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ORIGINAL ARTICLE

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A putative plant homolog of the yeast β-1,3-glucan synthase subunit *FKS1* from cotton (*Gossypium hirsutum* L.) fibers

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Abstract A novel plant gene *CFL1* was cloned from cotton (*Gossypium hirsutum* L.) fibers by expressed sequence tag (EST) database searching and 5′-RACE (rapid amplification of cDNA ends). This gene shows sequence homology with *FKS1* which has been identified as the putative catalytic subunit of the yeast β-1,3-glucan synthase. It encodes a protein (CFL1p) of 219 kDa with 13 deduced transmembrane helices and 2 large hydrophilic domains, one of which is at the N-terminus and the other in the internal region of the polypeptide. *CFL1* displays 21% identity and 41% similarity to *FKS1* at the amino acid level over its entire length, with 31% identity and 52% similarity for the hydrophilic central domain. Using RNA and protein blot analysis, *CFL1* was found to be expressed at higher levels in cotton fibers during primary wall development. *CFL1* also had a strong expression in young roots. Using a calmodulin (CaM)-gel overlay assay, the hydrophilic N-terminal domain of CFL1p was shown to bind to CaM, while the hydrophilic central domain did not. A putative CaM-binding domain, 16 amino acids long, was predicted in the hydrophilic N-terminal domain. Moreover, a product-entrapment assay demonstrated that a protein associated with an in vitro-synthesized callose pellet could be labeled by anti-*CFL1* antibodies. Our finding suggests that *CFL1* is a putative plant homolog of the yeast β-1,3-glucan synthase subunit *FKS1* and could be involved in callose synthesis.

Keywords β-1,3-glucan · Callose synthase · Cell wall · *Gossypium hirsutum* · Transmembrane protein

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Abbreviations CaM: calmodulin · CFL1p: the *CFL1* protein · DPA: days post-anthesis · EST: expressed sequence tag · FKS1p: the *FKS1* protein · RACE: rapid amplification of cDNA ends

Introduction

β-1,3-glucans are present in plants, algae, fungi, yeast, and bacteria (for a review, see Stone and Clarke 1992). Although they are major structural components of the fungal and yeast cell walls as well as storage polysaccharides in some algae and fungi, β-1,3-glucans ("callose") normally are produced only in specialized plant cells at particular developmental stages or at specific sites of higher plants. Callose is found not only in microsporogenic and megasporogenic tissues, pollen grains, pollen tubes, endosperm, plasmodesmatal canals, sieve plates, root hairs, but also appears transiently in the cell plate during cell division. Furthermore, it is synthesized rapidly and massively in response to wounding, pathogen infection, and stress. Various functions have been attributed to this polysaccharide, such as a matrix or scaffold for the deposition of other wall constituents, a permeability barrier, and a wall-strengthening substance (Stone and Clarke 1992). The pattern and timing of callose distribution and its physiological involvement suggest that it plays an important part in plant cell growth, differentiation, and response to extracellular perturbations. In addition, β-1,3-glucans are effective macrophage-activating and tumor-inhibiting agents (Battle et al. 1998).

In plants, the biosynthetic origin of callose is thought to be a high-molecular-weight protein complex located in the plasma membrane. Because of their relatively high and stable activity, presumptive membrane-bound callose synthases from several plants have been partially purified by biochemical approaches involving product entrapment and the use of polyclonal and monoclonal antibodies. Consequently, their multi-component features have been revealed (Bulone et al. 1995; Qi et al.

1995). Based on results from UDP-glucose affinity labeling, combined with other purification methodologies, a 55 kDa polypeptide was proposed as a candidate for the catalytic subunit (Frost et al. 1989; Delmer et al. 1991; Li et al. 1993; Dhugga and Ray 1994; McCormack et al. 1997); however, the precise polypeptide composition and structural nature of callose synthase has not been clarified to date. Although annexin and sucrose synthase have been suggested to participate in the interaction with callose synthase (Andrawis et al. 1993; Amor et al. 1995), no proteins have been unambiguously characterized at the molecular level and reported to be crucial for callose synthase activity.

Callose synthesis from plants has been studied concomitantly with cellulose synthesis. Experiments on in vitro cellulose synthesis using extracted plant membrane fractions have resulted only in callose formation or limited cellulose production (Kudlicka et al. 1995). Accordingly, the question of whether callose synthase shares subunits with cellulose synthase has been raised (Jacob and Northcote 1985; Delmer 1987). Recently, separation of callose and cellulose synthase activities on native gels was reported (Kudlicka and Brown 1997). Rosette-type complexes were found in the in vitro cellulose synthase fraction, while only single subunits were present in the callose synthase fraction. This suggests that the enzyme complexes for β-1,4- and β-1,3-glucan synthesis are different (also supported by the differential immunolocalization of cellulose and callose in pollen tubes and grains; Ferguson et al. 1998).

The understanding of callose synthase genes and their function among vascular plants still is highly speculative and with opposing viewpoints. Stone as well as Blanton (Stasinopoulos et al. 1999; Blanton et al. 2000) have suggested that *Agrobacterium* and *Dictyostelium* use *CesA* homologs to produce β-1,3-glucans. Other groups (Cui et al. 1999; Doblin et al. 2000; Hong et al. 2000) have proposed that plants may use *FKS1* homologs to produce callose.

β-1,3-Glucan synthesis in yeast has had several breakthroughs. First, *FKS1* was identified as an essential component of the yeast β-1,3-glucan synthase (Douglas et al. 1994). Although definitive proof of the gene function has not been presented, several lines of evidence suggest that this gene encodes the catalytic subunit of the enzyme. A second major development was the discovery of a pivotal role for GTP-binding protein Rho1p (Qadota et al. 1996). This protein was found to be an activator of the yeast β-1,3-glucan synthase, which explains the long-known observation that the enzyme activity is stimulated by sub-micromolar concentrations of GTP. Biochemical studies with plant tissues have not yet identified a possible *FKS1* protein homolog, leaving unresolved the question of whether plants use *FKS1* homologs to produce callose. The discovery that plant cellulose synthases contain homologs of the catalytic subunit of bacterial cellulose synthases makes it reasonable to search for *FKS1* homologs in plants (Pear et al. 1996).

In this report we describe the cloning of a novel plant gene *CFL1* for "cotton *FKS1*-like 1" from cotton, based on several plant expressed sequence tag (EST) clones homologous to *FKS1*. This plant gene shows significant homology to *FKS1* at the amino acid and transmembrane topology levels. Expression patterns of *CFL1* in different tissues and the protein's potential involvement in callose synthesis were also investigated.

Materials and methods

Plant materials

Cotton (*Gossypium hirsutum* L. Texas Marker-1) plants were grown in the greenhouse under the following light and temperature conditions: 14 h of daylight (incandescent and fluorescent lamps) at 32 °C and 10 h of darkness at 20 °C. Flowers were tagged on the day of anthesis for the later determination of the age of fibers. Locules were rapidly frozen and stored in liquid nitrogen.

Searches of the EST database

A BLAST search of the GenBank database was conducted with the the National Center for Biotechnology Information (NCBI) server. *FKS1* was divided into segments of 100 amino acids with 50 amino acids overlapping between two adjacent segments. Each segment was used as a query and subjected to the advanced BLAST search algorithm with "tblastn" for the program setting and "dbest" for the GenBank database setting. Emerging EST sequences with high probability under plus reading frames were conversely used as queries to search the GenBank protein database with "blastx" for the program setting to determine which proteins display homology to them. The query ESTs, to which *FKS1* and its fungal homologs claim precedence in the degree of sequence similarity over other proteins, were selected for further molecular characterization.

Isolation of a full-length cDNA

Total RNA from cotton fibers at 14 days post-anthesis (DPA) was extracted from frozen tissues according to Hughes and Galau (1988), and mRNA was further isolated from the total RNA. A cDNA library of primary-wall-stage (14 DPA) cotton fibers was constructed in the Uni-ZAP XR λ vector (Stratagene). A degenerate primer (5′-GAC/TTTC/TAGA/GGAC/TTGGACC/TAAC/TTGGCT-3′) was designed according to the conserved region in the selected ESTs and *FKS1*, and the codon usage in cotton. It was used as the 5′ primer together with the T7 3′ primer to amplify the corresponding cotton fiber cDNA fragments from the library. A 0.9-kb polymerase chain reaction (PCR) product was cloned. Northern blot analysis was then undertaken with the total RNA from primary-wall-stage cotton fibers to detect the size of the intact transcripts. To obtain the 5′-upstream sequence to the 0.9-kb cDNA fragment, the cDNA library was screened by the GeneTrapper cDNA-positive selection system (GIBCO BRL). A PAGE-purified oligonucleotide (5′-TTAACACCAATTCCGCCTCTG-3′) at the 5′-end of the 0.9-kb cDNA fragment was used as the capturing primer. Subsequently, 5′-RACE (rapid amplification of cDNA ends) was conducted to acquire the remaining 5′-end sequence with the Marathon cDNA amplification kit (Clontech). The primers (5′-TTAACACCAATTCCGCCTCTG-3′, 5′-TACCACCAACAGAAGCACAAC-3′) for 5′-RACE were 1 kb from 3′-poly(A). The expected 5-kb RACE product was cloned. Computer analysis of sequence data was performed using the ExPASy server (http://expasy.ch). Prediction of transmembrane topology was fulfilled with the SOSUI program of the GenomeNet server (http://www.genome.ad.jp). To determine the putative calmodulin (CaM)-binding region, the CFL1p sequence was subjected to

Chou-Fasman 2y and Robson-Garnier 2y structure analyses for α-helical regions using using Mac Vector (Version 6.5, Oxford Molecular). Potential regions were plotted on a helical wheel to determine if it was consistent with the basic, amphiphilic CaM-binding motif (Zielinski 1998).

Northern blot analysis

Total RNA was isolated from 7-, 12-, 14-, 18-, 24-, and 27-DPA cotton fibers, flowers, leaves, roots and stems. RNA (20 μg) from each sample was separated in a 1.2% denaturing agarose gel containing formaldehyde. After electrophoresis, RNA was transferred to a Bio-Rad Zeta-Probe membrane. The 0.9-kb fragment was labeled with α-[^{32}P]dCTP (111 TBq/mmol, 370 MBq/ml, Amersham) by the Prime-a-Gene random labeling system (Promega). The blots were exposed to X-ray films with intensifying screens at −70 °C for 2 days.

Antibody production

A pair of primers (5′-TCCGAATTCATGTCTCGAGCTGAGGAGCTA-3′, 5′-ACCAAGCTTCAACCGATGAAAACTATGGTAAAG-3′) was designed to amplify the hydrophilic N-terminal domain of CFL1p. The PCR product was cloned into pET21a (Novagen) with a His_6 tag fused at its C terminus. The recombinant plasmid was transformed into the BL21 (DE3) strain (Novagen). His-tagged recombinant proteins were purified using the pET system manual (Novagen). The elution fraction was then dialyzed against PBS (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, pH 7.5). Anti-CFL1p polyclonal antibodies were generated by Spring Valley Laboratories. IgGs were purified with the Avid-Chrom protein A antibody purification kit (Sigma).

Western blot analysis

The membrane fraction of cotton fibers was prepared by a modified procedure of Kudlicka and Brown (1997). The crude extract was filtered through a 210-μM mesh screen to remove cell walls, and the filtrate was centrifuged at 8,000 *g* over a 60% sucrose cushion to obtain the plasma membrane-enriched fraction at the interface of buffer and sucrose cushion. The membrane protein pellet was suspended in 50 mM Mops (pH 7.5) and 0.25 M sucrose, followed by a progressive solubilization protocol in which digitonin concentrations of 0.1% and 0.5% were applied. Protein was concentrated by ultrafiltration with Centriprep columns (Amicon). For immunoblotting, 40 μg of membrane protein was loaded onto 6% SDS polyacrylamide gel. After protein transfer, nitrocellulose membrane was blocked with 5% non-fat dry milk. Immunodetection was carried out using the ECL Western blotting system (Amersham) with 1:1,000 dilution of the CFL1 antibody and preimmune serum.

Calmodulin-gel overlay assay

Detection of CaM-binding using biotinylated CaM was performed as described by Fordham-Skelton et al. (1994) with some modifications. Avidin-alkaline phosphatase (Sigma) was used to detect biotinylated CaM. A 200-ng sample of protein was loaded into each lane and separated by 10% SDS-PAGE. The membrane was immersed in detection buffer [10 μg avidin-alkaline phosphatase, 50 mM Tris-HCl (pH 7.5), 50 mM $MgCl_2$, 200 mM NaCl, 0.5 mM $CaCl_2$, 0.1% Tween-20] and allowed to incubate at 30 °C for 30 min. The membrane was then washed with TBS buffer twice for 10 min each. 1-step BCIP/NBT substrate (Pierce) was added directly to the membrane to visualize the bands. Control experiments were run with 2 mM EGTA instead of 0.5 mM Ca^{2+}. The cDNA for *Arabidopsis thaliana* CaM in pET-5a was a gift from Dr. Zielinski at the University of Illinois at Urbana-Champaign. The protein was overexpressed and purified according to his procedure available at http://www.life.uiuc.edu/zielinski/. Calmodulin was biotinylated using the procedure described by Jun et al. (1996). Biotinamidocaproate N-hydroxysuccinimide ester and calcineurin were purchased from Sigma.

Product entrapment

A 100-μg sample of 0.5% digitonin-solubilized membrane protein (with the progressive solubilization protocol) was added to 10 mM Bis-Tris-Propane-Hepes (pH 7.6), 10 mM cellobiose, 1 mM $CaCl_2$ and 1 mM UDP-glucose in a volume of 500 μl. The reaction mixture was incubated at 25 °C for 2 h with gentle shaking. The β-1,3-glucan product with entrapped proteins was collected by centrifugation at 8,000*g* for 10 min. The pellet was resuspended in SDS-PAGE sample buffer, as water was not effective to solubilize trapped proteins into the supernatant. The suspension was boiled for 5 min followed by protein gel electrophoresis and Western blotting. The entrapment product was also resuspended in 50 mM Tris-HCl (pH 7.5) and 5-μl suspensions were mounted on transmission electron microscope grids, which were labeled with either the *CFL1* antibody at a dilution of 1:100 or an anti-callose antibody at a dilution of 1:50, and later with 10 nm protein A-gold particles. Then the samples were negatively stained with uranyl acetate and observed with a Philips 420 transmission electron microscope operating at 100 kV.

Results

Isolation of the *CFL1* gene

Based on the rice ESTs (D39285 and D39085) which display a strong and preferential sequence similarity to FKS1p, we cloned a corresponding full-length cDNA from cotton fibers. It has 6,123 bp with the longest open reading frame (ORF) of 5,700 bp (GenBank accession number AF085717). This ORF encodes a protein of 1,899 amino acids with a predicted molecular mass of nearly 219 kDa, which is very close to the size of FKS1p (215 kDa). Upstream of the putative ATG start codon, there are two in-frame stop codons TAG and TGA, indicating that the 5,700-bp ORF does not begin at a more distal site. Hence, the 6.1-kb cDNA encodes a full-length gene designated *CFL1*. Its 5′- and 3′-untranslated regions are 204 and 219 bp long, respectively.

When *CFL1* was used to search the GenBank protein database, *FKS1* and its fungal homologs emerged as the only proteins (not including hypothetical proteins predicted from plant genomic sequences) showing strong sequence similarity. Hydropathy plot analysis suggests that the putative CFL1p is an integral membrane protein with 13 predicted transmembrane helices, which is in good agreement with FKS1p (Fig. 1A,B). Like FKS1p, CFL1p (pI 8.42) contains a relatively large hydrophilic domain of 84 kDa (pI 6.13) in the central region, in addition to a smaller hydrophilic domain of 64 kDa (pI 6.78) at the N-terminus (Fig. 1C). Both proteins lack a cleavable signal sequence at the N-terminus. Under the rule proposed by Hartmann et al. (1989), the hydrophilic N-terminal domain of CFL1p presumably resides in the cytoplasm. The central

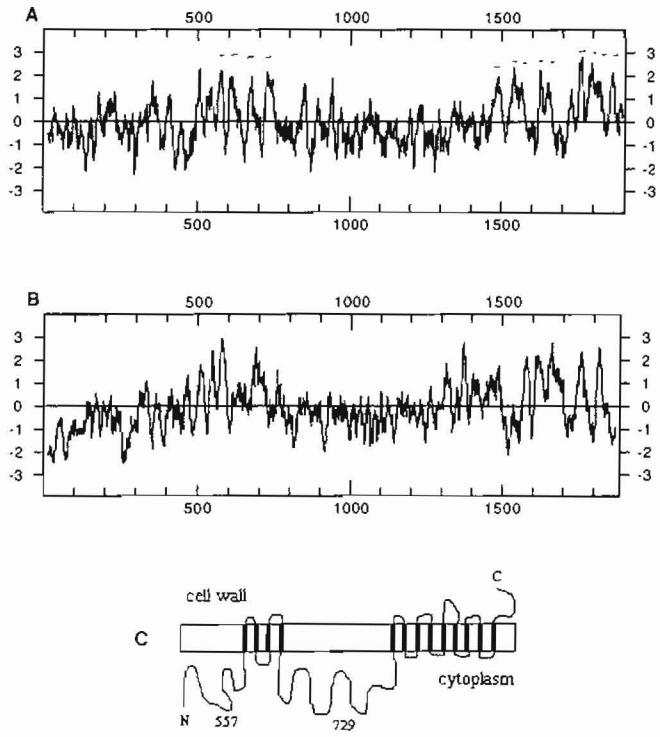

Fig. 1A–C Predicted transmembrane topology of CFL1p. **A** Kyte-Doolittle hydropathy plot of CFL1p from *Gossypium hirsutum*. The calculation was done with a window size of 15 amino acids. The number of amino acids is plotted on the *x*-axis, and hydropathy is plotted on the *y*-axis. Positive index values refer to hydrophobic regions of the protein. **B** Kyte-Doolittle hydropathy plot of FKS1p for comparison with that of CFL1p. Lines above peaks represent potential transmembrane domains. **C** Hypothetical orientation of CFL1p in the plasma membrane based on the prediction by the SOSUI program. 13 transmembrane helices are amino acids 558–580, 594–616, 657–679, 719–741, 1,471–1,492, 1,521–1,543, 1,549–1,570, 1,612–1,634, 1,636–1,658, 1,743–1,765, 1,773–1,795, 1,806–1,828, 1,846–1,868

domain is also predicted to be on the cytoplasmic side of the membrane. The overall hydropathy profiles suggest that CFL1p and FKS1p are strikingly analogous at the structural level, i.e. transmembrane topology.

Multiple sequence alignment reveals that the deduced amino acid sequence of *CFL1* displays significant homology to those of *FKS1* and its fungal homologs over its entire length (data not shown). The central cytoplasmic domain of CFL1p is more conserved compared with other regions of CFL1p. Table 1 summarizes the homology data from the comparison.

Interestingly, several consensus protein motifs are found in the CFL1p sequence. Many phosphorylation sites of cAMP/cGMP-dependent protein kinase, casein kinase II, protein kinase C and tyrosine kinase are present at various positions, as well as N-glycosylation, N-myristoylation sites, and a glycosaminoglycan attachment site.

There are now 12 potential *FKS1* homologs in *Arabidopsis* (for detailed information, see http://cellwall.stanford.edu/gsl/index.html); however, the exact copy number of *FKS1* homologs in *Arabidopsis*, the precise exon structure of these genes, and whether they actually undergo transcription, need to be verified.

Table 1 Percentage of conserved amino acids of the full-length *Gossypium hirsutum CFL1* and the central domain compared with *FKS1* (*S. cerevisiae*), *FKSA* (*A. nidulans*) and *CaCSG1* (*C. albicans*)

CFL1	*FKS1*		*FKSA*		*CaGSC1*	
	Identity	Similarity	Identity	Similarity	Identity	Similarity
Full length	21	41	21	40	22	42
Central domain	31	52	32	53	32	52

Northern blot analysis

As shown in Fig. 2, RNA samples from different cotton tissues all produced an approx. 6-kb band which is consistent with the size of *CFL1* cDNA. No other visible hybridization bands were found in the blot. A relatively strong hybridization signal was seen with RNA samples from the primary-wall-stage fibers, young roots and seedlings. The expression signal was weak with the secondary-wall-stage fibers, flowers, and leaves while a very low mRNA concentration was barely detected in 27 DPA fibers. Obviously, *CFL1* exhibits enhanced expression in young tissues.

Western blot analysis

In immunoblotting with membrane proteins solubilized by 0.5% digitonin, we detected a high-molecular-weight band of more than 200 kDa with the protein extract of 14 DPA fibers (Fig. 3, lane 2), whereas a similar signal

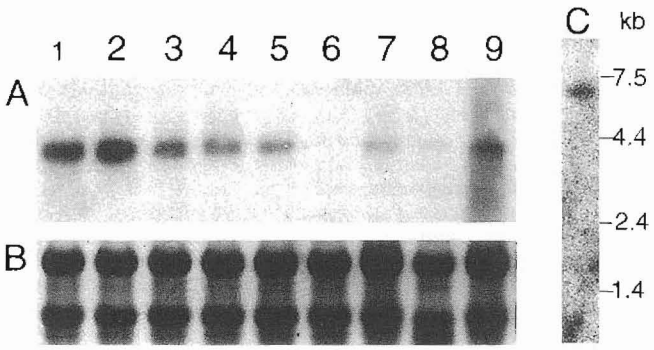

Fig. 2A–C Northern blot analysis of *CFL1* in different tissues of cotton. **A** RNA blot. **B** Ethidium bromide-stained gel to show equal loading in each lane. *Lane 1* 7-DPA fibers, *lane 2* 12-DPA fibers, *lane 3* 14-DPA fibers, *lane 4* 18-DPA fibers, *lane 5* 24-DPA fibers, *lane 6* 27-DPA fibers, *lane 7* flowers, *lane 8* leaves, *lane 9* roots **C** RNA blot with young cotton seedlings to demonstrate the size of *CFL1* mRNA. Migration of the RNA size standards is indicated on the right

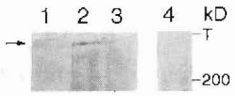

Fig. 3 Western blot analysis of CFL1p. *Lanes 1–3* CFL1 antibody, *lane 1* 14-DPA sample solubilized by 0.1% digitonin, *lane 2* 14-DPA sample solubilized by 0.5% digitonin, *lane 3* 24-DPA sample solubilized by 0.5% digitonin. *Lane 4* preimmune serum with the same sample as in lane 2. *T* represents top of the separating gel

was not distinguished with the 24 DPA fiber protein extract. The preimmune serum did not cross-react with the same sample. This suggests that the *CFL1* protein level was higher in the primary-wall stage (when *CFL1* mRNA level is high) than the secondary-wall stage (when *CFL1* mRNA level is low and cellulose synthesis is dramatically enhanced). Not surprisingly, when 0.1% digitonin-solubilized membrane proteins were applied, the signal could be barely recognized (Fig. 3, lane 1). Considering the number of transmembrane helices, 0.1% digitonin may not be efficient to solubilize CFL1p.

Calmodulin-binding assay

In our CaM-gel overlay assay, a band of 56 kDa corresponding to the N-terminal hydrophilic domain of CFL1p was detected (Fig. 4A), indicating that CaM binds to this region of CFL1p in the presence of Ca^{2+}. No band from the lane of the hydrophilic central domain of 80 kDa was found under the same conditions (Fig. 4A), indicating that this region does not bind to CaM. A 58-kDa band corresponding to the positive control, calcineurin (a CaM-binding phosphatase) was present, and no band was present at 61 kDa for cellulase as the negative control (Fig. 4A). No bands were apparent for any of the proteins in the absence of Ca^{2+} (Fig. 4B). In addition, a putative CaM-binding motif (VRAAVSALKHYRSLPK at amino acids 226–241; Zielinski 1998) was predicted from the hydrophilic N-terminal region and plotted on a helical wheel. The basic, amphiphilic α-helical pattern characteristic of conserved CaM-binding motifs was identified in this stretch of 16 amino acids. Basic and hydrophilic residues were dominant on one side of the α-helix, while hydrophobic residues were most abundant on the other side (Fig. 4C). Interestingly, this putative CaM-binding motif does not exist in the corresponding region of *FKS1* as there are two acidic amino acids (aspartic acid) that can disrupt the basic amphiphilic α -helical structure. This might explain why the yeast glucan synthase is not activated by Ca^{2+}. In addition, different *Arabidopsis FKS1* homologs display distinct similarity to *CFL1* in this 16-amino-acid domain in terms of the number of basic amino acids (data not shown). Whether this condition represents the wound-induced versus non-Ca^{2+}-regulated callose synthases remains to be studied.

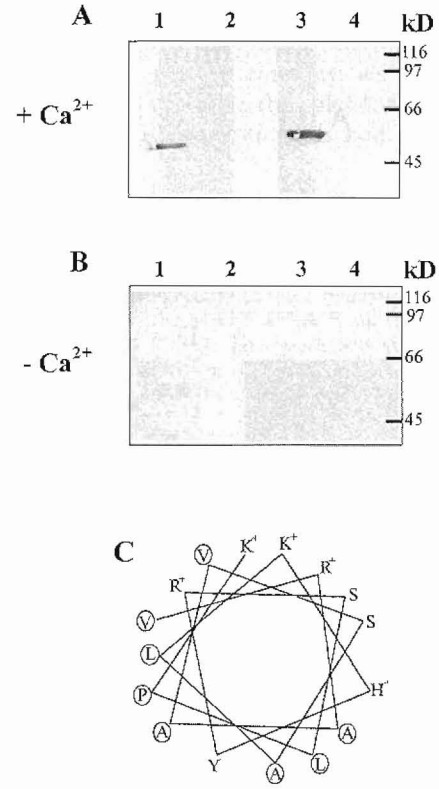

Fig. 4A–C Calmodulin binding of the hydrophilic N-terminal domain of CFL1p from cotton. CaM binding to the proteins was detected using avidin-labeled with alkaline phosphatase. **A** Gel overlay assay in the presence of 0.5 mM Ca^{2+}. **B** Gel overlay in the absence of Ca^{2+} with 2 mM EGTA. *Lane 1*, N-terminal domain of CFL1p (56 kDa), *lane 2* central domain of CFL1p (82 kDa), *lane 3* calcineurin (58 kDa, CaM-binding phosphatase), *lane 4* cellulase (61 kDa, negative control). **C** Helical wheel plot of the putative CaM-binding motif (VRAAVSALKHYRSLPK at amino acids 226–241). Basic amino acids are indicated with a positive charge and hydrophobic amino acids are circled

Product entrapment

Silver staining following SDS-PAGE of the resuspended callose pellet from the entrapment assay revealed a high-molecular-weight band larger than 220 kDa (Fig. 5A). Western blot analysis further showed that this high-molecular-weight band reacted with the *CFL1* antibody (Fig. 5A). This result suggests that CFL1p co-precipitated with the callose product and thus could be a component of the plant callose synthase. The disparity between the deduced molecular mass of CFL1p and the position of the band may be attributed to extensive covalent modification of CFL1p during fiber development such as glycosylation and myristoylation, and possibly protein aggregation during callose synthesis. In addition, callose may tightly associate with the enzyme (Antelo et al. 1998), and consequently retard the protein migration in the gel. The pellet labeled by an anti-callose antibody (Fig. 5C; also can be stained by aniline blue), was also labeled by the *CFL1* antibody (Fig. 5D,E). Controls with CBHI-gold which specifically labels

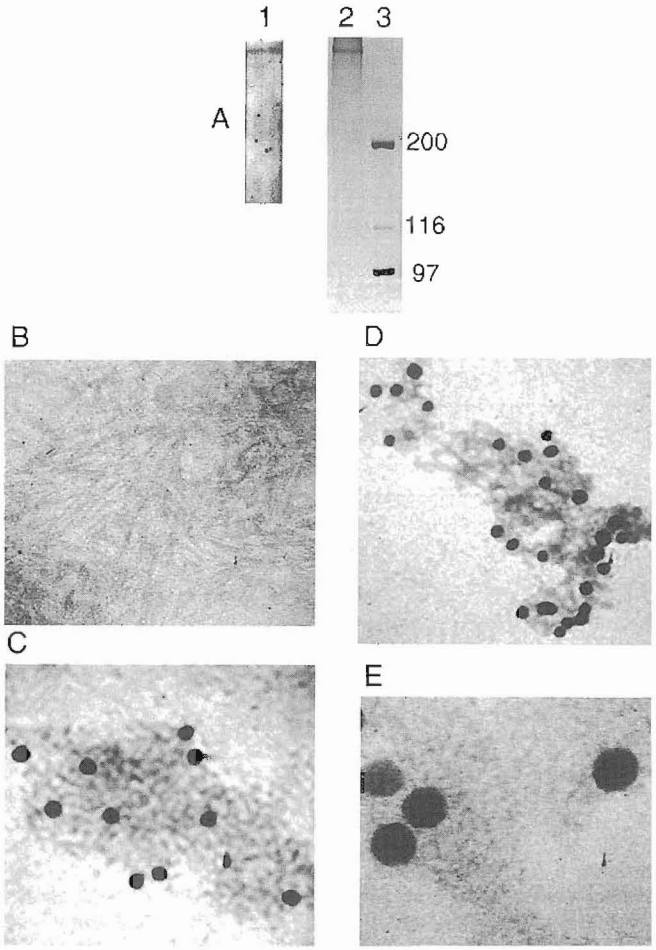

Fig. 5A–E Study of cotton fiber CFL1p using product entrapment. **A** SDS-PAGE. *Lane 1* Western blot analysis using CFL1 antibody, *lane 2* silver staining *lane 3* protein molecular weight standards. Several smaller polypeptides in SDS-PAGE are not shown here. **B** Electron micrograph (×96.000) showing the in vitro product of the entrapment assay. The morphology of the negatively stained material is characteristic of β-1,3-glucans. **C** Electron micrograph (×33,000) of the entrapment pellet labeled with an anti-callose antibody and protein A-gold particles. **D** Electron micrograph (×33,000) of the entrapment pellet labeled with *CFL1* antibody and protein A-gold particles. **E** Same as **D** but at higher magnification (×96,000) showing the gold particles appearing above the product which has the charactcristic callose morphology

cellulose, were negative with respect to the in vitro product reported here.

Discussion

The plant callose synthase is an intriguing enzyme. It is labile and has been proven difficult to purify by conventional techniques. Intensive effort has been made to identify the components of the enzyme through biochemical approaches. Although several polypeptides have been proposed as potential subunits of callose synthase, none of these has been shown to be homologous to *FKS1*. Our results indicate that the *CFL1* gene is a putative plant *FKS1* homolog.

Sequence comparison

The comparison between *CFL1* and *FKS1* is reminiscent of the sequence homology between plant and bacterial cellulose synthase catalytic subunits. The overall similarity between the cotton *CesA* and the *Acetobacter xylinum AcsAB* genes is less than 30%. The similarity is even lower in the N- and C-terminal regions (Pear et al. 1996); however, there are three internal segments, H-1, H-2, and H-3, where the sequence homology rises to over 50% at the amino acid level. Accordingly, the homology between *CFL1* and *FKS1* is comparable to that between *CesA* and *AcsAB*. Elicitation of the plant callose synthase differs considerably from the yeast counterpart in that the former is induced by micromolar levels of Ca^{2+} and millimolar concentrations of β-glucosides synergistically, while the yeast enzyme is activated by GTP. The sequence divergence between *CFL1* and *FKS1* may be indicative of distinct regulatory mechanisms for the two enzymes.

CFL1p shows similarity to FKS1p not only at the sequence level, but also at the transmembrane topology level. The central domain of CFL1p shares higher sequence homology to FKS1p than the entire length, a characteristic also found in fungal *FKS1* homologs. It is tempting to speculate that this region could be critical for the function of CFL1p and accordingly is more conserved than other parts of the protein. The consensus motif D,D,D,QXXRW (Saxena et al. 1995) involved in substrate binding and catalysis for progressive glycosyl transferases is not found in CFL1p or FKS1p. This can be explained in that either β-1,3-glucan synthase may actually utilize a different motif for substrate binding, or FKS1p and its homologs might not be the catalytic subunit. Instead, they might form a pore that exports nascent glucan chains across the plasma membrane, as their structure resembles that of some bacterial and eukaryotic transport proteins (Saier 1994).

Interestingly, protein bands of similar size to CFL1p in SDS-PAGE previously have been reported to be implicated in callose synthesis. Kudlicka and Brown (1997) found that a callose-synthesizing band in a native gel loaded with isolated plant membrane proteins barely entered the low-percentage separating gel. Polypeptides clearly larger than 200 kDa from cotton fibers were shown to be photolabeled by UDP-glucose in a Ca^{2+}-dependent manner (Delmer et al. 1991).

Expression of *CFL1*

In addition to sequence evidence, our Northern and Western blotting further provide clues for the function of *CFL1*. Not surprisingly, those EST sequences in the database that share similarity to *CFL1* were identified from roots, vascular tissues and seedlings, which contain a relatively high level of callose. Young roots consist of quickly dividing or expanding cells, and cotton fibers elongate rapidly at the primary-wall stage. Our

Northern blot analysis indicated that *CFL1* has the highest expression levels in these tissues. In agreement with RNA blot analysis, our immunoblotting further demonstrated that the cellular CFL1p concentration is higher in the fiber primary-wall stage than the secondary stage. It was previously reported that cotton fibers display maximal callose synthase activity around 18 DPA, at the time of onset of secondary wall synthesis (Maltby et al. 1979). Since *CFL1* mRNA is still relatively abundant in fibers between 14 and 18 DPA, the *CFL1* protein level at this stage may not decrease dramatically (our Western blot analysis is not extensive enough to judge where maximum protein levels occur). The oxidative burst (Potikha et al. 1999) during this period may be a key element leading to this phenomenon even though *CFL1* protein levels may not be high.

In terms of the 13 membrane-spanning segments, CFL1p is probably more hydrophobic than the catalytic subunit of cellulose synthase which contains 8 transmembrane domains. It has been reported that callose synthase is optimally solubilized by 0.5% digitonin (Kudlicka and Brown 1997), a concentration that was also effective for CFL1p in our work. Solubilization of cellulose synthase requires a lower digitonin concentration.

Calmodulin binding of CFL1p

Activation of callose synthases by Ca^{2+} has been well documented (Hayashi et al. 1987). Calmodulin is an important second messenger of the Ca^{2+} signal in plants and is regarded as the primary receptor for intracellular Ca^{2+} in all eukaryotes (Zielinski 1998). Upon wounding or pathogen attack, it is known that there is an uptake of Ca^{2+} (Kauss et al. 1991). Its binding to CaM may cause CaM to associate with the CFL1p N-terminal domain and trigger CFL1p catalytic activities by exposing the *CFL1* central domain to the cytoplasm or changing its conformation. Alternatively, CaM binding of CFL1p may increase the callose synthase affinity for UDP-glucose, diverting these same substrates from cellulose synthesis and thus making it more efficient for β-1,3-glucan synthesis. Calmodulin-binding substances have been reported to inhibit Ca^{2+}-mediated glucan synthase activities (Kauss et al. 1983). Although the addition of CaM isolated from bovine brain did not affect the in vitro glucan synthase activity, specific plant isoforms of CaM may be required for callose synthase activation.

At present, we also cannot draw any positive conclusions with respect to the functionality of a cellulose synthase performing the function of generating a β-1,3 glycosidic bond leading to callose. As mentioned above, there are opposing viewpoints and with varying degrees of evidence, none of which definitively settles this long-standing question. Mutations that inhibit cellulose formation in *Dictyostelium* also inhibit the formation of any glucan polymer, interpreted by Blanton et al. (2000) as evidence in support of the same enzyme controlling two different linkages. Perhaps bacteria and plants use dissimilar proteins for β-1,3-glucan biosynthesis. As the functional genomics picture emerges, we shall be in a much better position to define the roles for cellulose and callose biogenesis in eukaryotic cells and in particular, vascular plants.

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References

Amor Y, Haigler CH, Johnson S, Wainscott M, Delmer DP (1995) A membrane-associated form of sucrose synthase and its potential role in synthesis of cellulose and callose in plants. Proc Natl Acad Sci USA 92:9353–9357

Andrawis A, Solomon M, Delmer DP (1993) Cotton fiber annexins: a potential role in the regulation of callose synthase. Plant J 3:763–772

Antelo L, Cosio EG, Hertkorn N, Ebel J (1998) Partial purification of a GTP-insensitive (1→3)-β-glucan synthase from *Phytophthora sojae*. FEBS Lett 433:191–195

Battle J, Ha T, Li C, Della BV, Rice P, Kalbfleisch J, Browder W, Williams D (1998) Ligand binding to the (1→3)-beta-D-glucan receptor stimulates NFkappaB activation, but not apoptosis in U937 cells. Biochem Biophys Res Commun 249:499–504

Blanton RL, Fuller D, Iranfar N, Grimson MJ, Loomis WF (2000) The cellulose synthase gene of Dictyostelium. Proc Natl Acad Sci 97:2391–2396

Bulone V, Fincher GB, Stone BA (1995) In vitro synthesis of microfibrillar β-1,3-glucan by a ryegrass (*Lolium multiflorum*) endosperm β-1,3-glucan synthase enriched by product entrapment. Plant J 8:213–225

Cui X, Shin H, Brown RM Jr (1999) Abs # 184: A novel gene from cotton shows homology to the yeast β-1,3-glucan synthase subunit *FKS1*. [Abstr] American Society for Plant Physiology Annual Meeting, Baltimore

Delmer DP (1987) Cellulose biosynthesis. Annu Rev Plant Physiol 38:259–290

Delmer DP, Solomon M, Read SM (1991) Direct photolabeling with [^{32}P]UDP-glucose for identification of a subunit of cotton fiber callose synthase. Plant Physiol 95:556–563

Dhugga KS, Ray PM (1994) Purification of 1,3-β-D-glucan synthase activity from pea tissue: two polypeptides of 55 kD and 70 kD copurify with enzyme activity. Eur J Biochem 220:943–953

Doblin MS, de Melis L, Read S, Newbigin EJ, Bacic A (2000) The cloning of two putative β-glucan synthase genes from pollen tubes of *Nicotiana alata*. Abstract 310. American Society for Plant Physiology Annual Meeting, San Diego

Douglas CM, Foor F, Marrinan JA, Morin N, Nielsen JB, Dahl AM, Mazur P, Baginsky W, Li W, El-Sherbeini M, Clemas JA, Mandala SM, Frommer BR, Kurtz MB (1994) The *Saccharomyces cerevisiae FKS1* (ETG1) gene encodes an integral membrane protein which is a subunit of 1,3-β-D-glucan synthase. Proc Natl Acad Sci USA 91:12907–12911

Ferguson C, Teeri TT, Siika-aho M, Read SM, Bacic A (1998) Location of cellulose and callose in pollen tubes and grains of *Nicotiana tabacum*. Planta 206:452–460

Fordham-Skelton AP, Safadi F, Golovkin M, Reddy ASN (1994) A nonradioactive method for isolating complementary DNAs encoding calmodulin-binding proteins. Plant Mol Biol Rep 12:358–366

Frost DJ, Read SM, Drake RR, Haley BE, Wasserman BP (1989) Identification of the UDPG-binding polypeptide of (1,3)-β-glucan synthase from a higher plant by photoaffinity labeling with 5-azido-UDP-glucose. J Biol Chem 265:2162–2167

Hartmann E, Rapoport TA, Lodish HF (1989) Predicting the orientation of eukaryotic membrane-spanning proteins. Proc Natl Acad Sci USA 86:5786–5790

Hayashi T, Read SM, Bussell J, Thelen MT, Lin F-C, Brown RM Jr, Delmer DP (1987) UDP-glucose: (1→3)-β-glucan synthases from mung bean and cotton. Differential effects of Ca^{2+} and Mg^{2+} on enzyme properties and on macromolecular structure of the glucan product. Plant Physiol 83:1054–1062

Hong Z, Delauney AJ, Olson JM, Verma DPS (2000) Multiple forms of callose synthases in plants: callose synthase-1 interacts with UDP-glucosyltransferase and phraginoplastin on forming cell plate. Abstract 324, American Society for Plant Physiology Annual Meeting, San Diego

Hughes DW, Galau G (1988) Preparation of RNA from cotton leaves and pollen. Plant Mol Biol Rep 6:253–257

Jacob SR, Northcote DH (1985) In vitro glucan synthesis by membranes of celery petioles: the role of the membrane in determining the linkage formed. J Cell Sci [Suppl] 2:1–11

Jun T, Shupin W, Juan B, Sun D (1996) Extracellular calmodulin binding proteins in plants: purification of a 21-kDa calmodulin-binding protein. Planta 198:510–516

Kauss H, Köhle H, Jeblick W (1983) Proteolytic activation and stimulation by Ca^{2+} of glucan synthase from soybean cells. FEBS Lett 158:84–88

Kauss H, Waldmann T, Jeblick W, Takemoto JY (1991) The phytotoxin syringomycin elicits Ca^{2+}-dependent callose synthesis in suspension-cultured cells of *Catharanthus roseus*. Physiol Plant 81:134–138

Kudlicka K, Brown RM Jr (1997) Cellulose and callose biosynthesis in higher plants. I. Solubilization and separation of (1→3) and (1→4)-β-glucan synthase activities from mung bean. Plant Physiol 115:643–656

Kudlicka K, Brown RM Jr, Li L, Lee JH, Shin S, Kuga S (1995) β-Glucan synthesis in the cotton fiber. IV. In vitro assembly of the cellulose I allomorph. Plant Physiol 107:111–123

Li L, Drake RR Jr, Clement S, Brown RM Jr (1993) β-glucan synthesis in the cotton fiber. III. Identification of UDP-glu-binding components of β-glucan synthases by photoaffinity labeling with [β 32P]-5′N3-UDP-glucose. Plant Physiol 101:1149–1156

Maltby D, Carpita NC, Montezinos D, Kulow C, Delmer DP (1979) β-1,3-Glucan in developing cotton fibers. Structure, localization and relationship of synthesis to that of secondary wall cellulose. Plant Physiol 63:1158–1164

McCormack BA, Gregory ACE, Kerry ME, Smith C, Bolwell GP (1997) Purification of an elicitor-induced glucan synthase (callose synthase) from suspension cultures of French bean (*Phaseolus vulgaris* L.): purification and immnolocation of a probable M_r-65,000 subunit of the enzyme. Planta 203:196–203

Pear JR, Kawagoe Y, Schreckengost WE, Delmer DP, Stalker DM (1996) Higher plants contain homologs of the bacterial *CesA* genes encoding the catalytic subunit of cellulose synthase. Proc Natl Acad Sci USA 93:12637–12642

Potikha TS, Collins CC, Johnson DI, Delmer DP, Levine A (1999) The involvement of hydrogen peroxide in the differentiation of secondary walls in cotton fibers. Plant Physiol 119:849–858

Qadota H, Python CP, Inoue SB, Arisawa M, Anraku Y, Zheng Y, Watanabe T, Levin DE, Ohya T (1996) Identification of yeast Rho1p GTPase as a regulatory subunit of 1,3-β-glucan synthase. Science 272:279–281

Qi X, Tai CY, Wasserman BP (1995) Plasma membrane intrinsic proteins of *Beta vulgaris*. Plant Physiol 108:387–392

Saier MH Jr (1994) Computer-aided analyses of transport protein sequences: gleaning evidence concerning function, structure, biogenesis, and evolution. Microbiol Rev 58:71–93

Saxena IM, Brown RM, Fevre M, Geremia RA, Henrissat B (1995) Multidomain architecture of β-glycosyl transferases: implications for mechanism of action. J Bacteriol 177:1419–1424

Stasinopoulos SJ, Fisher PR, Stone BA, Stanisich VA (1999) Detection of two loci involved in (1–>3)-beta-glucan (curdlan) biosynthesis by *Agrobacterium* sp. ATCC31749, and comparative sequence analysis of the putative curdlan synthase gene. Glycobiology 9:31–41

Stone BA, Clarke AE (1992) Chemistry and biology of (1→3)-β-glucans. La Trobe University Press, Victoria, Australia, pp 365–429

Zielinski RE (1998) Calmodulin and calmodulin-binding proteins in plants. Annu Rev Plant Physiol Plant Mol Biol 49:697–725