Serine Palmitoyltransferase, a Key Enzyme for de Novo Synthesis of Sphingolipids, Is Essential for Male Gametophyte Development in Arabidopsis

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Sphingolipids are important signaling molecules involved in various cellular activities. De novo sphingolipid synthesis is initiated by a rate-limiting enzyme, serine palmitoyltransferase (SPT), a heterodimer consisting of LONG-CHAIN BASE1 (LCB1) and LCB2 subunits. A mutation in the Arabidopsis thaliana LCB1 gene, lcb1-1, was found to cause embryo lethality. However, the underlying molecular and cellular mechanisms remain largely unclear. Here, we report the identification of the fumonisin B₁ resistant1-2 (fbr1-2) mutant, an allele of lcb1-1. The fbr1-2 mutation, most likely an allele stronger than lcb1-1, was transmitted only through female gametophytes and caused the formation of abortive microspores. During the second pollen mitosis, fbr1-2 initiated apoptotic cell death in binculated microspores characteristic of nuclear DNA fragmentation, followed by cytoplasm shrinkage and organelle degeneration at the trinucleated stage. In addition, a double mutant with T-DNA insertions in two homologous LCB2 genes showed a phenotype similar to fbr1-2. Consistent with these observations, the FBR11/LCB1 expression was confined in microspores during microgametogenesis. These results suggest that SPT-modulated programmed cell death plays an important role in the regulation of male gametophyte development.

Sphingolipids are a class of complex lipids consisting of a sphingoid long-chain base (LCB) that is amide-linked to a fatty acid. Sphingolipids function as essential components of cellular membranes and important signaling molecules involved in a variety of cellular activities, including cell proliferation, cell differentiation, apoptosis, and stress responses (Spassieva and Hille, 2003; Sperling and Heinz, 2003; Worrall et al., 2003; Lynch and Dunn, 2004). The de novo sphingolipid synthetic pathway is highly conserved in eukaryotic organisms. The process is initiated by the condensation of Ser and palmitoyl-CoA to produce 3-ketosphinganine, catalyzed by Ser palmitoyltransferase (SPT; EC 2.3.1.50). Subsequently, 3-ketosphinganine is reduced to form sphinganine or dihydrosphingosine (dh-sph). In plant cells, a major fate of dh-sph is to be acylated to form ceramides catalyzed by ceramide synthase (Sperling and Heinz, 2003; Lynch and Dunn, 2004).

SPT and ceramide synthase are two key enzymes of the pathway. Whereas SPT is a heterodimer consisting of two subunits, LCB1 and LCB2, ceramide synthase is a multisubunit enzyme that can be competitively inhibited by AAL-toxin and fumonisin B₁ (FB₁), two fungal toxins that are structural analogs of dh-sph (Hanada, 2003; Sperling and Heinz, 2003; Lynch and Dunn, 2004). Genetic studies have revealed that mutations that affect sphingolipid metabolism are associated with a variety of developmental abnormalities and programmed cell death (PCD), as well as altered sensitivities to AAL-toxin or FB₁. In Arabidopsis (Arabidopsis thaliana), a mutation in the LCB1 gene causes embryo lethality (Chen et al., 2006). However, a weaker mutant allele in LCB1 (Shi et al., 2007) and a mutant in the LCB phosphate lyase gene (Tsegaye et al., 2007) do not show any developmental abnormalities, but cause altered sensitivity to FB₁. Moreover, mutations in genes encoding a sphingosine transfer protein (ACD11; Brodersen et al., 2002) and a ceramide kinase (ACD5; Liang et al., 2003) induce apoptotic cell death, thereby causing a pleiotropic phenotype. Interestingly, the eceriferum10 mutant, which displayed an altered sphingolipid level, also showed severe developmental abnormalities, including the formation of abortive pollens (Zheng et al., 2005). The latter result suggests that sphingolipids may be involved in the regulation of pollen development.

In Arabidopsis, pollen development has been well documented by microscopic studies (Owen and...
Makaroff, 1995). During male gametophyte development, a pollen mother cell undergoes meiosis to form tetrad cells containing microspores. After release from a tetrad, microspores undergo an asymmetric mitosis (pollen mitosis I) to form a binucleated pollen grain consisting of a vegetative cell and a generative cell. These two cells have distinctive developmental fates during later stages of male gametophyte development. Whereas the vegetative cell eventually forms the pollen tube on the stigma, the generative cell undergoes an additional round of mitosis (pollen mitosis II) to give rise to two sperm cells, leading to the formation of a trinucleated pollen grain (McCormick, 1993, 2004). During early stages of male gametophyte development, the male meiocyte death1 (mmd1) mutation causes apoptotic cell death, thus impairing male meiocyte meiosis. MMD1, encoding a PHD-domain protein, was therefore proposed to play a regulatory role in the checkpoint control during meiosis by repressing a male meiocyte cell death pathway (Yang et al., 2003). However, it is unclear whether a similar mechanism is involved in the later stages of male gametophyte development. In this study, we show that LCB1 and LCB2 genes are essential for male gametophyte development, likely involved in a sphingolipid-modulated PCD pathway during the second pollen mitosis.

RESULTS

Identification and Genetic Analysis of the fbr11-2 and fbr11-3 Mutants

In a previous study, we identified and characterized a mutant fumonisin B1 resistant11-1 (fbr11-1), which was a weak allele with a T-DNA insertion in the 3′-untranslated region (UTR) of LCB1 and had no detectable phenotype under normal growth conditions (Shi et al., 2007). To gain more insight into the FBR11 function, we identified two additional mutant alleles (SALK_097815 and SALK_077745; Alonso et al., 2003). Data presented below indicate that these two mutants are allelic to fbr11-1. Accordingly, we renamed these mutants as fbr11-2 and fbr11-3, respectively. In these two mutants, a T-DNA was inserted in exon 11 and intron 2 of the mutant genomes, respectively (Fig. 1A). In fbr11-2, the T-DNA insertion might cause the formation of a truncated protein containing the N-terminal 369 amino acid residues, lacking the C-terminal region of 113 amino acid residues, likely causing a null mutation. Alternatively, the T-DNA insertion might cause the formation of an unstable FBR11-T-DNA fusion transcript. In fbr11-3, however, a T-DNA was inserted inside intron 2, which did not affect the structures of exons 1 and 2 as well as the flanking splicing sites (Fig. 1A; Supplemental Fig. S1). Note that the T-DNA underwent rearrangement in fbr11-3, resulting in the duplication of the left border (LB) flanking both ends of the insertion in the mutant genome (Fig. 1A; Supplemental Fig. S1). Considering the T-DNA insertion positions and the phenotype severity (see below), fbr11-2 is most likely a mutant allele stronger than fbr11-3.

To obtain fbr11-2 mutant plants homozygous for the T-DNA insertion, we screened a population derived from self-pollinated FBR11/fbr11-2 heterozygous plants by PCR. However, we failed to recover any homozygous fbr11-2 mutant plants by screening of 444 individual plants. Among these progenies, 205 plants are heterozygous for the T-DNA insertion and 239 plants were wild type. This result suggests that fbr11-2 may cause a gametophyte-lethal phenotype. The fbr11-3 mutant allele, identical to the lcb1-1 mutant, showed an embryo-defective phenotype as previously reported (Chen et al., 2006). In a cross between FBR11/fbr11-2 (female) and FBR11/fbr11-3 (male), approximately one-fourth of F1 embryos showed defective development, indicating that these two mutants were allelic. More-specifically, the T-DNA insertion in the male meiocyte death1 (mmd1) mutant genome (Fig. 1A; Supplemental Fig. S1) resulted in the deletion of approximately 30 bp of intron 2. See Supplemental Figure S1 for more details on the insertion sites of fbr11-3. B, Pollen grains collected from anthers of the wild type, FBR11/fbr11-2, and fbr11-3/fbr11-2 carrying an FBR11 transgene (Comp.) that were stained with Alexander solution. Viable pollen grains were stained as purple and dead pollen grains as dark green. Bar = 20 μm. C, Scanning electron microscopy of pollen grains collected from wild-type and FBR11/fbr11-2 anthers. Approximately one-half of pollen grains were abnormally developed in FBR11/fbr11-2 anthers. Bars = 25 μm.

Figure 1. Characterization of the fbr11-2 and fbr11-3 mutants. A, Schematic map of the FBR11 gene. Black boxes and solid lines denote exons and introns, respectively. Putative untranslated regions are indicated as dashed lines. The T-DNA insertion positions in two fbr11 alleles are shown. Arrows denote the orientation of the LB. In fbr11-2, the insertion site for the right border (RB) is unclear as indicated by a question mark. In fbr11-3, insertion positions of the T-DNA, which had the LB at both ends of the insertion, were shown. The T-DNA insertion resulted in the deletion of approximately 30 bp of intron 2. See Supplemental Figure S1 for more details on the insertion sites of fbr11-3.
over, an FBR11 transgene fully rescued all developmental abnormalities in these two mutants (see below). These results indicate that fbr1l-2 and lcb1-1 (fbr1l-3) are allelic to fbr1l-1.

Because of the lethality of these mutations, fbr1l-2 and lcb1-1 (fbr1l-3) were maintained as heterozygous. Similar to that of fbr1l-1 (Shi et al., 2007), no apparent alterations of sphingolipids were detected in FBR11/fbr1l-2 plants (data not shown).

The fbr1l-2 Mutation Is Transmitted via Female Gametophytes

Data presented above suggest that fbr1l-2 may cause gametophytic lethality. To determine the nature of the mutation, we performed reciprocal crosses between the wild type and the FBR11/fbr1l-2 heterozygote. The transmission efficiency of the T-DNA insertion was 33.3% through the female gametophytes, lower than expected in wild-type plants (50%). However, essentially no transmission of fbr1l-2 through the pollen was found in the tested population (Table I). These results indicated that both the mutant allele and the T-DNA insertion were transmitted only through female gametophytes, but not male gametophytes.

In lcb1-1 (fbr1l-3), the T-DNA insertion showed slightly lower transmission efficiency through male gametophytes (41.4%; Supplemental Table S1), which was consistent with the observation that approximately 6% of pollens were abnormally developed in FBR11/fbr1l-3 anthers.

Abortive Pollen Development in fbr1l-2

Genetic analysis indicates that the fbr1l-2 mutation causes male sterility. We therefore examined the viability of the mutant pollens. Pollens collected from wild-type and FBR11/fbr1l-2 flowers were stained with Alexander solution, which stained mature viable pollen grains as purple and dead or dying ones as dark green (Alexander, 1969). In the wild type, the majority of examined pollen grains were viable (Fig. 1B) with occasional death. In FBR11/fbr1l-2 anthers, however, whereas only approximately one-half of pollen grains showed a staining pattern similar to that of the wild type, the remaining half were stained as dark green (Fig. 1B). Morphologically, the dead pollens were misshapen and smaller, which could easily be distinguished from wild-type pollens. Scanning electron microscopy showed that wild-type pollen grains had a uniform shape (Fig. 1C). By contrast, approximately 50% of pollens derived from FBR11/fbr1l-2 flowers had shrunken and collapsed shapes, and were smaller than wild-type pollens (Fig. 1C). Together with data obtained from the genetic analysis, we conclude that FBR11 is essential for pollen development.

To track the expression stage of the fbr1l-2 mutation, we followed pollen development in the mutant by microscopy. Semithin sections were prepared from anthers collected at various developmental stages of wild-type and FBR11/fbr1l-2 floral inflorescences, and then analyzed by light microscopy. No abnormality of pollen development was found in FBR11/fbr1l-2 anthers before stage 12 (Fig. 2, A and C; anther developmental stages were defined according to Sanders et al., 1999). However, fbr1l-2 pollens became aborted at stage 12 (Fig. 2, B and D). At this stage, an anther becomes bilocular after degeneration of septum below the stomium and contains trinucleated pollen grains that have undergone two rounds of mitosis. Therefore, the fbr1l-2 mutation appears to cause apparent abnormalities after the second pollen mitosis.

To confirm the light microscopic results, we further analyzed pollen development in FBR11/fbr1l-2 flowers by 4′,6′-diamidino-2-phenylindole (DAPI) staining. In the wild type, normal pollens at different developmental stages, including binucleated and trinucleated microspores, were observed (Fig. 3, A–D). In FBR11/fbr1l-2 anthers, no abnormality was observed in pollen grains at the uninucleated and binucleated stages (Fig. 3, E and F). At the trinucleated stage when the second mitosis is completed, a mixed population of pollen grains was observed. Approximately one-half of the pollen grains displayed a normal phenotype with three nuclei; the other half were smaller and abnormally developed (Fig. 3, G and H). The latter population showed two distinctive DAPI staining patterns. Whereas a portion of pollen grains had no detectable DAPI staining, others displayed a wild-type-like pattern with two or three nuclei (Fig. 3, G and H). In mature pollen grains, however, no DAPI signal or only diffused DAPI signal was detected in the mutant pollen grains that were swollen and misshapen (Fig. 3, I and J). These observations suggest that the degener-

Table 1. Genetic analysis of fbr1l-2

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eration of nuclei in \( fbr11-2 \) microspores occurs mainly at the trinucleated stage or after the second pollen mitosis.

Complementation of the \( fbr11-2 \) Mutant Phenotype by an \( FBR11 \) Transgene

To verify whether the observed abnormal development of microspores was caused by the T-DNA insertion in \( FBR11 \), we performed a genetic complementation experiment by crossing \( FBR11/fbr11-2 \) (male) plants with a transgenic plant carrying an \( FBR11 \) transgene (male; Shi et al., 2007). In F2 populations obtained from self-pollinated F1 plants, two of the 23 tested families were homozygous for both the \( fbr11-2 \) allele (assessed by PCR) and the \( FBR11 \) transgene.

Figure 2. The \( fbr11-2 \) mutant phenotype during anther development. Light microscopy of semithin sections prepared from wild-type or \( FBR11/fbr11-2 \) anthers at stages 11 and 12, as indicated on the top. Pollen developmental stages were defined according to Sanders et al. (1999). A and B, Wild-type anthers. C and D, \( FBR11/fbr11-2 \) anthers. E and F, \( lcb2a/lcb2a LCB2c/lcb2c \) anthers. In D and F, approximately one-half of pollen grains were aborted. A similar phenotype was observed in sections prepared from \( LCB2a/lcb2a lcb2c/lcb2c \) anthers. At stage 12, septum was degenerated in anthers (arrows). Bars = 25 \( \mu \)m.

Figure 3. Analysis of pollen development by DAPI staining. Pollen grains were released from anthers, stained with DAPI, and visualized under a light microscope equipped with (A, C, E, G, and I) or without (B, D, F, H, and J) a UV fluorescent filter. A and B, Wild type-derived microspores at the bicellular stage. C and D, Wild type-derived microspores at the tricellular stage. E and F, Microspores of the bicellular stage collected from \( FBR11/fbr11-2 \) anthers. No abnormal microspores were found at this stage. G and H, Microspores of the tricellular stage collected from \( FBR11/fbr11-2 \) anthers. Most microspores contained three nuclei. Abnormal development became apparent in approximately one-half of microspores (arrows). I and J, In matured pollens, three nuclei were found in the wild type, but only diffused nuclear signals in \( FBR11/fbr11-2 \). Bars = 20 \( \mu \)m.

SPT-Regulated PCD in Male Gametogenesis

(assessed by hygromycin resistance). In all tested F3 progenies of these two families, pollen development was normal (Fig. 1B), demonstrating that the FBR11 transgene fully complemented the male sterile phenotype. Similarly, the embryo-defective phenotype of the lcb1-1 (fbr11-3) mutant was fully rescued by the LCB1/FBR11 transgene, consistent with the results obtained from a previous study on this mutant allele (Chen et al., 2006).

Identification of lcb2 Mutants

We noticed that fbr11-2 and lcb1-1 (fbr11-3) showed different phenotypes during development, raising the possibility that the fbr11-2 phenotype is allele specific, rather than representing a general function of LCB1 or SPT. To test this possibility, we analyzed the function of LCB2, a second subunit of SPT. The Arabidopsis genome contains three copies of LCB2-related genes, At3g48780, At3g48790, and At5g23670, of which the former two are arranged as a tandem repeat, presumably originated from a duplication event. Hereafter, we refer to these three genes as LCB2a, LCB2b, and LCB2c, respectively. LCB2b appears to be a truncated form, lacking approximately 140 amino acid residues from the N terminus that is highly conserved across different kingdoms. Previous biochemical studies suggested that LCB2c was a functional LCB2 (Tamura et al., 2001; Chen et al., 2006). Overall, LCB2c shares 95% identity with LCB2a, suggesting that these two proteins may have a similar biochemical activity.

We identified two T-DNA insertional mutants in LCB2a (SALK_110242) and LCB2c (SALK_061472; Alonso et al., 2003), designated as lcb2a and lcb2c, respectively (Fig. 4A). Reverse transcription (RT)-PCR analysis did not detect any LCB2a or LCB2c expression in respective mutants homozygous for the T-DNA insertion (Fig. 4B). The lcb2a and lcb2c mutant plants had no detectable developmental defects under normal growth conditions.

LCB2 Genes Are Essential for Male Gametogenesis

Because plants homozygous for the T-DNA insertions in either LCB2a locus did not show apparent phenotype under normal growth conditions, we attempted to construct lcb2a lcb2c double mutants. In a screen for putative double mutants from an F2 population by PCR, we only recovered plants with two genotypes of lcb2a/lcb2a LCB2c/lcb2c and LCB2a/lcb2a lcb2c/lcb2c, and no double-mutant plants homozygous at both loci were identified. This result suggests that the double mutant is likely gametophyte or embryo lethal. When stained with Alexander solution, approximately 50% of pollens were inviable in anthers derived from lcb2a/lcb2a LCB2c/lcb2c or LCB2a/lcb2a lcb2c/lcb2c flowers (Fig. 4C). A similar observation was made by scanning electron microscopy (Fig. 4D). These results suggest that the lcb2a lcb2c double mutant is likely affected in male gametophyte development.

To genetically verify the above results, we performed reciprocal crosses of LCB2a/lcb2a lcb2c/lcb2c × lcb2a/lcb2a LCB2c/lcb2c and LCB2a/LCB2a lcb2c/lcb2c × lcb2a/lcb2a LCB2c/lcb2c. Similar to that of fbr11-2, the T-DNA insertions in both combinations were trans-
mitted only through female, but not male, gametophytes (Table II). We conclude from the above data that the lcb2a lcb2c double mutant is male gametophytic lethal. Again, similar to fbr11-2, lcb2a lcb2c showed reduced transmission efficiency of T-DNA through female gametophytes, indicating that SPT is also important for female reproductive development.

Light microscopic studies revealed that defective microspore development mainly occurred at stage 12 in lcb2a lcb2c (Fig. 2, E and F), similar to that in fbr11-2 (Fig. 2, C and D). We have also observed abortive lcb2a lcb2c microspores at stage 11 with a low frequency of 3% to 5%. The defective pollen development phenotype was fully rescued by transforming an LCB2a and an LCB2c transgene into lcb2a/lcb2a LCB2c/lcb2c and LCB2a/lcb2a lcb2c/lcb2c plants, respectively. In both cases, we have obtained transgenic plants homozygous for T-DNA insertions at both LCB2a and LCB2c loci (lcb2a/lcb2a lcb2c/lcb2c plants) that displayed normal pollen development in all tested transgenic lines (Fig. 4C), demonstrating that the observed abnormal pollen development was caused by T-DNA insertions in these two genes.

Taken together, these results indicate that the lcb2a lcb2c double mutant shows a phenotype similar to that of fbr11-2, suggesting that LCB1/FBR11 and LCB2 genes function similarly or in a linear pathway. In addition, these results also render it unlikely that the fbr11-2 phenotype is allele specific or caused by a dominant-negative effect (see “Discussion”).

Expression Pattern of LCB1/FBR11-GUS

To better understand its function, we analyzed the expression pattern of LCB1/FBR11. An LCB1/FBR11 promoter::GUS reporter construct was made and then stably transformed into wild-type plants. Consistent with the results of a previous study (Chen et al., 2006), the LCB1/FBR11::GUS expression was detected in most tissues and organs with different expression levels (Supplemental Fig. S2). During reproductive development, the FBR11::GUS expression was first detected in flowers at stage 11, and the GUS activity was restricted in sepalas and stigmas (Fig. 5, A and B). At stage 12, the expression domain was extended to petals and anthers.

In anthers at this stage, FBR11::GUS expression was specifically detected in pollen grains (Fig. 5, B and C). During embryogenesis, the FBR11::GUS expression was initiated approximately at the eight-cell stage and then throughout embryogenesis (Fig. 5D). Both LCB2a and LCB2c showed an expression pattern similar to FBR11 in floral organs/tissues. However, the expression of LCB2a and LCB2c was initiated in flowers at stage 10, slightly earlier than that of FBR11 (data not shown). These results suggest that LCB1/FBR11 plays an important role during male gametogenesis and embryogenesis.

Cellular Basis of Defective Microspore Development in fbr11-2 and lcb2a lcb2c

To reveal the cellular mechanism of the fbr11-2 and lcb2 mutations, we compared the ultrastructures of the mutant and wild-type pollen by transmission electron microscopy. In wild-type pollen sacs at stage 12, highly synchronized and well-developed pollen were observed. At the same developmental stage, approximately one-half of pollen grains were abnormally developed in FBR11/fbr11-2, lcb2a/lcb2a LCB2c/lcb2c, and LCB2a/lcb2a lcb2c/lcb2c anthers. The mutant pollen grains were collapsed and misshapen and the cytoplasm was shrunken (Fig. 6, A and B).

A wild-type pollen grain contained structurally well-defined nuclei, mitochondria, Golgi apparatus, oil bodies, and vacuoles (Fig. 6, C and D). By contrast, fbr11-2 (Fig. 6, E and F) and lcb2a lcb2c (Fig. 6, G and H) pollen grains had no distinctive structures of nuclei and organelles and had reduced numbers of oil bodies and vacuoles. In particular, most organelles were degenerated, resulting in no recognizable membrane systems in the mutant pollen. Compared to the wild type, the extine layer of fbr11-2 and lcb2a lcb2c pollen grains remained nearly normal. However, the intine layer of the mutant pollen grains was irregular and became degenerative. Overall, fbr11-2 and lcb2a lcb2c pollen grains showed a similar cellular phenotype (Fig. 6, E–H). These results suggest that fbr11-2 and lcb2a lcb2c mutations may trigger a cell death program, characteristics of condensed cytoplasm and degenerated organelles.

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*See Table I for technical details.*
**fbr11-2 and lcb2a lcb2c Mutations Initiate Apoptotic Cell Death during the Second Pollen Mitosis**

To determine whether or not cell death observed in fbr11-2 and lcb2a lcb2c pollens is involved in a PCD-related mechanism, we performed a TdT-mediated dUTP nick-end labeling (TUNEL) experiment to examine possible nuclear DNA fragmentation in wild-type and mutant pollens. Pollens prepared from wild-type, FBR11/fbr11-2, and LCB2a/lcb2a lcb2c/lcb2c flowers at stages 11 to 12 were used for the TUNEL experiment. In pollens prepared from wild-type flowers, no TUNEL-positive signals were detected (n > 2,000). However, a large population of microspores was TUNEL positive in preparations made from FBR11/fbr11-2 (17.1%; n = 1,273) and LCB2a/lcb2a lcb2c/lcb2c (20.8%; n = 315; Fig. 7). Notably, most TUNEL-positive microspores were in the binucleated stage (>98%) as revealed by DAPI staining. This result suggests that the onset of PCD occurs in binucleated microspores prior to apparent morphological abnormalities observed in trinucleated microspores (Figs. 2 and 3). Taken together, data presented in Figures 6 and 7 indicate that fbr11-2 and lcb2a lcb2c mutant microspores undergo apoptotic-type cell death, which is initiated in the binucleated stage before the completion of the second pollen mitosis.

**DISCUSSION**

SPT is a key enzyme in de novo biosynthesis of sphingolipids, catalyzing the first rate-limiting reaction. In this study, we present evidence showing that both subunits are essential for male gametophyte development. Mutations in the single-copied LCB1/FBR11 gene (fbr11-2) or double mutants in the highly homologous LCB2a and LCB2c genes, which presumably result in the lack or reduced de novo synthesis of sphingolipids, cause apoptotic-type cell death in microspores. Therefore, de novo synthesized sphingolipids play a critical role in pollen development, presumably by regulating a PCD event.

As the first rate-limiting enzyme, SPT is essential for growth and development in several eukaryotic organisms thus far documented (Perry, 2002; Hanada, 2003). In Drosophila, for example, a null mutation in LCB2 (the lace mutation) causes lethality during the first instar larval stage, whereas weak mutant alleles are able to grow into adults, but with defective development of various external organs (Adachi-Yamada et al., 1999).

In parallel to the observation made in Drosophila, a mutation in Arabidopsis LCB1, lcb1-1 (Chen et al., 2006), or fbr11-3 (this study), causes severe defects in early embryogenesis, suggestive of a conserved function of SPT in multicellular organisms.

In addition to its role in embryogenesis, we have revealed a novel function of SPT in male gametophyte development. Several lines of evidence obtained from the analysis of fbr11-2 and lcb2a lcb2c mutants suggest that SPT is essential for male gametogenesis. First, fbr11-2 can only be transmitted through female gametophytes, suggestive of male-germline lethality of the mutation. Second, approximately 50% of pollen grains in self-pollinated FBR11/fbr11-2 heterozygous plants were aborted, characteristic of male gametophytic sterility. Third, microscopic studies revealed that the hap-
loid fbr11-2 pollen grains displayed a variety of developmental abnormalities after the second pollen mitosis, which is well correlated with the expression pattern and timing of FBR11 during male gametogenesis. Fourth, these haploid fbr11-2 pollen grains clearly undergo apoptotic-type cell death, as revealed by transmission electron microscopy and TUNEL analysis. Finally, lcb2a lcb2c double mutants showed a phenotype similar to that of fbr11-2, suggesting that these two classes of genes function similarly during pollen development. More importantly, the fact that fbr11-2 and lcb2a lcb2c pollens show a similar cellular and molecular phenotype rules out the possibility that abnormal male microgametogenesis is an allele-specific phenotype of fbr11-2. Taken together, these observations suggest that de novo synthesis of sphingolipids is essential for male gametophyte development.

We noticed that the lcb1-1 mutation was mainly expressed during embryogenesis, although the cellular basis of the phenotype remained unknown. As mentioned before, because the lcb2a lcb2c mutant shows a phenotype similar, if not identical, to that of fbr11-2, it is unlikely that defective pollen development in fbr11-2 is allele specific. The phenotypic variations between fbr11-2 and lcb1-1 may be due to the different strengths of these two alleles. Several observations suggest that fbr11-2 is likely a stronger allele. First, whereas fbr11-2 showed full penetration of the mutant phenotype during microgametogenesis, only a small fraction of lcb1-1 microspores were aborted. Second, F1 progenies derived from the cross of fbr11-2 (female) and lcb1-1 (male) showed the lcb1-1 phenotype during embryogenesis. Third, it is possible that fbr11-2 is a dominant-negative mutation, due to possible formation of a truncated protein. However, the fbr11-2 mutation did not exert any detectable adverse effects on growth and development of FBR11/fbr11-2 heterozygous plants, thus disfavoring such an argument. Moreover, an FBR11 transgene fully rescues defects in pollen development as well as the sensitivity to FB1 (C. Teng, H. Dong, L. Shi, and J. Zuo, unpublished data) of the mutant, which is again inconsistent with the nature of a dominant-negative mutation. Last, in lcb1-1, a T-DNA was inserted inside intron 2 of FBR11/LCB1, but not in the junction between intron 2 and exon 3 as previously suggested (Chen et al., 2006; Fig. 5A). This configuration did not affect 5′- and 3′-splicing sites flanking the T-DNA insertion. Thus, it is most likely that lcb1-1 may maintain residual activity due to the removal of the T-DNA sequence during pre-mRNA splicing. Similar observations have been made in a number of mutants with a T-DNA in introns, such as the SALK_138092 line of the cer10 mutant (Zheng et al., 2005). Taken together, these results suggest that fbr11-2 is most likely a stronger mutant allele than lcb1-1, thereby showing a more severe phenotype during reproductive development.

Transmission electron microscopy reveals the presence of cell death in fbr11-2 and lcb2a lcb2c pollen grains, characteristics of shrunk cytoplasm, degenera-

Figure 6. Cellular defects of fbr11-2 and lcb2a lcb2c mutant pollens. Transmission electron microscopy of ultrathin sections of anthers prepared from wild-type and mutant flowers at stage 12. A and B, Pollen grains in a pollen sac derived from FBR11/fbr11-2 (A) and LCB2a/lcb2a lcb2c/lcb2c (B) anthers. Approximately one-half of normal (black arrows) and abortive (white arrows) pollen grains were observed. C, E, and G, Cross sections of wild-type (C), fbr11-2 (E), and lcb2a lcb2c (G) pollen grains. D, F, and H, Enlarged views in C, E, and G, respectively (boxed areas). Identical results were obtained from the analysis of lcb2a/lcb2a LCB2c/lcb2c anthers. N, Nuclei; Ex, exine layer; In, intine layer; Mt, mitochondrion; G, Golgi apparatus; O, oil bodies. Bars = 10 μm (A and B); 2 μm (C, E, and G); and 200 nm (D, F, and H).
Bars Teng et al. analysis of pollens prepared from
lcb2a/lcb2a lcb2c/lcb2c independent experiments. Similar results were obtained from the
stages during the two rounds of mitosis. In contrast
to these mutants, defective pollen mitosis have been characterized, in-
cluding sphingolipids. A number of mutants that displayed
defective pollen mitosis have been characterized, in-
cluding sidecar pollen (Chen and McCormick, 1996),
gemini pollen1 (Park et al., 1998; Twell et al., 2002), duo pollen1, duo pollen2 (Durbarry et al., 2005), and male sterility1 (ms1; Wilson et al., 2001). These mutations
affect male gametophyte development at various stages during the two rounds of mitosis. In contrast to these mutants, fbr11-2 and lcb2a lcb2c microspores show defective development at the trinucleated stage,
arguing that the SPT activity may not be required for the second pollen mitosis. However, TUNEL-positive
signals were predominantly detected in binucleated microspores, a stage before the completion of the
second mitosis. This result suggests that apoptotic cell death is initiated during the second mitosis in the
mutant microspores. Thus, de novo synthesized sphingolipids are essential for the second pollen mi-
tosis to prevent the initiation of apoptotic cell death, although morphological abnormalities become appar-
ent in a later developmental stage.

During male gametogenesis, PCD has been shown
as a major regulatory mechanism to control the develop-
mental fate of tapetal cells. The failure of initiation
of PCD causes delayed or no degeneration of tapetum,
thereby leading to abnormal pollen development, as
observed in the rice (Oryza sativa) tapetum degeneration
retardation mutant (Li et al., 2006) and the Arabidopsis
ms1 mutant (Wilson et al., 2001; Vizcay-Barrena and
Wilson, 2006). The absence of tapetal PCD, in turn, trig-
gers PCD in microspores (Vizcay-Barrena and Wilson,
2006). Therefore, the microspore-specific PCD pheno-
type observed in fbr11-2 and lcb2a lcb2c has distinctive
cellular and molecular mechanisms from those of ms1.
Because mutations in LCB1/FBR11 and LCB2 result in
apoptotic cell death, SPT activity likely acts to repress
PCD during the second mitosis. In this regard, SPT
appears to function similarly as MMD1, which was
proposed to repress apoptotic cell death during meio-
osis (Yang et al., 2003). Again, because of different
biochemical natures of MMD1 and SPT, it is unlikely
that a similar antiapoptotic mechanism is employed
during different stages of male gametophyte develop-
ment. In fbr11-2 and lcb2a lcb2c microspores, reduced
or lack of de novo synthesized sphingolipids may trig-
ger PCD. In agreement with this notion, the impaired
sphingolipid metabolism was demonstrated to induce
PCD in acd5 (Liang et al., 2003) and acd11 (Brodersen
et al., 2002) mutants. What is the biochemical basis of
sphingolipids in the regulation of microspore develop-
ment? We propose that de novo synthesized sphin-
golipids may act as signaling molecules to regulate the
cell cycle progression of the second pollen mitosis.
Failure of the cell cycle progression, due to reduced de
novo synthesis of sphingolipids, may consequently
trigger PCD. Alternatively, de novo synthesized sphin-
golipids may negatively regulate a PCD pathway, which
may be coupled with the cell cycle control during the
second pollen mitosis. A third possibility is that re-
duced or lack of de novo synthesized sphingolipids
blocks biogenesis of the cellular membrane, which, in
turn, triggers PCD in microspores. Currently, we are
investigating these possibilities by additional experi-
ments.

Sphingolipids have long been considered as major
signaling molecules to regulate cell division, cell differ-
entiation, and cell death. Our findings illustrate an im-
portant regulatory role of the sphingolipid-modulated
PCD in germline cell development. More specifically,
together with the observation made in the mmd1 mut-
ant, results presented in this study suggest that PCD
can be specifically initiated during male gametophyte
development at different stages, which may act as a
cellular surveillance mechanism to monitor the male
reproductive process. Therefore, PCD plays a more
general regulatory role in male reproductive develop-
ment than previously appreciated.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Genetic
Analysis of fbr Mutants

The Columbia ecotype of Arabidopsis (Arabidopsis thaliana) was used in this study. Unless otherwise indicated, plants were grown under a 16-h-light/8-h-dark cycle at 22°C in soil or on Murashige and Skoog medium (Murashige and Skoog, 1962) containing 3% Suc and 0.8% agar. The fbr11-1 mutant has been previously described (Shi et al., 2007). Other mutants (fbr11-2, lcb1-1, lcb2a, and lcb2c; Alonso et al., 2003) were obtained from the Arabidopsis Biological Resource Center.
Reciprocal crosses and genetic analysis of male gametophytic mutants were performed essentially as described (Johnson-Brosseau and McCormick, 2004). Transformation of Arabidopsis was done by vacuum infiltration (Bechtold et al., 1993).

Analysis of Nuclear DNA Fragmentation in Pollens

To prepare pollen grains for the TUNEL analysis, flower buds at appropriate stages were homogenized in phosphate-buffered saline (PBS) containing 4% paraformaldehyde, and the mixture was passed through a nylon filter (60 μm). The pollen suspension was fixed for at least 3 h at room temperature. After dehydration in 90% methanol and 10% 0.5 x EtGA (ME), pH 8.0, 4% paraformaldehyde in PBS (PP) gradients (ME-PP = 1.3; 1.1, and 3.1), and absolute ethanol twice, the sample was cleared in 100% xylene. After redrying in ethanol gradients (95%, 85%, 70%, 55%, and 35%), the sample was resuspended in 10 ml Tris-HCl, pH 7.4, and digested in 1 mg/ml proteinase K for 40 min at 37°C. After washing with PBS, the sample was subjected to TUNEL analysis using the In Situ Cell Death Detection kit according to the manufacturer’s instructions (Roche Diagnostics). The reaction was carried out at 37°C for 1 h in the dark, briefly washed with PBS, and then stained with 1 mg/mL DAPI for 5 min at room temperature. The sample was permanently mounted on a poly-Lys slide and analyzed under a fluorescent microscope or a confocal microscope (Olympus FV1000).

Light Microscopy and Electron Microscopy

To examine pollen viability, pollen grains were stained with Alexander solution (Alexander, 1969). The pollen nuclei were stained with DAPI (Park et al., 1998). Light microscopy was carried out as described previously (Feng et al., 2007) with minor modifications. Briefly, samples were fixed in formaldehyde-acetic acid (FAA) overnight. After dehydration in gradual ethanol series, the samples were embedded in historesin (Leica). Semithin sections (3 μm) were stained with 0.1% (w/v) aniline blue and then examined under a light microscope.

Transmission electron microscopy was carried out as previously described (Dong et al., 2007) with minor modifications. Samples were fixed in 2.5% glutaraldehyde, 2% paraformaldehyde, and 0.1 M sodium cacodylate, pH 7.4, at 4°C overnight, followed by post-fixation in 2% osmium tetroxide or 1% potassium permanganate for 1 h at room temperature. After dehydration in gradual ethanol series, the samples were embedded in Spurr’s resin (Sigma). Ultrathin sections (70 nm) were prepared and mounted on formvar-coated copper grids. The sections were stained with lead citrate and uranyl acetate, and then analyzed with a transmission electron microscope (model JEM-1200; JEOL). For scanning electron microscopy, samples were fixed, postfixed, and dehydrated as described above. Samples were critical-point dried in liquid CO2, mounted, sputter coated with gold particles, and then observed under a scanning electron microscope (model S-570; Hitachi) as described (Feng et al., 2007).

Molecular Manipulations

All molecular manipulations were carried out according to standard methods (Sambrook and Russell, 2001). Transgenic plants carrying an FBR11 transgene (in a pER8 binary vector) have been described elsewhere (Shi et al., 2007). pE8-LCB2a and pE8-LCB2b were constructed by a similar approach in which approximately 1.3 and 1.0 kb of the promoter sequences were in-frame with the putative translation start codon, was obtained by PCR. The PCR fragment digested by HindIII and XbaI was inserted into the same sites of pB123 (CLONTECH).

Analysis of gene expression by RT-PCR and real-time PCR was performed essentially as previously described (Sun et al., 2003; Dong et al., 2007). Genotyping of fbr11 and lcb2 mutants was performed by PCR with three primers as previously described (Dong et al., 2007). All primers used in this study are listed in Supplemental Table S2.

Analysis of GUS Activity

Histochemical analysis of GUS activity was performed as described (Jefferson et al., 1987). After staining at 37°C for 8 to 12 h, samples were cleared in 70% ethanol and photographed under a dissection microscope. For sections, the stained samples were fixed in FAA at 4°C overnight, and then embedded in Leica historesin. Semithin sections (3 μm) were cut and analyzed under a microscope.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. DNA sequence analysis of the T-DNA insertion site in the lcb1-1 (fbr11-3) mutant genome.

Supplemental Figure S2. Analysis of LCB1/FBR11::GUS expression in transgenic plants.

Supplemental Table S1. Genetic analysis of lcb1-1 (fbr11-3).

Supplemental Table S2. Primers used in constructs and assays.

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