Deregulation of the OsmiR160 Target Gene OsARF18 Causes Growth and Developmental Defects with an Alteration of Auxin Signaling in Rice

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MicroRNAs (miRNAs) control gene expression as key negative regulators at the post-transcriptional level. MiR160 plays a pivotal role in Arabidopsis growth and development through repressing expression of its target AUXIN RESPONSE FACTOR (ARF) genes; however, the function of miR160 in monocots remains elusive. In this study, we found that the mature rice miR160 (OsmiR160) was mainly derived from OsMIR160a and OsMIR160b genes. Among four potential OsmiR160 target OsARF genes, the OsARF18 transcript was cleaved at the OsMIR160 target site. Rice transgenic plants (named mOsARF18) expressing an OsMIR160-resistant version of OsARF18 exhibited pleiotropic defects in growth and development, including dwarf stature, rolled leaves, and small seeds. mOsARF18 leaves were abnormal in bulliform cell differentiation and epidermal cell division. Starch accumulation in mOsARF18 seeds was also reduced. Moreover, auxin induced expression of OsMIR160a, OsMIR160b, and OsARF18, whereas expression of OsMIR160a and OsMIR160b as well as genes involved in auxin signaling was altered in mOsARF18 plants. Our results show that negative regulation of OsARF18 expression by OsMIR160 is critical for rice growth and development via affecting auxin signaling, which will advance future studies on the molecular mechanism by which miR160 fine-tunes auxin signaling in plants.
In addition, OsmiR156 may target OsSPL16 to control grain size, shape, and quality. Furthermore, the gradually increased expression of OsmiR156 in leaves is important for leaf development. Overexpression of OsmiR172 represses expression of AP2-like genes, which consequently causes abnormal floral meristem identity and defects in flower organ and seed development. OsTIR1 and OsAFB2 are predicted targets of OsmiR393. Transgenic plants overexpressing OsmiR393a/b have defects similar to those observed in auxin signaling mutants. Although various miRNAs have been found in rice, it is imperative to study functions of individual OsmiRNAs in rice growth and development, particularly those of agricultural importance.

MiR160 is essential for plant growth and development. In Arabidopsis, miR160 targets AUXIN RESPONSE FACTOR 10 (ARF10), ARF16, and ARF17. Expression of the miR160-resistant version of ARF16 (miARF16) results in reduced fertility and less lateral roots. Plants expressing miARF17 exhibited pleiotropic defects in vegetative and reproductive development. Analyses using similar approaches have revealed that the miR160-controlled ARF10 is essential for seed germination and many post-embryonic growth and developmental processes through the regulation of both auxin and ABA signaling. Recent studies have shown the dormancy of miARF10 and miARF16 seeds is increased. In tomato, SlymiR160 and the SlymiR160a target SlyARF10 are required for floral organ and early fruit development. Moreover, in soybean, miR160 is involved in auxin and cytokinin signaling during nodulation. Although miR160 is conserved in plants, the role of miR160 in monocots is unknown. In this report, we examined expression of six OsMiR160 genes and found that the mature OsMiR160 was mainly derived from OsMIR160a and OsMIR160b genes. In addition, the OsARF18 transcript was cleaved at the OsRi160 target site. We generated transgenic rice plants (named mOsARF18) that expressed an OsRi160-resistant version of OsARF18. Phenotypic analyses revealed that mOsARF18 plants showed pleiotropic defects in growth and development. Furthermore, auxin treatment induced expression of OsMIR160a, OsMIR160b, and OsMIR160c, whereas expression of OsRi160a and OsRi160b as well as other genes involved in auxin signaling was altered in mOsARF18 plants. Our results support the idea that deregulation of the OsRi160 target gene OsARF18 leads to abnormal growth and development in rice through affecting the auxin signaling.

Results

Expression of OsMIR160 genes and OsRi160 target OsARF genes in rice. Rice has six OsMIR160 (OsMIR160a to OsMIR160f) genes. Four of six mature OsMIR160s (OsMIR160a to OsMIR160d) have identical sequences, while the other two (OsMIR160e and OsMIR160f) each differs by a single nucleotide. Our RT-PCR results showed that expression of OsMIR160a and OsMIR160b genes was significantly higher than that of the other four OsMIR160 genes in leaf (L), young inflorescence (YI), mature inflorescence (MI), and stem (ST) (Fig. S1b). Moreover, quantitative real-time RT-PCR (qRT-PCR) revealed that OsMIR160a and OsMIR160b genes had higher expression levels in leaf and inflorescence than that in stem and seedling (Fig. 1a, b). Our studies suggest that the mature OsMIR160 might be mainly derived from OsMIR160a and OsMIR160b genes.

Our analyses using the psRNATarget web server showed that OsARF8 (Os02g41800), OsARF10 (Os04g43910), OsARF18 (Os06g47150), and OsARF22 (Os10g33940) were potential OsRi160 target genes. Further phylogenetic analysis found that all four targets were similar to Arabidopsis ARF10 and ARF16, whereas OsARF18 had the highest similarity to the Arabidopsis ARF16 (Fig. 1g; Fig. S2). Our qRT-PCR results demonstrated that expression of OsARF8 mainly occurred in young and mature inflorescences (Fig. 1c), while OsARF10 was primarily expressed in seedling and stem (Fig. 1d). OsARF18 was predominantly expressed in leaf as well as young and mature inflorescences (Fig. 1e). Expression levels of OsARF22 appeared similar in all examined organs except in leaf (Fig. 1f). A previous study using the “degradome sequencing” approach has shown that cleavage frequencies associated with OsARF18 and OsARF22 are the highest, but frequencies for OsARF8 and OsARF10 are extremely low. We further identified that the OsARF18 transcript was cleaved at the OsRi160 target site using a gene-specific 5’ RACE (Fig. 1h). Therefore, our results suggest that OsARF18 (Os06g47150) is a promising target gene of OsMiR160.

Negative regulation of OsARF18 by OsMiR160 is essential for rice growth and development. To investigate the function of OsRi160 in rice, we chose its promising target OsARF18 to generate transgenic plants expressing an OsRi160-resistant version of OsARF18 (resulting plants named mOsARF18) in the Oryza sativa Japonica cv. Nipponbare background. Our qRT-PCR results showed that the expression of un-cleaved OsARF18 was significantly increased in leaf, stem, and inflorescence of three examined mOsARF18 transgenic lines (Fig. 2b). Eleven out of 19 mOsARF18 independent transgenic lines exhibited strong but similar defects in both growth and development, while the rest of lines showed mild phenotypes. Compared to wild-type plants, mOsARF18 lines were dwarf and formed less tillers (Fig. 3a, g, h). mOsARF18 plants produced short and rolled leaves (Fig. 3b, h, S3; Fig. 4a–c). mOsARF18 plants were also defective in reproduction, as indicated by abnormal flower and seed development (Fig. 3e–f). The lemma and palea did not enclose flowers (Fig. 3c). After fertilization, stamens remained attached to developing seeds, suggesting abnormal senescence of stamens (Fig. 3d). Moreover, mOsARF18 lines showed reduced seed setting (Fig. 3i). Although no change in seed length (Fig. 3e,f,j), mOsARF18 seeds had reduced width and weight when compared with wild type (Fig. 3e, f, k, l). Our results suggest that deregulation of OsARF18 results in abnormal growth and development in rice.

Cell division and differentiation were abnormal in mOsARF18 leaves. To further examine leaf defects in mOsARF18 plants, we analyzed the structure of the fifth mature leaf via cross-section. Compared with wild type, mOsARF18 lines produced rolled leaves (Fig. 4a–c). Bulliform cells are specialized epidermal cells between two vascular bundles on the adaxial blade. Bulliform cells are large and highly vacuolated, which are important for leaf rolling through turgor pressure regulation. Wild-type bulliform cells were arranged in groups of approximately 5 cells, among which the middle bulliform cell was larger than others along both sides (Fig. 4d, i).
Figure 1. Expression analysis of OsMIR160a, OsMIR160b, and OsmiR160 potential target OsARFs.
(a,b) Quantitative real time RT-PCR (qRT-PCR) results showing expression of OsMIR160a (a) and OsMIR160b
(b). (c–f) qRT-PCR results showing expression of OsmiR160 potential target genes: OsARF8 (c), OsARF10
(d), OsARF18 (e), and OsARF22 (f). S: seedling (7-day old), L: leaf, ST: stem, YI: young inflorescence, and MI:
mature inflorescence. Gene expression levels in other organs were normalized based on expression observed in
seedling. Stars indicate significant difference (P < 0.01). (g) An unrooted phylogenetic tree constructed by the
Maximum-Likelihood method showing miR160 target ARFs in Arabidopsis and rice. (h) The 5’ RACE result
showing that OsARF18 is a target of OsmiR160. The arrow points to the OsmiR160-directed cleavage site at the
OsARF18 transcript.
However, in \(mOsARF18\) leaves, we observed reduced number and size of bulliform cells, or bulliform cells were absent (Fig. 4e,f). Further statistical analysis showed that bulliform cell numbers between two vascular bundles in \(mOsARF18\) leaves were significantly reduced when compared with that of wild type (Fig. 4i). Numbers of total vascular bundles and abaxial epidermal cells between two vascular bundles were also decreased (Fig. 4d,g,h,j,k). In addition, with the exception of the bulliform cells, we did not observe significant size differences in other cells when comparing \(mOsARF18\) leaves with the wild-type. Thus, our results suggest that repression of \(OsARF18\) by \(Os\) \(miR160\) is important for epidermal cell division and bulliform cell differentiation during leaf development.

Starch accumulation during seed development was abnormal in \(mOsARF18\) plants. To study why \(mOsARF18\) plants produced small seeds, we examined starch accumulation in developing seeds. Our results demonstrated that endosperm in wild-type developing seeds contained many starch granules that were strongly stained by iodine (Fig. 4l), whereas endosperm in \(mOsARF18\) developing seeds contained smaller, weakly stained starch granules (Fig. 4m,n). In conclusion, \(mOsARF18\) plants were defective in starch accumulation during seed development.

The \(Os\) \(miR160\)-regulated \(OsARF18\) may control rice growth and development via affecting auxin signaling. ARFs play a primary role in auxin signaling. To test whether auxin signaling was affected in \(mOsARF18\) transgenic plants, we examined expression of representative genes known to be involved in auxin signaling in rice, including the \(AUXIN RESPONSIVE FACTOR\) gene \(OsARF2\), the auxin responsive gene \(OsGH3-1\), the \(AUX1-LIKE\) gene \(OsLAX2\) (\(OsAUX3\) or \(OsRAU2\); \(Os03g14080\)), the auxin efflux gene \(OsPIN1b\), and the auxin biosynthesis gene \(OsYUCCA2\) via qRT-PCR. Expression levels of all tested genes were significantly decreased in \(mOsARF18\) transgenic plant leaves (Fig. 5a–e). Thus, our results suggest that deregulation of \(OsARF18\) affects auxin signaling, which might cause abnormal growth and development in rice.
Figure 3. *mOsARF18* transgenic plants exhibited pleiotropic defects in growth and development. (a) Forty-day old wild-type (left), *mOsARF18*-3 (middle), and *mOsARF18*-5 (right) plants. Bar: 10 cm. (b) The fifth leaves of wild-type (left), *mOsARF18*-3 (middle), and *mOsARF18*-5 (right) plants. Bar: 1 mm. (c,d) Ten-DAP (Days After Pollination) wild-type (left), *mOsARF18*-3 (middle), and *mOsARF18*-5 (right) developing seeds. Bars: (c,d) 1 mm. Lemma and palea were removed in (d) to show attached stamens. (e,f) Wild-type (e) and *mOsARF18*-3 (f) mature seeds. Bars: (e,f) 1 mm. (g,h) Sixty-day old transgenic plants showed significantly (*P < 0.01*) decreased pant height (g) and tiller numbers (h) than that of wild type plants. (i–l) Mature transgenic plant seeds showed no difference in seed length (j), but significantly (*P < 0.01*) decreased seed setting (i), width (k) and weight (l).
We further tested whether auxin affected expression of OsMIR160a, OsMIR160b, and mOsARF18 target genes. Our qRT-PCR results showed that expression of OsMIR160a was significantly increased after 20 and 40-minute NAA treatment (Fig. 6a), while expression of OsMIR160b was significantly induced after 20-minute NAA treatment and then remained normal (Fig. 6b). NAA treatment also significantly induced expression of OsARF18 after 20 and 40 minutes (Fig. 6c) as well as expression of OsARF10 after 180 minutes (Fig. 6d). However, expression of OsARF8 (Fig. 6e) and OsARF22 (Fig. 6f) remained unchanged after NAA treatment.

Conversely, expression of OsMIR160a and OsMIR160b was found to be decreased in mOsARF18 plants (Fig. 6g,h), in which the expression of OsMIR160a was highly increased (Fig. 2b). Our results suggest that auxin up regulates expression of OsMIR160a, OsMIR160b, and OsARF18, whereas OsARF18 represses expression of...
OsMIR160a and OsMIR160b. Therefore, the positive regulation of OsMIR160a and OsMIR160b expression by auxin and the negative regulation of OsMIR160a and OsMIR160b by OsARF18 might be important for OsmiR160 to fine-tune auxin signaling in a negative feedback loop manner.

To test whether OsmARF18 seedlings are defective in auxin signaling, we treated OsmARF18 seedlings hydroponically with 1 μM of NAA for 7 days. We found that OsmARF18 seedlings produced significantly shorter primary roots than that of wild type (Fig. 7a,b,e). Primary root growth was significantly inhibited by NAA treatment in both wild type and OsmARF18 transgenic plants. Gene expression levels in OsmARF18-3, OsmARF18-5, and OsmARF18-8 were normalized based on expression observed in wild type. Stars indicate significant difference (P < 0.01).
Figure 6. Auxin upregulated expression of OsMIR160a, OsMIR160b, and OsMiR160 target genes, whereas OsARF18 suppressed the expression of OsMIR160a. (a–f) qRT-PCR results showing that expression changes of OsMIR160a, OsMIR160b, and OsMiR160 target genes in seedlings (7-day old) treated with NAA for 20, 40, and 180 minutes. Expression levels without NAA treatment (0 minute) were used to normalize expression with treatments. Stars indicate significant difference (P < 0.01). Expression of OsMIR160a was significantly induced after 20 and 40-minute treatment (a), while expression of OsMIR160b was significantly increased only after 20-minute treatment (b). Expression of OsARF8 (c) and OsARF22 (f) remained unchanged. Expression of OsARF10 was significantly induced after 180-minute treatment (d), whereas expression of OsARF18 was significantly increased after 20 and 40-minute treatment (e). (g,h) qRT-PCR results showing that expression of OsMIR160a and OsMIR160b was significantly decreased in mOsARF18 transgenic lines in comparison with wild-type plants. Gene expression levels in #3 (mOsARF18-3), #5 (mOsARF18-5), and #8 (mOsARF18-8) were normalized based on expression observed in wild type. Stars indicate significant difference (P < 0.01).
Figure 7. Effect of NAA treatment on lateral root formation. (a,c) Seven-day wild type (WT) seedlings without NAA (a) and with NAA (1 μM) (c) treatment (inset showing high magnification of roots in (c)). (b,d) Seven-day OsmARF18 seedlings without NAA (b) and with NAA (1 μM) (d) treatment (inset showing high magnification of roots in (d)). (e,f) Primary roots in WT 7-day seedlings were significantly (*indicating P < 0.01) longer than that of OsmARF18 seedlings. NAA treatment significantly (P < 0.01) inhibited primary root length of both WT and OsmARF18 seedlings, but the relative primary root length (percentage of root length between that treated and untreated) was similar. (g) Seven-day WT and OsmARF18 seedlings produced similar numbers of lateral roots. After NAA treatment, lateral root numbers in WT seedlings was significantly (P < 0.01) increased, whereas that in OsmARF18 seedlings was significantly (P < 0.01) decreased.
Discussion
Conserved and diverse roles of miR160 in plant growth and development. MiR160 is conserved throughout the plant kingdom from mosses to higher plants. Sequence similarity of miR160 is more than 80% among different species. Arabidopsis contains MIR160a, MIR160b, and MIR160c three genes which produce the same mature miR160. MiR160 targets AUXIN RESPONSE FACTOR 10 (ARF10), ARF16, and ARF17 three genes and each target gene has been conserved but some distinct functions in Arabidopsis. Plants expressing the miR160-resistant version of ARF10 (mARF10) exhibit pleiotropic defects that resemble phenotypes of some ABA and auxin defective mutants, while mARF16 plants have reduced fertility and less lateral roots. ARF10 and ARF16 are required for maintaining the expression of ABI3 gene, suggesting that ARF10 and ARF16 are involved in both auxin and ABA signaling or cross-talk between them. ARF17 plays a general role in both vegetative and reproductive development via modulating expression of early auxin response genes. SylmiR160 and the SylmiR160a target SylARF10 control ovary patterning, early fruit development and floral organ abscission in tomato, whereas the OsARF10 genes negatively regulate gene expression at the post-transcriptional level by binding to mRNA complementary to mOsARF18. OsARF18 control ovary patterning, early fruit development and floral organ abscission in tomato.

Negative regulation of OsARF18 by OsmiR160 is important for its normal function. miRNAs negatively regulate gene expression at the post-transcriptional level by binding to mRNA complementary sequences for RNA destabilization and translational inhibition in both plants and animals. One miRNA normally has multiple target genes. Over/ectopic-expression of normal miRNA-target gene usually does not cause a change in phenotype, because over/ectopic-expressed normal mRNAs can be still targeted by miRNAs for cleavage. Therefore, a primary approach for studying the function of miRNA and its target is to express the miRNA-resistant version of individual target gene. Employing 35S, Ubi, and ACTIN promoters, previous studies identified functions of many miRNAs and their target genes via expressing miRNA-resistant versions of target genes, such as 35S::mTCP3, 35S::mTCP4, 35S::mUC1, and 35S::mUC2 (Arabidopsis miR164 target genes), 35S::mAP2 (Arabidopsis miR172 target gene), 35S::mSylARF10 (tomato miR160 target gene), UBImGRF6 (rice miR396 target gene), as well as ACTIN::mOSHB1, ACTIN::mOSHB3 and ACTIN::mOSHB5 (rice miR166 target genes).

So far, no studies report that over/ectopic-expression of normal versions of miR160 target genes (ARF10, ARF16, and ARF17) causes phenotypes in leaf and flower development, but only plants expressing miR160-resistant versions of miR160 target genes (mARF10, mARF16, and mARF17) exhibit various phenotypes. In this study, we used the Ubiquitin (Ubi) promoter to drive mOsARF18 expression. To further rule out the possibility that the phenotype of mOsARF18 was caused by ectopic activity of the Ubi promoter, we generated ARF16, mARF16, OsARF18, and mOsARF18 Arabidopsis transgenic plants under the control of the Ubi promoter. Of 30 ARF16 and 32 OsARF18 transgenic plants that we obtained, none of them showed detectable defects in growth and development (Fig. S5a–c, f–h; Fig. S6a–c, f–h, k–m, p); however, mARF16 (32 out of 40) and mOsARF18 (20 out of 29) transgenic plants formed narrow and curled leaves, and had short stature (Fig. S5d, e, i, j). In addition, mARF16 and mOsARF18 plants produced abnormal flowers and a lower number of smaller seeds when compared to wild-type seeds. Collectively, miR160 plants produced conserved and diverse roles in plant growth and development. It will be interesting to test the loss-of-function of OsmiR160 and functions of other OsmiR160 target genes in rice growth and development.

Molecular mechanism of miR160 in fine-tuning auxin signaling. MiRNAs play a pivotal role in auxin signaling by negative regulation of ARFs. In Arabidopsis, miRNAs are involved in expression regulation of 8 of a total 23 ARFs (ARF2, 3, 4, 6, 8, 10, 16, and 17). MiR390-derived trans-acting-small interfering RNAs.
 Auxin promotes the release of OsARF activators or repressors via the auxin receptor SCF E3 ligase. The balance between OsARF activators and repressors decides up or down expression of OsMIR160 genes. Conversely, the change in abundance of mature OsMIR160 negatively regulates expression of its target OsARFs. OsARF activators and repressors might positively or negatively regulate expression of each other.

(ta-siRNAs) target ARF2, ARF3, and ARF4\(^{47,48}\). The tasiRNA gradient is important for establishing the normal patterning of ARF3 protein during leaf development\(^{49}\). During growth of lateral roots, miR390 affects production of tasiRNAs, and thus inhibits ARF2, ARF3, and ARF4\(^{49}\). Conversely, auxin activates ARF2, ARF3, and ARF4, which consequently influences the formation of miR390. Therefore, the regulatory network modulated by miR390 maintains normal expression of ARF2, ARF3, and ARF4. Negative regulation of ARF6 and ARF8 by miR167 is essential for anther and ovule development\(^{51}\). Moreover, miR160 negatively regulates expression of ARF10, ARF16, and ARF17\(^{26,22-24}\). In rice, OsMIR160s are predicted to regulate 11 of a total of 25 identified OsARFs\(^{52,53}\). Functional disruption of OsDCL4 causes increased expression of OsMIR165/166 and three OsARFs, which are orthologs of Arabidopsis ARF2, ARF3, and ARF4\(^{44}\). OsMIR160 is predicted to have four OsARF targets, including OsARF18 that was characterized in this study. OsMIR167 potentially targets OsARF6 (Os02g06910), OsARF12 (Os04g57610), OsARF17 (Os06g46410), and OsARF25 (Os12g41950), which are highly similar to Arabidopsis ARF6 and ARF8\(^{44}\). Together, miRNAs target a group of similar ARF genes in both monocots and dicots.

Auxin promotes the SCF\(^{TIR1/AFB}\) E3 ligase-mediated degradation of Aux/IAA proteins, which sequester ARFs\(^{55,56}\). Upon the perception of auxin, the released ARFs activate or suppress expression of a large set of auxin-responsive genes by binding to auxin response elements (AuxREs) in their regulatory regions. Based on transient expression studies and protein structures, among 23 ARFs in Arabidopsis, ARF5s-8, and 19 act as activators, while ARF1-4, 9-18, and 20-22 may function as repressors\(^{57-59}\).

Previous studies have implied that the feedback regulation between miRNA and its target ARF genes could provide a fine-tuning mechanism to regulate auxin signaling. During adventitious root development in Arabidopsis, expression of ARF6 and ARF8 is regulated by miR167 and miR160, whereas the abundance of ARF17 transcripts is controlled by miR160\(^{55}\). In addition, ARF6 and ARF8 activators as well as ARF17 repressors positively and negatively regulate the expression of each other. Thus, the delicate balance between miRNAs and ARFs is critical for auxin-regulated developmental processes. Our studies show that OsMIR160 negatively regulates the expression of OsARF18 by cleaving OsARF18 transcripts. Auxin induces expression of OsMIR160a, OsMIR160b, and OsARF18, whereas expression of OsMIR160a and OsMIR160b was suppressed by OsARF18. Our analyses revealed that promoters and 3′ regions of OsMIR160a and OsMIR160b had clusters of AuxRE cores and AuxRR cis elements (Fig. S4), which may create OsARF binding sites by which OsARF activators or repressors could regulate expression of OsMIR160a and OsMIR160b.

In our hypothetical model, auxin promotes the release of OsARF activators and repressors via the SCF\(^{TIR1/AFB}\) E3 ligase (Fig. 8). The balance between OsARF activators and repressors decides up or down expression of OsMIR160 genes, which consequently affects the abundance of mature OsMIR160. Conversely, OsMIR160 negatively modulates expression of its target OsARFs. OsARFs also mutually control their own expression. Future studies should focus on examining the feedback loop regulation between OsMIR160 and its target OsARFs, which might be important for fine-tuning highly dose-sensitive auxin signaling during rice growth and development.

Manipulating miRNAs and their target genes has demonstrated improvement of many important crop traits, including biomass yield, grain yield, fruit yield, nutritional quality, abiotic stress resistance (e.g. drought, salinity, cold, heat, oxidative stress, nutrient deprivation tolerance, and heavy metal detoxification), and biotic stress resistance (e.g. virus, bacteria, fungus, and nematode resistance)\(^{14,15,60,62}\). Our results showed that OsMIR160 played a pivotal role in rice growth and development via regulating auxin signaling. In particular, OsMIR160 is essential for leaf and seed development in rice. Leaf shape is important for photosynthesis, respiration, and transpiration. Moderate leaf rolling can enhance photosynthesis and stress responses by inhibiting water loss and radiant heat absorption, which, therefore, increases crop yield. Future studies on the molecular mechanism by which OsMIR160 modulates auxin signaling will lead to potential applications for improving crop agronomic traits.
Methods

Plant materials and growth condition. Rice (Oryza sativa L. Japonica Nipponbare) plants were grown in Metro-Mix 360 soil (Sun-Gro Horticulture, Agawam, MA, USA) supplemented with sand and iron in a walk-in growth chamber under a 12-hour light (28 °C)/12-hour dark (22 °C) photoperiod regime. Transgenic rice plants were generated at the Plant Transformation Facility at Iowa State University. In total, 124 mOsARF18 plants were obtained from 19 independent transgenic lines. For expression studies, seven-day old wild-type seedlings were treated with 1 μM of NAA for 20, 40, and 180 min with untreated 7-day old seedlings as control. For studying the effect of exogenous auxin on root development, geminated seeds were hydroponically grown in the 1/2 Kimura B nutrient solution (pH 5.6) containing 1 μM of NAA for 7 days with continuous light at 25 °C.

Vector construction and rice transformation. PCR reactions (Primers are shown in Table S1) were performed using the Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA). The OsARF18 cDNA was amplified from rice leaf cDNAs and then cloned into the pCR2.1 vector (Invitrogen, Grand Island, NY, USA), resulting in pCR2.1-OsARF18. Point mutations of OsARF18 were created by overlapping PCR to generate pCR2.1-mOsARF18. mOsARF18 was then subcloned into the modified pCambia1301 binary vector harboring the Gateway cassette sequence and the maize Ubiquitin (Ubi) promoter using the Gateway LR recombination II enzyme mix (Invitrogen, Grand Island, NY, USA).

For rice transformation, the mOsARF18 construct was introduced into the Agrobacterium strain EHA101. The callus induction (from mature embryos of Japonica cv. Nipponbare seeds), Agrobacterium infection, co-cultivation, selection of transformed calli, and plant regeneration were performed essentially as described previously63.

RT-PCR and real time qRT-PCR. Total RNAs were isolated from different rice tissues using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). After determining the RNA quantification by the NanoDrop 2000c (Thermo Scientific, Bannockburn, IL, USA), RNA reverse transcription was conducted using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA). Real-time PCR (DNA Engine Opticon 2 system, Hercules, California, USA) and data analysis were performed as previously described. Expression of OsMIR160a through OsMIR160f was examined by RT-PCR. Expression of OsMIR160a, OsMIR160b, OsARF8, OsARF10, OsARF18, and OsARF22, as well as other auxin signaling related genes was tested by real-time qRT-PCR. Three biological repeats were conducted and each value indicates the average with the standard error. All primer sequences are shown in Table S1.

5′ RACE. Using the GeneRacer™ kit (Invitrogen, Grand Island, NY, USA), a gene-specific 5′-rapid amplification of cDNA ends was conducted as described previously23. Gene-specific primers for OsARF18 (Os06647150) are shown in Table S1.

Semi-thin section analysis. Semi-thin sectioning was performed as described previously44,65. The fifth leaves of 6-week-old rice were fixed in 2.5% of glutaraldehyde and post-fixed with 1% of OsO4 at room temperature. Samples were dehydrated through a graded acetone series (10% increments) for 60 minutes each. Infiltrated with 20% of Spurr’s resin and then 40%, 60%, and 80% of Spurr’s resin every 3 hours. Following infiltration in three changes of 100% Spurr’s resin for 24 hours each, samples were finally embedded in 100% Spurr’s resin and polymerized at 60 °C overnight. Semi-thin sections (0.5 μm) were made using an RMC MT-7 ultramicrotome (Reichert-Jung, Depew, NY, USA) and were stained with 0.25% Toluidine Blue O. Images were photographed with an Olympus BX51 microscope equipped with an Olympus DP 70 digital camera (Olympus, Center Valley, PA, USA).

Histological detection of starch. Ten-DAP (Days After Pollination) seeds were fixed in FAA (50% ethanol, 10% formalin, 5% acetic acid). Following fixation, samples were dehydrated through an ethanol series, embedded in paraffin, and sectioned at 8 μm with a Spencer 820 microtome. Sections were dewaxed and stained with Lugol’s iodine solution (6 mM iodine, 43 mM KI, and 0.2 N HCl) for detection of starch granules. Images were photographed with an Olympus BX51 microscope equipped with an Olympus DP 70 digital camera (Olympus, Center Valley, PA, USA).

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Author Contributions
D.Z. and H.J. conceived and designed experiments. H.J. and Z.L. conducted experiments. D.Z. and H.J. wrote the paper.

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