The miR164-GhCUC2-GhBRC1 module regulates plant architecture through abscisic acid in cotton

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Summary

Branching determines cotton architecture and production, but the underlying regulatory mechanisms remain unclear. Here, we report that the miR164-GhCUC2-GhBRC1 (CUP-SHAPED COTYLEDON2) module regulates lateral shoot development in cotton and Arabidopsis. We generated OE-GhCUC2m (overexpression GhCUC2m) and STTM164 (short tandem target mimic RNA of miR164) lines in cotton and heterologous expression lines for gh-miR164, GhCUC2 and GhCUC2m in Arabidopsis to study the mechanisms controlling lateral branching. GhCUC2m overexpression resulted in a short-branch phenotype similar to STTM164. In addition, heterologous expression of GhCUC2m led to decreased number and length of branches compared with wild type, opposite to the effects of the OE-gh-pre164 line in Arabidopsis. GhCUC2 interacted with GhBRC1 and exhibited similar negative regulation of branching. Overexpression of GhBRC1 in the brc1-2 mutant partially rescued the mutant phenotype and decreased branch number. GhBRC1 directly bound to the NCED1 promoter and activated its transcription, leading to local abscisic acid (ABA) accumulation and response. Mutation of the NCED1 promoter disrupted activation by GhBRC1. This finding demonstrates a direct relationship between BRC1 and ABA signalling and places ABA downstream of BRC1 in the control of branching development. The miR164-GhCUC2-GhBRC1-GhNCED1 module provides a clear regulatory axis for ABA signalling to control plant architecture.

Keywords: miR164, GhCUC2, GhBRC1, ABA, lateral branch.

Introduction

Plant architecture, the physiological pattern of flower and lateral organ development, is a major determinant of planting area, cultivation pattern, grain productivity and planting efficiency in crops (Wang and Li, 2008). Xinjiang is currently the largest and highest-yielding cotton growing area in China, and compact plant architecture is required for cotton cultivars grown in this region to increase planting density and facilitate mechanical harvesting. Branches contribute to bud initiation, activation and expansion and sustained lateral growth. This process is regulated by a complex network of phytohormones and regulatory factors and ultimately governs aerial plant architecture and plant yield (Wang et al., 2018).

In recent years, some studies have identified the transcription factors that participate in initiation of axillary meristems, and subsequently, the formation of plant branches. The TCP transcription factor TEOSINTE BRANCHED1 (TB1) was found to participate in repressing bud development, resulting in decreased branching (Poza-Carrion et al., 2007). Transgenic rice plants overexpressing OsTB1 exhibited markedly reduced lateral branching, while an OsTB1 loss-of-function mutant, fine culm 1 (fc1), had enhanced lateral branching (Takeda et al., 2010). The tomato BLIND gene, encoding an MYB transcription factor, was reported to control the formation of lateral meristems (Schmitz et al., 2002). GRAS transcription factors, including LATERAL SUPPRESSOR (LAS) in Arabidopsis, lateral suppressor (LS) of tomato (Solanum lycopersicum) and MONOCULM1 (MOC1) in rice (Oryza sativa), were all found to regulate axillary meristem initiation (Greb et al., 2003). The Arabidopsis las mutant lost the ability to form lateral shoots during vegetative development. In rice, the moc1 mutant failed to develop tillers due to defects in axillary meristem formation, which is similar to the ls mutant tomato phenotype (Li et al., 2003; Schumacher et al., 1999). TIE1 promoted cotton branching via repressing the transcription activity of TCPs (Diao et al., 2019). Mutation of SP homologs promoted short-branching architecture in cotton, and dynamic changes in GhSFT and GhSP expression level regulated cotton monopodial and sympodial branching architecture (McGarry et al., 2016; Si et al., 2018). While these studies have provided major in-roads for understanding the regulation of branch development, a cohesive picture of the molecular mechanisms controlling shoot branching architecture is still unavailable.

NAC (e.g. NAM, ATAF1, ATAF2 and CUC2) proteins comprise one of the largest families of plant-specific transcription factors, which is present in a wide range of land plants and has more than 100 members identified in Arabidopsis (Olsen et al., 2005). Unsurprisingly, only a portion of NAC proteins has been studied to date, although genes in this family have been implicated in a diverse range of processes, including formation of secondary wall,
controlling flowering time, age-dependent senescence, seed germination and floral and vegetative development (Mathew and Agarwal, 2018; Mitsuda et al., 2005; Saad et al., 2013). Also, NAC proteins are important in the formation and maintenance of different meristem tissues and shoot branching development. NAC genes CUC2/CUC3 directly bind to the promoter of DA1 and activate its expression to control axillary meristem initiation and branching development (Li et al., 2020). CUC1 protein activates expression of LSH4 and its homolog LSH3 in shoot organ boundary cells and suppresses organ differentiation in the boundary region (Takeda et al., 2011). Overexpression of OsNAC2 contributes to tiller bud outgrowth and regulates shoot branching in rice (Mao et al., 2007). The expression patterns and mutant phenotypes of NAM, CUC1 and CUC2 suggest that their function is both similar and conserved in boundary specification and shoot apical meristem (SAM) formation (Chang et al., 2020; Cui et al., 2016; Raman et al., 2010; Vroemen et al., 2003; Wang et al., 2020).

NAC genes participate in auxin and abscisic acid (ABA) signal transduction and regulation of senescence (Guo and Gan, 2010), and ABA negatively regulates axillary bud growth in plants (Yao et al., 2011). Enhanced NCE63 expression led to ABA accumulation, thus suppressing bud and branching development. Reduced ABA level in buds increased branching in hb21, hb40 and hb53 triple mutants (González-Grandío et al., 2017). The complex regulation of NAC transcription factors includes microRNA (miRNA)-mediated cleavage of mRNAs (Wang et al., 2020). In Arabidopsis thaliana, the CUP-SHAPED COTYLEDON (CUC) genes CUC1 and CUC2, which are negatively regulated by miR164, were shown to be involved in SAM and boundary formation (Aida and Tasaka, 2006). In particular, zma-miR164e was demonstrated to cleave the mRNAs of Arabidopsis CUC1, CUC2 and NAC6 in vitro. Overexpression of zma-miR164e resulted in failure to form seeds and increased branch number in Arabidopsis via down-regulation of these genes (Liu et al., 2020). Although CUC2 participates in controlling the initiation of axillary meristems, no NAC genes have been identified to regulate cotton branching, and the direct molecular link between CUC2 and other architecture regulation genes including TCPs, NCEDs, etc. remains unknown.

Here, we show that GhCUC2 is cleaved by gh-miR164, and that GhCUC2 with mutated target sequence could not be cleaved by miR164. GhCUC2 physically interacts with a previously known lateral branch development-related protein GhBRC1 and activates its transcriptional activity to regulate lateral branch development (Diao et al., 2019). In addition, genetic analysis demonstrated that GhCUC2 and GhBRC1 negatively regulate branching, exerting the opposite effect of miR164. This study provides novel insights into the regulatory network of CUC2 genes lateral branch development in which a miR164-GhCUC2-GhBRC1-GhNAC6-1 regulatory module controls aerial plant architecture in cotton.

**Results**

RNA-seq and cluster analysis show differential GhCUC2 up-regulation in short-branch cotton

To mine the differentially expressed genes (DEGs) controlling branch length in cotton, we used RNA-Seq to investigate differences in the expression profiles within axillary buds (see Figure S1 for sampling position) among three short-branch cultivars (Shan 4080, Jin7, Changrongzhongmian) and three long-branch cultivars (Zhong24, Zhong6, TM-1) of upland cotton, Gossypium hirsutum. Correlation and clustering analysis among the six inbred cultivars revealed that the three short-branch and three long-branch materials, respectively, clustered together supporting that a set of specific DEGs participate in regulating cotton architecture differently between the two cultivar types (Figure 1a). To uncover the genetic underpinnings of branch length, we then identified the DEGs between the two architecture types (Figure 1b) and selected the DEGs with similar expression trends in the three short-branch materials but opposite expression trends in the long-branch materials for further analysis. This set was largely comprised of putative NAC family transcription factors, TCP family transcription factors and some HB family transcription factors (Table S1).

A recent report indicated that the CUC2/ CUC3-DA1-UBP15 regulatory module controls the initiation of axillary meristems, determining the number of lateral branches in Arabidopsis thaliana (Li et al., 2020). In addition, OsNAC2 has been reported to be involved in lateral shoot branching (Mao et al., 2007). In the light of the conserved function of CUC2 in shoot branching, we conducted a phylogenetic analysis of GhCUC2, AtCUC2, OsNAC2 and several other homologous genes, which showed that GhCUC2 is the closest homolog of AtCUC2, OsNAC2 and Petunia NAM (Figure 1c). Furthermore, the expression levels of GhCUC2 were significantly higher in short-branch cultivars than in long-branch cultivars, supporting a close correlation between GhCUC2 expression and cotton branch length (Figure 1d,e). We then carried out fluorescence in situ hybridization (FISH) assays to determine the tissue-specific expression patterns of GhCUC2, which revealed that it was preferentially expressed in the axillary bud (Figure 1f). These results showing preferential expression in axillary buds and differential up-regulation in short-branch materials together support that GhCUC2 may regulate the formation of lateral branches.

CUC2 mRNA cleavage is directed by miR164

To date, the majority of previously identified target genes of miR164-mediated regulation belong to the NAC gene family, including At-miR164-AtCUC1/CUC2/CUC3 in Arabidopsis (Lee et al., 2017), Ade-miR164-AdNAC6-7 in Actinidia spp. (Wang et al., 2020) and the NAC family genes OMTN1-OMTN6 in rice (Yu et al., 2014). However, miR164 targets in cotton have yet to be identified. To this end, we used degradome sequencing to accurately predict miR164 target genes and cleavage sites. Degradeom sequencing indicated a significant cleavage site located at nt position 517 in GhCUC2, and the predicted miRNA-mediated cleavage site matched the peak (Figure 2a). High base pairing between miR164 and the CUC2 genes suggested that miR164 can potentially establish regulatory module(s) with CUC2.

To obtain direct evidence that miR164 can mediate the cleavage of CUC2 mRNA, we constructed three vectors as follows: 35S::gh-pre164, 35S::GhCUC2:eGFP and 35S::GhCUC2m::eGFP. CUC2m was designed by introducing four synonymous nucleotides into the miR164 complementary region of CUC2 mRNA sequence (Figure 2b). Agrobacterium tumefaciens harbouring plasmid 35S::gh-pre164 and 35S::GhCUC2::eGFP were co-transformed into tobacco (Nicotiana benthamiana) leaves by infiltration, while co-transformation of 35S::CUC2m::eGFP and 35S::gh-pre164 served as negative controls. The fluorescence intensity of 35S::CUC2::eGFP gradually decreased commensurately with increasing gh-pre164 concentration (OD600 nm = 0.0–0.9) in the cells of tobacco leaves co-
Figure 1  RNA-seq and cluster analysis show differential GhCUC2 up-regulation in short-branch cotton. (a) Relative expression correlation analysis and cluster analysis between short- and long-branch cotton cultivars. (b) MA plot of differentially expressed genes (DEGs) for long-branch and short-branch cotton plants. (c) Unrooted phylogenetic tree of NAC transcription factors. Numbers between branches indicate bootstrap values based on 1000 replications. The red box indicates the CUC2 clade. Names and references for other NACs: Arabidopsis thaliana—ATAF1, ATER2 (Aida et al., 1997), AtNAC2 (He et al., 2005), AtNAC3 (Takada et al., 2001), AtNAM (Duval et al., 2002), CUC1, CUC2 (Takada et al., 2001), CUC3 (Vroemen et al., 2003), NAC1 (Xie et al., 2000), NAC2, NAP (Sablowski and Meyerowitz, 1998), TIP (Ren et al., 2000); Oryza sativa—OsNAC6 (Kusano et al., 2005; Ohnishi et al., 2005), OsNAC2 (Mao et al., 2007); Petunia—NAM (Souer et al., 1996). (d) GhCUC2 expression levels among long-branch and short-branch cotton plants based on RNA-seq. (e) qRT-PCR detection of relative expression of GhCUC2 among long-branch and short-branch cotton plants. (f) Fluorescence in situ hybridization (FISH) detection of GhCUC2 in different tissues. Blue, DAPI; Red, GhCUC2; Scale bars = 100 μm.
transferred with gh-pre164 and 35S::CUC2::eGFP (Figure 2c,d). However, no change in fluorescence intensity was observed in the tobacco leaves co-transformed with gh-pre164 and mutated 35S::CUC2m::eGFP (Figure 2e,f), regardless of increasing gh-pre164 concentration (OD600 nm = 0.0–0.9). We also performed 5’ RLM-RACE and found that the GhCUC2 target site is cleaved at position 11 starting from the 5’ end (Figure S2). Overall, these findings indicated that miR164 specifically cleaved CUC2 mRNA at the predicted target sequence and thereby suppressed the accumulation of CUC2 protein.

**miR164-CUC2 module regulates branching in cotton and Arabidopsis**

To assess the potential biological functions of miR164 and GhCUC2 in regulation of plant architecture, we generated transgenic Arabidopsis lines overexpressing miR164 (OE-gh-pre164), GhCUC2 (OE-GhCUC2) and GhCUC2m (OE-GhCUC2m) under the control of the CaMV35S promoter (Figure 3a). Their expression levels were confirmed using semi-quantitative PCR, which indicated successful overexpression in transgenic lines relative to wild type (WT; Figure 3d,e). The OE-gh-pre164 plants exhibited a significantly higher branch number than WT, while the OE-GhCUC2 and OE-GhCUC2m lines had significantly fewer branches than WT (Figure 3b). In addition, the majority of OE-GhCUC2m and OE-GhCUC2 transgenic seedlings produced similar numbers of lateral branches, although these lateral branches were significantly shorter on average in the OE-GhCUC2m line (P < 0.01) compared with those of the OE-GhCUC2 line (Figure 3c). These results indicated that CUC2m blocked the cleavage of miR164, which was supported by higher CUC2 transcript levels in the OE-GhCUC2m transgenic line compared with the OE-GhCUC2 line (Figure 3d,e), thus suggesting that higher CUC2 expression resulted in shorter branches.

To further investigate the putative biological function of GhCUC2 and miR164 in cotton, a CLCrV-based virus-induced gene silencing (VIGS) strategy was used to knock down the expression of GhCUC2 (pCLCrV::GhCUC2) and miR164 (short tandem target mimic RNA of miR164, STTM164), and to overexpress miR164 (pCLCrV::gh-pre164) in cotton. Plants with high silencing efficiency were selected for greenhouse cultivation, and their phenotypes were observed at 70 days (Figure S3). Compared with WT, the STTM164 plants exhibited significantly shorter branches (Figure 4a), while the pCLCrV::GhCUC2 and pCLCrV::pre164 plants had no obvious, visible differences from vector control plants (Figure 4b,c). We thus proposed that increased CUC2 expression in STTM164 plants potentially resulted in decreased branch length, whereas decreased CUC2 expression levels in pCLCrV::GhCUC2 and pCLCrV::pre164 plants had no effect on branch development.

Considering that CUC2 can be cleaved by miR164, while CUC2m disrupted CUC2 suppression by miR164, we subsequently overexpressed GhCUC2m in wild-type cotton to investigate its possible function in branch development. To generate these plants, we cloned the full-length CUC2m gene fragment into the pCambia2300 plant overexpression vector, then transferred the vector construct into WT cotton, resulting in six independent transgenic lines. Overexpression transgenic lines OE1, OE2 and OE3 were selected for further observation of CUC2-related phenotypes among progeny. Overexpression levels in the T₁ generation of these lines were evaluated by semi-quantitative PCR (Figure S4). We found that the branch lengths among the OE plants were significantly shorter compared with WT (Figures 4d,e, f and S4). Taken together, our data strongly suggest that miR164...
mediates the cleavage of CUC2 transcripts and that the CUC2 transcription factor regulates lateral branch development.

**GhCUC2 interacts with GhBRC1 in vitro and in vivo**

The BRC1 transcription factor has been shown to regulate plant development, including aspects of branch architecture (Alice et al., 2020; Diao et al., 2019). AtBRC1 was shown to arrest axillary bud development and prevent axillary bud outgrowth, while brc1 mutant plants produced more branches (Aguilar-Martinez et al., 2007). To analyse GhBRC1 function in branching development, we transformed plants from an Arabidopsis brc1 mutant line with GhBRC1 to observe whether it could rescue the high branch number phenotype. As expected, GhBRC1 overexpression in the brc1 mutant significantly decreased the rosette-leaf branch number by half, compared with that of the brc1 mutant (Figure 5a,b). GhBRC1 shares the conserved TCP domain with AtBRC1, thus supporting the conserved and essential role of BRC1 in regulating bud formation and shoot architecture (Figure S5).

We then performed FISH assays to examine the expression patterns of GhBRC1 in cotton and found that they were consistent with that of GhCUC2 expression (Figures 1e and 5c). We speculated that the short-branch phenotype in GhCUC2-overexpressing cotton plants may be related to an interaction between GhCUC2 and GhBRC1, and that they both participate in a common pathway to regulate branch development. To test this hypothesis, we conducted a yeast two-hybrid (Y2H) assay, which revealed a strong interaction between GhBRC1 and GhCUC2 (Figure 5d). We next performed a bimolecular fluorescence complementation (BiFC) assay. Agrobacterium tumefaciens cells carrying the GhBRC1-YFPN and GhCUC2-YFPC plasmids were co-infiltrated into the lower epidermis of tobacco leaves. After a 72-h incubation, a strong YFP fluorescence signal was detected (Figure 5e). In contrast, YFP fluorescence was undetectable in the negative control samples, confirming that GhBRC1 and GhCUC2 interacted in planta. We then used pull-down and Co-IP assays to further validate the interactions of the GhCUC2 proteins with GhBRC1. A direct physical interaction in vitro was also observed in GST pull-down assays (Figure 5g), and the Co-IP result also supported that GhCUC2 interacted with GhBRC1 in planta (Figure 5f).
GhCUC2 and GhBRC1 negatively regulate branching via activating NCED1 expression

TCP genes have been shown to regulate abscisic acid (ABA) signalling pathway genes. For example, ABI3 and ABI4 were up-regulated in OsTCP19 overexpressing plants (Mukhopadhyay and Tyagi, 2015). BRC1, itself a TCP family gene, together with the HB family genes, promotes the transcription of 9-CIS-EPOXICAROTENOID DIOXIGENASE3 (NCED3), a key enzyme in ABA biosynthesis, thereby leading to ABA accumulation and branching suppression (González-Grandío et al., 2017). We examined our RNA-Seq data for expression of native NCEDs in OE-GhCUC2m cotton plants, and also validated these expression data in OE-GhBRC1 Arabidopsis plants using semi-quantitative PCR (Figure S6). The results showed that the expression of NCED1 in both of these OE lines was increased, supporting that NCED1 was indirectly or directly regulated by CUC2 and BRC1.

To further elucidate whether NCED1 served as a direct target of GhBRC1 and GhCUC2, we used the Plant Regulation Data and Analysis Platform (http://plantregmap.cbi.pku.edu.cn/binding_site_prediction.php) to predict putative NCED1-binding sites in the ~2-kb promoter regions of these genes. This analysis revealed a GAGGAGACCACGT motif that could serve as a putative binding site in GhBRC1, while no binding motifs were detected in GhCUC2.

To further determine whether the expression of NCED1 is regulated by GhBRC1, the complete NCED1 motif-enriched fragment pNCED1 and a mutated fragment lacking the binding site, pNCED1m, were each cloned into a pAbAi plasmid for yeast one-hybrid (YTH) analysis. As indicated by the growth of yeast on the selective medium (–Leu+AbA), GhBRC1 could interact with pNCED1. By contrast, pNCED1m abolished binding by GhBRC1, indicating that NCED1 transcription was directly regulated by GhBRC1 (Figure 6a,b). In addition, an electrophoretic mobility shift assay (EMSA) using recombinant His-GhBRC1 fusion protein showed that GhBRC1 was able to bind to a NCED1 fragment (Figure 6c). Together, our results indicate that GhBRC1 positively regulates ABA content through direct activation of NCED1 expression.

Different from GhBRC1, transcriptional induction of NCED1 may be indirectly modulated by GhCUC2. In order to validate whether GhCUC2 and GhBRC1 function together as a transcriptional activator complex of NCED1 expression, we performed dual luciferase transactivation assays in N. benthamiana leaves. To this end, a ~2000 bp NCED1 promoter fragment was cloned into the pGreenII0800-LUC vector to generate the reporter construct proNCED1-LUC. The coding sequences (CDSs) of GhBRC1 and GhCUC2 were individually fused into a pCambia2300 vector to generate the 35S-GhBRC1 and 35S-GhCUC2 effector plasmids (Figure S7). Then, with the pCambia2300 empty vector serving as a control, the 35S-GhBRC1 and proNCED1-LUC constructs were co-infiltrated into N. benthamiana leaves. Analysis of LUC activity indicated that GhBRC1 directly activated the promoter of NCED1. proNCED1-LUC and 35S-GhCUC2 were also co-infiltrated with 35S-GhBRC1, resulting in extremely strong activation of the NCED1 promoter (Figure 6d,e).

Consistent with the induction of NCED1 expression, ABA content was significantly increased in axillary buds of STTM164 miR164 and GhCUC2 regulate branch length in cotton. (a) Representative images showing morphological and growth phenotypes of STTM164 cotton plants silenced for miR164 (right) and empty vector controls (left). Scale bar, 10 cm. (b) Representative images showing phenotypes of empty vector control, CUC2-silenced cotton and miR164 overexpression cotton. (c) Distribution of branch lengths of CUC2-silenced, WT, miR164-silenced and miR164 overexpression cotton lines. (d) Phenotypic analysis of GhCUC2m transgenic cotton plants. Scale bar, 20 cm. (e) Image of branch phenotype in WT and OE-GhCUC2m cotton plants. Scale bar, 2 cm. (f) Statistical analysis of branch length in WT and OE-GhCUC2m cotton plants.
and OE-GhCUC2m cotton plants, compared with WT plants (Figure S8). These results cumulatively suggest that GhCUC2 and GhBRC1 together activated NCED1 expression and ABA accumulation, and that this CUC2-mediated enhancement of GhBRC1 regulatory activity could boost local ABA signalling and response in axillary buds (Figure 7).

### Discussion

Shoot branching in plants arises from axillary meristems in the region between the SAM and leaf primordia and is a major component driving plant architecture. The initiation of axillary meristems is a crucial step for generating axillary branches, and several genes have been identified for their effects on axillary meristem initiation. However, except for SP, SFT and DREB1B (Ji et al., 2021; McGarry et al., 2016; Si et al., 2018), few genes controlling cotton branching architecture have been identified, and the molecular mechanism and genetic networks controlling this differentiation process during cotton branch development remain unknown. Here, we uncovered a genetic and molecular framework in which a miR164-GhCUC2-GhBRC1-GhNCED1 regulatory pathway controls the formation of axillary meristems, thereby determining plant architecture (Figure 7).
Phylogenetic analysis indicates that GhCUC2 is grouped into the same subclade with AtCUC2, OsNAC2 and petunia no apical meristem (NAM) proteins. These proteins are known to function in the development of the SAM and cotyledons, and to act in regulating lateral branch formation (Mao et al., 2007; Souer et al., 1996; Yu et al., 2020). In Arabidopsis thaliana, the CUC2/CUC3-DA1-UBP15 regulatory module controls the initiation of axillary meristems, which determines the number of lateral branches (Yu et al., 2020). The NAM gene of petunia is required for pattern formation in embryos and flowers, and embryos carrying the nam mutation fail to develop a SAM (Souter et al., 1996). Overexpression of OsNAC2 was found to increase tiller numbers in a dose-dependent way in rice, which is in agreement with the phenotype of the Ostil1 mutant (Mao et al., 2007). In this study, we found that GhCUC2 was preferentially expressed in axillary buds to regulate bud outgrowth in cotton. GhCUC2 transgenic overexpression lines showed reduced branch length, consistent with the phenotype exhibited by STTM164 plants. Furthermore, we observed that OE-GhCUC2 and OE-GhCUC2m displayed fewer axillary branches, as opposed to OE-pre164 in Arabidopsis, indicating that miR164-CUC2 regulatory module function is conserved in the initiation of plant axillary meristems.

ABA is well-established as a negative regulator of axillary bud growth in Arabidopsis, rice and soybean; studies examining a
wide variety of species showed that ABA abundance in buds is negatively correlated with bud development (Luo et al., 2019; Tamas et al., 1979; Yao and Finlayson, 2015). In Arabidopsis, mutation of the ABA biosynthetic genes NCED3 and ABA2 enhanced branching and confirmed an inverse correlation between bud growth potential and bud ABA levels (Reddy et al., 2013). ABA levels were reduced in the lower buds of hyperbranching mutants deficient for auxin signalling (AUXIN RESISTANT1) and BRANCHED1 (BRC1) function (Yao and Finlayson, 2015). BRC1 expression was not affected by exogenous ABA in buds, strongly suggesting that ABA signalling functions downstream of BRC1. However, the expression of TAA1, an indole-3-acetic acid (IAA) biosynthetic enzyme, and the auxin transporter PIN1, were both suppressed by ABA in buds, suggesting that ABA may inhibit bud growth in part by controlling IAA biosynthesis and transport locally (Yao and Finlayson, 2015). In our study, we analysed transcript levels of NCEDs, as well as that of auxin biosynthesis and transport genes, using three independent GhCUC2m overexpression cotton lines. We found that NCED1 was significantly up-regulated but PIN4 was significantly decreased in OE lines compared with WT, indicating that CUC2 regulates branch development via increasing the accumulation of ABA and inhibiting auxin transport. Moreover, manipulating miR164 levels or expressing cleavage-resistant CUC2m transcripts clearly demonstrated that miR164 functions as a negative regulator of ABA-mediated lateral branch development by controlling CUC2 mRNA levels.

In recent years, the network consisting of BRC1 and its downstream targets has been characterized. BRC1 has been called a branching integrator because it acts downstream of many other pathways that influence branching, including the strigolactone pathway (Braun et al., 2011), auxin (Aguilar-Martínez et al., 2007), ABA (González-Grandío et al., 2017), cytokinins (Stes et al., 2015), decapitation (Gao et al., 2016) and R:FR response (González-Grandío et al., 2013). A previous study showed that ABA affects branching independently of BRC1, and that BRC1 promoted the accumulation of ABA in lower buds. Therefore, ABA more likely acts downstream of BRC1 to transduce the effects of BRC1 function (Yao and Finlayson, 2015). In our study, genetic analyses and biochemistry assays suggested that GhBRC1 functions, at least in part, in a common pathway with CUC2 to regulate axillary meristem initiation. We found that GhBRC1 directly associates with the NCED1 promoter to induce its transcriptional expression, and consequently, ABA accumulation. Protein interactions between CUC2 and GhBRC1, as well as the similar phenotypes resulting from CUC2 and BRC1 overexpression in cotton and Arabidopsis, together support that GhBRC1 functions synergistically with GhCUC2 in regulating axillary meristem initiation. Furthermore, our study shows that BRC1 directly triggers an NCED1-mediated cascade, which ultimately boosts local ABA accumulation. Here, we provide evidence that CUC2-BRC1-NCED1 function in a common pathway to control the initiation of axillary meristems.

Taken together, our findings point to a genetic and molecular framework in which a miR164-CUC2-BRC1-NCED1 regulatory module mediates axillary meristem initiation to control lateral branch architecture in plants. This pathway enriched the regulatory network controlling cotton architecture and provided

Figure 7 Proposed model of how miR164-GhCUC2-GhBRC1 module regulates plant architecture through ABA in cotton. The number of dots indicates the content of ABA.
candidate genes for precisely modulating cotton architecture. This plant short-branch trait greatly improves cotton yield by allowing high planting density. Extensive planting globally of varieties with short branches would increase cotton fibre yield. Therefore, a short-branching trait represents an ideal plant type for cotton breeding.

Experimental procedures

Plants materials

All *Arabidopsis thaliana* lines used here were derived from the Columbia-0 genetic background (Col). *Arabidopsis* and *Nicotiana benthamiana* were grown in a controlled environment at 22°C with a relative humidity of 60% under long-day conditions (16 h light/8 h dark) with white light illumination. Transgenic cotton plants were grown under natural field conditions in Anyang, China. The phytotron, with 28/20°C day/night temperature, 55%–70% relative humidity and a 14/10 h light/dark cycle, was used to culture cotton seedlings before they were transplanted to fields.

RNA isolation and real-time PCR analysis

First-strand cDNA was synthesized from total RNA using the FastQuant RT Kit (Tiangen Biotech). qRT-PCR was performed using SuperReal PreMix Plus Kit (SYBR Green; Tiangen Biotech). Gene-specific primers were listed in Table S2. UBQ was used as an endogenous reference gene.

For miRNA quantification, total RNA was extracted as described above, a poly (A) tail was added to the 3' end of RNA, and reverse transcription was initiated using the miRcute miRNA First-Strand cDNA Synthesis Kit (Tiangen). qRT-PCR was performed using an ABI StepOne Plus instrument (Applied Biosystems, Inc., Carlsbad, CA), and the 2^{-ΔΔCT} method was used to calculate the relative expression level. U6 was used as an endogenous reference gene.

RNA-sequencing (RNA-seq) analysis

Three short-branch cultivars (Shan 4080, Jin7, Changrongzhongmian) and three long-branch cultivars (Zhong24, Zhong6, TM-1) were used as materials in this study. Total RNA was extracted, and RNA libraries were prepared by Beijing Genomics institute (BGI, Shenzhen, China). Three biological repeats were performed for the RNA-Seq experiment. The library products were sequenced using the Illumina HiSeq 2500 platform, generating paired-end reads. The raw data of RNA-seq were used to map reads to the reference genome TM-1 (AD1) genome NAU-NBI (Nanjing Agricultural University-Novogene Bioinformatics Technology) assembly v1.1 and annotated v1.1 (https://www.cottongen.org; Zhang et al., 2015), NCBI, PANTHER, GO, KEGG or domain search against Pfam. Differentially expressed genes (DEGs) were defined as those having at least twofold change in expression (false discovery rate, FDR < 0.05).

Degradome sequencing

Total RNA was extracted from cotton leaf (Zhong24) using Trizol reagent (Invitrogen, Carlsbad, CA). Approximately, 200 µg total RNA was used to construct the degradome library. In brief, a 5' RNA oligonucleotide adaptor ligated to the fragments containing 5'-monophosphates using T4 RNA ligase. The first-strand cDNA was synthesized by reverse transcription using the ligated products with SuperScript™ II Reverse Transcriptase (Invitrogen) and then PCR-amplified. Next, the PCR products were digested with Mme I, and the 20-bp ‘signatures’ were captured to ligate a 3' adapter via T4 DNA ligase. The ligated products were finally amplified and used for degradome sequencing on an Illumina HiSeq 2000 at the Beijing Genomics Institute (BGI), Shenzhen, China.

Prediction of miRNA164 targets and miRNA target cleavage sites

To validate the miRNA/target interactions predicted based on degradome sequencing, we experimentally analysed their transient co-expression in *N. benthamiana* leaves. We amplified CUC2 sequence from cDNA of cotton using a primer pair containing BamH I and Sac restriction sites as overhangs, and then, the amplified fragment was gel-purified and ligated into the pCamBia2300 (::GFP) vector. The mutated CUC2 variant CUC2m was produced by creating four mismatches in the miR164-binding sites without altering the amino acid sequences. The CUC2m sequence was also cloned in vector using the same method as for constructing the CUC2 vector. These vectors were delivered into *A. tumefaciens* GV3101 by the freeze-thaw method. We first pelleted and then gently resuspended the cells to the required final OD600 value and mixed the Agrobacterium suspension containing 35S::miR164 plasmid and 35S::CUC2 plasmid at a ratio of 1:1, then incubated for 1 h at room temperature. A syringe needle was used to force entry of the Agrobacterium suspension into the leaf. The plants were maintained at 20°C and shielded from direct light by placing them under a table surrounded with black plastic for 24 h. Plants were grown on soil in a 16 h light/8 h dark cycle at 20 °C, and the leaves were collected at approximately 72 h post infiltration for GFP fluorescence detection and RT-PCR.

RLM-RACE

To determine the miR164 cleavage site in CUC2, a RLM-RACE assay was performed with the RLM-RACE kit (Takara) according to the manufacturer’s instruction. Total RNA was extracted from tobacco leaves. Approximately, 2 µg RNA was ligated to the RNA Oligo adaptor, the ligated RNA was used to synthesize cDNA using M-MLV reverse transcriptase according to the manufacturer’s instructions. Two rounds of nested PCR were performed, after which the PCR products were inserted into a cloning vector for sequencing. The primers used in this assay are listed in Table S2.

Vector construction and plant transformation

We constructed all vectors using a one-step cloning method. This method uses a primer with a homologous arm and a double restriction site to amplify the gene fragment, which is then ligated to the vector with the same homologous arm and restriction site. All vectors were converted to *A. tumefaciens* strain GV3101/LB4404 using the freeze-thaw method.

pCambia2300 vector was used in this work to generate the overexpressing transgenic lines. gh-pre164 sequence was amplified from cotton genomic DNA, and CUC2 sequence was amplified from cDNA of cotton using primer pairs (Table S1). CUC2m sequence was synthesized, and then ligated into the vector pCambia2300 and confirmed by sequencing. *A. tumefaciens* containing gh-pre164, GhCUC2m and GhCUC2 plasmids were separately transformed into wild-type *Arabidopsis* (Col) by the floral dip method. Transgenic plants were selected with 50 µg/mL kanamycin on half-strength MS plates. OE-gh-pre164, OE-GhCUC2 and OE-GhCUC2m T2 single-copy lines were selected for the population study of lateral branching phenotype.

For cotton transformation, the detailed process was described in our previously published paper (Ge et al., 2015). Transformants with different expression levels were selected for further analysis.
VIGS

Virus-induced gene silencing was performed as described previously (Gao et al., 2011). A vector constructed with an miRNA precursor could result in high expression of the mature miRNA and a vector with an artificial small tandem target mimic (STTM) could lead to low expression of the mature miRNA. To create the miR164 overexpression vector pCCLCrV-miR164, the precursor of miR164 was amplified from total genomic DNA by PCR, verified by sequencing, and then introduced into the vector using the One-Step Cloning kit. For the miR164 suppression vector pCLCrV-STTM164, the STTM sequence of miR164 was designed and synthesized based on the same kit. A diagram of STTM164 vector is shown in Figure S9, and the primers are listed in Table S1. The vector was introduced into A. tumefaciens GV3101. The Agrobacterium cultures were pelleted and resuspended. After 3 h incubation at room temperature, Agrobacterium harbouring pCCLCrVVA was mixed with an equal volume of Agrobacterium harbouring pCCLCrVB. The mixed Agrobacterium solutions were infiltrated into the abaxial side of cotyledons of 7-day-old CR24 cotton seedlings using needleless syringes through small wounds, which were made on the surface of cotyledons or true leaves using small syringe needles. After the true leaves of the plants inoculated with CLCrV::CHL-expressing Agrobacterium showed a yellow phenotype, the young leaves from CLCrV::00, CLCrV::GhCUC2, STTM164, and CLCrV::pre164 plants were sampled for real-time PCR to check the interference efficiency and the expression levels of GhCUC2 and miR164 (Figure S3).

BiFC assays

The BiFC assays for protein interaction detection were performed in N. benthamiana leaves using A. tumefaciens-mediated transient expression. Candidate interacting proteins were separately fused with the N- or C-terminal parts of the yellow fluorescent protein and then infiltrated into 3-week-old N. benthamiana leaves using syringes. At 48 h post infiltration, tissues were imaged with an LSM780 confocal laser-scanning microscope. Six independent N. benthamiana leaves were observed for analyses, and three biological replications were performed.

GST pull-down and co-immunoprecipitation

GhCUC2 without signal peptides were cloned into pGEX-4T-3 vectors as baits. GhBRC1 without signal peptides were cloned into pET32a vectors as preys. All the constructs were transferred into the Escherichia coli BL21 (DE3) strain to produce recombinant proteins. The pGEX-4T-3 vector was used to express glutathione-S-transferase (GST) as a negative control. Bait and prey proteins were mixed at 4 °C for 6 h and then purified using glutathione-conjugatedagarose beads (17-5132-01; GE Healthcare, San Ramon, CA). Next, the proteins were separated by 12% SDS-PAGE and immunoblotted with anti-GST or anti-His antibody (ProteinTech, Rosemont, IL).

To validate the protein interactions of GhCUC2 and GhBRC1, co-immunoprecipitation (Co-IP) assays were performed as follows. 35S:Flag-GhCUC2 and 35S:HA-GhBRC1 were co-expressed in tobacco (N. benthamiana) leaves. Total proteins were isolated by homogenizing tissues with RIPA buffer 3 days after agroinfiltration. After centrifugation, the supernatant was incubated with 30 μL of HA-Myc-agarose conjugated beads (Sigma) for 3 h at 4 °C. The matrix beads were washed six times with immunoprecipitation buffer. All of the washing steps were carried out at 4 °C for 10 min and were followed by centrifugation at 1600×g. For immunoblot analysis, IP products and the input samples were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and the target protein was detected by Western blot using anti-FLAG (1 : 1000, Sigma-Aldrich F0804) or anti-HA (1 : 1000, Abcam ab32) antibodies.

Yeast experiments

Y2H assays were performed using the GAL4-based two-hybrid system. The coding sequences (CDS) of GhCUC2 were subcloned in-frame into pGADT7-Rec to generate pGAD-Preys. The coding regions of GhBRC1 were cloned in-frame into pGBK7 to generate pGBD-Baits. The corresponding primers are listed in Table S2. Then, pGBD-Baits and pGAD-Preys constructs were transformed into Y2HGold. Transformed cells were grown on synthetically defined (SD) medium lacking Leu or Trp for 4d. Then, the yeast cells were screened on synthetic dextrose selection medium lacking Leu, Trp, Hde and His (SD/-4).

In yeast one-hybrid (Y1H) experiments, the CDS of GhBRC1 was cloned into the pGADT7 vector. The putative GhBRC1 binding motif in the NCE1 promoter sequence was selected and ligated into the pAbAi vector. The combination of pGADT7:-GhBRC1 and the pAbAi-motif was transformed together into Y1HGld. Additionally, combinations of the empty vector pGADT7 and putative GhBRC1 binding motif, or mutated motif were also co-transformed into Y1HGld as negative controls. SD/Trp/Ura plates with appropriate nutrients were used to select the transformants, and positive clones were transferred to and grown on SD/Trp/Ura plates.

Dual-luciferase assay in Nicotiana benthamiana

The 2-kg GhNCE1 promoter sequence was cloned into the plant binary vector pGWB435, which was used as a reporter. The coding sequences of GhCUC2 and GhBRC1 were cloned into pCambia2300 vector, which was used as an effector. After sequencing, the verified plasmids were transformed into GV3101. The Agrobacterium harbouring reporter and effector constructs were co-infiltrated into N. benthamiana leaves. The empty pCambia2300 and pGWB435 vectors, the pCambia2300 vector with the reporter, and the effector with the pGWB435 empty vector were used as negative controls. Luciferase signals were captured and analysed using a Tanon 5200 Multi Chemiluminescent Imaging System (Tanon, Shanghai, China). Firefly luciferase (LUC) and Renilla luciferase (REN) activity levels were measured using a dual-luciferase reporter assay system (Promega). The measured LUC activity was normalized to that of REN.

Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assays were carried out as described previously (Smith and Delbarygossart, 2001). The amplified CDS regions of GhBRC1 (Table S2) were fused in-frame with His tags and transformed into E. coli BL21. Oligonucleotide probes were synthesized and labelled with biotin at their 5' ends (Invitrogen). EMSAs were performed using the LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific, Waltham, MA). The probes used in EMSA are listed in Table S2.

Fluorescence in situ hybridization

The partial-length cDNA fragments with fluorescent labels were synthesized by Sangon Biotech. The probe sequences for FISH are listed in Table S2. Western Blue plus 1 mL tetramisole was the substrate solution to reduce the hybridization background.
Sample preparation and FISH experiments were carried out as described previously with slight modifications (Jackson et al., 1994; Zhang et al., 2013).

**Extraction and quantification of endogenous hormone**

About 0.1 g samples were harvested from axillary buds of WT, STM164 and OE-GhCUC2 transgenic plants and used for measurement of hormone content. The extraction and quantification of endogenous hormones were performed using ELISAs by Shanghai Zhucai Biotechnology Co., Ltd. Three biological repeats were performed for each sample. Hormones were extracted by adding 500 μL cold (v/v) aqueous methanol to each sample, and samples were mixed on an overhead shaker in the dark overnight. The extraction was repeated with 1 mL cold 80% MeOH. After the second extraction, samples were pre-purified with 500 μL 80% aqueous methanol. Supernatants of the four extractions were pooled (Thermo Fisher Scientific). Tris-risermo Fisher Scientific) was then added up to 1 mL and samples were 1 : 10 diluted in 1x TBS for analysis.

**Statistical analysis**

All data were analysed using SigmaPlot 10.0 (Systat Software) and GraphPad Prism 5 (GraphPad Software) software. The averages and SD of all results were calculated, and ANOVA and Student-Newman-Kuels tests were conducted.

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**Conflict of interest**

The authors declare no conflicts of interest.

**Author contributions**

LG, GY and ZJ designed the research; ZJ, CY, DY, WY, ZY, LS, WX and MY performed the research; GY, ZJ and CY analysed the data; LG, GY and ZJ wrote the paper.

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