

The Regulation of Sporopollenin Biosynthesis Genes for Rapid Pollen Wall Formation¹[OPEN]

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Sporopollenin is the major component of the outer pollen wall (sexine). It is synthesized using a pathway of approximately eight genes in *Arabidopsis* (*Arabidopsis thaliana*). MALE STERILITY188 (MS188) and its direct upstream regulator ABORTED MICROSPORES (AMS) are two transcription factors essential for tapetum development. Here, we show that all the sporopollenin biosynthesis proteins are specifically expressed in the tapetum and are secreted into anther locules. MS188, a MYB transcription factor expressed in the tapetum, directly regulates the expression of POLYKETIDE SYNTHASE A (PKSA), PKSB, MALE STERILE2 (MS2), and a CYTOCHROME P450 gene (CYP703A2). By contrast, the expression of CYP704B1, ACYL-COA SYNTHETASE5 (ACOS5), TETRAKETIDE α -PYRONE REDUCTASE1 (TKPR1) and TKPR2 are significantly reduced in *ams* mutants but not affected in *ms188* mutants. However, MS188 but not AMS can activate the expression of CYP704B1, ACOS5, and TKPR1. In *ms188*, dominant suppression of MS188 homologs reduced the expression of these genes, suggesting that MS188 and other MYB family members play redundant roles in activating their expression. The expression of some sporopollenin synthesis genes (PKSA, PKSB, TKPR2, CYP704B1, and ACOS5) was rescued when MS188 was expressed in *ams*. Therefore, MS188 is a key regulator for activation of sporopollenin synthesis, and AMS and MS188 may form a feed-forward loop that activates the expression of the sporopollenin biosynthesis pathway for rapid pollen wall formation.

The cell wall is the fundamental support structure of a plant cell. The pollen wall is the most complicated wall of all plant cells. It is composed of the inner intine and the outer exine. The intine composition is similar to normal plant cell walls (Edlund et al., 2004). The exine is divided into the sexine and nexine. The nexine is a flat layer localized between the sculptured sexine and intine. Glycoproteins are essential components of the nexine layer (Lou et al., 2014; Jia et al., 2015). The sexine is mainly composed of complex chemical compounds of sporopollenin (Scott, 1994; Piffanelli et al.,

1998; Blackmore et al., 2007). The morphological pattern of sexine varies among species, which is the basis of the discipline of palynology (Blackmore et al., 2007). As one of the most complex structures of the extracellular matrix, the pollen wall plays an important role in protecting pollen against microorganism invasion and mediating species-specific pollen-stigma recognition during the pollination process.

Sporopollenin is a highly resistant biopolymer, which is thought to be composed of aromatics, phenolics, and long-chain aliphatic acids (Piffanelli et al., 1998; Ariizumi and Toriyama, 2011). The components of sporopollenin precursors are produced by and stored in tapetum cells after meiosis. They are subsequently transported out of the tapetum cells and deposited on the surface of microspores to form the sexine layer (Heslop-Harrison, 1962; Dickinson and Heslop-Harrison, 1968; Zhou et al., 2015; Xu et al., 2016). In *Arabidopsis* (*Arabidopsis thaliana*), eight proteins are reported to participate in the synthesis of sporopollenin precursors. Among them, ACYL-CoA SYNTHETASE5 (ACOS5) catalyzes mid-/long-chain fatty acids into fatty acyl-CoA, which is hydroxylated by members of the cytochrome P450 superfamily, CYP703A2 and CYP704B1. The hydroxylated products are catalyzed by the chalcone synthase family POLYKETIDE SYNTHASE A (PKSA) and PKSB into triketide and tetraketide α -pyrones, which are the substrates of TETRAKETIDE α -PYRONE REDUCTASE1

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(TKPR1) and TKPR2, respectively. As a fatty acyl reductase, MALE STERILE2 (MS2) catalyzes the palmitoyl acyl carrier protein into a fatty alcohol (Aarts et al., 1997; Morant et al., 2007; de Azevedo Souza et al., 2009; Dobritsa et al., 2009, 2010; Grienenberger et al., 2010; Kim et al., 2010; Chen et al., 2011). The resultant sporopollenin precursors were predicted to be synthesized in the tapetum and transported to the anther locule by the member of ATP-binding cassette transporter superfamily (ABCG26; Quilichini et al., 2010; Choi et al., 2011; Dou et al., 2011).

In Arabidopsis, several transcription factors are essential for tapetum development and pollen formation including two basic helix-loop-helix (bHLH) transcription factors, DYSFUNCTIONAL TAPETUM1 (DYT1) and ABORTED MICROSPORES (AMS), two MYB transcription factors, DEFECTIVE IN TAPETAL DEVELOPMENT AND FUNCTION1 (TDF1) and MALE STERILITY188 (MS188, also named MYB103 or MYB80), and a PLANT HOMEODOMAIN finger member MS1 (Wilson et al., 2001; Sorensen et al., 2003; Zhang et al., 2006, 2007; Zhu et al., 2008). *DYT1* is the most upstream regulator in tapetum development. *DYT1* affects many downstream genes related to tapetum development and pollen wall formation by directly regulating *TDF1* (Gu et al., 2014). *TDF1* directly regulates *AMS*, *AMS* regulates *MS188*, and *MS188* regulates *MS1* (Lou et al., 2014, 2018; J.Y. Lu, S.X. Xiong, W.Z. Yin, X.D. Teng, Y. Lou, J. Zhu, C. Zhang, J.N. Gu, Z.A. Wilson, and Z.N. Yang, unpublished data). Thus, these transcription factors form a genetic pathway (*DYT1*-*TDF1*-*AMS*-*MS188*-*MS1*) in controlling tapetum development. In this pathway, *AMS* can bind to the promoters of *CYP703A2*, *PKSB*, *TKPR1*, and *CYP704B1* in vivo (Xu et al., 2014). The *ams* mutant displays aberrantly enlarged tapetal cells and aborted microspores (Xu et al., 2010; Zhu et al., 2011). The pollen sexine is absent in the *ms188* mutant (Zhang et al., 2007). Recently, we found that *MS188* interacts with *AMS* to regulate the expression of *CYP703A2* (Xiong et al., 2016). The pollen wall is formed in a short time to protect the development of the microspore into mature pollen. It is uncertain how the tapetum regulates the sporopollenin synthesis pathway. In this work, we show that *MS188* acts as the major regulator to activate the sporopollenin synthesis pathway. As an upstream regulator, *AMS* may interact with *MS188* to form a feed-forward loop to facilitate the expression of sporopollenin synthesis genes for pollen wall formation.

RESULTS

Sporopollenin Synthesis Enzymes Are Localized in the Tapetum and Locules

During Arabidopsis anther development, *AMS* and *MS188* accumulate in tapetum cells at stages 6 to 9, in which the tapetal cells transfer into the secretory type and provide the sporopollenin materials for sexine

formation (Xiong et al., 2016). We confirmed this finding by GFP observation in current study (Fig. 1, A–J). The sporopollenin synthesis enzyme, *CYP703A2*, is localized in both the tapetal cells and anther locule at stages 7 through 9 (Xiong et al., 2016). The *MS2* protein accumulates in tapetal cells (Chen et al., 2011). To understand the expression pattern of other sporopollenin synthesis genes, we fused the *GFP* gene to the genomic sequences of *PKSA*, *PKSB*, *ACOS5*, *CYP704B1*, *TKPR2*, and *TKPR1* driven by their native promoters. The *TKPR1*-GFP construct was transferred to wild-type Arabidopsis plants to obtain transgenic lines. All other constructs were transferred to their corresponding mutants with heterozygous backgrounds. Transgenic lines with homozygous backgrounds were identified. The transgenes successfully complemented the fertility of the mutants (Supplemental Fig. S1). Hence, the GFP tagging of these genes did not interfere with their biological function. The transgenic plants were designated as *PKSA*-GFP, *PKSB*-GFP, *ACOS5*-GFP, *CYP704B1*-GFP, *TKPR1*-GFP, and *TKPR2*-GFP (Fig. 1, K–N1). Observations using confocal microscopy indicated that these sporopollenin synthesis proteins first accumulated in anthers at stage 7 and then decreased significantly at stage 10 (Fig. 1, K–N1). The expression pattern of these proteins overlap with that of *AMS* and *MS188* (Fig. 1, B, C, G, and H). The GFP fluorescent signals in transgenic plants appeared initially in the tapetal layer. When the microspores were released, the GFP signals were observed in both the tapetum and the locule and accumulated to high levels during the late stage of anther development. No GFP signals were detected inside the tetrads and free microspores (Supplemental Fig. S2). It has been reported that these genes are transcribed in tapetal cells (Aarts et al., 1997; Morant et al., 2007; de Azevedo Souza et al., 2009; Dobritsa et al., 2010; Chen et al., 2011), consistent with our results that indicate that the sporopollenin synthesis proteins are translated in the tapetum and gradually transported into the locules during anther development.

AMS and *MS188* Are Required for the Expression of Sporopollenin Synthesis Genes

DYT1, *TDF1*, *AMS*, *MS188*, and *MS1* form a genetic pathway that is important for tapetal development and pollen wall formation (Zhu et al., 2011; Gu et al., 2014; Lou et al., 2014, 2018; Fig. 2A). We analyzed the expression of sporopollenin synthesis genes in the mutants of these transcription factors. Results from microarray analysis showed that expression of *PKSA*, *PKSB*, *MS2*, and *CYP703A2* was suppressed in *dyt1*, *tdf1*, *ams*, and *ms188* mutants (Fig. 2B; Zhu et al., 2010; Li et al., 2017). Since *DYT1*, *TDF1*, and *AMS* are upstream regulators of *MS188*, it is likely that these sporopollenin synthesis genes are downstream of *MS188*. The expression of *CYP704B1*, *ACOS5*, *TKPR1*, and *TKPR2* was reduced in *dyt1*, *tdf1*, and *ams* mutants, but not in *ms188* (Fig. 2B). This indicates that these four genes are downstream of *AMS*. We utilized reverse transcription quantitative

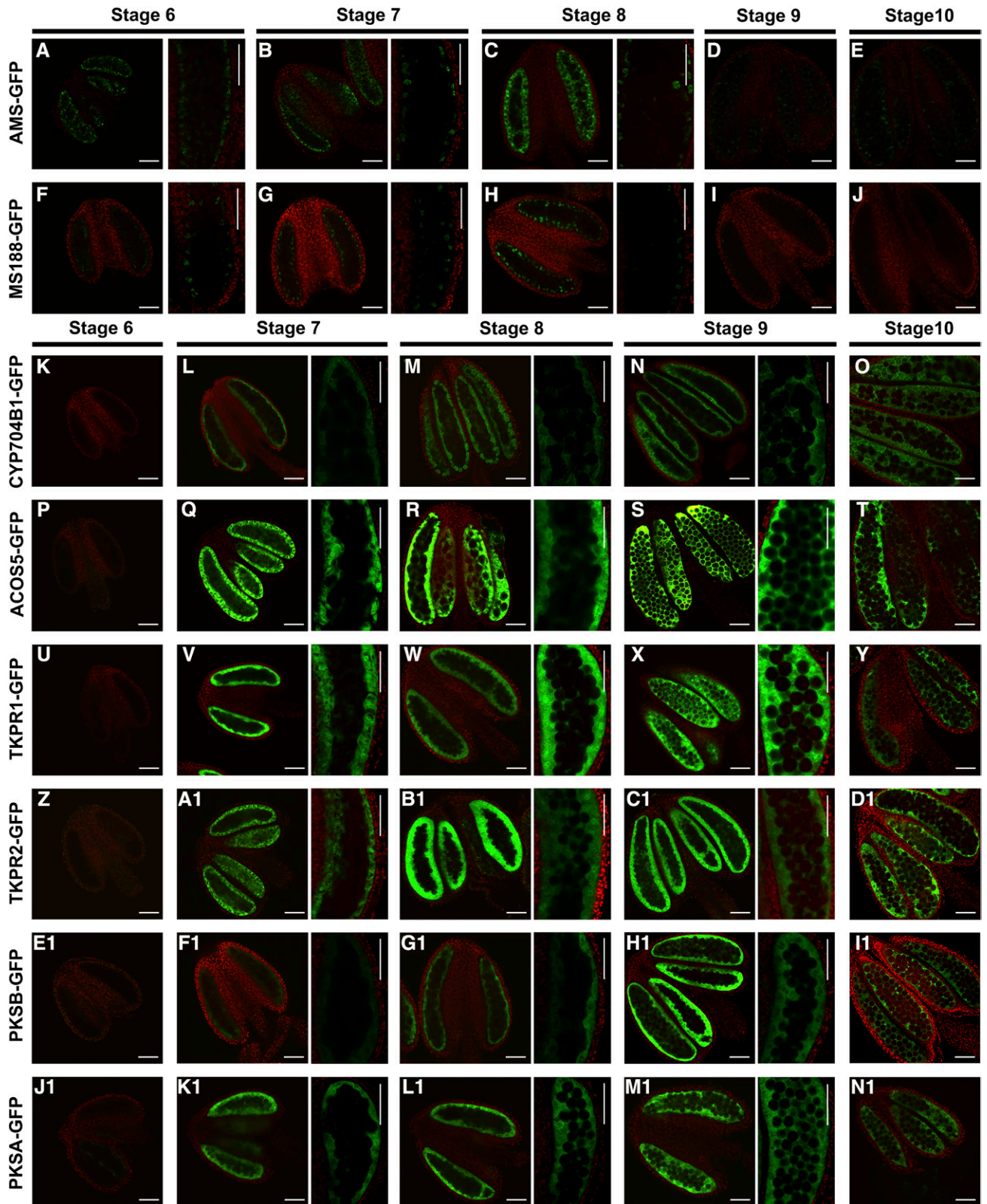


Figure 1. Protein localization of AMS-GFP, MS188-GFP, and sporopollenin synthesis protein-GFPs. Confocal images of the fluorescence of the AMS-GFP, MS188-GFP, CYP704B1-GFP, ACOS5-GFP, TKPR1-GFP, TKPR2-GFP, PKSB-GFP, and PKSA-GFP fusion proteins from stages 6 through 10. GFP expression (530 nm) is shown in the green channel, while chlorophyll autofluorescence (>560 nm) is shown in the red channel. A to C and F to H, The fluorescence of AMS-GFP and MS188-GFP at stages 6

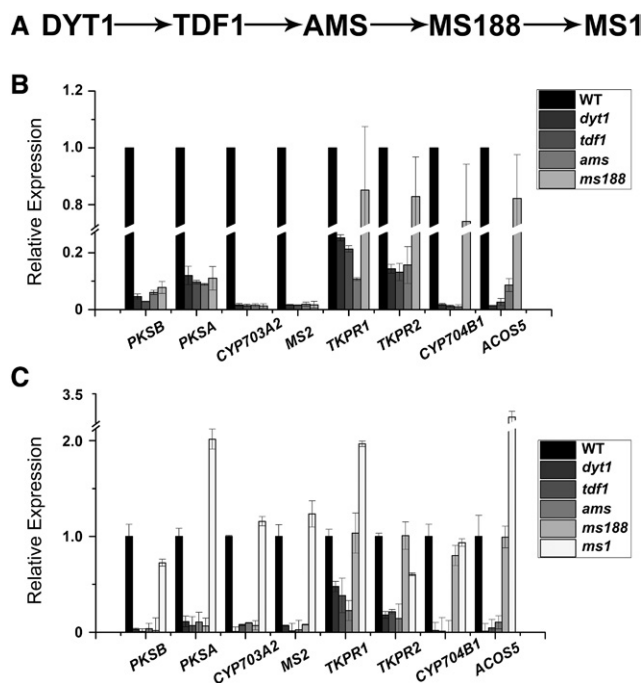


Figure 2. Expression of the sporopollenin synthesis genes in tapetal transcription factor mutants. **A**, The proposed hierarchical relationship between the tapetal transcription factors required for exine development. **B**, Results from microarray analysis of *PKSA*, *PKSB*, *CYP703A2*, *MS2*, *TKPR1*, *TKPR2*, *CYP704B1*, and *ACO5* expression in the inflorescences of wild type (WT), *dtf1*, *tdf1*, *ams*, and *ms188* mutants. **C**, RT-qPCR analysis of the above sporopollenin synthesis genes in the inflorescences of wild-type, *dtf1*, *tdf1*, *ams*, *ms188*, and *ms1* plants. *x* axis represents the genes, and *y* axis is shown as relative expression. SD is indicated as error bar.

PCR (RT-qPCR) analysis to further verify these data. The results of RT-qPCR were consistent with the microarray data (Fig. 2C). MS1 is thought to be the last transcription factor of the genetic pathway. RT-qPCR analysis showed that the expression of these genes was not affected in the *ms1* mutant (Fig. 2C). In summary, these results suggest that AMS and MS188 are two regulators closely related with the expression of sporopollenin synthesis genes.

MS188 Directly Activates the Expression of *PKSB*, *PKSA*, and *MS2*

MS188 encodes a member of the MYB family (Zhang et al., 2007). AACC is the core MYB binding motif

(Phan et al., 2011; Xiong et al., 2016). The promoter regions of *PKSA*, *PKSB*, and *MS2* genes all contain several MYB core binding motifs (Phan et al., 2011; Xiong et al., 2016). In a previous study, we obtained transgenic lines in which *Myc-tag* fused with *MS188* fully complemented the *ms188* phenotype (Xiong et al., 2016). We used this transgenic line to perform chromatin immunoprecipitation (ChIP) to understand whether MS188 was directly associated with these promoters. For each promoter, two probes spanning the MYB binding motifs (probe 1 and 2) and one probe in its coding region as a control were synthesized for analysis (Fig. 3, A–C). Our results showed that MYC-MS188 binding was stronger in the promoter regions of these genes than in the control coding regions (Fig. 3, D–F; Supplemental Fig. S3). Results from electrophoretic mobility shift assays (EMSA) showed that Maltose binding protein (MBP)-MS188 recombinant protein (Supplemental Fig. S4) bound to these cis-elements containing “AACC” core motifs. A 25-fold and 50-fold excess of unlabeled AACCT-containing probes was able to compete for the binding of MS188 to labeled probes (Fig. 3, G–I). Hence, MS188 is able to directly bind to the promoter sequences of *PKSA*, *PKSB*, and *MS2*. To analyze whether MS188 could activate the expression of these genes, a transient overexpression assay using *Arabidopsis* protoplasts was performed. The firefly luciferase (*LUC*) reporter gene individually driven by *PKSA*, *PKSB*, and *MS2* promoters and the *Renilla* gene driven by the 35S promoter were coconstructed as reporters, while *MS188* driven by the 35S promoter was used as an effector. When each reporter paired with the *p35S::MS188* effector were cotransformed into protoplasts, the amount of LUC luminescence driven by *PKSB*, *PKSA*, and *MS2* promoters increased, compared with the background level in the negative control (Fig. 3, J–L). These results indicate that MS188 directly binds to the promoters of *PKSA*, *PKSB*, and *MS2* to activate their expression.

MS188, Not AMS, Activates the Expression of *ACO5*, *CYP704B1*, and *TKPR1*

Through cytological analysis, MS188 was shown to be the key regulator for sexine formation (Zhang et al., 2007). However, results from microarray data and RT-qPCR analysis suggest that *ACO5*, *CYP704B1*, and *TKPR1* are downstream of AMS (Fig. 2, B and C). AMS can also bind to the promoters of *ACO5*, *CYP704B1*, and *TKPR1* (Xu et al., 2014; Supplemental Fig. S5). We further looked into this issue using a ChIP assay.

Figure 1. (Continued.)

through 8. Right, high-magnification images of GFP localization. D, E, I, and J, The fluorescence of AMS-GFP and MS188-GFP at stages 9 and 10. K, P, U, Z, E1, and J1, The fluorescence of CYP704B1-GFP, ACO5-GFP, TKPR1-GFP, TKPR2-GFP, PKSB-GFP, and PKSA-GFP at stage 6. L to N, Q to S, V to X, A1 to C1, F1 to H1, and K1 to M1, The fluorescence of CYP704B1-GFP, ACO5-GFP, TKPR1-GFP, TKPR2-GFP, PKSB-GFP, and PKSA-GFP at stages 7 to 9. Right, high-magnification images. O, T, Y, D1, I1, and N1, The fluorescence of CYP704B1-GFP, ACO5-GFP, TKPR1-GFP, TKPR2-GFP, PKSB-GFP, and PKSA-GFP at stage 10. Scale bars, 50 μ m.

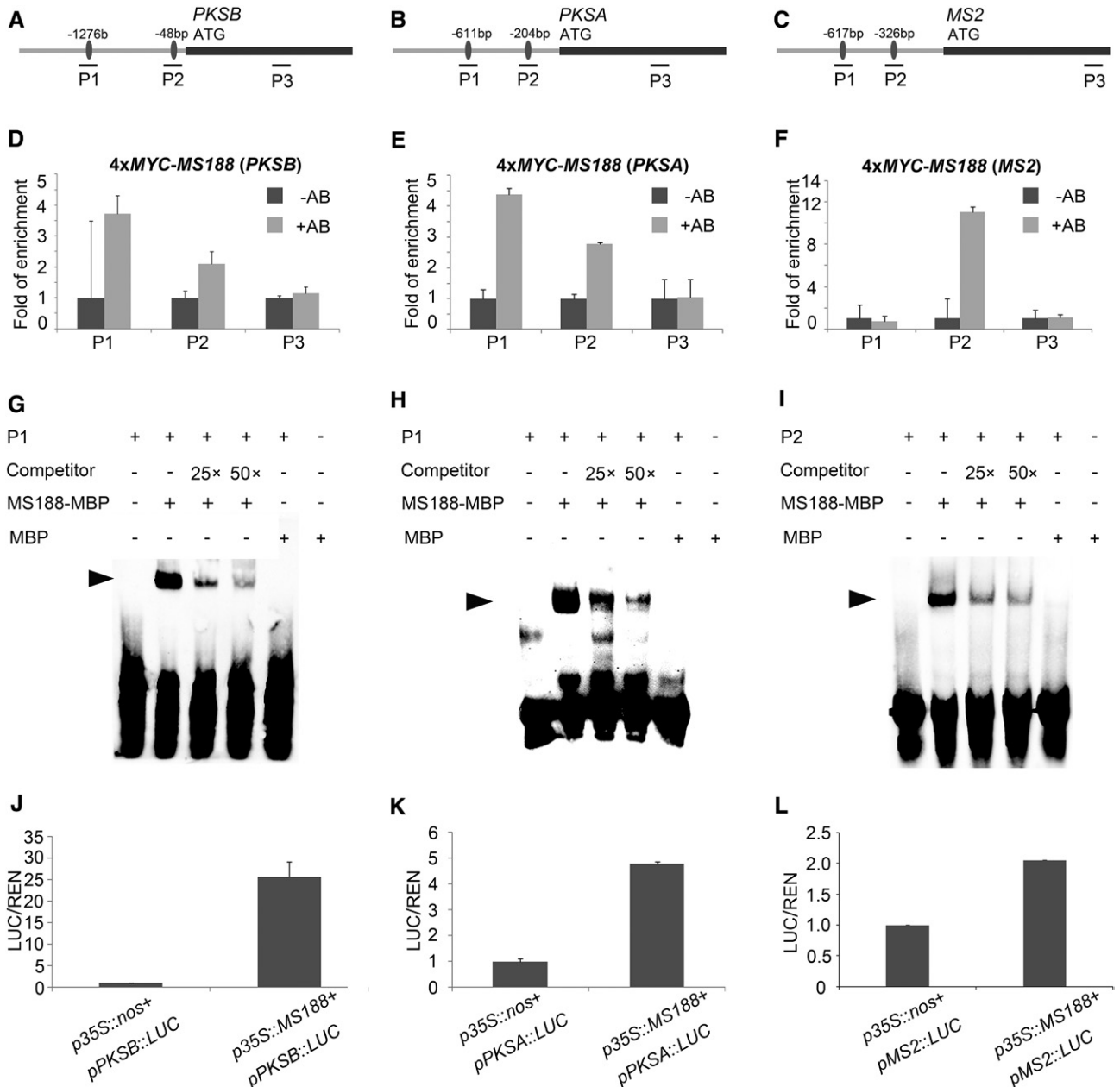


Figure 3. Transcription factor MS188 directly regulates the expression of *PKSA*, *PKSB*, and *MS2*. A to C, The designed probes of *PKSB*, *PKSA*, and *MS2*, respectively. Probe 1 (P1) and P2 contain the binding sites (black dots) of MS188, and P3 was designed in the coding region as a control. The black lines show the fragments of the probes for the ChIP-qPCR assay. D to F, The fragments of the above probes in *PKSB*, *PKSA*, and *MS2* promoters specifically amplified by ChIP-qPCR using *4xMYC-MS188* transgenic plant samples. The fold of enrichment is the average of three replicates, and SD is indicated as error bar. -AB, absence of antibody; +AB, presence of antibody. G to I, EMSA assay showing MS188 binding to probes in vitro. The first panel represents free probe, and the last panel indicates the mixture of free probe and MBP tag; both are used as negative controls. The shift band is indicated by the arrowhead, which is highlighted by the positive control of mixture of biotin-tagged probe and non-biotin-tagged probe and MBP-MS188. J to L, *p35S::MS188* was cotransformed with *pPKSB::LUC*, *pPKSA::LUC*, and *pMS2::LUC*, respectively, and a transient dual-luciferase assay was conducted in the *Arabidopsis* protoplasts. Three replicates were performed, and the y axis is shown as the ratio of *Luciferase/Renilla*. SD is indicated as error bar.

The promoter regions of *ACOS5*, *CYP704B1*, and *TKPR1* contain several MYB binding sites (Fig. 4, A–C). ChIP assay and EMSA showed that MS188 bound the

promoters of *ACOS5*, *CYP704B1*, and *TKPR1* (Fig. 4, D–F; Supplemental Figs. S3 and S6). Since both AMS and MS188 can bind to the promoters of these genes, a

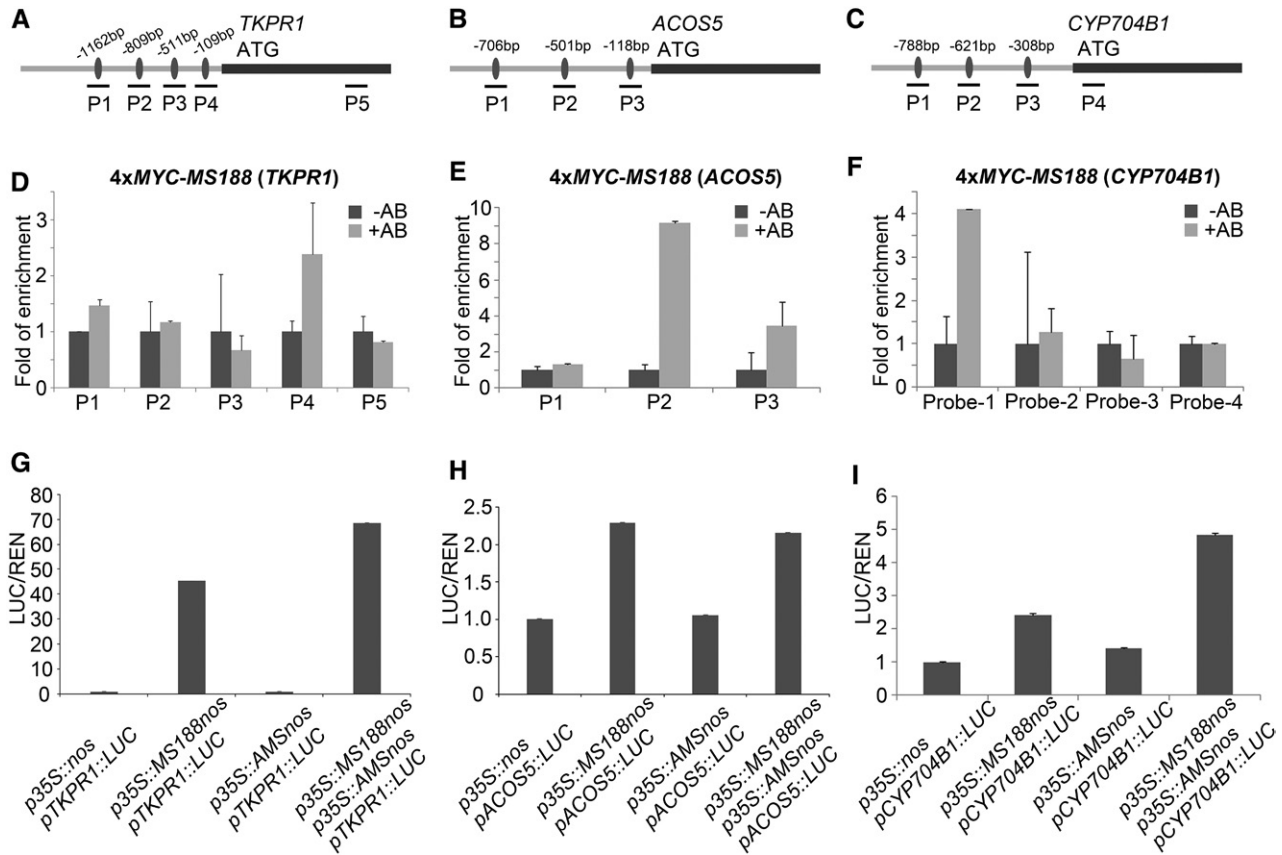


Figure 4. MS188 but not AMS activates the expression of *ACOS5*, *CYP704B1*, and *TKPR1*. A to C, The designed probes in the promoter and genomic regions of *TKPR1*, *ACOS5*, and *CYP704B1*, respectively. D to F, The fragments of the above probes in *TKPR1*, *ACOS5*, and *CYP704B1* promoters were specifically amplified by ChIP-qPCR using 4xMYC-MS188 transgenic plant samples. The fold enrichment is the average of three replicates (error bar is shown by sd). -AB, absence of antibody; +AB, presence of antibody. G to I, Transient dual-luciferase assays were performed in Arabidopsis leaf protoplasts. The *p35S::NOS* vector was used as negative control. The *p35S::MS188* and *p35S::AMS* were transformed into the protoplasts with reporter plasmids separately. The last column of three panels was cotransformation of *p35S::MS188*, *p35S::AMS*, and each reporter plasmid. sd is indicated as error bar.

dual-luciferase assay was performed to understand whether they could activate the expression of these genes. When the constructs of *pTKPR1::LUC*, *pACOS5::LUC*, and *p35S::AMS* were cotransformed into Arabidopsis leaf protoplasts, LUC activity was hardly induced (Fig. 4, G and H). The *pCYP704B1::LUC* could be slightly induced with *p35S::AMS* in the protoplasts (Fig. 4I). This indicates that AMS alone could hardly activate the expression of *ACOS5*, *CYP704B1*, and *TKPR1*. However, the LUC reporter driven by the promoters of the three genes above was strongly induced by MS188 protein (Fig. 4, G–I). Once *pCYP704B1::LUC* and *pTKPR1::LUC* were cotransformed with *p35S::AMS* and *p35S::MS188*, respectively, the luminescence signals increased (Fig. 4, G and I). For the cotransformation of *pACOS5::LUC* with *p35S::AMS* and *p35S::MS188*, LUC activity did not obviously increase (Fig. 4H). These results show that MS188 directly regulates the expression of *ACOS5*,

and AMS and MS188 synergistically activate the expression of *TKPR1* and *CYP704B1*.

Other MYBs, Together with MS188, Regulate the Expression of *CYP704B1*, *ACOS5*, *TKPR1*, and *TKPR2*

MS188 was shown to activate the expression of *ACOS5* and *TKPR1* in Arabidopsis protoplasts; however, their expression was not affected in the *ms188* mutant (Fig. 2, B and C). This may be caused by functional redundancy with other gene family members. To investigate this further, we made constructs of MS188 fused with a 12-amino acid SRDX motif (LDLDLELRGFA) driven by either the promoter of *MS188* or *AMS* (Fig. 5A). The chimeric protein with this motif was reported to act in a dominant manner to inhibit the activation of redundant transcription factors (Hiratsu et al., 2003). The two constructs were named *pMS188::MS188-SRDX*

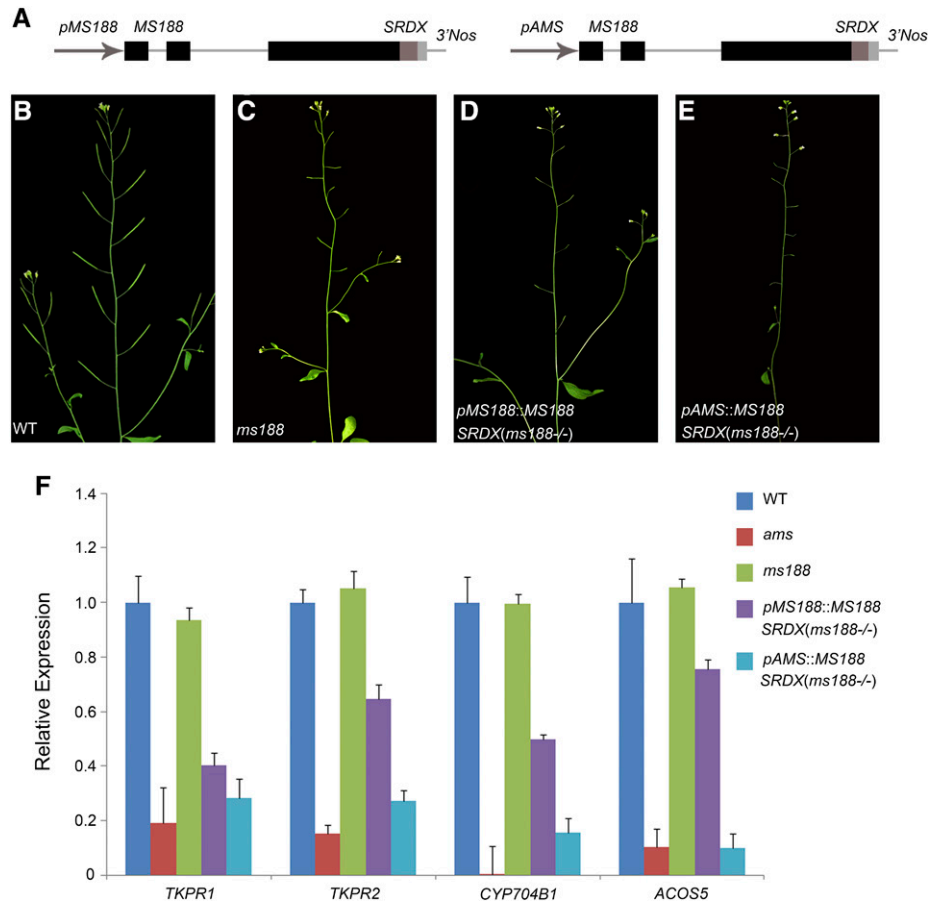


Figure 5. Redundant genes of the MYB family are involved in regulating the expression of sporopollenin synthesis genes. A, *pMS188::MS188-SRDX* and *pAMS::MS188-SRDX* constructs. B to E, Fertility comparison between wild-type (WT), *ms188*, *pMS188::MS188-SRDX* (*ms188*^{-/-}), and *pAMS::MS188-SRDX* (*ms188*^{-/-}) plants, respectively. F, Reverse transcription quantitative PCR (RT-qPCR) analysis of *TKPR1*, *TKPR2*, *CYP704B1*, and *ACOS5* expression in the inflorescences of WT, *ams*, *ms188*, *pMS188::MS188-SRDX* (*ms188*^{-/-}), and *pAMS::MS188-SRDX* (*ms188*^{-/-}) plants. *y* axis is shown as relative expression, and error bar is indicated as SD.

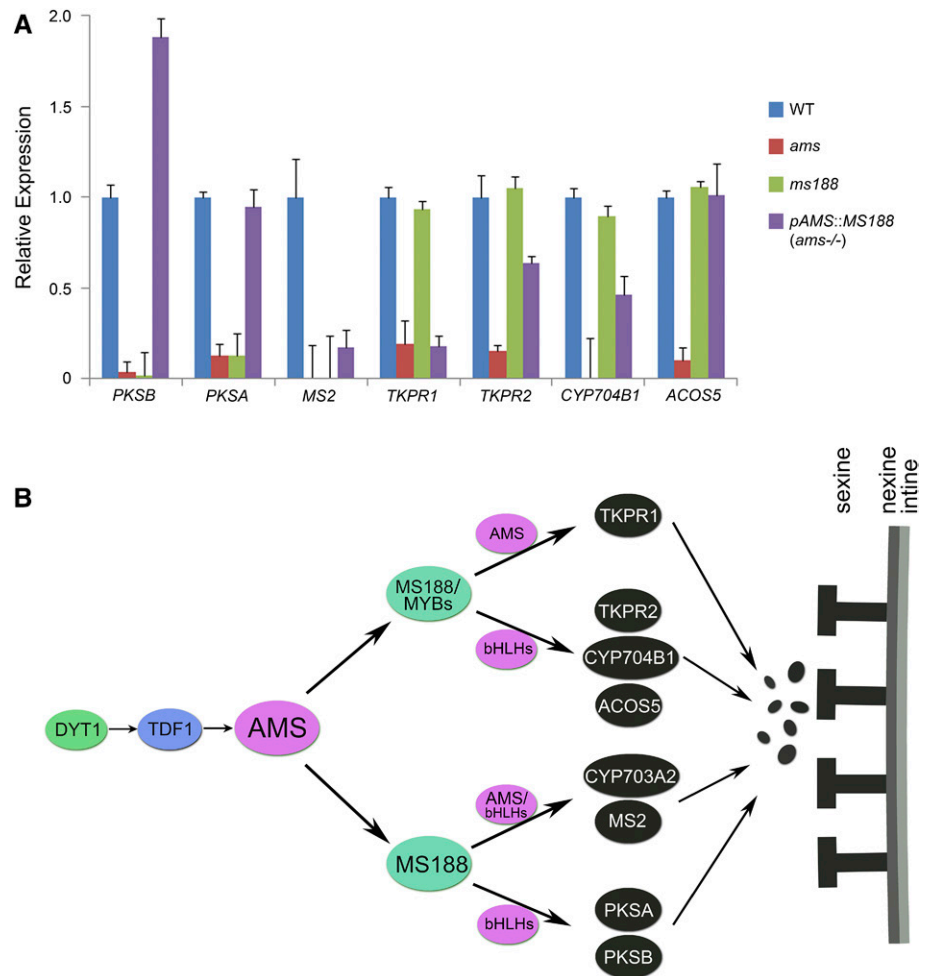
and *pAMS::MS188-SRDX* and were individually transformed into *MS188*^{+/-} heterozygous plants. Transgenic lines with the *ms188* homozygous background were identified (Fig. 5, B–E). In the *pMS188::MS188-SRDX* transgenic line, the expression of *CYP704B1*, *ACOS5*, *TKPR1*, and *TKPR2* was decreased compared with that in *ms188* (Fig. 5F). In the transgenic line of *pAMS::MS188-SRDX*, the transcript levels of *AMS* compared to wild-type plants were not affected (Supplemental Fig. S7A), but the expression of all these genes was further reduced compared with that in the *pMS188::MS188-SRDX* transgenic line (Fig. 5F). The expression level of these genes in the *pAMS::MS188-SRDX* line was close to that of the *ams* mutant (Fig. 5F). *AMS* acts upstream of *MS188*, and its expression is earlier than *MS188* (Zhu et al., 2011). The earlier expression of *MS188-SRDX* conferred by the *AMS* promoter may lead to a higher level of the chimeric *MS188-SRDX* protein thereby enhancing the repression of its targets.

These results indicate that other MYB transcription factors are also involved in regulating the expression of *CYP704B1*, *ACOS5*, *TKPR1*, and *TKPR2*.

The Expression of *MS188* in *ams* Rescues the Expression of Most Sporopollenin Synthesis Genes

In previous work, we obtained *pAMS::MS188* transgenic plants where *MS188* is expressed normally in the *ams* mutant background (Xiong et al., 2016). We used this transgenic line to analyze if *MS188* without *AMS* could activate the expression of sporopollenin synthesis genes. Of *PKSA*, *PKSB*, *MS2*, and *CYP703A2*, which were severely suppressed in both *ams* and *ms188* mutants (Fig. 2, B and C), the expression of *PKSA* and *PKSB* was fully restored in this transgenic line. The expression of *MS2* was partially restored (Fig. 6A), as was *CYP703A2* (Xiong et al., 2016). In *ams*, the expression of *CYP704B1*, *ACOS5*, and *TKPR2* decreased. The

Figure 6. Expression of MS188 in *ams* rescues the expression of sporopollenin synthesis-related genes. A, Reverse transcription quantitative PCR (RT-qPCR) analysis of *PKSB*, *PKSA*, *MS2*, *TKPR1*, *TKPR2*, *CYP704B1*, and *ACOS5* in the inflorescences of wild-type (WT), *ams*, *ms188*, and *pAMS::MS188* (*ams*^{-/-}) plants. y axis is shown as relative expression, and error bar is indicated as SD. B, A proposed model of MS188 acting as the main regulator together with AMS and other MYB family and bHLH family members to modulate sexine formation. For the genetic pathway (DYT1-TDF1-AMS-MS188-MS1) in the tapetal layer, MS188 is directly regulated by AMS and MS188 interacts with AMS to form a feed-forward loop to regulate the expression of sporopollenin synthesis genes. Some other MYB family and bHLH family members participate in the regulation process. The colorful ellipses indicate the transcription factors, and the black ones indicate the sporopollenin enzymes. The black circles represent the sporopollenin precursors.



expression of these genes was largely restored in the transgenic line (Fig. 6A). However, the expression of *TKPR1* did not show any recovery (Fig. 6A). These results demonstrate that MS188 acts as a major regulator to activate the expression of most genes for sporopollenin synthesis.

DISCUSSION

The Localization of Sporopollenin Synthesis Proteins Suggests the Synthesis of Sporopollenin Precursors in the Locule

In *Arabidopsis*, sporopollenin precursor synthesis involves at least eight genes that are expressed in the tapetal layer (Morant et al., 2007; de Azevedo Souza et al., 2009; Dobritsa et al., 2009, 2010; Grienenberger et al., 2010; Kim et al., 2010; Chen et al., 2011). Immunohistochemical localization under transmission electron microscope reveals that ACOS5, PKSA/B, TKPR1/2 are localized in tapetal cells (Lallemant et al., 2013). In the transgenic line with *CYP703A2-GFP* fully rescuing the *cyp703a2* phenotype, CYP703A2-GFP is

localized in both the tapetum and anther locule (Xiong et al., 2016). In this study, we used the same strategy to understand the expression pattern of sporopollenin synthesis genes during anther development. The protein products of these genes were initially detected in tapetal cells, then accumulated in the tapetum and anther locules (Fig. 1, K–N1). The expression pattern of these proteins is quite similar to that of CYP703A2. The similarity of their expression patterns supports that they belong to the same pathway of metabolic synthesis. Tapetal degeneration is first detected at stage 10, and is completed at stage 12 during anther development (Sanders et al., 1999). These proteins were observed in the locule at stage 8 (Fig. 1, M, R, W, B1, G1, and L1) indicating that they were secreted from the tapetum into the anther locule. It was proposed that the sporopollenin precursors are synthesized in the tapetal cells and transported onto the surface of microspores (Ariizumi and Toriyama, 2011; Quilichini et al., 2015). The high accumulation of sporopollenin synthesis proteins in locule suggests that sporopollenin precursors could be synthesized not only in the tapetum but also in the locule. The synthesis of sporopollenin precursors in the locule

may facilitate sporopollenin deposition on the microspore surface to form the sexine layer.

MS188 Plays a Key Role in Activating the Expression of Sporopollenin Synthesis Genes

In the genetic pathway (DYT1-TDF1-AMS-MS188-MS1), AMS and MS188 are two regulators important for the expression of sporopollenin synthesis genes. All eight genes were downregulated in *ams*, and four of them were suppressed in the *ms188* mutant (Fig. 2, B and C). In this work, we demonstrate that MS188 acts as a key regulator for sporopollenin synthesis gene expression based on the following evidence: (1) MS188 bound to the promoters of these genes (Figs. 3, D–I, and 4, D–F; Supplemental Fig. S6); (2) MS188, but not AMS, activated their transcription (Fig. 3, J–L, and 4, G and H); (3) without AMS, MS188 could activate the expression of most sporopollenin synthesis genes in vivo (Fig. 6A). The key role of MS188 for regulating sporopollenin synthesis is consistent with the phenotype of an absent sexine layer in the *ms188* mutant (Zhang et al., 2007). Among these sporopollenin synthesis genes, the expression of *ACOS5*, *CYP704B1*, and *TKPR1/2* were not affected in the *ms188* mutant (Fig. 2, B and C). A dominant suppression assay suggests that other MYB members play a redundant role to activate the expression of these genes (Fig. 5F). In Arabidopsis, MYB33 and MYB65 redundantly facilitate proper tapetal development. The tapetal cells of the *myb33 myb65* double mutant undergo hypertrophy during meiosis resulting in abortion of pollen development (Millar and Gubler, 2005). Additionally, MYB32 is reportedly involved in the regulation of phenylpropanoid pathways, affecting the composition of the pollen wall in Arabidopsis (Preston et al., 2004). These MYB family transcription factors might play a redundant role with MS188 to regulate sporopollenin synthesis for sexine formation.

AMS Interacting with MS188 May Form a Feed-Forward Loop to Highly Regulate Sporopollenin Synthesis

MYB family members frequently interact with bHLH transcription factors to regulate downstream genes during plant growth and development. In Arabidopsis, bHLH family members GLABROUS3 (GL3) and TRANSPARENT TESTA8 interact with MYB family members TRANSPARENT TESTA2, GL1, and MYB61 to regulate the expression of *DIHYDROFLAVONOL REDUCTASE* and *LEUCOANTHOCYANIDIN OXIDASE* for anthocyanin synthesis (Koes et al., 2005; Appelhagen et al., 2011). The GL3-GL1 complex is also required for root epidermal and trichome development (Schiefelbein, 2003; Zhao et al., 2008). bHLH-MYB complexes regulate the jasmonate-mediated stamen and seed maturation (Qi et al., 2015). The bHLH family member MYC2 interacts with MYB2 to regulate the gene expression of the abscisic acid-inducible pathway (Abe

et al., 2003; Kazan and Manners, 2013). MS188, a MYB gene family member, is the key regulator in activating the expression of sporopollenin synthesis genes for pollen wall formation. In the tapetum, AMS (bHLH family) is reported to interact with MS188 (MYB family; Xiong et al., 2016; Lou et al., 2018). AMS can bind to the promoters of several sporopollenin synthesis genes (Xu et al., 2014). It is likely that the activation of MS188 for sporopollenin synthesis requires its interaction with bHLH transcription factor AMS. During anther development, three other bHLH family members (bHLH010, bHLH089, and bHLH091) are also involved in tapetum development and male fertility (Zhu et al., 2015; Cui et al., 2016). Yeast two-hybrid assays showed that MS188 also interacted with both AMS and bHLH010 (Supplemental Fig. S8). Therefore, MS188 or other MYBs may form different combinations with AMS or other bHLHs to regulate the expression of relative sporopollenin synthesis genes as shown in the model (Fig. 6B). In this model, MS188 and other MYBs play a redundant role in activating the expression of *CYP704B1*, *ACOS5*, *TKPR1*, and *TKPR2*. Among them, AMS is critical for *TKPR1* expression because it could not be restored by MS188 in *ams* mutants. AMS and other bHLHs may also participate in activating the expression of *CYP704B1*, *ACOS5*, and *TKPR2*. MS188 was essential to activate the expression of *CYP703A2*, *PKSB*, *PKSA*, and *MS2*. As the expression of *CYP703A2* and *MS2* was partially restored by MS188 in *ams* mutants, AMS is important and other bHLHs play a partial redundant role to regulate their expression. The expression of *PKSB* and *PKSA* was fully restored, indicating that other bHLHs can replace AMS to regulate their expression (Fig. 6B).

Sporopollenin synthesis genes are highly and specifically expressed from stage 7 (late tetrad stage) to stage 10 during anther development (Fig. 1, C–H), when sporopollenin synthesis and deposition occur. The activation of the sporopollenin synthesis pathway by the key activator MS188 may facilitate the quick initiation of sporopollenin synthesis to protect developing microspores. AMS directly regulates *MS188* and interacts with MS188 (Xiong et al., 2016; Lou et al., 2018). This appears analogous to the regulatory relationship of TDF1 and AMS, in which TDF1 directly regulates *AMS* and interacts with *AMS* to form a feed-forward loop to facilitate downstream gene expression (Lou et al., 2018). In the tapetum, the expression of *TDF1* is relatively low (Lou et al., 2018). These two feed-forward loops may act together to achieve the high levels of expression of sporopollenin synthesis genes required for rapid formation of the pollen wall.

MATERIALS AND METHODS

Plants Growth Conditions

The Arabidopsis (*Arabidopsis thaliana*) Colombia ecotype was used for wild-type plants. The seeds were placed in the dark at 4°C for 2 d before germination

and then planted in a 22°C incubator with 16 h light and 8 h dark. Plant flowers were transformed with *Agrobacterium tumefaciens* containing the constructs in a binary vector, and the seeds obtained were screened on selective growth medium to obtain the transgenic plants. All T-DNA insertion mutant lines were provided by the Arabidopsis Biological Resource Center.

Expression Analysis

The RNA used for microarray and RT-qPCR analyses was derived from the inflorescences of wild-type, *dyl1*, *tdf1*, *ams*, *ms188*, and *ms1* plants. Three replicates of independently grown materials were used. The RNA was extracted using TRIzol (Invitrogen), purified using a RNeasy Mini kit (Qiagen) and used as a template for cDNA synthesis. Cy3- and Cy5-labeled cRNA molecules were synthesized (Agilent; 5184-3523) and hybridized onto a 44 K Arabidopsis oligo microarray (Agilent; 5188-5242). Signals were detected according to the manufacturer's protocol. The spots with 2-fold or more differential expression between the wild type and mutants (pValueLogRatio < 0.05; Flag = 0; S/n > 2.6) in three independent experiments were identified. RT-qPCR was carried out as previously described (Xiong et al., 2016). The primers were synthesized by Shanghai Genarray Biotech. The primer sequences are presented in Supplemental Table S1.

Protein Localization

The transgenic plants of *ACOS5-GFP*, *CYP704B1-GFP*, *TKPR1-GFP*, *TKPR2-GFP*, *PKSA-GFP*, and *PKSB-GFP* were constructed by fusing *GFP* with *ACOS5*, *CYP704B1*, *TKPR1*, *TKPR2*, *PKSA*, and *PKSB* driven by their native promoters, and the binary vector pCAMBIA1300 was used for the construction. The constructed plasmids were transferred into *A. tumefaciens* to infect the flower buds. The flower buds of the plants were harvested, and the anthers were isolated. A Zeiss laser confocal microscope with a 40× oil objective was used to observe GFP green fluorescence (Xiong et al., 2016).

ChIP

The *MS188-MYC* transgenic plants were planted and their buds were collected as ChIP experimental materials. The *MS188-MYC*-tagged proteins and chromatin forming complexes in buds were cross linked in formaldehyde containing buffer. The nuclei were isolated, and the chromatin was sheared with an ultrasonic apparatus. The majority of the DNA fragments with a size between 300 and 800 bp were incubated with salmon sperm DNA/protein agarose mix (Millipore) for 1 h, and the supernatants were incubated with polyclonal antibody against MYC (Millipore) at 4°C overnight. The antibody-protein/DNA complexes were precipitated with magnetic beads protein G (Invitrogen). After isolation and purification, the DNA samples were subjected to real-time PCR analysis. The primer information is shown in Supplemental Table S1.

EMSA

Induction and purification of MBP-*MS188* fusion protein was based on a published protocol (Xiong et al., 2016). The biotin-labeled and unlabeled DNA probes were designed based on the MYB family binding sites on the cis-elements of *PKSA*, *PKSB*, *MS2*, and *ACOS5* promoters (probe information in Supplemental Table S1). The EMSA was performed using the Light Shift Chemiluminescent EMSA Kit (Thermo Scientific). Reactions were performed in binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM DTT) at room temperature for 30 min. Images were taken with a Tanon-5500 Chemiluminescent Imaging System (Tanon, China).

Protoplast Transformation

The promoters of sporopollenin synthesis genes were inserted into the *pGreenII 0800-LUC* vector. Arabidopsis (Col-0) leaves grown for 28 d were harvested and protoplasts were prepared using cellulase (0.015 g/mL) and pectinase (0.0035 g/mL) to lyse the cell wall. The above constructs and the *p35S::MS188* construct were cotransfected into protoplasts in poly ethylene glycol solution (0.4g/mL PEG4000), and the cytoplasm was cultivated for 12 to 16 h of light treatment. After adding Passive Lysis buffer to lyse the protoplasts, the *LUC* substrate (Luciferase Assay Reagent II) was added to detect the firefly luminescence. The reaction was quenched, and then the Stop & Glo Reagent was added to test the *Renilla* luciferase. The above experiment

was performed using luciferase assay kit obtained from Promega (catalog no. E1910) according to the manufacturer's instructions.

Yeast Two-Hybrid Analysis

The yeast two-hybrid assay was achieved following the protocol of the Clontech two-hybrid system. The *MS188* proteins without the self-activation fragment were obtained from previous work (Xiong et al., 2016), and the *bHLH010* coding region was inserted into the pGADT7 plasmids. The *Saccharomyces cerevisiae* (yeast) strain AH109 was cotransformed with these constructs. The transformants were screened on the corresponding selective media of supplemented synthetic dextrose medium lacking Leu and Trp or supplemented synthetic dextrose medium lacking Leu, Trp, His, and adenine hemisulfate salt with *X-α-Gal*.

Accession Numbers

Microarray data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *DY11*, AT4G21330; *TDF1*, AT3G28470; *AMS*, AT2G16910; *MS188/MYB80/MYB103*, AT5G56110; *MS1*, AT5G22260; *ACOS5*, AT1G62940; *MS2*, AT3G11980; *CYP703A2*, AT1G01280; *CYP704B1*, AT1G69500; *PKSA*, AT1G02050; *PKSB*, AT4G34850; *TKPR1*, AT4G35420; *TKPR2*, AT1G68540; and *bHLH010*, AT2G31220.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. The mutants of sporopollenin synthesis genes and their transgenic complementary plants.

Supplemental Figure S2. Bright-field images of the tetrads of *AMS-GFP*, *MS188-GFP*, *CYP704B1-GFP*, *ACOS5-GFP*, *TKPR1-GFP*, *TKPR2-GFP*, *PKSB-GFP*, and *PKSA-GFP* at stage 7.

Supplemental Figure S3. Percentage input of the binding probes of *PKSA*, *PKSB*, *MS2*, *ACOS5*, *TKPR1*, and *CYP704B1* in ChIP assays.

Supplemental Figure S4. The expression and purification of *MS188* and *AMS* recombinant proteins.

Supplemental Figure S5. *AMS* directly binds to the promoters of *CYP704B1* and *ACOS5* in vivo.

Supplemental Figure S6. *MS188* directly binds to the promoters of *TKPR1* and *ACOS5* in vitro.

Supplemental Figure S7. RT-qPCR analysis of the expression of *AMS*, *MYB32*, *MYB33*, and *MYB65*.

Supplemental Figure S8. *MS188* interacts with *bHLH010* in yeast two-hybrid assays.

Supplemental Table S1. List of primers used in the study.

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LITERATURE CITED

- Aarts MG, Hodge R, Kalantidis K, Florack D, Wilson ZA, Mulligan BJ, Stiekema WJ, Scott R, Pereira A (1997) The Arabidopsis MALE STERILITY 2 protein shares similarity with reductases in elongation/condensation complexes. *Plant J* 12: 615–623
- Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. *Plant Cell* 15: 63–78
- Appelhaagen I, Jahns O, Bartelniewoehner L, Sagasser M, Weisshaar B, Stracke R (2011) Leucoanthocyanidin Dioxygenase in *Arabidopsis thaliana*:

- characterization of mutant alleles and regulation by MYB-BHLH-TTG1 transcription factor complexes. *Gene* **484**: 61–68
- Ariizumi T, Toriyama K** (2011) Genetic regulation of sporopollenin synthesis and pollen exine development. *Annu Rev Plant Biol* **62**: 437–460
- Blackmore S, Wortley AH, Skvarla JJ, Rowley JR** (2007) Pollen wall development in flowering plants. *New Phytol* **174**: 483–498
- Chen W, Yu XH, Zhang K, Shi J, De Oliveira S, Schreiber L, Shanklin J, Zhang D** (2011) Male Sterile2 encodes a plastid-localized fatty acyl carrier protein reductase required for pollen exine development in *Arabidopsis*. *Plant Physiol* **157**: 842–853
- Choi H, Jin JY, Choi S, Hwang JU, Kim YY, Suh MC, Lee Y** (2011) An ABCG/WBC-type ABC transporter is essential for transport of sporopollenin precursors for exine formation in developing pollen. *Plant J* **65**: 181–193
- Cui J, You C, Zhu E, Huang Q, Ma H, Chang F** (2016) Feedback regulation of DYT1 by interactions with downstream bHLH factors promotes DYT1 nuclear localization and anther development. *Plant Cell* **28**: 1078–1093
- de Azevedo Souza C, Kim SS, Koch S, Kienow L, Schneider K, McKim SM, Haughn GW, Kombrink E, Douglas CJ** (2009) A novel fatty Acyl-CoA synthetase is required for pollen development and sporopollenin biosynthesis in *Arabidopsis*. *Plant Cell* **21**: 507–525
- Dickinson HG, Heslop-Harrison J** (1968) Common mode of deposition for the sporopollenin of sexine and nexine. *Nature* **220**: 926–927
- Dobritsa AA, Shrestha J, Morant M, Pinot F, Matsuno M, Swanson R, Möller BL, Preuss D** (2009) CYP704B1 is a long-chain fatty acid omega-hydroxylase essential for sporopollenin synthesis in pollen of *Arabidopsis*. *Plant Physiol* **151**: 574–589
- Dobritsa AA, Lei Z, Nishikawa S, Urbanczyk-Wochniak E, Huhman DV, Preuss D, Sumner LW** (2010) LAP5 and LAP6 encode anther-specific proteins with similarity to chalcone synthase essential for pollen exine development in *Arabidopsis*. *Plant Physiol* **153**: 937–955
- Dou XY, Yang KZ, Zhang Y, Wang W, Liu XL, Chen LQ, Zhang XQ, Ye D** (2011) WBC27, an adenosine tri-phosphate-binding cassette protein, controls pollen wall formation and patterning in *Arabidopsis*. *J Integr Plant Biol* **53**: 74–88
- Edlund AF, Swanson R, Preuss D** (2004) Pollen and stigma structure and function: the role of diversity in pollination. *Plant Cell* **16** (Suppl): S84–S97
- Grienenberger E, Kim SS, Lallemand B, Geoffroy P, Heintz D, Souza CdeA, Heitz T, Douglas CJ, Legrand M** (2010) Analysis of TETRAKETIDE α -PYRONE REDUCTASE function in *Arabidopsis thaliana* reveals a previously unknown, but conserved, biochemical pathway in sporopollenin monomer biosynthesis. *Plant Cell* **22**: 4067–4083
- Gu JN, Zhu J, Yu Y, Teng XD, Lou Y, Xu XF, Liu JL, Yang ZN** (2014) DYT1 directly regulates the expression of TDF1 for tapetum development and pollen wall formation in *Arabidopsis*. *Plant J* **80**: 1005–1013
- Heslop-Harrison J** (1962) Origin of exine. *Nature* **195**: 1069–1071
- Hiratsu K, Matsui K, Koyama T, Ohme-Takagi M** (2003) Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in *Arabidopsis*. *Plant J* **34**: 733–739
- Jia QS, Zhu J, Xu XF, Lou Y, Zhang ZL, Zhang ZP, Yang ZN** (2015) *Arabidopsis* AT-hook protein TEK positively regulates the expression of arabinogalactan proteins for Nexine formation. *Mol Plant* **8**: 251–260
- Kazan K, Manners JM** (2013) MYC2: the master in action. *Mol Plant* **6**: 686–703
- Kim SS, Grienenberger E, Lallemand B, Colpitts CC, Kim SY, Souza CdeA, Geoffroy P, Heintz D, Krahn D, Kaiser M** (2010) LAP6/POLYKETIDE SYNTHASE A and LAP5/POLYKETIDE SYNTHASE B encode hydroxylalkyl α -pyrone synthases required for pollen development and sporopollenin biosynthesis in *Arabidopsis thaliana*. *Plant Cell* **22**: 4045–4066
- Koes R, Verweij W, Quattrocchio F** (2005) Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. *Trends Plant Sci* **10**: 236–242
- Lallemand B, Erhardt M, Heitz T, Legrand M** (2013) Sporopollenin biosynthetic enzymes interact and constitute a metabolon localized to the endoplasmic reticulum of tapetum cells. *Plant Physiol* **162**: 616–625
- Li DD, Xue JS, Zhu J, Yang ZN** (2017) Gene regulatory network for tapetum development in *Arabidopsis thaliana*. *Front Plant Sci* **8**: 1559
- Lou Y, Xu XF, Zhu J, Gu JN, Blackmore S, Yang ZN** (2014) The tapetal AHL family protein TEK determines nexine formation in the pollen wall. *Nat Commun* **5**: 3855
- Lou Y, Zhou HS, Han Y, Zeng QY, Zhu J, Yang ZN** (2018) Positive regulation of AMS by TDF1 and the formation of a TDF1-AMS complex are required for anther development in *Arabidopsis thaliana*. *New Phytol* **217**: 378–391
- Millar AA, Gubler F** (2005) The *Arabidopsis* GAMYB-like genes, MYB33 and MYB65, are microRNA-regulated genes that redundantly facilitate anther development. *Plant Cell* **17**: 705–721
- Morant M, Jørgensen K, Schaller H, Pinot F, Möller BL, Werck-Reichhart D, Bak S** (2007) CYP703 is an ancient cytochrome P450 in land plants catalyzing in-chain hydroxylation of lauric acid to provide building blocks for sporopollenin synthesis in pollen. *Plant Cell* **19**: 1473–1487
- Phan HA, Iacuone S, Li SF, Parish RW** (2011) The MYB80 transcription factor is required for pollen development and the regulation of tapetal programmed cell death in *Arabidopsis thaliana*. *Plant Cell* **23**: 2209–2224
- Piffanelli P, Ross JHE, Murphy DJ** (1998) Biogenesis and function of the lipidic structures of pollen grains. *Sex Plant Reprod* **11**: 65–80
- Preston J, Wheeler J, Heazlewood J, Li SF, Parish RW** (2004) AtMYB32 is required for normal pollen development in *Arabidopsis thaliana*. *Plant J* **40**: 979–995
- Qi T, Huang H, Song S, Xie D** (2015) Regulation of jasmonate-mediated stamen development and seed production by a bHLH-MYB complex in *Arabidopsis*. *Plant Cell* **27**: 1620–1633
- Quilichini TD, Friedmann MC, Samuels AL, Douglas CJ** (2010) ATP-binding cassette transporter G26 is required for male fertility and pollen exine formation in *Arabidopsis*. *Plant Physiol* **154**: 678–690
- Quilichini TD, Grienenberger E, Douglas CJ** (2015) The biosynthesis, composition and assembly of the outer pollen wall: A tough case to crack. *Phytochemistry* **113**: 170–182
- Sanders PM, Bui AQ, Weterings K, McIntire KN, Hsu YC, Lee PY, Truong MT, Beals TP, Goldberg RB** (1999) Anther developmental defects in *Arabidopsis thaliana* male-sterile mutants. *Sex Plant Reprod* **11**: 297–322
- Schiefelbein J** (2003) Cell-fate specification in the epidermis: A common patterning mechanism in the root and shoot. *Curr Opin Plant Biol* **6**: 74–78
- Scott RJ** (1994) *Molecular and Cellular Aspects of Plant Reproduction*. University Press, Cambridge, UK
- Sorensen AM, Kröber S, Unte US, Huijser P, Dekker K, Saedler H** (2003) The *Arabidopsis* ABORTED MICROSPORES (AMS) gene encodes a MYC class transcription factor. *Plant J* **33**: 413–423
- Wilson ZA, Morroll SM, Dawson J, Swarup R, Tighe PJ** (2001) The *Arabidopsis* MALE STERILITY1 (MS1) gene is a transcriptional regulator of male gametogenesis, with homology to the PHD-finger family of transcription factors. *Plant J* **28**: 27–39
- Xiong SX, Lu JY, Lou Y, Teng XD, Gu JN, Zhang C, Shi QS, Yang ZN, Zhu J** (2016) The transcription factors MS188 and AMS form a complex to activate the expression of CYP703A2 for sporopollenin biosynthesis in *Arabidopsis thaliana*. *Plant J* **88**: 936–946
- Xu J, Yang C, Yuan Z, Zhang D, Gondwe MY, Ding Z, Liang W, Zhang D, Wilson ZA** (2010) The ABORTED MICROSPORES regulatory network is required for postmeiotic male reproductive development in *Arabidopsis thaliana*. *Plant Cell* **22**: 91–107
- Xu J, Ding Z, Vizcay-Barrena G, Shi J, Liang W, Yuan Z, Werck-Reichhart D, Schreiber L, Wilson ZA, Zhang D** (2014) ABORTED MICROSPORES acts as a master regulator of pollen wall formation in *Arabidopsis*. *Plant Cell* **26**: 1544–1556
- Xu T, Zhang C, Zhou Q, Yang ZN** (2016) Pollen wall pattern in *Arabidopsis*. *Sci Bull (Beijing)* **61**: 832–837
- Zhang W, Sun Y, Timofejeva L, Chen C, Grossniklaus U, Ma H** (2006) Regulation of *Arabidopsis* tapetum development and function by DYSFUNCTIONAL TAPETUM1 (DYT1) encoding a putative bHLH transcription factor. *Development* **133**: 3085–3095
- Zhang ZB, Zhu J, Gao JF, Wang C, Li H, Li H, Zhang HQ, Zhang S, Wang DM, Wang QX** (2007) Transcription factor AtMYB103 is required for anther development by regulating tapetum development, callose dissolution and exine formation in *Arabidopsis*. *Plant J* **52**: 528–538
- Zhao M, Morohashi K, Hatlestad G, Grotewold E, Lloyd A** (2008) The TTG1-bHLH-MYB complex controls trichome cell fate and patterning through direct targeting of regulatory loci. *Development* **135**: 1991–1999

- Zhou Q, Zhu J, Cui YL, Yang ZN** (2015) Ultrastructure analysis reveals sporopollenin deposition and nexine formation at early stage of pollen wall development in Arabidopsis. *Sci Bull (Beijing)* **60**: 273–276
- Zhu E, You C, Wang S, Cui J, Niu B, Wang Y, Qi J, Ma H, Chang F** (2015) The DYT1-interacting proteins bHLH010, bHLH089 and bHLH091 are redundantly required for Arabidopsis anther development and transcriptome. *Plant J* **83**: 976–990
- Zhu J, Chen H, Li H, Gao JF, Jiang H, Wang C, Guan YF, Yang ZN** (2008) Defective in tapetal development and function 1 is essential for anther development and tapetal function for microspore maturation in Arabidopsis. *Plant J* **55**: 266–277
- Zhu J, Zhang G, Chang Y, Li X, Yang J, Huang X, Yu Q, Chen H, Wu T, Yang Z** (2010) AtMYB103 is a crucial regulator of several pathways affecting Arabidopsis anther development. *Sci China Life Sci* **53**: 1112–1122
- Zhu J, Lou Y, Xu X, Yang ZN** (2011) A genetic pathway for tapetum development and function in Arabidopsis. *J Integr Plant Biol* **53**: 892–900