular constituents in order to extend the life of the cell. The process of autophagy involves the engulfment of cytoplasmic components into vesicles, which are subsequently degraded into vacuole/lysosome. The autophagic response has been described in various patho-physiological conditions, including neurobiological disorders, cancer, and, more recently, in cardiovascular disease, and in the regulation of programmed cell death in yeast, mammals, and plants (Harrison-Lowe and Olsen, 2008). Although it has been studied extensively in yeasts and to some extent in mammals, it has nearly been overlooked in plants, as only a few reports have been published on the molecular or genetic aspects of autophagy. In plants, autophagy mediated by autophagosome (macroautophagy) and tonoplast (microautophagy) is obvious at different developmental stages and has been observed in vegetative tissues (macroautophagy), as well as during seedling germination and senescence (microautophagy) (Harrison-Lowe and Olsen, 2008). The accumulation of acidic vesicles (having autophagosomal characteristics) in carbohydrate-starved tobacco cell cultures provides evidence for the conservation of a critically essential autophagy pathway in plants (Moriyasu and Ohsumi, 1996).

Among other proteins involved in autophagy, AuTophGy-related (ATG) proteins are critical for autophagosome formation. In yeast, 15 ATG proteins are involved in autophagosome formation (Suzuki et al., 2001). Yeast autophagy protein 6 (ATG6/Vps30) has been reported to be essential for the formation of autophagosomes during starvation (Kametaka et al., 1998). ATG6 has been found in many eukaryotes, including mammals and plants, and has been assigned a conserved domain (PF04111). Mammalian homolog of yeast ATG6 is recognized as Beclin-1, which has been reported to interact with the Bcl-2 family, and is involved in the regulation of autophagy and anti-apoptotic pathways (Sinha and Levine, 2008). The plant ortholog of yeast ATG6 was first identified in *Nicotiana tobacum* during the screening of genes affecting hypersensitive-response-programmed cell death induced by tobacco mosaic virus; this suggests that ATG6 is a negative regulator of programmed cell death and also that it is responsible for controlling virus replication (Liu et al., 2005). No physiological defects were observed in ATG6-knockdown tobacco plants; however, *Arabidopsis* ATG6 (*AtATG6*) T-DNA

insertion mutants exhibited defects in pollen germination (Fujiki et al., 2007; Qin et al., 2007).

The relationship of ATG6 proteins with response to biotic stresses and some other physiological functions in plants has been well established; further comprehensive analysis is required to elucidate their behavior during abiotic stresses. In an attempt to explore ATG6 homology in plants, we designed a study for identifying and investigating the genome organization, phylogeny, comparative genomics, and expression profiles of rice *ATG6* (*OsATG6*) genes under normal growth conditions, abiotic stresses, and hormone treatments, via semi-quantitative reverse trancriptase-polymerase chain reaction (RT-PCR) and expression database. Additionally, we propose the physiological functions of *OsATG6* under abiotic stresses, and our findings are expected to serve as a basis for further studies on the OsATG6 family in rice.

MATERIAL AND METHODS

Plant materials and stress treatments

Rice seeds (*Oryza sativa* L. subsp *japonica* 'Nipponbare') were surface-sterilized with 0.1% $HgCl_2$, germinated, and grown in an incubator (Rana et al., 2012). Two-week-old seedlings were subjected to different abiotic stresses, including heat (42°C), cold (4°C), drought (20% PEG 6000, providing an osmotic potential of -0.54 MPa), and treatment with 0.1 mM abscisic acid (ABA). The seedlings were sampled at 0, 1, 3, 6, 12, and 24 h after each treatment and immediately stored at -80°C. For expression analysis in different tissues, plants were grown in natural conditions, and samples were collected at the booting stage.

Identification of the ATG6 domain in rice

AtATG6 protein was used as the query for applying basic local alignment search tool (BLAST) to the *O. sativa* genome. The resultant sequences were screened for genome annotation, as well as for Pfam, SMART, and InterPro ATG6 domain (accession Nos. Pfam: PF04111 and InterPro: IPR007243) created via hidden Markov model-based database searches, and processed for further study. Genomic and cDNA sequences of these proteins were retrieved from NCBI, and gene structure was predicted by FGENESH+ (http://linux1.softberry.com/berry.phtml). The chromosomal location of each *ATG6* gene in rice was determined from the rice physical map constructed by the International Rice Genome Sequencing Project (IRGSP) (http://rgp.dna.affrc.go.jp). Subcellular localization of the OsATG6 family was predicted by WoLF PSORT (Horton et al., 2006) and ProtComp (http://linux1.softberry.com/berry.phtml).

Multiple-sequence alignment and phylogenetic relationship

Alignment of rice or other plant/animal/yeast ATG6 protein sequences, as well as rice ATG6 domains, was performed using ClustalX v. 1.83 (Thompson et al., 1994) and viewed by the Jalview 2 software (Waterhouse et al., 2009). The phylogenetic tree was constructed with the MEGA5 program by using the neighbor-joining method (Tamura et al., 2011). Bootstrap test of phylogeny was performed with 1000 replicates using pair-wise deletion and the p-distance model.

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Test for selection and functional divergence analysis

The Codeml program of PAML package (Yang, 2007) based on Nielsen and Yang's method (Nielsen and Yang, 1998; Yang and Nielsen, 2002) was employed to estimate positive selection during the evolution of *ATG6* genes. Likelihood ratio statistics was used to evaluate the codon-substitution models, as described previously (Nielsen and Yang, 1998; Yang et al., 2000; Hashiguchi et al., 2007). In these analyses, the ratio (dN/dS) of non-synonymous (dN) to synonymous (dS) nucleotide substitution rate was used to measure the maximum likelihood estimates of the selection pressure (ω).

The DIVERGE software (version 2.0) (Gu and Vander Velden, 2002) was used to estimate type I functional divergence between the groups of the *ATG6* gene family through alignment and construction of phylogenetic trees of yeast, animal, and plant ATG6 protein sequences. The coefficient of functional divergence (θ), likelihood ratio test (LRT) and site-specific posterior analysis were estimated between the 2 groups.

Expression analysis of the *OsATG6* gene family and co-expressed genes in rice on the basis of microarray data

Growth-stage-specific expression of *OsATG6* genes was estimated using publicly available web-based tool with reclassified Affymetrix data (www.ricearray.org) (Jung et al., 2008). Data regarding gene expression under abiotic stresses (heat, cold, salt, and drought) GEO data series GSE14275 (heat) and GSE6901 (cold, salt, and drought) were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/geo). Analysis of co-expressed genes was accomplished through the rice oligo-array database (www.ricearray.org) at Pearson's correlation coefficient **(**PCC) cutoff (Jung et al., 2008).

Semi-quantitative RT-PCR

Total RNA from stress-treated and untreated rice seedlings were extracted using the EasySpin plant RNA extraction kit (Yuan Ping Hao Bio) according to the manufacturer protocol. The genomic DNA was removed using DNaseI (Takara, Japan) at 37°C for 15 min. The first strand cDNA was synthesized with 2 µg purified total RNA using the RT-PCR system (Promega, USA) according to the manufacturer protocol.

For semi-quantitative RT-PCR assays, rice housekeeping gene 18S-rRNA was used as the internal control. PCR was performed with 25 (18S rRNA) or 30 (*ATG6* genes) cycles (30 s at 94°C, 30 s at 63°C, and 20 s at 72°C) under the following conditions: 0.5 µL RT product was amplified in a 20-µL volume containing 2 µL 10X PCR buffer with $MgCl_2$, 0.25 µL 10 mM dNTPs, and 0.5 µL Taq polymerase (Tiangen, Beijing).

Analysis of stress-related *cis*-acting elements

Plant-specific *cis*-acting elements were analyzed in 1-kb region upstream of *OsATG6* genes. The *cis*-acting elements were predicted through the plant-specific *cis*-acting element database PLACE (http://www.dna.affrc.go.jp/PLACE/).

RESULTS

ATG6 homologs in the rice genome

A BLAST search at NCBI and UniProt using the AtATG6 protein as a query followed by screening through genome annotation and conserved domains (PF04111 and IPR007243) revealed 3 putative genes in rice with significant similarity to the ATG6 domain. Searches on the expressed sequence tag (EST) database revealed that all the genes were transcribed, while the cDNA sequences of *OsATG6a*, *OsATG6b*, and *OsATG6c* matched well with 11, 1, and 33 ESTs, respectively (Table 1). Genomic distribution analysis revealed that rice *OsATG6* genes were located on chromosomes 1 and 3 (Table 1). The exon-intron structure of rice *OsATG6* genes on the basis of the information obtained from the genomic DNA and cDNA sequences revealed 10 exons each in *ATG6a* and *ATG6c*, but only 1 in *ATG6b* (Figure 1). *In silico* analysis of subcellular locations showed that the 3 proteins *OsATG6a*, *OsATG6b*, and *OsATG6c* were localized to the chloroplast, nucleus, and mitochondria, respectively (Table 2).

Table 1. The *ATG6* genes in rice.

Gene	TIGR ID	Chromosome	Coordinates	Exons	No. of ESTs
OsATG6a	LOC_Os01g48920	Chr1	28,069,635-28,075,384	10	11
OsATG6b	LOC_Os03g15290	Chr3	8,368,285-8,369,916	1	1
OsATG6c	LOC_Os03g44200	Chr3	24,833,619-24,839,417	10	33

ESTs = expressed sequence tags.

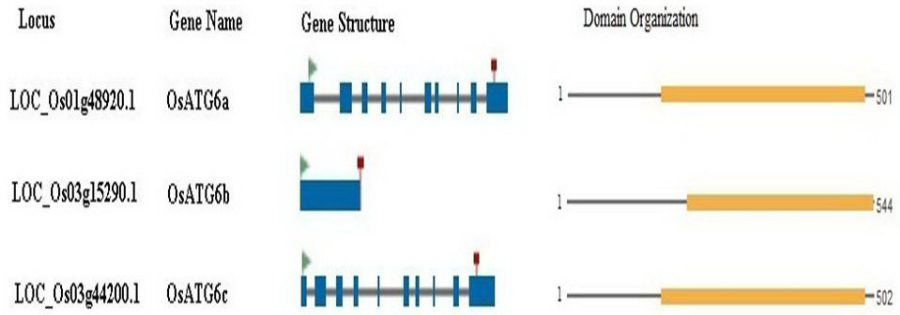

Figure 1. Gene structure and domain organization of *OsATG6* genes.

Table 2. *In silico* subcellular localization.

Protein	WoLFPSORT		ProtComp		Motif
	Cellular compartment	Score	Cellular compartment	Score	
ATG6a	Chloroplast	9.0	Chloroplast	2.3	Chloroplast transit peptide
ATG6b	Nuclear	6.0	Nuclear	2.6	Transmembrane segments
ATG6c	Nuclear	11.0	Mitochondria	1.2	

The multiple-sequence alignment of OsATG6 proteins with other plants, animals, and yeast counterparts showed higher similarity among themselves and to other plants than to animal/yeast homologs (Figure 2). Interspecies phylogenetic tree clustered the ATG6 proteins

into 3 distinct groups, i.e., GI (monocot plants), GII (dicot plants), and GIII (animals), while some of them (including yeast ATG6) did not cluster in any group. Three OsATG6 proteins clustered together and in the same group with monocot plants (Figure 3).

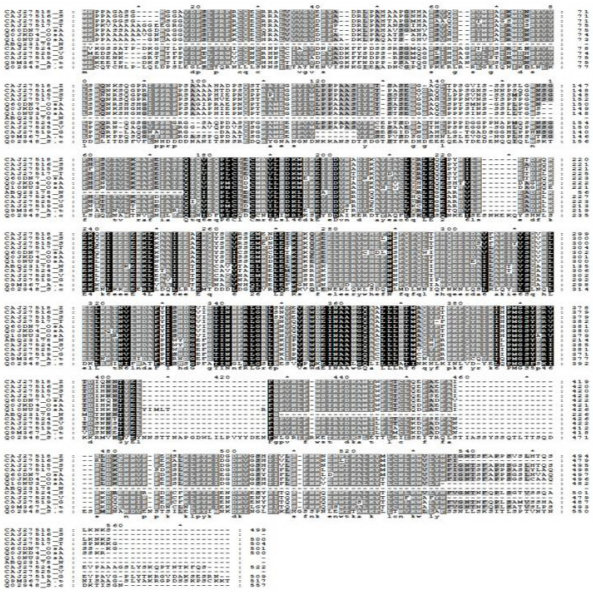

Figure 2. Multiple-sequence alignment of amino acid sequences of plant and yeast ATG6/Beclin-1 proteins.

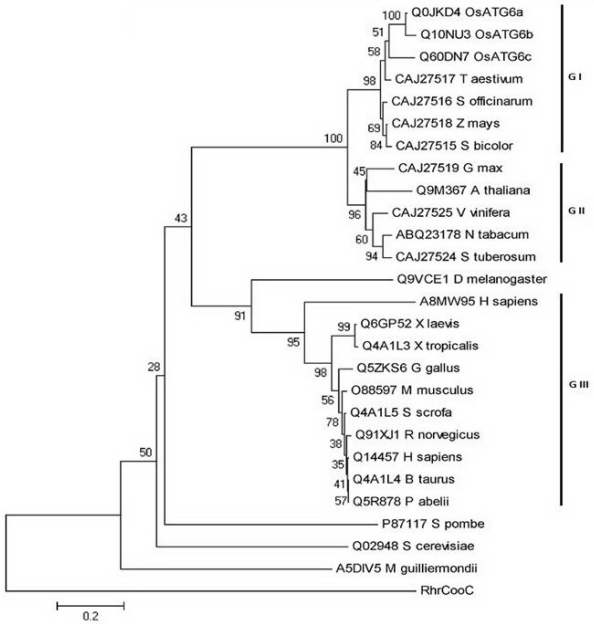

Figure 3. Phylogenetic relationship of plant, animal and yeast ATG6/Beclin-1 proteins. The tree was generated using the MEGA5 program by neighbor-joining method. The bootstrap values from 1000 replicates are indicated at each branch.

Evolution of the ATG6 family

The pattern of evolution of *ATG6* genes was investigated by Codeml program, and significance was estimated through the LRT. The ω estimates for ATG6 did not indicate positive selection; instead, they pointed towards purifying selection constraint. Different models were employed to investigate positive selection (Table S1). LRT comparison of the M3 model (discrete) to the M0 model (one-ratio) was significant ($2\Delta l = 114.43$, $P < 0.01$); however, none of the selected sites were significant ($P > 0.05$). A comparison of models M1a (neutral) to M2a (positive selection) and of M7 (beta) to M8 (beta + ω) also failed to detect significant positive selection sites, indicating selection constrains during ATG6 evolution. Branch-site model (Yang and Nielsen, 2002) was implemented to detect sites along specific positive selection. Estimated ω in model A was less than 1; hence, no positive selection was predicted (Table S1).

Further investigation of functional divergence was performed by the DIVERGE program (Gu and Vander Velden, 2002). Pair-wise comparison of ATG6 groups was performed to estimate the rate of amino acid evolution at each sequence position, and important amino acid residues potentially responsible for functional divergence were identified. The comparisons of GI to GII, GI to GIII, and GII to GIII were performed through coefficients of functional divergence (θ), and LRT showed significant functional constraints (Table S2). The posterior probability (processed with a suitable cutoff value) of each comparison based on site-specific profiles predicted amino acid residues responsible for functional divergence after gene duplication (Figure S1). For 180 aligned sites, an overall high posterior probability (0.7~0.8) was observed; therefore, a cutoff value of 0.9 was used to define the most effective sites. The comparisons of GI to GII, GI to GIII, and GII to GIII identified 5 (272, 278, 298, 397, 412), 8 (187, 194, 226, 271, 273, 298, 324, 397), and 9 (211, 215, 222, 226, 307, 324, 342, 389, 414) amino acid residues, respectively, as being important for functional divergence (Figure S1).

Expression profile of *OsATG6* genes

The expression profile of *OsATG6* genes in various rice tissues was investigated by using publically available microarray database. The rice *OsATG6a* showed high expression in stigma, ovary, and mature anther, while the expression level of *OsATG6c* was higher in internode and pith parenchyma. However, the remaining tissues showed very low or no expression (Figure S2). No Affymetrix probe was detected for *OsATG6b*. Semi-quantitative RT-PCR was performed to further analyze the expression pattern of *OsATG6* genes in different tissues. *OsATG6a* showed maximum expression in panicle; *OsATG6b* was expressed equally in all the tissues, and *OsATG6c* showed maximum expression in root, leaf and panicle (Figure 4).

Expression profiles of *OsATG6a* and *OsATG6c* under different abiotic stresses were analyzed by using GEO datasets GSE14275 (heat stress) and GSE6901 (cold, drought and salinity), which revealed that the *ATG6* genes were differentially regulated under different stresses (Figure 5).

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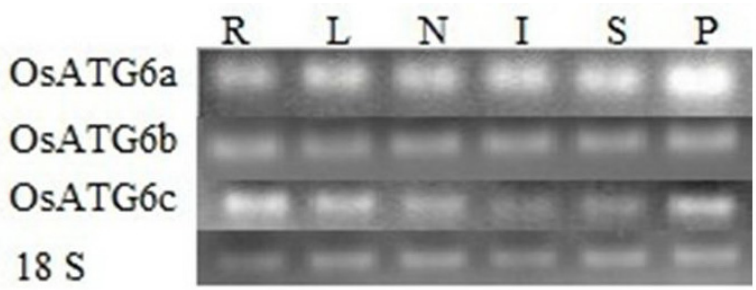

Figure 4. Expression profile of *OsATG6* genes in various rice organs based on semi-quantitative RT-PCR. Rice plants grown under natural conditions and samples were collected at the booting stage from different organs including, root (R), leaf (L), node (N), internode (I), leaf sheath (S), and panicle (P).

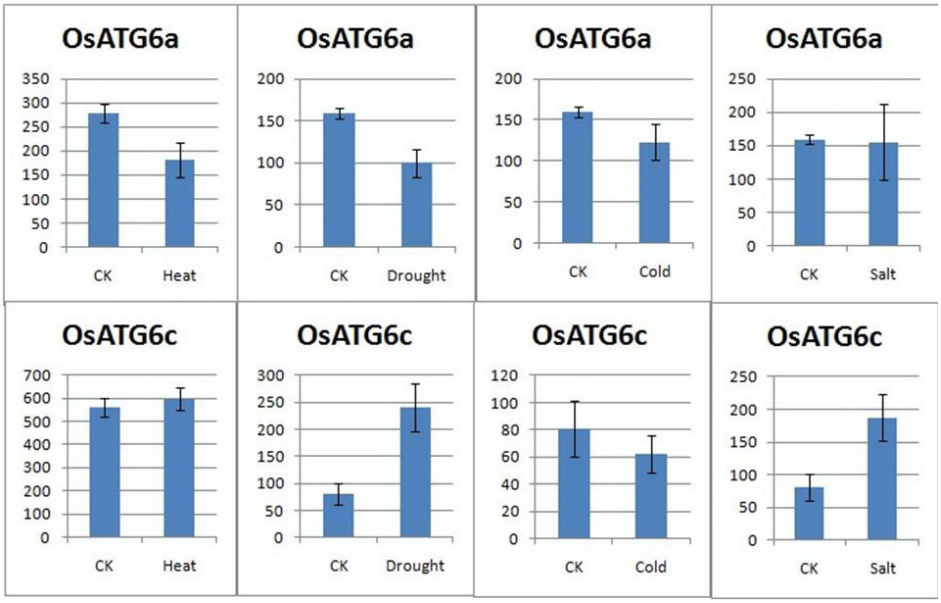

Figure 5. Expression profiles of *OsATG6* genes under different abiotic stresses based on rice microarray data from GEO database.

The expression pattern of *OsATG6* genes was further investigated under abiotic stresses, and their responses to cold, heat, drought (stimulated by 20% PEG6000), and ABA were analyzed by semi-quantitative RT-PCR. *OsATG6a* was upregulated by drought (12-24 h after treatments) and ABA (showed maximum expression at 3 h after treatments), but down-regulated by heat stress (24 h after treatments). However, *OsATG6b* was up-regulated by drought (12-24 h after treatments), and down-regulated by cold stress (3 h after treatments). On the other hand, *OsATG6c* was up-regulated by all stresses including heat (12-24 h after treatments), drought (12 h after treatments), cold (24 h after treatments), and ABA (3-6 h after treatments) (Figure 6).

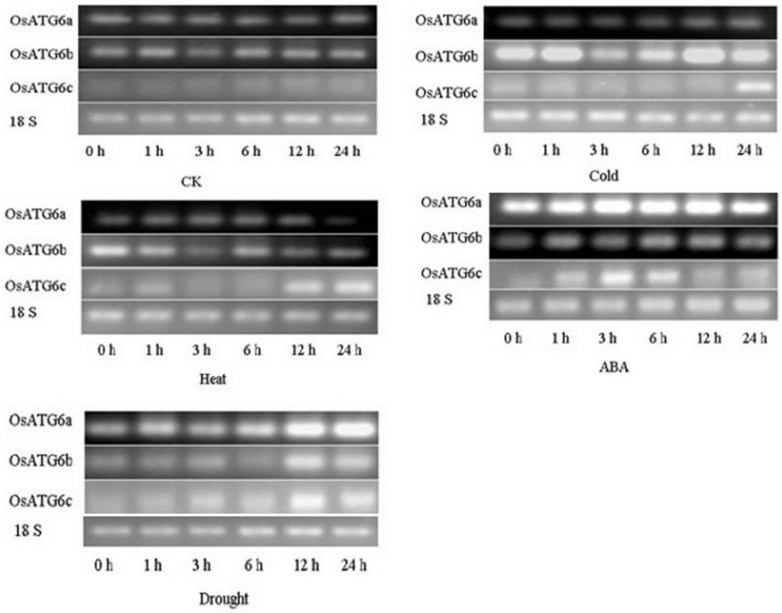

Figure 6. Expression profile of *OsATG6* genes under different abiotic stresses based on semi-quantitative real-time polymerase chain reaction.

Stress-related *cis*-acting elements and co-expressed genes

Since *ATG6* genes are involved in autophagy triggered by different stresses, it would be interesting to investigate the presence of stress-related *cis*-acting elements. Analysis of regions 1 kb upstream of the 3 *OsATG6* genes revealed stress-related *cis*-acting elements, including ABRE, WRKY71OS, MYBCORE, W-box, POLLEN1LELAT52, ARR1AT, MYCCONSENSUSAT, and PRECONSCRHSP70A in the promoter region of all the *OsATG6* genes (Table 3). To further understand the mechanism of action of *ATG6* genes, we analyzed the co-expressed genes under abiotic (heat, cold, and drought) stresses. The obtained data revealed that most of the co-expressed genes are either stress-related transcription factors, or involved in autophagy-related pathways, e.g., Myb transcription factor was highly co-expressed (PCC = 0.80) with *OsATG6a*. Gene ontology of the co-expressed genes revealed their involvement in transcription, DNA repair pathways and proteolysis, etc. (Table S3, Table S4, and Figure S3).

Table 3. *cis*-acting elements in 1-kb upstream region of rice ATG6 genes.

Gene/*cis*-acting element	*ATG6a*	*ATG6b*	*ATG6c*
ABRE	7	17	10
WRKY71OS	6	12	6
W-box	5	11	7
POLLEN1LELAT52	4	5	4
MYBCORE	4	8	3
ARR1AT	9	2	8
MYCCONSENSUSAT	8	12	4
PRECONSCRHSP70A	5	4	4
ACGTATERD1	0	22	12

DISCUSSION

Autophagy plays an important role in innate and adaptive immunity responses in yeast, animals, and plants (Cao and Klionsky, 2007). The proteins containing the ATG6 domain are involved in autophagy and have been found to be affected by biotic stresses in animals and plants (Liu et al., 2005). In this study, we have analyzed their relationship to abiotic stresses with special reference to plants. Keeping in view the importance of autophagy process and abiotic stresses for plants, *OsATG6* genes under different abiotic stresses were analyzed, and we describe the evolution of the *ATG6* gene family, identification and characterization of *ATG6* genes in *O. sativa*, as well as their expression profiles under different abiotic stresses and hormone (ABA) treatment.

Proteins containing the ATG6 domain that were found in plants, animals, and yeasts showed considerable functional homology. The species-specific clustering pattern of ATG6 proteins into GI together with other monocots suggested the absence of the role of duplication events during the divergence of monocots and dicots that occurred about 200 million years ago (Wolfe et al., 1989). Posterior probability analysis depicted the functional divergence between GI and GII proteins. A total of 5 critical amino acid residues were predicted to be involved in functional divergence. Three critical amino acid residues (positions 272, 278, and 298) were located in the N-terminal α-helix, suggesting that these evolutionary divergences probably generate group-specific binding sites for accessory proteins (Huang et al., 2008). Interestingly, all the critical residues were located in the ATG6 domain, which probably explains the diverse functions of this family, as observed in different animals and plants (Moriyasu and Ohsumi, 1996; Cao and Klionsky, 2007; Harrison-Lowe and Olsen, 2008). However, further experimental studies focusing on point mutation, protein-protein interactions, etc., will facilitate the confirmation of the involvement of these amino acid residues in the functional divergence of ATG6. The subcellular location of OsATG6 proteins in different cellular compartments further supports the results of functional divergence and was consistent with the findings of previous studies (Fujiki et al., 2007; Michiorri et al., 2010).

The higher accumulation of *OsATG6a* and *OsATG6c* mRNA in panicle (stigma, ovary, and mature anther) suggested that it might play an important role in the reproductive process and seed development, since their *Arabidopsis* counterpart *AtATG6* has been reported for its involvement in pollen tube germination (Fujiki et al., 2007; Harrison-Lowe and Olsen, 2008).

Abiotic stresses, such as heat, cold, drought, and hormone treatment might be of great importance in plants and are involved in provoking autophagy. Primary analysis using publically available microarray data showed that rice ATG6 were expressed differentially under heat, cold and drought stresses, indicating their involvement in abiotic stresses. Taking into account the functional divergence of the ATG6 family, ATG6 proteins are believed to be differently regulated under different stresses. Since the microarray data used in the analysis were obtained for a single time point and most of the changes induced by long-term stress stimuli cannot be detected by short time-course microarray (Chen et al., 2011), we further analyzed the expression of rice *ATG6* genes under exposure to abiotic stresses for different time periods. We found that *OsATG6* genes were expressed differentially under different stresses and that the expression pattern differed among the genes, suggesting a distinct stress-specific response. With regard to stress response, the maximum number of changes was observed in *OsATG6c*, which showed very low expression levels under normal conditions, while the expression changes mostly occurred over a long period in all the cases.

ABA has been reported to stimulate the production of hydrogen-peroxide in rice leaves, increase oxidative stress, and promote senescence (Hung and Kao, 2004). The expression pattern of *OsATG6c* observed under hormone treatment (ABA) first increased (maximum at 3 h after treatment) and then decreased to the basal level. These results point towards the involvement of *ATG6* genes in responses to abiotic stresses and hormone signaling and were consistent with previous reports on the differential expression of rice ATG homologs on exposure to salt and hormones (Xia et al., 2011). Our results are further supported by the finding that under abiotic stresses, some genes were co-expressed, resulting in the co-expression of stress-related transcription factors or genes involved in autophagy-related pathways (Table S1, Figure S1).

Cis-acting elements were analyzed in the promoter regions of *OsATG6* genes to further explore their stress responsiveness. We found that *OsATG6* genes contained multiple *cis*-acting elements related to different stresses, among which ABRE, MYBCORE, W-box, POLLEN1LELAT52, ARR1AT, MYCCONSENSUSAT, and PRECONSCRHSP70A. MYBCORE and W-box are important *cis*-acting elements for the proteins MYB and WRKY, which are involved in the regulation of stress-responsive genes (Meng et al., 2006; Mochida et al., 2006). The presence of these elements suggested that the expression of *OsATG6* genes may be stress induced. POLLEN1LELAT52, an important pollen-specific *cis*-regulatory element, discovered in the promoter region of the tomato let52 gene, has been reported to be expressed preferentially during pollen maturation (Sato and Yokoya, 2008). The presence of pollen-specific *cis*-regulatory element explains the importance of *OsATG6* genes during pollen maturation. ATG6 has also been reported as an essential protein for pollen germination in *Arabidopsis* (Qin et al., 2007). Another *cis* element, MYCCONSENSUSAT, found in the promoter regions, was involved in the dehydration stress response, which further supports our findings that indicated that all the 3 genes were upregulated under drought stress. ARR1AT is a binding element for the cytokinin response regulator ARR1, which is found in *Arabidopsis* (Ramalingam et al., 2006; Wu et al., 2007); it acts an upregulator of *ATG6* genes and was observed in rice leaves treated with cytokinin, thereby indicating the involvement of *ATG6* genes in the cytokinin signaling network (GEO dataset GSE6719; data not shown).

In conclusion, we identified 3 ATG6 homologs in rice and characterized them for evolution, functional divergence and expression. Our results showed the involvement of *ATG6* genes in abiotic stresses and hormone treatments, especially under drought and ABA. These results provide a basis for further detailed functional study of *OsATG6* genes in response to abiotic stresses; however, the biological roles and regulation mechanisms for OsATG6 proteins need to be further explored by using advanced techniques.

Supplementary material

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