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RESEARCH PAPER

BnMs3 is required for tapetal differentiation and degradation, microspore separation, and pollen-wall biosynthesis in *Brassica napus*

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Abstract

7365AB, a recessive genetic male sterility system, is controlled by *BnMs3* in *Brassica napus*, which encodes a Tic40 protein required for tapetum development. However, the role of *BnMs3* in rapeseed anther development is still largely unclear. In this research, cytological analysis revealed that anther development of a *Bnms3* mutant has defects in the transition of the tapetum to the secretory type, callose degradation, and pollen-wall formation. A total of 76 down-regulated unigenes in the *Bnms3* mutant, several of which are associated with tapetum development, callose degeneration, and pollen development, were isolated by suppression subtractive hybridization combined with a macroarray analysis. Reverse genetics was applied by means of *Arabidopsis* insertional mutant lines to characterize the function of these unigenes and revealed that *MSR02* is only required for transport of sporopollenin precursors through the plasma membrane of the tapetum. The real-time PCR data have further verified that *BnMs3* plays a primary role in tapetal differentiation by affecting the expression of a few key transcription factors, participates in tapetal degradation by modulating the expression of cysteine protease genes, and influences microspore separation by manipulating the expression of *BnA6* and *BnMSR66* related to callose degradation and of *BnQRT1* and *BnQRT3* required for the primary cell-wall degradation of the pollen mother cell. Moreover, *BnMs3* takes part in pollen-wall formation by affecting the expression of a series of genes involved in biosynthesis and transport of sporopollenin precursors. All of the above results suggest that *BnMs3* participates in tapetum development, microspore release, and pollen-wall formation in *B. napus*.

Key words: BnMs3, *Brassica napus*, exine development, gene expression, microspore release, recessive genetic male sterility, suppression subtractive hybridization, tapetum.

Introduction

The recessive genetic male sterility (RGMS) system plays an important role in hybrid seed production due to its amazing stability in maintaining sterility (complete male sterility) with no negative cytoplasmic effects. 7365AB, a RGMS system controlled by the *BnMs3* gene in *Brassica napus*, was identified by Huang *et al.* (2007). Presently, it has been widely used for heterosis in China because of its advantages in obtaining a 100% sterile population using a temporary maintainer line, in which there is no need to remove 50% of the fertile plants during commercial hybrid seed production of the F_1 generation (Xiao *et al.*, 2008). The cause of RGMS is attributed to the abnormal development of anthers. In *Arabidopsis*, anther development is a complicated process, which consists of two phases divided into 14 stages (Sanders *et al.*, 1999; Ma, 2005). During phase I, from stages 1 to 8, cells divide and differentiate to form anther tetra lobes. Each lobe contains five cellular layers from the exterior to the interior: the epidermis, endothecium, middle

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layer, tapetum, and microspore mother cell (Sanders *et al.*, 1999; Ma, 2005). Tapetal differentiation and microspore separation involved in this phase are essential for anther development. During phase II, after the microspores undergo mitosis to develop into pollen grains, mature pollen grains are released to the surface of anther by its dehiscence. Tapetal PCD (programmed cell death) and pollen-wall formation play important roles in phase II.

B. napus and *Arabidopsis* are both members of the *Brassicaceae* and they share a high level of similarity in exon sequences (85.64%) and genomic synteny regions (Cavell *et al.*, 1998). It has been reported that *Arabidopsis* genes and their orthologues in *Brassica* have approximately equivalent functions (Auger *et al.*, 2009; Zhang *et al.*, 2009). Therefore, studying gene functions during anther development of *B. napus* by using the public information resources from *Arabidopsis* is a valid strategy.

Several genes that are crucial for early anther development have been elucidated in *Arabidopsis*. *AGAMOUS* acts in the specification of stamen and carpels (Yanofsky *et al.*, 1990; Ito *et al.*, 2004). *SPOROCYTELESS* (*SPL*)/*NOZZLE* and *ROXY* control the archesporial cell differentiation (Schiefthaler *et al.*, 1999; Yang *et al.*, 1999; Xing and Zachgo, 2008). Tapetal/microsporocyte determination is regulated by the interaction of *TPD1* and *EMS1* (Canales *et al.*, 2002; Zhao *et al.*, 2002; Jia *et al.*, 2008). *DYSFUNCTIONAL TAPETUM 1* (*DYT1*), *ABORTED MICROSPORE* (*AMS*), *TAPETAL DEVELOPMENT FUNCTION 1* (*TDF1*), *AtMYB103*, and *MALE STERILITY 1* (*MS1*) play vital roles in tapetal development and PCD (Wilson *et al.*, 2001; Sorensen *et al.*, 2003; Zhang *et al.*, 2006, 2007; Zhu *et al.*, 2008). Moreover, according to several global transcriptome data for *spl*, *ems1*, and *ms1* mutants, models of the gene regulatory network for early anther development have been proposed in *Arabidopsis* (Wijeratne *et al.*, 2007; Yang *et al.*, 2007).

During anther development, the timely degradation of the tetrad inner wall (callose) and the outer wall (the primary cell wall of pollen mother cell) is critical for microspore release from tetrads (Rhee and Somerville, 1998). Callose, which is mainly composed of β-1,3-glucans, is degraded by callase (β-1,3-glucanase) (Frankel *et al.*, 1969; Stieglitz and Stern, 1973; Stieglitz, 1977). Several tapetum-specific expressed β-1,3-glucanase genes have been identified, such as *Tag1* from tobacco and *A6* from *B. napus* and *Arabidopsis* (Hird *et al.*, 1993; Bucciaglia and Smith, 1994). Premature expression of a β-1,3-glucanase using a tapetum-specific promoter in tobacco resulted in male sterility (Worrall *et al.*, 1992). In addition to callose degradation, that of the primary wall of pollen mother cell (PMC) is also required for microspore separation (Preuss *et al.*, 1994). The three quartet mutants (*qrt1*, *qrt2*, and *qrt3*) in *Arabidopsis* are defective in the PMC primary wall degradation and generate tetrad pollen (Rhee *et al.*, 2003; Francis *et al.*, 2006; Ogawa *et al.*, 2009).

The biopolymer sporopollenin is the major component of exine (Piffanelli *et al.*, 1998). It has been thought that sporopollenin is a complex polymer primarily made of a mixture of fatty acids and phenolic compounds (Piffanelli *et al.*, 1998). Therefore, biosynthesis of fatty acids and phenols is essential for exine formation. Recent studies have demonstrated that *FACELESS POLLEN 1* (*FLP1*), *NEF1*, *MS2*, *Acyl-CoA Synthetase 5* (*ACOS5*), *CYP703A2*, and *CYP704B1* are involved in the biosynthesis of lipid molecules during anther development (Aarts *et al.*, 1997; Ariizumi *et al.*, 2003, 2004; Morant *et al.*, 2007; de Azevedo Souza *et al.*, 2009; Dobritsa *et al.*, 2009; Shi *et al.*, 2011). *LESS ADHESIVE POLLEN 5* and *LESS ADHESIVE POLLEN 6*, encoding anther-specific chalcone synthase (CHS), play a role in the biosynthesis of both fatty acids and phenols (Dobritsa *et al.*, 2010). Apart from its biosynthesis, the export process of the sporopollenin precursor is also required for exine formation. In rice, it is likely that *Osc6* encodes a lipid transfer protein (LTP) to transfer lipid molecules from the tapetal cells to the pollen wall (Zhang *et al.*, 2010).

BnMs3, encoding a tapetally expressed Tic40 protein, has been isolated by a map-based cloning approach (Dun *et al.*, 2011). The *Bnms3* mutant shows abnormal tapetal cell enlargement and aborted microspores. The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay has revealed that the pattern of PCD in the tapetal cells of the *Bnms3* mutant is delayed (Dun *et al.*, 2011).

This study reports the functional significance of *BnMs3* in anther development of *B. napus*. Semi-thin sections reveal that the principal cause of male sterility in the *Bnms3* mutant is a defect in the transition of the tapetum to the secretory type. The results of cytochemical staining for the callose wall suggest that callose cannot be degraded in time. Subsequently, suppression subtractive hybridization (SSH) and macroarray analysis are used to identify differentially expressed genes between the *Bnms3* mutant and the wild-type plant, a few of which are associated with tapetum development, callose degradation, and pollen-wall biosynthesis. Furthermore, the information on *Arabidopsis* genes involved in the regulatory network of anther development is used to further understand the role of *BnMs3* in anther development and it reveals that *BnMs3* takes part in tapetal differentiation and PCD, microspore separation, and exine development by affecting the expression of a series of key genes involved in these pathways. The functional importance of the three differentially expressed genes in the *Bnms3* mutant is further analysed by reverse genetics using orthologues of *B. napus* genes in *Arabidopsis*, which indicates that *AtMSR02*, encoding an ATP-binding cassette (ABC) protein, is only required for exine development but not for cuticle formation in the anther. The work provides deeper insight into the role of *BnMs3* in tapetal function and pollen development in *B. napus*.

Materials and methods

Plant materials

This work used the RGMS two-type line (7365AB) as materials. The 7365AB line was maintained by full sib-mating (7365A×7365B), so that line 7365A (*Bnms3Bnms3*), which is male sterile, and line 7365B (*BnMs3Bnms3*), which is male fertile, are near-isogenic lines (NILs) differing only in the fertility trait. Seeds were sown in the rapeseed research field of Huazhong Agricultural University, Wuhan, China.

Bud lengths ranging from <1, 1–2, and 2–3 mm were collected separately for cDNA library construction. Anthers from bud lengths <3 mm were dissected for macroarray analysis. To investigate the validity of the cDNA macroarray analysis, buds <1, 1–2, 2–3, 3–4, 4–5, and >5 mm in size were harvested for Northern blot analysis. All the harvested samples were immediately deep frozen in liquid nitrogen and stored at –80 °C.

Light microscopy of 7365AB

Morphological observations of anther semi-thin sections were performed as described by Chen *et al.* (2009). Aniline blue staining was conducted as described by Zhang *et al.* (2007). Micrographs of fluorescence expression of callose were taken using a fluorescence microscope (Nikon Eclipse 80i) with appropriate filter under ultraviolet light.

SSH library construction and differential screening

The library was constructed using a SMART PCR cDNA Synthesis kit (Clontech, USA) combined with the PCR-Select cDNA Subtraction kit (Clontech), according to the manufacturer's recommendations. The cDNA inserts were amplified using the nested PCR primers 1 and 2R provided in the PCR-select cDNA Subtraction kit and 2 μl bacterial overnight culture as the template. PCR products of the expected lengths (8 μl) were evaluated on 1.2% agarose gel, purified by ethanol precipitation, dissolved in 20 μl distilled deionized water, transferred into 384-well microplates and dotted onto Hybond-N^+ nylon membranes (Amersham, UK) using a Biomek 2000 Laboratory Automation Workstation (Beckman Coulter, USA). The membranes (8×12cm) were arrayed into grids of 384 dots that contained triplicate dots. The membranes were denatured in 0.2 M NaOH for 15 min, neutralized in 0.5 M TRIS-HCl (pH 7.5) for 5 min, and rinsed in distilled water twice for 2 min. The cDNAs were permanently fixed onto the membranes by baking at 80 °C for 2 h and the filters were stored at –20 °C. The anther cDNAs from line 7365A or line 7365B were used as probes for membrane screening to isolate the differentially expressed genes. Probes were added to the hybridization solution, which contained 0.5 M NaH_2PO_4 buffer, 1 mM EDTA (pH8.0), 7% sodium dodecyl sulphate (SDS), and 0.6% bovine serum albumin. Hybridization was carried out overnight at 68 °C. The membranes were washed twice with 2×standard sodium citrate (SSC) and 0.1% SDS for 15 min at room temperature and then washed with 0.5×SSC and 0.1% SDS for 5 min at 68 °C.

The hybridized membranes were exposed using the Phosphor Screen system (FUJIFILM, Japan) for 5 h. The signals from the Phosphor plate were obtained using a FLA-5000 Plate/Fluorescent Image Analyser (FUJIFILM) and analysed with the software Multi Gauge version 3.0. On the basis of the results of the dot-blot hybridization, differentially expressed clones were selected for sequencing.

Sequencing and bioinformatic analysis

Differentially expressed colonies were picked from the subtractive cDNA library for sequence analysis using a ABI 3730 Genetic Analyzer (AuGCT Biotechnology, Beijing, China). Unigenes were obtained using SeqMan software from DNASTAR (Madison, WI). Similarity searches were performed using BLASTN in The *Arabidopsis* Information Resource (TAIR). Unigenes with BLASTN expectation values (E-values) $>10^{-5}$ were designated as having insignificant similarity and were analysed using the GenBank database with BLASTN at the NCBI dbEST network service (http://www.ncbi.nlm.nih.gov/BLAST/). Functional categories of unigenes were assigned according to the Munich Information Center for Protein Sequences (MIPS) catalogue of the *Arabidopsis thaliana* genome (Mewes *et al.*, 2008). If an individual protein was allotted to multiple functional categories, the most probable category (lowest *P*-value) was chosen (Table 1 and Supplementary Table S1, available at *JXB* online).

Northern blot analysis

Total RNA (20 μg) were separated by electrophoresis on a 1.2% formaldehyde agarose gel in 1×MOPS buffer, subsequently transferred onto Hybond-N^+ nylon membranes, finally fixed by baking at 80 °C for 2 h, and hybridized (Sambrook and Russell, 2001). The selected cDNA inserts were amplified using nested PCR primers 1 and 2R. About 25 ng of each purified PCR product was labelled using the Prime-a-Gene Labeling system (Promega, USA). The results were obtained using the Phosphor Screen system.

Real-time PCR analysis

Total RNA isolated from four bud stages (<0.5, 0.5–1, 1–2, 2–3 mm) for the lines 7365A and 7365B were used for real-time PCR analysis. First-strand cDNA was generated from 2 μg total RNA from each sample using MMLV reverse transcriptase (K1631, Fermentas) according to the manufacturer's instructions and the products were diluted 100-fold with sterilized ddH_2O and amplified by PCR using the SYBR Green Real-time PCR Master Mix (QPK-201, Toyobo) and in triplicate with the CFX96 Real-time system (Bio-Rad). The results were analysed using *BnACTIN7* (EV220887.1) as a control to normalize the expression data and with CFX Manager software according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The primer sequences for real-time PCR assay are shown in Table 2 and Supplementary Table S2.

Microscopy and analysis of Atmsr02

Seeds of the mutants *Atmsr02* (*BnMSR02* orthologue in *Arabidopsis*), *Atmsr42* (*BnMSR42* orthologue in *Arabidopsis*), and *Atmsr53* (*BnMSR53* orthologue in *Arabidopsis*) were taken from the SALK insertional mutant lines SALK_062317, SALK_144641, and SALK_121737, respectively. Seeds were grown in soil at 22 °C under a 16/8 h light/dark regime. PCR genotyping was used to identify homozygous insertion lines. Alexander staining was used to test pollen viability (Alexander, 1969). Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were performed as described by Yi *et al.* (2010).

Results

Phenotypic characteristics of the Bnms3 *mutant*

Semi-thin sections were used for both the *Bnms3* mutant and the wild type to observe anther defects in mutant and to identify the relationship between bud lengths and anther developmental stages. As a result, anthers from bud lengths <0.5, 0.5–1, and 1–1.5 mm represented the PMC stage (Fig. 1A,G), the meiosis stage (Fig. 1B,H), and the tetrad stage (Fig. 1C,I), respectively. Buds with lengths 1.5–2.0 mm showed microspore release from the tetrad (Fig. 1D), while anthers with bud lengths 2.0–4.5 mm only exhibited the uninuclear microspore stage (Fig. 1D,E). Bicellular microspores were examined in anthers with bud lengths 4.0–4.5 mm (Fig. 1F).

At the meiosis stage in microspore mother cells, the wild type and the *Bnms3* mutant showed no obvious differences in the anthers and the tapetal cells had two nuclei and were vacuolated (Fig. 1B,H). However, some differences in anther development were observed between the mutant and the wild type after meiosis. In the wild type, when the PMCs had finished meiosis and had entered the tetrad stage, the tapetal

Table 1. Co-down-regulated expressed genes clustered according to different assembly in the *Bnms3* mutant in *B. napus* and the *Atms1*, *Atspl*, and/or *Atems1* mutants in *A. thaliana*

Data for *ms1* were taken from Yang *et al*. (2007); data for *ems1/spl* were taken from Wijeratne *et al*. (2007). –, No data.

Cluster	Unigene no.	Locus	Description	Fold change in *ms1*	Fold change in *ems1/spl*
Cluster A	BnMSR72	AT1G67990	Caffeoyl-CoA 3-*O*-methyltransferase	−8.2	−9.4
	BnMSR73	AT3G52160	Beta-ketoacyl-CoA synthase family protein	−13.9	−15.3
	BnMSR20	AT1G71160	Fatty acid elongase 3-ketoacyl-CoA synthase 7	−4.4	−39.0
	BnMSR45	AT1G75940	Beta-glucosidase protein	−21.3	−5.9
	BnMSR42	AT5G65205	Short-chain dehydrogenase/reductase (SDR) family protein	−8.2	−2.0
	BnMSR69	AT1G76470	Cinnamoyl-CoA reductase family	−6.6	−18.7
	BnMSR39	AT2G03740	Late embryogenesis abundant domain-containing protein	−7.3	−5.4
	BnMSR12	AT3G10410	Serine carboxypeptidase-like 49 precursor	−2.5	−2.5
	BnMSR23	AT3G26125	Cytochrome p450 monooxygenase 86c2	−7.8	−2.1
	BnMSR04	AT3G51590	Lipid transfer protein 12	−51.9	−11.4
	BnMSR09	AT1G06260	Cysteine proteinase	−33.2	−2.5
	BnMSR38	AT4G28395	Lipid transfer protein	−10.8	−8.3
	BnMSR58	AT1G75910	Family II extracellular lipase 4	−43.1	−2.3
	BnMSR48	AT4G37900	Glycine-rich protein	−22.3	−2.4
	BnMSR53	AT5G62320	MYB99	−2.9	−2.5
	BnMSR13	AT1G61110	NAC domain containing protein 25	−11.5	−2.8
	BnMSR37	AT5G13380	Auxin-responsive GH3 family protein	−8.2	−3.7
	BnMSR63	AT1G68875	Expressed protein	−109.9	−15.4
	BnMSR65	AT5G48210	Expressed protein	−16.6	−5.2
Cluster B	BnMSR59	AT1G54540	Expressed protein	–	−2.7
	BnMSR54	AT1G33430	Galactosyltransferase family protein	–	−47.0
	BnMSR50	AT5G15960	Cold and ABA inducible protein kin1	–	−2.4
	BnMSR07	AT5G07230	Lipid transfer protein family protein	–	−50.6
	BnMSR52	AT4G31500	Cytochrome p450 monooxygenase 83b1	–	−2.5
	BnMSR11	AT4G20420	Tapetum-specific protein-related protein	–	−33.9
	BnMSR66	AT3G23770	Glycosyl hydrolase family 17 protein	–	−28.5
	BnMSR29	AT3G15400	ATA20	–	−21.6
	BnMSR02	AT3G13220	ABC transporter family protein	–	−52.2
Cluster C	BnMSR57	AT2G23800	Geranylgeranyl pyrophosphate synthase 2	−8.8	–
	BnMSR76	AT2G19070	Spermidine hydroxycinnamoyl transferase	−14.7	–
	BnMSR75	AT1G30020	Expressed protein	−22.1	–

Table 2. Genes and corresponding primers used for real-time PCR analysis in *B. napus* based on the information for genes involved in *A. thaliana* tapetal development and function (Zhu *et al*., 2008)

BnACTIN7 was used as a control.

Gene name	AGI accession	Length of *in silico* gene (bp)	Identity	E-value	Forward sequence (5′–3′)	Reverse sequence (5′–3′)	Length of products (bp)
BnSPL	AT4G27330	942	86%	5e−48	ACTTCAACGAGGCGACAAATCTTAC	CGGGAAAAATTCGTACTCCTTCA	101
BnROXY2	AT5G14070	413	90%	e−122	GAGCTCGACCTCCACCCTCAT	GAAGTGCCCCCGGAGAAGT	100
BnEMS1	AT5G07280	3702	82%	e−138	CTTGGTTGGTTGGGTTGTTCAGA	ACGTAGCATGGCCTGTTTGAAA	100
BnTPD1	AT4G24972	546	86%	2e−74	AGGCAGCGACCGAACCTATG	TGACGTGGATCCTCGAGATGTT	99
BnDYT1	AT4G21330	651	86%	e−148	TGTGCCTGTTGGGATTTGAGA	AGTCACCATCACATAGTCCCTGAAGT	108
BnTDF1	AT3G28470	972	92%	e−166	GTGAAGAACCACTGGAACACGAA	TGAATTCCGTAAGGACCTGAGAAAC	100
BnAMS	AT2G16910	511	88%	e−135	CGAGATGGTGCCAGCTGAAC	GGATTGAACCAGTTGCCATCTG	101
BnMYB103	AT5G56110	963	91%	0.0	CCACTACACTCAATCCTCCTCAAGTC	TCAACACGTTTCTTGGTGAGCAA	100
BnMS1	AT5G22260	1489	89%	0.0	ATGCCTCCACAAGAATGC	TCCCATCTAACACCAATCC	190
BnACTIN7	AT5G09810	734	90%	0.0	CGCGCCTAGCAGCATGAA	GTTGGAAAGTGCTGAGAGATGCA	101

cell walls were degraded and the tapetal cells shrank and were deeply stained with toluidine blue, which revealed that they had transformed into the secretory type (Fig. 1C). In contrast, the mutant tapetal cell walls remained intact and visible, and the tapetal cells continued to enlarge and vacuolate with reduced staining, meaning that the tapetal cells were unable to

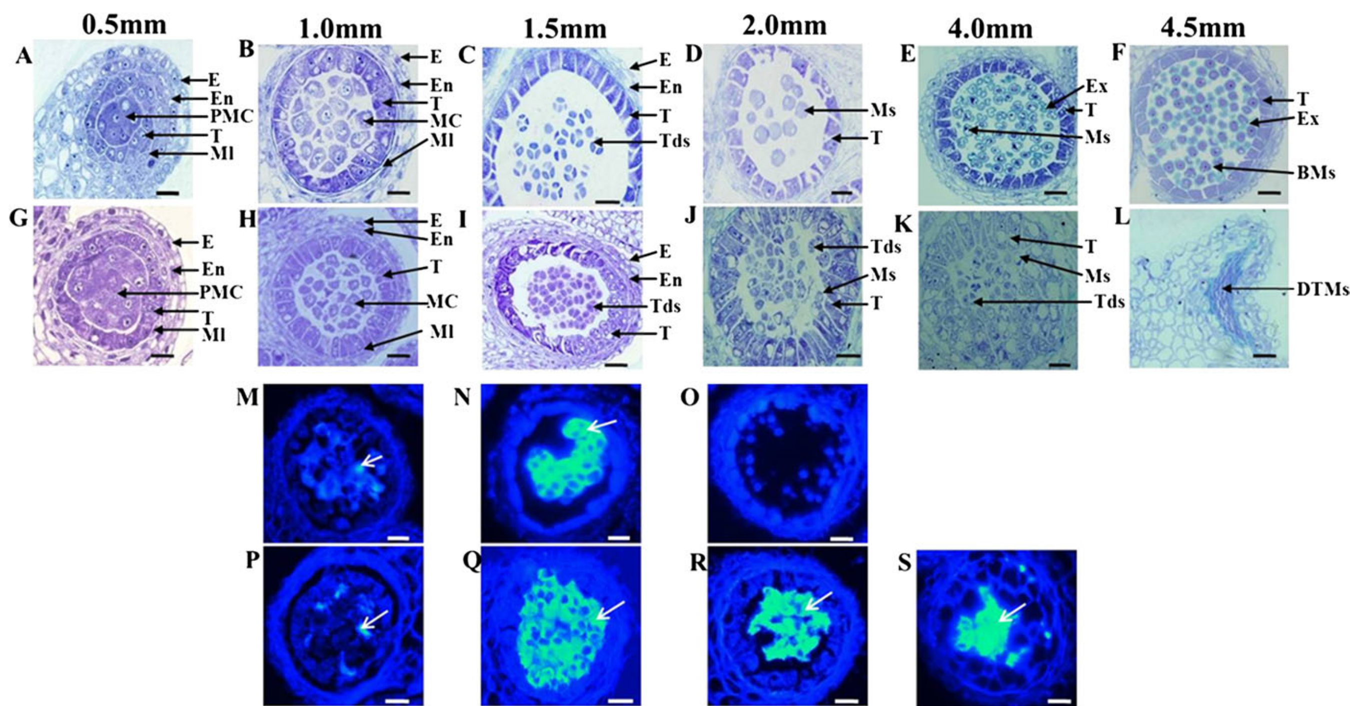

Fig. 1. Defects in *Bnms3* mutant anther development of *B. napus* in buds of different lengths (0.5, 1.0, 1.5, 2.0, 4.0, and 4.5 mm). (A–L) Comparison of anther development between the wild type (A–F) and the *Bnms3* mutant (G–L) by semi-thin sections, revealing that the principal cause of abnormal anther development in the *Bnms3* mutant is due to a defect in the transition of the tapetum to the secretory type, which is followed by abnormalities in microspore release and in pollen-wall formation in the few microspores that are released. (M–S) Cytochemical staining for callose wall in the wild type (M–O) and the *Bnms3* mutant (P–S) with aniline blue, with the fluorescence expression of callose under ultraviolet light (arrows), showing that callose persists longer in the *Bnms3* mutant than in the wild type and leads to the disruption of timely microspore release. BMs, bicellular microspore; DTMs, degenerated tapetum and microspore; E, epidermis; En, endothecium; Ex, exine ; MC, meiotic cell; Ml, middle layer; Ms, microspore; PMC, pollen mother cell; T, tapetum; Tds, tetrads. Bars, 25 μm.

transform into the secretory type at any time (Fig. 1I,J,K). During the uninucleate pollen stage, microspores were released from the tetrads in the wild-type anthers (Fig. 1D). Subsequently, microspore exine was formed (Fig. 1E). However, at this stage, the blockage of degradation of the callose surrounding the tetrads resulted in most of the microspores not being released, and the few that were released did not form normal microspore exine (Fig. 1J,K). During the bicellular pollen stage, the tapetal cells and microspores were degenerated instantly in the *Bnms3* mutant (Fig. 1L).

In order to further understand the abnormalities of microspore release in the *Bnms3* mutant, aniline blue staining was utilized to check the degradation of callose in both the wild-type and the mutant anthers. At the meiosis stage, callose fluorescence appeared in the locules of the wild type and the mutant (Fig. 1M,P) and became the clearest during the tetrad stage (Fig. 1N,Q). After the tetrad stage, callose fluorescence was still observed in the *Bnms3* mutant anthers but not detected in the wild type (Fig. 1O,R,S). This result exhibited that callose was not degraded in time in the *Bnms3* mutant.

Genes differentially expressed between lines 7365A and 7365B

To identify differentially expressed genes controlled by *BnMs3*, the subtracted library was constructed using SSH. For the subtracted library, tester cDNA was isolated from the wild type and driver cDNA was from the mutant. Furthermore, 768 clones were randomly chosen from the SSH library for amplification. Macroarray analysis was carried out to screen differentially expressed cDNAs (Supplementary Fig. S1). After scanning, a total of 120 clones showing obviously differential expression were selected for further analysis, 115 clones of which being successfully sequenced, with lengths from 130 bp to 1131 bp. Using SeqMan, 76 unique sequences were obtained from all of the expressed sequence tag (EST) assemblies.

Gene sequences were analysed using BLAST against sequences in TAIR. The 76 unigenes could be classified into two groups. The first group consisted of 68 (89.5%) unigenes with high similarity to genes in *Arabidopsis* (BLASTN; E-values $<10^{-5}$ for nucleic acids). The second group included eight unknown unigenes (10.5%) which showed no high similarities with any sequences in the TAIR database. However, by performing BLASTN of the eight sequences in NCBI, similar sequences expressed in anthers or flower buds were identified (Supplementary Table S1). These results reveal that a few novel genes are specifically expressed in the anther of *B. napus* and are affected by *BnMs3*.

To ascertain the validity of the cDNA macroarray analysis, the expression patterns of seven randomly picked

unigenes which were differentially expressed in lines 7365A and 7365B were compared using Northern blot analysis of buds of different lengths. The results were consistent with those from the macroarray analysis and indicated that there were two differential expression types at corresponding stages in the NILs (Fig. 2). Type I included four genes that were expressed only in line 7365B or showed a lower corresponding expression level in the mutant. Type II had three genes expressed in the buds of both NILs, but their expressions were delayed or repressed at the early stage of anther development in line 7365A (Fig. 2).

Functional categories of differentially expressed genes

To gain further insights into the functions of the differentially expressed genes, the 68 annotated down-regulated unigenes in the *Bnms3* mutant were classified into eight functional categories based on the information from MIPS (Fig. 3; Supplementary Table S1). The major functional groups of down-regulated unigenes were concerned with metabolism, lipid/fatty acid transport, and protein synthesis. The first main group comprised 20 unigenes related to metabolism, encoding enzymes such as cytochrome P450 monooxygenase, glyceraldehyde-3-phosphate dehydrogenase, fatty acid elongase 3-ketoacyl-CoA synthase 7, and caffeoyl-CoA 3-*O*-methyltransferase. The second main group included nine unigenes annotated as one ABC transport protein and eight LTPs involved in lipid/fatty acid transport. Moreover, six unigenes were associated with protein synthesis, such as translation elongation factor 2-like proteins and ribosomal proteins. The other unigenes were related to transport, protein fate, and stress-responsive proteins.

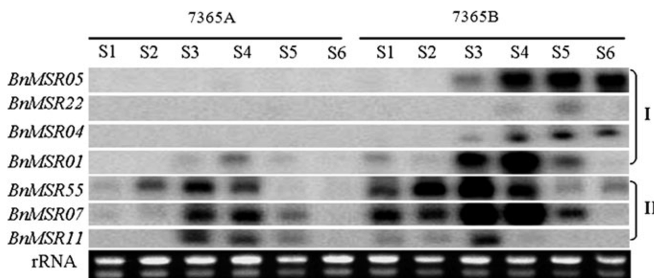

Fig. 2. Verification of macroarray results by Northern blot analysis in *B. napus*. Line 7365A represents the male sterile plant (the *Bnms3* mutant) and line 7365B represents the fertile plant (the wild type). S1–S6 represent the bud lengths <1, 1–2, 2–3, 3–4, 4–5, and >5 mm, respectively. I, type I expression type, genes expressed only in line 7365B or at a lower level in the mutant; II, type II expression type, genes expressed in the buds of both lines but expression was delayed or repressed at the early stage of anther development in line 7365A.

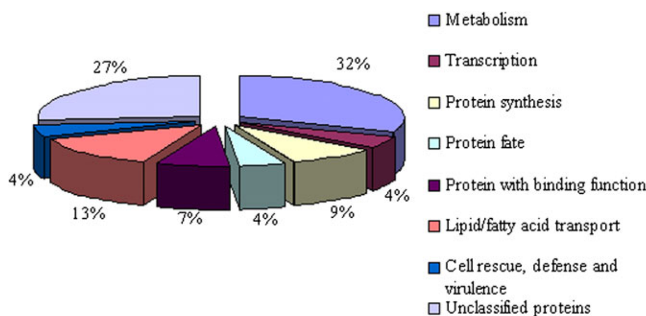

Fig. 3. Functional categorization of down-regulated unigenes contained in the *Bnms3* mutant according to the Munich Information Center for Protein Sequences classification. A total of 68 unigenes were grouped into eight functional categories and the percentages of gene transcripts in each group were listed.

Co-down-regulated genes in the Bnms3 *and* Arabidopsis *mutants*

Arabidopsis has been used as a model plant to study the molecular mechanisms in anther development (Ma, 2005; Wilson and Zhang, 2009). A large number of genes relevant to this process have been identified through microarray analysis in *Arabidopsis*, and there are several available transcription profile data of anther-development mutants, including SPL as a regulator of sporogenesis, EXCESS MICROSPOROCYTES 1 (EMS1) involved in the early tapetal cell initiation, and MALE STERILE 1 (MS1) essential for the late tapetal cell development and function (Alves-Ferreira *et al.*, 2007; Wijeratne *et al.*, 2007; Yang *et al.*, 2007). Since the *Bnms3* mutant showed abnormal tapetal cells and pollen exine in anther development, the present study compared the *Bnms3* mutant data set with that of *spl*, *ems1*, and *ms1* mutants in *Arabidopsis*. Consequently, out of 68 down-regulated unigenes in the *Bnms3* mutant, 41.2% (28 genes) were co-down-regulated in *spl* and/or *ems1* anthers, 32.4% (22 genes) were co-down-regulated in *ms1* anthers, and 19 (27.9%) genes were co-down-regulated in *ms1* as well as in *spl* and/or *ems1* anthers (Table 1, cluster A). It was concluded that several pathways common to all of the mutants were affected, such as lipid biosynthesis (fatty acid elongase 3-ketoacyl-CoA synthase 7), lipid transport (lipid transfer protein 12), hormone-responsive proteins (auxin-responsive GH3 family protein), and pollen coat proteins (family II extracellular lipase 4). Furthermore, it also indicated that nine out of 28 unigenes and three out of 22 unigenes, in *Atms1* (Table 1, cluster B) and *Atspl* and/or *Atems1* anthers (Table 1, cluster C), respectively, were not obviously down-regulated.

Expression changes of genes related to tapetal development

Semi-thin sections revealed that the *Bnms3* mutant tapetal cells were unable to transform into the secretory type. Among those down-regulated unigenes, *BnMSR29* (*ATA20*) was tapetum specific (Rubinelli *et al.*, 1998) but was only detected in the buds of the wild type as compared with the *Bnms3* mutant (Fig. 4). To date, several putative models for the transcriptional regulation of tapetal development and function have been proposed in *Arabidopsis*. Therefore, further clarification of the role of *BnMs3* in tapetal development was sought using real-time PCR analysis based on the gene

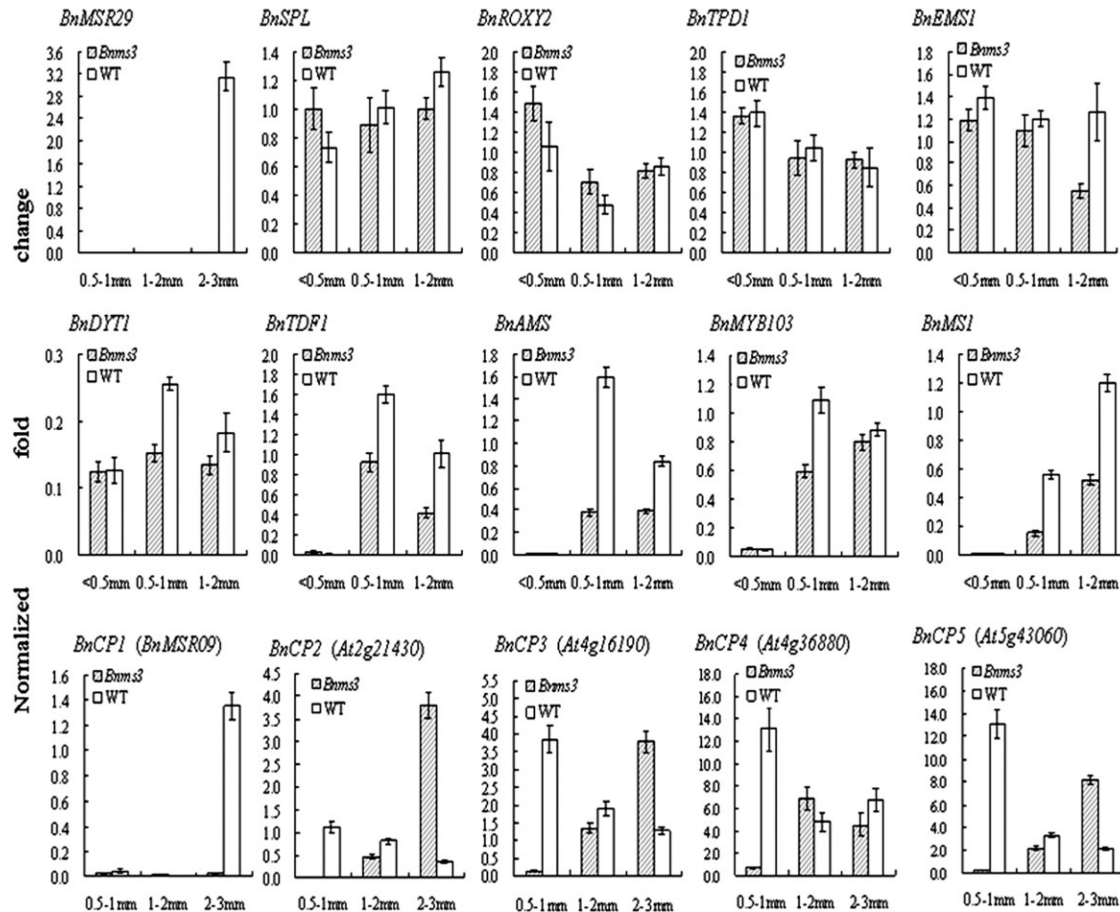

Fig. 4. Real-time PCR analysis of genes invovled in tapetal development and degradation between the *Bnms3* mutant and the wild type, using primers designed for *BnSPL*, *BnROXY2*, *BnTPD1*, *BnEMS1*, *BnDYT1*, *BnTDF1*, *BnAMS*, *BnMYB103*, and *BnMS1* according to the gene information of regulatory network of *Arabidopsis* tapetal development and function combined with the expressed sequence tag database in NCBI. *BnMSR29* and *BnCP1* were isolated from the suppression subtractive hybridization library. Sequences for *BnCP2*, *BnCP3*, *BnCP4*, and *BnCP5* (belonging to the cysteine protease family and related to tapetal programmed cell death were obtained using comparative genomics and the *Arabidopsis* electronic Fluorescent Pictograph browser. The expression levels of *BnDYT1* and its upstream genes *BnSPL*, *BnROXY2*, *BnTPD1*, and *BnEMS1* were unchanged at the pollen mother cell stage in the *Bnms3* mutant, whereas the activities of *BnTDF1*, *BnAMS*, *BnMYB103*, and *BnMS1* in the *Bnms3* mutant were significantly reduced at the post-meiotic stage of anther development. The expression of five cysteine protease genes was affected in the *Bnms3* mutant. Values are means±SD.

information from *Arabidopsis*. Comparative genomics was used to obtain the homologous *Brassica* ESTs and genome survey sequences using the full coding sequences of the corresponding key tapetal genes from *Arabidopsis* by BLASTN in NCBI. Subsequently, according to the conserved sequences of gene-contig assembly, gene-specific primers for real-time PCR were designed to match sequences close to their 3′ untranslated regions (Table 2). The data indicated that the expression levels of *DYSFUNCTIONAL TAPETUM 1* and its upstream genes, *SPL*, *ROXY2*, *TPD1*, and *EMS1*, were unchanged at the PMC stage in the *Bnms3* mutant, while those of *TDF1*, *AMS*, *MYB103*, and *MS1* were delayed in the *Bnms3* mutant buds at the post-meiotic stage of anther development (Fig. 4). These results suggested that the abortion of the *Bnms3* mutant emerged at the post-meiotic stage of anther development and *BnMs3* might affect tapetal development by affecting the expression of *BnTDF1*, *BnAMS*, *BnMYB103*, and *BnMS1*, which are associated with tapetal differentiation and/or PCD.

Recently, it has been shown that tapetum degeneration in *Bnms3* mutants is retarded (Dun *et al.*, 2011). It has been suggested that cysteine proteases may be acting as an effector of PCD in the wild-type tapetum. In the present research, the expression of a unigene encoding cysteine protease was down-regulated in the *Bnms3* mutant. In *Arabidopsis*, there are 30 genes encoding cysteine proteases (Beers *et al.*, 2004), which were analysed using comparative genomics and the *Arabidopsis* electronic Fluorescent Pictograph browser (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). Four other cysteine protease genes were chosen for real-time PCR analysis, for its orthologues ESTs revealed the presence of corresponding transcripts in anther, flower, and bud cDNA libraries in *Brassica*. The real-time PCR data showed that the expression patterns of these genes were divided into two clusters. Cluster A involved *BnCP2* (*At2g21430*), *BnCP3* (*At4g16190*), *BnCP4* (*At4g36880*), and *BnCP5* (*At5g43060*), whose expression levels were repressed at the post-meiotic stage when compared with wild type (Fig. 4). However, the

expression of *BnCP1* (*BnMSR09*), the one and only member of cluster B, was merely detected at the uninucleate stage of wild-type buds (Fig. 4).

Changes in expression of genes associated with microspore separation

It has been shown that callose cannot be degraded in time in the *Bnms3* mutant. In *Arabidopsis*, *A6* (belonging to β-1,3-glucanase family) is likely to be involved in callose dissolution (Zhang *et al.*, 2007; Zhu *et al.*, 2008). Among the down-regulated unigenes, *BnMSR66* encodes a β-1,3-glucanase and shares 85% nucleotide identity with *At3G23770*. Sequences of these genes were aligned using ClustalX version 1.8.3. Bucciaglia first isolated the *A6* gene of *B. napus* (Bucciaglia and Smith, 1994); however, on the basis of the sequences of *AT4G14080* and in EST database in NCBI, it lacked five amino acids at the N-terminal. A full-length coding sequence of *BnMSR66* was obtained using the analysis of *B. napus* EST fragments deposited in NCBI and the open reading frame of *AT3G23770* in combination with sequencing. *BnA6* cDNA contained a 1440-bp open reading frame encoding a polypeptide of 479 amino acids, while that of *BnMSR66* was 1431 bp encoding a polypeptide of 476 amino acids. On the basis of the alignment, all sequences contained a classic glycosyl hydrolase family 17 domain (belonging to the β-1,3-glucanase family motif) and an X8 domain, which is well defined as a class of carbohydrate-binding modules responsible for binding β-1,3-glucan (Fig. 5).

In the publicly available *Arabidopsis* electronic Fluorescent Pictograph browser, expression patterns of both *AtA6* and *AtMSR66* (*At3G23770*) showed high consistency from stages 9 to 11 in the anther, predominantly at stage 9 (Fig. 6A). To further confirm the function of *BnMs3* in callose dissolution, both *BnA6* and *BnMSR66* were selected for real-time PCR analysis. As a result, the expression level of *BnA6* was delayed while that of *BnMSR66* could not be detected in the *Bnms3* mutant at the post-meiotic stage as compared with the wild type (Fig. 6B). Hence, it was demonstrated that this study had found a novel β-1,3-glucanase gene (*BnMSR66*) which might be responsible for callose degeneration in anther development.

Apart from callose dissolution, that of the PMC pectic wall is also important for the microspore separation. There are three genes (*AtQRT1*, *AtQRT2*, and *AtQRT3*) whose mutants fail to undergo microspore separation with the release of viable pollen tetrads (Preuss *et al.*, 1994). To investigate the function of *BnMs3* in degrading the PMC wall, *BnQRT1*, *BnQRT2*, and *BnQRT3* were isolated by means of comparative genomics and chosen for real-time

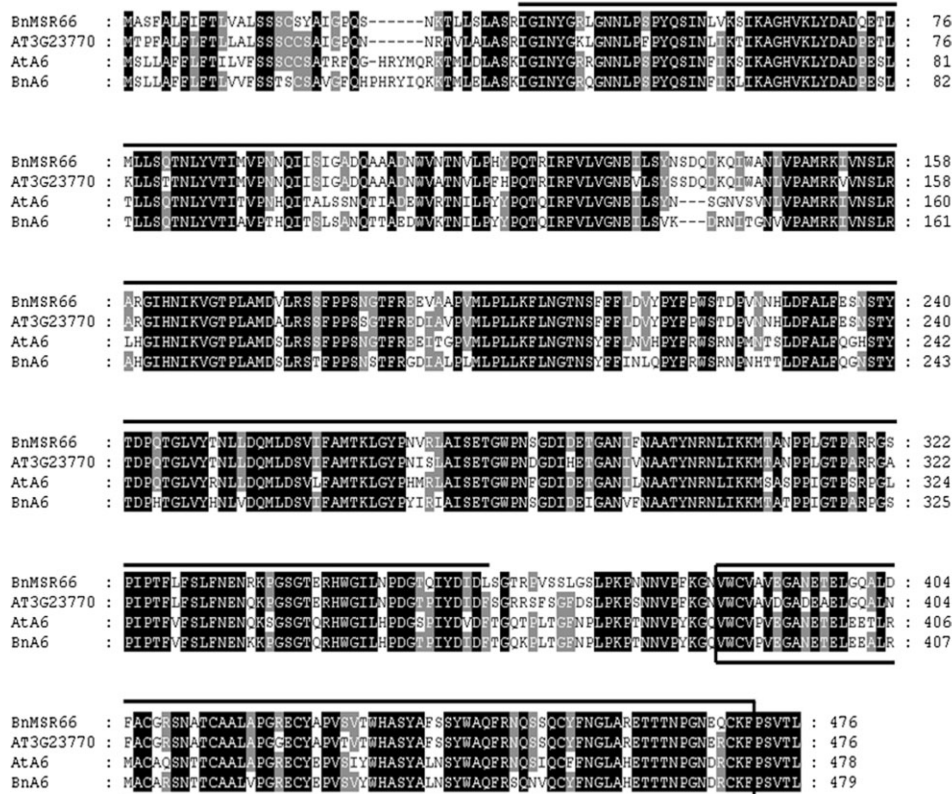

Fig. 5. Sequence analysis of BnA6 and BnMSR66, showing that *BnMSR66* and *BnA6* are both members of β-1,3-glucanase family and share high sequence identity. The amino acid sequences of BnMSR66 and BnA6 in *B. napus* and AT3G23770 and AtA6 in *Arabidopsis* were aligned using ClustalX version 1.8.3 (Jeanmougin *et al.*, 1998). Numbers show the positions of amino acid residues. Black shading indicates conserved residues and grey shading indicates sequence similarity. The putative glycosyl hydrolase family 17 motif sequences are marked by a straight lines. The X8 domain is boxed based on the result of Pfam analysis (http://www.sanger.ac.uk/Software/Pfam/; Finn *et al.*, 2010).

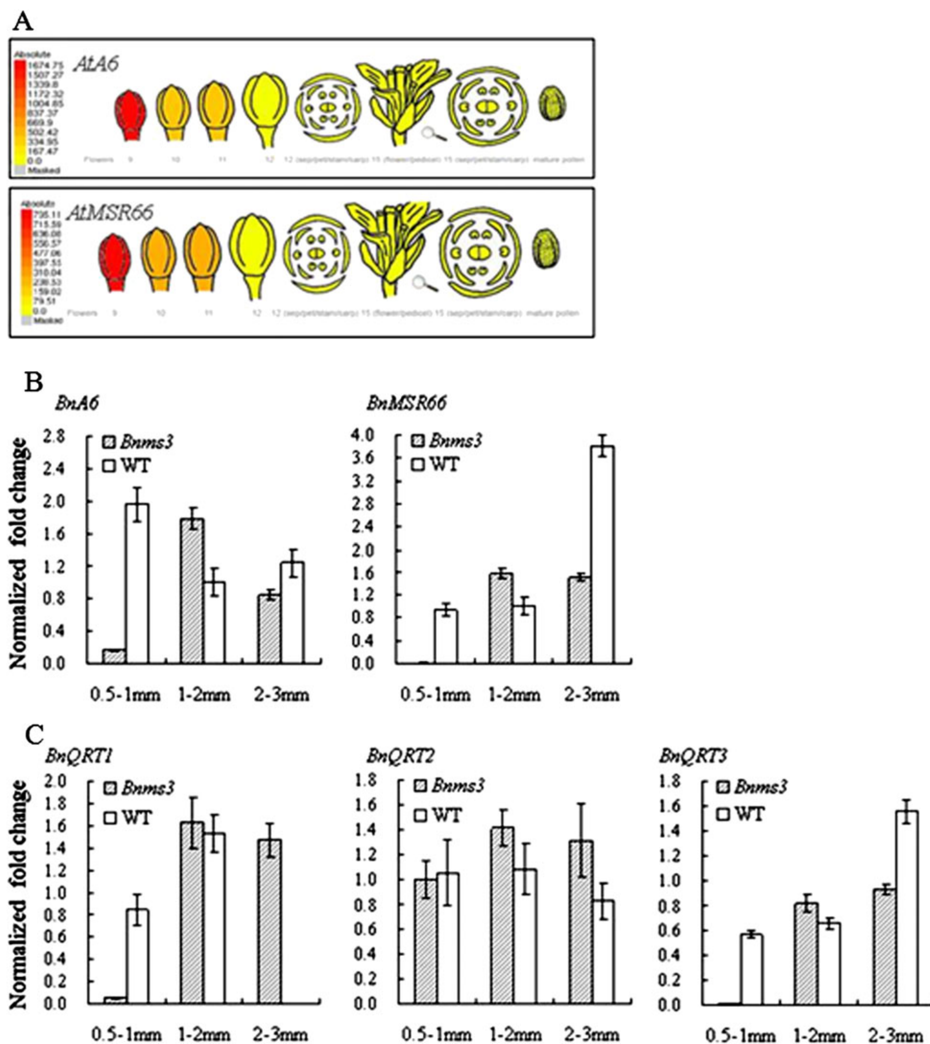

Fig. 6. Expression patterns of genes involved in microspore separation in *B. napus*. (A) Relative expression levels and patterns of *AtA6* and *AtMSR66* in flowers based on microarray data displayed in the *Arabidopsis* electronic Fluorescent Pictograph browser, showing that they have similar expression patterns; colour scale shows microarray signal level. (B) Real-time PCR analysis of callase-related genes *BnA6* and *BnMSR66* in the buds of the *Bnms3* mutant and the wild type, showing that expression of both is suppressed in the *Bnms3* mutant at the post-meiotic stage. (C) Real-time PCR analysis of *BnQRT1*, *BnQRT2*, and *BnQRT3* in the buds of the *Bnms3* mutant and the wild type, showing that expression of *BnQRT1* and *BnQRT3* were affected in the *Bnms3* mutant at the post-meiotic stage, while that of *BnQRT2* was unchanged at the three stages in both the mutant and the wild type.

PCR. The down-regulated expression of both *BnQRT1* and *BnQRT3* was initiated at the post-meiotic stage in the *Bnms3* mutant (Fig. 6C) while expression level of *BnQRT2* was unchanged at the three stages in both the mutant and the wild type.

Expression changes of genes involved in lipid metabolism

In the *Bnms3* mutant, callose dissolution was blocked, but there were still seldom microspores without pollen exine released from the tetrad according to our research. Lipid molecules are a main component of pollen wall. To explore the role of *BnMs3* in biosynthesis of lipid molecules, three unigenes obtained from the SSH library and eight ESTs acquired based on the corresponding information from *Arabidopsis* which participated in this process were analysed by using real-time PCR. According to the different stages at which their differential gene expression initiated, the expression patterns of them could be divided into three clusters. Cluster 1 included *BnNEF1* and *BnFLP1* which didn't show any expression changes at the three detected stages between the two materials (Fig. 7A, cluster 1). Cluster 2 was consisted of *BnCYP703A2*, *BnACOS5*, *BnCYP704B1*, *BnCHS2* (*At4g34850*), and *BnMS2*, showing the initially down-regulated expression in the *Bnms3* mutant at the post-meiotic stage (Fig. 7A, cluster 2). The three unigenes (*BnMSR20*, *BnMSR23*, and *BnMSR73*) and *BnCHS1* (*At4g00040*) were attached to cluster 3, which exhibited the down-regulated expression in the *Bnms3* mutant initiated at the uninuclear microspore stage (Fig. 7A, cluster 3). Among these differentially expressed genes, *BnCYP703A2*, *BnACOS5*,

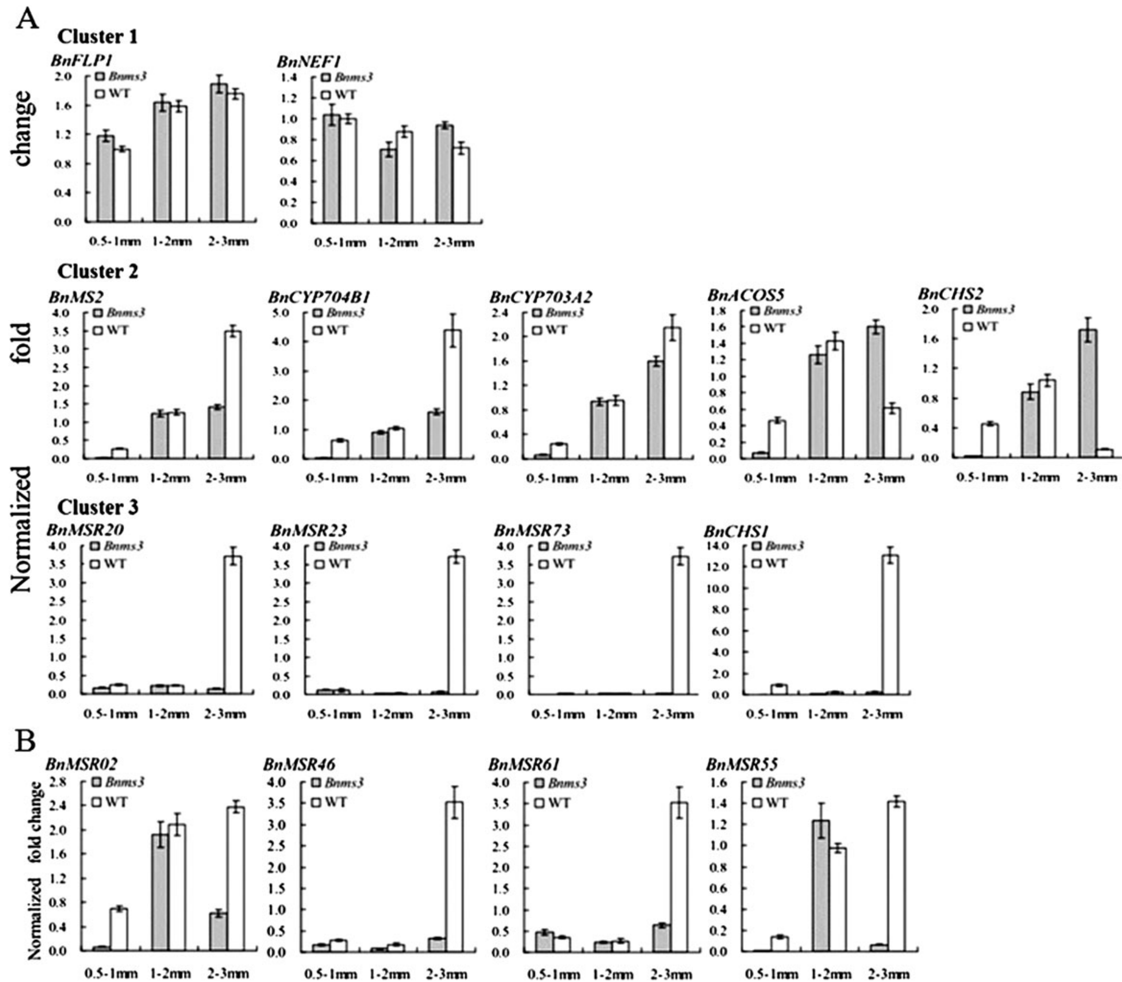

Fig. 7. Real-time PCR analysis of genes related to anther lipid metabolism in the wild type and the *Bnms3* mutant in *B. napus*. (A) According to the different stages at which differential gene expression initiated, the expression patterns of 11 genes involved in biosynthesis of lipid molecules could be divided into three clusters: cluster 1 did not show any gene expression changes at the three detected stages in the mutant and wild type; cluster 2 showed the initially down-regulated gene expression in the *Bnms3* mutant at the meiosis stage; cluster 3 exhibited initially down-regulated gene expression in the *Bnms3* mutant at the uninuclear microspore stage. (B) Real-time PCR analysis of four genes associated with transport of sporopollenin precursors in both the wild type and the *Bnms3* mutant, showing that the expression levels were all affected in the *Bnms3* mutant at their initial expression stage.

BnCYP704B1, *BnMS2*, and the three unigenes were relevant to biosynthesis of fatty acids, while *BnCHS1* and *BnCHS2*, which encode chalcone synthase, were involved in biosynthesis of both fatty acids and phenols in *Arabidopsis* (Dobritsa *et al.*, 2010). These results disclosed that *BnMs3* was associated with the biosynthetic pathways of fatty acids and phenolic compounds, which were required for exine development.

According to research on wax export from an epidermal cell to the cuticle, wax export consists of two steps of lipid export during pollen-wall formation (Samuels *et al.*, 2008). The first step is the transport of lipid molecules through the plasma membrane (PM) to the extracellular environment, and the second step is transport from the extracellular environment to the surface of microspores, forming pollen exine. *BnMSR02*, 648 bp in length, belongs to ABC transporter family, which is associated with transmembrane transport of lipid molecules and shares high gene sequence similarity (95.2%) with *AtABCG26* (*AT3G13220*). Real-time PCR analysis showed that the expression of *BnMSR02* was much lower in the *Bnms3* mutant when compared with that in the wild type during the post-meiotic period (Fig. 7B). It is likely that the anther-expressed LTPs may participate in the transport of sporopollenin precursors (lipid molecules) from the extracellular environment to the microspore during exine deposition (Zhang *et al.*, 2010). In the *Bnms3* mutant, the expression levels of eight putative LTPs were found to be altered, namely BnMSR55, BnMSR46, BnMSR24, BnMSR04, BnMSR07, BnMSR10, BnMSR61, and BnMSR38 (Supplementary Table S1). *BnMSR46*, *BnMSR55*, and *BnMSR61* were analysed by real-time PCR and their corresponding expression levels were reduced in the *Bnms3* mutant as compared with the wild-type plant (Fig. 7B). The expression patterns of several LTPs were different, based on the Northern blot and real-time PCR analysis combined with the microarray data from *Arabidopsis*. *BnMSR04* was only expressed in wild-type buds and its expression was initiated at

the uninucleate stage (Fig. 2). Expression of *BnMSR55* and *BnMSR07* was delayed in the *Bnms3* mutant at the post-meiotic stage of anther development (Fig. 2 and Fig. 7B). This suggested that lipids synthesized by the tapetal cells might be affected by *BnMs3* function and exported in different batches through different LTPs.

Three genes were selected for further analysis based on gene expression changes in the *Bnms3* mutant using transferred-DNA insertion mutants of *Arabidopsis* to understand their functions in anther development (Supplementary Table S2). As a result, only the *Atmsr02* mutant (*BnMSR02* orthologue in *Arabidopsis*) exhibited defects in pollen development (Fig. 8). In this mutant, the insertion occurred at 231 bp upstream of the predicted fifth exon of *AtABCG26* (Fig. 8I). The mutant phenotype was indistinguishable from that of the wild type in the heterozygous lines, but homozygous plants of the insertional mutant showed male sterility; hence, the mutation was recessive. The sterile mutant line displayed normal vegetative and floral development but small siliques and no seed yield (Fig. 8E). Pollen grains were absent on the surface of anthers or stigma in the mutant flowers as compared with the wild-type plant (Fig. 8F,G). Moreover, Alexander's stain showed that mature anthers of *Atmsr02* homozygous plants were empty (Fig. 8H). In *Arabidopsis*, ABCG subfamily transporters involved in the excretion of lipids through the PM have been previously reported (Bird, 2008), and lipids synthesized in the tapetal cells have two destinies, namely biosynthesis of sporopollenin and anther epidermis cutin (Li *et al.*, 2010). To deeply understand *BnMSR02* function, the

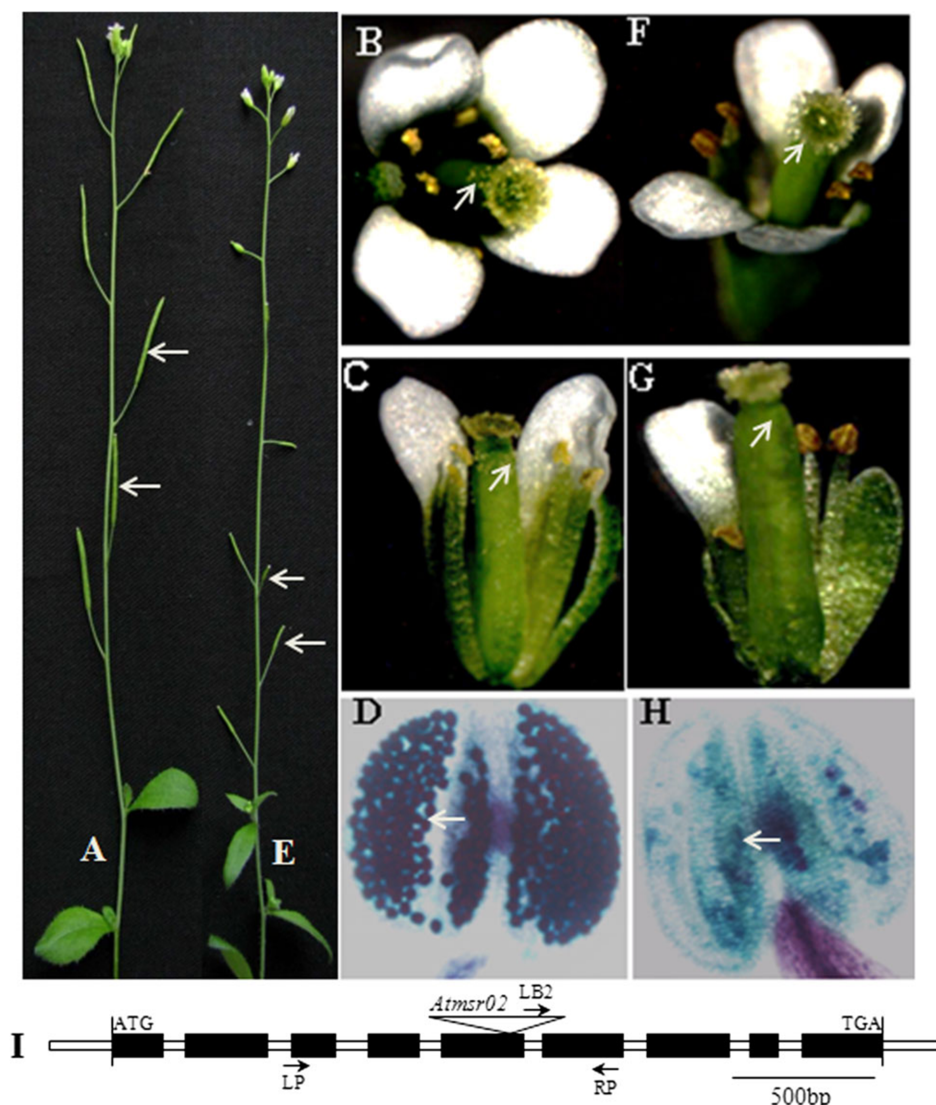

Fig. 8. Characterization of the *Atmsr02* transferred-DNA mutant in *A. thaliana*. (A) Wild-type plant, with normal silique development (arrows). (E) *Atmsr02* transferred-DNA mutant plant, with very small siliques containing no seeds (arrows). (B,C) Wild-type flowers, with many pollen grains displayed on the anther and stigma surfaces (arrows). (D) Wild-type anther by Alexander staining, with viable pollen grains (arrow). (F,G) *Atmsr02* transferred-DNA mutant flowers, with no pollen grains present on the surface of anther or stigma (arrows). (H) *Atmsr02* transferred-DNA mutant anther by Alexander staining, without viable pollen grains (arrow). (I) Diagrammatic representation of the *Atmsr02* transferred-DNA mutant.

present study used transmission and scanning electron microscopy to observe pollen exine and anther epidermis in the *Atmsr02* mutant (Fig. 9). The results indicated that free microspores were visible in the immature anthers of the *Atmsr02* mutant (Fig. 9A), but they had an abnormally smooth surface (Fig. 9C,E) and abnormal pollen exine (Fig. 9G). However, it was impossible to differentiate the cytological shape of anther epidermal cutin in the mutant from that of the wild type because of their cuticular ridges in common (Fig. 9K,L). These data suggested that there are two different pathways across the PM of tapetal cells for lipid transport for cuticles and pollen exine materials. *MSR02* is required for the transport of lipids related to sporopollenin precursors, forming pollen exine through PM of the tapetal cells, but not for that of cuticular lipid formation.

Discussion

In this research, cytological defects of the *Bnms3* mutant during anther development were investigated and SSH and macroarray approaches were used to identify genes affected by *BnMs3* using a NIL (line 7365AB). Results of both the cytological and differential expression analysis exhibited that *BnMs3* was associated with tapetal development, callose degradation, and pollen-wall formation. To gain a deeper insight into the function of *BnMs3* in these processes, *Brassica* orthologues of the *Arabidopsis* genes required for anther development were identified by comparative genomics and analysed by real-time PCR for both the *Bnms3* mutant and the wild type.

BnMs3 *influences tapetal differentiation and degradation*

Tapetum is a cell layer adjacent to the anther locule. There are two main tapetal types, designated as the secretory tapetum and the amoeboid tapetum, in the angiosperms (Pacini *et al.*, 1985; Murgia *et al.*, 1991). The secretory tapetum shows cytoplasm and nucleus shrinkage, loss of the cell wall, and reduction in the size of mitochondria (Pacini *et al.*, 1985; Parish and Li, 2010). Tapetal cells transform into the secretory type at the post-meiotic stage to secrete enzymes for the release of microspores from the tetrad and to provide nutrients for pollen development (Pacini *et al.*, 1985; Piffanelli *et al.*, 1998). Once the transition of tapetum is blocked in anther development in *Arabidopsis*, the tapetal cells become enlarged and abnormally vacuolated, which is followed by aberrant microspore separation and abnormal pollen-wall development (Zhang *et al.*, 2007; Zhu *et al.*, 2008). In *Arabidopsis*, *TDF1*, *AMS*, and *MYB103* are involved in this process. *AMS* encodes a basic helix–loop–helix protein and acts downstream of *TDF1* but upstream of *MYB103* (Sorensen *et al.*, 2003; Zhang *et al.*, 2007; Zhu *et al.*, 2008). Histological observation revealed that the critical cause of male sterility in the *Bnms3* mutant was attributed to a defect in the timely transition of the tapetum to the secretory type, which was similar to that of *Attdf1*, *Atam*s, and *Atmyb103* mutants. Moreover, the anther of *B. napus* is bigger than that of *Arabidopsis*, which made it easier to distinguish the

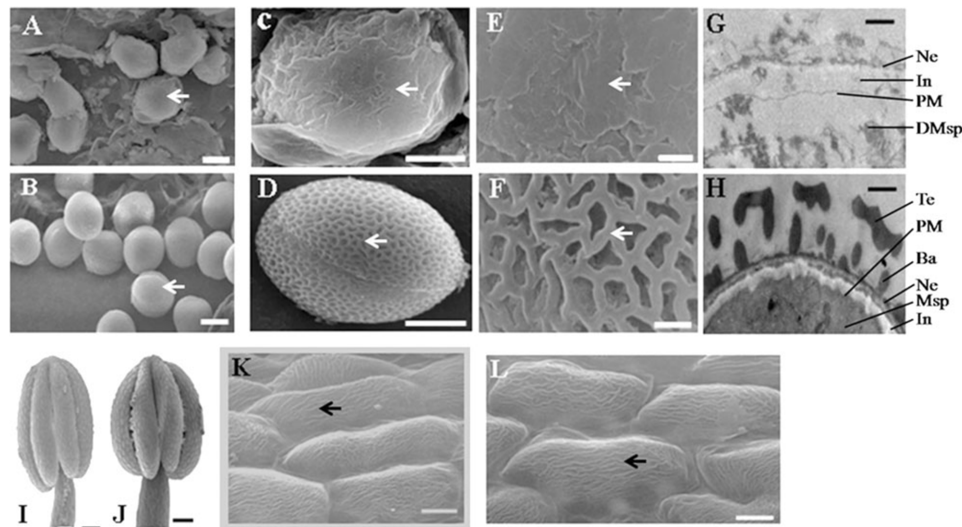

Fig. 9. Scanning and transmission electron microscopy (SEM, TEM, respectively) of pollen exine and anther epidermis from wild-type and *Atmsr02* transferred-DNA mutant *A. thaliana*. (A–F) SEM micrographs of pollen exine of (A,C,E) *Atmsr02* transferred-DNA mutant, showing smooth surfaces of the pollen grains (arrows), and (B,D,F) wild type, displaying a regular reticulate pattern (arrows). (G,H) TEM micrographs of the mature pollen wall of (G) *Atmsr02* transferred-DNA mutant, displaying abnormal exine layer on the pollen, and (H) wild type, showing an intact exine structure. (I,J) SEM micrographs of anthers of (I) *Atmsr02* transferred-DNA mutant and (J) wild type. (K,L) SEM micrographs of the outmost surface of the anther epidermis in (K) wild type and (L) *Atmsr02* transferred-DNA mutant, both exhibiting no apparent differences and having cuticular ridges (arrows). Ba, baculae; DMsp, degenerated microspore; In, intine ; Msp, microspore; Ne, nexine; PM, plasma membrane; Te, tectum. Bars=10 μm (A,B), 2.5 μm (C,D), 1 μm (E,F), 0.5 μm (G,H), 50 μm (I,J), 5 μm (K,L).

secretory type. Real-time PCR analysis demonstrated that the expression levels of *BnTDF1*, *BnAMS*, and *BnMYB103* were delayed at the post-meiotic stage of anther development in the *Bnms3* mutant. These data suggested that *BnMs3* shared the same pathway with *BnTDF1*, *BnAMS*, and *BnMYB103* in tapetal differentiation and might control the transition of the tapetal cells by affecting the expression of *BnMYB103* (Fig. 10A,B).

In flowering plants, degeneration of the tapetal cells is required for pollen-wall formation and is considered as the result of PCD (Wu and Cheung, 2000; Vizcay-Barrena and Wilson, 2006). The TUNEL assay has confirmed that tapetal PCD in *Atms1*, *Bncyp704b1*, *Ostdr* (*AtAMS* orthologue in rice), and *Bnms3* mutants was delayed or absent (Li *et al.*, 2006; Vizcay-Barrena and Wilson, 2006; Yi *et al.*, 2010; Dun *et al.*, 2011). Cysteine proteases are induced in PCD (Solomon *et al.*, 1999). *OsTDR* directly regulates a cysteine protease (*OsCP1*) associated with PCD (Li *et al.*, 2006). MS1, acting downstream of *AMS* and *CYP704B1*, also affects the expression of a cysteine protease gene, *At1g06260* (Yang *et al.*, 2007; Chen *et al.*, 2009; Xu *et al.*, 2010). The real-time PCR analysis revealed that the expression levels of *BnMS1*, *BnAMS*, and *BnCYP704B1* and five cysteine protease genes were affected in the *Bnms3* mutant at the corresponding initial expression stages of anther development. These results demonstrated that the *Bnms3* mutant exhibited abnormal tapetal degradation due to delayed tapetal PCD, which is affected by *BnMs3* by

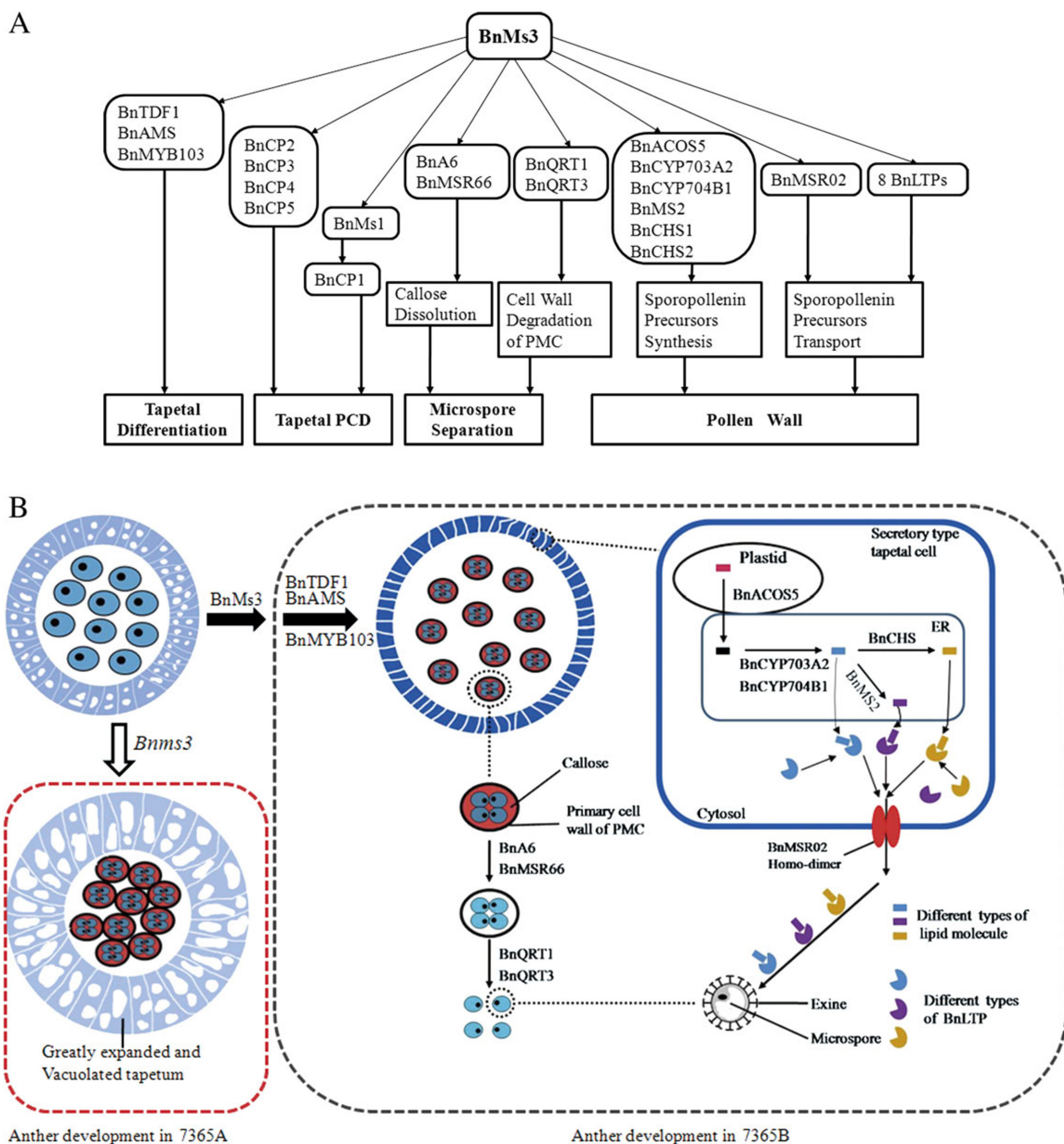

Fig. 10. Functional model of *BnMs3* in anther development of *B. napus*. (A) The functional network of *BnMs3* in tapetum and microspore development in *B. napus* based on the analysis of suppression subtractive hybridization and gene information from *Arabidopsis*. (B) Model for the role of *BnMs3* in tapetal function and pollen development in *B. napus* according to cytological observation, suppression subtractive hybridization, and gene information from *Arabidopsis*. 7365A and 7365B represent the *Bnms3* mutant and the wild type, respectively.

modulating the expression of four cysteine proteases at the tetrad stage and that of *BnMS1* to affect the expression of *BnCP1* (*At1g06260*) at the uninucleate stage (Fig. 10A).

BnMs3 *is essential for microspore separation*

In anther development, the PMC undergoes meiosis to form a tetrad, which is surrounded by the inner wall (the callose secondary cell wall) and the outer wall (the primary wall of PMC) (Rhee and Somerville, 1998). Therefore, degradation of the callose wall and primary wall of the PMC is a critical step during pollen development. The callose wall is made of β-1,3-glucan and is dissolved by callase (β-1,3-glucanase) secreted by the secretory tapetum (Frankel *et al.*, 1969; Stieglitz and Stern, 1973; Stieglitz, 1977). Several tapetum-specific expressed genes encoding β-1,3-glucanase have been cloned, such as *Tag1* in tobacco (Bucciaglia and Smith, 1994), *Osg1* in rice (Yamaguchi *et al.*, 2002), and *A6* from *B. napus* and *Arabidopsis* (Hird *et al.*, 1993). Silencing of *Osg1* using RNA interference resulted in disruption of callose degradation (Wan *et al.*, 2011). In transgenic tobacco, two pathogenesis-related β-1,3-glucanase genes were fused to the tapetum-specific promoters and the callose wall of the microspores dissolved prematurely, finally leading to male sterility (Worrall *et al.*, 1992; Tsuchiya *et al.*, 1995). These results imply that the timely degradation of the callose wall is crucial for microspore separation and that merely one β-1,3-glucanase is sufficient to form callase for degrading callose. The callose of the *Bnms3* mutant in this study was not degraded in time, according to aniline blue staining, which is consistent with results in *Attdf1* and *Atmyb103* mutants (Zhu *et al.*, 2010). Additionally, reduced expression of *A6* in *Attdf1* and *Atmyb103* mutants may result in abnormal callose degradation (Zhang *et al.*, 2007; Zhu *et al.*, 2008). This study isolated a novel β-1,3-glucanase (BnMSR66) in the *Bnms3* mutant, which included a glycosyl hydrolase family 17 motif and an X8 domain and had an expression pattern similar to that of *BnA6*. Both *BnMSR66* and *BnA6* showed reduced expression at the post-meiotic stage of anther development in the *Bnms3* mutant. Alignment of the sequences exhibited 73% identity at the amino acid level. Therefore, *BnMs3* may specifically control several β-1,3-glucanase genes that are likely to be functionally redundant in regulating callase activity in *B. napus* (Fig. 10A,B). The subsequent degradation of the PMC pectic wall is required for microspore separation (Rhee and Somerville, 1998). In *Arabidopsis*, *QRT1*, *QRT2*, and *QRT3* are involved in this process (Preuss *et al.*, 1994). Loss-of-function mutations of any one of the three *QRT* genes lead to the lack of microspores separation after meiosis and produce mature pollen tetrads (Rhee *et al.*, 2003; Francis *et al.*, 2006; Ogawa *et al.*, 2009). In the *Bnms3* mutant, the expression levels of *BnQRT1* and *BnQRT3* were down-regulated at the post-meiotic stage. These results indicate that *BnMs3* is also involved in controlling the degradation of the PMC pectic wall by affecting the expression of *BnQRT1* and *BnQRT3* (Fig. 10A,B).

BnMs3 *affects pollen-wall development*

The synthesis of pollen exine is a vital step during microspore development (Zinkl *et al.*, 1999). Since polymer sporopollenin largely composed of fatty acids and phenolic compounds are key components of pollen exine (Paxson-Sowders *et al.*, 1997; Piffanelli *et al.*, 1998), lipid metabolism and export are essential for exine formation (Piffanelli *et al.*, 1998; Ahlers *et al.*, 1999). In the *Bnms3* mutant, expression levels of five genes encoding putative enzymes involved in lipid metabolism were down-regulated, including two genes (*BnMSR20* encoding fatty acid elongase 3-ketoacyl-CoA synthase 7 and *BnMSR73* encoding beta-ketoacyl-CoA synthase) concerned with the biosynthesis of long-chain fatty acids, which plays an important role in pollen exine formation (Zhang *et al.*, 2008). Hydroxylation of fatty acids is also required for the exine formation, and several cytochrome P450 enzymes are associated with ω-hydroxylation of fatty acids (Mizutani and Ohta, 2010). It has been shown that CYP703A2 and CYP704B1 catalyse the in-chain hydroxylation of C10 to C14 and C16 to C18 fatty acids during pollen exine formation, respectively (Morant *et al.*, 2007; Dobritsa *et al.*, 2009; Li *et al.*, 2010). In the *Bnms3* mutant, the expression levels of both *BnCYP703A2* and *BnCYP704B1* were decreased. The conversion of fatty acids to fatty alcohols is required for lipid synthesis, which is principally important for pollen exine formation in flowering plants (Aarts *et al.*, 1997). *MS2*, encoding a fatty acyl carrier protein reductase associated with sporopollenin biosynthesis, is involved in this conversion during pollen development of *Arabidopsis* and rice (Aarts *et al.*, 1997; Shi *et al.*, 2011). The present study found that the expression of *BnMS2* was remarkably decreased at the post-meiotic stage of anther development in the *Bnms3* mutant. Moreover, chalcone synthase is able to use medium- to long-chain (C4–C20) fatty acyl-CoA as a substrate for generating phenylpropanoid (an ingredient of sporopollenin precursors), in which *At4g00040* (*CHS1*) and *At4g34850* (*CHS2*) are involved (Dobritsa *et al.*, 2010). The real-time PCR data in this study showed that the expression levels of *BnCHS1* and *BnCHS2* were affected in the *Bnms3* mutant. This suggested that both fatty acid and phenylpropanoid pathways were altered in the *Bnms3* mutant.

Apart from playing a role in the formation of pollen-wall materials, *BnMs3* also affects the export of lipid molecules from the tapetal cells to the surface of the microspores, which mainly consists of two steps (Samuels *et al.*, 2008). First, the lipid molecules synthesized in the tapetal cells are exported through the PM. ABCG11 and ABCG12 belong to ABC transporter protein family, which uses ATP hydrolysis to transport a variety of substances across biological membranes, and are required for wax transport across the PM in *Arabidopsis* (Pighin *et al.*, 2004; David *et al.*, 2007; Panikashvili *et al.*, 2007). In the *Bnms3* mutant, the expression of *BnMSR02* (*ABCG26*) was changed, and the *Atabcg26* mutant (*Atmsr02*) resulted in a male sterile phenotype due to microspore degradation, which was consistent with the data reported by Quilichini *et al.* (2010) and Choi *et al.* (2011). Fatty acids are required for both

cuticle and exine formation during anther development; consequently, transmission and scanning electron microscopy was conducted to analyse the cuticle and exine of the *Atabcg26* mutant. The cuticle was normal but the pollen exine was abnormal. Therefore it is concluded that *ABCG26* was only essential for lipid export of sporopollen precursors through the PM during pollen exine development. The second step of lipid transport is from the extracellular environment to the surface of microspores to form pollen exine (Samuels *et al.*, 2008; Zhang *et al.*, 2010). So far, LTP has been considered as an attractive candidate for this process (DeBono *et al.*, 2009). Expression levels of the eight genes putatively related to LTPs were apparently down-regulated in the *Bnms3* mutant. These results suggested that defects in the biosynthesis and transport of lipids, which led to fewer microspores being released from the tetrad and the abnormal pollen exine formation in the *Bnms3* mutant, made the pollen abortion more thorough (Fig. 10A,B).

A model for BnMs3 *function in anther development*

Cytological observation combined with the differential gene expression data from the macroarray has shown that *BnMs3* is involved in tapetal function and pollen development. According to putative models for the network of tapetum and pollen development in *Arabidopsis* and rice (Zhu *et al.*, 2008; Wilson and Zhang, 2009; Xu *et al.*, 2010), a few crucial genes from the models were analysed in this study using real-time PCR, which has provided a deeper insight into the role of *BnMs3* during anther development (Fig. 10A,B). All data indicated that *BnMs3* plays a specific role in the transition of the tapetal cells to the secretory type. Additionally, it is also required for microspore separation, lipid biosynthesis, export of pollen-wall materials, and pulling the trigger of tapetal PCD by affecting the expression of several key genes in *B. napus* (Fig. 10A,B).

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Differential screening of the subtracted libraries using macroarray analysis.

Supplementary Table S1. Homology analysis of differentially expressed sequences from the subtracted library.

Supplementary Table S2. Gene-specific primer pairs used in this study.

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