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# Identification of a Receptor Protein in Cotton Fibers for the Herbicide 2,6-Dichlorobenzonitrile

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## ABSTRACT

**The herbicide 2,6-dichlorobenzonitrile (DCB) is an effective and apparently specific inhibitor of cellulose synthesis in higher plants. We have synthesized a photoreactive analog of DCB (2,6-dichlorophenylazide [DCPA]) for use as an affinity-labeling probe to identify the DCB receptor in plants. This analog retains herbicide activity and inhibits cellulose synthesis in cotton fibers and tobacco cells in a manner similar to DCB. When cotton fiber extracts are incubated with [$^3$H]DCPA and exposed to ultraviolet light, an 18 kilodalton polypeptide is specifically labeled. About 90% of this polypeptide is found in the 100,000*g* supernatant, the remainder being membrane-associated. Gel filtration and nondenaturing polyacrylamide gel electrophoresis of this polypeptide indicate that it is an acidic protein which has a similar size in its native or denatured state. The amount of 18 kilodalton polypeptide detectable by [$^3$H]DCPA-labeling increases substantially at the onset of secondary wall cellulose synthesis in the fibers. A similar polypeptide, but of lower molecular weight (12,000), has been detected upon labeling of extracts from tomato or from the cellulosic alga *Chara corallina*. The specificity of labeling of the 18 kilodalton cotton fiber polypeptide, coupled with its pattern of developmental regulation, implicate a role for this protein in cellulose biosynthesis. Being, at most, only loosely associated with membranes, it is unlikely to be the catalytic polypeptide of the cellulose synthase, and we suggest instead that the DCB receptor may function as a regulatory protein for β-glucan synthesis in plants.**

Recent advances in genetic engineering of plants have established that directed modification of gene expression in higher plants is possible. Increasing efforts are being directed at identifying suitable target genes, the modification of which might lead to creation of plants with improved agronomic properties or which might exhibit novel forms of gene expression useful for gaining insights into basic metabolic process. The cell wall of plants, and particularly the polymer cellulose within that wall, represent a major sink for reduced carbon. Lacking useful mutants blocked in various aspects of cell wall synthesis, we know little about the flexibility of higher plants for surviving various modifications in wall structure. As an example, it would be useful to know to what extent cellulose:lignin ratios might be modified to enhance the quality of wood or to improve digestibility in forage crops. Changes in wall structure might also be expected to alter susceptibility of plants to disease or to modify growth characteristics. At present, only one gene responsible for synthesis of a cell wall component, that for the hydroxyproline-rich protein extensin, has been identified and cloned (4, 5). No genes involved in synthesis of the carbohydrate polymers of the wall have been identified, due primarily to the limited information available concerning the enzymes involved in plant cell wall synthesis (10). In the case of the most abundant polymer, cellulose, it has not yet been possible even to demonstrate convincing synthesis of this glucan *in vitro* (9). This report describes a strategy, alternative to enzyme characterization, for the identification of a polypeptide which may be involved in cellulose synthesis in higher plants.

The herbicide DCB[3] is now recognized as an effective and apparently specific inhibitor of cellulose synthesis in algae and higher plants (10). Applied *in vivo* in micromolar concentrations, DCB inhibits cellulose synthesis with little or no short-term effects on synthesis of noncellulosic polysaccharides (2, 17, 26, 28), nuclear division or DNA synthesis (15, 25), protein synthesis (15), respiration (26), or the *in vivo* labeling patterns of UDP-glucose, phospholipids, and nucleoside mono-, di-, and triphosphates (DP Delmer, unpublished data). It does inhibit cell wall regeneration and cytokinesis in protoplasts, presumably because these events require cellulose synthesis (15, 25), although a recent report indicating some effects on cytoplasmic organization in root hairs might indicate an effect on the cytoskeleton as well (23). DCB does not inhibit the synthesis *in vitro* by plant membrane preparations of large amounts of (1→3)-β-glucan or small amounts of (1→4)-β-glucan (2, 8), but these activities may not represent reactions involved in cellulose synthesis *in vivo* (9).

Since DCB appears to be so effective and specific in its mode of action, we reasoned that it might interact specifically with some polypeptide critically involved in cellulose synthesis. We therefore synthesized a photoreactive analog of DCB, DCPA, for use as an affinity-labeling reagent to detect such a receptor; under UV illumination, DCPA will decompose to a reactive nitrene, which should react and form a covalent link with any adjacent molecule. Preliminary experiments (8) indicated that [$^3$H]DCPA reacts specifically with an 18 kD polypeptide upon UV illumination of crude cotton fiber extracts. No labeling was observed if UV illumination was omitted, and labeling was substantially reduced in the presence of unlabeled DCB. We report here a more detailed characterization of this apparent receptor for DCB in plants and discuss possible roles for this polypeptide in the process of cellulose synthesis *in vivo*.

## MATERIALS AND METHODS

**Materials.** Cotton (*Gossypium hirsutum* Acala SJ-2) was grown in growth chambers as described by Meinert and Delmer (24). Cotton ovules with their associated fibers were cultured as described by Beasely and Ting (1). Suspension cultures of *Nico-*

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[3] Abbreviations: DCB, 2,6-dichlorobenzonitrile; DCPA, 2,6-dichlorophenylazide.

*tiana tabacum* L., line XD, were obtained from P. Filner of this Institute and grown on M-I-D medium (12). Suspension cultures of *Lycopersicon esculentum* VF-36 were obtained from B. Williams of this Institute and grown on a high-salt medium described by DuPont *et al.* (11). Both cell cultures were grown in the dark at 28°C on reciprocal shakers. *Chara corralina* was obtained from W. J. Lucas, University of California, Davis, and grown as described by Lucas and Shimmen (21). DCB was obtained from Fluka, Sepharose 4B from Pharmacia, and other standard reagent-grade chemicals were from Sigma. 2,6-Dichloro-[$^3$H]aniline was prepared on special request by New England Nuclear Corporation. U-[$^{14}$C]glucose, [$^{14}$C]-labeled protein mol wt standards, and Amplify were obtained from Amersham.

**Preparation of [$^3$H]DCPA.** 2,6-Dichloro-[$^3$H]aniline was purified by HPLC, converted to its diazo salt, and then reacted with sodium azide to produce [$^3$H]DCPA as described in detail by Cooper *et al.* (6). Following purification by HPLC, the [$^3$H]DCPA, in methanol, had a specific activity of approximately 2.4 Ci/mmol. Unlabeled DCPA was synthesized and purified similarly. [$^3$H]DCPA is stable for many months when stored in the dark in methanol at 4°C. The slow rate of decomposition (approximately 10% after several months) was monitored by TLC in the dark in n-hexane; decomposition products remained at or near the origin, while DCPA migrated at an $R_F$ of about 0.4. We have recently obtained some evidence for loss of tritium via exchange with protons in aqueous solution at low pH (<3).

**Effects of DCB and DCPA *in Vivo*.** Cotton ovules, with their associated fibers, were cultured for 17 d post-anthesis. The ovules and fibers were carefully removed from flasks and drained on paper towels, rinsed in culture medium lacking glucose, and 4 ovules per incubation were placed in 2 ml of the same medium lacking glucose with or without DCB or DCPA. DCB and DCPA were dissolved in dimethylsulfoxide and diluted into the medium to give the appropriate concentration. All incubations, including controls, were then adjusted to give a final concentration of dimethylsulfoxide of 0.4% (v/v). Following preincubation in the dark for 15 min at 30°C, [$^{14}$C]glucose (1.3 mM; 0.65 Ci/mol) was then added and incubation continued for 90 min. The ovules were then removed and washed in medium lacking glucose, and the fibers were removed from the ovules, frozen, and lyophilized. After weighing, noncellulosic material was solubilized by heating the fibers in acetic-nitric reagent and radioactivity in the insoluble cellulosic residue was determined as described in detail by Montezinos and Delmer (26). Tobacco cells, harvested 5 d after subculture, were filtered by gravity on Whatman No. 4 paper and washed 3 times with M-I-D medium lacking glucose. Aliquots (300 mg fresh weight) were then preincubated with or without inhibitors, and subsequently incubated with [$^{14}$C]glucose (1.9 mM; 2.8 Ci/mol) as described above. The cells were then collected by filtration on Whatman GF/A filters, washed in water, and radioactivity in cellulose determined as for cotton fibers.

**Labeling of Extracts with [$^3$H]DCPA.** Except where indicated, all extracts of cotton fibers were prepared using locules which were frozen in liquid $N_2$ immediately following harvest from bolls taken at 19 to 22 d post-anthesis. (Similar results were obtained using fibers harvested without freezing of locules.) Frozen fibers were removed from ovules and ground to a powder in liquid nitrogen. They were then extracted in a chilled mortar and pestle with 25 mM Hepes/KOH (pH 7.3) containing 2.5 mM $MgCl_2$, 0.5 mM phenyl methylsulfonyl fluoride, and 0.05 mM leupeptin, using 2 ml buffer for fibers derived from each locule harvested. The extract was then filtered through three layers of Miracloth. Labeling was either performed at this time or following centrifugation of the extract at 4°C for 45 min at 100,000*g* using a Beckman 80 Ti rotor. Membranes were resuspended to their original volume by homogenization in the extraction buffer described above. Although labeling was routinely performed immediately, recent experiments show that similar results can be obtained with extracts which have been stored at −80°C. Extracts of suspension-cultured tomato cells were prepared similarly, except that cells were harvested by gravity filtration on Whatman No. 4 paper, washed in extraction buffer lacking protease inhibitors, and then disrupted in extraction buffer containing $MgCl_2$ and inhibitors, using a French Press at 6000 p.s.i. *Chara* extracts were also prepared similarly using washed internode cells which were in the final stages of elongation; they were disrupted by chopping with scissors.

To label a crude extract, 100,000*g* supernatant, or membranes, under dim light, 25 μl of [$^3$H]DCPA in methanol (2 μCi; approximately 0.8 μM final concentration) were added per ml of sample, and this mixture was incubated in the dark at 4°C for 10 to 15 min. Samples were then placed in small plastic Petri dishes at 4°C and illuminated for 5 min using a Mineralight Short-Wave UV lamp (UVP Inc.) placed 3 cm above the sample, giving an intensity of approximately 400 to 500 μW/cm$^2$. (Analysis by TLC of the time course of decomposition of [$^3$H]DCPA under these conditions of illumination indicates that complete decomposition occurred within 1–2 min.) Any remaining reactive species were then quenched by addition of DTT to 10 mM. (We have recently shown that omission of this step gives similar results.)

**Analysis of Labeled Samples by PAGE.** For each lane analyzed by PAGE, a labeled sample (0.5 ml labeled crude extract, supernatant, or membranes containing 100–300 μg protein) was precipitated in four volumes of cold acetone containing 9 mM acetic acid and stored for at least 2 h at −20°C. The resulting precipitate was collected by centrifugation at 4000*g* for 5 min and resuspended with sonication in 25 to 50 μl of 0.125 M Tris-HCl (pH 6.8) containing 50 mM DTT and 10% (v/v) glycerol; samples for PAGE-SDS also contained 2% (w/v) SDS (Serva) and these were placed in a boiling water bath for 30 s following sonication. Prior to addition of DTT, protein in these samples was determined using a modification of the Lowry procedure (22). An aliquot of 5 to 15 μl of the sample was loaded per lane on 15% acrylamide gels prepared by the procedure of Laemmli (20) using a Hoeffer vertical mini-slab gel apparatus and 0.75 mm spacers. Acrylamide concentration in the stacking gel was 4.5%. Nondenaturing gels were prepared in similar fashion but lacked SDS in gels and running buffer. Following electrophoresis at 10 to 15 mamp per gel, gels were fixed for 30 min in 50% (v/v) methanol/10% (v/v) glacial acetic acid, then for 5 min in 10% methanol/5% acetic acid. The gels were then incubated with Amplify for 15 min, dried, and subjected to fluorography using Kodak X-OMAT film at −80°C. Under optimal labeling conditions, the 18 kD band was usually faintly visible within 24 to 48 h; good exposures usually required 2 weeks.

## RESULTS

**DCB Analog DCPA Mimics DCB in Its Effects *in Vivo* in Plants.** Structure-function studies with the herbicide DCB have indicated that analogs that retain a planar structure and halogen groups at positions 2 and 6 of the benzene ring retain herbicide activity (13, 14). This indicated to us that DCPA should therefore be a biologically active analog of DCB. Results shown in Table I confirm that unlabeled DCPA, like DCB, is an effective inhibitor of cellulose synthesis in cotton fibers and suspension-cultured tobacco cells. On a concentration basis, DCPA is almost as effective as the parent herbicide DCB, having a $K_i$ in the range of 0.5 to 1 μM for cotton fibers and about 10 μM for tobacco. In addition, extensive herbicide tests of DCPA using a variety of crop and weed plants indicate that DCPA is indistinguishable from DCB in its range and effectiveness as a pre-emergent herbicide (DP Delmer, G Cooper, unpublished data). Such re-

Table I. *Effects of DCB and DCPA on Cellulose Synthesis in Vivo*

All incubations were performed in the dark at 30°C in appropriate culture medium for cotton ovules and fibers (1) or tobacco cells (12), but lacking the normal carbon source. After 15 min preincubation with inhibitors, [$^{14}$C]glucose (1.9 mM, 2.8 Ci/mol for tobacco; 1.3 mM, 0.65 Ci/mol for cotton) was added and the incubation continued for 90 min. Radioactivity in cellulose was measured as described in "Materials and Methods" and Montezinos and Delmer (26).

| Inhibitor Added | Concentration | Radioactivity in Cellulose | |
|---|---|---|---|
| | | Cotton fibers | Tobacco suspension cultures |
| | μM | *cpm/mg dry wt fibers* | *cpm/mg fresh wt cells* |
| None | | 1998 | 302 |
| DCB | 1 | 895 | |
| | 3 | 426 | |
| | 10 | 279 | 143 |
| | 20 | 209 | |
| DCPA | 1 | 1087 | |
| | 3 | 733 | |
| | 10 | 356 | 147 |
| | 20 | 262 | |

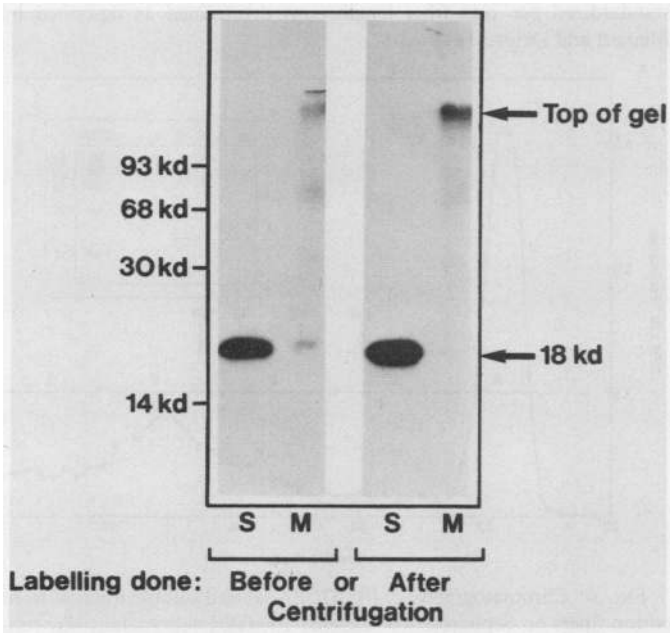


FIG. 1. Identification of 18 kD DCB receptor in cotton fibers. Figure shows a fluorogram of cotton fiber proteins separated by SDS-PAGE following labeling with [$^3$H]DCPA. S = supernatant obtained from centrifugation of crude extract at 4°C for 45 min at 100,000*g*. M = membrane fraction (pellet) obtained from such centrifugation.

sults support the notion that DCPA should interact well with the DCB receptor in plants.

**Identification of a DCB Receptor in Cotton Fibers.** Results shown in Figure 1 document that [$^3$H]DCPA preferentially interacts with, and upon UV illumination covalently couples to, an 18 kD polypeptide found in crude extracts of cotton fibers harvested at the time of onset of secondary wall cellulose synthesis, and that the majority of this polypeptide is found in the 100,000*g* supernatant of such extracts. The level of labeling of this polypeptide is pronounced, and we conclude it is specific for several reasons: for example, addition of DCB with [$^3$H]DCPA substantially reduces the label incorporated into this, but no other, band (8) (DP Delmer, unpublished data); and the protein has high affinity for DCPA since label saturates in this component, but in no other bands, at concentrations of DCPA between 1 and 5 μM (data not shown).

Nonspecific labeling does occur, particularly in the membrane fraction, as evidenced by the fact that, in terms of total radioactivity incorporated into all acetone-precipitable material, the membrane fraction contains about 3 times as much radioactivity as the supernatant. Upon prolonged exposure of the fluorograms, most of this label in the membrane fraction follows the pattern of Coomassie blue staining of the membrane tracks in the gels. Such enhanced nonspecific labeling in the membrane fraction may be due to the hydrophobic nature of DCPA and its preferential partitioning into the membranes. This may also explain why labeling of the soluble 18 kD component is more efficient if labeling is performed after the membranes are removed by centrifugation (see below). In addition to the nonspecific labeling in the membranes, about 10% of the total label found in the 18 kD polypeptide can be demonstrated in the membrane fraction, provided that labeling is carried out prior to centrifugation of the crude extract. The loss of ability to label the minor 18 kD component in the membrane following centrifugation could be explained by the presence of some factor in the supernatant required for efficient interaction of DCPA with its receptor. Some label of questionable specificity (see later section on developmental regulation and "Discussion") is also found in a membrane-associated component(s) which migrates through the stacking gel and focuses at the top of the separating gel. Inclusion of urea in the sample buffer has not resulted in enhanced mobility of this membrane-associated component.

$MgCl_2$ is routinely added to the homogenization buffer and is present during labeling, although its presence is apparently not absolutely required for interaction with DCPA (Fig. 2A). However, results of experiments shown in Figure 2 imply that some divalent cation may play a role in this interaction since the presence of EGTA (plus $Mg^{2+}$) or EDTA during labeling results in substantial reduction in ability to label the soluble 18 kD polypeptide, but has no effect on nonspecific labeling.

Interpretation of these results is complicated by several additional findings: (a) the inhibitory effect of chelators is more pronounced when labeling is performed prior to separation of membrane and soluble components, and inhibition is only observed for the soluble, and not the membrane-associated, 18 kD component (Fig. 2A); and (b) we have not been able to restore the capacity to label it in the presence of such chelators by readdition of $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, or $Zn^{2+}$, nor have we succeeded in removing any necessary divalent cation or other factor by passage of crude extracts through G-25 Sephadex; thus, following desalting on such a column equilibrated in 25 mM Hepes/KOH (pH 7.3), the 18 kD polypeptide elutes in the void volume and can be labeled successfully (not shown). We have noted, however, that purification steps such as DEAE-Sepharose or native gel electrophoresis followed by electroelution do result in loss of ability to detect any fraction which can be labeled by DCPA even though the prelabeled polypeptide can be recovered successfully from these purification steps. Thus, a possible role for a tightly bound divalent cation(s) and/or other factor in facilitating interaction of DCPA with its receptor is implied but not clarified to date. Studies with divalent cations do show, however, that the presence of 30 to 50 μM $Zn^{2+}$ ($Cl^-$ or $SO_4^{2-}$ salt; $SO_4^{2-}$ only shown) specifically inhibits labeling of the 18 kD soluble polypeptide (Fig. 2B).

**DCPA-Labeling of the 18 kD Polypeptide Parallels Rate of Cellulose Synthesis.** The fact that the majority of specific label is found associated with a soluble polypeptide indicates that it is highly unlikely that the 18 kD DCB receptor is a subunit of the

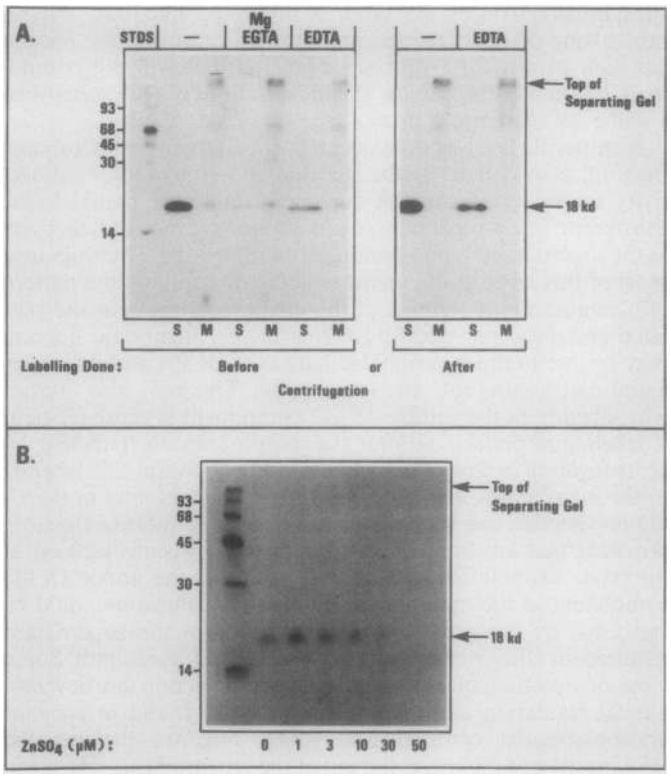


FIG. 2. Effects of divalent cations and/or chelators on labeling of DCB receptor. A, Fluorograms of cotton fiber proteins separated by SDS-PAGE following labeling with [$^3$H]DCPA in the absence of added divalent cations; or in the presence of 2.5 mM $MgCl_2$ and 5 mM EGTA; or in 5 mM EDTA. For this experiment, $MgCl_2$ was omitted from the standard homogenization buffer. B, Similar fluorogram showing the effect of various concentrations of $ZnCl_2$ on labeling of 100,000*g* cotton fiber supernatant. $MgCl_2$ was (2.5 mM) present in the homogenization buffer. The first, unmarked lane in this gel represents standard [$^{14}$C]protein markers (STDS). S and M are defined in the legend to Figure 1.

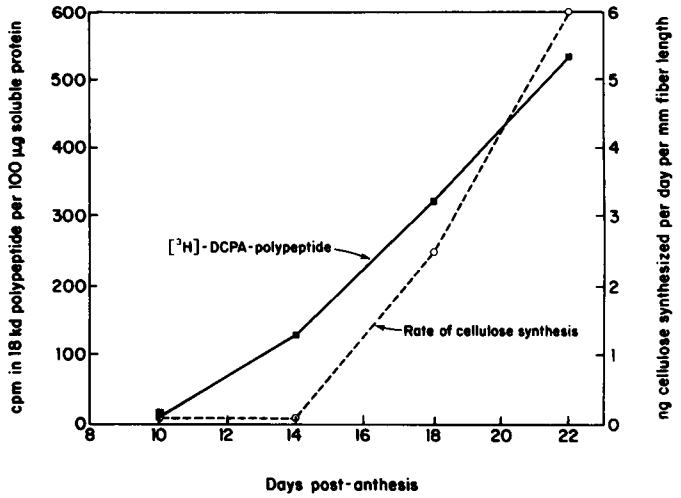


FIG. 3. Labeling of soluble 18 kD polypeptide in extracts derived from cotton fibers at various stages of fiber development. The 100,000*g* supernatants were prepared, labeled, and separated by SDS-PAGE as described in "Materials and Methods." Following fluorography, the area of the 18 kD polypeptide was cut from the dried gels and quantitated by liquid scintillation counting; cpm in this band were then standardized to cpm per 100 μg protein based on the original quantity of total protein separated in each lane of the gel. The rate of cellulose synthesis *in vivo*, standardized per mm fiber length, was determined as described by Meinert and Delmer (24).

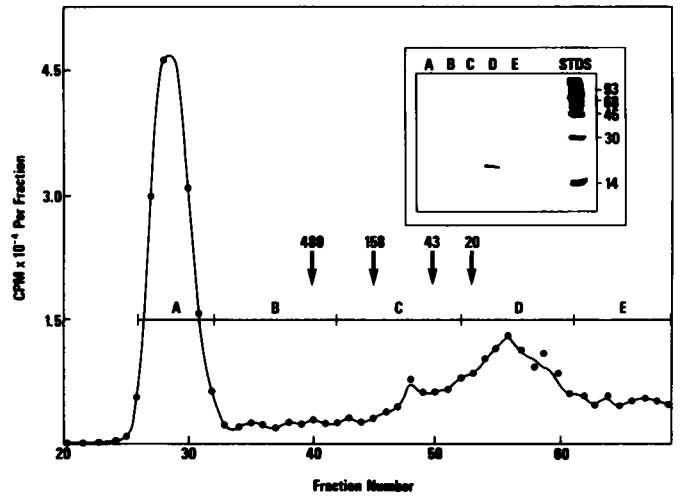


FIG. 4. Chromatography of [$^3$H]DCPA-labeled soluble fraction from cotton fibers on Sepharose 4B. Labeled 100,000*g* supernatant was concentrated by ammonium sulfate precipitation and separated at 4°C on Sepharose 4B column (2.2 × 32 cm) equilibrated in 25 mM HEPES/KOH (pH 7.2). Fractions (1 ml) were collected, pooled as indicated (A–E), precipitated with acetone, and analyzed by SDS-PAGE followed by fluorography (inset to figure). Arrows indicate the positions of elution of the molecular size markers: urease dimer (480 kD), aldolase (158 kD), ovalbumin (43 kD), and trypsin inhibitor (20 kD).

cellulose synthase, which is presumed to be an integral component of the plasma membrane (9). However, studies on the developmental regulation of the 18 kD polypeptide in developing cotton fibers do provide further support for the notion that it is somehow involved in the process of cellulose biosynthesis. These studies show that the ability to label this polypeptide increases with the increase in rate of cellulose synthesis *in vivo* in these fibers (Fig. 3). Labeled 18 kD polypeptide can barely be detected in elongating fibers when the rate of cellulose synthesis is low, and substantially increases at the onset of secondary wall cellulose synthesis. In two of three experiments, we also observed a concomitant increase in capacity to detect that labeled band in the membrane fraction which migrates at the top of the separating gel (not shown).

**Characterization of the DCB Receptor in Its Native State.** The labeled, soluble DCB receptor was found to precipitate following addition of ammonium sulfate to 70% saturation (not shown). When resolubilized and subjected to gel filtration on Sepharose 4B and various fractions pooled and analyzed by SDS-PAGE, it was found that the DCB receptor is <20 kD in its native state as well (Fig. 4, pool D). The quantity of label which elutes in the void volume (A) in such experiments was found to be variable, and the identity of this material remains uncertain; although precipitated by ammonium sulfate, it is not precipitated in acetone. We suggest that it might represent nonspecifically labeled polyphenolic material, which due to its hydrophobic nature might interact well with DCPA, be soluble in acetone, and the content of which is variable in our extracts. When the pooled fractions containing the DCPA-binding polypeptide (pool D) were concentrated and passed through a G-50 Sephadex column, the 18 kD polypeptide eluted between the void volume (≧30 kD) and a cytochrome *c* marker (mol wt 12,000).

In SDS-PAGE, the 18 kD polypeptide migrates in very close proximity to calmodulin from spinach or cotton fibers, and we considered that this polypeptide might be calmodulin, which is plausible in view of the proposed modulation of callose and

cellulose synthesis by $Ca^{2+}$ (9, 19). However, several results indicate that the DCB receptor, although similar in size and charge to calmodulin, is distinct from this regulatory polypeptide. First, purified spinach calmodulin, and a Coomassie blue-staining band of similar mobility from cotton fibers and presumed to be calmodulin, both show a distinct shift in mobility when SDS gels are prepared and run in buffer containing 1 mM $Ca^{2+}$ as opposed to similar gels run in 1 mM EDTA. This shift, which is characteristic of all calmodulins (3), is not seen for the labeled 18 kD polypeptide (not shown). Furthermore, in nondenaturing PAGE, the labeled 18 kD polypeptide clearly migrates in a position distinct from spinach calmodulin (Fig. 5). The 18 kD polypeptide, however, shows the properties, like calmodulin, of being both relatively small and acidic, since it migrates well below the majority of non-denatured cotton proteins in these 15% acrylamide gels.

**DCB Receptor in Organisms Other than Cotton.** Preliminary studies with internode cells of the cellulosic alga *C. corallina* and suspension-cultured cells of tomato indicate that a soluble polypeptide of about 12 kD becomes labeled upon incubation of 100,000*g* supernatants with [$^3$H]DCPA followed by UV illumination (Fig. 6). As for cotton fibers, label is also found at the top of the separating gel when the membrane fraction is analyzed.

## DISCUSSION

Using a photoreactive analog of DCB, we have detected an 18 kD polypeptide in developing cotton fibers which has the characteristics of a receptor for this herbicide. Since the majority of this polypeptide is found in the 100,000*g* supernatant of extracts, it seems highly unlikely that it represents the catalytic polypeptide of a cellulose synthase. A small proportion of the 18 kD polypeptide is found associated with the membrane fraction *in vitro*, and it is certainly possible that this receptor could be an easily dissociable membrane component *in vivo*. *In vivo* labeling coupled with the use of reversible cross-linking reagents may provide one future approach to studying the *in vivo* localization of this polypeptide.

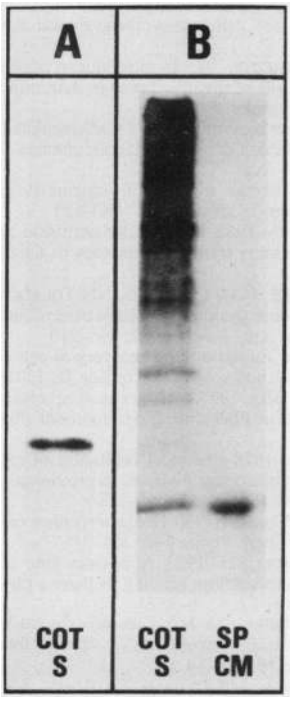


FIG. 5. Nondenaturing PAGE of [$^3$H]DCPA-labeled 100,000*g* cotton fiber supernatant. A, Fluorogram of labeled supernatant (COT S) separated on a 15% acrylamide nondenaturing gel. B, Parallel lanes containing labeled cotton fiber supernatant (COT S) or spinach calmodulin (SP CM) stained with Coomassie blue.

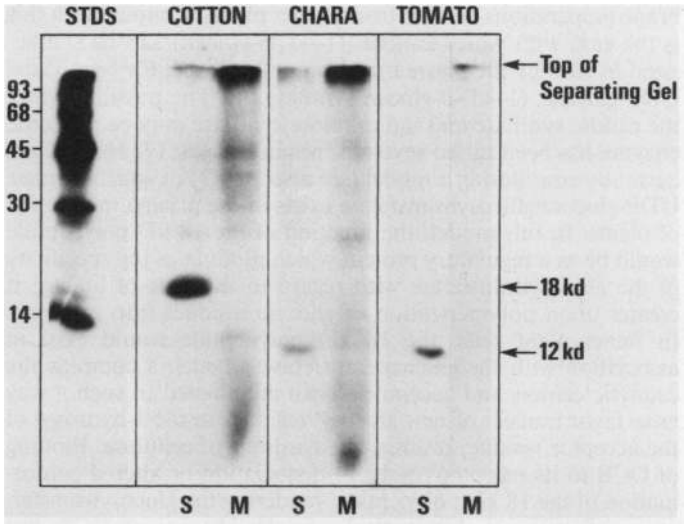


FIG. 6. Comparison of [$^3$H]DCPA-labeling patterns of extracts derived from cotton fibers, *C. corallina*, and suspension-cultured tomato cells. The 100,000*g* supernatants and membranes were prepared, labeled, and analyzed by SDS-PAGE followed by fluorography as described in "Materials and Methods." S = 100,000*g* supernatant; M = membrane fraction.

The receptor apparently exists as a monomeric 18 kD protein in its native state. Although similar in size and charge to calmodulin, it is clearly distinct from this protein. The pattern of developmental regulation of the 18 kD polypeptide in cotton fibers, coupled with the high affinity of the protein for the DCB analog, argue strongly that this protein plays some role in the process of cellulose synthesis *in vivo*.

At present, we have only detected the 18 kD polypeptide in cotton fibers harvested at or just before the onset of active secondary wall cellulose synthesis. The relationship, if any, of the 12 kD polypeptide found in *Chara* and tomato cells is not clear, although the pronounced labeling and soluble nature of this polypeptide suggests some relationship. Furthermore, we have recently selected a DCB-resistant line of these tomato cells, and preliminary experiments indicate that the capacity to label the 12 kD polypeptide is increased in these cells (DP Delmer, unpublished data). It is possible that the 12 kD polypeptide is a proteolytic fragment of an 18 kD polypeptide; we suggest this because we have observed on two occasions a shift in mobility of the cotton fiber protein to 12 kD upon prolonged standing of crude extracts, and we have recently observed that a highly purified preparation of the 18 kD polypeptide displays only two major bands on SDS gels—one at 18 kD and one at 12 kD. A minor labeled band at 12 kD is also seen in soluble fractions of cotton fiber extracts harvested at 10 d post-anthesis, an age prior to the onset of secondary wall deposition. Also uncertain is the significance, if any, of the label found in a membrane component(s) which focuses at the top of the separating gel in SDS-PAGE. Since the amount of labeling in this band, in two of three experiments, also increased with the onset of secondary wall cellulose synthesis, this might argue for a relationship to the 18 kD polypeptide. However, unlike the 18 kD polypeptide, the labeling of which is substantially reduced by the presence of unlabeled DCB (8), label in the membrane band was not affected by the presence of DCB (not shown).

In speculating on any potential function for the DCB receptor in plants, one is forced to consider the dilemma of the repeated inability to demonstrate cellulose synthesis *in vitro* using mem-

brane preparations derived from higher plants. Coupled with this is the ease with which callose ([1→3]-β-glucan) can be synthesized *in vitro* by the plasma-membrane-localized, $Ca^{2+}$-activated UDP-glucose: (1→3)-β-glucan synthase (9). The possibility that the callose synthase and the cellulose synthase may be the same enzyme has been raised several times in the past (7, 18). We are currently considering a model (see also Ref. 9) in which a single UDP-glucose:glucosyltransferase exists in the plasma membrane of plants. In this model, the function of the 18 kD polypeptide would be as a regulatory protein which modulates the specificity of the glucosyltransferase with regard to the type of linkage it creates upon polymerization of glucose residues into β-glucan. In intact plant cells, the 18 kD polypeptide would exist in association with the glucosyltransferase; in such a complex the catalytic centers and acceptor glucan are aligned in such a way as to favor transfer of new glucosyl residues to the 4-hydroxyl of the acceptor residue, resulting in synthesis of cellulose. Binding of DCB to its receptor results in dissociation or altered conformation of the 18 kD polypeptide, rendering the glucosyltransferase nonfunctional. Alternatively, in the absence of DCB, but upon influx of $Ca^{2+}$ and/or cell damage, the 18 kD dissociates from the complex, and the glucosyltransferase assumes a different conformation and functions as a callose synthase, transferring new glucosyl residues to the 3-hydroxyl of the acceptor. Such a model may also provide an explanation for the developmental regulation of the 18 kD polypeptide: changes in the rate of cellulose synthesis *in vivo* would be regulated, not by changes in the level of glucosyltransferase, but rather by changes in level of the 18 kD polypeptide. This type of regulation allows the cell to maintain a high level of potential callose synthase at all times, allowing it to respond quickly upon damage.

Arguing perhaps against such a model is the general finding of very rigorous specificity of glycosyltransferases with respect to the linkage formed during glycosyltransfer (30). There are, however, two notable exceptions: (a) a report of a highly purified α-fucosyltransferase which still retains the capacity to transfer fucose in either (1→3)- or (1→4)-linkage (29); and (b) the modulation of lactose synthase by the 14 kD acidic polypeptide lactalbumin (16). Both cases bear some resemblance to our model. Binding of lactalbumin to a Golgi-localized galactosyltransferase alters the acceptor specificity of the enzyme such that it prefers to transfer galactose to free glucose, resulting in the synthesis of lactose; in the absence of lactalbumin, *i.e.* prior to its induction by hormones during lactation, the enzyme transfers galactose to terminal GlcNAc residues on proteins passing through the Golgi. In this system, however, it should be noted that the linkage formed in either case is the same—(1→4)-β. Certainly the 18 kD polypeptide and lactalbumin bear some resemblance in size and charge properties and both are under developmental regulation. Clarification of the role of divalent cations in modulating interaction with DCB may offer other clues to function. In this regard, it may be of interest that lactalbumin is reported to contain a binding site for $Zn^{2+}$ (which inhibits labeling of the DCB receptor) and a distinct, high affinity site for $Ca^{2+}$ (27).

Obviously, other models for the function of the 18 kD polypeptide are possible. Since the cytoskeleton may be intimately involved in the orientation of cellulose microfibrils, this polypeptide could represent some component of that structure which participates in cellulose synthesis. Ultimate understanding of the function of this receptor must await its complete purification and characterization. If this polypeptide is found to play some role in the regulation of the rate of cellulose synthesis *in vivo*, then the gene which codes for it may well be an interesting target for genetic engineering.

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