GhBES1 mediates brassinosteroid regulation of leaf size by activating expression of GhEXO2 in cotton (Gossypium hirsutum)

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Abstract

Brassinosteroid (BR) is an essential phytohormone that controls plant growth. However, the mechanisms of BR regulation of leaf size remain to be determined. Here, we found that the BR deficient cotton mutant *pagoda1* (*pag1*) had a smaller leaf size than wild-type CRI24. The expression of *EXORDIUM* (*GhEXO2*) gene, was significantly downregulated in *pag1*. Silencing of *BRI1-EMS-SUPPRESSOR 1* (*GhBES1*), inhibited leaf cell expansion and reduced leaf size. Overexpression of *GhBES1.4* promoted leaf cell expansion and enlarged leaf size. Expression analysis showed *GhEXO2* expression positively correlated with GhBES1 expression. In plants, altered expression of *GhEXO2* promoted leaf cell expansion affecting leaf size. Furthermore, *GhBES1.4* specifically binds to the E-box elements in the *GhEXO2* promoter, inducing its expression. RNA-seq data revealed many down-regulated genes related to cell expansion in *GhEXO2* silenced plants. In summary, we discovered a novel mechanism of BR regulation of leaf size through *GhBES1* directly activating the expression of *GhEXO2*.

1. Introduction

Leaf size strongly influences the efficiency of photosynthesis in plants (Gonzalez et al. 2012). Brassinosteroids (BRs) are essential phytohormones that promote plant growth and a characteristic phenotype of BR-related mutants is reduced leaf size (Mao and Li 2020; Li and Chory 1997; Li et al. 1996; Praveena et al. 2020; Yang et al. 2011; Clouse and Sasse 1998; Nolan et al. 2017; Vert et al. 2005). In BR biosynthesis mutant *constitutive photomorphogenesis and dwarfism* (*cpd*), the leaves are small, round, and dark-green (Noguchi et al. 1999; Szekeres et al. 1996). Another BR-deficient mutant, *deetiolated-2* (*det2*), has leaves that are small and round and twice as numerous as the wild type (Azpiroz et al. 1998; Chory et al. 1991). The transmembrane receptor kinase brassinosteroid insensitive1 (BRI1), has been associated with BR responses (Guo et al. 2013). Mutations in *BRI1* result in BR-insensitivity and a morphological phenotype almost identical to that of the BR biosynthesis mutant *cpd* (Clouse et al. 1996). In rice, M107 is a gain-of-function mutant of the BR biosynthetic gene *P450 CYP724B1* with a typical BR excess phenotype, including long and narrow leaves with greatly increased leaf angles (Wan et al. 2009). In addition, the positive regulatory protein complexes of the BR signaling pathway reduced leaf angle 1 (*RLA1*) and BRASSINAZOLE RESISTANT1 (*OsBZR1*) have mutants with a distinct, erect leaf phenotype (Qiao et al. 2017). In *Gossypium hirsutum*, *GhPAG1* is highly homologous to *AtCYP734A1*, and its expression is activated in *pagoda1* (*pag1*) mutants exhibiting dwarfism and smaller leaf size due to inhibition of cell expansion (Yang et al. 2014).

BR regulates expression of downstream genes mainly through the transcription factor (TF) *BES1/BZR1* (BRI1-EMS-SUPPRESSOR 1/BRASSINAZOLE-RESISTANT 1) interacting with key elements, including E-box motif (CANNTG) and BR response element (BRRE; CGTGC/TG) (Li et al. 2018). *BES1/BZR1* can directly bind to the promoter regions of *CPD*, *dwarf4* (*DWF4*), and *BR-6-oxidase* (*BR6OX*) to regulate cell expansion in leaves (He et al. 2005). The *bes1-D* mutant, whose BES1 protein is widely expressed by an amino acid mutation, exhibits a constitutive BR response phenotype including long, bending petioles and curled leaves. Notably, multiple BR-induced genes are up-regulated or hyperresponsive to BR in *bes1-D*,

including *EXORDIUM* (*EXO*), which is described as an AtPhi-1 phosphate-induced protein (Yin et al. 2002). *BZR1/BES1* genes have been identified from many species such as Chinese cabbage (Wu et al. 2016), maize (Yu et al. 2018b), rice (Bai et al. 2007), and cotton (Liu et al. 2018) and they are reported to have a conserved role in regulating BR signal transduction pathway.

The *EXO* gene was first reported as a plant growth regulator expressed in tissues with high auxin concentrations (Farrar et al, 2003). In addition, as a BR response gene, *EXO* expression is induced after exogenous 2,4-epibrassinolide treatment in *Arabidopsis* (Schröder et al. 2009; Coll-Garcia et al. 2004). Proteomics approaches identified the EXO, EXL1, EXL2, EXL3, and EXL4 proteins as part of the cell wall proteome and determined that they promote plant growth, cell expansion, and carbon starvation response through mediating BR (Schröder et al. 2012; Schröder et al. 2009; Coll-Garcia et al. 2004). In *Arabidopsis*, rosette leaves became larger in *EXO* overexpression transgenic plants compared with wild type, and increased transcript levels of BR response genes were observed (Coll-Garcia et al. 2004). However, elongation of abaxial epidermal and palisade cells in the subepidermal layer were inhibited in the *exo* knockout mutant based on scanning electron microscopy (SEM) showing a diminished leaf and root growth phenotype and a diminished response to BL (Schröder et al. 2009). *OsEXO* knockout plants exhibited partial dwarfism and had smaller cells in the culms, promoting cell expansion by regulating microtubule organization (Aya et al. 2014). *EgEXO* overexpression in *Eucalyptus globulus* promoted plant height and increased leaf biomass. In addition, the presence of two G-boxes in the promoter region suggests that it is regulated by the *BES1* family of TFs (Sousa et al. 2020).

Cotton (mainly upland cotton, *G. hirsutum*) is a primary fiber crop and an excellent model to study polyploidization, genome evolution, and cell expansion (Malik et al. 2018; Ali et al. 2021). Cotton leaf size is an important agronomic trait that affects plant architecture, yield, and stress tolerance (Andres et al. 2016). Cotton leaf size has significant phenotypic diversity in crops and has a unique role in cotton development (Dolan and Poethig 1991). Only a few genes affecting cotton leaf development have been functionally characterized, such as *LMI1* (LATE MERISTEM IDENTITY1, an HD-Zip transcription factor) and *GhARF16* (Auxin response factor) (He et al. 2021; Andres et al. 2017). However, the mechanisms of cotton leaf development remain largely unknown.

Previous studies have shown that BR primarily regulates leaf size by affecting cell expansion, but the genetic mechanisms involved are unclear. In this study, the major TF *GhBES1* in the BR signaling pathway was shown to act as a key regulator that binds to the *GhEXO2* promoter and increases its expression, enhancing cell expansion and ultimately affecting leaf size. In brief, our data suggest that *GhEXO2*, the downstream target gene of *GhBES1*, functions in expanding cotton leaf cells in BR signaling to regulate leaf size.

2. Materials And Methods

2.1 Plant materials and growth conditions

Upland cotton (*Gossypium hirsutum*) mutant *pag1* (BR-defective) and the corresponding wild-type (WT) CRI24 were obtained from the Institute of Cotton Research of the Chinese Academy of Agricultural Sciences. In this study, cotton plant materials were grown in a greenhouse with a photoperiod of 16 hours light/8 hours darkness at 28°C. 1/2 Murashige and Skoog (MS) medium was used to germinate *Arabidopsis thaliana* seeds with long-day conditions at 23°C (16 hours light/8 hours darkness). The Columbia-0 ecotype of *Arabidopsis* was used as WT. *Arabidopsis* seeds were germinated on 1/2 MS medium followed by growth in a greenhouse with long-day conditions as described above.

2.2 Phylogenetic tree construction, conserved motif and promoter cis-acting elements analysis

A significantly down-regulated gene was identified in the unpublished cotyledon transcriptome data of *pag1*. We downloaded the protein sequences from the CottonFGD database (https://cottonfgd.org/). The retrieved protein sequences were used to identify eight *EXO* genes in *Arabidopsis* from The *Arabidopsis* Information Resource (TAIR) database (https://www.arabidopsis.org/). A phylogenetic tree was generated in MEGA 7 using the NJ (neighbor-joining) method (Saitou et al. 1987; Kumar et al. 1994). We performed conserved amino acid residue analysis via Jalview software after multiple alignments of the conserved Phi_1 domain and the signal peptide region using ClustalW (Waterhouse et al. 2009; Thompson et al. 2003). The protein domains were predicted with the online tool MEME (http://meme-suite.org/tools/meme) as described previously (Yu et al. 2018a; Wang et al. 2018). The promoter sequence 2,000 bp upstream of the start codon was downloaded from the cottonFGD database (https://cottonfgd.org/). The cis-acting elements in the promoter region were predicted on the web tools PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PlantTFDB (http://planttfdb.gao-lab.org/prediction.php).

2.3 BL induction treatment assay, RNA extraction and qRT-PCR analysis

For BL treatment, 10 μM/L BL to treat cotton seedlings growing the three-four leaf stage for 1, 3 and 5 hours. An equal volume of absolute ethanol (solvent) was added to deionized water as the Mock. Samples of plant tissues were immediately stored in liquid nitrogen, and the extracted RNA was stored at -80°C. Each experiment was conducted with three biological replicates. We extracted total RNA using the RNA prep Pure Plant Kit (TSINGKE, Beijing, China) based on the manufacturer's instructions. *EasyScript*® (One-Step gDNA Removal) (TransGen Biotech, Beijing, China) was used to synthesize cDNA from 1 μg of RNA. The internal controls were *Actin2* (AT3G18780.1) and *GhHistone3* (AF024716). AceQ qPCR SYBR Green Master Mix (Low ROX Premixed) (Vazyme, Nanjing, China) was used for Quantitative Real-time PCR (qRT-PCR) analysis on a Light-Cycler 480 (Roche Diagnostics, Germany). Values of relative expression patterns were calculated using the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen 2001). 2· Taq Plus Master Mix (Dye Plus) (Vazyme, Nanjing, China) was used for semi-quantitative RT-PCR analysis (Phillips et al. 1993).

2.4 Subcellular localization assay

We incorporated the full-length coding sequence of *GhEXO2* into a vector containing a GFP region to analyze the GhEXO2 protein expression localization. *Nicotiana benthamiana* leaves were co-injected with *Agrobacterium* GV3101 containing the GhEXO2-GFP vector coupled with p19. The tobacco plant was placed in the dark for 16 h then returned to the light. A confocal microscope (OLYMPUS FV1200) was used to laser scan the epidermal cells of infiltrated tobacco leaves after 48 hours of inoculation before which the leaves were stained with membrane dye FM 4–64 for 8 min for co-localization.

2.5 Generation of overexpression transgenic lines and virus-induced gene silencing

To verify the function of *GhEXO2*, we used *Arabidopsis* Col-0 plants to produce transgenic *GhEXO2* lines. To construct the overexpression vector, the full-length coding sequence of *GhEXO2* was amplified with gene-specific primers from cDNA and cloned into pCAMBIA-2300 with the 35S promoter. *Arabidopsis* plants were infected with *Agrobacterium* GV3101 containing 35S-*GhEXO2*- pCAMBIA-2300 vector. The inflorescences of *Arabidopsis* plants were dipped in an *Agrobacterium* suspension to produce transgenic *Arabidopsis* plants (Clough and Bent 1998). For VIGS (virus-induced gene silencing), 300 bp highly specific coding sequences were cloned into the PTRV2 vector, which was then ligated into GV3101 before injection into cotton plants (CRI24) (Dinesh-Kumar et al. 2003).

For overexpression and RNA interference vector, the *GhBES1* coding sequence was amplified with the corresponding primers, and separately constructed into pcambia-2300 and pbi121 vector driven by the 35S promoter. Notably, overexpression of the *GhBES1.4* coding sequence introduces a point mutational modifications designed by PrimerX (http://www.bioinformatics.org/primerx/cgi-bin/DNA_1.cgi), as we previously reported (Liu et al. 2018). Both vectors were transformed into *Agrobacterium* LBA4404. The hypocotyls of 6-day-old sterile cotton seedlings were infected with *Agrobacterium* LBA4404 as explants. Then, the transgenic plants were created in the greenhouse through callus induction, proliferation, embryogenic callus induction, embryo differentiation, and plant regeneration stages. PCR and qRT-PCR were used to detect transgenic plants at the genome and transcription level, respectively. All primers used were presented in Table S2.

2.6 Observation of cotton tissue morphology

The selected tissue was immersed in FAA fixation solution before dehydrating it with an alcohol gradient. The tissues were made transparent with xylene and then embedded in paraffin. The paraffin-embedded tissues were then sectioned into 10 micron thick slices (Leica Instruments GmbH, Wetzlar, Germany). The sections were stained with toluidine blue and observed under a Leica M165FC epifluorescence stereomicroscope (Leica Instruments GmbH). Each sample was repeated at least three times.

2.7 Electrophoretic mobility shift assay

EMSA was performed using the LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific, Shanghai, China) according to the previous description (Liu et al. 2019). Biotin-labeled probe containing E-box (CANNTG) and mutant probe (AAAAAA) from the *GhEXO2* promoters was incubated with purified

recombinant His-GhBES1 in binding buffer. Non-labeled probes were used as cold competitors. The reaction components were electrophoresed on a polyacrylamide gel using TBE buffer at 4°C. The DNA in the gel was electroblotted onto a nitrocellulose membrane and detected by ChemiScope 6300 (CLINX, Shanghai, China).

2.8 Transcriptional activation assay

The 2000 bp promoters of *GhEXO2*, both the natural promoter and that mutated in the E-box region, were separately inserted into the pGWB435 vector with the luciferase reporter gene. *Agrobacterium* GV3101 containing *GhEXO2*pro-LUC and *GhEXO2*pro-mutant-LUC were mixed with the above-mentioned *GhBES1*-pCAMBIA2300 vector co-injected into *N. benthamiana* leaves, and the empty vector pCAMBIA2300 was used as a control. The tobacco plants were grown in the dark for 24 h and then switched to a 16 h light/8 h dark cycle for 48 h at 23°C. The fluorescein signal was detected in the Tanon 5200 Multi automatic chemiluminescence/fluorescence image analysis system (Tanon, Shanghai, China).

2.9 Biolayer interferometry assay

The Octet RED96 System (ForteBio) was used to assess the kinetics of DNA-protein binding using streptavidin-coated biosensors. GhBES1 protein and biotin-labeled probe on *GhEXO2* promoter were used as described in the EMSA. After the sensor was immersed in the buffer, the biotinylated protein was bound according to Baseline, Loading and Wash, and then the binding of different sample wells and the sensor was detected according to Baseline, Association and Dissociation. All operations were as described in the manufacturer's instructions. Data were analyzed using OriginPro 2021 software.

2.10 RNA-sequencing and GO and KEGG analyses

For RNA-seq data analysis, the leaves of *GhEXO2* silenced cotton plants were collected 30 days after germination. Screening of clean reads was performed after the removal of low-quality and contaminated reads as well as adaptors. Next, we mapped clean reads in the GRAND database (http://grand.cricaas.com.cn/) by Hisat2. StringTie was used to measure the expression levels and the values were normalized by FPKM (fragments per kilobase of transcript per million fragments mapped) (Pertea et al. 2015). Raw reads count for coding genes were obtained using StringTie subscript prepDE.py (Kukurba and Montgomery 2015). R package 'EdgeR' was used to identify the differentially expressed genes (DEGs) between two groups (Robinson et al. 2010). To fine-tune the P-value for the false detection rate, Benjamini and Hochberg's approaches were used. DEGs were defined by fine-tuning P-value < 0.05 and absolute fold change value > 2. The GO and KEGG enrichments were conducted in the R package 'clusterProfiler' (Yu et al. 2012). RNA-seq data have been uploaded to the NCBI database, BioProject: PRJNA834593.

3. Results

3.1 Expression of the *GhEXO2* gene is down-regulated in *pag1* mutants

pag1 exhibited BR-defective related phenotypes, particularly in smaller leaf size (Fig. 1A) (Yang et al. 2014). Cytological observation and microscopic analysis showed that cell expansion was inhibited in the leaves of *pag1* mutant plants compared to wild-type (WT) CRI24 plants (Fig. 1B and Fig. S1A). A significantly down-regulated gene was identified in the hormone-related differentially expressed genes in cotyledon transcriptome data of *pag1*(Fig. S2). Sequence analysis showed that this gene has the highest similarity to *AtEXL2* and has a conserved phi-1 structural domain (Fig. S3 A), hence we named it *GhEXO2.* Expression of *GhEXO2* was monitored in cotton plants, including CRI24 and BR deficient mutant *pag1* in different tissues (roots, stem, leaves, and 20 days post-anthesis (DPA) fiber tissues). This gene had significantly down-regulated expression in *pag1* plants compared to CRI24 cotton plants at all experimental stages (Fig. 1C). Exogenous application of BL induced its expression, which decreased after the peak at 1 h of treatment (Fig. 1D). These results suggested that *GhEXO2* responds to BR treatment.

3.2 *GhBES1.4* promotes cotton leaf size and regulates *GhEXO2* expression

BES1 is the core TF regulating expression of downstream response genes in BR signaling to influence plant development and respond to environmental stress (Li et al. 2018). Our previous study identified 22 *GhBES1* genes in upland cotton and found that *GhBES1.4* was a functional gene mediating BR response (Liu et al. 2018). In order to explore if *GhBES1* participates in BR regulation of leaf size, we created *GhBES1* RNA interference and *GhBES1.4* overexpression transgenic cotton in CRI24. We selected several *GhBES1s* with high homology to *GhBES1.4* and verified the effectiveness of RNA interference in the *GhBES1*-RNAi plants (Fig. S4A and B). In addition, the transcription level of *GhBES1.4* was significantly increased in overexpression plants (Fig. S4C and D). Three independent transgenic lines with the most significant down-regulation in RNAi lines (lines 3, 7, and 8) and different levels of up-regulation in overexpression lines (lines 2, 4, and 8) were selected for further analysis. Suppressing the expression level of *GhBES1s* significantly reduced leaf size. In contrast, overexpression of *GhBES1.4* increased leaf size compared to the WT (Fig. 2A). Observation of cotton leaf morphology showed smaller cells in *GhBES1*-RNAi plants and bigger cells in overexpression *GhBES1.4* plants than WT (Fig. 2B and Fig. S1B). Moreover, qRT-PCR analysis showed that *GhEXO2* increased expression in *GhBES1.4* overexpression plants and decreased expression in *GhBES1*-RNAi plants (Fig. 2C and D). These results suggested that *GhBES1.4* positively regulated leaf size and induced the transcription of *GhEXO2*.

3.3 *GhEXO2* encodes a membrane protein containing BES1 binding sites in its promoter

Phylogenetic analysis showed that *GhEXO2* was highly homologous to *AtEXL2*, part of the EXO gene family (Fig. 3A and Fig. S3B). To elucidate the subcellular localization of GhEXO2, a construct containing GhEXO2-GFP was infiltrated into tobacco (*Nicotiana benthamiana*) leaves, which were subjected to confocal imaging microscopy. Confocal imaging microscopy confirmed the presence of GhEXO2-GFP in the cell membrane compared to the red cell membrane marker dye FM 4–64 (Fig. 3B). Analysis of the

2,000 bp promoter region upstream of *GhEXO2* start codon revealed some elements related to hormone response, including auxin (AuxRE), methyl jasmonate (MeJA, CGTCA-motif), and BR (E-box and G-box) (Fig. 3C). Furthermore, this E-box has been included in the BES1 binding motif multiple times as predicted by PlantTFDB (Tian et al. 2020). These findings confirmed that *GhEXO2* might have a function similar to *EXO* in *Arabidopsis* and that it is a potential target gene of *GhBES1*. These results indicated that GhEXO2 is a cell membrane-associated protein, which mediates BR signaling and may function in changing cotton leaf size through cell expansion.

3.4 Ectopic overexpression of *GhEXO2* in *Arabidopsis* promotes leaf size

Previous studies have shown that constitutive overexpression of *AtEXO* under 35SCaMV promoter control in *Arabidopsis* enhanced vegetative growth (Coll-Garcia et al. 2004), and that both *exo* knock-out mutant growth and cell size were reduced (Schröder et al. 2009). To identify the biological functions of *GhEXO2*, we ectopically overexpressed *GhEXO2* in *Arabidopsis* (Col-0). The resulting Col-0/*GhEXO2* transgenic lines had enhanced vegetative growth compared to WT (Col-0) plants, like *AtEXO* (Fig. 4A). Most obviously, the leaf area of Col-0/GhEXO2 transgenic plants was more than twice as large as in WT plants (Fig. 4B). Moreover, the high relative expression of *GhEXO2* in Col-0/*GhEXO2* transgenic plants was verified by semi-quantitative RT-PCR and qRT-PCR analysis (Fig. 4C and D) indicating the validity of the phenotype at the transcription level.

Expression analysis of cell elongation-related genes by qRT-PCR, including *AtAGP4*, *AtEXP5*, *AtKCS1*, and *Atδ-TIP*, was conducted in Col-0/*GhEXO2* transgenic plants and WT plants (Coll-Garcia et al. 2004). The results indicated that expression of all cell elongation-related genes was significantly up-regulated in three independent Col-0/*GhEXO2* transgenic lines compared to WT plants (Fig. 4E, F, G, and H). Among them, *AtAGP4*, *AtKCS1*, and *Atδ-TIP* were up-regulated by 2- to 8-fold in the three transgenic lines, and the expression level of *AtEXP5* was up-regulated by 15- to 20-fold. These gene expression results indicated that enhanced vegetative growth in Col-0/*GhEXO2* transgenic plants altering leaf size might result from cell expansion.

3.5 Silencing of *GhEXO2* suppresses leaf cell expansion in cotton

To further investigate the role of *GhEXO2* in the regulation of leaf size, we knocked down its expression through virus-induced gene silencing (VIGS) using the pTRV system. As predicted, significant suppression of *GhEXO2* expression and less vegetative growth was detected in silenced plants (Fig. 5A) as well as altered leaf size (Fig. 5B and Fig. S1C) compared to the vector control. Statistical analysis found that the leaf area of all three independent lines of *GhEXO2* silenced plants was significantly less than the vector control (Fig. 5E). The qRT-PCR analysis demonstrated that *GhEXO2* expression was significantly depressed in observed silenced lines relative to vector control, indicating that *GhEXO2* was effectively silenced in VIGS lines (Fig. 5C).

To fully understand the mechanism of altered leaf size in *GhEXO2* silenced plants, histological microscopic analysis was performed. Microscopic images of the leaves of *GhEXO2* silenced plants showed a more significant number of small cells than the leaves of vector control where the cells were large but in low numbers, indicating that silencing *GhEXO2* inhibited cell expansion and reduced leaf size (Fig. 5D). Similarly, cell expansion was inhibited in the leaves of *pag1* mutant plants compared to CRI24 plants. Further, the numbers of cells were measured in the same leaf area of both silenced and vector control plants under an electron microscope. All three lines of *GhEXO2* silenced plants showed a significantly greater number of cells compared to vector control (Fig. 5F). *GhEXO2* silenced plants showed smaller leaf sizes due to the inhibition of cell expansion compared with vector control. These findings followed our previous results. The overexpression of *GhEXO2* in *Arabidopsis* resulted in increased leaf size with higher LAI compared to WT plants (Fig. 4A).

3.6 *GhBES1.4* directly binds *GhEXO2* promoter to activate its expression

Cis-acting element analysis of *GhEXO2* promoter and detection of *GhEXO2* expression level in *GhBES1.4* transgenic plants indicate that *GhEXO2* may be a downstream target gene of *GhBES1.4*. To verify this hypothesis, an electrophoretic mobility shift assay (EMSA) was performed (Fig. 6A). The result indicated that GhBES1.4 fusion protein directly binds to the biotin-labeled probe but fails to bind to the mutant probe. Furthermore, non-labeled probes (cold competition probes) markedly reduced the binding efficiency of the biotin-labeled probe and GhBES1.4 fusion protein. The binding affinity in real-time of *GhBES1.4* and the E-box element in the *GhEXO2* promoter was measured by biolayer interferometry (BLI) to clarify their interaction (Fig. 6B). The fragment of the *GhEXO2* promoter had a stronger binding affinity to GhBES1.4 fusion protein compared with mutant fragments. It weakened with the decrease of the fusion protein concentration. These results indicated that *GhBES1.4* specifically binds to the E-box element in the *GhEXO2* promoter region *in vitro*.

However, it is unclear whether GhBES1.4 activates *GhEXO2* transcription *in vivo*. Promoters (2,000 bp) of *GhEXO2*, both natural and mutated in the E-box region, were separately inserted into the pGWB435 vector with the luciferase (LUC) reporter gene. Tobacco leaves co-infected with *GhEXO2*pro-LUC, and 35S-*GhBES1.4* showed brighter fluorescence signals, while leaves co-infected with *GhEXO2*pro-mutant-LUC and 35S-*GhBES1.4* had weaker fluorescence compared with the vector control (Fig. 6C). LUC activity analysis also indicated that the LUC reporter gene was activated by *GhBES1.4* driven by the E-box in the *GhEXO2* promoter (Fig. 6D). These results revealed that *GhBES1.4* directly binds to the E-box element in the promoter region of *GhEXO2* to activate its expression, which promotes cotton leaf size.

3.7 *GhEXO2* affects the expression of various cell expansion genes

To explore the mechanism by which *GhEXO2* in cotton altered leaf size, we performed RNA-seq analysis of *GhEXO2* silenced plants. A total of 919 genes were differentially expressed genes (DEGs); 595 genes (64.74% of total DEGs) were down-regulated while 324 genes (35.25% of all DEGs) were up-regulated

(Fig. 7A and Table S1). Here, *GhEXO2* integrated multiple pathways to induce plant growth and development with altered leaf size. Gene ontology (GO) enrichment analysis of RNA-seq data indicated that more genes (97 and 48 genes) enriched in cell wall organization and biogenesis and polysaccharide biosynthetic processes were down-regulated than up-regulated (only four and six genes), respectively (Fig. 7B and Table S1). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis indicated that more DEGs (55 genes) involved in plant hormone signal transduction were down-regulated than up-regulated (14 genes) in *GhEXO2* silenced plants (Fig. 7C and Table S1). Further, we generated a heatmap of expression of selected genes related to cell expansion, including fatty acid elongation, cell wall organization and biogenesis, cell periphery, cell wall biosynthetic process, BR biosynthesis, and MAPK pathway. Most of these selected genes were down-regulated in *GhEXO2* silenced plants (Fig. 7D).

Some key DEGs were selected to validate our results, and qRT-PCR analysis was conducted in two lines with significantly down-regulated *GhEXO2* expression levels. Except for *GhCPD* and *GhDWF4*, which are related to BR biosynthesis, the other seven genes are directly or indirectly related to cell expansion (Wanjie et al. 2005; Todd et al. 1999; Goh et al. 2012; Jensen et al. 2011; Lee et al. 2006; Liu et al. 2021a; Chung and Choe 2013). The results of the qRT-PCR analysis indicated that *GhKCS1*, *GhEXPA4*, *GhEXPA8*, *GhIRX15-L*, and *GhLNG1* were down-regulated while the two BR biosynthesis genes *GhCPD* and *GhDWF4* were up-regulated in *GhEXO2* silenced plants (Fig. S6). These qRT-PCR results followed the results of RNA-seq data analysis, indicating the validity of our findings.

4. Discussion

The leaf is an essential organ of plant photosynthesis and transpiration. Variability in leaf size is regulated by the external environment and localized delivery of the phytohormone (Nikolov et al. 2019). BRs are essential steroid hormones that function in different developmental processes in plants, chiefly in plant growth. Here, we demonstrated that BR regulates cotton leaf size by cell expansion. Further, GhBES1 mediates BR regulation of leaf size through directly activating the expression of *GhEXO2*, which was confirmed as a cell expansion regulator. In this study, we revealed the novel mechanism by which BR regulates leaf size.

4.1 BR regulates leaf size in plants

In previous studies, the BR signaling pathway was shown to be a relatively clear signal transduction pathway in plants, based on combining multiple research approaches (Guo et al. 2013; Kim et al. 2009; Kour et al. 2021; Li 2010). Analysis of BR deficient mutants and BR-related transgenic plants showed that BRs positively regulate leaf size in a variety of plants. In *Arabidopsis*, *dwf4* and *cpd* plants are reduced in stature, and have dark green colored leaves due exclusively to inhibition of cell elongation increasing chloroplast density (Szekeres et al. 1996; Azpiroz et al. 1998). In BR-insensitive mutant, *bri1*, applying exogenous BRs does not rescue the phenotype in an extreme dwarf with short rosette leaves (Clouse et al. 1996). In rice, *brd3-D* enhances *CYP734A4* expression and shows dwarf and erect leaf phenotypes. In addition, knockout of the *CYP734A4* gene by CRISPR/Cas9 rescues the *brd3-D* phenotype (Qian et al.

2017). In barley, the BR receptor gene *HvBRI1* mutant *uzu1* has an erect structure, an acute angle of the leaf blade, and a wavy leaf margin (Dockter et al. 2014). Overexpression of *GmMYB14* in soybean reduced the endogenous BR content, resulting in decreased plant height and smaller leaf area, and exogenous application of BL partially restored its phenotype (Chen et al. 2021). *GhPAG1*, a BR catabolic gene, produces BR deficient mutant (*pag1*) in cotton, which showed less fiber elongation, a dwarf phenotype, and markedly decreased leaf size due to inhibited cell elongation (Fig. 1A and B) (Yang et al. 2014). Thus, BR regulation of leaf size is conserved in plants.

4.2 BES1 is a critical factor for BR to promote leaf size

BES1 is a basic helix–loop–helix (bHLH) protein, a superfamily of TFs essential in plant growth and development. Activation of *BES1* causes curled leaves and long and curved petioles in *Arabidopsis bes1-D* mutant (Yin et al, 2002). Conversely, a hextuple mutant (*bzr-h*) that disrupts all BZR1s in *Arabidopsis* showed a phenotype that was more severely curled and crinkled, and had small and rounded leaves (Chen et al. 2019). In rice, BES1 promotes the bHLH TF *INCREASED LEAF INCLINATION 1* (*ILI1*) transcription by binding to its promoter region and significantly increasing leaf tilt (Zhang et al. 2009). In cotton, the bHLH TF *Fibre-related Protein 1* (*GhFP1*) promotes fiber elongation by directly binding to promoter sequences of BR synthesis genes to activate its expression (Liu et al. 2020). Our previous work found that *GhBES1.4* is a functional TF inducing BR response (Liu et al. 2018). In our study, inhibition of *GhBES1s* expression suppressed cell expansion and decreased leaf size. Overexpression of *GhBES1.4* showed larger leaf size via accelerating cell expansion in cotton (Fig. 2A and B). Therefore, BES1 functions directly in BR regulation of leaf size.

An important mechanism in BR signaling depends on the direct regulation of target genes by BZR1/BES1 via binding E-box (CANNTG) in the promoter (Wang et al. 2012; Xie et al. 2011; Li et al. 2018). Absence of *AtMYB30* function exhibited decreased BR responses, because BES1 binds to E-box to directly regulate the transcription of *AtMYB30* and activates downstream target genes to amplify BR signals via recruiting *AtMYB30* (Li et al. 2009). *BES1* directly binds to *MYELOBLASTOSIS FAMILY TRANSCRIPTION FACTOR-LIKE 2* (*MYBL2*), repressing its transcription to regulate cell elongation (Ye et al. 2012). In our study, a significantly down-regulated gene, *GhEXO2*, was found in *pag1* with the E-box in the promoter region, and exogenous application of BL induced *GhEXO2* expression (Fig. 1C, D, and 3B). *GhCaM7-like* was also downregulated in the *pag1* mutant as in a previous study (Yang et al., 2014). Silencing of *GhCaM7-like* expression suppressed fiber length and leaf size, and multiple E-boxes were predicted in the promoter region. These results indicated that *GhCaM7-like* is a potential target gene for BES1 (Cheng et al., 2016). In addition, silencing of *GhEXO2* resulted in inhibition of stem cell expansion similar to *pag1* and *GhCaM7-like*-silenced plants (Fig. S5A and B). Further, protein and DNA binding experiments *in vivo* and *in vitro* showed that *GhBES1.4* binds to the E-box motif in the promoter region of *GhEXO2* and activates its expression. Thus, *GhBES1* regulated leaf size and affected *GhEXO2* expression by activating its transcription (Fig. 2C and D). Overall, *BES1* mediated BR signaling affects leaf size by regulating downstream genes.

4.3 *GhEXO2* promotes cell expansion and affects cotton leaf size, thus mediating BR signal

EXO, a protein localized to the cell membrane, is closely associated with the cell wall and EXL1 and EXL2 in *Arabidopsis* and is expressed in dividing cells (Farrar et al. 2003; Schröder et al. 2009; Coll-Garcia et al. 2004). *Arabidopsis EXO/EXL* mediates plant growth and development through cell expansion as a downstream BR signaling gene, and overexpression in BR-deficient *dwf1-6* and *det2* mutants does not normalize the dwarf phenotype (Schröder et al. 2009; Coll-Garcia et al. 2004). In addition, exogenous application of auxin and BL strongly induced their expression, while cytokinin inhibited it (Farrar et al. 2003; Coll-Garcia et al. 2004). Further, *bes1-D*, a gain of function mutant of *BES1*, showed constitutive expression of *EXO* genes (Yin et al. 2002). 35S::*EXO* had larger rosette leaves and increased transcription levels of BR-responsive genes (*AtAGP4*, *AtEXP5*, *AtKCS1*, and *Atδ-TIP*) associated with cell walls (Coll-Garcia et al. 2004). *exo* knockout mutant showed a dwarfed phenotype with inhibited cell expansion, and reduced transcript levels of *AtKCS1* and *AtEXP5* recovered with increasing age (Schröder et al. 2009).

Our results demonstrate that *GhEXO2* promotes cotton leaf size by positively promoting cell expansion in the BR pathway. First, *GhEXO2*, like members of the *AtEXO* family, is a cell membrane-associated protein (Fig. 3B). The conserved Phi_1 domain showed maximum similarity with *Arabidopsis AtEXL2* (Fig. 3A, Fig. S3 A and B). These results imply that *GhEXO2* functions in the cell wall. Next, overexpression of *GhEXO2* in *Arabidopsis* showed enhanced leaf size (Fig. 4A). Higher expression patterns of cell elongation-related genes *AtAGP4*, *AtEXP5*, *AtKCS1*, and *Atδ-TIP* in transgenic lines indicated that *GhEXO2* induces enhanced leaf size through cell expansion (Fig. 4E, F, G, and H) like *AtEXO*. Furthermore, silencing *GhEXO2* by VIGS showed reduced leaf size and limited cell expansion (Fig. 5B and D). Therefore, *GhEXO2* acts as a cell expansion regulator to promote leaf size.

In addition, RNA-seq analysis in cotton indicated that silencing *GhEXO2* affects multiple pathway genes to suppress leaf size (Fig. 7B, C and D). First, in silenced *GhEXO2* plants, different genes were markedly affected in fatty acid elongation and cell wall organization or biogenesis pathways compared to *Arabidopsis* (Coll-Garcia et al. 2004; Shang et al. 2016). In the fatty acid elongation pathway, the *KCS1* gene is affected in both *Arabidopsis* and cotton, but other fatty acid elongation genes such as *KCS11* are also affected in cotton. In addition, *EXP5*, which is affected in *Arabidopsis*, is not a DEG, while *EXPA4* and *EXPA8* genes from the same family are significantly down-regulated in cell wall organization or biogenesis pathway. We also found that *CPD* and *DWF4*, which are reported to differ little in *Arabidopsis*, were enriched in the BR biosynthetic pathway in the up-regulated cotton DEGs. Moreover, we identified multiple genes that have not been reported in *Arabidopsis* that were enriched in the novel pathways. In the cell wall macromolecule biosynthetic process, the expression of the xylan biosynthesis gene *GhIRX15-L* and the longitudinal leaf cell elongation gene *GhLNG1* was down-regulated, which has not been found in *Arabidopsis*. This result may be due to interspecific differences between tetraploid cotton and diploid *Arabidopsis*.

KCS1, a BZR1 target gene involved in cell wall biosynthesis, has been reported to be an early BR-responsive gene (Sun et al. 2010). Expansion protein is the first discovered and most widely reported cell wall loosening protein. The alpha-expansin group members *GhEXPA4* and *GhEXPA8* enriched in cell wall organization contributed to early lint fiber initiation. Moreover, *GhEXPA8* improves mature cotton fiber length and micronaire value (Bajwa et al. 2015; Liu et al. 2021b). *CPD* and *DWF4* are key genes of BRs biosynthetic enzymes, which can be activated by feedback from reduced BR levels (Szekeres et al. 1996; Choe et al. 1998). *IRX15-L* encodes xylan biosynthesis, a major component of plant cell wall hemicellulose. *irx15 irx15-L* double mutants have irregular secondary cell wall edges and low xylan aggregation in fibroblasts. *LNG1* promotes polar elongation of cells and its dominant mutant *longifolia1-1D* (*lng1-1D*) presents a long petiole, narrow and extremely long leaf blade, and a serrated margin phenotype (Jensen et al. 2011; Lee et al. 2006). Taken together, these results imply that *GhEXO2* affects multiple pathways that influence leaf size by mediating cell expansion.

Collectively, we proposed a working model of BR to promote leaf size through cell expansion. In the BR signaling pathway, *GhBES1* affects cotton leaf size by binding to and activating the expression of the E-box element in the *GhEXO2* promoter region. Overexpression of *GhEXO2* promotes leaf size, and suppression of its expression inhibits leaf size by altering the expression of cell elongation-related genes (Fig. 8).

Declarations

Data Availability Statement

All datasets generated for this study are included in the article/**Supplementary Files**.

Author Contributions

All authors contributed to the study conception and design**. Shengdong Li:** Experimentation, writing. **Kun Xing:** Methodology, formal analysis. **Ghulam Qanmber:** Writing & editing. **Guoquan Chen:** Conceptualization, writing – review & editing. **Liu Le:** Software, data curation. **Mengzhen Guo:** Formal analysis. **Yan Hou:** Review & editing. **Lili Lu:** Methodology, data curation. **Zhao Liu:** Supervision. **Zuoren Yang:** Supervision.

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Conflict of Interest

The authors declare that they have no competing interests.

References

1. Ali F, Qanmber G, Li F, Wang Z (2021) Updated role of ABA in seed maturation, dormancy, and germination. Journal of Advanced Research
2. Andres RJ, Bowman DT, Jones DC, Kuraparthy V (2016) Major leaf shapes of cotton: Genetics and agronomic effects in crop production. J Cotton Sci 20(4):330–340
3. Andres RJ, Coneva V, Frank MH, Tuttle JR, Samayoa LF, Han S-W, Kaur B, Zhu L, Fang H, Bowman DT (2017) Modifications to a LATE MERISTEM IDENTITY1 gene are responsible for the major leaf shapes of Upland cotton (*Gossypium hirsutum L.*). Proceedings of the National Academy of Sciences 114 (1):E57-E66
4. Aya K, Hobo T, Sato-Izawa K, Ueguchi-Tanaka M, Kitano H, Matsuoka M (2014) A novel AP2-type transcription factor, SMALL ORGAN SIZE1, controls organ size downstream of an auxin signaling pathway. Plant Cell Physiol 55(5):897–912
5. Azpiroz R, Wu Y, LoCascio JC, Feldmann KA (1998) An *Arabidopsis* brassinosteroid-dependent mutant is blocked in cell elongation. Plant Cell 10(2):219–230
6. Bai M-Y, Zhang L-Y, Gampala SS, Zhu S-W, Song W-Y, Chong K, Wang Z-Y (2007) Functions of OsBZR1 and 14-3-3 proteins in brassinosteroid signaling in rice. Proceedings of the National Academy of Sciences 104 (34):13839–13844
7. Bajwa KS, Shahid AA, Rao AQ, Bashir A, Aftab A, Husnain T (2015) Stable transformation and expression of GhEXPA8 fiber expansin gene to improve fiber length and micronaire value in cotton. Front Plant Sci 6:838
8. Chen L-G, Gao Z, Zhao Z, Liu X, Li Y, Zhang Y, Liu X, Sun Y, Tang W (2019) BZR1 family transcription factors function redundantly and indispensably in BR signaling but exhibit BRI1-independent function in regulating anther development in *Arabidopsis*. Mol Plant 12(10):1408–1415
9. Chen L, Yang H, Fang Y, Guo W, Chen H, Zhang X, Dai W, Chen S, Hao Q, Yuan S (2021) Overexpression of GmMYB14 improves high-density yield and drought tolerance of soybean through regulating plant architecture mediated by the brassinosteroid pathway. Plant Biotechnol J 19(4):702–716
10. Choe S, Dilkes BP, Fujioka S, Takatsuto S, Sakurai A, Feldmann KA (1998) The DWF4 gene of *Arabidopsis* encodes a cytochrome P450 that mediates multiple 22α-hydroxylation steps in brassinosteroid biosynthesis. Plant Cell 10(2):231–243
11. Chory J, Nagpal P, Peto CA (1991) Phenotypic and genetic analysis of det2, a new mutant that affects light-regulated seedling development in *Arabidopsis*. Plant Cell 3(5):445–459
12. Chung Y, Choe S (2013) The regulation of brassinosteroid biosynthesis in *Arabidopsis*. CRC Crit Rev Plant Sci 32(6):396–410

13. Clough SJ, Bent AFJTpj (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. 16:735–7436
14. Clouse SD, Langford M, McMorris TC (1996) A brassinosteroid-insensitive mutant in *Arabidopsis thaliana* exhibits multiple defects in growth and development. Plant Physiol 111(3):671–678
15. Clouse SD, Sasse JMJAropb (1998) Brassinosteroids: essential regulators of plant growth and development. 49 (1):427–451
16. Coll-Garcia D, Mazuch J, Altmann T, Müssig C (2004) EXORDIUM regulates brassinosteroid-responsive genes. FEBS Lett 563(1–3):82–86
17. Dinesh-Kumar S, Anandalakshmi R, Marathe R, Schiff M, Liu Y (2003) Virus-induced gene silencing. Plant Functional Genomics. Springer, pp 287–293
18. Dockter C, Gruszka D, Braumann I, Druka A, Druka I, Franckowiak J, Gough SP, Janeczko A, Kurowska M, Lundqvist J (2014) Induced variations in brassinosteroid genes define barley height and sturdiness, and expand the green revolution genetic toolkit. Plant Physiol 166(4):1912–1927
19. Dolan L, Poethig RS (1991) Genetic analysis of leaf development in cotton
20. Farrar K, Evans IM, Topping JF, Souter MA, Nielsen JE, Lindsey K (2003) EXORDIUM–a gene expressed in proliferating cells and with a role in meristem function, identified by promoter trapping in *Arabidopsis*. Plant J 33(1):61–73
21. Goh H-H, Sloan J, Dorca-Fornell C, Fleming A (2012) Inducible repression of multiple expansin genes leads to growth suppression during leaf development. Plant Physiol 159(4):1759–1770
22. Gonzalez N, Vanhaeren H, Inzé D (2012) Leaf size control: complex coordination of cell division and expansion. Trends Plant Sci 17(6):332–340
23. Guo H, Li L, Aluru M, Aluru S, Yin Y (2013) Mechanisms and networks for brassinosteroid regulated gene expression. Curr Opin Plant Biol 16(5):545–553
24. He J-X, Gendron JM, Sun Y, Gampala SS, Gendron N, Sun CQ, Wang Z-Y (2005) BZR1 is a transcriptional repressor with dual roles in brassinosteroid homeostasis and growth responses. Science 307(5715):1634–1638
25. He P, Zhang Y, Li H, Fu X, Shang H, Zou C, Friml J, Xiao G (2021) GhARF16-1 modulates leaf development by transcriptionally regulating the GhKNOX2-1 gene in cotton. Plant Biotechnol J 19(3):548
26. Jensen JK, Kim H, Cocuron JC, Orler R, Ralph J, Wilkerson CG (2011) The DUF579 domain containing proteins IRX15 and IRX15-L affect xylan synthesis in *Arabidopsis*. Plant J 66(3):387–400
27. Kim T-W, Guan S, Sun Y, Deng Z, Tang W, Shang J-X, Sun Y, Burlingame AL, Wang Z-Y (2009) Brassinosteroid signal transduction from cell-surface receptor kinases to nuclear transcription factors. Nat Cell Biol 11(10):1254–1260
28. Kour J, Kohli SK, Khanna K, Bakshi P, Sharma P, Singh AD, Ibrahim M, Devi K, Sharma N, Ohri P (2021) Brassinosteroid signaling, crosstalk and, physiological functions in plants under heavy metal stress. Frontiers in Plant Science 12

29. Kukurba KR, Montgomery SBJCSHP (2015) RNA sequencing and analysis. 2015 (11):pdb. top084970
30. Kumar S, Tamura K, Nei MJB (1994) MEGA: molecular evolutionary genetics analysis software for microcomputers. 10 (2):189–191
31. Lee YK, Kim G-T, Kim I-J, Park J, Kwak S-S, Choi G, Chung W-I (2006) LONGIFOLIA1 and LONGIFOLIA2, two homologous genes, regulate longitudinal cell elongation in *Arabidopsis*. Development 133(21):4305–4314
32. Li J (2010) Regulation of the nuclear activities of brassinosteroid signaling. Curr Opin Plant Biol 13(5):540–547
33. Li J, Chory JJC (1997) A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. 90:929–9385
34. Li J, Nagpal P, Vitart V, McMorris TC, Chory JJS (1996) A role for brassinosteroids in light-dependent development of Arabidopsis. 272:398–4015260
35. Li L, Yu X, Thompson A, Guo M, Yoshida S, Asami T, Chory J, Yin Y (2009) Arabidopsis MYB30 is a direct target of BES1 and cooperates with BES1 to regulate brassinosteroid-induced gene expression. Plant J 58(2):275–286
36. Li Q-F, Lu J, Yu J-W, Zhang C-Q, He J-X, Liu Q-Q (2018) The brassinosteroid-regulated transcription factors BZR1/BES1 function as a coordinator in multisignal-regulated plant growth. Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms. 1861:561–5716
37. Liu D, Shi S, Hao Z, Xiong W, Luo M (2019) OsbZIP81, A Homologue of Arabidopsis VIP1, may positively regulate JA levels by directly targetting the genes in JA signaling and metabolism pathway in rice. Int J Mol Sci 20(9):2360
38. Liu L, Xie Z, Lu L, Qanmber G, Chen G, Li S, Guo M, Sun Z, Liu Z, Yang Z (2021a) Identification of BR biosynthesis genes in cotton reveals that GhCPD-3 restores BR biosynthesis and mediates plant growth and development. Planta 254(4):1–17
39. Liu W, Lv Y, Li X, Feng Z, Wang L (2021b) Comparative transcriptome analysis uncovers cell wall reorganization and repressed cell division during cotton fiber initiation
40. Liu Z, Qanmber G, Lu L, Qin W, Liu J, Li J, Ma S, Yang Z, Yang Z (2018) Genome-wide analysis of BES1 genes in *Gossypium* revealed their evolutionary conserved roles in brassinosteroid signaling. Sci China Life Sci 61(12):1566–1582
41. Liu ZH, Chen Y, Wang NN, Chen YH, Wei N, Lu R, Li Y, Li XB (2020) A basic helix–loop–helix protein (GhFP1) promotes fibre elongation of cotton (*Gossypium hirsutum*) by modulating brassinosteroid biosynthesis and signalling. New Phytol 225(6):2439–2452
42. Livak KJ, Schmittgen TDJm (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2 − ΔΔCT method. 25 (4):402–408
43. Malik W, Shah MSA, Abid MA, Qanmber G, Noor E, Qayyum A, Liang C, Guo S, Zhang R (2018) Genetic basis of variation for fiber quality and quality related biochemical traits in Bt and non-Bt colored cotton. Intl J Agric Biol 20:2117–2124

44. Mao J, Li JJIJoMS (2020) Regulation of Three Key Kinases of Brassinosteroid Signaling Pathway. 21:434012
45. Nikolov LA, Runions A, Gupta MD, Tsiantis M (2019) Leaf development and evolution. Curr Top Dev Biol 131:109–139
46. Noguchi T, Fujioka S, Choe S, Takatsuto S, Yoshida S, Yuan H, Feldmann KA, Tax FEJPp (1999) Brassinosteroid-insensitive dwarf mutants of Arabidopsis accumulate brassinosteroids. 121:743–7523
47. Nolan T, Chen J, Yin YJBJ (2017) Cross-talk of Brassinosteroid signaling in controlling growth and stress responses. 474:2641–266116
48. Pertea M, Pertea GM, Antonescu CM, Chang T-C, Mendell JT, Salzberg SL (2015) StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. Nat Biotechnol 33(3):290–295
49. Phillips R, Sarang M, Gibson N (1993) Semiquantitative measurement of gene-expression by rt-PCR-a cautionary tale. Int J Oncol 3(6):1097–1102
50. Praveena J, Dash S, Behera L, Rout GRJCJoAS (2020) Technology Brassinosteroids: A Multifunctional Phytohormone of Plant Development and Stress Responses.174–196
51. Qian W, Wu C, Fu Y, Hu G, He Z, Liu W (2017) Novel rice mutants overexpressing the brassinosteroid catabolic gene CYP734A4. Plant Mol Biol 93(1–2):197–208
52. Qiao S, Sun S, Wang L, Wu Z, Li C, Li X, Wang T, Leng L, Tian W, Lu T (2017) The RLA1/SMOS1 transcription factor functions with OsBZR1 to regulate brassinosteroid signaling and rice architecture. Plant Cell 29(2):292–309
53. Robinson MD, McCarthy DJ, Smyth GKJB (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. 26:139–1401
54. Saitou N, Nei, MJMb (1987) evolution The neighbor-joining method: a new method for reconstructing phylogenetic trees. 4 (4):406–425
55. Schröder F, Lisso J, Lange P, Müssig C (2009) The extracellular EXO protein mediates cell expansion in Arabidopsis leaves. BMC Plant Biol 9(1):1–12
56. Schröder F, Lisso J, Müssig, CJPs (2012) behavior Expression pattern and putative function of EXL1 and homologous genes in Arabidopsis. 7 (1):22–27
57. Shang B, Xu C, Zhang X, Cao H, Xin W, Hu Y (2016) Very-long-chain fatty acids restrict regeneration capacity by confining pericycle competence for callus formation in Arabidopsis. Proceedings of the National Academy of Sciences 113 (18):5101–5106
58. Sousa AO, Camillo LR, Assis ETC, Lima NS, Silva GO, Kirch RP, Silva DC, Ferraz A, Pasquali G, Costa MG (2020) EgPHI-1, a PHOSPHATE-INDUCED-1 gene from Eucalyptus globulus, is involved in shoot growth, xylem fiber length and secondary cell wall properties. Planta 252(3):1–17
59. Sun Y, Fan XY, Cao DM, Tang W, He K, Zhu JY, He JX, Bai MY, Zhu S, Oh E (2010) Integration of Brassinosteroid Signal Transduction with the Transcription Network for Plant Growth Regulation in Arabidopsis. Dev Cell 19(5):765–777

60. Szekeres M, Németh K, Koncz-Kálmán Z, Mathur J, Kauschmann A, Altmann T, Rédei GP, Nagy F, Schell J, Koncz C (1996) Brassinosteroids rescue the deficiency of CYP90, a cytochrome P450, controlling cell elongation and de-etiolation in *Arabidopsis*. Cell 85(2):171–182
61. Thompson JD, Gibson TJ, Higgins DGJCpib (2003) Multiple sequence alignment using ClustalW and ClustalX. (1):2.3. 1-2.3. 22
62. Tian F, Yang D-C, Meng Y-Q, Jin J, Gao G (2020) PlantRegMap: charting functional regulatory maps in plants. Nucleic Acids Res 48(D1):D1104–D1113
63. Todd J, Post-Beittenmiller D, Jaworski JG (1999) KCS1 encodes a fatty acid elongase 3-ketoacyl-CoA synthase affecting wax biosynthesis in *Arabidopsis thaliana*. Plant J 17(2):119–130
64. Vert G, Nemhauser JL, Geldner N, Hong F, Chory JJARCDB (2005) Molecular mechanisms of steroid hormone signaling in plants. 21:177–201
65. Wan S, Wu J, Zhang Z, Sun X, Lv Y, Gao C, Ning Y, Ma J, Guo Y, Zhang Q (2009) Activation tagging, an efficient tool for functional analysis of the rice genome. Plant Mol Biol 69(1–2):69–80
66. Wang L, Yang Z, Zhang B, Yu D, Liu J, Gong Q, Qanmber G, Li Y, Lu L, Lin Y (2018) Genome-wide characterization and phylogenetic analysis of GSK gene family in three species of cotton: evidence for a role of some GSKs in fiber development and responses to stress. BMC Plant Biol 18(1):1–21
67. Wang X, Zhang J, Yuan M, Ehrhardt DW, Wang Z, Mao TJTPC (2012) Arabidopsis microtubule destabilizing protein40 is involved in brassinosteroid regulation of hypocotyl elongation. 24:4012–402510
68. Wanjie SW, Welti R, Moreau RA, Chapman KD (2005) Identification and quantification of glycerolipids in cotton fibers: reconciliation with metabolic pathway predictions from DNA databases. Lipids 40(8):773–785
69. Waterhouse AM, Procter JB, Martin DM, Clamp M, Barton GJJB (2009) Jalview Version 2—a multiple sequence alignment editor and analysis workbench. 25:1189–11919
70. Wu P, Song X, Wang Z, Duan W, Hu R, Wang W, Li Y, Hou X (2016) Genome-wide analysis of the BES1 transcription factor family in Chinese cabbage (*Brassica rapa ssp. pekinensis*). Plant Growth Regul 80(3):291–301
71. Xie L, Yang C, Wang XJJoeb (2011) Brassinosteroids can regulate cellulose biosynthesis by controlling the expression of CESA genes in Arabidopsis. 62:4495–450613
72. Yang C-J, Zhang C, Lu Y-N, Jin J-Q, Wang X-LJMp (2011) The mechanisms of brassinosteroids' action: from signal transduction to plant development. 4 (4):588–600
73. Yang ZR, Zhang CJ, Yang XJ, Liu K, Wu ZX, Zhang XY, Zheng W, Xun QQ, Liu CL, Lu LL, Yang ZE, Qian YY, Xu ZZ, Li CF, Li J, Li FG (2014) PAG1, a cotton brassinosteroid catabolism gene, modulates fiber elongation. New Phytol 203(2):437–448. doi:10.1111/nph.12824
74. Ye H, Li L, Guo H, Yin Y (2012) MYBL2 is a substrate of GSK3-like kinase BIN2 and acts as a corepressor of BES1 in brassinosteroid signaling pathway in *Arabidopsis*. Proceedings of the National Academy of Sciences 109 (49):20142–20147

75. Yin Y, Wang Z-Y, Mora-Garcia S, Li J, Yoshida S, Asami T, Chory JJC (2002) BES1 accumulates in the nucleus in response to brassinosteroids to regulate gene expression and promote stem elongation. 109:181–1912
76. Yu D, Qanmber G, Lu L, Wang L, Li J, Yang Z, Liu Z, Li Y, Chen Q, Mendu V (2018a) Genome-wide analysis of cotton GH3 subfamily II reveals functional divergence in fiber development, hormone response and plant architecture. BMC Plant Biol 18(1):1–18
77. Yu G, Wang L-G, Han Y, He Q-Y (2012) clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS 16(5):284–287
78. Yu H, Feng W, Sun F, Zhang Y, Qu J, Liu B, Lu F, Yang L, Fu F, Li W (2018b) Cloning and characterization of BES1/BZR1 transcription factor genes in maize. Plant Growth Regul 86(2):235–249
79. Zhang L-Y, Bai M-Y, Wu J, Zhu J-Y, Wang H, Zhang Z, Wang W, Sun Y, Zhao J, Sun X (2009) Antagonistic HLH/bHLH transcription factors mediate brassinosteroid regulation of cell elongation and plant development in rice and *Arabidopsis*. Plant Cell 21(12):3767–3780

Figures

Figure 1

Leaf phenotype of BR deficient mutant *pag1* cotton plants and *GhEXO2* down-regulated in *pag1*. (A) Phenotype of WT and *pag1* leaf. (B) Microscopic imaging analysis for cell elongation in the leaves of WT and *pag1* cotton plants. (C) Relative expression pattern analysis of *GhEXO2* in cotton CRI24 and BR deficient mutant *pag1* plants. (D) Expression patterns of *GhEXO2* in response to BL treatment as determined by qRT-PCR analysis. Each experiment was performed with three biological repeats and the error bars indicate the standard deviation among these replicates. Student's *t*-test: *P<0.05, ***P<0.001.

Figure 2

The leaf phenotype of *GhBES1* transgenic cotton and microscopic imaging analysis. (A) Altered leaf phenotype of overexpression *GhBES1.4*, *GhBES1*-RNAi, and WT (CRI24) plants. (B) Microscopic imaging analysis for cell elongation in the leaves of overexpression *GhBES1.4*, *GhBES1*-RNAi, and WT (CRI24) plants. (C) qRT-PCR validation of *GhBES1.4* and *GhEXO2* transcript levels in overexpressed plants. (D) qRT-PCR validation of *GhBES1.4* and *GhEXO2* transcript levels in RNAi plants. Each experiment was performed with three biological repeats and the error bars indicate the standard deviation among these replicates. Student's *t*-test: *P<0.05, **P<0.01,***P<0.001.

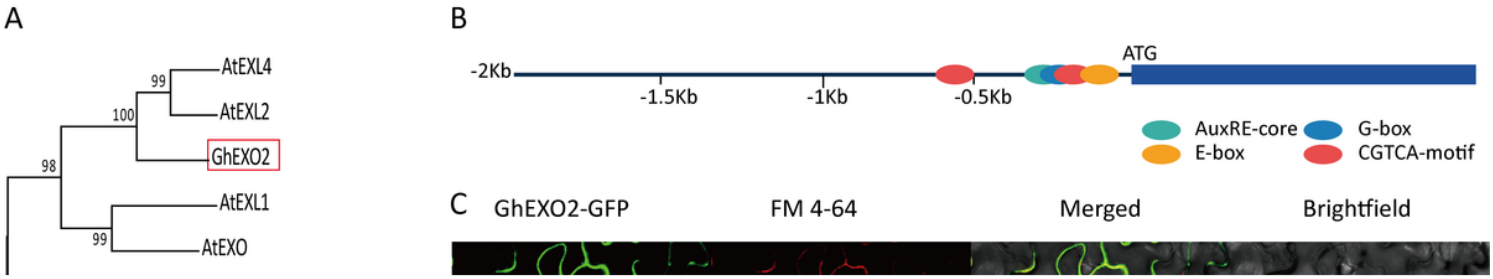

Figure 3

Analysis of *GhEXO2* characteristics. (A) Phylogenetic analysis of *GhEXO2* and *Arabidopsis EXO* genes indicated that *GhEXO2* is closely related to *AtEXL2* and *AtEXL4*. (B) Subcellular localization of GhEXO2 in tobacco. The subcellular localization of GhEXO2 was observed by the transient expression of GhEXO2 protein with a GFP tag in tobacco leaves and the images were observed as brightfield, fluorescence, and merged. (C) Analysis of cis-acting element of *GhEXO2* promoter.

Figure 4

Ectopic overexpression of *GhEXO2* in *Arabidopsis*. (A) Phenotype of ectopically overexpressed *GhEXO2*. (B) Leaf area in WT and GhEXO2 transgenic lines. (C) RT-PCR analysis for transgene validation in *GhEXO2* transgenic lines and wild type (WT). (D) qRT-PCR analysis of relative expression pattern analysis for transgene validation in *GhEXO2* transgenic lines and WT. (E, F, G, and H) qRT-PCR analysis of *AtAGP4*, *AtEXP5*, *AtKCS1*, and *Ata-TIP* genes in three independent Col-0/ *GhEXO2* transgenic lines and WT plants. Each experiment was performed in three biological repeats and the error bars indicate the standard deviation among these replicates. Student's t-test: *P<0.05, **P<0.01,***P<0.001.

Figure 5

Silencing of *GhEXO2* in cotton and microscopic imaging analysis. (A) Phenotype of *GhEXO2* silenced and control plants. (B) Altered leaf phenotype of *GhEXO2* silenced and control plants. (C) qRT-PCR analysis for the validation of *GhEXO2* gene silencing in cotton plants. (D) Microscopic imaging analysis for cell elongation in the leaves of *GhEXO2* silenced and control plants. (E) Graphical representation of leaf area in *GhEXO2* silenced and control plants. Each experiment was performed with three biological repeats and the error bars indicate the standard deviation among these replicates. Student's t-test: *P<0.05, **P<0.01.

Figure 6

Analysis of *GhBES1* and *GhEXO2* interactions *in vivo* and *in vitro*. (A) EMSA showed that GhBES1 protein binds to the E-box element of the *GhEXO2* promoter region. (B) Real-time binding analysis of GhBES1 protein to the E-box element of the *GhEXO2* promoter region. (C) Luc activity analysis of the interaction of the E-box element of the *GhEXO2* promoter and GhBES1. (D) Quantification of relevant Luc activities. The error bars represent the SD of three biological repeats. The asterisks indicated significant differences as determined by t-test (**P<0.01).

Figure 7

RNA-seq data analysis of *GhEXO2* silenced and control plants. (A) Volcano plot of differentially expressed genes (DEGs) (up- and down-regulated genes) in RNA-seq data. (B) Gene ontology (GO) enrichment analysis of DEGs of RNA-seq data. (C) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of RNA-seq data for DEGs. (D) Heatmap of expression of selected genes related to fatty acid elongation, cell wall organization and biogenesis, BR biosynthesis, cell periphery, cell wall biosynthetic process, and MAPK pathway.

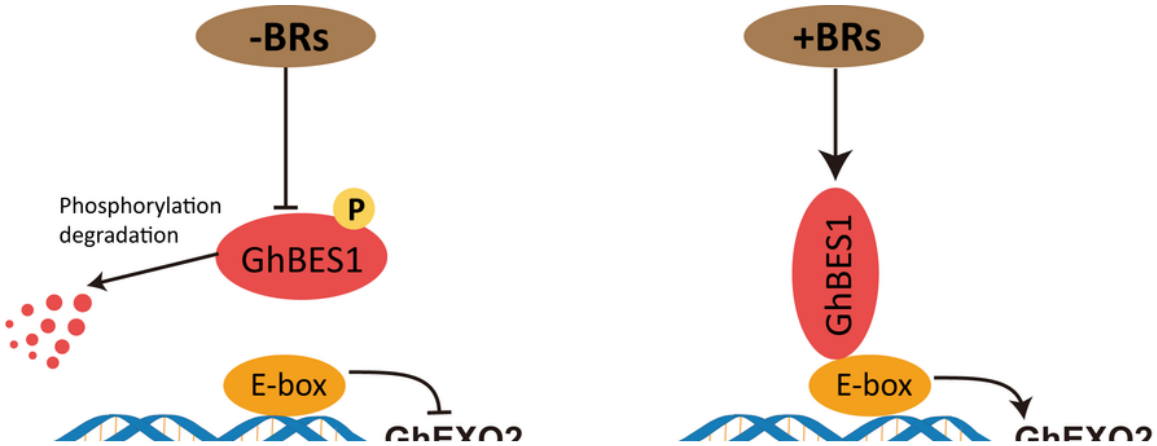

Figure 8

Proposed working model of *GhBES1* targeting *GhEXO2* to induce plant leaf size through cell expansion in BR signaling.

Supplementary Files

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