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ABORTED MICROSPORES Acts as a Master Regulator of Pollen Wall Formation in *Arabidopsis*[C][W][OPEN]

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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 α-pyrone synthases POLYKETIDE SYNTHASE A/LAP6 and B/LAP5 (PKSA/B) (Dobritsa et al., 2010; Kim et al., 2010), and TETRAKETIDE α-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 MICROSPORE (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., β-*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/SPOROCYTELESS, 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 (β-1,3-glucan) cell wall, which is subsequently degraded by tapetal β-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

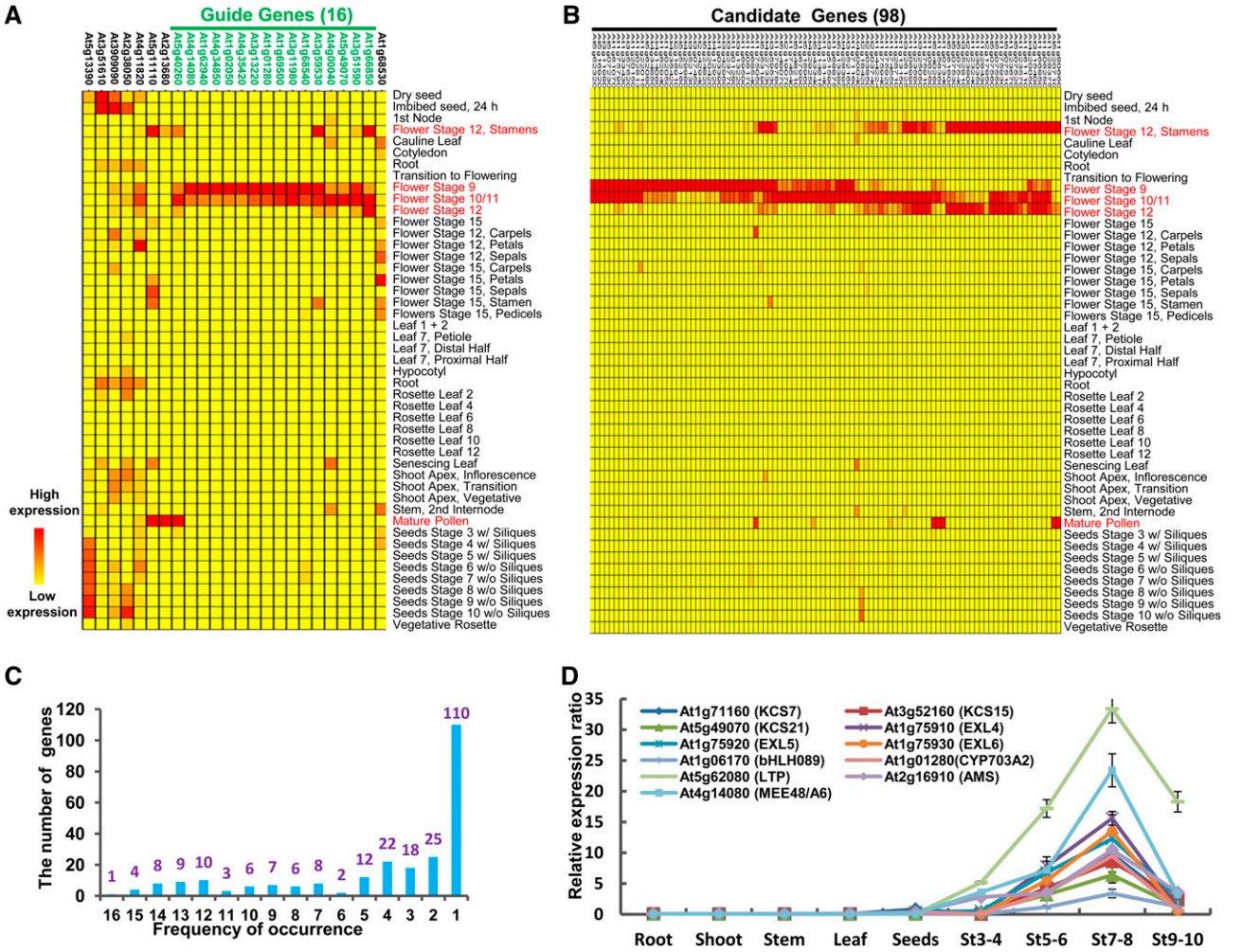

Figure 1. Coexpression Analysis of Candidate Genes Involved in Pollen Wall Formation.

(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 (±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

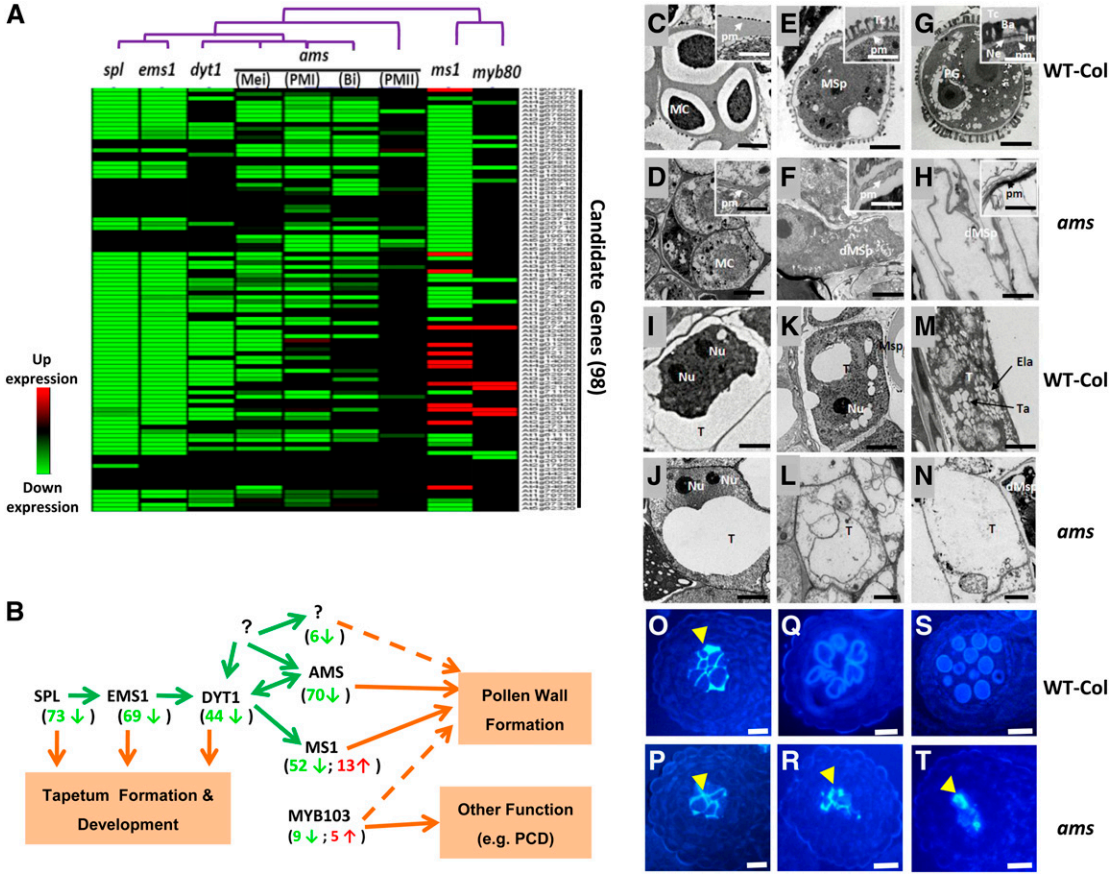

Figure 2. AMS Acts as a Key Transcriptional Regulator of Pollen Wall Formation in *Arabidopsis*.

(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 μm for **(C)** to **(H)** and 1 μm 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 μm.

(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 μm.

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 $NaNO_2$-Al $(NO_3)_3$ and Folin–Ciocalteau 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 *ams* Young Buds[a]				References
				Stage 6	Stage 8	Stage 9	Stage 10	
1	At4g14080	*A6*	Anther-specific protein 6	−3.58	0	0	0	This study
2	At4g20050	*QRT3*	Quartet 3	−2.50	−3.61	0	0	This study
3	At3g52160	*KCS15*	β-Ketoacyl-CoA synthase 15	−1.58	−1.81	0	0	This study
4	At5g49070	*KCS21*	β-Ketoacyl-CoA synthase 21	−1.01	−0.66	0	0	Xu et al. (2010)
5	At1g71160	*KCS7*	β-Ketoacyl-CoA synthase 7	0	−0.74	−0.64	−1.55	This study
6	At3g51590	*LTP12*	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	*WBC27*	ABC transporter	−2.57	0	−1.95	0	Xu et al. (2010)
10	At4g34850	*PKSB/LAP5*	Polyketide synthase B/Less adhesive pollen 5	−3.12	0	0	0	This study
11	At4g35420	*TKPR1/DRL1*	Tetraketide α-pyronereductase 1	−2.66	0	0	0	This study
12	At4g00040	*CHS*	Chalcone synthase	−0.45	−1.30	0	0	Xu et al. (2010)
13	At1g75920	*EXL5*	Family II extracellular lipase 5	−3.12	−3.58	−4.06	0	Xu et al. (2010)
14	At1g75910	*EXL4*	Family II extracellular lipase 4	−2.10	−2.08	0	0	This study
15	At1g75930	*EXL6*	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	*CYP704B1*	Cytochrome P450 704B1	−3.54	0	0	0	This study
18	At1g01280	*CYP703A2*	Cytochrome P450 703A2	−3.31	0	0	0	This study
19	At1g74540	*CYP98A8*	Cytochrome P450 98A8	−3.41	−3.31	−1.78	0	This study
20	At1g74550	*CYP98A9*	Cytochrome P450 98A9	−2.78	−3.11	−0.76	0	This study
21	At1g13140	*CYP86C3*	Cytochrome P450 CYP86C3	−1.77	−2.78	−1.57	−0.07	Xu et al. (2010)
22	At5g07520	*GRP18*	Glycine-rich protein18	−2.94	−3.91	−3.30	−2.51	This study
23	At5g07550	*GRP19*	Glycine-rich protein19	−3.12	−3.62	−2.54	−3.68	This study

[a]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 ≥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 β-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

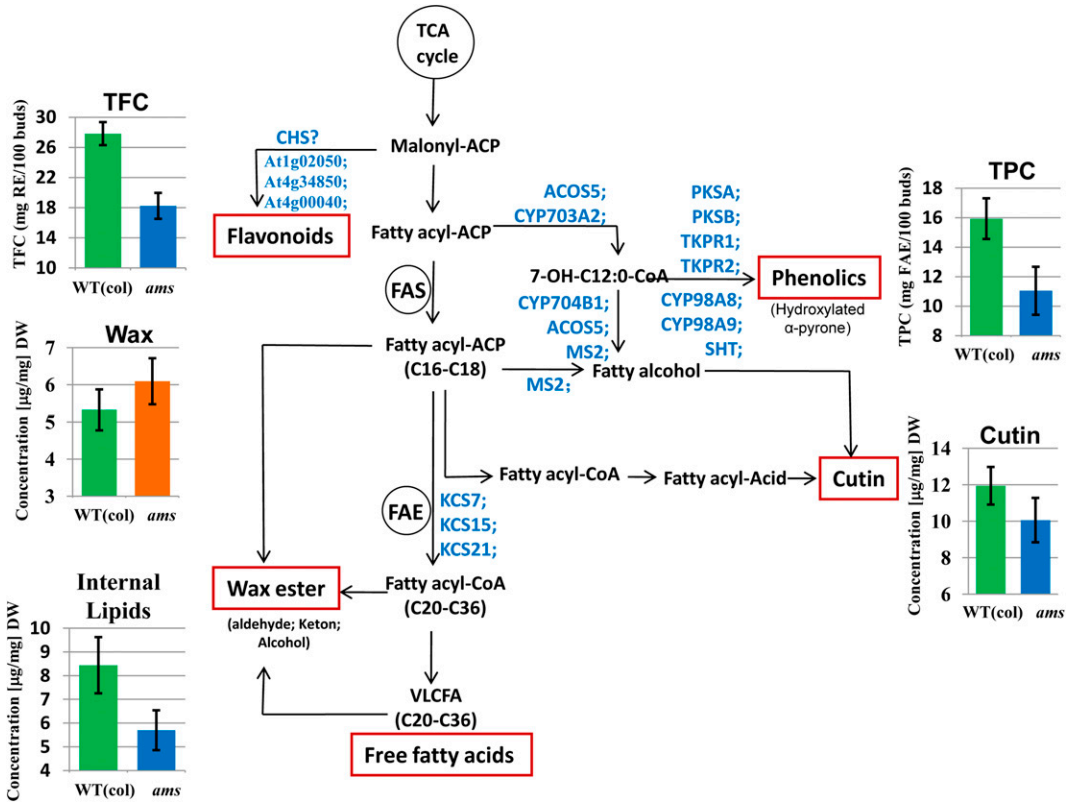

Figure 3. Metabolic Analysis of Wax, Cutin, Total Free Soluble Lipid, TPC, and TFC in Wild-Type and *ams* Buds.

The wax, cutin, and total soluble lipid amounts expressed as dry weight (μg/mg) in the wild type and *ams* flower buds. The TPC and TFC amounts relative to standard substance content in each of 100 buds. Error bars indicate SD (n = 5). Some genes (shown in blue on pathway) had been reported as being involved in these metabolic processes; these genes are down in *ams*, which lead to changes of the corresponding compounds.
[See online article for color version of this figure.]

formation, no obvious change in the expression of most primexine formation-associated genes was seen in our previous *ams* microarray analysis (Xu et al., 2010) (Supplemental Table 1).

Synthesis of Phenolic Compounds by CYP98A8 and CYP98A9 Is Essential for Pollen Wall Patterning

Phenol amides are major components of the pollen coat of all higher plants, but their function is still unknown. Phenolic compounds also are reported to be components of the pollen wall (Scott et al., 2004). *Arabidopsis CYP98A8* and *CYP98A9* were shown to catalyze oxygenation of phenol-amides, generating major pollen phenolic constituents (Matsuno et al., 2009). qChIP-PCR and expression analysis revealed that *CYP98A8* and its paralog *CYP98A9* were directly regulated by AMS (Figure 4A) (Matsuno et al., 2009). To further document the role of AMS in pollen development, null *cyp98A8/CYP98A9* RNAi double mutants from the T-DNA insertion line of *CYP98A8* and RNA interference (RNAi) line of *CYP98A9* were analyzed (Matsuno et al., 2009) (Figure 5A). No expression of the *CYP98A8* transcript and a very dramatic reduction of the *CYP98A9* transcript were observed in the homozygous null *cyp98A8/CYP98A9* RNAi line (Figure 5B). I_2-KI staining revealed that ~20% of its pollen grains were collapsed with impaired exine architecture. Furthermore, transmission electron microscopy (TEM) analysis indicated an abnormal exine layer with irregular baculum and tectum, and disordered tryphine deposition (Figures 5D and 5E). AMS thus also impacts pollen wall formation by regulating the biosynthesis of phenolic compounds.

AMS Regulates the Synthesis of Pollen Coat Composition

Hydration of desiccated pollen grains on the stigma is critical for pollination and is closely regulated by proteins and lipids of the pollen coat and stigma cuticle. qChIP-PCR analysis revealed that two pollen oleosin GRP-encoding genes (*GRP18* and *GRP19*) and three EXL family genes (*EXL4*, *EXL5*, and *EXL6*) are directly regulated by AMS (Xu et al., 2010) (Figure 4A). *Arabidopsis* includes six clustered *EXLs* with high similarity at the coding sequence level (Supplemental Figure 5G), which are assumed to be generated from a common ancestor (Mayfield et al., 2001). The eFP browser tool and hierarchical clustering analysis indicate that *EXL4*, *EXL5*, and *EXL6* are specifically expressed in flowers during pollen wall formation (Supplemental Figure 5F). *EXL4* expression was previously shown to be highly specific to anthers and was not detectable in microspores,

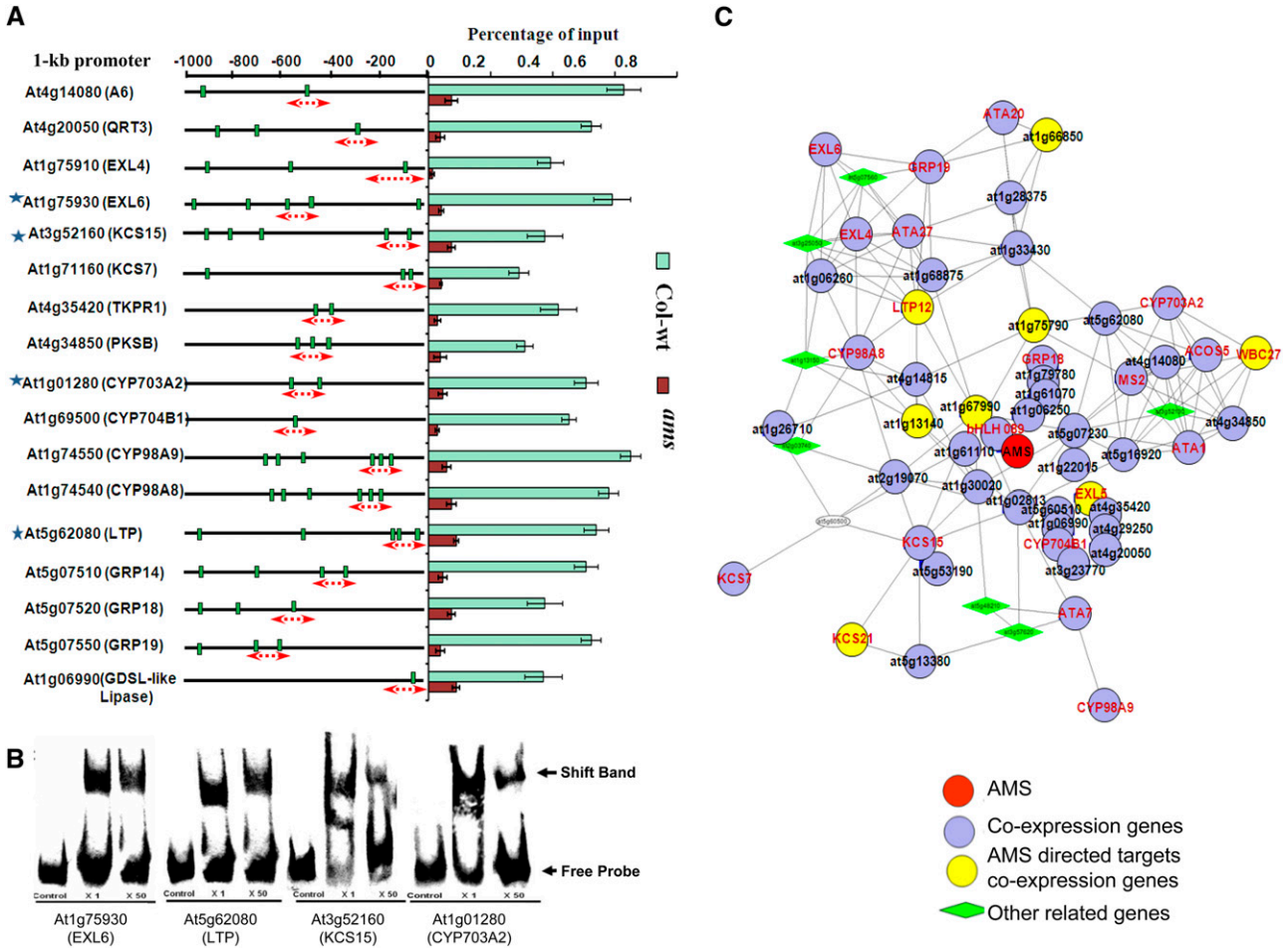

Figure 4. Analysis of the Direct Target Genes of AMS.

(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-β-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 *exl5* and *exl6* 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 *exl5* and *exl6* 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*, *exl5*, and *exl6* using *p*-nitrophenyl butyrate as a substrate (Updegraff et al., 2009) showed that *exl5* and *exl6* 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).

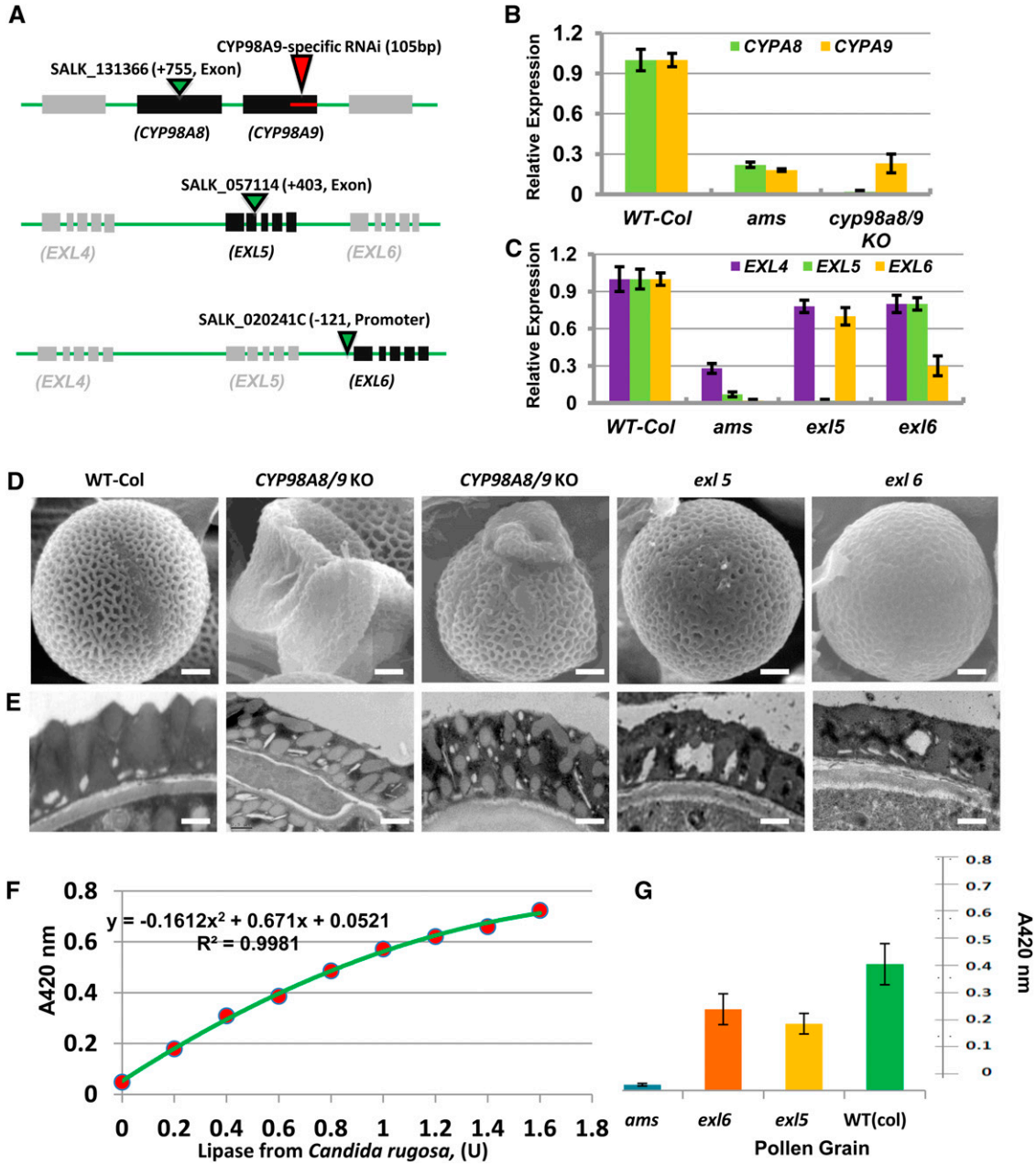

Figure 5. *CYP98A8*, *CYP98A9*, *EXL5*, and *EXL6*, Which Are Regulated by AMS, Are Required for Pollen Wall and Pollen Coat Development.

(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, *ms* 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 μm.

(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 μm.

(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.

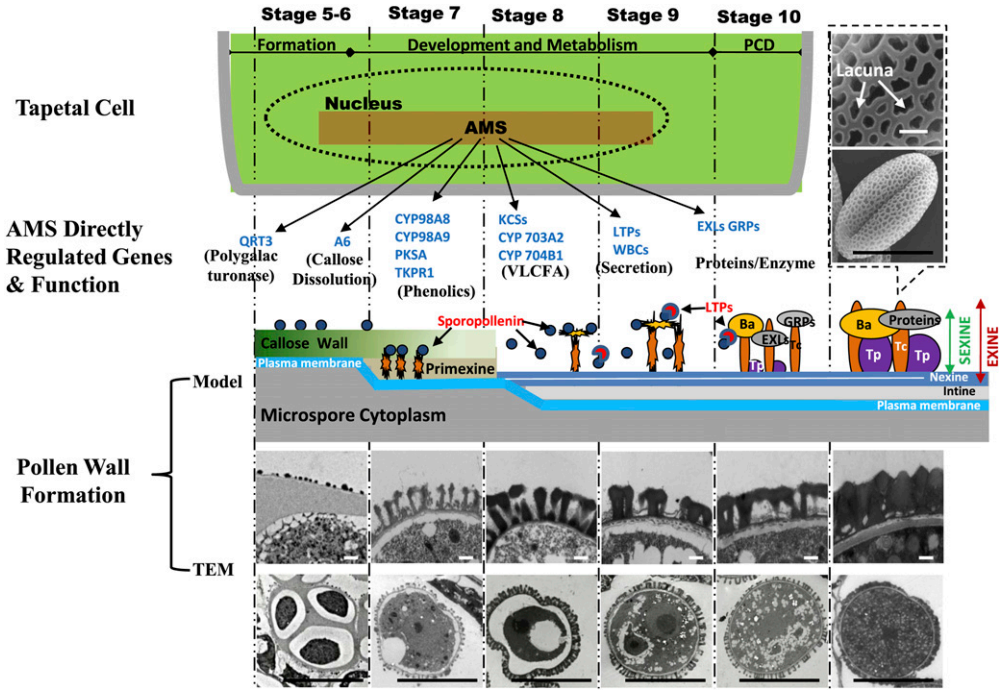

Figure 6. Proposed Scheme Showing the Central Role of AMS in Pollen Wall Formation.

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 μm.
[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 ω-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 myristoleicacid (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 ω-hydroxylated fatty acyl-CoAs to generate tri- and tetra-ketide α-pyrone compounds. *PKSB*/*LAP5* and *TKPR1*/*DRL1* encode enzymes that produce hydroxylated α-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, α-pyronepolyketides formed by PKSA and PKSB can be reduced by two tapetally expressed oxido-reductases, TKPR1/2. These form hydroxylated α-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 α-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 (Sturn et al., 2002). The Web-based Classification SuperViewer program (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 ΔCt values, and $2^{(-\Delta Ct)}$ as the percentage of input. All samples were run at least in duplicate.

I_2-KI Staining, Scanning Electron Microscopy, and TEM Analysis

I_2-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 (form 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–Ciocalteau Assay) and TFC of *Arabidopsis* Buds

Arabidopsis buds (0.5 g) were weighed in an EP tube and extracted with 500 μL 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 10,000*g* 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–Ciocalteau 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 ± 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), β-*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* α*-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 *CYPA8*, *CYPA9*, *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*, *dyt1*, *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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REFERENCES

Ahlers, F., Thom, I., Lambert, J., Kuckuk, R., and Rolf, W. (1999). 1H NMR analysis of sporopollenin from *Typha angustifolia.* Phytochemistry **50:** 1095–1098.

Alonso, J.M., et al. (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana.* Science **301:** 653–657.

Alves Ferreira, M., de Almeida Engler, J., Costa Miguens, F., Van Montagu, M., Engler, G., and de Oliveira, D.E. (1997). Oleosin gene expression in *Arabidopsis thaliana* tapetum coincides with accumulation of lipids in plastids and cytoplasmic bodies. Plant Physiol. Biochem. **35:** 729–739.

Ariizumi, T., and Toriyama, K. (2011). Genetic regulation of sporopollenin synthesis and pollen exine development. Annu. Rev. Plant Biol. **62:** 437–460.

Blackmore, S., Wortley, A.H., Skvarla, J.J., and Rowley, J.R. (2007). Pollen wall development in flowering plants. New Phytol. **174:** 483–498.

Bubert, H., Lambert, J., Steuernagel, S., Ahlers, F., and Wiermann, R. (2002). Continuous decomposition of sporopollenin from pollen of *Typha angustifolia L*. by acidic methanolysis. Z. Naturforsch. C. **57:** 1035–1041.

Canales, C., Bhatt, A.M., Scott, R., and Dickinson, H. (2002). EXS, a putative LRR 16 receptor kinase, regulates male germline cell number and tapetal identity and 17 promotes seed development in Arabidopsis. Curr Biol **12:** 1718–1727.

Chen, W., Yu, X.H., Zhang, K., Shi, J., De Oliveira, S., Schreiber, L., Shanklin, J., and 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.

de Azevedo Souza, C., Kim, S.S., Koch, S., Kienow, L., Schneider, K., McKim, S.M., Haughn, G.W., Kombrink, E., and Douglas, C.J. (2009). A novel fatty Acyl-CoA Synthetase is required for pollen development and sporopollenin biosynthesis in *Arabidopsis.* Plant Cell **21:** 507–525.

Dobritsa, A.A., Lei, Z., Nishikawa, S., Urbanczyk-Wochniak, E., Huhman, D.V., Preuss, D., and Sumner, L.W. (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.

Dobritsa, A.A., Shrestha, J., Morant, M., Pinot, F., Matsuno, M., Swanson, R., Møller, B.L., and 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.

Feng, B., Lu, D., Ma, X., Peng, Y., Sun, Y., Ning, G., and Ma, H. (2012). Regulation of the *Arabidopsis* anther transcriptome by *DYT1* for pollen development. Plant J. **72:** 612–624.

Grienenberger, E., Kim, S.S., Lallemand, B., Geoffroy, P., Heintz, D., Souza, Cde.A., Heitz, T., Douglas, C.J., and 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.

Heslop-Harrison, J. (1968). Pollen wall development. The succession of events in the growth of intricately patterned pollen walls is described and discussed. Science **161:** 230–237.

Hird, D.L., Worrall, D., Hodge, R., Smartt, S., Paul, W., and Scott, R. (1993). The anther-specific protein encoded by the *Brassica napus* and *Arabidopsis thaliana A6* gene displays similarity to beta-1,3-glucanases. Plant J. **4:** 1023–1033.

Jiang, J., Zhang, Z., and Cao, J. (2013). Pollen wall development: the associated enzymes and metabolic pathways. Plant Biol (Stuttg) **15:** 249–263.

Kai, K., Hashidzume, H., Yoshimura, K., Suzuki, H., Sakurai, N., Shibata, D., and Ohta, D. (2009). Metabolomics for the characterization of cytochromes P450-dependent fatty acid hydroxylation reactions in *Arabidopsis.* Plant Biotechnol. **26:** 175–182.

Kim, S.J., Son, K.H., Chang, H.W., Kang, S.S., and Kim, H.P. (2003). Tyrosinase inhibitory prenylated flavonoids from *Sophora flavescens.* Biol. Pharm. Bull. **26:** 1348–1350.

Kim, S.S., et al. (2010). LAP6/POLYKETIDE SYNTHASE A and LAP5/POLYKETIDE SYNTHASE B encode hydroxyalkyl α-pyrone synthases required for pollen development and sporopollenin biosynthesis in *Arabidopsis thaliana*. Plant Cell **22:** 4045–4066.

Knox, R.B., and Heslop-Harrison, J. (1970). Pollen-wall proteins: localization and enzymic activity. J. Cell Sci. **6:** 1–27.

Kouchi, H., and Hata, S. (1993). Isolation and characterization of novel nodulin cDNAs representing genes expressed at early stages of soybean nodule development. Mol. Gen. Genet. **238:** 106–119.

Li, H., Pinot, F., Sauveplane, V., Werck-Reichhart, D., Diehl, P., Schreiber, L., Franke, R., Zhang, P., Chen, L., Gao, Y., Liang, W., and Zhang, D. (2010). Cytochrome P450 family member *CYP704B2* catalyzes the ω-hydroxylation of fatty acids and is required for anther cutin biosynthesis and pollen exine formation in rice. Plant Cell **22:** 173–190.

Li, H., and Zhang, D. (2010). Biosynthesis of anther cuticle and pollen exine in rice. Plant Signal. Behav. **5:** 1121–1123.

Li, N., et al. (2006). The rice *tapetum degeneration retardation* gene is required for tapetum degradation and anther development. Plant Cell **18:** 2999–3014.

Matsuno, M., et al. (2009). Evolution of a novel phenolic pathway for pollen development. Science **325:** 1688–1692.

Mayfield, J.A., Fiebig, A., Johnstone, S.E., and Preuss, D. (2001). Gene families from the *Arabidopsis thaliana* pollen coat proteome. Science **292:** 2482–2485.

Mayfield, J.A., and Preuss, D. (2000). Rapid initiation of *Arabidopsis* pollination requires the oleosin-domain protein GRP17. Nat. Cell Biol. **2:** 128–130.

Morant, M., Jørgensen, K., Schaller, H., Pinot, F., Møller, B.L., Werck-Reichhart, D., and 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.

Niu, B.X., He, F.R., He, M., Ren, D., Chen, L.T., and Liu, Y.G. (2013). The ATP-binding cassette transporter OsABCG15 is required for anther development and pollen fertility in rice. J.Integr.Plant Biol. **55:** 710–720.

Obayashi, T., Hayashi, S., Saeki, M., Ohta, H., and Kinoshita, K. (2009). ATTED-II provides coexpressed gene networks for *Arabidopsis.* Nucleic Acids Res. **37** (Database issue)**:** D987–D991.

Phan, H.A., Iacuone, S., Li, S.F., and Parish, R.W. (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.

Pinot, F., and Beisson, F. (2011). Cytochrome P450 metabolizing fatty acids in plants: characterization and physiological roles. FEBS J. **278:** 195–205.

Qin, P., Tu, B., Wang, Y., Deng, L., Quilichini, T.D., Li, T., Wang, H., Ma, B., and Li, S. (2013). *ABCG15* encodes an ABC transporter protein, and is essential for post-meiotic anther and pollen exine development in rice. Plant Cell Physiol. **54:** 138–154.

Sanders, P.M., Bui, A.Q., Weterings, K., McIntire, K.N., Hsu, Y.C., Lee, P.Y., Truong, M.T., Beals, T.P., and Goldberg, R.B. (1999). Anther developmental defects in *Arabidopsis thaliana* male-sterile mutants. Sex. Plant Reprod. **11:** 297–322.

Schuler, M.A., and Werck-Reichhart, D. (2003). Functional genomics of *P450s.* Annu. Rev. Plant Biol. **54:** 629–667.

Scott, R.J. (1994). Molecular and Cellular Aspects of Plant Reproduction. (Cambridge, UK: Cambridge University Press).

Scott, R.J., Spielman, M., and Dickinson, H.G. (2004). Stamen structure and function. Plant Cell **16** (suppl.)**:** S46–S60.

Shi, J., et al. (2011). *Defective pollen wall* is required for anther and microspore development in rice and encodes a fatty acyl carrier protein reductase. Plant Cell **23:** 2225–2246.

Singleton, V.L., and Rossi, J.A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. **16:** 144–158.

Sorensen, A.M., Kröber, S., Unte, U.S., Huijser, P., Dekker, K., and Saedler, H. (2003). The *Arabidopsis ABORTED MICROSPORES* (*AMS*) gene encodes a MYC class transcription factor. Plant J. **33:** 413–423.

Stieglitz, H. (1977). Role of beta-1,3-glucanase in postmeiotic microspore release. Dev. Biol. **57:** 87–97.

Sturn, A., Quackenbush, J., and Trajanoski, Z. (2002). Genesis: cluster analysis of microarray data. Bioinformatics **18:** 207–208.

Tang, L.K., Chu, H., Yip, W.K., Yeung, E.C., and Lo, C. (2009). An anther-specific dihydroflavonol 4-reductase-like gene (*DRL1*) is essential for male fertility in *Arabidopsis.* New Phytol. **181:** 576–587.

Todd, J., Post-Beittenmiller, D., and Jaworski, J.G. (1999). *KCS1* encodes a fatty acid elongase 3-ketoacyl-CoA synthase affecting wax biosynthesis in *Arabidopsis thaliana.* Plant J. **17:** 119–130.

Updegraff, E.P., Zhao, F., and Preuss, D. (2009). The extracellular lipase *EXL4* is required for efficient hydration of *Arabidopsis* pollen. Sex. Plant Reprod. **22:** 197–204.

Upton, C., and Buckley, J.T. (1995). A new family of lipolytic enzymes? Trends Biochem. Sci. **20:** 178–179.

Wiermann, R., and Gubatz, S. (1992). Pollen wall and sporopollenin. Int. Rev. Cytol. **140:** 35–72.

Wilson, Z.A., and Zhang, D.B. (2009). From *Arabidopsis* to rice: pathways in pollen development. J. Exp. Bot. **60:** 1479–1492.

Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G.V., and Provart, N.J. (2007). An "Electronic Fluorescent Pictograph"

browser for exploring and analyzing large-scale biological data sets. PLoS ONE **2:** e718.

Xu, J., Yang, C., Yuan, Z., Zhang, D., Gondwe, M.Y., Ding, Z., Liang, W., Zhang, D., and Wilson, Z.A. (2010). The *ABORTED MICROSPORES* regulatory network is required for postmeiotic male reproductive development in *Arabidopsis thaliana.* Plant Cell **22:** 91–107.

Yang, C.Y., Vizcay-Barrena, G., Conner, K., and Wilson, Z.A. (2007). MALE STERILITY1 is required for tapetal development and pollen wall biosynthesis. Plant Cell **19:** 3530–3548.

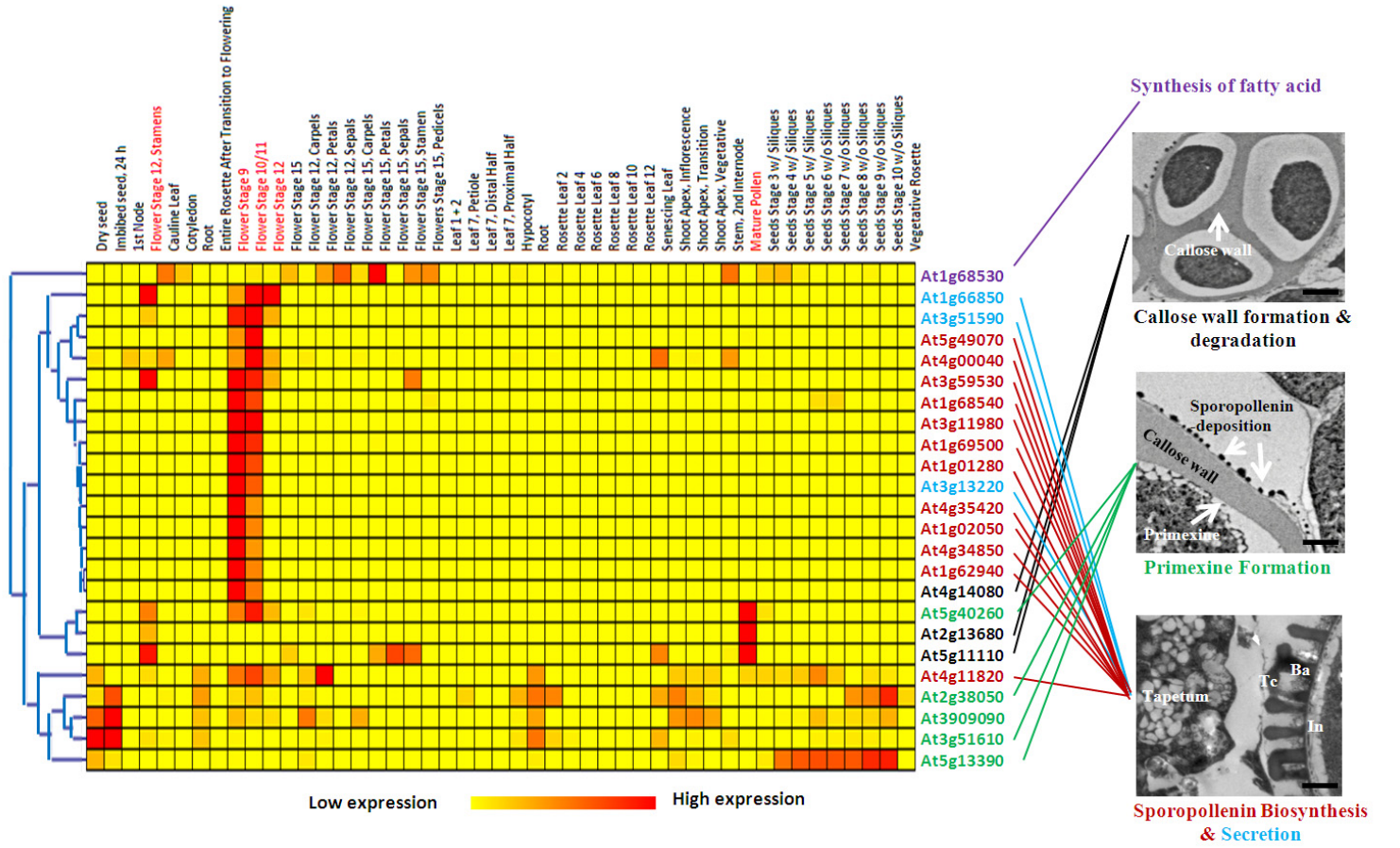
Zhang, D., Luo, X., and Zhu, L. (2011). Cytological analysis and genetic control of rice anther development. J. Genet. Genomics **38:** 379–390.

Zhang, W., Sun, Y.L., Timofejeva, L., Chen, C.B., Grossniklaus, U., and Ma, H. (2006). Regulation of *Arabidopsis* tapetum development and function by *DYSFUNCTIONAL TAPETUM* (*DYT1*) encoding a putative bHLH transcription factor. Development **133:** 3085–3095.

Zhao, D.Z., Wang, G.F., Speal, B., and Ma, H. (2002). The excess microsporocytes1 gene encodes a putative leucine-rich repeat receptor protein kinase that controls somatic and reproductive cell fates in the Arabidopsis anther. Genes Dev. **16:** 2021–2031.

Zhu, L., Shi, J., Zhao, G., Zhang, D., and Liang, W. (2013). Post-meiotic deficient anther1 (*PDA1*) encodes an ABC transporter required for the development of anther cuticle and pollen exine in rice. J. Plant Biol. **56:** 59–68.

Zimmermann, U., Schneider, H., Wegner, L., and Haase, A. (2004). Water ascent in tall trees: does evolution of land plants rely on a highly metastable state? New Phytol. **162:** 575–615.

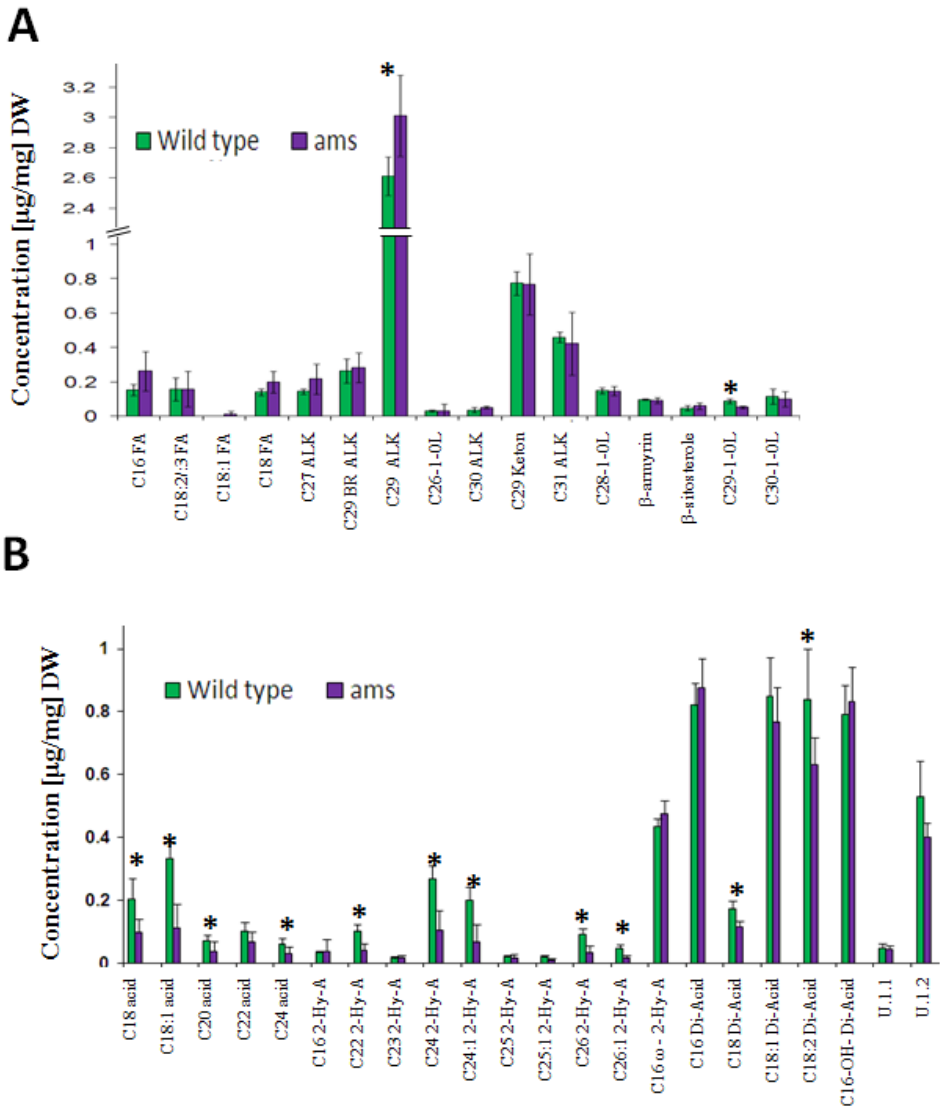

Dry seed
Imbibed seed, 24 h
1st Node
Flower Stage 12, Stamens
Cauline Leaf
Cotyledon
Root
Entire Rosette After Transition to Flowering
Flower Stage 9
Flower Stage 10/11
Flower Stage 12
Flower Stage 15
Flower Stage 12, Carpels
Flower Stage 12, Petals
Flower Stage 12, Sepals
Flower Stage 15, Carpels
Flower Stage 15, Petals
Flower Stage 15, Sepals
Flower Stage 15, Stamen
Flowers Stage 15, Pedicels
Leaf 1 + 2
Leaf 7, Petiole
Leaf 7, Distal Half
Leaf 7, Proximal Half
Hypocotyl
Root
Rosette Leaf 2
Rosette Leaf 4
Rosette Leaf 6
Rosette Leaf 8
Rosette Leaf 10
Rosette Leaf 12
Senescing Leaf
Shoot Apex, Inflorescence
Shoot Apex, Transition
Shoot Apex, Vegetative
Stem, 2nd Internode
Mature Pollen
Seeds Stage 3 w/ Siliques
Seeds Stage 4 w/ Siliques
Seeds Stage 5 w/ Siliques
Seeds Stage 6 w/o Siliques
Seeds Stage 7 w/o Siliques
Seeds Stage 8 w/o Siliques
Seeds Stage 9 w/o Siliques
Seeds Stage 10 w/o Siliques
Vegetative Rosette
At1g68530
At1g66850
At3g51590
At5g49070
At4g00040
At3g59530
At1g68540
At3g11980
At1g69500
At1g01280
At3g13220
At4g35420
At1g02050
At4g34850
At1g62940
At4g14080
At5g40260
At2g13680
At5g11110
At4g11820
At2g38050
At3909090
At3g51610
At5g13390
Low expression
High expression
Synthesis of fatty acid
Callose wall
Callose wall formation & degradation
Sporopollenin deposition
Callose wall
Primexine
Primexine Formation
Tapetum
Tc
Ba
In
Sporopollenin Biosynthesis & Secretion

Left

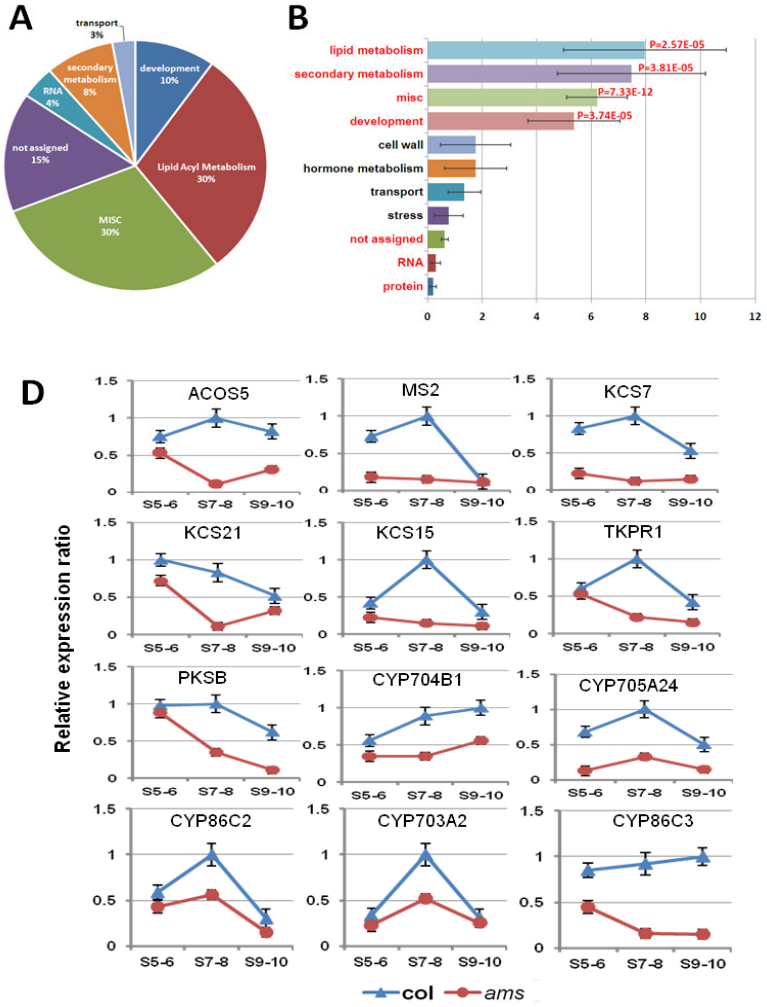
Right

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

The left shown the expression pattern and cluster analysis of pollen wall formation-related genes that have been demonstrated to be involved in pollen wall formation(detail formation in Supplemental Table 1). The right shown the key event in the development of pollen wall formation, The color of the genes locus (left) consistent with the color of the major developmental events in the process (right); Tc: tectum, Ba: bacula, In: intine. (Bars = 100 μm).



Supplemental Figure 2. Analysis of Wax and Cutin in the Wild-type and *ams* Buds. **A.** Wax constituents, amount dry weight (µg/mg). Error bars indicate SD (n = 5). **B.** Cutin monomers, amount dry weight (µg/mg). Error bars indicate SD (n = 5); T-Test analysis $P < 0.05$ indicated with a *. Compound names are abbreviated as follows: FA: fatty acids; ALK: alkanes; Hy-Acid: Hydroxy-Acid; C16 acid, hexadecanoic acid; C18 acid, octadecanoic acid; C20 acid, eicosanoic acid; C22 acid, docosanoic acid; C24 acid, tetracosanoic acid; C16-ω-HFA, 16-hydroxyhexadecanoic acid; C16 di-acid, hexadecane-(1,16)-dioic acid; C18 di-acid, octadecane-(1,18)-dioic acid; C18:1 di-acid, octadecene-(1,18)-dioic acid; C18:2 di-acid, octadecadiene-(1,18)-dioic acid; UI, unknown cutin monomer.

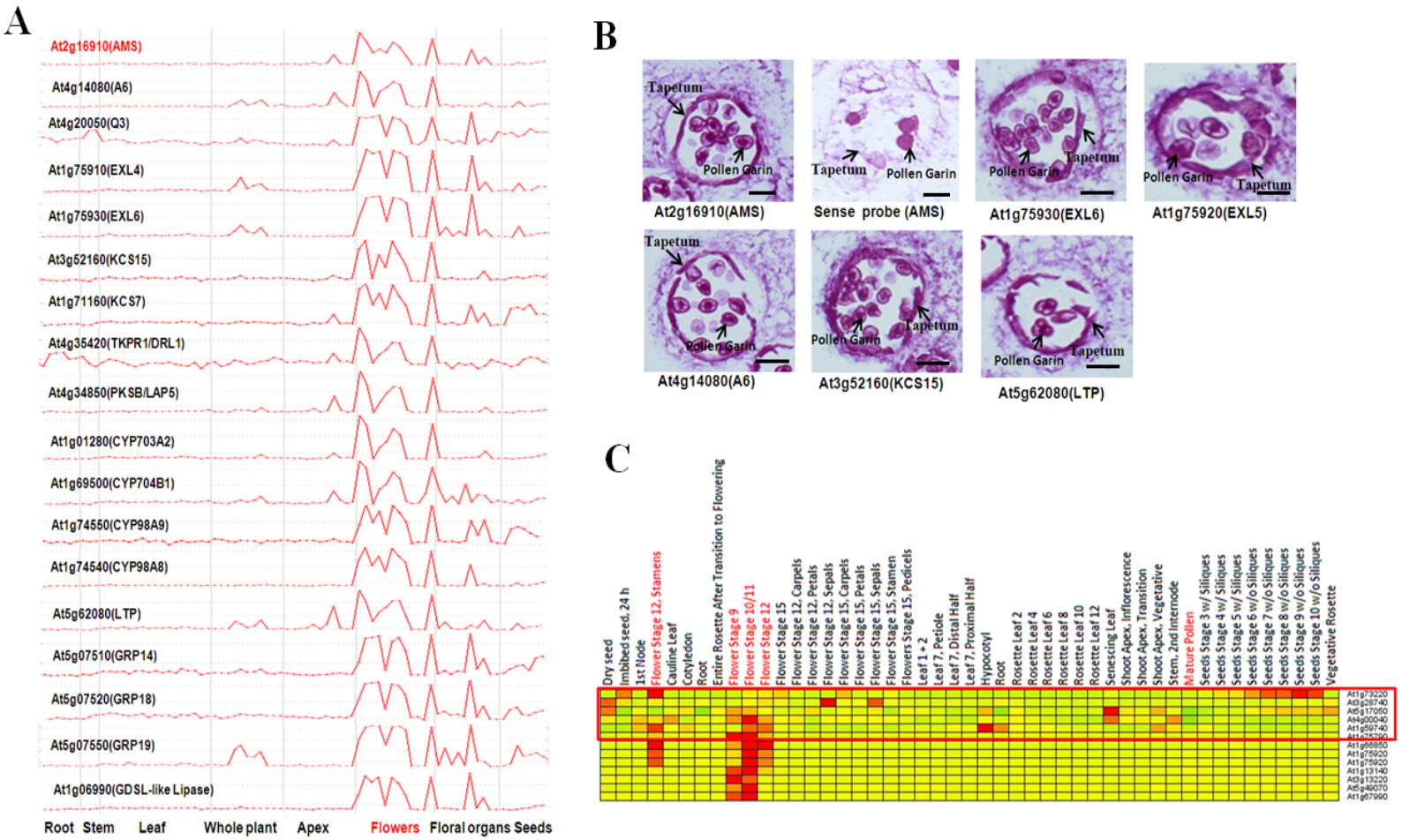


C

No	Locus	Gene Name	Description
Lipid Acyl Metabolism (21)			
1	At3g11980	*MS2*	MALE STERILITY 2;
2	At1g62940	*ACOS5*	ACYL-COA SYNTHETASE 5; 4-coumarate-CoA ligase
3	At1g23240		Caleosin-related family protein
4	At1g06990		GDSL-motif lipase
5	At1g06250		Lipase class 3 family protein
6	At1g75910	*EXL4*	Family II extracellular lipase 4
7	At1g75920	*EXL5*	Family II extracellular lipase 5
8	At1g75930	*EXL6*	Family II extracellular lipase 6
9	At3g13220	*WBC27*	ABC transporter family protein
10	At4g28395		ATA7; lipid transporter
11	At5g07230		Lipid transfer protein (LTP) family protein
12	At4g14815		Lipid transfer protein (LTP) family protein
13	At1g66850		Lipid transfer protein (LTP) family protein
14	At5g62080		Lipid transfer protein (LTP) family protein
15	At3g51590	*LTP12*	Lipid transfer protein 12 (LTP)
16	At1g71160	*KCS7*	3-KETOACYL-COA SYNTHASE 7
17	At3g52160	*KCS15*	3-KETOACYL-COA SYNTHASE 15
18	At5g49070	*KCS21*	3-KETOACYL-COA SYNTHASE 21
19	At5g07510	*GRP14*	GLYCINE-RICH PROTEIN 14
20	At5g07520	*GRP18*	GLYCINE-RICH PROTEIN 18
21	At5g07550	*GRP19*	GLYCINE-RICH PROTEIN 19
MISC (21)			
22	At1g01280	*CYP703A2*	CYTOCHROME P450
23	At1g13140	*CYP86C3*	CYTOCHROME P450
24	At1g28430	*CYP705A24*	CYTOCHROME P450
25	At1g69500	*CYP704B1*	CYTOCHROME P450
26	At1g74540	*CYP98A8*	CYTOCHROME P450
27	At1g74550	*CYP98A9*	CYTOCHROME P450
28	At3g26125	*CYP86C2*	CYTOCHROME P450
29	At1g06260		Cysteine proteinase, putative
30	At1g22015	*DD46*	Galactosyltransferase
31	At1g30350		Pectate lyase family protein
32	At1g33430		Galactosyltransferase family protein
33	At1g56360	*PAP6*	PURPLE ACID PHOSPHATASE 6
34	At1g75790	*SKS18*	SKU5 Similar 18
35	At1g75940	*ATA27*	Hydrolase, hydrolyzing O-glycosyl compounds
36	At3g25050	*XTH3*	XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 3
37	At3g42960	*ATA1*	ARABIDOPSIS TAPETUM 1
38	At4g14080	*MEE48/A6*	Maternal effect embryo arrest 48
39	At4g20050	*QRT3*	QUARTET 3; polygalacturonase
40	At4g29250		Transferase family protein
41	At5g16960		NADP-dependent oxidoreductase, putative
42	At5g20710	*BGAL7*	Beta-galactosidase 7
Secondary Metabolism (6)			
43	At1g02050		Chalcone and stilbene synthase family protein
44	At4g34850		Chalcone and stilbene synthase family protein
45	At1g67990	*TSM1*	TAPETUM-SPECIFIC METHYLTRANSFERASE 1
46	At1g68540		Oxidoreductase family protein
47	At2g19070	*SHT*	SPERMIDINE HYDROXYCINNAMOYL TRANSFERASE
48	At4g35420		Dihydroflavonol 4-reductase family

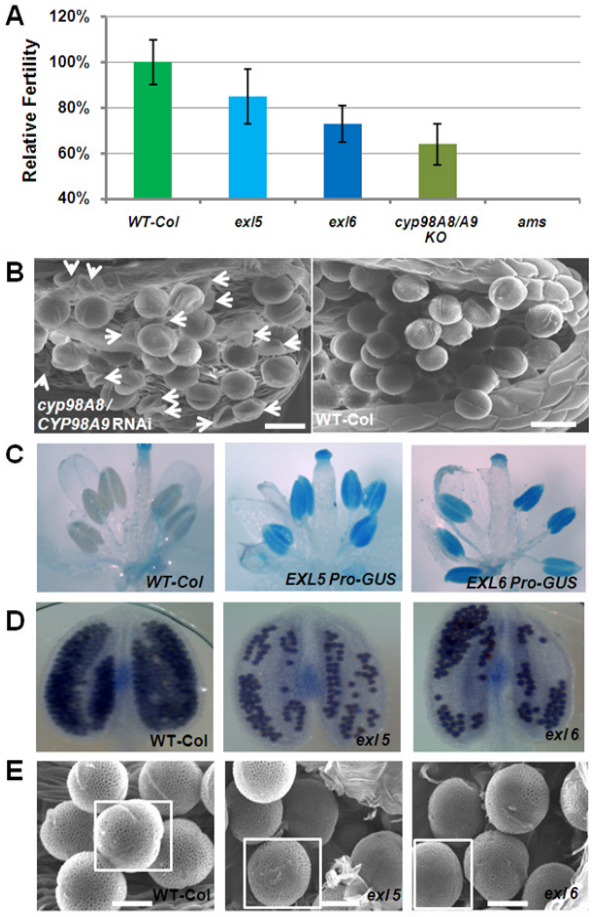
Supplemental Figure 3. Genes Related to Lipid Acyl Conversion, Metabolism and Miscellaneous enzyme (MISC) during Pollen Wall Formation Were Down-Regulated (> 2-Fold) in the *ams* Mutant.

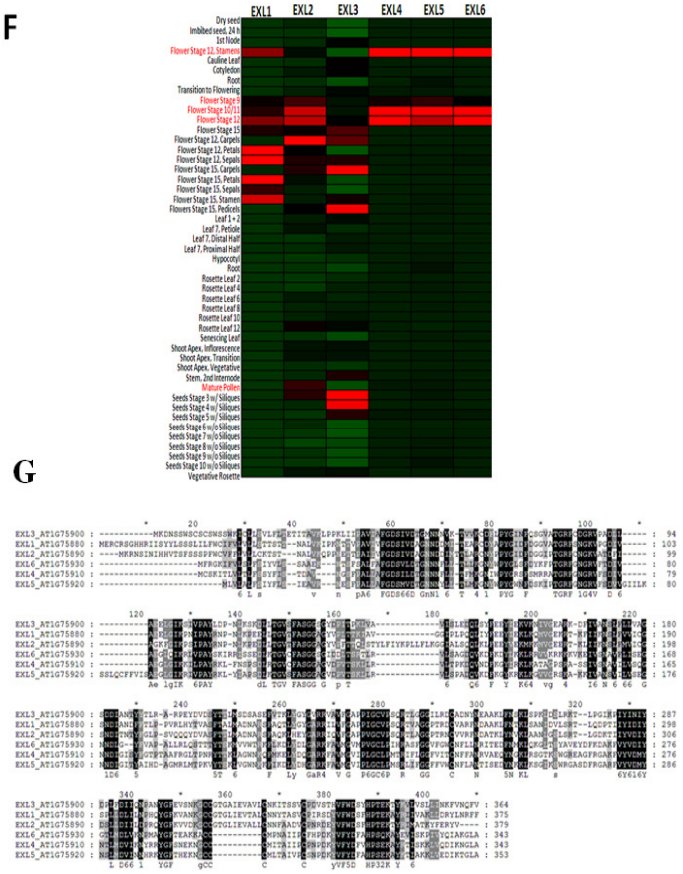
A. Summary of the MapMan classification of the 70 candidate genes co-expressed with *AMS*. MapMan analysis of genes was performed using the Bio-Array Resource (BAR) for Plant Biology website (http://bar.utoronto.ca/ntools/cgi-bin/ntools_classification_superviewer.cgi). **B.** The enriched MapMan categories (P< 0.01 highlighted with red letter) and their non-enriched parents were visualized using the 'Classification SuperViewer' program. The MapMan data were downloaded at http://mapman.gabipd.org/web/guest/mapmanstore (file Ath_AGI_TAIR9_Jan 2010.txt). **C.** Genes relative to pollen wall formation were down-regulated >2-fold in the *ams* mutant. **D.** Expression analysis of 12 genes in the wild type (WT) and the *ams* mutant at the 5-6, 7-8 and 9-10 anther stage using qRT-PCR. Error bars indicate SD, each with four biological repeats.


A
At2g16910(AMS)
At4g14080(A6)
At4g20050(Q3)
At1g75910(EXL4)
At1g75930(EXL6)
At3g52160(KCS15)
At1g71160(KCS7)
At4g35420(TKPR1/DRL1)
At4g34850(PKSB/LAP5)
At1g01280(CYP703A2)
At1g69500(CYP704B1)
At1g74550(CYP98A9)
At1g74540(CYP98A8)
At5g62080(LTP)
At5g07510(GRP14)
At5g07520(GRP18)
At5g07550(GRP19)
At1g06990(GDSL-like Lipase)
Root Stem Leaf Whole plant Apex Flowers Floral organs Seeds
B
Tapetum
Pollen Garin
At2g16910(AMS)
Sense probe (AMS)
At1g75930(EXL6)
At1g75920(EXL5)
At4g14080(A6)
At3g52160(KCS15)
At5g62080(LTP)
C
Dry seed
Imbibed seed, 24 h
1st Node
Flower Stage 12, Stamens
Cauline Leaf
Cotyledon
Root
Entire Rosette After Transition to Flowering
Flower Stage 9
Flower Stage 10/11
Flower Stage 12
Flower Stage 15
Flower Stage 12, Carpels
Flower Stage 12, Petals
Flower Stage 12, Sepals
Flower Stage 15, Carpels
Flower Stage 15, Petals
Flower Stage 15, Sepals
Flower Stage 15, Stamen
Flowers Stage 15, Pedicels
Leaf 1 + 2
Leaf 7, Petiole
Leaf 7, Distal Half
Leaf 7, Proximal Half
Hypocotyl
Root
Rosette Leaf 2
Rosette Leaf 4
Rosette Leaf 6
Rosette Leaf 8
Rosette Leaf 10
Rosette Leaf 12
Senescing Leaf
Shoot Apex, Inflorescence
Shoot Apex, Transition
Shoot Apex, Vegetative
Stem, 2nd Internode
Mature Pollen
Seeds Stage 3 w/ Siliques
Seeds Stage 4 w/ Siliques
Seeds Stage 5 w/ Siliques
Seeds Stage 6 w/o Siliques
Seeds Stage 7 w/o Siliques
Seeds Stage 8 w/o Siliques
Seeds Stage 9 w/o Siliques
Seeds Stage 10 w/o Siliques
Vegetative Rosette
At1g73220
At3g28740
At5g17050
At4g00040
At1g59740
At1g66850
At1g75920
At1g75920
At1g13140
At3g13220
At5g40070
At1g67990

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

A. Analysis using the Gene press Visualization Tool (http://jsp.weigelworld.org/expviz/). **B.** *In Situ* hybridization analysis for six genes in the anthers at stage 9, (Bars = 100 μm). **C.** Expression patterns of six out of the 13 direct targets of AMS reported by Xu et al. (2010), and of which seven genes do not have specific expression (in the red box region).


A
Relative Fertility
120%
100%
80%
60%
40%
WT-Col
exl5
exl6
cyp98A8/A9 KO
ams
B
cyp98A8/ CYP98A9 RNAi
WT-Col
C
WT-Col
EXL5 Pro-GUS
EXL6 Pro-GUS
D
WT-Col
exl 5
exl 6
E
WT-Col
exl 5
exl 6


F
EXL1
EXL2
EXL3
EXL4
EXL5
EXL6
G

Supplemental Figure 5. Role of *CYPA8, CYPA9, EXL5* and *EXL6* in Pollen Wall Development.

A. Pod seed setting rate statistics of the wild-type plant, the null *cyp98A8/CYP98A9* RNAi line, *exl5*, *exl6* and *ams*. **B.** Analysis of the outer surface structure of pollen grains from the wild-type plant and the null *cyp98A8/CYP98A9* RNAi double mutant by scanning electron microscopy (SEM), white arrows indicate abnormal microspores. (Bars = 100 μm). **C.** GUS-staining analysis showing the expression of *EXL5* and *EXL6* in developing anthers. **D.** Pollen grains of the wild-type plant, *exl5* and *exl6* analyzed by I_2-KI staining. **E.** Analysis of the outer surface structure of pollen grains from the wild-type plant, *exl5* and *exl6* mutant by SEM. white boxed regions indicate abnormal microspores, and amplification of the region in Figure 5D .(Bars = 100 μm). **F.** *EXL4*, *EXL5* and *EXL6* show similar expression patterns during anther development, but obviously distinct expression patterns from those of *EXL1*, *EXL2* and *EXL3*. **G.** Sequence alignment of *EXLs* (1-6).

Supplemental Table 1. Expression changes of genes known to be involved in pollen wall synthesis in the *ams* mutant.

Locus	Gene Name	Description	Fold Change in *ams* Young Buds*				References
			Mei	PMI	Bi	PMII	
Primexine Formation							
At3g09090	*DEX1*	Defective exine formation 1	0	0	0	0	Paxson-Sowders et al., 2001;
At5g13390	*NEF1*	No exine formation 1	0	0	0	0	Ariizumi et al., 2004;
At5g40260	*RPG1*	Ruptured pollen grain 1	-1.86	-1.98	-2.65	1	Guan et al ., 2008;
At5g22260	*MS1/HKM*	Male sterility 1/ Hackly microspore	0	0	0	0	Ariizumi et al ., 2005;Wilson et al ., 2001;
At3g51610	*NPU*	Noprimexineand plasma membrane undulation	0	0	0	0	Chang et al ., 2011;
At2g38050	*TDE1/ DET2*	Transient defective exine1/ De-etiolated 2	0	0	0	0	Ariizumi et al., 2008;
Callose wall formation							
At2g13680	*Cals5*	Callosesynthetase 5	0	0	0	0	Dong et al ., 2005;
At5g11110	*KNS2*	Kaonashi 2	0	0	0	0	Schnurr et al ., 2006;
At4g14080	*A6*	Anther-specific protein 6	-3.58	0	0	0	Hird et al., 2003;
Sporopollenin Biosynthesis							
At4g31380	*FLP1*	Faceless pollen 1	0	0	0	0	Rowland et al ., 2007;
At4g11820	*FKP1-1*	Flaky pollen1-1	0	0	0	0	Ishiguro et al., 2010;
At3g59530	*LAP3*	Less adhesive pollen 3	0	0	0	0	Dobritsa et al ., 2009;

At1g62940	*ACOS5*	Acyl-CoA synthase5	-2.78	0	0	0	De et al., 2009;
At3g11980	*MS2*	Male sterility 2	-4.13	1.87	0	0	Aartset al.,1997; Chen et al., 2011
At5g49070	*KCS21*	β-ketoacyl-CoA synthase 21	-1.01	-1.58	0	0	Xu et al., 2010;
At1g01280	*CYP703A2*	Cytochrome P450 703A2	-3.31	0	0	0	Morantal., 2007;
At1g69500	*CYP704B1*	Cytochrome P450 704B1	-3.54	0	0	0	Dobritsaet al., 2009;
At4g35420	*TKPR1/DRL1*	Tetraketidea-pyronereductase 1	-2.66	0	0	0	Tang et al., 2009; Etienne et al., 2010;
At1g68540	*TKPR2/CCRL6*	Tetraketidea-pyronereductase 2	-1.24	0	0	0	Etienne et al., 2010;
At4g00040		Chalcone synthase	-0.45	-1.3	0	0	Xu et al., 2010;
At1g02050	*PKSA/LAP6*	Polyketide synthase A/Less adhesive pollen 6	-2.71	0	1.13	0	Kim et al., 2010; Dobritsaet al., 2010;
At4g34850	*PKSB/LAP5*	Polyketide synthase B/Less adhesive pollen 5	-3.12	0	0	0	Kim et al., 2010; Dobritsaet al., 2010;
Sporopollenin Secretion							
At3g51590	*LTP12*	Lipid transfer protein12	-3.68	-2.98	-4.24	0	Xu et al., 2010;
At1g66850		Lipid transfer protein type 2	-4.08	-4.92	-4.1	-1.1	Xu et al., 2010;
At3g13220	*WBC27/ABCG26*	ABC transporter	-2.57	0	-1.95	0	Xu et al., 2010; Choi et al.,2011;Teagen et al., 2010;

Supplemental Table 2. Detailed wax and cutin compositions in the wild type and *ams* buds*.

Wax constituents	WT [µg/mg]		*ams*[µg/mg]		Chang VS. WT (Up %)
	Mean	SD	Mean	SD	
Long chain primary alcohols					
C26-1-OL	0.0325	0.0045	0.2093	0.0393	4.67%
C28-1-OL	0.0987	0.0039	0.0940	0.0176	-4.81%
C29-1-OL	**0.0903**	0.0131	**0.0536**	0.0063	-40.65%
C30-1-OL	0.0405	0.0042	0.0302	0.0109	25.57%
Subtotal	0.2621	0.0257	0.2117	0.0740	-19.19%
Fatty acids					
C16-FA	0.1545	0.0320	0.2640	0.1138	70.87%
18(2/3/1)-FA	0.1590	0.0668	0.1593	0.1026	0.19%
C18-FA	0.1421	0.0187	0.2004	0.063	41.03%
Subtotal	0.4556	0.1175	0.6237	0.2794	36.90%
Alkanes					
C27-ALK	0.1447	0.0168	0.2180	0.0879	50.66%
C29- BR ALK	0.263	0.070	0.2839	0.085	7.95%
C29-ALK	**2.6117**	0.1283	**3.0145**	0.2688	15.42%
C30-ALK	0.0379	0.0140	0.0524	0.0083	38.26%
C31-ALK	0.1493	0.0172	0.1469	0.0268	-1.61%
Subtotal	3.2066	0.2463	3.7157	0.4768	15.88%
Steroles					
ß-armyrin	0.0486	0.0161	0.0609	0.0173	25.31%
ß-sitosterol	0.1175	0.0442	0.1008	0.0439	-14.21%
Subtotal	0.1661	0.0603	0.1617	0.0612	-2.65%
Keton					

C29 Keton	0.7739	0.068	0.7674	0.1763	-0.84%
Total	**4.8643**	0.5178	**5.4802**	1.0677	12.67%

Identified cutin monomers	**WT (Col)[µg/mg]**		***ams*[µg/mg]**		**Chang VS. WT (Up %)**
	Mean	**SD**	**Mean**	**SD**	
Acid					
C16 Acid	1.8399	0.5963	1.1048	0.1964	-39.95%
C18 Acid	**0.2042**	0.0644	**0.0970**	0.0437	-52.50%
C18:1 Acid	**0.3326**	0.0536	**0.1125**	0.0764	-66.18%
C20 Acid	**0.0711**	0.0196	**0.0379**	0.0307	-46.69%
C22 Acid	0.1004	0.0286	0.0660	0.0349	-34.26%
C24 Acid	**0.0601**	0.0197	**0.0308**	0.0217	-48.75%
Subtotal	2.6083	0.7822	1.449	0.4038	-63.45%
2-Hydroxy-Acid					
C16 2-Hydroxy-Acid	0.0349	0.0041	0.0379	0.0388	8.60%
C22 2-Hydroxy-Acid	**0.1001**	0.0231	**0.0409**	0.0229	-59.14%
C23 2-Hydroxy-Acid	0.0159	0.0066	0.0179	0.0080	12.58%
C24 2-Hydroxy-Acid	**0.2683**	0.0418	**0.1042**	0.0645	-61.16%
C24:1 2-Hydroxy-Acid	**0.2008**	0.0407	**0.0664**	0.0566	-66.93%
C25 2-Hydroxy-Acid	0.0207	0.0036	0.0152	0.0128	-26.57%
C25:1 2-Hydroxy-Acid	**0.0196**	0.0039	**0.0089**	0.0048	-54.59%
C26 2-Hydroxy-Acid	**0.0920**	0.0167	**0.0335**	0.0221	-63.59%
C26:1 2-Hydroxy-Acid	**0.0470**	0.0126	**0.0152**	0.0092	-67.66%
Subtotal	0.7993	0.1531	0.3401	0.2397	-57.45%
ω-Hydroxy-Acid					
C16**ω**-Hydroxy-Acid	0.4353	0.0253	0.4747	0.0427	9.05%
Di-Acid					
C16 Di-Acid	0.8211	0.0700	0.8769	0.0924	6.80%
C18 Di-Acid	**0.1713**	0.0265	**0.1165**	0.0163	-31.99%

C18:1 Di-Acid	0.8491	0.1227	0.7663	0.1111	-9.75%
C18:2 Di-Acid	**0.8370**	0.1634	**0.6317**	0.0864	-24.53%
Subtotal	2.6785	0.3826	2.3914	0.3062	-10.72%
Mid-chain oxygenated					
C16-DiOH-Acid	4.0523	0.7430	4.1263	0.3323	1.83%
C16-OH-Di-Acid	0.7924	0.0931	0.8323	0.1104	5.04%
Subtotal	4.8447	0.8361	4.9586	0.4427	2.35%
Un-identified					
U.I.1	**0.0479**	0.0132	**0.0455**	0.0113	-5.01%
U.I.2	**0.5280**	0.1142	**0.4009**	0.0445	-24.07%
Subtotal	0.5759	0.1274	0.4464	0.0558	-22.49%
Total	**11.942**	2.3067	**10.060**	1.4909	-15.75%

1)* Data presented here is in dry weight (DW) μg/mg and represents the mean of five replicates;

2) $P < 0.01$ highlighted with bold font letter.

Supplemental Table 3. List of Primers Used in This Study.

Product Name	Primers	Used for
At3g15400	Forward: 5'-ATGGGACACTTTCTTTA-3' Reverse: 5'-CCTTATTACGATTATTTG-3'	qChIP-PCR
At4g14080	Forward: 5'-TATGAGTGGTCGTCTAA-3' Reverse: 5'- TTCTTTTGTGCTATGAT-3'	qChIP-PCR
At3g52160	Forward: 5'- ACGGAGATGATGGATTT-3' Reverse: 5'- TCCATTTTCACTAAGCA-3'	qChIP-PCR /EMSA
At1g71160	Forward: 5'- CAACAGACACGTAATGT-3' Reverse: 5'- TGAAAAGTGATAAAGGT-3'	qChIP-PCR
At1g01280	Forward: 5'-TAATCGACAAGTCAGAT-3' Reverse: 5'- GTAACCCGAGTTCAAGT-3'	qChIP-PCR /EMSA
At1g69500	Forward: 5'- GTTCGCTGGCTCATTTG-3' Reverse: 5'- TGTTTTATTAGTTCGTA-3'	qChIP-PCR
At1g74550	Forward: 5'- AACATCATCGTCCTTGG-3' Reverse: 5'- CTTTTCTCACACTATTTG-3'	qChIP-PCR
At1g22015	Forward: 5'- ACCAAACCAATCACAAA-3' Reverse: 5'-AGTTTAGGAAAAGTCTC-3'	qChIP-PCR
At1g33430	Forward-1: 5'- TCCGTATGGTCATTAGT-3' Reverse-1: 5'- TTCCTTTATTTCGCAAC-3'	qChIP-PCR
At1g33430	Forward-2: 5'-ATAACGTTCTTGATTCAGA-3' Reverse-2: 5'-AAGCTGGCTAGACAAAGC-3'	qChIP-PCR
At5g53190	Forward: 5'- TGATAACTCCAAGAACT-3' Reverse: 5'- AAAGAATACAATACCCA-3'	qChIP-PCR
At5g62080	Forward: 5'- TCAGCGTGGTGGATAGA-3' Reverse: 5'- AACGATGCCATTATTGC-3'	qChIP-PCR /EMSA
AT3G51590	Forward: 5'-CATTTTCCTTTGATGTCTCC-3' Reverse: 5'-GTAAATTGGACTGTCCTTGT-3'	qChIP-PCR
At1g74540	Forward: 5'-CCTTGTCGCTATCCTTTA-3' Reverse: 5'-GTGCTTTAGTTCTCATGTTGGA-3'	qChIP-PCR
At3g13220	Forward: 5'-GCTTTGATCTCGTTTATCTGTA-3'	qChIP-PCR

	Reverse: 5’-ATTGTTATGAATGTAGGCTTGT-3’	
AT1G75910	Forward: 5’- TTGTCTACATTTCGTCAG-3’ Reverse: 5’- TTAATTAGTGGTGCTTCT-3’	qChIP-PCR
AT1G75790	Forward: 5’- AGTAATAGCGACCATCT-3’ Reverse: 5’- ATTGTTACTCTGTTTGGA-3’	qChIP-PCR
AT1G75940	Forward: 5’- CTCCTACGACATTCCCATA-3’ Reverse: 5’- ACCGAGGAACAGAACTAAA-3’	qChIP-PCR
AT1G75930	Forward: 5’- GTCACGCTTCTGCTCAC-3’ Reverse: 5’- CTTATTTGGTCTTTCCTTTTTC-3’	qChIP-PCR
AT1G75920	Forward: 5’- GAGGCGGAGCGTATTTTC-3’ Reverse: 5’- ATGGACGGGATGTTGTG-3	qChIP-PCR
AT1G06990	Forward: 5’- AACTCAGGCGTCAGCAA-3’ Reverse: 5’- TAATCCCCTTGAACTGT-3’	qChIP-PCR
AT1G62940	Forward: 5’- CACGTGAAATCGAATAATCCA-3’ Reverse: 5’- TGTCATGTGTGTGCAAACCA-3’	qChIP-PCR
AT1G79780	Forward: 5’- GCCGAGTGGACGACCTA-3’ Reverse: 5’- ACAATCAGCGTGCCTCA-3’	qChIP-PCR
AT1G30020	Forward: 5’- TAAAGCCCATTAACTGA-3’ Reverse: 5’- AAGTTTCTATGGGTCTA-3’	qChIP-PCR
AT1G67990	Forward: 5’- AAATAAACACCTTTCAGC-3’ Reverse: 5’- CCTTTGTCAGGTAATCG-3’	qChIP-PCR
AT1G68875	Forward: 5’- GGCTTAGTTTGGGCTTTA-3’ Reverse: 5’- ATGCTTTTGTCATTAGTCAGG-3’	qChIP-PCR
AT1G13140	Forward: 5’- TGCAACAAAAGTGGAG-3’ Reverse: 5’- CATCGGACAGACAAAG-3’	qChIP-PCR
AT1G61110	Forward: 5’- TGAGGTATGTGCGTGATG-3’ Reverse: 5’- ATGCGAGAAGAAATAGGTG-3’	qChIP-PCR
AT1G06250	Forward: 5’- CTCTGAGGCGGTCTAATC-3’ Reverse: 5’- TAAACCTTTCTGGCACTG-3’	qChIP-PCR
AT1G02813	Forward: 5’- GGTTACATGGCCTGGTTA-3’ Reverse: 5’- GAACGGCTGAGATTTGA-3’	qChIP-PCR
AT1G26710	Forward: 5’- GGAAACCCGTACAACTAA-3’ Reverse: 5’- CACAAAGATGTTGGAAGC-3’	qChIP-PCR
AT1G61070	Forward: 5’- GTCTTCATTTAGCACCTTC-3’ Reverse: 5’- TTTGGTTCTTGCTCTATTC-3’	qChIP-PCR

AT1G66850	Forward-1: 5’- AACTTAAGCAATTGTACACA-3’ Reverse-1: 5’- TCCTTCTCCTCTTCCG-3’	qChIP-PCR
AT1G66850	Forward-2: 5’- TATTGGGCTATTGTAAAC-3’ Reverse-2: 5’- GCATTCCATAACGCAA-3’	qChIP-PCR
AT1G28375	Forward-1: 5’- ATACGGATAGTCTTGGTG-3’ Reverse-1: 5’- AATCGGACCATAAAAGCA-3’	qChIP-PCR
AT1G28375	Forward-2: 5’- CTTTTATGGTCCGATTG-3’ Reverse-2: 5’- GGTTGCATGTACTTGGTTG -3’	qChIP-PCR
AT2G19070	Forward: 5’- CTAAACCACGAGAAAA-3’ Reverse: 5’- GCTAAGGGAAACCTACCG-3’	qChIP-PCR
AT3G11980	Forward: 5’- AACACGAAGAAATGAGA-3’ Reverse: 5’- GTAAACCAAGAACAGAA-3’	qChIP-PCR
AT3G23770	Forward: 5’- AACCGTCGTAACTATTC-3’ Reverse: 5’- AGTGCTTTGGTGTTTC-3’	qChIP-PCR
AT3G42960	Forward: 5’- ATAGCCGTCAAATAGTC-3’ Reverse: 5’- TGCTCTTTACCTTTCC-3’	qChIP-PCR
AT4G34850	Forward: 5’- CTGCCAAACTTGATGAG-3’ Reverse: 5’- TCAATAGAAAGACAGCA-3’	qChIP-PCR
AT4G29250	Forward: 5’- AATAAGGTGCCCAGTAAA-3’ Reverse: 5’- GTTCATGAAAACCCCTA -3’	qChIP-PCR
AT4G35420	Forward-1: 5’- GAGTCGCAAAGTCCAG-3’ Reverse-1: 5’- AATGCAGCATCTACACC-3’	qChIP-PCR
AT4G35420	Forward-2: 5’- CCTGAAACCAAAGATATG-3’ Reverse-2: 5’- AAATAGTGGACAAGGA-3’	qChIP-PCR
AT4G28395	Forward: 5’- TTAAGAAGCAAGAAAGA-3’ Reverse: 5’- CGTTTCTAAGGTGGAG-3’	qChIP-PCR
AT4G14815	Forward: 5’- AAGTAAGTGTTTGGTG-3’ Reverse: 5’- GACGAAAATAAGGATGTG-3’	qChIP-PCR
AT4G20050	Forward: 5’- TCCAACTTCCGCATAC-3’ Reverse: 5’- GAGAATGGCTGAAGGTA-3’	qChIP-PCR
AT5G60500	Forward: 5’- AATCGGAGAAATTAAAA-3’ Reverse: 5’- TCTTCTAGGGTATTCA-3’	qChIP-PCR
AT5G49070	Forward: 5’- ACCGGACTCGCAGTGTAA-3’ Reverse: 5’- CATTATGGTGGCTTTTAC-3’	qChIP-PCR
AT5G16920	Forward: 5’- ATGACTCAACGTGACAAC-3’	qChIP-PCR

	Reverse: 5’- TAGATCACCAAAACATA-3’	
AT5G13380	Forward: 5’- AGATAAACCCGCAGTC-3’ Reverse: 5’- ATGAGTTTCCGACAGG-3’	qChIP-PCR
AT5G07230	Forward: 5’- ATTGTTGTCGTGGTTG-3’ Reverse: 5’- AATGCTTGATTAGTGG-3’	qChIP-PCR
AT5G07550	Forward: 5’- ATAAAAGGAGTAAAGGACA-3’ Reverse: 5’- TTAGTTTATGATTGTGGC-3’	qChIP-PCR
AT5G07520	Forward: 5’- TTTGGGGCTAATACTA-3’ Reverse: 5’- ATGAGAAGGCCGAGCA-3’	qChIP-PCR
Actin7F	Forward: 5’-TGGCCGATGGTGAGGATATT-3’	qRT-PCR
Actin7R	Reverse: 5’-AACGGCCTGAATGGCAACAT-3’	
AMS situ F	Reverse: 5’-aaGGATCCATCTTCCAGCTTCCATACCT-3’	In Situ
AMS situ R	Reverse: 5’ -aaGAATTCATACCCATCATTTCCTTGT-3’	
At5g62080si- F	Reverse: 5’ -aaGGATCCTAGCAAATGTGCAGGTATG-3’	In Situ
At5g62080 si-R	Reverse:5’-aaGAATTCGTTAGCTTGGCAACAATGAA-3’	
At3g52160 si-F	Reverse:5’-aaGGATCCAGATGGTGAATGGTGGAGTT-3’	In Situ
At3g52160 si-R	Reverse:5’-aaGAATTCACGAAGATTGACCCTGTGA-3’	
Salk_LBb1	Forward:5’-GCGTGGACCGCTTGCTGCAACT-3’	*ams* genotyping
DBAMS 1F	Reverse:5’-GAGTAATATGCAAAACTTGTTGG-3’	
DBAMS-7R	Reverse:5’-TGACTTCTTCTTGTACTTTGGATC-3’	
Salk_LBb2	Reverse:5’-AAGAGCTGAGCGAAGAGACGGTTGGG-3’	*cyp98a8* genotyping
DBCYP98A8-F	Reverse:5’-TCGGAAATTGTTGGTGTTAAAAGA -3’	
DBCYP98A8-R	Reverse:5’-GCACCCGCGGTTAACATG -3’	
CYP98A8 RT-F	Reverse: 5’-GCCATCGTTGAGAAAGAACATCTT-3’	qRT-PCR
CYP98A8 RT-R	Reverse: 5’-AACCCATTTGAGCCACCAGAC-3’	
CYP98A9 RT-F	Reverse: 5’-CACCGTTTGGCTTAAACGTCTT -3’	qRT-PCR
CYP98A9 RT-R	Reverse: 5’-CCGAGCCATGTGCTTCAT -3’	