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AtMYB103 is a crucial regulator of several pathways affecting *Arabidopsis* anther development

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Previous reports indicated that *AtMYB103* has an important role in tapetum development, callose dissolution, and exine formation in *A. thaliana* anthers. Here, we further characterized its function in anther development by expression pattern analysis, transmission electron microscopy observation of the knockout mutant, and microarray analysis of downstream genes. A total of 818 genes differentially expressed between *ms188* and the wild-type were identified by global expression profiling analysis. Functional classification showed that loss-of-function of *AtMYB103* impairs cell wall modification, lipid metabolic pathways, and signal transduction throughout anther development. RNA *in situ* hybridization confirmed that transcription factors acting downstream of *AtMYB103* (At1g06280 and At1g02040) were expressed in the tapetum and microspores at later stages, suggesting that they might have important roles in microsporogenesis. These results indicated that *AtMYB103* is a crucial regulator of *Arabidopsis* anther development.

***Arabidopsis*, *AtMYB103*, tapetum development, transcription factor, microarray**

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In flowering plants, anther development is divided into two distinct phases, termed microsporogenesis and microgametogenesis [1]. During microsporogenesis in *Arabidopsis*, stamen primordia cell-specification and differentiation occur and the morphology of the anther and the filament is established [2]. In microgametogenesis, the microspores gradually mature and then undergo an asymmetric mitosis to become a multicellular male gametophyte [3].

Pollen development depends on the normal function of haploid gametophytic cells, as well as the support of accessory diploid sporophytic cells, especially tapetal cells. The tapetum, which arises from secondary parietal cells and connective tissues, surrounds the developing reproductive cells and generates many proteins, lipids, polysaccharides, and other molecules to support pollen development [4,5]. Early cytological observation showed that tapetal cell protoplasts contain dense cytoplasm, two nuclei, and numerous vacuoles, reflecting the high metabolic activity of the tapetum [6]. Meiocytes and tapetal cells undergo active cell differentiation at an early stage of anther development, which depends on signal recognition by several leucine-rich repeat receptor-like protein kinases (LRR-RLKs) [7–9]. In addition, successful pollen wall development, especially exine wall formation, requires precise coordination of the microspore and tapetum. After the release of microspores from tetrads, the majority of exine components are derived

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from the tapetum and deposit on the microspores to become the sculptured exine wall [5,10]. Therefore, the tapetum plays critical roles in anther development.

Several transcription factors essential for tapetum development and function have been reported. *DYSFUNCTIONAL TAPETUM1* (*DYT1*) [11], *DEFECTIVE IN TAPETAL DEVELOPMENT AND FUNCTION1* (*TDF1*) [12], *ABORTED MICROSPORES* (*AMS*) [13], *AtMYB103* [14], and *MALE STERILITY 1* (*MS1*) [15,16] have been shown to be important for tapetal differentiation and function, and to participate in a gene regulatory network. Among them, *DYT1*, *TDF1*, and *AMS* are highly expressed in the tapetal layer at early stages and regulate tapetal differentiation. However, *AtMYB103* and *MS1* transcripts accumulate in the tapetum after meiosis (unpublished data). We previously isolated the male sterile *ms188* mutant and characterized the *AtMYB103* gene using a map-based cloning strategy. Morphological observation showed that the tapetum cell wall and protoplast degradation of *ms188* seems to be disturbed, and the callose dissolution is also altered. Most microspores collapse at the later stages, and surviving microspores are completely devoid of exine. Additionally, the expression of the callase-related gene *A6* and the exine synthesis gene *MS2* were downregulated in the *ms188* mutant [14]. Nevertheless, the metabolic pathways and candidate regulators for these biological processes are still not clear.

In the current study, we identified that *AtMYB103* is strongly expressed in tapetal cells during anther development. Knockout of *AtMYB103* leads to disrupted development and function of tapetal cells. In addition, a comparison of the gene expression profile of wild-type flower buds with that of the *ms188* mutant showed that several important metabolic and signaling pathways are blocked by loss-of-function of *AtMYB103*. These results indicated that *AtMYB103* acts as a crucial regulator to control anther development in *Arabidopsis*.

1 Materials and methods

1.1 Plant material

Arabidopsis mutant *ms188-1* (from the wild-type Landsberg *erecta*, Ler) was selected using an ethyl methane sulfonate (EMS) mutagenesis strategy as previously described [14]. Seeds were sown on vermiculite and allowed to vernalize for 3 d at 4°C. Plants were grown at 22°C under a 16-h-light/8-h-dark photoperiod.

1.2 Cytological analysis

For ultrastructure analysis, *Arabidopsis* buds from inflorescences were removed from Ler and *ms188* mutant plants, and fixed and embedded as previously described [14]. Ultrathin sections (50–70 nm thick) were observed with a Jeol JEM-1010 transmission electron microscope (TEM).

1.3 RNA extraction and real-time RT-PCR

Total RNA was isolated from floral tissue of mature soil-grown Arabidopsis plants using the Trizol reagent (Invitrogen, USA). First strand cDNA was synthesized from 5 μg of RNA using a poly-dT (12–18) primer, MMLV reverse transcriptase, and accompanying reagents, for 60 min at 42°C. The synthesized cDNAs were used as PCR templates. Quantitative real-time PCR was performed on an ABI PRISM 7300 detection system (Applied Biosystems, USA) using SYBR Green I master mix (Toyobo, Japan). Real-time PCR cycling parameters were 95°C for 5 min followed by 40 cycles of denaturation for 10 s at 94°C, and annealing and extension for 1 min at 60°C. The primers for real-time PCR are listed in Table S5 in the supplemental materials in the electronic version. β-Tubulin was used as a constitutive expression control.

1.4 Microarray analysis

Closed buds were collected from wild-type and *ms188* mutant plants and immediately frozen in liquid nitrogen. The collected tissues were stored at −80°C until the extraction of total RNA. The opened flowers and buds were discarded. Three replicates of independently grown materials were used. Total RNAs were isolated using the TRIzol reagent (Invitrogen, USA). This RNA was further purified using an RNeasy Mini kit (Qiagen, USA) and used as a template for cDNA synthesis. Cy3 and Cy5 Labeled cRNA were synthesized from 1 μg total RNA using Low RNA Input Linear Amplification and Labeling kit plus (Agilent, 5184–3523) and hybridized onto a 44 K *Arabidopsis* oligo microarray using the Gene Expression Hybridization kit (Agilent, 5188–5242). Signals were detected according to the manufacturer's protocol. The intensity of each spot was scanned sequentially for Cy3- and Cy5-labeled probes with a laser scanner (Agilent, G2565BA), at a resolution of 5 μm and a PMT of 100. The Feature Extraction software was used to normalize the overall intensity of the hybridized slide. Only those spots that displayed an expression ratio (*ms188*/wild-type) of <0.5 (for downregulated genes) and >2 (for upregulated genes), a Signal/ Noise value >2.6, and a pValueLogRatio <0.05 in three independent experiments were selected. The value quoted for gene expression was the average of the three independent experiments.

1.5 Categorization of expression patterns

The differentially expressed genes were classified according to their expression levels in by the 'e-fp' Browser. These genes were assigned as bud preferential (BP), which showed at least three-fold higher expression in early stage

buds compared with the highest level in other tissues; bud expressed (BE) if genes showed higher expression but less than three-fold. Similarly, late anther preferential (LAP) and pollen preferential (PP) mean that genes showed at least three-fold higher expression in late anthers (after stage 9) or in pollen grains relative to any other tissues. The expression of late anther expressed (LAE) and pollen expressed (PE) genes was higher but less than three-fold. Bud and late anther expressed (BAE) genes showed similar expression in buds and anthers (signal ratio >0.8), late anther and pollen expressed (APE) were similarly categorized.

1.6 RNA *in situ* hybridization

Non-radioactive RNA *in situ* hybridization was performed with the DIG (for digoxigenin) RNA labeling kit (Roche) and with the PCR DIG Probe Synthesis Kit (Roche, USA). Specific cDNA fragments of diverse genes were amplified using the primers listed in Table S6 in the supplemental materials in the electronic version. The PCR products were cloned into the pSK vector and confirmed by sequencing. Antisense and sense digoxigenin-labeled probes were prepared by *Eco*R I or *Bam*H I digestion and *in vitro* transcription using T3 or T7 RNA polymerase, respectively. Images were obtained using the Olympus BX-51 microscope.

2 Results and discussion

2.1 *AtMYB103* is strongly expressed in microsporocytes and the tapetum during anther development

A previous study showed that *AtMYB103* was expressed in the tapetum and middle layer of developing anthers [17]. To understand the detailed expression pattern of *AtMYB103*, quantitative real-time PCR analysis of total RNA extracted from various wild-type organs including the root, stem, leaves, inflorescence, and 7-day-old seedlings was performed. The results showed that *AtMYB103* is preferentially expressed in inflorescences and is slightly expressed in roots (approximately 11%) (Figure 1A). However, the expression of *AtMYB103* was barely detected in the stem, leaf, and seedlings (approximately 1%). Furthermore, RNA *in situ* hybridization experiments were performed to precisely delineate *AtMYB103* expression in anther. In *Arabidopsis*, anther development can be divided into 14 stages morphologically [2]. The *AtMYB103* transcript was initially detected in sporogenous cells and the tapetum at stage 4 (Figure 1B), and gradually increased in tapetal cells and microsporocytes (or meiocytes) at stages 5 and 6 (Figures 1C and D). Meanwhile, *AtMYB103* expression was barely detectable in other somatic tissues in anther, including the epidermis and endothecium. At the tetrad stage, *AtMYB103* transcripts were predominantly detected in tapetal cells (Figure 1E). During the microspore stage, the expression of

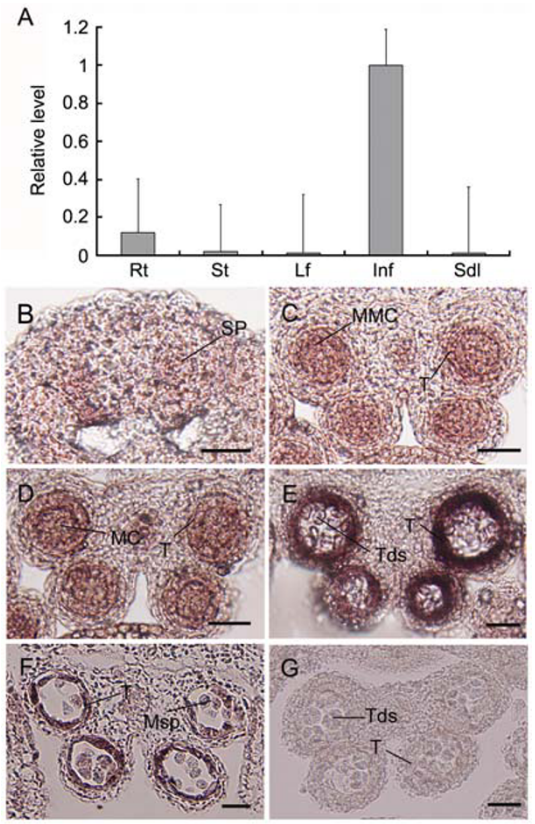

Figure 1 Expression analysis of *AtMYB103*. (A) Quantitative RT-PCR of RNA isolated from various tissues with *AtMYB103*-specific primer sets. Rt, root; St, stem; Lf, leaf; Inf, Inflorescence; Sl, seedling. (B)–(F) RNA *in situ* hybridization with an *AtMYB103* antisense probe. Anthers at stage 4 (B), stage 5 (C), stage 6 (D), stage 7 (E), and stage 9 (F). (G) *In situ* hybridization of *AtMYB103* transcripts within a tetrad stage anther using a sense probe. SP, sporocytes; MMC, Microspore mother cells; MC, meiosis cells; Msp, microspores; T, tapetum; Tds, tetrads. Bars=20 μm.

AtMYB103 was still detectable in the tapetum and microspores, but was reduced and compared with that at the tetrad stage (Figure 1F). In previous work, our results demonstrated that loss-of-function of *AtMYB103* altered tapetum development and pollen wall synthesis from tetrad stage in *Arabidopsis* [14]. Therefore, the expression pattern indicated that the *AtMYB103* plays its major role since the tetrad stage of anther development.

2.2 Defects in tapetal formation and secretion leads to aberrant exine formation in the *ms188* anther

To further determine the effects of *AtMYB103* on tapetal development and secretion of pollen wall materials, the anther sections of wild-type and *ms188* plants were analyzed by TEM. Consistent with previous observations, at early stages, no significant morphological differences were observed in anther wall layers and microspores between the wild-type and the mutant (data not shown). In wild-type anthers, the tapetal cells become polar secretory-type with a spongy shape at the tetrad stage (Figures 2A and C). The

tapetal cell protoplasts, which contain numerous vesicles, mitochondria, Golgi-bodies, and layers of rough endoplasmic reticulum (ER), appear to be highly active. In addition, cell wall degradation of tapetum has been initiated (Figure 2C). In *ms188*, however, the tapetal cytoplasm has a wider shape and is full of large vacuoles and the tapetal cell walls appear intact at this stage (Figures 2B and D), indicating that the transition of the tapetum to a secretory-type in *ms188* is affected. In the wild-type anther, once the microspores are released from the callose wall, clusters of small vesicles containing fibrillar material appear throughout the tapetal cytoplasm, some of which appear to fuse with the tapetal cell membranes and release fibrillar material (Figure 2E, arrows). By contrast, the cytoplasm of *ms188* contains several irregular enlarged vacuoles, but not the small vesicles (Figure 2F). At the late stage of microgametogenesis, lipid droplets are released into the locule in wild-type anthers, which serve as raw materials of the exine (Figure 2G). However, the protoplasts of mutant tapetal cells are almost degenerated at this stage (Figure 2H). Finally, the majority of microspores collapse and few defective microspores without exine layers survive in the locule of *ms188* [14]. Several previous reports showed that the primexine matrix, which is secreted by microsporocytes, acts as the footprint of the exine layer [18-20]. TEM observation demonstrated that the primexine matrix was regularly deposited outside the microspore plasma membrane in *ms188*, similar to that in the wild-type (Figures 2I and J). Therefore, the aberrant exine formation of *ms188* is due to the aborted tapetum development and secretory function, rather than the primexine formation.

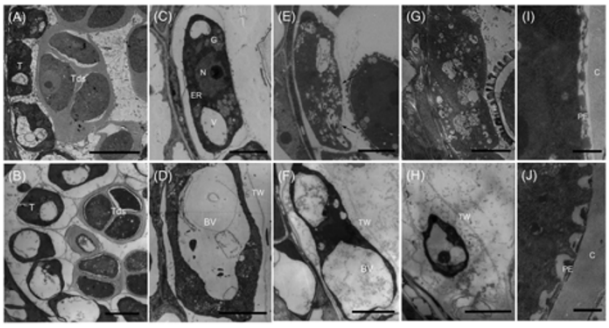

Figure 2 Ultrastructure of tapetum development and primexine deposition in wild-type ((A), (C), (E), (G) and (I)) and *ms188* ((B), (D), (F), (H) and (J)) plants. (A) and (B) Anther at the tetrad stage. Bars=10 μm. (C) and (D) Tapetum at the tetrad stage. The wild-type tapetum showed a spongy shape with numerous large and small vesicles, whereas the tapetal cell in *ms188* showed hypertrophy, with irregular large vacuoles. (E) and (F) Stage 8. Small vesicles containing fibrillar material occurred in the wild-type tapetum, but were not present in that of *ms188*. The black arrows indicate plastid inclusions and vesicles fused to the membrane for releasing material. (G) and (H) Tapetal cells at stage 9. The tapetal cytoplasm of *ms188* was almost degraded. (C)–(H) Bars=5 μm. (I) and (J) The primexine deposition of *ms188* is similar to that in wild-type plants. Bars=2 μm. T, tapetum; Tds, Tetrads; N Ms, microspore; ER, endoplasmic reticulum; G, Golgi-bodies; V, vesicles; BV, big vacuoles; N, nucleus; Pe, primexine; C, callose.

2.3 Microarray analysis of *AtMYB103*-dependent genes in flower development

To further reveal the regulatory role of *AtMYB103* during anther development, we performed transcript profiling of the *ms188* mutant. It is difficult to isolate *Arabidopsis* anthers at an early stage; therefore, we collected the closed buds and extracted RNAs for hybridization with three biological replicates from independently grown populations of wild-type plants and mutants, respectively. To focus on genes with a substantial extent of differential expression, only those exhibiting an expression ratio (*ms188*/wild-type) of <0.5 (for downregulated genes) and >2 (for upregulated genes) in mutant buds were selected for further analysis. A total of 1086 genes were selected and 818 (725 downregulated and 93 upregulated; see supplementary Tables S1 and S2 in the electronic version) of them were identified to be expressed in flower organs. These genes were classified into nine groups according to the 'Electronic Fluorescent Pictograph' (e-fp) Browser (several genes based on the genevestigator database) [21] (see materials and methods). In total, 42 genes were identified as bud preferential, 25 bud expressed, three bud and late anther expressed, 18 late anther preferential, 92 late anther expressed, 42 late anther and pollen expressed, 142 pollen expressed, and 263 pollen preferential. Additionally, 191 genes that were not detected in the 'e-fp' data sets were classified as not detected (Table 1). *AtMYB103* is strongly expressed from stage 6 to stage 8, with the highest expression level occurring at the tetrad stage (Figure 1), indicating that *AtMYB103* mostly functions during these stages. Thus, the differentially expressed genes of bud preferential, bud expressed, bud and late anther expressed (including some not detected genes) might be regulated by *AtMYB103*.

So far, dozens of genes involved in anther or pollen development have been characterized in *Arabidopsis*. Sixteen of these genes were significantly downregulated and one of them was upregulated in this experiment (Table 2). To verify the expression data from the microarray hybridization, we selected thirteen differentially expressed genes for quan-

Table 1 Expression location of differentially expressed genes in *ms188*

Cluster[a)]	Down	Up	Total
Bud preferential (BP)	35	7	42
Bud expressed (BE)	14	11	25
Bud and late anther expressed (BAE)	2	1	3
Late anther preferential (LAP)	18	0	18
Late anther expressed (LAE)	87	5	92
Late anther and pollen expressed (APE)	42	0	42
Pollen preferential (PP)	258	5	263
Pollen expressed (PE)	119	23	142
Not detected (ND)	150	41	191
Total	725	93	818

a) Expression location for differentially expressed genes was derived from *Arabidopsis* eFP Browser (Winter *et al*., 2007), classified as described in section 2.3.

titative RT-PCR analysis. These genes showed high fold changes and functional relevance, and included transcription factors (At1g77980, At2g41240 and At5g62320), callose synthase (At2g13680), glycosyl hydrolase (At3g23770), tapetum specific genes (At4g20420 and At4g28395), lipid metabolism (At2g19010, At3g51590, At4g11030), cytochrome P450 (At1g01280), chalcone synthesis (At4g34850), and an auxin-responsive gene (At5g13380). All RT-PCR data were consistent with the fold changes in the microarray experiments (Figure 3).

2.4 Functional classification of differentially expressed genes

The 818 differentially expressed genes selected in the microarray analysis were categorized into 24 functional groups according to MAPMAN tools [22], with manual adjustment when necessary (Table 3; for details, see supplementary Tables S3 and S4 in the electronic version). Because the number of down-regulated genes represents the overwhelming majority of differentially expressed genes, the group of down-regulated genes was selected for further analysis. Genes involved in cell wall synthesis and degradation (7.4% compared to approximately 2% among genes represented by in the microarray), lipid metabolism (2.9% relative to 1.5%), and signal transduction (10.3% compared with 3.9%) were over-represented in the down-regulated genes of *ms188* flower buds. These data indicated that *AtMYB103* might have a crucial role in regulating these pathways. Whereas genes related to DNA metabolism (0.69%), amino acid metabolism (0.41%), and glycolysis (0.14%) were under-represented, implying that *AtMYB103* has less of a function in regulating these vegetative pathways.

Table 2 Characterization of differentially expressed genes detected in this study

Gene name	Locus[a)]	Description	Expression/ localization	Fold change[b)] in *ms188* buds
CYP703A2	At1g01280	cytochrome P450 family protein	BP	–6.334
MS1	At5g22260	male sterility 1 protein	ND	–6.417
MYB99	At5g62320	myb family transcription factor	BP	–2.898
DUO1	At3g60460	myb family transcription factor	ND	–2.334
A9	At5g07230	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein tapetum-specific protein A9	BP	1.277
A6	At4g14080	glycosyl hydrolase family 17 protein/anther-specific protein (A6)	BP	–1.884
MS2	At3g11980	male sterility protein 2	BP	–5.887
AtPTEN1	AT5G39400	pollen specific phosphatase	PP	–2.388
ATA1	At3g42960	alcohol dehydrogenase (ATA1)	BP	–1.948
ATA7	At4g28395	lipid transfer protein/anther-specific gene ATA7	BP	–3.492
AGP11	At3g01700	arabinogalactan-protein (AGP11)	PP	–2.491
AGP6	At5g14380	hydroxyproline-rich glycoprotein family protein (AGP6)	PP	–3.839
AGL66	At1g77980	MADS-box family protein (AGL66)	PE	–2.001
AGL104	At1g22130	MADS-box family protein (AGL104)	PE	–2.325
	At2g40850	phosphatidylinositol 3- and 4-kinase	ND	–3.699
PIP5K5	At2g41210	phosphatidylinositol-4-phosphate 5-kinase	PE	–1.317
CalS5	At2g13680	1,3-beta-glucan synthase	PP	–1.841

a) Gene identification number; b) $\log_2$-fold difference of the mean expression ratios of three replicates for microarray assays.

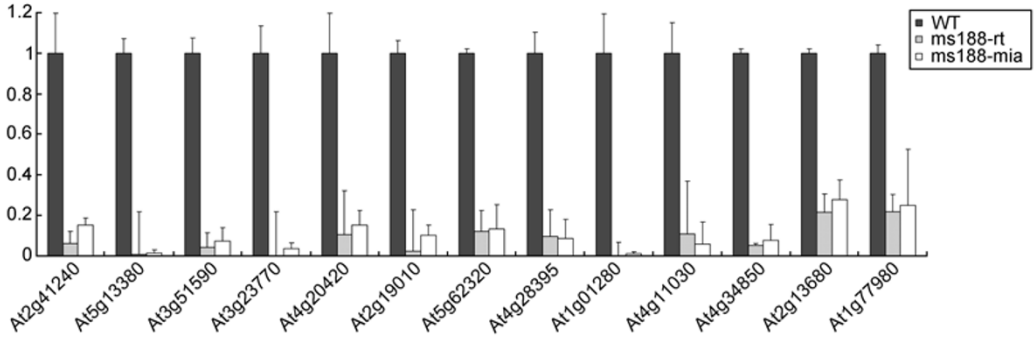

Figure 3 Quantitative RT-PCR analysis of selected genes in wild-type and the *ms188* flower buds. WT, expression of genes in the wild type; ms188-rt, expression of genes in *ms188* by real-time PCR; ms188-mia, expression of genes in the microarray experiment. β-tubulin served as a control.

Table 3 Functional classification of differentially expressed genes in *ms188*

Function	Down	Up	Total
CHO metabolism	9	3	12
Glycolysis	1	0	1
Glyoxylate cycle	1	0	1
TCA	4	0	4
Mitochondrial electron transport	1	0	1
Cell wall	52	0	52
Lipid metabolism	22	1	23
Amino acid metabolism	3	1	4
Metal handling	3	0	3
Secondary metabolism	15	2	17
Hormone metabolism	14	4	18
Stress response	26	3	29
Redox	7	0	7
Nucleotide metabolism	2	1	3
Biodegradation of Xenobiotics	2	0	2
Miscellaneous enzymes families	55	12	67
RNA processing and transcription	42	9	51
DNA synthesis and repair	5	0	5
Protein metabolism	55	22	77
Signal transduction	75	1	76
Cell	24	0	24
Development	16	1	17
Transport	51	4	55
Function unknown	240	29	269
Total	725	93	818

2.5 Expression of several kinase genes is affected by *AtMYB103* mutation

A total of 46 kinase genes were down-regulated in *ms188* flower buds (Table 4). Twenty-eight of these genes encode receptor-like kinases (RLKs), including nine LRR-RLKs, nine protein kinases, five calcium-dependent protein kinases, and four phosphatidylinositol phosphate (PIP) kinases. RLKs have been identified in various plant species and are suggested to have diverse roles in plant life cycles [23]. Leucine-rich repeat receptor-like kinases (LRR-RLKs) are the largest group of RLKs, with more than 200 members in *Arabidopsis* [24]. All the suppressed LRR-RLKs in *ms188* are pollen expressed or pollen preferential (Table 4). On the other hand, the expression of LRR-RLKs reported to function at the early stage of anther development [7-9] was not affected in *ms188*. This suggested that AtMYB103 indirectly regulates the signal transduction of anther development at the late stage. Calcium was shown to have a critical role in modulation of pollen tube growth and guidance [25–27], and calcium-dependent protein kinases and PIP kinases take part in Ca^{2+} signaling pathways in pollen tube growth [28,29]. Five calcium-dependent protein kinases and four PIP genes were identified among the downregulated genes in *ms188*. Most of them belong to pollen preferential or pollen expressed groups. One of them (PIP5K5) has an important role in pollen germination and pollen tube growth [30]. This suggested that these genes were involved with the signal transduction pathway of pollen germination or pollen tube growth. Because most of the *ms188* microspores undergo premature degradation before anther dehiscence, the calcium dependent signaling pathway in late pollen stages is probably suppressed by a secondary effect rather than by direct regulation by *AtMYB103*.

2.6 Cell wall modification related genes arrested by *AtMYB103* mutation

Cell wall structure is unique to plant cells, and the main components are cellulose and pectin [31]. In the *ms188* anther, the tapetal cell wall fails to degrade. Thus, a subset of down-regulated genes from diverse functional categories involved in cell wall formation was selected (Table 5). Twenty-four pectinase-related genes and two cellulose genes were suppressed in *ms188* (see Supplementary Table S3 in the electronic version). Among them, a cellulose family protein in the not detected group (At5g17500), a pectinase (At5g17200), and two pectate lyase family proteins (At4g22080 and At4g22090) in the bud preferential group, which were supposed to participate in the tapetal wall degradation, showed significant fold changes (Table 5). The investigation of these genes should help to understand tapetal cell wall degradation in anther development.

Exine formation contains several sequential processes, including lipid synthesis, sporopollenin biosynthesis, and lipid transport [5,32]. As the exine formation is defective in *ms188*, downregulated genes associated with these processes were selected. Twenty genes predicted to function in lipid metabolism and 15 genes related to secondary metabolism were identified (see Supplementary Table S3 in the electronic version). Among these genes, the anther-specific genes *MS2* and *ATA1* were reported to be involved with the conversion of fatty acids to fatty alcohol [33]. At3g52160, At5g49070, and At4g11030 are predicted to encode members of the fatty acid synthesis pathway, and At1g51260 and At3g11430 are predicted to encode acyltransferases. Two bud preferential expressed genes, At4g34850 and At1g02050, function in chalcone synthesis to produce the precursor of phenylpropanoid biosynthesis, and four genes encode cytochrome P450 enzymes that are supposed to synthesize the sporopollenin precursor [34]. Seven genes encode GDSL-like lipases catalyzing hydrolysis and synthesis of abundant ester compounds [35,36]. The above genes are all involved in lipid synthesis and sporopollenin biosynthesis, and they were suppressed in *ms188* (Table 5). In anther development, fatty acids and/or other sporopollenin precursors from the tapetum will be transferred to the microspore surface during exine deposition [37,38]. Five genes putatively related to lipid transfer were found to be downregulated. Two of them were bud preferential and other two members were late anther expressed. The microarray data illustrates that AtMYB103 is important for lipid synthesis, sporopollenin biosynthesis, and lipid transport progresses, which are essential for exine formation in anther development.

Table 4 Subset of down-regulated genes involved in kinase signal transduction

Locus[a)]	Description	Expression pattern	Fold change[b)] in *ms188* buds
At2g31500.1	calcium-dependent protein kinase	PP	–3.433
At2g38910.1	calcium-dependent protein kinase	PE	–2.906
At4g38230.1	calcium-dependent protein kinase	PP	–1.523
At5g12180.1	calcium-dependent protein kinase	PP	–3.919
At5g19360.1	calcium-dependent protein kinase	PP	–3.471
At4g20790.1	leucine-rich repeat transmembrane protein kinase	PE	–2.641
At1g50610.1	leucine-rich repeat transmembrane protein kinase	PP	–3.138
At1g72460.1	leucine-rich repeat transmembrane protein kinase	PE	–3.673
At1g78980.1	leucine-rich repeat transmembrane protein kinase	PE	–2.457
At2g07040.1	leucine-rich repeat transmembrane protein kinase	PP	–4.214
At3g13065.1	leucine-rich repeat transmembrane protein kinase	PP	–2.263
At3g42880.1	leucine-rich repeat transmembrane protein kinase	PP	–3.739
At5g20690.1	leucine-rich repeat transmembrane protein kinase	PP	–2.411
At5g45840.1	leucine-rich repeat transmembrane protein kinase	PP	–1.576
At2g40850.1	phosphatidylinositol 3- and 4-kinase family protein	ND	–3.699
At3g56600.1	phosphatidylinositol 3- and 4-kinase family protein	PP	–4.265
At1g01460.1	phosphatidylinositol-4-phosphate 5-kinase family protein	PP	–1.631
At2g41210.1	phosphatidylinositol-4-phosphate 5-kinase family protein	PE	–1.317
At4g20670.1	pollen coat receptor kinase	ND	–3.204
At1g49270.1	protein kinase family protein	PP	–3.267
At2g18470.1	protein kinase family protein	PP	–3.444
At3g04690.1	protein kinase family protein	PP	–2.738
At3g18810.1	protein kinase family protein	PP	–3.563
At4g28670.1	protein kinase family protein	PP	–2.199
At4g34440.1	protein kinase family protein	PP	–2.817
At4g39110.1	protein kinase family protein	PP	–3.339
At5g28680.1	protein kinase family protein	PP	–2.567
At3g21940.1	receptor protein kinase-related	ND	–3.770
At2g31620.1	receptor-like protein kinase-related	ND	–3.319
At3g21900.1	receptor-like protein kinase-related	ND	–3.141
At3g21910.1	receptor-like protein kinase-related	ND	–3.411
At3g21930.1	receptor-like protein kinase-related	LAP	–3.249
At3g21960.1	receptor-like protein kinase-related	LAE	–2.974
At3g21970.1	receptor-like protein kinase-related	LAE	–3.633
At3g21980.1	receptor-like protein kinase-related	ND	–2.759
At3g21990.1	receptor-like protein kinase-related	ND	–1.735
At3g22000.1	receptor-like protein kinase-related	LAE	–3.861
At3g22020.1	receptor-like protein kinase-related	LAE	–3.264
At3g22030.1	receptor-like protein kinase-related	ND	–3.682
At3g22050.1	receptor-like protein kinase-related	LAE	–3.913
At3g29040.1	receptor-like protein kinase-related	ND	–2.931
At3g29050.1	receptor-like protein kinase-related	ND	–3.724
At3g58310.1	receptor-like protein kinase-related	ND	–3.191
At4g20650.1	receptor-like protein kinase-related	PE	–2.295
At4g20680.1	receptor-like protein kinase-related	LAE	–4.331
At1g19090.1	serine/threonine protein kinase (RKF2)	PP	–2.486

a) Gene identification number; b) $\log_2$-fold difference of the mean expression ratios of three replicates for microarray assays.

2.7 Genes encoding transcription factors suppressed by *ms188*

Previous reports showed that transcription factors play crucial roles during anther development; therefore, we focused on genes encoding proteins with putative roles in transcriptional regulation. Among the downregulated genes in *ms188*, 27 genes coding for putative transcription factors (TFs) are identified according to the DATF database (http://datf.cbi.pku.edu.cn/) (Table 6). Among them, two MYB proteins (MYB99 and DUO1) control late tapetum development and male mitosis division [39,40]. MS1, a putative PHD-finger protein, which acts as a vital regulator for tapetum development and exine formation, is also suppressed in *ms188* [17,18]. MADS family TFs, AGL66 and AGL104, are reported to be involved in late stages of pollen

Table 5 Subset of cell wall modification enzymes arrested by *ms188*

Locus[a)]	Description	Expression pattern	Fold change[b)] in *ms188* buds
At5g17200.1	polygalacturonase (pectinase) family protein	BP	–3.373
At4g35670.1	polygalacturonase (pectinase) family protein	LAP	–4.328
At4g14080.1	glycosyl hydrolase family 17 protein	BP	–1.884
At3g23770.1	glycosyl hydrolase family 17 protein	BP	–4.737
At5g46940.1	pectin methylesterase inhibitor family protein	LAE	–2.545
At4g22080.1	pectate lyase family protein	BP	–3.139
At4g22090.1	pectate lyase family protein	BP	–1.990
At3g11980.1	male sterility protein 2 (MS2)	BP	–5.887
At1g51260.1	acyl-CoA:1-acylglycerol-3-phosphate acyltransferase	PP	–3.235
At3g52160.1	beta-ketoacyl-CoA synthase family protein	BP	–3.870
At5g49070.1	beta-ketoacyl-CoA synthase family protein	BP	–4.780
At4g11030.1	long-chain-fatty-acid--CoA ligase	LAE	–4.103
At4g04930.1	fatty acid desaturase family protein	PP	–3.190
At4g28395.1	lipid transfer protein	BP	–3.492
At3g51590.1	lipid transfer protein	BP	–3.752
At3g12545.1	lipid transfer protein-related	ND	–2.865
At3g11430.1	phospholipid/glycerol acyltransferase family protein	LAP	–4.336
At4g34850.1	chalcone and stilbene synthase family protein	BP	–3.680
At1g02050.1	chalcone and stilbene synthase family protein	BP	–3.182
At1g74540.1	cytochrome P450	BP	–1.788
At3g42960.1	alcohol dehydrogenase (ATA1)	BP	–1.948
At1g51410.1	cinnamyl-alcohol dehydrogenase	PE	–3.523
At2g45580.1	cytochrome P450 family protein	LAE	–3.375
At1g13140.1	cytochrome P450 family protein	BP	–2.238
At1g01280.1	cytochrome P450 family protein	BP	–6.334
At5g03600.1	GDSL-motif lipase/hydrolase family protein	LAE	–3.254
At4g30140.1	GDSL-motif lipase/hydrolase family protein	PE	–2.998
At2g19010.1	GDSL-motif lipase/hydrolase family protein	PE	–3.282
At2g04020.1	GDSL-motif lipase/hydrolase family protein	APE	–4.033
At1g06990.1	GDSL-motif lipase/hydrolase family protein	BAE	–2.385
At3g09930.1	GDSL-motif lipase/hydrolase family protein	LAP	–3.069
At5g03590.1	GDSL-motif lipase/hydrolase protein-related	LAP	–3.364
At4g08670.1	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	LAE	–1.803
At1g18280.1	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	LAE	–1.837
At5g51030.1	short-chain dehydrogenase/reductase (SDR) family protein	PE	–2.968
At5g65205.1	short-chain dehydrogenase/reductase (SDR) family protein	BP	–2.226
At5g02540.1	short-chain dehydrogenase/reductase (SDR) family protein	BE	–1.733

a) Gene identification number; b) $\log_2$-fold difference of the mean expression ratios of three replicates for microarray assays.

development and in pollen tube growth [41]. However, the functions of other suppressed TFs have not yet been investigated. Seven members of the zinc-finger (C2H2-type) family are downregulated in *ms188*. Two of them, At4g35610 and At4g35700, share 42% amino acid sequence identity in a distinct clade in the phylogenetic tree (Figure S1). Additionally, the predicted proteins of At2g17180 and At4g35280 share 55% sequence identity. The other three genes are relatively distant to one another, indicating that they might have different roles in *Arabidopsis*. These results suggest that C2H2 family TFs are potential regulators of the late stages of anther development.

Among the down-regulated transcription factors, ten of them were not detected in the 'e-fp' expression dataset. To assess expression patterns in the anther of these genes, we performed RNA *in situ* hybridization experiments with high fold change genes. A LOB domain-containing gene, At1g06280, was initially expressed at anther development stage 5, and the signal was increased significantly in tapetal cells during the tetrad stages. At stage 9, its expression was still detectable in the tapetum and microspores (Figures 4A–C). A zinc finger C2H2-type gene, At1g02040, was expressed in the tapetum and microsporocytes during several stages (Figures 4D and E). The expression patterns of these two genes are similar to that of *AtMYB103*, and the predicted promoters of these genes contain two MYB binding sites (ACCAAAC boxes). This result suggests that *AtMYB103* might regulate the expression of these genes *in vivo*. In addition, the *in situ* experiment showed that At4g35610 (encoding a zinc finger C2H2-type protein) was

Table 6 Transcription factors downregulated by *AtMYB103*[a)]

Locus	Description	Family	Expression pattern	Fold change in *ms188* buds
At1g02040.1	zinc finger (C2H2 type) family protein	C2H2	ND	–3.608
At1g04445.1	zinc finger (C2H2 type) family protein	C2H2	ND	–2.550
At1g04500.1	zinc finger CONSTANS-related	C2C2-CO-like	PE	–2.179
At1g06280.1	LOB domain family protein	AS2	ND	–5.975
At1g22130.1	MADS-box family protein	MADS	PE	–2.325
At1g26610.1	zinc finger (C2H2 type) family protein	C2H2	LAE	–2.384
At1g61110.1	no apical meristem (NAM) family protein	NAM	LAE	–2.163
At1g77980.1	MADS-box family protein (AGL66)	MADS	PE	–2.001
At2g13570.1	CCAAT-box binding transcription factor	CCAAT-HAP3	PE	–1.990
At2g17180.1	zinc finger (C2H2 type) family protein	C2H2	LAE	–3.466
At2g22750.1	basic helix-loop-helix (bHLH) family protein	bHLH	PE	–2.063
At2g41240.1	basic helix-loop-helix (bHLH) family protein	bHLH	ND	–2.718
At3g13850.1	LOB domain family protein	AS2	ND	–2.470
At3g28917.1	ZF-HD homeobox family protein	ZF-HD	ND	–1.282
At3g59960.1	SET domain-containing protein	PcG	ND	–1.673
At3g60460.1	myb family transcription factor DUO1	MYB	ND	–2.334
At4g04450.1	WRKY family transcription factor	WRKY	APE	–2.368
At4g15250.1	zinc finger (B-box type) family protein	C2C2-CO-like	LAP	–2.994
At4g22070.1	WRKY family transcription factor (WRKY31)	WRKY	LAE	–3.501
At4g35280.1	zinc finger (C2H2 type) family protein	C2H2	APE	–2.058
At4g35610.1	zinc finger (C2H2 type) family protein	C2H2	ND	–4.480
At4g35700.1	zinc finger (C2H2 type) family protein	C2H2	PP	–4.647
At5g22260.1	male sterility 1 protein contains PHD-finger	PHD	ND	–6.417
At5g27960.1	MADS-box family protein (AGL90)	MADS	PE	–2.400
At5g57660.1	zinc finger (B-box type) family protein	C2C2-CO-like	LAE	–1.269
At5g58010.1	basic helix-loop-helix (bHLH) family protein	bHLH	PE	–1.945
At5g62320.1	myb family transcription factor (MYB99)	MYB	BP	–2.898

a) Expression location for differentially expressed genes was derived from Arabidopsis eFP Browser (Winter *et al*., 2007). BP, bud preferential; PE, pollen expressed; LAE, late anther expressed; PP, pollen preferential; LAP, late anther preferential; APE, late anther and pollen expressed; ND, not detected.

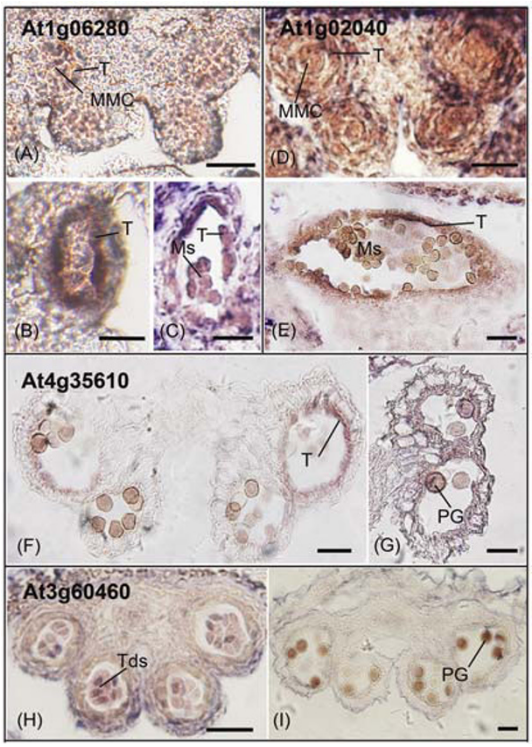

Figure 4 Spatial expression patterns of selected transcription factors (TFs) by *in situ* hybridization. (A)–(C) Expression patterns of At1g06280. (A) Expression is initiated in the tapetum and microsporocytes at an early stage. (B) An expression peak in tapeta at the tetrad stage. (C) Expression in tapeta and microspores in a late stage. (D)–(E) Expression patterns of At1g02040. (D) Expression is detected in the tapetum and microsporocytes within a 5 to 6 anther stage. (E) Expression in the tapetum and microspores in the late stage. (F)–(G) Expression of At4g35610 is only detected in the pollen and degraded tapetum in the late stage. (H)–(I) Expression of At3g60460 is associated with microspores and pollen grains. MMC, Microspore mother cells; Ms, microspores; PG, pollen grains; T, tapetum; Tds, tetrads. Bars=20 μm.

preferentially expressed in both the degraded tapetum and pollen during late anther stages (Figures 4F and G), suggesting partial functional redundancy with pollen preferential gene At4g35700, according to the phylogenetic analysis. The *DUO1* gene (At3g60460) is indeed expressed in microspores and pollens during late anther development (Figures 4H and I), which is consistent with its function in male gamete formation.

3 Conclusion

In this study, we analyzed the detailed expression pattern of *AtMYB103* in anther development. TEM observations indicated that the aberrant exine formation is because of abnormal tapetal development instead of primexine deposition. Furthermore, we analyzed gene expression in flower buds arrested by *AtMYB103* mutation using hybridization with *Arabidopsis* whole genome 44K Agilent microarrays. A total of 818 genes (93 up-regulated and 725 down-regulated) showed significant fold changes in the *ms188* buds during anther development. Functional classification showed that loss of function of *AtMYB103* caused the suppression of a large number of genes involved in signaling pathways and cell wall modification. *AtMYB103* also regulated a number of transcription factors. These results indicated that *AtMYB103* is a master regulator of biological processes occurring during the late stage of anther development, and provide useful information for further investigation of the gene regulatory network of anther development.

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