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ORIGINAL ARTICLE

Brassinosteroid is required for sugar promotion of hypocotyl elongation in *Arabidopsis* in darkness

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Abstract

Main Conclusion **Brassinosteroid is necessary for sugar promotion of *Arabidopsis* hypocotyl elongation in darkness, and sugar positively regulates BRASSINAZOLE RESISTANT1 (BZR1) at both transcription and protein levels.**

Sugar has the ability to induce *Arabidopsis* hypocotyl elongation in the dark, but the detailed mechanisms remain not well understood. Here, we report that the steroidal phytohormone brassinosteroid (BR) is involved in sugar promotion of hypocotyl elongation in the dark. Sugar-induced hypocotyl elongation was significantly repressed in the BR-deficient mutant *det2-1*, BR-insensitive mutant *bri1-5*, and wild-type plants (Col-0), but not in the BR-hypersensitive mutants *bzr1-1D* and *bes1-D* treated with the BR biosynthetic inhibitor brassinazole (BRZ). Sugar also up-regulated the expression of genes that are related to cell elongation in a BR-dependent manner, and this effect was more remarkable in *bzr1-1D* and *bes1-D* than in their corresponding wild types in the presence of BRZ, suggesting an important role of BZR1 and bri1-ems-suppressor 1 (BES1) in this process. Sugar treatment seems to have little effect on BR biosynthesis, but enhances the expression of *BZR1* and *BES1*, two transcription factors in BR signaling, in the dark. Furthermore, sugar treatment maintains higher BZR1 protein levels in plants grown in the dark. Collectively, our results indicate that BR is required for sugar promotion of hypocotyl elongation in darkness in *Arabidopsis*.

Keywords Brassinolide · Brassinosteroid · Dark · Hypocotyl elongation · Signaling · Sugar

Abbreviations

BES1	Bri1-ems-suppressor 1
BL	Brassinolide
BR	Brassinosteroid
bri1	*Brassinosteroid insensitive 1*
BRZ	Brassinazole
BZR1	Brassinazole resistant 1
CPD	Constitutive photomorphogenic dwarf
det2	*De-etiolated 2*
DWF4	Dwarf 4
EXPA8	Expansin A8
EXPL2	Expansin-like A2
Glc	Glucose
IAA19	Indole-3-acetic inducible 19
Mtl	Mannitol
PP2A	Protein phosphatase 2A
PRE1	Paclobutrazol resistance 1
PRE5	Paclobutrazol resistance 5

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qRT-PCR	Quantitative reverse transcription polymerase chain reaction
Suc	Sucrose
XTH18	Xyloglucan endotransglucosylase/hydrolase 18

Introduction

In plants, cell elongation and seedling growth are controlled by multiple environmental factors and endogenous hormones, including light, sugar, brassinosteroid (BR), gibberellin and auxin, but how these different signals coordinately regulate the same cellular and physiological responses remains an outstanding question to answer (Vert and Chory 2011; Eveland and Jackson 2012). In particular, sugar and BR are both required for hypocotyl elongation in response to darkness (Zhang et al. 2010; Stewart et al. 2011; Oh et al. 2012). However, how they cross talk to mediate this process is poorly understood.

Sugars are essential for the fundamental processes that are required for plant growth, and its metabolism and utilization must be coordinated with photosynthate availability, environmental cues, and the timing of key developmental programs (Lastdrager et al. 2014). Sugars, such as sucrose (Suc) and glucose (Glc), not only function to fuel cellular carbon and energy metabolism, but also act as signaling molecules and global regulators of gene expression required for plant growth and development including seed germination, floral transition, fruit ripening, embryogenesis, and senescence (Rolland et al. 2002, 2006; Smeekens et al. 2010). In the plant's daily life, the form and abundance of carbon are adjusted to meet the plant's metabolic needs. During daytime, excess fixed carbon is stored as starch in the chloroplasts of photosynthetic cells. At night, starch is broken down and gradually converted into Suc which travels from the leaves into the rest of the plant (Smith and Stitt 2007; Graf et al. 2010). The degradation of starch into Suc is highly correlated with growth and tightly regulated to prevent the plant from exhausting its resources (Smith and Stitt 2007). Suc is a dominant regulator of growth processes in plants, but its sensor proteins are yet to be identified (Lastdrager et al. 2014). *Arabidopsis* Hexokinase 1 (HXK1) is a Glc-phosphorylating enzyme that also serves as a Glc-sensing protein. The role of HXK1 as a Glc sensor and signal transducer is independent of its enzymatic function (Moore et al. 2003). During regulation of plant growth and development, cellular sugar signaling must be integrated with other growth regulatory pathways, particularly light and phytohormone signaling (Rolland et al. 2006; Eveland and Jackson 2012; Lastdrager et al. 2014).

Light can not only stimulate the production of sugars, but also act as a signal affecting plant growth and development throughout the entire life from germination to flowing. The development of plants in light is referred to as photomorphogenesis, whereas development in the dark is referred to as skotomorphogenesis (Chory 2010). Light switches the developmental program of seedlings from skotomorphogenesis to photomorphogenesis, causing inhibition of hypocotyl elongation, cotyledon opening and expansion, and chloroplast development. At present, the CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1)-based protein complex is considered to play a critical role in skotomorphogenesis in the dark. COP1, which is an ubiquitin E3 ligase, can constantly degrade a number of transcription factors that are required for development in light, such as the bZIP transcription factor LONG HYPOCOTYL5 (HY5) (Lee et al. 2007), but allows accumulation of others that promote etiolated growth, such as PHYTOCHROME INTERACTING FACTORS (PIFs), several of which have been shown to directly interact with light-activated phytochromes and subsequently be targeted for degradation via the ubiquitin–proteasome system (Leivar and Quail 2011). Hence, the COP1 and PIFs are regarded as positive regulators and HY5 as a negative regulator for hypocotyl elongation in *Arabidopsis*.

BRs are the steroidal hormones that are ubiquitously present in the plant kingdom. They are known to play key roles in light regulation of plant growth, as the BR biosynthetic mutants, such as *de-etiolated 2* (*det2*), show a light-grown morphology and express light-induced genes in the dark, and are defective in hypocotyl elongation response to darkness (Li et al. 1996). BR signaling is initiated from BR perception by the BR receptor BRASSINOSTEROID INSENSITIVE1 (BRI1), a transmemebrane leucine-rich repeat containing receptor-like kinase (LRR-RLK). BR binding to BRI1 results in fast activation of the receptor's intracellular kinase domain by means of phosphorylation and homodimerization (Zhu et al. 2013). Activated BRI1 heterodimerizes with BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1) and then activates two kinases, BRASSINOSTEROID-SIGNALING KINASE1 (BSK1) and CONSTITUTIVE DIFFERENTIAL GROWTH1 (CDG1), and a Ser/Thr phosphatase BRI-SUPPERSSOR1 (BSU1) that de-phosphorylates and thereby inactivates BRASSINOSTEROID INSENSITIVE2 (BIN2), a cytoplasmic GSK3/Shaggy-like protein kinase that negatively regulates BR signaling by phosphorylating and inactivating BRASSINAZOLE RESISTANT1 (BZR1) and BRI1-EMS-SUPPRESSOR1 (BES1, also named BZR2), two key transcription factors mediating BR responses (Wang et al. 2012; Zhu et al. 2013). In the presence of BR, BZR1 and BES1 become dephosphorylated and activated, presumably through the action of PROTEIN PHOSPHATASE 2A (PP2A) (Wang

et al. 2012). Constitutive active forms of BZR1 (*bzr1-1D*) and BES1 (*bes1-D*) suppress the photomorphogenesis-in-the-dark phenotypes of the BR-deficient or BR-insensitive mutants, indicating that BR promotes skotomorphogenesis through BZR1 and BES1 (Sun et al. 2010; Yu et al. 2011; Wang et al. 2012; Zhu et al. 2013).

Compared to the repression effects on plant growth in light (Rolland et al. 2002, 2006), sugar conversely promotes plant growth in the shade and dark (Moore et al. 2003; Zhang et al. 2010), suggesting that sugar regulates plant growth via different pathways in light and dark. PIFs have been identified to be required for Suc-promoted hypocotyl elongation in the dark (Liu et al. 2011; Stewart et al. 2011). In this study, we focused on the role of BRs in this process and found that BR biosynthesis and signaling were necessary for sugar-induced hypocotyl elongation in the dark. Sugar could not only up-regulate the expression of several key genes that are required for cell elongation in a BR-dependent manner, but also up-regulate the expression of *BZR1* and *BES1* expression. Furthermore, the BZR1 protein level in seedlings that were treated with sugar in the dark was significantly higher than that in control seedlings. Our results indicate that BR signaling is involved in sugar regulation of hypocotyl elongation, which is likely through BZR1- and BES1-dependent up-regulation of genes important for cell elongation.

Materials and methods

Plant materials and growth conditions

The materials of *Arabidopsis thaliana* (L.) used in this study include the mutants *det2-1*, *bri1-5*, *bzr1-1D* and *bes1-D*, and transgenic line *pBZR1::BZR1-CFP*, as described previously (Li et al. 1996, 2012; Wang et al. 2002; Yin et al. 2002). *det2-1*, *bzr1-1D* and *pBZR1::BZR1-CFP* are in Columbia-0 (Col-0) background, and *bri1-5* and *bes1-D* are in Wassilewskija (WS) and Ertkheim-2 (En2) backgrounds, respectively. Seeds were first surface sterilized with 20 % (v/v) bleach solution for 10 min, rinsed with sterile water three times, and then sown on 1/2 strength Murashige and Skoog (MS) media containing 0.68 % phytoblend without sugars. Seeds on media were cold treated at 4 °C for 3 days in the dark and then transferred to a growth chamber for germination and growth for 4 days under continuous light at 23 °C. For sugar or hormone treatment, the 4-day-old seedlings were transferred to new media with or without sugars or other chemicals, and then moved to darkness or left in light for the indicated time in each figure. When necessary, mannitol (Mtl) and the mock solutions such as dimethylsulfoxide (DMSO) and ethyl alcohol (EtOH) were used in the experiments as controls.

Hypocotyl length measurement

After the indicated time of growth and treatment, 25 seedlings were laid horizontally on an agar plate, photographed, and hypocotyl lengths were measured using the ImageJ software.

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Seedlings were harvested and ground in liquid nitrogen, and RNA was extracted using RNAiso Plus reagent (TaKaRa). The complementary DNA (cDNA) was synthesized using an oligo$(dT)_{18}$ primer and Moloney murine leukemia virus (M-MuLV) reverse transcriptase (Fermentas) at 42 °C for 60 min. Then the first-strand cDNA was synthesized from 1 μg of total RNA using PrimeScript RT reagent Kit containing gDNA eraser (TaKaRa). PCR reactions were performed using the CFX96 Real Time System (Bio-Rad) with SYBR Premix Ex Taq II Kit (TaKaRa), following the manufacturer's procedures. The raw data were analyzed with the CFX Manager Software (version 1.1), and the expression value of genes was normalized to *PP2A* to minimize variation in cDNA template levels. Relative expression levels were calculated using the comparative threshold (Ct value) method. Fold changes ($2^{-\Delta\Delta Ct}$) were expressed relative to the control. Mean values were obtained from three biological replicates. Primer sequences used for qRT-PCR are listed in Supplementary Table S1.

Transient expression assay

The transient expression assays were performed in *Arabidopsis* protoplasts as previously described (Yoo et al. 2007). To generate the reporter constructs, ~2 kb promoter sequences of *IAA19* and *XTH18* were, respectively, amplified from genomic DNA of *Arabidopsis* Col-0 and cloned into the *pGreenII 0800-LUC* vector (Hellens et al. 2005). Primers used for these plasmid constructions are listed in Supplementary Table S1. Preparation of *Arabidopsis* mesophyll protoplasts from 4-week-old Col-0, *det2-1*, WS and *bri1-5* seedlings grown under short photoperiod (12 h light/12 h dark) and subsequent transfections were performed as described (Yoo et al. 2007). After transfection, the protoplasts were cultured under dark for 12 h, and then the firefly luciferase (fLUC) and renilla luciferase (REN LUC) activities were measured using the Dual-Luciferase Reporter Assay System (Promega) by following the manufacturer's instructions. Relative fLUC activity was calculated by normalizing against the REN LUC activity. All the experiments were performed with three biological replicates.

Western blot assays

The Western blot assay for BZR1 protein level was performed as previously described (Li et al. 2012). Briefly, the transgenic *pBZR1::BZR1-CFP* seedlings were ground into a fine powder in liquid nitrogen, and 2 × SDS sample buffer was added at a ratio of 1:1 (1 μl of buffer for 1 mg of tissue powder) to extract the proteins. The protein extracts were heated at 70 °C for 10 min, centrifuged at 12,000*g* for 10 min at 4 °C, and the resulting supernatants were transferred to a new microfuge tube for subsequent analyses. SDS–polyacrylamide gel (10 %) electrophoresis was performed to separate the extracted proteins. After electrophoresis, proteins were transferred to a PVDF membrane (Millipore) with a semi-dry electrophoretic transfer cell (Bio-Rad) and immunodetected with a primary antibody against GFP (Clontech) to recognize BZR1-CFP. Membranes were developed with the SuperSignal West Pico Chemiluminescent substrate kit (Pierce Biotechnology) and visualized on X-ray films.

Statistical analysis

The significance of differences between data sets was evaluated using paired Student's *t* test using OriginPro8.0 software (OriginLab).

Accession numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: *PP2A* (At1g69960), *IAA19* (At3g15540), *PRE1* (At5g39860), *PRE5* (At3g28857), *XTH18* (At4g30280), *EXPL2* (At4g38400), *EXPA8* (At2g40610), *BZR1* (At1g75080), *BES1* (At1g19350) and *CPD* (At5g05690), *DWF4* (At3g50660).

Results

BR biosynthesis is necessary for sugar-induced hypocotyl elongation in darkness

To test the possible role of BR in sugar promotion of *Arabidopsis* hypocotyl elongation in the dark, Col-0 and *det2-1* seedlings were first grown in light for 4 days and then treated with 90 mM Suc in the dark for 3 days. As shown in Fig. 1a, Suc significantly induced hypocotyl elongation of Col-0 seedlings in the dark, whereas this effect of Suc was nearly abolished in the *det2-1* mutant. The time course experiment indicated that the hypocotyl length of Col-0 was only slightly increased with the progression of dark treatment, but this was significantly increased when plants were treated with 90 mM Suc, whereas the *det2-1* mutant was essentially insensitive to the Suc treatment (Fig. 1b). Alternatively, when the seedlings were treated with different concentrations of Suc (0–150 mM) for 2 days in the dark, the hypocotyl length of Col-0 was increased by 162.5 % (from 0.20 to 0.52 cm), but that of the *det2-1* mutant was only increased by 16.16 % (from 0.083 to 0.099 cm) (Fig. 1c). These results suggest that a normal BR biosynthesis in *Arabidopsis* seedlings is necessary for Suc-induced hypocotyl elongation. To further support this hypothesis, the wild-type Col-0 seedlings were treated with Suc and together with brassinazole (BRZ, a BR biosynthesis inhibitor) and/or brassinolide (BL, the most active form of BR). The results indicated that the promoting effect of Suc on hypocotyl elongation was not affected by BL treatment (Fig. S1), but decreased by BRZ treatment in a concentration-dependent manner (Fig. 1d). For instance, the hypocotyl length of Col-0 was decreased by 14.2 % with 0.1 μM BRZ treatment (from 0.53 to 0.46 cm), but by 26.4 % with 1 μM BRZ treatment (0.53–0.39 cm). However, when the plants were treated with BRZ (1 μM) together with BL (0.1 μM), the hypocotyl length was restored substantially (Fig. 1d), indicating that a proper level of BR in plants is necessary for Suc promotion of hypocotyl elongation. Similar results were observed in Glc-treated seedlings (Fig. 1d).

BR signaling is necessary for sugar-induced hypocotyl elongation

To determine whether BR signaling also plays a role in sugar regulation of hypocotyl elongation, the effects of sugar on hypocotyl elongation were assayed in the BR-insensitive mutant *bri1-5*, BR-hypersensitive mutant *bzr1-1D* and in their wild-types WS and Col-0, respectively. As the Suc concentration increased from 0 to 90 mM, the hypocotyl length of WS was increased by 43.1 % (from 0.29 to 0.51 cm), but only by 26.7 % in the *bir1-5* mutant (from 0.11 to 0.15 cm), indicating that a normal BR signaling is necessary for Suc promotion of hypocotyl elongation (Fig. 2b). A more obvious induction of hypocotyl elongation by Suc was seen in the *bzr1-1D* mutant. For example, with 30 mM Suc treatment, the hypocotyl length of *bzr1-1D* seedlings was increased by 100 % (from 0.19 to 0.38 cm), but that of its wild type (Col-0) was only increased by 50 % (from 0.18 to 0.27 cm) (Fig. 2b). But further increasing Suc concentration to 90 mM caused no differences in increase of hypocotyl lengths in Col-0 and *bzr1-1D* (Fig. 2b), presumably due to increased BR signaling in Col-0 under the high Suc concentration. Together, these results indicate that BZR1-dependent BR signaling plays a role in Suc induction of hypocotyl elongation. Consistent results were observed in Glc-treated seedlings (Fig. 2b).

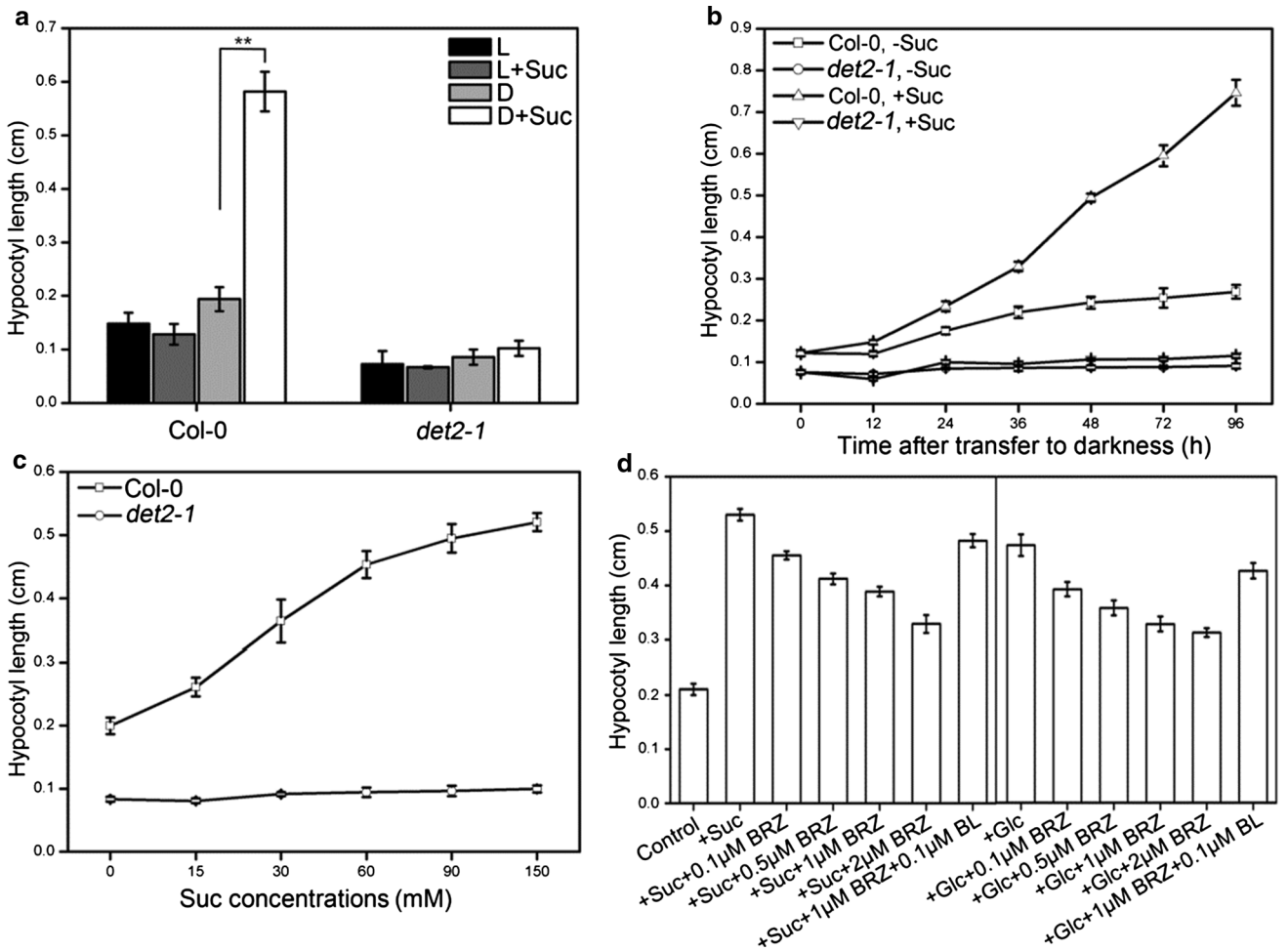


Fig. 1 Effect of BR on sugar-induced hypocotyl elongation in darkness. **a** Hypocotyl lengths of 4-day-old light-grown seedlings of Col-0 and *det2-1* that were treated with or without 90 mM sucrose (Suc) and then transferred to darkness or remained in white light for another 3 days. L, light; L+ Suc, treatment with 90 mM Suc under light; D, darkness; D+ Suc, treated with 90 mM Suc and transferred to darkness. *Double asterisks* represent the significant difference in hypocotyl lengths between D and D+ Suc at the level of $P < 0.01$ based on Student's *t* test. **b** Hypocotyl lengths of 4-day-old light-grown seedlings of Col-0 and *det2-1* that were treated with (+) or without (−) 90 mM Suc and then transferred to darkness for the indicated time (from 0 to 96 h). **c** Hypocotyl lengths of 4-day-old light-grown seedlings of Col-0 and *det2-1* that were treated with various concentrations of Suc (from 0 to 150 mM) for 2 more days. **d** Hypocotyl lengths of 4-day-old light-grown Col-0 seedlings that were treated with or without 90 mM Suc or 90 mM glucose (Glc) plus various concentrations of BRZ or BL as indicated for 2 more days. Each value is the mean of 25 seedlings ±SE

BZR1 has a close homolog BES1 (or BZR2). To further demonstrate the regulatory roles of BZR1 and BES1 in sugar-induced hypocotyl elongation in the dark, we assayed the effects of sugar on hypocotyl elongation in 4-day-old *bes1-D* and En2 seedlings (En2 is the wild type of *bes1-D*) together with *bzr1-1D* and Col-0 treated with 90 mM Suc plus 2 μM BRZ. As shown in Fig. 3, in the absence of BRZ, Suc could significantly induce hypocotyl elongation in all plants tested. However, with 2 μM BRZ treatment, less inhibition of hypocotyl elongation was seen in the *bzr1-1D* and *bes1-D* plants as compared to their corresponding wild types (Col-0 and En2, respectively) (Fig. 3a, b). To more clearly demonstrate the roles of BZR1 and BES1 in this process, we here used "Suc sensitivity" to reflect the difference of hypocotyl lengths in the presence and absence of Suc treatment. In the absence of BRZ, *bzr1-1D* and *bes1-D* exhibited similar sensitivities to Suc as compared to that of their corresponding wild types (Fig. 3c). In the presence of BRZ (2 μM), the "Suc sensitivity" of Col-0 and En2 decreased by 50.4 % (from 2.72 to 1.37) and 57.7 % (from 2.27 to 1.31), respectively, but it was only decreased by 21.1 % in *bzr1-1D* (from 2.75 to 2.17) and even increased by 10.0 % in *bes1-D* (from 2.38 to 2.64) (Fig. 3c). These results indicate that *bzr1-1D* and *bes1-D* are resistant to BRZ inhibition of sugar-induced hypocotyl elongation and suggest that BR signaling functions through BZR1 and BES1 to regulate this process.

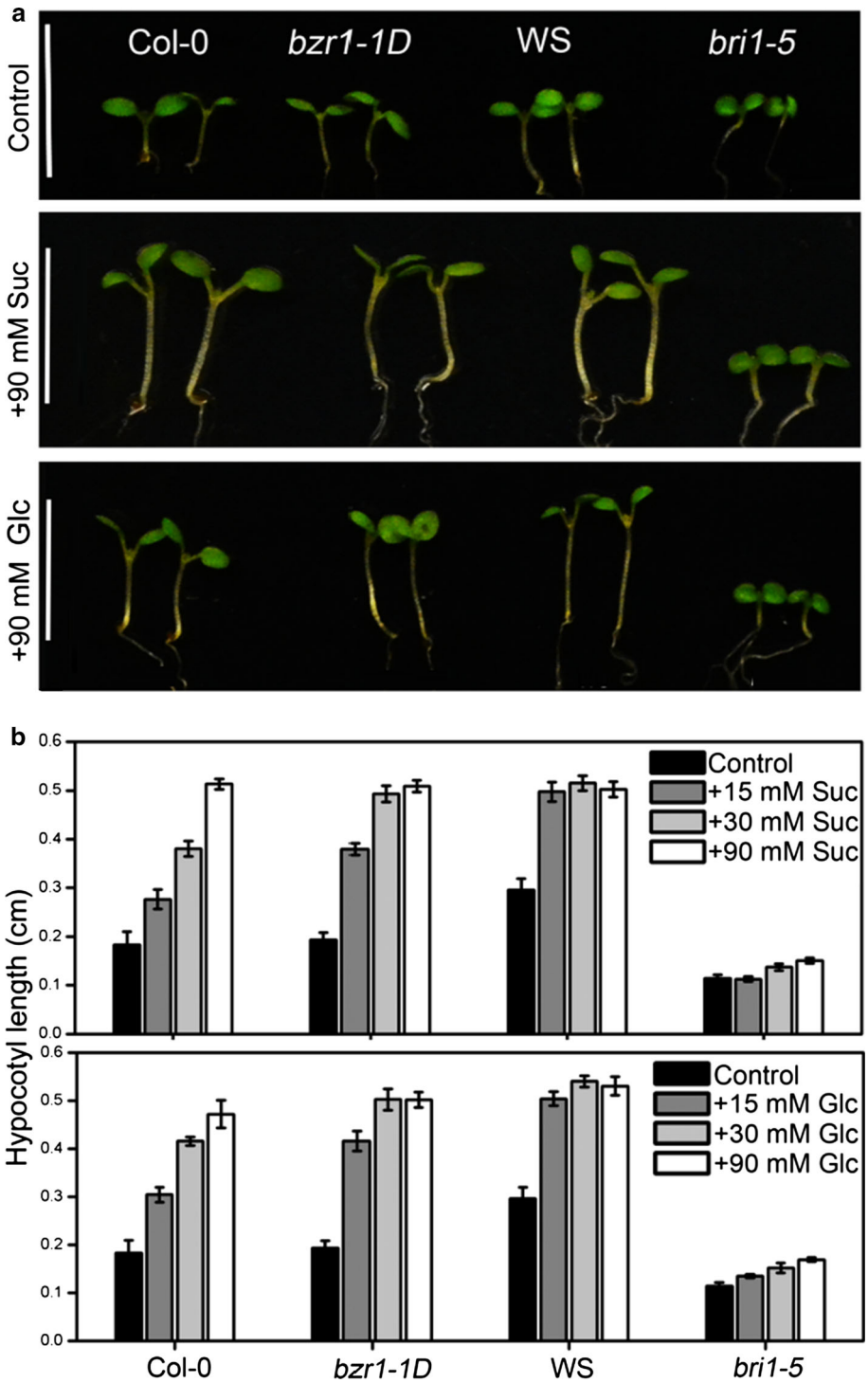


Fig. 2 Effect of BR signaling on sugar-induced hypocotyl elongation. **a** Representative images of 4-day-old light-grown seedlings of Col-0, *bzr1-1D*, WS, and *bri1-5* treated with or without 90 mM Suc or 90 mM Glc and then transferred to darkness for an additional 2 days. *Bar* 0.8 cm. **b** Hypocotyl lengths of plants in (**a**) treated with or without various concentrations of Suc or Glc for 2 days. Each value is the mean of 25 seedlings ±SE

BR and its signaling are necessary for sugar up-regulation of genes that are involved in cell elongation

To understand how sugar promotes hypocotyl elongation via BR signaling, the effects of sugar on the expression of several genes that are involved in cell elongation (*IAA19*, *PRE1*, *PRE5*, *XTH18*, *EXPL2*, and *EXPA8*) were assessed. As shown in Fig. 4, after Col-0 seedlings were treated with 90 mM Suc or 90 mM Glc for 24 h in the dark, the transcript levels of the above genes all increased significantly compared to the control, particularly *IAA19* and *XTH18*. However, similar treatment with mannitol (Mtl) did not induce, but suppressed the transcription of these genes

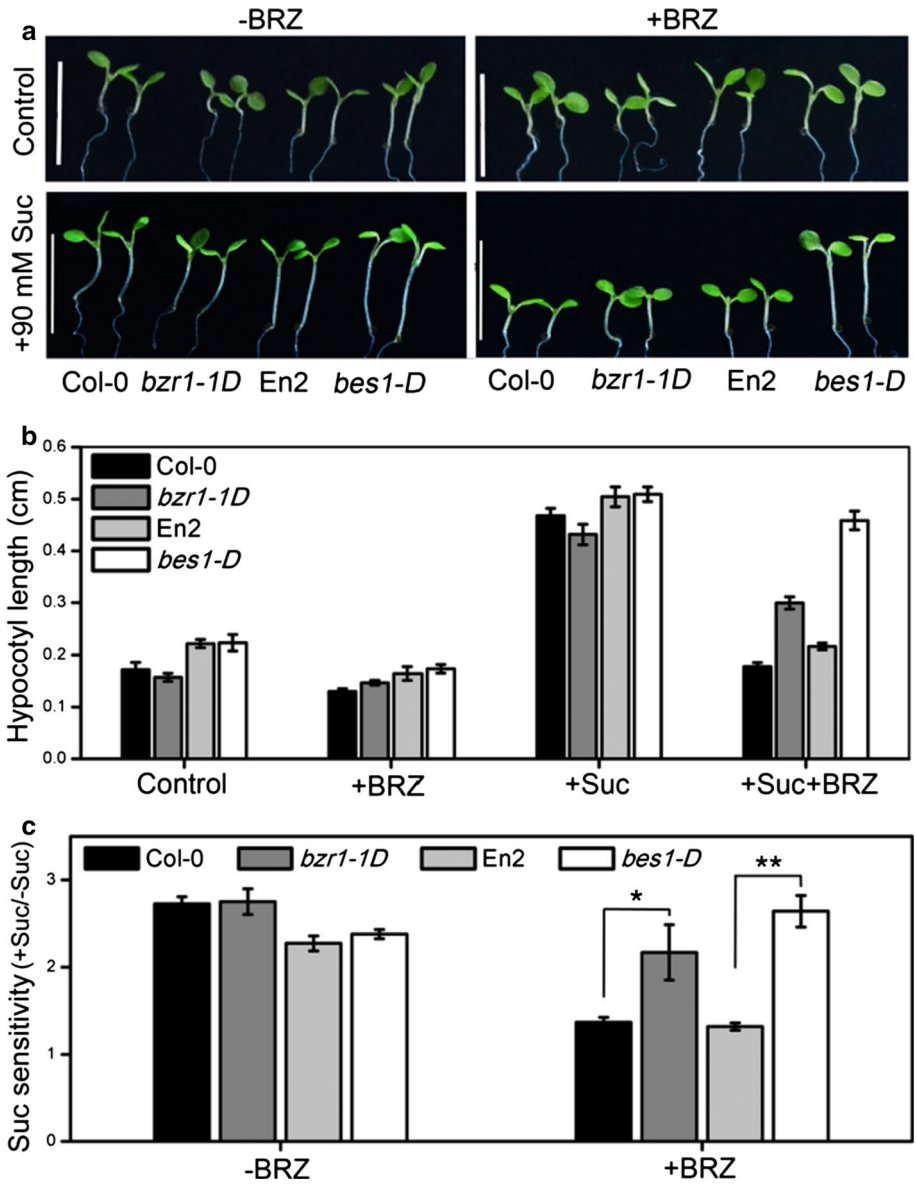


Fig. 3 Both BZR1 and BES1 are required for sugar-induced hypocotyl elongation in darkness. **a** Representive images of 4-day-old light-grown seedlings of Col-0, *bzr1-1D*, WS, and *bri1-5* treated with or without 90 mM Suc ± 2 μM BRZ for 2 days. *Bar* 0.8 cm. **b** Hypocotyl lengths of plants (**a**) treated with or without 90 mM Suc ± 2 μM BRZ for 2 days. **c** "Suc sensitivity" calculated from data in (**b**). Each value is the mean of 25 seedlings ±SE. *Single* and *double asterisks* indicate significance of differences at the levels of $P < 0.05$ and $P < 0.01$, respectively

(Fig. 4). These results suggest that the inducing effects of Suc and Glc on the expression of these genes in the dark are due to their signaling effects rather than by osmotic effects. In contrast, Suc had little effects on the expression of *IAA19*, *PRE1*, *PRE5*, and *XTH18* in seedlings grown under a continuous light condition (Fig. S2).

To further determine whether BR and its signaling are necessary for sugar up-regulation of cell elongation-related genes, we assayed the transcript levels of *IAA19* and *XTH18*, two genes that were significantly induced by sugars (Fig. 4), in 4-day-old Col-0, *det2-1*, WS, and *bri1-5* seedlings treated with or without 90 mM Suc in the dark. After treatment with Suc for 6 and 12 h, the transcript levels of both genes were significantly increased in the wild-types Col-0 and WS, but only slightly increased in *det2-1* and *bir1-5* mutants (Fig. 5a), indicating that BR biosynthesis and signaling are necessary for Suc up-regulation of *IAA19* and *XTH18*. To determine whether BZR1 and BES1 play roles during this process, transcript levels of *IAA19* and *XTH18* were assayed in the *bzr1-1D* and *bes1-D* mutants as well as their wild types (Col-0 and En2, respectively) that were treated with 90 mM Suc in the presence of 2 μM BRZ. The results indicated a much higher induction of both genes by Suc in *bzr1-1D* and *bes1-D* than in Col-0 and En2 (Fig. 5b), suggesting that BZR1 and BES1 are required for Suc induction of *IAA19* and *XTH18* genes.

To confirm the gene expression data in whole seedlings examined above, we further assayed the sugar effects on transcription of these genes in *Arabidopsis* protoplasts. Promoters of *IAA19* and *XTH18* genes were introduced into

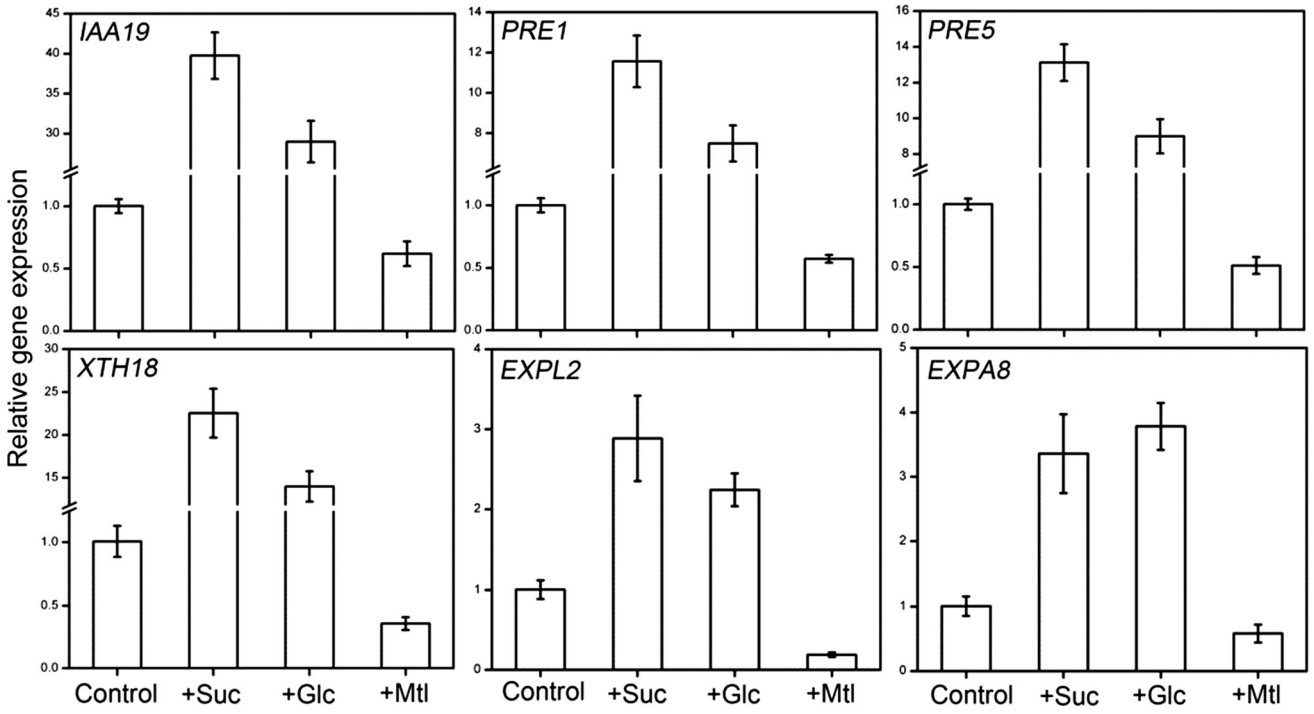


Fig. 4 Effects of Suc, Glc, and mannitol (Mtl) on the expression of *IAA19*, *PRE1*, *PRE5*, *XTH18*, *EXPL2*, and *EXPA8* genes in darkness. RNAs were extracted from 4-day-old light-grown Col-0 seedlings treated with 90 mM of Suc, Glc, or Mtl for 24 h in darkness. The relative transcript levels of the above genes were assayed by qRT-PCR. *PP2A* expression was used as the internal control. Each value is the mean of three biological replicates ±SE

the vector *pGreenII 0800-Luc* as reporters (Fig. 6a). After transformation, the protoplasts were treated with 45 mM Suc or 2 μM BRZ or 0.1 μM BL for 12 h in the dark, and then the relative fLUC activities were measured. The results showed that Suc treatment significantly increased the relative fLUC activity in Col-0 and WS protoplasts compared to the control, but only slightly in protoplasts of *det2-1* and *bir1-5* (Fig. 6b). In addition, application of BRZ treatment suppressed the promoting effect of Suc on fLUC activity in Col-0, but the suppression can be released by BL treatment (Fig. 6b). These data confirmed that BR biosynthesis and signaling are important for sugar up-regulation of *IAA19* and *XTH18* expression.

Effects of sugar on BR biosynthesis and signaling

The involvement of BR in sugar-promoted hypocotyl elongation in *Arabidopsis* made us ask another question, that is whether sugar can affect BR biosynthesis or signaling. To answer this question, we first analyzed the expression of *CPD* (*CONSTITUTIVE PHOTOMORPHOGENIC DWARF*) and *DWF4* (*DWARF4*), two rate-limiting BR biosynthetic genes, and that of *BZR1* and *BES1* genes by qRT-PCR in seedlings that were treated with 90 mM Suc. The results showed that Suc treatment up to 48 h did not cause significant changes in *CPD* and *DWF4* expression, but after 72 h's treatment their expression was significantly repressed compared to the untreated control (Fig. S3). While short-time treatment (6 h) of Suc only had slight effects on *BZR1* and *BES1* expression, longer treatment (12 and 24 h) markedly enhanced the transcript levels of both genes (Fig. 7a). For example, after 24 h of Suc treatment, the transcript levels of *BZR1* and *BES1* increased by 51.3 and 170.1 %, respectively. 90 mM Glc treatment also caused obvious changes in *BZR1* and *BES1* expression, but the effects were less significant than that of Suc treatment (Fig. 7a). Mtl was used as a negative control and it represses *BZR1* and *BES1* expression (Fig. 7a).

To test whether sugar affects the expression of BZR1 at the protein level, we next analyzed BZR1 protein abundance change during sugar treatment by Western blot analysis in the *pBZR1::BZR1-CFP* seedlings. The results showed that after treatments with 90 mM Suc for 12, 24, and 48 h, the BZR1 protein level was significantly higher in the Suc-treated seedlings than in the control (Fig. 7b). To determine whether this effect resulted from an up-regulation of *BZR1* transcription, the protein levels were determined in 4-day-old *pBZR1::BZR1-CFP* seedlings treated with three different sugars: 90 mM Suc, 90 mM Glc, and 90 mM Mtl for 24 h under both light and dark conditions. Interestingly, Suc and Glc showed no obvious effects on BZR1 protein level in light, whereas Mtl even

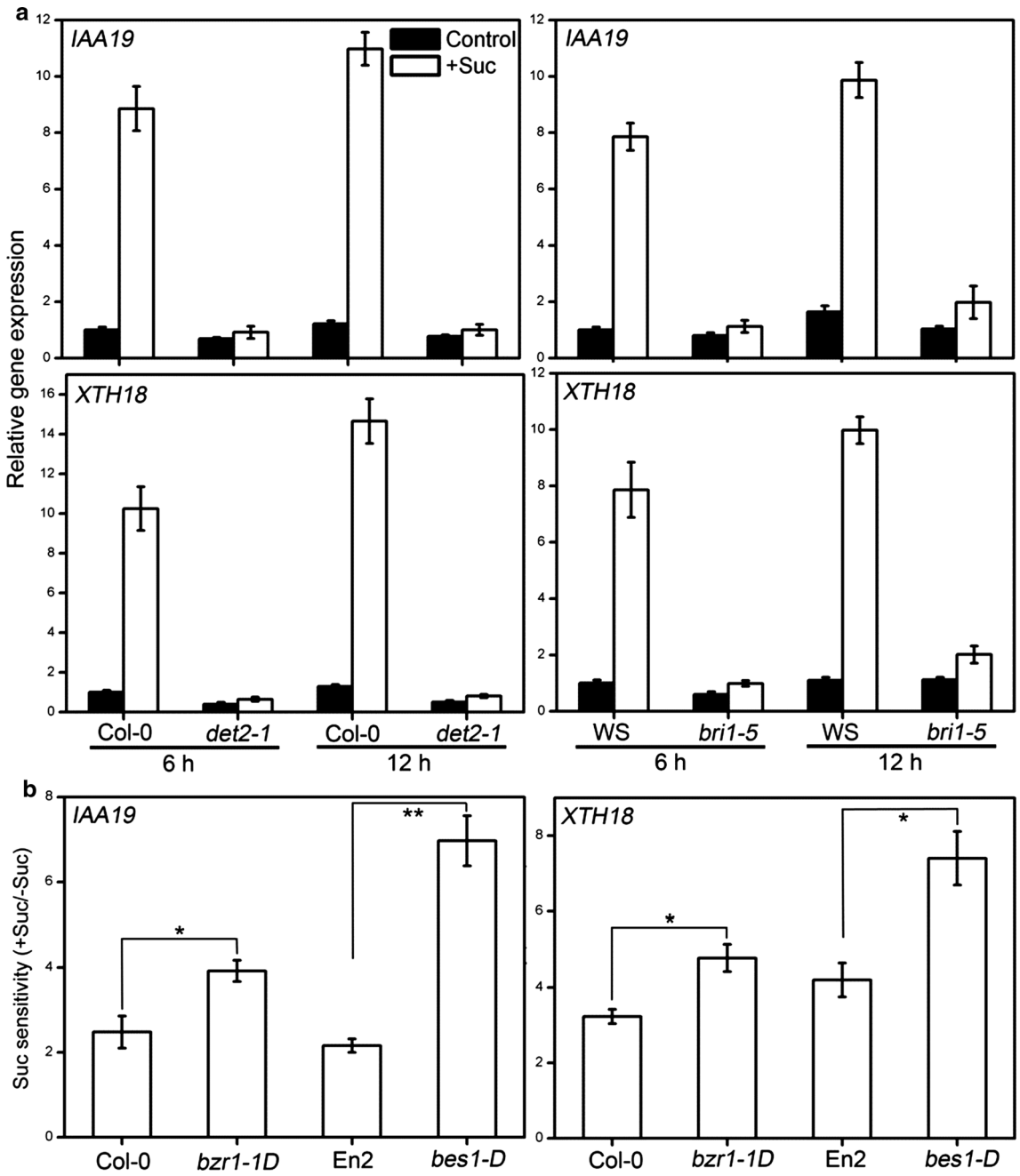


Fig. 5 BR and its signaling are necessary for sugar up-regulation of *IAA19* and *XTH18* in darkness. **a** Transcript levels of *IAA19* and *XTH18* genes in 4-day-old light-grown seedlings of Col-0, *det2-1*, WS, and *bri1-5* treated with or without 90 mM Suc for 6 and 12 h in darkness. *PP2A* expression was used as the internal control of qRT-PCR. **b** "Suc sensitivity" of 4-day-old light-grown seedlings of Col-0, *det2-1*; WS and *bri1-5* treated with or without 90 mM Suc in the presence of 2 μM BRZ for 12 h in darkness. The relative transcript levels of *IAA19* and *XTH18* were assayed by qRT-PCR, and then the "Suc sensitivity" was calculated. Each value is the mean of three biological replicates ±SE. *Single asterisk* and *double asterisks* represent the significance of differences at the levels of $P < 0.05$ and $P < 0.01$, respectively

had a repressive effect (Fig. 7c). However, when the seedlings were transferred to darkness for 24 h, the BZR1 level decreased remarkably in untreated control seedlings but not in sugar-treated seedlings (Fig. 7c), presumably due to the sugar inhibition of dark-enhanced BZR1 degradation. To strengthen this notion, the BZR1 protein levels were also tested in *pBZR1::BZR1-CFP* seedlings treated with 1 mM cycloheximide (CHX, an inhibitor of protein synthesis) in darkness. As shown in Fig. 7d, after blocking new protein synthesis by CHX treatment, existing BZR1 protein was still maintained at higher levels in the sugar-treated condition, suggesting that sugar had increased

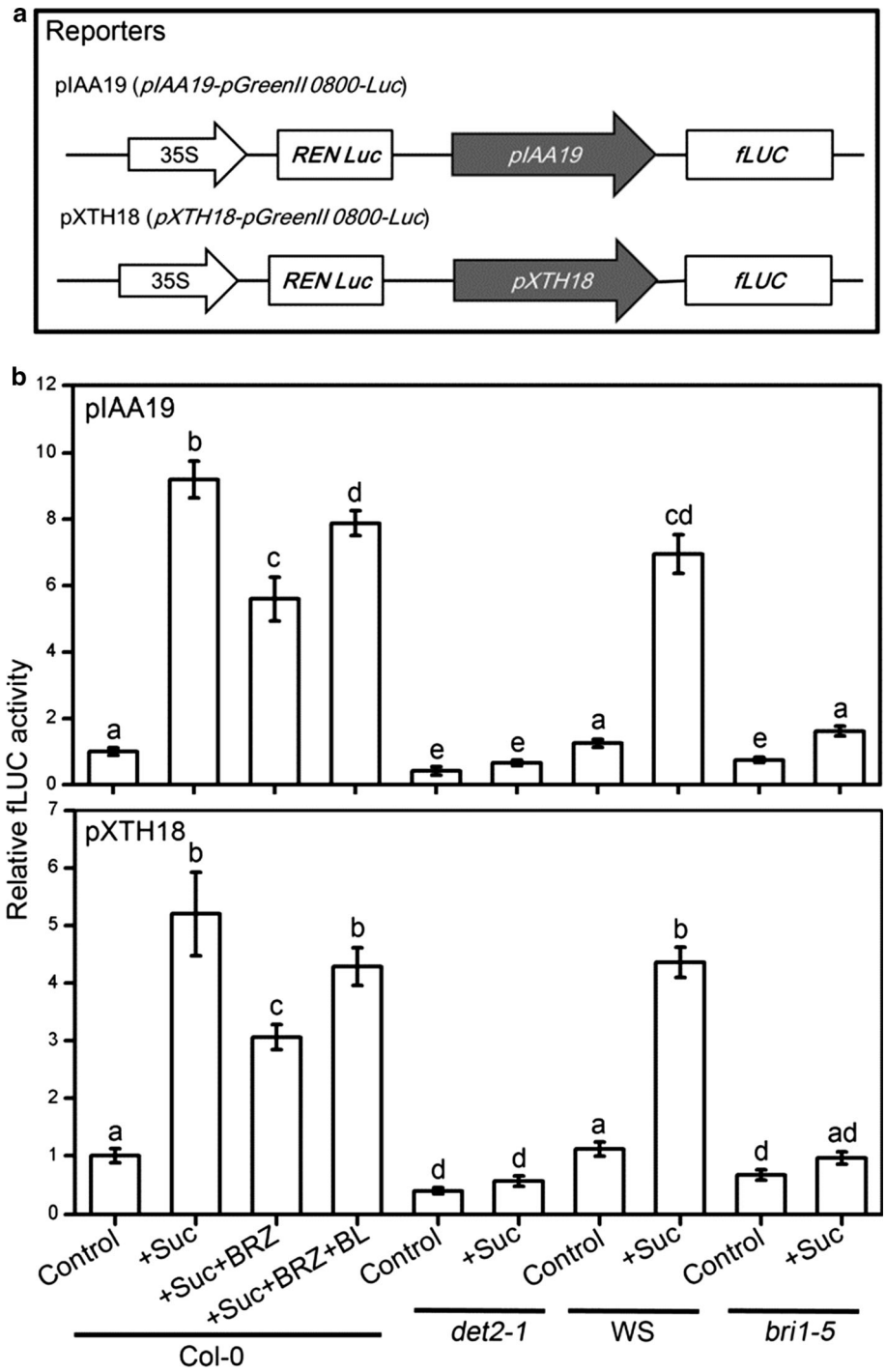
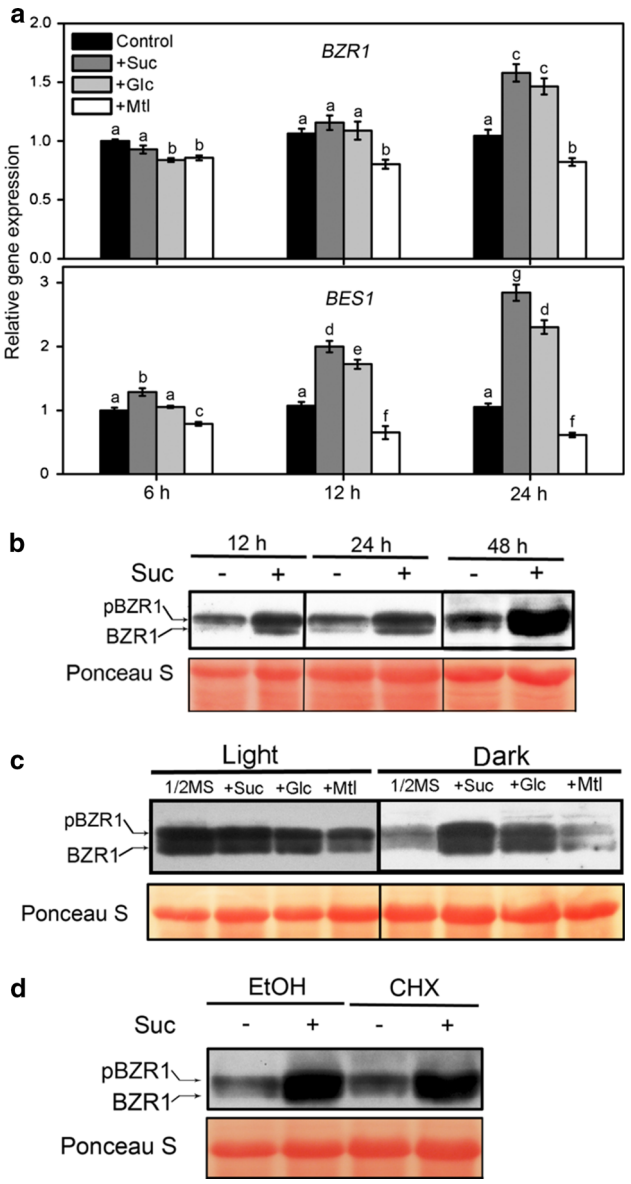


Fig. 6 Transient expression assays of sugar effects on the expression of *IAA19* and *XTH18* genes in *Arabidopsis* protoplasts. **a** Schematic maps of the constructs used in the transient expression assays. Promoters of *IAA19* and *XTH18* were individually inserted into *pGreenII 0800-LUC* vector to generate the reporter constructs. 35S, CaMV35S promoter; fLUC, firefly luciferase; REN LUC, Renilla luciferase; *pIAA19*, promoter of *IAA19*; *pXTH18*, promoter of *XTH18*. **b** Relative fLUC activities determined after the reporters were transformed into the protoplasts from 4-week-old short photoperiod-grown seedlings of Col-0, *det2-1*, WS, or *bri1-5* and then cultured for 12 h in darkness in the presence or absence of 45 mM Suc or other chemicals (2 μM BRZ or 0.1 μM BL). Each value is the mean of three biological replicates ±SE. *Different lowercase letters* on each *bar* represent significance of differences at the level of $P < 0.05$ according to the Student's *t* test

BZR1 protein stability. Although sugar could increase BZR1 protein abundance, it did not seem to affect BZR1's phosphorylation status (Fig. 7c). Only when the plants were treated with sugar together with BL, significant increase of BZR1 dephosphorylation was observed (Fig. S4). These results indicate that sugar induces hypocotyl elongation in the dark by increasing BZR1 protein abundance while not affecting its phosphorylation status.

Discussion

Sugars play central roles as signaling molecules in modulating plant growth, metabolism, and development (Eveland and Jackson 2012). A number of components that are involved in sugar sensing and signaling have been identified from mutant screens for altered responses to exogenous sugars during seed germination and early



◀**Fig. 7** Effects of sugars on *BZR1* and *BES1* expression at the transcriptional and protein levels. **a** Relative transcript levels of *BZR1* and *BES1* genes in 4-day-old light-grown Col-0 seedlings treated with or without 90 mM of Suc, Glc, or Mtl for 6, 12, and 24 h. The *PP2A* gene was used as an internal control of qRT-PCR. Each value is the mean of three biological replicates ±SE. **b** BZR1 protein level changes in 4-day-old light-grown *pBZR1::BZR1-CFP* seedlings treated with or without 90 mM Suc for 12, 24, and 48 h in darkness. The BZR1 protein was detected with Western blot analysis using an anti-GPF antibody that recognizes the BZR1-CFP fusions. **c** BZR1 protein level changes in 4-day-old light-grown *pBZR1::BZR1-CFP* seedlings treated with or without 90 mM of Suc, Glc, or Mtl for 24 h in the light or dark, and then the BZR1 protein levels were assayed by Western blot using the anti-GPF antibody. **d** Suc-induced BZR1 protein accumulation is not significantly affected by inhibition of new protein synthesis using cycloheximide (CHX). Proteins were extracted from 4-day-old light-grown *pBZR1::BZR1-CFP* seedlings treated with 90 mM Suc together with 1 mM CHX for 24 h in darkness, then the BZR1 protein level was assayed by Western blot analysis using an anti-GPF antibody. pBZR1 and BZR1 denote the phosphorylated and dephosphorylated forms of BZR1, respectively. Similar results were observed from three independent experiments and the one shown here is a representative one

seedling growth in *Arabidopsis* (Rolland et al. 2002, 2006). For example, *glucose insensitive* (*gin*) mutants fail to undergo growth arrest in the presence of inhibitory levels of glucose. Alternatively, *sucrose uncoupled* (*sun*), *sugar insensitive* (*sis*), and *impaired sucrose induction* (*isi*) mutations are allelic to *gin* loci, suggesting that these genes function at the interface of different sugar signaling pathways (Eveland and Jackson 2012). It should be noted, however, that the mutant screens were generally performed under normal growth conditions, including a long photoperiod (Rolland et al. 2002). Therefore, the role of light in sugar signaling is often ignored. Our recent studies as well as other reports have found that light signaling plays an important role during the sugar regulation of plant growth in the dark (Zhang et al. 2010; Liu et al. 2011; Stewart et al. 2011). In light, sugar generally inhibits seedling growth, including the inhibition of hypocotyl elongation; whereas in darkness, sugar conversely promotes hypocotyl elongation, suggesting a light dependence of sugar responses, possibly through sugar and light signaling cross talk. Indeed, several PIFs, which are known negative regulators of light signaling, have been found to be required for dark-dependent sugar promotion of hypocotyl elongation (Liu et al. 2011; Stewart et al. 2011).

Cross talks between sugar and various hormones in modulating critical aspects of plant growth have been reported in many studies (Rolland et al. 2006; Eveland and Jackson 2012). Plants that are defective in abscisic acid (ABA) or ethylene signaling tend to display altered sugar response phenotypes, and comparable mutant screens and subsequent genetic and functional analyses have revealed extensive overlaps between sugar, ABA, and ethylene signaling in the control of developmental processes, including seed germination and seedling growth (Gibson 2004, 2005; Rolland et al. 2006; Eveland and Jackson 2012). For sugar-induced hypocotyl elongation in the dark, our recent study indicated that the phytohormone gibberellin (GA) may play a role, as the application of paclobutrazol, a specific GA biosynthesis inhibitor, significantly impairs sucrose promotion of hypocotyl elongation (Zhang et al. 2010). In this study, we found that the steroidal hormone BR is also involved in this process. Sugar-induced hypocotyl elongation was nearly abolished in the BR-deficient *det2-1* mutant and was strongly inhibited by BRZ treatment (Fig. 1), indicating that a proper BR level in seedlings is required for sugar promotion of hypocotyl elongation in the dark. The role of BR in etiolated seedling growth has been well documented in

many reports (Li et al. 1996; Wang et al. 2002; Yin et al. 2002; Sun et al. 2010; Yu et al. 2011; Oh et al. 2012). In *Arabidopsis*, seedling growth in the dark is arrested in both BR-deficient and signaling mutants, suggesting a crucial role of BR in plant morphogenesis in the dark (Wang et al. 2012). The mechanisms of the BR regulation of hypocotyl elongation in etiolated seedlings have been often attributed to an up-regulation of genes that function to induce cell wall loosening or modification, which include *xyloglucan endotransglycosylase/hydrolase* (*XTH*) and *expansins* (*EXPs*). Some of these genes are direct targets of BZR1 and BES1, two key transcription factors in the BR signaling pathways (Sun et al. 2010; Yu et al. 2011; Oh et al. 2012). Sugar was found to significantly induce the expression of *IAA19*, *PRE1*, *PRE5*, *XTH18*, *EXPL2*, and *EXPA8* genes (Fig. 4), which are regulated by BR and are direct targets of BZR1 (Oh et al. 2012). Hence, sugar effects on these genes could be enhanced by BR signaling or vice versa. The promoting effect of sugars on *IAA19* and *XTH18* expression was almost abolished in the *det2-1* and *bri1-5* mutants, suggesting that BR level and signaling are critical for sugar up-regulation of cell elongation-related genes in the dark. This notion was supported by protoplast transient assays of *IAA19* and *XTH18* gene transcription (Fig. 6). In addition, we found that sugar-induced hypocotyl elongation and *IAA19* and *XTH18* gene expression were less inhibited by BRZ treatment in the *bzr1-1D* and *bes1-D* mutants than in their corresponding wild types (Figs. 3c, 5b), implying that the BRZ1- and BES1-dependent pathway is necessary for the sugar promotion of hypocotyl elongation.

BZR1 has recently been shown to be a key convergence point of BR cross talk with other signaling pathways during the regulation of plant growth. For example, BZR1 interacts with PIF4 to control a core transcription network, enabling co-regulation of plant growth by the steroid hormone and light signals (Oh et al. 2012). An interaction between BZR1 and RGA establishes a converging node of BR and GA signaling in controlling cell elongation and the regulation of plant growth (Li et al. 2012). Therefore, it is possible that BZR1 and/or BES1 may also function as integration nodes between sugar and BR signaling in the regulation of plant growth in the dark. An alternative mechanism for BZR1 and BES1 requirement for sugar-induced hypocotyl elongation is that some of the signaling components in sugar signaling may interact with BZR1 and/or BES1 through protein–protein interactions. We therefore tested by yeast two-hybrid assay the possible interaction between BZR1/BES1 and HXK1 (hexokinase 1), VHA-B1 (vacuolar H^+-ATPase B1), and RPT5B (19S regulatory particle of proteasome subunit), three factors that were shown to play key roles in Glc regulation of plant growth and likely function through interaction with other transcriptional factors (Cho et al. 2006). However, no interactions were observed between BZR1/BES1 and HXK1, VHA-B1, or RPT5B (data not shown). Whether BZR1 and BES1 interact with other factors in sugar signaling to modulate sugar-regulated hypocotyl elongation remain to be examined.

Another possibility for BR requirement in sugar-promoted hypocotyl elongation is that sugar could affect BR biosynthesis or signaling. Our results indicate that sugar seems to have less effect on BR biosynthesis in dark-grown seedlings as sugar treatment of up to 48 h did not cause significant changes in the expression of two key BR biosynthetic genes *CPD* and *DWF4*, and 72 h of treatment showed a repressive effect (Fig. S3). Also, in the presence of sugar, exogenous BL treatment could not enhance the sugar effect on hypocotyl elongation (Fig. S1). In contrast to *CPD* and *DWF4*, we found an inductive effect of sugar on transcripts of *BZR1* and *BES1*, two genes encoding for the BR transcription factors BZR1 and BES1, respectively (Fig. 7a). A more significant effect was observed in the change of BZR1 protein level, which was highly induced after the light-grown seedlings were transferred to the dark and treated with Suc (Fig. 7c). The increased BZR1 level in sugar-treated seedlings seems not completely due to increased *BZR1* transcription, as the sugar-induced increase of *BZR1* transcripts (Fig. 7a) was smaller and slower than that of BZR1 proteins (Fig. 7b). Therefore, it is possible that sugar affects BZR1 protein level post-transcriptionally, such as by repressing its degradation in the dark. Indeed, our data showed that Suc-induced BZR1 protein accumulation is not significantly affected by inhibition of new protein synthesis using CHX (Fig. 7d). Sugar seems to have little effect on BZR1 phosphorylation status, as under sugar treatment the phosphorylated form and dephosphorylated form of BZR1 increased proportionally (Fig. 7c). On the basis of these results, we conclude that sugar promotes hypocotyl elongation in the dark by increasing the total level of BZR1 protein and thus enhancing the expression of cell elongation-related genes. With this conclusion, one may ask another question: under light conditions the BZR1 protein level is also high (Fig. 7c), but why can sugar not induce hypocotyl elongation as observed in the dark? One explanation might be that in light, some positive factors in light signaling, such as HY5 and its homolog HYH, are active and may repress BZR1 activity and hypocotyl elongation (Lee et al. 2007; Chory 2010).

In summary, our results of this study revealed a novel role of BR in sugar promotion of hypocotyl elongation in the dark, which is mediated by BZR1 and BES1, two important transcription factors in BR signaling. Sugar could maintain a high abundance of BZR1 or BES1 proteins and thus up-regulate the expression of the genes required for cell elongation.

Author contribution YZ, YB, and JH conceived and designed the research. YZ, ZL, and JW conducted the experiments. YZ and ZL analyzed the data. YZ and JH wrote the manuscript. All of the authors read and approved the manuscript.

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