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Defective in Tapetal Development and Function 1 is essential for anther development and tapetal function for microspore maturation in Arabidopsis

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Summary

In Arabidopsis, the tapetum plays important roles in anther development by providing enzymes for callose dissolution and materials for pollen-wall formation, and by supplying nutrients for pollen development. Here, we report the identification and characterization of a male-sterile mutant, *defective in tapetal development and function 1* (*tdf1*), that exhibits irregular division and dysfunction of the tapetum. The *TDF1* gene was characterized using a map-based cloning strategy, and was confirmed by genetic complementation. It encodes a putative R2R3 MYB transcription factor, and is highly expressed in the tapetum, meiocytes and microspores during anther development. Callose staining and gene expression analysis suggested that *TDF1* may be a key component in controlling callose dissolution. Semi-quantitative and quantitative RT-PCR analysis showed that *TDF1* acts downstream of *DYT1* and upstream of *AMS* and *AtMYB103* in the transcriptional regulatory networks that regulate tapetal development. In conclusion, our results show that *TDF1* plays a vital role in tapetal differentiation and function.

Keywords: Arabidopsis, *TDF1*, anther development, tapetum development, callose wall, transcriptional regulatory network.

Introduction

In *Arabidopsis thaliana*, the anther, which is a bilaterally symmetrical structure with four lobes, yields mature pollen grains (Goldberg *et al.*, 1993). Cells divide and differentiate within each lobe to form four distinct somatic cell layers, which are designated from the exterior to the interior as the epidermis, endothecium, middle layer and tapetum (Goldberg *et al.*, 1993; Sanders *et al.*, 1999). Participation of both the developing haploid gametophytic cells and the accessory diploid sporophytic cells is necessary for the production of gametes in plants. The tapetum, which arises from secondary parietal cells and connective tissues, surrounds the L2-derived, developing reproductive cells (Goldberg *et al.*, 1993). It plays an important role in pollen development by contributing to microspore release, nutrition, pollen-wall synthesis and sporopollenin deposition (Mariani *et al.*, 1990; Pacini *et al.*, 1985; Piffanelli *et al.*, 1998; Stieglitz and Stern, 1973).

Several genes essential for tapetal development and function have been identified in Arabidopsis. *SPOROCYTELESS/NOZZLE* (*SPL/NZZ*), *BARELY ANY MERISTEM 1* (*BAM1*) and *BAM2*, *EXCESS MICROSPOROCYTES 1/EXTRA SPOROGENOUS CELLS* (*EMS1/EXS*), *SOMATIC EMBRYOGENESIS 1* (*SERK1*), *SERK2* and *TAPETUM DETERMINANT 1* (*TPD1*) all function at an early stage and determine tapetal formation (Albrecht *et al.*, 2005; Canales *et al.*, 2002; Colcombet *et al.*, 2005; Hord *et al.*, 2006; Schiefthaler *et al.*, 1999; Yang *et al.*, 1999, 2003, 2005; Zhao *et al.*, 2002). Mutation of *SPL* blocks sporocyte formation, and the *spl* mutant shows complete male and female sterility (Schiefthaler *et al.*, 1999; Yang *et al.*, 1999). *BAM1* and *BAM2* encode CLAVATA1-related Leu-rich repeat receptor-like kinases. The *bam1 bam2* double mutant displays developmental defects at the early anther stage and lacks somatic cell layers, which suggests that these genes promote cell division and differentiation (Hord *et al.*, 2006). *EMS1/EXS*, *SERK1*, *SERK2* and *TPD1* determine the differentiation of the microsporocytes and tapetal cells. Mutation of these genes causes the tapetal cell precursors to differentiate and develop into micro-

sporocytes instead (Albrecht *et al.*, 2005; Colcombet *et al.*, 2005; Yang *et al.*, 2003, 2005; Zhao *et al.*, 2002).

Following formation of tapetal cell layer, other genes such as *DYSFUNCTIONAL TAPETUM 1* (*DYT1*), *MYB33* and *MYB65* become important for tapetal differentiation and early functioning (Millar and Gubler, 2005; Zhang *et al.*, 2006). *DYT1*, which shows strong expression in the tapetum from late stage 5 to early stage 6, encodes a putative basic helix-loop-helix (bHLH) transcription factor, and is important for tapetum-specific gene expression. *DYT1* is a crucial component of a genetic network that controls anther development and function (Zhang *et al.*, 2006). In *myb33 myb65* double mutant anthers, the tapetum undergoes hypertrophy at the pollen mother cell stage, which results in pre-meiotic abortion of pollen development (Millar and Gubler, 2005).

At the late stage of tapetal development, the three regulatory genes *MALE STERILITY 1* (*MS1*), *ABORTED MICROSPORES* (*AMS*) and *AtMYB103* are essential for pollen formation and development. In the *ms1* mutant, the tapetum is abnormally vacuolated and microspores collapse soon after they are released from the tetrads. The MS1 protein shares strong homology with the PHD-finger motif (Wilson *et al.*, 2001). Recent research has shown that *MS1* plays a critical role in regulating exine formation and pollen coat development (Ito *et al.*, 2007; Vizcay-Barrena and Wilson, 2006; Yang *et al.*, 2007). *AMS* belongs to the *MYC* subfamily of bHLH genes, and mutation of *AMS* results in premature degeneration of both the tapetum and microspores (Sorensen *et al.*, 2003). *AtMYB103* is a member of the R2R3 *MYB* gene family, and is only expressed in the tapetum of developing anthers (Higginson *et al.*, 2003; Li *et al.*, 1999). Ablation of *AtMYB103* leads to complete male sterility because tapetal development, callose dissolution and exine formation in anther development are all disrupted (Zhang *et al.*, 2007).

Callose is synthesized in the microsporocytes of developing anthers, acting as a temporary wall between the primary cell wall and the plasma membrane to (i) prevent cell cohesion and fusion before the release of free microspores into the locule (Waterkeyn, 1962), (ii) protect developing microspores from the influence of the surrounding diploid tissues (Heslop-Harrison and Mackenzie, 1967), (ii) prevent premature swelling and bursting of the microspores, and (iv) act as a mould in which the exine pattern is formed on the mature pollen grain, according to a blueprint provided by primexine, soon after the completion of meiotic division (Stanley and Linskens, 1974; Waterkeyn and Beinfait, 1970; Zhang *et al.*, 2002). The major component of callose is a β-1,3-linked glace. Callose dissolution is a critical step in pollen development. At stage 7, callase is secreted from the tapetum to degrade callose, and then microspores are released into four locular spaces at stage 8 (Stieglitz and Stern, 1973). It has been reported that the *A6* gene is associated with callose dissolution in Arabidopsis (Hird *et al.*, 1993). Callose dissolution is therefore under strict regulation in anther development. However, the molecular mechanism of regulation has not been identified.

Here, we report the isolation and molecular characterization of a new Arabidopsis male-sterile mutant, *tdf1*. Knockout of *TDF1* severely impaired tapetal development and callose dissolution, which resulted in complete male sterility. *TDF1* was cloned using a map-based cloning strategy, and its function in anther development and pollen maturation was investigated. Our results indicate that *TDF1* acts downstream of *DYT1* and upstream of several genes that are essential for tapetal late-stage development and function, and showed that *TDF1* plays a key role in tapetum cell fate and function in Arabidopsis.

Results

Isolation of male-sterile mutant tdf1

To identify new genes important for anther development, we screened for male-sterile mutants among a population of Arabidopsis ecotype Landsberg *erecta* (L*er*) mutagenized using ethyl methane sulphonate (EMS). A sterile mutant line with normal vegetative and floral development but no seed yield was isolated (Figure 1b). No pollen grains were observed on the stigma and anthers of the mutant (Figure 1e,h). Backcrossing with wild-type pollen grains resulted in F_1 plants with normal fertility, suggesting that female fertility was not affected. The fertile and male-sterile plants segregated in the ratio 3:1 in the F_2 population, suggesting that the inherited male-sterile phenotype was caused by a single recessive Mendelian locus that was controlled sporophytically. This mutant was designated *defective in tapetal development and function 1* (*tdf1*) because of defects in its anther development, as described below.

Phenotypic characterization of tdf1

To analyze the anther development defects in the *tdf1* mutant, anther and pollen development in wild-type and mutant plants was compared using semi-thin anther sections. In Arabidopsis, anther development can be divided into 14 stages, based on morphological landmarks that correspond to cellular events visible under the light microscope (Sanders *et al.*, 1999). From stages 1–5, anther primordia undergo cell division to establish lateral structures, and microspore mother cells develop into meiotic cells. No detectable differences were observed between the *tdf1* mutant and the wild-type during stages 1–4 (data not shown for stage 1–3; data for stage 4 are shown in Figure S1). However, significant aberrations in anther development were observed after stage 5 in *tdf1*. The earliest defect observed in the mutant at this developmental stage was cell vacuolation in both the epidermis and the endothecium

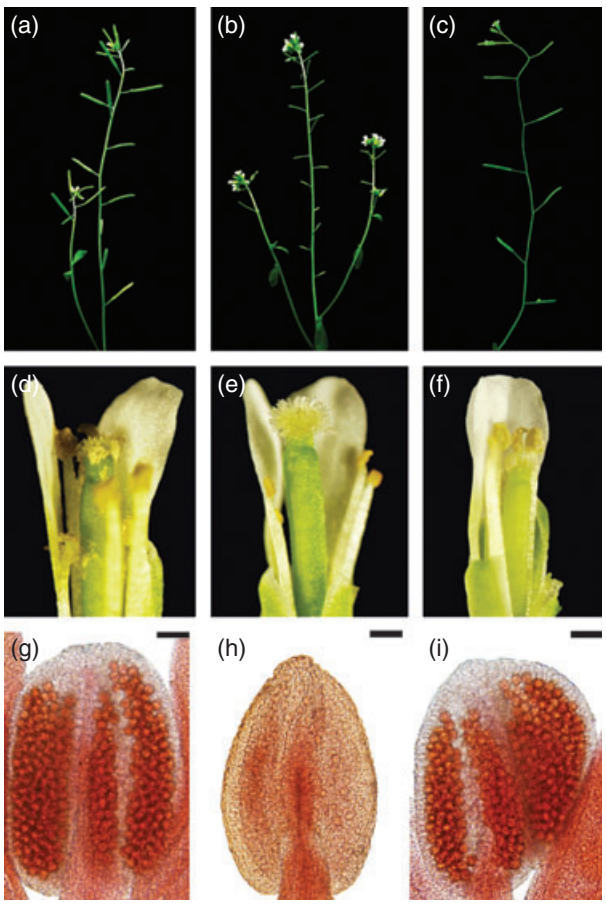

Figure 1. Phenotypes of the wild-type (L*er*), *tdf1* mutant and transgenic plants (complemented for *tdf1*).
(a) An L*er* plant, with normal fertility.
(b) A *tdf1* plant, with very small siliques containing no seeds.
(c) A *tdf1* plant containing the *TDF1* transgene.
(d) An L*er* flower.
(e) A *tdf1* flower, with no pollen grains present on the stigma.
(f) A flower of a *tdf1* plant containing the *TDF1* transgene.
(g) A wild-type anther, with viable pollen grains (stained).
(h) An anther from a *tdf1* plant without viable pollen.
(i) An anther from a *tdf1* plant containing the *TDF1* transgene, with a large number of viable pollen grains. Bar = 80 μm for (g)–(i).

(Figure 2a,b). The middle layer became vacuolated at stage 6 (Figure 2d), and the vacuolation remained visible in *tdf1* locules until the later stages (Figure 2j,l).

In wild-type anthers, tapetal cells formed a regular layer surrounding the locules (Figure 2c), and had a spongy appearance with clusters of small vesicles at stage 6, as seen by transmission electron microscopy (TEM), which indicated that polar secretory-type cells had been formed (Figure S2a). However, in the *tdf1* mutant, the tapetal cells were abnormally vacuolated and enlarged from stage 6, which resulted in an irregular layer with excessive division (Figure 2f). Moreover, TEM analysis showed that the characteristics of secretory tapetal cells were not observed in *tdf1* anthers at stage 6 (Figure S2b), which indicated that the transition of the tapetum to the secretory type was probably aberrant.

The number of tapetal cells was estimated in cross-sections of 60 and 67 independent locules from wild-type and *tdf1* mutant anthers, respectively. At stage 7, the wild-type had an average of 11.0 $\pm$ 1.0 tapetal cells per locule. By contrast, the average number of tapetal cells per locule in the *tdf1* mutant was 16.8 $\pm$ 1.5. These results suggest that tapetal cells undergo excessive division in the *tdf1* mutant.

The identity of the irregular cells was confirmed by RNA *in situ* hybridization on *tdf1* mutant tapetum sections using *ARABIDOPSIS THALIANA ANTHER7* (*ATA7*) RNA as a tapetum-specific probe (Figure 2m–p). The *ATA7* RNA probe hybridized strongly to the tapetal cells. The hybridization signal was much stronger in *tdf1* than in wild-type anthers at stage 7 (Figure 2n). However, after stage 7, the signal disappeared in the *tdf1* tapetum (Figure 2p), which indicated that the tapetum was impaired at later stages.

Developmental defects of microspores were also observed in the *tdf1* mutant. After meiosis, microspores of the wild-type formed tetrads that were surrounded by a thick callose wall. *In situ* hybridization on *tdf1* anther sections showed that tetrads could be formed in the *tdf1* mutant at this stage (Figure 2n). At stage 8, microspores were released from the tetrads after degradation of the callose wall in the wild-type, but the mutant tetrads remained enveloped by the highly vacuolated tapetum and the middle layer (Figure 2g,h). At later stages, the *tdf1* microspores collapsed and were degraded (Figure 2j,l). Thus, no pollen grains were formed in the locules, which resulted in a male-sterile phenotype.

Molecular cloning of the TDF1 *gene*

The *TDF1* locus was mapped in a genetic population generated by crossing *tdf1* and the Arabidopsis ecotype Columbia (Col). For first-pass mapping, a total of 20 insertion/deletion (In/Del) and SSLP markers were used (Table S1), and *TDF1* was linked to In/Del marker CIW11 on chromosome 3. Fine mapping was performed using a population with approximately 1600 male-sterile progenies. The mapping results indicated that the *TDF1* gene was located in a region of 35.5 kb between In/Del marker MZN14 and single nucleotide polymorphism (SNP) marker MFJ20SNP on the long arm of chromosome 3 (Figure 3a).

There are 11 putative genes in this region. Among them, At3g28470 encodes the putative transcription factor *AtMYB35* (data from the TAIR database, http://www.arabidopsis.org). *AtMYB35* was highly expressed in Arabidopsis anthers, as determined by comparison with expression data available in the Genevestigator database (http://www.genevestigator.ethz.ch). To establish whether the male-sterile phenotype was due to ablation of *AtMYB35*,

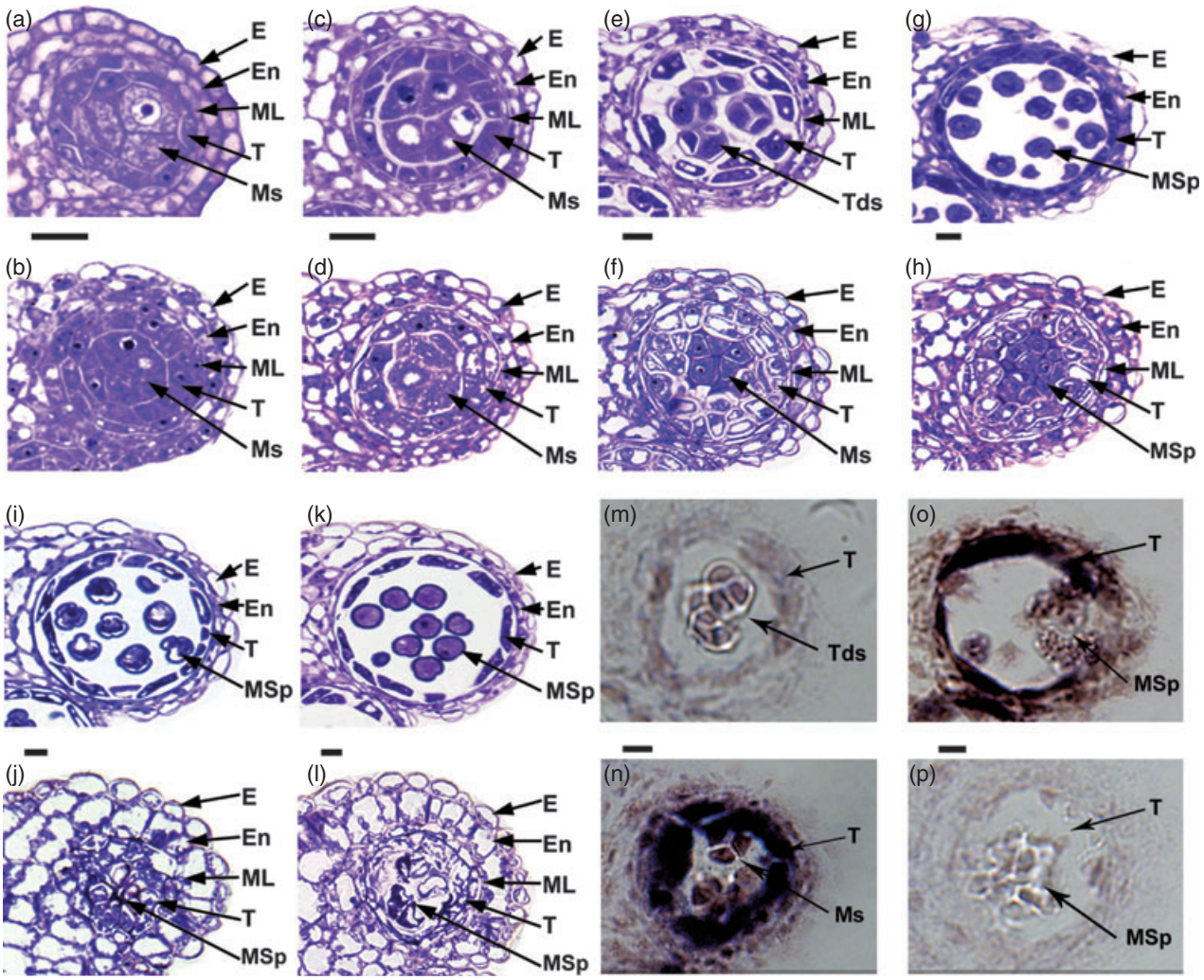

Figure 2. Anther development at stages 5–10 in the wild-type (L*er*) (a, c, e, g, i, k, m, o) and the *tdf1* mutant (b, d, f, h, j, l, n, p).
(a, b) Stage 5 anthers. Vacuolization occurred in more cells in the epidermis and endothecium of the *tdf1* mutant than in wild-type cells.
(c, d) Stage 6 anthers. Vacuolization was observed in tapetal cells in the *tdf1* mutant.
(e, f) Stage 7 anthers. Tapetal cell division was greater in the *tdf1* mutant than in the wild-type.
(g, h) Stage 8 anthers. Microspores were released from tetrads after the callose wall was degraded in the wild-type, but the mutant tetrads were enveloped by the highly vacuolated tapetum.
(i, j) Stage 9 anthers. The endothecium was enlarged and some of the microspores were degraded in the *tdf1* mutant.
(k, l) Stage 10 anthers. Microspores were completely collapsed and the endothecium was further enlarged in the *tdf1* mutant.
(m) Wild-type anther at stage 7, showing specific expression of *ATA7* in the tapetal cells.
(n) *tdf1* anther at stage 7, showing a strong *ATA7* RNA signal in the multi-layer tapetum.
(o) Wild-type anther at stage 8, showing specific expression of *ATA7* in the tapetal cells.
(p) *tdf1* anther at stage 8, showing that the *ATA7* probe signal has disappeared from the *tdf1* tapetum. E, epidermis; En, endothecium; ML, middle layer; Ms, meiocytes; Msp, microspores; T, tapetum; Tds, tetrads. Bar = 20 μm.

we cloned and sequenced genomic DNA and cDNA that corresponded to this gene from both *tdf1* mutant and wild-type plants. Sequence analysis revealed a CAA to TAA mutation in the *tdf1* mutant that caused translation to end prematurely in the third exon of *AtMYB35* (Figure 3b). This suggested that *AtMYB35* was the candidate gene for *TDF1*.

A genetic complementation experiment was performed to verify that *AtMYB35* corresponded to *TDF1*. The *AtMYB35* genomic fragment, which included 817 bp of the putative upstream promoter region and 106 bp of the downstream region, was amplified from the wild-type plant and cloned into pMD18-T. The fragment was then subcloned into the binary vector pCAMBIA1300. The resulting complementation construct was used to transform *tdf1*/+ heterozygous plants utilizing *Agrobacterium* LBA4404. One hundred and forty-four transformants that carried the complementation construct were obtained. PCR analysis using closely linked markers showed that 37 transgenic lines were *tdf1*/*tdf1*. Of

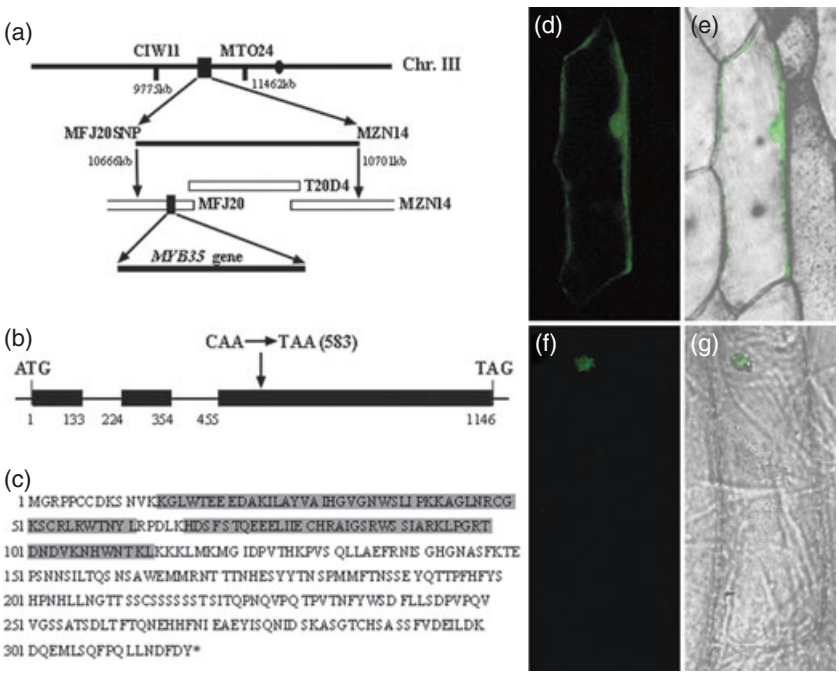

Figure 3. Molecular identification and protein sequence analysis of the *TDF1* gene, and nuclear localization of the TDF1–GFP fusion protein.
(a) Fine mapping of *TDF1* to a region of 35.5 kb between In/Del marker MZN14 and SNP marker MFJ20SNP on the long arm of chromosome 3.
(b) The *TDF1* gene structure and position of the nucleotide change in *tdf1*. The black boxes indicate exons of *TDF1*.
(c) The annotated amino acid sequence of the TDF1 protein, which contains 318 amino acid residues. Residues K14–L61 and H67–L112 (marked by gray shading) comprise the predicted conserved R2R3 MYB binding domains.
(d–g) Onion epidermal cells were bombarded with pCAMBIA1302-TDF1-GFP and a control construct pCAMBIA1302.
(d, e) The GFP protein was distributed throughout the cytoplasm and nucleus of the cell after bombardment with the control GFP construct.
(f, g) The TDF1–GFP fusion protein was detected only in the nucleus.

these, 16 lines had the same phenotype as the wild-type when grown to maturity, and showed recovered fertility with restored pollen development and normal siliques (Figure 1c,f,i). This validated the suggestion that *AtMYB35* was *TDF1*, and indicated that the 2.14 kb genomic fragment used for complementation contained sufficient genetic information for normal *TDF1* function.

TDF1 *encodes a putative MYB transcription factor (*AtMYB35*)*

The *TDF1* gene encodes an R2R3 MYB family transcription factor that comprises 318 amino acids with an estimated molecular weight of 35.8 kDa. The primary structure of *TDF1* was identical to the annotation in the TAIR database for *AtMYB35*, with three exons (133, 130 and 691 bp) and two introns (91 and 101 bp; Figure 3b). TDF1 shares homology in the N-terminal portion with the conserved MYB DNA-binding domain (Figure S3). Like most members of the MYB family, TDF1 consists of two repeats of 51 or 52 amino acids, each consisting of three α-helices, with the second and third forming a helix-turn-helix structure when bound to DNA (Kranz *et al.*, 1998). These Pfam DNA-binding domains were encoded by bases 14–61 and 67–112 in the coding sequence based on the annotation in the TAIR database (http://www.arabidopsis.org; Figure 3c). The results of a BLAST search indicated that the TDF1 protein shared highest similarity with the Arabidopsis AtMYB103 protein (Figure S3), which has previously been reported to be important for the secretory function of the tapetum (Zhang *et al.*, 2007).

We further analyzed the phylogenetic relationships between *TDF1* and other close homologues using the Clustal W program. An unrooted neighbor-joining tree showed that *TDF1* (At3g28470) and *AtMYB103* (At5g56110) from Arabidopsis, AAL84759 from *Sorghum bicolor*, Os04g0470600, OSIGBa0124N08 and LOC_Os03g18480 from *Oryza sativa*, and ABE78637 from *Medicago truncatula* formed a separate clade within a group of related members of the MYB-like gene family (Figure S4). Among them, ABE78637 and *TDF1* were identified as an orthologous pair. In Arabidopsis, *TDF1* and *AtMYB103* were most closely related to each other, which is in agreement with the results of the preliminary BLAST search.

The subcellular localization of TDF1 was investigated by construction of a translational fusion of *TDF1* to GFP, driven by the 35S promoter. Introduction of the *TDF1*–GFP fusion construct into onion epidermal cells by particle bombardment resulted in fluorescent signals localized in the nucleus (Figure 3f,g). In contrast, GFP alone was found throughout the control cells (Figure 3d,e). This result is consistent with the presumed function of TDF1 as a transcriptional regulator.

TDF1 *is preferentially expressed in both the tapetal cells and meiocytes*

RT-PCR analysis of total RNA extracted from various organs including roots, stems, leaves and inflorescences indicated that *TDF1* was highly expressed in inflorescences. No expression was detected in roots, stems or leaf tissues (Figure 4a), which indicated that *TDF1* was specifically expressed in the reproductive organs.

Spatial and temporal patterns of *TDF1* expression during anther development were determined by RNA *in situ*

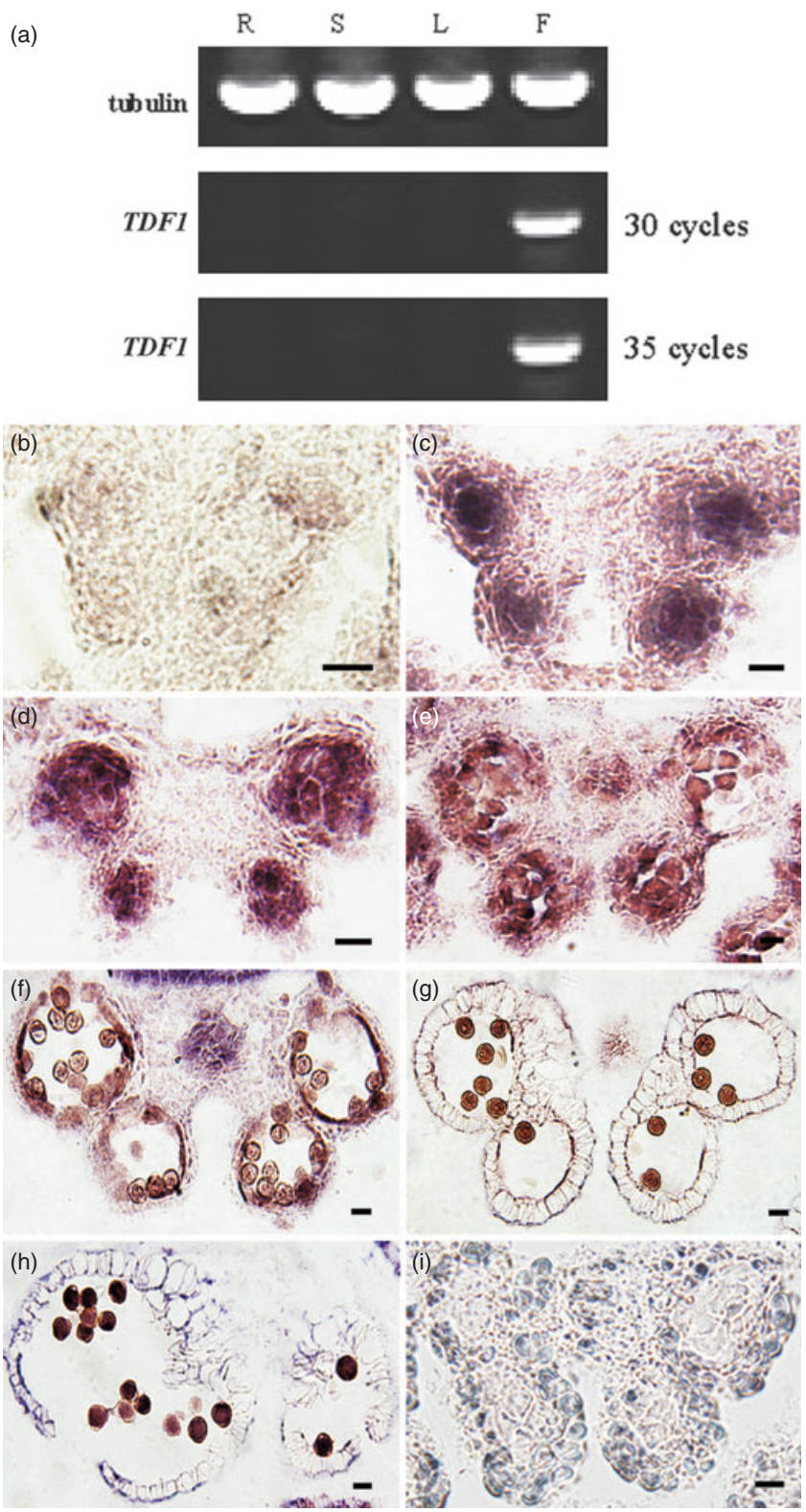

Figure 4. *TDF1* expression analysis.
(a) RT-PCR analysis of *TDF1* expression in various tissues using 30 or 35 cycles. F, inflorescence; L, leaf; R, root; S, stem.
(b–i) RNA *in situ* hybridization with a *TDF1* probe.
(b) TDF1 was barely expressed in the anther at stage 4.
(c–e) TDF1 was expressed predominantly in meiocytes, the tapetum and the middle layer at stages 5–7.
(f) *TDF1* was predominantly expressed in tapetal cells and microspores at late stage 8 and early stage 9.
(g, h) At the stage of pollen maturation and release, *TDF1* expression was solely detected in pollen grains.
(i) Sense control in a stage 6 anther. No *TDF1* signal was detected. Bar = 20 μm.

hybridization experiments with wild-type anther sections. A *TDF1*-specific DNA fragment was designed as a probe. *TDF1* RNA was hardly detectable at stage 4 (Figure 4b). From stages 5–7, *TDF1* transcripts were predominantly detected in meiocytes, tapetal and middle layer cells (Figure 4c–e). During these same stages, *TDF1* was expressed at relatively

low levels in other somatic tissues, including the epidermis and endothecium cells. At the microspore stage, *TDF1* was mainly expressed in the tapetum and microspores (Figure 4f). During pollen maturation and release, *TDF1* was only detected in pollen grains (Figure 4g,h). In the control, in which a sense probe was used for hybridization, only background signals were detected (Figure 4i). Therefore, *TDF1* expression was mainly associated with tapetal development and pollen formation in Arabidopsis anther development.

TDF1 *is required for callose dissolution*

In plants, microspores are released from tetrads after callose is degraded by callase, an enzyme produced by the tapetum (Stieglitz and Stern, 1973). As tapetal development is defective in the *tdf1* mutant, we performed callose staining in both wild-type and mutant anthers to establish whether callose degradation was affected in mutant plants. At stage 7, callose fluorescence could be clearly observed in the locules of both wild-type and *tdf1* mutant plants (Figure 5a,b). After stage 8, callose was not detected in the wild-type and was assumed to be completely degraded (Figure 5c,e). However, callose was still observed in *tdf1* anthers after stage 8 (Figure 5d,f), which indicated that callose is not degraded in *tdf1.*

Although gene(s) encoding callase have not been directly identified, it has been reported that the *A6* gene is involved in callose dissolution (Hird *et al.*, 1993). Therefore, expression of *A6* in the *tdf1* mutant was analyzed using RT-PCR. After amplification for 30 or 35 cycles, PCR products were obtained from the wild-type; however, no RT-PCR products from *A6* were detected in the *tdf1* mutant (Figure 5g). The expression level of *A6* in the *tdf1* mutant was only 0.38% of that in the wild-type (Figure 5h). These results indicated that *TDF1* is required for callose breakdown in Arabidopsis anther development.

TDF1 *acts downstream of* DYT1 *and upstream of* AMS *and* AtMYB103

To further elucidate the role of *TDF1* in tapetal function, we performed RT-PCR using RNA obtained from *tdf1* and several other previously reported male-sterile mutants. Previous studies have shown that *DYT1* plays an important role in differentiation and early function of the tapetum, and the expression of *TDF1* (*AtMYB35*) is significantly reduced in a *dyt1* mutant (Zhang *et al.*, 2006). We have previously identified an allelic mutant of *dyt1*, designated *dyt1-2*, from an EMS-mutagenized population (H.L. and Z.-N.Y., unpublished data). Here, we used *dyt1-2* to assess the relationship between *DYT1* and *TDF1*. RT-PCR analysis showed that

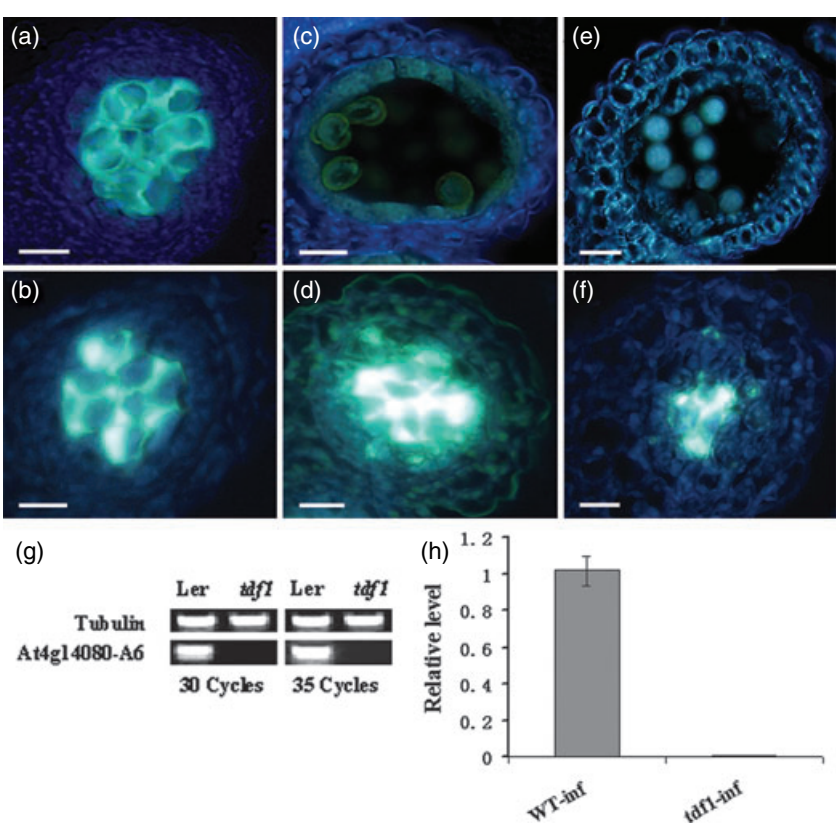

Figure 5. Analysis of callose dissolution and callase-related gene *A6* expression in the wild-type and *tdf1* mutant.
(a–f) Anther sections were stained with aniline blue and observed under UV.
(a, b) At stage 7, callose was observed in the locules of both the wild-type and the *tdf1* mutant.
(c, e) At late stage 8 and stage 10, callose was degraded and was not observed in the wild-type.
(d, f) At stages 8 and 10, callose was still observed in the *tdf1* anther.
(g) RT-PCR analysis of expression of the callase-related gene *A6* in the wild-type and *tdf1* mutant.
(h) Real-time PCR analysis. *A6* expression in the *tdf1* mutant was approximately 0.38% of the wild-type level. *tdf1*-inf, *tdf1* inflorescences; WT-inf, wild-type inflorescences. Bar = 20 μm.

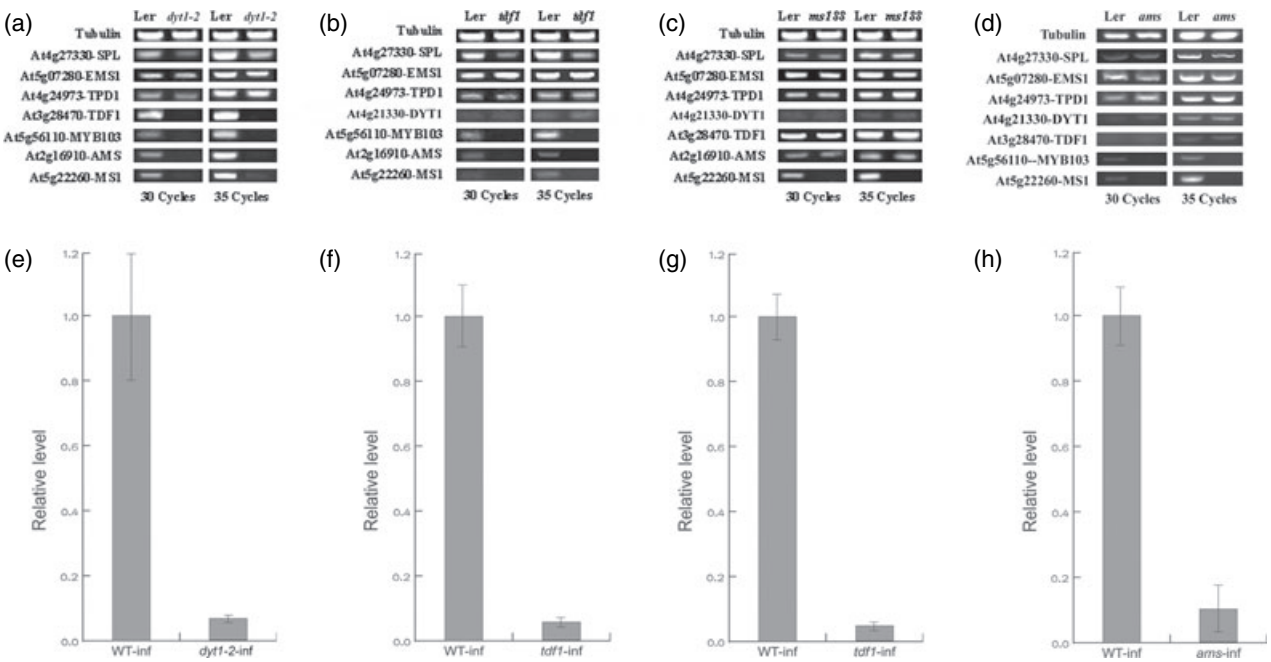

Figure 6. Determination of the gene regulatory network for Arabidopsis tapetal development.
(a–d) Expression of tapetal genes in the wild-type and *dyt1-2*, *tdf1*, *ms188* and *ams* mutants.
(e) Real-time RT-PCR analysis of *TDF1* expression in wild-type and *dyt1-2* backgrounds.
(f, g) Real-time RT-PCR analysis of *AMS* (f) and *AtMYB103* (g) expression in wild-type and *tdf1* backgrounds.
(h) Real-time RT-PCR analysis of *AtMYB103* expression in wild-type and *ams* backgrounds.

expression of *TDF1* was barely detectable in the *dyt1-2* mutant, as previously observed for *AtMYB35* in *dyt1* (Figure 6a) (Zhang *et al.*, 2006). Real-time RT-PCR analysis showed that the expression level of the *TDF1* gene in *dyt1-2* was only 8.0% of that in the wild-type (Figure 6e). These results corroborated the previous assumption that *TDF1* acts downstream of *DYT1*.

MS1, *MS2*, *AMS* and *AtMYB103* are essential for late tapetal function (Aarts *et al.*, 1997; Sorensen *et al.*, 2003; Wilson *et al.*, 2001; Zhang *et al.*, 2007). Previous studies have shown that *AMS* and *AtMYB103* act downstream of *DYT1* (Figure 6a) (Zhang *et al.*, 2006), and *MS1* and *MS2* act downstream of *AtMYB103* (Zhang *et al.*, 2007). Therefore, we investigated the relationship of *AMS* and *AtMYB103* with *TDF1*. In the *tdf1* mutant, *AMS* and *AtMYB103* were only minimally expressed (Figure 6b). Real-time RT-PCR showed that the expression levels of *AMS* and *AtMYB103* in *tdf1* were 7.0% and 6.3% of the wild-type expression levels, respectively (Figure 6f,g). This suggests that *AtMYB103* and *AMS* act downstream of *TDF1*. To further investigate the relationship between *AtMYB103* and *AMS*, we analyzed *AMS* expression in the *ms188* mutant (the *AtMYB103* mutant described by Zhang *et al.*, 2007). RT-PCR analysis showed that *AMS* expression was not changed significantly in *ms188* (Figure 6c). However, RT-PCR analysis showed that *AtMYB103* expression was dramatically reduced in *ams* inflorescences (Figure 6d). Real-time RT-PCR showed that *AtMYB103* expression in *ams* was 12.4% of that observed in the wild-type (Figure 6h). This indicates that *AtMYB103* acts downstream of *AMS*, and they both act downstream of *TDF1* in the same genetic pathway that regulates tapetal function in Arabidopsis.

Discussion

TDF1 *is required for tapetum development and function*

In this paper, we describe the characterization of a new gene, *TDF1*, which is essential for tapetal development and function in Arabidopsis. In the *tdf1* mutant, pollen development was aborted due to defects in tapetal function. The phenotypic characteristics of *tdf1* can be summarized as follows: (i) excessive periclinal division of tapetal cells, (ii) absence of transition of the tapetum to the secretory type, resulting in a loss of secretory function of the tapetum in *tdf1*, including a callose degradation defect and aberrant expression of a tapetal marker gene, (iii) aborted degeneration of the middle layer, and (iv) vacuolation and enlargement of the endothecial cells at the late stages of anther development, indicating a potential defect in anther wall development. *TDF1* was preferentially expressed in meiocytes (or microspores) and tapetal cells, and played a vital role in the transcriptional regulatory network of the tapetum (as discussed below). These results show that the

primary function of *TDF1* is regulation of tapetal cell differentiation and function.

Several genes have been reported to be important for tapetal differentiation and function, such as *DYT1*, *AMS* and *AtMYB103*. A previous report showed that ablation of *DYT1* leads to abnormal vacuolization in sporophytic layers as early as stage 4 (Zhang *et al.*, 2006). *DYT1* expression is preferentially detected in the precursors of the middle layer, tapetum and meiocytes at stage 4, and reaches its highest level in tapetal cells from stages 5 and 6 (Zhang *et al.*, 2006). These results indicate that *DYT1* is essential for early development of the tapetum. The present study showed that *TDF1* was highly expressed from stage 5–8, with the highest expression level occurring at stages 5 and 6 (Figure 4). Thus, the highest expression of both *DYT1* and *TDF1* occurred at similar stages. Furthermore, our preliminary microarray analysis showed that 70% of the downregulated genes in the *dyt1-2* mutant were also downregulated in *tdf1* (data not shown). These results suggest that these genes are close to each other in the gene network that regulates anther development, and *DYT1* may act mainly through *TDF1* in this regulation. The *AMS* gene encodes a putative MYC transcription factor, and is specifically expressed in the tapetum and microspores (Sorensen *et al.*, 2003), similar to *TDF1*. In this study, we found that *AMS* acted downstream of *TDF1* and upstream of *AtMYB103*. Morphological analysis of *ams* (an allelic mutant from SALK seed stocks) showed that features of the tapetal layer in the *ams* mutant resemble those in the *tdf1* mutant (data not shown). These results indicate that *AMS* and *TDF1* may play similar roles in the regulatory mechanism of tapetal development. Recently, it was reported that knockout of *AtMYB103* led to defects in tapetal cell-wall degradation that impaired the secretory function of the tapetum (Zhang *et al.*, 2007). Here, quantitative RT-PCR analysis showed that expression of *AtMYB103* was dramatically reduced in *tdf1*. The TEM results showed that the tapetal cells had not developed to the secretory type in the *tdf1* mutant (Figure S2). These results indicate that *TDF1* may promote the tapetal transition by positively regulating the expression of *AtMYB103*.

TDF1 *affects late functions of the tapetum in pollen development*

It is generally believed that the tapetum transforms a holocrine tissue during the meiosis stage to provide materials for microspore release from the tetrad and for exine formation, and acts as a supply of nutrients for pollen development (Pacini *et al.*, 1985; Piffanelli *et al.*, 1998). However, transition of the tapetum to the secretory type was blocked in *tdf1*. This suggests that the defect in tapetal development might disrupt the metabolically dynamic state and subsequent secretory function that is necessary for normal microspore development.

Tetrad formation and callose decomposition during and after meiotic division are unique features of male meiosis in plants (Scott *et al.*, 2004). In the anthers of petunia and *Lilium*, the expression and secretion of callase activity is under strict developmental control (Frankel *et al.*, 1969; Stieglitz and Stern, 1973). Although several candidate β-1,3-glucanase encoding genes such as *Tag1* and *A6* have been reported (Bucciaglia and Smith, 1994; Hird *et al.*, 1993), the genes encoding callase have not yet been identified, and the molecular mechanisms that regulate callase expression have not been described. It is quite possible that there is a complex regulatory network that controls callase activity in plants. In the *tdf1* mutant, callose was not degraded and the *A6* gene was barely expressed. *TDF1* may act as an important transcriptional regulator to control callase activity in Arabidopsis.

Another function of the tapetum is the secretion of sporopollenin, which is the major constituent of the exine layer (Scott, 1994). Sporopollenin is composed of a series of related polymers derived from long-chain fatty acids, oxygenated aromatic rings and phenylpropanoids. In Arabidopsis, the *MS2* gene is reported to be involved in sporopollenin synthesis and exine pattern formation (Aarts *et al.*, 1997). *MS2* acts downstream of *AtMYB103*, as shown by its minimal expression in the *ms188* mutant (Zhang *et al.*, 2007). In this study, both *AtMYB103* and *MS2* were only minimally expressed in the *tdf1* mutant (Figure 6b and Figure S5). This suggests that *TDF1* acts upstream of *AtMYB103* and *MS2*, and is likely to regulate exine formation in anther development.

Role of TDF1 *in the transcriptional regulatory network for tapetal development and function*

A putative model for the transcriptional regulation of tapetal development and function has previously been proposed (Wijeratne *et al.*, 2007; Zhang *et al.*, 2006). Here, using phenotypic and expression analysis in several mutants, we have verified and added new information to this model (Figure 7). Most of the genes in the network are involved in transcriptional regulation or signal transduction. As described above, *TDF1*, as a key regulator of tapetal development and function, acts downstream of *DYT1* and upstream of *AMS1* and *AtMYB103*. The role of *AtMYB103* is to regulate transition of the tapetum to the secretory type, and to control its subsequent function by regulating tapetal functional genes such as *A6* and *MS2*. Therefore, *TDF1* may partially fulfil its role in the tapetum through *AMS* and *AtMYB103*. As this model is based mainly on expression analysis, further experiments are needed to identify direct interactions between the genes. Moreover, characterization of new genes that participate in the network will facilitate our understanding of the molecular mechanisms that regulate tapetal development and function.

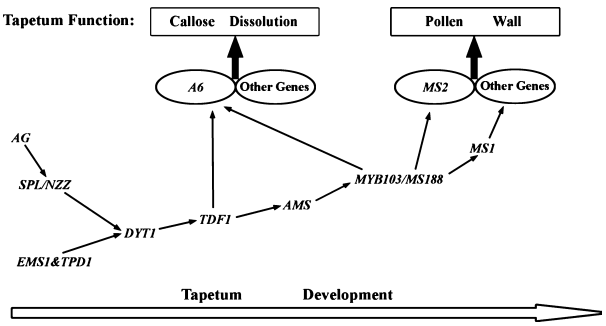

Figure 7. The gene regulatory network for Arabidopsis tapetal development and function.
The integrated gene regulatory network for Arabidopsis tapetum development is based on this study and previous work (Zhang *et al.*, 2006). The arrow at the bottom of the figure represents tapetal development and the two upper boxes represent the tapetal functions of callose dissolution and pollen wall development.

Experimental procedures

Plant materials

The Arabidopsis mutant *tdf1* (ecotype L*er*) was screened using an EMS mutagenesis strategy. Before phenotypic analysis, *tdf1* was back-crossed to wild-type L*er* three times. The plants were grown on soil at 22°C under a 16 h light/8 h dark photoperiod.

Mapping and cloning of the TDF1 *gene*

The F_2 population from a cross between *tdf1* (L*er*) and ecotype Col was used for mapping of the *TDF1* gene. For the first-pass mapping, SSLP and In/Del markers were used to localize *TDF1* to chromosome 3 (Table S1). For the fine mapping, six In/Del markers and one SNP marker for *TDF1* were generated using the Cereon database (http://www.arabidopsis.org/; Table S2), and a total of 1600 F_2 mutant plants were used. The *TDF1* locus was mapped to a 35.5 kb region between the markers MZN14 and MFJ20S using the molecular markers developed here. Genomic DNA and cDNA that corresponded to the candidate gene *AtMYB35* were amplified from both the *tdf1* mutant and the wild-type using primers 5′-CTATTTCTGTATCAAAAGGTGT-3′ and 5′- ATTAGGAGCCTTCAAGCA-3′. The PCR products were cloned into pMD18-T (Takara; http://www.takara-bio.com). The cloned DNA was sequenced using M13+ and M13– primers.

TDF1 *complementation experiment*

A 2.14-kb *TDF1* genomic fragment was amplified using LA *Taq* DNA polymerase (Takara) and the gene-specific primers 5′-TATTTCTGTATCAAAAGGTGT-3′ and 5′-CTGTTCTTCAATCTTCACC-3′. The PCR product was cloned into the pMD18-T vector (Takara). After verification by sequencing, the fragment was subcloned into the binary vector pCAMBIA1300 (CAMBIA; http://www.cambia.org) and introduced into *tdf1/+* heterozygous plants in the Col-0 background using the floral-dip method (Clough and Bent, 1998). Transformants were selected using 20 mg l^{-1} hygromycin. As there was minimal recombination in the genomic region comprising the *TDF1* gene and the closely linked In/Del markers by self-crossing, the T1 lines were genotyped to identify homozygous *tdf1/tdf1* plants by PCR analysis using the In/Del markers MFJ20 and MZN14.

Light microscopy

The process of fixation, dehydration and embedding was performed as described by Zhang *et al.*, 2007. An Olympus BX51 microscope (Olympus; http://www.olympus-global.com) with an Olympus DP70 digital camera was used for cytological observations.

Protein localization

Cellular localization of the TDF1 protein was examined by transient expression of a TDF1–GFP fusion protein. The complete *TDF1* coding sequence was amplified by PCR using two oligonucleotide primers, 5′-AGATCTGGGAAGACCTCCTTGTTGT-3′ and 5′-AGATCTTAATCGAAATCATTCAAGAGTT-3′ (*Bgl*II sites underlined), and cloned into pCAMBIA1302 (CAMBIA) to obtain pCAMBIA1302-TDF1-GFP. The pCAMBIA1302-TDF1-GFP plasmid was delivered into onion epidermal cells using a biolistic PDS-1000/He gene gun system (Bio-Rad, http://www.biorad.com/). The bombarded samples were kept overnight at 26°C and observed using a confocal laser scanning microscope (LSM 5 PASCAL; Zeiss, http://www.zeiss.com/).

RT-PCR and real-time PCR

Total RNA extraction, cDNA synthesis, RT-PCR and real-time PCR analysis were performed as described by Zhang *et al.* (2007). The primers for RT-PCR and real-time PCR are listed in Table S3 and S4, respectively. β-tubulin was used as a control for constitutive expression.

RNA in situ *hybridization*

Non-radioactive RNA *in situ* hybridization was performed using a digoxigenin (DIG) RNA labeling kit and PCR DIG probe synthesis kit (Roche; http://www.rochediagnostics.us) according to the manufacturer's instructions. A 335 bp *TDF1* cDNA fragment was amplified using *TDF1*-specific primers (forward 5′-CTGATGAAAATGGGGATA-3′, reverse 5′-TGGTTTGGCTGAGTGATA-3′). The PCR product was cloned into the pSK vector (Stratagene; http://www.stratagene.com) and confirmed by sequencing. Plasmid DNA was completely digested using *Hin*dIII or *Bam*HI, and used as a template for transcription with T3 or T7 RNA polymerase, respectively. Images were obtained using an Olympus DP70 digital camera.

Phylogenetic analysis of the TDF1 *subfamily*

The TDF1 protein sequence was used to search for the closest homologues using BLASTP, PSI- and PHI-BLAST programs. Multiple sequence alignment of full-length protein sequences was performed using CLUSTAL W online (http://www.ch.embnet.org/software/ClustalW.html), and this alignment was used to perform neighbor-joining analysis using Mega 3.1 (Kumar *et al.*, 2004). The numbers at the nodes represent percentage bootstrap values based on 1000 replications. The length of the branches is proportional to the expected numbers of amino acid substitutions per site.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Wild-type and *tdf1* anther sections at stage 4.

Figure S2. Transmission electron micrographs of cross-sections through wild-type and *tdf1* anthers.

Figure S3. Alignment of the MYB domain in the TDF1 protein with corresponding domains in proteins from various species that shared the highest homology with the TDF1 protein.

Figure S4. An unrooted neighbor-joining tree of MYB family genes with sequence similarity to *TDF1*.

Figure S5. RT-PCR and real-time PCR analysis of expression of four exine-related genes (*MS2*, *FLP1*, *DEX1* and *NEF1*) in the wild-type and *tdf1* mutant.

Table S1. Molecular markers used for first-pass mapping.

Table S2. Molecular markers used for fine-scale mapping.

Table S3. Primers for RT-PCR.

Table S4. Primers for real-time PCR.

Table S5. Genes downregulated in *tdf1* inflorescences tissue, as identified by microarray analysis.

Table S6. Genes upregulated in *tdf1* inflorescences tissue, as identified by microarray analysis.

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