CHAPTER 5

FROM CELLULOSE TO MECHANICAL STRENGTH: CARBON FLUX AND RELATIONSHIP OF THE CELLULOSE SYNTHASE GENES TO DRY MATTER ACCUMULATION IN MAIZE

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Abstract

Stalk strength in maize is primarily determined by the amount of cellulose in a unit length of the internode. An increase in cellulose concentration of the cell wall, aside from allowing simultaneous improvements in stalk strength and harvest index, will increase the value of stover as a feedstock for ethanol production. Sucrose synthase makes UDP-glucose, substrate for cellulose formation, from uridine diphosphate (UDP) and sucrose whereby it conserves the energy of the glycosidic bond. The alternative route of UDP-glucose formation through UDP-glucose pyrophosphorylase, in contrast, consumes two equivalents of uridine triphosphate (UTP), making it an energy-intensive process. *In vivo*, the reaction catalyzed by sucrose synthase operates in the direction of UDP-glucose formation because of deviation of the relationship between mass action ratio (*in vivo* ratio of products to substrates) and *Keq* from unity. A reduction in the amount of enzyme could be compensated by this mechanism without affecting the magnitude of net flux. Since cellulose is crystallized into microfibrils immediately after synthesis, the reaction of cellulose synthase is considered to be far from equilibrium. Cellulose synthase may thus exert considerable control on carbon flux into cellulose. We isolated 12 members of the *CesA* gene family from maize. Upon phylogenetic analysis, three of the maize *CesA* genes, *ZmCesA10-12*, clustered with the *Arabidopsis CesA* sequences that had previously been shown to be involved in secondary wall formation. These three genes were coordinately expressed across multiple tissues, suggesting that they might interact with each other to form a functional cellulose synthase complex. Isolation of the expressed *CesA* genes from maize and their association with primary or

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secondary wall formation has made it possible to test their respective roles in cellulose synthesis in different cell types through association genetics, mutational genetics, or a transgenic approach. This information would be useful in improving stalk strength in cereals.

Keywords

cellulose synthase, cell wall, CesA, flexural strength, flux control analysis, gene expression profiling, MPSS, phylogeny, secondary wall, stalk lodging, vascular bundles.

Abbreviations

cellulose synthase (*CesA*), expressed sequence tag (EST), irregular xylem(*Irx*), massively parallel signature sequencing (MPSS), weight (wt.).

1 INTRODUCTION

Cellulose is the major wall constituent in the supporting tissues of mature plant cells. The paracrystalline structure of cellulose, that results from energy minimization by the formation of inter- and intrachain hydrogen bonds, makes it mechanically the strongest known organic molecule on density basis (Niklas 1992). It is natural then that cellulose is the primary determinant of strength in structural tissues.

Stalk lodging, which results from mechanical failure of the stalk tissue anywhere below the ear node before harvest, results in significant yield losses in maize (Duvick and Cassman 1999). Lodging, also a problem in other cereal crops, is influenced by morphological traits as well as environmental conditions. An indicator of dry matter partitioning efficiency is harvest index, the ratio of the grain to the total aboveground biomass. Introduction of dwarfing genes into small grain cereals allowed for substantial increases in their harvest indices (Sinclair 1998). Dwarfing also made them less likely to lodge mainly by reducing torque on the top-heavy straw. This architectural alteration allowed for higher fertilizer inputs, resulting in increased biomass and thus grain production. In contrast, harvest index in maize has remained essentially unchanged at ~50% in the modern hybrids when compared with the older varieties (Russell 1985; Tollenaar and Wu 1999). Yield improvements in maize have thus been realized primarily from the increases in total biomass per unit land area. Most of the biomass increase has resulted from increased planting density although some can also be attributed to an increase in plant height. With increasing planting density, maize stalks become mechanically weaker and thus susceptible to lodging because of the resulting reduction in individual plant mass.

Identification of chemical constituents that contribute to mechanical strength and the corresponding molecular mechanisms responsible for their formation are prerequisite steps toward using the tools of genetic engineering to reduce the incidence of lodging in cereal crops. The biochemical approach was only partially successful in the isolation of polysaccharide synthases (Dhugga and Ray 1994, Dhugga 2005). In comparison, genomic technologies made it easier to isolate the gene families that affect cell wall formation and to associate their expression patterns with different tissues in the plant (Delmer 1999; Holland et al. 2000; Dhugga 2001; Richmond and Somerville 2001). This review will cover

the role of cellulose in stalk strength, flux of carbon into cellulose through cellulose synthases (*CesA*), alteration of cellulose formation in plants, and the roles different members of the *CesA* gene family from maize play in cellulose synthesis in different cell types as inferred from expression profiling.

2 ROLE OF CELLULOSE IN STALK STRENGTH

Dry matter in a unit length of the maize stalk explained approximately half of the variation in mechanical strength (Appenzeller et al. 2004). Structural dry matter, which was derived by removing the soluble contents from the total dry matter, accounted for approximately 80% of the mechanical strength and was thus a superior indicator of strength than total dry matter. A still greater proportion of the internodal flexural strength (85%) was explained when only cellulose content in a unit length of the stalk was considered. This finding is consistent with cellulose being the most abundant and strongest constituent of the cell wall, making up ~50% of the total dry matter in a mature corn stalk.

These results suggest that one of the avenues to improve mechanical strength of the maize stalk is to increase cellulose concentration in existing dry matter. Conversion of other polysaccharides and free sugars into cellulose is bioenergetically neutral so is not expected to adversely affect plant performance (Sinclair and de Wit 1975). *CesA* genes offer a suitable target for a biotechnological approach to accomplish this objective.

3 CARBON FLUX THROUGH CELLULOSE SYNTHASE

Kinetic information on cellulose synthesis is scarce in the literature. The in vitro studies reported are limited almost exclusively to experimental systems, such as bacteria and elongating cotton and flax fibers (Aloni et al. 1982; Delmer et al. 1993; Li et al. 1993). Li et al. (1993), analyzing cellulose synthase activity in cotton fibers, reported the K_m and V_{max} for UDP-glucose to be respectively 0.4 mM and 2.8 nmol•min^{-1}•mg protein^{-1}. If cellulose synthase exhibits similar properties *in vivo* then it must operate under substrate-saturated conditions since the concentration of UDP-glucose in the cytosol is 1–3 mM (Dancer et al. 1990; Krause and Stitt 1992; Winter et al. 1993, 1994).

True estimates of V_{max} for cellulose synthase, however, are still problematic. Delmer (1993, personal communications), for example, has noted that the rates for cellulose synthesis in vitro reported by Li et al. (1993) were no more than 5% of those occurring *in vivo* during primary wall synthesis.

An estimate of the carbon flux into cellulose could also be drawn based on an average value of net primary productivity (NPP) for crops, which can also be expressed on a leaf area basis. According to Leopold and Kriedemann (1975), a reasonable growth rate for sunflower leaves could be ~85 g dry wt•m^{-2}•week^{-1}. For maize, an average NPP value calculated from results published by Uhart and Andrade (1995) resulted in ~56 g dry wt•m^{-2}•week^{-1}. Averaging these two

figures and converting the units, the rate of dry matter deposition would amount to approximately 698 ng dry wt•cm^{-2}•min^{-1}. Considering that cellulose constitutes one third of the primary cell wall, approximately 30% of the increase in dry weight during growth would result from carbon allocation into the cellulose fraction of cell walls. This increase in dry mass can be expressed on a fresh weight basis by using an average value for specific leaf weight for maize of 0.009 g fresh wt•cm^{-2} (Barreiro 1999). Therefore, the rate of cellulose deposition in maize leaves expressed on a fresh weight basis would be approximately 160 nmoles of glucosyl units•g fresh wt^{-1}•min^{-1}. Flux through cellulose synthase measured *in vivo* in our lab using maize coleoptiles fed with radiolabeled sucrose at 30°C was 118 ± 37 nmoles of glucose•g^{-1} fresh wt•min^{-1} (N = 21) (Barreiro, unpublished). These results should be interpreted with caution since the accuracy of fluxes measured *in vivo* using radiolabeled substrates is limited by the estimation of the specific radioactivity within the enzyme microenvironment. However, these rates, estimated from NPP values or measured *in vivo*, were also consistent with fluxes ranging from 51 to 213 nmoles of glucose equivalents incorporated•g^{-1} fresh wt•min^{-1} obtained with simulations using a formal model incorporating complex kinetic equations and metabolic parameters determined experimentally (Barreiro 1999).

Sensitivity analyses run with a mathematical model in which simulated carbon fluxes through cellulose synthase are contrasted to changes in V*f* (limiting velocity in the direction of cellulose synthesis), indicate that fluxes are linear with respect to V*f* as expected for a condition in which the median substrate concentration is saturating (Barreiro 1999). According to these simulations, increases in the expression of cellulose synthases should correlate linearly with increases in cellulose production, providing that the substrate pool remains saturating at the steady state. Moreover, in reactions where the mass action ratio is far from the ratio of products to substrates in equilibrium, i.e., when the change in actual free energy of the reaction is different from zero, the flux is affected primarily by the enzymatic rate and not by the concentration of metabolites. Cellulose synthase is a multi-protein complex, however, and simultaneous up-regulation of genes encoding non-CesA complex members may be required for improved flux of carbon into cellulose.

4 ALTERATION OF CELLULOSE FORMATION IN PLANTS

In addition to its role as the primary determinant of tissue strength, a trait that is of significant interest in agriculture, cellulose constitutes the most abundant renewable energy resource on Earth. More than 200 million metric tons of stover is produced just from maize in the USA every year. About one-third of this could potentially be utilized for ethanol production (Kadam and McMillan 2003). The worldwide production of lignocellulosic wastes from cereal stover and straw is estimated to be ~3 billion tons per year (Kuhad

and Singh 1993). An increase in cellulose and reduction in lignin contents in the stover material is considered to be beneficial for ethanol production in biorefineries. Lignin is also a target for reduction because it is an undesirable constituent for silage digestibility and the paper industry (Hu et al. 1999; Li et al. 2003a).

Availability of the first plant *CesA* gene, which was isolated by sequencing only a few hundreds of the expressed sequence tags (EST) from developing cotton fibers at a stage when secondary wall was being deposited at a rapid rate (Pear et al. 1996), facilitated the isolation of similar sequences from other species based on homology, including a weakly related set of genes that was termed cellulose synthase-like (*Csl*) (Holland et al. 2000; Richmond and Somerville 2000; Wu et al. 2000; Hazen et al. 2002). Multiple copies of *CesA* have been identified in every plant species investigated. The *CesA* genes are believed to encode the catalytic subunits of the rosette, also referred to as terminal complex that is located in the plasma membrane (Kimura et al. 1999).

Characterization of several cellulose-deficient mutants of *Arabidopsis* allowed the isolation of a number of genes that affect cellulose synthesis. Some of these genes turned out to be members of the *CesA* gene family (Turner and Somerville 1997; Arioli et al. 1998; Taylor et al. 1999, 2000; Fagard et al. 2000; Desprez et al. 2002; Ellis et al. 2002; Cano-Delgado et al. 2003). Others encoded proteins for *N*-glycan synthesis and processing (Boisson et al. 2001; Lukowitz et al. 2001; Burn et al. 2002; Gillmor et al. 2002), a membrane-anchored β-1,4-endoglucanase, Korrigan (Nicol et al. 1998), a membrane-anchored protein of unknown function that might be a part of the cellulose synthase complex, Kobito (Pagant et al. 2002), and Cobra, a putative GPI-anchored protein, which upon being inactivated, dramatically reduced culm strength in rice (Li et al. 2003b). These genes encode proteins with diverse functions that influence cellulose synthesis either directly or indirectly. Precise functions of these proteins remain unknown, however.

Mutations in some of the *CesA* genes involved in primary wall formation caused severely altered phenotypes, which was expected given the role of primary walls in cell expansion starting early in development (Arioli et al. 1998; Fagard et al. 2000; Beeckman et al. 2002; Ellis et al. 2002). The mutant *CesA* genes involved in secondary wall formation affected the visual phenotype only slightly but caused a reduction in cellulose concentration in xylem cells, which was reflected in the diminished mechanical strength of the stem tissue (Turner and Somerville 1997; Taylor et al. 1999, 2000, 2003; Tanaka et al. 2003; Zhong et al. 2003).

Korrigan was originally identified as an extremely dwarf mutant with a lesion in a plasma membrane-associated β-1,4-endoglucanase (Nicol et al. 1998). The Korrigan protein is believed to be involved in the cellulose synthase complex that functions during primary or secondary wall formation (Nicol et al. 1998; Molhoj et al. 2002; Szyjanowicz et al. 2004). Its exact function remains to be determined although given its relationship to the cellulase type of hydrolases, it has been

postulated to play a role in terminating or editing the glucan chains emerging from the cellulose synthase complex before their crystallization into a cellulose microfibril (Matthysse et al. 1995; Nicol et al. 1998; Delmer 1999). Alternatively, it could cleave sterol from the sterol-glucoside primer that has been reported to initiate glucan chain formation for subsequent extension by the plant CesA complex (Molhoj et al. 2002; Peng et al. 2002). However, recent evidence does not support this role (Scheible and Pauly 2004).

Alteration of cellulose production in economically important plants using a transgenic approach has been the subject of only a few studies (Hu et al. 1999; Tang and Sturm 1999; Levy et al. 2002; Li et al. 2003a). An antisense approach used to reduce lignin content was reported to increase the proportion of cellulose in aspen stem wood (Hu et al. 1999; Li et al. 2003a). Aside from the fact that some of the apparent increase in cellulose level could be explained by the compensatory effects of nonlignin wall constituents, the estimate for cellulose concentration was derived from sugar composition and not from direct measurements of its crystalline form, making it difficult to discern the compounding effect of glucose present in other forms, such as free sugars. An interaction between cellulose and lignin deposition has also been recognized in *Arabidopsis* and rice. The *eli1-1 and eli1-2* mutants, defective in the *AtCesA3* gene, had reduced levels of cellulose and showed aberrant deposition of lignin in cells that do not normally become lignified (Cano-Delgado et al. 2000, 2003). Ectopic lignification was also observed in other mutants such as the *AtCesA1* mutant *rsw1-1* and *lion's-tail* (Hauser et al. 1995), or defective in *Korrigan* (Cano-Delgado et al. 2000).

5 MASS ACTION AND METABOLIC CONTROL

Sucrose synthase (SuSy) provides another potential pathway for controlling cellulose production. SuSy cleaves sucrose in the presence of uridine diphosphate (UDP) into UDP-glucose and fructose, thereby conserving the energy of the glycosidic bond:

$$\text{UDP-glucose} + \text{Fructose} \underset{v-1}{\overset{v+1}{\rightleftarrows}} \text{Sucrose} + \text{UDP}$$

Considering the free energy of hydrolysis of sucrose as $-6.6\,\text{Kcal}\bullet\text{mol}^{-1}$ and the energy of the α-D-glycosyl phosphate bond in UDP-glucose as $-7.6\,\text{Kcal}\bullet\text{mol}^{-1}$, the estimated change in free energy for the reaction in vitro results in $-1\,\text{Kcal}\bullet\text{mol}^{-1}$ in favor of sucrose synthesis (Cardini et al. 1955). The apparent equilibrium constant (*Keq*) can be calculated from its relationship to the standard free energy (ΔG^0) by the following equation:

$$\Delta G^0 = -2.303\, RT \log_{10} Keq$$

Where R equals 1.987×10^{-3} $\text{Kcal}\bullet\text{mol}^{-1}\bullet{}^\circ\text{K}^{-1}$ and T equals 25°C (298°K), then

$$Keq = 10^{\frac{-1\ \text{Kcal/mol}}{-1.36\ \text{Kcal/mol}}} = 5.44$$

This parameter falls within the range of values from 1.4 to 8 determined experimentally based on substrate concentrations at equilibrium (Cardini et al. 1955; Neufeld and Hassid 1963; Avigad 1964; Kruger 1997). Since this reaction is thermodynamically close to equilibrium, the net flux ($v = v^{+1} - v^{-1}$) will represent only a fraction of the unidirectional flux the enzyme could catalyze in the absence of products. *In vivo*, the net carbon flux through this enzyme will be a function of the relative size of substrate and product pools at the steady state according to the mass action ratio (Hess 1963; Bücher and Russman 1964). For the reaction catalyzed by SuSy:

$$\Gamma = \frac{[\text{Sucrose}][\text{UDP}]}{[\text{UDP} - \text{glucose}][\text{Fructose}]}$$

The net flux *in vivo* through SuSy can be altered by a relatively small shift in the mass action ratio away from its equilibrium position (*Keq*). To obtain net flux in favor of UDP-glucose formation, the reaction must be displaced from equilibrium such that Γ is higher than *Keq*. The disequilibrium $\left(\frac{\Gamma}{Keq}\right)$ ratio can be related to the change in free energy using the following equation (Rolleston 1972; Stitt 1989):

$$\Delta G = 2.303\ RT \log_{10}\left(\frac{\Gamma}{Keq}\right)$$

Thus, an increase in the divergence of mass action ratio *in vivo* will increase the change in free energy through the reaction. This disequilibrium ratio can also be linked to flux by the ratio of the forward and reverse reaction velocities according to the equation derived by Hess and Brand (1965) from a rate equation based on Michaelis-Menten kinetics:

$$\frac{v^{-1}}{v^{+1}} = \frac{\Gamma}{Keq}$$

Figure 5-1 shows the sensitivity analysis for changes in sucrose concentration and the ratio of reverse and forward reaction velocities. The parameters for Figure 5-1 were calculated using the ranges of substrate and product concentrations found in the literature. UDP-glucose was reported to vary between 1.4 to 3.2 mM (Dancer et al. 1990; Krause and Stitt 1992; Barreiro 1999). Reported fructose levels range from 0.1 to 1.5 mM (Winter et al. 1994; Krapp and Stitt 1995), however, the concentration of cytoplasmic fructose assumed in the calculations could be higher than the concentration found *in vivo* because of the existence of high fructokinase activity in the cytoplasm (Gardner et al. 1992; Renz and Stitt 1993; Renz et al. 1993). Cytosolic UDP concentrations vary from 0.35 to 1.3 mM (Isherwood and Selvendran 1970; Stitt 1989; Dancer et al. 1990; Barreiro 1999) and those for sucrose from 13 to 103 mM (Winter et al. 1993, 1994; Pilon-Smits 1995; Krapp and Stitt 1995; Barreiro 1999).

The cytoplasmic concentrations chosen for each metabolite are not only associated with the range found in the literature but also close to the ranges of apparent

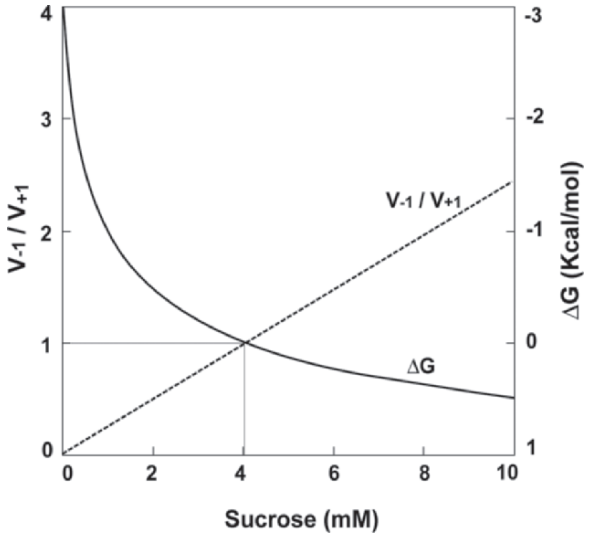

Figure 5-1. Sensitivity analysis of v − 1/v + 1 and change in free energy as a function of sucrose concentration. Negative values for the change in free energy indicate that the net flux of the reaction proceeds in the direction of sucrose synthesis

Km values for SuSy cited therein (Stitt and Steup 1985; Buczynski et al. 1993; Quick and Schaffer 1996; Barreiro 1999). We followed this approach since the optimal physiological concentration for substrates of a reaction displaced from equilibrium is at its *Km* (Rolleston 1972). For the sensitivity analysis the concentration of sucrose was varied, while the rest of the metabolites were clamped at the following concentrations: UDP-glucose 3 mM, fructose 0.1 mM, and UDP 0.4 mM.

The concentration gradient between metabolic pools on each side of the reaction could overcome the chemical bond energetic gradient, in favor of the mass action gradient, favoring sucrose hydrolysis in sink tissues. The threshold of sucrose concentration beyond which the net flux reverses is difficult to assess in a system with a dynamic equilibrium.

Under physiological conditions (assuming that the metabolite concentrations chosen for the calculations are similar at the steady state *in planta*), sucrose concentrations higher than ~4 mM could reverse the net flux of carbon through SuSy in favor of UDP-glucose synthesis due to mass action (Figure 5-1). If we assume that the physiological sucrose concentration within the cytoplasm remains near 50 mM (Barreiro 1999; Rohwer and Botha 2001) then the net flux of carbon through SuSy in sink tissues operates in the direction of UDP-glucose formation. This is consistent with experimental results using radioactive tracers (DeFekete and Cardini 1964; Milner and Avigad 1964; DeFekete 1969; Pavlinova 1971; Huber et al. 1996; Viola 1996; Geigenberger et al. 1997) and with simulations from a mathematical model built with a framework to simulate general pathways (Mendes 1993) and fitted with parameters determined experimentally

(Barreiro 1999). However, the reaction becomes readily reversible when the concentrations of sucrose within the enzyme microenvironment drop to values lower than ~4 mM (Figure 5-1).

Because of its proximity to equilibrium, the forward and reverse reactions catalyzed by SuSy will be affected in the same magnitude whenever the enzyme concentration is changed. As a consequence, the net direction of carbon flux through the enzyme will not be affected; therefore, increasing the expression of this enzyme per se may not increase the flux of carbon into the cellulose pool. On the other hand, decreasing the expression of SuSy, could cause a reduction of the UDP-glucose pool to the extent of limiting the cellulose synthetic rate. When this occurs *in vivo*, the UDP-glucose pool needs to be maintained by an alternative metabolic route that requires more energy to operate (Dhugga et al. 2002). Under stressful conditions, where SuSy expression may be severely attenuated, overexpression of the enzyme may help augment against yield losses.

An alternative metabolic route for UDP-glucose synthesis could be catalyzed by UDP-glucose pyrophosphorylase (UGPase). Both SuSy and UGPase are cytosolic enzymes and thus could contribute to the UDP-glucose pool in this compartment (Entwistle and ApRees 1988).

The metabolic path for UDP-glucose synthesis through UGPase, when considering sucrose as a precursor, has more intermediate steps than the route through SuSy. Whereas the formation of UDP-glucose through SuSy has only one step against a thermodynamic gradient, the route through UGPase has two: the phosphorylation of glucose, with a change in free energy of ~4.7 Kcal•mol^{-1}, and the conversion of glucose 6-P through glucose-1-P into UDP-glucose with a change in free energy of ~2.9 Kcal•mol^{-1}.

The available evidence, although scarce, suggests that the alternative route for the cytoplasmic UDP-glucose pool plays a relatively smaller role in comparison to SuSy. Carrot plants with an antisense version of the main form of SuSy had reduced SuSy activity in roots. Aside from having lower levels of UDP-glucose, these transgenic plants also had reduced levels of cellulose, starch, and total dry matter (Tang and Sturm 1999). Similarly, antisense downregulation of SuSy in potato tubers led to a reduction in cellulose formation (Haigler et al. 2001).

Figure 5-1 shows that the disequilibrium ratio (i.e., $v - 1/v + 1$) for the reaction catalyzed by SuSy can be influenced by a relative small shift in free energy away from equilibrium. Under normal growing conditions, manipulation of SuSy substrate levels to influence the magnitude of the net flux may therefore be a more effective way of controlling the quantity of cellulose deposited in the cell walls rather than altering its expression level.

6 THE CELLULOSE SYNTHASE GENE FAMILY

The *CesA* and *Csl* genes are generally expressed at a low level as judged from their occurrence in EST databases and from gene expression studies (Dhugga 2001). Yet, their transcripts can occur at higher frequencies in specific tissues,

for example, *GhCesA1* and *2* were identified amongst late-stage cotton fiber ESTs (Pear et al. 1996) and the mannan synthase gene was isolated from guar endosperm (Dhugga et al. 2004). In each case, the tissue where the ultimate product of the gene was being actively deposited was utilized to construct an EST database.

Genes involved in secondary wall formation are usually underrepresented in EST databases because many of the source cDNA libraries are derived from immature tissues. From a cDNA library from the transition zone of an elongating maize internode, a region in the stalk where the rate of secondary wall formation is higher than in the elongation zone, three additional full-length *CesA* genes *ZmCesA10*, *ZmCesA11*, and *ZmCesA12* were isolated (Appenzeller et al. 2004). These genes mapped to chromosomes 1, 3 and 7, respectively. Another gene, *ZmCesA7*, which was not previously assigned to any chromosome, mapped to chromosome 7 (Holland et al. 2000).

The deduced amino acid sequences of the three additional maize *CesA* genes were phylogenetically closer to the sequences from *Arabidopsis* that had previously been shown to be involved in secondary wall formation (Appenzeller et al. 2004). ZmCesA10, ZmCesA11, and ZmCesA12 grouped with AtCesA4 (Irx5), AtCesA8 (Irx1), and AtCesA7 (IrxX3), respectively, and are the probable orthologs of these genes (Figure 5-2). Likewise, OsCesA7, OsCesA4, and OsCesA9 are the orthologous sequences in rice (Tanaka et al. 2003), as are respectively the barley HvCesA4, HvCesA5/7 and HvCesA8 sequences (Burton et al. 2003). This suggests that the different subclasses of the CesA genes were formed early in higher plant evolution, before the divergence of monocots and dicots (Holland et al. 2000). Phylogenetic clustering of ZmCesA10-12 with the Irx sequences from *Arabidopsis* and their highest expression in the transition zone of the internode suggest that these genes are involved in secondary wall formation. Gene expression profiling studies described in the following section lend further support to this suggestion.

In comparison to ZmCesA10-12, the nine other maize sequences clustered in three groups: ZmCesA1, 2 and 3 form one group, ZmCesA4, 5 and 9 form another, and ZmCesA6, 7 and 8 form the third group (Figure 5-2). The first set of sequences lie in the same clade as AtCesA1 and 10, the second set clusters with AtCesA3 and the third set cluster with AtCesA2, 5, 6 and 9. Mutational analyses of *AtCesA1* (Arioli et al. 1998), *AtCesA3* (Scheible et al. 2001; Ellis et al. 2002; Cano-Delgado et al. 2003) and *AtCesA6* (Fagard et al. 2000; Desprez et al. 2002) have shown that these genes play a role in primary wall cellulose synthesis. Based on phylogenetic relationship, the maize CesA1-9 sequences may therefore be involved in primary cell wall formation; however, they are more divergent from their *Arabidopsis* relatives compared to ZmCesA10-12 (Appenzeller et al. 2004). Further, the maize sequences that cluster with AtCesA1, 3 and 10 appear to have duplicated at a higher rate than those that cluster with the other *Arabidopsis* sequences.

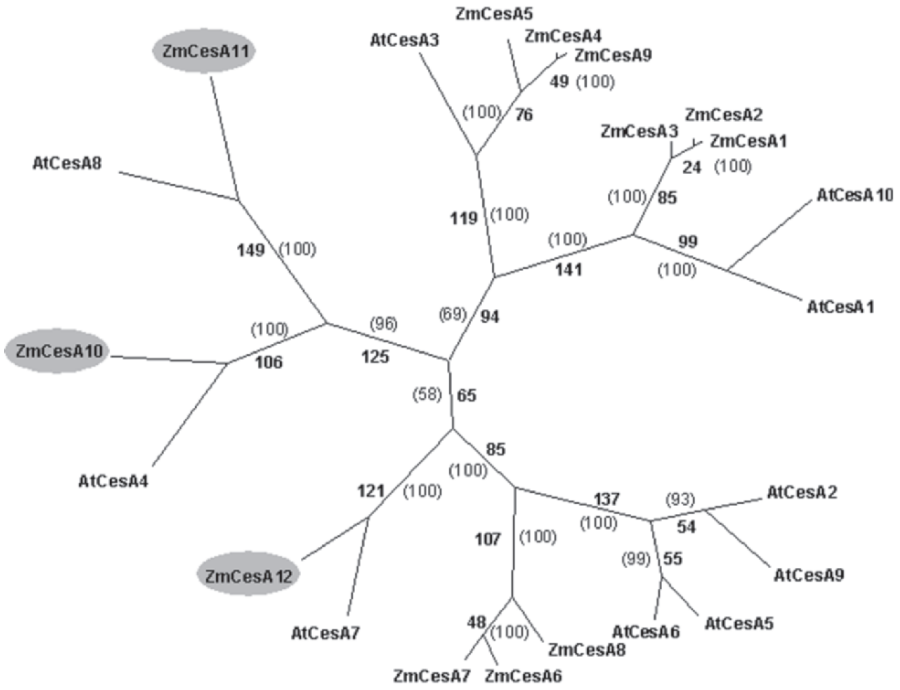

Figure 5-2. Unrooted single most parsimonious tree of the *CesA* proteins from maize and *Arabidopsis* determined by the Branch and Bound algorithm of PAUP (Swofford 1998). Branch lengths are proportional to the inferred number of amino acid substitutions, which are shown in bold font. Bootstrap values from 500 replicates (Felsenstein 1985) are represented as a percentage and are shown in parentheses. Reproduced with kind permission of Springer Science and Business Media from Appenzeller et al., 2004, Cellulose 11: 287–299, Fig. 2. © 2004 Kluwer Academic Publishers.

7 EXPRESSION ANALYSIS OF THE *ZmCesA* GENE FAMILY

Expression profiling of multiple tissues from a maize inbred line, B73, was carried out using massively parallel signature sequencing (MPSS) technology (Brenner et al. 2000; Brenner et al. 2000; Hoth et al. 2002; Meyers et al. 2002; Appenzeller et al. 2004). This technique entails cloning cDNAs onto synthetic beads such that each bead contains multiple copies of only one species of cDNA. Sequence tags 17–20 nucleotides long are then obtained from more than a million beads in parallel. Theoretically, the whole expressed genome is analyzed by the MPSS technology each time a library is screened for unique tags (Brenner et al. 2000a,b). Quantitative measures of the expression levels of different gene tags in the MPSS, as opposed to the ratios across paired tissues or treatments in the microarray-based platforms, combined with the depth of signature sequencing for each of the libraries make it possible to compare gene expression patterns across multiple, independent experiments. The technique is designed to select the 3′-most tag

from the cDNA. The abundance of each tag is a measure of the expression level of an individual *CesA* mRNA in the tissue the library is derived from.

ZmCesA1-8, with the exception of *ZmCesA2*, were expressed at different levels in the majority of the tissues (Appenzeller et al. 2004). In contrast, *ZmCesA10, 11* and *12* were selectively expressed in the stalk, a tissue rich in secondary wall. All three genes were the most highly expressed of the *CesA* genes in the stalk tissue. Secondary wall-rich cells account for ~80% of the dry matter in the maize stalk (K.S. Dhugga, unpublished). The expression pattern of *ZmCesA10-12* was therefore consistent with these genes being involved in cellulose synthesis for secondary wall formation. The observation that there are two groups of *CesA* sequences expressed mainly in primary or secondary wall forming cells has also been reported in barley (Burton et al. 2003) as well as *Arabidopsis* (reviewed in Doblin et al. 2002) and is likely to be a general phenomenon among plant species.

None of the *CesA* genes was detected in mature pollen grains (Dhugga 2001; Appenzeller et al. 2004). Doblin et al. (2001) also did not detect the expression of the *CesA* genes in *Nicotiana alata* pollen tubes. A *Csl* gene, *NaCslD1*, was the most highly expressed *Csl* gene in pollen tubes of *N. alata* and was specifically expressed in this tissue (Doblin et al. 2001). Based on this, it was suggested that, in addition to the *CesA* genes, *CslD* genes were possible candidates for making cellulose.

The pattern of expression of *ZmCesA10*, *11* and *12* was very similar across all the libraries studied. The expression pattern of all three genes paralleled the cellulose content in the three different tissues from an elongating internode, i.e., elongation zone, transition zone, and isolated vascular bundles (Appenzeller et al. 2004). Gene expression was lowest in the elongation zone, the tissue with the least amount of cellulose, followed by the transition zone with increased expression and cellulose content, with the vascular bundles having the highest expression and cellulose levels. *ZmCesA1* and *6–8* may be mainly responsible for making cellulose in the nonvascular, ground tissue cells in the maize stalk. These genes were also expressed in the vascular bundles where they may be involved in the formation of the walls of phloem elements as well as of fiber cells before the onset of secondary wall deposition (Appenzeller et al. 2004). Whereas *ZmCesA1, 7,* and *8* were expressed at a higher level in the elongation zone, *ZmCesA3, 5*, and *6* were expressed at a higher level in the transition zone.

ZmCesA6 showed maximal expression in leaves and was the most highly expressed *CesA* gene in this tissue (Appenzeller et al. 2004). Unopened leaves derived from a young plant at 4-leaf stage were dissected from the base and delineated as follows: cell division zone, elongation zone, and transition zone. Again, the expression of *ZmCesA6* was the highest of all the *CesA* genes in the leaf (Appenzeller et al. 2004). The expression of *ZmCesA6* was lowest in the cell division zone, increased in the expansion zone, and increased dramatically in the transition zone. *ZmCesA1*, *4*, *6–8,* and *10–12* had a similar type of expression pattern, with the levels of highest expression seen in the transition zone of the leaf. It is likely that *ZmCesA1* and *6–8* were responsible for making cellulose in leaf cells where primary wall synthesis was taking place such as mesophyll cells,

phloem elements, and fiber cells in vascular bundles before the onset of secondary wall formation. *ZmCesA10-12* seemed responsible for making cellulose in cells undergoing secondary wall synthesis in the vascular bundles. In contrast, *ZmCesA3* and *5*, the two other *CesA* genes expressed in the leaf, were expressed at their highest level in the cell division zone and were the most abundantly expressed of the *CesA* genes in this region of the leaf. Transcript levels declined in the expansion zone and were at their lowest level in the transition zone. This type of expression profile was suggestive of these genes participating in the synthesis of cellulose at the phragmoplast and possibly in the early stages of primary wall synthesis in elongating cells.

ZmCesA5 is also the highest expressed *CesA* gene in the developing endosperm (Appenzeller et al. 2004). It was previously proposed to be involved in mixed-linked glucan (MLG) formation in corn endosperm, where it is most highly expressed (Dhugga 2001). Unlike its role in cell elongation where MLG transiently accumulates in the wall, this polysaccharide is terminally deposited in the endosperm walls in cereal grains to varying degrees (Carpita 1996). This could be a remnant of MLG being the main source of carbohydrate storage in ancestral species before starch assumed this role (Dhugga et al. 2004). The expression of only six of the twelve *CesA* genes was detected in the endosperm, as observed in the earlier analysis. *ZmCesA5* was the most highly expressed of the *CesA* genes throughout the first 45 days of endosperm development, peaking at 12–20 days after pollination (Appenzeller et al. 2004). The pattern of *ZmCesA5* expression further supports a role for this gene in mixed-linked glucan synthesis. However, additional evidence is needed to substantiate this role. In addition, its role in cellulose formation cannot be ruled out.

Only three of the twelve maize *CesA* genes, *ZmCesA10, 11*, and *12*, appeared to be truly coordinately expressed. The expression of the remaining genes, where it occurred, was more overlapping in nature than coordinate (Appenzeller et al. 2004). The level of overlapped varied, however. The expression of *ZmCesA1, ZmCesA7* and *ZmCesA8* overlapped quite significantly. In contrast, the expression of *ZmCesA2* and *ZmCesA6* was independent of any of the other *CesA* genes. Whereas *ZmCesA2* was expressed in only 3 of the 76 libraries at a level exceeding 10 ppm, *ZmCesA6* was expressed in nearly all the libraries. *ZmCesA3* and *ZmCesA5* had similar expression patterns only with respect to one another and no other *CesA* gene. A lack of correlation of *ZmCesA2* and *ZmCesA6* with any of the *CesA* genes and moderate correlation among the other genes discussed above suggests that the relatively high correlation coefficients observed among some of the gene pairs may have biological relevance. Except *ZmCesA10-12* genes, which are expressed in the secondary wall forming cells, all the remaining genes appear to be largely expressed in the primary wall forming cells. Whether the clustering of the putatively primary wall forming genes based on their overlapping expression patterns into different groups has any functional relevance remains to be determined.

Dimerization of the CesA proteins has been proposed for the formation of a functional cellulose synthase complex (Scheible et al. 2001; Kurek et al. 2002).

All three of the *Arabidopsis* secondary wall forming CesA sequences (AtCesA8/Irx1, AtCesA7/Irx3 and AtCesA4/Irx5) have been reported to be involved in the formation of a functional cellulose synthase complex (Gardiner et al. 2003; Taylor et al. 2003). We propose that ZmCesA11, ZmCesA12 and ZmCesA10 have similar functional roles to these *Arabidopsis* proteins, respectively. Theoretically, only two subunits are necessary to provide juxtaposed catalytic sites for the formation of a β-glycosidic bond without having to rotate the chain after each bond is formed (Dhugga 2001). This could be accomplished either by a homo- or a heterodimer. That the combinations of CesA proteins in rosettes may be different between cell types of the same tissue and also amongst cells of the same type has also been proposed (Doblin et al. 2002). The significance of the presence of more than two CesA polypeptides in the same rosette still remains unclear.

8 RATIONALE FOR FUTURE TRANSGENIC WORK

According to the summation theorem of Metabolic Control Analysis (MCA) (Kacser and Burns 1973; Heinrich and Rapoport 1974; Kell and Westerhoff 1986; Cornish-Bowden et al. 1995; Fell 1996; Heinrich and Schuster 1996), changes in the concentrations of individual enzymes within a metabolic pathway tend to have little effect on metabolic fluxes and on the phenotype under most conditions (Thatcher et al. 1998). However, changes in individual enzyme concentrations could affect the size of metabolite pools even when the alterations in flux are minimal. Because of mass action effects, the change in a metabolic pool could lead to a shift of other coupled metabolites and change the flux at the nonequilibrium reactions in the pathway.

Metabolic simulations using a kinetic model suggest that the magnitude and net direction of carbon fluxes in the far-from-equilibrium reactions are influenced by changes in mass action relationships and total activity rather than by alteration in substrate affinity (Barreiro 1999). Since cellulose synthase is at the end of a metabolic route, and there is no other known branching path to bypass the synthesis of cellulose through this enzyme, it is likely to have a high flux control coefficient (the scaled partial derivative of a system variable such as flux) with respect to enzyme activities. If this is true *in vivo*, an increase in abundance of this enzyme should correlate with an increase in cellulose production, providing that the overexpressed enzyme is targeted in the proper amount to the appropriate subcellular location.

Overexpression of a cellulose-binding domain (CBD) has been reported to result in increased cellulose production in poplar (Levy et al. 2002). It was proposed that the CBD increased the rate of cellulose production by slowing down the rate of crystallization of the glucan chains into microfibrils, which is believed to limit the rate of cellulose synthesis in intact cells. This idea is based on the earlier finding that calcofluor white, a fluorescent dye commonly used to detect cellulose, interfered with the crystallization of the glucan chains into microfibrils (Haigler et al. 1980).

Atkinson (1977) pointed out that a limiting factor in cell structure is its solvation capacity. This limitation does not allow for each metabolite to fill the water volume of a cell to reach its operating concentration. According to Srere (1987), about 80% of the metabolic intermediates have just one use in the cell and this is possibly an evolutionary strategy to overcome the solvation capacity of the cell. An important consequence of this strategy is the metabolic organization through the formation of sequential multienzyme complexes with the concomitant channeling capabilities (Srere 1987). This view of cellular metabolism conceptualizes that, in order to be effective in metabolic control, an enzyme has to be expressed in the proper amount and that does not always correlate with the amount of transcript present (Siedow and Stitt 1998). Moreover, to participate in metabolic control, the participating enzymes need to be positioned in the adequate location to support the channel structure.

Since the control capacity at a particular steady state is distributed among the intervening enzymes, any attempt to engineer a change in the flux towards cellulose will need a careful study of the system and will probably involve the manipulation of more than one parameter (enzymes amount, kinetic constants, and metabolite pools). According to kinetic simulations, near-equilibrium reactions are more easily affected by mass action effects while enzymes catalyzing reactions that are far from equilibrium can alter the flux through modifications in their V*f* (Barreiro 1999). However, there are no fixed rules to alter metabolic systems at will but it is possible to engineer them by understanding the control coefficients involved and the local properties of the reactions involved. This will most likely involve altering the metabolic mass action ratios, modifying the activities of key enzymes, and stimulating the synthesis of end-products such as cellulose, to drive fluxes out of the intermediary metabolism.

9 SUMMARY

Cellulose in a unit length of the stalk below the ear node in maize is the main determinant of mechanical strength, a trait of considerable importance in agriculture. The majority of cellulose in the stalk is in the vascular bundles, which occur throughout the cross-section of the stalk but are densely packed among the peripheral sclerenchymatous cell layers collectively referred to as rind. Three of the twelve maize *CesA* genes, *ZmCesA10-12,* appear to be involved in secondary wall formation and the remaining nine in primary wall formation. The putatively primary wall forming genes can be grouped into different clusters based on their expression patterns, however, the functional significance of these groupings is not clear at this point. Availability of the secondary wall forming *CesA* genes has made it possible to isolate their promoter elements. These promoters will allow the expression of the *CesA* and other genes in specific cell types where cellulose could be increased at the expense of hemicellulose, soluble sugars and potentially lignin with the goal of improving stalk strength. Another opportunity would be to increase cellulose formation in the parenchymatous cells. In either

case, the harvest index must not be adversely impacted in order to maintain or increase grain yield while altering the composition of the existing biomass.

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