Functions of miRNAs in Rice

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Abstract As rice is both an important crop and a well-developed model for monocotyledonous plants, the study of miRNAs and their functions in this species is an active area of research. In this chapter, we summarize the progress that has been made in identifying miRNAs and their targets in rice. The identified miRNAs and their target mRNAs suggest that miRNAs function in a wide range of biological processes. The expression patterns of the miRNAs support their roles not only in development but also in response to environmental stresses. Demonstrations of biological functions for rice miRNAs are beginning to emerge and are detailed in this chapter.

1 Introduction

Rice is an important food crop. Having a relatively small genome (approximately 420 MB), it was among the earliest genomes sequenced (Goff et al. 2002; Yu et al. 2002). Coupled with extensive genomic resources such as EST collections and insertion mutant populations, this has led to rice becoming an important research species. The extensive synteny between rice and less tractable cereal crops such as wheat has meant that rice has also become a model system for cereal research. The availability of the rice genome sequence allowed identification of rice miRNA genes soon after the discovery of miRNAs in *Arabidopsis*. Numerous studies of rice

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miRNA populations and their biological roles have shown that they play important roles through the life cycle of the plant and in its response to environmental challenges.

2 Rice miRNAs and Their Target Genes

In the current miRBase (release 17), there are 491 rice miRNAs of 205 families (http://www.mirbase.org/; Table 1). These miRNAs were identified by sequencing of small RNA populations or by computational prediction using miRNAs first identified in other plant species. The first group of rice miRNAs to be identified (miR156–167 and miR169–172) was predicted computationally based on sequenced *Arabidopsis* miRNAs (Park et al. 2002; Reinhart et al. 2002) that are well conserved between dicotyledonous and monocotyledonous plants (Bonnet et al. 2004; Wang et al. 2004a). All of these bioinformatically predicted rice miRNAs were subsequently confirmed by small RNA cloning (Sunkar et al. 2005, 2008. Small RNA cloning followed by traditional Sanger sequencing played an important role in discovery of rice miRNAs, but this approach is biased toward miRNAs that are broadly and relatively highly expressed. miRNAs that are expressed at low abundance or only in certain cell types are not easily captured by this method (Jones-Rhoades et al. 2006). The development and application of next-generation high-throughput sequencing technology has greatly accelerated the discovery of miRNAs. It is now feasible to identify miRNAs with low expression, from specific tissues or induced by altered environmental conditions (Heisel et al. 2008; Li et al. 2010a; Lu et al. 2008; Morin et al. 2008; Sunkar et al. 2008; Xue et al. 2009; Zhu et al. 2008), and as a result, many more rice miRNAs have been identified (Table 1). There is often limited overlap between the newly identified miRNAs in different studies. This is a reflection of the differing tissue specificities or induction by environmental stimuli of the miRNAs, and it also suggests that new studies will identify further miRNAs.

2.1 Categories of Rice miRNAs

Rice miRNAs can be grouped into four categories based on their conservation in other plant species. Group I miRNAs (27 families) are conserved in at least one nonmonocotyledonous plant, Group II miRNAs (24 families) are conserved in at least one monocotyledonous plant, Group III miRNAs (145 families) are rice specific, and Group IV miRNAs (nine families) appear to be nonauthentic because they have not been found by high-throughput small RNA sequencing in any plant (Table 1).

Table 1 Rice miRNAs, target genes, and their functions

miRNA family	No. of family members	Identification method[a]	Conservation[b]	Target genes confirmed by degradome sequencing or 5′ RACE[c]	Functional investigation of miRNAs and their targets
MIR156	12	I, II[2,18]	I	***Os01g69830*** (I)[33] *Os02g04680* (I) (II) *Os06g45310* (I) (II) *Os07g32170* (III) (II) *Os11g30370* (I) (II) *Os02g07780* (II) (II) ***Os08g39890*** (III)[6,33] (***OsSPL14***) *Os06g49010* (III) (II) *Os09g32944* (II) *Os09g31438* (III) *Os05g48800* (III) *Os07g36500* (III)	Plants overexpressing of *MIR156b* or *MIR156h* were semidwarf and had increased number of tillers (Xie et al. 2006). Interruption of miR156-mediated *OsSPL14* repression leads to higher level of *OsSPL14*, less tillers, and highly branched panicles with more grains (Jiao et al. 2010; Miura et al. 2010)
MIR159	6	I, II[17,22]	I	***Os01g59660*** (I) (II)[15,33] (***OsGAMYB***) *Os06g40330* (II) (II) (*OsGAMYBL1*) *Os01g11430* (III) *Os10g05230* (III)	Overexpression of *MIR159a* causes shortened top internode, and shrunken, whitened anthers because of downregulation of *OsGAMYB* and *OsGAMYBL1* (Tsuji et al. 2006)
MIR160	6	I, II[2,18]	I	*Os06g47150* (I) (I) *Os10g33940* (I) (I) *Os02g41800* (II) (II) *Os04g43910* (II) (II) *Os04g59430* (I)	
MIR162	2	I, II[18,23]	I	*Os03g02970* (I)	

(continued)

Table 1 (continued)

miRNA family	No. of family members	Identification method[a]	Conservation[b]	Target genes confirmed by degradome sequencing or 5′ RACE[c]	Functional investigation of miRNAs and their targets
MIR164	6	I, II[2,18]	I	*Os06g46270* (I) (I) *Os08g10080* (I) *Os12g41680* (I) (I) ***Os02g36880*** (III) (I)[33] *Os04g38720* (III) (II) *Os03g50040* (I) *Os05g39650* (I) *Os12g05260* (III)	Overexpression of *MIR164b* causes semidwarfism and low fertility (Zhu et al. unpublished)
MIR166	14	I, II[2,18]	I	*Os03g01890* (I) (III) (*OSHB1*) *Os10g33960* (I) (II) (*OSHB2*) *Os12g41860* (I) (*OSHB3*) ***Os03g43930*** (I)[13] (*OSHB4*) *Os04g48290* (III) *Os04g57610* (II)	Overexpressing miR166-resistant versions of *OSHB1*, *OSHB3,* and *OSHB5* in Taichung 65 exhibited rolled and/or filamentous leaves (Itoh et al. 2008). Overexpressing miR166-resistant version of *OSHB1* in *shoot organization1* (*sho1*) partially restored the morphological defects of *sho1* seedlings (Nagasaki et al. 2007)
MIR167	10	I, II[2,18]	I	***Os04g57610*** (II)[13,29] *Os02g06910* (I) *Os06g46410* (I) *Os12g41950* (I) ***Os06g03830*** (I)[10] *Os07g33790* (I) *Os07g29820* (II) ***Os09g39420***[13]	Cultured rice cells delivered with artificial miR167 showed decreased mRNAs of *ARF8* and *OsGH3-2*, a rice IAA-conjugating enzyme (Yang et al. 2006a)
MIR168	2	I, II[8,22]	I	***Os04g47870*** (II)[33] ***Os02g45070*** (III)[33] *Os06g51310* (III) ***Os02g58490*** (III)[13,33]	

MIR169	17	I, II[18,22]	I	***Os02g53620*** (I) (II)[13] ***Os03g07880*** (I) (I)[10] ***Os03g29760*** (I) (II)[30] ***Os03g44540*** (I)[10] *Os03g48970* (I) *Os07g06470* (I) (III) *Os07g41720* (I) (II) ***Os12g42400*** (I) (I)[13,33]	
MIR171	9	I, II[17,25]	I	*Os02g44360* (I) (II) *Os04g46860* (I) (II) *Os02g44370* (III) (II) ***Os04g39864*** (I)[10]	
MIR172	4	I, II[17,22]	I	***Os05g03040*** (I) (II)[13] ***Os07g13170*** (III) (II)[32] ***Os04g55560*** (I) (II)[32] *Os03g60430* (III) *Os08g39630* (I) *Os01g04550* (I) ***Os06g43220***[32]	Overexpression of *MIR172b* but not *MIR172a* causes deformed spikelets (Zhu et al. 2009)
MIR319	2	I, II[8,23]	I	*Os07g05720* (III) (III) *Os03g57190* (III)	
MIR390	1	II[22]	I	*EU293144* (I) *AU100890* (I) *CA765877* (I)	
MIR393	2	I, II[8,22]	I	*Os05g05800* (I) (I) *Os04g32460* (I) (II) *Os03g36080* (II) ***Os04g58734*** (III)[10]	Overexpression of *MIR393* decreases salt and alkali stress tolerance (Gao et al. 2011)
MIR394	1	I, II[8,23]	I	*Os01g69940* (I) (II)	
MIR395	25	I, II[8,23]	I	*Os06g05160* (II)	

(continued)

Table 1 (continued)

miRNA family	No. of family members	Identification method[a]	Conservation[b]	Target genes confirmed by degradome sequencing or 5′ RACE[c]	Functional investigation of miRNAs and their targets
MIR396	9	I, II[8,23]	I	*Os02g47280* (I) (I) *Os03g47140* (I) *Os12g29980* (I) *Os04g51190* (III) *Os11g35030* (I) (I) *Os06g02560* (I) (II) *Os02g45570* (I) *Os02g53690* (III) *Os12g29980* (I) *Os04g48510* (III) *Os03g51970* (III) *Os01g32750* (III) ***Os11g35030*** (I)[10] *Os01g44230* (III) *Os06g29430* (I)	Overexpression of *MIR396c* causes decreased salt and alkali stress tolerance (Gao et al. 2010)
MIR397	2	I, II[8,23]	I	*Os11g48060* (III)	
MIR398	2	I, II[8,23]	I	*Os07g46990* (I) (II) *Os03g22810* (III) ***Os04g48410*** (II)[10]	
MIR399	11	I, II[8,23]	I	*Os05g48390* (III)	
MIR408	1	I, II[21,22]	I	*Os02g52180* (I) *Os04g46130* (I) *Os06g11490* (I) *Os08g37670* (I)	
MIR413–MIR426	1 for each	I[24]	IV		
MIR435	1	II[22]	III		

MIR437	1	II[22]	II	
MIR438, MIR440, MIR442, MIR443	1 for each	II[22]	III	
MIR439	10	II[22]	III	
MIR441	3	II[22]	III	
MIR444	6	II[22]	II	*Os08g33488* (I) ***Os02g36924*** (I) (I)[14] ***Os04g38780*** (I) (II)[14] ***Os02g49840*** (I) (II)[14] *Os02g49090* (I) ***Os03g63750*** (1)[10] *Os05g08410* (I) *Os08g33479* (III) ***Os02g13420*** (I)[10] *Os03g54084* (II) *Os05g47560* (II) *Os08g06510* (I)
MIR445	9	II[22]	III	
MIR446	1	II[22]	III	*Os02g29140* (III) *Os04g38450* (III) *Os06g19990* (III) *Os10g26720* (III)
MIR528	1	II[12]	II	***Os06g06050*** (I) (III)[10,33] *Os06g11310* (I) ***Os06g37150*** (I) (I)[33]
MIR529	2	II[12]	I	*Os06g47780* (I) *Os06g04270* (II)
MIR530	1	II[12]	I	*Os02g14990* (I)
MIR531	2	II[12]	III	
MIR535	1	I, II[1,23]	I	
MIR806	8	II[15]	III	

(continued)

Table 1 (continued)

miRNA family	No. of family members	Identification method[a]	Conservation[b]	Target genes confirmed by degradome sequencing or 5′ RACE[c]	Functional investigation of miRNAs and their targets
MIR807, MIR815	3 for each	II[15]	III		
MIR808	1	II[15]	III	*Os10g39970* (I) *Os12g40920* (I)	
MIR809	8	II[15]	III	*Os12g44250* (II) *Os04g08415* (I) *Os06g33210* (I) *Os10g40540* (I) *Os02g55480* (II) ***Os10g39520***[15]	
MIR810	2	II[15]	III		
MIR811	4	II[15]	III		
MIR812	15	II[15]	III	*Os03g22050* (II)	
MIR813, MIR816, MIR817	1 for each	II[15]	III		
MIR814	3	II[15]	III	*Os12g39380* (I)	
MIR818	5	II[15]	III	***Os09g10710***[15]	
MIR819	11	II[15]	III		
MIR820	3	II[15]	III	***^Os03g02010*** (II)[14,15,27]	
MIR821	3	II[15]	II (*S. bicolor*)		
MIR827	3	II[3]	I	***Os04g48390*** (II)[3,9] ***Os02g45520***[3]	
MIR1317, MIR1319	1 for each	II[16]	III		
MIR1318/1432	1	II[16]	II		
MIR1320	1	II[16]	III	*Os05g47550* (II)	
MIR1423	2	II[14]	III		

MIR1424, *MIR1427*, *MIR1429*, *MIR1430*,	1 for each	II[14]	III	
MIR1425	1	II[14]	III	*Os08g01640* (I)
				Os08g01650 (I)
				Os08g01870 (I)
				Os10g35230 (I)
				Os10g35240 (I) (I)[9,14,33]
				Os10g35436 (I)[9,14]
				Os10g35640 (I)[14,33]
				Os01g49580 (III)
				Os01g49614 (III)
				Os05g40700 (III)
MIR1426	1	II[14]	III	*Os05g30350* (I)
MIR1428	7	II[14]	III	***Os03g17980*** (III) (II)[31]
				Os08g37800[31]
MIR1431	1	II[14]	III	*Os02g16670* (I)
MIR1433	1	II[14]	II (*T. aestivum*)	
MIR1435	1	II[23]	II (*S. bicolor*)	
MIR1436	1	II[23]	II (*H. vulgare*)	*Os03g48010* (II)
MIR1437, *MIR1438*, *MIR1440*, *MIR1441*	1	II[23]	III	
MIR1439	1	II[23]	III	*Os09g33690* (I)
MIR1442	1	II[23]	III	*Os02g58670* (II)
MIR1846	5	II[31]	III	*Os06g14060* (I)
				Os09g07510 (I)
MIR1847	1	II[31]	III	*Os02g34990* (I)
				Os04g39170 (I)
MIR1848	1	II[31]	III	*Os01g49720* (I)
MIR1849	1	II[31]	III	

(continued)

Table 1 (continued)

miRNA family	No. of family members	Identification method[a]	Conservation[b]	Target genes confirmed by degradome sequencing or 5′ RACE[c]	Functional investigation of miRNAs and their targets
MIR1850	1	II[31]	III	*Os01g02360* (I) *Os09g32250* (I) *Os05g05140* (I) *Os01g05790* (I) *Os04g47410* (II) ***AK068321***[31]	
MIR1851–MIR1856	1 for each	II[31]	III		
MIR1857	1	II[31]	III	*Os11g45590* (III)	
MIR1858	2	II[31]	III		
MIR1859–MIR1860, MIR1864–MIR1872, MIR1874–MIR1875, MIR1877, MIR1879–MIR1881	1 for each	II[31]	III		
MIR1861	14	II[31]	III	***Os05g51790***[31]	
MIR1862	7	II[31]	III		
MIR1863	3	II[31]	I (*P. abies*)	^*Os06g38480*[27]	
MIR1873	1	II[31]	III	^*Os05g01790*[27]	
MIR1876	2	II[31]	III	^*Os07g41090*[27] ^*Os02g05890*[27]	
MIR1878	1	II[31]	II (*B. distachyon*)		
MIR1882	8	II[31]	III		
MIR1883	2	II[31]	III		

MIR1884	2	II[31]	III	*Os01g59720* (II) *Os01g64520* (III) *Os02g22610* (I) *Os03g10250* (I) *Os03g19380* (II) *Os11g34910* (II)
MIR2055	1	II[9]	III	
MIR2090–MIR2097, MIR2099, MIR2100, MIR2104, MIR2106	1 for each	II[28]	III	
MIR2098	1	II[28]	III	*Os03g23050* (I) (for 3p) *Os07g03279* (II) *Os07g03368* (II) *Os07g03458* (II) (for 5p)
MIR2101	1	II[28]	III	*Os12g08210* (I)
MIR2102	1	II[28]	III	*Os05g37460* (I)
MIR2103	1	II[28]	III	*Os03g52880* (I)
MIR2105	1	II[28]	III	*Os01g14040* (I) *Os01g25484* (I)
MIR2118	18	II[7]	I	
MIR2120, MIR2122, MIR2125	1 for each	II[4]	III	
MIR2121	2	II[4]	III	
MIR2123	3	II[4]	III	
MIR2124	9	II[4]	III	
MIR2275	2	II[7]	II (*Z. mays*)	
MIR2862	1	II[26]	III	
MIR2863	2	II[26]	III	

(continued)

Table 1 (continued)

miRNA family	No. of family members	Identification method[a]	Conservation[b]	Target genes confirmed by degradome sequencing or 5′ RACE[c]	Functional investigation of miRNAs and their targets
MIR2864–MIR2867, MIR2869, MIR2870, MIR2872, MIR2874–MIR2880, MIR2905	1	II[26]	III		
MIR2868	1	II[26]	III	*Os11g14140* (II)	
MIR2871, MIR2873	2 for each	II[26]	III		
MIR2906	2	II[5]	III		
MIR2907	4	II[5]	III		
MIR2918–MIR2932	1	II[19]	III		
MIR3979, MIR3981, MIR3982	1 for each	II[10]	III		
MIR3980	2	II[10]	III		
MIR5071–MIR5083	1 for each	I[20]	II		

[a] I—homology searching using miRNAs identified in other species; II—small RNA cloning. The paper that first reported the miRNA is shown. For the miRNAs that were first identified by homology searching, the paper that first confirmed their presence in rice is also shown

[b] I—conserved in rice and at least one nonmonocotyledonous plant; II—conserved in rice and at least one other monocotyledonous plant; III—rice specific; IV—supposed to be conserved in *Arabidopsis* and rice, but they have not been discovered by high-throughput sequencing in both species. For the rice miRNA families that are conserved only in one other plant species, the name of the species is given (based on the information available in miRBase)

[c] Target genes that were identified by degradome sequencing, confirmed by 5′ RACE or shown to be methylated, are shown. I, II, and III in parentheses indicate the classes of target as shown in the original papers [11] and [26] (the first and second pair of parentheses, respectively). Targets that were confirmed by 5′ RACE are bolded, and the reference that performed the confirmation is also shown. Targets starting with ^ are not cleaved but methylated by miRNA as shown in ref. [27]

[1] Arazi et al. (2005), [2] Bonnet et al. (2004), [3] Heisel et al. (2008), [4] Huang et al. (2009), [5] Jian et al. (2010), [6] Jiao et al. (2010), [7] Johnson et al. (2009), [8] Jones-Rhoades and Bartel (2004), [9] Lacombe et al. (2008), [10] Li et al. (2010a), [11] Li et al. (2010b), [12] Liu et al. (2005), [13] Liu et al. (2009), [14] Lu et al. (2008), [15] Luo et al. (2006), [16] Morin et al. (2008), [17] Park et al. (2002), [18] Reinhart et al. (2002), [19] Sanan-Mishra et al. (2009), [20] Schreiber et al. (2011), [21] Sunkar and Zhu (2004), [22] Sunkar et al. (2005), [23] Sunkar et al. (2008), [24] Wang et al. (2004b), [25] Wang et al. (2004a), [26] Wu et al. (2009), [27] Wu et al. (2010), [28] Xue et al. (2009), [29] Yang et al. (2006a), [30] Zhao et al. (2009), [31] Zhu et al. (2008), [32] Zhu et al. (2009), [33] Zhu et al. (unpublished)

2.1.1 Group I miRNAs and Their Targets

Of the 27 Group I miRNA families, 21 families (miR156, 159, 160, 162, 164, 166–169, 171, 172, 319, 390, 393–399, and 408) are well conserved among diverse angiosperms. At least three families (miR156, 160, and 166) are also conserved in gymnosperms, lycopods, and bryophytes, indicating an ancient origin of these miRNAs. Target genes of 11 conserved miRNA families (miR156, 159, 160, 164, 166, 167, 169, 171, 172, 319, and 396) encode transcription factors that have a conserved role in plant development. For example, miR172 functions in regulating the transitions between developmental stages and in specifying floral organ identity by regulating expression of *AP2*-domain-containing transcription factors in monocots and dicots (Zhu and Helliwell 2011). The Class III *HD-ZIP* transcription factors are targets of miR166. The rice genome contains five Class III *HD-ZIP* genes: *OSHB1* to *OSHB5*. All these five genes have the miR166 binding site, and four of them (*OSHB1* to *OSHB4*) have been confirmed to be regulated by miR166 (Table 1). Overexpression of miR166-resistant versions of *OSHB1*, *OSHB3,* and *OSHB5* exhibited rolled and/or filamentous leaves caused by loss-of-leaf polarity (Itoh et al. 2008). Mutations in Class III *HD-ZIP* genes, which reduce miR166 complementarity in *Arabidopsis* and maize, confer similar phenotypes (Juarez et al. 2004; McConnell et al. 2001). Further studies have shown that proper leaf polarity in rice and maize is established by a coordinated expression of miR166 and ta-siARF in opposing domains along the adaxial–abaxial axis (Nagasaki et al. 2007; Nogueira et al. 2007). These results suggest that conserved miRNAs play a similar role by targeting conserved genes in different plants, although nonconserved target genes have been identified (Li et al. 2010b).

Of the remaining six Group I miRNAs (miR529, 530, 535, 827, 1863, and 2118), only miR827 is conserved in *Arabidopsis* (Heisel et al. 2008; Lacombe et al. 2008), which targets *SPX*-domain-containing genes in rice and *Arabidopsis*. The *SPX*-domain is associated with proteins that have been implicated in transport or sensing of phosphate or nitrogen in *Arabidopsis* (Peng et al. 2007; Wang et al. 2004c). It is expected the two confirmed miR827 targets (*Os02g45520* and *Os02g48390*) could play a similar role in rice development. Homologs of miR529 and miR535 are found in moss (Arazi et al. 2005), and a homolog of miR1863 has been found in gymnosperms (*Picea abies*) (Yakovlev et al. 2010), suggesting that these miRNAs have an ancient origin but could have been lost in eudicots (miR529) or *Arabidopsis* (miR535) after their separation from the monocotyledonous plant lineage. No homolog of miR530 has been found in *Arabidopsis*, but it is conserved in several dicotyledonous and monocotyledonous plants.

2.1.2 Group II miRNAs and Their Targets

Group II, the monocot-conserved miRNAs, contains 24 miRNA families, of which miR444 and miR528 are well conserved in all monocotyledons investigated.

The miR444 family is encoded by four loci in the rice genome. A few features of this miRNA family make it unique. First, in each locus, the pri-miR444 is overlapping and antisense to its target MADS box gene. Second, the pre-miR444 is processed from a spliced pri-miR444 that possesses large introns. Third, more than one functional miRNA (overlapping or in phase) is processed from each pre-miR444 (Lu et al. 2008). Due to the natural antisense characteristics of pre-miR444 and its MADS targets, different miR444 variants from the same pre-miR444 are expected to cleave MADS target mRNAs. This was confirmed by recent degradome sequencing (Li et al. 2010b; Wu et al. 2009), indicating that different miR444 variants are functional, and the MADS genes are collectively regulated by miR444 variants at multiple positions with different cleavage frequencies. In both of the degradome sequencing studies (Li et al. 2010b; Wu et al. 2009), non-MADS targets were also found, and two of them were further confirmed by 5′ RACE (Li et al. 2010b), demonstrating that miR444, like some other deeply conserved miRNAs, can regulate targets unrelated to the conserved ones.

The miR528 family of rice contains a single miRNA (Liu et al. 2005), which is predicted to target several genes with unrelated functions (Li et al. 2010b; Wu et al. 2009; Xue et al. 2009), three of them (*Os06g06050*, *Os06g37150,* and *Os06g11310*) were confirmed by degradome sequencing and/or 5′ RACE (Table 1). *Os06g37150* encodes L-ascorbate oxidase, which catalyzes the oxidation of ascorbic acid to yield dehydroascorbate to regulate the apoplastic redox state and modulate plant growth and defense responses by regulating signal transduction cascades (Pignocchi et al. 2006). miR528 is highly expressed in the embryo compared to vegetative tissues (Xue et al. 2009; consistent with our P_{miR528}*::GUS* transgene results, Zhu et al. unpublished). miR528-mediated downregulation of *Os06g37150* has been observed in the embryo (Xue et al. 2009). This may be required to maintain a higher level of cell division in developing rice grains, because it has been found that accumulation of dehydroascorbate oxidized by L-ascorbate oxidase can trigger the arrest of cell division (Potters et al. 2000). miR528-mediated regulation of *OsCBP1* (Cu^{2+}-binding domain-containing protein 1, *Os07g38290*), one of the predicted miR528 targets (Wu et al. 2009), has not been confirmed in rice, but its sugarcane ortholog *SsCBP1* has been shown to be cleaved by miR528 (Zanca et al. 2010). Regulation of plant *CBPs* by miR528 seems to be conserved in monocotyledons and has evolved exclusively within the monocotyledons after their divergence from eudicots (Zanca et al. 2010). As for miR528, a few other miRNAs that are highly expressed in embryo also have target genes encoding copper-binding proteins that are downregulated in embryo, suggesting a role for these miRNAs in maintaining copper and reactive oxygen species homeostasis in the rice embryo (Xue et al. 2009).

Of the other Group II miRNAs, miR821, miR1433, miR1435, miR1436, miR1878, and miR2275 are only conversed in one other monocotyledonous plant (Table 1), whereas miR1318/1432 and miR437 are conserved across multiple monocotyledonous plants. With the exception of miR1436, all these miRNAs do not have targets confirmed by degradome sequencing or 5′ RACE (Table 1). The remaining 13 Group II miRNAs (miR5071–5083) are computational predictions

based on cloned barley miRNAs (Schreiber et al. 2011). Their presence in rice has not been confirmed by small RNA sequencing.

2.1.3 Group III miRNAs and Their Targets

The majority of annotated rice miRNAs appear to be species specific. They were identified based on small RNA cloning followed by traditional or high-throughput sequencing using various tissues, such as seedlings (Morin et al. 2008; Sunkar et al. 2005; 2008), seeds (Xue et al. 2009; Zhu et al. 2008) and callus (Luo et al. 2006), or using stress-treated samples (Huang et al. 2009; Li et al. 2010a; Lu et al. 2008). miRNAs were also identified from small RNA populations associated with AGO1 (Wu et al. 2009). As most of these miRNAs were identified from specific tissues or plants growing under altered growth conditions, it is expected that some of them will have a specific expression profile. For example, miR1428e is highly expressed in developing grains but not detected in roots (Zhu et al. 2008). However, for the majority of rice-specific miRNAs, their tissue specificity is yet to be explored.

The target genes of rice-specific miRNAs encode a wide range of proteins; some of these mRNAs have been shown to be cleaved by miRNAs (Table 1). Generally, the low expression level and/or tissue specificity of these miRNAs means that confirmation of miRNA-mediated regulation of putative target mRNAs is not as easy as for highly expressed conserved miRNAs. In addition to miRNA-mediated cleavage and translational repression, recent investigation in rice showed that 24-nt miRNAs were absent from the AGO1 complex but were loaded into AGO4 clade proteins to induce DNA methylation in *cis* at loci from which they are generated or in *trans* at their targets, suggesting that these miRNAs may transcriptionally regulate expression of themselves and their target genes (Wu et al. 2009, 2010). It is worthy of attention that *Os03g02010*, a target of miR820a, was subjected to both miRNA-mediated cleavage (Lu et al. 2008; Luo et al. 2006) and DNA methylation (Wu et al. 2010), presumably induced by 21-nt and 24-nt miRNA variants, respectively. This is consistent with our previous small-RNA-sequencing result for the *MIR820a* locus (Zhu et al. 2008), from which 94 reads of 21-nt and 2,208 reads of 24-nt miRNAs (the most abundant miRNA variant) were generated. This phenomenon may apply to many other *MIRNA* genes because different lengths of miRNA variants are generated from the same region of many pre-miRNAs.

miRNAs derived from exonic regions are rare but are found in RNA populations from human cancer cells (Lui et al. 2007). It was recently demonstrated in rice plants that miR3981 is processed from an exon region from a gene that encodes for a glyoxylase. More interestingly, by 5′ RACE, it was demonstrated that miR3981 targets its own precursor gene (Li et al. 2010a). Similarly, degradome sequencing has shown that miRNAs regulate their primary precursors (German et al. 2008).

3 miRNAs Are Required for Correct Growth and Development in Rice

miRNA-mediated gene regulation is implicated in most if not all major developmental steps of the plant life cycle, and the function of the well-conserved miRNA families seems to have been preserved across species (Chen 2009). For example, miR156, which is involved in shoot apical meristem (SAM) development, controls vegetative to floral phase transition in *Arabidopsis* and maize, and shoot branching in rice (Chuck et al. 2007; Jiao et al. 2010; Miura et al. 2010; Yang et al. 2010). Although specific functions of each conserved miRNA may differ across species, the key role of miRNAs in regulating plant development is undeniable, and plants affected in miRNA biogenesis or action show severe pleiotropic phenotypes.

In *Arabidopsis*, biogenesis of most miRNAs requires DCL1 for the two initial processing steps of the precursor (Bouche et al. 2006; Kurihara and Watanabe 2004). Loss-of-function *AtDCL1* mutants dramatically affect the accumulation of miRNAs and lead to a wide range of phenotypes, ranging from altered organ morphology to developmental arrest (McElver et al. 2001; Park et al. 2002; Reinhart et al. 2002; Schauer et al. 2002). Consistently, mutants of *AtAGO1*, an essential enzyme of the miRNA effector complex, display similar pleiotropic developmental defects associated with impaired miRNA function (Vaucheret et al. 2004).

In rice, RNA-interference-mediated silencing of *OsDCL1a* (*Os03g02970*), the homolog of *AtDCL1*, leads to abnormal shoot and root development with eventual growth arrest for the strongest RNAi lines (Liu et al. 2005). The aerial part of the plant shows severe dwarfism and rolled dark green leaves. The root system is dramatically affected, having reduced elongation and fewer adventitious roots together with the presence of ectopic chloroplasts. At a molecular level, RNAi-OsDCL1a plants show impaired accumulation of all seven mature miRNAs tested but none of the tested siRNAs, suggesting that OsDCL1a, like AtDCL1, is required for miRNA biogenesis. The phenotypes of RNAi-OsDCL1a can be therefore considered as direct consequences of a loss-of-miRNA function.

miRNA action in rice seems to be driven by at least four partially redundant AGO1-like proteins. Plants in which the *OsAGO1s* are silenced using an inverted repeat RNAi construct targeting all four AGO1 homologs (*OsAGO1a* (*Os02g45070*), *OsAGO1b* (*Os04g47870*), *OsAGO1c* (*Os02g58490*), and *OsAGO1d* (*Os06g51310*)) show pleiotropic phenotypes similar to those observed in RNAi-OsDCL1a, including dwarfism with narrow, rolled leaves, and developmental arrest for the strongest line (Wu et al. 2009). Accordingly, five previously confirmed targets of known miRNAs were shown to be upregulated in both RNAi-OsAGO1s and RNAi-OsDCL1a plants. The severe phenotypes associated with loss-of-miRNA function highlight the crucial role of miRNAs in the growth and development of the rice plant.

4 Expression of miRNAs and miRNA Biogenesis Machinery

4.1 Regulation of the miRNA Biogenesis Machinery

The initial step of plant miRNA biogenesis is the transcription of a *MIR* gene by RNA polymerase II. The primary transcript (pri-miRNA) forms an imperfect hairpin that is processed into a small RNA duplex in two or three steps by an enzyme complex containing at least three proteins: DICER-LIKE1 (DCL1), HYPONASTIC LEAVES1 (HYL1), and SERRATE (SE). The small RNA duplex, composed of the miRNA and its opposite strand, the miRNA*, is protected from exonuclease degradation through the methylation of its 3′ ends by HUA ENHANCER1 (HEN1). Exported outside the nucleus by HASTY (HST), the miRNA is then incorporated into an ARGONAUTE (AGO) protein to form the miRNA-induced silencing complex (miRISC) and trigger the posttranscriptional regulation of its target gene(s). A genome-wide analysis of the rice genome revealed the presence of homologs for all the currently known components of the miRNA biogenesis machinery, including eight *OsDCLs*, 19 *OsAGOs*, three *SE* (*Os06g48530*, *Os08g40560*, *Os02g05610*), four *HYL1* (*Os12g01916*, *Os05g24160*, *Os11g01869*, *Os01g56520*), and interestingly, only one copy of *HST* (*Os01g26160*) and *HEN1* (*Os07g06970*) homologs (data collected from Kapoor et al. 2008 and BLAST searches using the *Arabidopsis* gene sequences against the rice genome available at http://rice.plantbiology.msu.edu/index.shtml).

miRNA biogenesis in *Arabidopsis* requires *AtDCL1*, a gene with three homologs in rice: *OsDCL1a* (*Os03g02970*), *OsDCL1b* (*Os06g25250*), and *OsDCL1c* (*Os05g18850*) (Kapoor et al. 2008; Margis et al. 2006). Based on their sequence similarity and chromosome location none appear to be the result of tandem duplication. OsDCL1a is most closely related to AtDCL1, possessing all the six domains characteristic of the Dicer family; silencing this gene abolishes the biogenesis of miRNAs (Liu et al. 2005). On the other hand, *OsDCL1b* and *OsDCL1c* encode for smaller proteins containing only the RNaseIII and dsRB domains, matching the C-terminal region of OsDCL1a. Although these two domains are crucial for the recognition and spatial cleavage of dsRNAs (Zhang et al. 2004), both *OsDCL1b* and *OsDCL1c* are unlikely to be functionally redundant with *OsDCL1a*. Consistent with this, microarray analysis shows that *OsDCL1a* is ubiquitously expressed at all stages of the plant development with a peak of expression in the vegetative tissue, whereas *OsDCL1b* and *OsDCL1c* are only weakly expressed (Kapoor et al. 2008). However, *OsDCL1c* has a peak of expression in the panicle during the anthesis stage, suggesting a function distinct from *OsDCL1a*. It is also worth noticing that the silencing of *OsDCL4*, the closest homolog of *OsDCL1a* after *OsDCL1b* and *OsDCL1c*, does not affect the accumulation of any tested miRNAs (Liu et al. 2005). Taken together, these data strongly suggest that OsDCL1a is the primary dicer responsible for miRNA processing in rice.

Once processed, most of the mature miRNAs interact with AtAGO1 in *Arabidopsis*. Rice has five *AtAGO1* homologs: *OsAGO1a*, *b*, *c*, and *d* (with *OsAGO1a*

and *OsAGO1b* being segmentally duplicated) and *OsAGO10* (also known as *PIN-HEAD*, *Os06g39640*) (Kapoor et al. 2008). All of them possess the conserved metal chelating residues in the PIWI domain that are critical for endonuclease activity, suggesting that all encode for a functional enzyme. The four OsAGO1s probably have some functional redundancy since they mostly interact with the same known miRNAs (Wu et al. 2009). On the other hand, their spatiotemporal expression does not necessarily overlap (Fujita et al. 2010; Jain et al. 2007; Kapoor et al. 2008, http://www.plexdb.org/index.php) and may explain why a subset of miRNAs were found to differentially interact with OsAGO1a, b, and c. Here are a few striking examples of the differential expression among the *OsAGO1* family. During vegetative development, *OsAGO1b* and *c* are preferentially expressed in the shoot rather than the root. Later on in the developing anther, the expression constantly drops for all but *OsAGO1a* which is significantly upregulated in mature pollen. *OsAGO1b* and *OsAGO1d* are preferentially expressed in the top part of the ovary, but dramatically decrease just after fertilization. Finally, during seed development, the expression of *OsAGO1a* and *OsAGO1c* drops, whereas *OsAGO1b* and *OsAGO1d* gradually increase. These data clearly suggest that despite their similarity, the four OsAGO1s are probably involved, at least partially, in different biological processes. It has also been found that some miRNA*s accumulate in certain tissue more than their counterpart miRNA. miR390* is present at higher level in the embryo than miR390, suggesting that miRNAs and miRNA*s could function by association with different AGO1 family members (Xue et al. 2009). AGO10, which also belongs to the AGO1 family, has a miRNA-related function and is partially redundant with AGO1 in *Arabidopsis* (Mallory et al. 2009). Mutation of *AtAGO10* causes a critical alteration of the maintenance of the SAM (Lynn et al. 1999). Similarly in rice, *OsAGO10* is highly expressed in the SAM and is required for the maintenance of the indeterminate state of the SAM, suggesting a conserved function with *Arabidopsis* (Jain et al. 2007; Nishimura et al. 2002; http://www.plexdb.org/index.php). On the other hand, the proper development of the adaxial side of the leaves, which requires *AtAGO1* in *Arabidopsis*, seems to be dependent on *OsAGO10* in rice, suggesting that the specialization of AGO1 and AGO10 occurred after the split between monocot and dicot lineages (Kidner and Martienssen 2004; Nishimura et al. 2002; Yang et al. 2006b). Overall miRNA-mediated regulation in rice involves at least five AGO1 homologs, with potentially different tissue-specific functions.

Across species, the function of the miRNA pathway seems to be highly dependent on the *DCL1* and *AGO1* gene families. In the absence of AGO1, newly synthesized miRNAs are subject to degradation, suggesting that miRNA biogenesis and action need to be coupled in order for the miRNA to be functional. Interestingly, in tricellular mature pollen, *OsDCL1a* is not expressed, whereas *OsAGO1a* mRNA and two known miRNAs (miR166 and miR167) can be detected, suggesting that miRNAs previously processed and incorporated into miRISC can maintain their regulatory function in later developmental stages (Fujioka et al. 2008; Fujita et al. 2010; http://www.plexdb.org/index.php).

4.2 Regulation of miRNA Expression

While the overall control of the miRNA pathway depends on common enzymes, the specific function of a miRNA relies on its transcriptional regulation. Several mature miRNAs have been shown to accumulate preferentially in one or two tissues with a pattern that correlates with their associated function (Xue et al. 2009). The expression profile of a *MIR* gene provides valuable information in determining its role in plants. There is currently relatively little expression profile data for the rice miRNAs. However, the reduced cost and increased accessibility of next-generation high-throughput sequencing technology now allows generation of miRNA expression data at high sensitivity and resolution.

The *MIR* genes in rice are predominantly found in intergenic regions suggesting an autonomous control of their transcription, as opposed to pri-miRNA located in intronic regions that could be processed from the host gene transcript. Most of the *MIR* promoters possess *cis*-elements characteristic of RNA polymerase II activity (TATA-box, CAAT-box, and transcription start site), mainly located within 400 bp upstream of the pre-miRNA (Cui et al. 2009; Meng et al. 2009). The conserved *MIR* genes tend to contain more core promoter motifs than the nonconserved ones, suggesting that new pri-miRNAs gain their functionality by acquiring *cis*-regulatory elements in order to be specifically transcribed. Indeed some transcription factors, such as Auxin Response Factors (ARFs), may require the combination of a specific *cis*-element and the TATA-box in order to efficiently interact with the promoter (Inukai et al. 2005). In contrast, some *MIR* promoters are TATA-less; these can still be transcribed by RNA polymerase II but may belong to a different subclass of *MIR* genes expressed in more specific conditions (Bernard et al. 2010).

Regulation of rice miRNAs under abiotic stress conditions has been investigated by several research groups using different methods, including stem-loop RT-qPCR, mature-miRNA-specific RT-qPCR, miRNA microarray, Affymetrix GeneChip, and deep sequencing of small RNA libraries (Chen et al. 2009; Huang et al. 2009; Lv et al. 2010; Meng et al. 2009; Shen et al. 2010; Zhou et al. 2010). A large number of miRNAs, including most of the conserved miRNAs, have been shown to respond to abiotic stress. Six rice-specific *MIR* gene families have been identified in response to toxic exposure to the heavy metal cadmium (Cd^{2+}) (Huang et al. 2009).

Under drought stress during vegetative and reproductive stages, Zhou et al. (2010) showed that after a few days of treatment, nine miRNA families found in the rice genome were downregulated and four were upregulated. Two miRNA families, miR172 and miR396, were found to be consistently repressed at both developmental stages, suggesting an important role in drought response. Interestingly, one member of the miR393 family showed an opposite expression at the reproductive stage between 5 and 6 days of treatment.

Using a rice-specific miRNA microarray chip, Lv et al. found 15 miRNA families affected by cold stress (Lv et al. 2010). Five of those miRNA families (miR156, 168, 169, 171, and 319) were also deregulated under drought stress, with

miR169 showing an opposite response between both stresses. In comparison, two other groups found additional miRNA families showing a slight deregulation within a few hours after drought, salt, cold, and abscisic acid (ABA) treatments (Shen et al. 2010; Zhao et al. 2007). A majority of those miRNAs were deregulated in at least two stresses and were sensitive to ABA treatment, suggesting that a common regulatory mechanism exists in response to different abiotic stresses in rice (Shen et al. 2010). Additionally, 5% of the miRNAs in rice (miRBase release 10) have been shown to be sensitive to auxin, and expression of seven miRNA families was affected in *osaxr*, an auxin-resistant mutant (Meng et al. 2009). Interestingly, miR528, which was the only *MIR* gene sensitive to both auxin treatment and the *osaxr* mutation, has not been linked to any other abiotic stress.

Analysis of the promoter region of stress-sensitive *MIR* genes revealed the presence of multiple stress or phytohormone regulatory *cis*-elements (Lv et al. 2010; Meng et al. 2009; Shen et al. 2010; Zhou et al. 2010). Among the more represented are *cis*-element linked with ABA response ("ABRE"ACGTG, CAAACACC, CACCTCCA, CACGTGGC), early response to dehydration (ACGT), MYB transcription factor (CTAACCA, CNGTTR), salt response (CANNTG), gibberellin response ("P-box" CCTTTT, TATC-box), salicylic acid response, auxin response ("AuxRE" TGTCTC), and endosperm expression ("Skn-1" TRTCAT, "CGN4" TGAGTCA). So far, none of these regulatory elements has been functionally validated, and the diversity and elevated number of sequences found in the promoters make it difficult for any confident interpretation. Still, we can observe that almost all *MIR* genes that are drought-sensitive have the Skn-1 *cis*-element in their promoter (Zhou et al. 2010). Also, AuxREs are found more frequently in the promoters of auxin-related *MIR* genes (Meng et al. 2009).

Interestingly, most stress-sensitive miRNAs show dynamic expression patterns (as opposed to a constitutive response) within the first hours following the treatment (Lv et al. 2010; Shen et al. 2010; Zhao et al. 2007). So depending on the experimental protocol, some stress-sensitive miRNAs may have escaped detection or shown different responses. For example, Shen et al. (2010) observed an upregulation of miR408 at 20 min after treatment, whereas Zhou et al. (2010) observed a downregulation at 14 days. Additionally, the ability to detect different miRNA homologs is important since miRNAs that belong to the same family can show different stress–response patterns, an example of this is the miR169 family (Zhao et al. 2007). Indeed, precursors that encode for the same mature miRNA can also be expressed differently under the same stress. A good example is the response of miR171 family members to cold stress (Lv et al. 2010). In rice, there are nine *MIR171* genes (a–i), which encode for five different mature miRNAs. Under cold treatment, pri-miR171a/e/f/i are downregulated, whereas pri-miR171c/d/h are upregulated. Pri-miR171c/d and e/f encode for the same miRNA and so potentially target the same genes, suggesting that those precursors have different tissue specificity. This example illustrates the complexity of miRNA-mediated regulation during abiotic stress responses and highlights the need to investigate them in different tissues and at multiple time points.

An efficient way of coordinating the regulation of specific molecular processes is through the simultaneous expression of clustered miRNAs, a dominant

characteristic of animal *MIR* genes. Interestingly, the rice genome contains 18–24 miRNA clusters (reported in two different studies), which represent roughly 20% of the total miRNAs (Cui et al. 2009; Merchan et al. 2009). This suggests that coordinated expression of miRNAs via a polycistronic transcript may be an important biosynthesis pathway in rice. Indeed, two miRNA clusters have already been validated, the first located in an intergenic region encodes for two miRNA homologs (miR159a.1 and a.2) and the second encodes for three intronic miRNAs (miR1876, miR1884b, and miR1862d) that are cotranscribed with the host gene (Lacombe et al. 2008; Lv et al. 2010; Zhu et al. 2008). Additionally, some miRNA clusters originating from a gene duplication event may have conserved regulatory expression (Meng et al. 2009). Further study of the *MIR* clusters, particularly those containing nonhomolog miRNAs (*MIR172c-820d*, *MIR172b-806a*, *MIR399d-439h*, and *MIR171f-443*), will certainly provide new insights to investigate the connections between miRNA-regulated networks in rice.

Overall, the dynamic expression patterns of miRNAs in response to abiotic stress, the presence of multiple phytohormone-related *cis*-elements in their promoters, and the evidence of direct regulation of phytohormone-related transcription factors by miRNAs (such as miR160/miR167-targeting ARF and miR159-targeting GAMyb) suggest complex interconnections between miRNA and phytohormone regulatory networks. In rice, the miRNAs are likely to be early components in the cascade of regulation that spreads and specifies hormone responses during a stress or a developmental event.

5 Biological Functions of Rice miRNAs

As detailed above, many miRNAs (both conserved and nonconserved) have target mRNAs that have been confirmed cleaved at the predicted target site. However, how the regulation of these target mRNAs by miRNAs contributes to biological processes has only been defined in a few cases. This is particularly the case for members of the Group II and III miRNAs where no information is available from *Arabidopsis* studies.

It is well established from *Arabidopsis* studies that miR156 regulates the *SPL* genes. Rice plants overexpressing miR156 are semidwarf, have altered numbers of leaves, and have longer vegetative phase (Xie et al. 2006). The downregulation of miR156 expression as plants progress from the juvenile to the adult phase agrees with observations across multiple species that this miRNA has a conserved role in regulating phase changes. In contrast, miR172 is upregulated during rice development (Zhu et al. 2009) also consistent with a role in regulating developmental phase changes. miR172 targets a small group of genes encoding *AP2-like* transcription factors. Overexpression of miR172b leads to a delay in the transition from spikelet to floral meristem, changing the numbers and identities of floral organs (Zhu et al. 2009). The miR172b overexpression lines also had enlarged lodicules which prevent closing of the spikelets after flowering. Loss of the miR172 target site in

a barley *AP2-like* gene, *Cly*, leads to cleistogamous (closed) flowering; these results suggest that a balance of miR172-mediated repression is important for controlling the normal noncleistogamous phenotype (Zhu and Helliwell 2011).

A major target for plant breeders is improvement of plant architecture to optimize grain yields. The map-based cloning and characterization of a semidominant QTL (*IPA1*) revealed that it contains the miR156-targeted *SPL14* gene (Jiao et al. 2010). The basis of the QTL appears to be a mutation of the miR156 target site in the *SPL14* gene. This leads to increased abundance of the SPL14 protein. As a consequence, the number of tillers is reduced, and grain yield increased compared to wild type. This result suggests that identifying alleles with altered miRNA target sites may be a fruitful method of identifying useful phenotypic variation.

Recently, it was demonstrated that miR396 controls cell proliferation in *Arabidopsis* (Rodriguez et al. 2009). Rice plants overexpressing miR396c have reduced tolerance to salt and alkali stress (Gao et al. 2010). miR396 targets GRFs (growth regulating factors) that are important proteins in plant growth and development. Overexpression of rice miR396c caused the downregulation of different GRFs, which may confer the increased sensitivity of the plants to salt and alkali stress. It has also been reported that members of the highly conserved miR393 family have important roles in response to development and stress (Arenas-Huertero et al. 2009; Liu et al. 2008; Sunkar and Zhu 2004). In rice, miR393 expression shows a trend to decreased expression in response to either high salt or alkali treatments, while expression of the related miR393b is relatively constant (Gao et al. 2011). Rice plants overexpressing miR393 showed severe symptoms than wild type when treated with 150 mM of NaCl. Bioinformatic predictions suggest that miR393 targets rice genes that code for stress-related proteins (Gao et al. 2011), and thus, the reduction of miR393 under salt stress conditions may allow expression of stress–response proteins to increase.

6 Other miRNA-Like siRNAs in Rice

In addition to the miRNAs discussed above, a number of other siRNAs have been identified with miRNA-like properties but which differ in aspects of their biogenesis. One example is a putative mirtron identified from deep sequencing of grain small RNAs (Zhu et al. 2008). Mirtrons are miRNAs processed from spliced and debranched introns; they were originally defined in animal systems where intron splicing essentially replaces the need for processing of the pri-miRNA hairpin structure by Drosha (Ruby et al. 2007). In the case of the rice mirtron (miR1429.2), small RNA reads align exactly to each end of the intron of a putative homeobox gene which is predicted to form a miRNA-like stem-loop once debranched. miR1429.2 is predominantly expressed in developing grains, but does not have a demonstrated target gene, so whether this miRNA has a biological role is unclear.

A large number of phased siRNAs have been identified in rice; most of these appear to be generated by a similar mechanism to ta-siRNAs and are expressed specifically in the developing inflorescence (Johnson et al. 2009). Two examples of long hairpin structures that are transcribed and processed to phased 21-nt siRNAs have been identified in rice (Zhu et al. 2008). Among the small RNAs produced from these loci two have been shown to cleave target mRNA transcripts by 5′ RACE, indicating that these siRNAs can act to cleave target transcripts in a miRNA-like manner. At present, the biological significance of these miRNA-like small RNAs is unclear.

7 Conclusion

In conclusion, it is clear that as for other species, the rice genome encodes many miRNAs that potentially target a wide range of biological processes. To date, our understanding of the function of these miRNAs is largely limited to those that are conserved across species. The many rice-specific miRNAs tend to be present at low abundance, so many have very subtle or cell-specific roles that will require careful investigation.

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