A WIDESPREAD IMPACT ON SMALL RNAs AND GENE NETWORKS IN RICE MSP1/OSTDL1A MUTANTS, PARTNERS WITH KEY ROLES IN ANther DEVELOPMENT

by

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ABSTRACT

Dissection of the genetic pathways and mechanisms by which anther development occurs in grasses is crucial for both a basic understanding of plant development and for traits of agronomic importance like male sterility. In rice, MULTIPLE SPOROCYTES1 (MSP1), a leucine-rich-repeat receptor kinase, play an important role in anther development by limiting the number of sporocytes. OsTDL1a (a TPD1-like gene in rice) encodes a small protein which acts as a cofactor of MSP1 in the same regulatory pathway. In this study, we analyzed small RNA and mRNA changes in different stages of spikelets from wildtype rice, and from msp1 and ostdl1a mutants. Analysis across different stages of rice spikelets of the small RNA data identified miRNAs demonstrating differential abundances. miR2275 was depleted in the two rice mutants; this miRNA is specifically enriched in anthers and functions to trigger the production of 24-nt phased secondary siRNAs (phasiRNAs) from PHAS loci. We observed that the 24-nt phasiRNAs as well as their precursor PHAS mRNAs were also depleted in the two mutants. Based on comparisons of transcript levels across the spikelet stages and mutants, we identified 22 transcription factors as candidates to have roles specific to anther development, potentially acting downstream of the OsTDL1a-MSP1 pathway. An analysis of co-expression identified three Argonaute-encoding genes (OsAGO1d, OsAGO2b, and OsAGO18) that accumulate transcripts coordinately with phasiRNAs, suggesting a functional relationship. By mRNA in situ analysis, we demonstrated a strong correlation between the
spatiotemporal pattern of accumulation of these OsAGO transcripts with previously-published phasiRNA accumulation patterns from maize.
Chapter 1

INTRODUCTION

Rice, as a major crop, has been widely used as a monocot model species to explore the genetic basis of flower development in higher plants (Yoshida and Nagato, 2011). Rice anther development is one of the major topics studied in rice flower development, and changes in the cytological morphology in different developmental stages of rice anthers have been well-described (Zhang and Wilson, 2009; Zhang et al., 2011). Tapetum and microsporocyte specification is a crucial event in male fertility, occurring at early stages of anther development in plants; a number of genes have been discovered as regulators of cell fate specification (Zhang and Yang, 2014). For example, EXCESS MICROSPOROCYTES 1 (EMS1, or EXTRA SPOROGENOUS CELLS, EXS), a member of the leucine-rich repeat receptor-like kinase (LRR-RLK) family, specifies tapetal identity and limits the number of pollen mother cells (PMCs) in Arabidopsis (Canales et al., 2002; Zhao et al., 2002). The small secreted protein, TAPETAL DETERMINANT 1 (TPD1), was reported as a ligand of EMS1/EXS with a deterministic role in the cell fate of the tapetum (Jia et al., 2008).

The EMS1/EXS ortholog in rice is MULTIPLE SPOROCYTE (MSP1) (Nonomura et al., 2003), while the ligand protein TPD1 has two TPD1-like orthologs in rice, including OsTDL1A and OsTDL1B, among which OsTDL1A (also known as...
MICROSPORELESS2, MIL2) may interact with MSP1 (Zhao et al., 2008; Hong et al., 2012). Although msp1 and ostdl1a mutants display defects in anther and ovule development, both mutants show a phenotype of complete male sterility, while partially maintaining female fertility (Nonomura et al., 2003; Hong et al., 2012; Yang et al., submitted). MSP1, as a receptor-like kinase in an upstream signaling pathway, affects many other downstream genes involved in rice anther development. For example, a loss of function of MSP1 will largely downregulate the expression of other genes involved in rice anther development, such as Undeveloped Tapetum1 (UDT1) and Tapetum Degeneration Retardation (TDR) (Jung et al., 2005; Li et al., 2006).

Maize MAC1 is the ortholog of OsTDL1A, having similar functions in limiting archesporial cell proliferation in maize anther (Wang et al., 2012). Therefore, the OsTDL1A-MSP1 pathway plays a central role in early stages of rice anther development to simultaneously specify the tapetum and limit the number of pollen mother cells.

Small RNA pathways play roles in both flower development and gametogenesis in plants. Some conserved miRNAs appear to function similarly in flower development across different plant species, such as Arabidopsis, tomato, petunia, rice and maize, etc. (Luo et al., 2013). In rice, miR172 targets APETALA2 (AP2) genes controlling inflorescence architecture and spikelet meristem identity (Zhu et al., 2009; Lee and An, 2012). A number of rice SQUAMOSA Promoter Binding Protein-Like (OsSPL) genes, including OsSPL14, are targeted by miR156; this pathway has a role in flowering time, panicle architecture, grain yield, and other
developmental phenotypes (Xie et al., 2006; Jiao et al., 2010; Miura et al., 2010). Other miRNAs, such as miR159 and miR164 are also reported to be involved in rice floral development (Tsuji et al., 2006; Adam et al., 2011). In addition to miRNAs, trans-acting siRNAs (tasiRNAs), dependent on the activities of RNA-DEPENDENT RNA POLYMERASE 6 (RDR6) and DICER-LIKE 4 (DCL4), generated from noncoding transcripts like TAS3, play roles in both vegetative and reproductive development in both rice and maize by targeting genes encoding auxin response factors (ARFs). For example, the rice mutant of SHOOTLESS2 (SHL2), the ortholog of Arabidopsis RDR6, displays a severe phenotype of misregulation of adaxial-abaxial polarity patterning in both the lemma and anther (Toriba et al., 2010), while the maize mutant leafbladeless1 (lblr, a loss of function of an SGS3 ortholog, SUPPRESSOR OF GENE SILENCING 3) is defective in tasiRNA biogenesis and shows a pleotropic phenotype, including sterile male inflorescence (Nogueira et al., 2007). The RDR6-dependent small RNA biogenesis pathway not only produces tasiRNAs, but also yields two large populations of phased secondary siRNAs (phasiRNAs) in the reproductive tissues of monocots (reviewed in Fei et al., 2013). Data have suggested that these 21- and 24-nt reproductive phasiRNAs, triggered by miR2118 and miR2275 respectively, may play crucial roles in microgametogenesis in maize, because the accumulation of phasiRNAs shows highly stage-specific patterns in maize anther development (Zhai et al., 2015). In addition, the AGO protein MEIOSIS ARRESTED AT LEPTOTENE1 (MEL1), previously demonstrated to be essential for sporogenesis
in rice anthers (Nonomura et al., 2007), has recently been shown to recruit 21-nt phasiRNAs (Komiya et al., 2014).

In this study, we systematically characterized small RNA and mRNA changes across early developmental stages of rice spikelets in wildtype, *msp1* and *ostdl1a* backgrounds using deep sequencing data generated from both small RNA and strand-specific RNA-seq libraries. We showed comprehensive changes of miRNAs, phasiRNAs, and mRNAs in early stages of rice spikelet development. Importantly, the reproductive phasiRNAs displayed stage-specific expression patterns during early stages of anther development, suggesting that the timing of phasiRNA biogenesis is crucial in rice microsporogenesis. Furthermore, we identified three rice AGOs that are coordinately expressed with 21- and 24-nt phasiRNAs, in addition to MEL1.
Chapter 2

METHODS

2.1 Plant Materials and Growth Conditions

All the rice plants used in this study were in a genetic background of variety 9522, a japonica rice. The two male-sterile mutants, ostdl1a and msp1-4, are from a rice mutant library made by $^{60}$Co γ-ray radiation; the molecular details of these mutants are described in (Yang et al., submitted). Plants were grown in the paddy field of Shanghai Jiao Tong University.

2.2 Small RNA and RNA-seq Library Construction

For small RNA library construction, total RNA enriched for small RNA was extracted. The small RNA faction between 18- to 30-nt in length was collected by gel separation, then ligated to 5’ and 3’ adaptors and purified. These small RNAs were reverse transcribed by RT-PCR and finally amplified via PCR. For RNA-seq libraries, after the total RNA extraction and DNase I treatment, magnetic beads with oligo(dT) were used to isolate mRNA. Mixed with the fragmentation buffer, the mRNA was fragmented into short fragments. The cDNA was synthesized using the mRNA fragments as templates. Short fragments were purified for end repair and single nucleotide A (adenine) addition, ligated to adapters, and then the second strand was degraded using UNG (Uracil-N-Glycosylase). After agarose gel electrophoresis, the suitable fragments were selected for the PCR amplification as templates. All the small
RNA and RNA-seq libraries were sequenced on an Illumina HiSeq 2000 platform by BGI (BGI-Shenzhen, China).

2.3 Small RNA Data Analysis

Small RNA sequencing data were preprocessed by removing adapters, and then mapped to the version 7.0 of rice genome assembly from the Rice Genome Annotation Project Database using the program Bowtie (Langmead et al., 2009). Small RNA reads that were mapped to the tRNAs and rRNAs were filtered, and reads mapped to the rice genome from different libraries were normalized to reads per 5 million reads (RP5M) for comparisons. The Bioconductor (www.bioconductor.org) package ‘edgeR’ was used for small RNA differential analysis ($P < 0.05$, FDR < 0.05). Rice miRNA sequences were downloaded from miRBase (Release 21) (Kozomara and Griffiths-Jones, 2014). The R (www.r-project.org) package ‘pheatmap’ was used to represent the average abundance of miRNAs from three biological replicates. PHAS locus identification was performed using the same method described before (Zhai et al., 2011), with the same cutoff used in our earlier study in maize (Zhai et al., 2015). The overall phasiRNA abundance for each PHAS locus was calculated by summing up the normalized abundance of 21- or 24-nt small RNAs generated from each corresponding 21-PHAS and 24-PHAS locus.
2.4 RNA-seq and GO Enrichment Analysis

Paired-end RNA-seq reads (90 bp x 2) were mapped to the rice genome sequences allowing no more than 2 mismatches using ‘Tophat’ (Trapnell et al., 2009). The BAM files generated by ‘Tophat’ were sorted and indexed using ‘SAMtools’ (Li et al., 2009), and then visualized via Integrative Genomics Viewer (IGV) (Robinson et al., 2011). The program ‘Cufflinks’ was used for transcriptome assembly, differential analysis of gene expression, and FPKM value calculation (Trapnell et al., 2012). For differential analysis of gene expression, we used ‘q-value < 0.01’ and ‘fold change > 2’ as cutoff. Bar graphs and line charts representing FPKM values of gene expression were plotted using the Bioconductor package ‘cummeRbund’ (Trapnell et al., 2012). Heatmaps for RNA-seq data were generated using the R package ‘gplots’. Lists of differentially expressed genes were input into the webserver PlantGSEA for GO terms enrichment analysis (Yi et al., 2013). The cutoff used for significantly enriched GO terms is ‘$P < 0.05$’ and ‘FDR < 0.05’. The results of PlantGSEA were further visualized using REVIGO (Supek et al., 2011).

2.5 Microarray Data Analysis

Gene lists were input into the webserver Rice Oligonucleotide Array Database (Cao et al., 2012). Specific public microarray datasets were selected to acquire the abundance values of each gene. The normalized gene expression values were further visualized as heatmaps using the R package ‘pheatmap’.
2.6 *In Situ* Hybridizations

Freshly collected samples were fixed in FAA and dehydrated in a series of graded ethanol concentrations; these samples were then infiltrated with Histo-clear II, embedded in Paraplast Plus, and subsequently processed into 6-μm thick sections using a Leica RM2245 rotary microtome. Templates for RNA probe synthesis were amplified by PCR from the cDNA. Probes transcribed *in vitro* under T7 promoter with RNA polymerase, using the DIG RNA labeling kit (Roche). The RNA *in situ* hybridizations were carried out as described by Kouchi and Hata (1993) and Li et al. (2006). The forward and reverse RT-PCR primers are given below (from 5’ to 3’):

*OsAGO1d*, 5’-GCAATACCACCCACAAGGAC-3’ and 5’-

GGTTCCAATACTCCACTTCC-3’; *OsAGO18*, 5’-

CAGTATAACAGTACGGAACGC-3’ and 5’-TGTCATTACAACAGTACGGAGG-3’.
Chapter 3
RESULT

3.1 Comparative Analysis of Small RNAs in Spikelets of Wildtype and Mutant Rice

Small RNAs play crucial roles in mediating both transcription and translation, with different classes distinguishable by their distinct biogenesis pathways (Axtell, 2013). The recent discovery of reproductive phasiRNAs in monocots indicate that this special class of small RNAs may be important for male reproduction, although the underlying mechanism is still unelucidated (Johnson et al., 2009; Song et al., 2012; Zhai et al., 2015). To assess small RNA and mRNA changes across different stages of rice spikelet development and to understand how they are impacted by perturbation of the OsTDL1A-MSP1 pathway, we prepared small RNA and RNA-seq libraries from spikelets of wildtype rice cultivar 9522, and the mutantsmsp1-4 (“msp1” hereafter) and ostdl1a. We performed three biological replicates for each genotype and stage. The lengths of rice spikelets correspond to different anther developmental stages (Fig. 1). Specifically, stage 3 (0.15 to 0.2 mm), stage 5 (0.25 to 0.3 mm) and stage 7 (0.4 to 0.45 mm) of rice anthers correspond to 0.5 to 0.6 mm, 1.0 to 1.5 mm, and 2.5 to 3.0 mm rice spikelets respectively (Zhang et al., 2011) (Figure. 1A). Hereafter, we will refer to these sizes of rice spikelets (0.5 to 0.6 mm, 1.0 to 1.5 mm, and 2.5 to 3.0 mm) as stage 3, stage 5, and stage 7 spikelets, respectively.
Sequencing reads of small RNAs were aligned to the rice genome and normalized to 5 million (5M), and analyzed the distribution of lengths in different stages. The three replicates were nearly identical (Figure 1). The 24-nt small RNAs account for ~75% of all small RNAs at stage 3 in wildtype rice. Interestingly, with the development of spikelets, there is a shift in the predominant size class, with 21-nt small RNAs the largest proportion (nearly 50%) in the profile at stage 5. In stage 7, the proportion (~55%) of 24-nt small RNAs is once again larger than the 21-nt counterpart (<40%), but is far smaller than that in stage 3. The fluctuation of small RNA percentages in different sizes accompanied by spikelet development may represent shifts in small RNA biogenesis and transcriptome changes in rice reproductive tissues, perhaps consistent with prior reports in maize (Zhai et al., 2015). Similar to the wildtype spikelets, in the two mutants, changes in small RNA size proportions occurred more significantly in the transition from stage 3 to stage 5 than from stage 5 to stage 7. The only apparent difference was a reduction in the proportion of 21-nt small RNAs in the mutants in stage 5 spikelets (Figure 1B). To investigate this difference or others less readily apparent, we checked levels of small RNAs that are often 21-nt in length, miRNAs and phasiRNAs.
Figure 1. Small RNA size distribution in different developmental stages and backgrounds of rice spikelets. (A) Schematic representation of rice anther structures in different stages of spikelets of wildtype rice. Each layer of cells are indicated by arrows: epidermis (E); primary parietal cell (PPC); archesporial cell (Ar); endothecium (En); middle layer (ML); tapetum (Ta); sporogenous cell (Sp). Small RNA size distribution in different stages of wildtype rice spikelets are shown below. (B) Schematic representation of rice anther structures in different stages of spikelets of msp1 and ostd11a mutants. Excessive archesporial cell (eAr); unknown identity cell (UIC); excessive sporogenous cell (eSp). Small RNA size distribution in different stages of mutant spikelets are shown below.
3.2 miRNA Expression Patterns in Different Developmental Stages of Rice

Spikelets

A number of miRNAs have characterized roles in plant development, targeting several families of transcription factors or other development-related genes (Jones-Rhoades et al., 2006; Chen, 2009). miRNAs, such as miR156 and miR172, have been proven to control flower development at a post-transcriptional level in both Arabidopsis and rice (Aukerman and Sakai, 2003; Chen, 2004; Xie et al., 2006; Wu et al., 2009; Zhu et al., 2009; Jiao et al., 2010; Lee and An, 2012). Therefore, the expression patterns of miRNAs are important to our understanding of rice spikelet development.

Genome-wide differential analysis of miRNA expression was performed in wildtype rice across different stages of spikelet development. From stage 3 to stage 5, >60 miRNAs increased significantly, while only 18 miRNAs decreased at the same time period; from stage 5 to stage 7, the numbers of miRNAs with significantly different levels (up or down) were both fewer than 20 (Figure 2; Table 1).
Figure 2. miRNA expression in different developmental stages of spikelets in wildtype 9522. (A) Differentially expressed miRNAs in different developmental stages of spikelets of wild type rice. Only miRNAs with abundance greater than 50 RP5M are included in the heatmap. (B) 20 nt and 21 nt miR156 display distinct expression patterns; levels indicated as ‘20/21’ show the sum of abundance of the 20 and 21 nt isoforms. (C) Expression levels of miR156 target genes in different stages of rice spikelet development.
Table 1. Numbers of differentially abundant miRNAs, identified by pairwise comparisons of rice spikelets in different stages and backgrounds.^[1]

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^[1] Numbers indicate (by arrows) significantly up- and down-regulated miRNAs. (P < 0.05, FDR < 0.05). miRNAs are from miRBase release 21. “S3”, “S5”, and “S7” refer to stages 3, 5 and 7 of anthers, respectively.

Comparing different stages in the msp1 and ostdl1a mutant backgrounds, similar sets (both qualitatively and quantitatively) of differentially accumulating miRNAs were observed, suggesting that the male sterile phenotype of these mutants has a limited impact on miRNA levels, relative to wildtype. This was further confirmed by the observation that very few miRNAs were identified as significantly different in their levels when comparing two mutants with the wildtype (Table 1). Interestingly, among the few impacted miRNAs in the two mutants, miR2275, which triggers 24-nt phasiRNA production, was totally abolished.

Among the miRNAs differentially expressed in wildtype rice across the developmental stages, the level of miR164 showed an ~7-fold increase from stage 3 to stage 7, whereas miR172 showed a dramatic decline of ~4-fold from stage 3 to stage 5, then remained relatively steady to stage 7 (Figure 3A). Prior work using PARE/degradome data confirmed the targets of a number of miRNAs in rice (Li et al.,
From RNA-seq data, we obtained the expression levels of both miR164 and miR172 target genes. Transcript levels of the most abundant miR164 target (LOC_Os12g05260) decreased from 180 FPKM (stage 3) to 125 FPKM (stage 5), and the expression level increased slightly in stage 7 compared to stage 5, and a similar trend was observed for the target gene LOC_Os06g23650 (Figure 3B); these were the only two targets that showed an inverse relationship with miR164 abundance. Three miR172 targets showed an inverse correlation with miR172 levels (Figure 3C). miR156 showed an interesting pattern across developmental stages of rice spikelets: a 21-nt miR156 isoform increased gradually from stage 3 to stage 7, while the 20-nt miR156 firstly decreased from stage 3 to stage 5, and then increased at stage 7 (Figure 3A). The underlying mechanism is unknown, but it is possible that these two isoforms of miR156 are generated from different MIR156 genes, and are differentially regulated with distinct targets or cellular expression patterns, exerting distinct patterns of control on the expression of OsSPLs. Similar to miR164 and miR172, a subset of miR156 targets showed an inverse correlation with miR156 (Figure 3D).
Figure 3. miRNA and target transcript levels in different stages and backgrounds of rice spikelets.

(A) Abundances of miR164 and miR172 across the three stages, representing an average of the three replicates. (B) Levels of the mRNA targets of miR164 (validated in prior publications – see main text), as measured by RNA-seq. Values represent the average of three replicates. miR164 target gene expression. (C) Levels of predicted mRNA targets of miR172, measured as in panel B.
Overall, the abundance levels of miRNA target transcripts in the two mutants were quite similar to those in wildtype, indicating that mutations in the OsTDL1A-MSP1 pathway had a limited impact on rice miRNAs and their targets in spikelets (Figure 3B-D). Among the miRNA targets validated from prior work, only a subset had the expected inversely correlation with miRNA levels; thus, other factors may impact target transcript levels including both transcriptional (e.g. transcription factors and epigenetic regulation) and post-transcriptional (e.g. mRNA turnover or sequestration) factors. Taken together, these data indicate that miRNAs together with their target genes are dynamically modulated during early stages of rice spikelet development, and are largely independent of the OsTDL1A-MSP1 pathway.

3.3 Timing of PHAS Transcript Expression and PhasiRNA Production

PhasiRNAs, in addition to miRNAs, represent another class of small RNAs of great interest, because two populations are exclusively abundant in the reproductive tissues of monocots (Arikit et al., 2013). More specifically, miR2118 and miR2275 are triggers of 21-nt and 24-nt reproductive phasiRNAs, respectively, in both rice and maize (Song et al., 2012; Zhai et al., 2015). We checked the abundance levels of both miRNAs in different stages and backgrounds; miR2118 abundance peaked at stage 5 in wildtype spikelet libraries, reaching 320 reads per 5 million reads (RP5M), and then dropped to 130 RP5M at stage 7 (Figure 4A). Similar abundances were observed in msp1 and ostdl1a mutants, indicating that miR2118 is not impacted in both mutants, possibly explained by miR2118 accumulation in the epidermis of anthers (Zhai et al.,
2015), a cell layer apparently not defective in the msp1 and ostdlla mutants or in the mac1 maize mutant. In contrast to miR2118, in wildtype spikelets, miR2275 was not expressed in stage 3, and then increased in stage 5 (~85 RP5M) and stage 7 (~240 RP5M) (Figure 4B); miR2275, as mentioned above, is essentially absent in both msp1 and ostdlla mutants, at all stages.

We calculated both 21- and 24-nt phasiRNA abundances to see how phasiRNAs change during rice spikelet development and whether they are affected in the two mutants. Phasing analysis resulted in 1843 21-PHAS loci and 50 24-PHAS loci. By summing phasiRNA abundances from each PHAS locus, we obtained the overall phasiRNA abundances for both 21- and 24-nt phasiRNAs. In wildtype rice, the overall abundance of 21-nt phasiRNAs was ~120,000 RP5M in stage 3, increasing by >10 fold in stage 5, and then decreased by <30% in stage 7 in wildtype spikelets (Figure 4C). Compared to wildtype, 21-nt phasiRNAs had a very similar pattern in two mutants, although with a slightly lower total abundance in stage 5. The 24-nt counterparts had only a few hundred reads in stage 3 and stage 5, but then increased to more than 180,000 RP5M in stage 7 of wildtype spikelets (Figure 4D). Consistent with the observation that miR2275 is absent in msp1 and ostdlla mutants at all stages, 24-nt phasiRNAs were also diminished in both mutants. Considering the developmental defect in these mutants is largely in the anthers and not other tissues in the spikelets (Yang et al., submitted), we infer that the loss of 24-nt phasiRNAs is due to the defective anther development. Furthermore, as in maize (Zhai et al., 2015), rice 21-nt phasiRNAs initiate early stage in anther development, 24-nt phasiRNAs
appeared later coincident with or just before meiosis, and the absence of miR2275 and 24-nt phasiRNAs in msp1 and ostdl1a mutants is similar to maize mac1 (the ortholog of ostdl1a in maize).
Figure 4. miR2118, miR2275 and phasiRNA abundances in rice spikelets. Levels of miR2118 (panel A), miR2275 (panel B), miR2118-triggered 21-nt phasiRNAs (panel C), miR2275-triggered 24-nt phasiRNAs (panel D) in different stages and backgrounds of rice spikelets. (E) The percentage of 21-nt (above, blue slices with percentage numbers) and 24-nt phasiRNAs (below, orange slices with percentage numbers) out of the total population of 21-nt and 24-nt genome-matched small RNAs in wildtype rice spikelets.

We next calculated the proportion of 21- and 24-nt phasiRNAs in the entire genome-matched populations of either 21- and 24-nt small RNAs in rice spikelets (Figure 4E). We found that 21-nt phasiRNAs accounted for 24% of total 21-nt siRNAs in wildtype rice spikelets, increasing to a remarkable 74% of the total in stage 5 and then reduced slightly to 68% at stage 7. This compares to 60% of all 21-mers at the peak in maize (Zhai et al., 2015); this higher proportion in rice spikelets versus isolated maize anthers may reflect that there are ~4-fold as many genomic loci generating 21-nt phasiRNAs in rice compared to maize. The 24-nt phasiRNAs were almost absent at stages 3 and 5, followed by a substantial increase to 7% of the total at stage 7 (Figure 4E). This compares to 64% of the total 24-mers in isolated maize anthers (Zhai et al., 2015); this much lower proportion in rice may reflect that there are fewer loci, perhaps these are more weakly expressed, or perhaps the peak abundance of 24-nt phasiRNAs is outside of stages 3 to 7. Overall, these results reveal that both miRNA triggers and phasiRNAs are largely upregulated at specific stages in rice anthers.
Figure 5. 21-nt PHAS precursor transcripts peak coincident with their phasiRNA products. We examined a randomly selected 21-PHAS locus on rice chromosome 3 in wild type (9522) rice to assess the peak of abundance relative to the 21-phasiRNAs that peak at stage 5. Each dot is a small RNA; light blue are 21-nt sRNAs, green are 22-nt, orange are 24-nt. Yellow shaded regions are predicted DNA transposons; pink shaded regions are predicted retrotransposons; orange shaded regions are inverted repeats. The small pink box is an annotated miRNA. The strand-specific RNA-seq data is represented in an IGV screenshot; blue bars are top-strand reads, and red bars are bottom strand reads.
We next examined the phasiRNA precursors (PHAS transcripts) in the RNA-seq data to assess the correlation of phasiRNA and PHAS mRNA levels. As many 21-PHAS loci are found in large clusters in the rice genome (Johnson et al., 2009), we selected a representative 21-PHAS cluster on chromosome 3 for this analysis. Our earlier observations in wildtype rice showed a stage 5 peak for 21-nt phasiRNAs, which reduced slightly at stage 7 (Figure 4C); in the RNA-seq data at stage 3 and stage 5, 21-PHAS transcripts levels were generally consistent with phasiRNA production; however, at stage 7, 21-PHAS transcripts levels were very low (Figure 5) while phasiRNA levels were still high. Perhaps this is an indication that 21-nt phasiRNAs persist longer than their precursor transcripts, possibly for a prolonged role in later stages of spikelet development. As for msp1 and ostdl1a mutants, levels of neither 21-nt phasiRNAs nor 21-PHAS transcripts were impacted (stage 5 for both mutants is shown in Figure 6). Consistent with the precursor molecules consisting of a polyadenylated mRNA paired with an RDR6-derived antisense strand, the strand-specific RNA-seq reads were mapped to only one strand at a given PHAS locus (Figure 5).
Figure 6. 21-nt phasiRNAs and precursor transcripts were unaffected in both *msp1* and *ostdl1a* mutants at stage 5. A cluster of phasiRNA loci from rice chromosome 3 were measured in both the *msp1* and *ostdl1a* mutants; the wildtype data are shown in Figure 4. The RNA-seq data is an IGV screenshot; blue bars are top-strand reads, and red bars are bottom strand reads.
Figure 7. 24-nt phasiRNAs are strongly impacted in stage 7 spikelets of the two rice mutants. (A) RNA-seq data shown in an IGV-generated screenshot of the locus shown in panel B (Chr6: 25,434,100–25,437,750), for stage 5 and stage 7 spikelets of wildtype and stage 7 of the two mutants. Transcripts producing 24-nt phasiRNAs were highly abundant in Stage 7 of wild type 9522 anthers, but absent in the two mutants. (B) 24-nt phasiRNA levels increase substantially in stage 7 of wild type rice relative to stage 5, and are almost absent in stage 7 of the two mutants. Each row shows a different stage of spikelets as indicated at the left; the dots and colors are as described in Figure S3. Each column of images shows a different genotype, as indicated at the top.
An examination of the RNA-seq data for 24-PHAS loci showed a strong boost in transcript levels from stage 5 to stage 7 in wildtype rice anthers (Figure 7). However, unlike 21-PHAS loci, both 24-nt phasiRNAs and 24-PHAS mRNAs were absent in the RNA-seq data from msp1 and ostd11a mutants (Figure 7). In summary, sequencing data revealed the developmental modulation of phasiRNA biogenesis in rice anthers, with 24-phasiRNAs and their precursors disrupted in the msp1 and ostd11a mutants, perhaps reflecting an intimate association of these transcripts, anther development and microsporogenesis in rice.

3.4 Global Gene Expression Changes in Rice Spikelet Development

The process of flower development involves wide-ranging changes in the activity of gene regulatory networks, as revealed in Arabidopsis by microarray or high-throughput sequencing (Wellmer et al., 2006; Zhang et al., 2014; Ryan et al., 2015). Therefore, we performed differential analysis of our RNA-seq data from different wildtype stages of spikelets to provide comparable information about which transcripts accumulate during reproductive development in rice. We identified >2500 annotated genes that were significantly upregulated from stage 3 to stage 5; only ~800 genes were down-regulated using the same cutoff. An analysis for enrichment in gene ontology (GO) terms indicated upregulated genes were involved in a variety of processes from stage 3 to stage 5, such as ‘metabolic process’, ‘biosynthetic process’, ‘gene expression’, ‘photosynthesis’, ‘cellular component movement’, ‘peptide transport’ etc. (Figure 8A). Perhaps consistent with the burst in 21-nt phasiRNAs at
stage 5, upregulated genes were enriched in the category of ‘regulation of nucleobase-containing compound metabolic process’. In addition, ‘oxidation-reduction process’ was also observed, perhaps reflecting recent evidence that hypoxia determines germ cell fate in plants (Kelliher and Walbot, 2012). For the downregulated genes, other GO terms were enriched, such as ‘nitrogen compound metabolic process’, ‘cell cycle’, ‘mitotic M phase’ (Figure 8C). In stage 7, upregulated genes were enriched in additional categories such as ‘cellular protein modification process’, ‘macromolecule localization’, ‘signaling’, and metabolic processes for a few compounds, including alcohol, lipid, and amine (Figure 8B). At the same stage, other processes were downregulated, including genes labeled as ‘translation’, ‘photosynthesis’, ‘generation of precursor metabolites and energy’ (Figure 8D), which might be related to the completion of meiosis. In summary, our RNA-seq data contained evidence of widespread changes in mRNA abundances in the early stages of rice spikelet development.
A few genes are known to act downstream of OsTDL1A-MSP1 in rice (reviewed in Wang et al., 2013), but these analyses may not be exhaustive. We assessed whether the mRNA abundance of these genes known to be downstream was impacted in msp1 and ostdl1a mutants. The levels of UDT1 and GAMYB showed a ~2-
fold decrease in stages 5 and 7 compared to wildtype (Figure 9). TDR, a transcription factor downstream of UDT1, displayed a >10-fold decrease in both two mutants at stage 7 (Figure 9). To understand the global impact on gene expression in the msp1 and ostdl1a mutants, we performed genome-wide differential analysis of mRNA transcript levels comparing both mutants with wildtype, at stages 3 and 5 of spikelet development. This will potentially identify more genes downstream of MSP1 and OsTDL1A, perhaps like the case of UDT1, GAMYB, and TDR, as mentioned above.

Figure 9. mRNA levels of genes downstream of MSP1 and OsTDL1a in different stages and backgrounds or rice spikelets.
Global differential analysis showed highly similar gene expression patterns for
msp1 and ostdl1a mutants (Figure 9), not unexpected as these two proteins act in the
same pathway in anther development (Zhao et al., 2008). Among the downregulated
genes in mutants, we identified 474, 673, and 577 genes that are commonly
downregulated in msp1 and ostdl1a mutants at stages 3, 5, and 7, respectively (Figure
10A). We next assessed how many genes that may potentially encode proteins having
functions of DNA binding or transcription factor activity among them, as these are
important components of developmental pathways. Using PlantGSEA, a total of 51,
101, and 32 transcription factor (TF)-like genes were identified from the commonly
downregulated gene sets. To further confirm whether these TF-like genes have tissue-
specific expression patterns in rice, we examined their expression patterns using
public microarray datasets (GSE6893 and GSE13988). We found that 24 TF-like
genes were identified as specifically expressed in rice inflorescence and at early and
middle stages of anther development, including UDTI and TDR (Figure 10B-D).
These 24 genes can be categorized into seven groups according to their encoded DNA-
binding domains (Figure 10C). Among them, seven belong to the ‘helix-loop-helix
DNA-binding’ family, five belong to the MYB family, four belong to the AP2 family,
and two are in the ‘B3 DNA-binding domain containing’ family. The ‘homeobox
domain containing’ and WRKY families each have one gene, and four of the 24 genes
are putative transcription factors. These results indicate that a number of transcription
factors might function genetically downstream of the OsTDL1A-MSP1 pathway.
Future work focusing on these 24 TF-like genes might expand our understanding of
developmental networks in rice anther development.
Figure 10. Differentially expressed genes in *msp1* and *ostdl1a* mutant spikelets compared to wild type 9522 at different stages. (A) Expression levels of the union set of differentially expressed genes in *msp1* and *ostdl1a* mutants compared to wildtype rice. Venn diagrams below show commonly downregulated genes in both mutants at corresponding stage. (B) The 24 transcription factor-like genes identified as down-regulated in common in the mutants. These genes were validated as tissue- and stage-specific genes in rice anthers. (C) Categories of these identified 24 transcription factor-like genes. (D) Tissue specific expression of these TFs confirmed by public microarray datasets.
3.5 Co-expression Analysis Reveals Additional Reproduction-Associated AGOs

AGO proteins are core effector proteins in small RNA-mediated silencing pathways. Different small RNAs are preferentially recruited into specific AGOs, mainly determined by the 5’-terminal nucleotide of the small RNA (Mi et al., 2008). The ten AGO proteins of Arabidopsis are reasonably well studied, but the functional roles of the ~17 to 19 AGOs in grass genomes are less well described (Zhang et al., 2015). Moreover, it is still unclear which AGOs recruit the abundant reproductive phasiRNAs of grasses. A recent study in rice showed that the germline-specific AGO protein MEL1 associates with 21-nt phasiRNAs that have 5’-terminal cytosine (Komiya et al., 2014). In addition, the AGO(s) that recruits 24-nt phasiRNAs is still unknown, although ZmAGO18b is enriched in tapetum and germ cells in maize anthers (Zhai et al., 2014), where 24-nt phasiRNA accumulate (Zhai et al., 2015). Considering the dramatic changes of phasiRNA abundances across different stages of rice spikelet development, we hypothesized that AGO proteins that recruit phasiRNAs may show a gene expression pattern correlated with phasiRNA abundances. Therefore, we examined the expression of all AGOs in rice from stages 3 to 7 (Figure 11A). We found that MEL1 peaked at stage 5, the same stage as the peak of accumulation of 21-nt phasiRNAs; therefore, MEL1 expression was indeed correlated with phasiRNA production. Intriguingly, we found that OsAGO1d showed the same pattern as MEL1, suggesting a possible functional connection between the OsAGO1d protein and reproductive phasiRNAs (Figure 11A).
Figure 11. Abundance of OsAGO transcripts in different stages and backgrounds of anther development. (A) mRNA levels of all nineteen OsAGOs in wildtype rice, according to Zhang et al., (2015). OsAGO1d and OsAGO5c (MEL1) are highlighted in grey shading as they showed a similar pattern of abundance, distinct from other OsAGO genes, peaking at stage 5. (B) OsAGO3, OsAGO18, and OsAGO14 displayed substantial upregulation at stage 7 compared to stages 3 and 5. OsAGO3 and OsAGO18, but not OsAGO14, is defective in msp1 and ostd1la mutants at Stage 7. "***" and "**" indicate significant difference of p < 0.01 and p < 0.05 (Student’s t-test), respectively; "ns" indicates no significant difference.
We speculated that AGOs that accumulate in stage 7 could function with 24-nt phasiRNAs. Gene expression analysis showed that only three OsAGOs, including OsAGO2b, OsAGO5b, and OsAGO18, displayed a substantial upregulation at stage 7 compared to stages 3 and 5 (Figure 11B). Since 24-nt phasiRNA accumulation was deficient in msp1 and ostdll1a mutants, perhaps the result of defects in cell layers important for their biogenesis, it’s possible that the OsAGOs that load 24-nt phasiRNAs are similarly impacted in both mutants. We found that the levels of OsAGO2b and OsAGO18, but not OsAGO5b, were partially reduced in msp1 and ostdll1a mutants (Figure 11B), suggesting that OsAGO2b and OsAGO18 are candidates for roles with 24-nt phasiRNAs. To further confirm their specificity to anthers, we checked their expression patterns using published rice anther microarray datasets. Consistent with our RNA-seq data, OsAGO2b and OsAGO18, but not OsAGO5b, showed a meiosis-specific expression pattern in rice anthers (Figure 12).

Figure 12. Anther-specific expression patterns of OsAGO1d, OsAGO2b, OsAGO18, but not OsAGO5b.

To connect the temporal specificity of transcript accumulation with spatial patterns, we performed in situ hybridizations or examined published images for
selected rice AGO genes. Published images for OsAGO2b demonstrates its accumulation in sporocytes and wall layers of rice anthers at pre-meiotic stages, and in later meiosis stage, the transcript was restricted predominantly to the tapetum layer and microspores (Deveshwar et al., 2011). We performed in situ hybridizations in rice anthers to examine the transcript accumulation patterns of OsAGO1d and OsAGO18 (Figure 13). These results showed that OsAGO1d accumulates highly in the distal epidermis and primary parietal cells of the anther lobe at stage 3. This OsAGO1d pattern is reminiscent of miR2118, which accumulates in the distal cells of the epidermis in maize anthers (Zhai et al., 2015). In stage 5, OsAGO1d accumulates highly in the middle layer and the tapetal layer, which are both differentiated from the primary parietal cells. In stage 7, the level of OsAGO1d is much lower than that of stage 5 (Figure 13A). As for OsAGO18, its abundance was enriched in tapetal and sporogenous cells at stages 5 and 7 (Figure 13B). Therefore, the patterns of OsAGO1d and OsAGO18 across different developmental stages of rice anthers are consistent with the results of RNA-seq data from rice spikelets, and these OsAGO transcripts are highly correlated both spatially and temporally with reproductive phasiRNAs (comparing our in situ to those of the phasiRNAs in maize anthers, in Zhai et al., 2015). Taken together, these analyses suggest a possible functional connection between grass reproductive phasiRNAs and the three AGO proteins encoded by OsAGO1d, OsAGO2b, and OsAGO18.
Figure 13. RNA in situ hybridization of OsAGO1d and OsAGO18 in different stages and backgrounds of rice anthers. Expression patterns of OsAGO1d (A) and OsAGO18 (B), performed in stages 3, 5 and 7 of anthers, from either wildtype 9522 or the msp1 or ostdll1a mutants, as marked. Shown is one anther lobe. Cell layers are labeled, as in Figure 1. Scale bars indicate 20 μm.
Chapter 4
CONCLUSION

Like most complex developmental changes, rice spikelet development entails widespread transcriptional changes. Our small RNA and RNA-seq data provide a global view of such changes in wild type rice. Similar data for mutants in MSP1 and OsTDL1A, interacting proteins with crucial roles in initiating early stage reproductive development, demonstrate the impact of disordered cell specification in rice reproductive tissues.

miRNAs play important roles in plant development. We identified miRNAs with differential accumulation patterns during rice spikelet development, including conserved miRNAs (miR156, miR172, and miR164). Among these, the role of miR164 in development is not well studied, although it accumulates in spikelet and floral meristems (Adam et al., 2011). While variation in miRNA levels in different stages of rice spikelets suggests that miRNAs are active, one interesting observation from the msp1 and ostdl1a mutants is that levels of most miRNAs and their targets were largely not impacted in the mutants, despite the block in development. This suggests that these miRNA-involved gene silencing pathways are genetically independent or upstream of the OsTDL1A-MSP1 pathway.

Via interactions with OsTDL1A, the receptor-like kinase MSP1 phosphorylates substrate proteins to transduce signals during early stages of anther development. By transcriptional analysis of the msp1 and ostdl1a mutants, we confirmed an impact on transcription factors previously shown downstream and with
essential roles in rice anther development, namely GAMYB, UDT1, and TDR. Our analysis of these mutants identified an additional 22 transcription factor (TF)-encoding genes that were significantly downregulated in both mutants and specific to reproductive tissues, suggesting that these TFs are genetically downstream of OsTDL1a-MSP1 and may play roles in rice anther development. Therefore, our results provide a source of candidate genes for future functional studies that might help build up a more detailed gene regulatory network of rice anther development.

We also assessed phasiRNA production in rice spikelets. Two classes of phasiRNAs have distinct accumulation patterns in grass anthers (Zhai et al., 2015); as in maize, we show that rice phasiRNAs peak at specific stages during spikelet development. We showed that miR2118, the trigger of 21-nt phasiRNAs, accumulates to the highest level at stage 5 and drops severely in stage 7, while 21-nt phasiRNAs are relatively slightly retarded, peaking at stage 5 but decreasing only slightly at stage 7. As in maize, miR2275 peaks later (stage 7), the stage at which 24-nt phasiRNAs reached the highest abundance that we measured. In the msp1 and ostdl1a mutants, 24-but not 21-nt phasiRNAs were depleted - consistent with data from the maize mac1 (the ortholog of rice OsTDL1A) mutant (Wang et al., 2012). Therefore, the timing of phasiRNA biogenesis is conserved in rice and maize, two Poaceae evolutionarily separated by ~50 million years (Wolfe et al., 1989).

Considering that in the mutants, 24-nt phasiRNAs were impacted but miRNAs were largely not, we may be able to infer AGOs with roles in phasiRNA function. Compared to 10 AGOs in Arabidopsis, the rice and maize genomes encode more, 19
and 17 respectively (Zhang et al., 2015). AGO5 expression in Arabidopsis is specific to somatic ovule tissues, with a role in megagametogenesis (Tucker et al., 2012). A potentially conserved function of AGO5 in plant gametogenesis was shown in rice, as the AGO5 relative MEL1 (OsAGO5c) binds 21-nt reproductive phasiRNAs with 5’ C (Komiya et al., 2014). AGO1 in rice has four homologs (OsAGO1a, OsAGO1b, OsAGO1c, and OsAGO1d); OsAGO1a/b/c predominantly recruit miRNAs and other small RNAs with 5’-terminal uridine (Wu et al., 2009). Our RNA-seq data showed that rice OsAGO1d accumulates spikelets, synchronous with MEL1, making OsAGO1d a strong candidate for further functional analysis. In rice, OsAGO18 is induced upon viral infection, and was shown to confer resistance to viruses by sequestering miR168, suppressing OsAGO1 expression (Wu et al., 2015). Maize has two homologs of AGO18; ZmAGO18b is specific to the tapetum and germ cells (Zhai et al., 2014). Our data in rice spikelets showed that OsAGO18 and OsAGO2b transcripts increase substantially at stage 7, coincident with 24-nt phasiRNA accumulation. Decreased transcripts of OsAGO18 and OsAGO2b in msp1 and ostdl1a might be due to the loss of the tapetum cells in these mutants. Furthermore, in situ hybridization results of OsAGO1d, OsAGO18, and OsAGO2b showed that these OsAGOs have distinct expression patterns during rice anther development, suggesting that they may associate with either 21- or 24-nt phasiRNAs in a stage-specific manner. In summary, our results suggest a possible functional relationship between OsAGO1d, OsAGO18 and OsAGO2b, and grass reproductive phasiRNAs.
What might be the function of these AGO proteins loaded with phasiRNAs?
The functions remain unknown but the accumulation in grasses of 21- and 24-nt phasiRNAs before and during gametogenesis is intriguing. Prior work on MEL1 (associated with 21-nt phasiRNAs) suggests a role in histone modifications; histone H3 lysine 9 dimethylation (H3K9me2) was decreased in *mel1* mutant (Nonomura et al., 2007). Yet the reduced H3K9me2 in *mel1* could be an indirect effect of other epigenetic changes, as many chromatin modifications in plants and other organisms are intricately linked (Castel and Martienssen, 2013). Considering the lack of sequence complementarity of phasiRNAs and other regions in the genome (Zhai et al., 2015), it’s also possible that reproductive phasiRNAs act primarily in cis or impact cis-adjacent regions by a spreading mechanism. Overall, the coordinate accumulation of 21- and 24-nt phasiRNAs and several AGO transcripts in rice reproductive development suggests that more detailed and comprehensive analyses of DNA methylation and histone modifications are needed, particularly when coupled with mutants, in the manner that we have performed.
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