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ORIGINAL ARTICLE

The kinesin-13 protein BR HYPERSENSITIVE 1 is a negative brassinosteroid signaling component regulating rice growth and development

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Abstract

Phytohormones performed critical roles in regulating plant architecture and thus determine grain yield in rice. However, the roles of brassinosteroids (BRs) compared to other phytohormones in shaping rice architecture are less studied. In this study, we report that BR hypersensitive1 (BHS1) plays a negative role in BR signaling and regulate rice architecture. *BHS1* encodes the kinesin-13a protein and regulates grain length. We found that *bhs1* was hypersensitive to BR, while *BHS1*-overexpression was less sensitive to BR compare to WT. BHS1 was down-regulated at RNA and protein level upon exogenous BR treatment, and proteasome inhibitor MG132 delayed the BHS1 degradation, indicating that both the transcriptional and posttranscriptional regulation machineries are involved in BHS1-mediated regulation of plant growth and development. Furthermore, we found that the BR-induced degradation of BHS1 was attenuated in *Osbri1* and *Osbak1* mutants, but not in *Osbzr1* and *Oslic* mutants. Together, these results suggest that BHS1 is a novel component which is involved in negative regulation of the BR signaling downstream player of BRI1.

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Introduction

Rice (*Oryza sativa* L.) is a crucial crop for agriculture that provides stable food for billions of people worldwide. Thus, investigation of the genetic basis and molecular mechanism underlying rice yield regulation is of great significance. Rice architecture is an important factor for its yield (Gao et al. 2021), which is controlled by tillering number and angle, internodes elongation, panicle morphology, and leaf angle (Wang and Li 2008). Phytohormones play important roles in plant architecture (Tong and Chu 2018). BRs are a class of steroid hormones involved in multiple developmental and physiological processes, including vascular differentiation, reproductive development, photomorphogenesis, and stress responses (Izawa 2021; Sadura and Janeczko 2021).

Since the first discovery of brassins in rape pollen of *Brasssica napus* as the new hormones (Mitchell et al. 1970), many BR biosynthesis and signaling components have been identified in Arabidopsis through isolating BR-deficient mutants (Lv and Li 2020). BRI1 (BRASSINOSTEROID INSENSITIVE 1) is a receptor kinase that transduces steroid signals across the plasma membrane (Kinoshita et al. 2005; Li and Chory 1997; Wang et al. 2001). In the presence of BRs, BRs directly interact with the LRR domains of BRI1 and form a complex with SOMATIC EMBRYO-GENESIS RECEPTOR KINASE (SERK). BRI1 and SERK *trans*-phosphorylate each other to activate the BRI1 signaling pathway (Zhang et al., 2014). The phosphorylation cascade promotes the stability and activity of the plant-specific transcription factors BZR1 (BRASSINAZOLE RESISTANT 1) (Wang et al. 2002) and BES1 (BRI1-EMS-SUPPRESSOR 1) (Yin et al. 2002) by inhibiting the kinase activity of BIN2 (BRASSINOSTEROID INSENSITIVE 2) (He et al. 2002). BIN2 regulates stability and activity of the BZR1 and BES1 transcription factors which, in turn, regulate the expression of the BR-responsive genes and developmental events in plant (Planas-Riverola et al. 2019; Sun et al. 2010).

Compared to Arabidopsis (Lv and Li 2020), the knowledge about BR biosynthesis and signaling transduction in rice is relatively limited. As a key receptor of BR, OsBRI1 perceived the signal with its coreceptor OsBAK1 (Li et al. 2009) and transduced to GSK2 kinase (Tong et al. 2012) through OsBSK3 kinase (Zhang et al. 2016a). OsBZR1 directly binds to the promoter of DLT gene to behave as a transcriptional factor for BR response in rice (Tong et al. 2012). LIC, as an antagonistic transcription factor of OsBZR1, attenuated the BR signaling pathway. Moreover, its expression level was induced by high BR level through OsBZR1 and in turn suppressed the expression of OsBZR1 (Zhang et al. 2012). In rice genome, there are four homologous genes (*OsSERK1-4*) to the Arabidopsis *BAK1* gene and OsBAK1 (OsSERK1) is the closest homolog of the Arabidopsis counterpart (Li et al. 2009; Zhang et al. 2014). OsBAK1 forms a complex with OsBRI1 in the presence of BR. When the expression of *OsBAK1* was repressed, the OsBAK1-AS plants showed BR insensitive phenotype. In contrast, the OsBAK1-OE plants showed typical BR gain-of-function phenotypes, including enlarged lamina joint angle, stunted stature, and hypersensitivity to 24-epiBL (Li et al. 2009), suggesting that the function of OsBAK1 is similar to AtBAK1 (Nam and Li 2002). In addition, genetic analysis suggests OsBU1 acts downstream of OsBRI1 (Tanaka et al. 2009). Rice plants overexpressing BU1 showed enhanced bending of the lamina joint, increased grain size, and resistance to Brassinazole, an inhibitor of BR biosynthesis (Jang and Li 2017). The degradation of OsBRI1 requires the PP2A-mediated dephosphorylation that is specified by methylation of the phosphatase (Di Rubbo et al. 2011; Wu et al. 2011).

Kinesins are microtubule-based proteins with a conserved kinesin motor domain, and are involved in cell division and elongation (Lee et al. 2015). In plants, it has been found that kinesins regulate many developmental processes through hormone signaling (Li et al. 2012); however, the role of kinesins in BR signaling is still obscure. The earliest investigation of plant kinesin was conducted in tobacco phragmoplast (Asada et al. 1991). Subsequently, four Arabidopsis kinesins were identified by PCR-based cloning (KatA, KatB, KatC, KatD) (Liu et al. 1996; Mitsui et al. 1993, 1994; Tamura et al. 1999). Now, we know the Arabidopsis genome which contains at least 61 kinesin genes and they are divided into 10 of the 14 recognized subfamilies of kinesins, whereas three kinesins do not fall into any known family (Zhu and Dixit 2012). Among them, Kinesin-13a was reported to regulate the formation of secondary wall pits by promoting cortical microtubule depolymerization through bending microtubule precursor at the end of microtubule (Ems-McClung and Walczak 2010; Manning et al. 2007; Oda and Fukuda 2013). Mutations in AtKINESIN-13a (At3g16630) lead to the outgrowth of an additional branch in *Arabidopsis* trichomes; this outgrowth of an extra branch most likely involves additional cell-wall synthesis (Lu et al. 2005). Also, it is reported that intracellular activity of AtKINESIN-13a regulates cell expansion and wall architecture via THESEUS1 (Fujikura et al. 2014). In rice, previous studies have demonstrated that OsKinesin-13a deficiency causes small and round grains (Kitagawa et al. 2010). Subsequently, OsKinesin-13a (SGL) was shown to have transcriptional activity and mainly enriched in the nucleus in onion epidermal cells (Wu et al. 2014). The expression

levels of genes involved in gibberellin metabolic pathways were affected in the *sgl* mutant, suggesting SGL protein may be involved in regulating GA-related genes, that in turn, regulates grain length and plant height (Wu et al. 2014). Furthermore, Deng et al. found that OsKinesin-13a/SGL is involved in determination of glume size rather than caryopsis size, but it is still unclear how the developing caryopsis communicates with their developed glumes to coordinate its growth (Deng et al. 2015). It is noteworthy that the OsKinesin-13a protein is mainly localized on Golgi-derived vesicles and it promotes cell elongation via its microtubule depolymerizing activity that enhances microtubule turnover and facilitates vesicle transport (Deng et al. 2015).

In this study, we identified a rice mutant *bhs1* (*BR hypersensitive 1*), named based on its increased sensitivity to 24-epiBL. *BHS1* encodes an OsKinesin-13a protein, which was recognized previously as a positive regulator of grain length. BR suppresses the expression of *BHS1*; meanwhile, it promotes the degradation of BHS1 protein in a proteasome-dependent manner. Moreover, the feature of BHS1 degradation mediated by exogenous BR is attenuated by the deficiency of BR receptor D61 and its coreceptor BAK1, but not affected by BZR1 and LIC1. Collectively, our results suggested that BHS1 is a novel downstream player of BRI1 to mediate BR-regulated plant growth and development in rice.

Materials and methods

Plant materials and growth conditions

The *bhs1* mutant was identified from a screening in which ZH11 (Zhonghua11, *Japonica*) mature seeds were treated with ethyl methane sulfonate (EMS). The phenotype of the mutant was stably inherited for multiple generations under the field and greenhouse conditions in Hangzhou, Zhejiang, China. Rice plants were grown in an experimental field at the China National Rice Research Institute in Hangzhou under natural conditions or in a growth chamber under a 14 h light (30 °C)/10 h dark (24 °C) cycle (Chen et al. 2016).

Map-based cloning

To map the *BHS1* locus, the *bhs1* mutant was crossed to 9311 (*Indica*). A total of 2688 F2 mutant plants were used for genetic mapping. To fine-map *BHS1*, several simple sequence repeat (SSR) markers were generated. The *BHS1* locus was delimited to a 33 Kb region. The *bhs1* mutation was identified by PCR amplification of the *BHS1* genomic region from wild-type and *bhs1* mutant plants followed by sequencing.

Vector construction and rice transformation

For complementation analysis of the *bhs1* mutant, a 2460-bp CDS fragment was amplified from wild type. The fragment was then inserted into AHLG with ApaI and XbaI digestion. The AHLG vector is modified from pCAMBIA1300, which is driven by *actin* promoter and fusion a GFP-tag at C-terminal. The resulting plasmid was transformed into calli derived from the *bhs1* mutant using *Agrobacterium tumefaciens* strain EHA105.

The *bhs1-2*, *bak1*, *bu1*, and *bzr1* plants were generated using CRISPR/Cas9 as described previously (Wang et al. 2017) by the Biogle Company (Hangzhou, China). The sgRNA of *BHS1*: 5′-GGCAATTTCCTCAGTTTC GATGG-3′, sgRNA of *BAK1*: 5′-GGCGCGGGTGCTCGC CAACATGG-3′, sgRNA of *BU1*: 5′-GTCGTCGCGCTC CTCCGTGTCGG-3′, and sgRNA of *BZR1*: 5′- ACCTAC CGCAAGGTCAGTTCATGC-3′ were selected as targets. The sgRNA was created in the BGK03 vector containing Cas9 separately and then introduced into ZH11 rice calli by Agrobacterium (EHA105)-mediated transformation. Eight independent lines were obtained. To examine the function of CRISPR/Cas9 in vivo, genomic DNA was extracted from transgenic plants and amplified by PCR for sequencing (Supplemental Table 2) and used in the further investigation (Supplemental Fig. 6).

Phylogenetic analysis

Full-length protein sequences were used for phylogenetic analysis. The sequence of BHS1 and its homologues were downloaded from RGAP (http://rice.plantbiology.msu.edu/) and NCBI (https://www.ncbi.nlm.nih.gov/). The maximum-likelihood tree was constructed using MEGA7 (Kumar et al. 2016). The structure of the kinesin motor domain was predicted by standard template-based modeling at SWISS-MODEL (https://swissmodel.expasy.org/interactive).

GUS staining

To construct p*BHS1*::GUS, the 2207-bp promoter was amplified by PCR using primer pair pGUS-F and pGUS-R and then inserted into the pCAMBIA1301 vector. The vector was transformed into cv Nipponbare rice. For GUS staining, samples were permeabilized in 90% acetone for 1 h at − 20 °C and then washed twice with PBS buffer [0.01 M potassium phosphate buffer (pH 7.2)] before vacuuming for 5 min. The samples were incubated with X-Gluc at 37 °C for 3–5 h. The green tissues were de-colored with ethanol.

For cross-sections, samples of leaves, roots, and coleoptiles were fixed in FAA for 24 h at 4 °C and cut into 8-μm sections. The GUS staining images were taken using a Leica Light microscope (Li et al. 2008).

RNA extraction and quantitative RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen), and first-strand cDNA was synthesized using PrimeScript™ II 1st Strand cDNA Synthesis Kit with gDNA remover (TaKaRa). qRT-PCR was performed using SYBR® Premix Ex Taq™ II (TaKaRa) with a CFX96 Real-Time PCR Detection System (Bio-Rad). The rice Actin gene (LOC_Os03g50885) was used as an internal control (Guo et al. 2019). All the results presented were the means ± SD of three individual experiments. Primers used for qRT-PCR are listed in Supplementary Table 2.

Subcellular localization

The coding sequences of *BHS1* was amplified by PCR and inserted into the PGA3427 vector (Kim et al. 2009). The constructs were transiently expressed in protoplasts from cv Nipponbare cell suspensions by polyethylene glycol-mediated transformation. The protoplasts were incubated for 16 h prior to detection using a confocal laser scanning microscope (LSM 710, Zeiss, Germany) (Favery et al. 2004).

Treatments of different hormones

For different hormones treatments and gene expression analysis, rice plants (ZH11) were grown in 1/2 MS (Murashige and Skoog) in a growth chamber (28 °C, 14 h light/10 h dark) for 7 days. Then, they were immersed in liquid 1/2 MS medium containing different kinds of phytohormones for 2 h (ethanol was used as control, because the hormones used in our study are solved in ethanol), including BL (Brassinolide, 10 μM), GA3 (gibberellin, 10 μM), IAA (10 μM), JA (Jasmonic acid, 10 μM), ABA (10 μM), and 2, 4-D (10 μM). Twenty plants for each treatment were collected for RNA isolation. For BR response, de-hulled seeds were directly sowed on the 1/2 MS agar medium supplemented with 10 μM BL for different time. Seedlings were collected for RNA isolation (Liu et al. 2015).

BHS1 antibody production

Polyclonal antibody against BHS1 was raised in rabbit with purified BHS1 C-terminal protein at Wuhan Genecreate Biological Engineering Co., Ltd. CDS (1135–2454) of BHS1 were inserted into pET-B2M vector and the protein was purified with Ni–NTA agarose. Immunization was done with recombinant protein, and the quality was tested with expressed His-BHS1. The immune serum was purified using Protein G agarose following the instructions and used for immunoblotting analysis.

Immunoblot experiments

All the materials were ground into a fine powder in liquid nitrogen followed by extraction in protein extraction buffer containing 50 mM Tris pH 6.8, 4% SDS, 10% glycerol, 5% 2-mercaptoethanol, 20% bromophenol blue, and complete protease inhibitor (Sigma). Cellular debris was removed by centrifugation, and total protein concentration was quantified by Bradford assay and BSA as a standard. Proteins were separated by 10% SDS-PAGE and then transferred to nitrocellulose for immunoblotting experiments. Nitrocellulose membrane was blocked with 3% milk in TBST (0.8% NaCl, 0.02% KCl, 0.3% Tris, pH 7.4, 0.05% Tween20) for 30 min at room temperature. Excess milk was washed off with TBST. Blots were incubated in primary antibodies overnight at 4 ℃. Secondary antibodies were then applied for 1 h at room temperature. Blots were developed using the ECL solutions (Invitrogen).

BR sensitivity tests

The lamina joint assay by the micro-drop method was performed as described previously (Tong and Chu 2012). Ethanol (1 μl) containing 0, 10, 100, or 1000 ng of 24-epiBL was spotted onto the top of lamina after 2 day germination and 3 day growth at 30 ℃. Images were taken after 3 days incubation, and the angles of lamina joint bending were measured using IMAGEJ software. The lamina joint assay using excised leaf segments was performed as described previously (Wada et al. 1981). Synchronous seeds after 2 days germination were selected and grown in the dark for 8 days at 30 ℃. The entire segments comprising 1 cm of the second leaf blade, the lamina joint, and 1 cm of the leaf sheath were floated on distilled water for 24 h and then incubated in 2.5 mM maleic acid potassium solution containing various concentrations of 24-epiBL for 48 h in dark. Lamina joint angles were measured using IMAGEJ software. For coleoptile elongation analysis, rice seeds were germinated in 1/2MS solid medium with 0 or 1 μM 24-epiBL in the growth chamber at 30 ℃ in darkness for 3 days. Coleoptile length was measured using IMAGEJ software (Zhao et al. 2013). Each experiment was repeated three times independently.

In vivo degradation assay of BHS1

One-week-old rice seedlings were treated with or without 10 μM 24-epiBL and collected at different time points. To block proteasomal protein degradation, seedlings were pretreated with 40 μM MG132 (NO. T510313, Sangon Biotech)

for 2 h with Zhou's instruction (Zhou et al. 2013). Seedlings were collected for RNA isolation and total protein extraction.

Results

Isolation and characterization of rice *bhs1* mutant

To explore the roles of BR on rice development, we screened an EMS-induced mutant population with altered sensitivity to exogenous 24-epiBL and identified a BR HYPERSENSITIVE mutant (*bhs1*). Upon 24-epiBL treatment, the lamina joint bending angles increased dramatically in *bhs1* mutant compared with ZH11 (wild type, WT) with micro-drop assay (Fig. 1a, b). Moreover, the enlargement of lamina joint bending angles is correlated with the increased concentration of 24-epiBL (Fig. 1a, b). The lamina joint assay using excised leaf segments showed that the induced angles between laminae and sheaths in *bhs1* were much greater than that of WT (Fig. 1c, d). We further examined the coleoptile elongation of WT and *bhs1* seedling in response to 24-epiBL. As expected, the coleoptile elongation was promoted in the presence of 1 μM 24-epiBL in WT and the coleoptile elongation in *bhs1* was stronger than in WT (Fig. 1e, f). In addition, both the plant height and grain length of *bhs1* mutant are shorter than WT (Figure S1a, b; Table S1). Compared to WT, the grain width of the *bhs1* was significantly increased (Figure S1b; Table S1) and the 1000-grain weight of *bhs1* decreased about 33.3% (Table S1). We observed the epidermal cells of leaf sheath and found that the cells in *bhs1* mutant are decreased in length and increased in width (Figure S2), whereas the arrangement of cells in *bhs1* were as well-organized as that of WT (Figure S1c). The *bhs1* mutant

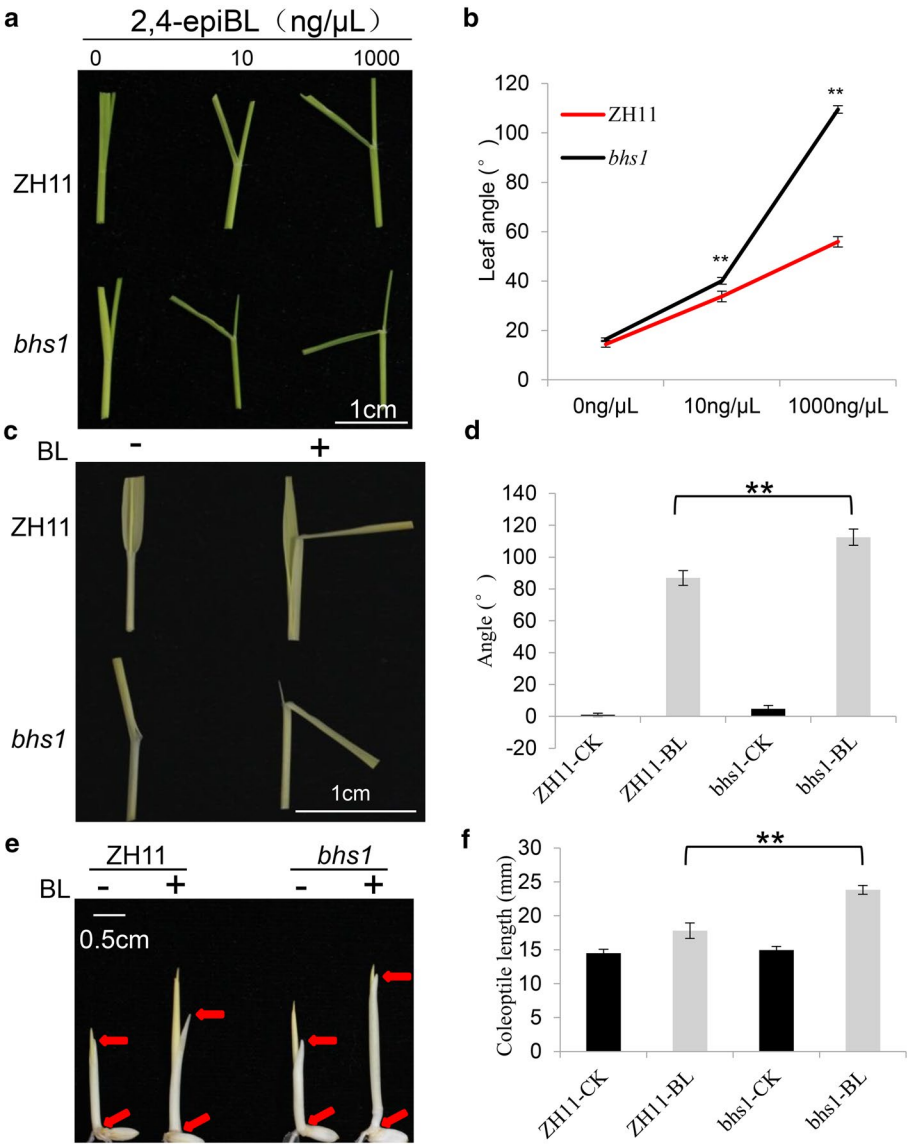

Fig. 1 Responses of wild-type and *bhs1* to 24-epiBL. **a** Comparison of the BR sensitivity of wild type (WT) and *bhs1* using the lamina inclination test in response to 24-epiBL. **b** The dose–response to 24-epiBL (ng/μL) of the bending angle in ZH11 (WT; black) and *bhs1* (red) plants. **c** Typical response of excised leaf lamina joint following 24-epiBL treatments in wild-type and *bhs1* plants. **d** Measurements of the lamina joints angles of wild-type and *bhs1* seedling in presence or absence of 1 M 24-epiBL. Data presented are means from 30 plants. **e** Effect of 24-epiBL on the coleoptile elongation in wild-type and *bhs1* seedlings. **f** Coleoptile length of wild-type and *bhs1* seedling in the presence or absence of 1 M 24-epiBL. The segment between two arrows indicates the coleoptile. Each experiment was repeated three times independently. Values are presented as means ± SD, and the statistically significant differences were determined by Student's *t* test ($n = 30$, $^{**}P < 0.01$)

was also defective in germination rate since the initiation of germination. Finally, approximately 50% of the *bhs1* mutant seeds arrested at the germination stage (Figure S1d). In summary, compared to WT, the *bhs1* mutant had altered sensitivity to 24-epiBL and showed deficiency in plant height, grain size, and seed germination.

Map-based cloning of *BHS1*

Segregation analysis indicated that the *bhs1* phenotype was controlled by a single recessive gene. To clone the *BHS1* gene, an F2 mapping population derived from a cross between *bhs1* (*Japonica* type) and the *Indica* rice 9311 was used for genetic mapping. The *BHS1* locus was initially delimited in an interval of ~ 14.4 cM on chromosome 5 between the two sequence tag site markers (STS) P3 and P4 (Fig. 2a). Then, additional 7 markers further narrowed *BHS1* down to a 33 Kb region (Fig. 2b, c). Within this region, 7 open-reading frames (ORFs) were predicted by Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/). Sequencing analysis revealed *bhs1* contained three nucleotide substitutions (CTGC for TTTT) on ORF4 (LOC_Os05g06280) (Figure S3a, b), which encodes OsKinesin-13a and had been identified by the previous investigations (Deng et al. 2015; Kitagawa et al. 2010; Wu et al. 2014). No mutations were found in other six ORFs. The predicted BHS1 in WT has 819 amino acids with 90.5Kd molecular weight. The nucleotide substitutions in *bhs1* were predicted to produce a premature stop codon and a truncated protein (Fig. 2d; Figure S3c). The kinesin motor domain has five motifs, namely N1 (P-loop), N2 (Switch I), N3 (Switch II), N4, and L2 (KVD finger), which plays significant role in intracellular transport of organelles and in cell division (Kitagawa et al. 2010). The truncated BHS1 protein would lack three α-helix and β-fold (including the N3 motif) (Figure S3d-f) (Kuhlman and Bradley 2019). Taken together, our results indicate that these mutations on *BHS1* are presumably responsible for the phenotypes in *bhs1* plant.

Rescue experiment and phylogenetic analysis

To test whether the mutation on *BHS1* is responsible for the defective phenotypes, we overexpressed BHS1–GFP fusion protein in *bhs1* background. In total, 15 transgenic lines (OEs) were obtained and two of lines (OE1 and OE2) were chosen for further analysis. The introduction of *BHS1–GFP* expression cassette was validated by specific PCR amplification (Fig. 3a). Moreover, the transcription of *BHS1* in OE1 and OE2 plants was significantly increased (Fig. 3b). The immunoblot analysis using an antibody specially recognized BHS1 C-terminal (Figure S4) revealed that the BHS1 protein was absent in *bhs1* mutant, but was abundant in the OE1 and OE2 lines (Fig. 3c). We further performed the BR sensitivity experiments including lamina inclination and

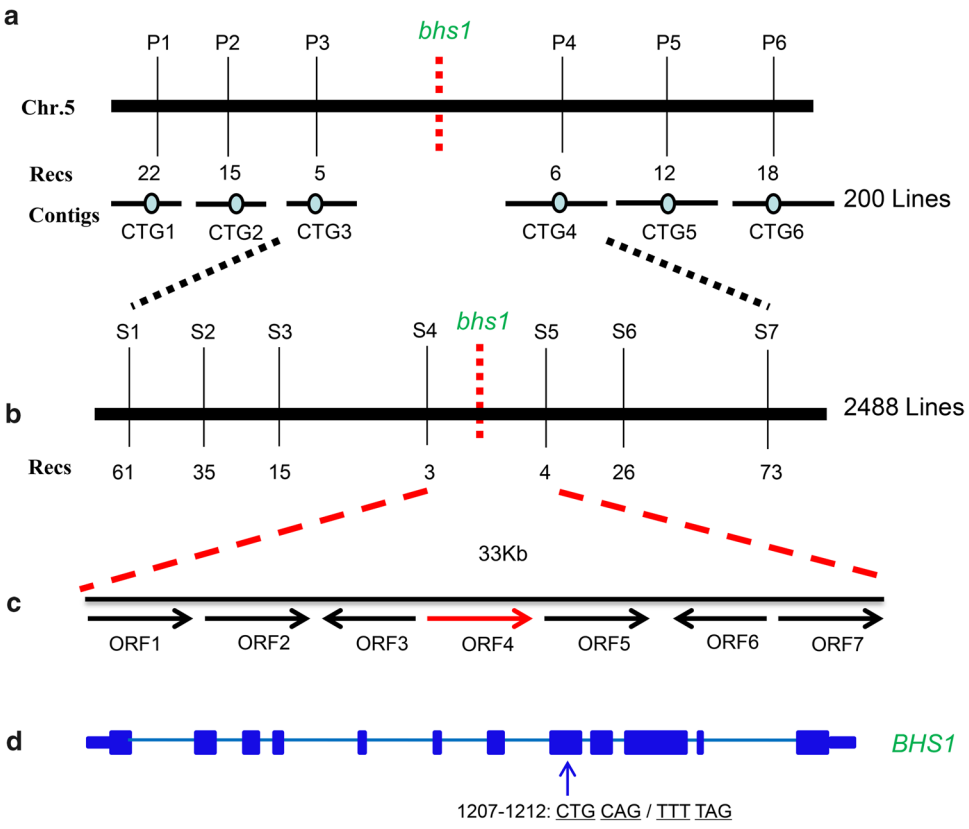

Fig. 2 Mapping of *BHS1*. **a** *BHS1* was mapped initially to the middle of rice chromosome 5 between markers P3 and P4. **b** Fine-mapping was taken and narrowed *BHS1* to a region of 33 Kb between markers S4 and S5. **c** In the 33 Kb region, there are 7 open-reading frames (ORFs). **d** The mutation sites in *BHS1*

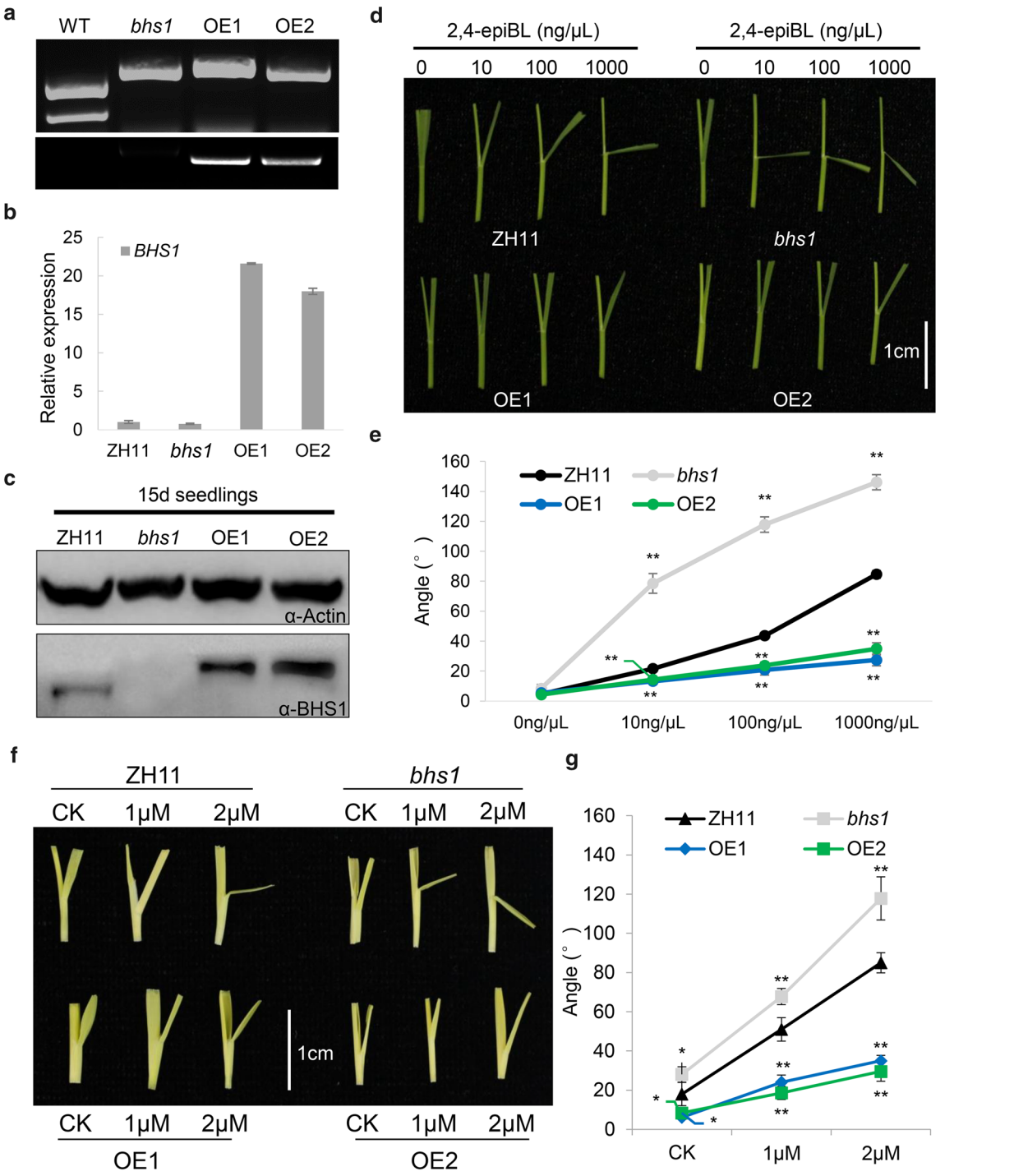

Fig. 3 Overexpression of BHS1. **a** Confirmation of the transgenic BHS1-overexpression construct into *bhs1* mutant background. Upper panel indicated materials' background confirmed by restriction enzyme (PstI) digestion; lower panel indicated the primers to amplify transgene fragment. **b** Expression of BHS1 in WT, *bhs1*, OE1, and OE2 plants analyzed by quantitative RT-PCR analysis. **c** Immunoblot analysis using anti-BHS1 antibody to show BHS1 protein levels at 15d seedling; Actin was used as the control. **d** Dose–response to 24-epiBL of the second lamina joint in wild-type (WT), *bhs1* mutant, and OE1 and OE2 seedlings. Scar bar = 1 cm. **e** Angles of the second lamina joint in wild-type (WT), *bhs1* mutant, and OE1 and OE2 seedlings responded to 24-epiBL (10, 100, 1000 ng/μL). **f** Typical response of the second leaf lamina joint in WT, *bhs1*, OE1 and OE2 plants to treatment with 24-epiBL. Scar bar = 1 cm. **g** Statistical analyses of lamina joint angles in WT, *bhs1*, and OE1 and OE2 plants. CK, negative control. Each experiment was repeated three times independently. Values are presented as means ± SD, and the statistically significant differences were determined by Student's t test ($n = 30$, $^{**}P < 0.01$, $^{*}P < 0.05$)

etiolated leaf lamina joint test with WT, *bhs1*, OE1, and OE2 plants (Fig. 3d–g). With the micro-drop assay, we found that the angles were gradually enlarged with the increased BR concentration in all three materials (Fig. 3d, e). The similar variation was also found in etiolated leaf lamina joint test (Fig. 3f, g). However, it should be pointed out that the angle enlargement upon 24-epiBL treatment is significantly increased in *bhs1* and reduced in OE1 and OE2 lines compared to WT, which is consistent with the previous hypothesis that BHS1 negatively responses to 24-epiBL. Moreover, the defects in *bhs1* including of plant height (Figure S5d, e), 1000-grain weight (Figure S5b), and seed size (Figure S5a, c) were all rescued in the OE1 and OE2 lines.

To verify the identity of *BHS1* further, we generated a *bhs1-2* allele using the CRISPR/Cas9 method, which introduced an additional C nucleotide into the tenth exon that led to a premature stop codon (Figure S6a, b). The *bhs1-2* mutant also shows a dwarf phenotype as *bhs1* (Figure S6c). Concurrently, we found that the expression of *BHS1* decreased slightly and BHS1 protein accumulation was depleted in *bhs1-2* (Figure S6d, e). Consistently, the lamina joint bending angles of *bhs1-2* increased dramatically upon 24-epiBL treatment compared with ZH11 (wild type, WT) within micro-drop assay (Figure S6f, g). Together, these data further supported that the defects in the *bhs1* were indeed caused by mutations in *BHS1*.

We investigated the possible relationships between plant kinesin-13 sequences and kinesin-13 homologs from animals by phylogenetic analysis (Figure S7). The phylogenetic analysis indicated that plant kinesin-13s were clustered a large group which was distinct from that of animal members. Plant kinesin-13s can be divided into two subfamilies (kinesin-13a and kinesin-13b), and each subfamily contains monocot and dicot sequences. BHS1 belonged to the monocot subgroup of kinesin-13a subfamily (Figure S7), which is consistent with previous findings (Deng et al. 2015).

Expression pattern and subcellular localization

To examine the spatial expression pattern of *BHS1*, qRT-PCR analysis of endogenous *BHS1* transcripts was performed using total RNA isolated from different WT tissues. As shown in Fig. 4a, *BHS1* was expressed in all the examined organs, including leaf blade, stem, leaf sheath, root, and panicle. Expression of *BHS1* in root was relatively low compared to other tissues. Similar results were obtained by immunoblot analysis using anti-BHS1 antibody (Fig. 4b). In addition, we found that in the *bhs1* mutant, expression of *BHS1* in the different nodes was much lower than WT (Fig. 4c).

To better determine the tissue specific expression pattern of *BHS1*, the 2200-bp *BHS1* promoter region was amplified and inserted into the 5′ end of the GUS reporter gene in the vector pCAMBIA1301. The resulting construct was transformed into WT calli. Histochemical staining for GUS activity in transgenic plants revealed that *BHS1* is mainly expressed in leaf (Fig. 4d), node (Fig. 4e, k), leaf lamina joint (Fig. 4f), and booting panicles but not in anther (Fig. 4g). Because the nodes and leaf lamina joint are critical for plant height and leaf inclination, the relatively high expressions of *BHS1* in nodes and lamina joint were consistent with the short plant height and enlarged leaf inclination phenotype of *bhs1*. When examined in more detail, *BHS1* was detected greatly in vascular bundle cells of small vein and also in phloem cells of central vein with a relatively weak expression in mesophyll cells (Fig. 4i), suggesting that *BHS1* may be involved in transportation of water, inorganic salts, and organic nutrients. Interestingly, before the leaf sheath elongation, *BHS1* is preferentially expressed in the thick tissue above the vascular bundle cells, while *BHS1* was not detected in outer cells of leaf sheath (Fig. 4h, j). Together, the expression pattern of *BHS1* is correlated well with its putative function in these organs.

To examine the subcellular localization of BHS1, a GFP fusion construct driven by the CaMV35S promoter was transiently expressed in rice protoplasts. The green fluorescence signal of BHS1::GFP completely overlapped with the cyan fluorescence signal of AtWAK2, which is an Endoplasmic reticulum-localized protein (He et al. 1999; Nelson et al. 2007) (Fig. 4i–p). In contrast, the GFP signal was ubiquitously distributed in cells with the 35S::GFP vector control in rice protoplast (Figure S8). These results suggested that BHS1 is localized in ER.

Expression of *BHS1* can be repressed by 24-epibrassinolide and alter expression of BR response genes

To further explore the role of *BHS1* in BR signaling pathway as well as its potential correlation with other hormones, we examined *BHS1* expression in WT seedlings under the treatment of various hormones. After 2-h application of hormones, we found that *BHS1* was specifically repressed by 24-epiBL and activated by ABA, but not affected by other hormones (Fig. 5a). We further performed time-course analysis of *BHS1* in WT plant with 24-epiBL application and the results showed that the expression of *BHS1* decreased rapidly after 1 h 24-epiBL treatment and retained only 10% after 24-h treatment (Fig. 5b), suggesting that BR suppressed the transcription of *BHS1*. To examine whether the 24-epiBL application also affect the translation of BHS1, we extracted total proteins from 24-epiBL treated WT plants to conduct immunoblot analysis. We found that the abundance of BHS1 protein was gradually decreased along with 24-epiBL treatment (Fig. 5c). Upon 24-h treatment, the abundance of BHS1 left only 5% compared with control, which indicated

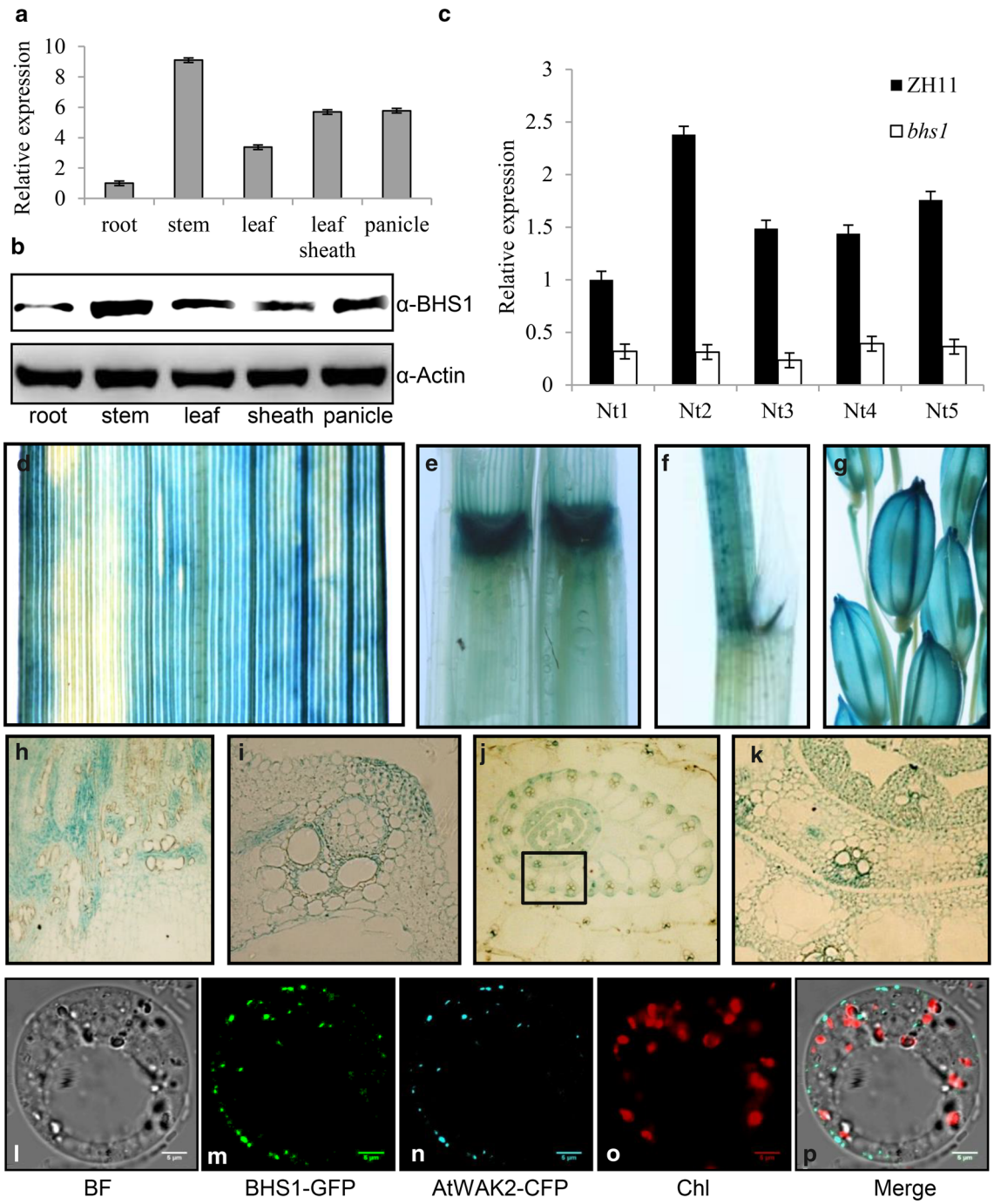

Fig. 4 Expression pattern and intracellular localization of *BHS1*. **a** Expression of *BHS1* in various organs analyzed by quantitative RT-PCR analysis. All the materials were harvested from a mature WT plant. **b** Immunoblot analysis of BHS1 in various organs in the wild type. **c** Expression pattern of *BHS1* in culm tissues. Nt1–Nt5 represent five nodes counted from top to bottom. **d–k** GUS staining of *pBHS1*::GUS transgenic line tissues. **d** Fully expanded first leaf; **e** full-length elongating internode. **f** leaf sheath; **g** spikelet; **h** microscopic observation of a cross-section of **e**. **i–k** Microscopic observation of a cross-section of **d**. Scale bars = 1 cm (**d–g**) and 2 mm (**h–k**). **l** Bright field. **m** The binary vector containing 35S::BHS1-GFP was transiently expressed in rice protoplasts. **n** The binary vector containing 35S::AtWAK2-CFP was transiently expressed in rice protoplasts. **o** Autofluorescence chlorophyll (red). **p** Merge image. Scale bars = 5 μm

that exogenous BR application might also induce BHS1 protein degradation. To validate this hypothesis, we then

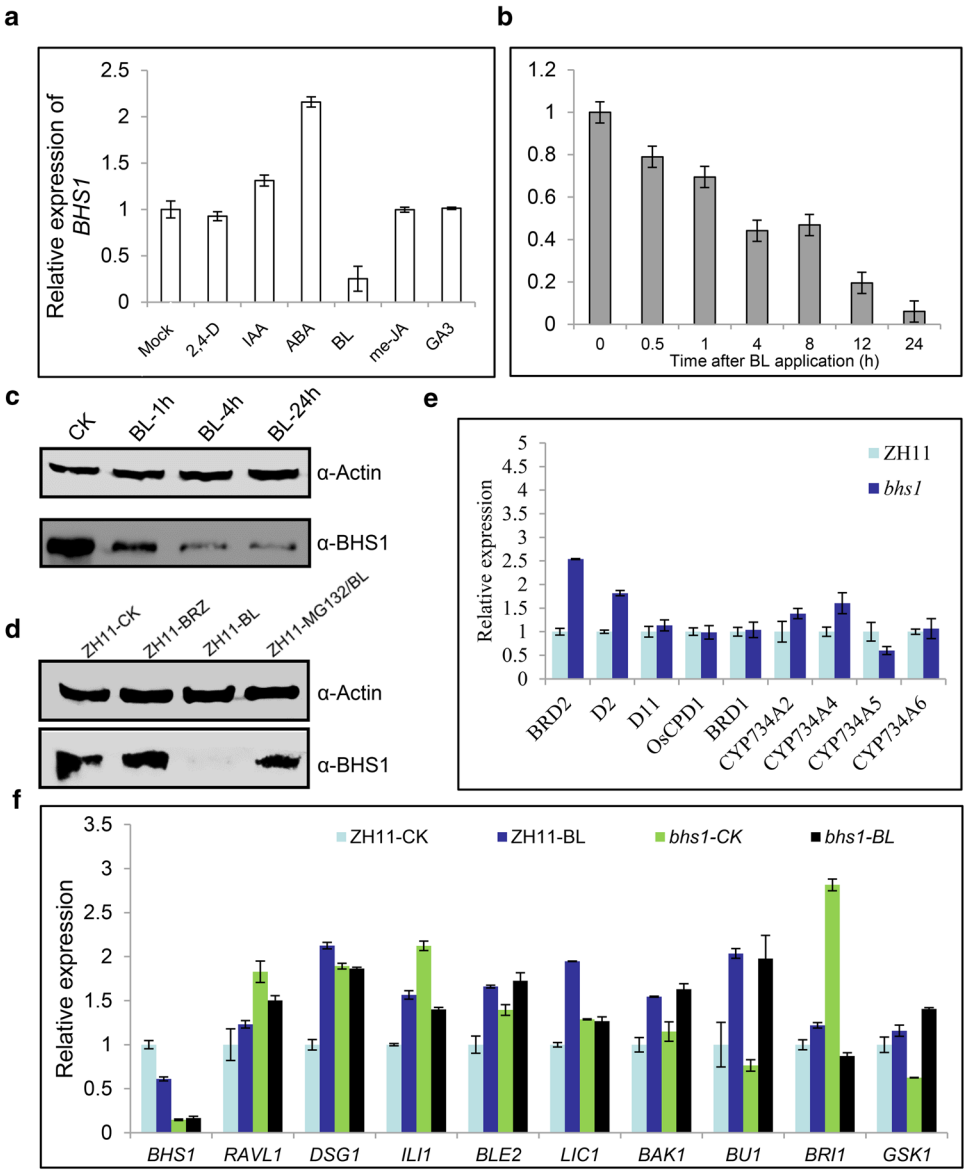

Fig. 5 *BHS1* is a BR response gene. **a** Relative expression level of *BHS1* under different phytohormone treatments. 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indole-3-acetic acid; ABA, abscisic acid; BL, brassinolide; me-JA, jasmonic acid; GA3, gibberellic acid. **b** Time-course response of *BHS1* to 10 μM 24-epiBL treatment. **c** Immunoblot analysis using anti-BHS1 antibody to show BHS1 protein levels after 24-epiBL treatment for different times; actin was used as the control. **d** BHS1 was degraded under 24-epiBL treatment in a proteasome-dependent manner. To block proteasomal protein degradation, One-week-old rice seedlings were pre-treated with 40 μM MG132 for 2 h and then treated with or without 10 μM 24-epiBL and 5 μM BRZ for 2 h. **e** Relative expression of BR biosynthetic genes (BRD2, D2, D11, OsCPD1, and BRD1) and BR catabolic genes (CYP734A2, CYP734A4, CYP734A5, and CYP734A6) in wild type and *bhs1*. **f** Relative expression of BR signaling genes in wild type and *bhs1*, respectively. CK, negative control. Actin was used as the control. Each experiment was repeated three times independently

analyzed the protein abundance of BHS1 from WT plants treated with 24-epiBL alone or combined with MG132, a 26S proteasome inhibitor. The BHS1 protein abundance in samples treated with 24-epiBL plus MG132 is more than that treated with 24-epiBL alone (Fig. 5d). In contrast, the application of BRZ, a specific brassinosteroid biosynthesis inhibitor (Sekimata et al. 2001), had little effect on BHS1 accumulation (Fig. 5d). These data indicated that the 26S proteasome machinery is indeed involved in BHS1 degradation upon 24-epiBL application.

To evaluate whether the BR biosynthesis and catabolism are altered in *bhs1*, we further performed qRT-PCR analysis of BR biosynthesis and catabolic genes, such as *BRD2*, *D2*, *D11*, *CPD1*, *BRD1* (Duan et al. 2006; Hong et al. 2003; Qin et al. 2018; Tanabe et al. 2005), and CYP734A (2, 4, 5, 6) (Sakamoto et al. 2011). The results showed that *BRD2* and *D2* were slightly upregulated in *bhs1* (Fig. 5e). Meanwhile, *D11, CPD1*, *BRD1*, and CYP734A (2, 4, 5, 6) were expressed in similar level between WT and *bhs1* (Fig. 5e). Therefore, the loss of function of *BHS1* did not have a major effect on the transcriptional regulation of genes related to BR synthesis and catabolic pathway. We then tested whether *BHS1* influences the expression of BR signaling genes. To this end, the expression of *BRI1*, *GSK1*, *LIC1*, etc. were analyzed in WT and *bhs1* with or without BR application (Tanaka et al. 2009; Tong and Chu 2012; Yamamuro et al. 2000; Zhang et al. 2009; Zhu et al. 2015). As shown in Fig. 5f, expression levels of these positive regulators of BR signaling (*RAVL1, DSG1, ILI1* and *BLE2*) in *bhs1* maintained a higher level than those in WT both with and without 24-epiBL, while expression levels of *BAK1* and *BU1* in *bhs1* were not significantly different from those in WT. On

the contrary, the upregulated expression of *LIC1* in WT, a negative factor in BR signaling pathway, is attenuated in *bhs1* mutant compared with WT. We also noticed that the expression of *BRI1* was elevated in *bhs1* without BR treatment, whereas the upregulation trend is abolished after BR application. Taken together, it is plausible that the mutation of *BHS1* might increase the sensitivity of BR signaling or responses mainly by regulating the expression level of BR signaling genes.

The regulation of BHS1 by exogenous BR depends on BRI1 and OsBAK1

Given that the abundance of BHS1 (both in transcriptional and posttranscriptional level) were gradually decreased along with 24-epiBL treatment (Fig. 5b, c) and the expressions of several BR-related genes were altered in *bhs1* (Fig. 5e, f), we asked whether BRI1 or BRI1-mediated BR signaling pathway is involved in the transcriptional and posttranscriptional regulation of BHS1 upon 24-epiBL treatment. To test that hypothesis, we characterized the mutant *d61-2* first, which kept a single amino acid substitution (Val to Met at 491th in protein sequences) (Figure S9a, b) (Yamamuro et al. 2000). It is consistent with previous report that the *d61-2* showed the dwarf and erect leave phenotypes (Figure S9c). Moreover, the expression level of *BRI1* in the *d61-2* mutant was slightly down-regulated at seedling stage and the *d61-2* mutant was less sensitive to BL than WT plants (Figure S9d, e). We then compared *BHS1* expression in 1-week-old seedlings of *d61-2* mutant. The quantitative RT-PCR results showed that *BHS1* transcripts were elevated in *d61-2* (Fig. 6a). We further performed time-course analysis of *BHS1* in WT and *d61-2* plants with 24-epiBL application and the results further revealed that the decreased expression of *BHS1* in WT after 24-epiBL treatment was attenuated in *d61-2* (Fig. 6b, c), suggesting that the transcriptional regulation of BHS1 upon BR treatment was dependent on BR receptor. To evaluate whether BRI1 is also required for BHS1 protein degradation upon BL application, we examined the BHS1 abundance in WT and *d61-2* mutant by the immunoblot assay and the results showed that, in *d61-2* mutant, more BHS1 protein accumulated than WT plants without BL application (Fig. 6d, e), which was consistent with the increased transcript level of *BHS1* in *d61-2* (Fig. 6a). In contrast, with 2,4-epiBL application, the rapid degradation of BHS1 in WT was blocked by the deficiency of BRI1 and the application of MG132 (Fig. 6e), suggesting that BRI1 and 26S proteasome machinery both involved in the BHS1 degradation process. To further clarify whether other BR signaling components are involving in the regulation of transcription and protein degradation of BHS1, we characterized several BR signaling mutants such as *Osbak1*, *Osbzr1, bu1* and *LICas1*, etc. (Fig. S10) and examined the level of BHS1 in these mutants' background. We found that the transcriptional and posttranscriptional levels of BHS1 were less affected in *Osbak1* with the BR treatment, whereas in *Osbzr1* and *LICas1* mutants' background, the change of BHS1 abundance with BR treatment is similar to WT (Fig. 6f–g), suggesting that the down-regulation of BHS1 after BR application is dependent on BR receptor complex (BRI1 and BAK1), but not affected by downstream player of BR signaling such as OsBZR1 and LIC. It should be pointed out that in *bu1* mutant, BHS1 was accumulated significantly both in transcriptional and posttranscriptional levels upon BR treatment. Taken together, our results suggested that *BHS1* is involved in BR signaling in rice, probably act as a negative regulator downstream of BRI1 affecting rice growth and development.

Discussion

Phytohormones such as brassinosteroids play important roles in plant growth and development, whereas the knowledge about BR signaling transduction and regulation in rice is relatively limited. In this study, we demonstrated that BHS1, a kinesin-13a protein, is involved in BR signaling in rice. First, *bhs1* showed BR hypersensitive phenotypes in term of lamina joint bending angle that is similar to BR-associated mutants (Fig. 1). Second, overexpression of *BHS1* reduced the sensitivity of plants to BR, which was lower than that of wild-type (Fig. 3). Third, in culms, *BHS1* has high expression level in the pulvinus (Fig. 4f), which is consistent with its function. Fourth, the transcript of *BHS1* is negatively regulated by either exogenous or endogenous BRs, and the degradation of BHS1 induced by BRs in WT is attenuated by OsBRI1 deficiency (Fig. 6d). These findings provide novel insights into BR signaling, and might have significant implications for improving plant architecture of monocot crops.

BHS1 co-localized with endoplasmic reticulum (ER)

In the post-genomic era, it is widely recognized that identification of the subcellular organelle localization and transport mechanisms of the encoded proteins are necessary for a fundamental understanding of their biological functions and the organization of cellular activity (Zhu et al. 2020). In term of kinesin proteins, several investigations had documented their subcellular locations. For animals, the high level of activity at the Golgi apparatus is sustained largely through the combined effects of microtubules, actin-microfilaments, and some intermediate filaments (Allan et al. 2002). Kinesins are not able to move along the microtubules in the conventional

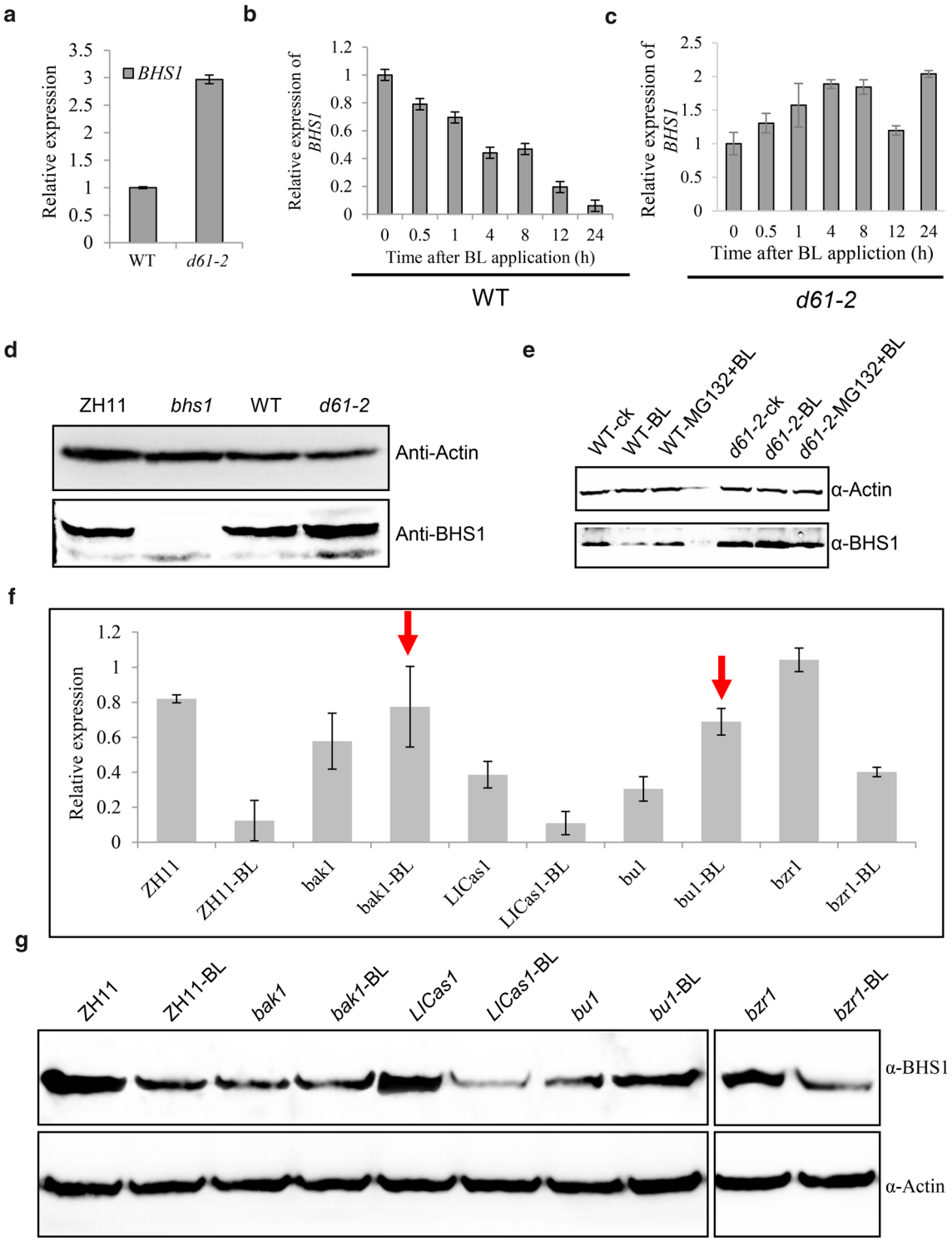

Fig. 6 Degradation of BHS1 depends on D61. **a** Relative expression of *BHS1* in wild-type (WT) and in *d61-2* plants. **b** Time-course response of *BHS1* to 10 μM 24-epiBL treatment in wild type (WT). **c** Time-course response of *BHS1* to 10 μM 24-epiBL treatment in *d61-2* plants. **d** Immunoblot analysis using anti-BHS1 antibody to show BHS1 protein levels in ZH11, WT, *bhs1*, and *d61-2*; Actin was used as the control. **e** Immunoblot analysis using anti-BHS1 antibody to show BHS1 protein levels in WT and *d61-2* under 24-epiBL treatment with and without MG132; Actin was used as the control. **f** Relative expression of *BHS1* in BR signaling mutants, respectively. Actin was used as the control. **g** Immunoblot analysis using anti-BHS1 antibody to show BHS1 protein levels in BR signaling mutants with and without 24-epiBL; CK, negative control. Actin was used as the control. Each experiment was repeated three times independently

form, but instead depolymerize microtubules from both ends (Ovechkina and Wordeman 2003). The completed Arabidopsis genome contains at least 61 genes encoding polypeptides with the kinesin catalytic core. Among these kinesins, AtKinesin-13A and AtKinesin-13B are two internal-motor kinesins (Reddy and Day 2001). As Lu et al. reported, AtKinesin-13A was co-localized with Golgi stacks in various Arabidopsis cells, indicating that AtKinesin-13A is a special plant internal-motor. However, because of the diverse cellular functions in which kinesins are involved, the localizations of kinesins in the rice cells would provide valuable information for many researchers. Previously, BC12 (a kinesin-4 protein) was found in both the nucleus and cytoplasm and associated with microtubule arrays during cell division (Zhang et al. 2010). Wan et al. proved that PSS1 protein (a kinesin-1 like protein) was predominantly localized in the cytoplasm in rice protoplast cells (Zhou et al. 2011). OsDLK (Dual Localization Kinesin), a kinesin-14 protein, was stably expressed in BY-2 cells decorating cortical microtubules, but also can shift into the nucleus of interphase cells. The nuclear import of this protein is strongly and reversibly promoted in response to cold (Xu et al. 2018). *STD1* encodes a phragmoplast-associated kinesin-related protein, which is localized to the phragmoplast mid-zone and regulates the lateral expansion of the phragmoplast (Fang et al. 2018). As plant-specific kinesin, kinesin-14 like protein O12 can interact with actin filament through its CH domain in plant cells (Umezu et al. 2011). However, as an atypical kinesin, BHS1 (OsKinesin-13A) might have feature of multicellular organelle localization. Using double-label immunofluorescence analysis, Deng et al. found previously that more than half of the OsKinesin-13A labeling was observed on vesicle-like structures. In addition, a small number of OsKinesin-13A labeling was also observed in cell membranes, Golgi stacks, and endoplasmic reticulum (Deng et al. 2015). In addition, the findings in onion epidermal cells by Wu et al. suggested that OsKinesin-13A (SGL) were mainly targeted to the nucleus, but also observed in other locations in the cells (Wu et al. 2014). In our study, we found that BHS1 was co-localized with endoplasmic reticulum (ER) marker protein AtWAK2 in rice protoplasts, suggesting that it is mainly localized on endoplasmic reticulum (ER) (Fig. 4; Figure S6). Previously, the investigations found that OsGH3-5, a BR signaling component, was localized in ER (Zhang et al. 2015). Combined with our data here, it seems that ER is an important organelle in BR signaling transduction. The inconsistent findings about BHS1/OsKinesin-13A localization might be caused by the different transformation receptor for fluorescence observation. It is also possible that the subcellular localization of OsKinesin-13A is flexible in different cell types or it is an intracellular shuttle protein, like OsDLK. Therefore, the determination of BHS1 localization with multiple methods in near future should be helpful to understand the cellular functions of BHS1.

Involvement of *BHS1* in BR signaling pathway

By 'forward' or 'reverse-genetics' approaches, several components of the BR signaling in Arabidopsis and other plants had been characterized (Asami et al. 2005). Because BR core components, like BRI1 and BZR1, etc., had also been identified in rice, suggesting that the core BR signaling pathway is conserved between rice and Arabidopsis (Bai et al. 2007; Gruszka 2020; Yamamuro et al. 2000). However, some difference may exist regarding to the signaling transduction components downstream of BRI1 receptor in Arabidopsis and rice plants. For example, two important components for BR signaling in Arabidopsis, PP2A (Protein Phosphatase 2A) and BSU (BRI1-Supressor1) phosphatases, are missing in rice genome. In contrast, the rice BR genes, such as OsLIC, OsDLT and OsTUD1, OsRAVL1, OsGW5, and OsPRA2, are not found in Arabidopsis (Gruszka 2020). Hence, isolation and characterization of additional rice BR components should be helpful for understanding BR signaling pathway and molecular breeding of rice. In rice, BR is initially perceived by the OsBRI1 receptor kinase (Nakamura et al. 2006; Yamamuro et al. 2000). Recent work has focused on the elucidation of the mechanisms of activation of BRI1 and of triggering of downstream actions (Gruszka 2020). OsBRI1 regulates OsBZR1, which in turn works with DLT to repress the expression of BR biosynthesis genes (Tong et al. 2009). Recently, a small GTPase (OsPRA2) was identified binding to the OsBRI1 receptor to repress its kinase activity. Accordingly, the transgenic plants with enhanced OsPRA2 activity leads to reduced sensitivity to the BR treatment, suggesting that OsPRA2 functions as negative regulator of the BR signaling (Zhang et al. 2016b). As far as we know, only a few players among the known BR signaling components showed their roles as negative BR regulator. In this study, *bhs1* mutant exhibits hypersensitivity to BR, including a larger angle of the lamina joint between the leaf blade and leaf sheath, longer coleoptile, and larger angle in etiolated leaf lamina joint. On the contrary, the plants overexpressing *BHS1* showed reduced sensitivity to BRs (Figs. 1, 3). Second, the transcriptional level of *BHS1* showed a notable reduction after 4 h 24-epiBL treatment (Fig. 5a, b). Third, BR-responsive genes showed altered transcript levels in *bhs1* mutant. Some positive regulators, such as *RAVL1, DSG1, ILI1,* and *BLE2*, have higher expression in *bhs1* than those in WT, which further proves that BHS1 is a negatively regulator of BR signaling (Fig. 5c, d). In addition, the occurrence

of the degradation induced by BRs in WT depends on BR receptor BRI1, BAK1 (Fig. 6), and 26S proteasome machinery (Fig. 5f). The deficiency of downstream player of BR signaling, such as BZR1 and LIC1, did not affect the degradation of BHS1 induced by BRs (Fig. 6f, g), suggesting that the degradation of BHS1 happened as early event. Previously, five mutants (*srs3*, TCM768, TCM2092, *sgl* and *sar1*) of *BHS1* were reported (Deng et al. 2015; Kitagawa et al. 2010; Wu et al. 2014). *srs3*, TCM768, TCM2092 all have a base substitution, but they occurred in different positions of the gene, resulting in different phenotypes due to unknown mechanisms (Wu et al. 2014). Wu et al. showed that *sgl* mutation causes defective GA synthesis and the GA response genes changed expression levels significantly in *sgl* mutant, which can respond to exogenous GA3 to rescue the dwarf phenotype. In addition, OsKinesin-13A affects cellulose microfibril orientation and cell elongation by promoting microtubule turnover and facilitating vesicle transport from the Golgi apparatus to the cell surface (Deng et al. 2015). Our work showed that BRs regulates the transcript level and protein stability of BHS1, thus regulating the expression levels of genes related to BR signaling pathway, resulting in the hypersensitive phenotype to BR of *bhs1* mutant (Figs. 1, 5, 6 and Fig. S11). Taken together, these observations indicate that the function of BHS1 was expounded in the aspect of plant hormone BR and might function with GA, etc. to regulate rice growth and development.

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Accession numbers: Sequence data from this article can be found in the GenBank data libraries under the following accession numbers: BHS1, LOC_Os05g06280; OsBRI1(D61), LOC_Os01g52050; D2, LOC_Os01g10040; D11, LOC_Os04g39430; OsCPD1, LOC_Os11g04710; BRD1, LOC_Os03g40540; BRD2, LOC_Os10g25780; DSG1, LOC_Os06g06090; BAK1, LOC_Os08g07760; LIC1, LOC_Os06g49080; ILI1, LOC_Os04g54900; BU1, LOC_Os06g12210; RAVL1, LOC_Os04g49230; GSK1, LOC_Os01g10840; BLE2, LOC_Os07g45570; ACTIN1, LOC_Os03g50885; AtWAK2, AT1G21270; CYP734A2, LOC_Os02g11020; CYP734A4, LOC_Os06g39880; CYP734A5, LOC_Os07g45290; CYP734A6, LOC_Os01g29150.

Author contribution statement YY, QQ, and ZY designed the research. ZY, WL, CF, LH, JY, and DG performed the experiments. XE, ZD, MD, and ZY analyzed the data. YY, WL, and ZY wrote the paper.

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Data availability statement The data that support the findings of this study are available from the corresponding author (Yanchun Yu) upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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