FINAL TECHNICAL REPORT

Cellulose Synthesizing Complexes in Vascular Plants and Procaryotes

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Abstract

Continuing the work initiated under DE-FG03-94ER20145, the following major accomplishments were achieved under DE-FG02-03ER15396 from 2003-2007: (a) we purified the acsD gene product of the *Acetobacter* cellulose synthase operon as well as transferred the CesA cellulose gene from *Gossypium* into *E. coli* in an attempt to crystallize this protein for x-ray diffraction structural analysis; however, crystallization attempts proved unsuccessful; (b) the *Acetobacter* cellulose synthase operon was successfully incorporated into *Synechococcus*, a cyanobacterium[2]; (c) this operon in *Synechococcus* was functionally expressed; (d) we successfully immunolabeled *Vigna* cellulose and callose synthase components and mapped their distribution before and after wounding; (e) we developed a novel method to produce replicas of cellulose synthases in tobacco BY-2 cells, and we demonstrated the cytoplasmic domain of the rosette TC; (f) from the moss *Physcomitrella*, we isolated two full-length cDNA sequences of cellulose synthase (PpCesA1 and PpCesA2) and attempted to obtain full genomic DNA sequences; (g) we examined the detailed molecular structure of a new form of non-crystalline cellulose known as nematic ordered cellulose (=NOC)[3].

Background

This grant was a continuation of DE-FG03-94ER20145 which operated from 1994 until 2003, the year DE-FG02-03ER15396 started. The earlier grant concentrated on purification of cellulose synthases and the production of cellulose I *in vitro.* The continuing research shifted focus somewhat to projects involving functional expression of cellulose synthases in cyanobacteria as well as expanding the mapping of the rosette TC complex which is the multimeric enzyme complex responsible for cellulose biosynthesis in higher plants.

Projects undertaken during the grant period

1. Protein purification studies

Our attempts to purify the acsD gene product of the *Acetobacter* cellulose synthase operon worked well but we were not able to obtain sufficient quantities for x-ray crystallization studies. Then we turned to a secondary approach expressing the CesA cellulose gene from *Gossypium* into *E. coli.* This approach yielded a greater quantity of purified protein, but since it was only a fragment of the complete operon, it was not possible to crystallize this protein although a number of attempts were made in the laboratory of Dr. Xiaogiang Wang of the Samuel Roberts Noble Foundation. Unfortunately, this project had to be abandoned, although it will be important in the future to obtain x-ray crystallographic data on cellulose synthase (note: to date, no lab has reported a successful x-ray analysis of cellulose synthase).

2. Transfer, incorporation, and functional expression of a partial cellulose synthase operon from *Acetobacter* into the cyanobacterium, *Synechococcus*[2]

One of the most successful research accomplishments during this grant period was the successful functional expression of cellulose in the cyanobacterium, *Synechococcus.* Interestingly, the cellulose was non-crystalline and of low molecular weight and had no physical characteristics of the wild type cellulose gene expressed in *Acetobacter.* Please refer to the publications by Brown, Nobles, et al, for details.

3. An immunocytochemical analysis of β-1,3 glucan (callose) and β-l,4 glucan (cellulose) biosynthesis in *Vigna radiata* upon wounding

In order to provide new insight into β-g1ucan synthesis in higher plants, we used a silver-enhanced, gold-secondary antibody immuno-location approach. We investigated the mechanisms for the switch from β-l,4-to β-l,3-glucan biosynthesis upon wounding. Antibodies against β-l,4-and β-l ,3-glucan synthases were used to locate (via high resolution light microscopy) these synthases before and after wounding of *Vigna radiata* hypocotyls. Within 5 min of wounding, β-l,4-glucan synthases which were densely localized on plasma membranes adjacent to the secondary walls at the wound site completely disappeared, and β-l,3-glucan synthases became labeled. The immuno-location of the β-1,3-glucan synthases in the secondary walls was in good accordance with the region where the β-1 ,4-glucan synthases were localized before wounding. Aniline blue was also utilized to visualize the deposition of callose upon wounding. Within 5 min of wounding, callose had accumulated in the corresponding region where the immuno-labeling or β-l,3-glucan synthase was detected after wounding. The β-1 ,3-glucan synthases were always detected from the sieve plate and plasmodesmata which are known to have constitutive synthesis of callose regardless of wounding. Secondary walls located distantly into the tissue away from the wound site were consistently labeled by the β-1,4-glucan synthase antibody even after wounding. Immuno-blot analysis clearly shows that the levels of β-l ,4-glucan synthase subunit Ces A decreased dramatically within 30 min, whereas the β-l,3 glucan synthase subunit CFLl levels increased significantly after wounding. The intensity of labeling reached a maximum at the wound site, and gradually decreased in correspondence with the distance from the wound site. When a protease inhibitor cocktail was applied upon wounding, neither the β-l,3-glucan

synthase appeared nor callose was deposited during the first 5 min of wounding. On the other hand, β-l,4glucan synthase was detected at the wound site, implying that activation or β-1 ,3-glucan synthase may rely on the degradation of the β-l,4-glucan synthase.

4. Investigations of the cytoplasmic domain of the rosette TC

One of the remaining enigmas in understanding cellulose biosynthesis centers on the rosette TC in higher plants. In the 1980s, we first discovered this complex and named it the rosette TC. The definitive freeze fracture labeling studies of Itoh, et al, laid to rest any doubt that the rosette TC was the site of cellulose biosynthesis; however, scientists did not have definitive evidence about which part of the rosette is the site of catalysis. The 6-fold symmetry of the rosette TC observed in freeze fracture is found in the interior of the plasma membrane and is not the catalytic site. Our earlier work with seconded materials (Kudlicka, K., Wardrop, A., Itoh, T., and R. M. Brown, Jr. 1987. Further evidence from sectioned material in support of a linear terminal complex in cellulose synthesis. *Protoplasma* **136**:96-103.) indicated that beneath the plasma membrane components in the cytoplasm lies the catalytic subunits. We wanted to learn if a similar case was evident for the rosette TC. We imaged the cytoplasmic domain of the rosette TC from in situ patches of plasma membrane isolated from tobacco BY-2 protoplasts. We partially extracted the plasma membrane lipids which allowed observations of cellulose microfibrils through the plasma membrane. The rosette TCs were identified on the basis of their association with the ends of the cellulose microfibrils. The cytoplasmic domain of the rosette TC is hexagonal and measures 45-50nm in diameter and extends about 30-35 nm deep into the cytoplasm. This new view of the rosette TC complex will be helpful in better understanding the function of this enzyme complex in polymerization and crystallization pathways leading to a native cellulose I microfibril.

5. Molecular biology of cellulose biosynthesis in the moss, *Physcomitrella*

The moss *Physcomitrella* has a smaller genome than higher plants and is thus better suited to study the genes involved in cellulose biosynthesis. Under the auspices of this grant, we undertook to sequence the CesA genes for cellulose biosynthesis from *Physcomitrella* in order to test knock-outs of various components to understand what is necessary for normal cellulose I microfibril assembly. Since *Physcomitrella* is haploid, the results of knock-out experiments could better reveal which genes are involved. This work was ongoing at the time this grant was concluded and continued for several years later to form the dissertation research of one of Dr. Brown's graduate students.

6. Investigating a new form of cellulose, nematic ordered cellulose [3]

For understanding the complex polymerization and crystallization steps leading to the biosynthesis of native cellulose, it is important to focus on other types of cellulose to better realize how the complex, native cellulose allomorph is generated. Thus, the discovery by Kondo et al (Kondo, T., Togawa, E. and R. M. Brown, Jr. 2001. "Nematic Ordered Cellulose"; A concept of glucan chain association. Biomacromolecules 2: 1324-1330) of a novel form of cellulose generated by solvent dissolution followed by slow removal of the solvent to prevent glucan chain aggregation, resulted in the formation of nematic ordered cellulose (NOC). Using high resolution transmission electron microscopy coupled with low dose of

the electron beam, we devised a method to directly observe single glucan chains in nematic ordered cellulose (see publication by Brown, et al 2007 below). This is the first observation of single glucan chains of cellulose. The parallel association of the glucan chains is relatively weak, allowing the uranyl acetate negative stain to penetrate, thus giving the first view of how glucan chains associate. The information gained from these studies has provided new knowledge to better understand the native polymerization/crystallization pathways in cellulose biosynthesis.

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[2] These projects have patent application **20080113413 Inventors: R. Malcolm Brown, Jr. and David R Nobles, Jr.** The University of Texas, Austin, Texas

[3] US Patent **7,335,882 Inventors: *Brown,* Jr.; R. Malcolm** (Austin, TX)**, *Barnes*; Zack** (Austin, TX)**, Sawatari; Chie** (Shizuoka, **JP**)**, Kondo; Tetsuo** (Kukuoka, **JP**)

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*Note. Several of these publications incorrectly cited DE-FG03-94ER20145 when, in fact, the work was performed under DE-FG02-03ER15396