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Molecules and Cells

OsATG10b, an Autophagosome Component, Is Needed for Cell Survival against Oxidative Stresses in Rice

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Autophagy degrades toxic materials and old organelles, and recycles nutrients in eukaryotic cells. Whereas the studies on autophagy have been reported in other eukaryotic cells, its functioning in plants has not been well elucidated. We analyzed the roles of *OsATG10* genes, which are autophagy-related. Two rice *ATG10* genes - *OsATG10a* and *OsATG10b* - share significant sequence homology (about 75%), and were ubiquitously expressed in all organs examined here. GUS assay indicated that *OsATG10b* was highly expressed in the mesophyll cells and vascular tissue of younger leaves, but its level of expression decreased in older leaves. We identified T-DNA insertional mutants in that gene. Those *osatg10b* mutants were sensitive to treatments with high salt and methyl viologen (MV). Monodansylcadaverine-staining experiments showed that the number of autophagosomes was significantly decreased in the mutants compared with the WT. Furthermore, the amount of oxidized proteins increased in MV-treated mutant seedlings. These results demonstrate that OsATG10b plays an important role in the survival of rice cells against oxidative stresses.

INTRODUCTION

Ubiquitination and autophagy are two separate mechanisms that degrade components, e.g., proteins and organelles, in eukaryotic cells. Whereas the former process breaks down a specific component that is tagged with ubiquitin, the latter is non-specific, causing bulk degradation (Kopitz et al., 1990). Autophagy transports aged organelles such as mitochondria and wastes generated by various metabolisms into the vacuole and lysosome for their degradation and re-use (Baba et al., 1994; Kwon and Park, 2008).

The autophagy-mediated degradation process has been well elucidated in yeast (Takeshige et al., 1992). Various waste products or aged organelles begin to be surrounded by a double-membrane pre-autophagosomal structure (PAS), which then expands to form an autophagosome. After maturation the autophagosome attaches to the vacuole and fuses with the vacuolar membrane. The internal materials are delivered and broken down by acid hydrolases; eventually, those degradation products are presumably transported back to the cytoplasm (Bassham et al., 2006; Schworer and Mortimore, 1979). It is thought that autophagy plays important roles in the survival of an organism by recycling and redistributing degraded materials, especially under conditions of nutrient starvation.

The autophagosome is formed by various autophagy-related proteins (ATGs) (Kametaka et al., 1996; Matsuura et al., 1997; Mizushima et al., 1998). Two conjugation pathways are involved during membrane expansion, a procedure similar to that of ubiquitin (Mizushima et al., 1998). ATG7 functions as an E1-like enzyme, and ATG3 and ATG10 as E2-like enzymes to deliver ATG8 and ATG12 to phosphatidyl-ethanolamine (PE) and ATG5, respectively. Both the ATG8-PE dimer and ATG12-ATG5 dimer are required for PAS formation (Tanida et al., 1999; Thompson et al., 2005). Although the ATG12-ATG5 conjugate functions as an E3-like enzyme that directly promotes ATG8-PE formation (Hanada et al., 2007), the detailed functions of that conjugation reaction remain veiled (Shao et al., 2007). Shintani et al. (1999) have observed in yeast that after ATG12 is activated by ATG7, the former is transferred to the Cys-133 residue of ATG10 to form an ATG12-ATG10 thioester, and then ATG12 is conjugated with ATG5. Phillips et al. (2008) also have reported that ATG10 is essential for ATG12-ATG5 conjugation in *Arabidopsis*. In rice, Su et al. (2006) have found that OsATG8 interacts with OsATG4, which functions as cysteine protease to cleave the C-terminal region of OsATG8.

Functional studies of autophagy under nutrient starvation have been performed in yeast, mammals, and plants (Doelling et al., 2002; Funakoshi et al., 1997; Hanaoka et al., 2002; Moriyasu and Ohsumi, 1996; Mortimore et al., 1983; Tsukda and Ohsumi, 1993). Additional roles have also now been proposed (Fujiki et al., 2007; Liu et al., 2005). Xiong et al. (2007a) have shown that RNAi transgenic plants of *AtATG18a* are hypersensitive to hydrogen peroxide and methyl viologen (MV), and

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accumulate more oxidized proteins, hence suggesting that autophagy helps to remove oxidized proteins that form under conditions of oxidative stress.

Here, we show differential expression patterns of rice *ATG10a* and *ATG10b.* In addition, we present a functional study of the *ATG10b* gene.

MATERIALS AND METHODS

Plant materials and growing conditions

A japonica cultivar, 'Dongjin', of rice (*Oryza sativa*) was used in this study. Seeds of *osatg10* and sibling WT plants were sterilized with 50% hypo-chlorite for 30 min, washed three times with sterile distilled water, and placed on an MS medium (Murashige and Skoog, 1962). One-week-old seedlings were acclimated for 1 d by opening the Petri dish lids.

Screening of *osatg10b* mutants from T-DNA tagging lines

T-DNA insertional mutants in the *OsATG10b* gene were obtained by searching the TES database (http://signal.salk.edu/cgi-bin/RiceGE) (An et al., 2003; Jeong et al., 2006). Homozygous progeny were identified by genotyping the seedlings, using gene-specific primers and a primer located in the inserted T-DNA.

Oxidative stress treatments

One-week-old seedlings grown on a solid MS medium were exposed to our stress treatments. For high salt, the seedlings were placed for 12 h on an MS medium containing 250 mM NaCl. Afterward, they were allowed to recover at room temperature. For the MV treatment, seedling roots were submerged into a 20 μM paraquat solution. After 1 day, those seedlings were transferred to distilled water for recovery. During all these treatments, seedlings were harvested at 3-h intervals for RNA isolation and real-time PCR analysis.

RT-PCR and quantitative real-time PCR

Samples were homogenized in a milling machine (Retsch, Germany), and total RNA was extracted with trizol reagent (Invitrogen, USA). First-strand cDNA synthesis and 33 reaction cycles of RT-PCR were conducted as described previously (Han et al., 2006). Primers were designed at different exons, enabling us to differentiate those cDNA products from genomic DNA contamination. Real-time PCR was performed in a Roche LightCycler II as previously described (Han et al., 2006), using a 20-μl solution containing 1 μl cDNA solution, 20 pmole of gene-specific primers, and 1× SYBR premix Ex Taq (Takara Shuzo, Japan). *OsActin1* mRNA served to normalize the relative expression levels of *OsATG10a* and *OsATG10b*.

Vector construction for localization and transient expression of the *OsATG10b::sGFP* gene

To construct the localization vector, we obtained the full-length cDNA clone of *OsATG10b* by PCR, using two primers: 5′-ggatccTTGTGATGGGAG GCTCCTCA-3′ and 5′- actagtCTCC-TGTGTTTTCAGTCCAG-3′ (small letters indicate *Bam*HI- and *Spe*I-recognized sequences, respectively). This amplified cDNA clone was inserted into the pBluescripts SK vector (Stratagene, USA), which was digested with *Eco*RV. The subclone was double-digested with *Bam*HI and *Spe*I, then introduced into the *Bam*HI/*Spe*I site in pGA3452, which contains the *sGFP* gene driven by the maize *ubiquitin* promoter. Protoplasts derived from the rice Oc cell line were used for our experiments on transient expression (Hattori et al., 1994). Protoplast preparation and transformation procedures were performed via the methods described by Woo et al. (2007) and Han et al. (2006).

Gus assay and microscopic analysis

Histochemical GUS staining was carried out according to the method of Jeon et al. (2000b). After staining, samples were washed and fixed with 70% ethanol. They were then observed with the naked eye and photographed. The procedure for histological observation was performed as described by Jung et al. (2006).

Monodansylcadaverine staining and microscopy

Root tissues from MV-treated seedlings were stained with 0.75 mM monodansylcadaverine (MDC) as previously described (Contento et al., 2005). They were observed under a Zeiss Axioplan II compound microscope equipped with an AxioCam HRC digital imaging system (Carl Zeiss, Germany). MDC-stained tissues were visualized with a DAPI-specific filter. Autophagosomes were counted as described by Xiong et al. (2007a).

Measurement of oxidized proteins

The aboveground (shoots) and underground (roots) tissues were collected from MV-treated seedlings to determine their amounts of oxidized proteins. Samples were homogenized in a Retsch milling machine, and total protein was obtained with an extraction buffer of 10 mM NaCl, 10 mM $MgCl_2$, 5 mM EDTA, 10 mM β-mercaptoethanol, 1 mM PMSF, and 25 mM Tris-HCl (pH 7.5). After the extracted protein was quantified with a Bradford (1976) assay, 500 μg of total protein was transferred into two new tubes. To one of those tubes, 100 ml of 2 M HCl containing 10 mM 2,4-Dinitrophenylhydrazine (DNPH) was added, while 1 ml of 2 M HCl was added to the other tube. Following incubation for 1 h at room temperature, the protein was precipitated with 10% TCA (final concentration) for 20 min on ice. After centrifugation at 13,000 rpm for 15 min, the pellets were washed with 1:1 ethanol:ethyl acetate, then dissolved in 20 mM sodium phosphate buffer (pH 6.5) containing 6 M guanidine-HCl. The difference in spectrums between the DNPH-treated sample and the HCl control was measured as described previously (Kim et al., 2005; Levine et al., 1990; Oliver et al., 1987).

RESULTS

Isolation of *OsATG10b* full-length cDNA

Two *ATG10*-like genes are located on rice Chromosomes 4 and 12. This occurrence is different from yeast and *Arabidopsis*, where *ATG10* is a single-copy gene (Fig. 1). We named these rice genes *OsATG10a* and *OsATG10b*. Comparison of their full-length cDNA clones with genomic DNA sequences revealed that *OsATG10a* is composed of five exons while *OsATG10b* comprises seven (Fig. 1A). Exon 3 in *OaATG10b* encodes 20 amino acid residues but is not present in *OsATG10a*. These amino acids are also found in the *Arabidopsis* ATG10 but not in yeast ATG10. Whether the two proteins are functionally different is unknown. The last two exons of *OsATG10b* appear to be derived from a single exon by the addition of a 441-bp intron. That last intron is not present in the genome of *Arabidopsis* or humans.

OsATG10a and *OsATG10b* encode for a protein of 198 and 222 amino acid residues, respectively, and are 75% identical (Fig. 1C). OsATG10s are moderately homologous (39-43%) to *Arabidopsis* AtATG10. However, the C domain among them is more conserved, especially the cysteine residue, which is necessary for binding to ATG12 (Shintani et al., 1999). In contrast, the rice ATG10s have significantly diverged from yeast ATG10 (11-13%).

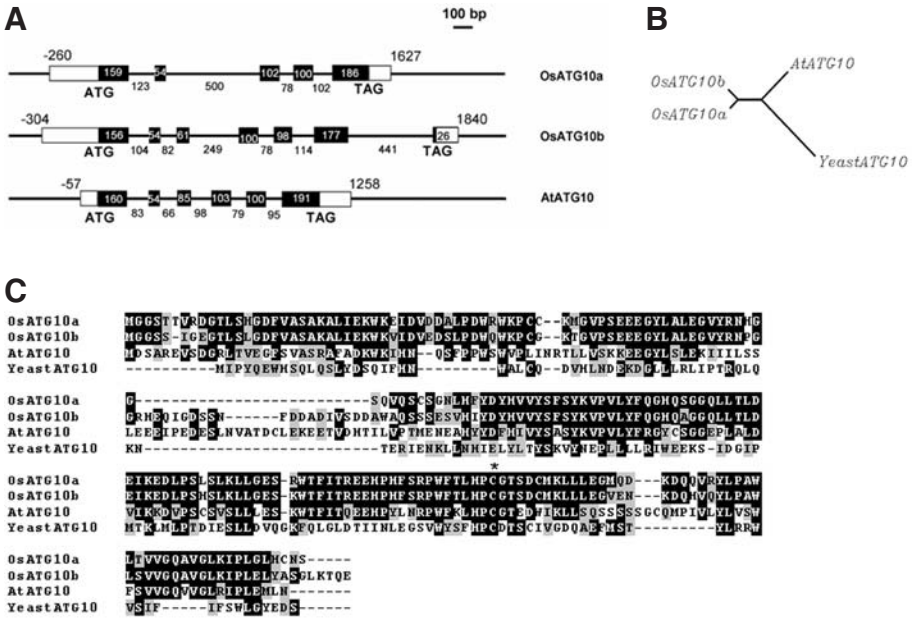

Fig. 1. (A) Comparison of genomic structures among *OsATG10a*, *OsATG 10b*, and *AtATG10*. Boxes are exons and lines between boxes are introns. Black boxes are coding regions; white boxes indicate untranslated regions. Numerals in black boxes and under lines indicate number of nucleotides in each exon and intron. (B) Phylogenic tree. (C) Alignment of amino acid sequences. Black boxes indicate identical amino acids; shaded regions are similar residues. Conserved cysteine residues required for binding to ATG12 are indicated by *.

Expression patterns of *OsATG10a* and *OsATG10b*

Expression profiles for *OsATG10a* and *OsATG10b* were studied by quantitative RT-PCR, using gene-specific primers. Patterns for those genes in 7-d-old seedlings were somewhat dissimilar (Fig. 2A). Although both were expressed in all samples examined, transcript of *OsATG10b* was more abundant in the leaves and maturation zone of the roots, whereas the level of *OsATG10a* transcript was higher in the leaf blades compared with other tissues. Because autophagy is known to play roles in degrading cellular components in old cells, we expected that the autophagous genes would be more actively expressed in the mature tissues compared with young cells. However, in 3-week-old plants, *OsATG10b* transcript levels were higher in the younger leaf blades, whereas *OsATG10a* expression was more or less similar regardless of tissue age (Fig. 2B).

Sub-cellular localization of OsATG10b

To locate it within a cell, OsATG10b protein was fused to the N-terminal region of sGFP and introduced into protoplasts isolated from Oc cells. We found that this fusion protein was localized in the cytosol (Fig. 3A), which was merged with that of mRFP (Figs. 3B and 3C). Our result is consistent with a previous report that ATG10 in yeast plays a role in the cytosol (Phillips et al., 2008).

Identification of T-DNA insertional mutants in the *OsATG10b* gene

To study the functional roles of *OsATG10b*, we obtained two mutant lines from T-DNA insertional populations (An et al., 2003; Jeon et al., 2000a; Jeong et al., 2006; Ryu et al., 2004). These alleles were designated *osatg10b-1* and *osatg10b-2* (Fig. 4A). T-DNA was introduced into the sixth intron in both alleles. Using RT-PCR, we demonstrated that the two are null alleles (Fig. 4B).

Because the direction of the *GUS* reporter gene within the T-DNA was the same as the direction of transcription in *osatg10b-1*, we examined whether the two genes form a translational fusion. During construction of the binary vector for T-DNA tagging, we included multiple splice acceptor sites in front of the *GUS* gene, allowing transcriptional and translational fusions regardless of the

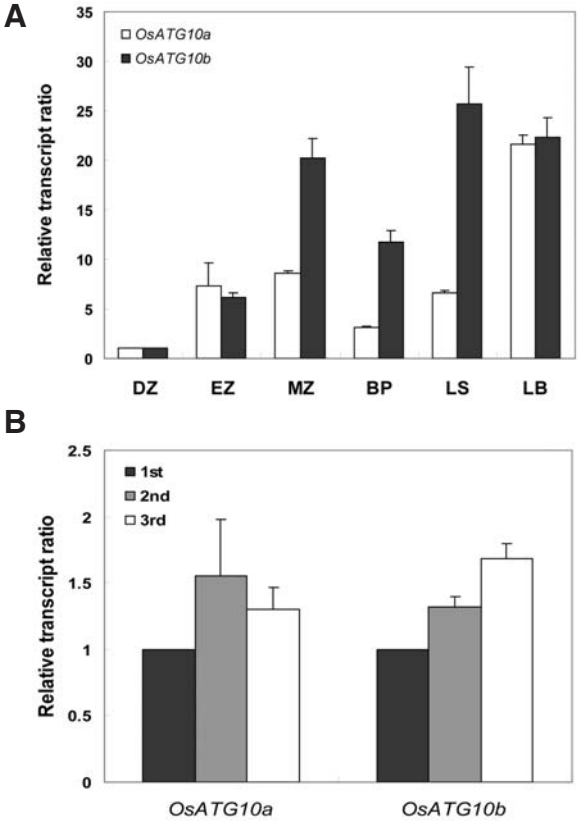

Fig. 2. Quantitative RT-PCR analyses of *OsATG10a* and *OsATG10b* transcripts. (A) Expression patterns in 1-week-old seedlings. Roots were dissected into division zone (DZ), elongation zone (EZ), and maturation zone (MZ). Shoots were separated into basal part (BP), leaf sheath (LS), and leaf blade (LB). (B) Expression patterns in 3-week-old seedlings. Blades were collected from 1st (oldest), 2nd, and 3rd (youngest) leaves. Relative transcript ratio implies *OsATG10* transcript levels normalized by *OsActin1* mRNA level.

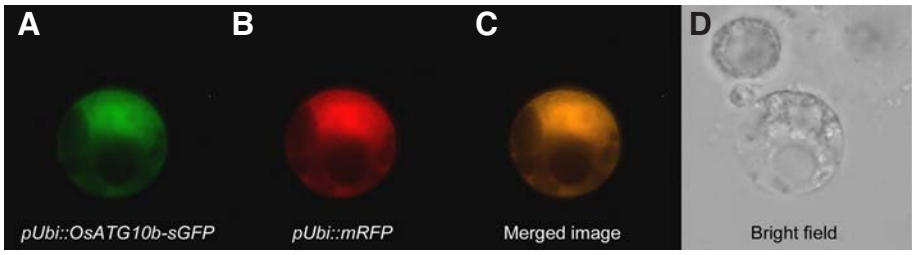

Fig. 3. Sub-cellular localization of OsATG10b::sGFP fusion protein. Protoplasts were derived from rice Oc cells and transfected with (A) *pUbiquitin::OsATG10b-sGFP* or (B) *pUbiquitin::mRFP.* (C) Merged image of A and B. (D) Image in bright field.

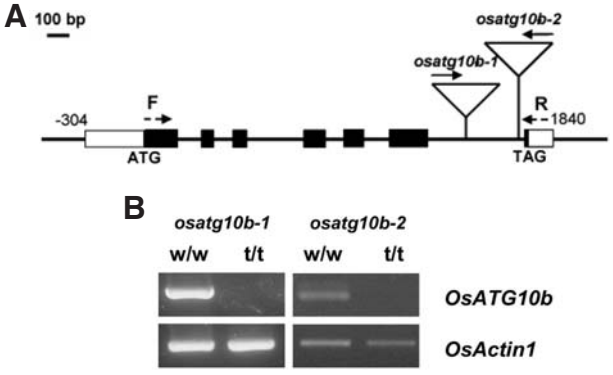

Fig. 4. T-DNA insertional mutants in *OsATG10b*. (A) Insertion positions within gene. T-DNA was inserted into 6th intron in both *osatg10b-1* and *osatg10b-2* alleles. Boxes indicate exons; lines are introns. Dotted arrows (F/R) indicate positions of primers used for RT-PCR. Arrows above triangles are *GUS* reporter. (B) RT-PCR analyses of *OsATG10b* expression. *OsActin1* was used to monitor equal loading. t/t = homozygous plant. w/w = sibling wild type.

insertion position (Jeon et al., 2000a). GUS analysis showed that the reporter gene was expressed in the *osatg10b-1* line (Fig. 5). Similar to our real-time PCR observations, GUS activity was detected in almost all the tissues from 7-d-old seedlings (Fig. 5A). In the roots, activity was higher in the tips (Figs. 5B and 5C). Among the three types of leaf samples, GUS intensity was similar (Fig. 5D). In 3-week-old plants, *OsATG10b* promoter-driven GUS activity was higher in the youngest leaf and very low in the oldest one, also consistent with our real time RT-PCR results (Fig. 5E). A cross section of the basal part of the plant showed that activity was strongly detected in both the young leaf and the phloem of the old leaf (Fig. 5F). In the former, GUS activity was observed in the mesophyll and phloem cells (Figs. 5G and 5H). These results indicate that the *OsATG10b* promoter is more active in young and active cells.

The *osatg10b-1* mutants are sensitive to stresses

Mutations of an autophagy component can cause senescence to accelerate under stress conditions (Xiong et al., 2007a; 2007b). Therefore, we examined whether our *osatg10b* mutants also had hypersensitive responses to stresses, such as from treatment with high salt or methyl viologen. Wild type and *osatg10b* mutants were germinated and grown on MS media. Under the normal conditions, the mutants were not different from WT (Figs. 6A and 6B). Seven-day-old seedlings were exposed to 250 mM NaCl and incubated at 30°C for 12 h. Afterward, they were displaced into distilled water for recovery. Compared with their wild-type siblings, the mutant seedlings recovered more slowly and some died (Figs. 6C and 6D). We also examined the effect of MV, an herbicide widely used for inducing oxidative stress. Seven-day-old seedlings were treated with 20 μM MV for 1 d, followed by recovery for 4 to 5 d. Whereas most wild-type siblings recovered after the treatment, none of the mutants recovered and all showed severe growth retardation and defects (Figs. 6E and 6F). These results indicate that the *OsATG10b* gene is necessary for conferring tolerance to such stresses.

Transcript levels for *OsATG10a* and *OsATG10b* in the leaf sheaths were not altered by MV (Fig. 6G). However, in the blades, expression was reduced by that treatment (Fig. 6H-6J). In the first (oldest) leaves, transcript levels of both genes were decreased; expression of *OsATG10b* slowly declined while that of *OsATG10a* was rapidly diminished (Fig. 6H). The *OsATG10a* transcript level was reduced to about one-half within 3 h of treatment. Transcript levels of *OsATG10a* also were rapidly decreased by MV in the second and third leaves, whereas those of *OsATG10b* were unaffected (Figs. 6I and 6J).

GUS analyses of the *osatg10b-1* plants produced similar results (Fig. 7). Staining intensity in the leaf blades slowly declined during MV exposure (Fig. 7A). In the roots, GUS activity remained only in the tips after that treatment (Fig. 7B).

The number of autophagosomes is decreased by MV treatment in *osatg10b* mutants

Most ATG proteins, including ATG10, function in the formation of autophagosomes (Patel et al., 2006; Shintani et al., 1999). Therefore, mutations in ATG genes should lead to a failure of autophagy generation. We counted the autophagosomes in root cells of 7-d-old seedlings via MDC-staining, which is routinely used for observing autophagy in plants and mammals (Biederbick et al., 1995; Contento et al., 2005). Autophagy was rare in either the WT plants or *osatg10* mutants before stress was applied (data not shown). After MV treatment, however, the number of autophagosomes rose significantly in both genotypes (Fig. 8A). However, the number in both mutant cells was only 30-35% of the amount found in WT (Fig. 8B).

Oxidized proteins are accumulated in MV-treated *osatg10b* mutants

Autophagy functions to transfer oxidized or modified proteins and organelles into the vacuole or lysosome (Bassham, 2007). Xiong et al. (2007a) have reported that more oxidized proteins are accumulated in *AtATG18a*-RNAi transgenic plants. We examined whether our mutations in *OsATG10b* also altered their ability to remove oxidized proteins. The 7-d-old seedlings of *osatg10b-1* and WT were treated with 20 μM MV and sampled at 12-h intervals to quantify oxidized proteins. For shoots of mutant plants, the amount of such proteins gradually increased up to 1.5 times in 12 h and about 2 times in 24 h. On the other hand, the amount of oxidized proteins in WT was slightly increased (about 1.2 times) in 12 h, but did not further increased in 24 h (Fig. 8C). Similar induction patterns were observed from the mutant roots (Fig. 8D). The MV treatment also increased the level of oxidized proteins in the *osatg10b-2* mutant (data not shown). This result indicates that the retardation of autophagosome formation in the *osatg10b* mutant

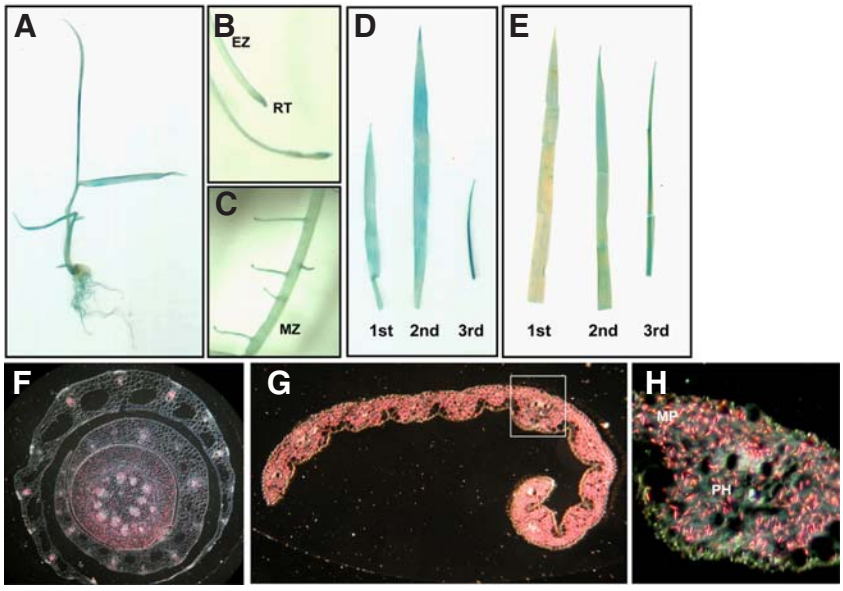

Fig. 5. GUS assay of *osatg10b-1* plants. (A) 1-week-old seedling. (B) Root tip (RT) and elongation zone (EZ) of (A) were magnified. (C) Maturation zone (MZ) of root in (A) was magnified. (D) Leaf blades of 1st, 2nd, and 3rd leaves from 1-week-old plant. (E) Leaf blades from 3-week-old seedling. (F) Cross section of basal part from 1-week-old seedling. (G) Cross section of leaf blade from 1-week-old seedling. (H) Magnification of vascular bundle region in (G). MP, mesophyll cells; PH, phloem.

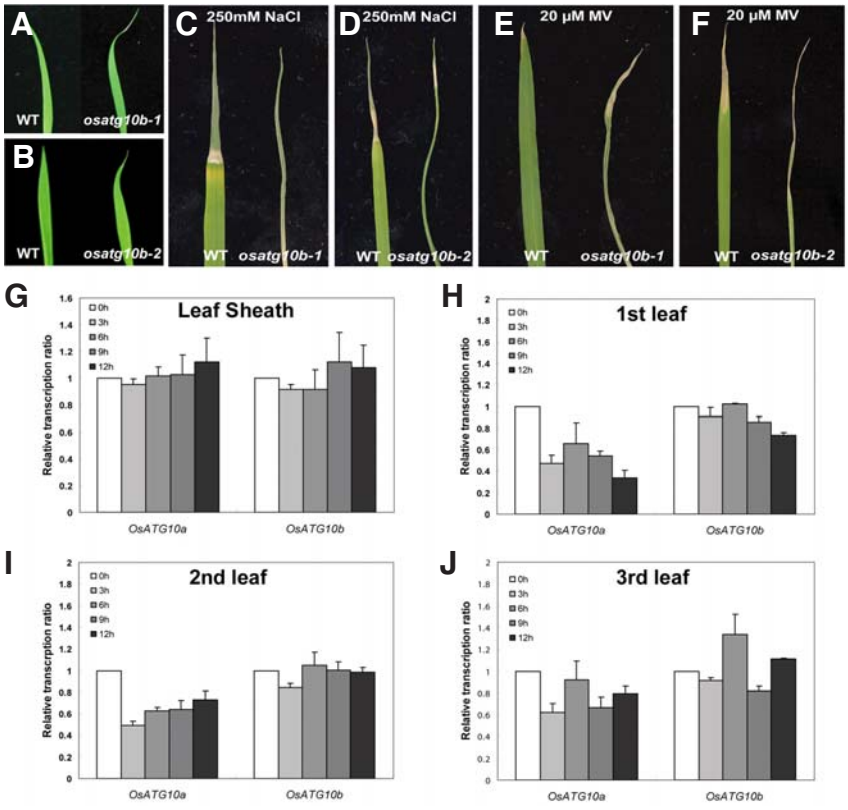

Fig. 6. Effect of high salt or methyl viologen on WT and mutants. Leaves of one-week-old *osatg10b* seedlings and wild-type siblings (A, B) were treated with 250 mM NaCl (C, D) or 20 μM MV (E, F). (G-J) Quantitative RT-PCR analyses of *OsATG10a* and *OsATG10b* transcript levels in leaf sheath (G) and leaf blades from 1st (H), 2nd (I), and 3rd (J) leaves during MV treatment. Samples were collected at 3-h intervals. Transcript levels were normalized by *OsActin1* mRNA level.

causes a decrease in the removal of oxidized proteins.

DISCUSSION

Until recently, autophagy has been mainly reported to function under starvation and stress. However, Slavikova et al. (2005) first observed autophagosome accumulation under normal growing conditions. Inoue et al. (2006) also reported that autophagy is constitutively present in *Arabidopsis* root tip cells. Similarly, we found here that *OsATG10* genes are ubiquitously expressed in rice. GUS analyses with *osatg10b-1* seedlings showed that *OsATG10b* is expressed during normal growth. Shao et al. (2007) have reported that no free ATG5 occurs in cells because it rapidly binds with ATG12 right after its translation. Phillips et al. (2008) also have noted that almost all ATG5 is present in the conjugated form regardless of plant age or nutritional status. This ATG12-ATG5 conjugation is caused by the action of ATG7 and ATG10, which indicates that the latter is

Fig. 7. Gus assay of *osatg10b-1* seedlings during MV treatment. (A) Whole seedlings stained for GUS activity at 3-h intervals. (B) Activity in mature zone of seedling roots.

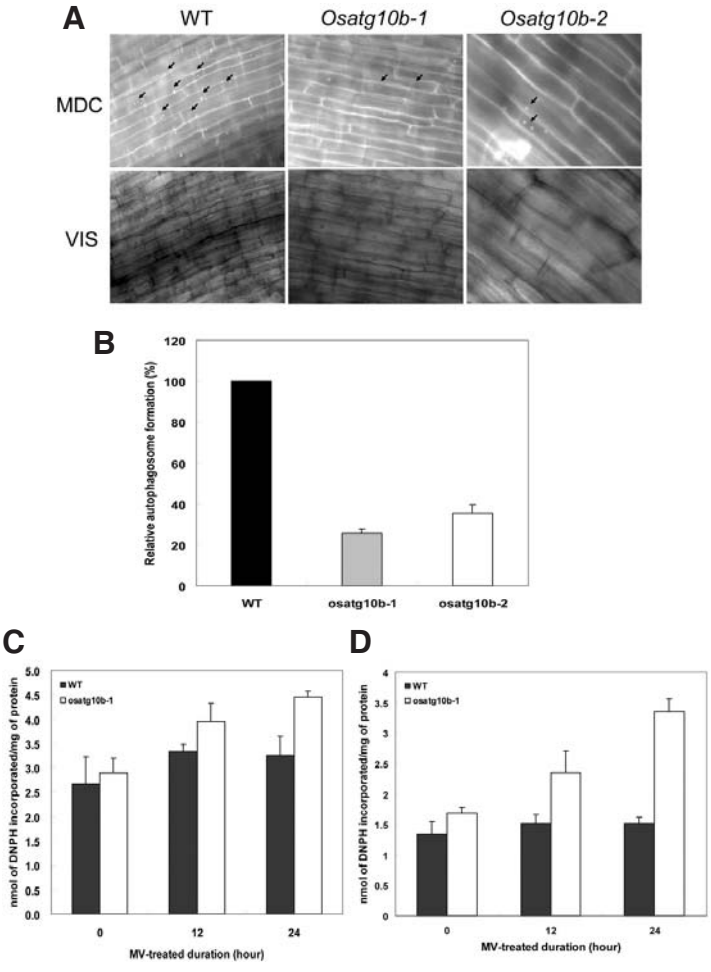

Fig. 8. Autophagy formation and oxidized protein levels. (A) Monodansylcadaverine staining of root tips from 1-week-old *osatg10b-1* and WT seedlings after treatment with 20 μM MV for 12 h. Stained samples were observed under fluorescent light (left) or visible light (right). Arrowheads indicate autophagosome. (B) Number of autophagosomes per root section. Average numbers were determined from 6 samples. (C, D) Levels of oxidized proteins in shoots (C) and roots (D). Seedlings treated with 20 μM MV were sampled at 12-h intervals and divided into shoots and roots. Five hundred micrograms of protein was used for measuring amount of oxidized proteins.

constitutively present for the rapid formation of that conjugate.

The *OsATG10b* gene is more active in young tissues, implying that plants prepare ATG10 in the young cells in order to readily form autophagy. Thompson et al. (2005) have observed that autophagy functions as a regulator for maintaining cell homeostasis. Defects in autophagy can trigger programmed cell death, or PCD (Phillips et al., 2008). Our histological analyses showed that *OsATG10b* is strongly expressed in the phloem tissue of old leaves. Organic molecules, such as sugars, amino acids, and certain hormones (Durrant and Dong, 2004; Kramer and Bennett,

2006), and even mRNAs and proteins (Banergee et al., 2006; Huang et al., 2005) are transported in the phloem through sieve tube elements. This re-mobilization is important for plant survival, especially under severe environmental conditions, including nutrient starvation and oxidative stresses. Therefore, OsATG10b may play essential roles in phloem cells by delaying PCD, allowing the effective movement of degraded materials such as amino acids, sugars, and hormones.

Our *osatg10b* mutants were hypersensitive to high salt and MV. Xiong et al. (2007b) have reported that *AtATG18a* RNAi plants accumulate hydrogen peroxide, even under normal conditions. We also observed that, even before MV treatment began, hydrogen peroxide and MDA levels were higher in the *osatg10b-1* mutants compared with the WT (data not shown). To examine whether those hypersensitive phenotypes are caused by a failure in autophagosome formation, we conducted MDC staining. The number of autophagosomes was remarkably decreased in the mutants, similar to that previously reported (Xiong et al., 2007a). However, about 30% of the autophagosomes still remained. Autophagosome formation in those *osatg10b* mutants is likely due to *OsATG10a*. The fact that knocking out *OsATG10b* reduced such formation demonstrates that those two genes are not functionally redundant. Although both were expressed in all organs examined, their expression patterns differed somewhat. For example, *OsATG10a* was rapidly decreased in the leaf blades during MV treatment compared with a slower decline for *OsATG10b*. Likewise, the two proteins might be functionally divergent. The 20 extra amino acid residues in OsATG10b may have additional functions not present in OsATG10a. Yoshimoto et al. (2004) have found that single mutants of *AtATG4* that exist as two copies in *Arabidopsis* do not cause phenotype alterations. Therefore, it is possible that OsATG10b is functionally different from OsATG10a.

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