Genetic Modification of Plant Cell Walls for Enhanced Biomass Production and Utilization

Investigators
Chris Somerville, Principal Investigator, Professor, Department of Biological Sciences, Stanford University and Director, Carnegie Institution, Department of Plant Biology; Jennifer Milne, Post-doctoral Scholar, Department of Biological Sciences, Stanford University; and Natalia Khitrov, Technician, Department of Plant Biology, Carnegie Institution

Abstract
The genes which encode the catalytic subunits of the Cellulose Synthase associated with the deposition of cellulose in the secondary cell wall in Arabidopsis, CESA4, 7, and 8, have been cloned under the control of an inducible promoter. Gene constructs were transformed into Arabidopsis and lines carrying the transgenes selected based on resistance to hygromycin. Reporter gene activity was measured in various aerial parts of the plant at 18 hour after application of the inducer, dexamethasone. All three genes were expressed in similar areas of the plant, including the vasculature of leaves, hydathones, sepals, and petals, and at the tips and base of pollen tubes. Plants will be available to be analyzed for changes in cellulose levels due to the expression of the CESA genes by the end of June.

Introduction
Plant cells walls are the primary source of terrestrial biomass. Any attempt to utilize biomass-based sources of energy must involve optimized production and utilization of plant cell walls. One of the principle components of plant cell walls is cellulose, a polymer of β-(1-4) linked glucose. This can be hydrolyzed to pure glucose and fermented to ethanol fuel. By modifying the normal biological control of cellulose synthesis, we think it possible to develop plants that accumulate more fixed carbon per unit land area per unit time. The percentage of cellulose to other wall polymers may also be increased, potentially making processing of the biomass more efficient.

The multi-subunit enzyme complex that makes cellulose in the model plant Arabidopsis thaliana, is composed of 36 protein subunits, figure 1. Each subunit makes one glucan chain and their proximity to one another in the plasma membrane leads to the formation of a strong, inextensible microfibril that is extruded into the cell wall. Our goal is to increase per unit area, per time, the amount of cellulose that is deposited by a plant. This project will test the hypothesis that cellulose synthesis is controlled at the level of gene expression by increasing expression of the genes that encode the components of the Cellulose Synthase. We have placed the CESA genes of the cellulose synthase of secondary cell walls under the control of a chemically inducible promoter in Arabidopsis and have observed induction, with dexamethasone, of reporter gene expression.
Figure 1: Schematic of Cellulose Synthase. The 36 subunit complex is embedded in the plasma membrane. Each protein subunit catalyzes the formation of a glucan chain. The 36 chains interact to form a microfibril that is extruded to the extracellular space and becomes incorporated into the cell wall. CESA4, 7, and 8, denote the protein catalytic subunits of the cellulose synthase complex encoded by genes CESA4, 7, and 8, respectively.
Background
The leaf cell walls of a dicot species such as Arabidopsis contain three major classes of polysaccharides: cellulose, hemicellulose, and pectins. Cellulose is present as long unbranched fibrils composed of approx 30-36 hydrogen-bonded chains of β-(1-4) glucose. Hemicelluloses are branched polysaccharides containing backbones of neutral sugars that can form hydrogen bonds to cellulose fibrils. Pectins are defined by the presence of uronic acids as major components. The amounts of the various polymers vary from one cell type to another for largely unknown reasons. Notably, the amount of cellulose may vary many-fold from one cell type to another. This is most readily evident in vascular tissue where the xylem cells exhibit “secondary cell walls” that form a second layer of deposition within the “primary wall”. Secondary walls are only deposited in cells that have stopped dividing and expanding.

In Arabidopsis, the leaf mesophyll cells have a composition such that cellulose comprises only 14% of the total wall mass. By contrast, in xylem cells, cellulose appears to form the majority of the mass. From these studies on Arabidopsis it appears that the amount of cellulose deposited is under developmental control and increased amounts of cellulose are not necessarily incompatible with cellular function.

A family of genes encoding the catalytic subunit of higher plant Cellulose Synthase, termed cesA, was identified by genomic methods on the basis of weak homology to bacterial cellulose synthase. The molecular characterization of mutants with defects in cell wall biogenesis confirmed the participation of the CESA proteins (Arioli et al., 1998; Fagard et al., 2000; Taylor et al., 1999). Arabidopsis has 10 CESA genes. Analysis of the tissue specificity of expression of various CESA genes combined with knowledge of the mutant phenotypes for several of the genes has led to the proposal that three or more different CESA proteins are required for formation of a functional cellulose synthase complex (Taylor et al., 2003). Direct evidence for formation of mixed complexes has been obtained by immuno-precipitation experiments (Taylor et al., 2000).

Co-expression analysis of the CESA genes has revealed further genes that may be involved in and required for cellulose synthesis (Persson et al., 2005). The mutant phenotypes of plants with knockouts in these genes are consistent with those of the cesa mutants, having xylem cells with walls that have collapsed inwards. Some of these mutants have reduced cellulose compared to wild type plants. Experiments are underway to characterize these mutants. Two of these may be involved in xylan synthesis (a hemicellulose polymer), which may be required for correct deposition of the cellulose microfibrils in the wall (Bauer et al., 2006). If these genes prove to be crucial for proper cellulose deposition, these too will be expressed under the control of an inducible promoter in the same plants as the CESA genes.
Results

Cloning of the cesA genes and transformation of Arabidopsis

Primers were designed for amplification of each CESA gene with restriction enzyme sites for cloning into the expression vector pH-TOP (Craft et al., 2005), and with the addition of a TAG sequence. CESA4 was cloned into the multiple cloning site of pH-TOP using unique restriction sites XhoI and KpnI; CESA7 using HindIII and XhoI; and CESA8 using SalI. A FLAG TAG was added to the 5' end of CESA4, a myc TAG to CESA7, and and HA TAG to CESA8. The constructs containing CESA4-FLAG and CESA7-myc and CESA8-HA have been sequenced, errors introduced by PCR corrected, sequenced again and transformed into plants.

Selection of plants containing CESA genes

Selection of plants containing each gene has been carried out and is continuing in order to obtain additional lines. Plants were plated out on standard growth medium supplemented with the antibiotic, hygromycin. Plants that showed resistance to hygromycin, by growing normally under light conditions, were transplanted to soil and grown under normal greenhouse conditions. At the four-week-old stage, gene expression was induced by topological application of dexamethasone to leaves and inflorescences.

Screen for gene expression using GUS assay

Leaves, and inflorescences of plants were excised from whole plants 18 hours after induction with dexamethasone. Tissue was placed in 6 well Petri dishes and histochemical GUS assays were performed as described in Jefferson (1987). Plates were placed in the dark at 37°C overnight. GUS staining solution was removed and plant tissues were cleared with 70% ethanol. Figure 2 shows the GUS staining patterns that were observed for the different lines carrying the genes, CESA4, 7, and 8.

Progress

Transgenic lines expressing each CESA gene required for the Cellulose Synthase complex associated with the deposition of cellulose in secondary cell walls have been obtained. These plants have yet to be crossed to obtain all three genes expressed under an inducible promoter in the same plant. Expression of the reporter gene, to which CESA expression is coupled, indicates the genes are expressed in similar places in all lines, suggesting that it will be possible to express all three genes in the same cell types in one plant. Due to the generation times of the plants we will be able to determine if the amount of cellulose made by a plant can controlled by the expression of these three necessary subunits of the cellulose synthase within the year. If this is the case, transferring this technology to energy crops seems feasible since orthologues of these genes have already been identified in a number of species including poplar. Increased cellulose in plants will allow an increase in unit area of stored carbon for use in the production of ethanol from cellulosic biomass, making this a more efficient process by reducing transportation costs. As a result, displacing fossil fuel use by this carbon neutral energy source could reduce CO2 emissions.
Figure 2: Dexamethasone-induced GUS expression in greenhouse-grown plants. (A-G) Four-week-old greenhouse-grown plants were treated by topological application of dexamethasone [20 uM in DMSO, with 2 ul Silwet, in 10 ul H2O] and samples were collected for histochemical staining 18 h later. A and B are from the same transgenic line transformed with CESA4; C, transgenic line with CESA7; D, E, and F, transgenic line with CESA8; and G, is a wild type induced control. H and I are the un-induced controls for CESA4 and 7.
Future plans

Additional plants will be screened for resistance to hygromycin and inducible reporter gene expression using the GUS assay. These lines will be analyzed for transcript levels, protein levels, and then changes in cellulose at various times after induction. Lines expressing each cesA gene will be crossed to give all three genes under the inducible promoter in the same plant. Several lines of each will be chosen to work with so that expression levels of each gene can be optimized. These plant lines will be further screened for accumulation of the CESA proteins.

Plants containing all three genes that are successfully expressed will be analyzed for cell wall composition and for effects on plant growth and development. If quantitative differences are observed, transmission electron microscopy will be used to determine the morphology of the cellulose. Other techniques such as immuno-labelling using specific antibodies to other polysaccharides will be used to determine the composition of the new wall material. In order to determine how the plants respond to the abnormal expression of the CESA genes we will examine the effects on gene expression using whole genome DNA chips.

If increased cellulose is obtained from chemical induction of the genes, we will test the feasibility of engineering enhanced cellulose under the control of developmental stage specific promoters. A number of suitable promoters have been identified through analysis of tissue specific gene expression for other purposes. Suitable promoters for expressing the CESA genes will be those that show high expression in non-dividing and non-expanding cells and no expression in expanding cells.

References


Contacts

Chris Somerville: crs@stanford.edu
Jennifer Milne: jmilne@stanford.edu