OsARF4 regulates leaf inclination via auxin and brassinosteroid pathways in rice

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Leaf inclination is a vital agronomic trait and is important for plant architecture that affects photosynthetic efficiency and grain yield. To understand the molecular mechanisms underlying regulation of leaf inclination, we constructed an auxin response factor (arf) rice mutant—osarf4—showing increased leaf inclination using CRISPR/Cas9 gene editing technology. OsARF4 encodes a nuclear protein that is expressed in the lamina joint (LJ) at different developmental stages in rice. Histological analysis indicated that an increase in cell differentiation on the adaxial side resulted in increased leaf inclination in the osarf4 mutants; however, OsARF4-overexpressing lines showed a decrease in leaf inclination, resulting in erect leaves. Additionally, a decrease in the content and distribution of indole-3-acetic acid (IAA) in osarf4 mutant led to a greater leaf inclination, whereas the OsARF4-overexpressing lines showed the opposite phenotype with increased IAA content. RNA-sequencing analysis revealed that the expression of genes related to brassinosteroid (BR) biosynthesis and response was different in the mutants and overexpressing lines, suggesting that OsARF4 participates in the BR signaling pathway. Moreover, BR sensitivity assay revealed that OsARF4-overexpressing lines were more sensitive to exogenous BR treatment than the mutants. In conclusion, OsARF4, a transcription factor in auxin signaling, participates in leaf inclination regulation and links auxin and BR signaling pathways. Our results provide a novel insight into leaf inclination regulation, and have significant implications for improving rice architecture and grain yield.

**KEYWORDS**
leaf inclination, auxin, OsARF4, rice, brassinosteroid

**Introduction**

Plant architecture is governed by a set of complex agronomic traits that determine grain yield and is a pivotal target of artificial selection for rice domestication. Rice tillering pattern, plant height, and leaf inclination are important factors influencing the plant architecture (Van Camp, 2005). Leaf inclination (also called leaf angle), which is the angle
between the leaf blade and the culm, is determined by the lamina joint (LJ). LJ, which is composed of a collar, ligules, and auricles, is a unique tissue that connects the leaf blade to leaf sheath in rice, and significantly contributes to the horizontal bending of the blade from the main axis. Division and expansion of the parenchyma and sclerenchyma cells in LJ affect its development, and leaf inclination is influenced by unequal cell division and elongation between the adaxial and abaxial sides of the LJ. Lack of cell expansion and longitudinal elongation of adaxial cells in the LJ results in erect leaves. In contrast, leaf inclination is enhanced by an increase in the expansion of cells on the adaxial surface (Duan et al., 2006). In rice, erect leaves have a higher leaf area index, which can increase the photosynthetic carbon assimilation rate by increasing light capture ability and nitrogen use efficiency to enhance total biomass and grain yield (Sakamoto et al., 2006). Therefore, revealing the molecular mechanism of genes involved in regulation of leaf inclination in rice is urgently required.

Phytohormones play an important role in regulating leaf inclination by changing the cytological structure of LJ. brassinosteroids (BRs), plant-specific steroid hormones, have a significant effect on the growth and development of the LJ. In rice, studies have shown that BR promotes division and elongation of parenchyma cells at the adaxial side of the LJ and proliferation of sclerenchyma cells at the abaxial side; this unbalanced development between the adaxial and abaxial cells of the LJ leads to leaf bending (Tanabe et al., 2005; Zhang L. Y. et al., 2009; Sun et al., 2015). Several studies have indicated that decreased BR biosynthesis and signal transduction could decrease leaf inclination, whereas excess BR results in increased leaf inclination (Zhang et al., 2014). The mutation of several cytochrome P450 genes involved in BR biosynthesis, such as OsD2, OsD11, and OsDWARF (Hong et al., 2003; Sakamoto et al., 2006; Li et al., 2013), causes shortening of parenchyma cells in the LJ, contributing to reduced leaf inclination and formation of erect leaves. Defective BR signaling genes—BAK1 and BZR1—also result in decreased leaf inclination (Bai et al., 2007; Li et al., 2009). BRL1/SMOS1 (REDUCED LEAF ANGLE1 SMALL ORGAN SIZE1) acts as an integrator, functioning with OsBZR1 and GSK2, and plays an important role in BR signal transduction and leaf inclination regulation in rice (Qiao et al., 2017). Furthermore, ILI1 (INCLINATION1) and IBH1 (IL11 binding bHLH) antagonistically regulate the elongation of parenchyma cells in the LJ by interacting with the transcription factor OsBZR1 involved in BR signaling (Zhang L. Y. et al., 2009). In addition, rice U-type cyclin CYCU41 regulates proliferation of sclerenchyma cells on the abaxial side of LJ in the BR-regulated pathway, which plays an important role in promoting the erectness of leaves (Sun et al., 2015). These results suggest that leaf inclination is closely related to BR biosynthesis and signal transduction in rice.

In addition to BR, auxin has been shown to play a negative role in controlling leaf inclination. Studies have shown that reducing auxin levels or signal transduction increases leaf inclination by promoting the elongation of parenchyma cells on the adaxial surface of the LJ (Du et al., 2012; Zhao et al., 2013; Zhang et al., 2015). Furthermore, loss of FIB (Fish Bone) function could reduce the endogenous indole acetic acid (IAA) content and polar auxin transport activity, leading to increased bending of the LJ (Yoshikawa et al., 2014). Auxin early response genes, such as AUX/IAA and GH3 family genes, the auxin receptor TIR1 (TRANSPORT INHIBITOR RESPONSE 1), and auxin response factor (ARF), have been reported to be associated with regulation of leaf inclination. The auxin response factor OsARF1 can inhibit the expression of OsGH3s, which encode IAA amino acid synthases by interacting with the repressor OsIAA1 (AUXIN/INDOLE3-ACETIC ACID 1) in the process of leaf inclination regulation (Wang et al., 2007). Additionally, overexpression of OsIAA1 results in an increased leaf angle (Song et al., 2009). Recent studies have shown that OsIAA6 regulates leaf inclination by inhibiting auxin signaling through interacting with OsARF1 in rice (Xing et al., 2022). The decrease in IAA content promotes elongation of parenchyma cells on the adaxial surface of the LJ, which is consistent with the result that the increase in GH3 expression levels increases leaf inclination. The increase in GH3-5 expression levels and the decrease in free IAA content also lead to increased leaf inclination in OsARF19-overexpressing rice plants (Zhang et al., 2015). LC1 (LEAF INCLINATION 1) encodes OsGH3-1 (an IAA amino synthetase) and promotes cell elongation at the LJ by decreasing the auxin content in rice (Zhao et al., 2013). LC3 (LEAF INCLINATION 3) interacts with LIPI1 (LC3-INTERACTING PROTEIN 1) to synergistically suppress auxin signaling, thus controlling leaf inclination (Chen et al., 2018). LPA1 (LOOSE PLANT ARCHITECTURE 1) influences the polar transport of auxin by regulating the expression of auxin transporter OsPIN1a and influences leaf angle regulation (Sun et al., 2019). Inhibition of the expression of auxin receptor OsTIR1 also leads to increased leaf inclination (Bian et al., 2012). In addition, OsARF6 and OsARF17 synergistically control flag leaf angle in response to auxin (Huang et al., 2021).

Both auxin and BR certainly play important roles in regulation of leaf inclination, which involves diverse factors and intricate regulatory networks. In this regard, we identified a novel gene OsARF4 (AUXIN RESPONSE FACTOR 4), mutants of which showed increased leaf inclination, whereas overexpressing lines had erect leaves. Histochemical analysis and scanning electron microscopy (SEM) results showed that the increase in leaf inclination was caused by cell differentiation on the adaxial surface. Moreover, OsARF4 was found to be involved in both auxin and BR signaling in regulation of leaf inclination. These results provide a new potential target for ideal architecture breeding and improving rice yield.

Materials and methods

Plant materials and growth conditions

Wild type variety of rice—Dongjin (Oryza sativa subsp. japonica, WT/DJ)—was used as a control in this study. All seeds were soaked in water in the dark for 3 days at 37°C, and then cultured in a nutrient solution (pH 5.4) and grown in the greenhouse under a 12 h light (30°C) and 12 h dark (24°C) cycle. Thereafter, rice
plants were grown in an experimental paddy field under natural conditions of the China National Rice Research Institute in Hangzhou or Hainan Yazhou Bay Seed Laboratory in Sanya, China.

### Construction and transformation of binary vectors

The *osarf4* mutants were obtained using CRISPR/Cas9 gene editing technology as described by Xie (Xie et al., 2015), and the selected target sites of *osarf4* mutants were AGGAGGCATC TCCCTCAGAG and AGTTCAAAAGGCTTGGTTGC. The full-length open reading frame (ORF) of OsARF4 was amplified from WT/DJ cDNA and then cloned into pCAMBIA1300-sGFP binary vector to construct the overexpression vector 35S:OsARF4-GFP. OsARF4 promoter was amplified from WT/DJ genomic DNA and then cloned into the pCAMBIA1301-GUS vector to generate proOsARF4:GUS vector. Agrobacterium strain EHA105 was used for transformation of the vector into WT/DJ. All primers used are shown in Supplementary Table 1.

### RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using TaKaRa MiniBEST Plant RNA Extraction Kit (TaKaRa, Kusatsu, Japan) and then reverse transcribed to obtain cDNA using FastKing RT Kit (with gDNase; Tiangen, Beijing, China) according to the manufacturer’s instructions. Quantitative real-time PCR (qRT-PCR) was performed using the 2*Easy Star Green Fast Mixture (Easy-Do, Zhejiang, China) and a CFX Connect™ Real-time System (Bio-Rad, Hercules, CA, United States). OsACTIN (LOC_Os03g50885) was used as an internal control. Three biological replicates were performed for each experiment. All primers used for qRT-PCR are shown in Supplementary Table 2.

### Subcellular localization of OsARF4

High-quality plasmids of 35S:OsARF4-GFP and the nuclear localization marker NSL-mCherry were transiently coexpressed in rice protoplasts using polyethylene glycol as previously described (Qiao et al., 2021). After overnight incubation in the dark at 28°C, fluorescence was observed using a two-photon confocal microscope (Zeiss LSM 710; Carl Zeiss, Oberkochen, Germany).

### β-Galactosidase (GUS) staining and activity analysis

The auxin reporter DR5:GUS was genetically transformed into WT/DJ, osarf4 mutants, and OsARF4-overexpressing lines. The LJs were treated with the GUS staining solution (10mM sodium phosphate buffer, pH 7.0; 10mM Na2EDTA; 1mM K3[Fe(CN)6]; 1mM K4[Fe(CN)6]; 0.5% Triton X-100; 20% methyl alcohol, and 0.5mg/ml X-Gluc) for 30 min at 37°C. Then, chlorophyll was removed by soaking the leaves in 70% ethanol. Images were collected using a stereo microscope Nikon SMZ 25 (Nikon Corporation, Tokyo, Japan). The positive proOsARF4-GUS transgenic lines were tested by GUS staining, and roots (3-day-old of seedlings), coleoptile (3-day-old of seedlings), and 2–10 week old of seedings of proOsARF4-GUS were used for expression pattern analysis. GUS activities in the LJs of WT/DJ, osarf4 mutants, and OsARF4-overexpressing lines were analyzed using the Plant GUS Elisa Kit1 according to the manufacturer’s instructions.

### Quantification of IAA contents

At 10 days after heading, the LJs of WT/DJ, osarf4 mutants, and OsARF4-overexpressing lines were ground into a powder using liquid nitrogen. IAA was extracted and its content was measured as previously described (Ljung et al., 2005; Shao et al., 2019; Qiao et al., 2021). Agilent 1,100 high-performance liquid chromatography was performed using a C18 reverse-phase column (250 mm × 4.6 mm, 5 μm).

### Scanning electron microscopy

Scanning electron microscopy was performed as previously described (Zhao et al., 2010; Wang et al., 2019). Briefly, at 10 days after heading, approximately 1 cm of LJs was excised from the flag leaves of WT/DJ, osarf4 mutants, and OsARF4-overexpressing lines. Thereafter, they were dehydrated in graded ethanol series (50–100%), dried in a critical point dryer for 2 h, and subjected to gold sputtering for 1 min. Samples were observed using an S-3000 N scanning electron microscope (Hitachi, Tokyo, Japan).

### Leaf inclination measurement and cytological analysis

Leaf inclinations between the sheaths and leaves of WT/DJ, osarf4 mutants, and OsARF4-overexpressing lines were photographed and measured at 10 days after heading. For paraffin sectioning, the LJs from flag leaves of WT/DJ, osarf4 mutants, and OsARF4-overexpressing lines were ground into a powder, dehydrated in graded ethanol series, and embedded in Paraplast Plus (Sigma, United States). Microtome sections (5 μm) were cut to obtain transverse and longitudinal sections using a rotary microtome (RM2245, Leica Microsystems, Hamburg, Germany) and stained with toluidine blue. Sections

1  http://www.ruixinbio.com/
were observed and photographed using a stereo microscope Nikon SMZ 25 (Nikon Corporation, Tokyo, Japan).

RNA sequencing analysis

At 10 days after heading, total RNA was extracted from LJs of flag leaves of WT/DJ, osarf4-1 mutant, and OE-OsARF4-1 overexpression line, and sequenced in BGI with three biological replicates (Shenzhen, China). The analytical methods and software analysis were performed according to previous studies (Zhang et al., 2020). Gene expression levels were expressed as transcript fragments per kilobase (FPKM) per million reads (Florea et al., 2013). Differentially expressed genes (DEGs) were detected using the DESeq2 package testing at |log2FC| > 2 and Q value <= 0.05. Additional detailed information is provided on the BGI official website. The RNA sequencing data were deposited in the NCBI Sequence Read Archive (SRA) with accession SRR20748275, SRR20748276, SRR20748277, SRR20748278, SRR20748279, SRR20748280, SRR20748281, SRR20748282, and SRR20748283.

BR sensitivity tests

Primary root (PR) inhibition analysis was performed as previously mentioned (Zhang et al., 2015). Seeds of WT/DJ, osarf4 mutants, and OsARF4-overexpressing lines were germinated at 37°C and then grown in a normal culture solution supplemented with 0, 0.01, 0.1, 1, or 10µm 24-epibrassinolide (24-eBL) for 7 days. Then, the PR length was measured. The coleoptile elongation test was performed as previously described (Duan et al., 2006). Seeds of WT/DJ, osarf4 mutants, and OsARF4-overexpressing lines were germinated and grown in a normal culture solution containing 0, 0.01, 0.1, 1, or 10µm 24-eBL for 7 days. Then, a camera (EOS 6D Mark II, Canon, Japan) was used to obtain a photograph to measure the coleoptile length. After 7 days of growth in a normal culture solution, WT/DJ, osarf4, and OsARF4-overexpressing plants were incubated with 24-eBL at different concentrations for 3 days. Thereafter, the leaf inclinations were measured as previously described (Zhang et al., 2015).

Results

OsARF4 is expressed in lamina joint and encodes a nuclear protein

Our previous study has found that the member of the OsARF family, OsARF19, controls rice leaf inclination positively (Zhang et al., 2015). Although OsARF4 and OsARF19 belong to the OsARF family, they exist in different clades. OsARF4 belongs to Class I subfamily, while OsARF19 is from Class II (Wang et al., 2007). OsARF4 is a putative transcriptional repressor with RD (repression domain) in its middle region (MR), while OsARF19 is a putative transcriptional activator with activation domain (AD; Shen et al., 2010). Furthermore, OsARF4 has a negative function in regulating grain size and grain weight in rice (Hu et al., 2018). In that case, whether OsARF4 also takes part in leaf inclination regulation? In order to make this question clearly, the related tests were performed. To determine the functional location of OsARF4, its expression pattern was first investigated. The promoter of OsARF4 was fused with the GUS gene and this construct was then transformed into WT/DJ calluses to obtain the pro:OsARF4-GUS transgenic plants. Pro:OsARF4-GUS transgenic plants were observed at different stages during 2–10 weeks using histochemical staining. The results showed that OsARF4 was expressed in LJ at different development stages, especially at the mature stage (Figure 1A). qRT-PCR further confirmed that OsARF4 was expressed at those stages in LJ (Figure 1B), consistent with the results of GUS staining, implying that OsARF4 may function in LJ in rice.

To explore the subcellular localization of OsARF4, the CDS of OsARF4 was fused with GFP and driven by the CaMV35S promoter. Transient co-expression of 35S:OsARF4-GFP and the nuclear marker NLS-mcherry in rice protoplasts showed that OsARF4 was localized in the nucleus (Figure 1C). Furthermore, OsARF4 was also localized in the nuclei of root cells in 35S:OsARF4-GFP transgenic lines (Supplementary Figure 1). These results indicate that OsARF4 encodes a nuclear protein.

OsARF4 negatively affects leaf inclination

Studies have shown that OsARF gene family members OsARF19, OsARF6, and OsARF1 were involved in regulating leaf inclination in rice (Zhang et al., 2015; Huang et al., 2021; Xing et al., 2022). To explore whether OsARF4 functions to control leaf inclination, two osarf4 mutants were constructed using CRISPR/Cas9 gene editing technology (Xie et al., 2015), and two OsARF4-overexpressing lines were obtained (Figures 2A,B). Compared with WT/DJ, osarf4-1 showed a 71-bp deletion in the eighth exon in the ORF (1834–1904bp) of OsARF4, which caused premature termination of protein translation, resulting in the formation of 627 amino acid protein instead of the 808 amino acid protein in osarf4-1. osarf4-2 showed a 56-bp deletion in the seventh exon in the ORF (1631–1,684 bp), which caused premature termination of protein translation resulting in the formation of a truncated protein with 564 amino acids instead of 808 amino acids. The protein structure in both mutants was changed (Figure 2A). The expression of OsARF4 was upregulated four and nine folds in OsARF4-1 and OsARF4-7-overexpressing lines, respectively, compared with that in WT/DJ (Supplementary Figure 2). Phenotypic observation showed that at the heading stage, leaf angles of osarf4 mutants (osarf4-1 and osarf4-2) were much...

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larger than that of WT/DJ, and the OsARF4-overexpressing lines (OE-OsARF4-1 and OE-OsARF4-7) showed a greater number of erect leaves than inclined leaves (Figure 2C). This was more evident in the flag leaves (reaching to ~85° in osarf4 mutants and ~18° in OsARF4-overexpressing lines compared with 28° in WT/DJ; Figures 2D, E).

To further verify the function of OsARF4 in controlling leaf inclination, we analyzed the expression of genes related to regulation of leaf inclination in the LJs of WT/DJ, osarf4 mutants, and OsARF4-overexpressing lines (Figure 2F). The transcriptional abundances of the genes OsARF19 and OsIAA1 were induced in the osarf4 mutants, whereas they were reduced in OsARF4-overexpressing lines compared with those in WT/DJ. In addition, OsTIR1 showed a opposite trend compared with OsIAA1. These results are consistent with those reported previously that OsARF19 and OsIAA1 positively regulate leaf inclination, and OsTIR1 negatively regulates leaf inclination (Song et al., 2009; Bian et al., 2012; Zhang et al., 2015), implying that OsARF4 might play a negative role in regulating leaf inclination.

Increased cell proliferation on the adaxial surface leads to increased leaf inclination in osarf4

Previous studies have shown that the LJ significantly contributes to the formation of leaf inclination, and the increased leaf inclination may cause alterations in LJ development (Yamamuro et al., 2000). Therefore, the collar length of the LJ at adaxial and abaxial epidermis in WT/DJ, osarf4 mutants, and OsARF4-overexpressing lines was measured. As shown in Figures 3A, B, the collar length of the LJ at adaxial surface of osarf4 mutants increased by 60%, whereas it decreased by 45% in OsARF4-overexpressing lines compared with that in WT/DJ. In contrast, there were no significant differences in the length at the abaxial surface between WT/DJ and osarf4 mutants or OsARF4-overexpressing lines. These results indicate that the main difference in leaf inclination in those lines was due to the imbalanced elongation between the adaxial and abaxial surfaces of LJ.
To identify the cellular mechanism underlying the regulation of leaf inclination by OsARF4, SEM analysis was conducted to observe microstructure morphology. In osarf4 mutant, there was much larger and formed a bulge in the adaxial surface of the LJ, whereas it was smoother in OsARF4-overexpressing lines compared to the smooth adaxial surface in WT/DJ (Figure 3C). These results further confirmed that the development of the LJ in osarf4 was altered and resulted in increased leaf inclination.

Previous studies have shown that aberrant cellular development, such as lack of the longitudinal cell elongation or increase in cell expansion at the adaxial sides in the collar, can lead to changes in leaf inclination (Duan et al., 2006; Zhao et al., 2010; Zhang et al., 2015; Guo et al., 2021; Tian et al., 2021). To further reveal the reason for the changed leaf inclination in the mutants and overexpression lines of OsARF4, we prepared paraffin sections with both transverse and longitudinal sections of the LJs of WT/DJ, osarf4 mutants, and OsARF4-overexpressing lines. The results indicate that the number of sclerenchyma cell layers on the adaxial surface increased in both transverse and longitudinal sections in osarf4 mutants and decreased in the OsARF4-overexpressing lines, compared with that in WT/DJ (Figure 3D–G), which illustrates that cell division, but not cell elongation, in the LJ resulted in increased leaf inclination in osarf4.
**OsARF4 regulates leaf inclination by controlling auxin distribution and content in lamina joints**

Auxin is an important plant hormone that plays a negative role in controlling leaf inclination (Du et al., 2012; Zhou et al., 2017). Studies have shown that *OsARF* regulates leaf angles induced by auxin (Zhang et al., 2015). To explore the role of auxin in *OsARF4*-mediated leaf inclination pathway, the relative expression level of *OsARF4* was detected under auxin treatment. It was found that auxin treatment could induce *OsARF4* expression (Supplementary Figure 3), indicating that the function of *OsARF4* might be positively regulated by auxin.

As the auxin response reporter, *DR5:GUS* has been widely used to study the distribution of endogenous auxin (Ulmasov et al., 1997; Qiao et al., 2021). To determine whether the changes in leaf inclination of *OsARF4* are influenced by the changes in auxin distribution, *DR5:GUS* was transformed into the *osarf4* mutants and *OsARF4*-overexpressing lines. GUS staining in LJs indicated that *DR5:GUS* expression in *osarf4* mutants was significantly reduced compared to the wild type and *OsARF4*-overexpressing lines. This suggests that the changes in auxin distribution might be involved in the regulation of leaf inclination by *OsARF4*.
FIGURE 4
Analysis of auxin distribution and content in LJs in WT/DJ, osarf4 mutants, and OsARF4-overexpressing lines. (A) GUS staining of DR5:GUS-WT/DJ, DR5:GUS-osarf4, and DR5:GUS-OE-OsARF4 transgenic lines in the LJs. Bars=500μm. (B) Quantification of GUS activity in DR5:GUS-WT/DJ, DR5:GUS-osarf4, and DR5:GUS-OE-OsARF4 transgenic lines. Ten biological replicates were evaluated. **p<0.01 indicates a significant difference in means ± SD compared with DR5:GUS-WT/DJ based on Student’s t-test. (C) Determination of auxin contents in LJs of WT/DJ, osarf4 mutants, and OsARF4-overexpressing lines. Five separate biological replicates were used in each test. **p<0.01 indicates significant differences from WT/DJ based on Student’s t-test.

mutants was markedly lower than that in WT/DJ but more intense in OsARF4-overexpressing lines (Figure 4A). Moreover, GUS activity was lower in osarf4 but higher in OsARF4-overexpressing lines, compared with that in WT/DJ (Figure 4B). Furthermore, the free auxin content in LJs of WT/DJ, osarf4 mutants, and OsARF4-overexpressing lines was measured. The results showed that the auxin content was lower and higher in osarf4 mutants and OsARF4-overexpressing lines, respectively, compared with that in WT/DJ (Figure 4C), implying that OsARF4 regulates leaf inclination by controlling local auxin levels in LJs.

RNA-seq analysis of WT/DJ, mutant and overexpression line of OsARF4

To analyze genes of OsARF4-mediated pathway that participate in the regulation of leaf inclination, transcriptome of the flag LJs from WT/DJ, osarf4 mutant, and OsARF4-overexpressing line was sequenced. A total of 32,938 genes were obtained in the RNA-seq analysis. Among those differentially expressed genes (DEGs), 70 genes were upregulated and 130 genes were downregulated between WT and osarf4 mutant; 1,229 genes were upregulated and 1,452 genes were downregulated between
the WT and OsARF4-overexpressing lines; and 12 genes were upregulated and 11 genes were downregulated between the osarf4 mutant and OsARF4-overexpressing line (Figures 5A,B). The results of gene ontology (GO) enrichment analysis demonstrated that genes related to auxin signal transduction and BR biosynthesis were significantly overrepresented (Figure 5C), which implies that OsARF4 might be involved in auxin signal transduction and BR biosynthesis. In previous studies, genes related to regulation of leaf inclination, including OsBZR2 (Min et al., 2019), OsREM4.1 (Li et al., 2021), OsNCED5 (Zhu et al., 2009), OsBZR1 (Bai et al.,
OsARF4 is sensitive to exogenous BR

In rice, BR has been proved to play a pivotal role in the determination of leaf inclination (Sakamoto et al., 2013; Zhang et al., 2015). To further confirm whether OsARF4 is involved in BR signaling in regulation of leaf inclination, the three classical BR sensitivity experiments were performed. In addition to LJs, OsARF4 was also expressed in primary root, lateral root, and coleoptile (Figure 1A; Supplementary Figure S5). Therefore, BR sensitivity experiments, PR elongation, coleoptile elongation, and the degree of leaf inclination tests were performed under treatments with different concentrations of 24-eBL (Figure 6). The PR growth was inhibited by BR treatments and this inhibition increased with the increased in BR concentration. Compared to the WT/DJ, osarf4 mutants were insensitive to exogenous BR treatments, whereas OsARF4-overexpressing lines were relatively more sensitive (Figures 6A,D). After BR treatments, the elongation of coleoptile increased in OsARF4-overexpressing lines and became more pronounced with the increase in BR concentration, whereas osarf4 mutants showed slight increase compared with that in WT/DJ (Figures 6B,D). The leaf inclination in OsARF4-overexpressing lines and osarf4 mutants increased by 8–80% and 3–20%, respectively, under treatments with different concentrations of 24-eBL (Figures 6C,D). Taken together, the OsARF4-overexpressing lines were more sensitive to exogenous BR treatments, whereas osarf4 mutants were insensitive, compared with WT/DJ.

Moreover, OsARF4 expression was induced by BR treatments (Figure 7A). When osarf4 mutants and OsARF4-overexpressing lines were treated with 0.1 μM 24-eBL (the optimum concentration selected from the above gradient treatment) for 3 days simultaneously, the leaf inclination of WT/DJ, osarf4 mutants, and OsARF4-overexpressing lines increased by 96, 38, and 390%, respectively (Figures 7B–C). Hence, OsARF4-overexpressing lines were more sensitive, whereas osarf4 mutants were insensitive to BR treatments, compared with WT/DJ. These results suggest that OsARF4 is related to regulation of BR signaling-mediated leaf inclination in rice.

Discussion

OsARF4 functions in regulating leaf inclination

As plant-specific B3-type transcription factors, ARFs play a role in activating or repressing the auxin response genes by specifically binding to auxin response element (AuxRE; Chandler, 2016). There are 25 ARFs in rice, many of which have been reported to execute multiple functions (Wang et al., 2007). For instance, OsARF12 and OsARF16 are associated with root development and phosphate homeostasis (Qi et al., 2012; Shen et al., 2013, 2014; Wang et al., 2014). OsARF25 is involved in controlling grain size, and its T-DNA insertion mutant has shorter grains (Zhang et al., 2018). OsmiR160 negatively regulates the expression of OsARF18 and improves rice growth and development via auxin signaling pathway (Huang et al., 2016a). OsARF6 participates in the miR167a-OsARF6-OsAUX3 module to regulate grain length and grain weight in rice (Qiao et al., 2021). OsARF6 and OsARF17 bind to the promoter of ILA1 to determine flag leaf blade angle (Huang et al., 2021). OsARF1 interacts with OsIAA6 to regulate leaf inclination in rice through auxin and BR pathway (Xing et al., 2022). In a previous study, the research of our laboratory have found that OsARF19 is involved in positive regulation of lamina inclination regulation by binding to the promotors of OsGH3-5 and OsBR11, through auxin and BR pathway (Zhang et al., 2015). In this study, we generated two osarf4 mutants using CRISPR/Cas9 gene editing technology, and two OsARF4-overexpressing lines using genetic transformation. Phenotype analysis showed that the flag leaf inclination increased by approximately 150% in osarf4 mutants and decreased by 44% in OsARF4-overexpressing lines after heading in rice (Figure 2; Supplementary Figure 2). Leaf inclination is mainly determined by the LJ, which is a unique tissue connecting the leaf blade and sheath in rice (Wang et al., 2020). GUS staining and qRT-PCR results indicated that OsARF4 was expressed in LJ at different developmental stages (Figures 1A,B), thus confirming that OsARF4 is involved in leaf inclination regulation. Interestingly, both OsARF4 and OsARF19 belong to the OsARF family, but they play converse roles in the regulation of leaf inclination in rice, because they were from different clades, namely, OsARF4 belongs to Class I with transcript repression domain while OsARF19 belongs to Class II with transcript activation domain (Wang et al., 2007; Shen et al., 2010), lead to they have different functions in leaf inclination regulation.
From a cytological perspective, the change in leaf inclination is attributed to the imbalance in the cell development of the adaxial and abaxial sides in LJs (Duan et al., 2006). In previous studies, most of the identified rice mutants with altered leaf inclination showed abnormal division and expansion of adaxial cells in the leaf collar (Nakamura et al., 2009; Zhang S. W. et al., 2009; Zhao et al., 2010; Ning et al., 2011). The leaf inclination is caused by the bending of the blade. From the perspective of a cytological structure, the most obvious change is the cell division and expansion at the adaxial side of the LJ (Wang et al., 2020). To illustrate the cytological structures of mutants and overexpression lines of OsARF4, the collar lengths in the adaxial and abaxial sides were measured. The results demonstrated that the asymmetric collar length in LJs alters the leaf inclination in the mutants and overexpression lines.
Furthermore, according to the cytological structural analysis of LJs, it was found that OsARF4 regulates leaf inclination through modulating adaxial cell division rather than cell expansion of the collar in LJs (Figure 3). The increase of leaf inclination in osarf4 is achieved by promoting the differentiation of the adaxial side parenchyma cells, which is mainly to promote the synthesis of genes related to cell wall such as the cyclin gene family members. The cell cycle-related genes OsCYCU4;1, OsCYCD2;1, OsCYCD6;1, and OsCYCD1;2 also changed differently in the RNA-seq analyses of WT/DJ, osarf4 mutant, and OsARF4-overexpressing line in LJs (Supplementary Figure 6). In addition, whether auxin has polar distribution in the adaxial side and abaxial side of LJs needs to be further explored.

**The change in auxin level in lamina joint might alter leaf inclination**

Auxin responses play a crucial role in plant growth and development by forming local concentration gradients. Auxin induces rapid transcriptional responses, incorporating a series of auxin early response genes (SABR, Aux/IAA, and GH3) and ARFs. It is known that auxin synthesis and signal transduction are related to the regulation of leaf inclination (Xu et al., 2021). Moreover, during early LJ development, high local auxin concentrations were formed due to auxin synthesis and polar transport, which will stimulate cell division. During LJ development, a decrease in IAA content will promote the elongation of parenchyma cells on the adaxial side of the LJ, which is consistent with the increase in GH3.
expression, thus increasing leaf inclination (Wang et al., 2007; Chen et al., 2018). The auxin signaling pathway is normally mediated by ARFs that regulate transcription in response to auxin in plants (Guilfoyle and Hagen, 2007). In this study, we have shown that OsARF4 regulated leaf inclination via altering the auxin distribution and content in LJ. As shown in Figure 4, the auxin distribution and GUS activity decreased in osarf4 mutants, they significantly increased in OsARF4-overexpressing lines compared with those in WT/DJ. The free auxin levels in LJs were consistent with this result, implying that the alteration of auxin level in LJ regulated by OsARF4 results in changes in leaf inclination. The RNA-seq analyses also revealed that some auxin-catabolism and auxin signaling-related genes such as OsGH3-1, OsGH3-2, OsGH3-5, OsAFB2, OsARF1, and OsIAA1 showed different expression levels in the LJs of WT/DJ, osarf4 mutant, and OsARF4-overexpressing line (Supplementary Figure 6).

**OsARF4 mediates auxin-BR crosstalk during regulation of leaf inclination**

Auxin and BR are two vital phytohormones that regulate leaf inclination in rice (Tong and Chu, 2018). Studies have shown that lacking of BR leads to an erect leaf phenotype because of the inhibition of elongation of parenchyma cells and division of sclerenchyma cells on the abaxial side of the LJ (Zhang L. Y. et al., 2009; Sun et al., 2015). Most BR-deficient and BR-insensitive mutants show altered BR sensitivity by altering auxin synthesis or signal transduction, and many auxin-related mutants also show altered BR responses by changing leaf inclination, thus suggesting crosstalk between BR and auxin during LJ development (Song et al., 2009; Zhao et al., 2013; Zhang et al., 2015). The signaling pathways of auxin and BR share multiple genes, which play important roles in plant growth and development (Vert et al., 2008). In rice, OsARF19 regulates leaf inclination by binding to the promoter region of OsBRI1 to connect auxin and BR signaling pathways (Zhang et al., 2015). The ds1 mutant shows decreased leaf angle owing to lower BR sensitivity, and DS1 participates in plant architecture regulation through regulating the expression of OsBRI1 via interacting with OsARF11 in rice (Liu et al., 2018). Therefore, BR and auxin genes act synergistically to co-regulate the development of adaxial cells in LJ (Liu et al., 2016). In this study, both auxin and BR induced the expression of OsARF4 (Figure 7A; Supplementary Figure 3). DR5:GUS staining and measurement of free IAA content showed that OsARF4 is involved in auxin signaling (Figure 4). RNA-seq analysis indicated that OsARF4 is related to BR biosynthesis and signaling (Figure 5). Furthermore, OsARF4-overexpressing lines were sensitive to BR treatments and had increased leaf inclination (Figures 6, 7B–D). These results indicate that OsARF4 acts as a bridge between auxin and BR signaling during the regulation of leaf inclination.

In addition, a previous study has reported that OsARF4 interacts with the rice GSK3-like kinase, OsGSK5/OsSK4I1, to negatively regulate grain size and weight in rice. And, osarf4 mutant was no effect on BR sensitivity in terms of leaf lamina inclination at the seedling stage (Hu et al., 2018). In this study, we observed that osarf4-1 and osarf4-2 had enlarged leaf inclination at the flag leaf of mature period (Figures 2C–D). As for BR sensitivity, the BR sensitivity experiments were treated with different concentrations 0, 0.01, 0.1, 1, and 10μM of 24-eBL. The above tests presented that osarf4 mutants are insensitive to exogenous BR treatments, while OsARF4-overexpressing lines are more sensitive not only in the degree of leaf inclination but also in BR elongation and coleoptile growth (Figure 6). Besides, the different editing sites and background plants from previous research might result in a little difference of regulatory mechanism (Hu et al., 2018).

Taken together, the semi-dwarf phenotype, characterized by erect leaves and shorter panicles, is an ideal phenotype for improving grain yield in high-density planting in rice (Morinaka et al., 2006; Sakamoto et al., 2006; Wu et al., 2008). Therefore, the regulation mechanism of leaf inclination can provide a theoretical basis for plant architecture breeding that would help develop highly productive rice varieties. In our study, evidence from genetic analysis, cellular biological observation, molecular biological analysis, and physiological experiments support the finding that OsARF4 regulates leaf inclination via auxin and BR signaling pathways in rice. Improving plant architecture is an important goal for breeding of high-yield rice varieties. Erect leaves can increase planting density, improve photosynthetic capacity, increase accumulation of photosynthetic products, and ultimately increase the grain yield of rice (Huang et al., 2016b). Our research uncovers a normal regulatory factor of leaf inclination and shows that a molecular bridge exists between auxin and BR signaling. These findings may help optimize plant architecture and increase yield of rice.

**Data availability statement**

The data presented in the study are deposited in the NCBI repository, accession number PRJNA862303.

**Author contributions**

YQ and QQ designed the experiments. JQ, YZ, SH, and SC performed experiments. YQ, QQ, ZG, and JQ analyzed the data. YQ, QQ, and JQ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.979033/full#supplementary-material

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