Identification of small RNAs in late developmental stage of rice anthers

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Small RNAs including microRNA (miRNA) and small interfering RNA (siRNA) are known as repressors of gene expression. There are many plant proteins involved in small RNA-mediated gene silencing, such as Dicer ribonucleases and RNA-dependent RNA polymerases. However, most of these proteins have been reported to be absent in the late developmental stage of the plant male gamete, pollen. In order to clarify the existence of the small RNAs during maturation of pollen, we cloned and sequenced small RNAs from rice anthers including tricellular pollen. From fifty six candidates of small RNAs, we identified two known miRNAs (miR166 and miR167), eight potential miRNAs, and ten putative heterochromatic siRNAs (hc-siRNAs). RNA gel blot analyses clearly showed that miR166 and miR167 were accumulated in the uninuclear pollen stage of anther development and remained until the tricellular pollen stage. Our cloning and RNA gel blot analyses of small RNAs led us to propose a possible function of small RNA-mediated gene regulation for the development of male gametes in rice.

Key words: anther, miRNA, rice, small RNA

In most eukaryotes, small RNAs of 18–24 nucleotides (nt), including microRNA (miRNA) and small interfering RNA (siRNA), regulate gene expression by mRNA degradation, translational repression or chromatin remodeling (Bartel, 2004). The miRNAs are processed by Dicer ribonucleases from partially folded stem-loop precursor RNAs, which are transcribed as single-strand non-coding RNA. A number of miRNAs in the eukaryote genome down-regulate their target genes accurately, leading to normal development. By similar mechanisms, siRNAs are processed from longer double-stranded (ds) RNAs and induce gene silencing. In plants, the majority of siRNAs are derived from heterochromatin, transposons, repetitive sequences and transgenes. Plant endogenous siRNAs are classified into three categories with characteristics of their functions and derivation, natural-antisense transcript-derived siRNAs (nat-siRNAs), trans-acting siRNAs (ta-siRNAs), and heterochromatic siRNA (hc-siRNAs) (Borsani et al., 2005; Allen et al., 2005; Lippman et al., 2004).

Recent considerable research of small RNAs in plants have revealed that small RNA-mediated gene silencing in plants involves several pathways, which are regulated by many proteins such as ARGONAUTE (AGO1 and AGO7), DICER-LIKE (DCL1, DCL2, DCL3, and DCL4), HYponastic LEAVES1 (HYL1), dsRNA-BINDING PROTEIN4 (DRB4), HUA ENHANCER1 (HEN1), HASTY (HST), RNA-DEPENDENT RNA POLYMERASE (RDR2, RDR6), SILENCING DEFECTIVE3 (SDE3), and SUPPRESSOR OF GENE SILENCING3 (SGS3) proteins (Vaucheret, 2006). Mutant analyses in Arabidopsis thaliana showed that plant development is influenced by proteins involved in small RNA-mediated gene silencing mechanism (Bohmert et al., 1998; Lu and Fedoroff, 2000; Chen et al., 2002; Telfer and Poethig, 1998; Jacobsen et
indicated that small RNA-mediated gene silencing posi-
tions important functions necessary for sustaining the
developmental cycle of plants. To date, several kinds of miRNAs
and siRNA have been cloned and are characterized in
*Arabidopsis* and rice, two major model plants (Llave et
al., 2002; Wang et al., 2004; Sunker et al., 2005b).

The male reproductive organ is one of the most drasti-
cally changing sites in plant development. In anthers, 
haploid microspores are produced through meiosis and
developed into mature pollen. The pollen development,
which is traditionally classified into 4 stages (uninuclear
microspore, bicellular pollen, tricellular pollen, mature
pollen grain), is regulated by a complex gene expression
in both gametophytic and sporophytic tissues (Yang and
Sundaresan, 2000; Kapoor et al., 2002; Durbarry et al.,
2005). Pina et al. (2005) conducted pollen transcriptome
in *A. thaliana*, and clarified an expression profile of gene
families involved in the small RNA-mediated gene sil-
cencing pathway, including AGO1, AGO7, DCL1, DCL2,
DCL3, HEN1, RDR2, RDR6, and SDE3. They showed that
all the genes except for AGO7 are expressed in uninu-
clear microspore and bicellular pollen (the early deve-
nmental stage). Also all the genes, except for AGO1,
were not expressed in tricellular microspore and mature
pollen grain (the late developmental stage), and AGO1
transcripts were identified in the tricellular pollen, sug-
gest that small RNAs might not function in the late
stage of pollen development. Because mature pollen
grains have many important biological roles, such as pol-
len germination, pollen-stigma interaction, pollen-tube
penetration, and double fertilization (Hulskamp et al.,
1995; Jiang et al., 2005; Dresselhaus, 2006), it is neces-
sary to determine whether small RNAs exist and function
in the late developmental stage of the male organ. In
this study, in order to clarify that small RNAs exist dur-
ing pollen maturation, we performed molecular cloning
and expression analysis of small RNAs in the tricellular
stage of rice anther.

Total RNA was isolated from anthers with tricellular
We constructed a small RNA library using the total RNAs
that range in size from 18 to 28 nt, with DynaExpress
miRNA Cloning Kit (BioDynamics Laboratory, Tokyo,
Japan) and TOPO TA Cloning Kit with pCR2.1 vector
(Invitrogen, Carlsbad, CA, USA). Subsequently, 864
randomly-selected clones were sequenced using ABI
PRISM 310 Genetic Analyzer (Applied Biosystems, Foster
City, CA, USA) and were characterized by BLAST ver.
2.2.15 (http://blast.ddbj.nig.ac.jp/top-j.html; Altschul et al.,
1997) and RAP (Rice Annotation Project)-BLAST sear-
ching (http://rapdb.dna.affrc.go.jp/tools/blast; Ohyanagi
et al., 2006). Out of the 864 selected clones, 169 were
able to be analyzed as data with high quality and
perfectly matched the rice genome. While 113 clones of
the 169 clones corresponded to degraded products of
rRNA and tRNA, most of the remaining 56 clones (48/56,
85.7%) were judged as small RNAs because the 48 clones
ranged in length from 18 to 24 nt as small RNAs (Fig.
1). The most frequent lengths were 21 and 24 nt (35.7%
and 17.9%, respectively), being consistent with the size
distribution of miRNAs reported in rice previously
(Sunker et al., 2005a). We categorized these 56 clones
with mfold program (http://frontend.bioinfo.rpi.edu/
applications/mfold/cgi-bin/rna-form1.cgi; Zuker, 2003)
and the results were as follows: two clones were known
miRNAs (miR166 and miR167) (Reinhart et al., 2002;
Sunker et al., 2005a), ten were putative siRNAs from
retrotransposon which might be categorized in hc-
siRNAs, eight were estimated as potential miRNAs by
stem-loop structures. Also none of nat-siRNAs were
matched to natural cis-antisense transcripts contained in
down in the dataset, the RAP database and the *Arabidopsis*
nat-siRNA dataset (Jin et al., 2008). The characteristics
of the 56 clones suggest the existence of small RNAs in the
late developmental stage of rice anthers. Nucleotide
sequence comparison among the 56 small RNAs in this
study showed that all of them were unique.

We further focused on validating existence of the small
RNAs identified with our sequence analysis though small-
RNA cloning was not saturated by the present small-scale
sequencing using the conventional cloning method. In
the case of miRNA, Lu et al. (2008) suggested that high-

![Fig. 1. Size distribution of 56 small RNAs cloned from rice
anthers at the tricellular pollen stage.](http://example.com/fig1.png)
Small RNAs in rice anthers at late stage

throughput deep-sequencing is essential for effective and confident annotating of non-conserved miRNA. They sequenced more than four million small RNAs from rice by using massively parallel sequencing, and found that most of the reported rice-specific miRNA candidates, except conserved miRNAs in Arabidopsis, were mis-annotated as miRNA even though they were predicted to form hairpin structures and confirmed by RNA gel blot analyses. Therefore, the miR166 and miR167, which were validated as conserved miRNAs with the RAP-DB, are available markers at present to ask if small RNAs occur in the late developmental stage of rice anthers. In our RNA gel blot analysis, both miR166 and miR167 were observed in anthers from the uninuclear microspore stage to the tricellular pollen stage (Fig. 2). Their accumulation was more abundant in anthers of the tricellular pollen stage than in leaves, indicating that these miRNAs might be derived from pollens, not from vegetative tissues of the anthers, such as epidermal cell layer. A target gene of miR166 encodes a homeodomain-leucine zipper (HD-ZIPIII) transcription factor, which regulates radial polarity of the leaves and stems of the shoot in Arabidopsis (Rhoades et al., 2002; Emery et al., 2003) and shoot apical meristem (SAM) formation in rice (Nagasaki et al., 2007). Identification of miR166 in the maturing pollen suggests that negative regulation of HD-ZIPIII is needed in the pollen development and that an accurate polarity of the pollen grains, which might affect an asymmetric division of microspore mitosis following organelle arrangement, would be maintained by the expression of the HD-ZIPIII regulated by miR166. miR167 is known to be highly expressed in floral organs of Arabidopsis, and target genes of miR167 are auxin response factor genes, ARF6 and ARF8 (Reinhart et al., 2002; Rhoades et al., 2002). In Arabidopsis, miR167 regulates anther development, and overexpression of miR167 causes a male sterile phenotype (Ru et al., 2006; Wu et al., 2006). These observations, suggesting the accurate regulation of maturing male gametes by miR167, are consistent with our data in which miR167 was highly accumulated during early developmental stage of anthers and until the tricellular pollen stage.

We conclude that small RNAs would have functions in the late developmental stage of the male organ in rice; they might be processed in the early stage of anthers and maintained until the later stage. Honys and Twel (2004) performed comprehensive transcriptome analysis of male gametophyte development in Arabidopsis, and found large-scale repression of various genes during transition from bicellular to tricellular pollen. This characteristic down-regulation of genes during pollen maturation, which increases the proportion of male gametophyte-specific transcripts, might be strictly regulated by small RNAs. Further analysis of small RNAs by massively parallel parallel sequencing will lead to identification of novel gene networks regulating the haploid male gametophyte development in plants.

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REFERENCE


Fig. 2. RNA gel blots of two known small RNAs, miR166 and miR167. Anther stage classification, which was divided into three stages by the number of cells in microspore, was followed by Tsuchiya et al. (1992). Ten micrograms of total RNA from roots (R), leaves (L), and anthers containing uninuclear microspores (1), bicellular pollens (2) and tricellular pollens (3) were resolved in a denaturing 10% polyacrylamide gel, transferred to a nylon membrane, and hybridized with a 32P end-labeled oligonucleotide complementary to miR166 or miR167. rRNA stained with ethidium bromide was used as loading controls.


