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GhBZR3 suppresses cotton fiber elongation by inhibiting very-long-chain fatty acid biosynthesis

Zemin Shi[1,2,†], Xia Chen[1,2,†], Huidan Xue[3,4,†], Tingting Jia[5,†], Funing Meng[1,2], Yunfei Liu[1,2], Xiaomin Luo[1], Guanghui Xiao[5,*] and Shengwei Zhu[1,*]

[1]*Key Laboratory of Plant Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China,*
[2]*College of Life Science, University of Chinese Academy of Sciences, Beijing 100049, China,*
[3]*School of Food and Biological Engineering, Shaanxi University of Science and Technology, Xi'an 710021, China,*
[4]*School of Ecology and Environment, Northwestern Polytechnical University, Xi'an 710012, China, and*
[5]*College of Life Sciences, Shaanxi Normal University, Xi'an 710062, China*

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*For correspondence (e-mails guanghuix@snnu.edu.cn; zhusw@ibcas.ac.cn).
†These authors contributed equally to this work.

SUMMARY

The BRASSINAZOLE-RESISTANT (BZR) transcription factor is a core component of brassinosteroid (BR) signaling and is involved in the development of many plant species. BR is essential for the initiation and elongation of cotton fibers. However, the mechanism of BR-regulating fiber development and the function of BZR is poorly understood in *Gossypium hirsutum* L. (cotton). Here, we identified a BZR family transcription factor protein referred to as GhBZR3 in cotton. Overexpression of *GhBZR3* in Arabidopsis caused shorter root hair length, hypocotyl length, and hypocotyl cell length, indicating that *GhBZR3* negatively regulates cell elongation. Pathway enrichment analysis from VIGS-*GhBZR3* cotton plants found that fatty acid metabolism and degradation might be the regulatory pathway that is primarily controlled by GhBZR3. Silencing *GhBZR3* expression in cotton resulted in taller plant height as well as longer fibers. The very-long-chain fatty acid (VLCFA) content was also significantly increased in silenced *GhBZR3* plants compared with the wild type. The *GhKCS13* promoter, a key gene for VLCFA biosynthesis, contains two *GhBZR3* binding sites. The results of yeast one-hybrid, electrophoretic mobility shift, and luciferase assays revealed that *GhBZR3* directly interacted with the *GhKCS13* promoter to suppress gene expression. Taken together, these results indicate that *GhBZR3* negatively regulates cotton fiber development by reducing VLCFA biosynthesis. This study not only deepens our understanding of GhBZR3 function in cotton fiber development, but also highlights the potential of improving cotton fiber length and plant growth using *GhBZR3* and its related genes in future cotton breeding programs.

Keywords: brassinosteroids, GhBZR3, VLCFA biosynthesis, cotton fiber.

INTRODUCTION

Cotton (*Gossypium hirsutum* L.) fiber is one of the most important natural raw materials in the textile industry. It originates from the epidermal cells of ovules and has been used as a model for studying cell elongation and development (Haigler et al., 2012; Kim & Triplett, 2001). It takes about 50 days for developing cotton fiber cells to fully mature. The whole process is divided into four stages, determined by days post-anthesis (DPA): fiber initiation, occurring approximately from −3 to 2 DPA; cell elongation, occurring approximately from 0 to 25 DPA; cell wall thickening, occurring approximately from 20 to 45 DPA; and cell maturation, occurring approximately from 45 to 50 DPA (Basra & Malik, 1984).

Plant hormones play critical roles in multiple developmental processes. It has been reported that various phytohormones are key regulators of cotton fiber development (Ikematsu et al., 2017; Li et al., 2015; Lu et al., 2018; Wang et al., 2011; Zhang et al., 2011). Gibberellin (GA) has a positive effect on both the initiation and elongation of cotton fibers. It also contributes to the deposition of secondary wall thickening of fibers (Ikematsu et al., 2017). Indole-3-acetic acid, a natural auxin, promotes fiber initiation by exogenously stimulating the ovule (Zhang et al., 2011). Brassinosteroids (BRs) are a class of plant steroidal hormones involved in diverse developmental processes during plant development, including cell division and

elongation, reproductive development, vascular tissue differentiation, photomorphogenesis and stress responses (Bajguz, 2007; Jung et al., 2010; Luo et al., 2010; Nemhauser et al., 2004; Yang et al., 2014). Moreover, BR is important for the development of cotton fiber cells (Luo et al., 2007; Qin et al., 2007; Shi et al., 2006). When cotton ovules are cultured *in vitro* with a low concentration of BR or with the BR biosynthesis inhibitor BRASSINAZOLE-RESISTANT (BRZ), BR significantly promotes the elongation of cotton fiber, but BRZ inhibits the development of cotton fibers (Shi et al., 2006; Sun et al., 2005). However, the specific molecular mechanism of BRZ regulation during the development of cotton fiber is not fully understood.

BRASSINAZOLE-RESISTANT (BZR) is a plant-specific transcription factor (TF) family in BR signaling. In Arabidopsis, the BZR TF family contains six genes, named *BZR1*, *BES1* and *BEH1–BEH4* (Sun et al., 2010; Yin et al., 2005; Yu et al., 2011). *BZR1* and *BES1* contain very similar DNA-binding domains. G-box and E-box *cis*-elements are highly enriched in the promoters of BZR1/BES1-responsive target genes. G-box elements contain two inverted repeats of the BZR1-targeted BR response element (BRRE, CGTGT/CG) core sequence, CGTG (Wang et al., 2013). BZR1 and BES1 are two key TFs that positively mediate BR responses. It has been shown that BZR1 and BES1 are involved in a variety of plant regulatory processes (Wang et al., 2013; Yin et al., 2002). Therefore, BZR1 and BES1 act as an integration hub in multi-signal-regulated plant growth and developmental processes, such as cell elongation (Li et al., 2018). The *Solanum lycopersicum* (tomato) BZR/BES TF SlBZR1 positively regulates BR signaling and salt stress tolerance in tomato and Arabidopsis (Jia et al., 2021). In *Pyrus bretschneideri* (pear), the BZR gene *PbBZR1* also acts as a transcriptional repressor for lignin biosynthetic genes in fruits (Cao et al., 2020). Overexpression of *BpBZR1* enhances salt tolerance in *Betula platyphylla* (Lv et al., 2020). In cotton, the expression of *Gh14-3-3* genes in fiber cells may alter the expression levels of BR-related and fiber growth-related genes, which consequently affect fiber development in cotton. This research indicated that cotton 14-3-3 proteins might be involved in the regulation of fiber initiation and elongation via their interaction with GhBZR1 to regulate the BR signaling pathway (Zhou et al., 2015). However, the functions of BZR TFs require further exploration in cotton.

Here, we cloned cotton *GhBZR3* and performed phenotyping of genetic systems with silenced *GhBZR3* in cotton and overexpressed *GhBZR3* in Arabidopsis. To analyze GhBZR3-regulated metabolic pathways, ovule samples treated with BRZ for 6 h and 12 h *in vitro* and 5-DPA cotton fibers from VIGS-*GhBZR3* plants were used for RNA-seq analysis. We found that the upregulated differentially expressed genes (DEGs) were significantly enriched in fatty acid biosynthesis. We also verified the expression patterns of downstream genes associated with *GhBZR3* to clarify how *GhBZR3* regulates fiber development by regulating the synthesis of very-long-chain fatty acids (VLCFA). These results indicate that *GhBZR3* negatively regulates cotton fiber development. Moreover, the expression of downstream *GhKCS13* is induced after *GhBZR3* is silenced, which leads to the accumulation of VLCFAs and promotes fiber development. This provides a direction for improving the quality of cotton fiber.

RESULTS

GhBZR3 is a *BZR1* family transcription factor

To explore the potential function of the BZR gene family in cotton fiber development, we downloaded the transcriptome data of *G. hirsutum* (TM-1) (Hu et al., 2019) from CottonFGD (https://cottonfgd.org) and further analyzed the gene expression values (Figure 1a; Table S1). The results showed that *GhBZR3* expression is lower than other *GhBZR* genes during the fiber developmental process. BZR acts as a negative feedback regulator of BR biosynthesis and inhibits fiber development (Shi et al., 2006; Wang et al., 2013). Thus, we speculated that *GhBZR3* might also play a role in the inhibition of cotton fiber development. In addition, we analyzed the expression profile of the *GhBZR3* in various tissues and during exposure to different abiotic stresses. Our results showed that *GhBZR3* is mainly expressed in the pistil, stem and leaf, and that the expression level of *GhBZR3* was significantly increased after 6 h of treatment with cold, 6 h of treatment with hot and 12 h of treatment with polyethylene glycol (PEG) (Figure S1).

The cDNA sequence length of *GhBZR3* is 960 bp and contains two exons and one intron. The molecular weight of *GhBZR3* was predicted to be 34.337 kDa, which had 38% and 68% amino acid sequence similarity to *AtBZR1* and *AtBEH4*, respectively. Interestingly, GhBZR3 contains typical features of BZR TFs, such as a DNA binding domain, a C-terminal domain and a nuclear localization signal sequence (NLS). Notably, the putative 14-3-3 binding site (RISNSAP) and PEST sequences are only present in *AtBZR1*, *AtBES1*, *GhBZR1* and *GhBZR2*, but not in *GhBZR3*, *GhBZR4* and *GhBZR5*. Thus, GhBZR3, GhBZR4 and GhBZR5 might have different functions in cotton (Figure S2). *GhBZR3* has the highest homology with AtBEH4 by systematic evolution analysis, which indicates divergence from the same ancestral gene in evolution (Figure S2).

Subcellular localization and transcriptional activity of GhBZR3

The subcellular localization of GhBZR3 was analyzed by expressing GhBZR3-GFP fusion proteins in transiently transformed *Nicotiana benthamiana* (tobacco) leaves. The

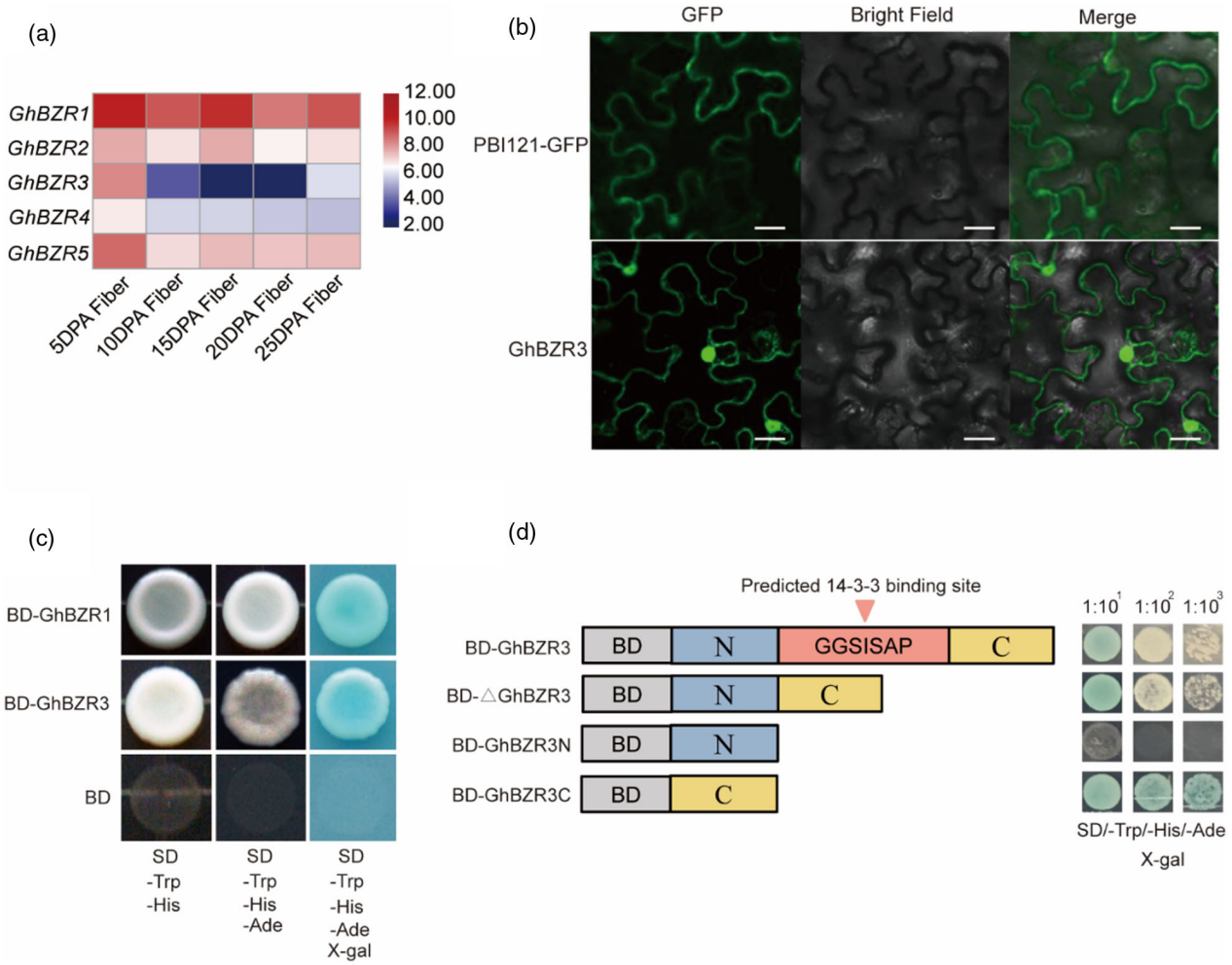

Figure 1. *GhBZR* gene characteristics in *Gossypium hirsutum* (TM-1).
(a) The expression of *GhBZR* genes in *G. hirsutum* (TM-1). (b) Fluorescence micrograph of GFP, brightfield and brightfield overlay images in transiently transformed *Nicotiana benthamiana* (tobacco) epidermal cells. Scale bars: 17.2 μm. (c) The transcriptional activity of the full sequence of GhBZR3 in yeast, GhBZR1 was used as a positive control. (d) The transcriptional activity of GhBZR3 without the 14-3-3 binding site and the N-terminal (blue box region) or C-terminal (yellow box region) of GhBZR3 in yeast.

results showed that GhBZR3 is mainly localized to the nucleus (Figure 1b). To study the transcriptional activation of full-length GhBZR3, *GhBZR3* was transferred into yeast cells for yeast one-hybrid analysis. As shown in Figure 1(c), GhBZR3 can grow on SD/–Trp/–His/–Ade selective medium and turns blue in the presence of X-α-galactosidase (X-gal), indicating that GhBZR3 confers transcriptional activity. The sequences of *AtBZR1*, *OsBZR1* and *GhBZR1* contain a complete phosphorylated 14-3-3 binding motif (RXXXpSXP), which thus affects its transcriptional activity (Aitken, 2006; Bai et al., 2007). A mutation within the RXXXpSXP motif was found in *GhBZR3*, suggesting that the transcriptional activation of *GhBZR3* might be different compared with *BZR1* genes. We further transferred the sequence of *GhBZR3* lacking the conserved 14-3-3 domain into yeast cells to investigate the transcriptional activity. As shown in Figure 1(d), loss of the 14-3-3 binding motif did not affect the transcriptional activity of GhBZR3, suggesting that GhBZR3 has a different regulatory mechanism. We also found that the N-terminus and C-terminus of GhBZR3 confer opposite transcriptional activities, where the C-terminus of GhBZR3 possessed transcriptional activity but the N-terminus exhibits inhibitory activity.

GhBZR3 negatively regulates cotton fiber development

To investigate the function of *GhBZR3* in cotton fiber development, *GhBZR3* was silenced using a virus-induced gene silencing (VIGS) strategy (Figure 2a). According to reverse transcription quantitative polymerase chain reaction (RT-qPCR) validation, the expression level of *GhBZR3* was significantly reduced in the VIGS plants (Figure 2b). Furthermore, the 45-DPA fiber length was measured from VIGS-*CK* and VIGS-*GhBZR3* lines (Figure 2c,d). The

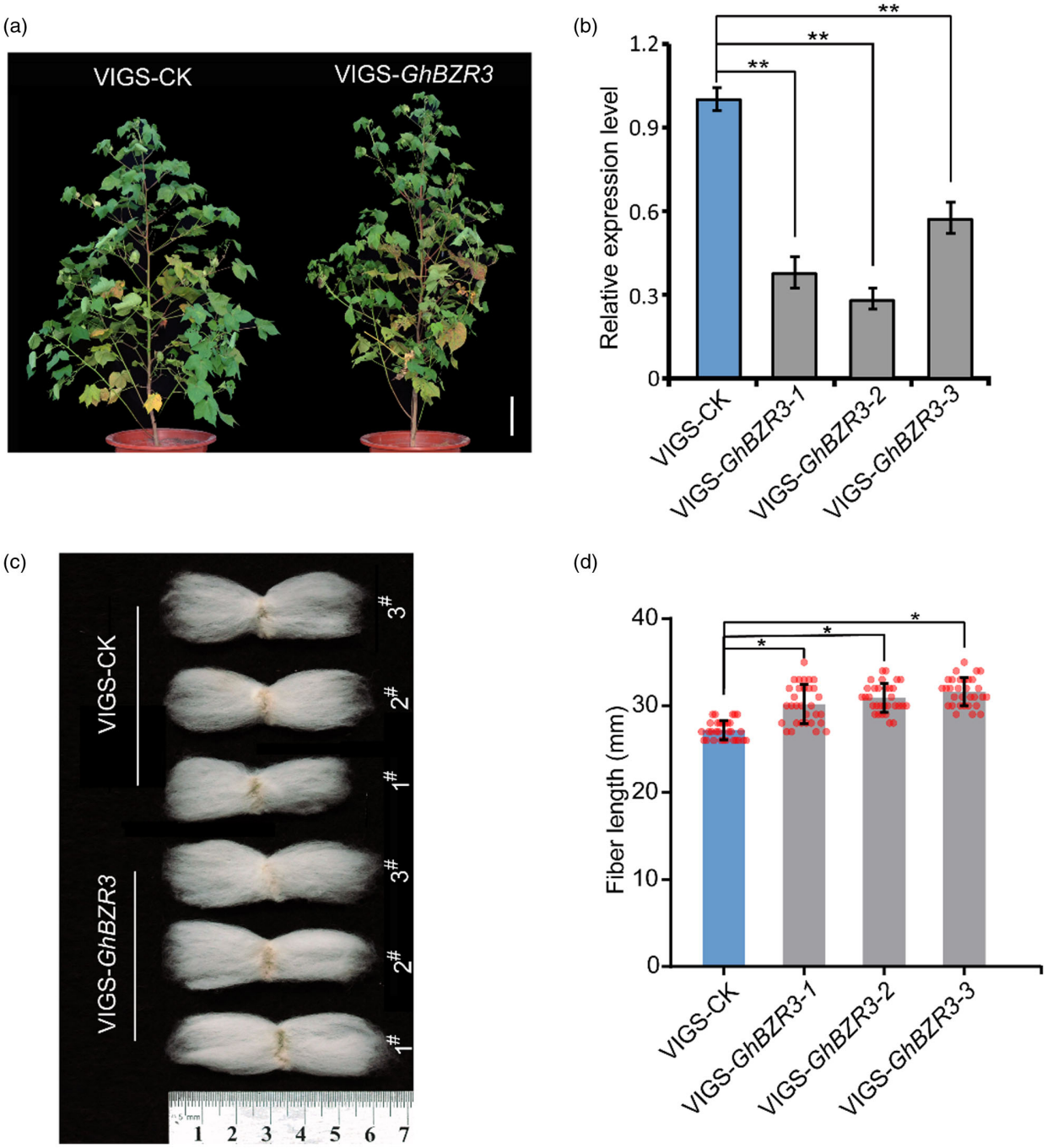

Figure 2. *GhBZR3* negatively regulates cotton fiber elongation.
(a) The phenotype of cotton after VIGS-*GhBZR3* and VIGS-CK. Scale bar: 20 cm. Representative seeds with attached fibers from the VIGS experiment. (b) The relative expression level of *GhBZR3* in VIGS-*GhBZR3*, VIGS-*GhBZR3-1*, VIGS-*GhBZR3-2* and VIGS-*GhBZR3-3* lines. (c, d) Statistical analysis of the fiber length in VIGS-*GhBZR3* (c) and VIGS-CK (d) plants. More than 30 plants were used for statistical analysis. Error bars indicate the standard error of the independent biological replicates. $*P < 0.05$, $**P < 0.01$.

average length of fibers from the *GhBZR3*-silenced plants reached 30.6 ± 0.16 mm, which was 2 mm longer than the control (28.3 ± 0.22 mm), suggesting that *GhBZR3* silencing promoted cotton fiber elongation. In addition, the *GhBZR3*-silenced plants had greater plant height and smaller angles between fruit branches and the main stem, compared with control plants (Figure S3).

Furthermore, to better characterize the biological function of GhBZR3, we generated *GhBZR3*-overexpression plants and *GhBZR3*-knockout plants using CRISPR/Cas9

and observed their phenotypes. After pedigree selection and qPCR analysis, three *GhBZR3*-overexpression lines and three *GhBZR3*-knockout lines were obtained via *Agrobacterium tumefaciens*-mediated transformation of the cotton cultivar Jin668 (Figure 3a,b). Compared with non-transgenic control plants, overexpression of *GhBZR3* significantly reduced fiber cell length (Figure 3c,d). In addition, overexpression of *GhBZR3* or knocking out *GhBZR3* expression did not affect the number of initiating fibers (Figure 3e–i). These results suggest that *GhBZR3* negatively regulates fiber cell elongation.

Overexpression of *GhBZR3* inhibits hypocotyl and root growth in Arabidopsis

Moreover, *GhBZR3* was overexpressed in Arabidopsis using the cauliflower mosaic virus (CaMV) *35S* promoter to further investigate the function of *GhBZR3*. The RT-qPCR results confirmed the overexpression of *GhBZR3* in transgenic lines of Arabidopsis (Figure 4a). Then, we measured the lengths of the hypocotyl, root hair and primary root from 5-day-old wild-type and *GhBZR3*-overexpressing plants. Compared with wild-type plants, the hypocotyl length (Figure 4b,d), primary root length (Figure 4c,e) and root hair length (Figure 4f) in *GhBZR3*-overexpressing plants were significantly shorter. To investigate whether these differences in organ and tissue lengths were attributable to defective cell elongation, we used propidium iodide (PI), which binds cell wall carbohydrates, to investigate cell morphology (McKenna et al., 2009). As shown in Figure 4(g), the length of hypocotyl cells from three *GhBZR3*-overexpressing transgenic lines was significantly shorter than in wild-type controls (Figure 4h), indicating that *GhBZR3* negatively regulates cell elongation in Arabidopsis.

Transcriptome identification and characterization of *GhBZR3* downstream genes

To further verify the potential mechanism of *GhBZR3* in regulating fiber development, ovules were treated with BRZ for 6 h and 12 h *in vitro*. The treated ovules and 5-DPA ovules (with fibers) from VIGS-*GhBZR3* plants were used for RNA-seq analysis. The results showed that there were 406 and 217 genes upregulated after 6 h and 12 h of BRZ treatment, respectively. By performing VIGS and RNA-seq in *GhBZR3* we found a total of 1571 upregulated genes, compared with the control (Figure 5a). The enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were substantially different for the DEGs in BRZ-treated or VIGS-*GhBZR3* materials. Compared with the control, fatty acid metabolism and degradation pathways were significantly enriched in the VIGS-*GhBZR3* ovules (Figure 5b). At the same time, we also found that fatty acid metabolism and degradation pathways were enriched in BRZ-treated cotton ($P < 0.001$) (Figure 5c). We further characterized the fatty acid profile of cotton fibers. This showed that the total VLCFA, C24 and C26 fatty acids were significantly increased in *GhBZR3*-silenced cotton fiber plants (Figures 5d,e,f and S4). Compared with wild-type plants, overexpression of *GhBZR3* reduced the accumulation of C24 and C26 fatty acids, whereas knocking out *GhBZR3* significantly increased the accumulation of C24 and C26 fatty acids (Figure S5). The results indicate that *GhBZR3* regulates fiber development by impacting fatty acid accumulation.

GhBZR3 directly binds to *GhKCS13* to reduce fatty acid synthesis

Four cotton *KCS* genes (*GhKCS2*, *GhKCS6*, *GhKCS12* and *GhKCS13*) are specifically and highly expressed in fibers (Qin et al., 2007). To explore the downstream regulatory genes of *GhBZR3*, RT-qPCR was used to detect the expression levels of these four *GhKCS* genes in both the wild type and the VIGS-*GhBZR3* silenced plants. The results showed that the expression levels of *GhKCS6*, *GhKCS12* and *GhKCS13* genes were significantly upregulated in VIGS-*GhBZR3* silenced plants, but the expression level of *GhKCS2* was only slightly different (Figure 6a). To screen for KCS proteins interacting with GhBZR3, the yeast one-hybrid technique was performed. We found that GhBZR3 could bind to the *GhKCS13* promoter region (Figure 6b). Using electrophoretic mobility shift assay (EMSA) and luciferase assays, it was confirmed that GhBZR3 interacted with the promoter element of *GhKCS13* (Figure 6c,d). Taken together, our results indicate that GhBZR3 can directly bind to the upstream promoter element of *GhKCS13* and inhibit its expression (Figure 6b,c,d).

GhKCS13 positively regulates fiber cell elongation in cotton

To investigate the function of *GhKCS13* in cotton fiber initiation and elongation, *GhKCS13* was silenced using the VIGS strategy. Compared with control plants (VIGS-CK), the expression level of *GhKCS13* was significantly reduced in *GhKCS13*-VIGS plants (Figure 7a). The mature cotton fiber length in *GhKCS13*-VIGS plants was significantly shorter than that of VIGS-CK, indicating that silencing *GhKCS13* inhibited cotton fiber elongation (Figure 7b,g). In addition, silencing *GhKCS13* expression did not affect the number of initiating fibers (Figure 7c–f, h). These results suggest that *GhKCS13* only regulates fiber cell elongation.

DISCUSSION

GhBZR3 is an atypical BES1/BZR1 family protein

Genetically redundant genes contribute to the health and stability of plants (Hall et al., 2007; Swarbreck et al., 2008). BZR1 and BES1 are key TFs in BR signal transduction (Lachowiec et al., 2018). They have a similar structure and

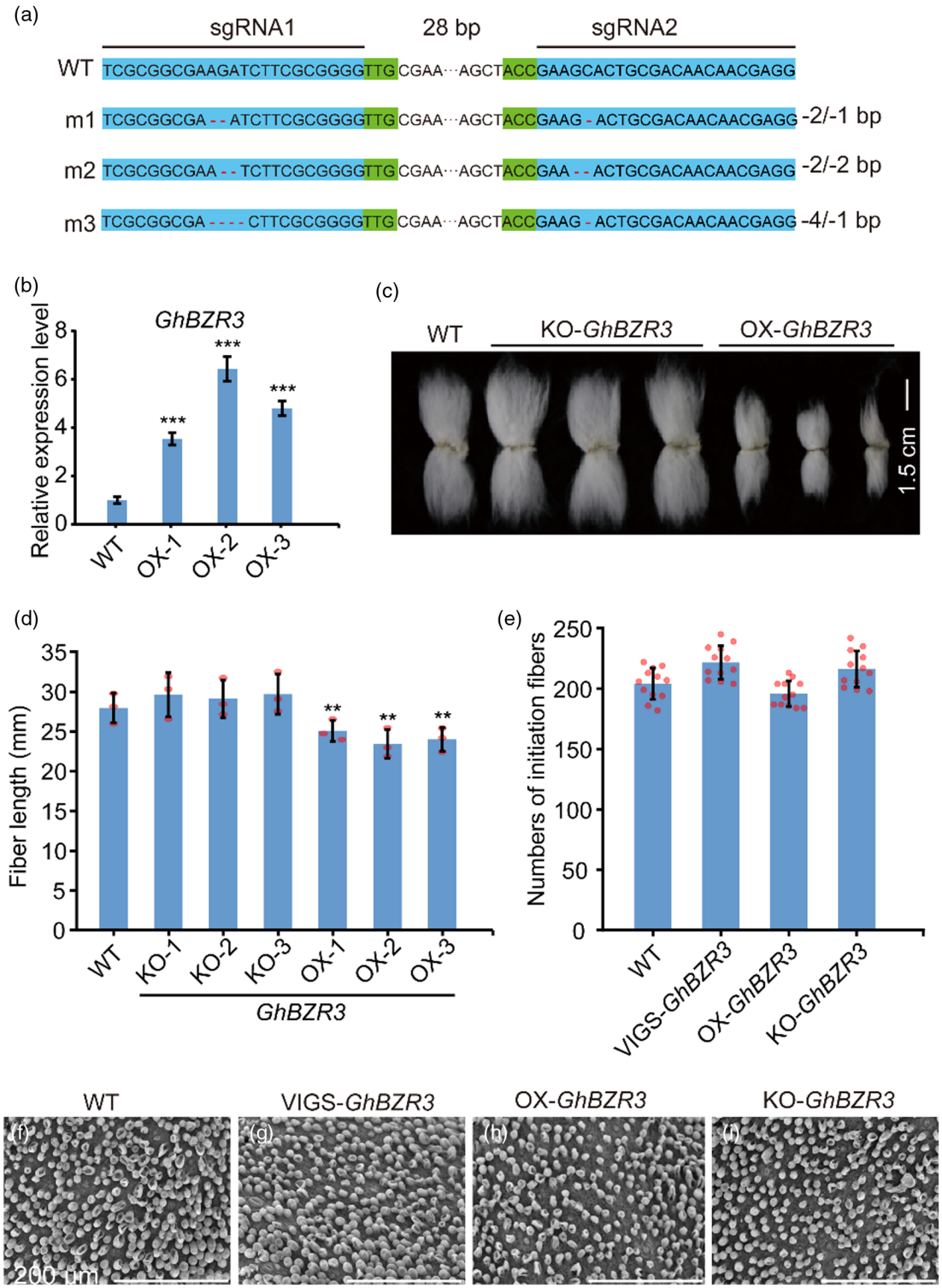

Figure 3. Overexpression of *GhBZR3* reduces fiber cell elongation.
(a) Sanger sequencing-based genotyping of *GhBZR3*-knockout lines obtained by CRISPR/Cas9 gene editing. Nucleotide deletions are indicated by the red dashes. (b) The relative expression level of *GhBZR3* in the wild type (WT) and transgenic plants overexpressing GhBZR3 (OX-*GhBZR3*). (c) Phenotype of cotton fibers in the wild type and OX-*GhBZR3* and *GhBZR3*-knockout (KO-*GhBZR3*) lines. Scale bar: 1.5 cm. (d) Statistical analysis of the fiber length in the wild type and OX-*GhBZR3* and KO-*GhBZR3* lines. (e) Statistical analysis of initial fiber cells from the wild-type, VIGS-*GhBZR3*, OX-*GhBZR3* and KO-*GhBZR3*. (f–i) Phenotypes of initiating fiber cells from the wild type, and VIGS-*GhBZR3*, OX-*GhBZR3* and KO-*GhBZR3* lines. Scale bars: 200 μm. More than 30 plants were used for statistical analysis. Error bars indicate the standard error of the independent biological replicates. $^{**}P < 0.01$, $^{***}P < 0.001$.

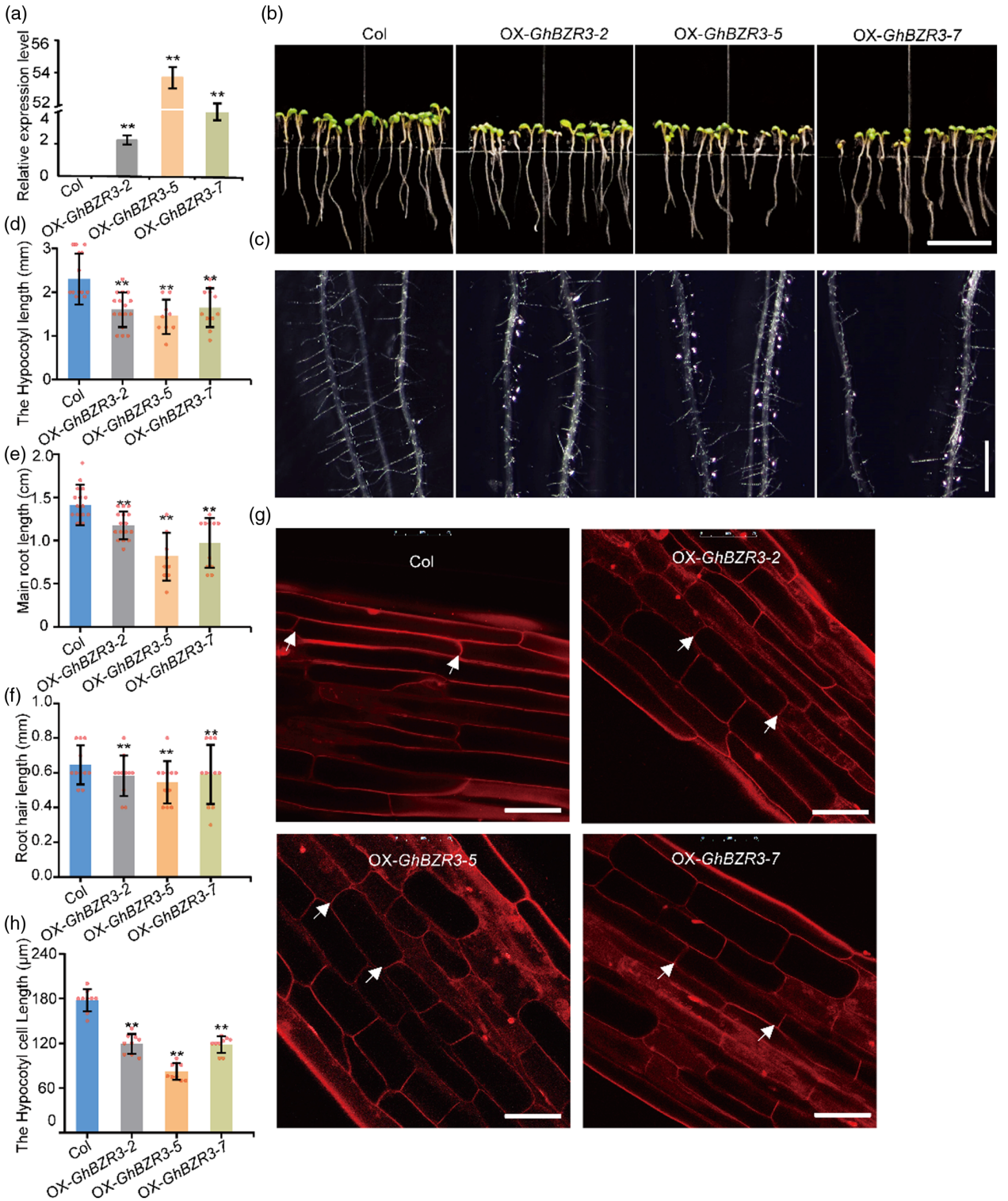

Figure 4. Overexpression of *GhBZR3* inhibits hypocotyl and root growth in Arabidopsis.
(a) The expression levels of overexpressed *GhBZR3* in Arabidopsis. (b) Comparison of hypocotyl and primary root growth in *GhBZR3*-overexpressed and wild-type plants. Scale bar: 1 cm. (c) Comparison of root hairs between wild-type and *GhBZR3*-overexpressed mutants. Scale bar: 0.6 cm. The statistics of the hypocotyl (d), primary root (e) and root hair (f) lengths in *GhBZR3*-overexpressed and wild-type plants. Error bars indicate the standard errors of three independent biological replicates. $^{**}P < 0.01$. (g) Microscopy of propidium iodide fluorescence in hypocotyls from the wild type and three *GhBZR3*-overexpressed mutants. Scale bars: 75 μm. (h) Statistics of hypocotyl cell length from wild-type and three *GhBZR3*-overexpressed mutants. Error bars indicate the standard errors of three independent biological replicates. $^{**}P < 0.01$.

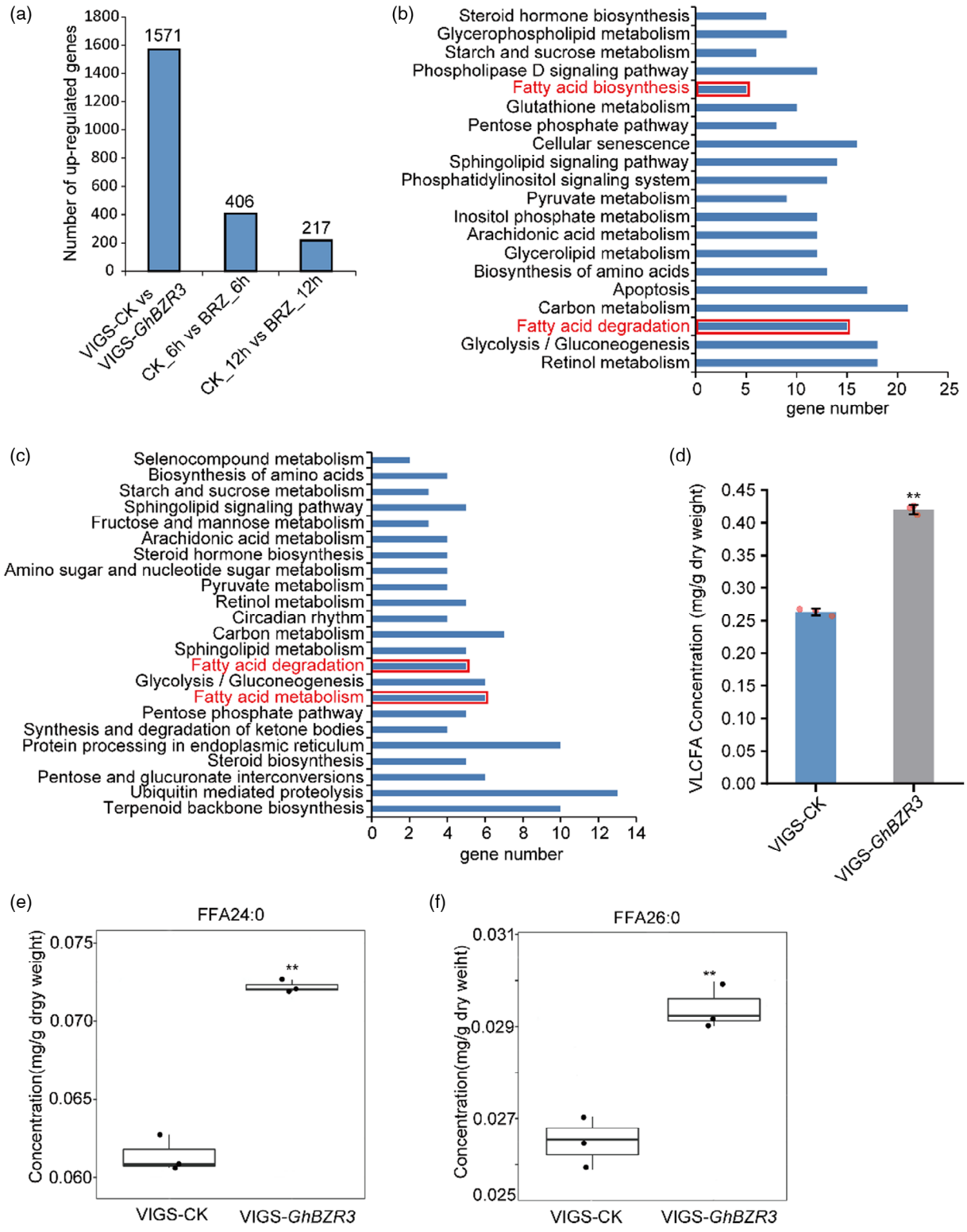

Figure 5. *GhBZR3* regulates downstream fatty acid synthesis.
(a) The number of upregulated differentially expressed genes (DEGs) in cotton after *GhBZR3* silencing, 6 h BRZ treatment or 12 h BRZ treatment. False discovery rate (FDR) corrected $P < 0.05$, $|\log2 \text{ (fold change)}| > 2$. (b) KEGG analysis showed enriched downstream regulatory pathways in cotton after *GhBZR3* gene silencing. $P < 0.001$. (c) KEGG analysis shows enriched downstream regulatory pathways in cotton following BRZ treatment. $P < 0.001$. (d) Silenced *GhBZR3* gene expression line total VLCFA content (C20–C28) in cotton fibers. $^{**}P < 0.01$. The silencing of *GhBZR3* increased the concentration of C26 (e) and C24 (f) VLCFAs in cotton fibers. FFA24:0, VLCFAC24; FFA26:0, VLCFAC26. $^{**}P < 0.01$.

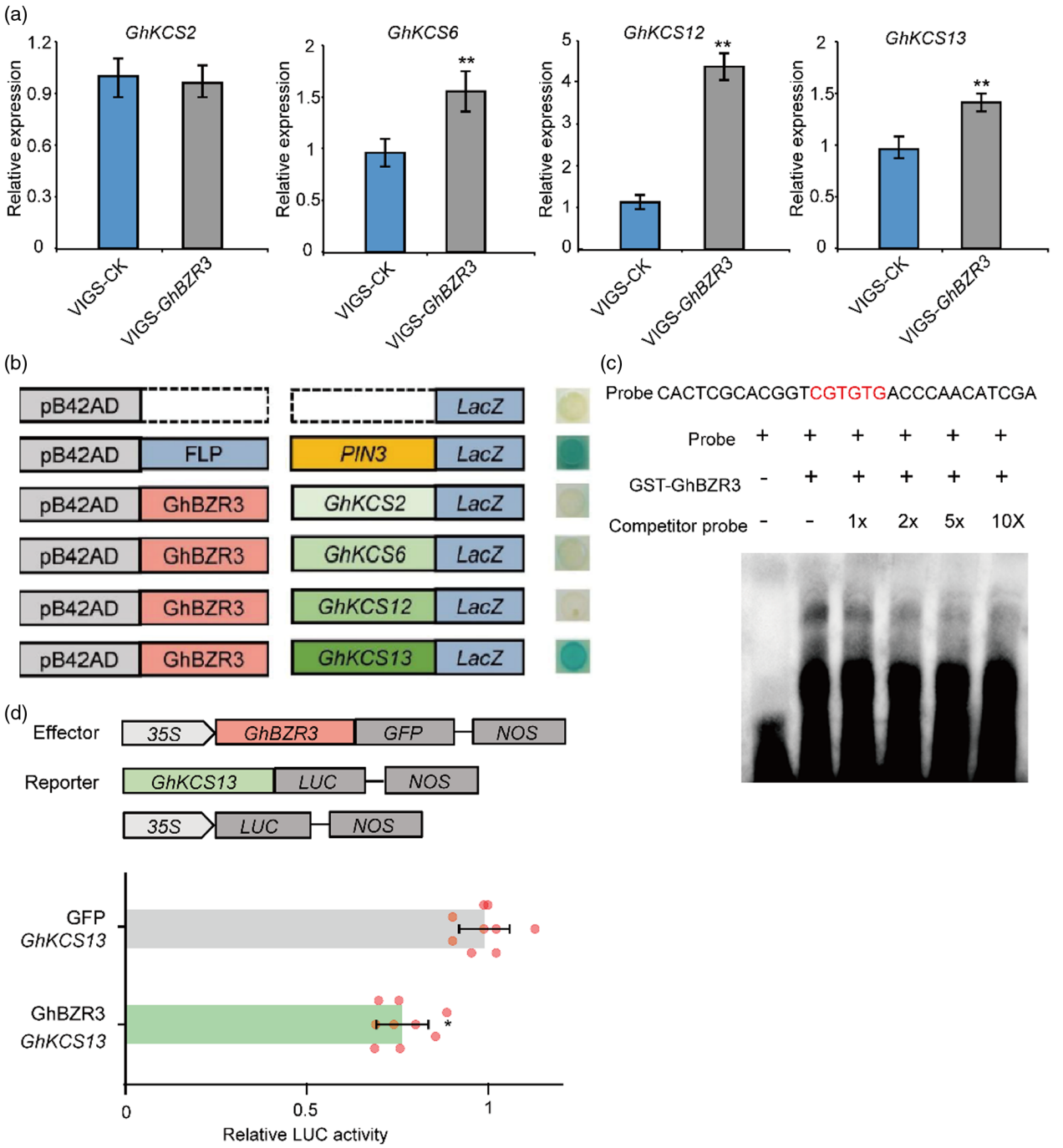

Figure 6. GhBZR3 binds to the *GhKCS13* promoter to regulate fatty acid synthesis.
(a) Expression of four *GhKCS* genes in VIGS-CK and VIGS-*GhBZR3* plants. (b) Testing the binding efficiency of GhBZR3 to the *GhKCS13* promoter via yeast one-hybrid assays. (c) EMSA and (d) luciferase assay, $*P < 0.05$, $**P < 0.01$.

contain a 14-3-3 binding site. Recent studies of BZR TFs show that BEH4 is central to cross-regulation among all BZR family members during hypocotyl development in Arabidopsis in the dark (Lachowiec et al., 2018). In cotton, *GhBZR3* is a homolog of *AtBEH4*, with a sequence similarity of 68% to *GhBZR1*. Similar results were observed by homology analysis (Figure S2). There are four domains in genes *GhBZR1–GhBZR5*: an N-terminal domain, a 14-3-3 protein binding site, a PEST conserved domain and a C-terminal domain (Figure S2). Previous studies showed that BIN2 and GSK3 regulate the binding activity of BZR1. Phosphorylation of the 14-3-3 binding site in BES1 facilitates

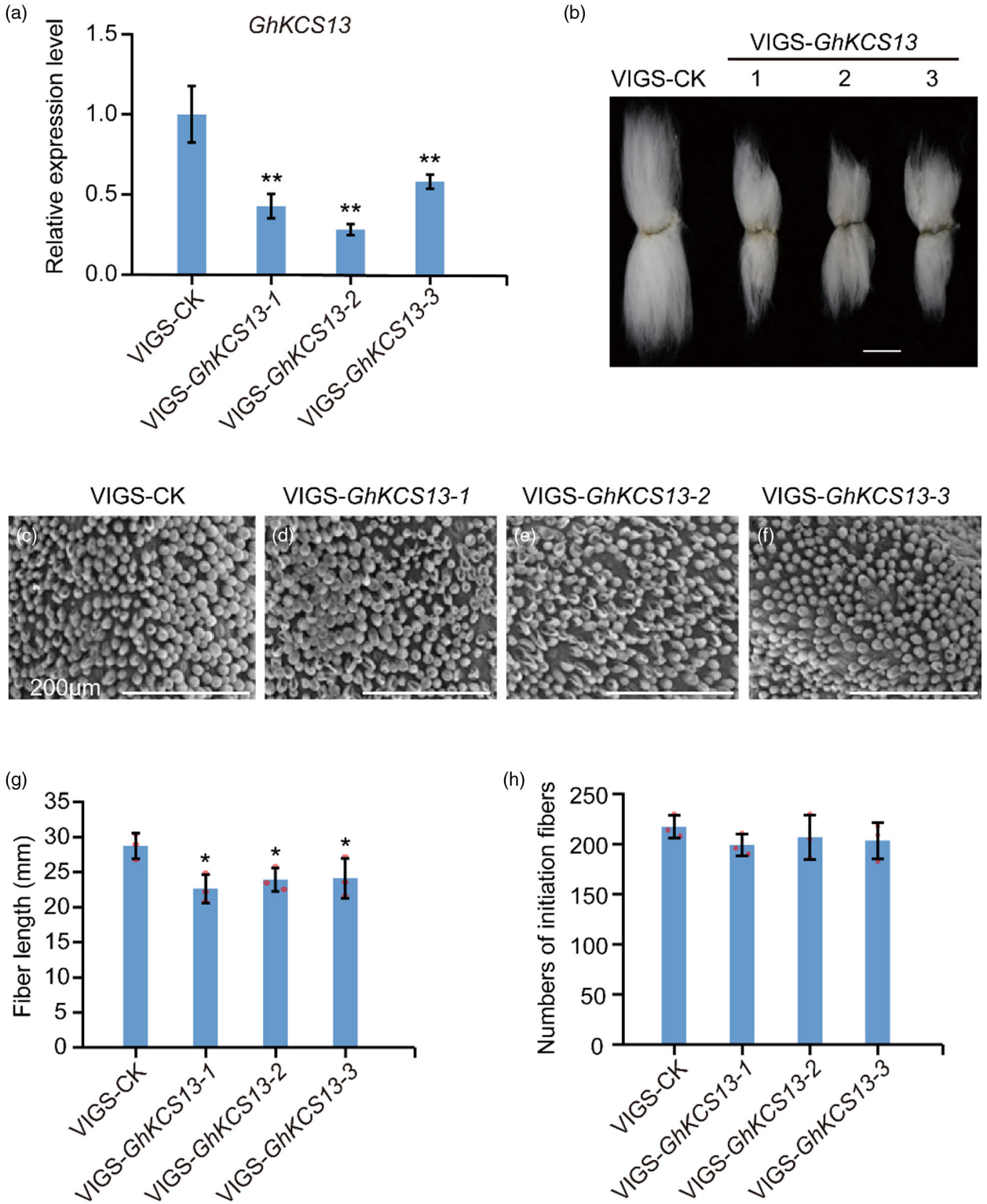

Figure 7. Silencing of *GhKCS13* reduces fiber cell elongation.
(a) The relative expression level of *GhKCS13* in CK and VIGS-*GhBZR3* lines. (b) Phenotype of the cotton fibers in CK and VIGS-*GhBZR3* lines. Scale bar: 1 cm. (c–f) Phenotypes of initiating fiber cells from CK and VIGS-*GhBZR3* lines. Scale bars: 200 μm. (g) Statistical analysis of the fiber length in CK and VIGS-*GhBZR3* lines. (h) Statistical analysis of initiating fiber cells from CK and VIGS-*GhBZR3* lines. More than 30 plants were used for statistical analysis. Error bars indicate the standard error of the independent biological replicates. $*P < 0.05$, $**P < 0.01$.

the precise regulation of BR target genes (Ryu et al., 2010), and thus it is considered an important regulatory mechanism in BZR family proteins. In *GhBZR3*, we found a mutation in the 14-3-3 binding site, with an arginine (Arg) replaced by a glycine (Gly). Using luciferase assays, the transcriptional activities of GhBZR3 without the 14-3-3 binding site and N-terminal (blue box region) or C-terminal (yellow box region) of GhBZR3 were detected in yeast. Our results show that the 14-3-3 binding motif did not affect the transcriptional activity of *GhBZR3* (Figure 1d), indicating that the 14-3-3 regulatory mechanisms in *GhBZR3* are different from that of *AtBZR1*, *AtBES1* and *OsBZR1*.

GhBZR3 plays a critical role in cotton fiber development

The BZR transcription factor family is involved in numerous physiological processes regulated by BR (Chen, Zhang, et al., 2019; Li et al., 2010; Song et al., 2009; Tong et al., 2014). For instance, the BZR1 TF plays an irreplaceable role in BR signaling, but it does not depend on BZR1 for regulating anther development in Arabidopsis (Chen, Gao, et al., 2019; Chen, Zhang, et al., 2019). In *Brassica rapa*, the BZR TF family is involved in the abiotic stress response (Saha et al., 2015). However, the regulatory mechanism of BZR remains unclear in cotton. In this study, VIGS-*GhBZR3* plants showed longer fibers (Figure 2c,d), higher plant heights and smaller fruit branches (Figure S3) compared with VIGS-CK. *GhBZR3* overexpression plants showed shorter hypocotyl length, root length, root hair length and hypocotyl cell length compared with the wild type (Figure 4). These results indicate that *GhBZR3* negatively regulates fiber development and plant growth in cotton.

GhBZR3 negatively regulates cotton fiber elongation by affecting VLCFA biosynthesis

The development of cotton fiber cells is a very complicated process involving the strict regulation of a large number of factors (Basra & Malik, 1984; Kim & Triplett, 2001; Wu et al., 2018). Fatty acid biosynthesis and elongation are upregulated biochemical pathways during cotton fiber cell elongation (Shi et al., 2006). The biosynthesis and transport of VLCFA (fatty acids of chain length greater than C20) are crucial for the elongation of fiber cells, (Qin et al., 2007). However, the mechanism of how to regulate VLCFA synthesis remains unclear in fiber development. To investigate the mechanism of VLCFA synthesis, we found four *GhKCS* genes, *GhKCS2*, *GhKCS6*, *GhKCS12* and *GhKCS13*, that are specifically overexpressed in cotton fiber cells (Qin et al., 2007). Previous studies have shown that those four *GhKCS* genes are involved in the synthesis of VLCFAs and promote cell elongation via enhancing ethylene synthesis (Qin et al., 2007; Xiao et al., 2016). In this study, except for *GhKCS2*, the expression levels of the other three *GhKCS*s were significantly upregulated after silencing *GhBZR3* (Figure 6a). GhBZR3 can directly bind to the upstream promoter elements of *GhKCS13* using yeast one-hybrid, EMSA and luciferase assays (Figure 6b,c,d), which is involved in VLCFA synthesis of C20 to C26 (Qin et al., 2007). These results suggest that *GhKCS13* is the direct downstream target gene of GhBZR3. Furthermore, we found that the content of VLCFAs significantly increased in cotton fibers upon silencing *GhBZR3* and in *GhBZR3*-knockout plants, but decreased in *GhBZR3*-overexpression lines (especially C24 and C26) (Figures 5d, e,f and S5). Although the expression levels of *GhKCS6* and *GhKCS12* were significantly upregulated after silencing *GhBZR3*, the yeast one-hybrid assay showed that GhBZR3 cannot directly bind to their promoter regions (Figure 6b), indicating that *GhKCS6* and *GhKCS12* might be the indirect downstream genes of GhBZR3. This hypothesis should be confirmed with more evidence in the future. These results indicate that *GhBZR3* negatively regulates the accumulation of VLCFAs. Therefore, the expression of downstream *GhKCS13* is induced after the silencing of *GhBZR3* and thereby results in an accumulation of VLCFAs and the promotion of fiber development. It provides a direction to regulate fiber development by regulating the content of fatty acids in cotton in the future cotton breeding process.

EXPERIMENTAL PROCEDURES

Plant materials

The *Arabidopsis thaliana* lines used here were derived from the Col-0 background. The *A. thaliana* and *N. benthamiana* plants were grown in a controlled environment at 22°C with a relative humidity of 60% under a 16-h light/8-h dark photoperiod. Transgenic cotton plants (Jin668) were grown in a pot. The glasshouse conditions were 28°C day/22°C night, 40%–60% relative humidity and a 14-h light/10-h dark cycle for culturing cotton seedlings before they were transplanted to pots.

Gene expression patterns, phylogenetic relationship determination and domain analysis

To identify BZR members in cotton, the *AtBZR1* sequence of Arabidopsis was used to retrieve the genome data of *G. hirsutum* (Hu et al., 2019) and the BZR sequences were obtained according to sequence similarity. *Gossypium hirsutum* data were derived from CottonFGD (https://cottonfgd.org) and the genome version is NBI v1.1. Finally, five BZR genes were obtained and named *GhBZR1*, *GhBZR2*, *GhBZR3*, *GhBZR4* and *GhBZR5* (Table S2). The transcriptome data of TM-1 were downloaded from CottonFGD. DNAMAN 4.0 (Lynnon Biosoft, https://www.lynnon.com) and SNAPGENE VIEWER (https://www.snapgene.com/snapgene-viewer) were used for multi-sequence alignment and the prediction of conserved domains, respectively. Gene expression levels were analyzed using the $\log_2$(FPKM+1) method. Gene expression patterns were visualized with heat maps prepared using R 3.3.0 (https://CRAN.R-project.org/package=pheatmap). MEGA 6.0 (https://www.megasoftware.net) was used to analyze BZR phylogenetic relationships from *A. thaliana*, *G. hirsutum* and *Oryza sativa* with the neighbor-joining statistical method and 1000 bootstrap replications.

Subcellular localization of GhBZR3

The full-length coding DNA sequence (CDS) of *GhBZR3* was amplified from total cDNA by PCR with the primers listed in Table S3. PCR products were digested with *Sma*I and *Bam*HI and then ligated into the pBI121-GFP vector using NEB T4 DNA ligase (NEB, https://international.neb.com). The pBI121-GhBZR3-GFP and pBI121-GFP vectors were transformed to *Agrobacterium tumefaciens* leaf and were further transiently transformed into 2-week-old tobacco leaves. The GFP signal was detected using a fluorescence microscope after 2 days (TCS SP5; Leica Microsystems, https://www.leica-microsystems.com).

Expression vector construction

The CDS of *GhBZR3* was cloned into pCAMBIA2300 overexpression vector. For *GhBZR3* gene editing, two single-guide RNAs (sgRNAs) were designed in the coding region and named sgRNA1 and sgRNA2. Using *GhBZR3* as a template, two fragments (containing the tRNA-sgRNA1 fusion and the gRNA-tRNA-sgRNA2 fusion) were obtained and fused with an overlapping extension PCR. Then, they were cloned into the pRGEB32 CRISPR/Cas9 vector. All constructed plasmids, GhBZR3- pRGEB32 and pCAMBIA2300-GhBZR3 vector were separately transformed into *Agrobacterium tumefaciens* strain GV3101 for cotton transformation. Three transgenic lines for each construct in overexpression lines and CRISPR/Cas9 lines were analyzed for the phenotype of interest and further experiments.

Overexpression of *GhBZR3* in *Arabidopsis thaliana*

The CDS of *GhBZR3* was cloned into pBI121 via the *Sma*I and *Kpn*I restriction sites. After that, this vector was transferred into *Agrobacterium tumefaciens* using electroporation. The floral-dip method was (Clough & Bent, 1998) used for the overexpression of *GhBZR3* in Arabidopsis. A stereoscope (M165 FC; Leica Microsystems) was used for the observation of Arabidopsis hypocotyls, roots and root hairs. The transgenic hypocotyls of Arabidopsis were measured using the PI method for 1 h and were observed on a fluorescence microscope (M165 FC; Leica Microsystems).

Yeast one-hybrid assay

For the yeast one-hybrid (Y1H) assay, the CDS of *GhBZR3* was cloned to the pGADT7 vector (Clontech, now TaKaRa, https://www.takarabio.com) via *Eco*RI and *Sma*I restriction sites. The primers used are listed in Table S3. BD-GhBZR1 and BD empty vectors served as positive (Zhou et al., 2015) and negative controls, respectively. The pGADT7 prey vector carrying the CDS of *GhBZR3* and those control vectors were transformed into yeast strain (Y1H) cells, as previously reported (Yuan et al., 2010). Equal numbers of transformed yeast cells were plated on SD/–Trp/–His and SD/–Trp/–His/–Ade medium plates containing 0.4 mg of X-gal and then incubated at 30°C for 2–6 days.

Electrophoretic mobility shift assay (EMSA)

The EMSA assay was performed as previously described using chemiluminescent EMSA kits (ThermoFisher Scientific, https://www.thermofisher.com). The promoter fragment of *GhKCS13* was labeled with a biotin-conjugated probe. GhBZR3 proteins were incubated with the labeled probe at 25°C for 1 h and were then separated by 8% native polyacrylamide gel electrophoresis (PAGE) (10 V cm^{-1}, 4°C) in 0.5X Tris-Borate-EDTA buffer. Non-labeled probes were used as cold competitors. Fluorescence was observed with an image scanner (FLA-9000; Fujifilm, https://global.fujifilm.com).

Luciferase assay

Cotton cotyledons were cut into thin strips, then soaked in enzymolysis solution (0.75% w/v Cellulase RS; 1.5% w/v Macerozyme R10; 20 mM $CaCl_2$; 0.6 M glycerol) at 28°C in the dark. Four hours after enzymolysis, a large number of fresh protoplasts were observed in the solution. The full-length *GhBZR3* CDS was cloned from cDNA and ligated into the pBI221 vector. The 2-kb promoter region upstream of the *GhKCS13* start site was cloned from DNA and ligated into the reporter vector to obtain pGreenII-0800:*GhKCS13*. The luciferase LUC was used as an internal reference for GUS activity detection. The effector pBI221:*GhBZR3* and reporter vectors pGreenII-0800:*GhKCS13* were transformed into *Agrobacterium* using the electrical stimulation method and GUS activity was detected by the Luciferase Assay System with Reporter Lysis Buffer (Yeasen Biotech Co., Ltd, https://www.yeasen-biotech.com).

RNA-seq data analysis

The 5-DPA wild-type ovules were collected and put on a liquid medium for the ovule culture experiment. Then, the wild-type cotton ovules were treated with BR inhibitor BRZ, which was directly added into the liquid medium, for 6 h or 12 h according to previous methods (Shi et al., 2006). We collected the fibers, which were carefully removed from the ovules using tweezers under a stereomicroscope and quickly frozen in liquid nitrogen for RNA isolation and fatty acids isolation. Also, 5-DPA fibers from *GhBZR3*-silenced plants (VIGS-*GhBZR3*) and control plants were collected according to the method above. Total RNA was extracted from the collected fibers and 2 μg of total RNA was used to construct RNA-seq libraries of cotton fibers. Sequencing was performed on an Illumina HiSeq2000 (Illumina, https://www.illumina.com) with three biological replications. The genes with fragments per kilobase of transcript per million (FPKM) $|\log2 \text{ (fold change)}| > 2$, $P < 0.05$, were identified as DEGs. For KEGG analysis, upregulated genes following *GhBZR3* gene silencing and BRZ treatment were submitted to KOBAS 3.0 (http://kobas.cbi.pku.edu.cn/anno_iden.php) and RNA-Seq raw data bioproject, ID PRJNA831601, in the National Center for Biotechnology Information (NCBI) sequence read archive (http://www.ncbi.nlm.nih.gov/bioproject/).

Fatty acid content measurement

Fatty acid profiling in *GhBZR3*-silenced plants was performed as previously described (Qin et al., 2007). The 5-DPA fibers were obtained from 10 μM BZR treatment after 6 h or 12 h and immersed in chloroform/methanol (2:1, v/v) for 1 min to remove surface waxes. Then, the samples were ground to powder in liquid nitrogen and extracted using 2.5% H_2SO_4 (v/v) in methanol. A known quantity of heptadecanoic acid (C17:0) was added into the extraction mixture to identify percentages for the quantification of fatty acids. Then, hexane was used for the extraction of fatty acid methyl esters. The Agilent 6890 N GC system (Agilent, https://www.agilent.com) with an HP 5975 mass selective detector (Agilent) coupled to a gas chromatography mass spectrometer (GC-MS) system was used for fatty acid detection and measurement, as described previously (Liu et al., 2015).

Virus-induced gene silencing

Virus-induced gene silencing (VIGS) was performed according to a previous study (Gu et al., 2014). A 279-bp fragment of *GhBZR3* (from +50 to 329 bp from the start codon) was cloned and ligated into the pCLCrV-A vector after digestion by *Spe*I. The vector

pCLCrV-*GhBZR3* and the helper vector pCLCrV-B were transformed into *Agrobacterium tumefaciens* strain (GV3101). *Agrobacterium* was propagated in YEP medium at 220 rpm for 12 h, followed by centrifugation at 5000 ***g*** for 10 min, according to a previous report (Gu et al., 2014). After the two cotyledons of cotton were fully unfolded (about 2 weeks old), *Agrobacterium* resuspension solution (10 mM 2-(*N*-morpholine)-ethanesulphonic acid (MES), pH 5.8, 10 mM $MgCl_2$ and 0.2 mM acetosyringone) was injected into cotyledons. At the same time, the plants injected with pCLCrV-CHLI500 served as the positive control. After that, the treated seedlings were kept in darkness overnight and grown in a 22°C, 16-h light/8-h dark glasshouse for 3 months.

Cotton transformation

For generating overexpression constructs, the *GhBZR3* coding sequence was driven by the *35S* promoter in pCAMBIA2300 (Zhan et al., 2021). For *GhBZR3* gene editing, two sgRNAs were designed to target the coding region. All vectors were transformed into *Agrobacterium tumefaciens* strain LBA4404 using the freeze–thaw method. Seeds (Jin668) were sterilized and cultured in a chamber without light for 5 days at 30°C. Their hypocotyl was used as explants for *Agrobacterium*-mediated transformation according to a previously published protocol (Wang et al., 2018). The transgenic plants were transferred into the soil with a 14-h light/10-h dark regimen at 25°C.

Transcription factor activity assay

The transcription factor activity assay was performed as described previously (He et al., 2021). The coding regions of *GhBZR3* were amplified by PCR and cloned into pRT-BD containing the GAL4-binding domain. All vectors were transferred into Y2H Gold and grown on SD/–Trp/–His/–Ade medium, with or without X-gal.

Microscopy assay

For the PI staining, Arabidopsis roots were dipped in methanol: acetic acid solution (5:1, v/v) for 24 h at room temperature about 25°C. The roots were then washed three times briefly with PBS and incubated in 1% periodic acid at room temperature for 30 min. Then, the roots were washed twice with PBS and incubated in Schiff's reagent containing PI (1 mg/ml; Sigma-Aldrich, https://www.sigmaaldrich.com) for 2 h. The roots were observed under a confocal laser scanning microscope at 488 nm (Rounds et al., 2011).

Fiber length measurement

The measurement of fiber length was performed according to a previously published method (Ma et al., 2018). In detail, a total of 25 naturally open bolls were harvested from each plot by hand. After ginning, 10–15 g of fiber from each sample was sent to the Cotton Fiber Quality Inspection and Testing Center of the Ministry of Agriculture, Anyang, China, and the fiber length was measured by the HVI1000 automatic fiber testing system.

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AUTHOR CONTRIBUTIONS

GX and SZ designed and supervised the research. ZS, XC and TJ performed the pivotal experiments. FM, YL and LM contributed the preparatory work. GX, SZ and HX were involved in many discussions. ZS, TL and HX wrote the article, with input from all co-authors.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest associated with this work.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the article and its supporting materials.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Expression profiling of GhBZR3 in various tissues and under abiotic stress.

Figure S2. BZR structure and evolutionary characteristics.

Figure S3. GhBZR3 regulates plant height and branch stem angles.

Figure S4. Liquid chromatography–mass spectrometry analysis of C24 and C26 VLCFAs in wild-type and *GhBZR3* transgenic cotton plants.

Figure S5. Statistical analysis of C24 and C26 VLCFAs in wild-type and *GhBZR3* transgenic cotton plants.

Table S1. The expression patterns of *BZR* genes in upland cotton (TM-1).

Table S2. Structural characteristics of the *GhBZR* genes.

Table S3. Primers used in this study.

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