Molecular characterization of genes essential for early development of germ cells in rice

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General Introduction

In flowering plants, the transition from the vegetative to the reproductive growth phase of is a critical developmental process. During the vegetative phase, the meristem at the shoot apex continuously generates leaves to make the upper plant body. Receiving the environmental changes such as temperature and day length, the vegetative meristem is transformed into a reproductive meristem, which either directly differentiates into flowers or remains meristematic to produce inflorescent brunches (inflorescence meristem) and multiple flowers at termini of brunches. Termination of the floral meristem becomes a signal to switch the reproductive organ to initiate the primordial plant germ cell, termed the archesporial cell. This drastic and qualitative change of the plant meristem might be accompanied by so many genetical and physiological changes, whereas most of them have yet been unknown. Of these complex and biologically important processes, this thesis will focus on several molecular mechanisms conducting the initiation and promotion of the plant germ cells development.

The plant system to generate germ cells is quite different from that of animals and insects. The germline cells in most animals diverge from somatic cells during early embryo development and remain a distinct germline stem cell population throughout their life to produce germline cells continuously (reviewed by Birnbaum and Alvarado 2008). In contrast, plants exhibit distinct two phases, vegetative and reproductive phases, and the plant germ line cells originate in flowers from the cells of a previous somatic lineage (Figure 1). During germline cell generation, animals and insects maintain the germline stem cell (GSC) and can continuously produce germline cells (reviewed by Morrison and Spradling 2008). The plants also maintain stem cells at a shoot apex and root tip. These proliferative tissues, termed meristems, continuously generate new cells to form lateral organs such as leaves, stems and roots. After the growth phase shifted to reproductive, the meristem becomes to generate inflorescent and floral organs continuously (reviewed by Birnbaum and Alvarado 2008). However, when the primordial plant germ cell (PGC) initiates within the

male and female reproductive organs, the floral meristem have already been terminated. To achieve a perennial reproduction, substitute for a self-renewal of animal GSCs, most of perennial plants produce new reproductive organs and germ cells at the tip of newly branched meristems. The renounce of the GSCs is another feature of plant reproduction.

Cell lineage of early germ cells development in plant

The stamen, the male reproductive organ and the anther primordium, initiates in the third whorl of the rice flower. The anther primordium comprises three layers, designated L1, L2 and L3 (reviewed by Goldberg et al. 1993). In rice the L1 layer gives rise to the epidermis and the stomium, which plays an important role in anther dehiscence. The L2 layer develops into the archesporial cells, which differentiate microsporangium and pollens. The L3 layer gives rise to the connective cells, vascular bundles, inner tapetum and circular cell cluster adjacent to the stomium (Raghavan 1988, Goldberg et al. 1993, Nonomura et al. 2003). The connective cells are positioned between microsporangia and vascular bundles, and degenerate during gametogenesis. The archesporial cells from the second layer undergo a series of mitotic periclinal divisions to differentiate the primary parietal cell (PPC) and the primary sporogenous cell (PSC) (Figure 2B). The PSCs undergo several mitotic divisions and differentiate into pollen mother cells (PMCs). The PPCs repeat periclinal divisions and generate endothecium, a middle layer and an outer-tapetum layer (Figure 2C). Each of the four-walled layers expands through anticlinal divisions. The tapetal cells are binucleated and serve as a nurse tissue to provide nutrition and pollen wall materials for gametophytes (reviewed by Scott et al. 2004). After completion of anther wall formation, the PMCs undergo pre-meiotic DNA synthesis (pre-meiotic S) and enter meiosis (Figure 2D).

On the other hand, a female archesporial cell is differentiated from a single hypodermal cell at the top of the nucellar primordium in the majority of flowering plants. In Gramineae species, including rice, a female archesporial cell elongates longitudinally and directly differentiates the megasporocyte or megaspore mother cell (MMC) (Davis, 1966;

Russell, 1979). The MMC then undergoes meiosis, resulting in the formation of four haploid spores.

Genes Important for Germ Cell Differentiation and Function in Arabidopsis

Several genes have been identified genetically that are critical for early anther development (reviewed by Ma 2005). One of the earliest acting genes is the *Arabidopsis SPOROCYTELESS* (*SPL*)/*NOZZLE* (*NZZ*) gene, which is important for early anther cell division and differentiation, both of which are required for sporogenesis (Yang et al. 1999, Schiefthaler et al. 1999). In *spl*/*nzz* mutant anthers, the formation of archesporial cells seems normal but subsequent cell divisions is defective, resulting in the absence of sporogenous cells and nonreproductive tissues, including the tapetum layer, and the *spl*/*nzz* mutants fail to produce pollen and are male sterile. The *SPL*/*NZZ* encodes a nuclear protein with some features of transcription factors, suggesting that it may regulate gene expression during early sporogenesis in the anther (Yang et al. 1999). Ito et al. (2004) show that the *AGAMOUS* (*AG*) gene promotes the expression of the *SPL*/*NZZ* and supports sporogenesis, indicating that a downstream of the *AG* signaling pathway is the *SPL*/*NZZ* pathway, required for plant germ-cell development. Subsequent to the action of the *SPL*/*NZZ*, the *EXCESS MALE SPOROCYTES1* (*EMS1*) gene (also called *EXTRA SPOROGENOUS CELLS* (*EXS*)) is required for normal formation of tapetal cells (Zhao et al. 2002, Canales et al. 2002). A defect in the *EMS1* gene causes an extra mumber of sporocytes and a lack of tapetal cells, and as a result, these mutants cannot produce any viable pollen grains and become male sterile. Molecular cloning indicates that the *EMS1*/*EXS* gene encodes a putative leucine-rich repeat receptor protein kinase that likely localizes to the cell surface (Zhao et al. 2002). Therefore, cell-to-cell communication is probably important for normal tapetal cell differentiation during anther development and the *EMS1*/*EXS* gene product is an important component of cell-to-cell communication. Yang et al. (2003) reported that a mutation in the *TPD1* (*TAPETUM DETERMINANT1*) gene causes the same phenotype as do the *ems1*/*exs* mutations: excess male sporocytes and lack of tapetum. The *TPD1* gene encodes a predicted small protein with a putative signal peptide for

secretion, suggesting that it is a ligand of the *EXS*/*EMS*. Recently, Jia et al. (2008) indicated that TPD1 serves as a ligand for the EMS1 receptor kinase to signal cell fate determination during plant sexual reproduction. They showed TPD1 interacts with EMS1 *in vitro* and *in vivo* by yeast two hybrid, pull-down and coimmunoprecipitation analyses (Jia et al. 2008). However, not so many plant genes have been identified in germ-cell initiation and sporogenesis stages before meiosis.

Genes Important for Germ Cells Development in Rice

As described above, several genes that function in specification of the male or female germ cells have been identified in *Arabidopsis*. In rice, the *MULTIPLE SPOROCYTES 1* (*MSP1*) are reported to be essential for early germ cell development (Nonomura et al. 2003). The *msp1* mutation gives rise to an excessive number of both male and female sporocytes. The *MSP1* is a member of the leucine-rich-repeat (LRR) receptor kinase gene family. The MSP1 closely related structurally and functionally to the EXS/EMS1 of *Arabidopsis*. However, they seem not to have completely same function; the *msp1* shows complete male sterility and partial female sterility, whereas *Arabidopsis exs*/*ems1* shows only male sterility. Recently, the *OsTDL1A*, a rice ortholog of *Arabidopsis TPD1*, was identified as a putative ligand of the MSP1 (Zhao et al.2008). OsTDL1A binds to the leucine-rich-repeat domain of MSP1 in yeast two-hybrid assay and bimolecular fluorescence complementation in onion cells. The *OsTDL1A*-RNAi driven by ubiquitin-gene promoter exhibited a phenotype of *msp1* mutant in the ovule, but not in the anther. The *exs*/*ems1* and the *tpd1* mutants do not exhibit a phenotype in the ovule in *Arabidopsis* (Canales et al., 2002, Zhao et al., 2002 and Yang et al., 2005), suggesting that *Arabidopsis* and rice differ in their control mechanism of germ cell development.

The *MEIOSIS ARRESTED AT LEPTOTENE 1* (*MEL1*) is also reported to be an important gene in maintenance of rice germ cells (Nonomura et al. 2007). The *mel1* mutant shows abnormalities in the morphogenesis of pollen mother cells and meiosis progression at early stages. Expression of the *MEL1* mRNA is limited in reproductive organs

(Nonomura et al. 2007). The *MEL1* gene encodes a protein of ARGONAUTE (AGO) family, defined by the presence of two conserved regions, the PAZ and PIWI domains (Nonomura et al. 2007). AGO proteins are integral players in small RNA-directed regulatory pathways and reported to function during miRNA- and siRNA- mediated regulation of development and stress responses, siRNA-mediated antiviral immune response, and siRNA-mediated regulation of chromatin structure and transposons (reviewed by Hutvagner et al. 2008, Vaucheret 2008, Mallory et al. 2008).

The AGO family members are grouped into two subfamilies, PIWI and AGO (Sasaki et al. 2003). Between two subgroups, PIWI subfamily members are expressed only in reproductive organs and/or the germline and they are associated with a distinct size of small RNAs, so-called PIWI-interacting RNAs (piRNA) (reviewed by O'Donnell et al. 2007). All of these Piwi orthologs conduct the maintenance of self-renewality of GSCs and/or normal development of germline cells. The number and diversity of AGO proteins greatly varies among organisms (reviewed by Hutvagner et al. 2008). A single AGO protein exists in *Schizosccharomyces pombe*. Two AGO and three PIWI proteins are found in *Drosophila melanogaster*. Four AGO and four PIWI proteins exist in *Homo sapiens*. Five AGO, four PIWI and 18 proteins are found in *Caenorhabditis elegans*. Annotation of *Arabidopsis thaliana* and *Oryza sativa* genomes revealed 10 and 18 genes, respectively, all of which correspond to proteins of the AGO group (Morel et al. 2002, Nonomura et al. 2007). This may represent that plant reproduction renounces to maintain GSCs, whereas the animal PIWI family proteins partly play a role in maintaining the GSCs. As mentioned above, the expression of the *MEL1* mRNA is limited in reproductive cells, the MEL is germ-cell specifies argonaute protein. An *Arabidopsis AGO5* (At2g27880) is the ortholog of the *MEL1*, according to phyrogenetic tree (Figure 3). However, the *ago5* mutants identified do not exhibit obvious developmental defects (Takeda et al. 2008, Katiyar-Agarwal et al. 2008). These observations suggest that no germ-cell specific AGO or another AGO bearing a redundant function in reproduction is exited in *Arabidopsis*.

The Goal of This Study

As mentioned above, the plant system to generate germ cells is quite different from that of animals and insects, and several genes that control early stages of germ-cell development have been identified, however, little is known about the molecular mechanism determining the germ cell fate during sexual reproduction in plants. Some genes of *Arabidopsis* are conserved in rice but some are not. For example, in flowering pathway, *FLOWERING LOCUS C* (*FLC*) (Michaels et al. 1999, Sheldon et al. 1999), a MADS-box gene, is a key regulator of the autonomous flowering and vernalization pathways in *Arabidopsis*, however, no apparent *FLC* orthologue has been found in rice. Further, no orthologues of the *VERNALIZATION1* (*VRN1*) and *VRN2* genes (Gendall et al. 2001, Levy et al. 2002) have been found in rice. Also as described above, in plant germ cell development, there are differences between rice and *Arabidopsis*. The *msp1* shows male and female sterility whereas *Arabidopsis exs/ems1* shows only male sterility, and no germ cell specific ARGONAUTE are exist in *Arabidopsis*. Thus, to study of rice reproduction and to compare it to that of *Arabidopsis* one will lead to well-understanding the mechanism of plant germ cell initiation and maintenance.

In this study, I planed to perform following examinations to understand the mechanism and gene regulation network in early germ cells development in rice.

1. Definition of early developing stages in reproductive organs and investigation of gene expression in rice

2. Spatial and temporal expression analysis of the *MEL1,* the *MSP1* and the *OsNZZ* genes in very early germ cell development

3. Search for the genes regulated by the *MEL1* gene by microarray analysis.

4. Analysis of a putative target gene of the MEL1 AGO identified by microarray analysis

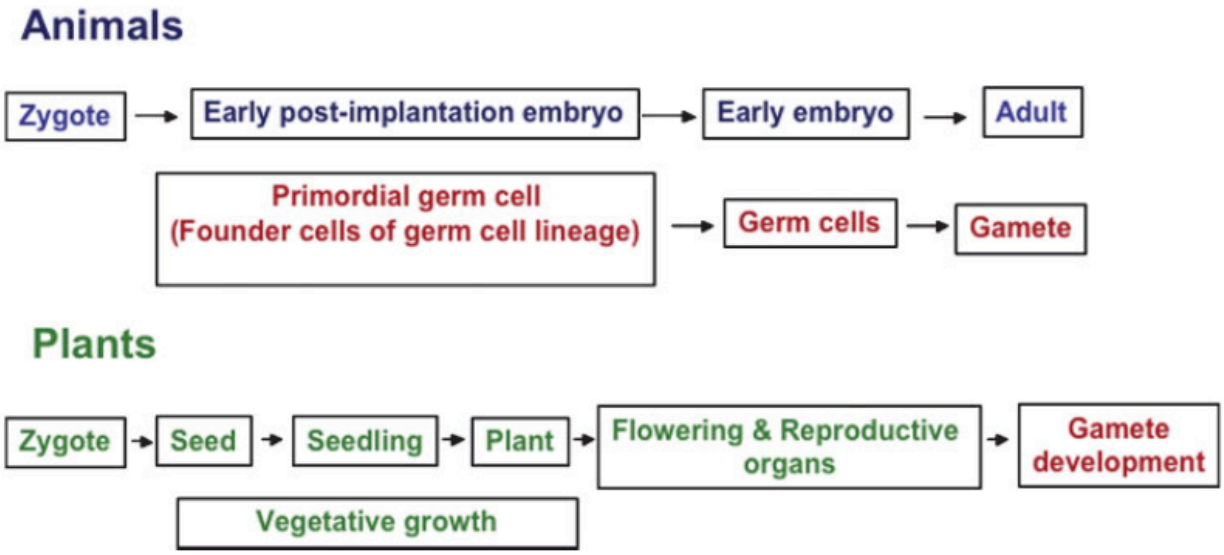


Figure 1. Outline of the landmark stages in the formation of germ lines in animals and plants

An early embryo formed from a zygote resulted from the fusion of male and female gametes generates not only all the parts of animal body (somatic cells) but also demarcates germ cells for the next generation. Note pre-meiosis establishment of germlines and the continuous production of functional male gametes are dependent on germ-line stem cells during the adult life of an animal. Meanwhile, in higher plants seeds are developed from double fertilization and have well developed embryos with endosperm when matured. Seed germinates to develop and differenciate whole architecture of plant in vegetative growth. In the transition stage from vegetative to flowering, formation of reproductive organs are initiated, followed by gamete development. Origin of gametes from gametophyte is a post-meiotic event. Gamete development within a reproductive organ is not a continuous process. (Shign et al. 2007)

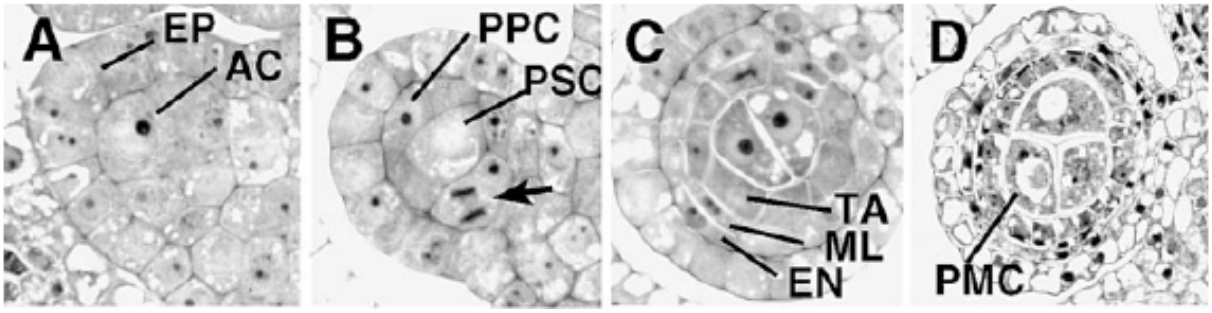


Figure 2. Sporogenesis and gametogenesis in rice anther

(A) The archesporial cells (AC) differentiate into the hypodermis of anthers. (B) The archessporial cells continue periclinal division (arrow) and develop primary parietal cell (PPC) and the primary sporogenous cell (PSC). (C) Formation of four-layered anther wall through periclinal divisions of PSC. (D) Formation of pollen mother cells (PMCs). PMCs enter into meiosis. EP; epidermis, EN; endocethium, Ml; middle layer; TA; tapetum. This figure is cited from Nonomura et al. 2003

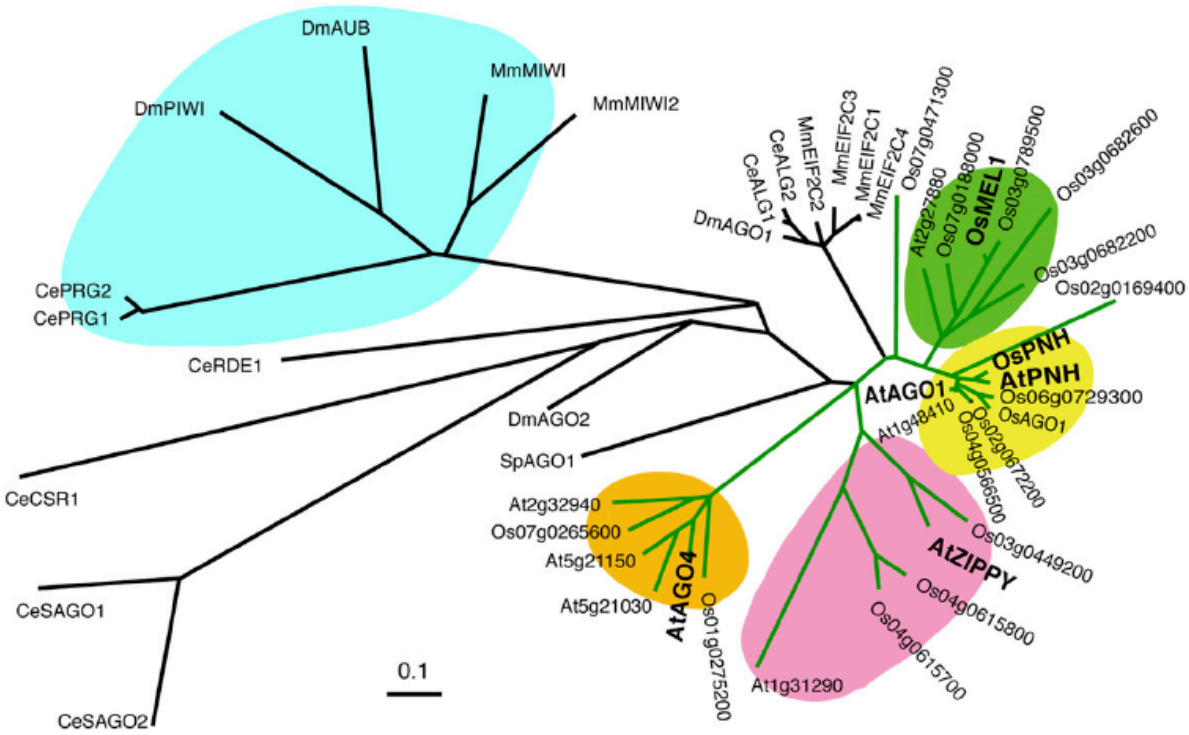


Figure 3. Phylogenetic tree of Argonaute family constructed using the peptide sequences of PAZ and PIWI domains

For plants, all AGO family members in *Oryza sativa* (Os) and *Arabidopsis thaliana* (At) were included (connected with green lines). In addition, functionally known AGOs from *Drosophila melanogaster* (Dm), *Caenorhabditis elegans* (Ce), *Mus musculus* (Mm), and *Schizosaccharomyces pombe* (Sp) were included (connected with black lines). The plant locus identifiers correspond to the *Arabidopsis* Genome Initiative codes for *Arabidopsis* and the Rice Annotation Project codes for rice. Accession numbers are as follows: NP_510322 (Ce ALG-1), NP_493837 (Ce ALG-2), CAA98113 (Ce PRG-1), AAB37734 (Ce PRG-2), AAF06159 (Ce RDE-1), NP_523734 (Dm AGO1), NP_476875 (Dm PIWI), AAF49619 (Dm AGO2), CAA64320 (Dm AUB), NP_067286 (Mm MIWI), AY135692 (Mm MIWI2), XP_001257025 (Mm EIF2C1), NP_694818 (Mm EIF2C2), NP_700451 (Mm EIF2C3), NP_694817 (Mm EIF2C4), AAD40098 (At PNH), ABO81950 (Os PNH), ABO81951 (Os AGO1), CAA19275 (Sp AGO1), NP_001040938 (Ce CSR-1), NP_504610 (Ce SAGO-1), and NP_490758 (Ce SAGO-2). The PIWI subfamily, which functions specifically in germline development in insects and animals, is highlighted in blue. The plant subfamilies Os MEL1 (AB297928), At AGO1 (AAC18440), At ZIPPY (AAQ92355) and At AGO4 (NP_565633) are highlighted in green, yellow, red, and orange, respectively. This figure is cited from Nonomura et al. (2007).

Section I

Gene expression profile analysis in early rice anther development

Introduction

In flowering plants, after the transition from vegetative growth to reproductive development, primordial germ cell initials differentiate from somatic cells reproductive organs of in flowers. Genetic analyses have demonstrated the function of genes in specifying plant germ cell identity, regulating cell division and differentiation and controlling meiosis. These genes encode a variety of proteins, including transcription factors, signal transduction proteins, and enzymes for the biosynthesis of hormones (reviewed by Ma et al. 2005). Although several genes have been reported in recent decades, much more findings await to be discovered and understood. In *Arabidopsis*, a lot of transcriptome data using microarrays for vegetative and reproductive organs have been compiled in the AtGenExpress database (Schmid et al. 2005), and they become fine tool for the identification of functional modules that control plant development. However, data for earlier stages, such as the spore mother cell developing stage and meiosis, have not compiled because of its tiny tissue size. On the other hand, although its genomes have been completely sequenced, the whole transcriptome profile of rice reproductive process has not yet been performed. In this study, the whole transcriptome profiles of plant reproductive process, including early stages that are difficult to be dissected in *Arabidopsis*, were established by using the Affymetrix rice genome array. All expression profiles were provided as a dataset of rice reproductive expression atlas.

Furthermore, comprehensive microarray analysis of rice reproductive organs in this section will give a useful information for rapid investigation of expression profile of genes regulated directly or indirectly by the *MEL1* Argonaute in the next section. Thus in this section, using the atlas data, the gene expression patterns of several genes that are highly expressed in early development stage were investigated.

Materials and Methods

Plant materials

The reproductive organs of rice (*O. sativa L. ssp. japonica cv.* Nipponbare) were collected from plants grown in paddy fields under normal condition at National Institute of Genetics, Mishima, Shizuoka, Japan.

Rice flower organs used for the analysis of gene expression profile

To grasp the global expression profile of the rice genes during reproductive organ development and fertilization in *O. sativa L. ssp. japonica cv*. Nipponbare, biological processes of flower development and fertilization, these biological processes were divided into 29 stages. The stage definition of samples was summarized in Table I- 1. Of 29 stages, five stages of anthers carrying the pollen mother cells in meiosis were dissected and analyzed in this study. Other 24 stages were examined by other members of Plant Genetics laboratory. Five different stages of meiosis were determined dependent on the anther length; An1; formation of archesporial cell, Mei1; Pre-meiotic S/G2, M1; meiosis (Leptotene), M2; meiosis (Zygotene to Pachytene), M3; meiosis (Diplotene to Tetrad). Stage ID5 contained meiotic cells from diplotene to tetrad (stage symbol; from mei5 to mei12), because these cells usually lost synchronization in development among six anthers in a spikelet (flower). Collected organs usually contained two or more tissues; for instance, the anther was composed of microspore cells, tapetum cell functions as a nursery of meiocytes and microspores, and outer anther wall cells. Before dissection of anthers, one of six anthers included in a flower was provided for acetic-carmine squashing on the slide glass, and observed under a light microscopy to judge whether the anthers contained the pollen mother cells at the appropriate meiotic stages.

Total RNA isolation and microarray experiment

The flower organs corresponding to each of 29 stages (Table I-1) were dissected, immediately frozen with liquid nitrogen, and stored at -80℃. This operation was shared with three persons including the author of this study. Total RNA was extracted with RNeasy plant mini kit (Qiagen, Valencia, CA, USA) according to manufacturer's protocol. A sample quality was assessed using the Agilent 2100 Bio analyzer and the Nano LabChip Kit (Agilent Technologies).

Microarray experiments

Affymetrix rice genome arrays constructed with almost all IRGSP Nipponbare gene sequences of 57381 including 25500 predicted genes were used for transcriptome analysis. The probe sets of Affymetrix Rice Genome Array were designed on 2004, based on NCBI UniGene Build #52, (May, 2004), GenBank mRNAs (July, 2004) and gene predictions from TIGR's osa1 version 2.0 release (April, 2004).

The Affymetrix protocol for one-cycle eukaryotic target preparation to prepare rice probe was as follows; 500 ng of total RNA was converted into single-stranded cDNA using SuperScript II and a T7-Oligo (dT) primer (Affymetrix). Second-strand cDNA was synthesized using dNTPs, second-strand reaction buffer, Escherichia coli DNA ligase, E. coli DNA polymerase I, and E. coli RNase H (all reagents were supplied by Affymetrix). The one-cycle cDNA synthesis was followed by a cleanup using Affymetrix GeneChip sample cleanup modules. Biotin-labeled cRNA was prepared using an Affymetrix in vitro transcription (IVT) labeling kit. After cleanup of the IVT products, the purified cRNA was fragmented to a size ranging from 35 to 200 bases using a fragmentation buffer at 94℃ for 35 min. The fragmentation of the labeled cRNA was confirmed before hybridization by running samples on the eGENE. 3.3 μg of the fragmented cRNA was mixed into a hybridization cocktail containing a hybridization buffer, B2 oligo control RNA (Affymetrix), herring sperm DNA, and BSA (Invitrogen, Carlsbad, CA). The solution was hybridized to a GeneChip at 45℃ for 16 h with a gentle rotation at 60 rpm. After the hybridization, the cocktail was removed from the GeneChip. The hybridized GeneChip was washed with two wash buffer provided by Affymetrix and stained with

streptavidine–phycoerythrin (Molecular Probes) using the Affymetrix Fluidics 450 wash station (Affymetrix Fluidics Protocol EUKGE_WS2v5). The GeneChips were immediately scanned with a GeneChip scanner 3000. Individual scans were quality checked for the presence of control genes and background signal values. Triplicate experiments were performed with independently collected samples for all stages.

Microarray data extraction, normalization and cluster analysis

For Affymetrix array data, CEL files produced by GCOS 1.3 (Affymetrix, Inc., Santa Clala, CA) were analyzed using the statistical software R with bioconductor package "affy". Signal intensities were extracted by expresso algorithm with parameters: bgcorrect.method = "mas", normalize = "F", pmcorrect.method = "pmonly", summary.method = "mas". Extracted signal intensities were introduced into GeneSpring 7.3.1 (Agilent) and scaled to 75th percentile per chip. To check data quality for stage specificity, hierarchical clustering technique (Cluster 3.0) was applied to array data, and the results were visualized using TreeView (Eisen et al. 1998). All 57381 probe sets were used for the expression profiling comparison, and log transformed normalized signal intensities were clustered using average linkage protocol. Based on these gene profiles, stage and sample reliability was confirmed.

Results and Discussion

Total RNA extraction and verification of microarray data

In this study, 98 microarray experiments with 25 reproductive stage/organs and 10 vegetative control tissues by Affymetrix rice genome array were performed (Table I-1). Organs and stages in rice anther development, pollination and embryogenesis process were divided into 25 categories, including the stages that have never been reported in *Arabidopsis*. In this study, I collected anthers of *O. sativa* at five different stages; An1; formation of archesporial cell, Mei1; Pre-meiotic S/G2, M1; meiosis (Leptotene), M2; meiosis (Zygotene to Pachytene), M3; meiosis (Diplotene to Tetrad). Stage ID5 contained meiotic cells from diplotene to tetrad (stage symbol; from mei5 to mei12) by confirming meiotic and gamete cell stages under a microscope as shown in Figure I-1. At least 1,200 anthers from 200 spikelets were used for each microarray reaction. To verify sample identity in a given stage and expression difference among stages, triplicate array data were compared with each other by drawing scatter plots with one to one profile, after obtaining all expression data. Most expression data of biological triplicate experiments in each stage coincided well with each other, however, several meiotic data showed some differences. To examine them more detail, hierarchical clustering technique was applied to all of microarray data. Several expression profiles of meiotic stages revealed to be interchangeable among meiotic stages of M1, M2 and M3. Those suspicious samples were deleted or relocated in correct cluster position. In these expression profiles, very similar expression levels among triplicate experiments and continuous changes in expression level between neighboring stages were observed. In addition, reproducibility of gene expression events in each stage and accuracy of stage definition was also verified using quantitative RT-PCR analysis and *in situ* hybridization (data not shown). *In situ* hybridization data clearly showed stage specific expression of examined genes in M1, M2, and M3 stages (data not shown). Thus, continuous and synchronous varying values of examined gene expression among reproductive stages revealed by microarray and quantitative RT-PCR analysis and stage identity examined by in situ hybridization experiments together strongly supported correct stage definition and

true expression variation.

Stage specific genes in pre-meiosis and meiosis stages

The number of specifically expressed genes in each stages of developing anther was summarized in Table I- 2. At early development stage (From An1 to M3), one stage specific genes were limited. Number of one stage specific genes was as follows; An1 and Mei1was 0; M1 and M2 was 1 and M3 was 54 (The genes were summarized in Table I- 3). Most genes are expressed in two or more stages at early development. Number of pre-meiosis (Stage ID; An1 and Mei1) or meiosis (Stage ID; M1, M2 and M3) stage specific genes were 24 and 189, respectively (Table I-2, Figure I-2). At pre-meiosis and meiosis stage, most genes were expressed from An1 to M1 (103 genes). The analysis of gene ontology (GO) for stage specific genes has been done to understand their functions. Designated GO terms to stage specific genes were summarized in Table I- 4. At pre-meiosis stage, 19 of 24 probes were hit to TIGR loci, but 12 of them (63 %) were not categorized to any functions. This result suggests that a large part of genes function in early reproductive development remains uncharacterized. At meiosis stage, many cell cycle-related genes were expressed (Figure I-3). SKP1 and F-box protein are well known as a SCF (Skp1-Cullin-F-box protein) complexes, and is involved in a number of aspects of plant growth and development, possibly through the ubiquitin-mediated proteolysis of proteins by the proteasome (Pozo and Estelle. 2000, Hershko and Ciechanover. 1998, Sullivan et al. 2003, Zheng et al. 2002). The *ARABIDOPSIS SKP1-LIKE* (*ASK1*) acts predominately from leptotene to pachytene, and the *ask1-1* mutation causes an increase in recombination frequency in *Arabidopsis* male meiosis (Wang et al. 2006). At meiosis stage, some transcription factor and transporter were also highly expressed, suggesting that various cell-to-cell signal transductions are occurring at this stage. From this analysis, however, it was revealed that genes identified as specific in early reproductive development stages have not been characterized yet. These genes might act as useful sources for further studies of early germ cell development in plant.

Expression patterns of genes essential or specific for rice germ cell development

Previously, Nonomura et al. (2003; 2007) reported that the expression of the *MEL1* and the *MSP1* genes was specific for early reproductive organ development by semi-quantative RT-PCR. To confirm the reliability of expression profiles of microarray datasets, the expression patterns of these two genes were investigated. The expression of the *MEL1* and the *MSP1* from microarray data clearly indicated that both genes expressed during early stages of anther development, well consistent to the results of Nonomura et al. (Figure I-4). Both the *MEL1* and the *MSP1* was highly expressed at early stages of anther development and their expression were limited, as expression of them did not detected in other tissues, such as leaf, shoot, root and generating callus. These results also support the reliability of microarray datasets.

The *MEL1* encodes ARGONAUTE that is known to be a key player in gene-silencing pathways guided by small RNAs. In atlas data, two argonaute genes (Os06g0729300 and Os03g0353900) were also expressed higher at early stage. These two AGO genes may have same function to the *MEL1*. Analysis of these two genes in comparison with *MEL1* function will be needed for further studies of early developmental events in reproduction.

In addition to two genes indicated above, the expression patterns of other meiosis-related genes reported so far (Nonomura et al. 2004, 2006, Deng et al. 2007, Zhao et al. 2008, Jung et al. 2005, Xie et al. 2006, Zhang et al. 2006, Chen et al. 2007) were also investigated. Four of them were specifically expressed in earlier and meiotic stages (Table I- 5, Figure I-5). *OsDMC1A*, the rice homologue of the yeast *DMC1*, plays an important role in recombination mediated homologous chromosome pairing and transcripts of the *DMC1A* were detected in globular PMCs (Kathiresan et al. 2001). *OsTDL1A* (*TPD1-like genes*) is the ortholog of *Arabidopsis TPD1*, and the ligand of the *MSP1* (Zhao et al. 2008). *UDT1* (*Undeveloped Tapetum 1*) is required for the differentiation of secondary parietal cells to mature tapetal cells, and the transcript level was higher in anthers than in other floral organs (Jung et al. 2005).

OsSPL14 is expressed stronger during very early stage of young panicles (Xie et al. 2006). Although much were not categorized to GO term, the specific genes that were found in this study may have the crucial function at early germ cells development stages.

This is the first detail and comprehensive genome-wide analysis throughout whole developmental stages of reproductive organ formation in plant. The first successful transcriptome datasets of early developing stages before tetrad are valuable and this would be a breakthrough to frontier studies. The *MEL1* and the *MSP1* were expressed in different tissue of anther; the expression of the *MEL1* was limited to sporogenous cells, whereas the expression of the *MSP1* was detected in anther wall cells (Nonomura et al. 2003; 2007). Recently, Hobo et al. (2008) and Suwabe et al. (2008) reported the organ specific expression data for microspores and tapetum layers of developing anthers by using laser micro-dissection experiments. They developed a microarray technique combined with laser microdissection and used to characterize separately the transcriptomes of the microspore/pollen and tapetum at 5 stages; meiosis (MEI), tetrad (TET), uninuclear microspore (UN), bicellular pollen (BC) and tricellular pollen (TC). Same analysis at earlier anther development will be needed to understand the gene regulation of early stages for early stage specific genes.

Table I-1. Stage and organ definition in rice reproductive phase and control vegetative phase.

Reproductive process	Stage ID	Stage Symbol[a]	Biological Event	Organ	Definition	Stage ID No.
Anther development	An1	Anther 1	Formation of hypodermal archesporial cells	Anther	Anther length[b]: 0.1-0.15 mm	1
	Mei1	Mei1	Pre-meiotic S/G2	Anther	Anther length: 0.2-0.45 mm	2
	M1	Mei2	Leptotene	Anther	Anther length: 0.4-0.55 mm	3
	M2	Mei3	Zygotene	Anther	Anther length: 0.45-0.65 mm	4
		Mei4	Pachytene	Anther	Anther length: 0.6-0.8 mm	
	M3	Mei5	Diplotene	Anther	Anther length: 0.75-0.85 mm	5
		Mei6	Diakinesis	Anther	Anther length: 0.7-0.9 mm	
		Mei7	Metaphase I	Anther	Anther length: 0.8-0.9 mm	
		Mei8	Anaphase I /Telophase I	Anther	Anther length:0.8-0.9 mm	
		Mei9	Interkinesis (Prophase II)	Anther	Anther length:0.8-0.9 mm	
		Mei10	Metaphase II	Anther	Anther length:0.8-0.9 mm	
		Mei11	Anaphase II /Telophase II	Anther	Anther length: 0.8-0.95 mm	
		Mei12	Tetrad	Anther	Anther length: 0.8-1.10 mm	
	P1		Uni-nuclear pollen	Anther	Anther length:1.1-2.2 mm	6
	P2		Bi-celullar pollen	Anther	Anther length: 2.2 mm	7
	P3		Tri-celullar mature pollen	Anther	Anther length: 2.2 mm	8
Pollination - Fertilization	St-0		Mature stigma	Stigma	0 min	12
	Ov-0		Mature ovary	Ovary	0 min	10
	Poll 1		Pollination	Stigma	5 min after pollination	13
	Ov-1		(equal to mature ovary)	Ovary	5 min after pollination	11
	Poll 2		Pollen tube growth	Pistil	15-25 min after pollination	14
	Fer1		Fertilization	Pistil	40-50 min after pollination	15
	Fer2		Zygote formation	Pistil	5-7 hrs after pollination	16
Embryogenesis	Em0		Embryo 0DAP	bottom 1/4 of ovary	0 DAP Embryo	17
	Es0		Endosperm 0DAP	top 3/4 of ovary	0 DAP Endosperm	22
	Em1		Embryo: Globular stage I	bottom 1/4 of ovary	1 DAP Embryo	18
	Es1		Endosperm 1DAP	top 3/4 of ovary	1 DAP Endosperm	23
	Em2		Embryo: Globular stage II	bottom 1/4 of ovary	2 DAP Embryo	19
	Es2		Endosperm 2DAP	top 3/4 of ovary	2 DAP Endosperm	24
	Em5		Embryo: SAM and radicle differentiation	bottom 1/4 of developing seed	3 DAP Embryo	20
	Es5		Endosperm 3DAP	top 3/4 of developing seed	3 DAP Endosperm	25
	Em7		Embryo: Second and third leaf formation	bottom 1/4 of developing seed	4 DAP Embryo	21
	Es7		Endosperm 4DAP	top 3/4 of developing seed	4 DAP Endosperm	26
Vegetative tissues	C0		growing callus	callus	0 day callus	30
	C2		2 days after regenaration	regenarating callus	2 days regenaration	31
	C4		4 days after regenaration	regenarating callus	4 days regenaration	32
	C6		6 days after regenaration	regenarating callus	6 days regenaration	33
	C8		8 days after regenaration	regenarating callus	8 days regenaration	34
	S		4 weeks after germination	shoot and SAM	4 weeks old	35
	YL		young leaf	4th leaf blade	4th leaf blade	36
	R		4th leaf growing stage	root	roots at 4th leaf stage	37

a. Stage symbol is coincided with the stage of Itoh et al. (2005)
b. Anther length was approximate index just for Nipponbare standard. Anther length of each stage is varied in different cultivar or species. Stages were decided by microscope observation for biological events.

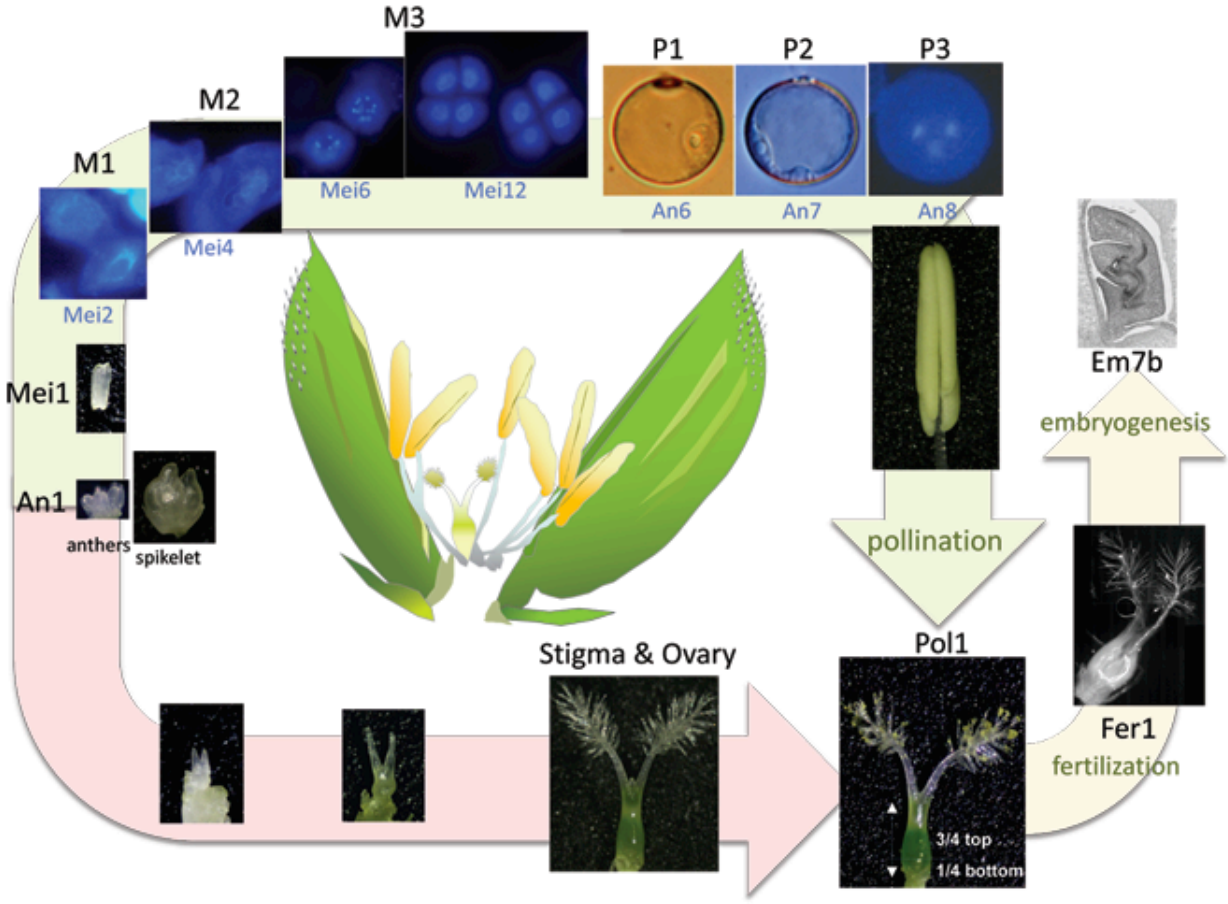


Figure I-1. Reproductive organs and stages used in this study

Morphology or cell images in anther, stigma and ovary in the representative stages are shown. Sizes of those organs are proportional to a 1.0 mm vertical bar indicated at the right of P-tri stage anther. Microspore cells and nuclei in several stages stained by DAPI (in blue color) are also shown. Bright blue nuclei with various chromosome configuration served as good indicators for stage definition. Biological event, organ and stage definition are coincided with those in Table I-1.

Table I-2. Number of specifically expressed genes in stages of developing anther

Stage		expressed until							
		An1	Mei1	M1	M2	M3	P1	P2	P3
expressed from	An1	0[a]	24	**103**[c]	11	16	15	10	55[b]
	Mei1		0	15	5	3	2	1	14
	M1			1	2	37	24	6	6
	M2				1	94	42	15	59
	M3					54	**184**	52	88
	P1						50	53	51
	P2							**203**	**564**
	P3								**740**

a. Figures in the thick square boxes denotes probe set number of individual stage specific genes. b. Figures in other boxes show gene numbers of stage group from the stage of leftmost column to that of the top column. C. Number of specific genes showing more than 100 in each stage/stage group was marked with bold letters.

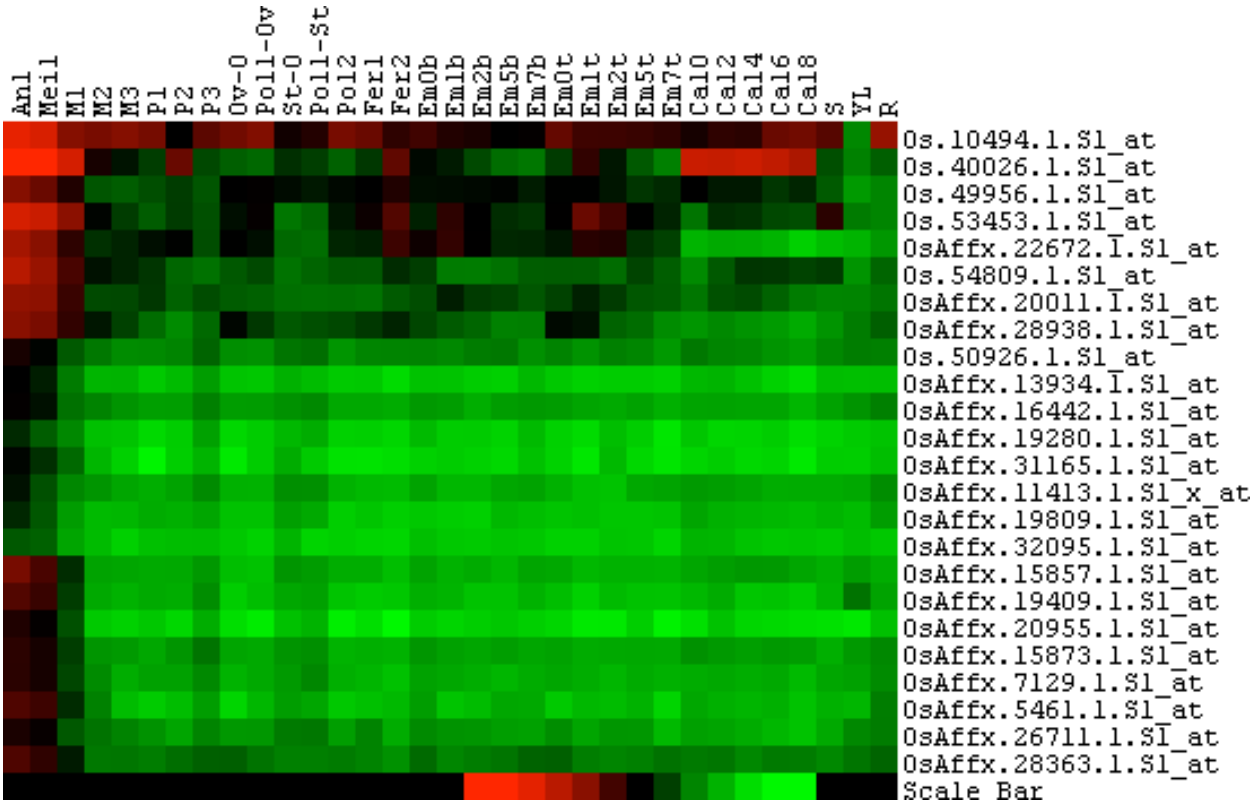
Table I-3. Genes expressed at only one stage

TIGR_loci	TIGR_description	RAP_loci	RAP_Description	stage	probe-Affymetrix
LOC_Os11g10890	expressed protein	Os11g0215300	Conserved hypothetical protein.	M1	OsAffx.7068.1.S1_at
LOC_Os04g55850	nuclease PA3,	Os04g0652700	Similar to Nuclease I.	M2	Os.26483.1.S1_at
LOC_Os08g34210	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	Os08g0440800	Glyceraldehyde-3-phosphate dehydrogenase.	M3	Os.17959.1.S1_a_at
LOC_Os12g12170	cytochrome b5 isoform 2	Os12g0223300	imilar to Outer membrane cytochrome b(5)	M3	Os.18642.1.S1_x_at
LOC_Os07g37090	ribosome inactivating protein	Os07g0556800	Ribosome-inactivating protein family protein.	M3	Os.22408.1.S1_at
LOC_Os03g49480	elongation of fatty acids protein 2	Os03g0701500	GNS1/SUR4 membrane protein family protein.	M3	Os.24106.1.S1_at
LOC_Os02g01980	anther-specific proline-rich protein APG precursor,	Os02g0110000	Lipolytic enzyme, G-D-S-L family protein.	M3	Os.24700.1.A1_at
LOC_Os01g14030	ramosa 2,	Os01g0242400	Lateral organ boundaries, LOB domain containing protein.	M3	Os.30523.1.S1_at
LOC_Os06g02360	SKP1-like protein 1A	Os06g0113800	Similar to Kinetechore (Skp1p-like) protein-like.	M3	Os.44828.1.A1_at
LOC_Os04g52320	QRT3	Os04g0613200	Virulence factor, pectin lyase fold family protein.	M3	Os.47317.1.S1_at
LOC_Os03g07140	male sterility protein 2,	Os03g0167600	Similar to Male sterility protein 2.	M3	Os.49681.1.S1_at
LOC_Os06g40880	polygalacturonase precursor	Os06g0611400	Virulence factor, pectin lyase fold family protein.	M3	Os.49993.1.S1_at
LOC_Os04g27950	expressed protein	Os04g0346800	EAR repeat containing protein.	M3	Os.54006.1.S1_at
LOC_Os02g57760	caffeic acid 3-O-methyltransferase	Os02g0823400	Similar to S-adenosyl-L-methionine: beta-alanine N-methyltransfera	M3	Os.54406.1.S1_at
LOC_Os03g40830	subtilisin-like protease precursor	Os03g0605300	Similar to Subtilisin-like protease	M3	Os.54563.1.S1_at
LOC_Os09g27940	spartic proteinase nepenthesin-1 precursor,	Os09g0452800	Peptidase A1, pepsin family protein.	M3	Os.54580.1.S1_at
LOC_Os08g34360	BABY BOOM 1	Os08g0442400	Similar to BABY BOOM.	M3	Os.54786.1.S1_at
LOC_Os04g48400	HOTHEAD precursor	Os04g0573100	Similar to Mandelonitrile lyase-like protein.	M3	Os.54795.1.S1_at
LOC_Os06g40550	ABC transporter-like protein	Os06g0607700	ABC transporter related domain containing protein.	M3	Os.54800.1.S1_at
LOC_Os04g18650	pathogenesis-related genes transcriptional activator PTI5	Os04g0257500	Similar to Transcription factor TSRF1.	M3	Os.55258.1.S1_at
LOC_Os09g10200	SKP1-like protein 1A	Os09g0273800	Fimbriata-associated protein	M3	Os.55370.1.S1_at
LOC_Os03g56974	latent nuclear antigen	Os03g0783100	Conserved hypothetical protein.	M3	Os.57386.1.S1_at
LOC_Os03g55960	calmodulin-like protein 41	Os03g0769500	EF-Hand type domain containing protein.	M3	Os.9049.1.S1_at
LOC_Os01g10760	hypothetical protein	Os01g0204800		M3	OsAffx.11001.1.S1_at
LOC_Os01g45400	RUB1	Os01g0641200	Ubiquitin domain containing protein.	M3	OsAffx.11477.1.S1_x_at
LOC_Os03g02700	HOTHEAD precursor	Os03g0118700	Alcohol oxidase family protein.	M3	OsAffx.12714.1.S1_at
LOC_Os03g02700	HOTHEAD precursor	Os03g0118700	Alcohol oxidase family protein	M3	OsAffx.12714.1.S1_x_at
LOC_Os08g03682	flavonoid 3-monooxygenase	Os08g0131100	Similar to Cytochrome P450.	M3	OsAffx.16813.1.S1_x_at
LOC_Os08g09360	hypothetical protein	Os08g0193000		M3	OsAffx.16914.1.S1_at
LOC_Os08g27210	expressed protein	Os08g0360700	Hypothetical protein.	M3	OsAffx.17184.1.S1_at
No TIGR-loci		No RAP-loci		M3	OsAffx.17435.1.S1_at
LOC_Os08g40440	dihydroflavonol-4-reductase	Os08g0515900	Conserved hypothetical protein.	M3	OsAffx.17435.1.S1_s_at
LOC_Os09g36380	hypothetical protein	Os09g0534300		M3	OsAffx.18011.1.S1_at
LOC_Os11g27030	MATH domain	Os11g0458600		M3	OsAffx.19034.1.S1_at
LOC_Os10g29750	snRNP protein	Os10g0434200	TRAF-like domain containing protein.	M3	OsAffx.20311.1.S1_x_at
LOC_Os01g43940	oligopeptide transporter 4	Os01g0630200		M3	OsAffx.21325.1.S1_at
LOC_Os03g11350	cytokinin-O-glucosyltransferase 3	Os03g0212000	UDP-glucuronosyl/UDP-glucosyltransferase family protein.	M3	OsAffx.2198.4.S1_at
LOC_Os03g61290	ATCHX19	Os03g0828600	Sodium/hydrogen exchanger family protein	M3	OsAffx.22646.1.S1_at
LOC_Os01g65710	hypothetical protein	Os01g0879300		M3	OsAffx.23928.1.S1_at
LOC_Os01g70600	GTP binding protein	Os01g0931700		M3	OsAffx.23990.1.S1_at
LOC_Os02g30060	3-oxoacyl-reductase, chloroplast precursor	Os02g0503500	Similar to 3-oxoacyl-[acyl-carrier-protein] reductase, chloroplast pre	M3	OsAffx.24506.1.S1_at
LOC_Os04g37570	aspartic proteinase nepenthesin-1 precursor	Os04g0448500	Peptidase A1, pepsin family protein.	M3	OsAffx.26364.1.S1_at
LOC_Os07g05150	SKP1-like protein 1A	Os07g0144800		M3	OsAffx.28281.1.S1_at
LOC_Os07g39640	RNA-binding protein 12	Os07g0585200		M3	OsAffx.28823.1.S1_at
LOC_Os07g39640	RNA-binding protein 12	Os07g0585200		M3	OsAffx.28823.1.S1_x_at
LOC_Os08g20200	male sterility protein 2	Os08g0298600	Male sterility C-terminal domain containing protein.	M3	OsAffx.29286.1.S1_at
LOC_Os09g10230	SKP1-like protein 1A	No RAP-loci		M3	OsAffx.29787.1.S1_at
LOC_Os09g16449	ATP-binding cassette transporter CGR1	Os09g0333500	Similar to PDR-like ABC transporter	M3	OsAffx.29871.2.S1_x_at
LOC_Os09g21580	F-box domain containing protein	Os09g0384100		M3	OsAffx.29966.1.S1_at
LOC_Os09g21580	F-box domain containing protein	Os09g0384100		M3	OsAffx.29966.1.S1_x_at
LOC_Os09g22460	F-box domain containing protein	Os09g0392200		M3	OsAffx.29976.1.S1_at
LOC_Os11g10920	carboxyl-terminal peptidase	Os11g0215600		M3	OsAffx.31532.1.S1_at
LOC_Os05g49790	expressed protein	Os05g0573600		M3	OsAffx.4671.1.S1_at
LOC_Os05g49790	expressed protein	Os05g0573600		M3	OsAffx.4671.1.S1_x_at
LOC_Os08g39000	expressed protein	Os08g0499400	Conserved hypothetical protein.	M3	OsAffx.6079.1.S1_at
LOC_Os11g03940	retrotransposon protein, putative, Ty1-copia subclass	Os11g0134100	Rhamnogalacturonate lyase family protein.	M3	OsAffx.7477.1.S1_at

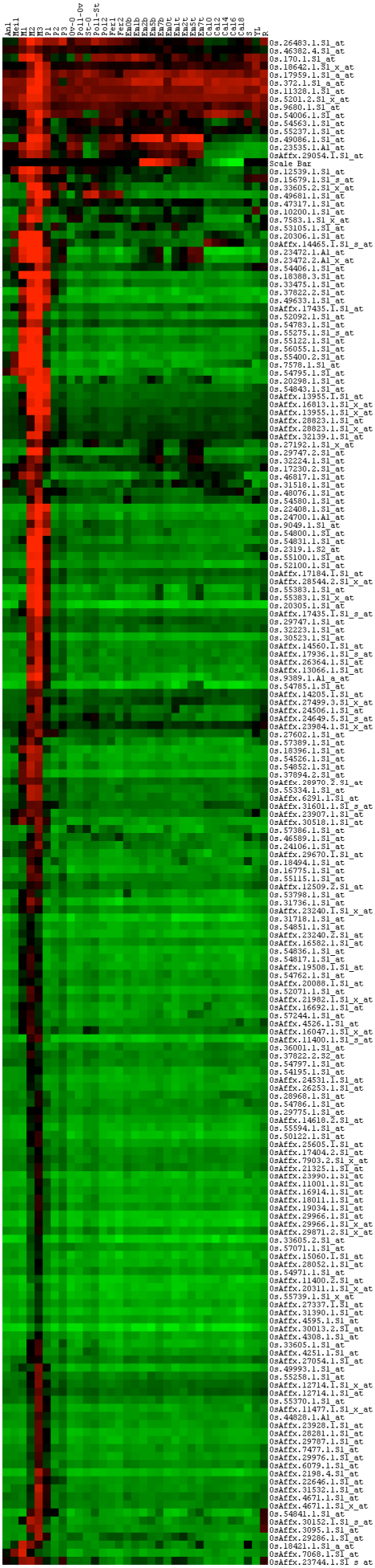
Figure I-2. Heat map view of the specific genes

Heat map of (A) An1-Mei1 specific genes and (B) M1-M3 specific genes. Red indicates higher, while green represents lower expression.

(A)



(B)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M1
M2
M3
P1
P2
P3
Ov-O
Pol1-Ov
Sc-O
Pol1-St
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Pe13
Em0b
Em1b
Em2b
Em5b
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Ca12
Ca14
Ca16
Ca18
St
Tl
R
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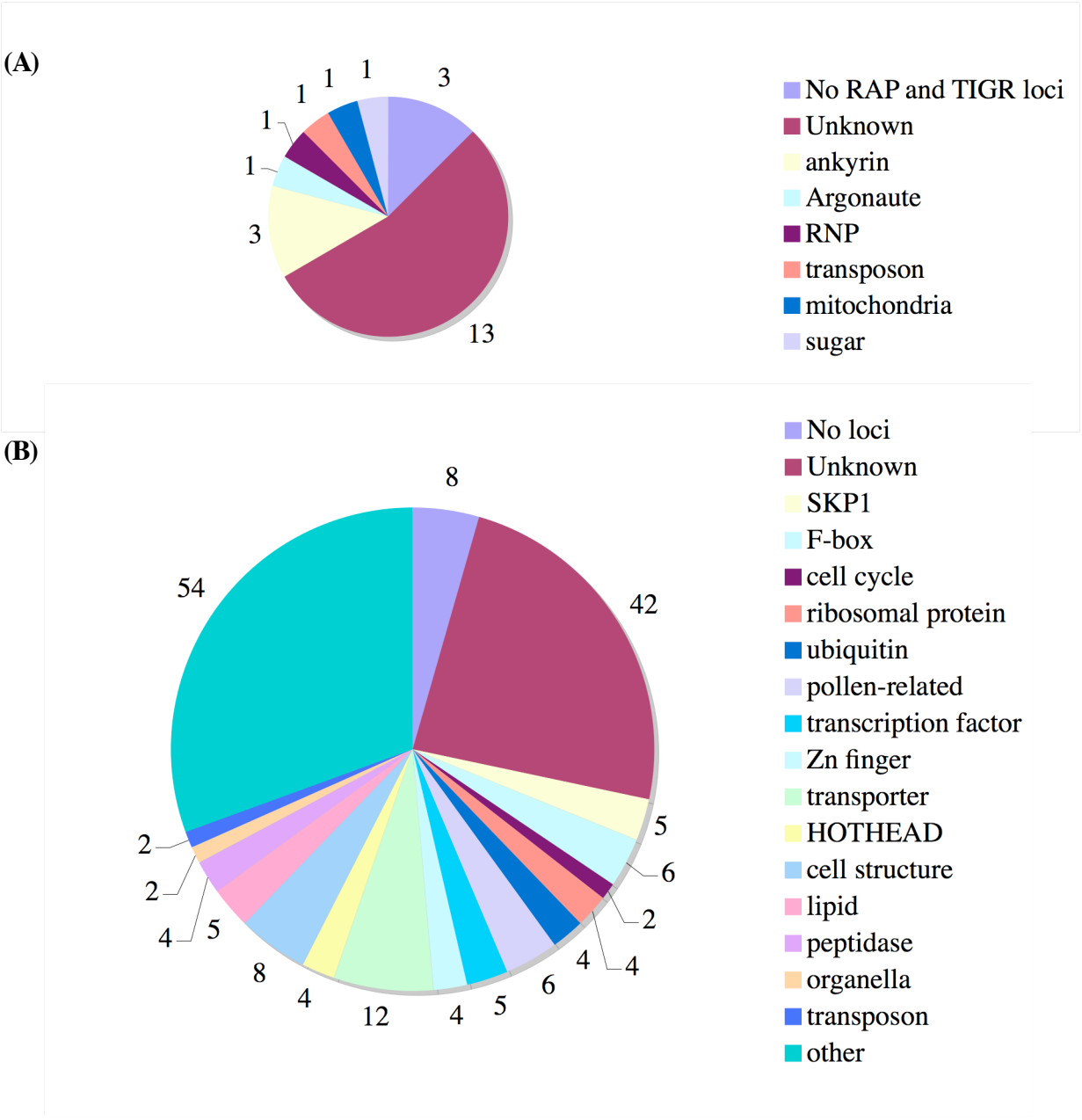


Figure I-3. The pie chart of stage specific genes (A) An1-Mei1 and (B) M1-M3

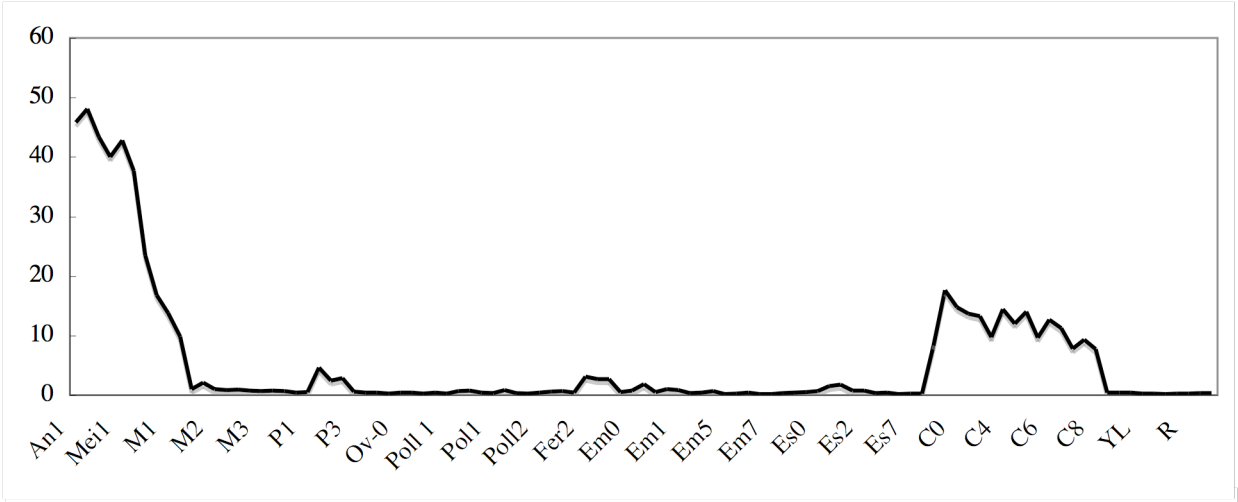
Genes expressed in pre-meiotic stages (A:An1-Mei1) or meiosis (B: M1-M3) were categorized. The number of stage specific genes were indicated.

Table I-4. GO terms of specifically expressed genes in reproductive tissues.

	Stages	An1_Mei1		M1_M3		P1_P3		Pollination _ Fertilization		Embryogenesis		All probe sets
		Number	%	Number	%	Number	%	Number	%	Number	%	Number
	Probe sets	24	0.04	189	0.33	1661	2.89	257	0.45	388	0.68	57381
	TIGR/MSU loci	19	0.05	182	0.50	1306	3.57	223	0.61	317	0.87	36583
BP : Biological Function	response to endogenous stimulus	2	0.06	16	0.45	142	4.03	**37**	**1.05**	**45**	**1.28**	3522
	cellular process	0	0.00	**33**	**1.03**	129	4.02	**32**	**1.00**	32	1.00	3205
	physiological process	0	0.00	11	0.39	103	3.65	**26**	**0.92**	**37**	**1.31**	2819
	signal transduction	0	0.00	9	0.33	**121**	**4.38**	**25**	**0.91**	**35**	**1.27**	2762
	response to stress	3	0.13	**18**	**0.79**	**97**	**4.26**	**24**	**1.05**	**38**	**1.67**	2278
	response to abiotic stimulus	0	0.00	13	0.64	**93**	**4.58**	**19**	**0.94**	**33**	**1.63**	2030
	biological_process	0	0.00	8	0.39	*54*	*2.66*	15	0.74	17	0.84	2028
	protein modification process	0	0.00	*1*	*0.05*	**94**	**4.81**	12	0.61	*9*	*0.46*	1956
	response to biotic stimulus	0	0.00	12	0.66	**84**	**4.62**	**20**	**1.10**	14	0.77	1818
	transcription	0	0.00	8	0.46	*28*	*1.62*	**23**	**1.33**	**36**	**2.08**	1729
	biosynthetic process	0	0.00	**21**	**1.37**	**69**	**4.49**	**20**	**1.30**	**25**	**1.63**	1537
	metabolic process	0	0.00	6	0.41	**68**	**4.70**	12	0.83	11	0.76	1448
	transport	1	0.08	**15**	**1.14**	**83**	**6.28**	10	0.76	7	0.53	1321
	protein metabolic process	0	0.00	**15**	**1.31**	36	3.15	5	0.44	7	0.61	1143
	amino acid and derivative metabolic process	0	0.00	9	0.92	44	4.49	10	1.02	**18**	**1.84**	980
	cellular component organization and biogenesis	0	0.00	2	0.22	**74**	**7.97**	8	0.86	6	0.65	929
	lipid metabolic process	0	0.00	5	0.57	31	3.53	**15**	**1.71**	**17**	**1.93**	879
	secondary metabolic process	0	0.00	**13**	**1.52**	37	4.32	9	1.05	**21**	**2.45**	857
	catabolic process	0	0.00	**11**	**1.44**	35	4.58	3	0.39	*2*	*0.26*	765
	response to external stimulus	0	0.00	7	1.05	**36**	**5.39**	**9**	**1.35**	8	1.20	668
	cell differentiation	0	0.00	4	0.61	*13*	*1.98*	6	0.91	4	0.61	658
	multicellular organismal development	0	0.00	4	0.63	26	4.08	5	0.78	**12**	**1.88**	637
	electron transport	0	0.00	4	0.65	**31**	**5.05**	7	1.14	**13**	**2.12**	614
	carbohydrate metabolic process	0	0.00	6	1.00	**41**	**6.80**	7	1.16	**14**	**2.32**	603
	flower development	0	0.00	3	0.56	*11*	*2.04*	1	0.19	6	1.11	540
	translation	0	0.00	2	0.38	*4*	*0.76*	1	0.19	1	0.19	529
	DNA metabolic process	0	0.00	1	0.19	**33**	**6.31**	3	0.57	2	0.38	523
	nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	0	0.00	1	0.20	*7*	*1.43*	2	0.41	1	0.20	491
	growth	0	0.00	2	0.47	9	2.10	4	0.93	3	0.70	428
	reproduction	0	0.00	**7**	**1.70**	18	4.37	3	0.73	**12**	**2.91**	412
	anatomical structure morphogenesis	0	0.00	1	0.29	*4*	*1.17*	0	0.00	3	0.88	342
	post-embryonic development	0	0.00	0	0.00	10	5.95	1	0.60	3	1.79	168
	cell death	0	0.00	2	1.26	3	1.89	2	1.26	3	1.89	159
	response to extracellular stimulus	0	0.00	0	0.00	**10**	**7.63**	1	0.76	2	1.53	131
	cell cycle	0	0.00	**10**	**8.55**	*0*	*0.00*	1	0.85	0	0.00	117
	generation of precursor metabolites and energy	0	0.00	0	0.00	**8**	**7.55**	0	0.00	3	2.83	106
	cell growth	0	0.00	0	0.00	**10**	**9.90**	0	0.00	0	0.00	101
	pollination	0	0.00	0	0.00	4	4.04	**3**	**3.03**	1	1.01	99
	cellular homeostasis	0	0.00	0	0.00	4	4.44	1	1.11	1	1.11	90
	pollen-pistil interaction	0	0.00	0	0.00	1	1.67	**3**	**5.00**	0	0.00	60
	embryonic development	0	0.00	0	0.00	2	4.17	0	0.00	0	0.00	48
	ripening	0	0.00	0	0.00	1	2.38	1	2.38	1	2.38	42
	photosynthesis	0	0.00	0	0.00	0	0.00	0	0.00	1	3.03	33
	regulation of gene expression, epigenetic	0	0.00	0	0.00	1	5.88	0	0.00	0	0.00	17
	cell communication	0	0.00	0	0.00	**1**	**100.00**	0	0.00	0	0.00	1
CC: Cellular Component	membrane	1	0.02	24	0.58	**198**	**4.83**	**37**	**0.90**	*24*	*0.58*	4103
	mitochondrion	1	0.03	*10*	*0.26*	140	3.58	22	0.56	31	0.79	3906
	nucleus	0	0.00	*7*	*0.20*	**146**	**4.13**	**37**	**1.05**	28	0.79	3533
	cytoplasm	0	0.00	8	0.28	**149**	**5.23**	**26**	**0.91**	24	0.84	2847
	cell wall	2	0.08	**20**	**0.83**	**130**	**5.37**	**30**	**1.24**	24	0.99	2419
	plastid	0	0.00	7	0.58	*29*	*2.38*	6	0.49	8	0.66	1217
	plasma membrane	0	0.00	*0*	*0.00*	34	3.54	**12**	**1.25**	7	0.73	961
	thylakoid	0	0.00	5	0.57	*14*	*1.59*	5	0.57	4	0.45	883
	intracellular	0	0.00	**9**	**1.26**	**44**	**6.16**	4	0.56	5	0.70	714
	nucleolus	0	0.00	3	0.45	**40**	**6.02**	**10**	**1.50**	3	0.45	665
	cytoskeleton	0	0.00	0	0.00	**43**	**10.70**	4	1.00	3	0.75	402
	cell	0	0.00	2	0.67	15	5.02	4	1.34	**9**	**3.01**	299
	cytosol	0	0.00	2	0.69	*3*	*1.04*	1	0.35	3	1.04	289
	ribosome	0	0.00	0	0.00	*2*	*0.69*	0	0.00	0	0.00	289
	extracellular region	0	0.00	2	0.75	**18**	**6.72**	3	1.12	**8**	**2.99**	268
	nuclear envelope	0	0.00	1	0.80	*0*	*0.00*	1	0.80	0	0.00	125
	vacuole	0	0.00	1	0.91	**17**	**15.45**	0	0.00	2	1.82	110
	endoplasmic reticulum	0	0.00	1	1.12	5	5.62	0	0.00	1	1.12	89
	Golgi apparatus	0	0.00	0	0.00	2	2.47	0	0.00	0	0.00	81
	peroxisome	0	0.00	1	1.35	0	0.00	1	1.35	2	2.70	74
	cellular_component	0	0.00	1	1.92	**5**	**9.62**	0	0.00	1	1.92	52
	nucleoplasm	0	0.00	0	0.00	1	2.38	0	0.00	1	2.38	42
	endosome	0	0.00	0	0.00	1	14.29	0	0.00	0	0.00	7
	lysosome	0	0.00	0	0.00	1	20.00	0	0.00	0	0.00	5
	proteinaceous extracellular matrix	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00	4
MF: Molecular Function	hydrolase activity	2	0.06	21	0.66	**193**	**6.08**	**46**	**1.45**	**37**	**1.17**	3172
	catalytic activity	0	0.00	**36**	**1.17**	113	3.69	**33**	**1.08**	34	1.11	3065
	protein binding	**5**	**0.17**	13	0.44	**124**	**4.22**	21	0.72	*14*	*0.48*	2936
	nucleotide binding	2	0.08	*3*	*0.11*	103	3.94	21	0.80	15	0.57	2616
	kinase activity	1	0.04	9	0.36	**113**	**4.56**	20	0.81	*13*	*0.52*	2478
	transcription factor activity	1	0.05	8	0.38	*55*	*2.58*	**27**	**1.27**	**38**	**1.78**	2133
	transferase activity	0	0.00	11	0.52	79	3.73	18	0.85	22	1.04	2120
	binding	0	0.00	9	0.48	**121**	**6.46**	17	0.91	13	0.69	1874
	DNA binding	0	0.00	12	0.76	*27*	*1.71*	15	0.95	**28**	**1.77**	1582
	molecular_function	0	0.00	9	0.58	56	3.64	12	0.78	**23**	**1.49**	1540
	transporter activity	1	0.07	**13**	**0.96**	**91**	**6.72**	13	0.96	10	0.74	1355
	carbohydrate binding	0	0.00	*1*	*0.10*	40	4.10	9	0.92	*3*	*0.31*	975
	nucleic acid binding	0	0.00	2	0.27	23	3.05	5	0.66	3	0.40	753
	structural molecule activity	1	0.14	5	0.71	17	2.42	2	0.28	5	0.71	702
	RNA binding	0	0.00	1	0.18	13	2.36	3	0.54	*0*	*0.00*	551
	receptor activity	0	0.00	1	0.19	13	2.45	6	1.13	2	0.38	530
	oxygen binding	0	0.00	2	0.40	12	2.38	3	0.60	**9**	**1.79**	504
	transcription regulator activity	0	0.00	2	0.45	10	2.24	6	1.35	**8**	**1.79**	446
	signal transducer activity	0	0.00	0	0.00	**30**	**6.91**	3	0.69	7	1.61	434
	lipid binding	1	0.32	**6**	**1.94**	13	4.21	2	0.65	6	1.94	309
	enzyme regulator activity	0	0.00	3	1.54	**23**	**11.79**	1	0.51	2	1.03	195
	translation factor activity, nucleic acid binding	0	0.00	2	1.30	2	1.30	2	1.30	1	0.65	154
	nuclease activity	0	0.00	1	0.72	6	4.35	0	0.00	0	0.00	138
	motor activity	0	0.00	0	0.00	**10**	**8.77**	1	0.88	0	0.00	114
	chromatin binding	0	0.00	0	0.00	3	10.71	0	0.00	1	3.57	28

Nnumber of probe sets that showed stage specific expression pattern and their percentage in all probe sets on the array were summarized in the first row. The second row indicated the number of corresponding TIGR/MSU loci and their percentage in all TIGR/MSU loci with probe sets. BP, CC and MF sections indicated counts of GO terms in each stage, and their percentage in all loci designated with corresponding terms on Affymetrix probes. Enriched and poor terms in each stage (one-tailed binominal test, P<0.05) were shown by bold and italic, respectively.

(A)



(B)

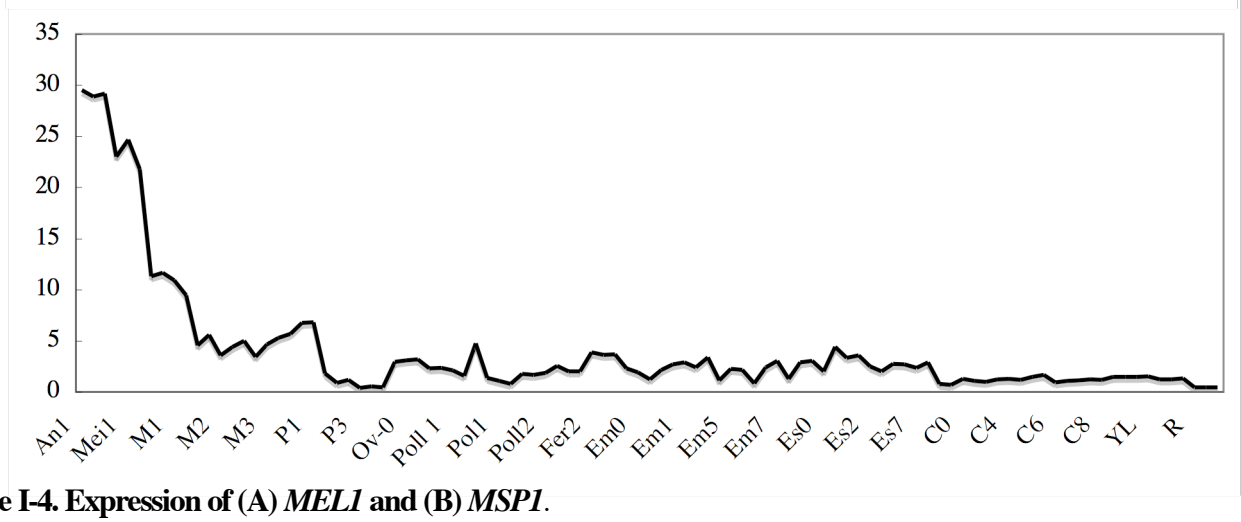


Figure I-4. Expression of (A) *MEL1* and (B) *MSP1*.

Vertical axis indicated normalized signal values in the microarray atlas. All 98 samples of our microarray experiment were displayed on horizontal axis from left to right according to the top to bottom stages in Table I-1, respectively.

Table I-5 Rice meiosis-related genes reported to date and shown their expression profiles

RAP_loci	TIGR_loci	probe-Affymetrix	Stage specificity		Reference
Os03g0800200	LOC_Os03g58600	Os.40026.1.S1_at	An1-M1	*MEL1*	Nonomura et al. 2007
Os01g0917500	LOC_Os01g68870	Os.23868.1.S1_at	An1-M1	*MSP1*	Nonomura et al. 2003
Os12g0143800	LOC_Os12g04980	Os.13051.1.S1_at	An1-M1	*OsDMC1*	Kathiresan et al. 2001
Os12g0472500	LOC_Os12g28750	Os.55667.1.S1_at	An1-M1	*OsTDL1A*	Zhao et al. 2008
Os07g0549600	LOC_Os07g36460	OsAffx.28779.1.S1_at	An1-M1	*UDT1*	Jung et al. 2005
Os08g0509600	LOC_Os08g3890	Os.6636.1.S1_at	An1-M1	*OsSPL14*	Xie et al. 2006
Os05g0580500	LOC_Os05g50410	Os.33045.1.S1_a_at	ubiquitous	*OsRad21-4*	Zhang et al. 2006
Os09g0553200	LOC_Os09g38030	Os.10118.1.S1_at	ubiquitous	*UGP1*	Chen et al. 2007
Os09g0506800	LOC_Os09g32930	Os.49778.1.S1_at	ubiquitous	*PAIR2*	Nonomura et al. 2006
Os03g0106300	LOC_Os03g01590	Os.49756.1.S1_at	ubiquitous	*PAIR1*	Nonomura et al. 2004

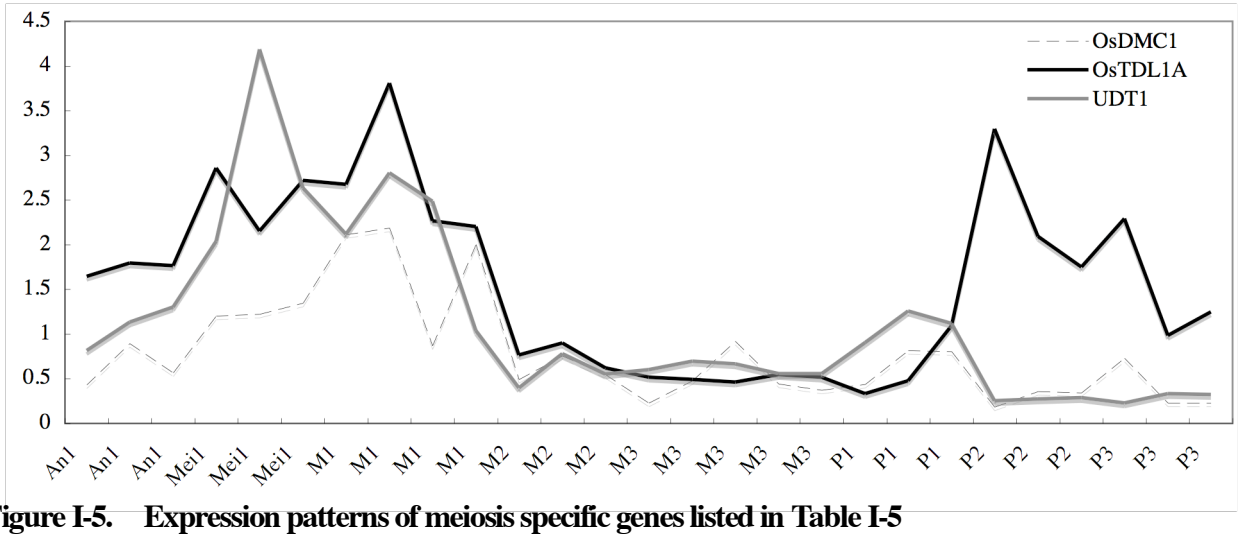


Figure I-5. Expression patterns of meiosis specific genes listed in Table I-5

The expression patterns of genes that show high expression levels at An1-M1 stages (listed up in Table I-5) were indicated. Vertical axis indicated normalized signal values in the microarray atlas. All 98 samples of our microarray experiment were displayed on horizontal axis from left to right according to the top to bottom stages in Table I-1, respectively.

Section II

Spatial and temporal expression of *MEL1*, *MSP1* and *OsNZZ* and their relationship

Introduction

In the life cycle of plants, the first event to switch their developmental phase from vegetative to reproductive is to form the inflorescent meristem from the shoot apical meristem. The inflorescent meristem of rice consecutively produces panicle axis and several dimension of panicle branches. At the end of each branch, the floral meristem, qualitatively converted from the inflorescent meristem, begins to form flower organs. Flowers of eudicots are organized into four concentric whorls of organs (sepals, petals, stamens, and carpels) that arise sequentially from the floral meristem. The third whorl in flowers differentiates six stamens and the fourth whorl differentiates a carpels. Current research in floral evolution and floral development has proposed its underlying framework 'the ABC model' of floral organ identity (Coen and Meyerowitz 1991). The ABC model posited that floral organ identity is controlled by three groups of genes with A, B and C functions; A in whorls 1 and 2, B in whorls 2 and 3, and C in whorls 3 and 4. They act in combination; A-function alone specifies sepal identity, A- and B-functions together control petal identity; B- and C-functions together control the identity of stamen, the male reproductive organ; C-function alone specifies the identity of carpel, the female reproductive organ. The archesporial cell, the primordial plant germ cell, initiates at hypodermal cell layer, the L2 layer, of primordia of male and female reproductive organs (Goldberg et al. 1993). Several genes that control the early stages of germ cells development have been identified through mutant analyses. However, the molecular network controlling these processes is yet poorly understood.

In *Arabidopsis*, a number of genes that function in specification of the male or female germ cells and meiosis have been identified. *Arabidopsis SPOROCYTELESS* (*SPL*) /*NOZZLE* (*NZZ*) is important for early germ cell differentiation (Schiefthaler et al. 1999, Yang et al. 1999). In *spl*/*nzz* mutant anthers, the formation of archesporial cells seems normal but subsequent cell division is defective, resulting in the absence of sporogenous cells and tapetum, and cause sterility. The *SPL*/*NZZ* mainly expresses at sporogenous cells, suggesting that the formation of the anther walls may be

dependent on signals from the developing microsporocytes. The *SPL/NZZ* encodes a novel transcription factor, suggesting that it may regulate gene expression during early sporogenesis in the anther. Ito et al. (2004) show that the *AGAMOUS* (*AG*) defined as a C-function protein binds to the regulatory region of a transcription of the *SPL/NZZ* gene, and promotes the sporogenous cell formation. This is a first case to prove that the *SPL/NZZ* pathway required for germ cell development is located at a downstream of the flower organ development. However, Schiefthaler et al. (1999) and Yang et al. (1999) reported that monocots lack the homolog of the *SPL/NZZ*.

The rice genes, *MULTIPLE SPOROCYTES 1* (*MSP1*) and *MEIOSIS ARRESTED AT LEPTOTENE 1* (*MEL1*), are essential for early anther development. The *msp1* mutation gives rise to an excessive number of both male and female sporocytes, resulting in complete male sterility and partial female sterility. The MSP1 protein has several distinct domains: a signal peptide, a putative leucine zipper motif, an LRR domain, a putative receptor domain composed of 32 leucine-rich repeats, a transmembrane domain, and a cytoplasmic serine/threonine protein kinase domain, suggesting a possibility that the MSP1 plays an important role in transferring an extracellular signal into the cytoplasm through the plasma membrane (Nonomura et al. 2003). The *OsTDL1A*, the rice ortholog of *Arabidopsis TPD1*, was recently identified as a putative ligand of the *MSP1* receptor kinase (Zhao et al. 2008). The *Arabidopsis TPD1* is thought to express mainly in sporogenous cells, and to be received by its receptor *AtEMS1* at the plasma membrane of sporogenous cells. These results also indicate that normal development of sporogenous cells is required for tapetum development.

The *mel1* mutant shows abnormalities in morphogenesis of pollen mother cells and meiosis progression at early stage. The *MEL1* gene encodes a protein of ARGONAUTE (AGO) family, defined by the presence of two conserved regions, the PAZ and PIWI domains (Nonomura et al. 2007). AGO proteins are integral players in small RNA-directed regulatory pathways and reported to function during miRNA- and siRNA- mediated regulation of development and stress responses, siRNA-mediated antiviral immune response, and siRNA-mediated regulation of

chromatin structure and transposons (reviewed by Hutvagner et al. 2008, Vaucheret 2008, Mallory et al. 2008). Phenotype of the *MEL1* mutant is only observed in male and female reproductive organs, in which meiosis is arrested at early stages. Expression of the *MEL1*mRNA is limited in reproductive organs, thus the MEL may acts as reproductive-cell specific argonaute protein (Nonomura et al. 2007).

The *MEL1* mRNA expression is already known to begin just after the archesporial cell initiation, which is quite earlier than differentiation of archespores into sporogenous and parietal cells (for details, see General Introduction). In this study, to confirm the spatial and temporal expression of the *MEL1* gene, mRNA *in situ* hybridization was performed on the anther sections with the *MSP1* gene as a cellular marker of young anther tissues.

In addition to the *MEL1* and the *MSP1*, a putative rice homolog of *Arabidopsis SPL/NZZ*, named *OsNOZZLE* (*NZZ*), and detected by a BLAST search (Figure II-1) in this study, was also provided for *in situ* expression analysis to compare the expression patterns of *Arabidopsis SPL/NZZ* and detect the relationship to that of the *MEL1* and the *MSP1*.

Methods

Oltholog search for Arabidopsis SPL/NZZ

The amino acid sequence of *Arabidopsis SPL/NZZ* gene (At4g27330) was used for blast search against rice cDNA, EST and genome sequences using DDBJ BLASTX search system. Resultant sequences were compared by full amino acid sequence aligment

Paraffin sections of the tissue

Observation of flower development was performed on standard paraffin sections as described by Hong et al. (1995). Various length of young panicles or flowers were fixed with 45 % ethanol, 15 % formaldehyde 5% acetic acid for 20 h at 4℃. The samples were substituted with xylene, embedded in Paraplast Plus. Microtome sections, 7 μm thick, were applied to glass slides coated with MAS (Matsunami glass industry, Tokyo, Japan).

In situ hybridization

The DNA fragment containing a sequence of the target gene was amplified by PCR, cloned into a pCRII-TOPO dual promoter system (Invitrogen) and transcribed in vitro from the T7 or SP6 promoter with RNA polymerases using the DIG RNA labeling kit (Roche Applied Science), and this mixture was prepared for hybridization. For all probes, cDNA fragments were amplified by PCR using primers specific to each gene as follows; 5'-CATTGTCTCAAGCAGAGTTAAGGC -3' and 5'-CCTGAAATCACCAAATACCG-3' for *MEL1*, 5'-ATCTCCAGGTTTTTAGGCTTTACG-3' and 5'-CTAGCAGGATGAAAAGCCAGAAAC-3' for *MSP1*. 5'-AGAGATGGTGCAGGAGCACG-3' and 5'-AGAAAGCAACAGGACAAGTTC-3' for *OsNZZ*.

In situ hybridization and immunological detection of the hybridization signals were performed as described by Kouchi

and Hata (1993). Tissue sections were deparaffinized in xylene, dehydrated through a graded ethanol series, and air-dried. The sections were incubated with 5 μg/mL proteinase K, 100 mM Tris-HCl, pH 7.5, and 50 mM EDTA at 37℃ for 30 min. Then, they were refixed with 4% paraformaldehyde at room temperature for 10 min, and incubated with 0.1 M triethanolamine and 0.5% acetic anhydride at room temperature for 10 min. Then, they were rinsed twice with double-distilled water for 5 min after each treatment. The sections were washed twice in 2x SSPE (1x SSPE is 0.115 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.4) for 5 min, dehydrated through a graded ethanol series, and hybridized with RNA probes in 50% deionized formamide, 0.3 M NaCl, 10% dextran sulfate, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM DTT, and 500 ng/μL poly(A) RNA (Wako Pure Chemical, Osaka, Japan) at 50℃ for 16 h. The sections were washed with 4x SSC (1x SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 50℃ for 5 min each and treated with 10 mg/mL RNase A at 37℃ for 30 min. They were then rinsed twice with 0.5x SSC at 50℃ for 20 min, rinsed twice with buffer 1 (150 mM NaCl and 100 mM Tris-HCl, pH 7.5) at room temperature for 5 min each, and incubated in blocking reagent of 5 mg / mL bovine serum (Wako Pure Chemical). Immunological detection of the hybridized probes was performed with anti-digoxigenin alkaline phosphatase (Roche) according to the manufacturer's recommendations. The sections were washed three times with buffer 1 for 10 min each, washed with buffer 3 (100 mM NaCl, 50 mM MgCl2, and 100 mM Tris-HCl, pH 9.5) at room temperature for 5 min, and incubated with 0.34 mg/mL nitroblue tetrazolium salt and 0.175 mg/mL 5-bromo-4-chloro-3-indolyl phosphate in buffer 3 for 12h. They were rinsed with double-distilled water, dehydrated through a graded ethanol series, mounted in Eukitt (O. Kindler, Freiburg, Germany), and photographed using a differential interference contrast microscope and a DP50 system (Olympus). A contrast of images was enhanced with Photoshop 7.0 (Adobe, Mountain View, CA).

Results and Discussion

The sporogenous cells subsequently grow into the sporocyte and undergo meiosis, and the parietal cells divide periclinally, form three-layered inner anther walls, inner-most of which differentiates into nursery cells, so called tapetum cells (See General Introduction). It is known that both the sporogenous cells and the parietal cells are derived from the archesporial cells, the plant primordial germ cells (reviewed by Goldberg et al. 1993).

Just after the completion of flower organ development, the *MEL1* mRNA signal first appeared within the anther and ovule primordia (Figure II-2A, Nonomura et al. 2007). This region corresponded to the hypodermal L2 layer, which is thought to generate the archesporial cells (Dawe and Freeling, 1990; Dellaporta et al. 1991). In contrast, no *MSP1* signal was detected in the reproductive organs in this stage (Figure II-2E). The *MSP1* is known to express after the sporogenous cells and the parietal cells, both of which differentiate from the archesporial cells (Nonomura et al. 2003). In this study, earlier expression of the *MEL1* than the expression of the *MSP1* and the *MEL1* mRNA expression started at the archesporial initials were clearly indicated. In the *MEL1* mutant, the primordial germ-cell initiation occurs normally, because the *MEL1* mutant germ cells indicate several critical features of the germ cells, such as quite larger volume of the cytoplasm and nucleus than that the somatic cells (Nonomura et al. 2007). Taken together with the result of this study, the *MEL1* expression is required not for the germ-cell initiation, but for the promotion of germ cell development and/or the maintenance of germ cells. After the archesporial cells differentiating into sporogenous cells and parietal cells, the *MEL1* signals were detected in sprogenous cells, but not detected in inner anther walls (Figure II-2B). In this stage, the *MSP1* also exhibited the strong signal in the inner anther walls (Figure II-2F). The *MEL1* signal disappeared before the pollen mother cells enter meiosis (Figure II-2D), whereas the *MSP1* expression was still observed in this stage (Figure II-2H). These results together with the previous study (Nonomura et al. 2007) indicate that *MEL1* mRNA expression is required for the maintenance and/or development of primordial plant germ cells,

sporogenous cells and sporocytes.

In this study, *in situ* hybridization of *OsNOZZLE* (*OsNZZ*), a putative rice ortholog of *Arabidopsis SPOLOCYTELESS/NOZZLE* was also performed. The *Arabidopsis SPL/NZZ* encodes a putative transcription factor carrying the MADS box-like sequence in the middle and the Myc-type helix-loop-helix dimerization domain in the carboxyl terminus (Yang et al. 1999). Search for a ortholog gene in the rice genome sequence identified one candidate as shown in Figure II-1, though similarity was low even at the amino acid level. This ortholog gene was designated as *OsNZZ*. Expression profile of this gene showed early stage expression in anther but also in other stages (Figure II-1).

Different from the *MEL1* or the *MSP1*, the *OsNZZ* mRNA was expressed both in developing anther wall layers and sporogenous cells (Figure II-2K), similar to that of *Arabidopsis SPL/NZZ* (Yang et al. 1999), but they were not detected in archesporial cells (Figure II-2I). Although their function is not the same as *Arabidopsis SPL/NZZ*, *OsNZZ* may have function in early germ cell development. I tried to seek the T-DNA- or Tos17-tagged mutant of *OsNZZ* genes on genome resource databases of rice, but unfortunately they were not registered in the public databases. Thus in the following experiments, I decided to focus on the functional analysis of the *MEL1* argonaute.

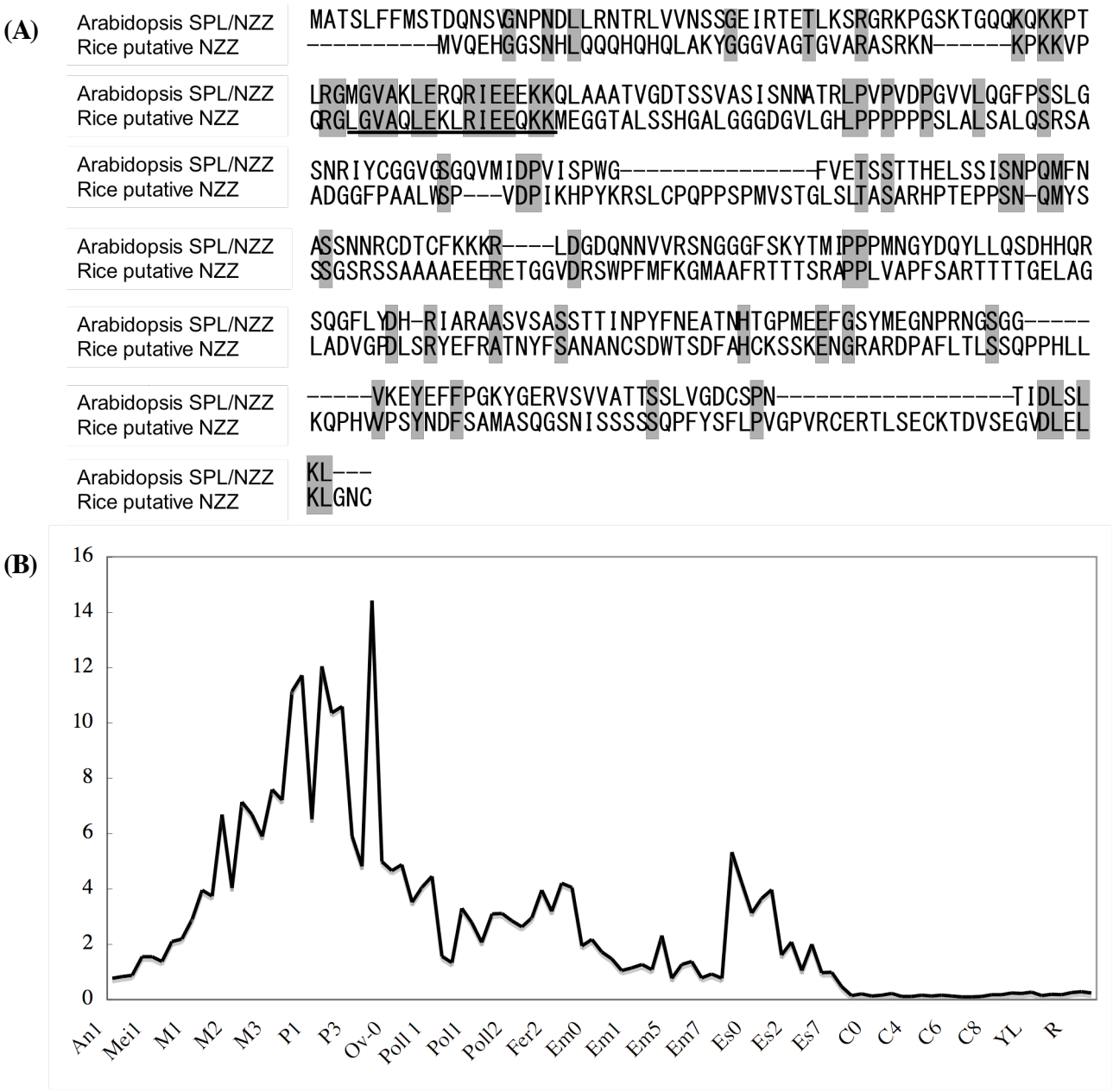


Figure II-1. Alignment of *Arabidopsis SPL/NZZ* and putative Rice *OsNZZ* and expression of *OsNZZ*

(A) Amino acid sequences of *Arabidopsis SPL/NZZ* and rice putative *NOZZLE* are shown. Sequences were aligned with the CLUSTAL W program (http://www.ddbj.nig.ac.jp). Identical amino acids conserved between two proteins are highlighted with grey boxes. Underlined sequence represents the putative MADS box signature. (B) Expression analysis of *OsNZZ* using expression profiles from the inhouse DB 'Rice Expression Atlas'. Vertical axis indicated normalized signal values in the expression atlas. All 98 samples of our microarray experiment were displayed on the horizontal axis from left to right according to the stages from top to bottom stages in Table I- 1, respectively.

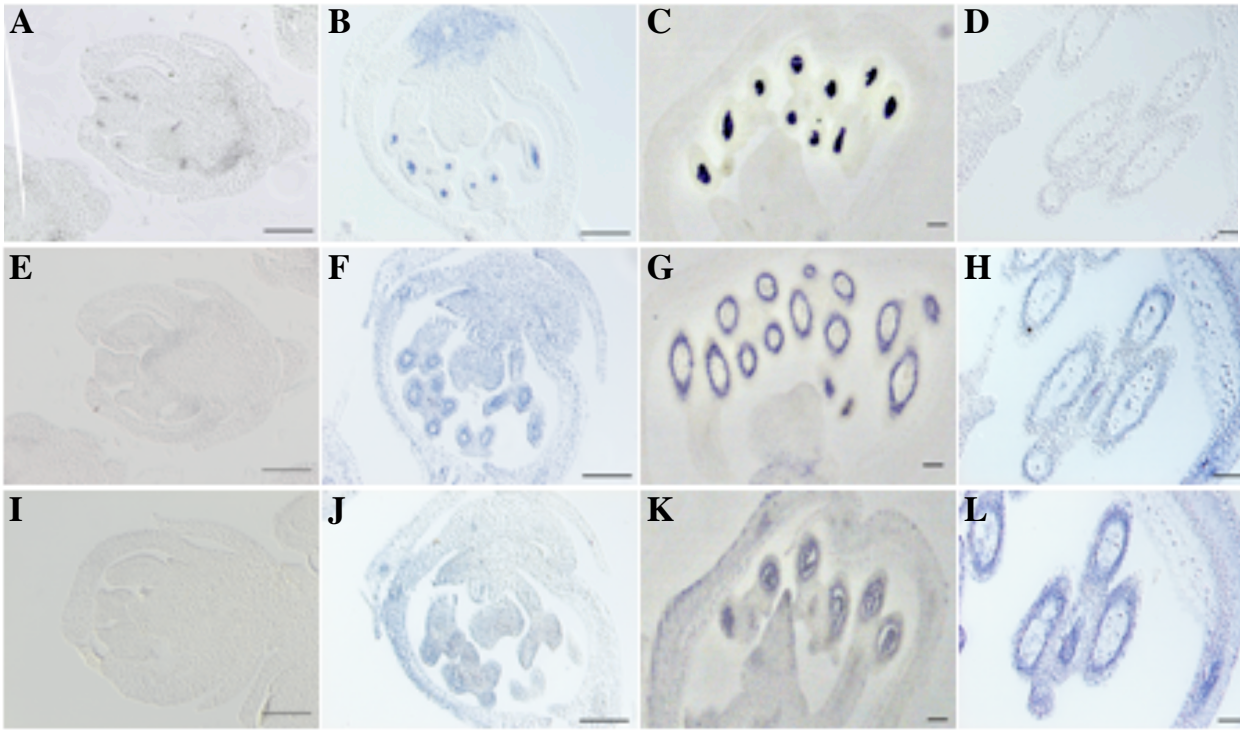


Figure II-2. Spatial and temporal expression of *MEL1*, *MSP1* and *OsNZZ*.

mRNA *in situ* hybridization of *MEL1* (A-D), *MSP1* (E-H), and *OsNZZ* (I-L). Three vertical photos (e.g. A, E, and I) were taken by using neighboring three serial sections derived from a same flower or anther. (A) The *MEL1* mRNA signal first appeared within the anther and ovule primordia, whereas no the *MSP1* and the *OsNZZ* signal was detected (E, I). (B) When the archesporial cells differentiating into sporogenous cells and parietal cells, the *MEL1* signals were detected in sprogenous cells. In this stage, the *MSP1* and the *OsNZZ* signal also began to appear at the hypodermal cells surrounding sporogenous cells (F, J). (C) When the anther wall was three-layered, the *MEL1* signal clearly marked only sporogenous cells. At this stage, the strongest signal of the *MSP1* was observed on the innermost layer attached to the sporogenous cells (G). On the other hand, the *OsNZZ* mRNA was expressed both in developing anther wall layers and sporogenous cells (K). (D) The *MEL1* signal was absent, before the pollen mother cells enter meiosis. The *MSP1* expression was still observed in this stage (H). At this stage, the *OsNZZ* was detected only in tapetum (L). Bars 100 μm.

Section III

Microarray analysis of *mel1-1* mutant

Introduction

The plant system to generate germ cells is quite different from that of animals and insects. One of representative differences is that animals and insects maintain the germline stem cell (GSC) and can continuously produce germline cells (reviewed by Morrison and Spradling, 2008). The plants also maintain stem cells at a shoot apex and root tip. These proliferative tissues, termed meristem, continuously generate new cells to form lateral organs such as leaves, stems and roots. After the growth phase shifted to reproductive, the meristem becomes to generate inflorescent and floral organs continuously (reviewed by Birnbaum and Alvarado 2008). However, when the primordial plant germ cell (PGC) initiates within the male and female reproductive organs, the floral meristem have already been terminated. To achieve a perennial reproduction, substitute for a self-renewal of GSCs, most of perennial plants produce new reproductive organs and germ cells at the tip of newly branched meristems as lateral organs.

It is known that the maintenance of the GSC identity requires specialized somatic cells adhered to the GSCs to regulate their microenvironment, termed the stem cell niche (Morrison et al. 1997, Spradling et al. 2001). For example, in the male germarium of Drosophila, a direct contact of the GSCs to terminal somatic cells, called hub cells, is essential to maintain the GSC identity (Tulina et al. 2001, Kiger et al. 2001). From the results of recent researches, the self-renewal ability of the GSC requires expression and accumulation of the germline specific ARGONAUTE protein Piwi (P-element induced wimpy testis) within the nuclei of hub cells (Cox et al. 1998). Another germline specific AGO of *Drosophila*, Aubergine, expresses within the cytoplasm of the GSC and its derivative germline cells (Findley et al. 2003). The orthologs of the *Drosophila* Piwi are generally found in other animals; for example, *Miwi*, *Miwi2*, and *Milli* in mouse (Kuramochi-Miyagawa et al. 2001, Carmell et al. 2007), *Ziwi* in zebrafish (Houwing et al. 2007), *PRG1* and *PRG2* in *C. elegans* (Batista et al. 2008, Das et al. 2008), and so on. All of these Piwi orthologs conduct the maintenance of self-renewality of GSCs and/or normal development germline cells.

The plant mechanism to control the size of the meristematic cell population is very similar to the mechanism to maintain the GSC in animals and insects (reviewed by Dinneny and Benfey, 2008).

In plant, shoot apical meristem (SAM) development is regulated by coordinate interactions of two major groups of interdependent signaling events, in which one group of signals maintaining stem cell identity is balanced with the other group of signals governing the initiation of lateral organs from the flanks of the SAM (Williams and Fletcher, 2005, Byrne 2006). Such signals from the adaxial domains of lateral organs are important for SAM formation (Eshed et al. 2001). Several key regulators, including class III homeodomain-leucine zipper (*HD-ZIP III*) transcription factors and miR165/166, mediate the signaling crosstalk (Kerstetter et al. 2001). In *Arabidopsis*, two *HD-ZIP III* genes, *PHABULOSA (PHB)* and *PHAVOLUTA (PHV)* control the establishment of adaxial (upper surface)–abaxial (lower surface) polarity, resulting in structurally and functionally specialized leaf surfaces. The *PHV* and *PHB* genes are normally expressed in leaf primordia cells near the shoot meristem to specify adaxial cell fate, and are turned off in zones that develop into abaxial cells at positions distant from the meristem, whereas in the abaxial side of the primordium, mRNA cleavage guided by miRNAs 165/166 prevents expression of *HD-ZIPIII* (Carrington et al. 2003). The function and expression pattern of the miRNAs that target *HD-ZIP III* were also shown to depend on *AGO1* (Kinder et al. 2004). In maize, adaxial/abaxial leaf polarity is established by an abaxial gradient of miR166, which spatially restricts the expression domain of HD-ZIPIII (Juarez et al. 2004). Specification of adaxial/abaxial organ polarity in maize also requires the activity of *leafbladeless1* (*lbl1*)(Timmermans et al. 1998). Nogueira et al (2007) reported that *lbl1* encodes a homolog of *SUPPRESSOR-OF-GENE-SILENCING3* (*SGS3*), which is specifically required for the biogenesis of trans-acting small interfering RNAs (ta-siRNAs), and *lbl1* acts on the adaxial side of developing leaves and demarcates the domains of HD-ZIP III and miR166 accumulation.

However, it is unknown whether AGO proteins have some roles in an initiation and early development of plant PGCs or not, in which the plant meristem have already been terminated.

ARGONAUTE (AGO) proteins are key players in all known small RNA-directed regulatory pathways. Eukaryotes produce numerous types of small RNAs, such as microRNAs (miRNA), small interfering RNAs (siRNA), PIWI-interacting RNAs (piRNAs) and 21U RNAs, and these RNA species associate with different types of AGO family members, such as AGO, PIWI. The plant model species *Arabidopsis* contains ten AGO proteins. Among them, *AGO1*, *AGO7*, and *AGO10* have been implicated in miRNA-guided gene repression (reviewed by Mallory et al. 2008). AGO7/ZIPPY is related to the ta-siRNAmediated regulatory mechanism (Peragine et al. 2004). In Rice, *SHOOTLESS2* (*SHL2*), *SHL4*/*SHOOT ORGANIZATION2* (*SHO2*), and *SHOOT ORGANIZATION1* (*SHO1*) encode orthologues of *Arabidopsis RNA-dependent RNA polymerase 6*, *AGO7*, and *DICER-like 4*, respectively, whose mutations affect leaf development through the trans-acting siRNA (ta-siRNA) pathway. Mutations in rice *SHL2*, *SHL4*/*SHO2*, and *SHO1* cause complete deletion or abnormal formation of the SAM (Nagasaki et al. 2007). Recently, Kapoor et al. showed a complete analysis of rice Dicer-like, Argonaute and RDR gene families including gene structure, genomic localization and phylogenetic relatedness. They also presented microarray-based expression profiling of these genes during 14 stages of reproductive and 5 stages of vegetative development and in response to cold, salt and dehydration stress (Kapoor et al. 2008). However, little is known about rice argonautes and their molecular function.

The rice gene, *MEIOSIS ARRESTED AT LEPTOTENE1* (*MEL1*), is reported to be essential for early germ cell development (Nonomura et al. 2007). Expression of the *MEL1* is limited in germ cells before meiosis and the *mel1* mutant shows abnormal shaped pollen mother cells and the mutant meiocytes are arrested at early stage(s).

The *MEL* gene encodes a protein of ARGONAUTE (AGO) family. The Argonaute family members are grouped into two subfamilies, PIWI and AGO (Sasaki et al. 2003). Between two subgroups, PIWI subfamily members are expressed only in germ cells and they are associated with piwi-interacting RNAs (piRNA) (reviewed by O'Donnell et al. 2007). The rice genome (*cv* Nipponbare) contains 18 AGO family members, including MEL1. Phylogenetic

analysis using the PAZ and PIWI domains for rice, *Arabidopsis*, *C. elegans*, *Drosophila melanogaster*, and mouse revealed that all plant AGOs were in the same branch that included several animal and yeast AGO members but were phylogenetically distinguished from the PIWI subfamily (Nonomura et al. 2007).

However, spatial and temporal expression of the *MEL1* in Section 2 revealed that the *MEL1* mRNA expressed specifically in primordial germ cells and sporocytes, but never in nursery cells. The result suggests that the MEL1 may acts as germ cell-specific Argonaute protein, similar to *Drosophila* PIWI, mouse MIWI and MILI, Zebrafish ZIWI and *C.elegans* PRG1 (Saito et al. 2006, Brennecke et al. 2007, Gunawardane et al. 2007, Grivna et al. 2006, Howing et al. 2007, Batista PJ et al. 2008, Das et al. 2008). This phylogenic analysis also suggests a possibility that the plant reproduction system which renounces to maintain self-renewal of GSCs does not require the Piwi-subfamily proteins.

In this study, to investigate the genes regulated by the MEL1 during sexual reproduction, microarray analyses were performed using 1cm young panicle and 0.4 mm anthers. To make the expression profiles more reliable, Rice Genome GeneChip Array (Affymetrix) and rice 44K oligo microarray (Agilent Technology) were used. From these expression profiles, the molecular functions and the candidate of target of the MEL1 will be discussed.

Materials and Methods

Plant materials

The gene expression profile at the initiation and early developing stage of germ cells was compared between the *mel1* mutant and the wild-type rice plant (*O. sativa L. ssp. japonica cv.* Nipponbare). The *mel1-1* mutation was induced by the insertion of *Tos17*, the endogenous retrotransposon of rice, into the *MEL1* coding region (Nonomura et al. 2007). In this study, the *mel1-1* mutant plants in the F2 generation after the four-times backcross with cv. Nipponbare were used. Both wild-type and mutant plants were grown in the paddy field in summer at Mishima, Japan.

Total RNA isolation

In this study, 1 cm panicle and 0.4 mm developing anthers were provided for RNA extraction. For the 0.4-mm anthers, to determine the developing stage precisely, one of six anthers from each floret was used for acetic carmine staining and microscopic observation of meiotic stages. The total RNA from 1 cm panicle and 0.4 mm anthers of three-independent wild-type and three-independent *mel1-1* plants was isolated by using the RNeasy plant mini kit (Qiagen, Valencia, CA, USA) according to the manufacture's instruction. To avoid the induction of responsible genes for wounding stress by picking with forceps, the isolated panicles were immediately frozen within the liquid nitrogen and stored at -80℃. A sample quality was assessed using the Agilent 2100 Bio analyzer and the Nano LabChip Kit (Agilent Technologies).

Microarray analysis

Rice Genome GeneChip Array contains approximately 48,564 japonica transcripts and 1,260 transcripts representing the indica caltivar. The Affymetrix protocol for one-cycle eukaryotic target preparation to prepare rice

probe was as follows; 1 μg of total RNA was converted into single-stranded cDNA using SuperScript II and a T7-Oligo (dT) primer (Affymetrix). Second-strand cDNA was synthesized using dNTPs, second-strand reaction buffer, Escherichia coli DNA ligase, E. coli DNA polymerase I, and E. coli RNase H (all reagents were supplied by Affymetrix). The one-cycle cDNA synthesis was followed by a cleanup using Affymetrix GeneChip sample cleanup modules. Biotin-labeled cRNA was prepared using an Affymetrix in vitro transcription (IVT) labeling kit. After cleanup of the IVT products, the purified cRNA was fragmented to a size ranging from 35 to 200 bases using a fragmentation buffer at 94℃ for 35 min. The fragmentation of the labeled cRNA was confirmed before hybridization by running samples on the eGENE. 5 μg of the fragmented cRNA was mixed into a hybridization cocktail containing a hybridization buffer, B2 oligo control RNA (Affymetrix), herring sperm DNA, and BSA (Invitrogen, Carlsbad, CA). The solution was hybridized to a GeneChip at 45℃ for 16 h with a gentle rotation at 60 rpm. After the hybridization, the cocktail was removed from the GeneChip. The hybridized GeneChip was washed with two wash buffer provided by Affymetrix and stained with streptavidine–phycoerythrin (Molecular Probes) using the Affymetrix Fluidics 450 wash station (Affymetrix Fluidics Protocol EUKGE_WS2v5). The GeneChips were immediately scanned with a GeneChip scanner 3000. Individual scans were quality checked for the presence of control genes and background signal values. Each microarray experiment was biologically replicated three-times using three-independent samples from wild-type or *mel1-1* panicles.

To make the microarray data more reliable, the rice 44K oligo microarray (Agilent Technologies, Palo Alto, CA, USA) was also used for gene expression profiling of the *mel1-1* mutant. The Agilent rice microarray chip contains 42, 000 probes composed of 60-bp oligonucleotides, which were synthesized on the basis of the public rice genome sequence database (Sasaki et al. 2002), the full-length rice cDNA database (Kikuchi et al. 2003) and the Rice Annotation Project (RAP) database (http://rapdb.dna.affrc.go.jp/). The method to extract total RNA used for the

Agilent microarray analysis was completely same with that for the Affymetrix analysis described above. Agilent oligonucleotide technology uses one-color analysis, in which the total RNA extracted were labeled by the cyanine-3-CTP (CTP-Cy3) (Agilent Technologies). 100 ng of total RNA a reaction were synthesized from in two steps as follows; in the first step, double stranded cDNA was synthesized with mouse Moloney Murine Leukemia Virus (MMLV) (Agilent Technologies) reverse transcriptase and an oligo (dT) T7 RNA polymerase promoter (Agilent Technologies). In the second step, the antisense cRNAs that were labeled by the CTP-Cy3 using the Agilent's Fluorescent Linear Amplification kit. Labeled cRNAs were purified using Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA), and fragmented to an average size by heating the samples at 60℃ for 30 min in a fragmentation buffer provided by Agilent. Hybridization was performed on rice-genome 44K oligonucleotide microarrays (Agilent) with reagents and protocols provided by the manufacturer at 65℃ for 17 h. After hybridization, the hybridized slides were washed using a commercial kit package (Agilent Technologies) and then scanned using Laser Scanner (Agilent Technologies).

Data analysis

For Affymetrix experiment, the data in the CEL files produced by GCOS 1.3 were extracted using statistical analysis software R 2.4.0 with the Bioconductor package 'affy'. Probe level data from CEL files were extracted with the function 'ReadAffy' in the package. The probe level data were processed with the function 'expresso' to obtain expression values for individual genes. In the data procession, the methods 'mas' was applied for background correction, the 'pmonly' for probe specific background correction, and again the 'mas' for summarization. The processed expression values for individual genes were imported into analysis software GeneSpring GX 7.3.1 (Agilent technologies, USA), and normalized to 75th percentile per chip (the 75th percentile values of each data set were set to 1).

For Agilent experiment, the feature extraction software provided by Agilent (version 9.5) was used to quantify the intensity of fluorescent images and to normalize results by subtracting local background fluorescence, according to the manufacturer's instructions.

Validation of microarray data with the quantitative real-time PCR

The quantitative real-time PCR was performed on cDNA using the Smart Cycler System (Cepheid, Sunnyvale, CA) to validate the results of the microarray analyses. The genes suggested to be significantly more or less expressed in *mel1-1* mutant than in the wild-type in the microarray analysis were subjected to the quantitative real-time PCR (qRT-PCR). The mRNAs were purified from the total RNA using Dynabeads oligo $(dT)_{25}$ (Dynal Biotech, Brown Deer, WI). The first-strand cDNA was synthesized with SuperScript II reverse transcriptase. qRT-PCR was carried out with specific primers for each genes (see below). The housekeeping genes, actin and ubiquitin was used for normalization of the quantity of the transcripts. A relative standard curve representing 4 steps of 10-fold dilution each of the actin or ubiquitin cDNA (1:10:100:1,000) was used for linear regression analyses of other unknown samples. After 30 sec at 95℃, each of 40 qRT-PCR cycles had 5 sec at 95℃ followed by 20 sec at 70℃ for extension (Smart Cycler; Cepheid, Sunnyvale, CA; Takara Ex Taq, R-PCR version). Real-time monitoring of PCR products was done with fluorescence of SYBR green I (Takara). The expression levels of specific genes was represented as ratios to that of actin or ubiquitin from the same master reaction. PCR primer pairs (5' to 3') used for each gene were as follows: AK102146, TCAATGGCTCTGTTTGGAAG and TGTCTCCAACATTGTCAGCA; AK058349, TTCTAGGCTCTAGCCGTCGT and TTACCGAAAGGAACCAGGAC; AK064061, TGATCGAGGTGATCGAAGG and AGGAAGGCCCTCTTGATTG; AK122162, ACCATCGCTTCTGCAGTTC and GAGAGGATCTTCTTGTCGGC; AK108249,

GGAAGAGTTGATGTTCGCAA and ACATCACAAGCTCGGACACC; AK120562,

GCTCTGTTCTTGGCCTTTGT and TTCTCCTCCTCTCTCGCCTT; AK069474,

CCGTCCCTATTCCAACAAGT and TATTCACTGAGGCTGCTTGG; AK060981,

ATGCTGGAGAAGCTGTGGGA and TCGCCATCTTTGTTGATGAC; AK068392,

CGAGAAGACCAACTGGATCA and CCGCCCTTCTTGTTGTAAAT; AK073848,

GGTGAAGGAGGACAACGACT and ATGTGCTGCAGGTTCATCAT

Results

To investigate genes regulated by the *MEL1* during early development of plant germ cells, the gene expression profile was compared between wild-type and *mel1-1* young panicle using Affymetrix Rice GeneChip Array and Agilent 44K rice microarray.

Up- and down-regulated genes in mel1-1 detected by Affymetrix GeneChip array

In the Affymetrix microarray, the number of probes whose intensity was more than 3-fold higher in the *mel1-1* mutant than in the wild-type was 349, and equal or less than 3-fold was 232 (Table III-1 and Appendix). The Affymetrix gene probe model containing miss-annotations were converted into public IDs of the full length cDNA (KOME; http://cdna01.dna.affrc.go.jp/cDNA/). In addition, to make the data more reliable, the probes which showed large experimental variation were removed by a standard t-test with a significance at the $P < 0.05$ level. The probes with the signal intensity less than 0.1 were also removed to minimize the effect of background noise. It is known that the reproducibility of the microarray data was extremely reduced in the probes with the signal intensity less than 0.1, in case of the 75-percentile normalization same as this study (Table III-1, Appendix). After the above-mentioned filtration steps, the genes up-regulated in the *mel1-1* mutant was estimated in 192, and the genes down-regulated was 185 (Table III- 1). The total 377 genes were provided for further analyses.

In the Affymetrix microarray, of 192 up-regulated genes in the *mel1-1* mutant, thirty-three (17%) were annotated to be the genes responsive for environmental stresses and/or hormone-responsive genes (Figure III-1, Table III-2). Especially, the former included 5 genes known to be up-regulated in the anaerobic condition, and the latter included 8 ethylene responsible genes, functioning in the downstream of the ethylene signaling pathway. Genes responsible for anaerobic condition and up-regulated in the *mel1-1* mutant were *vacuolar H+PPase* (*VP4*) (Os02g0802500), putative *early nodulin 93* (Os06g0142200), pyruvate dehydrogenase E1 component alpha subunit (Os12g0183100),

anaerobically inducible early2 (*Aie2*) (Os11g0112000) and dihydrolipoyllysine-residue acetyltransferase component of pyruvatedehydrogenase complex (Os01g0314300). In addition to these genes, *Usp* (Universal stress protein) (Os07g0673400) and *OsNAC6* (Os01g0884300) are responsible not only for anaerobic but also for various environmental stresses, and thought to bear unknown but important roles in stress responses in rice (Nakashima et al. 2007, Sauter et al. 2002).

Genes with the annotation of 'responsible for ethylene signaling pathway' and up-regulated in the *mel1-1* mutant were ethylene-responsive element binding protein 2, putative expressed (Os03g0183300), *ethylene response sensor1* (Os05g0525900 and Os08g0547300), MAP-KKK *CTR1*, downstream of ethylene receptor (Os07g0475900), pathogenesis-related, *ERF* domain-containing transcription factor (Os09g0286600), *ethylene-responsive transcription factor* 5(*AtERF5*) (Os09g0287000), *ethylene-responsive transcription factor 3* (Os08g0537900), and *DRE (dehydration responsive element)-binding transcription factor 2* (Os06g0222400). Ethylene is involved in many aspects of the plant life cycle, and was originally regarded as a stress hormone because its synthesis is induced by a variety of stress signals, such as wounding, drought and pathogen infection (Kende, 1993; Johnson and Ecker, 1998).

Futhermore, eleven (6 %) transposable element-like transcripts were also up-regulated in *mel1-1* mutant (Figure III-1, Table III-2). Those transcripts were transposon protein, putative, CACTA, En/Spm sub-class (Os10g0130500, Os10g0130500 and TIGR locus=LOC_Os12g26970), retrotransposon protein, putative, unclassified (Os01g0785300), transposon protein, putative, Mutator sub-class (Os05g0391050 and Os03g0402700), retrotransposon protein, CXC domain containing protein (TIGR locus=LOC_Os02g17440), retrotransposon protein, putative, Ty3-gypsy subclass (Os12g0185800 and Os10g0439400), RIRE10, sense (AK106483) and antisense transcripts (Os12g0186700) and transposon protein, putative, Ac/Ds sub-class (Os05g0414600) . It is reported that argonaute controls mobilization of retrotransposons in *Drosophila* and mouse and *C.elegans* (Kalmykova et al. 2005, Carmell et al. 2007).

On the other hand, 185 genes were down-regulated in *mel1-1* mutant. Thirty-four (18%) of them were predicted to concern the regulation of cell structure (Figure III-1, Table III-3). For example, Alpha-expansin *OsEXPA29* (Os06g0718100), *OsEXPA4* (Os03g0243700), *OsEXPA5* (Os02g0744200) and Beta-expansin *AtEXPB1* (Os01g0636500) are the key regulators of cell wall extension during plant growth and/or pollen tube elongation. Expansins are known to play an important role in regulating the development and response of plants to adverse environment (reviewed by Cosgrove DJ. 2000). The genes categorized to histone/chromosome and cell cycle include *Histone H3* (Os04g0419600), *Histone 2A* (Os03g0162200, Os03g0721900), *chromosome condensation regulator protein* (*RCC1*) (Os02g0684900), *DNA cytosine methyltransferase MET2a* (Os10g0104900), *Proliferating cell nuclear antigen (PCNA)* (Cyclin) (Os02g0805200), *Replication protein A* (Os02g0829100), *DNA replication licensing factor Mcm2* (Os11g0484300). They are thought to be key regulatior of cell division (Ohtsubo et al.1987, Teerawanichpan et al. 2003, Kimura et al. 2001, Ishibashi et al.2001).

Finally, four genes were predicted to concern reproduction specifically (Table III-3). They were annotated as follows ; *Male sterility protein 2* (Os09g0567500), *Pollen-specific kinase partner protein* (Os05g0560100), *Pollen Ole e 1 allergen* and extension domain containing protein (Os09g0508200), pollen-specific protein *SF3* (Os12g0510900). All of them are expressed especially in pollen, and thought to be essentilal for anther development (Aarts et al.1997, Kaothien et al. 2005, Alché et al. 2004, Baltz et al.1999).

Up- and down-regulated genes in mel1-1 detected by Agilent microarray

To make the microarray data more reliable, Agilent 44K oligo microarray was also used for gene expression profiling of the *mel1-1* mutant and those expression profiles from two different platforms were compared. The number of probes whose intensity was 3-fold or higher than it in the *mel1-1* mutant than in the wild-type was 358, and those 3-fold or less was 491 (Table III-1). However, after the same filtration step used in Affymetrix (see above), only 19

(down-regulated in *mel1-1*) and 4 (up-regulated in *mel1-1*) genes were considered to usable for further analysis. In contrast to Affymetrix GeneChip array, Agilent oligo microarray contains the probes for rice microRNA, however, all of them were excluded due to low signal intensity.

Of 19 down-regulated genes, six (31%) were unknown. Two heat shock protein (Os11g0187500, Os06g0219500) were down-regulated. Anther-specific proline-rich protein *APG (*Os02g0816200, Os06g0636600, Os02g0617400), which is expressed only in anther, especially tapetum (Roberts et al. 1993), and basic helix-loop-helix dimerisation region bHLH domain containing protein (Os06g0653200) was down-regulated both in Affymetrix and Agilent microarray, while an transcription factor, *OsSPL* (*SQUAMOSA* promoter-binding-like protein) (0s08g0509600) were up-regulated.

Genes detected in S/G2 stage

To compare the gene expression of WT and *mel1-1* in different germ cell development stages, gene expression profile at pre-meiotic S/G2 stage anther were also examined using both microarrays. At this stage, numbers of up- and down- regulated genes were limited compared to 1 cm panicle In Affymetrix microarray, 9 genes were down-regulated and 14 genes were up-regulated. In Agilent, microarray 23 genes were down-regulated and 11 genes were up-regulated. This result suggests that dynamic gene expression change in *mel1-1* occur at earlier stage than pre-meiotic S/G2 and expression profile.

In both microarray, of down-regulated genes in the *mel1-1* anther, many *SKP1* (Os07g0625600, Os07g0625500, Os07g0409500, Os07g0625400) genes were included (Table III-6). *SKP1* are well known as SCF (Skp1-Cullin-F-box protein) complexes, and is involved in a number of aspects of plant growth and development, possibly through the ubiquitin-mediated proteolysis of proteins by the proteasome (Pozo and Estelle. 2000, Hershko and Ciechanover. 1998, Sullivan et al. 2003, Zheng et al. 2002). The *Arabidopsis ASK1* (*Arabidopsis SKP1-like1)* gene encodes a homolog of

the human and yeast SKP1 proteins. *ASK1* is essential for male fertility in *Arabidopsis*, as *ask1-1* mutant was initially found to be defective in chromosome separation during male meiosis (Yang et al. 1999).

On the other hand, Myb, DNA-binding domain containing protein (Os01g0971800) and short highly repeated, interspersed DNA (Os02g0649300) were up-regulated in both microarray. Several additional transposable elements (Os03g0334400) were also up-regulated in *mel1-1*.in the S/G2 stage. However, gene number up- or down-regulated in S/G2 stage was much less than that detected in 1 cm young panicle. This result indicated that dynamic gene expression change in *mel1-1* occur at earlier stage than the pre-meiotic S/G2

Data confirmation by quantitative real-time PCR

I performed real-time quantitative RT-PCR to confirm and evaluate more quantitatively the expression differences predicted by microarray analyses. Eight up-regulated genes in the *mel1-1* by microarray were selected and examined their expression levels. Expression ratios between wild-type and *mel1-1* mutant analyzed by qRT-PCR data (Figure III-2) were coincided well with that detected in microarray analysis. The result indicated that expression profile data of individual genes observed in microarray should be highly reliable

Discussion

In this study, to investigate the genes regulated by the *MEL1* during early development of plant germ cells, the gene expression profiles was compared between wild-type and *mel1-1* young panicle (1 cm) and anthers (0.4 mm) at pre-meiotic stages using 55K Affymetrix Rice GeneChip Array and Agilent 44K rice microarray. To make the data more reliable and to minimize the effect of background noise, some filtration of microarray data were adopted. Due to low intensity (less than 0.1), most of probes were excluded. Agilent microarray consists of probes which represent 64 rice viral microRNAs from the Sanger database, but miRNA detection was also neglected because of low amount of small RNA sample was applied for analyses. Owing to the miRNA features of low molecular weight and low abundance, miRNA enrichment is an important step for the measurement of expression. The direct use of total RNA to profile miRNA expression should limit sensitivity because the relative abundance of small RNAs in a total RNA sample is less than 0.01% (Shingara et al. 2005). More suitable method for isolation of small RNA will be needed.

In 0.4-mm anthers including pre-meiotic pollen mother cells, numbers of up- or down- regulated genes were limited In Affymetrix microarray, 9 genes were down-regulated and 14 genes were up-regulated. In Agilent, microarray 23 genes were down-regulated and 11 genes were up-regulated. These numbers were much less than those detected in the pre-meiotic cells in 1 cm young panicle. This suggests that dynamic gene expression changes by the *mel1-1* mutation occur at earlier stage of germ-cell development than the pre-meiotic stages, and the expression profiles at the pre-meiotic stage may represent the secondary effects of primary changes of gene expressions. This result might correspond with the result of *in situ* hybridization in the Section II that the *MEL1* expression was limited to archesporial cells and early meiotic cells (Figure II-2).

In the Affymetrix microarray at 1 cm young panicle, 192 genes were up-regulated in the *mel1-1* mutant and 17 % of them were annotated to be the genes responsive for environmental stresses and/or hormone-responsive genes; the

former included 5 genes known to be up-regulated in the anaerobic condition, and the latter included 8 ethylene responsible genes. Anoxia is one of the major abiotic stresses that affect plant growth and development. Most of anoxia-related genes are reported that their expressions are induced by anaerobic condition. For example, seedlings anoxia evokes a marked increase in V-PPase transcript abundance, V-PPase immunoreactive protein in rice (Carystinos et al. 1995). The *Aie* (*anaerobically inducible early*) gene family is induced so early under anoxia in plants, and play an important role in plant metabolism under anaerobic conditions (Huq et al. 1999). In addition to them, some genes responsive to biotic and abiotic stress were also up-regulated in *mel1-1*. For example, rice transcription factor, *OsNAC6*, is induced by various stresses, such as salt, drought, ABA and wounding (Ohnishi et al. 2005, Nakashima et al. 2007).

Ethylene is the plant hormone involved in many aspects of the plant life cycle, including seed germination, root development and flower senescence and there is complicated signal transduction pathway related to ethylene. The components of the pathway for ethylene signal transduction were identified by genetic approaches (reviewed by Johnson et al. 2002, Chen et al. 2005, and Kendrick et al. 2008). Xu et al. (2006) and Fukao et al. (2006) reported that *Sub1A*, an ethylene-response-factor-like gene, determines submergence tolerance in rice and submergence-promoted ethylene production stimulates the accumulation of the *Sub1A* transcript in submergence-tolerant rice.

From these reports, up-regulation of the genes responsive for stresses and/or hormone-responsive genes in *mel1-1* mutant suggest that in *mel1-1* germ cell, absence of the *MEL1* may cause stressful condition, thus many stress-response genes and ethylene signaling-related gene are induced. The *MEL1* may inhibits stress responses in germ cells to disturb the accomplishment of precise germ cell division and meiosis.

Eleven (6 %) transposable element-like transcripts were also up-regulated in the *mel1-1* mutant. It was demonstrated that *Drosophila Piwi* mutations impact retrotransposon mobility; without functional *Piwi*, retrotransposons become abnormally active in both male and female germline (Sarot et al. 2004, Kalmykova et al. 2005). In *C.elegans*,

increased *Tc3* transposase mRNA in *piwi* mutants demonstrate that *Tc3* transposon silencing and associated siRNAs depend on *Piwi* (Das et al. 2008). In zebrafish, a low level or absence of transposon transcripts in wild-type gonads was detected, whereas in *ziwi* and *zili* (orthologous mutants of *piwi* of zebrafish) germ cells, an elevated level of transcripts could be detected. (Houwing et al. 2007, Houwing et al. 2008). These finding supports the idea that *MEL1* may also suppress the activity of transposable elements during rice germ-cell development. This raises another possibility that the ectopic activation of tansposable elements induces stressful conditions in the *mel1-1* mutant germ cells. However, the challenge of environmental stresses or supply of the phytohormone was reported to induce the activation of transposable elements (Grandbastien et al. 1998). Therefore it might be difficult to determine which is the primary event, stress-related gene expression or ectopic transposable element activation, in the *mel1-1* mutant germ cells.

On the other hand, in 185 genes down-regulated in the *mel1-1* mutant, many genes related to cell structure, such as glycoside hydrolase family protein and expansin were induced, and the genes relating to the cell cycle, such as cyclin and replication protein were included as well (Table III-3). Although it was unclear that the alteration of expression of these genes was whether a direct effect of *mel1-1* mutation or not, these results may indicated that in *mel1-1* mutant, the failure of pre-meiotic mitosis of sporogenous cells in the *mel1-1* mutant anther (Nonomura et al. 2007) was caused by down-regulation of these genes. Some genes were predicted to concern reproduction related genes. The down-regulation of pollen-related gene was also observed in the Agilent microarray; anther-specific proline-rich protein *APG* precursor was down-regulated. It is unknown that the down-regulation of these genes are direct or indirect effect of *mel1-1*, these results may suggest that some genes required for the pollen formation is expressed during early development of germ cells and that the normal *MEL1* function is required not only for the maintenance of archesporial cells and sporogenous cells, but also for the subsequent pollen formation.

At pre-meiotic S/G2 stage of young anthers, few genes were affected by the *mel1-1* mutation. However it was remarkable that multiple *SKP1* genes were down-regulated in the *mel1-1* mutant anthers (Table III-6). The

Arabidopsis ASK1 (*Arabidopsis SKP1-like1*) gene encodes a homolog of the human and yeast Skp1 proteins. *ASK1* is essential for male fertility in *Arabidopsis*, as *ask1-1* mutant was initially found to be defective in chromosome separation during male meiosis (Yang et al. 1999). Although most of up- and down-regulation of genes in the later stages of pre-meiotic germ-cell development might be secondary effects of *mel1-1* mutation, it is also possible that these remarkable secondary effects in gene expression might together act as causal genes for expressing mutant phenotypes. Hence, the comparison of gene expression profiles between WT and mutant could reveal useful cues to extract genes functioning in the reproduction events.

When the microarray results were compared between the Agilent and the Affymetrix microarray systems, several genes were common in both systems (Table III-5). An anther-specific proline-rich protein *APG* and a basic helix-loop-helix dimerisation region bHLH domain containing protein were down-regulated and an transcription factor, the *OsSPL* (*SQUAMOSA* promoter-binding-like protein) were up-regulated (Table III-5). Low redundancy between both systems may represent differences in probe preparation and genome annotation. It is sure that the genes commonly extracted in both systems can be the best reliable candidates affected by the *mel1-1* mutation during germ-cell development. It is noteworthy that one of above three genes, the *OsSPL* mRNA contains a putative target sequence of the plant microRNA, *miR156*. Therefore, the possibility that the *OsSPL* is a direct target of the *MEL1* Argonaute gene will be investigated in the next section.

Table III-1. Number of up- or down- regulated genes in *mel1-1* compared to wild-type

	Affymetrix						Agilent					
	1cm panicle			0.4mm anther			1cm panicle			0.4mm anther		
	total	filtered	≧0.1	total	filtered	≧0.1	total	filtered	≧0.1	total	filtered	≧0.1
WT>*mel1-1*	232	186	185	14*	9	9	491	76	19	64	29	23
mel1-1>WT	349	192	192	42*	14	14	358	29	4	599	63	11

Data were obtained by the result of 3 biological replicates, and normalized with the 75 percentile signal intensity.

Cut-off: 3-fold changes and p <0.05

*: ≥2.5 was adopted as a threshold.

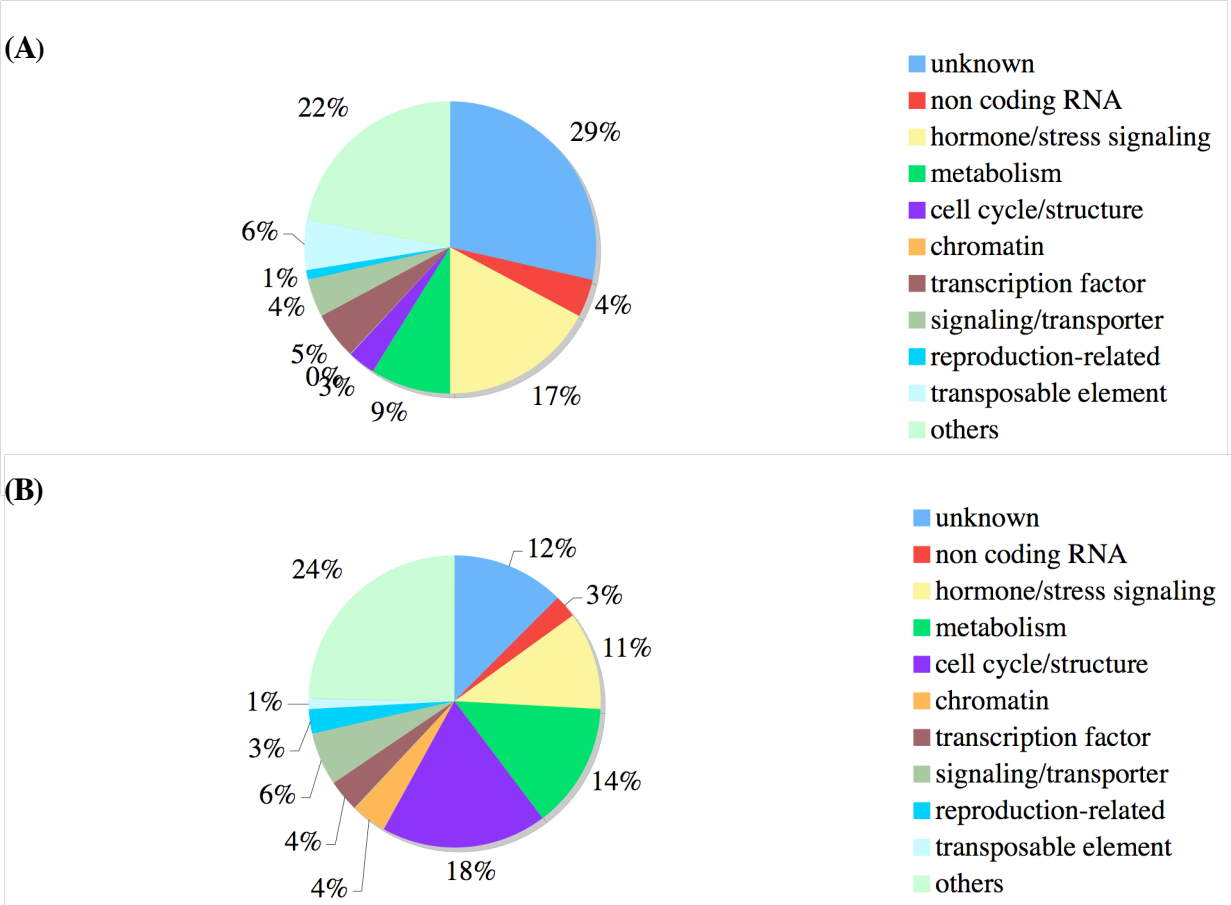


Figure III-1. Functional categories of the (A) up- or (B) down regulated genes in the *mel1-1* 1 cm young panicle

The pie chart shows the percentage of transcripts of up-regulated and down-regulated genes.

Table III-2. A gene list showing up-regulation in the *mel1-1* mutant of 1 cm young panicle

Category	RAP locus	TIGR locus	Description
anoxia	Os02g0802500	LOC_Os02g55890	vacuolar H+ PPase (VP4) (anoxia)
anoxia	Os06g0142200	LOC_Os06g04990	Putative early nodulin 93 (anoxia)
anoxia	Os12g0183100	LOC_Os12g08260	pyruvate dehydrogenase E1 component, alpha subunit (anoxia)
anoxia	Os11g0112000	LOC_Os11g02080	Aie2 (Anaerobically inducible early 2) protein
anoxia	Os01g0314300	LOC_Os01g21160	dihydrolipoyllysine-residue acetyltransferase component of pyruvatedehydrogenase complex (anoxia
cold	Os02g0790500	LOC_Os02g54820	trehalose-6-phosphate synthase (cold)
cold	Os08g0414700	LOC_Os08g31980	trehalose-6-phosphate synthase (cold)
salinity	Os03g0823400	LOC_Os03g60840	Bowman-Birk type trypsin inhibitor (salinity, drought, dormancy)
wound	-	LOC_Os04g54300	wound induced protein
various stress	Os01g0884300	LOC_Os01g66120	OsNAC6 (various stress)
various stress	Os07g0673400	LOC_Os07g47620	Usp (universal stress protein) protein (ethylene, anoxia)
ethylene	Os03g0183300	LOC_Os03g08500	ethylene-responsive element binding protein 2, putative, expressed
ethylene	Os05g0525900	LOC_Os05g45020	Similar to ERS1 (ethylene response sensor1)
ethylene	Os08g0547300	LOC_Os01g16170	Similar to ERS1 (ethylene response sensor1)
ethylene	Os07g0475900	LOC_Os07g29330	MAP-KKK CTR1, downstream of ethylene receptor
ethylene	Os09g0286600	LOC_Os09g11460	Pathogenesis-related, ERF domain-containing transcription factor
ethylene	Os09g0287000	LOC_Os09g11480	Similar to Ethylene-responsive transcription factor 5(AtERF5)
ethylene	Os08g0537900	LOC_Os08g42550	Similar to ethylene-responsive transcription factor 3 (AtERF3)
ethylene	Os06g0222400	LOC_Os06g11860	DRE (dehydration responsive element)-binding transcription factor 2
auxin	Os02g0818000	LOC_Os11g44810	auxin-repressed 12.5 kDa protein, dormancy-associated protein
auxin	Os09g0545280	LOC_Os09g37330	OsSAUR39 - Auxin-responsive SAUR gene family member
cellcyc	Os06g0594400	LOC_Os06g39370	Cyclin-like F-box domain containing protein
cellcyc	Os04g0571300	LOC_Os04g48270	Cyclin-like F-box domain containing protein
Transposon	-	LOC_Os12g26970	transposon protein, putative, CACTA, En/Spm sub-class
Transposon	Os01g0785300	LOC_Os01g57599	retrotransposon protein, putative, unclassified
Transposon	Os05g0391050	LOC_Os05g32480	transposon protein, putative, Mutator sub-class
Transposon	-	LOC_Os02g17440	retrotransposon protein, CXC domain containing protein
Transposon	Os12g0185800	LOC_Os12g08490	retrotransposon protein, putative, Ty3-gypsy subclass
Transposon	Os12g0186700	-	RIRE10, sense (AK106483) and antisense (this probe) transcripts
Transposon	Os10g0345150	LOC_Os10g20480	CACTA, En/Spm sub-class
Transposon	Os10g0439400	LOC_Os10g30350	retrotransposon protein, putative, Ty3-gypsy subclass
Transposon	Os10g0130500	LOC_Os10g04050	transposon protein, putative, CACTA, En/Spm sub-class
Transposon	Os03g0402700	LOC_Os03g28930	transposon protein, putative, Mutator sub-class
Transposon	Os05g0414600	LOC_Os05g34260	transposon protein, putative, Ac/Ds sub-class
Transcription factor	Os08g0509600	LOC_Os08g39890	Squamosa (AP1)-promoter binding-like protein 8 (SPL8), required for pollen sac development
reproduction	Os10g0499800	LOC_Os10g35650	Rf1 (Restorer of fertility1), mitochondrial precursor
reproduction	Os01g0719100	LOC_Os01g52110	Putative PGPD14 protein (pollen germination related protein)

Table III-3. A gene list showing down-regulation in the *mel1-1* mutant of 1 cm young panicle

Category	RAP locus	TIGR locus	Description	Fold change
cellstr	Os02g0816200	LOC_Os02g57110	anther-specific proline-rich protein APG precursor	5.59
cellstr	Os06g0636600	LOC_Os06g43030	anther-specific proline-rich protein APG	4.6
cellstr	Os02g0617400	LOC_Os02g40440	anther-specific proline-rich protein APG precursor	4.25
cellstr	Os06g0718100	LOC_Os06g50400	Alpha-expansin 29 (EXPA29)	5.55
cellstr	Os02g0744200	LOC_Os02g51040	Alpha-expansin OsEXPA5	4.63
cellstr	Os01g0636500	LOC_Os04g44780	Similar to Beta-expansin 1 precursor (AtEXPB1)	3.78
cellstr	Os03g0243700	LOC_Os05g39990	Alpha-expansin OsEXPA4	3.52
cellstr	Os05g0244500	LOC_Os05g15510	Glycoside hydrolase, family 5 protein	4.8
cellstr	Os05g0415700	LOC_Os05g34320	Glycoside hydrolase, family 20 protein	4.14
cellstr	Os06g0131500	LOC_Os06g04080	Glycoside hydrolase, family 17 protein	4.04
cellstr	Os02g0752200	LOC_Os02g51620	Similar to xylan beta-D-xylosidase	4.31
cellstr	Os04g0640700	LOC_Os04g54810	Alpha-L-arabinofuranosidase/beta-D-xylosidase isoenzyme like	4.16
cellstr	Os04g0137100	LOC_Os04g05050	Similar to Pectate lyase (Fragment)	3.93
cellstr	Os10g0160100	LOC_Os10g07290	Putative beta-1,3-glucanase	3.92
cellstr	Os01g0663300	LOC_Os01g47400	Putative mannan endo-1,4-beta-mannosidase	3.92
cellstr	Os01g0636500	LOC_Os01g44970	Similar to Polygalacturonase PG2	3.88
cellstr		LOC_Os12g43340	Similar to Actin-depolymerizing factor 6 (ADF-6) (AtADF6)	3.48
cellstr	Os07g0252400	LOC_Os07g14850	CESA6 - cellulose synthase	3.38
cellstr	Os01g0777900	LOC_Os01g57004	adhesive/proline-rich protein, putative, expressed	3.15
cellstr	Os01g0697100	LOC_Os01g50200	UDP-glucuronosyl/UDP-glucosyltransferase family protein	3.05
cellstr	Os11g0297800	LOC_Os11g19210	Similar to Beta-D-xylosidase	5.2
Hor/str	Os06g0266800	LOC_Os06g15620	Similar to GAST1, gibberellin-regulated family protein precursor	12.11
Hor/str	Os08g0460000	LOC_Os08g35760	auxin-binding protein ABP20 precursor, putative, expressed	9.7
Hor/str	Os02g0669100	LOC_Os02g44870	dehydrin COR410, putative, expressed (cold)	7.18
Hor/str	Os02g0782500	LOC_Os02g54140	Similar to Small heat stress protein class CIII	5.72
Hor/str	Os04g0673300	LOC_Os04g57720	Type A response regulator 6 (OsRR6) (cytokinin)	5.02
Hor/str	Os05g0460000	LOC_Os05g38530	heat shock cognate 70 kDa protein, putative, expressed	4.72
Hor/str	Os02g0132500	LOC_Os02g03960	ocs element-binding factor 1, putative, expressed (cold)	3.83
Hor/str	Os06g0729400	LOC_Os06g51320	Similar to Gibberellin-regulated protein 2 precursor	3.78
Hor/str	Os04g0526000	LOC_Os04g44440	Similar to Auxin-induced basic helix-loop-helix transcription factor	3.76
Hor/str	Os01g0840100	LOC_Os01g62290	Heat shock protein Hsp70 family	3.75
Hor/str	Os04g0662200	LOC_Os04g56680	OsSAUR22 - Auxin-responsive gene family member, expressed	3.75
Hor/str	Os03g0266900	LOC_Os03g16020	Low molecular mass heat shock protein Oshsp17.3	3.67
Hor/str	Os09g0461500	LOC_Os09g28690	gibberellin receptor GID1L2	3.64
Hor/str	Os10g0132300	LOC_Os10g04270	Similar to Jasmonate-induced protein	3.48
Hor/str	Os01g0184100	LOC_Os01g08860	Class II small heat shock protein	3.46
Hor/str	Os03g0159600	LOC_Os03g06360	LEA (late embryogenesis abundant) D-34 (drought, dormancy)	3.41
Hor/str	Os05g0436100	LOC_Os05g36050	Brassinosteroid Insensitive 1-associated receptor kinase 1 precursor	3.19
chrom	Os04g0419600	LOC_Os04g34240	Histone H3	7.14
chrom	Os03g0162200	LOC_Os03g06670	Putative histone H2A	4.18
chrom	Os02g0684900	LOC_Os02g45980	Similar to chromosome condensation regulator protein (RCC1)	3.73
chrom	Os10g0104900	LOC_Os10g01570	DNA cytosine methyltransferase MET2a	3.26
chrom	Os03g0721900	LOC_Os03g51200	histone H2A	3.04
cellcyc	Os02g0805200	LOC_Os02g56130	Proliferating cell nuclear antigen (PCNA) (Cyclin)	3.36
cellcyc	Os02g0829100	LOC_Os02g58220	Replication protein A 30kDa	3.27
cellcyc	Os11g0484300	LOC_Os11g29380	DNA replication licensing factor Mcm2	3.09
reprod	Os09g0567500	LOC_Os09g39410	male sterility protein 2, Similar to Fatty acyl coA reductase	4.06
reprod	Os05g0560100	LOC_Os05g48640	Similar to Pollen-specific kinase partner protein	4.02
reprod	Os09g0508200	LOC_Os09g32988	Pollen Ole e 1 allergen and extensin domain containing protein	3.9
reprod	Os03g0800200	LOC_Os03g58600	MEL1, putative argonaute protein	3.71
reprod	Os12g0510900	LOC_Os12g32620	pollen-specific protein SF3	3.35

Table III-4. Number of genes up- or down- regulated in *mel1-1* both in Agilent and Affymetrix microarray, or 1 cm panicle and 0.4 mm anther

The list of genes presented in this table was summarized in Table III-5.

		Affymetrix			Agilent			
		1cm	0.4mm		1cm		0.4mm	
			WT>mel	mel>WT	WT>mel	mel>WT	WT>mel	mel>WT
Affymetrix	1cm		4	1	2	1	0	1
	0.4mm				0	0	0	2
Agilent	1cm						0	0

Table III-5. The gene list of genes up- or down- regulated in *mel1-1* mutant both in Agilent and Affymetrix array, or 1 cm panicle and 0.4 mm anther

The number of genes listed in this table was summarized in Table III-4.

Affy1cm and	Affy0.4mm	Genbank	Fold change		Description
			Affy1cm	Affy0.4mm	
* WT>mel					
Os05g0460000	LOC_Os05g38530	AU075856	3.13	4.72	heat shock cognate 70 kDa protein, putative, expressed
Os03g0800200	LOC_Os03g58600	D42427	4.28	3.71	MEL1, Putative argonaute protein
Os02g0782500	LOC_Os02g54140	AK119261	3.01	5.72	Similar to Small heat stress protein class CIII
Os01g0840100	LOC_Os01g62290	CB633557	3.49	3.75	Heat shock protein Hsp70 family protein
* mel>WT					
Os06g0142200	LOC_Os06g04990	AK122162	12.82	2.74	Putative early nodulin 93

Affy1cm and	Agi1cm		Fold change		
			Affy1cm	Agi1cm	
* WT>mel					
Os06g0653200	LOC_Os06g44320	AK058439	4.00	3.62	Basic helix-loop-helix dimerisation region bHLH domain containing protei
Os02g0617400	LOC_Os02g40440	AK108108	4.25	3.2	anther-specific proline-rich protein APG precursor
* mel>WT					
Os08g0509600	LOC_Os08g39890	AK107191	4.37	3.01	Squamosa (AP1)-promoter binding-like protein 8 (SPL8)

Affy1cm and	Agi0.4mm		Fold change		
			Affy1cm	Agi0.4mm	
* mel>WT					
Os02g0671100	LOC_Os02g44990	AK100120	20.02	3.549	Cyclin-like F-box domain containing protein, MERMITE18E.245390

Affy0.4mm and	Agi0.4mm		Fold change		
			Affy0.4mm	Agi0.4mm	
* mel>WT					
Os01g0971800		AY581256	2.597	3.547	Myb, DNA-binding domain containing protein.
Os02g0649300		AK063685	2.537	6.34	Short highly repeated, interspersed DNA (Fragment), MERMITEA.244239

Table III-6. Genes up- or down- regulated in the *mel1-1* mutant in 1cm panicle or in 0.4 mm S/G2 anther

Agilent 1cm

mel1-1>WT

Category	TIGR locus	Description	Fold change
AK107161	Os08g0509600	SBP-domain protein	3.02

Agilent 0.4 mm

mel1-1>WT

Category	TIGR locus	Description	Fold change
	Os02g0649300	Short highly repeated, interspersed DNA (Fragment)	6.34
	Os02g0671100	Cyclin-like F-box domain containing protein,	3.54
	Os01g0971800	Myb, DNA-binding domain containing protein.	3.54

WT>*mel1-1*

Category	TIGR locus	Description	Fold change
SKP	Os07g0625600	Skp1-like, Fimbriata-associated protein	3.73
SKP	Os07g0409500	SKP1 component family protein.	3.07
SKP	Os07g0625400	SKP1 component family protein.	3.02
SKP	Os07g0625500	Osk5, Skp1-like, Fimbriata-associated protein (Fragment)	3.87

Affymetrix 0.4 mm

WT>*mel1-1*

Category	TIGR locus	Description	Fold change
SKP	Os07g0625600	putative Skp1	0.34

mel1-1>WT

Category	TIGR locus	Description	Fold change
transposon		RIRE2-like retrotransposon	3.14
transposon	Os03g0334400	transposon protein, putative, unclassified	2.51
transposon	LOC_Os12g33760	retrotransposon protein, putative, unclassified	2.78

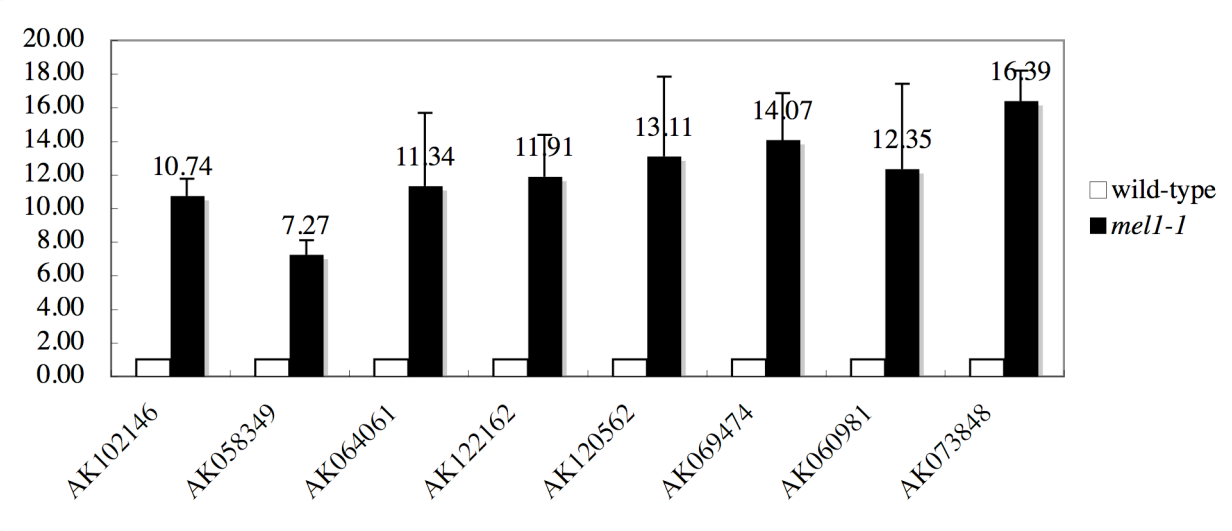


Figure III-2. Quantitative real-time PCR analysis of eight genes that were up-regulated in *mel1-1* mutant panicle

Comparison of expression profiles of for eight genes was indicated. The expression level of *RAc1* (*Rice Actin1*) gene was used as an internal standard. Relative ratio of each gene expression in the 1cm of panicles was calculated. Triplicate qRT-PCR experiments in each sample were carried out. Vertical bars indicate standard error.

Section IV

Molecular characterization of *OsSPL14* gene

Introduction

As indicated in the previous section, the gene expression profile compared between wild-type and *mel1-1* mutant detected up-regulation of one *SQUAMOSA* (*SQUA*) promoter-binding-like (*SPL*) genes *OsSPL14*, encoding a putative transcription factor, in *mel1-1* mutant in both Affymetrix (Fold change=4.37) and Agilent (=3.01) microarray (Table IV- 1).

The *SPL* genes represent a family of plant-specific transcription factors (Cardon et al. 1999). SPL proteins contain a highly conserved DNA-binding domain (*SQUA* promoter-binding protein [SBP] domain). *Antirrhinum majus SBP1* and *SBP2* have the *in vitro*-binding activity to the cis-element upstream of the gene *SQUA* (Klein et al., 1996). The *SQUA* gene is a member of the *MIKC* group of the *MADS*-box gene family that specifies flower meristem identity in *Arabidopsis* (Huijser et al., 1992; Jack, 2004). In the *Arabidopsis* genome, sixteen putative *SPL* genes were predicted based on sequence analysis, and several At*SPL* genes were thought to have roles in the regulation of plant development (Cardon et al., 1999). However, these *AtSPL* genes are different from *Arabidopsis SPL/NZZ*, described in section 2. The T-DNA insertion mutant of *AtSPL14* showed elongated petioles and enhanced leaf margin serration (Stone et al. 2005). *AtSPL8* was involved in the regulation of microsporogenesis, megasporogenesis, trichome formation on sepals, and stamen filament elongation (Unte et al. 2003). Mutants of *atspl8* exhibited a strong reduction in fertility. Computational analysis indicated that several *Arabidopsis SPL* genes were regulated by *miR156* family (Rhoades et al. 2002; Bonnet et al. 2004).

The *miR156* and its target genes are thought to be involved in some important developmental processes since overexpression of *AtmiR156* in *Arabidopsis* resulted in various phenotypic changes such as increased number of leaves, delayed flowering, and decreased apical dominance (Schwab et al., 2005). The constitutive overexpression of an *SPL3* cDNA, notably lacking most of the 3'- UTR, results in an early-flowering phenotype (Cardon et al. 1997). Previously,

nineteen *SPL* (Os*SPL*) genes were identified in the rice genome (Xie et al. 2006). Sequence and experimental analysis suggested that eleven Os*SPL* genes including *OsSPL14* were putative targets of *OsmiR156*. More than half of the Os*SPL* genes including most *OsmiR156*-targeted genes are predominantly expressed in the young panicles, whereas *OsmiR156* genes are predominantly expressed in the young shoots and leaves of rice. Overexpression of two *OsmiR156* (*OsmiR156b* and *OsmiR156h*) in rice resulted in severe dwarfism, strongly reduced panicle size, and delayed flowering, suggesting that *OsmiR156* and Os*SPL* target genes are involved in various developmental processes, especially the flower development of rice (Xie et al. 2006). Furthermore, they also reported that the *OsSPL14* expressed stronger during very early stage of young panicles (around 0.5 cm panicle length) than other tissues. These observations suggest that the *OsSPL14* should be directly targeted by the *miR156* microRNA.

In this study, whether the *OsSPL14* is one of the direct targets of the rice *MEL1 AGO*, the quantification of the *OsSPL14* expression in the wild-type and *mel1-1* mutant panicle. Next, expression of *miR156* was compared between the wild-type and *mel1-1* panicle. In addition, the *MEL1* specific antibody was applied to examine coimmunoprecipitation with *miR156*

Methods

Quantification of rice microRNA

To compare the expression of microRNAs in wild-type and *mel1-1* mutant panicle, quantitative real-time PCR (qRT-PCR) was employed by using the Applied Biosystems TaqMan microRNA Assays Protocol (Foster City, CA, USA). A two-step assay was performed in TaqMan-based real-time quantification of miRNAs. The first step involved a reverse-transcription (RT) reaction in which a stem-loop RT primer was used to reverse transcribe mature miRNAs to cDNAs. The second step involved real-time PCR, in which the expression level of miRNAs was monitored and quantified using quantitative real-time PCR that includes miRNA-specific forward primer, reverse primer and FAM dye-labeled TaqMan probes (Chen et al. 2005). In this study, the specific RT primer for the *Arabidopsis miR156* was used for the rice *OsSPL14* detection, and that for the Arabidopsis miR166 was used for the rice *HD-ZIP III* detection as a negative control, because these miRNA sequences were highly conserved in *Arabidopsis* and rice (Rhoades MW et al. 2002).

Reverse-transcription reaction

The miRNA RT reactions was done in the solution containing 10 ng of total RNAs, 0.25 mM each of dNTPs, 1 U of Multiscribe reverse transcriptase, 1.5 μl of RT buffer, and 3.8 U of RNase inhibitor, and nuclease-free water. The first RT reactions was performed with DNA Engine Tetrad (MJ Research, Incline Village, NV) according to a program was 30 min at 16℃, 30 min at 42℃, 5 min at 85℃ and then held at 4℃.

Real-time PCR reaction

Subsequent real-time quantative PCR reactions were performed using the Thermal Cycler Dice TP870AH system (Takara). Total 20 μL PCR reaction mixtures were prepared and each contained 1 μL TaqMan MicroRNA Assay

primers and probes, 10 μL TaqMan Universal PCR Master Mix, 1 μL of product from RT reaction, and nuclease-free water. The reactions were incubated in a 96-well plate at 95℃ for 10 min, followed by 45 cycles of 95℃ for 15 sec and 60℃ for 60 sec. After the completion of the real-time reactions, the threshold was manually set and the threshold cycle (Ct) was recorded. The Ct is defined as the fractional cycle number at which the fluorescence passes the fixed threshold (Chen et al. 2005). Experiment was biologically replicated three-times using three-independent samples from wild-type or *mel1-1* panicles.

Immunoprecipitation of MEL1 from rice anther tissues

1,200 rice anthers (anther length 0.4 mm) and 20 young panicles (1 cm) were frozen in liquid nitrogen, and then further ground in 100 μl of extraction buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% TritonX-100, protease inhibitor cocktail (SIGMA)). Insoluble material was removed by centrifugation (5 min at 16,000 g at 4℃. We then added 4 μg of anti-MEL1 antiserum per 100 μl and protein A micro beads to the supernatant, and carried out IP for 4 h at 4℃. The beads were then washed four times with 200 μl of extraction buffer. Immunoprecipitated RNA was purified from immunoprecipitates with phenol, phenol/chloroform and chloroform extraction.

Results

Expression analysis of OsSPL14 by quantitative real-time PCR

Both Affymetrix and Agilent microarray analyses strongly indicated that the *OsSPL14* gene, encoding a putative transcription factor, was up-regulated in the *mel1-1* mutant young panicles (Table IV-1). To confirm the microarray result of up-regulation of the *OsSPL14* in the *mel1-1* mutant young panicle, the quantative real-time PCR (qRT-PCR) was done using the *mel1-1* 1cm young panicle, same as the microarray analyses. The biological replication using three independent panicles indicated that the up-regulation of the *OsSPL14* in the *mel1-1* mutant was also confirmed in the qRT-PCR (Figure IV-1). To detect exact expression levels of *OsSPL14* and *MEL1* in successive developmental stages of wild-type panicles, the amount of transcripts of both genes were compared in 0.1-, 0.5-, 1.0-, 2.0- and 10.0-cm wild-type panicles (Figure IV- 2). The expression level of *OsSPL14* was higher than that of *MEL1* in the 0.1-cm and 0.5-cm younger panicles of the wild-type than those of the *mel1-1* mutant. In the 1 cm panicle, amount of both transcripts had become almost same level. As the panicles developed from 2 to 3 cm, the expression level of the *OsSPL14* was gradually decreased, however, in contrast, the *MEL1* expression became gradually increased. When the panicle length reached to 10 cm, the expression of both *MEL1* and *OsSPL14* was reduced. These data well corresponded to the Oryza Express expression profile data of microarrays as shown in the Section I (Figure IV- 3).

Accumulation of miR156 in mel1-1 mutant panicle

The qRT-PCR system of the Applied Biosystems was adopted to quantify the expression of microRNA using small amount of RNA samples, in which the unique stem-loop primer was used for the primary antisense cDNA elongation. The qRT-PCR analyses demonstrated that the level of accumulation of *miR156* was increased 6.7-fold more in the *mel1-1* mutants than in the wild-type young panicle (Figure IV-4). The *miR166*, which is generally expressed during the vegetative growth phase (Nagasaki et al. 2007) was also examined and revealed not to show a significant difference

between the wild-type and the *mel1-1* mutant panicles (Figure IV-4). The clear expression difference between *miR156* and *miR166* in early germ cell development and high accumulation of *miR156* in the *mel1-1* mutants strongly indicated that the *miR156* is one of good candidates of the target of the *MEL1* argonaute.

Detection of miR156 in co-immunoprecipitated (co-IP) sample

To verify a specific interaction of *miR156* with the MEL1 protein, an antibody for the MEL1 was applied and immnoprecipitation assay was performed using 1.0-cm panicles or 0.4-mm anthers of the wild-type and the *mel1-1* mutant, which included developing archesporial / sporogenous cells and pre-meiotic sporocytes, respectively. Co-IP RNA fracctions of above two stages were extracted and provided for qRT-PCR of with *miR156* specific primers. The *miR156* RNA detected in the co-IP fraction with the anti-MEL1 antibody 3.2-fold more than in that with the pre-immune serum (Figure IV-5). The detection level of *miR156* depended on that of the MEL1 protein, as the *miR156* coprecipitated with the MEL1 immune complex protein, but few or not with precipitates recovered using *mel1-1* mutant or preimmune serum (Figure IV-5). These data strongly suggest that the MEL1 forms a complex with the *miR156* microRNA in vivo.

Unexpectedly, when the *mel1-1* mutant panicles were used for the co-IP with the anit-MEL1 antibody, the *miR156* accumulation was also detected slightly lower than the wild-type panicles (Figure IV-5). In the previous study, the *MEL1* mRNA expression in the *mel1-1* mutant panicles was reduced about by 70-% less than that of the wild-type (Nonomura et al. 2007). The remained 30% transcript was an aberrantly shorter mRNA with a truncation of the exon 10, which included a *Tos17* insertion and was alternatively spliced out in the *mel1-1* mutant. This truncation occurred in-frame, and then the truncated *mel1-1* transcript possessed a potential to generate aberrant MEL1 proteins 24-amino-acid shorter at the middle than the wild-type protein (Nonomura et al. 2007). Indeed, when the co-IP fraction of the *mel1-1* mutant panicles with anti-MEL1 antibody was provided for the Western blot analysis, the signal intensity

of MEL1 proteins was almost same between the wild-type and the *mel1-1* mutant, whereas the crude extract before co-IP gave significantly a lesser intensity of the MEL1 signal in the *mel1-1* mutant (data not shown). These results indicated that the transcriptional and translatioal level of the MEL1 gene was significantly reduced in the *mel1-1* mutant, but the co-IP process would enrich a concentration of the *mel1-1* truncated proteins. This enrichment may be enough for making it difficult to distinguish the co-IP signal intensity of *mel1-1* mutant from that of the wild-type.

Discussion

The microarray qRT-PCR, and co-IP analyses in this study suggested that the *miR156* microRNA was one of good candidates of guide RNA molecules of the MEL1 AGO. The *OsSPL14*, encoding a putative transcription factor, was reported to carry a target site of the *miR156* microRNA (Xie et al. 2006), and was up-regulated in the *mel1-1* mutant young panicles in the previous section. The high expression of *OsSPL14* at the 0.5-cm very young panicles, previously reported by Xie et al. (2006), was also observed in this study (Figure IV-1). A subsequent expression profile of the *OsSPL14* was opposed to that of the *MEL1*. In the 0.1- and 0.5-cm young panicles, the amount of *OsSPL14* was larger than that of *MEL1*. During the progression of panicle development from 1.0 cm to 2.0 cm panicles, the amount of both genes became a reversal (Figure IV-2). These results raise a possibility that the *OsSPL14* is a direct target of the MEL1 AGO, and that an increase of the amount of MEL1 proteins enhances a degradation of the *OsSPL14* mRNA mediated by the *miR156* microRNA. It was also suggested that one of the roles of *MEL1* may be to inhibit the expression of the genes required for reproductive organ development, but not for the archesporial cell initiation within the wild-type panicles, such as the *OsSPL14* gene.

Recently, there are many reports of deep sequencing analysis of small RNA library (Ruby et al. 2006, Aravin et al. 2006, Girard et al., 2006; Grivna et al. 2006, Lau et al. 2006, Brennecke et al. 2007). For comprehensive understanding the rice MEL1 AGO function, the deep sequencing of small RNAs co-immunoprecipitated with MEL1 will be one of critical experiments to determine the function of MEL1 AGO on plant germ cell initiation and maintenance. Because biological function of *OsSPL14* has been unknown yet in rice, characterization of *OsSPL14* function in reproductive organ development should also be performed in future studies. To examine further, *in situ* hybridization of *OsSPL14* is underway. The phenotypic analysis of the *OsSPL14* mutant is now investigating. Recently, Andachi (2008) developed a new method to determine directly the target mRNA sequence of a microRNA by extracting the mRNA-microRNA

complex and amplifying the cDNA of the target with a reverse-transcription PCR, in which the microRNA was used as a primer. Application of this method to this study will lead to bring a direct evidence whether the *OsSPL14* is a direct target of the MEL1 AGO mediated by *miR156* microRNA or not.

Table IV-1. Expression level of the OsSPL14 in *mel1-1* mutant compared to that in wild-type

			Fold change	
RAP	TIGR	KOME	Affy1cm	Agi1cm
Os08g0509600	LOC_Os08g39890	AK107191	4.37	3.01

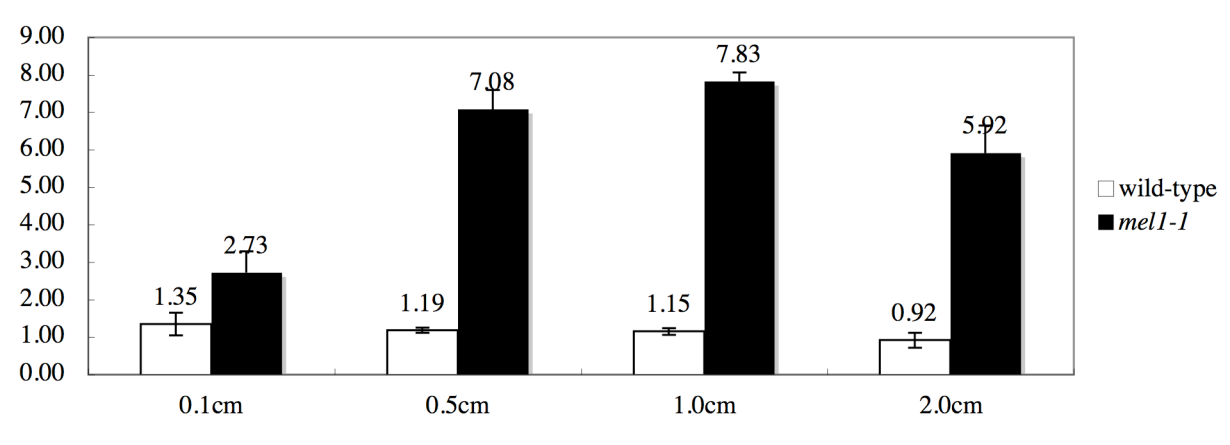


Figure IV- 1. Expression analysis of the *OsSPL14* by qRT-PCR

Expression levels of the *OsSPL14* in wild-type and *mel1-1* young panicle were compared. Y-axis indicates relative expression levels. The expression levels of triplicate qRT-PCR experiments in each sample were carried out. Vertical bars indicate standard error.

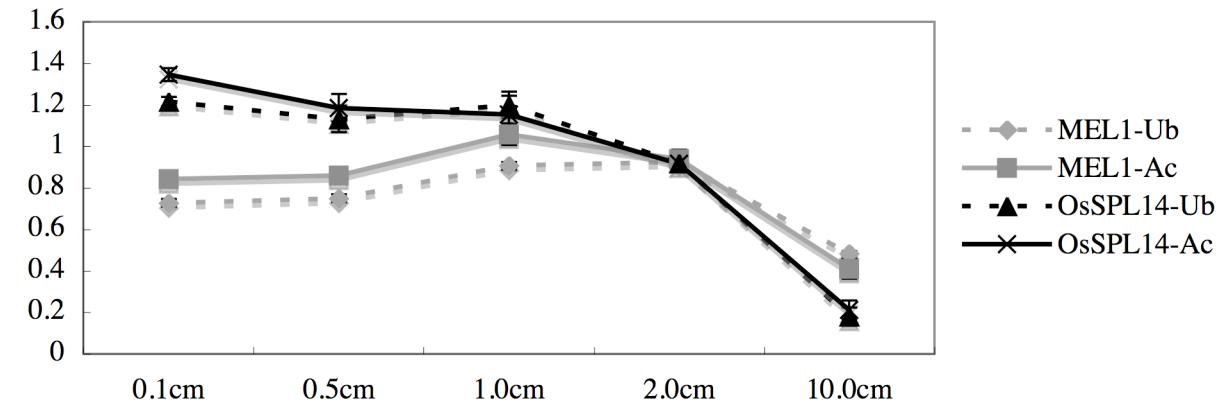


Figure IV- 2. Expression analysis of the *OsSPL14* and the *MEL1* genes by qRT-PCR.

Expressions of the *OsSPL14* and the *MEL1* in young panicles of various sizes (0.1 cm, 0.5 cm, 1.0 cm, 2.0 cm and 10.0 cm). *Rice Actin1* (for straight lines) gene and *Ubiquitin* (for dotted lines) were used as internal standards. Y axis indicates expression level.

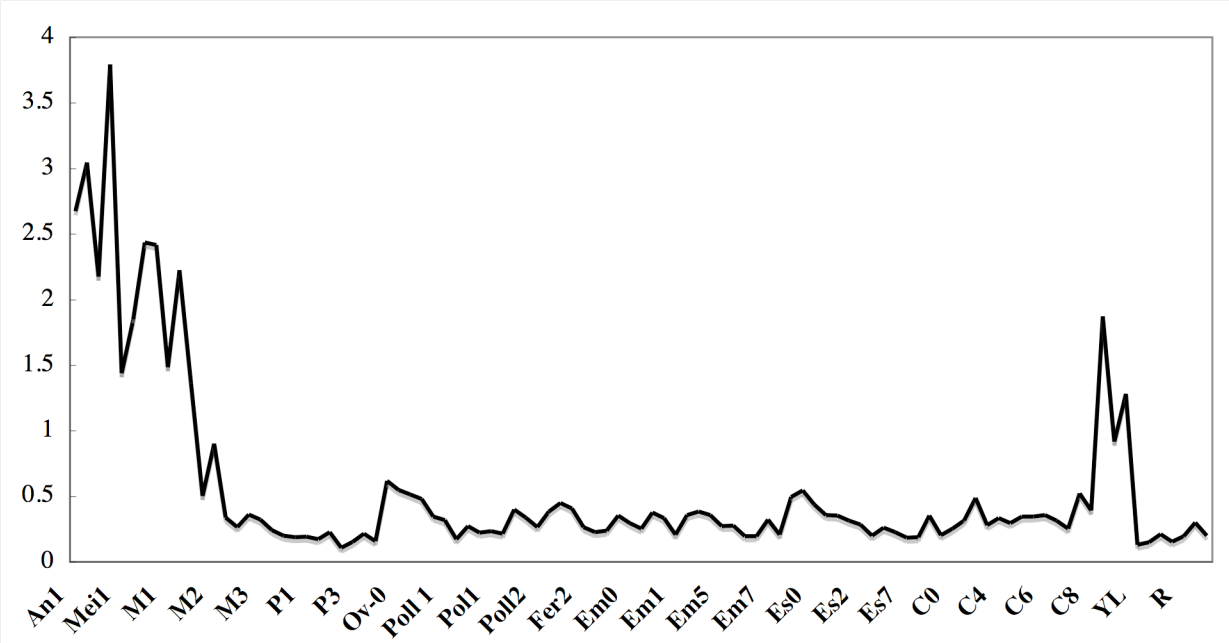


Figure IV- 3. Expression analysis of *OsSPL14* using expression profiles from the inhouse DB 'Rice Expression Atlas'

Vertical axis indicated normalized signal values in the expression atlas. All 98 samples of our microarray experiment were displayed on the horizontal axis from left to right according to the stages from top to bottom stages in Table I- 1, respectively.

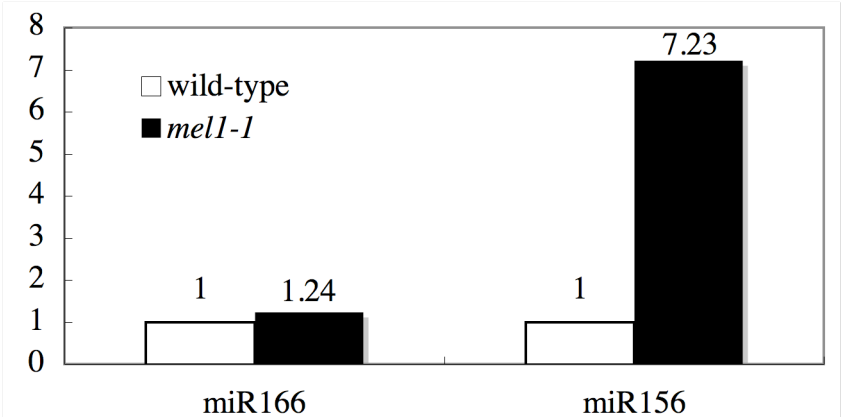


Figure IV- 4. Comparison assay as determined by quantitative realtime PCR of *miR156* levels in WT and *mel1-1* panicle

The *miR156* levels in young panicles of wild-type and *mel1-1* mutant. were indicated. The levels of *miR166* (left) and *miR156* (right) in wild-type (white bars) and *mel1-1* (black bars) were shown. Both *miR166* and *miR156* cDNA produced from 10 ng total RNA was included .

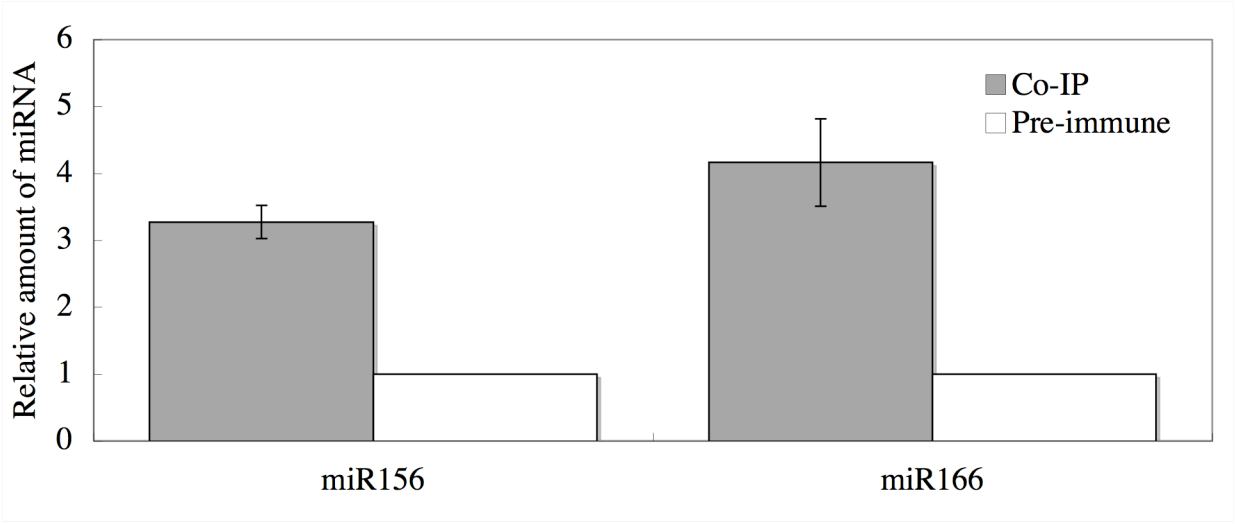


Figure IV- 5. Analysis of miRNA levels in co-IP samples by quantitative real-time PCR

The *miR156* and *miR166* levels in young panicles of co-IP samples were indicated. The levels of *mel1-1* co-IP (grey bars) and pre-immune (white bars) were indicated. Both miR166 and *miR156* cDNA produced from 10 ng total RNA was included in each. Vertical bars indicate standard error.

General discussion

In this thesis, I focused on the characterization of early expressing genes in germ cell development specifically in relation to MEL1 function. MEL1 is well characterized Argonaute gene that has essential functions in germ cell development identified in my laboratory. The rice MEL1 is only one available gene to use for investigating a genetic system conducting plant germ cell initiation and maintenance. A large portion of genes identified specific in very early stages in germ cell development had unknown function. However, comparison of expressed genes between wild-type and *mel1-1* mutant revealed many possible causal genes for *mel1-1* dysfunction, down- or up-regulated in *mel1-1* mutant. In these genes showing altered expression, I could find a highly probable target gene *OsSPL14* of MEL1 Argonaute recognition. *OsSPL14* is known to contain *miR156* sequence (Xie et al. 2006) and is revealed in this study to express specifically in early germ cell (Figure IV-3) and up-regulated much in *mel1-1* mutant (Figure IV-1). In addition, *miR156* also showed much up-regulated expression in *mel1-1* mutant (Figure IV-4). These results indicated that *OsSPL14* could be a strong candidate of direct target of the MEL1 AGO mediated by a plant specific microRNA *miR156* and have an essential function in germ cell development. In this consequence, MEL1 should act as a regulator of *OsSPL14* expression. However, another results also indicated that the repression of many stress- and hormone-related genes and repetitive sequences such as transposons and retrotransposons should be one of the important roles of MEL1 in germ cell development.

Spacial and temporal expression analysis revealed that the rice germ-cell specific AGO, MEL1, acts in the archesporial initial cells within the male reproductive organs (Figure II-2). The MEL1 in itself is not required for establishing the identity of archesporial cells, because even in the *mel1* mutant, the archesporial and sporogenous cells exhibited their cell features such as greater volume of nuclei and cytoplasms than somatic cells (Nonomura et al. 2007). Rather, the MEL1 is thought to act in the maintenance of archesporial- and sporogenous-cell identity by repressing the

genes required for reproductive organ development, but not for germ-cell initiation.

Small RNA molecules directly associated with the MEL1 AGO should be multiple. As a preliminary result, the cDNA library derived from co-IP and fractionation of small RNAs using anti-MEL1 antibody included that many kinds of repetitive sequences such as ribosomal RNAs and transposable elements (Dr. Niihama, personal communication). In addition, the co-IP and deep sequencing of small RNAs for the *Arabidopsis* ortholog *AGO5* of rice *MEL1* reveal that the *Arabidopsis AGO5* can associate with both 21- nt miRNAs and 24-nt siRNAs (Takeda et al. 2008). In this thesis, the rice MEL1 was suggested also to associate with miRNAs and probably siRNAs, generally generated in pathways of transposon repression, heterochromatinization, and so on (Kalmykova et al. 2005, Saito et al. 2006, Brower-Toland et al. 2007). It is clear that most of the genes up- and down-regulated in the microarray analysis in this thesis are secondary affected ones by the *mel1* mutation. To identify genes primarily affected by the *MEL1*, it will be necessary to give careful consideration to microarray data of this thesis with co-IP RNAs and public microarray data of rice reproduction.

The T-DNA tagged lines of the *AtAGO5* and *AtAGO9* displayed no mutated phenotype, even though both *AtAGO5* and *AtAGO9* express specifically in reproductive organs same as the rice MEL1 AGO (Takeda et al. 2007). Further analyses for molecular functions of the MEL1 AGO will contribute to understand how does the plant cell prepare its state for reproductive events such as meiosis and gametogenesis.

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