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Regulation of *Arabidopsis* tapetum development and function by *DYSFUNCTIONAL TAPETUM1* (*DYT1*) encoding a putative bHLH transcription factor

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In flowering plants, male fertility depends on proper cell differentiation in the anther. However, relatively little is known about the genes that regulate anther cell differentiation and function. Here, we report the analysis of a new *Arabidopsis* male sterile mutant, *dysfunctional tapetum1* (*dyt1*). The *dyt1* mutant exhibits abnormal anther morphology beginning at anther stage 4, with tapetal cells that have excess and/or enlarged vacuoles and lack the densely stained cytoplasm typical of normal tapetal cells. The mutant meiocytes are able to complete meiosis I, but they do not have a thick callose wall; they often fail to complete meiotic cytokinesis and eventually collapse. *DYT1* encodes a putative bHLH transcription factor and is strongly expressed in the tapetum from late anther stage 5 to early stage 6, and at a lower level in meiocytes. In addition, the level of *DYT1* mRNA is reduced in the *sporocyteless/nozzle* (*spl/nzz*) and *excess microsporocytes1/extra sporogenous cell* (*ems1/exs*) mutants; together with the mutant phenotypes, this suggests that *DYT1* acts downstream of *SPL/NZZ* and *EMS1/EXS*. RT-PCR results showed that the expression levels of many tapetum-preferential genes are reduced significantly in the *dyt1* mutant, indicating that *DYT1* is important for the expression of tapetum genes. Our results support the hypothesis that *DYT1* is a crucial component of a genetic network that controls anther development and function.

KEY WORDS: Pollen development, *DYT1*, *SPL/NZZ*, *EMS1/EXS*, Tapetum, bHLH Transcription factor

INTRODUCTION

In flowering plants, pollen grains are formed within the anther region of the male reproductive organ, the stamen (Ma, 2005). Molecular genetic studies have revealed that the B and C functions of the well-known ABC model together determine the stamen identity, whereas the C function alone specifies the carpel identity (Coen and Meyerowitz, 1991; Ma, 2005). In *Arabidopsis*, *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) are two essential B function genes, and *AGAMOUS* (*AG*) is required for C function (Coen and Meyerowitz, 1991; Ma, 2005). The *Arabidopsis* anther is a bilaterally symmetrical structure with four lobes. Each lobe comprises four distinct somatic cell layers from outer to inner: they are the epidermis, endothecium, middle layer and tapetum (Goldberg et al., 1993; Sanders et al., 1999). The tapetum surrounds meiocytes and generates many proteins, lipids, polysaccharides and other molecules necessary for pollen development (Goldberg et al., 1993). Accordingly, the tapetum is characterized by active protein synthesis and secretion, a high rate of energy metabolism, and expression of many tapetum-preferential genes (Dickinson and Bell, 1976; Hernould et al., 1998; Liu and Dickinson, 1989; Rubinelli et al., 1998; Scott et al., 2004; Taylor et al., 1998; Zheng et al., 2003).

Several genes have been identified that are required for normal anther development (Ma, 2005). For example, the *SPOROCYTELESS/NOZZLE* (*SPL/NZZ*) gene is required for cell type specification in both male and female reproductive organs (Schiefthaler et al., 1999; Yang et al., 1999). The *spl/nzz* mutant anthers lack the endothecium, middle layer, tapetum and meiocytes. Recently, Ito et al. (Ito et al., 2004) found that *SPL/NZZ* is a direct target of AG, and that ectopic expression of *SPL/NZZ,* independent of AG*,* induces the formation of anther locule with pollen grains (Ito et al., 2004). *SPL/NZZ* encodes a putative transcription factor (Schiefthaler et al., 1999; Yang et al., 1999), suggesting that *SPL/NZZ* regulates anther cell differentiation by activating downstream genes.

Furthermore, *EXCESS MICROSPOROCYTES1/EXTRA SPOROGENOUS CELLS* (*EMS1/EXS*) and *TAPETUM DETERMINANT1* (*TPD1*) are early (pre-meiosis) genes that encode a receptor-like protein kinase and a small protein, respectively, and act in the same genetic pathway to control the tapetal cell identity (Canales et al., 2002; Sorensen et al., 2003; Yang et al., 2003; Yang et al., 2005; Zhao et al., 2002). Recently, the *SERK1* and *SERK2* genes encoding closely related receptor-like protein kinases were shown to have redundant functions in controlling tapetum formation (Albrecht et al., 2005; Colcombet et al., 2005). *MALE STERILITY1* (*MS1*) and *ABORTED MICROSPORES* (*AMS*) act after meiosis and encode a PHD domain-containing protein and a bHLH transcription factor, respectively; they are essential for late stage functions of the tapetum (Ito and Shinozaki, 2002; Sorensen et al., 2003; Wilson et al., 2001). Remarkably, the 'early' gene *EMS1/EXS* is expressed in both stamen and gynoecium, whereas the 'late' genes *MS1* and *AMS* are anther specific. However, it is not known how the 'male-specific' *MS1* and *AMS* expression is regulated.

Here, we report the isolation of a new *Arabidopsis* mutant, which is male sterile and defective in tapetum differentiation and function. Because the mutant has an abnormal tapetum, we named the gene *DYSFUNCTIONAL TAPETUM1* (*DYT1*). *DYT1* encodes a putative bHLH transcription factor and is preferentially expressed in tapetal cells as early as anther stage 5, spatially similar to, but temporally earlier than, *MS1* and *AMS*. Furthermore, our results suggest that

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DYT1 probably acts downstream of *SPL/NZZ* and *EMS1/EXS*, and is required for normal expression of *AMS*, *MS1* and other tapetum-preferential genes. We propose that *DYT1* is a component of a genetic network for tapetum differentiation and function.

MATERIALS AND METHODS

Plant materials and growth

Arabidopsis thaliana plants in this study are in the Landsberg *erecta* (L*er*) background for all experiments with the exception of the mapping of the *DYT1* gene, for which we crossed *dyt1* with Columbia. The *spl* (Yang et al., 1999), *ems1* (Zhao et al., 2002) and *tpd1* (Yang et al., 2003) mutants were kindly provided by Drs W. Yang, D. Zhao and D. Ye, respectively. The *dyt1* mutant was isolated from the progeny of a *Ds* insertional line (ET4262). The *ems1 dyt1* double mutant was obtained by pollinating the *dyt1* pistil with pollen from an *ems1*/+ plant. Plants were grown under long-day conditions (16 hours light/8 hours dark) in a ~22°C growth room.

Characterization of mutant phenotypes

Plants were photographed with a Sony digital camera, DSC-F707 (Sony Corp., Tokyo, Japan). Flower pictures were taken using a Nikon dissecting microscope (Nikon Corp., Tokyo, Japan) with an Optronics digital camera (Optronics, Goleta, CA, USA). To determine pollen viability, mature anthers were stained with the Alexander solution (Alexander, 1969) and photographed under an Olympus BX-51microscope (Olympus, Tokyo, Japan) with a SPOT II RT camera (Diagnostic Instruments, Sterling Heights, MI, USA). Chromosome spreading and DAPI staining were performed as described (Ross et al., 1996). Wild-type and mutant inflorescences were collected and fixed as described (Zhao et al., 2002). Floral buds were embedded in Spurr's resin; semi-thin (0.5 μm) sections were made by using an Ultracut E ultramicrotome (Leica Microsystems, Nussloch, Germany), stained with 0.05% of Toluidine Blue O for 40 to 60 seconds, and photographed under the Olympus BX-51 microscope with the SPOT II RT camera.

Mapping and functional complementation

The *dyt1* mutation was found to be distinct from the *Ds* insertional locus (not shown). The *dyt1* mutant (in the L*er* background) was crossed with Columbia to obtain F1 and F2 seeds. About 500 F2 plants with mutant phenotypes were genotyped by using the SSLP and dCAPS markers (Li et al., 2001). Several predicted genes were found in the mapped region flanked by recombination events; these genes were amplified from wild-type and *dyt1* plants and sequenced. TAIL PCR (Liu and Whittier, 1995) was used to determine the sequences of a retrotransposon insertion in the *dyt1* mutant. To verify the *DYT1*-coding region, we used the primers oMC1872/oMC1873 (see Table 1 for all primer sequences), designed according to the annotated At4g21330 locus, to amplify wild-type L*er* cDNA.

To generate a complementation construct, a 2.7 kb wild-type genomic fragment was amplified by PCR using primers oMC2241 and oMC2242, and then cloned into a modified pCAMBIA1300, yielding pMC2969. The *Agrobacterium* strain GV1301 was transformed with the plasmid pMC2969, then used to transform *dyt1*/+ plants. The seeds of transformed plants were screened for hygromycin-resistant seedlings, which were transferred into the soil. The T1 plants were genotyped to identify homozygous *dyt1* plants with the T-DNA, using a primer (oMC1945) for the retrotransposon insertion at the *DYT1* locus and *DYT1*-gene-specific primer oMC1834. The oMC1823/oMC1873 and oMC2194/oMC1834 primers were used to identify the *dyt1* allele and the transgene, respectively.

RT-PCR and real-time PCR

A set of genes were selected for expression analysis by RT-PCR because they are known to be important for male reproduction: *SPL/NZZ* (Schiefthaler et al., 1999; Yang et al., 1999), *EMS1/EXS* (Canales et al., 2002; Zhao et al., 2002), *TPD1* (Yang et al., 2003; Yang et al., 2005), *AMS* (Sorensen et al., 2003), *MS1* (Ito and Shinozaki, 2002; Wilson et al., 2001), *MALE STERILITY2* (Aarts et al., 1997), *MALE STERILITY5* (Glover et al., 1998), *AtMYB32* (Preston et al., 2004), *AtMYB103* (Higginson et al., 2003), A6 and A9 (Sorensen et al., 2003), *AtMYB33* and *AtMYB65* (Millar and Gubler, 2005), *SOLO DANCERS* (*SDS*) (Azumi et al., 2002), and *ROCK-N-ROLLERS/AtMER3* (*RCK/AtMER3*) (Chen et al., 2005; Mercier et al., 2005). A second group of 16 genes was identified using microarray data of wild-type and *ems1* mutant anthers we had obtained in our laboratory (W.Z., Y.S., A. Wijeratne and H.M., unpublished). The genes that were expressed in the *ems1* anther at levels that were one half or less of those in the wild-type anthers were regarded as candidate tapetum-preferential genes because the *ems1* anthers lack tapetum. In addition, *UBQ1* and *AtMYB4* (Vannini et al., 2004) were used as constitutive expression controls. The primers for RT-PCR and relevant microarray data are provided in the Tables 1 and 2, respectively. The primers for real-time PCR are listed in Tables 1 and 3. The PCR and data treatment were carried out as described previously (Ni et al., 2004). Plant tissue was collected and quickly frozen in liquid nitrogen. The anthers at approximately anther stages 4 to 7 were collected under a dissection microscope. Total RNA was extracted using the RNeasy Plant Kit (Qiagen, Valencia, CA) from young inflorescences (approximately floral stage 1-10). 1-2 μg total RNA was used for reverse transcription according to the manufacturer's instruction to synthesize cDNA, which was used directly as PCR templates (Invitrogen, Carlsbad, CA).

RNA in situ hybridization

Non-radioactive RNA in situ hybridization was performed as described (Li et al., 2004). A 624 bp *DYT1* cDNA fragment was amplified using *DYT1*-specific primers with *Xba*I and *Bgl*II sites at the 5′ end: oMC2238 and oMC2239, respectively. The PCR product was confirmed by sequencing and cloned into the pGEM-T vector (Promega, Madison, WI) to yield plasmid pMC2949. Plasmid DNA was completely digested by *Xba*I or *Bgl*II and used as template for transcription with SP6 or T7 RNA polymerases, respectively (Roche, Mannheim, Germany). Images were obtained using the Olympus BX-51 microscope with the SPOT II RT camera and edited using PHOTOSHOP 5.0 (Adobe, San Jose, CA).

Overexpression of *DYT1*

A *DYT1* cDNA fragment was amplified using gene-specific primers oMC1872 and oMC1873 (Table 1), then cloned into pGEM-T vector to yield plasmid pMC2941. After verification by sequencing, the *DYT1* cDNA fragment was subcloned into pCAMBIA1300 downstream of the CaMV 35S promoter to produce the plasmid pMC2942. An *Agrobacterium* strain C3581 carrying pMC2942 was used to transform wild-type, *ems1*/+ and *spl*/+ plants. The transgenic plants were selected by hygromycin resistance and verified using PCR with oMC1872 and oMC1873. *EMS1* gene-specific primers oMC499 and oMC500, *SPL/NZZ* gene-specific primers oMC2044 and oMC2045, plus Ds5-specific primer oMC490 were used to identify *ems1* and *spl* heterozygous and homozygous plants, respectively. Paraffin sections were prepared as described for in situ experiments (above) and photographed as described for semi-thin sections.

Phylogenetic analysis of the *DYT1* subfamily

The protein sequences of the nine genes from group 9 of the *Arabidopsis* bHLH family, including *DYT1*, were used to search for the closest homologs in both the rice genome (http://tigrblast.tigr.org/euk-blast/index.cgi?project=osa1) and *Populus* genome (http://www.floralgenome.org/cgi-bin/tribedb/tribe.cgi) using both BLAST and TBLASTN programs with a cutoff of 1E-15 (Heim et al., 2003; Toledo-Ortiz et al., 2003). The multiple sequence alignment of full-length protein sequences was performed using ClustalX (Plate-Forme de Bio-Informatique, Illkirch Cedex, France) with a combination of GOP=4.0 and GEP=0.1. The bHLH domain region and additional conserved regions were aligned and used to perform neighbor joining (NJ) analyses with the 'pairwise deletion' option, 'P-distance' model and 1000 bootstrap replicates test using MEGA version 3.0 (Kumar et al., 2004) (http://www.megasoftware.net/index.html).

RESULTS

The isolation of a new male-sterile mutant

To identify new *Arabidopsis* genes important for anther development, we screened for male sterile plants among *Ds* transposon insertional lines. One line was found to segregate normal and sterile plants with an approximate 3:1 ratio. Pollination of the

Table 1. PCR primers in this study

oMC number	Sequence (5′ to 3′)	oMC number	Sequence (5′ to 3′)
271	CGATGAGGAATTCAAAGCGC	2083	CATACTCTGTTTCGCTTCGTTGC
272	CGTCTCCTTCACATTATAACC	2084	GTACTCGTAATTGGCGTCTCATCC
490	CGTTCCGTTTTCGTTTTTTACC	2085	CCATTCCCATGGTCCAAACC
499	AACAAACCCCGTCAGCTTTA	2098	GTTCCAAGGGCACAGGTACATG
500	ACCGGAGAAGTGGTTGTCAC	2099	GGAATGTTCCGTTCGATGGAG
509	GTGGATCCGATCAGAACCTGTATTTCTT	2100	ATGGTATCTCTAAAGTCCCTT
510	CGCTCGAGTTTAAGGTTATATGGCTCAT	2101	CTAGGCACTTATGCCACAATC
1533	GGTAACATTGTGCTCAGTGGTGG	2102	GCGACTTTGGGAATCTGAGTTG
1534	AACGACCTTAATCTTCATGCTGC	2103	GGAATAGTTGAAGCGCAGCTCC
1823	ACTCTAACTGAAATCACATTCAC	2104	GCACTCCAGCATCCATAATGAATC
1834	CAGAGCCATAAGCCGACAATG	2105	ATAGGTCAGAAGCGGCAGGTAAC
1872	ATGGGTGGAGGAAGCAGATTTC	2127	CGGTCATCGGTAGCAACAGTAAC
1873	TTATGGATTGCTTCTCATAACTTC	2128	CCTCTGCCTTTACGGTTAAGACC
1945	GTCAAGAGTTCCTCTCAACATCAC	2129	TTCACGTATGACCCGCTCTTTC
1963	TAGACGGTTTTTCGCCCTTTGACG	2130	ATTGGTCTATTACATCGCTCTCCG
1964	ACGCGCAATAATGGTTTCTGA	2194	GTCGGAGCTAAGATTCTACGATA
2044	CGTTGAGAGGAATGGGTGTAGC	2238	TCGAGCTCTCTAGAATGGGTGGAGGAAGCAGATTTC
2045	AAACTGAAGCTGCTCTAGCGATTC	2239	TCGGCGCGCCAGATCTTTATGGATTGCTTCTCATAACTTC
2046	ATTTGCTCTACGGCATGGCTC	2241	CATCCGCTCGTCAAGGTATAC
2047	GGTAAGGGAAAGTGTTGGCGTAG	2242	CGTGGAGTAGGGAAAGTCAGAG
2048	TCGCTTGTTCCCAGGATAACC	2280	TCATCCCTCGTCTACGCTCC
2049	TTCCAGCAACGAGTTCCTTACG	2281	GTGGCCAGAGTAAGACTCGCA
2050	TCGTGCTCCACCTGGAGTTTAC	2362	TCCAACGGTGGTGGATTTTC
2051	TCCTTGCTTTCTCTTGCTCGATAG	2363	TGGTCCCGTATGATTTGTTGC
2052	GAGGATTACAGAGGTCGCTGAG	2364	CTCATCTCCTTCAAGCGCTCA
2053	GAGCCTTCTTTCCTTTCATCC	2365	AGACACGTAACGCCAACCCA
2054	ACTTAACCGGTCATCAGGTCATG	2366	GGTATCGGCGACACTGCTCT
2055	CGATATCCATCGGTATTCGACTC	2367	CCAATTCTCTCCGGCTCTACA
2056	GGCGACTCATCGACCTATCAAC	2368	TTCCCATCTTCCAGCTTCCA
2057	GACCCAAGAAGTCATAACCAATGC	2369	ACCACGTTCTGATCTTCAGCG
2058	ACAGAGGGATTGATCCAACGAG	2370	AGGCTGAAGACGTTCGGAGA
2059	TGCTGCTACCTCCGACTACATC	2371	GGCTTCGATGGGCTCTTCTA
2060	AAAGGTTCGGTCCCACTTGG	2372	GCTGCCGTTCTCTTTGAAGTCT
2061	ATGTTTCCGGTCACAGGGATG	2373	AAGAGCGGCTACAGCAGCTT
2062	ATGGCGTTTACTCCGAAGATC	2374	AACCGACGAACAACGTTCCT
2063	TCACACGGCAGTCGATATACTG	2375	CCCAGTACGAATTAAGCGCG
2064	GATATTCTCTCGGTGGTGCTGC	2376	CTTGCTGCTATTCTCGTTGCC
2065	TGGCCAGTAATAGGACCAACTAGC	2377	GGCTGCTCGAAGAGCGTTAC
2066	AGCTATAATAACCGGAGGAGCAAG	2378	TGTGAATAAGCATGGTGAGGGT
2067	GAGGAGAGATGCTATTCACACGG	2379	TGTGCAGCCATACGTGCC
2068	TCGTGATCCGAGCACTAGATTG	2380	TGATGACCTTCATTGGAGCG
2069	AGACTTCTTCTGTGCCCTACCAAC	2381	GCGCCAGTTCCTAGCATGTT
2070	CAAGAAAGGTCTCTGGACCGAG	2382	ATTTGCTCGAACGGATCCAG
2071	GAAGATGTGGTTCCATTGAGCAG	2383	CCTGACTCCCATCGAATGAAG
2072	CAGGAAGCAATGGCATAGTTCAC	2384	CAGCACTCGTGTTCTTGTTCCT
2073	TCCTTGCTCATGCTACTTGGAAG	2385	ACGCTGAGATTCACACCGCT
2074	AGTAGTGGTTCCAATGCCATTCAC	2463	GCGGAATCATCGAGTTTAGCA
2075	AAGCAATGCCTCAGAGCCTATG	2464	TGCGTAACTCGTTGATGTAATCG
2076	CATTAAAGCAGGCCACGTCAAG	2465	GATCACGGTCATCGGTAGCA
2077	ACCGAATCGAGCATCTGGTCTAG	2466	TGCGCATACGATGGATCTTG
2078	TGTGATAAGTACGGCCCACTTGTC	2467	CTTGTGGCAACTTCATGGGA
2079	TTAGTGTGAAACTCGTCAACACGC	2468	TTGAGCACCAGACAACAATGG
2080	TCCAAACGTCGCAACGGTC	U1	ACCGGCAAGACCATCACTCT
2081	TCTCAGCTTCTGCTCCCACATG	U2	AGGCCTCAACTGGTTGCTGT
2082	AAAGGCCGAGTCAACTCAGTTATG		

The primers whose sequences have been shown in the text are not listed in this table. U1 and U2 are the RT-PCR primers for *UBQ1*.

mutant pistil with wild-type pollen indicated that the mutant is female fertile. The mutant was named *dysfunctional tapetum1* (*dyt1*) because of its anther defects (see below). The vegetative growth of the *dyt1* mutant appeared normal (Fig. 1A,B) and most mutant floral organs were also normal with the exception of shorter stamen filaments and smaller anthers (Fig. 1C,D). There were no pollen grains on the anther surface of opened flowers (Fig. 1G). Occasionally mutant siliques contained a few seeds (not shown), possibly owing to residual gene function (see below).

The *dyt1* mutant is defective in tapetum development

Detailed analyses were performed to understand the mutant developmental defects. Chromosome spread experiments were performed and revealed that normal meiotic features could be observed in the mutant meiocytes, demonstrating that meiotic nuclear divisions can proceed normally (Fig. 2). We also generated semi-thin anther sections to investigate the mutant anther development (Fig. 3). From anther stage 1 to 3, the *dyt1* anthers

Table 2. Microarray data of the genes selected in RT-PCR

Gene name	Locus	oMC	Av-WT	Av-ems1	Description
SPL	At4g27330	2044/2045	402.50	283.52	SPOROCYTELESS/NOZZLE
EMS1	At5g07280	509/511	1062.95	146.89	EXCESS MICROSPOROCYTES1/EXTRA SPOROGENOUS CELLS
TPD1	At4g24972	2046/2047			TAPETUM DETERMINANT1
AMS	At2g16910	2048/2049	348.88	9.39	ABORTED MICROSPORES
MS5	At4g20900	2050/2051	75.46	23.20	Male sterility MS5; pollenless3
MS5 like	At5g44330	2052/2053	90.26	1.51	MS5 family protein
MS1	At5g22260	2054/2055			Male sterile protein 1
AtMYB32	At4g34990	2056/2057	300.93	63.67	MYB-like protein myb-related protein Y49
AtMYB4	At4g38620	2058/2059	161.19	179.94	Putative transcription factor (MYB4)
MS2	At3g11980	2060/2061	690.78	12.25	Male sterility protein 2 (MS2)
LPT12	At3g51590	2062/2063	446.85	40.33	Lipid transfer protein
ATSTP2	At1g07340	2064/2065	174.90	60.65	Hexose transporter
ATA1	At3g42960	2066/2067	2090.38	29.80	Alcohol dehydrogenase (ATA1)
PAB5	At1g71770	2068/2069	823.25	106.96	Polyadenylate-binding protein 5
	At3g28470	2070/2071	758.75	22.98	Myb transcription factor
	At3g13220	2072/2073	794.97	14.42	ABC transporter
	At1g69500	2074/2075	1636.47	16.85	Cytochrome P450
	At3g23770	2076/2077	497.53	17.17	β-1,3-glucanase
	At1g01280	2078/2079	430.88	1.96	Cytochrome P450
FLF	At5g10140	2080/2081	251.54	20.77	MADS box protein FLOWERING LOCUS F (FLF)
	At3g60580	2082/2083	245.27	22.71	Zinc finger protein-like ZPT3-3, Petunia hybrida
ATMYB103	At5g56110	2084/2085	93.20	36.35	Regulate the tapetum and trichome development, anther specific gene
A6	At4g14080	2098/2099	3692.11	41.2	Glycosyl hydrolase family 17 protein / anther-specific protein (A6)
A9	At5g07230	2100/2101	5099.79	81.5	Protease inhibitor/seed storage/lipid transfer protein, tapetum-specific protein A9
MYB33	At5g06100	2102/2103	238.76	176.9	Myb family transcription factor (MYB33)
MYB65	At3g11440	2104/2105	335.48	203.4	Myb family transcription factor (MYB65)
	At1G06170	2127/2128	938.35	28.1	Basic helix-loop-helix (bHLH) family protein
	At2G31210	2129/2130	176.08	20.6	Basic helix-loop-helix (bHLH) family protein
SDS	At1g14750	271/272	80.48	27.18	SOLO DANCER, a putative cyclin
RCK/AtMER3	At3g27730	1963/1964	81.68	37.16	ROCK-N-ROLLERS/AtMER3, a ATP-dependent DNA helicase
UBQ1	At3g52590	U1/U2	4509.45	4007.97	UBIQUITIN EXTENSION PROTEIN 1 (UBQ1)

The genes were selected as described.
The microarray intensity value of the gene for the *ems1/exs* anther is at most half of that from the wild-type anther. Genes known to be important for early anther development are regulatory genes *SPL/NZZ, TPD1, AtMYB33* and *AtMYB65*. Constitutive controls are *AtMYB4* and the *UBQ1*.

appeared normal (data not shown). At stage 4, the mutant anther was similar to the wild-type anther in cell layer and cell number, but had a slightly different shape from the wild-type anther and was vacuolated in more cells than normal. In addition, the mutant sporogenous cells appeared more deeply stained than wild-type cells (Fig. 3A,B). At early stage 5, the *dyt1* anther lobe had four cell types interior to the epidermis, similar to the wild type. The wild-type anther at late stage 5 (Fig. 3E) was also vacuolated in more cells than earlier and had deeply stained meiocytes. Therefore, mutant anthers at stage 4 and early stage 5 exhibited these morphological features precociously (Fig. 3A-D).

In the wild-type anther at late stage 5 (Fig. 3E), the tapetum had significantly larger cells than earlier. In the mutant (Fig. 3F), additional vacuoles were observed in tapetal cells, with a reduction of the cytoplasm. The vacuoles in the wild-type tapetum at this time are fewer and smaller than those in the mutant. In addition, the mutant middle layer maintained its thickness with vacuolation, unlike the reduced thickness of the wild-type middle layer (Fig. 3E,F). At stage 6, a thick callose wall forms around the meiocytes (Fig. 3G). By contrast, the mutant meiocytes had very thin callose cell walls (Fig. 3G,H). At this stage, most of the mutant meiocytes were undergoing meiosis (Fig. 2), but some of them had collapsed. At stage 7 and stage 8, the completion of wild-type meiosis results in the formation of tetrads and then microspores, but mutant tapetal and middle layer cells swelled with expanded vacuoles and filled the center of the locules where the meiocytes had collapsed and degraded (Fig. 3I-L).

DYT1 encodes a putative bHLH transcription factor

To gain further insights into its function, we cloned the *DYT1* gene. The *dyt1* mutant was from a *Ds* insertional line, but the *dyt1* mutation was genetically separable from the *Ds* element (data not shown). To clone the gene, we mapped the *dyt1* locus by analyzing ~500 mutant F2 progenies from a cross between the *dyt1* mutant (L*er*) and Columbia wild-type plant. The mapping results indicated that the *DYT1* gene was on chromosome 4, between At4g21220 and At4g21360 (data not shown). We sequenced candidate genes in this region from both wild-type and the *dyt1* mutant, and found that only one locus, At4g21330, had an insertion mutation 109 bp upstream

Table 3. Primers used in real-time PCR

Gene name	Locus	oMC number	Product length
SPL	At4g27330	2362/2363	200
EMS1	At5g07280	2364/2365	101
TPD1	At4g24972	2366/2367	201
AMS	At2g16910	2368/2369	201
MS1	At5g22260	2370/2371	201
MYB33	At5g06100	2378/2379	201
MYB65	At3g11440	2380/2381	201
bHLH	At1g10610	2384/2385	201
DYT1	At4g21330	1872/1834	153
bHLH	At2g31210	2463/2464	201
bHLH	At1g06170	2465/2466	201
WD-40	At1g15850	2467/2468	201
ACT2	AT3G18780	1533/1534	108

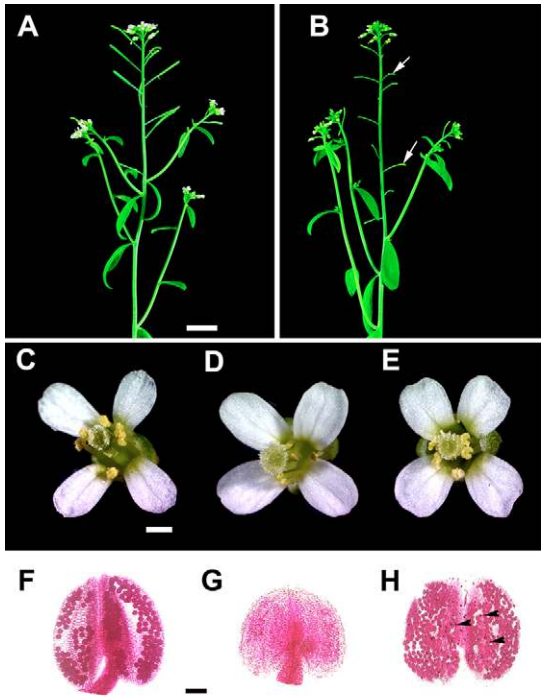

Fig. 1. Phenotypes of the wild type (L*er*), *dyt1* mutant and transgenic plants for complementation. (**A**) A L*er* plant. (**B**) A *dyt1* plant, with very small siliques (arrows). (**C**) A L*er* flower. (**D**) A *dyt1* flower. (**E**) A flower of the *dyt1* plant with the *DYT1* transgene. (**F**) A wild-type anther, with viable pollen grains (stained). (**G**) A *dyt1* anther, no viable pollen. (**H**) An anther from a *dyt1* plant with the *DYT1* transgene, with a large number of viable pollen grains and some microspores (arrowheads). Scale bars: 10 mm in A,B; 500 μm in C-E; 20 μm in F-H.

of the predicted translation initiation codon (Fig. 4A). We performed TAIL PCR to obtain the DNA at the 5′ and 3′ ends of the insertion and found that they each partially matched the same region of a putative retro-transposon At5g33382 in the Columbia genomic sequence. Further PCR and sequence analysis indicated that the insertion at the *DYT1* locus matched exactly and completely to a seemingly intact retro-transposon in the L*er* genome (W.Z., Y.S. and H.M., unpublished). To verify that At4g21330 is *DYT1*, we cloned an At4g21330 genomic fragment into a modified pCAMBIA1300 plasmid and used it to transform *dyt1*/+ plants. Fifty independent transgenic plants were analyzed; 12 lines were found to be homozygous for the *dyt1* insertion. All 12 lines were fertile, including nine lines with normal fertility (Fig. 1E,H), confirming that At4g21330 is the *DYT1* gene.

To determine the *DYT1* cDNA sequence, we performed an RT-PCR experiment with floral mRNA, and obtained a 624 bp product with an identical sequence to that indicated by the annotation. Additional RT-PCR experiments using primers that match sequences just beyond the annotated region yielded no product, confirming the annotated *DYT1*-coding region. The *DYT1* gene encodes a putative transcription factor of 207 amino acid residues with a basic helix-loop-helix (bHLH) domain. According to the annotation (http://www.arabidopsis.org), the bHLH domain spans the region from Phe_{29} to Gln_{78} (Fig. 4B) (Toledo-Ortiz et al., 2003). Interestingly, a preliminary search of public databases using the DYT1 protein sequence with the BLAST program showed that the DYT1 protein has the highest similarity to the *Arabidopsis* AMS protein (Sorensen et al., 2003).

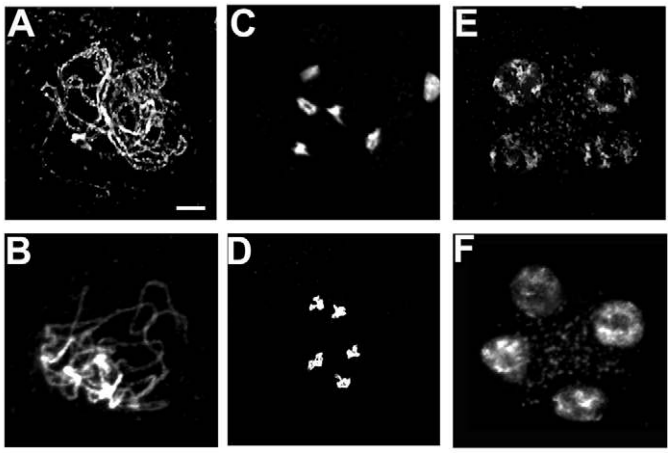

Fig. 2. Meiosis in L*er* and *dyt1* anthers. (**A**,**B**) Pachytene images of the L*er* (A) and *dyt1* (B) meiocytes with condensed chromosomes. (**C**,**D**) Diakinesis images of the L*er* (C) and *dyt1* (D) meiocytes, each with five bivalents of attached homologous chromosomes. (**E**,**F**) Telophase II images of the L*er* (E) and *dyt1* (F) meiocytes. Both show four decondensed chromosome clusters. Scale bar: 5 μm.

To gain additional insights into the phylogenetic relationship between *DYT1*, *AMS* and other close homologs, we performed phylogenetic analysis of bHLH genes, including *DYT1*, *AMS* and a recently reported rice gene, *UNDEVELOPED TAPETUM* (*OsUDT1*), which is required for normal tapetum development (Jung et al., 2005). The phylogenetic analyses with the bHLH domain region alone, with both bHLH domain and conserved regions (Fig. 4C), or with the full-length sequences yielded trees with very similar topologies (others not shown). Our result indicates that *DYT1*, *AMS*, *OsUDT1*, Os02g02820 and *PtDYT1-Like* (*Populus trichocarpa*) form a separate clade within a group of related members of the bHLH gene family. Among them, *AMS* and Os02g02820 are supported as an orthologous pair, as are *DYT1* and *PtDYT1-like*. In *Arabidopsis*, *DYT1* and *AMS* are most closely related to each other, in agreement with the preliminary BLAST results. The rice *OsUDT1* gene could be the ortholog of the *DYT1* and *PtDYT1-like* genes.

DYT1 is preferentially expressed in tapetal cells

To determine the *DYT1* expression pattern, we performed a real-time PCR experiment (Fig. 5A). *DYT1* expression was detected at a low level in young inflorescences with meiotic cells and in siliques, and at a high level in the wild-type anthers from stage 4 to 7, but not in vegetative tissues or in the post-meiotic stage-12 flower. In addition, RNA in situ hybridization experiments showed that *DYT1* expression was detectable in the floral meristem and early anther primordia (Fig. 5C), and in archesporial cells at stage 2 (not shown). From stage 4 to early stage 5, a weak signal was detected in precursors of the middle layer, tapetum and meiocytes (Fig. 5D). From late stage 5 (Fig. 5E,H) to early stage 6, the *DYT1* expression reached its highest level in the tapetum and is at a low level in meiocytes. However, at late stage 6, the *DYT1* expression signal was drastically reduced to background levels (Fig. 5F). The *DYT1* expression pattern is consistent to the observed tapetal defects in the *dyt1* mutant anther. In the gynoecium, a weak signal was detected (data not shown). In the *dyt1* anther, there was a low level expression, which was not specific to the tapetum (Fig. 5I). Therefore, the insertion upstream of the *DYT1* ATG codon caused a great

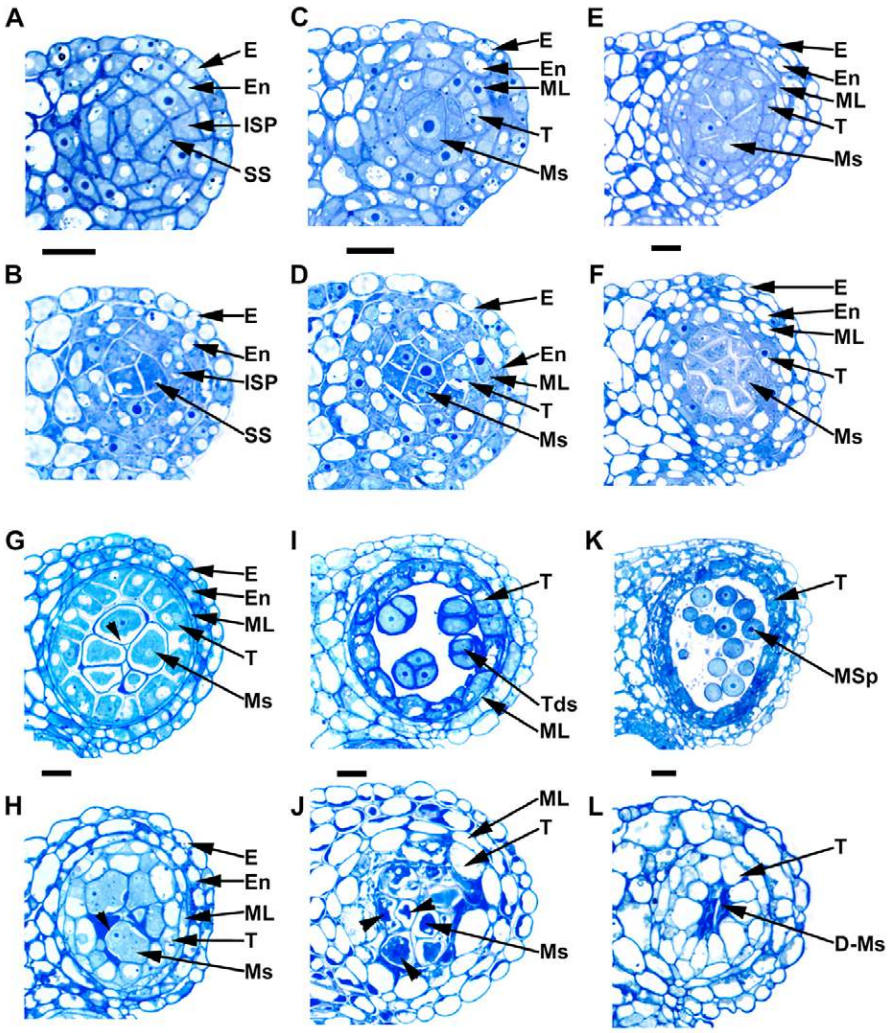

Fig. 3. Anther development from stage 4 to stage 8 in the wild type (L*er*) and *dyt1* mutant. Locules from anther sections: (A,C,E,G,I,K) wild type; (B,D,F,H,J,L) *dyt1* mutants. (**A**,**B**) Stage 4 anthers. (**C**,**D**) Late stage 4 or very early stage 5. Vacuolization in the *dyt1* mutant occurred in more cells and the vacuoles were larger than those in the wild type. (**E**,**F**) Stage 5 anthers, with more and larger vacuoles in the tapetum and middle layer of the *dyt1* anther (F) than the wild type (E). (**G**,**H**) Stage 6 anthers. The vacuolization in cells of the mutant tapetum and middle layer became more extensive. The mutant meiocytes had a much thinner callose layer around them (arrowheads). (**I**,**J**) Stage 7 anthers. Wild-type meiocytes undergo cytokinesis to form tetrads. In *dyt1* anther, the tapetum and middle layer cells were swollen and had excess vacuolization, and meiocytes generally collapsed before cytokinesis (arrowheads). (**K**,**L**) At stage 8, in the wild-type anther locules, microspores were released from the tetrad; in the *dyt1* anther, almost all meiocytes degenerated. E, epidermis; En, endothecium; ISP, inner secondary parietal cells; SS, secondary sporogenous cells; ML, middle layer; T, tapetum; Ms, meiocytes; Tds, tetrads; Msp, microspores; D-Ms, degenerated meiocytes. Scale bars: 10 μm.

reduction of the level of *DYT1* expression in the tapetum. The weak expression suggests that the *dyt1* allele may have some residual function, which might account for the occasional seeds that were observed.

DYT1 expression is positively regulated by *SPL/NZZ* and *EMS1/EXS*

To test whether the *DYT1* expression was affected by any known early anther development genes, we performed real-time PCR experiments with RNA from *spl* and *ems1* mutant floral buds. Our results showed that the *DYT1* expression was barely detectable in the *spl* inflorescences and that the expression level in the *ems1* inflorescences was only ~17% of the wild-type level (Fig. 5B). These results suggest that *DYT1* might be downstream of *SPL/NZZ* and *EMS1/EXS*. We performed RNA in situ experiments to verify this possibility. In the *spl* anther, little *DYT1* expression could be detected (Fig. 5J). A weak signal could be detected in the meiocytes in the *ems1* anther at late stage 5 (Fig. 5K) and early stage 6, but, unlike the wild-type anther, the strong tapetal signal was not found in the *ems1* anther (compare Fig. 5H with 5K). Therefore, the strong *DYT1* expression in the tapetum requires *EMS1/EXS*. Both the real-time PCR and the RNA in situ hybridization results indicate that *SPL/NZZ* is essential for *DYT1* expression and that *EMS1/EXS* promotes the high-level *DYT1* expression specific to the tapetum.

The expression of anther genes in the *dyt1* mutant is altered

The finding that *DYT1* encodes a bHLH-type putative transcription factor suggests that *DYT1* controls gene expression required for normal anther development. To test this hypothesis, we performed RT-PCR using primers for anther genes. We obtained results for a total of 32 genes (Fig. 6); among these, *SPL/NZZ*, *EMS1/EXS* and *TPD1* are known early anther development genes (Canales et al., 2002; Schiefthaler et al., 1999; Yang et al., 1999; Yang et al., 2003; Yang et al., 2005; Zhao et al., 2002). The other genes were chosen for their tapetum-preferential expression according to either previous reports or to gene expression data obtained in our laboratory (Table 2). We found that for 21 genes out of 32, the expression was significantly reduced in the *dyt1* mutant compared with the wild type (Fig. 6), indicating that indeed the normal expression of a large number of genes depends on the *DYT1* gene function. In particular, two regulatory genes, *MS1* and *AMS*, which are important for tapetum development (Ito and Shinozaki, 2002; Sorensen et al., 2003; Wilson et al., 2001), exhibited greatly reduced levels of expression, suggesting that *MS1* and *AMS* act downstream of *DYT1*. By contrast, some tapetum preferential genes, such as A6 and A9, were still expressed in the *dyt1* mutant at slightly reduced levels. In addition, the expression of *SPL*, *TPD1*, *EMS1/EXS*, *AtMYB33* and *AtMYB65* was not dramatically different in the *dyt1* mutant, indicating that their expression does

not require *DYT1*. To verify the RT-PCR results, selected genes were further analyzed using real-time PCR and the results (Fig. 6D) support the conclusion that *AMS* and *MS1* expression requires *DYT1* function.

In addition to the tapetum-preferential genes, we also tested the expression of two meiosis-specific genes: *SDS* and *RCK/AtMER3* (Azumi et al., 2002; Chen et al., 2005; Mercier et al., 2005). Although the expression level of *SDS* did not change, the expression of *RCK/AtMER3* was significantly reduced. *SDS* is known to act earlier than *RCK/AtMER3* in prophase I, suggesting that the *dyt1* mutation might affect the expression of late prophase I genes more than early prophase I genes.

DYT1 is not sufficient for tapetum development

Both *ems1/exs* and *dyt1* mutations affect tapetum development. Previous reports and our results suggest that *DYT1* acts downstream of *EMS1/EXS*. To test this further, we generated the *ems1 dyt1* double mutant and examined its early anther development. We found that the double mutant resembles the *ems1* mutant in that the double mutant anther also completely lacks the tapetum (Fig. 7), suggesting that indeed *EMS1/EXS* is upstream of *DYT1* in the same pathway. In addition, to test whether *DYT1* is sufficient to alter anther development, we generated transgenic plants carrying a *35S-DYT1* fusion in wild-type, *spl/nzz* and *ems1/exs* backgrounds. Transgenic lines with *DYT1* overexpression were identified by RT-PCR (Fig. 6E; data not shown) and analyzed for their anther morphology. In all cases, the *35S-DYT1* transgenic anthers had morphologies resembling those of the corresponding genotypes without the transgene (see Fig. S1 in the supplementary material). Therefore, the overexpression of *DYT1* was not able to suppress the *spl/nzz* and *ems1/exs* mutant phenotypes, indicating that other genes acting downstream of *SPL/NZZ* and *EMS1/EXS* are probably required for normal tapetum development. Because *DYT1* was found to be required for normal expression of a number of tapetum genes, we tested selected genes by real-time PCR to determine whether the *35S-DYT1* transgene was able to stimulate the expression of these genes. Our results indicate that the *35S-DYT1* transgene did not alter the expression of these genes substantially (Fig. 6E), consistent with the morphological results.

Our results and previous studies suggest that additional genes other than *DYT1* probably function downstream of *SPL/NZZ* and *EMS1/EXS* to promote tapetum development. It is possible that some of these genes might encode regulatory proteins. For example, the *AtMYB33* and *AtMYB65* genes are known to be important for tapetum development, and might also function downstream of *SPL/NZZ* and *EMS1/EXS*. To test these ideas, we examined the expression in wild-type and mutants of these genes and of other genes, which were identified as tapetum-preferential from our microarray data (W.Z., Y.S. and H.M., unpublished) and encode bHLH or WD-40 proteins. Real-time PCR results (Fig. 6F) indicate that the expression of two bHLH genes (At2g31210 and At1g06170) was greatly reduced in the *spl* mutant, and one of them (At2g31210) was expressed at a lower level in the *ems1* mutant. By contrast, the expression levels of *AtMYB33*, *AtMYB65* and two other genes were either nearly normal or increased.

DISCUSSION

DYT1 is important for tapetum development and function

Our analysis of the *dyt1* mutant phenotype indicates that *DYT1* is required for normal tapetum development following its formation. Furthermore, *DYT1* is expressed preferentially in the tapetum at late stage 5, consistent with its role in this layer. The *EMS1/EXS, SERK1/SERK2* and *TPD1* genes (Albrecht et al., 2005; Canales et al., 2002; Colcombet et al., 2005; Yang et al., 2003; Yang et al., 2005; Zhao et al., 2002) are important for the formation of the tapetal layer; therefore, these genes probably act at earlier stages than *DYT1*, as supported by the observed strong tapetal expression of *EMS1/EXS* and *TPD1* (Canales et al., 2002; Yang et al., 2003; Zhao et al., 2002)

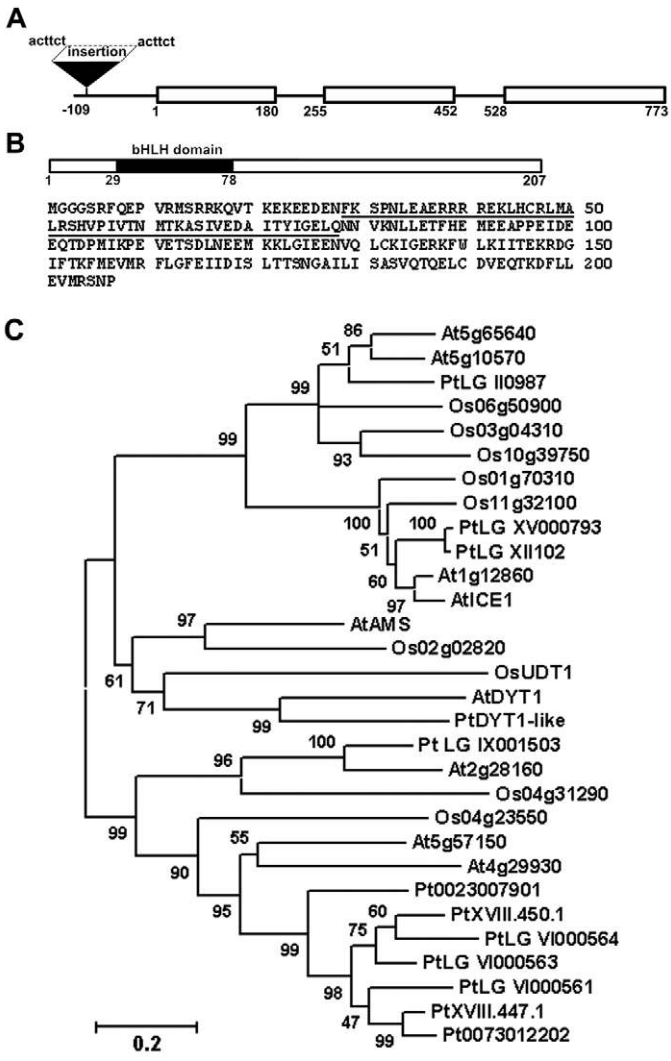

Fig. 4. The *DYT1* gene structure and annotated conserved domain. (**A**) The genomic region of the *DYT1* gene. The *dyt1* insertion is flanked by a direct repeat of 6 bp ACTTCT, which correspond to nucleotides 109-104 upstream of the annotated ATG (nucleotides 1-3) codon. The *DYT1* gene has three exons represented as white boxes. (**B**) The annotated amino acid sequence of the *DYT1* protein, with 207 amino acid residues. From Phe_{29} to the Gln_{78} is the conserved bHLH domain, which corresponds to the black region in the schematic box image and is underlined in the amino acid sequence. (**C**) An unrooted neighbor-joining tree of *Arabidopsis*, rice, and *Poplar* bHLH genes in the same subfamily as *DYT1*. Gene ID numbers starting with 'At' indicate genes from *Arabidopsis thaliana*; names of genes with functional information are given after the gene ID numbers. Gene ID numbers starting with 'Os' indicates genes from rice (*Oryza sativa*); UDT1 is shown as OsUDT1. 'Pt' indicates genes from poplar (*Populus trichocarpa*), with temporary names given according to gene ID from the Floral Genome Project. Bootstrap values are shown near the relevant nodes.

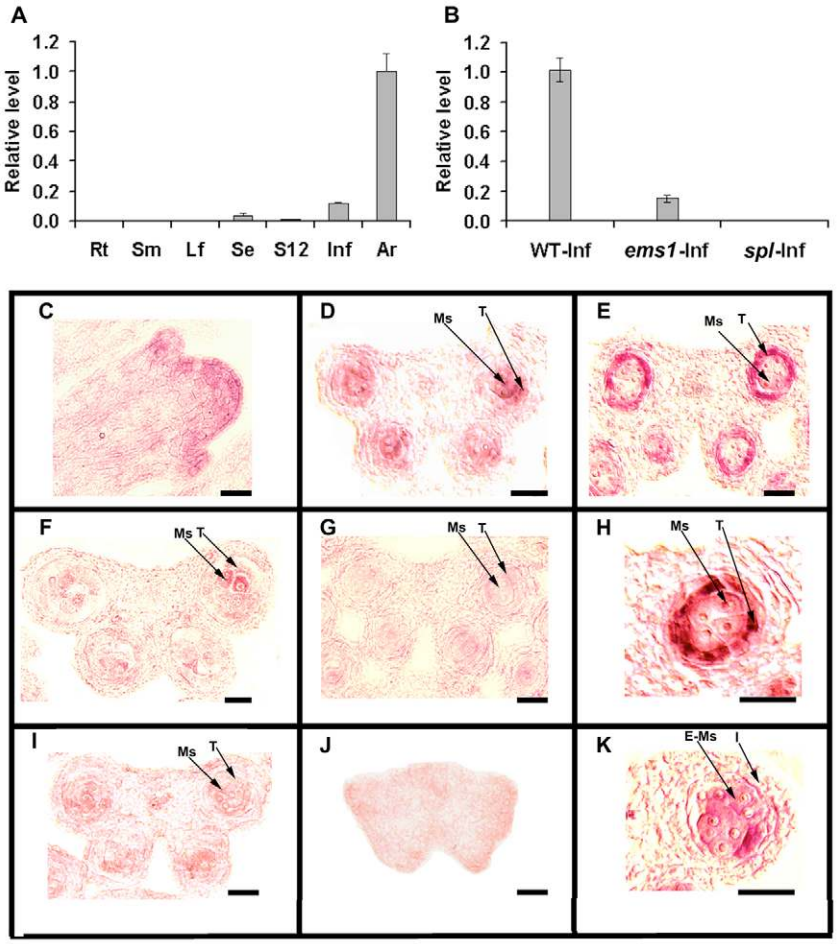

Fig. 5. The *DYT1* expression pattern.
(**A**) Detection of *DYT1* expression using real-time PCR in L*er* background. *DYT1* expression was not detected in any vegetative tissues or stage-12 flower, was detected at low levels in the young inflorescence and siliques, and was at the highest level in the anther. (**B**) Detection of *DYT1* expression using real-time PCR in L*er*, *ems1* and *spl* inflorescences. The *DYT1* expression was not detected in *spl*, but was detected in *ems1* at about 17% of the normal level. (**C-K**) RNA in situ hybridization with a *DYT1* probe. (C-F,H) *DYT1* expression in the L*er* background. (C) The *DYT1* signal was detected in the floral meristem. (D) An anther at stage 4 to early stage 5. The *DYT1* signal can be detected mainly within the newly formed tapetum and meiocytes. (E,H) At late stage 5, a strong signal is detected in the tapetal cell layer, whereas the signal in the meiocytes is much weaker. (F) At late stage 6, the *DYT1* signal is greatly reduced, with residual expression in some meiocytes. (I) A *dyt1* mutant anther at late stage 5; the *DYT1* signal is low and non-specific in the entire anther. (J) A *spl* mutant anther at late stage 5. The *DYT1* signal is at the background level. (K) An *ems1* mutant anther locule at late stage 5. Uniformly weak *DYT1* signal can be detected in meiocytes and little signal in cells surrounding the meiocytes. (G) The sense control with a L*er* late stage 5 anther. Only background signal is seen. Rt, root; Sm, stem; Lf, leaf; Se, silique; S12, stage 12 flower; Inf, inflorescence; Ar, anther; WT-Inf, wild-type inflorescence; *ems1*-Inf, *ems1* inflorescence; *spl*-Inf, *spl* inflorescence; T, tapetum; Ms, meiocytes; I, indeterminate cells; E-Ms, excess meiocytes. Scale bars: 20 μm in C-K.

prior to the strong *DYT1* expression in the tapetum. Our expression and double mutant analyses support the hypothesis that *DYT1* acts downstream of *SPL/NZZ* and *EMS1/EXS*.

AMS and *MS1* are also important for tapetum function, but they are required at post-meiotic steps (Ito and Shinozaki, 2002; Sorensen et al., 2003; Wilson et al., 2001). In *ams* anthers, meiosis is normal and microspores are formed; however, the newly formed microspores soon degenerate. In *ms1* anthers, pollen development is abnormal and no normal mature pollen is produced. Compared with *AMS* and *MS1*, *DYT1* acts at an earlier stage, before the completion of meiosis. Therefore, *DYT1* is required for a key step in tapetum development. In other words, tapetum development requires the combined activities of the *EMS1/EXS*, *SERK1/SERK2*, *TPD1*, *DYT1*, *AMS* and *MS1* genes: first *EMS1/EXS*, *SERK1/SERK2* and *TPD1* specify the tapetal cells as distinct from meiocytes at the time of the cell division that form the tapetal cells, then *DYT1* is required to promote correct tapetal cell fate for proper function, and finally *AMS* and *MS1* further regulate the tapetal cell function supporting normal microspore development.

DYT1 is required for normal levels of the expression of tapetum genes

As *DYT1* encodes a bHLH putative transcription factor, it is likely that it regulates the expression of tapetal genes. We found that the expression of a majority of tapetum-preferential genes tested depends on *DYT1*. The greatly reduced expression in the *dyt1* mutant of many tapetum-preferential genes, particularly those encoding transcription factors, supports the idea that *DYT1* is a key component of a regulatory step in normal tapetum development. The *Arabidopsis* bHLH gene family has over 140 members, making this the second largest gene family of transcription factors (Toledo-Ortiz et al., 2003). Although *DYT1* and *AMS* clearly have non-redundant and distinct functions, they are members of the same subfamily. Phylogenetic analysis performed here including the closest rice homologs of *DYT1* indicates that the rice gene, *OsUDT1*, is also a member of this subfamily and a putative *DYT1* ortholog. A mutation in the *OsUDT1* gene results in a defective tapetum (Jung et al., 2005), similar to the *dyt1* tapetum. In addition, the expression pattern of the *OsUDT1* gene (Jung et al., 2005) is different from that of *DYT1*. Therefore, this bHLH subfamily contains phylogenetically and functionally distinct members.

Some tapetum marker genes, such as A6 and A9, were expressed in the *dyt1* mutant at slightly reduced levels. It is possible that other genes are also important for the activation of some tapetum genes. In addition to *DYT1*, previous reports described mutants with similar phenotypes, such as *fat tapetum*, *gne1* and *gne4* (Sanders et al., 1999; Sorensen et al., 2002). Although the molecular nature of the *FAT TAPETUM*, *GNE1* and *GNE4* genes are unknown at this time, it is likely that additional loci are involved in defining the tapetal cell fate. In other words, *DYT1* is essential, but not sufficient, for the specification of the tapetum identity, as supported by our observation that overexpression of *DYT1* did not alter anther phenotypes in wild-type or mutant backgrounds. Recently, it was reported that the *AtMYB33* and *AtMYB65* genes redundantly facilitate tapetum development, with the double mutant having tapetum defects before the completion of meiosis (Millar and

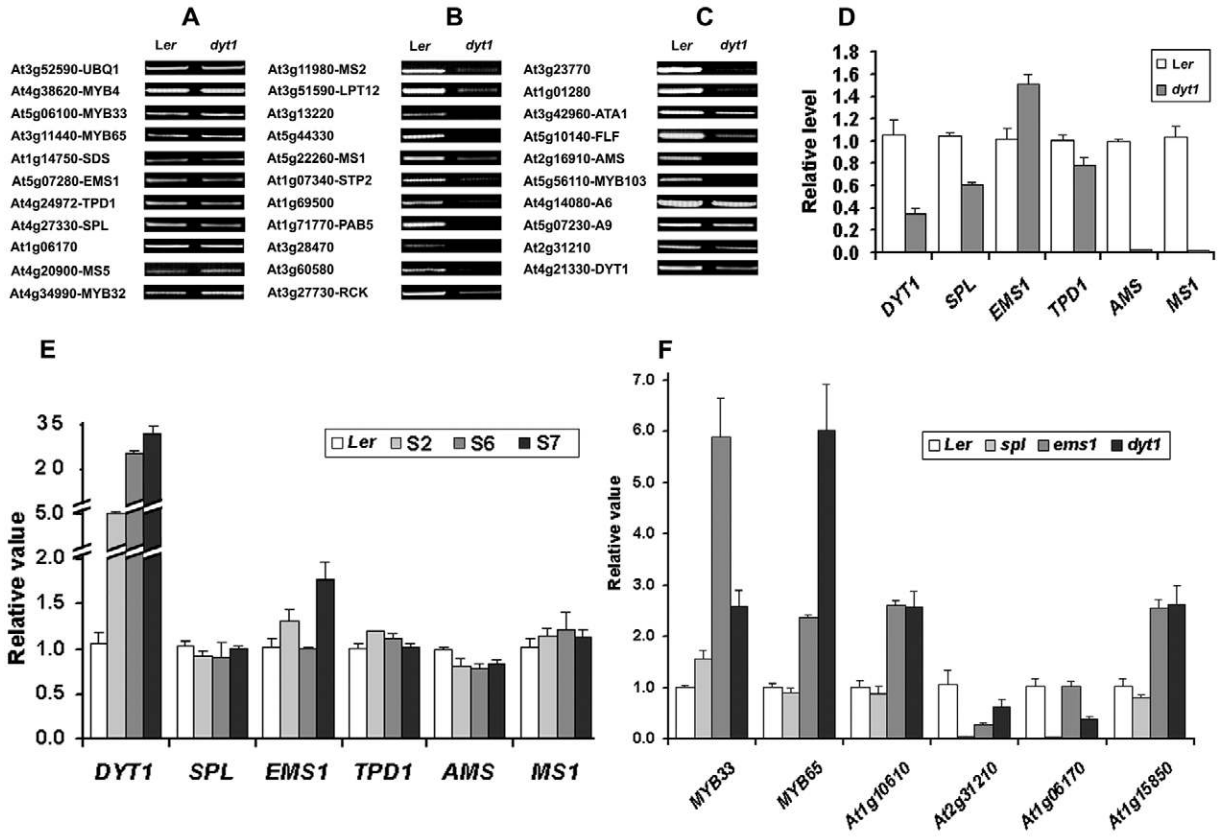

Fig. 6. Expression of anther and tapetum genes in the wild type and *dyt1* mutant. (**A**) Each of the 11 genes, including the controls *UBQ1* and *MYB4*, show either normal or increased expression in the *dyt1* mutant background compared with the wild type. (**B**,**C**) Twenty-one genes show significantly decreased expression in the *dyt1* mutant background. (**D**) Real-time PCR of selected anther genes in both wild-type and *dyt1* backgrounds. (**E**) Effects of overexpression of *DYT1* on selected anther genes. No significant effects were observed by *DYT1* overexpression, even though the *DYT1* expression levels were elevated. (**F**) Expression pattern of selected putative regulatory genes in *spl, ems1* and *dyt1* background.

Gubler, 2005). It is known that bHLH transcription factors can form homodimers or heterodimers with other bHLH proteins. In some cases, it has been shown that bHLH proteins can form complexes with MYB proteins and WD-40 proteins (Ramsay and Glover, 2005). It is possible that the AtMYB33 and AtMYB65 proteins may form heterodimers with DYT1 to regulate tapetum-preferential gene expression. This idea is consistent with our result that the expression of these two MYB genes is not altered in the *dyt1* mutant.

Our analysis of gene expression in the *spl* mutant suggest that two other genes (At2g31210 and At1g06170) encoding bHLH proteins might also act downstream of *SPL/NZZ* and that these bHLH genes might in turn regulate other genes. Indeed, an examination of upstream sequences (500 bp from the ATG codon) of 163 putative tapetum-preferential genes (identified from microarray data, W.Z., A. Wijeratne, Y.S. and H.M., unpublished) revealed that 143 of them have potential binding sites for bHLH proteins, and 69 genes have putative MYB-binding sites, with 59 genes have both types of elements. These observations support the hypothesis that bHLH and MYB proteins regulate overlapping but non-identical sets of tapetum-preferential genes. In particular, 18 out of 21 genes that exhibit reduced expression in the *dyt1* mutant have putative bHLH-binding sites; in addition, between 500-1000 bp upstream of the *AMS* ATG codon, there is a bHLH-binding consensus site. Further experiments are needed to test whether these genes are direct targets of DYT1 and/or other bHLH proteins.

DYT1 supports completion of meiosis

It is known that a functional tapetum is required for normal pollen development following meiosis, as shown by molecular ablation studies and the characterization of mutants such as *ams* (Aarts et al., 1997; Ito and Shinozaki, 2002; Sorensen et al., 2003; Wilson et al., 2001). In the *ems1/exs*, *serk1 serk2* and *tpd1* mutants, the tapetum is missing and excess meiocytes occupy the position of the tapetum (Albrecht et al., 2005; Colcombet et al., 2005; Yang et al., 2003; Zhao et al., 2002). Nevertheless, meiotic nuclear divisions still occur in the *ems1* mutant, indicating that the tapetum is not required for meiotic nuclear events. However, the *ems1* meiocytes do not undergo cytokinesis, suggesting that tapetum might be needed for the completion of meiosis. The *dyt1* mutant phenotypes provide further support for this idea. In addition to a morphologically abnormal tapetum, the *dyt1* mutant meiocytes were found to have thinner callose cell walls than normal, suggesting that normal tapetum function is needed for the formation of the callose wall. Nevertheless, *DYT1* expression was detected at a low level in the meiocytes and expression of some meiotic genes was reduced; therefore, it is possible that *DYT1* might also function in meiocytes.

A model for *DYT1* function and the control of tapetum identity

This and previous studies support a model for the genetic control of tapetum development and function (Fig. 8), although evidence for biochemical interactions is not yet available. *SPL/NZZ* is required

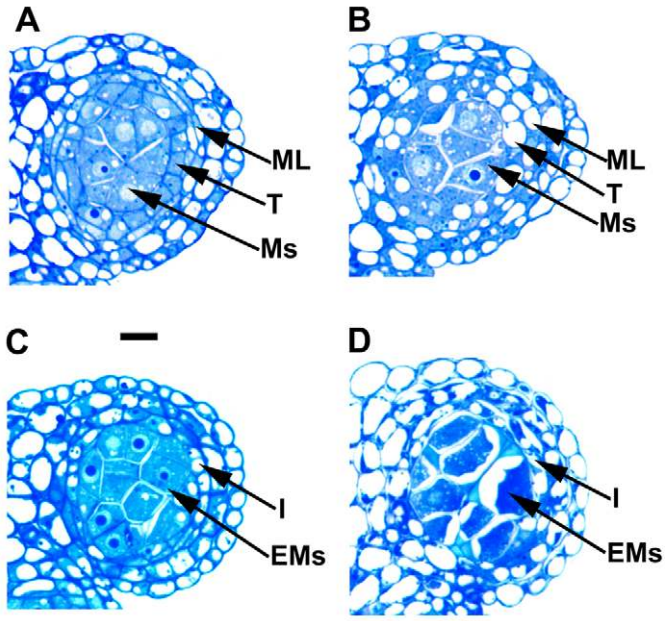

Fig. 7. The *ems1 dyt1* double mutant anther morphology at late stage 5. (**A**) Wild-type. (**B**) *dyt1*. (**C**) *ems1*. (**D**) *ems1 dyt1* double mutant. The double mutant lacks the tapetum. T, tapetum; ML, middle layer; Ms, meiocytes; E-Ms, excess meiocytes; I, indeterminate cells. Scale bar: 10 μm.

for the formation of sporogenous cells and surrounding somatic cell layers, including the tapetum (Yang et al., 1999). Recently, Ito et al. showed that *AG* is a direct activator of *SPL/NZZ* expression (Ito et al., 2004). *EMS1/EXS*, *TPD1* and *SERK1/SERK2* are required for the formation of tapetum (Albrecht et al., 2005; Colcombet et al., 2005; Yang et al., 2003; Zhao et al., 2002). In addition, phenotypic changes caused by *TPD1* overexpression are dependent on *EMS1/EXS* (Yang et al., 2005). We show here that *SPL/NZZ* and *EMS1/EXS* positively regulate expression of *DYT1*. The *AtMYB33* and *AtMYB65* genes are expressed in the tapetum and their expression does not require the *SPL/NZZ*, *EMS1/EXS* or *DYT1* gene.

In addition, it is likely that the genes that depend on *DYT1* for normal expression support the tapetum function that produces the enzymatic activities and materials needed for pollen development (Dickinson and Bell, 1976; Hernould et al., 1998; Liu and Dickinson, 1989; Rubinelli et al., 1998; Scott et al., 2004; Taylor et al., 1998; Zheng et al., 2003). Among the genes regulated by DYT1 are *AMS* and *MS1*, which also encode transcription factors that probably regulate late tapetum genes. Analysis of *DYT1* overexpression transgenic plants and expression studies in various mutants suggest strongly that other genes are needed for normal tapetum development. Phenotypic similarities suggest that *AtMYB33* and *AtMYB65*, as well as potentially others (such as *GNE1*) (Sorensen et al., 2002), may act at approximately the same step as *DYT1*. Therefore, *DYT1* is a crucial component at a key step in the regulatory network responsible for tapetum development and function (Fig. 8).

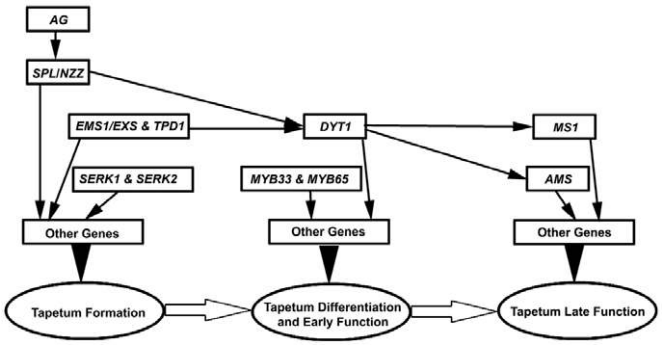

Fig. 8. A model for *DYT1* function and tapetum specification. The thin arrows indicate a positive genetic regulation. The arrowheads represent gene functions controlling a developmental stage. The open arrows indicate development from one stage to the next.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/16/3085/DC1

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