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Four *AUXIN RESPONSE FACTORs* downregulated by microRNA167 are associated with growth and development in *Oryza sativa*

Hai Liu[A], *Shenghua Jia*[A], *Defeng Shen*[A], *Jin Liu*[A], *Jie Li*[A], *Heping Zhao*[A], *Shengcheng Han*[A,B] *and Yingdian Wang*[A,B]

[A]Beijing Key Laboratory of Gene Resources and Molecular Development, College of Life Sciences, Beijing Normal University, 19, XinJieKouWai Avenue, Beijing 100875, China.
[B]Corresponding author. Email: gdbnu@bnu.edu.cn

Abstract. MicroRNA167 (miR167), as a conserved miRNA, has been implicated in auxin signalling by regulating the expression of certain auxin response factor (ARF) genes to determine the plant developmental process. Among the 10 *MIR167* genes of rice, the precursor structures derived from *MIR167a*, *MIR167b* and *MIR167c* produce miR167 with high efficiency. To explore the biological function of miR167 in rice, four of its predicted target genes, *OsARF6*, *OsARF12*, *OsARF17* and *OsARF25*, were identified *in vivo*. Although the expression levels of miR167 and its target *OsARFs* did not show an obvious negative correlation, the enhanced miR167 level in transgenic rice overexpressing miR167 resulted in a substantial decrease in mRNA levels of the four *OsARF* genes. Moreover, the transgenic rice plants were small in stature with remarkably reduced tiller number. These results suggest that miR167 is important for the appropriate expression of at least four *OsARFs*, which mediate the auxin response, to contribute to the normal growth and development of rice.

Additional keywords: auxin response factor, auxin signal, microRNA167, rice.

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Introduction

MicroRNAs (miRNAs) are small non-coding RNAs endogenously generated from stem-loop precursor transcripts (Reinhart *et al*. 2002; Carrington and Ambros 2003; Bartel 2004). In plants, miRNAs play a crucial role by negatively regulating the expression of target genes associated with a wide range of biological processes, including growth, development, nutrient metabolism and stress responses (Mallory *et al*. 2004; Navarro *et al*. 2006; Nikovics *et al*. 2006; Reyes and Chua 2007; Schwarz *et al*. 2008; Larue *et al*. 2009). To date, thousands of plant miRNAs have been identified and deposited in the miRNA database miRBase (Kozomara and Griffiths-Jones 2011). Many plant miRNAs are conserved and regulate homologous mRNA targets, mostly encoding transcription factors or other regulatory proteins (Jones-Rhoades *et al*. 2006). Moreover, many conserved miRNAs in angiosperms (e.g. miR156, miR160, miR166, miR167, miR169) are usually generated from multiple genomic loci, suggesting that they are parts of ancient and conserved regulatory modules with considerable functional significance (Axtell and Bowman 2008).

The auxin signal plays an indispensable role in various developmental processes and is normally initiated or mediated by the auxin response factor (ARF) family (Teale *et al*. 2006; Guilfoyle and Hagen 2007). Recently, the expression of some *ARF* genes has been demonstrated to be regulated by several conserved miRNAs (Meng *et al*. 2010*b*). In root cap formation and development of *Arabidopsis*, miR160 is essential to the regulation of the expression of *ARF10*, *ARF16* and *ARF17* (Mallory *et al*. 2005; Wang *et al*. 2005) and miR167 regulates the expression of *ARF6* and *ARF8* to affect female and male fertility and adventitious rooting (Ru *et al*. 2006; Wu *et al*. 2006; Gutierrez *et al*. 2009). In addition, miR390 is responsible for the biogenesis of tasiRNA-ARF, which may then affect lateral root development through modulation of the expression of *ARF2*, *ARF3* and *ARF4* in *Arabidopsis* (Allen *et al*. 2005; Williams *et al*. 2005; Yoon *et al*. 2010). These results suggest that several miRNAs are critical to mediate auxin signalling by restraining certain ARFs that control plant growth and development.

Rice is a model monocotyledonous crop plant with a fully sequenced genome. Recent studies have identified numerous miRNAs accumulating in different tissues or responding to different hormones or stresses in rice (Sunkar *et al*. 2005; Zhao *et al*. 2007; Zhu *et al*. 2008; Huang *et al*. 2009; Liu *et al*. 2009; Xue *et al*. 2009; Li *et al*. 2011). Moreover, the potential roles of several miRNAs have been proposed to be associated with auxin signalling in rice (Meng *et al*. 2010*b*). Among the 25 *OsARF* genes of rice (Wang *et al*. 2007), *OsARF6* and *OsARF12* have been predicted to be the targets of miR167 (Rhoades *et al*. 2002) and miR167 has been proposed to modulate cellular free auxin level through the miR167–*OsARF12*–*OsGH3*

pathway (Yang *et al*. 2006). Moreover, the regulation of *OsARF12* by miR167 is also important for root architecture (Meng *et al*. 2009; Qi *et al*. 2012). Nevertheless, the physiological functions of miR167 in the growth and development of rice are still not well understood.

In this study, we tested miR167 yield efficiencies of the stem-loop precursors derived from 10 *MIR167* genomic loci. The target genes of miR167 were predicted and validated *in vivo*. The expression of miR167 and its target *OsARFs* were also examined in rice. Furthermore, we generated transgenic rice overexpressing miR167. The ectopic accumulation of miR167 in rice significantly repressed expression of its target *OsARFs* and caused distinct growth phenotypes and developmental defects. This study broadens our understanding with respect to the regulatory roles of miR167 on OsARF transcription factors during rice growth and development.

Materials and methods

Plant materials and growth conditions

Rice (*Oryza sativa* L. ssp. *japonica* cv. Nipponbare) seeds were germinated in breeding trays at the beginning of each summer growing season. Four-leaf-stage seedlings were transplanted in a paddy field of the Experimental Station of Beijing Normal University, with basal dressing applied at a rate of 6.50 g N m^{-2}, 13.65 g P_2O_5 m^{-2} and 9.75 g K_2O m^{-2} (Zhou *et al*. 2010). Transgenic plants were grown in a greenhouse under the daily period of 14 h light at 28°C and 10 h dark at 20°C. Tobacco plants (*Nicotiana tabacum* L.) were germinated and grown in soil at 25°C in long-day conditions (16 h light/8 h dark) with white fluorescent lights (300 μmol m^{-2} s^{-1}) and RH of 65% in a growth chamber.

Samples from 3-week-old rice seedlings, adult leaf blades, mature flowers and developing caryopses after fertilisation were obtained and immediately frozen in liquid nitrogen for later RNA isolation. Six-week-old leaves of tobacco were used for the agroinfiltration experiment.

RNA isolation and miRNA gel blotting

Total RNAs were extracted from different rice samples and *Agrobacterium*-infiltrated tobacco leaves using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

For the miRNA gel blot analysis, total RNA was resolved on a urea-containing 15% polyacrylamide gel (PAGE), transferred to a Hybond-N^+ membrane (Amersham, Arlington Heights, IL, USA) and hybridised with a locked nucleic acid-modified oligonucleotide with a 3′-digoxigenin (DIG) label as the miR167 probe. Blots were detected using the enzyme-catalysed colour reaction (Válóczi *et al*. 2004; Ramkissoon *et al*. 2006). Then blots were stripped by washing with 1% SDS at 65°C and the membrane was reprobed for U6 snRNA with a 3′-DIG-labelled DNA oligonucleotide probe. The miR167 and U6 snRNA probes were commercially synthesised and HPLC-purified (TaKaRa). Information on all of the primers and probes used in this work is listed in Table S1, available as Supplementary Material to this paper.

Plasmid construction

DNA fragments containing the stem-loop precursors of rice *MIR167a* (MI0000676), *MIR167b* (MI0000677), *MIR167c* (MI0000678), *MIR167d* (MI0001109), *MIR167e* (MI0001110), *MIR167f* (MI0001111), *MIR167g* (MI0001112), *MIR167h* (MI0001113), *MIR167i* (MI0001114) and *MIR167j* (MI0001156) were amplified by PCR) from genomic DNA of rice. The open reading frames (ORF) of *OsARF6* (Os02 g0164900), *OsARF12* (Os04 g0671900), *OsARF17* (Os06 g0677800) and *OsARF25* (Os12 g0613700) were cloned from first-strand cDNA. A c-myc tag was introduced upstream of each *OsARF* ORF by PCR to produce the myc-tagged *OsARF* (*myc-ARF*). All of the products were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and named pT-MIR167 and pT-myc-ARF. Then the pT-myc-ARF plasmids were used as templates to construct each cleavage-resistant version of the myc-tagged *OsARF* (*myc-mARF*). Site-directed mutagenesis within the miR167 complementary region of each *OsARF* mRNA was performed with the QuickChange Multi Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). All of the mutation-generated products were cloned into the pGEM-T Easy vector (Promega) and designated pT-myc-mARF.

All of the plasmids of pT-MIR167, pT-myc-ARF and pT-myc-mARF were subcloned into the binary vector pCAMBIA1300 between the CaMV 35S promoter and the NOS 3′ poly (A) addition signal to generate *35S*::*MIR167a*, *35S*::*MIR167b*, *35S*::*MIR167c*, *35S*::*MIR167d*, *35S*::*MIR167e*, *35S*::*MIR167f*, *35S*::*MIR167g*, *35S*::*MIR167h*, *35S*::*MIR167i*, *35S*::*MIR167j*, *35S*::*myc-ARF6*, *35S*::*myc-mARF6*, *35S*::*myc-ARF12*, *35S*::*myc-mARF12*, *35S*::*myc-ARF17*, *35S*::*myc-mARF17*, *35S*::*myc-ARF25* and *35S*::*myc-mARF25*.

Agrobacterium tumefaciens *infiltration assay in tobacco*

The above binary constructs were introduced into the EHA105 strain of *Agrobacterium tumefaciens*. The *Agrobacterium* cultures induced with acetosyringone were injected into the abaxial surfaces of tobacco leaves as previously described (English *et al*. 1997). For the single vector infiltration, *Agrobacterium* culture was resuspended at OD_{600} = 0.8. For the co-infiltration experiments, the cultures containing *35S*::*MIR167b* (OD_{600} = 0.8) and each of the *35S*::*myc-ARFs* or *35S*::*myc-mARFs* (OD_{600} = 0.2) were mixed equally before infiltration. Tobacco leaves were harvested within 72 h after infiltration, then ground in liquid nitrogen for RNA isolation and protein extraction.

The expression level of miRNA in tobacco leaves was examined by RNA gel blotting; the accumulation for each *OsARF* transcript was determined by RT–PCR. DNA contaminants were removed from total RNA by digestion with RQ1 RNase-free DNase I (Promega). The DNA-eliminated RNA was reverse-transcribed with the SuperScript II Reverse Transcriptase (Invitrogen) and the oligo(dT) primer and then PCR was performed using gene-specific primers flanking the predicted cleavage site. The mRNA accumulation of tobacco *Elongation Factor1-α* (*EF-1α*) was used as an internal control (Schmidt and Delaney 2010). For protein immunoblotting, total protein was separated on 12% SDS–PAGE, transferred to the Immobilon-P membrane (Millipore, Bedford, MA, USA) and

immune-blotted with an anti-myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for each myc-tagged OsARF protein.

5′ RACE

5′ RACE was used to map the cleavage site within the mRNA sequence of each *OsARF*, as previously described (Llave *et al*. 2002). The poly(A)-tailed mRNA was purified from the total RNA of rice flowers before heading using the Oligotex Kit (Qiagen, Chatsworth, CA, USA). RNA ligase-mediated 5′ RACE was conducted with the GeneRacer Kit according to the manufacturer's instructions (Invitrogen). Gene-specific primers were designed and used for each RACE. The nested PCR product from each RACE reaction was gel-purified and cloned into the pGEM-T Easy vector (Promega) and at least 14 independent clones were sequenced.

Quantitative RT–PCR

The DNA-eliminated total RNA was reverse-transcribed with the oligo(dT) primer. Quantitative PCR was conducted with Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) on an ABI 7500HT system (Applied Biosystems). The relative amount of each *OsARF* was normalised to the *Ubiquitin5* (*UBQ5*) of rice (Jain *et al*. 2006).

Genetic transformation in rice

The genomic sequence of *MIR167b* in pT-MIR167b was subcloned into the binary vector pTCK303 driven by the maize *Ubi1* promoter (Wang *et al*. 2004). The constructed *Ubi*::*MIR167b* and the empty vector were introduced into *Agrobacterium* strain EHA105 and then transformed into rice embryonic calli as previously described (Ge *et al*. 2004). Transgenic calli were selected on half-strength Murashige and Skoog (1/2 MS) medium containing 50 mg L^{-1} hygromycin (Sigma, St Louis, MO, USA). Then the hygromycin-resistant transgenic seedlings were transplanted into soil and grown in the greenhouse.

Results

Efficiency of MIR167a, MIR167b *and* MIR167c *for miR167 yield*

According to the miRBase miRNA database (Kozomara and Griffiths-Jones 2011), the miR167 family of rice is encoded by 10 loci, *MIR167a–j*, which harbour different stem-loop precursors giving rise to the mature sequences of miR167. However, there is only one nucleotide difference in the 3′-end between miR167a–c and miR167d–j (Fig. 1*a*). To test whether the predicted stem-loop precursors derived from these *MIR167* genes could effectively produce mature miR167, the *Agrobacterium*-mediated infiltration assay was performed to express the genomic sequences containing the stem-loop region of each *MIR167* gene driven by the *Cauliflower mosaic virus* (CaMV) 35S promoter (*35S*::*MIR167a-j*) in tobacco leaves. Of the 10 rice *MIR167* genes, *MIR167a*, *MIR167b* and *MIR167c* produced much higher levels of miR167 (Fig. 1*b*). This suggests that the 10 genes have different efficiencies in miR167 production and that *MIR167a–c* is likely to play primary roles in rice.

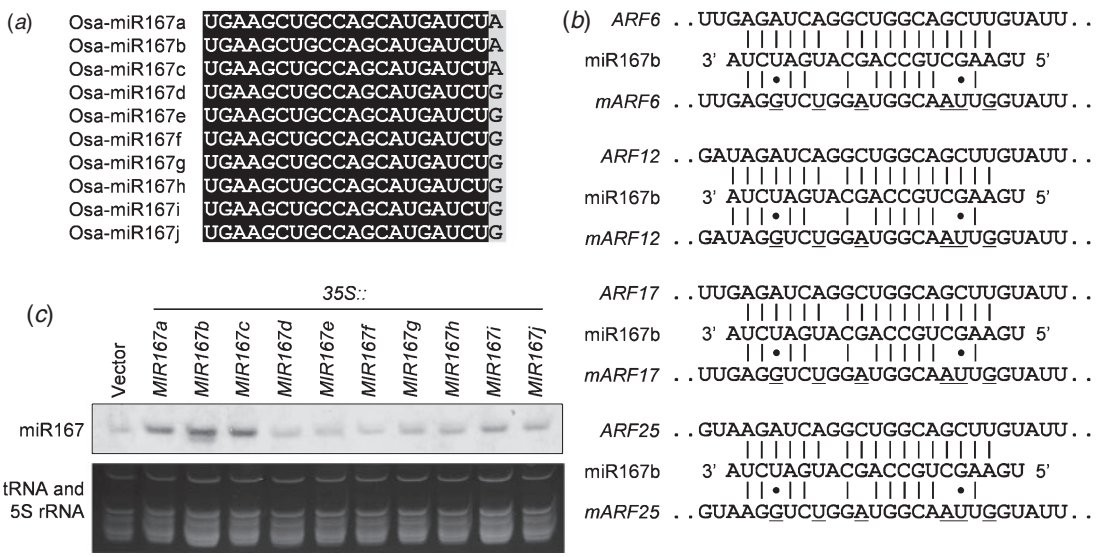

Fig. 1. Sequence and expression features of the miR167 family in rice and alignments of miR167 and *OsARFs*. (*a*) Alignment of the miR167 family members. (*b*) Ten *MIR167* genomic sequences containing the stem-loop structures were expressed behind the CaMV 35S promoter in tobacco leaves. The level of miR167 produced by each *35S*::*MIR167* was detected using RNA gel blot analysis. The ethidium bromide staining of small RNAs was used as loading controls. (*c*) Six silent mutations, which do not change the amino acid sequences, were introduced into the miR167-binding region of wild-type *OsARF6*, *OsARF12*, *OsARF17*, or *OsARF25* to produce miR167-resistant types of *OsARF* (i.e. *mARF6*, *mARF12*, *mARF17* and *mARF25* respectively). There were 3–4 mismatches between miR167 and the four wild-type *OsARFs*, whereas there were 9–10 mismatches between miR167 and the four mutation-modified *OsARFs*. The mismatched nucleotides that were artificially introduced are underlined and the predicted G-U base pairing is shown with dots.

Identification of the targets of miR167 in rice

For the purpose of investigating the mechanism of miR167 function, the potential target genes of miR167 were screened through the miRU web program (Zhang 2005). Nine genes were predicted to be the target candidates of miR167 (Table S2). Among them, four *OsARF* genes, *OsARF6*, *OsARF12*, *OsARF17* and *OsARF25*, exhibited high sequence complementarity to miR167 in almost the same manner (Table 1; Fig. 1*c*). We then focussed our attention on these four *OsARF* genes.

To obtain direct evidence that miR167 triggers the mRNA cleavage of these four genes, *Agrobacterium*-mediated co-expression was performed in tobacco leaves. A series of constructs were generated for the overexpression of each *OsARF* (*35S*::*myc-ARF6*, *35S*::*myc-ARF12*, *35S*::*myc-ARF17* and *35S*::*myc-ARF25*) and its cleavage-resistant form (*35S*::*myc-mARF6*, *35S*::*myc-mARF12*, *35S*::*myc-mARF17* and *35S*::*myc-mARF25*). The miR167 complementary region of each *myc-mARF* mRNA was disrupted by the introduction of six translationally silent mutations, which were expected to be able to disrupt miR167-specific binding (Fig. 1*c*). Then each of the *35S*::*myc-ARF*s or *35S*::*myc-mARF*s was expressed alone or co-expressed with *35S*::*MIR167b* in tobacco leaves. The co-expression of each *35S*::*myc-ARF* and *35S*::*MIR167b* caused a substantial decrease in transcript level of the respective *myc-ARF*, whereas the co-expression of each *35S*::*myc-mARF* and *35S*::*MIR167b* did not (Fig. 2). Consequently, protein accumulation of each myc-ARF could hardly be detected after the co-expression of the respective *35S*::*myc-ARF* and *35S*::*MIR167b*, whereas it was still intensely detected when co-expressing *35S*::*myc-mARF* with *35S*::*MIR167b* (Fig. 2).

To further prove that the four *OsARF* transcripts were cleaved by miR167 *in vivo*, 5′ rapid amplification of cDNA ends (5′ RACE) experiments were conducted to map the cleavage sites. The 5′ sequence of each *OsARF* cleavage product was consistent with the expected miR167 cleavage site (Fig. 3). These results provide direct evidence that miR167 triggers the cleavage of *OsARF6*, *OsARF12*, *OsARF17* and *OsARF25* mRNAs by binding to the complementary region.

Accumulation of miR167 and its target OsARFs *in rice*

For understanding the regulatory role of miR167 in relation to its target *OsARFs* during the growth and development of rice, we examined the expression status of miR167 and the four *OsARFs* in different tissues using RNA gel blot analysis and quantitative reverse transcription-polymerase chain reaction (RT–PCR) respectively. There was relatively specific accumulation of miR167 in the seedlings and flowers of rice (Fig. 4*a*). However, the mRNA level of each target *OsARF* did not exceed a 3-fold change among the examined tissues and its expression pattern was hardly correlated with that of miR167 (Fig. 4*b*). These results indicate that miR167 may mainly exert its effects during the development of seedlings and flowers, whereas the expression of the four *OsARFs* may only be repressed or restricted in more specific regions where miR167 intensely accumulates.

Table 1. Putative target genes of miR167 in rice based on miRU prediction

The sequence of miR167b was inputted into the miRU web program for target prediction (http://bioinfo3.noble.org/miRNA/miRU.htm, accessed August 2008). 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, 4). The *Arabidopsis* miR167 and mRNA dataset (TIGR Ath1 5) were used for homology analysis to reduce false positives

Predicted targets	Predicted functions	Score	Mismatch
Os02 g0164900	Auxin response factor 6	3.5	4
Os04 g0671900	Auxin response factor 12	2.5	3
Os06 g0677800	Auxin response factor 17	3.5	4
Os12 g0613700	Auxin response factor 25	3.5	4

Growth and development defects in transgenic rice overexpressing miR167

Further elucidation on the biological function of miR167 during rice growth and development was made by generating transgenic rice expressing the genomic sequence of *MIR167b* controlled by the maize *Ubiquitin1* (*Ubi1*) promoter (*Ubi*::*MIR167b*). Transgenic lines with an empty vector served as a negative control. Compared with the control lines, more than half of the *Ubi*::*MIR167b* lines displayed remarkable phenotypes, with a shorter stature and fewer tillers during vegetative growth (Fig. 5*a*, *c*; Table 2). RNA gel blot hybridisation was performed and a much higher accumulation of miR167 was detected in *Ubi*::*MIR167b* lines compared with the control lines (Fig. 5*e*). Simultaneously, quantitative RT–PCR showed that the transcript levels of *OsARF6*, *OsARF12*, *OsARF17* and *OsARF25* were all markedly reduced in the *Ubi*::*MIR167b* lines compared with the control lines (Fig. 5*f*). In addition, the transcript level of *OsARF9*, an *OsARF* unrelated to miR167-mediated regulation, did not change in the *Ubi*::*MIR167b* lines (see Fig. S1, available as Supplementary Material to this paper).

During the reproductive stage, the architecture of the *Ubi*::*MIR167b* flowers were almost identical to those of the control lines, whereas the panicles of the *Ubi*::*MIR167b* lines were shorter and had consequently fewer spikelets than those of the control lines (Fig. 5*b*, *d*; Table 2). However, fertility was less affected, as most of the spikelets turned into seeds in both lines at the time of maturation, even though *Ubi*::*MIR167b* produced slightly fewer seeds (Table 2). These results suggest that the phenotypic characteristics of the *Ubi*::*MIR167b* rice may partially, if not all, result from a defective auxin response due to impaired function of the four OsARFs and that miR167 is an important regulator for normal growth and development in rice.

Discussion

Although many plant miRNAs are transcribed by multiple loci among the angiosperms, their evolutionary features and the differences between each member of a miRNA family are little understood. Among the four *MIR167* genes in *Arabidopsis*, only *MIR167a* produces a high level of miR167 (Wu *et al*. 2006). Although there are 10 *MIR167* genes in the rice genome, our work demonstrated that *MIR167a*, *MIR167b* and *MIR167c* caused most miR167 production, suggesting that the stem-loop structures derived from these three genes might be more efficiently recognised or processed by Dicer-like1 (DCL1) to produce

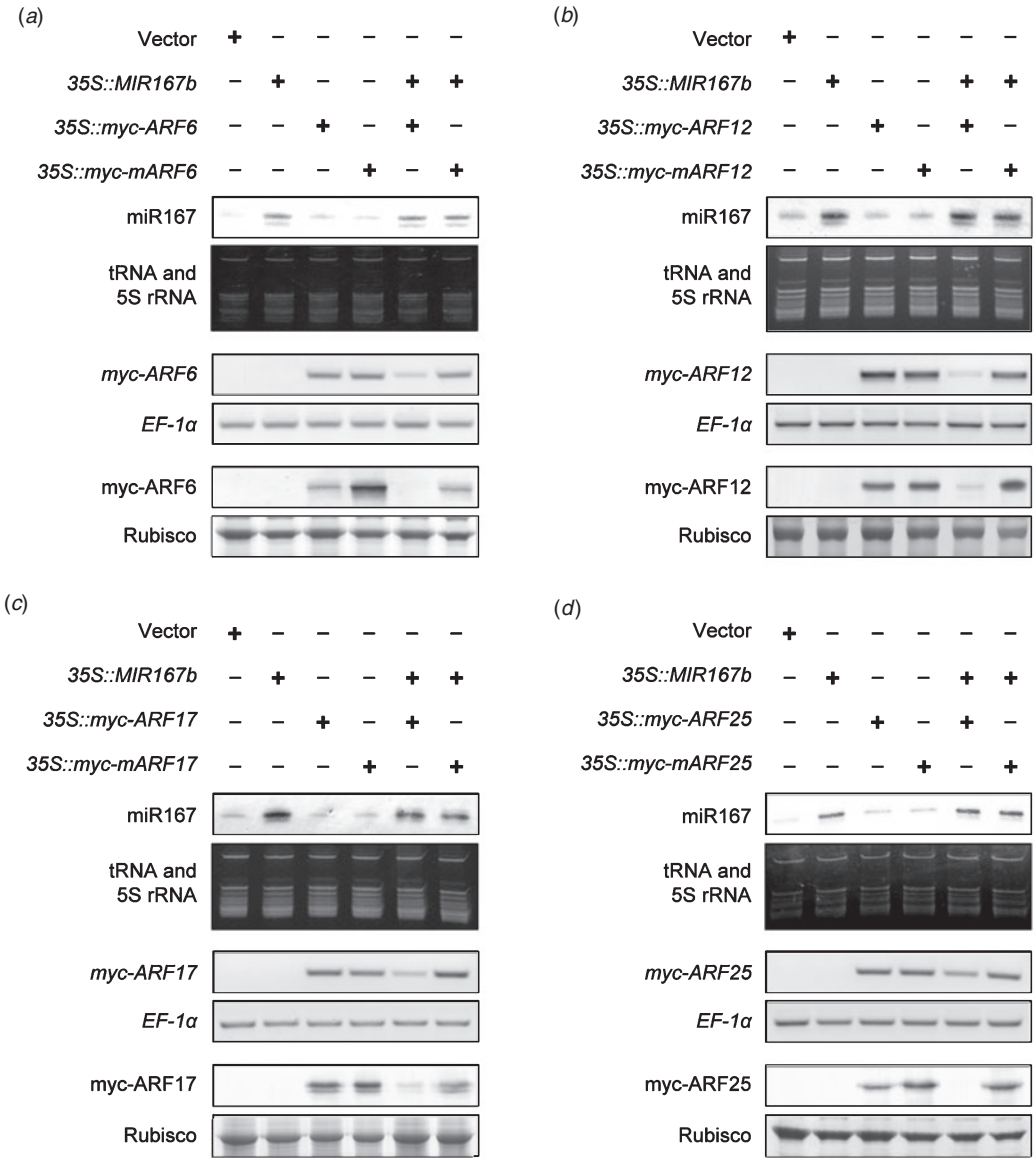

Fig. 2. Verification of four *OsARF* mRNAs as targets of miR167 by agroinfiltration assays. The effects of miR167 on the mRNA and protein stability of *myc-ARF6* and *myc-mARF6* (*a*), *myc-ARF12* and *myc-mARF12* (*b*), *myc-ARF17* and *myc-mARF17* (*c*) and *myc-ARF25* and *myc-mARF25* (*d*) are shown. The construct used for single or co-infiltration is indicated above each lane. The expression level of miR167 was examined by gel blot analysis from single or co-infiltrated tobacco leaves collected 72 h after infiltration (upper panels). Accumulation of the respective *myc-ARF* and *myc-mARF* transcripts was determined by RT–PCR (middle panels) and their protein abundance was detected by western blot (lower panels). The ethidium bromide staining of small RNAs and *EF-1α* were used as loading controls for miRNA hybridisation and RT–PCR respectively. Coomassie blue staining of the Rubisco band was used as a loading control for western blot.

mature miR167 than those in the other seven genes and that *MIR167a–c* may have been the primary functional *MIR167* genes during the evolution of miR167 in rice.

Previous studies have predicted that miR167 regulates *OsARF6* and *OsARF12* in rice (Rhoades *et al*. 2002). Through classical approaches for the prediction and validation of miRNA targets (Jones-Rhoades *et al*. 2006), we confirmed that not only *OsARF6* and *OsARF12* but also *OsARF17* and *OsARF25* are *in vivo* targets of miR167 in rice. Coincidently, phylogenetic analysis of 25 OsARFs of rice and 23 AtARFs of *Arabidopsis* revealed that OsARF6, OsARF12, OsARF17 and OsARF25 have the highest conservation with AtARF6 and AtARF8 (Wang *et al*. 2007), suggesting that miR167 function may have emerged before the divergence of monocots and eudicots, which happened ~100–115 million years ago (Chaw *et al*. 2004).

Many plant miRNAs have spatial or temporal expression patterns in different tissues or developmental stages and some of them are responsive to certain phytohormones or environmental conditions (Jones-Rhoades *et al*. 2006; Válóczi *et al*. 2006; Jung *et al*. 2009). Previous studies have indicated

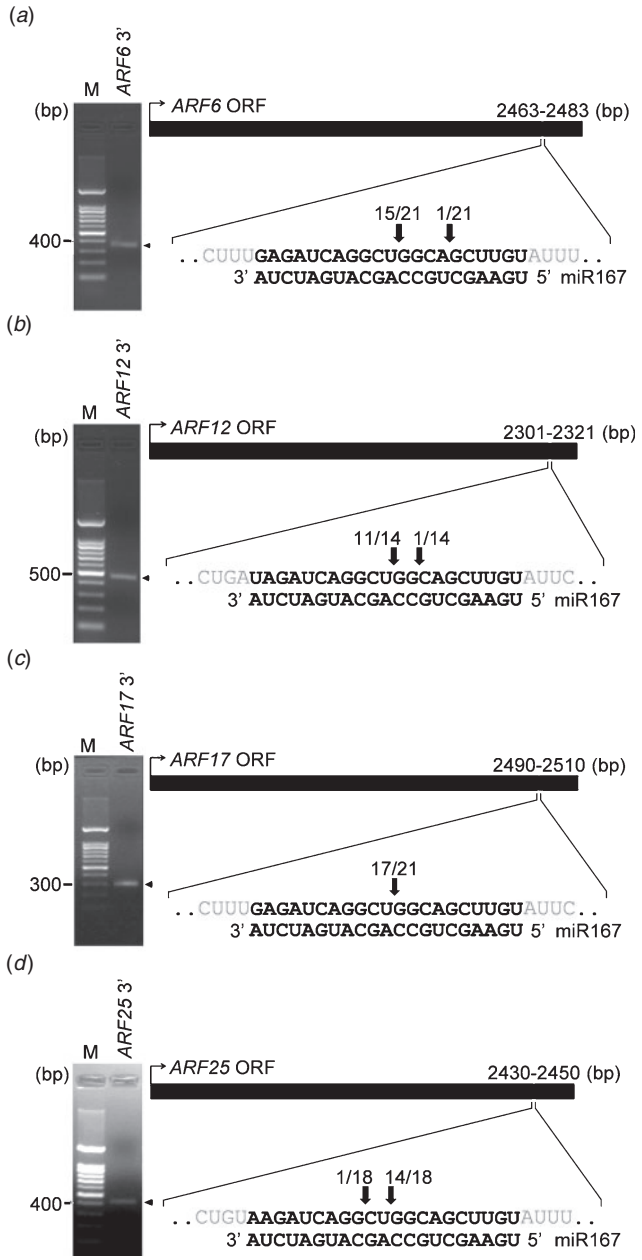

Fig. 3. Mapping of miR167 cleavage sites in four *OsARF* mRNAs determined by RNA ligase-mediated 5′ RACE. The thick black line represents the ORF of *OsARF6* (*a*), *OsARF12* (*b*), *OsARF17* (*c*) and *OsARF25* (*d*). The putative miRNA interaction site is shown as a grey box, with the nucleotide position within the ORF indicated. The sequence of the region complementary to miR167 is shown in the expanded diagram (right). Each gel-purified nested PCR product (arrowheads) is indicated on the ethidium bromide-stained agarose gel (left). The frequency of 5′ RACE clones corresponding to each cleavage site (arrows) is shown as a fraction, with the number of clones sequenced in the denominator (right). Lane M represents a 100-bp DNA ladder.

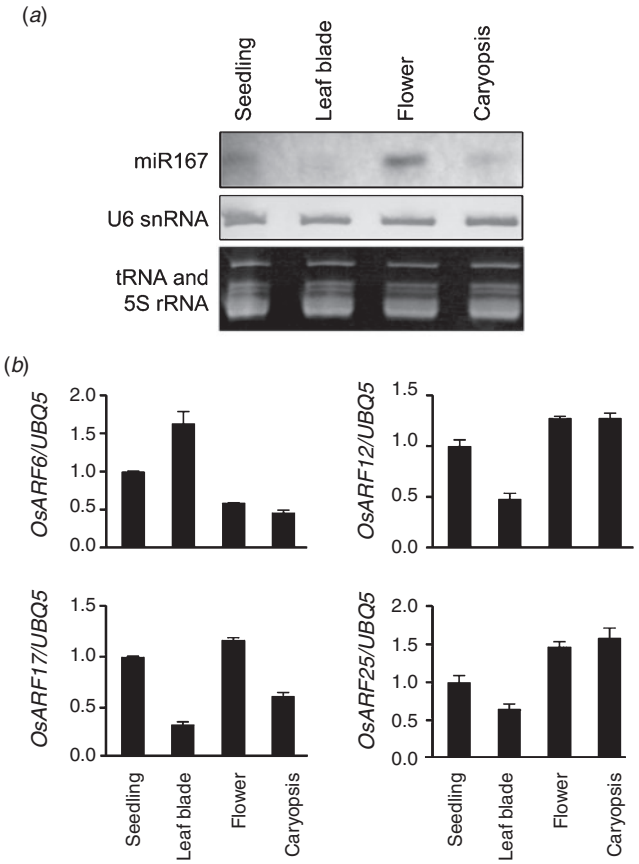

Fig. 4. Expression of miR167 and four *OsARFs* in rice. (*a*) The expression of miR167 in different tissues was detected using RNA gel blot analysis. U6 snRNA from gel blotting and small RNAs stained with ethidium bromide were used as loading controls. (*b*) The relative expression levels of *OsARF6*, *OsARF12*, *OsARF17* and *OsARF25* were detected by quantitative RT–PCR. The expression value in the seedling was used as a calibrator and the relative expression data were normalised to rice *UBQ5*. Error bars indicate s.d. ($n = 4$ PCR replicates).

that miR167 is upregulated under auxin treatment to repress the level of *OsARF12* transcripts in cultured rice cells and have predicted that auxin response elements (AuxREs) are frequently present in the promoters of rice *MIR167* genes (Yang *et al*. 2006; Meng *et al*. 2010*a*). Therefore, the distinct expression features of miR167 indicate that it can regulate the expression of target *OsARFs* mainly for the normal development of seedlings and flowers under the intrinsic auxin signal.

The ARF transcription factors can either activate or repress transcription according to the amino acid content in their middle region (MR) domains (Guilfoyle and Hagen 2007; Shen *et al*. 2010) and OsARF6, OsARF12, OsARF17 and OsARF25 are characterised as transcriptional activators. However, the auxin responses mediated by ARF activators are negatively regulated by the auxin/indole-3-acetic acid (Aux/IAA) proteins, which interact with ARFs to repress transcriptional activation (Weijers *et al*. 2005; Quint and Gray 2006). Recently, quantitative studies have indicated that all OsIAA proteins can interact with all OsARF activators in yeast (Shen *et al*. 2010). The overexpression or gain-of-function of several OsIAAs in rice resulted in phenotypes with decreased auxin response (Nakamura *et al*. 2006; Song *et al*. 2009; Zhu *et al*. 2012), which resembled that in *Ubi*::*MIR167b* rice. This indicates that the phenotypic characteristics of the

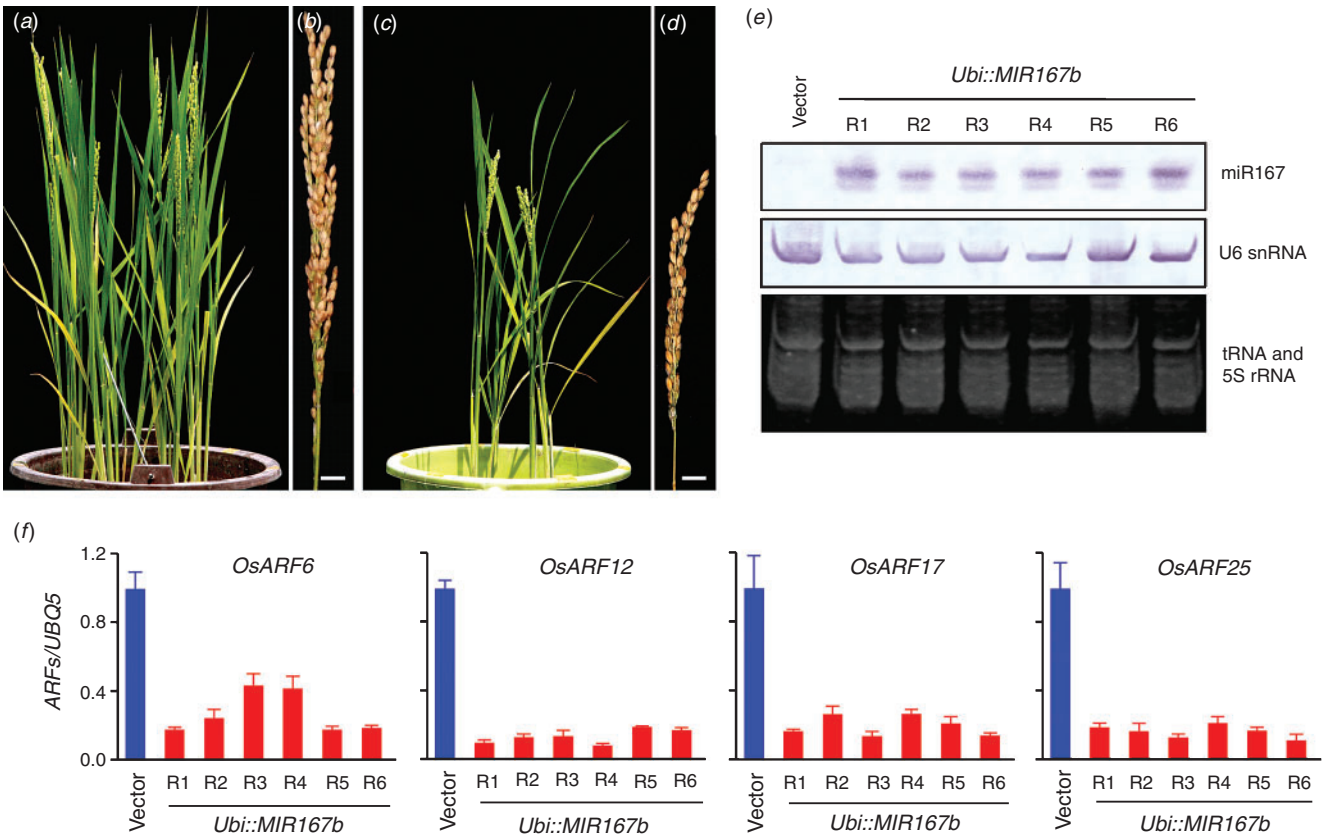

Fig. 5. Phenotypes of transgenic rice overexpressing miR167. (*a*, *c*) A representative overview of the empty vector control line and *Ubi*::*MIR167b* line at the heading stage. (*b*, *d*) The panicle architectures of the control line and *Ubi*::*MIR167b* line after ripening. The white scale bars represent 1 cm. (*e*) RNA gel blot analysis of miR167 in the control line and different *35S*::*MIR167b* lines (R1–R6). U6 snRNA and ethidium bromide staining of small RNAs were used as loading controls. (*f*) The mRNA levels of *OsARF6*, *OsARF12*, *OsARF17* and *OsARF25* in different *35S*::*MIR167b* lines were analysed by quantitative RT–PCR. The expression value in the control line was used as a calibrator and the relative expression data were normalised to rice *UBQ5*. Error bars indicate s.d. ($n = 4$ PCR replicates).

Table 2. Phenotype characteristics of transgenic rice

Values are means ± s.d. ($n \geq 30$). The data on tiller number were gathered at the heading stage. The data on plant height, panicle length and seed setting rate (ratio of total number of seeds produced per plant to total number of spikelets per plant) were obtained at maturity. The asterisks indicate that the value of *35S*::*MIR167* is significantly different from the value of vector control by independent-samples *t*-test (*, $P < 0.005$; **, $P < 0.001$)

Trait	Vector	*35S*::*MIR167*
Plant height (cm)	74.93 ± 4.18	61.98** ± 5.63
Tiller number per plant	7.21 ± 2.53	3.74** ± 1.66
Panicle length (cm)	18.21 ± 1.86	13.52** ± 2.70
Spikelet number per panicle	145.38 ± 30.6	85.87** ± 24.0
Seed setting rate (%)	83.03 ± 7.40	76.15* ± 10.05

Ubi::*MIR167b* rice – such as reduced plant height and tiller number – are a consequence of decreased auxin response resulting from the simultaneously impaired functions of four OsARFs by the constitutive overexpression of miR167. From our work, we conclude that the miR167-regulated expression of *OsARF6*, *OsARF12*, *OsARF17* and *OsARF25* is crucial for the normal intrinsic auxin response during rice growth and development. These results pave the way for further elucidation of the molecular behaviours of miR167 and its target *OsARFs* for the auxin signal response in rice.

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