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MOLECULAR IDENTIFICATION AND FUNCTIONAL ANALYSIS OF *BrbRI1* AS BRASSINOSTEROID RECEPTOR GENE IN *BRASSICA RAPA*

SHUHUA HUANG[1a], HAO WANG[1a], SUFU GAN[1a], MAHMOUD ABDALLA MAHMOUD HUSSEIN[1], QIANNAN WANG[1], XIN WANG[1], YANFENG ZHANG[1,2] AND XIAOFENG WANG[1*]

[1]College of Horticulture, Northwest A&F University, No.3, Taicheng Road, Yangling, Shaanxi 712100, China
[2]Hybrid Rapeseed Research Center of Shaanxi Province, Yangling, China
[a]Authors contributed equally to this work
Corresponding author's e-mail: wangxff99@nwsuaf.edu.cn; Tel.: +86-18092867224

Abstract

Brassinosteroids (BRs) are crucial phytohormones that have diverse functions in regulating plant growth and development. Brassinosteroid-insensitive1 (BRI1), an important plasma membrane-located leucine-rich repeat receptor kinase (LRR-RK), perceives BRs to initiate BR signal transduction, and is involved in altering plant architecture and increasing crop production. Here, three BRI1 proteins (BrBRI1-1, BrBRI1-2, and BrBRI1-3) from *Brassica rapa* exhibiting striking sequence similarity to *Arabidopsis* BRI1 (AtBRI1) were identified and characterized. Analysis of full-length sequences of *B. rapa* BRI1 (BrBRI1) proteins indicated that they contain an extracellular LRR domain, transmembrane domain, a juxtamembrane domain, and a cytoplasmic kinase domain similar to AtBRI1. The ectopic expression of these genes in the *Arabidopsis bri1-5*-weak mutant resulted in the full recovery of the *bri1* mutant to the wild-type. Furthermore, typical BR responsiveness including hypocotyl elongation, root growth, *CPD* expression, and BES1 dephosphorylation to exogenous BR treatment demonstrated a restored BR signaling pathway in transgenic lines. These data confirmed that BrBRI1 proteins function as BR receptors to mediate BR signaling. Our results provide a foundation for *B. rapa* molecular genetic breeding by regulating the BR signal transduction pathway through BrBRI1 proteins.

Key words: Brassinosteroids, *Brassica rapa*, *Br*BRI1 genes, Molecular breeding.

Introduction

Brassinosteroids (BRs) constitute important phytohormones that positively influence plant growth and development, such as the division and differentiation of cells, skotomorphogenesis, flowering, and senescence (Clouse, 2011). Mutants impaired in synthesizing, perceiving, or signal transduction of BRs consequently display dramatic growth defects, such as extreme dwarfism, reduced fertility, and abnormal morphology (Clouse, 1996; Szekeres *et al.*, 1996; Li & Chory, 1997; Noguchi *et al.*, 1999b; Ryu & Hwang, 2013).

BRs are recognized by BRI1 at the cell surface, which contains an extracellular leucine-rich repeat (LRR) domain separated by a unique 70 amino acid "island" between the 21st and 22nd LRR, a single transmembrane domain, a juxtamembrane domain, and a cytoplasmic kinase domain (Li&Chory, 1997; Friedrichsen *et al.*, 2000; Geldner *et al.*, 2007). Genetic and biochemical evidence has proved that BRI1 is indeed the receptor of BRs. Firstly, *bri1* null allele mutants are insensitive to exogenous application of BR and exhibit similar phenotypes to BR biosynthetic mutants (Clouse, 1996; Szekeres *et al.*, 1996; Choe *et al.*, 1998). Secondly, immunoprecipitated BRI1-Green Fluorescent Protein (GFP) fusion proteins can bind specifically to tritium-labeled brassinolide (BL), while mutations within the island region are greatly reduced by the activity of BRI1-binding BRs (Wang *et al.*, 2001). Thirdly, binding studies using biotin-tagged photoaffinity castasterone, ^{3}H-labeled BL (the most active BRs), and purified BRI1 protein fragments expressed *In vitro* showed that BRs boun directly to the island domain and adjacent flanking LRR22 (Kinoshita *et al.*, 2005). BRs recognized by the island region of BRI1 induce the release of a negative regulatory protein, BRI1 KINASE INHIBITOR 1 (BKI1), which interacts with the BRI1 kinase and suppresses its kinase activity (Wang & Chory, 2006; Jaillais *et al.*, 2011; Wang *et al.*, 2014a). Activated BRI1 then recruits and directly interacts with its co-receptor BRI1-Associated Receptor Kinase 1 (BAK1), which was independently discovered in a screen of the suppressor of a *bri1-5* mutant using the method of activation tagging and a screen of BRI1 interacting proteins using a yeast two-hybrid system (Li *et al.*, 2002; Nam & Li, 2002). Multiple phosphorylation sites of BRI1 and BAK1 have been identified via liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and a sequential transphosphorylation model between the BRI1/BAK1 complex was proposed (Wang *et al.*, 2005; Wang *et al.*, 2008). BAK1, also named SOMATIC Embryogenesis-Related Kinase 3 (SERK3) for its role in regulating somatic development, has four homologous genes in plants, and their functions in mediating BR signaling overlap (Karlova *et al.*, 2006; Geldner *et al.*, 2007; Gou *et al.*, 2012). Protein Phosphatase 2A (PP2A), a protein phosphatase, could be activated by the SUPPRESSOR OF *bri1* (SBI1) in methylation (Wu *et al.*, 2011). Recent research suggests that methylated PP2A could directly interact with BRI1, dephosphorylation, and the subsequent degradation of BRI1 and weakening of BR signaling (Wu *et al.*, 2011; Wang *et al.*, 2016b). In addition to BRI1, BRI1-LIKE Receptor Kinase 1 (BRL1), and BRI1-LIKE Receptor Kinase 3 (BRL3), close BR-binding relatives also constitute functional BR receptors that are able to recognize BRs to mediate BR signaling and have special roles in vascular organogenesis (Cano-Delgado *et al.*, 2004) and play organ-specific roles in the roots (Nakamura *et al.*, 2006).

Over the last two decades, genetic, proteomic, and biochemical approaches have clearly elucidated the brassinosteroid signal transduction from early stage BR identification in the plasma membrane to late BR response gene expression in the nucleus (Kim & Wang, 2010; Li, 2010; Clouse, 2011). To date, detailed BR signal transduction cascades have been established, which include BR binding BRI1 on the plasma membrane (Wang *et al.*, 2001; Kinoshita *et al.*, 2005), dissociation of the negative regulatory protein BKI1 (Wang & Chory, 2006; Jaillais *et al.*, 2011), transphosphorylation activation of the BRI1/BAK1 complex (Wang *et al.*, 2008), subsequent phosphorylation of two receptor-like cytoplasmic kinases Constitutive Differential Growth 1 (CDG1) and Brassinosteroid-Signaling Kinase 1 (BSK1) (Tang *et al.*, 2008; Kim *et al.*, 2011), activation of the BRI1 Suppressor 1 (BSU1) phosphatase (Mora-Garcia *et al.*, 2004; Kim *et al.*, 2009), deactivation of the Brassinosteroid-Insensitive 2 (BIN2) kinase (Li & Nam, 2002; Kim *et al.*, 2009), and accumulation of two dephosphorylated transcription factors Brassinazole-Resistant 1 (BZR1) and *BRI1*-EMS-Suppressor 1 (BES1) from the cytosol to the nucleus to regulate gene expression (He *et al.*, 2002; Wang *et al.*, 2002; Yin *et al.*, 2002; Jiang *et al.*, 2015; Yang *et al.*, 2017).

BRI1 homologs from several other crop species including tomato, rice, pea, soybean, and barley have been identified (Koka *et al.*, 2000; Yamamuro *et al.*, 2000; Montoya *et al.*, 2002; Chono *et al.*, 2003; Nomura *et al.*, 2003; Holton *et al.*, 2007; Wang *et al.*, 2014b; Peng *et al.*, 2016). *OsBRI1* have key roles in determining plant morphology. The *Osbri1* null mutant, *d61*, is stunted with a dwarfed stature, twisted leaves, and poor root system growth (Yamamuro *et al.*, 2000). *HvBRI1*, the homologue gene of BRI1 in barley, was cloned from a semi-dwarf mutant *uzu* with a single amino acid (His-857-Arg) substitution, which is responsible for a height reduction of 80% to 90% of its normal counterpart, but without any loss in fertility (Chono *et al.*, 2003). The tomato BR insensitive *curl3* (*cu3*) mutant exhibits extreme dwarfism, altered leaf morphology, and reduced fertility due to a nonsense mutation in the kinase domain, as observed in the *Arabidopsis* mutant *bri1* (Koka *et al.*, 2000; Holton *et al.*, 2007), while the genetic complementation of *cu3* with *SlBRI1* could restore the dwarf phenotype (Holton *et al.*, 2007). The ectopic over-expression of *GmBRI1* in *bri1-5* could complement the phenotype and restore the BR signaling pathway (Wang *et al.*, 2014b; Peng *et al.*, 2016). In combination, functional BRI1 receptors are not only indispensable for normal plant growth and developmental processes, but also possess enormous potential for crop genetic modification to increase crop yields and enhance crop resistance under biotic or abiotic stress.

The phosphorylation sites of AtBRI1 in *Arabidopsis* were detected and tremendous functional differences among multiple individual phosphorylation sites with respect to biological and biochemical characteristics were observed (Wang *et al.*, 2005; Wang *et al.*, 2008). Recently, the roles of phosphorylation sites of AtBRI1 in plant growth and development have been systematically characterized (Wang *et al.*, 2016a). For example, S1172A and S1187A mutants greatly promoted leaf growth, while T1039A, S1042A, and S1044A mutants dramatically decreased plant height. However, dephosphorylation at S1042 could significantly increase seed yield potential to approximately 30% per unit area (Wang *et al.*, 2016a). Regulating the phosphorylation states of specific BRI1 phosphorylation sites may be a practicable way to precisely and effectively control quantity and quality traits in crops.

Brassica rapa is an agronomically important species consisting of various widely cultivated subspecies with many vegetable, oilseed, and fodder types. Even though sufficient evidence has revealed the function and characterization of BR synthesis and signal transduction pathways in *Arabidopsis*, the effects of components of the BR signaling pathway on the growth and development of *B. rapa* are largely unknown even the basic knowledge on BRI1.

To improve traits relating to quantity and quality in *B. rapa* through regulating the phosphorylation states of specific BRI1 phosphorylation sites, three *BrBRI1* genes (*BrBRI1-1*, *BrBRI1-2*, and *BrBRI1-3*) obtained from Chinese cabbage (*B. rapa cv.* Qingjiang) were characterized in this study. The characterization of the *Br*BRI1 proteins could provide the theoretical foundation for the molecular genetic breeding of *B. rapa* by regulating the BR signal transduction pathway, and also provides a basis for later regulating the phosphorylation states of specific *Br*BRI1 phosphorylation sites to precisely improve agronomic characters in *B. rapa*.

Materials and Methods

Plant materials and growth conditions: *Arabidopsis thaliana* plants (WS2, *bri1-5*, and transgenic lines) used for petiole measurement and photographs were grown in glasshouse at 22°C with a 16-h-light/8-h dark photoperiod. Fifteen-day-old seedlings were grown on ½ Murashige and Skoog (MS) medium containing 1% sucrose and 0.8% agar at 22°C with a 16-h-light/8-h dark photoperiod for RNA isolation and BES1 probing.

Plasmid construction and transformation: The *BrBRI1-1*, *BrBRI1-2*, and *BrBRI1-3* genes were amplified from Chinese cabbage (*B. rapa* cv. Qingjiang) cDNA (primers for BrBRI1-1: BrBRI1-1F, 5'- CGGGGTACC ATGAACACTTTTCTAAGCGTCTT-3' and BrBRI1-1R, 5'-CTAGTCTAGAGAATTTTCCTTCGGGAACTTCTT-3'. For BrBRI1-2: BrBRI1-2F, 5'-CGGGGTACCATGAA GACTTTTCCAAGCTTC-3' and BrBRI1-2R, 5'-CTAGTCTAGAGAATTTTCCTTCGGGAACTTCTT-3'. For BrBRI1-3: BrBRI1-3F, 5'-CTAGTCTAGAATGAG AATGAAAACTTTCCCAAC-3' and BrBRI1-3R, 5'-AC GCGTCGACGAATTTTCCTTCGGGAACTTCTT-3'). These were subcloned into T-Vector pMD20 (Takara, Dalian, China). The fragments of *BrBRI1* genes verified by sequencing were cleaved from the T-Vector pMD20 and ligated into the p35S-Hyg binary vector using underlined enzyme cut sites (Takara, Dalian, China) to generate C-terminal GFP fusion. The constructs were introduced into *Agrobacterium tumefaciens* GV3101 component cells using electroporation. All constructs were infiltrated into the weak *bri1-5* mutants using the floral dipping method (Clough & Bent, 1998).

Subcellular localization: To determine the subcellular localization of BrBRI1-1, BrBRI1-2, and BrBRI1-3, *A.*

tumefaciens GV3101 containing the respective construct was cultured and infiltrated into the leaves of four-week-old *Nicotiana benthamiana* using a 1 mL injection syringe (Sparkes *et al.*, 2006). After 3 d infiltration, GFP fluorescence was detected with a fluorescence microscope (BX51, Olympus, Tokyo, Japan) under blue light.

Western blot analysis of plant proteins: To detect the expression levels of BrBRI1-GFP in independent transgenic plants, protein was extracted as previously described (Wang *et al.*, 2008). Protein concentration was determined and 6 μg protein was separated on 10% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a Polyvinylidene Fluoride (PVDF) membrane (Roche, Mannheim, Germany). The membrane was incubated overnight with primary mouse anti-GFP antibodies (1:2000) (Transgene, Beijing, China) coupled to a secondary anti-mouse horseradish peroxidase (1:20000) (Transgene, Beijing, China). Signals were visualized using a chemiluminescent substrate (Millipore, MA, USA).

BES1 phosphorylation*:* Seedlings were treated with 24-epiBL (Sigma-Aldrich, Saint Louis, USA) for 1.5 h before total proteins were extracted as described in (Gou *et al.*, 2012). Phosphorylation states of BES1 were analyzed by electrophoretic mobility on 15% SDS-PAGE, followed by detection with an anti-BES1 antibody (kindly provided by Yanhai Yin) and a secondary anti-rabbit horseradish peroxidase (1:5000) (Transgene, Beijing, China). 30-μg protein was used to perform the immunoblotting experiment.

Semi-quantitative RT-PCR: Total RNA was extracted from 15-d-old seedlings treated with either 1 μM 24-epiBL **(**Sigma-Aldrich**,** Saint Louis, MO, USA**)** or nothing for 1.5 h using RNAiso reagent (Takara, Dalian, China). 2 μg of total RNA predigested with DNase I (Thermo Scientific, Waltham, MA, USA) was used to synthesize the first-strand cDNA (Roche, Mannheim, Germany). The cDNA was then used for PCR amplification to detect the expression level of *Constitutive Photomorphogenesis and dwarfism* (*CPD*) with *ACTIN 2* (*ACT2*) as a reference gene. Gene primers used for *CPD* were 5'-GTTCTTATCCTGCTTCCATTTG-3' and 5'-CGAATCACTCTTCATTGCCC-3', and for *ACT2* were 5'-AACTCTCCCGCTATGTATGTCG-3' and 5'-GAGGT AATCAGTAAGGTCACGTCC-3'.

Root growth inhibition and hypocotyl elongation assay: To determine the effects of BR on root growth and hypocotyl elongation, seeds were germinated and grown on ½ MS plates containing 1% sucrose and 0.8% agar with different concentrations of 24-epiBL (Sigma-Aldrich, Saint Louis, USA). The plates were then placed vertically in the dark for 7 d. After photographing and hypocotyl length measurements, plates were then placed under the light until day 10 to record the root length.

Sequences analysis: Three BrBRI1 proteins and BrBRL1, BrBRL2, and BrBRL3 were retrieved from the *Brassica* database (http://brassicadb.org/brad). The alignment of multiple protein sequences was conducted using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/), and a phylogenetic tree was constructed using the neighbor-joining method in MEGA5.05 (http://www.megasoftware.net). Typical protein domains were analyzed using the following online databases: signal peptide (http://www.cbs.dtu.dk/services/SignalP/); LRR domain (http://lrrsearch.com/index.php?page=tool); and transmembrane and cytoplasmic region (http://www.cbs.dtu.dk/ services/TMHMM-2.0/). Conserved NGSM motifs in BrBRI1-1, BrBRI1-2, BrBRI1-3, BrBRL1, BrBRL2, and BrBRL3 proteins were detected using MEGA5.05.

Results

Sequence analysis and protein structure prediction of *BrBRI1* genes: A basic local alignment search tool (BLAST) search against the complete *B. rapa* protein database with the protein sequence of the *AtBRI1* gene as a query was conducted, and the homologs of *AtBRI1* in the *B. rapa* genome resulting in significant alignments were identified. Bra011862 with a score of 1,999 bits and an E-value of 0.0; *Bra*033615 with a score of 2,009 bits and an E-value of 0.0; and Bra010684 with 1,851 bits and an E-value of 0.0 localized in chromosomes 1, 6, and 8, respectively, and exhibited 85%, 85%, and 79% protein sequence identity with AtBRI1, respectively. All possessed remarkably similar protein sequences to *AtBRI1* and were therefore named *BrBRI1-1* (*Bra*011862), *BrBRI1-2* (*Bra*033615), and *BrBRI1-3* (*Bra*010684).

Previous studies reported that the *BRI1* genes in other species, such as *A. thaliana BRI1* (*AtBRI1*) (Li & Chory, 1997), *Solanum lycopersicum BRI1* (*SlBRI1*) (Montoya *et al.*, 2002), *Pisum sativum* L *BRI1* (*PsBRI1*)(Nomura *et al.*, 2003), *Hordeum vulgare BRI1* (*HvBRI1*) (Chono *et al.*, 2003), *Oryza sativa BRI1* (*OsBRI1*) (Nakamura *et al.*, 2006), *Zea mays BRI1* (*ZmBRI1*) (Kir *et al.*, 2015), *Brachypodium distachyon BRI1* (*BdBRI1*) (Feng *et al.*, 2015), *Triticum aestivum BRI1* (*TaBRI1*) (Singh *et al.*, 2016), and *Glycine max BRI1* (*GmBRI1*) (Peng *et al.*, 2016) all have no introns. To determine whether the *BrBRI1* genes contain introns, the genomic DNA was used for PCR amplification with designed primers including the start codon and stop codon. After sequencing, we confirmed that these three genes contained no introns and possessed large open reading frames of 3,585, 3,585, and 3,525 bp, respectively.

These three proteins encoding 1,194 (predicted molecular weight of 131 kD), 1,194 (131 kD), and 1,174 amino acids (129 kD), respectively, have the same functional domains as the AtBRI1 protein including an N-terminal signal peptide, 25 tandem LRR repeats with a potential BL binding region between the 21st and 22nd LRRs, a transmembrane domain to anchor the protein to the plasma membrane, an intracellular kinase domain that functions as a catalytic region and ends with a C-terminal extension, and all the domains are conserved among the BRI1 homologs (Fig. 1). High sequence conservation implied that the BrBRI1 proteins might possess biological functions similar to AtBRI1.

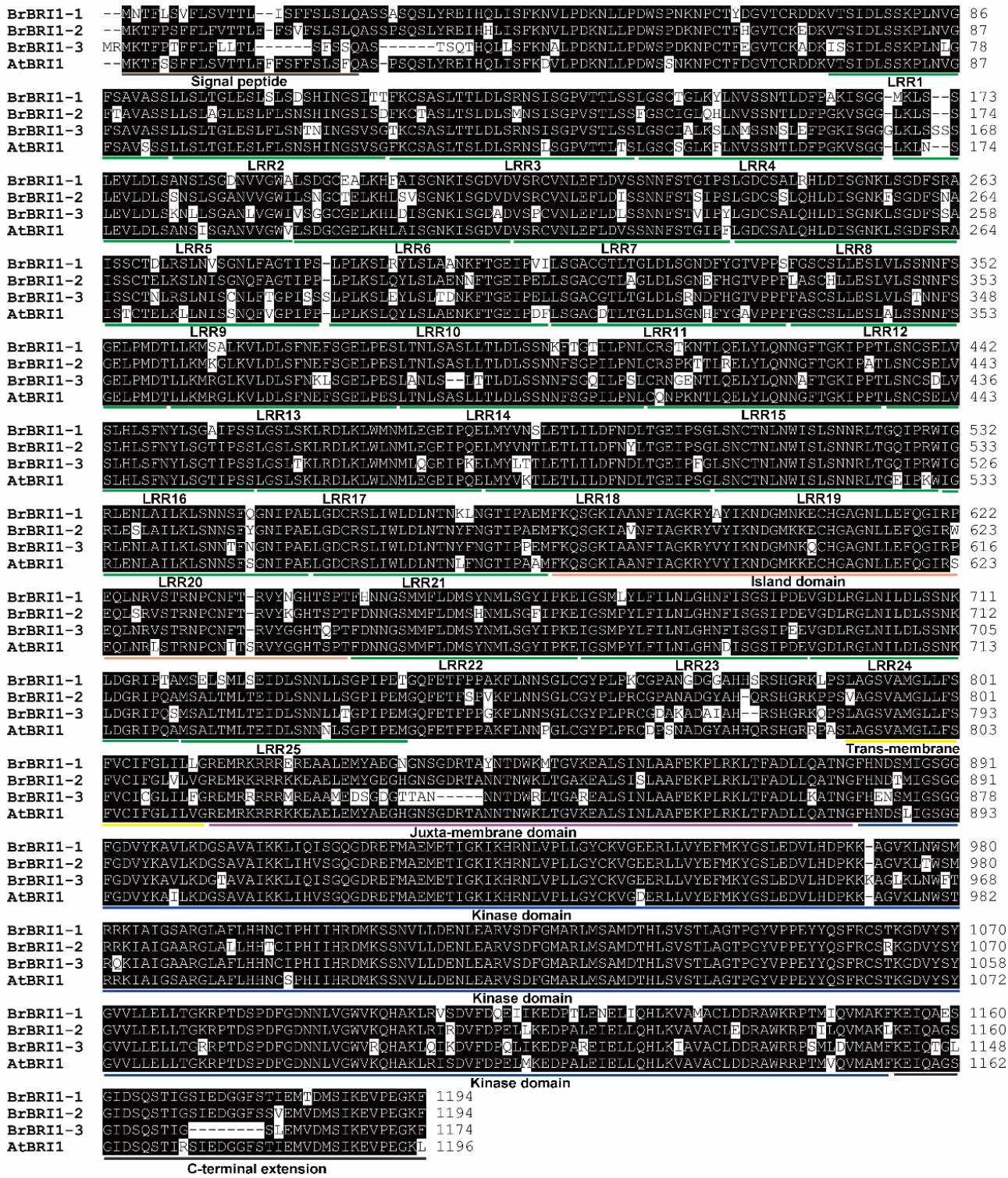

Fig. 1. Protein sequence comparison of BrBRI1 and AtBRI1 using ClustalW2. The predicted protein structural domains are underlined in the corresponding positions. The identical amino acid residues are highlighted in black.

Identification of *BrBRI1* genes homologous in *B. rapa*. The closest homologs of *BRI1* were identified in *Arabidopsis* (*AtBRL1*, *AtBRL2*, and *AtBRL3*) and rice (*OsBRL1*, *OsBRL2*, and *OsBRL3*) (Cano-Delgado *et al.*, 2004; Nakamura *et al.*, 2006). In *Arabidopsis*, AtBRL1 and AtBRL3, but not AtBRL2, are functional BR receptors that can associate with BL and compensate for the *bri1-301* mutant when overexpression is driven by the *AtBRI1* promoter (Cano-Delgado *et al.*, 2004). In rice, *OsBRL1* and *OsBRL3*, but not *OsBRL2*, which were highly expressed in the roots, were able to bind BL (Nakamura *et al.*, 2006). Three homologous genes of the *BrBRI1* genes (*BrBRL1*, *BrBRL2*, and *BrBRL3*) were also found in the *B. rapa* genome database. We aligned the *BrBRI1* genes with the *Arabidopsis* and rice BRLs, and discovered that BrBRL2 grouped into a clade with AtBRL2 and OsBRL2 (Fig. 2A).

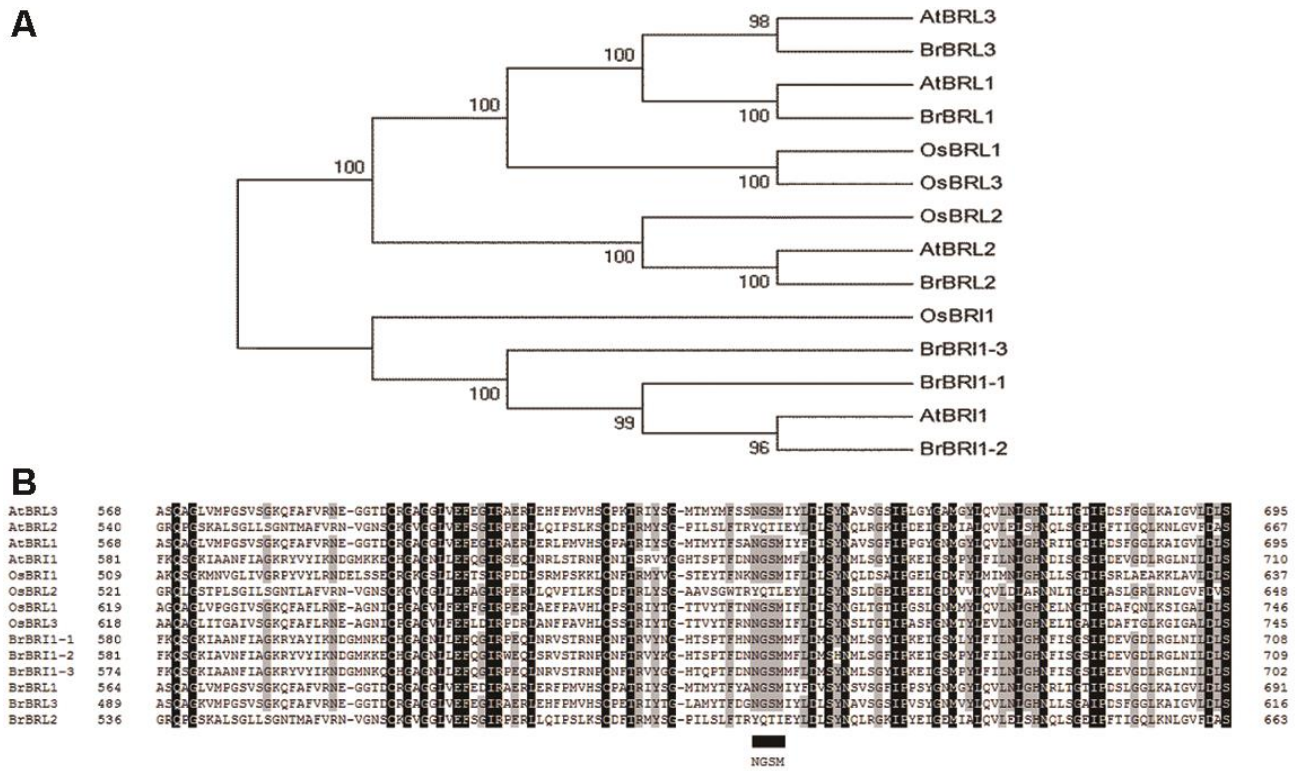

Fig. 2. The *B. rapa* genes homologous to *BrBRI1*. (A) Phylogenetic analysis of BRI1 and BRL proteins from *Arabidopsis*, *Oryza sativa*, and *B. rapa*. (B) Sequence alignments of the BR binding region of BRI1 and BRL proteins from the three species. The Genbank accession numbers are as follows: AtBRI1 (O22476); AtBRL1 (Q9ZWC8); AtBRL2 (Q9ZPS9); AtBRL3 (Q9LJF3); OsBRI1 (NP_001044077); OsBRL1 (NP_001062792); OsBRL2 (AAK52544); OsBRL3 (BAD01717); BrBRL1 (XP_009106844.1); BrBRL2 (XP_009101284.1); and BrBRL3 (XP_009146526.1).

Previous research has shown that the NGSM motif located in the unique island domain involving BR binding is highly conserved in known BRI1 proteins from multiple plants and in its homologues except BRL2 (Kinoshita *et al.*, 2005; Holton *et al.*, 2007). According to the amino acid alignments of the island domain, the underlined NGSM motif is highly conserved in BrBRI1 proteins and its homologs except AtBRL2, OsBRL2, and BrBRL2 (Fig. 2B). In combination, these observations led us to postulate that BrBRL1 and BrBRL3 are involved in BR perception, while BrBRL2 may possess distinct functions.

BrBRI1 proteins localized in the plasma membrane. The protein sequence predication results of the BrBRI1 proteins showed that they have an ectodomain, a transmembrane region, and a cytoplasmic region, which suggests that BrBRI1 proteins may constitute membrane proteins localized in the plasma membrane. To detect the subcellular localization of BrBRI1 proteins *In vivo*, 35S:BrBRI1-GFP constructs were generated and transformed into tobacco epidermal cells by agrobacterium-mediated transient transformation. As shown in Fig. 3, BrBRI1-1, BrBRI1-2, and BrBRI1-3 all possessed a similar localization compared with AtBRI1, which was mainly distributed in the plasma membrane, while the positive control, 35S:GFP, diffused over the entire cell.

BrBRI1 proteins restored the *Arabidopsis* weak *bri1* mutant phenotypes. To test if BrBRI1 proteins encode functional BR receptor proteins, three transgenic constructs containing the full-length BrBRI1 protein coding regions with a C-terminal fusing GFP tag, under the control of the CaMV 35S promoter, were transformed into the *Arabidopsis* weak *bri1-5* mutant (C69Y amino acid substitution) (Noguchi *et al.*, 1999a). The *bri1-5* mutant has a distinct phenotype from the wild-type (WS2), exhibiting intermediate dwarfism and a smaller rosette size, but with the ability to flower and fruit, which could be used for genetic transformation using the floral dip method, while this is not achievable with the *bri1* null mutant (Noguchi *et al.*, 1999a). As the striking sequence similarity between the *AtBRI1* and *BrBRI1* genes predicated the functional likeness, the *BrBRI1* genes were transformed into *bri1-5* to examine their capacity to rescue the weak *bri1-5* mutant. The phenotypic traits of multiple over-expressing transgenic lines were carefully assessed with wild-type and *bri1-5* as a control. One individual with the representative phenotype of each construct was selected and photographed (Fig. 4A and 4C). Western blot analysis with a specific GFP antibody was performed to ascertain the expression level of BrBRI1-GFP in the transgenic lines (Fig. 4B). Comparatively, the phenotype of the dwarf mutants had been successfully complemented by the BrBRI1 transgenes (Fig. 4C). Unlike the *bri1-5* plants exhibiting small curled rosette leaves and short petioles, the transgenic lines all possessed smooth leaves and longer petioles (Fig. 4F). The transgenic plants were similar in morphology to the wild-type plants with a taller plant height (Fig. 4A, 4C and 4F). Notably, the silique lengths of transgenic BrBRI1 lines were also significantly increased and similar to wild-type (Fig. 4D and 4G). Full morphological recovery of the defective phenotypes demonstrated that BrBRI1 proteins act as functional brassinosteroid receptors.

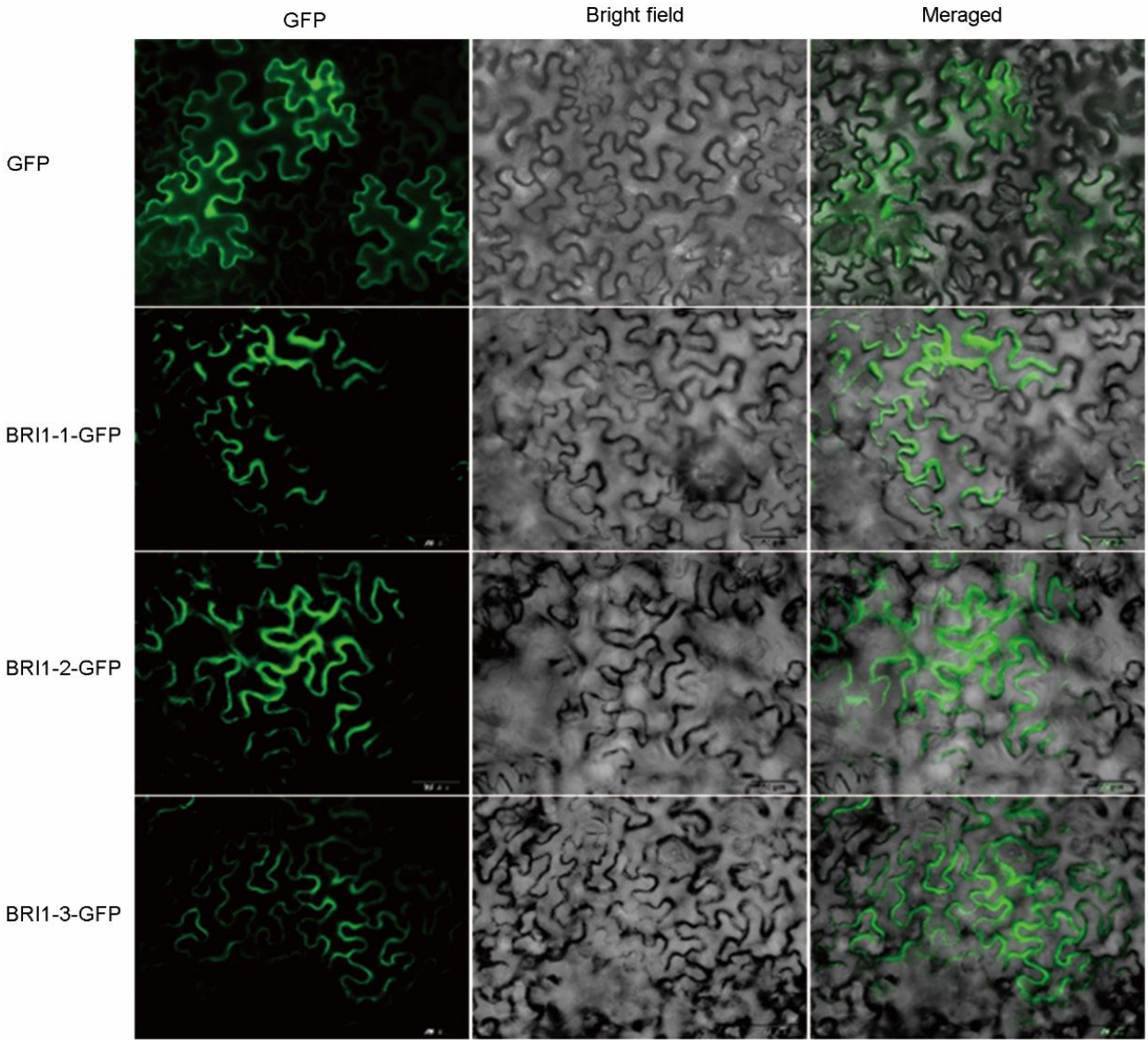

Fig. 3. Subcellular localization of BrBRI1-GFP. *Agrobacterium tumefaciens* GV3101 containing the 35S:GFP, 35S:BrBRI1-1-GFP, 35S:BrBRI1-2-GFP, and 35S:BrBRI1-3-GFP construct, respectively, were injected into tobacco leaves. Panels from left to right show the signals of green fluorescence, the image under bright field, and the overlay image. Scale bars represent 50 μm.

Expression of BrBRI1 proteins recovered BR signaling. In the wild-type, root and hypocotyl growth is inhibited at a high concentration of BR and is promoted at a low concentration of BR; and has also been reported that a BR-insensitive mutant reduced the ability to respond to exogenous BR (Clouse, 1996; Gou *et al*., 2012). To test whether the recovered phenotype seen in the *BrBRI1* overexpression lines arose from the recovery of BR sensitivity in the *bri1-5* mutant, a root inhibition assay and a hypocotyl elongation assay were performed. As shown in Fig. 5A and 5C, the *BrBRI1-1*, *BrBRI1-2*, and *BrBRI1-3* overexpression lines exhibited an approximately 79%, 71.7%, and 63% increase in root length without the application of exogenous BR compared to *bri1-5*, respectively, and were all sensitive to applied exogenous BR over a wide range of BR concentrations from 0 to 100 nM. The hypocotyl length of *BrBRI1-1*, *BrBRI1-2*, *and BrBRI1-3* overexpression lines increased 220%, 202%, and 183% compared to *bri1-5*, respectively, and exhibited the ability to respond to BR treatment at all applied concentrations, respectively (Figs. 5B and 5D). The sensitivity of the *BrBRI1* overexpression lines to BR treatment was similar to the wild-type, which corresponds with its phenotype.

It was reported that multiple genes were down-regulated in response to BR treatment via a negative feedback mechanism, and that the mechanism will be abolished when the BR signaling is blocked (Tanaka *et al*., 2005). To determine whether the recovered phenotype resulted from the recovery of BR signaling, the relative expression levels of BR-regulated genes using RT-PCR were analyzed. Compared with *bri1-5*, the expression level of *CPD* was dramatically decreased in 15-day-old *BrBRI1*-overexpression seedlings. After treatment with 1μM 24-epiBL for 1 h, the relative expression of *CPD* in BrBRI1-overexpression plants could not be detected due to its low transcript abundance, and the reduction was more severe than that in wild-type (Fig. 6A).

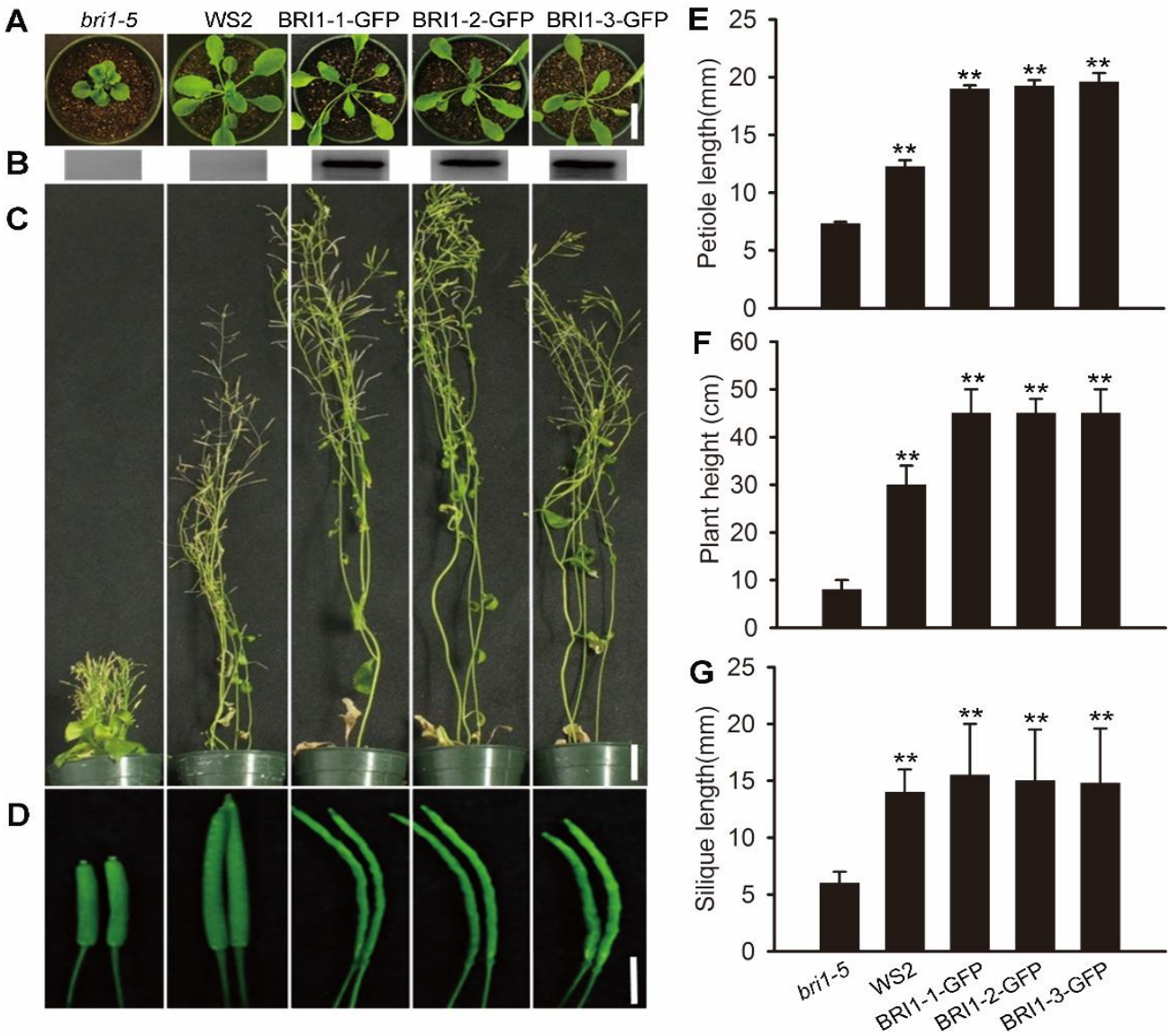

Fig. 4. BrBRI1 restored the *bri1-5* mutant phenotypes. (A) 35-day-old plants of *bri1-5*, WS2, and one independent transgenic line of each construct. Bar = 2 cm. (B) Western blot assays to validate the transgenic lines. (C) Seven-week-old plants of *bri1-5*, WS2, and one independent transgenic line of each construct. Bar = 2 cm. (D) Silique morphology comparison among *bri1-5*, WS2, and one independent transgenic line of each construct. Bar = 3 mm. (E) Transgenic plants exhibited longer petioles than those observed in plants corresponding to (A). (F) Transgenic plants exhibited higher plant height than those observed in plants corresponding to (C). Values are means of measurements from 10 seedlings. (G) Average silique length in seven-week-old plants corresponding to (D). Error bars = ±SEM; n = 20. Asterisks indicate mean values significantly different from *bri1-5* (Student's *t* test; **, $p<0.01$).

We next investigated the phosphorylation state of a BR-regulated protein, BES1, in BR signaling, which is phosphorylated when BR signaling is blocked or without BR treatment, and dephosphorylated under exogenous BL (Yin *et al.*, 2002). With BL treatment, the amount of dephosphorylated BES1 was more abundant in *Br*BRI1-overexpression plants compared with the *bri1-5* mutant, which has a strong resemblance to that of the wild-type plants (Fig. 6B).

Collectively, all the above data suggest that the rescued phenotypes of the *bri1-5* mutant and recovered BR signaling may be attributed to the functional BrBRI1 receptors.

Discussion

BRI1, a broadly expressed LRR-RLK X protein, functions significantly in regulating plant growth and development (Clouse, 1996; Friedrichsen *et al.*, 2000; Kim & Wang, 2010; Eremina *et al.*, 2016), and was first identified as a component involved in BR signal transduction by map-based cloning for the *brassinosteroid insensitive 1* (*bri1*) mutant (Li & Chory, 1997). Since its identification, numerous studies have supported that BRI1 perceives BRs and initiates early BR signaling (Wang *et al.*, 2001; Kinoshita *et al.*, 2005; Wang *et al.*, 2008). Due to the important biochemical function in BR signal transduction and biological roles in regulating plant growth and development, many *BRI1* homologues in other species have been identified and well-studied (Koka *et al.*, 2000; Montoya *et al.*, 2002; Nakamura *et al.*, 2006; Feng *et al.*, 2015; Singh *et al.*, 2016). However, the role of BRI1 in *B. rapa* is still unknown.

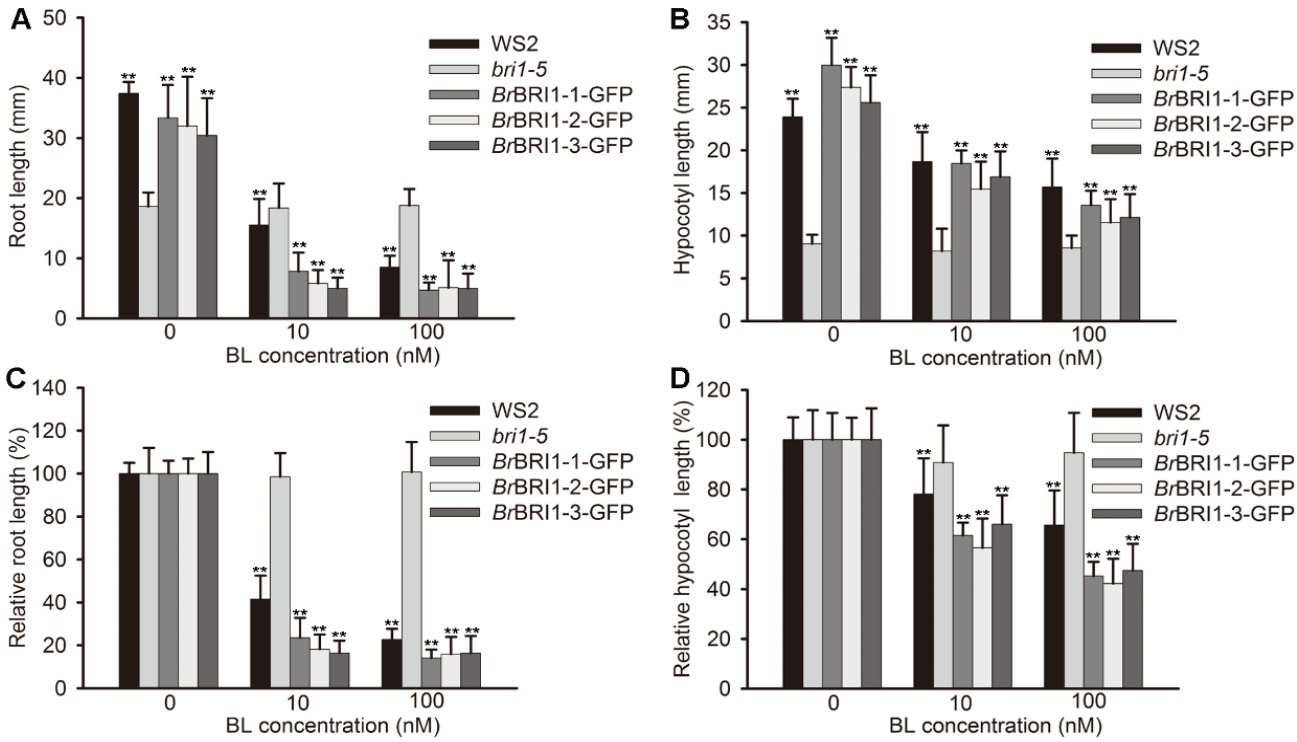

Fig. 5. Hypocotyl and root elongation in response to BL. Seedlings were grown on vertically placed ½ MS plates with different concentrations of 24-epiBL. (A) Root length in 10-day-old *Arabidopsis*. (B) Hypocotyl length in seven-day-old *Arabidopsis*. (C) Relative root length of seedlings corresponding to (A). (D) Relative hypocotyl length of seedlings corresponding to (B). Error bars = ±SEM; n = 20. Asterisks indicate mean values significantly different from *bri1-5* (Student's *t* test; **, $p<0.01$). Experiments were performed in triplicate with similar results.

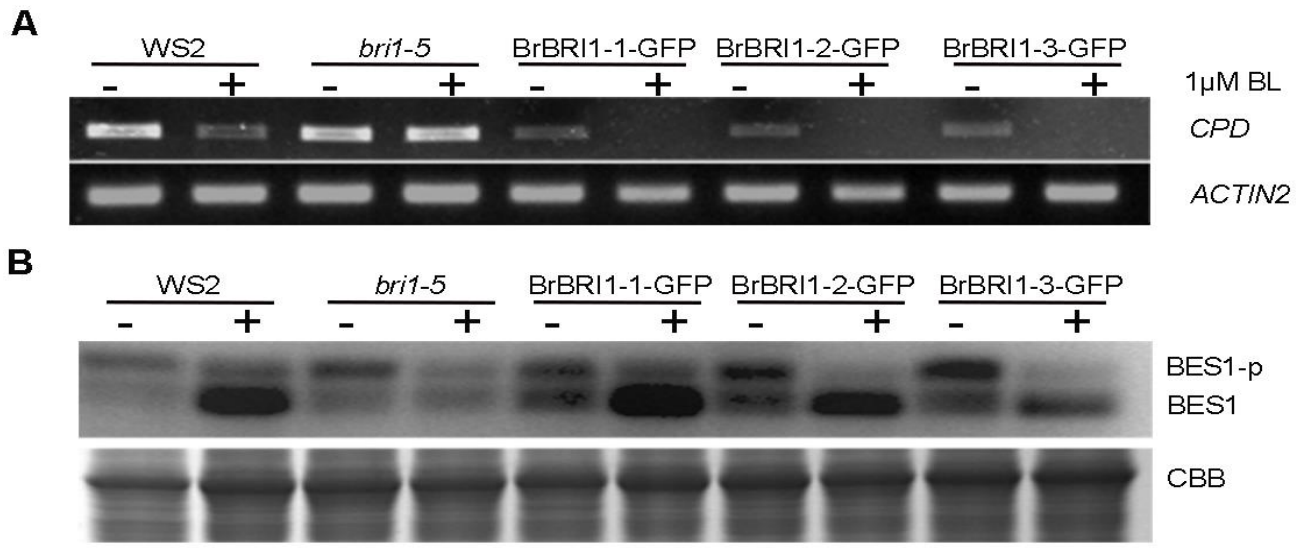

Fig. 6. BrBRI1 overexpression lines are sensitive to BL treatment. (A) RT-PCR analysis for *CPD* expression in *bri1-5* and transgenic lines treated with or without 1 μM 24-epiBL, respectively. *ACTIN2* was used as an internal control. (B) The accumulation of phosphorylated BES1 (BES1-p) and nonphosphorylated BES1 were analyzed by Western blot with the BES1 antibody. The seedlings with 0 or 1 μM 24-epiBL treated for 1.5 h were used for total protein extraction. The lower panel is the loading control.

In this study, three *B. rapa* BR receptor genes were identified and named *BrBRI1-1*, *BrBRI1-2*, and *BrBRI1-3*. Nucleotide acid sequence analysis indicated that none of them contain introns. Protein sequence analysis demonstrated that they have high similarity with AtBRI1, at 85%, 85%, and 79%, respectively, and all of them have 25 conserved LRR repeats, one island domain, a single trans-membrane domain, a juxtamembrane domain, a kinase domain, and a C-terminal region (Fig. 1). The subcellular localization analysis indicated that they mainly localized at the plasma membrane (Fig. 2). All these results suggest that *Br*BRI1 proteins might have a similar function to BRI1 in *Arabidopsis* (Li & Chory, 1997) and *Oryza sativa* (Nakamura *et al.*, 2006), namely in perceiving BRs and initiating BR signaling at the cell surface.

Considering that there are four BR receptor genes in *Arabidopsis* and rice, three *BrBRI1*-like genes were also identified in *B. rapa* and named *BrBRL1*, *BrBRL2*, and *BrBRL3*. Phylogenetic analysis showed that *BrBRL2* falls into the same clade with *AtBRL2* and *OsBRL2* (Fig. 2A). The amino acid alignment showed that BrBRL2 also has no conserved NGSM motif in the unique island domain, which is consistent with AtBRL2 and OsBRL2 (Fig. 2B). The above evidence indicates that BrBRL2 is unlikely to have the ability to perceive BRs and initiate BR signaling as reported AtBRL2 and OsBRL2 (Nakamura *et al.*, 2006).

Heterologous over expression of BrBRI1 proteins in the weak *Arabidopsis bri1-5* mutant totally complemented the defective *bri1-5* mutant phenotypes, not only in terms of plant height, but also with regard to petiole and silique length (Fig. 4), implying that BrBRI1 proteins are involved in BR signaling. According to the root inhibition assay, hypocotyl elongation assay (Fig. 5A), and the expression of *CPD* and the phosphorylation status of BES1 (Fig .6B), the BrBRI1 overexpression plants all exhibited sensitivity to the BR treatment, which was similar to that observed in the wild-type plants, indicating that BrBRI1 recovered the BR signaling blocked in the *bri1-5* mutant. Taken together, all the above-mentioned results confirmed that BrBRI1 proteins are functional BR receptors in plants.

Recently, significant progress has been made into influencing various important agronomic traits determining crop performance via the modification of brassinosteroid receptor kinase proteins. For example, reducing the expression of *OsBRI1* in rice could increase the seed production of transgenic lines by approximately over 30% at high density (Morinaka, 2006). The semi-dwarf phenotype in *uzu* barley is attributable to the H857R amino acid substitution in HvBRI1, which is also responsible for the increased seed production and enhanced resistance to lodging (Chono *et al.*, 2003). In addition to improving yield performance and abiotic stress, the mutation of HvBRI1 in *uzu* barley even strengthened its capability against a variety of pathogenic microbes (Ali *et al.*, 2014). Furthermore, this allele has been introduced into many cultivated species to obtain better agronomic traits (Dockter *et al.*, 2014). *Brachypodium distachyon* is a good model for cereals and temperate grasses for studying tolerance to various stresses (Brkljacic *et al.*, 2011). The RNAi mutants of *B. distachyon* with reduced *BdBRI1* expression showed stronger drought resistance than the wild-type (Feng *et al.*, 2015). The above research clearly suggests that further investigation and understanding of BrBRI1 protein-mediated molecular mechanisms and targeted modifying of BrBRI1 proteins will be helpful for the molecular breeding of *B. rapa* for yield, disease resistance, and tolerance to abiotic stresses.

Brassica napus, an important oilseed crops and an allodiploid species derived from the hybridization between *B. rapa* and *B. oleracea*, possesses the complete diploid chromosome sets of the parental genomes (Parkin *et al.*, 1995; Snowdon *et al.*, 2002; Nouroz *et al*., 2017). The characterization of BrBRI1 proteins will provide a foundation for the breeding of high-yielding, semi-dwarf rapeseed varieties through regulating the BR signaling and the phosphorylation states of specific BRI1s phosphorylation sites by the CRISPR/Cas9-mediated genome editing system (Feng *et al.*, 2013; Xing *et al.*, 2014; Cermak *et al.*, 2015) with the targets of *Br*BRI1 proteins.

Conclusion

BRI1, a receptor of BRs in BR signaling, functions significantly in regulating plant growth and developmental processes. In the current study, three *BRI1* genes (*BrBRI1-1*, *BrBRI1-2*, and *BrBRI1-3*) were cloned from *B. rapa*. The overexpression of *BrBRI1* genes in the BR-weak mutant *bri1-5* could rescue its phenotype to the wild-type and restore its BR signaling. Thus, BrBRI1 proteins function as BR receptors to mediate BR signaling.

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