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Cloning and expression analysis of novel *Aux/IAA* family genes in *Gossypium hirsutum*

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ABSTRACT

Members of the *Aux/IAA* gene family encode proteins that mediate the responses of auxin-regulated gene expression and regulate various aspects of plant morphological development. Here, we provide the first identification and characterization of nine cDNAs encoding the complete open reading frame (ORF) of the *Aux/IAA* family in cotton. These were designated *GhAux1* to *GhAux9* (*Gossypium hirsutum Aux/IAA*). The proteins encoded by these nine genes had either whole or partially conserved domains of the *Aux/IAA* superfamily, with sequence identity ranging from 14% to 69%. A pair of homeologs exists for each Aux/IAA in *G. hirsutum* acc. TM-1 with high identity both in ORF sequences and amino acid level. Tissue- and organ-specific analysis showed that transcripts of *GhAux1*, *GhAux2*, and *GhAux3* were abundant in vegetative organs, whereas *GhAux4*, *GhAux5*, *GhAux6*, and *GhAux7* were preferentially expressed in ovules on the day of anthesis. *GhAux8* and *GhIAA16* (previously reported) were also preferentially expressed during fiber developmental stages, especially *GhAux8* in fiber early elongation stages, and *GhIAA16* in fiber initiation and secondary cell wall thickening stage. *GhAux9* was specifically expressed in developing fibers. During the fiber initiation stage, except for *GhAux*3 and *GhAux*6, the expression of the other eight *GhAux*s in various lintless–fuzzless and linted–fuzzless mutants demonstrated that they were significantly up-regulated compared with linted–fuzzy TM-1.

1. Introduction

As a phytohormone, auxin plays important roles in many aspects of plant growth and developmental processes, such as apical dominance, tropism, and lateral root and flower formation (Friml, 2003). This wide range of effects is attributed to the role of auxin as a signal factor that activates a series of downstream pathways. In recent years, many auxin regulatory genes have been identified and characterized from different plant species. These genes have been grouped into three major classes: auxin/indole-3-acetic acid (Aux/IAA), growth hagen 3 (GH3), and small auxin-up RNA (SAUR) gene families (Guilfoyle et al., 1998). Auxin can rapidly and specifically alter the transcription levels of most of these genes without protein synthesis (Abel and Theologis, 1996). Moreover, molecular, genetic, and biochemical studies have shown that Aux/IAA proteins play central roles in auxin signal transduction (Guilfoyle et al., 1998; Leyser, 2002).

The *Aux/IAA* genes encode short-lived nuclear proteins, most of which contain four highly conserved domains. Domain I is an active repression domain that can inactivate auxin response factor (ARF) (Tiwari et al., 2004). Domain II confers instability to the Aux/IAA proteins (Ouellet et al., 2001; Worley et al., 2000). Domains III and IV mediate homo- and hetero-dimerization among the Aux/IAA proteins and auxin ARFs (Kim et al., 1997; Ulmasov et al., 1997). The promoter regions of auxin-responsive genes usually have auxin-responsive *cis*-elements (AuxREs) that can bind to ARFs to regulate auxin-mediated gene expression. The Aux/IAA proteins do not bind to AuxREs directly, but interact with ARFs to regulate auxin-responsive genes by controlling the activity of ARFs (Tiwari et al., 2003; Ulmasov et al., 1997). In the absence of auxin, the Aux/IAA proteins directly inhibit transcriptional activity of the auxin-responsive gene via interaction with ARFs via domains III and IV, which are conserved between the two protein families (Ulmasov et al., 1997). In the presence of auxin, the SCF component TIR1 or its associated proteins are affected such that the SCF^{TIR1} complex has enhanced interactions with Aux/IAA proteins, promoting polyubiquitination and proteasomal degradation of Aux/IAA proteins in an auxin-dependent manner (Kepinski and Leyser, 2005; Tan and Zheng, 2008). Auxin-induced degradation of Aux/IAA proteins frees ARFs, which then activates the transcription of auxin response genes. At the same time, the transcription of Aux/IAA is also activated by auxin, providing a negative feedback mechanism to modulate auxin activity (Tiwari et al., 2001; Ulmasov et al., 1999).

Abbreviations: Aux/IAA, Auxin/indole-3-acetic acid; GH3, Growth Hagen 3; SAUR, Small auxin-up RNA; ARF, Auxin response factor; AuxREs, Auxin-responsive *cis*-elements; SCF, Skp1-Cdc53/Cul1-F box protein; TIR1, Transport inhibitor response 1; DPA, Day post anthesis; CTAB, Cetyl-Trimethyl-Ammonium Bromide; Tail, Thermal asymmetric interlaced; NLS, Nuclear localization signal.

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Twenty-nine *Aux/IAA* genes have been identified in *Arabidopsis* and their functions are relatively clear. In *Arabidopsis*, it has also been found that a single *Aux/IAA* gene deletion has little impact on plant growth and development, but the gain-of-function mutants could cause pleiotropic phenotypes. When mutation occurs within a highly conserved amino acid sequence (VGWPPV) in domain II, the stability of the gene products may be altered, causing developmental defects related to auxin. For example, *axr2/iaa7*, *axr3/iaa17*, and *axr5/iaa1* mutants show reduced apical dominance and root gravitropism (Leyser et al., 1996; Timpte et al., 1995; Yang et al., 2004), *iaa28-1* and *slr/iaa14* mutants have fewer lateral roots and root hairs (Fukaki et al., 2002; Rogg and Bartel, 2001), and *shy2/iaa3* seedlings have short hypocotyls and up-curled leaves in the dark (Tian and Reed, 1999).

Cotton is one of the most important economic crops in the world. Auxin is known to play an important role in fiber development (John, 1999). However, little information about the Aux/IAA superfamily has been reported in cotton. Suo et al. (2002) identified a differentially expressed gene, *GhIAA16*, using a cDNA filter array containing over 20,000 clones randomly selected from an ovule cDNA library, constructed using ovule RNAs from wild-type cultivar Xuzhou142 and its corresponding fiberless mutant. Expression analysis revealed that *GhIAA16* transcripts peaked at 3 days pre-anthesis (−3 DPA) in the ovule, when cotton fibers start initiation, then decreased rapidly afterwards in the fiberless mutant, but maintained a stable expression in the wild-type ovule. In this study, using homology cloning methods, we identified nine novel cotton *GhAux* genes that belong to the Aux/IAA family, and further analyzed their phylogenetic relationship and expression patterns. This work may help us to understand the roles of *Aux/IAA* genes in auxin signal pathway in cotton, particularly in cotton fiber development.

2. Materials and methods

2.1. Plant materials

The following plant materials were used in this study: the genetic standard line Texas Marker-1 (TM-1), dominant naked seed N1, recessive naked seed n2, Xinxiangxiaoji linted–fuzzless (XinFLM), Xuzhou-142 lintless–fuzzless (XZ142WX), Xinxiangxiaoji lintless–fuzzless (XinWX), SL1-7-1, and MD-17. The mature fiber and seed phenotypes of these materials are shown in Table 1. All the materials were planted in the experimental field of Nanjing Agricultural University, Nanjing, Jiangsu Province, China.

The following samples were collected from TM-1: ovule and fiber mixtures at −3, 0, 2, and 5 DPA; fibers at 10, 15, 20, and 23 DPA; and root, stem, and leaf tissues. In the mutant lines, only −3, 0, and 1 DPA ovules were sampled. All samples with three replicates were immediately frozen in liquid nitrogen and stored in a −70 °C freezer before RNA extraction.

2.2. Total RNA isolation and synthesis of cDNA

Total RNA was extracted using the CTAB-acidic phenolic method (Jiang and Zhang, 2003). RNA samples were treated with DNase I (Ambion, Austin, TX, USA) according to manufacturer's instructions to remove trace contaminants of genomic DNA. Total RNA samples (1 μg per reaction) were reverse transcribed into cDNAs by avian myeloblastosis virus (AMV) reverse transcriptase, and the cDNAs were used as templates in subsequent Q-PCR reactions.

2.3. Identification of putative GhAux genes

The *Aux* domain proteins from *Arabidopsis thaliana* were used as query probes to search the *Gossypium hirsutum* EST database (http://www.ncbi.nlm.nih.gov/) using BLAST. The searched EST sequences were spliced with CAP3 to form contigs, which were used as probes to continue BLASTn searching until the contigs were no longer extended. Vector and low quality sequences were removed manually from the resulting hits. The remaining non-redundant sequences with the highest similarity to the query sequences were retained as putative *Aux* domain genes. In each contig, open reading frames (ORFs) were identified based on the amino acid sequences, and then classified by homology analysis using ClustalX (ftp://ftp-igbme.u-strasbg.fr/pub/ClustalX/).

2.4. Full-length ORF and genomic sequence cloning and confirmation

On the basis of the results of a bioinformatics analysis, TAIL and 3′ RACE methods were used to extend the 5′ and 3′ termini of the incomplete ORFs, respectively. TAIL-PCR was carried out according to the Liu and Huang (1998) method. All primer information, including that for degenerate primers, anchor primers, sequence-specific primers, and primers for full-length ORFs and RT-PCR is shown in Supplemental Tables S1 and S2.

Based on the cDNA sequences, gene-specific PCR primer pairs for each *Aux/IAA* were used for PCR-amplification in *G. hirsutum* acc. TM-1 genomic DNA. The PCR reaction was performed, amplicons were cloned and sequenced, and the subgenomes were confirmed as described (Zhu et al., 2011).

All primers were designed using the software Primer Premier 5.0 (Premier, Canada) and Oligo 6.0 (Cascade, USA). Oligo synthesis and DNA sequencing were performed by Nanjing Genscript Bio-Technology Co. Ltd (Nanjing, China).

2.5. Sequence analysis

BLAST searches for nucleic acid and protein sequences were performed via the GenBank database. ORFs were analyzed using DNAMAN software (Lynnon Biosoft, Quebec, Canada). Characteristics such as functional domains, molecular weights, isoelectric points, and amino acid signal peptides were predicted using the tools provided by the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov). Potential sites of phosphorylation and glycosylation were identified using the tools listed at http://www.cbs.dtu.dk/services/NetPhos and http://www.ncbi.nlm.gov/structure/cdd, respectively. Multiple sequence alignment was performed using the ClustalX program (Thompson et al., 1997) and GeneDoc software (Nicholas and Nicholas, 1997). An unrooted neighbor-joining tree

Table 1
Plant materials used in the experiment.

Accession	Abbr.	Phenotype	Origin
TM-1	TM-1	Linted–fuzzy	USDA-ARS, College Station, TX, USA
Dominant naked seed N1	N1	Fuzzless	USDA-ARS, College Station, TX, USA
Recessive naked seed n2	n2	Fuzzless	USDA-ARS, College Station, TX, USA
Xinxiangxiaoji linted–fuzzless	XinFLM	Fuzzless	Cotton Res. Inst, NAU, China
Xinxiangxiaoji lintless–fuzzless	XinWX	Fiberless	Cotton Res. Inst, CAAS
Xuzhou-142 lintless–fuzzless	XZ142WX	Fiberless	Xuzhou Res. Inst. Agri. Sci., China
MD17	MD17	Fiberless	USDA-ARS, College Station, TX, USA
SL1-7-1	SL1-7-1	Fiberless	USDA-ARS, College Station, TX, USA