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RESEARCH PAPER

Brassinosteroids can regulate cellulose biosynthesis by controlling the expression of *CESA* genes in *Arabidopsis*

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Abstract

The phytohormones, brassinosteroids (BRs), play important roles in regulating cell elongation and cell size, and BR-related mutants in *Arabidopsis* display significant dwarf phenotypes. Cellulose is a biopolymer which has a major contribution to cell wall formation during cell expansion and elongation. However, whether BRs regulate cellulose synthesis, and if so, what the underlying mechanism of cell elongation induced by BRs is, is unknown. The content of cellulose and the expression levels of the cellulose synthase genes (*CESA*s) was measured in BR-related mutants and their wild-type counterpart. The chromatin immunoprecipitation (CHIP) experiments and genetic analysis were used to demonstrate that BRs regulate *CESA* genes. It was found here that the BR-deficient or BR-perceptional mutants contain less cellulose than the wild type. The expression of *CESA* genes, especially those related to primary cell wall synthesis, was reduced in *det2-1* and *bri1-301*, and was only inducible by BRs in the BR-deficient mutant *det2-1*. CHIP experiments show that the BR-activated transcription factor BES1 can associate with upstream elements of most *CESA* genes particularly those related with the primary cell wall. Furthermore, over-expression of the BR receptor *BRI1* in *CESA1*, *3,* and *6* mutants can only partially rescue the dwarf phenotypes. Our findings provide potential insights into the mechanism that BRs regulate cellulose synthesis to accomplish the cell elongation process in plant development.

Key words: *Arabidopsis*, brassinosteroids, cell elongation, cellulose, cellulose synthase, transcription factor.

Introduction

Cellulose is the most abundant polysaccharide on earth, determines the orientation of cell expansion, and provides supporting material for plant cells (Pauly and Keegstra, 2008). The cellulose-formed microfibrils, consisting of a linear chain of several hundred to over 10 000 β-1, 4-linked glucan, are synthesized by a plasma membrane-localized cellulose synthase complex (CSC). In vascular plants, multiple cellulose synthase catalytic subunits are required for cellulose synthesis (Taylor *et al.*, 2000). The complex extrudes up to 36 individual cellulose chains that are bound together to form the cellulose microfibril (Wightman and Turner, 2010).

The *CESA* gene superfamily, which encodes the catalytic subunits of cellulose synthase, has been identified in hundreds of seed plant species and characterized in *Arabidopsis*. The *Arabidopsis* genome contains ten *CESA* genes that include two groups with known function and some members with uncertain function (Richmond and Somerville, 2000, 2001). One group, including *CESA1*, *CESA3*, and *CESA6*, is preferentially expressed in expanding tissues (Desprez *et al.*, 2002; Doblin *et al.*, 2002; Robert *et al.*, 2004). Mutants of *cesA1, cesA3*, and *cesA6* are dramatically dwarfed or seedling-lethal. The other group

Abbreviations: BR, brassinosteroid; CESA, cellulose synthase; CSC, cellulose synthase complex; epiBL, 2, 4-epi-brassinolide.

includes *CESA4, CESA*7, and *CESA8*, and mutants of *cesA4, cesA7*, and *cesA8* lack the characteristic secondary thickening in xylem (Scheible *et al.*, 2001; Taylor *et al.*, 2000, 2003). Although, the functions of the remaining *CESA2, CESA5*, *CESA9,* and *CESA10* genes are poorly understood, recent studies have suggested that some of them may be functionally redundant and may compensate for the function of other *CESA*s under different physiological conditions (Desprez *et al.*, 2007; Persson *et al.*, 2007).

Plants have evolved complicated regulatory mechanisms, including the expression of *CESA* genes, modification of CESA protein, and intracellular trafficking and deposition of CSC subunits in the plasma membrane (Hématy and Höfte, 2006) to control cellulose biosynthesis and assembling in cell walls. Previous genetic and molecular studies have revealed that a transcription factor cascade, including NAC and MYB families, controls secondary cell wall thickening in fibres, vessels, and anthers (Mitsuda *et al.*, 2005, 2007; Yang *et al.*, 2007; Taylor, 2008; Zhong *et al.*, 2008; Zhou *et al.*, 2009). However, only transcription factor NST1/SND1 is known to be involved in cellulose synthesis by positively regulating the expressions of *CESA7* and *CESA8* (Zhong *et al.*, 2007). In addition, it was recently reported that the signalling molecule nitric oxide can promote cellulose synthesis, leading to the increase in cellulose content in primary cell walls in tomato roots (Aragunde *et al.*, 2008). Because cellulosic polysaccharides are the major part of the above-ground biomass (except grains) for many plant species, and more than 30–40% of dry matter in plants is cellulose (Pauly and Keegstra, 2008; Vogel, 2008), it is reasonable to speculate that these related signals and the supply of cellulose are likely to play an important role in biomass accumulation in plants.

It is well known that many plant hormones, such as brassinosteroids (BRs), auxins, gibberellins, and cytokinins play pivotal roles in regulating plant growth and height via promoting cell elongation and/or cell division (Gonzalez *et al.*, 2009). During cell rapid elongation, new cell wall polymers need a large amount of cellulose deposition (Caderas *et al.*, 2000; Refrégier *et al.*, 2004). In some economically important crops, plant height is a major factor in determining above-ground biomass productivity (Alexopouloua *et al.*, 2008; Yuan *et al.*, 2008). However, it is unknown whether cellulose synthesis contributes to hormone-regulated plant growth and height.

BRs are one class of plant-specific steroid hormones that are involved in many aspects of plant growth and development (Li and Jin, 2006; Divi and Krishna, 2009), particularly in cell elongation. The biosynthetic pathway of BRs, which includes several key genes, such as *CPD, DWF4,* and *DET2,* has been established in *Arabidopsis* (Fujioka, 1999; Sakurai, 1999). More recently, many major components of hte BR signalling pathway have also been identified. BRs are perceived by a receptor-like kinase BRI1 (Li and Chory, 1997; Wang *et al.*, 2001). The BR signal can activate a preformed homodimer of BRI1 (Wang *et al.*, 2005), and induce the dissociation of a negative regulator BKI1 from the plasma membrane (Wang and Chory, 2006). Upon BRI1 activation, BSKs may be phosphorylated to inactivate a GSK3-like protein kinase BIN2 via an unkown mechanism (Tang *et al.*, 2008). BIN2 kinase can phosphorylate and inhibit the class of plant-specific transcription factors, BES1/BZR1, which can directly bind to E-box (CANNTG) and BRRE (CGTGT/CG) elements in the promoter regions of many target genes to regulate their expression (Yin *et al.*, 2002; He *et al.*, 2005).

Previous studies implied that BRs may affect cell wall polymer formation, and the dwarf phenotype of the BR mutants is mainly caused by the reduced cell size, not by cell number (Kauschmann *et al.*, 1996). In cotton, it was reported that BRs are required for fibre initiation as well as elongation of cultured cotton ovules (Sun *et al.*, 2005; Luo *et al.*, 2007). In *Arabidopsis*, BR-deficient or BR-perceptional mutants display dramatically dwarfed phenotypes (Li *et al.*, 1996; Szekeres *et al.*, 1996). In rice, reduction of *OsDwarf2*/*OsDwarf1*, which encodes a C-6 oxidase required for BR biosynthesis, caused a reduced elongation of the second internode and seed length (Hong *et al.*, 2005; Tanabe *et al.*, 2005). In maize, the *dwf1* mutant is severely stunted and its encoded protein has 86% similarity with the rice DWF1 (Tao *et al.*, 2004). By contrast, an increased BR level or activity can enhance plant size, biomass accumulation, and seed yield (Salas-Fernandez *et al.*, 2009). However, the molecular mechanisms by which cellulose synthesis coordinates with the enlarged cell size caused by BR signalling are poorly understood.

In this study, biomass accumulation and cellulose content were measured in the BR-related mutants and a *BRI1* overexpression line at different developmental stages, and it was found that BRs positively regulate biomass accumulation and cellulose content. The expression levels of the *CESA* genes were then measured by quantitative RT-PCR (qRT-PCR) and a *GUS* reporter driven by the *CESA* promoters, and it was discovered that BRs promote the expression of most *CESA* genes in the short term and in the long term. Further chromatin immunoprecipitation (ChIP) analysis demonstrated that BR-activated transcriptional factor BES1 can bind to the promoter regions of nine *CESA* genes *in vivo*. Using transgenic approaches, it was also found that over-expression of *BRI1* in some *CESA* mutants cannot completely rescue their dwarf phenotype. Our results support the suggestion that BRs promote the expression of most *CESA* genes, which may play an essential role in regulating biomass accumulation in *Arabidopsis*.

Materials and methods

Plant materials, growth conditions, and hypocotyl elongation assay

A. thaliana ecotype Columbia (Col-0) was the wild type. The *CESA* mutants *rsw1-1* (CS848759), *ixr1-1* (SALK_019756), and *prc1-1* (SALK_004587), and the T-DNA insertion mutants, *ct-2* (SALK_091570), *ct-5* (SALK_023353), and *CT-9* (SALK_049129) were obtained from the ABRC (*Arabidopsis* Biological Resource Center). Homozygous insertion lines were verified by PCR and RT-PCR. Plants were grown on 1/2 MS plates or soil under long day (16/8 h light/dark) at 23 °C. For the hypocotyl elongation

assay, seeds were planted on 1/2 MS plates, kept at 4 °C for 2 d, and then grown in the dark for 4 d or in the light for 7 d. Thirty to forty seedlings were measured for each genotype. For epiBL treatment, the 11-d-old light-grown seedlings were treated with 5 μM epiBL or DMSO (as a control) for 2 h.

Biomass and cellulose measurements

Thirty to forty aerial seedlings in different developmental stages were collected and dried at 60 °C overnight. Then the dry weight was recorded. The dry stem was ground into a fine powder in liquid nitrogen. The powder was treated as described by Updegraff (1969). Cellulose was quantified colorimetrically using the anthrone-sulphuric acid method (Laurentin and Edwards, 2003).

Plasmid construction

The 2000 bp region of each *CESA* gene was amplified with Col-0 genomic DNA and was cloned into the pCAMBIA1300 vector. To make BRI1 over-expression plants, *BRI1* was fused with GFP into the vector of pCAMBIA2300, the resulting construct was transformed into Col-0.

CESA gene expression pattern analysis

Histochemical staining of plants expressing p*CESA*::GUS reporters was performed as described by Jefferson (1987). Digital images were taken with a Leica MZ FLIII stereomicroscope (Leica Microsystems, Germany).

Gene expression analysis

Total RNA was extracted from young seedlings using an RNeasy mini kit (Tiangen, http://www.tiangen.com). For RT-PCR, 2 μg of total RNA was reverse-transcribed with Super-Script II reverse transcriptase (TAKARA, http://www.takara.com.cn) as described by the manufacturer. Equal amounts of cDNA were used for PCR with 30–35 cycles. For quantitative real-time PCR, SYBR master mix (Invitrogen) and a Bio-Rad iCycler quantitative PCR system were used as described by the manufacturer. A U-box gene (*at5g15400*) was used to normalize the data (5′-TGCGCTGCCAGATAATACACTATT-3′ and 5′-TGCTGCCCAACATCAGGTT-3′).

ChIP assay

ChIP experiments were performed as described in the UPSTATE chip kit (http://www.millipore.com/catalogue/item/17-295) with 11-d-old Col-0 seedlings. The BES1 antibody was used to precipitate chromatin, and the GFP antibody was used as a control. Equal amounts of starting plant material and ChIP products were used for the quantitative real-time PCR reaction. Primers from 5S rRNA (used as an internal control) and *CESA*s were used to detect the corresponding *CESA* promoters in the ChIP products. The ChIP assays were repeated at least three times, and typical results were presented. The means and standard deviations were calculated from three biological repeats.

Results

BR signal influences the above-ground biomass accumulation in Arabidopsis

BRs are a major class of growth-promoting hormones. The BR-deficient or perceptional mutants are smaller than the wild type, while over-expression of BR-biosynthetic genes or the BR receptor *BRI1* led to bigger plants (Li *et al.*, 1996; Szekeres *et al.*, 1996). Furthermore, the application of BRs to the BR-deficient mutant *det2-1* significantly induced hypocotyl elongation (see Supplementary Fig. S1 at *JXB* online) (Li *et al.*, 1996; Szekeres *et al.*, 1996; Yin *et al.*, 2005). To test the function of BRs in *Arabidopsis* further, the dry weight of the aerial parts of several genetic materials, including a weak allele of *bri1*, *bri1-301*, the biosynthetic mutant of *det2-1*, a *BRI1-GFP* over-expression line, and a *35S-BES1-GFP* over-expression line, and the wild type Col-0, was measured at five different developmental stages (Stage I: having nine rosette leaves, the stage with vigorous vegetative growth; Stage II: initiation of bolting; Stage III: having one primary inflorescence with four nodes; Stage IV: having three side shoots on the primary inflorescence stalk; Stage V: having two or more secondary inflorescences, the end of plant growth) (Fig. 1A). Although the dry weight per seedling was similar between these

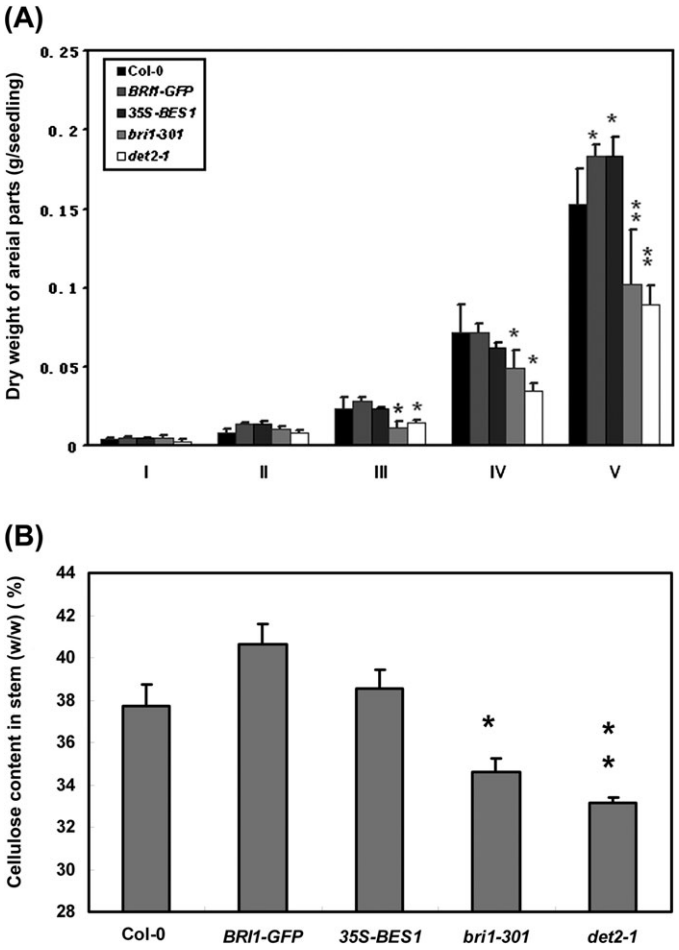

Fig. 1. BRs regulate biomass accumulation in aerial parts of *Arabidopsis*. (A) Biomass accumulation in aerial parts of BRs mutants at different developmental stages: I, having nine rosette leaves, the stage with vigorous vegetative growth; II, initiation of bolting; III, having one primary inflorescence with four nodes; IV, having three side shoots on the primary inflorescence stalk; V, having two or more secondary inflorescences, the end of plant growth. (B) Cellulose content in primary inflorescence stems of BR-related mutants at stages V. Data represent means (±SE) of four independent experiments. The asterisks indicate significant levels of $^{*}P$ <0.05 and $^{**}P$ <0.01, respectively.

mutants and the wild type at Stages I and II, it was found that, after bolting, there was a significant difference in the total dry weight among these materials. From Stage IV, the dry weights of *det2-1* and *bri1-301* were significantly less than that of the *BRI1-GFP* and *35S-BES1-GFP* lines. At Stage V, the dry weight per seedling of *det2-1* and *bri1-301* was only 0.1 g, while it was 0.18 g in the *BRI1-GFP* line. These results indicated that BR signalling is related to dry weight above ground.

Because cellulose usually takes a significant portion of the total biomass, it was also tested whether the cellulose content is correlated with the amount of total biomass among these materials. The cellulose content was measured in the stems at the final developmental stage and it was found that the cellulose content in *det2-1* and *bri1-301* was 8% and 12% lower than that that in wild type (P <0.05), respectively. Consistent with the biomass accumulation, the amount of cellulose in the *BRI1-GFP* line is 7% higher than that in the wild type (Fig. 1B), and in the *35S-BES1-GFP* line, the cellulose content is 3% higher than that in the wild type. These results suggest that BRs may promote cellulose biosynthesis, which contributes 30–40% of the total biomass accumulation (Pauly and Keegstra, 2008; Vogel, 2008).

BRs induce the expression of many CESA *genes*

To test whether the elevated cellulose content by BRs is caused by an elevated expression of *CESA* genes, the expression levels of the *CESA* genes were measured using qRT-PCR in the BR-related mutants and the wild type. Without a 2, 4-epi-brassinolide (epiBL) treatment, the expression levels of ten *CESA* genes in *det2-1* were reduced

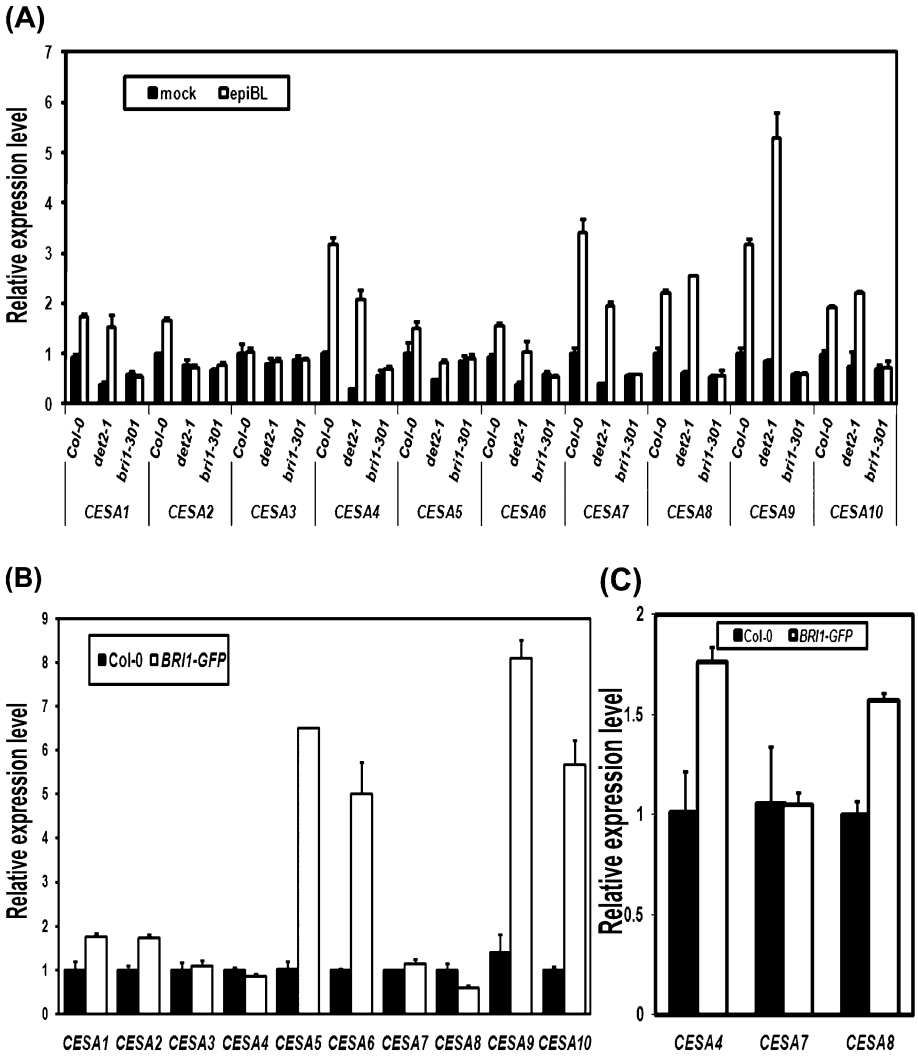

Fig. 2. The exogenously applied epiBL induces expression of *CESA* genes in *Arabidopsis*. These *CESA* genes include *CESA1*, at4g32410; *CESA2*, at4g39350; *CESA3*, at5g05170; *CESA4*, at5g44030; *CESA5*, at5g09870; *CESA6*, at5g64740; *CESA7*, at5g17420; *CESA8*, at4g18780; *CESA9*, at2g21770; and *CESA10*, at2g25540. (A) Relative expression levels of *CESA* genes in BR-deficient and BR-insensitive mutants. Quantitative real-time RT-PCR was conduced with total RNA from 9-d-old light-grown shoots with or without the treatment of 5 μM epiBL for 2 h. (B) Relative expression levels of *CESA* genes in the *BRI1-GFP* line. (C) Expression levels of *CESA4, 7,* and *8* in the first internode of primary inflorescence stems of the *BRI1-GFP* line. Quantitative real-time RT-PCR was performed using total RNAs from 9-d-old light-grown seedlings.

to about 50% of that in the wild type (Fig. 2A). After the application of epiBL to 9-d-old light-grown seedlings for 2 h, with the exception of *CESA2* and *CESA3* whose transcript levels were just slightly induced, the expression of the other *CESA* genes, including *CESA1, CESA4, CESA5, CESA6, CESA7, CESA8, CESA9*, and *CESA10*, were largely induced to approximately 1–5-fold higher than in the wild type and *det2-1* (Fig. 2A). While in the BR-insensitive mutant, *bri1-301*, the transcript levels of these *CESA* genes had no significant difference before and after epiBL treatment, indicating that BRs can rapidly induce the expression of *CESA*s through BR signalling.

The expression of these *CESA* genes was also tested in seedlings of the *BRI1-GFP* over-expression line. Compared with the wild type, the expression levels of *CESA1, CESA2, CESA5, CESA6, CESA9,* and *CESA10* in the *BRI1-GFP* line were much higher than in the wild type (Fig. 2B). While the expression of *CESA3, CESA4, CESA7,* and *CESA8* was not altered in the young seedlings of the *BRI1-GFP* line (Fig. 2B). Because *CESA 4, 7,* and *8* mainly synthesize cellulose used for the secondary cell wall, the expression levels of these genes was then measured in primary inflorescence stems at Stage V, which should contain more secondary cell wall, and it was found that, except for *CESA7*, the expression of *CESA4* and *CESA8* was significantly enhanced in the *BRI1-GFP* line (Fig. 2C). In summary, these results suggest that BR signalling enhances *CESA* gene expression for both primary and secondary growth.

To understand the biological function, expression pattern, and regulation of these *CESA* genes further, transgenic lines harbouring a beta-glucuronidase (*GUS*) reporter gene driven by the promoters of ten *CESA* genes in *Arabidopsis* were made. It was found that *CESA1, CESA2*, and *CESA6*, which showed similar expression patterns, were mainly expressed in the elongation zone of roots, in the veins of cotyledons and buds, and were weakly expressed in the leaves of light-grown seedlings (Fig. 3A, B, F). Because *CESA1* and *CESA6* are mainly involved in primary growth, this result indicates that *CESA2* may also participate in primary growth. The expression levels of *CESA3* and *CESA5* were hardly detected without BR treatment (Fig. 3C, E), and their expression was only detected in the root or shoot tips following BR treatment (Fig. 3J, L). *CESA9* was mainly expressed in the vasculature of cotyledons and shoot tips (Fig. 3G, N). Although *CESA4* was expressed in the veins of cotyledons (Fig. 3D), *CESA7* and *CESA8* were only expressed in the vascular tissue of flowers, stamens, and stems at the later developmental stages, and their expression was barely detected in young seedlings, further demonstrating their important role in secondary growth (see Supplementary Fig. S2 at *JXB* online). However, *CESA10* expression was not detected in young seedlings. These transgenic lines were also used to test whether *CESA* genes can be regulated by BRs. When these reporter lines were grown on medium containing 10 nm epiBL, compared with the untreated seedlings, the GUS-staining

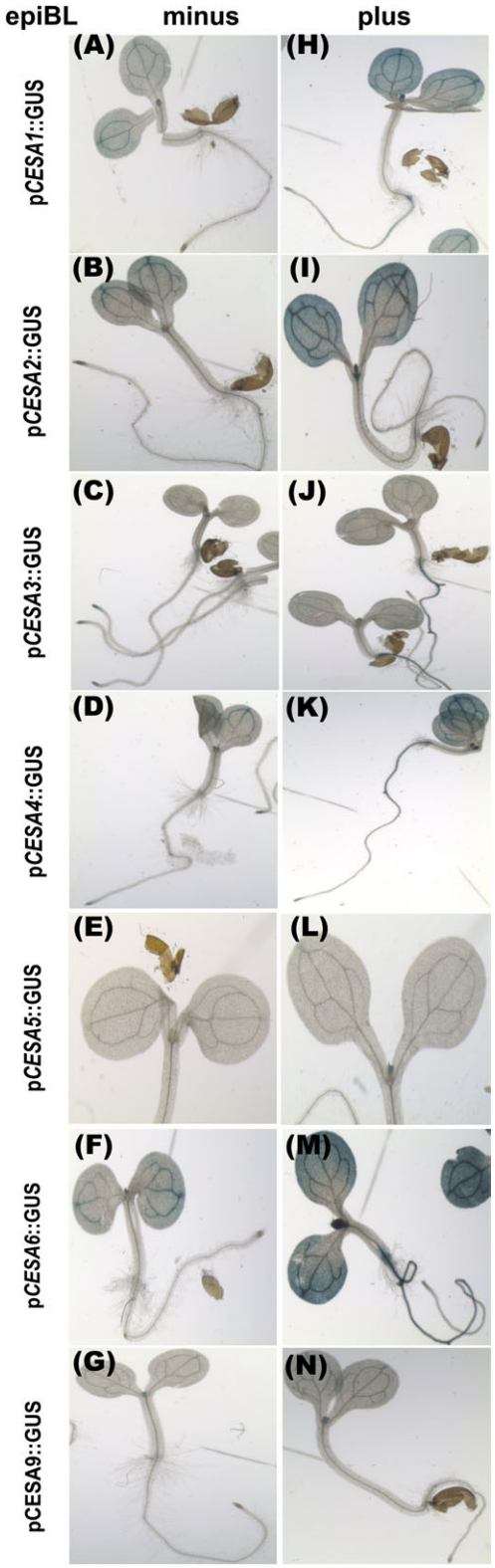

Fig. 3. The GUS staining in transgenic seedlings with *CESA* promoter-driven GUS reporters is enhanced on the medium containing epiBL. The expression pattern and regulation by BRs of *CESA* genes are shown by GUS-staining of *CESA* promoter::GUS reporters in 5-d-old light-grown seedlings on the 1/2 MS medium (A–G), or on the 1/2 MS medium containing 10 nm epiBL (H–N).

of all these transgenic lines was enhanced (Fig. 3H–N), which was consistent with our qRT-PCR analysis (see Supplementary Fig. S3 at *JXB* online). The expression of the *CESA4* gene in long-term epiBL treatment is slightly different from the pattern of GUS-staining (see Supplementary Fig. S3 at *JXB* online; Fig. 3D), so it is likely that the *CESA4* promoter (1.9 kb) is not long enough to drive the *GUS* reporter.

BR-activated transcription factor BES1 can associate with upstream elements of most CESA *genes* in vivo

To investigate whether the enhanced expression of these *CESA* genes by BR signalling is directly through the BR-activated transcription factor BES1, a ChIP assay was then used to test whether BES1 can bind to the promoter regions of these *CESA*s. The CANNTG E-box is a primary binding element of BES1 (Yin *et al.*, 2005), so RSTA (http://rsat.ulb.ac.be/rsat/) was used to predict how many potential binding sites of BES1 are present in the approximately 2000 bp region of each *CESA* promoter (see Supplementary Fig. S4A at *JXB* online). ChIP experiments were conducted with the anti-BES1 antibody and a control antibody, anti-GFP, and the promoter region of *ACT2* (*at3g18780*), which does not contain any predicted BES1 binding sites, was used as a negative control. The positive control was *at3g23770* (Ye *et al.*, 2010). qPCR was used to detect the enrichment of ChIP products in the promoter region containing E-boxes (Fig. 4; see Supplementary Fig. S4B–K at *JXB* online). It was found that BES1 can specifically pull-down DNA fragments from the promoter regions of nine *CESA*s, but not *CESA7*. One or more binding sites were found in promoter region of *CESA* genes, with an enrichment of 1.5–5-fold (Fig. 4; see Supplementary Fig. S4B–K at *JXB* online). Taken together, it was concluded that the transcriptional factor BES1 can regulate the expression of most *CESA* genes by binding to their promoters, mainly through the E-box elements.

CESA*s act downstream of BR signalling in plant development*

To determine whether BR signalling functions at upstream of *CESA*s to regulate cellulose synthesis, genetic analysis was conducted with some available *CESA* mutants: *rsw1-1*, a point mutation of *CESA1*; *ixr1-1,* a mutation of *CESA3*; *prc1-1*, a null mutant of *CESA6;* and *ixr2-1*, a weak allele of *CESA6* were obtained. The light-grown seedlings of these *CESA* mutants were slightly smaller than those of the wild type under normal growing conditions (Fig. 5A, D, G, J). Double mutants of *bri1-301* were then generated with some of these *CESA* mutants, including *bri1-301 rsw1-1, bri1-301 ixr1-1,* and *bri1-301 prc1-1*. All of these double mutants showed severely dwarf phenotypes (Fig. 5B, E, H, K), indicating that the *CESA* mutation can enhance the dwarf phenotype of *bri1-301*. Interestingly, although over-expression of the BR receptor *BRI1* in these *CESA* mutant backgrounds can make plants larger and enhance leaf petiole elongation, they were still smaller than *BRI1-OX* (Fig. 5C, F, I, L). These results suggested that the enhanced BR signalling cannot completely rescue the dwarf phenotype of the

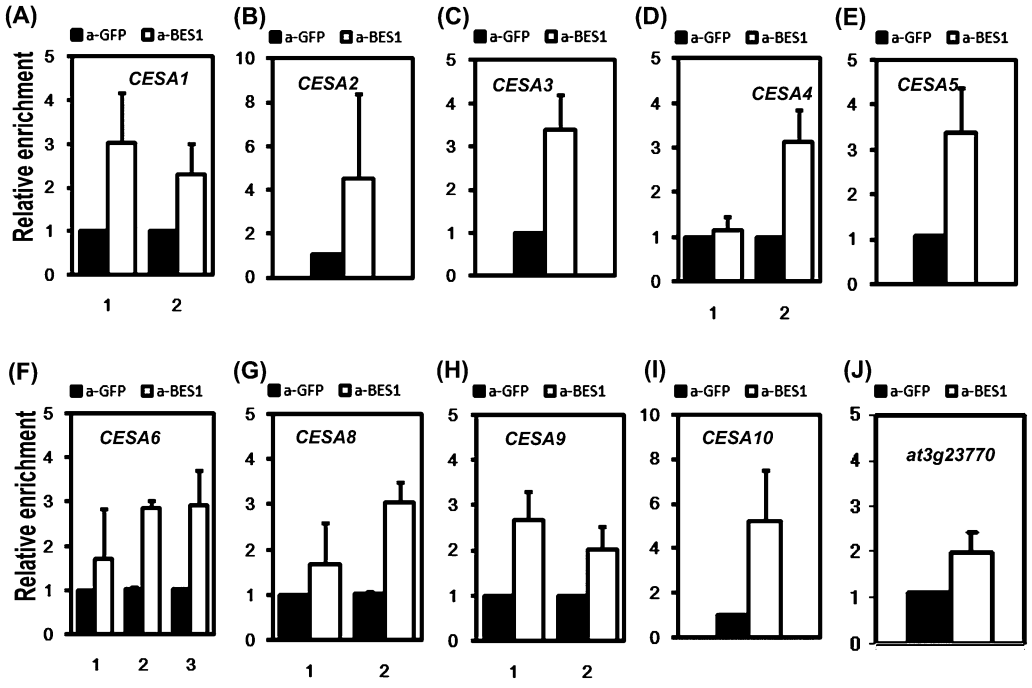

Fig. 4. BES1 can associate with the promoter regions of most *CESA* genes. The BES1 antibody and the GFP antibody (as a negative control) were used to immunoprecipitate chromatin prepared from 14-d-old light-grown Col-0 seedlings. Quantitative real-time PCR was performed using primers from the indicated positions of *CESA* promoters. The fold changes were calculated based on the change for anti-BES1 relative to anti-GFP, after normalization to 5S rRNA, an internal control. (A) *CESA1*; (B) *CESA2*; (C) *CESA3*; (D) *CESA4*; (E) *CESA5*; (F) *CESA6*; (G) *CESA8*; (H) *CESA9*; (I) *CESA10*; (J) the positive control *at3g23770*.

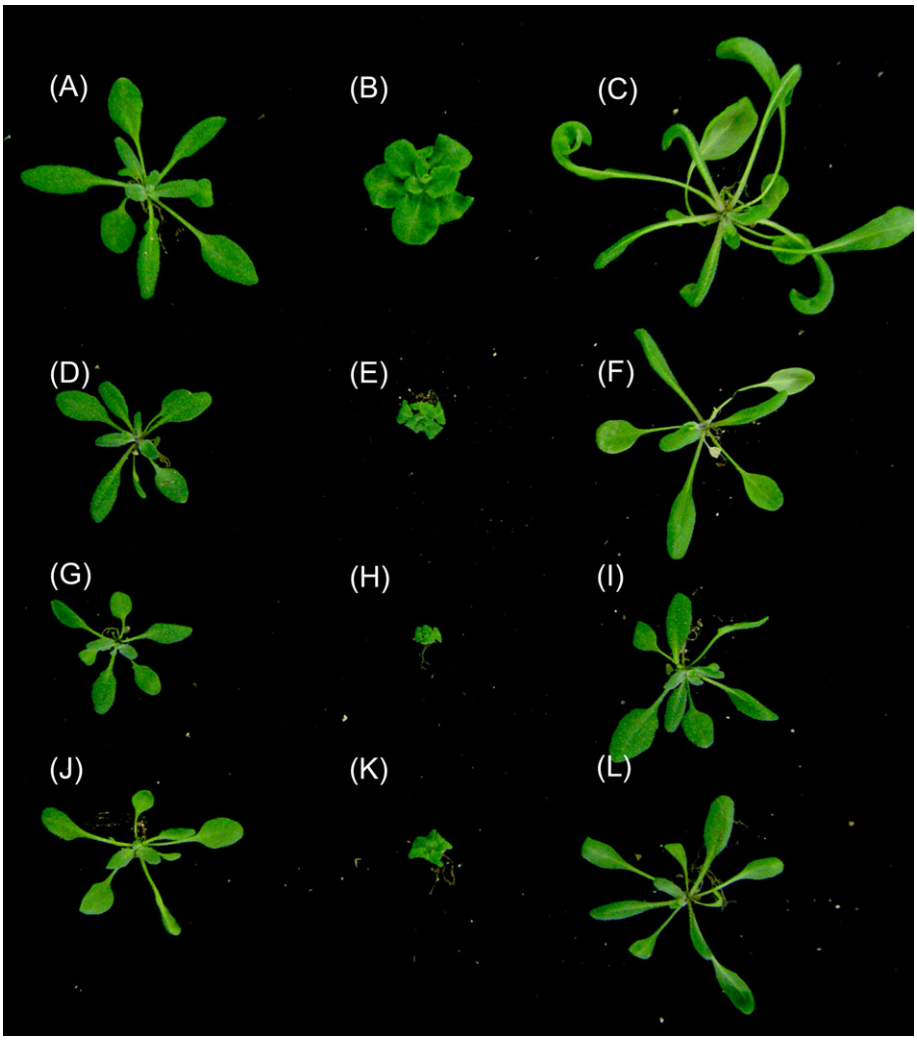

Fig. 5. The *CESA* mutant enhances the dwarf phenotype of *bri1-301*, and over-expression of *BRI1* in *CESA* mutants cannot completely rescue their dwarf phenotype. (A) Col-0; (B) the BR receptor mutant *bri1-301*; (C) the *BRI1-GFP* over-expression line; (D) *rsw1-1*, a *CESA1* mutant (Ala549Val); (E) *rsw1-1 bri1-301* double mutant; (F) the over-expression line of *BRI1-GFP* in *rsw1-1*; (G) *ixr1-1*, a *CESA3* mutant (Gly998Asp); (H) *ixr1-1 bri1-301* double mutant; (I) the over-expression line of *BRI1-GFP* in *ixr1-1*; (J) *prc1-1*, a *CESA6* mutant (Q720STOP); (K) *prc1-1 bri1-301* double mutant; (L) the over-expression line of *BRI1-GFP* in *prc1-1*.

CESA mutants, and a co-ordinated expression of many *CESA* genes downstream of BR signalling is required for plant development.

To test the ability of *CESA* mutants to respond to the applied BRs, the hypocotyl lengths of the wild type, *det2-1*, *rsw1-1, ixr1-1*, *prc1-1, ixr2-1,* and three T-DNA knockout mutants, *ct-2* (*CESA2*), *ct-5* (*CESA5*), and *ct-9* (*CESA9*) (Persson *et al.*, 2007; Desprez *et al.*, 2007) were measured with or without an epiBL treatment. Although the weak alleles of *rsw1-1, ixr1-1*, and *ixr2-1* can respond to BRs in the light or in the dark as indicated by a longer hypocotyl after the epiBL treatment, their hypocotyl was still shorter than that of the wild type (Fig. 6A, B), suggesting that *CESA1, 3,* and *6* are required for hypocotyl elongation promoted by epiBL. Moreover, the applied epiBL can largely promote hypocotyl elongation of *ixr2-1* under both light and dark conditions, but it can only slightly promote the hypocotyl elongation of its non-allele *prc1-1* in the light and cannot promote the hypocotyl elongation of *prc1-1* in the dark (Fig. 6A, B; see Supplementary Fig. S5 at *JXB* online), indicating that *prc1-1* was less insensitive to BRs both in light and dark. Because *CESA6* is mainly involved in cellulose synthesis in the primary cell wall (Richmond and Somerville, 2000, 2001), it was suggested that *CESA6* probably plays a key role in regulating BR-induced hypocotyl elongation and the knockout mutants *ct-2, ct-5* and *ct-9* were shown to have similar phenotypes to the wild type Col-0, suggesting that they are not essential in mediating BR-promoted cell elongation, or they are functionally redundant with other *CESA*s.

To investigate why the hypocotyl elongation of *prc1-1* is normal in the light, but dramatically shorter than the wild type in the dark, the gene expression of all *CESA* genes in

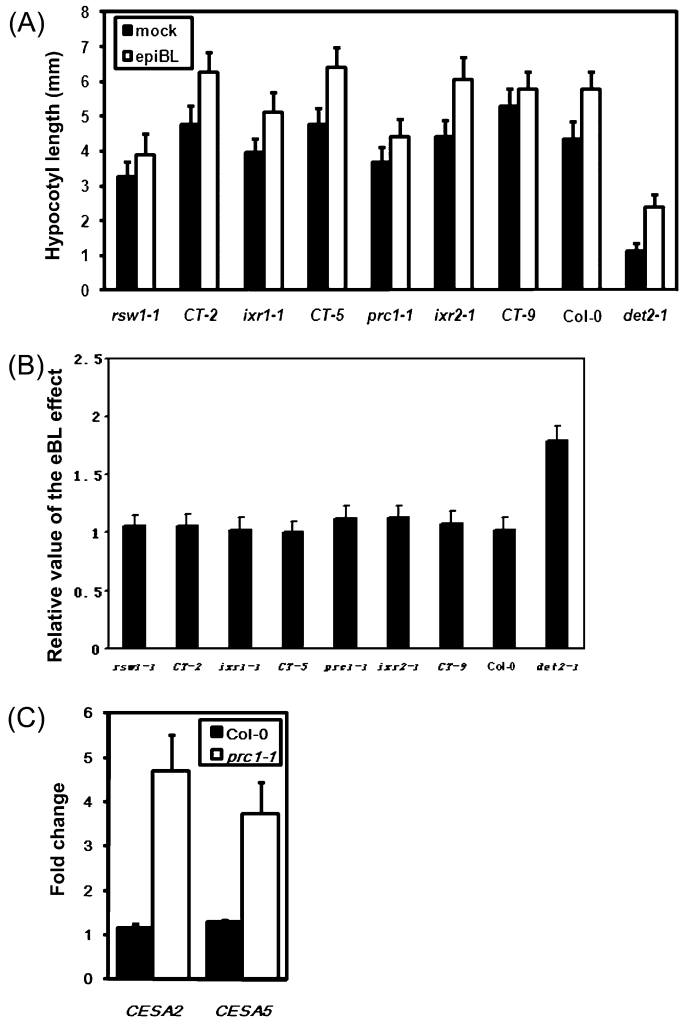

Fig. 6. *CESA* mutants show an altered BR response. (A) Hypocotyl length of 7-d-old light-grown seedlings of *rsw1-1*, *CT-2* (SALK_091570), *ixr1-1*, *CT-5* (SALK_023353), *prc1-1*, *ixr2-1*, *CT-9* (SALK_049129), Col-0, and *det2-1* grown on the 1/2 MS medium with or without 10 nM epiBL. Means and standard deviations were calculated from 30–45 seedlings. (B) The relative value of hypocotyl elongation of 4-d-old dark-grown seedlings in the absence or presence of 10 nM epiBL. (C) The fold changes of *CESA2* and *CESA5* expression in the light, compared to that in the dark. Quantitative real-time PCR was performed using 7-d-old light-grown seedlings and 4-d-old dark-grown seedlings of *prc1-1* and the wild type.

prc1-1 and the wild type was measured under both light and dark conditions. Previous studies have revealed that over-expression of *CESA2* and *CESA5* can partially rescue *prc1-1* (Desprez *et al.*, 2007; Persson *et al.*, 2007). In this study, the levels of *CESA2* and *CESA5* gene expression induced by BRs in the light is about 4.7-fold and 3.5-fold of that in the dark, respectively (Fig. 6C). Together with *CESA2* and *CESA5* being up-regulated with epiBL treatment (Fig. 2A), it is suggested that BR-induced expression of *CESA2* and *CESA5* in *prc1-1* may promote cellulose synthesis in order to keep the cell elongating in the light.

Discussion

In this study, several lines of evidence have been provided to conclude that BRs can induce the expression of most *CESA* genes. First, our data clearly demonstrated that BRs regulate the expression of multiple *CESA* genes in both the short term and the long term. Although most of the *CESA*s are induced by epiBL in 2 h, the *CESA* genes related to secondary cell wall synthesis were not induced in long-term treatments in young seedlings (Figs 2C, 3; see Supplementary Fig. S3 at *JXB* online). Furthermore, *CESA9* and *CESA10*, which are mainly expressed in flowers, also showed the similar induction (Fig. 3; see Supplementary Fig. S3 at *JXB* online). It is suggested that BRs regulate different sets of *CESA* expression at different developmental stages. In addition, the BR-related mutants, *bri1-301* and *det2-1* are both dwarf plants with a similar developmental speed, but the expression of *CESA*s in the two mutants differ in their response to the epiBL treatment (Fig. 2A), demonstrating that the BR signal is important for inducing *CESA* expression. When BR signalling was enhanced in the *BRI1-GFP* over-expression line, the expression of these *CESA* genes was significantly increased (Fig. 2B). Moreover, the dry weight of aerial parts and the cellulose content in the stems of BR's related materials (Fig. 1) suggested that the BR signal promotes above-ground biomass accumulation.

Our results also indicate that BRs may control both primary and secondary growth through regulating the expression of different sets of *CESA*s. During primary cell wall synthesis, the CSC, containing CESA1, CESA3, and CESA6, synthesizes cellulose microfibres mainly for cell enlargement and cell elongation. A previous study has shown that the dwarf phenotype of the BR mutants is mainly caused by a reduced cell size, not by cell number (Kauschmann *et al.*, 1996) and our results also proved that the non-allele of *cesA6*, *prc1-1* cannot respond to exogenous BRs. Therefore, the BR-induced *CESA* genes in young seedlings (Figs 2, 3) may be essential for hypocotyl elongation. Our data also indicate that these *CESA*s with unknown function, including *CESA2, CESA5*, and *CESA9*, also participate in BR-induced cell elongation in young seedlings. Furthermore, BRs also induce the expression of *CESA4* and *CESA8* involved in the secondary growth in the stem (Fig. 2C). Interestingly, at the early developmental stages, the dry weight above ground per seedling between the BR-related mutants and the wild type was not much different (Fig.1), but after bolting, the height and dry weight of the BR-deficient mutants were dramatically lower than those in the wild type. Thus, at the early stages, BR signalling can regulate the expression of the *CESA* genes mainly involved in primary growth to promote cell elongation, while at the later developmental stages; BR signalling may affect secondary growth by regulating *CESA4* and *CESA8* expression.

The BR-activated BES1 can regulate many developmental processes probably by controlling the expression of many structural and regulatory genes. Previous microarray data indicate that the *CESA5* (*at4g38850*) is up-regulated in

bzr1-D, a gain-of-function mutation of *BZR1* (He *et al.*, 2005). Microarray data also indicate that most *CESA* genes are induced solely following BR treatment (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi; http://www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp). Recently, it was reported that BRs can directly regulate the expression of many key genes involved in pollen and anther development (Ye *et al.*, 2010). In this study, the ChIP assay and genetic studies indicated that *CESA* genes act downstream in the BR signalling to regulate hypocotyl elongation and plant growth. Recently, a chip on chip study by two groups showed the interesting result that *CESA6* is the target of BZR1 (Sun *et al.*, 2010; Yu *et al.*, 2011), However, none of these *CESA* genes was identified as a BES1 target by Yu *et al.*'s study (Yu *et al.*, 2011). It suggested that data from the chip on chip assay may miss some important genes.

Like BRs, many other hormones, including auxins, gibberellins, and cytokinins, can also significantly promote plant growth and plant size. For instance, the enhanced expression of *AVP*, which encodes a vacuolar H^+-pyrophosphatase, and auxin-regulated genes, *ARGOS* and *ARF2* (in the auxin signalling pathway), apparently regulate plant size by promoting cell division (Hu *et al.*, 2003; Li *et al.*, 2005; Okushima *et al.*, 2005). In addition, over-expression of *GA20ox* required for GA biosynthesis can regulate plant size through regulating cell division and expansion (Coles *et al.*, 1999). Recently, a cytokinin binding protein HOG1 has been shown to promote leaf size and seed yield (Godge *et al.*, 2008). However, little is know about how the co-expressed genes related to cell wall expansion, cell wall formation, and the cell cycle, are regulated by various growth-promoting hormones. A large set of microarray data suggest that different hormones may regulate distinct sets of gene families in the same process of plant growth and development (Nemhauser *et al.*, 2006). Interestingly, according to our analysed chip data, it is suggested that most *CESA* genes can be induced by BRs, but other growth-promoting hormones do not have such broad effect on the expression of *CESA* genes (https://www.genevestigator.com/gv/user/serveApplet.jsp) (see Supplementary Fig. S6 at *JXB* online).

Based on our findings and previous observations, a model to illustrate the role of BRs in plant growth in *Arabidopsis* is proposed (Fig. 7). During primary growth, BR signalling activates the transcription factor BES1 and promotes BES1 to associate with the E-boxes of the *CESA1*, *CESA3*, and *CESA6* promoters to enhances their expression and cellulose biosynthesis in cell elongation. Following primary growth, the expression of *CESA4* and *CESA8*, which are also promoted by BES1, contributes to plant height and secondary growth. Apparently, almost all *CESA* genes for primary and secondary cell wall accumulation are regulated by BR signalling in order to provide adequate cellulose to sustain the architecture of enlarged cells. Without CESA6, the CSC cannot generate enough new cellulose for primary cell wall deposition and so the hypocotyl cannot elongate in the dark. In the light, the BR signal can regulate the expression of *CESA2, CESA5*, or *CESA9* and *CESA10*, which partially substitute for the function of *CESA6* and rescue the dwarf phenotype of *prc1-1*.

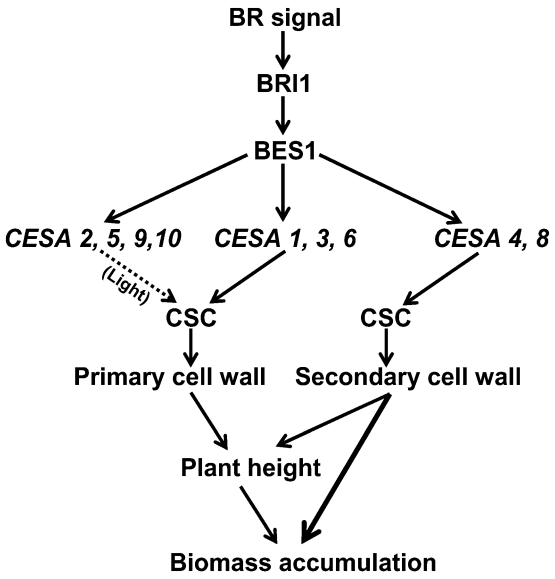

Fig. 7. A model to illustrate the mechanism of BR-regulated *CESA* expression in plant growth. The BR signalling activates the transcription factor BES1 and promotes its binding to the E-boxes of *CESA1*, *CESA3*, and *CESA6* promoters to enhance their expression during the primary growth. BES1 can also promote the expression of *CESA4* and *CESA8* for the secondary growth. These *CESA*s provide more cellulose to sustain the architecture of enlarged cells in cell elongation. BR signalling can regulate the expression of *CESA2, CESA5*, or *CESA9* and *CESA10*, which partially substituted for the function of *CESA6* and rescued the dwarf phenotype of *prc1-1* in the light.

This study on the mechanisms of BR regulating cellulose synthesis provides significant insights into the hormonal regulation of cellulose accumulation in *Arabidopsis*, but there are still many questions that remain to be studied further. First, many phytohormones can regulate plant size, but what their mechanisms are and how they cross-talk to regulate this process is still unknown. Second, it is known that different cellulose synthase complexes may function at different developmental stages and in different tissues. How cellulose biosynthesis is regulated, especially by BR, in different tissues and at different developmental stages is still poorly understood. Third, the applied epiBL can significantly induce the expression of *CESA7*, but BES1 cannot bind to the promoter region of *CESA7*, suggesting that the expression of *CESA7* may be regulated by other unknown mechanisms.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1. BRs induce hypocotyl elongation.

Supplementary Fig. S2. *CESA7, CESA8*, and *CESA10* were expressed in flowers or stems.

Supplementary Fig. S3. Expression of *CESAs* was enhanced in seedlings grown on the 1/2 MS medium containing BRs.

Supplementary Fig. S4. BES1 can associate with the upstream elements of C*ESA* genes.

Supplementary Fig. S5. The *CESA* mutants show an altered BR response.

Supplementary Fig. S6. Microarray data shows the regulation of *CESA* expression by various plant hormones. (https://www.genevestigator.com/gv/user/serveApplet.jsp).

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