Fertile *Arabidopsis cyp704b1* mutant, defective in sporopollenin biosynthesis, has a normal pollen coat and lipidic organelles in the tapetum

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Abstract  The exine acts as a protectant of the pollen from environmental stresses, and the pollen coat plays an important role in the attachment and recognition of the pollen to the stigma. The pollen coat is made of lipidic organelles in the tapetum. The pollen coat is necessary for fertility, as pollen coat-less mutants, such as those deficient in sterol biosynthesis, show severe male sterility. In contrast, the exine is made of sporopollenin precursors that are biosynthesized in the tapetum. Some mutants involved in sporopollenin biosynthesis lose the exine but show the fertile phenotype. One of these mutants, *cyp704b1*, was reported to lose not only the exine but also the pollen coat. To identify the cause of the fertile phenotype of the *cyp704b1* mutant, the detailed structures of the tapetum tissue and pollen surface in the mutant were analyzed. As a result, the *cyp704b1* mutant completely lost the normal exine but had high-electron-density granules localized where the exine should be present. Furthermore, normal lipidic organelles in the tapetum and pollen coat embedded between high-electron-density granules on the pollen surface were observed, unlike in a previous report, and the pollen coat was attached to the stigma. Therefore, the pollen coat is necessary for fertility, and the structure that functions like the exine, such as high-electron-density granules, on the pollen surface may play important roles in retaining the pollen coat in the *cyp704b1* mutant.

Key words: exine, pollen coat, sporopollenin, tapetum.

Introduction

The pollen surface is coated by the complex structure of the pollen wall. The pollen wall is composed of a mesh-like exine, the pollen coat in the crevices of the exine, and the intine derived from the cell wall in the innermost layer of the pollen wall. The pollen coat is involved in the attachment of insects for pollination (Piffanelli et al. 1998), attachment of the pollen to the stigma after hydration of the pollen (Hülskamp et al. 1995; Preuss et al. 1993), and self-incompatibility (Shiba et al. 2001). The exine acts as the protectant of the pollen from environmental stresses (Kim and Douglas 2013).

The exine is commonly divided into the outer sexine layer and inner nexine layer. The sexine layer, composed of the baculum and tectum, forms the characteristic mesh-like structure at the top of the nexine layer. In the tetrad stage, the primexine is formed by cellulose derived from microspores. Exine constituents are secreted by the tapetum, which is located on the innermost layer of the anther, into the anther locule, and deposited on the primexine (Kim and Douglas 2013). In the late tetrad stage, probaculae and protecta can be distinguished by transmission electron microscopy (TEM; Quilichini et al. 2014a). In the early uninucleate stage, microspores exhibit an exine that consists of baculae and tecta. In the bicellular pollen stage, characteristic lipidic organelles develop in the tapetum. These organelles mainly fill the tapetum in the late bicellular and early tricellular pollen stages (Quilichini et al. 2014a). In the tricellular pollen stage, the tapetum degrades, and the components of the cell are released in the anther locule. The components are deposited at the crevices of the mesh-like structure of the exine and form the pollen coat (Kim and Douglas 2013; Piffanelli et al. 1998; Quilichini et al. 2014a).

The pollen coat is rich in lipids and proteins. These components are produced and accumulate in characteristic lipidic organelles in the tapetum, namely...
the tapetosome and elaioplast. In *Brassica napus*, the tapetosome contains mainly triacylglycerols and wax esters, and the elaioplast contains sterol esters (Hernández-Pinzón et al. 1999; Wu et al. 1997, 1999). Sterol esters are one of the major components of the pollen coat (Hernández-Pinzón et al. 1999; Wu et al. 1999). Mutants defective in sterol biosynthesis show pollen coat-less phenotype and male sterility (Ishiguro et al. 2010; Jin et al. 2012; Kobayashi et al. 2018). These mutants also show abnormal phenotypes in the tapetosome and elaioplast (Ishiguro et al. 2010; Kobayashi et al. 2018). Furthermore, mutants deficient in pollen coat wax have dysfunctional pollen coat and abnormal lipidic organelles in the tapetum and show conditional male sterility (Aarts et al. 1995; Fiebig et al. 2000; Hülskamp et al. 1995; Jessen et al. 2011; Kobayashi et al. 2019; Preuss et al. 1993). These studies suggested that the pollen coat is necessary for male fertility.

The exine is composed of sporopollenin, a robust biopolymer. The sporopollenin is composed of a highly cross-linked polymer of hydroxylated fatty acids, aliphatic compounds, and, possibly, phenolics (Wang and Dobritsa 2018). Fatty acid modification steps and phenylpropanoid pathway are involved in sporopollenin biosynthesis (Quilichini et al. 2014b; Xue et al. 2020; Figure 1). The precursors of sporopollenin are biosynthesized in the tapetum, transported to the anther locule, and incorporated into the exine by polymerization on the pollen surface (Kim and Douglas 2013; Piffanelli et al. 1998). In *Arabidopsis*, mutants defective in the biosynthesis of sporopollenin precursors derived from fatty acids [acos5 (de Azevedo Souza et al. 2009), pksa/lap6 and pksb/lap5 (Dobritsa et al. 2010; Kim et al. 2010), cyp703a2 (Morant et al. 2007), cyp704b1 (Dobritsa et al. 2009), ms2 (Aarts et al. 1997; Chen et al. 2011), and tkpr1/drl1 (Grienenberger et al. 2010; Tang et al. 2009); Figure 1] have been isolated. These mutants are classified into two phenotypes. The first type shows complete male sterility, owing to microspore degradation [acos5 (de Azevedo Souza et al. 2009), pksa-1 pksb-3/lap5-1 lap6-1 double mutants (Dobritsa et al. 2010; Kim et al. 2010), and tkpr1-1/drl1-2 (Grienenberger et al. 2010; Tang et al. 2009)]. The other type is fertile [cyp703a2 (Morant et al. 2007) and cyp704b1 and ms2 (Dobritsa et al. 2009)]. The exine of these mutants commonly is defective as observed by TEM and scanning.
electron microscopy (SEM; Aarts et al. 1997; Chen et al. 2011; de Azevedo Souza et al. 2009; Dobritsa et al. 2009; Grienenberger et al. 2010; Kim et al. 2010; Morant et al. 2007). The fertile mutants (cyp703a2, cyp704b1, and ms2) have been reported to lack the pollen coat and the exine (Dobritsa et al. 2009; Morant et al. 2007). In contrast, the pollen coat-less mutants deficient in sterol biosynthesis showed severe male sterility (Ishiguro et al. 2010; Jin et al. 2012; Kobayashi et al. 2018). Although the mutants defective in the biosynthesis of sporopollenin precursors derived from fatty acids lack both the exine and the pollen coat, the mechanism of the fertile phenotype of these some mutants remains to be solved.

In a previous study, CYP704B1, which encodes an enzyme catalyzing the ω-hydroxylation of long-chain fatty acids, was reported to be the gene responsible for the mutation of the lack of a normal exine (Dobritsa et al. 2009). cyp704b1 showed a fertile phenotype, like wild-type (WT), although the pollen wall of this mutant was completely missing and replaced by a very thin layer of electron-dense material (Dobritsa et al. 2009). Here the detailed structures of the tapetum tissue and pollen surface in cyp704b1 were analyzed to identify the cause of the fertile phenotype of the mutant.

Materials and methods

Plant materials and growth conditions

WT (Col-0) and cyp704b1 (SAIL_1149_B03) seeds were sown on 1/2 MS agar medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 1.5% (w/v) sucrose and stored at 4°C for more than 2 days. After vernalization, plants were grown for 7 to 10 days on 1/2 MS agar medium and then transferred to soil and cultured until the flowering stage. All growth occurred under a 16 h light/8 h dark cycle at 23°C in a growth chamber.

Pollen adhesion assay

The fully matured stigma of the flower, eliminating the anther, was touched with the anther isolated from the fully opened flowers of WT and cyp704b1 plant. Then, 15 to 30 min after pollination, the pistil was harvested and fixed for TEM or SEM.

Microscopic analyses

TEM analysis was performed as described previously (Kobayashi et al. 2018). Briefly, inflorescences were soaked in 4% paraformaldehyde, and the anther or pollinated stigma samples were cut from the inflorescence. The samples were fixed with 4% glutaraldehyde and 4% paraformaldehyde and then postfixed with 2% OsO4 for 1 h. The fixed samples were run through an ethanol series and a t-butyl alcohol series. The samples were lyophilized, coated with platinum, and observed under an S-800 SEM (Jeol).

Results

cyp704b1 mutant has normal lipidic organelles in the tapetum and a pollen coat-like structure on the pollen surface

The pollen coat and tapetum of WT and cyp704b1 were observed in detail throughout the pollen developmental stages using TEM. The lower-magnification images are shown in Figure 2, and the higher-magnification images were embedded in Spurr resin, sectioned at 70 nm thickness, double stained with 4% (v/v) uranyl acetate and lead citrate, and observed under a JEM-1200 EX TEM (Jeol, Tokyo, Japan). At least three or more anthers were observed at each pollen developmental stage.

For light microscopy analysis, the samples prepared for TEM analysis were sectioned at 1 μm thickness, stained with toluidine blue solution, and observed.

For SEM analysis of pollen grains with an unfixed condition, the pollen grains were directly mounted on the specimen stubs using carbon tape and coated with platinum using a sputter coater. For SEM analysis of the pollen coat and pollen adhesion to stigma, the experimental procedure was performed as described previously (Kobayashi et al. 2019). Briefly, the mature anther or pollinated stigma samples were cut from the inflorescence and soaked in 2% glutaraldehyde. The samples were fixed with 2% glutaraldehyde for 2 h and then postfixed with 2% OsO4 for 1 h. The fixed samples were run through an ethanol series and a t-butyl alcohol series. The samples were lyophilized, coated with platinum, and observed under an S-800 SEM (Jeol).

Figure 2. TEM images of the developmental anther in WT (A–D) and cyp704b1 mutant (E–H). Male gametophyte development in the tetrad stage (A, E), early bicellular pollen stage (B, F), early tricellular pollen stage (C, G), and mature pollen stage (D, H). el, elaioplast; p, pollen; pc, pollen coat; pcl, pollen coat-like; t, tapetum; ta, tapetosome; tet, tetrad. Arrowheads, abnormal high-electron-density granules localized along the tapetal cell wall; arrows, abnormal high-electron-density granules localized on the pollen surface. Scale bar, 5 μm.
Exine-defective *cyp704b1* mutant has a functional pollen coat

Images are shown in Figure 3. In the tetrad stage, the smooth surface of microspores was shown in *cyp704b1* (Figure 2E), whereas the probaculum was shown on the WT microspore surface (Figure 2A). In the early bicellular pollen stage, the sexine layer was formed in WT microspores, and abnormal high-electron-density granules attached to the pollen surface of almost all microspores in *cyp704b1* (Figures 2B, F, 3B, G, Supplementary Figure S1A, C). Abnormal high-electron-density granules were shown not only at the pollen surface but also along the tapetal cell wall adjacent to the middle layer in *cyp704b1* from the early bicellular to the early tricellular pollen stage (Figures 2B, C, F, G, I, Supplementary Figure S1A, C). Similar results were obtained from at least three blocks that were observed. In the early bicellular and early tricellular pollen stages, the development of lipidic organelles, tapetosome, and elaioplast in the tapetum of *cyp704b1* was indistinguishable from that of WT (Figures 2B, C, F, G, 3A, C, F, H). In the mature pollen stage, the pollen coat was deposited on the pollen surface in WT (Figures 2D, 3E). In contrast, the pollen coat-like structure, the layer with a similar electron density to the WT pollen coat, was partially observed between abnormal high-electron-density granules on the pollen surface in *cyp704b1* (Figures 2H, 3I). Almost all pollens were evenly covered with exine and pollen coat in WT, but the pollens were partially covered with abnormal high-electron-density granules and pollen coat-like structure in *cyp704b1* (Supplementary Figure S1B, D). A structural difference was scarcely observed inside the microspores between WT and *cyp704b1* throughout the stages examined (Figure 2).

It is well known that the pollen coat is lost, and a hard structure, such as exine, can be noticed when the pollen is observed using SEM without fixation. To obtain information on abnormal high-electron-density granules, the pollen surface of WT and *cyp704b1* was observed using SEM without fixation. As a result, the mesh-like exine structure was observed in WT (Figure 4A). In contrast, the granular structure was partially observed, although the mesh-like exine structure was completely lacking in the collapsed *cyp704b1* (Figure 4D, E). Recently, a method was developed to observe the pollen coat surface by SEM after suitable fixation (Kobayashi et al. 2019). To observe the pollen coat, the pollen of WT and *cyp704b1* was analyzed using this method. In WT, the pollen coat filled the crevices of the mesh-like structure of the exine (Figure 4B, C). In contrast, a pollen coat-like structure covered the *cyp704b1* pollen surface entirely (Figure 4F, H) or partially (Figure 4G, I). The granule structure shown in Figure 4E was also observed on the *cyp704b1* pollen surface after fixation (Figure 4G, I).

**cyp704b1 pollen with a pollen coat-like structure adheres to the stigmatic papilla, like the WT pollen**

To investigate whether the pollen coat-like structure of *cyp704b1* functions normally, pollen adhesion was observed on the stigma. Mature pollen from the WT and *cyp704b1* anther were placed on unpollinated mature stigmas. Then, the stigmas were observed by microscopy after appropriate incubation.

As a result, the *cyp704b1* pollen adhered to the stigmatic papilla, like the WT pollen (Figure 5A, D). TEM analysis demonstrated the details of the adhesive surface between the pollen and the papilla. The pollen coat-like structure of *cyp704b1* was located on the adhesive surface between the pollen and the papilla, like the WT pollen coat (Figure 5B, C, E, F). Furthermore,
the adhesive surface between the pollen and the papilla was examined by SEM. The cyp704b1 pollen adhered to the papilla (Figure 6A, B, D, E), and the pollen tube was elongated from the cyp704b1 pollen to the papilla, like the WT pollen (Figure 6C, F).

**Discussion**

**Exine was defective in the cyp704b1 pollen**

CYP704B1 is involved in the biosynthesis of sporopollenin, a component of the exine. In a previous study, the cyp704b1 pollen lost the exine (Dobritsa et al. 2009). In this study, a TEM analysis of the anthers of the developmental stages of the pollen was performed. No normal exine formation was observed in the cyp704b1 pollen in all observation stages (Figures 2, 3B, D, E, G, I, J). These results were consistent with the data of the cyp704b1 pollen by Dobritsa et al. (2009). They also reported that the pollen wall of the cyp704b1 mutant was completely missing and replaced by a very thin layer of electron-dense material. However, high-electron-density granules were localized where the exine should be present (Figures 2B–H, 3B, D, E, G, I, J). Low-magnification observations and pollen surface observations showed that these high-electron-density granules attached to the pollen surface of almost all microspores in cyp704b1 but did not evenly cover the entire pollen, unlike WT exines (Figure 4, Supplementary Figure S1). Similar results were obtained from at least three blocks that were observed. The mechanisms demonstrating that the distribution of high-electron-density granules on the pollen surface is disproportioned are unsolved. Dobritsa et al. reported the images of cyp704b1 pollen grains stained with auramine O, which can bind specifically to exine. In the images of the pollen grains stained with auramine O of cyp704b1, there was a zebra pattern structure clearly different from the mesh-like structure in the WT despite the complete lack of the exine structure in the SEM and TEM analyses (Dobritsa et al. 2009). It was considered that the high-electron-density granules in our study might be identical to the structure stained by auramine O as reported by Dobritsa et al.

**Pollen coat was retained in the cyp704b1 pollen**

Mutants defective in the biosynthesis of the major lipidic components of the pollen coat, such as sterols or waxes, lost their pollen coat (Ishiguro et al. 2010; Jin et al. 2012; Kobayashi et al. 2018) or had abnormal pollen coat (Kobayashi et al. 2019). These mutants also showed abnormal development of lipidic organelles, the source of the pollen coat, in the tapetum (Ishiguro et al. 2010; Kobayashi et al. 2018, 2019). Although cyp704b1 was reported to be a pollen coat-less mutant, it showed normal development of these organelles, indistinguishable from WT (Figure 3A, C, F, H). Surprisingly, the pollen coat-like structure was observed in the cyp704b1 mutant (Figures 3E, J, 4B, C, F, H). This structure was embedded between high-electron-density granules on the pollen surface (Figures 3E, J, 4G, I) and was functional like the WT pollen coat (Figures 5, 6).
According to these results, the pollen coat-like structure of the cyp704b1 mutant is the pollen coat.

TEM images, which showed that the pollen wall of the cyp704b1 mutant was completely lost and replaced by a very thin layer of electron-dense material, were reported previously (Dobritsa et al. 2009). In this study, TEM images showed that the pollen coat between high-electron-density granules existed in the pollen surface of the cyp704b1 mutant (Figure 3E, J). The differences between the TEM images of the pollen wall of the cyp704b1 mutant may be caused by the differences in dehydration or growth conditions. Dobritsa et al. used acetone at the final step of dehydration, whereas propylene oxide was used at the same step in this study. The reason for the fertile phenotype of the cyp704b1 mutant is that the mutant pollen has a partially functional pollen coat.

It is known that when the pollen grains of exine-deficient mutants are analyzed by SEM, the unfixed pollen grain samples are easy to collapse by physical stimuli like in the vacuum condition (Figure 4D; Chen et al. 2011; Dobritsa et al. 2009; Grienenberger et al. 2010; Kim et al. 2010). In contrast, when pollen grains fixed with glutaraldehyde and paraformaldehyde are analyzed by SEM, the pollen grains of exine-deficient mutants can be observed without collapse (Dobritsa et al. 2009; Morant et al. 2007). However, no pollen coat was observed, although the pollen grains were fixed with glutaraldehyde and paraformaldehyde. The surface of the pollen coat was observed when the pollen grains were analyzed by SEM after two steps of fixation: the first step is the fixation of glutaraldehyde and the second step is the short-time fixation of OsO4 (Figure 4B, C, F–I; Kobayashi et al. 2019). These two fixation steps may help observe the pollen surface retaining the structure of the pollen wall by SEM.

Abnormal high-electron-density granules observed in the anther of the cyp704b1 mutant

In cyp704b1, abnormal high-electron-density granules were shown on the pollen surface and tapetum, especially on the edge of the cells adjacent to the middle layer (Figures 2F–H, 3G, I, J, Supplementary Figure S1). These granules may be composed of the polymerization and aggregation of abnormal intermembrane metabolites of sporopollenin precursors. Other mutants [nef1 (Ariizumi et al. 2004), abcg26 (Choi et al. 2011), and tde1/det2 (Ariizumi et al. 2008)] with a defective phenotype of the exine were reported to have abnormal high-electron-density granules at the edge of the tapetum adjacent to the middle layer. Some mutants (acos5, pksa pksb, and tkpr1) defective in the biosynthesis of sporopollenin precursors derived from fatty acids showed abnormal granules containing small fibrillar electron-dense core on the surface of the tapetum and microspores (Quilichini et al. 2014b). The localization of these abnormal granules was similar to that observed in the cyp704b1 mutant in this study. It cannot be asserted that these granules were identical structures because the observation and fixation conditions were different between the studies. However, in exine-defective mutants, abnormal granules were often observed in the tapetum and anther locule. A detailed study of these granules may reveal the transport mechanisms of the products for exine formation.

cyp704b1 mutant shows a fertile phenotype, unlike acos5

Mutants of exine formation, which are defective in the biosynthesis of sporopollenin precursors derived from fatty acids, can be classified into two types by the degree of male sterile phenotype. One type is the complete male sterile phenotype owing to the degradation of pollen such as acos5. The other is fertile, such as cyp704b1, although this fertile phenotype of mutants completely lost their pollen wall. However, in this study, the reason for the fertility of the cyp704b1 mutant is the retaining of the functional pollen coat in the mutant pollen. The high-electron-density granules on the cyp704b1 pollen surface may play a supporting role in retaining the pollen coat. CYP703A2, like CYP704B1, plays a role in the hydroxylation step of fatty acids in sporopollenin biosynthesis, and the mutant of this gene is the fertile phenotype, like cyp704b1. CYP703A2 catalyzes the in-chain hydroxylation of C10-14 fatty acids, such as lauric acid (Morant et al. 2007), whereas CYP704B1 catalyzes the ω-hydroxylation of long-chain fatty acids (Dobritsa et al. 2009). The apparent phenotype of double mutants, which is completely defective in these CYPs, is indistinguishable from single mutants (Dobritsa et al. 2009). In Arabidopsis, even without CYP703A2 and CYP704B1, the part of sporopollenin precursors may be biosynthesized to produce the structure that functions like the exine, such as high-electron-density granules on the pollen surface. In mutants of the complete male sterile phenotype [acos5 (de Azevedo Souza et al. 2009), pksa-1 pksb-3/lap5-1 lap6-1 double mutants (Dobritsia et al. 2010; Kim et al. 2010), and drl1-2 (Tang et al. 2009)], pollens are degraded in almost all anther locules of these mutants before pollen maturation. Because the genes responsible for the complete male sterile phenotype mutation may play a more crucial role for sporopollenin biosynthesis and exine formation than the genes responsible for the fertile mutants, the sterile mutants cannot keep their pollen shape. Considering the putative biosynthesis pathway of sporopollenin precursors (Figure 1), in acos5 mutants, the biosynthesis for sporopollenin precursors derived from fatty acids was completely stopped because fatty acyl-CoA esters were not synthesized by ACOS5 in the mutant. In contrast, in cyp704b1 and cyp703a2 mutants, the biosynthesis
pathway of sporopollenin precursors derived from fatty acids was not completely stopped because ACOS5 could catalyze the non-hydroxylated substrate (de Azevedo Souza et al. 2009), and the non-hydroxylated fatty acyl-CoA esters should be synthesized in these mutants. In proPpACOS6:ACOS5 transgenic Arabidopsis acos5 plants, which introduced the ACOS5 driven by the moss Physcomitrella promoter of the ortholog gene of ACOS5 into acos5, the round-shaped particles, similar to high-electron-density granules, were reported around the pollen. The viability and fertility of the pollen of these transgenic plants were weakly recovered (Li et al. 2019).

This report suggested that abnormal sporopollenin derived from insufficient fatty acyl-CoA esters form the round-shaped particles instead of the exine. Even if sporopollenin precursors that are not hydroxylated by CYP704B1 or CYP703A2 are difficult to polymerize, a structure, such as high-electron-density granules on the pollen surface, as observed in the cyp704b1 mutant, may be present in the cyp703a2 mutant as well as in the cyp704b1 mutant. The zebra pattern structures stained by auramine O were observed not only in the cyp704b1 pollen surface but also in that of cyp703a2 (Dobritsa et al. 2009). From these previous reports and these data, it was considered that the high-electron-density granules around the pollen are exine-like structures composed of insufficient sporopollenin precursor. Therefore, the pollen coat is necessary for fertility, and the structure that functions like the exine, such as high-electron-density granules, on the pollen surface may play important roles in retaining the pollen coat.

**Importance of hydroxylation by CYP704B1 is different in exine formation in higher plants**

Oryza sativa mutant for CYP704B2 (Li et al. 2010), B. napus S45A mutant for BnMS1 and BnMS2 (Yi et al. 2010), and Triticum aestivum triple mutant for Ms26s (Singh et al. 2017), which are mutants of the ortholog genes of CYP704B1, showed the complete male sterile phenotype. Arabidopsis cyp704b1 mutant showed a fertile phenotype (Dobritsa et al. 2009). These indicated that the importance of CYP704B, which catalyzes the hydroxylation step in sporopollenin biosynthesis, in Arabidopsis is different in other plants. Even if the structure, such as high-electron-density granules, on the pollen surface, as shown in Arabidopsis cyp704b1 mutant, also exists in rice cyp704b2, the structure may be insufficient for the maturation of the functional pollen, as the cyp704b2 pollen was collapsed without an obvious exine on its outer surface (Li et al. 2010). We hypothesize that higher plants have a quality-control system to remove the abnormal pollen with a defective exine. We consider that pollen with high-electron-density granules on the pollen surface can pass the system in Arabidopsis but may be degraded by the system in rice. Because rice and wheat partially perform wind pollination and rapeseed partially performs insect pollination, whereas Arabidopsis performs self-pollination completely, the quality-control system for pollen may be stricter in rice, wheat, and rapeseed than in Arabidopsis.

CYP704B evolved with land colonization and is highly conserved in higher plants (Dobritsa et al. 2009). A detailed observation of fertility and pollen development in cyp704b ortholog mutants of various higher plant species may clarify how the importance of the CYP704B in sporopollenin biosynthesis has changed with evolution.

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