The Arabidopsis COBRA protein facilitates cellulose crystallization at the plasma membrane

Nadav Sorek1,2, Hagit Sorek1, Aleksandra Kijac3, Heidi J. Szemenyei1,2, Stefan Bauer1, Kian Hématy4,5, David E. Wemmer1,3 and Chris R. Somerville1,2,6

1 Energy Biosciences Institute, University of California, Berkeley CA 94720
2 Plant and Microbial Biology Department, University of California, Berkeley CA 94720
3 Department of Chemistry, University of California, Berkeley CA 94720
4 INRA, Institut Jean-Pierre Bourgin, UMR 1318, ERL CNRS3559, Saclay Plant Sciences, RD10, F-78026 Versailles, France
5 AgroParisTech, Institut Jean-Pierre Bourgin, UMR 1318, ERL CNRS3559, Saclay Plant Sciences, RD10, F-78026 Versailles, France
6 Department of Arid Land Agriculture, Faculty of Meteorology, Environment and Arid Land Agriculture, King Abdulaziz University, Jeddah, Saudi Arabia

* Correspondence: nadavsorek@berkeley.edu

Address for correspondence: Nadav Sorek, Energy Biosciences Institute, University of California Berkeley, 1521 Berkeley Way, Berkeley CA 94704; phone 510-643-6265

Running title: The role of COBRA in cellulose synthesis
Capsule

Background –
The COBRA gene is highly coexpressed with cellulose synthase genes, but its function remains unclear.

Results –
COBRA localizes at the plasma membrane and binds glucan chains. NMR studies indicate structural defects in cellulose in the mutant despite normal polymerization rate.

Conclusions –
COBRA functions downstream of cellulose biosynthesis.

Significance –
This work suggests that alignment of glucan chains into cellulose fibrils is facilitated by one or more proteins.

Abstract

Mutations in the Arabidopsis COBRA gene lead to defects in cellulose synthesis but the function of COBRA is unknown. Here we present evidence that COBRA localizes to discrete particles in the plasma membrane and is sensitive to inhibitors of cellulose synthesis, suggesting that COBRA and the cellulose synthase complex reside in close proximity on the plasma membrane. Live-cell imaging of cellulose synthesis indicated that, once initiated, cellulose synthesis appeared to proceed normally in the cobra mutant. Using isothermal calorimetry, COBRA was found to bind individual β-1,4-linked glucan chains with a $K_A$ of 3.2μM. Competition assays suggests that COBRA binds individual β-1,4-linked glucan chains with higher affinity than crystalline cellulose. Solid-state nuclear magnetic resonance (SSNMR) studies of the cell wall of the cobra mutant also indicated that, in addition to decreases in cellulose amount, the properties of the cellulose fibrils and other cell wall polymers differed from wild type by being less crystalline and having an increased number of reducing ends. We interpret the available evidence as suggesting that COBRA facilitates cellulose crystallization from the emerging β-1,4 glucan chains by acting as a “polysaccharide chaperone”.

Introduction

Cellulose microfibrils comprise the core component of the cell walls that surround each plant cell, allowing a mode of growth based on turgor pressure (Somerville et al., 2004). In higher plants, cellulose is synthesized at the plasma membrane by the cellulose synthase complex (CSC) (Bashline et al., 2014; McFarlane et al., 2014), which is comprised of multiple cellulose synthase proteins (CESA), although their exact stoichiometry is unknown. Upon activation, the CESA proteins synthesize β-1-4 glucan chains (Doblin et al., 2002) which align with each other, and by hydrogen bonding, form the cellulose fibril (McFarlane et al., 2014). Cellulose synthase complex movement at the plasma membrane has been visualized by live imaging (Paredez et al., 2006), and a connection to cortical microtubules via the CSI1 protein has been demonstrated (Bashline et al., 2014; Li et al., 2012).

The mechanism by which individual glucan chains form cellulose microfibrils remains elusive. Because it is challenging to reconstruct active plant cellulose synthesis in vitro, genetic methods have been used to identify and characterize the genes for proteins that participate in cellulose synthesis and deposition (Ruprecht and Persson, 2012). KORRIGAN was one of the earliest genes that was implicated in cellulose synthesis (Nicol et al., 1998). KORRIGAN was shown to act as an endo-1,4-β-glucanase (Zuo et al., 2000), and it was also shown to have multiple effects on the composition of the cell wall (His et al., 2001; Szyjanowicz et al., 2004). Although the function of KORRIGAN is known and an interaction with the cellulose synthase complex has been shown, (Lei et al., 2014; Vain et al., 2014), the
role of KORRIGAN in cellulose formation is still unclear.

Co-expression analysis helped to identify several other genes required for different aspects of cellulose formation (Persson et al., 2005). Like CSI1 and KORRIGAN, chitinase-like (CTL) genes are co-expressed with the CESAs and were shown to be involved in cellulose formation (Sanchez-Rodriguez et al., 2012). CTL proteins were suggested to play a key role in establishing interactions between cellulose microfibrils and hemicelluloses, which are thought to coat the cellulose microfibrils.

**COBRA** is one of the genes that is most highly co-expressed with the primary CESA family (Persson et al., 2005). The Arabidopsis **COBRA** gene was originally identified in a screen for root defects (Benfey et al., 1993). In Arabidopsis, the COBRA family contains 11 members with different expression patterns. Of these genes, COBRA has the highest and most widely distributed expression (Brady et al., 2007).

Mutations in **COBRA** cause dramatic reduction in cellulose levels (Schindelmeier et al., 2001). Inspection of the orientation of cellulose fibrils in cobra mutants by electron microscopy suggested that the orientation of the cellulose fibrils was affected (Roudier et al., 2005). COBRA was shown to be glycoprophatidylinositol (GPI) anchored, as well as N-glycosylated (Roudier et al., 2005). GPI anchored proteins are typically targeted to the outer leaflet of the plasma membrane (Eisenhaber et al., 2003). Based on phase separation, COBRA was detected in both endomembranes as well as in the plasma membrane fraction, while immunolabeling demonstrated localization at the Golgi and the cell wall, but not at the plasma membrane (Roudier et al., 2005). However, Immunolabeling of the rice ortholog showed that COBRA localized mainly at the plasma membrane in young, developing tissue, and primarily in the cell wall in mature tissue (Liu et al., 2013). Therefore, it was suggested that its localization is dependent on tissue type and developmental stage.

The **COBRA** sequence harbors a putative cellulose binding motif (CBM) (Sato et al., 2010), and the rice COBRA CBM was shown to bind cellulose (Liu et al., 2013). COBRA was tested against a variety of cell wall polymers, and was found to bind specifically to cellulose. However, the binding was significantly higher when tested against a crude cellulose fraction compared to Avicel, a purified cellulose powder (Liu et al., 2013).

In this work, we succeeded in using live imaging to visualize COBRA and establish its presence in distinct particles at the plasma membrane. By using isothermal titration calorimetry (ITC) we showed that COBRA can bind individual β-1,4 glucan chains with an association constant (K_a) of 3.2±0.1 μM. Furthermore, COBRA binding to individual β-1,4 glucan chains competes with its binding to pure cellulose powder. These observations suggest that COBRA acts at the initial step of cellulose biosynthesis, when emerging individual chains align to form cellulose fibrils. Using Solid-state nuclear magnetic resonance (ssNMR) we characterize the cell wall of the cobra mutant in detail, demonstrating the effect of disrupting cellulose crystallization.

### Experimental Procedures

#### Molecular Cloning

All plasmids are listed in Table S1, Oligonucleotides are listed in Table S2. Gateway Cloning was performed according to the manufacturer’s instructions (Invitrogen). **COBRA** was subcloned into pGEX4T-2 with oligonucleotide primers NS173 and NS174 to create GST-His6-COBRA (pNS87). The **COBRA** CDS (lacking the signal peptide and GPI motif sequence) with the sequence CATCATCCAGGCCATCAT (encodes the FlAsH tag – cys-cys-pro-gly-cys-cys) inserted after Tyrosine 37 (FlAsH-COBRA) was synthesized by DNA2.0 (https://www.dna20.com/). FlAsH-COBRA was amplified using oligonucleotides NS29 and NS30 and cloned into pDONOR207 to create plasmid NS38. 2.5Kb of the **COBRA** promoter
The role of COBRA in cellulose synthesis

was amplified using oligonucleotides NS31 and NS33 and cloned into pDONOR P4-P1R to create plasmid NS29. Plasmids NS29, NS38 and NS60 were used to create the expression vector pCOBRA::FlAsH-COBRA:NOS in pH7 mu34GW (plasmid NS79) by multi gateway reaction.

Plant Growth Conditions

Arabidopsis ecotype Columbia seeds and various mutant lines were sterilized and germinated on Murashige and Skoog (MS) plates (one-half-strength MS salts, 0.8% agar, and 0.05% MES, pH 5.7). Seedlings were grown vertically on the agar at 22°C under continuous light for 5 d before being transferred to pots in a growth chamber at 22°C under a 16-h-light/8-h-dark cycle.

Confocal Microscopy

YFP-CESA6 imaging

For analyses of YFP-CESA6 proteins, seeds were germinated on MS agar plates and grown vertically in darkness for 5 days at 22°C. Seedlings were mounted between two coverslips in water. Imaging was performed on a Yokogawa CSUX1 spinning-disk system featuring the DMI6000 Leica motorized microscope and a Leica 100×/1.4 numerical aperture oil objective. YFP was excited at 488 nm, and a band-pass filter (510/20 nm) was used for emission filtering. Image analysis was performed using Metamorph (Molecular Devices) and Imaris (Bitplane) software.

Movies were collected at 5 days from randomly selected seedlings of the various genotypes. Movies were taken at ambient temperatures. An approximately equal number of acquisitions were attempted for each, with poor-focal-quality movies discarded during postprocessing.

Image analysis was performed with ImageJ (http://rsbweb.nih.gov/ij/) and Imaris software. Movies were first contrast enhanced in ImageJ, and a walking average of four frames was taken using the kymograph plugin for ImageJ. These steps were performed to improve the accuracy of automated particle recognition performed in subsequent steps. These images were opened in Imaris 6.2.1 and switched from Z-series to time series. The pixel size was set to 135 nm per pixel based on measurements from the microscope, and the time interval was set to 5 sec. The particle-recognition algorithm in Imaris was performed with a spot size of 250 nm. High-intensity signal was filtered to eliminate Golgi signal. Following this, the connected components program was run, which determines particle identity over several frames and converts a particle’s movement into tracks. All tracks present for less than 60 sec (12 frames) were discarded. The displacement and duration of the remaining tracks were exported to a spreadsheet, and their average velocity was calculated.

FlAsH-COBRA imaging

FlAsH labeling was done as reported previously (Estevez and Somerville, 2006). Seeds of FlAsH-COBRA were surface sterilized in 2.4% sodium hypochlorite with 0.1% SDS, washed four times in water, and suspended in 0.15% agar at 4°C in the dark for at least 2 days before planting. Seeds were germinated on vertical 0.8% agar plates containing 2.2 g/L Murashige and Skoog salts (Caisson Labs) and 0.6 g/L MES, (0.5× Murashige and Skoog medium) pH 5.6 in 24 h 120 μmol m⁻² s⁻¹ light at 21°C for 5 d. Seedlings were removed from the plate and placed in 1.5 mL microcentrifuge tubes containing 2 μM Lumio green (Invitrogen, # 12589-057) in liquid 0.5× Murashige and Skoog medium lacking agar for 60 min in the dark with gentle shaking. The seedlings were washed twice with Disperse Blue 3 (Invitrogen), and mounted on slides for observation as described in the YFP-CESA6 imaging section.

Isoxaben treatments.

Isoxaben treatments were carried out as previously reported (DeBolt et al., 2007). GFP-CESA3 and FlAsH-COBRA seeds were surface sterilized and plated as described in the GFP-CESA3 imaging section. Seedlings were treated for 30 minutes with 100 nM Isoxaben.

Plasmolysis
FlAsH-COBRA seedlings were plasmolyzed by incubation in 0.8 M NaCl for 10 min. Seedlings were mounted on microscope slides in the plasmolysis solution.

**Protein expression and purification**

*E. coli* DH5α was used for DNA propagation. For protein expression, pNS87 was transformed into *E. coli* BL21 CodonPlus DE3 RIL (Stratagene) cells. Cells were grown to OD600 of 0.6. Protein expression was induced by adding 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Protein extraction and purification was carried out with AKTA-prime (GE Healthcare, UK) using HisTRAP FF and GSTrap HP columns according to the manufacturer’s instructions (GE Healthcare, UK).

**Protein Immunoblots**

Anti-His6 antibodies (Sigma H1029) were used at a dilution of 1:3,000 together with blotting grade horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad, Hercules, CA). Detection was performed with SuperSignal West Pico Chemiluminescent Substrate (Pierce, cat#15168).

**Isothermal Titration Calorimetry**

ITC experiments were performed in NanoITC2G (TA instruments) (Todd and Gomez, 2001). Measurements were made at 21°C. The proteins were dialyzed extensively against 50 mM Tris-Cl buffer pH 7.5, and the cellohexaose was dissolved in the same buffer to minimize heats of dilution. Titrations were carried out in triplicate, and the errors are the S.D. of the means of these replicates. During a titration experiment the 50 μM protein sample stirred at 250 rpm in a 1.43 ml reaction cell was injected with 15 successive 15-μl aliquots of 750 μM cellohexaose at 300-s intervals. Integrated heat effects, after correction for heats of dilution, were analyzed by sigmoidal dose response + hill slope (SigmaPlot). The fitted data yielded the association constant (Ka), number of binding sites on the protein (n), and the enthalpy of binding (ΔH).

**COBRA, Avicel and cellohexaose competition assay**

Experiments were done based on (Walter and Schrempf, 2003). Proteins were incubated with Avicel PH-101 (Sigma 11365) (1 mg/ml) in 20 mM Tris-Cl buffer (pH 8) and 150 mM NaCl for 120 min. Avicel recovered by centrifugation was washed 3 times with 20 mM Tris-Cl buffer (pH 8) and 150 mM NaCl. Avicel-bound proteins were released by heating in SDS sample buffer for 5 min at 100 °C and subjected to SDS-PAGE.

**Cell wall analysis**

Seedlings grown vertically on plates were placed in the dark for 24 h to reduce starch levels before harvesting. The alcohol-insoluble residues (AIR) fraction was prepared by grinding 150 mg of seedling tissue in liquid nitrogen, followed by one extraction with 1 ml 70% ethanol and three extractions with 1 ml chloroform:methanol (1:1, v/v). The pellet was washed in 1 ml acetone and dried overnight under vacuum. Cellulose analysis was based on a modified version of Saeman hydrolysis according to Bauer and Ibáñez (2014). In brief, cellulose content was determined by subtracting hemicellulosic glucose released by hydrolysis with 4% sulfuric acid from total glucose released by a two-stage acid hydrolysis. From each sample, two 1 mg aliquots of AIR were prepared. The first was treated with 50 μl 72% sulfuric acid (room temperature (RT), 1 h) to disrupt the cellulose crystalline structure; sulfuric acid was then diluted to 4% by adding 1.4 ml H2O and the mixture was heated (121°C, 60 min). The second aliquot was treated with 1.45 ml 4% sulfuric acid shortly before heating (121°C, 60 min). After cooling at RT, the reaction mixture was vortexed, and a 1:50 dilution was used for HPLC analysis. Released monosaccharides were analyzed using an ICS-3000 HPLC system (Thermo Fisher Scientific) equipped with a pulse-amperometric detector (PAD). Samples were injected onto a 150 mm x 3 mm CarboPac PA20 column (Thermo Fisher Scientific) with a 50 mm x 3 mm guard column of the same material and eluted at 30°C with 2 mM potassium hydroxide at a flow rate of 0.4 ml/min. Cellulose content
(mg cellulose/mg dry tissue) was calculated by subtracting the amount of glucose released by 4% sulfuric acid hydrolysis from the amount of glucose released by 72% sulfuric acid treatment. For neutral sugar and uronic acid analysis, a 1 mg aliquot of AIR was hydrolyzed with 1.45 ml 4% sulfuric acid and the mixture was heated (121°C, 60 min). Samples were cooled and vortexed, and a 1:50 dilution was used for HPLC analysis as above. For uronic acids, samples were injected onto a 250 mm x 3 mm CarboPac PA200 column with a 30 x 3 mm guard column and eluted at 30°C using a 50 mM to 200 mM sodium acetate gradient (in 0.1 M NaOH) at 0.4 ml/min.

Cell wall structural analysis by $^{13}$C MAS SSNMR

All $^{13}$C SSNMR spectra were acquired on a 17.6 T (700 MHz $^1$H frequency) Bruker Avance spectrometer equipped with a 3.2 mm Efree triple resonance MAS probe from Bruker. The spectra were recorded at a 14 kHz magic angle spinning (MAS) rate and a temperature of 280 K. $^{13}$C chemical shifts were referenced externally to adamantane signal at 38.48 ppm (Morcombe and Zilm, 2003).

$^{13}$C direct polarization (DP) experiments with a long recycle delay, 20 s, were used for quantitative measurements. Spectra were acquired with a 48 ms acquisition time, 83 kHz $^1$H SPINAL-64 decoupling (Fung et al., 2000), and a 5 µs pulse width on $^{13}$C. $^{13}$C Chemical shifts were assigned based on reported literature assignments (Dick-Perez et al., 2011).

2D $^{13}$C-$^{13}$C chemical shift homonuclear correlation spectra were acquired with 50 ms dipolar-assisted rotational resonance (DARR) mixing time (Takeda et al., 2001). The data were acquired at 273 K, 14 kHz spinning frequency for ~60 hrs. Other acquisition parameters were 0.5 ms $^1$H-$^{13}$C CP contact time, 83 kHz $^1$H SPINAL-64 decoupling, 10 ms $t_1$ acquisition time (1024 Rows X 9.5 us) with States-TPPI detection, 48 ms $t_2$ acquisition time (5120 X 9.4 us), and a 2 s pulse delay. The data were processed with 0.28 ppm (50 Hz) net line broadening (Lorentzian-to-Gaussian apodization) and zero filled to 8192 (F2) X 2048 (F1) complex points before Fourier transformation. These experiments were performed to obtain through-space correlations and to observe changes in the cell wall structure.

Results

In order to visualize COBRA in live tissue, we created two transgenic lines expressing YFP-COBRA under the native promoter. In the first line, YFP was inserted after Pro42, five amino acids after the predicted secretion peptide cleavage site. In the second line, the YFP tag was inserted before the GPI omega site, at Pro427. In both lines we used 2.5Kb upstream of the ATG as a promoter sequence. However, these constructs did not complement the phenotype of the mutant in the 10 independent lines examined, implying the fusion protein was non-functional.

Thus, in order to visualize COBRA in live tissue we used FlAsH labeling, which involves the modification of the COBRA protein by the addition of a small peptide motif that binds derivatives of fluorescein (Estevez and Somerville, 2006). The tetracysteine motif (Cys-Cys-Pro-Gly-Cys-Cys) was placed immediately after the predicted secretion peptide cleavage motif at Tyr37. The FlAsH-COBRA construct expressed under the control of the native COBRA promoter complemented the cob-6 phenotype, implying functionality of FlAsH-COBRA. Due to low penetration of the FlAsH dyes into hypocotyl cells, FlAsH-COBRA imaging was done in elongating root cells in 5 day old light grown seedlings (Figure 1). FlAsH-COBRA was found to localize mainly at the plasma membrane in a punctate pattern (Figure 1A). Plasma membrane localization of FlAsH-COBRA was further confirmed by plasmolysis (Figure 1B). Unfortunately, the FlAsH tag bleached rapidly during laser illumination so we were not able to obtain useful measurements of FlAsH-COBRA movement. However, we observed movement of the particles, although they appeared to move more slowly than the GFP-CESA particles and were significantly less abundant (Figure 1A).

To further investigate the association between COBRA and the cellulose synthase
The role of COBRA in cellulose synthesis

COBRA contains a putative cellulose binding motif (CBM) (Roudier et al., 2005), and it was recently shown that the purified CBM region from Brittle Culm1, a COBRA-like protein from rice, can bind cellulose (Liu et al., 2013). The fact that COBRA contains a GPI anchor (Roudier et al., 2005) implies that this association occurs on the plasma membrane outer leaflet. Therefore, we tested if COBRA could interact with individual β-1,4 glucan chains. We expressed COBRA from Arabidopsis in E. coli without the secretion peptide and the GPI motifs that are cleaved during protein maturation. Binding, affinity, and stoichiometry of COBRA for cellohexaose was determined by isothermal titration calorimetry (ITC) (Figure 2). From these results, the number of ligand molecules bound to COBRA (n), the association constant (Kₘ) and the binding enthalpy (ΔH) were evaluated. Monomeric carbohydrate-binding proteins generally have a 1:1 ratio with their oligosaccharide ligands (Lis and Sharon, 1998). Similarly, COBRA was also found to bind one molecule of cellohexaose per protein molecule (n = 0.94±0.02). The enthalpy of binding, ΔH, is negative with a value of -8.2±0.4 Kcal mol⁻¹ (Figure 2) implying that the reaction is exothermic. The binding association constant of COBRA to cellohexaose was found to be 3.2±0.1 μM, which is similar to other proteins containing CBMs (Boraston et al., 2004).

Since COBRA was shown to bind cellulose (Liu et al., 2013), we tested the affinity to crystalline cellulose versus the affinity to individual glucan chains (Figure 3). Avicel is a 95% pure crystalline cellulose with an average particle size of 50 μm. Because Avicel is not soluble, competition assays with cellohexaose were carried out using pull down assays (Walter and Schrempf, 2003). COBRA was incubated for 2 hours at 21°C with Avicel in the presence of increasing concentrations of cellohexaose. The samples were analyzed using an anti-His₆ antibody, the washed pellet representing COBRA bound to Avicel, and the soluble fraction COBRA not bound to Avicel (Fig3). It is important to bear in mind that in this type of experiment, the individual glucan chains are soluble, while in planta they would be constrained by attachment to the cellulose synthase complex and/or hydrogen bonding to

complex, seedlings expressing FlAsH-COBRA were treated with the cellulose synthase complex inhibitor, isoxaben (Brabham and Debolt, 2012). Isoxaben is thought to specifically act on the cellulose synthase complex since point mutations in CESA3 confer resistance to the drug (Scheible et al., 2001). Treatment of seedlings with 100 nM isoxaben for 45 minutes caused almost complete depletion of the cellulose synthase complex from the plasma membrane (Paredez et al., 2006) (Figure 1C). In order to detect the different focal planes, we used the outer epidermal cell wall autofluorescence as a reference point, the focal plane below (200nm) was referred to as plasma membrane, and 3 μm below this was referred to as the focal plane of the cytoplasm (Figure 1C, lower scheme). Isoxaben treatment of seedlings expressing FlAsH-COBRA showed a similar phenomenon, as the signal from the plasma membrane depleted, and FlAsH-COBRA could be detected only within the cells (Figure 1C).

The similar effect of isoxaben on both GFP-CESA and FlAsH-COBRA puncta suggests a relationship between the two proteins. One possibility is that the presence of COBRA on the plasma membrane is dependent upon the presence of the CESA complex.

The effect of the cob-6 mutation on cellulose synthesis was visualized by live-cell imaging of cellulose synthase complexes labeled with YFP (Figure 1D). The average velocity of the cellulose synthase complex in 5 day-old seedlings was 225 nm/min in WT and 289 nm/min in cob-6 (Fig1E). The average apparent lifetime of the CESA6-YFP-labelled complex in the cob-6 background, measured as “tracks” in time-averaged images, was shorter, but the directionality of the tracks was unaffected and linear movement was maintained (Figure 1D). Thus, the basis for the cellulose deficiency was not apparent from direct observation of cellulose synthesis. These sets of data suggest that COBRA acts at the plasma membrane, adjacent to the cellulose synthase complex. Therefore, we tested whether COBRA could interact with the product of the cellulose synthase complex, β-1,4-linked glucan (Figure 2).
The role of COBRA in cellulose synthesis

cellulose microfibrils. In the absence of cellohexaose, there is no detectable COBRA in the unbound portion (Figure 3, 0 μM C6). As mentioned above, the COBRA K_A for cellohexaose was found to be 3.2 μM (Figure 2), so we tested a range of cellohexaose concentrations below and above the K_A. When 1 μM of cellohexaose was added to the reaction, a considerable fraction of COBRA remained in the soluble fraction, and this fraction increased when 2 μM of cellohexaose was added to the reaction. With addition of 3 μM of cellohexaose, more protein was detected in the soluble fraction compared to the Avicel bound fraction, and with 30 μM of cellohexaose, 10 fold above the K_A, COBRA was detected only in the soluble fraction and undetectable in the Avicel bound fraction (Figure 3).

Along with the localization results, this finding raises the possibility that COBRA may act at the stage where individual β-1,4 glucan chains have emerged from the complex and are being assembled into cellulose fibrils.

We characterized the effect of mutation in COBRA on the cell wall composition (Figure 4) and structure (Figures 5 and 6). cob-6 has a 50% reduction in the amount of cellulose (figure 4A), and fewer overall glucan chains in the cell wall fraction (Figure 5). It has been shown that the reduction of cellulose in the cobra mutant is accompanied by overproduction of starch, preventing osmotic stress by the excess of glucose that can’t be incorporated into the cell wall (Dai et al., 2011). Analysis of galacturonic acid (GalA) levels revealed a significant increase in the cobra mutant (figure 4B), and Arabinose (Ara) and galactose (Gal) levels also were increased (figure 4C). Galacturonic acid forms the backbone of the pectic polymers, and arabinose and galactose are abundant in the pectin side chains, implying a significant increase of the pectic fraction in the cobra mutant.

In order to further characterize the cellulose fibrils that form in the cobra mutant, we used Magic-angle-spinning (MAS) solid-state NMR (SSNMR) spectroscopy (Figure 5 and 6). Magic-angle-spinning solid-state NMR spectroscopy has already proven to be a feasible technique to investigate the structure and intermolecular interactions of polysaccharides in intact plant cell walls (Dick-Perez et al., 2011; Foston, 2014; Mansfield et al., 2012). Previous SSNMR studies have yielded important structural information on primary Arabidopsis cell walls (Dick-Perez et al., 2012; Harris et al., 2012; Wang et al., 2012).

We used SSNMR to further analyze the molecular structure of the polysaccharides in WT and cob-6 dark grown seedlings grown in liquid ½MS media with 13C glucose (Dick-Perez et al., 2011) to obtain detailed information about crystalline cellulose structure. Spectra were measured quantitatively by 13C direct polarization SSNMR using a long recycle delay (Figure 5A). The 1D 13C spectra show that the amount of cellulose in cob-6 is considerably lower than in WT. Furthermore, the ratio between the intensity of the interior C4 peak (iC4) to surface C4 peak (sC4) in cellulose (Harris et al., 2012) was measured and indicates that cob-6 has 16% lower crystallinity relative to WT (Table 4). In addition, we observed the peak at 88 ppm which is indicative of a degree of crystallinity that is intermediate between the surface and interior cellulose (Harris et al., 2012).

When a short relaxation delay was utilized to increase the intensities of dynamic polysaccharides, the 88 ppm peak intensity was preferentially enhanced in cob-6 relative to WT. We measured 2D 13C-13C CP-DARR spectra to detect through-space contacts between carbons (Figure 5B and C). Qualitative inspection of the 2D spectra shows that the relative intensities of hemicellulose and pectin cross signals is significantly higher in cob-6 compared to WT. We also observed that cob-6 is not only rich in pectin, but the amount of non-esterified galacturonic acid is significantly higher than in WT. A comparison of the 2D spectra revealed additional cross peaks in the cob-6 spectrum, which are absent in the WT spectrum even at lower counter levels (Figure 6). Those cross peaks align at 95.7 ppm, which is characteristic of the anomeric carbon chemical shift of a glucose monomer or cellulose reducing end. The specific chemical shifts we observed suggest that these peaks arise from a β-glucose reducing
end. This indicates that there is a higher concentration of short glucan chains in the \textit{cob-6} mutant than in WT, high enough to enable detection.

\textbf{Discussion}

COBRA was one of the first proteins suggested to be involved in cellulose formation (Schindelman et al., 2001) but its role has remained obscure. Previous immunoelectron microscopy studies suggested that COBRA was primarily located in the cell wall (Liu et al., 2013; Roudier et al., 2005), though the protein carries a motif that would be expected to lead to attachment of a GPI anchor. We found, by live-cell imaging, that COBRA was located in particles in the plasma membrane but did not observe any labeling in the cell wall. These particles are potentially the same as those containing CESAs, although since there are a fewer number of COBRA particles than CESA containing particles, we infer that cellulose synthase complexes would not always be associated with COBRA. Also, we observed an difference between the average velocity of movement of the COBRA-containing particles and the CESA-containing particles, suggesting that, if COBRA associates with cellulose synthase, it would be a slow-moving subset of CESA particles or a transient interaction such as initiation of microfibril synthesis (see speculative model as figure 7). The apparent discrepancy in localization may be due to an effect of the developmental stage of the tissue, as observed by Liu et al. (2013). They suggested that COBRA may be released from the membrane in older tissues by developmentally regulated hydrolysis of the GPI anchor.

Live-cell imaging of the cellulose synthase complex in the \textit{cob-6} mutant did not reveal any obvious defect in the rate of cellulose polymerization in the mutant once polymerization was initiated, nor any apparent difference from the wild type in the orientation of cellulose deposition. The disorganization of cellulose observed by electron microscopy (Roudier et al., 2005) may not be due to \textit{cobra} per se, but rather reflect an altered manner by which the defective microfibrils shift during expansion. There is also no apparent difference in the abundance of cellulose synthase complexes. Thus, we infer that the reduced amount of cellulose measured in the \textit{cobra} mutants, as well as the increased number of cellulose-associated reducing ends evident by NMR, reflect an alteration in a process downstream of glucan polymerization.

The evidence that COBRA is in the plasma membrane (Figure 1) and can bind cellulose and individual glucan chains (Figure 2 and 3) leads us to hypothesize that COBRA functions after glucan polymerization at the step of cellulose formation in which individual chains emerge from the complex and are aligned to form cellulose fibrils (Figure 7). Thus, we propose that COBRA is a kind of "polysaccharide chaperone" that facilitates cellulose crystallization. We hypothesize that, in the absence of COBRA, crystallization takes place with lower fidelity leading to the formation of partially "amorphous cellulose" which leads to the swollen cells that are characteristic of \textit{cobra} mutants. The reduction in amount of cellulose may be due to the defect in ability to control turgor-induced cell expansion, or to degradation of amorphous cellulose by cellulases, perhaps by KORRIGAN. The apparent reduction in dimensions of cellulose microfibrils inferred from NMR of cellulose from the \textit{cob-6} mutant is consistent with this hypothesis. The particulate organization of COBRA, evident from the live cell imaging, presumably reflects accumulation of many COBRA proteins in close proximity. This organization is compatible with the idea that many COBRA molecules would be required in close proximity during cellulose formation because many glucan strands must be co-crystallized. In view of our observation that COBRA does not appear to be associated with the CESA complexes, we propose that COBRA acts transiently at the initiation of cellulose microfibrils to align the glucans as they emerge from the CSC, after which the process does not require COBRA participation. Because it is GPI-anchored, COBRA may help keep the growing cellulose microfibril at the plasma membrane during the initial stages of microfibril formation.
If COBRA does not dissociate from the glucan chains, it could eventually be displaced into the cell wall by newly synthesized cellulose fibrils. Cellulose-synthesizing bacteria do not have detectable homologs of COBRA. However, these bacteria do not incorporate cellulose into cell wall structures and the fibrils are smaller than those from higher plants and not coated with hemicellulose. Most importantly perhaps, plant cellulose microfibrils are tightly appressed between the site of synthesis in the plasma membrane and the adjacent cell wall whereas bacterial microfibrils are not. This may create a tight bend in the glucan chains as they emerge from the plant cellulose synthase, which may impose a need for the proposed COBRA activity that the bacterial process lacks. Alternatively, the presence in plant cell walls of hemicellulose polysaccharides, that hydrogen-bond to cellulose, may impose a need for shielding of the newly synthesized glucans during the process of crystallization to form cellulose microfibrils.

Acknowledgments

We thank Trevor Yeats for helpful advice and for his comments on the manuscript, and Ana B. Ibáñez, Abigail A Landers and Erin Imsand for advice and excellent technical assistance. This work was supported by grants from the Energy Biosciences Institute to CRS and DEW. NS was the recipient of Postdoctoral Award number FI-434-2010 from the Binational Agricultural Research and Development Fund.

References

1. Bashline, L., Lei, L., Li, S., and Gu, Y. (2014). Cell wall, cytoskeleton, and cell expansion in higher plants. Mol Plant 7, 586-600.
2. Bauer, S. and A. B. Ibáñez (2014). "Rapid determination of cellulose." Biotechnol, Bioeng. doi: 10.1002/bit.25276.
3. Benfey, P.N., Linstead, P.J., Roberts, K., Schiefelbein, J.W., Hauser, M.T., and Aeschbacher, R.A. (1993). Root development in Arabidopsis: four mutants with dramatically altered root morphogenesis. Development 119, 57-70.
4. Boraston, A.B., Bolam, D.N., Gilbert, H.J., and Davies, G.J. (2004). Carbohydrate-binding modules: fine-tuning polysaccharide recognition. Biochem J 382, 769-781.
5. Brabham, C., and Debolt, S. (2012). Chemical genetics to examine cellulose biosynthesis. Front Plant Sci 3, 309.
6. Brady, S.M., Song, S., Dhugga, K.S., Rafalski, J.A., and Benfey, P.N. (2007). Combining expression and comparative evolutionary analysis. The COBRA gene family. Plant Physiol 143, 172-187.
7. Dai, X.X., You, C.J., Chen, G.X., Li, X.H., Zhang, Q.F., and Wu, C.Y. (2011). OsBC114 encodes a COBRA-like protein that affects cellulose synthesis in rice. Plant Mol Biol 75, 333-345.
8. Dick-Perez, M., Wang, T., Salazar, A., Zabotina, O.A., and Hong, M. (2012). Multidimensional solid-state NMR studies of the structure and dynamics of pectic polysaccharides in uniformly C-13-labeled Arabidopsis primary cell walls. Magn Reson Chem 50, 539-550.
9. Dick-Perez, M., Zhang, Y., Hayes, J., Salazar, A., Zabotina, O.A., and Hong, M. (2011). Structure and interactions of plant cell-wall polysaccharides by two- and three-dimensional magic-angle-spinning solid-state NMR. Biochemistry 50, 989-1000.
10. Dobkin, M.S., Kurek, I., Jacob-Wilk, D., and Delmer, D.P. (2002). Cellulose biosynthesis in plants: from genes to rosettes. Plant Cell Physiol 43, 1407-1420.
11. Eisenhaber, B., Wildpaner, M., Schultz, C.J., Borner, G.H.H., Dupree, P., and Eisenhaber, F. (2003). Glycosylphosphatidylinositol lipid anchoring of plant proteins. Sensitive prediction from sequence- and genome-wide studies for arabidopsis and rice. Plant Physiology 133, 1691-1701.
12. Estevez, J.M., and Somerville, C. (2006). FIAsh-based live-cell fluorescent imaging of synthetic peptides expressed in Arabidopsis and tobacco. Biotechniques 41, 569-574-574.
13. Foston, M. (2014). Advances in solid-state NMR of cellulose. Curr Opin Biotechnol 27, 176-184.
14. Harris, D.M., Corbin, K., Wang, T., Gutierrez, R., Bertolo, A.L., Petti, C., Smilgies, D.M., Estevez, J.M., Bonetta, D., Urbanowicz, B.R., et al. (2012). Cellulose microfibril crystallinity is reduced by mutating C-terminal transmembrane region residues CESA1(A903V) and CESA3(T942I) of cellulose synthase. Proc Natl Acad Sci USA 109, 4098-4103.
15. His, I., Driouch, A., Nicol, F., Jauneau, A., and Hofte, H. (2001). Altered pectin composition in primary cell walls of korrigan, a dwarf mutant of Arabidopsis deficient in a membrane-bound endo-1,4-beta-glucanase. Planta 212, 348-358.
16. Lei, L., Zhang, T., Strasser, R., Lee, C.M., Gonneau, M., Mach, L., Vernhettes, S., Kim, S.H., Cosgrove, D., Li, S., et al. (2014). The jiaoya01 Mutant Is an Allele of korrigan1 That Abolishes Endoglucanase Activity and Affects the Organization of Both Cellulose Microfibrils and Microtubules in Arabidopsis. Plant Cell 2601-2616.
17. Li, S.D., Lei, L., Somerville, C.R., and Gu, Y. (2012). Cellulose synthase interactive protein 1 (CSI1) links microtubules and cellulose synthase complexes. Proc Natl Acad Sci USA 109, 185-190.
18. Lis, H., and Sharon, N. (1998). Lectins: Carbohydrate-Specific Proteins That Mediate Cellular Recognition. Chem Rev 98, 637-674.
19. Liu, L.F., Shang-Guan, K.K., Zhang, B.C., Liu, X.L., Yan, M.X., Zhang, L.J., Shi, Y.Y., Zhang, M., Qian, Q., Li, J.Y., et al. (2013). Brittle Culm1, a COBRA-Like Protein, Functions in Cellulose Assembly through Binding Cellulose Microfibrils. Plos Genet 9(8) e1003704.
20. Mansfield, S.D., Kim, H., Lu, F.C., and Ralph, J. (2012). Whole plant cell wall characterization using solution-state 2D NMR. Nat Protoc 7, 1579-1589.
21. McFarlane, H.E., Doring, A., and Persson, S. (2014). The cell biology of cellulose synthesis. Annu Rev Plant Biol 65, 69-94.
22. Morcombe, C.R., and Zilm, K.W. (2003). Chemical shift referencing in MAS solid state NMR. J Magn Reson 162, 479-486.
23. Nicol, F., His, I., Jauneau, A., Vernhettes, S., Canut, H., and Hofte, H. (1998). A plasma membrane-bound putative endo-1,4-beta-D-glucanase is required for normal wall assembly and cell elongation in Arabidopsis. Embo J 17, 5563-5576.
24. Paredez, A.R., Somerville, C.R., and Ehrhardt, D.W. (2006). Visualization of cellulose synthase demonstrates functional association with microtubules. Science 312, 1491-1495.
25. Persson, S., Wei, H., Milne, J., Page, G.P., and Somerville, C.R. (2005). Identification of genes required for cellulose synthesis by regression analysis of public microarray data sets. Proc Natl Acad Sci U S A 102, 8633-8638.
26. Roudier, F., Fernandez, A.G., Fujita, M., Himmelspach, R., Borner, G.H.H., Schindelman, G., Song, S., Baskin, T.I., Dupree, P., Wasteneys, G.O., et al. (2005). COBRA, an Arabidopsis extracellular glycosyl-phosphatidyl inositol-anchored protein, specifically controls highly anisotropic expansion through its involvement in cellulose microfibril orientation. Plant Cell 17, 1749-1763.
27. Ruprecht, C., and Persson, S. (2012). Co-expression of cell-wall related genes: new tools and insights. Front Plant Sci 3, 83.
28. Sanchez-Rodriguez, C., Bauer, S., Hematy, K., Saxe, F., Ibanez, A.B., Vodermaier, V., Konlechner, C., Sampathkumar, A., Ruggeberg, M., Aichinger, E., et al. (2012). Chitinase-like1/pom-pom1 and its homolog CTL2 are glucan-interacting proteins important for cellulose biosynthesis in Arabidopsis. Plant Cell 24, 589-607.
29. Sato, K., Ito, S., Fujii, T., Suzuki, R., Takenouchi, S., Nakaba, S., Funada, R., Sano, Y., Kajita, S., Kitano, H., et al. (2010). The carbohydrate-binding module (CBM)-like sequence is crucial for rice
The role of COBRA in cellulose synthesis

CWA1/BC1 function in proper assembly of secondary cell wall materials. Plant Signal Behav 5, 1433-1436.

30. Scheible, W.R., Eshed, R., Richmond, T., Delmer, D., and Somerville, C. (2001). Modifications of cellulose synthase confer resistance to isoxaben and thiazolidinone herbicides in Arabidopsis lxr1 mutants. Proc Natl Acad Sci U S A 98, 10079-10084.

31. Schindelman, G., Morikami, A., Jung, J., Baskin, T.I., Carpita, N.C., Derbyshire, P., McCann, M.C., and Benfey, P.N. (2001). COBRA encodes a putative GPI-anchored protein, which is polarly localized and necessary for oriented cell expansion in Arabidopsis. Gene Dev 15, 1115-1127.

32. Szyjanowicz, P.M.J., McKinnon, I., Taylor, N.G., Gardiner, J., Jarvis, M.C., and Turner, S.R. (2004). The irregular xylem 2 mutant is an allele of korrigan that affects the secondary cell wall of Arabidopsis thaliana. Plant Journal 37, 730-740.

33. Todd, M.J., and Gomez, J. (2001). Enzyme kinetics determined using calorimetry: A general assay for enzyme activity? Analytical Biochemistry 296, 179-187.

34. Vain, T., Crowell, E.F., Timpano, H., Biot, E., Desprez, T., Mansoori, N., Trindade, L.M., Pagant, S., Robert, S., Hofte, H., et al. (2014). The cellulase KORRIGAN is part of the Cellulose Synthase Complex. Plant Physiol 1521-1532.

35. Walter, S., and Schrempf, H. (2003). Oligomerization, membrane anchoring, and cellulose-binding characteristics of AbpS, a receptor-like Streptomyces protein. J Biol Chem 278, 26639-26647.

36. Wang, T., Zabotina, O., and Hong, M. (2012). Pectin-Cellulose Interactions in the Arabidopsis Primary Cell Wall from Two-Dimensional Magic-Angle-Spinning Solid-State Nuclear Magnetic Resonance. Biochemistry 51, 9846-9856.

37. Zuo, J.R., Niu, Q.W., Nishizawa, N., Wu, Y., Kost, B., and Chua, N.H. (2000). KORRIGAN, an arabidopsis endo-1,4-beta-glucanase, localizes to the cell plate by polarized targeting and is essential for cytokinesis. Plant Cell 12, 1137-1152.
**Figure legends**

**Figure 1** – **COBRA is localized at the plasma membrane, is sensitive to isoxaben, and when mutated disrupts cellulose synthesis.** A) FlAsH-COBRA and GFP-CESA3 were visualized in the hypocotyl elongation zone of 5 day old dark grown Arabidopsis seedlings. FlAsH-COBRA showed a punctate pattern of labeling in the plasma membrane focal plane. B) Plasma membrane localization was verified by plasmolysis with 0.8 M NaCl, which causes shrinkage and condensation of the cytoplasm and vacuole, and detachment of the plasma membrane from the cell wall (arrow head). C) Treatment of seedlings with 100 nm isoxaben caused clearance of GFP-CESA3 particles from the plasma membrane resulting in intracellular accumulation. The same observation was made for FlAsH-COBRA treated with isoxaben. D) Time-lapse confocal images of YFP-CESA6 in hypocotyl cells of 5 day-old etiolated Col-0 (left) and cob-6 (right) plants were compared. Each image is an average of 36 frames taken at 5 second intervals (n = 9). CESA complex velocities were measured using Imaris. Data passed normality and equal distribution tests after natural log transformation. The complex velocity in cob-6 (289±162nm/min) was found to be significantly different from that of wild type (225±147 nm/min), (t-test, p < 0.01). Aside from the differences in the velocity rate, in cob-6, cellulose synthesis occurs in a relatively linear direction. Scale bars are 5μm in A, B, and D and 10 μM in C.

**Figure 2** – **Isothermal titration calorimetry of COBRA binding to cellohexaose.** The upper plot shows the raw heats of binding, and the lower plot shows the integrated heats of binding, excluding dilution effects. Titrations were carried out at 21°C, by the addition of 750 μM cellohexaose to 50 μM COBRA.

**Figure 3** – **COBRA competition assay with crystalline cellulose (Avicel) and individual glucan chains (cellohexaose).** Pull down assays of full length COBRA and Avicel were performed with increasing concentrations of cellohexaose. Without cellohexaose, COBRA binds to Avicel. 1μM of cellohexaose was enough to prevent a significant fraction of COBRA from binding to Avicel. With 3 μM of cellohexaose, which represented COBRA’s binding association constant of 3.2 μM. In 30 μM cellohexaose, a 10 fold increase over the Kₐ, COBRA could not be detected in the fraction bound to Avicel.

**Figure 4** – **Cell wall composition of WT and cob-6.** A) Measurement of cellulose. B) Analysis of galacturonic acid (GalA). C) Monosaccharide analysis. In A and B, asterisks represent P<0.05 by T-TEST and in C asterisks represent P<0.05 by 2 way ANOVA coupled to Tukey test.

**Figure 5** – **1D and 2D SSNMR of WT and cob-6 cell wall.** A) Quantitative ¹³C DP-MAS SSNMR spectra of WT and cob-6 cell walls. B and C) ¹³C-¹³C chemical shift correlation spectra with 50 ms DARR mixing of B) WT and C) cob-6. Assignments are indicated by color for cellulose (red), hemicelluloses (blue) and pectin (green). Yellow Rectangles indicate polysaccharides whose amounts changed in cob-6 compared to the WT and relative to the amount of cellulose.

**Figure 6** - ¹³C-¹³C chemical shift correlation spectra of WT and cob-6 acquired with 50 ms DARR mixing time. The spectra from figure 4 plotted at lower contour levels, emphasizing the cross section at
~96 ppm in the cob-6 spectrum. The cross section signals have been assigned to the β-glucose reducing end units, which can be detected when the degree of polymerization of cellulose is sufficiently low.

Figure 7 – Speculative model for the stages of cellulose formation – T0) Cellulose synthase complex (CSC) is targeted and integrated into the plasma membrane. T1) COBRA is recruited to the vicinity of the cellulose synthase complex. Upon activation of the cellulose synthase complex, individual β1-4 glucose chains emerge from the complex. T2) during the beginning of cellulose synthesis, the β1-4 glucose chains (in the model only 2 are drawn, for simplicity) are bound by COBRA proteins, one glucan chain by one molecule of COBRA protein. COBRA facilitates the interaction of the individual chains into a cellulose fibril. T3) If COBRA does not dissociate from the cellulose microfibrils it would eventually be found in the cell wall as newly synthesized cellulose fibrils displace older fibrils.
### Figures and tables

#### Table 1 - Plasmids Used in This Study

| Plasmid name            | Description                                      | Source                        |
|-------------------------|--------------------------------------------------|-------------------------------|
| pGEX4T-2                | N terminus GST expression                        | GE Healthcare                 |
| PCR8                    | Entry vector                                     | Invitrogen                    |
| pDONOR P4-P1R           | Promoter entry vector                            | http://www.psb.ugent.be/      |
| pDONOR 207              | 1\(^{st}\) gene entry vector                     | http://www.psb.ugent.be/      |
| pDONOR P2R-P3           | 2\(^{nd}\) gene entry vector                     | http://www.psb.ugent.be/      |
| pH7 mu34GW              | Plant expression vector                          | http://www.psb.ugent.be/      |
| NS29                    | **COBRA** 2.5Kb promoter in pDONOR P4-P1R        | This study                    |
| NS38                    | FlAsH-COBRA in pDONOR207                         | This study                    |
| NS60                    | NOS terminator in pDONOR P2R-P3                   | Carroll et al., 2012          |
| NS79                    | pCOB::FlAsH-COBRA::NOS in pH7 mu34GW             | This study                    |
| NS85                    | His6-COBRA in PCR8                               | This study                    |
| NS87                    | GST-His6-COBRA in pGEX4T-2                       | This study                    |
Table 2 - Primers Used in This Study

| Primer name | Sequence (5’ to 3’) | Product of target |
|-------------|---------------------|-------------------|
| NS29        | GGGGACAAGTTTGTACAAAAAAAGCAGGCT ATGGAGTCTT TCTTC | 5’ FLAsH-COBRA into p207 |
| NS30        | GGGGACCACCTTTGTACAAGAAAGCTGGT TTAGGCAGAGAAGAA | 3’ FLAsH-COBRA into p207 |
| NS31        | GGGGACAACTTTGTATAGAAAGAAGTTGGATCTTGATGATGAATGGAA | 5’ COBRA promoter into pDONOR P4-P1R |
| NS33        | GGGGACTGCTTTTTTTGTACAAAAACTTGGTTTTAATACTCTTGATGATC | 3’ COBRA promoter into pDONOR P4-P1R |
| NS173       | CTCGAGAACATCATCATCATCATCATCATCTTTACTTCGACAGAAGCATA | 5’ His6:COBRA CDS, no SP and no GPI seq |
| NS174       | GCTCGAGTCAGGGAAGAAGGAGGTATTACTTCGACAGAAGCATA | 3’ COBRA CDS, no SP and no GPI seq |
| NS180       | TTTGTGCTCCACCATACTCC | COBRA LP |
| NS181       | AAGCAAAAGACCTTTCTCTCTTC | COBRA RP |
| Lbb1        | ATTTTGCCGATTTCCGAAC | cob-6 LP |
Table 3. Transgenic Plant Lines Used in This Study

| Transgenic line       | Genotype                                      | Source                  |
|-----------------------|-----------------------------------------------|-------------------------|
| cob-6                 | cobra                                         | SALK_051906             |
| YFP:CESA6             | pCESA6::YFP:CESA6, prc-/-                    | Paredez et al., 2006    |
| GFP:CESA3             | pCESA3::GFP:CESA3, eli1-1-/-                 | Carroll et al., 2012    |
| YFP:CESA6 x cob-6     | pCESA6::YFP:CESA6, prc-/-; cob-6-/-          | This study              |
| FlAsH-COBRA line1    | pCOBRA::FlAsH-COBRA, cob-6-/-                | This study              |
| FlAsH-COBRA line2    | pCOBRA::FlAsH-COBRA, cob-6-/-                | This study              |
| FlAsH-COBRA line3    | pCOBRA::FlAsH-COBRA, cob-6-/-                | This study              |
Table 4 - Relative intensities of interior and surface cellulose C4 signals from $^{13}$C DP-MAS spectra.

| Genotype | I(iC4) 88.5-91.5 ppm | I(sC4) 84-86 ppm | I(iC4)/I(sC4) | Normalized |
|----------|---------------------|-----------------|---------------|------------|
| WT       | 36.4E9              | 54.5E9          | 0.67          | 1          |
| cob-6    | 20.2E9              | 36.0E9          | 0.56          | 0.84       |
The role of COBRA in cellulose synthesis

Figure 1

A. Plasma membrane focal plane

B. Plasmolysis

C. Plasma membrane focal plane

D. YFP-CESA6 WT

D. YFP-CESA6 cob-6

E. Graph showing particles number vs. nm/min
Figure 3

|       | C6     | 0μM    | 1μM    | 2μM    | 3μM    | 6μM    | 30μM   |
|-------|--------|--------|--------|--------|--------|--------|--------|
| cobra | 10μg   | 10μg   | 10μg   | 10μg   | 10μg   | 10μg   | 10μg   |
| Avicel| 1mg    | 1mg    | 1mg    | 1mg    | 1mg    | 1mg    | 1mg    |

Soluble

Avicel bound
Figure 4

A. Cellulose

\[ \text{Cellulose} \]

\[ \mu g \text{Glu/mg of AIR} \]

WT \hspace{1cm} cob-6

B. Pectin backbone

\[ \text{Pectin backbone} \]

\[ \mu g \text{Gal/Mg of AIR} \]

WT \hspace{1cm} cob-6

C. Monosaccharides

\[ \mu g \text{sugar/mg of AIR} \]

Man, Fuc, Ara, Xyl, Rha, Gal, Glu

WT \hspace{1cm} cob-6
The role of COBRA in cellulose synthesis

Figure 5

A. **WT**
   - cob-6
The role of COBRA in cellulose synthesis
The Arabidopsis COBRA protein facilitates cellulose crystallization at the plasma membrane
Nadav Sorek, Hagit Sorek, Aleksandra Kijac, Heidi J. Szemenyei, Stefan Bauer, Kian Hematy, David E. Wemmer and Chris R. Somerville

J. Biol. Chem. published online October 20, 2014

Access the most updated version of this article at doi: 10.1074/jbc.M114.607192

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts