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Short Communication

Nutrient starvation differentially regulates the autophagy-related gene *GmATG8i* in soybean seedlings

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Abstract Autophagy functions in bulk degradation of proteins and organelles for nutrient recycling. Recent evidence suggests that a set of autophagy-related (*ATG*) genes are induced under starvation, senescence and various abiotic stresses. To understand how these genes function in plants, expression profiles of soybean *ATG* homologs, *GmATG8s*, *GmATG4*, *GmATG9*, *GmATG12* and *GmATG18a*, were examined using seedlings of *Glycine max* (L.) Merr. cv. Fukuyutaka) subjected to various nutrient conditions. Among them, *GmATG8i* mRNA was induced at higher level than any other *ATG*s under starvation. Immuoblot with a specific antibody raised against GmAtg8i indicated that endogenous GmAtg8i associates to microsomes of soybean seedling extracts. In the presence of vacuolar protease inhibitors, significant accumulation of anti-GmAtg8i was observed and mRNA level of *GmATG8i* increased in higher level than in the absence of the inhibitors. These results suggest that in soybean gene expression of *ATG8i* is regulated by both environmental nutrient conditions and intracellular nutrient recycling *via* proteolysis in vacuole.

Key words: ATG, autophagy, nutrient, proteolysis, soybean, starvation.

Autophagy is a bulk degradation system of intracellular components. In all eukaryotic organisms, autophagy has essential roles for homeostasis in most normal cells in preventing the accumulation of protein aggregates and defective cellular substructures (Moriyasu and Hillmer 2000; Reggiori and Klionsky 2002; Wang and Klionsky 2003). Moreover, various environmental cues (such as starvation, high temperature, hormonal stimulation and microbial invasion) or intracellular stress (such as damaged organelles and senescence) stimulate autophagy process (Aubert et al. 1996; Moriyasu and Ohsumi 1996; Doelling et al. 2002). Autophagy is enhanced under starvation not only in mammalian and yeast, but also in plant (Chen et al. 1994), possibly involving recycling of amino acids and other elements necessary for basic metabolism and biosynthetic pathway.

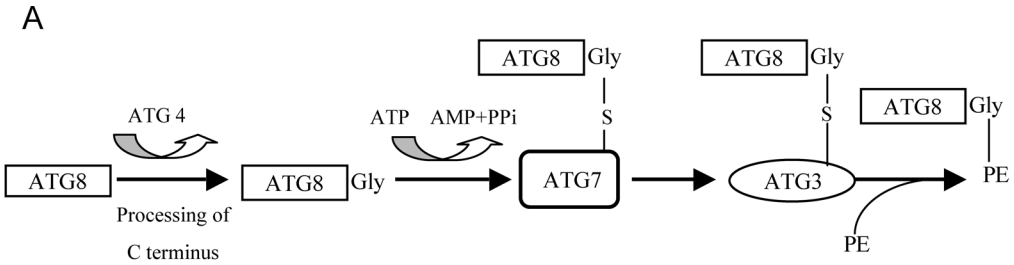
Genetic studies using budding yeast, *Saccharomyces cerevisiae*, have unveiled a set of novel genes involved in autophagy, many of which participate in the direct cytoplasm-to-vacuole transport by generating autophagosome (Harding et al. 1996; Baba et al. 1997). One of membrane-binding proteins, necessary for autophagy, is Atg8 protein (formerly known as Apg8/Aut7) (Klionsky et al. 2003). Atg8 is a ubiquitin-like protein that is firstly processed by the cysteine protease Atg4, removing the carboxyl terminal arginine residue and exposing a glycine residue at the carboxyl terminal end. Processed Atg8 is then activated by conjugating to an ubiquitin-activating enzyme (E1)-like enzyme Atg7 through a thioester bond and subsequently transferred to the ubiquitin-conjugating enzyme (E2)-like enzyme Atg3 via a new thioester bond. Finally, Atg8 is covalently conjugated to phosphatidylethanolamine (PE) on an autophagosome membrane via an amide bond between the carboxyl terminal glycine of Atg8 and PE (Figure 1A). Upon completion of autophagosome formation, the amide bond linking Atg8 to PE may be cleaved by the Atg4 protease (Kirisako et al. 1999, 2000) thus allowing the free Atg8 to participate in a new cycle of autophagosome formation.

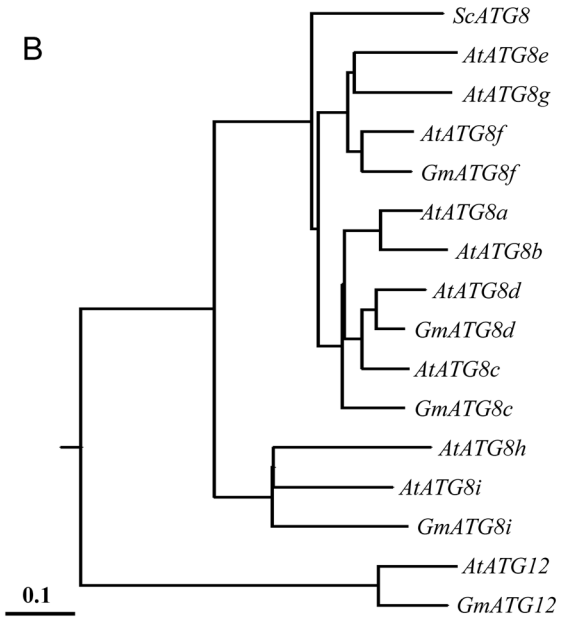
In contrast to *S. cerevisiae*, which has a single *ATG8*

Abbreviations: ATG, autophagy-related gene; GST, glutathione S-transferase; MBP, maltose-binding protein; PMSF, phenylmethylsulfonyl fluoride; RT-PCR, reverse transcription polymerase chain reaction; TOR, target of rapamycin; 1xTBS, 25 mM Tris-HCl [pH7.5] containing 150mM NaCl.

The nucleotide sequence reported in this paper have been submitted to DDBJ under accession numbers, AB453309 (*GmATG8i*) and AB453310 (*GmATG8d*).

This article can be found at http://www.jspcmb.jp/





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                *
GmATG8i         MGGSSSFRDEYTLEQRLGESRDIVAKYPDRVPVIVERYAKCDLPELEKKKYLVPRDLSVGH
AtATG8i           MK--KEQ---DE--A---E-I----T-I---A-K-C-T---AI----F-----M---Q
AtATG8h         MGIVVK--K-QFSSDE--K--NNI-------I---I-K-SNA---DM--N-------MT---
GmATG8c           MAKT--KLQHP--R-QA-ASR-RE-----I-----KAERS-I-DID-------A--T--Q
AtATG8c           MAN---KL-HP--R-QI--SR-RE-----I------AERS-V-NID-------A--T--Q
GmATG8d           MAK---KM-HP--R-QA-ASR-RE-----I------AE-S-V--ID-------A--T--Q
AtATG8d           MAI---KH-HP--K-QA-AAR-RE-----I------AE-S-V-DIDR------A--T--Q
YeastAPG8          MKST-KS--PF-KRKAE-E-ADRFKN--I---C-KAE-S-I--ID-R-----A--T--Q

                                                                        ↓
GmATG8i           FIHILSSRLSLPPGKALFVFVKNTLPQTANMMDSVYRSFKDEDGFLYMYYSTEKTFGSYIM   122
AtATG8i           --Y---A--H-S---------N-------AL-----E-Y--D---V--C--S----G       117
AtATG8h           ---M--K-MQ-D-S-------H-------SR---L-NT--E-------C-------G       120
GmATG8c           -VYVVRK-IK-SAE--I---IN----P--AL-SAI-EEN--Q------T--G-N--G-H     119
AtATG8c           -VYVVRK-IK-SAE--I---------P--A--SAI-DEN---------T--G-N--GLV     119
GmATG8d           -VYVVRK-IK-S-E--I-I----I--P--A--SAI-EEN---------T--G-N--GLVF    120
AtATG8d           -VYVVRK-IK-S-E--I-I----I--P---I-SAI-EEH---------S--G-N--GIFF    120
YeastAPG8         -VYVIRK-IM---E--I-I--ND---P--AL-SAI-QEH--K-----VT--G-N--GR      119
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Figure 1. A Schematic representation of ubiquitin-like modification in autophagy pathway. Atg8 precursor is processed by Atg4 and then activated by E1-Atg7 and E2-Atg3 enzyme and finally conjugated to phosphatidylethanolamine (PE) which appears to promote formation of autophagic vesicles. (B) Phylogenic trees of Atg8s and Atg12s in soybean *Glycine max* (Gm), *Arabidopsis thalania* (At) and budding yeast *Saccharomyces cerevisiae* (Sc). (C) Deduced amino acid sequences of GmATG8i and alignment with related sequences. The dashes in the sequences indicate identical amino acid to those of GmAtg8i. An underline and an astarisk (*) indicate N-myristoyl motif and the N-terminal glycine residue which is putatively modified for N-myristoylation, respectively. Arrow indicates the C-terminal Glycine residue which is processed by Atg4 cyctein protease. Accession numbers and TC numbers for soybean ATG homologs are described in the text. An accession number of yeast ATG8 and Arabidopsis Gene Index numbers (http://compbio.dfci.harvard.edu/tgi/) for Arabidopsis ATG homologs are: ScATG8, AY692870; AtATG8a, AT4G21980; AtATG8b, AT4G04620; AtATG8c, AT1G62040; AtATG8d, AT2G05630; AtATG8e, AT2G45170; AtATG8f, AT4G16520; AtATG8g, AT3G60640; AtATG8h, AT3G06420; AtATG8i, AT3G15580; AtATG12(ATG12a), AT1G54210. The deduced amino acid sequences were aligned by using the CLUSTALW program (http://align.genome.jp/).

gene in its genome, the *Arabidopsis thaliana* genome encodes nine *ATG8* (*AtATG8a-i*) orthologs. This suggests that autophagy in plants is more complicated (Hanaoka et al. 2002). To assess involvement of autophagy in starvation response of soybean, a set of *ATG* homologs of *GmATG8s*, *ATG4*, and *ATG12* which are involved in ubiquitin-like conjugation pathway was analysed. *ATG9* and *ATG18a*, considered to be essential for membrane recruitment to autophagosome, were also examined. Immunoblot with specific antibody indicated that soybean Atg8i was significantly accumulated in the presence of vacuolar protease inhibitors under starvation in different profiles.

Eight sets of one week-old seedlings soybean (*Glycine max* [L.] Merr. cv Fukuyutaka) grown in distilled water and sands were used. When the length of hypocotyls reached 6 to 8 cm, the seedlings were pre-incubated at 25°C in the nutrient-rich medium for 24 h after removing of cotyledons. After that the seedlings were transferred and grown under various nutrient conditions and harvested at intervals of 0, 8 and 24 h. For nutrient-rich treatment, the seedlings were incubated in a medium of 10 mM MOPS-KOH [pH7.4] supplemented with 2% of sucrose, 10 mM of KNO_3, 2 mM of $MgSO_4$ and 10 mM of KH_2PO_4. For a starvation treatment, the seedlings were incubated in a nitrate- and sucrose-depleted medium that had been prepared by replacing KNO_3 with KCl. For the protease inhibitor treatment, the seedlings were incubated in the nitrate- and sucrose-depleted medium supplemented with 1 mM of phenylmethylsulfonyl fluoride (PMSF, serine protease inhibitor), 10 μg ml^{-1} of leupeptin (cysteine protease inhibitor), 100 μM of E64-d (membrane permeable cysteine protease inhibitor) (PEPTIDE Inc., Osaka, Japan) and 10 mM of quinacrine for alkalization of vacuolar pH, at final concentrations.

BLAST search using *A. thaliana APG* genes (also known as *ATG*) in the Soybean Gene Index (SGI) at DFCI (http://compbio.dfci.harvard.edu/tgi/) identified *GmATG8c* (SGI tentative consensus (TC) 218668), *GmATG8d* (TC216643; Genbank accession number, AB453310), *GmATG8f* (TC210141), *GmATG8i* (TC207133; Genbank accession number, AB453309), *GmATG4* (TC228796), *GmATG9* (GenBank accession no. AM085508), *GmATG12* (TC228894) and *GmATG18a* (TC229926). A set of specific primers for a soybean actin gene (Genbank accession no. V00450) were used as positive control. RT-PCR was performed with total RNA from seedlings by using M-MLV reverse transcriptase (RT) and ExTaq DNA polymerase (TaKaRa, Tokyo, Japan) according to the manufacturer's manuals with gene specific primers shown in Table 1. To synthesize cDNAs, a 20 μl of reverse transcription reaction mixture containing 1 μg of total RNA, 2.5 μM oligo-$dT_{15–18}$, each 0.5 mM dNTPs, 40 units of cloned RNase inhibitor and 200 units of RT in the standard buffer condition were incubated at 42°C for 60 min after annealing. PCR was performed by the thermal cycler PC-816 (ASTEC, Fukuoka, Japana) with a 20 μl of reaction mixture containing 1 μl of cDNA sample, each 200 μM dNTPs, each 400 nM 5′- and 3′-primers, and ExTaq DNA polymerase (TaKaRa) in PCR reaction buffer under following thermal cycle conditions: initial denaturing at 94°C for 2 min, followed by 3-steps of 25 cycle denaturing at 94°C for 30 s, annealing at 61°C for 30 s, extending at 72°C for 40 s and then final extending at 72°C for 5 min. The cycle number for *GmATG4* and *Gm ATG18a* were 25 and or *GmATG8s*, *GmATG9*, *GmATG12* were 28, respectively. The amplified products were visualized by FluorChem (AlphaInnotech, San Leandro, CA, USA) after electrophoresis in 1.5% agarose gels and staining in ethidium bromide.

To prepare glutathione S-transferase (GST)-fused GmAtg8i, maltose binding protein (MBP)-fused GmAtg8i and MBP-GmAtg8d, pGEX-*GmATG8i*, pMal-*GmATG8i* and pMal-*GmATG8d* were constructed as following. PCR fragments coding *GmATG8i* and *GmATG8d* were amplified with KOD plus DNA polymerase (TOYOBO, Tokyo, Japan), soybean cDNA and specific primers sets (Table 1) and then digested with *Eco*RI and *Sal* I for *GmATG8i* and *Bam* HI and *Sal*I for *GmATG8d*, respectively. The *GmATG8i* fragment was ligated to pGEX4T-1 (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) or pMal-c (New England Bio labo, NE, USA), which were digested with *Eco*RI and *Sal* I, by Ligation Kit ver. 2 (TaKaRa). The *GmATG8d* fragment was ligated to pMal-c digested with *Bam* HI and *Sal*I by Ligation Kit ver.2. The cDNAs of *GmATG8i* and *GmATG8d* cloned in pMal vectors were sequenced by an ABI Prism 310 DNA

Table 1 Primers used for RT-PCR analysis and cloning of *GmATG8d,i*

	Forward primers	Reverse primers
GmATG4	5′-GGCAAACCAGGTGCTTCAACATAC-3′	5′-ATGCCAGGGATCAACCAAACTTGC-3′
GmATG8c	5′-GACCATGGCCAAAAGCCCCTTCAAGCT T-3′	5′-ATTGAGCTCCATAATTTGAAGAGAATGCAC-3′
GmATG8d	5′-TTCGGATCCATTGCCATGGGCAAGAGCTCA-3′	5′-GATGGTCGACTGTCACGACGGATTATT TCA-3′
GmATG8f	5′-TCTGGATCCTCTTCACGAGGAAGAATGACA-3′	5′-TGATGTCGACTACCGATACAAATTTA GAG-3′
GmATG8i	5′-CCGGAATTCGTTGAGCTG CACAACAACCTA-3′	5′-GATAGTCGACACTTCACAAAGTGTTGGATA-3′
GmATG9	5′-ATTTGGATCCCATGATGTGAATCTG-3′	5′-CCT GTCGACAGCAAGAACCTCCTAA-3′
GmATG12	5′-TTGGGATCCCTGAGGACAATGTCTTCTGAA-3′	5′-AATGTCGACCCCCCTAAAGTAAAACC-3′
GmATG18	5′-ATGTCTCAAACCCTCGGTTCCGATGAG-3′	5′-GGAGCTGGCGGTTGCAAGCAACCTGCC-3′
Actin	5′-GCGTGATCTCACTGATGCCCTTAT-3′	5′-AGCCTTCGCAATCCACATCTGTTG-3′

sequencer with a Big Dye Terminator Cycle Sequencing Kit ver. 1.1 (Applied Biosystems, Foster City, CA, USA).

To express GST-GmAtg8i, MBP-GmAtg8i and MBP-GmAtg8d were expressed in *E. coli* BL21DE3 (Stratagene, La Jolla, CA, USA) containing pGEX-*GmATG8i*, pMal-*GmATG8i* or *d*, by adding isopropyl ß-D thiogalactopyranoside (IPTG) into the cultures at a final concentration of 0.1 mM when OD_{600} of *E. coli* culture reached 0.5. The *E. coli* cells (250 ml, 2xYT media) were harvested by centrifugation at 10,000×*g* for 15 min. The *E. coli* pellet was then resuspended in lysis buffer containing 1 mM PMSF, 0.5 mM benzamidine, 1xTBS, 5 mM EDTA, 0.5% Triton X-100 and 0.05 % ß-mercaptoethanol, and the cells were disrupted by sonication (Handy Sonic model UR-20P, TOMY SEIKO, Tokyo, Japan). GST-GmAtg8i, MBP-GmAtg8i and -GmAtg8d were purified from extracts of *E. coli* according to manufacturer's (GE Healthcare Bioscience) instruction manuals. After centrifugation of the mixture at 10,000×*g* for 15 min at 4°C, the resultant supernatants was mixed with 100 µl (50% (v/v) bed volume) of glutathione sepharose CL4B beads for GST-GmAtg8i and amylose bead for MBP-GmAtg8i or d and then rotated at 4°C for 2 h. The recombinant protein-bound beads were washed with 10 ml of washing buffer containing 1xTBS, 1 M NaCl, 0.1% Triton X-100 and 0.05% ß-mercaptoethanol 6 times. Then, GST-GmAtg8i was eluted from glutathione sepharose beads by adding washing buffer containing 10 mM glutathione. Resultant GST-GmAtg8i was inoculated into a rabbit to raise anti-GmAtg8i specific antibody (IWAKI, Fukuoka, Japan). MBP-GmAtg8i- or -GmAtg8d-bound beads were stored at −30°C.

Protein extract was prepared from 1 week old seedlings (about 1 g) under various nutrient treatments by homogenization on an ice-cooled motor with liquid nitrogen and mixed with 3 ml of lysis buffer containing 1xTBS, 10 mM EDTA, 5% glycerol, 0.2% ß-mercaptoethanol, 1 mM PMSF, 10 µg ml^{-1} leupeptin and 1 mM benzamidine. The resultant extracts were centrifuged at 5,000×*g* for 5 min at 4°C to prepare a post-nuclear supernatant. Then, the resulting 5,000×*g* supernatant was successively centrifuged at 100,000×*g* for 1 h at 4°C to prepare a 100,000×*g* pellet as microsomal fraction and a 100,000×*g* supernatant as soluble fraction. Protein concentrations were determined by measuring OD_{595} with Bio Rad protein assay kit (Bio Rad, Hercules, CA, USA) using 1 mg ml^{-1} bovine serum albumin as a standard.

For immunoblot, polypeptides that had been separated by SDS-PAGE were electro-transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) in blotting buffer containing 25 mM Tris, 0.05% SDS and 20% methanol at 10 V cm^{-1} for 2 h. Then, the membrane was incubated in blocking buffer containing 1xTBS, 3% skim milk for 1 h for blocking and then incubated in blocking buffer supplemented with primary antibody anti-GmAtg8i antibody (1/1,000 dilution) and 0.05% Tween20 for 2 h at 4°C. After washing in 1xTBS containing 0.05% Tween20, the membrane was incubated in blocking buffer supplemented with horse raddish peroxidase labeled anti rabbit antibody (1/5,000 dilution (v/v))(GE Healthcare Bio-Sciences) for 1 h. Immuno-reactive signals were visualized by ECL Plus kit (GE Healthcare Bio-Sciences) and FluorChem. A competition assay with GST-GmAtg8i recombinant protein was carried out to confirm the specificity of the antibody against the endogenous GmAtg8i polypeptide. After starvation and protease treatment of soybean seedlings, protein samples (each 20 µg) of 100,000×*g* supernatant and 100,000×*g* pellet were subjected to SDS-PAGE with 15% polyacrylamide gel and electroblot onto PVDF membrane. TBS-milk (3 ml) containing anti-GmAtg8i antibody (3 µl, 1/1,000 dilution) was incubated with 20 µg of GST-GmATG8i recombinant protein as a competitor for 2 h before incubation of the PVDF membrane. Other procedures of immunoblot were performed as control.

A phylogenic analysis of deduced amino acid sequences of *ATG8* homologs using AtAtg12 and GmAtg12 as an out-group showed that *AtATG8a–g* and *GmATG8c*, *d*, *f* can be classified into one clade of Atg8 subfamilies with 68 to 91% amino acid similarities including yeast Atg8 and that GmAtg8i is grouped into another clade of Atg8 subfamilies with AtAtg8h and AtAtg8i (Figure 1B). In addition, GmAtg8i has high homology (70.4% and 64.7%) with AtAtg8i and AtAtg8h, respectively. GmAtg8i possesses extra amino acids at the conserved glycine end while AtAtg8i and AtAtg8h have a glycine residue at their carboxyl terminus without the extra amino acid (Hanaoka et al. 2002; Yoshimoto et al. 2004). Alignment of the deduced amino acids of *GmATG8i*, *AtATG8i*, *AtATG8h*, *GmATG8c*, *AtATG8c*, *GmATG8d*, *AtATG8d* and yeast *ATG8* indicates indicates that GmAtg8i, AtAtg8i and AtAtg8h share high similarity in those amino acid sequences distant from other Atg8s. The deduced amino acid sequence of also shows that GmAtg8i has an unique motif which myristoylation consensus motif (M-G-X-X-X-S/T-) at the N-terminus (Thompson Jr and Okuyama 2000), suggestting that GmAtg8i is myristolyated on the glycine residue at N-terminus (Figure 1C). N-myristoylated proteins such as calcineurin B-like molecule appeared to be localized onto microsomes (Imamura et al. 2008). Therefore, it is possible that GmAtg8i associates to microsomes.

It was examined whether expression of *GmATG*s is regulated in response to various nutrient conditions (nutrient rich, starvation and starvation with protease inhibitor) by RT-PCR. The expression patterns were

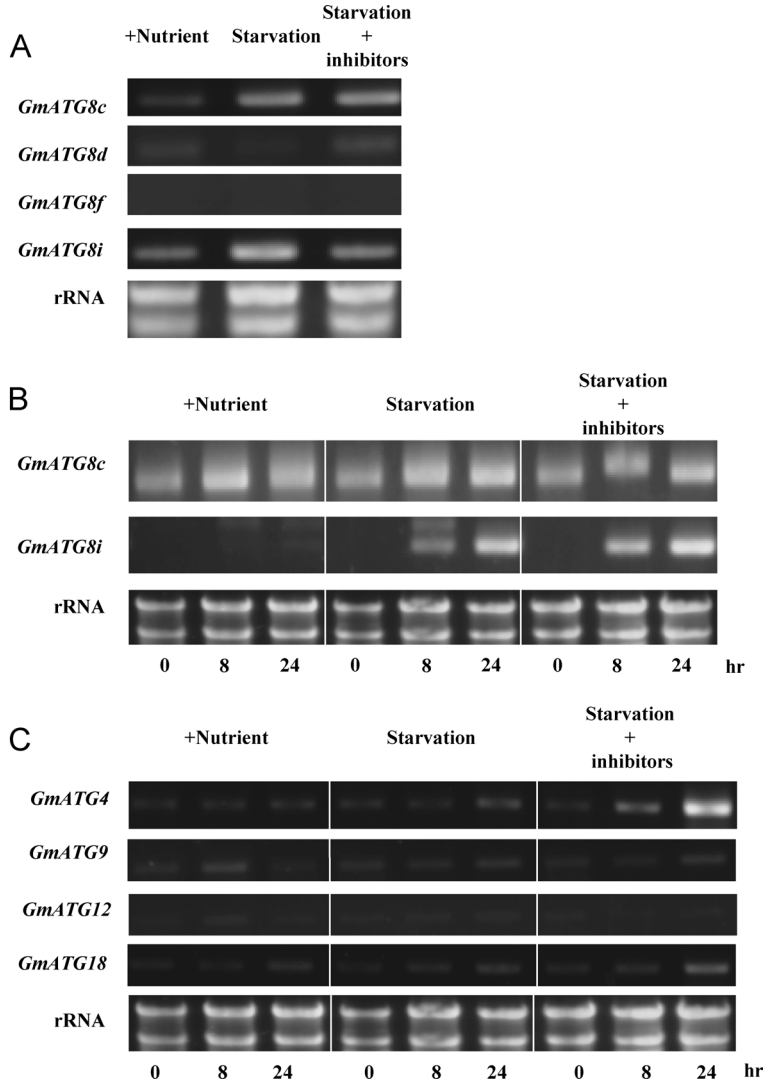

Figure 2. Starvation induced autophagy related genes in soybean seedlings. (A) The expression of the mRNA levels of homolog *GmATG8s*. (B,C) The expression patterns of soybean *ATG*s. Soybean seedlings were transferred from nutrient rich medium to the media of starvation and starvation with protease inhibitors, for the indicated times.

varied among soybean *ATG8* isoforms. *GmATG8c* and *GmATG8i* were significantly induced in response to various nutrient conditions while the expression levels of *GmATG8d* was weak and *GmATG8f* was marginal in any nutrient conditions tested, comparing with those of *GmATG8c* and *GmATG8i* at the same cycle number in RT-PCR (Figure 2A).

Next, the expression profiles of *GmATG8c* and *GmATG8i* of 1-week-old seedlings (after removing cotyledon) were examined at different time intervals after incubation in starvation media. *GmATG8i* mRNA increased significantly both under starvation and starvation with protease inhibitors. Expression of *GmATG8c* was observed under favorable growth condition and following exposure to starvation or starvation with protease inhibitors (Figure 2B). On the other hand, expression of *GmATG4* mRNA was stimulated only in the presence of protease inhibitors under starvation whereas expression levels of the *GmATG9*, *12*, *18a* were not altered under these conditions (Figure 2C).

It was examined if anti-GmAtg8i antibody cross-reacts specifically with GmAtg8i using recombinant MBP-GmAtg8i and MBP-GmAtg8d (Figure 3A). Immuno-reactive signals were detected with MBP-Atg8i and GST-GmAtg8i, but not which MBP nor MBP-GmAtg8d. The observation is consistent with that an anti-AtAtg8i antibody specifically recognizes AtAtg8i and AtAtg8h, but not AtAtg8a, b, c, d, e, f, g (Yoshimoto et al. 2004). When 100,000×*g* supernatant and 100,000×*g* pellet prepared from seedling incubated in sucrose and nitrate starved media containing protease inhibitors were subjected to immunoblot, an immunoreactive signal at 14 kDa detected in 100,000×*g* pellet sample with immunoblot in control condition but not with immunoblot in the presence of competitor protein, excessive GST-GmAtg8i (Figure 3B), suggesting that the immunoreactive

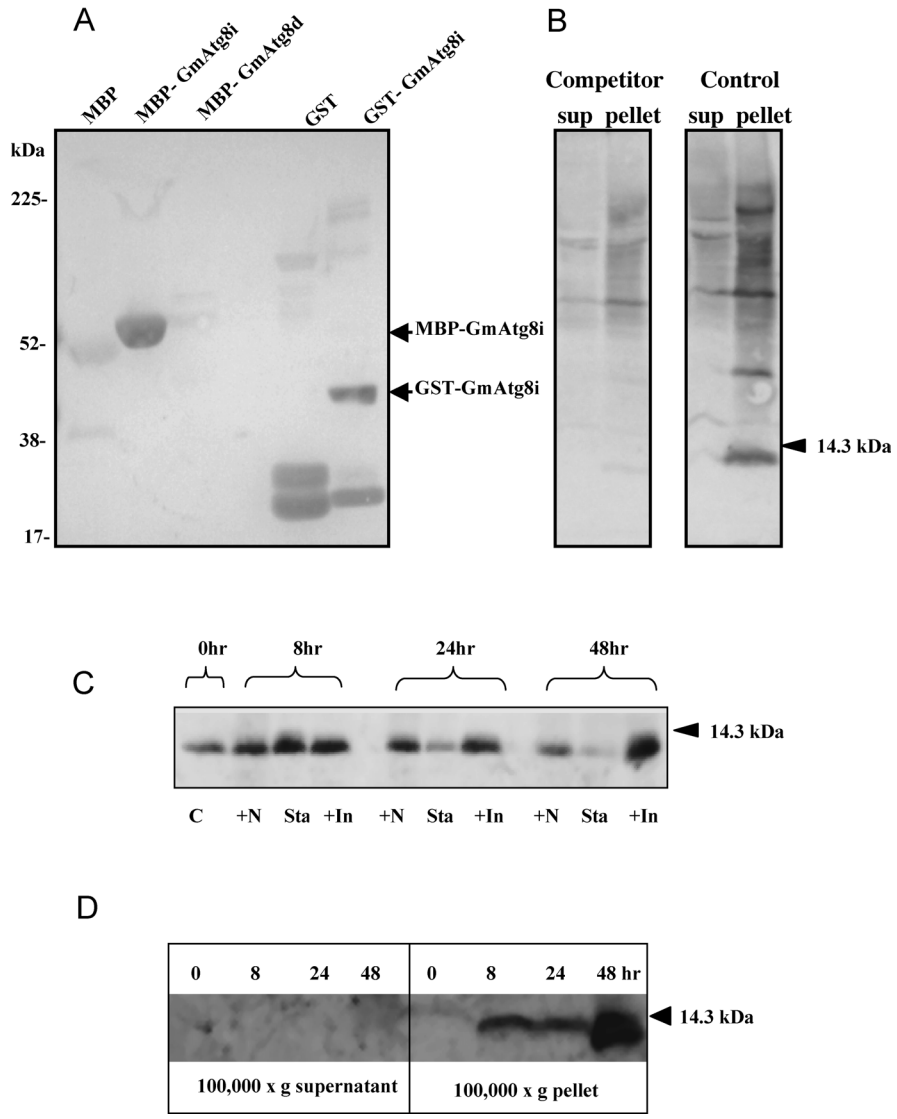

Figure 3. Accumulation of GmAtg8i in soybean seedlings under various nutrient treatments. (A) Cross-reactivity of an anti-GmAtg8i antibody with MBP-GmAtg8i and -GmAtg8d expressed in *E. coli*. Immuno-reactive signal with the anti-GST-Atg8i antibody were specifically detected on polypeptides (each 2.5 μg protein) of GST-GmAtg8i, MBP-GmAtg8i but not to MBP nor MBP-GmAtg8d. (B) Immunoblot of 100,000×*g* supernatanat and 100,000×*g* pellet by anti-GmAtg8i antibody was carried out in the presence of competitor, GST-GmAtg8i recombinant protein (left) and in control condition (right). (C) Immunoblot was carried out with the microsomal fractions after seedlings were treated in different nutrient treatments. (D) Immunoblot was carried out with soluble and microsomal fractions of soybean seedlings in the presence of protease inhibitors under starvation treatment at various time courses. C; control, +N; rich nutrient, Sta; starvation, +In; starvation with protease inhibitors. Each 50 μg protein per lane of soybean extracts were subjected to SDS-PAGE.

signal at 14 kDa is the endogenous GmAtg8i polypeptide.

Intracellular distribution of GmAtg8i, subcellular fractionation was investigated by immunoblot using the antibody. After 1-week-old seedlings were incubated in different nutrient conditions at various time courses, homogenates of harvested seedlings were subjected to successive centrifugation to prepare 100,000×*g* supernatant and 100,000×*g* pellet (see materials and methods). Immuno-reactive signals were detected at 14 kDa in 100,000×*g* pellets of each samples (Figure 3C). However, the protein accumulation was more abundant when immunoblotted in the presence of protease inhibitors under starvation (Figure 3C, lanes: +In, 24 and 48 h) although accumulation of GmAtg8i protein under starvation (without protease inhibitors) decreased as incubation got prolonged (Figure 3C, lanes: Sta, 24 and 48 h). Protease inhibitors are supposed to suppress degradation of autophagosomes in vacuole, resulting in accumulation of its cytoplasmic inclusions as autophagic bodies within the central vacuole. On the other hand, almost all GmAtg8i-related protein was degraded possibly by proteases under starvation treatment without protease inhibitors.

When 100,000×*g* supernatant and 100,000×*g* pellet were prepared from soybean seedlings incubated in starvation media with protease inhibitor at different time

courses, immunoreactive signals were predominantly detected in 100,000×*g* pellets as incubation time got prolonged while immuno-reactive signals were not detected in 100,000×*g* supernatant (Figure 3D). This indicates that the GmAtg8i is tightly associated with microsomal fraction. In several experiments, the signals of GmAtg8i in 100,000×*g* pellet were weak (as in Figure 3C, 0 h) or in faint level (as in Figure 3D, right panel, 0 h) just after pre-incubation. It is conceivable that consumption and/or uptake of sucrose and nitrate by seedlings resulted in mild deficiency of nutrients in media, which might affect on levels of GmAtg8i at 0 h. The observation that GmAtG8i associated to 100,000×*g* pellet (Figure 3B, D) is consistent with the speculation that N-myristoylation of *GmATG8i* predicted by the amino terminal consensus motif recruit GmAtg8i onto microsomes (Figure 1C).

Autophagy plays important roles in recycling various cellular constituents especially under starvation or environmental stresses (Dunn 1994; Kim and Kilonsky 2000). The molecular mechanisms of autophagy have been well characterized in yeast, mammals and *A. thaliana*. Until now, however, there have been no study on soybean *ATG*s. According to ubiquitin-like conjugation system (conjagation of Atg8 to PE) (Figure 1A) involving autophagosome formation, soybean *ATG8*s share significantly high similarities in the deduced amino acid sequences to those of *A. thaliana ATG8*s (Figure 1B). As shown in phylogenic tree analysis, GmAtg8i has an amino acid sequence with a little difference from those of other GmAtg8 proteins, GmAtg8c, d, f, and belongs to another clade including AtAtg8i and AtAtg8h among a phylogenic tree of Atg8 family (Figure 1B). This suggests that soybean and *A. thaliana* have similar molecular mechanisms utilizing multiple Atg8-related proteins, different from yeast possessing a single *ATG8*. This prediction has been further supported by the demonstration that different *GmATG8s* homologs were expressed in various levels under different nutrient treatments of 1 week-old seedlings (Figure 2A). Among them, *GmATG8i* mRNA was significantly induced both in sucrose- and nitrate-starvation medium and in starvation medium containing vacuole protease inhibitors (Figure 2B). *GmATG8c* was expressed in all nutrient treatments whereas expressions of *GmATG8d* and *GmATG8f* were undetectable by RT-PCR with 33 cycles in all nutrient treatments (data not shown). It was reported that combination of sucrose starvation and protease inhibitors generates autophagosomes in vacuoles of *Arabidopsis* and yeast (Ohsumi 2001; Rose et al. 2006). In *Arabidopsis*, all *AtATG8s* were ubiquitously expressed in every tested organ but the mRNA level was much higher only in leaves of 4–5 week old wild type plant under nitrogen starvation (Hanaoka et al. 2002; Slavikova et al. 2005). This may suggest that different *ATG8*s are functionally assigned according to tissue, cell developmental age or stress conditions in each organ of whole plant.

Atg4 protein is required for the procession of Atg8 to expose the carboxyl terminus at the glycine residue (Figure 1A). *GmATG4* mRNA was induced only when incubated with starvation medium containing protease inhibitors. Transcripts of *GmATG9* and *GmATG18a* which are involved in delivery of membrane components to the formation of autophagosome were faintly detected under various nutrient treatments (Figure 2C). In *A. thaliana*, *AtATG18a* mRNA is up-regulated under starvation while *AtATG18b*, *-c* and *-d* were expressed ubiquitously throughout plant organs and their expression levels did not alter in response to starvation (Xiong et al. 2005). In soybean, mRNA level of *GmATG18a* did not change in response to starvation but marginally did in the present of protease inhibitors (Figure 2C).

Moreover, it is interesting that GmAtg8i was significantly accumulated when incubated in starvation medium containing protease inhibitors (Figure 3C) because proteolysis was inhibited by E64-d, leupeptin and vacuolar alkalization with quinacrine even though *GmATG8i* mRNA was induced. Whereas Atg4 has significant similarity to cysteine protease, it was reported that E-64 does not inhibit processing activity of Atg4s in yeast and trypanosoma (Alvarez et al. 2008). Inoue et al. (2006) reported that E-64 treatment resulted in accumulation of autolysosomes, plant-specific autophagosome-like structure, suggesting E-64 did not inhibit formation of autophagosome but inhibited degradation by vacuolar proteases. It was reported that the serine protease inhibitor, PMSF, results in the accumulation of membrane bounded parts of the cytoplasm, the autophagic bodies, in the vacuole in yeast (Takeshige et al. 1992). Similarly, treatments of leupeptin and E64-d on tobacco cells resulted in accumulation of particles of cytoplasm in the vacuole because plant specific vacuolar processing enzymes necessary for maturation of proteases were effectively inhibited by the cystein protease inhibitors (Moriyasu and Ohsumi 1996). Without starvation of sucrose, E-64 did not induce accumulation of autolysosomes in *Arabidopsis* root as control (Inoue et al. 2004). It is conceivable that treatment of vacuolar protease inhibitors is not enough to induce autophagy and accumulation of GmAtg8i in plant cells incubated with sucrose-rich medium. On the other hand, it is possible that starvation enhanced both autophagy process and proteolysis of the GmAtg8i on autophagosomes. That is a reason why the immunoreactive signals of the GmAtg8i decreased apparently whereas expression of the mRNA level of *GmATG8i* was up-regulated as incubation got prolonged under starvation treatment (Figures 2B, 3C).

As observed in yeast, the treatment of protease inhibitors on soybean resulted in a significant accumulation of the GmAtg8i in 100,000×*g* pellet (Figure 3D). Atg8i undergoes conjugation to PE on the surface of membrane structures in 100,000×*g* pellet before the formation of autophagosomes (Yoshimoto et al. 2004). It is conceivable that GmAtg8i associating to autophagosomes comes to be rapidly degradated in vacuoles once autophagy is accelerated under starvation.

Under the sucrose- and nitrate-starvation condition (Figure 4A), the starvation signal was transmitted for autophagosome generation and then the transcript level of *GmATG8i* increased which in turn, nonselective degradation of intracellular proteins was promoted by vacuolar hydrolases and the regenerated amino acids and lipids via degradation were transported from vacuole to the cytoplasm for recycling. Consequently, recycling of amino acid maintains intracellular amino acid pool. It is conceivable that a putative nutrient sensing molecule (Sensor X in Figure 4A) monitoring intracellular amino acid pool affect starvation-induced autophagy and expression of *GmATG8i* by feedback regulation. In the other hand, treatment of soybean seedlings with cysteine protease inhibitors (E64-d and leupeptin), a serine protease inhibitor (PMSF) and quinacrine (for alkalization of vacuolar pH) inhibited both protein degradation (Figure 4B). Then GmAtg8i and autophagosomes were highly accumulated without degradation, leading to suppression of amino acids recycling. Concomitantly, resultant severe nutrient starvation and depletion of amino acid pool leads to further activation of autophagy process, such as significant induction of *GmATG8i*. Recent studies of autophagy in animal cells and yeast have revealed that TOR (target of rapamycin) kinase is a nutrient sensing molecule which regulates autophagy process by monitoring intracellular amino acid pool and that TOR kinases are essential for the pleiotropic biological processes such as transcription, translation, development and stress resistance, etc. (Deprost et al. 2007) in eukaryotic cells. TOR kinases have been involved in control of cell growth in *Arabidopsis* and green alga. The Sensor X in Figure 4 may be assumed to be a plant TOR homolog. However, there is no experimental evidence at present that TOR regulates autophagy in plants (Diaz-Troya et al. 2008), mainly due to that plant TOR appeared to be resistant to rapamaycin which is the most strong tool for analysis of TOR in autophagy process. By searching for a soybean TOR homolog, we identified partial cDNA sequences putatively coding TOR-like kinase (TC265041 in SGI and accession number BI700698 in GenBank). It is necessary to examine if the endogenous TOR activity in soybean is changed under various nutrient conditions affecting autophagic process. Cloning and biochemical characterization of a soybean TOR homolog should be important to address the question.

In conclusion, the present study unveiled that soybean isoforms of *ATG8*s and *ATG*4 are differentially regulated

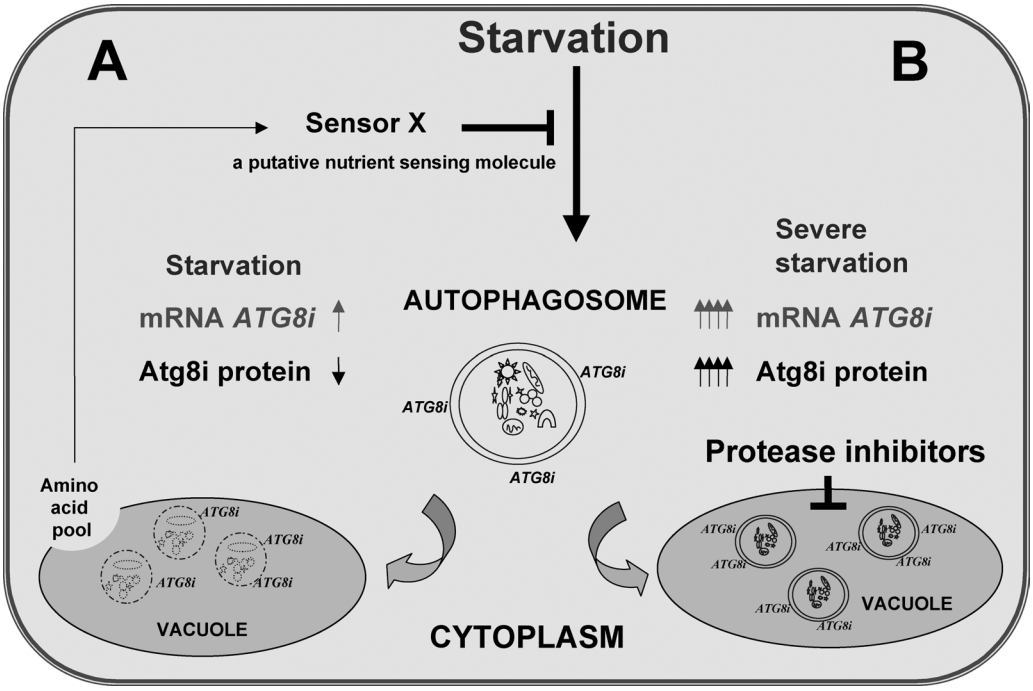

Figure 4. Schematic representation of autophagy function under nutrient starvation and protease inhibitors treatment. The starvation signal is transmitted for autophagosome formation, upregulation of *ATG8* and then protein degradation occurs (A). The resultant nutrients activate Sensor X (a putative nutrient sensing molecule) and in turn it suppresses autophagy process. Under the protease inhibitors (B), however, it block proteolysis and high accumulation of Atg8 proteins. It prevents recycling of amino acids, inactivates the Sensor X and in turn enchances autophagy process.

by nutrient starvation in media and/or depletion of intracellular amino acid pool by protease inhibitors. While other *GmATGs* mRNA are not significantly changed under various starvations, *GmATG8i* and *GmATG4* are up-regulated at mRNA level under severe starvation, and therefore potentially have specific roles in starvation-induced autophagy. Recently, we observed that expression of ethylene synthesis- and ethylene signal-related genes are stimulated under starvation accompanied with induction of *ATG*s in soybean (Nang Myint Phyu Sin Htwe et al. 2008). It is conceivable that expression pattern of *ATG*s in reproductive organs is regulated by phytohormones involving developmental stages. Here, we expect that autophagy is involved in leaf senescence and/or recycling of nutrients in sink-source-relationship. In addition, our present study shows a possibility that detection of GmAtg8i protein and *GmATG8i* mRNA is utilized for monitoring temporal/spacial specific induction of autophagy process in soybean plants.

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