Cytochrome P450 Family Member CYP704B2 Catalyzes the \(\omega\)-Hydroxylation of Fatty Acids and Is Required for Anther Cutin Biosynthesis and Pollen Exine Formation in Rice

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INTRODUCTION

Male reproductive development in flowering plants is a complex biological process starting from the formation of the anther with differentiated tissues, in which haploid microspores/pollen are generated, and then followed by anther dehiscence and pollination. When anther morphogenesis is complete, the meiotic cells (also called microsporocytes) at the center of each anther lobe are surrounded by four somatic layers, which are the epidermis, the endothecium, the middle layer, and the tapetum from the surface to the interior (Goldberg et al., 1995). The development of viable pollen grains within the anther is a prerequisite for the propagation of flowering plants, which requires the cooperative interaction of sporophytic and gametophytic genes (McCormick, 1993; Goldberg et al., 1995; Scott et al., 2004; Ma, 2005).

To ensure reproductive success, plants have established two barriers for protecting the microspore/pollen grain from various environmental and biological stresses. One is the anther wall with its four somatic layers, in particular, the epidermal layer covered by a cuticle. After anther dehiscence, the exine, forming the outer wall of the pollen, acts as another major barrier offering pollen grains high resistance to environmental stresses (Piffanelli et al., 1998). The biochemical nature of the anther surface and pollen exine remains elusive due to the technical limitation of purifying and obtaining a large quantity of materials for analysis. Furthermore, sporopollenin, forming the pollen/spore exine, is highly insoluble, hard to degrade, and exceptionally stable (Brooks and Shaw, 1978; Ahlers et al., 2003). Until now, only aliphatic polyhydroxy compounds and phenolic OH groups were identified in sporopollenin of Typha angustifolia pollen by NMR spectroscopy analysis (Ahlers et al., 2000, 2003). The formation of the pollen exine largely relies on the secretory role of the tapetum (Pacini et al., 1985; Shivanna et al., 1997), which starts depositing lipidic precursors onto the surface of microspore-derived cellulosic primexine shortly after tetrad release, leading to the formation of the sculptured exine (Piffanelli et al., 1997; Blackmore et al., 2007). In later pollen developmental stages, the tapetum produces and secretes lipidic components of pollen coat/tryphine into exine cavities (Piffanelli et al., 1997; Blackmore et al., 2007).

Recent forward and reverse genetic approaches greatly facilitated our understanding of the molecular regulation of the formation of aliphatic biopolymers, such as sporopollenin and...
epicuticular waxes, during the anther development (Ma, 2005; Wilson and Zhang, 2009). Several Arabidopsis thaliana mutants, such as male sterile2, defective exine1, no exine formation1, and faceless pollen-1, display defects in pollen exine synthesis and deposition (Aarts et al., 1997; Fiebig et al., 2000; Paxson-Sowders et al., 2001; Ariizumi et al., 2003, 2004). MALE STERILITY2 (MS2) is expressed in the tapetum during pollen development in Arabidopsis; MS2 is a putative fatty acyl reductase that is supposed to convert fatty acids into fatty alcohols for production of sporopollenin (Aarts et al., 1997). Recently, the Acyl-CoA Synthetase5 (ACOS5) gene was found to function in a conserved biochemical pathway for biosynthesis of medium-chain fatty acyl-CoA during pollen wall development, and the Arabidopsis acos5 mutant does not form obvious pollen exine (Souza et al., 2009). Arabidopsis CYP703A2 is expressed mainly in microspores and tapetal cells. The cyp703A2 mutants display abnormal sporopollenin deposition and a partial male-sterile phenotype. Biochemical tests revealed that this CYP703 enzyme has the activity of fatty acid hydroxylase catalyzing the in-chain hydroxylation of C10 to C14 fatty acids required for pollen exine formation (Morant et al., 2007). The rice (Oryza sativa) Wax-Deficient Anther1 (WDA1) gene was shown to be expressed mainly in the epidermal cells of anther walls, and WDA1 was proposed to participate in the biosynthesis of very-long-chain fatty acids for the establishment of the anther cuticle and pollen exine (Jung et al., 2006). However, the mechanism underlying the synthesis of cutin monomers in the anther cuticle and sporopollenin of pollen exine remains less understood, particularly in the important crop plant rice.

Here, we report the key role of CYP704B2 in synthesizing the anther cuticle and pollen exine during anther development in rice. The cyp704B2 mutant is male sterile with undeveloped anther epidermal cuticle and aborted pollen grains without obvious exine. In agreement with this defect, the cyp704B2 anther had a large reduction of cutin monomers. The recombinant CYP704B2 protein catalyzed the hydroxylation of palmitic acid and unsaturated C18 fatty acids in the \( \omega \) position of the carbon chain. Interestingly, potential orthologs of rice CYP704B2 are found among the EST or genomic databases of bryophytes, pteridophytes, and spermatophytes, suggesting that CYP704B2 is involved in a conserved and ancient pathway for synthesis of cutin and sporopollenin during plant male reproductive and spore development.

RESULTS

Isolation and Phenotypic Analyses of the cyp704B2 Mutant

To identify new rice genes regulating male fertility, we generated a rice mutant library using the japonica subspecies 9522 background (O. sativa ssp. japonica) by treatment with \( ^{60} \text{Co} \gamma \)-ray (280 Gy) and screened for male sterile mutants (Chu et al., 2005; Liu et al., 2005; Chen et al., 2006; Wang et al., 2006). One mutant line displayed complete male sterility and normal female development confirmed by reciprocal cross analysis. All of the F1 progeny were fertile with an approximate 3:1 ratio for phenotype segregation in the F2 plants (fertility:sterility = 78:23), supporting that this mutation most likely occurs at a single recessive locus. We named this mutant cyp704B2 because of a deletion of the putative CYP704B2 gene detected in the mutant by map-based cloning approach (see below). The cyp704B2 mutant exhibited normal vegetative development (Figure 1A). Like wild-type plants, the cyp704B2 plants developed normal panicles and

![Figure 1. The Phenotype Comparison between the Wild Type and cyp704B2.](image-url)

(A) Comparison between the wild type (left) and cyp704B2 (right) after heading.
(B) Comparison between a part of the wild-type panicle showing dehisced anthers (left) and a part of the cyp704B2 mutant panicle (right) showing smaller anthers and only few whitened flowers (arrow) at pollination stage.
(C) The wild-type flower (left) and the mutant flower (right) before anthesis.
(D) Comparison of wild-type (left) and cyp704B2 (right) flower organs after removal of the palea and lemma.
(E) \( I_2-KI \) staining of the pollen grains within the anther of wild type (left) and cyp704B2 (right).

gl, glume; le, lemma; lo, lodicule; pa, palea; pi, pistil; st, stamen. Bars = 1 mm.
floral organs (Figures 1B to 1D), but the mutant anthers appeared smaller and light yellow without pollen grains (Figures 1D and 1E).

Defects of Anther Wall and Pollen Exine Development in cyp704B2

To determine the anther morphological defects in the cyp704B2 mutant, anther transverse sections were further examined. Based on the cellular events visible under the light microscope and previous classification of anther development (Feng et al., 2001; Itoh et al., 2005), we recently further delineated rice anther development into 14 stages (Zhang and Wilson, 2009). From stages 1 to 5, the anther primordia differentiate and form the characteristic anther structure with pollen mother cells, somatic cells, and connective and vascular tissues. Compared with the wild type, the cyp704B2 pollen mother cells appeared to undergo normal meiosis (Figures 2A and 2F) and form tetrads of haploid microspores from the early stage 8 to the late stage 8 (Figures 2B and 2G). In agreement with this, normal chromosome separation during meiosis and in the derived tetrads was observed by 4',6-diamidino-2-phenylindole staining in the cyp704B2 mutant (see Supplemental Figure 1 online).

Subsequently, cyp704B2 anthers displayed obvious morphological abnormalities. At stage 9, young microspores were released from the tetrad, tapetal cells became more condensed and deeply stained, and the middle layer appeared degenerated and almost invisible in the wild type (Figure 2C). Although the cyp704B2 microspores appeared normal, the tapetal cells seemed less condensed at this stage (Figure 2H). At early stage 10, vacuolated and round shaped microspores with visible exines were observed in the wild type, and the degradation of the tapetum continued (Figure 2D). By contrast, the tapetal cells in cyp704B2 were swollen and lightly stained, and the microspore became less vacuolated and collapsed without an obvious exine on its outer surface (Figure 2I). At the late stage 10, the wild-type tapetal layer nearly disappeared and it was weakly stained. Vacuolated microspores with abundant exine depositions were visible (Figure 2E). Unlike those of wild-type plants, cyp704B2 microspores were aborted, and only remnants remained at the center of the anther locule (Figure 2J). In addition, the mutant middle layer and tapetum expanded until they occupied the space of locule (Figure 2J).

To gain a more detailed observation on the defects in cyp704B2, anther samples were investigated using transmission electron microscopy (TEM) (Figure 3; see Supplemental Figure 2 online). Consistent with the observations of the light microscopy sections, at late stage 8, the middle layer of wild-type anthers began to degenerate, and the tapetal cells appeared vacuolated, with some electron-opaque primary orbicules or Ubisch bodies being extruded onto the peripheral side of the tapetum (Figure 3A; see Supplemental Figures 2A and 2I online). Meanwhile, the initiation of primexine formation was observed on the microspore surface of the wild type within the tetrad (Figure 3I; see Supplemental Figure 2R online). At this stage, the microspore development of the cyp704B2 mutant was similar to that of the wild type (Figure 3M; see Supplemental Figure 2V online), but the tapetal layer developed abnormal and smaller extruding orbicules on its peripheral region in the cyp704B2 mutant (Figure 3E; see Supplemental Figures 2E and 2M online). At stage 9, within wild-type tapetal cells, the vacuoles were reabsorbed and the cytoplasm

Figure 2. Transverse Section Analysis of the Anther Development in the Wild Type and cyp704B2.
Locules from the anther section of wild type (A to J) and cyp704B2 (F to J) from stage 8 to stage 10. DMsp, degenerated microspores; E, epidermis; En, endothecium; M, middle layer; Msp, microspores; PMC, pollen mother cell; ST, swollen tapetal layer; T, tapetal layer; Tds, tetrads. Bars = 15 μm.
(A) and (F) Early stage 8.
(B) and (G) Late stage 8.
(C) and (H) Stage 9.
(D) and (I) Early stage 10. The tapetal cells were swollen, the microspore started being degraded, and there was no visible exine profile on the microspore in cyp704B2 (I) compared with the wild type (D).
(E) and (J) Late stage 10. The cells of the middle layer and tapetum expanded unceasingly, and degraded microspores were observed in the mutant (E).
Figure 3. TEM Analysis of Anthers in the Wild Type and cyp704B2.

(A) to (D) Cross sections of the wild-type tapetum at late stage 8 (A), stage 9 (B), early stage 10 (C), and late stage 10 (D).

(E) to (H) Cross sections of the cyp704B2 tapetum at late stage 8 (E), stage 9 (F), early stage 10 (G), and late stage 10 (H).

(I) to (L) The pollen exine development of the wild type from late stage 8 to late stage 10.

(M) to (P) The defective pollen exine development of cyp704B2 from late stage 8 to late stage 10.

(Q) to (T) The outer region of anther epidermis in the wild type from late stage 8 to late stage 10.

(U) to (X) The outer region of anther epidermis in cyp704B2 from late stage 8 to late stage 10.

AOr, abnormal orbicule; Ba, bacula; C, cuticle; CW, cell wall; DMsp, degenerated microspore; DPE, degenerated pollen exine; E, epidermis; En, endothecium; ER, endoplasmic reticulum; Ex, exine; M, middle layer; Msp, microspores; N, nucleus; Ne, nexine; Or, orbicule; PE, prim-exine; PT, peritapetal region; ST, swollen tapetal layer; T, tapetal layer; Tds, tetrads; Te, tectum. Bars = 1 μm in (A), (B), and (E), 2 μm in (C) and (G), 5 μm in (D), 10 μm in (H), 200 nm in (N) and (P), and 500 nm in (I), (J), (K), (L), (M), (O), and (Q) to (X).
appeared condensed, with many electron-dense orbicules extruded onto the locular side of the tapetum (Figure 3B; see Supplemental Figures 2B and 2J online). This indicates that materials, which can be speculated to be sporopollenin components, are actively transported from the sporophytic tapetum to gametophytic microspores. As a result, the primary structure of the exine, composed of the nexine and the baculum, was observed at the surface of the wild-type microspores released from the tetrads at this stage (Figure 3J; see Supplemental Figure 2V online). In the cyp704B2 mutant, the tapetal cells seemed slightly less stained (Figure 3F; see Supplemental Figures 2F and 2J online). Furthermore, no obvious orbicules from the tapetal layer were observed (Figure 3F), and the sporopollenin precursor seemed to be randomly deposited onto the microspore surface of the cyp704B2 mutant (Figure 3N; see Supplemental Figure 2V online). At early stage 10, the innermost somatic tapetal layer became condensed and the nucleus was degraded (Figure 3C; see Supplemental Figures 2C and 2K online). In addition, the amount of densely stained sporopollenin precursors carried by spheroid orbicules that were actively synthesized in the tapetum reached a maximum, and after being deposited, they were polymerized to form a regular exine structure with nexine, baculum, and tectum on the microspore surface (Figures 3C and 3K; see Supplemental Figure 2S online). Different from the wild type, the cyp704B2 tapetal cells showed hypertrophy with weakly stained cytoplasm and intact nuclei (Figure 3G; see Supplemental Figures 2G and 2O online). There were still no orbicules accumulating at the locular side of the tapetum, and thinner and abnormal exine structure appeared on the surface of the collapsing microspores (Figures 3G and 3O; see Supplemental Figure 2W online), suggesting that the cyp704B2 tapetum had metabolic defects in synthesis and transport of sporopollenin precursor components. At late stage 10, the wild-type anther wall became degenerated, displaying protrudent hair-like structures on the epidermis (Figure 3T). This indicated that lipophilic materials (cutin and wax) diffused to the surface of the anther cell wall. However, even though the cyp704B2 anther epidermis developed a cell wall structure, it was weakly stained, suggesting decreased amounts of lipophilic materials deposited to the outer epidermal cell wall during these stages (Figures 3V and 3W). Surprisingly, all the cell walls of the middle layer, the endothecium, and the epidermis of the mutant also displayed a weak staining (see Supplemental Figures 2G and 2O online). Furthermore, the cyp704B2 cuticle was weakly stained from stage 9 to the early stage 10 (Figures 3V and 3W). Particularly, at late stage 10, the whole anther structure looked degenerated in the cyp704B2 mutant (see Supplemental Figure 2H online), and the thickness of the cell wall and cuticle was reduced greatly in the mutant (Figure 3X). This observation suggests that abnormal synthesis/supply of lipophilic molecules for anther epidermal cuticle formation occurs in the cyp704B2 anther.

In agreement with these TEM observations, we noted reduced cell length and width in the cyp704B2 anther epidermis compared with the wild type by scanning electron microscopy examination (Figures 4A and 4B). Moreover, we observed a normal outermost linear-shaped surface of the wild-type anther (Figure 4C), and the pollen developed the exquisite exine pattern and a visible germination pore (Figure 4D). However, a glossy and smooth anther surface (Figure 4E) and shrunken

![Figure 4](image-url)

**Figure 4.** Observation of the Anther and Pollen Grain in the Wild Type and cyp704B2 by Scanning Electron Microscopy. (A) and (B) Wild-type (A) and cyp704B2 (B) anthers at stage 13. (C) and (E) The outmost surface on epidermis of the wild-type (C) and cyp704B2 (E) anthers at stage 13. (D) and (F) The pollen in wild type (D) and cyp704B2 (F) at stage 10. GP, germination pore. Bars = 20 μm in (A) and (B), 10 μm in (C), and 5 μm in (D) to (F).
microspores (Figure 4F) in cyp704B2 were observed as a result of the undeveloped cuticle/epicuticular structures and lack of obvious pollen exine.

**CYP704B2 Is Required for Synthesis of Cutin Monomers**

The phenotypic defects of anther cuticle and pollen exine suggested that the cyp704B2 anther had an alteration of biosynthesis or transport of cuticle components as well as lipidic sporopollenin components. To gain further insights into anther wax and cutin biosynthesis, we measured the compositions of the chloroform-extractable cuticular wax and the aliphatic cutin monomers in the anthers of both the wild type and cyp704B2 by gas chromatography–mass spectrometry (GC-MS) and gas chromatography–flame ionization detection (GC-FID) (Bonaventure et al., 2004; Franke et al., 2005). We developed an approach to calculate the surface area of the anthers, and these calculated values of surface area were plotted against the weight of each sample (see Supplemental Figure 3A online). From GC-FID analysis, we observed that the total wax amount per mm² of anther in the cyp704B2 mutant was very close to that of the wild type (Figure 5A).

The cutin polyester from delipidated anthers was transesterified to analyze anther cutin monomer compositions (Bonaventure et al., 2004; Franke et al., 2005). An earlier study by Jung et al. (2006) indicated that the applied transesterification conditions did not release cutin-like monomers out of isolated pollen; therefore, the identified monomers were released from the epidermal cutin of anthers by this method. In the wild-type anther, we detected ~0.824 µg of cutin monomers per mm² anther epidermis. By contrast, only 0.0586 µg/mm² was detected in the epidermis of the cyp704B2 anther, which corresponds to >90% reduction of the total cutin amount compared with the wild type (Figure 5A; see Supplemental Table 1 online). In wild-type anthers, the dominating aliphatic cutin monomers in the methanolysate were palmitic acid, 16-hydroxy-palmitic acid, 9 (10),16-dihydroxy-palmitic acid, 18-hydroxy-oleic acid, and 9,10-epoxy-18-hydroxy-oleic acid (Figure 5B) and compounds reported as unidentified rice cutin monomers (URCMs) in rice anthers (Jung et al., 2006). We have tried to identify the URCM1, URCM2, and URCM3 peaks, but did not succeed. However, the mass spectra of URCMs suggested that these three peaks are likely unsaturated compounds and most probably are hydroxylated. In contrast with the wild type, a large reduction of all aliphatic cutin monomers was observed in the cyp704B2 anther (Figure 5B; see Supplemental Figures 4B and 4C online). Therefore, from the chemical analysis of cutin and wax, we assumed that the mutant gene is involved in the formation of precursors needed for the establishment of anther cutin and pollen exine during rice anther development.

**Map-Based Cloning of CYP704B2**

To identify the mutant gene, we used a map-based cloning approach. Based on primary mapping, we mapped this gene to a genetic distance of 16.8 and 17.9 centimorgans with CL6-4 and SJ301 markers on chromosome 3 (Figure 6A). To localize the mutant gene more precisely, 2700 individuals of F2 mapping population were analyzed using a set of primer pairs (see Supplemental Table 2 online). Finally, this mutant gene was located between two markers SJ622 and LH301, defining a DNA region of 116 kb, and these two markers were located in the BAC clones OSJNBA0026H19 and OSJNBA0091P11, respectively. No recombinant was found using another marker CL8-1 (Figure 6A). By PCR amplification using the forward primer 3F and reverse primer 2R (Figure 6B) and sequencing analysis, we found in the mutant a DNA fragment deletion of 3102 bp, which included 258 bp of the 3’ coding region of Os03g07260, 1732 bp of the genomic region of Os03g07250, and 1112 bp of the intergenic region between the two genes (Figure 6B). Os03g07260 was predicted to encode a tetratricopeptide repeat
domain containing protein, and Os03g07250 was predicted to encode a cytochrome P450 CYP704B2 protein (http://drnelson.utmen.edu/rice.html).

To determine which gene is responsible for the main defects of anther development in cyp704B2, two constructs were generated by PCR amplification from the BAC clone (OSJN-Ba0091P11): one carrying a 3.8-kb wild-type genomic fragment for Os03g07250/CYP704B2 using the primer pair 1F and 2R, and another one containing the fragment of 4 kb for Os03g07260 from the primer pair 4F and 3R (Figure 6B). Among the transgenic plants obtained from transformed calli induced from the flower of the cyp704B2 mutant, only the construct including Os03g07250 was able to restore pollen fertility and normal anther epidermal surface in the homozygous mutant plants (see Supplemental Figure 4 online). These results confirmed that the deletion of Os03g07250 is responsible for the developmental defects found in this mutant.

To verify the gene structure of the CYP704B2 gene, we determined exon-intron junctions by sequencing the cDNA fragment amplified by RT-PCR. Comparison with the genomic sequence indicated that CYP704B2 contained four exons and three introns (Figure 6B). CYP704B2 putatively encodes a cytochrome P450 protein with 545 amino acids containing a transmembrane segment of 23 amino acids (VTSFPVAGHKLIAIFLVLSW) in the N-terminal region (Figure 6C).

CYP704B2 Regulates Anther Development

CYP704B2 Belongs to an Ancient and Conserved Family among Terrestrial Plants

Each plant species has a large number of P450 members with a diversity of biological functions in biosynthetic and catabolic pathways (Schuler and Werck-Reichhart, 2003). To place CYP704B2 in the P450 evolutionary tree and gain information on its potential function, we used the full-length CYP704B2 protein as the query to search for its closest relatives in EST databases. A total of 39 putative ESTs and annotated protein sequences that are related to rice CYP704B2 were obtained from 19 different species, in addition to rice and Arabidopsis, belonging to moss, pteridophytes, gymnosperm, and angiosperms (see Supplemental Table 3 online). Along with the six and two members of the CYP704A subfamily encoded by the genome of rice and Arabidopsis, respectively (Figure 7), these P450s belonged to four families all from the CYP86 clan: CYP86, CYP94, CYP704, and CYP96. Several members in the CYP86 clan have been shown to catalyze fatty acid hydroxylation in vitro (Le Bouquin et al., 1999; Wellesen et al., 2001; Greer et al., 2007; Hofer et al., 2008). No putative CYP704 ortholog was identified in green algae, and CYP704Bs are encoded by a single gene subfamily in rice, Arabidopsis, and other land plants (Nelson et al., 2004) (black dots in Figure 7). Furthermore, most of the CYP704B subfamily members were found to be expressed in the reproductive organs, such as inflorescences, male organs, and spores, among plants from bryophyte to angiosperms (see Supplemental Table 3 online) (Zhang et al., 2006). Altogether, these observations suggest an essential and conserved function of CYP704B2 in fatty acid oxygenation during plant male reproductive development.

Figure 6. Cloning and Analysis of CYP704B2.

(A) Fine mapping of the CYP704B2 gene on chromosome 3. Names and positions of the markers are noted. cM, centimorgan.

(B) A schematic representation of the exon and intron organization of Os03g07250 and Os03g07260 in the deletion region. +1 indicates the putative starting nucleotide of translation, and the stop codon (TGA) is +1635. Black boxes indicate exons, and intervening lines indicate introns. The region between the two long arrows indicates the deleted fragment. Numbers indicate the exon length (base). Short arrows indicate the primer locations.

(C) The amino acid sequence of CYP704B2. The predicted transmembrane region is boxed, and the region of the deletion in the mutant is underlined.

CYP704B2 Is Specifically Expressed in the Tapetum and Microspores

The main morphological defects of the cyp704B2 mutant occur in anther development, while there is no obvious phenotype during vegetative growth. To confirm that the function of CYP704B2 is...
confined to the anther development, we further investigated expression of CYP704B2. According to the analysis of the Massive Parallel Signature Sequence data (Nobuta et al., 2007), CYP704B2 was predicted to be expressed in immature rice panicles. In addition, recent expression profile data from laser microdissection–mediated microarray analysis showed that the CYP704B2 gene was expressed in both the tapetum and microspores in the developing anther (Hobo et al., 2008). Consistently, our RT-PCR analysis also revealed that CYP704B2 was expressed in the anther from stage 8 to stage 10, but not in the root, stem, leaf,

Figure 7. Protein Phylogeny of the CYP704 Family and Related P450 Enzymes.
A maximum likelihood analysis was performed using MEGA 3.1 based on the alignment given in Supplemental Data Set 1 online of CYP704B2 with the most similar P450 sequences from rice (Os), Pinus taeda (Pta), P. patens (Pp), Zea mays (Zm), Medicago truncatula (Mt), Arabidopsis (At), Vitis vinifera (Vv), Ceratopteris richardii (Cr), and Populus trichocarpa (Pt), CYP97B2 from rice and CYP703A2 from Arabidopsis were used as outgroups. Bootstrap values are percentage of 1000 replicates. Black dots show the CYP704B subfamily members.
palea/lemma, and pistil (Figure 8A). CYP704B2 expression started at stage 8, reached the highest level at stage 10, and then dropped to an undetectable level at stage 13 (Figure 8A).

In transgenic plants expressing the CYP704B2pro:GUS fusion (for expression of the β-glucuronidase [GUS] marker protein driven by the promoter region of CYP704B2), GUS staining was specifically detected in the anther from stage 8 to stage 10, and no expression was found in the palea, lemma, and pistil (Figures 8B and 8C). When observed by phase-contrast microscopy using cleared mounted anthers (Willemsen et al., 1998), only the tapetal layer and microspores showed GUS staining (Figures 8D and 8E). All data therefore converge to indicate a specific function of CYP704B2 in the tapetal cells and microspores essential for anther and pollen development.

CYP704B2 Catalyzes ω-Hydroxylation of C16 and C18 Fatty Acids

To determine the CYP704B2 metabolic activity, CYP704B2 was heterologously expressed in the Saccharomyces cerevisiae strain WAT11 stably modified to express the Arabidopsis NADPH P450 Reductase1 under the control of the galactose-inducible promotron GAL10-CYC1 (Pompon et al., 1996). Due to the steep decrease of cutin monomers in the cyp704B2 anther and clustering of CYP704B2 with the reported fatty acid hydroxylases (Benveniste et al., 1998; Wellesen et al., 2001), fatty acids (C12:0, C16:0, C18:1, C18:2, C18:3, and epoxystearic acid) likely involved in cutin synthesis were tested as substrates of CYP704B2 in recombinant yeast microsomes.

Figure 9 shows the radioactivity profiles obtained after incubation of oleic acid (C18:1) with microsomes from yeast expressing CYP704B2 as analyzed by thin layer chromatography. Addition of NADPH in the incubation led to the formation of a major radioactive peak 1 (Figure 9B), which is not present without NADPH (Figure 9A). It was not formed when microsomes were boiled or in incubation with microsomes from yeast transformed with an empty plasmid (data not shown). This peak contains only one metabolite, which was identified by GC-MS analysis after purification and derivatization as 18-hydroxyoleic acid. In order to study the substrate specificity of CYP704B2, saturated and unsaturated fatty acids with different chain lengths were incubated at a concentration of 100 μM with microsomes of the transformed yeast. As shown in Figure 9C, CYP704B2 was able to metabolize palmitic, oleic, linoleic, and linolenic acids, and

Figure 8. The Expression Pattern of CYP704B2 by RT-PCR and GUS Assay.

(A) Spatial and temporal expression analyses of CYP704B2 by RT-PCR. Rice Actin1 (OsActin1) expression was used as a control. Pa/Le: palea and lemma; Pre-M, the flowers before stage 8 of anther development; M, early stage 8; T, late stage 8; Y, stage 9; V, stage 10; H, the stage 11 to 13 anther; GDNA, genomic DNA.

(B) GUS expression (blue staining) patterns in the heterozygous spikelets of the CYP704B2pro::GUS transgenic line at various stages. 1, Flowers before stage 8 of anther development; 2, early stage 8; 3, late stage 8; 4, stage 9; 5, early stage 10; 6, late stage 10; 7, stage 13.

(C) GUS staining in the flower shown at stage 9 after removal of the palea and lemma.

(D) Phase-contrast microscope analyses of the cleared mounted anther at stage 10.

(E) Magnified image of the top region in (D).

E, epidermis; En, endothecium; Msp, microspores; T, tapetal layer. Bars = 0.5 mm in (B) and 20 μm in (C) to (E).
GC-MS analysis of the products indicated that they were all monohydroxylated in omega of the fatty acid carbon chain. Neither lauric acid (C12:0) nor epoxystearic acid was metabolized by CYP704B2. The turnover number of CYP704B2 with C16 and C18 fatty acids as substrates was similar to that of Arabidopsis CYP704B1 (Dobritsa et al., 2009) and those previously reported for CYP94A1, CYP709C1, and CYP94C1 (Tijet et al., 1998; Kandel et al., 2005, 2007).

To further investigate the in planta substrate and products of CYP704B2, lyophilized methanol extracts from cyp704B2 anthers were tested as substrates for the recombinant enzyme (Morant et al., 2007). Fatty acids with different carbon chain lengths, including palmitic, palmitoleic, oleic, and linoleic acids and others, were detected by GC-MS in the anther methanol extract incubated with yeast-expressed CYP704B2 in the absence of NADPH (Figure 10A). After incubation in the presence of NADPH, five new metabolites were observed (Figure 10B), three of them fragmenting as 16-hydroxypalmitoleic acid, 16-hydroxypalmitic acid, or 18-hydroxyoleic acid (Figure 10B). Two metabolites (1 and 2) present only after incubation with NADPH could not be identified from their fragmentation profiles. Although high levels of linoleic acid were detected in the incubation samples (Figure 10), no corresponding hydroxylated linoleic acid product was detected after incubation with recombinant CYP704B2 as judged by comparing the fragmentation pattern and retention time to reference compounds generated by CYP94A1 (Pinot et al., 1999).

To clarify this inconsistency with the observed conversion of radiolabeled linoleic acid by yeast-expressed CYP704B2, we analyzed directly the anther methanol extract. Surprisingly, the mass spectra of the major peaks for C18:1 and C18:2 fatty acids indicated that they were methylated (when omitting the methylation and trimethylsilylation steps before GC-MS). C16:0 was, however, detected as a free fatty acid. This was not related to the extraction process and was confirmed after dichloromethane extraction of the anthers. The C16:1 and C18:1 fatty acids converted in the assay with the anther extract were thus those found in yeast microsomes (Tuller et al., 1999) as recently described by Dobritsa et al. (2009). On the other hand, the hydroxylated C16:0 in the assay was of mixed yeast and anther origin. Our data thus indicate that methylated fatty acids are not metabolized by recombinant CYP704B2 and confirm hydroxylation of free fatty acids. They also suggest that C16:0 might be the only and physiological CYP704B2 substrate present in the anther extract.

The possibility that acyl-CoAs could be the in vivo substrates of CYP704B2 was also considered. A very fast hydrolysis of CoA esters was observed upon incubation with yeast microsomes. It was thus not possible to determine if they can be substrates for CYP704B2.

**DISCUSSION**

**CYP704B2 Is Required for Microspore Exine and Anther Development in Rice**

Regulation of the fertility of pollen grains is critical for rice breeding, particularly for hybrid rice production. In rice breeding, many cytoplasmic and nuclear genic mutations leading to male sterility lines are considered of agricultural importance for the production of hybrids to improve yield. This study identified a cytochrome P450 protein, CYP704B2, and showed that it plays an important role in rice postmeiotic anther development. Our morphological and biochemical analysis of the cyp704B2 mutant suggests that abnormal lipidic metabolism in the mutant anther affects tapetum and microspore development and the biosynthesis of essential precursors of the pollen wall and anther epidermal cutin (Figures 2, 3, and 5B). The cyp704B2 tapetum could not undergo normal degradation and displayed no obvious cytological features of tapetum degeneration, such as cytoplasmic shrinkage and nucleus breakage, from stage 8 to stage 10. Additionally, cyp704B2 developed no obvious orbicules along...
the locular side of the tapetum, indicating defects in synthesis and transport of sporopollenin precursors from the tapetum to the surface of microspores for exine formation in the mutant. The formation of the pollen wall starts from the outer surface of the microspore. The precursors of sporopollenin from the tapetum are transported and deposited onto the structure of primexine on the microspore plasma membrane for exine formation. Therefore, the cooperative action between the tapetum and microspore is critical for pollen wall synthesis (Bedinger, 1992). This is supported by the fact that several genes regulating pollen exine development have been shown to be expressed in both tapetal cells and microspores (Wang et al., 2002; Morant et al., 2007; Guan et al., 2008; Aya et al., 2009). Similarly, \textit{CYP704B2} is expressed in both the tapetum and microspores. The observed pollen defect of \textit{cyp704B2} is thus likely not only associated with aberrant tapetal development but also with the microspore itself. The resulting failure of pollen wall formation and abortion of microspores in \textit{cyp704B2} led to male sterility. \textit{CYP704B2} is highly expressed in the tapetal layer and microspores from stage 8 to stage 10. Despite its expression pattern being apparently restricted to the tapetum and microspores, \textit{CYP704B2} is surprisingly critical for cuticle development on the surface of epidermal cells. At late stage 10, undeveloped cuticle and no epicuticular structure existed on the anther surface of the \textit{cyp704B2} mutant (Figures 3 and 4). However, the epicuticular structures of the leaf, stem, and palea/lemma of \textit{cyp704B2} were very similar to those of the wild type (see Supplemental Figure 5 online), indicating the indispensability of \textit{CYP704B2} in anther development. The anther cuticle structure defect can be explained by the biochemical evidence that the synthesis of cutin monomers, such as 16-hydroxypalmitic and 18-hydroxystearic acids, and their derivatives and precursors, were largely reduced in the \textit{cyp704B2} anthers (Figure 5A; see Supplemental Table 1 online). A lack of the cutin matrix may impede the proper incorporation of intracuticular and epicuticular waxes into the cuticle framework even though biosynthesis of waxes was not obviously blocked in the mutant, leading to a glossy and smooth anther surface.

The observation of concomitant defects of the anther cuticle and microspore exine in the null mutants suggests that a common biosynthetic pathway for cutin and sporopollenin precursors likely exists in anthers (Figures 3 and 5). \textit{CYP704B2} belongs to the CYP86 clan of P450 oxygenases that include the CYP86 subfamily involved in fatty acid hydroxylation and in several cases confirmed to be involved in the biosynthesis of cutin or suberin. The phylogeny thus suggested that the function of the \textit{CYP704} family is possibly related to the synthesis of cutin or suberin monomers. Sporopollenin is described as a biopolymer derived from oxygenated fatty acids and phenylpropanoids (Ahlers et al., 2000, 2003). Therefore, it was expected that sporopollenin, suberin, and cutin would share similar aliphatic constituents, polymerization, and function (Dominguez et al., 1999). The cellular and biochemical analyses of \textit{CYP704B2} confirms this hypothesis and demonstrates that cutin and

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**Figure 10.** GC-MS Chromatograms of the Incubation of CYP704B2 with Rice Anther Extracts in Absence or Presence of NADPH.

The GC profiles of substrates and metabolites obtained after incubation of methanol extracts from anthers with microsomes from yeast expressing \textit{CYP704B2}. Addition of NADPH (B) in the incubation media led to the formation of five metabolites that are not present without NADPH (A). Metabolites denoted by 1 and 2 have not been identified by mass spectra. C16:0, palmitic acid; C16:1, palmitoleic acid; C18:1, oleic acid; C18:2, linoleic acid; 16-OH C16:0, 16-hydroxy-palmitic acid; 16-OH C16:1, 16-hydroxy-palmitoleic acid; 18-OH C18:1, 18-hydroxy-oleic acid.
sporopollenin share common structural elements and biosynthetic steps during anther development. Because of the lack of mature pollen grains in cyp704B2 and the technical limitation in analyzing chemical resistant sporopollenin, we could not analyze the pollen wall composition in the mutant. The products of CYP704B2 are, however, good candidates as aliphatic constituents of sporopollenin, according to the pollen wall composition of T. angustifolia (Ahiers et al., 2000, 2003). Thus, the future work on CYP704B2 may help to reveal how common aliphatic metabolic pathway(s) contribute to anther cuticle formation and pollen exine development.

As the innermost sporophytic layer within the anther wall, the tapetum undergoes active lipid metabolism and timely degradation, which is crucial for microspore development and pollen maturation as well as anther cuticle formation (Pacini et al., 1985; Shivanna et al., 1997; Jung et al., 2006; Li et al., 2006; Zhang et al., 2008). Cytological analyses of several male sterile mutants of rice tapetum-expressed genes Tapetum Degredation Retardation and Undeveloped Tapetum1 indicated that defects in tapetal differentiation and development affected the degradation and development of other anther somatic layers (Jung et al., 2005; Li et al., 2006). Together with our data, it suggests that the precursors synthesized by the fatty acid hydroxylase in the tapetal cells or microspores can be transported within the anther and transferred to the epidermis to support anther cuticle development. Other genes, such as rice WDA1, preferentially expressed in anther epidermal cells, are also necessary for the synthesis of anther cuticle and pollen exine (Jung et al., 2006).

**CYP704B2 Belongs to a Conserved and Ancient Subfamily of Fatty Acid Hydroxylases**

The evolutionary mechanism behind plants colonizing land still remains a challenging question for biologists. The biopolymer sporopollenin of the exine constitutes of spores and pollen serves a pivotal role in the adaptation to land by plants (Blackmore et al., 2007). The cuticle is a thin hydrophobic layer continuously coating the outermost surface of the primary aerial organs, such as leaves, stems, flowers, and fruits. It plays key biological functions, such as limiting uncontrolled nonstomatatal gas, water, and solute loss (Riederer and Schreiber, 2001; Burghardt and Riederer, 2006), protecting plants from UV damage (Barnes and Cardoso-Vilhena, 1996; Holmes and Keiller, 2002; Pfundel et al., 2006), mediating the interactions of plants with insects and pathogens (Craver and Gurr, 2006; Leveau, 2006; Muller, 2006), and determining organ identity by preventing organ fusion (Lolle et al., 1998; Sieber et al., 2000). Even though the plant cuticle structure varies among species, organ types, and developmental stages, all cuticles are complex bio-polymers with two types of lipophilic materials, cutin and waxes (Kerstiens, 1996; Jeffree, 2006; Riederer and Muller, 2006; Koch and Enskiat, 2008; Samuels et al., 2008). The framework of the plant cuticle is determined by the cutin matrix, which is an insoluble polymeric composed of hydroxylated and epoxy C16 and C18 fatty acids (Kolattukudy, 2001; Heredia, 2003; Nawrath, 2006). By contrast, waxes belong to different substance classes (i.e., alkanes, alcohols, ketones, and wax esters composed of C24-C34 long-chain fatty acids and alcohols), which are embedded and superimposed within the cuticle and can be easily extracted by organic solvents (Kunst and Samuels, 2003).

In the plant kingdom, the CYP86 clan of P450 enzymes that is phylogenetically related to the animal and microbial fatty acid hydroxylases includes five families: CYP86, CYP94, CYP96, CYP730, and CYP704 (Nelson, 1999; Nelson et al., 2004). Three of those are related to lipid oxygenation. To date, all the reported members of the CYP86A and CYP94 subfamilies in Arabidopsis could catalyze oxygenation of fatty acids in vitro (Benveniste et al., 1998; Wellesen et al., 2001; Duan and Schuler, 2005; Kandel et al., 2007; Rupasinghe et al., 2007). Arabidopsis CYP96A15 (MAH1) was reported to be involved in the synthesis of wax as a mid-chain alkane hydroxylase (Greer et al., 2007). The CYP86, CYP94, and CYP704 families are represented in the ancestral land plant genome of Physcomytrella patens. The most expanded of these families in the moss is CYP704, mostly represented by the CYP704B subfamily. The CYP86 clan, and the CYP704B subfamily in particular, thus seems to have played an essential role in land colonization. This would be in good agreement with a role in the establishment of protective barriers against dessication and for water, gas, and solute exchange with the aerial environment. In agreement with this hypothesis, recombinant CYP704B2 shows fatty acid hydroxylase activity (Figures 9 and 10).

C16 and C18 fatty acids have been demonstrated to be the main substrates and units for plant cutin monomer synthesis (Pinot et al., 1992; Heredia, 2003; Heredia-Guerrero et al., 2008). Several P450 candidates were suggested to contribute to the synthesis of such monomers. Those include, for example, CYP86B2, CYP94C1, or CYP94A1 from Arabidopsis that were shown to catalyze omega oxygenation of saturated and unsaturated fatty acids of different chain lengths (C12 to C18) (Tijet et al., 1998). In wheat (Triticum aestivum), on the other hand, CYP709C1 was shown to hydroxylate C12 to 18 fatty acids at the omega-1 and omega-2 positions, with cis-9,10-epoxysearic acid as the best substrate (Kandel et al., 2005).

The activity of recombinant CYP704B2 measured in vitro is in the same range as those previously described for other plant fatty acids hydroxylases. It is interesting to note that CYP704B2, however, exhibits a different substrate specificity. It does not metabolize lauric acid or 9,10-epoxysearic acid, which is often the best substrate of plant fatty acid hydroxylases in vitro (Pinot et al., 1999; Kandel et al., 2005; Kandel et al., 2007). CYP704B2 more specifically ω-hydroxylates C16 and C18 saturated and unsaturated fatty acids. Consistent with this, we detected a large decrease of cutin monomers in the cyp704B2 anther by GC-FID analysis. The derivatives of palmitic, palmitoleic, and oleic acids are major components of cutin in plants (Heredia, 2003; Heredia-Guerrero et al., 2008; Pollard et al., 2008). It is interesting to note that unhydroxylated fatty acids were decreased in the cutin monomers of cyp704B2. This may either result from a decrease in cutin polymerization due to insufficient ω-hydroxylated fatty acids in the mutant anthers (Pollard et al., 2008) or from feedback regulation in the mutant. In support of the latter hypothesis, the development of the whole anther was obviously affected in the mutant.

Another phylogenetically unrelated P450 enzyme that is also highly conserved in land plants, CYP703A2 in Arabidopsis, was...
recently reported to catalyze in-chain hydroxylation of C10 to C14 fatty acids, with a preference for lauric acid. While the expression pattern of CYP703A2 in Arabidopsis is similar to that of CYP704B2 and to that of its Arabidopsis ortholog CYP704B1, the cyp703A2 mutant displays defective pollen development but only partial male sterility was reported, and there was no alteration in anther cutin development (Morant et al., 2007). Recent observations revealed that the putative ortholog of CYP703A2 in rice, CYP703A3, is also expressed in both the tapetum and microspores at about the same stages as CYP704B2. Insertional lines of rice CYP703A3 displayed aborted microspores with exine defects, leading to completely male sterile plants (Aya et al., 2009). Together, these findings suggest that the two conserved genes are required for converting distinct substrates in the aliphatic lipid pathway for sporopollenin synthesis. Interestingly, both CYP703 and CYP704B are conserved among land plants (Figure 7) (Morant et al., 2007). This probably reflects the critical need for spore protection against dessication via a sporopollenin-like biopolymer (Zetzsche et al., 1937; Blackmore, 2007). A comparative analysis of the functions of these genes might be helpful in understanding the conserved mechanism of spore/pollen wall development.

**The Role of CYP704B2 in Anther Cutin and Sporopollenin Synthesis**

C16 and C18 fatty acids are synthesized de novo in the plastid and transported to the endoplasmic reticulum for cutin monomer formation and/or elongation to very-long-chain fatty acids for wax biosynthesis (Harwood, 1988; Kunst and Samuels, 2003). According to the previous analyses of cutin and sporopollenin-defective mutants (Schnurr et al., 2004; Kurdyukov et al., 2006; Kannangara et al., 2007; Morant et al., 2007; Souza et al., 2009), in combination with the mutant phenotype and biochemical function of CYP704B2, we propose a model for cutin and sporopollenin biosynthesis during anther development (Figure 11). The ω-hydroxy-palmitic, -palmitoleic, and -oleic acids formed by CYP704B2 play a central role in the development of cuticular and sporopollenin polyesters according to this model. Within the endoplasmic reticulum, fatty acids with C16 and C18 as precursors are hydroxylated at the ω-carbon by CYP704B2. The resulting ω-hydroxylated fatty acids then contribute to the essential steps of the lipid metabolism/transport required for normal tapetum development and provide building precursors of sporopollenin units and cutin monomers during anther development. The ω-hydroxy palmitic and oleic acids were described as cutin monomers (Heredia, 2003; Heredia-Guerrero et al., 2008). Other monomers, such as 9(10),16-dihydroxy-palmitic acid, 9(10),18-dihydroxy-stearic, and 9,10-epoxy-18-hydroxy-stearic acids or dioic acids, can be obtained by the concerted action of other ω- or in-chain hydroxylases, epoxidases, epoxide hydrolases, or dehydrogenases (Le Bouquin et al., 1999; Kurdyukov et al., 2006; Kandel et al., 2007; Sauveplane et al., 2009). In addition, the acyl-CoA synthetases, such as LACS or ACOS5, can possibly regenerate fatty acyl-CoA ester for conversion by a fatty acyl reductase into fatty alcohol, which is necessary for pollen exine formation (Aarts et al., 1997; Schnurr et al., 2004; Kannangara et al., 2007; Souza et al., 2009). Subsequently or simultaneously, the cutin precursors are likely to be transported by lipid transport proteins to the anther epidermal cell wall for cuticle formation. Alternatively, some of these intermediates are transported to the locule as constituents of sporopollenin.

In conclusion, we identified CYP704B2 as a previously unknown gene in rice, encoding a cytochrome P450 protein. The deletion of CYP704B2 disturbs the pollen exine and anther cuticle development by blocking the ω-hydroxylation of fatty acids. This CYP704B2 belongs to a conserved and ancient cytochrome P450 family in moss and seed plants, suggesting that the ω-hydroxylation pathway by CYP704B2 is a key step for anther cuticle and spore/pollen exine formation during plant evolutionary history.

**METHODS**

**Mutant Materials and Growth Conditions**

All plants (Oryza sativa) were grown in the paddy field of Shanghai Jiaotong University. The F2 mapping population was generated from a
cross between the cyp704B2 mutant (japonica) and Guan Lu Ai (indica) (Chu et al., 2005; Liu et al., 2005; Chen et al., 2006; Wang et al., 2006). In the F2 population, male sterile plants were selected for gene mapping.

Characterization of the Mutant Phenotype

Plants and flowers at mature stage were photographed with a Nikon E995 digital camera. The wild-type and cyp704B2 mutant anthers were immersed into I2-KI solution and centrifuged for a short time to make the I2-KI solution enter into the anther for pollen starch staining, then photographed with a microscope (Leica DM2500). The cyp704B2 anther was stained by 0.2% 4',6-diamidino-2-phenylindole n-hydrate to observe the chromosomes. Observation of anther development by semithin sections and TEM was performed as described by Li et al. (2006). Scanning electron microscopy was performed as described by Zhang et al. (2008).

Cloning of CYP704B2 and Complementation

The Nipponbare BAC (OSJNBA0091P11) containing CYP704B2 and Os03g07260 was used as the template to amplify a 3.8-kb wild-type genomic DNA fragment for CYP704B2 with a 1231-bp 5’ upstream region, 1635-bp genomic region (including introns), and 649-bp 3’ stream region using the primer pair 1F, 5’-aaaaGTACCAAGTTGAAGAGA-GAAGGTGGA-3’ and 2R, 5’-aaaaCTGAGTTGTGGACACACGAG-GTAAG-3’, and a 4-kb wild-type genomic DNA fragment for Os03g07260 with a 692-bp 5’ region, 1611-bp genomic region, 1294-bp 3’ region using primers 4F, 5’-aaaaGTACCCACGTCTACCTCAACTCCATC-3’ and 3R, 5’-aaaaCTCCAGCTCTGACTGGAAGAAGATGA-3’. Then, the two DNA fragments were cloned into the vector pCAMBIA1301:GUS (kindly provided by Richard Jefferson) by restriction enzyme sites (underlined) to produce two plasmids, pCAMBIA1301:CYP704B2 and pCAMBIA1301: Os03g07260, respectively. These two plasmids were introduced into the cyp704B2 mutant plants by Agrobacterium tumefaciens-mediated transformation individually. The pollen grains of transgenic lines were assayed by I2-KI staining, and their anther cuticular structures were observed by scanning electron microscopy. The mutant background of transgenic lines, the segregation of fertility plants and sterility plants was 88:17; fertility:1 sterility. In the T1 generation of transgenic mutant plants by I2-KI staining, and their anther cuticular structures were observed by scanning electron microscopy. The mutant background of transgenic lines, the segregation of fertility plants and sterility plants was 88:17; fertility:1 sterility. In the T1 generation of transgenic lines, the segregation of fertility plants and sterility plants was 88:17; fertility:1 sterility. In the T1 generation of transgenic

RT-PCR and Promoter Fusions

Total RNA was isolated using Trizol reagent (Generay) as described by the supplier from rice tissues: roots, shoots, leaves, lemma/palea, pistils, and anthers at different stages. The stages of anthers were classified into categories according to the spikelet length (Feng et al., 2001). After treatment with DNase (Promega), 0.3 μg RNA was used to synthesize the oligo(dT) primed first-strand cDNA using the ReverTra Ace-a-First Strand cDNA synthesis kit (Ferment). One microliter of the reverse transcription product was subsequently used as the template in an RT-PCR reaction. A 1.23-kb upstream region of the CYP704B2 gene was digested from the complementation construct pCAMBIA1301:CYP704B2 by BamHI and Ncol. The CYP704B2 promoter was subcloned into pCAMBIA1301: GUS cut by the same restriction enzyme sites. The pCAMBIA 1301: CYP704B2 promoter was introduced into calli of the wild-type rice by A. tumefaciens transformation. GUS activity was visualized by staining the root, stem, leaf, and different stage flowers from heterozygous spikelets of transgenic lines, overnight in X-Gluc solution (Willemsen et al., 1998), and then tissues were then cleared in 75% (v/v) ethanol. The treated anthers were immersed into the clearing reagent (8 g hydrate chloraldehyde, 1mL glycerol, and 3 mL deionized water) for 20 min and then mounted into the slide and photographed using a phase-contrast microscope (Leica DM2500).

Analysis of Anther Wax and Cutin Constituents

Wax and cutin from anthers was analyzed as described previously (Jung et al., 2006). Surface area was determined from pixel numbers in microscopy images, assuming a cylindrical body for rice anthers. Three to six milligrams of freeze-dried anther material corresponding to 52 to 280 mm2 anther surface area was submersed in 700 μL chloroform for 1 min. The resulting chloroform extract was spiked with 10 μg of tetracosane (Fluka) as internal standard and transferred to a new glass vial. The solvent was evaporated under a gentle stream of nitrogen. Compounds containing free hydroxyl and carboxyl groups were converted to their trimethylsilyl ethers and esters with 20 μL of bis-(N,N-trimethylsilyl)-tri-fluoroacetamide (Sigma-Aldrich) in 20 μL of pyridine for 40 min at 70°C before analysis by GC-FID (Agilent 6890N gas chromatograph) and GC-MS (Agilent gas chromatograph coupled to an Agilent 5973N quadrupole mass selective detector). Monomers were identified from their electron ionization-mass spectrometry spectra (70 eV, m/z 50 to 700) after GC separation (column 30 mm × 0.25 mm × 0.1 μm film thickness [DB-1; JandW Scientific]; on-column injection at 50°C, oven temperature of 2 min at 50°C, increasing at 40°C/min to 200°C, 2 min at 200°C, increasing at 3°C/min to 310°C, 30 min at 310°C, and helium carrier gas at 2 mL/min). Quantification of wax monomers was accomplished with an identical GC system combined with a flame ionization detector.

Analysis of the monomer composition of the anther polyester was performed as described by Franke et al. (2005). To ensure complete extraction of all soluble lipids, anthers that had been used in the wax extraction were re-extracted in freshly added 1 mL of chloroform/methanol (1:1 v/v) for several hours. This extraction step was repeated four times before the anthers were finally dried over silica. The delipidated anthers were then depolymerized using transesterification in 1 mL of 1 N methanolic HCl for 2 h at 80°C. After the addition of 2 mL of saturated NaOH and 10 μg of dotriacontane (Fluka) as an internal standard, the hydrophobic monomers were subsequently extracted three times with 1 mL of hexane. The organic phases were combined, the solvent evaporated, and the remaining sample derivatized as described above. GC-MS and GC-FID analysis were performed as for the wax analysis using a modified GC temperature program: on-column injection at 50°C, oven temperature of 2 min at 50°C, increasing at 10°C/min to 150°C, 1 min at 150°C, increasing at 3°C/min to 310°C.

Phylogenetic Analysis

The full-length amino acid of CYP704B2 and most similar 36 sequences identified via BLAST search were aligned with the Muscle tool (Edgar, 2004) using the default parameters. CYP97C2 from rice and CYP703A2 from Arabidopsis thaliana were added for rooting purposes. The alignment (in the Supplemental Data Set 1 online) was used to construct a maximum likelihood tree with MEGA 3.1 (Kumar et al., 2004) using the following parameters: poisson correction, pairwise deletion, and 1000 bootstrap replicates.

Heterologous Expression and Enzymatic Analysis of CYP704B2

Full-length CYP704B2 cDNA was isolated using the primers forward, 5’-AAAGGATCCATGGAAGGCCCCGATAGA-3’; reverse, 5’-AAAG-GGATCCATGGAAGGCCCCGATAGA-3’, and enzyme, 5’-AAAGGATCCATGGAAGGCCCCGATAGA-3’. The restriction enzyme sites BamHI and KpnI (underlined) and shifted into the shuttle vector pYeDP60 (kindly provided by Denis Pompon). Subsequently, this construct was transformed into Saccharomyces cerevisiae strain WAT11 engineered to coproduce Arabidopsis NADPH P450 reductase-1 (Pompon et al., 1998). Yeast cultures were grown, and CYP704B2 expression was induced as described by Pompon et al. (1998) from one isolated transformed colony. After growth, cells were harvested by centrifugation.
and manually broken with glass beads (0.45 mm diameter) in 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and 600 mM sorbitol. The homogenate was centrifuged for 10 min at 10,000g. The supernatant was centrifuged for 1 h at 100,000g. The pellet consisting of microsomal membranes was resuspended in 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 30% (v/v) glycerol with a Potter-Elvehjem homogenizer and stored at −30°C. The volume of resuspension buffer was proportional to the weight of yeast pellets, and microsomes extracted from 6 g of yeast were resuspended in 3 mL of buffer. All procedures for microsomal preparation were performed at 0 to 4°C.

The cytochrome P450 content was measured by carbon monoxide difference spectrometry (Omura and Sato, 1964). Microsomes (20 pmoles of P450) isolated from yeast expressing CYP704B2 were incubated (30 min) in 20 mM sodium phosphate, pH 7.4, containing 1 mM NADPH and 100 μM radiolabeled fatty acids of different chain lengths (Perkin-Elmer). The media were directly spotted on TLC plates after incubation with a mixture of diethyl ether/light petroleum (boiling point, 40 to 60°C)/formic acid (50:50:1, v/v/v).

After elution from silica, metabolites generated were methylated with diazomethane and trimethylsilylated with N,O-bistrimethylsilyl trifluoroacetamide containing 1% (v/v) trimethylchlorosilane (1:1; v/v) and then subjected to GC-MS analysis performed on a gas chromatograph (Agilent 6890 Series) equipped with a 30-m capillary column with an internal diameter of 0.25 mm and a film thickness of 0.25 μm (HP-5MS). The gas chromatograph was combined with a quadrupole mass selective detector (Agilent 5973N). Mass spectra were recorded at 70 eV.

The metabolite generated by recombinant CYP704B2, in incubation with palmitic acid, was eluted from silica with 10 mL of diethyl ether, methylated with diazomethane, and then trimethylsilylated and subjected to GC-MS analysis as described above. Mass spectrum showed ions at m/z (relative intensity %) 73 (23%) [CH3]3Si+, 75 (29%) [CH3]3Si=O, 103 (14%) [CH3OSi(CH3)2], 146 (9%) [CH2=C=O]+([Si(CH3)3]+), 159 (6%) [CH3-O=C=O]+([Si(CH3)3]+CH2CHOH), 311 (100%) (M-47) [loss of methanol from the (M-15) fragment], 325 (9%) (M-31) [loss of OCH3 from the methyl ester], and 343 (32%) (M-15) (loss of CH3 from TMSi group). This fragmentation pattern is identical to the one obtained with authentic 18-hydroxypalmitic acid (M = 358g/mol).

After elution from silica, metabolite generated by recombinant CYP704B2 in incubation with oleic acid was methylated with diazomethane and then trimethylsilylated and subjected to GC-MS analysis as described above. Mass spectrum showed ions at m/z (relative intensity %) 73 (100%) [CH3]3Si+, 75 (17%) [CH3]3Si=O, 103 (69%) [CH3OSi(CH3)2], 146 (2%) [CH2=C=O]+([Si(CH3)3]+), 159 (4%) [CH3-O=C=O]+([Si(CH3)3]+CH2CHOH), 335 (51%) (M-47) [loss of methanol from the (M-15) fragment], 353 (8%) (M-31) [loss of OCH3 from the methyl ester], 369 (9%) (M-15) (loss of CH3 from TMSi group), and 382 (2%) (M). This fragmentation pattern is characteristic of derivatized 18-hydroxylinolenic acid (M = 380 g/mol).

In vitro enzyme assays with rice anther extracts were performed as described previously (Morant et al., 2007). Total lipids from anthers (37 mg) were extracted in 1 mL of methanol at 60°C. After centrifugation, the methanolic phase was kept in a 2-mL glass vial at 4°C. For microsomal incubation, 5 μL were transferred to assay in a glass vial, and the methanol was evaporated with argon. Microsomes (20 pmoles of P450) isolated from yeast expressing CYP704B2 were added and incubated (30 min) in 20 mM sodium phosphate, pH 7.4, containing 1 mM NADPH in a final volume of 100 μL. Control experiments were performed in the absence of NADPH. Incubation media were then extracted with 500 μL of diethyl ether, which was then evaporated with argon. Derivatized 16-hydroxypalmitic and 18-hydroxyoleic acids were characterized as described above. Mass spectrum of metabolite with retention time of 42.6 min showed ion at m/z (relative intensity) 73 (100%) [CH3]3Si+, 75 (96%) [CH3OSi(CH3)2]+, 146 (12%) [CH2=C=O]+([Si(CH3)3]+), 159 (22%) [CH3-O=C=O]+([Si(CH3)3]+CH2CHOH), 309 (41%) (M-47) [loss of methanol from the (M-15) fragment], 325 (9%) (M-31) [loss of OCH3 from the methyl ester], 327 (2%) (M-29), 341 (9%) (M-15) (loss of CH3 from TMSi group), and 356 (3%) (M). This fragmentation pattern is characteristic of 16-hydroxy-palmitoleic acid (M = 356g/mol). The derivatized metabolite with retention time of 45.4 min has not been identified.

Accession Numbers

Sequence data from this article for the cDNA and genomic DNA of CYP704B2 can be found in the GenBank/EMBL data libraries under accession numbers NM_001055627 and AC073556, respectively. Accession numbers for the sequences used in the phylogenetic analysis are on the tree in Figure 7.

Supplemental Data

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

Supplemental Figure 1. Meiosis of PMCs Is Normal in the cyp704B2 Mutant.

Supplemental Figure 2. Analysis of the Anther and Pollen Development by Transmission Electron Microscopy.

Supplemental Figure 3. Analyses of Cutin Monomers in the Wild-Type and cyp704B2 Anthers.

Supplemental Figure 4. The Phenotype of the Flower, Anther Surface, and Pollen Grains in the Complemented Line.

Supplemental Figure 5. Scanning Electron Microscopy Observation of the Surface Structures of the Leaf, Stem, and Pala in the Wild Type and the cyp704B2 Mutant.

Supplemental Table 1. Detailed Cutin Compositions of the Wild-Type and cyp704B2 Anthers.

Supplemental Table 2. Primer Sequences Used in the Mapping and Gene Cloning.

Supplemental Table 3. The Putative Members of CYP704B2 Family from Other Species in EST Databases.

Supplemental Data Set 1. Text File of the Protein Alignment among Rice CYP704B2 and Other Members in the Phylogenetic Tree.
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