

OsATG7 is required for autophagy-dependent lipid metabolism in rice postmeiotic anther development

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Abbreviations: DAGs, diacylglycerols; GFP, green fluorescent protein; NB, *Nipponbare*; OsC6, anther specific protein 6; PCD, programmed cell death; PCs, phosphatidylcholines; PtdIns3K, phosphatidylinositol 3-kinase; TAGs, triacylglycerols; TEM, transmission electron microscopy

In flowering plants, the tapetum, the innermost layer of the anther, provides both nutrient and lipid components to developing microspores, pollen grains, and the pollen coat. Though the programmed cell death of the tapetum is one of the most critical and sensitive steps for fertility and is affected by various environmental stresses, its regulatory mechanisms remain mostly unknown. Here we show that autophagy is required for the metabolic regulation and nutrient supply in anthers and that autophagic degradation within tapetum cells is essential for postmeiotic anther development in rice. Autophagosome-like structures and several vacuole-enclosed lipid bodies were observed in postmeiotic tapetum cells specifically at the uninucleate stage during pollen development, which were completely abolished in a retrotransposon-insertional *OsATG7* (autophagy-related 7)-knockout mutant defective in autophagy, suggesting that autophagy is induced in tapetum cells. Surprisingly, the mutant showed complete sporophytic male sterility, failed to accumulate lipidic and starch components in pollen grains at the flowering stage, showed reduced pollen germination activity, and had limited anther dehiscence. Lipidomic analyses suggested impairment of editing of phosphatidylcholines and lipid desaturation in the mutant during pollen maturation. These results indicate a critical involvement of autophagy in a reproductive developmental process of rice, and shed light on the novel autophagy-mediated regulation of lipid metabolism in eukaryotic cells.

Introduction

Metabolic regulation and a supply of nutrients are essential for developmental processes in both plants and animals. In flowering plants, the anther consists of 4 distinct cell layers comprising the epidermis, endothecium, middle layer, and the tapetum. The tapetum is known to act as a supplier of metabolites and nutrients to developing microspores, pollen grains, and the pollen coat.¹ Lipid bodies containing triacylglycerols

(TAGs) in the tapetum are also necessary for pollen maturation and pollen-tube elongation as a supplier of lipid components,^{2,3} and some factors involved in the regulation of lipid metabolism in the anther have been suggested by recent bioinformatic and transcriptomic analyses.^{4,5} Though the programmed cell death (PCD) of the tapetum⁶ is one of the most critical and sensitive steps for fertility and is affected by various environmental stresses, their regulatory mechanisms remain mostly unknown.

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Autophagy, a major catabolic pathway in eukaryotic cells, plays a role in the recycling of proteins and metabolites, including lipids, by delivering cytoplasmic components and organelles to degradation compartments such as lysosomes and vacuoles^{7,8} and is involved in many physiological processes including abiotic and biotic stress responses in plants.^{9,10} Lipophagy is a type of selective autophagy that has been suggested to contribute to lipid metabolism through the breakdown of intracellular lipids. Defects in lipophagy have been linked to important metabolic disorders such as fatty liver, obesity, and atherosclerosis in animals.^{11,12} However, the direct involvement of lipophagy during development processes as well as autophagy-mediated regulation of lipid metabolism has not previously been addressed in all eukaryotes.

Autophagy is essential for developmental processes in many eukaryotes, including preimplantation in mice and dauer development in nematodes.¹³⁻¹⁵ In plants, autophagy has been suggested to be involved in senescence and fertile floret development under the nutrient-starved conditions.¹⁶⁻¹⁸ However, the life cycle of the autophagy-defective mutants in *Arabidopsis* is normal, and direct involvement of autophagy during normal reproductive development has not yet been addressed in plants.¹⁰ Though the *atg6* mutant of *Arabidopsis* shows gamete male sterility,¹⁹ ATG6 is one of the components of the class III phosphatidylinositol 3-kinase (PtdIns3K) complex and the mutant phenotype is similar to that of other mutants of the PtdIns3K complex,²⁰ indicating that the sterile phenotype of *atg6* is attributed to other functions of PtdIns3K than autophagy.

In the present study, we have discovered that autophagy-defective mutants of rice show complete sporophytic male sterility. Multidisciplinary analyses including molecular genetics, electron microscopy, and lipidomics revealed that autophagy in tapetum cells of anthers plays critical roles in pollen maturation and male reproductive development in rice.

Results and Discussion

Isolation of autophagy-deficient mutants in rice

To analyze the physiological role of autophagy in developmental processes in rice, we isolated a mutant (*Osatg7-1*) of *OsATG7* (locus ID: Os01g0614900, Fig. S1A), a sole rice homolog of *ATG7*, from retrotransposon *Tos17*-insertional mutant pools.²¹ We evaluated autophagy activity in the *Osatg7-1* mutant using biochemical and imaging approaches. *ATG7* is specific to autophagy and possesses E1-like activity in the ATG12 conjugation system that is essential for autophagosome biogenesis.²² First, the OsATG12–ATG5 conjugate was detected in the wild type but not the mutant using immunoblot analysis with an anti-OsATG5 antibody (Fig. S1C). The conjugate formation was restored by introduction of the wild-type *OsATG7* gene but not the *OsATG7* (C to S) mutant gene, which contains a mutation in the catalytic site and is predicted to be enzymatically inactive (Fig. S1D). Second, to monitor autophagy in vivo, we generated wild-type and *Osatg7-1* cultured cells expressing the green fluorescent protein (GFP)-ATG8 fusion protein, a marker

for autophagosomal membranes.^{23,24} Under sucrose-starved conditions, most GFP-ATG8 was delivered into the vacuolar lumen in the wild-type cells, whereas no GFP-ATG8 was detected in the vacuole in the *Osatg7-1* mutant (Fig. 1A). Furthermore, cultured cells of *Osatg7-1* showed more severe growth retardation under sucrose starvation than the wild type (Fig. 1B), which is consistent with the phenotypes of autophagy-deficient *Arabidopsis atg* mutants²⁴⁻²⁶ and indicates that autophagy is defective in the *Osatg7-1* mutant.

Quantitative polymerase chain reaction (qPCR) analysis showed that *OsATG7* messenger RNA (mRNA) was expressed in mature leaves, shoots, roots, and suspension-cultured cells, as well as reproductive tissues (Fig. S1B). We also consulted the microarray-expression database (Rice XPro; <http://ricexpro.dna.affrc.go.jp/GGEP/index.html>), which showed *OsATG7* expression throughout several developmental stages. Recent expression profiling using a laser microdissection-mediated microarray revealed expression of *OsATG7* in the tapetum,⁵ suggesting that *OsATG7* is expressed throughout the life cycle and that the *Osatg7-1* mutant is deficient in autophagy in all developmental tissues including the tapetum.

Critical role of autophagy in rice reproductive development and its significance in crop production

Next, we investigated the effect of autophagy disruption on growth and development in rice. The rates of seed germination and vegetative growth of the *Osatg7-1* mutant plants were comparable to those of wild type when grown in a greenhouse or paddy field (Fig. S2A). By contrast, the *Osatg7-1* plants grown in a paddy field showed slower heading (Fig. 1C; Fig. S2B) and flowering (Fig. S2C and S2D) accompanying the delay of anther development including pollen maturation (data not shown), and had slightly larger spikelets (Fig. 1E and F). Surprisingly, unlike *Arabidopsis atg* mutants, the *Osatg7-1* mutant exhibited a sterile phenotype (Fig. 1D; Fig. S3A; Table S1) and limited anther dehiscence under normal growth conditions (Fig. S3B). The stomium, an essential structure for anther dehiscence,²⁷ did not show significant difference between the wild type and the mutant (data not shown), suggesting that the defects in anther dehiscence is attributed to other critical steps. These *Osatg7-1* mutant phenotypes were complemented by the expression of *OsATG7* (Fig. S3B–S3D). We also identified another mutant allele (*Osatg7-2*) of *OsATG7* (3A-03442; cv *Donjing*) and a mutant line defective in *OsATG9* (2D-00675; cv *Hwayoung*) from the T-DNA-tag lines of the POSTECH Biotech Center (Kyungbuk, Korea; Fig. S1A), both of which were sterile (Fig. S4A and S4B), indicating that autophagy plays critical roles in rice reproductive development. Overall, the present findings using the autophagy-defective mutants demonstrate the important role of autophagy in plant reproductive development and crop production.

Autophagy is required for male reproductive development in rice

To characterize the fertility phenotype of the *Osatg7-1* mutant, we performed reciprocal crossing analysis. Mutant plants produced seeds when fertilized with the pollen of wild type (cv NB; Table S2). Moreover, the structure of the ovary at the flowering stage was similar between the wild type and

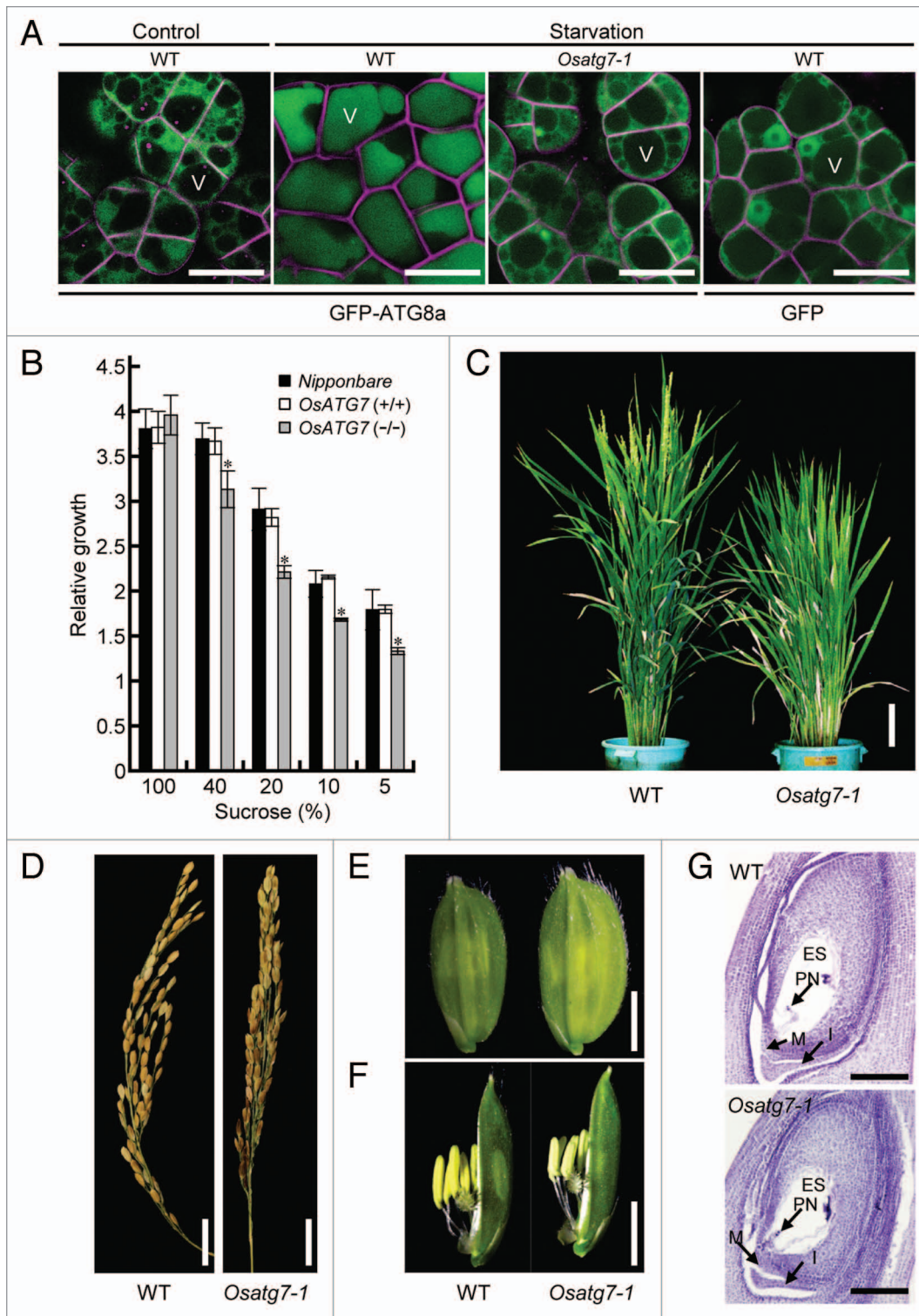


Figure 1. The *Osatg7-1* mutant exhibits a sterility phenotype. **(A)** In vivo imaging of autophagy in cultured rice cells. The accumulation of GFP-ATG8 in vacuoles in the presence of concanamycin A in wild-type and *Osatg7-1* mutant cells under sucrose-starved conditions. Scale bar: 20 μ m. V, vacuole. **(B)** The effects of autophagy disruption on cell growth under sucrose starvation in cultured rice cells. The growth of cultured cells is shown for 7 d. The relative growth level at 0 d (fresh weight, 0.5 g) was standardized as 1. Data are the mean \pm standard error (SE) for 3 independent experiments. * $P < 0.01$; significantly different from the controls. **(C)** The *Osatg7-1* mutant shows slower heading. Plants were grown for 90 d. Scale bar: 10 cm. **(D–F)** Comparison of wild-type and *Osatg7-1* mutant phenotypes at various reproductive stages; **(D)** panicles, **(E)** spikelet, and **(F)** flower organs. The *Osatg7-1* mutant exhibited a sterile phenotype. **(G)** Ovary development appeared normal in the *Osatg7-1* mutant. Ovaries from the wild type and *Osatg7-1* mutant at the flowering stage were stained with hematoxylin and observed under a microscope. Scale bar: 100 μ m. ES, embryo sac; PN, polar nucleus; M, micropyle; I, integument.

the mutant (Fig. 1G), indicating that the sterility phenotype is caused by the male gametophyte.

We then assessed pollen maturation. Mature pollen stained by iodine accounted for approximately 95% and 45% of the total pollen in the wild type and mutant, respectively (Fig. 2A and B). Undeveloped mutant pollen was confirmed by toluidine blue staining (Fig. S5A). At the flowering stage, the coat structure of many pollen grains in *Osatg7-1* was undeveloped (Fig. S5B) and

there was less accumulation of lipid bodies in *Osatg7-1* mutant than wild-type pollen (Fig. 2C), indicating that the defects in *Osatg7-1* pollen maturation occur at the flowering stage. Some mutant pollen was stained by the iodine reagent, and the pollen coat appeared normal. We therefore checked the germination activity of mutant pollen, and observed germination in only 0.74% of the *Osatg7-1* pollen grains compared with more than 60% of wild-type grains (Fig. 2D; Fig. S5C). These results suggest that

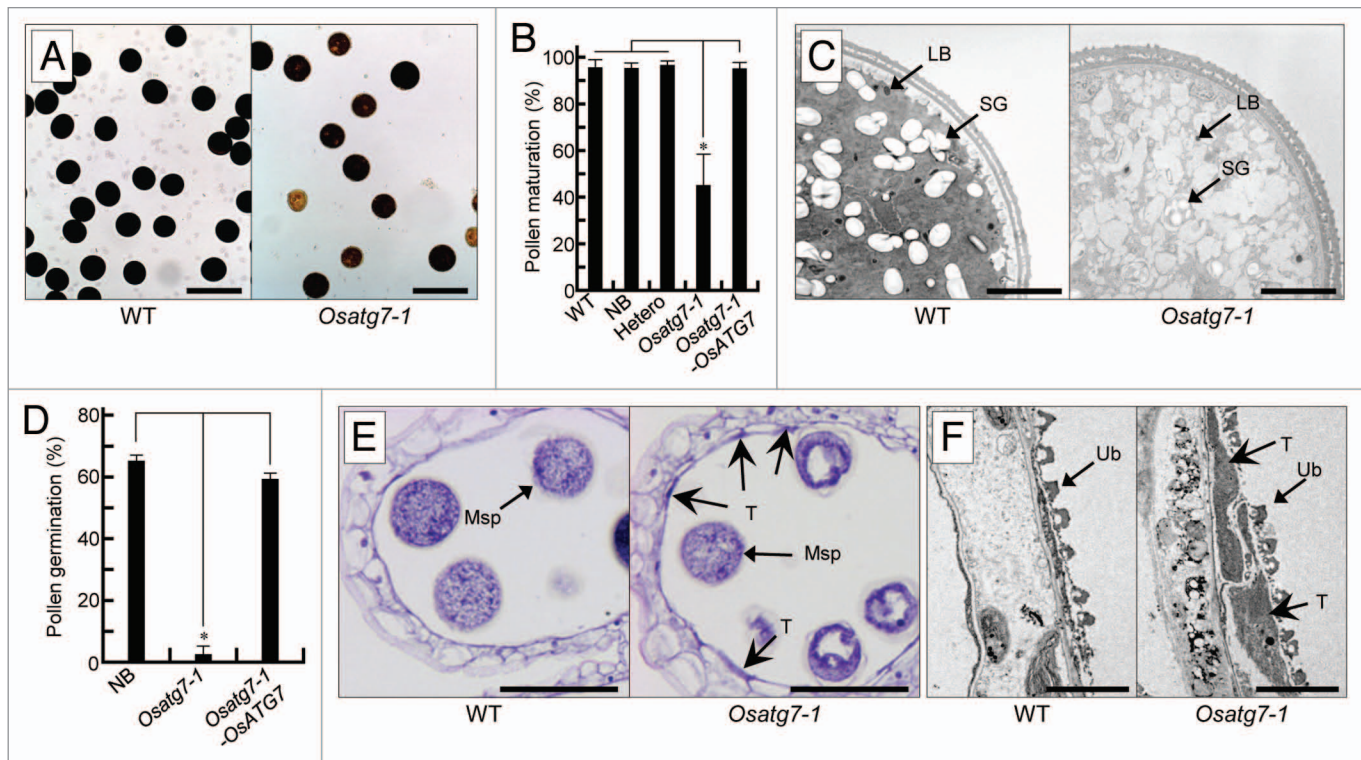


Figure 2. Autophagy is required for male reproductive development in rice. (A) Pollen grains from the wild-type and *Osatg7-1* mutant stained with I_2 -KI solution. Scale bar: 100 μ m. (B) Viable pollen grains shown in (A) were quantified. Data are mean \pm SD; n = 3 independent samples. * $P < 0.01$; significantly different from the controls. (C) The ultrastructure of pollen in the wild-type and *Osatg7-1* mutant at the flowering stage. Scale bar: 5 μ m. LB, lipid body; SG, starch granule. (D) In vitro pollen germination rate. Pollen grains from NB, *Osatg7-1*, and complementation line (*Osatg7-1-OsATG7*) anthers at the mature stage were assayed. Data are mean \pm SD; n = 3 independent samples. * $P < 0.01$; significantly different from the controls. (E) Transverse section analysis of wild-type and *Osatg7-1* anthers stained with hematoxylin at the flowering stage. Scale bar: 50 μ m. (F) Ultrastructure of the tapetum in the wild-type and *Osatg7-1* mutant at the flowering stage. Scale bar: 3 μ m. Msp, microspore; T, tapetum.

the pollens of autophagy-defective mutants are premature due to critical defects in the anther during pollen maturation. The ratio of mature pollens in the heterozygous plants was similar to that in the wild type (Fig. 2B), and the pollination of the wild-type (cv NB) stigmas with heterozygous pollens led to a normal segregation rate in the next generation (Table S3). These results suggest that the immature pollen phenotype of the *Osatg7-1* mutant is attributed to defects in parental tissues or organs. The autophagosome-like structures observed in the tapetum cells were not detected in the pollen during developmental processes (Fig. 2C and data not shown).

Autophagy in postmeiotic tapetum cells in rice

The tapetum acts as a supplier of metabolites and nutrients to developing microspores and pollen grains.¹ To investigate whether autophagy takes place in the rice tapetum under normal growth conditions, we performed transmission electron microscopy (TEM) analysis (Fig. 3). As shown in Figure 3B, no obvious autophagosome-like structures were detected in the cytoplasm at the tetrad stage in rice anthers. By contrast, at the uninucleate stage, autophagosome-like structures were clearly observed in the cytoplasm (Fig. 3B and C). Numerous vacuole-enclosed dense globular bodies, lipid bodies, were also found at the uninucleate stage (Fig. 3D). Thereafter, at the bicellular stage, some multilamellar bodies, presumably

intermediate structures of autophagosomes,²⁸ were observed in the cytoplasm (Fig. 3E). Moreover, vacuoles fused with the lipid bodies that could be degraded by autophagy, appeared in the tapetum (Fig. 3B). Finally, the tapetum disappeared at the mature stage (Fig. 3A). These results suggest induction of autophagy at the uninucleate stage in postmeiotic tapetum cells in rice.

We next performed TEM analysis to determine the dynamics of autophagy in the tapetum during pollen maturation. At the tetrad stage, no obvious differences were apparent in the intracellular structure between the wild type and *Osatg7-1* mutant (Fig. 3; Fig. S6). In the uninucleate stage, however, few double-membrane autophagosome-like structures and the vacuole-enclosed dense globular bodies were observed in the cytoplasm of the mutant (Fig. 3F and G; Fig. S6). More lipid body-like structures remained in the cytoplasm in the mutant than in the wild type at the bicellular stage (Fig. 3F and G). Intracellular organelles such as plastids and mitochondria, which have been reported to be degraded by autophagy,¹⁸ were also clearly observed in the cytoplasm of the mutant even at the bicellular stage (Fig. 3B; Fig. S6). These results indicate that autophagy is active in the tapetum, which may be involved in the degradation of intracellular components such as plastids and lipid bodies during pollen maturation.

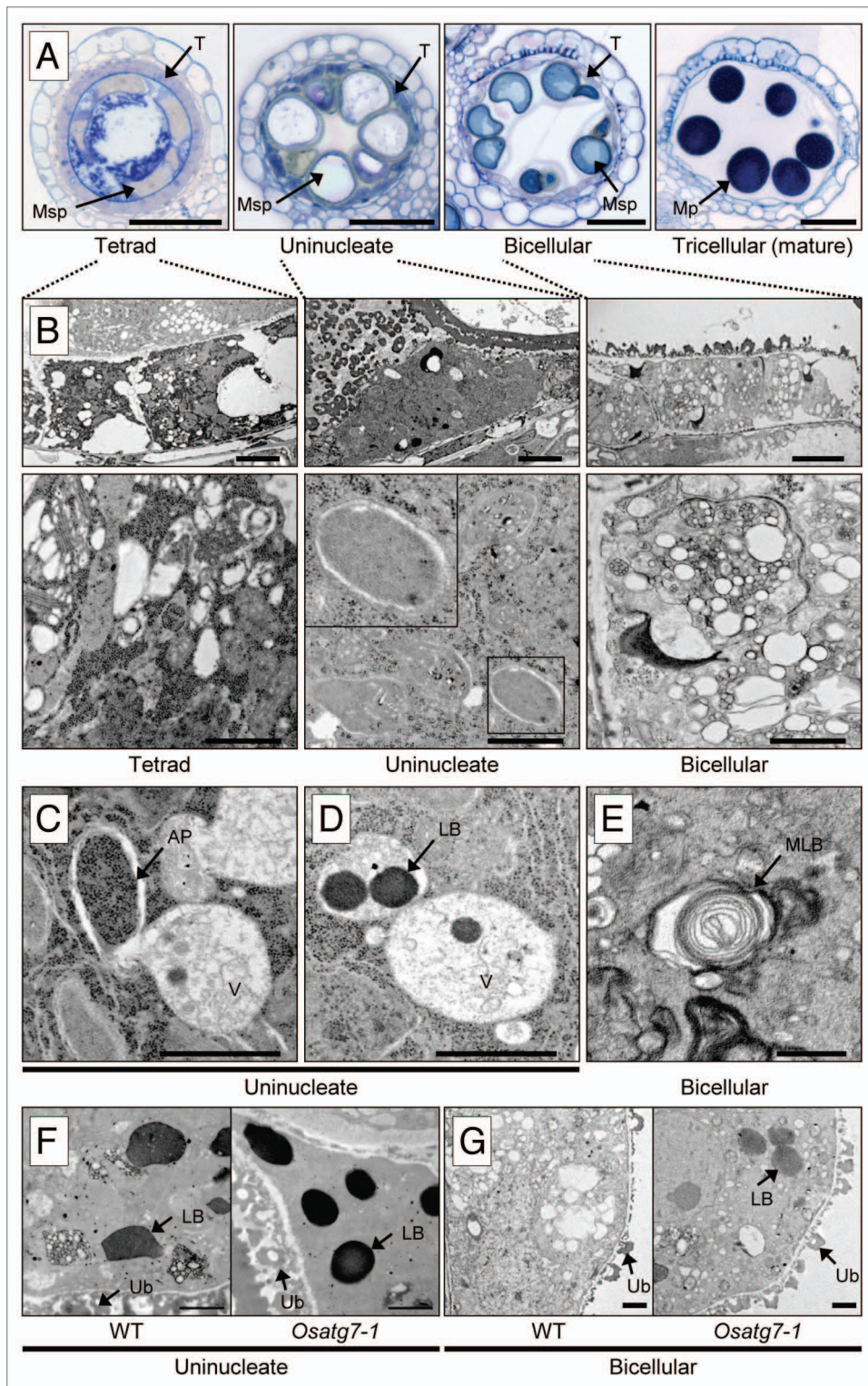


Figure 3. Autophagy occurs in tapetal cells during the male reproductive phase. **(A)** Transverse section analysis during rice anther development. Samples taken at the 4 stages of anther development, and stained with toluidine blue. MP, mature pollen; Msp, microspore; T, tapetum. Scale bar: 50 μm . **(B–E)** The ultrastructure of the tapetum during anther development by TEM analysis. **(B)** Autophagosome-like structures and many vacuole-enclosed lipid bodies were observed in the cytoplasm at the uninucleate stage. Thereafter, at the bicellular stage, many characteristic intermediate structures including vacuoles fused with lipid bodies appeared in the tapetum. Scale bar: (upper) 3 μm , (lower) 1 μm . **(C–E)** Close-up images of the cell in **(B)**. Scale bar: 1 μm . AP, autophagosome; LB, lipid body; V, vacuole; MLB, multi-lamellar body. **(F and G)** Ultrastructure of the tapetum at reproductive stages in the wild-type and *Osatg7-1* mutant. Samples taken at uninucleate (1n; **F**) and bicellular (2n; **G**) stages of anther development, respectively. Scale bar: 1 μm . LB, lipid body; Ub, Ubisch body.

pollen germination activity in the mutant (Fig. 2D). To investigate the roles of tapetal autophagy in lipid metabolism of pollen grains at the flowering stage, crude lipid fractions were extracted from the mature anthers mainly occupied by pollen grains, and subjected to lipid profiling by LC-MS.²⁹ TAGs are the main components stored in lipid bodies within pollen grains,³⁰ and essential for normal pollen development.³¹ The amounts of TAGs and diacylglycerols (DAGs), intermediary metabolites in TAG synthesis, were significantly lower in the mutant than in the wild type (Fig. 4A and B). Desaturation of acyl groups of TAGs involves editing of phosphatidylcholine (PC) through the Lands cycle.³² Interestingly, the total amount of PCs was similar between the wild type and the mutant (Fig. 4C), whereas the amount of highly desaturated PCs (34:3 and 36:6) was significantly lower in the mutant than the wild type (Fig. 5A). The level of lysoPC (16:0), a possible intermediary metabolite in the Lands cycle pathway, also decreased in the mutant (Fig. 5B), suggesting that autophagy may play roles in the PC editing through the Lands cycle pathway. The decrease in some highly desaturated TAGs

Involvement of autophagy in the regulation of lipid metabolism and nutrient supply in anthers

Lipid bodies inside the pollen grains are necessary for its maturation and pollen-tube elongation.^{2,3} In fact, fewer lipid bodies and starch grains were accumulated in the pollen grains of *Osatg7-1* mutant than the wild type at the flowering stage (Fig. 2C; Fig. S5B), which is consistent with reduced

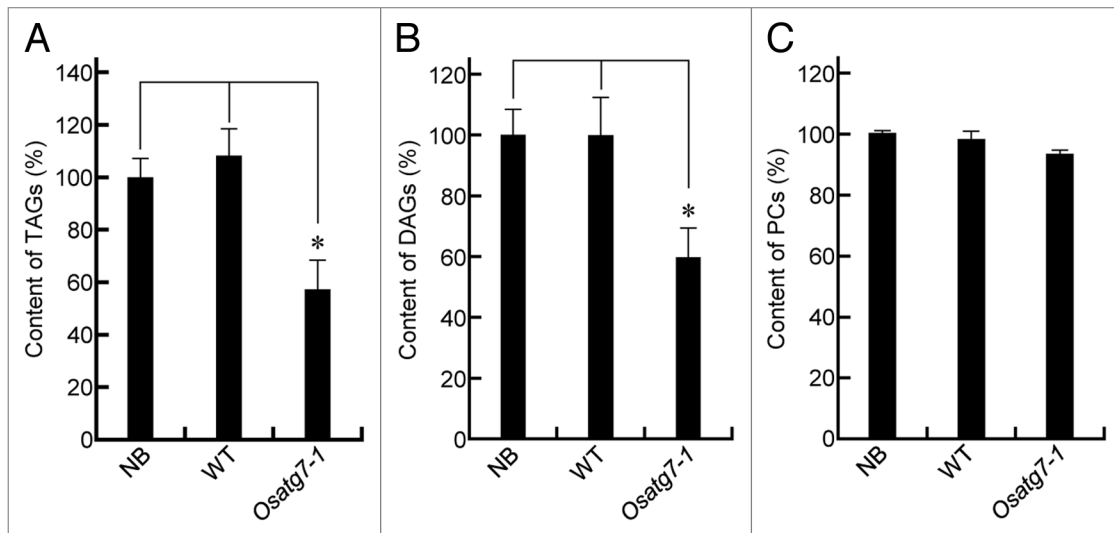


Figure 4. Autophagy contributes to lipid metabolism during tapetum and pollen maturation. (A–C) Levels of individual lipid molecules (A) integrated value of TAGs, (B) integrated value of DAGs, and (C) integrated value of PCs in the wild-type and *Osatg7-1* mutant were expressed as relative values against the sum of the peak areas of lipid molecules with the same polarity in the wild type. Samples (mature anthers) were taken from each plant. * $P < 0.01$; significantly different from the control.

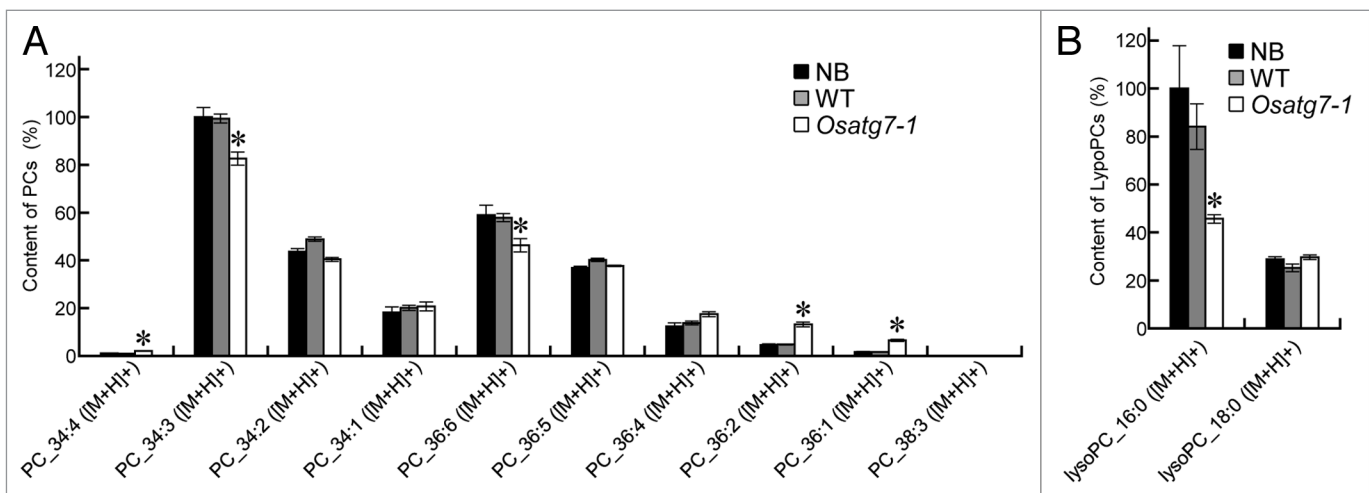


Figure 5. Composition of phosphatidylcholines (PCs) and lysophosphatidylcholines (LysoPCs) of the anthers at the flowering stage. Levels of individual lipid molecules (A) PC, and (B) LysoPC in the wild-type and *Osatg7-1* mutant were expressed as relative values against the sum of the peak areas of lipid molecules with the same polarity in the wild type. Samples (mature anthers) were taken from each plant. * $P < 0.01$; significantly different from the control.

and DAGs in the *Osatg7-1* mutant (Fig. 6) can be accounted for by impairment in the PC editing. On the other hand, the level of de novo DAG (34:1), synthesized by the Kennedy pathway,³² was comparable to those of the wild type (Fig. 6B). These results suggest that tapetal autophagy contributes to lipid metabolism of pollen grains, especially the Lands cycle-mediated PC editing and desaturation in rice anthers, which affects the pollen maturation including coat formation.

A lipid transfer protein of rice, anther specific protein 6 (OsC6), is specifically expressed in tapetal cells and is able to transport lipid molecules such as fatty acids from tapetal cells to developing microspores. The RNA interference (*RNAi*)-suppression lines

of *OsC6* contain more lipid bodies in tapetal cells than in the control during pollen maturation, and show decreased pollen fertility.³³ These phenotypes of the *OsC6*-suppressed plants were similar in part to those of *Osatg7-1* mutant, suggesting that the immature pollen phenotype of *Osatg7-1* mutant is attributed to the defects in the transport of materials including lipids from the tapetum cells to developing microspores.

Lipophagy has recently been suggested to be involved in lipid droplet degradation in animals.¹¹ However, the direct involvement of lipophagy during developmental processes as well as autophagy-mediated regulation of lipid metabolism had remained mostly unknown in plants. The present results suggest that autophagy in

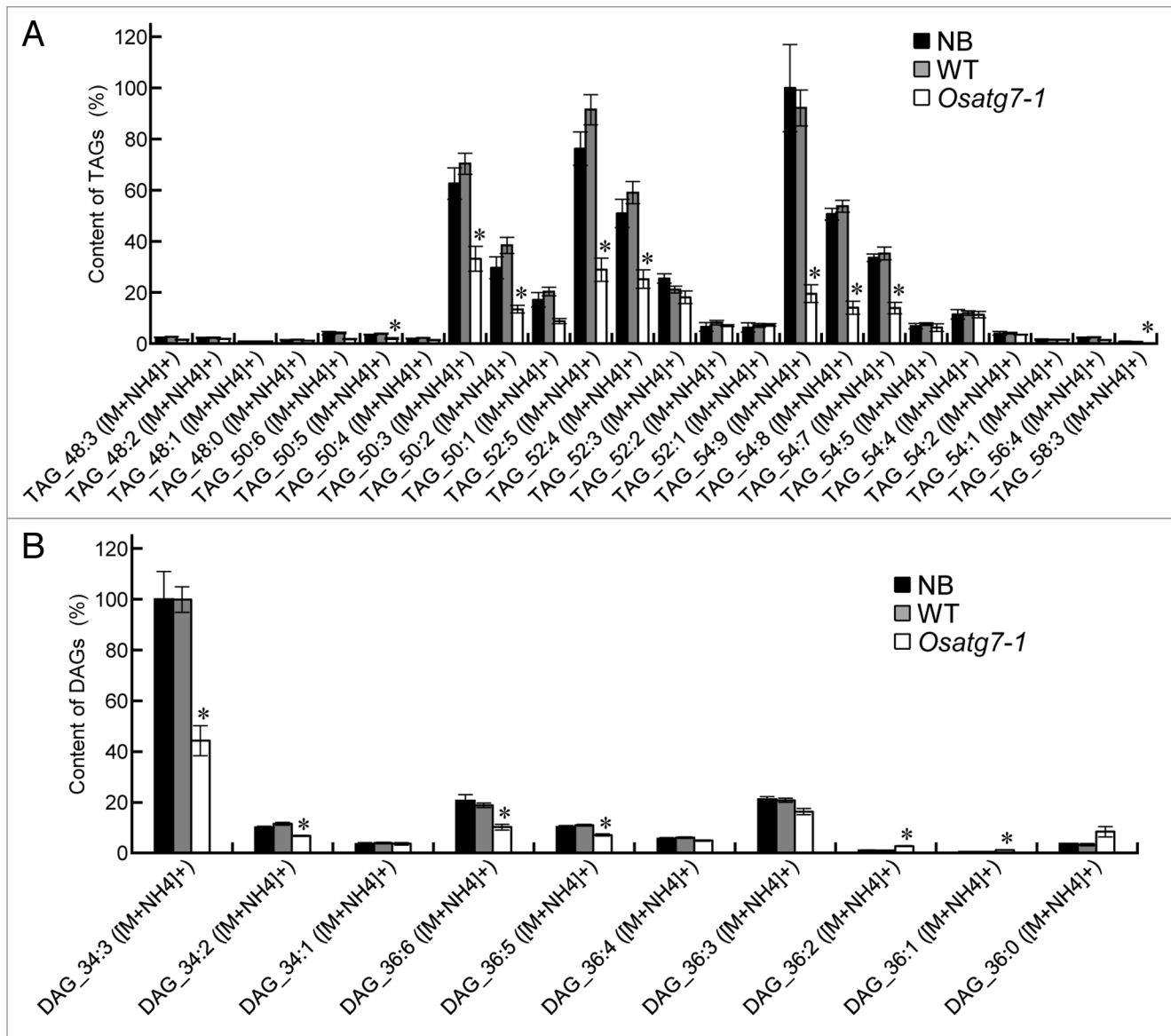


Figure 6. Composition of triacylglycerols (TAGs) and diacylglycerols (DAGs) of the anthers at the flowering stage. Levels of individual lipid molecules (A) TAG, and (B) DAG in the wild-type and *Osatg7-1* mutant were expressed as relative values against the sum of the peak areas of lipid molecules with the same polarity in the wild type. Samples (mature anthers) were taken from each plant. * $P < 0.01$; significantly different from the control.

the tapetum may be involved in the degradation of lipid bodies and regulation of lipid metabolism during pollen development, and shed light on the importance of autophagy-mediated regulation of lipid metabolism in development not only in plants but in all eukaryotic cells. Autophagy has also recently been suggested to contribute to the turnover of peroxisomes,³⁴ which are known to be involved in the regulation of lipid metabolisms.³⁵ Possible involvement of autophagy in the regulation of lipid metabolism is an emerging important future subject of research.

Possible involvement of autophagy in the degradation and programmed cell death of the tapetum

During pollen development, the tapetum is degraded to supply metabolites, nutrients, and sporopollenin precursors to developing microspores. Defects in the degradation of the tapetum cause abnormal formation of the pollen coat and pollen

grains, resulting in severe male sterility.¹ The *Osatg7-1* mutant showed limited anther dehiscence (Fig. S3B), which could also contribute to its sterility.

The dense thin layer connected with the orbicules was only observed by both the transverse section and TEM analysis in the *Osatg7-1* mutant even at the flowering stage (Fig. 2E and F, arrows), suggesting that the tapetal cell layer thoroughly disappeared in the wild-type, whereas partially remained in the mutant. It has been proposed that the tapetum is degraded by PCD during the later stages of pollen maturation in plants.^{1,6} Autophagy is also involved in PCD in animals.^{36,37} Autophagic cell death is characterized by the occurrence of double-membrane autophagosomes within the dying cells that remove the cell remnants.³⁸ In *Drosophila melanogaster*, destruction of the salivary glands and digestive tract is mediated by a dramatic

upregulation of autophagy prior to and during cell death during metamorphosis.³⁹ Autophagy may therefore contribute, at least in part, to tapetum degradation in rice. Future detailed TEM/imaging analyses of various tapetum developmental stages under environmental stress conditions in the *Osatg7* mutants may reveal a novel mechanism for autophagy-mediated PCD and its physiological significance in rice.

Concluding Remarks

In hybrid rice breeding, male sterility lines with cytoplasmic and/or nuclear mutations are of agricultural importance for the production of hybrids to improve yield.⁴⁰ We here demonstrated that autophagy-defective mutants of rice show complete sporophytic male sterility, and suggest the involvement of tapetum autophagy in the regulation of lipid metabolism and nutrient supply in anthers. Importantly, the life cycle of the autophagy-defective mutants in *Arabidopsis* were found to be normal. The most significant difference of the tapetum between monocots and dicots is that monocots do not form tapetosomes for lipid transport in tapetal cells, while dicots have lipidic tapetosomes. It may suggest a critical difference in the development of lipidic components in the pollen grains between monocots and dicots. Future TEM/imaging analyses of tapetum in various developmental stages in other plant species including *Arabidopsis* along with genetic analyses may reveal a novel function of autophagy in lipid metabolism and its physiological significance during postmeiotic anther development.

Materials and Methods

Plant materials and growth conditions

Surface-sterilized seeds of rice, *Oryza sativa* L. cv NB, *Donjing* and *Hwayoung*, were germinated on MS medium⁴¹ containing 0.8% agar and grown for 10 d in a growth chamber under long-day conditions (16 h light/8 h darkness, 28 °C). Seedlings were transplanted into soil and grown in a greenhouse (16 h light/8 h darkness, 28 °C and 60% humidity) or paddy field.

To generate cultured cells of *Osatg7*-knockout and wild type (WT), in which the insertion of *Tos17* in *OsATG7* was removed by heterozygous segregation, seeds were placed onto callus-inducing medium. The calli were suspension-cultured at 25 °C in a liquid L medium⁴² containing 2,4-D (0.5 mg L⁻¹) (Wako Pure Chemical, 119-0563) in the dark, and subcultured in fresh medium every 7 d. Cells were filtered through a 20-mesh screen every 2 wk to produce fine aggregates. In our experiments, NB, *Donjing*, *Hwayoung*, and WT plants as well as cultured cells were used as controls.

TEM analysis

Samples were fixed in a 0.05 M cacodylate buffer (pH 7.4) containing 2% glutaraldehyde and 4% paraformaldehyde for 3 h in an ice bath. Specimens were rinsed in the same buffer and post-fixed in 2% osmium tetroxide for 3 h on ice. Dehydrated specimens were embedded in epoxy resin (Epon 812; Nissin

EM, 341). Ultrathin sections (70 to 80 nm) were made with an LKB-2088 ULTRATOME V (LKB) using a diamond knife, and mounted on 200-mesh copper grids (Nissin EM, 2632). Sections were stained with 2% uranyl acetate and lead citrate, and observed with a JEM-1200EX transmission electron microscope (JEOL Ltd.) operating at 80 kV.

RNA isolation and RT-PCR

Total RNA was isolated using Sepasol reagent (Nacalai Tesque, 30486-56) in accordance with the manufacturer's protocol, and quantified using a spectrophotometer. First-strand complementary DNA (cDNA) was synthesized from 3 µg total RNA with an oligo-dT primer (Life Technologies, 18418-012) and reverse transcriptase (Promega, M3682). PCR amplification was performed with an initial denaturation at 95 °C for 3 min followed by 30 cycles of incubation at 95 °C for 60 s, 55 °C for 90 s, and 72 °C for 40 s using specific primers for *OsATG7*. *OsActin1* (locus ID: Os03g0718100) was used as a quantitative control.⁴³ Aliquots of individual PCR products were resolved by agarose gel electrophoresis, and visualized using ethidium bromide staining and exposure to UV light.

OsATG-null mutants

Large populations (39,744 lines) of rice (*O. sativa* cv NB) mutants generated by *Tos17*-mediated mutagenesis²¹ were made available by the National Institute of Agrobiological Resources (NIAS; Tsukuba, Japan). *Tos17*-mediated mutagenesis details have been described previously.²¹ Using this technique, we isolated one mutant line (NC7558) by PCR screening. *Tos17* insertion in the mutant (*Osatg7-1*) was confirmed by DNA gel-blot analysis, and its exact position was determined by sequencing. Plants homozygous for the *Osatg7*-null allele were confirmed by the isolation of total RNA followed by reverse transcription (RT)-PCR using the following primer set: forward primer 5'-TCAAGCTGGA CGTCCTCGG-3' and reverse primer 5'-TTGCTTCAGG CACATAATCA GG-3'. *OsActin1* was used as a quantitative control.⁴³

Another allele (3A-03442; cv *Donjing*) and a mutant line defective in *OsATG9* were also isolated from the T-DNA-tag lines of the POSTECH Biotech Center.^{44,45} The primer (5'-GTGGTAGTAA GAATGGAAC CAC-3') annealing to the T-DNA left border was used together with the *OsATG7*-specific (5'-AACAATTGTC CAGTCCCAGG-3') and *OsATG9*-specific (5'-AATGCATCGT AGCCCTTTTG-3') primers for the identification of putative mutant lines. T-DNA insertions in the mutants were confirmed by DNA gel-blot analysis, and exact insertion positions were determined by DNA sequencing.

Establishment of transgenic rice cell lines stably expressing GFP-AtATG8a

The GFP-AtATG8a fusion construct (pBI121 binary vector) was kindly provided by K Yoshimoto (Institut National de la Recherche Agronomique, Paris, France). The construct was introduced into rice calli using *Agrobacterium*-mediated transformation.⁴⁶ Transformed calli were screened by hygromycin selection (50 µg/mL; Nacalai Tesque, 07296-24); transgenic plants were then regenerated. Transgenic cell lines derived from T2 plants were used for various analyses.

In vivo imaging of autophagy

Six-d-old cultured rice cells expressing the GFP-AtATG8a protein were incubated in a sucrose-free medium⁴² for 3 d, and then incubated for an additional 16 h in the presence of 1 μ M concanamycin A (Sigma, C9705). The control was set at time 0.

All images were observed using an LSM 5 EXCITER confocal fluorescence microscope with a 40 \times objective lens (Carl Zeiss). For all experiments, laser intensity was adjusted to the lowest level that retained a significant signal-to-noise ratio. The fluorescent styryl membrane probe FM 4-64 (Life Technologies, T-13320) was kept as a 17-mM stock solution in sterile water, and used at a final concentration of 4.25 μ M. Cultured rice cells were treated with FM 4-64 for 10 min and washed twice with the wash buffer at room temperature to label the plasma membrane.

Immunoblot analyses

A rabbit polyclonal anti-OsATG5 antibody was generated to detect the OsATG12-ATG5 conjugate. The coding region of the amino (N)-terminal half domain of OsATG5 (M1-C190) was amplified using the following sequence-specific primers: M1-C190F, 5'-CACCATGGCG GCGCAGCGCG ACGA-3'; and M1-C190R, 5'-GCAATCCTCT TCGAATGGTC-3'. A fusion protein consisting of the domain fused to the histidine-tag in the pDEST17 vector (Life Technologies, 11803-012) was transformed into *E. coli* BL21-AI (Life Technologies, C6070-03). Inclusion bodies with the recombinant protein were obtained after induction at 37 °C for 6 h, and were resolved using a preparative 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. A ground polyacrylamide gel slice of the fusion protein was checked using tandem mass spectrometry (MS/MS) analysis, and used to immunize rabbits by intradermal injections.

For immunoblotting analysis, crude protein samples were separated by 10% SDS-PAGE gel, and blotted onto a PVDF membrane. The membrane was blocked in 1 \times TTBS buffer (10 mM TRIS-HCl, 150 mM NaCl, and 0.05% Tween-20, pH 7.5) with 5% nonfat milk overnight at 4 °C. Blots were incubated with the affinity-purified anti-OsATG5, and then with horseradish peroxidase (HRP)-linked anti-rabbit immunoglobulin G. Bands were detected using chemiluminescent HRP substrate (Merck Millipore, WBKLS0050) and the chemiluminescent analyzer, LAS3000 (GE Healthcare).

Pollen viability

Pollen maturity was investigated with I₂-KI staining (0.2% iodine and 2% potassium iodine) for starch accumulation. More than 200 pollen grains were counted in each examination under bright-field microscopy.

Pollen-germination assay

Pollen grains from anthers at the mature stage during flowering were immediately placed on Ishikawa's liquid germination medium⁴⁷ [0.01% H₃BO₃, 0.1% Ca(NO₃)₂ · 4 H₂O, and 15% sucrose in sterilized water (pH 5.7)] and incubated at room temperature in a humid chamber. After 1 h, more than 200 pollen grains were counted in each examination under bright-field microscopy. Pollen grains that developed normal tubes longer than the grain diameter were considered as "germinated."

Growth assay of rice cultured cells

The cell-growth assay was performed as described by Kurusu et al.⁴⁸ Seven-d-old cultured cells were transferred to a 50-mL tube containing sucrose-free L medium and preincubated for 3 h. Cells (fresh weight, 0.5 g) were transferred to L medium⁴² with standard (100%) or low sucrose conditions (40, 20, 10, and 5%). After culturing for 0 and 7 d, the fresh weight of cells was measured.

Real-time RT-PCR quantification

Total RNA was extracted from various tissues of rice plants as well as cultured cells. Real-time RT-PCR assays were performed as described by Kurusu et al.⁴⁹ Real-time PCR was performed using an ABI PRISM 7300 sequence detection system (Life Technologies) with SYBR Green real-time PCR Master Mix (Toyobo, QPS-201) and the following *OsATG7*-specific primers: *OsATG7*-RealF, 5'-GATCGTGACA GCCCAAAAGC A-3'; and *OsATG7*-RealR, 5'-GACCAAGTGG ACCCTCACTG C-3'.⁵⁰ Relative mRNA levels were calculated using the standard curve method and normalized to corresponding *OsEF1 α* (locus ID: Os03g0177500) gene levels.⁵¹ Standard samples of known template amounts were used to quantify PCR products.

Complementation analysis

The PAC clone P0410E01 encompassing the *OsATG7* locus was obtained from NIAS. A DNA fragment (9.5 kb) of the *OsATG7* genome region was amplified by PCR using the PAC clone as a template and the following primer sets (KpnI and SmaI sites are underlined): KpnI-*OsATG7*F, 5'-CGGGGTACC GTCACAACAC TTACATCGGG TTTCATTGC-3'; and SmaI-*OsATG7*R, 5'-AAACCCGGG GAAGAGAGGA AGAAGCAGAG GACGC-3'. The resulting product was cut with KpnI and SmaI, and inserted into the KpnI-*Apa*I site of the pPZP2H-lac binary vector.⁵² To make the C to S mutant, the following primer sets were used for PCR amplification: *OsATG7*F(C910S), 5'-GAACACTGGA TCAACAAAGT ACAGTAACAC GGCCT-3'; and *OsATG7*R(C910S), 5'-AGGCCGTGTT ACTGTACTTT GTTGATCCAG TGTTC-3'. The construct and control vector were introduced into *Osatg7* calli using *Agrobacterium*-mediated transformation.⁴⁶ Transformed calli were screened by hygromycin selection (50 μ g/mL); transgenic calli were then regenerated. Transformed cultured cells and plants were used for various analyses.

Lipidome analysis

Lipidome analyses were performed as described by Okazaki et al.²⁹ High-resolution electrospray ionization mass spectra were acquired in both positive and negative ion modes. Unless stated otherwise, all the lipid profile data was analyzed based on the data recorded in positive ion mode, except for fatty acids which are detected in negative ion mode. Peak areas of individual lipid molecules were calculated based on the *m/z* values of their molecular-related ions. The ions used for the calculation of peak areas are as follows: [M+H]⁺ for PG, [M+NH₄]⁺ for PE, [M+H]⁺ for lysoPC, [M+NH₄]⁺ for TAG, and [M-H]⁻ for fatty acid.

Statistical analyses

Significance was determined using an unpaired Student *t* test at *P* < 0.01.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/autophagy/article/28279

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