1 **Original Article**

2 **Full title:**

3 **A barley homolog of yeast ATG6 is involved in multiple abiotic stress responses and stress**
4 **resistance regulation**

5

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Abstract:

Autophagy is a highly conserved degradation pathway among eukaryote cells, which can recycle damaged or unwanted cell materials upon encountering stress conditions. As a key component of the Class III PI3K kinase complex, ATG6/Beclin-1 is essential for autophagosome formation. In this study, we isolated a putative *HvATG6* gene in barley genome. The protein encoded by *HvATG6* shares high sequence identity to ATG6 orthologs in rice and wheat, and has a typical autophagy-specific domain containing segments of repeated β -sheet- α -helix. The expression of HvATG6 protein restored the appearance of autophagosomes in yeast *atg6* mutant, indicating that HvATG6 complements the deficiency of yeast ATG6 protein in autophagy. Punctate florescence signals, considered as the PAS for autophagosome initiation, were observed in the cytoplasm of cells when HvATG6-GFP fusion construct was transformed into barley protoplast. Furthermore, the expression of *HvATG6* was upregulated by various abiotic stresses including dark, H₂O₂ treatment, nitrogen deficiency, high salinity, drought, low temperature and toxic aluminum. Knockdown of *HvATG6* in barley leaves through barley strip mosaic virus (BSMV)-induced gene silencing led to accelerated yellowing under dark and H₂O₂ treatments. Based on the above findings, we propose that barley ATG6 plays the similar role as other plant ATG6 orthologs, and might be involved in stress-induced autophagy process.

Abbreviations

ATG: Autophagy-related gene; BSMV: Barley stripe mosaic virus; EV: Empty vector; GFP: Green fluorescent protein; PAS: Pre-autophagosome structures; PI3K: Phosphatidylinositol 3-kinase; VIGS: Virus-induced gene silencing.

Kew words: Autophagy, PI3K, ATG6, abiotic stress, VIGS, barley.

Introduction

Through their lifetime, plants have to continuously respond to fluctuations in growth conditions. Abiotic stresses, such as low temperature, high salinity, drought, and nutrient deprivation, affect the growth and development of plants, and even result in a huge amount loss of crop production (Crisp et al., 2016). Plants have evolved sophisticated protection mechanisms to survive in unfavorable environments (Sewelam et al., 2016). One of the key pathways that mediate stress-induced metabolic adaptation and damage control is autophagy (Liu and Bassham, 2012). Autophagy is a ubiquitous and highly conserved degradation process in eukaryotes, by which the damaged or redundant organelles and proteins generated in hostile environment can be sequestered into double-membrane structures called autophagosomes, and subsequently fuse with vacuoles for digesting and recycling (Xie and Klionsky, 2007). Recent studies have indicated that autophagy can be stimulated by multiple forms of abiotic stress, and autophagy-defective plants usually exhibit accelerated senescence, and are more susceptible to stress conditions compared with wild-type (WT) plants (Bassham, 2007; Doelling et al., 2002; Hanaoka et al., 2002; Liu et al., 2009; Xiong et al., 2005).

Through mutagenesis studies, more than 30 autophagy-related (ATGs) genes have been identified in yeast; most of which have homologs in plant (Kim et al., 2012). The products of these genes can be divided into several functional groups: the Atg1-Atg13 kinase complex, Atg9 and associated proteins, phosphatidylinositol 3-kinase (PtdIns3K) complex, and two ubiquitin-like conjugation systems (Mizushima et al., 2011). ATG6, which is a core component of PI3K complex, has been postulated to play an indispensable role in autophagy initiation (Kihara et al., 2001; Obara and Ohsumi, 2011). Yeast

1 ATG6/VPS30 (vacuolar protein sorting 30) forms the PI3K complex I with VPS34, VPS15 and ATG14
2 to recruit some of other ATGs for autophagosome formation (Reggiori and Klionsky, 2013). In
3 addition, yeast ATG6, along with VPS38, VPS34, and VPS15, which compose the PI3K complex II, is
4 involved in the vacuolar protein sorting pathway (Kihara et al., 2001). Beclin 1, the mammalian
5 homolog of yeast ATG6, can interact with anti-apoptotic Bcl-2-like proteins and function in tumor
6 suppression, suggesting that the ATG6-associated PI3K complex is not autophagy-specific (Fu et al.,
7 2013; Kang et al., 2011; Pattingre et al., 2005). Plant homologs of yeast ATG6 play essential roles in
8 stress-induced autophagy, for *AtATG6* antisense *Arabidopsis* plants were sensitive to nutrient starvation,
9 and virus-induced gene silencing of *NbATG6* in tobacco (*Nicotiana benthamiana*) enhanced yellowing
10 of leaves under dark-induced senescence (Liu et al., 2005; Patel and Dinesh-Kumar, 2008). Also,
11 *AtATG6* and *NbATG6* have been found to be required for pathogen-induced autophagy and regulation
12 of programmed cell death (PCD) (Liu et al., 2005; Patel and Dinesh-Kumar, 2008). In wheat, the
13 expression of *TaATG6s* was induced by the fungus *Blumeria graminis* f. sp. *tritici* (*Bgt*), the causal
14 agent of powdery mildew. Knockdown of *TaATG6s* significantly enhanced the basal resistance of
15 susceptible plants, indicating a role of autophagy genes in wheat immune responses to fungal
16 pathogens (Yue et al., 2015). Another characteristic of ATG6s in plant is that they have diverse
17 physiological functions besides autophagy. Several independent reports have shown that the function of
18 *AtATG6* is essential for pollen germination, and disruption of *AtATG6* by T-DNA insertion resulted in
19 increased anthocyanin production, short roots, early leaf senescence, dwarfism, fewer flowers and low
20 fertility (Fujiki et al., 2007; Harrison-Lowe and Olsen, 2008; Patel and Dinesh-Kumar, 2008; Qin et al.,
21 2007).

22 To date, the physiological functions of autophagy and their underlying mechanisms have been

1 reported in *Arabidopsis*, tobacco, rice, and even common wheat (Fujiki et al., 2007; Harrison-Lowe and
2 Olsen, 2008; Rana et al., 2012; Xia et al., 2011; Yue et al., 2015). However, in barley (*Hordeum*
3 *vulgare*), which is the fourth important cereal crop for human food and animal feed, related studies are
4 still limited. Here, we searched ATG candidates in barley genome database through sequence
5 conservation based on ATG orthologs reported in *Arabidopsis*, rice, maize and wheat, including ATG6,
6 ATG8, ATG10, ATG12, ATG7 and ATG5. Among them, only a putative ATG6 protein in barley has
7 been confirmed through functional complementation test in yeast, and its protein subcellular
8 localization is consistent with the characteristic of known ATG6 proteins reported in other species.
9 Therefore, we chose this ATG6 candidate for further study. According to previous studies, it was
10 expected that the expression of *ATG6* would vary during barley development and in response to diverse
11 stress treatments, and deficiency in *ATG6* would affect sensitivity of barley plant to unfavorable
12 environments. Our results indicated that the function of ATG6 in autophagy is highly conserved across
13 different plant species, and might be implicated in barley stress response and resistance regulation.

14

15 **Methods**

16 **Plant materials and growth conditions**

17 Seeds of barley (*Hordeum vulgare* L. cv Golden Promise) were surface-sterilized, rinsed, and then
18 placed on moist filter papers in 9 cm culture dishes, and kept at 24°C in dark for 36 h. Germinated
19 Seeds with emerged radicles were grown under a 24°C, 16 h light/8 h dark photoperiods in a controlled
20 climate chamber.

21 For hydroponics, germinated seeds were transferred to normal nutrient solution as described (NiSO₄
22 was not included) (Shavrukov et al., 2012) for 5-7 days until the second leaf appeared, and then added

1 200 mM NaCl in the solution for high salinity, 20% PEG 6000 for drought stress, 10, 30, 50 mM H₂O₂
2 for oxidative stress, or 2 μM /10 μM/30 μM AlCl₃ (pH 4.3) for aluminum stress, respectively. For cold
3 treatment, seedlings were transferred to a 4°C refrigerator. For dark treatment, leaves of 7 d-old barley
4 seedlings were covered with aluminum foil for 3 d.

5 Soil culture method was selected for nitrogen deficiency treatment. Two germinated seeds were
6 transferred into air-dried sand packed into clear plastic bags in 1 kg portions added basal nutrients
7 (Genc et al., 2007), while deleting NH₄NO₃ for nitrogen deficiency. Plants were watered daily with
8 double deionized water to 12% (w/w) moisture content throughout the experiment. The sand bags can
9 be put into polyethylene-lined cylindrical PVC pots with approximated dimension of 6.5×30 cm
10 (diameter×depth).

11

12 **Identification and sequence analysis of barley ATG6**

13 Barley *ATG6* candidates were identified by BLASTP in barley database
14 (<http://webblast.ipk-gatersleben.de/barley/viroblast.php>) using the ATG6 protein sequences from
15 *Arabidopsis* and rice as queries. The more detail gene information can be found in website of Ensembl
16 plants (http://plants.ensembl.org/Hordeum_vulgare/Info/Index). The coding sequence of *HvATG6* gene
17 was amplified through PCR using cDNA as templates, which was generated from total RNA extracted
18 from barley seedlings. Primers used were listed in Table S1. The amplified products were inserted into
19 pENTR/D-TOPO (Invitrogen) for sequencing.

20 Sequence alignment was performed using Vector NTI. Conservative domains and protein secondary
21 structure were predicted in SMART (<http://smart.embl-heidelberg.de>) and SWISS-MODEL
22 (<http://swissmodel.expasy.org/interactive>). Phylogenetic tree was constructed in MEGA 4 by the

1 Neighbor-joining method (bootstrap test with 1000 replicates). The motifs in ATG6 were identified
2 through the online MEME software (<http://meme.sdsc.edu/meme/meme.html>), with an expected
3 *e*-value of less than 2×10^{-30} .

5 **RNA extraction and RT-PCR analysis**

6 Total RNA was isolated from harvested tissue samples using TRIZOL (Takara) method and subjected
7 to RNase-free DNase I digestion and purification. First-strand cDNA was synthesized using reverse
8 transcription kit (PrimeScript[®] RT Reagent kit, Takara).

9 The expression patterns of barley *ATG6* under various abiotic treatments were detected by
10 quantitative real-time PCR (qRT-PCR) with gene-specific primers. qRT-PCR was conducted in a 10 μ l
11 2 $\times$ SYBR Premix Ex Taq I (Takara) mixture, and the reaction was performed on an *Mastercycler ep*
12 *realplex*² system (Eppendorf). Each cDNA sample was repeated in triplicate. Relative expression levels
13 were calculated using the $2^{-\Delta\Delta CT}$ method with the amplification of the barley *α -tubulin* gene or *UBQ* as
14 an internal control. Primers used were listed in Table S1.

16 **Subcellular localization of HvATG6 protein fused with GFP**

17 The *35S:HvATG6-GFP* vector was constructed using gateway technology (Invitrogen). The ORF of
18 barley *ATG6* gene was amplified using the KOD DNA polymerase (KOD-Plus-Neo, TOYOBO). The
19 PCR fragment was inserted into the entry vector pENTR/D-TOPO, and then was integrated into
20 destination vector pUGW5 by an LR reaction using LR enzyme (Invitrogen). The recombinant vector
21 was verified by sequencing. Primers used were listed in Table S1.

22 Barley protoplasts were prepared as described in our previous work (Bai et al., 2014). The

1 35S:HvATG6-GFP vector was introduced into protoplasts cells using 40% (W/V) PEG 4000. GFP
2 fluorescence was visualized by fluorescence microscopy (Nikon ECLIPSE *Boi*, Japan).

3

4 **Complementation test of yeast *atg6* mutant**

5 The ORF of barley *ATG6* gene was amplified as *KpnI*-*NotI* fragment using the KOD DNA polymerase
6 (KOD-Plus-Neo, TOYOBO). The resulting PCR fragment was digested by *KpnI* and *NotI* restriction
7 endonuclease and ligated into the yeast expression vector pYES2 (Invitrogen) in which inducible
8 expression of inserted gene was driven by the yeast *GALI* gene promoter. Recombinant vector was
9 sequenced to prove the correct cDNA insertion. Primers used were listed in Table S1.

10 Autophagy complementation test was performed according to Fujiki et al (2007) and Yue et al
11 (2015). Recombinant vectors were introduced into the *atg6* mutant yeast cells according to the
12 LiAc-induced method. Positive transformants were screened on SD-Ura medium (MINIMAL SD BASE
13 GAL/RAF 37 g/L, -Ura DO Supplement 0.77 g/L, Clontech), and then cultured with shaking at 30°C in
14 SC medium to mid-log phase ($OD_{600} = 1$). Cells were then collected, washed and incubated for another
15 5 h in nitrogen-deficient medium (MINIMAL SD BASE GAL/RAF 37 g/L) that added 1 mM
16 phenylmethylsulfonyl fluoride (PMSF) to induce autophagy and accumulate autophagic bodies in the
17 vacuoles. Then the cells were observed using a differential interference contrast microscope (Nikon
18 ECLIPSE *Boi*, Japan).

19

20 **BSMV-induced barley gene silencing**

21 The BSMV-induced barley gene silencing was performed according to Yuan et al (2011). The BSMV
22 pCa- γ bLIC derivatives were constructed using the provided pCa- γ bLIC vector. Barley *PDS* gene was

1 chosen for assessment of gene silencing efficiency, since silencing of *PDS* leads to an apparent
2 longitudinal white streaks in the leaves of barley seedlings. The 297 bp of *HvPDS* (GenBank accession:
3 AY062039.1) and 263 bp fragment of *HvATG6* were obtained by RT-PCR using primers listed in
4 Table S1. PCR products and the *Apa* I-linearized pCa- γ bLIC vector were treated respectively at room
5 temperature with T4 DNA polymerase (Takara) in 1 $\times$ reaction buffer containing only 5 mM dATP (for
6 PCR products) or dTTP (for pCa- γ bLIC vector) for 30 min to generate sticky ends, and then incubated
7 at 75°C for 10 min to terminate the reaction. 200 ng PCR products and 20 ng pCa- γ bLIC were then
8 mixed at 66°C for 2 min and cooled to room temperature to obtain the BSMV pCa- γ bLIC derivatives.
9 Subsequently, 10 μ l aliquots were used for transformation into *Escherichia coli* Trans 10 (Transgene,
10 Beijing, China), and the positive transformants were verified by colony PCR and endonuclease
11 digestions.

12 The agroinfiltration of *N. benthamiana* and viral inoculation of cereals were conducted according to
13 Yuan et al (2011). The BSMV pCaBS- α , pCaBS- β and pCa- γ bLIC (or its derivatives) were
14 transformed together into *A. tumefaciens* strain GV3101 by electroporation. Single colonies of
15 transformed GV3101 were grown in 3 ml LB overnight, then inoculated in 50 ml LB containing
16 rifampicin (25 μ g/ml) and kanamycin (100 μ g/ml) and grown at 30°C for about 16 h with constant
17 shaking. The *A. tumefaciens* cells were pelleted, washed and resuspended in infiltration buffer (10 mM
18 MgCl₂, 10 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 5.2, and 0.1 mM acetosyringone) to a
19 final OD₆₀₀ of 0.7, and incubated at room temperature for at least 3 h. For agroinfiltration, equal
20 amounts of bacteria harboring pCaBS- α , pCaBS- β and pCa- γ bLIC (or its derivatives) were mixed, and
21 infiltrated into four to eight *N. benthamiana* leaves immediately above the cotyledons with a 1ml
22 needleless syringe.

1 After maintenance in a growth chamber for 5 to 12 days postinfiltration (dpi), the infiltrated leaves
2 were harvested, ground in 20 mM Na-phosphate buffer (pH7.2) containing 1% celite, and the sap was
3 mechanically inoculated onto the two-leaf stage of barley. Barley gene silencing experiment consisted
4 of four groups: the normal culture group (which was not infiltrated with BSMV), and the other three
5 groups including BSMV: γ , BSMV:*PDS*, BSMV:*ATG6* (which were infiltrated with *Agrobacterium*
6 mixtures harboring pCaBS- α , pCaBS- β , and pCaBS- γ bLIC/pCaBS- γ bLIC-*PDS*/pCaBS- γ bLIC-*ATG6*
7 respectively). When BSMV:*PDS*-treated leaves developed longitudinal white streaks, total RNA was
8 extracted from BSMV-treated plants. Semi- quantitative PCR was conducted to evaluate the efficiency of
9 gene silencing. For those gene knockdown plants, green sections were excised, floated on deionized
10 water at room temperature in the dark or in 40 mM H₂O₂, and photographed after 5 days incubation.
11 Then the chlorophyll content in leaves was determined spectrophotometrically (Patel and
12 Dinesh-Kumar, 2008).

13

14 **Results**

15 **Identification of barley *ATG6* gene and sequence analysis**

16 To search the *ATG6* homolog in barley genome, we used the protein sequences of *Arabidopsis* and rice
17 counterparts as queries (Bassham, 2007; Thompson and Vierstra, 2005; Xia et al., 2011). One gene
18 MLOC_71271 in Ensembl plants, which located on chromosome 3 in barley, was identified as a
19 putative *ATG6* and was designated as *HvATG6*. *HvATG6* contains 10 exons and 9 introns, and encodes
20 a peptide with 504 amino acids (Fig 1A). Phylogenetic tree analysis of *ATG6* proteins in plants showed
21 that several monocot *ATG6*s including *HvATG6* can be grouped into the same clade (Fig 1B). The
22 protein sequence of *HvATG6* shares 96%~97% of similarity to TaATG6a-6c from wheat, 70%-85% to

OsATG6a-6c from rice and 55% to AtATG6 from *Arabidopsis*. To reveal the structural characteristics of ATG6 proteins, motif analysis was carried out through the online MEME software. As shown in Fig 1C, the pattern of motif composition and location is similar among ten ATG6 proteins we tested. Five conserved motifs were found for the plant ATG6 protein sequences, two among them (Motif 1 and Motif 3) are shared with ScATG6.

The β -sheet- α -helix repeats in yeast ATG6 and mammalian Beclin 1 have been shown to be a pivotal structure involved in autophagic process (Huang et al., 2012; Levine et al., 2015; Noda et al., 2012). Through protein secondary structure prediction, we found that the carboxyl terminal of HvATG6 protein has four β -sheets and four α -helices to form two β -sheet- α -helix repeats (Fig 2).

HvATG6 rescues autophagy deficiency in yeast *atg6* mutant

ATG6 homologs in *Arabidopsis* and wheat can restore autophagy in yeast *atg6/vps30* mutant (Fujiki et al., 2007; Yue et al., 2015). To investigate whether HvATG6 is a functional homolog of yeast ATG6, a yeast vector containing the entire CDS of *HvATG6* was transformed into *atg6/vps30* mutant yeast cells. After about 5 h of nutrient deprivation, numerous autophagic bodies accumulated in both wild type and *atg6/vps30* mutant cells expressing HvATG6 protein, but not in *atg6/vps30* mutant cells (Fig 3A).

HvATG6 forms autophagosome-like punctate structure in barley protoplast cells

Previous studies have reported that fluorescence signals of GFP-fused ATG6s were located in the punctate structures in *Arabidopsis* and wheat cells, which were generally considered as the pre-autophagosomal structure (PAS) during the process of autophagy (Fujiki et al., 2007; Yue et al., 2015). In order to determine the subcellular localization of barley ATG6 protein, GFP fusion construct

1 of *HvATG6* under the control of the *Cauliflower mosaic virus* 35S promoter was transformed to barley
2 protoplasts. The fluorescence signals from *HvATG6*-GFP were observed in the cytoplasm as bright dots
3 (Fig 3B). While in cells transformed with *35S:GFP* alone, such punctuate structure was not found (Fig
4 3B). In the meantime, we used rice SPCH protein as another control, which located in nucleus in barley
5 protoplasts as expected (Fig 3B) (Bai et al., 2014).

6

7 **Analysis of *HvATG6* expression in response to abiotic stresses**

8 To monitor the expression pattern of *HvATG6* under various abiotic stresses, real time qPCR was
9 performed using samples of 7-day-old barley seedlings. Dark and H₂O₂ treatment promoted the
10 expression of *HvATG6* (Fig 4E, F). Additionally, the transcriptional level of *HvATG6* gene was
11 upregulated moderately under the treatments including nitrogen deficiency, high salinity, drought and
12 low temperature (Fig 5). The treatments we tested were associated with phenotypic changes of
13 seedlings such as leaf yellowing, root elongation inhibition and plant dwarf (Fig 4A-D and Fig 5A, D).
14 We also detected the expression level of *HvATG6* in seedlings exposed to toxic Al³⁺. The cultivar we
15 used was Golden Promise (GP), which was Al³⁺ sensitive. Al³⁺-induced cell death can be observed in
16 root tip through Evans blue staining (Fig 5G). It showed that 4 h exposure of 10 μM and 30 μM Al³⁺
17 greatly induced the transcription of *HvATG6*, especially in the roots (Fig 5H). We then tested the
18 accumulation of *HvATG6* transcripts in different sections of root, such as root tip (RT), the middle part
19 of root (RM) and root base (RB). The most significant variation of transcripts was observed in the root
20 tip, which is proved to be the major target site of Al toxicity (Fig 5I).

21

22 ***HvATG6* gene silencing leaves are more susceptible to dark and oxidative stresses**

1 To further evaluate the biological importance of *HvATG6*, *HvATG6* knockdown plants were generated
2 by the BSMV induced gene silencing method (Fig 6A). Knockdown efficiency was detected by
3 semi-quantitative PCR. The transcript level of *HvATG6* and PDS were greatly decreased in
4 BSMV:*HvATG6* and BSMV:*HvPDS* plants, compared with mock and EV-treated plant (Fig 6B). The
5 leaf sections in confirmed *HvATG6* knockdown plants were then excised and subjected to dark stress.
6 When compared with control plants (BSMV:γ), *HvATG6* knockdown plants displayed an apparently
7 accelerated chlorosis (Fig 6C) and a corresponding decrease of chlorophyll content after 5 days of dark
8 treatment (Fig 6D). The detached leaves were subjected to H₂O₂ treatment as well. As shown in Fig 6E,
9 stress-induced leaf yellowing was more severe in *HvATG6*-knockdown leaves compared with that in
10 mock control and BSMV:γ treated leaves.

11

12 **Discussion**

13 As a key component of the Class III PI3K kinase complex, ATG6/Beclin-1 is essential for
14 autophagosome formation. Previous studies have identified several plant ATG6 orthologs, including
15 one ATG6 member in *Arabidopsis* and tobacco respectively, three in rice and three in common wheat
16 (Fujiki et al., 2007; Xia et al., 2011; Yue et al., 2015). In this study, we isolated a putative ATG6 in
17 barley, which shares high sequence and motif identity to ATG6 orthologs in other plant species,
18 especially wheat and rice (Fig 1). Structural analysis proved that HvATG6 has a typical
19 autophagy-specific domain containing segments of repeated β-sheet-α-helix, just as yeast ATG6 and
20 mammalian Beclin 1 (Fig 2). The expression of HvATG6 protein restored the appearance of
21 autophagosomes in yeast *atg6* mutant, indicating that HvATG6 could complement the deficiency of
22 yeast ATG6 protein in autophagy (Fig 3A). Moreover, the expression of HvATG6-GFP fusion protein

1 produced punctate fluorescence signals, which were regarded as the PAS for autophagosome initiation
2 (Fig 3B). Based on the above results, we proposed that HvATG6 might play an essential role in
3 autophagy biogenesis in barley. Further study to test whether HvATG6 colocalizes with ATG8-GFP
4 fusion protein, a well-developed marker for autophagy occurrence in plant cells by the specific labeling
5 of autophagosomes, will help us to further verify the role of ATG6 in barley autophagy (Fujiki et al.,
6 2007).

7 The function of ATG6 in development and stress responses was further investigated through
8 expression profile analysis. During heading stage, the expression level of *HvATG6* in spike of Golden
9 Promise was increased gradually (Fig S1A, B). Based on RNA-seq database of barley (cv. Morex)
10 (<https://ics.hutton.ac.uk/morexGenes/>), it was found that the expression of *HvATG6* displays a specific
11 spatial-temporal pattern, with the highest expression in 3rd internode (developing tillers at six leaf
12 stage) and lowest level in developing grain (15 DPA) (Fig S1C). These data indicated that ATG6 might
13 be involved in the regulation of normal development in barley.

14 Furthermore, we showed that multiple abiotic stress treatments like dark, H₂O₂ treatment, nitrogen
15 deficiency, high salinity, drought and aluminum toxicity led to an significant upregulation of *HvATG6*
16 transcript level (Fig 4 and Fig 5). This was agreement with previous studies in wheat and rice,
17 implicated that the expression of *ATG6* is sensitive and responsive to the change of environmental
18 conditions (Rana et al., 2012; Xia et al., 2011; Yue et al., 2015). Remarkably, in seedlings of Golden
19 Promise, which is a high sensitive cultivar to aluminum stress, the expression of *HvATG6* was elevated
20 by more than 3 times in the roots compared with that in non-treatment control (Fig 5H, I). These data
21 supported a potential role of autophagy in plant response to various environmental cues. Using
22 autophagy-specific marker, some previous studies have provided direct evidence for occurrence of

1 stress-induced autophagy in plant cells. Autophagosomes induced by salinity, osmotic stress and
2 nutrient starvation were observed in *Arabidopsis* roots using monodansylcadaverine (MDC) fluorescent
3 dye (Liu et al., 2009; Patel and Dinesh-Kumar, 2008). In wheat, the LysoTracker Red-stained
4 punctuate structures were observed in leaves when inoculated with the fungus (Bgt) (Yue et al., 2015).
5 The number of autophagosomes was dramatically reduced under treatment conditions in *ATG*-deficient
6 or knockdown plants, further indicated that autophagy is associated with plant stress response (Phillips
7 et al., 2008; Yoshimoto et al., 2004).

8 We noticed that most abiotic treatments we tested resulted in root growth inhibition, leaf yellowing
9 and cell death (Fig 4, Fig 5). Leaf senescence is one of the escape strategies adopted by plants to cope
10 with environmental stresses they encountered. An early event during leaf senescence is the degradation
11 of chloroplasts, so that the mobile nitrogen resulting from chloroplast disassembly can be recycled and
12 supplied to the sink organs, flowers and seeds (Ishida et al., 2008). Previous studies demonstrated that
13 autophagy functions in the non-selective catabolism of chloroplast and photosynthetic proteins during
14 stress-induced leaf yellowing (Ishida et al., 2008; Wada and Ishida, 2009). During leaf senescence,
15 Rubisco, the most abundant stromal protein in the chloroplasts, is released from chloroplasts into the
16 cytoplasm as small double-membrane bodies termed Rubisco-containing bodies (RCBs), and then is
17 transported to the central vacuole for degradation by autophagy. Dark-induced chloroplast degradation
18 and RCB formation were impaired in autophagy-defective mutants (Wada and Ishida, 2009). Moreover,
19 autophagy has been reported to contribute to starch degradation in leaf at night, to support respiration
20 and metabolism (Wang et al., 2013). Therefore, *ATG6*-dependent autophagy might be required for
21 nutrient remobilization through chloroplast degradation, which helps plant cells survive under
22 unfavorable growth conditions.

1 In addition to its role in nutrient recycling, autophagy is also involved in degrading oxidized proteins
2 upon encountering oxidative stress. The autophagy-defective RNAi-*AtATG18a* plants are more
3 sensitive to methylviologen treatment and accumulate higher levels of oxidized proteins (Xiong et al.,
4 2007); similar phenomena have also been observed in a rice mutant *atg10* (Shin et al., 2009). To
5 elucidate the biological roles of HvATG6 in stress response, we generated knockdown plant through
6 BSMV-based VIGS method, and found that detached leaves from *HvATG6* knockdown plants
7 displayed an accelerated senescence under dark stress and H₂O₂ treatment (Fig 6). It demonstrated that
8 ATG6 is required for autophagy process in barley triggered by abiotic stress, at least dark and oxidative
9 stress. On the other hand, it is well known that many biotic stresses, such as H₂O₂ treatment, toxic Al
10 treatment and high salinity can induce reactive oxygen species (ROS) production. Combined with the
11 observation that *HvATG6* expression level was induced by these treatments, we proposed that
12 autophagy might negatively regulate oxidative-induced cell death through inhibiting ROS
13 accumulation, which is supported by the evidence that autophagy-defective mutants in *Arabidopsis*
14 accumulate higher levels of ROS during leaf senescence (Yoshimoto et al., 2009).

15 Collectively, we identified a barley homolog of yeast ATG6 and verified its function in autophagy.
16 The expression of *HvATG6* is induced by kinds of abiotic stresses, and knockdown of *HvATG6*
17 enhanced sensitivity of barley leaves to dark and oxidative stresses. These results reflect the importance
18 of autophagy in plant adapting to stress conditions, implying that autophagy might be a target for
19 improvement of abiotic stress resistance in barley breeding.

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Figure legends

Fig. 1. Sequence analysis of a putative ATG6 protein in barley. A. Diagram model of *HvATG6* gene. Black boxes, white boxes and fold lines represent coding regions, untranslated regions and introns, respectively. The predicted proteins amino acid (aa) length for *HvATG6* is shown at right. B. Phylogenetic tree of ATG6 families among barley and other species. The thickened line means that these proteins can be classified into one subfamily. The gene IDs encoding ATG6 are including *HvATG6* (MLOC_71271.1) from *Hordeum vulgare*, *TaATG6a* (AGW81783.1), *TaATG6b* (AGW81784.1), *TaATG6c* (AGW81785.1) from *Triticum aestivum*, *OsATG6a* (Os01g0681400), *OsATG6b* (Os03g0644000), *OsATG6c* (Os03g0258500) from *Oryza sativa*, *ZmATG6* (NP_001105883.1) from *Zea mays*, *AtATG6* (AT3G61710) from *Arabidopsis thaliana*, *ScATG6* (NP_015205.1) from *Saccharomyces cerevisiae*. C. Motif analysis of the ATG6 proteins. Motif locations were shown with combined block diagrams.

Fig. 2. Structural analysis of C-terminal amino acid sequence of *HvATG6*. A. Overall structure of C-terminal amino acid sequence of *HvATG6*. Red tubes represent α helix, cyan arrows represent β sheets. B. Sequence alignment of ATG6 homologs from several organisms. Predicted secondary structural elements are indicated above the sequences.

Fig. 3. Functional analysis and subcellular localization of *HvATG6* protein. A. *HvATG6* rescues the autophagic defects of yeast *atg6* mutant cells. Black arrows represent the autophagic bodies in vacuole. Scale bar = 5 μ m. B. Subcellular localization of three proteins in barley protoplasts. GFP was localized in intracellular compartments when transformed with *35S::GFP*. *HvATG6*-GFP was targeted to the

1 PAS-like structure in cytoplasm. White arrows indicate thePAS-like structure. OsSPCH-GFP was
 2 targeted to nucleus. Images of GFP, GFP merged with chlorophyll autofluorescence (Merged) and
 3 bright field are shown. Scale bar = 20 μ m.

4
 5 **Fig. 4.** The expression patterns of *HvATG6* under dark and H₂O₂ treatment. A. Phenotype of leaves of
 6 15 d-old seedlings after control (left) or dark treatment for 5 d (right). B-C. Leaves of 6 d-old seedlings
 7 were treated with 0 mM (B) and 50 mM H₂O₂ for 2 d (C). D. 6 d-old seedlings were grown in nutrient
 8 solution described in Methods supplied with 0 mM (left) or 30 mM H₂O₂ for 2 d (right). Scale bar=1
 9 cm. E-F. Expression analysis of *HvATG6* gene in response to dark (E) and H₂O₂ treatment (F). The
 10 relative expression level of *HvATG6* was normalized to the barley *UBQ* gene, with the expression level
 11 in non-treated sample (control) set as 1.0. Data shown are mean $\pm$ SE of 3 biological replicates.
 12 Asterisks indicate significant difference from the control, as determined by ANOVA (*, $p < 0.05$; **,
 13 $p < 0.01$).

14
 15 **Fig. 5.** The expression patterns of *HvATG6* under different abiotic stresses. A. Phenotype of barley
 16 seedlings under control (left) and nitrogen deprivation (right) for 15 d. Scale bar = 2cm. B. Expression
 17 analysis of *HvATG6* gene in response to N deficiency. Control showed in white bar, N-lack-15 d
 18 showed in green (leaf) or brown (root) bar. The expression level of *HvATG6* in root sample under
 19 control was set as 1. C. Expression analysis of *HvATG6* gene in response to cold (4°C) treatment. D.
 20 Phenotype of barley seedlings after treatment of 0 mM (left) or 200 mM NaCl for 5 d (right). Scale
 21 bar=4 cm. E. Relative expression of *HvATG6* under 200 mM NaCl treatment. Root showed in brown
 22 bar, leaf showed in green bar. F. Relative expression of *HvATG6* gene in response to 20% PEG

1 treatment. G. Evans blue stain of barley root samples under control (left) and 10 μM AlCl_3 treatment
2 (right). Scale bar=1 cm. H. The expression of *HvATG6* in root and leaf samples under toxic Al^{3+}
3 treatment for 72 h. Root showed in brown bar, leaf showed in green bar. The expression level of
4 *HvATG6* in root under control was set as 1. I. The expression of *HvATG6* in different segments of root
5 sample under 10 μM AlCl_3 treatment for 4 h. The relative expression level of *HvATG6* was normalized
6 to the barley *α -tubulin* gene, with the expression level in non-treated sample (control) set as 1.0. Data
7 shown are mean $\pm$ SE of 3 biological replicates. Asterisks indicate significant difference from the
8 control, as determined by ANOVA (*, $p < 0.05$; **, $p < 0.01$).

9
10 **Fig. 6.** *HvATG6* knockdown leaves exhibited an accelerated dark and oxidative stress-induced
11 yellowing. A. The phenotype of the third leaf at 15 days post inoculation with the corresponding
12 BSMV constructs. B. Semi- quantitative PCR analysis of *HvATG6* and *HvPDS* in mock, EV-treated and
13 BSMV-treated plants. The *18S rRNA* gene was used as an internal control. C. The phenotype of
14 detached leaves before (left) and after 5 days of dark treatment (right). D. The chlorophyll content in
15 detached leaf segments after 5 days of dark treatment. Mock represents the non-treated barley seedlings.
16 BSMV: γ or BSMV:*ATG6* represent the barley leaves inoculated with BSMV containing pCaBS- γ bLIC
17 or pCaBS- γ bLIC-*ATG6*, respectively. Means with different letters are significantly different ($P < 0.05$
18 by Duncan's multiple range test). E. *HvATG6* knockdown leaves exhibited an accelerated oxidative
19 stress-induced yellowing. Left, before treatment; right, after 4-d treatment of 40 mM H_2O_2 . Scale bar =
20 1 cm.

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