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Expression analysis of phytohormone-regulated microRNAs in rice, implying their regulation roles in plant hormone signaling

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Twenty-two conserved miRNAs were chosen to investigate the expression pattern in response to phytohormone treatments, in which the effects of five classic plant hormone stresses were surveyed in *Oryza sativa.* The results showed that 11 miRNAs were found to be dysregulated by one or more phytohormone treatments. The target genes of these miRNAs were validated in vivo and their expression profiling were revealed. We also analyzed the promoter regions of the 22 conserved miRNAs for phytohormone-responsive elements and the existence of the elements provided further evidences supporting our results. These findings enable us to further investigate the role of miRNAs in phytohormone signaling.
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1. Introduction

Plant growth and development involves with the integration of many environmental and endogenous signals that determine plant form together with the intrinsic genetic program. Fundamental to this process are several growth regulators which we collectively called the plant hormones or phytohormones [1]. For many years, phytohormones have been considered to fall into five classes: auxins; gibberellins (GAs); cytokinins; abscisic acid (ABA) and ethylene. More recently, a variety of additional classes such as jasmonic acid (JA) and brassinosteriods (BRs) have been added [1]. Each of these classes acts at low concentrations to regulate almost every aspect of plant growth and development to some degree [2]. Phytohormones not only act to control many aspects of plant growth and development but also response to the environment stresses [3]. Their signaling pathways can be effectively controlled by modulation of positive and negative regulators during plant growth and development [4]. Among these, small RNA was recently found to be a new growth regulator involved in plant hormone signaling. Recent reports indicated that the disturbance of small RNA pathways enhanced abscisic acid response in *Arabidopsis* [5] and silencing of RNA-directed RNA polymerase 1 (*RdR1*) makes *Nicotiana attenuata* highly susceptible to insect herbivores through reducing JA while enhancing ethylene levels [6]. These regulatory small RNAs can be classified into microRNA (miRNA) and short interfering RNA (siRNA) [7].

MicroRNAs (miRNAs) are single-stranded endogenous small RNAs of about 22nt in length and are processed from typical stem-loop precursors by the Dicer-like (DCL) family of enzymes in plants [8]. They have been identified in both animals and plants. Up to now, miRNAs have proven to be involved in many functional processes in plants by targeting mRNAs for cleavage or translational repression such as leaf development [9–11], shoot and root development [12–14], floral development [15,16] and stress responses [17,18]. Plant miRNAs have also been considered to regulate miRNA and siRNA biogenesis and function [15,19,20].

The first report linking miRNAs and hormone signaling was that the hyl1-1 mutant displayed impaired responses to auxin, ABA and cytokinin [21]. After this, gibberellic acid (GA) has been shown to modulate miR159 levels during another development [22], and auxin induction of miR164 to clear *NAC1* mRNA to reset auxin signaling [12]. Furthermore, miR160 has been shown to regulate expression of an auxin response transcription factor, *ARF17* [23] and miR159 is induced by ABA to cleave two MYB factors (*MYB33* and *MYB101*) mRNAs during Arabidopsis seed germination [17]. Finally, the predicted targets for miRNAs include several mRNAs which are involved in hormone responses, such as transport inhibitor response 1 (*TIR1*), a negative regulator in auxin signaling [17,24,25] and the latest research showed that *TCP* (*TEOSINTE BRANCHED/CYCLOIDEA/PCF*), the targets of mir319, control biosynthesis of the hormone jasmonic acid [26]. However, the roles of miRNAs in plant hormone signaling are still largely

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unexplored and up to now no miRNAs have been identified to be involved in cytokinin or ethylene signaling.

Thus, in this study, 22 conserved miRNAs were sought and used to analyze the expression under different phytohormone treatments in rice. Better understanding miRNA functions in rice development can help us to deal with abiotic stresses to improve rice yield.

2. Materials and methods

2.1. Plant materials and growth conditions

Mature seeds of Indica rice were used in the experiments. Seeds were soaked in distilled water for 2 days in room temperature. Then the seeds were embedded in gauze for another 1 day in room temperature for germination and were used in the following hormone experiments.

Germinated seeds were placed in gauze and transferred to salver for incubation in room temperature. After three days or longer, different concentrations of plant hormones were sprinkled on the leaves as water source and distilled water as the control. The different concentrations of phytohormones used in our experiment were: auxin: 0, 0.1, 1, 5 mg/l; GA: 0, 0.25, 2.5, 25 mg/l; cytokinin: 0, 0.5, 5, 50 mg/l; ABA: 0, 1, 10, 100 mg/l; ethylene: 0, 0.5, 5, 50 mg/l. Plant hormone solution was renewed every day. About 1-week-old rice seedlings were sprinkled with different concentrations of plant hormone solution for 4, 8 h and even longer until noticeable differences in height of young seedling were observed: ABA for 72 h; GA for 36 h; 6-BA, 2,4-D and ethylene for 48 h. The seedlings with different treatment time were collected for RNA extraction.

2.2. RNA isolation and northern blot analysis

Total cellular RNAs were extracted from seedling of Indica rice with different treatments using guanidine thiocyanate/phenol–chloroform method (Wang et al. [27]). Low-molecular-weight RNA was subsequently isolated by precipitation in PEG8000/NaCl as previously described [27].

Blot hybridization analysis was performed as described [27] with some modifications. Forty micrograms of low-molecular-weight RNA was run on denaturing 10% polyacrylamide gel, and then electrophoretically transferred to Hybond-N$^+$ membranes (Amersham Biosciences), followed by UV light irradiation for 4 min and baked at 80 °C for 50 min. The DNA probes complementary to small RNA sequences were end labeled with [γ-^{32}P]ATP. The membranes were pre-hybridized for at least 1 h and then hybridized overnight at a temperature of 37–42 °C. below the calculated dissociation temperature (Td). After being washed three times with washing buffer (2×SSC, 0.5% SDS) at 25–30 °C, filters were exposed to a phosphor screen and visualized by Typhoon 8600 imager (Amersham Biosciences). All probes used in our experiment were listed as follow: miR156b: 5′-GTGCTCACTCTCTTCTGTCA-3′; miR159a: 5′-CAGAGCTCCCTTCAATCCAAA-3′; miR160a: 5′-TGGCATACAGGGAGCCGGCA-3′; miR162a: 5′-CTGGATGCAGAGGTTTATCGA-3′; miR166a:5′-GGGGAATGAAGCCTGGTCCGA-3′; miR167f: 5′-CAGATCATGC TGGCAGCTTCA-3′; miR168a: 5′-GTCCCGATCTGCACCAAGCGA-3′; miR169l: 5′-CAGGCAAGTCATCCTTGGCTA-3′; miR171b: 5′-GATATTGGCACGGCTCAATCA-3′; miR172a: 5′-ATGCAGCATCATCAAGATTCT-3′; miR319a: 5′-GGGAGCACCCTTCAGTCCAA-3′; miR393b: 5′-AGATCAATGCGATCCCTTTGGA-3′; miR394: 5′-GGAGGTGGACAGAATGCCAA-3′; miR395d: 5′-GAGTTCCCCCAAACACTTCAC-3′; miR396c: 5′-AAGTTC AAGAAAGCTGTGGAA-3′; miR397a: 5′-CATCAACGCTGCACTCAATG A-3′; miR398a: 5′-AAGGGGTGACCTGAGAACACA-3′; miR399a: 5′-CAGGGCAATTCTCCTTTGGCA- 3′; miR413: 5′-GTGCAGAACAAGTGAAACTAG-3′; miR414: 5′-GGACGATGATG ATGGGATGA-3′; miR416: 5′-ACTTGAACAGTGTACGGACGAA CA-3′; miR418: 5′-CGTCATTTCATCATCACATTA-3′.

2.3. 5′ RACE

Total RNA was extracted from rice seedling. Poly(A)$^+$ mRNA was subsequently enriched using the Oligotex mRNA Kit (Qiagen). 5′ rapid amplification of cDNA ends (5′ RACE) was carried out with GeneRacer Kit (Invitrogen) as described [28]. Briefly, first round PCR was performed with GeneRacer 5′ primer and gene-specific primer, followed by second round PCR with Gene-Race 5′ nested primer and gene-specific nested primer. The PCR products were gel-purified, cloned and sequenced.

2.4. Clustalx software alignment

The whole set of Arabidopsis and rice mature miRNA sequences were extracted from the miRBase (Release 10.0, August 2007; http://microrna.sanger.ac.uk) and were used in clustal alignment. The alignments were limited to match at least 18nt and at most allowing 3nt for sequence variations between the two plant species.

2.5. PLACE prediction for cis-acting elements

About 2000bp sequence upstream of the 23 miRNAs pre-stem-loop were extracted from TIGR Rice Genome Annotation (http://www.tigr.org/tdb/e2k1/osa1/data_download.shtml) for *cis*-acting element analysis. The 2000bp sequence was, respectively, scanned by PLACE (http://www.dna.affrc.go.jp/PLACE/), a database of nucleotide sequence motifs found in PLAnt *cis*-acting regulatory DNA Elements.

2.6. Quantitative real-time polymerase chain reaction

Total RNA was reversely transcribed by using primescript™ RT regeant kit (Takara, Japan) to generate cDNA. Real-time PCR was carried out using the SYBR Premix Ex Taq™ (Takara, Japan) for detection of PCR products. The β-actin was chosen as a reference gene. The real-time PCR was performed according to manufacturer's instruction (Takara, Japan) and the resulting melting curves were visually inspected to ensure specificity of product detection. Quantification of the gene expression was done with comparative CT method. Experiments were performed in triplicate and the results were represented by their mean ± standard Derivation (S.D.).

3. Results

3.1. 22 miRNAs conserved in Arabidopsis thaliana (Arabidopsis) and Oryza sativa (rice) were chosen for expression analysis in response to phytohormones

It has been demonstrated that plant conserved miRNAs play fundamental roles in plant development and are regulated under diverse stress conditions [29]. In this study, the miRNAs conserved in Arabidopsis and rice were sought for expression analysis in response to phytohormone treatments. To identify miRNA homologs between Arabidopsis and rice, the whole set of Arabidopsis and rice mature miRNA sequences were extracted from the miRBase (Release 10.0, August 2007; http://microrna.sanger.ac.uk) and were used in multiple sequence alignment. The alignments were limited to match at least 18nt and at most allowing 3nt for sequence variations between these two species. At last 22 conserved miRNAs including 18 miRNA families were found (Table 1). Our

Table 1
22 miRNAs conserved in Arabidopsis and rice and their responses to five classes of phytohormone treatments.

miRNA	Auxin	GA	Cytokinin	ABA	Ethylene
miR156b	–	–	–	–	–
miR159a	–	–	–	–	↓
miR160a	–	–	–	–	–
miR162a	–	–	–	–	–
miR166a	–	–	–	–	–
miR167f	–	–	–	↓	–
miR168a	↓	–	–	–	–
miR169l	↑	–	–	↓	–
miR171b	–	–	–	–	–
miR172a	–	–	–	–	–
miR319a	–	↑	↓	↓	–
miR393b	–	–	–	–	–
miR394	–	–	–	–	↑
miR395d	–	–	–	–	–
miR396c	–	–	–	–	–
miR397b	–	–	–	–	–
miR398a	–	–	–	–	–
miR399a	–	–	–	–	–
miR413	–	–	–	–	–
miR414	–	–	–	–	–
miR416	–	–	–	–	–
miR418	–	–	–	–	–

(↑) Up-regulated in response to the treatment; (↓) down-regulated in response to the treatment and (–) no significant difference.

results were consistent with a recent report by Sunkar and Jagadeeswaran [29]. Of the 18 miRNA families, most are conserved in large number of diverse plant species except for 4 miRNAs (miR413, miR414, miR416 and miR418) only conserved between Arabidopsis and rice.

3.2. Identification and expression analysis of phytohormone-responsive miRNAs

To identify and investigate the expression of the 22 conserved miRNAs in rice, northern blot analysis was performed with low-molecular-weight RNAs isolated from young seedlings treated with five classes of plant hormones as described in Section 2. We screened time points for miRNAs beginning to response to different phytohormone treatments and found that the earlier time for miRNAs respond to auxin, GA, cytokinin and ethylene was at 8 h while for ABA treatment was at 4 h.

As indicated in Fig. 1, a set of miRNAs showed down-regulation while some miRNAs showed up-regulation when subjected to different classes of hormone treatments, i.e. miR167, miR319 were down-regulated by ABA while miR413 was up-regulated by this treatment. In response to ethylene miR159 was down-regulated while miR394 was firstly down-regulated and finally up-regulated; miR319 and miR166 were down-regulated by GA while miR159 was up-regulated in this involvement; miR319 and miR172 were down-regulated by 6-BA. From these results, we could find that miR319 and miR159 showed regulation simultaneity by more than one class of plant hormones. For instance, miR319 was simultaneously regulated by ABA, GA and 6-BA while miR159 was also simultaneously regulated by ethylene and GA. The responses of miR319 to ABA, miR159 to GA and miR168 to auxin in rice were consistent with previous studies in other species, e.g. miR319 was found to be down-regulated by ABA treatment in Arabidopsis [30], miR159 was upregulated by GA in Arabidopsis [22] and miR168 was involved in auxin signaling in nodule development in soybean roots [31]. Notably, most miRNAs expression pattern was the same for different time point treatments except that miR168 and miR169 were firstly down-regulated in the earlier treatment and then up-regulated at 48 h by auxin. The situation was the same for miR394 by ethylene, 11 of 22 miRNAs remained unaffected by these five classes of plant hormones (Table 1).

3.3. Predication and verification of potential targets of phytohormone-regulated microRNAs in rice

In order to understand more about the roles of miRNAs in phytohormone signaling, we sought to identify potential targets silenced by phytohormone-regulated microRNAs. An web server miRU, which based on penalty scoring system for bulges and mismatches in the target site, together with possible conservative

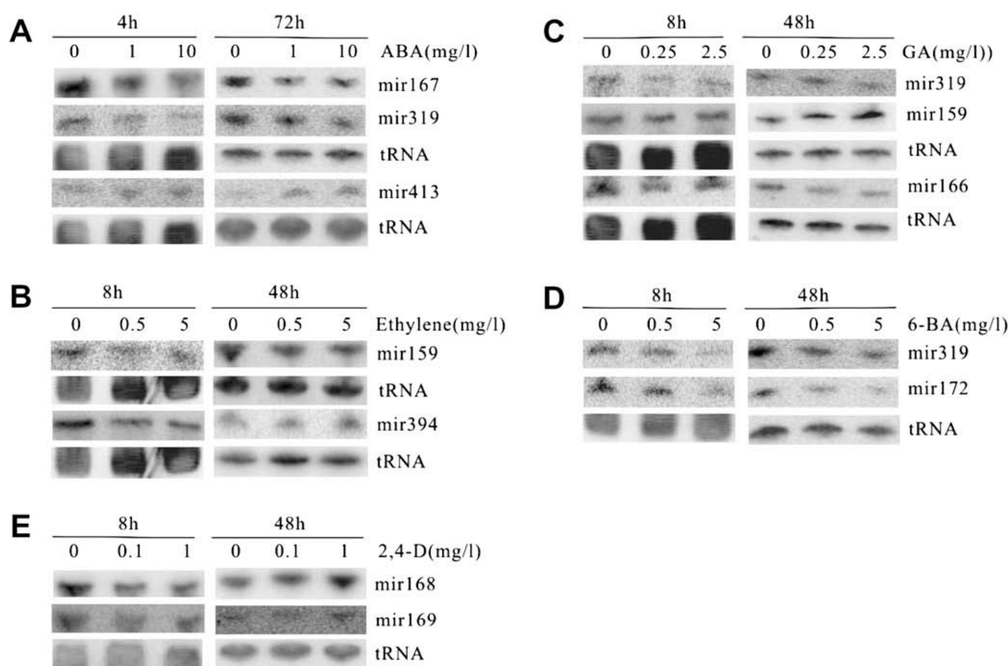

Fig. 1. Expression patterns of miRNAs in rice under different phytohormone treatments. Total RNAs were extracted from about one-week-old rice seedlings subjected to different treatments for 4 h, 8 h and even longer. Different concentrations of hormones were used. MiRNAs expression patterns were analyzed by northern blot using 40 μg of low-molecular-weight RNA; tRNA was loaded as control.

Table 2
Putative target genes of the 11 phytohormone-responsive miRNAs in rice.

miRNA	Target protein(s)	Predicted functions
miR159a	Os05g41166	Myb family transcription factor
	Os01g59660	Myb family transcription factor
	Os04g46384	DUO1
	Os03g47949	Ubiquitin–protein ligase E3C
	Os11g05540	GTPase activating protein 1
miR162a	Os03g02970	Dicer
miR167f	Os07g29820	NBS-LRR disease resistance protein
	Os04g57610	Auxin response factor 8
	Os09g39420	HIRA
miR168a	Os02g584920	PINHEAD protein
miR169g	Os02g53620	Nuclear transcription factor Y subunit A-3(NF-YA3)
	Os12g42400	Nuclear transcription factor Y subunit A-2(NF-YA2)
miR169l	Os02g53620	Nuclear transcription factor Y subunit A-3(NF-YA3)
	Os12g42400	Nuclear transcription factor Y subunit A-2(NF-YA2)
miR166a	Os05g03040	Floral homeotic protein APETALA2
miR172a	Os03g01890	Rolled leaf1
	Os03g43930	Class III HD-Zip protein 4
miR319a	×	×
miR394	×	×
miR413	Os04g30010	OsWAK45

×: No targets were predicted for mir319a and mir394.

targets in another genome [32], was applied for predicting plant miRNA target candidates in this study. All phytohormone-regulated microRNAs were submitted to miRU web server (http://bioinfo3.noble.org/miRU.htm) with default parameters and the target dataset was TIGR Rice Genome mRNA (OSA1 release 5, 01/23/2007). The number of mismatch was restricted to 5 or less including G: U pairs with no mismatch in the position between the 10th and 11th nt of the miRNA (data not shown). The results were shown in Table 2. To investigate whether some important predicted targets of the phytohormone-regulated microRNAs were authentic and were cleaved in vivo, we performed 5′ rapid amplification of cDNA ends (5′ RACE) analysis to confirm the predictions. MiRNA-directed cleavage products were found by PCR using specific primers to the target of interest.

As shown in Fig. 3, targets of miR168a, miR167f, miR169l, miR172a and miR166a were validated. No targets of miR394 and miR319 were predicted in rice and no targets were validated for miR413. As for miR159, the predicted targets in rice have been validated previously [33].

3.4. Real-time PCR analysis of expression profiling of the corresponding targets of phytohormone-regulated microRNAs

To further validate the regulation roles of miRNAs in phytohormone signaling, we also analyzed the expression profiling of corresponding targets of phytohormone-regulated microRNAs. Results showed that the expression levels of five in vivo validated targets, *AP2* (the target of miR166), *GAMYB* (the target of miR159), *HD-ZIP* (the target of miR172), *PINHEAD* (the target of miR168) and *HIRA* (the target of miR167) were just contrary to the expression levels of their corresponding miRNAs. As shown in Figs. 1 and 2, when miRNAs levels were down-regulated the levels of their corresponding targets were up-regulated and the negative expression patterns between miRNAs and their targets further validated the involvement of miRNAs in phytohormone signaling. However, the expression levels of *NF-YA* was exceptional, with the same expres-

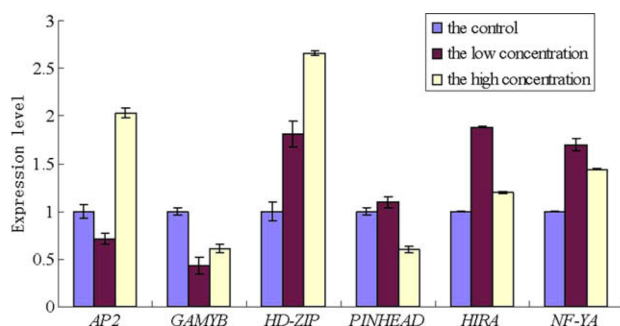

Fig. 2. Expression profiling analysis of the targets of phytohormone-responsive miRNAs under different hormone treatments. Data represents the mean vaules ± S.D. of three replicates, 'blue' represents the control; 'red' represents the low concentration of corresponding treatments and 'yellow' represents the high concentration of corresponding treatments.

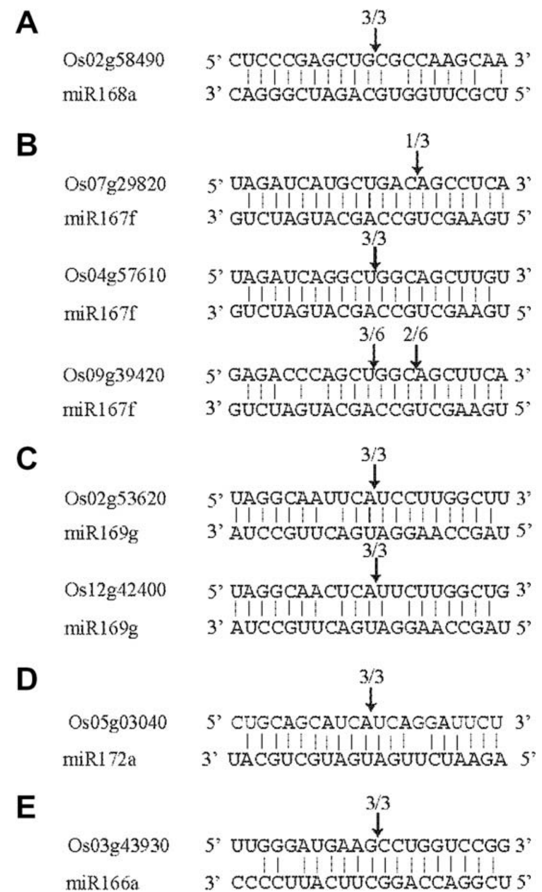

Fig. 3. Identification of miRNA-guided cleavage products. 5′ RACE was performed to map the cleavage sites of miRNA targets. The arrow in the diagram indicated the cleavage sites. The numbers refer to the frequency of clones. (A) Os02g58490, PINHEAD protein. (B) Os07g29820, NBS-LRR diaease resistance protein;Os04g57610, auxin response factor 8; Os09g39420,HIRA. (C) Os02g53620,-nuclear transcription factor Y subunit A-3; Os12g42400, nuclear transcription factor Y subunit A-2. (D) Os05g03040, floral homeotic protein APETALA2. (E) Os03g43930, class III HD-Zip protein 4.

Table 3
The phytohormone-related *cis*-acting elements analysis of 22 conserved miRNAs.

miRNA	Site name	Loc(-bp)
miR156b	GARE	1111
miR166a	GARE	637
miR171b	GARE	1787
miR319a	GARE	1794
miR393b	GARE	1743
miR397b	ABRE	1392
miR416	GARE	1978
	GARE	1054

ABRE: ABA-responsive element; GARE: gibberellin-responsive element.

sion pattern as its corresponding miRNA (mir169, Fig. 2), which was contrary to our expectation. This may be due to the large members of NF-Y subunit family and their combinatorial roles between each subunit.

3.5. Identification of potential and specific phytohormone-responsive elements in the promoters of the 22 conserved miRNAs

Phytohormones play important roles in plant development through regulating the expression of many genes transcriptionally, and the *cis*-acting elements have been shown to be involved in specific plant hormone-induced gene expressions. For example, gibberellin-responsive element (GARE) (TAACAG/AA) was necessary and sufficient to confer GA inducibility [34] and ABA-responsive element (ABRE) (Py-ACGTGGTC) functions as a *cis*-acting element and is involved in ABA-responsive gene expression [35]. To further elucidate the inducibility of these phytohormones, in this study, we investigated the potential hormone-responsive elements in the promoter regions of the 22 conserved miRNAs. About 2000bp sequence upstream of the 22 miRNAs pre-stem-loop were extracted from TIGR Rice Genome Annotation (http://www.tigr.org/tdb/e2k1/osa1/data_download.shtml) for *cis*-acting element analysis. Each of the 2000bp extracted sequence was, respectively, scanned by PLACE (http://www.dna.affrc.go.jp/PLACE/), a database of nucleotide sequence motifs found in PLAnt *cis*-acting regulatory DNA Elements [36]. Of the 22 miRNAs analyzed, 6 miRNAs have GAREs in their up-stream regions and one miRNA has ABRE (Table 3). The presence of GAREs or ABRE in the promoter regions of these miRNAs suggests that these miRNAs might be regulated by GA or ABA and are likely to play a role in hormone signaling pathways in rice. Among the 11 phytohormone-regulated miRNAs identified, only two miRNAs (miR166a and miR319a) were found GAREs in their promoter regions. The existence of GAREs at the promoter regions of miR166a and miR319a genes, combining with the evidences that these two miRNAs were responding to plant hormone treatments, suggested that both of the two miRNAs expression levels might be regulated directly by GA through GAREs. The results also showed that there exists phytohormone-responsive miRNAs without hormone related *cis*-acting elements while some miRNAs with *cis*-acting elements were not regulated by phytohormones, suggesting the complicated regulation network between miRNAs and phytohormone signaling. However, it is not excluded that these phytohormone-responsive miRNAs with *cis*-acting elements might be regulated by phytohormones indirectly. Further research is still needed to shed light on the complicated regulation network.

4. Discussion

MicroRNAs (miRNAs) are endogenous small non-coding regulatory RNAs that play important roles in plants by targeting mRNAs for cleavage or translational repression [8]. Though many recent reports propose that miRNAs regulate key components of hormone signaling pathways and further regulate hormone homeostasis and related developmental processes, the relationship between miRNA expressions and phytohormone responses remain to be explored. In this study, 22 conserved miRNAs were chosen to investigate the expression pattern in response to five classic phytohormone treatments and 11 miRNAs were found to be dysregulated by one or more of the five plant hormone treatments. By exploring

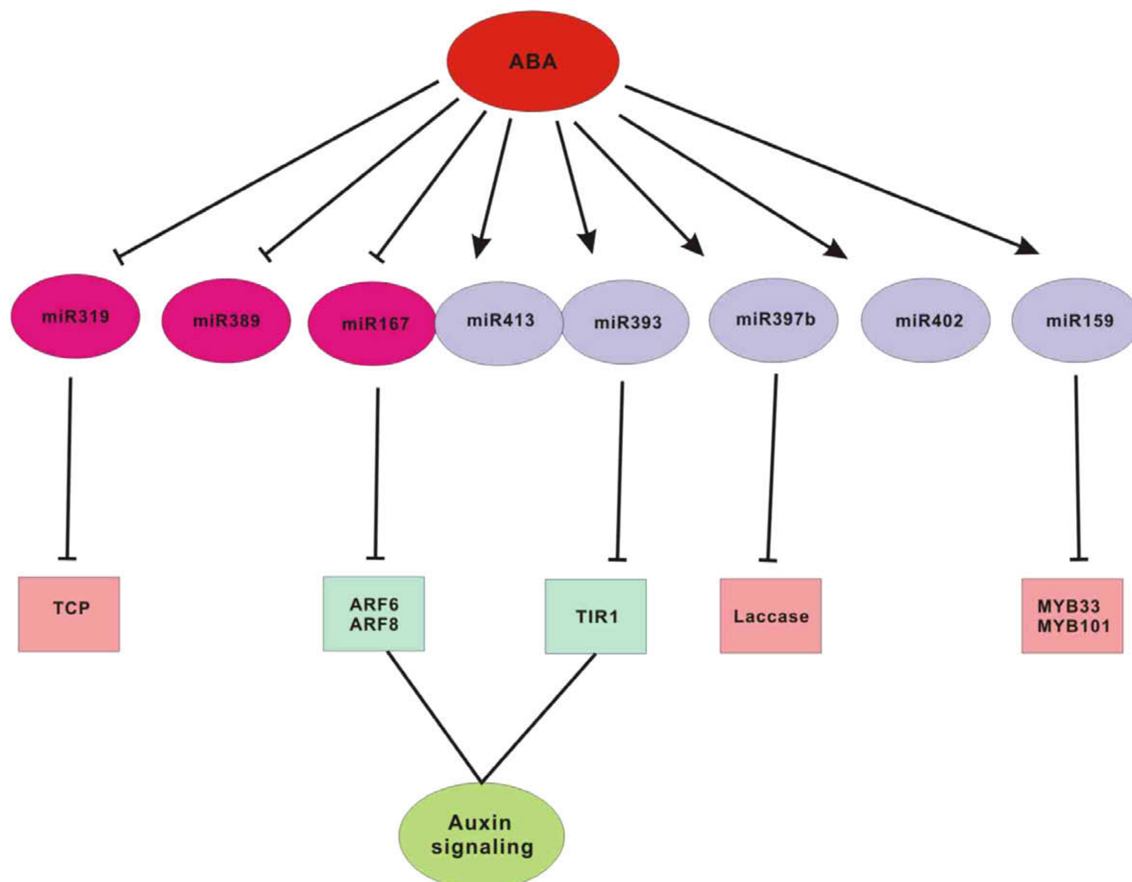

Fig. 4. Regulation of the interaction between miRNAs and ABA signaling. Arrows show simultaneous effect in the pathway; nail shape represents repression.

the correlations between the expression patterns of miRNAs and phytohormone signaling, we are able to further investigate the role of miRNAs in phytohormone signaling.

Up to now, no miRNAs have been shown to be involved in ethylene signaling and cytokinin signaling. Notably, as shown in Fig. 1B, miR159 expression level decreased while miR394 expression level firstly decreased and finally slightly increased when subjected to ethylene treatment in rice and these were the first two miRNAs that were found to be regulated by ethylene. We also found that miR172 and miR319 were down-regulated when subjected to 6-BA treatment (Fig. 1D). They are all suggested to be involved in regulating phytohormone signaling. Further examination of the regulatory interactions between these miRNAs and their target genes will reveal insights into the complex interplay between phytohormone signaling and miRNAs in regulating plant development.

Abscisic acid (ABA) and gibberellin (GA) are another two phytohormones that play pivotal roles in plant development. At present, several miRNAs have already been verified to be involved in ABA signaling or GA signaling. Here, we found three other miRNAs (miR167 and miR413) were regulated by ABA (Fig. 1A), suggesting that they may be involved in stress-responsive gene expression and stress adaptation because ABA is a plant hormone which protects plant from a variety of environmental stress [37]. Among these three miRNAs, miR167 was obviously down-regulated by ABA and miR162 was just slightly down-regulated whereas miR413 was up-regulated by ABA. A schematic relationship between all known miRNA and ABA signaling was summarized in Fig. 4. Additionally, another two miRNAs (miR166 and miR319) were down-regulated by GA (Fig. 1C) and the promoter regions analysis of miR166a and miR319a showed that they all have one GARE. This suggests that miR166 and miR319 might be regulated directly by GA through GARE and further suggested their regulation roles in GA signaling.

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