OsTIR1 and OsAFB2 Downregulation via OsmiR393 Overexpression Leads to More Tillers, Early Flowering and Less Tolerance to Salt and Drought in Rice

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Abstract

The microRNA miR393 has been shown to play a role in plant development and in the stress response by targeting mRNAs that code for the auxin receptors in Arabidopsis. In this study, we verified that two rice auxin receptor gene homologs (OsTIR1 and OsAFB2) could be targeted by OsmiR393 (Os for Oryza sativa). Two new phenotypes (increased tillers and early flowering) and two previously observed phenotypes (reduced tolerance to salt and drought and hyposensitivity to auxin) were observed in the OsmiR393-overexpressing rice plants. The OsmiR393-overexpressing rice demonstrated hyposensitivity to synthetic auxin-analog treatments. These data indicated that the phenotypes of OsmiR393-overexpressing rice may be caused through hyposensitivity to the auxin signal by reduced expression of two auxin receptor genes (OsTIR1 and OsAFB2).

Introduction

Plant architecture, fertility and flowering time are important in the genetic improvement of rice [1]. These traits are generally controlled by multiple genes and are influenced by the environment. Recently, plant microRNAs (miRNAs) have been shown to play important regulatory roles in plant development and response to environmental stresses by targeting mRNAs for miRNA cleavage, mRNA decay or translational repression [2,3]; therefore, they may be useful for molecular breeding.

Many miRNAs regulate organ development from embryo to leaves in plants [2,4]. In rice, OsSPL14 controls plant architecture and affects grain yield, and it is regulated by OsmiR136 [1]. Upregulation of OsmiR172 induced a loss of spikelet determinacy and floral organ abnormalities in rice [5]. Many miRNAs also regulate phase changes in plants [2,6]. Arabidopsis miR172, miR159, miR156 and miR171 regulate flowering time and floral patterning [2,7,8]. Many miRNAs are involved in the stress responses of plants against different environmental factors [3,9,10,11] and also have important roles in plant signal transduction systems (such as miR393 and miR167) [12,13,14] and the production of Ta-siRNA, an endogenous trans-acting siRNA [15].

Many microRNA gene families are evolutionarily conserved across all major plant lineages [16]. As one of the conserved miRNA families in plants, miR393 genes have been found in different plant species including rice [4,17,18]. Although miRNA precursors (pre-miRNAs) of miR393 vary with plant species, the length (21 bp) and the sequence of the mature miRNA are conserved among different species [4,17]. The microRNA miR393 has been shown function via the auxin pathway by posttranscriptional regulation of auxin receptors [4,12,17]. In Arabidopsis, miR393 targets mRNAs that code for the auxin receptors (TIR1, AFB2 and AFB5) [4,12]. The microRNA miR393 has been found to have a role in leaf development [4] and normal development [14] and in response to pathogen attack [12] and salt stress [17,18,19]. In rice, OsmiR393 was recently found to play an important role in response to salt stress [17].

However, the targeting genes of OsmiR393 have not been determined in rice. Apart from the response to salt, other effects of OsmiR393 on rice growth and development are still unknown, as is
whether it is a potential target for rice molecular breeding. We constructed OsmiR393 overexpressing transgenic rice to answer these questions. Our results indicated that OsmiR393 could target two rice auxin receptor genes (OsTIR1 and OsAFB2), and two new functions were identified through OsmiR393 overexpression. An increase in tillers and early flowering were caused by overexpression of OsmiR393, in addition to a decreased tolerance to salt and hyposensitive to auxin.

Results

1. Seven rice genes were predicted to be putative target genes of OsmiR393

To identify the potential target genes of OsmiR393, the web-based prediction program miRU (Plant microRNA Potential Target Finder, http://bioinfo3.noble.org/miRNA/miRU.htm) was used, and seven candidate genes were obtained from the rice genome. Information regarding these seven genes is listed in Table 1. There were at least three mismatch nucleotides in the target sites of the seven mRNAs compared to the 21 nucleotides of OsmiR393. Of the seven candidate genes, the functions of five genes have been predicted; however, the functions of LOC_Os04g58734 and LOC_Os03g36080 have not been predicted. Two genes (LOC_Os04g32460 and LOC_Os05g05800) were highly homologous with the auxin receptors OsAFB2 and OsTIR1, respectively; the similarity of OsAFB2 to Arabidopsis AFB2 and OsTIR1 to Arabidopsis TIR1 were 80% and 77%, respectively (Table 1). The other three genes (LOC_Os03g59290, LOC_Os10g39790 and LOC_Os05g41010) showed significant similarity to the GRF1-interacting factor, magnesium transporter and prolyl-4-hydroxylase, respectively.

2. Overexpression of OsmiR393 reduced the expression levels of two auxin receptor gene homologs

To investigate whether OsmiR393 could target the above predicted genes, we generated transgenic rice plants that overexpress OsmiR393. Expression of the DNA sequence containing the mature folded structure of OsmiR393 was driven by the constitutive 35S promoter. This construct was introduced into the rice variety Zhonghua 11 through Agrobacterium-mediated transformation [20]. Sixty independent transgenic plant lines were confirmed to have successfully integrated 35S:OsmiR393 into the rice genome using PCR amplification with the pair of primers 35S:OsmiR393-2. These data suggested that OsmiR393 could target the OsTIR1 and OsAFB2 mRNAs.

When the plants were grown in a natural paddy, q-RT-PCR results showed that the expression levels of OsmiR393 was the highest in the roots and flag leaves among the five organs tested at the booting stage (Figure 1A). In addition, the expression of OsmiR393 was significantly upregulated by salt and drought in the leaves, but not in the roots (Figure 1B), when using at least a two-fold threshold for expression changes. Our experiments also verified that the expression of OsmiR393 in four independent transgenic lines was stably upregulated in the flag leaves of the first (T1) (data not shown) and the second (T2; Figure 2) generation of transgenic plants. These indicated that the OsmiR393 transgene is inherited persistently in rice; therefore, these four transgenic lines were used in subsequent experiments. The expression of OsmiR393 in the four transgenic rice plants was upregulated at least by two fold compared to the two controls, which were the wild-type rice variety Zhonghua 11 (Z11) and the transgenic rice plants with empty vector pCAMBIA1301 (Figure 2).

To confirm whether the transcripts of the seven putative target genes were downregulated in the transgenic plants overexpressing OsmiR393, real-time RT-PCR was performed to measure the transcript abundance of the seven putative target genes using the primer pairs listed in Table S1. We first investigated the organ expression of these candidates by semi-quantitative RT-PCR (Figure 3). Then, we used the organs that showed the highest expression for the corresponding candidate genes to monitor gene expression in the OsmiR393-overexpressing rice plants. Among the seven candidate genes, only two genes (OsAFB2: LOC_Os04g32460 and OsTIR1: LOC_Os05g05800) were repressed in the homozygous and heterozygous lines of the T1 and T2 OsmiR393-overexpressing rice plants (Figure 4). To detect actual cleavage of the candidate genes in the OsmiR393-overexpressing lines, 5’ RACE was used to monitor the cut fragments of the mRNAs of OsAFB2 and OsTIR1; 5’ RACE detected the expected cleavage fragments of OsAFB2 and OsTIR1 mRNAs in the OsmiR393 over-expressing rice plants (Figure S1). These data suggested that OsmiR393 could target the OsAFB2 and OsTIR1 mRNA.

OsAFB2 is an alternatively spliced gene with at least two corresponding mRNA forms (AK072338: OsAFB2-1 and AK100862: OsAFB2-2; Table 1). Both alternatively spliced mRNA transcripts (OsAFB2-1 and OsAFB2-2) were downregulated in the OsmiR393-overexpressing rice plants, but the OsAFB2-1 mRNA was reduced further than the OsAFB2-2 mRNA (Figure 4). In addition, based on data analysis of the rice chip-expression results, 35S:OsmiR393 could target the OsTIR1 and OsAFB2 mRNAs.

Table 1. Putative target genes for OsmiR393 in rice predicted based on the sequence complementarity.

| Gene ID     | Gene name  | Description                                           | Similarity (%) | Full-length cDNA | Target site alignment | Actual target |
|-------------|------------|-------------------------------------------------------|----------------|------------------|-----------------------|--------------|
| OsmiR393    |            |                                                       |                |                  |                       |              |
| LOC_Os04g32460| OsAFB2     | Auxin signaling f-box 2                                | 80.38          | (At3g26810)      | GAGAGAAGGCUAGCC1      | Yes          |
| LOC_Os04g58734| OsTIR1     | Protein transport inhibitor response (auxin receptor) | 76.82          | (At1g62980)      | GAGAGAAGGCUAGCC1      | No           |
| LOC_Os05g05800| OsAFB2-1   |                                                       |                |                  |                       |              |
| LOC_Os05g36080| OsAFB2-2   |                                                       |                |                  |                       |              |
| LOC_Os03g52230| OsGRF1     | GRF1-interacting factor 3                              | 54.92          | (At4g00850)      | UGGUGAAGGCUAGCC1      | No           |
| LOC_Os10g39790| OsMGT6     | Magnesium transporter                                  | 68.81          | (At3g59870)      | UGGUGAAGGCUAGCC1      | No           |
| LOC_Os05g41010| Prolyl-4-hydroxylase |                                        | 81.61          | (At5g18900)      | UGGUGAAGGCUAGCC1      | No           |

The putative target genes for OsmiR393 were predicted using the web-based program (http://bioinfo3.noble.org/miRNA/miRU.htm).

A Mismatch nucleotides in target mRNAs with OsmiR393 are underlined.
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were kept in the dark for 48 h. The seedlings were kept at 4°C for 4 h between folds of tissue paper at 28°C. For salt treatment, the seedlings were kept in a 250 mM NaCl solution for 4 h. For drought treatment, the seedlings were dried of abiotic stress. For salt treatment, the seedlings were kept in a 250 mM NaCl solution for 4 h. For drought treatment, the seedlings were dried.

Figure 1. Relative expression levels of OsmiR393 in different organs under normal growth conditions and under abiotic stresses. Expression levels of OsmiR393 were normalized to the reference gene snRNA U6 [48], whose expression level was defined as 1. A. RNA was extracted from the booting rice plants, which were grown in the natural field. B. Expression levels of OsmiR393 in response to abiotic stress. For salt treatment, the seedlings were kept in a 250 mM NaCl solution for 4 h. For drought treatment, the seedlings were dried for 4 h between folds of tissue paper at 28°C. For cold treatment, the seedlings were kept at 4±1°C for 4 h. For dark treatment, seedlings were kept in the dark for 48 h.

database (RiceXPro: http://ricexpro.dna.affrc.go.jp/index.html), the signal intensity of the OsaAFB2-1 mRNA was much higher than that of the OsaAFB2-2 mRNA. These data indicated that OsaAFB2-1 may be the main transcript of OsaAFB2 in rice.

Both target genes (OsaAFB2 and OsaTIR1) of OsmiR393 were classified as auxin receptor homologs, but their functions have not yet been experimentally demonstrated in rice. Homologous analysis showed that OsaAFB2 and OsaTIR1 had a high similarity to Arabidopsis AFB2 (At1g12820) and TIR1 (At3g62980) (Table 1), which had been found as auxin receptors involved in primary and lateral root growth inhibition in response to nitrates [21,22]. Spatial-expression results showed that the two genes were differentially expressed; OsaTIR1 was only expressed in the flag leaves, but OsaAFB2 was expressed in root, stems, flag leaves, panicles and shoot sheaths at the booting stage. In addition, the two alternatively spliced mRNAs of OsaAFB2 showed different expression patterns (Figure 3). One alternatively spliced mRNA (OsaAFB2-1, AK072338) of OsaAFB2 was expressed in the stems, flag leaves and panicles, whereas the other alternatively spliced mRNA (OsaAFB2-2, AK100862) was expressed in all tested organs (Figure 3).

3. Overexpression of OsmiR393 resulted in an increase in tillers and early flowering

To investigate the newly discovered functions of OsmiR393 in rice, we compared the OsmiR393-overexpressing rice plants to the wild-type rice variety Zhonghua 11 and transgenic rice plants with the empty vector pCambia1301. In the natural field, two new phenotypes (increased tillers and early flowering) in the OsmiR393-overexpressing rice plants were found. The flowering (heading) time of the OsmiR393-overexpressing rice plants was earlier by one week than the control rice plants (Figure 5A). Tillers of the OsmiR393-overexpressing rice plants were increased by at least 50% compared to the control plants (Figure 5B, C).

Our results also confirmed the negative role of OsmiR393 in the tolerance of abiotic stresses, which had been found in Arabidopsis and rice [17,19]. Growth of the OsmiR393-overexpressing seedlings was repressed by one-day drought treatments (Figure 6A). OsmiR393-overexpressing rice plants grew slowly compared with the control plants in nutrient solution [23] containing 100 mM NaCl (Figure 6B). Treatment with 250 mM NaCl repressed the seed germination of the OsmiR393-overexpressing rice compared to the controls (Figure 6C). These data indicated that the OsmiR393 negatively regulates salt and drought tolerance.

Figure 2. Verification of the 22-bp mature-OsmiR393 overexpression in OsmiR393-overexpressing transgenic rice lines by real-time RT-PCR. RNA was extracted from the flag leaves of the second generation of the transgenic rice plants. The expression level of OsmiR393 in wild-type rice ZH11 was set as one; p1301 is the transgenic ZH11 with the empty vector. The OsmiR393-overexpressing transgenic lines 7.5 and 31.2 are homozygous, and the lines 10.4 and 6.6 are heterozygous. These lines are the same as in Figures 3–10; snRNA U6 [48] served as a reference gene for the detection of miRNAs.

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Figure 3. Organ-specific expression analysis for the predicted target genes of OsmiR393 by semi-quantitative RT-PCR. Total RNA was isolated from booting rice plants grown in the natural field. Rice Actin1 was used as an internal control. OsaAFB2-1 and OsaAFB2-2 are two alternatively spliced mRNAs of OsaAFB2. The term “cycles” indicates the PCR cycle number used in RT-PCR reactions.

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4. Overexpression of OsmiR393 repressed the auxin signaling pathway

As previously documented in Arabidopsis, miR393 silences its target genes TIR1 and AFBs, which encode auxin receptors in Arabidopsis [4,12]. To examine whether the roles of OsmiR393 in rice are similar with Arabidopsis through the auxin signaling pathway, the responses of the OsmiR393-overexpressing rice plants to auxin were investigated. We found that overexpression of OsmiR393 lead to a reduction in the transcript of the two auxin receptor gene homologs (Figure 4). However, the IAA content analysis in the flag leaves showed no difference between the OsmiR393-overexpressing rice plants and the controls (Figure 7). These data indicated that the synthesis of IAA in the OsmiR393-overexpressing rice plants did not change.

Compared to the controls, the OsmiR393-overexpressing rice plants exhibited auxin hyposensitivity. Mature seed callus induction of OsmiR393-overexpressing rice was hyposensitive to 2,4-D (Figure 8A–D). The size of the callus was smaller than the controls (Figure 8B, C) and the callus-inducing rate of OsmiR393-overexpressing rice seeds was lower than the controls (Figure 8D). However, no significant differences were found in the germination ratios of the OsmiR393-overexpressing rice and the controls under different NAA treatments (data not shown). NAA treatments exerted an influence on root growth. Under the inhibitory growth concentration of NAA, more roots (Figure 9B,C,E) and longer main roots (Figure 9B–D) of OsmiR393-overexpressing rice plants were observed in 0.001 and 0.0001 mg/L NAA treatments compared with controls. However, when they were germinated in water (Figure 9A), the root length and root numbers of OsmiR393-overexpressing rice plants were lower than that of the controls (Figure 9D, E). These data may indicate that the roots of the OsmiR393-overexpressing rice plants are less responsive to normal physiological concentrations of NAA. These responses to NAA suggested that the auxin receptors might be damaged by overexpression of OsmiR393.

5. Expression analysis of OsAFB2 or OsTIR1 downstream genes related to tillering and flowering time

To form a chain of OsmiR393 on the phenotype via the auxin signaling pathway, we compared the expression of two auxin...
transporter genes (OsAUX1: LOC_Os05g37470 and OsLAX1: LOC_Os01g63770) [13] in OsmiR393-overexpressing lines and the controls using the quantitative RT-PCR. However, the expression of only one gene (OsAUX1) was downregulated in the OsmiR393-overexpressing rice (Figure 10A) compared with the controls when using a two-fold change threshold. These data indicated that OsmiR393 could regulate the auxin transport via OsAUX1. To determine the mechanism by which OsmiR393 controls tillering and flowering, the expression levels of genes related with tillering (Figure 10C, D) and flowering time (Figure 10E, F) of rice were compared among the OsmiR393-overexpressing lines and the controls. The data showed that overexpression of OsmiR393 could downregulate expression levels of OsTB1 (LOC_Os03g49880; Figure 10D), which is a negative regulator for lateral branching and inhibits tillering in rice [24]. However, OsmiR393 did not regulate expression levels of MOC1, which enhances rice tillering [25]. The expression of two genes (OsHd1 and OsMADS50) [26] related to flowering time was not affected by OsmiR393 (Figure 10E, F).

**Discussion**

1. Prediction of target genes of OsmiR393 requires experimental confirmation

Many computational predictions of miRNA target genes have been conducted [2,27,28]. The microRNA miR393 has been identified in diverse species including monocotyledons and dicotyledons [12,17]. Two mature miR393 isoforms, OsmiR393 and OsmiR393b, arising from two primary transcripts, have been identified in rice and Arabidopsis (http://www.mirbase.org/cgi-bin/query.pl?term=miR393). Previous studies indicated that miR393 targets the auxin receptors in Arabidopsis and rice [4,12]. In rice, candidate OsmiR393-targeted genes have been predicted [17], but they have not been experimentally investigated. Although the program miRU predicted that seven genes in the rice genome are candidate target genes of OsmiR393 (Table 1), only two genes (OsAFB2 and OsTIR1) were actually targeted by OsmiR393 (Figure 3) in the OsmiR393-overexpressing rice plants. These data indicated that the target genes predicted by computational programs should be experimentally validated.

The microRNA miR393 regulates plant growth and development by regulating target genes, which encode F-box proteins such as TIR1, AFB2 and AFB3 in Arabidopsis [4,12,29]. Those F-box proteins, especially TIR1, act as auxin receptors and play crucial roles in controlling the expression of genes associated with the auxin signaling pathway, which is implicated in various aspects of plant growth and development [30,31,32]. Our results indicated that rice OsmiR393 could also target the mRNAs of two auxin receptor gene homologs in the overexpressing-OsmiR393 transgenic rice plants (Figure 3), suggesting that the regulation pathway

Figure 6. Response to drought and salt treatments. A. Growth was affected by drought stress. The 6-day seedlings of OsmiR393-overexpressing rice and controls were removed from Hoagland’s Solution for one day and then re-cultured in Hoagland’s Solution for 4 days. B. Images were taken on the fifth day after 10-day seedlings were placed in 100 mM NaCl. C. The germination ratio was calculated after the seeds germinated in 150 and 200 mM NaCl for 15 days; ** indicates significance by the Duncan’s multiple range tests at the 1% level.
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Figure 7. Auxin (IAA) contents in OsmiR393-overexpressing rice plants. The rice was grown in the field during the rice-growing season, and IAA concentrations in flag leaves were measured.
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of mir393 might be conserved among the monocot and dicot species through regulation of the transcript abundance of the auxin receptor genes. It has been previously reported that mature mir393 sequences are a canonical microRNA conserved among different plant species such as Arabidopsis, rice, maize, poplar, Medicago and Brassica napus [9,17,33,34,35]. The conservation in mir393 itself and the recognition sites of its target genes suggest that mir393 functions in a similar way among different plants, including rice.

2. Functions of OsmiR393 and its target genes

In high-yielding semidwarf rice, early flowering and high tillering capacity are considered beneficial for the grain yield of rice [1]. Several gene mutations showing a relationship to the regulation of tillering have been found in rice. Mutations in some genes decrease rice tillers including moc1 [25] and htd-1 [36]. Some mutations increase tillers including Hdl1 [37] and D10 [38]. Flowering time is controlled by many quantitative trait loci in rice [39]. Hdl1 [40] and Ghd7 [41] repress floral transition strongly under long-day (LD) conditions, but Ehd1 promotes floral transition [42]. We found that overexpression of OsmiR393 in rice caused significant changes in two yield-associated traits, increased tillers and early flowering (Figure 5).

OsmiR393 overexpression reduced the mRNA abundance of OsTIR1 and OsAFB2 (Figure 3) but did not change the content of IAA (Figure 7). The OsmiR393-overexpressing rice also decreased sensitivity to two synthetic auxin analogs (Figure 8, 9). Auxin acts through the TIR1 auxin receptor protein [43]. Upon auxin binding, TIR1 recruits specific transcriptional repressors (the Aux/IAA repressors) for ubiquitination by the SCF complex [43]. This process leads to the degradation of the Aux/IAAs repressors by the proteasome. The degradation of the repressors leads in turn to the potentiation of auxin response factors and AFB-mediated transcription of specific genes in response to auxins [44]. Our results indicated that OsmiR393 regulated the abundance of OsTIR1 and OsAFB2 mRNA (Figure 3), which are involved in this auxin pathway.

The two new phenotypes (increased tillers and early flowering) of OsmiR393-overexpressing rice plants might arise from the hypersensitivity to auxin. Auxin induces shoot apical dominance; the axillary buds are inhibited by auxin [45]. However, the OsmiR393-overexpressing plant showed more tillers (Figure 5B) indicating that the inhibitory effect is removed, and the growth of lateral buds is enhanced at a high concentration of auxin. Auxin might also play a minor role in the initiation of flowering (Figure 5A).

3. A gene chain model from OsmiR393 to rice tillering via auxin signaling

Similar to Arabidopsis, the posttranscriptional repression of OsTIR1/OsAFB genes by OsmiR393 alters rice auxin responses (Figure 4). Here, we found that an auxin transporter gene (OaUX1) and a rice tiller inhibitor gene (OsTB1) were repressed by the overexpression of OsmiR393 (Figure 10). Therefore, we propose a pathway for the controlling of rice tillering by OsmiR393 (Figure 11). OsmiR393 represses the expression of OsTIR1 and OsAFB, which further represses the expression of OaUX1 (Figure 10A). Therefore, less auxin is transported to the auxiliary buds resulting in low-levels of auxin in the auxiliary buds. The low levels of auxin in the auxiliary buds induce downregulation of OsTB1 (a tillering inhibitor) [24] (Figure 10D). The downregulation of OsTB1 promotes auxiliary bud development and finally results in an increase in tillers in the OsmiR393-overexpressing rice plants (Figure 5).

4. Implications for rice breeding

Tillering ability and flowering time control are often associated with yield-related traits of rice; therefore, both traits are very important agronomic traits for the genetic improvement of rice. OsmiR393 overexpression could improve the tillering and early

Figure 8. Effects of 2,4-dichlorophenoxyacetic acid (2, 4-D, a synthetic auxin analog) on callus induction. Callus induction under 2 mg/L (A), 3 mg/L (B) and 4 mg/L (C) of 2,4-D. Callus-inducing rates of OsmiR393-overexpressing rice seeds were much lower than that of controls, and they easily generated buds and roots. Bar = 0.5 cm. D. Statistics of callus-inducing rate after 21 days of cultivation; * and ** indicate significance by the Duncan’s multiple range tests at the 5% or 1% level, respectively. doi:10.1371/journal.pone.0030039.g008
flowering of rice (Figure 5), which are positive goals for molecular breeding of rice. However, a previous study [17] and this study also found that OsmiR393 was negatively involved in the salt and drought stress responses. The OsmiR393-overexpressing rice plants were less tolerant to NaCl or drought treatments (Figure 6). These may cause a dilemma regarding the use of OsmiR393 in rice molecular breeding, which could increase tillers and early flowering but could also impair the stress tolerance.

Materials and Methods

1. Vector construction

Rice mature OsmiR393 sequences (accession: MIMAT0000957) were downloaded from miRBase (http://www.mirbase.org). A vector with artificial microRNAs (amiRNAs) OsmiR393 was constructed following the procedures from the website (http://wmd3.weigelworld.org/downloads/modify_pNW55.pdf) and the descriptions by Warthmann [46]. Briefly, the three resultant fragments (amplification from pNW55 with the primers designed by the website for the rice miR393 sequence) were gel purified (TransGen Biotech, Lot #E30719) and fused by a PCR reaction with the two flanking primers G-4368 (with the restriction site Bgl II) and G-4369 (with the restriction site Afl II) using 1 μl of the mixture of the three previous PCR products as a template. The fusion product of 554 bp was cloned into pGEM-T Easy Vector (Promega, USA). After verification by sequencing, the constructs were digested with Bgl II and Afl II and transferred to the binary vector pCambia1301 (pCambia, Australia) by replacing the gus site. In pCambia1301, the expression of the transgene is driven by the 35S CaMV promoter. The Ami3939 plant expression vector and the control vector pCambia1301 were transformed into Agrobacterium tumefaciens strain EHA105.

2. Rice transformation and trait records

The rice variety Zhonghua11 (japonica) was transformed by Agrobacterium-mediated transformation [20] and selected with hygromycin. All regenerated T₀ transgenic plants were genotyped using the primer O27F (within the CaMV 35S promoter) and G4369 (Table S1). Plants were grown in the South China Botanical Garden fields under normal growth seasons. The number of total tillers was determined after transplanting on the indicated days, and the flowering time was compared with the controls.

3. Target gene analysis of OsmiR393 with bioinformatics

The prediction of target genes was performed by the web-based prediction program miRU (Plant microRNA Potential Target Finder, http://bioinfo3.noble.org/miRNA/miRU.htm). The TIGR Rice Genome mRNA database and putative targets with an expectation score of ≤3 for each of 20 nucleotides were selected; other options were set to default. The gene expression was also analyzed in the chip-expression database (RiceXPro: http://ricexpro.dna.affrc.go.jp/index.html).

4. RT-PCR/qRT-PCR

4.1. Small RNA extraction and reverse transcription

Small RNA was extracted from rice using an RNAiso kit for small RNA (Takara, Cat # D340A) and digested with DNase I (Takara, Code: D2215) according to the product manuals. Reverse transcription was performed with a cDNA Synthesis Kit (Promega, Cat # M1701) in combination with stem-loop RT-PCR technique [47]. Briefly, the reaction was performed with 1 μg of total RNA, 2 μL 0.05 μM RT primer mix (0.05 μM OsmiR393 RT primer and U6 RT primer each) in a volume of 12.5 μl. The
reaction was incubated at 65°C for 5 min and then in ice-water for at least 2 min to ensure the formation of stem-loops and combination with miRNA from the miRNA-Primer Mix. Then, 4 μl of the 5× M-MMLV Reverse Transcriptase buffer, 1 μl M-MMLV, 2 μl 10 mM each dNTPs, 0.5 μl RNase inhibitor (0.25 U/μl) were added to the 12.5 μl miRNA-Primer Mix for a total reaction volume of 20 μl. The reaction was incubated at 25°C for 25 min, 42°C for 30 min and 70°C for 10 min to inactivate the reverse transcriptase. To reduce the high abundance effect on PCR amplification, the reverse transcription mixture was diluted 20 times with water.

4.2. RNA extraction for total RNA and reverse transcription. Total RNA was extracted from rice with Trizol (Invitrogen). The total RNA reverse transcription reaction was performed with a two-step RT-PCR kit (Promega, Cat.# M1701) on 2 μg of total RNA according to the product manual.

4.3. Expression detection by real-time quantitative PCR. Gene expression was analyzed by quantitative real time RT-PCR and semi-quantitative RT-PCR using the primers listed in Table S1. PCR amplification was performed in the presence of the double-stranded DNA-specific dye SYBR Green (Takara) and monitored in real time with the 7500 RT-qPCR system (Applied Biosystems). Rice Actin1 (LOC_Os03g50885) served as the standard for normalizing the expression of predicted target genes in semi-quantitative RT-PCR. snRNA U6 [48] served as a reference gene for the detection of miRNAs in quantitative real time RT-PCR.

PCR amplification reactions were performed in a volume of 20 μl containing 10 μl of TaqMan Master Mix SYBR Premix Ex Taq (Takara), 1 μl of 20 times diluted cDNA and 0.8 μl each of forward and reverse primer (10 μM each). Cycling conditions were as follows: 95°C for 30 sec, followed by 40 cycles of 95°C for 10 sec and 61°C for 20 sec. PCR reactions were performed on the 7500 RT-qPCR system (Applied Biosystems). Raw Cq values were calculated using the SDS software v.2.0.1 using automatic baseline settings and a threshold of 0.2.

![Figure 10. Expression comparison of genes downstream of OsAFB2 and OsTIR1 that control the tillers and flowering time.](image)

Figure 10. Expression comparison of genes downstream of OsAFB2 and OsTIR1 that control the tillers and flowering time. Total RNA was isolated from the booting rice plants grown in field. Rice Actin1 was used as an internal control. Two auxin transporter homologs (A, B), an enhancing tiller gene (C), a tillering inhibitor (D) and two genes promoting flowering (E, F) of rice were compared. doi:10.1371/journal.pone.0030039.g010

![Figure 11. A gene chain model from OsmiR393 to tillering via auxin signaling.](image)

Figure 11. A gene chain model from OsmiR393 to tillering via auxin signaling. Upregulation of OsmiR393 represses expression of two auxin receptors (OsAFB2 and OsTIR1). Downregulation of OsAFB2 and OsTIR1 then leads to reduced expression of an auxin transporter (OsAUX1). Low levels of OsAUX1 results in a decrease of auxin transported to the auxiliary buds. The low auxin in auxiliary buds reduces expression of a tillering inhibitor (OsTB1). Finally, the downregulation of OsTB1 promotes the tillering of rice. doi:10.1371/journal.pone.0030039.g011
5. Extraction, purification and determination of indole-3-acetic acid (IAA)

The analysis of indole-3-acetic acid (IAA) was performed according to Kuraishi et al. [49] and Agar et al. [50]. The frozen sample (2 g) was powdered in liquid nitrogen. The isolation process for IAA followed a previously published protocol [49,50]. The hormone extracts were injected into WATERS2695 High Performance Liquid Chromatography (HPLC, American) to measure IAA. A standard IAA sample was obtained from Sigma-Aldrich (St. Louis, MO).

6. Effect of 2,4-dichlorophenoxyacetic acid (2, 4-D) on callus induction

Sterilized seeds of rice were inoculated on MS medium containing 2, 3 and 4 mg/L of 2,4-D [51]. The cultures were incubated at 26±2°C in the dark. After 21 days of inoculation, callus generation rates induced from the different mature seeds were calculated and images were acquired.

7. Response of seeds and seedlings to auxin treatments

The seeds were soaked in 0, 0.001 and 0.0001 mg/L NAA overnight. Subsequently, the soaked seeds were transferred to a petri dish with a filter paper soaked with different concentration of NAA with high humidity and then incubated in the dark at 26±2°C. After 10 days of germination and growth, root numbers, root numbers, root length and seedling length were measured and images were acquired.

8. Salt and drought treatments

To examine the effects of salt stress on germination, the rice seeds were germinated in NaCl solution for 15 days. The seedlings (3 mm in length) were calculated as the germinated seeds. For drought treatment, the 6-day seedlings were removed from the Hoagland’s Solution [23] for 1 day and then re-cultivated in Hoagland’s Solution for 4 days.

9. Statistical analysis

All the data were analyzed from at least 30 plants for each trait with SAS software. Statistical analysis was performed with Duncan’s multiple range tests.

Supporting Information

Figure S1 Monitor cleavage of OsAFB2 and OsTIR1 miRNA by 5’-RACE. Total RNAs were extracted from the wild type rice ZH11 and OsmiR393 overexpressing line. A. The target site schematic diagram of OsAFB2 and OsTIR1 miRNAs by OsmiR393 and the expected sizes of 5’-RACE with their gene specific primers. B. Image of 5’-RACE. (TIF)

Table S1 Primer sequences used in this study. (DOC)

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Author Contributions

Conceived and designed the experiments: KFX MYZ. Performed the experiments: RW KFX XJO ZMF JD CET. Analyzed the data: KFX RW YQW CET MYZ. Contributed reagents/materials/analysis tools: RW KFX XJO. Wrote the paper: MYZ KFX CET.
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