Planta (2009) 230:883–898
DOI 10.1007/s00425-009-0994-3

ORIGINAL ARTICLE

Genome-wide survey of rice microRNAs and microRNA–target pairs in the root of a novel auxin-resistant mutant

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Received: 31 May 2009 / Accepted: 10 July 2009 / Published online: 5 August 2009

Abstract Auxin is one of the central hormones in plants, and auxin response factor (ARF) is a key regulator in the early auxin response. MicroRNAs (miRNAs) play an essential role in auxin signal transduction, but knowledge remains limited about the regulatory network between miRNAs and protein-coding genes (e.g. ARFs) involved in auxin signalling. In this study, we used a novel auxin-resistant rice mutant with plethoric root defects to investigate the miRNA expression patterns using microarray analysis. A number of miRNAs showed reduced auxin sensitivity in the mutant compared with the wild type, consistent with the auxin-resistant phenotype of the mutant. Four miRNAs with significantly altered expression patterns in the mutant were further confirmed by Northern blot, which supported our microarray data. Clustering analysis revealed some novel auxin-sensitive miRNAs in roots. Analysis of miRNA duplication and expression patterns suggested the evolutionary conservation between miRNAs and protein-coding genes. MiRNA promoter analysis suggested the possibility that most plant miRNAs might share the similar transcriptional mechanisms with other non-plant eukaryotic genes transcribed by RNA polymerase II. Auxin response elements were proved to be more frequently present in auxin-related miRNA promoters. Comparative analysis of miRNA and protein-coding gene expression datasets uncovered many reciprocally expressed miRNA–target pairs, which could provide some hints for miRNA downstream analysis. Based on these findings, we also proposed a feedback circuit between miRNA(s) and ARF(s). The results presented here could serve as the basis for further in-depth studies of plant miRNAs involved in auxin signalling.

Keywords Auxin response element · Auxin response factor · Auxin signalling · Microarray · MicroRNA–target pairs · Rice root

Abbreviations

α-NAA	α-Naphthalene acetic acid
ARF	Auxin response factor
AuxRE	Auxin response element
EMS	Ethyl methanesulfonate
GO	Gene Ontology
IAA	3-Indole acetic acid
MiRNA	MicroRNA
MT	*osaxr* mutant
osaxr	*Oryza sativa auxin resistant*
TF	Transcription factor
TSS	Transcription start site
WT	Wild type

Introduction

Auxin plays a central role in plant growth and development (Leyser 2002), and the mechanisms of auxin-mediated gene regulation have been intensively studied in *Arabidopsis*

Electronic supplementary material The online version of this article (doi:10.1007/s00425-009-0994-3) contains supplementary material, which is available to authorized users.

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thaliana (Dharmasiri and Estelle 2004; Teale et al. 2006). Auxin response factors (ARFs) are the key transcription factors (TFs) in the early auxin response. They bind specifically to the auxin response elements (AuxREs) and modulate the transcription of the early auxin response genes (Guilfoyle and Hagen 2007).

MicroRNAs (miRNAs) are RNA species about 21 nucleotides in length, involved in post-transcriptional regulation mostly through their cleavage effects on target transcripts in plants (Voinnet 2009). In Arabidopsis, certain miRNAs are involved in ARF-mediated auxin signalling. For instance, *miR167* represses *ARF6* and *ARF8* at the post-transcriptional level (Ru et al. 2006; Wu et al. 2006), and ARF8 can modulate the transcription of certain *GH3*-like genes (Tian et al. 2004). These *GH3*-like genes catalyse the conjugation between endogenous auxin and specific complexes (e.g. amino acids), suggesting their essential role in balancing the level of active 3-indole acetic acid (IAA) (Staswick et al. 2005). This *miR167-ARF8-GH3* pathway is conserved in rice (Yang et al. 2006). So far, 243 rice miRNAs have been discovered (miRBase Release 10.1) (Griffiths-Jones et al. 2006), whereas the understanding of their regulatory role remains limited.

Most research on plant miRNAs has focused on their downstream regulatory pathways, but little has been reported on their transcriptional mechanisms. Lee et al. (2004) reported that RNA polymerase II transcribes human miRNAs which suggests that miRNAs and protein-coding genes may share similar transcriptional mechanisms. *Cis*-acting element studies of miRNA promoters are still restricted to bioinformatics analysis (Zhou et al. 2007). Comparative analysis of both the miRNA and protein-coding gene promoters has uncovered five *cis*-acting elements (e.g. AuxREs) in miRNA promoters, and a negative feedback model between miRNAs and TFs has been proposed (Megraw et al. 2006). However, there has been little systematic analysis of rice miRNAs involved in auxin signalling, and the auxin-specific regulatory network between miRNAs and their targets remains to be determined.

Here, a novel auxin-resistant rice mutant with plethoric root defects was used to investigate miRNA expression patterns by microarray analysis. A number of miRNAs showed greatly reduced auxin sensitivity in the mutant compared with the wild type, consistent with the auxin-resistant phenotype of the mutant. Analysis of miRNA duplication and expression patterns suggested the evolutionary conservation between miRNAs and protein-coding genes. We selected promoters of the auxin-related miRNAs for in-depth analysis. A number of potential miRNA–target pairs were identified in the rice roots based on the comparative analysis of miRNA and protein-coding gene expression datasets, which could serve as a repository for in-depth miRNA downstream analysis. These findings led us to propose a feedback circuit between miRNA(s) and ARF(s), revealing the complexity of the regulatory network between miRNAs and their targets involved in auxin signalling.

Materials and methods

Mutant library generation and screening

Rice (*Oryza sativa* L. ssp. *indica* cv. Kasalath, kindly provided by Hongxuan Lin (Shanghai Institute of Plant Physiology and Ecology, Shanghai Institute for Biological Sciences, The Chinese Academy of Sciences, China), seeds were treated with 0.6% (V/V) ethyl methanesulfonate (EMS, Sigma, Shanghai, China) solution for 12 h at 26°C in a shaker. The treated seeds were rinsed with distilled water at least six times for 4 h in the shaker, and then the seeds were germinated in distilled water for 2 days. Seed germination rate was assessed to optimise the EMS treatment intensity. The seedlings were grown in the field for harvesting, and the first-generation seeds were used for mutant screening after being germinated in distilled water for 2 days. After germination, seedlings were transferred to plastic nets floating on Yoshida nutrient solution (Yoshida et al. 1976) in plastic pots. The seedlings were grown in a growth chamber under 30°C (day)/22°C (night) and 12-h light (450 μmol photons m^{-2} s^{-1})/12-h dark regime. Ten-day-old seedlings were screened based on their root phenotypes.

Seedling treatments and physiological experiments

Seeds were germinated in distilled water for 2 days. After germination, seedlings were transferred to plastic nets floating on Yoshida nutrient solution (Yoshida et al. 1976) in plastic pots. Seedlings were grown in a growth chamber under 30°C (day)/22°C (night) and 12-h light (450 μmol photons m^{-2} s^{-1})/12-h dark regime. For miRNA microarray analysis and Northern blot, 7-day-old seedlings were treated with 30 μM IAA for 3 h. For microarray analysis of protein-coding genes, 7-day-old seedlings were treated with 10 μM IAA for 3 h. For real-time quantitative PCR, 7-day-old seedlings were treated with 10 μM α-naphthalene acetic acid (α-NAA), 10 μM IAA, or 10 μM 2, 4-D for 3 h. For auxin sensitivity analysis, seedlings were treated with 1 μM α-NAA for 7 days.

Real-time qRT-PCR analysis

Real-time qRT-PCR was performed by using the Universal Probe Library and LightCycler480 Probe Master Kit on the LightCycler480 machine (Roche, Shanghai, China), following the manufacturer's instructions. The amplification

programme was performed at 95°C for 10 s, 60°C for 25 s, and 72°C for 1 s. A triplicate quantitative assay was performed on each cDNA sample. The relative quantification of each sample was determined by normalisation to the amount of rice actin cDNA detected in the same sample. The primers for *OsIAA20* were 5′ CATCCTCGGCTCA TACGC 3′ (forward) and 5′ ATCGTGCCCATCCTCTTG 3′ (reverse). The primers for rice actin were 5′ CAACACC CCTGCTATGTACG 3′ (forward) and 5′ CATCACCAGA GTCCAACACAA 3′ (reverse).

MicroRNA microarray assay

Total RNA was isolated from 7-day-old seedling roots by using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's manual. The microarray assay was performed by LC Sciences (http://www.lcsciences.com). The assay started with a 2–5 μg total RNA sample, which was size fractionated using a YM-100 Microcon centrifugal filter (Millipore, Bejing, China). [Note: Although the miRVana kit (Ambion, Austin, TX, USA) is widely used for low-molecular weight RNA (<200 nt) isolation, no significant difference exists using the YM-100 filter as indicated by the technical support of LC Sciences, Hangzhou, China.]

The small RNAs (<300 nt) isolated were 3′-extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly(A) tail for subsequent fluorescent dye staining; two different tags (Cy3 and Cy5) were used for our dual-sample experiments. Hybridisation was performed overnight on a μParaflo microfluidic chip using a micro-circulation pump (Atactic Technologies, Houston, TX, USA) (Gao et al. 2004; Zhu et al. 2007). On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide coding segment complementary to the target miRNA or other RNA (control or customer-defined sequences) and a spacer segment of polyethylene glycol extending the coding segment away from the substrate. The detection probes were made by in situ synthesis using PGR (photo-generated reagent). Each chip contained 115 probes for detecting all known miRNAs in rice with 24 repeats. The hybridisation melting temperatures were balanced by chemical modifications of the detection probes. Hybridisation was performed with 100 μL 6× SSPE buffer (0.90 M NaCl, 60 mM Na_2HPO_4, 6 mM EDTA, pH 6.8) containing 25% formamide at 34°C. Subsequent hybridisation detection used fluorescence labelling with tag-specific Cy3 and Cy5 dyes. Hybridisation images were collected using GenePix 4000B (Molecular Device, Sunnyvale, CA, USA) and digitised using Array-Pro image analysis software (Media Cybernetics, Bethesda, MD, USA). Data were analysed by first subtracting the background and then normalising the signals using a LOWESS filter (Bolstad et al. 2003). For two-colour experiments, the ratio of the two sets of detected signals ($\log_2$ transformed, balanced) and *P* values of the *t* tests were calculated; differentially detected signals were those with *P* values <0.01.

Affymetrix microarray assay

Total RNA was isolated from 7-day-old seedling roots by using Trizol (Invitrogen, Beijing, China) according to the manufacturer's manual. RNA samples were processed according to the procedure recommended by Affymetrix's manual. Single-stranded and then double-stranded cDNA were synthesised using the SuperScript double-stranded cDNA synthesis kit (Invitrogen). A portion of the resulting double-stranded cDNA served as the template for generating biotin-tagged cRNA using the GeneChip IVT labelling kit (Affymetrix, Shanghai, China). A total of 15 μg of the resulting biotin-tagged cRNA was fragmented to a size range of 35–200 bases following the instructions. Subsequently, 10 μg of this fragmented target cRNA was hybridised at 45°C with rotation for 16 h to probe sets present on an Affymetrix rice genome array. The GeneChip arrays were washed and then stained using streptavidin–phycoerythrin on an Affymetrix Fluidics Station 450 followed by scanning on a GeneChip Scanner 3000. Hybridisation data were analysed using GeneChip Operating Software 1.2 and dChip software (Li and Wong 2001).

MicroRNA Northern blot

Total RNA was isolated from 7-day-old seedling roots using Trizol (Invitrogen) according to the manufacturer's instructions. A total of 30 μg of total RNA was used for loading and were dissolved in a 15% (w/v) Tris-borate-EDTA PAGE gel with 8 M urea by electrophoresis at 300 V for 5 h. The low-molecular-weight RNAs on the PAGE gel were then electro-blotted onto a Hybond-N^+ membrane (Amersham Biosciences, Hong Kong). The membrane was UV cross-linked and hybridised using Denhardt's solution (USB Corp., Cleveland, OH, USA). Antisense oligonucleotide probes were prepared by end-labelling with ^{32}P-γATP using T4 polynucleotide kinase (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's instructions. Hybridisation was carried out at 50°C, and the blot was reused after washing and stripping. The original protocol was obtained from Bartel's lab, see (http://web.wi.mit.edu/bartel/pub/protocols.html) for details.

Clustering analysis

Data normalisation was carried out using a cyclic LOWESS method (Bolstad et al. 2003). The normalisation served to remove system-related variations, such as sample amount variations, different labelling dyes and signal gain

differences of scanners so that biological variations could be validly revealed. Among 243 miRNAs (miRBase Release 10.1), 118 members with detectable expression data for 12 experimental combinations (two samples × two treatments × three biological replicates) were recruited, and the expression data averaged from three biological replicates were used for clustering analysis. Then, the miRNA expression profile was sorted using a hierarchical clustering method (Eisen et al. 1998) and the heatmap was drawn with R language (Ihaka and Gentleman 1996). For the clustering analysis, we used the program heatplus provided by Bioconductor (http://www.bioconductor.org).

Duplication and expression pattern analysis

All miRNAs in the clustering analysis were marked on the rice chromosomes except for *osa-miR156j*, *osa-miR166j*, *osa-miR396e* and *osa-miR444*, which had no available locus information (miRBase Release 10.1). Gene duplication segments (500 kb) containing these miRNAs were retrieved from TIGR (http://www.tigr.org/tdb/e2k1/osa1/segmental_dup/index.shtml). MicroRNAs with similar expression patterns, located in duplication segment pairs, were linked. Because the expression levels of miRNAs with the same mature sequences were indistinguishable, they were considered to have the same expression pattern.

MicroRNA promoter selection and *cis*-acting element analysis

MiRNA promoters were obtained mainly following by the rule mentioned previously (Zhou et al. 2007). First, when a precursor (pre-) miRNA [all sequence information on pre-miRNAs including the 5′ first nucleotide was retrieved from miRBase, miRBase Release 10.1; http://microrna.sanger.ac.uk/cgi-bin/sequences/mirna_summary.pl?org=osa)] and its closest upstream gene were unidirectional—if the distance between them was longer than 2,400 bp—the 2,000-bp sequence upstream of the pre-miRNA was retrieved. Otherwise, the sequence between the site 400 bp downstream of the upstream gene and the first nucleotide of the miRNA precursor was used. Second, when a pre-miRNA and its closest upstream gene were convergent—if the distance between them was longer than 4,000 bp—the 2,000-bp sequence upstream of the precursor was obtained. Otherwise, the sequence from the precursor to the middle point between the upstream gene and the precursor was retrieved. AuxREs in miRNA promoters were searched by our in-house tool written with Perl language, and the distribution patterns of other *cis*-acting elements were analysed by using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) (Lescot et al. 2002). These results were used for further analysis.

Distribution pattern analysis of TSS, TATA-box and CAAT-box

Both the transcription start site (TSS) and the TATA-box in miRNA promoters were searched by using TSSP (http://www.softberry.com/berry.phtml?topic=tssp&group=programs&subgroup=promoter) (Shahmuradov et al. 2003). Considering the result, the CAAT-box was selected from the search results produced by PlantCARE (Lescot et al. 2002). The distribution patterns of the three *cis*-acting elements were depicted by scatter plot using R language (Ihaka and Gentleman 1996) and simulated by the trend curves using the non-linear-fitting *loess* method (Cleveland and Devlin 1988).

Target prediction and functional classification

Target prediction was performed by using miRU (http://bioinfo3.noble.org/miRNA/miRU.htm) (Zhang 2005) and CSRDB (http://sundarlab.ucdavis.edu/smrnas/) (Johnson et al. 2007). To obtain comprehensive results, the stringency of the miRU prediction was set to be the lowest [score for each 20 nt: 3.5, G:U wobble pairs: 7, indels: 2, other mismatches: 5, and dataset 1: TIGR Rice Genome mRNA (OSA1 release 5, 01/23/2007)]. Putative targets were classified based on their functions by using Gene Ontology (GO) annotations. GO annotations were mainly obtained from TIGR (http://rice.plantbiology.msu.edu/data_download.shtml), and about 55% of the targets were annotated. Some GO annotations were obtained from GRAMENE (http://www.gramene.org/protein/index.html#browse). BLAST provided by Gene Ontology was used (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi?search_constraint=terms&action=replace_tree) to annotate the remaining targets according to the information for Arabidopsis. Finally, 123 genes remained unannotated. A Perl script was developed to obtain the GO annotations of a gene from Gene Ontology. The targets were classified mainly based on the first offspring node annotations belonging to molecular function, biological process, or cellular component, depicted with pie charts. Because many targets have two or more GO hits, the number of total hits can be bigger than that of the total targets. The percentage was derived from specific hits divided by total hits.

Results

MicroRNA microarray analysis in the auxin-resistant mutant

A rice (*O. sativa* L. ssp. *indica* cv. Kasalath) mutant with plethoric root defects was isolated from an EMS-generated mutant library. It was impaired in lateral root, adventitious

Fig. 1 The root phenotype of the auxin-resistant rice mutant *osaxr*. **a** Seven-day-old seedlings of wild type (*WT*) (*left*) and *osaxr* (*right*) under normal culture conditions. The lateral root and adventitious root defects of *osaxr* are shown. **b** The root phenotype of 7-day-old WT under 1 μM α-naphthalene acetic acid (α-*NAA*) treatment. **c** The root phenotype of 7-day-old *osaxr* under 1 μM α-NAA treatment. **d** The root hairs of 7-day-old WT under 1 μM α-NAA treatment. **e** The root hairs of 7-day-old *osaxr* under 1 μM α-NAA treatment. **f** The root cap structure of 7-day-old WT under normal culture conditions. **g** The root cap structure of 7-day-old *osaxr* under normal culture conditions. **h** Real-time qRT-PCR analysis of *OsIAA20* expression in rice roots. The relative expression levels of *OsIAA20* were averaged from three biological replicates. The *error bars* represent standard errors. *CK* normal culture conditions, *NAA* 10 μM α-NAA treatment for 3 h, *IAA* 10 μM 3-indole acetic acid treatment for 3 h, 2, 4-D 10 μM 2, 4-dichlorophenoxyacetic acid treatment for 3 h

root and root cap development (Fig. 1a, g). For wild-type (WT) seedlings, the root elongation was greatly inhibited and the root hairs proliferated towards the root tip under 7-day 1 μM α-NAA treatment (Fig. 1b, d); however, these features were not observed in the mutant (Fig. 1c, e). Moreover, the induction of *OsIAA20* (Jain et al. 2006), an early auxin response gene, was remarkably inhibited in the mutant after transient auxin treatment (Fig. 1h). Based on the phenotype characterised above, the mutant was designated as *osaxr* (*O. sativa* auxin resistant).

So far, a number of miRNAs have been reported to be involved in auxin signalling or root development (Guo et al. 2005; Ru et al. 2006; Wu et al. 2006; Yang et al. 2006). To investigate the genome-wide expression patterns of miRNAs in *osaxr*, we performed a microarray study. Among 243 miRNAs (miRBase Release 10.1) (Griffiths-Jones et al. 2006), 118 members with detectable expression data for 12 experimental combinations (two samples × two treatments × three biological replicates) were clustered (Fig. 2). Based on their expression patterns, all of the miRNAs could be classified into eight major clusters (Fig. 2). We selected the miRNAs (*osa-miR164abf*, *osa-miR167d-j*, *osa-miR171g* and *osa-miR390*) with significantly changed expression patterns in *osaxr* for Northern confirmation (Fig. 3).

[Since not all 12 expression data of *osa-miR171g* were present according to the microarray data (Table S1 in Electronic Supplementary Material), *osa-miR171g* was not included in the clustering analysis (Fig. 2).] The results were consistent with our microarray data.

Auxin sensitivity of rice miRNAs

In WT, 33.1% of the clustered miRNAs were sensitive (over 1.5-fold change compared with mock treatment) to exogenous IAA treatment (Table S1). At the twofold change threshold, only 5.1% were identified as auxin sensitive. Consistent with previous results in Arabidopsis (Mallory et al. 2005), IAA treatment did not significantly influence the auxin-related rice homologous miRNAs, such as *osa-miR160*, *osa-miR164*, and *osa-miR167*. Interestingly, many auxin-sensitive rice miRNAs revealed by our analysis are not implicated in the auxin-signalling pathway; instead, they or their homologues, such as *osa-miR395* and *osa-miR169*, are reported to be implicated in nutrition metabolism or stress response (Jones-Rhoades and Bartel 2004; Chiou 2007; Sunkar et al. 2007; Zhao et al. 2007). In *osaxr*, the number of auxin-sensitive miRNAs was greatly reduced (3.4% with 1.5-fold change compared with mock treatment; 0.8% with twofold change and only *osa-miR528* was observed) (Table S1).

Duplication and expression pattern analysis of rice miRNAs

All of the miRNAs in the clustering analysis were included for the duplication and expression pattern assay. Protein-coding gene duplication segments containing these miRNA genes were retrieved from TIGR (http://www.tigr.org/tdb/e2k1/osa1/segmental_dup/index.shtml). Our results showed that certain members of the miR156, miR159, miR160, miR166, miR167, miR168, miR169, miR171, miR319, miR394, miR397, and miR806 families with similar expression patterns were located in the duplication segment pairs (Fig. 4). Potential polycistrons formed by two or more miRNAs were scattered on the rice chromosomes (Fig. 4), and most consisted of the same family members.

osa-miR159c and *osa-miR159d* with a similar expression pattern cluster within a region of <10 Kb. *osa-miR159ab* and *osa-miR159f* also had a similar expression pattern, but they were far from each other on the same chromosome (Fig. S1 in Electronic Supplementary Material and Fig. 4). The expression patterns of *osa-miR156k* and *osa-miR156l* on different chromosomes were also quite uniform (Fig. 2). With respect to the miRNAs from different families, *osa-miR160a–d* and *osa-miR171b–f* had a similar expression pattern, as did *osa-miR171h* and *osa-miR390* (Fig. 2).

MicroRNA promoter characterisation

First, the promoters of abnormally expressed miRNAs in *osaxr* (Table 1) were recruited (Data S1) following the rule proposed by Zhou et al. (2007). The first residue of pre-miRNA was defined as the boundary for promoter collection. We used TSSP (Shahmuradov et al. 2003) to search for the TSS and the TATA-box, and used PlantCARE (Lescot et al. 2002) to search for the CAAT-box. The results indicated that the relative distributions of the three *cis*-acting elements in these miRNA promoters (data available in Table S3) were quite similar to those of the RNA polymerase II-dependent protein-coding genes in eukaryotic organisms (Breathnach and Chambon 1981) (Fig. 5).

Cis-acting elements in miRNA promoters were searched by PlantCARE (Lescot et al. 2002). Certain *cis*-acting elements are family specifically distributed (Table 1). The MSA-like element involved in cell cycle regulation was present in the miR160, miR169, and miR528 families, while the Motif I, implicated in root-specific expression, was present in the miR167 and miR169 families. The NON-box element involved in meristem specific activation was found only in *osa-miR159e*.

For auxin-specific *cis*-acting element analysis, typical AuxREs were recruited, and their distribution patterns were characterised (Table 1). We identified DR5-related sequences (Ulmasov et al. 1995) in the promoters of *osa-miR160f* and *osa-miR164a* (Table 1). Previous experiments showed that the proper combination of GAGACA and the TATA-box could be bound by OsARF1 specifically, whereas TGTCTC alone could not (Inukai et al. 2005). This information opens up the possibility that in addition to typical AuxREs, co-elements are also required for ARF binding in some cases. Therefore, five short AuxRE variants were combined with the TATA-box to generate five combinations with relatively high ARF-binding specificity and probability. *osa-miR171b* and *osa-miR171c* had at least one of the five combinations of AuxREs and the TATA-box located in similar upstream regions, as did *osa-miR164a*

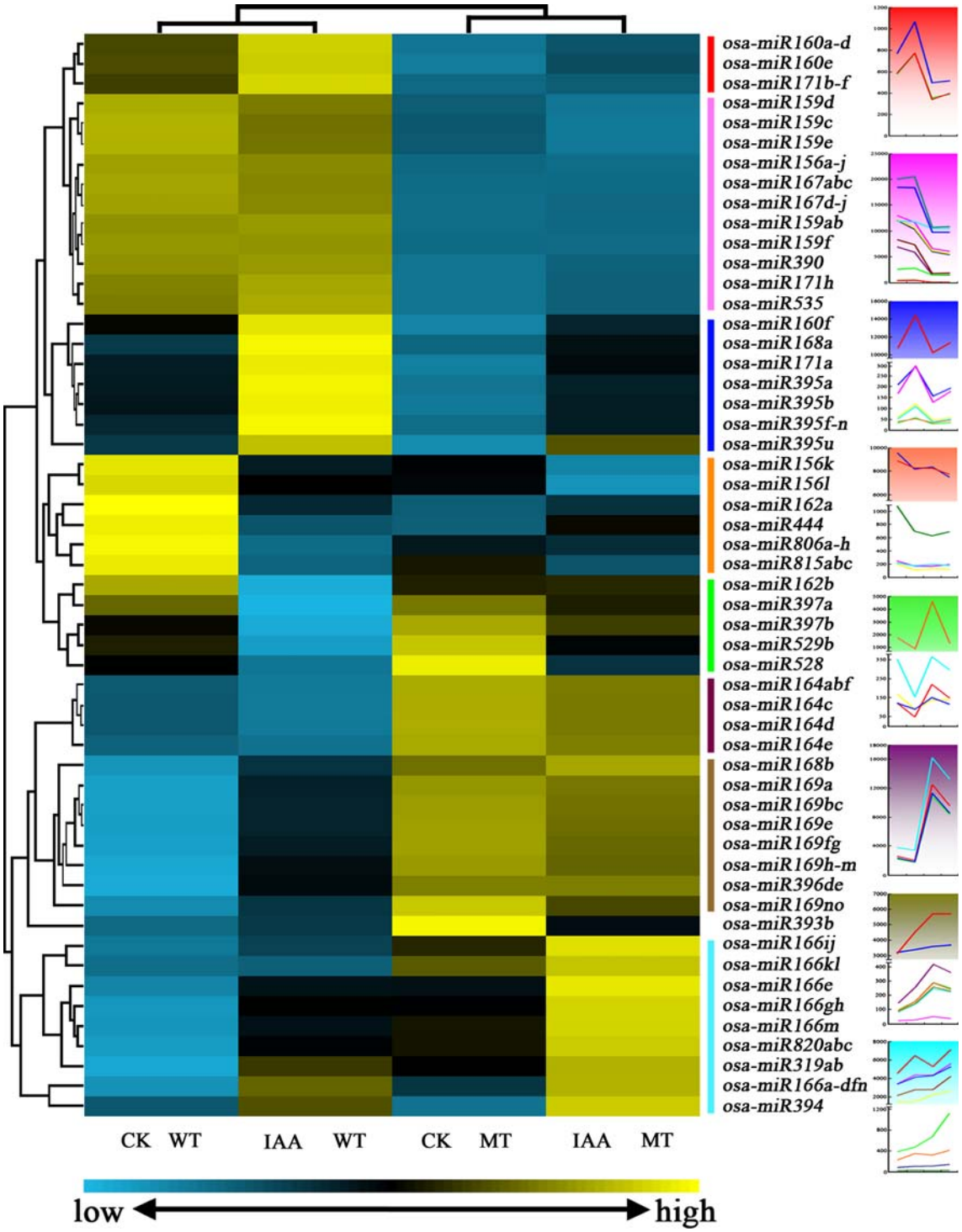

Fig. 2 Clustering analysis of microRNA expression data. The miRNA microarray data (Table S1) were sorted by hierarchical clustering method (Eisen et al. 1998). The intensity of *blue* and *yellow* colour indicates the relative expression levels. The clustering result was sorted into eight major clusters with *colour bars* marked on vertical axis. The histograms simulating the expression patterns of the corresponding clusters are presented on the *right*. *CK* normal culture conditions, *IAA* 30 μM IAA treatment for 3 h, *WT* wild type, *MT osaxr* mutant

and *osa-miR164f* (Table 1). *osa-miR171h* and *osa-miR390* exhibited similar expression patterns (Fig. 2), and they also had combinations of AuxREs and the TATA-box with similar distribution patterns. These results led to the possibility that auxin-specific *cis*-acting elements play a regulatory role in miRNA transcription under certain conditions.

To demonstrate that AuxREs have a specifically high occurrence in the auxin-related miRNA families (Table 1),

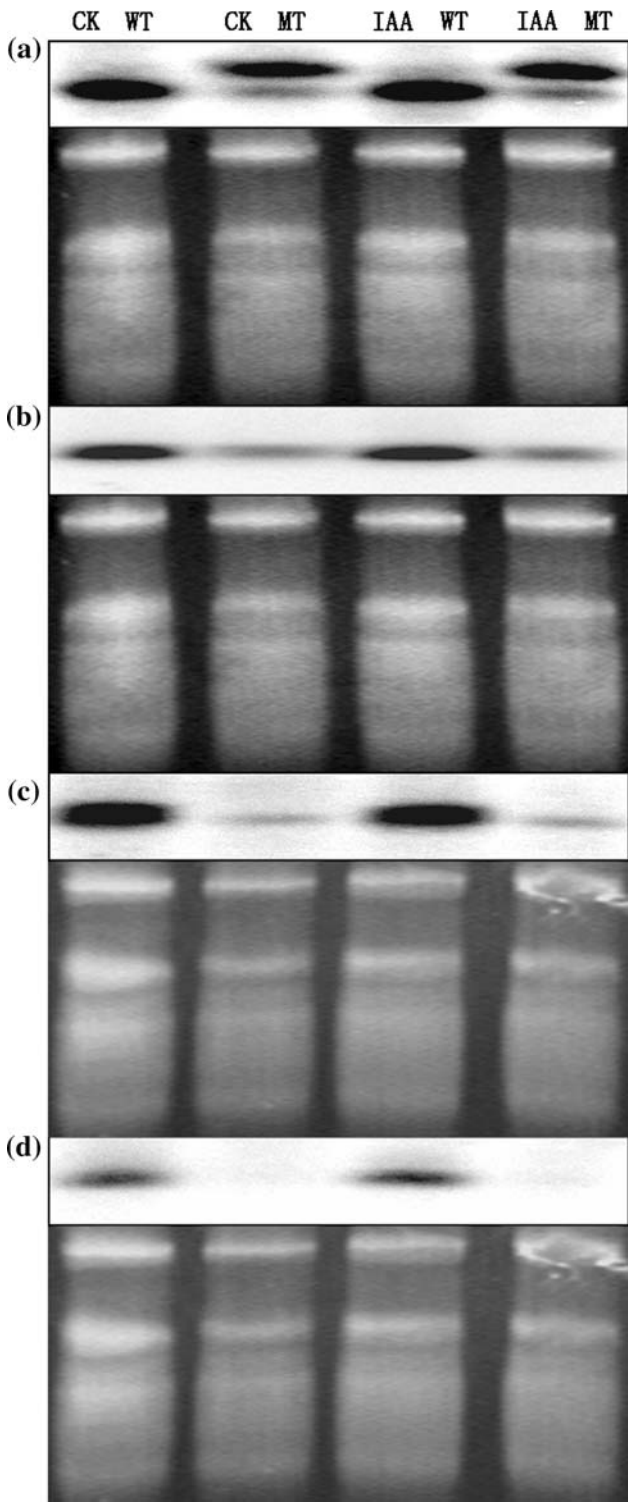

Fig. 3 Verification of microRNA microarray data by Northern blot. **a** *osa-miR164abf*. **b** *osa-miR167d–j*. **c** *osa-miR171g*. **d** *osa-miR390*. Ethidium bromide-stained 5S rRNAs/tRNAs were used as loading controls. *CK* normal culture conditions, *IAA* 30 μM IAA treatment for 3 h, *WT* wild type, *MT osaxr* mutant

all rice miRNA promoters (data S1) were recruited for the AuxRE distribution frequency calculation (Table S4). The results indicated that AuxREs occurred more frequently in auxin-related miRNA families than in other miRNA families (5.69 AuxREs per each auxin-related miRNA promoter vs. 4.42 AuxREs per each additional miRNA promoter) (Fig. 6a, b). This difference was significant (*t* test, $P = 0.0163$). When only DR5, DR5R, ER9 and the five combinations of AuxREs and the TATA-box were analysed, the difference was even more significant (0.56 AuxREs per each auxin-related miRNA promoter vs. 0.21 AuxREs per each additional miRNA promoter; *t* test, $P = 0.0091$) (Fig. 6c, d).

MicroRNA–target pairs and potential feedback circuit between microRNA(s) and ARF(s)

All rice miRNA targets were predicted by miRU (Zhang 2005) and CSRDB (Johnson et al. 2007). We classified the targets into three categories based on TIGR GO annotations (http://rice.plantbiology.msu.edu/data_download.shtml): molecular function, biological process and cellular component (Fig. S2). Considering molecular function, 4.13% of the targets were identified as having RNA polymerase II TF activity, and 0.98% of the targets were identified as being involved in auxin response in biological process. Both of the target groups contained ARFs.

To investigate the expression patterns of miRNA targets, we performed microarray analysis for protein-coding genes (Table S5). Considering the major cleavage effect of plant miRNAs on their target mRNAs, our comparative analysis of miRNA and protein-coding gene expression datasets revealed a number of reciprocally expressed miRNA–target pairs. Fifteen pairs were found under normal culture conditions (Table 2a), and 33 pairs were found under IAA treatment (Table 2b).

Yang et al. (2006) reported that *OsARF6* (*LOC_Os02g06910*) and *OsARF12* (*LOC_Os04g57610*) were genuine targets in cultured rice cells. Thus, *OsARF6* is an in vivo target of the miR167 family in rice. It is also strongly supported by the fact that *OsARF6* is upregulated in *osaxr* with repressed expression of the miR167 family (Fig. S3). The promoters of some miR167 family members have specific AuxREs with high ARF-binding potential (Table 1), suggesting that OsARF6 probably regulates the transcription of certain miR167 family member under specific conditions. Thus, we propose a feedback circuit between the miR167 family and *OsARF6* (Fig. S4).

Discussion

A number of microRNAs are expressed abnormally in *osaxr*

Our clustering analysis showed that many miRNAs were abnormally expressed in *osaxr*, including those with

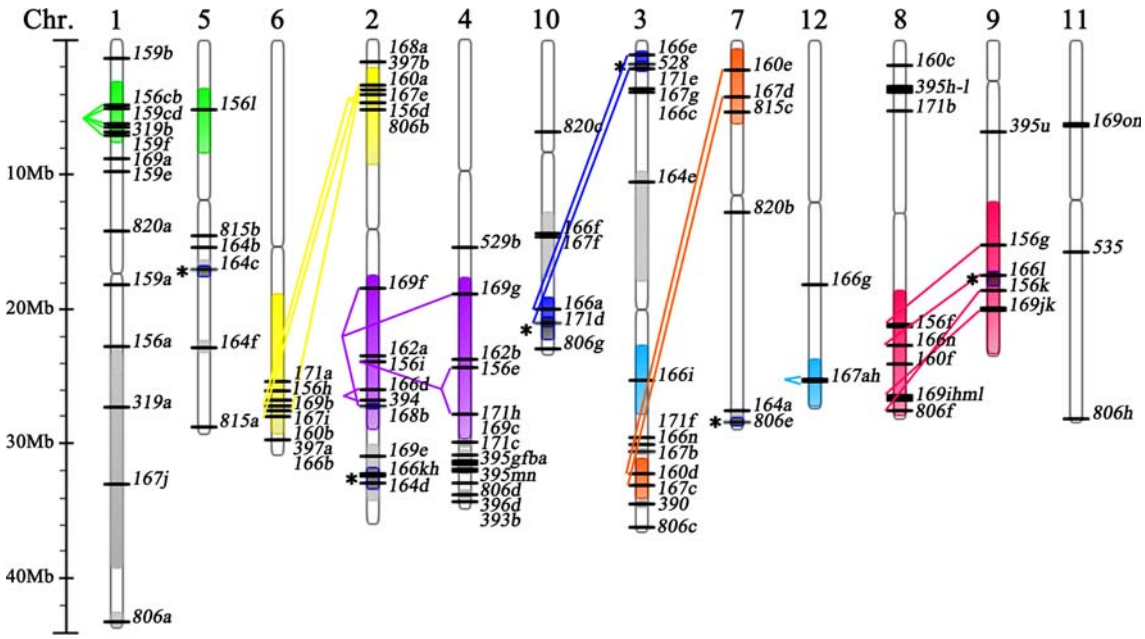

Fig. 4 Duplication and expression pattern analysis of rice microRNAs. All of the miRNAs in clustering analysis (Fig. 2) were marked on the rice chromosomes according to their locus information (miRBase Release 10.1). All duplication segments containing these miRNAs were retrieved from TIGR (http://www.tigr.org/tdb/e2k1/osa1/segmental_dup/index.shtml). The duplication segment pairs are shown with the same colours. In the duplication segment pairs, the miRNAs with similar expression patterns were linked. Both *light* and *dark grey* duplication segments are odd ones, whose counterparts (not shown) possess no analysed miRNAs. *Dark grey* segments overlapping with other segments were marked with *asterisks*. The *scale bar* on the *left* indicates the physical distance approximately. The *number* on the *top* of each chromosome represents the corresponding chromosome number

significantly changed expression levels or reduced auxin sensitivity compared with WT (Fig. 2). Many targets of these miRNAs are TFs involved in auxin signalling or root development (Table 1). Consistent with the microarray data, the Northern results showed that *osa-miR167d-j*, *osa-miR171g* and *osa-miR390* were intensively repressed in *osaxr* (Fig. 3b–d) and that their putative targets were ARFs, *Scarecrow*-like TFs and *TAS3*, respectively. Two bands appeared on the blot of *osa-miR164abf* (Fig. 3a), and the upper band was consistent with the microarray data. In Arabidopsis, the two-band expression pattern of the miR164 family was also observed and was organ-specific (Dunoyer et al. 2004). Guo et al. (2005) reported that *ath-miR164* directed *NAC1* mRNA cleavage to downregulate the auxin signal for lateral root development. Considering the lateral root defect of *osaxr*, we suggest that the upper band represents the functional *osa-miR164abf*, whereas the lower band represents low-weight molecules with unknown function or only the non-specific hybridisation signal. Thus, the higher expression level of *osa-miR164abf* in *osaxr* is a reasonable explanation for its lateral root defect. However, this hypothesis requires experimental validation.

MicroRNA auxin sensitivity is greatly reduced in *osaxr*

The number of auxin-sensitive miRNAs in *osaxr* was greatly reduced compared with WT. This finding indicates that miRNAs in the roots of *osaxr* are much less sensitive to exogenous auxin, consistent with the auxin-resistant phenotype of the mutant (Fig. 1c, e). In addition, a number of auxin-sensitive miRNAs in WT are not involved in the auxin-signalling pathway. Instead, they or their homologues in Arabidopsis, such as *osa-miR395* and *osa-miR169*, are involved in nutrition metabolism or stress response (Jones-Rhoades and Bartel 2004; Chiou 2007; Sunkar et al. 2007; Zhao et al. 2007). Considering the widespread signal interactions between hormones and nutrition or stress in plants, the results suggest that miRNAs may also mediate these signal interactions.

Some rice miRNAs share an overlapping duplication history with protein-coding genes

Duplication and expression pattern analysis show that certain members of the miR156, miR159, miR160, miR166, miR167, miR168, miR169, miR171, miR319, miR394, miR397 and miR806 families with similar expression patterns are located in the duplication segment pairs of rice protein-coding genes (Fig. 4). Base on the data provided by TIGR (http://www.tigr.org/tdb/e2k1/osa1/segmental_dup/index.shtml), the putative duplicated protein-coding genes within the duplication segment pairs usually have analogous functions or even are the same family members. Thus, rice miRNAs and protein-coding genes may share an

Table 1 Expression data, putative targets and *cis*-acting element search results of miRNAs abnormally expressed in *osaxr*

MiRNA	MiRNA expression data (average)				Putative target families[a]	Promoter *cis*-acting element analysis[b]
	CK WT	IAA WT	CK MT	IAA MT		
osa-miR159a	20,103	20,500	10,698	10,846	MYB transcription factor	AuxRR-core[c](−245); TGA-element[j](−16, −1780); TGTCTC[m](−1568)
osa-miR159b						–
osa-miR159c	12,008	10,335	6,042	5,400		MBS[f](−612, −842, −918); TGTCTC(−713)
osa-miR159d	12,951	11,626	6,612	6,099		–
osa-miR159e	12,174	10,588	6,126	5,549		MBS(−846, −1342, −1977); NON-box[i](−403); TGTCTC(−666)
osa-miR159f	18,486	18,371	9,744	9,733		CCGTCC-box[e](−1203); MBS(−811, −1770)
osa-miR160a	580	771	356	392	Auxin response factors	MBS(−247); TGTCTC(−1713)
osa-miR160b						MBS(−32, −52, −1233); MSA-like[h](−341)
osa-miR160c						CAT-box[d](−1081, −1127, −1749, −1766); CCGTCC-box(−1248); TGTCTC(−811, −1689); TGTCTC*TATA-box[q](−1689)
osa-miR160d						CCGTCC-box(−509, −1541); MBS(−580, −1234); TGTCTC(−291, −1631)
osa-miR160e	587	774	343	396		CAT-box(−347); TGTCTC(−133)
osa-miR160f	213	299	160	196		MBS(−258, −368, −861); TGTCTC(−158, −152); DR5[n](−158)
osa-miR164a	2,232	1,744	10,954	8,374	NAC domain proteins	CAT-box(−386); CCGTCC-box(−1592, −1666); MBS(−552, −612, −1083, −1217, −1653); TGTCTC(−262, −175); DR5(−262); TGTCTC*TATA-box(−175)
osa-miR164b						AuxRR-core(−1373); CAT-box(−322, −797); MBS(−270); AATAAG*TATA-box[t](−1518)
osa-miR164f						MBS(−430, −1616, −1641); TGTCTC(−245); GAGACA*TATA-box[p](−250)
osa-miR164c	2,624	2,032	12,559	9,596		MBS(−534)
osa-miR164d	2,356	1,837	11,301	8,593		CCGTCC-box(−556); MBS(−343, −379, −616, −1267, −1588, −1852)
osa-miR164e	3,797	3,431	16,255	13,249		CAT-box(−216); CCGTCC-box(−289, −527); MBS(−1141, −1191); dOCT[k](−1891); TGTCTC(−382)
osa-miR167a	6,896	5,890	1,471	1,475	Auxin response factors	CAT-box(−1070); MBS(−449); TGA-element(−1662); TGTCTC(−1676)
osa-miR167b						MBS(−390); TGTCTC(−402); AATAAG*TATA-box(−790)
osa-miR167c						TGTCTC(−1680)
osa-miR167d	8,313	7,349	1,743	1,834		CAT-box(−276); MBS(−294, −480, −1348); TGTCTC(−1428); TGTCAC*TATA-box[s](−441)
osa-miR167e						CAT-box(−83, −162); MBS(−1690); MRE[g](−1146, −1960); TGTCTC(−1563, −151, −35)
osa-miR167f						MBS(−1030); motif I[l](−376); TGTCTC(−930, −1087)
osa-miR167g						AuxRR-core(−496); MBS(−1548); MRE(−1431); TGA-element(−407, −647); TGTCTC(−1026); TGTCTC*TATA-box(−1026)
osa-miR167h						CAT-box(−249); CCGTCC-box(−1040); MBS(−855)

Table 1 continued

MiRNA	MiRNA expression data (average)				Putative target families[a]	Promoter *cis*-acting element analysis[b]
	CK WT	IAA WT	CK MT	IAA MT		
osa-miR167i						AuxRR-core(−1342); CAT-box(−101, −1300); MBS(−1322); TGTCTC(−124)
osa-miR167j						AuxRR-core(−653, −1561); CCGTCC-box(−676); MBS(−478, −555, −750, −822, −1242); TGA-element(−592, −1823); TGTCTC(−1387)
osa-miR171a	172	304	130	181	GRAS domain transcription factors (SCARECROW-like)	CAT-box(−442); MBS(−164, −227, −311, −394, −1036, −1097); TGA-element(−1271); TGTCTC(−691, −1771)
osa-miR171b	772	1,066	498	516		AuxRR-core(−18); CAT-box(−153, −1430); CCGTCC-box(−1794); MBS(−68, −223, −847); MRE(−1707); TGA-element(−83); TGTCTC(−1407); TGTCTC*TATA-box(−1407); AATAAG*TATA-box(−1285)
osa-miR171c						CAT-box(−708, −1521); TGA-element(−677, −1059); TGTCTC(−1440); GAGACA*TATA-box(−1445)
osa-miR171d						CCGTCC-box(−776); MRE(−938); TGTCTC(−601)
osa-miR171e						AuxRR-core(−231); CCGTCC-box(−1360); MRE(−660); TGA-element(−472); TGTCTC(−109, −474, −599, −1451)
osa-miR171f						MBS(−503, −831, −1075); TGA-element(−155); TGTCTC(−1420)
osa-miR171g	402	459	15	33		MBS(−748, −1507); TGTCTC(−647, −832, −1754); AATAAG*TATA-box(−273)
osa-miR171h	369	446	83	97		CAT-box(−469); MBS(−507, −645); TGTCTC(−855); TGTCAC*TATA-box(−567)
osa-miR390	472	508	36	45	Leu-rich repeat proteins; TAS3	CAT-box(−588); MBS(−335, −738, −882, −924); TGTCTC(−331, −694); TGTCTC*TATA-box(−331)
osa-miR528	1,758	911	4,608	1,329	–	CCGTCC-box(−901); MSA-like(−278)

– no information available, *CK* normal culture conditions, *IAA* 30 μM IAA treatment for 3 h, *WT* wild type, *MT osaxr* mutant

[a] The results are based on references (Llave et al. 2002; Allen et al. 2005; Guo et al. 2005; Wang et al. 2005; Williams et al. 2005; Ru et al. 2006; Wu et al. 2006; Yang et al. 2006; Liu et al. 2007; Reyes and Chua 2007) and miRU prediction

[b] Double-stranded miRNA promoters were collected for *cis*-acting element analysis

[c] AuxRR-core: *cis*-acting element involved in auxin responsiveness

[d] CAT-box: *cis*-acting element related to meristem expression

[e] CCGTCC-box: *cis*-acting element related to meristem specific activation

[f] MBS: MYB binding site involved in drought-inducibility

[g] MRE: MYB binding site involved in light responsiveness

[h] MSA-like: *cis*-acting element involved in cell cycle regulation

[i] NON-box: *cis*-acting element related to meristem specific activation

[j] TGA-element: auxin-responsive element

[k] dOCT: *cis*-acting element related to meristem specific activation

[l] motif I: *cis*-acting element root specific

[m–t] AuxREs. TATA-box: TATA(A|T)A(T|A)(A|G). Asterisk represent any nucleic acid compositions <100 nt. '|' means 'or'. The annotations of 3–12 were adopted from PlantCARE

overlapping duplication history to some extent. Recently, Axtell and Bowman (2008) reported that many miRNA family members arose through genome-wide duplication events. Maher et al. (2006) demonstrated that both miRNAs and genes had high birth and death rates during duplication; thus, they might not differ from each other. In addition, recent results have shown that the evolutionary history of miRNA families seems to be similar to that of the protein-coding gene families in plants (Li and Mao 2007). Our results are consistent with these reports to some extent. In Arabidopsis, several miRNA genes originated by inverted duplication of their target genes (Allen et al. 2004).

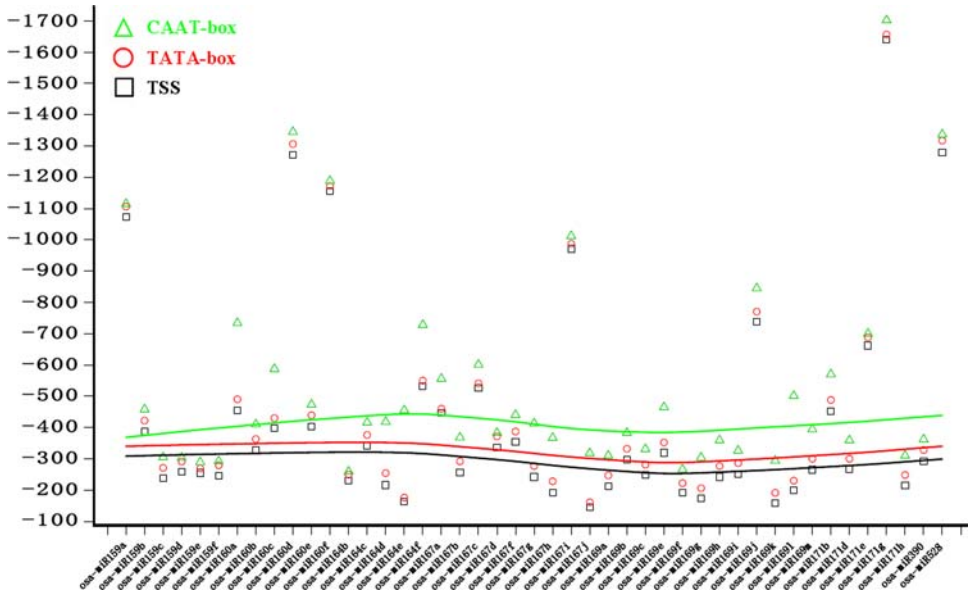

Fig. 5 The basic characteristics of microRNA promoters are similar to those of the RNA polymerase II-dependent protein-coding genes. The promoters of abnormally expressed miRNAs in *osaxr* (Table 1) were recruited for the analysis. The distribution patterns of the TSS, the TATA-box and the CAAT-box in the miRNA promoters (Table S3) were depicted by scatter plot and simulated by trend lines using *loess* method (Cleveland and Devlin 1988). The *Y*-axis represents the distance from the 5′ end of the pre-miRNAs

Whether the same mechanism exists in rice needs to be further elucidated.

The chromosome distribution shows that most potential miRNA polycistrons are made up of the same family members, which is consistent with recent observations (Cui et al. 2009). They probably arise from tandem duplication events. In addition, the miRNA clusters with similar expression patterns, such as *osa-miR159c* and *osa-miR159d*, may share common promoters. In contrast to a previous finding (Jiang et al. 2006), in the current work, we found that miRNAs within the same families, such as the miR159 family, share quite uniform expression patterns.

AuxREs have a highly specific occurrence in auxin-related miRNAs

Our TSS, TATA-box and CAAT-box distribution analysis showed that the rice miRNA promoters share similar characteristics with the RNA polymerase II-dependent protein-coding gene promoters (Fig. 5) (Breathnach and Chambon 1981). Although Breathnach and Chambon (1981) characterised non-plant eukaryotic promoters, our result is consistent with a previous report that RNA polymerase II transcribes most miRNAs (Lee et al. 2004). We observed that the distributions of the three *cis*-acting elements were consistently upstream-shifted. The explanation is that primary miRNA is transcribed upstream of the boundary that we defined for promoter collection. In addition, our results also support the hypothesis proposed by Zhou et al. (2007) that miRNA core promoters are close to precursor miRNAs.

Auxin-specific *cis*-acting element analysis showed that TGTCTC, a typical AuxRE, existed in most miRNA families because of its poor sequence specificity (Table S4). DR5 can confer auxin responsiveness to the minimal −46 CaMV 35S promoter (Ulmasov et al. 1995, 1997). DR5-related sequences are present in the promoters of *osa-miR160f* and *osa-miR164a* (Table 1), which have homologs in Arabidopsis that play key roles in auxin signalling (Guo et al. 2005; Wang et al. 2005; Liu et al. 2007).

To demonstrate that the AuxREs have a highly specific occurrence in the auxin-related miRNA families (Table 1), all rice miRNA promoters (data S1) were recruited for the AuxRE distribution frequency calculation (Table S4). Our results demonstrated that AuxREs were more frequently present in the promoters of auxin-related miRNAs.

Potential miRNA–target pairs in rice roots

Comparative analysis of miRNA and protein-coding gene expression datasets revealed a number of reciprocally expressed miRNA–target pairs. Fifteen pairs were found under normal culture conditions (Table 2a), and 33 pairs were found under IAA treatment (Table 2b). Similar to Arabidopsis (Gustafson et al. 2005; Wang et al. 2005; Wu et al. 2006; Liu et al. 2007), the miR160 and miR167 families in rice have corresponding ARF targets. *ath-miR390* positively regulates *AtTAS3* to produce *trans*-acting small interfering RNA (ta-siRNA), which has *AtARF2*, *AtARF3* and *AtARF4* as targets (Allen et al. 2005; Gustafson et al. 2005; Williams et al. 2005). We retrieved the sequence information for ta-siRNA encoded by *OsTAS3* (Williams et al. 2005) for target prediction and identified *OsARF2*, *OsARF3* and *OsARF4* as the putative targets of *OsTAS3*. *ath-miR164* regulates several *NAC* domain-encoding genes involved in auxin signal transduction for lateral root development (Xie et al. 2000; Guo et al. 2005; Gustafson et al. 2005). Four *osa-miR164–NAC* pairs were predicted under

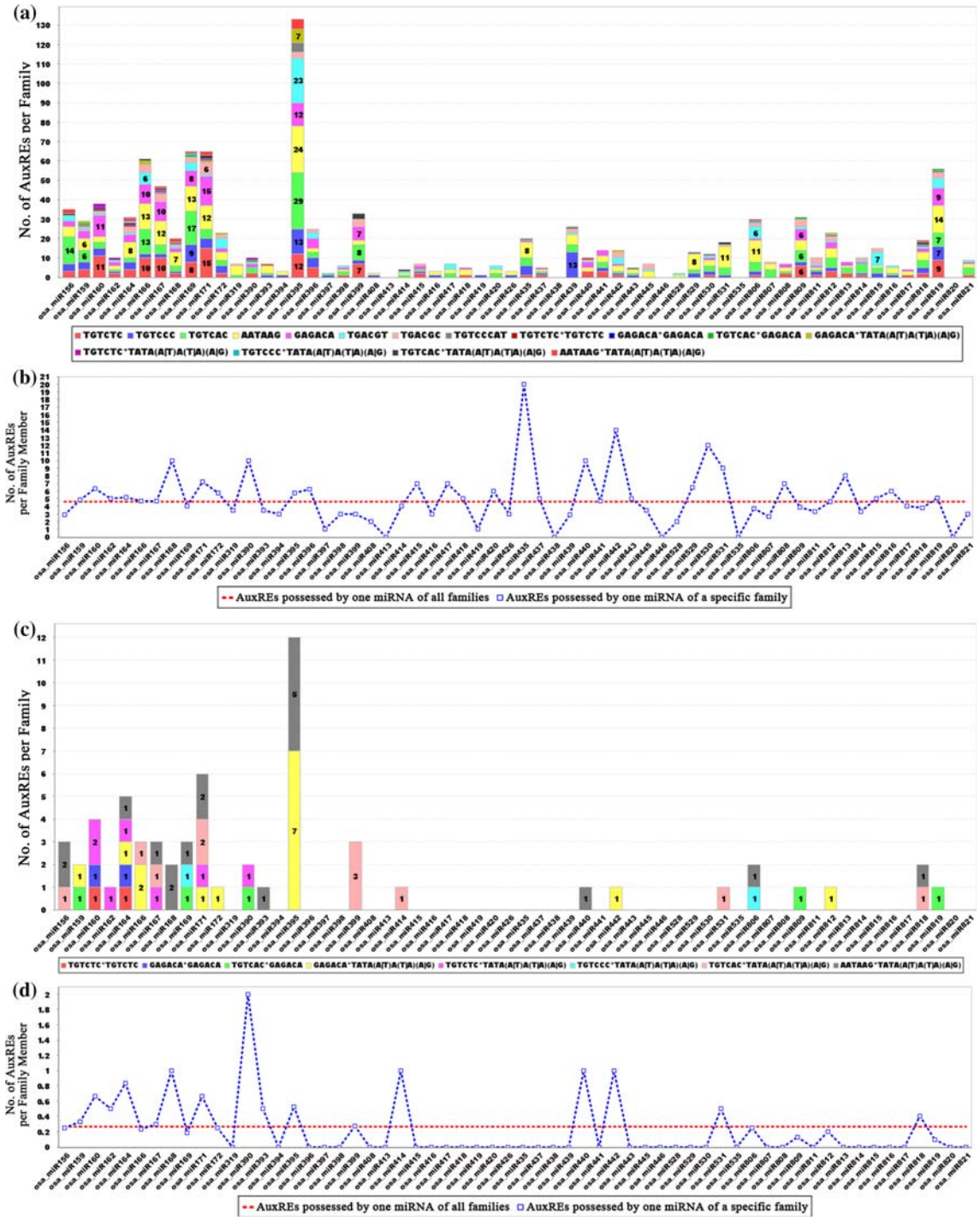

Fig. 6 AuxREs have specifically high occurrence in the promoters of the auxin-related microRNAs. **a** The numbers of the 16 AuxREs possessed by each miRNA family. **b** The average numbers of the 16 AuxREs possessed by one miRNA in each family were calculated and depicted with *blue squares*. *Red dash line* represents the average numbers of the 16 AuxREs possessed by one miRNA. **c** The numbers of the eight AuxREs with relatively high ARF-binding specificity and potential possessed by each miRNA family. **d** The average numbers of the eight AuxREs possessed by one miRNA in each family were calculated and depicted with *blue squares*. *Red dash line* represents the average numbers of the eight AuxREs possessed by one miRNA

both normal culture conditions and IAA treatment (Table 2). Our prediction results additionally showed that *osa-miR396abde* targeted *LOC_Os03g51970* (Table 2), consistent with a previous report (Sunkar et al. 2005). *ath-miR408* is involved in copper utilisation (Abdel-Ghany and Pilon 2008). Interestingly, four *osa-miR408*–target pairs were predicted under IAA treatment, and all of the targets were copper related (Table 2b). Homologous targets of the

Table 2 Potential miRNA–target pairs with reciprocal expression patterns in rice roots

miRNA	miRNA CK $\log_2$(MT/WT)[a]	Target CK $\log_2$(MT/WT)[b]	TIGR locus name	TIGR annotation[c]	miRU[d]	CSRDB
a. Normal culture conditions						
osa-miR164c	2.259	−2.184	LOC_Os02g36880.1–3	NAC domain protein NAC5	Yes	Yes
osa-miR164c	2.259	−6.644	LOC_Os09g36490.1	Collagen, type IV, alpha 5		Yes
osa-miR164a/b/f	2.295	−2.184	LOC_Os02g36880.1–3	NAC domain protein NAC5	Yes	Yes
osa-miR164d	2.262	−2.184	LOC_Os02g36880.1–3	NAC domain protein NAC5	Yes	Yes
osa-miR164d	2.262	−2.000	LOC_Os01g59120.1	Cyclin IaZm	Yes	
osa-miR164d	2.262	−1.737	LOC_Os09g30486.1	Fasciclin-like arabinogalactan protein 7 precursor		Yes
osa-miR164e	2.098	−2.184	LOC_Os02g36880.1–3	NAC domain protein NAC5	Yes	Yes
osa-miR528	1.390	−1.737	LOC_Os01g21070.1	Endoglucanase 1 precursor	Yes	
osa-miR528	1.390	−4.322	LOC_Os01g03620.1	Copper ion binding protein	Yes	
osa-miR169a	1.569	−2.474	LOC_Os03g11900.1	Sugar transport protein 8		Yes
osa-miR396d/e	0.850	−2.556	LOC_Os03g51970.1	Growth-regulating factor 1	Yes	
osa-miR166i/j	0.819	−1.599	LOC_Os03g10440.1	1,4-Beta-xylanase	Yes	
osa-miR397b	0.862	−2.644	LOC_Os03g16610.1	L-Ascorbate oxidase precursor	Yes	
osa-miR397b	0.862	−3.184	LOC_Os12g15680.1	L-Ascorbate oxidase precursor	Yes	
osa-miR806a-h	−0.588	1.700	LOC_Os04g48200.1	Cytochrome P450 87A3	Yes	
miRNA	miRNA IAA $\log_2$ (MT/WT)[a]	Target IAA $\log_2$ (MT/WT)[b]	TIGR locus name	TIGR annotation[c]	miRU[d]	CSRDB
b. IAA treatment						
osa-miR164c	2.240	−2.556	LOC_Os06g49660.1	Taxadien-5-alpha-ol *O*-acetyltransferase	Yes	Yes
osa-miR164c	2.240	−3.474	LOC_Os02g36880.1–3	NAC domain protein NAC5	Yes	Yes
osa-miR164c	2.240	−1.786	LOC_Os11g05190.1	Phytosulfokines 2 precursor	Yes	
osa-miR164c	2.240	−5.059	LOC_Os09g36490.1	Collagen, type IV, alpha 5		Yes
osa-miR164a/b/f	2.264	−2.556	LOC_Os06g49660.1	Taxadien-5-alpha-ol *O*-acetyltransferase	Yes	Yes
osa-miR164a/b/f	2.264	−3.474	LOC_Os02g36880.1–3	NAC domain protein NAC5	Yes	Yes
osa-miR164a/b/f	2.264	−1.786	LOC_Os11g05190.1	Phytosulfokines 2 precursor	Yes	
osa-miR164a/b/f	2.264	−1.599	LOC_Os11g05820.1	Transposon protein, Mutator sub-class	Yes	
osa-miR164d	2.226	−2.556	LOC_Os06g49660.1	Taxadien-5-alpha-ol *O*-acetyltransferase	Yes	
osa-miR164d	2.226	−3.474	LOC_Os02g36880.1–3	NAC domain protein NAC5	Yes	
osa-miR164d	2.226	−1.599	LOC_Os11g05820.1	Transposon protein, Mutator sub-class	Yes	
osa-miR164d	2.226	−1.889	LOC_Os02g43170.1	Salt tolerance-like protein	Yes	
osa-miR164d	2.226	−1.786	LOC_Os08g01480.1	Cytochrome P450 71C4		Yes
osa-miR164e	1.949	−2.556	LOC_Os06g49660.1	Taxadien-5-alpha-ol *O*-acetyltransferase	Yes	
osa-miR164e	1.949	−3.474	LOC_Os02g36880.1–3	NAC domain protein NAC5	Yes	
osa-miR164e	1.949	−1.786	LOC_Os08g01480.1	Cytochrome P450 71C4	Yes	
osa-miR396a/b	1.798	−4.059	LOC_Os03g51970.1	Growth-regulating factor 1	Yes	
osa-miR397b	1.606	−2.943	LOC_Os03g16610.1	L-Ascorbate oxidase precursor	Yes	
osa-miR397b	1.606	−2.837	LOC_Os12g15680.1	L-Ascorbate oxidase precursor	Yes	
osa-miR397b	1.606	−2.000	LOC_Os01g63180.1	L-Ascorbate oxidase precursor	Yes	
osa-miR166i/j	1.254	−2.556	LOC_Os03g10440.1	1,4-Beta-xylanase	Yes	
osa-miR397a	0.948	−2.943	LOC_Os03g16610.1	L-Ascorbate oxidase precursor	Yes	Yes
osa-miR397a	0.948	−2.837	LOC_Os12g15680.1	L-Ascorbate oxidase precursor	Yes	Yes
osa-miR397a	0.948	−3.322	LOC_Os01g15810.1	Peroxidase 9 precursor	Yes	
osa-miR397a	0.948	−2.000	LOC_Os01g63180.1	L-Ascorbate oxidase precursor	Yes	Yes
osa-miR408	1.185	−2.644	LOC_Os06g15600.1	Chemocyanin precursor	Yes	

Table 2 continued

miRNA	miRNA IAA $\log_2$ (MT/WT)[a]	Target IAA $\log_2$ (MT/WT)[b]	TIGR locus name	TIGR annotation[c]	miRU[d]	CSRDB
osa-miR408	1.185	−1.599	LOC_Os06g11490.1	Blue copper protein precursor	Yes	Yes
osa-miR408	1.185	−3.059	LOC_Os01g03530.1	copper ion binding protein	Yes	
osa-miR408	1.185	−2.059	LOC_Os02g43660.1	Blue copper protein precursor	Yes	
osa-miR166k/l	0.800	−3.322	LOC_Os10g31690.1	Glycine-rich cell wall structural protein 2 precursor	Yes	
osa-miR160a-d	−0.976	2.498	LOC_Os04g37990.1	Sugar transport protein 5	Yes	
osa-miR160e	−0.966	2.498	LOC_Os04g37990.1	Sugar transport protein 5	Yes	
osa-miR171h	−2.207	1.664	LOC_Os03g19070.1	Long cell-linked locus protein	Yes	

CK normal culture conditions, *IAA* 30 μM (for miRNAs) or 10 μM (for protein-coding genes) IAA treatment for 3 h, *WT* wild type, *MT osaxr* mutant

[a] Mean expression levels averaged from three biological replicates were used for the calculation; miRNAs with more than 1.5-fold changed expression levels in *osaxr* compared with the wild type were selected

[b] Mean expression levels averaged from two biological replicates were used for the calculation; protein-coding genes with more than threefold changed expression levels in *osaxr* compared with the wild type were selected

[c] Gene annotations from TIGR (Version 5)

[d] miRU prediction was performed with the lowest stringency

miR396 and miR408 families have been demonstrated in Arabidopsis (Gustafson et al. 2005; miRNA target information in Arabidopsis can be viewed at http://asrp.cgrb.oregonstate.edu/db/download.html); thus, these miRNA–target pairs can serve as a repository for further experimental analysis of specific miRNA–target regulatory relationships.

Feedback regulatory models between miRNAs and TFs have been established in animals and humans (Tsang et al. 2007). In this study, we investigated the feedback regulation involved in auxin signalling in rice, and based on our results, we proposed a feedback circuit between the miR167 family and *OsARF6* (Fig. S4). Although this feedback model requires further validation, it reflects the widespread feedback regulatory loops between miRNAs and TFs in plants.

Acknowledgments This study is supported by 973 Program (2005CB120901), NSFC (30771326), 863 Program (2008AA10Z125), and Program for NCET.

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