TITLE PAGE - TREES CONTAINING BUILT-IN PULPING CATALYSTS

A Final Report to the United States Department of Energy, Agenda 2020 Program: A Technology Vision and Research Agenda for America's Forest, Wood, and Paper Industry. Agenda 2020 Environmental Performance Research Task Group

Program / Project Title: Trees Containing Built-In Pulping Catalysts
Program / Project Identification No: DE-FC07-97ID 13538
Project Duration: August 18, 1997 – August 18, 2000 (project was extended 1 year)

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RESEARCH AREA(S) IN THE RFP TO WHICH THE WORK WAS TARGETED:
Environmental Performance: #8 - reducing emissions of odorous gasses: other areas impacted include #1 - mill closure, #7 - pulping processes capable of producing easily bleachable, low kappa pulps at acceptable yields, and # 14 - extending the useful life of wood-based building materials. This proposal also addresses high-priority research areas within the Agenda 2020 Capital Effectiveness and Sustainable Forestry programs.

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EXECUTIVE SUMMARY: Several hardwood and softwood trees were analyzed for the presence of anthraquinone-type molecules. Low levels of anthraquinone (AQ) and anthrone components were detected using gas chromatography-mass spectroscopy and sensitive selected-ion monitoring techniques. Ten out of seventeen hardwood samples examined contained AQ-type components; however, the levels were typically below ~6 ppm. No AQs were observed in the few softwood samples that were examined. The AQs were more concentrated in the heartwood of teak than in the sapwood.

The delignification of pine was enhanced by the addition of teak chips (~0.7% AQ-equivalence content) to the cook, suggesting that endogenous AQs can be released from wood during pulping and can catalyze delignification reactions.

Eastern cottonwood contained AQ, methyl AQ, and dimethyl AQ, all useful for wood pulping. This is the first time unsubstituted AQ has been observed in wood extracts. Due to the presence of these pulping catalysts, rapid growth rates in plantation settings, and the ease of genetic transformation, eastern cottonwood is a suitable candidate for genetic engineering studies to enhance AQ content. To achieve effective catalytic pulping activity, poplar and cottonwood, respectively, require ~100 and 1000 times more for pulping catalysts.

A strategy to increase AQ concentration in natural wood was developed and is currently being tested. This strategy involves "turning up" isochorismate synthase (ICS) through genetic engineering. Isochorismate synthase is the first enzyme in the AQ pathway branching from the shikimic acid pathway. In general, the level of enzyme activity at the first branch point or committed step controls the flux through a biosynthetic pathway. To test if the level of ICS regulates AQ biosynthesis in plant tissues, we proposed to over-express this synthase in plant cells.

A partial cDNA encoding a putative ICS was available from the random cDNA sequencing project carried out with *Arabidopsis thaliana*. We used this putative plant ICS gene fragment to isolate and sequence a full-length ICS cDNA from *Arabidopsis thaliana*. The putative full-length cDNA encodes for a 569 amino acid protein of ~62kDa. This sequence represents the first full-length ICS cDNA isolated from a plant. When inserted into *E. coli*, our isolated cDNA over-expressed ICS protein in the insoluble inclusion bodies.

A plant expression vector containing the ICS cDNA, NP II for selection on the antibiotic kanamycin, and duplicated 35S-cauliflower mosaic virus promoter were inserted into *Agrobacterium tumefaciens* strain GV3101. Transformation experiments for insertion of these foreign genes into *Populus deltoides* 'C175' resulted in eight lines able to regenerate shoots and grow roots in the presence of kanamycin. Plants from these eight lines have acclimated to growth in sterile soil and will be moved to a greenhouse environment in spring 2001. Non rooted shoots from each line are currently being multiplied by shoot culture. When enough shoot tissue and/or greenhouse plant stem tissue is available, AQ analysis will be done and compared with non transformed control tissue.

BACKGROUND PRIOR TO PROJECT START: Kraft pulping, the dominant chemical pulping process in the world, uses sodium sulfide (NASH) and sodium hydroxide to degrade the lignin component in the wood, while maintaining the fibrous carbohydrate materials. While the process obviously has several advantages, there are also disadvantages, including odor emissions and the dark color of the resulting pulp. High levels of bleaching chemicals are needed to brighten the pulp, and the resulting bleach effluents contain large amounts of organic material that is an environmental concern.

Anthraquinone (AQ) Pulping. AQ can be used at <0.1% levels to improve pulping productivity and lower environmental impact (1). AQ increases pulping rates and product yields, reduces chemical recovery bottlenecks, and facilitates lignin removal during pulping, resulting in fewer organic effluents during bleaching (1). It can be used alone (soda/AQ) or with a kraft process. In the latter case, the NASH content of the liquor can be reduced, and fewer odorous emissions occur. Other AQ-based pulping processes include alkaline sulfite/AQ in water or aq. methanol (ASAM) and soda/AQ/aq. methanol (organocell) (2, 3, 4). These systems offer advantages mentioned above; however, widespread use of anthraquinone has been hindered by AQ cost, which in past years has ranged from $1.50 – 4.50 / lb. The current cost is about $2.50 / lb. (formulated) to pulp mills. The AQ cost would virtually disappear if pulpwood naturally contained AQ. Our long-term research goal is to genetically modify trees to produce AQ components that would catalyze their own pulping (or that of a mixture of woods).

Preliminary Results. Approximately two years before this project began; IPST started to explore the feasibility of genetically engineering *Pinus taeda*, loblolly pine, for the production of natural anthraquinone(s) for self-catalyzed pulping. Some plant species naturally produce high levels of AQ-type materials. Often the AQ substances in plants are highly oxygenated; such structures do not make good pulping catalysts (5). However, *Tectona grandis*, teak, produces wood that contains 0.33% 2-methyl AQ along with other AQs. We have extracted teak with organic solvents and have tested the pulping activity of the extract. At 0.05% addition (based on 2-methyl AQ content), the teak extract delignifies pine under soda pulping conditions to the same extent as either 0.1% industrial 2-methyl AQ or AQ alone. Thus, the extract contains several active components. Further research in our laboratory has enabled us to start callus tissue cultures from teak stem tissue. This callus produces 2-methyl AQ.

Natural AQs have not been examined in typical pulpwood trees. If trees can be genetically engineered to contain pulping catalysts in the wood at concentrations suitable for pulping, wood that contains catalysts can be mixed and pulped with wood that does not contain catalyst, eliminating the need to add costly commercial anthraquinone. In addition, since 2-methyl AQ is repellent to termites, genetically engineered trees should resist termite attack. The fact that teak already produces 2-5 times the concentration of catalyst needed for pulping recommended it as an initial focus for our work.

Genetic Engineering of Forest Trees. It is now becoming routine to genetically engineer plant species for improved value. Our laboratory has been successful in transferring foreign genes into *Populus deltoides* (eastern cottonwood) (6). While forest trees such as loblolly pine are more recalcitrant, our team at IPST is actively working on methods to introduce foreign DNA into loblolly pine. Our laboratory has abundant experience in the tissue-culture tools needed for successful gene transfer. We were one of a few laboratories in the world capable of repeatedly producing trees from somatic embryos of loblolly pine (7). 'This project seeks to integrate existing IPST expertise in pulping chemistry, plant physiology, and molecular biology to provide a tree whose enhanced character will be of direct use to the American pulp and paper industry.

Biosynthesis of Plant Anthraquinones. Plants contain at least two pathways for biosynthesis of anthraquinones. One is the acetate polymalonate pathway that leads to polyketyl-like AQs, e.g., emodin, which often have a characteristic substitution pattern indicative of their biosynthesis from acetyl-CoA and malonyl-CoA (8). This pathway has been shown to participate in the biosynthesis of AQs in *Rumex* species and *Rhamnus* species. The other pathway (Figure 1) branches from the shikimic acid pathway starting with isochorismic acid and α-ketoglutaric acid forming o-succinylbenzoic acid which in turn leads to menaquinone (vitamin K-2), napthaquinones, and anthraquinones (9).

Other than in teak, the presence of AQs in forest trees is unknown. To get an overview of the naturally occurring AQs, we proposed to identify AQs present in the wood of commercially important trees such as cottonwood and loblolly pine. Additionally, a survey of other commercially important hardwood trees grown in a variety of regions was proposed.

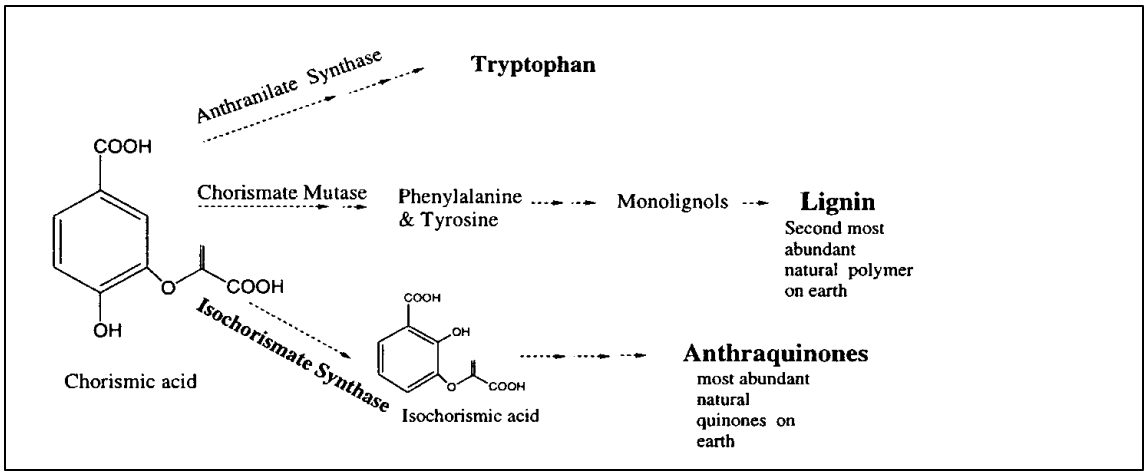

Figure 1. The competition of anthranilate synthase, chorismate mutase, and isochorismate synthase on chorismic acid for synthesis of aromatic acids and secondary metabolites.

No direct evidence exists about which pathway is involved in the biosynthesis of AQs useful for pulping. Two reasons suggest that the shikimic acid-succinylbenzoic acid is the primary pathway. First, the substitution pattern of the most desirable pulping anthraquinones is not like that for polyketyl-derived AQs. Second, AQs useful for pulping are quite abundant, 0.33% of the weight of teakwood. Since the flux through the shikimic acid pathway to chorismic acid is already high for lignin precursor synthesis in developing xylem tissues, it is likely that the relatively large amounts of AQs come from this pathway. Thus, we hypothesize that the rate-limiting step for AQ biosynthesis in xylem tissue is likely to be at the branch point of chorismic acid from the shikimate pathway and the AQ pathway and will be regulated by the activity of isochorismate synthase.

PROJECT OBJECTIVE: Our objective was to engineer a fast-growing tree, such as cottonwood, that contains high levels of AQs (0.1-0.5%). If successful one pound of genetically engineered AQ-containing cottonwood, for example, would catalyze pulping of nine pounds of unengineered wood such as other hardwood species or loblolly pine. The AQ-rich wood could be blended with a non-AQ wood to promote the pulping of both. The choice of species to be engineered will likely control the rate of release of AQs from wood. Cottonwood pulps faster than pine, so the release of AQs from one species to the next should be rapid. To be successful this project needed to: (1) demonstrate if natural AQs are released rapidly enough to catalyze pulping, (2) identify a genetic engineering strategy to produce sufficient catalyst levels in a tree of commercial interest, and (3) carry out experiments to test that strategy.

PROJECT MILESTONES:

1. Run pulping study with teak chips to demonstrate AQ release from chips.
2. Continue study of AQs present in teak extract.
3. Commence survey of AQs present in 10 commercial hardwood trees and loblolly pine.
4. Repeat assay in Milestone 1 using chips from AQ-containing species identified in Milestone 3.
5. Isolate a complete sequence of Arabidopsis cDNA for isochorismate synthase.
6. Clone isochorismate synthase DNA into appropriate bacterial vector; conduct complementation assay.
7. Clone isochorismate synthase cDNA into plant expression vector.
8. Transfer (for over-expression) isochorismate synthase DNA (in plant expression vector) to teak callus, Arabidopsis (model plant), and target tree (cottonwood or commercial hardwood with natural AQs).
9. Assay AQ concentration in transformed teak callus, model species (Arabidopsis), and target tree.

EXPERIMENTAL APPROACH AND RESULTS:

Release of AQs from Chips. First, there remains a question of whether the catalyst in wood will be released during pulping. All of our past studies were with extracted AQ. We will pulp pine in the presence of teakwood and determine the effectiveness of the catalyst(s) to migrate from the wood to the liquors. Increased loblolly pine pulp yields with lower kappa numbers should occur in the presence of teak chips. We expect the catalyst to migrate from the teak chips.

Two pulping studies have been carried out, one of a practical nature, the other more theoretical (experimental details can be found in Dimmel et al., 2000; 10). Previously, we showed that adding a teak extract to a pine cook accelerated the delignification process (11). In a new set of experiments, we examined whether 2-methyl AQ would diffuse out of teak and catalyze the pulping of pine. As the data shows in Figure 2, increasing the amount of teak chips added to southern pine chips resulted in a lower kappa number (lignin content) pulp. (The kappa contribution of the teak was subtracted out.) The data suggests that endogenous AQs in wood can be released from wood chips during critical phases of the pulping and can catalyze delignification reactions.

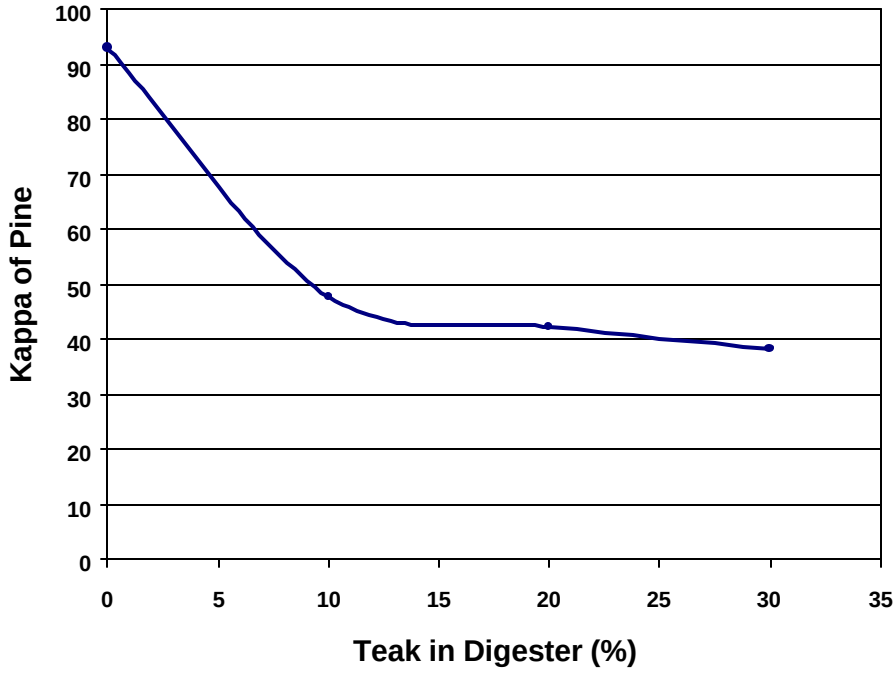

Figure 2. Effect of teak addition to the cooking of pine (10).

Study of AQs Present in Teak Extract. Analysis of several teak species from India and Indonesia gave 2-methyl AQ contents of 0.3 to 1.3% (12). To confirm these levels and identify other abundant AQs, we exhaustively extracted the sawdust from a piece of commercial teak lumber with toluene and toluene-ethanol mixtures until the extract had no color; chemical isolation and characterization details can be found in Leyva et al, (11). The combined extracts were evaporated to yield an oil with 6.7 weight % yield. Analysis of the extract residue by gas chromatography (GC) indicated the presence of 5.0 weight % 2-methyl AQ, along with several other quinones. Thus, the 2-methyl AQ content of the purchased teak was 0.33%; six different extractions were conducted, and the yields were consistent.

Analysis of the extract by GC-mass spectroscopy (MS) gave the data shown in Figure 3. The signal at 25.5 min was identified as 2-methyl AQ by comparison to the GC-MS of commercial 2-methyl AQ. Structures corresponding to the signals at 28.8 and 32.8 min were assigned based on a good match to the reported MS of the structures shown (13). Other structures were tentatively assigned based on the apparent molecular ions in the MS and the known presence of these materials in teak; however, the spectral characteristics contained flaws that led to some uncertainties. Not shown in Figure 3, but noticeable in GC analyses of several extracts, were small signals that have retention times corresponding to authentic samples of 1-methyl and 2,3-dimethyl AQ.

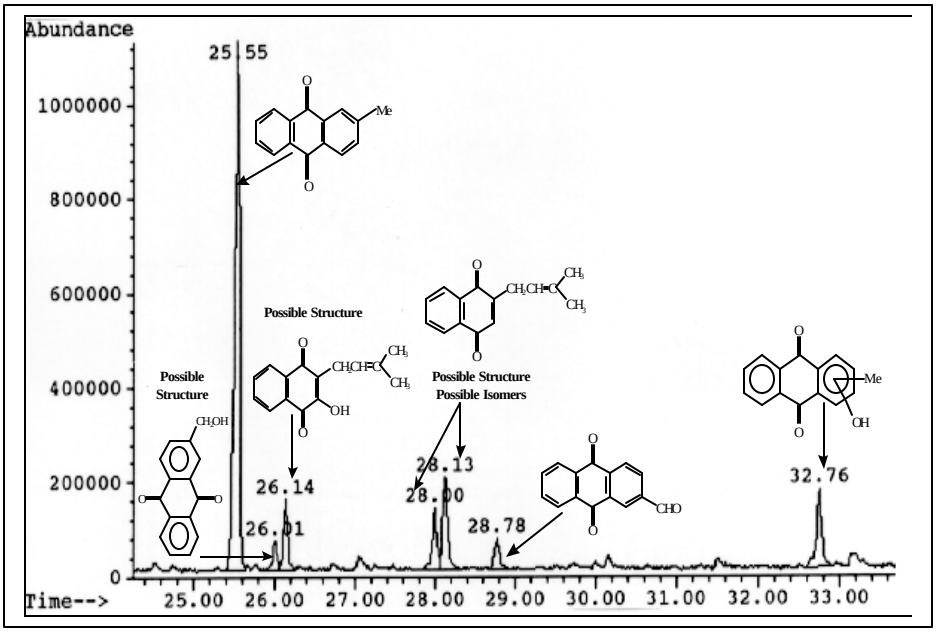

Figure 3. Chromatogram of teak extract showing the most abundant compounds and their structures or possible structures (11).

While additional characterization studies are needed, it is apparent that the extract contains several potentially active AQ components, the major one being 2-methyl AQ. The efficacy of quinones as soda pulping catalysts is reported (5) to be: AQ, 1.00; 2-methyl AQ, 1.04; 1-methyl AQ, 0.90; 2,3-dimethyl AQ, 1.05; various dihydroxy AQs 0.4-0.7; 2-methyl-4, 5-dihydroxy AQ, 0.56; 2-methylnaphthaquinone and 2-hydroxynaphthaquinone, 0.44. In general, napthaquinones and oxygenated AQs display poorer pulping activities. The reported effectiveness of the catalysts was determined at a relatively high level of material (1%). One of the catalysts, 2,6/7-dimethyl AQ, reported to have an efficacy of ~1 at 1%, has a value of ~2 (twice as active as AQ) at typical pulping doses (0.025-0.1%)(5). This may be the case for several of the other studied compounds.

Analyses of teakwood annual rings from a fresh-frozen wood sample of a 14-year-old tree showed no 2-methyl AQ present in rings 1-4 (sapwood). As sapwood converted to heartwood between rings 5 and 6, 2-methyl AQ became detectable. All heartwood rings (6-14) contained 2-methyl AQ. Sandermann and Simatupang have reported a similar trend for teak (14). Antibodies for 2-methyl AQ and dimethyl AQ were prepared and used to

attempt identification of the cell type(s) storing AQ. Antibody staining produced variable (and un-repeated) results indicating that 2-methyl AQ is located in the heartwood parenchyma cells.

Survey AQs Present in Commercial Hardwood Trees and Loblolly Pine. Our genetic engineering goal is to increase flux through the pathway that leads to AQ. Increasing AQ levels in trees is easier when trees already contain the biosynthetic pathway and necessary genes for producing AQs. To identify a suitable candidate tree for gene transfer (and demonstrate the applicability of the strategy to other species), we conducted a study to determine the levels of AQs in a select number of trees. In addition, we hoped to address the question of whether there might be a correlation between anthraquinone content and ease of pulping a particular wood species.

Analysis Techniques. Finely chipped wood samples were extracted with chloroform. Concentrated extract was then analyzed by gas chromatography-mass spectrometry (GC-MS). Standard samples of AQ, 2-methyl anthraquinone (2-MeAQ), 2,3-dimethylanthraquinone (2,3-DiMeAQ), 2,6/7-dimethylanthraquinone (2,6/7-DiMeAQ) were first run to get GC retention times. In addition, we looked for the isomers of hydroxy, methyl anthraquinone (HOMeAQ), a component in teak (11). Our analysis of teak was relatively simple, since the level of the major component, 2-MeAQ, was ~5% of the organic extract (11). The new wood samples had ~2500 lower amounts of AQs, requiring more sensitive analytical techniques.

There are several ways to perform and analyze GC-MS data. The most common is to obtain a total ion chromatogram; a mass spectrum over a range of ion masses (i.e., 50-350) is recorded roughly every half second during the GC run. The data, stored in a computer file, provides whole mass spectra of the eluting compounds. Alternatively, one can use the data to examine only key ions as a function of time; this is referred to as selective ion monitoring (SIM). For example, you can ask to see the chromatogram for ions 208, 222, and 236 (the molecular ions of AQ, MeAQ, and DiMeAQ). If you get a 208 signal at the same retention time as AQ, you would conclude that the signal was due to AQ in your sample.

In our case, we frequently saw mass signals corresponding to a particular AQ component, along with several other mass signals due to one or more components eluting at the same time. In addition, SIM for m/z 208, for example, showed signals at several GC retention times, two of which were close to the retention time of anthraquinone. Thus, looking simply for the molecular ion of a particular species presented some ambiguities. It was important to examine a GC signal for all of the major key mass ions associated with a particular anthraquinone and to make certain that the relative ratios of the key ions were in agreement with a known spectrum of the compound. Table 1 lists the major ions associated with the AQs of interest.

Table 1. Molecular ions typical of anthraquinone fragmentation patterns.

COMPOUND	SIM FOR PEAK IDENTIFICATION	SIM FOR QUANTIFICATION
AQ	208, 180, 152	208, 180, 152
2-MeAQ	222, 207,194,166, 165	222, 194, 165
2,3-DiMeAQ	236, 235, 221, 208, 178, 165	236, 208, 165
HOMeAQ	238, 223, 210, 209, 182, 181, 152	

To identify the presence of AQ components in a wood extract, we ran a total ion GC-MS, followed by an SIM analysis of the collected data, looking specifically for those ions listed in Table 1. A positive assignment was made when we saw all the key ions in the right relative abundance and at the right retention time for a particular anthraquinone.

The next challenge was to quantify the AQ levels in the sample, even though they may co-elute with other components. This required that we run a second spectrum in which the mass selective detector was preset at the beginning of the acquisition to detect specific key ions, not a whole range of ions. With a limited selection, we collected only the ion information of interest and dwelt on each ion about 100 times longer than the norm mode. The advantages of this SIM technique are much improved sensitivity and an ability to overcome interference. The selected ions were the abundant ones present in the various AQ compound mass spectra (Table 1). Prior to analyzing an extract sample, we prepared standard solutions of AQ compounds and 2,6/7-DMAQ (the internal

standard) in $CHCl_3$, ran their GC-MS in a SIM data-acquisition mode, and calculated response factors from the select major ions (Table 1). We chose an internal standard that we knew was not present in the wood extracts, although it was similar to the compounds being studied.

Identification of AQs in Wood Extracts. The presence of select anthraquinones in the extracts was confirmed by matching GC retention times and mass spectra to standards, as outlined above. The extracts contained an abundance of GC signals, most of which we did not attempt to identify; some of these may have been other AQs. It should be pointed out that the presence of polyhydroxylated AQs, of which there may have been many (15), were of little interest to us since such compounds are relatively poor pulping catalysts (5). Our focus was primarily on those structures listed in Table 1 that we knew would be good pulping catalysts (5). Each GC component that showed a key ion was examined thoroughly using the total ion mode. Isomeric compounds such as a methoxylated or ethylated AQ were not evident.

However, of particular interest to us was a relatively strong GC signal with a retention time 20 seconds longer than AQ. It was observed in several cottonwood varieties, poplar, walnut, red oak, elm, and red maple and had a strong m/z 208 signal (same as AQ), a strong 165 signal, and a modest 180 signal, but not a 152 signal. The 180 (M-28 mass units) is a signature signal of the AQs, and the 165 is strong in the case of methylated AQs (see Table 1). Few organic compounds expel CO (28 mass units) in their MS. Anthraquinone shows three main ions of similar intensity, corresponding to the molecular ion (208) and fragment ions at 180 (loss of CO) and 152 (loss of a second CO), Figure 4 (10). The lack of a m/z 152 signal for the unknown component might indicate that the compound has only one CO unit. These facts, together with the appearance of a weak m/z 193 (M-15, loss of a methyl) signal, raised the possibility that the unknown was a methylanthrone, Figure 4. Losses of methyl groups from similar structures are not very pronounced, namely 5% and 18% for 1- and 2-methyl AQ, respectively.

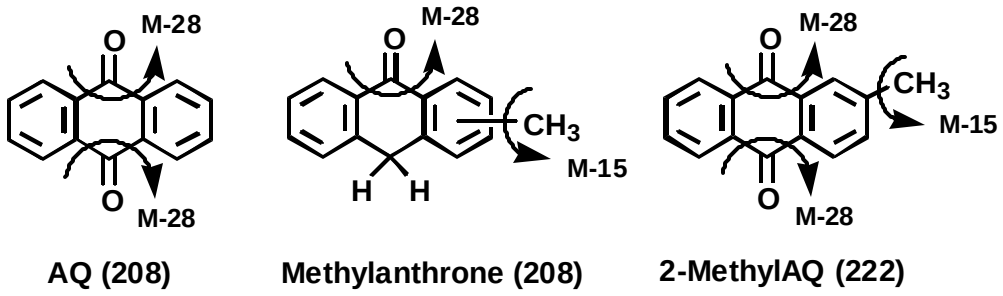

Figure 4. Proposed MS fragmentation processes of component structures (10).

Having an anthrone compound in the wood extracts heightened our interest since anthrone has good pulping catalysis activity (5). Since methylanthrones are not commercially available, we synthesized several mono- and dimethyl-isomers by reducing the corresponding methyl-substituted AQs with $NaBH_4$ (16). Reductions of 1- and 2-methyl AQ gave mixtures of 1-, 2-, 3-, and 4-methylanthrone. The only missing structure in the series, 10-methylanthrone, was not prepared because its MS was already available (17). Its spectrum displays a large M-15 signal (methyl loss stable benzyl ion); our unknown exhibited only a small M-15 mass spectral signal.

The synthesized anthrones showed modestly intense mass spectral signals (~40% of the molecular ion, base peak signal) corresponding to loss of a methyl group from the molecular ion and extremely weak m/z 180 (M-28) and modest m/z 178 signals. Since our unknown displayed just the opposite trends, it is most likely not a methyl anthrone. Moreover, the GC retention times of the synthesized methylanthrones did not match that of the unknown signal. Interestingly, an extract from an experimental poplar tree contained 1- or 4-methylanthrone (undistinguished isomers from our synthesis), along with 1-methyl AQ. Structure verification was apparent from comparison of GC retention times and mass spectra to authentic samples.

Quantification of AQs in Wood Extracts. Using the SIM GC-MS techniques described above, we analyzed wood extracts from (a) purchased, extensively dried lumber, (b) fresh or frozen wet wood samples, and (c) air-dried fresh chips (Table 2). Ten out of seventeen hardwood trees examined contained AQs; none of the three softwood

trees examined had detectable levels of AQs. Except for teak at ~0.7% extract (11), the levels of naturally occurring AQs in the hardwood samples were very low in the ppm-ppb range.

Our extensive analyses of several cottonwood species indicated quite varied levels of AQs between different species; the content ranged from 0-6 ppm. We observed no AQs in seven hardwood trees and all three softwood trees examined (the species indicated by footnotes g and h in Table 2). For these woods, we performed a total ion GC-MS of the extract, followed by an SIM analysis of the data and did not observe key ions at the retention time for a particular anthraquinone. A second, highly sensitive GC-MS acquisition, run to detect specific key ions, was not performed.

There were higher AQ levels in wet vs. dried chips of the same wood sample. This finding could be explained by partial evaporation of AQs due to drying, better distribution of the AQs into $CHCl_3$ in the presence of water, and/or a more open wet wood structure that allows better penetration of the $CHCl_3$. For dry samples, we consider the levels of observed AQs to be minimum values.

A poplar hybrid grown on an eastern Washington plantation was peculiar in that it was the only one to show 1-methyl AQ and 1/4-methylanthrone in its extract in rough amounts of ~6 and ~10 ppm, respectively. The levels of AQs were second in abundance to that of teak.

Table 2. Component levels in the whole wood samples.

Sample	**Type**	**AQ PPB**	**2-MeAQ PPB**	**DiMe AQ PPB**	**Sum of AQs PPB**
red oak	Wet	81	801	807	1689
red maple	Lumber	1196	293	327	1816
Elm	Lumber	300	725	1377	2402
Walnut	Lumber	219	257	86	562
e. cottonwood (EC)	Lumber	298	448	1264	2010
EC Stoneville[a]	Air-dried	n.d	22	n.d.	22
EC Ky[b]	Air-dried	8	66	n.d.	75
EC Ky[b]	Wet	31	90	n.d.	121
EC clone[c]	Air-dried	n.d.	70	n.d.	70
EC clone sap[d]	Wet	n.d.	92	n.d.	92
EC clone heart[e]	Wet	n.d.	89	n.d.	89
EC clone C175	Wet	197	618	n.d.	815
Poplar	Air-dried	n.d.	2045[f]	n.d.	2045
Poplar	Wet	n.d.	5686[f]	n.d.	5686
Other samples[g]	Lumber	n.d.	n.d	n.d.	n.d.
Other samples[h]	Air-dried	n.d.	n.d	n.d.	n.d.

[a]eastern cottonwood Stoneville 66, 11 yrs; [b]eastern cottonwood Kentucky Wild, 18 yrs; eastern cottonwood clone, Stoneville unidentified selection, 24 yrs, whole sample[c], sapwood[d], heartwood[e]; [f]1-methylanthraquinone; [g]normal GC-MS analysis of hickory, chestnut, blackjack oak, aspen, white spruce; [h]normal GC-MS analysis of *Eucalyptus viminalis* and *E. camadulenis*, Douglas-fir, loblolly pine, and eastern cottonwood Kentucky Wild, 30 yrs.

Repeat Assay in Milestone 1 Using Chips from AQ Containing Species Identified in Milestone 3. It is well known that softwoods are more difficult to pulp than hardwoods, a difference typically attributed to lignin content and type. However, could hardwoods pulp faster, in part, because they contain natural pulping catalysts? Based on our studies, we know that hardwoods contain low levels of simple AQs, probably much too low to have a positive impact on pulping. But could the collective concentrations of all the catalytic material present be enough to have an influence?

We attempted to answer this question by comparing the pulping of extracted and unextracted cottonwood. Since pulping catalysts should be removed by extraction, we expected delignification of the extracted

wood to be more difficult, resulting in higher kappa numbers. Naturally occurring surfactants such as fatty acids that would be removed during the extraction process might aid delignification as well. To determine their influence, we performed a control in which a Douglas-fir extract was added to an extracted-cottonwood cook. Douglas-fir contained no detectable amounts of simple AQs. As a further control, we also added the extract of cottonwood to the cook of an extracted cottonwood to make certain that physical factors such as moisture removal during extraction did not play a role. The amount of extract added to these cooks was equal in weight to what had been removed from the cottonwood.

The experiment was complicated by the fact that we were working with 0.55g samples. Multiple pulping runs, roughly 50 kraft and soda cooks, were performed to estimate the variability in the experimental design. We have had good success with the small cooks in other studies. The cooks were done on (a) cottonwood, (b) extracted cottonwood, (c) extracted cottonwood and its extract, and (d) extracted cottonwood with a Douglas-fir extract. The soda cooks were expected to show more differences than the kraft cooks, since the catalyst would have a higher impact in the soda case. In general, the differences in kappa numbers were small and within our experimental error for the (a)-(d) samples in each set of cooks. We are left to conclude that the natural level of catalysts in cottonwood is too small to have an impact.

We also examined two other wood species: elm and red oak. The data presented in Figure 5 (10) indicate that the extracted wood is more difficult to delignify under identical pulping conditions. However, control experiments (extractives add-backs) were not done in this case, so it is difficult to say with certainty if the effect observed is due to AQ removal.

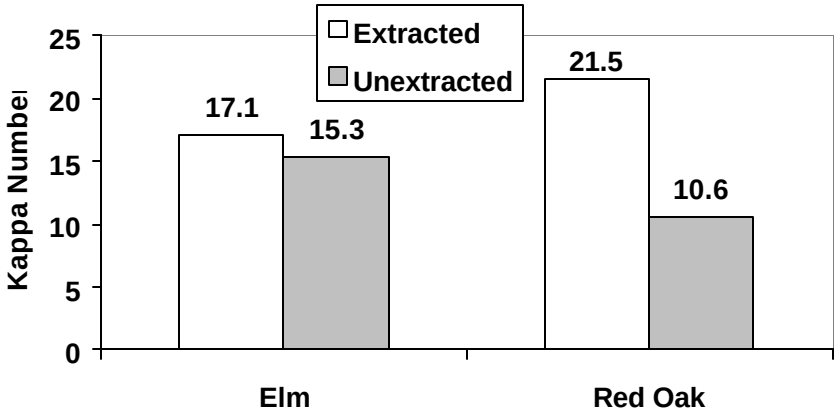

Figure 5. Kappa numbers of pulps obtained from identical kraft cooks (10).

A Global Strategy for Increasing the Level of Anthraquinones in Plant Tissues. In general, the level of enzyme activity at the first branch point or committed step controls the flux through a biosynthetic pathway. For example, in *E. coli* the level of isochorismate synthase regulates the amount of chorismate that is used for menaquionone and enterobactin C biosynthesis (18). In plants, the first branch point from the shikimate pathway to AQ biosynthesis is catalyzed by the enzyme isochorismate synthase, which converts chorismate to isochorismate (9). To test if plant AQ biosynthesis is regulated by the level of isochorismate synthase, we propose to over-express this synthase in plant cells. A partial cDNA encoding a putative isochorismate synthase is available from the random cDNA-sequencing project carried out with *Arabidopsis thaliana*. This partial cDNA shows a high degree of sequence similarity (41% identity over 220 amino acids) to the Haemophilus homolog of the *E. coli* entC gene that codes for isochorismate synthase. Starting with this clone we planned to isolate and sequence a full-length cDNA of the putative isochorismate synthase from *Arabidopsis thaliana*. To prove that this plant cDNA encodes an active isochorismate synthase, we planned to express it in an *E. coli* strain mutated in its isochorismate synthase gene to see if it would complement the mutant phenotype (19). If as expected this cDNA encodes for an active isochorismate synthase then we planned to place the cDNA under control of the strong 35S CaMV promoter and 3' terminator for over-expression in plants. To test the efficacy of this strategy we

planned to use teak callus, which makes 2-methyl AQ, or callus from a pulpwood tree with commercial interest. Eastern cottonwood became our target plant for isochorismate synthase over-expression with the finding that it contains several suitable pulping catalysts. If the level of isochorismate synthase is limiting for the biosynthesis of AQs, then we expect the AQ levels to increase in the transformed plant tissue above the levels detected in nontransformed tissue.

Isolate a Complete Sequence of Arabidopsis cDNA for Isochorismate Synthase. Isochorismate synthase (ICS) has been well studied and cloned from many prokaryotic and eucaryotic organisms. The study of this enzyme in plants lags behind other organisms. With a nested PCR strategy we synthesized a full-length ICS cDNA from an Arabidopsis cDNA library. Three nested primers (UP1, UP1a, and UP2a) were designed (Figure 6) according to the sequence of the partial fragment of isochorismate synthase. Yes1 and Yes2 primers corresponding to the λYes vector were also designed. This approach yielded a cDNA that was ~2000 base pairs long and encoded a protein of 503 amino acids with a predicted molecular weight of 55,368 Daltons. We thought this cDNA was a full-length cDNA encoding isochorismate synthase from Arabidopsis. However, recent evidence from *Catharanthus roseus* suggested that our initial cDNA was not full length and was missing potentially important amino terminal sequences (20). With further work we isolated the additional 5' end of the cDNA encoding the amino terminal region. Analysis of this protein sequence shows that it contains all of the characteristics of a transit peptide essential for directing the ICS enzyme in plastids. The putative full-length cDNA encodes for a 569 amino acid protein of ~62kDa. This full-length cDNA sequence was deposited in the gene bank (21).

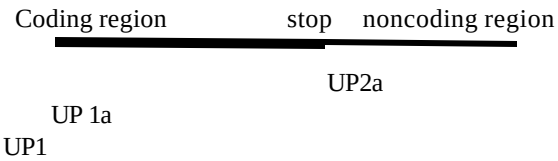

Figure 6. Arabidopsis isochorismate synthase DNA fragment and designed nested primers.

Expression studies, indicated by gel blotting, show that ICS mRNA and protein are present in leaves as well as roots. The mature protein of 59-60 kDA suggests that the transcript peptide is cleaved off the precursor that is predicted to be 62.7 kDA. Theoretical analyses of transit peptide sequence suggest that the ICS enzyme will be located in the chloroplast stroma. Wounding but not NAA or cold stress increases ICS mRNA accumulation in leaves (Figures 7 and 8).

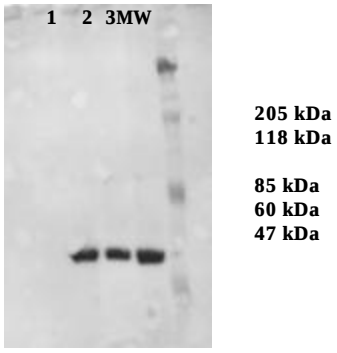

Figure 7. Protein gel blot with anti-ICS antibodies of total soluble protein from Arabidopsis leaves. (1) uninduced leaves, (2) 6-hour wounded leaves, (3) 12-hour wounded leaves.

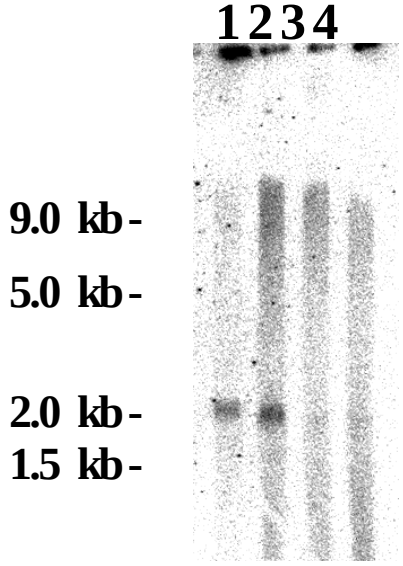

Figure 8. RNA gel blot of ICS (1) untreated leaves, (2) wounded leaves, 12h; (3) NAA, treated leaves, 12h; (4) cold-stressed leaves, 12h.

Clone ICS DNA into Appropriate Bacterial Vector, Conduct Complementation Assay. We have successfully over-expressed the Arabidopsis ICS protein in *E. coli.* The expressed protein was localized in the insoluble inclusion bodies and was not active in vitro. Mutants deficient in the *E. coli* ICS could not be obtained. Thus, no complementation test could be performed. However, relatively large amounts of the expressed protein were obtained and used to produce polyclonal antibodies in rabbits. These polyclonal antibodies have been shown to be of high titer in ELISA assays and react specifically to the ICS protein in protein gel blot analyses (Figure 7). This confirms our prediction of the reading frame and allows us to test in vitro the possibility that this cDNA will encode for an enzyme that catalyzes the conversion of chorismate to isochorismate in plant tissue.

Clone Isochorismate Synthase cDNA into Plant Expression Vector. We reconstructed a probable full-length cDNA containing the putative plastid transit sequence and inserted this "full-length" ICS cDNA (under the control of a duplicated 35S CaMV promoter and the TEV translational enhancer into a binary vector, pBIN-ICS. We then transformed it into *Agrobacterium tumefaciens* strain GV3101. This construct also encodes the gene, NP II, for kanamycin resistance, which acts as a selection agent for transformed tissue. PBIN-ICS is shown in Figure 9.

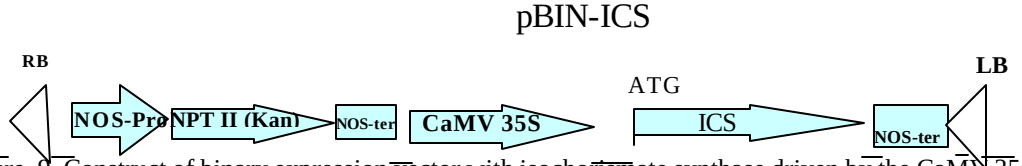

Figure. 9. Construct of binary expression vector with isochorismate synthase driven by the CaMV 35S promoter.

Transfer ICS DNA into Teak Callus, Arabidopsis, and/or Target Tree. We decided to focus our efforts on increasing the natural production of cottonwood AQs by over-expression of the isolated ICS cDNA. This decision was based on the following: (1) eastern cottonwood produces several excellent AQ pulping catalysts; (2) IPST has experience in the genetic transformation of eastern cottonwood; and (3) the AF&PA /DOE Agenda 2020 Program recently placed an emphasis on the improvement of fast-growing cottonwood trees.

Three transformation experiments for eastern cottonwood clone C175 were executed, two with the plastid transit sequence containing the ICS construct and one with the ICS construct that lacks the plastid transit sequence. Eight lines of putatively transformed tissue were developed. All lines formed shoots and subsequently grew roots on media containing kanamycin indicating presence of the kanamycin gene. Both plant regeneration and rooting in the presence of the selection antibiotic, kanamycin, are strong indicators that tissue carries the desired foreign genes. To date we have been unsuccessful in proving that these lines contain the ICS gene. PCR analysis with NP II primers indicates the presence of this gene in the lines; however, only two of these lines contain PCR products with the ICS primers.

At this point the project has used all of its funding. However, we are continuing two approaches to confirm if AQs are elevated in the transformed lines: (1) to grow sufficient shoot tissue to assay for AQs by GC/MS and (2) to produce several greenhouse-grown trees for each line and to assay tissue AQ content when size is sufficient. Comparison of AQ levels between transformed and control (nontransformed) shoot and whole plant tissues will indicate if our genetic engineering strategy was successful. If increased anthraquinone content is detected we will seek additional funding for further molecular biology analyses of transformed and control tissues.

Assay AQ Concentration in Transformed Teak Callus, Model Species (Arabidopsis), and Target Tree.
Two cottonwood shoots have been regenerated from each line, rooted, and transferred to sterile soil. Containers were closed with "Sun Caps", a clear membrane with inserts that allow container humidity content to reduce with time. Most of these plants have now acclimated to the soil/reduced humidity environment and are now showing new height growth. Plants will be transferred to a greenhouse in spring 2001 and grown to sufficient size for wood sampling and anthraquinone tissue analysis. Anthraquinones have also been quantified in the non-transformed parent shoot culture line.

To our surprise, nontransformed control shoots grown in our protocol showed unsubstituted anthraquinone content of 1874 ppb. As indicated above, the next step is to grow and collect enough shoot material to assay each putatively transformed line for AQ content. If AQ analyses of either putatively transformed shoots or greenhouse plants show AQ content greater than nontransformed controls, then we will return to analysis of the gene(s) present in lines with stimulated AQ production.

Funding for this project ended August 18, 2000. Research activity to complete this project will proceed slowly when small amounts of funding are available.

BENEFITS TO THE INDUSTRY IF THE RESEARCH YIELDS PROMISING RESULTS: If wood AQ content is sufficiently increased, the pulp and paper industry will benefit. On a worldwide basis, during 1990 nearly 30% of paper and paperboard capacity and 34% of pulp capacity was located in the U.S. (22). During 1995 approximately 66 million tons of wood pulp were produced in North America. Over 54 million tons (84%) were produced as chemical pulps, the rest falling into thermomechanical and semichemical categories. Assuming a conservative AQ-related increase in yield of l%, we calculate approximately 1/2 million additional tons of chemical pulp could have been generated using AQ technology. Assuming a value of $400 per ton of pulp, AQ pulping has the potential to increase product value by over $200 million. Today, the limiting factor for increased use of AQ technology is the cost of AQ. The commercial availability of fast-growing trees containing economic levels of pulping catalysts will have several significant impacts on competitiveness of the U.S. pulp & paper industry and the U.S.

1. A low-cost supply of AQs will become available in the wood itself, thus eliminating a cost bottleneck for the use of AQ pulping. Trees containing catalysts could be combined with normal trees in ratios varying with species and process needs.
2. Pulp yields will increase causing a decrease in pulping costs.
3. Capital equipment will be used more effectively. AQ pulping improves efficiency of capital equipment by improving pulping productivity, lowering environmental impacts, and simplifying the chemical recovery system.
4. Trees with built-in pulping catalysts will be easily pulped and require little if any sulfur delignification agents creating a substantial reduction in odor emissions.

5. Methods for gene isolation, transfer, and regulation in forest trees and knowledge of anthraquinone synthesis chemistry will be very useful in further modifications of trees for higher value. Success in this project will encourage further tree improvements through biotechnology.

Genetically engineered trees containing pulping catalysts can readily be integrated with current forestry operations in the U.S. Once elite seedlings have been created they may be moved into the field using conventional practices. No additional training or equipment will be required. The initial creation of high-value trees is the principal limiting factor. While this project is oriented towards the Agenda 2020 environmental performance programs, it also meets goals within the capital effectiveness and sustainable forestry programs. Additional expected benefits include the development and use of methods to target genetic engineering of wood-forming tissues and an improved understanding of AQ synthesis and pulping chemistry.

PUBLICATIONS RESULTING FROM THIS RESEARCH:

1. Dimmel, D., J. MacKay, G. Pullman, E. Althen, and R. Sederoff. 2000. Improving Pulp Production with Raw Material Changes. Submitted to TAPPI Pulping/Process & Product Quality Conference. November 5-8, 2000, Boston, MA.
2. Dimmel, D. R., P. I. Sklar, K. E. Crews, and G. S. Pullman. 2000. Pulping Catalysts in Trees. Journal of Wood Chemistry and Technology, 20 (3) 225-242.
3. Leyva, A., Dimmel, D. R., and Pullman, G. S. 1998. Teak Extract as a Catalyst for the Pulping of Loblolly Pine. Tappi Journal. 81(5): 237-240.
4. Meng, H., G. Pullman, and G. F. Peter. 1998. Cloning of a Plant Isochorismate Synthase (Accession No. AF078080) (PGR98-214). Plant Physiol. 118: 1536

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