


SHORT COMMUNICATION



Autophagy-mediated regulation of phytohormone metabolism during rice anther development

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ABSTRACT

Autophagy has recently been shown to be required for postmeiotic anther development including anther dehiscence, programmed cell death-mediated degradation of the tapetum and pollen maturation in rice. Several phytohormones are known to play essential roles during male reproductive development including pollen maturation. However, the relationship between phytohormone metabolism and autophagy in plant reproductive development is unknown. We here comprehensively analyzed the effect of autophagy disruption on phytohormone contents in rice anthers at the flowering stage, and found that endogenous levels of active-forms of gibberellins (GAs) and cytokinin, trans-zeatin, were significantly lower in the autophagy-defective mutant, *Osatg7-1*, than in the wild type. Treatment with GA₄ partially recovered maturation of the mutant pollens, but did not recover the limited anther dehiscence as well as sterility phenotype. These results suggest that autophagy affects metabolism and endogenous levels of GAs and cytokinin in rice anthers. Reduction in bioactive GAs in the autophagy-deficient mutant may partially explain the defects in pollen maturation of the autophagy-deficient mutant, but tapetal autophagy also plays other specific roles in fertilization.

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Autophagy is an evolutionarily conserved system for the degradation of intracellular components through vacuoles/lysosomes and has been shown to play essential roles in growth, development and survival of eukaryotic cells.¹ Intracellular components are enveloped by autophagosomal membranes and fuse with the vacuoles/lysosomes, where they are broken down by lytic enzymes. These changes are referred to as autophagic flux.²



In many eukaryotes, autophagy is required for normal development, e.g. for dauer development in nematodes and preimplantation in mice.³⁻⁵ In plants, autophagy has been suggested to be involved in seed development and germination, photomorphogenesis, chloroplast maturation, mineral nutrition, hormonal responses, pathogen resistance, stress protection, senescence, and fertile floret development under nutrient-limiting conditions.⁶⁻⁹ However, mutants of *Arabidopsis* and maize defective in autophagy exhibit normal life cycles, and little is known regarding the roles of autophagy during normal reproductive development in plants.^{10,11}


Rice mutants defective in autophagy, *Osatg7-1* and *Osatg9*, show sporophytic male sterility and limited anther dehiscence under normal growth conditions, suggesting that autophagy is crucial for sexual reproductive development of rice.^{12,13} Pollens of *Osatg7-1* mutant are premature due to significant defects in the anther during pollen maturation. Autophagosomes emerge in

the tapetum during the uninucleate stage in the wild type but not in *Osatg7-1* mutant, indicating that autophagy is induced at the uninucleate stage in the postmeiotic tapetum cells and may be involved in the catabolism of intracellular components such as plastids and lipid bodies during pollen maturation.^{12,14}

Several phytohormones are known to play essential roles during the male reproductive development in rice.¹⁵ Autophagy-deficient mutants of *Arabidopsis* such as *atg5* have been shown to over-accumulate salicylic acid (SA) in leaves, and many of their phenotypes can be explained by the excess amount of SA.¹⁶ However, relationship between autophagy and phytohormone metabolism in plant reproductive development is unknown.

In the present study, we conducted comprehensive analyses for phytohormone levels of anthers at the flowering stage in the *Osatg7-1* mutant and wild type, in which the insertion of *Tos17* in *OsATG7* was removed by heterozygous segregation, and investigated the effect of autophagy disruption on phytohormone contents. Endogenous levels of bioactive gibberellins (GAs; GA₁, GA₄ and GA₇) in anthers were lower in the *Osatg7-1* mutant than in the wild type and cultivar *Nipponbare* (NB) as controls at the flowering stage (Table 1). A cytokinin (trans-zeatin; tZ) also decreased in the mutant, while no significant difference was observed for other phytohormones including SA, jasmonic acid (JA), auxin and abscisic acid (ABA)

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Table 1. Phytohormones in *Nipponbare* (NB), wild type and *Osatg7-1* anthers at the flowering stage.

hormones (pmol/gFW)	NB	wild-type	<i>Osatg7-1</i>
tZ	5.26 ± 0.91	4.74 ± 0.40	2.29 ± 0.16*
IAA	32200 ± 10000	30500 ± 18300	57600 ± 20300
GA ₁	48.09 ± 5.51	38.00 ± 16.87	4.78 ± 1.16*
GA ₄	4740 ± 119.4	4860 ± 545.3	3170 ± 684.3**
GA ₇	1110 ± 79.4	1030 ± 119.5	672 ± 128.4**
GA ₁₂	63699 ± 4816	85200 ± 4770	10400 ± 2310**
ABA	17.76 ± 3.34	11.51 ± 2.42	16.49 ± 3.46
SA	7520 ± 3100	7010 ± 2880	7410 ± 1740
JA	430 ± 63.35	532 ± 59.14	735 ± 216.21

Data represent the mean ± SD of 3 ≥ plants.

* $P < 0.05$,

** $P < 0.01$; significantly different from both NB and the wild type.

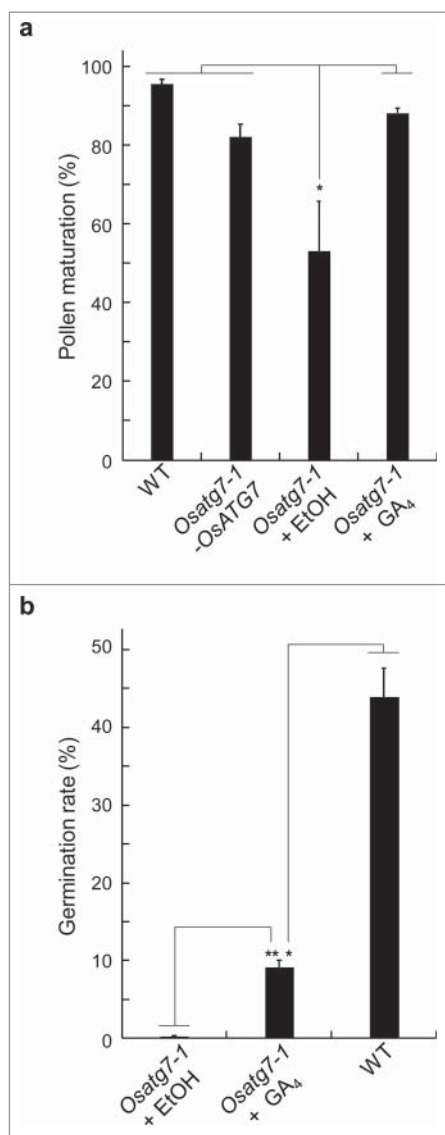


Figure 1. Effects of active GA on pollen maturation and germination in rice autophagy-deficient mutant. (a) Pollen grains from the wild type (WT), complementation plant (*Osatg7-1-OsATG7*) and *Osatg7-1* mutant were stained with I₂-KI solution and quantified. (b) Pollen grains from the *Osatg7-1* anthers at the flowering stage were germinated for 1 h in pollen germination medium, and then observed and quantified by bright-field microscopy. Data indicates means ± SD of 10 independent samples. The average was determined from 200 pollen grains per each experiment. * $P < 0.05$, ** $P < 0.01$; significantly different from the control using an unpaired Student's *t*-test.



Figure 2. The effect of active GA on anther dehiscence in rice autophagy-deficient mutant. Anthers from the *Osatg7-1* at the flowering stage were observed by bright-field microscopy. Scale bar: 0.5 mm.

between the wild type and the mutant (Table 1). Unlike the case in *Arabidopsis* leaves,¹⁶ SA content was not affected by the disruption of autophagy in anthers (Table 1).

Mild defects in GA biosynthesis have been shown to impair pollen function in rice.¹⁷ To investigate the effect of bioactive GA during anther development in autophagy deficient mutant, rice plants at the pollen mother cell (PMC) stage were sprayed with 10⁻⁵ M GA₄, and then both the pollen maturation and germination activity were assessed. Treatment with GA₄ recovered the maturation of mutant pollens at the flowering stage (Fig. 1a). In contrast, the germination activity of mutant pollens was partially recovered by the treatment of bioactive GA (Fig. 1b). Moreover, the limited anther dehiscence as well as sterility phenotype of the mutant was not recovered by the addition of bioactive gibberellin, GA₄ (Fig. 2). These results suggest that autophagy affects phytohormone contents in rice anthers at the flowering stage, and the reduction in bioactive GAs may explain in part the defects in the pollen function in the *Osatg7-1* mutant. GAs play important roles in seed germination and anther development in several plant species. GA deficiency or insensitivity causes abnormal development of anthers and leads to male sterility in tomato, petunia, *Arabidopsis*, and rice.¹⁸⁻²² All these studies suggest that GA is essential for normal development of tapetal cells and pollen.

As pollens develop, the tapetum is broken down to provide nutrients, metabolites, and sporopollenin precursors to the developing microspores. Defects in tapetal degradation have been suggested to result in the development of abnormal pollen coats and grains, leading to severe male sterility.²³⁻²⁶ Additionally, the appropriate temporal regulation of tapetal programmed cell death (PCD) is vital for normal pollen development. The signal initiating tapetal PCD has been suggested to be first produced during the tetrad stage.²⁷ GA was previously shown to control tapetum degradation.^{18,19} A delay in tapetal breakdown and a switch from PCD to necrosis in the tapeta were observed in an *ms1* mutant in *Arabidopsis*.²⁸ Pollen wall deposition and subsequent microspore degeneration failed in a rice mutant in which tapetal degeneration and PCD was retarded.^{25,26}

As shown in Table 1, disruption of autophagy significantly reduced endogenous levels of the precursor GA₁₂ as well as

bioactive GAs in rice anthers at the flowering stage (Table 1). Autophagy may affect the *ent*-kaurene pathway that supply the precursor of bioactive GAs in anthers. The *ent*-kaurene products are synthesized in plastids by *ent*-copalyl diphosphate synthase (CPS) and kaurene synthase (KS).^{29,30} Autophagy plays important roles in the quality control of plastids including elimination and turnover of photodamaged chloroplasts.³¹ Accumulation of abnormal plastids in anther cells of autophagy-deficient mutant may explain the lower activity of the *ent*-kaurene pathway in anther cells, suggesting the significance of autophagy in the quality control of plastids in anthers. Our present research indicates a critical role of autophagy in developmental process of crops, and shed light on the novel autophagy-mediated regulation of GA metabolism in plastids and tapetal PCD in rice reproductive development.

Jasmonic acid (JA) has been thought to play an important role in regulating anther dehiscence. Mutations in genes that participate in JA biosynthesis cause a failure or delay in anther dehiscence and can result in male sterility.^{32,33} Rice mutants defective in autophagy (*Osatg7-1* and *Osatg9*) show limited anther dehiscence under normal growth conditions.¹² However, Endogenous JA level was not affected by the disruption of autophagy during anther development (Table 1). In fact, the limited anther dehiscence of the *Osatg7-1* mutant was not recovered by the addition of MetJA at the flowering stage (Fig. S1), indicating that the defect in anther dehiscence and the male sterile phenotype in the *Osatg7-1* mutant may not explain by the reduction of JA content.

Comprehensive analyses of phytohormones in rice anther showed that endogenous levels of bioactive cytokinin tZ (trans-zeatin) was also lower in the *Osatg7-1* mutant than in the wild type at the flowering stage (Table 1). However, the physiologic function of cytokinin during anther development remains largely unclear. Overexpression of cytokinin oxidase/dehydrogenase (CKX) under the control of anther specific promoter have been suggested to result in male sterility in maize.³⁴ Interestingly, ectopic-expression of Arabidopsis ATG8 protein reduces shoot anthocyanin accumulation in response to cytokinin feeding to the roots,³⁵ implying the participation of ATG8 protein in cytokinin-regulated root-shoot communication in Arabidopsis. Moreover, external application of cytokinin leads to the autophagosome formation in Arabidopsis root cells.³⁵ Autophagy may participate in the control CK content during anther development, and the potential role of CK in the regulation of anther development is an important topic for future research.

Disclosure of potential conflicts of interests

The authors declare that they have no competing interests.

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