The GhREV transcription factor regulate the development of shoot apical meristem in cotton (Gossypium hirsutum)

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Abstract

Background: Manual topping is a routine agronomic practice for balancing the vegetative and reproductive growth of cotton (Gossypium hirsutum) in China, but its cost-effectiveness has decreased over time. Therefore, there is an urgent need to replace manual topping with new approaches, such as biological topping. In this study, we examined the function of GhREV transcription factors (a class III homeodomain-leucine zipper family, HD-ZIP III) in regulating the development of shoot apical meristem (SAM) in cotton with the purpose of providing candidate genes for biological topping of cotton in the future.

Results: We cloned four orthologous genes of AtREV in cotton, namely GhREV1, GhREV2, GhREV3, and GhREV4. All the GhREVs expressed in roots, stem, leaves, and SAM. Compared with GhREV1 and GhREV3, the expression level of GhREV2 and GhREV4 was higher in the SAM. However, only GhREV2 had transcriptional activity. GhREV2 is localized in the nucleus; and silencing it via virus-induced gene silencing (VIGS) produced an abnormal SAM. Two key genes, GhWUSA10 and GhSTM, which involved in regulating the development of plant SAM, showed about 50% reduction in their transcripts in VIGS-GhREV2 plants.

Conclusion: GhREV2 positively regulates the development of cotton SAM by regulating GhWUSA10 and GhSTM potentially.

Keywords: Cotton, Revoluta (REV), Shoot apical meristem (SAM), Virus induced gene silencing (VIGS)

Background

All aerial organs (leaves, stems, flowers, and germline) of plants are derived from the shoot apical meristem (SAM), which is the basis of aboveground biomass sources for crops. The primordia of an organ arises from the periphery of the SAM and develops into leaves at the vegetative growth stage, or flowers at the reproductive growth stage (Pautler et al. 2013). In dicotyledonous angiosperms, the SAM can be divided into three zones, the central zone (CZ), the organizing center (OC) and the peripheral zone (PZ). The central zone contains three layers (L1-L3) of pluripotent stem cells. Directly underneath the CZ lies the OC, which is a zonation with signals regulating stem cell maintenance. The daughter cells from the CZ are laterally displaced into the PZ, where they proliferate and ultimately differentiate during organogenesis (Bäurle and Laux 2003; Soyars et al. 2016).

The class III homeodomain-leucine zipper (HD-ZIP III) family of transcription factors (TFs) is unique to the plant kingdom; it plays important roles in regulating embryo patterning, meristem formation, organ polarity, vascular development, and meristem function (Mcconnell et al. 2001; Du and Wang 2015; Bustamante et al. 2016; Shi et al. 2016). The HD-ZIP III family of Arabidopsis consists of five members, including REVOLUTA (REV), PHABULOSA (PHB), PHAVOLUTA (PHV), CORONA (CNA) and ATHB8 (Baima et al. 1995; Green et al. 2005). All of these HD-ZIP III proteins possess the HD-ZIP domain containing a homeodomain (a leucine zipper domain acting on DNA binding and protein dimerization), a steroidogenic acute regulatory protein lipid transfer domain (START), and a MEKHLA domain (Ponting and Aravind 1999; Mukherjee and Bürglin

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2006). Studies with loss-of-function alleles of the HD-ZIP III family members reveal that loss of *REV* gene can lead to apparent defects in apical and axillary meristem development, such as the lack of axillary meristematic tissue, reduced branches, and underdeveloped or even sterile flower structure (Talbert et al. 1995; Otsuga et al. 2001). The *rev/phb/phv* triple mutant shows enhanced defective phenotype, indicating the functional redundancy of *REV*, *PHB* and *PHV* in regulating SAM formation (Emery et al. 2003). *ATHB8* and *CAN* antagonize *REV* in certain tissues, but overlap with *REV* in other tissues (Prigge et al. 2005).

Cotton (*Gossypium hirsutum*) is an important economic crop with an indeterminate growth habit. In order to help balance its vegetative and reproductive growth, manual topping (removal of growth tips) of the main stem is often performed during cotton production in China. However, due to the decreasing labor force and higher labor cost in recent decades, there is a pressing need to develop more efficient techniques, such as biological topping, to replace manual topping. In this study, we cloned and identified four homologs of *AtREV* genes in cotton (*GhREV1*, *GhREV2*, *GhREV3*, and *GhREV4*), and found that *GhREV2* is a key regulator of the development of SAM. The results shed light on developing biological measures to control growth of the main stem of cotton.

Materials and methods
Plant materials and growth conditions
Gossypium hirsutum cv CCRI 41 and Xinshi 17 were used in this study to perform the *Agrobacterium*-mediated virus-induced gene silencing (VIGS) and quantitative real-time polymerase chain reaction (qRT-PCR) assays. Seeds were germinated in sand and transfered into a pot with 5 L Hoagland's solution (12 seedlings per pot) after 4 days. The experiment was carried out in a greenhouse at $24 \pm 2\,°C$ (day)/$20 \pm 2\,°C$ (night), 60% relative humidity, and $400\ \mu mol \cdot m^{-2} \cdot s^{-1}$ light with a 14 h (light)/10 h (dark) photoperiod. The nutrient solutions were changed every 4 days. *Arabidopsis* seedlings were grown in a chamber with $22\,°C$, 60% relative humidity, and $80\ \mu mol \cdot cm^{-2} \cdot s^{-1}$ light with a 14 h (light)/10 h (dark) photoperiod for protoplast transient assays.

Protein phylogenetic tree and sequence analysis
The Basic local alignment search tool (BLAST) in Cotton-Gen (http://www.cottongen.org) was used to search the HD-ZIP III homologs in cotton, the corresponding amino acid sequence was downloaded. The phylogenetic tree of HD-ZIP III homologs in cotton and *Arabidopsis* was built using the neighbor-joining method in MEGA5. Sequence comparative analysis was aligned using multiple sequence alignment (http://multalin.toulouse.inra.fr/multalin/).

Extraction of RNA and qRT-PCR
Cotton seedling samples were collected for tissue-specific expression of *GhREVs* at the sixth leaf stage. Shoot apex samples of VIGS-ed cotton were collected after VIGS-*GhCLA1* plants showing complete bleaching of the first and second true leaves. The samples were immediately frozen in liquid nitrogen and stored at $-80\,°C$. Total RNA was isolated from the samples using a Rapid Extraction Kit for plant RNA (Aidlab N09, Beijing, China), then reversely transcribed into cDNA. The expression of *GhREVs*, *GhWUS10A*, and *GhSTM* in the plants was detected by qRT-PCR. Primers used are listed in Additional file 1: Table S1.

Transcriptional activity assay
The effector and reporter constructs were used to detect transcriptional activity of GhREVs. The reporter includes four copies of GAL4 upstream activation sequence (UAS), a minimal 35S promoter (TATA box included), and a luciferase reporter gene. The effectors contained the GAL4 DNA-binding domain with *AtDB5* (negative control), or with *AtWRKY29* (positive control) or individual *GhREVs* under the control of the 35S promoter. *GhREV1*, *GhREV2*, *GhREV3*, and *GhREV4* were cloned into GAL4 vector via restriction enzyme cloning using *Nco*I and *Stu*I, respectively. UBQ10-GUS was added as an internal control for transfection efficiency. The activity of luciferase reporter was detected by an enzyme standard instrument (Power Wave XS2, BioTek, America) after 12 h incubation.

Subcellular localization
The subcellular localization of *GhREV2* protein was performed in *Arabidopsis* protoplasts. Full length cDNA of *GhREV2* was cloned via restriction enzymes using *Sma*I and *Kpn*I into the pSuper1300 vector to generate pSuper::*GhREV2*-GFP. The fused constructs were transformed or co-transformed into protoplasts for 12 h. The fluorescence was examined by a confocal microscopy (ZEISS710, Carl Zeiss, Germany).

Agrobacterium-mediated VIGS
A 330 bp cDNA fragment of *GhREV2* was amplified and cloned into pYL156 (pTRV:*RNA2*) vector. The primers are listed in Additional file 1: Table S1. Plasmids of binary TRV vectors pTRV:*RNA1* and pTRV:*RNA2* (Ctrl, *GhCLA1*, *GhREV2*) were transformed into *Agrobacterium tumefactions* strain GV3101 by electroporation. *Agrobacterium* strains were cultured for VIGS assays as previously described (Mu et al. 2019). The mixtures of *Agrobacterium* strains were infiltrated into two fully expanded cotyledons using a needle-less syringe (Li et al. 2015).

Data analysis

Data was pooled across independent repeats. The statistical analyses was performed using one-way analysis of variance (ANOVA), and treatment means were compared using Duncan's multiple range test at $P < 0.05$.

Results

Phylogenetic analysis of HD-ZIP III family

The HD-ZIP III family in *Arabidopsis* has been well characterized (Byrne 2006; Youn-Sung et al. 2008; Turchi et al. 2015). The full amino acid sequence of the HD-ZIP III family members in *Arabidopsis* was used as the query for a BLAST analysis against the *G. hirsutum* National Biological Information (NBI) protein database (https://www.cottongen.org/blast/). Phylogenetic analysis showed 18 putative HD-ZIP III members in cotton (Fig. 1a), including eight AtREV paralogs, four genes located in the D subgenome and other four genes in the A subgenome. They were named as *GhREV1A* and *GhREV1D* (*Gh_A05G0892* and *Gh_D05G0975*), *GhREV2A* and *GhREV2D* (*Gh_A03G0276* and *Gh_D03G1290*), *GhREV3A* and *GhREV3D* (*Gh_A08G1765* and *Gh_D08G2109*), and *GhREV4A* and *GhREV4D* (*Gh_A13G2011* and *Gh_D13G2409*) (Fig. 1b), respectively. These GhREVs

share 95%–99% similarity in their amino acid sequence. In addition, each GhREV shares more than 82% amino acid identity and 90% cDNA sequence similarity with *Arabidopsis* REV. Because of the high similarity of A subgenome and D subgenome (Fig. 1b), *GhREVsA* and *GhREVsD* cannot be distinguished by RT-PCR. Thus, we named *GhREVsA/D* as *GhREV1*, *GhREV2*, *GhREV3* and *GhREV4*, respectively, in the following work.

Spatial and temporal expression pattern of *GhREVs* genes

The expression levels of genes tend to be correlated with their biological functions. Total RNA of roots, stem, leaf, and shoot apex were extracted from cotton seedlings at the cotyledon stage and at the 2nd, 4th, 6th, and 8th leaf stages. Quantitative real-time PCR (qRT-PCR) was performed to determine the temporal and spatial transcription expression patterns of *GhREVs*. The results showed that *GhREV* genes were expressed in all of the tested tissues, higher in the stem and SAM (Fig. 2). *GhREV2* and *GhREV4* showed higher expression levels than *GhREV1* and *GhREV3* in roots, leaves, and the SAM, while the stem possessed more *GhREV3* transcript in addition to *GhREV2* and *GhREV4* (Fig. 2). Considering the temporal expressing pattern of *GhREVs* in roots (Fig. 2a), leaves (Fig. 2c), and shoot apex
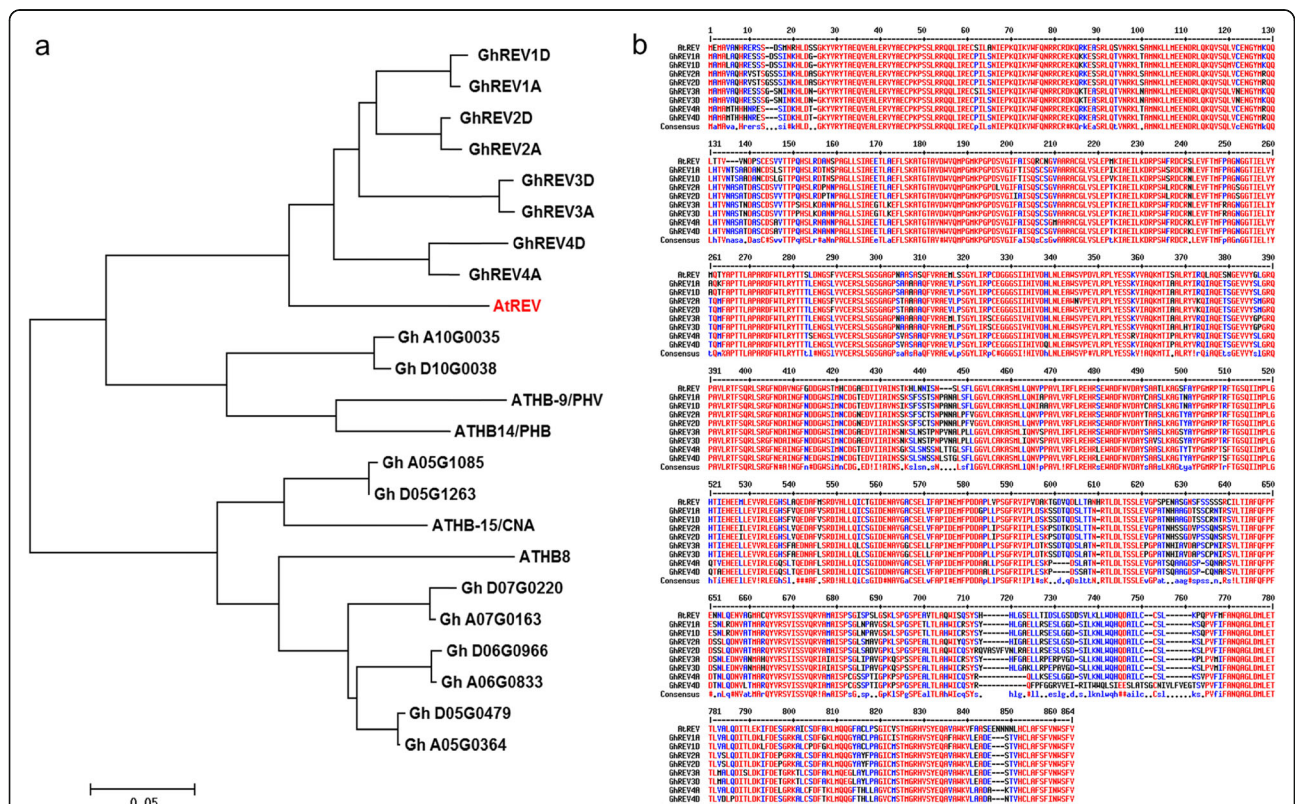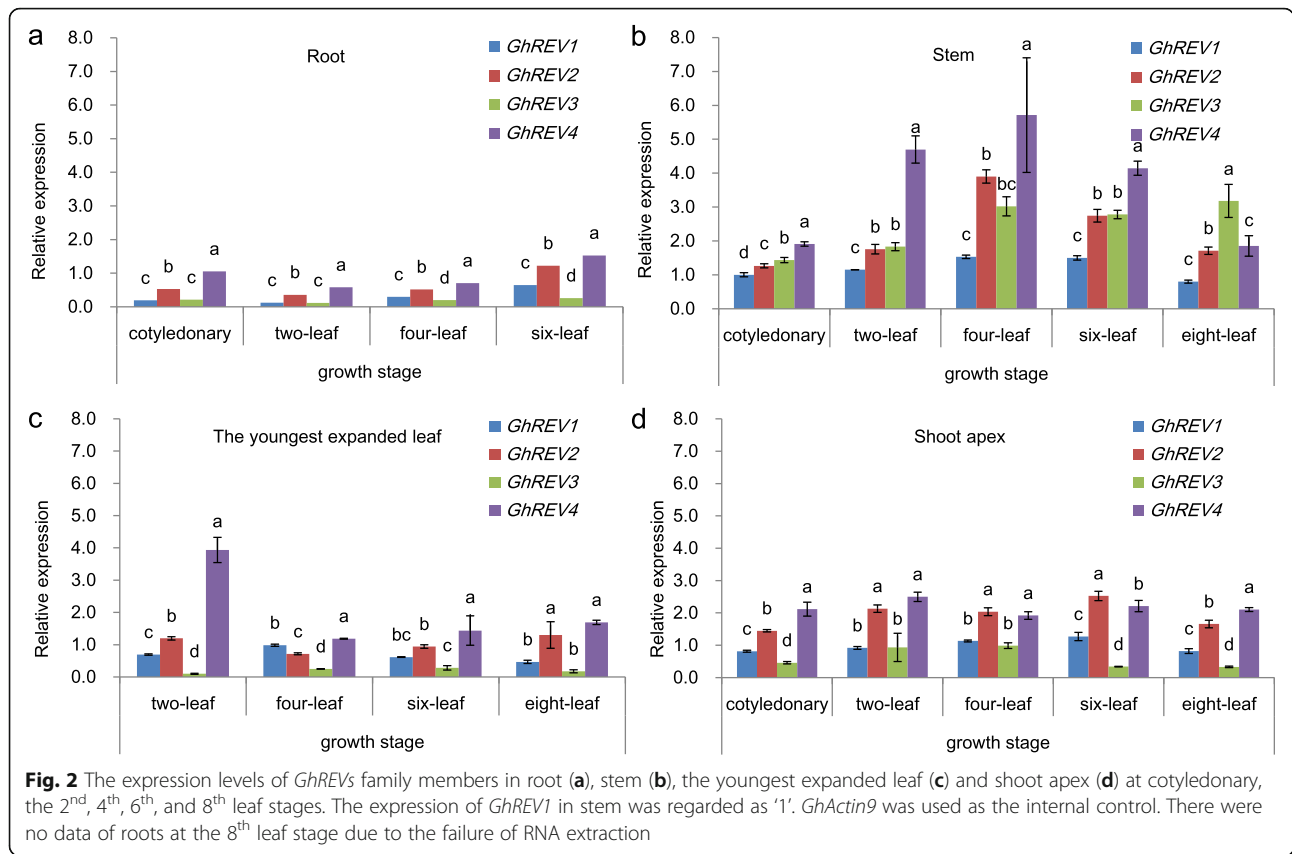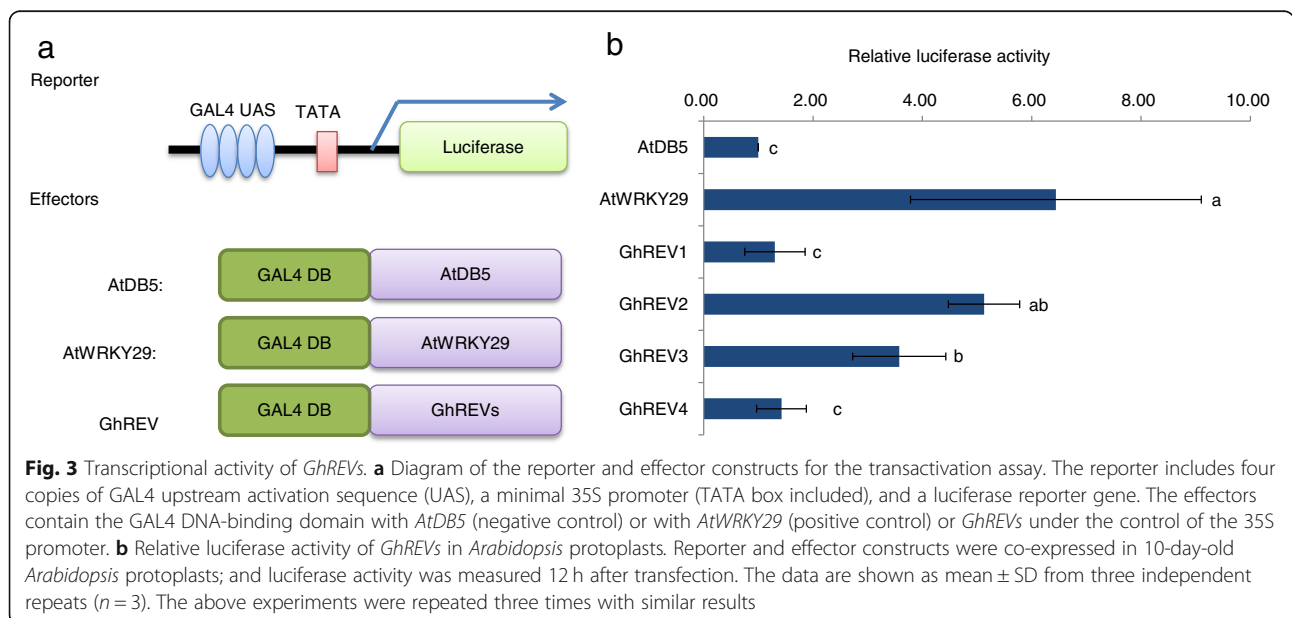

Fig. 1 Sequence analysis of *Gossypium hirsutum* REVOLUTA (GhREV). (a) Phylogenetic tree of HD-ZIP III family in both cotton and *Arabidopsis*. The tree was drawn to scale with branch lengths in the same unit. (b) The amino acid sequence alignment of REV in cotton and *Arabidopsis*

Fig. 2 The expression levels of *GhREVs* family members in root (**a**), stem (**b**), the youngest expanded leaf (**c**) and shoot apex (**d**) at cotyledonary, the 2nd, 4th, 6th, and 8th leaf stages. The expression of *GhREV1* in stem was regarded as '1'. *GhActin9* was used as the internal control. There were no data of roots at the 8th leaf stage due to the failure of RNA extraction
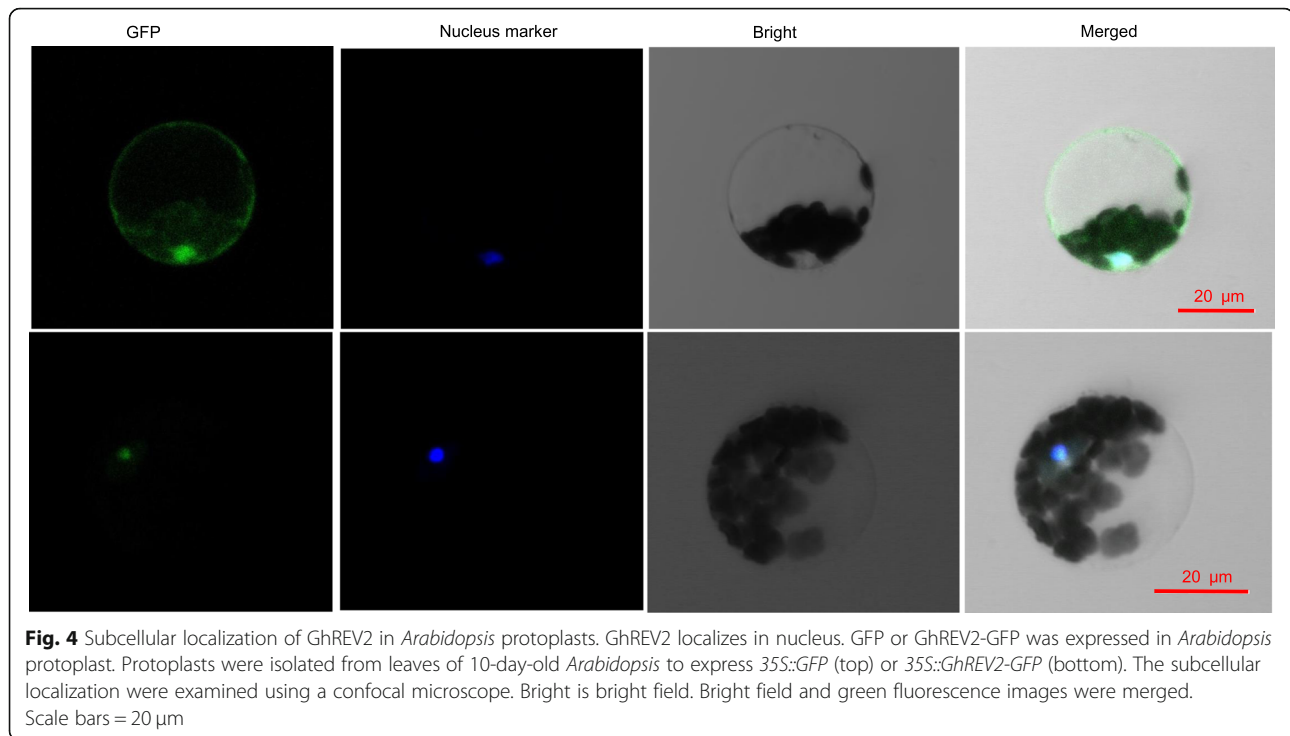
(Fig. 2d), there were no obvious and explicit differences from the cotyledon stage to the 6th or 8th leaf stage. For the stem, we observed that the expression level of *GhREV2* and *GhREV4* peaked at the 4th leaf stage, while *GhREV3* peaked at the 8th leaf stage (Fig. 2b).

GhREV2 and GhREV3 act as transcriptional activators
To determine whether GhREVs confer transcriptional activity, we carried out an *Arabidopsis* protoplast-based transactivation assay (Fig. 3a). Compared with the negative control, GhREV2 and GhREV3 significantly

Fig. 3 Transcriptional activity of *GhREVs*. **a** Diagram of the reporter and effector constructs for the transactivation assay. The reporter includes four copies of GAL4 upstream activation sequence (UAS), a minimal 35S promoter (TATA box included), and a luciferase reporter gene. The effectors contain the GAL4 DNA-binding domain with *AtDB5* (negative control) or with *AtWRKY29* (positive control) or *GhREVs* under the control of the 35S promoter. **b** Relative luciferase activity of *GhREVs* in *Arabidopsis* protoplasts. Reporter and effector constructs were co-expressed in 10-day-old *Arabidopsis* protoplasts; and luciferase activity was measured 12 h after transfection. The data are shown as mean ± SD from three independent repeats (*n* = 3). The above experiments were repeated three times with similar results

Fig. 4 Subcellular localization of GhREV2 in *Arabidopsis* protoplasts. GhREV2 localizes in nucleus. GFP or GhREV2-GFP was expressed in *Arabidopsis* protoplast. Protoplasts were isolated from leaves of 10-day-old *Arabidopsis* to express *35S::GFP* (top) or *35S::GhREV2-GFP* (bottom). The subcellular localization were examined using a confocal microscope. Bright is bright field. Bright field and green fluorescence images were merged. Scale bars = 20 μm

activated the luciferase reporter. The activation activity of GhREV2 was similar to that of AtWRKY29 (Asai et al. 2002; Li et al. 2017) (Fig. 3b).

Subcellular localization of GhREV2

To determine the subcellular localization, GhREV2 was fused with the C-terminus of green fluorescent protein (GFP) and transformed into *Arabidopsis* protoplasts. The empty GFP construct was driven by the cauliflower mosaic virus *35S* promoter and expressed in the cytoplasm, nucleus, and plasma membrane of the protoplasts. The fluorescence signals derived from the *GhREV2-GFP* construct were observed only in nucleus (Fig. 4).

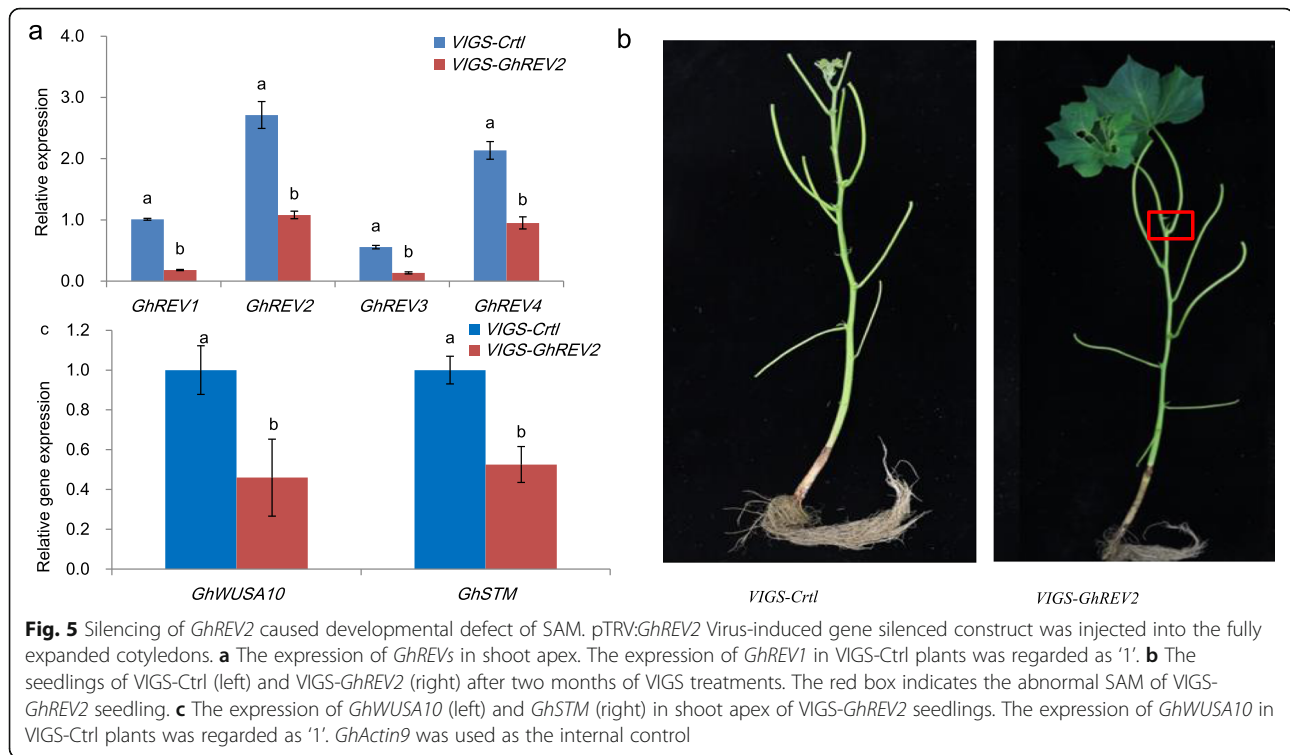### Silencing of *GhREV2* causes developmental defect in cotton SAM

To characterize the function of *GhREV2*, we silenced it in cotton seedlings via the Tobacco rattle virus (TRV)-based VIGS system. After the VIGS-*GLA1* plants showed albino phenotype, the relative expression levels were assessed using qRT-PCR. The data showed that not only *GhREV2* but also *GhREV1*, *GhREV3* and *GhREV4* were silenced compared with those in the control due to the high similarity of *GhREV* genes. The silencing efficiency of *GhREVs* all exceeded 55% (Fig. 5a).

After two months after the plants were treated with the VIGS system, the VIGS-*GhREV2* plants exhibited an abnormal SAM, but not the VIGS-Ctrl plants (Fig. 5b). For the possible mechanism of this abnormality, we determined the relative expression level of *WUSCHEL* (*GhWUSA10*) and *SHOOT MERISTEM-LESS* (*GhSTM*), two key genes involved in SAM development, found that the transcripts of both *GhWUSA10* and *GhSTM* decreased by 50% in VIGS-*GhREV2* plants (Fig. 5c).

Discussion

Similar to *AtREVs* in *Arabidopsis*, *GhREVs* are expressed in various tissues in cotton (Fig. 2). We speculate that *GhREVs* may also be involved in the formation of vascular bundles (Ramachandran et al. 2016), in the establishment of leaf polarity (Kim et al. 2010; Xie et al. 2014), and in the differentiation of the SAM (Lee and Clark 2015; Mandel et al. 2016). The expression of GhREV2 and GhREV4 in the SAM was significantly higher than GhREV1 and GhREV3 (Fig. 2), indicating that GhREV2 and GhREV4 may act mainly in the development of the shoot apex.

In addition, double luciferase reporter assays showed that only GhREV2 and GhREV3 possess transcriptional activity (Fig. 3). Based on the spatio-temporal expression patterns, GhREV3 may acts as a positive TF in the stem to regulate the development of vascular tissues, while GhREV2 may play a major role in regulating the SAM.

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Fig. 5 Silencing of *GhREV2* caused developmental defect of SAM. pTRV:*GhREV2* Virus-induced gene silenced construct was injected into the fully expanded cotyledons. **a** The expression of *GhREVs* in shoot apex. The expression of *GhREV1* in VIGS-Ctrl plants was regarded as '1'. **b** The seedlings of VIGS-Ctrl (left) and VIGS-*GhREV2* (right) after two months of VIGS treatments. The red box indicates the abnormal SAM of VIGS-*GhREV2* seedling. **c** The expression of *GhWUSA10* (left) and *GhSTM* (right) in shoot apex of VIGS-*GhREV2* seedlings. The expression of *GhWUSA10* in VIGS-Ctrl plants was regarded as '1'. *GhActin9* was used as the internal control

Although *GhREV4* showed higher expression levels than *GhREV1* and *GhREV3* in all tested tissues, it does not function as a transcriptional activator. Moreover, GhREV2 was located in the nucleus (Fig. 4), as expected with its TF function.

Owing to the high homology of *GhREVs*, the silencing of GhREV2 also reduced the expression level of other family members to a certain extent. However, the transcriptional activity assay showed that only *GhREV2* and *GhREV3* had transcriptional activity, and the expression of *GhREV3* was less in the SAM. Therefore, we speculated that *GhREV2* plays the major function in controlling the development of the SAM.

Extensive molecular genetic studies have identified key regulators and networks that operate in the SAM processes across species. It is known that the homeodomain of WUSCHEL (WUS) TF is essential for the maintenance of stem cells in plant SAM. *WUS* expresses in the OC (Mayer et al. 1998), and then enters the CZ and activates the transcription of CLAVATA3 (CLV3) (Yadaw et al. 2012; Daum et al. 2014). In turn, CLV3 can repress *WUS* expression. These events form a negative feedback loop that guarantees the dynamic size adjustment of the stem cell niches in the SAM (Clark 1997; Schoof et al. 2000; Lenhard and Laux 2003; Gaillochet and Lohmann 2015). In addition, the SHOOTMERISTEMLESS (STM)

is a member of the KNOX family and it prevents stem cell differentiation by inhibiting the expression of organ-forming factors ASYMMETRIC LEAVES1 (AS1) and AS2 in the CZ (Katayama et al. 2010). The mutation of STM can lead to premature termination of the stem and meristem. That is in parallel with WUS-CLV3 pathway (Clark et al. 1996; Endrizzi et al. 2010). Importantly, it has been reported that HD-ZIP III family, including REV and PHB, can strongly interact with B-type ARABIDOPSIS RESPONSE REGULATORs (ARRs) to activate *WUS* (Zhang et al. 2017). In this study, we found that the expression of *GhWUSA10* and *GhSTM* is explicitly suppressed in VIGS-*GhREV2* plants, indicating that GhREV2 may function together with GhWUSA10 and GhSTM to regulate the development of cotton SAM.

Conclusion
The results in this study indicate that GhREV2, a nuclear localized transcriptional activator, positively affects the development of cotton SAM, potentially by modulating the transcripts of *GhWUSA10* and *GhSTM*.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s42397-020-0045-y.

Additional file 1: Table S1. Primer sequences.

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Authors' contributions

Tian XL and Li ZH conceived and designed the study; An J and Yang DD carried out the experiments, analyzed and interpreted the data; Yang DD, Li FJ and Tian XL prepared the manuscript. Eneji AA revised the manuscript. All authors read and approved the final version of manuscript.

Availability of data and materials

No other data related to this study is available at this time.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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