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Molecular cloning and expression analysis of *GhLOF* genes in upland cotton (*Gossypium hirsutum* L.)

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ABSTRACT. Shoot branching, i.e., the timing and position of shoot growth, determines to a large extend the pattern of plant architecture, and is the result of the integration of a plant's genetic background and environmental cues. Many genes that are involved in the formation and outgrowth of axillary buds have been cloned, but the exact mechanism is still unclear. Branching pattern is an important agronomic trait in many crops, including cotton. In the present study, we cloned four genes from cotton, and designated them as *GhLOF1/2/3/4*. Sequence analysis revealed that all four genes shared conserved protein domains with *LATERAL ORGAN FUSION* (*LOF*) from *Arabidopsis* and *TRIFOLIATE* (*Tf*) from tomato. Phylogenetic analysis revealed that *GhLOF3* and *GhLOF4* were close to *Tf* because of their similar expression patterns, whereas *GhLOF1* and *GhLOF2* were differentially expressed.

Key words: *Gossypium hirsutum* L. (upland cotton); *GhLOF* genes; Shoot branching; RACE; Expression patterns

INTRODUCTION

The determination of plant architecture is a complex progress involving the integration of environmental and developmental cues. Two groups of mitotic cells, the root apical meristem at one end and the shoot apical meristem at the other, constitute the basic apical-basal axis of the plant during embryogenesis, and multiple new growth axes are formed from the secondary axillary meristems during post-embryogenesis, which determine a mature plant's architecture (McSteen and Leyser, 2005).

The pattern of shoot branching, i.e., spatial and temporal patterns of shoot initiation and the angle and orientation of shoot growth, determines to a large extent the aerial shape of the plant. Two developmental stages, the formation of axillary meristems and the subsequent axillary bud outgrowth, are involved in shoot development (Shimizu-Sato and Mori, 2001), and many researches have tried to elucidate the mechanisms of the two stages. It has been demonstrated that three phytohormones, auxin, cytokinin, and strigolactone, participate in the regulation of axillary bud outgrowth, while auxin and strigolactone act as inhibiters and cytokinin acts as a promoter (Cheng et al., 2013). The mechanism underlying axillary meristem initiation is unclear, despite the fact that a set of pathway transcription factors have been identified from branching-deficient mutants, including: 1) the NAC domain protein CUP-SHAPED COTYLEDON3 (CUC3) from *Arabidopsis* (Vroemen et al., 2003; Hibara et al., 2006); 2) the GRAS domain protein LATERAL SUPPRESSOR (LAS) from *Arabidopsis* (Greb et al., 2003), and its homolog Ls from tomato (Schumacher et al., 1999), as well as MONOCULM1 (MOC1) from rice (Li et al., 2003); 3) R2R3 MYBs LATERAL ORGAN FUSION1/2 (LOF1/2) (Lee et al., 2009) and REGULATOR OF AXILLARY MERISTEMS (RAX1/2/3) (Keller et al., 2006; Müller et al., 2006) from *Arabidopsis*, and their respective homologs TRIFOLIATE (Tf) (Naza et al., 2013) and BLIND (Bl) (Schmitz et al., 2002) from tomato; 4) the bHLH protein REGULATOR OF AXILLARY MERISTEM FORMATION (ROX) from *Arabidopsis* (Yang et al., 2012), and its orthologs LAX PANICLE1 (LAX1) from rice (Komatsu et al., 2001, 2003) and BARREN STALK1 (BA1) from maize (Gallavotti et al., 2004).

The modification of plant architecture constitutes a critical aspect in plant breeding. The most significant example is the Green Revolution (Peng et al., 1999), during which the introduction of new dwarf wheat and rice varieties into agriculture significantly enhanced grain productivity. A branching pattern is an important agronomic trait of crops, including cotton, as it is related to whether the crop is suitable for mechanized harvesting. In addition, during cotton plant development, pinching redundant vegetative shoots is laborious. However, little is known about the molecular basis of cotton branching, and few relevant genes have been cloned; therefore, it would be informative to understand the mechanism involved in order to modify plant architecture using biotechnology.

Two R2R3 MYB-like transcription factors, LOF1 and LOF2, have been identified in *Arabidopsis*, functioning in both lateral organ separation and axillary meristem formation. A homologous gene with similar functions (*Tf*) has been cloned from tomato, and *tf* mutants exhibit defects in their compound leaf development and shoot branching (Naza et al., 2013). In this study, we cloned four *LOF* homologous genes from upland cotton (*Gossypium hirsutum* L.), designated *GhLOF1* to *GhLOF4*, and compared their sequences with *LOF1*, *LOF2*, *Tf*, and other MYB-like transcription factor genes from cotton, and analyzed their expression patterns in different tissues.

MATERIAL AND METHODS

Plant material

Cotton seeds were germinated at 28°C in a growth chamber for about a week, and were then transplanted into soil and grown in a greenhouse at the Agriculture and Biology School of Shanghai Jiao Tong University, Shanghai, China. Apical tips, roots, stems, and leaves were harvested and stored at -80°C for RNA extraction.

Total RNA and DNA isolation and synthesis of the first-strand cDNA

Total RNA was extracted using a Quick RNA isolation Kit (Huayueyang, China) from different tissues of one cotton plant, following the manufacturer protocol. Agarose gel electrophoresis was performed to check the integrity of the RNA, while the concentration was obtained by spectrophotometer (DU-640, Beckman, USA) analysis. DNaseI (Takara, Japan) was added to remove genomic DNA. Genomic DNA was extracted using a QuickExtract™ Plant DNA Extraction Solution (Tiangen Biotech, China) and checked with agarose gel electrophoresis. First-strand cDNA was synthesized using a One-Step RNA PCR Kit (Takara). Two pairs of DP (degenerate primers; LOFDP-F1/2 and LOFDP-R1/2) (Table 1), were designed according to the conserved regions of the *LOF* genes and their homologous genes in other species, and a polymerase chain reaction (PCR) was conducted using apical tip cDNA as the template.

PCR amplification was performed using the following parameters: 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min for 35 cycles. The product was purified using a PCR Purification Kit (Tiangen Biotech) following the manufacturer protocol, and ligated into a pMD18-T vector (Takara) for sequencing.

Rapid amplification of cDNA ends (RACE)

Four different gene fragments that were homologous to *LOF* and related genes from other species were obtained, and were named *GhLOF-CS1*, *GhLOF-CS2*, *GhLOF-CS3*, and *GhLOF-CS4*. 3'- and 5'-RACE were carried out using a SMARTer™ RACE cDNA Amplification Kit (Clontech, USA), according to manufacturer instructions, to gain the full length of these four genes. For each fragment, a GSP (gene-specific primer; GhLOF-GSPF and GhLOF-GSPR) and an NP (nested primer; GhLOF-NPF and GhLOF-NPR) (Table 1) were designed to perform the PCR amplification using 3'/5'-RACE-Ready cDNA as a template, and the PCR procedure was: 95°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min for 35 cycles. The product was purified using a PCR Purification Kit (Tiangen Biotech) following the manufacturer protocol, and ligated into a pMD18-T vector (Takara) for sequencing.

Sequence analysis

The opening reading frame (ORF) was determined using the ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). A multiple-sequence alignment was performed using the DNAMAN software package. The MEGA software (version 4.1) was used to construct phylogenetic trees; the tree nodes were evaluated by bootstrap analysis with 1000 replicates.

Expression analysis by real-time PCR

Total RNA from the roots, leaves, stems, and apical tips of one cotton plant was separately extracted, examined, and treated, as described above. Real-time quantitative PCR analysis was performed with SYBR® Green (Takara) in an FTC-3000™ System Real-time Quantitative Thermal Cycler (Funglyn Biotech, Canada). The 25-μL reaction mixture contained 2.0 μL cDNA, 1.0 μL (10 μM) of each of the primers GhLOF-RTF and GhLOF-RTR (Table 1), 8.5 μL sterile water, and 12.5 μL SYBR® Green PCR mix. The reverse transcription PCR conditions were as follows: 95°C for 2 min, followed by 40 cycles at 95°C for 20 s, 60°C for 15 s, and 72°C for 20 s, with an extension time of 10 min at 72°C at the end of the last cycle. The *Ubiquitin* gene was amplified as the endogenous control, with the primers UBQ-F and UBQ-R (Table 1). The relative expression ratios of the target genes in different tissues were calculated based on the comparative $2^{-\Delta\Delta CT}$ method, and the experiments were conducted with three independent replicates.

Table 1. Polymerase chain reaction (PCR) primers used in this study.

Primer	Sequence (5'-3')	Utilization
LOFDP-F1	AGAGGNCATTGGACNCCNGCNGAAGAT	Degenerate primers for core sequence amplification
LOFDP-F2	CAAAANTGGAACCTNATAGCTGA	
LOFDP-R1	TGTTCTCTANANTTNCNNGCCAT	
LOFDP-R2	ACATGCCANTGGTTCTTNACTGNATT	
GhLOF1-GSPF	TCAGCTTGCAGACTTGTGTGTTT	Primers for 5'- and 3'-RACE
GhLOF1-NPF	ATAGAGCTTATGGAAACAAATGGGCAA	
GhLOF1-GSPR	GCCATTGGTTCTTAACTGGATTATCTG	
GhLOF1-NPR	ACATGCCATTGGTTCTTAACTGGA	
GhLOF2-GSPF	ATGGATGGCAGGGAAGAGTTGTAGA	
GhLOF2-NPF	AAGAATTAACAAGAGGAATTTTACGG	
GhLOF2-GSPR	CCTCGCCCATTTGTTACCATGC	
GhLOF2-NPR	AACTCTTCCCTGCCATCCATGCAA	
GhLOF3-GSPF	CCCCTACTGTCTCAATATTTCGGAT	
GhLOF3-NPF	TCAAATGTTTCACCCCACATGACCA	
GhLOF3-GSPR	GATAATGCCTAGTCGTCTATCCGAA	
GhLOF3-NPR	ATTGTTCTCTATACTTCCTAGCCAT	
GhLOF4-GSPF	TCAGGTGCTGTTGGTGGTA	
GhLOF4-NPF	TGATGAAGAAGAGGAGAGGCT	
GhLOF4-GSPR	TCCACTGTTCTCAGCAGGATACCTT	
GhLOF4-NPR	GGTCTTATTTGTGCCCACTGACTCG	
GhLOF1-F	ATGGAGGTCTTAAAACCC	Primers for amplification of open reading frames
GhLOF1-R	CTAAGCTCCTACTCCAAG	
GhLOF2-F	ATGGAACAAAGTAATAAA	
GhLOF2-R	TTAAGAAGCTCTTCCAAC	
GhLOF3-F	ATGAATAGGCTCAGCTGT	
GhLOF3-R	TCATGTGGCTCCTACTCC	
GhLOF4-F	ATGACCATGATTACGAAT	
GhLOF4-R	TCAAGAAGCTCCAACTCC	
GhLOF1-RTF	TCAAGGAAGAACAGGTAAAAGCTG	Primers for reverse transcription PCR
GhLOF1-RTR	AGCCTGTTCTCTATATTTCCTCGAC	
GhLOF2-RTF	TTGAAGGAAGATCAGGGAAGAGTT	
GhLOF2-RTR	TCGTCAAACCCAAGCCTTTAGGGA	
GhLOF3-RTF	GGAAGGAAGATCAGGTAAGACCTG	
GhLOF3-RTR	TGTTCGGAGTTTCTTCCATC	
GhLOF4-RTF	GTTCTTCAAATGGGTCCACCACGTA	
GhLOF4-RTR	AGAAAATCAATGAAGCGCATGTTGT	
UBQ-F	GCCGACTACAACATCCAGAAGG	Endogenous control primers
UBQ-R	AGACGCAGGACCAGATGAAGAG	

RESULTS

Isolation of four *LOF* homologs from *G. hirsutum* L.

Four *LOF* homologous genes were isolated from upland cotton and designated as *GhLOF1/2/3/4*. Sequence alignments of coding sequence (CDS) with genome DNAs showed that these four genes were structurally similar to each other, and all of them contained two introns (Table 2).

Table 2. Structures of the four genes.

	CDS length (bp)	gDNA length (bp)	Amino acid length	Intron 1		Intron 2	
				Length (bp)	Position* (bp)	Length (bp)	Position* (bp)
GhLOF1	969	1154	323	82	260	103	697
GhLOF2	942	1194	314	125	308	127	701
GhLOF3	1299	1763	433	158	465	306	994
GhLOF4	1152	1538	384	254	461	132	898

*Nucleotide position in coding sequence (CDS).

Multiple-sequence alignment and phylogenetic analysis

Nucleotide sequence analysis revealed that *GhLOF1* was 52.59, 50.05, and 57.90% identical to *LOF1*, *LOF2* (*Arabidopsis*), and *Tf* (tomato), respectively, while *GhLOF2* was 50.32, 48.19, and 51.71% identical, *GhLOF3* was 51.28, 48.63, and 58.19% identical, and *GhLOF4* was 51.31, 46.58, and 52.65% identical, respectively. The multiple-amino acid sequence alignment revealed that all four deduced GhLOF proteins shared the conserved domain with LOF1, LOF2, and Tf (Figure 1). Five other MYB-like transcription factor genes, homologous to *RAX1/2/3* (*Arabidopsis*) and *Bl* (tomato), were cloned in our laboratory and designated as *GhRAX1/2/3/4/5* (data not shown). To analyze the phylogenetic relationships among the *GhLOF*s and other MYB-like transcription factor genes from *G. hirsutum* L. and other species, phylogenetic trees were constructed that included the protein sequences of GhLOFs, GhRAXs, two RAX/BLIND homologs from *G. hirsutum* L. that were deposited in the National Center for Biotechnology Information (NCBI) (GhBLIND1 and GhBLIND2), LOF1 and LOF2 from *Arabidopsis*, and Tf from tomato, based on the neighbor-joining method. As shown in Figure 2a, the RAX/BLIND homologs and the LOF homologs in *G. hirsutum* L. were divided into different subgroups, suggesting that functional divergence had occurred. Relationship analysis among the GhLOFs, LOF1/2, and Tf revealed that LOF1/2 from *Arabidopsis* fell into a different subgroup from the GhLOFs and Tf, while GhLOF3 and GhLOF4 were close to Tf (Figure 2b).

Expression patterns of *GhLOF*s in *G. hirsutum* L.

Real-time PCR was employed to quantify the transcription levels of the four *GhLOF* genes in different tissues of upland cotton, including the roots, stems, leaves, and apical tips. As shown in Figure 3, the highest transcription level of *GhLOF1* was detected in the stem, *GhLOF2* was highly expressed in the stem and in the leaves, and the transcripts of *GhLOF3* and *GhLOF4* had mainly accumulated in the apical tips.

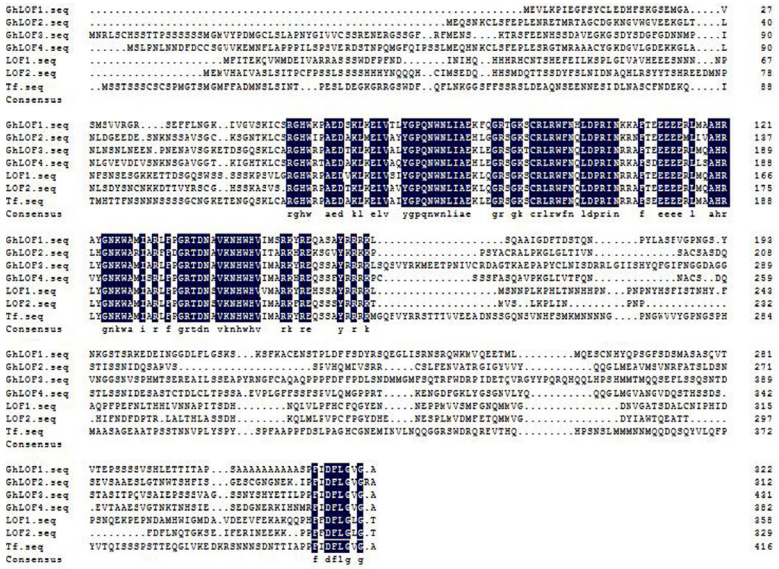

Figure 1. Multiple-amino acid sequence alignment of GhLOFs with LOF1/2 from *Arabidopsis* and Tf from tomato. GenBank registration numbers: LOF1, AEE30736.1; LOF2, AEE34949.1; and Tf, AGI17582.1.

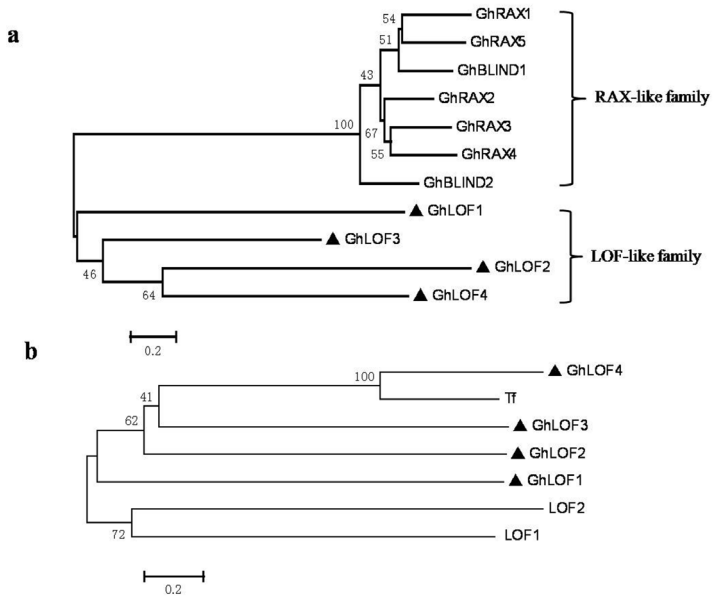

Figure 2. Phylogenetic relationships between GhLOFs and other MYB-like transcription factors from *Gossypium hirsutum* L. and other species. **a.** Phylogenetic relationships of LOF-like proteins and RAX-like proteins in *G. hirsutum* L. **b.** Phylogenetic relationships between GhLOFs and their homologs from *Arabidopsis* and tomato. Amino acid sequences used for the alignment included LOF1 (AEE30736.1) and LOF2 (AEE34949.1) from *Arabidopsis*; GhRAX1/2/3/4/5, GhBLIND1 (ADT80570.1), and GhBLIND2 (ADT80571.1) from *G. hirsutum* L., and Tf (AGI17582.1) from *Solanum lycopersicum*.

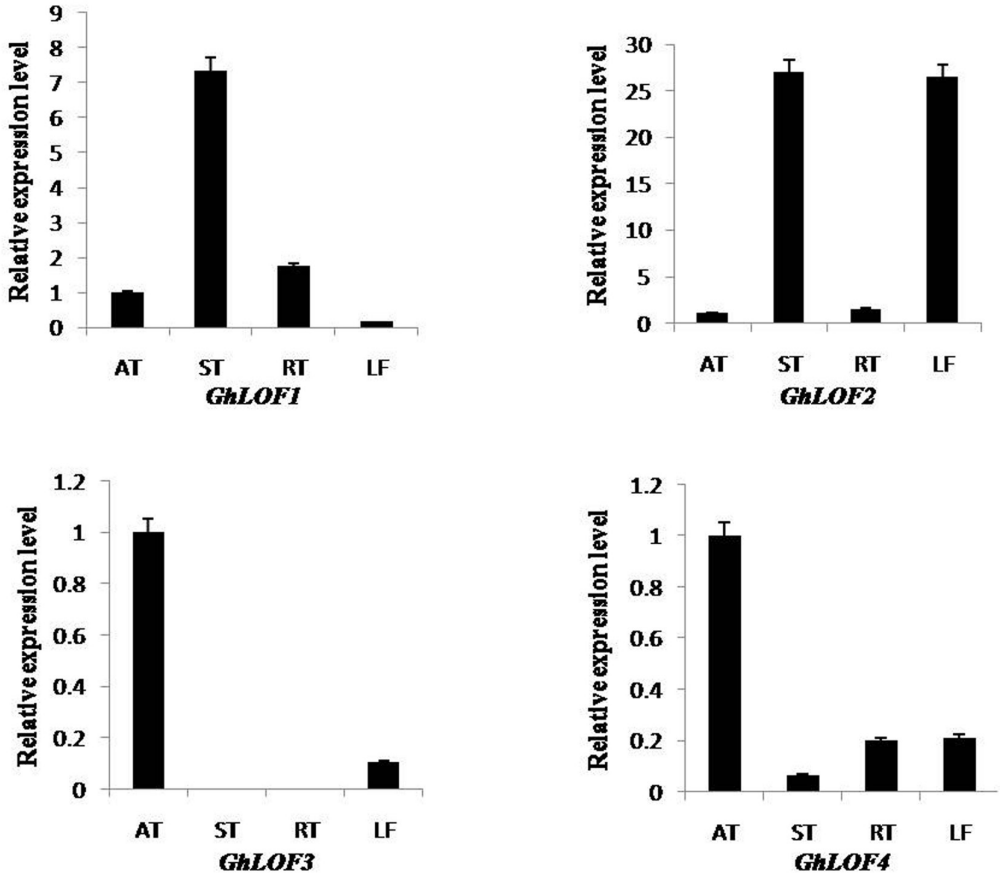

Figure 3. Expression analysis of *GhLOF*s in the apical tips (AT), stems (ST), roots (RT), and leaves (LF) of *Gossypium hirsutum* L. The data are reported as means (N = 3) and standard deviation, represented by error bars.

DISCUSSION

The elucidation of the mechanism of branch development is an important and intriguing aspect of plant science. The formation of axillary meristems and the subsequent axillary bud outgrowth are two key stages during shoot development. It has been known for decades that auxin and cytokinin influence the activity of axillary buds, while auxin inhibits the outgrowth of the buds and cytokinin works antagonistically (Müller and Leyser, 2011). In 2008, a novel phytohormone, strigolactone, was confirmed as the third endogenous regulator of shoot branching that functionally represses bud outgrowth, and that interacts with auxin and cytokinin (Gomez-Roldan et al., 2008; Umehara et al., 2008). Some models have been proposed that highlight the interaction of these phytohormones, and their roles in integrating endogenous and exogenous cues in the regulation of bud outgrowth, but the exact mechanism is still under debate. The molecular basis underlying the formation of axillary buds is even more unclear,

although many relevant genes have been cloned from *Arabidopsis* and other species.

In this study, we cloned four genes from upland cotton (*G. hirsutum* L.) that were homologous to the *LOF*s in *Arabidopsis* and *Tf* in tomato. The ORFs of these four *GhLOF*s were 969, 942, 1299, and 1152 bp in length, and were predicted to encode putative peptides of 323, 314, 433, and 384 amino acids, respectively. Analysis of the genomic DNA showed that all four genes had two introns, structurally similar to the *LOF*s and *Tf*. Multiple-amino acid sequence alignments revealed that all of these putative GhLOF proteins were highly homologous to the LOFs and Tf within the conserved domains. *RAX*s, classified as another gene family in *Arabidopsis*, are also engaged in the formation of axillary meristems, and expression analysis of *RAX1* in a *lof1* mutant has shown that *RAX1* works downstream of *LOF1* (Lee et al., 2009). Five *GhRAX*s were cloned in our laboratory, and a multiple-amino acid sequence alignment showed that they were homologous to the *RAX*s in *Arabidopsis*. To analyze the relationship between these two families and *GhLOF*s in cotton with their homologs in other species, phylogenetic trees were constructed. In Figure 2, *GhLOF*s and *GhRAX*s were categorized into two groups, which suggests that a functional divergence occurred between these two families. The subsequent analysis of *GhLOF*s with their homologs from *Arabidopsis* and tomato showed that *LOF1* and *LOF2* fell into a different subgroup to *GhLOF*s and *Tf*. This may have resulted from the species gap, because *GhLOF3* and *GhLOF4* are close to *Tf*, which was validated by expression analysis. The expression patterns of *GhLOF3* and *GhLOF4* were similar to that of *Tf*, which also predominantly accumulated in the shoot apical, whereas *GhLOF1* and *GhLOF2* were different, suggesting that they function in different developmental processes.

Cotton is one of the most important global commercial crops, and is a primary source of natural fiber. It is planted in more than 80 countries by about 20 million farmers, and occupies approximately 5% of the world's arable land (Sunilkumar et al., 2006; Wang et al., 2012). Branching pattern is an important agronomic trait of cotton, and understanding the molecular basis of cotton branching will help develop compact cotton varieties that are suitable for mechanized harvesting, through the modulation of the plants' architecture. Four *GhLOF* genes were cloned in this study, and further complementary experiments with *Arabidopsis* mutants should be conducted to clarify the exact functions of these four genes, and manipulations of the gene sequences or transcription levels such as RNAi, TALEN, or CRISPR, could be conducted, in order for cotton to adapt to agricultural mechanization.

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