ISSN 1009－3095 *Journal of Zhejiang University SCIENCE* V. 3, No. 5, P. 594－599, Nov. －Dec., 2002
http://www.periodicals.com.cn; http://www.zju.edu.cn/English
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A suppressed gene in integument cells of a fiberless seed mutant in upland cotton*

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Received Sept. 16, 2001; revision accepted Jan. 29, 2002

Abstract: A fiberless seed mutant (fl) was identified in a commercial cotton (*Gossypium hirsutum* L.) variety Xu-Zhou 142 (FL). This phenotype is associated with lack of fiber cell initiation in the outer integument of the ovule, as was characterized by analysis of genes related to fiber differentiation and development. Two genes, fl-E6 and FL-E6, were cloned from fl-integument cells and FL-fiber or integument cells, respectively. Compared with FL-E6, fl-E6 showed a dramatic change in nucleotide sequence: (1) FL-E6 contained a tandem repetitive sequence in which GGCTCA (Gly-Ser) is repeated five times between the 82nd and the 93rd codon from the first ATG codon, while in fl-E6 the same sequence is repeated four times; (2) The fl-E6 gene encodes a polypeptide of 241 amino acids but lacks two codons between the 90th and 93rd codon and three between the 171st and 174th relative to FL-E6; (3) There are also 12 nucleotide substitutions which would result in 7 amino acid differences between fl-E6 and FL-E6. Analysis of RT-PCR and Northern Blot showed that expression of the fl-E6 gene is suppressed in the fl-integument cells, but highly expressed in FL-fiber cells. The difference between fl-E6 and FL-E6 may be associated with lower expression of fl-E6 in the fl-integument cells. Searches of protein databases with the FL-E6 gene sequence showed similarity to the protein backbones of two arabinogalactan-proteins (AGPs), one from the filtrate of suspension-cultured cells of *Pyrus communis* (AGPPc2) and the other from *Nicotiana alata* (AGPNa2). Although the function of the FL-E6 protein in differentiation and development of cotton fiber cells is not known, the data indicate that the mutation of fl-E6 gene from FL-E6 gene may inhibit the fiber cell initiation from epidermal cells of the outer integument of the ovule.

Key words: Upland cotton, Fiberless seed mutant, Integument cells, cDNA, Arabinogalactan proteins
Document code: A **CLC number:** Q812; S603.2

INTRODUCTION

Cotton is the most extensively used fiber for textile production. Cotton fiber is a differentiated single epidermal cell of the outer integument of the ovule and has four growth phases: initiation, elongation (primary cell wall synthesis), secondary cell wall synthesis, and maturation. Mature fiber is made up of cellulose (89%), water (7%), proteins (0.9%), pectins (0.9%), mineral substances (0.1%), wax (0.7%), and small amount of organic acids, sugars, and pigments (Basra et al., 1984; Ryser, 1985; Arthur, 1990). Characterization of mRNAs and the corresponding genes predominantly expressed in fiber may be useful in correlating various proteins to their specific functions in fiber cells. In this regard, some genes involved in cotton fiber development have been identified and characterized in recent years (Rinehart et al., 1996; John et al., 1992, 1995a, 1995b). E6, one of the first genes preferentially expressed in cotton fiber cells, was isolated from a cotton variety Coker 312 (*Gossypium hirsutum* L.) by John and Crow (1992). It was reported that the E6 promoter could be used to modify fiber properties through genetic engineering, although its function is not yet known (John et al., 1996a).

There is a fiberless seed (fl) mutation in cotton, which is associated with lack of fiber cell initiation in the ovule epidermis. This mutant is useful for studying the mechanisms of cotton

* Project supported by the National Natural Science Foundation of China (Nos. 39770473 and 30170058), National Program of Plant Gene Transfer (J00-B-002-10), National High Technology Research and Development (863) Program of China (No. 2001AA212081), and Research Program of Ministry of Education of China (No. 0114)

fiber differentiation and development. In this paper, we present the results of characterization of a fiberless seed mutant (lacking fibers), obtained by comparative analysis of DNAs amplified from the fl mutant and a normal (FL) ovule, by polymerase chain reaction (PCR) using oligonucleotide primers targeted to the E6 gene. There was a dramatic change in nucleotide composition and expression of fl-E6 gene compared to the FL-E6 gene. These observations may be helpful in understanding the initial molecular events in fiber development.

MATERIALS AND METHODS

Plant Materials

Two lines of cotton (*Gossypium hirsutum* L.), Xu-Zhou-142 as the wild type (FL) and fl-xz-142 as the mutant (fl), were grown during the fall production season in 1998. Cotton boll age was determined by tagging the petioles of a flower when it was fully opened. Ovules were excised from bolls at different developmental stages. The young ovules were separated into fibers, integuments (seed coats), and embryos; they were frozen in liquid nitrogen and then stored at −70℃ until used for RNA or DNA preparation according to Wang (2000). All samples were treated this way unless otherwise specified.

Cloning and sequencing of PCR amplification products

For reverse transcription PCR (RT-PCR) analysis, the first-strand cDNA was synthesized using 5 μg of total RNA at 42℃ for 2 h with oligo(dT) and SuperScript (SuperScript Preamplification System from Gibco BRL). PCR was performed with final volumes of 25 μl containing 0.6 unit of AmpliTaq (Perkin-Elmer), 50 mmol/L dNTPs, 0.4 mmol/L $MgCl_2$, and 10 pmol/L gene-specific primers with the first-strand cDNA. The gene-specific primers, 5'-ATGCGCAAGCTTTGGCTTCCTCACCAAAAC-3' and 5'-GTCGATCCATGGGTTCGAACTCT TCCTC-3', were designed from the nucleotide sequence of the E6 gene preferentially expressed in cotton fiber cells (John et al., 1992). In order to provide high fidelity amplification, Pfu DNA polymerase was applied to amplify the E6 gene from total genomic DNA. The A-tailing reaction was then performed for blunt ended PCR fragments generated by Pfu. Both of cDNA generated by Taq in RT-PCR and A-tailed genomic DNA fragment by Pfu in PCR were cloned into the pGEM® -T Easy Vector and sequenced by an ABI PRISM™ 377 DNA Sequencer using the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PERKIN ELMER).

Northern Blot analysis

Ten μg total RNAs from long fibers, integument with short fibers (fuzz) and integument with no short fibers were fractionated by electrophoresis in denaturing formaldehyde agarose gel, and transferred to a nylon membrane by capillary diffusion. The membrane was baked at 80℃ for 2h and probed with ^{32}P-labeled E6-cDNA. The blots were hybridized (1×10^8cpm/μg; 5×10^5cpm/ml) and washed under stringent conditions (0.1×SSC, 52℃). Autoradiography was done at −70℃ for 24 h.

RESULTS

FL-E6 gene in normal cotton line (XZ−142)

Xu-Zhou 142 (FL) is a commercial cotton variety planted in China and shows a typical pattern of upland cotton in differentiation and development of fiber cells. Double-stranded cDNAs or DNA fragments were synthesized from total RNA of 5 DPA (days post-anthesis) fibers or genomic DNA of embryos from normal cotton line (FL) by RT-PCR or PCR amplification using E6 gene primers, and then cloned into the T-vector. We randomly selected two cDNA clones, FL-E6-1 and FL-E6-12, and one genomic DNA clone, FL-E6-5, and sequenced these plasmid inserts. Comparison of the three nucleotide sequences showing they were identical indicated that there was no intron in the protein coding region of the FL-E6 gene and also provided strong evidence that both Pfu and Taq DNA polymerases used in this experiment were producing high fidelity products. The nucleotide sequence of FL-E6, as shown in Fig. 1, contains an open reading frame of 771 bases and would result in a peptide of 246 amino acids. When compared with the E6 gene cloned from Coker 312 (*Gossypium hirsutum* L.) by John et al. (1992), the homologous gene, FL-E6, from XZ-142 con-

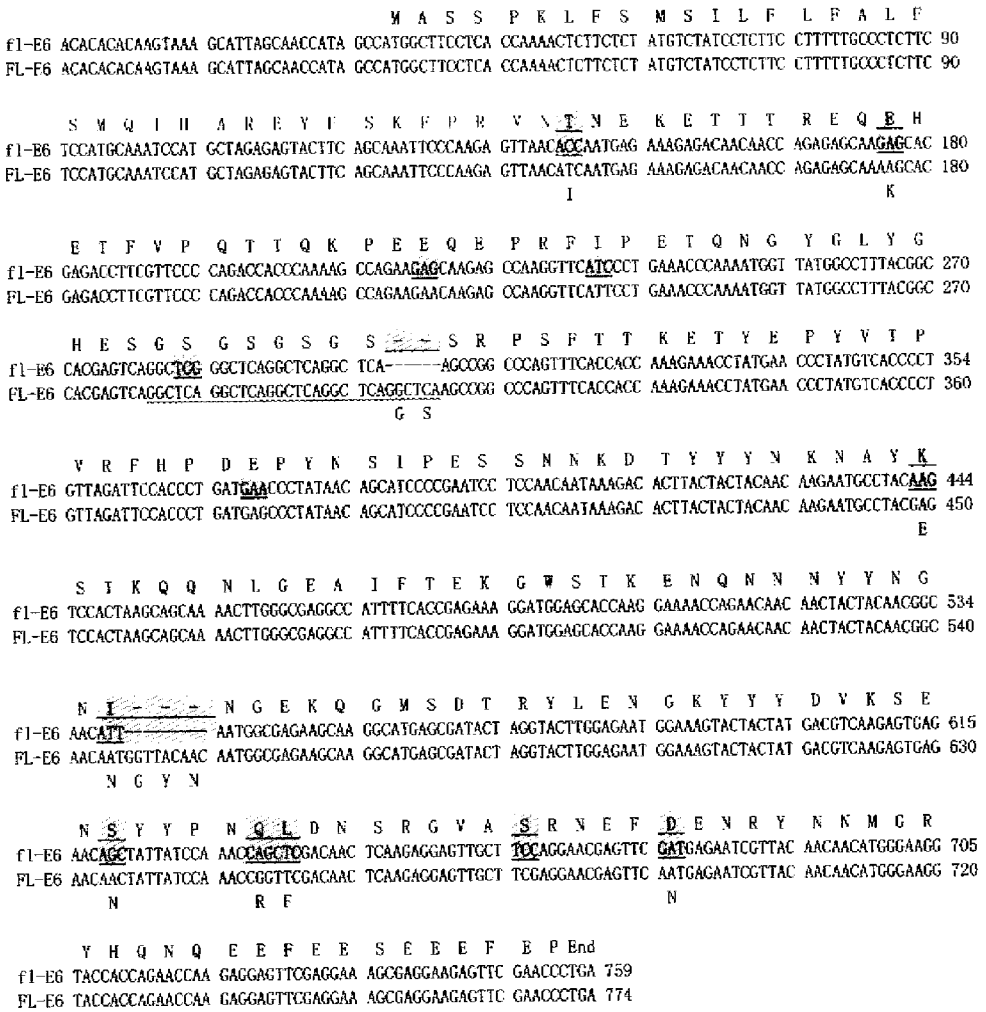

Fig. 1 Nucleotide and deduced amino acid sequences of fl-E6 and FL-E6 genes. Codons and amino acids from fl-E6 which differ from FL-E6 are underlined and shaded. A tandem repetitive sequence is indicated by a wavy line

tained a tandem repetitive sequence in which GGCTCA (Gly-Ser) was repeated five times between the 82nd and the 93rd codon, while in Coker E6 gene it was present only once. This situation was observed also in the fl-E6 gene (Fig. 1) and in other fiber-mutant cotton lines (Wang et al., 2001). However, the remaining nucleotide sequence of FL-E6 was identical to the Cocker E6 gene.

Searches of protein databases (GenBank, Jan. 15, 2000) with the FL-E6 sequence revealed significant similarity to the protein backbones of two arabinogalactan-proteins (AGPs), one from the filtrate of suspension-cultured cells of *Pyrus communis* (AGPPc2) and the other from *Nicotiana alata* (AGPNa2), which are quite different from the 'classical' AGP backbones (Mau et al., 1995). The deduced amino acid sequences of FL-E6 and AGPNA2 are aligned in Fig. 2 (AGPPc2 not shown). The predicted protein of FL-E6 in residues 71-230 showed 37.11% identity to AGPNa2. Position 177-200 (EKQGMSDTRYLENGKYYYDV KSEN) in FL-E6 protein was particularly well conserved with AGPNa2 and AGPPc2. It is noteworthy that more than 40% of protein encoded by FL-E6 consists of glutamate, serine, asparagine and tyrosine. However, it contains only one tryptophan and no cysteine residues.

Fig. 2 Alignment of the deduced amino acid sequence from the *G. hirsutum* XZ－E6 and *Nicotiana alata* AGP-Na2. The middle alignment represents conserved residues

fl-E6 gene in fiberless seed mutant (fl-xz-142)

fl-xz-142 is a mutant (fl) that occurred in a commercial cotton variety Xu-Zhou 142 (FL). Although plants of the two genotypes show an overall similar pattern in growth rate, development, and timing of flowering, there is a striking difference between them in seed development. The developing FL seeds are covered with young fibers (long and short fibers), whereas the fl seeds show no detectable fiber growth, except for a few rudimentary fibers detectable only under a dissecting microscope (Fig. 3). The size and shape of the seeds and fruits are similar in both genotypes.

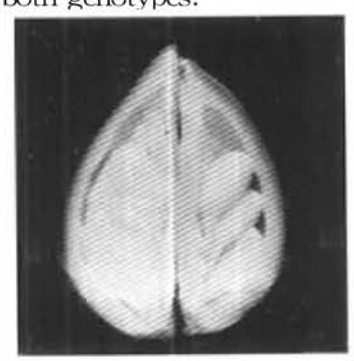

Fig. 3 A comparison of cotton seeds in bolls between normal line (Xu-Zhou 142) a fiberless seed mutant (fl-xz-142). Left: normal cotton (FL) seed are covered with fibers. Right: mutant (fl) seeds show no fiber

DNA fragments homologous to the FL-E6 gene were amplified only from genomic DNA, not from the integument cell RNA of fl-xz-142. Compared with the FL-E6 gene, the fl-E6 gene has a dramatic change in nucleotide sequence as shown in Fig. 1. The open reading frame of the fl-E6 gene contains 705 bases, and is shorter by 15 bases than that of the FL-E6 gene and longer by 9 bases than that of Coker E6 gene. The fl-E6 gene encodes a polypeptide of 241 amino acids, but lacks two codons between the 90th and 93rd codon and three between the 171st and 174th in comparison with the FL-E6 gene. In addition, there are 12 nucleotide substitutions and these have resulted in 7 amino acids differences (2.9% of total amino acids) between fl-E6 gene and FL-E6 gene.

Expression of fl-E6 gene

In order to measure the levels of E6-mRNAs in the fiber or integument cells of ovules at the stage of fiber initiation (0-5 DPA), total RNAs from fiber (FL-fiber), integument with short fibers (FL-integument) and integument with no fibers (fl-integument) were used to synthesize cDNAs by reverse transcription-PCR. As shown in Fig. 4-A, the E6-cDNA could be synthesized only from mRNAs in the cells of FL-fiber or FL-integument; there was no detectable E6-cDNA in the cells of fl-integument. However, when the RNA Northern Blot was hybridized with fl-

E6-cDNA, a very weak signal was produced in the line of fl-integument (Fig. 4-B). Taken together, this suggests that the expression of fl-E6 gene is preferentially suppressed in the integument cells of the fiberless seed, and such suppression was coincident with the mutation of fl-E6 gene. Fig. 4-A also shows that the concentration of E6 mRNA is higher during the late primary cell wall (10 DPA) and early secondary cell wall synthesis stages (15 DPA).

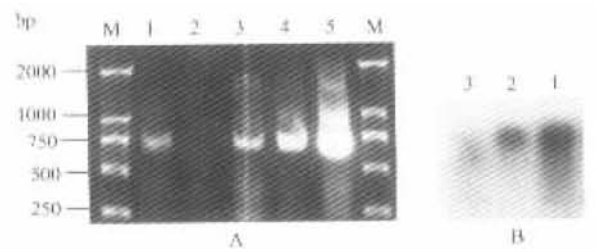

Fig. 4 Tissue and temporal expression of E6 gene in normal (FL) and mutant (fl) cotton. (A) 8μl cDNA synthesized from 5 μg total RNA by RT-PCR was electrophoresed on the agarose gel. Lane 1 and 2: 5 DPA FL-integument and fl-integument cDNAs; Lane 3, 4 and 5: 5, 10 and 15 DPA FL-fiber cDNAs; Lane M: DNA markers. (B) Northern blot containing total RNA (10 μg) from 5 DPA FL-fiber(1), FL-integument(2) and fl-integument(3) was hybridized to ^{32}P-labeled fl-E6 cDNA insert. The blot was hybridized (1×10^8cpm/μg; 5×10^5cpm/ml) and washed under stringent conditions (0.1×SSC, 52℃). Autoradiography was done at −70℃ for 24 h

DISCUSSION

Concentrations of FL-E6 mRNAs are very high during late primary cell wall and early secondary cell wall synthesis stages. A direct role of FL-E6 in the cellulose synthesis can be ruled out since the maximum rate of cellulose deposition in fibers occurs between 26-28 DPA. Therefore, the antisense experiment by John (1996b) demonstrated that E6 gene is not critical to the change of fiber properties which are directly influenced by the cellulose synthesis. However, a number of other carbohydrate components are synthesized early in fiber development (Basra et al., 1984) and FL-E6 can be a candidate for the biosynthesis or degradation of any of these polysaccharides during the initiation of cotton fiber cells.

Search of protein databases with the FL-E6 sequence showed similarities to the protein backbones of two arabinogalactan-proteins (AGPs). AGPs are a family of proteoglycans that are widely distributed throughout the plant kingdom. The majority of the AGPs characterized so far have a protein content of less than 10% and contain more than 90% carbohydrate (for review, see Kreuger et al., 1996; Nothnagel, 1997). The synthesis of AGPs with high level of carbohydrate may be associated with a function of the FL-E6 protein for the biosynthesis or degradation of any of these polysaccharides. But it is not clear whether the initiation of cotton fiber cells is triggered by FL-E6 protein or AGPs.

Currently, AGP's precise function(s) remains speculative, but evidence is accumulating to suggest that AGPs play important roles in plant development, cell division, cell elongation and morphogenesis (Kreuger et al., 1996). Some direct evidence of involvement in differentiation are given by the fact that addition of nanomolar quantities of specific AGPs to old cell cultures which have lost embryogenic potential can restore the ability to produce somatic embryos (Kreuger et al., 1996; Nothnagel, 1997). AGPs can react with $(\beta$-D-Glc$)_3$ Yariv reagent. Treatment with Yariv reagent caused inhibition of root growth of Arabidopsis thaliana seedlings. Treated roots (*Arabidopsis*) exhibited numerous bulging epidermal cells which phenocopies the *reb*1 (root epidermal cell bulging) mutant with lower levels of AGPs. Therefore, the *reb*1 phenotype appears to be a result of defective or missing root AGPs, which strongly indicates a function of AGPs in the control of root epidermal cell expansion (Ding et al., 1997). Yariv reagent also perturbs cell wall assembly in lily pollen tube and this further emphasizes the role of AGPs in the deposition of cell wall subunits within the previously synthesized cell wall (Roy et al., 1998). It is worthy to consider whether the initiation of cotton fiber cells is similar to initiation of root epidermal cell expansion.

Compared with the XZ-E6 gene, the fl-E6 gene has major changes in nucleotide sequence. Analysis of RT-PCR and Northern Blot showed that the fl-E6 gene was suppressed in the fl-in-

tegument cells, but was highly expressed in the FL-fiber cells. The difference of fl-E6 gene in sequence relative to the FL-E6 gene may be associated with lower expression of the fl-gene in the fl-integument cells. Alternatively, the fiberless seed mutant phenotype is apparently a result of defective or missing FL-E6 protein similar to the *reb*1 phenotype due to defective arabinogalactan protein. Although the function of the FL-E6 protein in differentiation and development of cotton fiber cells is not known, the data imply importance of the FL-E6 protein in fiber cell initiation.

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