The mevalonate pathway but not the methylerythritol phosphate pathway is critical for elaioplast and pollen coat development in Arabidopsis

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Abstract Pollen coat components are derived from tapetum cells, which contain elaioplasts derived from plastids and tapetosome derived from the endoplasmic reticulum. In Brassica napus, the main neutral lipids in the elaioplast and tapetosome have been reported to be sterol ester and triacylglycerol, respectively. Isopentenyl pyrophosphate, the structural component of sterol, is produced via the cytosolic mevalonate (MVA) and plastidic methylerythritol phosphate (MEP) pathways. Although these two pathways are compartmentalized, partial cross-talk between them has been reported. To investigate the contribution of these two pathways in elaioplast formation, we characterized mutant pollen of these two pathways. We observed the anthers of male sterile hmg1-1 and atipi1 atipi2 mutants ultrastructurally, which were deficient in MVA pathway enzymes. hmg1-1 and atipi1atipi2 showed a shrunken elaioplast inner granule at the bicellular pollen stage. Conversely, in the cla1-1 mutant, which showed a defective MEP pathway, elaioplast development was normal. The pollen of hmg1-1 and atipi1atipi2 was coatless, whereas cla1-1 had a pollen coat. These results indicate that the MVA pathway but not the MEP pathway is critical for elaioplast development though the organelle is derived from plastids.

Key words: gametophyte, isoprenoid, sterol, tapetosome, tapetum.

A mature male gametophyte is covered by exine and a pollen coat. The main component of exine is a sporopollenin polymer that comprises lipids and phenolic derivatives. Exine guards pollen grains chemically and physically (Piffanelli et al. 1998). Conversely, the pollen coat mainly comprises lipids and protein and it has many properties, such as attaching to vector insects to facilitate pollen transmission (Piffanelli et al. 1998), attaching to the dry stigmatic surface to start pollen hydration (Hülskamp et al. 1995; Preuss et al. 1993), and self-incompatibility (Shiba et al. 2001).

The pollen coat is derived from tapetum cells at the innermost cell layer of the anther wall, which contains the elaioplast and tepetosome (Hernández-Pinzón et al. 1999). The elaioplast is an organelle derived from plastids, and the tapetosome is an organelle derived from the endoplasmic reticulum (ER). In Arabidopsis, these organelles develop in tapetum cells when a male gametophyte is in the bicellular pollen stage (Quilichini et al. 2014). In the tricellular pollen stage, tapetum cells disappear and a pollen coat forms at the microspore surface. It is considered that the pollen coat contains sterol ester as the major natural lipid and contains phospholipid as a polar lipid. Various sterol types exist in the pollen of different plant species (Villette et al. 2015). Lipid profiles of tapetum organelles are very different between the elaioplast and tapetosome. In a previous report, the major natural lipids of elaioplasts were identified as sterol ester and wax ester, while that of the tapetosome was triacylglycerol in Brassica napus (Hernández-Pinzón et al. 1999). Arabidopsis knock-down mutants of acetoacetyl CoA synthase (AACT2) (Jin et al. 2012) and HMG-CoA synthase (HMGS) (Ishiguro et al. 2010) also show a pollen coatless phenotype and male sterility. AACT2 and HMGS are biosynthetic enzymes up-stream of sterol biosynthesis.

Sterol is a kind of isoprenoid. Isoprenoid compounds comprise isopentenyl diphosphate (IPP)
The MVA pathway is critical for elaioplast development. The elaioplasts are derived from plastids (Hernández-Pinzón et al. 2002; Nagata et al. 2002), although sterol is known as the cytosolic MVA pathway derivative (Newman and Chappell 1999), although sterol is known as the cytosolic MVA pathway derivative (Newman and Chappell 1999).

Sterol accumulation in elaioplasts is strange, and the mechanism is unclear, differences in the genetic lines between them may cause this phenotypic variation.

In anthers, HMG1 was expressed in the tapetum and microspores, while the expression of HMG2 appeared only in microspores (Suzuki et al. 2009). The hmg1-1 mutant showed an inhibition of pollen germination or pollen tube elongation (Suzuki et al. 2004). Besides the RNAi mutant of AACT2, hmgfs/fkp1 mutant, and hmg1 mutant (Ishiguro et al. 2010; Jin et al. 2012; Suzuki et al. 2004), the atipi1atipi2 mutant (Okada et al. 2008) has been isolated as a male sterile mutant, which is defective in the MVA pathway. Isopentenyl pyrophosphate isomerase (IPI) catalyzes the isomerization reaction from IPP to DMAPP. Two genes encoding IPI, IPI1 and IPI2, exist in the Arabidopsis genome. DMAPP is necessary for isoprenoid biosynthesis as an enzyme substrate, such as farnesyl diphosphate synthase and geranylgeranyl diphosphate synthase.

clal-1 is a null mutant of the first enzyme of the MEP pathway, 1-deoxy-D-xylulose-5-phosphate synthase 1 (Estévez et al. 2000). In this mutant, the accumulation of chlorophyll and carotenoids is totally inhibited in the chloroplast and this mutant shows an albino phenotype (Mandel et al. 1996). In this study, we investigated the role of the MEP pathway on elaioplast development using Arabidopsis flowers of clal-1. We observed the developmental stage of microspores in these mutants and demonstrated the contribution of the MVA pathway but not the MEP pathway on elaioplast development.

We observed and compared the ultrastructure of anthers between wild type (WT) and male sterile mutants of the MVA pathway, hmg1-1 and atipi1atipi2. In the bicellular pollen stage, electron-dense granules in the elaioplast were shrunken in hmg1-1 and atipi1atipi2 (Figure 2A–H). The area of high electron density in the elaioplast were shrunken in hmg1-1 and atipi1atipi2 and atipi1atipi2 hmg1-1 mutants of the MV A pathway, the RNAi mutant of AACT2, hmgfs/fkp1 mutant, and hmg1 mutant (Ishiguro et al. 2010; Jin et al. 2012; Suzuki et al. 2004), the atipi1atipi2 mutant (Okada et al. 2008) has been isolated as a male sterile mutant, which is defective in the MVA pathway. Isopentenyl pyrophosphate isomerase (IPI) catalyzes the isomerization reaction from IPP to DMAPP. Two genes encoding IPI, IPI1 and IPI2, exist in the Arabidopsis genome. DMAPP is necessary for isoprenoid biosynthesis as an enzyme substrate, such as farnesyl diphosphate synthase and geranylgeranyl diphosphate synthase.

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viable probably because 7% WT IPI activity remained in atipi1atipi2 (Okada et al. 2008). However, we demonstrated that 7% WT IPI activity in atipi1atipi2 may be insufficient for normal differentiation of the elaioplast and formation of the pollen coat. These results were also consistent with the results of hmg1-1 (Ishiguro et al. 2010).

The tapetosome also showed an abnormal phenotype in hmg1-1, atipi1atipi2 (Figure 2A–H), and hmg1-1 (B, D, I, L). These results suggest that the cytosolic MVA pathway contributed to the formation of a pollen coat, elaioplast, and tapetosome. A sterol-deficient mutant, such as defective mutants of the MVA pathway, may also show a pollen coatless phenotype because sterol ester, the main component of a pollen coat, was decreased.

The next question is the importance of the products from the MEP pathway in the elaioplast. To investigate the contribution of MEP pathway to elaioplast development, we performed an electron microscopic analysis of the microspore in cla1-1. Most cla1-1 homozygous mutants showed a lethal seedling phenotype when grown on Murashige and Skoog (MS) agar. However, when the seedlings were transferred to 1/2 MS agar supplemented with 1.5% sucrose in a large agripot, only some that grew to the flower stage were obtained (Figure 3A–D). Mature cla1-1 plants showed a sterile phenotype wherein the primary shoot terminated at some buds. At the bicellular pollen stage, the elaioplast and tapetosome of cla1-1 were comparable to that of WT (Figure 2A, C, I, K, and Figure 3E, F). In the tricellular pollen stage, the pollen coat formed normally (Figure 2I, K, and Figure 3G, H), although almost all gametophytes had abnormal vacuoles (Figure 3G) or were crushed in cla1-1 (data not shown). At least the male gametophytes were indistinguishable between WT and cla1-1 at bicellular pollen stage, while normal development may be prevented thereafter in cla1-1 (Supplementary Figure). From these results, we considered that the sterile cla1-1 phenotype may have been caused by an abnormal gametophyte.

These results suggest that the MEP pathway, unlike the
The MV A pathway is critical for elaioplast or pollen coat. In the tricellular pollen stage, there were many abnormal gametophytes in the clal-1 mutant. This result indicated that the clal-1 mutant had no ability for normal pollen development after the tricellular pollen stage, suggesting that the MEP pathway is important in the formation of the gametophyte but not of the elaioplast.

It has been reported that sterol ester accumulates in the elaioplast in *B. napus* (Hernández-Pinzón et al. 1999). The abnormal elaioplast, pollen coatless microspores, and male sterility phenotypes were observed not only in *hmg5/kpl1-1* (Ishiguro et al. 2010) but also in both *hmg1-1* and *atipi1atipi2* in this study. Our results strongly support the previous hypothesis that sterol ester in the elaioplast is derived from the cytosolic MVA pathway, even though the elaioplast is derived from plasts. Two possibilities have been proposed: (1) MVA and sterol biosynthesis pathways localize in a special organelle, which is the elaioplast and (2) sterols are produced in cytosol and then transported to the elaioplast. The possibility of (1) may be low as there are more than 11 steps in sterol synthesis from acetyl CoA (Suzuki and Muranaka 2007). Although it is necessary that enzymes with more than 11 synthesis steps are imported to the elaioplast, these 11-step enzymes do not have a plastid-transfer signal. On the contrary, it was reported that ER covered the proplastid in the process of formation of elaioplast from proplastid in *Olea europaea* (Pacini and Casadoro 1981). It is likely that sterols were transported to the elaioplast from ER. The trafficking of intermediary isoprenoid metabolites between plastids and cytosol has been reported (Hemmerlin et al. 2003; Kasahara et al. 2002; Nagata et al. 2002). Elucidation of the mechanisms how MVA pathway products accumulate in plastids may reveal the developmental mechanisms of the elaioplast in pollen growth stages.

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**Appendix**

**Materials and methods**

**Plant growth condition**

Seeds were sown on 1/2 MS medium (Wako Pure Chemical Industries, Ltd., Japan) supplemented with 1.5% (w/v) sucrose and stored at 4°C for more than 2 day. After vernalization, WT (WS, Col-0), *hmg1-1* (Suzuki et al. 2004), and *atipi1atipi2* (Okada et al. 2008) plants were grown for two weeks on 1/2 MS agar medium and then transferred to soil. *clal-1* (Mandel et al. 1996) plants were grown for two weeks on 1/2 MS agar and then transferred to 1/2 MS agar in an agripot. These plants were cultured for 40–50 day. All growth occurred under a 16:8 h light:dark cycle at 23°C in a growth chamber.

**Electron microscopic analysis**

For transmission electron microscopy, the samples were fixed with 4% glutaraldehyde and 4% paraformaldehyde in a 20 mM sodium cacodylate buffer, pH 7.0, at 4°C overnight. They were then washed with the same buffer for 4h at 4°C. Then, they were post-fixed with 2% OsO₄ in a 20 mM sodium cacodylate buffer at 4°C overnight. The fixed samples were run through an alcohol series and embedded in Spurr resin. Ultra-thin sections (70 nm thick) were cut with a diamond knife on an ULTRACUT E ultra-microtome (Leica, Wien, Austria) and transferred to Formvar-coated grids. They were double-stained with 4% (v/v) uranyl acetate for 15 min and with lead citrate solution for 7 min. After washing with distilled water, the samples were observed using a JEM-1200 EX transmission electron microscope (Jeol, Tokyo, Japan).

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