Plant Mol Biol (2017) 93:197–208
DOI 10.1007/s11103-016-0558-4

Novel rice mutants overexpressing the brassinosteroid catabolic gene *CYP734A4*

Wenjing Qian[1,2] · Chao Wu[1,3] · Yaping Fu[1] · Guocheng Hu[1] · Zhengquan He[2] · Wenzhen Liu[1]

Received: 17 April 2016 / Accepted: 29 October 2016 / Published online: 4 November 2016
© Springer Science+Business Media Dordrecht 2016

Abstract

Key message **Moderate overexpression of *CYP734A4* improves grain number per main panicle and seed setting rate.**

Abstract Brassinosteroid (BR) homeostasis and signaling are crucial for plant growth and development. *CYP734A* genes encode cytochrome P450 monooxygenases that control the level of bioactive BRs by degrading BRs. However, fertile plants overexpressing *CYP734As* have not been reported in rice. Here, we isolated a novel semi-dominant mutant *brd3-D*, in which T-DNA was inserted approximately 4 kb upstream of the *CYP734A4* gene (GenBank Accession AB488667), causing its overexpression. The mutant is characterized by dwarfism, small grains, and erect leaves and is less sensitive to brassinolide-induced lamina joint inclination and primary root elongation. However, increased grain number per main panicle and improved seed setting rate were also found in heterozygous *brd3-D*. To our knowledge, these traits have not been reported in other BR deficient mutants. Quantitative real-time PCR analysis indicated that phenotypic severity of the *brd3-D* mutant is positively correlated with the *CYP734A4* transcription level. In accordance with the increased expression of *CYP734A4*, a lower castasterone (a rice BR) content was detected in the *brd3-D* mutants. Knockout of *brd3-D* by using the CRISPR/Cas9 system rescued the mutation. In addition, transgenic plants overexpressing *CYP734A4* with the 35S enhancer mimicked the *brd3-D* phenotypes, confirming that moderate overexpression of the *CYP734A4* gene can improve grain number per main panicle and the seed setting rate in rice. Further studies showed that overexpression of *CYP734A4* influences the expressions of multiple genes involved in the BR pathway, and the expression of *CYP734A4* is induced by exogenous brassinolide, confirming the negative regulatory role of *CYP734A4* in the BR pathway. *CYP734A4* might provide a useful gene resource for developing new high-yielding rice varieties.

Keywords Rice · Brassinosteroids catabolic gene · *Brd3-D* mutant · *CYP734A4* overexpression · Grain number · Seed setting rating · Erect leaf

Introduction

Brassinosteroids (BRs) are plant steroid hormones that are widely distributed in plants. Thus far, more than 69 types of BRs have been isolated from different plants, among which castasterone and brassinolide play roles in many biological processes, including plant growth and development, cell division and elongation, vascular bundle differentiation, photomorphogenesis, male fertility, flowering,

Wenjing Qian and Chao Wu have contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s11103-016-0558-4) contains supplementary material, which is available to authorized users.

✉ Zhengquan He
zhq_he@163.com

✉ Wenzhen Liu
lwzzju@163.com

[1] State Key Laboratory of Rice Biology, China National Rice Research Institute, Hangzhou 310006, China

[2] Biotechnology Research Center, China Three Gorges University, Yichang 443002, China

[3] Institute of Horticulture, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, China

senescence and seed germination (Bajguz 2011; Gudesblat and Russinova 2011). They can also improve a plant's ability to withstand low temperature, high temperature, salt stress and other adverse conditions (Koh et al. 2007).

BR biosynthesis occurs in a complex metabolic network and can be divided into three steps: (1) formation of campestanol from campesterol, (2) formation of castasterone from campestanol, and (3) formation of brassinolide from castasterone. Recently, several rice BR biosynthesis genes functioning in the second step have been isolated. *BRD2* is involved in converting 24-methylenecholesterol to campesterol, the precursor of BRs (Hong et al. 2005). D11 and OsDWARF4, which are redundant proteins, function in C-22 hydroxylation, the rate-limiting step in BR biosynthesis (Sakamoto et al. 2006; Tanabe et al. 2005). The *BRD1* gene encodes BR-6-oxidase, which regulates multiple C-6 oxidation steps from the late to the early C-6 oxidation pathway (Hong et al. 2002; Mori et al. 2002). The D2 protein catalyzes the conversions from 6-deoxoteasterone to 3-dehydro-6-deoxoteasterone and from teasterone to 3-dehydroteasterone in the late step of the BR biosynthesis pathway (Hong et al. 2003).

The endogenous level of bioactive BRs is also maintained by inactivation of the bioactive forms. In contrast to BR biosynthesis, fewer advances were made in BR catabolism in plants. Several BR catabolic genes, including *BAS1, CYP72C1, UGT73C5* and *UGT73C6*, have been found in *Arabidopsis*. Overexpression of these genes led to BR-deficient phenotypes (Husar et al. 2011; Nakamura et al. 2005; Neff et al. 1999; Takahashi et al. 2005). However, little is known about rice catabolic genes. Four *BAS1* homologues, including *CYP734A2, CYP734A4, CYP734A5* and *CYP734A6*, but not the *CYP72C1* ortholog, have been found in rice. These genes appear to be upregulated in response to brassinolide (Thornton et al. 2011; Sakamoto et al. 2011). In vitro analysis showed that these CYP734As were multifunctional and multisubstrate P450s, which inactivate all C-22 hydroxylated BRs, including 6-Deoxocathasterone, Cathasterone, and their downstream products (Sakamoto et al. 2011). Transgenic rice overexpressing *CYP734As* driven by the rice *actin1* promoter showed serious defects in vegetative growth and did not produce floral organs (Sakamoto et al. 2011). Loss of function of *CYP734A6* leads to a strongly bending lamina phenotype (Park et al. 2006).

Extensive genetic and biochemical studies in *Arabidopsis* have identified almost all major BR signaling components. Most components of the BR signaling pathway are conserved between *Arabidopsis* and rice. However, some BR signaling components in *Arabidopsis* have not been identified in rice. In addition, a number of rice-specific components, including OsLIC, OsDLT and OsTUD1, have also been identified (Zhang et al. 2014). In rice, OsBRI1 and OsBAK1 coperceive BRs at the plasma membrane (Li et al. 2009; Nakamura et al. 2006; Yamamuro et al. 2000). BR binding to OsBRI1 promotes association with OsBAK1, and inactivates OsGSK1 and OsGSK2, which are orthologues of *Arabidopsis* BIN2 that functions as a key negative regulator of BR signaling (Koh et al. 2007; Tong et al. 2012). OsGSK1 and OsGSK2 repress the activity of OsBZR1, OsLIC and DLT by phosphorylation. *OsBZR1* inhibits the expression of *OsLIC* and *DLT*, whereas *OsLIC* also inhibits the expression of *OsBZR1* (Bai et al. 2007; Tong et al. 2012; Wang et al. 2008). OsILI1 and OsIBH1 interact antagonistically with each other and function downstream of OsBZR1. *OsBZR1* induces *OsILI1*, but represses *OsIBH1* expression, whereas *OsLIC* directly represses *OsILI1* to oppose the action of *OsBZR1* (Zhang et al. 2009, 2012). 14-3-3 proteins bind phosphorylated OsBZR1 to retain OsBZR1 in the cytoplasm (Bai et al. 2007). *OsMDP1* play a negative regulatory role in BR signaling. In *OsMDP1*-deficient plants, expression of *OsXTR1* was enhanced and that of *OsXTH1* was suppressed. *OsXTH1* and *OsXTR1* encode xyloglucan endotransglycosylase, which is necessary for cell elongation (Duan et al. 2006). *BU1* encoding a helix-loop-helix protein is a positive regulator of BR response (Tanaka et al. 2009). D1 and TUD1 act together to mediate BR signaling; however, the connection between D1 and OsBRI1-mediated BR signaling remains unknown (Hu et al. 2013; Wang et al. 2006). OsGSR1 activates BR synthesis by directly interacting with *BRD2* (Wang et al. 2009). Os*SPY* and *RAVL1* coordinate the expression of genes involved in BR signaling and biosynthesis (Je et al. 2010; Shimada et al. 2006).

In this study, a semi-dominant mutant, *brd3-D*, with a BR-deficient phenotype was identified as the first fertile mutant caused by overexpression of a rice BR catabolic gene, *CYP734A4*. Different from other BR deficient mutants, the heterozygous *brd3-D* mutant displayed improved grain number per main panicle and seed setting rate. Knockout of *CYP734A4* using the CRISPR/Cas9 system rescued the *brd3-D* mutant phenotype. Furthermore, transgenic plants overexpressing *CYP734A4* with the 35S enhancer mimicked the heterozygous *brd3-D* phenotype, confirming that moderate overexpression of the *CYP734A4* gene can increase grain number per main panicle and the seed setting rate in rice. Our results showed that controlling the BR level by BR catabolism could be useful for increasing grain yield in crop plants.

Materials and methods

Plant materials and phenotypic analysis

The *brd3-D* mutant with the genetic background of *japonica* rice cultivar Zhonghua11 was isolated from a T-DNA

insertion library containing 15000 transgenic rice lines (Zhu et al. 2001). F2 generation plants from a cross of the mutant and wild type parent Zhonghua11 were used for genetic analysis. All rice plants were grown under natural conditions in the paddy field at the China National Rice Research Institute (119°57′E, 30°03′N). The above-mentioned rice materials were obtained from the State Key Laboratory of Rice Biology, China National Rice Research Institute.

F2 plants from the cross of the *brd3-D* mutant and its original parent, and T1 plants from *NO-1* transgenic lines were used for phenotypic analysis. The plant height, internode length, panicle length, grain length and effective tiller number, grain number per main panicle, seed setting rate, and thousand seed weight were investigated. Data were analyzed with IBM SPSS Statistics 20 software.

Hygromycin resistant assay

To determine whether the mutant phenotype was caused by T-DNA insertion, a hygromycin resistant assay was performed. Fresh blades from the T_1 generation of the *brd3-D* mutant line were sheared into 2–4-cm segments and placed in two Petri dishes containing a solution of 0.5 mg/L 6-BA and 50 mg/L hygromycin. Care was taken to ensure the segments were in direct contact with the hygromycin solution. The experiment was carried out at approximately 26 °C under 12-h light/12-h dark cycles for 3 days (Wang and Waterhouse 1997).

Isolation and sequencing of T-DNA flanking sequence

Thermal asymmetric interlaced PCR (TAIL-PCR) was used to amplify the rice genome sequence flanking the T-DNA insertion site (Liu et al. 1995). Specific primers complementary to the T-DNA left border sequences were designed to amplify the genome sequence flanking the T-DNA insertion site. In addition, four arbitrary degenerate primers were synthesized (Supplemental Table 2). The tertiary TAIL-PCR products were recovered, cloned into a pMD18-T carrier after purification, and then sequenced by the Huada Gene Research Institution (Shenzhen, Guangdong, China).

DNA extraction and PCR-based co-segregation analysis

Fresh leaves of F_2 generation plants were used for extracting rice total DNA according to the modified method of Lu and Zheng (Lu and Zhen 1992). Three primers were designed to study whether the *brd3-D* phenotype co-segregated with the T-DNA insertion (Supplemental Table 2; Fig. 3b). The PCR reaction was carried out as follows: denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, annealing at 56 °C for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for 7 min.

Quantitative real-time PCR analysis

Total RNA was extracted from various plants using the Qiagen RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). First-strand cDNAs were synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN, USA). The cDNAs were assayed by quantitative real-time PCR using SYBR Green or TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on the Applied Biosystems 7900 Real-Time PCR System. The relative expression levels of each transcript were obtained by normalization to the *OsACT1* gene. PCR was carried out as follows: preheating at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. No reverse transcription control and no template control were included in all qRT-PCR experiments and no non-specific amplification was seen. The primers and probes used for quantitative real-time PCR analysis are listed in Supplemental Table 2.

Plasmid construction and rice transformation

For obtaining mutants with loss of function of *CYP734A4*, a CRISPR/Cas9 vector targeting the *CYP734A4* gene was constructed. A 20-bp fragment of the *CYP734A4* gene was assembled into the intermediate vector SK-gRNA. The gRNA was then inserted into the CRISPR/Cas9 binary vector pC1300-Cas9 (Wang et al. 2015). For the construction of the overexpression vector, two overlapped fragments containing the promoter and whole coding region of *CYP734A4* and the 35S enhancer were amplified by PCR and inserted into the p130035SSI-X binary vector (Fig. 6a). The two constructs were introduced into rice using the *Agrobacterium tumefaciens*-mediated genetic transformation method (Wu et al. 2010).

Lamina joint bending assay

The lamina joint bending assay was performed at the 3-leaf stage. One microliter of ethanol containing 0, 10, 1000 ng of 24-epiBR was spotted on the joint between the lamina and leaf sheath of homozygous *brd3-D*, the wild type and *d1*. After incubation for 4 days, the angle between the lamina and leaf sheath was measured. Five plants were measured in each treatment.

The influence of 24-epiBR on primary root elongation

Seeds were soaked in water for 1 day and germinated at 30 °C in darkness for 2 days, and then grown in

nutrient solutions with different concentrations (0, 10, 100, 1000 nM) of 24-epiBR. The primary root length was measured after the seedlings were cultured for 4 days at 28 °C under an 18-h photoperiod.

Results

Morphological and genetic characterization of the *brd3-D* mutant

The *brd3-D* mutant was identified from a transgenic rice line that displayed a dwarf phenotype typical of BR-deficient mutants. The progeny of *brd3-D* segregated into discrete groups of wild type, weak, and severe dwarf plants (Fig. 1a), suggesting that the parental dwarf mutants were heterozygous for a semi-dominant mutation. The homozygous mutant was further crossed with its wild type parent Zhonghua11. All F1 generation plants exhibited the mutation phenotype. The separation of wild type, weak, and severe dwarf plants in the F2 generation showed a good fit to a 1:2:1 segregation ratio (normal:weak:severe = 109:206:85, $X^2 = 1.69 < X^2_{0.05} = 5.99$). The results showed that the mutation was controlled by a single semi-dominant gene.

The phenotype of the mutant was further characterized using F2 generation plants under natural field conditions. Similar to most BR-related mutants, the *brd3-D* mutant had crinkly, dark green and erect leaves (Fig. 1a, b). In comparison with the wild type, both homozygous and heterozygous *brd3-D* mutants exhibited significantly reduced plant height (Fig. 1a, c). The height of the homozygous and heterozygous mutant was reduced on average to about 56.0 and 75.0%, respectively, that of the wild type plants (Fig. 1c, Supplemental Table 1). A further survey showed that all internodes of *brd3-D* were shortened significantly, but the homozygous *brd3-D* had little specific reduction in the second internode (Supplemental Table 1, Fig. 1c), which is similar to *d61*, a BR-insensitive mutant (Yamamuro et al. 2000). Although plant height was substantially reduced, there was no significant difference in panicle length between the homozygous mutant and wild type, and the panicle of the heterozygous mutant was even

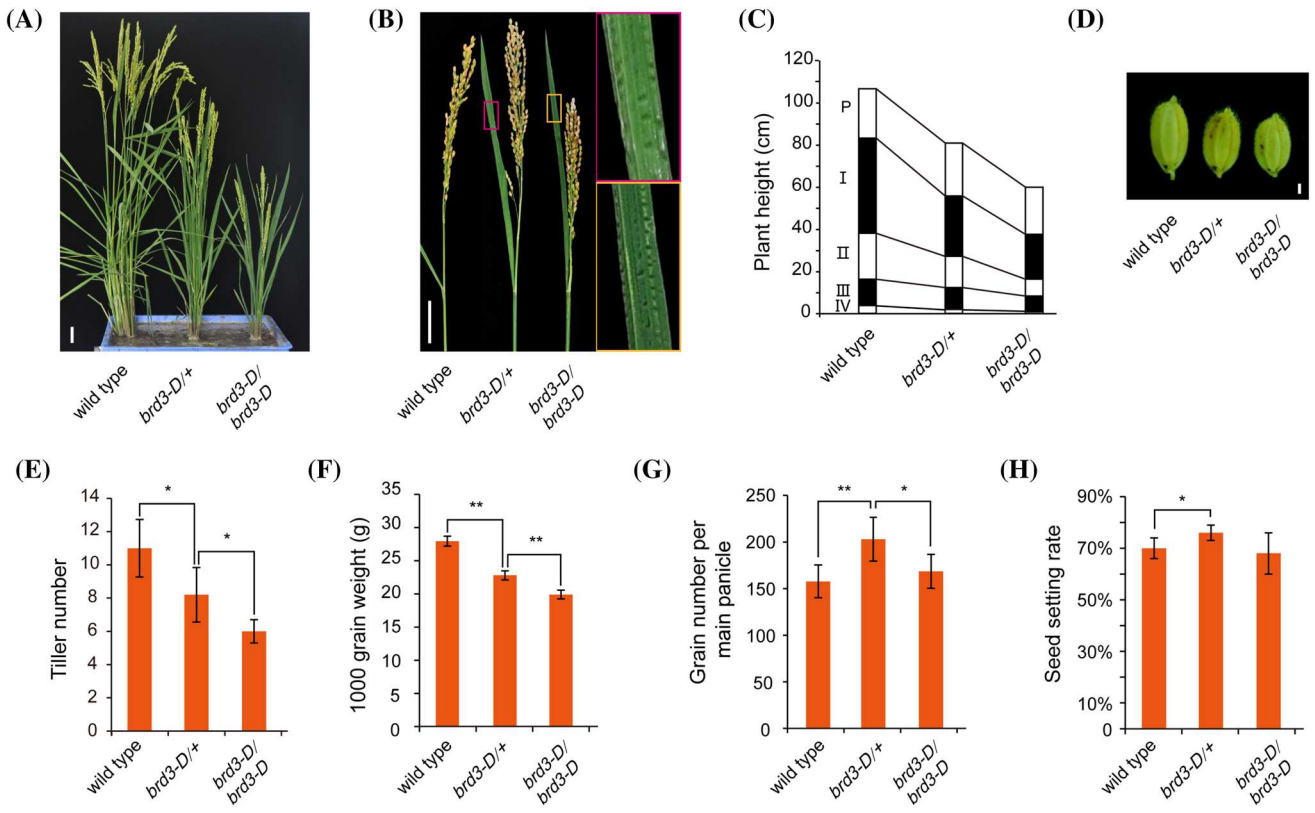

Fig. 1 Phenotype of the *brd3-D* mutant. **a** Phenotypes of the wild type and *brd3-D* heterozygous (*brd3-D*/+) and homozygous (*brd3-D*/*brd3-D*) mutants at the flowering stage. *Bar* 5 cm. **b** Panicle morphology and leaf angle of the wild type and *brd3-D*. *Bar* 5 cm. **c** Schematic representation of the elongation patterns of internodes of the wild type and *brd3-D*. *P* panicle, I–IV internodes from top to bottom. **d** Seed morphology of the wild type and *brd3-D*. *Bar* 1 mm. **e** Tiller number from the wild type and *brd3-D*. **f** Thousand grain weight of the wild type and *brd3-D*. **g** Grain number per main panicle of the wild type and *brd3-D*. **h** Seed setting rate of the wild type and *brd3-D*. Values are means of ten biological replicates (*P < 0.05, **P < 0.01)

longer than that of the wild type (Fig. 1c, Supplemental Table 1). The grain of *brd3-D* was significantly smaller than that of the wild type (Fig. 1d). The number of effective panicles per plant was reduced significantly by 45.6% in the homozygous mutant and 25.5% in the heterozygous mutant (Fig. 1e). The thousand grain weight was reduced by 28.8% in the homozygous mutant and reduced by 18.4% in the heterozygous mutant (Fig. 1f). Remarkably, although there was no significant difference between the homozygous mutant and wild type, the heterozygous mutant had a significantly higher grain number per main panicle (Fig. 1g). We also found that the heterozygous mutant had a slightly higher seed setting rate than the wild type (Fig. 1h). Generally, most traits of the *brd3-D* mutant were similar to those of BR-deficient mutants; however, improved grain number per main panicle and seed setting rate have not been reported in other BR-deficient mutants.

brd3-D displayed less sensitivity to 24-epiBR

Since some traits of the *brd3-D* mutant were similar to the BR-deficient mutants, we further examined whether *brd3-D* was sensitive to BR. Previous studies have shown that a low concentration of BR promotes root elongation, but high concentrations of BR inhibit root growth in rice (Yamamuro et al. 2000). Therefore, we measured the length of the roots under different concentrations of 24-epiBR. The result showed that 1 nM 24-epiBR promoted root elongation, but 10 nM or higher concentrations of 24-epiBR (24-epibrassinolide) inhibited root growth in the wild type and *brd3-D*. However, the *brd3-D* mutant showed less sensitivity to high concentrations of 24-epiBR than the wild type (Fig. 2a).

Lamina joint inclination is one of the most sensitive BR responses in rice (Zhang et al. 2009). To further confirm that *brd3-D* is a less sensitive BR mutant, a lamina joint inclination assay was performed. When treated with different concentrations of 24-epiBR, both *brd3-D* and wild type plants showed enhanced bending of the lamina joint in a dose-dependent manner. However, in contrast to the wild type, the degree of lamina joint bending of *brd3-D* was lower under all treatments. The lamina joint bending of *brd3-D* at 1000 ng/μL was almost the same as that of the untreated wild type (Fig. 2b). The results further suggest that the *brd3-D* mutation reduces BR responses in rice.

T-DNA insertion co-segregates with the *brd3-D* mutant

Transgenic plants expressing the hygromycin phosphotransferase gene can be identified by a hygromycin resistant assay (Wang and Waterhouse 1997). To determine whether the mutation phenotype is caused by T-DNA insertion, 120 T1 generation plants of the *brd3-D* mutant line were used for co-segregation analysis according to

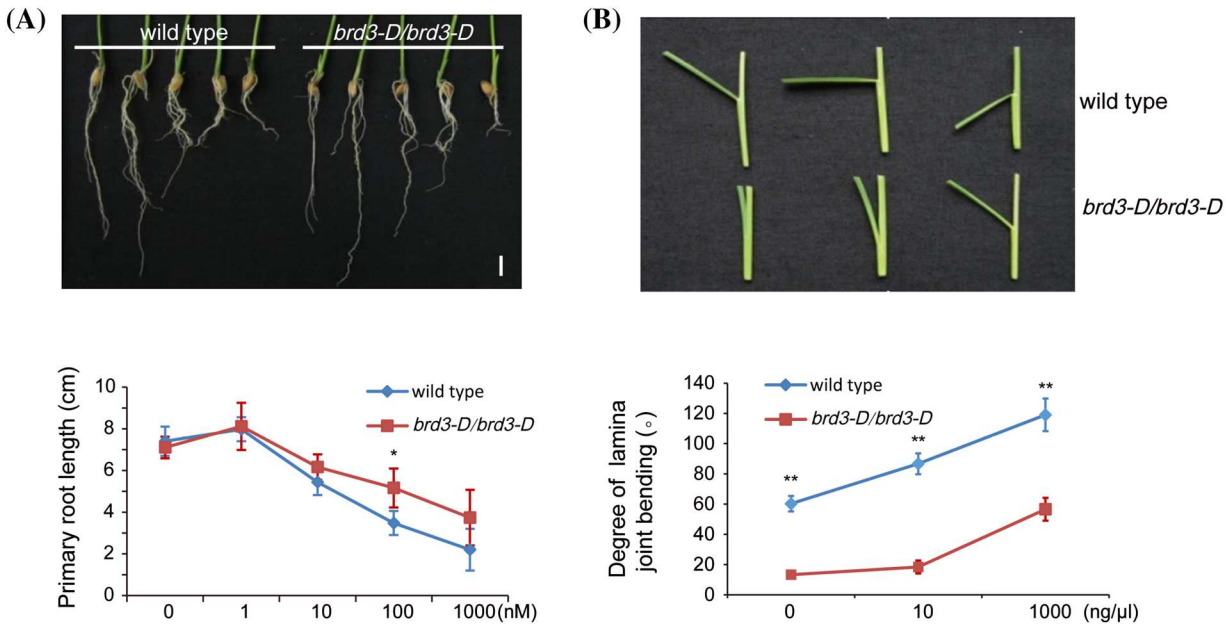

Fig. 2 Response of the *brd3-D* homozygous mutant to 24-epiBR. **a** Primary root elongation response to 24-epiBR. The wild type and homozygous *brd3-D* were treated with different concentrations of 24-epiBR (from *left to right*: 0 nM, 1 nM, 10 nM, 100 nM, 1000 nM). Primary root length was measured on day 4 after germination. *Bar* 1 cm. **b** The dose response to 24-epiBR of the bending angle in the wild type and homozygous *brd3-D* mutants at the 3-leaf stage (from *left to right*: 0 ng/μL, 10 ng/μL, 1000 ng/μL). Values are the means ± SD of five biological replicates. (*P < 0.05, **P < 0.01)

the leaf response to hygromycin. The segments from the *brd3-D* mutant remained green after 3 days in the hygromycin solution, while all the leaves from the wild type exhibited brown, necrotic, water-soaked lesions under the same condition (Supplemental Fig. 1a). The results suggest that the mutation phenotype could be caused by T-DNA insertion.

Since the mutation could be caused by a T-DNA insertion, we used the TAIL-PCR method to obtain the genomic sequence flanking the T-DNA insertion in the *brd3-D* mutant (Liu et al. 1995). The tertiary TAIL-PCR products were cloned into pMD18-T vectors and then sequenced (Supplemental Fig. 1b). Sequence analysis indicated that the T-DNA was inserted approximately 4 kb upstream of the *CYP734A4* gene of the PAC clone P0457B11, which is located on the sixth chromosome. Both the annotation results of the RiceGAAS system and alignment between the full-length cDNA (002-114-A02) and the genomic sequence of *CYP734A4* showed that the *CYP734A4* gene contains five exons and four introns (Fig. 3a). The *CYP734A4* full-length cDNA encodes a cytochrome P450 family protein of 538 amino acid residues, which inactivates C-22 hydroxylated BRs in vitro (Sakamoto et al. 2011).

Ninety F_2 generation plants were genotyped to further study whether the *brd3-D* phenotype co-segregated with the T-DNA insertion (Fig. 3b). Homozygous mutants were also found to be homozygous for the T-DNA insertion because only the 500-bp bands were amplified by the P1 and P2 primer pairs (Fig. 3c). Normal phenotype plants were found with no T-DNA insertion because only the 623-bp bands were amplified by the P1 and P3 primer pairs (Fig. 3c). Both the 500-bp and 623-bp bands were amplified from heterozygous mutants, which showed their heterozygous nature for the T-DNA insertion (Fig. 3c). These results further suggest that the *brd3-D* phenotype is caused by the T-DNA insertion.

Expression of the *CYP734A4* gene is increased drastically in *brd3-D*

In the *brd3-D* mutant, T-DNA carrying cauliflower mosaic virus 35S enhancer sequences on its right border, was inserted approximately 4 kb upstream of the *CYP734A4* gene. Therefore, we speculated that the 35S enhancer sequence may enhance the transcription of *CYP734A4* in *brd3-D*. The mRNA expression levels of the *CYP734A4* gene in different organs of the wild type and *brd3-D* heterozygous and homozygous mutants were quantified by

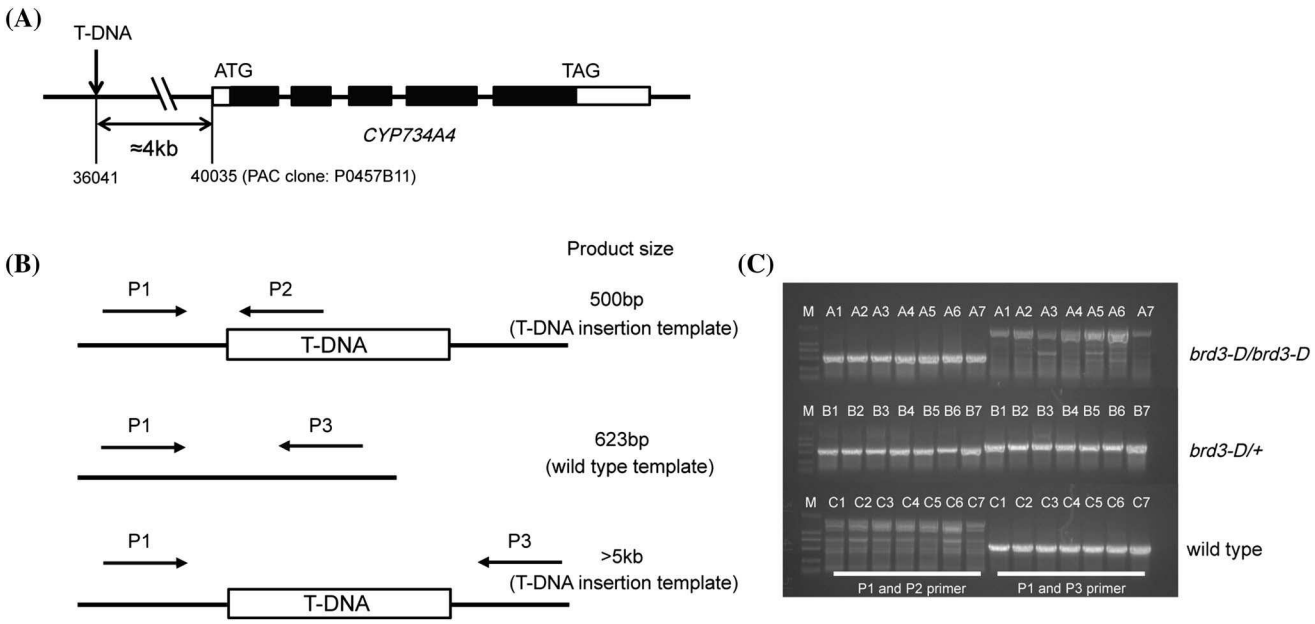

Fig. 3 Schematic diagram of *brd3-D* and cosegregation analysis of T-DNA and the *brd3-D* phenotype. **a** Schematic diagram of the *brd3-D* gene. T-DNA was inserted approximately 4 kb upstream of the *CYP734A4* gene, which contains five exons and four introns. Exons are indicated with boxes, coding region with filled boxes, and introns with lines between boxes. **b** Schematic diagrams of the genotyping. *P1* forward primer from rice genome flanking the left end of the T-DNA, *P2* reverse primer in T-DNA, *P3* reverse primer from rice genome flanking the right border of the T-DNA. The P1 and P2 primers produced a 500 bp PCR fragment from the T-DNA insertion DNA template; P1 and P3 primers produced a 623-bp PCR band from the wild type DNA, while no PCR band was obtained from the T-DNA insertion template because the expected fragment was too large to amplify. **c** Genotyping of F2 plants derived from a cross between *brd3-D* and its parent Zhonghua11. A1-A7, Homozygous mutants (*brd3-D*/*brd3-D*); B1-B7, Heterozygous mutants (*brd3-D*/+); C1-C7, wild type plants; *M* marker

real-time RT-PCR analysis. In the wild type, the highest expression level of *CYP734A4* was found in the leaf blades, followed by roots, leaf sheath, node, panicle and internode (Fig. 4a). Compared with the wild type, the expression levels of *CYP734A4* in all organs were significantly increased in the *brd3-D* mutant. *CYP734A4* in the leaf blade was increased by about 38 fold in the *brd3-D* homozygous mutant and by about 22-fold in the heterozygous mutant (Fig. 4b). Moreover, *CYP734A4* in both the *brd3-D* homozygous and heterozygous mutants showed a similar expression pattern as that in the wild type (Fig. 4a, b). The results suggested that the T-DNA insertion resulted in overexpression of the *CYP734A4* gene in *brd3-D*, and the severity of the *brd3-D* phenotype was positively correlated with the expression level of *CYP734A4* in the transgenic line.

Because the *CYP734A4* gene is involved in BR catabolism, we quantified the contents of castasterone and brassinolide, which are the most potent BRs in plants. As expected, in comparison to the wild type, the castasterone content was reduced by 94.31, 94.32, 94.02% in leaf blade, stem, and panicle in the homozygous *brd3-D* mutant, respectively (Fig. 4c). A previous study showed that there

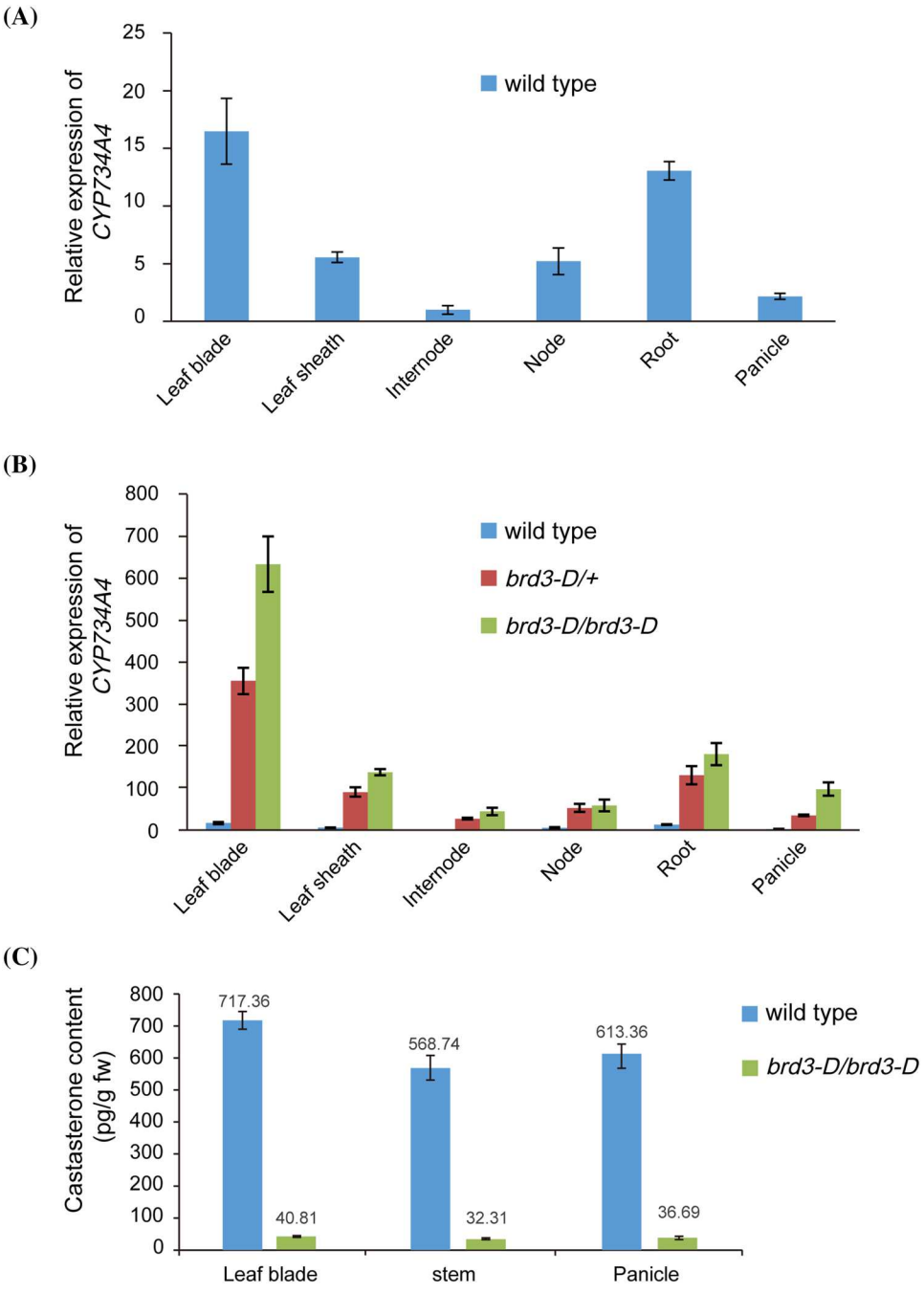

Fig. 4 The expression profile of *CYP734A4* and BR contents in the wild type and *brd3-D*. **a** The expression levels of *CYP734A4* in different organs of the wild type. **b** The transcript levels of *CYP734A4* among different organs of the wild type, heterozygous, and homozygous *brd3-D*. The transcript levels of *CYP734A4* in the internode of the wild type were arbitrarily set to 1. Values are the mean ± SD of three biological replicates. **c** Castasterone and brassinolide contents in the leaves of the wild type and homozygous *brd3-D*

is no or little brassinolide in rice (Nakamura et al. 2006). Similarly, we did not detect any brassinolide in either the wild type or the homozygous *brd3-D* mutant.

Knockout of the *CYP734A4* gene by CRISPR/Cas9 rescues the *brd3-D* phenotype

To elucidate the effects of reduced *CYP734A4* expression, a CRISPR/Cas9 vector targeting *CYP734A4* was constructed (Wang et al. 2015) and transformed into the wild type cultivar Zhonghua11 using the *Agrobacterium tumefaciens*-mediated genetic transformation method. Five homozygotes with frameshift mutations in the *CYP734A4* gene were identified by sequencing. However, none of these plants showed obvious changes in leaf morphology, leaf angles or seed sizes compared with those of the wild type plants (Supplemental Fig. 2). BLASTp analysis showed that CYP734A4 had high identity with CYP734A2 (78%), CYP734A6 (69%) and CYP734A5 (58%) in rice (Supplemental Fig. 3). Therefore, it is suggested that the unaltered phenotype under reduced expression of *CYP734A4* could be due to the functional redundancy of *CYP734As* in rice.

To prove that overexpression of the *CYP734A4* gene is responsible for the *brd3-D* phenotype, the CRISPR/Cas9 construct targeting *CYP734A4* was also transformed into homozygous *brd3-D* mutants. A frameshift mutation in the *CYP734A4* gene restored the homozygous *brd3-D* plant to a wild type phenotype (Fig. 5a, b). We therefore concluded that *CYP734A4* is the gene responsible for the *brd3-D* mutation.

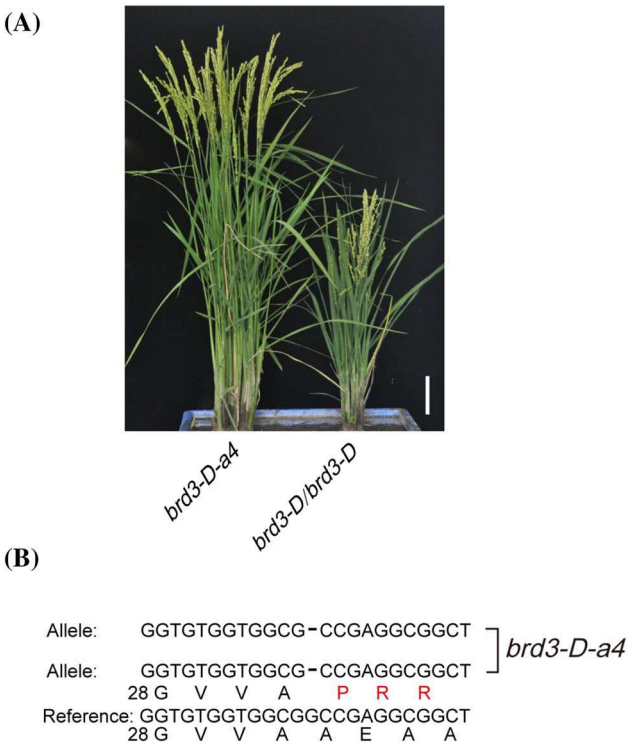

Fig. 5 Knockout of the *CYP734A4* gene with the CRISPR/Cas9 system rescues the *brd3-D* phenotype. **a** Phenotypic differences between the homozygous *brd3-D* with a frameshift mutation in the *CYP734A4* gene (*brd3-D-a4*) and the homozygous *brd3-D* plant (*brd3-D/brd3-D). Bar* 10 cm. **b** Partial nucleotide sequence of the *brd3-D-a4* and wild type alleles. 28: the amino acid number from the translation start site. Amino acid changes in *brd3-D-a4* are represented in *red* in comparison to the reference gene

Overexpressing *CYP734A4* with the 35S enhancer mimicked the *brd3-D* phenotypes

Phenotypic analysis of the *brd3-D* mutant showed that the moderate overexpression of *CYP734A4* improved the grain number per main panicle, panicle length, and seed setting rate. To further confirm the function of *CYP734A4*, a T-DNA construct containing *CYP734A4* with the 35S enhancer was generated and transformed into the rice cultivar Nipponbare (Fig. 6a). One transgenic line with a mild phenotype was selected for detailed analysis. In these transgenic plants, the expression level of *CYP734A4* was increased significantly (Fig. 6b). These transgenic plants showed reduced plant height (Fig. 6c) and lamina joint bending (Fig. 6d). These plants also exhibited an increase in total grain number (Fig. 6e) and filled grain number per main panicle (Fig. 6f), panicle length (Fig. 6g), and seed setting rate (Fig. 6h), but a decrease in the thousand grain weight (Fig. 6i, j). These results suggested that manipulating the expression of *CYP734A4* can reduce lamina joint angle and improve grain number and seed setting rate in rice.

CYP734A4 plays a role in regulating BR homeostasis in vivo

BR homeostasis is controlled by both BR biosynthesis and BR catabolism. Exogenous BR reduces the expression of BR biosynthesis genes, while BR deficiency induces their expression (Tong et al. 2009). However, little is known about the role of BR catabolic genes on BR homeostasis in rice. To investigate the in vivo function of *CYP734A4* in regulating BR homeostasis, we monitored changes in *CYP734A4* expression in response to 1000 nM 24-epiBR treatment using quantitative real-time RT-PCR. The mRNA levels in the wild type were dramatically increased 8 h after BR treatment and by more than 50 times at 48 h (Fig. 7a). Consistently, expressions of BR biosynthetic genes, including *BRD2, D2* and *OsDWARF4*, were significantly increased and expressions of BR catabolic genes, including *CYP734A5* and *CYP734A6*, were significantly reduced in the *brd3-D* mutant (Fig. 7b). These results confirmed that *CYP734A4* is involved in regulating BR homeostasis

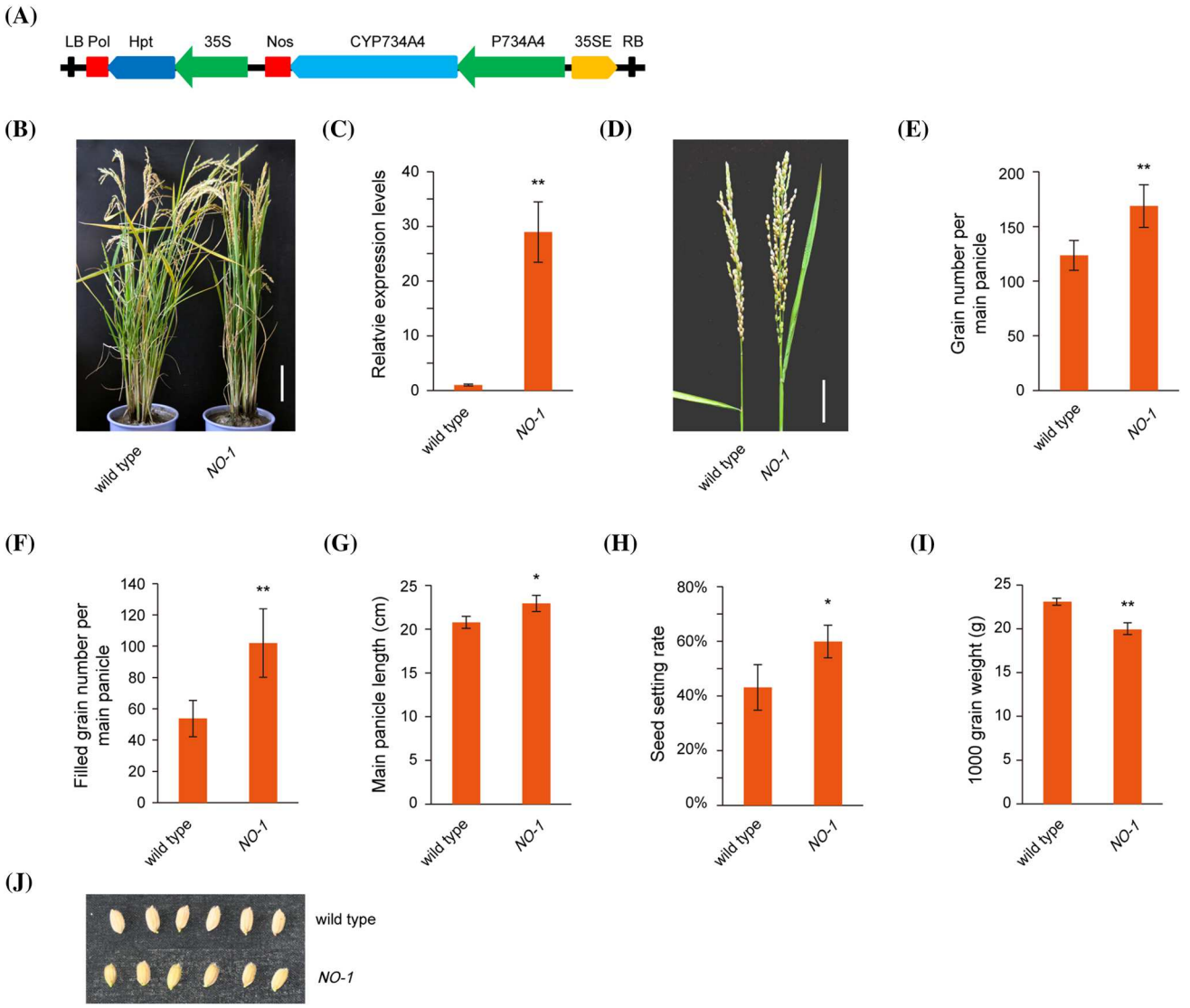

Fig. 6 Overexpression of *CYP734A4* under the control of the wild type CYP734A4 promoter together with the CaMV 35S enhancer recapitulates the *brd3-D* phenotype. **a** Schematic diagrams of the overexpression construct *pN208OX*. *Pol* PolyA terminator, *Hpt* hygromycin phosphotransferase gene, *Nos* nos terminator, *CYP734A4* cDNA sequence coding for CYP734A4, *P734A4* rice *CYP734A4* promoter, *35SE* 35S enhancer. **b** Gross morphology of *NO-1* (a transgenic positive plant from the T1 generation) and the wild type (a transgenic negative plant from the T1 generation). *Bar* 10 cm. **c** Real-time PCR analysis results of the *CYP734A4* transcripts in the leaf blade of *NO-1* and the wild type. The levels of the *CYP734A4* transcript in the wild type plants were arbitrarily set to 1. Values are the mean ± SD of three replicates. **d** Panicle morphology of *NO-1* and the wild type. *Bar* 5 cm. **e** Grain number per main panicle of *NO-1* and the wild type. **f** Filled grain number per main panicle of *NO-1* and the wild type. **g** Main panicle length of *NO-1* and the wild type. **h** Seed setting rate of *NO-1* and the wild type. **i** Thousand grain weight of *NO-1* and the wild type. **j** Grain morphology of *NO-1* and the wild type. Values are the means of five biological replicates (*$P < 0.05$, **$P < 0.01$)

in vivo. We also found that the expression of *BRD1* was decreased in *brd3-D* (Fig. 7b), suggesting that *BRD1* transcripts could be regulated in a feed-forward manner.

Overexpression of *CYP734A4* affects expression of BR signaling genes

To further investigate the underlying molecular basis of the *brd3-D* phenotype, we tested the effects of the *brd3-D* mutation on the BR signaling genes through quantitative real-time PCR. OsBRI1 and OsBAK1 mediate BR signaling at the plasma membrane (Li et al. 2009; Nakamura et al. 2006). The expression of *OsBRI1* and *OsBAK1* was increased in *brd3-D* (Fig. 7c). In rice, the OsBZR1 protein mediates feedback inhibition of BR biosynthetic genes (Bai et al. 2007). Interestingly, a decreased expression of *OsBZR1* was found in *brd3-D* (Fig. 7c). *OsBZR1* inhibits the expression of *OsLIC*. Consistently,

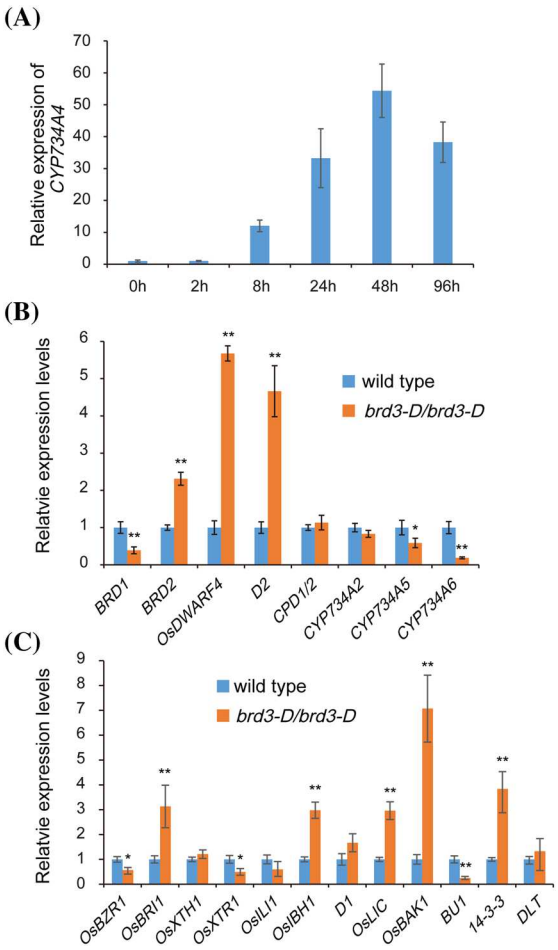

Fig. 7 *CYP734A4* regulates BR homeostasis in vivo. **a** Expression of *CYP734A4* in response to 24-epiBR in the wild type. 2-month-old rice plants were incubated in solutions of 1000 nM 24-epiBR and leaves were collected at 0, 2, 8, 24, 48, 96 h after treatment. The levels of the *CYP734A4* transcript at 0 h were arbitrarily set to 1. **b** Real-time PCR analysis results of the transcripts of BR biogenesis and BR catabolism-related genes in the leaves of the wild type and homozygous *brd3-D*. **c** Real-time PCR analysis results of the transcripts of genes related to BR signaling in the leaves of the wild type and homozygous *brd3-D*. The transcript levels of all tested genes in the wild type were arbitrarily set to 1. Values were obtained from three independent experiments

expressions of *OsLIC* were enhanced in *brd3-D* (Fig. 7c). *OsBZR1* induces *OsILI1*, but represses *OsIBH1* expression. In *brd3-D*, the expression of *OsIBH1* was increased (Fig. 7c). It has been reported that the expression of *OsXTH1* is decreased and that of *OsXTR1* is increased in *OsMDP1*-deficient plants that exhibited enhanced lamina joint inclination (Duan et al. 2006). Interestingly, the expression of *OsXTR1* was decreased in *brd3-D* (Fig. 7c). We also found elevated levels of *14-3-3* and decreased levels of *BU1* (Fig. 7c). These results suggested that a complicated regulation mechanism underlies the BR signaling pathway.

Discussion

Control of BR catabolism and BR homeostasis is important for plant growth and development. However, little is known about BR catabolism, and fertile plants overexpressing BR catabolic genes have not been reported in rice. In the present study, a semi-dominant mutant *brd3-D* with a BR-related dwarf phenotype was isolated from a rice T-DNA insertion library and the T-DNA was inserted approximately 4 kb upstream of the BR catabolic gene, *CYP734A4*. Gene expression analysis revealed that the expression of *CYP734A4* dramatically increased in the *brd3-D* mutant, and the severity of the *brd3-D* phenotype was correlated with the expression level of the *CYP734A4* gene. Transgenic *brd3-D* mutants harboring a frameshift mutation in the *CYP734A4* gene rescued the mutation. In addition, transgenic plants overexpressing full-length *CYP734A4* cDNA under the control of the wild type *CYP734A4* promoter together with the CaMV 35 S enhancer recapitulated the *brd3-D* phenotype. These results demonstrated that overexpression of the *CYP734A4* gene was responsible for the *brd3-D* phenotype. The expression of *CYP734A4* was dramatically increased by treatment with 24-epiBR in the wild type, and the expressions of BR biosynthetic genes were significantly increased (Fig. 7a, b). These results suggest that *CYP734A4* plays a role in modulating bioactive BR levels in vivo.

In *Arabidopsis*, it has been reported that *CYP734A1/BAS1* is involved in BR degradation, and overexpression of *BAS1* caused a BR-deficient phenotype (Neff et al. 1999; Turk et al. 2005). In rice, four *BAS1* homologues, including *CYP734A2, CYP734A4, CYP734A5, CYP734A6*, have been found. All four rice CYP734As showed high identities with BAS1 (ranging between 50.5 and 60.2%). In vitro analysis showed that rice CYP734As can catalyze inactivation of BRs (Sakamoto et al. 2011). Furthermore, the present study demonstrated that overexpression of the *CYP734A4* gene resulted in the *brd3-D* phenotype, such as dwarfism, small grains, erect and dark green leaves, high grain number per main panicle, and high seed setting rate (Fig. 1). BAS1 and CYP734As exhibit high sequence identity and share a similar function, suggesting that the mechanisms controlling BR inactivation are conserved in monocots and dicots.

Previously, BR was shown to have opposing effects on root meristem size, depending on its site of action (Vragović et al. 2015). Our results showed that the *brd3-D* mutant, different from other BR-deficient mutants, exhibits increased grain number per main panicle and an improved seed setting rate. It is possible that the spatiotemporal overexpression of *CYP734A4* caused specific BR degradation, which resulted in the *brd3-D* phenotype. In addition, the *brd3-D* phenotype might also be caused by inactivation of a

series of intermediate products for BR biosynthesis. It cannot be excluded that *CYP734A4* plays a role in the metabolism of other steroids that affect plant architecture.

Rice BR-related mutants showed distinctive internode elongation features. In the mild BR mutants, including *d11, d2*, and *d61-1*, the second internodes were completely stunted but the other internodes were elongated (Hong et al. 2003; Tanabe et al. 2005; Yamamuro et al. 2000). The *brd2* and *d61-2* mutants with intermediate phenotypes had a specific reduction from the second to the fourth internodes (Hong et al. 2005; Yamamuro et al. 2000). The *brd1-1* and *brd1-2* mutants with severe phenotypes showed elongation at the neck internode (Hong et al. 2002). In the heterozygous *brd3-D* mutants, the length of each internode was almost uniformly shortened (Fig. 1c), resulting in an elongation pattern similar to that of the wild type plant. However, the homozygous *brd3-D* exhibited little specific reduction in the second internode (Fig. 1c; Supplemental Table 1), but the phenotype was far less conspicuous than that of *d11, d2*, and *d61-1*. Therefore, the internode elongation pattern of *brd3-D* is different from those of previously isolated rice BR-related mutants.

Different from mutants deficient in BR biosynthesis, which exhibit supersensitivity to 24-epiBR, the *brd3-D* mutant was less sensitive to 24-epiBR. Because the *brd3-D* mutant displayed significantly higher transcription of the *CYP734A4* gene, we inferred that an increased *CYP734A4* mRNA level produced more CYP734A4 protein in this mutant, which resulted in 24-epiBR degradation and less available 24-epiBR to bind the BR receptor in *brd3-D*.

Both the overproduction and a mild deficiency of BR can enhance grain yields under certain conditions. Increased BR levels in specific tissues produce more and heavier seed, thus increasing per-plant grain yields (Wu et al. 2008). In contrast, an overall reduction in BR levels results in lower per-plant grain yields due to reduced seed number and size. However, reduced BR levels or sensitivity make leaves more erect. Erect leaves enable planting at higher densities and increase the grain yield per plot (Morinaka et al. 2006; Sakamoto et al. 2006). These results suggest that manipulating BR levels by catabolism can improve crop yield. Our results revealed that overexpression of *CYP734A4*, a catabolic gene, showed a phenotype with erect leaves in the *brd3-D* and *NO1* mutants. Surprisingly, different from previously reported BR-deficient or insensitive mutants, the heterozygous *brd3-D* and *NO1* mutants also exhibited increased seed setting rate and number of seeds in the main panicle (Figs. 1g, h, 6e, h). These traits are valuable for increasing seed yield. Therefore, *CYP734A4* might provide a useful gene resource for developing new high-yielding rice varieties.

Acknowledgements This work was supported by grants from the National Natural Science Foundation of China (31271686), the Important National Science & Technology Specific Projects for Breeding New Transgenic Varieties in China (2014ZX08001-004 and 2014ZX08010-004) and the Central Public-interest Scientific Institution Basal Research Fund (2012RG002-6).

Author contributions WL and ZH conceived the project and designed the experiments. WQ, CW, GH, YF and WL performed experiments; WQ and WL analyzed data and wrote the manuscript.

References

Bai MY, Zhang LY, Gampala SS, Zhu SW, Song WY, Chong K, Wang ZY (2007) Functions of OsBZR1 and 14-3-3 proteins in brassinosteroid signaling in rice. Proc Natl Acad Sci USA 104:13839–13844

Bajguz A (2011) Brassinosteroids-occurrence and chemical structures in plants. In: Brassinosteroids: a class of plant hormone, 1st edn. Academic Press, New York, pp 1–28

Duan K, Li L, Hu P, Xu SP, Xu ZH, Xue HW (2006) A brassinolide-suppressed rice MADS-box transcription factor, OsMDP1, has a negative regulatory role in BR signaling. Plant J 47:519–531

Gudesblat GE, Russinova E (2011) Plants grow on brassinosteroids. Curr Opin Plant Biol 14(5):530–537

Hong Z, Ueguchi-Tanaka M, Shimizu-Sato S, Inukai Y, Fujioka S, Shimada Y, Takatsuto S, Agetsuma M, Yoshida S, Watanabe Y, Uozu S, Kitano H, Ashikari M, Matsuoka M (2002) Loss-of-function of a rice brassinosteroid biosynthetic enzyme, C-6 oxidase, prevents the organized arrangement and polar elongation of cells in the leaves and stem. Plant J 32:495–508

Hong Z, Ueguchi-Tanaka M, Umemura K, Uozu S, Fujioka S, Takatsuto S, Yoshida S, Ashikari M, Kitano H, Matsuoka M (2003) A rice brassinosteroid-deficient mutant, *ebisu dwarf (d2)*, is caused by a loss of function of a new member of cytochrome P450. Plant Cell 15:2900–2910

Hong Z, Ueguchi-Tanaka M, Fujioka S, Takatsuto S, Yoshida S, Hasegawa Y, Ashikari M, Kitano H, Matsuoka M (2005) The Rice *brassinosteroid-deficient dwarf2* mutant, defective in the rice homolog of *Arabidopsis* DIMINUTO/DWARF1, is rescued by the endogenously accumulated alternative bioactive brassinosteroid, dolichosterone. Plant Cell 17:2243–2254

Hu X, Qian Q, Xu T, Zhang Y, Dong G, Gao T, Xie Q, Xue Y (2013) The U-box E3 ubiquitin ligase TUD1 functions with a heterotrimeric G alpha subunit to regulate Brassinosteroid-mediated growth in rice. PLoS Genet 9:e1003391

Husar S, Berthiller F, Fujioka S, Rozhon W, Khan M, Kalaivanan F, Elias L, Higgins GS, Li Y, Schuhmacher R et al (2011) Overexpression of the UGT73C6 alters brassinosteroid glucoside formation in *Arabidopsis thaliana*. BMC Plant Biol 11:51

Je BI, Piao HL, Park SJ, Park SH, Kim CM, Xuan YH, Huang J, Do Choi Y, An G, Wong HL, Fujioka S, Kim MC, Shimamoto K, Han CD (2010) *RAV-Like1* maintains brassinosteroid homeostasis via the coordinated activation of *BRI1* and biosynthetic genes in rice. Plant Cell 22:1777–1791

Koh S, Lee SC, Kim MK, Koh JH, Lee S, An G, Choe S, Kim SR (2007) T-DNA tagged knockout mutation of rice *OsGSK1*, an orthologue of *Arabidopsis BIN2*, with enhanced tolerance to various abiotic stresses. Plant mol Biol 65:453–466

Li D, Wang L, Wang M, Xu YY, Luo W, Liu YJ, Xu ZH, Li J, Chong K (2009) Engineering *OsBAK1* gene as a molecular tool to

improve rice architecture for high yield. Plant Biotechnol J 7:791–806

Liu YG, Mitsukawa N, Oosumi T, Whittier RF (1995) Efficient isolation and mapping of Arabidopsis thaliana T-DNA insert junctions by thermal asymmetric interlaced PCR. Plant J 8:457–463

Lu YJ, Zhen KL (1992) A simple method for DNA extraction of rice. Chin J Rice Sci 6:47–48 (**in Chinese**)

Mori M, Nomura T, Ooka H, Ishizaka M, Yokota T, Sugimoto K, Okabe K, Kajiwara H, Satoh K, Yamamoto K, Hirochika H, Kikuchi S (2002) Isolation and characterization of a rice dwarf mutant with a defect in brassinosteroid biosynthesis. Plant Physiol 130:1152–1161

Morinaka Y, Sakamoto T, Inukai Y, Agetsuma M, Kitano H, Ashikari M, Matsuoka M (2006) Morphological alteration caused by brassinosteroid insensitivity increases the biomass and grain production of rice. Plant Physiol 3:924–931

Nakamura M, Satoh T, Tanaka S, Mochizuki N, Yokota T, Nagatani A (2005) Activation of the cytochrome P450 gene, *CYP72C1*, reduces the levels of active brassinosteroids in vivo. J Exp Bot 56:833–840

Nakamura A, Fujioka S, Sunohara H, Kamiya N, Hong Z, Inukai Y, Miura K, Takatsuto S, Yoshida S, Ueguchi-Tanaka M, Hasegawa Y, Kitano H, Matsuoka M (2006) The role of *OsBRI1* and its homologous genes, *OsBRL1* and *OsBRL3*, in rice. Plant Physiol 140:580–590

Neff MM, Nguyen SM, Malancharuvil EJ, Fujioka S, Noguchi T, Seto H, Tsubuki M, Honda T, Takatsuto S, Yoshida S, Chory J (1999) *BAS1*: a gene regulating brassinosteroid levels and light responsiveness in *Arabidopsis*. Proc Natl Acad Sci USA 96:15316–15323

Park W, Kim HB, Kim WT, Park PB, An G, Choe S (2006) Rice *bending lamina 2* (*bin2*) mutants are defective in a Cytochrome P450 (CYP734A6) gene predicted to mediate brassinesteroid catabolism. J Plant Biol 49(6):469–476

Sakamoto T, Morinaka Y, Ohnishi T, Sunohara H, Fujioka S, Ueguchi-Tanaka M, Mizutani M, Sakata K, Takatsuto S, Yoshida S, Tanaka H, Kitano H, Matsuoka M (2006) Erect leaves caused by brassinosteroid deficiency increase biomass production and grain yield in rice. Nat Biotechnol 24:105–109

Sakamoto T, Kawabe A, Tokida-Segawa A, Shimizu B, Takatsuto S, Shimada Y, Fujioka S, Mizutani M (2011) Rice CYP734As function as multisubstrate and multifunctional enzymes in brassinosteroid catabolism. Plant J 67:1–12

Shimada A, Ueguchi-Tanaka M, Sakamoto T, Fujioka S, Takatsuto S, Yoshida S, Sazuka T, Ashikari M, Matsuoka M (2006) The rice *SPINDLY* gene functions as a negative regulator of gibberellin signaling by controlling the suppressive function of the DELLA protein, SLR1, and modulating brassinosteroid synthesis. Plant J 48:390–402

Takahashi N, Nakazawa M, Shibata K, Yokota T, Ishikawa A, Suzuki K, Kawashima M, Ichikawa T, Shimada H, Matsui M (2005) *shk1-D*, a dwarf *Arabidopsis* mutant caused by activation of the *CYP72C1* gene, has altered brassinosteroid levels. Plant J 42:13–22

Tanabe S, Ashikari M, Fujioka S, Takatsuto S, Yoshida S, Yano M, Yoshimura A, Kitano H, Matsuoka M, Fujisawa Y, Kato H, Iwasaki Y (2005) A novel cytochrome P450 is implicated in brassinosteroid biosynthesis via the characterization of a rice dwarf mutant, *dwarf11*, with reduced seed length. Plant Cell 17:776–790

Tanaka A, Nakagawa H, Tomita C, Shimatani Z, Ohtake M, Nomura T, Jiang CJ, Dubouzet JG, Kikuchi S, Sekimoto H, Yokota T, Asami T, Kamakura T, Mori M (2009) *BRASSINOSTEROID UPREGULATED1*, encoding a helix-loop-helix protein, is a novel gene involved in brassinosteroid signaling and controls bending of the lamina joint in rice. Plant Physiol 151:669–680

Thornton L, Peng H, Neff M (2011) Rice CYP734A cytochrome P450s inactivate brassinosteroids in Arabidopsis. Planta 234(6):1151–1162

Tong H, Jin Y, Liu W, Li F, Fang J, Yin Y, Qian Q, Zhu L, Chu C (2009) DWARF AND LOW-TILLERING, a new member of the GRAS family, plays positive roles in brassinosteroid signaling in rice. Plant J 58:803–816

Tong H, Liu L, Jin Y, Du L, Yin Y, Qian Q, Zhu L, Chu C (2012) DWARF AND LOW-TILLERING acts as a direct downstream target of a GSK3/SHAGGY-like kinase to mediate brassinosteroid responses in rice. Plant Cell 24:2562–2577

Turk EM, Fujioka S, Seto H, Shimada Y, Takatsuto S, Yoshida S, Wang H, Torres QI, Ward JM, Murthy G, Zhang J, Walker JC, Neff MM (2005) *BAS1* and *SOB7* act redundantly to modulate *Arabidopsis* photomorphogenesis via unique brassinosteroid inactivation mechanisms. Plant J 42(1):23–34

Vragović K, Sela A, Friedlander-Shani L, Fridman Y, Hacham Y, Holland N, Bartom E, Mockler TC, Savaldi-Goldstein S (2015) Translatome analyses capture of opposing tissue-specific brassinosteroid signals orchestrating root meristem differentiation. Proc Natl Acad Sci USA 112(3):923–928

Wang MB, Waterhouse PM (1997) A rapid and simple method of assaying plants transformed with hygromycin or PPT resistance gene. Plant Mol Biol Rep 15:209–215

Wang L, Xu YY, Ma QB, Li D, Xu ZH, Chong K (2006) Heterotrimeric G protein alpha subunit is involved in rice brassinosteroid response. Cell Res 16:916–922

Wang L, Xu Y, Zhang C, Ma Q, Joo SH, Kim SK, Xu Z, Chong K (2008) OsLIC, a novel CCCH-type zinc finger protein with transcription activation, mediates rice architecture via brassinosteroids signaling. PLoS One 3:1–12

Wang L, Wang Z, Xu Y, Joo SH, Kim SK, Xue Z, Xu Z, Chong K (2009) *OsGSR1* is involved in crosstalk between gibberellins and brassinosteroids in rice. Plant J 57:498–510

Wang C, Shen L, Fu YP, Yan CJ, Wang KJ (2015) A simple CRISPR/Cas9 system for multiplex genome editing in rice. J Genet Genomics 42(12):703–706

Wu CY, Trieu A, Radhakrishnan P, Kwok SF, Harris S, Zhang K, Wang J, Wan J, Zhai H, Takatsuto S, Matsumoto S, Fujioka S, Feldmann KA, Pennell RI (2008) Brassinosteroids regulate grain filling in rice. Plant Cell 20:2130–2145

Wu C, Fu YP, Hu GC, Si HM, Cheng SH, Liu WZ (2010) Isolation and characterization of a rice mutant with narrow and rolled leaves. Planta 232:313–324

Yamamuro C, Ihara Y, Wu X, Noguchi T, Fujioka S, Takatsuto S, Ashikari M, Kitano H, Matsuoka M (2000) Loss of function of a rice brassinosteroid insensitive1 homolog prevents internode elongation and bending of the lamina joint. Plant Cell 12:1591–1606

Zhang LY, Bai MY, Wu J, Zhu JY, Wang H, Zhang Z, Wang W, Sun Y, Zhao J, Sun X, Yang H, Xu Y, Kim SH, Fujioka S, Lin WH, Chong K, Lu TG, Wang ZY (2009) Antagonistic HLH/bHLH transcription factors mediate brassinosteroid regulation of cell elongation and plant development in rice and *Arabidopsis*. Plant Cell 21:3767–3780

Zhang C, Xu Y, Guo S, Zhu J, Huan Q, Liu H, Wang L, Luo G, Wang X, Chong K (2012) Dynamics of brassinosteroid response modulated by negative regulator LIC in rice. PLoS Genet 8:e1002686

Zhang C, Bai MY, Chong K (2014) Brassinosteroid-mediated regulation of agronomic traits in rice. Plant cell rep 33:683–696

Zhu ZG, Xiao H, Fu YP, Hu GC, Yu YH, Si HM, Zhang JL, Sun ZX (2001) Construction of transgenic rice populations by inserting the maize transponson Ac/Ds and genetic analysis for several mutants. Chinese J Biotech 17:288–292 **[In Chinese with English abstract]**