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Evidence for a Cyclic Diguanylic Acid-Dependent Cellulose Synthase in Plants

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Because numerous attempts to detect an activity for a cellulose synthase in plants have failed, we have taken a different approach toward detecting polypeptides involved in this process. The uniqueness of the structure and function of cyclic diguanylic acid (c-di-GMP) as an activator of the cellulose synthase of the bacterium *Acetobacter xylinum* makes it an attractive probe to use in a search for a c-di-GMP receptor that might be involved in the process in plants. Direct photolabeling with ^{32}P-c-di-GMP has been used, therefore, to identify in plants two membrane polypeptides of 83 and 48 kD derived from cotton fibers that possess properties consistent with their being components of a c-di-GMP-dependent cellulose synthase. Based upon several criteria, the 48-kD species is proposed to be derived by proteolytic cleavage of the 83-kD polypeptide. Both polypeptides bind c-di-GMP with high affinity and specificity and show antigenic relatedness to the bacterial cellulose synthase, and the N-terminal sequence of the 48-kD polypeptide also indicates relatedness to the bacterial synthase. Ability to detect both cotton fiber polypeptides by photolabeling increases markedly in extracts derived from fibers entering the active phase of secondary wall cellulose synthesis. These results provide a basis for future work aimed at identifying and characterizing genes involved in cellulose synthesis in plants.

INTRODUCTION

Cellulose is a polysaccharide of great commercial importance and is the polymer synthesized in most abundance by higher plants. The regulation of the timing, quantity, and orientation of deposition of cellulose microfibrils in plant cell walls determines to a great extent patterns of cell expansion and thus, ultimately cell and tissue shape, as well as providing strength to the plant. Regrettably, no activity for a higher plant cellulose synthase has been identified (for review, see Delmer, 1987), nor is any information available in higher plants concerning the genes that control the process. However, a cellulose synthase has been well characterized in the bacterium *Acetobacter xylinum* and requires a unique activator, 3′,5′-cyclic diguanylic acid (c-di-GMP) (Ross et al., 1987; for review, see Ross et al., 1991). To date, no evidence has been available concerning a role for this activator in higher plants. However, several plant membrane polypeptides were recently shown by Mayer et al. (1991) to cross-react with an antibody against the product of the *A. xylinum* bacterial cellulose synthesis B (*bcsB*) gene, identified as the second of four genes of a cellulose synthase operon (Wong et al., 1990). This finding suggests that the pathway for cellulose synthesis might be strongly conserved during evolution and that the use of probes such as antibodies or the unique activator of the bacterial synthase, c-di-GMP, might offer a fruitful alternative approach for identifying components of the plant synthase.

Because cotton fibers are plant cells programmed to exhibit unusually high rates of cellulose synthesis in vivo, we have used this system to search for a receptor for c-di-GMP. We report here the identification of two membrane polypeptides that bind c-di-GMP and that possess properties consistent with their being components of a c-di-GMP-dependent cellulose synthase. The finding that these polypeptides show homology with a subunit of the bacterial cellulose synthase provides an indication that the pathway has been relatively conserved during evolution; because genes coding for the synthase and other functions required for the process have recently been cloned from *A. xylinum* (Wong et al., 1990; Saxena et al., 1990, 1991), it would now seem feasible to attempt the cloning of similar genes from plants using strategies based upon the assumption of conserved sequence homology.

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RESULTS

Detection of c-di-GMP-Binding Polypeptides

Membranes were prepared from cotton fibers harvested at the onset of secondary wall cellulose synthesis, and proteins solubilized from these membranes with digitonin were used for labeling studies. The data of Figure 1 show that two polypeptides of 83 and 48 kD can indeed be photolabeled with ^{32}P-c-di-GMP (lane 3) from among the numerous membrane polypeptides detected by Coomassie Brilliant Blue R 250 staining (lane 4). No labeling was detected in the absence of UV irradiation (lane 1), nor when the proteins were heated at 100°C for 2 min before labeling (lane 2).

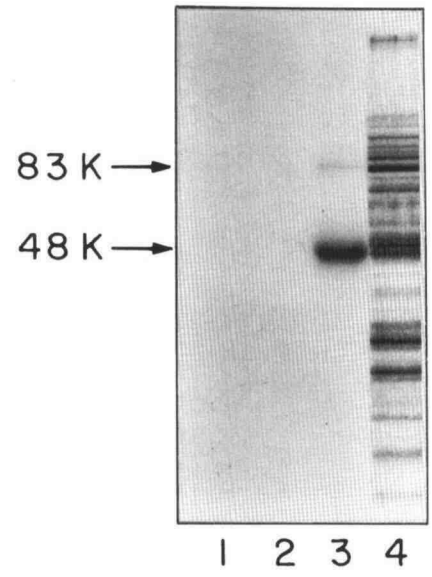

Figure 1. Direct Photolabeling of Cotton Fiber Membrane Polypeptides with ^{32}P-c-di-GMP.

Of the total digitonin-solubilized membrane proteins derived from cotton fibers entering the stage of active secondary wall cellulose synthesis (18 days postanthesis), two polypeptides of 83 and 48 kD can be photolabeled with ^{32}P-c-di-GMP (lane 3). Control samples not subjected to UV irradiation (lane 1) or that were heated 2 min at 100°C before labeling (lane 2) showed no labeling. Lane 4 shows the Coomassie blue pattern of the total membrane proteins separated by SDS-PAGE.

Antigenic Relatedness and Sequence Homology with the Bacterial Cellulose Synthase

Mayer et al. (1991) recently showed that several species of higher plants, including cotton fibers, possess membrane polypeptides that show cross-reaction with a polyclonal antibody prepared against a 561-amino acid peptide encoded by a cloned fragment of the *A. xylinum bcsB* gene, which Wong et al. (1990) concluded codes for the catalytic subunit of the cellulose synthase. Mayer et al. (1991) have further shown that this subunit has a binding site for c-di-GMP. The data of Figure 2, obtained using the same antibody as that used by Mayer et al. (1991), indicate that the polypeptides in cotton fibers that bind c-di-GMP are the same as those that show cross-reaction with this antibody. In further studies to understand the relationship of the 83- and 48-kD polypeptides to each other, we observed that digitonin-solubilized forms of these two polypeptides do not comigrate during gradient centrifugation. Labeling of gradient fractions with ^{32}P-c-di-GMP revealed that the 48-kD species migrated considerably faster than the 83-kD polypeptide (Figure 2, right panel). However, when these fractions were probed using the polyclonal antibody against the *A. xylinum bcsB* gene product, the pattern of specific reaction correlated well with the pattern of c-di-GMP labeling (Figure 2, left panel). A control blot using preimmune serum as first antibody also showed the faint reactions seen for the low molecular weight bands in fractions 1 and 2, but no reaction at all was seen in the higher molecular weight regions (not shown). Because the antibody preparation used in these studies was generated using a single, cloned gene product as antigen, the cross-reaction of both cotton fiber polypeptides suggests that these two species are antigenically related to each other as well as to the bacterial synthase and that the 48-kD species may be a proteolytic fragment of the 83-kD polypeptide.

To explore further these relationships, N-terminal sequence analysis was performed on the 48-kD polypeptide, and the results are shown in Figure 3. By examining the amino acid sequence deduced from the *bcsB* gene cloned by Wong et al. (1990), we found a sequence about halfway into the translated region that shows at least 45% homology with the N-terminal sequence of the cotton fiber 48-kD polypeptide; the homology rises to 55% if the comparison also includes amino acids showing group homology. Homology could be even greater because, in the cotton fiber 48-kD polypeptide, we could tentatively identify glutamic acid (D) at the residues that corresponded to the two glutamic acid residues of the bacterial sequence, but have not included them in Figure 3 because the identification was not definitive. We note that a comparison of the sequence of the *bcsB* gene cloned by Wong et al. (1990) with an analogous gene more recently cloned by Saxena et al. (1991) from a different strain of *Acetobacter* shows only 68% overall homology; furthermore, the comparison reveals less than 40% homology in the specific region examined in Figure 3, which is slightly less than the minimum homology observed between the cotton sequence and the corresponding one in the *bcsB* gene.

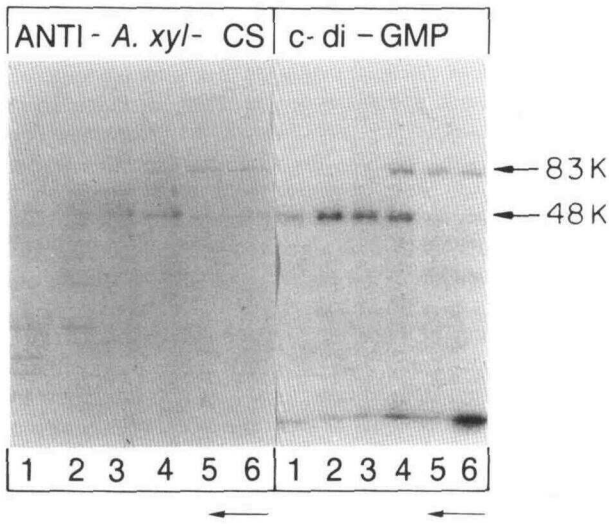

Figure 2. Photolabeling and Antibody Probing of Cotton Fiber Membrane Proteins Separated by Glycerol Gradient Centrifugation.

Solubilized membrane proteins were derived from fibers harvested 20 days postanthesis. Following centrifugation, fractions were collected, photolabeled, and separated by SDS-PAGE. The right panel shows labeling of concentrated gradient fractions with ^{32}P-c-di-GMP. The left panel shows the reaction of blotted proteins from the same fractions with antibody against the *A. xylinum bcsB* gene product (anti-*A.xyl*-CS). Arrows indicate the direction of centrifugation.

The fact that the N-terminal sequence of the cotton fiber 48-kD polypeptide shows homology only with a specific internal sequence of the bacterial polypeptide further indicates that the 48-kD species may be a proteolytic fragment of a larger gene product. We also note that a proline (P) residue is found in the bacterial sequence immediately preceding the proposed cleavage site; because proline residues create turns in polypeptide chains, this could create a loop in a relatively hydrophilic region that might be more susceptible to proteolytic attack.

Affinity and Specificity of Interaction with c-di-GMP

The data of Figures 4 and 5 show that the affinity and specificity of labeling of the cotton fiber polypeptides with ^{32}P-c-di-GMP further support the conclusion that these polypeptides are involved in cellulose synthesis. Proteins concentrated from fraction 4 of the gradient shown in Figure 2, where the 83- and 48-kD polypeptides are both present, were used for these experiments. Calculations from the data plotted in Figure 4, which correlate the relative binding of c-di-GMP to these polypeptides as a function of c-di-GMP concentration, yield an apparent K_a (defined here as the concentration required for half-maximal binding) for c-di-GMP of 24 and 13 μM for the 83- and 48-kD polypeptides, respectively. Although these values are about 1 order of magnitude higher than those required for binding (Mayer et al., 1991) and activation (Ross et al., 1990) of the bacterial synthase, they are nevertheless within the concentration range of c-di-GMP found in vivo in *A. xylinum* (Ross et al., 1991).

Figure 5 shows results of experiments in which specificity was examined by assessing the ability of related compounds to compete for c-di-GMP binding. This was determined by quantifying the intensity of labeling of the two polypeptides using 10 μM ^{32}P-c-di-GMP in the presence of a 10-fold molar excess of the unlabeled, related compounds. Under such conditions, if any of the compounds tested also bind with any significant affinity, then, when present in 10-fold excess, they should severely compete with the labeled probe and substantially reduce labeling intensity. Cyclic-di-IMP, which is an effective analog for binding (Mayer et al., 1991) and activation (Ross et al., 1990) of the bacterial synthase, decreases the labeling intensity of both the 83- and 48-kD polypeptides to approximately the same extent as that obtained with a similar excess of unlabeled c-di-GMP. However, other cyclic nucleotides that are inactive in the bacterial system (Ross et al., 1990) are much poorer competitors for labeling of both cotton fiber polypeptides. 5′-GMP, which perfectly mimics half of the symmetrical c-di-GMP molecule, is an effective competitor, particularly for the 48-kD species.

Developmental Regulation

Cellulose synthesis in cotton fibers is known to be a developmentally regulated process in which the fibers of each boll undergo a >100-fold synchronous increase in

bcsB	L	R	E	P	D	S	T	L	-	D	Q
48 K	M	R	E	I	X	S	V	L	X	X	Q

Figure 3. Comparison of Bacterial and Cotton Fiber Peptide Sequences.

The N-terminal sequence of the cotton fiber 48-kD c-di-GMP-binding polypeptide is compared with that of an internal sequence (residues 457 to 466; Wong et al., 1990) of the *A. xylinum bcsB* gene product. Sequences in black boxes indicate perfect homology, whereas those in white boxes indicate homology within a chemically similar group of amino acids. X indicates that identification was either not definitive (at the fifth and tenth residues of the cotton sequence that were only tentatively identified as D) or that no amino acid was found to increase in level for that cycle of sequencing (ninth residue of the cotton sequence).

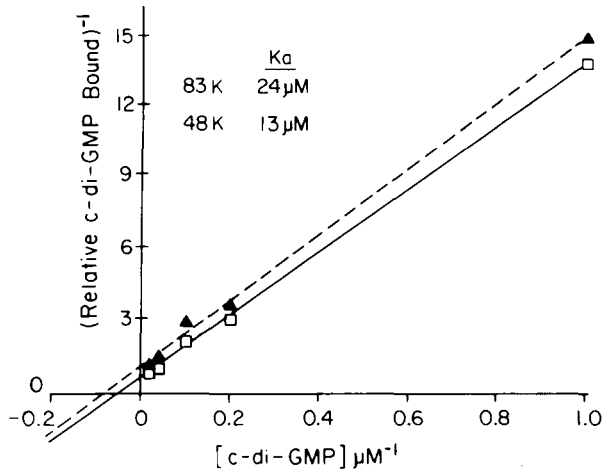

Figure 4. Affinity of the 83- and 48-kD Polypeptides for c-di-GMP.

From the data shown on the Lineweaver-Burk plot, which assesses relative binding as a function of c-di-GMP concentration, a K_a for c-di-GMP of 24 μM was calculated for the 83-kD polypeptide (▲- - -▲) and 13 μM for the 48-kD polypeptide (□——□).

the rate of cellulose synthesis as they enter the transition from primary to secondary cell wall deposition (Meinert and Delmer, 1977). Therefore, it seems logical to expect that the level of at least some of the polypeptides involved in this process may markedly increase during this transition. The results shown in Figure 6 do indeed demonstrate that the ability to detect labeling of both of the cotton fiber c-di-GMP-binding polypeptides was strongly dependent upon the developmental stage at which fibers were harvested. Neither species can be detected in young fibers engaged only in primary wall synthesis; both are first detected as fibers enter the transition to secondary wall synthesis, and the ability to label the 48-kD species further increased markedly in older fibers most actively engaged in secondary wall synthesis. Coomassie blue staining of these gels also showed a corresponding appearance and subsequent increase in the level of a moderately abundant 48-kD polypeptide that had a mobility identical to that of the labeled species (not shown).

DISCUSSION

Based upon affinity and specificity of interaction with c-di-GMP, as well as antigenic relatedness and partial sequence homology with the bacterial synthase, the 83- and 48-kD polypeptides meet important criteria for being components of a higher plant c-di-GMP-dependent cellulose synthase. The marked increase in ability to detect these polypeptides as fibers undergo the transition to secondary wall cellulose synthesis also strongly supports a role for these species in the process. Because an increase in abundance is also detected by Coomassie blue staining (at least for the 48-kD polypeptide), this implies (but does not prove) that developmental regulation of cellulose synthesis in vivo may well be at gene level. Therefore, a system such as cotton fibers, which displays marked regulation of the pathway, should be a good one for attempts to identify and study the regulation of the gene(s) coding for these polypeptides. The inability to label either species during the stage of primary wall synthesis does not necessarily indicate that these polypeptides are absent or nonfunctional at this time because the rate of cellulose synthesis in vivo is less than 1% of that occurring during secondary wall synthesis (Meinert and Delmer, 1977), and, if these polypeptides were present in a correspondingly lower level, then lack of detection would more likely be due to the insensitivity of the detection technique.

The finding that the specific region of sequence homology between the N-terminal region of the 48-kD polypeptide and the deduced amino acid sequence of the *bcsB* gene product was found at a position about halfway into the translated region of the *bcsB* gene, supports the conclusion that the 48-kD polypeptide is a C-terminal cleavage product of a larger polypeptide. Based upon the deduced amino acid composition of the *bcsB* gene product, cleavage at this region (residue 457) would yield a C-terminal fragment of about 40 kD, which compares reasonably well with the size of the 48-kD polypeptide as

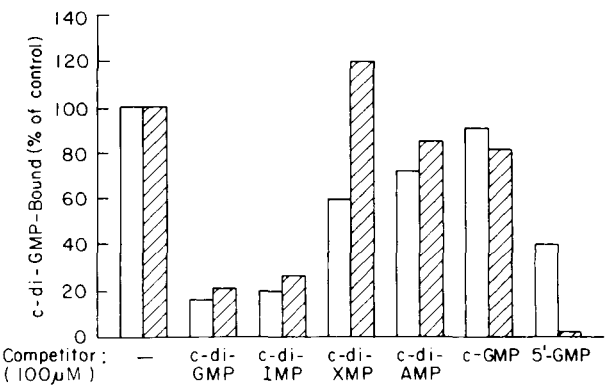

Figure 5. Specificity of Interaction of the 83- and 48-kD Polypeptides with c-di-GMP.

Specificity was assessed by the ability of a 10-fold molar excess of the unlabeled compounds indicated on the x axis to repress labeling with 10 μM ^{32}P-c-di-GMP compared with a control reaction (—), which contained no unlabeled competitor. Open bars refer to data for the 83-kD polypeptide; hatched bars represent data for the 48-kD polypeptide. X, xanthosine.

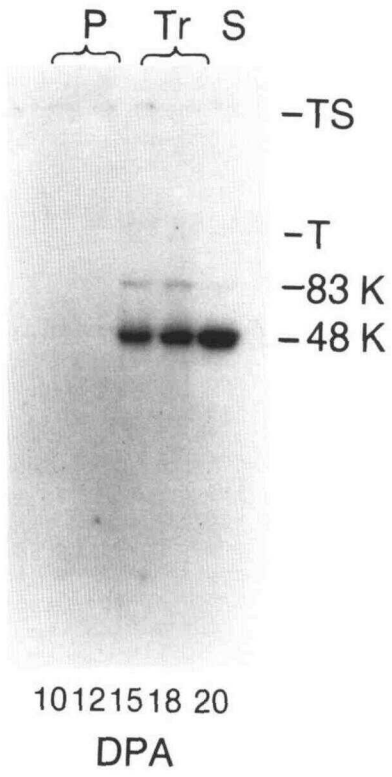

Figure 6. Developmental Pattern of Photolabeling of Cotton Fiber Membrane Polypeptides with ^{32}P-c-di-GMP.

Equal amounts of protein (15 μg) were analyzed for the various ages indicated. Stages of primary (P), transition (Tr), and secondary (S) cell wall synthesis are indicated. DPA, days postanthesis at which bolls were harvested; TS, top of stacking gel; T, top of separating gel.

estimated by SDS-PAGE. The similar affinity and specificity of c-di-GMP binding, antigenic relatedness, and pattern of developmental regulation of the 83- and 48-kD polypeptides suggest that they are related to each other, and, based upon the data available at present, the most logical conclusion is that the 48-kD species is a C-terminal fragment derived by proteolytic cleavage of the 83-kD polypeptide. If so, this would be analogous to the case in *A. xylinum*, wherein the major c-di-GMP-binding polypeptide present in purified synthase preparations was found to be a 67-kD polypeptide which, based upon sequence homology and antigenic relatedness, was concluded to be derived by proteolytic cleavage of a C-terminal 67-kD fragment from a larger product of the *bcsB* gene (Mayer et al., 1991). Although the cotton fiber 48-kD polypeptide may represent an analogous cleavage product, we have not yet found any specific protease inhibitor that can influence the labeling patterns either in cotton fibers or in *A. xylinum*.

These results suggest that renewed efforts to detect synthase activity in vitro should concentrate on conditions that include the presence of c-di-GMP during assay. We note that, at the most active stage of secondary wall cellulose synthesis, the ratio of 48-kD to 83-kD polypeptides labeled is highest (Figure 6). If the smaller 48-kD c-di-GMP-binding species found here is indeed a proteolytic fragment, then cleavage activity appears to be highest at this stage of development. If the 48-kD species is nonfunctional, then a search for conditions that inhibit this cleavage may favor preservation of activity in vitro. Alternatively, the cleavage may represent some natural processing event leading to the active enzyme; certainly an analogous cleavage product has been detected in purified preparations of the active bacterial synthase (Mayer et al., 1991).

The question arises as to whether one or both of the c-di-GMP-binding polypeptides of cotton fibers also contain a binding site for the synthase substrate UDP-Glc. Delmer et al. (1991) have found that an 84-kD membrane polypeptide is the most heavily labeled of several in cotton fibers that can be photolabeled with ^{32}P-UDP-Glc. Because that work identified a distinct 52-kD polypeptide as being the likely catalytic subunit of the other major UDP-Glc–β-glucan synthase, callose synthase, it was suggested that the 84-kD polypeptide might be the catalytic subunit of the cellulose synthase. We have observed that, during SDS-PAGE, this UDP-Glc-binding polypeptide migrates coincident with, or sometimes slightly slower than, the 83 kD c-di-GMP-binding species described herein; furthermore, in some, but not all, experiments, we have found that addition of micromolar levels of unlabeled c-di-GMP can enhance the labeling with ^{32}P-UDP-Glc; the reasons for this variability in the effect of c-di-GMP are still being explored. However, the c-di-GMP- and UDP-Glc-binding species do not exactly comigrate during gradient centrifugation (Y. Amor and D. Delmer, unpublished data), implying that they are distinct species. We note that Lin et al. (1990) identified in *A. xylinum* a polypeptide of similar size (83 kD) that bound azido-^{32}P-UDP-Glc in their most purified preparations of the cellulose synthase. This group recently cloned a gene using probes based upon its amino acid sequence (Saxena et al., 1990), and this gene bears notable homology to the *bcsA* gene cloned by Wong et al. (1990). In contrast, using a purified synthase preparation from a different strain of *A. xylinum*, Mayer et al. (1991) reported that a 54-kD fragment, presumably derived by cleavage of the *bcsB* gene product, binds UDP-Glc. Thus, further studies will be necessary to determine the relatedness between synthase subunit(s) that bind substrate and activator.

Finally, our results imply a strong conservation during evolution of the pathway for cellulose synthesis and its mode of regulation. The results further point to the exciting possibility that genes involved in cellulose synthesis can be identified in plants using sequence information derived

from the cotton fiber polypeptides and/or clones of the related genes from *A. xylinum* (Saxena et al., 1990, 1991; Wong et al., 1990) as heterologous probes.

METHODS

Isolation and Solubilization of Membrane Proteins

Bolls of cotton (*Gossypium hirsutum* Acala SJ-2) were harvested at the ages indicated in the text from field-grown plants in Israel in the summer of 1990. The locules were immediately excised, rapidly frozen in liquid N_2, and stored at −80°C until use. Fibers were separated from such locules and ground to a fine powder under liquid N_2, then extracted at 4°C in 50 mM Hepes/KOH, pH 7.3, containing 5 mM EDTA, filtered through three layers of Miracloth (Calbiochem) and centrifuged at 100,000*g* for 45 min. For the developmental studies, 5 mM $MgCl_2$ and 1 mM $CaCl_2$ were substituted for EDTA in both the extraction and solubilization buffers. This was done because it was recently found that better quantitative recovery of the labeled polypeptides was obtained under these conditions. The resulting membrane pellets were resuspended, homogenized, and briefly placed in a sonic bath at 4°C in the same buffer containing in addition 1% (w/v) digitonin (Calbiochem). Digitonin-solubilized proteins were obtained by centrifuging these extracts for 1 hr at 100,000*g*, and these were used for photolabeling.

Synthesis of and Labeling with c-di-GMP

^{32}P-c-di-GMP was synthesized enzymatically from α-^{32}P-GTP (Amersham Radiochemical Centre, Amersham, United Kingdom) and purified as described by Ross et al. (1987) and Mayer et al. (1991). Photolabeling was carried out at 4°C in reactions of 20 μL containing 15 μg of digitonin-solubilized proteins, 50 mM Hepes/KOH, pH 7.3, 10 mM $MgCl_2$, and 0.03 μM ^{32}P-c-di-GMP (1.6 Ci/μmol). Samples were irradiated for 20 min (determined to be the optimal time of labeling) with a Mineralight short-wave (254 nm) UV lamp held 4 cm above the samples. Five microliters of 4 ×-SDS-PAGE sample buffer containing 20% (v/v) mercaptoethanol was added, the samples were heated 2 min at 100°C, and proteins were separated by SDS-PAGE using the procedure of Laemmli (1970). Details concerning gel size and composition, and procedures for fixation, staining, and autoradiography are described by Delmer et al. (1991). This reference also includes a discussion of the use of direct photolabeling of plant proteins with nucleotide derivatives. Basically, the technique involves irradiation-induced generation of free radicals from the UV-absorbing nucleotide probe, which then leads to covalent coupling of the probe to its nearest-neighbor molecule. Exposure time for autoradiograms was generally 1 to 4 days, but in some cases was extended to enhance the intensity of bands when quantitative measurements were desired.

Gradient Centrifugation and Antibody Probing of Proteins

Digitonin-solubilized proteins were separated by centrifugation in a 15% to 30% glycerol gradient as described by Delmer et al. (1991). Collected fractions were concentrated at 4°C 20-fold using an Amicon pressure dialysis cell with a YM-10 filter and photolabeled as described above. Rabbit polyclonal antibody was prepared against a 561-amino acid peptide encoded by a 1683-bp DNA region of the *Acetobacter xylinum* cellulose synthase *bcsB* gene expressed in and isolated from *Escherichia coli* as described by Mayer et al. (1991). Protein gel blotting using a 1:1000 dilution of this antiserum and goat anti-rabbit alkaline phosphatase (Sigma) as second antibody was performed as described by Elthon and McIntosh (1987).

Affinity and Specificity of Labeling with c-di-GMP

For determination of the affinity of polypeptides for c-di-GMP, proteins concentrated from fraction 4 of the gradient shown in Figure 2 were photolabeled as described above using varying concentrations and specific activity of ^{32}P-c-di-GMP. This was achieved by using a constant concentration of 0.03 μM ^{32}P-c-di-GMP with increasing concentrations of unlabeled c-di-GMP, which resulted in progressive lowering of the intensity of labeling. The resulting autoradiograms of separated proteins were analyzed with a Model SL-TRFF scanning densitometer (Biomed Instruments, Fullerton, CA) to quantify results in which the scanned area was proportional to the radioactivity bound for each polypeptide. The area values were converted to "relative c-di-GMP bound" using calculations based upon the specific activity of ^{32}P-c-di-GMP present during labeling. For assessing the specificity of interaction with c-di-GMP, photolabeling conditions were similar to those above except that ^{32}P-c-di-GMP was present in all samples at 10 μM (1 μCi per reaction); the control contained no added competitor, whereas other reactions contained 100 μM of the unlabeled compound indicated. Results were quantified by scanning densitometry of the labeled polypeptides. c-di-IMP, c-di-XMP (X, xanthosine), and c-di-AMP were synthesized and purified as described by Ross et al. (1990).

N-Terminal Amino Acid Sequence Analysis

The 48-kD polypeptide, identified by its labeling with ^{32}P-c-di-GMP, reaction with the antibody against the bacterial synthase, and marked increase in abundance at the onset of secondary wall synthesis, was excised from the appropriate region of SDS-PAGE gels of separated digitonin-solubilized proteins (20 days postanthesis). (Excision of the corresponding region from separated proteins derived from fibers harvested at 10 days postanthesis showed recovery of only minute traces of protein in this region.) The excised bands were pooled, rerun on SDS-PAGE, and blotted onto Immobilon membranes (Matsudaira, 1987). An estimated 10 μg was subjected to N-terminal sequencing using an Applied Biosystems model 470A gas-phase microsequencer as described by Speicher (1989). As a precaution against artifactual N-terminal blockage, all gels were prepared a day in advance of use, and the running buffer contained 0.1 mM sodium thioglycollate. Sequencing was carried out at The Bletterman Laboratory of Macromolecular Research at The Hebrew University Medical School.

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