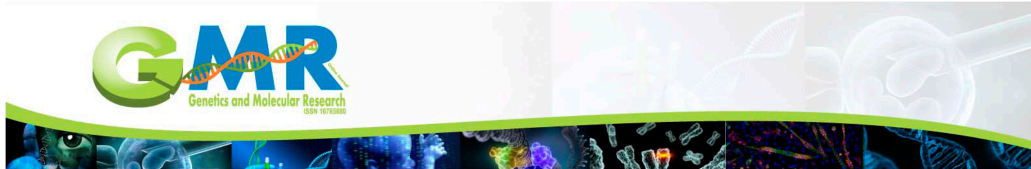

Molecular cloning and expression analysis of five *GhRAX*s in upland cotton (*Gossypium hirsutum* L.)

T.C. Dai[1,2] and Z.M. Wang[1,2]

[1]Plant Science Department, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai, China
[2]Key Laboratory of Urban Agriculture (South), Ministry of Agriculture, Shanghai, China

Corresponding author: Z.M. Wang
E-mail: zmwang@sjtu.edu.cn

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ABSTRACT. The formation of axillary meristems in leaf axils is a prerequisite for the development of lateral shoots, which largely contribute to plant architecture. Several transcription factor-encoding genes, including *CUC3*, *RAX*, *LAS*, *LOF1*, and *ROX*, have been cloned by screening for axillary meristem mutants in *Arabidopsis thaliana*. These genes will facilitate our understanding of the mechanisms underlying axillary meristem development. In this study, we report the cloning of five genes from cotton (*Gossypium hirsutum* L.) that are orthologous to *A. thaliana REGULATORS OFAXILLARY MERISTEMS* (*RAX*) and tomato *Blind* (*Bl*), and they are designated *GhRAX1*, *2*, *3*, *4*, and *5*. Sequence analyses indicated that all five genes shared conserved protein domains with *RAX* and *Bl*. Phylogenetic analyses of protein sequences revealed that GhRAX2/3/4 were close to RAX1, whereas GhRAX1 and GhRAX5 were close to RAX3. Expression patterns of these genes in different tissues were also analyzed using real-time PCR.

Keywords: *Gossypium hirsutum* L.; *GhRAX*s; Shoot branching; RACE; Expression patterns

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INTRODUCTION

Shoot branching (i.e., timing, position, and extent of lateral shoot growth) largely determines plant architecture, and it is the result of the integration of plant genetic background, developmental stage, and environmental cues. The axillary meristem (AM), which maintains a group of pluripotent stem cells, is the starting point of shoot branching. During post-embryonic development, AMs located in the axils of leaf primordia form axillary buds, some of which remain active, while others go dormant. However, the dormant buds retain the capacity to grow out. The active buds give rise to phytomers comprising a stem segment, a node bearing one or more leaves or leaf-like structures, and one or more remaining AMs in each leaf axil (Sussex, 1989). The above process is repeated by new AMs.

Phytohormones regulate bud outgrowth during development. At the beginning of the last century, researchers discovered that the removal of the shoot apex (decapitation) could activate lateral buds and promote branching (Snow, 1929), a phenomenon known as "apical dominance" (Cline, 1997). It was later determined that auxin is the substance in the shoot apex that inhibits bud outgrowth. Cytokinin was also identified as a regulator of bud develop that functions contrarily to auxin (Sachs and Thimann, 1967). A third phytohormone, strigolactone, which functions as an inhibiter of bud activity, was subsequently identified (Gomez-Roldan et al., 2008; Umehara et al., 2008) due to unremitting research on mutants with enhanced branching in *max* (*Arabidopsis thaliana*), *rms* (pea), *d* or *hdt* (rice), and *dad* (petunia) (Kebrom et al., 2013; Janssen et al., 2014; Waldie et al., 2014).

In addition to bushy mutants with genes involved in strigolactone synthesis and perception, other mutants with barren branching were also identified based on lateral meristem absence. Moreover, some of these genes have been cloned in *A. thaliana* [e.g., *LAS* (Greb et al., 2003), *RAX1/2/3* (Keller et al., 2006; Muller et al., 2006), *LOF1/2* (Lee et al., 2009), and *ROX* (Yang et al., 2012)], tomato [e.g., *Ls* (Schumacher et al., 1999), *Blind* (Schmitz et al., 2002), and *Tf* (Naza et al., 2013)], rice [e.g., *MOC1* (Li et al., 2003) and *LAX1/2* (Komatsu et al., 2001, 2003; Tabuchi et al., 2011)], and maize [e.g., *BA1* (Gallavotti et al., 2004)]. Regarding transcription factors, *LAS/Ls/MOC1* are orthologs that encode GRAS family transcription factors, *Tf/LOF1/2* and *Blind/RAX1/2/3* are orthologs encoding MYB transcription factors, and *ROX/BA1/LAX1* encode orthologous bHLH transcription factor proteins.

Cotton (*Gossypium* spp) is one of the most important commercial crops and primary sources of natural fiber for the textile industry (Sunilkumar et al., 2006; Wang et al., 2012). The modification of plant architecture is an important part of cotton breeding, and the pinching of redundant vegetative shoots is a laborious method, which is now a limitation of cotton cultivation. Although the aforementioned genes have been cloned and simple models have been suggested (Raman et al., 2008; Yang et al., 2012), little is known about the molecular basis of cotton branching.

Previous studies demonstrated that three orthologs, *A. thaliana RAX1/2/3*, were involved in axillary meristem formation during vegetative and reproductive stages (Muller et al., 2006). *RAX1* is the most predominant, and its mutant with defective branching patterns is orthologous to *blind* in the tomato (Schmitz et al., 2002). However, single *rax2* and *rax3* mutants were undistinguishable from the wild type.

In this study, we cloned five *RAX* orthologous genes from upland cotton (*Gossypium hirsutum* L.) designated *GhRAX1* to *GhRAX5*, respectively. We then compared their sequences to *RAX1/2/3*, *Bl,* and other MYB-like transcription factor genes, and subsequently analyzed their expression patterns in different tissues.

MATERIAL AND METHODS

Plant material

Preparation of plant material has been previously described (Dai and Wang, 2015). Briefly, cotton seeds were germinated, transplanted into soil, and grown in the greenhouse at Shanghai Jiao Tong University. Samples of apical tips, roots, stems, and leaves were collected and stored at -80°C for RNA extraction.

Extraction of total RNA and DNA

Total RNA was extracted from various tissues using a Quick RNA isolation kit (Huayuyang, China) according to manufacturer instructions. Agarose gel electrophoresis and spectrophotometer (DU-640, Beckman, USA) analyses were conducted to check RNA integrity and concentration followed by DNaseI (Takara, Japan) treatment to remove genomic DNA. The DNA Quick Plant System (Tiangen Biotech, China) was used to extract genomic DNA, and DNA quality was also examined using the above methods.

Synthesis of the first cDNA strand

The first cDNA strand was synthesized using the one-step RNA PCR kit (Takara). PCR was conducted to gain the homologous segments of *RAX*s in cotton using two pairs of degenerate primers, RAXDP-F1/2 and RAXDP-R1/2 (Table 1), which were designed according to the conserved regions of *RAX*s and their homologs in other species (Schmitz et al., 2002; Keller et al., 2006; Muller et al., 2006). PCR parameters were as follows: 35 cycles of 95ºC for 30 s, 55ºC for 30 s, and 72ºC for 1 min. Product examination, purification, and ligation into the pMD18-T vector were conducted as previously described (Dai and Wang, 2015).

Rapid amplification of cDNA ends (RACE)

Sixty clones of proper length were sequenced, among which five different gene fragments were obtained and named *GhRAX-CS1*, *GhRAX-CS2*, *GhRAX-CS3*, *GhRAX-CS4,* and *GhRAX-CS5*. Sequenced amplified fragments were used as templates for 3′ and 5′ RACE to gain the full length of these genes using a SMARTer™ RACE cDNA Amplification Kit (Clontech, USA) according to manufacturer instructions. For 3′ RACE, a gene-specific primer GhRAX-GSPF and a nested primer GhRAX-NPF (Table 1) were designed to perform PCR amplifications using 3′ RACE-Ready cDNA as a template, whereas 5′ RACE was conducted with GhRAX-GSPR and GhRAX-NPR (Table 1). The PCR parameters were as follows: 35 cycles of 95ºC for 30 s, 55ºC for 30 s, and 72ºC for 1.5 min. The products were examined, purified, and ligated into vectors as previously described (Dai and Wang, 2015).

Sequence analysis

After the RACE experiments, the ORFs of the amplified genes were determined using the ORF Finder of NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Multiple sequence alignment was performed to analyze the homology of the genes with *RAX*s and other *RAX*-like genes using

the DNAman software package. Phylogenetic trees were constructed using the MEGA software version 4.1, and the tree nodes were evaluated by bootstrap analysis with 1000 replicates.

Table 1. PCR primers used in this study.

Primers	Sequences (5'-3')	Utilization
RAXDP-F1	ATGGGWAGRGCWCCRTGTTG	Degenerate primers for core sequence amplification
RAXDP-F2	AGGKCCWTGGTCKCCWGA	
RAXDP-R1	AWGAGYTTYTKAGCYTNGTGT	
RAXDP-R2	GACCACCTGCTWCCWAT	
GhRAX1-GSPF	GTGAAGAAAGGTCCATGG	Primers for 5' and 3' RACE
GhRAX1-NPF	GCCTCAGAAGATAGGGCT	
GhRAX1-GSPR	CTGCAATTATAGACCATCTG	
GhRAX1-NPR	TTTTAAGCCTCGTGTTCCAG	
GhRAX2-GSPF	GTGAAGAAAGGGCCATG	
GhRAX2-NPF	CCCTCAAAAAGCTGGTCTG	
GhRAX2-GSPR	CGTCATCAGTGAATTCACCA	
GhRAX2-NPR	CAGCTATTATTGACCATCTG	
GhRAX3-GSPF	AAGGGCCATGGTCCACTGA	
GhRAX3-NPF	AAGCTGGTCTGAAAAGATGTGG	
GhRAX3-GSPR	TATCTTCCTCGTCAGTGAAT	
GhRAX3-NPR	ACTGAGCAGCTATTATTGAC	
GhRAX4-GSPF	TGTGAAGAAAGGACCTTGG	
GhRAX4-NPF	CCCTCACAAAGCTGGTCTGAAAAG	
GhRAX4-GSPR	CACCATGCTTAATGTTGGGC	
GhRAX4-NPR	ATCTGCTCCCAATGGTAGCA	
GhRAX5-GSPF	GTGAAAAAAGGTCCATGGTCACC	
GhRAX5-NPF	GCCTCAGAAAATTGGTCTC	
GhRAX5-GSPR	GCAAAGAGATCCAGTTTCCA	
GhRAX5-NPR	GATAGTTCAACCATCTTAAA	
GhRAX1-F	ATGGGGAGAGCTCCTTGCTGTGAC	Primers for amplification of ORFs
GhRAX1-R	TTACACCGCCCATGTACAGT	
GhRAX2-F	ATGGGGAGAGCTCCTTGTTGT	
GhRAX2-R	CTAGTAGTAATAGCAGTACA	
GhRAX3-F	ATGGGGAGAGCTCCTTGCTGCGAC	
GhRAX3-R	TCAGTAGTAGAAGTACATGA	
GhRAX4-F	ATGGGGAGGGCTCCTTGCTGT	
GhRAX4-R	TCAGTAGTAATAATACATGA	
GhRAX5-F	ATGGGTAGAGCTCCTTGCTGTG	
GhRAX5-R	TTACTGCAAGCTGATACGAT	
GhRAX1-RTF	CTGGAATGGTGAGCCAACCC	Primers for RT-PCR
GhRAX1-RTR	CCGTGTTGTTCATGGAATTC	
GhRAX2-RTF	CATCACTATACGAAGACAG	
GhRAX2-RTR	CAAAACCCTGAAAACCCAT	
GhRAX3-RTF	CATGAGCATGACTTCTCAA	
GhRAX3-RTR	TTCACCGGATAATACT	
GhRAX4-RTF	CGTATTATTATACTCCAA	
GhRAX4-RTR	CACAAAAGCCCTGAAAATT	
GhRAX5-RTF	TGGTCTATTATTGCTGC	
GhRAX5-RTR	ATATAACAATCCCATT	
UBQ-F	GCCGACTACAACATCCAGAAGG	Endogenous control primers
UBQ-R	AGACGCAGGACCAGATGAAGAG	

Expression analysis by real-time PCR

RNA from roots, leaves, stems, and apical tips of one cotton plant was evaluated by real-time quantitative PCR using SYBR-Green (Takara) in a FTC-3000TM System Real-time Quantitative Thermal Cycler (Funglyn Biotech, Canada) for each gene, and the *Ubiquitin* gene was used as the endogenous control. The 25-μL reaction mixture contained 2.0 μL cDNA, 1.0 μL 10 μM of each primer (GhRAX-RTF and GhRAX-RTR; Table 1), 8.5 μL sterile water, and 12.5 μL

SYBR-Green PCR mix. The RT-PCR parameters were as follows: 95ºC for 2 min followed by 40 cycles of 95ºC for 20 s, 60ºC for 15 s, and 72ºC for 20 s, with an extension time of 10 min at 72ºC at the end of the last cycle. The relative expression ratios were calculated for each gene in different tissues based on the comparative $2^{-\Delta\Delta CT}$ method, and the experiments were conducted with three independent replicates.

RESULTS

Isolation of five *RAX* Orthologs from *G. hirsutum* L.

Five *RAX* orthologs (*GhRAX1/2/3/4/5*) in upland cotton were isolated, and the sequences were submitted to GenBank. Although their lengths differed, their structures were similar, and all contained two introns located at almost the same position in the CDS (Table 2).

Table 2. Structures of the five *GhRAX*s.

	CDS length (bp)	gDNA length (bp)	Amino acid length	Intron1		Intron2	
				Length (bp)	Position* (bp)	Length (bp)	Position* (bp)
GhRAX1	1083	1267	361	96	134	88	262
GhRAX2	903	1139	301	79	135	157	266
GhRAX3	969	1447	323	96	135	382	262
GhRAX4	948	1290	316	92	135	250	262
GhRAX5	624	784	208	85	135	75	263

*Nucleotide position in CDS.

Multiple sequence alignment and phylogenetic analysis

Nucleotide sequence alignments of *GhRAX*s with *RAX*s (*A. thaliana*) and *Bl* (tomato) were listed in Table 3. Multiple amino acid sequence alignments of GhRAXs with RAXs and BLIND showed that all five of the deduced proteins shared conserved domains with RAXs and BLIND (Figure 1). *LOF1*, *LOF2* (*A. thaliana*), and *Tf* (tomato) were also orthologous MYB-like transcription factor genes whose mutants also showed axillary meristem defects. Moreover, of these four orthologous genes in cotton were cloned in our laboratory and designated *GhLOF1/2/3/4* (Dai and Wang, 2015).

Table 3. Nucleotide sequence identity of five *GhRAX*s with *RAX1/2/3* and *Bl* (%).

	RAX1	*RAX2*	*RAX3*	*Bl*
GhRAX1	50.15	51.62	54.10	50.98
GhRAX2	52.87	62.25	54.25	59.84
GhRAX3	53.65	58.33	51.42	54.50
GhRAX4	57.17	59.22	50.49	56.39
GhRAX5	56.09	60.42	61.70	55.61

To analyze the phylogenetic relationships of these MYB-like transcription factor genes, phylogenetic trees were constructed using GhLOFs, GhRAXs, two RAX/BLIND orthologs from *G. hirsutum* L. deposited in NCBI (GhBlind1 and GhBlind2), RAXs from *A. thaliana*, BLIND from tomato, and CaBLIND from pepper. The results showed that RAX/BLIND orthologs and LOF orthologs in *G. hirsutum* L. were separated into different subgroups, suggesting functional divergence (Figure 2A).

The phylogenetic analysis of RAX-like proteins indicated that GhRAX2/3/4 and RAX1/2 belonged to the same subgroup, while GhRAX1 and GhRAX5 were closely related to RAX3 from *A. thaliana* (Figure 2B).

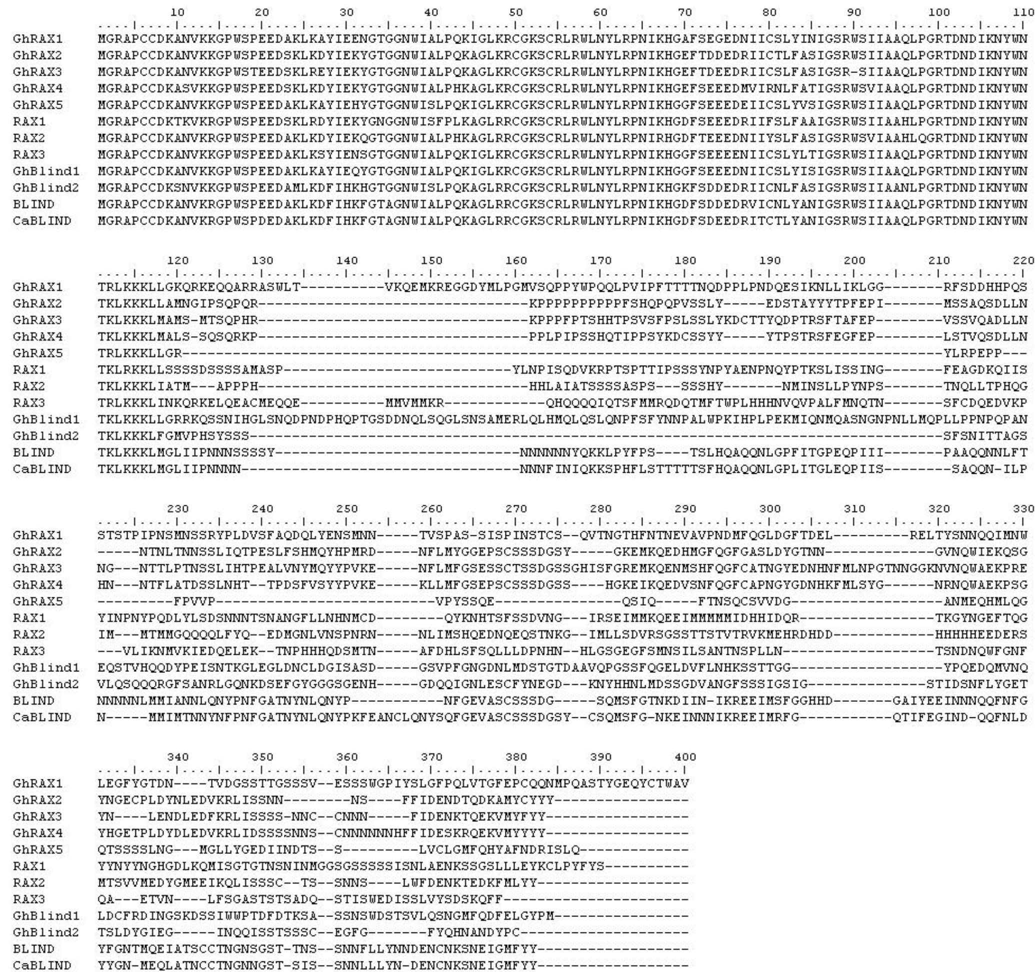

Figure 1. Multiple amino acid sequence alignment of GhRAXs with RAX1/2/3 from *Arabidopsis thaliana*, BLIND from tomato, CaBLIND from pepper, and GhBlind1/2 from cotton. GenBank accession Nos.: RAX1 (AED93106.1), RAX2 (AEC09315.1), RAX3 (AEE78577.1), BLIND (AAL69334.1), CaBLIND (JF496586.1), GhBlind1 (ADT80570.1), and GhBlind2 (ADT80571.1).

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

Real-time PCR was employed to quantify transcription patterns of *GhRAX*s in roots, stems, leaves, and apical tips. As shown in Figure 3, all of the *GhRAX*s were highly expressed in the stems and roots, but *GhRAX1/3/5* had higher levels in roots. The expression patterns of these genes

corresponded with those of *RAX1/2/3* in *A. thaliana,* which accumulated most in roots with much lower expression levels in apical tips (Muller et al., 2006). It remains unclear why these genes predominantly accumulated in roots, because none of the mutants showed distinct morphological root defects in *A. thaliana*.

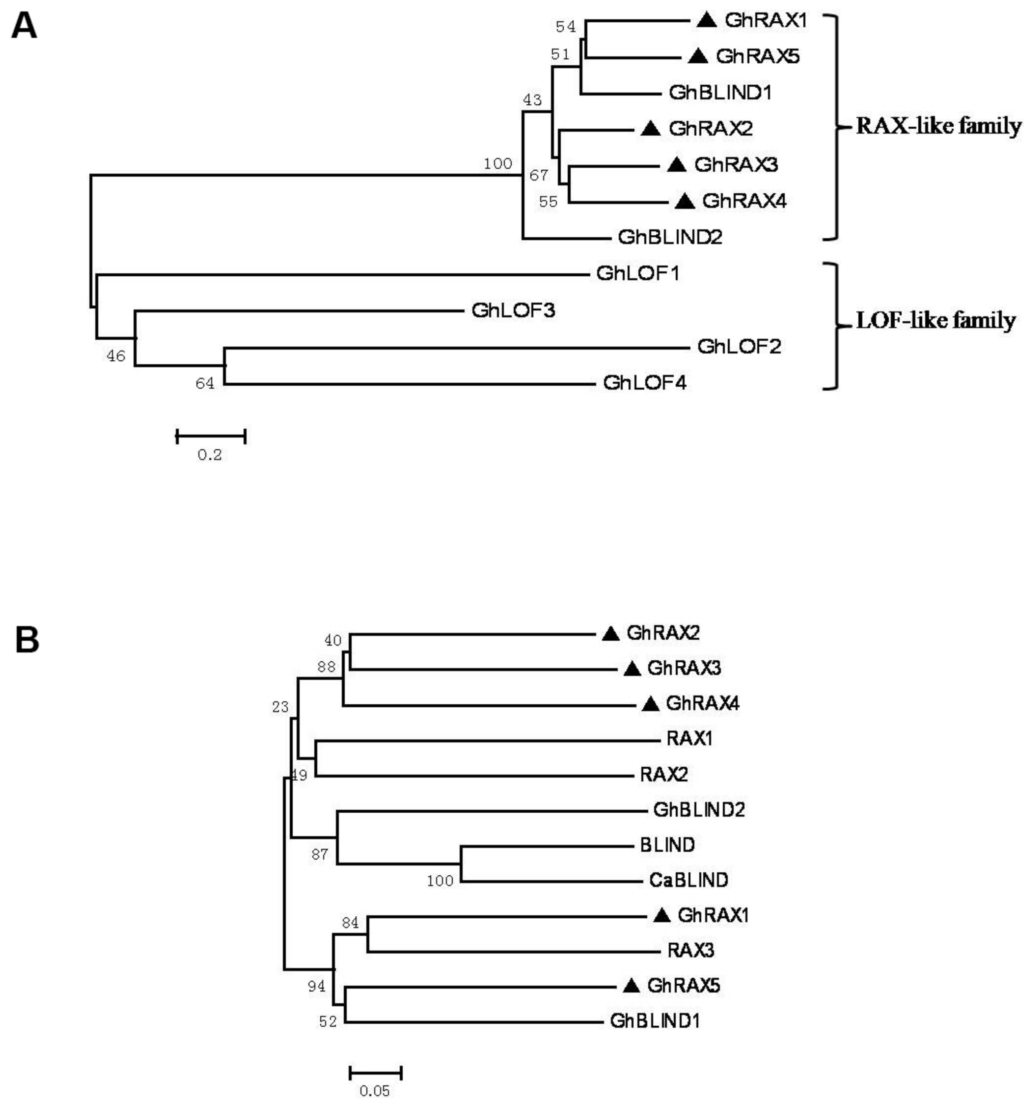

Figure 2. Phylogenetic relationships of GhRAXs with other MYB-like transcription factors from *Gossypium hirsutum* L. and other species. **A.** Phylogenetic relationships of RAX-like proteins and LOF-like proteins in *G. hirsutum* L. **B.** Phylogenetic relationships of GhRAXs and their orthologs from other species. Amino acid sequences used for alignment include RAX1 (AED93106.1), RAX2 (AEC09315.1), and RAX3 (AEE78577.1) from *Arabidopsis thaliana*, BLIND (AAL69334.1) from tomato, CaBLIND (JF496586.1) from pepper, and GhBlind1 (ADT80570.1) and GhBlind2 (ADT80571.1) from cotton.

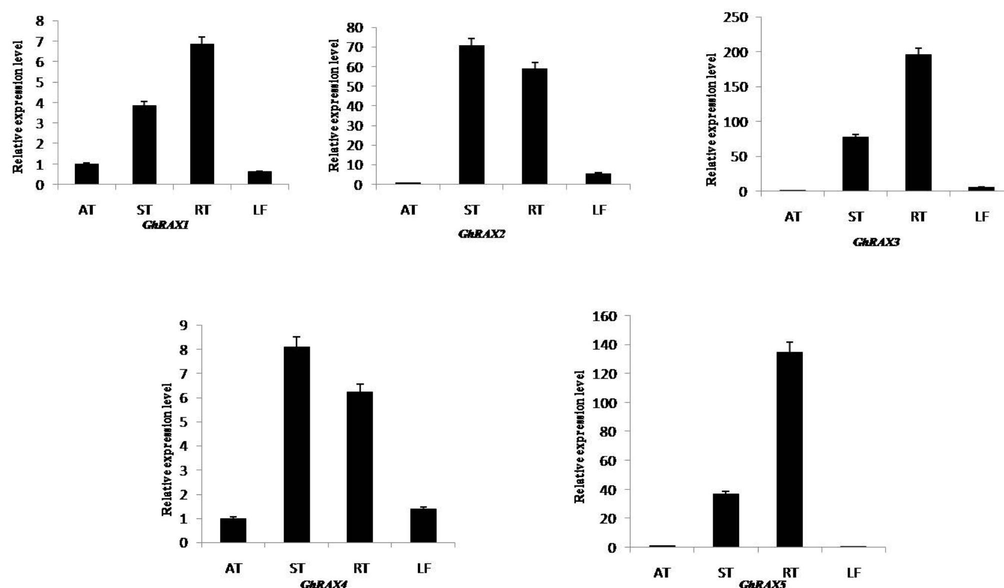

Figure 3. Expression analysis of *GhRAX*s in the apical tips (AP), stems (ST), roots (RT), and leaves (LF) of *Gossypium hirsutum* L. Data are the mean values (N = 3), and error bars represent the standard deviation.

DISCUSSION

The development of plant architecture is a complex process determined by endogenous genetic information, phytohormones, and environmental cues. Two phases are crucial to the development of shoot branches: the formation of axillary meristems and the outgrowth of axillary buds. Various models have been proposed, but the mechanisms underlying the latter stage were clarified after strigolactone was identified as the third phytohormone, which functions together with auxin and cytokinin to affect bud outgrowth (Domagalska and Leyser, 2011; Kebrom et al., 2013). However, much less is known about the molecular basis of axillary meristem formation, despite information from genes that were cloned from different species. For example, in *A. thaliana*, *LAS*, *RAX1*, *CUC3*, *LOF1*, and *ROX* all encode transcription factors essential for axillary meristem formation, but the target genes of these transcription factors are still unknown; however, they may be further identified using ChIP-seq analyses. *LAS* and *CUC3* are different from *RAX1* and *ROX* in that their mutants showed concaulescent phenotypes, in which axillary shoots from cauline leaves were fused to the main stem beside defects in axillary meristem formation. *In situ* hybridizations in the shoot tip demonstrated that *LAS* and *CUC3* accumulated in a band-shaped region at the axil of leaf primordia, while transcripts of *RAX1* and *ROX* were restricted to a ball-shaped region where the axillary meristem was formed (Greb et al., 2003; Hibara et al., 2006; Muller et al., 2006; Yang et al., 2012). These results indicate that the four genes were involved in axillary meristem formation, and that *LAS* and *CUC3* were involved in the formation of lateral organ boundaries. The *lof1* mutant showed fusions of cauline branches with cauline leaves, and it suppressed the formation of accessory bud meristems, located at the axils of lateral branches and cauline leaves,

which contribute little to the plant architecture (Lee et al., 2009). It seems that the function of *LOF1* differs slightly from the other four genes in *A. thaliana*. However, its ortholog *Tf* in tomato plays a critical role in axillary meristem formation. Intriguingly, the branching defects of almost all of these mutants were strongly diminished or absent under long-day conditions, indicating an influence of the environment on meristem formation.

In this study, we cloned five genes from upland cotton, which are orthologous to *RAX*s in *A. thaliana* and *Blind* in tomato. The ORFs of these five *GhRAX*s are 1083, 903, 969, 948, and 624 bp long, and they are predicted to encode putative peptides of 361, 301, 323, 316, and 208 amino acids, respectively. Analyses of genomic DNA showed that all five genes have two introns that are structurally similar to *RAX*s and *Blind*. Multiple amino acid sequence alignments showed that all five putative GhRAX proteins were highly homologous to RAXs and BLIND within the conserved domains. *LOF*s, classified as another gene family in *A. thaliana*, are also involved in the formation of axillary meristems, and the expression analysis of *RAX1* in the *lof1* mutant showed that *RAX1* functioned downstream of *LOF1* (Lee et al., 2009). Four *GhLOF*s were previously cloned in our laboratory, and a multiple amino acid sequence alignment showed that they were orthologous to the *LOF*s in *A. thaliana*. Phylogenetic trees were constructed to analyze the relationship of the two families in cotton and the *GhRAX*s with their orthologs from other species. *GhRAX*s and *GhLOF*s were categorized into two groups, suggesting functional divergence of the two families. Subsequent analysis of *GhRAX*s with their orthologs from *A. thaliana* and tomato showed that *GhRAX2/3/4* had a close relationship with *RAX1/2*, whereas *GhRAX1* and *GhRAX5* were close to *RAX3* from *A. thaliana*. A single *rax1* mutant, all double *rax1/2/3* mutants, and a triple mutant showed branching pattern defects, especially under short-day conditions. However, high-level expression of these genes in the roots of the mutants did not induce distinguishable root morphology from the wild type. The underlying mechanism requires further exploration. The expression patterns of *GhRAX*s were similar to those of *RAX*s, and their transcription levels were predominant in the root, indicating their possible functions beyond the acceleration of axillary meristem formation.

The genetic modification of plant architecture is of great importance to plant improvement. For example, the Green Revolution (Peng et al., 1999) has drastically increased the yield of wheat and rice. Branching pattern is another critical characteristic of crops. However, the underlying mechanisms of branch control remain largely unknown, and this lack of knowledge prohibits genetic improvement of branching patterns through artificial modification of genes involved in branching control. Cloning and further functional investigations of genes controlling cotton branching will facilitate our understanding of the mechanisms and the genetic manipulation of plant architecture needed to develop compact cotton varieties for mechanical harvest. Five *GhRAX*s were cloned in this study, and further complementary experiments with *A. thaliana* mutants should be conducted to clarify the exact functions of these genes. Moreover, the direct modulation of gene sequences or gene transcription, including RNAi, TALEN, or CRISPR (Sunilkumar et al., 2006; Gaj et al., 2013), can be adopted for this purpose.

Conflicts of interest

The authors declare no conflict of interest.

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