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ORIGINAL RESEARCH

Molecular Cloning and Expression of cDNA Encoding the Cysteine Proteinase Inhibitor from Upland Cotton

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Abstract A cDNA encoding a novel cysteine proteinase inhibitor (CPI) was isolated from a gland mutant Xiangmian-18 of upland cotton during the pigments gland forming stage. The cDNA comprises 378 bp and encodes 125 amino acid residues with molecular mass of 13.8 kDa. It contains the conserved motif of cysteine protease inhibitors and belongs to the cystatin superfamily (Gln-Val-Val-Ala-Gly). The deduced amino acid sequences of the domains are highly similar to the normal upland cotton (96.8%). SDS-PAGE and western hybridization analysis showed that the expressed recombinant protein was recombinant CPI. The inhibitory activity of recombinant CPI was 46 u/μg which was measured by inhibiting the protease activity of papain. RT-PCR results indicated that the expression level of developing gland stage was higher than that of undeveloped gland stage.

Keywords Cysteine proteinase inhibitor · cDNA cloning · Expression · RT-PCR · Western blotting

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Introduction

Cysteine proteinase inhibitors (CPI) act as inhibitors of cysteine proteinases. Cysteine proteinase inhibitors of plant origin have been grouped into the fourth cystatin family, the "phytocystatins". The molecular mass of phytocystatins ranges from 12 to 16 kDa (Barrett 1987). Phytocystatin families contain three essential structural motifs, a conserved G in the N-terminal region, a highly conserved Q-V-V-A-G motif in a central loop segment, and a conserved W in the second half of the protein (Arai et al. 1991). It is widely assumed that phytocystatins perform a defensive role in plants because of their effects on exogenous proteinases such as those produced by insects and nematodes (Liang et al. 1991). The defense role of phytocystatins is not only demonstrated in vivo by their ability to significantly affect larval development (Kuroda et al. 1996) and in vitro by their ability to inhibit insect gut proteinases (Liang et al. 1991), but also shown by transgenic plants expressing CPI gene, which blocks cell death (Belenghi et al. 2003) and defends against nematodes, insects, slugs, and viruses (Gutierrez-Campos et al. 1999; Walker et al. 1999; Atkinson et al. 2004; Alvarez-Alfageme et al. 2007). In addition, antifungal and antimite activities have also been described for some phytocystatins (Pernas et al. 1999; Martinez et al. 2003; Yang and Yeh 2005; Christova et al. 2006).

Generally, phytocystatins are only present in storage organs, and their synthesis might be induced by cell damage that contributes to the complex defense mechanisms of plants (Zhu et al. 1999). In seeds, phytocystatin mRNAs show an expression pattern similar to that of major seed storage proteins (Abe et al. 1992). Phytocystatins have also played an important role in programmed cell death by regulating cysteine protease activities in the modulation of

protein turnover (Solomon et al. 1999; Belenghi et al. 2003). In addition, phytocystatins have been supplemented as a stabilizer in food processing and considered for other biotechnological applications (Gutierrez-Campos et al. 1999; Arai et al. 2002).

Up to now, phytocystatin genes have been cloned and over-expressed in many plants, including rice (Kondo et al. 1990), Chinese cabbage (Lim et al. 1996), soybean (Botella et al. 1996), American chestnut (Bernadette et al. 2002), pineapple stem (Shyu et al. 2004), taro (Yang and Yeh 2005), and *Arabidopsis thaliana* (Zhang et al. 2008). However, there was only one coding sequence of CPI gene found in cotton (*Gossypium hirsutum*) and lack of abundant discussion. In the study presented in this paper, the molecular cloning, heterologous expression, the bioinformatics analysis, and the expression detection of cDNA encoding CPI from Xiangmian-18 cotton (Zhang et al. 2001) were reported.

Materials and Methods

Materials

Xiangmian-18 (glandless seed but glanded plant) and Chuan-2802 (normal glanded cotton) were obtained from the National Research Center for Hybrid Cotton of China (Changde, China) and the Cotton Institute of the Sichuan Academy of Agricultural Sciences (Jianyang, China), respectively.

Total RNA Extraction

Cotton seeds were disinfected in 70% (*v*/*v*) ethanol then dipped in sterilized water, and began to bud in the plates containing sterilized filter paper and water. The pigment gland begins to develop at 36 h after budding. Total RNA was isolated by total plant RNA extraction kit (Watson Biotech, Shanghai China) according to the manufacturer's instructions. The purity and concentration of each RNA sample were detected by agarose gel electrophoresis and ultraviolet spectrophotometer.

Cloning of *cpi* Gene

A total of 12 μl reaction volume was generated containing 4 μl (5 μg) total RNA, 5 μl 10 pmol μl^{-1} Oligo(dT)20, and 5 μl RNase free H_2O, then incubated at 65°C for 5 min and placed on ice for at least 1 min. The first-strand cDNA synthesis mixture was prepared as follow: 4 μl 5×RT-PCR buffer, 2 μl 10 mmol/l dNTPs, 1 μl RNase inhibitor and 1 μl ReverTra Ace. The mixture was added into the 12 μl reaction volume prepared previously and mixed well. The first-strand cDNA synthesis mixture was incubated at 42°C for 60 min and terminated at 85°C for 5 min.

The primary synthesized ss-cDNA was used as template for PCR amplification by using *cpi* gene specific primers. CPI-F: 5′-CGGATCCAGAGATATGGCGAAAGC-3′ and CPI-R: 5′-CAAGCTTGACTACTCCTTTTAATGA-3′were designed based on *cpi* gene of normal upland cotton (*G. hirsutum*, GenBank accession number: EF643506), the underlined sequences are the *Bam*HI and *Hind*III restriction sites, respectively. The reaction system was as follows: template 1 μl, 10×Taq buffer 2 μl, dNTP mix (2.5 mmol) 1 μl, primer R 1 μl, primer F 1 μl, Taq DNA polymerase 0.2 μl, and ddH_2O 11.8 μl, the total volume was 20 μl. PCR was performed with the following program: 94°C for 4 min followed by 31 cycles of 94°C 30 s, 57°C 30 s, 72°C 30 s, and a final extension for 5 min at 72°C. The amplified cDNA fragments were isolated by agarose gel electrophoresis and then purified. The purified fragment was cloned into the pMD18-T vector (Takara Biotech Co., Ltd.) and then transferred into the *Escherichia coli* DH5α. The inserted fragment was sequenced using standard M13 sequencing primers.

Expression and Enzyme Activity Detection of Recombinant CPI

The recombinant plasmid pMD18-CPI was digested with *Bam*HI and *Hind*III. The inserted fragment was isolated and ligated into *Bam*HI and *Hin*dIII digested plasmid pET32a (+) expression vector and then transferred into *E. coli* BL21 (DE3) cells (Novagen). The recombinant plasmid was denominated as pET-CPI and then the inserted sequence was confirmed by sequencing and restriction enzyme digesting. The confirmed clone was grown in ampicillin-supplemented LB media to an OD600 of 0.6–0.8 at 30°C and 180 rpm, and then induced with isopropyl-beta-D-thiogalactopyranoside (IPTG). Each 4 ml of culture samples from different inducing times were destroyed by the ultrasonic method, and the pellets of the cells were collected by centrifuge at 12,000 rpm. SDS-PAGE was performed with 4% (*v*/*v*) stacking gel and 15% (*v*/*v*) separating gel. The proteins were visualized by Coomassie Brilliant Blue staining then recorded by Gel Doc 2000 (Bio. Rad).

To detect the activity of recombinant CPI, 5 μL (5 mg/mL) casein was added into the test tubes at 37°C for 10 min, then 30 μL papain and proper volume of supernatant (100 μL) were added into the test tubes. One hundred mM phosphate buffer (pH6.5, 0.3 M KCl, 0.1 mM EDTA, and 1 mM DTT) was added until the volume of enzyme solution reached 300 μL and then incubated at 37°C for 20 min. After incubation, 3 ml of Coomassie brilliant blue was added and incubated at 37°C for 20 min, the absorbance was measured at

595 nm. Inhibitory activity was calculated by the following formula:

$$^{\Delta}OD595 = OD595 - OD595'$$

$^{\Delta}$OD595 indicates the value of absorbance changes caused by recombinant CPI; OD595 indicates absorbance after 20 min incubation when adding the recombinant CPI; OD595′ indicates the value of absorbance before adding recombinant CPI (Ma et al. 2003)

Western Blotting Analysis

For Western blot analysis, recombinant protein samples were subjected to SDS-PAGE and then electrically transferred onto PVDF membrane. The membrane was blocked with membrane confining liquid (Tiangen) at room temperature for 1 h and then incubated with a 1:2,000 dilution of anti-His antibody at room temperature for 1 h. After washing 3–5 min with PBST (137 mmol l–1 NaCl, 2.7 mmol L^{-1} KCl, 10 mmol L^{-1} Na_2HPO_4, 2 mmol L^{-1} K_2HPO_4, 0.1% Tween-20, pH7.4), the blot was incubated with a goat anti-mouse IgG conjugated to horseradish peroxidase in membrane confining liquid (diluted 1:200). The blot was washed 5 min with PBST for three times. Lastly, the blot was incubated in the dark with Pro-Light HRP Detection Kit, and the result was recorded by Gel Doc 2000 (Bio. Rad).

Expression Pattern of Cotton *cpi* Gene

The mRNA expression level of *cpi* gene in different stages of Xiangmian-18 and Chuan-2802 was detected by RT-PCR method. Total RNA of undeveloped and developing stage were extracted and the ss-cDNAs were synthesized by the same method described in the previous step. All ss-cDNA were used as the templates in RT-PCR analysis by the same primers used in *cpi* gene amplification. The 18S rRNA gene (GenBank accession number: L24145) was used as the intra-reference gene (Forward primer 18S-1: 5′-TCGTAGTTG GACTTAGGGTGGG-3′, Reverse primer 18 S-2: 5′-CAA ATGCTTTCGCAGTTGTTCG-3′). The experiments were repeated three times then the density ratio of the target cDNA band and corresponding 18S rRNA band was carried out by Quantity One program (Bio-Rad). Results were generally represented as the mean±the standard error from the values of three independent tests. Groups of data were compared by one-way ANOVA. A difference between means was considered significant at a value of $P<0.05$.

Sequence Analysis of *cpi* Gene

The sequenced *cpi* gene was compared with the GenBank database at the National Center for Biotechnology Information (NCBI; Bethesda, MD) using the Blastx algorithm (Altschul et al., 1997). The amino acid sequence was deduced from *cpi* gene nucleic acids sequence by DNAMAN software. Function-point analysis was conducted by online tools motifs-PROSITE (http://cn.expasy.org/tools/scanprosite/). The conserved domain was searched against the NCBI database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The CPI transmembrane helix was predicted by online service (http://www.cbs.dtu.dk/services/TMHMM/). The signal peptide of CPI was predicted using SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). The secondary structure of CPI was predicted by online service (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_hnn.html).

Results

Cloning of *cpi* Gene from Xiangmian-18 Upland Cotton

The cDNA encoding *cpi* gene was amplified by RT-PCR method from a gland mutant Xiangmian-18 upland cotton during the pigments gland forming stage. An open reading frame spans over 378 bp and encodes a protein of 125 amino acid residues with a calculated molecular mass of 13.8 kDa and an isoelectric point of 9.87 (Fig. 1). The DNA sequence of *cpi* gene was then deposited in GenBank (Accession no. EU710847).

Sequence Analysis of *cpi* Gene

Blastn analysis indicated that the sequence similarity between *cpi* gene of Xiangmian-18 and *cpi* gene of normal upland cotton was 98.7% in nucleic acid sequence, only five nucleic acids were different. Blastx analysis showed that the deduced amino acids of CPI in the mutant Xiangmian-18 are highly similar to the normal upland cotton, except difference of four amino acids, with a similarity of 96.8%.

Further analysis of cotton CPI amino acid sequence implied that it contains a signal peptide in its N-terminal (Fig. 1). The protein with the signal peptide is generally secreted to the ecto-cell; this may be important in the role of cytokines, which has potential application, and a complete conserved domain. This result suggested that mature CPI could be produced through cleavage at the conserved VDG-LG sites in the N-terminal regions in CPI. The results of function-point analysis showed that the predicted ‘KQVVAGIKYYLEIK’ (79–92 sites) polypeptide amino acid sequence was an active site of cotton CPI, which gives further proof that the cloned sequence was the *cpi* gene. The complete conserved domain CPI in its C-terminal shows that the protein belongs to the cystatin

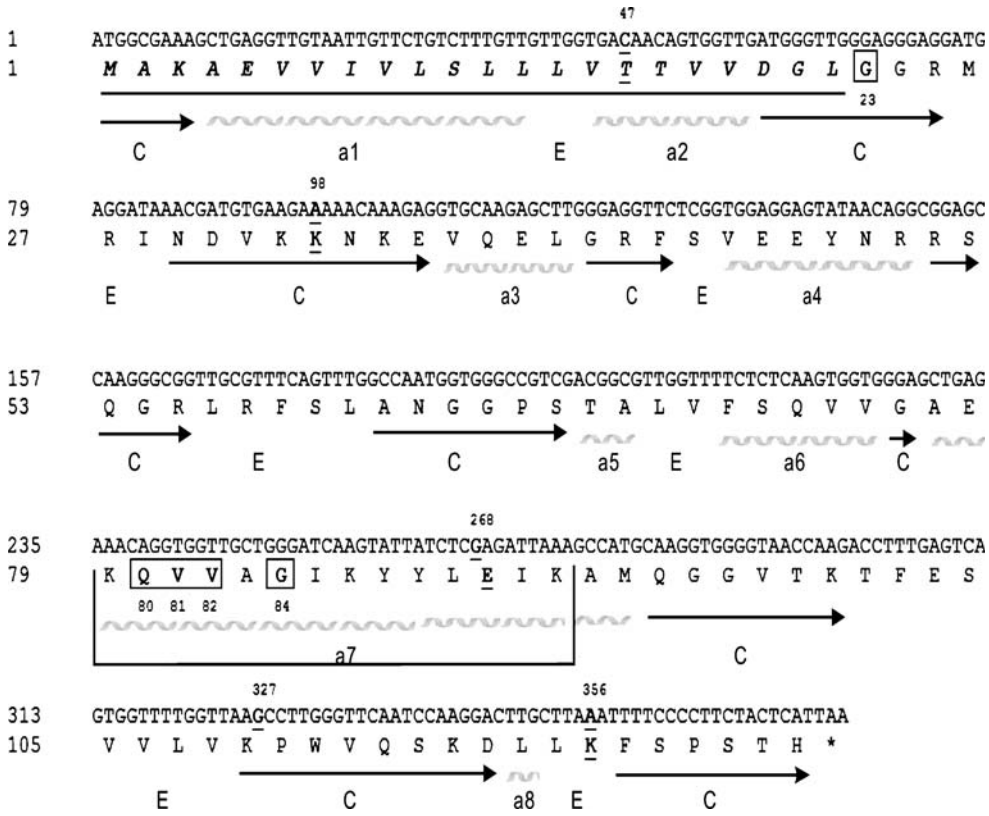

Fig. 1 The cDNA sequence, deduced amino sequence, and the sequence analysis of *cpi* gene from Xiangmian-18 cotton. Five putative predicted active sites (G23, Q80, V81, V82, G84) are in *bold* and *boxed*; the signal peptide is shown in *bold* and *italics*; the locations of the different nucleic acids and amino acid between cotton mutant (Xiangmian-18) and normal upland cotton are *bold* and *underlined*; the secondary structural elements [α1-8-Alpha helixes, *E* extended strand and *C* random coil] are indicated below the sequence. Conserved domain is range from 23–122 amino acids. The *asterisk* (*) denotes stop codon

superfamily (Fig. 1). Secondary structure prediction indicated that there are several α-helixes and β-sheets in this CPI protein sequence (Fig. 1).

Expression and Western Blotting of the Recombinant CPI

The results indicated that both restriction enzyme digestion and PCR amplification of recombinant plasmid pET-CPI can produce a 378 bp length DNA fragment (results not shown), and the subsequently sequenced results also showed that the inserted fragment was *cpi* gene. To characterize the properties of CPI, the recombinant plasmid pET-CPI was transformed into *E. coli* strain BL21-(DE3) and then recombinant CPI protein was induced by IPTG. The estimated molecular mass of recombinant CPI (31 kDa) was confirmed by SDS-PAGE (Fig. 2). The larger size of the fusion protein is due to the N-terminal leader peptide of 17 kDa encoded by the expression vector. The results of fermentation showed that expression of *cpi* gene in *E. coli* can be achieved under the conditions of 30°C, IPTG 1 mmol L^{-1}, time for 24~36 h. The result of Western Blotting analysis showed that the expressed recombinant protein is CPI (Fig. 2).

The inhibitory activity of CPI was investigated using papain as the proteolytic enzyme. Figure 3 shows the titration curve for casein with papain and CPI. To include the amount of inhibitor for the abscissa, △OD595 was used for the longitudinal coordinates. Inhibition curve analysis identified that the amount of inhibitor was less in 10 μg, with the increase in CPI concentration, inhibition vitality was enhanced, showing a good linear relationship, the curve equation was $y=0.0458\times-0.0027$ ($R^2=0.9936$). When the inhibitor is greater than the amount of 10 μg, the curve does not comply with the above equation; this may be due to competitive inhibition. Activity by inhibiting the terms of the restructuring activity of CPI was 46 u/μg.

Expression Analysis of *cpi* Gene by RT-PCR

The transcription pattern of the *cpi* gene was detected by semiquantitative RT-PCR assay at two stages of gland

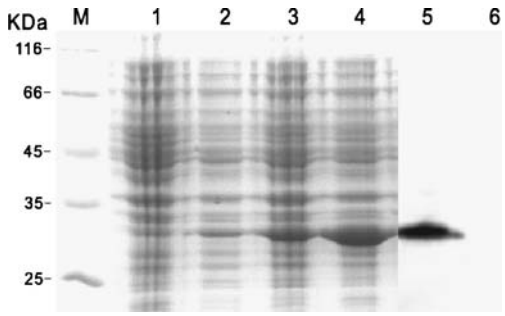

Fig. 2 SDS-PAGE analysis of the recombinant CPI. SDS-PAGE (15%) was loaded with *E. coli* extract expressing the CPI before (lane *1*) and after 1, 3 and 5 h IPTG induction (lanes *2*, *3*, *4*). Western blotting analyses with (lane *5*) and without IPTG induction (lane *6*). Molecular weight markers in KDa are indicated on the *left*

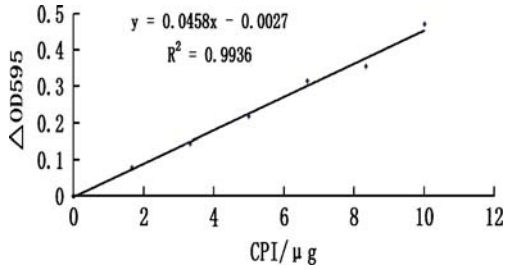

Fig. 3 Curve of CPI inhibitory activity. A fixed amount of papain was mixed with increasing amounts of CPI and residual enzyme activity was assayed. Casein was used as the substrate

development in Xiangmian-18 and Chuan-2802 cotton. The results indicated that the gene was expressed at both undeveloped stage and developing stage (Fig. 4a). Along with the development of the pigment gland, the mRNA levels in the developing stage of gland were increased about 1.5- to twofold compared with that in the undeveloped stage of the gland, which suggested that the CPI played a role in gland development and related defense system of cotton (Fig. 4b).

Discussion

CPI and its function in plants has been reported in recent years, CPI is involved in development, defense responses and programmed cell death in plants (Kuroda et al. 1996; Fernandes et al. 1999; Solomon et al. 1999), and it was also applied in transgenic plants (Alvarez-Alfageme et al. 2007; Gutierrez-Campos et al. 1999). However, there have been no comprehensive studies to characterize *cpi* gene in upland cotton (Gossypium).

Gossypium species are characterized by their lysigenous glands containing terpenoid aldehydes (TAs), the important secondary phytoalexins consisting mainly of gossypol, which constitute one of the plant's significant defense systems against pests and diseases (Cai et al. 2004; Townsend et al. 2005). In the past, the research was mainly focused on the biosynthesis of gossypol (Townsend et al. 2005), as the storage organ of gossypol and other TAs, the pigments gland began to attract the attention of some researchers (Zhu et al. 1999; Chang et al. 2007), but the regulatory mechanism between the storage organ gland and its inclusions TAs including gossypol in cotton has not been disclosed up to the present.

Besides the important role in defense systems in cotton (Cai et al. 2004; Agrawal and Karban 2000; Akingbemi et al. 1996), in vitro gossypol is toxic to nonruminant animals and humans, and severely limits the use of cotton seed as a food source (Cai et al. 2004; Townsend et al. 2005). The mutant Xiangmian-18 is a new gland-type of upland cotton (*G. hirsutum*), which is bred by crossing of tetraploid-dominant glandless cotton *Gossypium barbadense* with glanded *G. hirsutum* (Combet et al. 2000), with character similar to delayed pigment gland morphogenesis (Zhu et al. 1999), the leaves of Xiangmian-18 appear to be normal glanded, but its seed is glandless with very low gossypol levels which is below the international food standard (Zhang et al. 2001).

In order to study the regulatory mechanism between the gland and gossypol, a full length cDNA of CPI was cloned from a mutant cotton Xiangmian-18, and the nucleotide and amino acid sequences as well as the bioinformatics characters of it were analyzed. The deduced polypeptide sequence contained 125 amino acid residues with a molecular mass of 13.8 kDa and an isoelectric point of 9.87. The predicted 'KQVVAGIKYYLEIK' polypeptide amino acid sequence was an active site of cotton CPI (Fig. 1). As in other cystatins, the central Q-V-V-A-G sequence, considered to be one of the binding sites of cystatins, was present in CPI sequence of the Xiangmian-

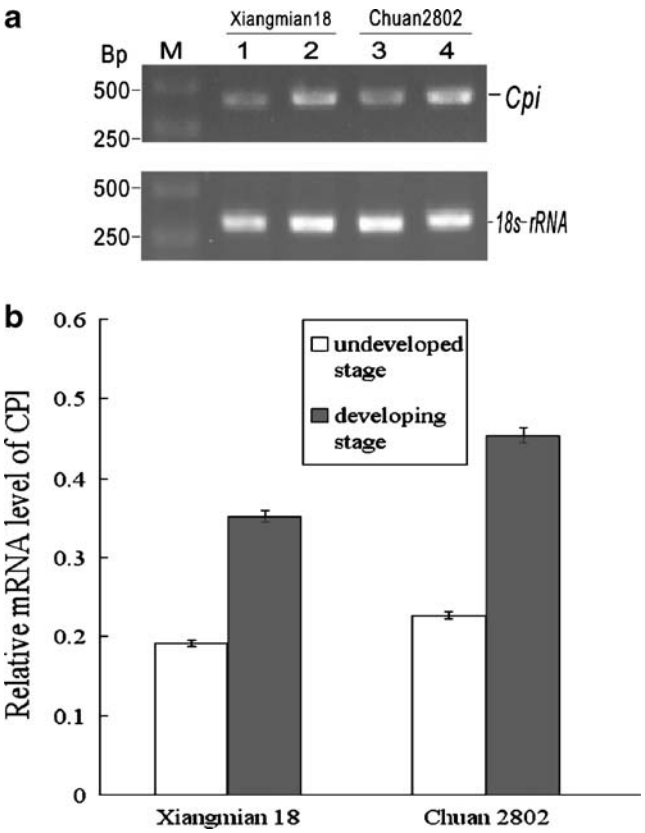

Fig. 4 Detection the mRNA expression level of *cpi* gene at different developing stage of cotton. **a** RT-PCR detection of *cpi* gene: lane *M* DNA marker; lanes *1* and *3* indicates undeveloped gland stage; lanes *2* and *4* indicates developing gland stage. **b** Quantified analysis of the expression level of *cpi* gene: data are presented as a percentage of mRNA level at undeveloped stage and developing stage of gland. Results were generally expressed mean±standard error (ER) from values in three independent tests. Groups of data were compared by one-way ANOVA, $P<0.05$, $n=3$

18. This result indicates that the cloned cDNA sequence from Xiangmian-18 was indeed a CPI. The inhibitory activity of recombinant CPI was measured by inhibiting the protease activity of papain. The inhibitory activity of recombinant CPI was 46 u/μg.

The expression level of the *cpi* gene was different between the undeveloped stage and developing stage of gland in Xiangmian-18 and Chuan-2802, the mRNA level of the *cpi* gene increased with the development of cotton pigment gland. These results suggest that the novel CPI also plays a pivotal role in the developing of gland and related defense system of this mutant upland cotton (Tan et al. 1995). We also found that the mRNA expression level of Chuan-2802 (a normal upland cotton) was higher than Xiangmian-18 (mutant upland cotton); this phenomena reflects the truth that the expression level of CPI gene was down-regulated in the mutant breed. Future efforts to unveil the regulatory mechanism between pigment gland and gossypol of cotton will help clarify these unique aspects.

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