WBC27, an Adenosine Tri-phosphate-binding Cassette Protein, Controls Pollen Wall Formation and Patterning in Arabidopsis

Xiao-Ying Dou1, Ke-Zhen Yang1, Yi Zhang2, Wei Wang1, Xiao-Lei Liu1, Li-Qun Chen1, Xue-Qin Zhang1 and De Ye1∗

1State Key Laboratory of Plant Physiology and Biochemistry, College of Biological Sciences, China Agricultural University, Beijing 100193, China
2Key Laboratory of Biotechnology and Crop Quality Improvement, Ministry of Agriculture, Southwest University, Chongqing 400716, China
∗Corresponding author
Tel(Fax): +86 10 6273 4839; E-mail: yede@cau.edu.cn
Available online on 18 November 2010 at www.jipb.net and www.wileyonlinelibrary.com/journal/jipb
doi: 10.1111/j.1744-7909.2010.01010.x

Abstract

In flowering plants, the exine components are derived from tapetum. Despite its importance to sexual plant reproduction, little is known about the translocation of exine materials from tapetum to developing microspores. Here we report functional characterization of the arabidopsis WBC27 gene. WBC27 encodes an adenosine tri-phosphate binding cassette (ABC) transporter and is expressed preferentially in tapetum. Mutation of WBC27 disrupted the exine formation. The wbc27 mutant microspores began to degenerate once released from tetrads and most of the microspores collapsed at the uninucleate stage. Only a small number of wbc27-1 microspores could develop into tricellular pollen grains. These survival pollen grains lacked exine and germinated in the anther before anthesis. All of these results suggest that the ABC transporter, WBC27 plays important roles in the formation of arabidopsis exine, possibly by translocation of lipidic precursors of sporopollenin from tapetum to developing microspores.


Introduction

In flowering plants, the male gametes are packed in a pollen grain with a special cell wall, usually called pollen wall. The pollen wall is composed of an intine layer and an exine layer (Heslop-Harrison 1971; Piffanelli et al. 1998). The intine is the inner layer of pollen wall, adjacent to pollen plasma membrane. It consists of cellulose, pectin, and various proteins produced by the microspore itself (Heslop-Harrison 1971). The exine is the outer layer of the pollen wall. It protects the pollen grain from various environmental harms and bacterial attacks, and plays a role in the recognition between pollen grain and female stigmatic cells (Zinkl et al. 1999). The exine is mainly composed of sporopollenin (Scott 1994) and usually decorated with a complex pattern of spines and ridges (Heslop-Harrison 1971; Piffanelli et al. 1998). The sporopollenin constituents are mainly derived from the tapetum. At the early stage of pollen development, the tapetum secretes lipidic precursors of sporopollenin to surfaces of microspores, which form a sculptured exine. At the later stages of pollen development, the tapetum deposits the lipidic components of pollen coat tryphine into the exine cavities (Pacini et al. 1985; Goldberg et al. 1993) to form a complete exine.

A number of genes have been identified to be involved in exine formation (Aarts et al. 1997; Paxson-Sowders et al. 2001; Ariizumi et al. 2003; Ariizumi et al. 2004; Morant et al. 2007; Guan et al. 2008; Zhang et al. 2008; de Azevedo Souza et al. 2009; Dobritsa et al. 2009; Li et al. 2010; Zhang et al. 2010). These genes can be classified into three types. The first type of the genes includes those that are involved in biosynthesis...
of exine materials. For example, *MALE STERILITY 2* (*MS2*) encodes a putative fatty acid reductase that is involved in sporopollenin biosynthesis, possibly by reducing very long chain fatty acids to fatty alcohol. Mutation in *MS2* led to a defect in exine formation and male sterility (*Aarts et al. 1997*). *faceless pollen-1* (*FLP1*) encodes a protein that is involved in biosynthesis of typhine, sporopollenin and wax. The *flp1* mutant had abnormal pollen grains with a distorted exine (*Ariizumi et al. 2003*). *CYP703*, belonging to P450 family, catalyzes in-chain hydroxylation of lauric acid, which forms building blocks for sporopollenin synthesis in pollen. The *Arabidopsis CYP703A2* knockout mutant lines showed abnormal pollen development and a partial male-sterile phenotype (*Morant et al. 2007*).

The second type of genes are those that are involved in deposition patterning of sporopollenin. For example, mutation in *DEFECTIVE IN EXINE FORMATION 1* (*DEX1*) that encodes a calcium-binding protein significantly delayed and reduced the deposition of primexine, leading to random deposition of sporopollenin on the plasma membrane. As a result, no normal exine was formed in the *dex1* pollen grains (*Paxson-Sowders et al. 2001*). The third type of gene may participate in translocation of exine materials from tapetal cells to microspores. *OsC6* in rice, which encodes a lipid transfer protein, plays a crucial role in the development of lipidic orbicules and pollen exine. Mutation of *OsC6* caused a defect in the development of orbicules, leading to reduction in male fertility (*Zhang et al. 2010*). Many genes that share homology with the transporter genes, which were found in other species, have been identified in the *arabidopsis* genome (www.arabidopsis.org). Thus far, however, none of them has been demonstrated to be actually involved in transport of exine materials from tapetal cells to microspores.

Adenosine tri-phosphate (ATP)-binding cassette (ABC) transporters are known to play roles in translocation of a broad range of substances across biological membranes by ATP hydrolysis (*Garcia et al. 2004*; *Jung et al. 2006*; *Sugiyama et al. 2006*; *Luo et al. 2007*; *Rea 2007*). Furthermore, several lines of evidence have indicated that the WBC/ABCG subfamily of ABC-transporter proteins was involved in lipid transport (*Sanchez-Fernandez et al. 2001*; *Chen et al. 2003*; *Xu et al. 2004*; *Wang et al. 2006*; *Rowland et al. 2007*). For example, knockout of *CERS/ABCG12* and *WBC11* from the WBC subfamily of *Arabidopsis* ABC transporter genes (*Sanchez-Fernandez et al. 2001*) decreased wax load in stems and accumulation of intracellular lipidic inclusion (*Bird et al. 2007*). Besides, studies have also shown that the members, *ABCG5*, *ABCG8* and *ABCG2* in human ABCG subfamily play roles in lipid transport (*Xu et al. 2004*; *Wang et al. 2006*). These studies implied that the WBC-transporters might participate in transport of lipidic precursors of sporopollenin from tapetum to microspores. However, which WBC-transporter is involved in lipids transport from tapetum to microspores remains unknown.

In this paper, we report the characterization of a mutation, *wbc27-1*, in ABC-transporter gene *WBC27*, belonging to the WBC/ABCG subfamily. *WBC27* is preferentially expressed in tapetal cells at the stage of pollen wall formation. Knockout of *WBC27* gene by T-DNA insertion disrupted the exine formation, leading to degeneration of pollen grains. Our results suggest that *WBC27* plays a role in exine formation, possibly by functioning as a lipid transporter that may be involved in the translocation of lipidic precursors of sporopollenin from tapetum to developing microspores.

**Results**

**Isolation of *wbc27-1* mutant**

We screened for the WBC/ABCG subfamily genes that are highly or specially expressed in anther by searching microarray databases (www.arabidopsis.org) (*Ito et al. 2007*; *Yang et al. 2007*; *Zhang et al. 2007*; *Zhu et al. 2008*). A candidate gene, *At3g13220* (*WBC27*) (**Figure 1A**), was identified prolifically expressed during early anther development. To characterize the biological functions of *WBC27*, we searched the T-DNA insertion mutants (*wbc27*) from Arabidopsis Biological Resource.
Center (ABRC) germplasm stock (http://www.arabidopsis.org). Three T-DNA insertion lines in WBC27 were obtained from ABRC. Only one line called SALK_062317 was confirmed as actually having T-DNA insertion in WBC27. Sequencing of the flanking sequences confirmed that the T-DNA was inserted in the fifth exon of WBC27 (Figure 1A). It was named as wbc27-1. Real-time polymerase chain reaction (PCR) analysis showed that the T-DNA insertion had knocked out the expression of WBC27 (Figure 1B). The plants homozygous for wbc27-1 mutation were male sterile (Figure 2B). We examined the pollen grains in wbc27-1 and wild type mature anthers by Alexander staining (Alexander 1969). A wild-type anther could produce a large number of viable pollen grains (Figure 2G), while only few viable pollen grains were found in a wbc27-1 anther (Figure 2H). No significant abnormality in other vegetative parts was found in wbc27-1. To determine whether wbc27-1 mutation affected female gametophytic function, the wbc27-1 homozygous plants were used as female to cross with wild type plants. The wbc27-1 homozygous pistils pollinated with wild type pollen grains all gave rise to full seed set, indicating that wbc27-1 mutation did not affect female gametophytic functions. The resulting F1 plants were fully fertile. In F2 generation, the plants segregated in a ratio of 3 (152) fertile to 1 sterile (49). Among the 152 fertile plants, 50 plants had no T-DNA insertion and 102 plants had T-DNA insertion in WBC27, as revealed by PCR. In F3 generation, the plants without T-DNA insertion all were fertile, while those with T-DNA insertions segregated in a ratio of 2 fertile to 1 sterile. All these results indicated that wcb27-1 was a male sterile mutant generated by a single-locus of recessive mutation and tightly linked to the T-DNA insertion in WBC27 gene.

To determine that the wbc27-1 phenotype was caused by the insertion of T-DNA in WBC27, a 4 318-bp genomic DNA fragment of At3g13220 (WBC27), including the promoter region (1 034 bp) upstream of the start codon ATG, encoding region, and transcription terminal region (392 bp), was cloned into a Ti-plasmid vector pCAM1300. The resulting complementation construct was introduced into wbc27-1 heterozygous plants (wbc27-1/+ ) using an Agrobacterium-mediated infiltration method (Clough and Bent 1998). In total, we obtained 165 independent T1 transformants. PCR assays confirmed that they had T-DNA insertion and carried the complementation construct. Fifty-five out of the 165 transgenic plants were homozygous for wbc27-1 mutation. Thirty-seven out of the 55 transgenic plants were as normal as wild type (Figure 2C,F,I). In T2 generation, the transgenic plants selected by hygromycin were all fully fertile, while the non-hygromycin-selected plants were segregated into two groups, fertile and sterile plants. These results indicate that the phenotype of wbc27-1 was caused by the T-DNA insertion in At3g13220.

Phenotypic characterization of wbc27-1 mutant

In arabidopsis, anther development has been divided into 14 stages, according to morphological landmarks of cellular events (Sanders et al. 1999). We first dissected the wbc27-1 anthers at different anther developmental stages and compared them with wild type anthers. wbc27-1 microspores developed normally until the anther developmental stage 7 (Figure 3D). The visible aberration on wbc27-1 pollen grains occurred at the anther developmental stage 8 after release of microspores.
WBC27 is Required for Exine Formation

Figure 3. Development of anthers and microspores in wild type (Col) and wbc27-1 mutant.

(A–C, G–I) Sections of the anthers from a wild type Col plant. (D–F, J–L) Sections of the anthers from a wbc27-1 plant. (A, D) Stage 7. (B, E) Stage 8. (C, F) Stage 9. (G, J) Stage 10. (H, K) Stage 11. (I, L) Stage 12. The arrows indicate the debris of degenerated microspores. E, epidermis; En, endothecium; ML, middle layer; Msp, microspores; Pg, pollen grains; RM, remnants of microspores; T, tapetum; Tds, tetrads. Scale bars = 50 µm.

from tetrads. At this stage, wild type microspores have an angular shape (Figure 3B), while wbc27-1 pollen grains were round and turgid (Figure 3E). At the anther developmental stage 9, wild type microspores started to form exine and had vacuoles (Figure 3C), while wbc27-1 microspores lacked vacuole (Figure 3F). At the anther developmental stages 10 and 11, more and more pollen grains were degenerated in wbc27-1 mutant anthers (Figure 3J,K). At the anther developmental stage 12, wild type anther was filled with pollen grains (Figure 3I). In contrast, the wbc27-1 anthers mainly contained the debris of the degenerated microspores (Figure 3L), indicating that most of the microspores had been degenerated in wbc27-1 anther before they developed into mature pollen grains. DAPI (4, 6-diamidino-2-phenylindole) staining assays further confirmed the degeneration of wbc27-1 microspores once released from tetrads. Nucleates of wbc27-1 microspores at the early tetrad stage were as normal as those of wild type microspores (Figure S1A, S1D, S1G and S1J). The defect occurred at the later tetrad stage in wbc27-1 mutant. After the microspores were released from tetrads, no DAPI stain signal was detected in most wbc27-1 microspores (Figure S1B, S1E), which had become shrunken and been degenerated (Figure S1C and S1F). All of these results suggested that the male sterility of wbc27-1 mutant resulted from degeneration of microspores.
Figure 4. Scanning electron microscopy (SEM) observation of pollen grains and anthers from wbc27-1 and wild type plants.

(A–D, I–L) From wbc27-1 mutant.
(E–H) From wild type (Col).
(A–H) At the uninucleate microspore stage.
(I, J) At the bicellular pollen stage.
(K, L) At the tricellular pollen stage. The white arrow indicates the patchy architectures of wbc27-1 pollen grains. Pt, pollen tube.
Scale bars = 100 µm in (A), (E) and (I); 10 µm in (B), (F) and (J); 7.5 µm in (C) and (G); 25 µm in (K); 3 µm in (D) and (H); 8.5 µm in (L).

We further observed the pollen grains using scanning electronic microscopy (SEM). In wbc27-1 immature anthers, most of the developing microspores appeared morphologically abnormal at the early uninucleate stages. Some of them had started to degenerate at this stage (Figure 4A,B). This was consistent with the result obtained by DAPI staining as described above. At the bicellular pollen stage, the wbc27-1 pollen sacs were filled with cell debris from degenerated microspores (Figure 4J). Occasionally, few survival pollen grains could be found remaining in such pollen sacs (Figure 4K). In contrast, a large number of pollen grains were formed in a wild type anther (Figure 4E,G). In addition, the survival wbc27-1 pollen grains germinated before anthesis (Figure 4K,L). Finally, the wbc27-1 anthers did not have any functional pollen grain at the mature stage.

To understand whether the survival wbc27-1 pollen grains had a normal wall, we first examined their surface morphology by SEM. As shown in Figure 4C,D, the surfaces of wbc27-1 pollen grains were nearly smooth. Patchy architectures frequently appeared on the surface of wbc27-1 pollen grains (Figure 4C,D). Comparison with the exine of a wild type pollen grain, which has a characteristic ridged surface (Figure 4G,H), indicated that the wbc27-1 pollen grains were defective in exine formation.

Transmission electron microscopy (TEM) was then applied to investigate the ultrastructures of the defective mutant pollen walls from the tetrad stage to the bicellular pollen stage by comparison with those of wild type exine. At the tetrad stage, a part of wild type microspore plasma membrane became invaginated and formed an undulant surface on which primexine matrix, the precursor of exine, was formed. Meanwhile, deposition of sporopollenin occurred on the outer surface of callose wall (Figure 5D,E). In wbc27-1 microspores at the tetrad stage, the plasma membrane could become invaginated and formed a regular undulant structure as well as primexine matrix. However, no sporopollenin was deposited on the callose wall (Figure 5A,B). At the later tetrad stage, the probacula emerged
with deposition of sporopollenin on the primexine matrix in wild type (Figure 5E), while probacula formation did not occur in wbc27-1 microspores (Figure 5B). At the early uninucleate microspore stage, in wild type, the probacula became enlarged with deposition of more sporopollenin (Figure 5F). Then, at the later uninucleate microspore stage, the bacule and tectum were formed as probacula, became elongated and expanded. In contrast, wbc27-1 microspores lacked probacula, bacula and tectum. The exine was not initiated (Figure 5C,G). In wild type pollen grains at the bicellular pollen stage, the intine, bacula and tectum became larger and thicker, which led to formation of a sculptured exine wall (Figure 5K). In contrast, no exine was formed in wbc27-1 mutant pollen grains (Figure 5H,I,L). These results further suggest that the degeneration of wbc27-1 mutant pollen grains may be caused by a defect in or lack of exine.

During arabidopsis pollen development, callose is actively produced in two periods. One is at the anther developmental stages 5 to 7, and other is during pollen germination. Defect in callose production could affect pollen development (Nishikawa et al. 2005). To determine whether callose formation was affected by wbc27-1 mutation, we stained the developing wbc27-1 and wild type anthers with aniline blue and observed them under UV irradiation. At the anther developmental stages 5 to 7, the wbc27-1 microspores were normally encased by the callose wall as in wild type (Figure 6A,B,D,E). The callose wall also could be degraded to release the microspores as demonstrated by that callose stain fluorescence was not detectable in wild type and wbc27-1 (Figure 6C,F) at stage 8. We examined the expression of a callose dissolution-indicative marker, A6 gene that is actively expressed at this stage (Hird et al. 1993). The real-time PCR assay showed that the A6 mRNA level in wbc27-1 was not significantly different from that in wild type (Figure S2). This result suggested that wbc27-1 mutation did not affect callose production during pollen development. Therefore, we concluded that the degeneration of wbc27-1 microspores was not correlated to callose production.

From anther developmental stage 11 to stage 12, we occasionally found that some wbc27-1 mutant anthers contained few pollen grains that could be stained by aniline blue (Figure 6H,I). In contrast, none of the pollen grains in wild type anthers at the same stage were stained by aniline blue (Figure 6K,L) although the wild type anther was filled fully with viable pollen grains. Callose is largely produced in germinating pollen grains and growing pollen tubes. Therefore, these pollen grains in the wbc27-1 mutant anther had germinated before anthesis. This was consistent with the result obtained from SEM observation, as described above.

The phenylpropanoid, which is a part of sporopollenin, can emit fluorescence under UV irradiation (Morant et al. 2007). In wild type, the microspores could have emitted weak fluorescence under UV irradiation stage 8 (Figure 6F) although the

Figure 5. Transmission electron microscopy (TEM) observation of wbc27-1 and wild type exine(A–C, G–I, L) From wbc27-1. (D–F, J–K) From wild type (Col).

(A, D) At the early tetrad stage. (B, E) At the late tetrad stage.
(C, F) At the early uninucleate microspore stage. (G, J) At the later uninucleate microspore stage.
(H, K) At the bicellular pollen stage.
(I, L) Showing the germinating wbc27-1 pollen grains before anthesis.

The white arrows in (A) and (B) indicate that no sporopollenin was deposited on the callose wall; the white arrows in (D) and (E) indicate that the sporopollenin was deposited on the callose wall. The black arrows in (F) indicate the probacula. Ba, bacula; In, intine; Msp, microspore; TC, tectum; P, Pollen; Pt, pollen tube. Scale bars = 2 μm in (A–B), (D–E); 1 μm in (C), (F–G) and (J); 10 μm in (H), (K) and (L); 0.5 μm in (I).
deposition of sporopollenin on plasma membrane of microspores just occurred. In contrast, the fluorescence was not detectable in the wbc27-1 mutant microspores at this stage (Figure 6C). After anther developmental stage 9, as the exine became mature, the fluorescence became stronger and stronger in wild type anther locules. However, no exine fluorescence signal appeared at all on the wbc27-1 pollen grains at the later stages until maturity (Figure 6G,H,I). This result implied that the component of sporopollenin in the wbc27-1 exine layer was absent or had been altered.

Tapetal cells are important for pollen wall formation. To understand whether the disruption of pollen wall formation was caused by a defect of tapetal cells, we examined the development of tapetum in wbc27-1 with TEM by comparison with wild type. At the tetrad stage, both wild type and wbc27-1 tapetal cells had many (small and large) electron-translucent vesicles and a small number of fine fibrillar materials. No significant difference in the ultrastructures of tapetal cells was observed between wild type and wbc27-1 (Figure S3A and S3E). At the early uninucleate microspore stage, the cytoplasm of both wild type and wbc27-1 tapetal cells was heavily stained and the large vacuoles had disappeared. Instead, many small vesicles appeared throughout the tapetal cytoplasm and many fibrillar materials were released into the locules from the tapetum. The plastids also appeared in both wild type and wbc27 tapetal cells (Figure S3B and S3F). At the later uninucleate microspore stage, the plastids fully developed into relatively translucent elaioplasts in wild type and wbc27-1 mutant (Figure S3C and S3G). At this stage, the tapetal cells had become a typical polar secretory-type cell. Such cells contained a large number of trafficking vesicles that were fused with the plasma membrane in both wild type and wbc27-1 (Figure S3C and S3G). At this stage, the wbc27-1 mutant tapetal cells were more highly vacuolated (Figure S3C and S3G), compared with wild type. Then, the wbc27-1 tapetal cells started to disintegrate (Figure S3D and S3H) at the bicellular pollen stage. These results suggested that wbc27-1 mutation could affect tapetal cells at the later developmental stages.

**WBC27 encodes a plasma membrane-localized ABC transporter-homologous protein**

The WBC27 mRNA was predicted to encode an ABC transporter-homologous protein of 685 amino acids with an ABCG-EPDR domain and an ABC2-membrane domain (Figure 7A). The ABC2-membrane domain has six transmembrane regions (http://www.cbs.dtu.dk/services/TMHMM-2.0). The arabidopsis genome contains more than 120 open reading frames (ORFs) that have potential for encoding ABC transporter proteins. These proteins can be classified into 13 subfamilies. WBC27 belongs to the WBC/ABCG (white/brown complex) subfamily that has 28 members (previously reported as 29 proteins, Figure 7B). A phylogenetic analysis showed that WBC27 was clustered together with WBC28 and WBC23 (Figure 7B). WBC27 had 43% identity and 61% similarity of amino acid sequence to WBC23, and 41% identity and 59% similarity to WBC28. WBC27 also shared a high identity of amino acid sequence to WBC/ABCG proteins from other species, such as animal, rice and grape. Specifically, WBC27 had 75% identity and 86% similarity of amino acid sequence to VITISV_035231 (CAN73913) from *vitis vinifera*, and 60% identity and 74% similarity to Os06g060607700 (EA237593) from *Oryza sativa* (Figure S4).

WBC subfamily proteins were predicted to be plasma membrane proteins. To determine whether WBC27 is localized on plasma membrane, we generated a GFP-WBC27 fusion protein expression construct under the control of the 35S promoter. The construct was introduced into onion epidermal
cells by particle bombardment. The green fluorescent protein (GFP) fluorescent signal was detected on plasma membrane (Figure 8A–C). In the control, naked GFP signal was found throughout the cells (Figure 8D–F). To further confirm the GFP-WBC27 localization to the plasma membrane, the construct of p35S::GFP-WBC27 was introduced into Nicotiana benthamiana epidermal cells by Agrobacterium infiltration (Voinnet et al. 2003). The GFP fluorescence was observed in the periphery of the cells and was coincident with membrane-specific FM4–64 stain signal (Bolte et al. 2004). Merge of the GFP-WBC27 signal with FM4–64 signal showed that they were overlapped on the plasma membrane (Figure 8G–I). This result indicated that WBC27 was localized on plasma membrane, consistent with the presumed function of the ATP-binding cassette (ABC) transporter protein.

**Figure 7. Molecular characteristic of WBC27 protein.**

(A) Structure of WBC27 protein, which shows the positions of two predicted functional domains, ABCG-EPDR domain and ABC2-membrane domain.

(B) A phylogenic analysis of the WBC subfamily of proteins.

**WBC27 is preferentially expressed in tapetal cells**

The expression pattern of WBC27 was determined by real-time PCR and promoter activity assay. Real-time PCR was performed using the RNAs extracted from different tissues with WBC27 specific primers. The results showed that the WBC27 transcripts were detected mostly in developing flower buds, but not or rarely in roots, leaves, stems, green siliques and mature flowers (Figure 9A). To determine the spatial and temporal expression pattern of WBC27 during anther development, a 1.0 kb-promoter region upstream of the start codon of WBC27 was fused to glucuronidase (GUS) reporter gene and introduced into wild type arabidopsis plants. Twelve independent pWBC27::GUS transgenic plants were obtained. In six out of 12 transgenic lines, GUS stains were restricted to developing flowers from the anther developmental stage 7 to stage 11 (Figure 9C,D). To more precisely determine the exact cell type where WBC27 was expressed in anthers, we further investigated the expression pattern of WBC27 in detail by sectioning of GUS-stained anthers. No GUS stain was found in the sections of anthers before the anther developmental stage 6 (Figure 9E). The GUS activity was initially detected in the tapetum at the anther developmental stage 7 (Figure 9F). After the anther developmental stage 8, the GUS activity was found highly in the tapetum, lasting until the anther developmental stage 10 (Figure 9G–I). The GUS activity was weakened at the anther developmental stage 11 (Figure 9J) and disappeared in the anthers at the anther developmental stage 12 (Figure 9K). Meanwhile, to validate the GUS reporter data, RNA in situ hybridizations were also performed with sections of developing wild-type anthers using the WBC27 anti-sense RNAs as a probe. WBC27 mRNA was hardly detected before stage 7 (Figure S5A). From anther developmental stages 7 to 11, WBC27 RNAs were found predominantly in tapetal cells (Figure S5B–D), and then was undetectable (Figure S5E) at anther developmental stage 12, compared with the control in which only background signals were detected using the WBC27 sense RNA probe for hybridization (Figure S5F). In summary, WBC27 was expressed mainly in tapetal cells from the anther developmental stages 7 to 11 during arabidopsis anther development.

**The expression of WBC27 was drastically decreased in tdf1 and ams mutants**

In arabidopsis, several transcription factor and regulatory genes have been reported to be involved in anther development. To investigate whether the expression of WBC27 was regulated by any of these genes, we measured the relative expression level of WBC27 in the mutants by real-time PCR, which was previously reported as defective in tapetal functions during anther development, by comparison to its expression
level in wild type plant. We divided these mutants into two groups. The first group included those such as sporocyte-less/nozzle (spl/nzz), tapetum determinant 1 (tpd1), excess microsporocytes 1extra sporogenous cell (ems1/ems), which were involved in cell fate determination during early anther development (Schieffthaler et al. 1999; Yang et al. 1999; Zhao et al. 2002; Yang et al. 2003). The second group contained those that were involved in pollen development and in which corresponding genes were expressed in tapetal cells, such as defective in tapetal development and function 1 (tdf1), aborted microspores (ams) and male sterility 1 (ms1) (Zhu et al. 2008).

The results are summarized in Figure 10. In the first group of mutants, the WBC27 mRNA levels in spl, ems1-2 and tpd1 flowers were counted as 21%, 40% and 12.5% of the WBC27 mRNA level in wild type plants, respectively.

In the second group of mutants, the expression level of WBC27 gene in tdf1 mutant was only 5% of that in wild type (Figure 10), indicating that the tdf1 mutation dramatically inhibited the expression of WBC27. The mutant (SALK_152147) in AMS gene was obtained from ABRC (Arabidopsis Biological Resource Center) mutation resource (www.arabidopsis.org), which was also called ams-2, allelic to ams. The phenotype of ams-2 was identical to ams (Sorensen et al. 2003). Real-time PCR results showed that the expression of WBC27 in ams-2 inflorescences was drastically decreased to 11% of that in wild type (Figure 10). The expression level of WBC27 in ms1-1 was reduced to 81% of that in the wild type (Figure 10). In summary, the expression of WBC27 was drastically inhibited by tdf1 and ams-2 mutations, but only slightly affected by ms1-1 (Figure 10).

Discussion

WBC27 plays important roles in arabidopsis exine formation

The WBC27 encodes a putative ABC-transporter protein and is expressed prolifically in tapetal cells at the anther developmental stages 7 to 11 when exine is formed. Mutation in WBC27 disrupted formation of exine, but did not affect the differentiation of tapetal cells and meiosis of microsporocytes. The wbc27-1 pollen grains lacked exine and were eventually degenerated, leading to a male sterility. All of these results we report suggest that WBC27 plays important roles in pollen development.

Supply of materials from tapetum to microspore involves two steps. The first is the production of the materials in tapetum, and the second is translocation of the materials from tapetum to microspores. Several genes have been identified as being involved in the biosynthesis of exine components, such as FLP1, MS2, CYP703A2, NO EXINE FORMATION 1 (NEF1) and RUPTURED POLLEN GRAIN1 (RPG1) (Aarts et al. 1997; Paxson-Sowders et al. 2001; Ariizumi et al. 2003, 2004; Morant et al. 2007; Guan et al. 2008). Recent study shows that the lipid transfer proteins (LPTs), such as type I LTP(At3g51590) and type II LTP (At1g66850) in arabidopsis and OsC6 in rice, are involved in exine formation (Xu et al. 2010; Zhang et al. 2010). Mutations in these genes all caused defects in the formation of exine. WBC27, identified in this study, is a new candidate for the transporter that may be involved in translocation of exine materials from tapetum to microspores. WBC27 is expressed mainly in tapetum at the anther developmental stages 7 to 11 and may function specifically on pollen wall formation. In wbc27-1 pollen grains, deposition of sporopollenin was disrupted as exine was not formed. Therefore, we propose that WBC27 is a novel candidate that may play roles in transport
of the precursors of sporopollenin to myesicrospores, which may transport the substrate differently from those of type I LTP (At3g51590) and type II LTP (At1g66850).

In addition, wbc27-1 also was defective in tapetal cells and control of pollen germination. In wbc27-1, the tapetal cells were differentiated and developed normally at least until the tetrad microspore developmental stage. The secretory tapetum was formed at the later stages. The obvious abnormality of tapetal cells occurred at the later microspore developmental stage. At this stage, more vacuoles were emerged in wbc27-1, indicating
WBC27 is a candidate for lipid transporter functioning on translocation of lipids from tapetum to surfaces of microspores

As described above, the Arabidopsis genome encodes more than 120 ABC-transporter proteins including WBC27. Several studies have shown that WBC family ABC transporters mediate lipids excretion (Sanchez-Fernandez et al. 2001; Xu et al. 2004; Wang et al. 2006; Bird et al. 2007), implying that WBC27 might be a candidate for lipid transporter in Arabidopsis. In plants, the sporopollenin in exine is mainly made from the lipidic precursors. Some studies have suggested that the lipids transport from the tapetum to microspores through two transport means. One is through vesicular transport, the other is to use transporter as lipid carrier (Vizcay-Barrena and Wilson 2006). In wbc27-1 mutant, the secretory tapetal cells were formed. This indicated that the vesicular transport was normal and implied that the transport of lipidic precursors of sporopollenin from tapetum to microspores unlikely was blocked by the defect in vesicular transport. It was likely blocked by a defect in the transporter instead. Therefore, WBC27 may involve the transport of precursors of sporopollenin from tapetum to microspores. However, the kinds of lipidic materials that WBC27 could carry remain unknown.

An ABC transporter should have at least two sets of nucleotide binding folds (NBF) and transmembrane domains (TMD) to function normally. Theoretically, a half-molecule transporter protein will function by dimerization (Garcia et al. 2004; Jung et al. 2006; Sugiyama et al. 2006; Rea 2007). This implied that it may form a homodimer or a heterodimer with other proteins from the same family. It will be worth investigating whether it could form a homodimer or a heterodimer. Nevertheless, more evidence is needed to address this question.

The WBC27 expression is under control of the known transcription factors TDF1 and AMS during anther development

The expression of WBC27 was decreased in several mutants that were involved in anther development. Therefore, it is likely controlled directly or indirectly by the corresponding genes. The SPL/NZZ that encodes a MAD-box transcription factor is involved in earlier anther development (Schieflhuber et al. 1999; Yang et al. 1999). Mutation in SPL/NZZ disrupted the formation of microsporocyte and tapetum. TPD1 encodes a predicted secreted protein and EMS1 encodes a receptor protein kinase. Mutations in these two genes all caused the transformation of tapetal cells into microsporocytes, leading to lack of tapetum in the anthers (Zhao et al. 2002; Yang et al. 2003). Therefore, reduction of WBC27 expression in spl/nzz, tpd1 and ems1 could be caused by absence of tapetal cells since WBC27 is expressed preferentially in tapetal cells. However, it does not exclude the possibility that TPD1 and EMS1 directly or indirectly control the expression of WBC27, since they are all expressed in the tapetal cells at the stages when WBC27 is expressed (Yang et al. 2003).

In Arabidopsis, a number of other transcription factor genes have been identified to regulate the late development and functions of tapetal cells. Mutations of these genes result in male sterility. Recently, a working model for the transcriptional regulation of tapetal function and exine formation has been proposed (Zhang et al. 2007; Zhu et al. 2008). DYT1 is most highly expressed in tapetal cells and microspores at the anther developmental stages 4 to 7 and TDF1 is expressed in tapetal cells with the highest level at the anther developmental stages 5 to 6. Mutation in DYT1 affected the expression of TDF1. Therefore, TDF1 was placed downstream of DYT1 (Zhang et al. 2007; Zhu et al. 2008). The WBC27 is expressed mostly...
in tapetal cells at the anther developmental stages 7 to 11, overlapping with DYT1 and TDF1. The mRNA level of WBC27 was largely decreased in tdf1 mutant. This indicates that WBC27 may act downstream of TDF1. WBC27 expression was also drastically decreased in am5-2 mutant. WBC27 is likely to act downstream of AMS. Recent study has demonstrated that AMS can bind the promoter of WBC27 and positively regulates the expression of WBC27 (Xu et al. 2010). All of these results suggest that the expression of WBC27 in tapetal cells is under the control of transcript factor AMS (Xu et al. 2010).

Materials and Methods

Plant materials and mutants isolation

The Arabidopsis thaliana mutants used in this study were of Colombia (Col-0) and landsberg erecta (Ler) backgrounds. The wbc27-1, ms1-1 and am5-2 mutant seeds were obtained from the Arabidopsis Biological Resource Center (ABRC). The tdf1 seeds were the presents from Dr Zhongnan Yang. The T-DNA insertion sites in the mutant genomes were verified by PCR. The wbc27-1 mutant was back-crossed to wild type (Col-0) three times before being used for phenotypic characterization. The seeds were pre-germinated on MS-salt agar plates at 22°C under the same light cycle as for seeds growing LA. After verification by sequencing, the fragment was subcloned into binary vector pCAMBIA1300 (CAMBIA, Canberra, Australia, http://www.cambia.org) and introduced into the plants heterozygous for wbc27-1 mutation (wbc27-1/+). The resulting fragment was amplified using the primers (5′-GAACCCAGCAGTTCTTGCT-3′ and 5′-TGAGGGATGGCTTAACTTGGAA-3′). The resulting fragment was subcloned upstream of the GUS reporter gene in pCAMBIA1300 Ti-derived binary vector. The resulting construct was introduced into wild-type arabidopsis plants. Transformation and GUS activity analysis were performed as described by Yang et al. (2003, 2009).

Analyses of WBC27 expression pattern

The seedlings, roots, leaves, unopened flowers and inflorescence materials were harvested from 2-week-old seedlings and 4-week-old flowering plants, respectively. The total RNAs were extracted with cetyltrimethylammonium bromide (CTAB) solution (Yu and Goh 2000). The quantitative RT-PCR analysis was performed as described by Yang et al. (2009) with WBC27-specific (5′-TGAGCGCCAGAGGAGT3′ and 5′-GGTAGCCCTGAAGCGGAT3′) and 18S primers following the supplier’s instruction.

The promoter fragment of WBC27 was amplified using rTaq (TaKaRa) with the gene-specific primers (5′-GATCATACCAGGACATTGG3′ and 5′-TTATCTTCTTATTCTGGTC-3′). The resulting fragment was cloned into the pBluescript II KS(-) vector (Stratagene; http://www.stratagene.com) and verified by sequencing. The resulting plasmid DNAs were completely linearized using BamHI or SalI, and used as templates for preparation of antisense and sense WBC27 RNA probes. Half of the labeled products were hydrolyzed to 150 bp by alkaline treatment. A mixture of hydrolyzed and non-hydrolyzed RNA probe was used for the hybridization. Non-radioactive RNA in situ hybridization was performed as described by Shi et al. (2005). The hybridization signal images were viewed and recorded using Leica DM2500 microscopy (Wetzlar, Germany).
Acknowledgements

We thank Zhongnan Yang for the seeds of tdf1 and ms188-1 mutants. This work was supported by research grants from the National Natural Science Foundation of China (30530060 and 30970274), the Ministry of Sciences and Technology (2007CB108700 and 2007CB947603) and the Ministry of Education (B06003).

Received 7 Sept. 2010 Accepted 9 Nov. 2010

References


Paxson-Sowders DM, Dodrill CH, Owen HA, Makaroff CA (2001) DEX1, a novel plant protein, is required for exine pattern formation


(Co-Editor: Weicai Yang)

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) staining assay on wbc27-1 and wild type microspores.
Figure S2. The relative expression levels of A6 in wbc27-1, compared to wild type (Col).

Figure S3. Transmission electron microscopy (TEM) observation of wbc27-1 and wild type tapetum development.

Figure S4. A multiple alignment of proteins, which share a high similarity with WBC27 protein.

Figure S5. The expression pattern of WBC27, revealed by *in situ* hybridization.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.