

# ABORTED MICROSPORES Acts as a Master Regulator of Pollen Wall Formation in *Arabidopsis*<sup>C|W|OPEN</sup>

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Mature pollen is covered by durable cell walls, principally composed of sporopollenin, an evolutionary conserved, highly resilient, but not fully characterized, biopolymer of aliphatic and aromatic components. Here, we report that ABORTED MICROSPORES (AMS) acts as a master regulator coordinating pollen wall development and sporopollenin biosynthesis in *Arabidopsis thaliana*. Genome-wide coexpression analysis revealed 98 candidate genes with specific expression in the anther and 70 that showed reduced expression in *ams*. Among these 70 members, we showed that AMS can directly regulate 23 genes implicated in callose dissociation, fatty acids elongation, formation of phenolic compounds, and lipidic transport putatively involved in sporopollenin precursor synthesis. Consistently, *ams* mutants showed defective microspore release, a lack of sporopollenin deposition, and a dramatic reduction in total phenolic compounds and cutin monomers. The functional importance of the AMS pathway was further demonstrated by the observation of impaired pollen wall architecture in plant lines with reduced expression of several AMS targets: the abundant pollen coat protein extracellular lipases (EXL5 and EXL6), and CYP98A8 and CYP98A9, which are enzymes required for the production of phenolic precursors. These findings demonstrate the central role of AMS in coordinating sporopollenin biosynthesis and the secretion of materials for pollen wall patterning.

## INTRODUCTION

The pollen wall, a multilayer specialized cell wall surrounding the pollen grains, not only provides mechanical protection for male gametophytes from desiccation, environmental stresses, and microbial attacks, but is also essential for various aspects of pollination, including pollen adhesion, hydration, and germination (Scott et al., 2004). The outer layer, called exine, is composed principally of sporopollenin, a highly resistant biopolymer derived from fatty acids, phenylpropanoids, phenolics, and traces of carotenoids (Ahlers et al., 1999). Exine morphology varies among species (Blackmore et al., 2007), and this complex, and sometimes species-specific patterning, combined with its durability, enable its use in paleontological and forensic analyses. Sporopollenin is highly conserved across distant phylogenetic groups of embryophytes, fossilized green algae, and higher plants, suggesting that it has remained evolutionary conserved since the initial colonization of land by embryophytes. Despite this, due to

its insolubility and chemical resilience, its biochemistry and biosynthesis still remain largely uncharacterized (Blackmore et al., 2007).

The anther, the higher plant male reproductive organ, comprises four somatic cell layers surrounding the sporogenous cells, which will subsequently form the mature pollen (Scott et al., 2004). The tapetum, the innermost layer, serves as a nutritive tissue that provides metabolites, nutrients, and lipidic sporopollenin precursors for pollen development. Tapetal cells are known to transcribe genes involved in pollen wall biosynthesis and patterning, and in the secretion of the pollen coat, or tryphine, which contains flavonoids, phenolamides, carotenoids, lipids, and proteins that cover the surface and cavities of the exine (Jiang et al., 2013).

Recently, biochemical and genetic evidence revealed that several tapetally expressed enzymes, such as plastid-localized fatty acyl ACP reductase, *Arabidopsis thaliana* MALE STERILITY2, and rice (*Oryza sativa*) DEFECTIVE POLLEN WALL (Chen et al., 2011; Shi et al., 2011), fatty acid hydroxylases, such as CYP703A (Morant et al., 2007) and CYP704Bs (Li and Zhang, 2010), fatty acyl-CoA ester synthase ACYL-COA SYNTHETASE5 (de Azevedo Souza et al., 2009), hydroxyalkyl  $\alpha$ -pyrone synthases POLYKETIDE SYNTHASE A/LAP6 and B/LAP5 (PKSA/B) (Dobritsa et al., 2010; Kim et al., 2010), and TETRAKETIDE  $\alpha$ -PYRONE REDUCTASE1 and 2 (TKPR1/2) (also called DIHYDROFLAVONOL 4-REDUCTASE LIKE1 [DRL1]) (Tang et al., 2009; Grienenberger et al., 2010) play important roles in synthesizing sporopollenin precursors. Moreover, ABC transporters

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such as WBC27/ABCG26 in *Arabidopsis* (Xu et al., 2010) and POST-MEIOTIC DEFICIENT ANTHER1/ABCG15 in rice (Niu et al., 2013; Qin et al., 2013; Zhu et al., 2013) are involved in the transport of sporopollenin precursors across the hydrophilic cell wall from the tapetum to the microspore surface.

In higher plants, a number of conserved transcription factors have been shown to be associated with tapetal function and pollen development (Wilson and Zhang, 2009; Zhang et al., 2011); however, the mechanisms that regulate pollen wall biosynthesis and secretion remain elusive. ABORTED MICRO-SPORE (AMS), a basic loop-helix-loop (bHLH) tapetum-specific transcriptional factor, has been shown to affect the expression of genes involved in the transport of lipids, flavonol accumulation, substrate oxidation, methyl modification, and pectin dynamics (Xu et al., 2010). The *ams* mutant displays abnormally enlarged tapetal cells and aborted microspore development (Sorensen et al., 2003; Xu et al., 2010).

In this work, we uncover the central role that AMS plays in coordinating the biosynthesis and secretion of materials for pollen wall biosynthesis. Using genome-wide transcriptional analysis, combined with biochemical and functional validation, we show that AMS acts as a principal coordinator of pollen wall formation by directly regulating target genes associated with separation of the microspore mother cell, microspore compartmentalization, and callose dissociation within the tetrad, sporopollenin precursor synthesis (fatty acid elongation and hydroxylation and synthesis of phenolic compounds) and transport, as well as pollen coat formation.

## RESULTS

### Genome-Wide Identification of Candidate Genes Required for Pollen Wall Development

To understand the precise molecular mechanism underlying pollen wall formation in this study, we used coexpression analysis to show that 98 genes are closely associated with pollen wall development. We determined the expression profiles (Figure 1A) of previously characterized genes involved in callose wall formation, primexine formation, or sporopollenin biosynthesis (Ariizumi and Toriyama, 2011) (Supplemental Figure 1 and Supplemental Table 1) to generate a list of 16 genes that are specifically expressed during various stages of pollen development (Figure 1A). These 16 genes were then used as the query for a genome-wide coexpression analysis to identify additional genes associated with pollen wall development from public microarray data sets. Using a cutoff threshold of 0.6 for pairwise Pearson correlation coefficients in the public expression database ATTED-II, a total of 251 genes with a frequency of occurrence of 1059 times were identified as coexpressed with the 16 “guide genes,” among them, 43.8% (110/251) genes appeared only once with one guide gene, while 98 genes were present more than four times with 16 guide genes (Figure 1C). Furthermore, their expression pattern was confirmed as consistent with the guide genes using the *Arabidopsis* eFP browser (Figure 1B). Additionally, 11 genes with functional relevance, i.e.,  $\beta$ -Ketoacyl-CoA synthases (*KCSs*; *KCS7*, *15*, and *21*), *Lipid*

*Transfer Protein (LTP)*, *Extra Cellular Lipases (EXLs; EXL4*, *5*, and *6)*, *Anther-specific protein6 (A6)*, *CYP450* encoding genes (*CYP703A2*), *AMS*, and *AtbHLH089*, were further validated by quantitative RT-PCR (qRT-PCR) as highly expressed during stage 9 of anther development (Figure 1D). Anther staging in *Arabidopsis* refers to Sanders et al. (1999).

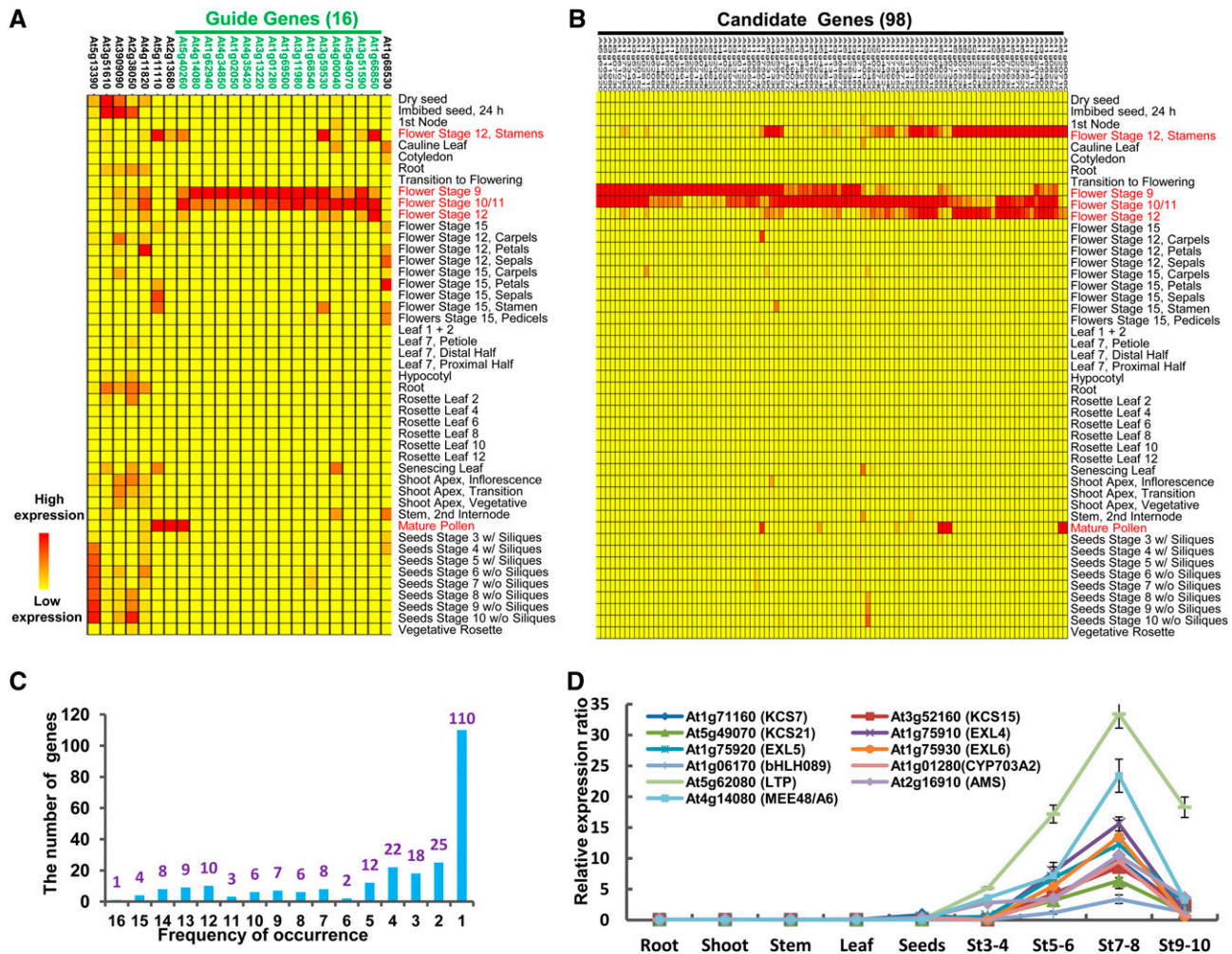
### AMS Acts as a Key Transcriptional Regulator in Pollen Wall Patterning

Among the 98 genes associated with pollen wall development were six transcription factors (*bHLH089*, *ANAC025*, *WUS/WUSCHEL*, *SPL/SPOROCTELESS*, *MYB99*, and *AMS*) possibly associated with specifying/determining tapetal fate and development. Zhang et al. (2006) reported that *SPL*, *EMS1*, and *DYT1* might regulate anther development via the expression of *AMS* and thereby indirectly affect pollen wall formation (Zhang et al., 2006). We compared the expression of these 98 genes in the microarray data of *spl* (Zhang et al., 2006), *dyt1* (Feng et al., 2012), *ams* (Xu et al., 2010), *myb80* (Phan et al., 2011), *ms1* (Yang et al., 2007), and *ems1/exs* (Canales et al., 2002; Zhao et al., 2002) and showed that the expression of most of these 98 candidate genes had been changed in *spl* (73 downregulated), *ems1* (69 downregulated), *ams* (70 downregulated), *dyt1* (44 downregulated), and *ms1* (52 downregulated; 13 upregulated), while the expression of only a few genes was changed in *myb80* (nine downregulated; five upregulated) (Figures 2A and 2B). Given the earlier role of *SPL*, *EMS1/EXS*, and *DYT1* during anther development, our analyses support the key role of *AMS* in pollen wall development (Figures 2A and 2B). Previously, we reported that *AMS* affects the expression of 549 genes in *ams* buds, and 13 directly regulated genes are involved in the transport of lipids, oligopeptides, and ions, fatty acid synthesis and metabolism, flavonol accumulation, substrate oxidation, methyl modification, and pectin dynamics (Xu et al., 2010). Interestingly, seven of the 13 genes were included in these 98 candidate genes (Table 1).

### *ams* Mutants Have Defects in Microspore Compartmentalization and Callose Dissolution

The *ams* mutant failed to produce functional pollen; after pollen mother cell meiosis, abnormal tetrads were frequently observed (Figure 2D) and the tapetal cells were highly vacuolated (Figures 2J to 2N), in contrast to the condensed cytoplasm of wild-type tapetum (Figures 2I to 2M). Compartmentalization of the microsporocyte during meiosis and initial primexine wall patterning occurs within a specialized callose ( $\beta$ -1,3-glucan) cell wall, which is subsequently degraded by tapetal  $\beta$ -1,3-glucanase(s) (callase) (Stieglitz, 1977). The callose wall was clearly visible in the wild type (Figure 2C), while only a weakly staining, ambiguous boundary was visible between the *ams* microspores (Figure 2D). Reduced callose accumulation occurred in the *ams* anther locule (Figure 2P), which subsequently showed delayed, abnormal breakdown (Figures 2R and 2T).

After the formation of free microspores, wild-type tapetal cells actively secrete sporopollenin precursors onto the primexine (a microfibrillar matrix consisting mainly of cellulose) on the outer



**Figure 1.** Coexpression Analysis of Candidate Genes Involved in Pollen Wall Formation.

**(A)** Sixteen guide genes were selected from the 26 genes that have been demonstrated to be involved in pollen wall formation by forward genetics analysis.

**(B)** Using coexpression analysis, 98 candidate genes were shown to be specifically expressed during pollen wall development.

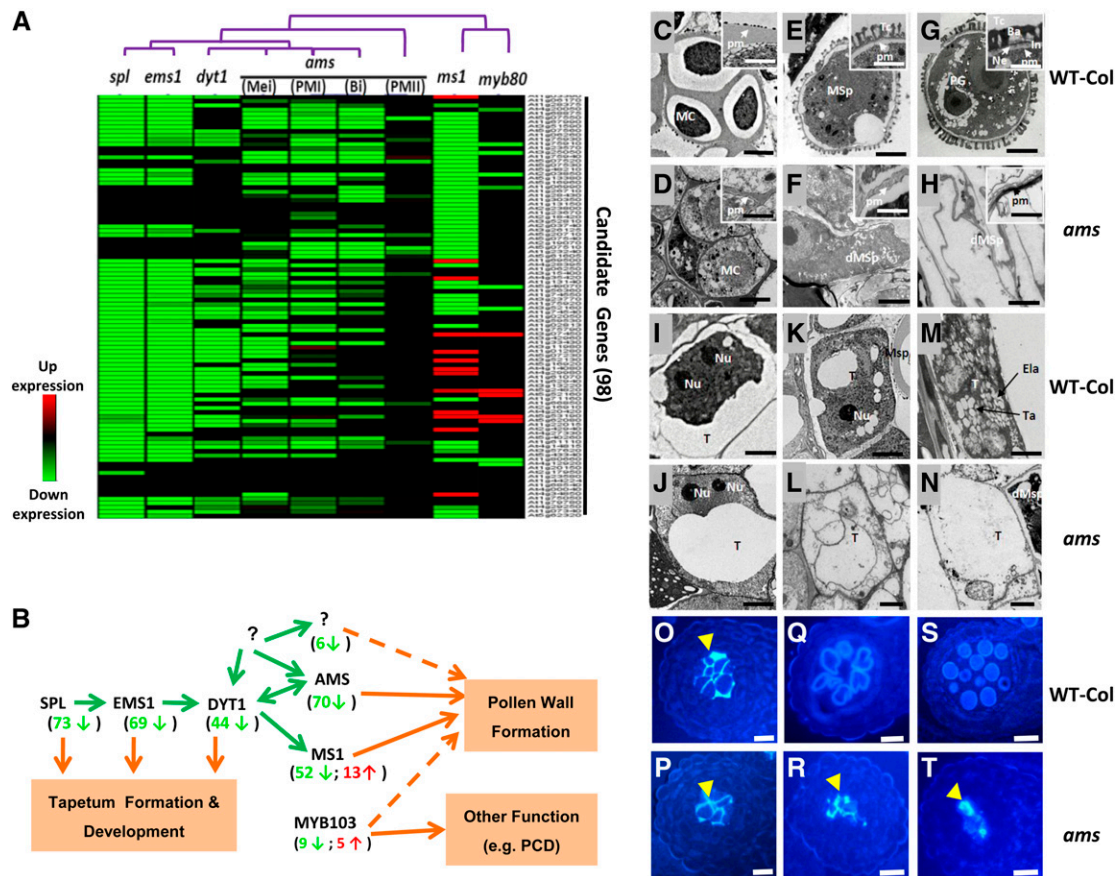
**(C)** About 43.8% of the genes (110/251) only matched one guide gene during the coexpression analysis, and the average frequency of each gene among 98 genes matching guide genes was more than 4.

**(D)** Expression analysis of 11 selected genes by qRT-PCR analysis. Expression profiles of At1g71160 (*KCS7*), At3g52160 (*KCS15*), At5g49070 (*KCS21*), At1g75910 (*EXL4*), At1g75920 (*EXL5*), At1g75930 (*EXL6*), At1g06170 (*bHLH089*), At1g01280 (*CYP703A2*), At5g62080 (*LTP*), At2g16910 (*AMS*), and At4g14080 (*MEE48/A6*) as determined by qRT-PCR using total RNA from shoot (15 d), root (15 d), stem (15 d), leaf (15 d), seed, and anther samples. The relative amount of amplified product was normalized against *At-ACT2* transcripts. Each value represents the average of two or more biologic replicate experiments in which each value is the mean of three separate qRT-PCR reactions ( $\pm$ SE).

surface of microspore. Ultimately, the pollen grains form an exine with a distinct tectum, bacula, and nexine (Figures 2E and 2G). However, although *ams* mutant microspores formed a primexine, no obvious accumulation of lipidic sporopollenin precursors was seen, with eventual collapse of microspores occurring (Figures 2F and 2H). Active lipid synthesis was evident in the wild-type tapetum, which appeared to be condensed and degenerated, with disintegration of the cell membrane and accumulation of lipidic tapetosomes and elaioplasts (Figure 2M). In contrast, *ams* tapetal cells were severely swollen, with large vacuoles and few lipidic tapetosomes and elaioplasts (Figure 2N).

### AMS Controls the Synthesis of Lipidic and Phenolic Components and Flavonoids for Pollen Wall Formation

The defective pollen wall and absence of obvious tapetosomes and elaioplasts in the *ams* tapetal cells suggest the *ams* mutant may have defects in synthesizing chemical precursors of pollen wall development. Gas chromatography–mass spectrometry and gas chromatography–flame ionization detection (GC-FID) were used to measure the lipidic compositions of cuticular waxes, cutin monomers, and total soluble lipids in buds at the tetrad and early microspore stages. GC-FID analysis indicated



**Figure 2.** AMS Acts as a Key Transcriptional Regulator of Pollen Wall Formation in *Arabidopsis*.

**(A)** and **(B)** Comparison of expression in male sterile mutants of 98 genes that may be involved in pollen wall formation. Mei, meiosis; PMI, pollen mitosis I; Bi, bicellular; PMII, pollen mitosis II; PCD, programmed cell death.

**(C)** to **(H)** TEM analysis of pollen wall formation in the wild type (Columbia-0 [Col]) and *ams*. Stage 7 anthers (**(C)** and **(D)**), stage 8 anthers (**(E)** and **(F)**), and stage 10 anthers (**(G)** and **(H)**). Insets are a higher magnification of pollen exine showing tectum, bacula, and nexine. Msp, microspores; dMsp, degenerated microspores; pm, plasma membrane; PG, pollen grains. Bars = 100  $\mu$ m for **(C)** to **(H)** and 1  $\mu$ m for the insets.

**(I)** to **(N)** TEM analysis of tapetal cells of the wild type (Col-0) and *ams*. Stage 7 anthers (**(I)** and **(J)**), stage 8 anthers (**(K)** and **(L)**), and stage 10 anthers (**(M)** and **(N)**) showing obvious accumulation of lipidic tapetosomes (arrow) and elaioplasts (arrow) in wild-type tapetal cells (**(M)**), and no detectable lipidic tapetosomes and elaioplasts in *ams* tapetal cells (**(N)**). T, tapetal cells; Nu, nucleus; Ela, elaioplasts; Ta, tapetosomes; Msp, microspores; dMsp, degenerated microspores. Bars = 5  $\mu$ m.

**(O)** to **(T)** Callose staining in wild-type (Col-0) and *ams* anthers. Stage 7 anthers (**(O)** and **(P)**), stage 9 anthers (**(Q)** and **(R)**), and stage 10 anthers (**(S)** and **(T)**) showing the delayed callose degradation in *ams* (arrow). Bars = 100  $\mu$ m.

that total wax in the *ams* buds increased by 12.67% (Figure 3; Supplemental Figure 2). In particular, C29 alkane was significantly increased ( $P < 0.05$ ) (Supplemental Figure 2A). Whereas total cutin decreased by 15.8% (Figure 3; Supplemental Table 2), mainly due to the significant reduction in the levels of acids (C18, C18:1, C20, and C24), 2-hydroxy-fatty acids (C22, C24, C24:1, C25:1, C26, and C26:1) and fatty di-acids (C18 and C18:2) (Supplemental Figure 2B).

Phenol amides and flavonoids are major components of the pollen coat and possibly also the pollen wall (Matsuno et al., 2009). Total flavonoid content (TFC) and total phenol content (TPC), determined using  $\text{NaNO}_2$ -Al ( $\text{NO}_3$ )<sub>3</sub> and Folin-Ciocalteu reagent analysis, respectively, were reduced in *ams* buds (30.6 and 34.4%) (Figure 3), indicating that AMS also regulates the

biosynthetic pathways for lipidic, phenolic, and flavonoid compounds in the pollen wall and coat. This is supported by transcriptomic analysis that suggested that 21 genes implicated in lipid acyl metabolism, six in secondary metabolism and 21 miscellaneous enzyme encoding (MISC) genes are down-regulated (Supplemental Figures 3C and 3D) in *ams* buds. Thirteen of these 21 genes were previously shown to be involved in pollen wall development (Figure 3).

#### AMS Is Directly Associated with the Promoters of Genes Related to Pollen Wall Development

Analysis of the 1-kb upstream promoter region of the putative AMS target genes showed significant enrichment for E-box

**Table 1.** Expression Changes of Genes (Downregulated >2-Fold in the *ams* Mutant) Directly Regulated by AMS

No.	Locus	Gene Name	Description	Fold Change in <i>ams</i> Young Buds <sup>a</sup>				References
				Stage 6	Stage 8	Stage 9	Stage 10	
1	At4g14080	<i>A6</i>	Anther-specific protein 6	-3.58	0	0	0	This study
2	At4g20050	<i>QRT3</i>	Quartet 3	-2.50	-3.61	0	0	This study
3	At3g52160	<i>KCS15</i>	$\beta$ -Ketoacyl-CoA synthase 15	-1.58	-1.81	0	0	This study
4	At5g49070	<i>KCS21</i>	$\beta$ -Ketoacyl-CoA synthase 21	-1.01	-0.66	0	0	Xu et al. (2010)
5	At1g71160	<i>KCS7</i>	$\beta$ -Ketoacyl-CoA synthase 7	0	-0.74	-0.64	-1.55	This study
6	At3g51590	<i>LTP12</i>	Lipid transfer protein 12 (LTP12)	-3.68	-2.98	-4.24	0	Xu et al. (2010)
7	At1g66850		LTP family protein	-4.08	-4.92	-4.10	-1.09	Xu et al. (2010)
8	At5g62080		LTP family protein	-3.22	-2.50	0	0	This study
9	At3g13220	<i>WBC27</i>	ABC transporter	-2.57	0	-1.95	0	Xu et al. (2010)
10	At4g34850	<i>PKSB/LAP5</i>	Polyketide synthase B/Less adhesive pollen 5	-3.12	0	0	0	This study
11	At4g35420	<i>TKPR1/DRL1</i>	Tetraketide $\alpha$ -pyronereductase 1	-2.66	0	0	0	This study
12	At4g00040	<i>CHS</i>	Chalcone synthase	-0.45	-1.30	0	0	Xu et al. (2010)
13	At1g75920	<i>EXL5</i>	Family II extracellular lipase 5	-3.12	-3.58	-4.06	0	Xu et al. (2010)
14	At1g75910	<i>EXL4</i>	Family II extracellular lipase 4	-2.10	-2.08	0	0	This study
15	At1g75930	<i>EXL6</i>	Family II extracellular lipase 6	0	-4.82	0	-1.08	This study
16	At1g06990		GDSL-like lipase/Extracellular lipase	0	0	-2.78	0	This study
17	At1g69500	<i>CYP704B1</i>	Cytochrome P450 704B1	-3.54	0	0	0	This study
18	At1g01280	<i>CYP703A2</i>	Cytochrome P450 703A2	-3.31	0	0	0	This study
19	At1g74540	<i>CYP98A8</i>	Cytochrome P450 98A8	-3.41	-3.31	-1.78	0	This study
20	At1g74550	<i>CYP98A9</i>	Cytochrome P450 98A9	-2.78	-3.11	-0.76	0	This study
21	At1g13140	<i>CYP86C3</i>	Cytochrome P450 CYP86C3	-1.77	-2.78	-1.57	-0.07	Xu et al. (2010)
22	At5g07520	<i>GRP18</i>	Glycine-rich protein18	-2.94	-3.91	-3.30	-2.51	This study
23	At5g07550	<i>GRP19</i>	Glycine-rich protein19	-3.12	-3.62	-2.54	-3.68	This study

<sup>a</sup>Microarray data presented here are from Xu et al. (2010).

binding motifs (CANNTG) (Xu et al., 2010), with 17 genes containing more than five E-box motifs in this region and 25 genes containing  $\geq 3$  motifs, suggesting direct regulation by AMS. To confirm this, we employed quantitative chromatin immunoprecipitation PCR (qChIP-PCR) analysis using an AMS-specific antibody (Xu et al., 2010) and observed that the promoter regions of 17 genes, including *A6*, *QUARTET3* (*QRT3*), *EXL4*, *EXL5*, *EXL6*, *KCS7*, and *KCS15*, two GLYCINE-RICH PROTEIN encoding genes (*GRP18* and *GRP19*), four CYP 450 encoding genes (*CYP703A2*, *CYP704B1*, *CYP98A8*, and *CYP98A9*), *At5g62080* (*LIPID TRANSFER PROTEIN* [*LTP*]), *At1g06990* (*GDSL-like Lipase*), *PKSB/LAP5*, and *TKPR1/DRL1*, from the 55 well-annotated genes from the set of 70 genes showing reduced expression in *ams*, were enriched by AMS (Figure 4A). Furthermore, electrophoretic mobility shift assay (EMSA) confirmed the binding of AMS to the promoter of *LTP* (*At5g62080*), *EXL5*, *KCS15*, and *CYP703A2* (Figure 4B). Moreover, analysis using the GeneCAT tool suggested that AMS and the coexpressed genes form a connective regulatory network (Figure 4C). Among the genes showing coexpression with AMS were seven genes, including *KCS21*, *WBC27*, *LTP12*, and *CYP86C3*, that are putatively associated with lipid transport, fatty acid synthesis and metabolism, substrate oxidation, methyl modification, and pectin dynamics, and were previously identified as direct targets of AMS by qChIP-PCR (Xu et al., 2010). Six of the 13 previously identified direct targets of AMS reported by Xu et al. (2010) do not show a significant correlation of expression with AMS, as revealed by our coexpression analysis. Furthermore, available expression data from the websites indicate that these six genes

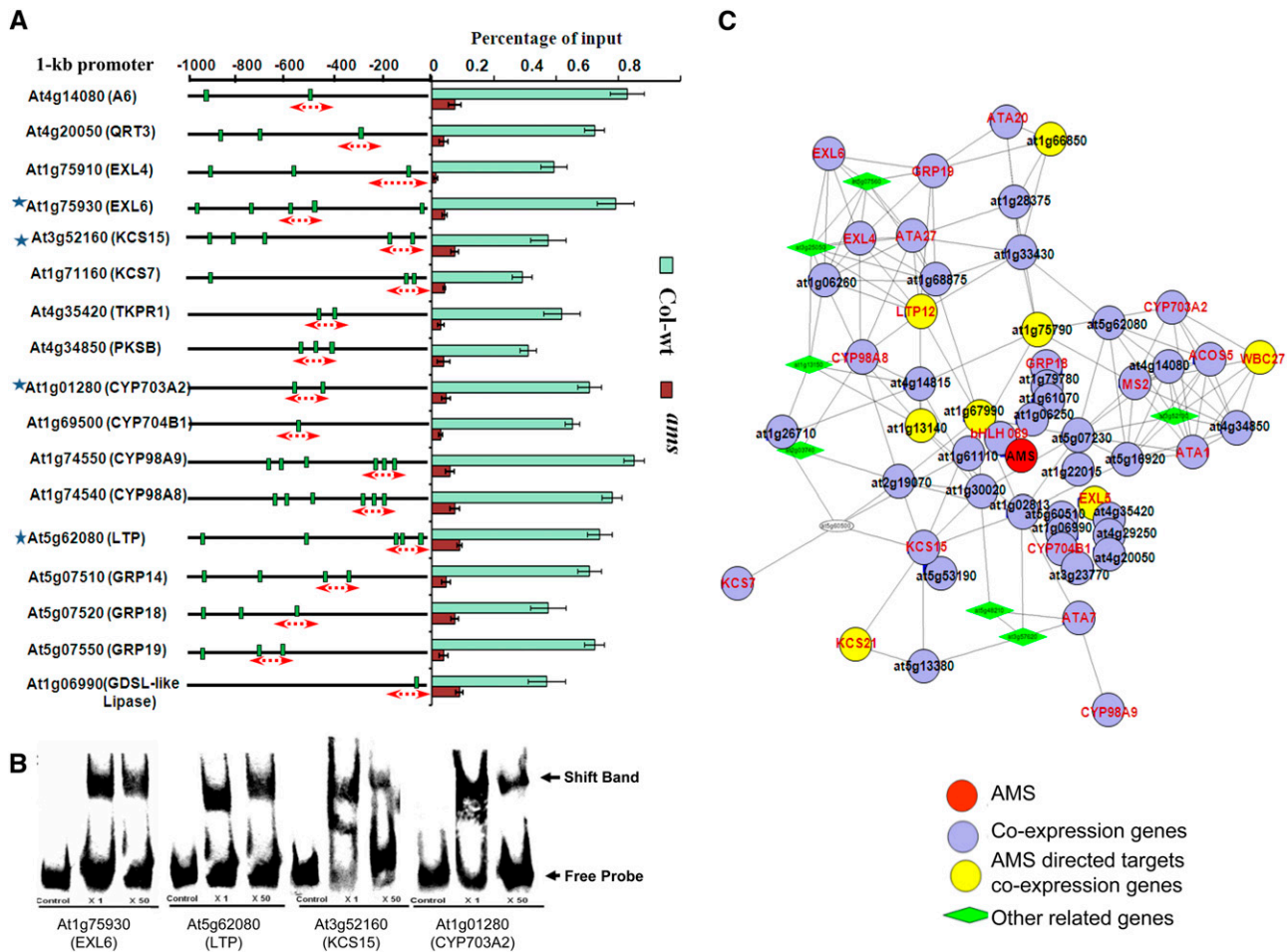
(Supplemental Figure 4C) are expressed in other tissues as well as anthers, suggesting that they may be regulated by additional transcription factors besides AMS. Thus, we did not include these six genes in this study, even though we cannot exclude the possibility that they may directly regulate the formation of the pollen wall. Therefore, together with the previously identified seven targets associated with pollen wall development, AMS may directly regulate the expression of 23 of the 98 genes identified as linked to pollen wall development (Table 1), suggesting that AMS forms a key regulatory hub in pollen wall biosynthesis (Figure 4C, Table 1).

#### AMS Regulates Dissociation of the Pollen Mother Cell Wall

The callose wall surrounding the microspore is subjected to enzymatic digestion by  $\beta$ -1,3-glucanase secreted from the tapetum after the tetrad stage. *A6* is thought to encode a callase that plays a key role in callose dissolution during microspore release in *Arabidopsis* (Hird et al., 1993). In this study, we showed that AMS may determine the configuration of microspores in tetrad and callose degeneration by regulating the expression of *A6* (Figures 2O to 2T). The callose layer is thought to serve as a temporary wall for microspores that facilitates microspore separation during meiosis and supports primexine formation. Additionally, it may provide both the sugar source for microspore development and a stress factor that acts to compress and flatten the upper ends of the probacula for tectum formation (Heslop-Harrison, 1968; Knox and Heslop-Harrison, 1970).

Consistent with the major role of AMS in regulating callose wall formation and degradation, and subsequent pollen wall





**Figure 4.** Analysis of the Direct Target Genes of AMS.

**(A)** qChIP-PCR analysis revealed the direct association of AMS with the promoters of 17 genes. DNA recovered from immunoprecipitation was used as templates for PCR using primer pairs (the double-headed arrows) spanning the putative AMS binding sequences in the upstream regions of candidate genes. Stars indicate the validation of the binding activity by AMS using EMSA. Data presented here represent the mean of five biological replicates.

**(B)** EMSA of the binding of recombinant AMS onto the promoter fragments. DNA oligomers containing a consensus AMS recognition sequence found in the upstream regions of each of the candidate genes were labeled with digoxin and used as probes.

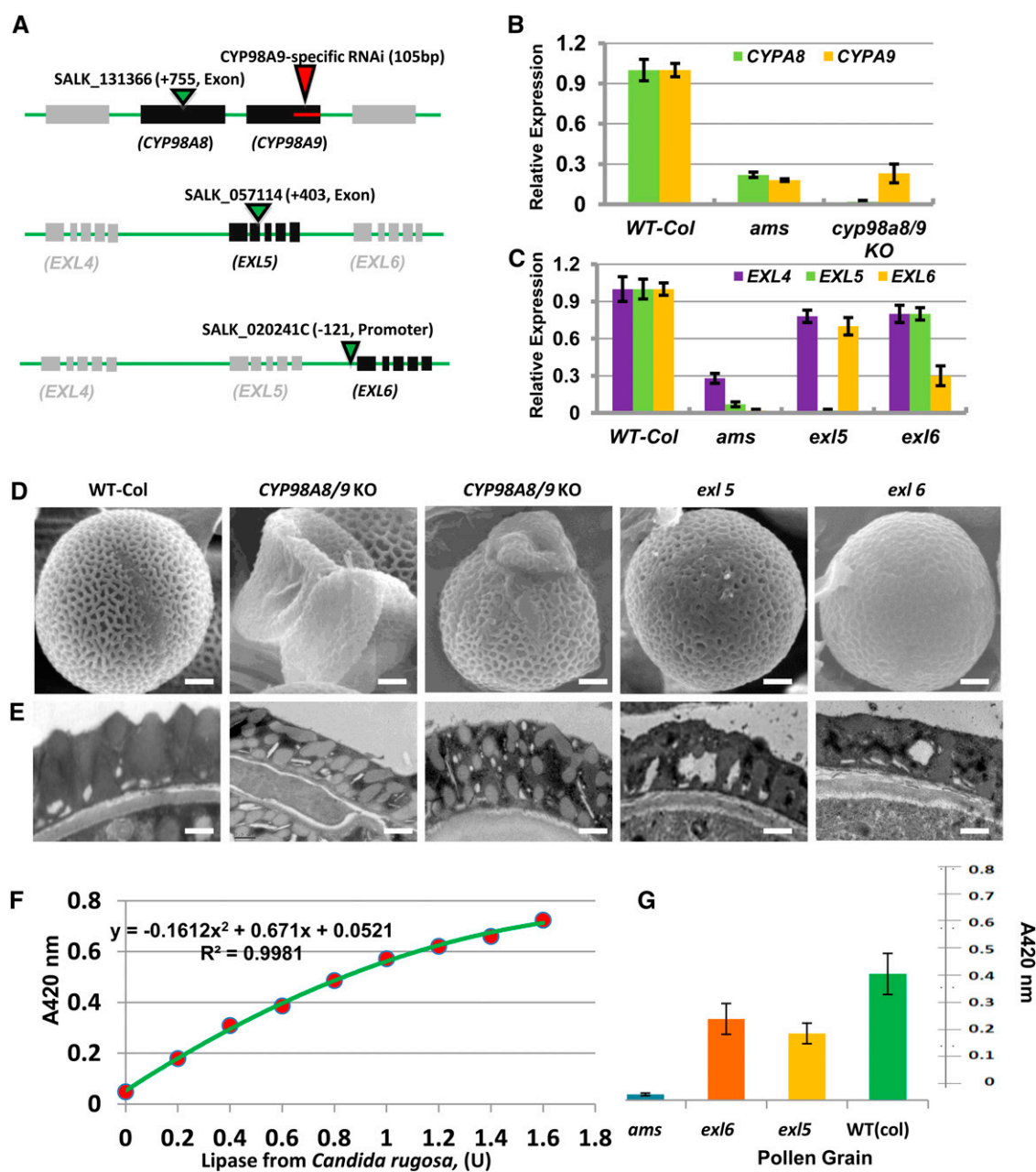
**(C)** A network composed of AMS and correlated genes that are putatively involved in microspore compartmentalization in the tetrad, callose degeneration, lipid metabolism, and pollen exine formation and are direct targets of AMS, as determined using the AtGenExpress visualization tool on the GeneCAT website (<http://genecat.mpg.de>).

pollen, or other plant tissues (Zimmermann et al., 2004). Promoter- $\beta$ -glucuronidase and RNA in situ hybridization analyses indicate that *EXL5* and *EXL6* are expressed in the tapetum and that the protein is present in the tapetum and pollen coat (Supplemental Figure 5C). This suggests that AMS is also a regulator of the expression of the pollen coat proteins that mediate the early contact between pollen grains and the stigma that are required for successful adhesion, hydration, and germination.

Null *ex15* and *ex16* mutants (Figure 5A), as indicated by qRT-PCR from the T-DNA insertion line of *EXL5* (+403, second exon) and *EXL6* (-121, promoter), displayed a partial lack of exine patterning, resulting in a smooth outer surface (Figure 5D; Supplemental Figure 5E). Furthermore, TEM analysis showed

that *ex15* and *ex16* pollen exhibited an irregular baculum and tectum, and disordered tryphine deposition (Figure 5E).

EXLs contain a predicted family II lipase domain and perform acyl transfer reactions in extracellular environments (Upton and Buckley, 1995). *EXL4* is enzymatically active during pollen development as the pollen coat is deposited onto pollen grains, creating the complement of lipids required for pollen hydration (Updegraff et al., 2009). Esterase activity, as measured using pollen grains from *ams*, wild type/*col*, *ex15*, and *ex16* using *p*-nitrophenyl butyrate as a substrate (Updegraff et al., 2009) showed that *ex15* and *ex16* pollen grains had significantly reduced esterase activity (Figures 5F and 5G). As expected, *ams* had low esterase activity due to the lack of pollen wall (Figures 5F and 5G).



**Figure 5.** *CYP98A8*, *CYP98A9*, *EXL5*, and *EXL6*, Which Are Regulated by AMS, Are Required for Pollen Wall and Pollen Coat Development.

**(A)** Schematic representation of the null *cyp98A8/CYP98A9* RNAi double mutants derived from SALK-131366 T-DNA (green arrow) in *CYP98A8* and RNAi (red arrow) in *CYP98A9*; SALK-057114 T-DNA (green arrow) in *EXL5*; and SALK-020241C T-DNA (green arrow) in *EXL6*. Green line represents the genome, black block represents the exons.

**(B)** qRT-PCR of *CYP98A8* and *CYP98A9* expression in the wild type, *ams* null mutant, and null *cyp98A8/CYP98A9* RNAi buds. Expression was normalized to *ACTIN7* and presented relative to wild-type expression levels. Error bars represent sd of three biological replicates.

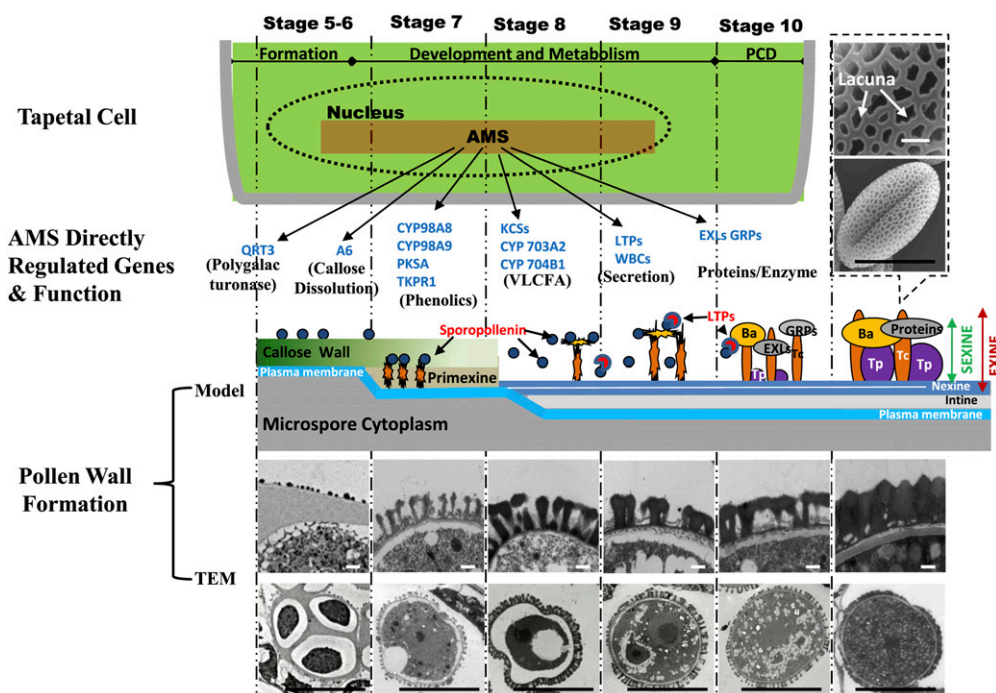
**(C)** qRT-PCR analysis of the expression of *EXL4*, *EXL5*, and *EXL6* in the wild-type, *ams*, *exl5*, and *exl6* buds during anther development at stage 8. Expression was normalized to *ACTIN7* and presented relative to wild-type expression levels with three biological replicates; error bars represent sd.

**(D)** Analysis of the outer surface structure of pollen grains from the wild type, the null *cyp98A8/CYP98A9* RNAi double mutant, and *exl5* and *exl6* mutants by scanning electron microscopy. Bars = 10  $\mu$ m.

**(E)** TEM analysis of the pollen wall from the wild type, the null *cyp98A8/CYP98A9* RNAi double mutants, and the *exl5* and *exl6* mutants. Bars = 500  $\mu$ m.

**(F)** and **(G)** The standard curve of lipase activity **(F)** and lipase activity of pollen grains of *ams*, *exl5*, *exl6*, and the wild type **(G)**. The values were obtained under  $A_{420}$  wavelength measured, and assays were performed with three biological replicates.





**Figure 6.** Proposed Scheme Showing the Central Role of AMS in Pollen Wall Formation.

AMS acts as a key transcriptional regulator that modulates the expression of genes associated with microspore compartmentalization and callose degeneration (*A6* and *QRT3*), fatty acid/phenolic synthesis, elongation, metabolism and transport (*KCS7*, *KCS15*, *KCS21*, *CYP703A2*, *CYP704B1*, *CYP86C3*, *CYP98A8*, *CYP98A9*, *PKSB/LAP5*, *TKPR1/DRL1*, *LTPs*, and *WBC27*), and pollen coat formation (*EXL4*, *EXL5*, *EXL6*, *GRP18*, and *GRP19*). The micrographs refer to various developmental events of pollen wall in *Arabidopsis*. WBCs, White/Brown Complex subfamily. Bars = 30  $\mu$ m. [See online article for color version of this figure.]

## DISCUSSION

Biochemically, sporopollenin is recognized as one of the most resistant biopolymers in nature because it is highly resistant to prolonged desiccation and various stresses as well being insoluble in the strongest acids, bases, and oxidizers. A wealth of biochemical characterization attempts suggest that sporopollenin consists of complex biopolymers derived mainly from long-chain fatty acids and phenolic compounds (Scott, 1994; Bubert et al., 2002) and that sporopollenin has been preserved unchanged for >400 million years ago (Wiermann and Gubatz, 1992). An *Arabidopsis* fatty acid elongase gene, *KCS1*, shares a high degree of sequence identity to *FATTY ACID ELONGASE1*, which encodes a 3-ketoacyl-CoA synthase. *KCS1* is essential for wax biosynthesis via catalyzing very-long-chain fatty acid synthesis in vegetative tissues (Todd et al., 1999). We reveal here that AMS is able to regulate the transcription of genes involved in fatty acid elongation such as *KCS7*, *KCS15*, and *KCS21* (Figure 4A, Table 1). Furthermore, in situ analysis indicates that *KCS15* and At5g62080 (*LTP*) are expressed in tapetal cells, similar to *AMS* (Supplemental Figures 4A and 4B).

In higher plants, cytochromes P450 (P450s) play essential crucial roles in both primary metabolism and a wide variety of specialized metabolic processes, including the pathways for production of the precursors of biopolymers, such as oxygenated fatty acid derivatives and lignin monomers (Schuler and

Werck-Reichhart, 2003; Pinot and Beisson, 2011). *CYP703As* and *CYP704Bs* belong to P450 families specific to land plants under high purifying selection (Li et al., 2010). *CYP703s* and *CYP704Bs* from *Arabidopsis* and rice are specifically expressed in the tapetal cells and microspores (Morant et al., 2007; Li et al., 2010), and when mutated cause distorted pollen exine and male-sterile phenotypes. Recombinant *CYP703A2* in yeast cells catalyzes the in-chain hydroxylation of medium-chain saturated fatty (Morant et al., 2007). In contrast, heterologously expressed *Arabidopsis* *CYP704B1* and rice *CYP704B2* in yeast catalyze the  $\omega$ -hydroxylation of long-chain fatty acids (Dobritsa et al., 2009; Li et al., 2010). *CYP86C3* belongs to the *CYP86C* subfamily, which has four members in *Arabidopsis*; however, the biological functions of this subfamily remain unclear. Recently, recombinant *CYP86C3* was shown to act as a short-chain fatty acid hydroxylase with substrates of lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), and myristoleic acid (C14:1) (Kai et al., 2009). In this study, we found that AMS directly regulates the transcription of *CYP703A2*, *CYP704B1*, and *CYP86C3* (Table 1, Figure 4A), suggesting that AMS is a critical regulator of the hydroxylation of mid-chain and long-chain fatty acids during pollen exine biosynthesis. Consistent with this, we observed a reduction of hydroxylated lipidic monomers in *ams* buds (Figure 3).

Similarly, *Arabidopsis* *CYP98A8* and *CYP98A9* were shown to be expressed in tapetal cells and to be required for oxygenated

phenolamide formation (Matsuno et al., 2009). We provide additional evidence that these genes are regulated by AMS and contribute to pollen wall formation. In *Arabidopsis*, *PKSA* and *PKSB* encode plant-type III polyketide synthases, which catalyze the condensation of malonyl-CoA units with mid-chain and  $\omega$ -hydroxylated fatty acyl-CoAs to generate tri- and tetra-ketide  $\alpha$ -pyrone compounds. *PKSB/LAP5* and *TKPR1/DRL1* encode enzymes that produce hydroxylated  $\alpha$ -pyronepolyketide compounds, which are thought to serve as sporopollenin monomers (Grienenberger et al., 2010; Kim et al., 2010). *PKSA* and *PKSB* are transiently expressed specifically in tapetal cells, and the *pksa pksb* mutant lacks an obvious exine, causing complete male sterility. Furthermore,  $\alpha$ -pyronepolyketides formed by *PKSA* and *PKSB* can be reduced by two tapetally expressed oxido-reductases, *TKPR1/2*. These form hydroxylated  $\alpha$ -pyrone compounds that serve as sporopollenin precursors. Interestingly, in this study, we observed that AMS is capable of triggering the formation of sporopollenin precursors, hydroxylated  $\alpha$ -pyrone, and phenolic compounds by directly binding to the promoters of *PKSB*, *TKPR1*, *CYP98A8*, and *CYP98A9* (Figures 4A and 6).

Emerging evidence suggests that pollen coat EXLs in combination with GRP play a role in the initial steps of pollination, namely, hydration on the stigma (Updegraff et al., 2009). Previous reports revealed that six tandem duplicated genes (*GRP14*, -16, -17, -18, -19, and -20) encode the pollen coat GRPs that are mainly expressed in the tapetum and pollen coat (Alves Ferreira et al., 1997; Mayfield et al., 2001; Kim et al., 2003). Furthermore, it has been reported that *GRP17* is required for the rapid initiation of hydration on the stigma (Mayfield and Preuss, 2000). Mutations of one GRP result in no obvious phenotype, indicating the possible functional redundancy of these genes in *Arabidopsis* (Mayfield et al., 2001). In this study, we showed that AMS directly regulates the expression of pollen coat protein, EXLs, and GRPs, which are implicated in the pollen exine and pollen coat formation and subsequent pollination (Figures 4 and 6).

In short, these data support a diverse and critical role for AMS during pollen development, which includes a direct transcriptional regulatory role in the separation of microspore mother cells, dissolution of the callose layer of tetrads, and subsequent sporopollenin biosynthesis and pollen coat formation (Figure 6). An AMS ortholog (TDR) is functionally conserved in rice (Li et al., 2006), further suggesting that the biochemical regulation of sporopollenin production may be transcriptionally regulated across both monocot and dicot plants via a limited number of key transcriptional regulators. This work provides insight into the transcriptional control of the synthesis of the durable pollen wall in plants.

## METHODS

### Plant Material

Seeds of the SALK insertional mutant lines SALK\_152147, SALK\_057114, and SALK\_020241C (SIGnal; Alonso et al., 2003) and Columbia were obtained from the Nottingham Arabidopsis Stock Centre. Phenotypic analysis of the SALK\_152147 mutant confirmed that the pollen development defects seen in the *ams* mutant (Sorensen et al., 2003; Xu et al.,

2010) were also observed in the *ams* SALK knockout. Phenotypic analysis was performed using the null *cyp98A8/CYP98A9* RNAi double mutants derived from a T-DNA insertion line of *CYP98A8* and an RNAi line. The SALK\_057114 and SALK\_020241C mutants of *EXL5* and *EXL6* were confirmed to have pollen development defects. Primers for RT-PCR and genotyping are listed in Supplemental Table 3.

### Coexpression Analysis in *Arabidopsis thaliana* and Cluster Analysis

The *Arabidopsis* genes in the manually curated abstracts/full text articles that contain experimental evidence for pollen wall biosynthesis genes were used as bait genes to query the ATTED-II database (Obayashi et al., 2009). The expression pattern analysis was performed using the *Arabidopsis* eFP browser (<http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi>) (Winter et al., 2007). Microarray data analysis employed Genesis (1.7.5) software (Stum et al., 2002). The Web-based Classification SuperViewer program ([http://bar.utoronto.ca/ntools/cgi-bin/ntools\\_classification\\_superviewer.cgi](http://bar.utoronto.ca/ntools/cgi-bin/ntools_classification_superviewer.cgi)) was used to search for differential distributions of Gene Ontology and biological terms within the correlated genes.

### Chromatin Immunoprecipitation and qRT-PCR

Experimental plant material and preparation of specific antibodies were as described by Xu et al. (2010). For qChIP-PCR and RT-PCR, oligonucleotides used for the experiments are listed in Supplemental Table 3. Quantification involved normalization of each immune precipitation sample, Ct subtraction of the Ct of the input control in the same immune precipitation to obtain  $\Delta$ Ct values, and  $2^{(-\Delta Ct)}$  as the percentage of input. All samples were run at least in duplicate.

### I<sub>2</sub>-KI Staining, Scanning Electron Microscopy, and TEM Analysis

I<sub>2</sub>-KI and callose staining and scanning electron microscopy were performed as described previously (Chen et al., 2011), and the samples treated with adjusted dehydration and fixation as described by Xu et al. (2010).

### Analysis of Bud Waxes, Internal Lipids, and Cutin

Wax, internal lipid and cutin extraction, and gas chromatography–mass spectrometry and GC-FID analyses were performed as described previously (Chen et al., 2011; Shi et al., 2011) with slight modifications. Small buds (from the tetrad stage to early microspore stage) were collected; each sample contained ~2 to 3 g dry matter from five individual plants.

### Determination of TPC (Folin–Ciocalteu Assay) and TFC of *Arabidopsis* Buds

*Arabidopsis* buds (0.5 g) were weighed in an EP tube and extracted with 500  $\mu$ L of 75% (v/v) ethanol at room temperature for 24 h; then, the extracts were sonicated for 2 h. After extraction, the mixture was centrifuged at 10,000g for 10 min to give the supernatant. The crude extracts were diluted 10 times with 75% (w/w) ethanol before the assay. Total phenolics were determined using Folin–Ciocalteu reagents with some modification (Singleton and Rossi, 1965). Reagent blank using distilled deionized water was also prepared. TPC was quantified using a calibration curve obtained from measuring the absorbance of known concentrations of ferulic acid standard solution. The result was calculated as ferulic acid equivalent per 1 g *Arabidopsis* buds and reported as mean value  $\pm$  sd. All samples were analyzed in triplicate.

The determination of flavonoids was performed according to the colorimetric assay of Kim et al. (2003). Distilled deionized water was used as a reagent blank. TFC was calculated by extrapolating the absorbance

of the reaction mixture using a standard curve of rutin. The experiment was repeated thrice and the TFC was expressed as equivalent to rutin (RE) in milligrams/grams of *Arabidopsis*.

### In Situ Hybridization

RNA hybridization and immunological detection of the hybridized probes were performed according to the protocol of Kouchi and Hata (1993). All primers used to amplify probes for in situ hybridization are listed in Supplemental Table 3.

### EMSA

The recombinant GST-AMS protein was prepared as previously described by Xu et al. (2010). The DNA fragments containing the E-box of the regulatory region of target genes were generated using PCR amplification with the primers listed in Supplemental Table 3.

### Accession Numbers

Sequence data from this article for the cDNA and genomic DNA of AMS can be found in the GenBank/EMBL data libraries under accession numbers NM\_127244.4 and NC\_003071, respectively. Loci and their accession numbers for the genes in this article are as follows: *Defective Exine Formation1* (At3g09090), *No Exine Formation1* (At5g13390); *Ruptured Pollen Grain1* (At5g40260); *Male Sterility1/Hackly Microspore* (At5g22260), *No Primexine and Plasma Membrane Undulation* (At3g51610), *Transient Defective Exine1/De-etiolated2* (At2g38050), *Callose Synthetase5* (At2g13680), *A6* (At4g14080), *ABC Transporter WBC27* (At1g66850),  $\beta$ -ketoacyl-CoA Synthase 21 (At5g49070), *Polyketidesynthase A/Less adhesive pollen6* (At1g02050), *AtbHLH089* (At1g06170), *Polyketide Synthase B/Less Adhesive Pollen5* (At4g34850), *Male Sterility2* (At3g11980), *ATA20* (At3g15400), *ATA7* (At4g28395), *Tetraketide  $\alpha$ -pyronereductase2* (At1g68540), *ATSTP2* (At1g07340), *Tetraketidea-pyronereductase1* (At4g35420), *CYP704B1* (At1g69500), *CYP703A2* (At1g01280), and *Acyl-CoA Synthase5* (At1g62940).

### Supplemental Data

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

**Supplemental Figure 1.** Analysis of Genes Involved in Pollen Wall Formation.

**Supplemental Figure 2.** Analysis of Wax and Cutin in the Wild Type and *ams* Buds.

**Supplemental Figure 3.** Genes Related to Lipid Acyl Conversion, Metabolism, and Miscellaneous Enzyme (MISC) during Pollen Wall Formation Were Downregulated (>2-Fold) in the *ams* Mutant.

**Supplemental Figure 4.** Expression Analysis of 14 AMS Direct Target Genes Involved in Pollen Wall Formation.

**Supplemental Figure 5.** Role of *CYP8A*, *CYP9A*, *EXL5*, and *EXL6* in Pollen Wall Development.

**Supplemental Table 1.** Expression Changes of Genes Known to Be Involved in Pollen Wall Synthesis in the *ams* Mutant.

**Supplemental Table 2.** Detailed Wax and Cutin Compositions in the Wild Type and *ams* Buds.

**Supplemental Table 3.** Primers Used in This Study.

**Supplemental Data Set 1.** Coexpression Analysis of 98 Candidate Genes Involved in Pollen Wall Formation in *Arabidopsis* and Expression in the Microarray Data of *spl*, *dyl1*, *ams*, *myb80*, *ms1*, and *ems1/exs*.

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### AUTHOR CONTRIBUTIONS

J.X., Z.D., and G.V.-B. carried out experiments. J.S., W.L., and Z.Y. conceived the study, supervised the work, and analyzed the data. D.W.-R., L.S., and Z.A.W. participated in project discussions and wrote the article. D.Z. designed experiments, analyzed data, and wrote the article.

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