

# WITHDRAWN: A BIN2-like1 Protein Confers Dwarf by Interacting with BZR1 of Brassinosteroid Signaling in Allotetraploid Brassica Napus

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## EDITORIAL NOTE:

The full text of this preprint has been withdrawn by the authors while they make corrections to the work. Therefore, the authors do not wish this work to be cited as a reference. Questions should be directed to the corresponding author.

# Abstract

Brassinosteroids (BRs) are steroid hormones that play essential roles in plant growth and development. In this study, we identified a new dwarf mutant in *Brassica napus*. By map-based cloning, *BnaC04.BIL1* (*BnaC04g41660D*) gene, a *BIN2-like1* (BIL1) encoding a GLYCOGEN SYNTHASE KINASE 3 (GSK3-like) protein kinase, was isolated. To date, how BIL1 involves in BR signal transduction remains uncovered. Genetic transformation experiments confirmed that the *BnaC04.BIL1* is responsible for the plant dwarf phenotype in the *Bndwarf2* mutants. Overexpression of *BnaC04.BIL1* not only reduced plant height, but also resulted in compact plant architecture. Using CRISPR/Cas9, two sgRNAs were designed to target *BnaC04.BIL1* gene. The gene editing experiments generated mutations of *BnaC04.BIL1* sequence, which were stably transmitted to successive generations, and lead to restoration of plant height and plant architecture. The molecular mechanism of *Bndwarf2* dwarfing was further verified by Y2H and BiFC assays. Results shown that a Thr187Ser amino acid substitution residing in the conserved region promotes the interaction between *BnaC04.BIL1*<sup>Mut</sup> with *BnaBZR1*, thus enhances the negative regulation of plant growth. The genetic and molecular evidence clarifies first the *BnaC04.BIL1* can sharply change plant architecture in natural plant accessions in allotetraploid, and provides new insights into the molecular mechanisms of BR signaling.

## Key Message

*BnaC04.BIL1*, a *BIN2-like1* (BIL1) encoding a GLYCOGEN SYNTHASE KINASE 3 (GSK3-like) protein kinase was isolated, which confers dwarf by interacting with BZR1 of brassinosteroid signaling in allotetraploid *Brassica napus*.

## Introduction

Oilseed rape (*Brassica napus* L.) is one of the most important oil crops worldwide, and provides high-quality edible oil for human diets, protein-rich feed for animals, and raw materials for industrial processes. Breeding its cultivars with dwarf or semi-dwarf phenotype is a major objective in the genetic improvement because dwarfing architecture can be helpful to increase harvest index and enhance lodging resistance (Hedden 2003). To find available germplasm or genes associated with dwarfing plant type for *B. napus* breeding, some efforts have been carried out. For example, the dwarfness-associated genes in *B. napus*, including *DS-1* (Liu et al. 2010), *ndf-1* (Li et al. 2011), *DS-3* (Zhao et al. 2017), *DS-4* (Zhao et al. 2019), *G7* (Cheng et al. 2019), *BnaDwf.C9* (Wang et al. 2020), have been positioned or identified. Additionally, the *Bndwf1* was fine-mapped on the A9 chromosome to a 152-kb interval (Wang et al. 2016a). However, the molecular mechanism(s) underlying the development of the dwarf phenotype in *B. napus* remain elusive for ideal plant type breeding due to absence of successfully applied cultivar in vast oilseed rape production region.

Brassinosteroids (BRs) are important sterol hormones that regulate plant developmental and growth processes through a signaling cascade from the BR receptor to the expression of BR target genes (Clouse

et al. 1996; He et al. 2002). Researches during the past several decades have accumulated extensive knowledge of BR biosynthesis and the signaling pathways in model plants (Belkhadir and Jaillais 2015; Guo et al. 2013; Tanabe et al. 2005; Wang et al. 2014; Yamamuro et al. 2000), such as *Arabidopsis* and rice. It is well documented that BRs are perceived extracellularly by the BR-INSENSITIVE1-BRI1-ASSOCIATED KINASE1 (BRI1-BAK1) (Li et al. 1997; Li et al. 2002; Nam and Li 2002) complex. Afterwards, the binding between BRs and BRI1-BAK1 complex could initiate signal transduction to BRASSINAZOLERESISTANT1/BRI1-EMS-SUPPRESSOR1 (BZR1/BES1) (Wang et al. 2002; Yin et al. 2002) through CONSTITUTIVE DIFFERENTIAL GROWTH1 (CDG1) (Kim et al. 2011) and BR SIGNALING KINASE1 (BSK1) (Tang et al. 2008), then BRI-SUPPRESSOR1 (BSU1) (Mora-Garcia et al. 2004), BRASSINOSTEROID INSENSITIVE2 (BIN2) (Choe et al. 2002; Li and Nam 2002), as well somehow PROTEIN PHOSPHATASE 2A (PP2A) (Tang et al. 2011). The transcriptional factor BES1/BZR1 affects plant growth and development in various aspects through the regulating expression of thousands of BR responsive genes. Among these genes, glycogen synthase kinase-3 (GSK3)-like kinase BIN2 is a key suppressor that regulates plant growth and development by determining the phosphorylation status of BES1 and BZR1 (Choe et al. 2002; Li and Nam 2002; Wang et al. 2002; Yin et al. 2002). GSK3-like kinases are a highly conserved Ser/Thr kinases that are implicated in a wide range of cellular and developmental processes (Woodgett 2001). In *Arabidopsis*, the GSK3/SHAGGY-like gene family has 10 gene members that can be classified into four subgroups (Jonak and Hirt 2002). In this family, the *Arabidopsis* GSK3-like kinase (AT4G18710, BIN2/UCU1/DWF12/AtSK21) which belongs to the group II, has activity to negatively regulate the BR signal transduction by phosphorylating BZR1/BES1 (Choe et al. 2002; He et al. 2002; Li and Nam 2002). The gain-of-function *bin2* mutant was discovered to be insensitive to BRs in *Arabidopsis*, and has the shaggy phenotypic characteristic of dwarfing architecture. It also confers curved leaves, and an impaired cell elongation (Perez-Perez et al. 2002). The coding sequence of the *BIN2* gene, substitutes consecutive glutamate residues in the highly conserved TREE domain, which results in the negatively regulating growth by phosphorylating the BES1 and BZR1 proteins, that result in the degradation of BZR1 to reduce its activity (Li and Nam 2002). Based on sequence similarity of BIN2 with its two closest group II *Arabidopsis* homologs, BIN2-Like1 (BIL1) and BIN2-Like2 (BIL2), which belong to the AtSKs group (Jonak and Hirt 2002). It was further suggested that BIL1 and BIL2 may also be involved in BR signaling. Overexpression of BIL1 or BIL2 gene driven by their native promoters in wild-type *Arabidopsis* plants exhibits the dwarf phenotype (Yan et al. 2009). However, the evidence of BIL1 and BIL2 genes involved in BR signal transduction is still insufficient, and the mechanism of plant dwarf phenotype caused by overexpression of *BIL1* and *BIL2* genes remains to be elucidated. Therefore, it is urgent to further explore their participation and even related mechanism.

In this study, a pure dwarf mutant, *Bndwarf2*, was found in advanced selfing generation in a nearly pure line CB1501-1 in *B. napus*. To expedite this study, the dwarf gene *BnaC04.BIL1* was isolated using map-based cloning. The *BnaC04.BIL1* gene encoding a GSK3-like kinase, belongs to GSK II subfamily. Genetic transformation experiments confirmed that the *BnaC04.BIL1* was responsible for the plant dwarf phenotype in the *Bndwarf2* mutants. The molecular mechanism of *Bndwarf2* dwarfing was verified by Y2H and BiFC assays demonstrating that a Thr187Ser amino acid substitution residing in the highly

conserved region of *BnaC04.BIL1* promoted the interaction between *BnaC04.BIL1<sup>Mut</sup>* with *BnaBZR1*. Overall, this study clarifies the role of *BnaC04.BIL1* in the regulation of plant height, which may help to improve lodging resistance in oilseed rape, and therefore provides new insights into the molecular mechanisms of BR signaling in allotetraploid.

## Materials And Methods

### Plant materials

A pure dwarf mutant, *Bndwarf2* was found in advanced selfing generation in a nearly pure line CB1501-1 in *B. napus* by our lab. The populations for mapping the *BnDWARF2* locus, were generated from the crosses between *Bndwarf2* and the canola variety Zhpngshuang 11 (ZS11). All oilseed rape materials were grown in growth chamber and the fields of the Jiangpu Agricultural Experimental Station at Nanjing Agricultural University.

Tobacco was grown in growth chamber. The illumination period was 14 hours with temperature at 26°C and 10 hours with temperature at 20°C. When tobacco leaves at 5-leaf stage were used for the gene subcellular localization and bimolecular fluorescence complementary assay (BiFC).

### Map-based cloning

SNP and SSR markers were used to map the dwarf gene. 70 dwarf plants, 24 tall plants and parents from F<sub>2</sub> population were genotyped using a Brassica 60 K SNP Bead Chip Array (Illumina, Inc), which have a total of 52,157 SNP markers. The SNP genetic map was constructed by JoinMap 4.1 mapping software (Ooijen et al. 2006), then the *BnDWARF2* locus was primarily mapped onto physical and genetic map. The mapping interval sequence was downloaded from the *Brassica napus* Genome Browser (<http://www.genoscope.cns.fr/brassicanapus/cgi-bin/gbrowse/colza/>). Using this genomic sequence, SSR marker primers were designed by aid of SSR Hunter 1.3 (Li et al. 2005), and Primer Premier 5.0 (Singh et al. 1998). A total of 318 polymorphic SSR markers were obtained. These SSR markers helped to fine-map the *BnDWARF2* locus using a size-enlarged populations comprised of F<sub>2:3</sub> plants.

To identify genes associated to the dwarf trait, sequence of the fine mapping interval was obtained from the *Brassica napus* Genome Browser for reference to next-step experiments. Then, all of the genes in the fine mapping interval were cloned from *Bndwarf2* and parent ZS11. And, the resulting sequences were aligned using ClustalX 1.83 and GeneDoc software. The specific primers of the genes are listed in Table S6.

### Sequence analysis

The *B. napus* BIL1 genes were obtained by screening the *B. napus* Genome Browser (<http://www.genoscope.cns.fr/brassicanapus/>) with known *A. thaliana* BIL1 gene as a query. The Conserved Domain Database was used to search the protein functional in the National Center for

Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>). Predicted *A. thaliana* BIL1 amino acid sequences were obtained from the TAIR website (<http://www.arabidopsis.org/Blast>). Moreover, the protein sequences of other species were obtained from the NCBI using the *A. thaliana* BIL1 protein sequence as a query. All obtained protein sequences were aligned using ClustalX 1.83 (Crooks et al. 2004). Additionally, a phylogenetic tree was constructed using MEGA 7.0 (Kumar et al. 2016) with maximum likelihood method, and the bootstrap values were estimated with 1000 replicates. The functional protein association network was analyzed by STRING (<https://string-db.org/cgi/>).

## RNA extraction and qRT-PCR

Total RNA was extracted from various samples using TRIzol reagent (Sigma; <http://www.sigmaaldrich.com/>). First-strand cDNA synthesis was carried out using a Reverse Transcription System (Takara, Tokyo, Japan). The cDNA was used as the template for qRT-PCR analysis with specific primers (Table S6). The qRT-PCRs were carried out with SYBR Green Real-time PCR Master mix using a CFX96-2 PCR machine (BIO-RAD, USA). Relative expression levels were calculated using the  $2^{-\Delta\Delta C_t}$  method with Actin as an internal control.

## Subcellular localization

The coding sequence of *BnaC04.BIL1* (BnaC04g41660D) and *BnaBZR1* (BnaCnng34980D) were amplified using two pairs of primers GFP-BnaC04.BIL1-F/R and GFP-BnaBZR1-F/R (Table S6) and inserted into pA7-GFP entry vector. The recombinant plasmid BnaC04.BIL1-GFP, BnaBZR1-GFP, and the empty vector pA7-GFP were introduced into tobacco leaf cells by the particle bombardment method. The tobacco leaf cells were then bombarded by PDS-1000/He (Bio-Rad, USA), with an 1100 psi split membrane and gold particles coated with the plasmid DNA. After bombardment, the tobacco leaf cells were incubated on MS medium in a dark chamber at 28°C for 16 h. Fluorescence was observed using a LSM780 confocal microscopy imaging system (Zeiss, Germany).

## Plant transformation

The 1223-bp *BnaC04.BIL1* open reading frame was amplified from *Bndwarf2* using the primers *BnaC04.BIL1*-F/R (Table S6) and cloned into the *Xba*I-*Bam*HI sites of the overexpression pBI121 vector with CaMV35S promoter to construct the 35S::*BnaC04.BIL1*-pBI121 plasmid. The plasmid was introduced into *Agrobacterium tumefaciens* strain EHA105 by a heat shock method. The positive *A. tumefaciens* were transformed into ZS11 as previously described (Tan et al. 2011). Seeds were collected, followed by screening of the transgenic plants.

## CRISPR/Cas9 target locus selection and construct assembly

In order to target *BnaC04.BIL1*, the sequence-specific sgRNAs were designed using the web-based tool CRISPR-P 2.0 (<http://crispr.hzau.edu.cn/CRISPR2/>). The S1 target sites in ORF 5' terminal and the S2 in the conserved region for *BnaC04.BIL1* were selected, and the specific sgRNA primers are listed in Table

S6. The two targets were assessed using PCR and Sanger sequencing of *Bndwarf2* to ensure that no polymorphisms existed between the sgRNAs and the corresponding target sequences.

The binary pYLCRIPSR/Cas9 multiplex genome targeting vector system (pYLCRIPSR/Cas9P 35S-H), which was provided by Prof. Yaoguang Liu (South China Agriculture University), in which Cas9p is driven by the cauliflower mosaic virus 35S promoter (P35S), and four plasmids with sgRNA cassettes driven by the promoters of AtU3b, AtU3d, AtU6-1, and AtU6-29. This system was used for construct assembly according to a method previously described by Ma et al. (2015). The resulting constructs contained a Cas9p expression cassette, sgRNA expression cassettes with target sequences.

## Y2H assays

For the Y2H analysis, *BnaC04.BIL1<sup>Mut</sup>* and *BnaC04.BIL1<sup>WT</sup>* were cloned into the pGADT7 vector, and *BnaBZR1* was cloned into the pGBKT7 vector, resulting in AD (Activation Domain)-*BnaC04.BIL1<sup>Mut</sup>*, AD-*BnaC04.BIL1<sup>WT</sup>*, and BD (Binding Domain)-*BnaBZR1*, respectively. The yeast cells were cultured on synthetic defined medium (SD)/-Trp-Leu and SD/-Trp-Leu-His-Ade containing 5-Bromo-4-Chloro-3-Indolyl- $\alpha$ -D-Galactoside (X-a-gal) at 30°C for 3 d in the dark. SD/-Trp-Leu is yeast culture medium without Trp and Leu. SD/-Trp-Leu-His-Ade is culture medium without Trp, Leu, His, and Ade. The vector plasmid used in the positive control was pGADT7-T/pGBKT7-53, and the negative control was pGADT7/pGBKT7-Lam. The PCR primers used for Y2H assays are listed in Table S6.

## Bimolecular Fluorescence Complementarity (BiFC) assay

For the BiFC analysis, the cDNA of *BnaBZR1* was cloned into *Bam*H I site of pSPYNE vector, and *BnaC04.BIL1<sup>Mut</sup>* and *BnaC04.BIL1<sup>WT</sup>* were cloned into *Bam*H I site of pSPYCE vector to generate the *BnaBZR1*-YFPn, *BnaC04.BIL1<sup>Mut</sup>*-YFPc, and *BnaC04.BIL1<sup>WT</sup>*-YFPc fusion proteins, respectively (Waddt et al. 2008). The plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101. *A. tumefaciens* cells containing each construct were prepared and mixed to an OD<sub>600</sub> of 0.6:0.6 and transformed into tobacco leaf cells. YFP fluorescence was observed using the LSM780 confocal microscopy imaging system (Zeiss, German) 40 to 48 h after infiltration. The PCR primers used for the BiFC assays are listed in Table S6.

## RNA-sequencing analysis

The *Bndwarf2* mutants were evaluated by RNA sequencing, with the ZS11 plants serving as the control. Seedlings were grown in pots containing peat: vermiculite (1:1 v/v). Because the *Bndwarf2* showed wrinkled leaves, shorter petioles, and shorter hypocotyls, the whole plants of 6-week-old were harvested, and immediately frozen in liquid nitrogen, then stored at -80 °C until analyzed. Total RNA was extracted using Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The RNA was used to construct sequencing libraries that were analyzed with the HiSeq 2500 platform (Illumina, San Diego, CA). Paired-end clean reads were aligned to the *B. napus* "*Darmor-bzh*" reference genome (Chalhoub et al. 2014) using HISAT2.2.4 (Kim et al. 2015). For each transcription region, a FPKM

(fragment per kilobase of transcript per million mapped reads) value was calculated with StringTie v1.3.1 (Pertea et al. 2015). Differential expression analysis of *Bndwarf2* and ZS11 (three biological replicates per sample) were performed using DESeq2 software (Love et al. 2014). Genes with a false discovery rate < 0.005 and absolute fold change  $\geq 2$  were considered DEGs.

The GO annotation of DEGs was performed using the Gene Ontology database (<http://www.geneontology.org/>), and GO terms with corrected  $P < 0.05$  were considered to be significantly enriched. In addition, DEGs were analyzed using the KEGG database (Kanehisa et al. 2000), and KEGG enrichment pathways of DEGs were determined using edge R package (Robinson et al. 2010).

## Results

### Characterization of the *Bndwarf2* mutant

A pure dwarf mutant, *Bndwarf2* was obtained in advanced selfing generation in a nearly pure line CB1501-1 in *B. napus*. The *Bndwarf2* mutant showed an obvious dwarf and etiolated phenotype after the germination in the dark at 24°C for 6 d in comparison with the cultivar Zhongshuang 11 (ZS11, a conventional *B. napus* cultivar) that was used as a parent for map-based cloning gene responsible for the dwarfism, or as wild type (Fig. 1a). At seedling stage, the *Bndwarf2* mutant plants had shorter hypocotyls and shorter petioles (Figs. 1b, c). The leaves of *Bndwarf2* mutants showed darker green, thickened, and wrinkled leaves, and had significant higher Chl a, Chl b, and Chl contents than those of ZS11 (Table S1). At flowering stage, the *Bndwarf2* mutant showed significant difference in plant height from ZS11 (Fig. S1). While at maturity stage, the *Bndwarf2* mutant showed dwarf stature ( $33.62 \pm 1.12$  cm) with no apical dominance, that was significantly lower than that for ZS11 ( $193.54 \pm 4.80$  cm) (Fig. 1d). The siliques of *Bndwarf2* mutants were significantly shorter compared to that of ZS11 (Fig. 1e). In addition, the *Bndwarf2* mutants had smaller seeds, lower 1000-seeds weight and compact plant architecture (Fig. 1f, Table S2). The  $F_1$  plants ( $105.30 \pm 5.16$  cm) generated by cross of ZS11 with *Bndwarf2* were in-between that of ZS11 and *Bndwarf2*. The observed multiple morphological abnormal of *Bndwarf2* mutants showed similar characteristics in appearance to the other BR-related mutants, such as *bri1* (Clouse et al. 1996), *dwf12* (Choe et al. 2002), *bin2* (Li and Nam 2002), and *ucu1* (Perez-Perez et al. 2002).

### Map-based cloning

To investigate the genetic regulation mechanism for *Bndwarf2*, the  $F_1$  (ZS11  $\times$  *Bndwarf2*) and  $RF_1$  (*Bndwarf2*  $\times$  ZS11) plants were obtained by crossing *Bndwarf2* with ZS11, all had the dwarf trait, indicating that dwarf trait was controlled by dominant genes. The phenotypic segregation ratio of dwarf plants to tall plants in the  $F_2$  population was in a Mendelian model of 3:1 (209 dwarf plants vs. 78 tall plants,  $<_{0.05}$ ). Among 289  $BC_1$  individuals, 139 as dwarf types and 150 as tall types, also approximately fitted an expected Mendelian inheritance ratio of 1:1 (dwarf plants vs. tall plants). In subsequent segregating  $F_{2:3}$  populations, the genetic regulation was confirmed (Table S3). These results indicated

that the dwarf trait was controlled by a dominant nuclear gene, which was named as *BnDWARF2* in the subsequent study.

To map *BnDWARF2*, 94 plants (70 dwarf plants and 24 tall plants) from the F<sub>2</sub> population were used for single nucleotide polymorphism (SNP) marker genotyping. Although the chip (Illumina, Inc) has 52,157 SNP markers, only 7457 polymorphic markers were used to construct the SNP genetic linkage map after removing the invalid markers. The *BnDWARF2* locus was located on C04 chromosome between the SNP marker M33367 and M35244 (Fig. 2a). To fine map the *BnDWARF2* locus, 318 primer pairs of simple sequence repeat (SSR) markers were designed to uniformly cover the preliminary mapping interval. A further 889 individuals from the F<sub>2:3</sub> populations, finally narrowed down the *BnDWARF2* locus to a 34.62-kb region between SSR markers S3 and S4 (Fig. 2b). No other markers to further narrow the mapping interval were found for this mapping population and its parents. A total of 5 putative genes were localized in the 34.62-kb region according to the gene annotation of the *B. napus* reference genome (Fig. 2c). Sequence cloning was performed for the mapping interval, and the results showed that only *BnaC04g41660D* (*BnaC04.BIL1*) gene had 10 SNPs differences between ZS11 and *Bndwarf2*. The *BnaC04.BIL1* had two amino acid residues substitutions at aa-187 (Thr-to-Ser mutation, named Thr187Ser) and aa-399 (Gln-to-His mutation, named Gln399His) (Fig. 2e).

### Sequence analysis of *BnaC04.BIL1*

*BnaC04.BIL1* contains a 1233-bp open reading frame (ORF) with 11 introns and has 3 copy genes in *B. napus* (Fig. 2d, Fig. S2). *BnaC04.BIL1* is a homologous gene of the *Arabidopsis* AT2G30980 gene, which encodes a GSK3-like protein kinase (Charrier et al. 2002). The conservative domain analysis showed that the amino acid sequence 65-357 was the conserved domain of STKc\_GSK3, and Thr187Ser is in the conserved domain (Fig. 2e). The amino acid multiple sequence analysis showed that *BnaC04.BIL1* gene had a series of amino acid residues conserved in GSK3 kinase, such as GSK3 domain signature SYICSR and plant-specific TREE motif (Fig. S2). It was perfectly aligned with the genes for GSK3/Shaggy kinases with regarding to a series of amino acid residues such as the GSK3 signature SYICSR within domain VIII that was absent from MAP kinase sequences (Dornelas et al. 1999). The E-K mutation in the highly conserved TREE motif is thought to preventing the BR-mediated BIN2 inhibition (Peng and Li 2003), thus resulting in the increased BIN2 stability (Peng et al. 2008; Vert and Chory 2006). The phylogenetic tree clustering and construction were analyzed by MEGA 7.0 selection Neighbor-joining method. The results showed that *BnaC04.BIL1* gene and *Arabidopsis* *BIN2* gene were homologous, belonging to GSK3 II subfamily (Fig. S2). These indicated that *BnaC04.BIL1* protein might also be interacting with *BnaBZR1* to negatively regulate BR signaling transduction. Further bioinformatics analysis suggested that the protein interaction network of *BnaC04.BIL1* showed that the *BnaC04.BIL1* has binding domains with other proteins between aa-20 and aa-369 (Fig. S2), which was consistent with previous reports (Kim et al. 2009). The Gln399His mutation is not in the conserved domain, but in C-terminal. Accordingly, it suggested that the Thr187Ser mutation may be responsible for affecting the interaction with *BnaBZR1*.

### Expression patterns of *BnaC04.BIL1* and the subcellular localization

To explore the possible function of *BnaC04.BIL 1* gene from *Bndwarf2* mutant in different tissues, the transcription levels of *BnaC04.BIL 1* in leaves, roots, hypocotyl, stems, buds, flowers, siliques, and seeds were analyzed. The qRT-PCR analysis showed that the *BnaC04.BIL 1* gene was expressed in all tissues, which indicated that *BnaC04.BIL 1* expressed constitutively (Fig. 3a). The expression level of *BnaC04.BIL 1* was higher in leaves, hypocotyls, siliques, and seeds, while its level in buds and stems were lower.

Previous research showed that *Arabidopsis* BIN2 (Ryu et al. 2010) and rice BZR1 (Bai et al. 2007) were localized in the nucleus. To define the subcellular location of expression, pA7-GFP, *BnaC04.BIL1*-GFP, and *BnaBZR1*-GFP constructs were then introduced into the tobacco leaf cells by the particle bombardment method. The merged image of *BnaC04.BIL1*-GFP and nuclear localization signal (NLS)-mCherry signals showed that *BnaC04.BIL 1* was localized to the nucleus, with the merged image of *BnaBZR1*-GFP showing that *BnaBZR1* was localized in the nucleus and cell membrane (Fig. 3b). The result showed the *BnaC04.BIL 1* gene functions in the nucleus.

### **Overexpression of *BnaC04.BIL 1* leads to plant dwarf**

To investigate *BnaC04.BIL 1* functioning in plant height, a construct was generated by inserting a 1233 bp *BnaC04.BIL 1* ORF fragment from *Bndwarf2* into the vector pBI-121 under the control of the CaMV35S promoter. The construct was introduced into ZS11 plants by *Agrobacterium*-mediated transformation. The plant height trait was compared between the ZS11 and OE-*BnaC04.BIL 1* (OE-*BIL 1*) transgenic plants by overexpressing the *BnaC04.BIL 1* gene. Notably, plant height in the OE-*BIL 1* transgenic plants was similar to the expected *Bndwarf2* phenotype with obvious dwarf stature; meanwhile, the transgenic plants also displayed dramatically smaller seeds than the ZS11 plants (Figs. 4a, 4c, Table S4). At the seedling stage, the OE-*BIL 1* transgenic lines displayed darker green and wrinkled leaves compared to those of ZS11 (Fig. 4b). These results suggest that the *BnaC04.BIL 1* gene not only controls the plant height, but also regulates the seed size. It follows that, the yield of per OE-*BIL 1* transgenic plants showed a significantly reduction compared to that of ZS11 (Table S4). The T<sub>2</sub> progeny plants were examined from six T<sub>1</sub> transgenic lines in growth chamber, which showed the expected Mendelian inheritance ratio of 3:1 in T<sub>2</sub> progeny (dwarf vs. tall plants,  $\chi^2_{0.05, 1} = 3.84$ ;  $P > 0.05$ ; Table S5). The T<sub>2</sub> progeny plants displayed perfect co-segregation between the transgene and the dwarf phenotype. Consistently, the expressions of *BnaC04.BIL 1* gene in homozygous T<sub>3</sub> lines (OE-*BnaC04.BIL 1* transgenic genes) were significantly higher than those of ZS11 plants (Fig. 4c). These results confirmed that the *BnaC04.BIL 1* is the causal mutation for the dwarfism and controls smaller seeds, which were also observed in *Bndwarf2*.

### **Knockout of *BnaC04.BIL 1* restored plant height**

An *CRISPR-BnaC04.BIL 1* (CR-*BIL 1*) construct for *BnaC04.BIL 1* was transformed into *Bndwarf2* plants by using *Agrobacterium*-mediated transformation. According to a PCR examination using construct-specific primers (Table S6), some T<sub>1</sub>-positive transgenic lines were selected for verification by Sanger DNA sequencing of the target sites. The T<sub>1</sub>-positive plants displayed the expected tall phenotype within apical dominance at maturity (Fig. 5a), suggesting that the plant height of *Bndwarf2* were restored (Table S4).

The transgenic CR-*BIL1* plants showed dramatically larger seeds than the *Bndwarf2* mutants (Fig. 5b). These results further verified that the *BnaC04.BIL1* was responsible for the plant dwarf phenotype.

To obtain stable mutant lines, nine independent T<sub>1</sub> editing lines of *BnaC04.BIL1* were self-pollinated to produce T<sub>2</sub> and T<sub>3</sub> progeny. Genetic analysis of the T<sub>2</sub> progeny plants from four positive plants was also performed, showing the expected Mendelian inheritance ratio of 1:3 in T<sub>2</sub> progeny (dwarf vs. tall plants; Table S5). The targeted mutations of progeny from these T<sub>1</sub> lines were further verified with a direct sequence analysis of the PCR products of the target sites. Sequencing of the mutated region revealed that various mutations, including mutation and deletion of different nucleotides, were produced in proximity to target sites in all selected lines. A total of nine T<sub>3</sub> lines with homozygous mutations in *BnaC04.BIL1* were detected, including three S1 target (in ORF 5' terminal) mutants and six S2 target (in the conserved region) mutants (Fig. 5c). All of these homozygous mutations were detected at the target sites within *BnaC04.BIL1* as predicted, and caused frameshifts that resulted in non-functional proteins. These results suggested that the loss-of-function of *BnaC04.BIL1* leads to the restored plant height of *Bndwarf2*.

### **BnaC04.BIL1 interacts with BnaBZR1**

In order to investigate whether the mutation(s) in conserved domain of *BnaC04.BIL1* promotes or attenuates the interaction with *BnaBZR1* protein, we tested the effect of the Thr187Ser substitution on the interaction between *BnaC04.BIL1* and *BnaBZR1* using yeast two-hybrid (Y2H) assays. The Y2H assays showed that *BnaC04.BIL1*<sup>Mut</sup> could interact with *BnaBZR1*, but *BnaC04.BIL1*<sup>WT</sup> cannot interact with *BnaBZR1* (Fig. 6a). As expected, these results clearly suggested that the Thr187Ser amino acid substitution residing in the conserved region affected the interaction between *BnaC04.BIL1* with *BnaBZR1*.

For further verification, bimolecular fluorescence complementation (BiFC) analysis was used to determine whether *BnaC04.BIL1* interacts with *BnaBZR1* *in vivo*. In *Nicotiana benthamiana* leaf epidermal cells co-expressing the C-terminal half of yellow fluorescent protein (YFP) fused to *BnaC04.BIL1*<sup>Mut</sup> or *BnaC04.BIL1*<sup>WT</sup>, and the N-terminal half of YFP fused to *BnaBZR1*. We observed that the strong YFP fluorescence appeared in the cell membrane and nucleus when *BnaC04.BIL1*<sup>Mut</sup> interacts with *BnaBZR1* (Fig. 6b), whereas other combinations had none YFP fluorescence in cells. These results further confirmed that unlike the response of *BnaC04.BIL1*<sup>WT</sup>, *BnaC04.BIL1*<sup>Mut</sup> could interact with *BnaBZR1*.

### **Transcriptome analysis**

To further explore the possible mechanism underlying dwarf and determine whether other metabolic pathways were also influenced in the *Bndwarf2* mutant, differentially expressed genes (DEGs) between *Bndwarf2* plants and ZS11 plants were detected using RNA sequencing technology. A total of 365,670,572 clean reads from six samples (three duplications for *Bndwarf2* and ZS11) were obtained (Table S7). Ultimately, a total of 7,270 DEGs were isolated from the *Bndwarf2* and ZS11 plants.

Compared with the ZS11 lines, 3,625 genes were up-regulated and 3,645 genes were down-regulated in the *Bndwarf2* mutants (Table S8). GO analysis showed that the biological process category contained 21 terms, and molecular function category contained 12 terms and cellular component contained 12 (Table S9). In the biological process category, the top three terms were in the metabolic, cellular, and single-organism processes. In the molecular function category, the top three terms were binding, catalytic activity, and transporter activity. In the cellular component category, the top three terms were cell, cell part, and membrane (Fig. S3). These results indicated that *Bndwarf2* mutation seriously affected the plants cell elongation and development.

Furthermore, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis showed that a total of 129 pathways were affected in the *Bndwarf2*, and particularly, the biosynthesis and signal transduction of BR seemed to be destroyed in dwarf plants (Table S10). The related DEGs included 15 genes related to the BR biosynthesis pathway (Table S11) and 6 genes related to the BR signal transduction (Table S12), all of which exhibited up-regulated expression in *Bndwarf2* plants, compared to ZS11. For example, three *BnaBZR1* genes were increased (Table S12), which can affect plant growth and development in various aspects through the regulating downstream genes expression.

## Discussion

Plant type improvement is a major goal for crop breeding, where plant height is an important growth habit that is a fundamental yield determining trait in crops. In the 1960s and 1970s, the dwarf trait genes (*Rht1* and *sd1*) were introduced into wheat and rice that were crucial to the first “Green Revolution” (Hedden 2003; Khush 2001). The semi-dwarf genes result in a shortened culm with improved lodging resistance and a greater harvest index (Islam and Evans 1994). However, there are few studies with respect to dwarf oilseed rape. Because of the lower mechanization level of oilseed rape production and few varieties suitable for mechanization harvest, oilseed rape production faces severe challenge.

Most of our knowledge about BIN2 functions came mostly from gain-of-function results. For example, genetic screening in *Arabidopsis* for BR-insensitive dwarf mutants resulted in the isolation of eight gain-of-function *bin2* alleles (Choe et al. 2002; Li and Nam 2002; Perez-Perez et al. 2002). However, no single gain-of-function mutation for *BIL1* or *BIL2*, two closest homologs of *BIN2* encoding group II AtSKs, has been isolated. Although previous report found that transgenic *Arabidopsis* plants overexpressing *BIL1* or *BIL2* gene confer the dwarf phenotype (Yan et al. 2009), it remains unknown whether *BIL1* or *BIL2* might also participate in BR signaling, similar to the function of *BIN2* (Choe et al. 2002; Perez-Perez et al. 2002). In our study, a gain-of-function mutation for *BIL1* in oilseed rape has been discovered, and most importantly, it exhibits the BR-insensitive dwarf phenotype. For example, the *Bndwarf2* mutant displayed the BR signaling phenotypes: shorter hypocotyls, shorter petioles, wrinkled leaves, smaller seeds, and obvious dwarf compared with the ZS11 (Fig. 1, Fig. S1, Tables S1, S2). These characteristics were similar to the phenotypes of BR-insensitive mutants such as *bri1* (Clouse et al. 1996), *dwf12* (Choe et al. 2002), and *ucu1* (Perez-Perez et al. 2002). Through map-based cloning, the BnaC04.BIL1 was identified to be a *BIN2-Like1* (*BIL1*), showing a Thr187Ser amino acid substitution residing in the conserved region (Fig. 2,

Fig. S2). Genetic transformation experiments confirmed that the BnaC04.BIL1 was responsible for the plant dwarf phenotype in the *Bndwarf2* mutants. Overexpression of *BnaC04.BIL1* under the background of ZS11 reduced plant height compared with ZS11 (Fig. 4, Table S4). This result was consistent with previous reports, showing that overexpressing *BIL1* gene confers the dwarf phenotype in *Arabidopsis* (Yan et al. 2009). Meanwhile, a CRISPR/Cas9 vector knockdown of *BnaC04.BIL1* showed that the plant height of *Bndwarf2* was restored (Fig. 5, Table S4). The genetic evidence clarifies the *BnaC04.BIL1* can sharply change plant architecture in natural plant accessions in allotetraploid.

Furthermore, our study provides evidence that BnaC04.BIL1 can interact with BnaBZR1, thus negatively regulating BR signaling in allotetraploid *Brassica napus*, similar to the function of BIN2 on BR signaling in *Arabidopsis* (Choe et al. 2002; Li and Nam 2002). First, the protein interaction network of BnaC04.BIL1 showed that the BnaC04.BIL1 has binding domains with other proteins between aa-20 and aa-369 (Fig. S2), which was consistent with previous reports, showing the BSU1 interacts with BIN2 on Tyr200 in *Arabidopsis* (Kim et al. 2009; Kim et al. 2017). Second, the subcellular localization analysis demonstrated that BnaC04.BIL1 exists in the nucleus (Fig. 3). Consistently, the *Arabidopsis* BIN2 functioned in nucleus to negatively regulate BR signaling (Ryu et al. 2010). Third, the biosynthesis and signal transduction of BR were destroyed in dwarf plants (Table S10, Table S11, Table S12). And, since the *BnaBZR1* genes were increased in *Bndwarf2* mutants (Table S12), we further speculated that these genes can affect plant growth and development in various aspects through the regulating downstream genes expression (Oh et al. 2012; Wang et al. 2012; Nolan et al. 2018). In fact, previous results revealed that many genes regulated by BZR1 and/or BES1, and some proteins interacting with BZR1/BES1, were closely associated with the BR signaling (Guo et al. 2013; Li 2010). Fourth, Y2H and BiFC assays confirmed that the Thr187Ser amino acid substitution residing in the conserved region affected the interaction between BnaC04.BIL1 with BnaBZR1 (Fig. 6), thus negatively regulating the BR signal transduction (Li and Nam 2002; Perez-Perez et al. 2002). The genetic and molecular evidence clarifies first the *BnaC04.BIL1* can sharply change plant architecture in natural plant accessions in allotetraploid, and provides new insights into the molecular mechanisms of BR signaling.

Further study has identified *Bndwarf2*, a dwarf and compact mutant in *B. napus*, and the dwarf trait is controlled by a semi-dominant nuclear gene (Table S3). The plant height of F<sub>1</sub> derived from the cross of *Bndwarf2* with the tall parent, decreased by about 50% compared to that of tall plant (Table S2). Particularly, the *Bndwarf2* displayed an extreme reduction in height at maturity ( $33.62 \pm 1.12$  cm), which is different from the previously reported dwarf mutants in *B. napus* (Foisset et al. 1995; Wang et al. 2016a; Wang et al. 2016b; Zeng et al. 2011; Zhao et al. 2017). For example, the dwarf mutant *BnC.dwf* only had 95 cm, and the dwarf locus was controlled by a recessive gene (Zeng et al. 2011). And, the dwarf trait of *Bndwf1* mutant (80 cm) was controlled by a dominant gene (Wang et al. 2016a). The F<sub>1</sub> plants have compact properties such as shortened branch, shortened gap between siliques, shortened gap between branches and dwarfing plant height by *BnDWARF2* gene (Fig. 1, Fig. S1, Table S2). This finding implicates that the plant architecture of homozygous or heterozygous individuals derived *Bndwarf2* mutant is compact (Fig. 1). This kind of compact architecture can be undoubtedly helpful to

increase planting density, enhance lodging resistance and increase planting density, therefore the compact plant architecture is ideal for machinery production of oilseed rape.

The germplasm *Bndwarf2* that is sparse lacks strong vigorous growth habit. However, the compact plant architecture can be used in hybrid cultivar development in which the compact type and hybrid vigor can be combined well. This is helpful to breeding of variety breeding with the objectives such as high-yield, good quality and suitable for machinery. On the other hand, the growth vigor in pure line or cultivar may be improved in some genetic background. Some reports have demonstrated that the genes in BIN2 regulation network can also interact with BIN2, leading to improvement of the growth inhibition caused by BIN2 gene overexpression caused by natural biological accession state or by transgenics (He et al. 2019; He et al. 2020; Ling et al. 2017; Sun et al. 2018). We speculate that some gene may interact with *BnaC04.BIL1* to attenuate its role in limit growth vigor as that the *Arabidopsis* homolog BIN2 crosstalk experiments have shown. Furthermore, expressions of some regulator genes may probably alter the expression level of *BnaC04.BIL1* that is constitutively expressed in the various organs, and reduced expression level may improve the growth vigor.

## Declarations

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### Author contribution statement

MY and RG designed the research. MY, SW, WL, WC, YW, XJ, PC, and PC performed the experiments. JH analyzed the SNP and sequencing data. RG advised on the experiments. MY wrote the manuscript. WS and RG revised the manuscript. All authors read and approved of the final manuscript.

### Conflict of interest

All authors declare that they have no conflict of interest.

### Consent to participate

Not applicable

### Consent for publication

All authors contributed to the study and approved the final version for submission.

### Ethics approval

We declare that these experiments complied with the ethical standards in China.

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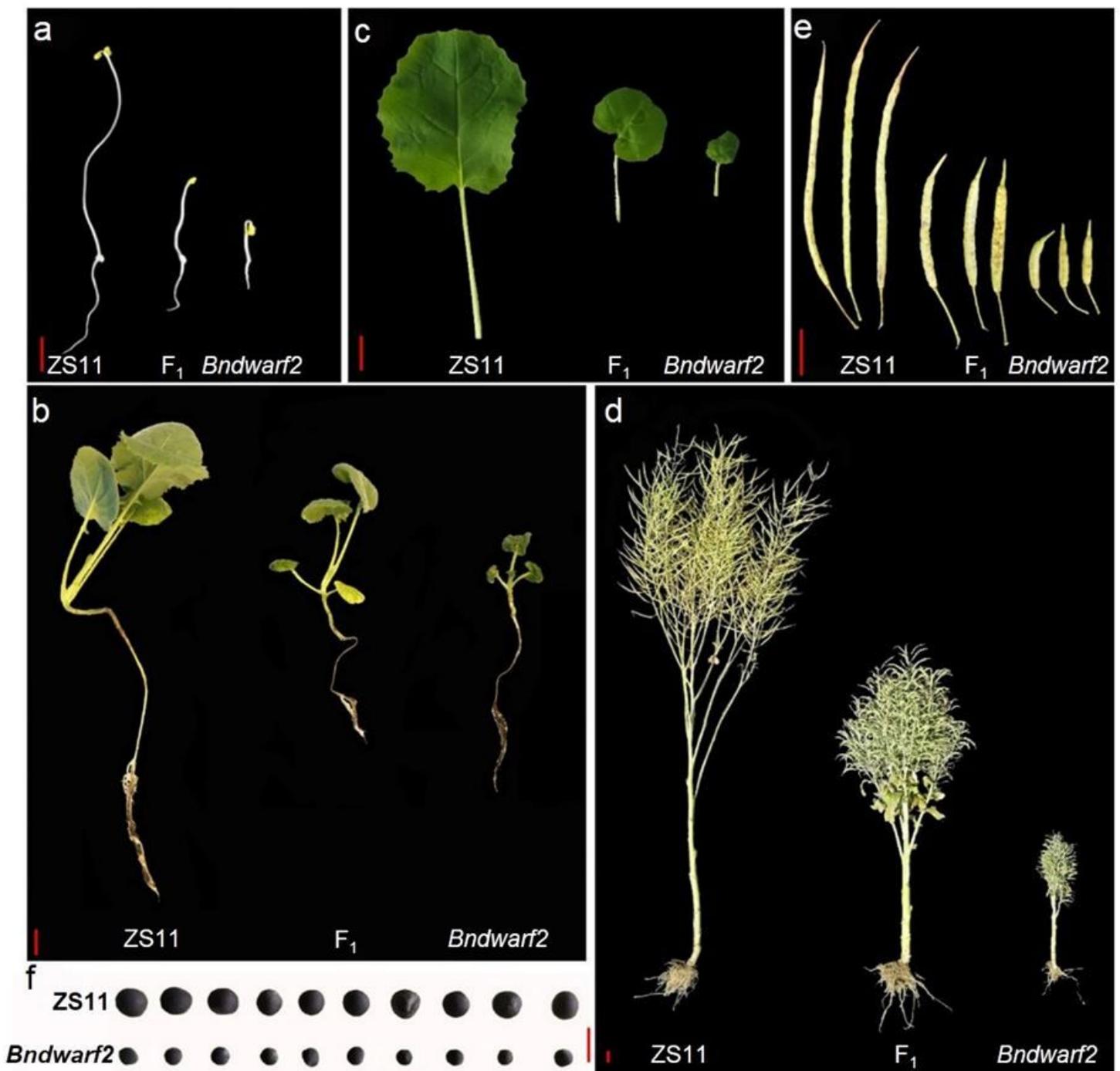
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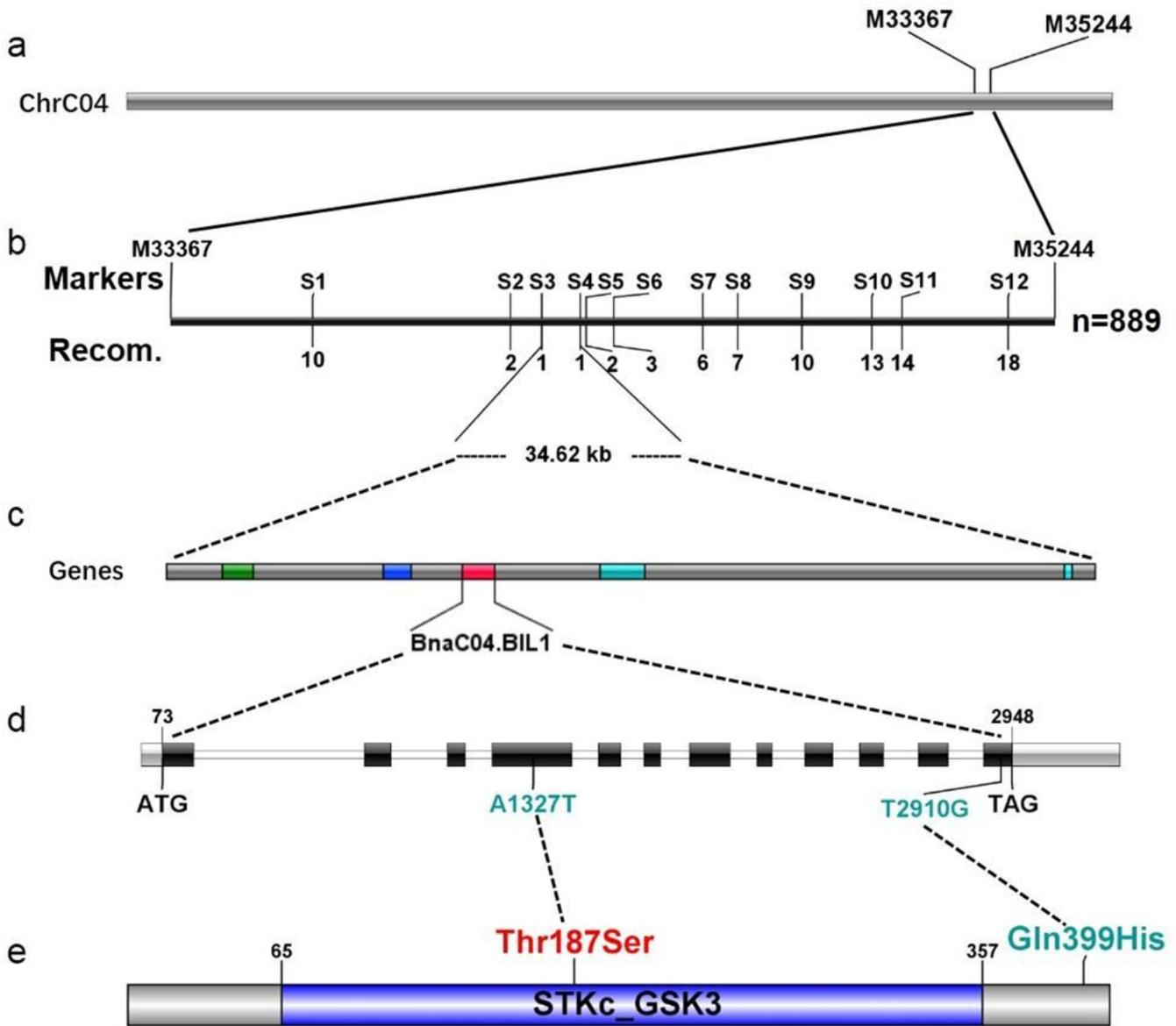
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## Figures



**Figure 1**

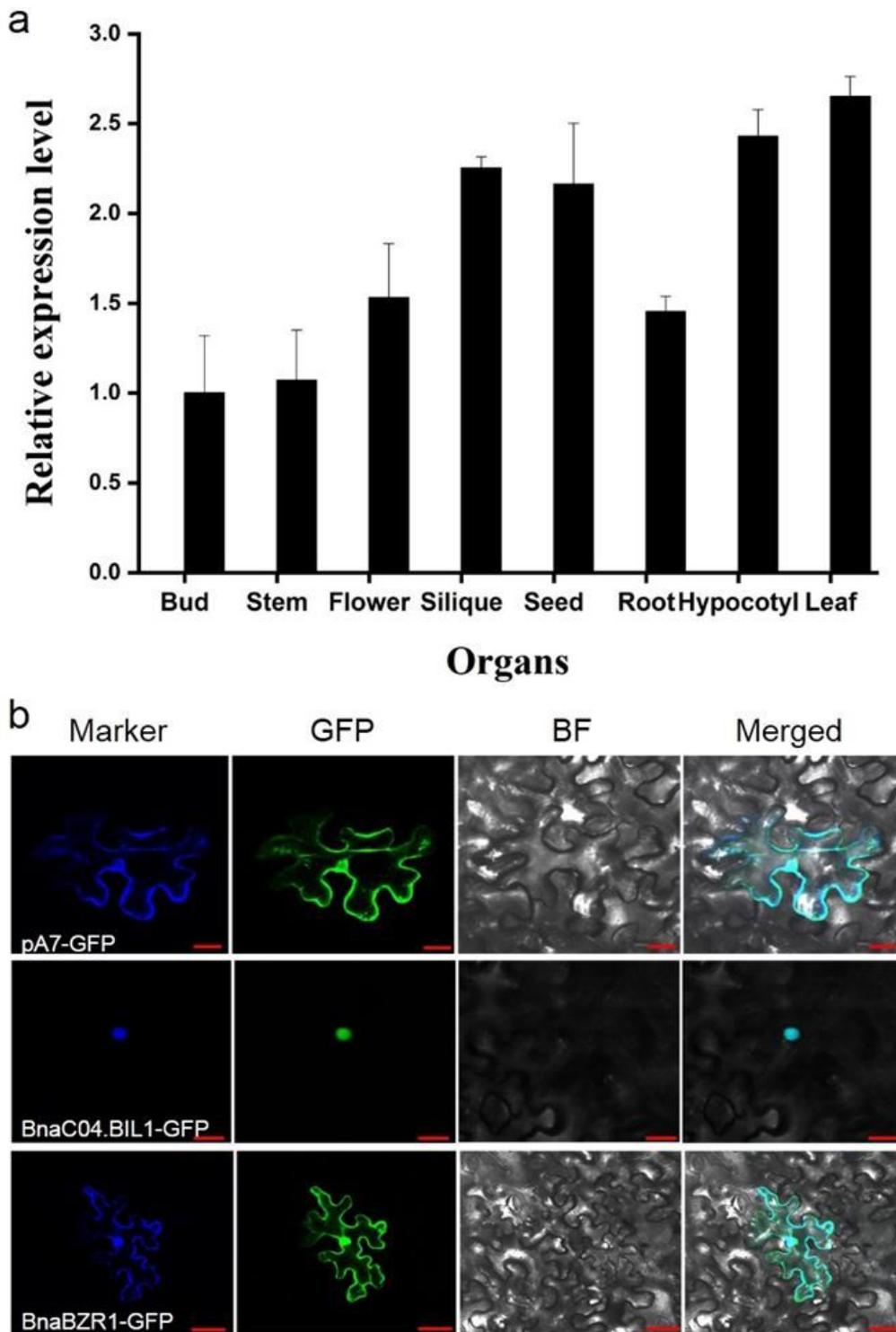
Phenotypic comparison among ZS11, F1, and Bndwarf2 mutant. a The root length and hypocotyl length of ZS11 (left), F1 (middle), and Bndwarf2 (right) in the dark for 6 d. b The performance of ZS11 (left), F1 (middle), and Bndwarf2 (right) at seedling stage. c The petioles comparison at seedling stage. d The plant height comparison at maturity stage. e The siliques comparison of at maturity stage. f The seeds comparison of ZS11 (up) and Bndwarf2 mutant (down). Bars = 2 cm



**Figure 2**

Map-based cloning of BnDWARF2. a The BnDWARF2 locus was mapped primarily on C04 chromosome between the SNP markers M33367 and M35244. b The BnDWARF2 locus was fine-mapped in the 34.62 kb region between SSR markers S3 and S4. The numerals indicate the number of recombinants. c The genes in the mapping interval. d The gene structure and the mutation sites in BnaC04.BIL1. e The protein

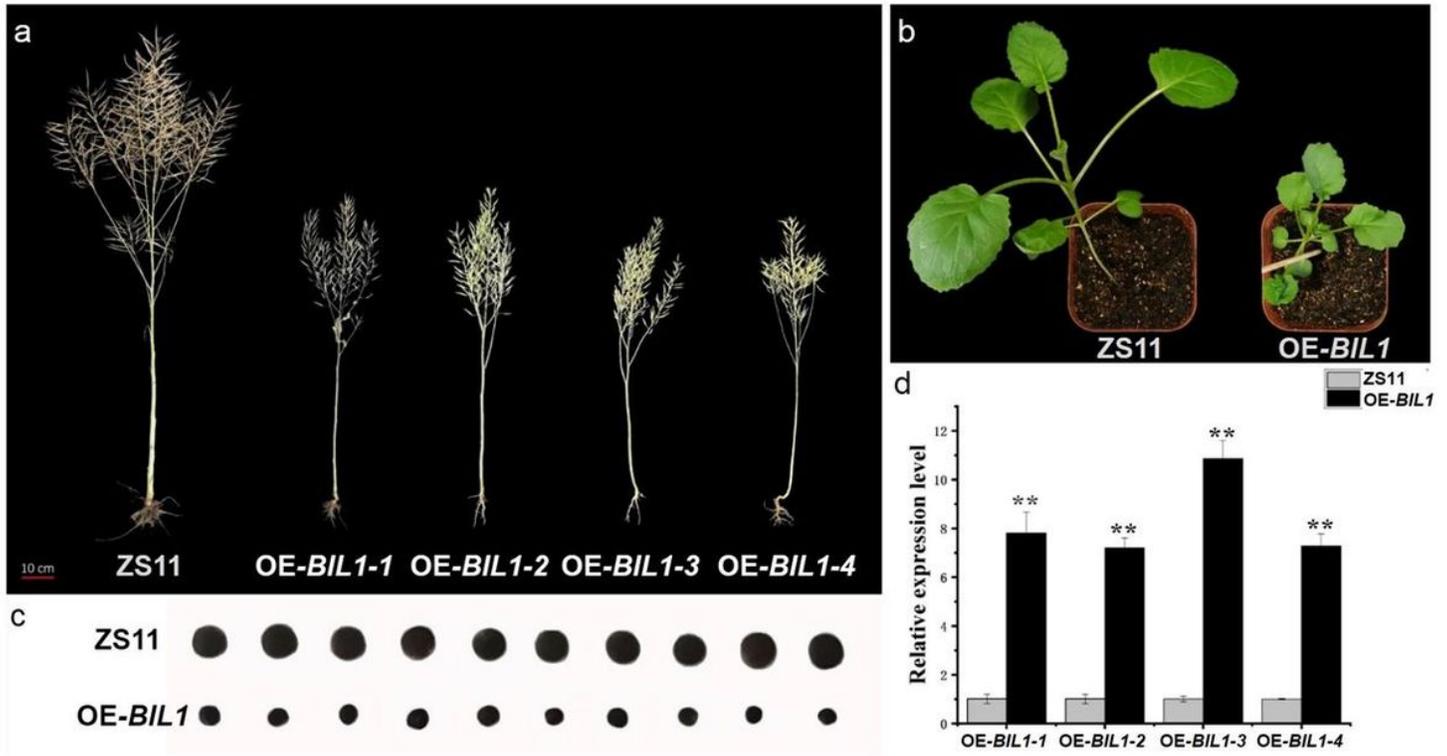
structure and the mutation sites of the BnaC04.BIL1 protein, and the STKc\_GSK3 superfamily domain was predicted. Solid lines show the position of the amino acid transition



**Figure 3**

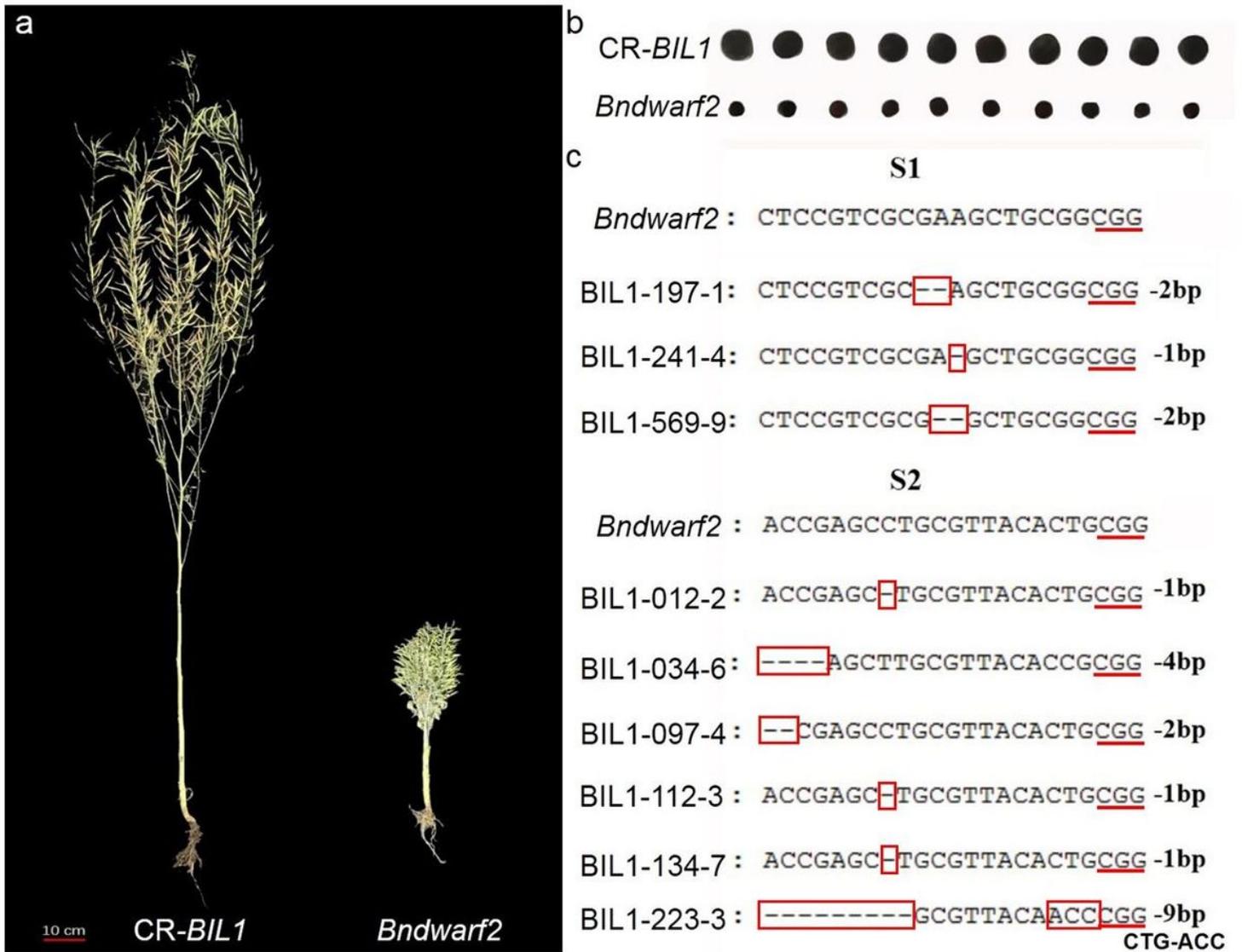
Expression pattern of BnaC04.BIL1 and subcellular localization of its encoding protein. a Expression pattern of BnaC04.BIL1 detected by qRT-PCR in bud, stem, flower, silique, seed, root, hypocotyl, and leaf from Bndwarf2. The BnActin gene was used as a reference gene and the expression level of bud was set

to 1. The bud, stem, and flower samples are from flowering stage. The silique samples are from podding stage. The seed samples are from maturity stage. The root and hypocotyl samples are from 7-day-old seedlings grown on medium, and the leaf samples are from seedling stage. b Subcellular localization of BnaC04.BIL1 and BnaBZR1 proteins in tobacco leaf cells. Plasmids pA7-GFP, BnaC04.BIL1-GFP, and BnaBZR1-GFP were introduced into tobacco leaf cells by particle bombardment, respectively. Bars = 20  $\mu$ m



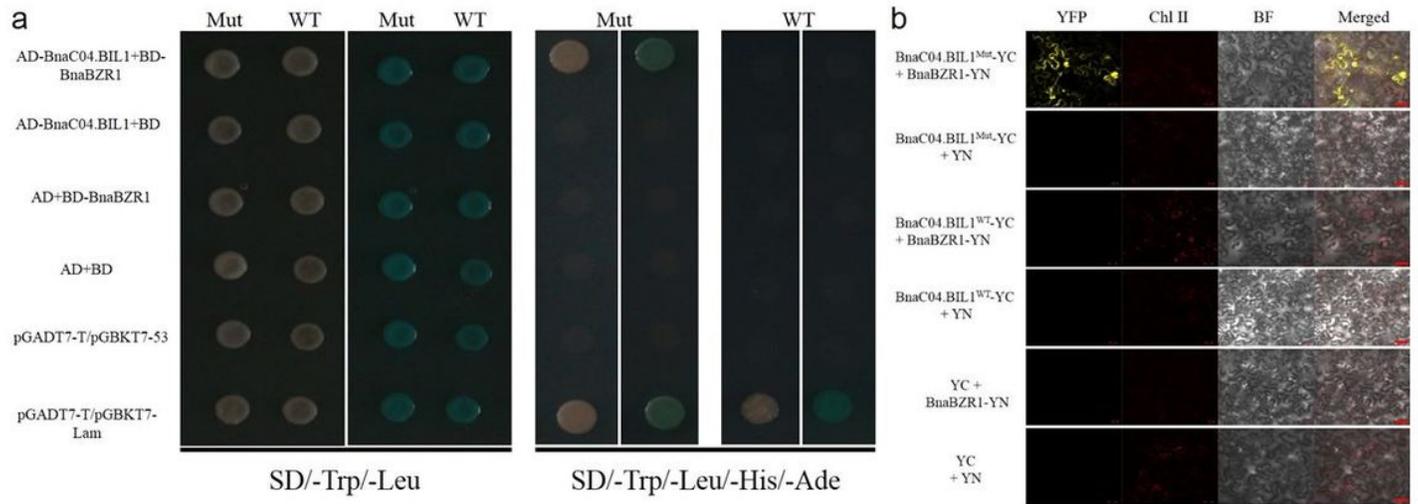
**Figure 4**

Phenotype comparison and qRT-PCR analysis between the ZS11 and OE-BIL1 transgenic plants. a The phenotype of ZS11 (left) and OE-BIL1 (right) transgenic plants at maturity stage. b The phenotype of ZS11 (left) and OE-BIL1 (right) transgenic plants at seedling stage. c The seeds of ZS11 (up) and OE-BIL1 (down) plants. d qRT-PCR analysis of BnaC04.BIL in ZS11 and OE-BIL1 transgenic lines



**Figure 5**

Phenotypic and molecular comparisons between *Bndwarf2* and CR-BIL1 transgenic plants. a The phenotype of CR-BIL1 (left) transgenic plants and *Bndwarf2* mutant (right) at maturity stage. b The seeds of CR-BIL1 (up) plants and *Bndwarf2* mutant (down). c The mutation of CR-BIL1 transgenic plants in T3 generation. The PAM is underlined, and nucleotide mutations and deletions are marked with red, with details labeled at right. S1 indicates the sgRNA primer in ORF 5' terminal, and S2 indicates the sgRNA primer in the conserved region



**Figure 6**

BnaC04.BIL1 interacts with BnaBZR1. a Y2H analysis. b BiFC analysis. The YFP fluorescence signals and autofluorescence signals from chloroplasts are pseudo-colored as yellow and red, respectively. YN indicates YFP-N terminal; YC indicates YFP-C terminal. Bars = 20  $\mu$ m

## Supplementary Files

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