Biotech enhanced levels of syringyl lignin improves *Eucalyptus* pulping efficiency

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Abstract

The word “lignin” is derived from the Latin word “lignum” meaning wood, and, indeed, lignins form an essential component that ranges from 15% to 36% of the dry weight of the woody stems of arborescent gymnosperms and angiosperms. Not only lignin quantity is important, lignin composition is also critical. The composition of a lignin in relation to its basic phenylpropane units is an important parameter in pulpwood production in view of delignification rates, chemical consumption and pulp yields. In hardwood, lignins are composed of syringyl (S) and guaiacyl (G) units in varying ratios, but syringyl lignins show higher reactivity in kraft pulping than guaiacyl lignins. Modification of lignin has therefore been a target for genetic engineering because costly and noxious chemicals are used in chemical pulping to remove it from cellulose and hemicelluloses. Many genes involved in lignin biosynthesis have been manipulated in model species, and some experiments have been performed in trees. In our research, we investigated the impact of overexpressing a coniferaldehyde 5-hydroxylase (cald5H) gene on growth and wood chemistry in nearly 100 independent lines of four year old, field-grown transgenic *Eucalyptus.* This gene is an essential step in the formation of syringyl lignin, and over-expression of this gene was expected to increase the S/G ratio of the wood without causing a change in total lignin content. A small set of lines were selected based on their growth and shift in S:G ratio for more extensive analysis. The S:G ratio of the selected four year old lead lines was 3.1 while the control trees had a S:G ratio of 2.3. Pulp yield results demonstrated an increase of up to 2.8% yield for a 17 kappa number. Chemical consumption of alkali was also influenced showing a decrease of 15% for the line with highest SG ratio. The advent of genetic engineering has meant that transgenic trees with altered lignin composition could dramatically improve pulping efficiency and the environmental impact of effluent from paper mills while reducing productions costs.

Keywords: eucalyptus; SG ratio; lignin; transgenic; chemical composition; kraft pulping; pulp yield.

Introduction

In Brazil, for kraft pulp production the dominant wood raw material are *Eucalyptus* species such as *E. grandis*, *E. urophylla*, *E. saligna* and hybrids between these species. In addition to its high wood production rate, *Eucalyptus* also produces a wood that requires mild pulping and bleaching conditions compared to other hardwoods, making this trees genus widely used in the pulp and paper industry.

Today, *Eucalyptus* is the largest single global source of market pulp. The major interest in eucalyptus wood comes from its low production cost in certain regions, mainly because of high forest productivity and high pulping yield. However, more recently the outstanding quality of eucalyptus fibers has been recognized. The use of bleached eucalyptus fibers to manufacture paper grades previously made only with bleached softwoods is growing quickly. The ongoing scientific and technical advances achieved in production of eucalyptus fibers, from forest to product, and the increasing understanding of their applications in various paper grades, have made these the preferred fibers worldwide. Unlike mixed-species pulps, single species offer specific benefits and well-defined properties. In this regard, eucalyptus pulp fibers produced from clonal plantations have emerged as the most desirable fibers in the market, not only for the production of tissue and printing and writing papers, but also for the manufacture of “new products.” (1).

Although the worldwide demand for pulp continues to increase, the kraft process has already been optimized to minimize consumption of energy and chemicals. Clearly, the key to improving pulp production is the development of wood with novel properties, such as low lignin content or a higher proportion of reactive lignin, that could sharply lower the kraft energy and chemical intensity limits (2).

Pulp mills around the world are continuously looking for cost reduction in chemicals required for the process and also for raw material wood, which represents at least 40% of the total cost production of pulp. One of the ways of decreasing costs in a pulp mill is by improving wood properties such as wood density and pulp yield. It can be made through traditional breeding and also through biotechnology in which introduced genes can reduce the total lignin content, or modify lignin structure, resulting in a transgenic tree which produces wood that is easier to pulp. It is important to mention that biotech enables trait improvements that cannot quickly or easily be accomplished via traditional breeding.

Lignin is an aromatic heteropolymer derived primarily from the hydroxycinnamyl alcohols p-coumaryl, coniferyl, and sinapyl alcohol, which give rise to p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) subunits, respectively. Lignin is one of the three main components of plant secondary cell walls and plays important roles in vascular plant growth by contributing to structural rigidity, facilitating water transport, and providing a defensive barrier against pathogens. Lignin monomer composition varies among plant species. Although S lignin has arisen more than once during evolution, it does not appear to exist in the majority of gymnosperms or in ferns, whereas angiosperms deposit S units in lignin polymers in secondary cell walls (3).

Lignins have attracted significant research attention because they represent a major obstacle in chemical pulping, forage digestibility, and processing of plant biomass to biofuels. These industries would benefit from processing biomass with either less lignin or a lignin that is easier to degrade. Ultimately, our knowledge of lignin biosynthesis needs to be valorized to improve plant varieties for end-use applications, such as pulping, forage digestibility, or conversion to biofuels, either through genetic engineering or by exploiting natural variation. Several studies, some using field-grown transgenic trees, have demonstrated that lignin engineering can be beneficial for pulping (4).

The general picture that emerges from two decades of studies on the individual roles of the monolignol biosynthetic genes is that downregulation of PAL, C4H, 4CL, HCT, C3H, CCoAOMT, CCR, and, to a lesser extent, CAD, have a prominent effect on lignin content. Lignin composition (H/G/S) can be engineered as well. HCT- and C3H-downregulated plants are strikingly enriched in H units, which are a minor component of typical wild-type lignin. Downregulation of F5H results in lignin essentially composed of G units, whereas F5H overexpression can result in plants with lignins almost entirely composed of S units. COMT downregulation reduces S units and leads to the incorporation of 5-hydroxyconiferyl alcohol into the polymer. Finally, hydroxycinnamaldehydes are incorporated as a result of CAD downregulation, particularly into angiosperm lignin (4).

Coniferaldehyde 5-Hydroxylase (Cald5H) gene is an essential step in the formation of syringyl lignin, and is lacking in conifers. Over-expression of this gene is expected to increase the S/G ratio of the wood without causing a change in total lignin content.

Li et al. (5) says overexpression of the CAld5H gene specifically induced SG ratio augmentation. Results suggest that the sense over-expression of CAld5H triggering an accelerated syringyl lignin deposition induces a rapid maturation or lignification of stem secondary xylem cells. Importantly, regulation in trees of CAld5H enzymes, which all are downstream of coniferaldehyde, has not resulted in lignin quantity change. Rather, it affects the overall lignin structure.

The objective of this study is to evaluate the impact of overexpressing a coniferaldehyde 5-hydroxylase (cald5H) gene in *Eucalyptus* trees assessed at four years old in a trial located in Brazil.

Experimental

97 transgenic lines of *Eucalyptus* overexpressing a coniferaldehyde 5-hydroxylase (cald5H) gene, was assessed for: growth, wood chemistry and pulping in a 4-year old trial. This study was conducted in two phases: Phase one was an investigative study of the S/G ratio of the transgenic lines compared to the control. For that, 164 trees were harvested, representing 37 transgenic lines and also the control, which is the non transgenic clone. Between three to five replicates were analyzed for each line; The second phase represents a more in-depth study on the effect of the S/G ratio on the chemical composition of the trees and its impact on pulping. For this work, 68 trees were analyzed representing 12 transgenic lines and the control (non transgenic). Analyses were done with three to five replicates per line on the transgenic lines and the control had ten replicates.

Trees were sampled, debarked and sent Viçosa University for sample preparation and analysis. Chips were prepared in a pilot-scale chipper. The ABNT NBR-11941 method was used for wood density analysis (6). Insoluble and soluble lignins were measured according to Gomide and Demuner (7) and Goldschmid (8), respectively. The lignin syringyl/guaiacyl ratio was determined by high-performance liquid chromatography (HPLC) after oxidation of sawdust with nitrobenzene, as described by Lin and Dence (9).

Kraft pulping experiments were carried out in laboratory forced circulation batch digesters. The conditions were as follows: liquor-to-wood ratio (L/Kg), 4:1; sulfidity, 30%; final temperature, 165°C; time to final temperature, 70 minutes; time at 165°C, 60 minutes. Active alkali (%, as NaOH) was varied in order to attain similar degree of delignification, expressed as kappa number. An alkali charge curve was established after exploratory analyses aiming a 17 kappa number in the middle of the delignification curve. Four charges were applied for each sample. Alkali charge, pulp yield, viscosity and residual alkali were determined through regression curves for a Kappa number 17 (10). TAPPI procedures were used for kappa number and pulp viscosity determinations (11).

Results and Discussion

Eucalyptus wood-fiber characteristics may vary substantially among the various species and clones as well as with age. By acting on these parameters and the pulping process, it is possible to prepare eucalyptus pulps that are of good quality and suitable for most paper-manufacturing applications (1).

Industrially, wood quality for kraft pulping is assessed by the following main characteristics: density; fiber morphology (coarseness, fiber distribution, and other fiber properties); and lignin, extractives, and xylan contents. More recently, wood uronic-acids content and lignin syringyl/guaiacyl (S/G) ratio have been added to this list (1).

In this section, results of chemical composition and pulping of transgenic *Eucalyptus* overexpressing the Cald5H gene will be presented.

Forest yield and SG ratio – First selection

Four year old growth data was used to select the lines to go for S/G ratio analysis. From 97 lines produced, 94 were in the field trial for growth assessment. Of these lines, 20 were selected based on growth results (not statistically different from the control) and 18 lines were selected for scientific purposes, in order to check how the S/G ratio behaved when some loss in growth was noted. In total 164 trees were sampled and analyzed for S/G ratio. Results are presented in Figure 1.

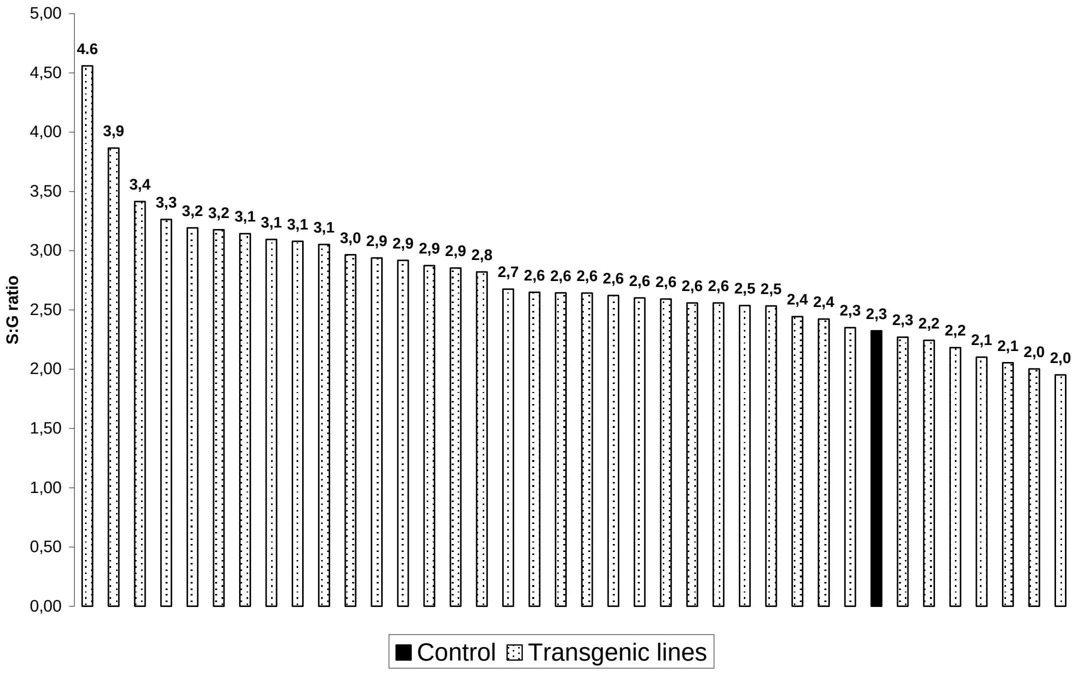

Figure 1. SG ratio – 4 year old data

Based on the S/G results, it is possible to state that the gene worked effectively by increasing the S/G ratio of most of the lines evaluated by up to 96% when compared to the control. Results obtained ranged from 2.0 up to 4.6.

These results were important to conduct the next step of the research that would be chemical analysis and pulping studies to verify the impact of the higher SG ratio in pulping.

Chemical composition and Pulping – Second selection

For this part of the research the 12 best lines were selected based on growth and S/G ratio results. Criteria used to make the selection of these lines were: 1) growth is not statistically different from the control; 2) wood density is not statistically different from the control. It is important to note that cald5H gene is supposed to alter the composition of the lignin. By assuming that we would not expect differences in wood density.

Chemical composition

Total lignin content did not differ between the transgenic lines and the control, but we can see significant differences in the results for soluble and insoluble lignin content and for SG ratio presented in Figure 2.

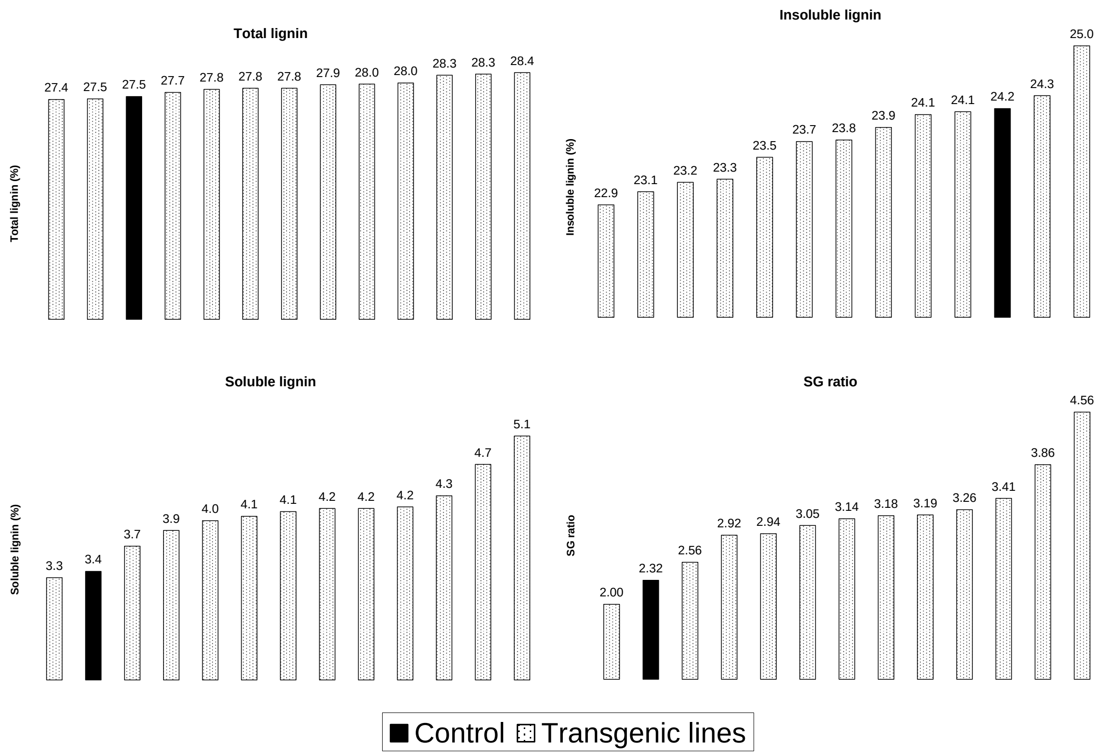

Figure 2. Total, soluble and insoluble lignin content and SG ratio of lead lines

As a result of the transformation using the gene cald5H, we found a decrease in the insoluble lignin, and an increase of the soluble lignin as well as the S/G ratio. An important aspect is that the results are very consistent, with most of the lines evaluated showing similar changes in lignin, which supports gene efficiency.

For insoluble lignin, the results ranged from 22.9 up to 25.0%, compared with 24.2% for the control line.

For soluble lignin, which is easier to dissolve during pulping, we note an increase of up to 50%, with results between 3.3 and 5.1. Considering all the transgenic lines, average increase for soluble lignin was 23%.

The S/G ratio of the transgenic lines shows an average increase of 36.7%, and an increase of 96% can be attributed to the line with the highest S/G ratio.

Syringyl type units, due to the presence of the two methoxyl groups in positions 3 and 5 of the aromatic nuclei, are known to be more reactive than the guaiacyl counterparts. Additionally, the extent of lignin recondensation occurring in the alkaline or acidic reaction media is hindered by the presence of the additional methoxyl group in the S units, when compared to G ones. When the S:G ratio of wood and kraft pulp lignins is plotted against the chemical charges used in the pulping and bleaching, an interesting correlation was observed, confirming the effect of the syringyl units in the pulping and bleaching ability (12).

Kraft Pulp Production

Woods were kraft pulped to similar degree of delignification (kappa number 17), using regression curves obtained with different charges of alkaline chemicals. Representative results of the pulping experiments carried out are presented in Figure 3.

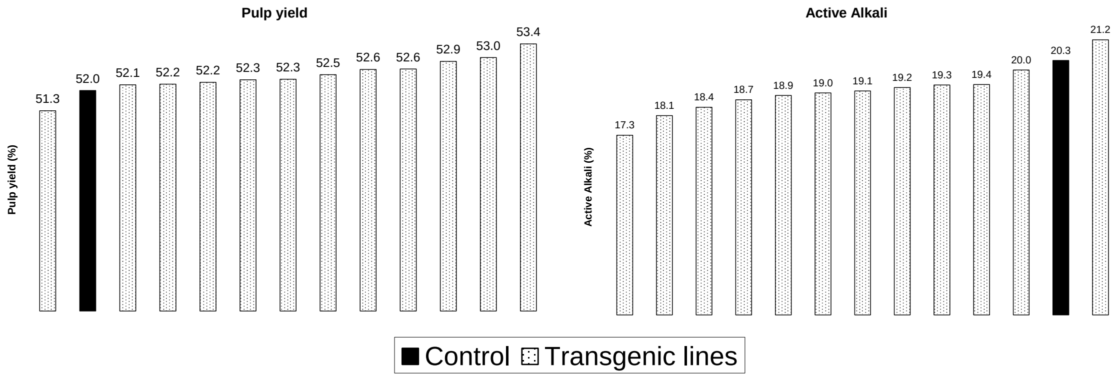

Figure 3. Pulp yield and Alkali consumption of lead lines

Potential lines have shown an average pulp yield increase of 0.9% as an effect of a higher S/G ratio. The increase in pulp yield reached the higher value for the line with higher S/G ratio, with 2.8% increase in pulp yield. Values obtained in the transgenic lines ranged between 51.3 and 53.4% for pulp yield, while the control has a pulp yield equal to 52%.

Active alkali consumption was decreased by around 6% on average due to the easiness of pulping trees with higher S/G ratio, with the best line for pulping showing 15% decrease in alkali consumption.

Neto et al. (12) says the relative ease of wood pulping and pulp bleaching was shown to be determined by the structural features of lignins rather than by their relative content in woods and pulps. The ease of pulping and bleaching, correlated with syringyl:guaiacyl (S:G) units ratio, as well as with the proportion of non-condensed:condensed (nC:C) units ratio. In general terms, the lower pulping and bleaching chemical charges required by *Eucalyptus* species and, particularly, by *E. globulus*, may be explained by the structural features of their lignins, namely the high proportion of syringyl units, low degree of condensation and higher content a β-O-4 structures particularly in unbleached pulp.

Pilate et al. (13), presented results demonstrating that the modified poplar wood properties of CAD- and COMT-antisense trees were maintained over four years in the field, in two different locations. Kraft pulp was done under varying conditions to determine the optimum chemical charge for good delignification of wood from different lines. The authors report that ASCAD21, the line with lowest CAD activity, required a lower amount of chemicals than wild-type wood to reach the same kappa number. Savings obtained in alkali charge were 6%. For the same line, the screened pulp yield was satisfactory even at low alkali charge, which means this line can provide more pulp with fewer chemicals.

In principle, the higher the lignin S/G ratio, the better should be the wood pulp ability, because syringyl lignin is more reactive in kraft pulping. Many workers have found that the S/G ratio correlates negatively with effective alkali demand and positively with pulping yield. Unfortunately, the eucalyptus wood species grown in Brazil present a rather narrow range of variation (two or three to one) of lignin S/G ratio among clones, and therefore good correlations between this ratio and pulping yield are usually hard to observe (1).

Results presented in this paper state that through genetic engineering, S/G ratio of Brazilian clones can be increased in order to improve pulping performance.

Conclusions

Results obtained in this study are very exciting once it proves that genetic engineering results in truly potential commercial materials that provide real gains in a pulp mill. Savings in chemicals as alkali charge and an increase in pulp yield in a pulp mill represent millions of dollars per year.

A potential negative impact of high-S/G-ratio lignin is a decrease in black liquor heating value since syringyl lignin is more oxygenated than its guaiacyl counterpart and gives rise to black liquor of lower heating value. This matter has to be better analyzed to determine the real, if any, impact in pulp mills.

It has also been proven that eucalyptus woods with high S/G ratios provide kraft pulps that are easier to bleach at a given kappa number. Bleaching studies with trees from this trial will be carried on and published soon.

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