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Running title: OsmiR396d integrates BR and GA singling in rice

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OsmiR396d miRNA affects gibberellin and brassinosteroid signaling to regulate plant architecture

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One sentence summary: The microRNA396d connects OsBZR1 with OsGRFs to regulate the signal of brassinosteroid and gibberellin for controlling the key agriculture yield traits in rice.

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ABSTRACT

Genetic improvement of plant architecture is one of the strategies for increasing the yield potential of rice (*Oryza sativa*). Although great progress has been made in the understanding of plant architecture regulation, the precise mechanism is still an urgent need to be revealed. Here, we report that over-expression of *OsMIR396d* in rice results in semi-dwarf and increased leaf angle, a typical phenotype of BR enhanced mutant. OsmiR396d is involved in the interaction network of BR and GA signal. In *OsMIR396d* over-expression plants, BR signaling was enhanced. In contrast, both the signaling and biosynthesis of GA were impaired. BRASSINAZOLE-RESISTANT1 (OsBZR1), a core transcription activator of BR signaling, directly promoted the accumulation of OsmiR396d which controlled BR response and GA biosynthesis by regulating the expression of different target genes respectively. *GROWTH REGULATING FACTOR 6* (*OsGRF6*), one of OsmiR396d targets, participated in GA biosynthesis and signal transduction, but was not directly involved in BR signaling. This study provides a new insight into the understanding of interaction between BR and GA from multiple levels on controlling plant architecture.

INTRODUCTION

One of the major goal for cereal crop breeding is to increase grain yields. An ideal plant architecture is a concept for high grain yield in rice breeding (Khush, 2013). Moderate plant height and erect leaves are two of the main factors for ideal plant architecture in rice (Yuan, 2001). In the 1960s, the introduction of semi-dwarf gene *sd1* greatly increased rice yields throughout Asia, and brought about the "green revolution", as a result of increased lodging resistance and harvest index in the semi-dwarf *sd1* mutant (Spielmeyer et al., 2002). Leaf angle has progressively decreased due to genetic improvement of *japonica* and *indica* rice cultivars in China in recent decades (Yang et al., 2006; Wu et al., 2007; Hao et al., 2010). Erect leaves can capture more sunlight and store more nitrogen, thus improving grain filling and increasing rice yield (Sinclair & Sheehy, 1999). For example, the erect leaf rice mutant, *osdwarf4-1*, which is partially deficient in biosynthesis of brassinosteroid (BR), can improve biomass production and

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grain yield under dense planting conditions without extra fertilizer (Sakamoto et al., 2006).

Gibberellin (GA) and brassinosteroid (BR) are two predominant plant hormones that determine plant height and leaf angle by regulating cell growth (Tong et al., 2014; Zhang et al., 2014). GA is perceived and bound by the soluble protein GIBBERELLIN INSENSITIVE DWARF1 (GID1), which results in GID1 conformation change, thus the interaction of GID1 and DELLA proteins (GA response repressors) is promoted (Ueguchi-Tanaka et al., 2007; Hedden & Sponsel, 2015). The GA-GID1-DELLA complex recruits an F-box protein (SLY1 in *Arabidopsis* and GID2 in rice), and subsequently DELLA is ubiquitinated by SCF E3 ubiquitin ligase and then degraded through the proteasomal degradation pathway. Following this, GA signal transduction is released from repression by DELLA proteins and cell elongation is promoted (McGinnis et al., 2003; Sasaki et al., 2003; Hirano et al., 2010).

BR signal transduction pathway is mainly shared in *Arabidopsis* and rice (Zhang et al. 2014). BR is perceived by leucine-rich repeat receptor-like kinases BRASSINOSTEROID-INSENSITIVE 1 (BRI1) and its co-receptor BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) (Clouse et al., 1996; Yamamuro et al., 2000; Li et al., 2002; Wang, et al., 2008). Then, the signal is passed along to a key negative regulator, BRASSINOSTEROID-INSENSITIVE 2 (BIN2), which is dephosphorylated by BRI1 SUPPRESSOR 1 (BSU1) (Li et al., 2001; Mora-Garcia et al., 2004; Kim et al., 2011). In rice, the dephosphorylation of GSK3/SHAGGY-like Kinase (OsGSK2) (a rice homolog of *Arabidopsis* BIN2) releases its suppression on BRASSINAZOLE-RESISTANT1 (OsBZR1), LEAF AND TILLER ANGLE INCREASED CONTROLLER (OsLIC), DWARF AND LOW-TILLERING (OsDLT), OVATE Family Protein 8 (OsOFP8) and REDUCED LEAF ANGLE1 (RLA1) to activate BR response and increase leaf angle (Bai et al., 2007; Wang, L et al., 2008; Tong et al., 2009; Tong et al., 2012; Zhang et al., 2012; Yang et al., 2016, Qiao et al., 2017). The kinase activity of OsGSK2 can also be directly inhibited by membrane protein GW5 (QTL for grain width and weight on chromosome 5), which is a calmodulin-binding protein (Liu et al., 2017).

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The crosstalk network between BR and GA is quite complicated. BR and GA interact not only at biosynthesis regulation level, but also at signaling level. And their crosstalk differs depending on hormone concentrations, development stages, tissues and species (Tong & Chu, 2016; Unterholzner et al., 2016). BR signaling defected mutants are impaired in GA biosynthesis in *Arabidopsis* and rice (Tong et al., 2014; Unterholzner et al., 2015). Expression of GA biosynthesis gene *GA20ox1* under the promoter of *BRI1* in *bri1-301* mutant can rescue most of its defects (Unterholzner et al., 2015). *Arabidopsis* BES1 and rice OsBZR1 can bind the promoters of GA biosynthesis or inactivation genes to regulate GA biosynthesis (Tong et al., 2014; Unterholzner et al., 2015). On the other hand, GA regulates BR biosynthesis at transcription level. OsSPY, a negative regulator of GA signaling, negatively regulates BR biosynthesis (Shimada et al., 2006). OsGSR1, a positive regulator of GA signaling, activates BR synthesis by directly interacting with BR biosynthesis enzyme, DWF1 (Wang et al., 2009). There is also a physical interaction between BR and GA at the signaling level. The GA signaling negative regulator, DELLA protein, interacts with BZR1/BES1 and inhibits their function. Thus GA can release the inhibition of DELLA on BZR1/BES1 to induce downstream BR responses in *Arabidopsis* (Bai et al., 2012; Gallego-Bartolome et al., 2012; Li et al., 2012). Although, great progresses have been made in this aspect, the knowledge we get now is far from enough to understand the interaction network of BR and GA. More related research work need to be carried out.

MicroRNAs (miRNAs) also participate in hormone regulation and plant development, especially in integrating distinct agricultural traits (Tang & Chu, 2017). miR396 represents one of the most deeply conserved miRNA families in plants (Liu et al., 2009). Many studies have been performed in *Arabidopsis* and rice focusing on miR396 and its targets, *GROWTH REGULATING FACTORS* (*GRFs*). In rice, OsmiR396 and its targets, *OsGRFs*, regulate leaf development, plant height, meristem function, flowering time, inflorescence architecture, and seed size (Luo et al., 2005; Kuijt et al., 2014; Liu et al., 2014; Che et al., 2015; Duan et al., 2015; Gao et al., 2015). OsGRF4 interacts with OsGIFs to control seed size through BR signaling, and its transcription activity can be directly regulated by OsGSK2 (Che et al., 2015; Duan et al., 2015). OsGRF6 is involved in floral organogenesis by regulating the expression of

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JMJD2 family jmjC gene 706 (*OsJMJ706*) and *Crinkly4 receptor-like Kinase* (*OsCR4*) (Liu et al., 2014). OsGRF6 also modulates inflorescence architecture through the IAA regulating pathway (Gao et al., 2015).

It is well established that OsmiR396 targets *OsGRFs* to function in the regulation pathway of multiple hormones such as GA, BR, and auxin (van der Knaap et al., 2000; Che et al., 2015; Gao et al., 2015). However, little is known about how miR396 is regulated in plant development. Here, we show that *OsMIR396d* is involved in the BR signaling transduction pathway and is directly activated by the key transcription factor OsBZR1. *OsMIR396d* also participates in the regulation of GA signaling by modulating the expression of *OsGRF6*, which is independent of BR signaling. Our findings reveal that the OsmiR396d can combine the BR and GA regulation pathway to control rice plant architecture.

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RESULTS

Over-expression of *OsMIR396d* changed rice plant architecture

Our previous study showed that *OsMIR396d* over-expression transgenic plants (miROE) exhibited abnormal florets. OsmiR396d target genes, *OsGRF6* and *OsGRF10*, were involved in regulating floral organ identity and husk opening (Liu et al., 2014). In miROE lines, the third leaf angles were increased to two times more than that of Zhonghua 10 (ZH10) at the seedling stage (Fig. 1A, B). The increased leaf angle phenotype was also observed on the top three leaves of miROE lines at the seed filling stage (Fig. 1B). miROE8 had the highest expression level of OsmiR396d among the transgenic lines (Liu et al., 2014) and exhibited the largest leaf angle. The angles of top third leaf of ZH10, miROE2, miROE5, miROE8, and miROE10 were 17°, 29°, 30°, 36°, and 32° respectively (Fig. 1B).

The height of miROE plants was also significantly decreased compared with ZH10 (Fig. 2A). The data showed that the plant height of miROE was only 70-80% of that of ZH10, and every internode of miROE was shortened compared with ZH10 (Fig. 2B, C). The fifth internode of miROE exhibited severe growth retardation in particular (Fig. 2D). The fifth internode length was decreased from 2.6 cm in ZH10 to about 1.0 cm in miROE lines. The relative lengths of each internode (percentage of each internode length to total culm length) had no obvious differences between miROE and ZH10, except that the fifth internode was reduced from 2.6% in ZH10 to about 1.5% in miROE (Fig. 2E).

The collar adaxial cell size was enlarged in *OsMIR396d* over-expression plants

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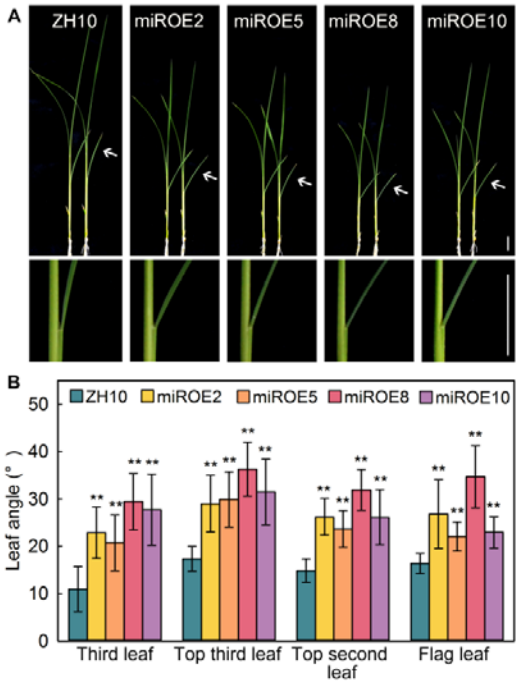

Figure 1. The leaf angle of *OsMIR396d* over-expression plants was increased.

A. The third leaf angle of miROEs was specifically increased. Bar = 1 cm.

B. Statistical analysis of leaf angles. The third leaf angle was measured at the seedling stage and the angles of the top three leaves were measured at the seed filling stage. Error bars indicate SD for at least fifteen plants. Asterisks indicate $P < 0.01$ (**) compared with wild type in the Student's t test analysis.

The promotion of lamina inclination is one of the most typical BR responses in rice, and it is often caused by proliferation or expansion of collar adaxial cells (Wada et al., 1981; Cao & Chen, 1995; Sun et al., 2015). To determine the cell structure basis for increased leaf angle of miROE, the adaxial surface and longitudinal sections of miROE8 and ZH10 lamina joints were observed using scanning electron microscopy (SEM). The lamina joint adaxial side of miROE8 was obviously expanded compared to ZH10 (Fig. 3A, D), and the cell size in the adaxial region of miROE8 was much larger than that of ZH10 (Fig. 3B, C, E, F). Especially, miROE8 cells were expanded nearly one fold larger than ZH10 along the proximal-distal axis (Fig. 3G). In contrast, the cell layers in the lamina joint adaxial side were not distinct between miROE8 and ZH10 at both proximal-distal axis (half frame indicated regions in Fig. 3B and E) and adaxial-abaxial axis. Therefore, these results indicated that the increased leaf angle of miROE might mainly result from enlarged cell size at the adaxial side of the lamina joint.

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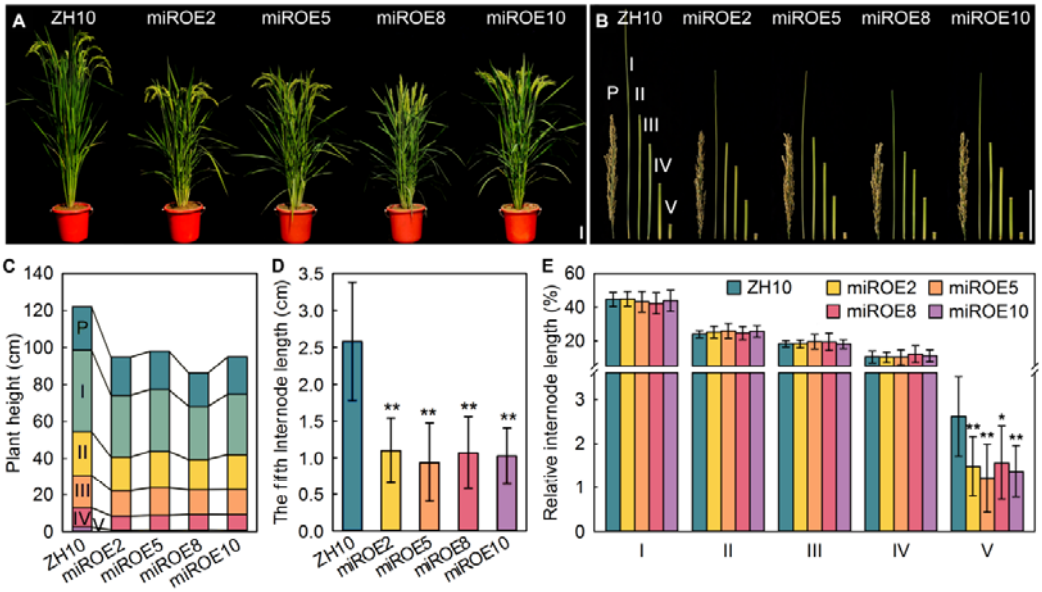

Figure 2. The plant height and length of internodes in *OsMIR396d* over-expression plants were reduced.

A. The phenotype of miROEs and ZH10 at the seed filling stage. Bar = 5 cm.

B. The panicle and internodes of miROEs and ZH10. Bar = 5 cm.

C. Results of statistical analysis of plant height and internode length of miROE lines and ZH10. More than fifteen mature plants were used in each analysis.

D. Results of statistical analysis of fifth internode lengths of miROEs and ZH10. Error bars indicate SD for at least fifteen plants. Asterisks indicate $P < 0.01$ (**) compared with ZH10 in the Student's t test analysis.

E. Relative internode length of miROEs and ZH10. The data shows each internode length relative to the total culm length. Error bars indicate SD for at least fifteen plants. Asterisks indicate $P < 0.05$ (*) and $P < 0.01$ (**) compared with ZH10 in the Student's t test analysis.

Cell elongation and division were arrested in *OsMIR396d* over-expression plants

To figure out the reason for the semi-dwarf phenotype of miROE plants, the cell sizes in the second leaf sheaths were analyzed. Compared with ZH10, the cell length of miROE8 was slightly decreased (Fig. 4A, B). In addition, the cell cycle progression was analyzed by flow cytometry using root apical cells. The results showed that the DNA content of miROE8 was decreased compared to ZH10 (Fig. 4C). Thus, miROE possessed fewer cells in the G2/M phase. The mitotic index, a measure for the proliferation status of a cell population (Simpson et al., 1992), was about 9.4% in miROE8, which was significantly lower than that of ZH10 (16.4%) (Fig. 4D). Therefore, the semi-dwarf phenotype of miROE plants was caused by both impaired cell elongation and reduced cell division.

OsmiR396d is involved in the BR signaling pathway

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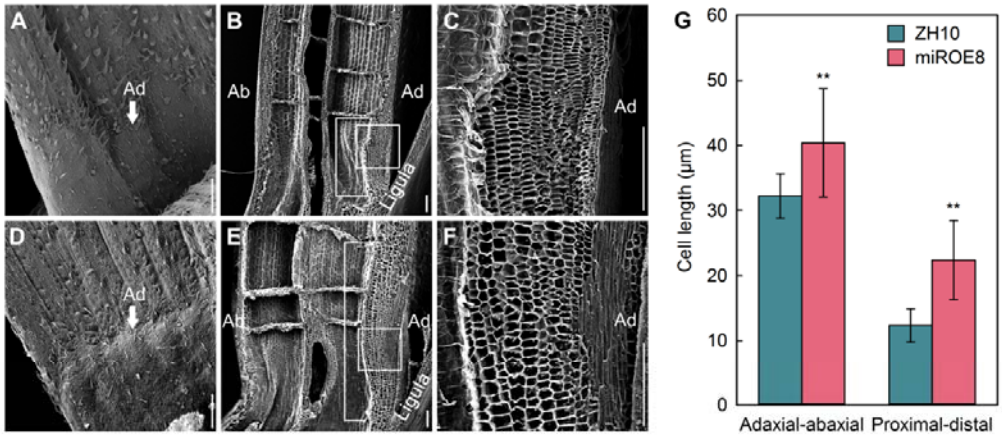

Figure 3. Scanning electron microscopic examination of the leaf lamina joint.

A, B. Adaxial surface of the leaf lamina joint in ZH10 (A) and miROE8 (B). Arrows indicate the increased growth of cells.

C, D. Longitudinal sections of the leaf lamina joint of ZH10 (C) and miROE8 (D). Half frame indicated regions were used to calculate cell layers.

E, F. Close-up of regions denoted by rectangles in (C) and (D) respectively. Ad, adaxial; Ab, abaxial. Scale bars = 200 μm.

G. Statistical analysis of cell length in (C) and (F). Cell length along the adaxial-abaxial axis and proximal-distal axis were measured respectively. Error bars indicate SD for at least 100 cells. Asterisks indicate $P < 0.01$ (**) compared with ZH10 in the Student's t test analysis.

The increased leaf angle and semi-dwarf phenotypes of miROE plants are similar to the phenotypes of BR signal-enhanced rice plants, such as *OsLIC* antisense lines (Zhang et al., 2012) and *ili1-D* mutant (Zhang et al., 2009). Therefore, it is possible that OsmiR396d is involved in positive regulation of BR signaling. To investigate this possibility, the effects of OsmiR396d on BR signaling were examined by leaf angle and root length growth of miROE8 and ZH10 in the presence of 24-epibrassinolide (24-eBL). The leaf angle of miROE8 was increased more efficiently than that of ZH10 when exogenous 24-eBL was applied (Fig. 5A). The root growth of miROE8 was also more sensitive to 24-eBL compared to ZH10 (Fig. 5B). These 24-eBL treatment results implied that OsmiR396d positively regulated BR signaling in rice. To figure out the points of BR signaling impacted with *OsMIR396d* over-expression, the expression levels of several BR regulation related genes were compared between miROEs and ZH10. The qRT-PCR result showed that the positive regulatory genes of BR signaling, *OsBRI1* and *OsDLT1,* were upregulated in miROEs, while the negative regulatory gene of BR signaling *OsLIC* was downregulated (Supplemental Fig. S1). This result indicated that OsmiR396d might regulate multiple steps of the BR signaling pathway.

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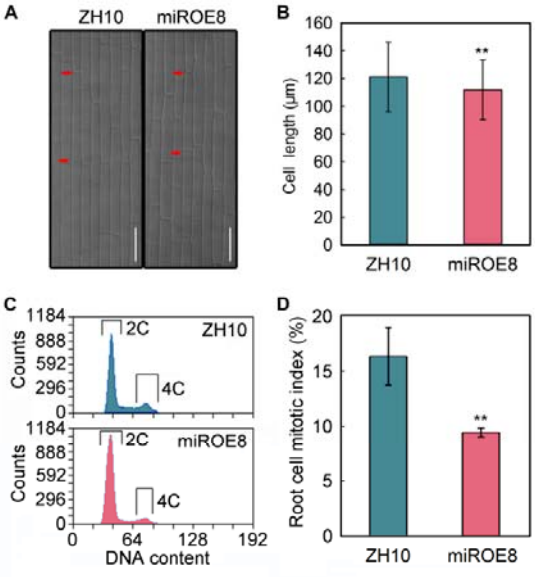

Figure 4. Cell length and cell division analysis.

A. The second leaf sheath cells of 2-week-old seedlings of miROE8 and ZH10. Bars = 50 μm.

B. The statistical analysis of cell length in the second leaf sheath. Error bars indicate SD for more than 400 cells. Asterisks indicate $P < 0.01$ (**) compared with ZH10 in student's t test analysis.

C. The DNA content of miROE8 and ZH10. After the seeds germinated for 3 days at 28°C, the root tips were harvested. Cell nuclei (10,000) were stained with 1 μg mL^{-1} DAPI and analyzed by flow cytometry. 2C and 4C represent the DAPI signals that correspond to nuclei with different DNA contents.

D. Cell mitotic index in root apical meristem in miROE8 and ZH10. The results were from three independent replications. Error bars indicate SD. Asterisks indicate $P < 0.01$ (**) compared with ZH10 in the Student's t test analysis.

How BR influences *OsMIR396d* expression was also investigated. The response of *OsMIR396d* to 24-eBL was detected by qRT-PCR. Compared with the control, the expression level of *OsMIR396d* was upregulated under 24-eBL treatment and reached the highest level after treatment for 3 h (Fig. 5C). Consistently, the transcript level of several *OsGRFs* decreased after BR treatment for 3 h (Supplemental Fig. S2). Furthermore, the expression level of *OsMIR396d* in BR signaling enhanced or impaired rice plants was checked. The qRT-PCR results showed that the expression of *OsMIR396d* was increased in the BR-signal enhanced *OsLIC* antisense line (LIC-AS) (Zhang et al., 2012) and the *OsBZR1* over-expression line (BZR1-OE), but decreased in the BR-signal reduced *OsBZR1* RNAi line (BZR1R) (Bai et al., 2007) (Fig. 5D). These results indicated that *OsMIR396d* transcription was induced by both exogenous BR treatment and endogenous BR signal.

***OsMIR396d* is a direct target of OsBZR1**

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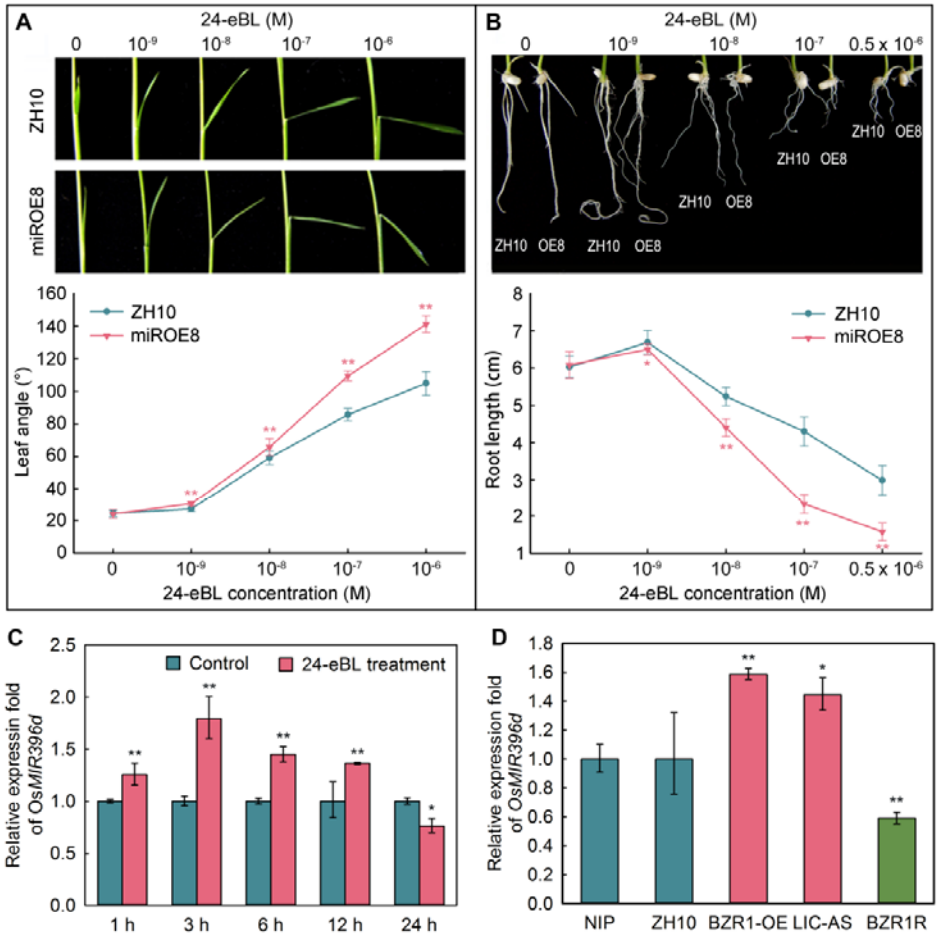

Figure 5. OsmiR396d is involved in BR signaling.

A. Lamina joint inclination assay of miROE8 and ZH10. The second lamina joints of one-week-old seedlings grown under dark condition were cut off and treated with different concentrations of 24-eBL for three days. Error bars indicate SD for at least fifteen plants. Asterisks indicate $P < 0.05$ (*) and $P < 0.01$ (**) compared with ZH10 in the Student's t test analysis.

B. The seminal root growth of miROE8 and ZH10 under treatment with different concentrations of 24-eBL. The seminal root length was measured after seedlings were grown in 24-eBL containing 1/2 MS medium for one week. Error bars indicate SD for at least fifteen plants. Asterisks indicate $P < 0.05$ (*) and $P < 0.01$ (**) compared with ZH10 in the Student's t test analysis.

C. The expression pattern of OsMIR396d under treatment with 24-eBL. Two-week-old ZH10 seedlings were treated with 1 μM 24-eBL. Whole plants were sampled 1, 3, 6, 12 and 24 h after treatment. The plants sampled from the normal growth condition were used as control. *UBQUITIN1* was used as a reference gene. Error bars indicate SD for three replicates. Asterisks indicate $P < 0.05$ (*) and $P < 0.01$ (**) compared with control in the Student's t test analysis.

D. The expression of *OsMIR396d* in *OsLIC* antisense plants (LIC-AS), *OsBZR1* RNAi plants (BZR1R), and OsBZR1 over-expression (BZR1-OE) plants. LIC-AS and BZR1R were under the background of ZH10 and BZR1-OE was under the background of Nipponbare (NIP). *UBQUITIN1* was used as reference gene. Error bars indicate SD for three replicates. Asterisks indicate $P < 0.05$ (*) and $P < 0.01$ (**) compared with wild-type ZH10 or NIP respectively in the Student's t test analysis.

208 The expression of *OsMIR396d* was upregulated in BZR1-OE plants and

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209 downregulated in BZR1R lines (Fig. 5D). It was reported that transcription factor

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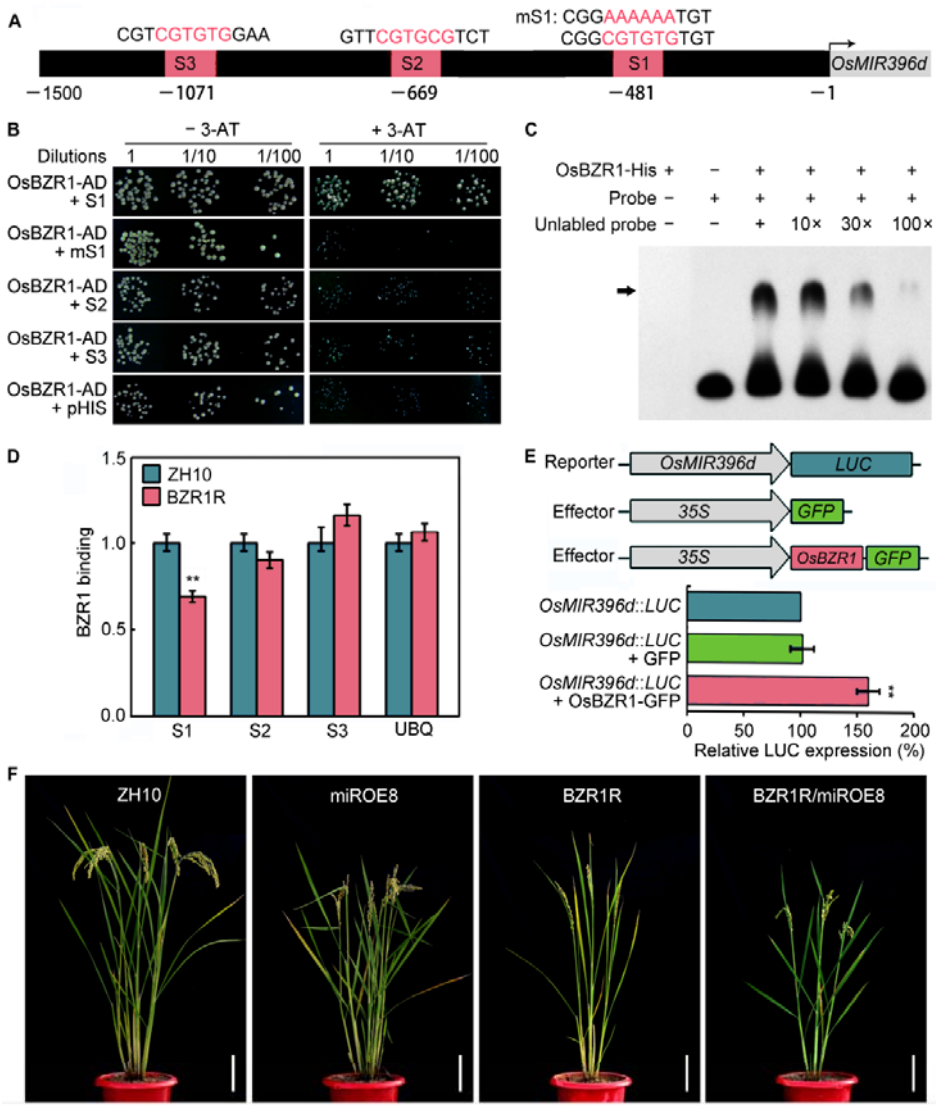

Figure 6. OsBZR1 directly activates the expression of *OsMIR396d*.

A. Localization of three putative OsBZR1 binding sites S1, S2, and S3 in the OsMIR396d promoter.

B. Yeast one-hybrid analysis of OsBZR1 binding the promoter of OsMIR396d. Empty pHIS vector was used as negative control.

C. EMSA analysis of OsBZR1 binding the promoter of OsMIR396d. S1-containing DNA fragment was labeled with biotin. The arrow indicates the complex of OsBZR1-His and the DNA probe.

D. ChIP-qPCR analysis of OsBZR1 binding the promoter of OsMIR396d using OsBZR1 antibody to enrich DNA. The UBIQUITIN DNA fragment was used as an internal control. Error bars indicate SD for three replicates. Asterisks indicate $P < 0.01$ (**) compared with ZH10 in the Student's t test analysis.

E. Transactivation assay of OsBZR1 with the luciferase reporter system. Error bars indicate SD for three replicates. Asterisks indicate $P < 0.01$ (**) compared with control in the Student's t test analysis.

F. The phenotype of OsBZR1 RNA interference transgenic plants (miROE8/*BZR1*R) under the background of miROE8. Bar = 10 cm.

210 BZR1 binds to CGTGT/CG element (He et al., 2005; Zhang et al., 2012). The 1.5 kb

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sequence of the *OsMIR396d* promoter was screened, and three putative BZR1 binding sites were found at -479 (S1), -667 (S2), and -1069 (S3) bp upstream of the pre-miR396d transcription start site (Fig. 6A). These results implied that *OsMIR396d* is potentially targeted by the transcription factor OsBZR1.

To test the direct regulation of OsBZR1 to *OsMIR396d*, yeast one-hybrid assay was carried out. The data showed that OsBZR1 could bind the S1 site but not the S2 or S3 site. The mutation of S1 sites led to disappeared binding ability (Fig. 6B). The EMSA assay further confirmed this possibility. The His-tagged OsBZR1 protein could physically bind the S1 site containing DNA fragment *in vitro*, and the excess unlabeled competitive probe could effectively inhibit the biotin probe bound by OsBZR1 (Fig. 6C). Furthermore, OsBZR1 bound the promoter of *OsMIR396d in vivo* was analyzed through ChIP-qPCR via the antibody against OsBZR1. The S1-containing DNA fragment was significantly decreased in BZR1R plants compared with ZH10, while the amount of S2 and S3-containing DNA fragments was not significantly different between BZR1R and ZH10 (Fig. 6D). In addition, activity of luciferase was used as a reporter to detect the effect of OsBZR1 on *OsMIR396d* expression in *Arabidopsis* protoplasts. The data showed that the luciferase activity of *OsMIR396d::LUC* transfected protoplasts was increased when OsBZR1-GFP was co-expressed (Fig. 6E). This result suggested that OsBZR1 positively regulates the expression of *OsMIR396d*.

To explore the genetic relationship of *OsMIR396d* and *OsBZR1*, the *OsBZR1* RNAi vector was transformed into miROE8 to get double transgenic plants (BZR1R/miROE8) (Supplemental Fig. S3). The leaf angle of BZR1R/miROE8 was increased compared with BZR1R or ZH10 (Fig. 6F; Supplemental Fig. S4A, B). This result suggested that the function of OsBZR1 might be partially dependent on OsmiR396d. Additionally, miROE8 was crossed with BR receptor *OsBRI1* mutant *d61* (Nakamura et al., 2006). The leaf angles were significantly increased in the progenies of miROE8/*d61* compared with the parents (Supplemental Fig. S4). This result further confirmed that *OsMIR396d* was involved in the BR signal regulated leaf angle development process. These biochemical and genetic evidences indicate that *OsMIR396d* was one direct target gene of OsBZR1.

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OsmiR396d is involved in the GA regulation pathway

The *OsMIR396d* over-expression plants displayed decreased internode length, especially the shortened fifth internode (Fig. 2). This characteristic was similar to GA deficient mutants such as *d18*, *d35*, and *sd1* (Itoh et al., 2004). The cross progenies miROE8/*d61* showed not only increased leaf angle but also reduced plant height compared with their parents (Supplemental Fig. S4). These phenotypes implied that OsmiR396d was possible to control plant height through GA signaling in addition to the BR-dependent pathway.

To examine if OsmiR396d controls plant height through GA signaling, the GA sensitivity of miROEs was analyzed by measuring the second leaf sheath length under GA treatment. The results showed that the second leaf sheath length of both miROE8 and ZH10 were distinctly elongated under GA treatment (Fig. 7A). However, by comparison, the leaf sheath of miROE8 was less sensitive to GA_3 than that of ZH10. The expression levels of GA signaling and biosynthesis related genes were also checked in miROEs and ZH10. Results from qRT-PCR showed that the transcription levels of GA signaling pathway gene *OsGID2* and several GA biosynthesis pathway genes (including *OsCPS1*, *OsKO2*, *OsGA20ox1*, and *OsGA20ox3*) were apparently reduced in miROEs compared with ZH10 (Fig. 7B). The expression pattern of *OsMIR396d* after treatment with paclobutrazol (PAC, GA biosynthesis inhibitor) or GA_3 was also analyzed in ZH10 seedlings. The result showed the expression of *OsMIR396d* was typically repressed by continuous PAC treatment, but was upregulated after treatment with a high concentration of GA_3 (Supplemental Fig. S5). These findings suggested that OsmiR396d participates in the GA signaling pathway and regulates internal GA biosynthesis as well.

OsmiR396d targeted *OsGRF6* to regulate the GA pathway

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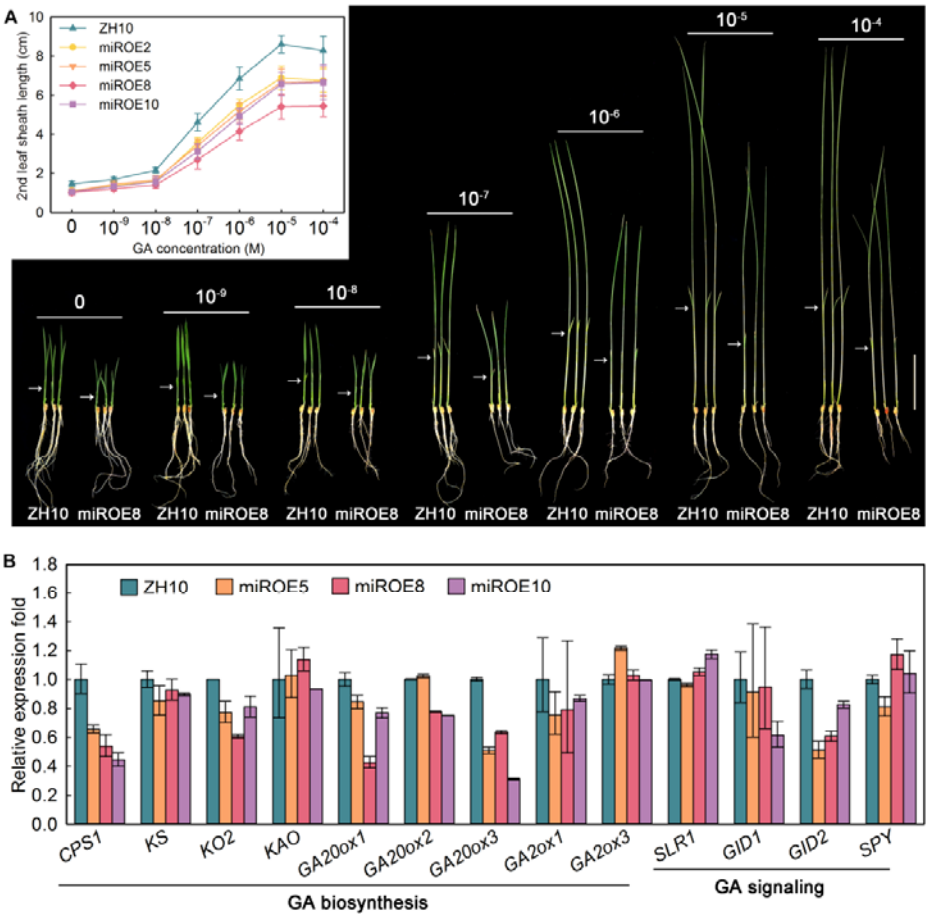

Figure 7. GA signaling and biosynthesis were impaired in *OsMIR396d* over-expression plants.

A. The phenotype of miROE8 and ZH10 seedlings growth under different concentrations of GA_3 treatment. The seeds were treated with 10 μM PAC for 2 days before cultivation in nutrient solution containing the indicated concentration of GA_3. After 10 days of growth, the seedlings were photographed. The measurements of the second leaf sheath are shown. Error bars indicate SD for at least fifteen plants. Bar = 5 cm.

B. The expression of GA pathway related genes in miROE and ZH10. *UBQUITIN1* was used as reference gene. Error bars indicate SD for three replicates.

GRFs are predicted to be the targets of miR396 (Jones-Rhoades & Bartel, 2004; Wu et al., 2009). *OsGRF6,* one target of OsmiR396d, was involved in floral organogenesis under the regulation of OsmiR396d (Liu et al., 2014). We were also interested in determining whether *OsGRF6* participates in BR and GA signaling in a similar manner as *OsMIR396d*. Our previous data showed that the plant height of *osgrf6* mutant was decreased compared with wild type Dongjin (DJ) (Liu et al., 2014). *OsGRF6* antisense

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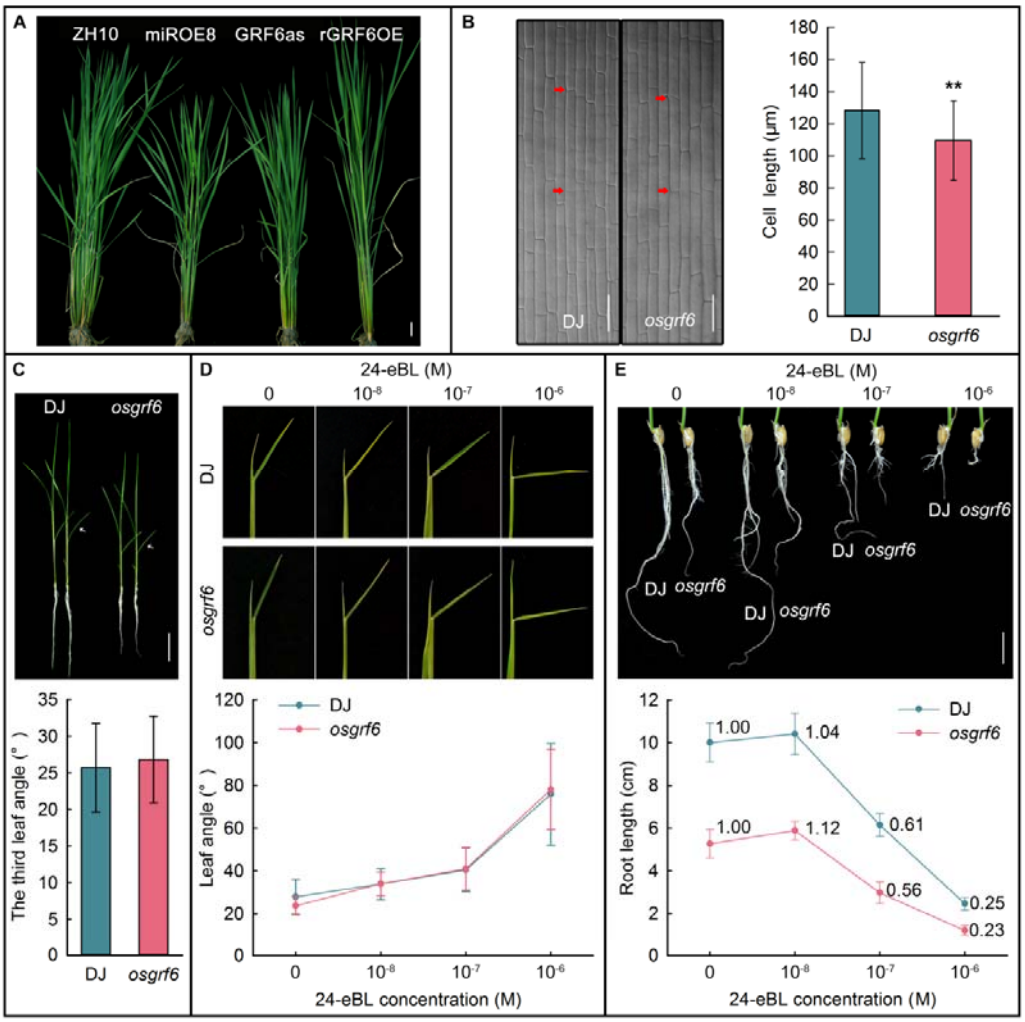

Figure 8. BR signaling was not affected in *osgrf6* mutant.

A. The phenotype of *OsGRF6* antisense transgenic plant (GRF6as) and the OsmiR396d-resistant form of *OsGRF6* transgenic plant (rGRF6OE). Bar = 5 cm.

B. The second leaf sheath cells of 2-week-old seedlings of *osgrf6* and DJ. The cell length was measured. Error bars indicate SD for more than 400 cells. Asterisks indicate $P < 0.01$ (**) compared with DJ in the Student's t test analysis. Bar = 50 μm.

C. The leaf angle of *osgrf6* was not changed. The third leaf angle was measured. Error bars indicate SD for at least fifteen plants.

D. Lamina joint inclination assay of *osgrf6* and DJ. Error bars indicate SD for at least fifteen plants.

E. The seminal root growth of miROE8 and ZH10 under treatment with different concentrations of 24-eBL. Error bars indicate SD for at least fifteen plants.

transgenic plants (GRF6as) were also moderately reduced compared with wild type (ZH10) plants, while the OsmiR396d-resistant form of *OsGRF6* transgenic plants (rGRF6OE) had a slightly increased plant height (Fig. 8A). Microscopy data further showed that the cell length of the *osgrf6* second leaf sheath was significantly shortened

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compared with DJ (Fig. 8B). In contrast, the leaf angle of *osgrf6* was very similar to DJ (Fig. 8C). These results suggested that OsmiR396d might target *OsGRF6* to regulate plant height but nearly not affect leaf angle.

In lamina inclination assay, the result showed that the sensitivity of *osgrf6* to 24-eBL was not apparently different from that of DJ (Fig. 8D). In seminal root growth experiment, the root length of *osgrf6* mutant was much shorter than that of DJ under normal growth condition, so we used relative root length to do the analysis following the method used by Tong et. al (2014), and the root length under normal growth condition was used as reference. The relative root length of *osgrf6* was 0.56 and that of DJ was 0.61 at 10^{-7} M 24-eBL (Fig. 8E). This result also showed that there was no apparent distinction in BR sensitivity between *osgrf6* and DJ. Above results indicated that *OsGRF6* might not be directly involved in the BR signaling mediated lamina joint development and root growth processes.

To determine if *OsGRF6* participates in GA regulation, *osgrf6* and DJ seeds were pretreated with 10^{-5} M PAC (to inhibit endogenous GA biosynthesis) or water (control) for 2 days. Then the germinated seeds were planted in nutrient solution containing 10^{-5} M PAC and/or 10^{-5} M GA_3 as indicated in the Fig. 9A. The second leaf sheath of *osgrf6* was significantly shorter than DJ after the treatment for 10 days. When treated only with PAC, there was no difference in second leaf sheath length between *osgrf6* and DJ. However, this difference appeared again when GA_3 was added into the nutrient solution (Fig. 9A, B). This result implied that GA signaling or biosynthesis was likely impaired in *osgrf6*. The reduced sensitivity of *osgrf6* to GA_3 was further confirmed by treating *osgrf6* and DJ with different concentration of exogenous GA_3 (Supplemental Fig. S6). The genes that were downregulated in miROEs were also analyzed in *osgrf6*. Results from qRT-PCR showed that the expression levels of *OsCPS1*, *OsGA20ox1*, *OsGA20ox3* and *OsGA3ox2* were also downregulated in *osgrf6* compared with DJ (Fig. 9C).

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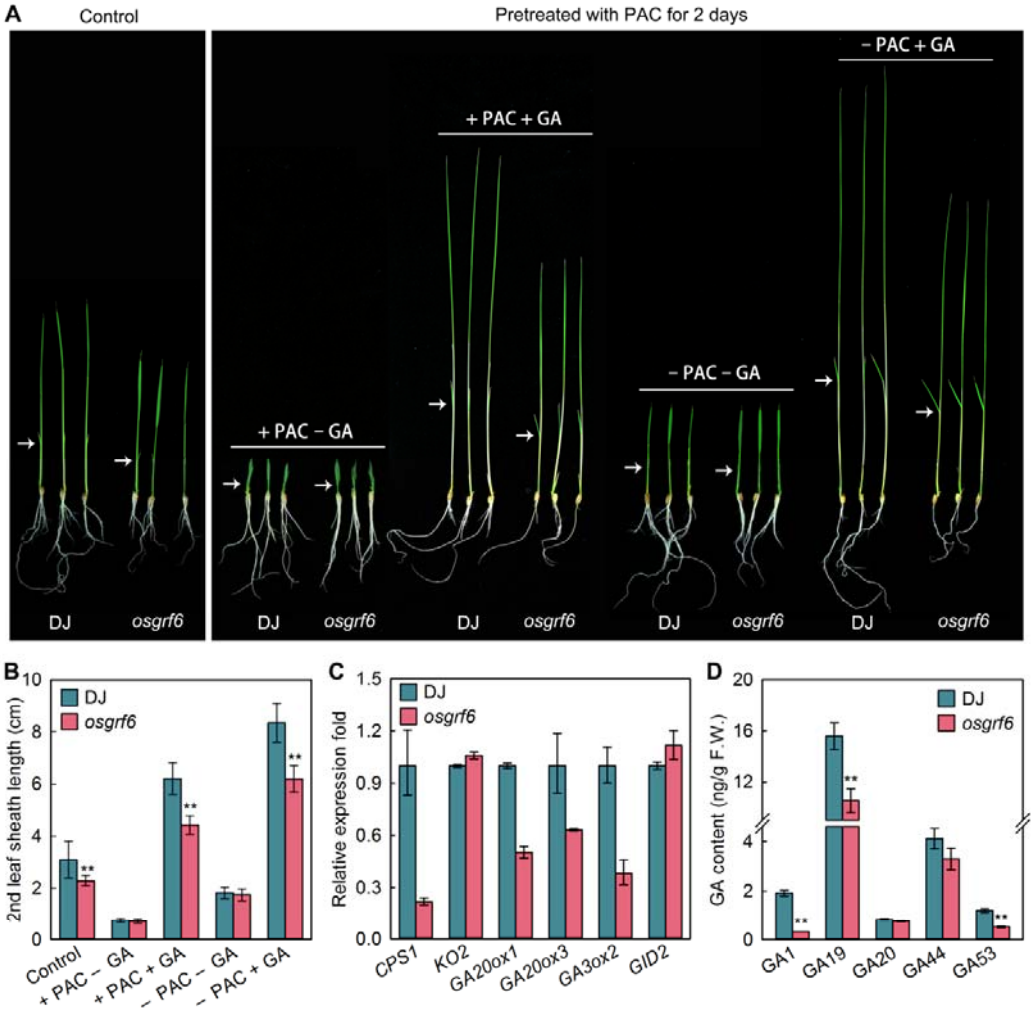

Figure 9. GA signaling and biosynthesis are impaired in the *osgrf6* mutant.

A. *osgrf6* and DJ seedlings treated with PAC or GA_3. The seeds were treated with water (control) or 10^{-5} M PAC for 2 days, and then germinated seeds were planted in PAC and/or GA_3 containing culture solution for 10 days. Bar = 5 cm.

B. The statistical result of the second leaf sheath length of *osgrf6* and DJ grown under PAC and/or GA_3 treatment. Error bars indicate SD for at least fifteen plants. The relative leaf sheath length between DJ and *osrf6* was calculated and shown. Asterisks indicate $P < 0.01$ (**) compared with DJ in student's t test analysis.

C. The expression of GA pathway related genes in *osgrf6* and DJ. *UBQUITIN1* was used as reference gene. Error bars indicate SD for three replicates.

D. Quantification of endogenous GAs in *osgrf6* and DJ. Two-week-old seedlings were sampled. Error bars indicate SD for three replicates. Asterisks indicate $P < 0.01$ (**) compared with DJ in the Student's t test analysis.

To confirm that GA biosynthesis was impaired in *osgrf6*, endogenous GAs were quantified. The results showed that GA_1, GA_{19}, and GA_{53} level were significantly decreased in *osgrf6* (Fig. 9D). These results demonstrated that *OsGFR6* was involved in the regulation of both GA signaling and biosynthesis.

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DISCUSSION

BR and GA are vital hormones in plant that influence various developmental processes, such as cell division, cell elongation, flowering, leaf senescence, and seed germination (Greenboim-Wainberg et al., 2005; Vriet et al., 2013; Fariduddin et al., 2014; Sakata et al., 2014; Hedden & Sponsel, 2015). BR and GA interact with each other at both biosynthesis level and signaling level to form a potential complex network (Wang et al., 2009; Zhang et al., 2012). Here, we report that OsmiR396d participates in this interaction network of BR and GA. OsBZR1, as a node of BR signaling pathway, can directly activate the expression of *OsMIR396d* to repress the expression of *OsGRF6* for regulating GA signaling and biosynthesis (Fig. 10).

OsBZR1 targets *OsMIR396d* to control leaf angle through OsGRF4 mediated BR response

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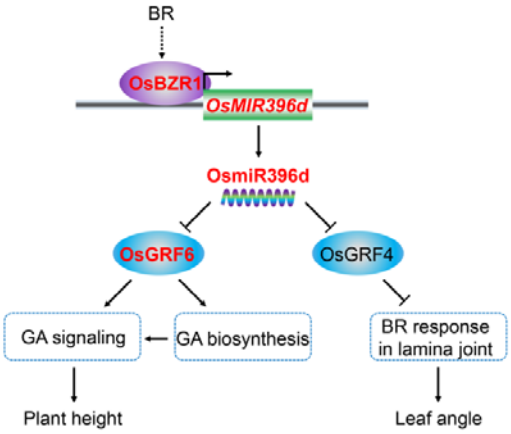

Figure 10. A proposed model for OsmiR396d controlling rice plant architecture. *OsMIR396d* is activated by OsBZR1, which is the key regulator transcription factor of BR signaling. On the right side, OsmiR396d regulates leaf angle by repressing the expression of *OsGRF4*. On the left side, OsmiR396d is involved in GA signaling and biosynthesis by regulating the expression of *OsGRF6* to control rice plant height.

In this study, we propose that OsmiR396d is involved in the BR signaling pathway based on data with four lines. Firstly, over-expression of *OsMIR396d* in rice resulted in increased leaf angle and semi-dwarf phenotype (Fig. 1, 2), which was similar to the phenotype of rice plants with accumulated BR or with enhanced BR signal (Tanaka et al., 2009; Tong et al., 2009; Zhang et al., 2009; Tong et al., 2012; Zhang et al., 2012). Secondly, we demonstrated that the CGTGT/CG element in the *OsMIR396d* promoter was directly bound by OsBZR1 using yeast one-hybrid experiment andEMSA assay *in vitro*, also ChIP-qPCR detection *in vivo*. The results of the luciferase reporter assay indicated that OsBZR1 could activate *OsMIR396d* expression (Fig. 6). Coincidently, the *OsMIR396d* transcript level was increased in *OsBZR1* over-expression plants, but was decreased in *OsBZR1* RNAi plants (Fig. 5D). Thirdly, in the double transgenic plants BZR1R/miROE8, the leaf angle was increased compared with BZR1R (Fig. 6F; S4), which further confirmed that *OsMIR396d* was possibly epistatic to *OsBZR1*. Finally, the miROE8 seedlings with a decreased *OsGRF4* transcript level (Liu et al., 2014) were sensitive to BR (Fig. 5). In addition, near-isogenic line carrying GL2 (NIL-GL2) plants, which possessed enhanced *OsGRF4* expression, showed impaired sensitivity to BR in lamina joints (Che et al., 2015). Therefore, OsmiR396d, under the regulation of OsBZR1, may control rice leaf angle by regulating the expression of *OsGRF4*.

Our data support *OsMIR396d* is directly controlled by *OsBZR1* on the transcription

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level, but we also noticed that over-expression of *OsMIR396d* in OsBZR1 RNAi background only partially rescue the phenotype of OsBZR1i plant. There are three potential reasons for this phenomenon. Firstly, OsBZR1 is not only involved in BR signaling, but also participates in some other biological processes. There are thousands of target genes of OsBZR1, such as *OsLIC*, *DLT*, *IBH1*, *ILI1* and so on (Tong & Chu, 2009; Zhang *et al.*, 2009; Sun *et al.*, 2010; Zhang *et al.*, 2012). *OsMIR396d* is just one of its targets, so *OsMIR396d* alone is not enough to explain all phenotypes of OsBZR1i plant, even the phenotypes specific to BR response. Secondly, *OsMIR396d* is not only controlled by OsBZR1 but also controlled by other transcription factors, such as unknown GA response factors mentioned below. Thirdly, there are twelve putative targets (*OsGRFs*) of OsmiR396d, and they are involved in different development progress. Some of them are not involved in OsBZR1 regulated signaling pathway. For example, OsGRF3 and OsGRF10 regulate meristem function through repressing the expression of KNOX gene *OsKN2* (Kuijt *et al*., 2014), OsGRF6 is involved in IAA regulation to control panicle size (Gao *et al.*, 2015), OsGRF6 and OsGRF10 also influence flower organ development through binding to the promoters of *OsJMJ706* and *OsCR4* (Liu *et al*., 2014). So OsBZR1 and OsmiR396d are not in a single signaling pathway, but in a complex network system, their function just partially overlay with each other. In this study, we only reveal one kind of connections between OsBZR1 and OsmiR396d through biochemical and genetic approaches.

OsmiR396d regulates *OsGRF6* to control rice plant height through GA pathway

The plant height of BZR1R/miROE8 and miROE8/*d61* were shorter than both parent plants (Fig. 6F, S4). The additive genetic effect between *OsMIR396d* and these BR signaling pathway genes may result from the fact that OsmiR396d and its target genes are not only involved in BR signaling but also in other hormone responses. Our experiments showed that over-expression of *OsMIR396d* caused enhanced BR signal and reduced GA signal in rice seedlings (Fig. 5, 7). In the *osgrf6* mutant, the BR sensitivity was similar to wild type (Fig. 8), however, both GA signaling and biosynthesis were impaired (Fig. 9). Furthermore, the expression of both *OsMIR396d*

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and *OsGRF6* were regulated by GA. *OsMIR396d* expression level was downregulated by treatment with GA biosynthesis inhibitor PAC, but was upregulated by treatment with high concentrations of GA. However, the *OsGRF6* transcript level was changed in an opposite way to *OsMIR396d* (Supplemental Fig. S5). These results indicate that OsmiR396d controls rice height by directly regulating *OsGRF6* expression and GA signaling.

OsmiR396d integrates different hormone regulation pathway

Our results showed that *OsMIR396d* transcription was induced by both exogenous BR and GA, and *OsMIR396d* can be directly activated by OsBZR1. Based on a previous report that the protein level of OsBZR1 decreased under 10^{-5} M GA_3 treatment (Tong et al., 2014), the transcription level of OsBZR1's target gene *OsMIR396d* should decrease under the same GA_3 concentration. However, qRT-PCR results showed that the transcripts of *OsMIR396d* were increased (Supplemental Fig. S5). These conflicting results implied that there might be another GA response factor, but not OsBZR1, which also controlled the transcription of *OsMIR396d* in GA signal. As one of OsmiR396d targets, *OsGRF4* functions in the BR response but not in GA signaling to control seed size and leaf angle (Che et al., 2015; Duan et al., 2015). Here, we reported another target of OsmiR396d, *OsGRF6*, was involved in GA biosynthesis regulation and signal transduction to regulate plant height, but did not directly participate in the BR response to control leaf angle. In addition, OsmiR396-OsGRF6 was also involved in auxin signaling to determine panicle architecture (Gao et al., 2015). Therefore, *OsMIR396d* could be regulated by BR,GA and auxin respectively, and controls various yield traits through different downstream targets.

OsmiR396d positively regulates BR signaling but negatively regulates GA function

Although BR and GA coordinately regulate plant development, they are distinct from each other in the specific biological processes. GA powerfully promotes cell elongation in a great range of concentrations, while BR only moderately promotes cell elongation under specific physiological concentrations (Tong *et al.*, 2014). BR is a master controller of leaf angle, while GA has only limited effect on this aspect (Tong

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& Chu, 2016). Here, we report that OsmiR396d positively regulate BR singling mainly focused on leaf angle, and OsmiR396d negatively control GA biosynthesis and signaling mainly focused on plant height. Furtherly, we found that *OsMIR396d* expression is also differentially regulated by different concentrations of GA. The expression of *OsMIR396d* is downregulated by low GA conditions, while upregulated by high GA conditions (Supplemental Fig. S5). It indicated that the repression of GA biosynthesis by OsmiR396d is attenuated when GA signaling is weak, and is enhanced when GA signaling is strong. It hints OsmiR396d possibly participate in the feedback regulation of GA biosynthesis. In addition, *OsMIR396d* expression is upregulated under exogenous 1 μM BR treatment (Fig. 5C). It suggested that the repression of GA biosynthesis by OsmiR396d will became stronger when BR signaling is greatly enhanced.

Improving agronomic traits through manipulating miR396-GRF regulated pathways

Rice miR396 family and their targets control various yield traits via multiple phytohormone signals. OsmiR396-OsGRF6 controls plant height and panicle size (Gao *et al.*, 2015), while OsmiR396-OsGRF4 controls leaf angle and seed size (Che *et al.*, 2015; Duan *et al.*, 2015). So it's possible to improve these traits at the same time through regulating the expression of multiple OsmiR396 family members or their targets. Enhancing the expression of *OsGRF4* and *OsGRF6* directly may cause more spikelet, increased seed size, erect leaves and slightly higher plant height, which may be close to the concept of ideal plant architecture for high grain yield breeding. Now, it is also possible to create OsmiR396d resistant *OsGRF4* and *OsGRF6* alleles through CRISPR–Cas9 nickase-cytidine deaminase fusion system (Zong *et al.*, 2017) to convert C to T in OsmiR396 targeted DNA regions. Additional approach may also be achieved through combining different members of OsmiR396 deleted "non-transgenic" mutants by CRISPR-CAS9 technology (Feng *et al.*, 2013) to improve rice yield.

In this study, an important component of the BR signaling, OsBZR1, was identified as the first direct upstream regulator of *OsMIR396d*. OsmiR396d can integrate the BR

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and GA signal interaction network to control rice plant architecture by repressing the expression of multiple target genes, such as *OsGRF4* and *OsGRF6*, which participate in BR and GA regulation, respectively (Fig. 10). Effective regulation of the expression of *OsMIR396* and its target genes may not only increase spikelet number (Gao et al., 2015) and seed size (Che et al., 2015; Duan et al., 2015), but also improve plant architecture.

MATERIALS AND METHODS

Plant materials and growth conditions

Rice (*Oryza sativa*) *Japonica* cultivars Zhonghua 10 (ZH10), Nipponbare (NIP), and Dongjin (DJ) were used in this study. Transgenic plants were produced in our laboratory, including: *OsMIR396d* over-expression lines (miROE); *OsGRF6* antisense plants (GRF6as); the OsmiR396d-resistant form of *OsGRF6* transgenic plants (rGRF6OE); *OsLIC* antisense plants (LIC-AS); *OsBZR1* over-expression plants (BZR1-OE); and *OsBZR1* RNAi interference plants (BZR1R) (Bai et al., 2007; Zhang et al., 2012; Liu et al., 2014). The T3 generation of double transgenic plants BZR1R/miROE8 and the F3 generation of cross progenies miROE8/*d61* were used in this study. The *osgrf6* mutant from the Dongjin background was obtained from the Rice Functional Genomics Express Database of Korea (Jeong et al., 2002). Mutant *d61* was kindly provided by Makoto Matsuoka (Yamamuro et al., 2000). Rice plants used in this study were grown in a greenhouse at 30°C/25°C (day/night) or in the field located in Beijing under natural conditions.

Hormone treatment

For BR treatment, rice seeds were sterilized with 5% $NaClO_3$ and grown in 1/2 Murashige and Skoog (MS) medium (M519, Phytotech, China) with different concentrations of 24-epibrassinolide (24-eBL) (CE5051, Coolaber, China) for one week and the seminal root length was measured. The lamina inclination experiment was carried out according to Zhang et al. (2012).

To detect GA sensitivity, rice seeds were first soaked in 10^{-5} M paclobutrazol (PAC)

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(CP8121, Coolaber, China) for 2 days to inhibit endogenous GA biosynthesis, and then germinated seeds were planted in Yoshida's culture solution (Yoshida et al., 1976) with various concentrations of GA_3 (G8040, Solarbio, China). After 10 days of growth, the length of the second leaf sheath was measured, and the seedlings were used to detect the transcription levels of *OsMIR396d* and *OsGRF6*.

Microscopy observation

For scanning electron microscopy (SEM) analysis, the lamina joints of *OsMIR396d* over-expression plants and wild type ZH10 at heading stage were excised to image the adaxial surface according to Wang et al. (2008). To determine the cell length of second leaf sheaths, surface cells in the same position of the second leaf sheaths of miROE, *osgrf6*, and wild type ZH10 and DJ 2-week-old seedlings were torn off and observed under Differential Interference Contrast (DIC) microscopy according to Li et al (2011).

Flow cytometric analysis of cell cycle progression

For flow cytometry, wild type and miROE seeds were germinated for 7 days in petri dishes (diameter, 20 cm) on filter paper with sterilized water in the dark at 28°C. Root apical tips (1 mm) were excised and immediately collected in chilled chopping buffer, and chopped with a single-edged razor blade in a glass petri dish (diameter, 5 cm) on ice. Chopping buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM 4-morpholinepropane sulfonate, and 1 mg ml^{-1} triton X-100, pH 7.0) was used to release cells from the chopped tissues. The DNA content of individual cells was determined by flow cytometry. Cell nuclei were prepared for FACSAriaTM by staining with 2 μg ml^{-1} 4',6-diamidino-2-phenylindole (DAPI). Each Root apical tip sample was prepared three times and subjected to FACS Caliber cytometry (BD Bioscience, USA) three times. Ten thousand nuclei were measured per analysis as previously described (Ma et al., 2009).

RNA extraction and qRT-PCR

Total RNA was extracted from 2-week-old rice seedlings using the Trizol RNA extraction kit according to the user manual (15596, Invitrogen, USA). First-strand

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cDNA was then synthesized from 1-2 μg total RNA using the FastQuant RT Kit (with gDNase) (KR106, Tiangen, China) according to the manufacturer's instructions. qRT-PCR analyses were carried out using the SYBR Green Master Mix (QPK-201, TOYOBO, Japan) on the Mx3000P real-time PCR System (Stratagene, USA) according to the manufacturer's instructions. Rice *UBIQUITIN* was used as internal reference, and gene expression levels were normalized to the expression level of *UBIQUITIN*. The expression level of *OsMIR396d* was analyzed as described by Liu et al. (2014). All primers are described in Table S1.

ChIP-qPCR

Two grams of leaves from 2-week-old rice seedlings of wild type (ZH10) and *OsBZR1* RNAi plants (BZR1R) were used for chromatin immunoprecipitation (ChIP) assay; the experiment was performed as described in He et al. (2005). The OsBZR1 antibody was used for immunoblot and ChIP analysis. The immunoprecipitated DNA content was determined by qPCR. Three replications were performed independently. The primers used in ChIP assays are listed in Table S1.

Yeast one-hybrid assays

Yeast one-hybrid assays were used to check binding of OsBZR1 to the *OsMIR396d* promoter according to a previous study (Chen et al., 2013). The wild-type putative OsBZR1 binding sites, S1, S2, S3 and mutated site S1 (mS1) of the *OsMIR396d* promoter tandem repeat sequences (S1, CGTCGTGTGGAA; S2, GTTCGTGCGTCT; S3, CGGCGTGTGTGT; and mS1, CGTAAAAAAGAA) were placed upstream of the minimal promoter in pHISi-1 vector. The full-length coding region of *OsBZR1* was fused to the pGAD424 vector. pGAD424-*OsBZR1* was then transformed into the yeast strain YM4271, which carries the reporter gene *HIS3* under the control of wild-type S1/S2/S3 or mS1. The transformed yeast cells were selected on SD/–His/–Leu medium containing 0, 15, 30, 45 and 60 mM 3-AT (a competitive inhibitor of the *HIS3* gene product) by standard protocols (Clontech, USA) and 15 mM 3-AT was used as the final concentration for screening.

EMSA

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The full-length cDNA of *OsBZR1* was fused into the *Kpn*I and *EcoR*I sites of vector pColdTM TF DNA. The fusion protein was purified with Ni Sepharose High Performance purification column (17-5268, Amersham, Sweden) according to the product manual. Biotin end-labeled DNA fragment of the *OsMIR396d* promoter was prepared by PCR amplification using 5'-biotin labeled primers, 5'-CGGGATCGTGCAATTCTCA-3' and 5'-TAAATAGCGGGAGGAGATAACC-3'. EMSA assay was performed using the LightShift Chemiluminescent EMSA Kit (20148, Thermo, USA) according to the manufacturer's instructions. Briefly, the reaction mixtures (20 μL) for EMSA contained 2 μg purified OsBZR1, 20 fmol biotin end-labeled target DNA, 2 μL 10× Binding Buffer, 1 μL 1μg μL^{-1} Poly (dI•dC) and double-distilled water. The binding reactions were incubated at room temperature for 20 min and electrophoresed on a 10% native polyacrylamide gel, then transferred to a nylon membrane (S4056, Millipore, USA) in 0.5× TBE buffer at 380 mA for 60 min. Biotin-labeled DNA was detected by chemiluminescence.

Transient assays for activation activity *in vivo*

The transcriptional activity assay was carried out in the transient-transformed protoplast prepared from 4-week-old *Arabidopsis* seedlings of the Columbia line grown in short day conditions as described previously (Lin et al., 2007). For the specific binding and activating activity of OsBZR1 to the *OsMIR396d* promoter assay, full-length cDNA of *OsBZR1* were fused into the pBI221-GFP vector driven by the CaMV 35S promoter to generate pBI221-OsBZR1-GFP. The pBI221-GFP vector was used as the negative control. The *OsMIR396d* promoter was amplified to generate the *OsMIR396d::LUC* reporter gene. The plasmid carrying the *GUS* gene under the control of the CaMV 35S promoter was used as a normalization control. Values represent means ± SD of three technical replicates.

GA quantification

For GA quantification of Dongjin and *osgrf6* plants, 3 grams of 2-week-old seedlings were harvested and frozen in liquid nitrogen. Quantification of endogenous GAs was performed as described previously (Li et al., 2011).

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SUPPLEMENTAL DATA

Supplemental Figure S1. The expression level of several BR related genes in miROEs.

Supplemental Figure S2 The expression pattern of *OsGRFs* under 24-eBL treatment.

Supplemental Figure S3 The expression of *OsBZR1* in miROE8 and BZR1R/miROE double transgenic lines.

Supplemental Figure S4 Genetic relationship of *OsMIR396d* and *OsBZR1* or *D61*.

Supplemental Figure S5 The expression pattern of *OsMIR396d* and *OsGRF6* under PAC or GA_3 treatment.

Supplemental Figure S6 The sensitivity of *osgrf6* to GA was decreased compared with DJ.

Supplemental Table S1. List of primers used in this study.

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FIGURE LEGENDS

Figure 1. The leaf angle of *OsMIR396d* over-expression plants was increased.

A. The third leaf angle of miROEs was specifically increased. Bar = 1 cm.

B. Statistical analysis of leaf angles. The third leaf angle was measured at the seedling stage and the angles of the top three leaves were measured at the seed filling stage. Error bars indicate SD for at least fifteen plants. Asterisks indicate $P < 0.01$ (**) compared with wild type in the Student's t test analysis.

Figure 2. The plant height and length of internodes in *OsMIR396d* over-expression plants were reduced.

A. The phenotype of miROEs and ZH10 at the seed filling stage. Bar = 5 cm.

B. The panicle and internodes of miROEs and ZH10. Bar = 5 cm.

C. Results of statistical analysis of plant height and internode length of miROE lines and ZH10. More than fifteen mature plants were used in each analysis.

D. Results of statistical analysis of fifth internode lengths of miROEs and ZH10. Error bars indicate SD for at least fifteen plants. Asterisks indicate $P < 0.01$ (**) compared with ZH10 in the Student's t test analysis.

E. Relative internode length of miROEs and ZH10. The data shows each internode length relative to the total culm length. Error bars indicate SD for at least fifteen plants. Asterisks indicate $P < 0.05$ (*) and $P < 0.01$ (**) compared with ZH10 in the Student's t test analysis.

Figure 3. Scanning electron microscopic examination of the leaf lamina joint.

A, B. Adaxial surface of the leaf lamina joint in ZH10 (A) and miROE8 (B). Arrows indicate the increased growth of cells.

C, D. Longitudinal sections of the leaf lamina joint of ZH10 (C) and miROE8 (D). Half frame indicated regions were used to calculate cell layers.

E, F. Close-up of regions denoted by rectangles in (C) and (D) respectively. Ad, adaxial; Ab, abaxial. Scale bars = 200 μm.

G. Statistical analysis of cell length in (C) and (F). Cell length along the adaxial-abaxial axis and proximal-distal axis were measured respectively. Error bars indicate SD for at least 100 cells. Asterisks indicate $P < 0.01$ (**) compared with ZH10 in the Student's t

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test analysis.

Figure 4. Cell length and cell division analysis.

A. The second leaf sheath cells of 2-week-old seedlings of miROE8 and ZH10. Bars = 50 μm.

B. The statistical analysis of cell length in the second leaf sheath. Error bars indicate SD for more than 400 cells. Asterisks indicate $P < 0.01$ (**) compared with ZH10 in student's t test analysis.

C. The DNA content of miROE8 and ZH10. After the seeds germinated for 3 days at 28°C, the root tips were harvested. Cell nuclei (10,000) were stained with 1 μg mL^{-1} DAPI and analyzed by flow cytometry. 2C and 4C represent the DAPI signals that correspond to nuclei with different DNA contents.

D. Cell mitotic index in root apical meristem in miROE8 and ZH10. The results were from three independent replications. Error bars indicate SD. Asterisks indicate $P < 0.01$ (**) compared with ZH10 in the Student's t test analysis.

Figure 5. OsmiR396d is involved in BR signaling.

A. Lamina joint inclination assay of miROE8 and ZH10. The second lamina joints of one-week-old seedlings grown under dark condition were cut off and treated with different concentrations of 24-eBL for three days. Error bars indicate SD for at least fifteen plants. Asterisks indicate $P < 0.05$ (*) and $P < 0.01$ (**) compared with ZH10 in the Student's t test analysis.

B. The seminal root growth of miROE8 and ZH10 under treatment with different concentrations of 24-eBL. The seminal root length was measured after seedlings were grown in 24-eBL containing 1/2 MS medium for one week. Error bars indicate SD for at least fifteen plants. Asterisks indicate $P < 0.05$ (*) and $P < 0.01$ (**) compared with ZH10 in the Student's t test analysis.

C. The expression pattern of *OsMIR396d* under treatment with 24-eBL. Two-week-old ZH10 seedlings were treated with 1 μM 24-eBL. Whole plants were sampled 1, 3, 6, 12 and 24 h after treatment. The plants sampled from the normal growth condition were used as control. *UBQUITIN1* was used as a reference gene. Error bars indicate SD for three replicates. Asterisks indicate $P < 0.05$ (*) and $P < 0.01$ (**) compared with control

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in the Student's t test analysis.

D. The expression of *OsMIR396d* in *OsLIC* antisense plants (LIC-AS), *OsBZR1* RNAi plants (BZR1R), and OsBZR1 over-expression (BZR1-OE) plants. LIC-AS and BZR1R were under the background of ZH10 and BZR1-OE was under the background of Nipponbare (NIP). *UBQUITIN1* was used as reference gene. Error bars indicate SD for three replicates. Asterisks indicate $P < 0.05$ (*) and $P < 0.01$ (**) compared with wild-type ZH10 or NIP respectively in the Student's t test analysis.

Figure 6. OsBZR1 directly activates the expression of *OsMIR396d*.

A. Localization of three putative OsBZR1 binding sites S1, S2, and S3 in the OsMIR396d promoter.

B. Yeast one-hybrid analysis of OsBZR1 binding the promoter of OsMIR396d. Empty pHIS vector was used as negative control.

C. EMSA analysis of OsBZR1 binding the promoter of OsMIR396d. S1-containing DNA fragment was labeled with biotin. The arrow indicates the complex of OsBZR1-His and the DNA probe.

D. ChIP-qPCR analysis of OsBZR1 binding the promoter of OsMIR396d using OsBZR1 antibody to enrich DNA. The UBIQUITIN DNA fragment was used as an internal control. Error bars indicate SD for three replicates. Asterisks indicate $P < 0.01$ (**) compared with ZH10 in the Student's t test analysis.

E. Transactivation assay of OsBZR1 with the luciferase reporter system. Error bars indicate SD for three replicates. Asterisks indicate $P < 0.01$ (**) compared with control in the Student's t test analysis.

F. The phenotype of OsBZR1 RNA interference transgenic plants (miROE8/*BZR1*R) under the background of miROE8. Bar = 10 cm.

Figure 7. GA signaling and biosynthesis were impaired in *OsMIR396d* over-expression plants.

A. The phenotype of miROE8 and ZH10 seedlings growth under different concentrations of GA_3 treatment. The seeds were treated with 10 μM PAC for 2 days before cultivation in nutrient solution containing the indicated concentration of GA_3. After 10 days of growth, the seedlings were photographed. The measurements of the

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second leaf sheath are shown. Error bars indicate SD for at least fifteen plants. Bar = 5 cm.

B. The expression of GA pathway related genes in miROE and ZH10. *UBQUITIN1* was used as reference gene. Error bars indicate SD for three replicates.

Figure 8. BR signaling was not affected in *osgrf6* mutant.

A. The phenotype of *OsGRF6* antisense transgenic plant (GRF6as) and the OsmiR396d-resistant form of *OsGRF6* transgenic plant (rGRF6OE). Bar = 5 cm.

B. The second leaf sheath cells of 2-week-old seedlings of *osgrf6* and DJ. The cell length was measured. Error bars indicate SD for more than 400 cells. Asterisks indicate $P < 0.01$ (**) compared with DJ in the Student's t test analysis. Bar = 50 μm.

C. The leaf angle of *osgrf6* was not changed. The third leaf angle was measured. Error bars indicate SD for at least fifteen plants.

D. Lamina joint inclination assay of *osgrf6* and DJ. Error bars indicate SD for at least fifteen plants.

E. The seminal root growth of miROE8 and ZH10 under treatment with different concentrations of 24-eBL. Error bars indicate SD for at least fifteen plants.

Figure 9. GA signaling and biosynthesis are impaired in the *osgrf6* mutant.

A. *osgrf6* and DJ seedlings treated with PAC or GA_3. The seeds were treated with water (control) or 10^{-5} M PAC for 2 days, and then germinated seeds were planted in PAC and/or GA_3 containing culture solution for 10 days. Bar = 5 cm.

B. The statistical result of the second leaf sheath length of *osgrf6* and DJ grown under PAC and/or GA_3 treatment. Error bars indicate SD for at least fifteen plants. The relative leaf sheath length between DJ and *osrf6* was calculated and shown. Asterisks indicate $P < 0.01$ (**) compared with DJ in student's t test analysis.

C. The expression of GA pathway related genes in *osgrf6* and DJ. *UBQUITIN1* was used as reference gene. Error bars indicate SD for three replicates.

D. Quantification of endogenous GAs in *osgrf6* and DJ. Two-week-old seedlings were sampled. Error bars indicate SD for three replicates. Asterisks indicate $P < 0.01$ (**) compared with DJ in the Student's t test analysis.

Figure 10. A proposed model for OsmiR396d controlling rice plant architecture.

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OsMIR396d is activated by OsBZR1, which is the key regulator transcription factor of BR signaling. On the one hand, OsmiR396d regulates leaf angle by repressing the expression of *OsGRF4*. On the other hand, OsmiR396d is involved in GA signaling and biosynthesis by regulating the expression of *OsGRF6* to control rice plant height.

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