The ZmMYB84-ZmPKSB regulatory module controls male fertility through modulating anther cuticle—pollen exine trade-off in maize anthers

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Summary
Anther cuticle and pollen exine are two crucial lipid layers that ensure normal pollen development and pollen-stigma interaction for successful fertilization and seed production in plants. Their formation processes share certain common pathways of lipid biosynthesis and transport across four anther wall layers. However, molecular mechanism underlying a trade-off of lipid-metabolic products to promote the proper formation of the two lipid layers remains elusive. Here, we identified and characterized a maize male-sterility mutant pksb, which displayed denser anther cuticle but thinner pollen exine as well as delayed tapetal degeneration compared with its wild type. Based on map-based cloning and CRISPR/Cas9 mutagenesis, we found that the causal gene (ZmPKSB) of pksb mutant encoded an endoplasmic reticulum (ER)-localized polyketide synthase (PKS) with catalytic activities to malonyl-CoA and midchain-fatty acyl-CoA to generate triketide and tetraketide α-pyrones. A conserved catalytic triad (C171, H320 and N353) was essential for its enzymatic activity. ZmPKSB was specifically expressed in maize anthers from stages S8b to S9-10 with its peak at S9 and was directly activated by a transcription factor ZmMYB84. Moreover, loss function of ZmMYB84 resulted in denser anther cuticle but thinner pollen exine similar to the pksb mutant. The ZmMYB84-ZmPKSB regulatory module controlled a trade-off between anther cuticle and pollen exine formation by altering expression of a series of genes related to biosynthesis and transport of sporopollenin, cutin and wax. These findings provide new insights into the fine-tuning regulation of lipid-metabolic balance to precisely promote anther cuticle and pollen exine formation in plants.

Introduction
In flowering plants, pollen grains develop within anthers and the developmental process requires well-coordination between sporophytic and gametophytic tissues (Ma, 2005; McCormick, 1993). To protect pollen grains from biotic and abiotic stresses, plants develop two lipid barriers, anther cuticle and pollen exine. Anther cuticle covers the outer surface of anther epidermis, and pollen exine is the outermost layer of pollen wall (Shi et al., 2015). The normal male fertility requires the proper formation of the two lipid layers during anther and pollen development, and any defects in this process generally fail to produce normal microspores or pollen grains, ultimately resulting in genic (nuclear) male sterility (GMS; Wan et al., 2020). Therefore, the molecular mechanism underlying anther cuticle and pollen exine formation has inspired decades of scientific inquiry. Pollen exine is primarily made of sporopollenin (Ariizumi and Toriyama, 2011), and anther cuticle is mainly composed of cutin polymers and cuticular waxes (Yeats and Rose, 2013). The main chemical compositions of these three type materials are lipids and their derivatives that are synthesized and secreted mainly in anther tapetum, the innermost somatic cell layer of anther wall, and then transported onto the outer surfaces of microspores and anthers (Quilichini et al., 2014; Wan et al., 2020). Previous studies have shown that pollen exine formation shares certain common lipid biosynthetic pathways with anther cuticle formation in plant anther tapetum, such as de novo fatty acid synthesis in plastids and critical lipid biosynthesis in endoplasmic reticulum (ER; Ariizumi and Toriyama, 2011; Shi et al., 2015; Wan et al., 2020; Wang et al., 2022). Thus, a precise coordination of lipid metabolism and allocation exists between anther cuticle and pollen exine formation. Actually, many lipid-metabolic GMS genes involved in sporopollenin biosynthesis have been found to participate in cutin and wax biosynthesis in plants (Wan et al., 2020). For example, rice Defective Pollen Wall (OsDPW) and maize Male sterility 25 (ZmMs25) encoding fatty acyl carrier protein (ACP) reductases are required for biosynthesis of sporopollenin, cutin and wax (Shi et al., 2011; Zhang et al., 2021). Acyl-CoA synthetase12 (ACOS12) in rice and ACOS5 in maize encode fatty
acetyl-CoA synthetases and play essential roles in pollen exine and anther cuticle formation (Li et al., 2016; Liu et al., 2022). CYP703A3 in rice and ABNORMAL POLLEN VACUOLATION1 (APV1) in maize encode cytochrome P450 fatty acid hydrolases that are essential for pollen exine and anther cuticle formation (Somarathne et al., 2017; Yang et al., 2014). No Polen 1 (NPT) in rice and IRREGULAR POLLEN EXINE1 (IPET) ZmMs20 in maize encode glucose-methyl-choline oxidoreductases and regulate pollen exine and anther cuticle formation (Chen et al., 2017; Wang et al., 2019). ZmMs30 in maize encoding a GDSL lipase is required for pollen exine and anther cuticle formation (An et al., 2019). On the contrary, many lipid-metabolic GMS genes primarily involved in cutin and/or wax biosynthesis also take part in sporopollenin biosynthesis, for example OsGPAT3 in rice and ZmMs33 in maize encoding glycerol-3-phosphate acyltransferases (GPAT; Li et al., 2022; Men et al., 2017; Xie et al., 2018; Zhu et al., 2019, 2020). Rice Wax-deficient anther1 (OsWDA1) encoding an aldehyde decarbonylase (Jung et al., 2006) and rice OsABCG26 encoding an ATP binding cassette transporter G26 (Zhao et al., 2015). However, how plants balance the distribution of these lipids and their derivatives in anthers and whether a lipid-metabolic trade-off between anther cuticle and pollen exine formation exists to promote their normal development and male fertility remain elusive.

Polyketide synthases (PKSs) catalyse sequential condensation of malonyl-CoA with an acetyl-CoA starter and are classified into three types (I, II and III) based on their protein structures (Austin and Noel, 2003; Koduri et al., 2010; Shimizu et al., 2017). Previous studies have shown that Arabidopsis POLYKETIDE SYNTHASE B (AtPKSB) and its ortholog POLYKETIDE SYNTHASE 2 (OsPKS2) in rice participate in the phenylpropanoid pathway (Kim et al., 2010; Zhu et al., 2017), which is a key component of the lipid-metabolic network of sporopollenin precursor biosynthesis and is essential for pollen exine formation (Wan et al., 2020). Recombinant AtPKSB and OsPKS2 can generate triketide and tetraketide α-pyrones that are major components to synthesize sporopollenin precursors (Kim et al., 2010; Zhu et al., 2017). AtPKSB mutant exhibits abnormal exine pattern and-patterning and slightly male sterility (Kim et al., 2010), while loss-of-function of OsPKS2 results in defective pollen exine and anther cuticle and complete male sterility (Zhu et al., 2017). These results suggest that AtPKSB and OsPKS2 have conserved and diversified functions in male reproductive development. Nevertheless, the molecular mechanism of their maize orthologs underlying anther and pollen development has not yet been revealed.

Here, we identified and characterized a GMS gene, ZmPKSB, encoding a polyketide synthase B essential for normal male fertility in maize. Loss of ZmPKSB function results in delayed tapetal degeneration, denser anther cuticle, thinner pollen exine and complete male sterility. ZmPKSB is directly activated by ZmMYB84. Both pksb and myb84 mutations can alter the expression patterns of nine sporopollenin-related and nine cutin- and wax-related genes; the altered lipid metabolite profiles in pksb and myb84 anthers further confirm that the ZmMYB84-ZmPKSB regulatory module controls a trade-off to balance lipid biosynthesis and supplies between anther cuticle and pollen exine formation. These findings deepen our understanding on the fine-tuning process of anther and pollen development in plants.

Results

Maize pksb mutant exhibits complete male sterility with dense anther cuticle but thin pollen exine

By screening an ethyl methane sulfonate (EMS)-induced mutant library of maize inbred line B73 in our laboratory, we obtained a complete male-sterility mutant, which was designated pksb on the basis of its ZmPKSB mutation (see below). The pksb mutant exhibited normal vegetative growth and female fertility, but fewer exerted and smaller anthers compared with its wild type (WT), and failed to produce viable pollen grains at mature stage (Figure 1a).

Maize anther and pollen development can be divided into 14 stages (S1–S14; Wan et al., 2019). To investigate the cytological defects in pksb anthers, we performed transverse section analysis of WT and pksb anthers from stages S5 to S12, and found that anther and microspore development proceeded similarly between WT and pksb anthers until stage S9 (Figures 1b and S1a). From stages S10 to S12, WT microspores underwent vacuolization and gradually accumulated starch, ultimately forming round and mature pollen grains. In contrast, pksb microspores developed abnormally in terms of shape and size, failed to accumulate starch and finally collapsed (Figure 1b).

Scanning electron microscopy (SEM) observations of anthers from stages S6 to S12 showed there were no obvious differences of anther inner surface and microspores between WT and pksb before stage S10 (Figure S1b). From stages S10 to S12, although the anther inner surface of pksb mutant was comparable with that of WT, pksb microspores adhered with each other and displayed defective vacuolization and starch accumulation compared with WT microspores (Figure S1b). At stage S13, pksb anthers exhibited a denser three-dimensional reticulate cuticle, larger Ubisch bodies and collapsed pollen grains with irregular and rough pollen wall surface compared with WT anthers (Figure 1c). These findings indicate that the pksb mutation affects development of anther cuticle, Ubisch bodies and pollen grains in maize.

To observe more detailed cytological defects of pksb mutant, we performed transmission electron microscopy (TEM) of WT and pksb anthers from stages S9 to S12 and found that there was no detectable difference between pksb and WT at stage S9 (Figure 2a). From stages S10 to S12, pksb anthers showed thicker tapetum, delayed tapetal degeneration, expanded and collapsed microspores, larger Ubisch bodies and thinner pollen exine compared with WT anthers (Figure 2a). Notably, pksb anthers displayed obviously denser reticulate and thicker finger-like structure anther cuticle at stages S11 and S12 compared with WT anthers by SEM and TEM observations (Figure 2b,c). Taken together, the pksb mutation results in the delayed tapetal degeneration, enlarged Ubisch bodies, denser anther cuticle but thinner pollen exine and ultimately complete male sterility.

Map-based cloning and molecular characterization of ZmPKSB gene

To identify the causal gene of pksb mutant, map-based cloning of pksb locus was carried out by using an F2 population derived from the cross between pksb mutant and maize inbred line Zheng 5B. All the F1 progenies were male fertile, and F2 individuals segregated with a male fertile-to-sterile ratio of approximately
$3:1 \ (\chi^2 [3:1] = 0.185, P > 0.05)$, indicating that the *pksb* locus is a recessive mutation with single-gene inheritance (Figure S2a).

Based on a maize 60K microarray analysis, the region of 20–60 Mb on chromosome 7 was selected as the candidate interval harbouring *pksb* locus (Figure S2b), and then, the *pksb* locus was
primarily mapped to a 3.2-cM genetic region between markers umc1978 and bnlg1792 by using 48 fertile and 48 sterile F2 individuals (Figure 3a). Furthermore, it was narrowed down to a 752.1-kb interval between markers M3 and M6 using a larger F2 population including 1050 plants. Seven ORFs were predicted in this interval based on the B73 maize reference genome (AGPv3; Figure 3a). Based on RNA-sequencing (RNA-seq) data analysis of B73 anthers, only GRMZM2G108894 was found to be highly expressed during anther development and displayed a fluctuated expression pattern with a peak at stage S9 (Figure S2c). DNA

Figure 2 TEM and SEM analyses of WT and pksb anthers from stages S9 to S12. (a) TEM observation of anther wall, microspores, Ubisch bodies and exine in WT and pksb mutant. SEM (b) and TEM observations (c) of anther outer surface in WT and pksb mutant. Ba, bacula; Cu, cuticle; Cw, cuticle wall; E, epidermis; En, endothecium; F, foot layer; ML, middle layer; Mp, mature pollen; Msp, microspore; Ta, tapetum; Te, tectum; Ub, Ubisch body.
sequencing showed that a 1-bp deletion in the second exon of GRMZM2G108894 in pksb mutant compared with WT resulted in a frame-shift mutation and premature stop codon (Figure 3b). The requirement of GRMZM2G108894 for male fertility was confirmed by the male-sterility phenotypes of its three knockout lines we generated using CRISPR/Cas9 (Figures 3c and S2,d,e) and by the allelic tests between two CRISPR/Cas9 knockout lines (ZmPKSB-Cas9-1 and -2) and the pksb mutant which showed nearly 1 : 1 segregation ratio of male sterile to fertile F1 plants (Figure 3d). GRMZM2G108894 was predicted to encode a polyketide synthase. Therefore, the 1-bp deletion of GRMZM2G108894 (thereafter termed as ZmPKSB) is responsible for the male-sterility phenotype of pksb mutant.

To explore the phylogenetic evolution of ZmPKSB, we performed amino acid sequence alignment of ZmPKSB and its 20 orthologs from 17 plant species and found that the CoA binding motifs and active sites were conserved between ZmPKSB and its orthologs (Figure S3). The phylogenetic analysis showed that ZmPKSB and its orthologs were grouped into three clades. Clade I consists of 10 orthologs from monocots such as ZmPKSB and rice OsPKS2 (Os07g0411300; Kim et al., 2010; Zhu et al., 2017), clade II is composed of nine orthologs from dicots such as Arabidopsis ATPPKSB (AT4G34850; Kim et al., 2010; Zhu et al., 2017), and clade III contains two orthologs from Hypericum perforatum and Physcomitrium patens (Figure 3e). In addition, microsynteny analysis showed that ZmPKSB and its flanking genes displayed good synteny in sorghum, millet, rice and Brachypodium distachyon, but diverged from those in wheat, barley and Arabidopsis (Figure S4a; Table S1). Therefore, although the structure and function of ZmPKSB and its orthologs are relatively conserved in multiple plant species, the chromosomal regions harbouring ZmPKSB and its orthologs display good synteny only in several monocot species.

Quantitative real-time PCR (qPCR) was performed to investigate the spatiotemporal expression pattern of ZmPKSB in maize different tissues or organs. As a result, ZmPKSB transcript was only detected in anthers from stages S9b to S9-10 and peaked at stage S9 (Figures 3f and S4b), suggesting that ZmPKSB is an anther-specific expressed GMS gene. In pksb anthers, ZmPKSB transcript was nearly undetectable from stages S7 to S13, indicating that pksb is a null mutant (Figures 3g and S4c). In addition, a subcellular localization analysis in maize protoplasts showed that ZmPKSB was localized in ER (Figure 3h). Furthermore, TUNEL assays to examine DNA fragmentation showed that tapetal TUNEL signals appeared localized in ER (Figure 3h). Furthermore, TUNEL assays to examine DNA fragmentation showed that tapetal TUNEL signals appeared localized in ER (Figure 3h). Consequently, ZmPKSB is an anther-specific GMS gene with peak expression at stage S9, encodes an ER-localized protein containing the conserved CoA binding motifs and active sites and is essential for tapetal PCD, pollen development and male fertility in maize.

**ZmPKSB is directly activated by a transcription factor (TF) ZmMYB84**

To investigate the regulatory mechanism of ZmPKSB, we analysed another RNA-seq data of four maize inbred lines including B73, M6007, Oh43 and W23 (An et al., 2019; Jiang et al., 2021a,b; Zhai et al., 2015). Our results showed that the expression pattern of a TF encoding gene ZmMYB84 overlapped with that of ZmPKSB from stages S8a to S10, especially sharing the common peak at stage S9 (Figures 4a and S5a; Table S2). This result was confirmed by qPCR analysis (Figures 4b and S5b). Given that ZmMYB84 is required for maize male fertility (Jiang et al., 2021a, b), we speculate that ZmMYB84 may regulate expression of ZmPKSB. As expected, qPCR results showed that ZmPKSB transcript was nearly undetectable in myb84 mutant anthers (Figures 4c and S5c). Meanwhile, the expression pattern of ZmMYB84 was not obviously altered in pksb anthers (Figure S5d). Thus, the expression of ZmPKSB depends on TF ZmMYB84.

To test whether ZmPKSB is a direct target gene of ZmMYB84, we performed a transient dual-luciferase reporter (TDLR) assay and an electrophoretic mobility shift assay (EMSA), and found that the promoter of ZmPKSB was activated by ZmMYB84 (Figure 4d). Moreover, ZmMYB84 bound to the promoter of ZmPKSB (Figure 4e). Notably, a denser anther cuticle was observed in myb84 anthers from stages S11 to S13 (Figure 4f) similar to that of pksb anthers (Figure 2b), and the cutin and wax contents of myb84 anthers were significantly higher than those of WT anthers at stages S13 (Figure 4g), suggesting that ZmMYB84 controls anther cuticle formation through regulating ZmPKSB, which constitutes the ZmMYB84-ZmPKSB regulatory module. Collectively, ZmPKSB is directly activated by ZmMYB84, and the ZmMYB84-ZmPKSB regulatory module controls lipid biosynthesis for anther cuticle and pollen exine formation (Figure 4h).

**ZmPKSB functions as a polyketide synthase with its enzymatic activities dependent on a catalytic triad**

Based on the Maize Genetics and Genomics Database (http://www.maizegdb.org/), ZmPKSB was annotated as a putative chalcone synthase 1 (CHS1) belonging to a subclade of plant-specific type III PKS superfamily and encoded a 417-amino acid protein containing a CHS_ N domain, a CHS_ C domain and a putative catalytic triad (C171, H320 and N353; Figure 5a). Homology modelling of ZmPKSB revealed that the putative catalytic triad formed an active centre (Figure 5b), suggesting that these three residues might play essential roles in ZmPKSB catalytic activity.
activity. Then, we expressed and purified recombinant proteins of ZmPKSB-MBP and its three single amino acid substitution variants (C171A-MBP, H320F-MBP and N353A-MBP) from Escherichia coli (Figure 5c,d).

It is well-known that type III PKSs generally use fatty acyl-CoA as a starter substrate to carry out sequential condensation with malonyl-CoA to generate structurally divergent products (Austin and Noel, 2003). Thus, activities of the recombinant ZmPKSB-
MBP and its three variants were tested in the presence of C16:0-CoA and C18:0-CoA as the starter substrates and malonyl-CoA as the extender molecule. When the ZmPKSB-MBP recombinant protein was used as a catalytic enzyme, two major reaction products were detected and identified as triketide and tetraketide α-pyrones by UPLC-MS/MS (ultra-performance liquid chromatography–tandem mass spectrometry) analysis (Figure 5e, f). In contrast, when the three variants (C171A-MBP, H320A-MBP and N253A-MBP) were used as catalytic enzymes, no reaction product was detected (Figure 5e), demonstrating that the catalytic triad is indispensable for the enzymatic activities of ZmPKSB. Taken together, ZmPKSB is an active polyketide synthase with its enzymatic activities dependent on a catalytic triad and can use fatty acyl-CoA esters as starter substrates to produce triketide and tetraketide α-pyrones.

The ZmMYB84-ZmPKSB regulatory module controls a trade-off between anther cuticle and pollen exine formation

The aberrantly thin pollen exine in pksb anther (Figure 2a) implied that sporopollenin biosynthesis and transport might be blocked by pksb mutation. To test this hypothesis, we measured expression alterations of nine sporopollenin-related genes between WT and pksb anthers from stages S7 to S13 by qPCR (Figure 6a). These nine genes include seven reported GMS genes (ZmACO55, ZmIPE1, ZmMs45, ZmMs25, ZmDFR1, ZmDFR2 and ZmABCG26; Jiang et al., 2021b; Liu et al., 2022; Wan et al., 2020; Zhang et al., 2021) and two orthologs (ZmPKSA-1 and ZmPKSA-2) of the reported GMS genes OsPKS1 and AtIPKSA (Kim et al., 2010; Zhu et al., 2017). The qPCR results showed that the expression of all nine genes peaked at stage S9 in WT anthers, while significantly reduced in pksb anthers (Figures 6a and 6a). Considering that the deposition of a large amount of sporopollenin on microspore surface starts at stage S9 and mainly occurs at stage S10 (Figure 2a), the aberrantly thin pollen exine of pksb most likely results from the significant downregulation of all nine genes by pksb mutation at stage S9. Together, ZmPKSB is required for pollen exine formation through modulating the expression of sporopollenin-related GMS genes.

Given that pksb exhibited the denser anther cuticle at stages S11 and S12 by SEM and TEM observations (Figure 2b,c), it seems plausible that biosynthesis and transport of cutin and wax compositions for anther cuticle formation might be increased by pksb mutation. To confirm this, we measured expression alterations of three cutin-related genes (ZmHCT12, ZmLTL1 and ZmABCG11) and six wax-related genes (ZmCER8, ZmCER1, ZmGL2, ZmCL3929, ZmKCAS-4 and ZmGL8) between WT and pksb anthers from stages S7 to S13 by qPCR (Figure 6b1,b2). Notably, all nine cutin/wax-related genes were significantly up-regulated in pksb anthers at stage S10 compared with WT anthers (Figures 6b1,b2 and S6b,c). Anther cuticle formation starts at stage S10, and the three-dimensional reticulate cuticle obviously appears at stage S11 (Figure 2b). Thus, the aberrantly dense anther cuticle of pksb mutant is consistent with the significant upregulation of all nine cutin/wax-related genes induced by pksb mutation at stage S10.

Next, we measured the contents of anther cuticular cutin and wax as well as their constituents and monomers at stage S13 by GC–MS. The total content of cutin in pksb anthers was increased by 22.9% and that of wax in pksb anthers was significantly increased when compared to those in WT anthers (Figure 6c; Table S3). In addition, the amounts of major constituents and monomers of wax and cutin were significantly increased in pksb anthers compared with WT anthers (Figure 5; Table S3). These results are consistent with the denser anther cuticle and upregulation of cutin/wax-related genes in pksb anthers. Thus, ZmPKSB is indispensable for the proper anther cuticle formation in maize through modulating the expression of cutin/wax-related genes.

Considering that ZmMYB84 directly activates ZmPKSB transcription and that myb84 anthers display the aberrantly thin pollen exine and dense anther cuticle as well as the increased cutin and wax contents similar to pksb anthers (Figures 2 and 4; Fang et al., 2022), we performed qPCR analysis to determine whether ZmMYB84 regulates these above 18 sporopollenin- and cutin/wax-related genes. As expected, expression of all nine sporopollenin-related genes was hardly detectable in myb84 anthers from stages S6 to S12 (Figure 6d), indicating that ZmMYB84 controls pollen exine formation through regulating ZmPKSB and these nine sporopollenin-related genes. In addition, these nine cutin/wax-related genes displayed various expression alterations in myb84 anthers and were significantly up-regulated at more than half of anther developmental stages from S6 to S12 (Figure 6e1,e2), suggesting that the regulation of cutin/wax-related genes by ZmMYB84 is relatively complex, and ZmMYB84 partly regulates anther cuticle formation.

Taken together, a working model was proposed to illustrate how the ZmMYB84-ZmPKSB regulatory module controls a trade-off of lipid-metabolic products individually used for anther cuticle and pollen exine formation (Figure 7). ZmPKSB is activated by ZmMYB84. ZmPKSB affects biosynthesis of sporopollenin precursors and cutin/wax monomers in anther tapetal cell through modulating expression of sporopollenin biosynthetic GMS genes (e.g. ZmACO55, ZmIPE1, ZmMs45, ZmMs25, ZmDFR1, ZmDFR2, ZmPKSA-1 and ZmPKSA-2) and cutin/wax biosynthetic genes such as ZmHCT12, ZmLTL1, ZmCER8, ZmCER1, ZmGL2, ZmCL3929, ZmKCAS-4 and ZmGL8. Subsequently, these lipid-metabolic products are transported by lipid transporters (e.g. ZmABCG26 and ZmABCG11) to form normal anther cuticle and pollen exine. The balance of lipid distribution for anther cuticle and pollen exine formation is disturbed in pksb and myb84 mutant anthers, ultimately resulting in the denser anther cuticle and thinner pollen exine.

**Discussion**

Trade-off is generally regarded as an absolute restriction on the direction of evolution in plants, and is involved in two or more traits which need and share one type of energy and substance for plant growth and development as well as stress responses (Pease and Bull, 1988; Sterns, 1989; Wan et al., 2021). Trade-off that occurs in plants is often caused by resource restrictions, including the well-known growth–defence trade-off and grain size-number trade-off (Guo et al., 2018; Huot et al., 2014). Pollen exine and anther cuticle are mainly composed of specific lipidic substances including sporopollenin, cutin and wax. Given that sporopollenin precursors and cutin and wax monomers share certain common lipid synthetic pathways in plant anthers and that the total amount of lipidic substances synthesized in anthers are largely constant in a certain class of plant species (Wan et al., 2020; Wang et al., 2022), it seems plausible that a trade-off of lipid-metabolic products to be individually used for anther cuticle and

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pollen exine formation exists in plant anthers. However, molecular mechanism underlying this kind of trade-off has been yet unexplored.

Our results indicate that loss of ZmPKSB function not only causes the negative correlation of cytological phenotypes between pollen exine and anther cuticle formation (Figure 2), but also leads to the negative correlation of expression pattern changes between sporopollenin-related genes and cutin/wax-related genes (Figure 6a,b), implying that a trade-off may exist between anther cuticle and pollen exine at the cytological and transcriptional levels. Specifically, nine sporopollenin-related genes were significantly down-regulated in pksb compared with WT anthers at stage S9 (Figure 6a). Downregulation of these nine genes can impair biosynthesis and transport of sporopollenin precursors, and further results in the thinner pollen exine in pksb mutant, which is demonstrated by the cytological phenotypes in Figure 2a. In contrast, nine cutin- and wax-related genes are significantly up-regulated in pksb anthers at stage S10 (Figure 6b), suggesting that more cutin and wax monomers may be produced in pksb anthers. Consistently, the amounts of anther

Figure 4  ZmPKSB is activated by ZmMYB84 and the ZmMYB84-ZmPKSB regulatory module controls lipid biosynthesis for anther cuticle and pollen exine formation. (a) Expression patterns of the ZmMYB84 and ZmPKSB genes in WT (B73) anthers from stages S5 to S12 based on RNA-seq data. Data represent mean ± SD, n = 2–4. (b) Expression patterns of the ZmMYB84 and ZmPKSB genes in WT (B73) anthers from stages S5 to S12 by qPCR analysis. Data represent mean ± SD, n = 9. (c) Expression pattern change in ZmPKSB in myb84 anthers compared with WT anthers from stages S6 to S12 based on qPCR analysis. Data represent mean ± SD, n = 9. (d)Transient dual-luciferase reporter (TDLR) assay of ZmPKSB promoter activity activated by ZmMYB84 in maize protoplasts. Data represent mean ± SD, n = 3. (e) EMSA showing ZmMYB84 binding to the ccaacc box in ZmPKSB promoter in vitro. (f) SEM observation of anther cuticle in WT and myb84 mutant form stages S9 to S13. (g) Analysis of anther cutin and wax contents in WT and myb84 anthers at stage S13. Data represent mean ± SD, n = 3. (h) A proposed model of the ZmMYB84-ZmPKSB regulatory module, which is required for lipid biosynthesis for maize anther cuticle and pollen exine formation. For (d and g), * and *** indicate the significant levels of P < 0.05 and P < 0.001 by a two-tailed Student’s t-test, respectively.
cutin and wax in pksb anthers are obviously higher than those of WT anthers (Figure 6c). Thus, the lipid-metabolic products, originally used to form pollen exine, were transferred to use for anther cuticle formation.

ZmPKSB has the conserved polyketide synthase activity in vitro (Figure 5) and participates in the phenylpropanoid pathway for biosynthesis of sporopollenin precursors (Wan et al., 2020). According to the lipid-metabolic networks underlying anther cuticle and pollen exine formation (Wan et al., 2020), the block of the phenylpropanoid pathway induced by loss of ZmPKSB function likely results in the excessive accumulations of midchain- and ω-OH fatty acyl-CoAs (C16 and C18) substrates, which are used for biosynthesis of more cutin and wax monomers. This hypothesis is proved by metabolic profiles of lipids (Figures 6c and S7; Table S3).

Moreover, previous studies showed that ω-OH fatty acids are the common substrates for biosynthesis of anther cutin monomers and pollen sporopollenin precursors (Djukanovic et al., 2013; Dobritsa et al., 2009; Li et al., 2010; Yi et al., 2010). Collectively, ZmPKSB functions as a controller to modulate the allocation of lipidic substances between pollen exine (sporopollenin precursors) and anther cuticle (cutin and wax monomers) possibly through affecting the homeostasis of ω-OH fatty acids. Loss of ZmMYB84 function (myb84) results in the denser anther cuticle but thinner pollen exine (Figure 4f; Fang et al., 2022), in agreement with the cytological phenotypes of pksb mutant (Figure 2). Similarly, expression of nine sporopollenin-related genes and ZmPKSB is nearly undetectable in myb84 compared with WT anthers, indicating that ZmMYB84 plays a crucial role in activating these 10 genes for pollen exine formation (Figures 4c and 6d). In contrast, expression of nine cutin- and wax-related genes is increased in myb84 compared with WT anthers at more than half of anther developmental stages from S6 to S12 (Figure 6e), which is consistent with the cytological phenotype (the denser anther cuticle) of myb84 mutant (Figure 4f) and the increased contents of cutin and wax in myb84 anthers (Figure 4g).

In addition, ZmPKSB is directly activated by ZmMYB84 (Figure 4). ZmMs25, one of the above nine sporopollenin-related genes, is also directly activated by ZmMYB84 and functions in multiple lipid-metabolic pathways for anther cuticle and exine formation in maize (Zhang et al., 2021). Therefore, ZmMYB84 and ZmPKSB take full synchronization actions in anther cuticle and pollen exine formation at the cytological, transcriptional and lipid-metabolic levels, and the ZmMYB84-ZmPKSB regulatory module controls a trade-off of lipid-metabolic products individually used for anther cuticle and pollen exine formation. The physiological function of this trade-off is to balance biosynthesis and transport of sporopollenin precursors and cutin/wax monomers in the context of anther and pollen development.

OsMs188 and AtMs188, the orthologs of ZmMYB84, act as master TFs to directly regulate expression of many sporopollenin-related GMS genes including OsPKS2 and AtPKSB during anther development in rice and Arabidopsis (Han et al., 2021; Wang et al., 2018). However, according to the previous studies, the MYB-PKS regulatory module modulating anther cuticle—pollen exine trade-off is not found in both plants. First, loss-function of OsMS188 causes defective anther cuticle and pollen exine unlike to that of the opks2 mutant at a cytological level (Xiang et al., 2020; Zhu et al., 2017). Second, based on cytological observation, mutations in AtMS188 and AtPKSB result in thinner pollen exine compared with their WT, but whether they also lead to defective anther cuticles has not been found (Kim et al., 2010; Zhang et al., 2007). Considering that anther cuticle of Arabidopsis pksa-1/pksb-3 double mutant appears normal by TEM observation (Kim et al., 2010), atpkSB single mutant may not affect anther cuticle formation. Finally, it is still unclear whether OsMS188-OsPKS2 and AtMS188-AtPKSB take full synchronization actions in anther cuticle and pollen exine formation at transcriptional and lipid-metabolic levels. Therefore, the ZmMYB84-ZmPKSB regulatory module controlling a trade-off between anther cuticle and pollen exine formation may be unique in maize.

Based on the cytological observations, mutations of many GMS genes involved in transcriptional regulation and lipid metabolism in maize and rice result in various defects in anther cuticle and pollen exine formation, which can be divided into five types. Type I exhibits anther cuticle and thinner exine compared with WT. Most of GMS mutants belong to this type and include ms25-6065 (Zhang et al., 2021), ms33-6038 (Li et al., 2022; Xie et al., 2018; Zhu et al., 2019, 2020), ms30-6028 (An et al., 2019) and apv1 (Somaratne et al., 2017) in maize and dpw (Shi et al., 2011), gpat3 (Men et al., 2017), cyp703a3–2 (Yang et al., 2014) and tdr (Li et al., 2006) in rice. Type II contains rice myb80/bm1 (Xiang et al., 2020), tip3 (Yang et al., 2019) and pksb (this study) mutants that display denser anther cuticle but thinner exine compared with their WT. Type III includes maize zmms-1 mutant with absent anther cuticle but thickened exine (Singleton and Jones, 1930). Rice pks1 (Shi et al., 2018), abcg3 (Chang et al., 2018) and dmd1 (Ren et al., 2020) mutants belong to type IV and have normal anther cuticle but thinner exine compared with WT. Mutants in Type V display denser anther cuticle but normal exine compared with WT and include pk52 (Zhu et al., 2017), gelp34 (Yuan et al., 2020), ptc1 (Li et al., 2011) and csa (Zhang et al., 2010) in rice. According to the phenotypic changes, it seems plausible that the trade-off between anther cuticle and pollen exine formation may exist in GMS mutants of types II and III.

Another tapetum plays an essential role in supporting gametogenesis, and abnormal tapetal PCD often results in plant male sterility (Liu et al., 2021; Niu et al., 2013). The tapetal PCD in
ZmMYB84-ZmPKSB module controls male fertility
pksb anthers is delayed (at stage S11) compared with WT anthers (at S10; Figure S2f). The delayed PCD likely makes the tapetum more time and space to synthesize cutin and wax precursors, resulting in the denser anther cuticle from stages S11 to S13. In addition, Ubisch bodies are sporopollenin carrier granules that are formed on the tapetum surface and involved in pollen exine formation (Shi et al., 2015). The size and structure of Ubisch bodies in pksb mutant are similar to those of WT at stages S9 and S10, while Ubisch bodies in pksb mutant exhibits larger than those in WT at stages S11 and S12. Nevertheless, pksb mutant still has the thinner pollen exine due to the blocked biosynthesis of sporopollenin precursors (e.g. triketide and tetraketide α-pyrones) that are catalyzed and synthesized by ZmPKSB. This finding implies that function of Ubisch bodies may also relate to anther cuticle (cutin and wax) formation. The underlying mechanism needs further investigation.

**Materials and methods**

**Plant materials, growth conditions and phenotype characterization**

Maize inbred line B73 and the parental lines (Hi A and B) of hybrid Hi II were obtained from the Maize Genetics Cooperation Stock Center (http://maizecoop.cropsci.uiuc.edu). Maize CRISPR/Cas9 knockout line myb84 was produced previously and maintained in our laboratory (Jiang et al., 2021a,b). All plants were grown at the experimental stations of the University of Science and Technology Beijing (USTB) in Beijing and Sanya, China. The T2 transgenic plants were grown in a greenhouse under long-day conditions (16 h/8 h (day/night) at 26 °C/22 °C). Images of the tassels and anthers were captured with a Canon EOS 700D digital camera and a SZX2-ILB stereomicroscope (Olympus, Tokyo, Japan), respectively. Pollen grains stained with 1% I2-KI solution were photographed using BX-5SF microscope (Olympus).

**Cytological analysis and microscopy**

The light microscopy of transverse sections, SEM and TEM analyses of maize anthers were performed as described previously (An et al., 2020), and the images were captured using a BX-53 microscope (Olympus), a HITACHI S-3400N scanning electron microscope (Hitachi, Tokyo, Japan) and a HITACHI H-7500 transmission electron microscope (Hitachi), respectively.

**Genetic analysis and map-based cloning of pksb locus**

An F2 mapping population was generated from the cross of pksb mutant and maize inbred line Zheng S8. Genetic analysis, primary mapping and fine mapping of pksb locus were performed according to the method described previously (Zhang et al., 2018). Seven candidate genes in the narrowed down 75Z.1-kb interval were sequenced and compared between WT and pksb mutant to find the candidate gene of ZmPKSB. All SSR markers and PCR primers used in this study are listed in Table S4 (similarly hereinafter).

**RNA-seq data analysis**

The RNA-seq data of maize inbred lines B73, M6007, Oh43 and W23 were obtained from the previous studies (An et al., 2019; Jiang et al., 2021a,b; Zhai et al., 2015). Gene expression levels were estimated as described previously (Jiang et al., 2021a,b).

**Plasmid construction and maize transformation**

For mutagenesis of ZmPKSB, a CRISPR/Cas9 plasmid with two gRNAs was constructed based on the pBUE411 vector (Xing et al., 2014). The CRISPR-P 2.0 (http://crispr.hzau.edu.cn/CRISPR2/) was used to choose specific gRNAs targeting the coding sequence of ZmPKSB, and off-target analysis of gRNAs was carried out on the Website (http://www.rgenometools/cas-offinder/). For assembly of the two gRNAs, the PCR fragment was amplified based on pCBC-MT172 and the purified PCR fragment was cloned into a Bsal-digested pBUE411 vector.

The CRISPR/Cas9 plasmid was introduced in Agrobacterium tumefaciens strain EHA105 and transformed into immature embryos of hybrid Hi II following the previously published protocols (Frame et al., 2002). The positive transformants were selected by PCR amplification using primers Bar-F and Bar-R. The genotyping of transgenic progenies was performed as described previously (Jiang et al., 2021a,b).

**Allelism test of ZmPKSB-Cas9 knockout lines and pksb mutant**

The allelism test was performed by using F2 progenies generated from the crosses of two homozygous knockout lines of GRMZM2G108894 (ZmPKSB-Cas9-1 and -2) with pksb heterozygous plants (ZmPKSB/pksb). The ratio of male fertile-to-sterile plants was analysed, and if the segregation ratio fits to 1 : 1, they are allelic gene.

**Phylogenetic and microsynteny analysis**

Twenty orthologues of ZmPKSB in 17 plant species were obtained from the GRAMENE website (http://www.gramene.org). A phylogenetic tree was constructed using MEGA7.0 software with the Maximum likelihood method (Kumar et al., 2016). The alignment of amino acid sequences was performed with DNAMAN6.0 software.

For microsynteny analysis, the neighbouring genes of ZmPKSB were identified in the B73 maize reference genome (AGPv3), and the flanking genes of ZmPKSB orthologues in sorghum, millet, rice, barley, wheat and Brachypodium distachyon were downloaded from Phytozome V12 (https://phytozome.jgi.doe.gov/pz/portal.html; Table S1). Microsynteny of ZmPKSB was determined by multiple sequence alignments around flanking genes of ZmPKSB or its orthologues in six monocots and one eudicot.

**Subcellular localization of ZmPKSB**

The corresponding full-length CDS of ZmPKSB was amplified by PCR and fused in-frame with the N-terminal of GFP and then inserted into the expression vectors pUC19 via in-fusion method. The ER marker mCherry-HDEL was created by adding the sequence encoding HDEL to the 3′ C-terminus. The ER marker mCherry-HDEL was created by adding the sequence encoding HDEL to the 3′ C-terminus. The alignment of amino acid sequences was performed with DNAMAN6.0 software.

The corresponding full-length CDS of ZmPKSB was amplified by PCR and fused in-frame with the N-terminal of GFP and then inserted into the sequence encoding HDEL to the 3′ C-terminus. The alignment of amino acid sequences was performed with DNAMAN6.0 software.

The corresponding full-length CDS of ZmPKSB was amplified by PCR and fused in-frame with the N-terminal of GFP and then inserted into the sequence encoding HDEL to the 3′ C-terminus. The alignment of amino acid sequences was performed with DNAMAN6.0 software.

**TUNEL assay**

Anthers from WT and pksb mutant were collected and prepared to generate paraffin sections at different developmental stages. The selected paraffin sections were dewaxed in xylene and rehydrated in an ethanol series (95%, 80%, 50%, 30% and 0%). DNA fragmentation was detected by TUNEL assay using a TUNEL kit (DeadEnd™; Fluorometric TUNEL System; Promega, Madison, Wisconsin, USA) according to the manufacturer’s instructions. Signals were observed under a fluorescence confocal scanner microscope (TCS-SP8; Leica, Wetzlar, Hessen, Germany).
Quantitative real-time PCR (qPCR) analysis

Total RNA was isolated using TRIzol reagent (Invitrogen, Waltham). The cDNA was synthesized using 5X All-In-One RT MasterMix (abm, Vancouver, British Columbia, Canada) following the manufacturer’s protocol. qPCR was conducted on a QuantStudio 5 Real-Time PCR system (ABI, Foster, California) using TB Green™; Premix EX Tag™; (TakaRa, Kusatsu, Japan).

Figure 6 ZmMYB84 and ZmPKSB affect pollen exine and anther cuticle formation by altering expression of sporopollenin-, cutin- and wax-related genes. (a) Expression pattern changes in nine sporopollenin-related genes in WT and pksb mutant anthers from stages S7 to S13. (b) Expression pattern changes of three cutin-related genes (b1) and six wax-related genes (b2) in WT and pksb mutant anthers from stages S7 to S13. (c) Analysis of anther wax and cutin contents per unit surface area in WT and pksb mutant at stage S13. Anther cutin and wax contents were increased in pksb mutant compared with WT. Data represent mean ± SD of three independent experiments. ** indicates $P < 0.01$ by a two-tailed Student’s t-test, respectively. (d) Expression pattern changes of nine sporopollenin-related genes in WT and myb84 anthers from stages S6 to S12. (e) Expression pattern changes of three cutin-related genes (e1) and six wax-related genes (e2) in WT and myb84 anthers from stages S6 to S12. For (a, b, d and e), error bars indicate SD, and each reaction had three biological replicates with three technical repeats. *, **, and *** indicate the significant levels of $P < 0.05$, 0.01 and 0.001 (Student’s t-test, n = 9), respectively.

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and ZmCyanase (Zm00001d032736) were used as the internal controls. Each sample had three biological replicates with three technical replicates. The amplification data were calculated by the $2^{-\Delta\Delta Ct}$ method, and results were shown as means ± SD.

**Transient dual-luciferase assay**

The promoter region of ZmPKSB (1530 bp before ATG) was cloned into the pEASY-LUC vector by homologous recombination to generate proZmPKSB: LUC plasmid used as a reporter. The CDS region of ZmMYB84 (GRMZM2G108894) was inserted into pRTBD vector to generate 35S-Ω: ZmMYB84 construct used as an effector. Subsequent maize protoplast transformation and the relative LUC activity measurement were described as previously (An et al., 2020).

**Electrophoretic mobility shift assay (EMSA)**

The full-length CDS of ZmMYB84 was fused with N-terminal of MBP and cloned into the vector pMCSG7. The fusion vector transformation, proteins purification and EMSA assay were performed as described previously (An et al., 2020).

**Protein purification and enzymatic analysis**

Amino acid sequence alignment was used for prediction analysis of the ZmPKSB conserved active-site residues and CoA binding motifs (Mizuuchi et al., 2008). The CDSs of ZmPKSB and its three substitution mutations (C171A, H320A and N353A) were cloned into pMCSG7 vector. The vector construction and protein purification were performed as described previously (Zhang et al., 2021). Malonyl-CoA and fatty acyl-CoA (C16:0-CoA and C18:0-CoA) were used as substrates of ZmPKSB and its mutations to verify the activity of polyketide synthase, and the enzymatic activity analysis and reaction product detection were performed as described previously (Zhu et al., 2017).

**Analysis of anther cutin and wax**

WT and pksb mutant anthers at stage S13 were collected and freeze-dried. To precisely calculate the amounts per unit of surface area, we plotted the anther dry weight against anther surface area according to Shi et al. (2011). The extraction and GC–MS analysis of anther wax and cutin were performed as described previously (An et al., 2020).

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**Conflict of interest**

The authors declare no conflicts of interest.

**Author contributions**

X.W. and X.A. designed research and supervised the project. X.L. and Y.J. performed plasmid construction, genetic transformation, CRISPR/Cas9 assay, qPCR analysis, enzyme activity assay and lipidomics analysis. X.L., R.X. and F.L. carried out phenotypic and cytological observations and TUNEL assay. J.W. analyzed the data. X.L., C.F. and S.Z. performed gene
mapping. C.F. and L.Z. performed TDLR and EMSA assays. X.L., S.W., Y.J., X.A. and X.W. wrote and revised the paper.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Cytological observation of WT and pksb mutant.

Figure S2 Map-based cloning of maize pksb locus, mutation sites in three CRISPR/Cas9 knockout lines of ZmPKSB, and TUNEL assay of tapetal cells.

Figure S3 The amino acid sequence alignment of ZmPKSB and its orthologs from 17 plant species.

Figure S4 Microsynteny analysis of ZmPKSB and its orthologs in different plant species and qPCR analysis of ZmPKSB.

Figure S5 Expression analysis of ZmPKSB and ZmMYB84 based on RNA-seq data or qPCR analyses of maize anthers.

Figure S6 Expression analysis of sporopollenin-, cutin- and wax-related genes in WT and pksb anthers from stages S7 to S13.

Figure S7 Analysis of anther wax and cutin contents in WT and pksb mutant at stage S13.

Table S1 The microsynteny analysis of ZmPKSB and its orthologs in different plant species.

Table S2 Transcriptional levels of the investigated genes in anther RNA-seq data at different stages.

Table S3 The detailed compositions of cutin and wax.

Table S4 The SSR markers and PCR primers used in this study.