SHORT COMMUNICATION

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microRNA access to the target helicases from rice

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Major classes of small RNAs include microRNAs (miRNAs) and small interfering RNAs (siRNAs). miRNAs are single-stranded RNA molecules of around 22 nucleotides in length. Most miRNAs show imperfect homology with their targets. The biogenesis mechanisms of miRNAs are different for plant and animals. Silencing of genes by miRNAs may serve as an appropriate tool to speed-up analyses of gene functions in a post-genomic era. We have identified in silico a set of miRNAs that control helicase gene expression by regulating its mRNA stability and translation in rice. Our analyses revealed that several rice helicases have distinct miRNA specificities. Such analyses will be a prerequisite to refining our understanding of target selection and regulation of helicase gene expression by miRNAs in rice. Further, we discuss recent findings on miRNA gene family and its gene structure, criteria for miRNA annotation, and on miRNA biogenesis that involve transcription, processing, and maturation of miRNAs.

A single stranded RNA of ~22 nucleotides in length generated from an endogenous transcript containing a local hairpin structure using RNAse-III-type enzyme Dicer is defined as miRNA.[1] The miRNAs by base pairing with target mRNAs causes mRNA cleavage or translation repression and thus they function as guide molecules in post-transcriptional gene silencing. Recent findings indicated that the degradation of mRNA in both plants and animals can be induced by miRNAs.[2] The miRNAs are implicated to play vital role in protein degradation, signal transduction, plant development, stress response and pathogen invasion. Studies on miRNA have broadened our understanding of gene expression, plant genetic engineering and plant pathogenesis related molecular investigations. Recent evidence demonstrates that plant viral diseases can be modulated by miRNAs.[3]

The studies have revealed that miRNAs play vital role in cell proliferation, organ development and control of developmental timing and other diverse regulatory pathways.[4] Thus complex regulatory networks are established by interactions of miRNA and their targets. In this article we have analyzed complete set of rice helicases for their miRNA targets. The helicase gene family comprises 115 genes in rice.[5] We also discuss recent findings on miRNA gene family and its gene structure, criteria and annotation of miRNA and on miRNA biogenesis, that involve transcription, processing and maturation of miRNAs.

In Silico Analysis of Interaction between Target Helicases and miRNAs in Rice

A single and highly complementary target sites are required to regulate the transcripts by plant miRNAs. Target sites are predominantly found in coding regions, but can be located in UTRs. Each miRNA is thought to possess a limited number of mRNA targets. Interestingly, the transcription factors those involved in developmental regulation and cell differentiation acts as known targets of plant miRNAs.[6]

Our earlier studies have shown the presence of 115 loci that encode for helicase proteins in the rice genome.[5] The genomic sequences for these helicases were downloaded from rice.plantbiology.msu.edu. Individual sequence was imported in psRNA Target: A Plant Small RNA Regulator Target Analysis Server (bioinfo3.noble.org/psRNATarget/). The miRNAs for their target helicases with expection scores ≤3.5 are shown in **Table 1**. From the list of 115 loci, we found 17 helicases to be miRNA targets with expection ≤3.5 (**Table 1**). The **Figure 1** shows diagrammatic representation of the complementary region for these 17 target helicases and their miRNAs. For the remaining helicases, the expection range was 4–5 (**Sup. Tables 1–3**). No complementary region between miRNA and target helicase was detected for the following loci, LOC_Os11g09060, LOC_Os01g07080, LOC_Os03g19960, LOC_Os06g48750, LOC_Os01g53580, LOC_Os04g14810, LOC_Os07g48270 (**Sup. Tables 1–3**).

Annotation of miRNAs

The vital roles of miRNAs in hormone signaling, stress adaptation and in development is reported for both animal and plant kingdoms.[7] The nematode lin-4 and let-7 have provided the paradigm for the function of miRNA. A large and complex small RNA population is found in plants within which miRNAs are often a minority. By contrast, miRNAs form the majority of most of the small RNAs in the vertebrates and flies. The plant-specific

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Table 1. A list of rice helicases as targets for miRNAs with expection ≤3.5

					Alignment region	
S. no.	**Gene name**	**Loci**	**miRNA Acc.**	**Expection**	**miRNA**	**Target**
	RNA helicases					
1	DBH	LOC_Os04g40970	osa-miR164e	2.5	21 to 2	2642 to 2661
2	DDX46	LOC_Os08g05810	osa-miR414	3	21-2	613–632
3	DDX47	LOC_Os03g46610	osa-miR414	3.5	21-1	1335–1355
4	DDX49	LOC_Os07g43980	osa-miR2094-5p	3.5	21-2	519–538
5	DHX8	LOC_Os06g09280	osa-miR531b	3.5	20-1	203–222
6	DHX16	LOC_Os05g32370	osa-miR1846a-3p	3.5	21-2	480–499
7	DHX36	LOC_Os03g53760	osa-miR1319	3.5	24-5	2516–2536
8	NRH2	LOC_Os03g61220	osa-miR396a	3.5	21-2	803–822
9	PRP5	LOC_Os08g06344	osa-miR414	2	21-2	1030–1049
10	RNAhA	LOC_Os01g56190	osa-miR414	3.5	21-2	3064–3083
11	U5.snRNP 200 kDa	LOC_Os02g01740	osa-miR395o	3	21-2	4069–4088
	DNA helicases					
12	ABP	LOC_Os06g33520	osa-miR414	0	21-2	442–461
13	MCM3	LOC_Os05g39850	osa-miR1860-3p	3.5	22-3	1385–1404
14	RecQSim	LOC_Os05g05810	osa-miR1854-5p	2	21-2	2553–2572
	Others					
15	CTD2	LOC_Os02g43460	osa-miR414	2.5	21-2	1408–1427
16	HADfp	LOC_Os03g53760	osa-miR1319	3.5	24-5	2516–2536
17	helicase C6F12.16c	LOC_Os11g07500	osa-miR408	3.5	21-1	172–192

RNA polymerase IV/RNA polymerase V (PolIV/PolV)-dependent short interfering RNAs (siRNAs) are responsible for the more complex pool of plant small RNAs. Plants also produce secondary siRNAs. The annotation of miRNAs becomes challenging due to these diverse endogenous siRNA populations.

Many studies were conducted to identify miRNAs in plants. As a result, several miRNAs have been discovered in model plants like Arabidopsis and rice. The miRNAs were also identified in other plant species, such as *Zea mays*, *Sorghum bicolor*, *Gossypium hirsutum*, *Brassica napus*, *Vitis vinifera*, *Solanum lycopersicum* and *Populus tricocarpa*. Furthermore, vital role for miRNAs were predicted in moss *Physcomitrella patens* and unicellular green alga *Chlamydomonas reinhardtti*. Following miRNA identification, they are deposited in miRBase, the miRNA database (www.mirbase.org/index.shtml). An increasing number of miRNAs have been identified and deposited in miRbase. As listed in miRBase, 199 miRNA sequences have been reported in *Arabidopsis thaliana*, 447 in *Oryza sativa*, 375 in *Medicago truncatula*, 170 in *Zea mays*, 148 in *Sorghum bicolor*, 137 in *Vitis vinifera* and >2,000 have been annotated among all plants.

The psRNATarget server accepts miRNA/ta-siRNA mature sequences and target candidates as inputs. It reports all potential complementary regions between miRNA/ta-siRNA and target sequences. In addition to search for potential targets of user-submitted small RNA in published transcript libraries, psRNATarget added two new search functions. The psRNATarget is able to (1) search whether the user-submitted sequences are potential targets of published miRNA/ta-siRNA sequences, and (2) search user-submitted small RNA against user-submitted target candidates. The psRNATarget server automatically synchronizes with miRBase to keep the published miRNA dataset up to date. The number of published miRNAs deposited in psRNATarget includes, 224 in *A. thaliana*, 383 in *M. truncatula*, 496 in *O. sativa*, 237 in *P. trichocarpa* and 319 in *Z. mays*.

Criteria for miRNA Classification

In practice, followed by the discovery of small RNA by cDNA cloning, the following criteria must be met to classify it as a miRNA. First, the expression of miRNA should be confirmed by northern blot hybridization. Second, in one arm of the hairpin precursor, the small RNA sequence should be present which should lack the large internal loops or bulges. Usually ~60–80 length precursors are found in animals, however the lengths are more variable in plants.[6] Third, the phylogenetic conservation of small RNA should be considered. The conservation should be seen at sequence level in the precursor hairpin, usually this extent of conservation is less than in the mature miRNA segment. Last, the precursor should accumulate in the presence of reduced Dicer function. The use of this criterion is still not in practice since depleting Dicer in certain cell types is hampered with technical difficulties.[6]

Gene Structure and microRNA Gene Transcription

An indication that most miRNAs are located in the intergenic regions came from the studies on early annotation of the genome position of miRNAs. However, in the intronic regions of known

genes a sizeable minority of miRNA population was also found.[8] The miRNA genes can be transcribed from their own promoters,[9] and that the clustered miRNAs are generated as polycistronic primary transcripts (pri-miRNAs).[10]

The RNA polymerase II (pol II) mediates the transcription of miRNA genes.[9] Similar to other genes that are transcribed by pol II, the expression profiles have indicated that miRNAs are under elaborate control during development and in various tissues.[6] A plasmid that contains a pri-miRNA segment under the control of a heterologous pol II promoter can generate a fully functional mature miRNA.[10] Three lines of direct evidence in connection to miRNAs transcription were recently reported. First, both cap structures and poly (A) tails were present in pri-miRNAs.[9] Second, the sensitivity of miRNA transcription activity to alpha-amanitin at concentration that specifically inhibits pol II, but not pol I or pol III was confirmed.[9] Last, using the chromatin immunoprecipitation assay, the physical association of pol II with the promoter of miR-23a~27a~24-2 was demonstrated.[9]

On the basis of genomic locations, the miRNA genes are grouped into the following categories:

- exonic miRNA (in non-coding transcription units)
- intronic miRNA (in non-coding transcription units)
- intronic miRNA (in protein-coding transcription units)

It is now generally accepted that splicing occurs first followed with processing of pri-miRNA and that the processing of intron lariats results in the release of pre-miRNAs. Similar expression profiles of the host transcript and miRNAs indicate that these miRNAs are transcribed as part of the long transcription units.[8]

miRNA Biogenesis

Slightly different mechanisms of miRNA biogenesis exist in plants and animals. In plants, miRNAs are transcribed into primary miRNA transcripts (pri-miRNA) with polymerase II (polII). Further processing of pri-miRNA also occurs in the nucleus with a ribonuclease III-like nuclease (DICER-LIKE 1, DCL1). This process yields a precursor miRNA (pre-miRNA) and ultimately a mature miRNA:miRNA* duplex. Following its export to the cytoplasm, the duplex gets unwound and incorporated into the RNA-induced silencing complex (RISC) complex.[4] The complex is then guided by the miRNA to its specific target mRNA. In animals, the final step of maturation is mediated in the cytosol by Dicer.

Maturation of miRNA

Two simple observations lead to the formulation of the current model for miRNA maturation. First, transcription of miRNAs leads to the generation of long primary transcripts which are trimmed into hairpin intermediates (pre-miRNAs) and subsequently cleaved into mature miRNAs. Second, the first and second processing and their catalytic activities are compartmentalized in the nucleus and cytoplasm, respectively. Therefore, for the cytoplasmic processing to occur, the nuclear export of pre-miRNA is necessary.

```
miRNA   21 AGUGCACGGGACGAAGAGGU 2    (osa-miR164e)
           : :  : :  : ::: ::: : :. : :::
Target 2642 UCUCGAGCCCUGCUUUUCCA 2661 (LOC_Os04g40970)

miRNA   21 CUGCUACUACUACUCCUACU 2    (osa-miR414)
           : :: ::  ::  :: :: : :: .: :.
Target  613 GACGACGACGAUGAGGGUGG 632 (LOC_Os08g05810)

miRNA   21 CCUGCUACUACUACUCCUACU 1    (osa-miR414)
           :  :: ::  ::: : :: :: ::  ::
Target 1335 GAACGAAGAUGAUGAGGAAGA 1355 (LOC_Os03g46610)

miRNA   21 UGGGUCCUCGGAUCGUCGGU 2    (osa-miR2094-5p)
           . : .: :::  :: :: :: .: :: :
Target  519  GCUCAGGUGCCUCGCGGCCA 538 (LOC_Os07g43980)

miRNA   20 GCCGUGCGUCGGGGCCGCUC 1    (osa-miR531b)
           ::: : .: :. . ::    ::: :: ::
Target  203  CGGCGCGUGGCGGCGGCGAG 222 (LOC_Os06g09280)

miRNA   21 GCCGCUCCUCUUGCCCCAGU 2    (osa-miR1846a-3p)
           :: ::::  ::  .  ::: :: :::
Target  480  CGGCGACGACGCCGGGGUCA 499 (LOC_Os05g32370)

miRNA   24 UAUAUAAUGUC- UACGGCCAA 5  (osa-miR1319)
           ::: : :: ::  ::  : : :: .: :: :
Target 2516 AUAUAUUAAAGGAUGCUGGUU 2536 (LOC_Os03g53760)

miRNA   21 UCAAGUUCUUUCGACACCUU 2  (osa-miR396a)
           . :: : .. : :. :. :  :: :: :: :
Target  803 GGUUUGAGGAGGAUGUGGAA 822 (LOC_Os03g61220)

miRNA   21 CUGCUACUACUACUCCUACU 2    (osa-miR414)
           ::: ::  :: :: :: :: :: . :: .
Target 1030 GACGAGGAUGAUGAGGGUGG 1049 (LOC_Os08g06344)

miRNA   21 CUGCUACUACUACUCCUACU 2    (osa-miR414)
           :: . .: :: :: ::  :: :: : :.
Target 3064 GAUAGUGAUGAAGAGGAUGG 3083 (LOC_Os01g56190)

miRNA   21 UCAAGGAGGUUUGUGAAGUA 2  (osa-miR395o)
           : ::  :: :. :: :: :: . :: ::
Target 4069 AGUGCCUUCAAACAUUUCAA 4088 (LOC_Os02g01740)

miRNA   21 CUGCUACUACUACUCCUACU 2    (osa-miR414)
           :: :: :: :: :: :: :: :: : :::
Target  442 GACGAUGAUGAUGAGGAUGA 461  (LOC_Os06g33520)

miRNA   22 UCUUUUGGAUCGAAGGUCUA 3  (osa-miR1860-3p)
           : :: :  : . . .: :: :: ::: ::
Target 1385 AGAACAUUGGGCUUCCAGAU 1404 (LOC_Os05g39850)

miRNA   21 GGUUAGAUGUUUAAAGUGGU 2    (osa-miR1854-5p)
           : ::  : :: ::: .. :: : ::: ::
Target 2553 CCA CUCUACAGGUUUCACCA 2572 (LOC_Os05g05810)

miRNA   21 CUGCUACUACUACUCCUACU 2    (osa-miR414)
           ::  : :: :: : ::: ::  :: :::
Target 1408 GAAGAUGAUGAUGAUGAUGA 1427 (LOC_Os02g43460)

miRNA   24 UAUAUAAUGUC- UACGGCCAA 5  (osa-miR1319)
           ::: :: : ::  : :  : : ::. : :: :
Target 2516 AUAUAUUAAAGGAUGCUGGUU 2536 (LOC_Os03g53760)

miRNA   21 CGGUCCCUUCUCCGUCACGUC 1   (osa-miR408)
           ::: :  : ::  ::: : :. :: :::
Target  172  GCCAAGGACGAGGCGGUGCAC 192 (LOC_Os11g07500)
```

Figure 1. The sequence alignment of miRNAs and target helicases. The miRNA accession numbers and locus IDs are given in parenthesis. The nucleotide positions for the genomic sequences and miRNAs are indicated. Refer to **Table 1** for more details.

Processing in the Nucleus by Drosha

The pri-miRNAs (primary transcripts) that are usually several kilobases long and contain a local hairpin structure are generated by the transcription of miRNA genes. The precursor of miRNA (pre-miRNA) is released after cleavage of the stem-loop structure by the nuclear RNase III Drosha.[11]

Drosha (~160 kDa) is conserved in animals. For catalysis of the Drosha, two tandem RNase III domains (RIIIDs) and a double-stranded RNA-binding domain (dsRBD) are crucial.[12] The central region of the protein, adjacent to the RIIIDs, is also essential for pri-miRNA processing.[12] The Drosha forms a large complex, the Microprocessor complex, of ~500 kDa in *Drosophila melanogaster*[13] and ~650 kDa in humans[14] respectively, where it interacts with its cofactor, the DiDeorge syndrome critical region gene 8 (DGCR8) protein in humans (also known as Pasha in *D. melanogaster* and *Caenorhabditis elegans*).[12,13] The DGCR8/Pasha is a ~120 kDa protein that contains two dsRBDs. A putative WW domain is also found which interacts with specific proline-rich sequences. It is believed that DGCR8/Pasha assist Drosha in substrate recognition; however, its exact biochemical role is currently unclear.[12,13]

Nuclear Export of pre-miRNA by Exportin-5

The pre-miRNAs are exported to the cytoplasm after the nuclear processing by Drosha where they undergo the second processing step by Dicer (another RNase III enzyme) to generate the final ~22 nucleotide product. The crucial step in miRNA biogenesis is the nuclear export of pre-miRNAs. One of the nuclear transport receptors, exportin-5, mediates the export of pre-miRNA.[14] A significant reduction, but not the complete loss, of mature miRNA results from RNA interference (RNAi)-mediated knock down of Drosha, exportin-5 or Dicer.[11,14]

Cytoplasmic Processing by Dicer

Dicer is found in almost all eukaryotic organisms such as *Schizosaccharomyces pombe*, plants and animals, hence is considered to be a highly conserved protein. There are multiple Dicer homologues in some organisms that are often assigned to take on distinct roles. For example, the Dicer-1 is needed for pre-miRNA cleavage whereas Dicer-2 is needed for siRNA generation in *D. melanogaster*.[15] In addition to two RIIIDs and a dsRBD, a long N-terminal segment that contains a DEAD-box RNA helicase domain, domain of unknown function (DUF283), and Piwi-Argonaut-Zwille (PAZ) domain are present in Dicer protein. The group of highly conserved proteins known as Argonaute also contains the PAZ domain.

The ~22-nucleotide miRNA duplexes, also called as the short-lived cleavage products, do not persist in the cell for long. Usually, one strand of this duplex remains as a mature miRNA whereas, the other strand disappears. Studies on siRNA duplexes show that the relative thermodynamic stability of the two ends of the duplex determines which strand is to be selected.[16] In miRNA duplexes, the miRNA* is degraded whereas, the selection of mature miRNA depends on the weakest 5'-end base pairing. The strand with relatively unstable base pairs at the 5' end typically remains (for example, G:U pair versus G:C pair).[16] The same rule is thought to be applicable to miRNA.

Biogenesis of microRNA in Plants

The Drosha-dependent stepwise processing is applicable only to animal cells. This is because the homologues of Drosha and DGCR8/Pasha have not been identified in plants till date. Usually, the stem-loops of plant miRNA precursors are longer than animal pre-miRNAs and the plant miRNA precursors are structurally quite diverse. It has been demonstrated by genetic studies that for miRNA accumulation in Arabidopsis, the DCL1, one of the four Dicer-like proteins [also known as CARPEL FACTORY (CAF)] is important.[17,18] The mature ~22-nucleotide miRNA might be generated in the nucleus in plants due to nuclear localization of DCL1 protein.[18] The plant miRNAs, similar to animal miRNAs, might be processed in a stepwise manner wherein DCL1 is solely responsible for all processing steps.

The amino acid sequence identity established the HASTY (HST) as a plant homologue of exportin-5. The mutant HST showed pleiotropic effects indicating that this protein might be involved in miRNA biogenesis.[19] An indication that HST functions as a nuclear export receptor came from the findings that showed a reduced accumulation of most miRNAs in the background of HST loss-of-function mutant.[20] A two dsRBD-containing nuclear protein, the HYL1,[21] and a protein with a dsRBD and a methyltransferase domain, HEN1[17] might be additional miRNA biogenesis factors.

Regulation of miRNA Expression

The regulation of miRNA expression can occur at multiple steps of RNA biogenesis. A control at the level of development and/or tissue specific signaling for most miRNAs was demonstrated based on the expression profiling studies.[6] A control at the post-transcriptional level was studied for some miRNAs.[22]

The Seed 'Rule'

The seed rule for the bona fide miRNA target sites states that contiguous Watson-Crick base-pairing to the 5' miRNA nucleotides 2–7 is required for activity.[23] The evidence that base-pairing at the 5' end of miRNAs is important for target recognition came from the miRNA target site mutagenesis studies in human cells, *D. melanogaster* and *A. thaliana*.[24] The introduction of mismatches into the seed region of miRNA-mRNA duplex can be used for target site validation. The introduction of seed rule has helped to characterize biologically important miRNA functions since several miRNA-mRNA interactions obey the seed rule.[25]

The studies on target site and miRNA mutagenesis have shown that the seed region is crucial for miRNA targeting in plants, while the pairing of the central nucleotides is necessary for efficient slicing.[26] The studies on forward-genetic screens

have demonstrated that factors beyond seed and central pairing can be important in planta. These findings were corroborated from the evidence that targets from miRNA control can be released by single-nucleotide changes at positions paired to miRNA nucleotides 16 and 19.[27]

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Note

Recent work has identified a Dicer-independent miRNA biogenesis pathway that uses Argonaute2 (Ago2) slicer catalytic activity.[28, 29]

Supplementry materials can be found at: www.landesbioscience.com/journals/psb/article/12801

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