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Higher plants contain homologs of the bacterial *celA* genes encoding the catalytic subunit of cellulose synthase

(cotton/rice/glucan/*Acetobacter xylinum*/*Gossypium hirsutum*)

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ABSTRACT **In spite of much effort, no one has succeeded in isolating and characterizing the enzyme(s) responsible for synthesis of cellulose, the major cell wall polymer of plants. We have characterized two cotton (*Gossypium hirsutum*) cDNA clones and identified one rice (*Oryza sativa*) cDNA that are homologs of the bacterial *celA* genes that encode the catalytic subunit of cellulose synthase. Three regions in the deduced amino acid sequences of the plant *celA* gene products are conserved with respect to the proteins encoded by bacterial celA genes. Within these conserved regions, there are four highly conserved subdomains previously suggested to be critical for catalysis and/or binding of the substrate UDP-glucose (UDP-Glc). An overexpressed DNA segment of the cotton *celA1* gene encodes a polypeptide fragment that spans these domains and binds UDP-Glc, while a similar fragment having one of these domains deleted does not. The plant *celA* genes show little homology at the N- and C-terminal regions and also contain two internal insertions of sequence, one conserved and one hypervariable, that are not found in the bacterial gene sequences. Cotton *celA1* and *celA2* genes are expressed at high levels during active secondary wall cellulose synthesis in developing cotton fibers. Genomic Southern blot analyses in cotton demonstrate that *celA* forms a small gene family.**

Numerous efforts have been directed toward the study of synthesis of cellulose (1,4-β-D-glucan) in higher plants. However, hampered by low rates of activity *in vitro*, the cellulose synthase of plants has resisted purification and detailed characterization (for reviews, see refs. 1 and 2). Aided by the discovery of cyclic-di-GMP as a specific activator, the cellulose synthase of the bacterium *Acetobacter xylinum* can be easily assayed *in vitro* and has been purified to homogeneity, and a catalytic subunit has been identified (for reviews, see refs. 2 and 3). Furthermore, an operon of four genes involved in cellulose synthesis in *A. xylinum* has been cloned (4–7). Characterization of these genes indicates that the first gene, termed either *BcsA* (7) or *AcsAB* (6), codes for the 83-kDa subunit of the cellulose synthase that binds the substrate UDP-Glc and presumably catalyzes the polymerization of glucose residues to form 1,4-β-D-glucan (8). The second gene (*B*) of the operon is believed to function as a regulatory subunit binding cyclic-di-GMP (9), and recent evidence suggests that the *C* and *D* genes may code for proteins that form a pore allowing secretion of the polymer and control the pattern of crystallization of the resulting microfibrils (6). Recent studies with another Gram-negative bacterium, *Agrobacterium tumefaciens*, have also led to cloning of genes involved in cellulose synthesis (10, 11). The proposed pathway of synthesis differs in some respects in *A. tumefaciens* from that of *A. xylinum*. In *A. tumefaciens*, a *celA* gene showing significant homology to the *BcsA*/*AcsAB* gene of *A. xylinum* is proposed to transfer glc from UDP-Glc to a lipid acceptor; other gene products may then build up a lipid oligosaccharide that is finally polymerized to cellulose by the action of an endoglucanase functioning in a synthetic mode. In addition, homologs of the *celA, B,* and *C* genes have been identified in *Escherichia coli*, but, as this organism is not known to synthesize cellulose *in vivo*, the function of these genes is not clear (2).

These successes in bacterial systems opened the possibility that homologs of the bacterial genes might be identified in higher plants. However, experiments in a number of laboratories utilizing the *A. xylinum* genes as probes for screening plant cDNA libraries did not lead to the identification of similar plant genes. Such lack of success suggests that, if plants do contain homologs of the bacterial genes, their overall sequence homology is not very high. Recent studies analyzing the conserved motifs common to glycosyltransferases using either UDP-Glc or UDP-GlcNAc as substrate suggest that there are specific conserved regions that might be found in plant homologs of the catalytic subunit (referred to hereafter as celA). In one of these studies, Delmer and Amor (2) identifed a motif common to many such glycosyltransferases, including the bacterial celA proteins. An independent analysis (6) also concluded that this motif was highly conserved in a group of similar glycosyltransferases. Extending these studies further, Saxena *et al.* (12) presented an elegant model for the mechanism of catalysis for enzymes such as cellulose synthase that have the perceived problem of synthesizing consecutive residues that are rotated approximately 180° with respect to each other. The model invokes independent UDP-Glc binding sites and, based upon hydrophobic cluster analysis of these enzymes, the authors concluded that three critical regions in all such processive glycosyltransferases contain a conserved Asp residue, whereas a fourth region contains a conserved QXXRW motif. The first Asp residue resides in the motif as previously analyzed (2, 6).

The developing cotton fiber is an excellent system for studies on cellulose biosynthesis as these single cells develop synchronously in the boll and, at the end of elongation, initiate the synthesis of a nearly pure cellulosic cell wall. During this transition period, synthesis of other cell wall polymers ceases and the rate of cellulose synthesis is estimated to rise nearly 100-fold *in vivo* (13). In our continuing efforts to identify genes critical to this phase of fiber development, we have initiated a program sequencing randomly selected cDNA clones derived from a library prepared from mRNA harvested from fibers at the stage in which secondary wall synthesis approaches its maximum rate [approximately 21 days post-anthesis (dpa)]. We report herein the discovery of two cotton genes that show

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Abbreviations: dpa, days post-anthesis; GST, glutathione *S*-transferase.
Data deposition: The sequences reported in this paper have been deposited in the GenBank data base (accession nos. cotton *celA1*, U58283; cotton *celA2*, U58284; and rice *celA*, D48636).
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enhanced expression at the time of onset of secondary wall synthesis in the fiber. The sequences of these two cDNA clones, termed *celA1* and *celA2*, while not identical, are highly homologous to each other and to a sequenced rice EST clone found in the dBEST databank. The deduced proteins also share signifigant regions of homology with the bacterial celA proteins. Coupled with their high level and specificity of expression in cotton fibers at the time of active cellulose synthesis, as well as the ability of an *E. coli* expressed fragment of the *celA1* gene product to bind UDP-Glc, these findings support the conclusion that these plant genes are true homologs of the bacterial *celA* genes.

MATERIALS AND METHODS

Isolation and Sequencing of cDNA Clones. We used an unamplified cDNA library prepared in the Lambda Uni-Zap vector (Stratagene) using cDNA derived from poly(A)$^+$ mRNA prepared from fibers of *Gossypium hirsutum* Acala SJ-2 harvested at 21 dpa, the time at which secondary wall cellulose synthesis is approaching a maximal rate (13). Approximately 250 plaques were randomly selected from the cDNA library, the phages were purified, and the plasmids were excised from the phage vector and transformed into *E. coli*. The resulting clones/insert sizes were determined on 0.8% agarose gels (DNA inserts less than 600 bp were excluded). Plasmid DNA inserts were randomly sequenced using an Applied Biosystems model 373A DNA sequencer. Rice EST clone S14965 was obtained from Y. Nagamura (Rice Genome Research Program, Tsukuba, Japan). A series of deletion mutants were generated and used for DNA sequencing analysis at the Weizmann Institute of Science (Rehovot, Israel).

Northern and Southern Blot Analyses. Cotton plants (*G. hirsutum* cv. Coker 130) were grown in the greenhouse, and tissues harvested at the times indicated were frozen in liquid N_2. Total cotton RNA and cotton genomic DNA was prepared and subjected to Northern and Southern analyses as described (14).

UDP-Glc Binding Studies. To construct a glutathione *S*-transferase (GST)–celA1 protein fusion, a 1.6-kb DNA *celA1* DNA fragment containing a putative cytoplasmic domain between the second and third transmembrane helices was amplified by PCR using the primers ATTGAATTCCTGGGTGTTGGATCAGTT and ATTCTCGAGTGGAAGGGATTGAAA in a reaction mixture containing 1 ng of plasmid DNA (clone 213) as template. The amplified fragment was unidirectionally cloned into the *Eco*RI and *Xho*I sites of the GST expression vector pGEX4T-3 (Pharmacia), generating a fusion protein (GST–CS) spanning Ser-215 to Leu-759 of the cotton celA1 protein. Two cotton *celA1* gene internal *Pst*I sites within the plasmid pGST-CS were used to generate the deletion mutant pGST-CSΔU1, which lacks 196 amino acids (and the U1 binding region) between Val-252 and Ala-447.

For the UDGP binding assays, α-^{32}P-labeled UDP-Glc was prepared as described (15). The two fusion proteins, GST–CS and GST–CSΔU1, were expressed in *E. coli* and purified from inclusion bodies (16). Proteins were suspended in sample buffer and heated to 100°C for 5 min, and approximately 50 ng of the two fusion protein products and molecular weight standards (Bio-Rad) were subjected to SDS/PAGE using 4.5% and 7.5% acrylamide in the stacking and separating gels, respectively (17). After electrophoresis, protein transfer to nitrocellulose filters was carried out in transfer buffer (25 mM Tris·HCl/192 mM glycine/20% methanol). The filter was briefly rinsed in deionized H_2O, incubated in PBS buffer for 15 min, and then stained with Ponceau-S in PBS buffer. After washing in deionized H_2O, protein was further renatured on the filter by incubation in PBS buffer for 30 min and used directly for binding assays. All binding buffers contained 50 mM Hepes·KOH (pH 7.3), 50 mM NaCl, and 1 mM DTT. In addition, binding buffers contained 5 mM $MgCl_2$ and 5 mM EGTA (buffer Mg^{2+}/EGTA), 5 mM EDTA (buffer EDTA), or 1 mM $CaCl_2$ and 20 mM cellobiose (buffer Ca^{2+}/CB). Binding reaction was carried out in 7 ml containing ^{32}P-labeled UDP-Glc (10^7 cpm) at room temperature for 3 h with constant shaking. Filters were washed separately for three 5-min periods in 20 ml of washing buffer (50 mM Hepes·KOH, pH 7.3/50 mM NaCl), briefly dried, and analyzed on a Bioimaging analyzer BAS1000 (Fugi).

RESULTS

Identification, Differential Expression, and Genomic Analysis of Cotton *celA* Genes. During the course of screening and sequencing of random cDNA clones from a cotton fiber cDNA library prepared from RNA collected approximately 21 dpa, we discovered two cDNA clones with small blocks of 5 or 6 amino acids that exhibited homology with the proteins encoded by the bacterial *celA* genes. Clone 213 appeared to be a full-length cDNA, whereas another distinct cDNA clone, 207, appeared to be a partial clone. These two clones were partially homologous at the nucleotide and amino acid levels and designated *celA1* and *celA2*, respectively. These clones were then used as probes for Northern blot analysis to determine their differential expression in cotton tissues and developing cotton fiber. Fig. 1 shows the expression pattern of the *celA1* gene when the entire *celA1* cDNA is used as a probe. The *celA1* gene encodes a mRNA of approximately 3.2 kb and is expressed at high levels in developing fibers, beginning at approximately 17 dpa, the time at which secondary wall cellulose synthesis is initiated (13). The gene is also expressed at low levels in all other cotton tissues, most notably in root, flower, and developing seeds. Since conserved regions of the *celA1* and *celA2* cDNAs are 70–80% homologous at the nucleotide level, gene-specific probes were designed (using the hypervariable regions described in Fig. 3) to distinguish the specific expression patterns of *celA1* and *celA2*. These gene-specific probes revealed expression patterns (data not shown) for the two genes very similar to that shown in Fig. 1, except that a very low mRNA level was also detected in the primary wall phase of fiber development (5–14 dpa) for the *celA2* gene when the blots were overexposed. The *celA2* gene-specific probe also detected a 3.2-kb mRNA, analogous in size to the

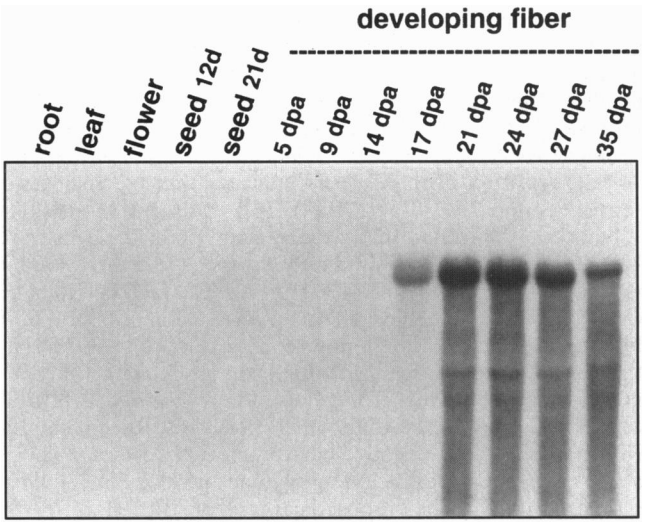

FIG. 1. Northern blot analysis of *celA1* gene in cotton tissues and developing fiber. Total RNA (10 μg) from each tissue was loaded per lane. Blots were prepared and probe preparation and hybridization were performed as described (14). The entire *celA1* cDNA insert was used as a probe in this experiment. Exposure time for the autoradiogram was 7 h at −70°C.

mRNA specified by the gene for *celA1*. mRNAs for both genes exhibit a characteristic turnover pattern similar to other mRNAs expressed in the later stages of cotton fiber development (J.R.P., unpublished observations). This observed turnover pattern for the celA mRNAs is not a result of the integrity of the mRNA preparations as these mRNAs are subjected to rapid degradation prior to RNA extraction (14). We estimate that both cotton *celA* genes are expressed in developing fiber approximately 500 times their level of expression in other cotton tissues and that they constitute 1–2% of the fiber mRNA at 24 dpa.

To estimate the number of *celA* genes in the cotton genome, Southern blot analysis was performed utilizing both *celA* cDNAs independently as probes (Fig. 2). Although the two cotton genes are approximately 65% homologous at the nucleotide level over their entire length, there are regions of homology (the H1, H2, and H3 regions described below), and it was thought these regions could be useful in identifying other cotton celA genes. Fig. 2 indicates that the *celA1* cDNA probe will hybridize, albeit weakly, to the *celA2* genomic equivalent and vise versa. The *Hin*dIII restriction pattern for both genes and cDNA probes is particularly discriminating with respect to the number of potential cotton *celA* genes. There are also a number of other weakly hybridizing bands in these digests, and from these blots, we estimate that the cotton *celA* genes constitute a small gene family (three to four genes).

Homology of Plant and Bacterial *celA* Gene Products. In addition to the two similar cotton *celA* genes, we have also found homologous sequences in the dBest data base[§] for 23 rice and 8 *Arabidopsis* ESTs. The rice clone having the longest insert (GenBank accession no. D48636) was obtained and sequenced; the homology comparisons with bacterial proteins reported herein also include results with the rice *celA*. Fig. 3 shows the results of a multiple alignment of the deduced amino acid sequences from the three plant *celA* genes and four bacterial *celA* genes from *A. xylinum* (*AcsAB* and *BcsA*), *E. coli*, and *A. tumefaciens*. Fig. 4 shows hydropathy plots (18) of cotton celA1 aligned with two bacterial celA proteins and serves as a more general summary of the overall homologies.

Of the plant genes, only the cotton *celA1* appears to be a full-length clone of 3.2 kb. It has an open reading frame that could encode for a polypeptide of 109,586 Da with a pI of 6.4 and four potential sites for N-glycosylation. Comparison of the N-terminal region of cotton celA1 with bacterial genes indicates that the plant protein has an extended N-terminal similar in length and hydropathy profile, but with low amino acid sequence homology to the *A. tumefaciens* celA protein. In general, sequence homology of plant and bacterial proteins in both the N-terminal and C-terminal regions is low. However, although overall similarity comparing plant to bacterial proteins is less than 30%, we have identified three homologous regions, designated H-1, H-2, and H-3, where the sequence homology rises to 50–60% at the amino acid level. Interspersed between these regions of homology are two plant-specific regions not found in the bacterial proteins. Sequences in the first of these insertions are highly conserved in the plant proteins (P-CR), while the second interspersed region seems to be a hypervariable regions (HVR), for there is considerable sequence divergence among the plant celA proteins analyzed.

None of the plant or bacterial celA proteins contains obvious signal sequences, even though they are presumably transmembrane proteins (4). However, the overall hydropathy profiles suggest two potential transmembrane helices in the N-terminal and six in the C-terminal region of the cotton celA1 that could anchor the protein in the membrane (see arrows Fig. 4 and also Fig. 5*A*). The amino acid sequence positions for these predicted transmembrane helices are A (aa 169–187), B (aa 200–218), C (aa 759–777), D (aa 783–801), E (aa 819–837), F (aa 870–888), G (aa 903–921), and H (aa 933–951). The central portions of the proteins are more hydrophilic and are predicted to reside in the cytoplasm and contain the site(s) of catalysis. More detailed inspection of these hydrophilic stretches reveals four particularly conserved subregions (marked U-1 through U-4 on Figs. 3 and 4) that contain the conserved Asp residues (in U-1, U-2, and U-3) and the motif QXXRW (in U-4) that have been proposed (12) to be involved in substrate binding and/or catalysis.

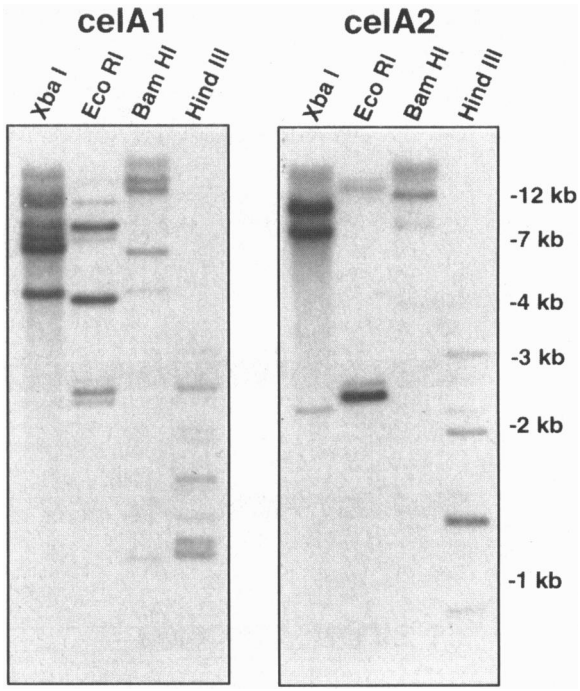

FIG. 2. Cotton genomic DNA analysis for both the *celA1* and *celA2* cDNAs. DNA (10–12 μg) was digested with the designated restriction enzymes and separated by electrophoresis on 0.9% agarose gels. Probe preparation and hybridization conditions were as described (14). The entire *celA1* and *celA2* cDNAs were utlized as probes. Exposure time for the autoradiograms was for 3 days at −70°C.

Binding of UDP-Glc. Further evidence that the proteins encoded by these plant genes are celA homologs comes from our demonstration that a DNA segment encoding the central region of the cotton celA1 protein, overexpressed in *E. coli*, binds UDP-Glc. We subcloned a 1.6-kb fragment of the cotton *celA1* clone to create a hybrid gene that encodes GST fused to the *celA1* sequence encoding aa 215–759 of the celA1 protein (Fig. 5*A*). This region spans U-1 through U-4 that are suspected to be critical for UDP-Glc binding. As a control, another GST fusion was created using a 1.0-kb fragment that had the U-1 region deleted and might not bind UDP-Glc. The fusion proteins were overexpressed in *E. coli*, purified, and shown to have the predicted sizes of approximately 87 and 64 kDa, respectively (Fig. 5*B*). The purified proteins and three standards were subjected to SDS/PAGE and blotted to nitrocellulose. Blotted proteins were suspended in buffer that facilitates partial folding and incubated with [^{32}P]UDP-Glc to test for binding (Fig. 5*B*). As predicted, the 87-kDa GST–celA1 fusion does indeed bind UDP-Glc in a Mg^{2+}-dependent manner, while the shorter fusion with the U-1 domain deleted did not show any binding (although not observed in the experiment shown, in some experiments very weak labeling in

[§]The following GenBank accession nos. were identified as showing homology with cotton *CelA-1*. For rice: D48636, D41261, D40691, D46824, D47622, D47175, D41766, D41986, D24655, D23732, D24375, D47732, D47821, D47850, D47494, D24964, D24862, D24860, D24711, D23841, D48053, D48612, D40673; for *Arabidopsis*: T45303, T45414, H76149, H36985, Z30729, H36425, T45311, A35212.

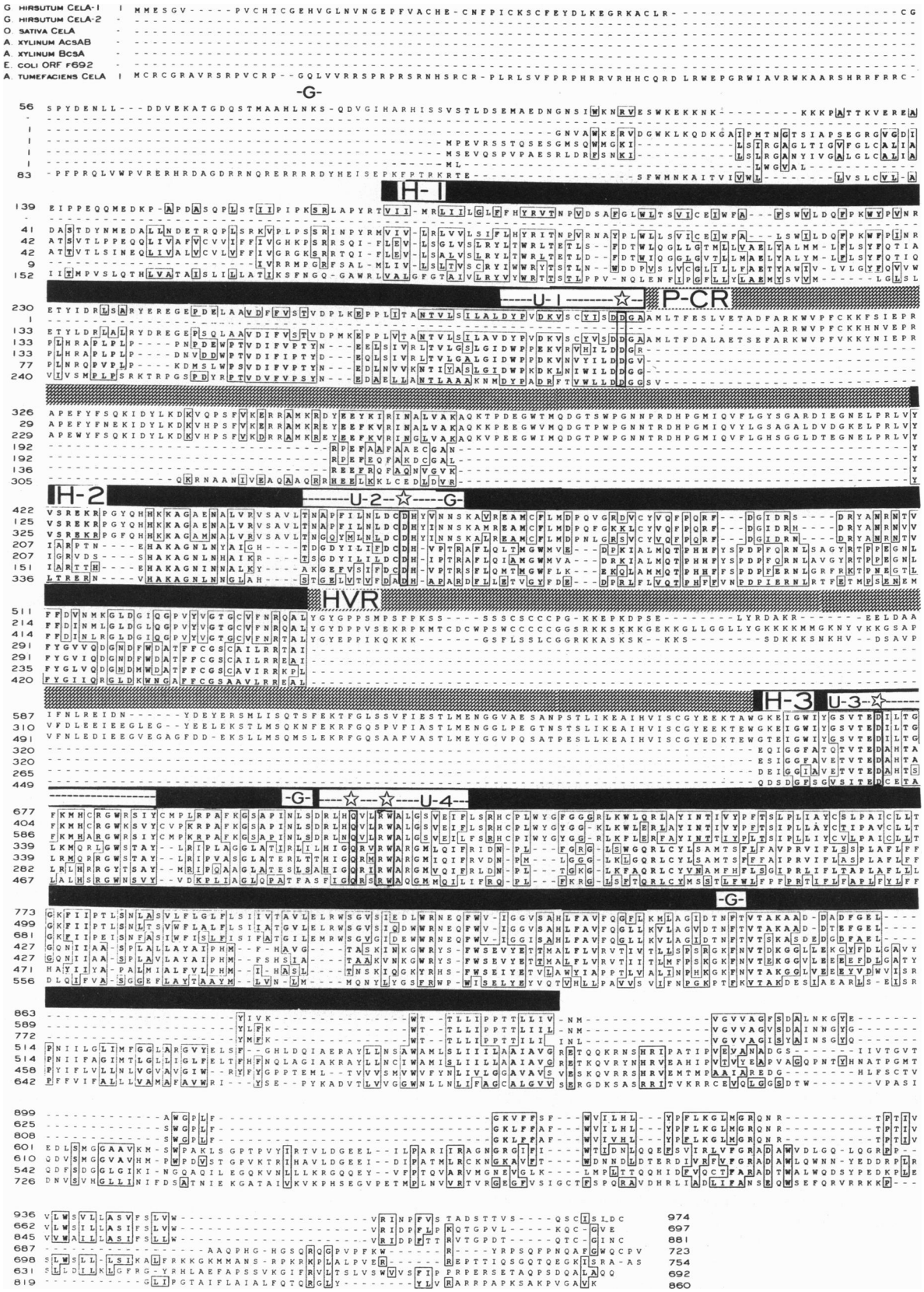

FIG. 3. Multiple alignment of deduced amino acid sequences of plant and bacterial celA proteins. Analyses were performed by Clustal analysis using the LASERGENE MULTALIGN program (DNAstar, Madison, WI) with gap and gap-length penalties of 10 and a PAM250 weight table. Residues are boxed and shaded when they show chemical group similarity in four out of seven proteins compared. H-1, H-2, and H-3 regions (solid bars) are indicated where homology between plant and bacterial proteins is highest. The plant proteins show two regions (shaded bars) that are not present in bacterial proteins. One region (P-CR) is conserved among the plant *celA* genes, while a second region is hypervariable (HVR) between plant genes. The presence of the P-CR and HVR regions led to inaccurate alignments when the entire proteins were compared; the optimal alignments shown here were thus performed in five seperate blocks. Regions U-1 through U-4 are predicted to be critical (*Legend continues on opposite page.*)

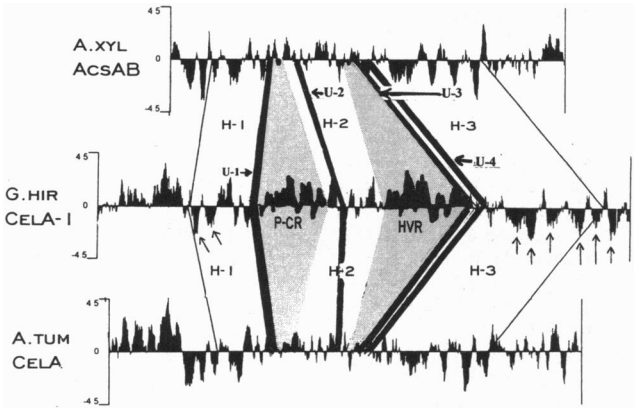

FIG. 4. Kyte–Doolittle hydropathy plots of cotton celA1 aligned with those of two bacterial celA proteins. Alignments and designations are based upon those noted in Fig. 3. The hydropathy profiles shown were calculated using a window of 7, whereas a window of 19 was used for predictions of transmembrane helices that are indicated by the arrows.

the presence of Ca^{2+} could be observed). As further controls, note that the molecular weight standards BSA and ovalbumin, proteins lacking UDP-Glc binding sites, show no interaction with UDP-Glc, while phosphorylase *b*, an enzyme known to bind UDP-Glc (19), binds this substrate.

DISCUSSION

Two cotton genes, *celA1* and *celA2*, have been shown to be highly expressed in developing cotton fibers at the onset of secondary wall cellulose synthesis. Our comparisons indicate that these genes and the rice *celA* gene encode polypeptides that have three regions of homology with bacterial celA proteins, both in terms of amino acid sequence and hydropathy. The fact that these homologous stretches are in the same sequential order as in the bacterial celA proteins and also contain four subregions predicted to be critical for substrate binding and catalysis (12) indicates that the plant genes encode true homologs of bacterial celA proteins. Furthermore, the pattern of expression in cotton fibers as well as our demonstration that at least one of these highly conserved regions is critical for UDP-Glc binding also support this conclusion. Primary wall hemicellulose synthesis ceases as secondary wall synthesis commences in the fiber, and there are only two possible β-glucans synthesized in fibers at the time these genes are highly expressed, namely, callose and cellulose (20). The following data strongly argue against the plant *celA* genes coding for callose synthase: (*i*) callose synthase binds UDP-Glc and is activated in a Ca^{2+}-dependent manner (2), whereas the celA1 polypeptide fragment containing the UDP-Glc binding site preferentially binds UDP-Glc in a Mg^{2+}-dependent manner, similar to bacterial cellulose synthase (9); (*ii*) the timing of callose synthesis *in vivo* in developing cotton fiber (20) does not match the expression of the cotton celA genes (Fig. 1); (*iii*) comparison of the *celA* gene sequences with those of suspected 1,3-β-glucan synthase genes from yeast (21) indicate no significant homology. However, it is still premature to rule out the possibility that the celA protein may encode both activities, as hypothesized some years ago (22, 23). We also lack sufficient biochemical information to speculate whether the plant celAs might be responsible for direct polymerization of glucan from UDP-Glc as proposed for *A. xylinum* or whether they may catalyze synthesis of a lipid-Glc precursor as proposed for the celA protein of *A. tumefaciens.*

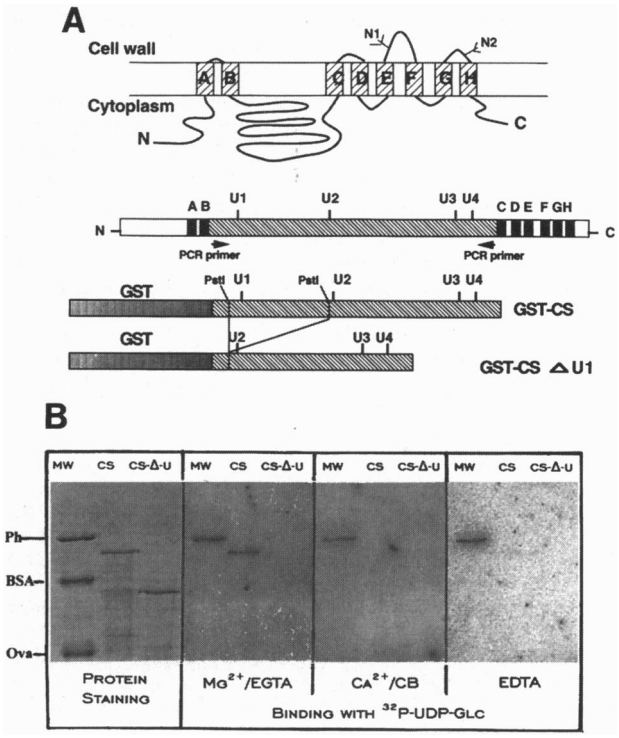

FIG. 5. GST–cotton celA1 fusion protein (spanning the U1 through U4 regions) expressed in *E. coli* binds UDP-Glc *in vitro*. (*A*) Hypothetical orientation of the cotton celA1 protein in the plasma membrane and indicates the cytoplasmic region containing the subdomains U-1 to U-4. GST fusion constructs for celA1 fragments spanning the region between the potential transmembrane helices (A through H) were prepared. The purified and blotted celA1 fusion protein fragments were tested for their ability to bind [^{32}P]UDP-Glc (*B*). MW refers to the molecular weight markers while CS and CS-Δ-U refer to the full-length and deleted GST–celA1 fusion polypeptides. The left panel shows proteins stained with Coomassie blue while the other three panels show representative autoradiograms using the different binding conditions (Mg^{2+}/EGTA, Ca^{2+}/CB, and EDTA). Ph, BSA, and Ova refer to the molecular weight standards phosphorylase *b* (97 kDa), bovine serum albumin (68 kDa), and ovalbumin (43 kDa), respectively.

In addition to their similarities, the plant celA genes show several very interesting divergences from their bacterial ancestors that may account for the previous lack of success to detect plant cDNA clones using bacterial probes. However, a BLAST search of protein data banks (24) using the entire protein sequence of cotton celA1 always showed highest homology with the bacterial cellulose synthases. Of particular interest is the insertion of two unique, plant-specific regions designated P-CR and HVR. These regions are clearly not artifacts of cloning as they are observed in both cotton genes as well as the rice *celA* gene. The three plant proteins show a high degree of amino acid homology to each other throughout most of their length, diverging only at the N- and C-terminal ends and the very interesting HVR region. It is tempting to speculate that the HVR region may confer some specificity of function; the highly charged and cysteine-rich nature of the first portion of HVR could make this region a potential candidate for interaction with specific regulatory proteins (e.g., cytoskeletal elements) or for redox regulation. In addition, we note the presence of several cysteine residues near the N- and C-terminal regions of the protein that may serve as substrates for palmytolylation and also help anchor the protein in the membrane (25).

for UDP-Glc binding and catalysisin bacterial celA proteins; the predicted critical Asp residues and QXXRW motif are boxed and starred, respectively. Potential sites of N-glycosylation are indicated by -G-.

In summary, the finding of these plant celA homologs potentially opens up an exciting chapter in research on cellulose synthesis in higher plants. This finding is of particular significance since biochemical approaches to the identification of plant cellulose synthase have proven extremely frustrating. One obvious challenge should be to gain definitive proof that these genes are truly functional in cellulose synthesis *in vivo*. Other promising goals should be to identify other components of a complex that might interact with celA, such as that proposed for sucrose synthase (26), and/or a regulatory subunit that binds cyclic-di-GMP (9, 27) or glycosyltransferases (10, 11).

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