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The basic helix-loop-helix transcription factor OsBLR1 regulates leaf angle in rice via brassinosteroid signalling

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Abstract

Leaf angle is a key factor in plant architecture and crop yield. Brassinosteroids (BRs) regulate many developmental processes, especially the leaf angle in monocots. However, the BR signalling pathway is complex and includes many unknown members. Here, we propose that *Oryza sativa BRASSINOSTEROID-RESPONSIVE LEAF ANGLE REGULATOR 1* (*OsBLR1*) encodes a bHLH transcription factor, and positively regulates BR signalling to increase the leaf angle and grain length in rice (*Oryza sativa* L.). Lines overexpressing *OsBLR1* (*blr1-D* and *BLR1-OE-1/2/3*) had similar traits, with increased leaf angle and grain length. Conversely, *OsBLR1*-knockout mutants (*blr1-1/2/3*) had erect leaves and shorter grains. Lamina joint inclination, coleoptile elongation, and root elongation assay results indicated that these overexpression lines were more sensitive to BR, while the knockout mutants were less sensitive. There was no significant difference in the endogenous BR contents of *blr1-1/2* and wild-type plants. These results suggest that OsBLR1 is involved in BR signal transduction. The *blr1-D* mutant, with increased cell growth in the lamina joint and smaller leaf midrib, showed significant changes in gene expression related to the cell wall and leaf development compared with wild-type plants; furthermore, the cellulose and protopectin contents in *blr1-D* were reduced, which resulted in the increased leaf angle and bent leaves. As the potential downstream target gene of OsBLR1, the *REGULATOR OF LEAF INCLINATION1* (*OsRLI1*) gene expression was up-regulated in *OsBLR1*-overexpression lines and down-regulated in *OsBLR1*-knockout mutants. Moreover, we screened OsRACK1A as an interaction protein of OsBLR1 using a yeast two-hybrid assay and glutathione-S-transferase pull-down.

Key message

The overexpression of *OsBLR1* caused increased leaf angle and grain length, whereas *OsBLR1*-knockout mutants had the opposite phenotypes. OsBLR1 is a member in BR signalling pathway.

Keywords Brassinosteroid signalling · Grain length · Leaf angle · OsBLR1 · OsRACK1A

Introduction

As one of the most important crops worldwide, rice is a model species for studying monocots. Plant architecture is a key factor in rice yield (Khush and Gupta 2013). The leaf angle, which is the angle between the stem and adaxial side of the blade, is a key component of rice leaf architecture. Erect leaves maximise their carbon earnings through more effective absorption of photosynthetic residues (Zhu et al. 2008; Song et al. 2013). In addition, crops with erect leaves can grow normally at increased plant densities with no additional fertiliser, increasing yield and economic benefits (Sinclair and Sheehy 1999). Recent studies have shown that plant hormones such as brassinosteroids (BRs), auxin,

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and gibberellin are the main factors that regulate leaf angle (Luo et al. 2016).

BRs, as a class of steroid hormones, are widespread, have high physiological activity, and regulate a series of developmental and physiological processes, including cell differentiation, reproductive development, photomorphogenesis, and stress responses (Bishop and Koncz 2002; Fukuda 2004). In rice, BRs positively regulate grain size and leaf angle (Zhang et al. 2014a). BR signal transduction has been studied systematically in *Arabidopsis thaliana*. BRI1 recognises the BR signal, which causes separation from BKI1 (Nam and Li 2002; Wang and Chory 2006), and then the BRI1 Associated receptor Kinase 1 (BAK1) binds to BRI1, and the phosphorylated BRI1-BAK1 complex activates BR-signalling kinases (BSKs), which causes the activated BSKs to phosphorylate BSU1 (Tang et al. 2008). On dephosphorylation of BSU1, BIN2 loses its phosphorylation activity on the transcription factor BZR1/BES1, which leads to the accumulation of BZR1 and BES1 in the nucleus (Ryu et al. 2007, 2010). These two key transcription factors ultimately regulate the expression of downstream genes and affect plant development (Wang et al. 2002; Yin et al. 2002). In rice, some BR signalling proteins have been studied. The BR signal is activated by the dephosphorylation of GSK3/SHAGGY-like kinase (OsGSK2, a homologue of BIN2 in rice), which releases its suppression on BRASSINAZOLE-RESISTANT1 (OsBZR1) and DWARF AND LOW-TILLERING (OsDLT) and ultimately increases the leaf angle (Bai et al. 2007; Tong and Chu 2009; Tong et al. 2012). However, the BR signalling pathway is complex, and the functions of many BR-related proteins in rice are unclear.

The bHLH transcription factor family proteins have diverse functions in plants and are involved in growth, stress resistance, biosynthesis, and signal transduction (Jiang et al. 2009; Oikawa and Kyozuka 2009; Ogo et al. 2010). The typical bHLH transcription factor has a highly conserved domain, which comprises approximately 60 amino acids with two functionally distinct regions: the DNA binding region (basic region) and the helix1-loop-helix2 region (HLH region) (Atchley et al. 1999). bHLH transcription factors play an important role in regulating BR signalling. In *Arabidopsis thaliana*, BES1 interacts with BIM1 (bHLH protein) to bind the E-box of target genes and promote target gene expression (Yin et al. 2002). AIF2 (bHLH protein) interacts with BIN2 to participate in the BR signalling pathway and the overexpression of AIF2 represses the expression of growth-promoting genes, which results in growth retardation (Kim et al. 2017). In rice, ILI1 dimerises with IBH1 (bHLH protein) to regulate the leaf angle under BR induction (Zhang et al. 2009). OsBUL1 (HLH protein) interacts with OsBC1 (bHLH protein) and LO9-177 to form a heterotrimer complex, which responds to BR signalling and regulates leaf angle in rice (Jang et al. 2017). There are approximately 167 members of the bHLH transcription factor family in rice (Li et al. 2006), and studies on the relationships between BR and bHLH proteins are insufficient. Research to identify new bHLH proteins in BR signalling is essential for a deep understanding of BR.

The Receptor for Activated C-Kinase 1 (RACK1) protein family contains the tryptophan-aspartate domain WD40 (Guo and Chen 2008). In plants, RACK1 regulates a series of physiological processes, including seed germination, root and leaf growth, and stress responses (Ullah et al. 2008; Guo et al. 2009). RACK1 family members include RACK1A, RACK1B, and RACK1C, of which RACK1A is the most important (Guo and Chen 2008). RACK1A is involved in many hormone signal pathways in *Arabidopsis thaliana* (Islas-Flores et al. 2015). The RACK1A mutant is insensitive to gibberellin and BR and exhibits a variety of developmental defects affecting seed germination, leaf production, and flowering (Chen et al. 2006). In rice, OsRACK1A (*Os01g0686800*) regulates seed germination by taking part in abscisic acid and H_2O_2-mediated signalling (Zhang et al. 2014b). Recently, it was reported that rice OsRACK1A mutants are shorter (Chen et al. 2019). However, the study of the multifunctional protein OsRACK1A is at an early stage and needs more research in rice.

In this study, we revealed that OsBLR1 (*Os02g0705500*), as a typical bHLH protein (Li et al. 2006), is a positive regulator in BR signalling. The overexpression of *OsBLR1* in rice caused an increased leaf angle, grain length, and BR sensitivity, and the opposite phenotypes appeared in *OsBLR1*-knockout mutants. We performed additional preliminary studies of the mechanism of OsBLR1 in BR signalling.

Materials and methods

Plant materials and growth conditions

The wild-type Dongjin (DJ, *Oryza sativa* L. ssp. *japonica* cv.) and T-DNA insertion mutant seeds of *OsBLR1* were purchased from the Korean rice mutant library (https://signal.salk.edu/). Homozygous mutants were identified by two rounds of PCR with three primers (T-DNA border primer: RB-R, Gene primers: BLR1-LP-F and BLR1-RP-R). For homozygous mutants, we could clone the T-DNA border sequence, but could not clone the genome sequence by Gene primers. The T-DNA (pGA2772) insertion copy number analysis was carried out for four hypothetical homozygous mutants by Southern blot, and the specific sequence for 512 bp of T-DNA was marked with digoxin for detection (Zoonbio Biotechnology, Nanjing, China). To generate *OsBLR1*-overexpression plants, the full-length CDS sequence of *OsBLR1* was linked to the pTCK303 plasmid to construct the Ubi:OsBLR1 vector. Then, the vector was

transformed into *Agrobacterium tumefaciens* (EHA105) and introduced into Nipponbare (NIP, *Oryza sativa* L. ssp. *japonica* cv.) using the callus genetic transformation method. Positive lines were detected by hygromycin screening and reverse transcription-quantitative PCR (RT-qPCR) analysis. To construct the loss-of-function rice plants of *OsBLR1* using the CRISPR/Cas9 technology, two protospacer adjacent motif (PAM) site sequences (PAM1: ACCGTCGAG AGCCTCTGCCAGGG, PAM2: AGAGCAACAGCATCT GCAAGAGG) were located in the first and second exons of *OsBLR1* were selected. Following, the sgRNAs (sgRNA1: CCGTCGAGAGCCTCTGCCA, sgRNA2: GAGCAACAG CATCTGCAAG) were linked to the pBWA (V) H2S-cas9pl (osu3)i plasmid to construct the Cas9-OsBLR1 vector. Then, the vector was introduced into NIP in the same method. The homozygous mutants were obtained by gene segregation after the seed propagation of T0-T1 generation. The mutation types were analyzed by sequence alignment of *OsBLR1* between mutants and wild type.

For disinfection, seeds were soaked in 5% NaClO solution for 15 min. These sterilized seeds were then sown on 1/2 MS agar medium containing 0.8% phytagel (pH 5.7) and germinated in darkness at 30 °C for 2 days. The germinating seeds were transferred to a 28 °C/25 °C (16 h/8 h) light incubator for culture in water or to a greenhouse for soil culture. Tobacco (*Nicotiana benthamiana*) seeds were provided by our laboratory. Tobacco seeds were sown evenly into culture soil (nutritious soil:vermiculite = 3:1) and cultured in a 26 °C/24 °C (16 h/8 h) light incubator.

Gene expression analysis

The Trizol method was performed to extract total RNA, and using the FastKing RT Kit (with gDNase) (Tiangen, Beijing, China) for reverse transcribed of cDNA from mRNA. The cDNA was used as the template for semi-quantitative reverse transcription PCR (SqRT-PCR) and RT-qPCR. *β-ACTIN* was used as the reference gene. For RT-qPCR, the SYBR Green Taq Ready Mix (Tiangen, Beijing, China) and the Bio-Rad CFX96 Real-Time System C1000 Thermal Cycler were used for expression analysis. The following data analysis formula referred to $R = 2^{-[\Delta C_t \text{ sample} - \Delta C_t \text{ control}]}$, where R refers to the relative expression level, ΔC_t sample is the D-value of gene Ct value to internal reference *β-ACTIN* Ct value in experimental group, and ΔC_t control is the D-value of gene Ct value to *β-ACTIN* Ct value in the control group. The RT-qPCR data represent the mean ± SE (n = 3). The data were compared by Student's *t*-test. *$p < 0.05$, **$p < 0.01$.

Subcellular localization analysis

The full-length *OsBLR1* coding region without a stop codon was linked to pBI221-GFP plasmid to construct pBI221-OsBLR1-GFP vector. pBI221-OsBLR1-GFP and pBI221-GFP plasmids were transformed into EHA105 and spread on YEP medium (10 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl, 50 μg/ml Ampicillin, 50 μg/ml Rifampin, 15 g/l Agar for solid medium), and incubated at 28 °C for 3 days. Shaking the positive clone bacteria in YEP medium and resuspending it with MMA (10 mM MES, 10 mM $MgCl_2$, 0.2 mM Acetosyringone, pH 5.6) to $OD_{600} = 0.1$. The mixture was injected into tobacco for transient expression, and then incubated in darkness at 25 °C for 2.5 days. Observation was performed by LSM 710 Laser Confocal Microscope (Carl Zeiss AG, Germany).

Scanning electron microscopy and paraffin section

The germinated seeds were transplanted into the soil and grew to filling stage at 30 °C. For Scanning electron microscopy, the lamina joint tissue of flag leaf (2 cm of length) was excised for imaging the adaxial surface. Plant tissues were fixed in 2.5% glutaraldehyde solution, and then in a graded ethanol series. After desiccation for 3 h, sprayed with gold powder, and observed by scanning electron microscope. For paraffin section, the flag leaf tissue distance from stem 3 cm (0.5 cm of length) were placed in FAA solution (50% alcohol:formalin (37–40% formaldehyde):glacial acetic acid; 18:1:1). After dehydration in a graded ethanol series (70%, 80%, 90% and 100%), followed by embedding. The samples were dissected and then observed by light microscope. Parts of these experiments were performed by Servicebio company (Servicebio technology CO, Wuhan, China).

Measurement of endogenous BRs, cellulose and protopectin contents

blr1-D and wild-type DJ plants (> 50 seeds) grew in a greenhouse for two months. For endogenous BR measurement, the leaf and stem tissue (> 2 g) were mixed. The samples were rapidly frozen and ground to powder and then dissolved in methanol. The endogenous BRs contents were determined by electrospray ionisation/high-performance liquid chromatography/tandem mass spectrometry (ESI-HPLC–MS/MS) (ProNetsBio, Wuhan, China), and the measure method referred to Wu et al. (2013) and Xin et al. (2013). For endogenous BR measurement of wild-type NIP-1/2, *blr1-1* and *blr1-2*, we sampled at 5 cm in the vicinity of the lamina joint and performed measurement in the same method. For cellulose and protopectin measurement, leaf tissue (> 0.3 g) was used for crude extraction and digestion with sulfuric acid, and a determination experiment was performed by Comin Company (Suzhou Comin biotechnology, Suzhou, China).

Exogenous BR treatment analysis

For lamina joint inclination assay, sterilized rice seeds were grown in the dark at 30 °C for 9 days. We obtained equally sized samples by excising approximately 2 cm of tissue near the lamina joint. The samples were floated on distilled water containing various concentrations of 24-Epibrassinolide (eBL, a bioactive form of BRs, dissolved in ethanol) at 30 °C for 30 h in darkness. Leaf angle was measured by Image J software. This experiment was carried out under dim light and repeated three times. For coleoptile elongation assay and root elongation assay, the sterilized seeds grew on 0.4% phytagel medium with different concentrations (0, 1, 10 μM) of eBL at 30 °C for 4.5 days in darkness, and then measured the length of the coleoptile and the primary root. For RNA extraction, 10-day-old seedlings were sprayed with 10 μM eBL, and then the leaves were harvested at various time points. For phenotypic analysis of BR treatment in living plants, different concentrations of eBL solution was applied to specific tissue using transfer liquid gun, and then the plants were cultured in greenhouses at 30 °C.

RNA-Seq analysis

Samples were collected from the leaves of DJ and *blr1-D* at the tillering stage (2 months), and two biological replicates were carried out. The construction and sequencing of the cDNA library were based on the BGISEQ-500 platform (BGI Genomics, Shenzhen, China). The clean reads obtained by sequencing data filtering were compared with the reference genome IRGSP-1.0. The data of DJ and *blr1-D* were normalized, and genes whose expression differences were more than two-fold increased were selected and analysed by the differentially expressed genes (DEG)-seq method (Wang et al. 2010). The DEGs were classified according to Gene Ontology (GO) annotation (https://geneontology.org/). The phyper function of R software was used for enrichment analysis and FDR correction of the p-value. FDR ≤ 0.01 was regarded as significant enrichment. The selected differential genes were blasted using the RAP-DB database (https://rapdb.dna.affrc.go.jp/index.html) and the NCBI database (https://www.ncbi.nlm.nih.gov/). The heatmap was plotted by MeV4.9.0 software.

Yeast two-hybrid (Y2H) assay

The full-length CDS sequence of *OsBLR1* was linked to the pGBKT7 plasmid to construct the OsBLR1-pGBKT7 vector. OsBLR1-pGBKT7 plasmid was transformed into *yeast* AH109 for toxicity and auto-activation detection. For yeast two-hybrid library screening assay, OsBLR1-pGBKT7 plasmid was used as bait and mated with a rice seedling yeast two-hybrid library (constructed by Shanghai biogene biotechnology company, Shanghai, China), and then the mated culture was spread on the SD/-Leu/-Trp, SD/-Leu/-Trp/-His and SD/-Leu/-Trp/-His/-Ade plates for positive screening. Finally, the positive clones were detected by Lac Z assay. The selected positive clones were analysed by PCR and sequencing alignment with databases (NCBI and RAP-DB). For the yeast two-hybrid verification assay, the CDS sequence of *OsRACK1A* was cloned into the pGADT7 plasmid to construct the OsRACK1A-pGADT7 vector, OsBLR1-pGBKT7 and OsRACK1A-pGADT7 plasmids were co-transferred into *yeast* AH109 and spread on SD/-Leu/-Trp, SD/-Leu/-Trp/-His, SD/-Leu/-Trp/-His/-Ade and then the Lac Z assay was performed. pGBKT7-P53 + pGADT7-largeT was used as a positive control and pGBKT7-Laminc + pGADT7-largeT was used as a negative control.

Glutathione-S-transferase (GST) pull-down assay

The CDS sequence of *OsBLR1* was linked to the pGEX-4T1 plasmid to construct the OsBLR1-pGEX-4T1 vector. OsBLR1-GST and GST proteins were expressed in *Escherichia coli* Rosetta (DE3) after induction by 0.5 mM isopropyl-b-D-thiogalactopyranoside (IPTG) and purified by Glutathione SepHarose™ 4B (GE Healthcare, Boston, USA). The similar method was performed to construct the OsRACK1A-pET-30a vector. OsRACK1A-His protein was expressed in *Escherichia coli* Rosetta (DE3) after induction by 0.8 mM IPTG and purified by Ni SepHarose™ 6 Fast Flow (GE Healthcare, Boston, USA). For GST-pulldown assay, glutathione beads containing 0.5 mg OsBLR1-GST or GST purified proteins were incubated with 0.5 mg OsRACK1A-His protein in pull-down buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA and 1 mM DTT) at 4 °C for 5 h. The beads were washed three times with wash buffer (20 mM Tris–HCl, pH 7.5, 300 mM NaCl, 1 mM EDTA, 1 mM DTT and 0.1% (w/v) TritonX-100). The proteins were eluted from the beads using elution buffer (20 mM Tris–HCl, pH 7.5, 300 mM NaCl, 1 mM DTT and 15 mM Glutathione), and then boiled in 2 × sodium dodecyl sulphate (SDS) buffer for 5 min. The mixture was isolated in a 10% SDS–polyacrylamide gel. We analysed the results by Coomassie brilliant blue R-250 staining and Western blot analysis using anti-GST and anti-His antibodies (Abmart, Shanghai, China).

Results

Overexpression of *OsBLR1* increased leaf angle and grain length

In the prior researches on the expression profile of gene, we found that *OsBLR1* was highly expressed in the leaf and was mainly located in the nucleus (Fig. S1a, b, and c). To explore the function of *OsBLR1* in the leaf, we screened T-DNA insertion mutants of *OsBLR1*, and obtained the homozygous, single-copy T-DNA insertion mutant *blr1-D* by PCR and Southern blotting (Fig. S2a and b). Sequencing analysis indicated that the reverse insertion site of Ti plasmid containing the 35 s enhancer (Jeong et al. 2006) in *blr1-D* was 624 bp upstream from the initiation codon of *OsBLR1*, and the Ti plasmid was located between *OsBLR1* and *Os02g0705600* on chromosome 2 (Fig. 1a). RT-qPCR data showed that the expression of *OsBLR1* was significantly increased, while that of *Os02g0705600* was unchanged in *blr1-D* (Fig. 1b), which demonstrated that *blr1-D* was an *OsBLR1*-overexpression

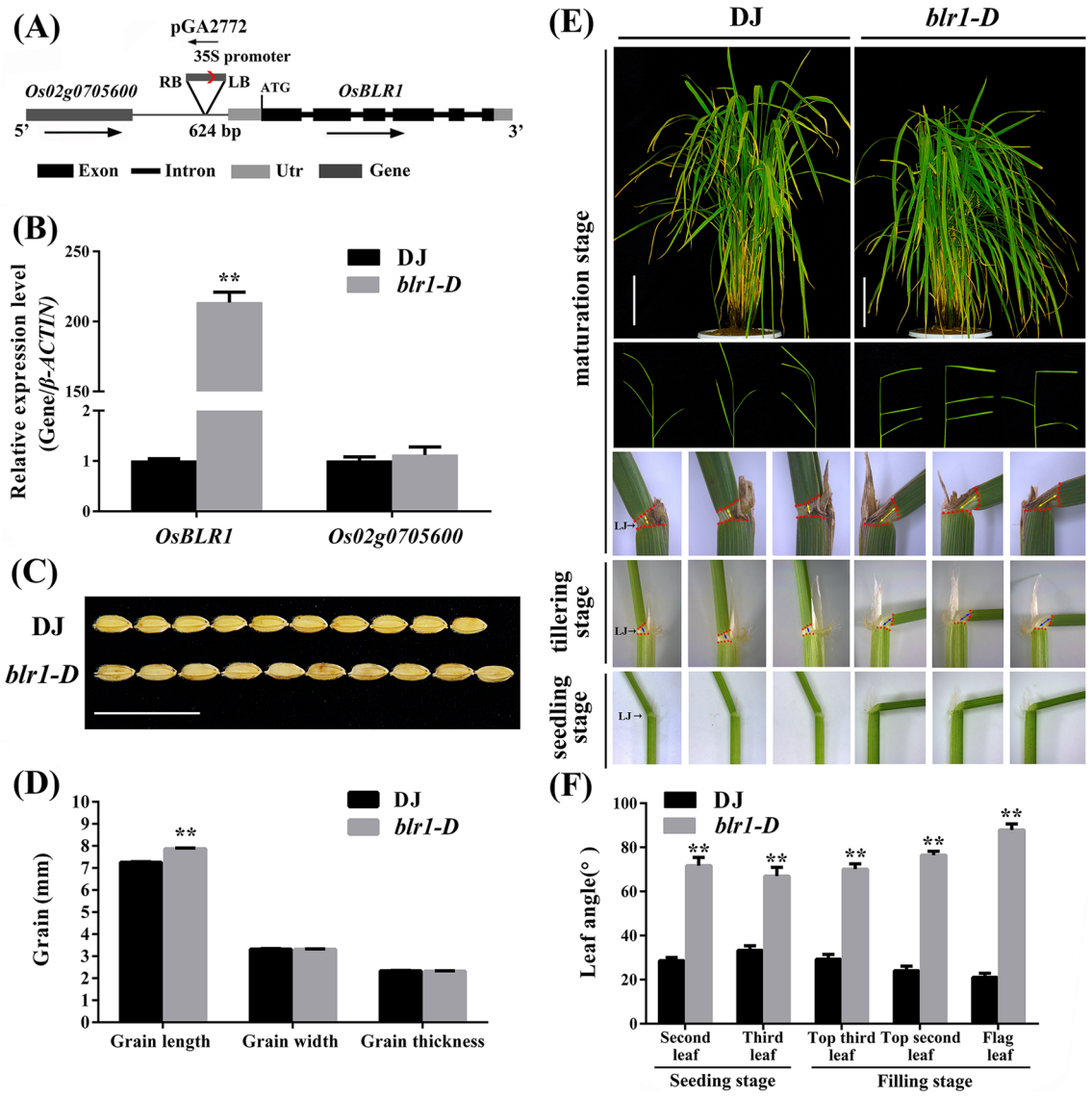


Fig. 1 The T-DNA insertion mutant of *OsBLR1* (*blr1-D*) had increased leaf angle and grain length. **a** The reverse insertion site of the Ti plasmid between *OsBLR1* and *Os02g0705600* was located at 624 bp upstream of *OsBLR1*. **b** Expression analysis of *OsBLR1* and *Os02g0705600* in *blr1-D* by RT-qPCR. Data represent the mean ± SE (n = 3). **p < 0.01, Student's *t*-test. **c** The grain (T3 generation) photos of DJ and *blr1-D*. Bar: 2 cm. **d** Statistical analysis of grain length, grain width and thickness in DJ and *blr1-D*. Data represent the mean ± SE (n = 15). **p < 0.01, Student's *t*-test. e Phenotypic photos of DJ and *blr1-D* during different development stages. LJ: Lamina joint. Bar: 20 cm. f Statistical analysis of leaf angle in DJ and *blr1-D*. Data represent the mean ± SE (n = 10). **p < 0.01, Student's *t*-test

line. The phenotype of *blr1-D* was characterised using T3 generation plants. We found that *blr1-D* had larger grains than the wild-type DJ (Fig. 1c). Although the grain length of *blr1-D* increased significantly, there were no significant changes in grain width or thickness (Fig. 1d). *blr1-D* had a dramatically increased leaf angle during the seedling, tillering, and maturation stages compared with DJ, and had drooping leaves that appeared bent and brittle (Fig. 1e). At the seedling stage (4-week-old rice), the angles of the second and third leaves of DJ were 27° and 32°, respectively, while the corresponding angles of *blr1-D* were 72° and 67°. At the filling stage, the angles of the top third leaf, the top second leaf and the flag leaf of DJ were 29°, 24°, and 21°, respectively, while the corresponding angles of *blr1-D* were 70°, 76°, and 87° (Fig. 1f).

To verify the function of *OsBLR1*, we transformed wild-type rice (NIP) with a construct of the Ubi promoter driving the expression of *OsBLR1* cDNA and obtained three overexpression lines after hygromycin screening and expression analysis: *BLR1-OE-1*, *BLR1-OE-2*, and *BLR1-OE-3* (Fig. 2a, b). The T2 generation overexpression lines had larger grains than NIP (Fig. 2c); the grain length of *BLR1-OE-1/2/3* was longer, but there were no significant changes in grain width or grain thickness (Fig. 2d). At the tillering stage, *BLR1-OE-1/2/3* showed a significant increase in leaf angle compared with NIP (Fig. 2e). The leaf angle of NIP was 28°, while those of *BLR1-OE-1*, *BLR1-OE-2*, and *BLR1-OE-3* were 62°, 59°, and 68°, respectively (Fig. 2f). In addition, compared with the wild type, the 1000-grain weight, and tiller number of the *OsBLR1*-overexpression lines were significantly increased (Fig. S3a and b). Considering the

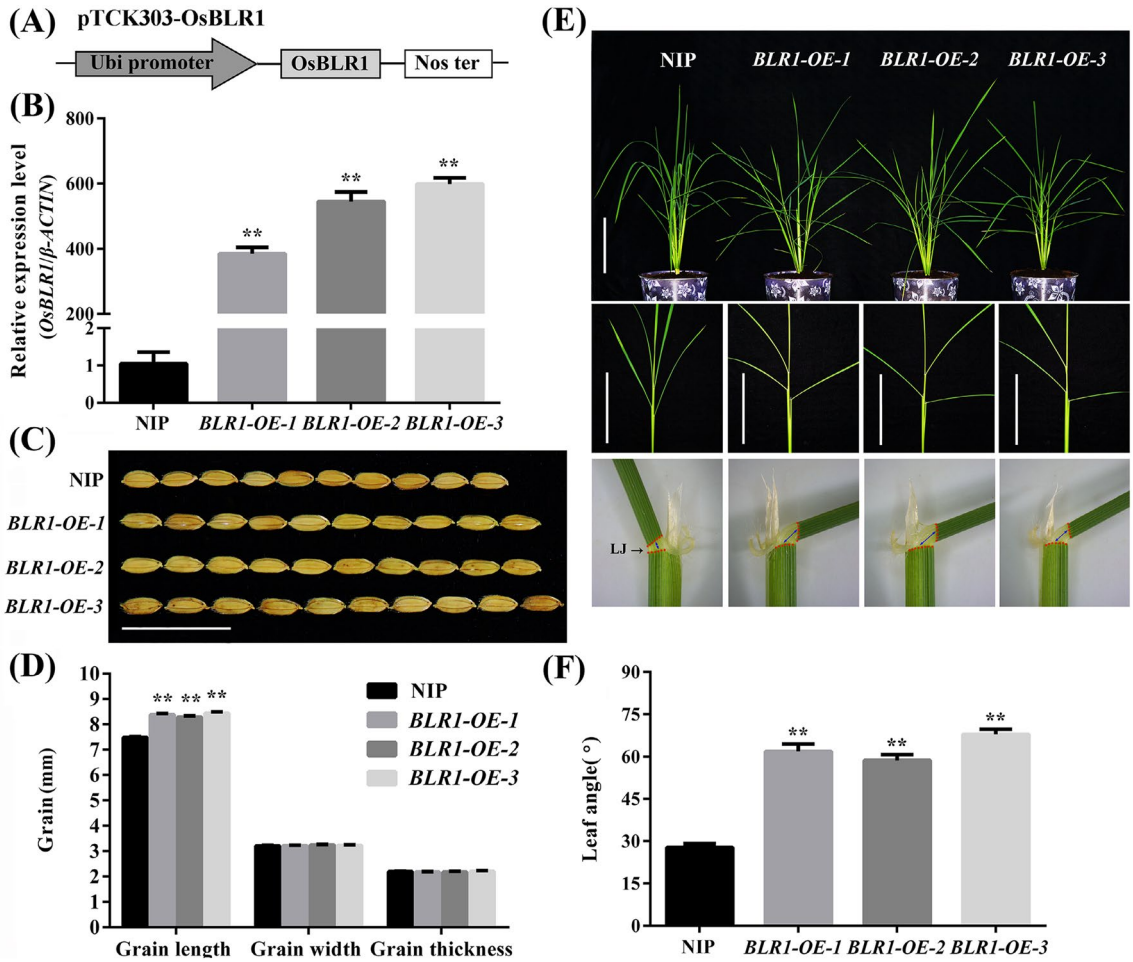


Fig. 2 *OsBLR1*-overexpression lines (*BLR1-OE-1/2/3*) had increased leaf angle and grain length. **a** The Ubi:OsBLR1 construct model in pTCK303-OsBLR1 plasmid. **b** RT-qPCR analysis of the expression of *OsBLR1* in *BLR1-OE-1/2/3* and NIP. Data represent the mean ± SE (n = 3). **p < 0.01, Student's *t*-test. **c** The grain (T2 generation) photos of *BLR1-OE-1/2/3* and NIP. Bar: 2 cm. **d** Statistical analysis of grain length, grain width and thickness in *BLR1-OE-1/2/3* and NIP. Data represent the mean ± SE (n = 15). **p < 0.01, Student's *t*-test. **e** Phenotypic photos of NIP and *BLR1-OE-1/2/3* during the tillering stage. *LJ* lamina joint. Bar: 20 cm. **f** Statistical analysis of the leaf angle in NIP and *BLR1-OE-1/2/3*. Data represent the mean ± SE (n = 10). **p < 0.01, Student's *t*-test

consistent phenotypes in *blr1-D* and *BLR1-OE-1/2/3*, we inferred that *OsBLR1* increased the leaf angle and grain length in rice.

Knockout of *OsBLR1* decreased leaf angle and grain length

To understand the function of OsBLR1, we generated *OsBLR1*-knockout mutants using the CRISPR/Cas9 technique. The target PAM sequences were located in the first and second exons of *OsBLR1*. Ultimately, we obtained three mutants with altered OsBLR1amino acid sequences, and named *blr1-1*, *blr1-2*, and *blr1-3* (Fig. 3a and S4). Interestingly, these mutants had significantly smaller grains than NIP due to the grain length decreased significantly (Fig. 3b); however, there was no significant difference in grain width or thickness (Fig. 3c). *blr1-1*, *blr1-2*, and *blr1-3* had erect leaves (Fig. 3d). The leaf angle of NIP was 28°, while those of *blr1-1*, *blr1-2*, and *blr1-3* were 13°, 16°, and 14°, respectively (Fig. 3e). Considering the opposite phenotypes in the overexpression lines and knockout mutants of *OsBLR1*, we confirmed that OsBLR1 functions to increase leaf angle and grain length. Interestingly, compared with the wild type, the 1000-grain weight, and tiller number of *blr1-1*, *blr1-2*, and *blr1-3* were decreased (Fig. S3a and b). This confirmed that OsBLR1 functions not only to increase leaf angle and grain length, but also 1000-grain weight, and tiller number.

OsBLR1 transduced the BR signalling

We found that the phenotypes of increased leaf angle and stem elongation in *blr1-D* seedlings were consistent with wild-type rice seedlings treated with eBL (Fig. S5a–d). Because the transgenic lines of OsBLR1 had these typical phenotypes, especially in the leaf angle, we speculated that OsBLR1 was involved in either the biosynthesis or signal

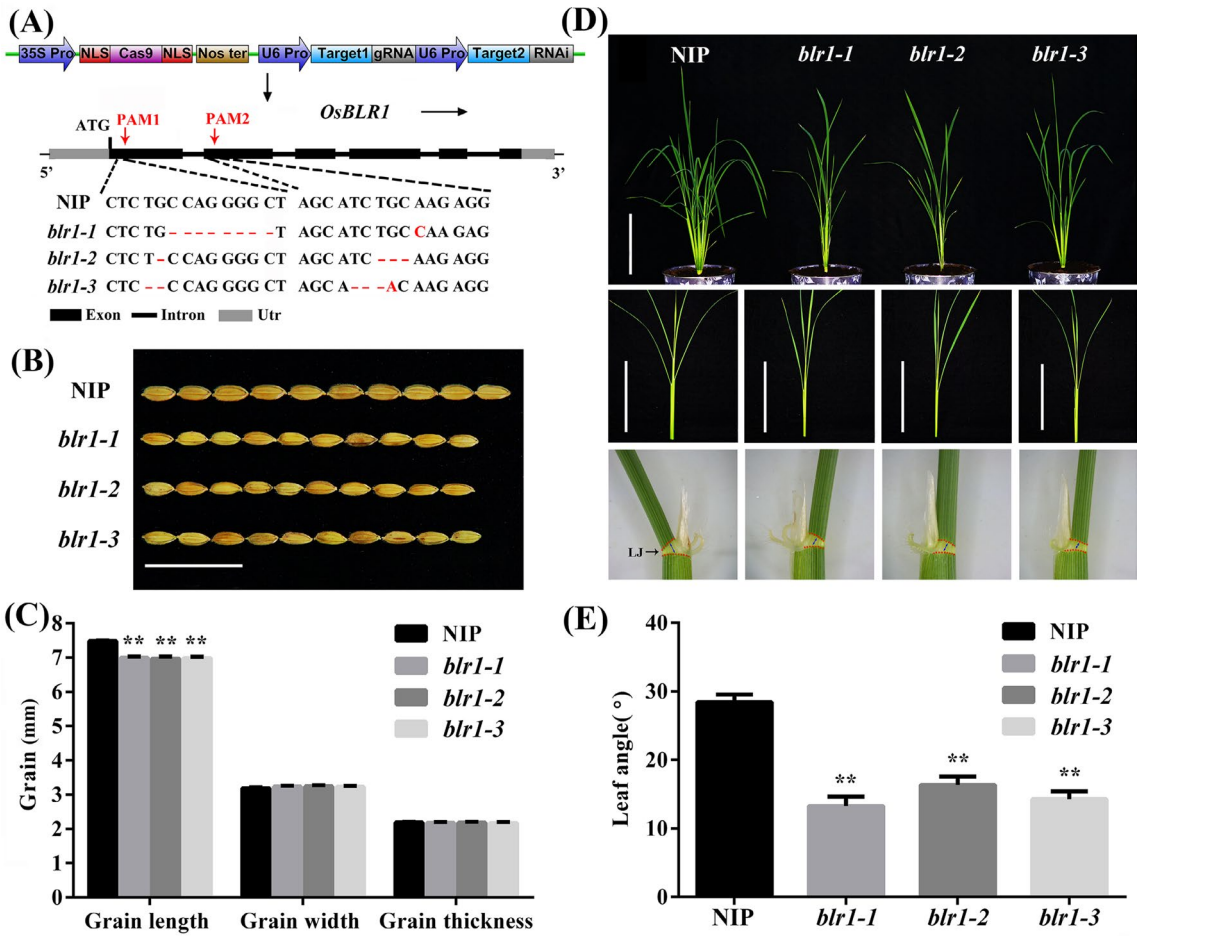


Fig. 3 *OsBLR1*-knockout mutants had erect leaves and shorter grains. **a** The Cas9-OsBLR1 construct model and mutation types in PAM site sequences of *blr1-1/2/3*. **b** The grain (T2 generation) photos of *blr1-1/2/3* and NIP. *LJ* lamina joint. Bar: 20 cm. **c** Statistical analysis of grain length, grain width and thickness in *blr1-1/2/3* and NIP. Data represent the mean ± SE (n = 15). **p < 0.01, Student's *t*-test. **d** Phenotypic photos of NIP and *blr1-1/2/3* during the tillering stage. *LJ* lamina joint. Bar: 20 cm. **e** Statistical analysis of leaf angle in NIP and *blr1-1/2/3*. Data represent the mean ± SE (n = 10). **p < 0.01, Student's *t*-test

transduction of BR. Lamina joint inclination is one of the most sensitive responses to BR in rice and has been used as a sensitive bioassay for BR (Yamamuro et al. 2000). First, we performed a lamina joint inclination assay using eBL to study the relationship between OsBLR1 and BR. This showed that the leaf angle changed in a dose-dependent manner, and *blr1-D* reached the maximum bending angle at 1 μM eBL, while the wild-type DJ reached the maximum bending angle at 10 μM (Fig. 4a, b). Similarly, the wild-type NIP reached the maximum bending angle at 10 μM eBL, but the corresponding concentration in *BLR1-OE-1/2/3* was 5 μM (Fig. 4c, d). However, in living plants, *blr1-1/2/3* mutants did not show significant bending, even at 100 μM eBL (Fig. 4e, f). The BR signalling pathway was blocked in these knockout mutants.

Next, we performed coleoptile elongation and root elongation assays. In the wild-type plants, the coleoptile length was elongated in an eBL dose-dependent manner and the root was wavy and its elongation was inhibited by high concentrations of eBL (1 and 10 μM). The coleoptile and root elongation responses to exogenous BR were enhanced in the overexpression lines (*blr1-D* and *BLR1-OE-1*) and decreased in the knockout mutants (*blr1-1* and *blr1-2*) (Fig. 5a–c). The above findings suggested that the sensitivity to BR in *OsBLR1*-knockout mutants was reduced, whereas it was increased in *OsBLR1*-overexpression lines. Then we measured the contents of endogenous BRs, the brassinolide (BL),

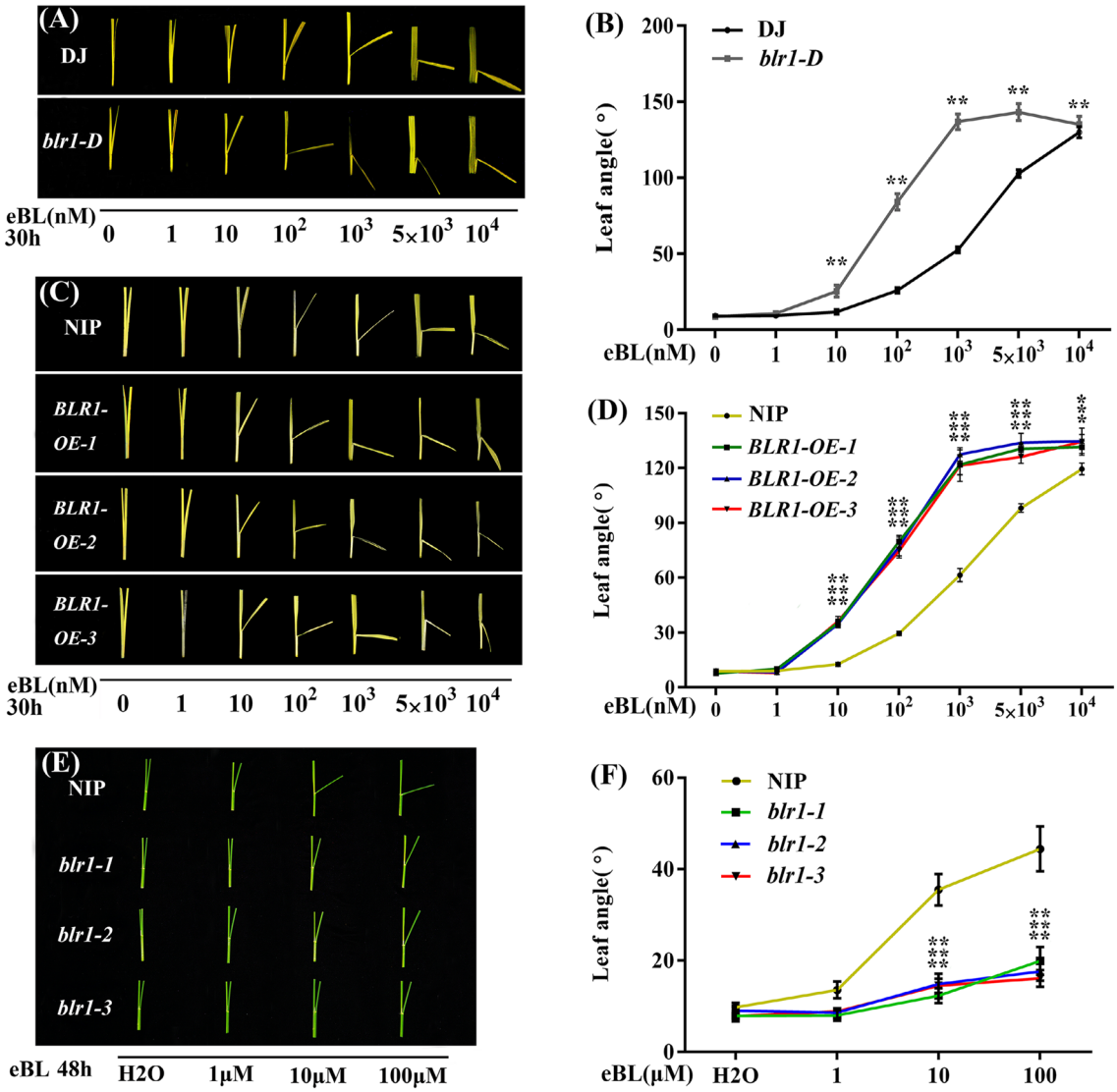


Fig. 4 Lamina joint inclination assay in wild type and *OsBLR1*-transgenic lines. **a**, **c** and **e** The lamina joint inclination assay using different concentration of eBL in *blr1-D*, *BLR1-OE-1/2/3*, *blr1-1/2/3* and wild-type. **b**, **d** and **f** Statistical analysis of the data in **a**, **c** and **e**. Data represent the mean ± SE (n > 3), *p < 0.05, **p < 0.01, Student's *t*-test

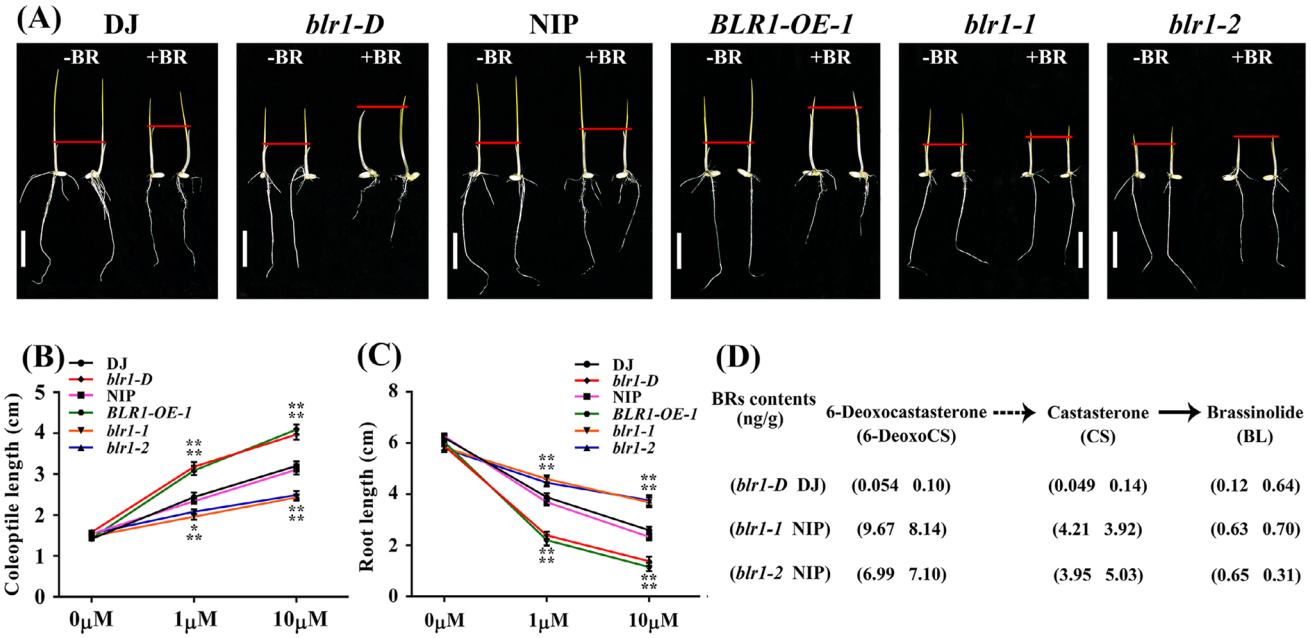


Fig. 5 The measurement of sensitivity to eBL (coleoptile elongation and root elongation assays) and contents of endogenous BRs of *OsBLR1*-transgenic lines. **a** Seedlings of wild type and transgenic lines of *OsBLR1* were photographed after growing in medium in the presence (+) or absence (−) of 1 μm BR (eBL) for 4.5 days. Bar: 2 cm. **b**, **c** Statistical analysis of coleoptile elongation and root elongation under different concentration of BR treatment. Data represent the mean ± SE (n = 10), *p < 0.05, **p < 0.01, Student's *t*-test. **d** The contents of BL, CS and 6-DeoxoCS in wild type, *blr1-D* and *blr1-1/2* by ESI-HPLC–MS/MS method. In parentheses, the value of the mutant was on the left and the wild type on the right

castasterone (CS), and 6-deoxocastasterone (6-DeoxoCS) contents in *blr1-D* were lower than in DJ, whereas there were no significant differences in NIP, *blr1-1*, and *blr1-2* (Fig. 5d). Accordingly, we inferred that OsBLR1 affects the signal transduction but not biosynthesis of BR.

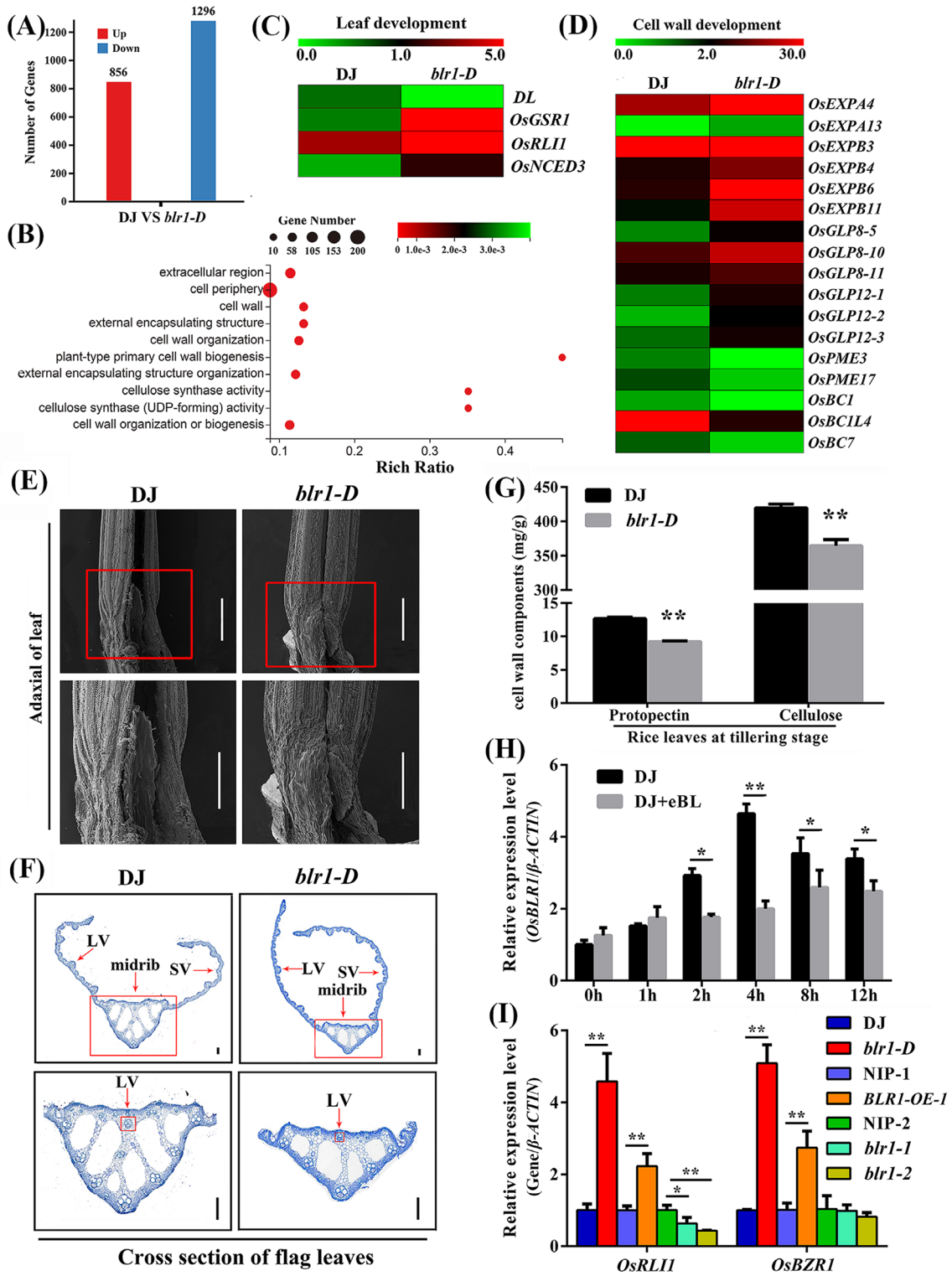
OsBLR1 regulated leaf development and BR signalling

RNA-Seq analysis was performed to analyse the molecular mechanism of OsBLR1 function in leaf development. DEG-seq analysis showed that approximately 2152 genes were significantly changed in *blr1-D* compared with DJ (Fig. 6a). The GO annotation classification results of these differentially expressed genes (DEGs) indicated that many genes were associated with cell development, leaf development, and hormone signalling. After the GO enrichment analysis, the ten terms with the smallest Q-values were selected; these terms were closely related to cell development, especially cell wall development (Fig. 6b). Among leaf development genes, the expression of *DL* was down-regulated, while the expression of *OsRLI1*, *OsGSR1*, and *OsNCED3* were up-regulated (Fig. 6c). Among cell development genes, the expression of *OsEXPs* and *OsGLPs* in *blr1-D* were up-regulated. However, the expression of the pectin methylesterases *OsPME3* and *OsPME17*, and the cellulose synthases *OsBC1*, *OsBC1L4*, and *OsBC7* were significantly down-regulated (Fig. 6d).

Additionally, *blr1-D* showed increased cell growth on the adaxial side of the lamina joint and had a smaller leaf midrib on scanning electron microscopy and paraffin section observations (Fig. 6e, f). The cellulose and protopectin contents in leaves of *blr1-D* were reduced by 13.2% and 27.2%, respectively, compared with DJ (Fig. 6g). Moreover, the expression of *OsBLR1* was down-regulated by 10 μM eBL (Fig. 6h). To explore the function of OsBLR1 in the BR signalling, we analysed the expression of a series of marker genes in the BR signal network in the OsBLR1 transgenic lines. Of these, *OsBZR1* and *OsRLI1* were up-regulated in the *OsBLR1*-overexpression lines, whereas *OsRLI1* was down-regulated in the *OsBLR1*-knockout mutants; however, the expression of *OsBRI1*, *OsGSK2*, *OsDLT*, and *OsLIC* were unchanged in the OsBLR1-transgenic lines and wild type (Figs. 6i and S6). Above results suggested that OsBLR1 regulates leaf development and transduces BR signalling.

OsBLR1 interacted with OsRACK1A

To function, transcription factors usually interact with other proteins. To investigate the OsBLR1 binding proteins, we performed Y2H screening with a rice seedling yeast library using the full-length OsBLR1 protein as bait.



The pGBKT7-OsBLR1 bait plasmid was nontoxic to yeast cells, but self-activated, and 20 mM 3-AT completely inhibited the self-activation of OsBLR1 (Fig. 7a). After mating pGBKT7-OsBLR1 with the yeast library, 22 strong interactive proteins were obtained after screening SD-THL, SD-THLA, and LacZ detection (Fig. S7). OsRACK1A was one of the candidate proteins, and has been reported to respond to multiple hormone signals. To verify the interaction between OsBLR1 and OsRACK1A, we co-transformed pGADT7-OsRACK1A with pGBKT7-OsBLR1 into yeast

◂**Fig. 6** OsBLR1 regulated leaf development and BR signalling. **a** Genes regulated by *OsBLR1* using RNA-Seq. **b** Analysis of the top 10 GO terms in the smallest Q value using GO enrichment of DEGs in *blr1-D*. **c** Heatmap showing the fold changes in gene expression involved in leaf development in *blr1-D*. **d** Heatmap showing the fold changes in gene expression involved in cell wall development in *blr1-D*. Log_2 (fold changes) are represented by a colour scale from green (down-regulated) to red (up-regulated). **e** The contents of protopectin and cellulose in the leaves of *blr1-D* and DJ. Data represent the mean ± SE (n = 3). **p < 0.01, Student's *t*-test. **f** The increased cell growth on the adaxial side of the lamina joint in *blr1-D* shown by scanning electron microscopy. Bar: 200 μm. **g** Histological section analysis of the cross section of blade in *blr1-D* and DJ. Bar: 200 μm. LV: large vascular, SV: small vascular. **h** The expression of *OsBLR1* at different points in time under the induction of eBL (10 μM). i The expression s of *OsRLI1* and *OsBZR1* in the *OsBLR1*-transgenic lines. Data represent the mean ± SE (n = 3). *p < 0.05, **p < 0.01, Student's *t*-test

(AH109). The yeast cells grew well in both SD-THL and SD-THLA medium, and LacZ detection showed blue colour (positive results) (Fig. 7b). We further proved the interaction in a GST pull-down experiment, SDS-PAGE and Western blot data showed that GST protein could not pull down OsRACK1A-His protein, but OsBLR1-GST protein could pull down OsRACK1A-His protein (Fig. 7c). We treated rice plants with 10 μM eBL and found that the expression of *OsRACK1A* was up-regulated by eBL (Fig. 7d); moreover, OsRACK1A was up-regulated in the *OsBLR1*-overexpression lines (Fig. 7e). Accordingly, we speculated that OsRACK1A interacts with OsBLR1 and is up-regulated by BR.

Discussion

OsBLR1 is a typical member of the bHLH transcription factor family, but its specific function and mechanism in rice are unknown. In this study, we demonstrated the involvement of OsBLR1 in BR signalling by constructing and identifying transgenic lines of *OsBLR1*. OsBLR1 transduces the BR signal to increase leaf angle and grain length. OsBLR1 regulated the expression of *OsDL*, *OsEXPs*, *OsGLPs*, *OsBC1*, *OsBCIL4*, and *OsRLI1*, which affected leaf development, cell wall development, and BR signalling, resulting in lower pectin and cellulose contents, a smaller midrib, and decreased vascular bundles in leaves. We also screened OsRACK1A as an OsBLR1-binding protein using Y2H and GST pull-down.

OsBLR1 transduces the BR signal to increase leaf angle and grain length

We studied the function of OsBLR1 using transgenic lines, including overexpression lines (*blr1-D* and *BLR1-OE-1/2/3*) and knockout mutants (*blr1-1/2/3)*. The overexpression lines had increased leaf angles and grain lengths, while the knockout mutants had erect leaves and shorter grains. Considering the opposing phenotypes in the overexpression lines and knockout mutants, we inferred OsBLR1 to be a positive regulator of leaf angle and grain length. Similarly, the overexpression lines *OsbHLH153*, *OsbHLH173*, and *OsbHLH174* has increased leaf angles (Dong et al. 2018). Our data provide new experimental evidence that deepens our understanding of the function of bHLH transcription factors in regulating leaf angle and grain length.

Mutants with overdoses of BRs or enhanced BR signalling exhibit a dramatically increased leaf angle. BR-related mutants, such as *d2-1* (loss-of-function of *CYP90D2*) (Hong et al. 2003), *d61-1* (loss-of-function of *OsBRI1*) (Yamamuro et al. 2000), and *brd3-D* (loss-of-function of *OsCYP734A4*) (Qian et al. 2017), have erect leaves, and *brd3-D* has smaller grains. A significant increase in leaf angle can be induced by promoting rice BR biosynthesis or signal transduction (Zhang et al. 2014a). Given that the *OsBLR1* mutants had typical phenotypes similar to those of *d2-1*, *d61-1*, and *brd3-D*, we first performed lamina joint inclination, coleoptile elongation, and root elongation assays to measure the BR sensitivity of OsBLR1-transgenic lines. This showed that *blr1-1/2/3* were less sensitive to eBL, whereas *blr1-D* and *BLR1-OE-1/2/3* were hypersensitive to eBL. The phenotypes of the lines sensitive or insensitive to BR are similar to those of the *d61*, *dlt*, and *m-qgl3* mutants (Yamamuro et al. 2000; Tong et al. 2009; Gao et al. 2019). Next, we measured the BL, CS, and 6-DeoxoCS contents. There was no significant difference between the wild type and *OsBLR1* mutants and, surprisingly, we detected BL in rice. The BL contents in NIP and DJ were 0.70, 0.31, and 0.64 ng/g, respectively. This is the first direct evidence of BL in rice. We measured the BL content using ESI-HPLC–MS/MS method, which is more sensitive to BL than the gas chromatography/mass spectrometry method used previously (the original BL data are shown in Supplementary 2). Based on these results, we speculated that OsBLR1 transduces the BR signal to increase leaf angle, but not biosynthesis. The function of OsBLR1 is similar to those of *OsILI1* and *OsBU1* (*OsbHLH172*), which have been reported to participate in BR signalling to regulate leaf angle (Tanaka et al. 2009; Zhang et al. 2009).

OsBLR1 regulates leaf development and BR signalling

Many reports have shown that BRs regulate cell elongation to promote lamina joint bending, such as BR functional enhancement lines: *ili1-D* (*ILI1* high expression mutant), *OsBUL1D* (*OsBUL1* mutant), and *RLI1-OE-3* (*OsRLI1* high expression line) (Zhang et al. 2009; Jang et al. 2017; Ruan et al. 2018). We observed increased cell growth on the adaxial side of the lamina joint by scanning electron microscopy,

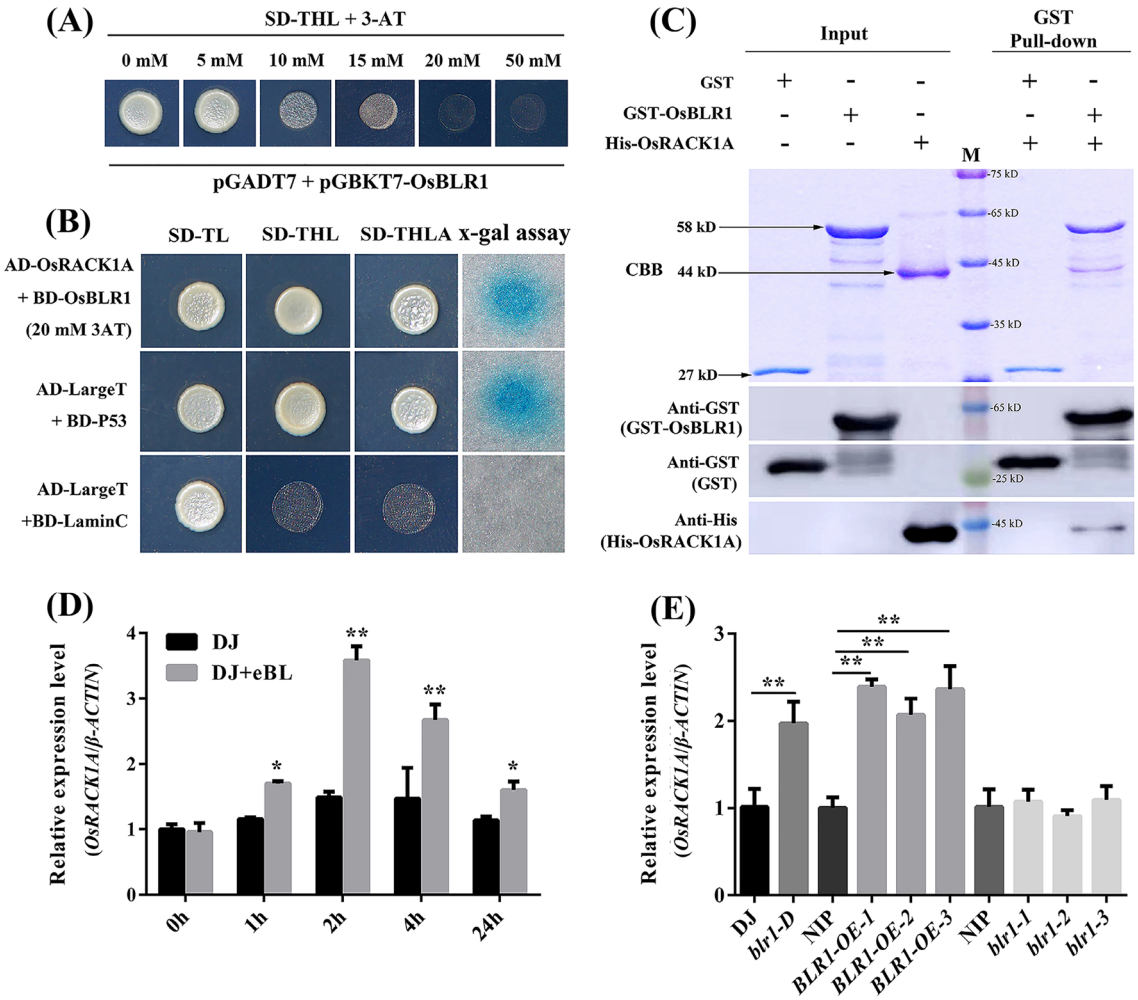


Fig. 7 OsBLR1 interacted with OsRACK1A. **a** 20 mM 3-AT completely inhibits the self-activation of OsBLR1. **b** Y2H assay indicates OsBLR1 interacts with OsRACK1A. OsBLR1-pGBKT7 + OsRACK1A-pGADT7 as the experimental group, pGBKT7-P53 + pGADT7-largeT as the positive control and pGBKT7-Laminc + pGADT7-largeT as the negative control. SD-TL: SD/-Leu/-Trp, SD-THL: SD-Leu/-Trp/-His, SD-THLA: SD/-Leu/-Trp/-His/-Ade. **c** GST pull-down assay provides evidence for the interaction between OsBLR1 and OsRACK1A. *M* protein marker. **d** The expression of *OsRACK1A* in leaves in the induction of eBL. **e** The expression of *OsRACK1A* in *OsBLR1*-transgenic lines. Data represent the mean ± SE (n = 3). *p < 0.05, **p < 0.01, Student's *t*-test

which led to the lamina joint bending in *blr1-D*. We also found that the leaf midrib had developmental defects in *blr1-D*, which is similar to *DL* mutants that form an abnormal midrib and have drooping leaves (Nagasawa et al. 2003). We performed RNA-Seq to study the molecular mechanism of OsBLR1. The results showed that *DL*, as a positive regulatory factor of midrib development, was significantly down-regulated in *blr1-D*, which may result in the smaller midrib of *blr1-D*. We also found that the development of vascular bundles was impaired in the midrib compared with DJ. In addition, the transcription of *OsEXPs* and *OsGLPs* was significantly up-regulated in *blr1-D*. *OsEXPs* regulate stem and leaf development by facilitating cell wall extension (Li et al. 2002). *OsGLPs* promote cell wall modification and reconstruction by regulating the H_2O_2 content (Bolwell et al. 1995). These factors promote cell development in *blr1-D*. However, the cellulose and protopectin contents in the leaves of *blr1-D* were significantly decreased. RNA-Seq data showed that the expression of pectin methylesterase and cellulose synthetase genes, such as *OsBC1* and *OsBC1L4*, was significantly decreased (Dai et al. 2011; Liu et al. 2013). We speculate that these changes led to lower pectin and cellulose contents and abnormal cell walls, which may ultimately result in the reduced vascular bundle tissue in the leaves of *blr1-D*. These factors are important for the increased leaf angle and drooping leaves in *blr1-D*.

In addition, RNA-Seq and RT-qPCR data showed that the expression of *OsRLI1* was up-regulated in *OsBLR1*-overexpression lines, but down-regulated in *OsBLR1*-knockout mutants. We know that OsRLI1 positively regulates leaf

inclination by regulating lamina joint cell elongation. The *rli1* mutants have reduced leaf angles and *RLI1* overexpression results in increased leaf angles (Ruan et al. 2018); the phenotypes of the *OsRLI1* mutants are similar and consistent with the *OsBLR1* mutants. We propose that *OsRLI1* is a downstream gene of OsBLR1 in BR signalling, but this hypothesis needs further study.

OsBLR1 combines with OsRACK1A

In this study, we demonstrate an interaction between OsBLR1 and OsRACK1A using Y2H and GST pull-down assays. Recently, some studies have shown that the MYB-bHLH-WD40 complex plays a key role in BR signalling (Cheng et al. 2014). In *Arabidopsis thaliana*, the sensitivity of the *RACK1A* mutant to exogenous BR is decreased (Chen et al. 2006); this is consistent with the reduced sensitivity to BR in *blr1-1/2/3*. In this study, RT-qPCR showed that the expression of *OsRACK1A* was up-regulated by exogenous BR. The function of OsRACK1A is complicated, and the function of the interaction between OsBLR1 and OsRACK1A in BR signalling needs further study.

Author contributions W.Z. and Q.Z. provided funds; W.Z. and K.W. conceived and designed the experiments; K.W. performed the major experiments and data analysis; W.Z. and K.W. discussed the results and drafted the manuscript; M.L. performed the identification of *blr1-D*; Y.C. performed the coleoptile elongation and root elongation assays; B.Z. assisted the Y2H screening.

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