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Research paper

Determination of male strobilus developmental stages by cytological and gene expression analyses in Japanese cedar (*Cryptomeria japonica*)

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The molecular mechanisms that control male strobilus development in conifers are largely unknown because the developmental stages and related genes have not yet been characterized. The determination of male strobilus developmental stages will contribute to genetic research and reproductive biology in conifers. Our objectives in this study were to determine the developmental stages of male strobili by cytological and transcriptome analysis, and to determine the stages at which aberrant morphology is observed in a male-sterile mutant of *Cryptomeria japonica* D. Don to better understand the molecular mechanisms that control male strobilus and pollen development. Male strobilus development was observed for 8 months, from initiation to pollen dispersal. A set of 19,209 expressed sequence tags (ESTs) collected from a male reproductive library and a pollen library was used for microarray analysis. We divided male strobilus development into 10 stages by cytological and transcriptome analysis. Eight clusters (7324 ESTs) exhibited major changes in transcriptome profiles during male strobili and pollen development in *C. japonica*. Two clusters showed a gradual increase and decline in transcript abundance, respectively, while the other six clusters exhibited stage-specific changes. The stages at which the male sterility trait of Sosyun was expressed were identified using information on male strobilus and pollen developmental stages and gene expression profiles. Aberrant morphology was observed cytologically at Stage 6 (microspore stage), and differences in expression patterns compared with wild type were observed at Stage 4 (tetrad stage).

Keywords: cDNA microarray, conifer, *Cryptomeria japonica*, male reproduction.

Introduction

Male strobili are one of the most important organs for conifers, and many studies associated with seed orchard management have reported on pollen development (Owens and Blake 1985, Bonnet-Masimbert and Webber 1995, Owens 2004, 2006, 2008, Fernando 2013). Reproduction in many conifers begins 5–10 years after planting (Williams 2009), and the number of male strobili and female cones fluctuates from year to year. Most conifers rarely bear male strobili or female cones in their juvenile period without artificial treatment to stimulate reproductive onset (Williams 2009). Male strobilus and female cone induction in many Pinaceae has been achieved through application of a gibberellin (GA_4 and GA_7) mixture in combination with various cultural treatments (Owens and Blake 1985). In addition to GA application, the effects of girdling and root pruning on induction of male strobili and female cones have also been studied (Pharis 1975, Owens and Blake 1985, Bonnet-Masimbert and Webber 1995, Almqvist 2003).

Male strobilus and pollen development in conifer species have been investigated, especially with respect to morphological traits (Hashizume 1962, Williams 2009). In *Pinus taeda*, the timing and pattern of initiation and differentiation of male strobili have

been investigated (Greenwood 1980), and male strobilus development was divided into six stages based only on morphological traits, without observation of cytological traits (Bramlett and Bridgwater 1989, Williams 2009). Harrison and Slee (1992) reported on pollen cone differentiation in *Pinus caribaea* based on a microscopic study. The reproductive biology, especially with a focus on reproductive cycle and seed production, of western white pine (*Pinus monticola* Dougl.), lodgepole pine (*Pinus contorta* Dougl.), and western white larch (*Larix occidentalis* Nutt) has been reported (Owens 2004, 2006, 2008), and Fernando (2013) has reviewed these studies. Pollen and pollen wall development have also been well studied in conifers (Kurmann 1989, Rowley et al. 2000, Uehara and Sahashi 2000, Fernando et al. 2005, Owens et al. 2008). In addition to these cytological analyses, further studies that consider male strobilus development in connection with the genes involved are necessary to understand male reproduction in conifers.

Conifer species in which reproductive onset can be controlled with easy treatment are desirable for studies on reproductive biology. Male reproductive organs have been well studied in *Cryptomeria japonica* D. Don, Japanese cedar (Futamura et al. 2008, Moriguchi et al. 2012, Tsubomura et al. 2012, Ujino-Ihara et al. 2012, Kurita et al. 2013), because GA_3 treatment has a strong effect on male reproductive onset. GA_3 treatment of seedlings, even of 1-year-old individuals, enables male strobilus and female cone set (Hashizume 1959). Male strobili are easily induced by GA_3 application in July, as are female cones from the end of July to the end of August (Hashizume 1959). Male strobilus and female cone formation are initiated about 1–2 months after GA_3 treatment, and later development is similar to natural development (Hashizume 1962).

Fortunately, male-sterile mutants in *C. japonica* have been discovered from natural stands, and they have been classified into several types (*ms-1*, *ms-2*, *ms-3*, *ms-4*) based on cytological observation and results of test crosses (Saito et al. 1998, Hosoo et al. 2005, Ueuma et al. 2009, Miyajima et al. 2010, Miura et al. 2011, Moriguchi et al. 2014). Some male-sterile mutants are due to nuclear mutations controlled by a pair of recessive genes (Taira et al. 1999, Moriguchi et al. 2014). The characterization of male-sterile mutants in *C. japonica* may lead to understanding of the molecular mechanisms of male strobilus and pollen development.

The molecular mechanisms controlling reproductive onset in conifers are largely unknown, and genes related to male strobilus and pollen development have not yet been characterized, with some exceptions, including MADS-box genes (Katahata et al. 2014), *FT* (*FLOWERING LOCUS T*)*-like* genes (Klintenäs et al. 2012), gibberellin metabolism genes (Niu et al. 2014), and *LEAFY* (Dornelas and Rodriguez 2005, Shiokawa et al. 2008). In recent years, expressed sequence tags (ESTs) have been obtained on a large scale in *C. japonica* (Futamura et al. 2008, Mishima et al. 2014, Nose and Watanabe 2014). Expressed sequence tags expressed in male reproductive organs have been obtained from full-length cDNA libraries, and ~20,000 ESTs are available to the public (Futamura et al. 2008). Transcriptome analysis using these ESTs may allow characterization of each stage in the male strobilus developmental process. In this study, our objectives were (i) the classification of male strobilus developmental stages by cytological and transcriptome analysis, and (ii) the determination of the aberrant stage or stages in the newly discovered male-sterile mutant 'Sosyun' to understand genes related to male strobilus and pollen development.

Materials and methods

Plant material and sampling

One wild type clone (WT1, 7-year-old seedling) was used for cytological analysis and another wild type clone (WT2, Usui2, 17-year-old graft) and a male-sterile mutant (MT, Sosyun, 4-year-old cutting) were used for cytological and gene expression analysis. They were planted in Hitachi, Ibaraki, Japan (36°69N, 140°69E; elevation 52 m).

On 4 July 2011, these individuals were sprayed with 100 ppm GA_3 (Kyowa-Hakko, Japan) to promote setting of male strobili. Sampling of male strobili began at the end of August, when they started to develop, and continued to the end of the following March, the pollen dispersal season. At each sampling time, twigs of ~3 cm in length with male strobili were collected from the top of the shoots of each tree. The materials for gene expression analysis were stored at −80 °C, and the materials for cytological analysis were fixed in a solution of formalin : acetic acid : alcohol (FAA, 5 : 5 : 90 by volume) and stored at 4 °C until use.

Cytological analysis

Male strobili of ordinary size were collected from the twigs fixed in FAA and were soaked in water for a week. Since the size of male strobili of *C. japonica* varied within one cluster, extremely large or small strobili were eliminated by eye. The samples were prepared following the modified method of the Kawamoto system (Kawamoto and Shimizu 2000). The samples were frozen in cooled hexane and freeze-embedded with 4–5% carboxymethyl cellulose in the coolant. A specially prepared adhesive film, Cryofilm (Leica, Tokyo, Japan), was fastened to the cut surface of the samples. Longitudinal sections ~7 μm thick were cut from the embedded blocks using a Leica CM3050S research cryostat (Leica, Bensheim, Germany). The sections were stained with hematoxylin and eosin for cytological observation. The sections were observed under a Leica Leitz DMR microscope (Leica). Male strobilus developmental stages were defined by observation.

Suppression subtractive hybridization

A third wild type clone (WT3, Ohtawara1, 11-year-old graft) and a male-sterile mutant (MT, Sosyun, 11-year-old graft) were used

for suppression subtractive hybridization (SSH) libraries. At the beginning of July 2006, these individuals were sprayed with 100 ppm GA_3, and sampling of male strobili began on 19 September and continued to 17 October 2006. At each sampling time, twigs of ~3 cm in length with male strobili were collected from the top of the shoots of each tree. The materials were stored at −80 °C until use. A PCR-Select cDNA Subtraction kit (Takara Bio Inc., Kusatsu, Japan) was used for SSH. First, we constructed four male strobili cDNA pools (cDNA pools 1, 2, 3 and 4); these were respectively derived from the pollen mother cell (PMC) stage of WT3, the PMC stage of MT, the tetrad stage of WT3 and the tetrad stage of MT. Then we made four SSH libraries in the following way. Library SSH_A was constructed using cDNA pool 1 (tester) and cDNA pool 2 (driver). Library SSH_B was constructed using cDNA pool 3 (tester) and cDNA pool 4 (driver). Library SSH_C was constructed using cDNA pool 2 (tester) and cDNA pool 1 (driver). Library SSH_D was constructed using cDNA pool 4 (tester) and cDNA pool 3 (driver). Double-stranded cDNA was synthesized and digested with restriction enzyme *Rsa*I. Tester cDNAs were divided into two groups, one ligated to specific adaptor 1 in the Takara kit and the other to specific adaptor 2R. Each denatured adaptor-ligated tester mix was separately hybridized with a single-stranded driver cDNA for 8 h at 68 °C to enrich non-pairing sequences unique to the tester cDNA pool. A second hybridization using single-stranded driver cDNA as well as the products of the first hybridization from both tester aliquots (adaptor 1-linked and adaptor 2R-linked molecules) was performed for 16 h at 68 °C. Products from the second hybridization were diluted in 200 μl of dilution buffer of this kit, heated at 68 °C for 7 min, and stored at −20 °C. Cloning and sequencing were carried out according to Kurita et al. (2011). Finally, 278, 184, 281 and 180 ESTs were obtained from the SSH_A, SSH_B, SSH_C, and SSH_D library, respectively. In total, 923 ESTs were submitted to the DNA Data Bank of Japan (HX970307 through HX971791).

Microarray chip design

A microarray chip was designed according to the Roche Nimblegen protocol. A custom microarray comprising a total of 75,160 oligonucleotide probes was designed using the EST sequences in the ForestGEN public database (Forest EST and Genome database, http://forestgen.ffpri.affrc.go.jp) and the ESTs collected from SSH libraries of male strobili (Kurita et al. 2011) and libraries of MT and WT3 male strobili (described above).

We chose 18,080 ESTs from the male reproductive organ library (Futamura et al. 2008) and pollen libraries of the ForestGEN database. The male reproductive organ library was constructed using male strobili collected from August to mid-November (Futamura et al. 2008). This library might contain samples of the early and middle stages of male strobilus development and the pollen library might contain samples of the late stages. From the SSH libraries, 1129 ESTs were selected. Altogether, 3773 probes were based on the ESTs from SSH libraries and 68,061 probes were based on the ESTs from the ForestGEN database. The ESTs were spotted in triplicate or quadruplicate on the array. The array comprised 19,209 individual ESTs. Using the TAIR8 blastX algorithm (http://www.arabidopsis.org/index.jsp), 18,688 ESTs were annotated and 521 ESTs had no hits.

RNA preparation and microarray analysis

Male strobili were collected from each twig and were observed by microscope according to the methods previously described. Then, each sample stage was defined and strobili from each stage used for total RNA isolation. Total RNA was isolated using Plant RNA Isolation Reagent (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. This RNA was reacted with recombinant DNase I (Takara Bio Inc.) and purified using an RNeasy plant mini kit (Qiagen, Hilden, Germany). The quality of RNA was checked using an Agilent 2100 bioanalyzer and RNA 6000 Nano Kit (Agilent Technologies, Tokyo, Japan) before cDNA synthesis. Three biological replicates of each sample were used in the analysis. Double-stranded cDNA was synthesized from 10 μg of total RNA using a SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen) and labeled with Cy3 random nonamers with a One-Color DNA Labeling Kit (Roche Nimblegen, Madison, WI, USA). The following steps were carried out with equipment and software from the same manufacturer. The custom 4 × 72K array was incubated at 42 °C for 18 h in a Hybridization System 4 (Roche Nimblegen), and washed at room temperature. The microarray slide was scanned at 2 μm resolution using an MS 200 Microarray Scanner (Roche Nimblegen), generating corresponding 532 nm TIFF images. The data were imported into Nimblescan software (Roche Nimblegen) to quantify the signal intensities of the spots on the image. The microarray design and data have been submitted to the National Center for Biotechnology Information Gene Expression Omnibus database (GSE64663).

Data processing and statistical analyses

Normalization of signal intensity was carried out with Nimblescan software. Expressed sequence tags with raw signal intensity <100 in two-thirds of all samples were excluded. The array data were normalized according to the Robust Multichip Average algorithm. The datasets were normalized through two following normalization steps using the Subio platform (Subio Inc., Kagoshima, Japan), log-based transformation of the data, and global normalization (to the 75th percentile). We calculated the average $\log_2$ ratios at each time point, and excluded ESTs with expression levels that hardly varied (between −1.0 and 1.0) through all stages. One-way analysis of variance (Benjamini–Hochberg false discovery rate <0.05) was executed to identify ESTs with expression levels that varied for at least one time

point. Clustering and categorizing were performed on the Subio platform. We selected the annotated ESTs with low *E*-values (*E*-value <1e−5) and categorized them using a database of Clusters of Orthologous Groups (COG) from seven eukaryotic genomes (Tatusov et al. 2003).

Real-time PCR

Primer pairs were designed for each sequence using Primer3 software (Rozen and Skaletsky 1999), and the details of these primers are shown in Table S1 available as Supplementary Data at *Tree Physiology* Online. For SYBR Green real-time PCR assays, the amplification efficiency of all primer pairs was optimized with genomic DNA from the WT2 clone using the StepOnePlus Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific).

Total RNA (500 ng in a final volume of 20 µl) extracted from male strobili at each stage was reverse-transcribed using the High Capacity RNA-to-cDNA Kit (Applied Biosystems) according to the manufacturer's protocol. Quantitative real-time PCR was performed on the total RNA samples using Power SYBR Green PCR Master Mix (Applied Biosystems) on the StepOnePlus Real-Time PCR System. PCR mixtures were prepared according to the manufacturer's instructions and contained 300 nM of both forward and reverse gene-specific primers and 2 µl of the 50-fold diluted reverse transcription reaction (total 1 ng) in a final volume of 20 µl. All reactions were heated to 95 °C for 10 min; this denaturation step was followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The PCR products were subjected to melting curve analysis; the conditions were incubation at 60–95 °C with a temperature increment of 0.3 °C s^{-1}. We used *C. japonica* ubiquitin as an internal control gene, amplified with specific primers (Nose and Watanabe 2014). Relative expression was measured by the $\Delta\Delta C_t$ method (Pfaffl 2001). The experiments were conducted three times. The specificity of each amplification was checked by melting analysis.

Results

Cytological analysis of C. japonica male strobilus development

We divided *C. japonica* male strobilus development into 10 stages by observation of longitudinal sections of WT2 male strobili (Figure 1). Male strobili of *C. japonica* develop from axils. In late August, the point when the scale primordia were visible in the axils was defined as Stage 1 (Figure 1a and f). At the end of September, following scale formation, the microsporangia were differentiated on the basis of the appearance of scales (Stage 2, Figure 1b and g). Microsporangia wall, middle layer, tapetum and PMCs were recognized from the end of September to the beginning of October (Stage 3, Figure 1c and h). Pollen mother cells entered meiosis (Stage 4, Figure 1d and i), and meiosis was completed and tetrads developed (Stage 5, Figure 1j) in the middle of October. The callose wall surrounding the tetrads degenerated and the microspores were released into the microsporangium (Stage 6, Figure 1k and p). The tapetum degenerated at the end of October (Stage 7, Figure 1l and q), and fibrous bands formed in the microsporangial wall at the beginning of November (Stage 8, Figure 1r and s). Pollen mitotic division occurred and mature pollen grains developed in late December (Stage 9, Figure 1t and u). Dehiscence occurred and pollen grains were released in March (Stage 10, Figure 1v).

The correspondence between *C. japonica* male strobilus developmental stages and *Arabidopsis* anther developmental stages (Sanders et al. 1999) is shown in Table 1. In *Arabidopsis thaliana*, primary parietal and primary sporogenous layers are derived from archesporial cells and further divisions of each layer generate the secondary parietal layers and sporogenous cells, respectively, at Stage 3. Stage 2 of *C. japonica* corresponded to Stage 3 of *A. thaliana*. Microspore mother cells appearing at Stage 5 in *A. thaliana* corresponded to Stage 3 of *C. japonica*. Entering meiosis, formation of tetrads and appearance of microspores occurs at Stages 6–8 in *A. thaliana*, corresponding with *C. japonica* pollen developmental Stages 4–6, respectively. Stages 7 and 8 in *C. japonica*, tapetum degeneration to appearance of fibrous bands, corresponded with Stages 10 and 11 of *A. thaliana*, respectively. Pollen mitotic division occurs at Stage 11 in *A. thaliana* and at Stage 9 in *C. japonica*. While the pollen grains of *C. japonica* have two cells at Stage 9 (Figure 1u), the anther contains tricellular pollen grains at Stages 12 in *A. thaliana*. Pollen release occurs at Stage 13 in *A. thaliana* and at Stage 10 in *C. japonica*. Stages 14 and 15 of *A. thaliana* are stamen senescence, and therefore these stages were not observed in *C. japonica*.

The correspondence between morphological development of *C. japonica* and *P. taeda* male strobili (Bramlett and Bridgwater 1989, Williams 2009) is also shown in Table 1. In the *P. taeda* male strobilus developmental classification, at Stage 1 male strobili are encased in bud scales at tips of vegetative shoots; at Stage 2 the individual male strobilus emerges from its bud scales; at Stage 3 the male strobilus exudes liquid when pressed; at Stage 4 pollen release starts; at Stage 5 pollen release reaches a maximum; and at Stage 6 pollen release is completed (Williams 2009). Stages 1 and 2 of *C. japonica* corresponded to Stages 1 and 2 of *P. taeda*, respectively. Stages 3–9 and Stage 10 of *C. japonica* corresponded to Stage 3 and Stages 4–6 of *P. taeda*, respectively.

Gene expression analysis

Gene expression patterns during male strobilus development were examined by microarray analysis to identify ESTs that were differentially expressed among the 10 stages. Principal component analysis was performed on the expression patterns of 8079 ESTs. The developmental stages were clustered into four groups, Stages 1–3, Stages 4–7, Stage 8 and Stages 9–10 (Figure 2).

The 8079 ESTs were sorted into several clusters according to their expression profiles during male strobili and pollen

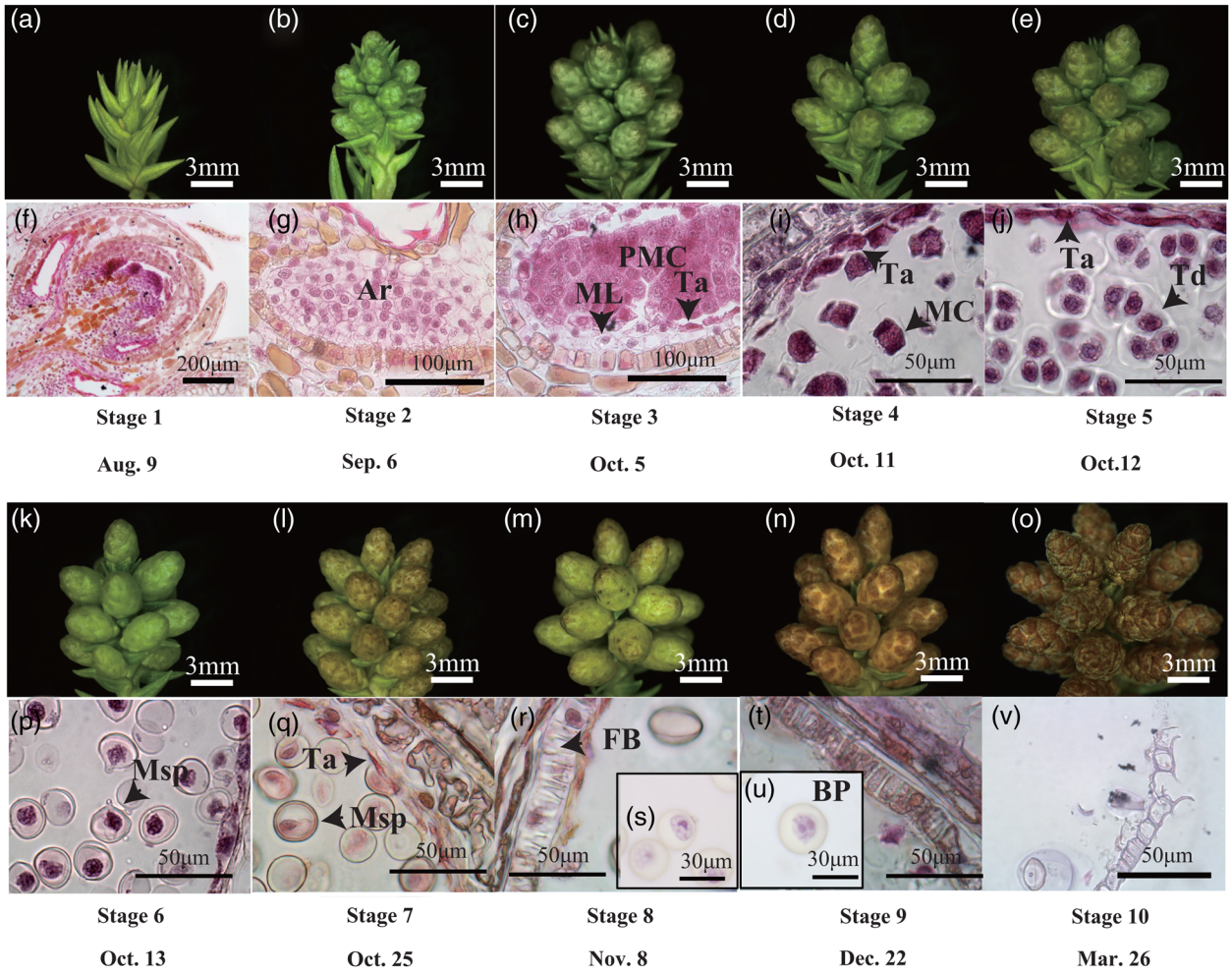

Figure 1. Development of *C. japonica* male strobili and pollen. (a–e) Longitudinal section of male strobili at Stages 1–5; (f–j) male strobilus at Stages 1–5; (k–o) male strobilus at Stages 6–10; (p–v) male strobilus at Stages 6–10; sections were stained with eosin and hematoxylin. Ar, archesporial cell; BP, bicellular pollen; FB, fibrous band; MC, meiotic cell; ML, middle layer; Msp, microspore; PMC, pollen mother cell; Ta, tapetum; Td, tetrad.

Table 1. Cytological characterization of the 10 developmental stages of male strobili of *C. japonica*.

C. japonica male strobilus stages and cytological characterization	Anther stage of *Arabidopsis* (Sanders et al. 1999)	Microsporangiate strobilus development of *P. taeda* (Williams 2009)
1. The scale primordia are visible in the axils	—	1
2. The archesporial cells are differentiated in the base of the scales	3	2
3. The intact tapetum layer is well defined	5	3
4. Microspore mother cells enter meiosis	6	3
5. Meiosis completed; tetrads of micropsores free within each locule	7	3
6. Callose wall surrounding tetrads degenerates and individual microspores released	8	3
7. Tapetum degeneration initiated	10	3
8. 'Fibrous bands' appear in endothecium	11	3
9. Pollen mitotic division occurrs	11–12	3
10. Dehiscence; pollen release	13	4–6

development using the Subio platform tree clustering tool (Pearson correlation, Figure 3). Eight major clusters (7324 ESTs) were further examined (Figures 3 and 4). Two clusters, C2 and C6, showed a gradual increase and decline; these included the majority of all profiles (58.5%). Other profiles showed stage-specific expression patterns. Four clusters exhibited a transient increase at multiple stages (C1, C3, C5 and C7). C1 had transient increases in the late developmental stages, 8 and 9. Two clusters (C3 and C7) showed a transient increase at the middle developmental stages, 3–8. C5 exhibited a transient

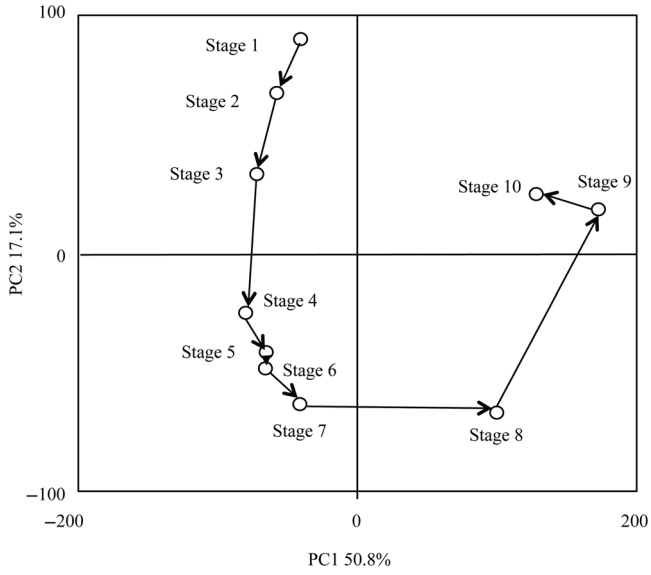

Figure 2. Principal component analysis of 10 stages in male strobilus development for 8079 ESTs differentially expressed among stages.

increase at the early developmental stages, 2–4. C8 showed a transient decrease at Stage 9. C4 showed a transient increase in the early and late stages, 1 and 10. Of the total 7324 ESTs, 38.5% (C1 and C2) were expressed only in the late developmental stages, and 46.0% (C3, C5, C6 and C7) were expressed in the early and middle developmental stages.

A total of 4198 ESTs can be assigned a putative function based on *E*-value <1e−5, and were classified into 26 functional categories. The ratio of each category to each cluster was calculated (Figure 4). In C1 the ratio of category [A], 'RNA processing', was higher than in the other clusters. The ratio of category [I], 'Lipid transport', in C3 and the ratio of category [D], 'Cell cycle control, cell division, chromosome partitioning', in C5 were high compared with other clusters. The ratio of category [G], 'Carbohydrate transport and metabolism', in C7 and C8 was higher than in other clusters.

Expressed sequence tags that changed in expression fourfold or more at a single stage ($P < 0.05$) compared with the other nine stages were identified. As a result, 15 ESTs at Stage 1,

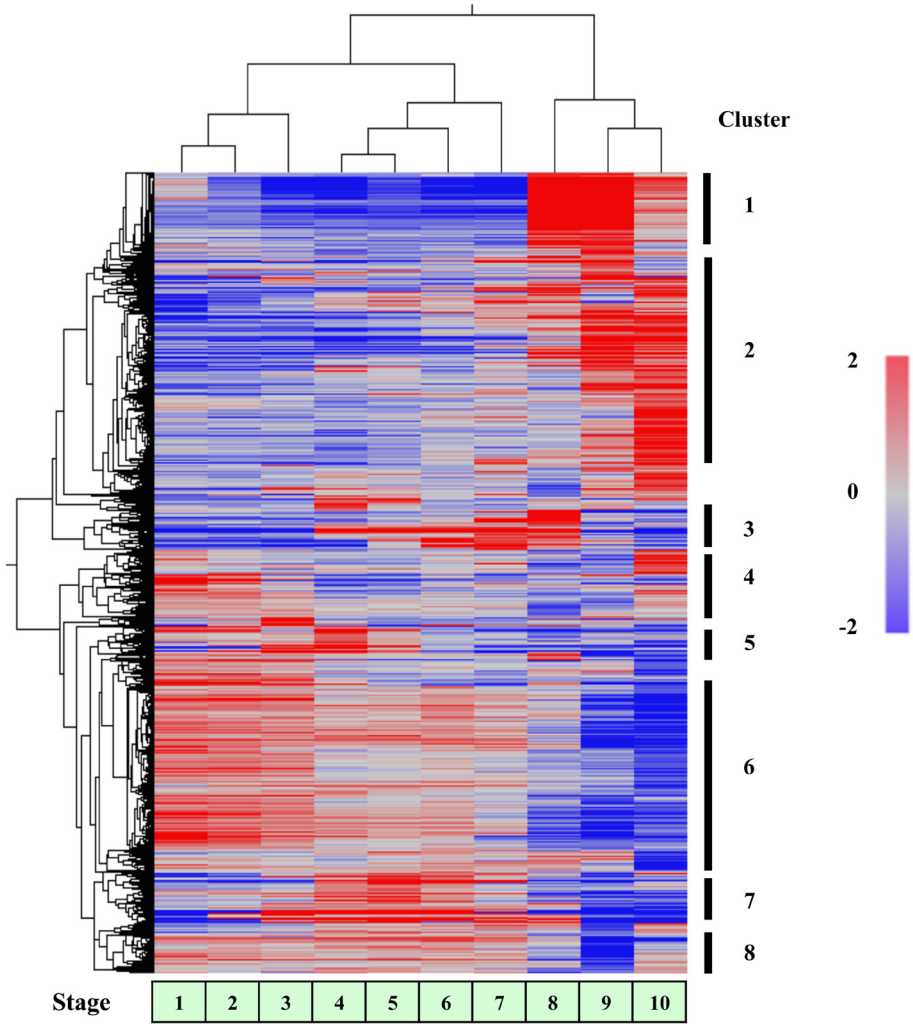

Figure 3. Tree clustering analysis of expression of 8079 ESTs in developing WT2 male strobili. Numbered bars on the right indicate distinct clusters of ESTs used for further analysis.

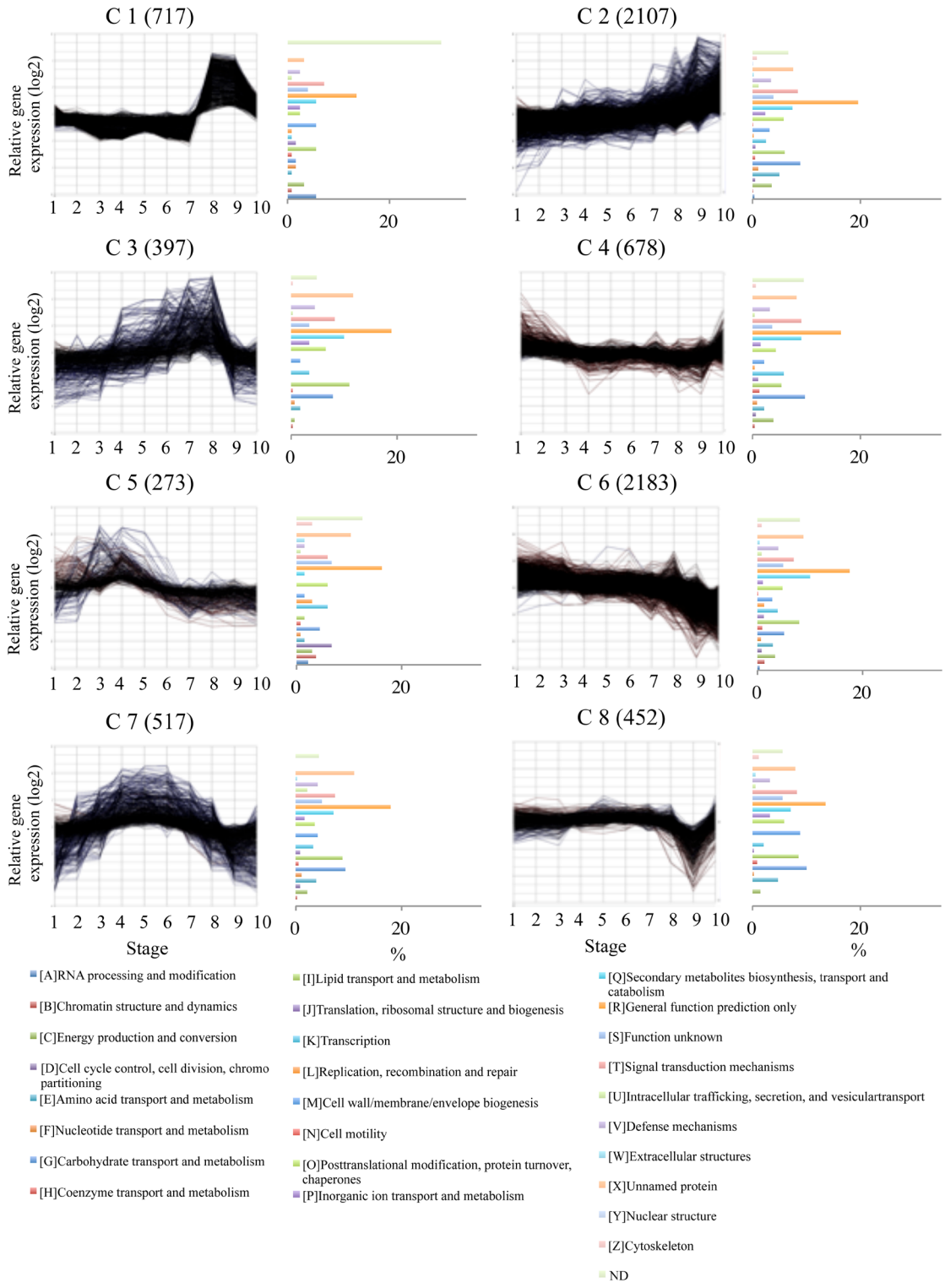

Figure 4. Category distribution in the eight expression clusters. Clusters were obtained for 7324 EST expression profiles using the Pearson correlation method. Cluster number and number of transcripts belonging to each cluster are labeled on each chart. In the histogram of the functional category distribution, each category is expressed as the percentage of the number of ESTs belonging to that cluster.

three ESTs at Stage 2, 35 ESTs at Stage 3, 15 ESTs at Stage 4, two ESTs at Stage 5, 19 ESTs at Stage 6, 24 ESTs at Stage 7, 79 ESTs at Stage 8, 59 ESTs at Stage 9, and 222 ESTs at Stage 10 were identified (see Table S2 available as Supplementary Data at *Tree Physiology* Online). The expression of these ESTs was confirmed by real-time PCR at all stages (Figure 5 and Figure S1 available as Supplementary Data at *Tree Physiology* Online).

Cytological and gene expression analysis of male-sterile mutant male strobili

Male strobili of MT were compared with WT2 during Stages 3–6, the pollen developmental stages (Figure 6). At Stages 3–5, when PMCs developed to tetrads, the MT phenotype was similar to that of WT2. At Stage 6, the degenerating callose wall and pollen release period, an abnormal phenotype was observed in MT microsporangia (Figure 6h). The tetrads of MT were not

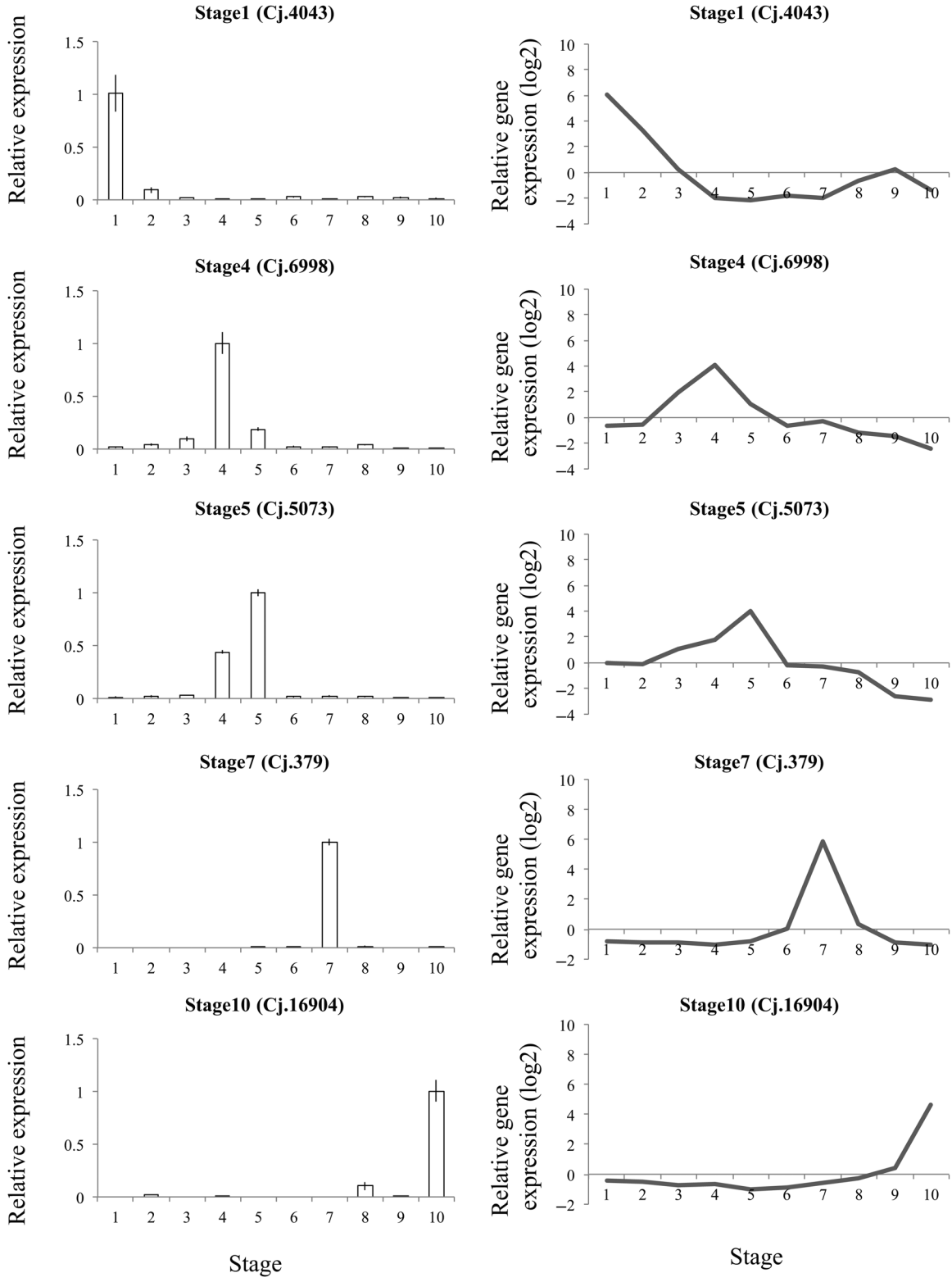

Figure 5. Real-time PCR analysis of five ESTs showing stage-specific expression. Bar graphs show relative expression from real-time PCR and line graphs show processed signals from microarray analysis. Error bar shows the standard deviation.

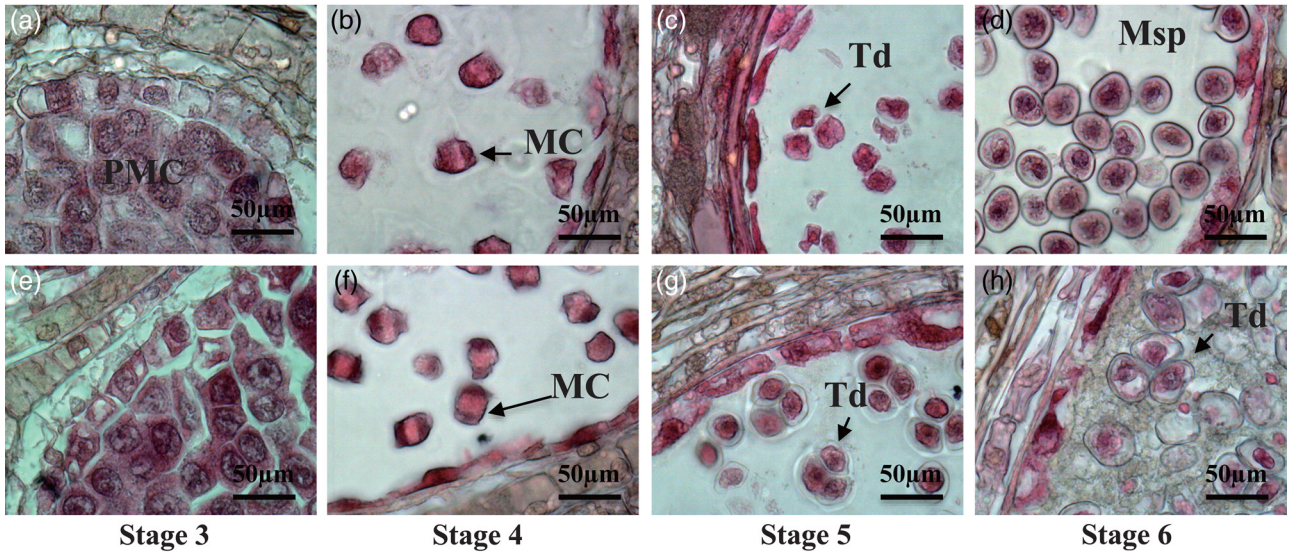

Figure 6. Male strobili and pollen development of WT2 and MT at Stages 4–6. (a–d) Longitudinal sections of wild type male strobili at Stages 4–6; (e–h) longitudinal sections of mutant type male strobili at Stages 4–6; MC, meiotic cell; Msp, microspore; PMC, pollen mother cell; Td, tetrad.

separated and they had thinner walls than the microspores of WT2. The cytoplasm of tetrads of MT was slightly expanded. The microsporangia of MT were filled with debris and the tetrads were embedded in them. The debris remained until Stage 10, and the microspores did not develop further.

Similarly, gene expression of MT during Stages 3–6 was analysed by microarray. Principal component analysis and clustering was performed on the expression patterns of the 8079 ESTs differentially expressed among the 10 stages in WT2 (see Figures S2 and S3 available as Supplementary Data at *Tree Physiology* Online). The changes in gene expression among stages of MT were similar to those of WT2, with slight differences at Stages 4–6 (see Figures S2 and S3 available as Supplementary Data at *Tree Physiology* Online).

We identified the ESTs for which expression changed at least fourfold ($P < 0.05$) between MT and WT2 at each developmental stage (Table 2 and Table S3 available as Supplementary Data at *Tree Physiology* Online). The largest number of upregulated and downregulated ESTs was recognized at Stage 5 (105 and 152 ESTs, respectively), compared with expression in WT2. The number of upregulated or downregulated ESTs at any stage was 188 and 209, respectively, and the total number of ESTs that changed in expression fourfold at any stage was 395. Of 109 ESTs downregulated at Stage 4, 92 were also downregulated at Stage 5, and 70 ESTs were downregulated from Stages 4 to 6. Details on upregulated and downregulated ESTs at Stages 4–6, omitting the ESTs with high *E*-values, are shown in Tables 3 and 4. Of the 46 downregulated ESTs, 14 were related to terpenoid biosynthesis, four belonged to the CYP450 family and three were disease resistance responsive family proteins and *O*-methyltransferase. Of the upregulated ESTs, four were identified as a disease resistance family protein.

Table 2. The number of ESTs at each stage with ≥ fourfold change in expression compared with WT2.

Stage	Fourfold up in MT	Fourfold down in MT
3 (PMC)	95	64
4 (MEI)	97	109
5 (TED)	105	152
6 (MSP)	103	110
3–6	40	32
4–6	46	70

Discussion

Male strobilus development of C. japonica based on cytological and transcriptome analysis

The process of male strobilus development (Hashizume 1962), pollen wall development (Uehara and Sahashi 2000), and histological comparison of fertile and sterile pollen development (Hosoo et al. 2005, Ueuma et al. 2009) have been reported in *C. japonica*. However, previous reports did not focus on gene expression or molecular analysis. Based on cytological observation and transcriptome analysis, male strobilus development of *C. japonica* can be divided into 10 stages. It took 8 months from male strobilus initiation to pollen maturation, as previously reported (Hashizume 1962). Identification of the stages by cytological analysis is difficult from Stages 3 to 6, because it takes only a few days to progress through them. In addition, even for the same tree and the same clusters, pollen developmental stages are slightly different among strobili. In comparison with *P. taeda* male strobilus development (Williams 2009), almost all stages were categorized as Stage 3 (Table 1). Internal cytological observation of the microsporangium is necessary for male strobilus developmental stage classification, in addition to external morphological traits. In *Pinus banksiana*, the

Table 3. Expressed sequence tags downregulated at Stages 4–6 in MT compared with WT2, omitting the ESTs with high *E*-values.

Measurement ID	TAIR	Annotation	*E*-value
Cj_ES_Contig_0091	AT4G23340.1	2-Oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	2.00e–13
Cj.13983	AT5G05600.1	2-Oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	7.00e–12
Cj.11285	AT4G05040.4	Ankyrin repeat family protein	6.00e–13
Cj.11004	AT5G24960.1	Cytochrome P450, family 71, subfamily A, polypeptide 14	2.00e–21
Cj_ssh_male_e_026	AT5G36110.1	Cytochrome P450, family 716, subfamily A, polypeptide 1	3.00e–67
Cj_ssh_male_e_198	AT5G36110.1	Cytochrome P450, family 716, subfamily A, polypeptide 1	7.00e–29
Cj.26959	AT2G45580.1	Cytochrome P450, family 76, subfamily C, polypeptide 3	2.00e–30
Cj.21151	AT2G21100.1	Disease resistance-responsive (dirigent-like protein) family protein	6.00e–17
Cj.20835	AT5G42500.1	Disease resistance-responsive (dirigent-like protein) family protein	2.00e–08
Cj.8130	AT5G42500.1	Disease resistance-responsive (dirigent-like protein) family protein	2.00e–19
Cj.17487	AT5G22500.1	Fatty acid reductase 1	4.00e–26
Cj.20412	AT1G08510.1	Fatty acyl-ACP thioesterases B	2.00e–26
Cj.25774	AT1G08510.1	Fatty acyl-ACP thioesterases B	1.00e–27
Cj.19951	AT5G51950.2	Glucose-methanol-choline (GMC) oxidoreductase family protein	3.00e–37
Cj.11111	AT4G33790.1	Jojoba acyl CoA reductase-related male sterility protein	4.00e–40
Cj.13061	AT3G53880.1	NAD(P)-linked oxidoreductase superfamily protein	1.00e–41
Cj.1269	AT5G04950.1	Nicotianamine synthase 1	3.00e–23
Cj.12365	AT1G09240.1	Nicotianamine synthase 3	1.00e–30
Cj.15578	AT4G35160.1	*O*-methyltransferase family protein	5.00e–14
Cj.6619	AT4G35160.1	*O*-methyltransferase family protein	3.00e–12
Cj.21447	AT1G77520.1	*O*-methyltransferase family protein	4.00e–17
Cj.11351	AT4G24340.1	Phosphorylase superfamily protein	2.00e–44
Cj_AE_Contig_0085	AT5G15780.1	Pollen Ole e 1 allergen and extensin family protein	1.00e–09
Cj.4624	AT5G15780.1	Pollen Ole e 1 allergen and extensin family protein	2.00e–10
Cj.2222	AT5G17170.2	Rubredoxin family protein	4.00e–26
Cj.2343	AT1G21200.1	Sequence-specific DNA binding transcription factors	3.00e–23
Cj.2987	AT4G16740.2	Terpene synthase 03	8.00e–50
Cj.2096	AT1G70080.1	Terpenoid cyclases/protein prenyltransferases superfamily protein	6.00e–11
Cj.14040	AT1G79460.1	Terpenoid cyclases/protein prenyltransferases superfamily protein	2.00e–17
Cj_ssh_male_e_239	AT5G48110.1	Terpenoid cyclases/protein prenyltransferases superfamily protein	7.00e–09
Cj_ssh_male_e_020	AT4G02780.1	Terpenoid cyclases/protein prenyltransferases superfamily protein	3.00e–16
Cj_ssh_male_e_009	AT3G29410.1	Terpenoid cyclases/protein prenyltransferases superfamily protein	2.00e–17
Cj_ssh_male_e_187	AT1G79460.1	Terpenoid cyclases/protein prenyltransferases superfamily protein	5.00e–40
Cj.36	AT3G25810.1	Terpenoid cyclases/protein prenyltransferases superfamily protein	2.00e–27
Cj.21863	AT1G33750.1	Terpenoid cyclases/protein prenyltransferases superfamily protein	2.00e–19
Cj.8750	AT4G02780.1	Terpenoid cyclases/protein prenyltransferases superfamily protein	5.00e–22
Cj.11599	AT4G02780.1	Terpenoid cyclases/protein prenyltransferases superfamily protein	2.00e–32
Cj.8462	AT1G79460.1	Terpenoid cyclases/protein prenyltransferases superfamily protein	8.00e–25
Cj.8670	AT1G79460.1	Terpenoid cyclases/protein prenyltransferases superfamily protein	3.00e–38
Cj.2241	AT4G02780.1	Terpenoid cyclases/protein prenyltransferases superfamily protein	7.00e–18
Cj.16674	AT1G15780.1	Unknown protein	2.00e–20
Cj.25366	AT2G36835.1	Unknown protein	6.00e–18
Cj.21025	AT1G52825.1	Unknown protein	5.00e–21

process of sporopollenin formation and microsporangial development has been reported (Dickinson and Bell 1972), and so has pollen cone and pollen development of eastern white pine and lodgepole pine (Owens 2004, 2006). From PMCs to microspore formation, *C. japonica* and pine are quite similar in time period and morphological traits. Since mature pollen grains have two cells in *C. japonica*, late pollen development is different from that of pine, which contains four to five cells (Fernando et al. 2010).

In angiosperms, such as *A. thaliana* and *Oryza sativa*, developmental stages of anthers and pollen are well characterized by cytological and transcriptome analysis (Regan and Moffatt 1990, Smyth et al. 1990, Sanders et al. 1999, Ikeda et al. 2004, Wang et al. 2005, Li et al. 2006) and are useful for investigation of mutants. Smyth et al. (1990) divided the flower development of *A. thaliana* into 12 stages based on observation using an electron microscope. Sanders et al. (1999) divided anther development of *A. thaliana* into 15 stages using a light microscope, and Regan and Moffatt (1990) divided pollen development into 10 stages based on staining and microscopic observation. Ikeda et al. (2004) categorized spikelet development of rice. These developmental stages have been used to determine the stage at which aberrant traits can be observed in mutants. These morphological

Table 4. Expressed sequence tags upregulated at Stages 4–6 in MT compared with WT2, omitting the ESTs with high *E*-values.

Measurement ID	TAIR	Annotation	*E*-value
Cj.11098	AT5G12270.1	2-Oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	3.00e–21
Cj.21820	AT1G77380.1	Amino acid permease 3	2.00e–27
Cj.6636	AT2G41890.1	Curculin-like (mannose-binding) lectin family protein/PAN domain-containing protein	2.00e–22
Cj.10292	AT2G34930.1	Disease resistance family protein/LRR family protein	7.00e–26
Cj.12287	AT2G34930.1	Disease resistance family protein/LRR family protein	2.00e–14
Cj.25224	AT5G45050.2	Disease resistance protein (TIR-NBS-LRR class)	5.00e–25
Cj.14439	AT1G69550.1	Disease resistance protein (TIR-NBS-LRR class)	6.00e–19
Cj.10817	AT5G65350.1	Histone 3 11	3.00e–11
Cj.5057	AT2G39050.1	Hydroxyproline-rich glycoprotein family protein	9.00e–06
Cj.14392	AT5G55250.2	IAA carboxylmethyltransferase 1	2.00e–12
Cj.3467	AT1G51980.2	Insulinase (peptidase family M16) protein	9.00e–27
Cj.3691	AT2G37050.3	Leucine-rich repeat protein kinase family protein	1.00e–13
Cj.12169	AT5G64410.1	Oligopeptide transporter 4	2.00e–56
Cj.13917	AT1G63770.5	Peptidase M1 family protein	1.00e–17
Cj.6976	AT1G74180.1	Receptor like protein 14	8.00e–10
Cj.14309	AT3G11010.1	Receptor like protein 34	8.00e–23
Cj.19053	AT3G12000.1	S-locus related protein SLR1, putative (S1)	1.00e–28
Cj.13990	AT5G67360.1	Subtilase family protein	1.00e–09
Cj.21905	AT4G01870.1	tolB protein-related	1.00e–23
Cj.13548	AT5G15390.1	tRNA/rRNA methyltransferase (SpoU) family protein	1.00e–33
Cj.26297	AT4G27570.1	UDP-glycosyltransferase superfamily protein	1.00e–12
Cj.9453	AT5G64190.1	Unknown protein	4.00e–32

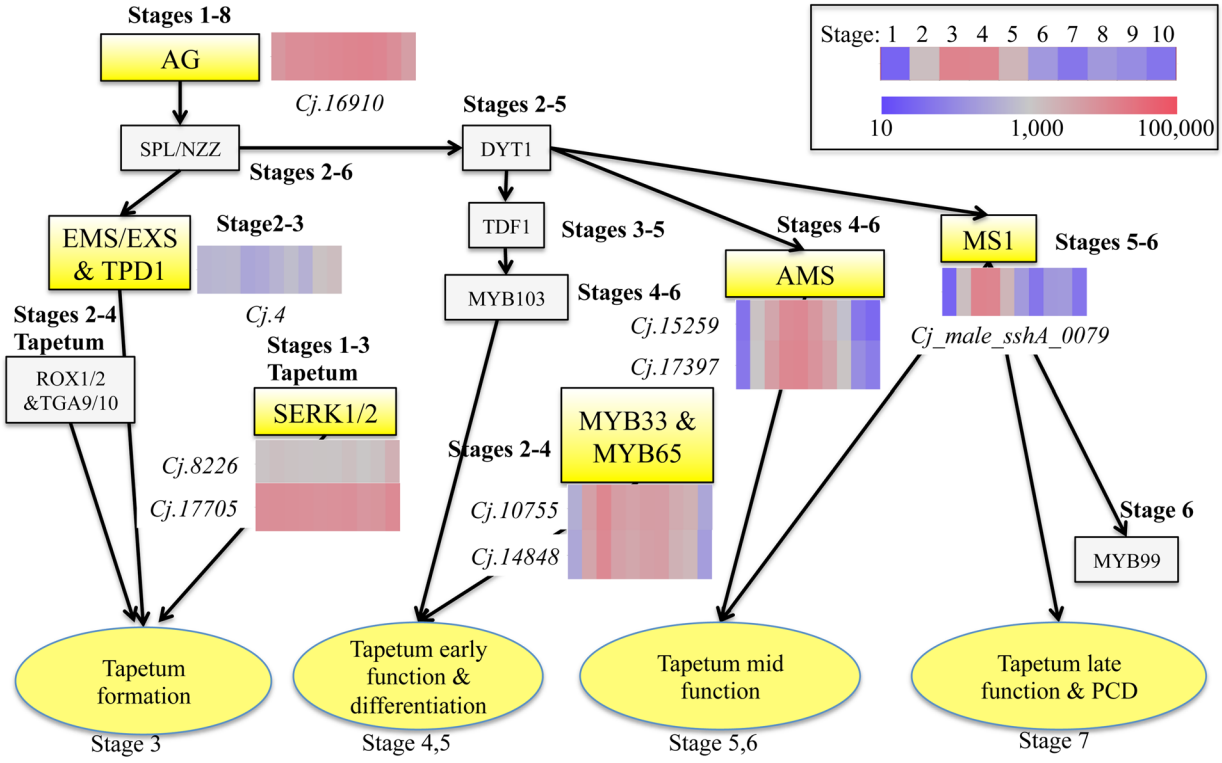

Figure 7. Proposed gene regulation network for tapetum development in *Arabidopsis* and the expression patterns of candidate ESTs in *C. japonica*. Stage numbers next to genes are expected expression stages presumed from the comparison between *Arabidopsis* and *C. japonica*. This figure is adapted from Huang et al. (2011) and Parish and Li (2010).

developmental stages have often been cited by other studies to compare morphological traits or transcriptomes of mutants and wild type to investigate anther developmental genes.

Angiosperms and gymnosperms are quite different in the late stages of pollen development and pollen tube growth (Dickinson and Bell 1972, Fernando et al. 2005). In this study,

the process of male strobilus and pollen development of *C. japonica*, which occurs from Stage 3 (tapetum differentiates) to Stage 6 (microspores are released from tetrads) based on cytological observations, was similar to anther development of *A. thaliana*. In *A. thaliana*, genes related to anther development are well characterized and their functions have been analysed (Parish and Li 2010, Huang et al. 2011), while the genes related to male strobilus development in conifers are still unknown. Some genes already characterized in conifers, e.g., *A9* (Kurita et al. 2013), *LEAFY* (Shiokawa et al. 2008) and gibberellin metabolism genes (Niu et al. 2014), are highly conserved among angiosperms. We investigated the expression patterns of *C. japonica* ESTs homologous to *A. thaliana* genes involved in anther development (Figure 7). Arabidopsis mutants defective in these genes are male-sterile. *AG* is a C-function gene required for stamen identity (Bowman et al. 1991, Liu and Fan 2013). *EMS/EXS* (Canales et al. 2002) and *TPD1* (*TAPETAL DETERMINANT1*) (Yang et al. 2003) are genes encoding leucine-rich repeat receptor-like kinases (LRR-RLKs), and regulate development of archesporial cells from the L2 layer. *SERK1/2* (Colcombet et al. 2005) also encodes LRR-RLKs, but *SERK1/2* is not regulated by *SPL/NZZ* (*NOZZLE/SPOROCYTELESS*) (Liu et al. 2009), as opposed to *EMS*. *MYB33* and *MYB65* act redundantly to facilitate tapetal development and may play a role in tapetal starch mobilization in the seed aleurone layer (Millar 2005, Wilson and Zhang 2009). *AMS* (Sorensen et al. 2003) and *MS1* (Wilson et al. 2001) are regulated by *DYT1* (*DYSFUNCTIONAL TAPETUM1*) and are expressed in the tapetum (Wilson and Zhang 2009). *MS1* expression starts in the tapetum as the callose wall separating the tetraspores begins to degrade in *A. thaliana* (Yang et al. 2007). *AG-like* gene, *SERK-like* gene, *MYB33&65-like* genes, *AMS-like* gene and *MS1-like* gene were similar to gene expression in addition to sequence across species. It is likely that these genes play a major role for tapetum development also in *C. japonica*.

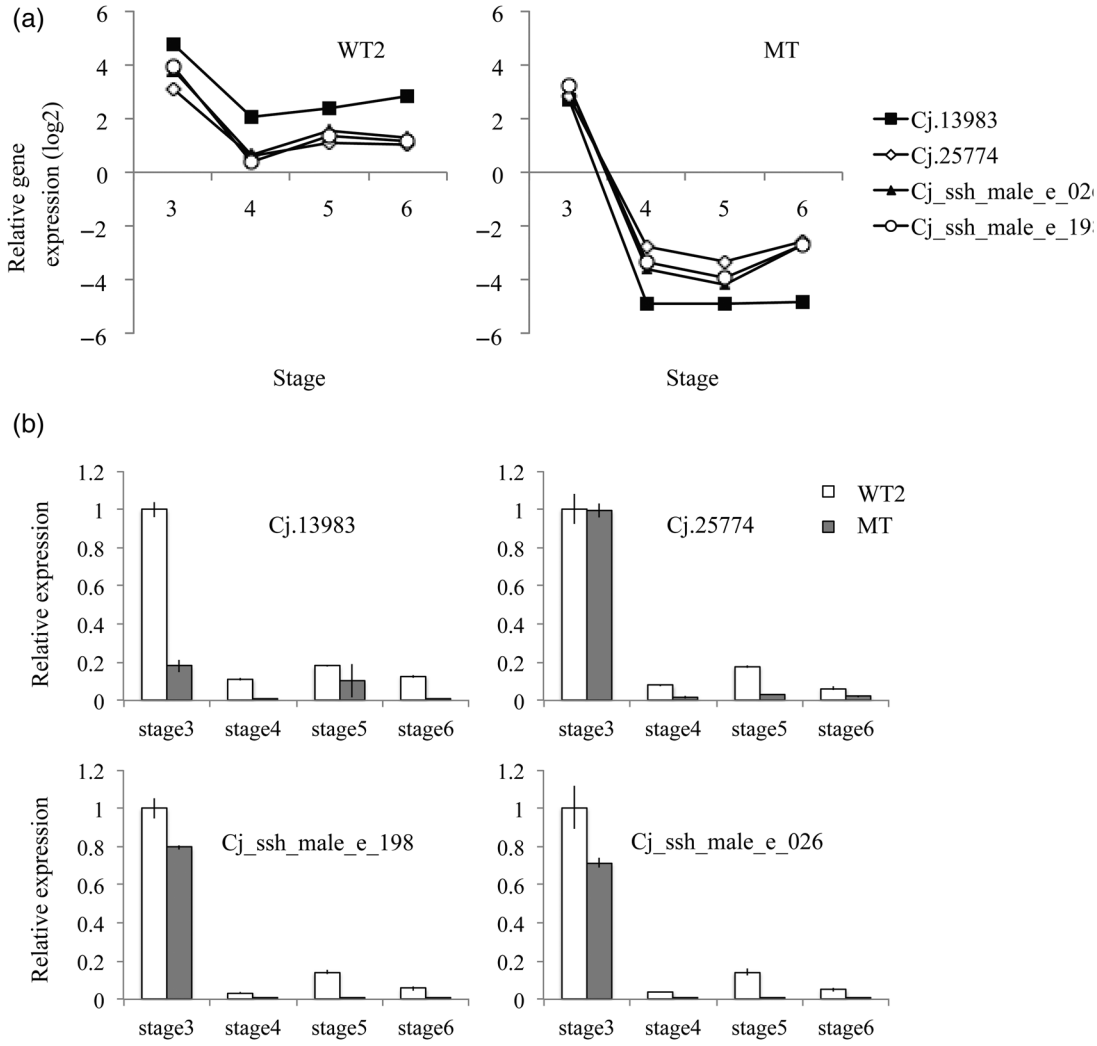

Figure 8. Four ESTs with expression remarkably downregulated in MT at Stages 4–6. (a) Microarray analysis, (b) real-time PCR analysis. Error bar means standard deviation.

Cytological and transcriptome analysis of male-sterile mutant pollen

In Sosyun, which is a male-sterile mutant, abnormality of the microsporangium was observed at Stage 6 by cytological analysis. The microsporangium of Sosyun filled with debris, presumably secreted from the tapetum. The abundant debris is regarded as the cause of pollen grains not being released from the microsporangium. Saito et al. (1998) reported that microspores gradually enlarged after individual microspores separated from tetrads in the male sterile mutant 'Toyama MS', classified as *ms-1*. In 'Shindai1', classified as *ms-2*, and 'Shindai5', classified as *ms-3*, microsporogenesis proceeds normally but the microspores clump by the time of pollen release (Yoshii and Taira 2007). In four clones of male-sterile mutant lines (Fukushima-1, Fukushima-2, Shindai-11 and Shindai-12) with abnormal tetrads, the release of Ubisch bodies from the tapetum is not observed after callose dissolution, and the amount of translucent amorphous substances gradually increases (Miura et al. 2011). Sosyun is considered to be a similar type to male-sterile mutants reported in Miura et al. (2011) because of the presence of debris in the microsporangium. The gene expression pattern of Sosyun differed from WT2 at Stages 4–6. Therefore, the difference in its gene expression pattern at Stage 6 appears to be correlated with the abundant debris in the microsporangium. Some ESTs were downregulated at Stage 4 in both WT2 and Sosyun (Figure 8). However, from Stages 4 to 6, they were not expressed at all in Sosyun, while they were slightly expressed in WT2. The protein annotations of these ESTs are 2-oxoglutarate and Fe (II)-dependent oxygenase superfamily protein, CYP450 family and fatty acyl-ACP thioesterases B (Table 3). There are no reports of these genes being directly related to male strobilus development, but they are likely related to pollen sac development.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

Conflict of interest

None declared.

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