AN ABSTRACT OF THE DISSERTATION OF

Steven L. Voelker for the degree of Doctor of Philosophy in Wood Science and Forest Science presented on May 22, 2009.

Title: Functional Decreases in Hydraulic and Mechanical Properties of Field-grown Transgenic Poplar Trees Caused by Modification of the Lignin Synthesis Pathway Through Downregulation of the 4-Coumarate:Coenzyme A Ligase Gene

Abstract approved:

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Barbara Lachenbruch Frederick C. Meinzer

Society uses massive quantities of wood fiber in production of paper, and demand for fiber is projected to increase further as production of biofuels from fermentation of plant cellulosic materials increases. Because these end uses generally require the costly step of removing of lignin, wood with reduced or more easily extracted lignin has long been a goal of plant biotechnologists. However, there is little information on how reduction of lignin affects wood properties, tree development, and survival, especially in field environments. We studied a gene that had been previously reported to substantially reduce lignin content and improve biomass production in poplar. An antisense, xylem expressed version of the *Pt4CL1* gene that encodes 4-coumarate:coenzyme A ligase (4CL) was inserted into hybrid poplar (*Populus tremula x alba*, INRA 717-1B4) and the growth and physiology of 14 transgenic lines (i.e., independent gene insertion events) was assessed over two growing seasons. Transgenic lines had 30-70% reductions in 4CL RNA expression in young shoots. This corresponded to 5-45% reduction in lignin as indicated by total monomer release through thioacidolysis and/or nitrobenzene oxidation. Only three transgenic lines with modest (>10%) reductions in lignin content sustained adequate growth and had normal tree form. Trees from five lines with severely reduced lignin formed up 24-60% of the

stem cross-sectional area in brown colored wood that was essentially non-conductive to water, presumably due to the ectopic deposition of non-lignin phenolics and associated tyloses that occluded vessels. Across all genotypes, the transgenic lines had up to a three-fold increase in tensionwood, 40% lower modulus of elasticity, 25% lower modulus of rupture, 45% reduced resistance to xylem embolism (P_{50}), and a 60% increase in stem taper. Comparable patterns in wood density in lines that were lower in lignin by up to 9% by mass was compensated for by a 3% increase in polysaccharide content associated with tensionwood and a 6% increase in the deposition of extractives. Taken together, these data suggest that extensive field testing, ecophysiology, and wood quality evaluations are critical components of research and development on lignin-modified tree crops.

Functional Decreases in Hydraulic and Mechanical Properties of Field-grown Transgenic Poplar Trees caused by Modification of the Lignin Synthesis Pathway through Downregulation of the 4-Coumarate:Coenzyme A Ligase Gene

by
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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes the release of my dissertation to any reader upon request.

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CONTRIBUTION OF AUTHORS

Dr. Steve Strauss oversaw the initiation of this research and its original design. He also provided interpretation of the data. Dr. Barb Lachenbruch and Dr. Rick Meinzer provided insight on study design and interpretation of the data and contributed to the writing and editing of each thesis chapter. Dr. Michael Jourdes conducted lignin and extractive analyses. Dr. Ann Patten provided excellent photos of the wood coloration in the poplars and interpretation of data. Dr. Norman Lewis and Dr. Laurence Davin provided comments on Chapter 2 and interpretation of the data. Dr. Peter Kitin provided expertise in microscopy including images shown in Chapters 2 and 3. Catherine Ma and Olga Shevchenko propagated the trees and conducted RNA expression analyses, respectively.

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CHAPTER 1: INTRODUCTION

NEW PERSPECTIVES ON LIGNIN: ENERGY, BIOTECHNOLOGY AND ANCIENT ATMOSPHERIC GAS COMPOSITIONS

Wood is an abundant, renewable natural resource and its utilization for pulp or in biofuel production requires the costly and energy-intensive step of degrading lignin in woody cell walls. Lignin is often characterized as a polyphenolic substance that encrusts the space between cellulose microfibrils in plant cell walls, but the function of lignin is more than just a "space-filler". Lignin is generally thought to have evolved to reinforce the tracheids of early land plants and was soon after (a few millions of years) increasingly used by plants as a stiffening agent in cell walls, allowing some early plants to adopt arborescent growth forms to outcompete their relatively unlignified neighbors for light. Lignin is therefore crucial for sealing off tracheary elements from air in the surrounding atmosphere as well as for transfering stresses between cells and within cell walls that all have unevenly distributed material properties. Lignin has a greater carbon density than polysaccharides and is also 75-100% more metabolically expensive than cellulose (Lewis and Yamamoto 1989; Loomis and Amthor 2000). Therefore, despite its expense, all extant arborescent plants (including palms and grasses such as bamboos) utilize lignin to the degree that it constitutes approximately 20% of their cell wall dry mass.

Proper experimental investigation of the multiple important functions of lignin for secondary growth - that is unconfounded by variation in plant development - requires alteration to one or a few small sets of genes amidst a massive genome (often within poplars, *Populus* spp.). Indeed, many sets of genes can influence the biosynthesis of lignin. However, at the outset of this type of research the "4CL" (4-coumarate:CoA-Ligase) gene family was prominently identified by numerous researchers as a potential candidate for effectively governing the normal program for lignification. This study and others now suggest that much work still needs still to be done to significantly reduce lignin concentrations without incurring severe consequences for plant function. In Chapter 2 I provide an overview of our tests to determine if hybrid poplars containing a 4CL transgene (grown for two years in a field trial) can be used to reduce lignin, and if so

determine if that lowered carbon demand by lignin can be effectively utilized by primary metabolism for greater growth or increased cellulose deposition. In Chapter 3 I detail the range in phenotypic plasticity of growth form of trees varying in lignin content by comparing the field-grown unstaked trees to greenhouse-grown staked trees. I also compare traits determining the "hydraulic architecture" of these trees that are relevant for estimating whole plant water use and drought tolerance. The following chapters I hope will provide 1) useful knowledge for determining the value of lowering lignin for woody biomass crop production, and 2) a deeper understanding of lignin biosynthesis and its relationships with other aspects of tree physiology.

Because discussion in the following chapters generally takes place at the level of genes, cell walls, wood or whole trees, I only allude to their broader importance rather sparsely. It is therefore my intention to set out in this introductory chapter a set of brief commentaries to provide a context in which to frame the studies that follow. These commentaries start with the bottom line, how many dollars potentially could be saved during pulp production if lignin content of the furnish were able to be reduced. Secondly, I discuss the broader interest in lignin, biotechnology and biofuels by analyzing internet search data. In the final two commentaries I discuss what may be the most important and underappreciated aspect of lignin, its pivotal role in determining global carbon storage and the evolution of our atmospheric composition over the past 450 million years.

Economic consequences of reducing lignin

Society and industry share a common interest in increasing energy efficiency by reducing lignin concentrations in feedstocks used for paper or second generation biofuel production. At the current annual consumption of 130 million tons of chemically treated wood pulp (http://faostat.fao.org/default.aspx) and 2008 prices of near $700 US dollars per ton (http://www.paperage.com/foex/pulp.html), the total annual value of this pulp was nearly 90 billion dollars. Assuming that a reasonably modern pulp mill uses about 875 kWh to remove lignin and bleach each ton of raw wood fiber (Klugman et al. 2007a, 2007b), approximately one billion kWh in electricity consumption (or about 100 million US dollars at $0.1 per kWh) might be saved on an annual basis for each 1% global reduction in lignin of feedstocks used for paper. These savings do not include any reductions in expenditures related to chemical and waste water treatment costs. Though

simplistic, these calculations demonstrate the tremendous potential economic and environmental benefits for designing trees for more efficient utilization in paper or biofuel feedstocks. If lignin concentrations were reduced by just 10% globally (out of 100%=current lignin levels), there could be a savings of one billion dollars, a substantial decrease in the paper industry's carbon footprint, and fewer noxious chemicals entering rivers that often serve as the water supplies of cities downstream.

From biofuel to biotech: tracking interest with internet search data

The internet search engine Google, has recently made available their weekly records (2004-present) of internet search volume for key words among various countries and states (within the U.S.). These data, though imperfect and only representing the interests of those who have regular access to the internet, may nonetheless be useful for tracking or even interpreting potential shifts in the broader interest in certain issues. Here I use a simple algorithm to correct these data to calculate what I hypothesize to be more accurate trends in public interest. To put some trends related to the scientific findings discussed in later chapters into context, I use examples where the variation in searches (and thus apparent interest) is clearly determined by either well known seasonal phenomena or recent socioeconomic trends. All data presented are from global averages of internet users. These Google trend data are no doubt biased towards the northern hemisphere and western culture. However, this actually simplifies interpretations. These data are maybe most exciting because they represent just an inkling of the potential information that this database may hold that can help science better understand society.

In discussing internet search trends I must first discuss the need for de-trending, similar to the methods that are used in dendrochronology and for some aspects of climatology. Many time series data may include low-frequency variation that owe to phenomena that may be real or an artifact. An example of a real shift is when there are subtle changes in the behavior of a system, whereas artifactual trends are often introduced when data collection or standardization methods change incrementally over time. Both of these types of changes are often seen in meteorological data and it may be in the interest of a researcher to remove either or both, depending on the question they wish to answer. An example of the former is when a weather station originally placed in a rural setting is progressively surrounded by a warmer urban setting. An example of the latter occurs

when larger scale records of precipitation are compared over time using instrumentation that has become more precise (so as to record rainfall amounts when previous gauges might record "trace"), and thus result in an artificial trend towards increasing annual precipitation.

In the case of internet search data, there is no clear answer for why long-term shifts occur, but a long-term decline was obvious in a number of search terms I believed should have been relatively stable over time. Because Google rates their search volume numbers on a per capita internet user basis, this suggests that terms in English may in fact be losing ground as the internet becomes available to non-English speaking users at a greater rate. To quantify and remove this unwanted variation I needed to somehow construct a "long-term composite trend" for standardization. For this purpose I averaged together a number of terms that I hypothesized to have little dependence on socioeconomic forcings and also apparently had weak seasonal phases. The terms I included were as follows: cats, dogs, internet, music, nature, plastic, science, society, stapler, technology and wood. The polynomial equation fit to this long term average started at about 90 at the beginning of 2004 and ended at about 60 in Mid-April of 2009 (Figure 1.1, top). The internet search data presented and discussed herein have had this 30% reduction in internet searches removed by subtraction and then were re-standardized so that the peak value over the periods 2004-2009 was equal to 100. It should be noted before the discussion, that a sharp peak in the long-term composite mean is apparent at the end of November 2007. This occurs as a 100% value for some apparently unrelated search datasets, but is not apparent in other closely related search terms. It is therefore likely that there are a few anomalous data calculation problems that have not yet been fixed by Google. This single datapoint had little influence on the de-trending curve, but may be seen in a few of the results from search terms.

I will start out by discussing the term that is on the tip of everyone's tongue right now, "recession". The current economy, both in the U.S. and globally is in a recession. The international monetary fund, or IMF, recently reported that the global economy is projected to shrink by 1.3% in the next year for the first time in about 60 years. Internet searches that used the word "recession" peaked between October 2008 and January 2009 with a short-lived, likely anomalous peak in January of 2007 also apparent. In another

interesting example, a very different pattern is seen in the volume of searches for "builders". As expected, there are drastic seasonal dynamics as those looking for builders peak in the northern hemisphere spring and summer and drop precipitously each fall with lows each holiday season. More relevant and interesting is the difference between seasonal peaks. This long term trend can be seen to peak in 2005 and 2006 before ebbing slightly in 2007, being below 2004 levels in 2008 and being at levels equivalent to the normal "Christmas holiday low" during the recent months of 2009.

In transitioning towards search terms relevant to forestry and wood science I plotted internet searches for the term "biofuels" in parallel with the actual baseline regular unleaded gas prices as documented by the U.S. Dept. of Energy (averaged from New York Harbor, Gulf Coast and Los Angeles refineries; http://www.eia.doe.gov/emeu/international/prices.html#Motor). Not surprisingly these trends track each other closely over most of the past four years. However, even though gas prices have dropped, internet searches for biofuels have reached a plateau that is about 40% higher in 2009 than at the same time in 2004. This demonstrates that at present, interest in developing biofuel technologies has not been quenched by lower gas prices alone.

Next I compare two crucial components to basic plant biology that differ in their seasonality of interest or lack thereof. While searches for "pollen" have a seasonal cycle, searches for "lignin" show no seasonal patterns (Figure 1.2). Searches for pollen may represent an amazing seasonal swell in the interest plant sexual reproduction, but more likely lies in the timing of pollen release of many plants in the northern hemisphere and the resulting human misery induced by allergic reactions caused by these microgametophytes. Interestingly, it could be that Google trends data for searches about pollen (when spatially explicit) may some day track the seasonality of pollen dispersal as climate change causes shifts in plant phenological patterns. Lignin, on the other hand, does not usually cause allergies and is probably doomed to be underappreciated by human kind for the foreseeable future.

The final set of three search terms "genetically modified", "transgenic" and "biotechnology" or "biotech" (the latter two being averaged together) were chosen because they all offer relevant descriptions of the trees or the technology used in the

following chapters. Those interested in searching these terms may do so for purposes ranging from scientific, to macro-economic, to self-interest related to the fear or disdain of any of these phenomena (be it well-informed or otherwise). Each of these terms has a varying seasonal component whereby genetically modified is highly seasonal and biotechnology has little to no seasonality excepting the acute Christmas lows. All of the above search terms obviously share a similar long-term decline in their use. This is slightly lesser for biotechnology or biotech than for genetically modified. Because these terms can and often do elicit diametrically opposed feelings in different people, there could be some very interesting implications for differences in trends between words that are predominately used by different interest groups. It is conceivable that those internet users who search for "genetically modified" organisms could best be characterized as leaning towards the perception that further transgenic research is unwarranted. It is also conceivable that the majority of those who searched for "biotechnology" or "biotech" may be scientists and economists who have a positive perception of those terms. If these generalizations are true, then these data suggest that the field of biotechnology may have weathered the worst of the controversy surrounding its right to exist. Alternatively, it could be that biotechnology is no longer seen by the public as the boon it once was. Certainly, a few more years of data or more specific analyses (country by country) would provide more refined insights for this line of inquiry.

Lignin in plants, and its influence on oxygen and carbon dioxide in the atmosphere

Key events are herein described that span the last 450 million years leading up to the development of our current atmospheric oxygen and carbon dioxide concentrations. Based on the events emphasized here I argue that the evolution of lignin set the stage for a series of further evolutionary events critical for the radiation of plants and the corresponding development of our current atmosphere. I identify six periods relevant for this discussion. Each of these six periods corresponds to the numbers located at the top of Figure 1.3. Many of the events associated with these periods have been recounted in greater detail elsewhere (see Beerling 2007 and those citations listed in the caption of Figure 1.3).

1. Plants invade the land, but their limited abundance limits their impact on atmospheric gas concentrations.

2. Evolution of the cuticle, stomata and a vascular system spurs the radiation of plants, sequestering CO_2 in wood and soils (Raven 1977, Sperry 2003). Large trees with deep roots develop, increasing weathering (Mosbrugger 1990, Raven and Edwards 2001). In turn, weathering increases delivery of K, Mg and Ca to marine systems and results in their precipitation as carbonates and increased burial of carbon in the ocean. This burial rate was not excessive in absolute terms, but because fungi capable of decomposing organic carbon in lignin and other phenolics (an oxidative process) had not yet evolved, oxygen builds up in atmosphere at unprecedented rates (Robinson 1990).
3. Massive stocks of organic carbon and high $[O_2]$ set the stage (or the dinner table) for the evolution of first fungal decomposers (basidiomycetes). As atmospheric $[O_2]$ rose above 13%, wildfires would have tended to become more abundant and larger. Burial of charcoal could initially create a positive feedback loop causing further rises in $[O_2]$, however increasing wildfire frequency and severity must eventually limit the amount of steady state biomass in any ecosystem. Thus the highest atmospheric $[O_2]$, will provide a negative feedback by increasing fire to a fuel-limited stage (i.e. fire is can no longer propagate across the landscape when the plants that fuel it have been removed by fires that are too frequent – see Guyette et al. 2002 for an example of human-induced ignitions rather than $[O_2]$-induced ignitions promoting fuel-limitation), slow chemical weathering rates and reduced inorganic carbon burial in the oceans (see Bowman et al. 2009 and references therein).
4. The advent of vessels in angiosperms increases hydraulic efficiency, allowing plants to spend more water to gain carbon during low $[CO_2]$ periods and efficiently cool larger leaves in regions with warm temperatures (Osborne et al. 2004; Sperry 2006). The rather low recalcitrance to decomposition of angiosperm tissues (low in lignin, and C:N ratio) and the progressive decline in the steady state organic carbon stocks as basidiomycetes evolve and spread cause a net rise in $[CO_2]$ between about 250 to 100 million years ago.
5. Angiosperms gain in dominance, but the evolution of torus-margo pitting occurs in gymnosperms at this time. Indeed, torus-margo pitting allows tracheids to conduct water more efficiently while providing a greater degree of safety to the xylem stream (Pitterman et al. 2005; Domec et al. 2008). The evolution of this trait, which is present in nearly every extant gymnosperm suggests it may have been key in fostering greater competition with angiosperms in resource-rich environments that then drove maximum tree heights to increase towards their modern day limits. The dotted lines for maximum tree height represent a provisional hypothesis proposed here that maximum tree heights did not undergo a smooth transition towards those we know today. The location of the second abrupt increase in maximum tree height was placed just after the date of the first known torus-margo pitting in the fossil record.
6. The closing of the Isthmus of Panama and opening of the Drake Passage caused dramatic shifts in ocean circulation patterns that in turn drove ice sheet formation at the poles, gradually cooling the globe. Glacial cycles increase abiotic weathering (transport of glacially derived rock dust to the oceans) while ever-increasing angiosperm dominance has been proposed to induce greater biotic

weathering (Volk 1989). Stabilization of ocean currents in conjunction with few (or short-lived) strategic advancements by either plant or decomposer communities has led to less abrupt shifts in atmospheric [CO_2] and [O_2] and the gradual advance towards modern concentrations.

If the sequence of events outlined above did play out as hypothesized, then it could be argued that the evolution of lignin, a key step in the radiation of land plants may have been necessary to set up low [CO_2] conditions increasing the adaptive value of greater hydraulic efficiency and favoring the rise of vessel-bearing angiosperms. By chance then, through the further interplay of various plant and fungal evolutionary advances, a rather stable atmospheric composition has been brought about whereby oxygen is in great enough supply to support life as we know it without being so high as to fuel both greatly accelerated rates of decomposition as well as greater potential for massive wildfires to sweep across the landscape. As noted by Beerling (2007), although trees are often thought of as the source of global oxygen concentrations, the amount of oxygen in the atmosphere would barely be affected if all the trees on earth were cut down at once. However, any evolutionary advantage that favored greatly enhanced decomposition rates of biomass has the potential to not only release great amounts of carbon into the atmosphere, but also drive oxygen to levels much lower. If this were to occur it would take millions of years for steady state conditions once again prevail. The intervening dynamics would be devastating to life as we know it in a much hotter, low oxygen world.

Modern carbon storage resulting from lignin versus polysaccharides

Plants and forests in particular comprise a large part of global carbon storage (Fischlin et al. 2007). Arborescent plants generally contain 20-40% lignin by mass, making this polyphenolic molecule an important component to global carbon storage. Moreover, lignin has a very high C:N ratio and near-random structure that renders its biodegradation dependent on certain environmental conditions favorable for basidiomycetes to effectively oxidize and break down this complex molecule. Lignin in plants and their respective distributions across different biomes are therefore important in determining what the steady state carbon stores are in soils across the earth. These considerations have led some researchers to suggest the “mass of lignin in the biosphere is second only to the mass of cellulose” (Peter and Neale 2004), or stated more

specifically, “next to cellulose, they [lignins] are Nature’s second most abundant organic substances” (Davin et al. 2008). These sweeping statements, however, were made without any citations suggesting there may be little quantitative basis to judge their veracity. Although statements about the global masses of lignin versus cellulose may seem rather inconsequential, a firm basis for these statements should nonetheless promote better understanding of how recent evolution of this macromolecule (as compared to Rubisco, the enzyme required for photosynthesis) has changed the course of earth’s history by favoring woody plants. This turn of events succeeded in changing global biogeochemical cycles to such a degree that resulting climates have not only continued to support diverse plant assemblages but also promoted human occupation, agriculture and the development of modern societies. Therefore, inasmuch as one can glean numbers on such massive global phenomena from the diverse literature data sources that are required, I have taken it upon myself to attempt a perhaps crude, preliminary estimation of global carbon storage deriving from the most basic terrestrial plant-derived compounds.

Carbon pools were largely estimated from data synthesized in the most recent reports by the Intergovernmental Panel on Climate Change (Fischlin et al. 2007). Lignin, phenolic and polysaccharide contents of biomass at these biome levels are unknown and thus I made crude estimates, given my knowledge of their vegetation. In the scaling of this carbon the relative components add up to 97%, because a constant of 3% was removed to account for wall structural proteins. Soluble sugars and starches were not considered here as they are only a few percent of the dry mass of vegetation and are the most labile carbon compounds that are quickly respired into CO_2 during decomposition.

The same IPCC data syntheses were used for total soil carbon estimates. These data take into account many studies of soil carbon densities and account for soil depths to 3m. Lignin does not survive for long in soils in its native polymerized form but the resulting phenolic products of its degradation, though difficult to define in absolute terms, are nonetheless an important component of the stable organic fraction of soil (Rasse et al. 2006; Bahri et al 2008). Therefore, because the fate of lignin-derived compounds is uncertain, upland soil pools were assigned similar estimates as the vegetation type inputs they were formed by. There are large uncertainties in the amount of carbon in soils, especially wetland soils from different regions. For wetland soil carbon estimates we

refer to those synthesized by Mitra et al. (2005) and split these overall estimates into three broad climatic classes. These classes were thought necessary to capture the inputs of different vegetation types, in that mosses dominate peatland formation at higher latitudes and have no lignin but are often >20% by mass phenolics (Graham et al. 2004).

Global coal stocks that are "recoverable" were estimated after the most recent report by British Petroleum (BPP 2007). Because these estimates are "recoverable", they are certainly significant underestimates of total coal carbon storage, but to what percentage is unknown. Given the oxygen contents required for degradation of lignin and phenolics (>5%), the substrates undergoing later stages of peatification and coalification are known to consist almost entirely of cutans, lignins, tannins and other phenolics (Stout et al. 1988). Moreover most coal was deposited during the Devonian, Carboniferous and Permian periods by pteridophytes, lycophytes and pro-gymnosperms that had large periderms estimated to be 30× greater in mass for a given stem diameter than that of modern trees (Phillips et al. 1985; Bowyer et al. 2003). It should be noted, however, that even this 30-fold difference is small compared to some of the earliest land plants, whose primary xylem strand was centrally located and only consisted of a few percent of the cross-sectional area (Rowe and Speck 2004). Since bark generally has greater lignin and phenolic contents than wood in most modern taxa, this would only have added to the rate of carbon burial, especially at this time before lignin-degrading basidiomycetes were abundant (Timell 1962; Robinson 1990). Carbon densities were assigned as follows: lignin, phenolics and fatty acids = 67%; polysaccharides= 47%; anthracite and bituminous coal 92%; sub-bituminous and lignite coal = 72%.

During photosynthesis inorganic carbon is initially fixed into organic carbon as a sugar, which is then used to synthesize polysaccharides, lignins and other structural compounds that largely determine global carbon storage. A substantial portion of the global net annual carbon fixation is by phytoplankton, which do not contain lignin. Organic carbon of dead phytoplankton is efficiently captured by heterotrophic organisms, subsequently respired as it moves up the food chain and eventually remineralized in the ocean if not released to the atmosphere as a gas. This results in only about 2 Pg of carbon per year being buried in ocean sediments and only about 0.2 Pg per year being buried as organic carbon (Sarmiento and Gruber 2006). This buried organic carbon would tend to

be degraded further by bacteria even after reaching the ocean floor. Only a small portion of terrestrial carbon (<1Pg/year) is transferred from land to the ocean via rivers. In this case, few if any heterotrophic organisms in oceans can effectively use carbon sediments derived from lignin whereas bacteria may continue degrading polysaccharides even in the anoxic deposition zones of alluvial fans outside the mouths of large rivers. This process would tend to result in a net sink of lignin-derived carbon located in marine systems that would partially offset burial of organic carbon derived from polysaccharides. Globally, these components of organic carbon sinks in marine systems are both small and uncertain compared to terrestrial estimates and were therefore not included in Table 1.1.

The results of this inventory were in fact surprising because the polysaccharide component is equal to 52% of carbon and the lignin component 31%. If it is assumed that "hemicelluloses" are 40% of structural polysaccharides and the remainder is cellulose, then cellulose-derived carbon only represents only 32% of the carbon inventoried here. Consider then that the coal and the soil pools are very conservative estimates of lignin-derived carbon. In this case, it seems likely that in comparison to cellulose, the mass of lignin-derived carbon is greater! Doubtless, carbon storage derived from polysaccharide synthesis is less than that originally synthesized by the phenylpropanoid pathway.

Closing thoughts on the importance of lignin:

This introductory chapter underscores the importance of plants, and lignin in particular, for beneficially modifying our atmosphere in the past and continuing to moderating our atmosphere in response to our profligate use of fossil carbon. To help reduce our dependence on fossil carbon it will require those scientists across academic and applied fields to harness the full potential of plants and technology to more efficiently provide structure, fiber and energy. Increasing this efficiency may involve altering lignin biosynthesis in some crop plants. Lignin is so crucial to plant form and physiological function that altering its biosynthesis will have many unknown effects. Therefore this and the following chapters represent just one small step towards achieving a greater and more refined understanding of lignin.

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Figure 1.1. Trends (2004-2009) in selected socioeconomic keywords using Google internet search engine records and the base price of unleaded gasoline in the U.S. All search data plotted here were de-trended with the polynomial regression line fit to the long-term composite trend shown in the top panel and described in detail in the text.

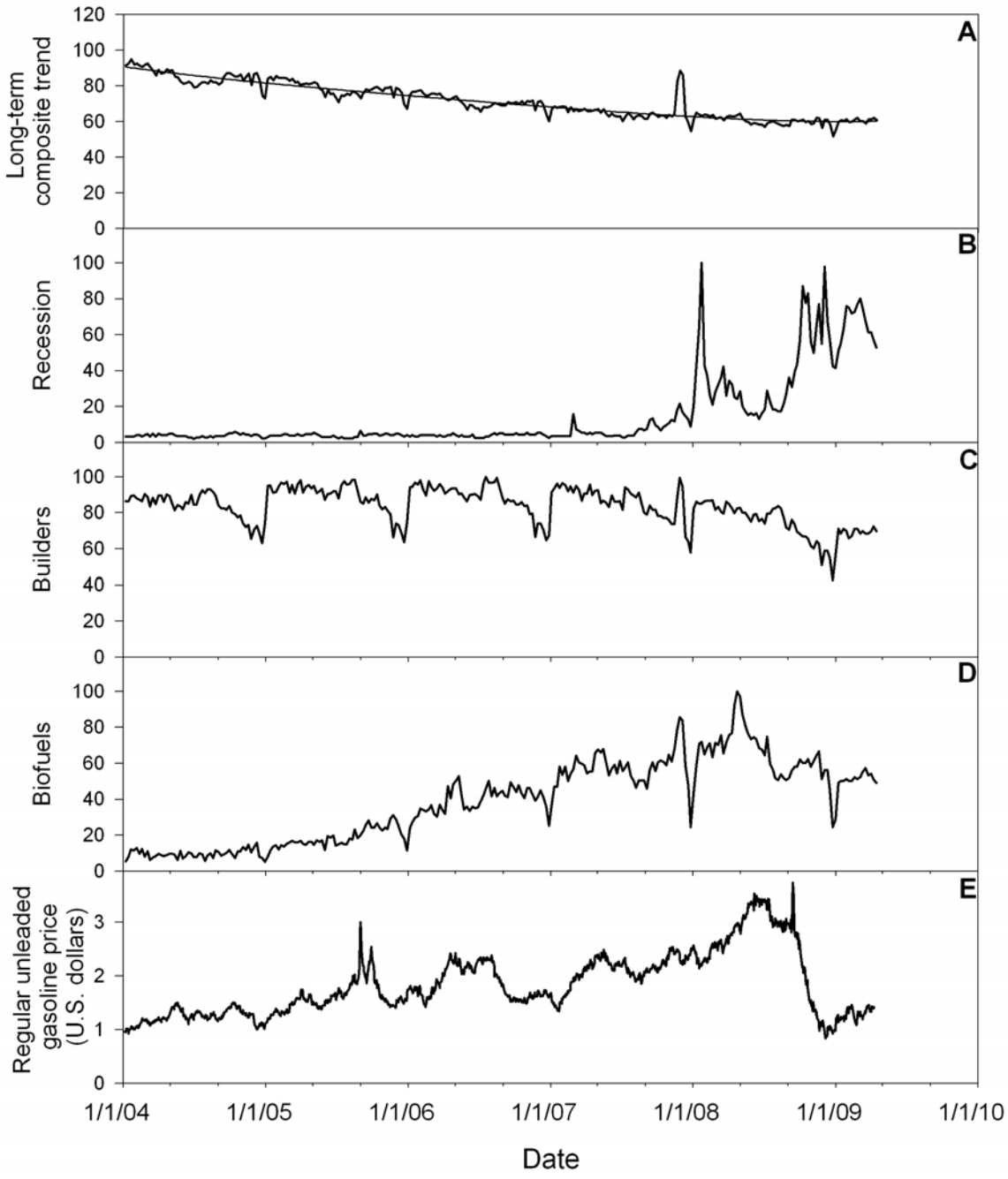

Figure 1.2. Trends from 2004-2009 in selected biological keywords using Google internet search engine records. All search data plotted here were de-trended as shown in Figure 1.1 and discussed in the text.

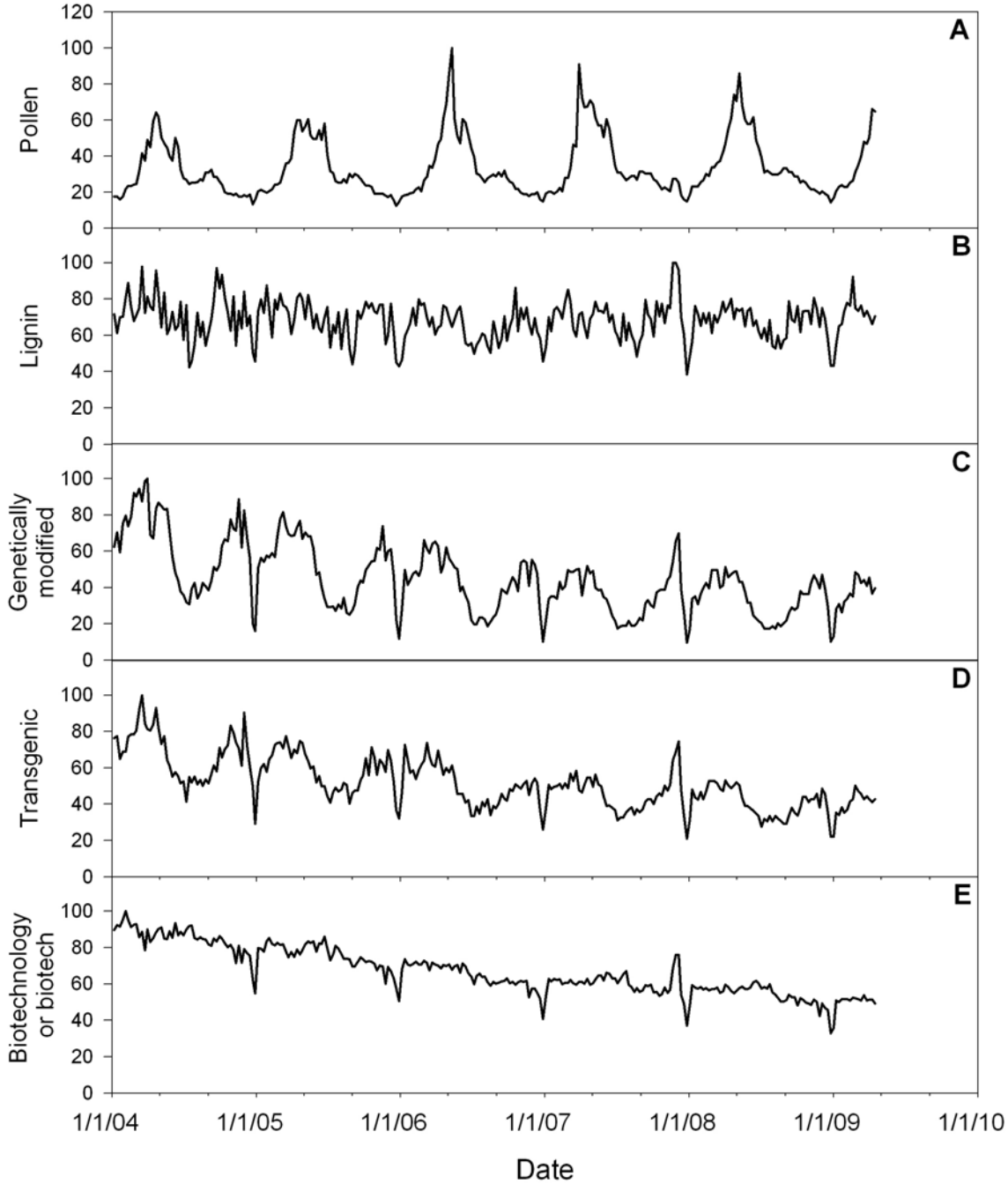

Figure 1.3. Trends in atmospheric oxygen, carbon dioxide, plant evolution and basidiomycete abundance. Data shown here are largely adapted from Niklas 1985 (tracheid diameter), Mosbrugger 1990 (tree height), Robinson 1990 (basidomycete abundance), Sperry 2003 ([CO_2] and torus-margo pitting), Osborne et al. 2004 (gymnosperm leaf area) and Berner 2007 ([O_2]). The range shown for atmospheric gases is 12-30% for [O_2] and 280-5000 ppm for [CO_2]. Maximum tree height as well as angiosperm leaf area and vessel diameters are known from their beginning and ending, but the intervening period represent hypothetical approximations of how the progression in these traits may have occurred.

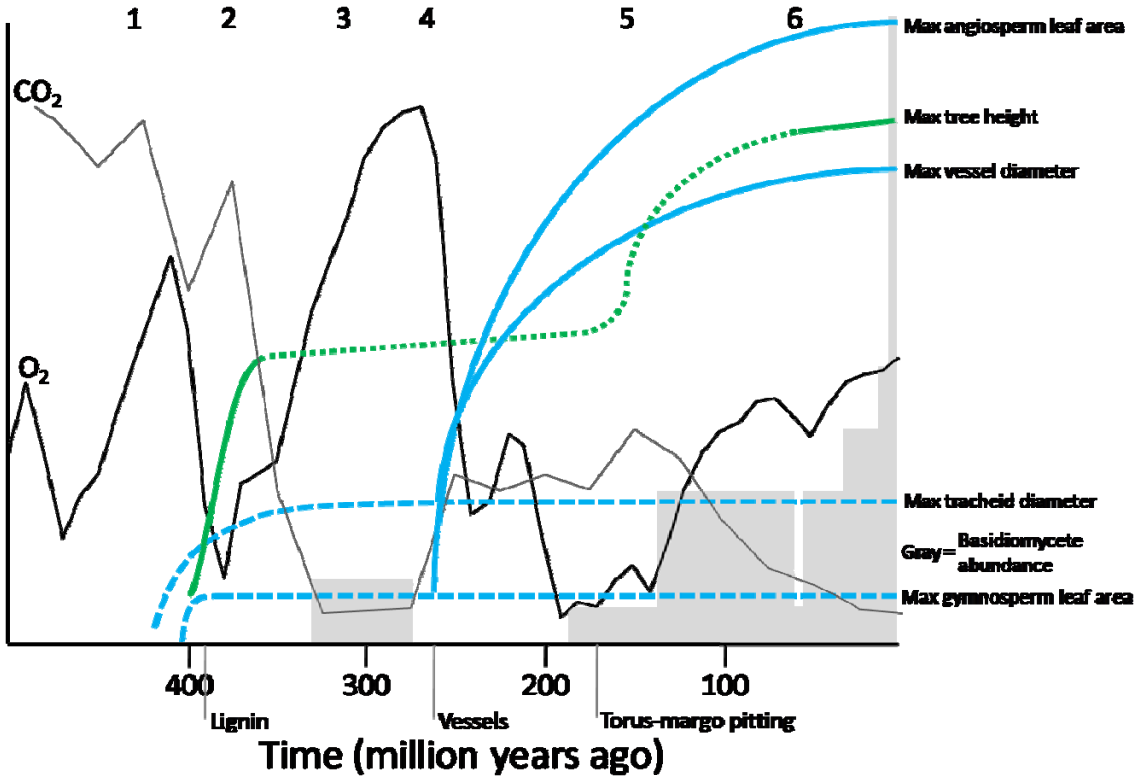

Table 1.1. Estimates of modern terrestrial carbon storage by lignin and polysaccharides.

Biome for biomass	Biomass carbon (Pg)	Lignin (%)	Phenolics & fatty acids (%)	Poly-saccharide (%)	Lignin carbon (Pg)	Phenolics & fatty acid carbon (Pg)	Poly-saccharide carbon (Pg)
Tropical rain forests	351	18	5	74	42.3	11.7	116.7
Temp. Decid. Forest	150	18	5	74	18.1	5.0	49.9
Boreal forest	67	25	5	67	11.2	2.2	20.2
Med. & open forest	25	18	6	73	3.1	1.0	8.4
Desert and scrub	22	18	6	73	2.7	0.9	7.3
Trop. Grasslands & Savannas	86	8	4	85	4.6	2.3	32.9
Temprerate Grasslands	16	8	4	85	0.9	0.4	6.1
Croplands	13	8	4	85	0.7	0.3	4.9
Tundra	5	25	70	85	0.4	2.1	3.8
Biomass sub-total	735				84	26	250
Specific percentage					23	7	69
Biome for upland soils	Soil carbon (Pg)						
Tropical rain forests	708	18	5	74	214.4	59.5	433.7
Temp. Decid. Forest	268	18	5	74	81.1	22.5	164.1
Boreal foreests	159	25	5	67	63.3	12.7	83.4
Med. & open forest	134	18	6	73	40.2	13.4	80.2
Desert and scrub	217	18	6	73	65.1	21.7	129.9
Trop. Grasslands & Savannas	357	8	4	85	53.1	26.5	277.4
Temprerate Grasslands	182	8	4	85	27.0	13.5	141.2
Cropland	261	8	4	85	38.8	19.4	203.1
Tundra	150	5	25	67	11.9	59.5	78.4
Yedoma	507	10	15	83	75.3	112.9	318.6
Permafrost	408	10	15	83	60.6	90.9	256.5
Upland soil sub-total	3350				731	453	2166
Specific percentage					22	14	65
Latitude of wetland/peatland soil	Soil carbon (Pg)						
Boreal	346.5	10	60	27	41.6	249.6	55.3
Temperate	46.2	40	30	27	22.2	16.6	7.4
Tropical	69.3	50	20	27	41.6	16.6	11.1
Wetland/peatland soil sub-total	462				105	283	74
Specific percentage					23	61	16
Coal type	Coal carbon (Pg)						
Anthracite and bituminous	440.5	79	14	4	348.0	61.7	17.6
Sub-bituminous and lignite	309.8	74	17	6	229.3	52.7	18.6
Recoverable coal sub-total	750				577	114	36
Specific percentage					79	16	5
Grand total	5292				1497	876	2527
Specific percentage					31	18	52

CHAPTER 2:

REDUCED GROWTH, WEAKER WOOD AND DISCOLORED XYLEM ACCOMPANY SEVERE LIGNIN REDUCTIONS IN POPLARS CONTAINING A 4CL-TRANSGENE

ABSTRACT

Society depends heavily on wood for energy, fiber, and as a structural material. Ways to reduce the lignin content of wood have long been sought because this polyphenolic compound is the principal cause of recalcitrance to chemical pulping and fermentation of wood for the production of paper and biofuels, respectively. On the other hand, wood structural properties important for tree development and survival may be compromised if lignin content is reduced. Initial studies of poplars (*Populus* spp.) with antisense downregulation of the *Pt4CL1* gene family that encodes 4-coumarate:coenzyme A ligase (4CL) to reduce lignin content have not included the rigorous phenotyping necessary to evaluate the potential of this technology for large-scale biomass production. To begin to clarify what level of 4CL downregulation, if any, could most effectively optimize short-rotation biomass production, a two-year field trial of 14 transgenic lines and an empty vector control line from a single clone of a hybrid white poplar was carried out. Only transgenic lines with modest (15%) reductions in lignin content sustained adequate growth and tree form. Trees with severely reduced lignin contents formed brown colored wood that was less conductive to water due to the ectopic deposition of phenolics and tyloses formation. These trees also had reduced wood stiffness and strength and increased stem taper despite wood densities being similar to controls.

INTRODUCTION

The unique physico-chemical properties of xylem cell walls have evolved to fulfill the requirement that tracheary elements resist stresses generated by hydrostatic tensions during periods of transpiration while the matrix of woody tissue simultaneously resists stresses associated with plant self-support and wind-loading (Raven 1977; Niklas 1992; Boyce et al. 2004). Transgenic plants provide the opportunity to understand the degree to which cell walls and lignification may be altered and to evaluate the resulting physiological and biomechanical consequences. There is long-standing enthusiasm for reducing lignification of cell walls or changing native lignin monomeric compositions

(i.e. shifting the ratios of syringyl [S], guaiacyl [G] and p-hydroxyphenyl [H] lignins) to improve utilization of plant feedstocks for pulp, bioethanol and forage (Porter et al. 1977; Miller et al. 1983; Schubert 2006; Jørgensen et al. 2007; Yang and Wyman 2007; Foust et al. 2008; Weng and Chapple 2008). In addition to the more recent interest in biofuels, the ever-increasing consumption of wood fiber (currently at 130 million metric tons annually) underscores the urgent need for continued improvements in wood production, quality and utilization efficiency. Towards this end we have used hybrid poplars containing a 4-coumarate:coenzyme A ligase (4CL) transgene to learn how altered lignification can affect tree productivity, stem form, wood mechanical properties, extractive contents and tree hydraulic architecture.

Although carbon flux toward H,S or G monolignols must be determined in part by metabolic networks regulating phenylalanine production, the entry point to the phenylpropanoid pathway has more traditionally been considered to occur where phenylalanine ammonia lyase (PAL) catalyses the deamination of phenylalanine. The product, cinnamic acid, is then converted into *p*-coumaric acid by the cytochrome P450 monooxygenase cinnamic acid 4-hyroxylase (C4H). Located just after C4H, 4CL is necessary for the formation of flavonoids and all three monolignols. Like any of the phenylpropanoid pathway enzymes, the severe downregulation of 4CL will eventually reduce cell wall lignin content.

Angiosperms have so far been found to have two or more isoenzymes that make up the 4CL gene family. These isoenzymes have differing degrees of substrate affinities and can generally be grouped into two classes; Class 1 isoenzymes are most highly expressed in the xylem and thought to be associated with monolignol biosynthesis. Class 2 isoenzymes, though not missing from the xylem, are most highly expressed in green tissues and are thought to be associated with the synthesis of secondary metabolites important for various aspects of plant defense (Hu et al. 1998; Ehlting et al. 1999; Harding et al. 2002; Lindermayer et al. 2002; Hamada et al. 2004; Hamberger and Hahlbrock 2004; Costa et al. 2005; Endler et al. 2008). Poplar 4CL isoenzymes are encoded by two sets of paralogs resulting from genome-wide duplication; Poptr4CL1/5 belonging to Class 1, Pptr4CL3/4 are of unknown functional significance and Poptr4CL2 is a Class 2 gene (Hu et al. 1998; Harding et al. 2002; Tsai et al. 2006). Because the 4CL

step is located just before the branching of carbon flow to monolignols versus other secondary metabolites and each species has two classes of 4CLs that apparently divert carbon to these separate metabolic endpoints, we hypothesize that constitutive down-regulation of 4CL results in two distinct types of transgenic lines. One type favors shunt pathways for carbon flux that cause the build-up of secondary metabolites while other lines would tend to reduce total carbon demand for lignin biosynthesis. We also hypothesize that little carbon can be re-routed to polysaccharide biosynthesis even under conditions of reduced carbon demand for lignin biosynthesis and little carbon shunted towards secondary metabolites.

Initially greeted with optimism, 4CL-downregulation of poplars was reported to result in trees with up to 45% less lignin, increased cellulose contents and increased growth (Hu et al. 1999). These results led Hu et al. (1999) to hypothesize that compensatory deposition of cell wall polysaccharides resulted from reduced carbon demand for lignin synthesis. However, plants are unknown to have the capacity to re-direct carbon to primary metabolism when the process of monolignol assembly is altered (Anterola and Lewis 2002). Therefore this interesting hypothesis, supported by data from Hu et al. (1999) and Li et al. (2003), deserves to be tested more completely in other species and growth environments.

More recent analyses found that 4CL-downregulation did not enhance tree growth (Li et al. 2003; Hancock et al. 2007, 2008). Li et al. (2003) contend that these apparently contrary results stem from the use of a xylem specific rather than a constitutively expressed promoter. Moreover, comprehensive reviews make clear that other than Hu et al. (1999) there are no reports of transgenic trees modified in lignin content with increased growth (Anterola and Lewis 2002; Davin et al. 2008a,b). Metabolic profiling has yielded insights on cross-talk between primary and secondary metabolic sinks, but total carbon demand by the phenylpropanoid sinks was not greatly reduced by down-regulating later enzymatic steps in the monolignol pathway (Dauwe et al. 2007). Rather the data were interpreted to show that sensing of reduced cell wall integrity resulted in a considerable phenylpropanoid sink that could not be satisfied in the absence of a full complement of enzymatic machinery necessary for normal lignin deposition.

Evidence for potential controls on carbon flux through the phenylpropanoid pathway suggests PAL, C4H and *p*-coumaroyl 3′-hydroxylase (C3H) are all important (Anterola et al. 1999, 2002). However, most enzymes downstream of C4H, including 4CL, are regularly in overabundance in all but the most severely altered transgenics, generally characterized by pleiotropic phenotypes and evidence of shunt pathway effects (Anterola and Lewis 2002). Therefore the goal of producing trees that are suitable for woody biomass production by reducing lignification through 4CL down-regulation rests upon the assumption that carbon flux towards lignin and non-lignin phenolics will be reduced in tandem. There is as yet no simple means of predicting how effectively this can be accomplished for a gene family with multiple isoenzymes. Further complexities would be introduced when demand for the many non-lignin phenylpropanoid compounds varies according to developmental stages or environmental stresses, causing genome-wide feedbacks on regulation of primary and secondary metabolism (Dixon and Paiva 1995; Dauwe et al. 2007; Davin et al. 2008a).

None of the previous research on 4CL-downregulated trees has tested xylem functionality or wood mechanics. The few reports of wood mechanical properties from transgenic trees with altered lignin (Köhler and Spatz 2002; Koehler and Telewski 2006) are inconclusive regarding whether trees with reduced lignin content and presumably structurally weakened wood may be viable for biomass production. This uncertainty demands more complete phenotyping in field trials that can provide realistic assessments of tree performance. Such testing will also advance knowledge of carbon fluxes through the phenylpropanoid pathway that will be crucial for a more successful development and deployment of the next generation of transgenic plants. Herein we report results from a field trial that should help reconcile some previously divergent results regarding the effectiveness of 4CL suppression for reducing lignin content without incurring negative pleiotropic effects.

METHODS

Plant genotypes and bacterial strain

Hybrid white poplars (*P. tremula* × *P. alba, INRA-France 717-1B4;* all female plants) were used for all transformations. Forty- to fifty-day-old, *in vitro* grown plantlets served as explant sources. Micro-cuttings of 717-1B4 were initially cultured on hormone-

free, half strength Murashige and Skoog medium (MS) (Murashige and Skoog 1962). Shoot cultures were maintained on MS at 25° C under a 16-h photoperiod [fluorescent tubes (TL70, F25T8/TL735, Philips) at a photon flux density of 45 μmol m^{-2} s^{-1}]. The antisense *Pt4CL1* construct was generated by fusing the *Pt4CL1* cDNA coding sequence in an antisense orientation with respect to a duplicated-enhancer cauliflower mosaic virus 35S promoter (*Agrobacterium strain* C58 and gene construct were provided by Dr. Vincent Chiang at North Carolina State University).

Plant transformation

Agrobacterium cells harboring the binary vector were grown for 24 h in Luria Butani (LB) medium (Weigel and Glazebrook 2002) supplemented with 50 mg/l rifampicin, 50 mg/l kanamycin, and 50 mg/l gentamycin on an orbital shaker at 28°C and 250 rpm. Cells were pelleted by centrifugation at 3,500 rpm (1,992 RCF) for 30– 40 min and resuspended in *Agrobacterium* induction medium (IM) (Han et al. 2000) to achieve an OD600nm of 0.5–0.6. Inter-nodal stem segments (3–4 mm in length) and leaf discs (4 mm in diameter) were wounded with multiple fine cuts and incubated in *Agrobacterium* suspension for 1 h. The inoculated explants were co-cultivated in callus-induction medium (CIM) [MS supplemented with 10 μM naphthaleneacetic acid (Sigma, St. Louis, MO) and 5 μM N6-(2- isopentenyl) adenine (Sigma)] at 22°C in darkness for 2 days. Explants were washed following Han et al. (2000) and transferred to CIM containing 50 mg/l kanamycin and 200 mg/l timentin for 21 days. Shoots were induced on SIM medium (MS containing 0.2 μM TDZ, NOR-AM Chemical Co.,Wilmington, DE), 100 mg/l kanamycin, and 200 mg/l timentin (GlaxoSmithKline Inc., Research Triangle Park, NC] for 2 to 3 months. For shoot elongation, explants were transferred onto MS containing 0.1 μM 6-benzylaminopurine (Sigma), 100 mg/l kanamycin, and 200 mg/l timentin. Regenerated shoots were rooted and micropropagated after 30 days on half-strength MS supplemented 0.5 μMindole-3-butyric acid (Sigma) and 25 mg/l kanamycin. To ensure transformation events were independent, a single clone per individual explant was selected for further propagation after confirmation of transgene presence by polymerase chain reaction (PCR).

Genomic DNA isolation and PCR amplification

Genomic DNA was isolated from young poplar leaves using a Plant DNAeasy Kit (Qiagen, Valencia, CA). Approximately 25–50 ng of poplar DNA was used as a template for PCR. Transgene presence was confirmed using 4CL-specific primers (5_-CAGGAATGCTCTGCACTCTG-3_ and 5_-ATGAATCCACAAGAATTCAT-3_) to amplify 1.6-kp product. The PCR conditions used for 30 cycles were: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The PCR products were separated on 1% agarose gel and stained with ethidium bromide.

Plant preparation and field trial establishment

PCR positive events were propagated with half strength MS medium under the same conditions as shoot cultures. Fifty- to sixty-day-old plantlets were transferred to small pots (5.71×8.25 cm) with soil and grown in a greenhouse for two months (April-May, 2005), grown for another 2 months (June- July, 2005) in tubular pots (6.99×24.75 cm) under the same16 h/8 h photoperiod with supplemental lighting. A total of 14 transgenic events (i.e., independent gene insertions) with 10-17 ramets plus 108 non-transgenic empty vector controls were so produced. Plants were then moved to a coldframe for three months of acclimatization (August-October, 2005).

The field trial was conducted just outside Corvallis, Oregon (44.65º N, 123.3º W, 140 m elevation). Mean annual precipitation at the site is 130 cm, but June through September is usually very dry. The frost-free period ranges from 160-210 days, with mean maximum and minimum temperatures over the period of 23.2ºC and 8.6 ºC. Soil at the site is a well-drained, silty clay loam in the top 15cm that transitions to clay at a depth of 40 cm. To ensure no significant drought stress was incurred at the site, all trees were regularly hand-watered during the 2006 growing season and permanent drip irrigation was installed for the 2007 growing season. The planting arrangement was a randomized complete block with 10-15 ramets from each of 14 transgenic lines and control line planted at a spacing of three meters between trees. To minimize the influence of competing vegetation, the bare soil surrounding each tree was covered with shade cloth and an herbicide treatment was applied to rows between trees at the beginning of the 2007 growing season.

4CL expression

The expression of the two endogenous 4-coumarate:CoA ligase *4CL1* genes was assessed by qRT-PCR. In May 2007 developing xylem tissues from six control trees and four ramets per transgenic line were sampled. From these tissues a modified *Qiagen* RNA extraction protocol was used (Busov et al. 2003). All RNA samples were treated with DNaseI (TURBO DNA-free kit, *Ambion*) to avoid genomic DNA contamination. First strand synthesis of cDNA from 1 ug of total RNA for all samples was carried out according to SuperScript. III First-Strand Synthesis System for RT-PCR (*Invitrogen*) general guidelines. The reverse transcription reactions were aliquoted and diluted 10 times, and 1 uL was used as a template for the PCR reactions.

A nucleotide database query was performed using BLASTN to search the *P. trichocarpa* genome against the *4CL1*gene sequence from *P. tremuloides* (GenBank Accession number AF041049) (*Pt4CL1*) (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html). Two gene sequences were identified which share 94% sequence similarity, and 89% amino acid identity: 4CL1-1, the most similar to *Pt4CL*, grail3.0100002702 (JGI annotation *Ptr4CL3*) and 4CL1-2 *fgenesh4_pg.C_LG_III001773* (JGI annotation *Ptr4CL2*) (GenBank Accession number EU603298).

Primers were designed specifically for poplar 4CL1-1 and 4CL1-2 genes. The 3' UTR's were verified using total RNA from stem tissues of untransformed control trees (GeneRacer kit, Invitrogen, Carlsbad, CA, USA). Based on the sequences obtained, primers specific for each gene were designed for qRT-PCR (Table 2.1). Samples were run with four replicates on two different plates using Platinum SYBR Green QPCR Super Mix-UDG (Invitrogen Corp.) on an Mx3000p real-time PCR system (Stratagene, La Jolla, CA, USA).

Final concentration of the primers was 0.5 μM. Conditions for all of the PCRs were as follows: 50^{o}C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 30 s, 61°C for 30 s, and 72°C for 30 s. The transcript levels of 4CL1-1 and 4CL1-2 and housekeeping genes were determined from standard curves of the control sample sequentially diluted five times. The amounts of 4CL1-1 and 4CL1-2 were then divided by the housekeeping reference amounts to obtain normalized level of expression of *4CL1-1* and *4CL1-2* genes.

Transgenic poplar performance and physiology

Tree height and basal diameter were measured in November of 2005 (planting), 2006 and 2007. At the end of 2007, six control trees and three or four trees that spanned the range in tree size for each transgenic event were harvested to determine allometric estimates of oven-dried aboveground biomass. These relationships were then used with diameter and height measurements to compare biomass estimates among lines. All other trees were eventually harvested during 2008 to compare other traits.

Estimation of brown wood

Among transgenic lines, wood color varied from being indistinguishable from that of controls, to light pink throughout, to a mottled appearance with red-brown patches next to control-colored wood. The cross-sectional area in "brown wood", as distinct from the light pink color was estimated near the base of each tree by overlaying a grid of dots on a transparent plastic sheet over three cross-sections from each tree at three heights (stem base, 20 and 40 cm) and recording the relative frequency of brown wood as compared to the entire cross-sectional wood area.

Wood chemical constituents

Three trees from each line were randomly chosen for chemical characterization. For each tree, basal stem sections were used for both lignin and extractive analyses. Each sample was debarked, cut into small pieces and freeze-dried for 48 h. These samples were ground in a Waring blender in liquid nitrogen and then ball milled [Fritsch planetary mill, Pulverisette with agate bowls and balls (Gilson Company, Worthington, OH, USA)] for 3 h to obtain an homogenous powder. Aliquots of powder (~1 g) were submitted to sequential extraction to generate the CWR (Cell Wall Residue) used for lignin degradation analysis as previously described (Jourdes et al. 2007). [Note: in cases of brown or red wood, this coloration remained after the extraction procedure.] Lignin contents and monomeric composition of CWR were estimated using the alkaline nitrobenzene oxidation and thioacidolysis (Rolando et al.1992; Blee et al. 2001) methods, respectively.

For wood extractive identification, powdered stem wood (~5 mg), from a control tree and line 713, were individually extracted with methanol-water (10ml, 80:20, v/v) by sonication for 10 min at room temperature. Crude extracts were centrifuged (3000 × g) for 10 min, with each supernatant (8 ml) individually dried under a stream of nitrogen.

Residue was then redissolved in MeOH-H_2O (1 ml, 80:20, v/v) and passed through a syringe filter (0.2 μm pore size, NALGENE), with aliquots (1 μl) subjected to HPLC/MS analyses as described below.

Chromatographic separations and detections were carried out using a Waters ACQUITY Ultra-Performance Liquid Chromatography (UPLC) system, coupled with diode array and mass spectrometric (Thermo Finnigan, APCI mode) detection. An ACQUITY BEH column (C18, 50 × 2.1 mm, 1.7 μm particle size, Waters) with a Vanguard pre-column (5 mm × 2.1, 1.7 μm particle size, Waters) were employed at a flow rate of 0.3 μl/min. The solvent system employed a combination of A (water/acetic acid, 97:3, v/v) and B (acetonitrile) in A:B (95:5) for 6 min, with linear gradients to A:B (60:40) in 6.5 min, to A:B (55:45) in 6 min, and finally to B (100%) in 2 min, with the latter being held for 2 min.

Extractive and cellulose contents were determined gravimetrically. From each of four transgenic trees and seven control trees, stem wood was ground to a fine powder using a Dremel® tool (Robert Bosch Tool Corp., Racine, WI). From lines forming "brown wood", brown and white colored patches were ground and analyzed separately. Each sample of ground wood (~1 g, air-dried wood) was heat-sealed in a polyester filter bag (mesh size 25 μm, ANKOM Technology, Macedon, NY). Lipids and volatile extractives were removed by soxhlet extraction for 24 h (toluene/ethanol, 75:25, v/v) and then repeated with pure ethanol. Soluble sugars were extracted in a 90ºC water bath for 6 hours. To determine α-cellulose, lignin and hemi-celluloses were removed by treatment with $NaClO_2$ and CH_3COOH solution kept at 70ºC and pH<4 and then purified in a NaOH solution.

Wood mechanical properties

For initial mechanical testing of wood, small branches were excised, (<10 mm diameter, June 2006) from each line. Dried samples were stored at ASTM standard 60% relative humidity, preserving wood at 12% moisture content. Three-point dynamic bending tests were then conducted on branch sections using a portable mechanical tester fitted with a 45 kg load cell (In-Spec 2200, Instron, Norwood, MA). In 2008 a larger mechanical testing system (Sintech Model 1/G, MTS Systems Corp. fitted with a Sensotec 230 kg load cell Model 41/571-07, Honeywell International Inc.) was used to

carry out further three-point bending tests on sections of main stems (most > 10-mm-diameter). Diameter inside the bark at each end and total length were recorded for each sample to calculate taper. The span tested for each sample was between 16 to 20 times the estimated mid-point diameter (under bark). The modulus of elasticity (MOE) and modulus of rupture (MOR) for each sample were calculated for a tapered beam following Maki and Kuenzi (1965). Bark was not removed for bending tests because its influence should be negligible for comparisons among groups. For formal statistical comparisons of wood properties among lines where stem diameter was not constant we compared relative values of MOE calculated for each sample relative to the regression-predicted values for that same diameter in the control samples tested.

Estimation of tension wood

Tensionwood was estimated with two methods. From the base of trees harvested for biomass measurements, a razor blade was used to make hand-sections located along a radial path across the stem cross-section (pith to bark). Hand-sections were stained with safranin and astra-blue following Jourez et al. (2001) to distinguish the gelatinous "G-layers" of tension wood fibers (Figure S1). Using a light microscope three radial scans were conducted across hand-sections to visually estimate the relative radial position (to the nearest 5%) where patches of Astra blue-stained tension wood fibers began and ended. The resulting distance and frequency distributions were used to estimate the percentage of the xylem cross-sectional area formed as tension wood fibers assuming stems were circular in cross-section. Cut-ends of those trees not harvested for biomass were quantified for tension wood using the same method as described above for brown wood. Tensionwood patches were identified in this way because of their lighter color and smooth and shiny appearance as compared to surrounding normal wood fibers (Badia et al. 2006; Barbacci et al. 2008; Figure S2.1).

To determine if fiber cell wall area differed among lines, we analyzed images from the same hand-sections described above. Within each image four sub-sections composed exclusively of sclerenchyma fibers without any tensionwood were selected. Sub-sections for image analysis were at least three cells wide and included no partial walls of ray cells. With the ImageJ program (http://rsbweb.nih.gov/ij/), each sub-section was converted to a binary image and the number and size of fibers and the percentage of

fiber lumen area were determined after digitally "cleaning" to remove any foreign material embedded during slide preparation. Percentage of fiber cell wall area was calculated by subtracting the percentage lumen area from 100.

Visualization of in vivo xylem dye ascent

Xylem dye tracer experiments were conducted in September 2008. At dawn, apparently healthy branches of control and transgenic line 712 were excised under water to avoid the introduction of embolisms to the xylem stream. Branches were transported with their bases in water to the lab, transferred to a beaker containing acid fuchsin and placed in sunlight under a fan. To preserve the localization of dye within water-conducting vessels, each of the stems was snap-frozen in liquid nitrogen as soon as acid fuchsin (red coloration) was observed to reach the distal-most leaf petiole on each branch. Cryo-fixed branch segments were planed at – 10 to – 30° C on a sliding cryo-microtome and observed at -30° C with an epi-fluorescence microscope (Nikon E400, Tokyo, Japan) equipped with a cryo-stage (Kitin et al., *manuscript in preparation*). Images were recorded with a digital CCD camera (Q Imaging, Micropublisher 5.0 RTV).

Statistical analyses

Least-squares regression methods were used to assess relationships between tree form, size and wood mechanical properties. To compare trait values among lines (controls and transgenic events) we conducted analysis of variance tests. Traits were first compared with a global ANOVA (PROC GLM, SAS version 9.2, SAS Institute Inc. Cary, NC, USA) to determine if significant variation among lines existed. Further analyses compared means among lines with Tukey HSD tests to control for Type 1 experiment-wise error. For wood density ANOVA was employed to test for the contribution to the observed variation of event, sample diameter and event × sample diameter.

RESULTS

Characterization of transgenic poplar growth, wood properties and wood anatomy

4CL1-1 and 4CL1-2 shared 90% protein similarity and also had similar expression patterns among various tissues. Average lignin content, as indicated by trends in total monomer release by thioacidolysis and nitrobenzene oxidation (Table 2.2, S2.1) showed substantial variability and was non-linearly correlated with RNA expression

(Figure 2.1). Lignin contents declined precipitously below 4CL1-1 and 4CL1-2 RNA expression levels of 40 and 70%, respectively. NBLAST scores indicated that expression of 4CL1-1 in xylem was nearly four times greater than that of 4CL1-2, so that the greatest reductions in lignin content were associated with weighted mean RNA expression values below 50% of controls (Figure 2.1, bottom panel).

Variation in biomass and form was great among transgenic lines grown in the field for two years. Some transgenic lines were very similar in form to controls and had normal or pink colored wood throughout their stems, except near branch junctions, where patches of brown wood were often observed upon pruning for biomass measurements. The stems of five transgenic lines (150, 350, 671 and 712 and 713) regularly formed patches of brown colored wood throughout the main stems (Figure 2.2). The abundance of this brown wood also increased distally (S. Voelker, *personal observation*). Brown wood formation was often associated with irregular cambial activity as demonstrated by the irregular shape of stem cross-sections (Figure 2.2). The brown wood lines often exhibited shoot dieback late in the growing season (September). The subsequent re-initiation of shoots after dieback often left these lines with more of a shrubby appearance. Across transgenic lines, stem taper was one of the traits most consistently and significantly different than controls (Table S2.1). The main stems of brown wood lines were also more tapered than the controls whereas the "normal wood" transgenic lines were transitional in form (Figure 2.3).

Lignin content of controls, as indicated by total monomer release was qualitatively greater than each of the transgenic lines. However, with only three replicates, just the brown wood lines differed significantly from controls (Figure 2.1, Table S2.1). Thioacidolysis and nitrobenzene oxidation showed that control and transgenic lines had similar lignin S/G ratios, except for a trend toward lower S/G ratios in the lines with the lowest total lignin content and greatest incidence of brown wood (Table 2.2). These same three low lignin, or brown wood lines also had substantially higher H-lignin content (Table 2.2). Preliminary identification of non-lignin phenolics (i.e. extractives and coloration associated with brown wood) with HPLC analysis of methanol extracts of brown wood revealed prominent peaks for flavonoids including naringenin and dihydrokaempferol (Figure 2.4). After extraction the wood remained

discolored. Though there was little distinction between the extractive contents of controls and the white or pink colored wood of normal transgenic lines, brown wood patches were characterized by increased volatile (toluene/ethanol soluble) and hot water extractive contents in (Figure 2.5, Table S2.2). Among lines, cellulose contents were variable, with no apparent relationship with brown wood formation or lignin contents (Figure 2.5, Tables S2.1 and S2.2).

To account for size-related variation, we calculated a relative MOE or MOR value of each transgenic stem segment tested relative to the predicted values for controls (see *Methods*, Figure S2.2). Using this approach, a number of the transgenic lines were found to differ significantly from controls in stiffness and strength (Table S2.1). Brown wood lines were initially lower in wood density, but each group reached a similar plateau at stem diameters greater than about 1cm diameter (Figure S2.3). Although event did not describe a significant portion of the variation in wood density, an event × diameter interaction was detected ($P<0.01$). That wood densities of stems >1cm in diameter did not differ among lines is consistent with our findings that the percentage of cross-sectional area in fiber walls was also very similar (Table S2.1).

A number of traits related to plant performance varied in a non-linear or threshold-type pattern with lignin content (Figure 2.6, Table S2.1). Biomass production and brown wood formation had threshold responses corresponding to a 15% reduction in lignin (Figure 2.6) or a 60% reduction in 4CL RNA expression (*data not shown*). At this moderate 15% reduction in lignin, tensionwood increased from about 5 to 10% of wood area, wood strength (MOR) was reduced by about 20% and wood stiffness (MOE) was decreased by about 30% (Figure 2.6, Table S2.1). Tensionwood occurrence should not be directly tied to the phenylpropanoid pathway, but should be promoted by increased bending stresses associated with reductions in wood stiffness. Among lines, MOE was highly correlated with tension wood incidence ($R^2=0.84$, $P<0.01$, *see values in Table S2.1*).

Shoot dieback and visualization of water flow

After observing regular branch dieback in brown wood lines (Figure 2.7), we hypothesized dieback to have resulted from effects associated with the ectopic deposition of phenolics within vessels. To test this hypothesis we conducted dye trace experiments

to visualize whether brown wood carried less water. Dye was observed in nearly all of the vessels of branches of control trees, whereas in brown wood dye was only observed in vessels near the pith and not in most of the normal appearing or collapsed vessels (Figure 2.8). It is likely that reductions in conductivity were caused not only by the ectopic deposition of extractives, but that brown wood formation also was associated with other symptoms normally associated responses to pests and pathogens or heartwood formation. Upon close inspection of tangential sections of wood (rather than cross-sections used to quantify wood anatomy), we observed thin-walled tyloses throughout the length of most vessels within brown wood but these were rare to absent in normal wood (Chapter 3).

DISCUSSION

Lignin contents, brown wood and plant performance

Downregulation of 4CL resulted in up to a 45% reduction in lignin content as indicated by total momomer release of "uncondensed" aromatic units by both thioacidolysis and nitrobenzene oxidation (Figure 2.1; 2.6; Table 2.2; S2.1). This range in lignin contents was similar to those of other studies of poplars containing a 4CL transgene (Hu et al. 1999; Li et al. 2003; Table 2.2). However, a surprising contrast to other studies of poplars with 4CL downregulation, transgenics with the lower total monomer yields were associated with a shift towards reduced Syringyl/Guaiacyl ratios and a corresponding incorporation of H-lignin (Table 2.2), suggesting that the changes to 4CL in this study may have caused more drastic departures from the expected patterns of carbon flux through the pheylproplanoid pathway. Pinpointing the exact point of 4CL downregulation or lignin contents at which this apparent shunt pathway for carbon flux occurred is difficult, but drastic shifts in the values of most traits were undoubtedly associated with a reduction in lignin content (Figure 2.6). Patchy brown wood formation (Figure 2.2) increased dramatically, nearing 60% of the stem cross-sectional area as lignin content was reduced by 15% (Figure 2.6). The brown wood lines thus appeared to experience "misregulation" or shunt pathway effects, as separate from the normal transgenics. Biomass accumulation of the normal transgenics was similar to controls, suggesting pleiotropic effects associated with brown wood occurrence rather than lower lignin per se was the cause of drastic growth reductions of transgenic lines with the lowest lignin contents.

Tree form and wood properties

Regardless of wood color, transgenics lines tended to be shorter for a given diameter than controls (Figure 2.3, 2.6). This trend in tree form among lines was similar to that of wood properties in that wood stiffness and strength were both moderately lower in normal transgenics and reduced further in brown wood lines (figure 2.6). This is precisely the pattern we hypothesized to occur because lower wood stiffness would transmit greater wind-induced bending stresses to the cambium, which would then compensate by increasing cell division rates at the base of the trees to foster a more rigid whole plant form (Telewski 2006). For the same reasons, our hypothesis that trees with lowered wood stiffness would tend to synthesize greater tensionwood was supported (Figure 2.6).

Compared to controls, 4CL-downregulated poplars had similar cellulose contents (Figure 2.5; Table S2.1) fiber cell wall area (Table S2.1) and wood density (Figure S2.3) even though lignin contents were substantially reduced in some transgenic lines. This combination suggests that in the most severely affected lines either some unknown constituents were deposited within cell lumens and or filled the space unoccupied by normal lignin. It is certain that in brown wood, abundant soluble non-lignin phenolics were deposited in cell lumens (Figure 2.5; Table S2.2) as well as incorporated into cell walls as indicated by the brown coloration. However there is little support for substantial increases in polysaccharide content outside of what would be expected due to increased tensionwood. Similar to Caihong et al. (2004), low lignin lines had only small increases in cellulose content (Figure 2.5; Table S2.1) and hemicellulose content (*data not shown*).

Cellulose microfibrils have much greater stiffness than individual wood fibers (Sakurada et al. 1962; Niklas 1992) and tensionwood fibers themselves are nearly pure cellulose oriented close to their vertical axis. Therefore, if lignin had a relatively small role in wood mechanics, transgenic lines with reduced lignin contents and elevated tensionwood would be expected to have higher, rather than lower strength and stiffness than normal wood. Although the cellular and tissue-level mechanism(s) responsible for lower wood stiffness and strength in low-lignin plants are unknown, a few scenarios seem likely. After wood density is taken into account, much of the remaining variation in wood stiffness is related to the orientation of microfibrils (often termed microfibril angle)

within the S2 layer of wood fibers (Page et al. 1983; Keckes et al. 2003). Microfibril angle should not have been affected by 4CL downregulation. However, an altered lignin matrix is likely to have caused a lower frequency of covalent bonds between lignin and hemicelluloses necessary to transfer shear stresses between cellulose microfibrils. Lignin has long been recognized to be most important in resisting compression stresses in wood. Therefore, reduced lignin content would result in a greater proportion of bending stress being transferred to the tension side of a stem where the role of lignin in stress transfer between cells would be most important. In low-lignin wood, lower MOE and MOR would be expected to result from compromised cell-to-cell connections in the middle lamellae as well as from fibril slippage within the cell walls from voids in lignin assembly and fewer bonds to hemicelluloses. In considering the biomechanics of plant tissues, the role of lignin has often been overshadowed by the truly incredible strength of cellulose. However, our data demonstrate that lignin is undoubtedly crucial for the properties of cellulose to be even in part manifested at greater than nano-scales from cell wall to tissue to wood.

Assuming that the results obtained for one- and two-year-old wood in this study are applicable to older stems (or at least more so than for seedlings grown in a greenhouse), then reduced wood stiffness and strength could affect industrial biomass production because relatively long rotation ages increase the risk of incurring critical bending loads induced by wind and ice storms. In the case of open grown trees, the observed reductions in wood stiffness may be compensated for by stems that are more tapered and thus stable in the face of wind loading (Figure 2.5, Table S2.1). For a light-demanding tree species like poplar, forest canopy closure increasingly limits light availability, diminishes average wind loading and results in trees with a less tapered form (Oliver and Larson 1996). In our field trial open growth conditions (10×10m spacing) fostered average values of height / diameter of 50-90, which are low compared to the 150-170 found in very crowded stands (1×1m spacing; DeBell et al. 1997). For woody biomass crops, spacing and its effects on trees with reduced lignin and thus wood stiffness could therefore be a key limitation to productivity that has not yet been investigated.

Is there compensatory carbon flow between primary and secondary metabolism?

The carbon costs of biosynthesis of S, G and H lignins are greater than polysaccharides because of lignin's 75-100% greater metabolic energy requirement and 50% greater carbon density (Lewis and Yamamoto 1989; Loomis and Amthor 2000; Amthor 2003). If it were indeed possible to reduce or redirect the carbon allocated to native levels of lignification, the tradeoffs between metabolic endpoints might be tailored to sequester carbon in cell wall components that are more efficiently utilized by many industrial applications. One set of hypotheses suggests that 4CL downregulation can cause both increased growth and cellulose deposition (Hu et al. 1999). Similar to results obtained with plants without secondary growth (Kajita et al. 1996; Lee et al. 1997), as well as other tree species (Caihong et al. 2004; Wagner et al. 2009), 4CL-downregulation of poplar resulted in no detectable growth enhancement (Li et al. 2003; Hancock et al. 2007, 2008). Lignification is no doubt associated with growth, but growth is a trait controlled by complex source-sink interactions among metabolic endpoints. In fact, many genes associated with lignin biosynthesis were correlated with growth of interspecific backcrosses of *Eucalyptus*, but 4CL was not one of them (Kirst et al. 2004). Collectively, these results clearly demonstrate that growth stimulation is not an expected outcome of 4CL downregulation.

Analyses offered in support of the hypothesis of simple carbon source-sink reciprocity between primary and secondary metabolism (i.e. Hu et al. 1999; Li et al. 2003) are not easily reconciled with our results (Figure 2.5, Table S2.2) and those of Caihong et al. (2004). Both the data presented here and those from Caihong et al (2004) used cell wall polysaccharide mass percentages based on extractive-free cell wall residue, whereas those from Hu et al. (1999) and Li et al. (2003) specified their polysaccharide mass percentages on a dry wood basis. When our data are plotted on a dry wood basis along with those of Hu et al. (1999) and Li et al. (2003) there is a significant relationship that could be construed as weak support of the compensatory regulation of cellulose and lignin deposition (Figure S2.5). However, after being corrected for extractive contents, our cell wall polysaccharide data along with those of Caihong et al. (2004) exhibit no significant trends associated with lignin content (Figure S2.5). Similar to the patterns shown by Patten et al. (2007), lignin downregulation was associated with increases in reaction tissue or tensionwood (Figure 2.6, Table S2.1). Although Pilate et al. (2004)

reviewed evidence suggesting that the middle lamella and S1 layers of tensionwood cells have normal lignin concentrations, the contribution of a thick and unlignified G-layer would result in these cells having greater cellulose contents than normal fibers (Timell 1969; Meier 1985; Nishikubo et al. 2007). Given a 24% increase in cellulose content of tensionwood as compared to normal wood (Meier 1985) and the observed increases in tensionwood in poplars from the present study (i.e. 5 to 18% of fibers), cellulose contents would have been increased by about 3% by tensionwood alone.

If there is carbon reciprocity between primary and secondary metabolism, then the converse pattern in carbon allocation might be expected, in which genes up- or downregulated for cell wall polysaccharide synthesis would cause compensatory changes in lignin contents. Stems of *Arabidopsis* mutants with cellulose contents as low as 17% of control plants had no corresponding change in phenolic content (Turner and Somerville 1997). Additionally, poplars upregulated in cell wall polysaccharide deposition by 11.2% and 10.8% had lignin contents decreased by 5.8% or increased by 2%, respectively (Park et al. 2003; Shani et al. 2004). For the normal amounts of cellulose and lignin found in poplars, these differences in lignin content are both substantially lower than the approximately 14% reduction in lignin that would be expected given a simple compensatory shift of carbon among metabolic endpoints.

We are not suggesting there to be no interactions between primary and secondary metabolism. In fact, when trees were grown in CO_2 concentrations of 1200 versus 400 ppm, genes from stem tissues involved in both cell wall formation and flavonol biosynthesis were clearly downregulated while genes associated with lignin biosynthesis were up-regulated (Druart et al. 2006). Taken together, the above evidence suggests little carbon is simply re-routed between phenylpropanoid and cell wall polysaccharide metabolisms and the weak associations that exist are limited to complex interactions with source-dink dynamics. To better characterize interactions between primary and secondary metabolic endpoints there is a need for more careful measurement of extractive and cell wall residue contents of controls versus mutants in monolignol or cell wall polysaccharide synthesis as affected by experimental changes in CO_2 levels or nitrogen availability.

Altered lignification and xylem discoloration

The phenylpropanoid pathway is responsible for lignins, lignans, flavonoids, benzoic acid, salicylic acid, coumarins and an array of other phenolics (Lewis and Yamamoto 1989; Dixon and Paiva 1995; Lewis et al. 1998; Winkel-Shirley 2001; Tsai et al. 2006). These can impart pigmentation, provide protection from reactive oxygen species, signal stress responses and act as constituitive or inducible defenses in various woody and soft tissues (Dixon and Paiva 1995; Winkel-Shirley 2001; Filkowski et al. 2004; Pietarinen et al. 2006; Tsai et al. 2006).

The build-up of phenolics associated with brown wood formation may be due in part to an amplified stress response and up-regulation of secondary metabolite production as a result of a cinnamic acid build-up being utilized for salicylic acid production due to a 4CL-downregulated constriction in the phenylpropanoid pathway (e.g. Dixon and Paiva 1995;Shirasu et al.1997; Shah 2003). However, naringenin and dihydrokaempferol, both flavonoids, were the main compounds isolated from methanol extracts of discolored brown wood (Figure 2.4). The overabundance of dihydrokaempferol is curious, because this compound is usually undetectable in poplar, apparently because of the efficient metabolic conversion of dihydrokaempferol to dihydroquercitin or kaempferol and thereby to flavonol glycosides, pelargonidin and anthocyanin derivatives (see Tsai et al. 2006). Therefore the detection of this compound is evidence that somehow, excess carbon was shunted into flavonoid production. This notion suggests that hydroxycinnamic acids that would otherwise be ligated by one of the class 1 4CL isoenzymes (toward assembly of S- or G-monolignols) were apparently ligated by a class 2 4CL isoenzyme toward a pool of *p*-Coumaroyl CoA destined for flavonoid biosynthesis and/or *p*-Coumaryl aldehydes that will end up as H-lignin. It seems likely that an amplified stress response resulting from a build-up of salicylic acid may have prompted up-regulation of the class 2 4CL isoenzyme as was demonstrated for inducible defensive responses to wounding in poplar leaves (Kao et al. 2003). If there existed separate pools of *p*-Coumaroyl CoA for monolignol assembly versus secondary metabolism (i.e. spatially segregated within the cell), this stress response and resulting isoenzyme-specific up-regulation could explain the abundance of dihydrokaempferol in brown wood (Figure 2.4) and why the lines with the lowest lignin concentrations formed the most H-lignin (Table 2.2).

Shunt pathways resulting in abnormally pink, red or brown xylem have been found in other 4CL mutants (Kajita et al. 1996; Caihong et al. 2004) but are also found when enzymes either upstream or downstream of 4CL have been altered (Porter et al. 1978; Miller et al. 1983; Baucher et al. 1996; Ralph et al. 1997; Tsai et al. 1998; LaPierre et al. 1999; Meyerman et al. 2000; Pilate et al. 2002; Jourdes et al. 2007; Leple et al. 2007). Cinnamyl alcohol dehydrogenase (CAD) downregulation producing red xylem was demonstrated to result from small amounts of sinapaldehyde entering the xylem cell wall region (Laskar et al. 2009) This pigmentation could readily be removed with MeOH:1% HCl (Jourdes et al. 2007) – a procedure generally utilized for anthocyanin floral pigment removal. Such solubilization behavior is indicative that this pigmentation was not part of the lignin macromolecule. In contrast, we found the brown or red coloration to remain after the same extraction procedure, suggesting that structural components other than the flavonoids identified entered the wood. It is notable that the mottled brown or red wood coloration associated with reduced S/G ratios we documented (Figure 2.2; Table 2.2) was very similar in both respects to mutant poplars downregulated in caffeic acid O-methyltransferase (CAOMT), in which it was demonstrated that cinnamic aldehydes were imparting coloration to the lignin. Further investigation is underway to better identify the compounds imparting the brown and red coloration.

Some flavonoids are known for their modulation of P-glycoproteins necessary for normal auxin transport (Peer and Murphy 2007). Although “collapsed” tracheary elements that we observed and that have been reported in other low lignin mutants (Turner and Somerville 1997; Coleman et al. 2008; Wagner et al. 2009) likely result in part from compromised lignification, abnormal auxin concentrations during development may also have contributed to their malformation (Besseau et al 2007). Bessau et al. (2007) used hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT) downregulated *Arabidopsis* to demonstrate that concomitant silencing of chalcone synthase (CHS) activity reduces carbon flow towards secondary metabolites and restored normal form to the otherwise dwarf phenotypes. This was accomplished apparently by both reducing flavonoid concentrations in the xylem, but still resulted in carbon being shunted from *p*-Coumaroyl CoA toward H- rather than S-lignin. Thereby downregulation

of CHS may allow further reductions in lignin content with lignin that was more amenable to chemical degradation without severe pleiotropic effects. However, it may be difficult in poplar because CHS is encoded by at least six CHS and seven CHS-like genes (Tsai et al. 2006).

Altered lignification effects on water flow and branch dieback

Poplar clones used for wood or biomass production tend to be sensitive to water supply (Shock et al. 2002) and yet it is estimated that the demand for wood fiber will require the expansion of poplar plantings to less than ideal sites (Tuskan 1998). Therefore, increasing poplar wood production, while at the same time increasing water use efficiency, will require the strategic assessment of traits that confer both rapid growth as well as drought tolerance (Tschaplinski et al. 2006). This knowledge is relevant for transgenic trees with altered lignin because poplars closely regulate their stomata to avoid xylem embolism. In cases in which xylem design or stomatal control are not taken into account, greater embolism and losses in xylem conductivity could be expected to lead to branch dieback (Cochard et al. 1996; Sparks and Black 1999; Rood et al. 2000). If compromised cell walls result in xylem embolism at less negative hydrostatic pressures than those genetically determined for stomatal closure, plants are at greater risk of "catastrophic embolism" that would lead to severe dieback or even tree death (Sperry and Tyree 1988; McDowell et al. 2008).

Resistance to embolism was decreased in C3H-downregulated poplars that were reduced in total lignin contents and G-lignin 55% as compared to controls. (Coleman et al. 2008). Though transgenic xylem was more susceptible to embolism, it was unclear if these results owed most greatly to unintended changes in vessel ultrastructure (i.e. deformed and collapsed vessels), a decrease in total lignin content that weakened the wood matrix, or decreased G-lignin that specifically compromised vessel cell walls. More thorough tests are necessary before any conclusions can be drawn about how altered lignification may influence xylem resistance to embolism. Our own data, however, suggest that reduced lignification (without major pleiotropic effects or drastic reductions in G-lignin) caused increased vulnerability to xylem embolism (Chapter 3).

Brown wood lines lines, having with the greatest reductions in lignin underwent shoot dieback each year in the late summer (Figure 2.7). Desiccation of distal leaves

occurred so abruptly after necroses appeared that no yellowing, or apparent remobilization of cell contents was observed. Although summers in this region are often dry, trees were well-watered, excluding the possibility that these symptoms were caused exclusively by a combination of "weak" xylem and poor stomatal control over water loss. Because distal portions of affected shoots were characterized by near complete brown wood formation (S. Voelker, *personal observation*), we hypothesized that impaired vessel function associated with brown wood caused increased hydrostatic tensions which eventually lead to catastrophic embolism and dieback.

In our dye trace experiments, for branches of comparable size it took about 20-30 minutes for dye to reach distal-most petioles of control branches whereas it took 60-90 minutes for branches of line 712 (a transgenic line characterized by the lowest lignin contents and greatest brown wood occurrence). Through dye localization by a careful cryo-fixing procedure, xylem functionality was inferred by the magnitude of dye flow reduction between brown wood of line 712 as compared to control wood (Figure 2.8). Vessels within brown wood patches often were occluded with tyloses (cell-wall outgrowths of parenchyma cells that fill the vessel lumen), whereas wood from the control line lacked tyloses (P. Kitin, *personal observation*). In a complementary study of these trees we found brown had conductivity reduced by 100-500-fold as compared to normally colored wood from the same trees (Chapter 3). Taken together, these results and observations strongly suggest that both tyloses and the progressive deposition of phenolics associated with brown wood formation restricted wood conductivity, increased hydrostatic tensions and caused embolism and shoot dieback late in the summer.

CONCLUSIONS

Consistent with the notion that severe perturbations to the phenylpropanoid pathway will eventually mediate a cascade of negative pleiotropic effects, only lines with modest reductions in lignin content sustained adequate growth and tree form. These findings thus begin to clarify the limitations to this first generation of transgenic systems for poplars. Hopefully such findings and new insights will spur more incisively designed research aimed not simply at further reductions in lignin, but towards more precise regulation of metabolic carbon fluxes that do not seriously affect cell wall properties, tree growth and development.

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Table 2.1. The sequences of primers used for qRT-PCR assay.

Primer name	**Sequence (from 5' end to 3')**	**Size of PCR product**
717-4CL1-1-F02	GCCAGGCATATAACTGAAG	87 bp
717-4CL1-1-R01	AACACTACAATGATTCGCAAG	
717-4CL1-2-F02	CTTGAGAGAGAGGTTGGCA	117 bp
717-4CL1-2-R02	AGGACTCAATGTAAGGTTTTCTC	
aUbq-F03	CTCTTTTGAAGTTGGTGTTTGC	75 bp
aUbq-R03	TCCAATGGAACGGCCATTAA	

Table 2.2. Lignin monomer release from each line by thioacidolysis and nitrobenzene oxidation (mean ±SD from n=3 trees). Both methods used extractive-free cell wall residue (CWR). S=syringyl, G=guaiacyl and H=*p*-hydroxyphenyl lignins.

Analysis	Line	H (μmol/g)	S (μmol/g)	G (μmol/g)	Total (μmol/g)	S/G	H/T
Thioacidolysis	Control	2.0 (±1.1)	174.2 (±3.4)	319.6 (±6.5)	495.9 (±11.0)	1.83	0.004
	224	2.3 (±0.5)	155.3 (±4.5)	330.8 (±14.9)	488.4 (±19.9)	2.13	0.005
	90	2.2 (±0.2)	147.6 (±12.6)	313.5 (±31.1)	463.4 (±43.9)	2.12	0.005
	17	1.7 (±0.4)	144.4 (±3.2)	313.9 (±10.2)	460.1 (±13.7)	2.17	0.004
	210	4.6 (±0.4)	143.4 (±0.2)	290.4 (±5.9)	438.4 (±6.5)	2.03	0.010
	209	3.0 (±0.2)	150.7 (±3.8)	298.8 (±29.9)	452.5 (±33.8)	1.98	0.007
	115	3.1 (±0.8)	135.6 (±5.8)	278.2 (±5.5)	416.9 (±12.1)	2.05	0.007
	204	2.6 (±0.6)	135.8 (±23.0)	295.6 (±14.8)	433.9 (±38.5)	2.18	0.006
	225	6.1 (±3.1)	145.5 (±14.1)	259.2 (±15.5)	410.8 (±32.7)	1.78	0.015
	640	3.7 (±1.2)	149.3 (±5.3)	284.0 (±22.4)	436.9 (±28.9)	1.90	0.008
	713	4.1 (±2.0)	146.6 (±5.6)	284.7 (±23.6)	435.3 (±31.3)	1.94	0.009
	150	4.1 (±2.7)	133.0 (±10.6)	273.9 (±21.4)	411.0 (±34.7)	2.06	0.010
	671	10.9 (±1.8)	110.4 (±9.9)	210.8 (±23.4)	332.1 (±35.0)	1.91	0.033
	350	14.7 (±1.4)	94.5 (±8.6)	174.1 (±12.5)	283.2 (±22.5)	1.84	0.052
	712	20.3 (±2.6)	74.6 (±9.6)	107.0 (±15.2)	201.9 (±27.4)	1.43	0.101
Nitrobenze oxidation	Control	21.1 (±4.7)	150.2 (±16.5)	306.3 (±19.9)	499.4 (±41.1)	2.04	0.042
	224	27.6 (±2.5)	121.0 (±6.6)	284.1 (±23.4)	453.7 (±32.4)	2.35	0.061
	90	26.6 (±1.5)	122.6 (±8.3)	294.8 (±17.8)	467.2 (±27.5)	2.40	0.057
	17	26.4 (±6.7)	125.2 (±9.9)	294.9 (±13.0)	469.6 (±29.6)	2.36	0.056
	210	34.8 (±4.8)	130.9 (±2.4)	298.0 (±17.0)	484.4 (±24.2)	2.28	0.072
	209	27.9 (±1.8)	125.2 (±5.4)	284.3 (±33.3)	458.6 (±40.4)	2.27	0.061
	115	25.3 (±1.8)	134.8 (±14.7)	303.1 (±40.3)	483.1 (±56.8)	2.25	0.052
	204	28.8 (±1.6)	127.3 (±3.7)	278.9 (±17.4)	455.4 (±22.7)	2.19	0.063
	225	37.0 (±4.4)	136.7 (±10.9)	272.2 (±27.3)	465.0 (±42.5)	1.99	0.080
	640	29.3 (±4.4)	118.6 (±9.3)	254.1 (±31.3)	421.9 (±45.0)	2.14	0.069
	713	26.0 (±6.0)	122.0 (±5.3)	256.0 (±34.2)	423.1 (±45.5)	2.10	0.061
	150	27.9 (±10.3)	125.0 (±9.9)	264.5 (±41.6)	438.7 (±61.8)	2.12	0.064
	671	36.4 (±7.1)	106.8 (±21.5)	204.9 (±30.9)	367.7 (±59.5)	1.92	0.099
	350	43.4 (±11.0)	106.6 (±13.5)	174.3 (±21.2)	345.6 (±45.7)	1.64	0.125
	712	55.5 (±6.9)	115.2 (±1.7)	144.5 (±26.5)	336.0 (±35.1)	1.25	0.165

Figure 2.1. Lignin content in relation to RNA expression of 4CL1-1, 4CL1-2 isoenzymes. Weighted means of 4CL1-1 and 4CL1-2 are shown in the bottom panel. Lignin contents (held relative to control values) are total monomer release from thioacidolysis and nitrobenzene oxidation (see Table 2.2). Triangle=control, circles=normal transgenics, squares=brown wood transgenics.

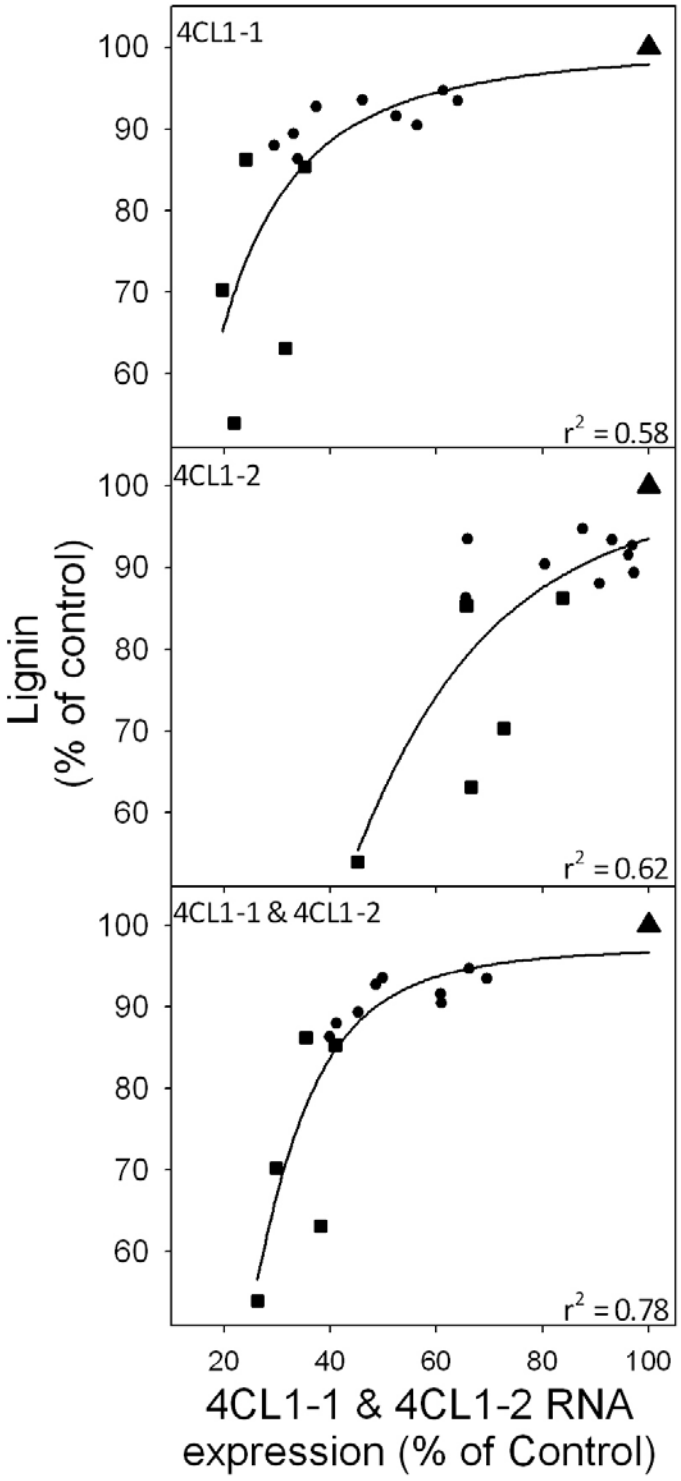


Figure 2.2. Examples of a transgenic line that performed well and the natural coloration of freshly harvested brown versus control wood. Left: a tree from transgenic line 225 pictured Sept. 2008. Center: freshly harvested stems of line 350 (reddish-brown) as compared to control wood. Right: Examples of wood color in transverse sections of the control line (top), line 225 (middle) and line 350 (bottom).

Figure 2.3. Comparison of tree form among control, brown wood and normal transgenics. Each tree is represented by two points as measured at the end of the 2006 and 2007 growing seasons.

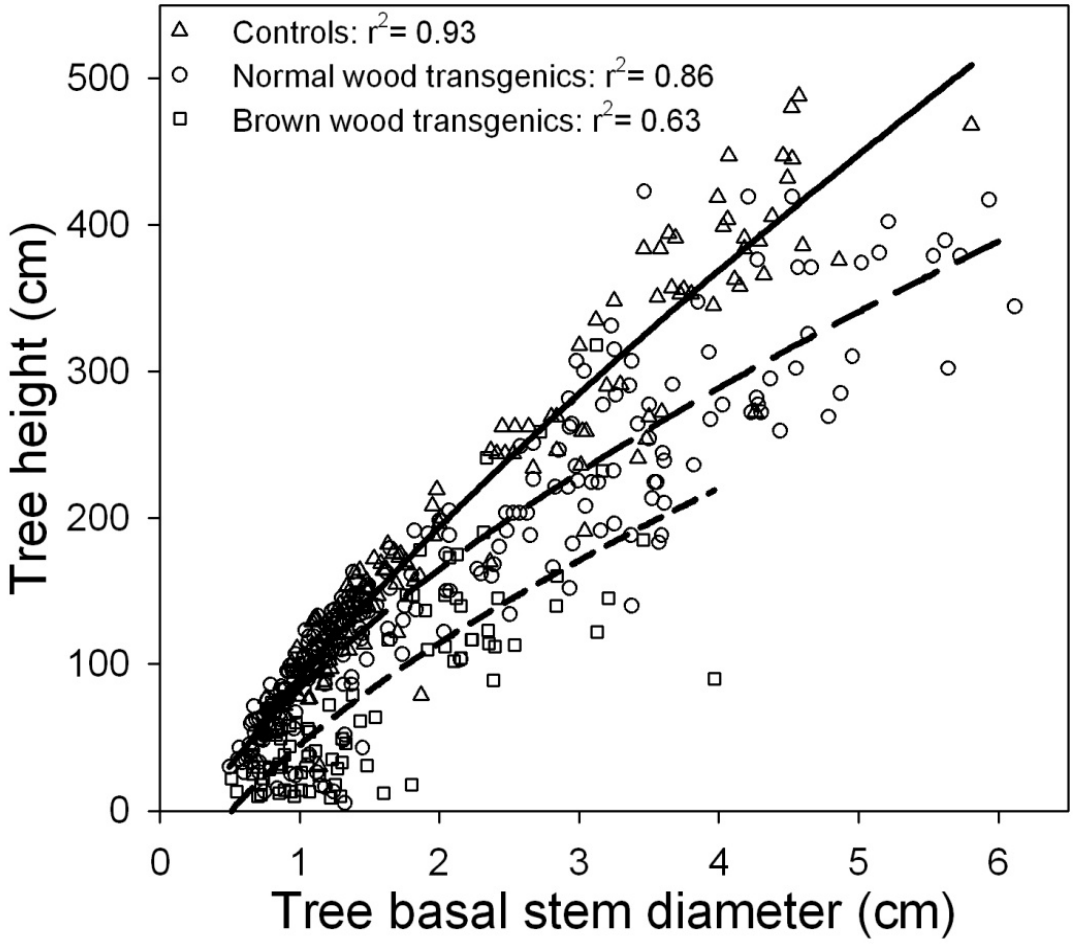
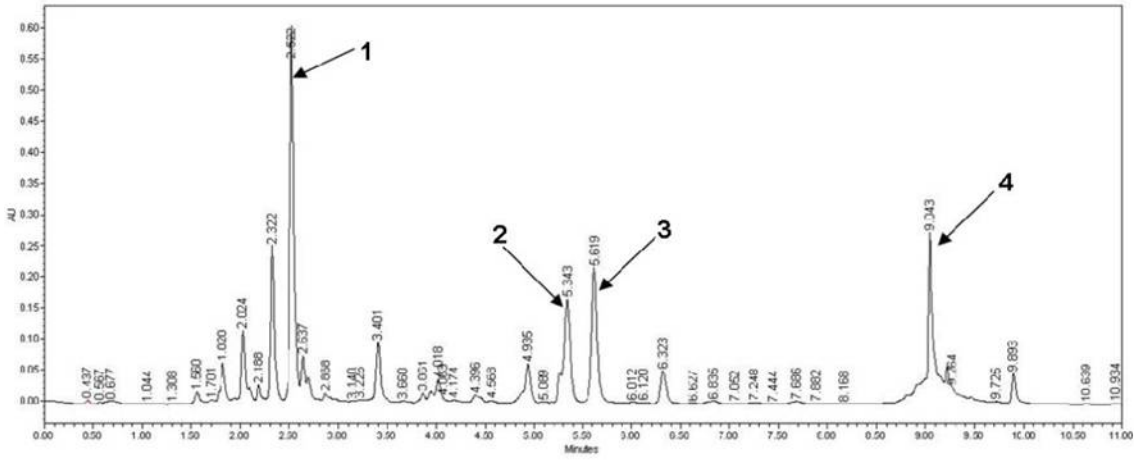
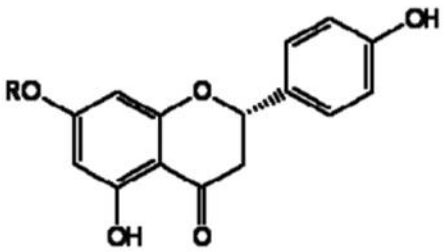
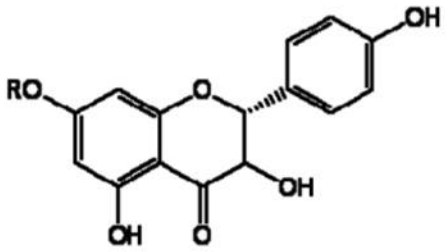


Figure 2.4. Extractive identification from brown wood of line 713. Number labels pointing to peaks correspond to the numbers of the chemical constituent labels below the structures.





1: R=H, Naringenin
2: R= glucose, Naringenin-7-*O*-glucoside



3: R=H, Dihydrokaempferol
4: R= glucose, Dihydrokaempferol-7-*O*-glucoside

Figure 2.5. Comparison of extractive and cellulose contents among control wood, normal transgenic wood and brown transgenic wood. Errors bars = 1SE are from 4-7 trees from each line (see *Methods*).

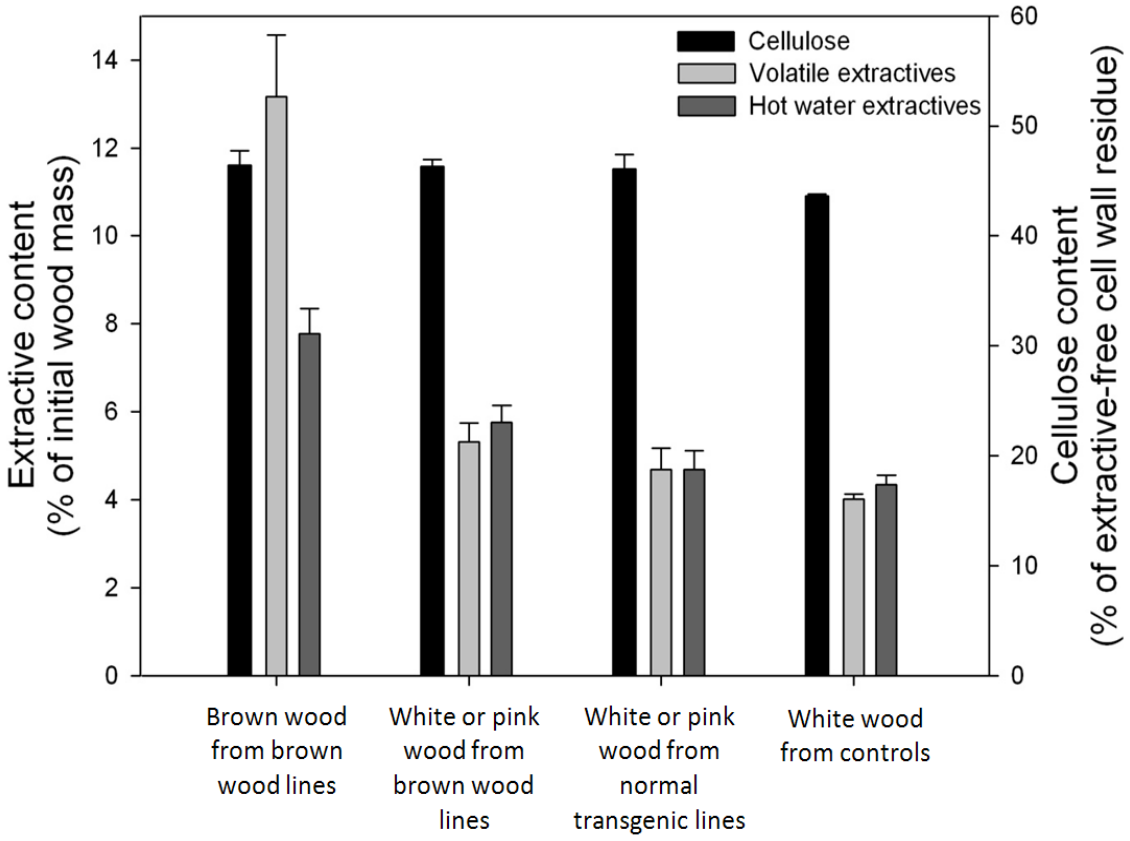


Figure 2.6. Selected performance-related traits plotted against stem lignin concentrations. Percentages of brown wood and tensionwood xylem fibers were measured on five or more trees per line. For MOR and MOE the percentage of the predicted values for a given diameter for the control lines are used to compare among lines (see Figure S2.2). Means and variation for each trait×line combination shown are given in Table S2.1. Symbols as in Figure 2.1.

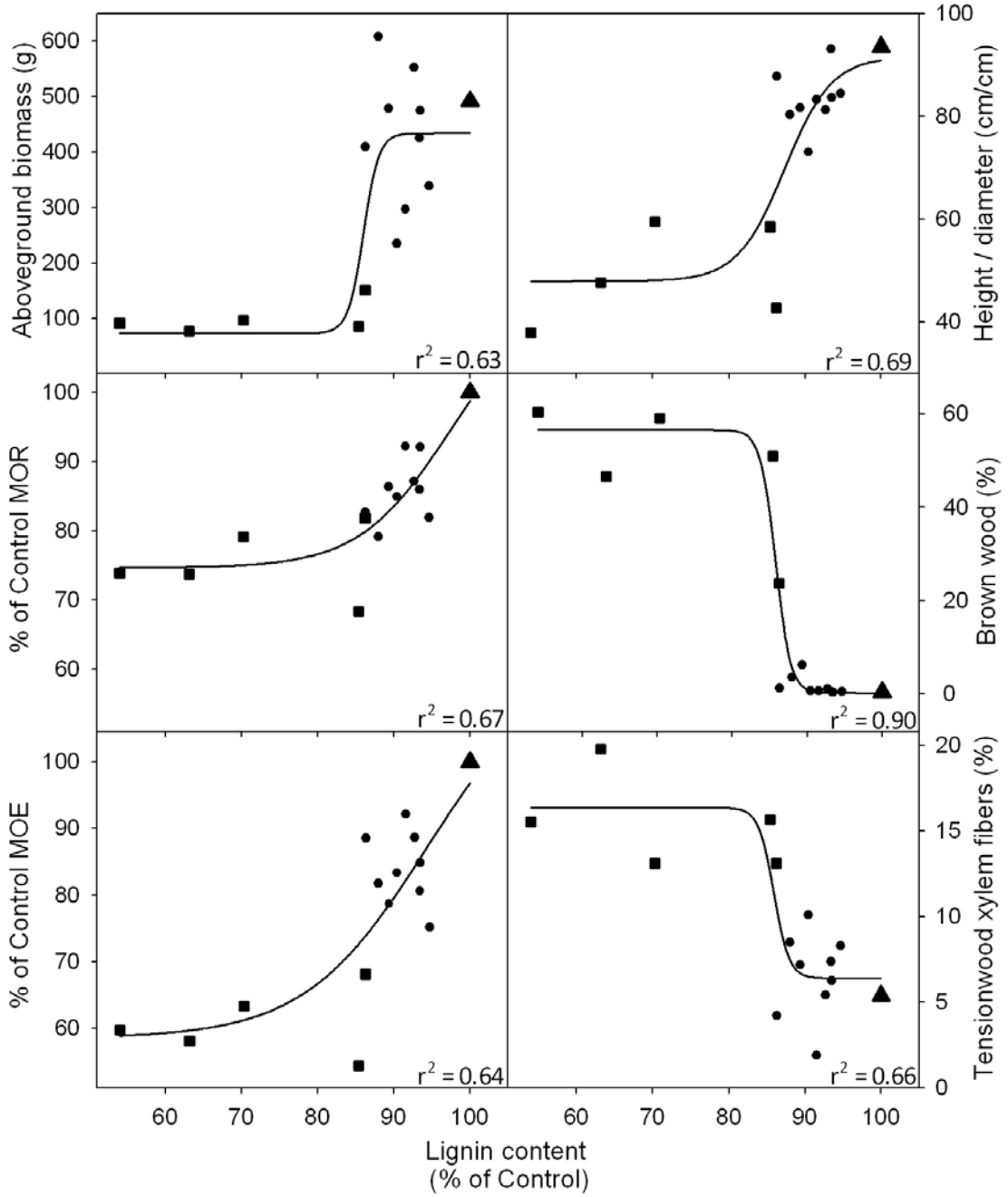
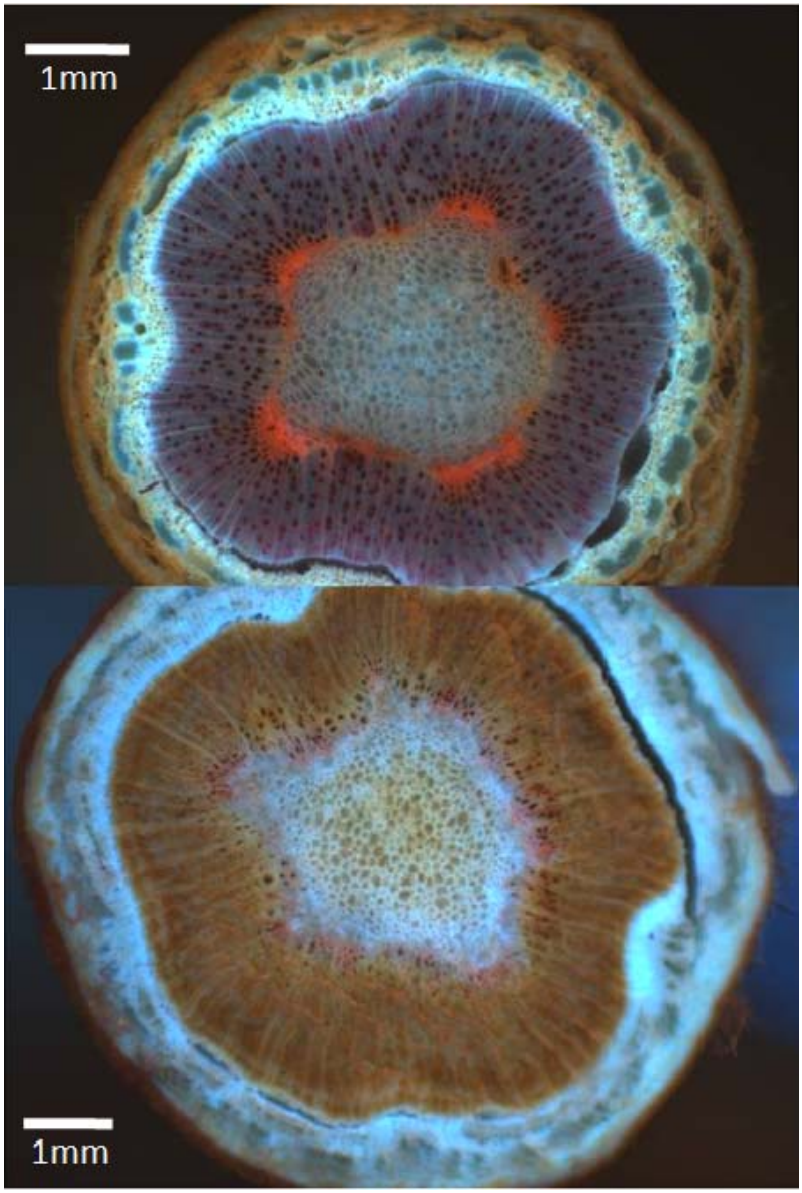


Figure 2.7. Example of the rapidity of shoot dieback progression in reduced lignin, brown wood transgenic line 712. Top picture: morning of Sept. 10, 2008, necrotic areas observed on tips of basal leaves. Bottom picture: morning of Sept. 12, 2008, basal leaves on shoot were completely wilted and distal leaves (not shown) were completely desiccated. Despite being well watered, dieback and desiccation occurred so quickly that leaves were unable to senesce normally to permit translocation of leaf constituents to other parts of plant.

Figure 2.8. Fluorescence microscopy photomicrographs of a dye trace experiment comparing a control and brown wood branch. Most all vessels were dye-filled in the control line (upper), whereas dye was only faintly observed within a few earlywood vessels next to the pith in the brown wood of line 712 (lower).



CHAPTER 3:

ALTERED XYLEM HYDRAULICS, BIOMECHANICS AND BIOMASS ACCUMULATION IN POPLARS CONTAINING A 4CL-TRANSGENE

ABSTRACT

There are few published studies that detail effects on functional traits of the downregulation of enzymatic steps within the phenylpropanoid pathway leading to lignin in poplars (*Populus* spp.). This study examined functional traits related to xylem function, water transport, resistance to embolism, stem taper, stem material properties, shoot dieback, and mortality in a control and 14 transgenic lines of high-yielding hybrid poplars with antisense downregulation of the *Pt4CL1* gene family that encodes 4-coumarate:coenzyme A ligase (4CL). Two year old poplars were characterized after being grown either as free-standing trees in the field or staked in the greenhouse. Across lines we found substantial variation in stem form, but the range in phenotypic plasticity was greatly restricted for those transgenic lines with the lowest lignin contents. Field-grown trees modified their stem taper, thus compensating for wood with reduced stiffness. In contrast, trees grown affixed to stakes all neared the critical height for elastic buckling. Trees with reduced lignin also had reduced growth efficiencies (i.e. biomass / leaf area) and the most affected lines had a patchy, or mottled brown wood coloration. Transgenic lines with reduced lignin were more vulnerable to embolism, and those forming brown wood had reduced specific conductivity, increased mortality and increased late-summer shoot dieback. New methods using dye uptake experiments followed by cryo-sectioning and then fluorescence microscopy or confocal microscopy showed brown wood to be associated with the deposition of non-lignin phenolics within vessels and fibers, and frequently to have tyloses as well. We suggest lignin reductions will invariably alter basic tree form and function such that woody crop biomass production will need to carefully consider what lignin content may be considered optimal over the long term.

INTRODUCTION

Much effort has been invested in the molecular and quantitative chemical characterization of trees with altered lignification, including those with 4-coumarate:coenzyme A ligase (4CL) downregulation (Hu et al. 1999; Leple et al. 2000; Tsai et al. 2006; Meyermans et al. 2007; Dauwe et al. 2008; Wagner et al. 2009). In contrast, the characterization of the form of these mutants and of their mechanical and physiological functioning has received much less attention, and is usually described only qualitatively if at all. This lack of rigor in describing form, biomechanics, and physiology is defensible for certain research goals. However, if low-lignin trees are sought for industrial uses, such information is needed to understand the tradeoffs that may be inherent in finding a balance between the positive impact of modest lignin reductions on pulping or biofuel production efficiency and their eventual negative impact on the structural integrity of the xylem and the plant form (Davin et al. 2008a; Chapter 2). There is as yet no model to determine this balance point because modulation of enzymatic steps within the phenylpropanoid pathway can alter plant functional attributes directly or indirectly. Some direct effects should result from shifts in lignin content, but the indirect, unintended effects can be genome-wide and more significant to normal plant function (Anterola and Lewis 2002; Dauwe et al. 2007, Coleman et al. 2008; Wagner et al. 2009; Chapter 2). Advancing the transgenic technology of altered lignification toward yielding tangible benefits to society will thus require a more comprehensive approach to distinguish phenotypes with true potential from those that may not thrive under realistic field conditions.

Lignin is an important component of secondary cell walls where it provides significant strength. Lignin in wood is in its highest concentration in cell corners and the compound middle lamella (Saka and Goring 1985), where it functions to bond cells together. It has been estimated that the shear modulus of the middle lamella is around three-fold greater than that of the secondary wall (Mark 1967). Once a fracture forms toward the outer edge of a cell wall it could conceivably propagate under lower loads if lignification had been altered. Thereby, reduced strength of cell-to-cell and fibril-to-fibril connections in plants with lower lignin could lead to failure under loads induced by wind or hydrostatic tensions in the xylem. The 4CL isoenzymes of the phenylpropanoid

pathway are involved in production not only of lignins, but also of lignans, flavanoids, salicylic acid and an array of other polyphenolic compounds and thus must be in part responsible for determination of the carbon allocation to the production of these products (Lewis et al. 1998; Tsai et al. 1998; Lindermayer et al. 2002; Tsai et al. 2006; Davin et al. 2008b; Chapter 2). Therefore reductions in lignin through reduced 4CL enzyme activity should compromise the strength of vessels and stems as well as alter the biosynthesis of secondary metabolites whose potential roles in stress signaling and in complex interactions with hormones such as auxin during cambial cell development is as yet not fully understood.

The current study was undertaken to determine the functional phenotypes of 14 transgenic events from a single high-yielding hybrid poplar (*Populus* spp.) clone with antisense downregulation of *Pt4CL1* gene family that encode 4CL isoenzymes. These trees were grown in the field versus staked in a greenhouse to compare phenotype under different biomechanical demands on their xylem. The primary goal of this research was to characterize the mechanical, hydraulic, and growth phenotypes of these lines in relation to their lignin concentrations. We hypothesized that plants with low lignin would have: a) reduced resistance to embolism but increased tissue water storage than those with normal lignin contents; b) wood that has lower modulus of elasticity and modulus of rupture than wood with normal lignin contents, and c) more stem taper than plants with normal lignin contents.

METHODS

Plant materials

Hybrid white poplars (*P. tremula* × *P. alba, INRA-France 717-1B4;* all female plants) were used for all transformations. Forty- to fifty-day-old, *in vitro* grown poplar plantlets served as explant sources. Micro-cuttings were initially cultured on hormone-free, half strength Murashige and Skoog medium (MS) (Murashige and Skoog 1962). Shoot cultures were grown at 25° C under a 16-h photoperiod. The antisense *Pt4CL1* construct was generated by fusing the *Pt4CL1* cDNA coding sequence in an antisense orientation with respect to a duplicated-enhancer cauliflower mosaic virus 35S promoter (*Agrobacterium strain* C58 and gene construct were provided by Dr. Vincent Chiang at North Carolina State University).

Agrobacterium cells harboring the binary vector were grown for 24 h in Luria Butani (LB) medium supplemented with 50 mg/l rifampicin, 50 mg/l kanamycin, and 50 mg/l gentamycin on an orbital shaker at 28°C and 250 rpm. The cells were pelleted by centrifugation at 3,500 rpm (1,992 RCF) for 30-40 min and then resuspended in sufficient *Agrobacterium* induction medium. Inter-nodal stem segments (3–4 mm in length) and leaf discs (4 mm in diameter) were wounded with multiple fine cuts and incubated in *Agrobacterium* suspension. The inoculated explants were co-cultivated in callus-induction medium [MS supplemented with 10 μM naphthaleneacetic acid (Sigma, St. Louis, MO) and 5 μM N6-(2- isopentenyl) adenine (Sigma)] at 22°C in darkness for 2 days. Transformed explant calli were grown for 21 days and shoots induced by culturing on MS medium containing 0.2 μM TDZ (NOR-AM Chemical Co.,Wilmington, DE), 100 mg/l kanamycin, and 200 mg/l timentin (GlaxoSmithKline Inc., Research Triangle Park, NC]. Explants were subcultured at 3- to 4-week intervals for 2-3 months. For shoot elongation, explants were transferred onto MS medium containing 0.1 μM 6-benzylaminopurine (Sigma), 100 mg/l kanamycin, and 200 mg/l timentin. The regenerated shoots were rooted on half-strength MS medium supplemented with 0.5 μMindole-3-butyric acid (Sigma) and 25 mg/l kanamycin. After 30 days, rooted shoots were micropropagated on the same medium. To ensure transformation events were independent, only a single clone per individual explant was propagated after PCR confirmation of transgene presence. Controls were propagated in the same manner as transgenic lines, but contained an empty vector rather than a 4CL transgene.

Genomic DNA isolation and PCR amplification

Genomic DNA was isolated from young poplar leaves of each of the transgenic events and the control using a Plant DNAeasy Kit (Qiagen, Valencia, CA). Approximately 25–50 ng of poplar DNA was used as template for the polymerase chain reaction (PCR). The transgene presence was confirmed by using primers specific for 4CL (5_-CAGGAATGCTCTGCACTCTG-3_ and 5_-ATGAATCCACAAGAATTCAT-3_) to amplify 1.6-kp product. The PCR conditions used for 30 cycles were: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The PCR products were separated on 1% agarose gel and stained with ethidium bromide.

Experimental tree growth conditions

The fifty- to sixty-day-old, *in vitro* grown poplar plantlets described above were grown in a greenhouse from April-July 2005. This yielded a total of 14 transgenic events (i.e., independent gene insertions) with 10-17 ramets plus 108 non-transgenic controls. Plants were moved to a coldframe for acclimatization from August-October 2005 before planting late in 2005 at a field site just outside of Corvallis, Oregon. The planting arrangement was a randomized complete block with 3 m between trees. To minimize the influence of competing vegetation, we placed 1 m^2 of shadecloth around the base of each tree at planting, and applied an herbicide between the trees at the beginning of the 2007 growing season.

In February 2007, before the second year of growth, two individuals from each transgenic line (except line 90 because it had the fewest trees, n=10) and four individuals from the control line were randomly selected for placement in an unheated greenhouse. These trees were transferred to large pots (about 66 L of soil volume per tree) full of a local sandy loam. Each tree was watered and fertilized regularly. The main stem was affixed to a 1.5 x 3 cm wooden stake from heights of about 0.5 and up to 5m from the pot soil-level at weekly intervals (Fig. 1). The stakes themselves were guyed to the bases of the pots with steel wires to prevent their movement. Trees were fixed between the stake and the steel guy wire using numerous plastic zip-ties that prevented any significant sway below the growing tip. Trees were moved within the planting arrangement once per month to minimize any effects of spatial variation in growth conditions. The roof and sides of the greenhouse opened automatically when indoor temperatures exceeded 29º C and closed when temperatures dropped below 20 º C. This arrangement permitted trees to grow in near full sunlight with wind shelter and some temperature control.

4CL expression

In May 2007 the expression of the two endogenous 4-coumarate:CoA ligase *4CL1* genes was assessed by qRT-PCR for the developing xylem tissues along the main stem of field-grown trees. We used one sample each from six controls and four ramets per transgenic line. From these tissues a modified *Qiagen* RNA extraction protocol was used (Busov et al. 2003). All RNA samples were treated with DNaseI (TURBO DNA-free kit, *Ambion*) to avoid genomic DNA contamination.

First-strand synthesis of cDNA from 1 ug of total RNA for all samples was carried out according to SuperScript III First-Strand Synthesis System for RT-PCR (*Invitrogen*). The reverse transcription reactions were aliquoted and diluted 10 times, and 1 uL was used as template for the PCR reactions. A nucleotide database query used BLASTN to search the *P. trichocarpa* genome against the *4CL1*gene sequence from *P. tremuloides* (GenBank Accession number AF041049) (*Pt4CL1*) (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html). Two gene sequences were identified which share 94% similarity of DNA sequences, and 89% amino acid identity: 4CL1-1, the most similar to *Pt4CL*, grail3.0100002702 (JGI annotation *Ptr4CL3*), and 4CL1-2 *fgenesh4_pg.C_LG_III001773* (JGI annotation *Ptr4CL2*) (GenBank Accession number EU603298).

Primers were designed specifically for both poplar 4CL1-1 or 4CL1-2 genes as follows. The 3' UTR of both *4CL* genes was verified using total RNA from stem tissues of untransformed control trees (GeneRacer kit, Invitrogen, Carlsbad, CA, USA). Based on the sequences obtained, primers specific for each gene were designed for qRT-PCR (Table 1). Final concentration of the primers was 0.5 uM. Four replicates of each sample were run on each of two different plates using Platinum SYBR Green QPCR Super Mix-UDG (Invitrogen Corp.) on an Mx3000p real-time PCR system (Stratagene, La Jolla, CA, USA). Conditions for all of the PCRs were as follows: 50^{o}C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 30 s, 61°C for 30 s, and 72°C for 30 s.

Lastly, the transcript levels of 4CL1-1, 4CL1-2 and housekeeping genes for each transgenic event were determined from standard curves of the control sample sequentially diluted 5 times. The amounts of 4CL1-1 and 4CL1-2 were then divided by the housekeeping reference amounts to obtain normalized level of expression of *4CL1-1* and *4CL1-2* genes.

Wood chemical constituents

Three trees from each line were randomly chosen for lignin analyses. After being freeze-dried for 48 h, samples were ground in a Waring blender in liquid nitrogen and then ball milled [Fritsch planetary mill, Pulverisette with agate bowls and balls (Gilson Company, Worthington, OH, USA)] for 3 hrs to obtain a homogenous powder. Aliquots of powder (~1 g) were submitted to sequential extraction to generate the CWR (Cell Wall

Residue) used for lignin analyses following Jourdes et al. 2007. Lignin contents were estimated from total monomeric release by both alkaline nitrobenzene oxidation (Rolando et al.1992) and thioacidolysis (Blee et al. 2001).

Extractive and cellulose contents of stem sapwood were determined gravimetrically as follows. From lines forming "brown wood", brown and white wood were kept separate. About 1 g of oven-dried wood was enclosed in heat-sealable polyester filter bags (ANKOM Technology, Macedon, NY) that were also oven dried and masses determined. After each of the following steps, the bags were oven-dried and re-weighed to determine mass losses. A blank bag was included in each batch (i.e. one per transgenic line) so corrections could be made for some slight mass losses associated with bag degradation. Extractives were removed by soxhlet extraction with 75:25 toluene and ethanol (v/v) for 24 h and then again in pure ethanol for another 24 h followed by a hot water bath for 6 h and thorough rinsing with deionized water. To determine α-cellulose contents, extracted cell wall residue was treated with a solution of $NaClO_2$ and CH_3COOH kept at 70ºC and pH<4 and then purified in a NaOH solution and thoroughly rinsed with deionized water. After this treatment only α-cellulose should remain in the bag.

Transgenic poplar performance and physiology

Tree height and basal diameter were measured at planting and at the end of the 2006 and 2007 growing seasons. At the end of the 2007 growing season several field-grown trees from each line and all of the greenhouse trees were harvested to estimate of oven-dried aboveground biomass. Biomass of primary stems, branches and leaves were determined separately for each harvested tree. During late August 2007 all leaves were counted on each of the trees that were later harvested. We excised each 5^{th} leaf from small trees or each 10^{th} leaf on larger trees, scanned leaves for area with a LI-3100C leaf area meter (LI-COR Biosciences, Lincoln, NE), and then estimated leaf area per tree.

Tree mortality was recorded just after leaf-flush in May of 2006 and 2007 and again in November of 2007 just before trees were harvested for biomass. In early September 2006, we first observed branch dieback in distal branches and this continued to progress until leaf fall. In November 2006, the occurrence of dieback was recorded for each tree. A similar level of dieback occurred in September of 2007 and 2008 but no data

were collected (often dieback occurred in the same trees as previous years; S. Voelker, *personal observation*).

Estimation of brown wood

Among transgenic lines, wood color varied in appearance from indistinguishable from controls, to consistently light pink throughout, to appearing "brown" or mottled red-brown. The cross-sectional area in brown wood in stems, as distinct from the light pink color, was estimated near the base of each tree by overlaying a transparent grid of dots over three cross-sections from each tree at three heights (stem base, and approximately 20 and 40 cm from the stem base) and recording the relative frequency of brown wood as compared to the entire cross-sectional wood area. Transgenic lines that had, on average, more than 10% of their stem cross-sectional area colored brown were designated as 'brown wood lines'. The brown wood lines were identified as 150, 350, 671, 712 and 713 while the normal transgenic lines were identified as 17, 90, 115, 204, 209, 210, 224, 225 and 640.

Visualization of wood anatomy and in vivo xylem dye ascent

Xylem dye trace experiments were conducted in the field in September 2008 on branches of control plants and transgenic line 712 (that tended to most regularly form brown wood). At dawn, apparently healthy branches of control and line 712 were excised under water to avoid the introduction of embolisms to the xylem stream. They were transported to the laboratory under water, and then once there, their ends were re- cut and placed in a beaker of 0.2 % acid fuchsin that had previously been filtered. To preserve the localization of dye only within water-conducting vessels, each of the branches was frozen in liquid Nitrogen as soon as the acid fuchsin (red coloration) was observed in the most distal petiole. Cryo-fixation stops dye from diffusing far from functioning vessels (Sano et al. 2005, Umebayashi et al. 2007). Cryo-fixed branch segments were planed in the frozen state at – 10 to – 30° C on a sliding cryo-microtome and observed at -30° C with an epi-fluorescence microscope (Nikon E400, Tokyo, Japan) equipped with a cryo-stage (Kitin et al., *manuscript in preparation*). Images were recorded with a digital CCD camera (Q Imaging, Micropublisher 5.0 RTV). Several samples were instead freeze-dried after the cryo-planing, and then observed at room temperature with the epi-fluorescence microscope (emission filters LP 420 and LP 520) or a confocal microscope (LSM, Carl

Zeiss 510) using a single track, triple channel imaging with 405, 488, and 543 laser lines and emission filters (BP 420-480, BP 505-530, and LP 590).

Cryo-fixed branches were brought slowly from -8 °C to 4 °C, to room temperature. Transverse or longitudinal sections (40-60 μm in thickness) were cut the same day on a sliding microtome. The sections were passed through 30%, 50%, and 95% ethanol series to remove extractives, and stored in 50% ethanol. For fluorescence microscopy staining, a drop of 0.5% calcofluor (Calcofluor white M2R, Sigma Chemical Co., St. Louis, Mo.) was added over each section and left under dark for 30 min. Then the sections were mounted in 50% glycerin on microscope slides and observed with fluorescence or confocal microscope as described above. Calcofluor stain, a fluorescent agent that binds to cellulosic material, made tyloses appears bright whitish green while lignified tissue contrasts as dark red (see Figure S3.1).

Quantification of xylem hydraulic and anatomical traits

In late August 2007 as part of the plant harvests, we measured specific conductivity (k_s, kg m^{-1} $s^{-1}MPa^{-1}$) of wood from the main stem for three trees from each transgenic line and six control trees in the field, and all trees in the greenhouse. Two xylem segments from the base of each tree were measured for wood specific conductivity. A measure of xylem transport efficiency, k_s can be estimated according to Darcy's law as

$$k_s = \frac{Ql}{A\Delta P},$$

where Q is the volume flow rate (m^3s^{-1}), l is the length of the segment (m), A is the cross-sectional area of the segment (m^2), and ΔP is the pressure difference across the segment (MPa). In stems less than 1 cm in diameter, the entire segment was used, cut to a length of 12 cm. From larger stems, two segments were chiseled to dimensions of approximately 1×1×12 cm. Chiseled segments were submerged in pH 2 HCl solution and placed under vacuum overnight to remove embolisms. Segment ends were re-cut under water with a razor blade, and segment dimensions were then measured with digital calipers. Segments chiseled from stems were tightly wrapped in parafilm (Pechiney Plastic Packaging, Inc.) to reduce variability in k_s due to radial leakage.

Next, maximum k_s was measured by perfusing fresh, filtered HCl solution through refilled, embolism-free segments at a pressure head of approximately 0.006 MPa. The solution was near 20 °C for all measurements. It entered one end of the sample through a latex tube, and exited through another latex tube fitted tightly over the segment and that was attached to a pipette used to estimate efflux rate. Volume flow rate, Q, was estimated from the mean time required for the solution to pass at least four successive marks on a 1or 0.1 mL pipette attached to the distal end of the efflux tube. To estimate the sufficiency of water supply by stem xylem to the leaves we estimated $k_{\text{leaf specific}}$. This measure was calculated by multiplying k_s by the stem sapwood area of the tree (m^2) and then dividing by the total leaf area (m^2).

We made transverse sections by hand with a razor blade from each of the k_s samples for anatomical analyses. Images were obtained using a light microscope with 20× or 40× objective lenses leading to a total magnification of 200× and 400×. Digital images photographed with light microscopy were used to estimate vessel lumen areas (later converted to diameters assuming circularity) and vessel frequencies with ImageJ software (http://rsbweb.nih.gov/ij/). A series of six digital images distributed radially across the 2007 growth ring of each k_s sample were used for vessel measurements. To equitably represent the contribution of vessels located along a radial transect, one image was located in the first one third of the ring, two were located in the center one third of the ring and the last three in the outer one third of the ring. With knowledge of average vessel lumen diameters and vessel frequencies we calculated theoretical conductivity ($k_{s\ \text{theoretical}}$) based on the Hagen-Poiseuille equation (Zimmerman 1983).

Xylem vulnerability to embolism for each line was determined using the air injection method (Cochard et al. 1992). A two-ended pressure collar was used to inject N_2 gas at pressures of 0.6, 1.2, 1.8, 2.4 and 3.4 MPa for each segment on which k_s was measured. After each round of pressurization, stem segments were wrapped in a wet paper towel and allowed to equilibrate before k_s was measured again. After each k_s measurement samples were re-cut under water with a fresh razor blade and their length recorded. Xylem vulnerability curves were generated by plotting the percent loss of conductivity from the initial maximum value of k_s as a function of the pressure applied. A total of 780 k_s measurements were made. The pressure causing 50% loss of conductivity

(P_{50}) was calculated using a polynomial regression fit to the pressure data (y-axis) plotted against percent loss in conductivity data (x-axis) on each of six stem samples per transgenic line and twelve samples from the control line. Means for each line were calculated from the predicted P_{50} values from each stem sample.

To estimate moisture contents, trees harvested for biomass measurements were stripped of all leaves early in the morning, cut into sections and quickly placed in plastic bags for transport. At the lab, the fresh mass of large stem sections were weighed, oven dried and re-weighed. On a subsample of lines, we carefully removed the bark of fresh stem segments spanning a range of diameter and both bark and xylem components weighed separately for fresh mass and then oven-dried and weighed separately again. The relationship between whole stem and wood-only moisture contents as a function of diameter was then used to estimate wood moisture contents without removal of the bark from the remaining whole-stem samples. No differences were found in the size-related scaling of wood to whole cross-section moisture content among the control line, a normal wood line and a brown wood line so the data were pooled to provide an overall relationship.

Tree biomechanics

To calculate wood stiffness (MOE, the modulus of elasticity) and wood strength (MOR, the modulus of rupture) we subjected stem sections with bark intact to standard three-point dynamic bending tests. All stem sections >5 mm diameter were from "main stems" that were near vertically aligned, whereas stem sections < 5mm generally came from small side branches. Because the samples varied in size, the larger samples may have included 2 years of growth while smaller samples generally included just one year of growth. For stem samples <10 mm diameter we used a portable mechanical testing device (Instron® In-Spec 2200, equipped with a 45 kg load cell). For stem samples >10 mm diameter we used a larger testing machine (Sintech Model 1/G, MTS Systems Corp., fitted with a Sensotec 230 kg load cell Model 41/571-07, Honeywell International Inc.). We recorded the span tested for each sample, which was 16 to 20 times the sample's estimated mid-point diameter. We calculated taper from the diameter inside bark at each end and total length. Average pith diameter to the 4^{th} power was subtracted from the average inner bark diameter to the 4^{th} power for calculation of the second moment of

inertia of a hollow cylindrical cross-section of wood. Simple moduli for each sample were calculated assuming a non-tapered beam and then a correction for taper was applied following the formulas in Maki and Kuenzi (1965). All samples were tested at 12% wood moisture content.

To compare an index of biomechanical function for trees that differed in both material properties and form we estimated safety factor for buckling as the critical buckling height (H_{crit}, Greenhill 1881) divided by the actual height of an individual tree. This Greenhill equation estimates a critical height (H_{crit}) for elastic buckling of a uniform column. Although this model oversimplifies tree form, it has been shown to approximate well the height at which a tree of a given material and geometry becomes unstable and buckles under its own self loading (Holbrook and Putz 1989). Greenhill's formula is to the following:

$$H_{crit} = 1.26 \times (E/W)^{1/3} \times (D)^{2/3}$$

where

E= apparent modulus of elasticity (Pa),

W = green wood density (i.e., density of wood and water, N/m^3) and

D = basal diameter of the tree (m).

For the data presented here, D was measured just above the basal flare associated with the root collar, 5-10 cm above the soil surface. Green densities were estimated for each line, not each tree.

Statistical analyses

Relationships between traits were characterized with least-squares regression models. With few *a priori* hypotheses regarding the form of the relationships between these traits, we used linear, exponential or sigmoidal equations that best fit the data. To compare many functional traits, we calculated coefficients of determination (r^2) between each of 28 traits and their composite, standardized means for each transgenic line and controls (Table 3.1, 3.2). Values for each trait×line combination were standardized by subtracting the mean value across all lines for that trait and dividing each of these by the standard deviation among lines for the same trait. This resulted in each trait×line value represented by a certain standard deviation away from a mean of zero for each trait (positive or negative). Two traits, fiber cell wall area and α-cellulose content were not

significantly correlated ($P<0.05$) with the composite means among lines and were therefore removed from the composite mean and not included in further analyses. For consistency within these comparisons we only used standard linear regressions ($y = a + bx$) to calculate r^2 values. Analysis of variance was used to compare how variation in selected traits was determined by transgenic line, treatment (i.e. staked versus field) and their interaction (PROC GLM, SAS version 9.2, SAS Institute Inc. Cary, NC, USA).

RESULTS

Tree form, growth, and mortality

Field-grown trees were characterized by a more or less typical tree form as compared to the staked trees that were often mistaken for vines or referred to as "beanstalk trees" by some passersby (Figure 3.1). Unstaked trees had much more stem taper than did staked trees, as seen from the plots of height vs. stem diameter (Figure 3.2). During 2007 field-grown trees grew on average 146 cm in height (max=346), whereas staked trees grew on average 333 cm (max=452 cm). Controls, normal transgenics and brown wood transgenics each showed distinct trajectories of height versus diameter in the field, but not when staked in the greenhouse (Figure 3.2). Brown wood lines tended to have the lowest lignin contents and were so named because on average, about 25-60% of the xylem in their main stems was characterized by the patchy infusion of phenolic substances (Chapter 2). Field-grown trees averaged nearly three times more allocation to branch biomass than staked trees, though this range in phenotypic plasticity difference was constrained in the low-lignin brown wood lines that tended towards a shrubby form (S. Voelker, *personal observation*).

For field-grown trees, there was a sigmoidal relationship between aboveground biomass and leaf area across all lines (Figure 3.3). Though the control line was not the largest of the lines tested, it had the greatest growth efficiency, or biomass per leaf area (Figure 3.3). Growth efficiency was slightly lower than the control for the normal transgenics, and was uniformly low for the low-lignin brown wood lines. Lower total biomass and growth efficiency in brown wood lines was associated with an increased frequency of shoot dieback each late summer. These brown wood lines also had higher mortality rates than did five of the seven transgenic lines that had normal-colored wood (Figure 3.4, Table 3.1).

Plant hydraulics and brown wood

A line characterized by regular brown wood occurrence (line 712) was selected for visualizing the pathways of water movement *in vivo.* We had previously determined that acid fuchsin traveled in fewer vessels and more slowly in brown wood of Line 712 as compared to controls (Chapter 2). Here we show that acid fuchsin dye taken up by excised branches of Line 712 was found in earlywood vessels and some malformed vessels halfway across the growth ring, but none was observed in the "collapsed" latewood vessels (Figure 3.5). It was not clear why dye was transported through some malformed vessels and not others in the brown wood. To determine if vessels were obstructed with some substance, we took photos through a fluorescence microscope of a different branch from line 712 and a branch from a control tree that had undergone the same acid fuchsin uptake. Again, nearly all vessels of the control branchcontained dye (red), but only the earlywood vessels of the brown wood contained the dye in line 712 (Figure 3.6). Additionally in line 712, confocal microscopy revealed an unknown substance that filled many of the vessels in the middle region of the growth ring adjacent to bands of collapsed vessels nearer to the cambium (Fig. 3.6, blue color). In normal wood, cell walls of vessels, fibers and parenchyma fluoresced more or less green (i.e, a mix of cell wall polysaccharides=yellow and phenolics such as lignin=blue). In contrast, vessel and fiber cell walls in brown wood xylem (that were collapsed in tangential bands) tended to fluoresce yellow-brown, especially near the cambium. In addition to the extractives seen in brown wood we also regularly observed tyloses throughout the length of most vessels (Figure S3.1).

Across transgenic lines there were significant relationships between lignin content and functional traits related to xylem hydraulics (Figure 3.7). Both k_s and theoretical k_s, (the latter being based entirely on anatomical measurements, see *Methods*) declined with decreasing lignin content. The relationship between lignin content and k_s was driven by the brown wood lines: below a threshold lignin content, k_s was severely reduced. Moisture content had a strong inverse relationship with lignin content, with all values equaling or exceeding that of the controls. The overall relationship of P_{50} (the negative hydrostatic pressure causing a 50% loss in k_s) to lignin content was also significant, but

was the only relationship whose fit was improved by removing data from the brown wood lines.

Theoretical k_s was strongly correlated with measured k_s among field-grown and staked trees (Figure 3.8, top). However, this relationship did not necessarily indicate that changes in xylem anatomy were responsible for lower k_s associated with brown wood occurrence. The percentage of theoretical k_s (i.e. observed k_s / theoretical k_s), yields a clearer picture of realized conductive efficiency in relation to the theoretical maximum (Figure 3.8, bottom). The average value of percentage of theoretical k_s for diverse angiosperms is 44% (Hacke et al. 2006); all samples in the current study were lower than this average, but the brown wood lines were dramatically lower with values approaching 0%.

To better determine the effect of brown wood on conductive efficiency, we plotted observed k_s / theoretical k_s against the relative amount of brown wood in the samples on which k_s was measured for each line (Figure 3.9). Brown wood was associated with drastic reductions in conductive efficiency among both field-grown and staked poplars.

The leaf area/sapwood area (LA:SA) of staked trees was more than double that of the field-grown trees, both when sapwood areas included brown wood ('unadjusted', top panel, Fig. 3.10) or when the brown wood area had been subtracted out from the sapwood area ('adjusted', lower panel, Fig. 3.10). When brown wood was excluded from the sapwood area, the brown wood lines tended to have greater LA:SA ratios for both field-grown and staked trees.

Functional phenotyping across many traits

For each line, mean values across 28 traits were standardized relative to the control to clarify the relative roles of lignin levels versus brown wood occurrence in governing the variation observed among lines (Table 3.1, 3.2). These calculations showed a broad range of standardized values across the normal colored transgenic lines (0.68 to 0.19), but the controls (1.00) and the brown wood transgenics (-0.62 to-1.54) differed substantially (Table 3.1). When ranked according to coefficient of determination (Table 3.2), the order of the traits can be interpreted as reflecting how consistently the traits are affected by altered lignification across the lines. Several traits, such as biomass:leaf area,

lignin content, tensionwood, height to diameter ratio (H:D ratio) and MOE tended to be related to overall means even when broken out among controls, normal transgenics and brown wood transgenics (Table 3.2). If lignin content analyses had been measured on more than three trees per line and integrated more across developmental stages, as were the rest of the traits, it undoubtedly would have moved higher in the overall ranking.

Comparisons of field-grown and staked poplars

Most traits measured both in the field and in the greenhouse showed significant variation overall and among lines (Table S3.1). Of more interest is where treatment effects or treatment×line interactions did and did not occur. Both total aboveground biomass and leaf area of field-grown trees tended to be slightly greater on average than staked trees (*data not shown*) such that growth efficiency, or biomass produced per unit leaf area was not significantly different between staked and field-grown trees (Table 3.2). We do not report values for xylem vulnerability to embolism or moisture contents for staked trees (see Table S3.1) because an inadvertent shutdown of the greenhouse irrigation system at the end of the growing season caused severe drought stress (leaves wilted) for some trees.

The largest [treatment] effects of staking for form were found in H:D ratio, LA:SA and branch biomass (Table 3.3, S3.1). Despite LA:SA being doubled in staked trees (Figure 3.10, Table S3.1), the specific leaf area, or leaf area / leaf mass of staked trees was significantly greater than field-grown trees (15% ±SE=5%, *data not shown*), suggesting some compensation in photosynthetic potential. There was also a large increase in k_s of staked trees that was only partially offset by their increased LA:SA, as indicated by the moderate but still significant increase of $k_{leaf\ specific}$ of staked trees (Table 3.3, S3.1). The increased theoretical k_s (Table 3.3, S3.1) and the large increases in k_s in staked trees were both due to increased vessel lumen diameters, but little change in vessel frequencies (*data not shown*).

Somewhat surprisingly, neither tensionwood nor MOE decreased in staked trees or showed significant treatment×line interactions (Table 3.3). MOR, however, was significantly lower in staked trees. Buckling safety factors, which take both stem taper and MOE into account (see *Discussion*) were about two to four times higher on average

in field-grown than staked trees (Figure 3.11, Table 3.3, S3.1). MOR showed a weak treatment×line interaction, whereas safety factors did not (Table 3.3).

DISCUSSION

Functional phenotyping across many traits

We documented a large range of phenotypic variation related to levels of 4CL downregulation, which in turn caused lowered lignin and related pleiotropic effects in the most severely affected lines. For most traits, much of the variation was driven by these pleiotropic effects, and not by the decreased lignin content itself. Our use of 14 transgenic lines allowed us to show that at lignin reductions of more than 15% of control values pleiotropic effects became increasingly apparent. Therefore this suggests that labeling 4CL down-regulated lines as "mis-regulated" is not warranted. Rather, there is a tipping point that corresponded to 15% lignin reductions, after which carbon was increasingly shunted toward secondary metabolites (Chapter 2) that in turn caused a positive feedback loop involving negative physiological and anatomical consequences associated with brown wood formation.

The relative importance of controls versus normal transgenics versus brown wood transgenics in determining the overall phenotypic variation in can be summarized by comparison of each trait to the overall score among a large number of traits (Table 3.1, Table 3.2). With respect to tree function, growth efficiency (biomass produced per leaf area) ranked as the trait most greatly affected overall and ranked high among each sub-set of data. This finding contrasts sharply with the idea that lowering lignin will enhance growth of poplars (Hu et al. 1999). Even if several of the most affected lines were labeled as 'mis-regulated', all of the transgenic lines, even the ones with normal appearing wood, had lower growth efficiency than the control line (Figure 3.2). This result is important, but perhaps should not be surprising considering that dwarfism often results from altered lignification (Anterola and Lewis 2002; Wagner et al. 2008) and that plants often bolster their cell walls with non-structural phenolics when developing cells sense that the surrounding wood is structurally insufficient (Dauwe et al. 2007). Traits ranked 2nd, 6th and 7th (height to diameter ratio, MOE and tensionwood) are all related in that lower wood stiffness may cause developing xylem cells to sense increased mechanical strains and thus increase cell divisions basipetally and form more reaction wood (Telewski

2006). Traits ranked 3rd-5th, brown wood, total nitrobenzene oxidation monomer release and shoot dieback, indicate that lowered total lignin contents were associated with dieback. Traits 8th-10th help explain how this dieback likely occurred - in that severe reductions to k_s, and theoretical k_s by deposition of phenolic compounds and tyloses formation would have limited water supply to the leaves. Despite the fact that total thioacidolysis and nitrobenzene oxidation were not ranked highest overall, both of these traits were in the top ten and correlated strongly with each of the sub-groups shown, suggesting most trait variation was driven by the non-linear effects of 4CL downregulation on lignin content.

Plant hydraulics and brown wood

Dye trace experiments combined with cryo- and fluorescence microscopy together demonstrated that brown wood impacted the hydraulic function of the trees with the lowest lignin levels through blockages in vessels and if not "collapsed", then crumpled conducting cell walls (Figures 3.5, 3.6). The pattern of lighter yellow fluorescence of cell walls near the cambium is consistent with a delayed program of lignin deposition. In an apparent sensing and response to altered lignification and presumably weaker cell walls, the blue-green colored fluorescence of cell contents indicates soluble phenolics were infused into fiber and vessel lumens (Figure 3.6). The buildup of non-lignin phenolics in cell walls led Dauwe et al. (2007) to hypothesize that a similar scenario had occurred when other phenylpropanoid steps were downregulated. Ray cells in brown wood of line 712 also appeared bright blue, indicating they too were filled with phenolic substances. This pattern suggests phenolic substances in fiber and vessel lumens were directed to cells after they had left the cambial zone, as is observed during the infusion of flavonoids, lignans and other phenolics during the normal deposition of heartwood extractives (Taylor et al. 2002, also see Chapter 2).

Across all lines there were significant relationships between lignin content and traits known to determine xylem safety, efficiency and water storage (Figure 3.7). The lower k_s lower in brown wood than normal wood could be explained potentially by the increased incidence of blockages. In contrast, theoretical k_s is not affected by phenolic deposits, tyloses or other occlusions, so the lower theoretical k_s of brown wood than normal wood indicates some inherent reductions in conductivity in low-lignin lines.

These may have been due in part to size-related trends, in which smaller stems tend to have lower specific conductivity (Tyree et al. 1991).

Water storage is an important component of diurnal water supply for trees (Meinzer et al. 2003, 2006) and as measured by wood moisture content, varies inversely with the amounts of cell wall material as well as the amount of gas present (in embolized cells, intercellular air spaces and dissolved in liquid). For the poplar stems in the present study, given the range of wood densities and moisture contents measured, and assuming a constant cell wall material density, water occupied 39-57% of the wood volume, solids 24-29% and gas 19-34%. Because wood density did not differ appreciably among lines (Chapter 2), the negative relationship between stem moisture content and lignin content must have been caused by more water being stored in cell lumens or the basic density of the woody cell wall material being less in low lignin lines. Either reduced lignification or increasing tensionwood could decrease the average basic cell wall density although this has never been reported. Lignin, being only 12-22% of the woody cell wall material in these poplars, could only make up about 2.5-5.0% of the stem volume and be responsible for less than 4% out of the 25% variation in moisture content among lines. The proportion of wood volume in tensionwood fibers is difficult to establish because the G-layers deform when mounted for light microscopy. Nonetheless, reasonable estimates of proportion of wood volume occupied by tensionwood and associated decreases in basic cell wall density can explain less than 3% of the 25% variation in moisture content. Therefore, 6% or less of the variation in moisture content was explainable due structural changes to cell walls while the remaining ~20% would need to result from increased water stored in cell lumens of low lignin and brown wood forming lines. We attribute much of this remaining trend in water storage to the effective isolation of water in lumens of cells surrounded by brown wood and extractives that have very low conductivity.

This study is the first to provide substantial evidence that the degree of lignification in wood can affect the resistance to embolism in vessels. Evidence for this is seen in the trend of P_{50} values across the control and normal transgenic lines as compared to lignin contents (Figure 3.7). The resistance of xylem to embolism is often discussed in terms of P_{50}, which is the negative xylem pressure causing a 50% loss in k_s (Pammenter and Vander Willigen 1998; Wheeler et al. 2005; Sperry et al. 2006; Jacobsen et al. 2007).

Indeed, a previous study determined low-lignin transgenic poplar wood to have less negative P_{50} values than wood from the control line (Coleman et al. 2008). In the study by Coleman et al. (2008), only the most affected transgenic line was compared to the wild type so that it was unclear to what extent this result owed to low-lignin of the wood as a whole, a parallel reduction in guaiacyl lignin that tends to be localized in vessels (Saka and Goring 1985; Nakashima et al. 2008), or the irregular "collapsed" ultrastructure of the vessels formed in that transgenic line. P_{50} may not be a representative measure of embolism resistance across all lines because the patchy nature of brown wood and its effects on water flow through stems (Figures 3.5, 3.6) likely caused a different population of vessels to be tested than those transgenic lines that did not form brown wood. In addition, initial k_s values after embolism removal were so reduced in some brown wood samples that even the smallest of measurement errors could have resulted in great variability in the percent loss in conductivity and P_{50} values calculated later. As such, the regression relating P_{50} to lignin across all lines was not significant.

There are two plausible mechanisms by which reduced lignin could cause wood to be more vulnerable to air-seeding of embolisms. In the first scenario, altered lignification may result in larger or more abundant voids in the cell wall. Micro-fractures might then be more readily propagated under the hoop stresses that bend vessel walls inward when the xylem stream is under tension (or when pressurized, as conducted for our treatments, see *Methods*). Thus, where cell walls are compromised air could more readily seed through the wall, resulting in embolism of water columns under *in vivo* hydrostatic tensions. A second scenario envisages the edges of pit membranes, made of randomly oriented cellulose microfibrils, being stretched and or ruptured so as to cause increased porosity. This might occur where microfibrils are not adequately adhered to lignin within the middle lamellae next to the pit chamber or if the entire wall and pit chamber complex were bent inward towards the lesser of the pressures between two adjacent gas-filled versus water-filled vessels.

The calculation of theoretical k_s uses anatomical measurements to determine the maximum potential conductivity of a collection of vessels assuming they are perfectly round capillaries (Zimmerman 1983). In so doing, the resistance due to inter-vessel pit

fields and vessel perforation plates is not accounted for and the measured k_s of the same wood should always be lower. Although theoretical k_s is not very realistic in absolute value, when compared to measured k_s values of the same wood, an unbiased measure of constraints on water transport efficiency can be obtained. This strategy has been used to show that an average efficiency of only 44% was achieved across a diverse set of hardwood species (Hacke et al. 2006). We found measured k_s to be strongly related to theoretical k_s for trees growing in both the field and greenhouse environments, suggesting that the variation in hydraulic functioning of these trees was largely determined by similar mechanisms (Figure 3.8, top). More telling of what drove most of the variation in xylem conductivity was the tendency of brown wood lines to have ratios of measured k_s: theoretical k_s that approached 0% (Figure 3.8, bottom; Figure 3.9). The overall hydraulic efficiency at a given percentage of brown wood was greater in staked trees than unstaked trees (Figure 3.9) as a result of larger average vessel diameters but no change in vessel frequencies (*data not shown*). These results demonstrate that in both field or staked growing environments conduit sizes and frequencies in brown wood lines were adequate to have supplied water to the leaves but vessels contained in sections of brown wood were rendered almost completely non-functional.

The above results in combination with knowledge of sapwood area and total leaf area can be used to draw inferences about whole plant water status. Since we demonstrated that brown wood severely restricted k_s, we also calculated leaf area to sapwood area ratios that excluded brown wood from the sapwood area (Figure 10). This more realistic estimate of functional sapwood area in relation to transpirational demand tended to be greater in brown wood lines by an average of about 20% in the field and 55% for staked trees. Without compensation by stomatal closure, the tension gradients in brown wood lines would have been increased in direct proportion to the increase in leaf area to sapwood area (i.e. 20-55% greater). In combination with decreased resistance to embolism and increased tension gradients, the pattern of greatly increased shoot dieback in the brown wood lines (Figure 3.4) can be considered one of the few documented cases of catastrophic, or runaway xylem embolism (*sensu* Sperry and Tyree 1988). Because of their poor growth, transgenic lines with brown wood would not be considered for large scale production of biomass. Nevertheless, these trees with the greatest alterations to the

program of lignification were well watered and still underwent dieback. This dieback, coupled with our findings of increased susceptibility to embolism suggests there is a need for in-depth characterization of drought tolerance if low-lignin transgenic plants are to be deployed for large scale production without irrigation.

Comparisons of field-grown and staked poplar biomechanics

The poplars investigated here used two distinct mechanisms to compensate for reductions in stiffness and strength of wood with lower lignin contents. Because stiffness was less in trees with reduced lignin, increased bending stresses would have been perceived most greatly near the cambium at the base of the tree such that this signal is likely to have locally up-regulated cell division to increase stem taper as well as form more tensionwood fibers (Morgan and Cannell 1994; Telewski et al. 2006). To include changes in both stiffness and taper we estimated the structural stability by estimating H_{Crit} of field and staked trees of each line using the Greenhill (1881) model (see *Methods*). H_{Crit} is the height at which an untapered column of wood of a known stiffness will buckle if bent from vertical due to elastic instability (for example most climbing vines separated from their support would bend over to lie on the ground). Evolution has generally resulted in trees being over-engineered for their own self-support by a certain factor to avoid buckling under mechanical loading by wind, snow and ice. Niklas (1994) found that trees can be very plastic in their form, but on average the buckling safety factor was about 4.66 (H_{Crit} / tree height =4.66). There is a propensity, however for safety factors of sub-canopy saplings or very dense stands to be reduced (King 1986, King et al. 2008) due to both reduced light and wind-induced sway (Jacobs 1954; Holbrook and Putz 1989). Safety factors of the field-grown poplars compensated for their reduced stiffness by increasing their taper so that controls and the normal transgenics were centered just below Niklas' (1994) average value of 4.66 (Figure 3.11). Brown wood lines increased their taper but were on the low end of the safety factors observed. In contrast, staked trees approached the H_{Crit} threshold, all having safety factors lesser than those documented for trees in natural growth environments (King 1986; Niklas 1994).

The Greenhill's equation for H_{crit} (see *Methods*) predicts that the mechanical stability of trees is more sensitive to stem diameter than to the material properties. Because variation in stem taper strongly influences tree biomechanics, much of the

resistance to mechanical loading is conferred by a trees height to diameter ratio (H:D). In staking these fast-growing poplars during an entire season of growth it was hypothesized that by a combination of increased H:D ratio and reduced lignin, we could produce tree phenotypes that approached or even attained greater heights than H_{Crit} by reducing wind-induced sway and the resulting stresses transmitted to secondary cambial cells. We also hypothesized that staked trees would form less tensionwood and afford us the opportunity to determine the role of lignin in wood mechanics as separate from the confounding influence of tensionwood. With respect to the latter hypothesis, tensionwood of staked trees was not significantly different than field-grown trees (Table 3.3). This was a curious result because MOR was significantly lower in staked trees (Table 3.3). With respect to the first hypothesis, the staked trees all approached H_{Crit}, but not a single line exceeded H_{Crit}, suggesting low-lignin trees may have somehow sensed an upper limit to the H:D ratio (Figure 3.11). Holbrook and Putz (1989) were able to grow trees that exceeded H_{Crit}, but only by staking combined with shading out 90% of light to lateral branches along the length of the trees. For our field-grown trees versus staked poplars, H:D ratios ranged from 38-94 and 95-199 respectively (Table S3.1). How though, does this range in phenotypic plasticity compare to other woody growth forms, and to "normal" poplar trees, and what might these results mean for survival?

One instructive example of great phenotypic plasticity in a woody plant has been observed in western poison oak (*Toxicodendron diversilobum* T. &G Greene) that can grow as a vine when supported or as a shrub when unsupported. Average H:D ratios of two-year old poison oak were 43.5 for unsupported shrubs and 91.4 for supported vines (Gartner 1991). Interestingly, these H:D ratios correspond to field-grown and staked transgenic poplar lines with the lowest lignin concentrations (Table S3.1). Moreover, the trends of wood stiffness with stem diameter for unsupported, shrub-form poison oak (Gartner 1991) were similar to the values measured for brown wood lines (Chapter 2). For naturally regenerated cottonwoods across a range of growing environments, H:D ratios were found to peak near 100 when trees were 10-30cm in diameter Colbert et al. (2002), while in naturally regenerated aspen stands trees peaked in H:D above 130 at diameters of 5-10 cm (King 1981). Intensive cultivation of poplars (1×1m spacing) has even resulted in plantings with H:D ratios of 150-170 (Debell et al. 1997). Mechanical

testing of whole trees demonstrated that birches (*Betula* spp.) characterized by a H:D ratio of 120 tended to break at half the critical wind speed of those with a H:D ratio of 80 (Peltola et al. 1999). Extrapolation of these same data suggests that a H:D ratio of 160 would cause a windspeed of <1 m/s to cause stem breakage without the shelter of an accompanying canopy to deflect and diffuse wind gusts (Peltola et al. 1999). Because trees modify their cell types and cambial division rates to shift both material properties and form according to stresses sensed, this scenario is surely unrealistic. Nonetheless, these data and the above discussion demonstrate the strong selective pressure towards maximizing H:D ratios, or "risking" a minimal safety factor in crowded stands where bending stresses from wind are lesser and competition for light is greater.

CONCLUSION

After more than a decade of research, evidence for what hydraulic and biomechanical limitations may exist for transgenic poplars with low lignin had scarcely been quantified. Our results indicate that reductions in lignin content of (woody) crops, even if produced without pleiotropic effects, will have less resistance to embolism during drought stress. Furthermore, our data strongly suggest that the stand densities necessary for the profitable production of woody biomass will tend to enforce reductions in stem taper during development that will result in greater risk of wind and ice damage in trees with reduced wood stiffness owing to lowered lignin. To what extent woody crops grown for biomass in plantations with 7-40 year rotations can be expected to survive wind and ice storms with lignin contents reduced to 10-15% by mass is an open question warranting further investigation. Therefore more comprehensive considerations of "optimal" lignin concentrations for woody biomass production are still necessary.

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FIGURES

Figure 3.1. Contrasts in tree form between field-grown unstaked poplars and greenhouse-grown staked poplars. The photograph in the field (transgenic line 225) was taken in late August 2008 while that in the greenhouse was taken in early August 2007.

Figure 3.2. Height vs. diameter of unstaked field-grown and stakedgreenhouse-grown hybrid poplar trees. Each line respresents one individual tree, with the lower point measured at the end of 2006 after one year of growth in the field, and the upper point measured at the end of 2007. Staked trees were moved back into the greenhouse in winter of 2006-07. Field grown trees are open symbols; staked trees are filled symbols. Triangles=control, circles=normal transgenic lines and square=brown wood transgenic lines.

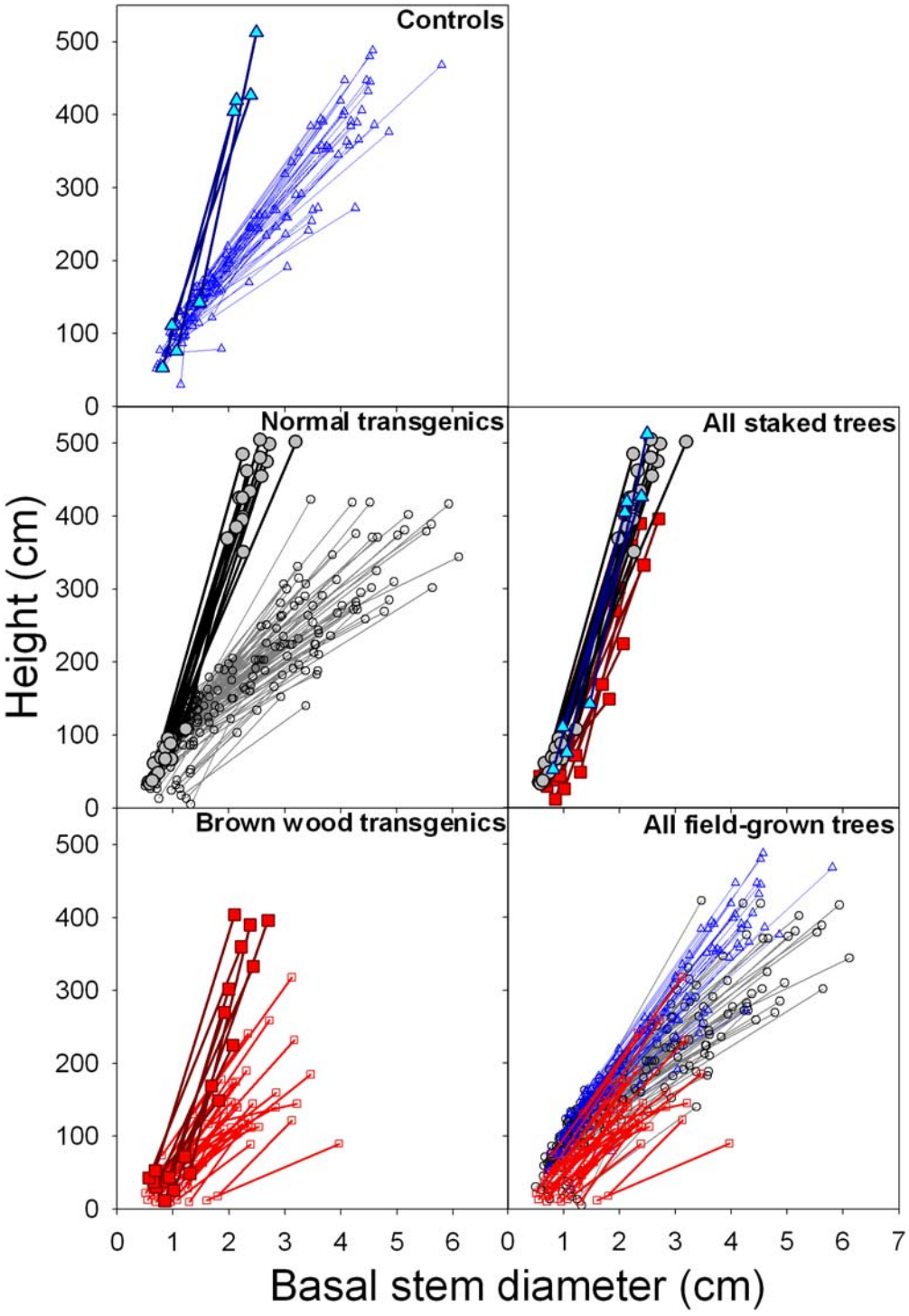


Figure 3.3. Variation in biomass as related to leaf area and biomass:leaf area as related to lignin content. Each point is the mean from one line from field-grown trees only. Biomass = oven-dry aboveground biomass.

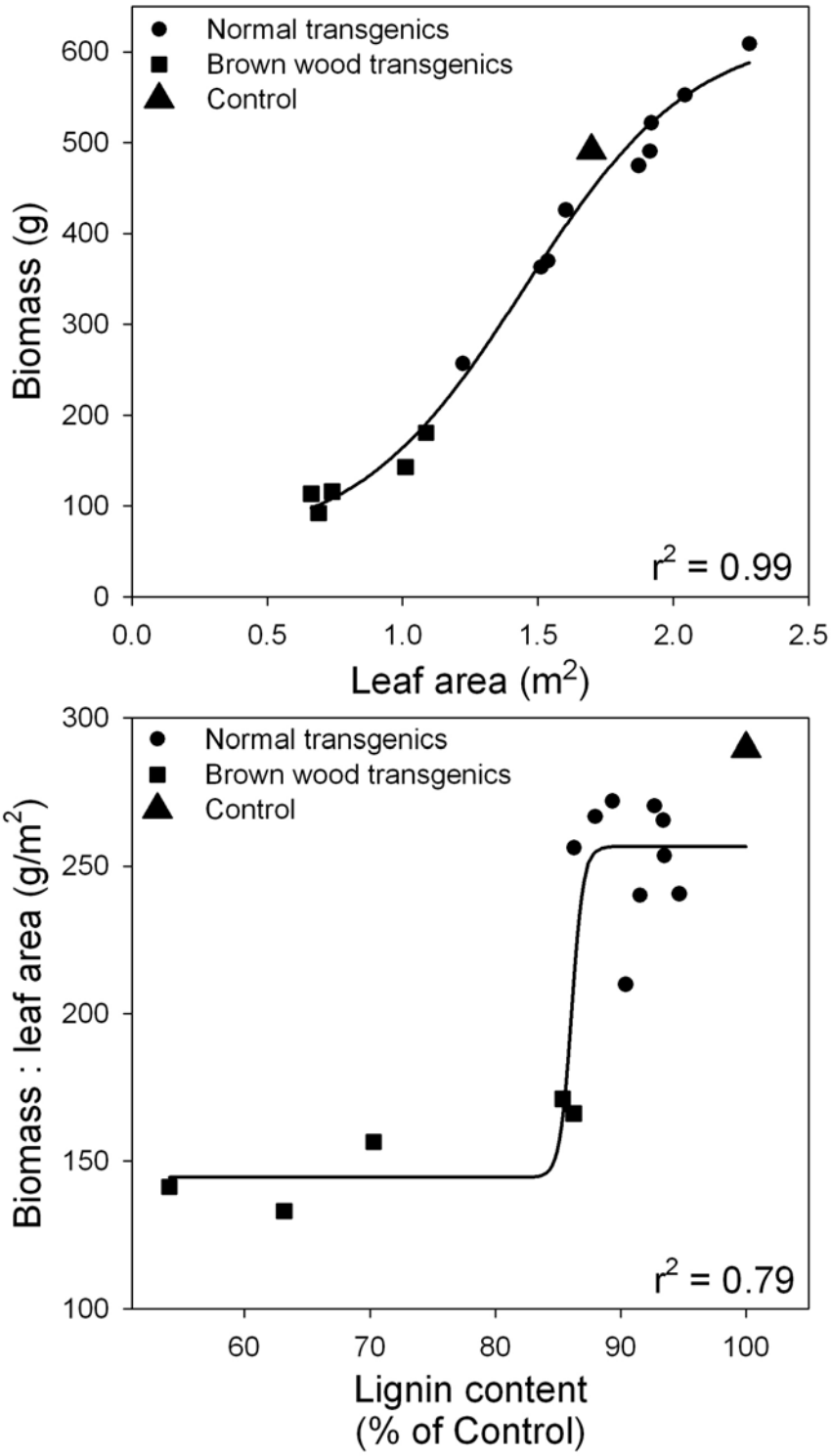


Figure 3.4. Patterns of shoot dieback frequency and cumulative mortality (±SE) among control, normal transgenic and brown wood transgenic lines. Shoot dieback was tallied in 2006 and cumulative mortality is from the entire period after planting in the field (Nov. 2005-Nov. 2007).

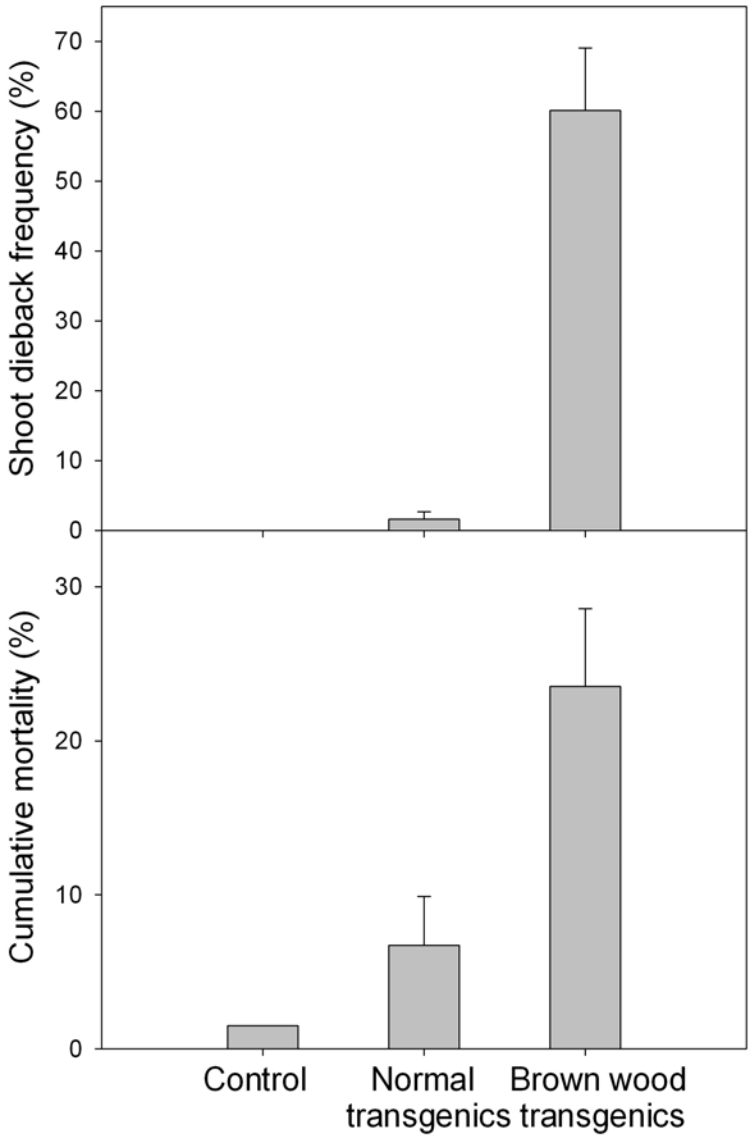


Figure 3.5. Example of dye flow and patchy formation of collapsed vasculature in line 712. Cryo-fluorescence of a well hydrated branch of tree 712-2. Dye (red color) was only observed in early wood vessels. White arrows indicate collapsed vessels, which increased in frequency towards the cambium (i.e. later in the growing season). Inset is from multiple stitched, focused images of the cambial region.

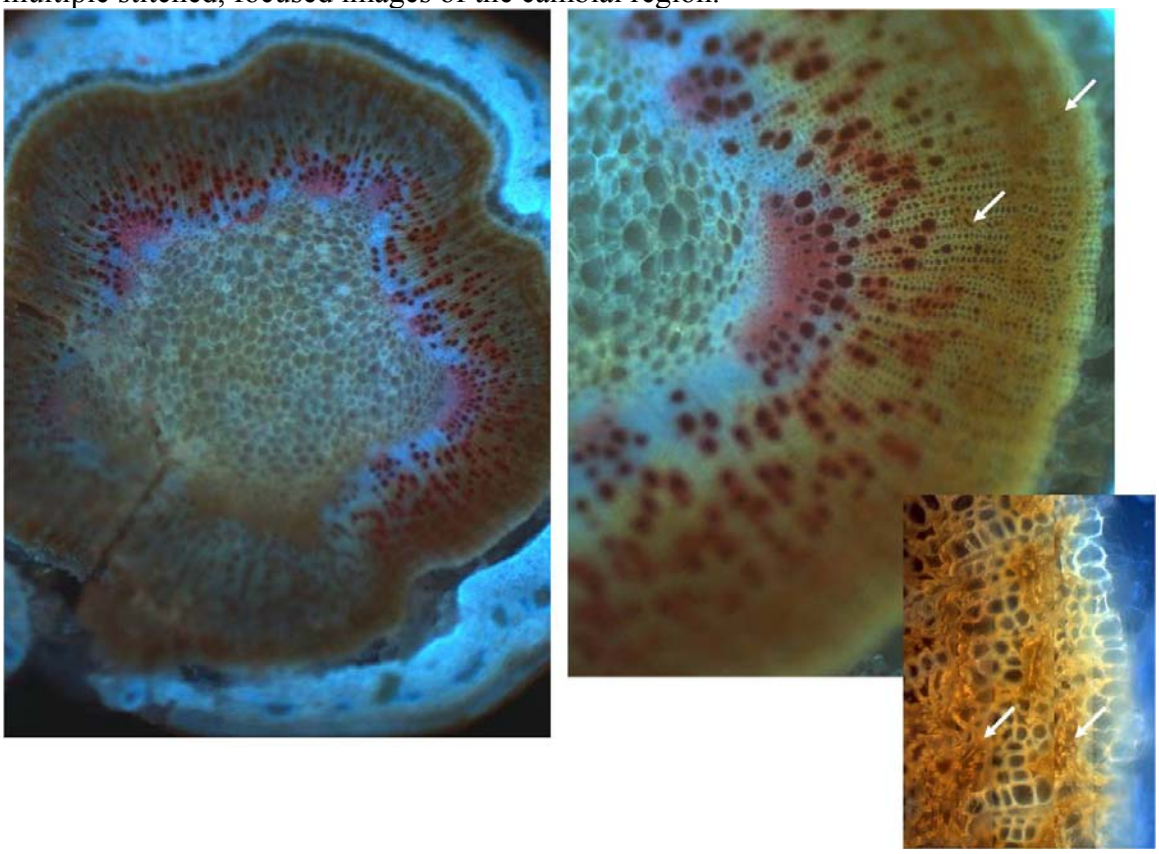


Figure 3.6. Confocal microscopy of a dye trace experiment on a control branch (left) and a branch characterized as “brown wood” from line 712-2 (right). The image at left shows dye (red color) occurring in most vessels of the control, whereas vessels outside the earlywood in line 712 were occluded with a blue fluorescent material, likely a phenolic secondary metabolite. A similar substance is also apparent in the parenchyma of line 712.

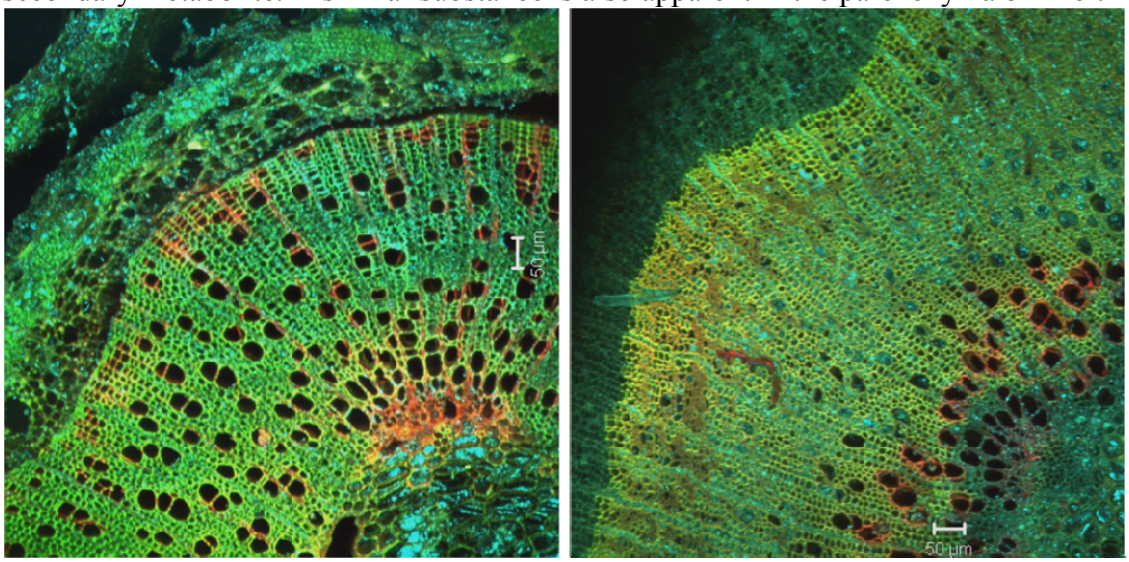


Figure 3.7. Hydraulics-related traits plotted against lignin content. Triangles=control, circles=normal transgenic lines and square=brown wood transgenic lines.

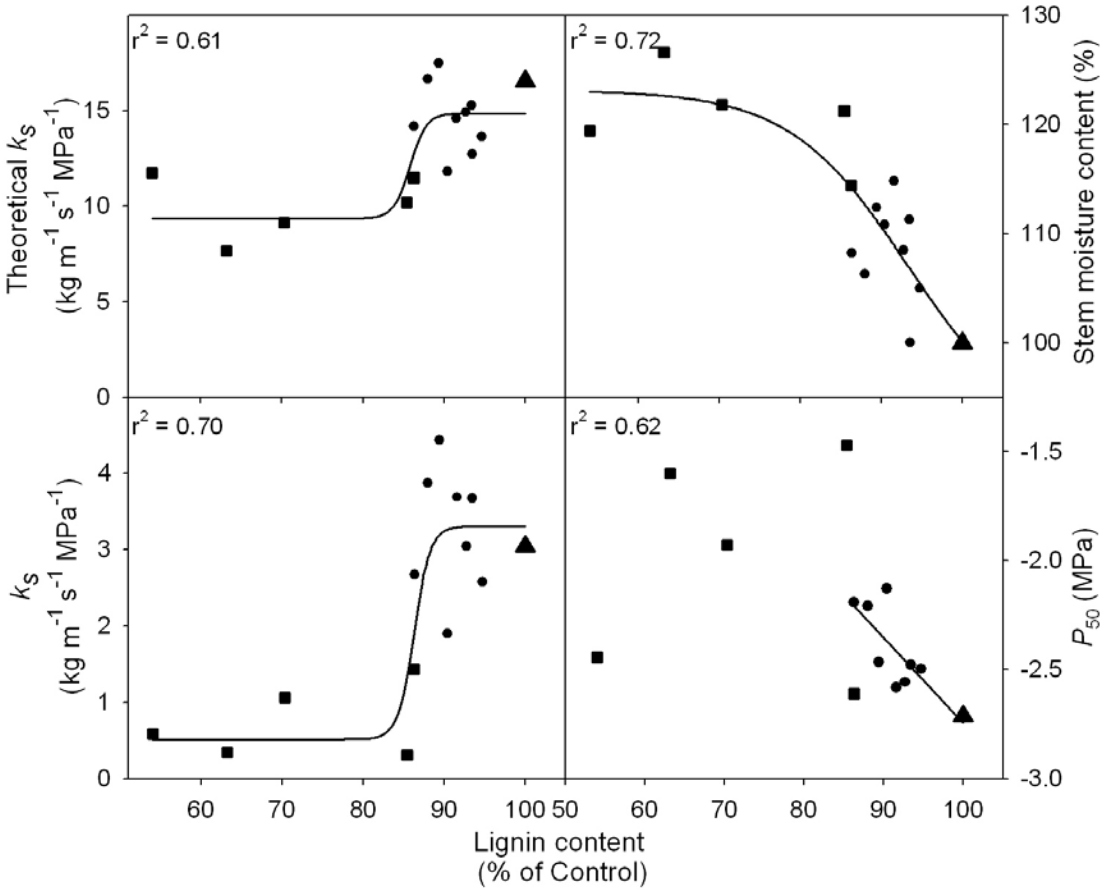


Figure 3.8. Relationship of measured k_s to theoretical k_s and to percentage of theoretical k_s. Percentage of theoretical k_s is a measure of conductive efficiency. Mean ± SE of conductive efficiency of angiosperms is shown as a dotted line and shaded region (from Hacke et al. 2006). Triangles=control, circles=normal transgenic lines and square=brown wood transgenic lines.

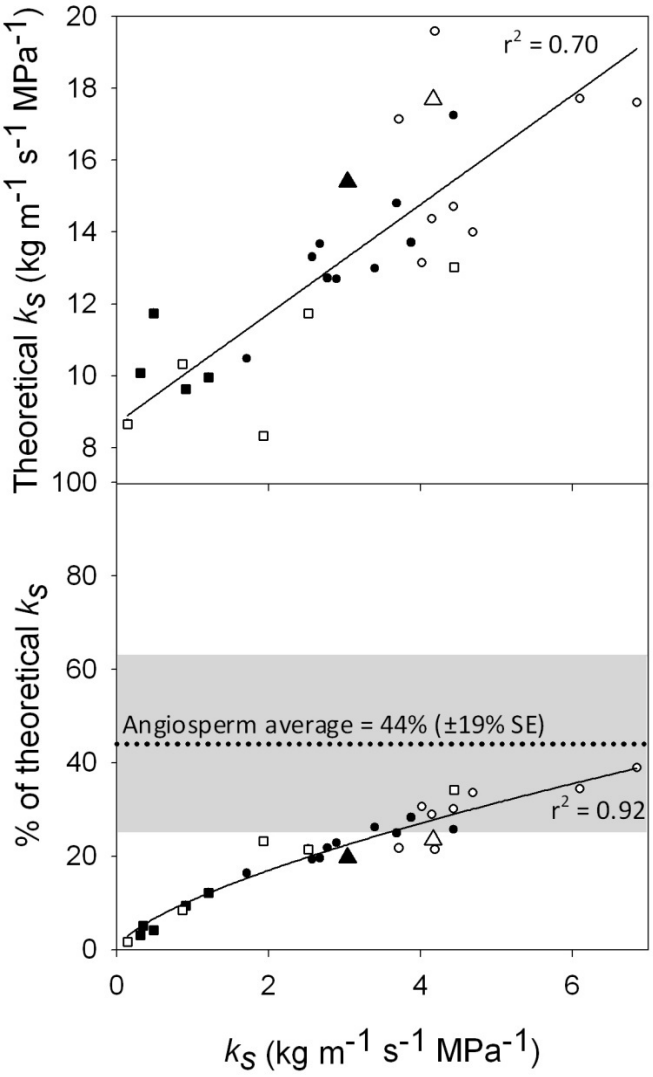


Figure 3.9. Influence of brown wood formation on conductive efficiency of field-grown staked poplars. Brown wood formation in each point is the mean value within those stem segments sampled for k_s measurement in each line**.** Field grown trees are open shapes while staked trees are filled shapes. Triangles=control, circles=normal transgenic lines and square=brown wood transgenic lines.

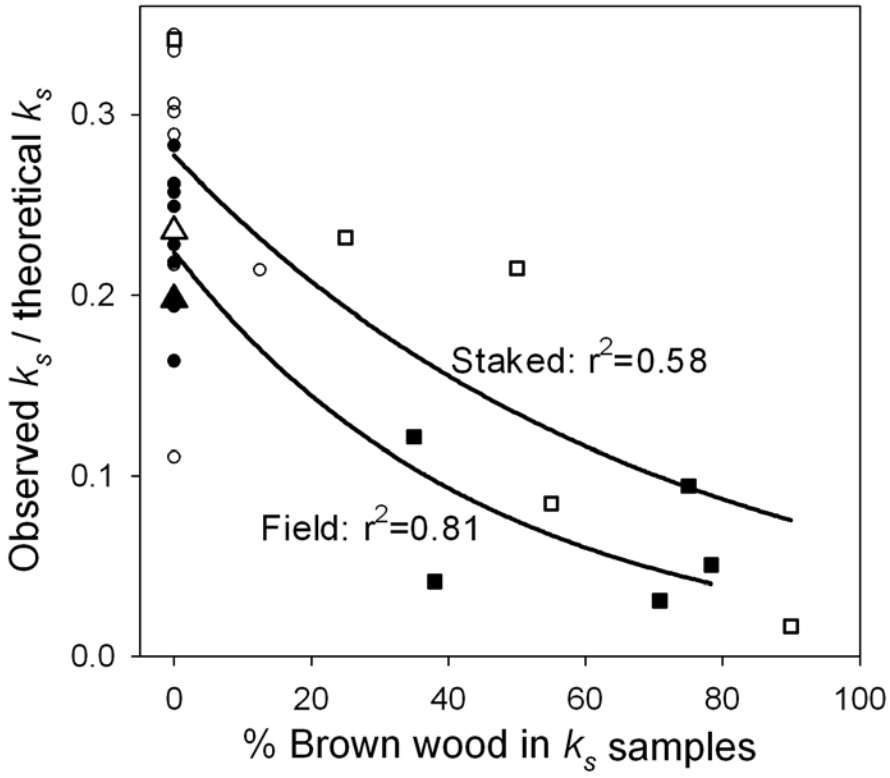


Figure 3.10. Leaf area and sapwood area ratios for field-grown and staked poplars. Because brown wood was essentially non-conductive the lower panel shows LA:SA adjusted by average % brown wood that occurred in main stems of each line. Inset bar graphs show overall means and one standard error across lines. Controls = triangle, normal transgenics = circles and brown wood lines = squares.

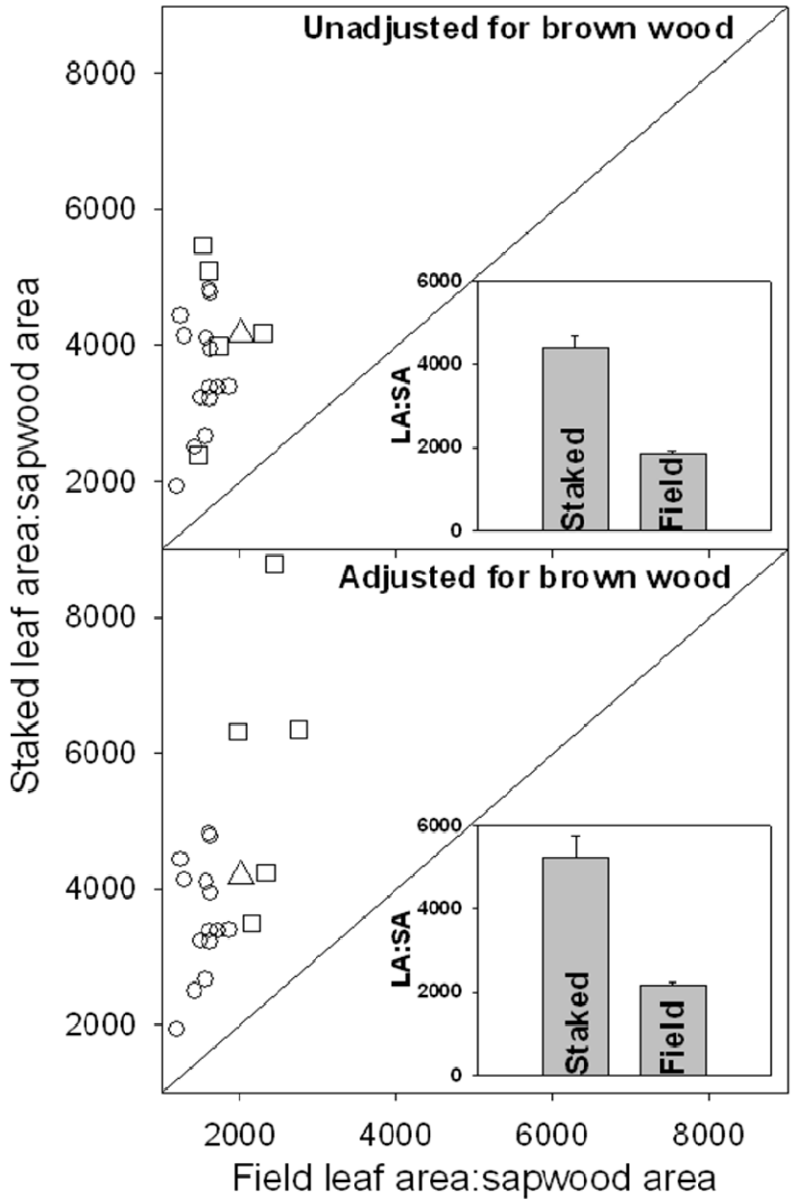
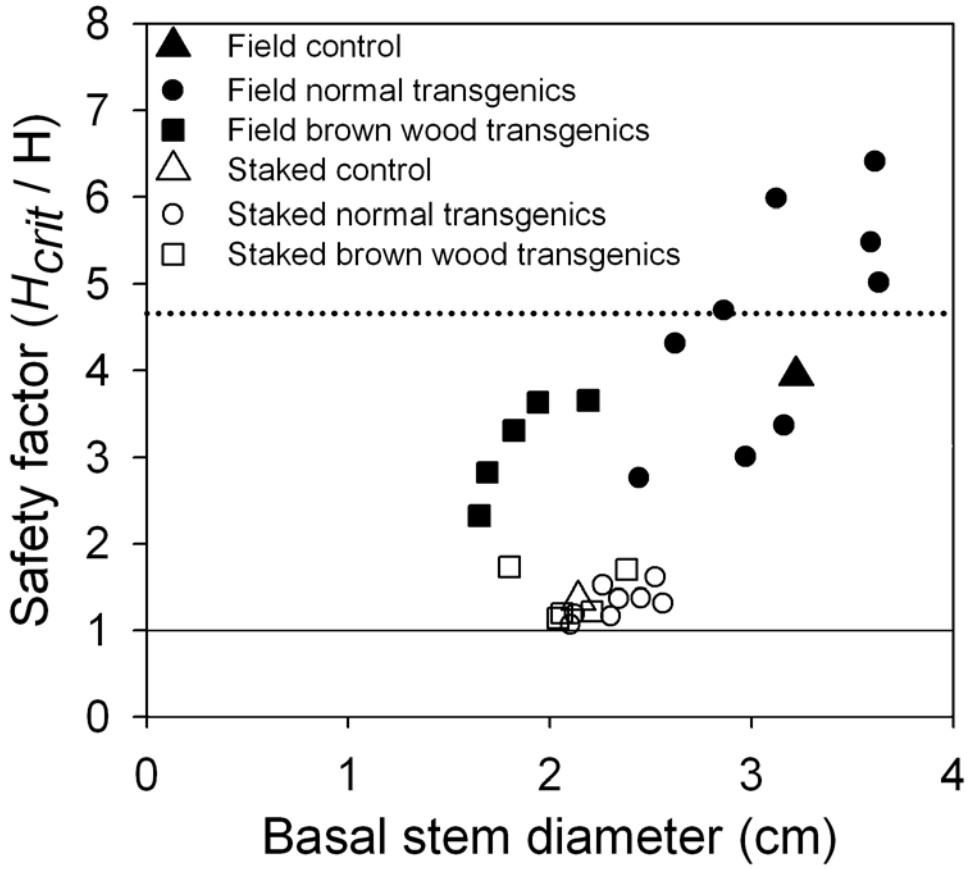


Figure 3.11. A comparison of wood mechanical properties between field-grown and staked trees. Each point is the mean from one transgenic line. The dotted line indicates the average safety factor of 4.66 (range from 1.5 to 8.5) reported by Niklas (1994) across tree species.



TABLES

Table 3.1. Comparison of lines with normal-colored and brown-colored wood for standardized means across traits (see methods), mortality and dieback. Traits used to calculate means within lines (±1 SD) are listed in Table 3.2. Cumulative mortality (2005-2007) and leader dieback (2006 only) included all trees (field-grown and staked trees).

	Line	Average score across 28 traits		Cumulative mortality (%)	Leader dieback (%)
Normal wood lines	Control	1.00	(±0.72)	1.5	0.0
	210	0.68	(±0.45)	0.0	0.0
	17	0.59	(±0.47)	18.2	0.0
	90	0.58	(±0.59)	8.3	0.0
	204	0.53	(±0.51)	0.0	0.0
	209	0.52	(±0.49)	0.0	7.1
	224	0.45	(±0.46)	0.0	0.0
	225	0.32	(±0.62)	0.0	0.0
	640	0.29	(±0.60)	16.7	0.0
	115	0.19	(±0.55)	8.3	7.1
Brown wood lines	713	-0.62	(±0.68)	16.7	78.6
	671	-0.65	(±0.54)	16.7	50.0
	150	-1.00	(±0.53)	25.0	83.3
	350	-1.34	(±0.49)	16.7	42.9
	712	-1.54	(±0.86)	36.4	64.3

Table 3.2. Coefficients of determination relating individual traits to the overall standardized means across 28 traits, listed in rank order. The overall standardized means within lines are listed in Table 1. Bold values are significant at P<0.05. Calculation of means included field-grown and staked trees, otherwise only field-grown trees were used. Nit. Ox.= nitrobenzene oxidation; S = syringil G = guiacyl and H = *p*-hydroxyphenyl monomer release.

Trait	Overall r^2 (n=15)	All transgenics r^2 (n=14)	Control & normal transgenics r^2 (n=10)	Brown wood transgenics r^2 (n=5)
Biomass / leaf area	**0.91**	**0.90**	**0.53**	0.54
Height / diameter	**0.88**	**0.87**	**0.42**	0.24
Brown wood formation	**0.86**	**0.87**	0.03	0.23
Total Nit. Ox.	**0.85**	**0.83**	0.29	**0.93**
Leader dieback	**0.85**	**0.86**	0.12	0.09
MOE	**0.83**	**0.82**	0.32	0.37
Tensionwood formation	**0.83**	**0.83**	0.14	0.53
k_s / Theoretical k_s	**0.83**	**0.88**	0.02	0.63
Total Thioacidolysis	**0.79**	**0.77**	**0.54**	**0.97**
k_s	**0.77**	**0.79**	0.06	0.60
Total biomass	**0.75**	**0.74**	0.15	0.42
Volatile extractives	**0.74**	**0.72**	0.17	0.00
Moisture content	**0.71**	**0.67**	0.18	0.22
MOR	**0.70**	**0.69**	**0.65**	0.36
Cumulative mortality	**0.68**	**0.66**	0.18	0.47
H / Nit. Ox.	**0.67**	**0.64**	0.31	**0.97**
Hot water extractives	**0.67**	**0.65**	0.01	0.23
Nit. Ox. S/G	**0.64**	**0.76**	0.00	**0.93**
H / total thioacidolysis	**0.64**	**0.65**	0.28	**0.90**
% branch biomass	**0.60**	**0.55**	0.30	0.01
4CL1-2 RNA expression	**0.59**	**0.54**	0.26	**0.77**
Theoretical k_s	**0.57**	**0.53**	0.20	0.00
Leaf area / sapwood area	**0.53**	**0.53**	0.00	0.23
$k_{leaf\ specific}$	**0.48**	**0.44**	0.01	0.01
4CL1-1 RNA expression	**0.41**	**0.39**	**0.40**	0.06
Thioacidolysis S/G	**0.34**	**0.49**	0.00	**0.72**
Buckling safety factor	**0.33**	**0.39**	0.05	0.27
P_{50}	0.28	0.22	**0.52**	0.00

Table 3.3. Selected ANOVA results for traits compared within and between field-grown unstaked trees and staked greenhouse-grown trees.

Trait	Source	D.F.	F	P	Trait	Source	D.F.	F	P
Height to diameter ratio	Overall	28	41.20	<.01	Specific leaf area	Overall	28	4.24	<.01
	Treatment	1	894.93	<.01		Treatment	1	40.97	<.01
	Line	14	14.33	<.01		Line	14	2.23	0.02
	Trt. × Line	13	4.47	<.01		Trt. × Line	13	3.58	<.01
Brown wood	Overall	28	19.01	<.01	$k_{leaf\ specific}$	Overall	28	2.95	<.01
	Treatment	1	1.34	0.25		Treatment	1	34.46	<.01
	Line	14	35.60	<.01		Line	14	2.60	0.01
	Trt. × Line	13	2.51	<.01		Trt. × Line	13	0.90	0.56
k_s	Overall	28	14.04	<.01	Tension wood	Overall	28	2.76	<.01
	Treatment	1	67.38	<.01		Treatment	1	0.72	0.40
	Line	14	20.41	<.01		Line	14	4.76	<.01
	Trt. × Line	13	3.090	<.01		Trt. × Line	13	0.76	0.70
MOR	Overall	27	9.45	<.01	Safety factor	Overall	28	2.36	<.01
	Treatment	1	80.07	<.01		Treatment	1	44.93	<.01
	Line	14	10.67	<.01		Line	14	1.06	0.42
	Trt. × Line	13	2.14	0.01		Trt. × Line	13	0.50	0.91
Leaf area to sapwood area ratio	Overall	28	7.27	<.01	Biomass per unit leaf area	Overall	28	2.20	<.01
	Treatment	1	162.89	<.01		Treatment	1	2.95	0.09
	Line	14	1.18	0.31		Line	14	2.83	<.01
	Trt. × Line	13	1.87	0.05		Trt. × Line	13	1.46	0.13
Branch biomass	Overall	28	5.94	<.01	Theoretical k_s	Overall	28	1.89	0.01
	Treatment	1	122.2	<.01		Treatment	1	4.85	0.03
	Line	14	2.50	0.01		Line	14	2.97	<.01
	Trt. × Line	13	0.69	0.76		Trt. × Line	13	0.50	0.92
MOE	Overall	27	4.79	<.01	Total biomass	Overall	28	1.77	0.01
	Treatment	1	1.63	0.20		Treatment	1	4.30	0.04
	Line	14	7.76	<.01		Line	14	2.95	<.01
	Trt. × Line	13	1.58	0.10		Trt. × Line	13	0.30	0.99

CHAPTER 4:
CONCLUSION

It is necessary to remove lignin from wood in the production of most paper and in the pre-treatment steps leading to the production of cellulosic bio-ethanol. Therefore, with the aim of reducing lignin in wood, the primary goal of this study was to determine the optimal level of 4CL down-regulation assuming that tradeoffs for lowered lignin would include compromised wood properties, altered tree form and reduced survival. Secondarily, it was a goal of this study to determine the mechanisms governing the pleiotropic effects that were bound to occur given a change to the metabolic pathway leading to a major sink for carbon.

This study demonstrated that constitutive 4CL downregulation in poplar did indeed reduce lignin content of wood in a non-linear fashion. Pleiotropic or unintended effects did occur, but it was initially surprising to me that they did not include increased growth or cellulose content and also drastically reduced tree growth at lignin-levels only 10-20% lower than those of the control line. In retrospect, after learning much more about lignin biosynthesis, as well as how monolignol formation is so closely tied to secondary phenolic compounds, these results of shunt pathways for carbon within the phenylpropanoid pathway should not have been surprising. Specific results of this sort of shunt pathway for carbon were discolored wood, extractive deposition and reduced conductivity to water. Only one previous publication reporting on 4CL downregulated poplars mentions wood discoloration and none mention the stunted phenotypes we observed in the lowest lignin lines. These differences may be because all of the previous poplars were grown for only one season in a greenhouse. If so, such differences among studies should underscore the need to test transgenic trees under real-world conditions for multiple years. In contrast with initial findings on 4CL-downregulated poplars, the trees here did not have enhanced growth or cellulose content. Small increases in cellulose or hemicelluloses were largely attributable to three-fold increases in tensionwood. This finding is noteworthy because the relationship between lowered lignin and tensionwood formation has not been previously reported.

The foremost evolutionary roles of lignin are thought to be the sealing of water-conducting conduits from the atmosphere during plant transpiration and the provision of

increased stiffness to wood supporting arborescent growth forms. Yet no study in 10 years of transgenic research on low-lignin poplars has reported clear results that relate lignin content to xylem vulnerability to embolism, nor lignin content and wood stiffness. The poplars studied here, with lowered lignin but little brown wood, had xylem with increased vulnerability to embolism without any pleiotropic effects; thereby providing the first report on the effect of lignin content on this aspect of drought tolerance. This study also is the first study to report reduced wood stiffness in transgenic wood with low lignin. Because of lower wood stiffness, trees grown in the field and characterized by lower lignin altered their growth form so that they were more tapered. This increase in taper compensated for reduced wood stiffness such that the whole-tree biomechanical stabilities of transgenic lines were reasonably comparable. The trees I grew staked in the greenhouse found that although taper was still greater than controls in low-lignin lines, the differences between transgenics and controls was substantially less than in the trees that were field-grown. This finding provides a compelling argument for why greenhouse studies are likely inadequate to accurately judge if tree form of low-lignin transgenics will provide for sustained woody biomass production once canopy closure occurs. Taken together the findings of this study suggest more attention needs to be paid to testing functional traits. Both wood stiffness and eco-physiology need more rigorous assessment if this technology is to be feasible for large-scale woody biomass production. It is my opinion this could best be done by growing low-lignin trees for three- to five-years using spacing studies (both mixed and single-genotype stands) to determine how tree form is affected by competition for light versus the need for biomechanical stability.

It is unknown whether low-lignin wood has previously been tested for wood stiffness or drought tolerance but the results not published. It is alarming that 10 years of research on low-lignin transgenic trees has occurred without this type of very basic information being reported in the literature. I think that this lack of information may stem from most efforts on lignin research having been focused on understanding its biosynthesis (and establishing primacy in better resolving certain aspects of the phenylpropanoid pathway). Basic research of this sort is undoubtedly important goal, yet most studies of lignin justify their research by the need to produce trees that will be more amenable to industrial production and utilization of plant biomass. If this were really the

case, should not the functional aspects of low-lignin trees be just as important as understanding the biochemistry of lignification? In this sense, I think that a fevered rush for primacy in any field dominated by rapidly evolving technologies and short-term funding cycles (not just molecular biology) can be a double-edged sword and that at times ambition may best be tempered in favor of more circumspect enthusiasm. Fortunately though, rapid progress made in any field should usually outweigh the potential negative consequences of some research becoming entrained within an unproductive current of scientific inquiry.

The simplest solution to identify current knowledge gaps such as the one this study aimed to fill is to promote or advocate for a scientific culture that encourages broad interdisciplinary thinking. A task such as this is probably difficult to achieve in industrial settings in which scientific groups may work in parallel with confidential datasets, but may be feasible in an academic setting where there may be better control on the needless multiplication of efforts on basic research. Even within academia, confidentiality must be sought to a greater degree in some cases where the protection of intellectual property is difficult. However, when this fortification of intellectual borders is taken too far, the resulting fiefdom of scientific thought and practice can become too hierarchical, segregated, specialized, canalized or even myopic.

As pointed out above, this study has provided valuable insights on tree functional traits associated with reduced wood lignin content. Yet, my own study could have been improved substantially. In pursuit of knowledge scientists must routinely make decisions regarding where their efforts are best focused and this process may occasionally even surpass the importance the initial study design. I was involved in little of the original study design for testing the 4CL poplars but did make choices regarding traits that needed to be measured, the number of trees, and in what order. These choices, in turn affected the time available for research needed to interpret and communicate our results.

There are three choices that most clearly could have been made differently and in so doing made this study stronger. Foremost, is it would have been worthwhile to have completed earlier a more thorough review of the phenylpropanoid pathway so I could have better predicted results, planned comparisons and known which tests were best left for an outside lab to conduct. Because chemistry has always been my least favorite

subject of the basic sciences, this part of the research was the most frustrating task and the one most easily put off when more data needed to be collected or analyzed. Secondly, it would have been worthwhile to have determined Klason lignin and α-cellulose contents on my own. If I had made a better effort at this as soon as I finished biomass measurements, the analyses and interpretation data collected on other traits would have had to rely less on outside laboratories. Moreover, α-cellulose submitted for analyses of the stable isotope ratios of carbon would likely have been completed, thereby providing insights into whether brown wood induced reductions in wood specific conductivity affected stomatal conductance. Third, I planned to compare moisture contents and P_{50} values of staked trees to the field-grown trees but because I did not fully secure the greenhouse irrigation system from being tampered with, the plants were droughted and these comparisons were thus of little value.

In practice, decision making is always more difficult as each scientist's personal obligations are many and it may seem best at any particular moment for one to stay within the comfort zone delineated by their closest peers. As I move on in my scientific career, it seems it will serve me well in the long-term to keep reaching out to try and involve colleagues from far-ranging disciplines. In peer-review this may mean looking not only to senior scientists for advice, but also to involve younger and diverse scientists with fewer vested interests or potential conflicts of interest to help provide perspective on issues where scientific camps have long been entrenched. At a personal level, these efforts at inclusiveness will mean trying to maintain effective communication and solid relationships with those scientific peers at a number of institutions that may refer me to someone else for advice as well as offer advice directly. After all, a chance meeting by not only oneself, but also one's colleagues, may play as large a role in the direction of a career as all the study and preparation that goes into a Ph.D. degree.

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APPENDIX 1
CHAPTER 2 SUPPLEMENTAL MATERIAL:

Table S2.1. Selected traits (mean ±SD) measured for each of 14 transgenic lines and controls. Lines are arrayed according to their total lignin contents as determined from three trees by monomer release of both thioacidolysis and nitrobenzene oxidation treatments of extracted cell wall residue. All other trait means were determined from at least five trees. Brown wood and tensionwood values are percentages of the xylem cross-sectional area. Bold values were significantly different ($P<0.05$) than controls according to Tukey HSD tests.

Line	Lignin (% of Ctrl)	4CL1-1 RNA expression	4CL1-2 RNA expression	Oven-dry aboveground biomass (g)	Brown wood (%)	Tension-wood (%)	Height to diameter ratio	MOE (% of Ctrl)	MOR (% of Ctrl)	Fiber wall cross-sect. area (%)
Control	100 (±5)	100 (±13)	100 (±24)	605 (±434)	0 (±1)	5 (±6)	94 (±14)	100 (±18)	100 (±15)	61 (±5)
224	95 (±6)	61 (±20)	88 (±28)	459 (±257)	1 (±1)	8 (±6)	84 (±16)	75 (±8)	**82** (±9)	61 (±7)
90	93 (±8)	46 (±8)	66 (±15)	597 (±763)	0 (±1)	6 (±4)	84 (±14)	85 (±24)	92 (±11)	58 (±5)
17	93 (±5)	64 (±11)	93 (±27)	537 (±317)	0 (±1)	7 (±11)	93 (±10)	81 (±26)	86 (±13)	59 (±6)
210	93 (±3)	**37** (±12)	97 (±32)	701 (±555)	1 (±2)	5 (±5)	**81** (±13)	89 (±28)	87 (±14)	59 (±5)
209	92 (±8)	52 (±24)	96 (±47)	430 (±352)	1 (±2)	2 (±2)	**83** (±9)	92 (±25)	92 (±12)	60 (±4)
115	90 (±7)	56 (±11)	80 (±9)	326 (±262)	1 (±2)	10 (±7)	**73** (±26)	83 (±25)	85 (±15)	60 (±7)
204	89 (±7)	**33** (±24)	97 (±42)	634 (±482)	6 (±17)	7 (±3)	**82** (±15)	79 (±17)	86 (±7)	61 (±4)
225	88 (±9)	**29** (±4)	91 (±25)	766 (±588)	4 (±10)	9 (±8)	**80** (±18)	82 (±33)	**79** (±13)	60 (±5)
640	86 (±9)	**34** (±11)	66 (±8)	565 (±399)	1 (±2)	4 (±5)	88 (±15)	89 (±31)	**83** (±16)	60 (±4)
713	86 (±9)	**24** (±3)	84 (±14)	237 (±212)	**24** (±31)	13 (±10)	**43** (±14)	**68** (±15)	**82** (±7)	61 (±4)
150	85 (±11)	**35** (±13)	66 (±13)	**144** (±117)	**51** (±37)	**16** (±10)	**59** (±19)	**54** (±17)	**68** (±11)	59 (±4)
671	**70** (±13)	**20** (±13)	73 (±37)	**158** (±98)	**59** (±34)	13 (±8)	**60** (±18)	**63** (±9)	**79** (±13)	61 (±5)
350	**63** (±11)	**32** (±5)	66 (±15)	**132** (±111)	**47** (±30)	**20** (±18)	**48** (±15)	**58** (±19)	**74** (±14)	61 (±4)
712	**54** (±12)	**22** (±4)	45 (±14)	**180** (±144)	**60** (±33)	**16** (±11)	**38** (±27)	**60** (±24)	**74** (±16)	59 (±5)

Table S2.2. Cellulose and extractive contents (mean ±SD) by transgenic line by brown wood presence or absence. No error estimates were included for wood color-weighted extractives because mean brown wood percentages within lines were used to calculate these estimates (see Table S1). CWR is oven-dry, extractive-free cell wall residue and DW is oven-dry initial wood mass including extractives. Controls n=7, normal wood n=4 and brown wood n=3 trees. Volatile extractives are those determined by soxhlet extraction with toluene/ethanol (see *Methods*).

Line	Brown wood α-cellulose (% of CWR)	Normal wood α-cellulose (% of CWR)	Brown wood volatile extractives (% of DW)	Normal wood volatile extractives (% of DW)	Brown wood hot water extractives (% of DW)	Normal wood hot water extractives (% of DW)	Color weighted α-cellulose (% of CWR)	Color weighted vol. ext. (% of DW)	Color weighted hot water ext. (% of DW)
Control		43.6 (±1.0)		4.0 (±0.6)		4.3(±1.1)	43.4	4.0	4.3
224		47.1 (±2.9)		4.0 (±0.5)		4.0(±0.5)	46.8	4.0	4.0
17		45.4 (±1.1)		4.9 (±0.5)		5.3(±1.0)	43.0	4.9	5.3
90		43.2 (±2.6)		4.0 (±0.9)		4.1(±0.6)	45.3	4.0	4.0
209		47.1 (±0.5)		5.7 (±0.6)		5.8(±0.8)	46.7	5.6	5.7
210		46.2 (±1.5)		4.2 (±0.6)		3.9(±0.9)	45.7	4.1	3.9
640		50.3 (±1.1)		4.0 (±0.4)		4.4(±0.9)	49.7	3.9	4.4
713	40.2 (±1.0)	42.4 (±1.1)	19.5 (±0.4)	5.9 (±2.8)	8.0(±2.0)	6.9(±1.7)	41.9	9.1	7.2
204		44.5 (±2.1)		4.6 (±1.1)		5.2(±0.8)	41.8	4.3	4.9
115		41.9 (±2.4)		5.0 (±0.9)		4.0(±1.0)	41.7	5.0	4.0
225		49.3 (±1.7)		6.4 (±1.4)		5.5(±0.3)	47.3	6.2	5.3
150	47.1 (±1.4)	47.5 (±1.9)	11.2 (±1.7)	5.4 (±1.4)	8.5(±2.9)	4.4(±0.8)	47.6	8.3	6.5
671	49.0 (±0.7)	48.5 (±1.6)	9.5 (±3.2)	5.9 (±1.5)	6.2(±1.1)	5.6(±2.1)	48.8	8.0	6.0
350	43.8 (±2.8)	46.3 (±2.4)	16.2 (±5.2)	5.5 (±1.5)	7.4(±0.6)	6.3(±0.7)	45.1	10.5	6.8
712	52.0 (±4.4)	46.8 (±0.9)	9.5 (±2.6)	3.9 (±0.5)	8.7(±1.6)	5.6(±1.6)	49.9	7.3	7.5
Only brown wood means	46.4 (±4.9)	46.3 (±2.7)	13.2 (±5.2)	5.3 (±1.9)	7.8(±2.1)	5.8(±1.7)	46.6	8.6	6.8
Only normal trans. means		46.1 (±3.3)		4.7 (±1.2)		4.3(±1.2)	45.4	4.7	4.6

Figure S2.1. Examples of methods used for tensionwood estimates. Light microscopy examples show patterns of safranin-stained (red=lignified) cell walls and Astra blue-stained "g-layers" within tensionwood fibers. The top left photomicrograph shows the boundary at the edge of a patch of tensionwood while the lower left photos are both within a contiguous patch of tensionwood fibers. The green box from the left center image frames the area pictured in the lower left. Green arrows point to brown colored extractives deposited within vessels and fiber cells. The photos at right picture a dried, roughsawn cross-section of a control stem. These pictures demonstrate the banded, patchy pattern of tensionwood formation (light colored regions in lower right quadrant of stem) versus the dull appeareance of the surrounding normal wood. The graph on the lower right shows tensionwood means (± 1SE) within each poplar line for a given method. The regression line (r^2=0.43, P=0.008) falls near the 1:1 line indicating there was no significant bias of grouping the data from both methods for our analyses.

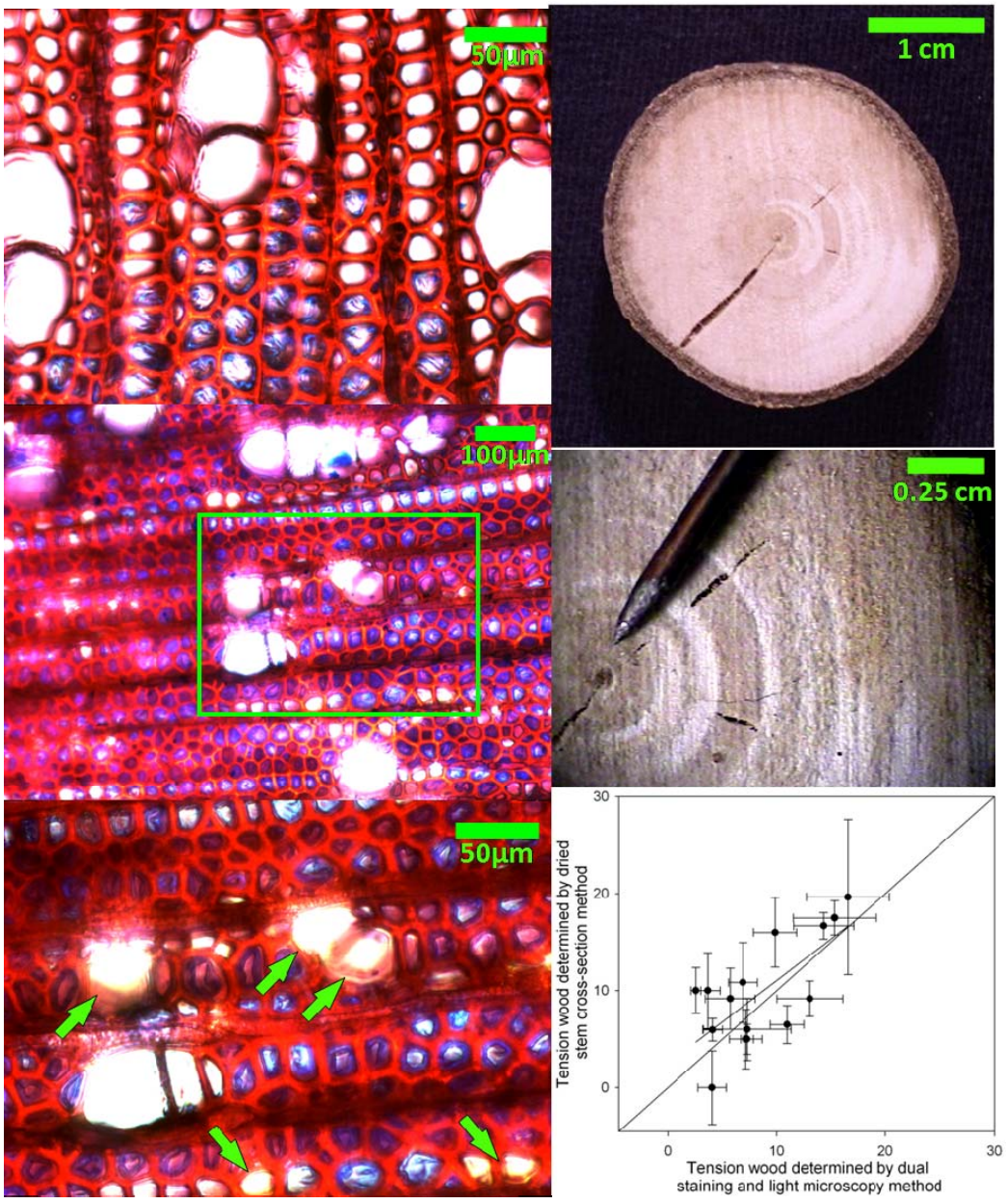


Figure S2.2. Modulus of elasticity (MOE) and modulus of rupture (MOR) of control, normal wood transgenics and brown wood transgenics by sample diameter. MOE is a measure of wood stiffness and MOR is a measure of wood strength.

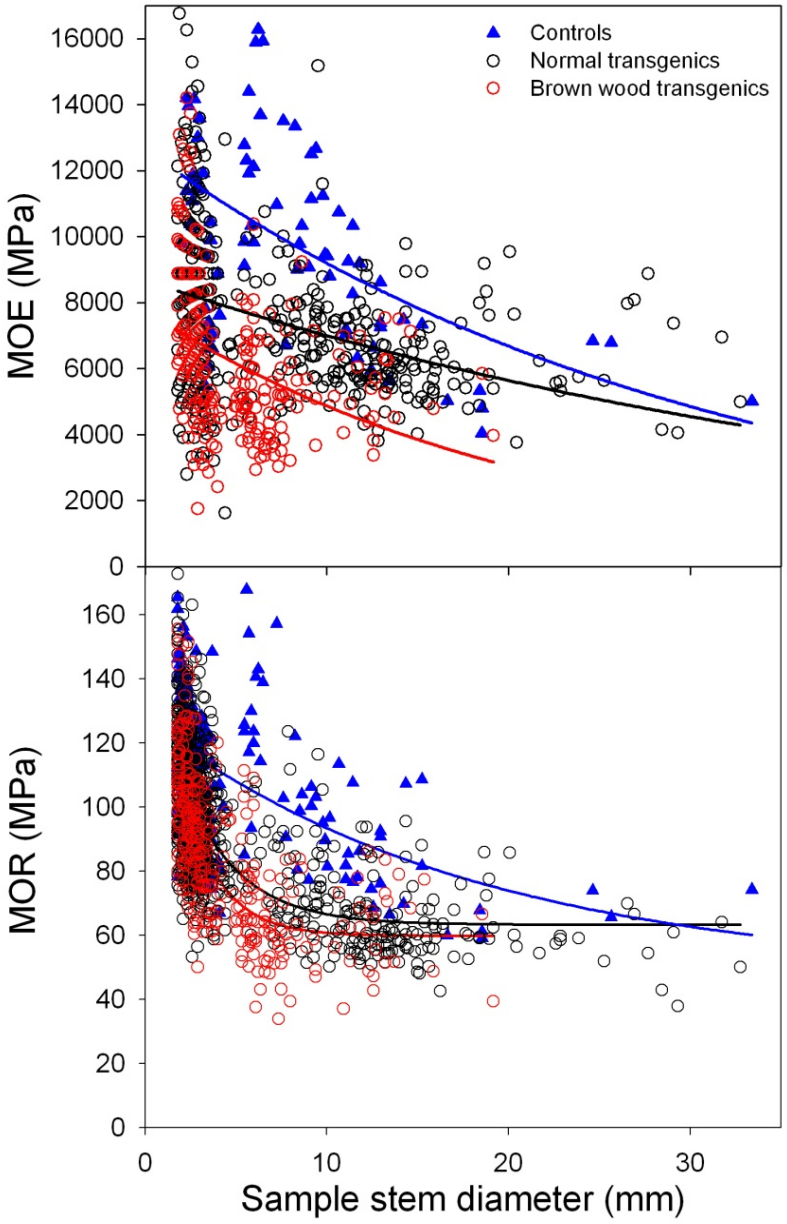


Figure. S2.3. Wood density of control, normal wood transgenics and brown wood transgenics by sample diameter. Note that both axes are on a log scale. Wood densities appear to diverge in smallest sample diameters. We attribute this to a progressive reduction in rates of lignification as compared to cell division with suppression of 4CL and lignin content (see Figure S2.4). This initially reduced wood density is eventually mediated by an increase in the deposition of extractives.

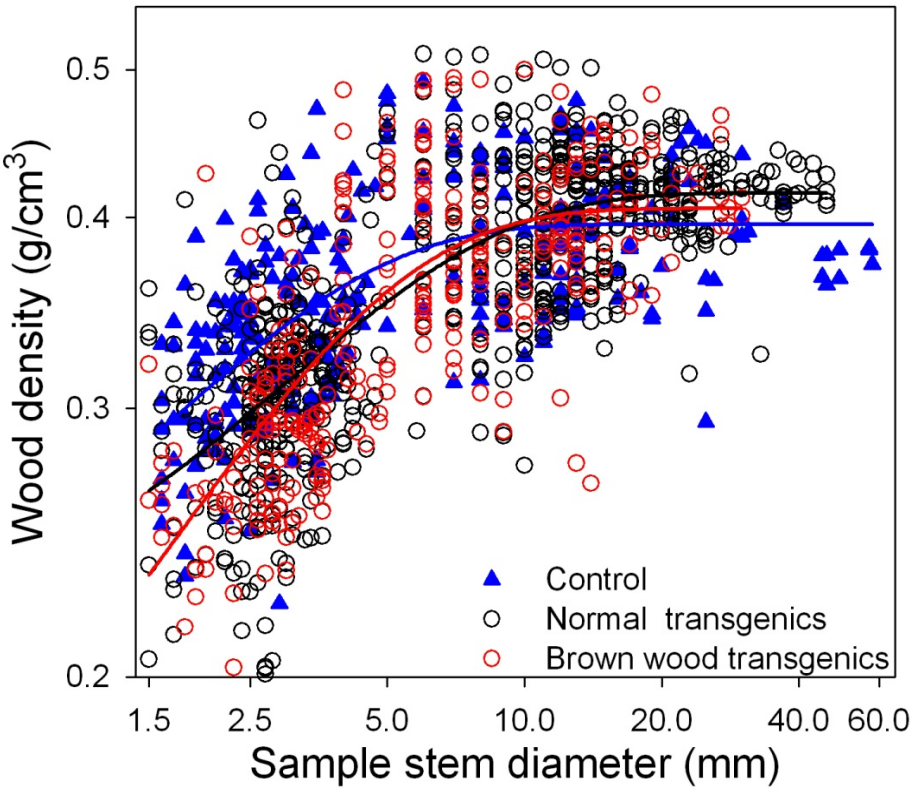


Figure S2.4. Comparison of lignification patterns using Astra-blue and safranin staining. The photos are aligned by cambial regions at the bottom of each photo for line 17 (left, average of 95% of control lignin) and line 712 (average of 43% of control lignin). The vessels and fibers of line 712 are obviously malformed or "collapsed". However this is the case throughout cell development and before negative hydrostatic pressures would have been realized by developing cells, suggesting that "collapse" is a direct result of an altered program of lignification. Line 17 is characterized by an abrupt transition to red, safranin-stained (lignified) xylem that is also very consistent in its distance from the cambium. In contrast, line 712 was often characterized by patchy safranin staining that is less intensely red (red= phenolics, most of these likely to be lignin). The lignification of line 712 also began a greater number of cells from the cambium (the phloem and bark were not present in this photo). Because the developing xylem of line 712 is stained with Astra-blue and not safranin, as observed for tensionwood g-layers (see Figure S1), these regions must have little or no lignin and be composed largely of cellulose and/or hemicelluloses. Overall these patterns suggest that developmental patterns of lignification were slower in the low-lignin transgenic lines.

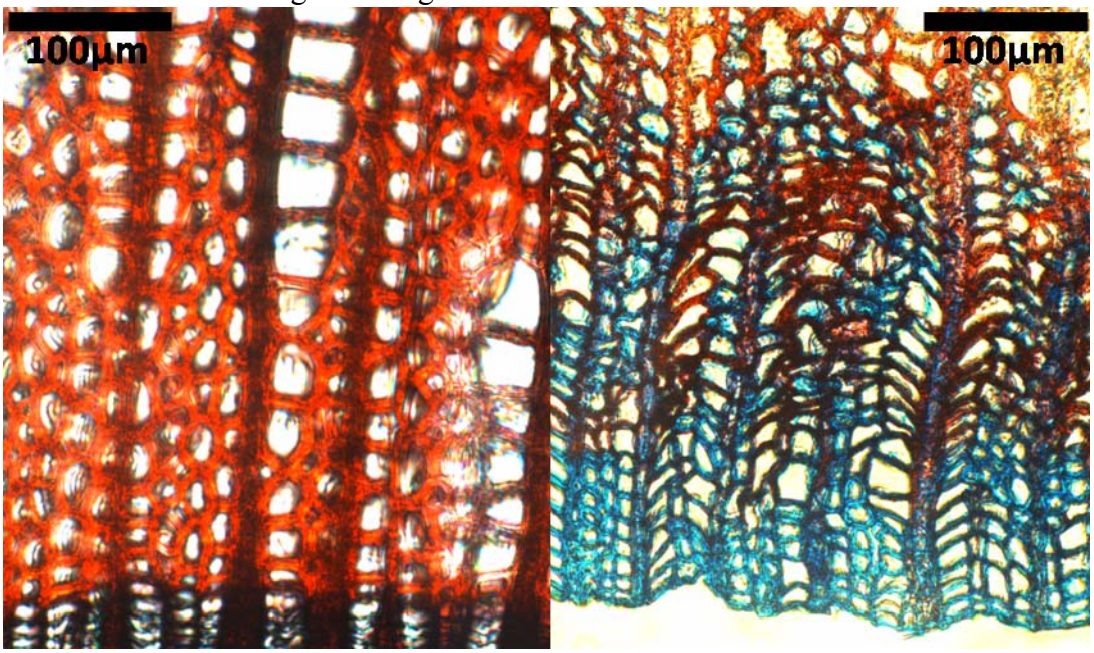
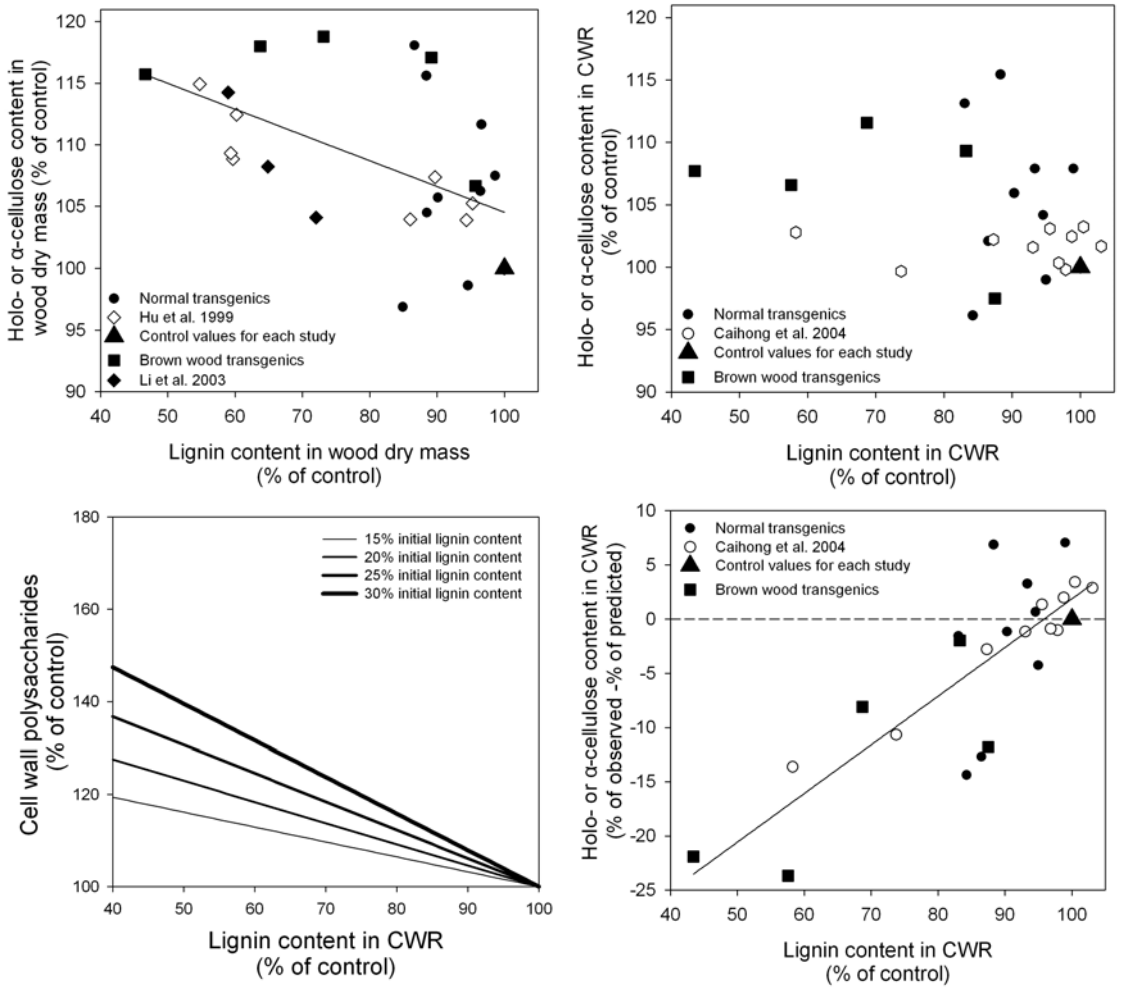


Figure S2.5. Comparison of cell wall polysaccharides as related to lignin levels. Top left panel: on a percent dry wood basis, including extractives, the data overall show that cellulose content tends to increase as lignin content decreases (r^2 =0.29, P<0.01). Data from Hu et al. 1999 and Li et al. 2003 are from 4CL-downregulated aspen (*P. tremuloides* Michx.) using CaMV 35S and 4CL-specific promoters, respectively. Top right panel: on an extractive-free cell wall residue basis (CWR), no overall relationship was found between holo- or α-cellulose and lignin contents (r^2 =0.07, P =0.35). For the purposes of estimating the magnitude of reciprocal carbon transfer between metabolic endpoints, lignin has a greater carbon density and metabolic costs. It has been reported that approximately 0.83 g of cell wall polysaccharides or 0.47 g of lignin can be synthesized for each 1.00 g of sucrose (Loomis and Amthor 2000). Thereby the hypothetical maximum increase in cell wall polysaccharides for a given decrease in lignin content can be predicted for wood with a given initial lignin content (see bottom left panel). The bottom right panel displays differences between the predicted (dotted line, corresponding to initial lignin contents that vary slightly) and the observed data, indicating that the hypothesis of increasing cell wall polysaccharide content with decreasing lignin contents is not supported (solid regression line across all data, r^2 =0.69, P <0.01).



APPENDIX 2:
CHAPTER 3 SUPPLEMENTAL MATERIAL

Table S3.1. Selected trait means (±SD) for field-grown and staked trees. Thioacidolysis lignin content values were measured on trees from the field (n=3). Two trees per transgenic line and four control trees were grown staked in the greenhouse. Units for k_s, theoretical k_s and $k_{leaf\ specific}$ are of kg m^{-1} s^{-1} MPa^{-1}. Bold values were significantly different than controls (P<0.05, Tukey HSD).

	Line	Lignin Content (% of Ctrl)	k_s	Theoretical k_s	$k_{leaf\ specific}$	Leaf to sapwood area ratio	Buckling safety factor	Branch biomass (%)	Height to diameter ratio	Stem moisture content (% of Ctrl)	P_{50} (MPa)
Field	Control	100 (±5)	3.0 (±0.6)	15.4 (±8.5)	0.7 (±0.5)	2006 (±537)	3.9 (±2.1)	39 (±10)	94 (±14)	100 (±5)	-2.7 (±0.7)
	210	93 (±3)	2.9 (±0.5)	12.7 (±5.4)	0.5 (±0.4)	1880 (±508)	6.4 (±2.9)	43 (±3)	93 (±10)	108 (±7)	-2.6 (±0.2)
	17	93 (±5)	3.4 (±1.2)	13.0 (±5.7)	1.1 (±0.9)	1939 (±989)	3.4 (±1.7)	45 (±15)	83 (±9)	111 (±6)	-2.7 (±0.9)
	204	89 (±7)	4.4 (±0.8)	17.2 (±9.3)	0.5 (±0.3)	1925 (±573)	5.5 (±2.0)	43 (±3)	**82** (±15)	112 (±5)	-2.5 (±0.2)
	90	93 (±8)	2.8 (±0.0)	12.7 (±6.1)	0.3 (±0.2)	2175 (±567)	6.0 (±2.5)	49 (±1)	81 (±13)	100 (±2)	-2.1 (±0.1)
	209	92 (±8)	3.7 (±0.4)	14.8 (±5.3)	0.7 (±0.4)	2023 (±127)	4.3 (±2.0)	47 (±6)	**84** (±16)	**115** (±7)	-2.9 (±0.4)
	224	95 (±6)	2.6 (±0.6)	13.3 (±8.0)	0.6 (±0.3)	1748 (±1028)	3.0 (±0.2)	43 (±7)	84 (±14)	105 (±2)	-2.5 (±0.5)
	225	88 (±9)	3.9 (±1.2)	13.7 (±7.3)	0.5 (±0.2)	1910 (±617)	5.0 (±2.2)	41 (±3)	**80** (±18)	106 (±6)	-2.2 (±0.2)
	640	86 (±9)	2.7 (±0.8)	13.7 (±7.1)	0.3 (±0.2)	2294 (±750)	4.7 (±0.9)	46 (±4)	88 (±15)	108 (±8)	-2.2 (±0.1)
	115	90 (±9)	1.7 (±0.7)	10.5 (±4.0)	0.9 (±0.7)	1927 (±820)	2.8 (±1.5)	52 (±5)	**73** (±26)	111 (±11)	-2.1 (±0.3)
	713	86 (±9)	**1.2** (±1.1)	10.0 (±4.5)	0.2 (±0.2)	1597 (±857)	3.7 (±1.6)	**63** (±4)	**43** (±14)	114 (±11)	-2.5 (±0.2)
	671	**70** (±13)	**0.9** (±0.6)	9.6 (±6.8)	0.2 (±0.1)	1727 (±313)	3.6 (±1.1)	55 (±2)	**60** (±18)	**122** (±15)	-1.9 (±0.1)
	150	85 (±11)	**0.3** (±0.4)	10.1 (±4.3)	0.1 (±0.1)	1903 (±1011)	2.3 (±0.7)	49 (±6)	**59** (±19)	**121** (±8)	**-1.5** (±0.5)
	350	**63** (±11)	**0.4** (±0.2)	7.0 (±1.7)	0.1 (±0.0)	1467 (±1302)	3.3 (±0.4)	52 (±5)	**48** (±15)	**127** (±14)	**-1.6** (±0.2)
	712	**54** (±12)	**0.5** (±0.4)	11.7 (±7.1)	0.3 (±0.3)	1518 (±584)	2.8 (±0.5)	**61** (±6)	**38** (±27)	**119** (±15)	-2.6 (±0.7)
Staked	Control		4.2 (±0.9)	17.7 (±6.7)	1.44 (±0.3)	4171 (±215)	1.4 (±0.1)	10 (±3)	194 (±10)		
	210		3.7 (±1.2)	17.1 (±5.5)	1.11 (±0.2)	3974 (±410)	1.5 (±0.1)	25 (±10)	166 (±11)		
	17		6.8 (±1.4)	17.6 (±6.4)	1.94 (±0.0)	5063 (±871)	1.4 (±0.2)	7 (±2)	189 (±6)		
	204		6.1 (±0.1)	17.7 (±7.1)	1.64 (±0.5)	5896 (±1841)	1.6 (±0.5)	28 (±16)	167 (±10)		
	90										
	209		4.1 (±0.8)	14.4 (±7.6)	1.26 (±0.3)	4166 (±30)	1.2 (±0.1)	13 (±3)	199 (±17)		
	224		4.7 (±0.2)	14.0 (±6.2)	1.13 (±0.1)	3083 (±174)	1.3 (±0.1)	14 (±2)	198 (±1)		
	225		4.2 (±0.7)	19.6 (±6.9)	1.11 (±0.2)	3289 (±549)	1.4 (±0.1)	15 (±0)	182 (±6)		
	640		4.4 (±0.3)	14.7 (±7.2)	1.56 (±0.2)	4186 (±88)	1.2 (±0.0)	12 (±10)	185 (±4)		
	115		4.0 (±2.0)	13.1 (±4.6)	1.36 (±0.5)	4867 (±175)	1.1 (±0.1)	19 (±0)	182 (±5)		
	713		4.4 (±0.4)	13.0 (±5.7)	1.69 (±0.3)	5103 (±228)	1.2 (±0.1)	23 (±8)	178 (±15)		
	671		0.1 (±0.1)	8.7 (±4.7)	0.09 (±0.1)	3991 (±537)	1.7 (±0.1)	42 (±20)	95 (±13)		
	150		0.9 (±0.2)	10.3 (±5.2)	0.28 (±0.0)	5948 (±1230)	1.2 (±0.2)	26 (±9)	139 (±2)		
	350		1.9 (±0.1)	8.3 (±3.4)	0.54 (±0.1)	2382 (±1226)	1.7 (±0.3)	21 (±7)	156 (±9)		
	712		2.5 (±2.5)	11.7 (±9.1)	0.88 (±0.9)	5474 (±128)	1.1 (±0.1)	32 (±26)	126 (±26)		

Figure S3.1. Example of tyloses formation observed in a vessel located in a brown wood patch of line 712. The vessel pictured is from a tangential section of wood that had been extracted with ethanol and stained with calcofouor (see *Methods*). The cell walls of the vessel are red colored, indicating the presence of phenolics including lignin. Green-fluorescence, including tyloses indicate cell walls dominated by cellulose.