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TRANSFORMACION PARA REDUCIR EL CONTENIDO DE LIGNINA, Y AUMENTAR EL CONTENIDO DE CELULOSA EN CONIFERAS

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TRANSFORMATION FOR INCREASING CELLULOSE CONTENT AND MODIFYING LIGNIN IN CONIFERS

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SUMMARY

The commercial use of ligno-cellulose (or wood) from forest trees has prompted a number of biotechnology groups to explore aspects of wood biosynthesis which might be manipulated to improve product quality and yield. Increasing cellulose and growth has attracted relatively little attention from the perspective of genetic engineering. An approach to increasing cellulose and biomass has been developed by increasing the substrate for cellulose synthesis (UDP-glucose). This has been achieved by expressing a bacterial enzyme, UDP-glucose pyrophosphorylase, *in planta*. Highly significant increases in total biomass and cellulose have been shown in tobacco and transformants of both conifer and broadleaf trees are now under evaluation.

To date, most work on wood modification has focused on reducing lignin content by suppression of the biosynthetic enzymes responsible for mono-lignol synthesis. The assumption is that reduced lignin content will result in greater ease of pulping. This might be manifested through lower mechanical energy requirements or reduced chemical usage. Ancillary benefits may include greater pulp yield and reduced effluents. An alternate approach, that of modifying the lignin polymer, is being pursued in conifers. This has the potential advantages of maintaining the functional integrity of lignin in the tree while still realizing the benefits in the pulp mill.

INTRODUCTION

Both the cellulose and, to a greater extent the lignin portion of wood have attracted experimental work on genetic transformation. It has generally been assumed that growth and yield are complex, quantitative traits controlled by many genes and hence the primary tool for their improvement would be through conventional selection. However, we present evidence that the creation of an increased sink for photosynthate in the xylem may enable increases in cellulose biosynthesis and hence, biomass accumulation to be achieved. This approach relies upon increasing the level of the UDP-glucose substrate for cellulose biosynthesis.

There have been a number of approaches to altering lignification. The principal rationale for most of this work has been to reduce the expression of enzymes key to the biosynthesis of lignin precursors (using antisense technology) to reduce overall lignin content. Thus far, enzymes in the pathway for which this approach has been tested include phenylalanine ammonia lyase (PAL), cinamyl alcohol dehydrogenase (CAD) and O-methyl transferase (OMT) (see Figure 1). An alternate approach to reduce lignin content is to alter the levels of monomers to generate a lignin polymer which is more easily hydrolyzed. This approach, if successful, may have the advantage of maintaining a biologically important level of lignin for physical and biological protection while still providing lignin which is readily pulped. The goal of either approach is to provide more efficient pulping properties which result in reduced energy and chemical cost as well as increased cellulose yield.

AN APPROACH TO INCREASING CELLULOSE BIOSYNTHESIS BY ALTERED CARBON ALLOCATION

Increasing harvestable plant yield is a major goal of all plant breeding efforts. In fiber-producing crops, the economic value of this yield is directly related to the amount, location, and length of the cellulose fibers. It has been suggested that cellulose content and fiber yield is limited by the amount of substrate, or sugars, produced during photosynthesis. However, numerous studies provide evidence that although crucial for plant growth and survival, the availability of carbohydrates derived from photosynthesis is not a major limiting factor in cellulose synthesis. Thus there exists a substantial opportunity to increase fiber yield by creating a sink for this existing photosynthate in cells high in cellulose. Sucrose, the major form of translocatable carbohydrate produced during photosynthesis, is translocated to sink tissue where it is converted to other compounds such as starch or cellulose. By creating a sink for these carbohydrates in cellulose producing cells, excess photosynthate can be diverted to these cells where the default pathway for their use would be the conversion into cellulose, thereby increasing cellulose content in the plant. This would have a huge impact in forestry, because whether harvesting for lumber or fiber, the product is cellulose. The benefit of increased cellulose is not limited to forestry, as there are numerous other fiber crops including sisal, cotton, and hemp.

Despite the fact that the amount of photosynthates in the plant are not a primary limitation in cellulose content, the rate of photosynthesis plays a large role in the overall growth of a plant. One element in the control of photosynthesis in the plant is the feedback-inhibition of photosynthesis by photosynthetic products, such as starch, sucrose and hexose sugars. Goldschmidt and Huber (1992) tested the effect of girdling the leaves of crop plants and demonstrated that the build-up of starch and other products of photosynthesis actually inhibited the rate of photosynthesis. These findings, and others (Sonnewald & Willmitzer 1992), indicate the photosynthetic rate, and ultimately plant growth, may be directly correlated with the rate that photosynthates are drawn away from the leaf, or the rate of biosynthetic degradation in the leaves. The degradation of photosynthates occurs primarily in cells/tissues that are actively growing (meristematic or young tissues) or in tissues where photosynthates are utilized for storage or structural components (sink tissues). Therefore, altering the rate that carbohydrates are translocated to these sink tissues (altering carbon allocation) would not only increase overall plant growth (remove inhibitors of photosynthesis), but also increase the amount of storage (starch) or structural components (cellulose).

A striking example of the benefits of altering carbon allocation has been demonstrated in potato. By increasing the synthesis and accumulation of ADP-glucose in the tuber, starch synthesis increased which significantly increased dry matter content. In fact, this resulted in a 25% increase in tuber yield. The increase in ADP-glucose in the tuber was accomplished by genetically engineering the

potato with a bacterial ADP-glucose pyrophosphorylase gene controlled by a tuber-specific promoter (Shewmaker and Stalker 1992).

Increasing cellulose biosynthesis through increased UDP-glucose accumulation

Similar to ADP-glucose being a substrate for starch synthesis, UDP-glucose (UDPG), is a substrate for cellulose biosynthesis in both bacteria and higher plants (Delmer 1987, Delmer & Amor 1995). Several bacterial genes which encode the enzyme UDP-glucose pyrophosphorylase (UGPase), responsible for the synthesis of UDPG, have been isolated (Ross et al. 1991). *Acetobacter xylinum*, which is among the bacteria from which this gene has been isolated (Valla et al. 1989), produces substantial quantities of cellulose. The relatively high K_m value for the product UDPG (0.44mM) versus the substrates UTP, glucose-1-P and PPi (0.28, 0.23, 0.27mM) makes this enzyme favorable for increasing UDPG levels because the reaction is strongly favored in the direction of UDPG synthesis. This is supported by the high levels of UDPG which accumulate in the cell (Ross et al. 1991).

This is in contrast to a UGPase cDNA which has been cloned from potato (Katsube et al. 1990). The potato enzyme has a K_m for UDPG which is substantially lower than that for the substrates UDP and glucose-1-phosphate (Nakano et al. 1989), meaning that the accumulation of high UDPG levels would be difficult with the plant enzyme. It is also important to note that these workers have confirmed the absence of a signal for intra- or extra-cellular targeting in plants. The enzyme kinetics data, lack of signal sequences, and the fact that unlike the plant gene, the bacterial UGPase gene is not strongly inhibited by UDPG accumulation, suggest that the bacterial UGPase gene is an excellent target gene to increase UDPG levels in plants. Additional rationale for using a bacterial gene for modification of cellulose biosynthesis is that, aside from its availability, the bacterial gene is less likely to lead to co-supression of the native UGPase genes. This is based on the observation that sequence similarity is one basis for co-suppression often observed in transgenic plants (Finnegan and McElroy 1994).

Specific applications for increased cellulose synthesis include numerous crops with diverse uses and growth habits. In forestry, wood production is influenced by a combination of physiological and biochemical processes governed by substantial genetic variation. This has lead to the theoretical consideration of limitations on increasing yield due to fundamental constraints on energy supply (Farnum et al. 1983). Despite such limitations, increases in tree growth of 50 to 300% are possible depending on the tree species and growing environment. Clearly, improving energy capture, conversion of radiant energy, and altering carbon allocation within the plant are promising areas for tree improvement. Increasing cellulose content by the manipulation of UDPG levels can achieve such gains.

The primary portion of the tree responsible for lumber and fiber yield is the stem. Therefore, the creation of sink tissue for UDPG in the stem would: 1) reallocate photosynthate from the leaves to the stem removing any inhibition by these compounds on photosynthesis, and 2) provide a high level of the primary building block (UDPG) for cellulose synthesis in the cells of the stem which are the main cells (xylem cells) responsible for cellulose content in the plant. A high UDPG content in the xylem cells would encourage the conversion of UDPG to cellulose by way of a default pathway for the cell to utilize the excess UDPG. This strategy necessitates the expression of the introduced UGPase gene specifically in the xylem cells in the stem.

Ellis et al. (1994) have shown that interior spruce transformed with the CaMV 35S linked to a β-glucuronidase (GUS) gene exhibits histochemically-detectable GUS expression predominantly in vascular tissue of spruce. Furthermore, when expression was followed throughout several growing seasons in the field, expression levels coincided with maximal periods of stem growth. On this basis, initial assessment of the effects of engineered bacterial UGPase on stem growth in plants were carried out using the CaMV 35S promoter. In addition to CaMV 35S, to specifically direct UGPase expression to wood forming tissues, a xylem specific promoter from a parsley 4-coumarate lyase (4CL) gene is also being tested.

Tobacco transformation and characterization of UGPase expression

To test the concept of using increased UGPase expression to enhance cellulose biosynthesis, this gene was cloned from *Acetobacter xylinum* and placed between a CaMV 35S promoter and *nos* terminator sequence. *Agrobacterium* containing the UGPase construct was used to infect leaf discs of *Nicotiana tabacum* c.v. *xanthii,* following standard tobacco transformation procedures.

The stable transformation of tobacco with the UGPase gene was first confirmed by PCR and later by Southern Blot analysis. More than 42 independent transformed plants (T_0) have rooted and grown in both sterile MS medium and in soil. Seeds from 24 T_0 plants were harvested and used to generate T_1 plants. T_1 plants were grown in soil following germination on kanamycin (150 mg/ml). Segregation of kanamycin resistant T_1 plants followed expected segregation patterns.

Phenotypes of plants transformed with UGPase linked to CaMV35S

Although considerable variation existed between individual transformed lines, transformed plants from selected lines exhibited up to 10-15% increased height growth over control plants. More importantly, significant increases in biomass were observed in all plant parts from these transformed lines when compared to control plants. Examination of the variation among individual transformed lines revealed that about 20% of the transformed lines tested had significant increases in biomass of up to 30% greater than controls. The proportion of lines with significantly altered

phenotype was observed consistently in different experiments and was beyond the range observed with control lines derived from independent isolated shoot cultures.

Targeting the over-expression of UGPase to the xylem.

Using a parsley 4 coumerate:coenzyme A ligase (4CL) promoter to target the over-expression of UGPase to the xylem/vascular system, increased biomass was observed specifically in the stem. This is in contrast to the over-expression of UGPase controlled by CaMV 35S where the increase in biomass was due to increased biomass in the roots, leaves and stems. This clearly demonstrates the ability to manipulate the expression of transgenes to target increases in biomass and cellulose to the stems.

In addition to biomass, significant increases in height growth were also observed in T_0 tobacco transformed with the 4CL-UGPase construct. These increases were not observed until the plants started rapid growth.

Application of UGPase gene to forest trees

Having established proof of concept in tobacco, BC Research Inc. has introduced this gene into both conifer and broad leaf trees. Both constitutive promoters and xylem directed promoters are being used in conjunction with the *A. xylinum* gene. Currently, approximately 40 poplar transformed lines and 20 spruce transformed lines are under evaluation.

AN APPROACH TO ALTERED LIGNIN IN CONIFEROUS TREES.

Lignin is a cell wall component present in vascular plants, ferns and club mosses. Lignin decreases the permeability of cells, contributes to strength and rigidity of the stem, and protects microfibrils from chemical, physical, and biological attack (Bugos et al. 1991). Despite its advantage to the plant, lignin greatly affects the agroindustrial uses of plants. Lignin content and composition alters digestibility and dietary conversion in herbaceous crops and is undesirable in the conversion of wood into paper and pulp (Campbell and Sederoff 1996).

Although lignin can contribute up to 25% of the mass of wood, from a pulp and paper viewpoint, lignin does not contribute to the usable biomass in pulping and hence is waste. More importantly, the extraction of lignin during chemical pulping is a costly and difficult process, involving chemical removal. In fact, there is a negative correlation between the amount of lignin removed and fiber yield with chemical pulping. Therefore, because the removal of lignin from fibers is a major cost, the modification of both lignin content and composition is a major focus of several labs world wide. Of importance is that trees with altered lignin, either decreased content or modified to reduce the energy needed to extract the lignin,

could allow the allocation of additional resources to the production of pulpable biomass with decreased costs.

Chemically, lignin is a highly complex network of phenylpropanoid units derived from the oxidative polymerization of one or more of three monolignol precursors. Due to the variety of linkages formed between these precursors during polymerization, lignin polymers are highly heterogeneous. This heterogeneity in lignin and the linkages formed during polymerization have a large influence on the pulping characteristics of wood. For example, the presence of the C-5 methoxylated syringyl units make hardwood lignin easier to hydrolyze during pulping, while a higher proportion of condensed ρ-hydroxyphenyl units make hydrolysis more difficult (Campbell and Sederoff 1996). This is due in part to the unprotected C-5 group characteristic of ρ-hydroxyphenyl and guaiacyl units typical of softwoods, accounting for the relatively slower delignifying rate of the softwoods (Chiang and Funaoka 1990).

Any modification of this complex polymer requires an understanding of the metabolic pathway. Fortunately, many steps in the lignin biosynthetic pathway are well understood (Davin et al. 1992) and therefore, there are numerous reports on the modification of the phenylpropanoid pathway by genetic engineering. One example is the sense suppression of PAL by a bean *PAL2* gene in tobacco. These experiments demonstrated that PAL activity becomes rate-limiting to lignin deposition when levels are 3- to 4-fold lower than in wild-type plants (Bate et al. 1994). While PAL may hold promise for use in engineered lignin modification, it has been suggested that due to its key role in general phenylpropanoid metabolism, the interruption of PAL synthesis would also affect other biochemical pathways. In contrast, the activity of CAD, an enzyme well downstream in the lignin biosynthetic pathway, can be reduced to 10% of normal levels and still have no effect on the quantity of lignin, although clear qualitative differences are observed (Halpin et al. 1994). From these, and other studies [OMT (Dwivedi et al. 1994; Ni et al. 1994), F5H (Bell-Lelong et al. 1997), and peroxidase (Lagrimini et al. 1990)] on the manipulation by genetic engineering of key enzymes in the lignin biosynthetic pathway, it is clear that lignin modification is possible.

However, in contrast to the above studies, where the emphasis was the reduction of enzyme activity, our approach is to introduce a gene encoding the enzyme ferulate 5-hydoxylase (F5H), the enzyme which catalyzes the final C-5 ring-hydroxylation of ferulic acid (Figure 1). We and others (Campbell and Sederoff 1996) believe that the lack of this enzyme in gymnopserms may account for the lack of syringal lignin in this huge group of plants. Due to the fact that the presence of syringal lignin plays a role in the easier removal of angiosperm lignin during pulping relative to gymnosperms lignin, we are investigating whether expressing this gene in gymnosperms could make lignin removal less costly in gymnosperms.

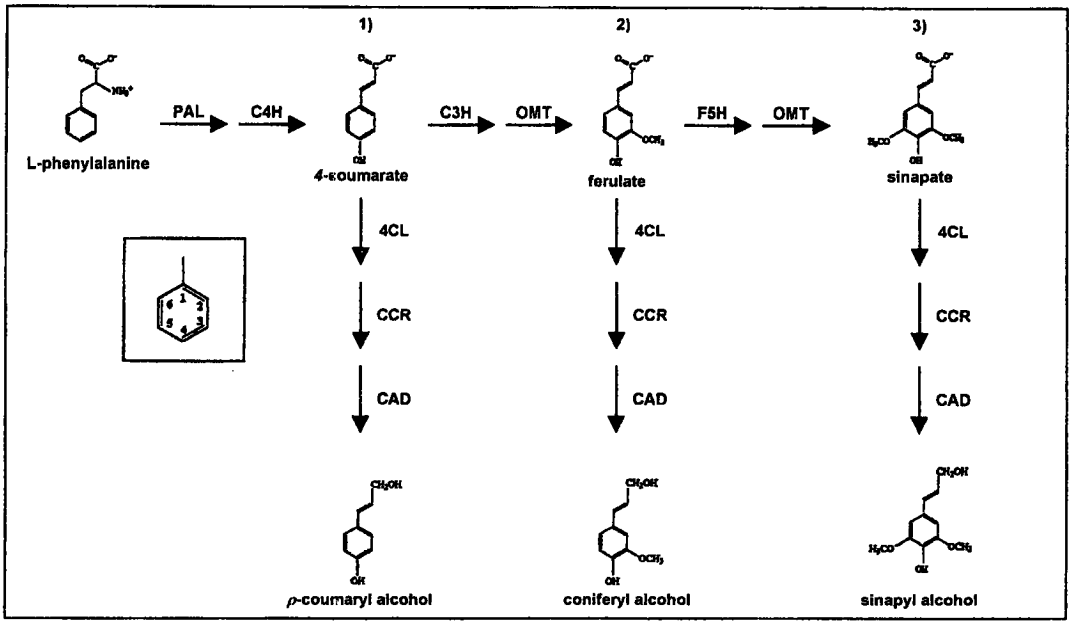

Figure 1. The basic lignin biosynthetic pathway. Enzyme abbreviations are described in the text. F5H, the focus of this proposal is highlighted. Inserted box indicates numbering in the phenyl ring. Adapted from Campbell and Sederoff (1996).

Progress with assessment of F5H gene in spruce

In collaboration with Dr. Clint Chapple, Purdue University, we have inserted numerous *Arabidopsis* F5H constructs into spruce. Research currently ongoing at BC Research Inc. has utilized a genomic clone of F5H linked to the CaMV 35 promoter. Transformation of spruce embryogenic clones were carried out using the biolistic method (Ellis 1993). Over 500 transgenic plants from numerous lines containing this construct are currently being grown in the greenhouse. Height growth of the genomic-F5H transgenic lines are similar to the controls and lignin analysis on 2 year-old wood will be done this winter. Preliminary results suggest that relative to the expression of this construct in tobacco, expression of the genomic F5H construct in spruce is relatively low. Due to this, current emphasis is on the use of a cDNA construct as well as other promoters, particularly promoters which may offer xylem specific expression.

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