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Identification of novel stress-regulated microRNAs from *Oryza sativa* L.

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ABSTRACT

MicroRNAs (miRNAs) are a type of small non-coding RNA found in eukaryotes. They play a key role in gene expression by down-regulating gene expression and are involved in the environment stress response in plants. Although a large number of miRNAs have been identified from *Arabidopsis*, few studies have focused on *Oryza sativa* miRNAs, especially on stress-related miRNAs. Five cDNA libraries of small RNAs from rice seedlings treated with cold, dehydration, salinity, and abscisic acid (ABA), as well as wild-type seedlings, were constructed. Seven rice novel miRNAs were identified by Northern analysis, and their expression patterns under different stress treatments were determined. Results showed that the expression of several novel miRNAs was regulated by one or more stress treatments. Our identification of novel stress-related miRNAs in rice suggests that these miRNAs might be involved in rice stress response pathways.

Introduction

A class of small RNA molecules called microRNAs (miRNAs) has been identified in recent years. MiRNAs are endogenous non-coding RNAs, are 20–22 nucleotides long, and regulate gene expression in eukaryotes ranging from animals to plants [1–6]. In both animals and plants, miRNAs are transcribed by RNA polymerase II into long primary transcripts (pri-miRNAs) [7]; in animals, pri-miRNAs are trimmed in the nucleus into miRNA precursors (pre-miRNAs) by an RNase III-like enzyme called Drosha [8,9]. The pre-miRNAs are then exported to the cytoplasm and were then cleaved to generate mature miRNAs by Dicer [10–12]. However, it seems that there is no Drosha ortholog in plant genomes. The plant Dicer homolog is likely to have the Drosha function, but its localization is unclear [12]. Mature miRNAs are incorporated into the RNA-induced silencing complex (RISC) endonuclease, which seems to negatively regulate the genes required for several developmental processes by promoting RISC-mediated degradation of target mRNAs or the inhibition of target mRNA translation [13].

MiRNAs have been identified by cloning [4,14,15], computational approaches [3,16,17,18] and by genetic screens [19]. The identification of the entire set of miRNAs and their target genes from model organisms is of fundamental importance to the understanding of regulatory networks and gene silencing mechanisms. Four Dicer-like enzymes (DCL1–DCL4) are encoded in the genome of *Arabidopsis thaliana* [20]. It has been shown that DCL1 is involved in miRNA accumulation [21]. However, there is no *dcl1* mutant available for rice. A recent study has shown that the loss of function of *OsDCL1* transformants could be used to identify miRNAs [22].

Recent evidence indicates that miRNAs are involved in biotic and abiotic stress responses in plants. The first such role of miRNAs in plants was described by Sunkar and Zhu [4] in *Arabidopsis*, they found several miRNAs are rather up-regulated or down-regulated by abiotic stresses. Sunkar and Zhu cloned short RNAs from *Arabidopsis* seedlings exposed to different abiotic stresses and identified several miRNAs with differential expression patterns [4]. For example, miR393 was strongly up-regulated by cold, dehydration, high salinity and abscisic acid (ABA) treatments. Furthermore, miR319c, miR389a, miR397b and miR402 were regulated by different abiotic stress treatments to varying degrees in *Arabidopsis* [4]. Nearly at the same time, Jones-Rhoades and Bartel [3] investigated miR395 was up-regulated upon sulfate starvation in *Arabidopsis*, and miR395 targets the ATP sulfurylases genes as well as *AST68* (encoding a sulfate transporter) [3,23]. Subsequently, the stress-involved miRNAs were reported. MiR399 was shown to down-regulate *UBC24* mRNA accumulation and to be involved in plant responses to Pi starvation *in planta* [24,25]. MiR393 has also been shown to inhibit the expression of *TIR1* to down-regulated auxin signaling and seedling growth under abiotic stress conditions [4,26]. Moreover, miR159 was shown to involved in hormone signaling and dehydration responses in *Arabidopsis* [27,28]. However, these observations were all shown in

Abbreviations: miRNA, microRNA; bp, base pairs; GC, guanine-cytosine; PCR, polymerase chain reaction; UTL, untranslated region; MPSS, massively parallel signature sequencing.

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Arabidopsis, few stress-related miRNAs have been discovered in rice. Among miRNAs discovered in rice, only two have been found to be related to abiotic stress, miR393 and miR169g, both up-regulated by dehydration [29].

To explore the potential roles of regulatory small RNAs in abiotic stress tolerance and gene regulation, we constructed five libraries of small RNAs from rice seedlings treated with cold, dehydration, salinity, and abscisic acid (ABA), as well as wild-type seedlings. Through library sequencing and analysis, we found new candidate miRNAs. A miRNA array was used to analyze the expression profiles of candidate miRNAs under different stress treatments. The expression patterns of the miRNAs under different stress treatments were verified by Northern blotting. Seven novel miRNAs were identified, which may respond to and be regulated by stress in rice. Our results have important implications for gene regulation under abiotic stress and also contribute to the understanding of the functions of miRNAs in rice.

Results

Cloning of miRNAs from rice

To identify novel miRNAs from rice, we generated five independent small RNA libraries with size range of 18–28 nucleotides from untreated 2-week rice seedlings and 2-week rice seedlings treated with ABA, 4 °C, dehydration, and NaCl, respectively. After sequencing, about 3900 individual small cDNA sequences between 18 and 28 nucleotides in length were isolated (Table 1).

The ~3900 small cDNAs were used to predict *Oryza sativa* miRNAs and eliminate redundancy. A total of 74 new miRNA candidates were predicted in the cloned small RNA libraries as well as seven previously identified rice miRNAs (Table 2). Several guidelines have been proposed for miRNAs annotation [6]. The miRNA precursors should contain stable and conserved stem-loop structures that can be predicted by Mfold [30]. Seventy-four small RNAs could be regarded as candidate miRNAs according to the miRNA annotation criteria [6].

MicroRNA array analysis

To reduce false discovery rate of miRNAs among the predicted candidate miRNAs and identify the expression pattern of the newly candidate miRNAs, we performed microarray analysis using these candidate miRNAs. Seventy-four candidate miRNAs were used in the microRNA array construction, and low-molecular-weight RNAs of different stress treated rice seedlings, as well as untreated ones, were used for screening. The expressions of all the candidate miRNAs were analyzed in paired stress treated and untreated rice seedlings with SAM software. Microarray analysis indicated that some miRNAs were significantly over-expressed in treated seedlings with fold changes >1.5, of which 3 miRNAs were over-expressed under ABA treatment, 14 miRNAs were over-expressed under drought treatment, 7 miRNAs were over-expressed under NaCl treatment, and no miRNA over-expressed under cold treatment. Among them, eight candidate miRNAs were shown to be up-regulated under stress treatments (Table 3).

Table 1
New rice candidate miRNAs predicted by computation.

Treated condition	Sequenced number	Predicted miRNAs no.
ABA (100 μM) 24 h	215	7
NaCl (30 mM) 24 h	1089	21
Cold (4 °C) 24 h	1090	36
Drought 24 h	865	14
Untreated	629	16
Total	3888	76

Table 2
Cloning verification of previously predicted miRNAs in rice.

miRNA	miRNA sequence (5′-3′)	Cloning frequencies in libraries
OsmiR156 a→f	UGACAGAAGAGAGUGAGCAC	ABA (1)
OsmiR159 a,b	UUUGGAUUGAAGGGAGCUCUG	Cold (1)
OsmiR167 a→j	UGAAGCUGCCAGCAUGAUCUA	Cold (1)
OsmiR169 f→m	UAGCCAAGGAUGACUUGCCUA	Cold (2)
OsmiR171 b→f	UGAUUGAGCCGUGCCAAUAUC	ABA (1), Cold (4), NaCl (1)
OsmiR172 a,b,d	AGAAUCUUGAUGAUGCUGCAU	Cold (1), NaCl (1)
OsmiR319 a,b	UUGGACUGAAGGGUGCUCCC	Control (1)

Meanwhile, fewer under-expressed miRNAs/candidate miRNAs were identified than over-expressed ones. Eight miRNAs were under-expressed under ABA treatment, four miRNAs were under-expressed under cold treatment, two miRNAs were under-expressed under drought treatment, and four miRNAs were under-expressed under NaCl treatment, and in all of them, the change was <1.5-fold. Our 15 down-regulated candidate miRNAs under stress treatments are shown in Table 3.

Identification of seven new miRNAs from rice

To identify the candidate miRNAs, especially those over-expressed or under-expressed candidate miRNAs under stress treatments on the miRNA chip, Northern blot hybridization was carried out. DNA oligonucleotides complementary to candidate miRNAs sequences were synthesized and used as first round probes. The candidate miRNAs presented in the Northern blot hybridization were selected and used for second round Northern blot hybridization. DNA oligonucleotides identical to candidate miRNAs sequences were

Table 3
Differentially regulated miRNAs in stress treated rice seedlings.

miRNA	Expression location	Fold	Localfdr (%)	Regulated pattern
cd miR1	ABA	2.2	17.2	Up-regulated
	Drought	3.1	1.9	Up-regulated
cd miR2	NaCl	1.6	6.9	Up-regulated
cd miR3	NaCl	3.3	0.3	Up-regulated
cd miR4	NaCl	2.0	1.4	Up-regulated
cd miR5	NaCl	2.3	2.0	Up-regulated
cd miR6	ABA	1.5	25.1	Up-regulated
	NaCl	2.1	0.5	Up-regulated
miR2006	ABA	2.7	11.8	Up-regulated
	Drought	3.3	0.0	Up-regulated
	NaCl	1.7	2.7	Up-regulated
cd miR7	NaCl	1.7	1.8	Up-regulated
cd miR8	ABA	0.61	7.0	Down-regulated
	Cold	0.61	0.0	Down-regulated
cd miR9	Drought	0.49	0.7	Down-regulated
cd miR10	NaCl	0.52	0.0	Down-regulated
miR2003	NaCl	0.65	13.2	Down-regulated
cd miR11	ABA	0.65	9.3	Down-regulated
cd miR12	ABA	0.57	0.0	Down-regulated
cd miR13	ABA	0.63	0.3	Down-regulated
miR2004	ABA	0.63	13.6	Down-regulated
cd miR14	ABA	0.47	17.2	Down-regulated
	Cold	0.67	0.0	Down-regulated
cd miR15	ABA	0.60	14.2	Down-regulated
cd miR16	Cold	0.60	0.0	Down-regulated
miR2005	NaCl	0.65	11.6	Down-regulated
miR2007	Drought	0.44	2.1	Down-regulated
cd miR17	NaCl	0.65	12.9	Down-regulated
cd miR18	ABA	0.56	0.0	Down-regulated
	Cold	0.66	0.0	Down-regulated

A summary of SAM analysis of paired stress treated and untreated rice seedlings. Mean of fold changes; localfdr, local false discovery rates. *q* value (%) of all miRNAs was 0. cd miR: candidate miRNA.

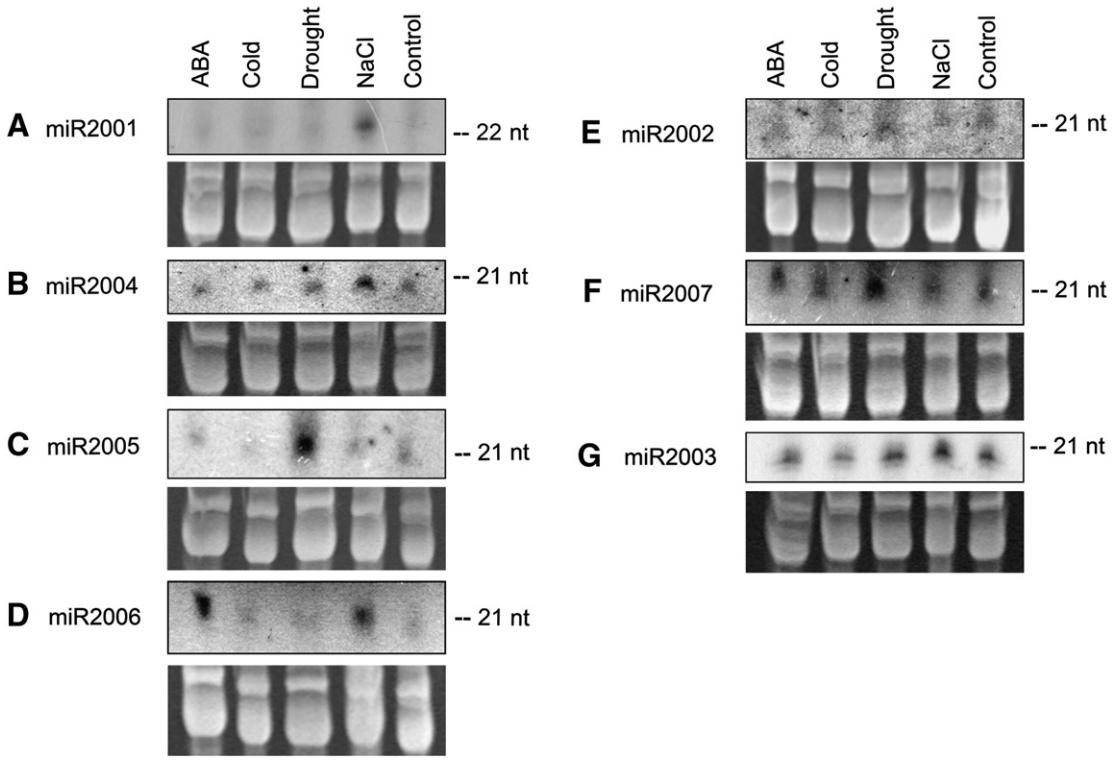

Fig. 1. Detection of the miRNAs by Northern blot with oligonucleotide probes complementary to miRNA sequences. The samples were from rice seedlings, as well as seedlings with different treatments, including ABA, cold, drought and NaCl. The 5S rRNA and tRNA bands were visualized by ethidium bromide staining of gels and served as loading control. Each probe is listed.

synthesized and used as second round probes. According to the miRNA annotation criteria [6], mature miRNAs should be detected by Northern blotting, and an observation that ~22 nt RNAs are produced from both a sense and an antisense transcript is evidence of siRNAs, our candidate miRNAs that show no small RNA signal in the second round Northern blot hybridization are regarded as miRNA.

Table 4
Novel rice miRNAs identified by cloning.

miRNA	miRNA sequence	Length (nt)	Folback arm/nt	Chromosome	Distance to nearest gene	Orientation
miR2001a	CCCAGCUUGAGAAUCGGGCGGC	22	5′/66	2	4898nt upstream to Os02g47060	Sense
miR2001b			5′/66	9	33038nt upstream to Os09g01002	Sense
miR2001c			5′/66	9	4646nt downstream to Os09g00998	Sense
miR2001d			5′/66	9	12574nt downstream to Os09g00998	Sense
miR2001e			5′/66	9	9254nt upstream to Os09g01002	Sense
miR2002a	AACGGGCCGCCGCACUGCUGG	21	3′/206	3	3571nt upstream to Os03g51350	Sense
miR2002b			3′/78	6	1437nt downstream to Os06g43590	Anti-sense
miR2003a	CCGGCCCCGAACCCGUCGGCU	21	5′/93	2	7498nt downstream to Os02g47040	Sense
miR2003b			5′/95	9	31185nt upstream to Os09g01002	Sense
miR2003c			5′/95	9	6500nt downstream to Os09g00998	Sense
miR2003d			5′/95	9	14428nt downstream to Os09g00998	Sense
miR2003e			5′/95	9	7401nt upstream to Os09g01002	Sense
miR2004a	GACCGCAUAGCGCAGUGGAUU	21	5′/168	3	261nt downstream to Os03g17230	Sense
miR2004b			5′/184	5	153nt upstream to Os05g02640	Sense
miR2005a	GGCAGCCGAGCGAGGGCCUCGG	22	3′/65	3	1157nt downstream to Os03g42410	Sense
miR2005b			3′/65	11	516nt upstream to Os11g12830	Sense
miR2005c			5′/88	11	367nt upstream to Os11g20542	Sense
miR2005d			3′/66	12	796nt upstream to Os12g21550	Sense
miR2006a	GUGGCUGUAGUUUAGUGGUGA	21	3′/151	1	345nt downstream to Os01g21610	Sense
miR2006b			3′/74	2	3121nt downstream to Os02g26400	Anti-sense
miR2006c			3′/194	3	31nt downstream to Os03g18200	Sense
miR2006d			3′/101	3	670nt upstream to Os03g03390	Anti-sense
miR2006e			3′/81	4	2375nt downstream to Os04g21780	Anti-sense
miR2006f			3′/80	7	345nt downstream to Os07g32420	Sense
miR2006g			5′/74	7	3478nt downstream to Os07g44060	Sense
miR2006h			5′/143	10	666nt upstream to Os10g38934	Sense
miR2007a	UCCACUGAGAUCCAGCCCCGC	21	3′/81	2	8884nt downstream to Os02g47040	Sense
miR2007b			3′/81	9	1408nt upstream to Os09g00998	Sense
miR2007c			3′/81	9	29799nt upstream to Os09g01002	Sense
miR2007d			3′/81	9	7886nt downstream to Os09g00998	Sense
miR2007e			3′/81	9	13943nt upstream to Os09g01002	Sense
miR2007f			3′/81	9	6015nt upstream to Os09g01002	Sense

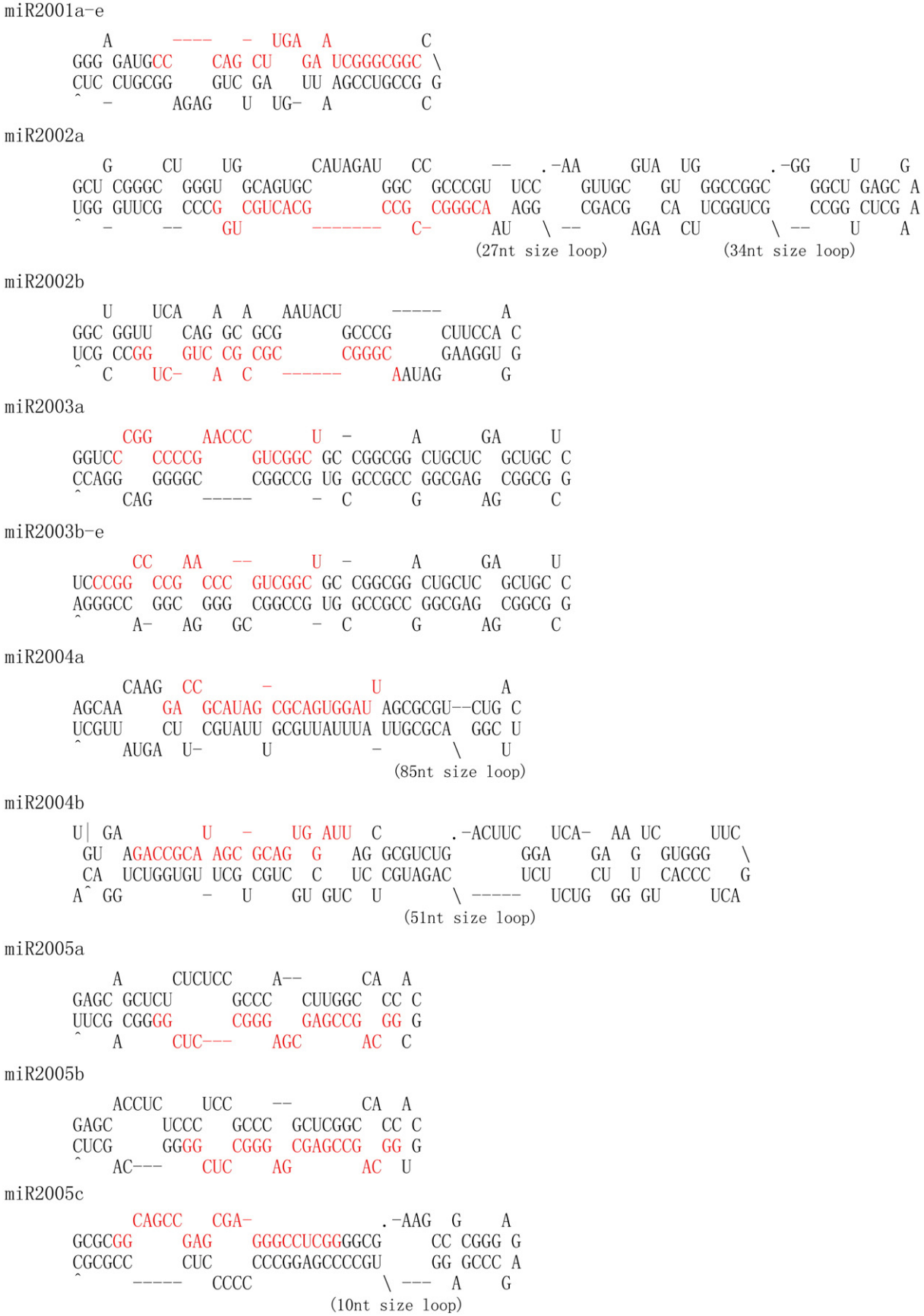

Fig. 2. Stem-loop structures of putative miRNA precursors. The miRNA sequences are in red and underlined. The actual size of the precursors is not identified experimentally and maybe slightly shorter or longer than represented.

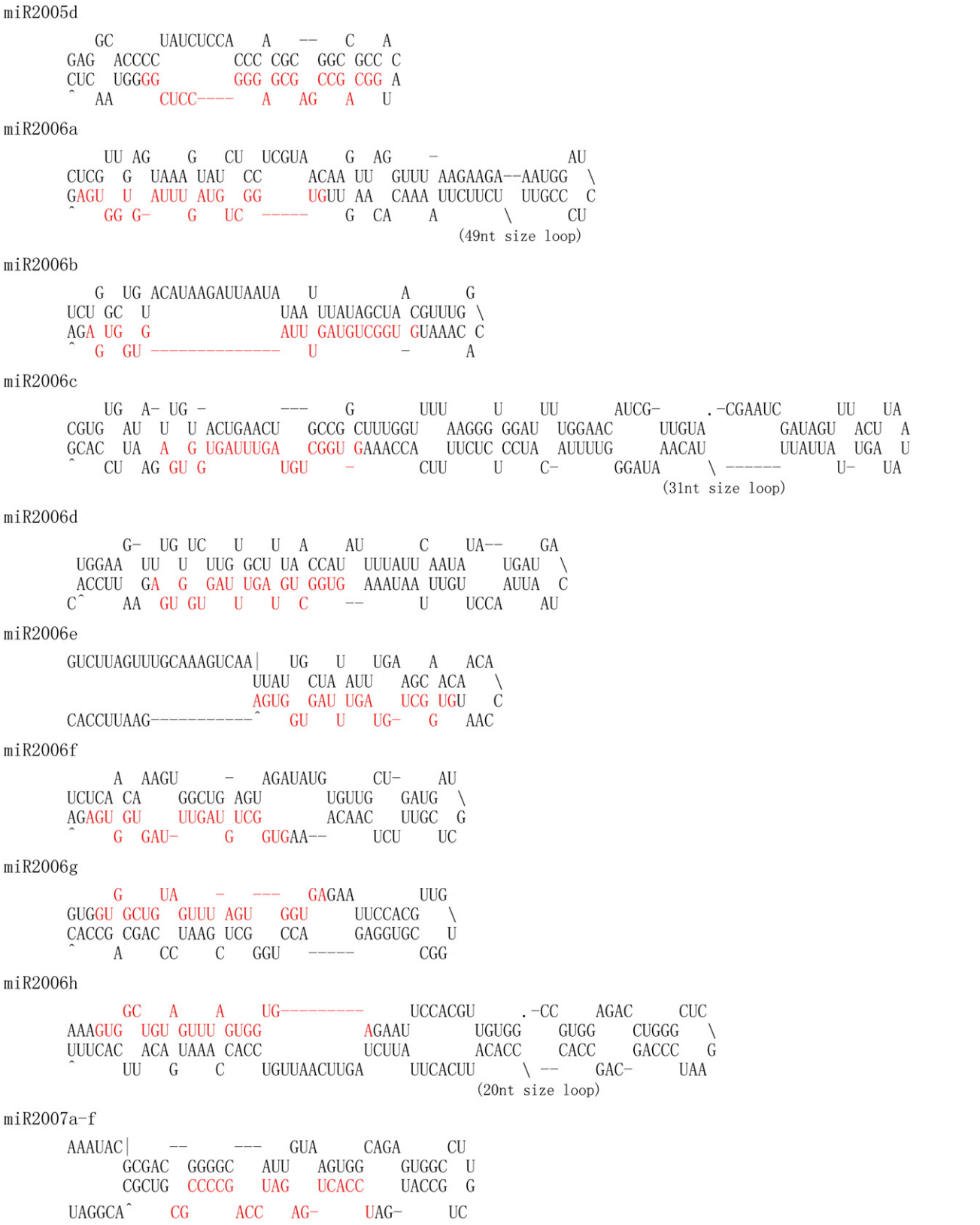

Fig. 2 (*continued*).

After two rounds of Northern blot hybridization, seven candidate miRNAs were identified as bona fide miRNAs (detected in the first round of Northern blot hybridization and undetected in the second round). The expression patterns of the seven miRNAs under different stress treatments were observed (Fig. 1). Moreover, according to the current annotation criteria [31], a new miRNA should be confirmed using a dcl-1 mutant, these seven small RNAs cannot be detected in a rice *DCL1* transgenic plant, confirming they are miRNAs (data not shown). Therefore, we conclude that miR2001, miR2002, miR2003, miR2004, miR2005, miR2006 and miR2007 are seven novel miRNAs

from rice (Table 4) (miR2005 and miR2007 were deposited as osa-miR2906 and osa-miR2907 in miRBase database). To determine whether these novel miRNAs are conserved in rice, their expression was analyzed in *Arabidopsis* by Northern blotting. None of these seven novel miRNAs can be detected in *Arabidopsis* (data not shown). The observation that of these seven novel miRNAs are not conserved in *Arabidopsis* suggests that these miRNAs may play specific roles in rice.

Expression patterns of the novel rice miRNAs under different stress treatments

To test whether these candidate miRNAs were regulated by abiotic stress, RNA gel blot analysis was performed on 2-week-old seedlings without stress treatment or treated with ABA, cold, drought or NaCl. We found that the expression of several novel miRNAs is regulated by one or more stress treatments (Fig. 1). Two novel miRNAs, miR2001 and miR2004, are strongly up-regulated by NaCl treatments (Figs. 1A and B). miR2005 is strongly up-regulated by dehydration (Fig. 1C) and miR2006 is strongly up-regulated by ABA and NaCl treatment (Fig. 1D). miR2002 and miR2007 are slightly up-regulated by drought treatment (Figs. 1E and F), and they were expressed under all stress treatments as well as in the control. Furthermore, miR2003 is not only up-regulated but also down-regulated by different stress treatments, showing a complex expression pattern under stress treatments. miR2003 is slightly up-regulated by NaCl, but slightly down-regulated by cold (Fig. 1G). This is the first time that a miRNA has been shown to have opposite expression patterns by different stress treatments. Interestingly, miR2003 has two mature miRNA products of different sizes. The two mature miRNA products were detected by Northern analysis (Fig. 1G) giving lengths of 20 and 21 nt, respectively. This kind of expression pattern is similar to miR156 [32]. The complex expression patterns of the novel miRNAs indicate that they are functional in the stress response pathways of rice.

Genomic organization of the miRNAs

To further investigate the seven novel miRNAs, genomic analysis was performed. The genomic locations of the novel miRNA genes in rice are shown in Table 4. The seven newly identified miRNAs correspond to 32 loci. Hairpin structures can be predicted for all these 32 loci using miRNA surrounding sequences (Fig. 2). All of these novel miRNAs have at least two loci in the genome, and miR2006 has eight loci (Table 4). Our analysis of the genomic positions of the novel miRNA genes shows that all localize to intronic regions, twenty-eight correspond to introns of protein-coding genes in the sense orientation, and four correspond to introns in the antisense orientation.

Discussion

The rice miRNA sequences are catalogued in the miR Base database (http://microrna.sanger.ac.uk). More than thousand of rice miRNAs have been predicted, and nearly half of them have been identified by experiment. This number increased in the recent years, as more expressed miRNAs are discovered. For instance, the genome-wide analysis for discovery of rice miRNAs was carried out [18]. However, the experimental evidence for rice miRNA identification is confined to few reports [14,15,22,18,33]. Recently, the reports about new miRNAs in monocot species were increased apace [34,35], whereas none of them related to the expressed miRNAs under abiotic stresses. So far, several reports showed that plant miRNAs are involved in some abiotic stresses [3,4,24,25]. The first such role of miRNAs was described by Sunkar and Zhu [4] in *Arabidopsis*, but the discovery of such miRNAs is lacking in rice. For these reasons, we generated four small RNA libraries, from rice plants subjected to 4 abiotic stresses, to find novel rice miRNAs and miRNAs related to stress. Plant miRNAs have been predicted or confirmed to regulate genes encoding various types of proteins by changing their expression. For instance, over-expression of miR156 leads to delayed flowering under short day by regulating its target protein [27]. In this study, from our small RNA libraries, we found seven novel rice miRNAs, and their expression pattern under different stress conditions showed they were regulated under at least one abiotic stress. This indicated that the seven novel miRNAs are probably involved in stress response pathways, and we hypothesize that the abiotic stress-related rice miRNAs might modulate some transcription factors to help resist environmental stresses.

To identify the rice miRNAs, three methods were used. Firstly, after predicting the candidate rice miRNAs, we used a microRNA array to briefly identify the real/genuine miRNAs among candidate ones. Candidate miRNAs whose hybridization signal was under our threshold value (one third of the candidate miRNAs on the chip) were discarded and the remaining candidate miRNAs were identified. Secondly, after predicting the potential fold-back precursor structure of our cloned novel candidate miRNAs, we chose the candidate miRNAs located in one arm of the hairpin. According to the criteria of microRNA annotation previously reported [6], we detected the expression of these candidate miRNAs by Northern blot hybridization. MicroRNAs come from a single-molecule fold-back structure; RNAs that are processed by Dicer from a hybrid between two antiparallel transcripts are siRNAs and not miRNAs [6]. Thus, two round Northern blotting was carried out with sense or antisense miRNA probe. An observation that ~21-nt RNAs are produced from both a sense and an antisense transcript is evidence of siRNAs. However, according to the recent miRNA annotation criteria [31], the miRNA should be confirmed using a dcl-1 mutant. Thirdly, we validated the candidate miRNAs in rice *DCL1* plants. In *Arabidopsis*, the maturation of the miRNA from the pri-miRNA is a stepwise process that involves a Dicer-like protein, DCL1. DCL1 is likely to be the key enzyme that processes pre-miRNA to the mature miRNA [36]. It has been demonstrated that OsDCL1 is required for miRNA processing and rice normal development [22]. To confirm the rice miRNAs, they were detected in *OsDCL*1 by Northern blotting. Five candidate miRNAs were detectable in *OsDCL1*. After these steps, seven novel rice miRNAs were identified in this paper.

Besides our 74 rice candidate miRNAs, there were 222 known miRNAs of other species on the miRNA chip (after deleting redundant high homology sequences). All the down-regulated miRNAs in treated rice seedlings were our candidate miRNAs. Most of the up-regulated miRNAs in treated rice seedlings were candidate miRNAs. The ratios of up-regulated and down-regulated (candidate) miRNAs on the chip under different stress treatments are shown in Table 5. Most (candidate) miRNAs were up-regulated in drought treated seedlings, while low temperature had the smallest effect. This indicates that the candidate miRNAs were specifically expressed in rice and/or their expression was environmentally induced. At the same time, most of the known miRNAs on the chip were lower expressed under stress-treatments. The expression of osa-miR172 was hardly detected by Northern blot analysis and, same as the results of miRNA array, showed it was not environmental induced. Besides, several known miRNAs on the chip were highly expressed under drought stress treatment. Ath-miR395b had been reported previously by Matthew

Table 5
The ratio of deregulated miRNAs on the chip.

Stress treatment	Ratio of up-regulated miRNAs[a]	Ratio of down-regulated miRNAs[a]
ABA	1.0 %	2.5 %
Cold	0	1.3 %
Drought	4.5 %	0.6 %
NaCl	2.2 %	1.3 %

[a] The number of deregulated miRNAs (including candidate miRNAs) after SAM analyze/the number of all miRNAs and candidate miRNAs on the chip (314).

[3] and corresponded to sulfate starvation; it showed 42-fold higher expression under drought stress on the miRNA chips. Osa-miR159 showed to be regulated by gibberellin [27] and showed 3.5-fold higher expression under drought stress treatment. Osa-miR164 was induced by auxin to clear NAC1 mRNA to reset auxin signals [37], and it was 6-fold higher expressed under drought stress treatment on the miRNA chips. Additionally, other experiments were carried out with this plant miRNA array, and the results showed several our novel miRNAs were regulated by different developmental stage in other species (data not shown). From these results, we conclude that the plant miRNA array is a potent tool that can be applied to other plant species.

Plant miRNAs function on post-transcriptional gene regulation by targeting mRNA for degradation or repression [7]. They modify the plants by regulating the expression of their target genes. To further understand our novel miRNAs, we predicted the potential targets of the novel miRNAs. We used a developed computational method by aligning miRNA sequences with target mRNA sequences using the TimeLogic implementation of the Smith-Waterman nucleotide-alignment algorithm [16]. Forty-two unique rice genes were identified to be feasible targets for seven novel rice miRNAs, because there are highly stringent complementarities with four or fewer mismatches between the miRNAs and their targets (Table 6). However, no miRNA was perfect matched in the antisense orientation to the mRNAs of protein-coding genes. By comparing the miRNAs expression pattern with their predicted targets' expression pattern in rice MPSS (massively parallel signature sequencing), we found the expression of two novel miRNAs under different stress treatments increased as the expression of the target mRNA fell (though this does not prove a cause and effect relationship). miR2004 was strongly up-regulated by NaCl treatment, while its predicted target Os10g33460 which encoded a putative oxidoreductase shown down-regulated by salt treatment in MPSS. In addition, miR2005 was strongly up-regulated by drought treatment, while its predicted targets Os03g15690 and Os01g13740 shown down-regulated by drought treatment in MPSS. This suggest us the predicted target mentioned above were probably be the real target of novel miRNAs. This method of prediction of the miRNA targets is a useful tool in the validation of miRNA targets and is feasible for investigating the function and mechanism of plant miRNAs.

Table 6
Prediction of rice novel miRNA targets.

microRNA	Target protein family	Target gene names (no. of mismatches)
miR2001	Hypothetical protein	Os10g22830 (3)
	Protein GPR107 precursor, putative, expressed	Os04g42960 (4)
miR2002	Hypothetical protein	Os09g14640 (3)
	Caltractin, putative	Os09g15680 (3)
	Mitogen-activated protein kinase kinase kinase 2, putative, expressed	Os02g21700 (4)
	YGGT family protein, expressed	Os03g08080 (4)
	Protein TOC75, chloroplast precursor, putative, expressed	Os03g16440 (4)
	Expressed protein	Os09g36890 (4)
	Reticuline oxidase precursor, putative, expressed	Os06g35650 (4)
	Hypothetical protein	Os07g13090 (4)
	Peroxidase 35 precursor, putative, expressed	Os03g55420 (4)
	Expressed protein	Os03g47190 (3)
	LRX2, putative, expressed	Os06g49100 (3)
	Protein MONOCULM 1, putative	Os04g35250 (4)
	MYB transcription factor TaMYB1, putative	Os06g14700 (4)
	Cell Division Protein AAA ATPase family, putative, expressed	Os07g09480 (4)
	Transferase, putative, expressed	Os03g53360 (4)
miR2003	Expressed protein	Os01g04010 (3)
	HEAT repeat family protein, expressed	Os01g40250 (3)
	Ribosomal protein S11 containing protein, expressed	Os03g26860 (3)
	BGGP Beta-1-3-galactosyl-O-glycosyl-glycoprotein, putative, expressed	Os03g48560 (3)
	Hypothetical protein	Os03g58560 (4)
	NAC domain-containing protein 90, putative, expressed	Os11g45950 (4)
miR2004	EMB2745, putative, expressed	Os01g42620 (4)
	Exonuclease, putative, expressed	Os04g55700 (4)
	Lectin receptor-type protein kinase, putative, expressed	Os02g42780 (4)
	FAD binding domain containing protein, putative, expressed	Os04g29090 (4)
	Serine/threonine-protein kinase receptor precursor, putative	Os09g37890 (4)
	Hypothetical protein	Os11g03190 (4)
	Oxidoreductase, putative, expressed	Os10g33460 (4)
	Peroxidase 52 precursor, putative	Os06g35490 (4)
miR2005	Nitrate and chloride transporter, putative, expressed	Os10g08850 (3)
	Expressed protein	Os03g40770 (3)
	Phosphate carrier protein, mitochondrial precursor, putative, expressed	Os03g15690 (4)
	Myb-like DNA-binding domain, SHAQKYF class family protein, expressed	Os01g13740 (4)
miR2006	Hypothetical protein	Os01g57520 (3)
	Conserved hypothetical protein	Os10g38680 (4)
miR2007	DNA binding protein, putative, expressed	Os09g31470 (3)
	Cyclin delta-2, putative, expressed	Os06g12980 (3)
	F-box domain containing protein	Os11g33210 (4)
	Cell Division Protein AAA ATPase family, putative, expressed	Os12g24320 (4)
	Cell Division Protein AAA ATPase family, putative, expressed	Os12g44210 (4)

Materials and methods

Plant materials and stress treatments

The seeds (*O. sativa* L. ssp. *Japonica* cv 9522) were stimulated to break dormancy and germinated. Uniformly germinated seeds were sown in plates, immersed in water for 1 d at room temperature and 1 d at 37 °C, and then transferred to grown chamber with a 16-h light and 8-h dark photoperiod at 25 °C. Two-week-old seedlings were treated with 100 μM ABA, 4 °C, dehydration (exposed to dry air), or 300 mM NaCl individually for 24 h. Untreated seedlings were used as controls. Entire seedlings were collected and immediately transferred into liquid nitrogen. The frozen seedlings were used for generation of small RNA libraries, the miRNA array and for Northern hybridization.

Construction of rice miRNA library

Total RNA was extracted and enriched for small-sized RNAs using polyethylene glycol precipitation as described [38,39]. Cloning of miRNAs was performed as described [40,5,22]. In brief, ~300 μg of small RNAs were resolved through two lanes on a denaturing 15% polyacrylamide-7 M urea gel. Labeled Decade Marker System (Ambion) was used as a size marker. RNAs of 18–28 nt were recovered. A 3′ adapter (5′ dephosphorylated) (5′-pUUUctgtaggcaccatcaat-it-3′; uppercase, RNA; lowercase, DNA; p, phosphate; it, inverted deoxythymidine) and a 5′ adapter (5′-atcgtaggcaccUGAAA-3′; uppercase, RNA; lowercase, DNA) were ligated to the small RNAs. Reverse transcription was performed using the RT primer (5′-ATTGAT**GGTGCC**TACAGAAA-3′; bold, Ban I site). This was followed by PCR using the reverse (RT primer) and forward (5′-ATCGTAGGCACCAGAAA) primers. The second PCR product was purified and digested with Ban I. The digested products were concatamerized using T4 DNA ligase and recovered from the agarose gel. Fragments spanning the size range of 500–800 bp were excised. The ends of concatamers were tailed with one adenosine nucleotide and subsequently ligated into a commercial Promega pGEM-T easy vector using T4 DNA ligase for DNA sequencing. The small RNA libraries

were sequenced by BGI (Beijing, China) with 3730 DNA Analyzer. Adaptor sequences enclosing the inserts were trimmed, and the inserts were normalized to the sense direction. We discarded all inserts shorter than 16 bp. The remaining sequences were scanned for duplicates, and a non-redundant set was created.

Computational prediction of candidate miRNAs

Prediction of *O. sativa* miRNAs was carried out using criteria similar to that described previously [16,41]. Cloned small RNAs were first compared with the Rfam non-coding RNA database using BLAST to remove remnants of non-coding RNAs other than miRNAs and then mapped to the rice genome using a Perl Script. Small RNAs originating from intergenic and intronic regions were collected for miRNA prediction. Genomic sequences surrounding each small RNA locus (with lengths ranging between 70 and 350 nt) were extracted, using a 20-nt increment each time (assuming miRNAs could originate from either the 5′ or 3′ arm of their hairpin-shaped precursors) and subjected to Mfold for RNA secondary structure prediction [42]. Small RNAs whose precursor sequences possess qualified hairpin-shaped secondary structure were selected as candidate miRNAs.

Microarray methods and data analysis

In addition to our predicted 74 miRNA sequences, we also integrated the mature plant miRNA sequences downloaded from the miRNA Registry (http://microrna.sanger.ac.uk; miRBase Release 7.1, accessed January 2006). There are 308 miRNAs on the microarray after deleting redundant high homology sequences. miRNA probes were designed to be fully complementary to their predicted mature miRNAs and PolyT was added to the 5′-end to concatenate up to 40 nt with C6 5′-amino-modifier. Oligonucleotide probes were synthesized by Invitrogen (Shanghai, China) and dissolved in DNA Spotting Solution (CapitalBio Corp., Beijing, China) at a concentration of 40 mM and printed on the aldehyde-modified glass slides (CapitalBio) in triplicate using a SmartArray™-136 microarrayer (CapitalBio). RNA labeling, microarray hybridization, and array scanning were performed as previously described [43]. In brief, 25 mg of total RNA was used to isolate the low-molecular-weight RNA (LMW RNA) using PEG solution precipitation. Subsequently, LMW RNAs were labeled with Cy3-CU using RNA ligase and hybridized with the microarrays. Finally, hybridization signals were detected and quantified. Two independent RNAs for each sample were hybridized with the microarrays separately. Hybridization intensity values from individual array were filtered and the global median normalized. Significantly expressed miRNAs were identified as those above the negative control (blank spotting solution) adding 2 standard deviations and with a $P<0.01$ using Student's *t*-test combined the two slide hybridization and triplicate spots for each probe on one slide. To determine whether the miRNAs were significantly differentially expressed, significance analysis of microarrays (SAM, version 2.1) was performed using two-class unpaired comparison in the SAM program.

Northern blot analysis

Small RNA was isolated from different stress-treated seedlings as well as untreated ones according to the protocol described previously [39]. 50 μg small RNA was loaded per lane and resolved on a denaturing 15% polyacrylamide gel and transferred electrophoretically to Hybond N^+ (Amersham) membranes by using a Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). Membranes were UV cross-linked (150 mJ, GS Gene Linker UV Chamber, Bio-Rad) and baked for 2 h at 80 °C. DNA oligonucleotide probes were synthesized (Invitrogene, Shanghai) and labeled with γ-^{32}P-ATP (Furui Co.) using T4 polynucleotide kinase (Fermentas). Membranes were prehybridized in 7% SDS, 50% deionized formamide, 250 mM NaCl, and 125 mM sodium phosphate buffer (pH 7.2) at 42 °C for at least 1 h and hybridized with ^{32}P-end-labeled oligonucleotide probes overnight. Membranes were washed twice with 2×SSC and 0.2% SDS at 42 °C. The membranes were briefly air dried and then subjected to autoradiography.

Targets prediction of candidate miRNAs

The target prediction of *O. sativa* miRNAs was carried out using criteria similar to that described previously [16,41]. The putative target sites of all miRNA candidates were identified by aligning miRNA sequences to the annotated gene sequences of *O. sativa* using the TimeLogic implementation of the Smith-Waterman nucleotide-alignment algorithm and a Perl script. The scoring system we used is as follows: mismatches and single-nucleotide bulges were each scored as 1, G:U pairs were each scored as 0.5. The scores were doubled if mismatches, G:U pairs and bulges were located at positions 2–13 as counted from the 5′ end of an miRNA [44]. Genes with a mispair score <3 were selected as putative miRNA targets.

MPSS analyse

The locus number of predicted targets was inputted to the rice MPSS (http://mpss.udel.edu/rice), and the results of 17 bp signatures were downloaded and trimmed. Compared the stress treated data (NCL, NCR, NDL, NDR, NSL, NSR) with non-treated data (NYL, NYR), regarded the number of leaves plus the one of roots as a signal value, the expression patterns of target genes under stress treatments were gained. Compared miRNA expression pattern with its predicted targets expression pattern under stress treatments, chose the pairs showed opposite expression patterns, and the target was regarded as miRNA target.

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