



Comparison of fertile and sterile male gametogenesis in *Cryptomeria japonica* D. Don

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Received: 25 October 2018 / Revised: 11 March 2019 / Accepted: 12 March 2019
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Abstract

Cryptomeria japonica D. Don is one of the most important conifers for forestry; however, the pollinosis caused by this species is the most prevalent allergy in Japan. Male-sterile mutants have attracted attention as countermeasures to prevent pollen dispersion. Histological and transcriptomic analyses were conducted on one of the *C. japonica* male-sterile mutant lines, Toyama 1. Histological examination of pollen development revealed that the lamellar structure of the endexine was not observed in pollen of Toyama 1 and that the microspore was disrupted during the tetrad stage. To determine the mechanism causing male sterility in *C. japonica*, we analyzed gene expression in normal and sterile male strobili derived from the progeny of Toyama 1. A microarray of 22,882 low-redundancy sequences was designed from *C. japonica* cDNAs for this experiment. Microarray analysis revealed that the expression pattern of genes in male strobili during pollen development was very similar between fertile and male-sterile individuals. We confirmed 32 genes that were expressed at a lower level in sterile male strobili than in fertile ones at the stage when the disrupted microspores were observed in the sterile mutant. Nine of these 32 genes showed similarity to those involved in carbohydrate metabolic process, phosphorylation, and transmembrane transport. Other genes showed similarity to those involved in regulation of transcription, signal transduction, protein modification, and transport of ions and amino acids. These results suggest that the gene responsible for the Toyama 1 mutant may be involved in the network regulating pollen wall formation.

Keywords *Cryptomeria japonica* · Male gametogenesis · Male sterility · Pollen development · Pollen wall

Introduction

Cryptomeria japonica D. Don belongs to Cupressaceae sensu lato and is one of the most commercially important conifers in

Japan because of its high productivity and utility. However, the allergic disease caused by its pollen is a severe public health problem in Japan. A meta-regression analysis on an epidemiological survey predicted that 24.5% of the general

Communicated by Y. Tsumura

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s11295-019-1335-8>) contains supplementary material, which is available to authorized users.

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population in urban areas suffers from pollinosis caused by pollen of *C. japonica* (Kaneko et al. 2005). The male-sterility trait in *C. japonica* represents valuable breeding material to reduce airborne pollen in the future.

The first genetic male-sterile tree of *C. japonica*, called “Toyama 1” and controlled by a major recessive gene (*ms1*), was discovered in 1992 (Taira et al. 1993). Other types of male-sterile trees, called “Shindai 1” and “Shindai 5” were also reported that possess different nuclear recessive genes involved in the male-sterility traits (Yoshii and Taira 2007). Until 2010, 23 individuals have been selected in total as male-sterile mutants; morphological characterization and investigation of their genetic traits continues (Saito 2008; Saito 2010). The cytological study of male-sterile *C. japonica* based on light microscopy showed that the process of microspore development differs among male-sterile mutants (Saito et al. 1998). Saito et al. (1998) reported that microspores in Toyama 1 gradually enlarged following the release of individual microspores from tetrads, fused together, and finally collapsed in the microsporangia. Microsporogenesis in Shindai 1 and Shindai 5 produced normal microspores that began to make contact and clump during the uninuclear microspore stage (Yoshii and Taira 2007). In Shindai 3, the microspores were not released from the tetrads and subsequently degraded (Ueuma et al. 2009). Shindai 2 and Shindai 10 are meiotic mutants that produce tetrads containing microspores abnormal in size and number. These two mutants are both male and female sterile (Hosoo et al. 2005; Kawana et al. 2006). Transmission electron microscopy was performed using four male-sterile mutants (Fukushima 1, Fukushima 2, Shindai 11, and Shindai 12) and their endoexines were found to be undeveloped at the tetrad stage with microspores not yet released from the tetrad (Miura et al. 2011).

In recent years, the gene regulatory networks controlling the pollen development process have gradually been clarified in angiosperm species, especially in *Arabidopsis* and rice (Wilson and Zhang 2009; Tian et al. 2010; Twell 2011). The analysis of male-sterile mutants revealed many genes involved in pollen development processes such as the establishment of anther cell layers, tapetal development, male gametophyte generation, and pollen wall formation (Twell et al. 2006; Ariizumi and Toriyama 2007; Borg et al. 2009; Zhao 2009; Ge et al. 2010). Transcriptome analyses have also identified pollen-specific genes and gene networks downstream of male-sterility genes (Honys and Twell 2004; Pina et al. 2005; Alves-Ferreira et al. 2007; Carlsson et al. 2007; Ito et al. 2007; Chen et al. 2009; Kang et al. 2008; Xu et al. 2010). In conifers, various types of sterile mutants have been selected in several species, including *C. japonica* (Andersson 1947; El Maâtaoui and Pichot 2001; Wilson and Owens 2003; Chen et al. 2006). Genes expressed in male strobili and pollen were also isolated using a subtracted library or collections of expressed sequence tags (EST) (Walden et al. 1999; Futamura et al. 2006, 2008;

Chu et al. 2011). Recently, Tsubomura et al. (2016) described gene expression of wild type and male-sterile mutant of *C. japonica* using microarray analysis. However, the gene responsible for the sterile mutant has not been identified.

In this study, we compared the ultrastructural changes and expressed genes in male strobili between fertile and another type of male-sterile mutant Toyama 1 plants using a microarray experiment. Preliminary results of microarray analysis in this study were previously reported (Futamura et al. 2009). We also examined the relationship between gene expression and histological changes during pollen development.

Material and methods

Plant materials

Male strobili of *C. japonica* were collected from Toyama 1 half-sib F_2 pedigree. F_1 was generated by crossing between Toyama 1 and fertile elite trees (Mio 4 and Higashinishihara 2). F_2 generation was created by crossing F_1 sibling of Toyama 1 and Mio 4 as seed parent, and F_1 sibling of Toyama 1 and Higashinishihara 2 as pollen parent. Three-year old plants of F_2 generation were planted in the nursery of FFPRI (N 36° 00' E 140° 08') in 2005. The development of strobili was initiated by treatment with 290 μ M gibberellic acid (GA_3) at the end of July. We collected male strobili from male-sterile and fertile trees at 7-day intervals from the beginning of September to mid-November between 2005 and 2008. The length and dry weight of male strobili derived from male-sterile and fertile trees were determined at the end of October 2008. We measured the length of each 50 male strobili from seven fertile and sterile trees. The dry weight of each 100 male strobili from seven fertile and sterile trees was also measured. A part of each sample was fixed for observation by light and electron microscopy and the rest was immediately frozen in liquid nitrogen and stored at -80°C until use. Mature pollen in microsporangia was observed with a KH-7700 (Hirox, Tokyo, Japan) digital microscope using the multi-focus and image assembly functions. Total RNA was extracted from each set of strobili using a modified CTAB method and purified with the SV Total RNA Isolating System (Promega, Madison, WI) (Futamura et al. 2008). We used 0.5 to 1.0 g (fresh weight) of male strobili from each individual to extract total RNA samples for microarray and quantitative real-time PCR (qRT-PCR). Although the developmental stages of male strobili were not fully synchronized, each developmental stage was confirmed by microscopy. Transverse sections of 10 male strobili derived from each tree were observed under a light microscopy to confirm the developmental stages. RNA samples for microarray and qRT-PCR were classified at each of four developmental stages: pre-meiosis (PM), meiosis (MEI),

tetrads (TET), and unicellular microspores (UM) based on the result of the observation by microscopy.

Light and electron microscopy

Samples for light microscopy were fixed with FAA containing 3.7% formaldehyde solution, 5% (v/v) glacial acetic acid, and 50% (v/v) ethanol before being dehydrated through a graded ethanol series and finally embedded in Paraplast Plus (Sherwood Medical, St. Louis, MO). Sections (4 μ m thick) were cut on a microtome and placed on glass microscope slides. The sections were deparaffinized in xylene, rehydrated through a graded ethanol series, and stained with Mayer's hematoxylin and eosin before being again dehydrated through an ethanol series and xylene then mounted with Malinol (Muto Pure Chemicals, Tokyo, Japan) for microscopic examination.

Samples for transmission electron microscope analysis were fixed in 4% (w/v) paraformaldehyde and 2% glutaraldehyde in 5 mM sodium cacodylate buffer at pH 7.4. These specimens were stored overnight at 4 °C and then post-fixed in 2% osmium tetroxide in 5 mM sodium cacodylate buffer for 4 h. Following dehydration through an ethanol series, the specimens were infiltrated in Quetol 651 resin (Nissin EM Co., Tokyo, Japan) and propylene oxide with a ratio of 3:7 and hardened at 60 °C for 48 h. Semi-thin sections (1.5 μ m) were stained with 1% toluidine blue O. Ultrathin sections (70 nm) were obtained using a 2088 Ultratome V (LKB Bromma, Bromma, Sweden) and double stained with 2% uranyl acetate and lead stain solution (Sigma Chemical Co., St. Louis, MO). Observation and capturing of images were performed with a JEM-1200EX electron microscope (JEOL, Tokyo, Japan) at 80 kV and a CCD camera (Olympus Veleta).

Microarray analysis

To prepare the probe set for the microarray, we collected previously reported information for ESTs and cDNAs of interest. The ESTs were derived from male strobilus (accession nos. BY877900-BY914001, AU298712-AU299544, BW993917-BW996365), female strobilus (AU299545-AU300308), needle (DC429774-DC429778), vegetative bud (BW996366-BW997181), sapwood (BB940565-BB941614), pollen (BJ936638-BJ940292, DC429611-DC429773), and seedling (AU036727-AU036912, DC429408-DC429610) (Tsumura et al. 1997; Ujino-Ihara et al. 2000, 2003, 2005; Futamura et al. 2006, 2008; Yoshida et al. 2007). The sequences of six cDNAs that were not included the EST pool were also added for preparation of the probe set (*MADS2*, AF097747; *CjNdh*, AB074568; allergen No.121, AB197670; *Cry j 3.7*, AB212218; putative ORFX, AB254812; prolyl 4-hydroxylase, alpha subunit, AB254817). The total number of ESTs and cDNAs were 56,306, of which 39,324 were derived from

the cDNA library of male strobilus. We assembled *C. japonica* EST information using the CAP3 program with default conditions (Huang and Madan 1999). The sequences of ESTs were assembled into 10,655 tentative contigs and 12,227 singletons. A total of 365,959 60-mer probes were selected from both strands of these 22,882 sequences. The number of total probe sets was 45,753. The chosen probes were synthesized on glass-based arrays using a maskless array synthesizer (Nuwaysir et al. 2002). Total RNA preparations were labeled and each sample was individually hybridized to the array by Roche NimbleGen (Madison, WI), using standard single-dye labeling and hybridization protocols. We prepared two different data sets to perform microarray analysis. One data set has RNA from each of seven fertile and male-sterile plants at four developmental stages, whereas the other set has three pooled RNAs that contains six-to-eight different fertile and sterile individuals at three developmental stages. We tried to reduce the influence of individual variation and evaluate the efficiency of pooling biological samples in microarray experiments because there were some discussions about pooling samples (Kendzierski et al. 2005; Kainkaryam et al. 2010). Expression values derived from microarray data were generated using quantile normalization (Bolstad et al. 2003) and the Robust Multichip Average (RMA) algorithm (Irizarry et al. 2003). The microarray dataset has been deposited in the Gene Expression Omnibus database (Accession: GSE33499). Differentially expressed genes were identified using the moderated *t*-statistic (Smyth 2004) and correcting for multiple testing using the false discovery rate (FDR) (Benjamini and Hochberg 1995). Hierarchical clustering was performed using *pvc* (Suzuki and Shimodaira 2006).

DNA sequencing

cDNA clones were picked from bacterial cultures stored as glycerol stocks (Futamura et al. 2006, 2008). Plasmid DNA was isolated from 1.5 ml of culture using the Wizard Plus SV Miniprep System (Promega). The BigDye Terminator v3.1 Cycle Sequencing Kit and 3130xl Genetic Analyzer (Life Technologies, Grand Island, NY) were used for sequencing. Complete sequencing of target clones was performed using primer walking. Functional annotation was carried out using the BLASTX program. We used the Pfam database (Punta et al. 2012) for a domain search and TAIR10 (<http://www.arabidopsis.org/>) for a similarity search to *Arabidopsis* proteins as databases. Gene ontology (GO) was assigned based on the results of the similarity search.

cDNA synthesis and qRT-PCR

cDNA synthesis reactions were made for the staged male strobili (PM, MEI, TET, and UM, see Plant materials) using six individuals each of fertile and sterile trees. RNA (2 μ g) was

used as the template for the cDNA synthesis reaction with an AffinityScript QPCR cDNA Synthesis Kit (Stratagene, La Jolla, CA), according to manufacturer instructions. First-strand cDNA was synthesized in a 20- μ l reaction mixture containing 10 μ l cDNA synthesis master mix (2 $\times$), 1.7 μ l of oligo (dT) primer, 0.3 μ l of random primer, and 1 μ l AffinityScript RT/RNase Block enzyme mixture. The reaction mixture was incubated at 25 °C for 5 min to allow primer annealing, at 42 °C for 15 min to allow cDNA synthesis, and at 95 °C for 5 min to terminate reverse transcription. Finally, the reaction tubes were stored at –20 °C for long-term storage until analysis.

qRT-PCR was carried out with the Stratagene Mx3000P QPCR system. A 15- μ l reaction volume contained 7.5 μ l SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA), 0.3 μ l each of the forward and reverse primers at 10 μ M, 4 μ l of 20-fold diluted cDNA template reaction mixture, and 2.9 μ l of DNase-free water. PCRs were initiated with denaturation at 95 °C for 30 s, followed by 40 cycles of amplification at 95 °C for 5 s, and annealing and extension at 57 °C for 15 s. The PCR cycles were followed by a dissociation curve analysis to confirm the single-PCR product by heating from 57 to 95 °C. Each PCR was performed in duplicate.

We selected nine control genes (three kinds of *PPlase*, *GAPDH*, *actin*, *ubiquitin*, *RPS18*, *RPL8*, and *eIF*) from the cDNA library of male strobili as candidate control genes for normalization (Supplemental Table 1). The expression levels of these genes during pollen were confirmed by qRT-PCR and assessed using the geNorm program (Vandesompele et al. 2002); *eIF* was then selected as the most suitable control gene for normalization. The expression value of *eIF* varied within 2-fold during different developmental stages and individuals. Descriptive statistical analysis was performed for numerical data. The $2^{-\Delta\Delta CT}$ method was used to calculate the expression level of respective genes. The level of *eIF1* was used for normalization to correct for the amount of total RNA added and the amount of inhibition during the reverse transcription process.

The mean expression level was calculated using cDNA sets derived from six individuals each of fertile and sterile trees of Toyama 1 half-sib F₂ pedigree. The primer sets are shown in Supplemental Table 2. The primers for *CjMALE1* are as follows: forward—GTT TGG TGG CAT TGG TTG TAG; reverse—GCA TTC AGG AGT TGG GTC TTG.

Results

Pollen development in fertile and sterile microsporangia

Fertile and male-sterile individuals from Toyama 1 half-sib F₂ pedigree were used as plant materials in this study. The male strobili of Toyama 1 male-sterile mutants are known to not

have any marked morphological properties relative to fertile plants (Saito et al. 1998). We verified the length and weights of male strobili derived from male fertile and sterile individuals (Supplemental Fig. 1). The results showed that significant differences were observed among individuals and that these differences were not associated with differences in fertility.

Saito et al. (1998) reported that microspores of Toyama 1 male-sterile mutants of *C. japonica* were swollen following the release of individual microspores from TETs (Saito et al. 1998). We observed the pollen development of fertile and male-sterile mutant plants of the Toyama 1 pedigree using light and electron microscopy to clarify the cytological defects in detail. The development of microspores in male-sterile mutants was comparable to fertile plants before the formation of TETs (Fig. 1a, b, f, g); however, the TETs of male-sterile mutants began to collapse and degrade (Fig. 1h–j). No mature pollen was observed in microsporangia of the mutant at the time of pollen dispersion (Fig. 1j).

Ultrathin sections of fertile microsporangia at the TET stage showed that the ectexinous granular layer and lamellated endexine were constructed between the callose wall and plasma membrane (Fig. 2a, b). The innermost lamellated endexine attached to the microspore plasma membrane (Fig. 2b). The microspore surface coat layer was present between the initial ectexine and endexine. Conversely, the pollen wall was disrupted and the cellular content had leaked in some microspores of sterile TETs (Fig. 2d). The lamella structure of the endexine was not detected in sterile TETs (Fig. 2e). The broad periplasmic space between the plasma membrane and the ectexine, corresponding to the microspore surface coat, was observed in sterile TETs. Fine fibrillar materials were observed in this gap and the granules of ectexine were merged together. Tapetal cells in sterile microsporangia appeared similar to those in fertile microsporangia; however, the exocytosing material released from tapetal cells was different (Fig. 2c, f). In fertile microsporangia, granular pro-orbicular bodies were released from tapetal cells, while beak-like structures were detected in the materials released from sterile tapetal cells.

Assessment of developing male strobili used for transcriptome

We used whole male strobili of fertile and sterile trees to clarify transcriptome profiling of male gametogenesis in *C. japonica*. The male gametogenesis stage was confirmed using light microscopy. We prepared two data sets for microarray analysis (see Material and Methods; Fig. 3). The male strobili picked from September 22 to October 5 contained only the pollen mother cells before MEI and are, therefore, classified as PM. From October 10 to 24, the timing of MEI and the transition to TET and separated microspore varied between individuals and the position of male strobili in a tree.

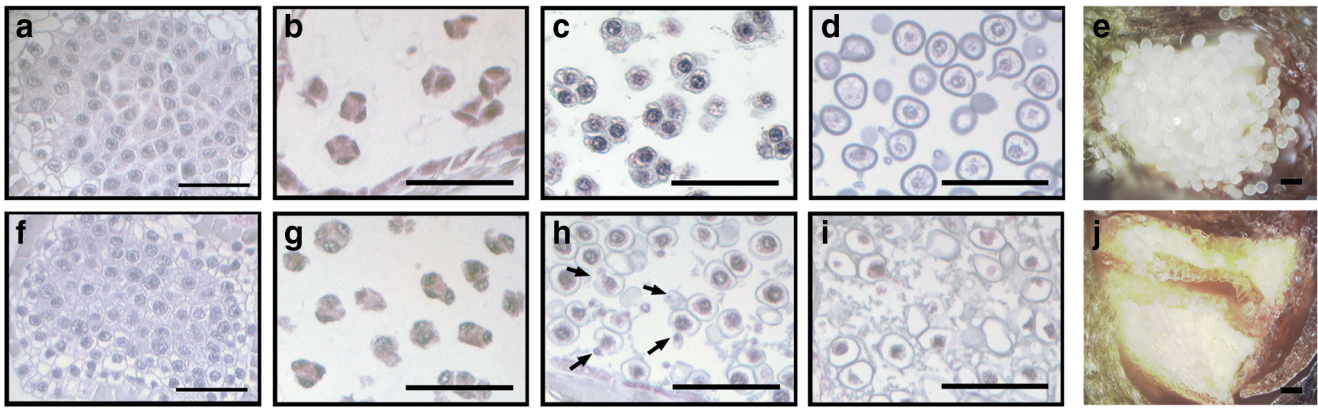


Fig. 1 The developmental steps of male gametophytes from a fertile plant (**a–e**) and a male-sterile mutant (**f–j**). Transverse sections of microsporangia were stained with hematoxylin and eosin (**a–d**, **f–i**). Mature pollen in microsporangia was observed by digital microscopy (**e**, **j**). Microspore mother cells before meiosis (**a**, **f**) and during meiosis (**b**, **g**) appear similar in both fertile and sterile male strobilus. Sterile

tetrads (**h**) collapsed (black arrows) and almost all sterile microspores were degraded (**i**). Observation by digital microscopy at the stage immediately before pollen dispersion (**e**, **j**) indicated that microsporangia of male-sterile mutant possessed no granular pollen grains. Scale bar = 50 μ m

Individual samples during this period were divided into two groups: in one group about 70% of observed microsporangia

contained pollen mother cells undergoing MEI and in the other group about 40% of observed microsporangia contained

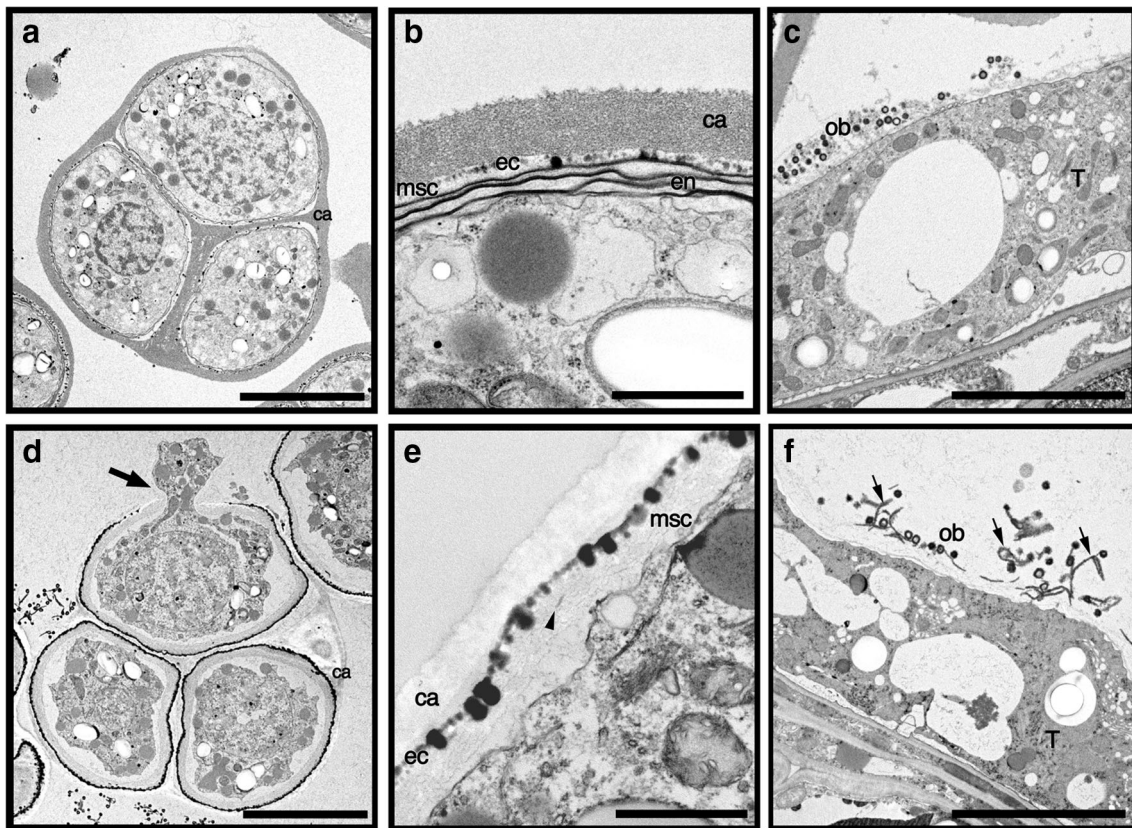


Fig. 2 Transmission electron micrographs of microspores and tapetal layers at the tetrad stage in fertile (**a–c**) and sterile (**d–f**) microsporangia. **a** Fertile tetrads surrounded by the callose wall. **b** Exine formation of fertile microspores at the tetrad stage. **c** Tapetal layer in fertile microsporangium. Pro-orbicular bodies were released from tapetal cells into the loculus. **d** Sterile tetrads surrounded by the callose wall. The black arrow indicates the disruption of the pollen wall and leakage of cellular contents. **e** Exine development of sterile microspores.

Lamellated endexine was deficient and a periplasmic space appeared between the granular ectexine and the plasma membrane. The arrowhead indicates the fibrillar material in the microspore surface coat. **f** The tapetal layer in sterile microsporangium. The beak-like structures of the pro-orbicular bodies are indicated by the arrows. ca, callose wall; ec, ectexine; en, endexine; msc, microspore surface coat; ob, pro-orbicular bodies. Bars = 10 μ m in **a** and **d**, 1 μ m in **b** and **e**, 5 μ m in **c** and **f**

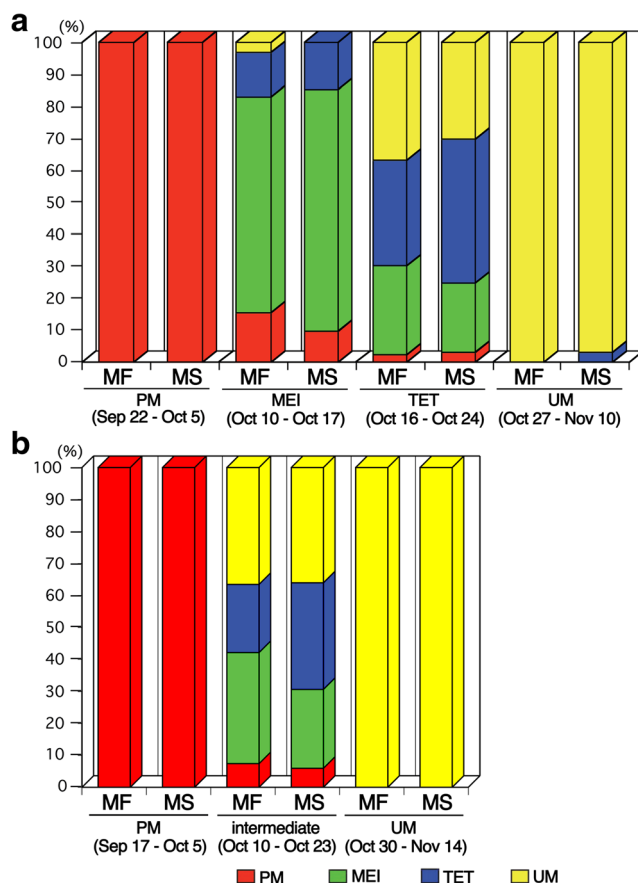


Fig. 3 Microarray analysis of the pollen developmental stage in microsporangia. **a** Four developmental stages of each of seven male-fertile (MF) and male-sterile (MS) individuals. **b** Three developmental stages of pooled fertile and sterile male strobili. Transverse sections of 10 male strobili derived from each tree were observed under a light microscope and stages of male gametogenesis were counted per microsporangium because the pollen developmental stages sometimes varied in even one male strobilus. We examined four microsporangia per one male strobilus, counting 280 microsporangia in total (7 individuals $\times$ 10 male strobili $\times$ 4 microsporangia) at each stage in Fig. 3a. We picked up one individual from each fertile and male-sterile trees in 2005, two individuals each in 2006, and four individuals each in 2007 without individual duplication among years. For pooled samples, we mixed six individuals from each fertile and male-sterile trees in 2006, eight individuals each in 2007, and seven individuals each in 2008. The RNA samples are pooled in each year. They include a few individual duplication among years. The stages of pollen development were confirmed as shown above. The average ratio of each stage of male gametogenesis was shown in stacked bar charts

TETs. The male strobili of MEI stage were collected from October 10 to 17, and those of TET stage were collected from October 16 to 24. For the mixed samples, the male strobili samples collected from October 10 to 23 contained about 30% pollen mother cells during MEI, about 27% TETs, and about 36% free UMs. The male strobili collected in this period were pooled as intermediate stage. After October 30, almost all male strobili contained only free UMs.

It is known that the *CjMALE1* promoter is activated in the male strobilus, especially in the tapetum (Kurita et al. 2013).

To examine the difference of tapetum development between male fertile and sterile individuals through gene expression, we confirmed the expression of *CjMALE1* using qRT-PCR (Supplemental Fig. 2). Our experiment revealed that the expression level of *CjMALE1* was over 1000 times higher than those of seed, seedling, young stem, root, female strobilus, and pollen (Supplemental Fig. 2A), as shown in previous report (Kurita et al. 2011). The expression of *CjMALE1* in male strobili showed a peak level at the TET stage that corresponds to the most active stage of the tapetum, and no difference in expression between fertile and sterile male strobili was observed through differential developmental stages (Supplemental Fig. 2B).

Microarray analysis using information from *C. japonica* ESTs

We prepared a novel microarray of *C. japonica* to clarify transcriptome profiling throughout male gametogenesis in fertile and sterile male strobili by utilizing EST information (see Materials and methods). The microarray data indicated extremely high correlation in relative expression levels between fertile and sterile male strobili. When seven individuals at four developmental stages were used for microarray analysis, the comparisons between the fertile and sterile male strobili achieved correlation coefficients of 0.994, 0.994, 0.996, and 0.986 for the PM, MEI, TET, and UM stages, respectively (Fig. 4). Moreover, no probe sets were identified as changing in sterile male strobili at the stages of PM, MEI, and TET with an FDR of less than 5%. Ninety-three probe sets showed alteration of RNA transcripts with at least 2-fold changes at the UM stage in the sterile strobili (Supplemental Dataset 1). Among these probe sets, only four (4.3%) were upregulated. Sixty-seven probe sets showed expression changes of more than 4-fold, all of which were downregulated (Fig. 5). Conversely, when three pooling samples at three developmental stages were analyzed, the correlation coefficients of each developmental stage between the fertile and sterile strobili were 0.986, 0.991, and 0.964, respectively (Supplemental Fig. 3). Furthermore, 302 probe sets showed changes in expression levels of more than 2-fold at the UM stage (Supplemental Dataset 2). In the earlier stages, no probe sets showed significant differences in expression between sterile and fertile strobili with an FDR of less than 5%. Among the 302 probe sets, 52 probe sets (17%) showed upregulated expression in sterile male strobili. Transcripts corresponding to 172 probe sets were downregulated and those corresponding to 18 probe sets were upregulated 4-fold (Fig. 5). There were no probe sets that showed altered expression at a 5% FDR between the fertile and the sterile male strobili at earlier stages. These results indicated that it is difficult to detect genes with different expression in relation to the fertility at the stages before the collapse of all microspores is observed under

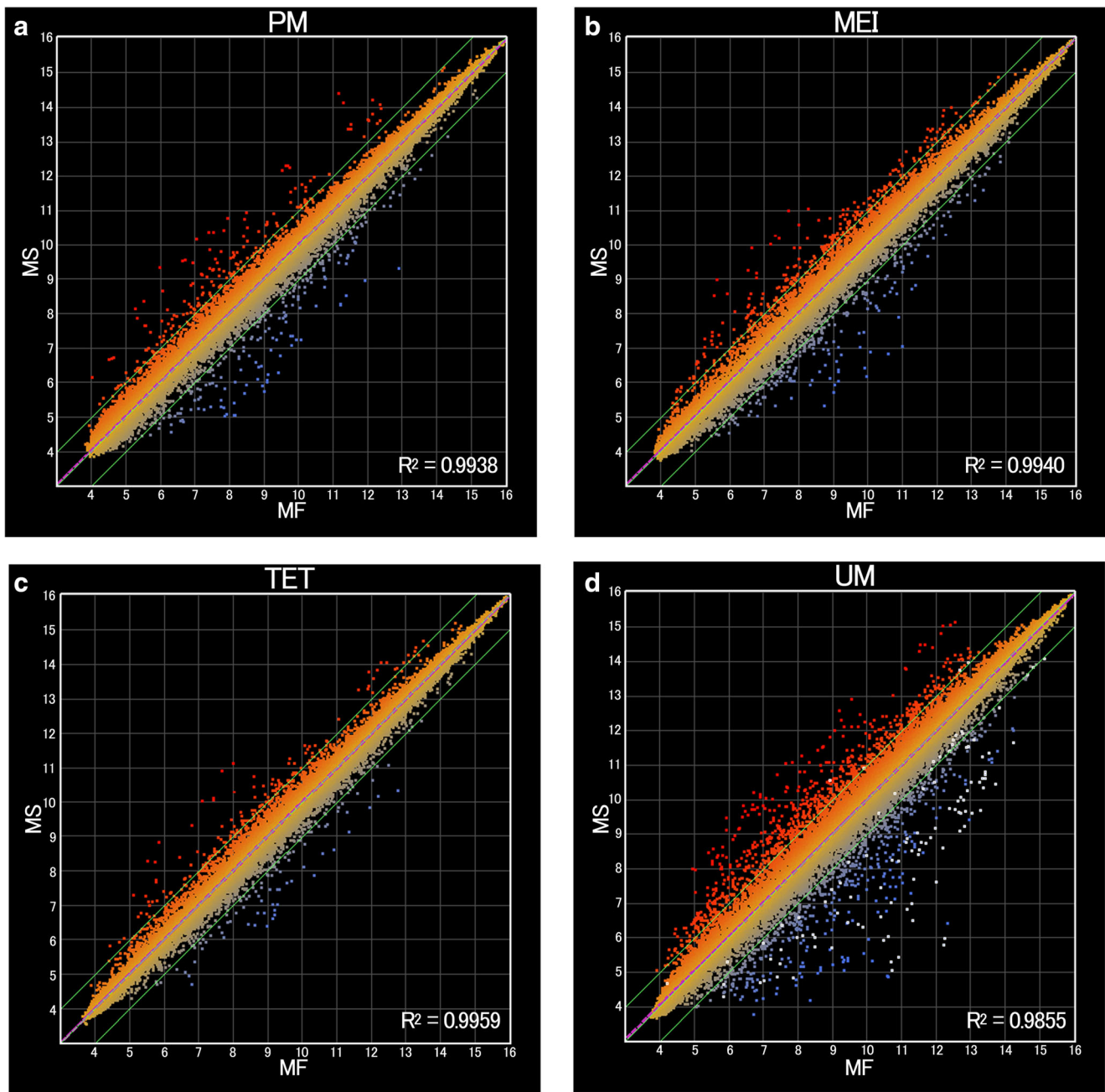


Fig. 4 Scatter plots of signal intensity values (log 2) comparing relative gene expression in pairs of male fertile (MF) and sterile (MS) strobili derived from seven individuals at different developmental stages. The gene expression in male strobili was measured by microarrays for **a** PM, **b** MEI, **c** TET, and **d** UM stages. The detailed state of microsporogenesis at each stage is shown in Fig. 3a. X-axis and Y-axis represent the normalized signal values for the MF and MS strobili, respectively (log 2

scaled). The expression levels of individual genes were normalized using Robust Multichip Average (RMA) and genes co-expressed in pairs of transcriptome datasets were plotted. White blots indicate the probe sets that show significantly different expression levels ($FDR < 0.05$) between male fertile and sterile strobili. The number in each figure is the coefficient of determination between male fertile and sterile strobili

microscopy. In fact, most of the genes affected by sterility were downregulated.

To clarify the relationship between the specificity of expression at each developmental stage and fertility, we performed bootstrap hierarchical cluster analysis using the pvclust software. The dendrogram showed that there are

clusters consisting of samples at the same developmental stage although some groups contained a few samples that did not fit the representative developmental stage (Supplemental Fig. 4). The fertile and sterile samples were not clustered separately in the groups consisting of samples at the PM, MEI, and TET stages. The sterile samples belonging to UM showed a weak

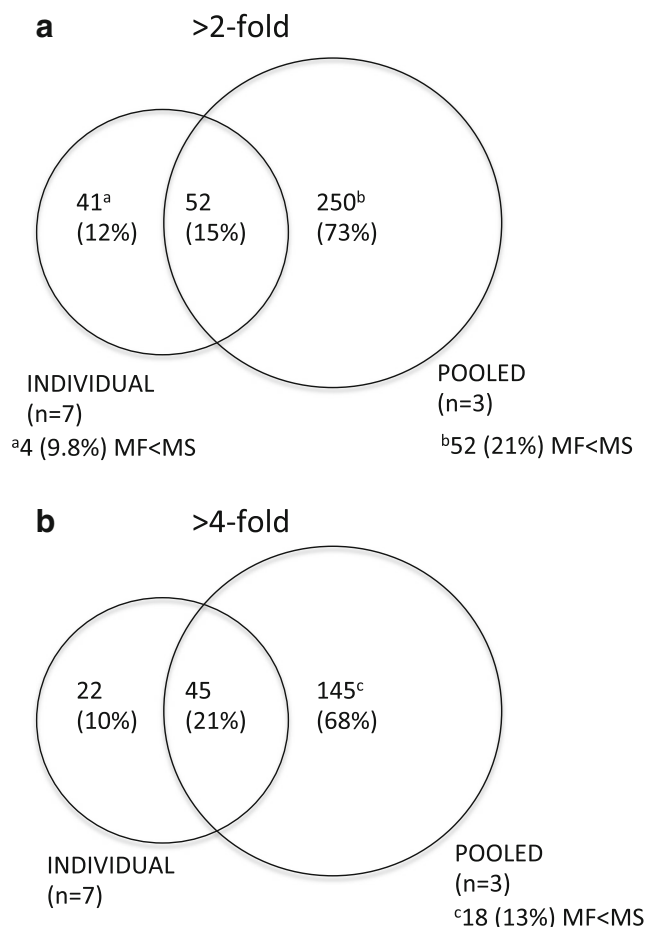


Fig. 5 Overlap of probe sets that changed between the fertile and sterile male strobili at the UM stage. **a** The number of probes sets that showed greater than 2-fold changes. **b** The number of probe sets that showed greater than 4-fold changes. ^a Four of the 41 probe sets showed higher values in sterile male strobili than in fertile samples. ^b Fifty-two of the 250 probe sets showed higher expression in sterile male strobili. ^c Eighteen of the 145 probe sets showed higher expression in sterile male strobili. There were no probe sets that showed a more than 4-fold increase in expression in the sterile male strobili using seven individuals

tendency to form clusters in comparison to other samples. This result may indicate that a quantitative shift in transcriptome profiling arises during the transition through developmental stages, but that the difference depending on fertility is not significant until the developmental stage when the collapsed microspores were obvious in sterile male strobili and all microspores had been released from TETs in fertile strobili.

Characterization of genes with differential expression between the fertile and sterile male strobili

The microarray data indicated that there are a number of genes differentially expressed between the fertile and sterile male strobili at the stage shortly after the release of microspores. We compared the probe sets identified as being differentially expressed genes between the seven individuals and three

pooled samples (Fig. 5). The percent overlap between the two approaches was about 15% for greater than 2-fold changes and about 21% for greater than 4-fold changes. About half of the probe sets varied by more than 2-fold when using seven fertile and sterile individuals overlapped with the results of those from the three pooled samples. We focused on the 52 overlapping probe sets (Supplemental Dataset 3) and attempted to obtain the corresponding cDNAs. All these probe sets showed lower expression in the sterile male strobili than the fertile ones at the UM stage. Among the 52 probe sets, 20 probe sets belonged to pairs of the overlapping sense-antisense transcripts derived from 10 genes. Moreover, some probe sets were derived from partial sequences belonging to identical genes. Finally, the 52 probe sets could be summarized in 32 cDNA clones. We selected original clones and sequenced their full-length cDNAs to ensure availability of their whole sequence. Overall, 24 cDNAs contained full-length open reading frames (ORFs) for deduced proteins, five cDNAs contained partial ORFs (Table 1), and three cDNAs encoded ORFs of less than 100 amino acids and so were classified as cDNAs with small ORFs (sORFs) (Basrai et al. 1997; Hanada et al. 2007). The putative proteins derived from these sORFs showed no similarity to conserved domains and known proteins in databases. Of the 29 cDNAs encoding full-length and partial ORFs, 26 showed similarity to *Arabidopsis* genes and were classified into six groups based on GO category, namely, regulation of transcription, signal transduction, transport, carbohydrate metabolic process, protein metabolic process, and protein modification process. Three cDNAs showed similarity to genes encoding proteins of unknown function. As for four pairs of cDNAs, identical functional annotations (C2H2-type zinc finger protein, AP2/B3-like transcriptional factor family protein, polygalacturonase gene family protein, fructokinase) were assigned as the predicted amino acid sequences showed similarity to the same genes or genes of the same families.

Confirmation of gene expression patterns associated with male sterility

To clarify the expression pattern of selected genes during gametogenesis in fertile and sterile individuals, we performed qRT-PCR using male strobili at four developmental stages (Fig. 6). The expression of the above 32 genes was detected in male strobili at the UM stage; all of these genes exhibited lower expression in the sterile strobili than the fertile ones. This result is consistent with that of the microarray analysis, indicating that the expression profile obtained by microarray is reliable.

We applied hierarchical clustering to the expression values of these genes to examine their expression patterns during gametogenesis (Fig. 6) and found that the 32 genes were divided into three groups. Group I contained five genes that

Table 1 Selected genes whose expression was affected by the male-sterile mutation

Probe sets ID	Accession no.	cDNA clone ID	Nucleotide length of cDNA (bp)	Longest ORF ^{ss} (aa)	MS/MF at UM stage	Tentative designation (estimated by Pfam domain and similarity search)	AGI code	E value
Regulation of transcription								
FSUGI0001S00005525	AB728801	CMFL040_G19	1154	204	0.407	AUX/IAA family	AT1G04100	1E-15
FSUGI0001S00019361	AB728802	CMFL035_D21	1229	336	0.228	C2H2-type zinc finger protein	AT1G13290	2E-84
FSUGI0001S000021265	AB728803	CMFL047_L13	1896	514	0.088	C2H2-type zinc finger protein	AT1G68480	6E-08
FSUGI0001S00004542	AB728804	CMFL014_I14	3342	801	0.146	AP2/B3-like transcriptional factor family protein	AT3G24650	6E-57
FSUGI0001S000021242	AB728805	CMFL047_J01	1695	373	0.342	AP2/B3-like transcriptional factor family protein	AT3G18990	6E-09
Signal transduction								
FSUGI0001S000021137	AB728806	CMFL046_P08	1449	330	0.184	Leucine-rich repeat family protein	AT5G61240	1E-121
Transport								
FSUGI0001S00016527	AB728807	CMFL017_C19	2069	524	0.025	Putative phosphate transporter	AT2G38940	0
FSUGI0001S00004753	AB728808	CMFL017_C14	900	> 240	0.046	Sugar efflux transporter for intercellular exchange (MtN3/saliva gene family)	AT5G62850	6E-48
FSUGI0001S00016794	AB728809	CMFL019_A02	2059	313	0.065	Transmembrane amino acid transporter protein	AT1G77380	1E-156
FSUGI0001S00006948	AB728810	CMFL039_J03	1151	328	0.046	ZIP zinc transporter	AT3G12750	2E-72
FSUGI0001S00019437	AB728811	CMFL035_M03	1966	551	0.354	Natural resistance-associated macrophage protein	AT1G80830	0
FSUGI0001S00016349	AB728812	CMFL016_B11	3322	534	0.161	Sodium/hydrogen exchanger family	AT3G17630	0
Carbohydrate metabolic process								
FSUGI0001S00007545	BY894314	CMFL046_A01	459	> 110	0.003	Glycosyl hydrolase family 3 (similar to b-xylosidase)	AT1G02640	4E-14
FSUGI0001S00013070	BB940663	Cj04A12	414	> 137	0.070	Glycosyl hydrolase family 32 (similar to cell wall-type invertase)	AT1G55120	1E-27
FSUGI0001S00019289	AB728813	CMFL034_M21	1349	322	0.064	Glycosyl hydrolases family 28 (polygalacturonase gene family)	AT1G23460	2E-60
FSUGI0001S00005997	BY888322	CMFL029_K03	408	> 109	0.044	Glycosyl hydrolases family 28 (polygalacturonase gene family)	AT1G80170	2E-42
FSUGI0001S00018950	AB728814	CMFL032_J21	1304	365	0.057	Aldose 1-epimerase	AT3G17940	1E-101
FSUGI0001S00003741	AB728815	CMFL007_H23	1482	345	0.099	Fructokinase	AT4G10260	1E-116
FSUGI0001S00014204	AB728816	CMFL002_H20	2017	276	0.074	Fructokinase	AT4G10260	3E-95
Protein metabolic process								
RSUGI0002S00006343	AB728817	CMFL045_J22	1705	484	0.060	Serine carboxypeptidase	AT2G33530	1E-116
Protein modification process								

Table 1 (continued)

Probe sets ID	Accession no.	cDNA clone ID	Nucleotide length of cDNA (bp)	Longest ORF ^a (aa)	MS/MF at UM stage	Tentative designation (estimated by Pfam domain and similarity search)	AGI code	E value
FSUGI0001S00021035	AB728818	CMFL046_C08	1822	407	0.054	U-box protein (E3 ubiquitin ligase)	AT2G35930	1E-76
FSUGI0001S00016619	AB728819	CMFL017_L24	932	> 224	0.544	RING finger protein (E3 ubiquitin ligase)	AT5G60710	2E-29
FSUGI0001S00022437	AB728820	CP11498	1375	359	0.063	Glycosyltransferase family 17 (β -1,4-N-acetylglucosaminyltransferase family)	AT1G12990	1E-144
FSUGI0001S00016483	AB728821	CMFL016_O15	1194	101	0.085	S-locus glycoprotein family	AT2G19130	4E-17
FSUGI0001S00014995	AB728822	CMFL007_K23	1206	300	0.422	Putative cyclin-dependent kinase B	AT2G38620	1E-110
RSUGI0002S00003812	AB728823	CMFL007_E16	1418	365	0.116	Protein kinase	AT4G05200	3E-036
Biological process unknown								
FSUGI0001S00014074	AB728824	CMFL001_H24	1354	305	0.120	Protein of unknown function (DUF1191)	AT3G08600	1E-28
FSUGI0001S00007022	AB728825	CMFL013_O01	1359	368	0.107	TRICHOME BIREFRINGENCE-like protein	AT3G54260	3E-86
FSUGI0001S00004768	AB728826	CMFL016_O17	880	198	0.059	Protein of unknown function (DUF1218)	AT4G31130	2E-36
No hit (sORFs)								
FSUGI0001S00006825	BY892834	CMFL041_O22	570	99	0.002	No hit		
RSUGI0002S00008731	AB728827	CMFL014_C06	1209	63	0.029	No hit		
FSUGI0001S00022109	AB728828	CP10049	1316	54	0.141	No hit		

^a The number of amino acid residues corresponds to the longest ORF that starts with ATG in each case. The inequality sign was imposed in case the cDNA was incomplete

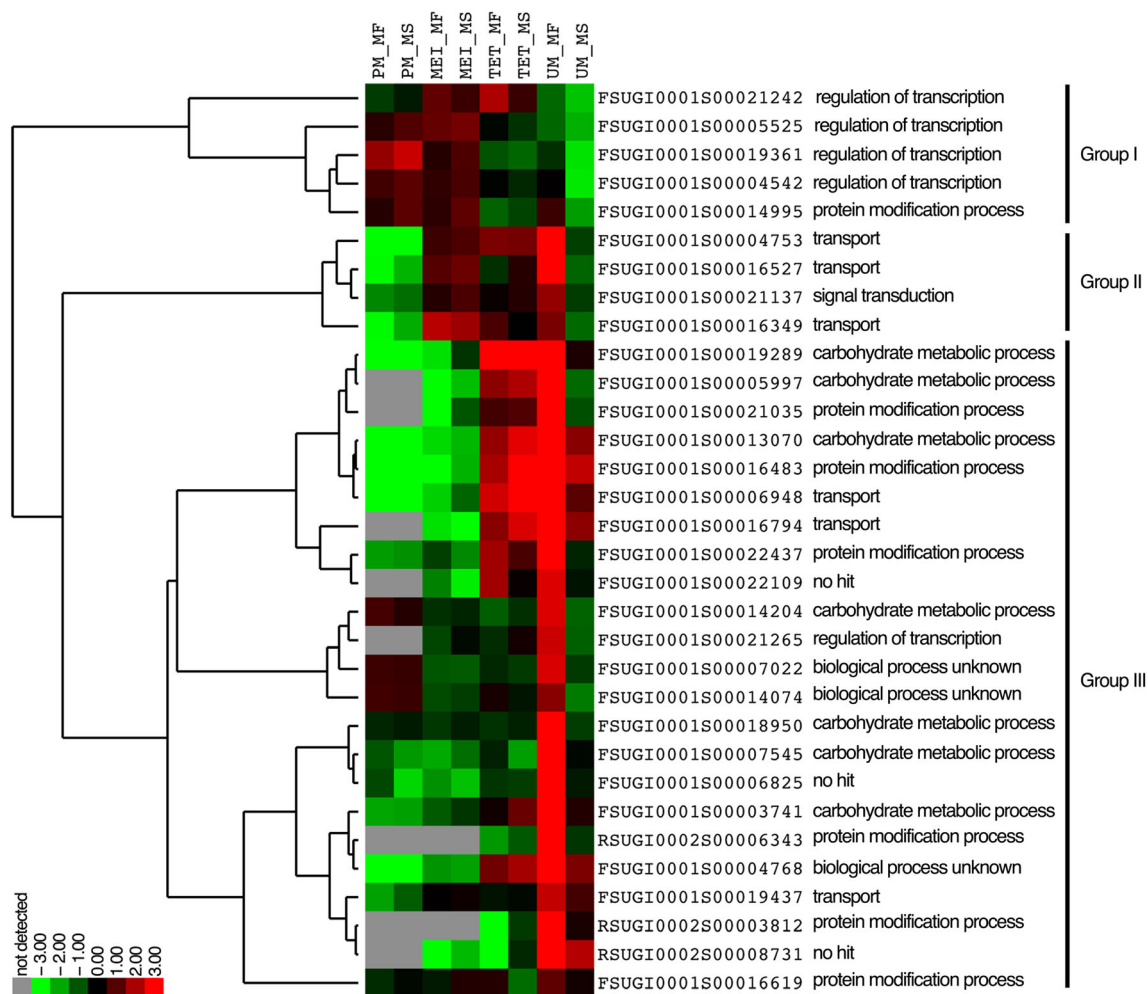


Fig. 6 Clusters of genes differentially expressed in male strobili at the UM stage. A total of 32 genes were selected and qRT-PCR was performed using RNA derived from male strobili at four developmental stages. The normalized expression values were clustered by hierarchical clustering

and displayed as a heat map. The stages on the horizontal axis are defined in Fig. 3. Primer sequences used for qRT-PCR experiments are shown in Supplemental Table 2

show relatively high expression at the PM and MEI stages. Two of these genes showed increased expression at the UM stage after low expression at TET stage. In sterile male strobili, the expression of these genes decreased at the UM stage. The genes in this group showed similarity to an AUX/IAA family protein, C2H2 type zinc finger protein, AP2/B3-like transcriptional factor family protein, and cyclin-dependent kinase B. Four of these five genes were categorized into the regulation of transcription category. Group II contains four genes upregulated at the MEI, TET, and UM stages. In sterile male strobili, high-level expression of these genes was observed at the MEI and TET stages as in fertile strobili; however, the level decreased at the UM stage. The genes in this group showed similarity to a MtN3/saliva gene family protein, phosphate transporter, sodium/hydrogen exchanger family protein, and leucine-rich repeat family protein. Three of these four genes were categorized into the transport category. Group III contains 23 genes whose expression increased at the TET and UM

stages or only at the UM stage. In sterile male strobili, the increase in expression at the UM stage was less dramatic, in contrast with the fertile strobili. Twenty of these 23 genes showed similarity to known proteins belonging to six functional groups. Seven of these genes belonged to the carbohydrate metabolic process category and encoded a β -xylosidase, cell wall-type invertase, polygalacturonase, aldose 1-epimerase, and fructokinase.

Discussion

In this study, we examined the changes in ultrastructure and gene expression in male strobili during pollen development in fertile and male-sterile *C. japonica*. The lamellar structure of the endexine was not observed in pollen of male-sterile mutants and the microspore was disrupted at the TET stage (Fig. 2). Thirty-two genes that were expressed at a lower level

in sterile male strobili than in fertile ones at the UM stage were identified (Table 1). These 32 genes showed similarity to those involved in regulation of transcription, signal transduction, modification of proteins, transport of ions and amino acids, carbohydrate metabolic processes, phosphorylation, and transmembrane transport. Therefore, the gene responsible for the sterile mutant may be involved in the network responsible for pollen wall formation.

The pollen wall can be divided into the exine and the intine. The exine of *C. japonica* consists of the outer granular ectexine and the inner lamellated endexine. The initial granular ectexine and lamellated endexine form on the surface of the plasma membrane at the TET stage while the microspores are enclosed by the callose wall (Uehara and Sahashi 2000). The most striking feature of sterile TETs is the defective endexine that results in leaking of the cellular contents (Fig. 2d, e). The absence of the endexine is consistent with a previous report that used four male-sterile mutants (Miura et al. 2011); however, to our knowledge, this is the first report of the leakage of cellular contents. During the normal development of microspores, the papilla begins to protrude at the TET stage and is covered with a single lamella (Uehara and Sahashi 2000). The leakage of cellular contents in sterile microspores seemed to occur where the papilla began to protrude. This suggests that endexine plays an important role in maintaining the shape of the microspore during pollen development. The disappearance of the endexine is obvious in some plant species (Stone et al. 1981); in gymnosperms, the lamellated endexine sometimes extends all around the grain and sporopollenin is deposited on both sides of the lamellae (Rowley et al. 2000) while in angiosperms, the membranes are short, lamellation is often limited at the apertures, and sporopollenin accretion is not apparent (Guédès 1982). Both the endexine and ectexine are formed during the TET period in gymnosperms, while in angiosperms, only the ectexine is formed in this period while the endexine forms during the free spore phase (Kurmman 1989). The *C. japonica* male-sterile mutant used in this report had defective lamellated endexine produced during the TET period. This is the characteristic feature of gymnosperm gametogenesis and so the study of the genetic mechanism underlying the male sterility may help to clarify which developmental process are conserved and which differ between gymnosperms and angiosperms.

In the earliest stages of conifer pollen wall development, a coating material is deposited between the plasma membrane of the microspores and the surrounding callose wall (Fig. 2b; Kurmann 1990). The coating material is called the microspore surface coat and is considered to act as a template for the ectexine (Kurmman 1989). In sterile TETs, fine fibrillar materials were observed in the microspore surface coat and the ectexine granules were merged (Fig. 2e). The ectexine granules of *C. japonica* do not merge together and the shapes of the granules can be distinguished through normal pollen

development (Uehara and Sahashi 2000). In conifers, granular exine structure was confirmed in Araucariaceae, Cupressaceae, Cephalotaxaceae, and Taxaceae (Kurmman 1992). *Sciadopitys*, belonging to Sciadopityaceae, is known to produce merged ectexine granules (Uehara and Saiki 2011). The granules of the initial ectexine of sterile TETs were similar to those of *Sciadopitys*. The glycocalyx in the microspore surface coat acts as a scaffold for the future exine (Gabarayeva et al. 2011; Gabarayeva and Grigorjeva 2013). Our results suggest that the abnormal glycocalyx might have an effect on the accumulation of initial sporopollenin and result in the merged granules of ectexine as occurs in *Sciadopitys*. Moreover, we showed that the shape of pro-orbicular bodies released from the tapetum differs between fertile and sterile individuals. During normal wall development, spherical pro-orbicular bodies were formed in the tapetal cells and exported into the locule by exocytosis (Fig. 2c). Conversely, a long “beak” was attached to the pro-orbicular bodies released from sterile tapetum (Fig. 2f). These structures were called amorphous substances and were observed in sterile microsporangia as one of the characteristics of sterility during the TET stage (Ueuma et al. 2009; Miura et al. 2011). In *Pinus sylvestris*, the pro-orbicular bodies with a long beak were released at the MEI phase and were followed by the release of spherical ones after the TET period (Rowley and Walles 1987). The sterile pro-orbicular bodies resembled the immature ones observed in *P. sylvestris*. The pro-orbicular bodies were coated with glycocalyx, implicating the involvement of sporopollenin accumulation (Rowley and Walles 1987). Therefore, the abnormal accumulation of sporopollenin occurs around glycocalyx elements in sterile microsporangia and leads to the formation of merged granules of the initial ectexine and pro-orbicular bodies with a long beak not normally seen during microsporogenesis of *C. japonica*.

At present, a number of mutants have been identified that are involved in exine formation in *Arabidopsis* and rice (Ariizumi and Toriyama 2011). These studies have suggested that pollen exine development is dependent on three major developmental processes, namely, primexine formation, callose wall formation, and sporopollenin synthesis (Ariizumi and Toriyama 2007, 2011). The Toyama 1 mutant appeared to have deficiencies in the primexine formation and/or sporopollenin synthesis processes that resulted in the collapse of microspores. The exine in gymnosperm pollen is considered to be homologous with that in angiosperm pollen (Guédès 1982; Kurmann 1989). We performed the microarray analysis to determine whether the molecular mechanism of exine formation is similar between gymnosperms and angiosperms. Microarray technology has been recognized as a powerful tool to measure mRNA abundance on a genomic scale. We developed a novel microarray of *C. japonica* to identify differentially expressed genes in sterile male strobili. Recently, Tsubomura et al. (2016) reported the information of

expressed genes of wild type and sterile male strobili using three biological replicates between wild type and male-sterile mutants. We developed a novel microarray independently and used another type of male-sterile mutants as plant material. We compared the probe sets identified as differentially expressed relative to fertile male strobili in the pooled and individual analyses using half-sib F2 pedigree. Three different biological replicates were used in the pooled analysis and seven biological replicates were used in the individual analysis. We selected 93 probe sets using individual samples and 305 probe sets using pooled samples that showed alteration of RNA transcripts at the UM stage. Among these probe sets, 52 probe sets overlapped. When we conduct transcript profiling using microarrays, pooling is a common means to reduce both the cost and complexity of data analysis. Our results indicate that while pooling analysis can be used but many repetitions are required to identify candidate genes with high reliability.

The microarray analysis indicated that there were no significant differences in gene expression between fertile and sterile male strobili at the stages before the release of microspores. The observation of pollen development revealed the morphological aberrations in TETs. More detailed studies such as tissue microdissection may be required to reveal the differences in gene expression at this stage. Our results using whole male strobili detected genes with significant differences at the UM stage. In this stage, microspores were released from TETs in normal male strobili and all microspores collapsed in sterile male strobili. Finally, we confirmed 32 genes that were expressed at a lower level in sterile male strobili than in fertile ones at the stage when the disrupted microspores were observed in the sterile mutant (Table 1). Some of these genes are related to transcription, transport of materials, and carbohydrate metabolic processes. The histological examination of pollen development revealed that the most striking difference between fertile and sterile male strobili during pollen development is the formation of pollen wall. Therefore, these genes may be involved in the network regulating pollen wall formation.

Similar functional genes have been reported to be responsible for the *Arabidopsis* mutants. In *Arabidopsis*, mutations in *DEFECTIVE IN EXINE PATTERN FORMATION 1* (*DEX1*), *NO EXINE FORMATION 1* (*NEF1*), *MALE STERILITY 1/HACKLY MICROSPORE* (*MS1/HKM*), and *RAPTURED POLLEN GRAIN 1* (*RPG1*) disrupted the membrane structure of the pollen wall, apparently resulting in decreased exine synthesis and random deposition of sporopollenin (Paxson-Sowders et al. 1997, 2001; Ariizumi et al. 2004, 2005; Guan et al. 2008). *MS1/HKM* contains the plant homeodomain (PHD)-finger motif and the leucine zipper motif, suggesting that it functions as a transcription factor (Wilson et al. 2001; Ito and Shinozaki 2002; Ariizumi et al. 2005; Ito et al. 2007; Yang et al. 2007). While the actual protein functions of *DEX1*, *NEF1*, and *RPG1* have not yet been determined precisely, these proteins are considered to

function in exine pattern formation and stabilization of the cell integrity of the plasma membrane (Paxson-Sowders et al. 2001; Ariizumi et al. 2004; Guan et al. 2008). Additionally, previous studies in different plant species have shown that carbohydrate metabolism is essential for anther and pollen development (Dorion et al. 1996; Goetz et al. 2001). Cell wall invertase genes are strongly expressed in the tapetum and pollen (Goetz et al. 2001; Proels et al. 2003; Koonjul et al. 2005; Oliver et al. 2005; Hirsche et al. 2009) and their inhibition or downregulation would limit the provision of the hexoses required for both tapetal and pollen development (Dorion et al. 1996; Goetz et al. 2001). Pollen fructokinases are assumed to play a role in pollen germination and tube growth by providing fructose-6-phosphate for glycolysis or through conversion to UDP-glucose to support the biosynthesis of cell wall materials (Karni and Aloni 2002). Thus, our confirmed 32 genes represent candidate genes responsible for the *C. japonica* sterile mutant.

Microarray analysis revealed that the expression pattern of genes in male strobili during pollen development was very similar between fertile and male-sterile individuals. We identified 32 genes that were expressed at a lower level in sterile male strobili than in fertile ones at the stage when the disrupted microspores were observed in the sterile mutant. Moriguchi et al. (2012) succeeded in the construction of a high-density linkage map for identifying markers that are tightly linked to a major gene for male sterility. We could not conclusively identify the gene responsible for the Toyama 1 mutant in this study and the previous one (Moriguchi et al. 2012). At present, the Forestry and Forest Products Research Institute (FFPRI) is attempting to obtain the entire genome sequence of *C. japonica*. Therefore, in the near future, we will be able to compare the genome sequences of fertile and male-sterile *C. japonica* to narrow down our proposed candidates and identify the gene responsible for the Toyama 1 mutant. The role of this gene could then be confirmed by rescuing the phenotype of the Toyama 1 mutant using transgenic *C. japonica*.

Acknowledgments This research was supported in part by a Grant-in-Aid (Development of Technologies for Control of Pollen Production by Genetic Engineering) from the Forest Agency of Japan.

Data archiving statement The probe set for the microarray were prepared from previously reported *C. japonica* ESTs and cDNAs. The ESTs were derived from male strobilus (accession nos. BY877900-BY914001, AU298712-AU299544, BW993917-BW996365), female strobilus (AU299545-AU300308), needle (DC429774-DC429778), vegetative bud (BW996366-BW997181), sapwood (BB940565-BB941614), pollen (BJ936638-BJ940292, DC429611-DC429773), and seedling (AU036727-AU036912, DC429408-DC429610). The sequences of six cDNAs were also added for preparation of the probe set (*MADS2*, AF097747; *CjNdy*, AB074568; allergen No.121, AB197670; *Cry j* 3.7, AB212218; putative ORFX, AB254812; prolyl 4-hydroxylase, alpha subunit, AB254817). The microarray dataset has been deposited in the Gene Expression Omnibus database (GSE33499).

We confirmed 32 cDNA clones that were expressed at a lower level in sterile male strobili than in fertile ones at the stage when the disrupted microspores were observed in the sterile mutant. Twenty-eight of 32 cDNA clones were re-sequenced to obtain full-length inserts. These cDNA sequences were submitted to GenBank (AB728801-AB728828). The accession numbers of the other four cDNA fragment were BB940663, BY888322, BY892834, and BY894314.

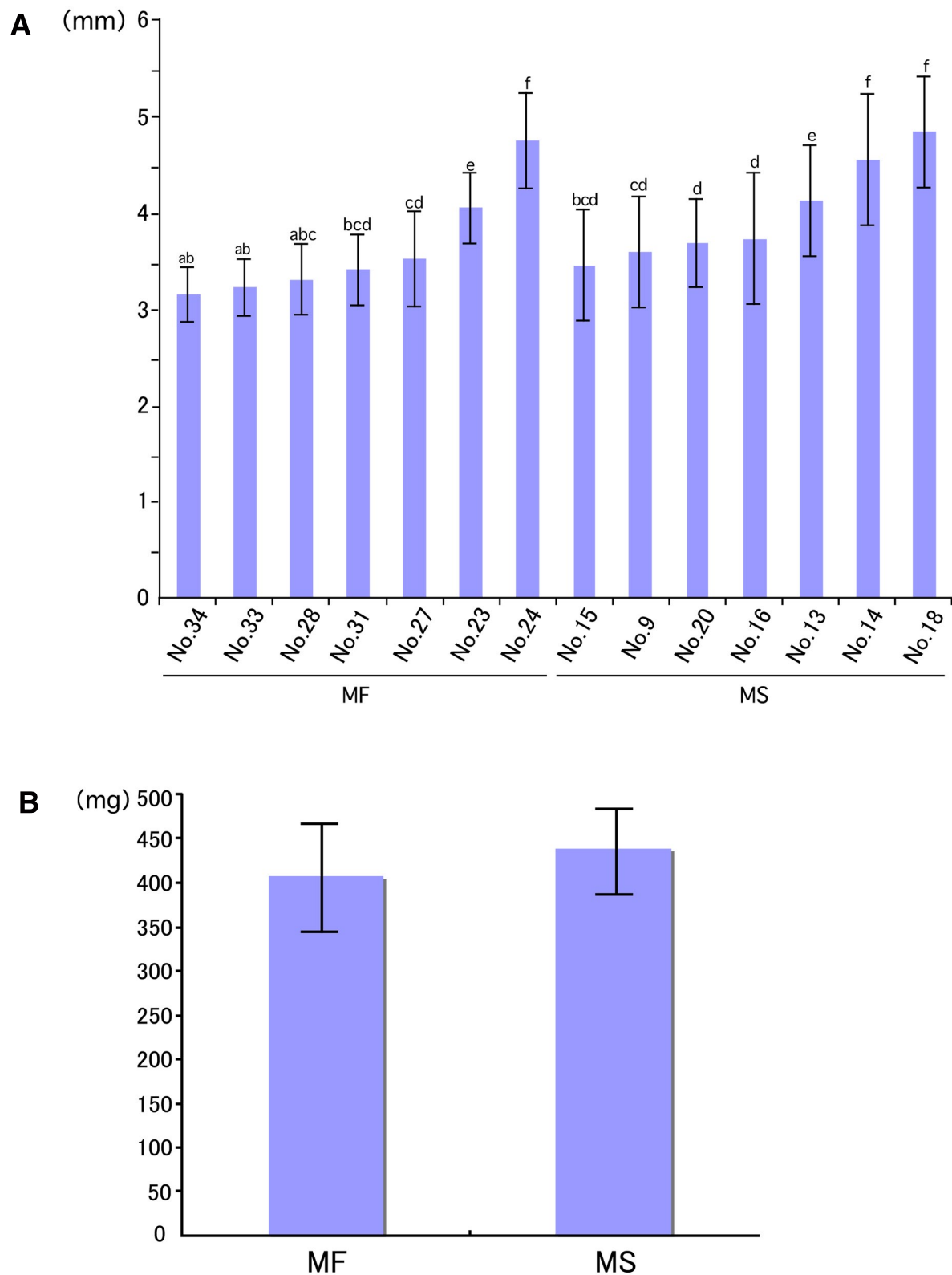
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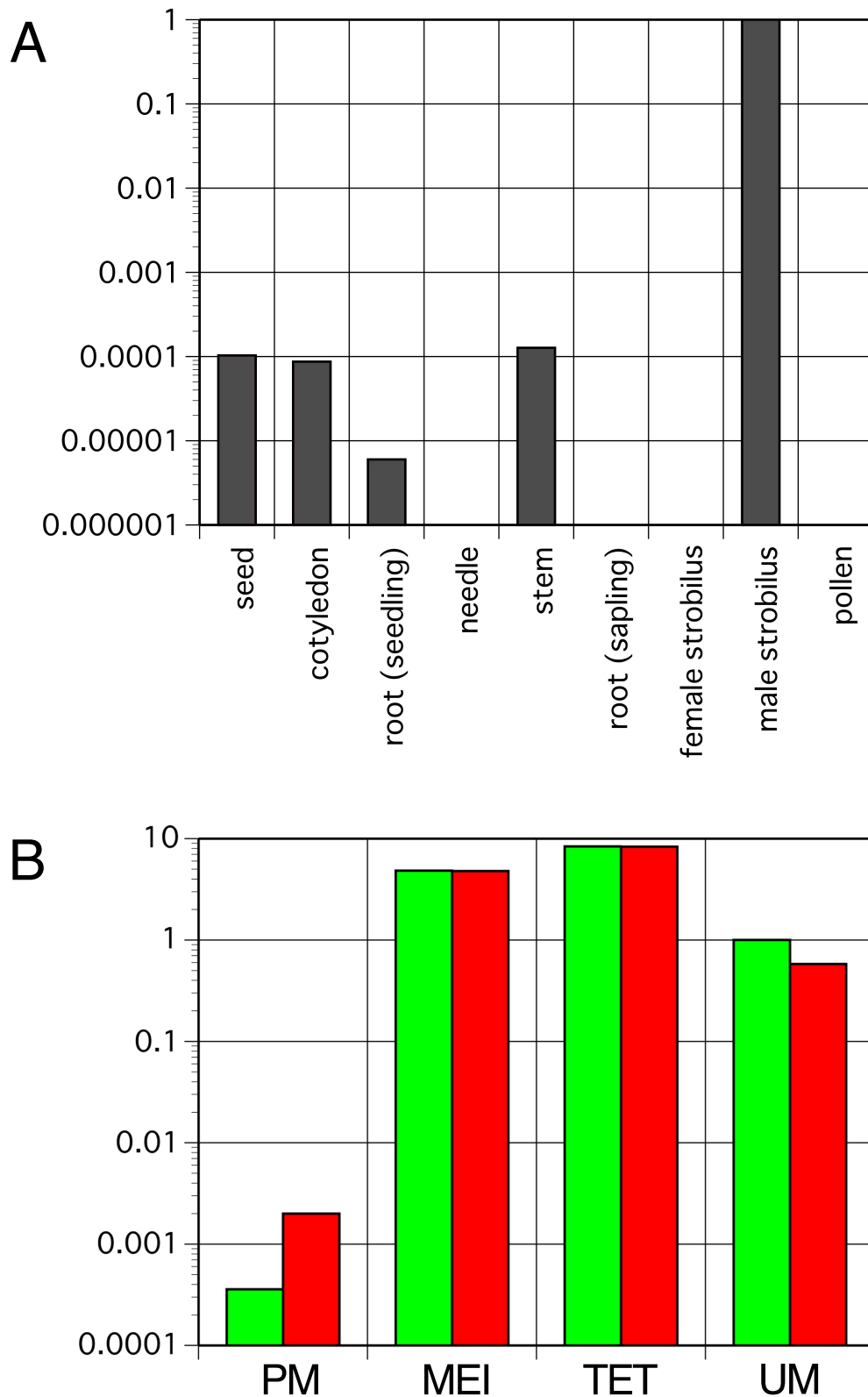


Supplementary Fig. 1

Comparison of length and dry weight of male strobili derived from fertile (MF) and sterile (MS) trees.

A. Length of male strobili from fertile and sterile trees. Vertical bars indicate the mean length $\pm$ SE of fifty male strobili derived from each individual. Those bearing different letters differ significantly at $P < 0.05$.

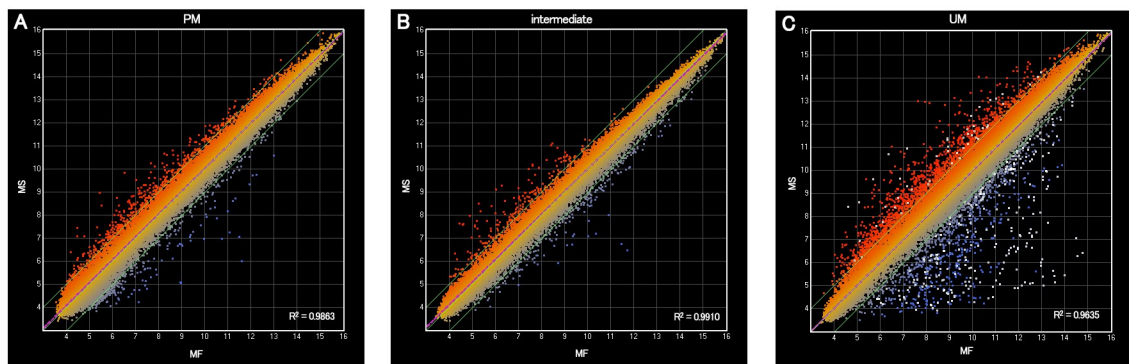
B. The dry weight of male strobili from fertile and sterile trees. Vertical bars indicate the mean dry weights $\pm$ SE of a hundred male strobili from seven fertile and sterile trees, respectively. There were no significant differences between fertile and male-sterile trees. Male strobili were collected at the end of October when sterile microspores were degraded.



Supplementary Fig. 2

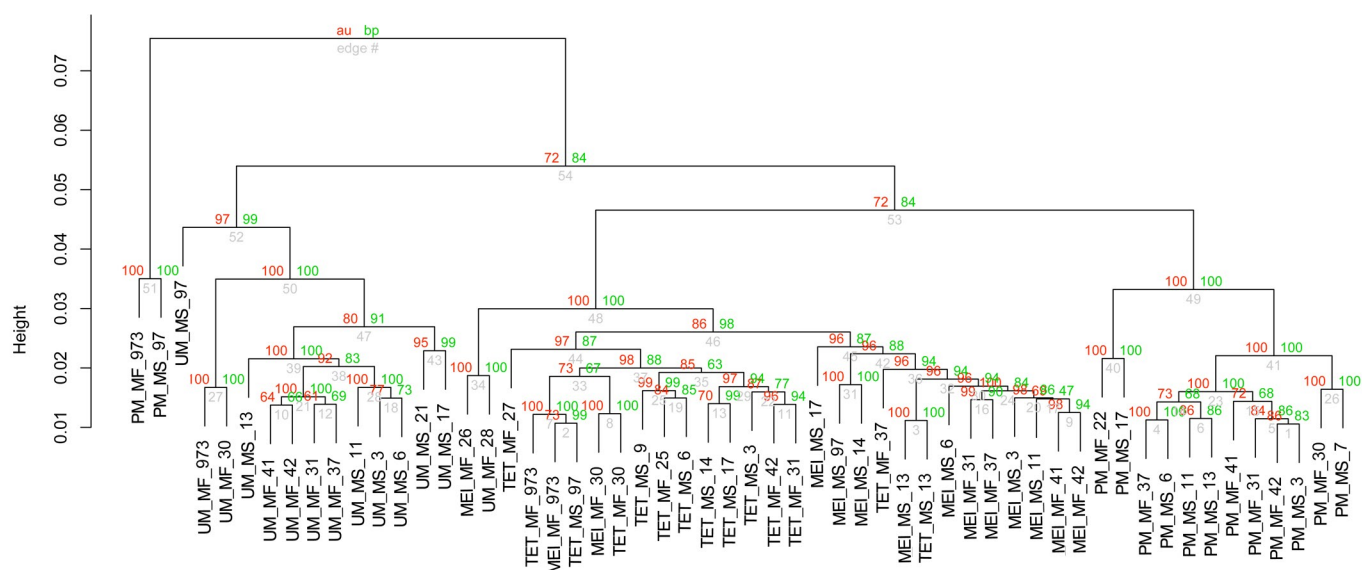
Expression level of *CjMALE1* mRNA.

A. Transcript levels in various organs. The information of plant materials was shown previously (Futamura et al. 2005). B. Transcript levels at various stages in male strobili. Green bars and red bars indicate the transcript levels in fertile and sterile strobili, respectively. Quantitative real-time RT-PCR was used for the determination of transcript levels. The y-axis indicates the ratio of expression and is represented on a logarithmic scale. The transcription levels were normalized with the eIF gene and the level of *CjMALE1* mRNA in fertile male strobili at the UM stage was set at 1.0. Six individuals each for fertile and male-sterile trees were used in this study. The SD values were 0.00120, 1.00230, 16.0, 15.8, 27.8, 27.5, 3.41 and 1.97 in order from the left to the right.



Supplementary Fig. 3

Scatter plots of signal intensity values (log 2) comparing relative gene expression in pairs of male fertile (MF) and sterile (MS) strobili derived from each of three pooled RNAs at different developmental stages. The detailed state of microsporogenesis at each stage is shown in Fig. 3B. The expression levels of individual genes were normalized using RMA and genes co-expressed in pairs of transcriptome datasets were plotted. White blots indicated the probe sets that show significantly different expression levels (FDR < 0.05) between male fertile and sterile strobili. The number in each figure means the coefficient of determination between male fertile and sterile strobili.



Supplementary Fig. 4

Hierarchical clustering of expression pattern in 56 male strobili of seven fertile and sterile individuals at four developmental stages.

The expression pattern data were derived from microarray analysis with 45,753 probe sets that were clustered using pvclust. Values at branches are approximately unbiased (AU) p-values (left), bootstrap probability (BP) values (right), and cluster labels (bottom).

Reference

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