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Microarray-based screening of the microRNAs associated with caryopsis development in *Oryza sativa*

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Abstract

Plant microRNAs modulate diverse developmental processes by regulating expression of their target genes. To explore potential miRNA-guided gene regulation in developing rice (*Oryza sativa* L.) caryopses, a miRNA microarray was used to identify miRNAs present at the different developmental stages. We found that 27 miRNAs, of which 16 were conserved miRNAs, were present in developing caryopses. High expression levels were detected for miR159, miR167, and miR530 at the morphogenesis stage and for miR169, miR435, and miR528 at the stage of accumulation of metabolites. Next, 26 target genes were predicted for seven of the detected miRNAs and the expression profiles of these miRNAs and their corresponding target genes were examined in developing caryopses. Our results suggest that the miRNAs and their target genes examined at the two distinct stages could contribute to the developmental progress of rice caryopses in concert with phytohormone signalling.

Additional key words: nutrient storage, morphogenesis, rice.

Introduction

Rice is not only a model monocotyledonous plant with a fully sequenced genome but it is also a key cereal crop with agronomical importance. The major part of a mature rice caryopsis is composed of the embryo and the endosperm which is filled with storage nutrients. For decades, efforts have been made to elucidate the processes of embryogenesis and endosperm development in rice (Hoshikawa 1993, Olsen 2004, Itoh *et al.* 2005). Although a number of genes associated with the development of rice caryopsis have been studied by different approaches (Ishimaru *et al.* 2005, Barroco *et al.* 2006, Song *et al.* 2007, Wang *et al.* 2008, Weng *et al.* 2008, She *et al.* 2010), the regulatory mechanisms of the expression of these genes remain unclear.

Recent evidence has shown that plant miRNAs play crucial roles in a variety of biological processes including growth, development, metabolism, and stress responses (Reinhart *et al.* 2002, Jones-Rhoades *et al.* 2006, Yang *et al.* 2007, Chuck *et al.* 2009). A large number of plant miRNAs have been identified by size-fractioned cloning, computational prediction, and high-throughput sequencing (Rhoades *et al.* 2002, Jones-Rhoades and Bartel 2004, Sunkar and Zhu 2004, Wang *et al.* 2004, Adai *et al.* 2005, Sunkar *et al.* 2005, 2008). Based on the identified miRNAs, it was suggested that plant miRNAs are common and function in diverse processes of growth and development.

The development of rice caryopsis involves a series of precise developmental events. After fertilisation, cell division and morphological differentiation progress rapidly in both the embryo and the endosperm. Most morphogenetic events in the embryo and endosperm are completed by about 10 d after fertilisation (DAF). After that, the embryo progresses into the maturation and dormancy and the endosperm undergoes intense collection of reserve substances such as starch and proteins (Hoshikawa 1993, Itoh *et al.* 2005). Therefore, the course of caryopsis development can be roughly divided into two distinct stages: the morphogenesis stage before 10 DAF and the stage involving the accumulation

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Abbreviations: ABA - abscisic acid; ARF - auxin response factor; ASO - *L*-ascorbate oxidase; DAF - days after fertilisation; DAH - days after heading; GAP - GTPase activating protein; GPCR - G protein-coupled receptor; LRR - leucine-rich repeat; PCD - programmed cell death; snRNA - small nuclear RNA.

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of metabolites after 10 DAF. Based on the complexity of gene expression and regulation at the two stages, it is suggested that certain miRNAs are likely to be involved in regulation of the expression of genes related to the development of rice caryopsis.

Recently, several studies have focused on the identification of miRNAs to clarify the expression mechanisms of genes mainly involved in stress responses and grain development in rice. It has been reported that 11 miRNAs were regulated by phytohormones and 24 miRNAs were induced by drought, heavy metals, and oxidative stresses (Zhao *et al.* 2007, Huang *et al.* 2009, Liu *et al.* 2009, Li *et al.* 2010). Furthermore, it was demonstrated that miR159 regulates two MYB factor genes during anther development in rice (Tsuji *et al.* 2006). To date, at least 21 rice miRNAs have been reported to be conserved among angiosperms and many of these are predicted to target mRNAs encoding transcription factors (Jones-Rhoades *et al.* 2006, Axtell and Bowman 2008) suggesting that these conserved miRNAs may have an irreplaceable physiological role in gene regulation networks. Additionally, numerous miRNAs, including most of the conserved miRNAs, have been found to accumulate in grains (Zhu *et al.* 2008, Xue *et al.* 2009). Nevertheless, functional studies on miRNAs in rice caryopses are still limited.

To understand the physiological functions of miRNAs and their target genes with respect to the morphogenesis and metabolite accumulation of developing caryopses, we carried out a miRNA microarray assay using caryopses at these two stages. The expression of 27 miRNAs was detected in developing caryopses but only eight of them showed differential expression between two stages. The expression patterns of these eight miRNAs and their corresponding targets were examined.

Materials and methods

Rice (*Oryza sativa* L. ssp. *japonica* cv. Nipponbare) plants were grown in a paddy field under normal conditions (Zhou *et al.* 2010). At different days after heading (DAH), developing caryopses were sampled and immediately frozen in liquid nitrogen and stored at -80 ºC for later RNA extraction. The stages of the caryopses obtained at 5 DAH and 12 DAH were defined as the morphogenesis stage and the metabolite accumulation stage, respectively. Total RNA was extracted using *TRIzol* reagent according to the manufacturer's instructions (*Invitrogen*, Carlsbad, CA, USA). Contaminating DNA was then digested with *RQ1* RNase-free DNase I (*Promega*, Madison, WI, USA). To perform miRNA microarray analysis and miRNA reverse transcription polymerase chain reaction (RT-PCR), low-molecular-mass (LMM) RNA was prepared from total RNA by PEG precipitation as previously described (Thomson *et al.* 2004).

Based on the miRNA database (http://www.mirbase.org/), a miRNA microarray with 308 non-redundant probes was constructed as previously described (Jian *et al.* 2010). LMM RNAs derived from the caryopses of two developmental stages were labelled with Cy3 by RNA ligase and then used for microarray hybridisation at 42 °C overnight. Microarray hybridisation was performed in duplicate as previously described (Liu *et al.* 2008). Data analysis was performed as described by Jian *et al.* (2010). After the hybridization signals were gathered, the intensity values from each microarray were filtered and normalised against the global mean (*i.e.*, the average of all miRNAs). The miRNAs with mean intensity values >1000 and P values < 0.01 according to Student's t-test were defined as significantly expressed miRNAs. To identify the miRNAs that were differentially expressed between the two distinct stages, a significance analysis of microarrays (*SAM, v. 2.1*) was performed using a two-class unpaired comparison with a criterion of fold change > 2 and false discovery rate (FDR) < 5 % (Tusher *et al.* 2001).

Real-time RT-PCR of miRNAs was performed as previously described (Chen *et al.* 2005). Briefly, the LMM RNA was reverse-transcribed with *SuperScript*™ II reverse transcriptase (*Invitrogen*). A gene-specific primer mix for each miRNA and U6 small nuclear RNA (snRNA) was used for reverse transcription. PCR for each miRNA was carried out using *Power SYBR® Green PCR Master Mix* (*Applied Biosystems*, Foster City, CA, USA). The relative amount of miRNA was normalised to the level of U6 snRNA. To perform RT-PCR of target genes, total RNA was reverse-transcribed using an oligo(dT) primer. PCR for each target gene was performed using gene-specific primers flanking the predicted cleavage site within the mRNA sequence. The rice *actin* gene was used as an internal control. Varying numbers of cycles, ranging from 26 to 35, were run according to the abundance of each mRNA.

The targets for each miRNA were predicted using the *miRU* web program developed by Zhang (2005). The sensitivity of prediction was increased with a relatively low stringency (score for each 20 nt, 3.5; G:U wobble pairs, 2; indels, 1; other mismatches, 3). Information on conserved miRNAs and the mRNA dataset of *Arabidopsis* were used for homology analysis to reduce false positives in the target prediction of the conserved rice miRNAs.

Results

To identify miRNAs in developing caryopses, a miRNA microarray constructed with 308 probes was hybridised with RNAs derived from the developing caryopses of two distinct stages. A total of 27 miRNAs were detected. Of these, 16 miRNAs were confirmed to be conserved miRNAs. Although most of the detected miRNAs exhibited approximately unchanged expression levels, eight miRNAs showed significant differences in expression between the two distinct stages: the expression levels of miR167, miR169, miR390, miR435, miR528, miR530, and miR2001 were higher at 12 DAH than that at 5 DAH, and the expression level of miR159 was lower (Table 1).

The expression of these eight miRNAs was examined in developing caryopses from 0 to 20 DAH using semi-quantitative RT-PCR and real-time RT-PCR (Fig. 1).Two types of miRNA expression profiles were observed in developing caryopses. Before 8 DAH, high expression was detected for miR159, miR167 and miR530, particularly for miR159 and miR167 just after heading (Fig. 1*A*,*B*,*G*). After 8 DAH, high expression was confirmed for miR169, miR390, miR435, miR528 and miR2001, and especially for miR169, miR435 and miR2001 at about 16 DAH (Fig. 1*C*,*D*,*E*,*F*,*H*).

To investigate the physiological functions of the 8 detected miRNAs in developing caryopses, their target candidates were screened by *miRU* web program. The program predicted 26 target genes for seven of the miRNAs, but no target gene for miR435 was predicted (Table 1). The target genes of miR159 and miR167, the

Table 1. Prediction of target candidates for miRNAs identified by microarray analysis. § - Nomenclature of auxin response factors in rice was based on the genome-wide analysis by Wang *et al*. (2007);* - this target was newly predicted in this work.

miRNA	Fold change (12 DAH/5 DAH)	Predicted or validated target	Putative target function	Reference
osa-miR159	0.39	*Os01g59660*	MYB transcription factor	Luo *et al*. 2006
		Os05g41166	MYB transcription factor	Liu *et al*. 2009
		Os04g46384	MYB transcription factor	Liu *et al*. 2009
		Os03g47949	E3 ubiquitin–protein ligase	Liu *et al*. 2009
		Os11g05540	GTPase activating protein	Liu *et al*. 2009
osa-miR167	2.14	*Os04g57610*	auxin response factor 12 §	Liu *et al*. 2009; Yang *et al*. 2006
		Os09g39420	HIRA protein	Liu *et al*. 2009
		Os07g29820	NBS-LRR disease resistance protein	Liu *et al*. 2009
		Os02g06910	auxin response factor 6 §,*	
		Os06g46410	auxin response factor 17 §,*	
		Os12g41950	auxin response factor 25 §,*	
osa-miR169	2.19	*Os03g29760*	HAP2-like transcription factor	Li *et al*. 2010
		Os12g42400	HAP2-like transcription factor	Liu *et al*. 2009; Li *et al*. 2010
		Os02g53620	HAP2-like transcription factor	Liu *et al*. 2009
		Os07g41720	HAP2-like transcription factor	Xue *et al*. 2009
		Os03g44540	HAP2-like transcription factor *	
		Os07g06470	HAP2-like transcription factor *	
osa-miR390	2.49	*Os02g10100*	LRR protein	Sunkar *et al*. 2005
osa-miR435	3.34	unknown	unknown	
osa-miR528	3.47	*Os08g36420*	metal cation transporter	Li *et al*. 2010
		Os06g06050	F-box-LRR protein	Li *et al*. 2010
		Os06g37150	*L*-ascorbate oxidase	Xue *et al*. 2009
		Os08g04310	plastocyanin-like protein	Xue *et al*. 2009
		Os07g38290	plastocyanin-like protein *	
osa-miR530	2.15	*Os03g15600*	unknown	Xue *et al*. 2009
osa-miR2001	2.05	*Os10g22830*	unknown	Jian *et al*. 2010
		Os04g42960	G protein-coupled receptor	Jian *et al*. 2010

miRNAs showing high expression at the morphogenesis stage, mainly encode MYB proteins and auxin response factors (ARFs). Among the miRNAs showing high expression during metabolite accumulation (miR169, miR390, and miR2001), the target genes encode HAP2-like transcription factors, leucine-rich repeat (LRR) protein, and G protein-coupled receptor (GPCR), respectively, and those of miR528 encode a metal cation transporter, F-box-LRR protein, *L*-ascorbate oxidase (ASO), and plastocyanin-like proteins.

The accumulation profiles for the above predicted target genes during caryopsis development were determined by RT-PCR (Fig. 2). The mRNA levels of most of the miR159, miR167, and miR169 target genes were relatively abundant in developing caryopses of all that were examined. Remarkably, the transcripts of the target genes of miR159 and miR2001, *Os11g05540* and *Os04g42960*, respectively, were only observed in developing caryopses before about 2 DAH. Otherwise, the target genes of miR159, *Os01g59660*, and *Os03g47949*, showed high expression at 12 - 16 DAH, and that of miR169, *Os07g41720*, exhibited elevated expression after about 12 DAH.

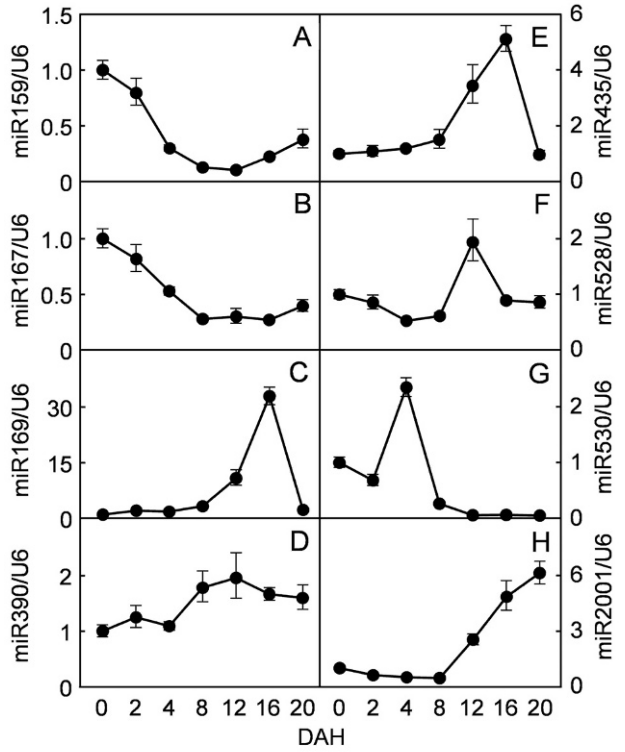

Fig. 1. Expression profiles of miRNAs during the development of rice caryopsis. Expression levels were detected in developing caryopses for osa-miR159 (*A*), osa-miR167 (*B*), osa-miR169 (*C*), osa-miR390 (*D*), osa-miR435 (*E*), osa-miR528 (*F*), osa-miR530 (*G*), and osa-miR2001 (*H*) using real-time RT-PCR. The relative miRNA amounts (means ± SD) were normalised to the level of U6 snRNA using the ddCt method. DAH - days after heading.

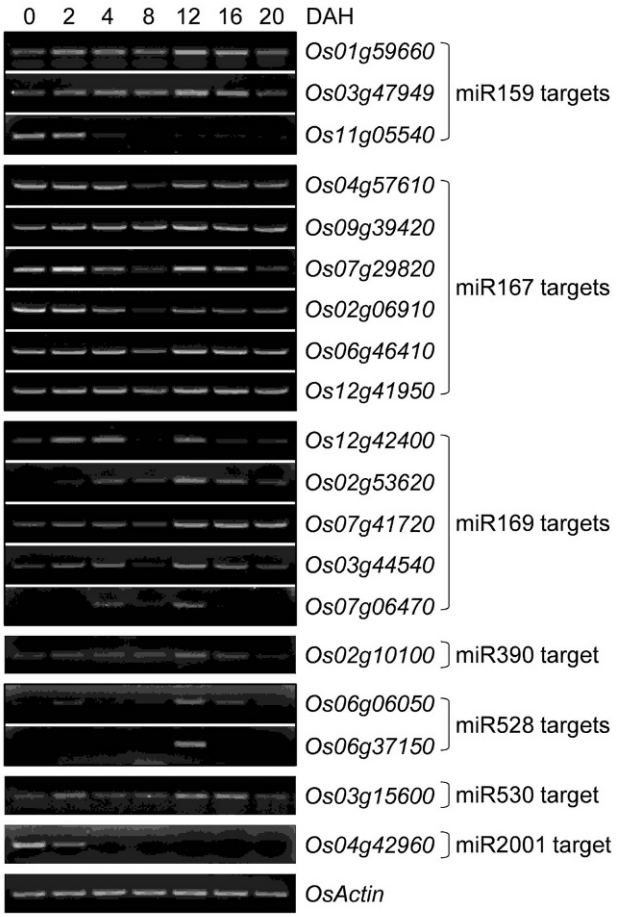

Fig. 2. Expression patterns of the predicted target genes during the development of rice caryopsis. The accumulation of transcripts produced by the target candidates of seven miRNAs detected in developing caryopses were determined by semi-quantitative RT-PCR. The rice *actin* gene was used as the internal control. DAH - days after heading.

Discussion

Rice caryopses are the most important organs for nutrient storage, and their developmental status usually firstly depends on the cell proliferation and differentiation and later on the metabolite accumulation. Several researchers have demonstrated that many miRNAs are involved in the stress response and grain development in rice (Zhao *et al.* 2007, Zhu *et al.* 2008, Huang *et al.* 2009, Liu *et al.* 2009, Xue *et al.* 2009, Li *et al.* 2010). This study revealed

that at least 16 conserved and 11 non-conserved miRNAs are present in developing caryopses. High expression of miR159, miR167, and miR530 was observed at the morphogenesis stage, and high expression of miR169, miR435, and miR201 was found during metabolite accumulation. These data suggest that the miRNAs present at the two distinct stages post-transcriptionally regulate their target genes to contribute to the developmental progress of rice caryopses.

Gene-regulation networks are the basis of the complicated but meticulous developmental course of cereal caryopses. In this work, the target genes of miR159, *Os01g59660*, *Os05g41166*, and *Os04g46384*, were predicted to encode MYB transcription factors. *Os01g59660* was confirmed to be *GAMYB* which mediates programmed cell death (PCD) in both the tapetum and the aleurone in rice (Kaneko *et al.* 2004, Tsuji *et al.* 2006, Aya *et al.* 2009, Liu *et al.* 2010). Several lines of evidence indicate that ethylene content is positively correlated with endosperm PCD but ethylene downregulates the miR159 level in cereals (Young *et al.* 1997, Liu *et al.* 2009). Together, the data suggest that the decrease of miR159 level at the metabolism stage is responsible for the release of *MYB* target genes probably involved in the endosperm PCD during the maturation progress of rice caryopses.

In this work, multiple *HAP2-like* genes encoding the components of CCAAT-box binding transcription factors were predicted as targets of miR169. Several miR169 family members have been implied to be induced by abscisic acid (ABA) in rice (Zhao *et al.* 2009). However, it is unknown whether miR169 and its *HAP2-like* target genes are regulated by ABA and are involved in the development progress of rice caryopses. Elevated ABA content is usually required not only for the drought response but also for seed maturation including the grain-filling and dormancy (Itoh *et al.* 2005, Gutierrez *et al.* 2007). The enhanced accumulation of miR169 and its target gene (*Os07g41720*) examined in this study suggests their contribution to the maturation progress of rice caryopsis probably through ABA signalling. In contrast to the expected negative correlation between miRNA and its potential targets, miR169 and *Os07g41720* exhibited a positive correlation in their expression patterns. Such positive correlation suggested a fine-tune regulation of the expression of *Os07g41720* by miR169 through a feedback mechanism (Nikovics *et al.* 2006, Cartolano *et al.* 2007).

The predicted target genes of miR159 and miR2001, *Os11g05540* and *Os04g42960* encode a putative GTPase-activating protein (GAP) and GPCR. Inte-restingly, we observed a positive expression correlation for miR159 - *Os11g05540* and a negative expression correlation for miR2001 - *Os04g42960* in developing caryopses. Thus GAP and GPCR, which are likely to be differently regulated by miR159 and miR2001, may be involved in the early morphogenesis events through signalling by certain G proteins.

In conclusion, we presented a microarray analysis to reveal the miRNA-mediated gene regulation during the rice caryopsis development. Although further experi-mental data are still needed for the rationalisation of miRNA involvement in gene regulation, the results suggest that the accumulation changes of several differential miRNAs are related to the progression from morphogenesis to metabolite accumulation and aid in understanding the functions of miRNAs and their target genes during the development of rice caryopses.

Supplementary data are available online:
Fig. S1
http://cls.bnu.edu.cn/portals/0/documents/SupplementaryFig.1.pdf
Table S1
http://cls.bnu.edu.cn/portals/0/documents/Liu_SupplementaryTable1.xls
Table S2
http://cls.bnu.edu.cn/portals/0/documents/Liu_SupplementaryTable2.xls

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