

Chapter 35

The Role of Peroxisomes in Plant Reproductive Processes

Shino Goto-Yamada, Shoji Mano, and Mikio Nishimura

Abstract Peroxisomes are ubiquitous organelles found in eukaryotic cells. These organelles perform important metabolic functions required for normal development. Peroxisomes play crucial roles in flowering plants, functioning in such processes as lipid metabolism, photorespiration, and the production of phytohormones, rendering these organelles indispensable for normal plant growth. In addition to functioning in vegetative tissues, recent studies have shown that peroxisomes play essential roles in reproductive processes. This chapter describes the analysis of peroxisomes in gametophytes, using the approach of organelle visualization in reproductive tissues, which reveals organelle distribution in gametophytes and the dynamics of peroxisomes in pollen cells. Studies using mutants defective in peroxisomal functions demonstrate the importance of peroxisomes to pollen fertility and pollen tube elongation in pistils. We also summarize the relationship between peroxisomal function and reproductive processes in plants. Peroxisomes may contribute to pollen fertility by generating energy for pollen tube growth and producing signaling molecules required for pollen tube elongation and orientation. Moreover, peroxisomes may be involved in male–female gametophyte recognition.

Keywords Fluorescent protein • Peroxisome • Plant • Reproduction

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35.1 Introduction

Peroxisomes are single membrane-bound organelles that are ubiquitous in eukaryotic cells. As peroxisomes contribute to lipid metabolism via β -oxidation activities in various organisms, including animals and plants, these organelles are essential for survival. Peroxisomes have a variety of functions, such as polyamine metabolism (Nishikawa et al. 2000; Wu et al. 2003; Kamada-Nobusada et al. 2008), the synthesis of various secondary metabolites (Imazaki et al. 2010; Meijer et al. 2010), and vitamin production (Babujee et al. 2010; Tanabe et al. 2011), in addition to lipid metabolism, which demonstrates that peroxisomes are involved in biological processes at various developmental stages. Recent analyses using various mutants showed that defects in peroxisomal function result in the failure of reproductive processes (Footitt et al. 2007; Boisson-Dernier et al. 2008; Wu et al. 2010). This chapter focuses on the relationship between peroxisomal functions and reproductive processes in plants.

35.2 Peroxisomes in Higher Plants

35.2.1 *Peroxisomal Functions During Plant Development*

In plants, peroxisomes appear in various tissues as globular organelles of approximately 1–1.5 μm . Several types of peroxisomes are found in plants, including glyoxysomes and leaf peroxisomes (Kamada et al. 2003). Plant peroxisomes adapt to environmental and developmental changes (Nishimura et al. 1993). Peroxisomes in etiolated cotyledons are referred to as glyoxysomes. These organelles are responsible for the conversion of fatty acids to sucrose, which provides energy for seedling growth after germination (Fig. 35.1a). In photosynthetic leaves, a functional transition from glyoxysomes to leaf peroxisomes takes place (Fig. 35.1b) (Nishimura et al. 1993). Leaf peroxisomes contain enzymes that function in the glycolate pathway and are active during photorespiration. This function is indispensable for scavenging by-products produced by photosynthesis and is required for normal plant growth (Tolbert 1981). Moreover, plant peroxisomes contribute to the generation and detoxification of reactive oxygen species (ROS), the biosynthesis of plant hormones, such as jasmonate and auxin, and the metabolism of polyamines (Kamada-Nobusada et al. 2008). Studies of various *Arabidopsis* mutants with defective peroxisomes have demonstrated that peroxisomal functions and biogenesis are essential for plant growth (Hayashi et al. 1998, 2002; Mano et al. 2004, 2006; Goto et al. 2011).

Because all peroxisomal proteins are encoded by the nuclear genome, peroxisomal proteins are translated on free ribosomes in the cytosol. These proteins are then transported to peroxisomes when their peroxisomal targeting signals 1 or 2 (PTS1 or PTS2) are recognized by the cytosolic receptors PEROXIN 5 (PEX5) and PEX7, respectively, which direct the proteins to the peroxisomal membrane. Constitutive expression of the gene encoding the PTS1 fused green fluorescent protein (GFP) construct under the control of the CaMV 35S promoter results in the

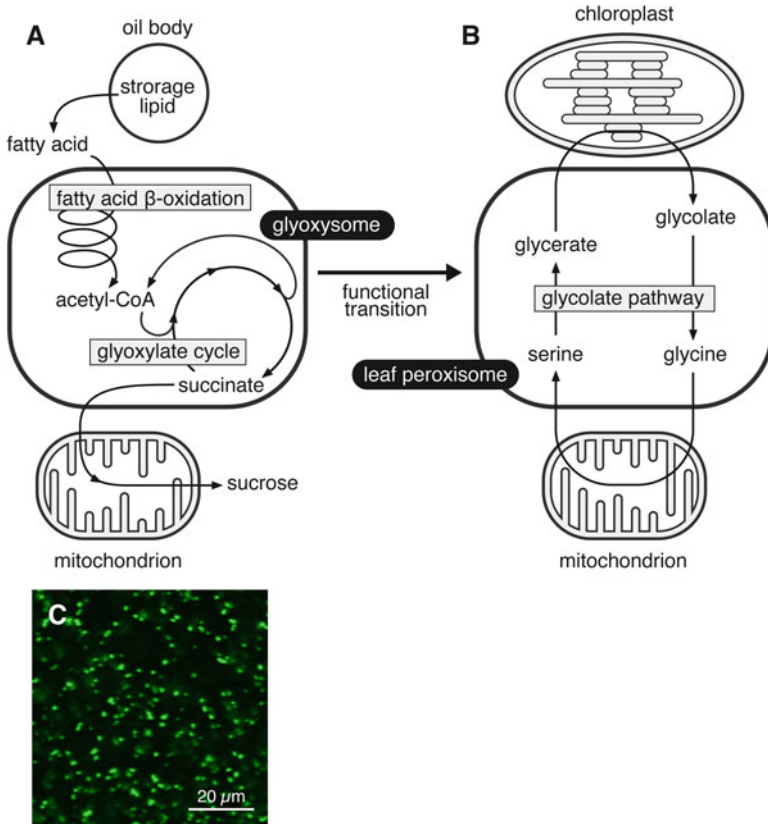


Fig. 35.1 Plant peroxisomes. (a, b) Metabolic pathways in peroxisomes. Lipid metabolism in glyoxysomes (a) and photorespiration in leaf peroxisomes (b). After the plant receives light, a functional transition from glyoxysomes to leaf peroxisomes occurs. (c) GFP-labeled peroxisomes in leaves of transgenic *Arabidopsis* expressing *GFP-PTS1* under the control of the CaMV 35S promoter

transport of this protein to the peroxisomes; GFP in the peroxisomes is detectable in almost all cells when visualized under a fluorescence microscope (Fig. 35.1c) (Mano et al. 2002). This visualization system is useful for a variety of experiments, including the observation of peroxisome dynamics, such as morphology and movement, and the isolation of mutants (Mano et al. 2004, 2006, 2011; Goto et al. 2011).

35.2.2 Peroxisomes in Plant Reproductive Tissues

Electron microscopic observation has revealed that peroxisomes exist in both vegetative and reproductive tissues. Many vesicles thought to be oil bodies are present in pollen grains, and peroxisomes are found among them (Fig. 35.2a, e). Pollen peroxisomes appear as spherical structures, and they are smaller than peroxisomes in vegetative tissues, with an average size of approximately 500 nm. To understand the

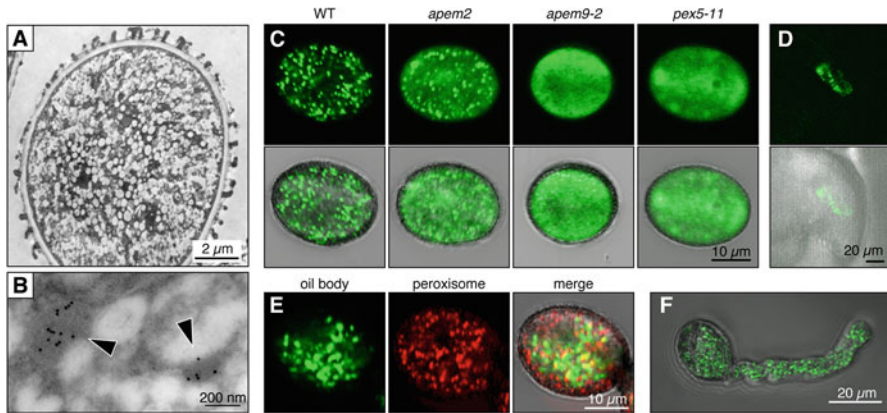


Fig. 35.2 Peroxisomes in reproductive tissues. (a) Electron micrograph of Arabidopsis pollen. (b) Immunoelectron micrograph of pollen from transgenic Arabidopsis harboring *Lat52p:GFP-PTS1*. Arrowheads indicate peroxisomes, which were immunogold labeled with anti-GFP antibodies. (c) GFP fluorescence pattern in wild-type and peroxisome-defective mutants *apem2*, *pex5-11*, and *apem9-2*, which express *GFP-PTS1*. The *pex5-11* and *apem9-2* represent the T-DNA insertion lines SALK_018579 and SALK-132193, respectively. Both mutants are unable to produce homozygotes, and heterozygotes produce two types of pollen that exhibit different phenotypes, including wild-type-like spherical structures and mutant structures exhibiting cytosolic fluorescence; only pollen grains of the mutant are shown in this figure. (d) Peroxisomes in egg cell of female gametophyte. Peroxisomes were visualized using *DD45p:GFP-PTS1*, with the expression of *GFP-PTS1* under the control of the *DD45* promoter. (e) Localization of oil bodies and peroxisomes in pollen. Oil bodies were visualized with GFP-labeled oleosin, a membrane protein present in oil bodies. Peroxisomes were visualized with TagRFP-PTS1. (f) Subcellular localization of peroxisomes in elongating pollen tube

temporal and spatial behavior of peroxisomes during reproductive processes, we generated transgenic Arabidopsis-expressing peroxisome-targeted fluorescent protein in pollen and egg cells using the *Lat52* and *DD45* (Twel et al. 1991; Steffen et al. 2007) promoters, respectively. Because the CaMV 35S promoter does not function properly in these cells, *Lat52* and *DD45* promoter-controlled *GFP-PTS1* (*Lat52p:GFP-PTS1* or *DD45p:GFP-PTS1*) was used to visualize peroxisomes in pollen and egg cell, respectively. Using these constructs, we could easily observe peroxisomal movement and distribution during the reproductive processes (Fig 35.2c,d). In mature pollen grains, the peroxisomes are quite immobile, but after pollen germination, the peroxisomes move in the direction of pollen tube growth (Fig. 35.2f). The peroxisomes then move through the pollen tube that reaches the female gametophyte.

35.3 Key Genes of Peroxisome Biogenesis Also Function in Pollen Cells

In flowering plants and in animals, peroxisomal functions are essential for normal development and survival. In humans, peroxisome biogenesis disorders, including Zellweger syndrome, are fatal genetic disorders (Fujiki 2000). In plants, defects of

peroxisomal biogenesis and function result in various defects, including the failure to germinate without sucrose (Hayashi et al. 1998; Hayashi et al. 2002), dwarfism (Mano et al. 2004, 2006; Goto et al. 2011), and embryonic lethality (Schumann et al. 2003; Sparkes et al. 2003; Fan et al. 2005). Moreover, recent studies have revealed that defects in peroxisomes result in a decrease of pollen fertility and failures in gametophyte recognition (Boisson-Dernier et al. 2008), indicating that explication of peroxisomal functions is important for understanding reproductive processes in plants.

As reported previously, we isolated a number of *Arabidopsis apem* (*aberrant peroxisome morphology*) mutants expressing *35Sp:GFP-PTS1* that had different patterns of GFP fluorescence from the parental GFP-PTS1 plants (Mano et al. 2004, 2006, 2011; Goto et al. 2011). To understand the role of peroxisomes in pollen fertility, we expressed the *Lat52p:GFP-PTS1* transgene in various mutants defective in peroxisomal biogenesis and function, including the *apem* mutants. Of these mutants, both *apem2* and *apem9* have mutations in the *PEX* genes, which are key genes of peroxisome biogenesis. *APEM2* and *APEM9* encode PEX13 and plant-specific factor, respectively, and both proteins are involved in peroxisomal protein transport. Therefore, in both mutants, GFP-PTS1 protein is accumulated in the cytosol as well as in peroxisomes (Mano et al. 2006; Goto et al. 2011). As shown in Fig. 35.2c, the pattern of GFP fluorescence in the pollen cells of both mutants is similar to that in vegetative tissues; GFP fluorescence is observed in the cytosol, whereas GFP is observed only in peroxisomes in the wild-type background. A similar pattern is observed in the pollen of a mutant defective in *PEX5*, which encodes a receptor of peroxisomal matrix protein and is essential for peroxisomal protein transport. As shown in Fig. 35.2c, GFP-PTS1 protein is accumulated in the cytosol in pollen cells of the *pex5-11* mutant. These results show that peroxisomal protein transport functions in pollen peroxisomes and that peroxisome biogenesis factors, such as PEXs, act in pollen cells.

35.4 Effects of Peroxisome Defects on Male Gametes

To examine the contribution of peroxisomal function to reproductive process, the peroxisome-defective *apem9* mutant was employed. First, to investigate the fertility of gametes in peroxisome-defective mutants, reciprocal crosses of *apem9-2/+* with wild-type Columbia (Col) were performed. When *apem9-2/+* pollen was used to pollinate Col pistils, only 14.6 % [transmission efficiency (TE)=17.1, $n=96$] of the seeds had the *apem9-2/+* genotype, whereas when *apem9-2/+* pistils were pollinated with Col pollen, 45.5 % (TE=83.3, $n=44$) of the seeds had the *apem9-2/+* genotype (Table 35.1). These results show that the transmission of the mutation through the female gametophyte is not impaired, and *apem9* ovules are fertile. However, pollen development and function are severely affected in the *apem9* mutant.

Table 35.1 Reciprocal crosses between peroxisome mutants and wild-type plants

Mutant name	Gene	TE _{female} (%) ^a	TE _{male} (%) ^a	Reference
<i>pex5_11</i> ^b	<i>PEROXIN5</i>	110.0	30.2	This study
<i>apem9-2</i> ^c	<i>APEM9</i>	83.3	17.1	This study
<i>amc/apem2</i>	<i>PEROXIN13</i>	82.8	45.3	Boisson-Dernier et al. (2008)
<i>cts-1</i> ^d	<i>COMATOSE</i>	93.9	21.6	Footitt et al. (2007)
<i>cts-2</i> ^d	<i>COMATOSE</i>	111.3	10.3	Footitt et al. (2007)

^aTransmission efficiencies (TE) were calculated according to Howden et al. (1998)

^b*pex5_11* represents SALK_018579

^c*apem9-2* represents SALK-132193

^d*cts-1* and *cts-2* are allelic to *ped3/pxa1*, which is defective in peroxisomal ABC transporter

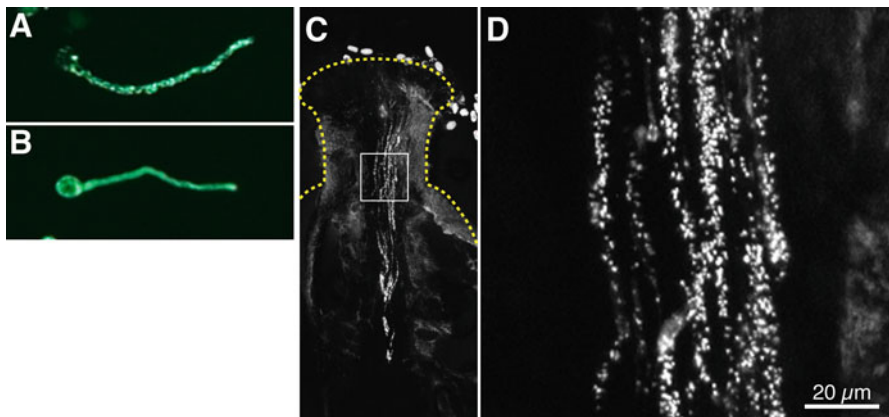


Fig. 35.3 Analysis of pollen tube elongation in *apem9-2* mutant and wild-type plant. (a, b) Fluorescence images of elongating wild-type (a) and *apem9-2* (b) pollen tubes grown in vitro. (c) In vivo pollen germination assay. Mutant *apem9-2/+* pollens were used to pollinate a wild-type pistil, and GFP fluorescence was observed with a laser-scanning confocal microscope 1 h after pollination. (d) Magnified image of the area enclosed by the white box in c

Pollen fertilization occurs via the following steps: (1) pollen maturation, (2) pollen germination and elongation of the pollen tube, (3) guidance of the pollen tube to the ovule, and (4) fertilization with the ovule. To determine whether the reduced male transmission of the *apem9-2* mutation was caused by a pollen germination defect, and to identify which step(s) in fertilization are disturbed in *apem9-2* pollen, an in vitro pollen germination assay was performed, and the numbers of germinated wild-type (+) and *apem9-2* pollen were counted. There was no obvious difference in the number of germinated pollen grains between wild-type and *apem9-2* pollen, indicating that *apem9-2* pollen matured successfully, as did the wild-type pollen, and had the ability to germinate under artificial germination conditions (Fig. 35.3a,b). However, in the pollen germination assay in vivo, in which pollen was germinated on wild-type stigmas, the *apem9-2* pollen tubes, unlike the wild-type pollen tubes, failed to elongate in the top parts of the pistils within 1 h of pollination (Fig. 35.3c,d). Based on these results, we determined that the defect in

the *APEM9* gene may decrease the efficiency of pollen fertility caused by a delay in pollen tube elongation in vivo or a failure of pollen tube penetration into the papilla cells, indicating that peroxisome biogenesis and function are required for pollen fertility.

35.5 Relationship Between Peroxisomal Function and Reproduction

In addition to the *apem9* mutant, other peroxisome mutants also show defects in pollen fertility. Table 35.1 shows the results of reciprocal crosses between peroxisome mutants. Although the female TEs are almost normal in these mutants, the male TEs are obviously decreased. These results indicate that peroxisomal functions are required for pollen fertility. This section reviews the relationship between peroxisomal functions and reproductive processes, including pollen fertility.

35.5.1 Peroxisomal β -Oxidation and Pollen Fertility

Fatty acid β -oxidation is one of the major metabolic processes that occur in peroxisomes. Fatty acid β -oxidation is required for the metabolism of lipids, as well as the conversion of fatty acid derivatives into phytohormones, such as auxin and jasmonic acid (JA) (Wain and Wightman 1954; Sanders et al. 2000; Stintzi and Browse 2000). JA plays a crucial role as a signaling molecule during plant growth and development and in plant responses to biotic and abiotic stress. Moreover, JA contributes to reproductive processes. Peroxisome-targeted OPR3 (12-oxophytodienoic acid reductase 3) is one of the enzymes required for JA biosynthesis (Sanders et al. 2000; Stintzi and Browse 2000). The Arabidopsis *opr3* mutant shows the male-sterility phenotype, resulting from defects of sufficient elongation of the anther filament, normal anther dehiscence, and the production of viable pollen (Sanders et al. 2000; Stintzi and Browse 2000). These results indicate the importance of peroxisomal β -oxidation in plant reproductive processes.

Another important aspect of fatty acid β -oxidation is lipid metabolism in seeds. Oilseed plants, such as Arabidopsis and pumpkin, convert seed-storage lipids to sucrose during postgerminative growth as energy sources before the plant begins photosynthesis (Fig. 35.1a). Triacylglycerols are accumulated as storage lipids in organelles known as oil bodies, which are surrounded by oleosin in seeds. Similarly, numerous oil bodies and peroxisomes are observed in pollen grains when examined by electron (Fig. 35.2a) and fluorescence microscopy (Fig. 35.2e). The similarity between seeds and pollen suggests that fatty acid β -oxidation provides energy during pollen germination and/or pollen tube elongation. Indeed, the lack of CTS (COMATOSE), an ABC transporter that contributes to the uptake of fatty acids into peroxisomes, results in the suppression of pollen tube elongation (Footitt et al. 2007),

whereas this mutant pollen tube can elongate in artificial pollen germination medium containing sucrose. The phenotype for suppression of pollen tube elongation is also seen in other mutants defective in fatty acid β -oxidation, such as KAT2 (3-keto acyl-CoA thiolase), LACS6, and LACS7 (long-chain acyl-CoA synthetase 6 and 7), which demonstrates the importance of peroxisomal fatty acid β -oxidation in pollen fertility and pollen tube elongation (Footitt et al. 2007).

35.5.2 Peroxisome Biogenesis and Male–Female Gametophyte Recognition

A recent study has revealed that peroxisomes are involved in male–female gametophyte recognition (Boisson-Dernier et al. 2008). The *amc* (*abstinence by mutual consent*) mutant was originally identified as a mutant with defective in male–female gamete recognition. In addition to decreased pollen fertility, the *amc* mutant exhibits impaired fertilization only when both male and female gametophytes have mutations in *AMC*. In wild-type plants, pollen germinates after it attaches to a pistil. The pollen tube then elongates, reaches one of the two synergid cells of the female gametophyte, and ruptures to release two sperm cells. However, the *amc* mutant shows impaired pollen tube reception when an *amc* pollen tube reaches an *amc* female gametophyte, resulting in the continued growth of the pollen tube in the synergid cell. As the pollen tube fails to rupture, the sperm cells are not released, and homozygous mutants never develop. Interestingly, the *AMC* gene is allelic to *apem2* (Mano et al. 2006). Therefore, the *amc* mutant is defective in one of the peroxisome biogenesis factors, PEX13, which is required for peroxisomal protein transport. The *AMC/PEX13* gene is transiently expressed in both male and female gametophytes during fertilization (Boisson-Dernier et al. 2008). As PEX13 is one of the peroxisome biogenesis factors, these results indicate that peroxisome biogenesis plays an unexpected key role in gametophyte recognition.

35.5.3 Peroxisome-Derived ROS and NO and Reproduction

ROS and nitric oxide (NO) are signaling molecules; peroxisomes are involved in the production of these compounds. A recent study has revealed that peroxisome-targeted PAO (polyamine oxidase) contributes to pollen tube elongation in association with ROS production (Wu et al. 2010). This study illustrates that PAO regulates pollen tube elongation during the oxidation of spermidine, one of the polyamines, and generates hydrogen peroxide (H_2O_2), which triggers the opening of the hyperpolarization-activated Ca^{2+} -permeable channels in pollen. The Arabidopsis genome has five isoforms of PAO, and three of these (PAO2, PAO3, and PAO4) are localized to peroxisomes (Kamada-Nobusada et al. 2008). The *pao3* mutant exhibits

suppressed pollen tube elongation and decreased seed numbers, indicating the importance of peroxisome function, combined with ROS production, in plant reproduction. The details of how PAO-mediated H_2O_2 production contributes to pollen fertility will become clearer in future studies. In addition, Prado et al. reported that NO is generated in peroxisomes in the pollen tube, and NO regulates pollen tube orientation in vitro (Prado et al. 2004). Although the activity of NOS (nitrogen oxide synthase) is detected in peroxisomes isolated from pea (*Pisum sativum*) (Barroso et al. 1999), peroxisome-targeted NOS has not been identified yet. The metabolic process leading to NO production is still largely unclear and should be investigated in the future.

35.6 Perspectives

This chapter describes how plant peroxisomes contribute to pollen development, pollen tube elongation, and male–female gametophyte recognition. Although some details about how peroxisomes function during reproduction remain to be elucidated, the importance of peroxisomes in reproductive processes has become clear through the analysis of peroxisome mutants. Defects in peroxisomal biogenesis and metabolism impair sperm development and fertility in *Drosophila* and other animals as well as in plants (Huyghe et al. 2006; Nenicu et al. 2007; Chen et al. 2010; Nakayama et al. 2011), suggesting that peroxisomes may function in the reproductive processes of various organisms in a similar manner.

In mammalian cells, the β -oxidation is localized in both peroxisomes and mitochondria. Long- and medium-chain fatty acids were metabolized in peroxisomes, whereas short-chain fatty acids were metabolized in mitochondria but not in peroxisomes. In plants, however, the activity of β -oxidation is present only in peroxisomes. Therefore, we have to consider the difference along with the similarity of peroxisomal functions between plants and animals. Transdisciplinary investigation that concerns both plant and mammalian peroxisomes will clarify the similarities or differences of peroxisomal functions in the reproductive process among various organisms.

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