Journal of Plant Biology (2023) 66:233–242
https://doi.org/10.1007/s12374-023-09387-4

RESEARCH ARTICLE

Usefulness and Molecular Mechanism of Seed-Specificity Introduced by *AtBZR1* and *AtBES1* to Improve Seed Yield and Quality in *Arabidopsis thaliana*

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Received: 29 November 2022 / Revised: 25 January 2023 / Accepted: 31 January 2023 / Published online: 11 February 2023

Abstract

This study generated the transgenic *Arabidopsis* lines *pAt5g54000-AtBZR1::Col-0* and *pAt5g54000-AtBES1::Col-0*, in which *Arabidopsis BZR1* and *BES1* (*AtBZR1* and *AtBES1*) were seed-specifically expressed with *pAt5g54000*, a seed-specific promoter. Semi-quantitative RT-PCR and *GUS*-staining analysis demonstrated that the inserted *AtBZR1* and *AtBES1* were concentrated in seeds in siliques of transgenic plants. Seed number, length, width, and mass increased in the *pAt5g54000-AtBZR1::Col-0* and *pAt5g54000-AtBES1::Col-0* mutants compared to untransformed *Arabidopsis*. The endogenous levels of primary metabolites, such as carbohydrates, proteins, and lipids, in transgenic seeds were also higher than those in wild-type seeds, indicating that both seed size and quality are improved by seed-specific expression of *AtBZR1* and *AtBES1* in *Arabidopsis*. In both transgenic *Arabidopsis* seeds, relative to wild-type seeds, the expression of positive regulatory genes involved in determining seed size, such as *SHORT HYPOCOTYL UNDER BLUE1* (*AtSHB1*), *MINISEED3* (*AtMINI3*), and *HAIKU2* (*AtIKU2*), was increased by up-regulation of *AtBZR*1 and *AtBES1* as well as down-regulation of *ABA Deficient 2* (*AtABA2*) and *ABA Insensitive 5* (*AtABI5*). This result suggests that AtBZR1- and AtBES1-mediated signaling pathways such as *AtBZR1/AtBES1* → *AtSHB1* → *AtMINI3* → *AtIKU2* and *AtBZR1/AtBES1* → *AtABA2*, and/or *AtABI5* → *AtSHB1* → *AtMINI3* → *AtIKU2* increase the yield and quality of seeds in transgenic *Arabidopsis*. Taken together, our findings demonstrated the usefulness and applicability of seed-specific introduction of *AtBZR1* and *AtBES1* encoding key transcription factors in brassinosteroid signaling to improve seed yield and quality in *Arabidopsis*.

Keywords *Arabidopsis thaliana* · AtBES1 · AtBZR1 · Brassinosteroid · Seed-specific expression · Seed yield and quality

Introduction

Brassinosteroids (BRs) play various regulatory roles in plant growth and development (Bajguz et al. 2020; Clouse 2011; Clouse and Sasse 1998). BRs are recognized by the membrane-localized receptor kinase brassinosteroid-insensitive 1 (BRI1), promoting dimerization between BRI1 and its co-receptor BRI1-associated receptor kinase 1 (BAK1) (Li et al. 2002; Nam and Li 2002). In turn, BRI1/BAK1 phosphorylates brassinosteroid-signaling kinases and BRI1 suppressor 1 suppresses the kinase activity of brassinosteroid-insensitive 2 (BIN2), a negative regulator in BR signaling (Li et al. 2001). The inactivation of BIN2 leads to the accumulation of BR transcription factors, such as brassinazole-resistant 1 (BZR1) and BRI1 EMS suppressor 1 (BES1) in the nucleus (He et al. 2002; Yin et al. 2002). These transcription factors ultimately control the transcription of BR target genes to regulate plant growth and development (Kim and Russinova 2020; Kim and Wang 2010).

BRs play crucial roles as positive regulators in the determination of seed size, suggesting that they are ideal targets to improve seed production in plants, especially grain yields in monocotyledonous crops (Wu et al. 2008; Zhang et al. 2014). However, the high cost of BR synthesis discourages their direct application to plants (Tong and Chu 2012). In response to these limitations, modulating genes associated with BR biosynthesis and signaling is a promising approach to enhance BR

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activity and improve seed production in plants (Li et al. 2018; Sahni et al. 2016; Shimada et al. 2003).

In previous studies in *Arabidopsis* (*Arabidopsis thaliana*), we found that the over-expression of *cytochrome P450 85A2 (AtCYP85A2)*, which encodes a bi-functional enzyme for BR 6-oxidase/brassinolide (BL) synthase, could be used to synthesize biologically active BRs such as castasterone (CS) and BL using the universal promoter *35S*, thereby increasing seed yields compared to that of wild types (Kim et al. 2005). Recently, we also found that *AtCYP85A2* introduced by a seed-specific *pAt5g54000* promoter (*pAt5g54000-AtCYP85A2::Col-0*) increased the seed size of *Arabidopsis* more effectively compared to a *35S-AtCYP85A2* mutant (Yeon et al. 2022). In *Brachypodium distachyon*, a model plant for monocotyledonous crops, a significant increase in grain yield by seed-specific expression of *AtCYP85A2* was also demonstrated in a transgenic line (*pAt5g54000-AtCYP85A2::Bd21-3*) (Roh et al. 2021). In rice (*Oryza sativa*), seed-specifically expressed *DWARF4* (*OsDWARF4*), an upstream biosynthetic gene for rate-limiting BR biosynthesis, increased grain yield (Li et al. 2018). Therefore, increasing BR activity via the seed-specific introduction of BR biosynthetic genes appears to be a promising method to improve seed yield in both monocotyledonous and dicotyledonous plants.

The endogenous levels of CS and BL increased in seeds of *pAt5g54000-AtCYP85A2::Col-0* and *pAt5g54000-AtCYP85A2::Bd21-3*, thus enhancing down-stream BR signaling and increasing the yield and quality of seeds in transgenic plants (Roh et al. 2021; Yeon et al. 2022). This suggests that genes involved in BR signaling may also be good candidates for molecular manipulation of BR activity to improve seed productivity. However, the ability of BR-signaling genes to increase seed yield and quality remains largely unexplored. Therefore, our study sought to generate a transgenic *Arabidopsis* line in which *AtBZR1* and *AtBES1*, which encode major transcription factors in BR-regulated determination of seed size, were seed-specifically introduced. The physiological function and molecular mechanisms through which AtBZR1/AtBES1-mediated BR signaling promotes seed yield and quality in transgenic *Arabidopsis* were also investigated in this study. Our results thus provide key insights into the applicability of BR-signaling genes in molecular breeding to promote grain productivity in economically relevant monocotyledonous crops.

Materials and Methods

Plant Materials and Growth Conditions

A. thaliana Columbia-0 (Col-0), *pAt5g54000-AtBZR1::Col-0*, and *pAt5g54000-AtBES1::Col-0* were characterized in this study. The seeds were sterilized twice with 70% EtOH (*v/v*), rinsed with distilled water, and incubated at 4 °C for 2 days. The washed *Arabidopsis* seeds were then sown on 0.5X Murashige–Skoog (MS) medium containing 1% sucrose (*w/v*) and 0.8% agar (Phytagel; Sigma, St. Louis, MO, USA). The plants were then grown in a growth chamber (Vision Scientific, Seoul, Korea) under a 16 h light/8 h dark cycle at 22 °C during the light period and 20 °C during the dark period.

Preparation of *pAt5g54000-AtBZR1::Col-0* and *pAt5g54000-AtBES1::Col-0*

A seed-specific expression construct was produced to generate *pAt5g54000-AtBZR1::Col-0* and *pAt5g54000-AtBES1::Col-0*. The seed-specific construct was generated as described in a previous study (Yeon et al. 2022). The *AtBZR1* and *AtBES1* coding sequences (CDS) were amplified using Ex-Taq polymerase (Takara Bio, Shiga, Japan). Primers were designed by incorporating the BamHI restriction enzyme site into the *AtBZR1* CDS and the AscI and EcoRI restriction enzyme sites into the *AtBES1* CDS. The sequences of the primers used in this study are listed in Supplementary Table 1. The amplified *AtBZR1* CDS and *AtBES1* CDS were cloned into the pTA-Topo vector (MGmed, Seoul, South Korea). The promoter of *At5g54000* (*pAt5g54000*), a seed-specific promoter, was introduced into the binary vector pCAMBIA1381. pAt5g54000/pCAMBIA1381, *AtBZR1*/pTA-Topo, and *AtBES1*/pTA-Topo were digested with restriction enzymes (New England Biolabs, Ipswich, MA, USA). *AtBZR1* and *AtBES1* CDS were introduced into pAt5g54000/pCAMBIA1381 using the T4 DNA ligase (Takara Bio). Using DNA sequencing, the expected sequences of pAt5g54000-AtBZR1/pCAMBIA1381 and pAt5g54000-AtBES1/pCAMBIA1381 were confirmed and then introduced into *Agrobacterium tumefaciens* (GV3101) with an electroporator (Bio-Rad, Hercules, CA, USA). Using the floral-dip method (Clough and Bent 1998), the seed-specific construct (pAt5g54000-AtBZR1 and pAt5g54000-AtBES1) was introduced into Col-0. Hygromycin was used for the selection of transformed seeds.

Semi-quantitative Real-Time PCR (Semi-qRT-PCR) and Quantitative Real-Time PCR (qRT-PCR) Analysis

According to the manufacturer's instructions, total RNA was extracted using TRI reagent (Invitrogen, Carlsbad, CA, USA) and cDNAs were synthesized from 1 µg of the total RNAs with an MMLV-reverse transcription system (Promega, Madison, WI, USA). First, semi-qRT-PCR was performed to confirm the *AtBZR1* and *AtBES1* expression levels for each tissue in the transgenic plants. The same amount of cDNA from each tissue was used as a template. The

reference gene *UBQ5* and the genes of interest (*AtBZR1*, *AtBES1*) were amplified using rTaq polymerase (ELPIS, Seoul, South Korea) with a total of 20 thermal cycles (95 °C for 10 s, 58 °C for 10 s, and 72 °C for 10 s). The qRT-PCR experiments were performed using the CFX96TM Real-Time PCR Detection System and iQ SYBR Green Supermix (Bio-Rad). qRT-PCR was then conducted using the same amount of cDNA as with the template. The thermal cycling program consisted of an initial denaturation step at 95 °C for 3 min, followed by 45 cycles at 95 °C for 10 s, 50 °C for 15 s, and 75 °C 15 s. *PP2A* was used to normalize the expression level of the target genes. The primers used for semi-qRT-PCR and qRT-PCR are listed in Supplementary Table 1.

Histochemical *GUS* Staining

Histochemical *GUS* staining was performed using the established methods (Weigel and Glazebrook 2002). Five-week-old *Arabidopsis* were harvested to confirm seed-specific expression in the transgenic plant. The stems, roots, leaves, siliques, and seeds of harvested *Arabidopsis* were fixed overnight in cold 90% acetone. The samples were then incubated in staining buffer (2 mM 5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, and 0.2% triton X-100 in 50 mM Na_2HPO_4 [pH 7.2]). Under a vacuum, the sample contained in the staining solution was incubated on ice for 30 min. The samples were further incubated overnight at 37 °C. Afterward, the samples were sequentially incubated in 20%, 35%, and 50% ethanol for 20 min each, then de-stained, and finally transferred into a fixative solution (50% ethanol, 10% acetic acid, and 5% formaldehyde). The final incubation was performed for 20 min. The prepared samples were washed with 70% ethanol and observed under a dissecting microscope (Olympus SZ-PT).

Quantification of Endogenous BRs and ABA in *Arabidopsis* Seeds

The quantification of endogenous BRs was performed as previously described (Roh et al. 2020). Arabidopsis Col-0, *pAt5g54000-AtBZR1::Col-0*, and *pAt5g54000-AtBES1::Col-0* were harvested at 6 weeks of age, after which the seeds were isolated. Active BRs were then purified from 50 g of each plant part. Reversed-phase high-performance liquid chromatography (HPLC) (SenshuPak, C_{18}) was performed on the sample after purification by solvent partitioning, silica chromatography, and C_{18} chromatography under the following conditions: MeCN-water gradient of 45% MeCN for 0–20 min, 45–100% MeCN gradient for 20–40 min, and 100% MeCN for 40–70 min. The flow rate was 2.5 mL min^{-1} and fractions were collected every minute. Authentic BL and CS were detected at 13/14 and 22/23 min in this HPLC condition, respectively. The corresponding fractions were collected for both active BRs, after which they were dried and combined before being analyzed via capillary gas chromatography (GC)-selected ion monitoring (SIM)/mass spectrometry (MS) after bismethaneboronation.

GC-SIM/MS analyses were conducted using a Hewlett-Packard 5973 mass spectrometer (electron impact ionization, 70 electron voltage; Agilent, Santa Clara, CA, USA) connected to a 6890 gas chromatograph fitted with a fused silica capillary column (HP-5, 0.25 mm × 15 m, 0.25-μm film thickness; Agilent). Helium was used as a carrier gas at a 1 mL min^{-1} flow rate. The sample was subjected to GC–MS in on-column injection mode. The oven temperature was maintained for 2 min at 175 °C and elevated to 280 °C at a 40 °C min^{-1} rate. The final oven temperature was maintained at 280 °C for 15 min. For methaneboronation, the samples were dissolved in pyridine containing 2 mg mL^{-1} methaneboronic acid (Sigma) and incubated at 80 °C for 20 min.

ABA measurements were performed via enzyme-linked immunosorbent assay (ELISA) using previously described methods (Moon et al. 2021). Twenty-day-old *Arabidopsis* seedlings were harvested, and 3 g of harvested samples were ground with liquid nitrogen. The ground sample was then extracted three times with 30 mL of 80% MeOH. The extracted sample was concentrated with a rotary evaporator, and solvent partitioning was performed with ethyl acetate and PI buffer (pH 2.5). The ethyl acetate-soluble fraction was collected, dried, and applied to a Sep-Pak C_{18} column cartridge (Waters Co., Milford, MA, USA). Several conditioning steps with H_2O and MeOH were performed to equilibrate the column, and the final equilibrium was maintained with 50% MeOH. After sample injection, ABA was eluted by adding 5 mL of 50% MeOH to the column. The ABA-containing fraction was then collected and dried. The sample obtained from the Sep-Pak C_{18} cartridge column was analyzed by ELISA using the Phytodetek ABA Test Kit (Agdia, Eikhart, IN, USA, PDK09347/0096) according to the manufacturer's instructions.

Quantification of Carbohydrates, Lipids, and Proteins in *Arabidopsis* Seeds

The measurement of soluble carbohydrates, proteins, and lipids was carried out using previously described methods (Yeon et al. 2022). A total of 100 *Arabidopsis* seeds were used for the quantitative analysis of each substance. The Starch Assay Kit (Abcam, Cambridge, United Kingdom) was used to measure starch content according to the manufacturer's instructions. The soluble protein content in the seeds was analyzed as described by Focks and Benning (1998). Seed lipid content was assessed as described by Mishra et al. (2014). Each experiment was conducted by

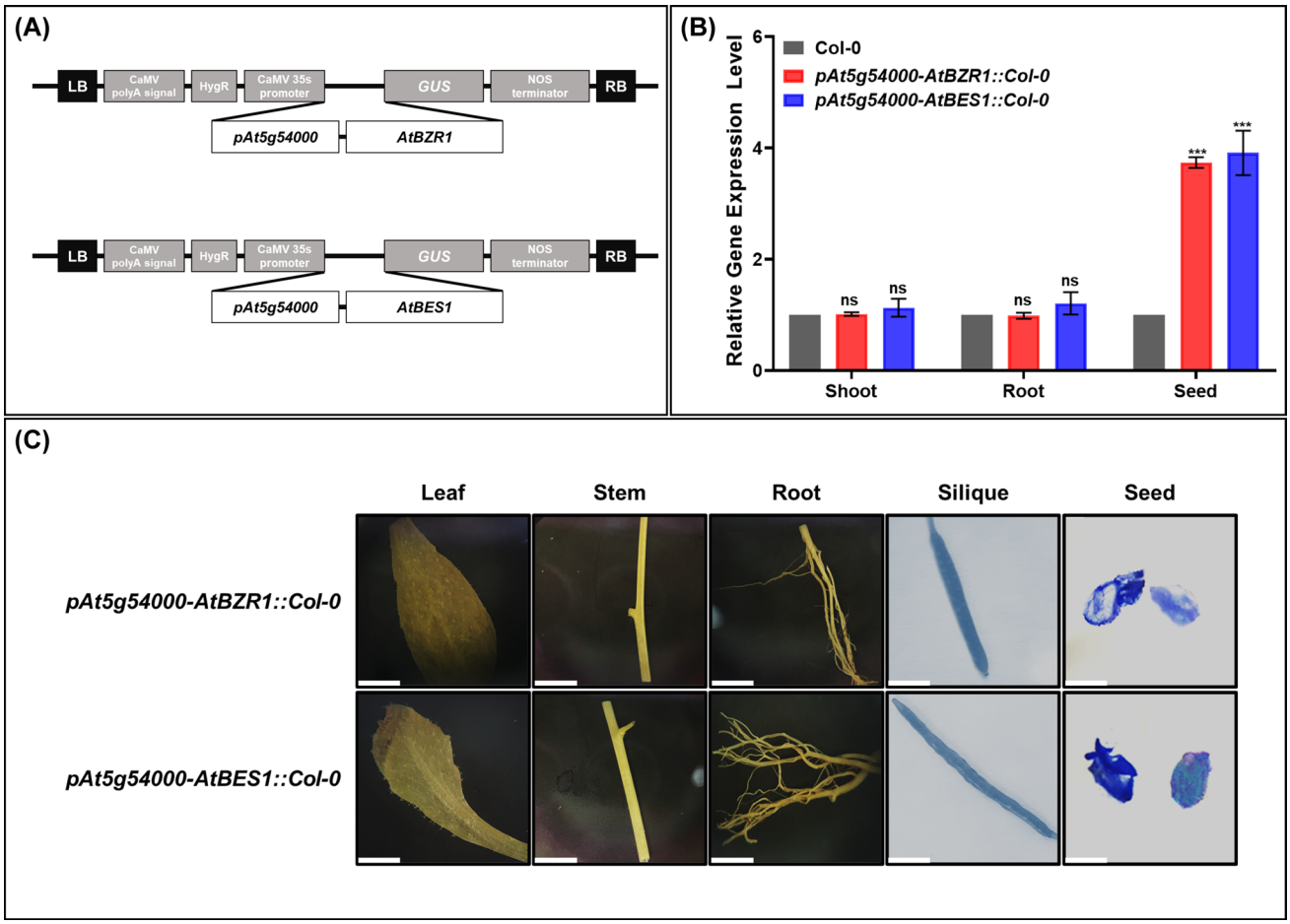


Fig. 1 Seed-specific introduction of *AtBZR1* and *AtBES1* into transgenic *Arabidopsis*. **A** Genetic map of *AtBZR1* and *AtBES1*-expressing construct driven by an *Arabidopsis* seed-specific promoter, *pAt5g54000*. CaMV polyA signal, cauliflower mosaic virus polyadenylation signal; HygR, hygromycin resistance gene; CaMV 35S promoter, cauliflower mosaic virus 35S promoter; *GUS*, *β-glucuronidase* gene; NOS terminator, nopaline synthase terminator; LB, left border; RB, right border. **B** Semi-quantitative RT-PCR analysis of *AtBZR1* and *AtBES1* expression in shoots, roots, and seeds of wild-type and transgenic plants. The data were obtained from three different lines of *pAt5g54000-AtBZR1::Col-0* and *pAt5g54000-AtBES1::Col-0* transgenic plants. *AtBZR1* and *AtBES1* expressions were only detected in the seeds of transgenic plants. The asterisks indicate the statistical significance determined via Student's *t* test: ***($P < 0.001$), *ns* not significant. **C** *GUS*-staining in roots, stems, leaves, siliques, and seeds of *pAt5g54000-AtBZR1::Col-0* and *pAt5g54000-AtBES1::Col-0* transgenic plants. *GUS* activity was concentrated in the siliques and seeds of transgenic plants. The white scale bar in **C** represents 1 cm in leaf, stem, root, and silique and 500 µm in seed

measuring absorbance after color development, and starch, protein, and lipids were measured at 570, 595, and 530 nm, respectively.

Statistical Analysis

A total of 200 seeds from Col-0, *pAt5g54000-AtBZR1::Col-0*, and *pAt5g54000-AtBES1::Col-0* were examined to measure their length and width using an optical microscope. Phenotypic changes were determined based on the width, length, and mass of the seed. Pair-wise comparisons between groups were conducted via Student's *t* test and the Shapiro–Wilk test was performed to test for normality. Multiple comparisons were conducted via a one-way analysis of variance (ANOVA) followed by the Holm–Sidak test.

Results

An *Arabidopsis* seed-specific promoter, *pAt5g54000*, was cloned into a binary vector. The *AtBZR1* and *AtBES1* genes were then cloned into the vector containing *pAt5g54000* (Fig. 1A). The obtained construct was transferred into *A. tumefaciens* and introduced into the wild type (*Col-0*), after which antibiotic-resistant seeds were collected. Semi-qRT-PCR analysis of expression of *AtBZR1* and *AtBES1* in the transgenic *Arabidopsis* (*pAt5g54000-AtBZR1::Col-0* and

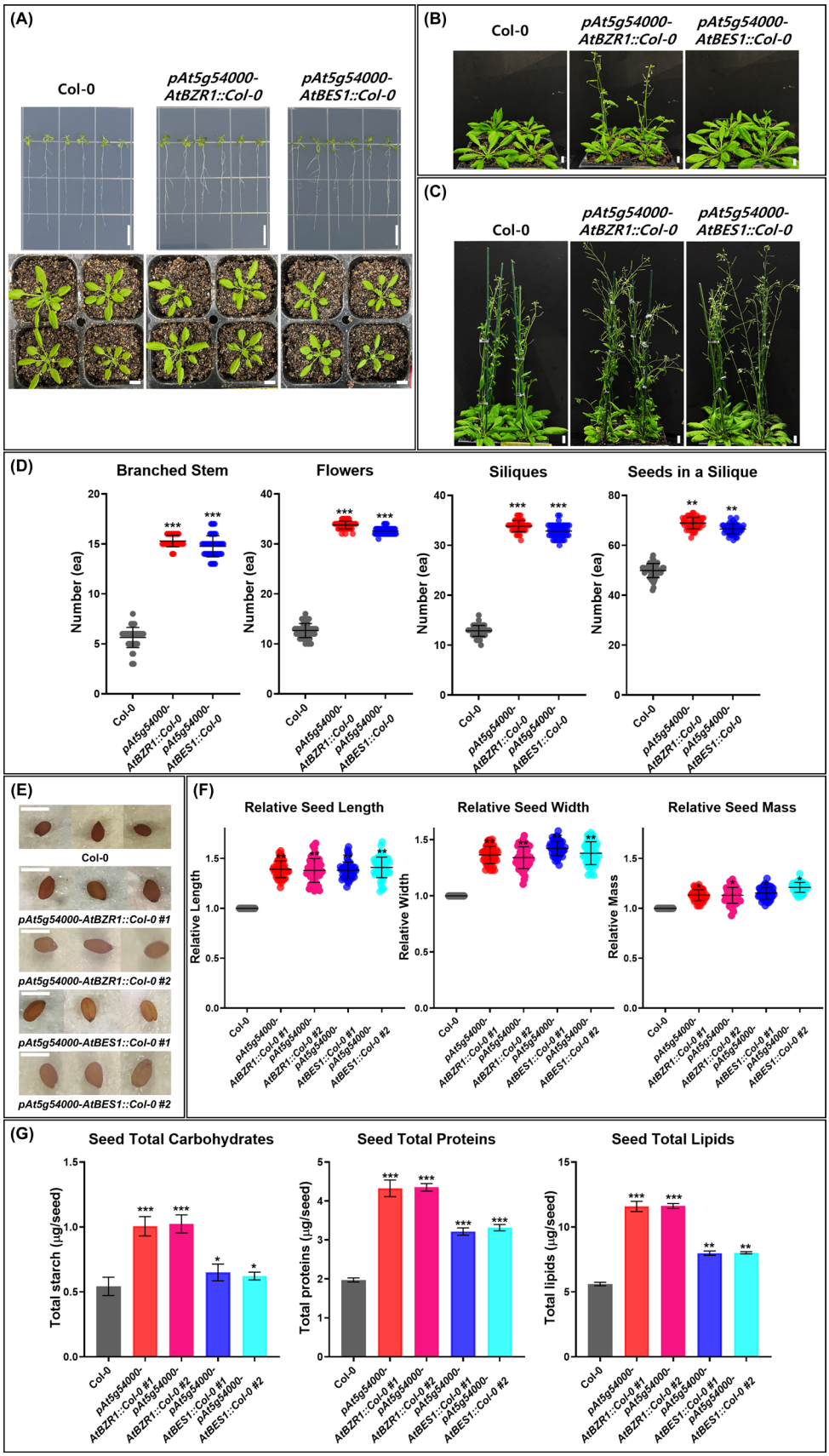
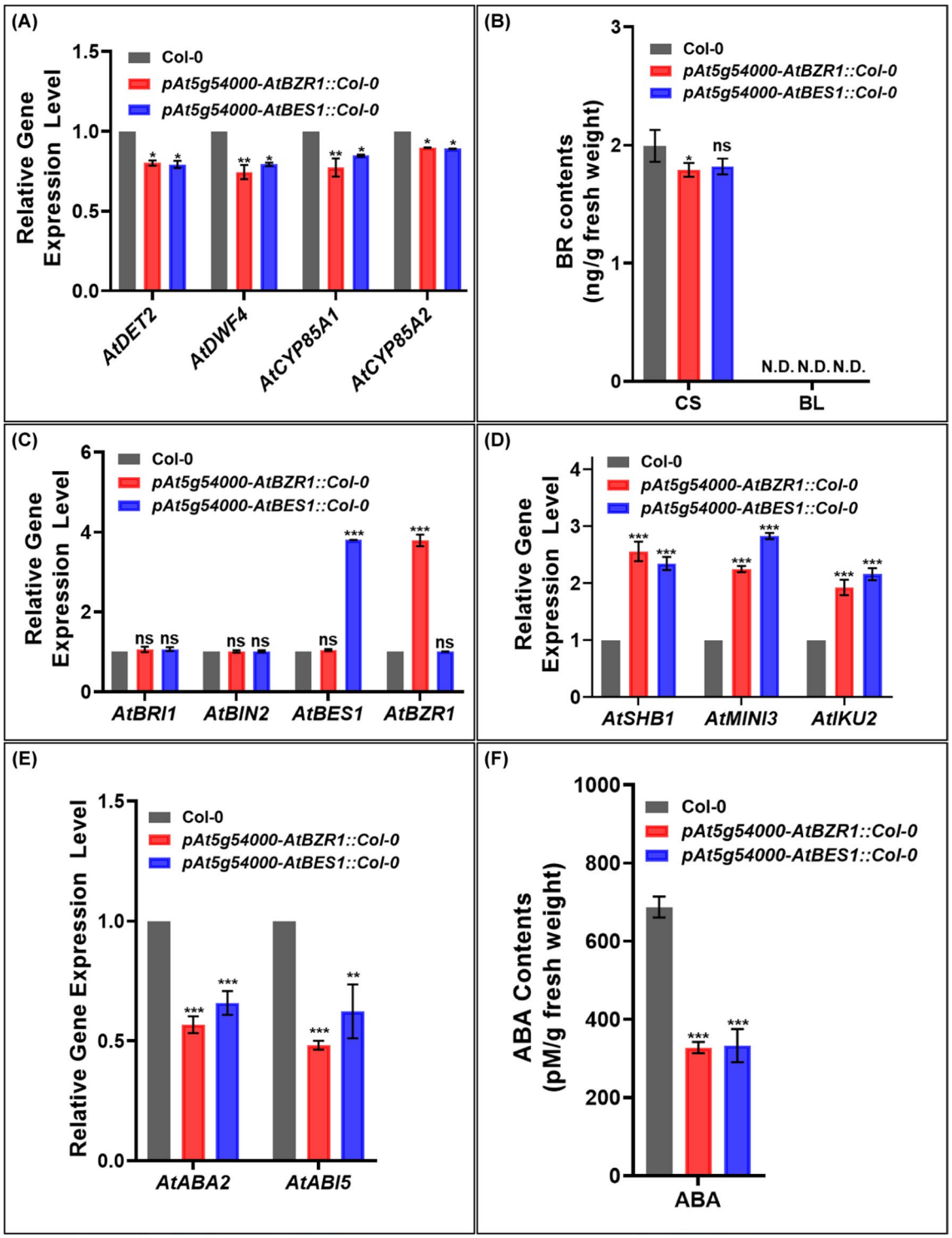


Fig. 2 Comparison of the growth and development of wild-type, *pAt5g54000-AtBZR1::Col-0,* and *pAt5g54000-AtBES1::Col-0* transgenic *Arabidopsis*. **A** Images of seedling and early rosette plant growth in the wild type and *pAt5g54000-AtBZR1::Col-0* and *pAt5g54000-AtBES1::Col-0* mutants. **B** Images of 3-week-old rosette plants of the wild type, *pAt5g54000-AtBZR1::Col-0*, and *pAt5g54000-AtBES1::Col-0.* **C** Images of 6-week-old intact plants of the wild type, *pAt5g54000-AtBZR1::Col-0*, and *pAt5g54000-AtBES1::Col-0.* **D** Number of branched stems, flowers, siliques, and seeds in siliques of the wild type, *pAt5g54000-AtBZR1::Col-0*, and *pAt5g54000-AtBES1::Col-0.* The white scale bar in **A** through **C** indicates 1 cm. **E** Images of mature seeds of the wild type, *pAt5g54000-AtBZR1::Col-0*, and *pAt5g54000-AtBES1::Col-0.* The white scale bar in **E** indicates 1 mm. **F** Length, width, and mass of mature seeds in the wild type, *pAt5g54000-AtBZR1::Col-0*, and *pAt5g54000-AtBES1::Col-0.* The data are represented as means ± SE calculated from at least 50 seeds independently collected from wild types and mutants. **G** Total carbohydrates, proteins, and lipids in seeds obtained from the wild type, *pAt5g54000-AtBZR1::Col-0*, and *pAt5g54000-AtBES1::Col-0.* For each analysis, 100 seeds of each line were used, and the experiments to analyze the endogenous content of each compound were carried out five times. The error bars indicate the standard errors. The asterisks in **D**, **F**, and **G** indicate statistical significance determined via Student's *t* test: *($P < 0.05$), **($P < 0.01$), and ***($P < 0.001$)



pAt5g54000-AtBES1::Col-0) indicated that the expression level of both *AtBZR1* and *AtBES1* was unchanged in the shoot and root but was significantly up-regulated in seeds (approximately 3.7 times and 3.9 times higher, respectively) compared to the wild-type levels (Fig. 1B). In the *GUS* report system, *GUS* activity was not detected in the root, stem, and leaf, whereas strong *GUS* activity was found in the siliques and seeds of both transgenic *Arabidopsis* (Fig. 1C). Therefore, our results confirmed the seed-specific introduction of *AtBZR1* and *AtBES1* into *pAt5g54000-AtBZR1::Col-0* and *pAt5g54000-AtBES1::Col-0*, respectively.

◂**Fig. 3** Changes in BR- and ABA-related gene expression and endogenous levels of BRs and ABA in *pAt5g54000-AtBZR1::Col*-0 and *pAt5g54000-AtBES1::Col-0* seeds. **A** Expression of BR biosynthetic genes in wild type, *pAt5g54000-AtBZR1::Col-0,* and *pAt5g54000-AtBES1::Col-0* seeds. **B** Endogenous level of active BRs in wild type, *pAt5g54000-AtBZR1::Col-0*, and *pAt5g54000-AtBES1::Col-0* seeds. BRs were quantified via GC–MS/SIM analysis with 3 g of seeds from the wild type, *pAt5g54000-AtBZR1::Col-0*, and *pAt5g54000-AtBES1::Col-0*. *CS* castasterone, *BL* brassinolide, *N.D.* not detected. **C** Expression of BR-signaling genes in seeds of the wild type, *pAt5g54000-AtBZR1::Col-0*, and *pAt5g54000-AtBES1::Col-0*. **D** Expression of *AtSHB1, AtMINI3*, and *AtIKU2* in seeds of the wild type, *pAt5g54000-AtBZR1::Col-0*, and *pAt5g54000-AtBES1::Col-0.* **E** Expression of *AtABA2* and *AtABI5* in seeds of the wild type, *pAt5g54000-AtBZR1::Col-0*, and *pAt5g54000-AtBES1::Col-0.* **F** Endogenous level of ABA in seeds of the wild type, *pAt5g54000-AtBZR1::Col-0*, and *pAt5g54000-AtBES1::Col-0.* Quantification of ABA was performed by ELISA analysis with 3 g of seeds from the wild type, *pAt5g54000-AtBZR1::Col-0*, and *pAt5g54000-AtBES1::Col-0*. The data in **A**, **C**, **D**, and **E** were obtained from at least three independent experiments and are presented as the mean ± SE. The data in **B** and **F** were obtained from three independent experiments and are presented as the mean ± SE. *ns* not significant. The asterisks in **A** through **F** indicate the statistical significance determined via Student's *t* test: *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$), and *ns* not significant

Compared to the wild type, *pAt5g54000-AtBZR1::Col-0* and *pAt5g54000-AtBES1::Col-0* exhibited no significant differences in the growth and development of seedlings and early rosette stages (Fig. 2A). In 3-week-old plants, the bolting and growth of inflorescent stems in both transgenic plants were accelerated, which promoted the growth and branching of inflorescent stems in 6-week-old *pAt5g54000-AtBZR1::Col-0* and *pAt5g54000-AtBES1::Col-0* (Fig. 2B–D). In terms of reproductive organ growth, the number of flowers, siliques, and seeds in a silique was greatly increased in *pAt5g54000-AtBZR1::Col-0* and *pAt5g54000-AtBES1::Col-0*. After maturation, the length, width, and weight of the seeds were increased, resulting in larger and heavier seeds in both transgenic *Arabidopsis* lines compared to their wild-type counterpart (Fig. 2E, F). The endogenous levels of primary metabolites, such as carbohydrates, proteins, and lipids, in the seeds of *pAt5g54000-AtBZR1::Col-0* and *pAt5g54000-AtBES1::Col-0* were higher by as much as approximately twofold compared to the wild type (Fig. 2G). Taken together, our findings demonstrated that both *pAt5g54000-AtBZR1::Col-0* and *pAt5g54000-AtBES1::Col-0* exhibited improvements in both seed yield and quality.

In seeds of *pAt5g54000-AtBZR1::Col-0* and *pAt5g54000-AtBES1::Col-0,* the expression of BR biosynthetic genes such as *AtDET2*, *AtDWF4, AtCYP85A1*, and *AtCYP85A2* was slightly down-regulated compared to that in the wild type (Fig. 3A). GC–MS/SIM analysis revealed that the endogenous level of active BRs, CS, and BL in transgenic seeds was slightly lower than that in wild-type seeds (Fig. 3B), indicating that the inserted *AtBZR1* and *AtBES1* weakly down-regulated the biosynthesis of BRs in seeds of *pAt5g54000-AtBZR1::Col-0* and *pAt5g54000-AtBES1::Col-0.* Nevertheless, the expression levels of BR-signaling genes, such as *AtBRI1*, *AtBIN2*, and *AtBES1*, in the transgenic line were similar to those in the wild type (Fig. 3C), suggesting that BR signaling, except activation of AtBZR1 and AtBES1, appears to be unaffected in the transgenic seeds. The increase in seed size appears to be regulated by the down-stream target genes, most likely *AtSHB1*, *AtMINI3*, and *AtIKU2*, which have been reported to act as positive regulators of seed size and mass in *Arabidopsis* (Jiang et al. 2013; Li et al. 2019). In fact, enhanced expression of *AtBZR1* and *AtBES1* increased the expression of *AtSHB1, AtMINI3*, and *AtIKU2* in seeds of *pAt5g54000-AtBZR1::Col-0* and *pAt5g54000-AtBES1::Col-0* (Fig. 3D), suggesting that the AtBZR1- and AtBES1-regulated signaling pathway of *AtSHB1* → *AtMINI3* → *AtIKU2* plays a role in increasing seed size in the transgenic *Arabidopsis*.

ABA is known as a negative regulator of seed size in plants (Finkelstein 2010; Li and Li 2016; Orozco-Arroyo et al. 2015). In *Arabidopsis, ABA Deficient 2 (AtABA2)*, which is involved in ABA biosynthesis, and *ABA Insensitive 5* (*AtABI5*), which encodes a major transcription factor in ABA signaling, are down-stream target genes for AtBZR1 (Skubacz et al. 2016; Yang et al. 2016). *AtABA2* and *AtABI5* expression is down-regulated by direct binding of AtBZR1 to the promoter, which reduces the expression of *AtSHB1, AtMINI3*, and *AtIKU2* by AtABI5 and ultimately increases *Arabidopsis* seed size (Yang et al. 2016). In this study, *AtABA2* expression was down-regulated in seeds of *pAt5g54000-AtBZR1::Col-0* and *pAt5g54000-AtBES1::Col-0,* which decreased the endogenous levels of ABA in the transgenic lines relative to the wild type (Fig. 3E, F). *AtABI5* expression was also down-regulated in the transgenic seeds, suggesting that both biosynthesis and signaling were suppressed compared to the untransformed seeds. Coupled with the aforementioned up-regulation of *AtSHB1, AtMINI3*, and *AtIKU2,* our findings suggested that seed-specific expression of *AtBZR1* and *AtBES1* can also increase seed size through down-regulation of ABA signaling via inhibition of *AtABA2* and *AtABI5* to activate expression of *AtSHB1*, *AtMINI3*, and *AtIKU2* in the transgenic *Arabidopsis*.

Discussion

AtSHB1, AtMINI3, and AtIKU2 operate in the same pathway to determine seed size in *Arabidopsis* (Zhou et al. 2009). They are positively regulated by BRs, and *AtSHB1* and *AtIKU2* are direct target genes of AtBZR1 (Jiang et al. 2013). When AtBZR1 binds to the promoter of *AtSHB1* and

AtIKU2, the expression of *AtSHB1, AtMINI3*, and *AtIKU2* is up-regulated and this is accompanied by an increase in seed size, suggesting that AtBZR1 can directly activate the pathway of AtSHB1 → AtMINI3 → AtIKU2 in the plant (Jiang et al. 2013). Unlike BRs, ABA negatively controls regulatory gene expression in *Arabidopsis* (Huang et al. 2017). AtABI5 binds to the promoter of *AtSHB1*, which down-regulates the expression of *AtSHB1* and its down-stream regulatory genes, resulting in seed size reduction in the plant (Cheng et al. 2014). AtBZR1 directly binds to the *AtABI5* promoter, resulting in the down-regulation of the AtABI5 transcription factor to increase seed size in *Arabidopsis* (Jiang et al. 2013; Jiang and Lin 2013; Luo et al. 2005; Sundaresan 2005). Therefore, AtBZR1 can also indirectly regulate the pathway of AtSHB1 → AtMINI3 → AtIKU2 via AtABI5-mediated ABA signaling in the plant. Recently, we found that AtBES1 can directly bind to the promotor of *AtABI5* in *Arabidopsis* (data to be published elsewhere). However, unlike AtBZR1, AtBES1 cannot directly bind to the promoter of *AtSHB1* (Jiang et al. 2013; Sun et al. 2010; Yu et al. 2011), suggesting that up-regulation of *AtSHB1*, *AtMINI3*, and *AtIKU2* by AtBES1 occurs through down-regulation of ABA signaling via *AtABI5*, but not by direct regulation of *AtSHB1* in *Arabidopsis*.

As mentioned above, the ability of AtBZR1-mediated signaling to increase seed size is relatively well established. However, changes in seed size in a dominant mutant of *AtBZR1, bzr1-D*, were not significant compared to those in other BR-related mutants (Jiang et al. 2013; Kim et al. 2021). This suggests that AtBZR1 is not a key transcription factor in BR-induced *Arabidopsis* seed size increases. However, our finding demonstrated that over-expressed *AtBZR1* in seeds increased seed size while maintaining high seed quality in Arabidopsis. The *AtBZR1-D* is an active version of *AtBZR1*. Therefore, seed-specific expression of *AtBZR1-D* (e.g., *pAt5g54000-BZR1-D*) will be more effective than that of *AtBZR1* (e.g., *pAt5g54000-BZR1*) in improving seed size, which can more clearly demonstrate the importance of *AtBZR1* in BR-induced increases in seed yield of Arabidopsis. Recently, we found that AtBES1 also plays an important role in BR-induced *Arabidopsis* seed yield increases (data to be published elsewhere). This indicates that both AtBZR1- and AtBES1-mediated BR signaling are important in determining seed size and that they function additively or competitively in the seed development of *Arabidopsis*.

The expression level of *AtBZR1* in *pAt5g54000-AtBZR1::Col-0* and *AtBES1* in *pAt5g54000-AtBES1::Col-0* was higher than that in *pAt5g54000-AtCYP85A2::Col-0* (Supplementary Fig. 1A). In addition, the expression of *AtSHB1*, *AtMINI3*, and *AtIKU2* in *pAt5g54000-AtBZR1::Col-0* and *pAt5g54000-AtBES1::Col-0* was activated compared to those in *pAt5g54000-AtCYP85A2::Col-0*, which increases the seed size in *pAt5g54000-AtBZR1::Col-0* and *pAt5g54000-AtBES1::Col-0* compared to that in *pAt5g54000-AtCYP85A2::Col-0* (Supplementary Fig. 1B). This suggests that seed-specific expression of BR signaling genes may be more effective than that of BR biosynthetic genes for seed size improvement in *Arabidopsis*. Nevertheless, the effect of seed-specificity introduced by *AtCYP85A2* on seed size was comparable to that of *AtBZR1* and *AtBES1*, indicating that seed-specific manipulation of both BR biosynthetic genes and BR-signaling genes are suitable biotechnical approaches for increasing seed yield and quality. To examine whether simultaneous expression of both biosynthetic and signaling genes in seeds can more strongly promote seed quality and yield, double-mutant *pAt5g54000-AtCYP85A2::Col-0* × *pAt5g54000-AtBZR1::Col-0/pAt5g54000-AtBES1::Col-0* and *pAt5g54000-AtCYP85A2-AtBZR1/AtBES1::Col-O* transgenic *Arabidopsis* are currently being constructed.

Monocotyledonous crops generally contain a BR transcription factor, such as OsBZR1 in rice, ZmBZR1 in corn (*Zea mays*), HvBZR1 in barley (*Hordeum vulgare*), and TaBZR1 in wheat (*Triticum aestivum*), indicating that BR-induced increases in grain yield are mediated by BZR1 transcription factors in monocotyledonous plants (Groszyk and Szechyńska-Hebda 2021; Liu et al. 2019; Zhang et al. 2020). A previous study demonstrated that the heterologous introduction of *Arabidopsis AtCYP85A2* into *B. distachyon*, a monocotyledonous model plant, successfully promoted grain yield and quality (Roh et al. 2021). Therefore, the seed-specific expression of *AtBZR1* established in our study could be applied to promote grain yield and quality in monocotyledonous crops. Alternatively, seed-specific over-expression of native *BZR1* in monocotyledonous plants is also a promising method for increasing BR activity to promote grain yield and quality in monocotyledonous crops.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12374-023-09387-4.

Acknowledgements We would like to thank Prof. Jeong Sheop Shin (Korea University, Seoul, Republic of Korea) for providing the seed-specific promoter construct *pAt5g54000/pBI101*. This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean Government's Ministry of Science, ICT, and Future Planning (2021R1A2C1007516 to S.-K. Kim).

Author Contributions S-KK, JR, and YEL planned the experiments; JR, YEL, and C-HP carried out the experiments; S-KK, JR, and YEL analyzed the data; S-KK, JR, and YEL wrote and revised the manuscript for publication.

Data Availability Statement The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials. Raw data that support the findings of this study are available from the corresponding author, S-K Kim, upon reasonable request.

Declarations

Conflict of Interest The authors have no potential conflicts of interest to disclose.

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