

# Monitoring autophagy in rice tapetal cells during pollen maturation

Shigeru Hanamata<sup>1,2,3,†</sup>, Jumpei Sawada<sup>1,†</sup>, Bunki Toh<sup>1</sup>, Seijiro Ono<sup>4</sup>,  
Kazunori Ogawa<sup>1</sup>, Togo Fukunaga<sup>1</sup>, Ken-Ichi Nonomura<sup>4</sup>, Takamitsu Kurusu<sup>1,2,5,\*</sup>,  
Kazuyuki Kuchitsu<sup>1,2,\*\*</sup>

<sup>1</sup>Department of Applied Biological Science, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan; <sup>2</sup>Imaging Frontier Center, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan; <sup>3</sup>Graduate School of Science and Technology, Niigata University, 2-8050 Ikarashi, Niigata 950-2181, Japan; <sup>4</sup>Plant Cytogenetics Laboratory, National Institute of Genetics, 1111 Yata, Mishima, Shizuoka 411-8540, Japan; <sup>5</sup>Department of Mechanical and Electrical Engineering, Suwa University of Science, 5000-1 Toyohira, Chino, Nagano 391-0292, Japan

\* E-mail: kurusu@rs.sus.ac.jp Tel: +81-266-73-9826 Fax: +81-266-73-1230

\*\* E-mail: kuchitsu@rs.noda.tus.ac.jp Tel: +81-4-7122-9404 Fax: +81-4-7123-9767

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**Abstract** We have previously shown that autophagy is required for post meiotic anther development including programmed cell death-mediated degradation of the tapetum and pollen maturation in rice. However, the spatiotemporal dynamics of autophagy in the tapetum remain poorly understood. We here established an *in vivo* imaging technique to analyze the dynamics of autophagy in rice tapetum cells by expressing green fluorescent protein-tagged AtATG8, a marker for autophagosomes. 3D-imaging analysis revealed that the number of autophagosomes/autophagy-related structures is extremely low at the tetrad stage (stage 8), and autophagy is dramatically induced at the uninucleate stages (stage 9–10) throughout the tapetal cells during anther development. The present monitoring system for autophagy offers a powerful tool to analyze the regulation of autophagy in rice tapetal cells during pollen maturation.

**Key words:** autophagy, imaging, pollen maturation, rice tapetal programmed cell death.

## Introduction

Rice (*Oryza sativa* L.) is the staple food for more than half of the world's population, making it the world's most important food crop. Due to the importance of this plant in agriculture and scientific research, elucidating the mechanism of pollen development in rice is of considerable significance.

Reproductive development is accompanied by drastic changes in metabolism for the plentiful supply of nutrients and thus requires its appropriate regulation. In flowering plants, anthers exhibit four-layered structure composed of epidermis, endothecium, middle layer, and tapetum. Of these layers, the tapetum provides metabolites and nutrients to pollen grains, microspores, and pollen coat during their development (Ariizumi and Toriyama 2011). The tapetum includes triacylglycerol (TAG)-containing lipid bodies, which supply essential lipid components during pollen maturation (Li-Beisson

et al. 2010; Murphy 2012).

As pollens develop, the tapetum is degraded to provide nutrients, metabolites, and sporopollenin precursors to the developing microspores from stage 7 to 11. Defects in tapetal degradation have been suggested to result in the development of abnormal pollen coats and grains, leading to severe male sterility (Ariizumi and Toriyama 2011; Ku et al. 2003; Li et al. 2006; Zhang et al. 2008). Tapetal degradation is tightly regulated, and characteristic features of programmed cell death (PCD) such as chromatin condensation, cell shrinkage, endoplasmic reticulum (ER) swelling, mitochondrial persistence have been reported (Rogers et al. 2005).

Autophagy is an evolutionarily conserved system for degradation of intracellular components through the vacuoles/lysosomes and has been shown to play essential roles in growth, development, and survival of eukaryotic cells (Mizushima et al. 2010). Intracellular components are enveloped by the autophagosomal membrane and

Abbreviations: ATGs, autophagy-related genes; CLSM, confocal laser scanning microscope; ER, endoplasmic reticulum; GFP, green fluorescent protein; PCD, programmed cell death; PFA, paraformaldehyde; TAG, triacylglycerol; TEM, transmission electron microscopy.

<sup>†</sup> These authors contributed equally to this work.

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fuse with the vacuoles/lysosomes, where they are broken down by lytic enzymes. These changes are referred to as autophagic flux (Kurusu et al. 2016; Yoshii and Mizushima 2017).

More than 30 known autophagy-related genes (ATG) have been identified in yeast, many of which are conserved in most eukaryotes, including animals and plants (Yoshimoto and Ohsumi 2018). The formation of autophagosomes requires two ubiquitin conjugation-like reactions with ATG12 and ATG8. The C-terminal glycine-residue of autophagy-related protein 8 (ATG8) is essential for conjugation reactions and the formation of autophagosomes (Mizushima et al. 2011). The green fluorescent protein (GFP)–ATG8 fusion protein has been used as a marker for monitoring the entire process of autophagy in animals and fungi (Klionsky et al. 2007) as well as in plant cells (Contento et al. 2005; Hanamata et al. 2013; Thompson et al. 2005; Toyooka et al. 2006; Yoshimoto et al. 2004). Autophagosomes were detected as ring-shaped and punctate structures containing GFP–ATG8 in the cytosol, which increased under nutrient starvation and stress conditions (Toyooka et al. 2006; Yoshimoto et al. 2009).

Rice mutants defective in autophagy, *Osatg7-1*, *Osatg7-2*, and *Osatg9*, show sporophytic severe male sterility in normal growth conditions, suggesting that autophagy is crucial for sexual reproductive development as well as phytohormone metabolism of rice (Hanamata et al. 2014; Kurusu et al. 2014, 2017). Pollens from the *Osatg7-1* mutant are premature due to significant defects in the anther during pollen maturation. Of note, autophagosomes as well as autophagy-related structures including multilamellar bodies, presumably intermediate structures of autophagosomes, and dense globular bodies enclosed within the vacuoles emerge in the tapetum in the wild-type but not in the *Osatg7-1* mutant, as determined by transmission electron microscopy (TEM) analysis (Hanamata et al. 2014; Kurusu et al. 2014), suggesting that autophagy contribute to tapetal degradation and PCD in rice (Kurusu and Kuchitsu 2017). Little is known, however, on the molecular links and the timing between the spatiotemporal dynamics of autophagy and tapetal PCD. Moreover, TEM is not suitable for quantification, but quantitative assessment techniques of autophagy in the tapetum have not yet been available.

We here established an *in vivo* imaging technique to analyze the dynamics of autophagy in rice tapetum cells by expressing GFP–ATG8 fusion protein, a marker of autophagosomes, and revealed that autophagy is dramatically induced at stage 9–10 throughout the tapetal cells during pollen maturation.

## Materials and methods

### *Establishment of transgenic rice plants stably expressing GFP–ATG8 specifically in the tapetum*

To visualize the dynamics of autophagy in rice tapetal cells at various developmental stages, we generated transgenic rice lines expressing GFP–AtATG8a (GFP–ATG8) fusion protein (Yoshimoto et al. 2004) under the control of *ETERNAL TAPETUM1* (*EAT1*) and *ANTHER SPECIFIC PROTEIN 6* (*Osg6B/OsC6*) promoters, which were mainly expressed in the tapetum during anther development stages (Ono et al. 2018; Yokoi et al. 1997; Zhang et al. 2010) (Figure 1A). The GFP–*AtATG8a* fusion construct (pBI121 binary vector) was kindly provided by Dr. Koki Yoshimoto (Meiji University, Kawasaki, Japan).

The *EAT1* promoter and terminator linked to GFP–*AtATG8a* fragment was constructed as follows. The pPZP–*EAT1*pro–GFP–*NOSter* plasmid was constructed from pPZP–*EAT1*pro–*EAT1*–GFP (Ono et al. 2018) by inverse PCR using primers 5′-CTT GCT CACCAT GGA TCC TTT GGC AAA ACA GTG CTA GGC-3′ and 5′-CTG TTT TGCCAA AGG ATCCAT GGT GAG CAA GGG CG-3′, and by self-circularization using an In-Fusion HD Cloning Kit (TaKaRa, Japan). The *AtATG8a* fragment was amplified from a construct containing GFP–*AtATG8a* with the following primers: 5′-GCT GCCCGA CAA CCA CTA CCT G-3′ and 5′-TAC TTG TAC AGA GCA ACG GTA AGA GAT CCA AAAG-3′ (*Bsr*GI site is underlined). The *AtATG8a* PCR product was digested with *Bsr*GI and subcloned into the *Bsr*GI site of pPZP–*EAT1*pro–GFP–*NOSter* (Figure 1B). The binary vector pSMA35H2-NG was kindly provided by Dr. Hiroaki Ichikawa (NARO, Tsukuba, Japan).

The *Osg6B/OsC6* promoter (Accession No. GenBank: D21160.1) linked to GFP–*AtATG8a* fragment was constructed using PCR-based cloning. Briefly, the *Osg6B/OsC6* promoter fragment was amplified by PCR from rice genome DNA (cv. *Nipponbare*) with the following primers: 5′-CCG GAT ATC GAA TTC TTT TTT TTA CAC AGT TCA AAG TG-3′ and 5′-acagctcctgcacctgctcacatGCT AGC TTA ATT AGC TTT GG-3′ for *Osg6B/OsC6* containing the overlapping region of GFP–*AtATG8a* (*EcoRV* site is underlined, and lowercase indicates the overlapping region). The GFP-fused *AtATG8a* fragment was amplified from a construct containing GFP–*AtATG8a* with the following primers: 5′-attaaccaaagctaatagctagcATG GTG AGC AAG GGC GAG GA-3′ and 5′-CGGAGCTCTCA AGC AAC GGT AAG AGA TCC AAA AGT GTT C-3′ for GFP–*AtATG8a* containing the overlapping region of *Osg6B/OsC6* (*SacI* site is underlined, and lowercase indicates the overlapping region). The PCR products were linked by PCR using the following primers: 5′-CCGGATATCGAA TTC TTT TTT TTA CAC AGT TCA AAG TG-3′ and 5′-CGGAGCTCTCA AGC AAC GGT AAG AGA TCC AAA AGT GTT C-3′. The resulting product (*Osg6B/OsC6*–GFP–*AtATG8a*) was digested with *EcoRV* and *SacI* and then cloned into the binary vector pSMA35H2-NG (Chen et al. 2016)

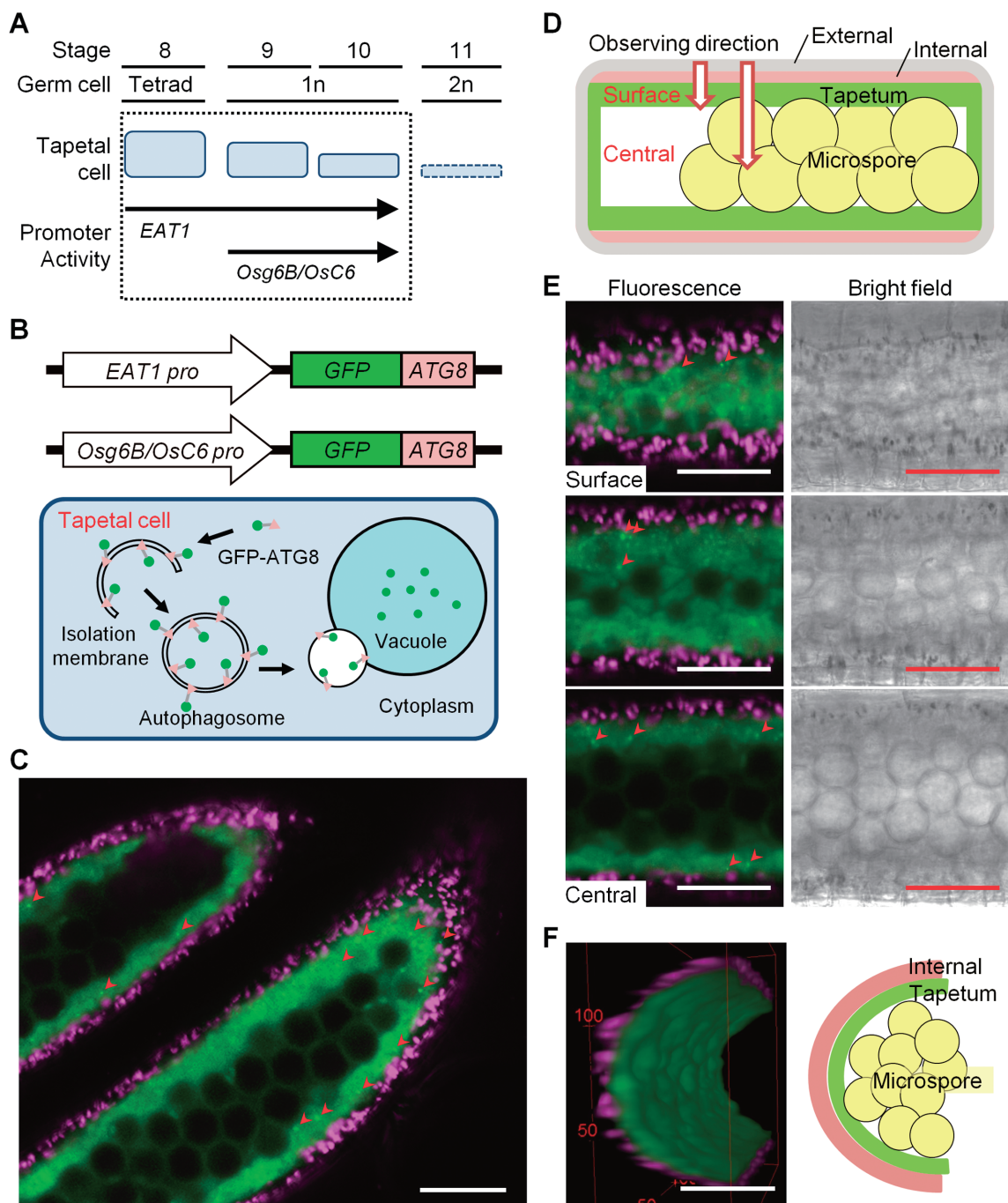


Figure 1. Imaging and quantitative characterization of autophagy based on GFP-ATG8 protein in rice tapetum during anther development and pollen maturation. (A) A schematic diagram of developmental stages of rice anthers. Tapetal cells are gradually degraded from stage 8 until 11 by programmed cell death. Stages when the activity of the two tapetum-specific promoters are active are shown. (B) A schematic diagram of visualization of the autophagy using GFP-ATG8 fusion proteins under the control of tapetum-specific promoters, *EAT1* and *Osg6B/OsC6*. ATG8 is incorporated into the isolation membrane and the autophagosome. Autophagosomes are fused with the vacuole, where ATG8 is degraded. (C, E, F) Visualization of the dynamics of autophagosomes/autophagy-related structures in rice tapetum using transgenic plants stably expressing GFP-ATG8 under the control of *EAT1* promoter. Confocal fluorescence images were obtained using a CLSM. Green and magenta fluorescence indicate GFP and autofluorescence of chlorophyll, respectively. Arrowheads indicate punctate signals of GFP-ATG8 (autophagosomes/autophagy-related structures). Scale bars: 50  $\mu\text{m}$ . Similar images were also observed in plants stably expressing GFP-ATG8 under the control of *Osg6B/OsC6* promoter (data not shown). (C) Cross-section of rice anthers containing immature microspores. Data are representative of three experiments. (D) A schematic diagram of a cross section of rice anther. (E) Z-Stack images of anthers from the surface to the central area. (F) 3-Dimensional reconstruction of the tapetum undergoing autophagy at stage 10 in rice anther based on the Z-Stack images. Figures were generated by rotating the cell 360° horizontally. Refer to the Supplementary Movie S1 for 3-dimensional visualization. Green signal represents GFP, red signal represents autofluorescence of chlorophyll in the endothecium.

Table 1. Anther groups defined for imaging analyses.

Sample stages	Stage 8	Stage 9	Stage 10	Stage 11
Anther length (mm)	0.8–0.9	0.9–1.0	1.1–1.5	1.6–1.8
Colors	Transparent	Transparent	Transparent/Whitish yellow	Yellow

using the *SacI* site and a *HindIII* site blunted with T4 DNA polymerase.

Each construct was introduced into rice calli derived from *OsATG7* (+/–) hetero seeds as well as *Nipponbare* using *Agrobacterium*-mediated transformation. Transformed calli were screened by hygromycin selection (50 µg ml<sup>-1</sup>) and transgenic plants were then regenerated. Transgenic cell lines derived from T<sub>2</sub> plants were genotyped and used for various analyses.

### Plant materials and growth conditions

Surface-sterilized seeds of transgenic rice lines (*Oryza sativa* L. cv. *Nipponbare*) were germinated on MS medium (Murashige and Skoog 1962) containing 0.8% agar and grown for 10 days in a growth chamber under long-day conditions (16-h light/8-h darkness, 28°C). Seedlings were transplanted into soil and grown in the greenhouse.

*Tos17*-insertional rice *Osatg7-1* mutant (*OsATG7*-/-), wild-type (*OsATG7*+/+), and heterozygous (*OsATG7*+/-) plants were selected in seed pools obtained from heterozygous lines expressing *GFP-AtATG8* by genomic PCR using the following primer mixture: *OsATG7* forward primer 5'-CAT ACT ACC ACC TCA GCT TGC TAG-3', *Tos-17* forward primer 5'-ACT ATT GTT AGG TTG CAA GTT AGT TAA GA-3', and *OsATG7* reverse primer 5'-GCA TTC AGG AAA ACC TCG TAT CG-3' for *OsATG7*. HPTII forward 5'-GAT GTA GGA GGG CGT GGA TAT GTC-3' and HPTII reverse 5'-CTT CTA CAC AGC CAT CGG TCC AGA-3' were used for hygromycin phosphotransferase II detection.

### In vivo imaging of autophagy

Imaging analysis of rice anther was performed by a modification of the method described by (Ono et al. 2018). Rice spikelets were fixed in phosphate buffered saline without calcium and magnesium (PBS (-)) buffer containing 4% paraformaldehyde (PFA) for 3 h and washed 6 times in PBS (-) buffer for 2 h. Anther samples of different developmental stages were separated based on the length and color of the anthers (Table 1) (Zhang et al. 2011). To visualize the fluorescence of GFP, whole anthers were mounted on slides in PBS (-) buffer, and images of autophagosomes/autophagy-related structures were observed using an LSM5 EXCITER confocal fluorescence microscope with a 40× objective lens (Carl Zeiss, Germany). For all experiments, the laser intensity was adjusted to the lowest level that retained a significant signal-to-noise ratio. Images were processed using the Zeiss LSM Image Browser (Carl Zeiss, Germany) and the ImageJ software. 3D-image processing was carried out using the 3D viewer of ImageJ software. Z-Stack images obtained by CLSM were reconstructed

into a 3-dimensional model.

## Results and discussion

To visualize the dynamics of autophagy in rice tapetal cells during pollen maturation, we established transgenic rice plants stably expressing the GFP-ATG8 under the control of tapetal specific promoters, *EAT1* or *Osg6B/OsC6* (Figure 1A, B). As shown in Figure 1C, GFP fluorescence was detected specifically within the cytosol of tapetal cells stably expressing GFP-ATG8 under the control of *EAT1* promoter. Similar images were also observed in plants stably expressing GFP-ATG8 under the control of *Osg6B/OsC6* promoter (data not shown). We also performed Z-stack-imaging analysis to determine the spatiotemporal expression of GFP-ATG8 in rice anthers (Figure 1D). As shown in Figure 1E and F, GFP signals were detected in the inner layer of the chlorophyll autofluorescence, and autophagosomes/autophagy-related structures were clearly observed throughout the tapetum at stage 10 (Supplementary Movie S1), indicating that the GFP-ATG8 protein was expressed specifically in the tapetum in anthers.

We applied this technique to analyze the spatiotemporal dynamics of autophagy in the tapetum during tapetal PCD in the wild type plants as well as the autophagy deficient mutant *Osatg7-1* (Kurusu et al. 2014). ATG7 is specific to autophagy and possesses E1-like activity in the ATG12 conjugation system that is essential for autophagosome biogenesis (Li and Vierstra 2012). A few punctate signals of GFP-ATG8 were observed at the tetrad stage (stage 8), and an increase in punctate signals was observed from early uninucleate stage (stage 9) to the late uninucleate stage (stage 10) (Figure 2). Increase in the number of autophagosomes/autophagy-related structures of tapetum at stage 10 was also confirmed by the transgenic plants stably expressing GFP-ATG8 under the control of *Osg6B/OsC6* promoter (Figure 3; wild-type). In contrast, no increase in punctate signals was detected in the *Osatg7-1* mutant at stage 10 (Figure 3; *Osatg7-1*), indicating that punctate signals of GFP-ATG8 were derived from autophagy-related structures such as the autophagosomes.

In the *Osatg7-1* mutant in which tapetal PCD is delayed in comparison with the wild-type, intracellular organelles such as plastids and mitochondria, which could be degraded by autophagy, are clearly observed in the cytoplasm even at the bicellular stage (stage 11) (Kurusu et al. 2014). Moreover, some multilamellar

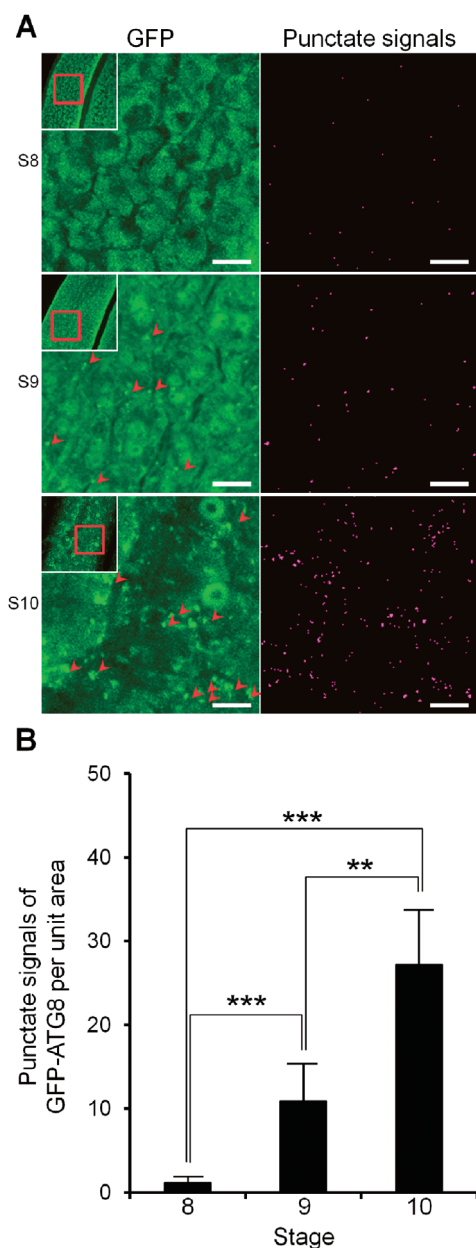


Figure 2. Quantitative monitoring of autophagosomes/autophagy-related structures in the tapetal cells in each developmental stage during anther development and pollen maturation. (A) Visualization of the dynamics of autophagosomes/autophagy-related structures in rice tapetum during anther development using a transgenic plant stably expressing GFP-AtATG8a under the control of *EAT1* promoter. Pictures were obtained by the maximum intensity projection (Left panels). Extraction of the punctate signals of GFP-ATG8 from left panels was performed by rolling ball background subtraction algorithm of the ImageJ software, then thresholds of all images were adjusted using Yen algorithm (Right panels). Arrowheads indicate punctate signals of GFP-AtATG8a. Scale bars: 10  $\mu$ m. (B) Levels of autophagosomes/autophagy-related structures from the tetrad (stage 8) to the uninucleate stages (stage 9 and 10) in the tapetum. To quantify the levels of autophagosomes/autophagy-related structures, the number of GFP punctate signals (arrowheads) per unit area were counted at the indicated stages using analyze particles plugin of the ImageJ software. Data are means  $\pm$  SD;  $n=5$  independent samples. \*\* $p<0.005$ , \*\*\* $p<0.0005$ ; significantly different from each indicated stage data.

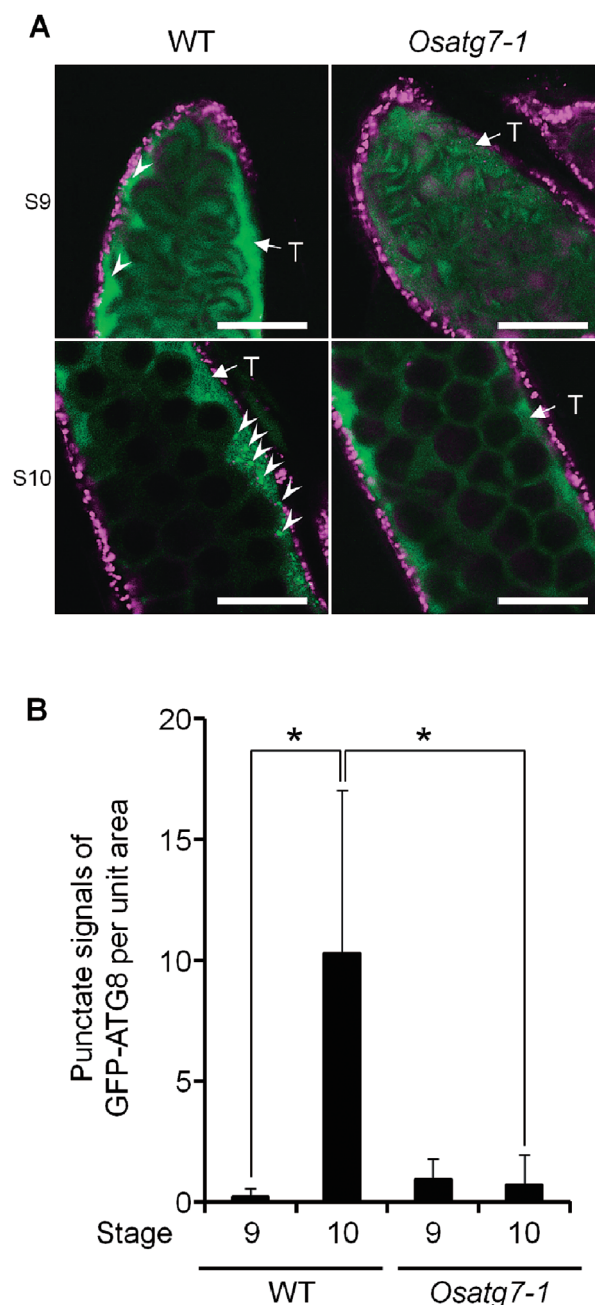


Figure 3. Numbers of autophagosomes/autophagy-related structures in the tapetal cells of the wild-type and the *Osatg7-1* mutant. (A) Fluorescence images of the autophagosomes/autophagy-related structures in rice tapetum using a transgenic rice plant stably expressing GFP-AtATG8a under the control of *Osg6B/OsC6* promoter. Green and magenta fluorescence indicate GFP and autofluorescence of chlorophyll, respectively. These fluorescence images show one slice containing the tapetal cells obtained by CLSM. Arrowheads indicate punctate signals of GFP-AtATG8a. Scale bars: 50  $\mu$ m. T, tapetum. (B) Levels of autophagosomes/autophagy-related structures at the uninucleate stages (stage 9 and 10) in the tapetum. To quantify the levels of autophagosome formation, the number of GFP punctate signals (arrowheads) per unit area were counted at the indicated stages. Data are the means  $\pm$  SE of three independent experiments. \* $p<0.05$ ; significantly different from the wild-type.

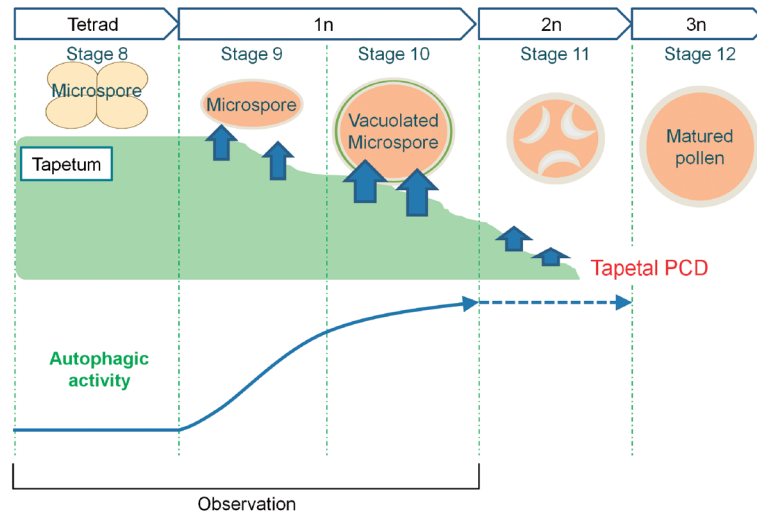


Figure 4. Schematic diagrams of the induction of autophagy at specific stages for rice tapetal degradation. The tapetum, the innermost of the four sporophytic layers of the anther wall directly contacts with the developing gametophytes and acts as a nutritional source for the development of microspore by undergoing degeneration triggered by PCD from stage 8 to 11. Unbroken and broken arrows indicate established and hypothetical links, respectively.

bodies, presumably intermediate structures of autophagosomes (Hariri et al. 2000), were observed in the cytoplasm of the wild-type (Kurusu et al. 2014), indicating that autophagy is activated simultaneously in the tapetum from early uninucleate stage (stage 9), which may be involved in the degradation of intracellular components such as plastids and mitochondria during pollen maturation.

Appropriate temporal regulation of tapetal PCD is vital for normal pollen development. The signal initiating tapetal PCD has been suggested to be first produced during the tetrad stage (Kawanabe et al. 2006). The proper timing of tapetal PCD is tightly controlled by an evolutionarily conserved transcriptional network mediated by several key transcription factors in *Arabidopsis* and rice (Niu et al. 2013; Ono et al. 2018; Phan et al. 2011). Recently, the expression pattern of *ATG* genes imply their unique roles in some critical stages of sexual plant reproduction, such as gametophyte development, gametogenesis, and embryogenesis (Zhou et al. 2015). The proper timing of tapetal PCD may be tightly controlled by the specific expression pattern of *ATGs*. The potential role of autophagy in the regulation of the tapetal transcriptional network is an important topic for future research.

In the present study, we have discovered that the number of autophagosomes/autophagy-related structures is extremely low during early PCD/tetrad stage (stage 8), and autophagy is dramatically induced at stage 9 throughout the tapetal cells during pollen maturation (Figure 4). Since, the *Osatg7-1* mutant exhibits a dense thin layer of tapetal tissue that came into contact with the orbicules, which is not observed in the wild-type even at the flowering stage (Hanamata et al. 2014), the activation

of autophagy in tapetal cells may play a key role in complete tapetal breakdown in rice. Further imaging analyses of the tapetum using the system established here expressing GFP-ATG8 under the control of various tapetum-specific promoters in other developmental stages along with genetic analyses and characterization of the molecules associated with the autophagy-related structures may reveal novel functions of autophagy and their physiological significance during anther development in rice.

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