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BRIEF COMMUNICATION

Expression of a phenylcoumaran benzylic ether reductase-like protein in the ovules of *Gossypium hirsutum*

R.B. TURLEY*

USDA-ARS, Crop Genetics and Production Research Unit, Stoneville, MS 38776, USA

Abstract

Two dimensional polyacrylamide gel electrophoresis (2D-PAGE) was used to identify differentially expressed proteins in wild-type (DP 5690) and fiberless (SL 1-7-1) cotton ovules. One protein, designated V2 was unique to ovules of the fiber producing DP 5690 line. The protein was purified from 2D-PAGE of 4 d post anthesis DP 5690 ovules and partially sequenced. The short amino acid sequence was nearly identical to the deduced amino acid sequence for cotton phenylcoumaran benzylic ether reductase (PCBER) protein. A consensus sequence was assembled from ESTs encoding cotton PCBER genes, primers were designed, and a full length gene was amplified from plasmid DNA from a 72 h etiolated cotton cotyledon library. The polymerase chain reaction generated a 950 bp product with unique *Eco*RI (5') and (3') *Kpn*I restriction sites for directional insertion into the expression vector pPICZA. Nucleotide sequencing was performed, and the full length coding region was 924 bp encoding a protein of 308 amino acids. The molecular mass and pI measured (2D PAGE) were similar to the theoretical protein.

Additional key words: cotton, fiber, ovule development, polyacrylamide gel electrophoresis, polymerase chain reaction.

Phenylcoumaran benzylic ether reductase (PCBER) is a member of the NmrA or the NmrA-like family (pfam05368) which include isoflavone reductase (IFR) and pinoresonol-lariciresinol reductase (Gang *et al*. 1999, Karamloo *et al*. 2001). All these reductases are related in the biosynthesis of important phenylpropanoid-derived plant defense compounds including several lignans (Lewis and Davin 1999, Dixon 1999, Karamloo *et al*. 2001, Repka 2001, Shoji *et al.* 2002, Tannert *et al*. 2003,). Plant PCBER was reported to be up-regulated by abiotic stress (Nam *et al*. 2003, Vander Mijnsbrugge *et al*. 2000), the circadian clock (up-regulated before subjective dawn, Harmer *et al.* 2000), and reported to be strongly associated with phenylpropanoid biosynthesis in young xylem, phloem and differentiating xylem ray parenchyma cells (Vander Mijnsbrugge *et al*. 2000, Kwon *et al*. 2001). In poplar, the expression of PCBER is correlated with active growth (Vander Mijnsbrugge *et al*. 2000). If this pattern holds true in other plants, PCBER would be a common protein constituent in most growing plant organs.

Two dimensional PAGE was used to identify changes in the protein profiles of cotton ovules in both a fiberless and a wild-type fiber producing line during the fiber initiation stage (Turley and Ferguson 1996). It was observed that at least 37 different proteins were developmentally, or varietally regulated during fiber initiation on ovules. One of these proteins, designated V2, was partially sequenced and found to be a cotton PCBER. This PCBER was up-regulated only in the wild type cotton ovule. One explanation may be that the PCBER is induced by *GhMybs* which are structurally similar to *R2R3-Myb* factors implicated in the up-regulation of proteins involved in phenylpropanoid biosynthesis (Loguercio *et al*. 1999). This is the first report characterizing cotton PCBER and differential regulation in a fiber producing and a fiberless line during early fiber development.

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Abbreviations: 2D PAGE - two dimensional polyacrylamide gel electrophoresis; DP - delta and pine land; EST - expressed sequence tags; IFR - isoflavone reductase; PCBER - phenylcoumaran benzylic ether reductase; PCR - polymerase chain reaction; SDS PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

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* Fax: (+662) 686 5218, e-mail: rick.turley@ars.usda.gov

Vector *TOPO TA pCR®2.1 TOPO* and *TOP10 electrocomp*™ cells were obtained from *Invitrogen* (Carlsbad, CA, USA). The *QIAquick* gel extraction kits were purchased from *Qiagen* (Valencia, CA, USA). The PCR primers were synthesized by *Integrated DNA Technologies* (Skokie, IL, USA). Inbred lines of cotton (*Gossypium hirsutum* L.), DP 5690 and SL 1-7-1, were used in this study. All plants were grown in the field and ovule ages were determined as described by Turley and Ferguson (1996).

The protein designated V2 by Turley and Ferguson (1996) was purified by 2D PAGE with modifications to their protocol. These modifications included electrophoresis of 500 µg of protein from 4 d post anthesis (DPA) ovules of DP 5690 in 4 IEF tube gels (2.0 mg total) with dimensions of 4 mm i.d. and 140 mm long. Focusing and SDS-PAGE were as described by Turley and Ferguson (1996), however, staining was performed using standard Coomassie blue R-250. The V2 protein was excised and collected in an individual tube. These gel pieces were loaded into a single well of a 15 % sodium dodecyl sulphate (SDS)-PAGE and electrophoresed overnight. SDS-PAGE gels were stained with Coomassie blue and protein band was sequenced at the Protein Structure Laboratory (University of California, Davis, CA, USA) using in gel trypsin digestion, HPLC purification, followed by the sequencing of one of the HPLC peaks (Edman degredation) with an *ABI470* sequencer. The short amino acid sequence was compared with other sequences using the *tblastn* program with the expressed sequence tags (EST) of others database with the "limit to entrez queries" set to *Gossypium* (http://ncbi.nlm.nih.gov/BLAST/). Numerous ESTs were identified and four cDNA sequences were compiled to make a consensus sequence for the V2 protein.

A full length clone encoding the PCBER protein was amplified from cDNA of a 72 h etiolated cotton cotyledon library (Ni and Trelease 1991). Two PCR primers (*Integrated DNA Technologies*) were designed to amplify the full length clone with unique *Eco*RI (5') and (3') *Kpn*I restriction sites (underlined) for directional insertion into expression vectors. The primers for the sense strand was 5'-GGAGCGAGGAATTCATGGCT GAAAAGAGCAAGGTT-3' and the anti-sense was 5'-ACGACAGGGGTACCTCAAACAAAATGACTGA GGCC-3'. Amplification using PCR was performed with 92 °C for 3 min, 35 cycles of 92 °C for 30 s and 53 °C for 1 min, 70 °C for 30 min and 4 °C for 5 min. The PCR was performed using the *DyNAzyme EXT* DNA polymerase kit (*New England Biolabs*, Beverly, MA, USA) and generated a 950 bp product. The PCR product was TA cloned into a *TOPO TA pCR®2.1 TOPO* vector and positive transformants selected by blue/white screening. Plasmid DNA was isolated using the standard alkali lysis followed by precipitation by polyethylene glycol as described by Sambrook *et al*. (1989). The full length cDNA clone was sequenced in both directions, and the protein was confirmed and analyzed for M_r and pI using *ExPASy* proteomic tools *Translate ProtParam*, (http://us.expasy.org/tools/), and *BLASTN* and *BLASTP* (http://www.ncbi.nlm.nih.gov/).

Differentially regulated proteins in cotton ovules were previously evaluated before, during and after the fiber initiation stage in a fiberless (SL 1-7-1) and a wild-type (DP 5690) line of cotton by 2D PAGE (Turley and Ferguson 1996). One protein from the 37 proteins different between the lines was designated the V2 (second varietal difference found). The V2 protein was expressed in wild-type ovules at all ages from 3 d before anthesis to 4 DPA, but was not found at any age, or in any replication of the protein profiles of the fiberless line during this same period (Fig. 1). This work was

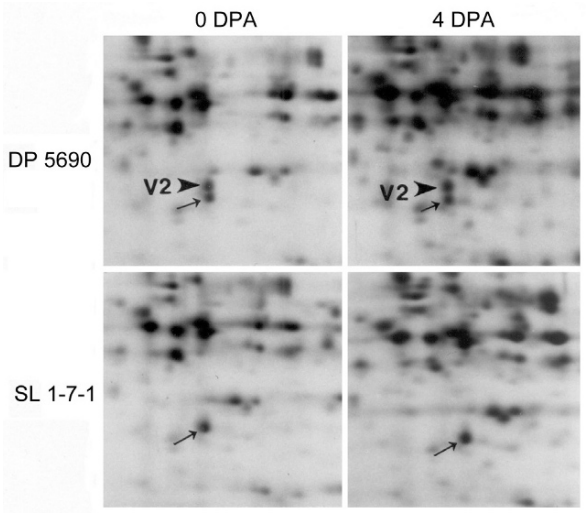

Fig. 1. Two dimensional PAGE of ovule proteins at 0 and 4 DPA from a wild-type fiber producing cotton (DP 5690) and a fiberless cotton (SL 1-7-1). *Stick arrow* pinpoints a common protein in all four gels for easy comparison. The *solid arrowhead* pinpoints the PCBER (V2) in both 0 and 4 DPA panels of wild-type line of DP5690.

Table 1. Comparison of measured and deduced protein parameters of cotton PCBER. Standard one letter abbreviations for amino acids with differences in amino acids in bold print and "-" representing indeterminate amino acids.

Feature	Comparisons
Protein sequence 1 comparison:	AQI - - AVEAEGIPYTYVPAN**S**FA/
sequenced/deduced sequence	AQIRRAVEAEGIPYTYVPAN**C**FA
Consensus sequence derived from EST's	CO083275.1, CO127815.1,
Accession numbers	DW226959.1 and CO492530.1
Cotton species	*G. hirsutum, G. arboreum, G. raimondii*
Measured M_r/theoretical M_r [kDa]	36.9/33.8
Measured pI/theoretical pI	5.72/5.76

reconfirmed in a preliminary study comparing the protein profiles of 4 DPA ovules from SL 1-7-1 and DP 5690 on immobilized pH gradient gels (*BioRad*, Hercules, CA, USA). However, these immobilized 2D PAGE did not focus as well and had fewer proteins than the original tube gels. Also compared in this study were two other fiberless lines, MD 17 (Turley and Kloth 2002) and XZ 142 w (Zhang and Pan 1991), and another wild-type line XZ 142. The study indicated that the fiberless cotton lines were deficient in the V2 protein (data not shown).

The protein was purified and prepared for sequencing by scaling up 2D PAGE to use 0.5 mg protein and wider bore IEF tubes during focusing, followed by SDS-PAGE and Coomassie staining. Protein spots (V2) were excised from the SDS-PAGE and sequenced. The amino acid sequence for a single fragment was determined to be AQI - -AVEAEGIPYTYVPANSFA where the "-" indicated the "no calls" and the underline S was a problematic read by the sequencer (Table 1). This amino acid sequence was compared to the translated *NCBI est_others* database with the "limits to entrez queries" set to *Gossypium*. Four cDNA clones were selected from the database (Table 1), and the consensus sequence was determined and used to produce primers.

The PCR primers were designed to produce a full length coding region of the V2 protein from a 72 h etiolated cotton cotyledon phagemid library (Ni and Trelease 1991). Nucleotide sequencing was performed, and the full length coding region was found to be 924 bp encoding a protein of 308 amino acids (Table 1). The theoretical molecular mass and pIs were calculated using *ExPASy* program *ProtParam* (Table 1) and compared to the reported molecular mass and pI of the V2 protein from 2D PAGE (Turley and Ferguson 1996). The measured molecular mass was approximately 3 kDa larger than the theoretical mass for the V2 protein (Table 1). This increase in mass may indicate a possible post-translation modification or, for some unknown reason, the protein's movement in the gel is retarded. The measured pI was almost identical to the calculated theoretical pI (Table 1).

A BLASTP comparison for the deduced protein sequence indicated that the V2 protein was a member of the pfam05368 (NmrA or the NmrA-like family) with the two most similar clones being a PCBER (3e-134; AAF64174.1) from *Forsynthia* × *intermedia*, and a isoflavone reductase-like protein (3e-134; CAI56333) from *Vitris vinifera*. The *BLASTN* comparison of the full length nucleotide sequence to *NCBI est_others* database indicated that the PCBER transcript (E values of 0.0) could be found in a variety of cotton tissues including cotyledons, stems, leaves, developing cotton fiber, drought stressed seedlings, and roots infected with *Fusarium oxysporum* (Dowd *et al*. 2004, Haigler *et al*. 2005, Shi *et al*. 2006, Taliercio *et al*. 2006, Udall *et al*. 2006). Finding transcripts in almost every major organ in the cotton plant indicates that PCBER is an important component in cotton development. This expression pattern could fit the putative role of cotton PCBER in the phenylpropanoid biosynthesis in lignifying cells like xylem, as has previously been reported in poplar (Vander Mijnsbrugge *et al*. 2000). The question remains, what is the role of PCBER in the fiber. The PCBER transcript was found in 5 to 10 DPA and 20 DPA fiber indicating a metabolic role for these proteins in fiber. Cotton fibers do not produce lignin and it is unknown if they produce the known PCBER substrates, dehydrodiconiferyl and dihydrodehydrodiconiferyl alcohol (Karamloo *et al.* 2001).

Van der Mijnsbrugge *et al.* (2000) reported there was a strong expression of PCBER in the seed coat which is likely due to structural lignification as well as the production of protective lignans. Therefore, cotton ovules used in this study would be an ideal location for the expression of PCBER. The ovule is actively growing and xylem and phloem are being produced as the immature seed expands. The lack of this protein (or its post-translational modification) in the fiberless line becomes an enigma. One explanation is that PCBER protein in the fiberless line may be expressed at a level below the threshold of detection on gels, or possibly it is expressed in a modified form. Another explanation is that recent work on the *R2R3 Myb* gene family indicates that many of these genes are involved in the regulation of phenylpropanoid biosynthesis, precursors of PCBER substrates (Loguercio et al. 1999). Many of these same members of the *R2R3 Myb* gene family have been implicated in the initiation and growth of trichomes (Serna and Martin 2006). Whatever the explanation for the presence of PCBER in the wild-type lines and

absence in the fiberless lines, further work is needed to identify the role of PCBER in cotton fiber initiation and growth, and if there is a relationship between *Myb* genes and up-regulation of cotton PCBER.

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