ANNUAL PROGRAM REVIEW

FOREST BIOLOGY

October 14-15, 1998

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FOREST BIOLOGY FALL PAC REVIEW
AGENDA

Wednesday, October 14, 1998 (Seminar Room)

10:00 A.M.	Welcome, introduction, antitrust statement	McCullough

F-010 (SOFTWOODS) DUES FUNDED CONSORTIUM PROJECTS
(1.1 Professional, 4 Support Staff)

10:15	F-010 Mass Clonal Propagation of Improved Conifers Summary of Accomplishments Since Last Meeting, Goals, Personnel, Grants,	Pullman
10:30	Softwood Embryogenesis	
	Initiation	Pullman
	Genetic Analysis of Initiation	MacKay
	Culture Survival & Multiplication	Peter
	Embryo Development	Montello
	Plating Process Optimization	Vales
	Conversion Update	Montello
12:00	Lunch	
1:00	Zygotic Embryogenesis	
	Levels of Free Amino Acids	Zhang
	Full term embryo Ion analysis (NH_4, NO_3, anion)	Pullman
	Zygotic vs. Somatic Germination-Partial Drying	Peter
2:00	Gene Expression During Embryogenesis	
	Introduction	Cairney
	Suspensor-specific genes	MacKay

EXTERNALLY FUNDED & STUDENT PROJECTS RELATED TO F010

2:40	Gene Expression During Embryogenesis	
	Zygotic & Somatic	Cairney
	Expression analysis of stage specific genes	Cairney
3:10	Break	
3:20	Vegetative Expression of Floral Genes in Loblolly Pine	
	Summary	Cairney

F-011 DUES FUNDED CONSORTIUM PROJECTS (0.4 Professional, 1.0 Support Staff)

3:40	F-011 Fundamental Biological Mechanisms: better fibers & faster growth rates Summary of Accomplishments Since Last Meeting, Goals, Personnel, Grants	Peter
4:20	Cellulose Synthesis	Peter

EXTERNALLY FUNDED PROJECTS RELATED TO F011

4:40	Towards Trees with Built-In Catalysts	Meng
5:30	Dinner & Speaker – Biotechnology Patenting - Dr. John McDonald from Jones & Askew	

Thursday, October 15, 1998 (Seminar Room)

8:00 A.M. Coffee and Donuts

NON-DUES FUNDED CONSORTIUM PROJECTS RELATED TO F011 CONTINUED

8:15	Microfibril Angle Measurement (student research)	Benton/Peter

NEW PERSONEL, GRANTS ACTIVITY, STUDENT RESEARCH, PUBLICATION ACTIVITY AND DISCUSSION OF DUES-FUNDED CONSORTIUM RESEARCH

8:35	Grant Proposal Activity, Student Research, Publications, IPST meeting host	Pullman, Peter MacKay, Cairney
9:00	Comments on Research Programs, Questions, Discussion, Issues	PAC
10:15	Break	
10:30	Continued discussion	
11:00	IPST Intellectual Property Policy	Baum
12:00	Adjourn (Lunch will be available at 12:00)	

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SOFTWOODS

IPST DUES FUNDED RESEARCH CONSORTIUM
1998-1999

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS

Status Report for Project F010

Gerald Pullman
John Cairney
Gary Peter
John MacKay
Xiarong Feng
Barbara Johns
Shannon Johnson
Paul Montello
Christina Perfetti
Heidi Schindler
Teresa Vales
Yalin Zhang

October 14-15, 1998

DUES-FUNDED PROJECT SUMMARY

Project Title:	MASS CLONAL PROPAGATION OF IMPROVED CONIFERS
Project Code / Project Number:	SFTWD / F-010
PAC:	FOREST BIOLOGY
Project Duration:	FY 1998-1999
Project Staff	
Faculty/Senior Staff:	Jerry Pullman, John Cairney, Gary Peter, John MacKay
Staff:	Barbara Johns, Paul Montello, Christina Perfetti, Teresa Vales, Cielo Castillo, Yalin Zhang, open, temporary help
Project Funding:	
FY 98-99 Budget:	$440,000
Allocated as Matching Funds:	11%
Time Allocation	
Faculty/Senior Staff:	1.3
Support:	4.2
Supporting Research	
M.S. Students:	1
Ph.D. Students:	2
External:	$144,377

RESEARCH LINE/ROADMAP:

Area:	Improved Forest Productivity
Research Line:	Improve the fiber productivity of North American lands so that they are competitive in the world pulpwood market.
Road Map:	Develop improved trees via genetic manipulation.

BENEFITS TO INDUSTRY:

- Will provide methodology to multiply high-value loblolly pine trees
  Increasing growth rate
  Increasing wood uniformity
- Will provide foundation for genetic engineering of loblolly pine

PROJECT OBJECTIVE: Develop reliable cell & tissue culture systems for the mass clonal propagation of genetically improved softwoods.

PROJECT BACKGROUND:

A continued supply of low cost, high quality raw materials is essential for the future success of the U.S. forest products industry. The continual loss of forest lands to urban growth, continued and new environmental regulations, and the rapid growth occurring in competitive forest plantations abroad put intense pressure on U.S. companies to increase the yield of wood per acre. If the industry is to grow, it must sustain reliable low cost sources of raw materials. The clonal propagation of high value forest trees from breeding and genetic engineering programs has the potential to help meet future industry needs by increasing forest yields and improving raw material uniformity and quality.

GOALS FOR FY 98-99:

1. Improve initiation protocol to meet target of 35%.
    - Run, evaluate and report winter initiation experiments.
    - Run, evaluate and report summer initiation experiments.
    - Begin characterization of environmental & genetic variability during initiation.
    - Improve initiation survival
2. Improve quality of and ability to monitor early-stage embryos in liquid media.
    - Identify stains to distinguish and monitor live embryos during automated counting
    - Develop metals analysis-based maintenance medium
3. Test hypotheses for causes of culture decline
4. Metals analysis of somatic and zygotic embryos.
    - Analyze somatic embryos grown on analysis-based media modifications.
    - Analyze additional zygotic embryo & female gametophyte tissue over embryo development cycle.
5. Evaluate new cultures for performance in improved protocols.
6. Improve maturation protocol.
    - Optimize live embryos per plate
    - continue work on analysis-based improvements
    - continue work on ABA / osmoticant control of embryo quality
    - re-implement, improve, and standardize ABA analysis for somatic & zygotic tissues
7. Establish 50+ seedlings from each of 5 genotypes in a plantation setting.
8. Continue work on analysis of osmoticants in zygotic embryos and female gametophyte tissue.
    - amino acids
    - sugars
9. Complete development of a set of high quality cDNA libraries representing staged loblolly pine zygotic embryo development.
10. Investigate role of suspensor during embryo development
    - isolate tissue specific genes expressed in the suspensor
    - investigate function & role of major suspensor genes
11. Gene expression during conifer embryogenesis.

- Identify marker genes which are active at specific stages of zygotic embryo development.
- Clone and sequence gene fragments.
- Use information to expand usefulness of these markers, applying them to somatic genotypes.
- Repeat steps above for somatic embryos.
- Begin to determine where (anatomically) in embryo specific genes are expressed.
- Develop reliable quantification tools / protocols for assaying gene expression
- Demonstrate reproducibility of results for Dot Array Southern
- Monitor gene activity under different tissue culture conditions
    - relate to embryo quality
    - compare to zygotic embryo gene activity patterns

EXTERNALLY FUNDED RESEARCH COMPLEMENTING F010 GOALS

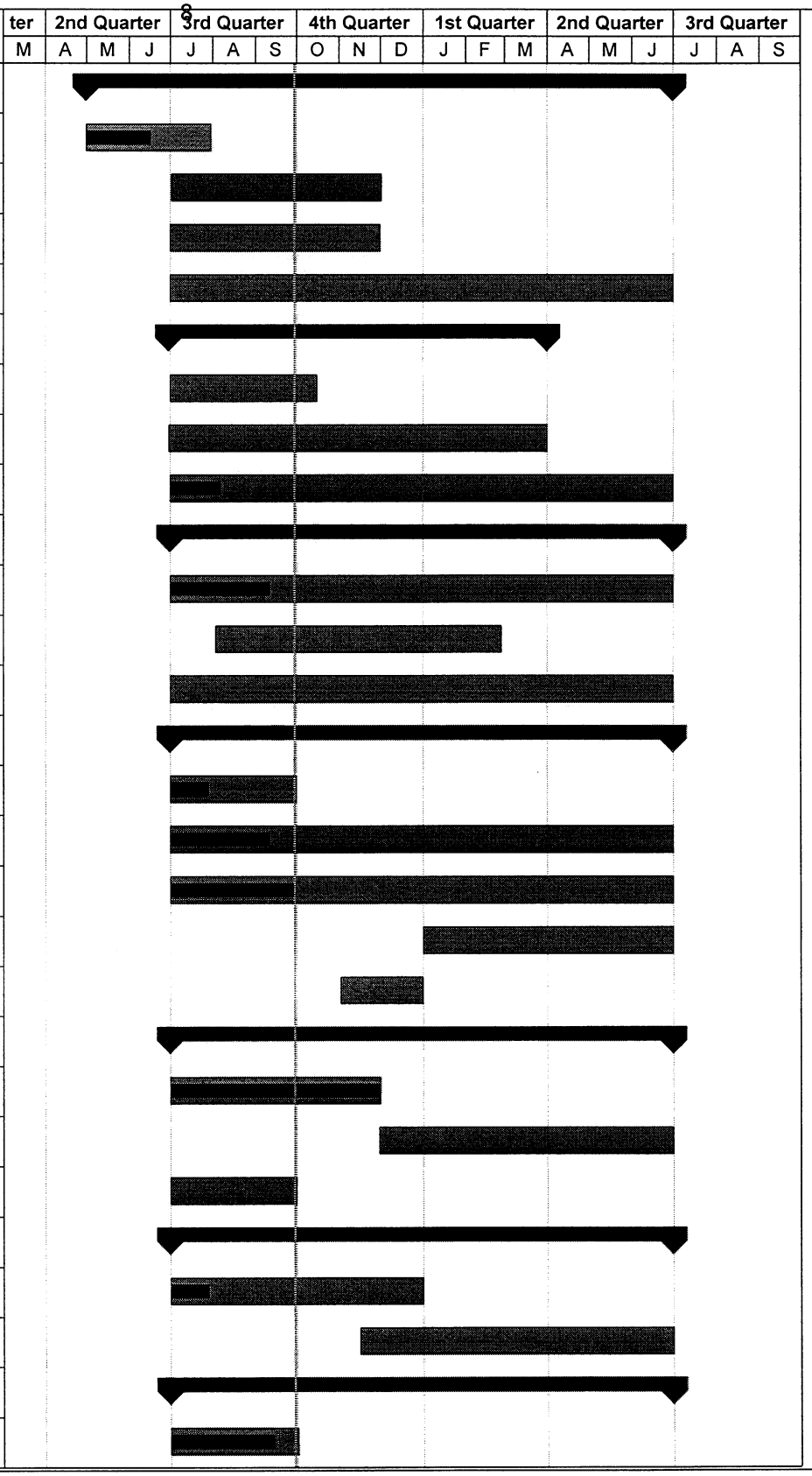
- Ensuring Fiber Supply Through Biotechnology: New Tissue Culture Tools from Gene Expression Studies. Tip[3]. Nanfei Xu, G. Pullman, J. Cairney ($144,377)

DELIVERABLES:

Report describing results and research progress in the fall of 1998.
Annual report describing results and research progress in the spring 1999.

SCHEDULE (See Attached Timeline)

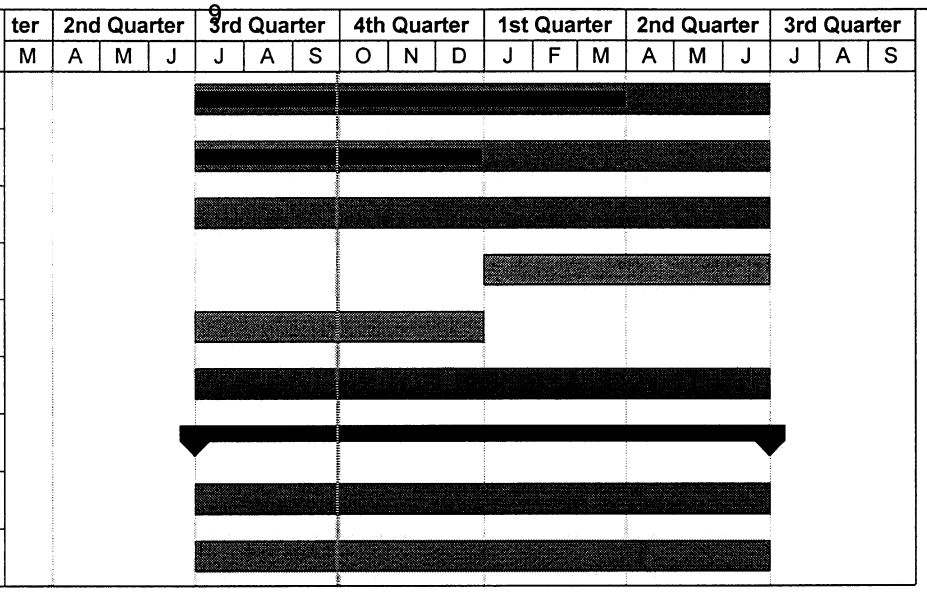
ID	Task Name
1	**Initiation at 35%**
2	Run & Evaluate Winter Expts.
3	Run & Evaluate Summer Expts
4	Begin characterization of environmental & genetic variability
5	Improve initiation survival
6	**Improve quality of & ability to monitor early-stage embryos in liquid media**
7	Identify stains to distinguish & monitor live embryos during
8	Develop metals analysis-based maintenance medium
9	**Test hypotheses for causes of culture decline**
10	**METALS ANALYSIS OF Somatic and Zygotic Embryos**
11	Analyze somatic embryos grown on metals analysis-based media modificatins
12	Analyze additional zygotic embryo & female gametophyte tissue over embryo
13	**Evaluate new cultures for performance in improved protocols.**
14	**Improve maturation protocol.**
15	Optimize live embryos per plate
16	Continue work on analysis-based improvements
17	Continue work on ABA / osmoticant control of embryo quality
18	Re-implement, improve, and standardize ABA analysis for somatic
19	Establish 50+ seedlings from each of 5 genotypes in a plantation setting.
20	**Continue work on analysis of osmoticants in zygotic embryos and**
21	Amino acids
22	Sugars
23	**Complete development of a set of high quality cDNA libraries representing**
24	**Investigate role of suspensor during embryo development**
25	Isolate tissue specific genes expressed in the suspensor
26	Investigate function & role of major suspensor genes
27	**Gene expression during conifer embryogenesis.**
28	Identify marker genes which are active at specific stages of zygotic embryo



Project: F010 Goals Gant Chart
Date: Tue 9/29/98

Task
Progress
Milestone
Summary
Rolled Up Task
Rolled Up Milestone
Rolled Up Progress

ID	Task Name
29	Clone and sequence gene fragments.
30	Use information to expand usefulness of these markers,
31	Repeat steps above for somatic embryos.
32	Begin to determine where (anatomically) in embryo specific
33	Develop reliable quantification tools / protocols for assaying gene
34	Demonstrate reproducibility of results for Dot Array Southern
35	Monitor gene activity under different tissue culture conditions
36	Relate to embryo quality
37	Compare to zygotic embryo gene activity patterns



Project: F010 Goals Gant Chart
Date: Tue 9/29/98

Task
Progress
Milestone
Summary
Rolled Up Task
Rolled Up Milestone
Rolled Up Progress

IPST-DFRC F010

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS - INITIATION

Gerald Pullman, Yalin Zhang, Nazima Allaudeen, Anneil Basnandan, Sean Coy, Chris Culbreth, Jessica Halprin, Allison Snow, Shannon Johnson

Summary

Optimization of the cytokinin level has resulted in statistically significant increases in initiation rates. This increase has resulted from two experiments during winter and summer 1998 with tests for eight cone collections. Ovules from additional cone collections currently on the improved medium show early initiation rates (8 week observations) consistent with the averages seen in the two experiments.

Introduction

The initiation of an embryogenic culture or embryo suspensor mass (ESM) is the first step in cloning the embryo(s) from a valuable conifer seed. The process in loblolly pine starts with an immature seed. The seed is sterilized and the seed coat removed to expose the ovule or female gametophyte which contains the early-staged embryo(s). The whole female gametophyte (megagametophyte) is placed on a chemically defined medium and incubated in the dark at 22-24 C. The process of initiation then occurs in several phases: extrusion of zygotic embryos, formation of the first somatic embryos, and multiplication of somatic embryos. The results that we report are for successful progression through these three steps resulting in at least three visible somatic embryos emanating from a zygotic embryo(s).

Initiation Improvements

Our program continually strives to reach our target of 35% loblolly pine initiation among many families. Last spring (March 1998 PAC Meeting) we reported that the addition of abscisic acid and silver nitrate improved initiation rates. This hypothesis was based on the fact that Renee Kapik's doctoral thesis showed significant levels of ABA present during the period of early embryo development when natural cleavage polyembryony (multiplication) occurs. This suggested that embryo initiation and maintenance may be improved by the addition of ABA. This hypothesis was confirmed over two years of IPST research resulting in an improved initiation medium. Other laboratories also experimented with the addition of abscisic acid to initiation medium over the past several years. Patent # 5,677,185 was granted to Westvaco Corporation for the use of ABA during somatic embryo initiation for a list of *Pinus* species. New Zealand researchers (Jenny Aitken-Christie of Baker Holding Company) also applied, a few days after Westvaco, for a world patent on a similar concept for initiation in conifers or other woody species.

With this improved initiation medium we proceeded this winter and summer to further improve the medium. The increased initiation rates provided an opportunity to test several

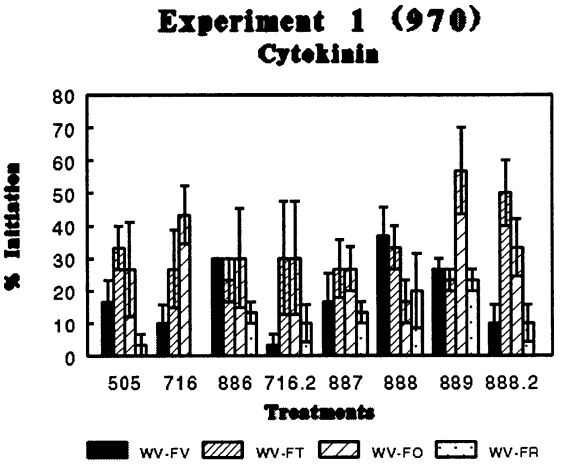
hypotheses that had been promising in the past but never yielded statistically significant increases. One hypothesis was that with the addition of activated carbon in the initiation medium we had not successfully optimized the cytokinin levels. Through modifying the cytokinin level in the initiation medium we have now further increased initiation rates. We will attempt to document the key IPST experiments that lead to this improvement in our initiation system. Unless otherwise mentioned all experiments are modifications as described of media 505 and 716 (505 + 1ppm Abscisic acid + 20 mM silver nitrate). Table 2 shows the composition of medium 505, 716, and modifications.

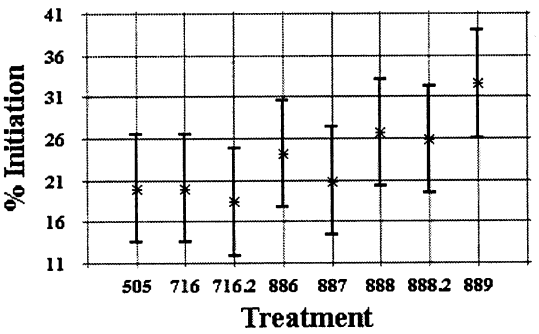
Experiments and Results

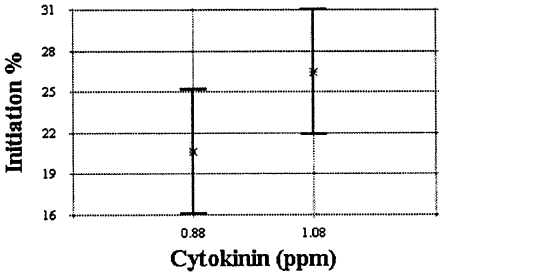
Experiment 1 (970, Winter 1998). Medium 505 was tested in a 2 x 4 factorial design with two levels of cytokinin (0.88 and 1.08 ppm) and four treatments testing no ABA or silver nitrate, inclusion of ABA and silver nitrate, inclusion of ABA and silver nitrate with cyclic GMP, and ABA and silver nitrate but wrapped with a gas permeable tape for increased water loss. Initiation results show no increase in initiation due to ABA / silver nitrate, cGMP, or alternative wrapping materials. However, initiation showed a (statistically) significant (P=0.07) increase with the change in cytokinin from 0.88 to 1.08 ppm. Across the four cone collections tested initiation increased from 20.6% to 26.5% with the increase in cytokinin concentration.

Experiment 970

Media	ABA	AgNO3	Cytokinin	cGMP	Tape
505	0 ppm	0 mM	0.88 ppm	0 µM	parafilm
716	1	20	0.88	0	parafilm
886	1	20	0.88	10	parafilm
716.2	1	20	0.88	0	3M Tape
887	0 ppm	0 mM	1.08 ppm	0 µM	parafilm
888	1	20	1.08	0	parafilm
889	1	20	1.08	10	parafilm
888.2	1	20	1.08	0	3M Tape

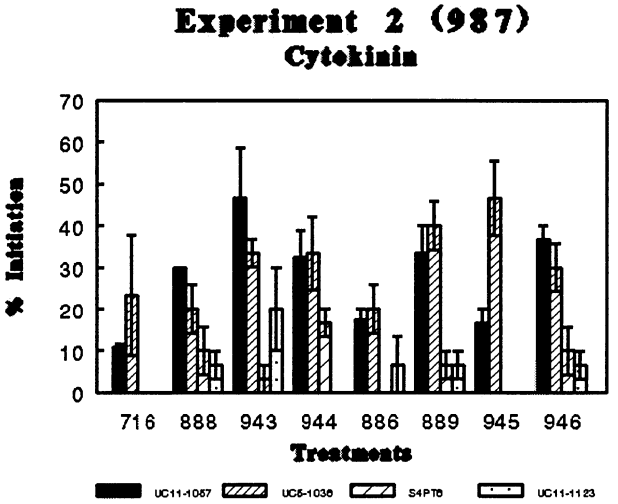


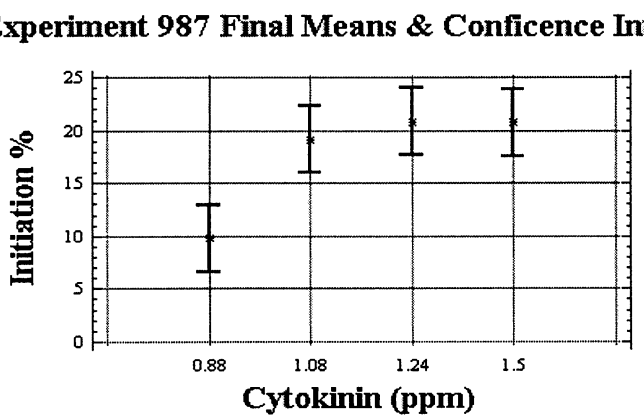




Experiment 2 (987, Summer 1998). This experiment again used a 2 x 4 factorial design to test two levels of cGMP (0 and 10 μM) against four levels of cytokinin (0.88, 1.08, 1.24, and 1.50 ppm). This experiment was our first Summer 1998 experiment and has proceeded long enough for a final evaluation. Results indicate that cyclic GMP did not cause a change in initiation rate. However, again increasing cytokinin caused initiation rates to increase. Results showed increased initiation with all levels of raised cytokinin. Cytokinin levels of 1.08 to 1.5 approximately doubled initiation when compared to 0.88 ppm cytokinin.

Experiment 987				
Media	Cytokinin (ppm)	ABA (ppm)	AgNO3 (mM)	cGMP (μM)
716	0.88	1.0	20	0
888	1.08	1.0	20	0
943	1.24	1.0	20	0
944	1.50	1.0	20	0
886	0.88	1.0	20	10
889	1.08	1.0	20	10
945	1.24	1.0	20	10
946	1.50	1.0	20	10





Additional Experiments. Eight-week initiation ratings for medium 889 are listed in Table 1 for an additional 4 cone collections. Initiation percentages are again favorable compared to previous years.

1997-1998 Winter and Early Summer Initiation Frequencies and Comparison with Past Years
Table 1 shows a summary of initiations for media 505 and media improvements this winter and for the first Summer 1998 initiation experiments. Cones from ½ sib families were collected from 13 families. All families contained early zygotic embryos at stages 2-4 that were suitable for experimentation. All families were able to initiate cultures with initiation rates ranging from 7 to 57%. Table 1 also compares initiation rates for medium 505 during 1995, 1996, and 1997 for 32 loblolly pine mother trees. Average initiation for the 13 families tested on medium 889 was 27.5% which compares favorably with 8.5% and 7.4% initiation on medium 505 during 1996 and 1997 respectively.

Table 1. Loblolly pine cone collection mother trees with 1995, 1996, and 1997 and 1998 initiation rates on medium 505 and improved medium.

Tree Identification	Initiation % 1995	Initiation % 1996	Initiation % 1997	Initiation % 1998		
	Medium 505	Medium 505	Medium 505	505	716	889
BC-1 (S4PT6)		6.7%			0	6.6
BC-2	10%	0.0%				
BC-3		3.2%				
BC-5		9.3%				
BC-8						
BC-9	17%	10.7%				
C7-2			0			
C7-88			1.7			
C7-100			0			
C8-76			4.8			
C10-14			0			
C10-38			6.7			
UC5-1036	32%	7.9%	3.3		23.3	40
UC5-1507			8.9			
UC7-1037	10%					
UC7-1051		4.5%	3.3			
UC10-1027	33%	13.8%				
UC10-5	3.3%					
UC10-33	12%					
UC11-1055		3.3%	2.2			8wk, 20
UC11-1057		15.3%	23.7		10.7	33.3
UC11-1066		10.0%				
UC11-1069		4.4%				
UC11-1123			1.3		0	6.6
UC18-1212			23.6			
F-2	11%					
FO				26.7, 17.4	43.3	56.7, 50
FR				3.3	0	23.3
FT				33.3, 16.7	26.7	23.3, 33.3
FU				13.3		16.7
FV				16.7	10	26.7
G-2						
H-2, 3, 4		6.7%	26.7			H4, 40
I-2, 3	15%	3.3%	12.2			
J-2, 3		7.0%	10.3			
K-2, 3		19.0%	8.9			
L-4						20
M 9-1019						23.3
Overall	16%	7.4%	8.5%			27.5

BC = Boisie Cascade, C = Champion, UC = Union Camp, WV = Westvaco, M = Mead

Table 2. Media composition for initiation medium 505, 716, and 889.

Components	505	716	889
NH_4NO_3	200.0	200.0	200.0
KNO_3	909.9	909.9	909.9
KH_2PO_4	136.1	136.1	136.1
$Ca(NO_3)_2 \bullet 4H_2O$	236.2	236.2	236.2
$MgSO_4 \bullet 7H_2O$	246.5	246.5	246.5
$Mg(NO_3)_2 \bullet 6H_2O$	256.5	256.5	256.5
$MgCl_2 \bullet 6H_2O$	101.7	101.7	101.7
KI	4.15	4.15	4.15
H_3BO_3	15.5	15.5	15.5
$MnSO_4 \bullet H_2O$	10.5	10.5	10.5
$ZnSO_4 \bullet 7H_2O$	14.668	14.668	14.668
$Na_2MoO_4 \bullet 2H_2O$	0.125	0.125	0.125
$CuSO_4 \bullet 5H_2O$	0.1725	0.1725	0.1725
$CoCl_2 \bullet 6H_2O$	0.125	0.125	0.125
$AgNO_3$	--	3.398	3.398
$FeSO_4 \bullet 7H_2O$	13.9	13.9	13.9
Na_2EDTA	18.65	18.65	18.65
Maltose	15,000	15,000	15,000
myo-Inositol	20,000	20,000	20,000
Casamino Acids	500	500	500
L-Glutamine	450	450	450
Thiamine•HCL	1.0	1.0	1.0
Pyridoxine•HCL	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5
Glycine	2.0	2.0	2.0
NAA	2.0	2.0	2.0
BAP	0.45	0.45	0.55
Kinetin	0.43	0.43	0.53
Activated Charcoal	50	50	50
ABA*	--	1.0	1.0
GMP*	--	--	10uM
Gelrite	2,000	2,000	2,000
pH	5.7	5.7	5.7

* = is added filter sterilized.

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS

Genetic Control of Initiation and the Number of Zygotic Embryos in the Seed.

John MacKay
Heidi Schindler
Christina Perfetti
Jerry Pullman
Yalin Zhang

Summary

Initiation of somatic embryogenic cultures has become increasingly successful however variation between seed sources poses potential challenges to the development of robust methods applicable to a wide range of pine genotypes. This report explores genetic variation of the number of zygotic embryos per seed and its potential effect on initiation. The overall goal of this work is to identify factors (genetic or other) that control initiation and thus gain insights that will help improve initiation methods. Our preliminary assessment indicates that the number of zygotic embryos per seed is usually positively correlated with initiation rate. Genetic variation for this trait between open pollinated families is extensive, suggesting strong genetic control. Other non-genetic factors may also contribute to the variability, but less extensively.

BACKGROUND AND RATIONALE

Initiation of somatic embryogenic cultures from immature seed of loblolly pine has been increasingly successful in recent years, with average initiation rates between 8% and 15%. However in many cases, initiation rates of different seed sources vary significantly. While some sources initiated quite readily others appear recalcitrant. It is usually inferred that much of this variation is due to the genotype of the seed, inherited from parent trees. A few studies carried out in spruce support the hypothesis that the response to initiation treatments is indeed under genetic control (e.g. Park et al., 1993). In pine, individual mother trees were characterized as producers of seeds with either low, moderate or high initiation rates (Becwar and Chesik, 1994). Variation in initiation rates poses a challenge to the development of tissue culture methods that are sufficiently robust to succeed with a wide range of genotypes. It may also provide an opportunity to identify the driving forces that control initiation rates. In other words, by identifying the factors (genetic or other) that cause initiation rates to vary, it may be possible to develop improved methods that will give higher success rates with a greater number of seed sources.

Genetic analysis of the response to initiation protocols could provide insights needed to develop tissue culture methods that will effectively capture an increased number of desirable genotypes. Quantitative genetic analyses of initiation, although desirable, are technically demanding and very time consuming. Becwar *et al.* (1997) showed that the number of zygotic embryos present in the seed at the time of initiation may be correlated with the initiation rate and, therefore could be a driving force that controls initiation. This trait can be rapidly assessed and does not require tissue culture. Our goal is to explore if this trait could be used to help focus the genetic analyses of initiation.

Polyembryony in pine

Multiple embryos (polyembryony) are formed early during development of pines seed, although only one usually reaches maturity. Several archeagonia (usually 1 to 4) are

formed in each seed and thus there can be several fertilization events (simple polyembryony). Soon after fertilization, each of the resulting embryos undergoes cleavage, and goes from one to four embryos. This is referred to as cleavage polyembryony and has been reported to generate up to a total 16 embryos per seed in loblolly pine. The resulting cohort of embryos develops more or less synchronously until one emerges as the dominant embryo and the other (subordinate) embryos will not develop beyond stage 2 (20 -30 cells).

SPECIFIC OBJECTIVES

1. Make a preliminary assessment of the impact of the number of embryos on initiation
2. Assess the variation in the number of embryos per seed between and within diverse seed sources
3. Examine potential environmental sources of variation for the number of embryos
4. Follow the evolution of the number of embryos during normal development

METHODS

Plant material. The number of post cleavage embryos per seed was counted by extracting the developing embryo tissues from 10 to 20 seed per cone and staining on a microscope slide in 2% aceto-carmine for 10 minutes or more. For each seed the stage of the dominant embryo and the number of embryos (dominant and subordinate) were determined by using a light microscope (50 X magnification). To ensure uniformity of developmental stage, two or three cone collections were obtained and staged; the data were obtained from seed collected when a maximum of seed were at developmental stages between 2 and 4 (except for experiment 5).

Experiment 1. Impact of embryo numbers on initiation. We selected three initiation studies carried out during the winter of 1998 (by G. Pullman and collaborators) with cones collected in Brazil (provided by Westvaco). For each seed source, the number of embryos was counted in 15 seed. The correlation coefficient between number of embryos per seed and initiation rate on the control medium (medium 716) or the mean initiation rate across all tested media were determined for each experiment. The detail of these initiation studies are presented elsewhere (see report on Initiation).

Experiment 2. Survey of genetic variability. Open pollinated cones were collected from twenty different mother trees grown on a single site (Wayne Co., GA, provided by TTC). Fifteen seed (from a single cone) were analyzed from 14 of those mother trees: 7-100, 8-120, 1-516, 3-35, 3-7, 1-76, 1-14, 15-42, 3-1, 18-102, 1-11, 1-526, 3-17.

Experiment 3. Site to site variability. Cones were collected from two or three sites for four mother trees: S4PT6 (2 sites near Livingston, TX; Bullock Co., GA), 7-100 (Santa Rosa Co., FL; Wayne Co., GA), 7-56 (Wayne Co., GA; Terrell Co., GA, Santa Rosa Co., FL), 5-1036 (Wayne Co., GA; Belville, GA). Fifteen seed (from a single cone) were analyzed for each site used for each mother tree.

Experiment 4. Crown position. Cones were collected from the upper part of the crown and the lower part of the crown. Data were obtained from ten seed from two cones of each part of the crown. S4PT6 (trees from 2 sites near Livingston, TX), 9-11, 7-100, 7-56 (Santa Rosa Co., FL).

Experiment 5. Evolution during the growth season. Cones were collected weekly from tree 5-1036 starting on June 22 ( ) and, tree S4PT6 starting of July 6 (Livingston, TX). Data were collected from 2 cones (15 to 20 seed each).

RESULTS AND DISCUSSION

Preliminary observation of the impact of embryo numbers.

Preliminary analysis of the number of embryos per seed carried out in three initiation experiments (winter of 1998) showed a trend toward higher initiation rates in seed lots with higher mean number of embryos (Figure 1). There was a moderate to high positive

correlation between these two variables, when each experiment was considered separately. Experiments using a greater number of seed sources and larger sample sizes are needed to confirm and generalize this result.

Genetic variation of the number embryos among open-pollinated seed sources

The number of post cleavage embryos per seed was highly variable, ranging from two to 20 embryos per seed (Figure 2). Previous reports have indicated that *P. taeda* (loblolly pine) may produce up to 4 archeagonia and consequently up to 16 post cleavage embryos (Skinner, 1992) but as many as 5 to 7 archaegonia in other pines (Ferguson, 1904). We have observed as many as 16 to 20 embryos, in several seed from three distinct mother trees. These data indicate that certain loblolly pine mother trees most likely produce up to 5 archeagonia per seed, thus increasing the number of post cleavage embryos significantly.

The seed source (open pollinated family) was a highly significant source of variation in all experiments. To assess the extent of variation between o-p families, embryos were counted in seed collected from 13 clones grown on a single site (Wayne Co, GA, provided by TTC). The mean number of embryos ranged from 4.4 ± 1.0 to 12.2 ± 0.95 (Table 1, Figure 3). Variation within individual seed lots was also extensive but not sufficient to mask the variation between seed lots. These data suggest the number of zygotic embryos per seed is under genetic control.

The site or geographic region in which the ramets were grown had a small effect on the number of embryos per seed (Figure 4). Cones collected from the same mother tree but growing in two or three different locations had similar numbers of embryos, but significant variation was observed in some cases. Although only a few genotypes and locations were examined, genotype of the mother tree appears to contribute more significantly to the variation than the location or site of the tree. The design of this experiment does not allow to estimate the relative contributions of these sources of variation.

Variation within an individual tree – Effect of position within the crown.

A comparison of cones collected from the upper and lower parts of the crown in five trees (four of them growing on the same site) showed a trend toward fewer embryos per seed in the lower part of the tree (Table 2, Figure 5). The position in the crown had a statistically significant effect and the overall average was 8.5 ± 0.38 for the upper crown cones and 7.5 ± 0.39. This result may be explained in part by the increased incidence of inbreeding in the lower part of the crown, which may lead to embryo abortion and thus decrease the total number of viable zygotic embryos in the seed. The increased inbreeding does not generally lower seed set (Dave Branlit, pers. comm.) because multiple fertilizations allows to overcome abortion of individual embryos. In addition, cone to cone variation within one region of the mother tree was small, therefore a sample of one or two cones is representative of the whole tree (except for cones from the lowest part of the crown).

Variation over time / during development.

The number of embryos per seed was followed weekly in two seed sources and declined very gradually from early in the growing season (Figure 6). The decline in number could begin shortly after cleavage. The number of embryos declined more sharply toward the end of the embryo development, when the dominant embryo reached stage 8 to stage 9. Overall the number of embryos per seed within a cone remained variable throughout the season. As might be expected, these data show that the developmental stage of the seed may impact significantly on the number of embryos.

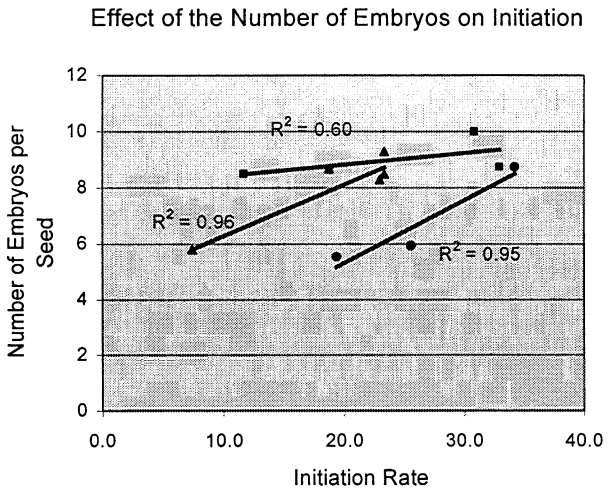


Figure 1. Effect of the number of embryos on initiation rate, in three initiation experiments. Each data point represents the mean initiation rate and mean number of zygotic embryos per seed of a single seed source (o-p family). Trend lines and correlation coefficients (R^2) are given for each tissue culture experiment separately.

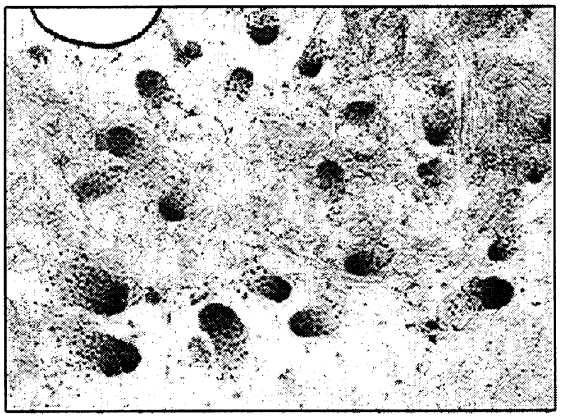


Figure 2. Microscopic view of the aceto-carmine stained mass of immature embryos from a single see (50X magnification)

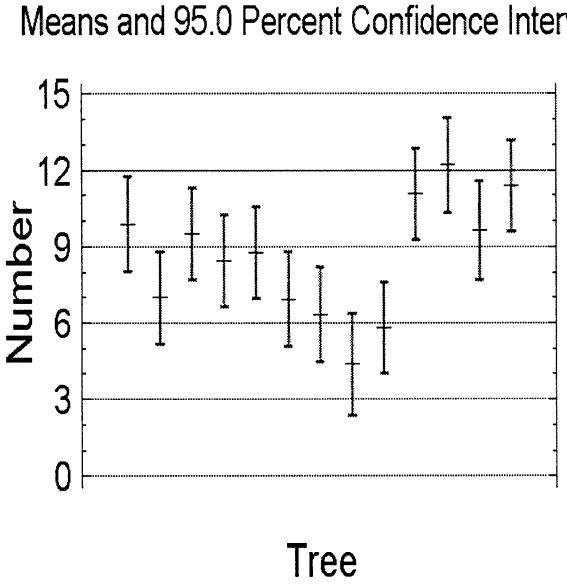


Figure 3. Number of zygotic post cleavage embryo per seed from 14 mother trees of diverse genotypes (each represented by a mean and 95% C.I.) and grown on the same site.

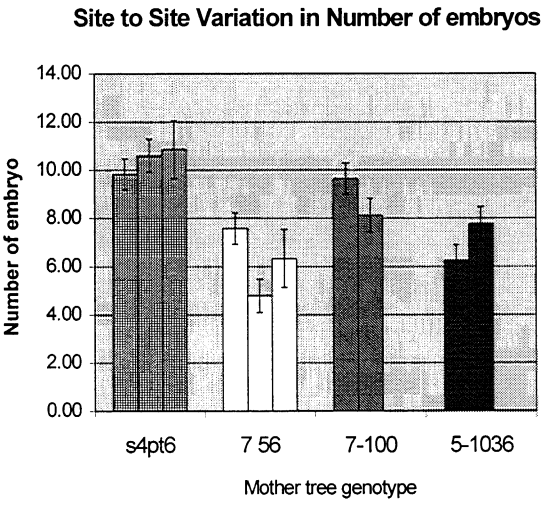


Figure 4. Effect of site or geographic region on the number of zygotic embryos per seed. Each bar represents the mean number of embryos for an individual site.

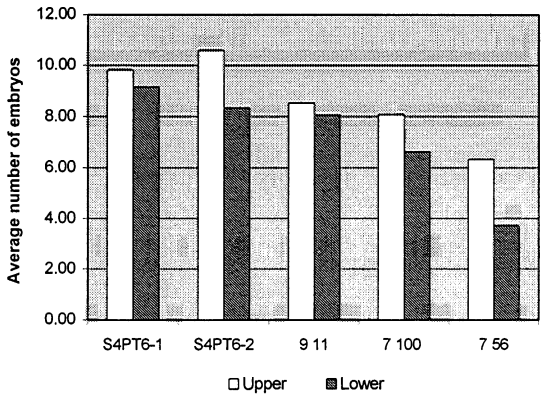


Figure 5. Effect of crown position on the number of zygotic embryos per seed. Each bar is the mean of two cones in the upper crown or two cones in the lower crown.

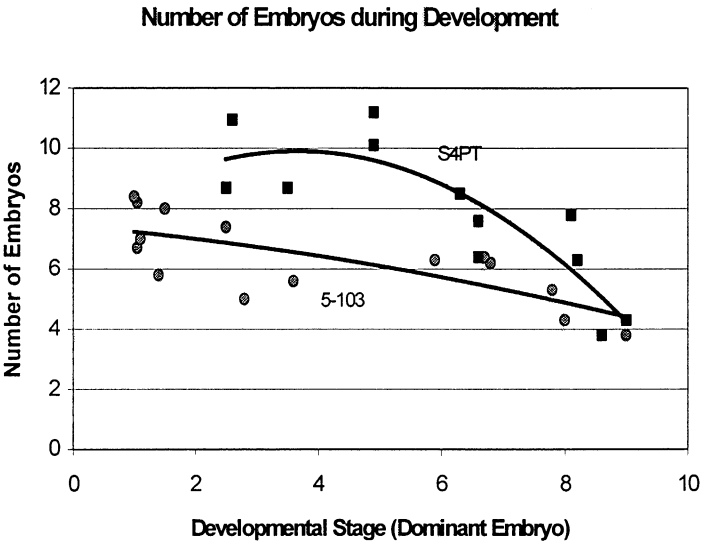


Figure 6. Evolution of the number of embryos per seed during natural development. The stage of each data point was determined by calculating the averaged stage of each weekly collection

Table 1. Analysis of variance table for the number of embryos per seed from 14 mother trees

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
Genotype	982.67	12	81.89	6.11	<0.0001
RESIDUAL	2492.75	186	13.40		
TOTAL (CORRECTED)	3475.43	198			

Table 2. Analysis of variance table for the number of embryos per seed in 2 crown positions and 5 trees

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
Genotype	354.24	4	88.56	7.08	<0.0001
Position (Up vs Low crown)	51.31	1	51.31	4.11	0.044
RESIDUAL	2200.0	176	12.5		
TOTAL (CORRECTED)	2640.71	181			

CONCLUSIONS

Our results show that the number of post cleavage zygotic embryos per seed varies considerably among open pollinated families. Our preliminary findings and previous reports (Becwar et al., 1997) indicate that higher number of embryos per seed correlate with higher initiation rates, thus families with more embryos per seed may initiate more successfully. We are currently carrying out experiments to (1) better characterize and confirm the correlation between these two traits and, (2) better understand the genetic basis of initiation. Initiation is dependent on several factors including use of appropriate tissue culture procedures and media and, appropriate developmental stage of the embryo. Assessment of the number of embryos per seed may provide an additional piece of information to ensure success of initiation. For example, we have found cases where there was variation between cone collections coming from the same mother tree grown on different sites or cones collected in different parts of the crown. Although tissue culture results are needed to confirm the usefulness of this parameter, it could help select seed collections that are best suited for initiation.

ACKNOWLEDGMENTS

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MASS CLONAL PROPAGATION OF IMPROVED CONIFERS

Survival of Initiated Cultures after Direct Transfer to Liquid Media

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SUMMARY

The first step after somatic embryo initiation is to multiply the somatic embryos for cryostorage and maturation. Previous results in our lab have shown that the rates of long-term survival of newly initiated cultures on multiplication media were 22% in 1995 and 33% in 1996 (Pullman et al., Annual Review 1998 pp. 15). In 1997, it was found that by transferring newly initiated cultures directly into liquid media multiplying cultures were established for 42% of the cultures, but only 38 cultures were tested. We have now direct transferred 123 new initiates from initiation media into liquid multiplication media. The overall rate of establishment of multiplying cultures was 32%. However, the success of directly transferring the newly initiated somatic embryo culture into liquid media is strongly correlated with its starting mass. 88% of the cultures with a starting mass of ≥ 0.2 g survived. This result suggests that the limitation for culture survival may not be related to transfer shock but rather that the initiated culture had previously ceased multiplying.

INTRODUCTION

A significant problem in employing somatic embryogenesis for germplasm capture, long-term storage and multiplication of elite *Pinus taeda* genotypes has been initiating somatic embryos. Intact ovules with early stage zygotic embryos are sterilized and placed onto initiation media. The somatic embryos initiate from the zygotic embryos and continue to proliferate. The scale up process occurs when the initiated somatic embryo mass is transferred to multiplication media. This transfer needs to be successful to capture the germplasm by cryostorage and to produce seedlings from these somatic embryos. We have found that many of our "initiated" cultures are lost upon transfer to multiplication media.

To overcome this loss and reduce the time spent on transfers, we have investigated the frequency of capturing a new initiate after direct transfer to liquid multiplication media (Pullman et al., PAC Annual Review 1998 pp. 15). Initial results from 34 cultures suggested that the rates of survival in liquid were 42% compared to survival rates of 25-33% in previous years. We have now determined the success rate of new initiates after direct transfer into liquid multiplication media for 123 additional lines. We found that an overall success rate of directly starting a liquid culture with 9-week-old initiates was 32%. However, the success rate of direct transfer was ~90% with initiates that had grown to a mass of ≥0.2 g fresh weight.

* Atlanta High School Talented and Gifted Students

MATERIALS AND METHODS

Media

Liquid multiplication media 16 was used in all experiments. Table 1 shows media composition.

Methods

After the 9-week initiation period initiates were weighed and placed into the flask with the appropriate amount of media 16. A mass to volume ratio was 1 gm: 9 ml was used. The minimal volume of media was 1 ml due to culture vessel size used. The culture vessels were 250 ml flasks or 25 ml flat tissue culture vessels typically used for mammalian tissue culture (VWR cat. #29186-024). The cultures were shaken at 100 rpm in the dark at 22°C.

Each week the cultures were checked for growth and additional liquid media was added if growth had occurred. Final scoring was done after nine weeks of growth.

Table 1
Media 16

Components	Mg/L
NH4NO3	603.8
KNO3	909.9
KH2PO4	136.1
Ca(NO3)2•4H2O	236.2
MgSO4•7H2O	246.5
Mg(NO3)2•6H2O	256.5
MgCl2•6H2O	101.7
KI	4.15
H3BO3	15.5
MnSO4•H2O	10.5
ZnSO4•7H2O	14.4
Na2MoO4•2H2O	0.125
CuSO4•5H2O	0.125
CoCl2•6H2O	0.125
FeSO4•7H2O	6.95
Na2EDTA	9.33
Sucrose	30,000
myo-Inositol	1,000
Casamino acids	500
L-Glutamine	450
Thiamine•HCl	1
Pyridoxine•HCl	0.5
Nicotinic acid	0.5
Glycine	2
2,4-D	1.1
BAP	0.45
Kinetin	0.43
pH	5.7

RESULTS & DISCUSSION

Experimental Design

Four separate initiation experiments, two from the 98 winter and two from the 97 summer, were used as the source of initiates. Each initiation experiment contained ovules from 4 separate cone collections. The largest experiment included 73 initiates that were chosen randomly from one initiation experiment and transferred directly into liquid media 16. For three experiments, initiates were chosen randomly and in one experiment only large masses (> 0.15 g) were transferred to test whether good growing initiates established liquid cultures more readily.

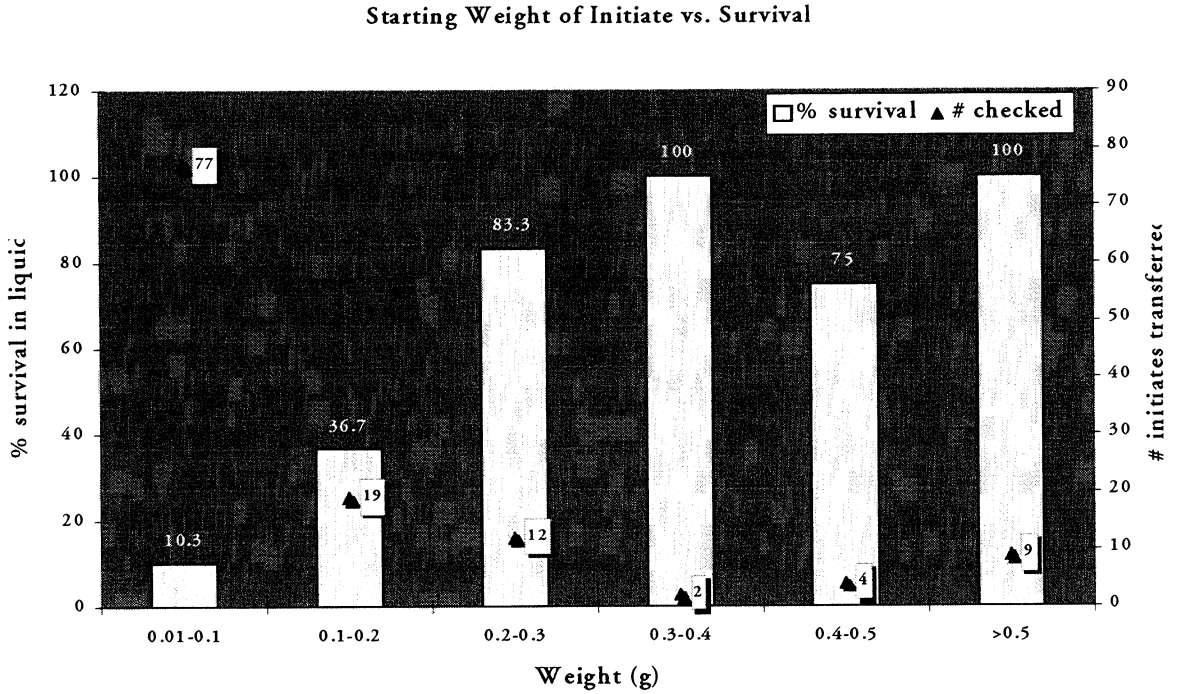


Figure 1. Summary of Liquid Culture Establishment versus Starting Mass The numbers above the bars indicate the percentage of initiates that grew in liquid culture. The diamonds and numbers next to them indicate the number of initiates transferred in that weight range.

Directly transferring 9-week-old, newly initiated somatic embryo masses into liquid media was successful with 32% (40/123) of all the cultures tested. Figure 1 shows that there is a strong correlation between the starting size of the somatic embryo initiation mass and its ability to grow in liquid. Somatic embryo initiations that grew to a mass of $\geq$ 0.1g had a success rate of 68%, (32/47); $\geq$ 0.15g a rate of 80%, (28/35); and $\geq$ 0.2g (24/27) had an 89% chance of being established directly in liquid cultures. This suggests that transfer to liquid is limited by the amount of embryo proliferation that has occurred, and some initiations stop growth shortly after forming the first somatic embryos.

This general correlation suggests that in practice initiated somatic embryos should not be transferred into liquid media until they reach a starting mass of $\geq$ 0.15-0.2g. To test this practical guideline we transferred 10 newly initiated somatic embryo lines that had reached a starting mass of 0.18 – 1.2g into media 16. Each of these lines was established in liquid culture, a success rate of 100%.

Interestingly there were some notable exceptions to this general trend. Figure 2 shows the distribution of successful and unsuccessful establishments by starting weight for the largest experiment (73 initiates). The smallest starting mass that established directly in liquid was 0.03g. This means that not all of the small masses have the same proliferation potential. Likewise, some initiations that apparently grew well on initiation media reaching 0.2g or even 0.45g did not establish proliferating cultures in liquid media 16.

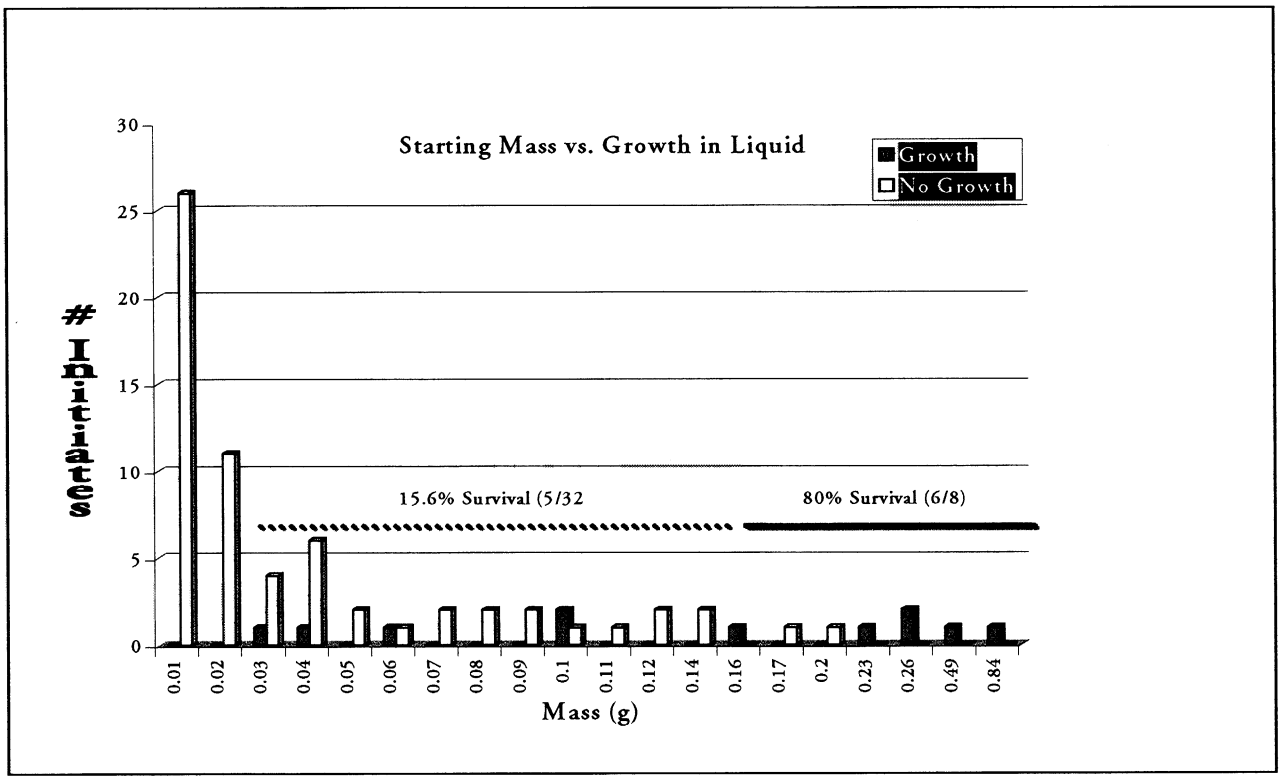


Figure 2. Survival of Initiates in Liquid Media The number of individual somatic embryo cultures that survived after transfer into liquid media related to the starting mass of the newly initiated culture. The data are all from one experiment. The dark bars represent no growth and the light bars represent growth in liquid culture.

CONCLUSIONS

1. In practice initiates should reach a mass of ≥ 0.15g before transfer into liquid media 16.

2. In the experiments presented here, ~85% of the initiates after 9 weeks have a mass < 0.15g. This suggests that a significant limiting factor for implementing somatic embryogenesis for lots of genotypes is the proliferation of early stage somatic embryos. Observations of initiations leads us to suggest that the initiation media induces somatic embryos to form from a reasonable number of, zygotic embryos. However, many of the initiated embryos either do not proliferate at all, only proliferate very slowly or proliferate for a short period and then stop proliferating on initiation media.

3. This data supports the notion that we need to conceptually breakdown "initiation" into 3 stages:
   1. extrusion
   2. somatic embryo induction/formation
   3. proliferation of somatic embryos on initiation media or maintenance of competency to proliferate on multiplication media

4. It also suggests that changes to the initiation as well as to the multiplication media that promote the proliferation of early stage embryos might improve the rate of successfully starting a liquid culture.

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS - EMBRYO DEVELOPMENT IMPROVEMENTS BASED ON ELEMENTAL ANALYSIS OF FEMALE GAMETOPHYTE, ZYGOTIC AND SOMATIC EMBRYO TISSUES

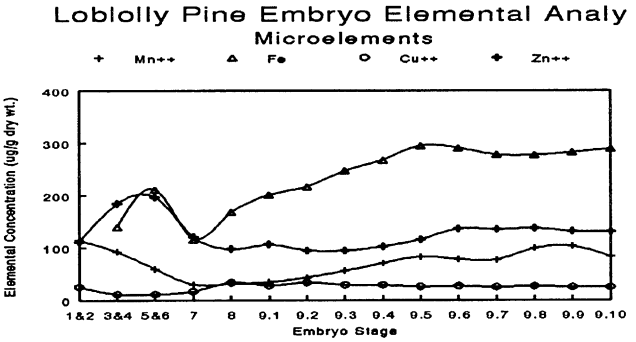
Gerald Pullman
Paul Montello
Xiaorong Feng

Summary

Metal analyses of full term zygotic embryo and female gametophyte tissue were compared to analyses of our most developed somatic embryos. Major differences in some elemental compositions were observed. Metal analyses were also done weekly throughout the developmental sequence for female gametophytes and zygotic embryos. These analyses showed changing compositions over time for the various elements measured. Based on these observations a series of experiments on media adjustment for each step in the somatic embryo protocol are ongoing. To date statistically significant improvements in embryo yield have been documented due to increases in iron in the development and maturation medium. A three fold increase in the level of iron in development and maturation medium caused a repeatable statistically significant increase in yield with a marginal increase in visual embryo quality.

Introduction

Table 1 shows a comparison of elemental analyses for zygotic and somatic tissues with the ratio of somatic / zygotics. Analyses of elemental composition of somatic embryos and comparison to zygotic targets show that somatic embryos contain 191 times the sodium, 17 times the boron, 3 times the calcium, and 1.8 times the potassium vs. the target levels of zygotic embryos. Somatic embryos are also deficient in elemental content for copper (10%), iron (21%), phosphorous (46%), magnesium (50%), and manganese (66%). Zinc and sulfur appear to be on target. Based on these findings a series of experiments was begun to tests modifications in the media to produce somatic embryos which better match zygotic embryo elemental compositions. The following experiments targeted iron levels in the development and maturation step but experiments have also been performed or are in progress to modify the initiation, maintenance and germination media.



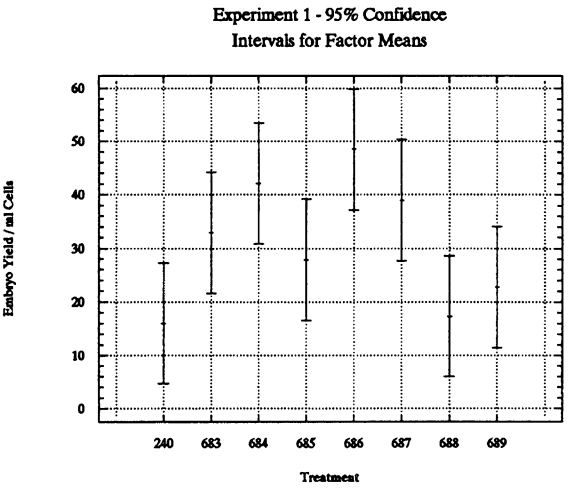
Materials and Methods

25 ml of development and maturation media was dispensed into 100x15 mm petri dishes for each treatment (media formulation listed in Table 2, to make iron variations multiply the modified level by the amount listed for both $FeSO_4 \bullet_7 H2O$ and Na_2EDTA). Suspension culture stocks of each genotype were poured into sterile 300ml graduated cylinders. Cells were settled for twenty minutes after which the supernatant was removed. One ml of cells was pipetted and spread evenly on sterile black filter paper (Ahlstrom, Inc., Mt. Holly Sprints, PA) in the petri plate of media. There were four replications for each treatment of several genotypes (depending on the experiment). Cells were subcultured monthly by transferring the black filter paper covered in cells to fresh development medium. After four months embryo production observations were recorded. When size differences in somatic embryos were apparent embryo weight observations were made. Quality control observations were taken for both osmolality and pH of fresh media.

Results

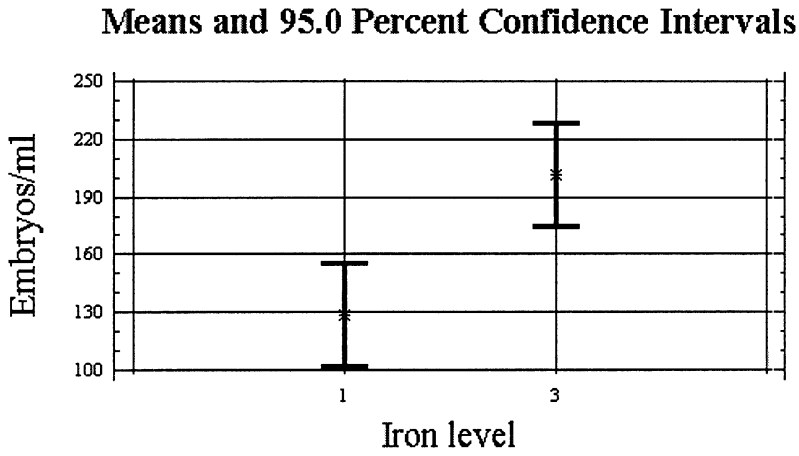
Experiment 1 (918): In this experiment Medium 240 was modified to contain altered levels of copper, iron, or boron. Results show that doubling the iron permits more embryos to advance to the cotyledonary stage of development when compared to the control. These data were presented in the prior PAC Report and lead to changing the boron level in our standard medium. The iron level is the focus of this report.

Media	Copper	Iron	Boron
240	1x	1x	1x
683	2x	1x	1x
684	5x	1x	1x
685	1x	2x	1x
686	1x	1x	1/2x
687	1x	1x	1/5x
688	1x	1x	1/10x
689	2x	2x	1/5x



Experiment 2 (933): In this experiment the iron level was raised to 1.5x and 2x of the control level in Medium 240. Embryo production was improved in both experimental treatments when compared to the control. The germination rate was elevated for embryos that were exposed to raised iron during development and maturation.

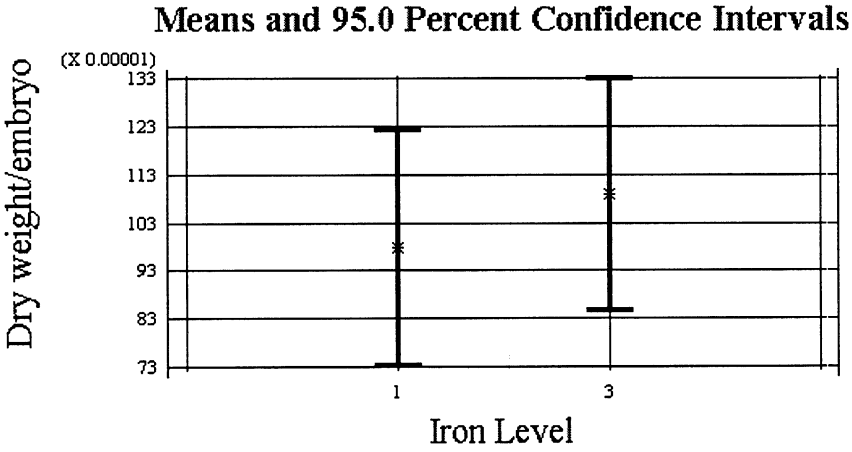
Experiment 3 (963): In this experiment cells were plated on development and maturation Medium 752 (control). The experimental treatment tripled the iron of Medium 752. Statistically significant differences in embryo production were observed. A slight increase in somatic embryo dry weight was detected.



Analysis of Variance for Embryo Yield Experiment 3 - Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Main Effects					
A: Iron	2.900492E-7	1	2.90492E-7	14.44	0.0002
Residual	0.000065713	93	7.06591E-7		
Total (Corrected)	0.0000660035	94			

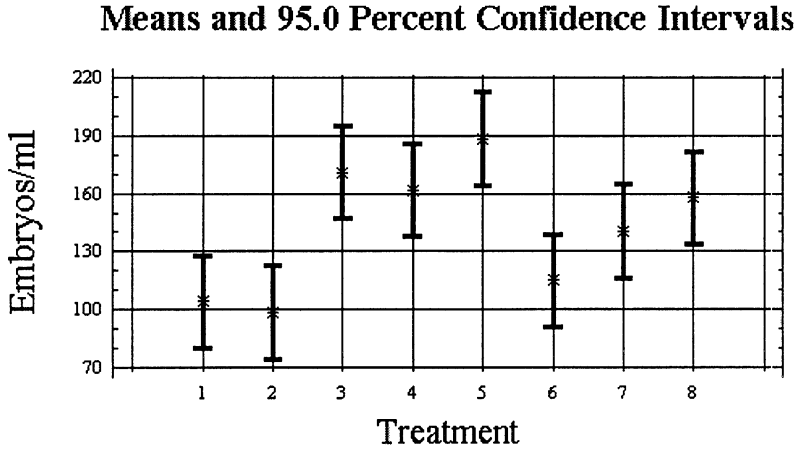
All F-ratios are based on the residual mean square error.

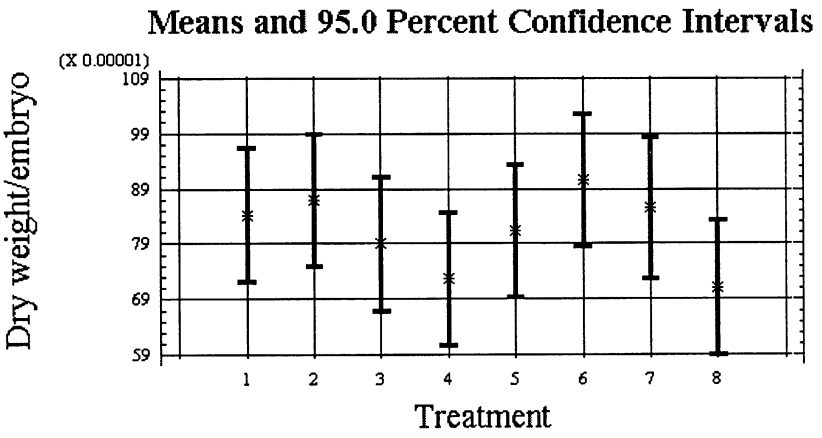


Experiment 4 (966): In this experiment a full factorial of 8 treatments focused on increasing iron over time as we see in natural embryo development. The control was Medium 752. The chart below shows that several treatments were significantly better than the control for producing cotyledonary embryos.

Treatments:

#)	Transfer 1	Transfer 2	Transfer 3
1	control control	control	
2	2x Fe	2x Fe	2x Fe
3	2.5x Fe	2.5x Fe	2.5x Fe
4	3x Fe	3x Fe	3x Fe
5	4x Fe	4x Fe	4x Fe
6	3x Fe	4x Fe	4x Fe
7	3x Fe	4x Fe	5x Fe
8	3x Fe	5x Fe	6x Fe





Analysis of Variance for Embryo Yield Experiment 4- Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Main Effects					
A: Treatment	122338.0	7	17476.9	7.39	0.0000
B: Genotype	1.76908E6	3	589692.0	249.36	0.0000
Residual	276682.0	117	2364.81		
Total (Corrected)	2.1681E6	127			

All F-ratios are based on the residual mean square error.

Conclusions

These data show that an increase in the iron level in the development and maturation medium allows a statistically significant increase in the number of cotyledonary embryos produced. However, the change in dry weight per embryo is not statistically different when compared to the control. Considering all four experiments, iron raised to three times that of the control level yields the highest quantity of somatic embryos produced per ml. Furthermore, it is interesting to note that a three fold increase in iron coincides well with the increase found in zygotic embryos through development.

Table 1. Comparison of elemental compositions for zygotic female gametophyte, zygotic embryo, and somatic embryo tissues with along with the ratio for each element found in somatic / zygotic embryos.

Metal	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
Gametophyte	227	71	#####	3	20	149	19	12215	5388	#####	4	5132	9112	256
Zygotics	81	231	#####	1.8	27.4	130	4.5	16246	2466	#####	6.9	7609	12075	182
Somatics	54	49			2.8	124	78	7449	2713		1315	3820	22204	551
Ratio Som/Zyg	0.66	0.21	#####	0	0.10	0.95	17.2	0.46	1.1	#####	191	0.50	1.8	3.0

Table 2. Media compositions for maintenance (16), control (240), and improved (752) development and maturation media.

Components (mg/l)	16	240	752
NH4NO3	603.8	200	200
KNO3	909.9	909.9	909.9
KH2PO4	136.1	136.1	136.1
Ca(NO3)2•4H2O	236.2	236.2	59.05
MgSO4•7H2O	246.5	246.5	246.5
Mg(NO3)2•6H2O	256.5	256.5	256.5
MgCl2•6H2O	101.7	101.7	101.7
KI	4.15	4.15	4.15
H3BO3	15.5	15.5	7.75
MnSO4•H2O	10.5	10.5	10.5
ZnSO4•7H2O	14.4	14.4	14.4
Na2MoO4•2H2O	0.125	0.125	0.125
CuSO4•5H2O	0.125	0.125	0.125
CoCl2•6H2O	0.125	0.125	0.125
FeSO4•7H2O	6.95	6.95	6.95
Na2EDTA	9.33	13.9	13.9
Maltose	0	20000	20000
Sucrose	30,000	0	0
PEG 8,000	0	130,000	130,000
myo-Inositol	1,000	20,000	20,000
Casamino acids	500	500	500
L-Glutamine	450	450	450
Thiamine•HCl	1	1	1
Pyridoxine•HCl	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5
Glycine	2	2	2
2,4-D	1.1	0	0
NAA	0	0	0
BAP	0.45	0	0
Kinetin	0.43	0	0
ABA		5.2	5.2
Activated Charcoal	0	0	0
Gelrite	0	2,500	2,500
TC Agar	0	0	0
pH	5.7	5.7	5.7

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS

Optimizing the Percent of Embryos That Develop Into Cotyledonary Embryos by Altering Embryo Plating Densities and ABA Concentration

Teresa Vales
Gary Peter

SUMMARY

It is a long-term goal of our program to develop a tissue culture system that will support the production of high quality somatic embryos in a cost-effective way. The experiments presented in this report provide data that further us toward this goal by showing that a) there are nutritional and/or hormonal limitations in our present tissue culture system and that by decreasing the density of cells plated these limitations can be overcome, b) a higher percent of early stage embryos from liquid cultures will develop into cotyledonary embryos when ABA is optimized relative to the starting embryo number.

INTRODUCTION

Preliminary experiments have indicated that plating density may be a factor that needs to be addressed in our tissue culture system. In the Spring 98 PAC report (Peter, Vales, p.47), an experiment was described in which plating either 1.0 or 0.5 ml of settled cells onto a maturation plate produced similar amounts of cotyledonary embryos per plate. The experiment showed 2.7 times as many of the early stage embryos plated, developed into cotyledonary embryos at the lower density. An increase in the percent of early stage embryos grown in liquid media, which ultimately develop into cotyledonary embryos is seen as an improvement.

There are several hypotheses that would explain why embryo yield increases at lower densities. One simple hypothesis is that nutrient and/or ABA is limiting when too many embryos are plated. This report presents data that investigate the possibility of nutritional and hormonal deficiencies due to too many cells on a plate in our present system where 1ml of settled cells is typically added per maturation plate containing 19.6uM ABA. Results for five genotypes indicate that 1ml cell density is not optimal. Decreasing the cell density to a range between 0.25-0.5ml increases the percent of early stage embryos that develop. That range changes with the ABA concentration used as well as the frequency of subculturing. It was speculated that experiments using different cell plating densities could be used as a tool to establish a range of embryo plating amounts for each specific ABA concentration and amount of media.

The data in this report also indicates that there can be too few embryos on a plate. There are two hypotheses that can explain this observation. At low cell densities, the percent of embryos developing may be low due to inhibition from excess ABA. On the other hand, perhaps development is dependent on the secretion of factors from other cells present or on specific cell-cell interactions, which would be altered when there are low amounts of cells on a plate (De Jong et. Al. (1993) Plant Cell 5, 615).

Experiment 1: Effect of Plating Density Variations on Embryo Development

Method: Cells were settled from suspension cultures and diluted 1/10, 1/20, and 1/40 with media 16. Several aliquots were taken from the dilutions and stained with fluorescein diacetate (0.5ug/ml) which will stain only live cells. This staining allowed the number of initial live embryos plated to be counted. Various amounts of the dilutions from 5-40mls were added to a vacuum filter apparatus to produce plates containing 0.25, 0.5, or 1ml of settled cells. Weak suction was applied to remove the media and spread the settled cells uniformly over the black filter paper. For a non-vacuum applied control, settled cells were diluted by one quarter and by one half. One ml of these dilutions as well as 1ml of undiluted settled cells were applied to each plate producing a final of 0.25, 0.5, and 1 ml respectively. The cells were then spread with the pipette. The black filter papers were then placed onto plates containing 20mls of media 752; plates were wrapped in parafilm and placed in the dark. Plates were subcultured monthly onto freshly poured media 752. At the end of three months, the number of cotyledonary embryos per plate was determined. The results from all plates for a given cell amount were averaged together then divided by the initial embryo number plated to determine the percent of embryos that developed.

RESULTS Table 1

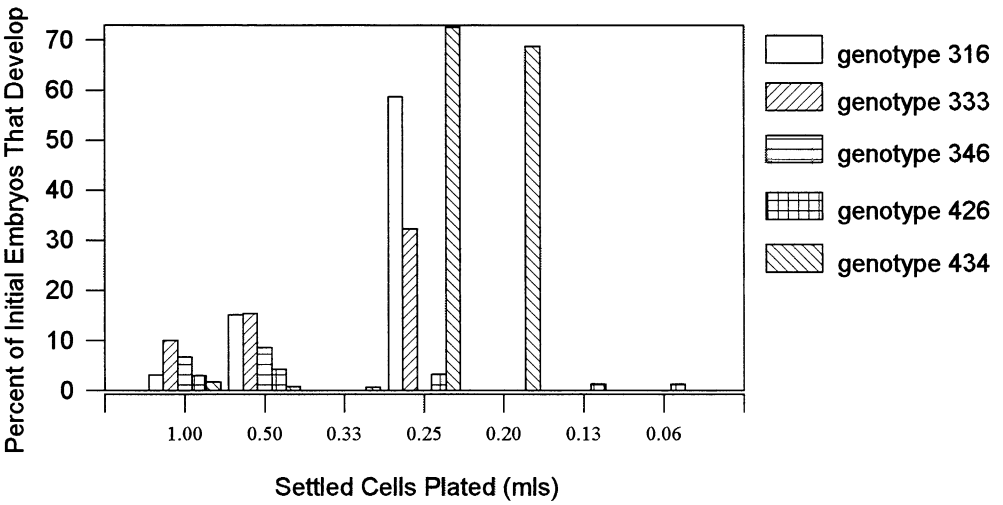
Vacuum-Applied Controls (19.6uM ABA; Subcultured Monthly)

Genotype	Cell Amount Plated	% Embryos Developed
333	1ml	10.0
316	1ml	3.03
346	1ml	6.66
426	1ml	2.97
434	1ml	1.65
333	0.5ml	15.3
316	0.5ml	15.1
346	0.5ml	17.6
426	0.5ml	4.26
434	0.5ml	0.754
434	0.33ml	0.653
333	0.25ml	32.2
316	0.25ml	58.6
426	0.25ml	3.14
434	0.25ml	72.6
434	0.20ml	68.7
426	0.125ml	1.35
426	0.06ml	1.28

Table 1 shows the results from this experiment (genotypes 316, 346, and 333) as well as control treatments from the next two experiments for comparison. The results demonstrate that for all genotypes used that a higher percent of embryos develop into cotyledonary embryos when plated cell densities are less than 1ml. For genotypes 316, 333, and 434, a cell density of 0.25mls was optimal

with respect to the highest percent of initial embryos plated developing into cotyledonary embryos. However, for genotypes 426 and 346, the optimal cell density appeared to be 0.5mls. Figure 1 shows a graphical representation of the data in the table.

Figure1.



Experiment 2: Optimizing Embryo Plating Densities With Respect to ABA Concentration

Method: Settled cells from genotype 434 were diluted 1/20 in media 16. Several aliquots were taken from the dilutions and stained with FDA (0.5ug/ml) and the live embryos were counted. Either 4, 5, 6.6, 10, or 20 mls of the dilution were added to a vacuum filter apparatus to produce plates having final settled cell amounts of 0.2, 0.25, 0.33, 0.5, and 1ml respectively. Weak suction was applied to remove excess media and spread the cells uniformly over the black filter paper. The black filter papers for each cell density were placed on plates containing media 752 with increasing ABA amounts of 19.6-98 uM. (There were 5 replicates per ABA concentration). Plates were wrapped in parafilm and placed in the dark. They were subcultured monthly for three months at the end of which the number of cotyledonary embryos was determined. The number of embryos produced was divided by the initial number of embryos plated to get the percent that developed.

Experimental Design

Settled Cells Plated	uM ABA
1ml	19.6 uM
1ml	29.4 uM
1ml	49.0 uM
1ml	98.0 uM
0.5ml	19.6 uM
0.5ml	29.4 uM
0.5ml	49.0 uM
0.5ml	98.0 uM
0.33ml	19.6 uM
0.33ml	29.4 uM
0.33ml	49.0 uM
0.33ml	98.0 uM
0.25ml	19.6 uM
0.25ml	29.4 uM
0.25ml	49.0 uM
0.25ml	98.0 uM
0.20ml	19.6 uM
0.20ml	29.4 uM
0.20ml	49.0 uM
0.20ml	98.0 uM

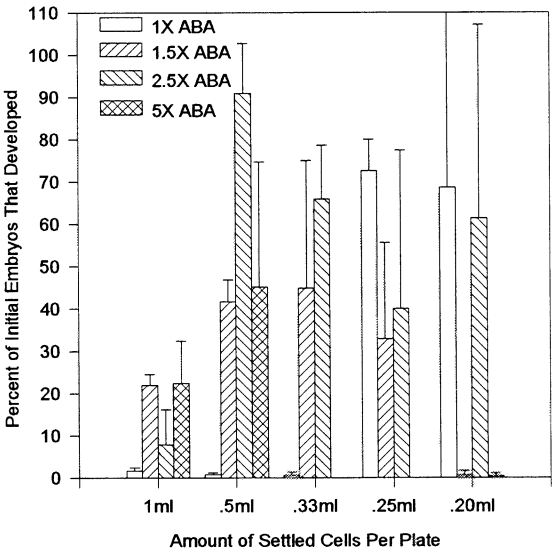


Figure 2

RESULTS AND DISCUSSION

The results reported by Pullman and Feng in the spring 1998 PAC Report, 41-46, show that increasing ABA levels in the 2nd and 3rd months improved embryo yield and quality when 1 ml of cells was plated. The results from the experiments presented here also show that ABA can be limiting throughout our standard maturation protocol in which 1ml of settled cells is added per plate (Figures 2 & 3).

At this point it is hard to completely separate nutritional requirements from ABA requirements, though it is clear that ABA limitation does play a role. When one ml of settled cells are put onto plates which contain varying amounts of ABA, the percent of embryos that develop into cotyledonary embryos is quite low when 19.6uM is used and ten to twenty times higher on the plates having greater than 29.4uM ABA (Figure 3). When 0.5mls of settled cells are plated, the 19.6uM treatment remains low indicating an ABA deficiency because when the ABA is elevated to 29.4 uM the percent of embryos developing also increases. There seems to be an optimal concentration of ABA when 0.5ml settled cells are plated and this appears to be 49 uM. When the concentration of ABA is elevated to 98 uM the percent of embryos developing decreases implying that too much ABA can inhibit embryo development. On plates where 0.33mls of cells are plated, the 19.6uM treatment still has a low percent of embryos developing relative to the other ABA amounts, however it is slightly higher than the 0.5 and 1ml plates at the same ABA concentration.

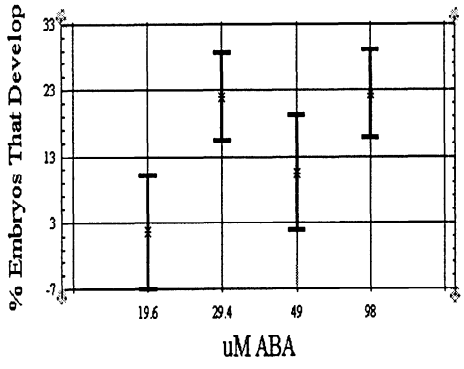
This may imply nutritional as well as ABA limitations. The optimal ABA concentration was still around 49 uM, however the percent development was not as high as with the 0.5ml plates. Perhaps the optimum is somewhere between 29 and 49 uM. Again, 98 uM is inhibitory, though even more so than the 0.5ml treatment. When 0.25mls of cells are plated the optimum ABA concentration changes to 19.6 uM. The percent of embryos developing into cotyledonary embryos is lower in all of the higher ABA concentrations, which again implies that too much ABA can inhibit embryo development. A similar effect was seen on the plates containing 0.20ml settled cells. An ABA concentration of 19.6uM was optimal and any higher ABA amount appeared to be inhibitory to embryo development. It is unclear however why there is a slight peak in percent development at 49 uM ABA in these plates. Table 3 shows the number of cotyledonary embryos that developed per plate at each plating density.

These results support the hypothesis that a specific initial cell amount to ABA concentration ratio needs to be determined for a high percentage of early stage embryos to develop into cotyledonary embryos. Thus far, this experiment was done using only one genotype. Future experiments will attempt to define the optimal ratio of initial embryo number to ABA concentrations on several other genotypes. A comparison of this ratio for many genotypes should provide a more rational basis for optimizing the large-scale production with specific genotypes.

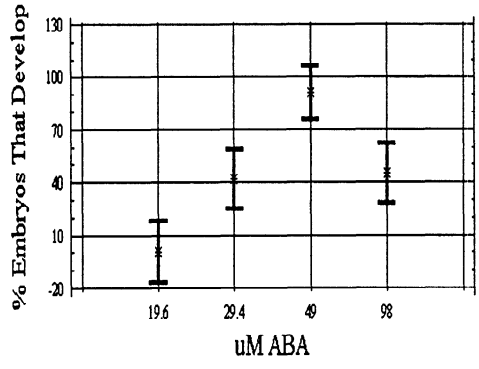
Table 3.

Number of Cotyledonary Embryos After Three Months on Media 752								
Amount	**[ABA]**	**# 1**	**# 2**	**# 3**	**# 4**	**# 5**	**AVG**	**SD**
1 ml	**19.6 uM**	4	8	11			7.67	3.51
1 ml	**29.4 uM**	86	116	97	99	112	102	12.1
1 ml	**49.0 uM**	8	56	82			48.7	37.5
1 ml	**98.0 uM**	118	47	63	149	144	104	46.8
.5 ml	**19.6 uM**	2	3	1	1		1.75	0.957
.5 ml	**29.4 uM**	109	86	105	87		96.8	12.0
.5 ml	**49.0 uM**	178	214	213	198	253	211	27.5
.5 ml	**98.0 uM**	50	154	43	174		105	68.4
.33 ml	**19.6 uM**	0	0	2	2		1.00	1.15
.33 ml	**29.4 uM**	69	122	41	104		84.0	36.1
.33 ml	**49.0 uM**	120	105	74	106		101	19.4
.33 ml	**98.0 uM**	0	0	0	0	0	0.00	0.00
.25 ml	**19.6 uM**	76	84	85	98	78	84.2	8.61
.25 ml	**29.4 uM**	33	30	77	46		46.5	21.5
.25 ml	**49.0 uM**	24	22	54	13		28.3	17.8
.25 ml	**98.0 uM**	0	0	0	0	0	0.00	0.00
.20 ml	**19.6 uM**	108	90	35	22		63.8	41.7
.20 ml	**29.4 uM**	0	0	1	0	2	0.600	0.894
.20 ml	**49.0 uM**	23	32	56			37.0	17.1
.20 ml	**98.0 uM**	1	0	0	1	0	0.400	0.548

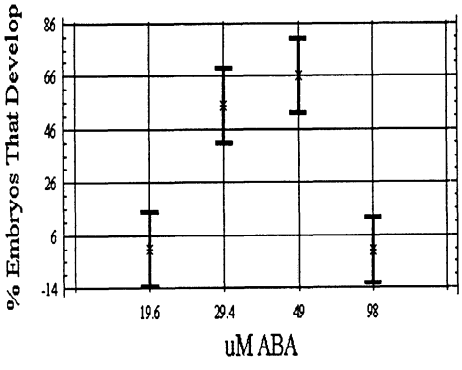
1ml Settled Cells



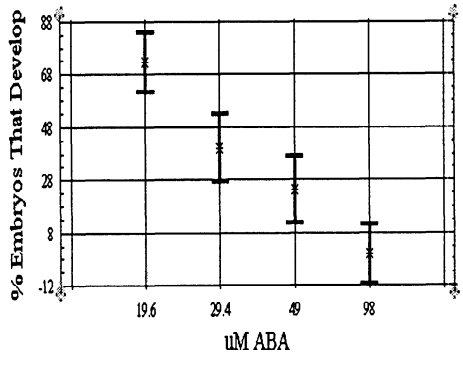
0.5mls Settled Cells



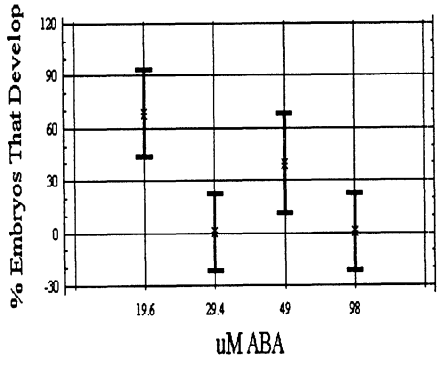
0.33ml Settled Cells



0.25ml Settled Cells



0.20ml Settled Cells



Media compositions (mg/l) for maintenance (16) and maturation media (752).

Components	16	752
NH4NO3	603.8	200
KNO3	909.9	909.9
KH2PO4	136.1	136.1
Ca(NO3)2•4H2O	236.2	59.05
MgSO4•7H2O	246.5	246.5
Mg(NO3)2•6H2O	256.5	256.5
MgCl2•6H2O	101.7	101.7
KI	4.15	4.15
H3BO3	15.5	7.75
MnSO4•H2O	10.5	10.5
ZnSO4•7H2O	14.4	14.4
Na2MoO4•2H2O	0.125	0.125
CuSO4•5H2O	0.125	0.125
CoCl2•6H2O	0.125	0.125
FeSO4•7H2O	6.95	6.95
Na2EDTA	9.33	13.9
Maltose	0	20000
Sucrose	30,000	0
PEG 8,000	0	130,000
myo-Inositol	1,000	20,000
Casamino acids	500	500
L-Glutamine	450	450
Thiamine•HCl	1	1
Pyridoxine•HCl	0.5	0.5
Nicotinic acid	0.5	0.5
Glycine	2	2
2,4-D	1.1	0
NAA	0	0
BAP	0.45	0
Kinetin	0.43	0
ABA		5.2
Activated Charcoal	0	0
Gelrite	0	2,500
TC Agar	0	0
pH	5.7	5.7

Experiment 3: Effects of Altering ABA concentration, Frequency of Subculture and Cell Density on the Percent of Embryos that Mature

Mass Clonal Propagation of Improved Conifers: Field Establishment of Somatic Embryo Derived Loblolly Pine Seedlings

Jerry Pullman, Paul Montello, Mike Cunningham

Summary

On January 22, 1997 thirty five somatic embryo derived loblolly pine seedlings, initiated in summer 1994, from open pollinated ovules from tree UC10-1003 were established in a field plot at the Union Camp Ogeechee Forest in Tattnall County Georgia. To date plants have shown 100% survival and normal growth. Average tree height after 2 growing seasons in the field is 5.2 feet for somatic seedlings vs. 8.6 feet for nearby loblolly pine seedlings planted at the same time.

Loblolly pine seeds that originated from tree UC10-1003 of the Union Camp Corporation were initiated during 1994 initiation trials. The somatic embryos that resulted subsequently were allowed to undergo conversion and germination. The seedlings spent approximately 1 year in the greenhouse. 35 of these seedlings were delivered to the Union Camp Bellville Georgia location. On January 22, 1997 they were established in the field. The study was laid out in four rows. Rows 1-3 have 9 trees whereas Row 4 has 8 trees. The spacing between seedlings is 10'x6'. The study plot is marked with a post at each corner and a flag pin at each tree.

IPST Somatic Seedling				UC Seedling	
Tree	HT	Tree	HT	Tree	HT
1	5.2	21	5.2	1	8.8
2	6.3	22	3.0	2	9.7
3	4.6	23	5.4	3	10.2
4	3.3	24	6.8	4	8.2
5	5.3	25	5.5	5	7.3
6	5.0	26	5.6	6	7.8
7	3.4	27	5.5	7	10.0
8	5.3	28	filler	8	7.4
9	5.7	29	6.6	9	7.7
10	5.8	30	5.9	10	7.7
11	5.8	31	4.9	11	9.1
12	6.6	32	4.9	12	8.2
13	6.5	33	4.3	13	7.0
14	5.0	34	4.8	14	9.3
15	4.8	35	5.0	15	8.0
16	5.6	36	5.2	16	11.0
17	5.6			17	10.0
18	5.5			18	8.0
19	4.4			19	8.3
20	3.5			20	7.3

As of September 1998 the trees displayed 100% survival in the field. The average height of the 35 trees is 5.2 feet. The tallest tree is 6.8 feet and the shortest is 3.0 feet. For a reference

point, 20 nearby traditionally generated loblolly pine seedlings that were planted at about the same time were measured. The average height of these seedlings is 8.6 feet. It is speculated that the differences in height may be due to a slow start in the first growing season due to somatic seedling residency in our greenhouse for 1 year.

We would like to thank Mike Cunningham, Jerome Martin, Randy Purvis, and Paul Winski at Union Camp for the establishment and care of these seedlings.

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS

Functional Analysis of *Pinus taeda* Zygotic Embryo Germination: The Effect of Partial Drying and The Acquisition of Desiccation Tolerance

Gary Peter
Teresa Vales
Sarah Sward
John Cairney
Nanfei Xu
Gerald Pullman

SUMMARY

Previously, we tested the ability of immature zygotic embryos to germinate without a partial drying treatment (Peter et. al., 1997/98 PAC Reports). The results showed that immature zygotic embryos at stage 7 were capable of germinating albeit at a low frequency and that root emergence was delayed similar to our somatic embryos. Stage 8 and 9.1 zygotic embryos germinated with higher frequencies, up to ~30% at stage 9.1. Partial drying treatments are known to promote the germination of embryos that have acquired at least some level of desiccation tolerance. We are now testing the effectiveness of a partial drying treatment on enhancing the germination of stage 7-9.2 zygotic embryos. Initial germination data from undried zygotic embryos confirm last years results with ~50% of the stage 9.2 embryos germinating within 19 days in the light. At present it is too early to score the effect of partial drying on germination frequencies, but this data will be presented at the PAC meeting.

What stage of zygotic embryo development desiccation tolerance is acquired was functionally assessed by comparing the survival of embryos with and without a partial drying treatment. Preliminary results show that desiccation tolerance is attained by stage 8. Interestingly, the steady state level of mRNAs related to ABA induced, desiccation tolerance genes shows that they are highly expressed by stage 5 in zygotic embryos. This molecular data suggests that some aspects of the desiccation tolerance program is activated by stage 5. The expression pattern of these desiccation tolerance mRNAs in somatic embryos is similar to zygotic embryos which supports the functional data that our somatic embryos acquire some level of desiccation tolerance. However, thus far partial drying treatments of our somatic embryos have not promoted germination, though most somatic embryos survive the partial drying treatments. These results suggest that somatic embryo germination is inhibited by factors that cannot be overcome by partial drying.

INTRODUCTION

To obtain a better understanding of the germination potential of our somatic embryos, last year we tested the ability of immature zygotic embryos to germinate without a partial drying treatment (Peter et al., 1997/98). The results showed that immature zygotic embryos at stage 7 were capable of germinating albeit at a low frequency and that root emergence was delayed similar to our somatic embryos. Stage 8 and 9.1 zygotic embryos germinated with higher frequencies, up to ~30% at stage 9.1. The results also showed that the root apical meristem is formed around stage 4 when the shoot apical meristem is formed during zygotic embryogenesis. Importantly, the emergence of the root apical meristem limited zygotic germination and this appears to limit somatic embryo

germination. Although, comparisons of the fresh weight, dry weight, and patterns of gene expression between zygotic and somatic embryos suggested that with our protocols loblolly pine somatic embryos develop to stages 8-9.1 these functional data suggest that our somatic embryos probably do not miss any major early developmental steps but just do not develop past stages 7-8.

Somatic embryos developed with our current protocol germinate at a low frequency and do so in reverse sequence when compared to mature zygotic embryos. Mature zygotic embryos germinate when the root emerges before or coincident with the shoot. In contrast, when somatic embryos germinate the cotyledons green first, the shoot emerges and then only much later if at all does the root appear. The reason(s) for this backward pattern and low germination frequency of our somatic embryos is unclear but include: 1) the embryos being too immature, 2) root emergence is inhibited by some component of the maturation media, e.g., by ABA or PEG (Rudder, Becwar 1998) or 3) a partial drying treatment has not been used.

Partial drying treatments are known to promote the germination of somatic embryos that have acquired at least some level of desiccation tolerance (Kermode & Bewley, 1985; Kermode & Bewley, 1989; Roberts et. al., 1990). We now have tested the effectiveness of a partial drying treatment on enhancing germination of stage 7-9.2 immature zygotic embryos in *Pinus taeda.* This report describes preliminary data and more complete data will be presented at the PAC meeting.

MATERIALS AND METHODS

Genotype S4PT6 ovules were surface sterilized with H_20_2 and the immature zygotic embryos were dissected from the female gametophytes. Results from last year indicated that this treatment did not kill the dissected embryos. Undried zygotic embryos were placed immediately onto germination plates. In general, up to ten embryos from each stage were placed on one germination plate. This germination media did not contain exogenous hormones (Table 2, attached at the end of this report). Following our normal germination protocol, the embryos were then cultured in the dark for 7 days and subsequently shifted to continuous white light. An embryo was considered to have germinated when it contained both a root and a shoot.

Partial drying of the embryos was carried out in the center dry wells of a microtiter plate (Roberts et. al., 1990). The wells on the edge were filled with sterile water and the plates were wrapped with parafilm. Partial drying was for 17 days. All embryos after being placed on germination plates were incubated in the dark for 7 days and then transferred to the light following our standard germination protocol. Embryos were visually scored for having a shoot only, root only or were considered to have germinated if they possessed both a root and a shoot.

RESULTS

Germination of Undried Immature Zygotic Embryos

Figure 1 shows the germination frequency of undried S4PT6 zygotic embryos after 19 days in the light. The more mature the zygotic embryos, the higher the germination frequencies: stage 7 (0%), stage 8 (4%), stage 9.1 (22%), and stage 9.2 (48%). This general trend was also observed last year with a different genotype, UC 5-1036, and the results are reproduced in Figure 2. This shows that the ability to germinate without a partial drying treatment improves with increasing maturity. In addition, the ability of the root to emerge first improves with increasing maturity, confirming that root emergence limits germination of undried immature embryos (Figures 1 & 2).

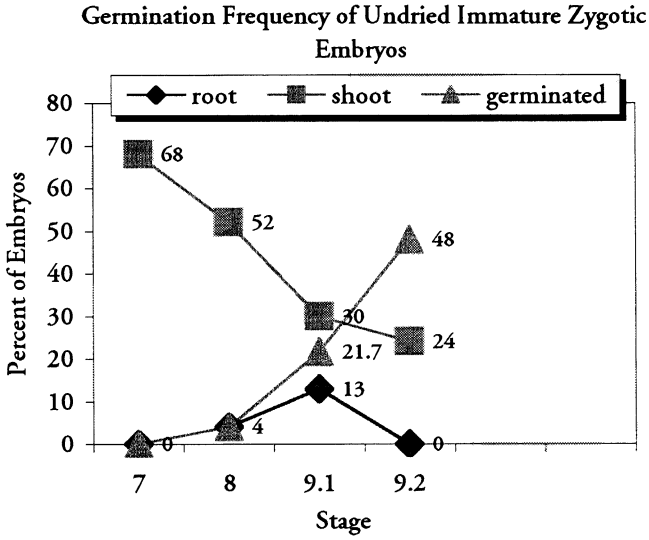


Figure 1. The frequency of germination of undried S4PT6 immature zygotic embryos.

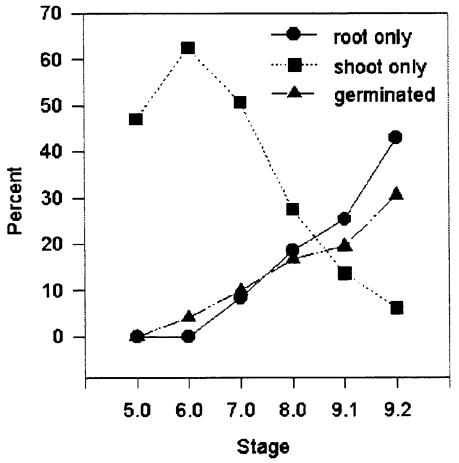


Figure 2. Germination frequencies of undried UC 5-1036 immature zygotic embryos obtained in 1997

Germination of Partially Dried Immature Zygotic Embryos

Partial drying treatments are thought to stimulate germination of somatic and zygotic embryos (Kermode & Bewley, 1985; Kermode & Bewley, 1989; Roberts et. al., 1990). To test this hypothesis we carried out partial drying treatments on loblolly pine immature zygotic embryos before transferring them to germination media. The zygotic embryos were dried in a high humidity environment for 17 days and then placed on germination media. These treatments lead to a 30-70% decrease in fresh weight in our hands as well as others (Becwar, et. al., 1995). These embryos have been in the light for only 7 days and will be scored and the data presented at the PAC meeting. If partial drying is effective then we expect to see an increase in the germination frequency after this treatment.

Acquisition of Drying Tolerance in Zygotic and Somatic Embryos

Functional Evidence

To determine when during the development of loblolly pine zygotic embryos at least some desiccation tolerance is acquired, we compared the survival of undried and dried embryos. If the

cotyledons greened and the embryo expanded it was considered to have survived. Table 1 shows preliminary data for the survival of undried and dried embryos. Note that survival of undried embryos was close to 100% for all stages; whereas survival was strongly reduced only in stage seven embryos after partial drying. The data for the survival of dried embryos was taken early and therefore is considered preliminary. This data will be reassessed and updated for the presentation at the PAC meeting.

Table 1. Survival of Zygotic Embryos to Partial Drying Treatment

Stage	Undried[1]	Partially* Dried
7	88% (22/25)	29% (7/24)
8	96% (24/25)	76% (19/25)
9.1	100% (23/0)	90% (17/19)
9.2	100% (37/0)	93% (25/27)

1. Data after 20 days in light

* Preliminary data after 3 days in light

Molecular Evidence

A family of late embryogenesis related proteins (LEA) that are expressed in the drying seed are proposed to play an important role in desiccation tolerance. LEA gene expression during embryogenesis is induced by ABA. We have identified three LEA genes from previous targeted cloning efforts with differential display (Fall PAC report 1997). The patterns of steady state mRNA levels for these three pine LEA genes during zygotic (Figure 3) and somatic (Figure 4) embryogenesis are shown. Figure 3 shows that during zygotic embryo development all three genes are induced at stage 5. Their expression stays relatively high until stage 8 when it decreases. Finally, the genes are expressed again in the late phase, stages 9.4 –9.10, as expected. The expression of these genes reflects changes in the level of ABA during zygotic development observed for *Pinus taeda* (Figure 5). Since tolerance to partial drying treatments is acquired by stage 8 for zygotic embryos, if these LEA gene products are important for desiccation tolerance then it is their early expression at stages 5-7 that is important. Figure 4 shows that during somatic embryo development the three LEA genes are expressed even earlier, stages 3-6. The earlier expression of these genes in somatic embryos could

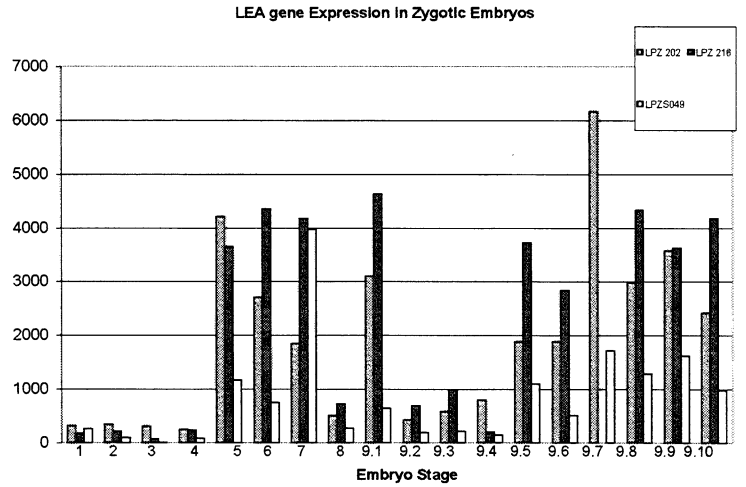


Figure 3. Steady state mRNA levels of three *Pinus taeda* LEA genes at zygotic embryo stages 1-9.10

reflect higher levels of ABA and osmolality in our tissue culture than is observed in the natural seed (Kapik, 1994) (Figure 5). Finally since the somatic embryos do not mature past stage 9.1 the second phase of LEA gene expression is not observed.

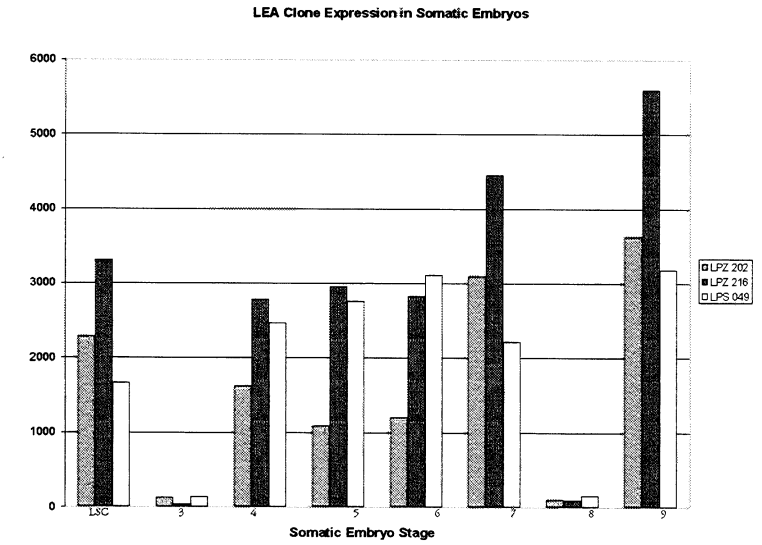


Figure 4.. Steady state mRNA levels of three *Pinus taeda* LEA genes at somatic embryo stages 1-9.1.

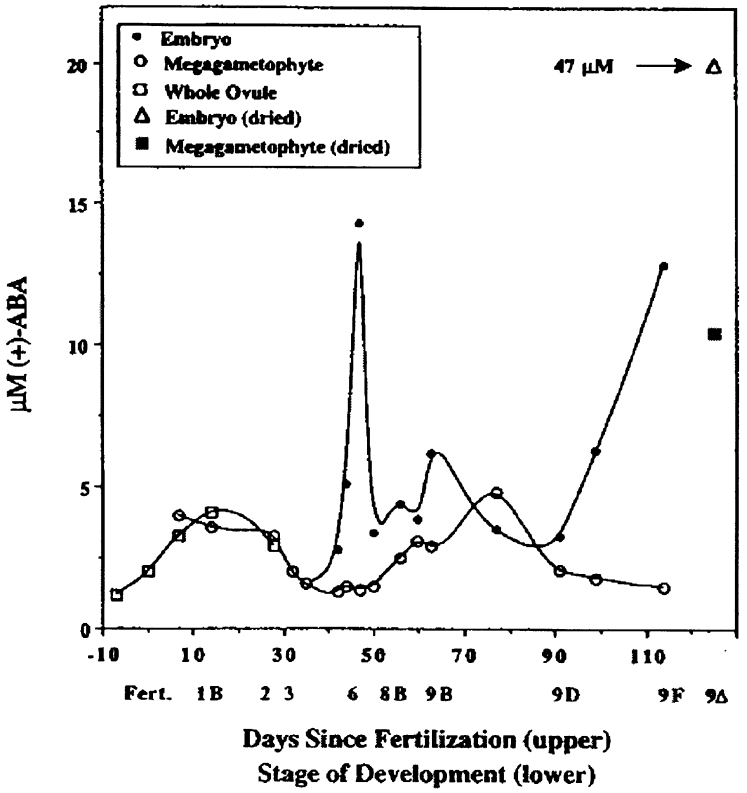


Figure 5. (+) ABA levels in 1993 zygotic whole ovules, embryos and mega-gametophytes on a uM basis from WA_{93}. Stages 9A, 9B etc are equivalent to 9.1,9.2 etc (Kapik, PhD dissertation, IPST 1994).

CONCLUSIONS

1. Immature zygotic embryos are capable of germinating with reasonable frequencies without partial drying treatments. Stage 7 embryos are competent for root emergence and the percent of embryos that germinate increases with increasing maturity.
2. Immature zygotic embryos acquire tolerance to partial drying treatments by stage 8.
3. Our most mature somatic embryos are tolerant to partial drying treatments, although this treatment did not promote germination, suggesting that other factors that can not be overcome by partial drying inhibit germination.
4. The results show that the expression of these LEA genes can be used as an index for the acquisition of desiccation tolerance of somatic embryos. The induction of LEA genes by stage 7 could predict the likelihood that the embryos would survive partial drying treatments.

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Table 1. Media composition of germination media.

Components	397 mg/l
NH4NO3	206.3
KNO3	1170
KH2PO4	85
Ca(NO3)2•4H2O	0
CaCl2•2H2O	220
MgSO4•7H2O	185.5
Mg(NO3)2•6H2O	0
MgCl2•6H2O	0
KI	0.415
H3BO3	3.1
MnSO4•H2O	8.45
ZnSO4•7H2O	4.3
Na2MoO4•2H2O	0.125
CuSO4•5H2O	0.25
CoCl2•6H2O	0.0125
FeSO4•7H2O	13.93
Na2EDTA	18.65
Maltose	0
Sucrose	20,000
PEG 8,000	0
myo-Inositol	100
Casamino acids	0
L-Glutamine	0
Thiamine•HCl	1
Pyridoxine•HCl	0.5
Nicotinic acid	0.5
Glycine	2
2,4-D	0
NAA	0
BAP	0
Kinetin	0
ABA	0
Activated Charcoal	2,500
Gelrite	
TC Agar	8,000
pH	5.7

MASS CLONAL PROPAGATION OF IMPROVED CONIFERS

Differential gene expression in the suspensor of zygotic embryos - Update

John MacKay
Heidi Schindler
Christina Perfetti

Jerry Pullman

SUMMARY

The goal of this research is to investigate suspensor biology in zygotic embryos and to use the information learned from zygotic embryos to improve the quality of somatic embryos by adapting tissue culture conditions. This specific report is an update of gene isolation experiments aimed at identifying genes that are differentially expressed in the suspensor of immature zygotic embryos. The purpose of these gene isolation experiments is to gain insights into suspensor biology and to potentially identify markers of suspensor development. Genes that are specifically or more abundantly expressed in the suspensor are being isolated from cDNA libraries that were enriched for such genes by subtractive hybridization. A few hundred cDNAs were thus isolated and many of these cDNAs are confirmed to be more abundant in the suspensor. A few seed storage protein genes were very abundant in our cDNA libraires. These genes may help uncover a nutritional role for the suspesnsor in embryo development of pines.

INTRODUCTION

The working hypothesis for these studies is that the suspensor plays an important, yet poorly understood, role in embryogenesis of loblolly pine both for zygotic and somatic embryos. Therefore, the formation and early development of **somatic embryos** should require suspensor functions, which may be uncovered by investigating the role of the suspensor in **zygotic embryos**. A better understanding of the role and development of the zygotic suspensor could allow us to better assess the effect of culture conditions and, make improvements to the initiation and multiplication phases of somatic embryogenesis. A better understanding of embryo development at early stages may also be essential to improving the quality of late stage embryos.

In angiosperm plants, the suspensor is required for early embryo development and, three different roles have been attributed to the suspensor (Cionini, 1987).

- Mechanical support of the embryo in the corrosion cavity of the seed
- A nutritional role to sustain embryo growth
- Potential regulation of embryo growth and development through the synthesis of growth regulators.

These roles highlight the importance of the suspensor in angiosperms but have only been partially verified in conifers. As a first step toward assessing the biological role of the suspensor in pine, we have undertaken experiments to identify genes that are differentially expressed in the suspensor of immature zygotic embryos.

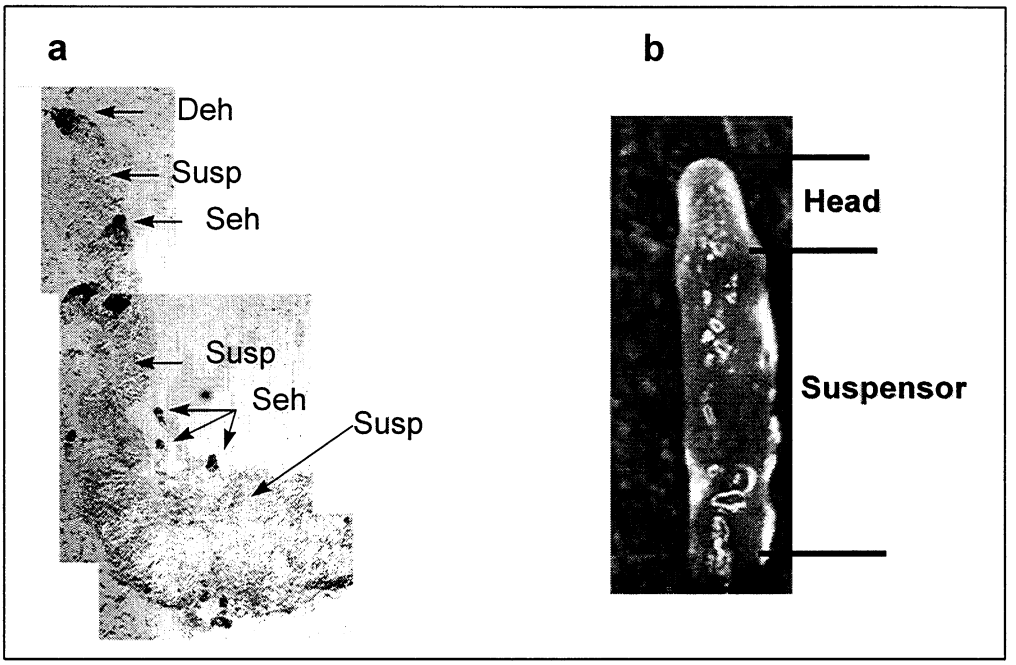

Figure 1. The suspensor of immature loblolly pine zygotic embryos. a. Stage 2 embryo stained to reveal the dominant and subordinate embryos supported by the suspensor mass, Deh: Dominant embryo head; Seh: Subordinate embryo head; Susp: Suspensor; b. Unstained stage 3 embryo.

RESULTS

Previous results

In March 1998, we reported

- Isolation of tissues and RNA from embryo head, suspensor and megagametophyte
- Construction of suspensor enriched cDNA libraries by subtractive hybridization
- Identification of abundantly expressed suspensor genes by blot analysis
- Sequence analysis of suspensor cDNAs

Verification of tissue specificity

We have continued to verify the tissue specificity of expression of the cDNA sequences of our subtracted libraries, by using reversed Southern blot analysis. A subset of 240 cDNA clones were screened with probes made from embryo head cDNA and embryo suspensor cDNA separately. The results from each probe were compared to determine the relative abundance of each cDNA in the suspensor and in the embryo head. A ribosomal RNA gene was used as an internal standard to attempt to normalize signal intensities from different tissue types. We have identified 110 cloned cDNAs that were more abundant in the suspensor. Future experiments are needed to analyze more clones, verify repeatability using other genotypes and include comparison to the megagametophyte.

Gene identification

Sequence analysis of a small number of the cDNAs has identified several genes that give strong matches with gene sequences in databases (see table). Many of the genes identified are conifer seed or embryogenesis related proteins.

DATABASE MATCH		Probability score
Gene	**Organism**	
Albumin 1 (seed storage protein)	White pine	10^{-86}
Albumin 3 (seed storage protein)	White pine	10^{-112}
Albumin 4 (seed storage protein)	White pine	10^{-110}
Alpha tubulin	Barley	10^{-61}
Globulin (seed storage protein), white pine	White pine	10^{-35}
Hin 1 gene (defense related)	Tobacco	10^{-10}
Legumin (seed storage protein)	Douglas fir	10^{-90}
Vicilin (seed storage protein)	White spruce	10^{-138}
PRE87 (embryogenesis, unknown function)	Radiata pine	10^{-145}
Cysteine proteinase inhibitor (defense)		10^{-21}

Abundance of major storage proteins in the cDNA libraries.

Sequence analysis of cDNAs revealed that cDNA fragments of a few genes had been cloned several times, specifically storage proteins appeared to represent a large pool of the cDNAs isolated through subtractive hybridization. To estimate the frequency of these cDNAs in our libraries, 340 cDNAs were hybridized with probes made from four of the major seed proteins we had isolated. The result is an estimate of the abundance or frequency of each of those genes in our libraries (see table). The abundance of albumin, globulin and legumin RNA transcripts in the supsensor provides a clear indication that storage proteins are expressed in the suspensor during early development. The information gained from this exeperiment will help to avoid redundancy in future sequencing experiments. These gene probes can also be used to more efficiently isolate other genes of low abundance in this set of cDNAs.

Gene	Frequency in libraries
Albumin 3-4 *	11%
Globulin	12%
Legumin	9%
PRE87	2%

*Albumin 3 and albumin 4 are two distinct but very similar genes in pine, the probe used in this experiment hybridized to both and did not discriminate between the two genes.

Experiments in progress.

- Verification of gene expression results in other genotypes
- Additional screening of the subtracted stage 3 and stage 4 libraries
- Development of storage protein probes as potential markers for embryo development
- Analysis of gene expression in somatic embryogenic lines.

DISCUSSION AND CONCLUSIONS

Several of the genes we have isolated and sequenced appear to encode seed storage proteins. Recently it was reported that the suspensor of *Vicia faba* (field bean) has a significant but transient accumulation of storage proteins (Panitz et al., 1995) which most likely contributes to the nutritional role of the suspensor. The accumulation of storage protein has also been observed in immature zygotic embryos of spruce but no report details the putative localization of these proteins (Flinn et al., 1993). Enhanced expression of these cDNAs in the suspensor needs to be confirmed in other loblolly pine genotypes and, using other methods. If confirmed, our results indicate that suspensor functions characterized in angiosperms could be directly applied to pine. Libraries developed by these experiments can serve as laboratory resource to isolate full length cDNAs or as probes (see Ciavatta *et al.* Report).

There are several ways in which the isolation and characterization of genes expressed in the suspensor can provide useful information to modify somatic embryogenesis methods with the ultimate goal to improve embryo quality. From the knowledge gained by careful studies of gene expression, molecular and biochemical markers could be developed to assess embryo (suspensor) development and growth in the early stages of somatic embryogenesis. Markers of suspensor development and function may also be useful to follow the evolution and fate of different cell types in somatic cultures, during culture cycling or culture decline. Knowledge of the function of the suspensor specific genes may also help develop specific hypothesis to improve embryo growth and development. For example, in this study, we have identified several cDNAs encoding putative storage proteins. Although this finding does not establish the accumulation or abundance of these proteins in the suspensor, it suggests that further studies could be directed to verify the synthesis of some of these storage proteins by suspensor cells in somatic embryogenesis. We are developing a collection of several storage protein gene probes that can be used as a resource to analyze storage protein expression at any stage of development. Selected storage proteins or their genes could be used as markers to explore how tissue culture treatments may effect their synthesis and mobilization.

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John Cairney
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Gary Peter
Lin Ge
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Vincent Ciavatta

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GENE EXPRESSION STUDIES WITH DEVELOPING EMBRYOS: THE EFFECT OF ABA TREATEMENT

Nanfei Xu,
Xiarong Feng,
Gerald Pullman
John Cairney

Abstract

Changes during embryo development are subtle and difficult to observe visually, but molecular events are dramatic and conspicuous. A fundamental understanding of gene expression patterns during embryo development would allow more detailed and informative comparison and monitoring of somatic and zygotic embryos. We believe that identifying normal and abnormal gene activity will provide stage-specific markers and hypotheses to improve somatic embryos. We have demonstrated previously that the techniques of differential display and DNA arrays permit us to view the activity of hundreds of genes over the course of embryo development. In this current report we apply the tools which we have developed to monitor gene activity during tissue culture modifications which alter embryo quality. We demonstrate that the same genes are affected in two different genotypes. We compare the activity of these genes over the course of development in somatic and zygotic embryos.

Introduction

Developing and improving tissue culture protocols is a lengthy and costly process. The traditional literature-based trial-and-error approach is most often used successfully; however, at some point embryo yield and quality improvements become increasingly difficult to obtain. Additional approaches such as studying natural embryos provides insight which permit the hormonal (Kapik 1994), nutritional, and physical (Pullman 1997) conditions found in vivo to be mimicked in vitro. An understanding of how medium changes over time, the effects of activated carbon adsorption and pH (Van Winkle et al. 1997) can further protocol development. Yet, new ways to improve protocols are still needed.

The program of gene expression which results in embryo development is largely unknown but our recent studies have revealed much about what is common and different between zygotic and somatic embryos in this regard. We have charted the expression of hundreds of genes during development and, cloned and sequenced over four hundred cDNAs. One of the goals of this work was to use gene expression studies to monitor the effect of prtocol changes on embryos and relate gene activity in Somatic embryos to that in Zygotic embryos. We wished to determine firstly, whether protocol changes which improved embryo quality made gene expression patterns in somatic embryos more like those in zygotic embryos. Secondly, we wished to determine whether the rapid gene expression assays could act as a predictor of a successful protocol change, and thus could be used to accelerate process improvement. The experiments reported here monitored gene expression

changes in response to a variety of growth regimes in the presence of ABA for different periods of time.

Materials and Methods

Zygotic and somatic loblolly pine embryos. Loblolly pine cones were collected weekly from a breeding orchard near Lake Charles, Louisiana, and shipped on ice to IPST. Embryos were excised and evaluated for developmental stage (Pullman et al. 1994). Stage 9 embryos were separated by the week they were collected - 9.1 (week 1), 9.2 (week 2), etc. Staged zygotic embryos were sorted into vials partially immersed in liquid nitrogen and stored at -70°C. Somatic embryos for loblolly pine were initiated as described by Becwar et al. (1995) or with minor modifications. Somatic embryos were grown, selected, and staged as described by Pullman et al. (1994) and stored at -70°C.

Molecular techniques. Differential Display was carried out essentially according to Liang and Pardee (1992). To allow the extraction of mRNA from minute amounts of embryo tissue, we developed a magnetic bead extraction method (Xu et al. 1997). cDNA bands from differentially expressed genes were excised from gels, cloned, and their differential expression confirmed using methods modified in our laboratory (Xu et al. 1997, Cairney et al. 1997). The southern blotting procedure described by Cairney et al. (1998) was used to prepare membranes containing quadrate blots for several hundred differentially expressed cDNAs. Resulting autoradiographs were scanned and digitized.

Results and Discussion

DNA arrays (also known as Gene Arrays, DNA chips, and in our case, Dot Array Southerns) permit the evaluation of the expression of hundreds or thousands of genes within an organism in response to altered growth conditions (Schena 1996, Marshall and Hodgson 1998, Ramsey 1998). Gene arrays may be comprehensive, consisting of a library of essentially unselected genes, cDNAs, primers (Marshall and Hodgson 1998), or may be more focused, consisting of selected clones, such as described in this paper (Xu et al 1997, Cairney et al 1997). The choice will depend on the nature of the experiment and the sensitivity of the detection system. A large amount of information can be gained which allows the effect of process alterations on mRNA accumulation to be viewed. The metabolic progress of an embryo through development may then be followed. Tissues can be subdivided and assays conducted to reveal expression in different locations. Such assays can be carried out without knowledge of the function or even the identity of the genes being assayed. Synthesis of protein from transcripts is not assayed in this system or in differential display. Post-transcriptional regulation of expression must be determined by other experiments, however assays which provide information on the differential accumulation of mRNA are instructive of themselves and provide materials for further study.

To demonstrate the ubiquity or uniqueness of a gene expression profile in development, we can avail ourselves of the ability of related genes to cross-hybridize in Dot Array Southerns. If we use RNA from different genotypes or tree species, and hybridize at lower stringency, we can view cross-hybridizing mRNA and note their identity, time of appearance, and quantity compared to the

loblolly pine controls. This technique might be applied to quite divergent species. Our sequence analysis shows very strong homologies between certain pine cDNAs and cDNAs from Arabidopsis, Tomato, Tobacco, etc. (Xu et al. unpublished). Such assays may forge a link between studies in tractable model plants, such as Arabidopsis, and commercially important plants such a pine or maize. In Arabidopsis, developmental mutants have been isolated but their overall effect is not known. By using our pine cDNA array systems we may be able to evaluate the effect of a mutation on embryo metabolism and physiology.

ABA Treatments

The results from earlier experiments showed that increasing ABA concentrations from 5.2mg/L to 10.4mg/L at week 5-16 on maturation medium increased the number of embryos. In zygotic embryos, the ABA concentration changes as the embryos develop. The ABA concentration is low for the first few stages of embryo development, increases dramatically at middle stage an thean drops at late stage. In this experiment embryos were isolated after treatments shown in Table 1, RNA was extracted and probes made for use in DNA arrays as described above.

Table 1. Treatments applied to developing embryos, for genotypes 314 and 333.

	Week 1-4		Week 5-8		Week 9-12		Week 13-16	
Treatment	ABA mg/L	Media	ABA mg/L	Media	ABA mg/L	Media	ABA mg/L	Media
A	5.2	752	5.2	752	5.2	752		
B	5.2	752	10	796	10	796		
C	5.2	752	5.2	752	5.2	752	5.2	752
D	5.2	752	10	796	10	796	10	796
E	5.2	752	10	796	10	796	5.2	752

In the panels shown in Figure 1, the quartets of dots represent four identical spots containing a particular cDNA clone. The intensity of the signal (light gray, dark gray etc) reflects how much probe has been bound and thus how much mRNA for that particular gene was in the embryo. Comparing two quartets from Figure 1 we see that in A, Box 1 we have a certain intensity of signal, in B Box 1 the intensity is slightly lower. From this we conclude that in treatment B, higher level of ABA, there is less mRNA for that particular gene in the embryo (indicated by a downward pointing arrow). We know which cDNA we spotted there, clone LPZ 049 which has strong homlogy to Starch Synthase, so from this data we can begin to fashion hypotheses about the reduction in the synthesis of Starch Synthase in Stage 8 embryos in response to ABA. Table 2 shows the identity of the other clones boxed in Figure 1. DNA arrays thus provide insight into gene activity in Tissue Culture and permits testable hypotheses to be constructed.

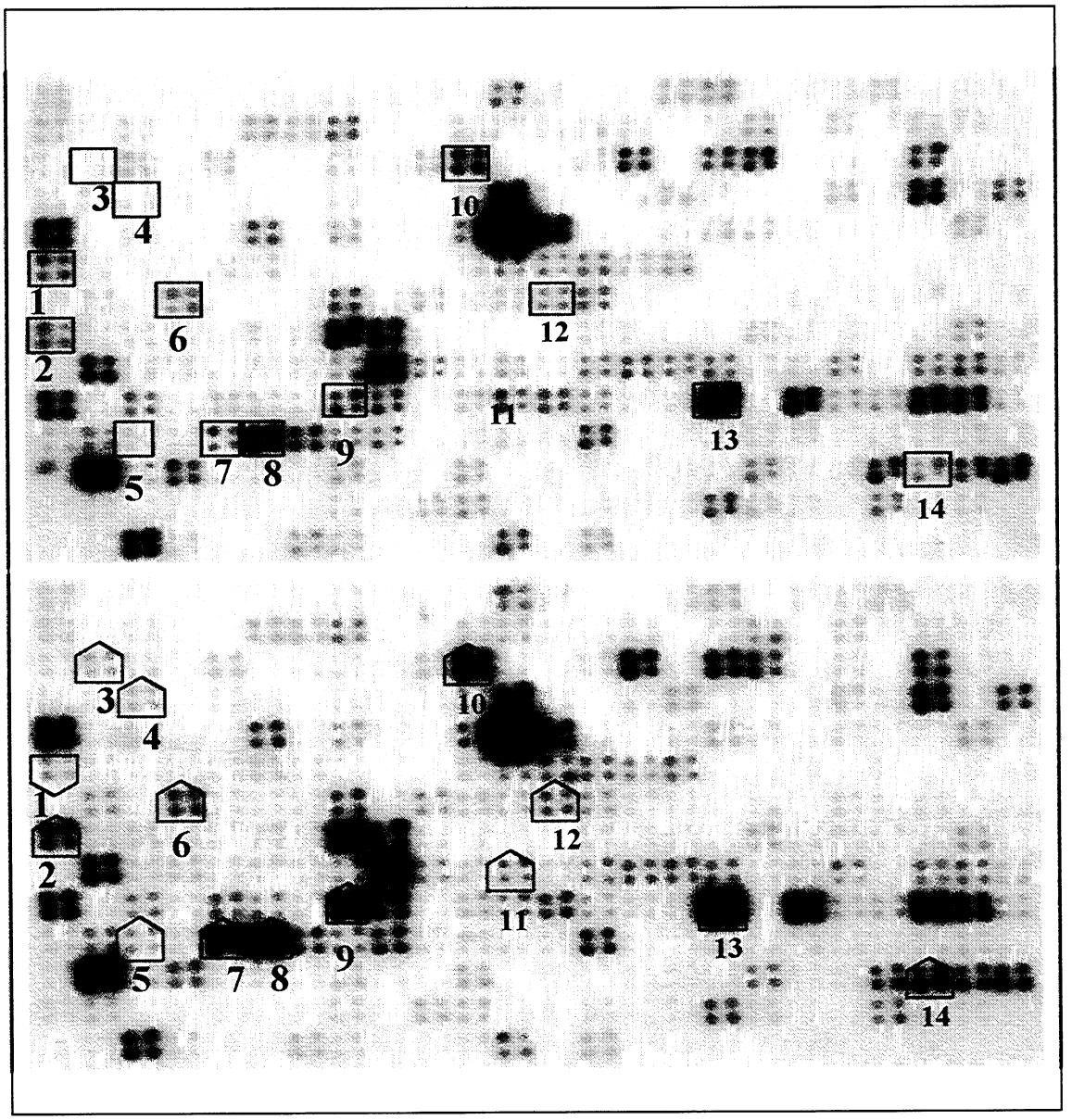

Figure 1. Detection of gene expression by high density array Southern hybridization for loblolly pine genotype 333 after 12 weeks on two maturation media. Top, 5.2 mg/L ABA (Treatment A, Table 1); bottom 10 mg/L ABA (Treatment B, Table 1). Arrows up indicate increased gene expression in the 10 mg ABA treatment; arrow down, expression lower in 10 mg ABA treatment. The same clones are boxed in both panels.

Table 2. Identity of Clones Boxed in Figure 1

Number on Autorad	Original Clone Number	Clone Identity 1/
1	LPZ 049	dH; Starch synthase
2	LPZ 107	pL; Yeast protein in SMY1-MUD2 intergenic region
3	LPS 064	Novel
4	LPS 092	dM; Arabidopsis cDNA clone AR192
5	LPZ 213	Novel
6	LPZ 080	pM,dM; acyl-CoA-binding protein
7	LPZ 215	Novel
8	LPZ 216	pL; late embryogenesis abundant protein
9	LPZ 186	Novel
10	LPS 074	Novel
11	LPZ 153	Novel
12	LPZ 091	dL; mRNA for LMW heat shock protein
13	LPZ 202	pL; late embryogenesis abundant protein
14	LPZ 270	pH; Heat shock protein 70

1/ pH:, pM:, pL:, homlogy of translated peptide at hight level (BLAST score>200), middle level (BLAST score 150-200), and low level (BLAST score 80-149).
dL:, dM:, dL:, homology of DNA sequence at high level (BLAST score > 400), middle level (BLAST score 300-399) and low level (BLAST score 200-299)

When the experiment is repeated with a different Genotype (Genotype 314) the same clones respond to the treatment (Fig.2). The image quality of Figure 2 is not as clean as Figure 1 however the same pattern as seen for Genotypes 333 and 314. Allowing for differences in exposure, four additional clones in Genotype 314, indicated by arrows, may be responding to ABA. This result indicates that the DNA array assay can provide an overview of gene activity which and that this information is repeatable for other genotypes.

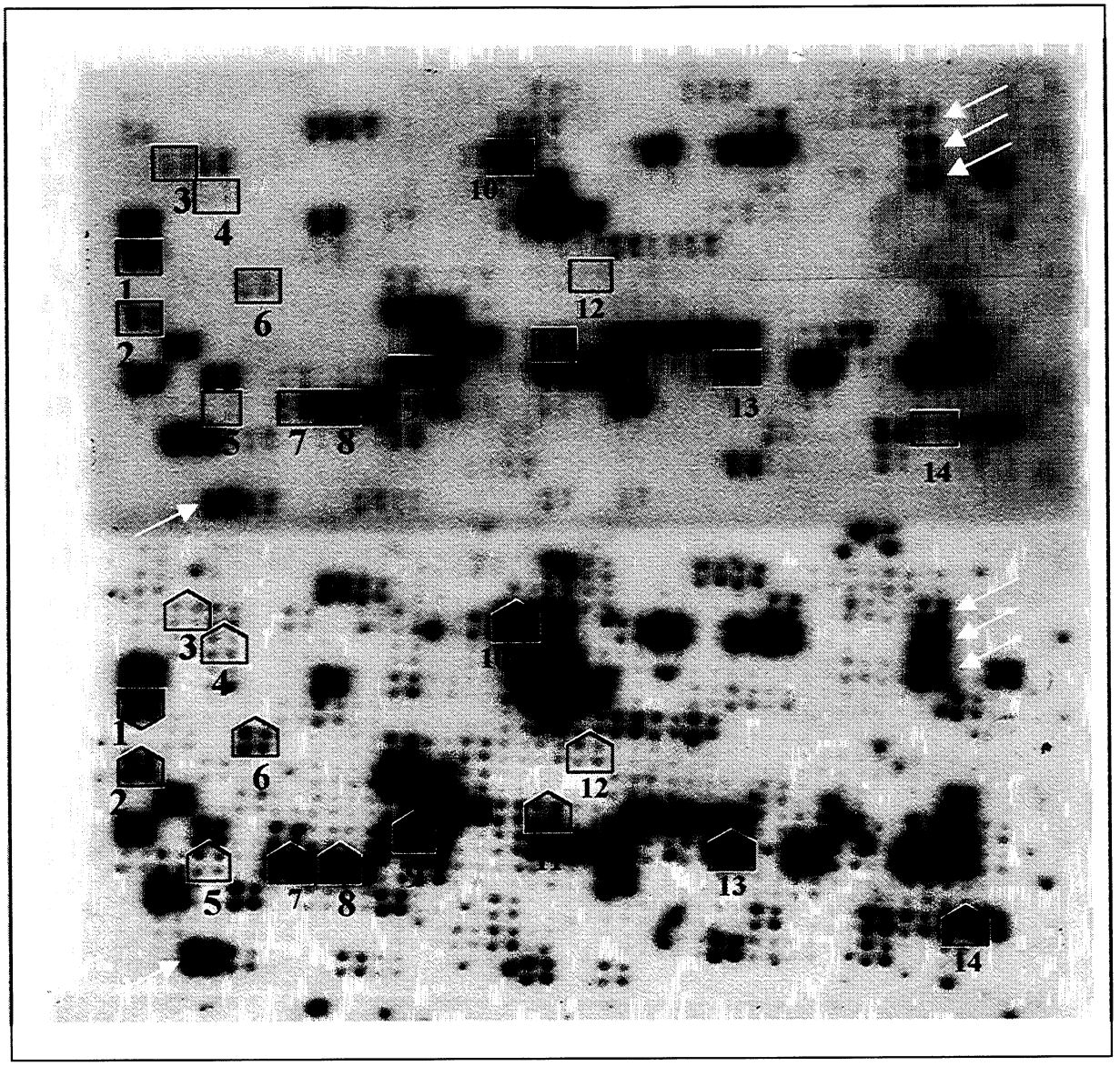

Figure 2. Detection of gene expression by high density array Southern hybridization for loblolly pine genotype 314 after 12 weeks on two maturation media. Top, 5.2 mg/L ABA (Treatment A, Table 1); bottom 10 mg/L ABA (Treatment B, Table 1). Arrows up indicate increased gene expression in the 10 mg ABA treatment; arrow down, expression lower in 10 mg ABA treatment. The same clones are boxed in both panels and the boxed clones are the same as those in Fig.1. The arrows indicated clones whose expression appears to be elevated by ABA in Genotype 314 but apparently not elevated in Genotype 333.

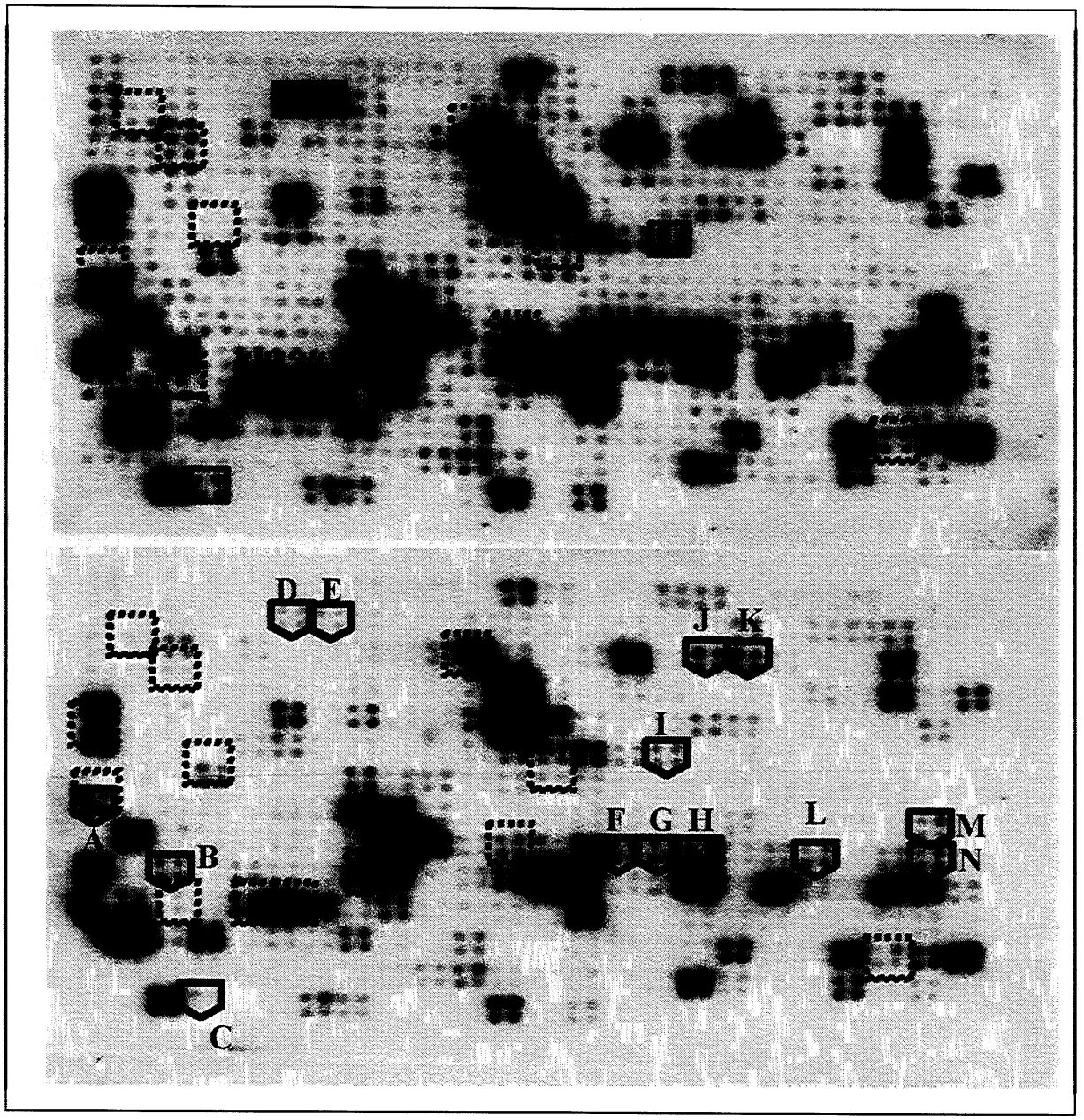

Figure 3. Detection of gene expression by high density array Southern hybridization for loblolly pine genotype 314 after 16 weeks on two maturation media. Top Panel;Treatment D, Table 1; embryos maintained on 10 mg/L ABA throughout experiment. Bottom panel (Treatment B, Table 1). 10 mg/L ABA until Wk 13-16 when media changed to 5.2mg/L ABA. Hatched boxes are the clones which were induced by ABA in Fig.1. Solid boxes show clones which are affected by the drop in ABA.

Table 3: Clones whose expression is reduced in embryos exposed to lower ABA concentration (see Fig.3.)

Letter On Autorad	Origian Clone Number	Clone Identity 1/
A	LPZ 107	pL; Yeast protein in SMY1-MUD2 intergenic region
B	LPZ 175	pL; nitrate reductase
C	LPZ 307	Novel
D	LPS 42	Novel
E	LPS 43	Novel
F	LPZ 157	Novel
G	LPZ 150	pL: Gibberelin regulated protein GASA5
H	LPZ 162	pL: Arabidopsis ERECTA gene
I	LPZ 67	Novel
J	LPS 80	pL: Xenopus laevis Etr-3, dL: lobloly pine cDNA clone 2345e
K	LP 81	Novel
L	LPZ 167	Novel
M	LPZ 138	Novel
N	LPZ 171	pL: adenylyl cyclase isoform DAC9

1/pH:, pM:, pL:, homlogy of translated peptide at hight level (BLAST score>200), middle level (BLAST score 150-200), and low level (BLAST score 80-149).
dL:, dM:, dL:, homology of DNA sequence at high level (BLAST score > 400), middle level (BLAST score 300-399) and low level (BLAST score 200-299)

In Figure 3 the effect of reducing ABA concentration in the final three weeks of culture is observed. The clones which were induced by elevated ABA, for the most part do not, appear to be affected by the lower ABA level. The expression of fourteen genes is reduced by growth on the media containing lower ABA levels. Their identities are given in Table 3. Once more, we gain an insight into variation in the induction of different genes and may use this information to guage the metabolic state of the embryo, make measurements and design experiments to confirm these hypotheses, and ultimtely we may design new, improved, culture protocols.

Achievements and Problems with Our Current Technology:

We have been using DNA Arrays to evaluate gene expression over the course of pine embryo development (PAC report , March 1998) and in response to different media conditions such as ABA levels (Figs in this report) and Boron and Calcium levels (PAC Report March 1998). The information given in this reports is generated by gridding cDNA clones on a membrane, exposing them to a radioactive probe generated from RNA isolated from embryos growing under a given condition and exposing the membrane to X-ray film. The image on the X-ray film is then scanned and using Gel-Pro software, the intensity of each dot is quantified. This latter step will assign

numbers to each dot permitting a quantitative evaluation of the degree to which a specfic mRNA changes in the embryo. This latter approach is a powerful tool in gene expression analysis but currently, we are limited by the tools available to us.

Currently we are attempting to quantify expression by an analysis of autoradiographic images. The detection aspect of this technology has several severe shortcomings. 1) This is an indirect evaluation dependent on the sensitivity of the X-ray film and assumes that signals that are being compared are within the linear range of the film. 2) Saturation of signals prevents accurate assignment of values to strongly expressed genes. 3) Problems of 'bleed' of intense signals into the field of less intense signals requires us to expose membranes for different periods of time then compare signal intensity using a conversion formula. 4) The use of radioisotopes is increasingly expensive, requiring special facilities and training plus costly disposal. The processing of data is very labor-intensive an time consuming.

The **Storm®Image Analyzer (Molecular Dynamics)** provides solutions to these problems. 1) Data can be collected directly, without the intervention of film, 2) Hybridization levels can by determined directly, irrespective of their intensity. 3) No 'bleed' of signals due to medium saturation 4) Adoption of non-radioisotope protocols is possible. Quantification of Northern and Western blots is possible with this system. 5) Plate assays can be conducted using this system. 6) The flexibility of the system permits three detection modes: Storage Phosphor Autoradiography (Radioisotope detection), Direct Fluorescence, and Chemifluorescence. The Storm®Image Analyzer is being used for **microarray analysis by a number of companies, research institutes and universities (See Molecular Dynamics Homepage** http://www.mdyn.com/default.htm).

An accurate evaluaion of gene expression is essential if we are to capitalize on the work carried out over the past four years and establish a genomics program whose aim is the improvement of somatic embryogenesis. Genomics programs such as we propose are currently being funded to levels of several hundred million dollars by agricultural/biotechnology programs. The focus of these commercial programs will be food crops. The potential of the genomics approach which we have developed over the past years is enormous but to exploit it we need modern, sesitive and accurate equipment.

A summary of the experiments aimed at improving somatic embryogenesis experiments, proposed using the Storm®Image Analyzer are as follows:

- Quantification of Gene Expression using pine cDNA Arrays and RNA from Pine Somatic Embryos Stages 1-9. Currently the success of a Pine Tissue Culture Protocol can only be gauged after several months. There is no method to monitor the progress of an embryo through development other than visual inspection. Our cloning of 400 cDNAs from genes differentially expressed during development (and continual supplementation of this collection with new clones) permits us to follow the sequential induction of gene expression during development and identify expression markers. The success of this evaluation depends on the accuracy with which we can determine gene expression. Clarifying and establishing profiles of expression demands consistency and reproducibility. Faith in the accuracy of the measurement is thus essential to the utility of the assay.

- Quantification of Gene Expression using pine cDNA Arrays and RNA from Pine Zygotic Embryos Stages 1-9.10. The ability to produce a loblolly pine somatic embryo which resembles a zygotic pine embryo, in terms of size, shape and capacity to germinate, is goal of may industrial and academic research programs. Unfortunately we know very little about the molecular biology of pine embryogenesis. Our preliminary results with differential display (Xu et al 1997, Cairney et al 1997)

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Westvaco Company (1998) Forest Focus 22, 10-13.
Xu, N., Johns, B., Pullman, G., Cairney, J. (1997). Rapid and Reliable Differential Display Results from Minute Amounts of Tissue: Mass Cloning and Characterization of Differentially Expressed Genes from Loblolly Pine Embryos. Plant Mol. Biol. Reporter, 15 (4): 377-391.

Early-Stage-Specific Gene Expression in Loblolly Pine Zygotic and Somatic Embryos: cDNA Cloning, Sequencing and Expression Analysis

Vincent Ciavatta, Gerald Pullman and John Cairney

The goal of this project is to uncover genes expressed early in embryo development and gather evidence to determine their functions. To this end, several early-abundant expressed sequence tags (ESTs) were identified by differential display. During the spring PAC, a dot southern and a northern blot along with preliminary GenBank search results for the EST das6 were presented. The purpose of this report is to provide an update on the cDNA cloning and expression analysis of das6. In addition, northern blots for two more ESTs that show preferential expression in early stage embryos are presented.

We wish to develop tools that permit us to determine, quickly and unambiguously, the metabolic conditions of an embryo. Such tools would allow us to monitor embryogenesis more closely, thus the effect of different tissue culture modifications on advancing the embryo through development could be viewed with greater accuracy; minor advances, not discernable by eye, could be seen.

One of the goals of our gene expression studies with pine embryos has been to identify stage-specific markers; genes that are active at a certain, clearly defined, stage of development. When mRNA for such a gene was observed we could then conclude that the embryo was at a certain stage of development. A definition of development based on gene expression is more closely related to the metabolic state of the embryo and thus would be a more valuable and precise index of embryo development than that current descriptive definitions based on morphology. In work reported in the PAC Report March 1998, we described differential display experiments which resulted in the cloning of cDNAs which were present at the earliest stages of development, Stages 1-3. We have cloned a number of these bands and conducted Northern blots which confirm that these mRNAs are present early in development. Most of the cDNAs which we obtained, were partial copies of the mRNA, much of the 5' region of the molecule was missing, a normal occurrence for cDNAs isolated from differential display. In the present report we describe experiments designed to determine the remainder of the sequence of one clone, das6, and experiments to isolate a full-length cDNA from a library. Expression data, nothern blots is provided on other, early-expressed clones.

I. 3' RACE and 5' RACE of das6

The RACE (rapid amplification of cDNA ends) techniques were used to get near-full length mRNA sequence information of the message that contains das6. Because the das6 clone did not contain the 3' end of the message, both 5' RACE and 3' RACE were required to capture the entire mRNA sequence. The resulting sequence information (two walks towards the 5' end + the original differential display fragment + one walk towards the 3' end) was assembled into an open reading frame (Figure 1.) The message is approximately 1650 to 1700 nucleotides, a size which is consistent with the size calculted from Northern analysis (PAC Report March 1998). The longest open reading froma encodes a protein that is 321 amino acids long. This gene is referred to below as LPdes.

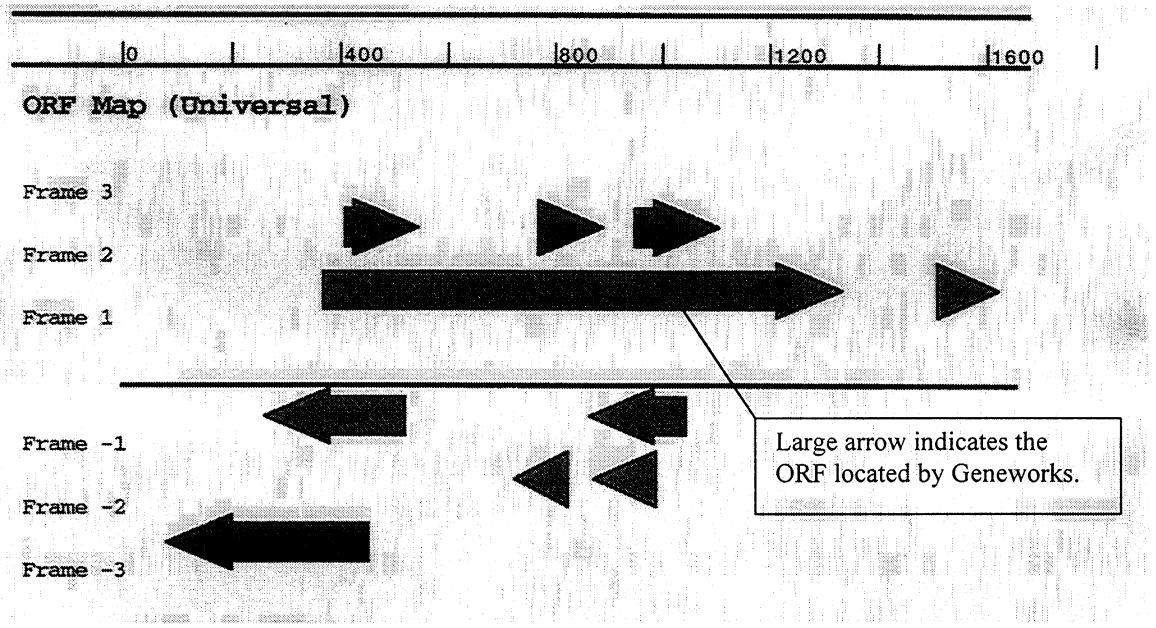


Figure 1. Open reading frame map of LPdes. This map was constructed with the help of the software program Geneworks. The large arrow represents the LPdes coding region that spans from nucleotide 370 (first nucleotide in start codon) to nucleotide 1333 (last nucleotide in stop codon).

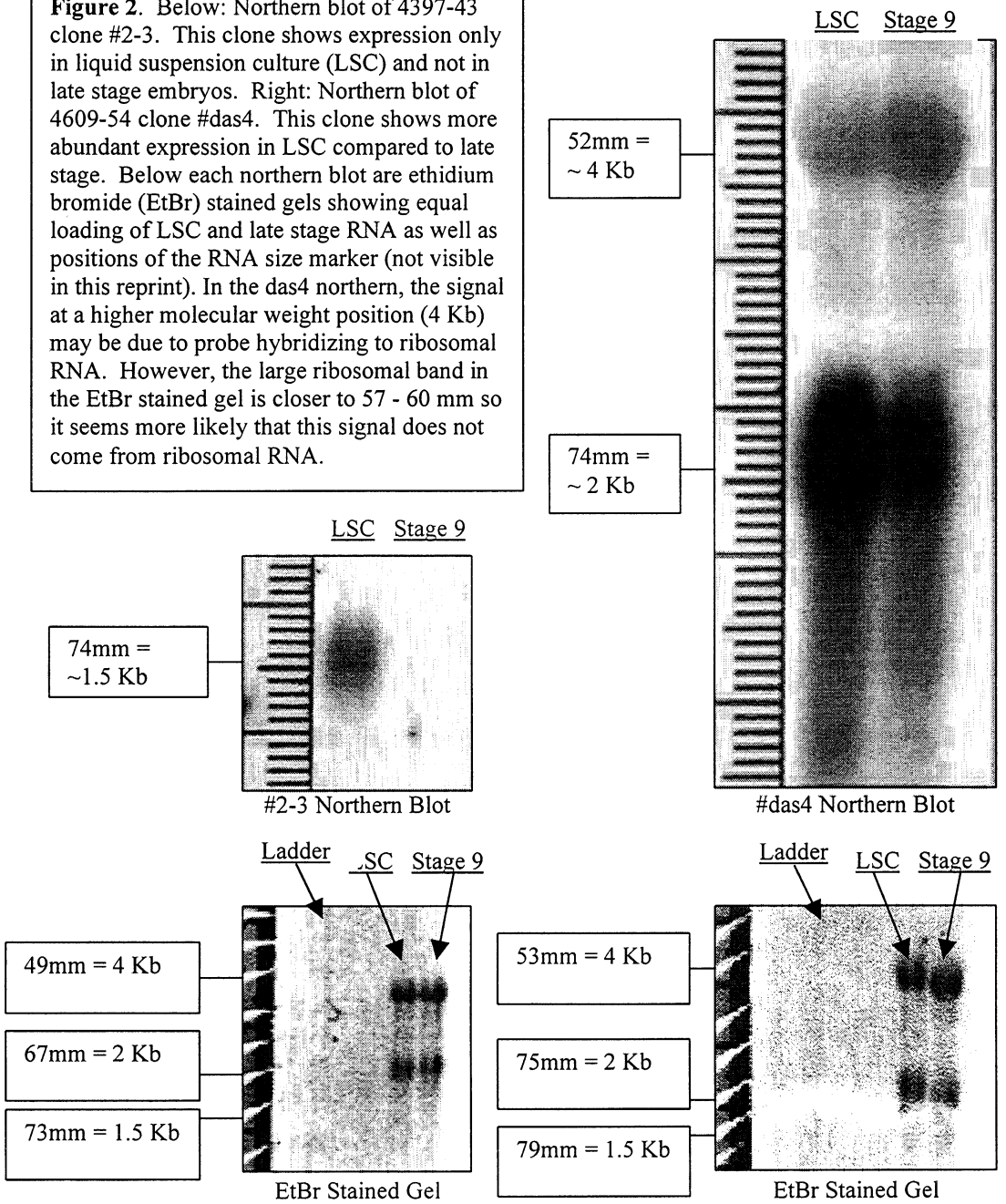
II. Northern Blots for 4397-43 Clone #2-3 and 4609-54 Clone #das4

In addition to das6, two other ESTs have had differential expression confirmed by northern blot (Figure 2). These are 4397-43 clone #2-3 and 4609-54 clone #das4.

III. GenBank Searches for 4397-43 #2-3, and 4609-54 #s das6 and das4

GenBank searches for the 3 ESTs revealed strong amino acid sequence homology of das6 to developmentally regulated genes in Drosophila, mice and humans, while clones #2-3 and #das4 showed no significant homology to archived nucleotide or amino acid sequences. Once sequence information from 5' RACE and 3' RACE clones was used to construct the ORF of LPdes, a GenBank search was conducted. This search corroborated the earlier homology to the Drosophila, mouse, and human genes. It also revealed that the LPdes predicted amino acid sequence aligns correctly with 3 histidine motifs, $HX_{(3-4)}H$, $HX_{(2-3)}HH$, and $HX_{(2-3)}HH$, that are characteristic of membrane fatty acid desaturases or membrane hydrocarbon hydoxylases from organisms such as *Mus musculus, Homo sapiens, Brassica napus, Arabidopsis thaliana, Pseudomonas oleovorans* and *Agrobacterium aurantiacum.*

Figure 2. Below: Northern blot of 4397-43 clone #2-3. This clone shows expression only in liquid suspension culture (LSC) and not in late stage embryos. Right: Northern blot of 4609-54 clone #das4. This clone shows more abundant expression in LSC compared to late stage. Below each northern blot are ethidium bromide (EtBr) stained gels showing equal loading of LSC and late stage RNA as well as positions of the RNA size marker (not visible in this reprint). In the das4 northern, the signal at a higher molecular weight position (4 Kb) may be due to probe hybridizing to ribosomal RNA. However, the large ribosomal band in the EtBr stained gel is closer to 57 - 60 mm so it seems more likely that this signal does not come from ribosomal RNA.



III. Capturing Full-Length cDNAs from a SMART cDNA Library

While we have assembled a composite sequence for the das4 clone, this is compiled from the sequences of three separate molecules. Further work, such as expression and characterization of the protein, or gene transfer experiments, will require a single cDNA containing all the sequence information in the mRNA. Conventional methods for isolating full-length cDNAs require cDNA synthesis, ligation of adapters, cloning into an appropriate vector, followed by library screening. The process is lengthy and fairly expensive. Most of our clones from Differential Display are partial sequences so most would have to be taken through the library screening process.

We wished to develop a more rapid method for isolating full-length cDNA clones. An uncloned 'library' of cDNA molecules has been prepared from the Head, Suspensor and Megagametophyte of early stage embryos by John Mackay using the SMART-PCR system (PAC Report March 1998). Preliminary PCR experiments showed that the das4 sequence was present in the Head and Suspensor library.). The cDNAs in the library have a common linker at the end of the molecules thus may be amplified by PCR. If one were able to select a specific sequence from the library, then it could be re-amplified and cloned without recourse to cloning into a vector and screening.

Work is currently being done to develop a general method for retrieving full-length SMART (Clontech) cDNA clones using only an EST clone. A successful method should permit rapid cloning of full-length cDNAs containing 4609-54 #das4, 4397-43 #2-3 and other ESTs without any need for radioactivity or 5'/3' RACE. The following is a skeleton of the procedure being developed. The approach for retrieving cDNA clones is based on solution hybridization between a specific biotinylated probe and the cDNA of interest (Figure 3).

- Probes are synthesized from EST clones or RACE clones by PCR with biotinylated d-UTP.
- Following denaturation and hybridization, the hybrids are captured with streptavidin-coated iron beads. The iron beads are then immobilized with a magnet and several washes are used to remove unwanted cDNAs.
- Finally, hybrids are eluted by high temperature incubation. Due to the linkers at both ends of SMART cDNA molecules, the eluted cDNA can be amplified by PCR using primers complementary to the SMART linkers.
- Products of PCR are electrophoresed to examine effectiveness of the enrichment.
- The PCR products are either cloned directly or the enrichment procedure is repeated on the PCR products prior to cloning.
- Colony PCR is then used to find clones that contain the cDNA of interest.

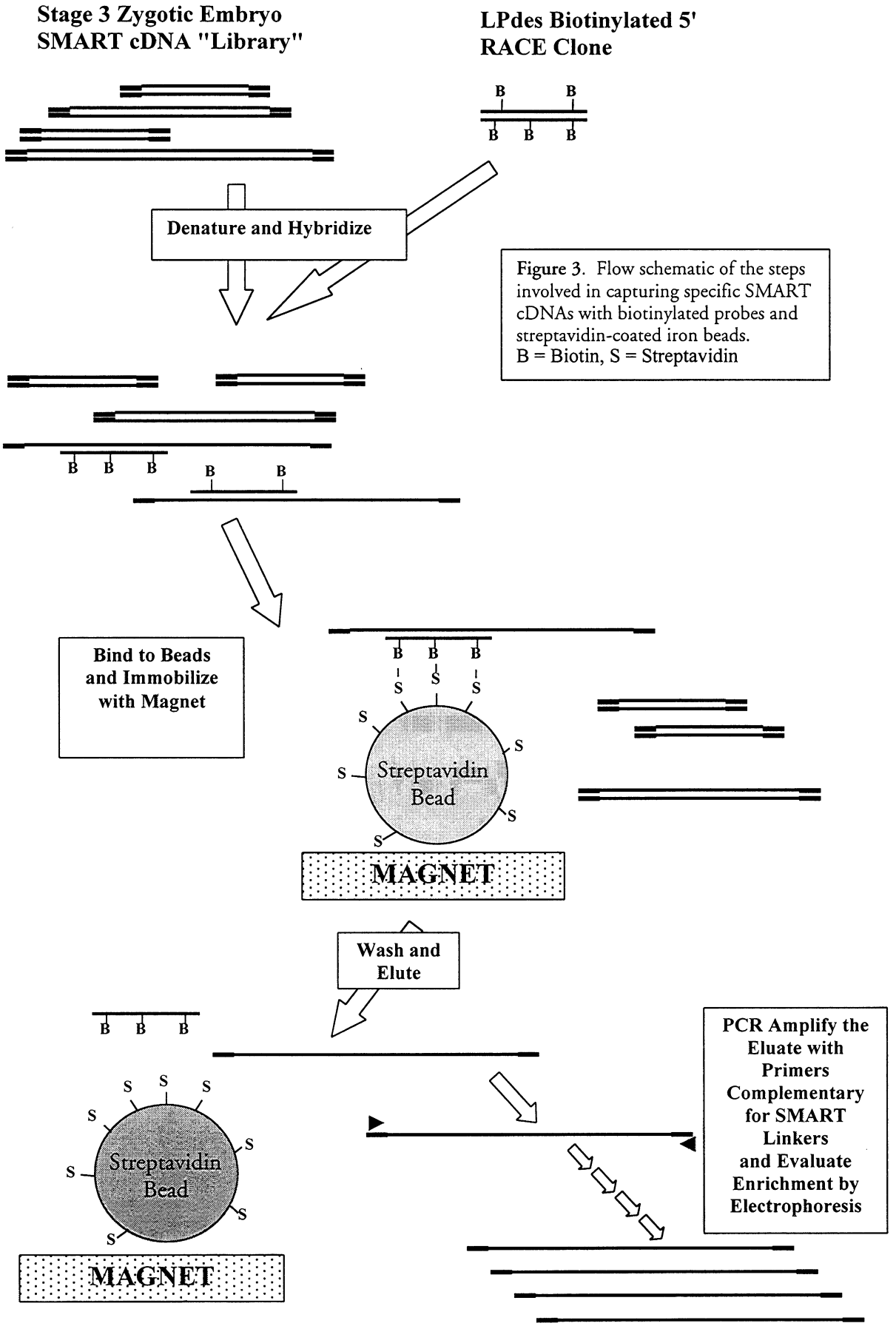


Figure 3. Flow schematic of the steps involved in capturing specific SMART cDNAs with biotinylated probes and streptavidin-coated iron beads.
B = Biotin, S = Streptavidin

IV. Future Objectives

Full-Length cDNAs: The next primary objective is to get full-length cDNA clones for the three ESTs: LPdes, 4609-54 #das4, and 4397-43 #2-3. In addition to allowing further insights through homology comparisons, full-length clones are necessary for *in vitro* translation which will be undertaken in the next phase of the project: expression analysis.

Expression Analysis: In keeping with the goals of the project, evidence will be gathered to help determine the function of candidate genes. Expression analysis will include mRNA localization and protein localization. RT-PCR and northern blots for various tissues (needles, roots, stems, flowers, etc) and embryo *in situ* hybridizations will be used to determine presence of mRNAs. Protein localization in embryos will be performed by immunoassays using antibodies raised against *in vitro* translated proteins.

Promoter Analysis: Promoters for candidate genes will be cloned from a Loblolly Pine Genome Walker (Clontech) library assembled by Dr. Ranjan Perera. Promoter - reporter gene constructs will be assembled and used in transformation experiments in Arabidopsis, Cottonwood, or Loblolly Pine to gather information on promoter function and tissue specificity.

Identification and Cloning of cDNAs for Novel Female-, Male- and Vegetatively Expressed Genes

Lin Ge, Gerald Pullman, and John Cairney

Abstract

A joint project with UGA, sponsored by the GA Consortium, funded research on reproductive genes in trees. IPST researchers have succeeded in isolating previoulsy uncharacterized genes which display exclusive male-, female- or vegetative expression. Three male-specific genes have been cloned, over 20 other male and female-specific genes are being characterized.

Introduction

A recently completed GA Consortium-funded project was focused on the use of molecular techniques to control flowering. Work with angiosperms has identified key genes in floral development which if manipulated appropriately can prevent the formation of flowers or accelerate flower formation. Each of these characteristics has a practical application. The prevention of flower formation will prevent the dissemination of pollen on transgenic trees, and engineering sterility is a strategy which is being considered as an environmental safeguard when transgenic plants are put in the field. The engineering of early flowering could have benefits for conventional breeding programs and could be employed as a testing strategy to determine the efficacy of the sterility programs.

A number of candidate genes for the sterility programs described above have been cloned and patented. One of the goals of our project was to identify and isolate novel genes which are expressed exclusively in female, or male tissue. Here we report the successful isolation and cloning of novel female and male-specific genes.

Differential Display With RNA from Male, Female and Vegetative Flowers

Loblolly pine flower tissues and vegetative shoots were collected in the time period of Sept. 1997 to June 1998. Total RNAs were isolated from female flower buds, male flower buds and vegetative shoots respectively. Differential Display was conducted using Primers T_{12}MG and AP-4 (Genehunter). The experiments were conducted at two or three different RNA concentrations to confirm the reproducibility of the band. Several differentially expressed transcripts were noted (Fig.1). When a band was observed at more than one RNA concentration, these cDNAs were excised from the gel and cloned into vectors for further Analysis.

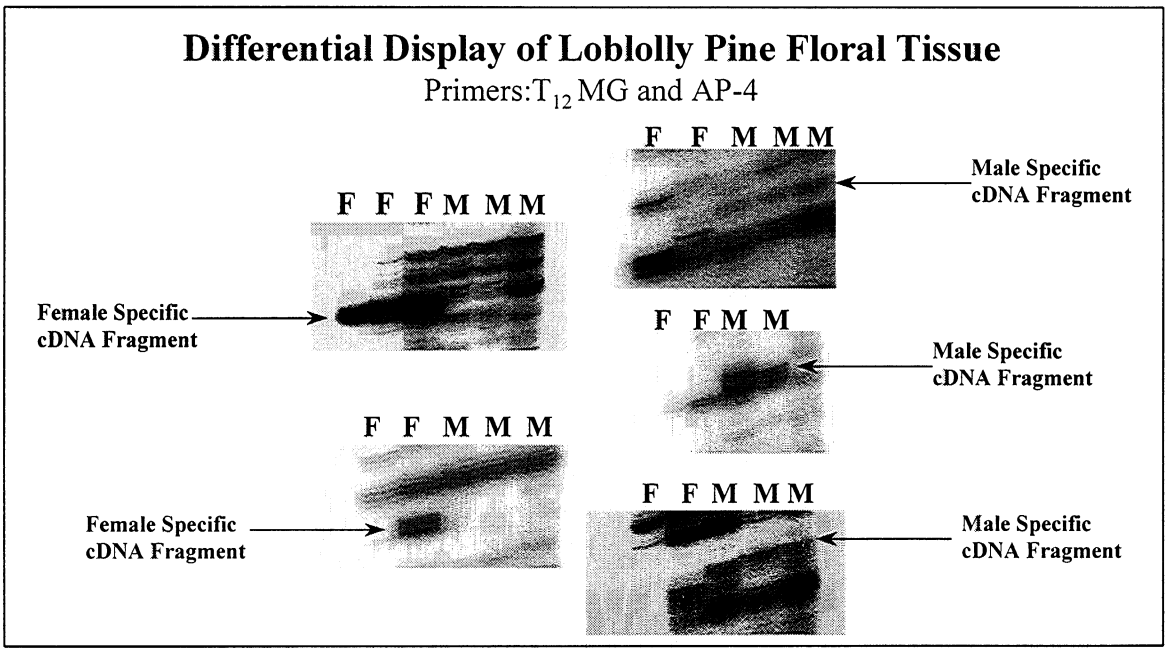


Figure 1. Differential Display Results using primer T12MG and AP-4 for Male-, Female- tissue Total RNA.

Construction of a Loblolly Pine cDNA Library Enriched in Female Flower-Specific Transcripts and a cDNA Library Enriched in Male Flower-Specific Transcripts.

An efficient method for selection of differentially expressed mRNAs is the construction of subtractive cDNA libraries. We have employed this technique and succeeded in isolating hundreds of cDNA clones of female and male expressed genes.

RNA was isolated as described above. After checking the quality of total RNA by formaldehyde agarose gel, mRNAs were isolated by Dynabeads (dT)25. Using these mRNAs and Smart PCR cDNA Synthesis Kit (Clontech), cDNAs were synthesized. Two subtractive hybridization reactions were carried out, (1) by subtracting vegetative shoot cDNAs and male flower cDNAs from female flower cDNAs (female-specific library), and (2) by subtracting vegetative shoot cDNAs and female flower cDNAs from male flower cDNAs (male-specific library). In this way, cDNAs for genes expressed specifically or more abundantly in loblolly pine female flower tissue were isolated and similarly transcripts abundant in male flower tissue were obtained.

The cDNAs arising from subtraction were cloned into plasmid vectors for further analysis.

Preliminary Results of Screening the Female Flower and Male Flower cDNA libraries

1. Colony PCR was used for preliminary screening both plasmid cDNA libraries, 90% of the plasmids contained inserts, ranging in size from 300 bp to 2200 bp.

2. Northern Blotting were carried out by using three randomly selected male clones and three randomly selected female clones as probes.

Male clones showed very specific expression in male flower tissue, but not in female flower tissue and vegetative shoots (see Fig. 2) even on long exposure (Fig. 3). These three male-specific clones were sequenced and found to be novel sequences.

Female clones showed very weak hybridization to and no clear pattern could be discerned for theses three clones, possibly due to the low expression of these three clones.

3. To further check the quality of these subtracted cDNA libraries, more Northern Blotting will be carried out by using randomly selected clones as probes from both libraries. Also high density array techniques are being used as a rapid screen to confirm differential expression in these libraries.

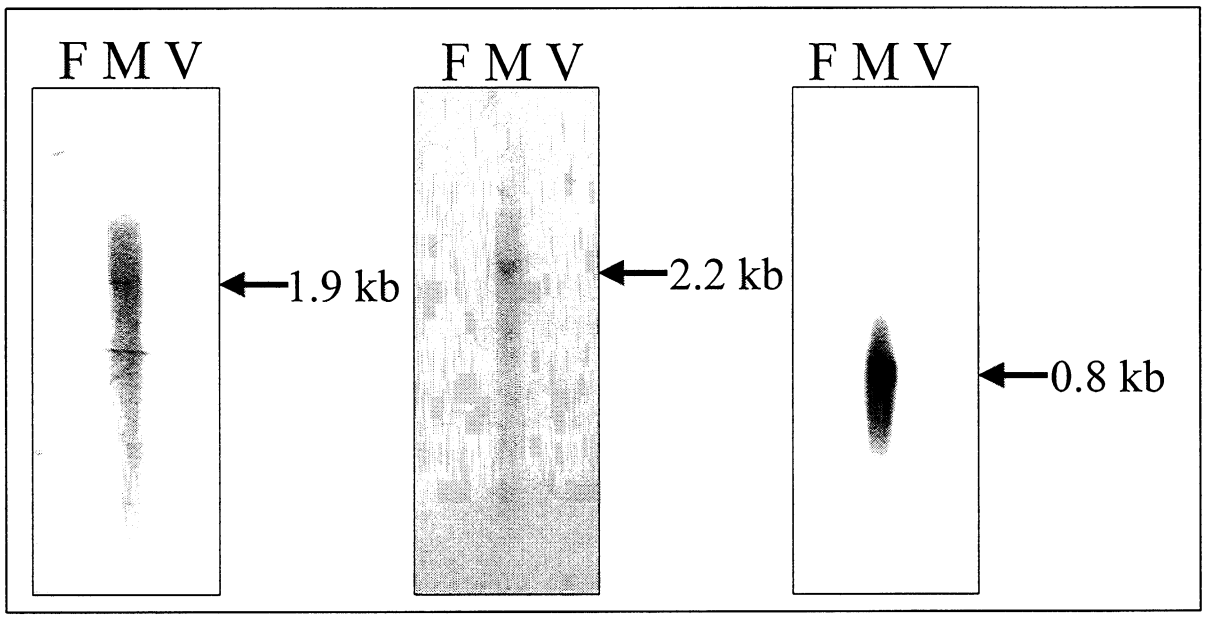


Figure 2. Northern Blotting of Three Male-Flower-Specific cDNAs. Ten micrograms of female flower tissue RNA (F), male flower tissue RNA (M) and vegetative shoot RNA were hybridized with 32P-labeled male flower clone-1 (left picture), clone-2 (middle picture), and clone-3 (right picture)

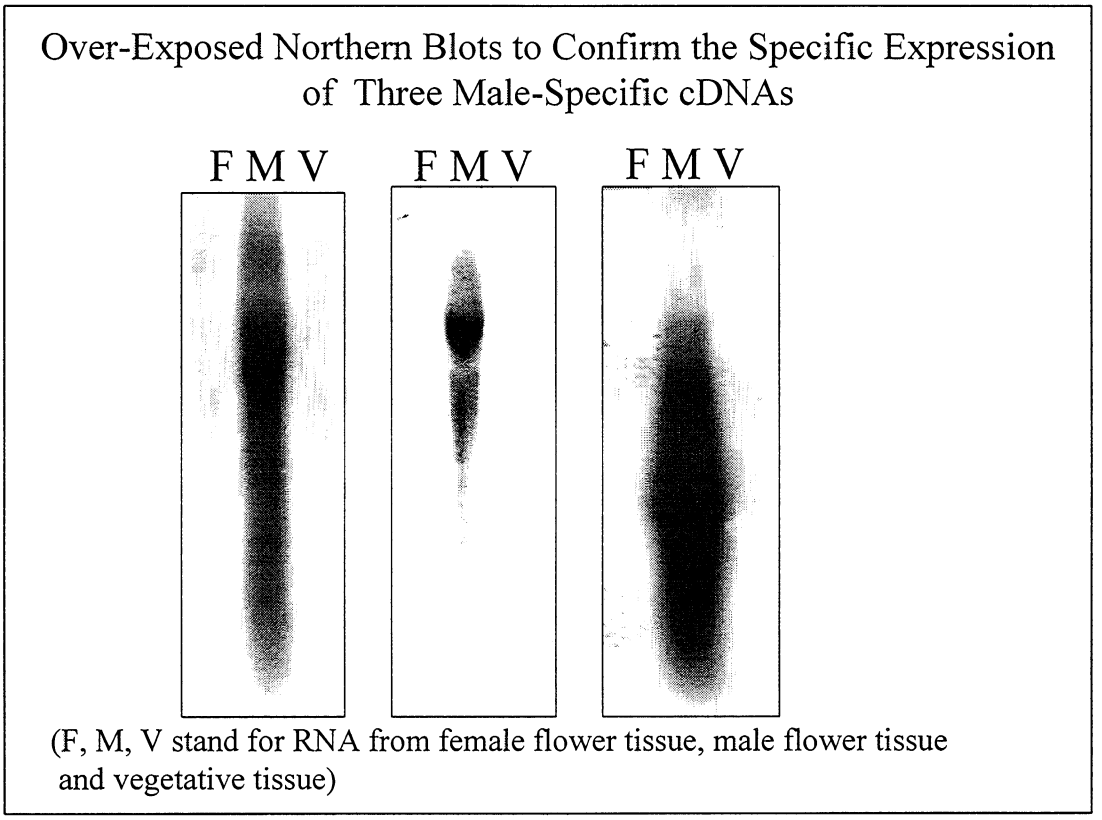


Figure 3. Long exposure of blots from Fig.2. Shows that there is not female or vegetative expression of these three genes.

Conclusion

To date, 20 cDNA clones of genes which are specific to male or female tissue have been isolated. The three male-specific clones tested and sequenced, are novel, thus would be available for use in a reproductive engineering program, since not previously reported, are not covered by existing patents.

FIBER PROPERTY MODIFICATION

IPST DUES FUNDED RESEARCH CONSORTIUM
1998-1999

FUNDAMENTAL BIOLOGICAL MECHANISMS: IMPROVED STEM GROWTH RATES AND FIBER PROPERTIES

Status Report for
Project F011

Gary Peter
John Cairney
John MacKay
Gerald Pullman
Douglas Benton
Cielo Castillo
Huabin Meng

October 14-15, 1998

DUES-FUNDED PROJECT SUMMARY
1998-1999

Project Title:	Fundamental Biological Mechanisms: Improved Stem Growth Rates and Fiber Properties
Project Code/Project Number:	HRDWD/F011
PAC:	Forest Biology
Division:	Chemical and Biological Sciences
Project Duration:	6 Months
Project Staff	
Faculty/Senior Staff:	Gary Peter, John Cairney, John MacKay, Gerald Pullman
Staff:	Postdoctoral Fellow to be hired
FY 97-98 Budget:	120,000
Allocated as Matching Funds:	10%
Time Allocation	
Faculty/Senior Staff:	0.4
Support:	1.0
Supporting Research	
M.S. Students:	1
Ph.D. Students:	1 starting Jan. 99
External:	$271,845

RESEARCH LINE/ROADMAP:

Area	Improved Forest Productivity
Research Line	Develop fibers with properties similar to or better than northern softwood and Eucalyptus that can be grown in most regions of North America.
Road Map	Develop fundamental understanding of secondary wall differentiation and stem growth

BENEFITS TO INDUSTRY:

- Faster growing trees with better fiber properties

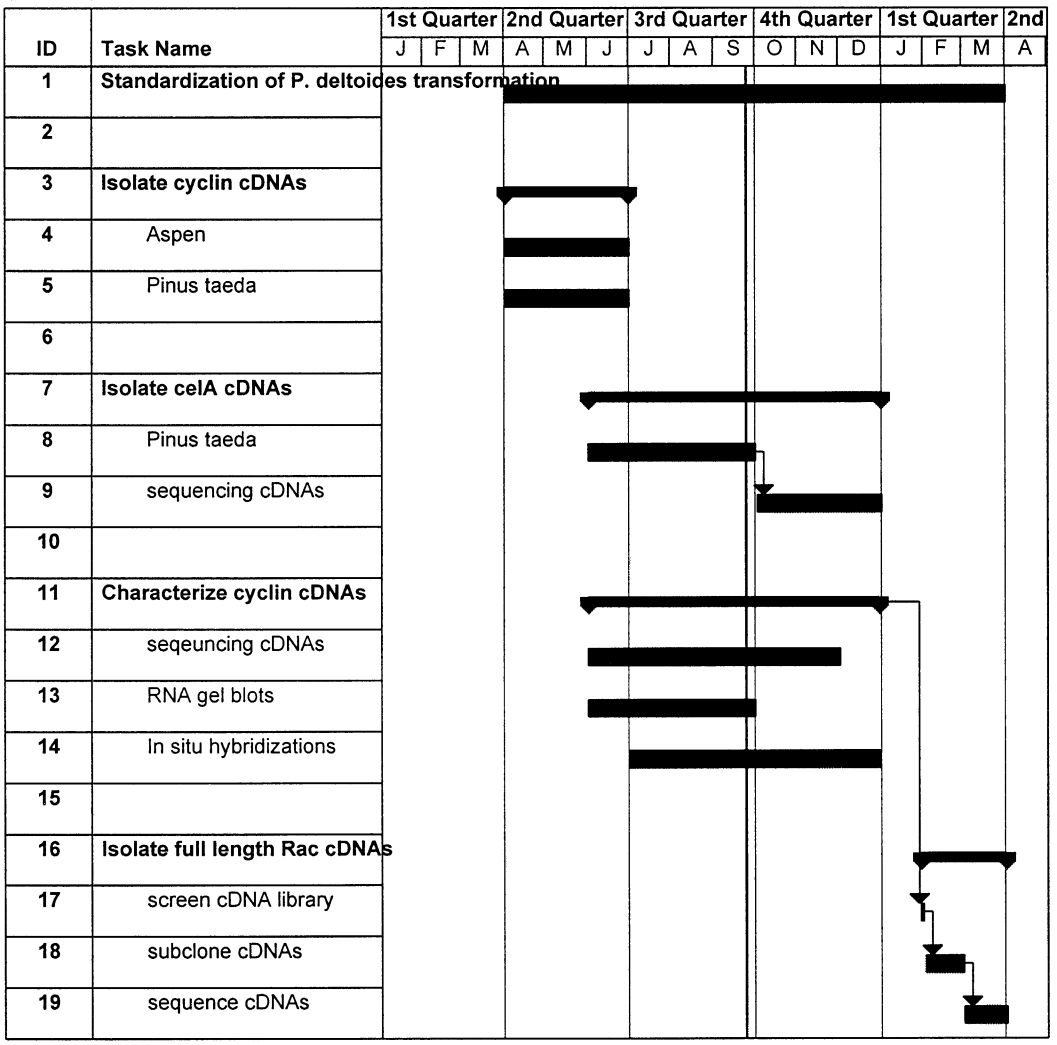
PROJECT OBJECTIVES:

This project has three broad objectives: 1) increase the growth rate of the stem, 2) improve fiber properties for value added paper products and 3) improve the processing characteristics of wood to decrease environmental impacts while increasing fiber yield and quality. Since our understanding of the mechanisms that regulate cambial cell growth rate, xylem fiber and secondary cell wall properties is so limited, to accomplish these goals a more fundamental understanding of the biochemical, cellular and molecular genetics in each of these areas must be obtained first. Although we would like to begin in parallel a coordinated effort in each of these areas, the limited funds in this project dictate that we work on them in sequential order based upon prioritization's. Our efforts toward this project will accelerate once additional moneys are obtained

GOALS FOR FY 98-99:

1. Complete standardization of tissue culture and transformation methods for *Populus deltoides* C175.
2. Isolate cyclin cDNAs that are expressed in the cambial meristems of loblolly pine and/or Poplar.
   A) Structurally characterize cDNAs
B) Begin experiments to characterize their patterns of expression within the cambial meristem
C) Begin to determine spatial patterns of gene expression within the stem for specific cyclins.
3. Isolate celA genes from differentiating xylem from loblolly pine.
A) Structurally characterize cDNAs
   B) Begin experiments to characterize their patterns of expression within differentiating xylem
   C) Initiate biochemical studies into the mechanisms of cellulose synthesis and interaction with cytoskeletal elements.
4. Isolate full length rac GTP-binding protein cDNAs from *Z. elegans*.
   A) Begin structural characterization of cDNAs (This work will be done depending upon progress made on the above goals)

SCHEDULE:



The mechanism(s) that regulate microfibril angle is the fiber property that we have decided to investigate more thoroughly in the F011 project. Microfibril angle is determined by the orientation and distribution of cellulose synthase complexes within the plasma membrane during microfibril formation. DFRC goal 3 is to isolate the cellulose synthase in order to develop reagents that should facilitate studies of the interaction of the cellulose synthase complex with microtubules and other cytoplasmic structures. DFRC goal 4 is to isolate and elucidate the role(s) that small GTP-binding protein Rac homologs play in cellular polarity and cytoskeletal organization are addressing the mechanisms that control cortical microtubule organization. To complement this goal, with student work and external funding we have developed a rapid method method for measuring microfibril angle in single fibers.

EXTERNALLY FUNDED RESEARCH COMPLEMENTING F011 GOALS

- *Environmental and Genetic Regulation of Microfibril Angle in Southern Pines*
  TIP^3 G. Peter & T. Faust (UGA) ($27,000 IPST & 13,000 UGA)

- *Trees Containing Built-in Pulping Catalysts*
  AF & PA/DOE Agenda 2020 G. Pullman, D. Dimmel, J. Cairney, G. Peter ($175,845)

- *Genetically Modified Lignin in Pine: Structure and Properties*
  USDA R. Sederoff (NCSU) & J. MacKay ($24,000 IPST & $31,000 NCSU)

- *Genetic Transformation Methods for Southern Pine*
  TIP^3 G. Pullman, G. Peter, J. Cairney & S. Merkle, J. Dean (UGA) ($55,000 IPST & $50,000 UGA)

DELIVERABLES:

Report describing results and research progress in the fall of 1998.
Annual report describing results and research progress in the spring 1998.

FUNDAMENTAL BIOLOGICAL MECHANISMS: IMPROVED FIBER PROPERTIES AND RATES OF WOOD CELL FORMATION

Cellulose Biosynthesis

Gary Peter
Chris Ricker

SUMMARY

The synthesis of β-1,4-glucan (cellulose) is critical for plant growth and development. The mechanical properties of cellulose together with its abundance in the primary and secondary cell walls make it the most important polymer in the plant cell wall. Many important questions remain about the biochemical mechanisms for cellulose synthesis and cellulose organization. The objective of this report is to summarize current information about cellulose synthesis in plant cells, and to update our progress toward answering some of the important questions related to cellulose synthesis in secondary cell walls of the secondary xylem of woody plant species.

INTRODUCTION

Overview

Cellulose chains are homopolymers of ~2000-25,000 β-1,4 linked glucose residues. Cellulose synthesis can be conceptually separated into three phases chain initiation, chain elongation or polymerization and chain crystallization to form microfibrils. Cellulose chains initiate with cellobiose and grow by the transfer of glucose from UDP-glucose to the reducing end. The elongating chains are probably pushed through the plasma membrane and 36 to 1200 individual chains crystallize during or shortly after synthesis into bundles termed microfibrils. These microfibrils are deposited on the cell surface in the cellulose I form. In cellulose I, the chains crystallize parallel, whereas in cellulose II the chains are antiparallel. Cellulose II is the thermodynamically more stable form of crystalline cellulose and is the form present in rayon; however, cellulose II is not present in nature. Note: amorphous cellulose or noncrystalline cellulose is not synthesized in the plant it is produced only by damage and hydrolytic enzyme activity. In plants, cellulose synthesis, initiation, elongation and crystallization are proposed to occur on the same multisubunit cellulose synthase complex located in the plasma membrane (Delmer, 1987; Brown, 1996).

The Cellulose Synthase Complex – Cellular Organization

Analysis of freeze fracture replicas of plant plasma membranes by transmission electron microscopy showed particles shaped into rosettes (Delmer, 1987; Brown, 1996). These rosettes are enriched in regions of the cell surface that are actively synthesizing cellulose and are located at the ends of the cellulose chains. This led to them being called terminal complexes and the hypothesis that these rosette particles represent the cellulose synthase complex (Delmer, 1987; Brown, 1996). These rosettes are hexagonal in most plants. A direct connection between these membrane bound and cortical microtubules has not been observed. This is paradoxical because there is general agreement that cortical microtubules orient cellulose microfibril synthesis. During secondary cell

wall formation in primary xylem the rosettes are found almost exclusively where the cortical microtubules are located under the developing secondary thickenings (Delmer, 1987; Brown, 1996). Some of the current models which try to explain this paradox suggest that the cortical microtubules are tightly bound to the plasma membrane and that they form channels through which the movements of the cellulose synthase rosettes are restricted. In these models the rosettes are proposed to move forward away from the existing chain by the energy of cellulose crystallization (Delmer, 1987; Brown, 1996). It should be noted that the lack of an observable linkage to cortical microtubules does not preclude a labile one that is difficult to detect by the microscopy methods employed. Further, it is possible that additional mechanisms, e.g., actin filaments and existing cellulose microfibrils, also play a role in orienting the newly forming cellulose microfibrils.

Biochemical Analysis of Cellulose Synthesis

The cellulose synthase complex from higher plants has not been purified to any significant extent in a biochemically active form. The proposed multisubunit nature of the membrane bound enzyme and the complexities of the synthetic reactions (initiation, elongation and crystallization) have limited the successes of this approach (Brown, 1996). The numerous published reports in this area of research reflect the effort given towards purifying the cellulose synthase complex; however, the only generally accepted results are that most of the radiolabeled UDP-glucose that is incorporated *in vitro* is recovered as β-1,3-glucose or callose and not cellulose (Delmer, 1987; Brown, 1996). Newer more specific and sensitive assays for cellulose based upon the specificity of cellulose binding domains from β-1,4-glucanases are just beginning to be employed (Brown, 1996).

Isolation of Plant Genes Involved in Cellulose Biosynthesis - celA

Sequence similarity was used to identify the first *celA* genes in plants. cDNAs from differentiating cotton fibers were randomly sequenced and two genes, *celA1* and *celA2*, with significant similarity to bacterial celA genes were found (Pear *et. al.*, 1996). The bacterial *celA* genes code for the catalytic subunit involved with β-1,4-glucan chain polymerization (Wong et al., 1990, Matthyse et al.,1995). This genomics style approach was based upon the belief that the amino acid sequence would be conserved to a recognizable degree with the bacterial *celA* genes identified from *Acetobacter xylinum* and *Agrobacterium tumefacians*. It was also shown for the cotton *celA1* and *celA2* that when the most highly conserved domain was expressed in *E. coli*, it bound radiolabeled UDP-glucose (Pear *et. al.*, 1996). This provided good evidence that celA codes for proteins that could be involved with cellulose synthesis. However, more direct functional data was not presented.

The strongest evidence that *celA* like genes in plants code for proteins involved in cellulose biosynthesis comes from the recent isolation of the RSW1 gene, a *celA* like gene (Arioli *et. al.*, 1998). RSW1 is a mutant of *Arabidopsis thaliana* that is specifically reduced in cellulose synthesis, accumulates noncrystalline β-1,4-glucan and has widespread morphological abnormalities. Interestingly, this point mutation does not inhibit β-1,4-glucan chain synthesis but rather crystallization. These results confirm that the *celA* genes in plants code for a subunit of the cellulose synthase complex. Other genes associated with the complex have yet to be identified. However, detailed electron microscopy work suggests that sucrose synthase may be part of the complex (Amor *et. al.*, 1995).

There is a Family of celA Genes in Plants

It is now clear that there is a family of *celA* genes in plants (Arioli *et. al.*, 1998). For cotton, the *celA1* and *celA2* genes are 65% identical and DNA gel blots indicate a small gene family with 3-5 members (Pear *et. al.*, 1996). In *Arabidopsis* there are at least 3 genes (RSW1, Ath-A and Ath-B)

(Arioli *et. al.*, 1998). In addition, there is a much larger set of genes that belong to the cellulose-synthase like class. The genes in this class are much more diverged from the celA genes (Cutter, S. & C. Sommerville, 1997). For rice, 2 genes have been identified with significant sequence similarity with *celA* genes (Arioli *et. al.*, 1998). The significance of multiple genes is uncertain. Aside from the importance of the cellulose synthase genes and the need for some redundancy in these essential genes, two general possibilities exist for a small family of *celA* genes: 1) the amino acid sequences have diverged enough to give specialized functions and 2) the need for differences in transcriptional regulation of cellulose synthesis during development or stress.

Cellulose Synthase Genes from Woody Species

Partial length cDNAs that show significant similarity to *celA* genes have been isolated from the secondary xylem of *Populus tremuloides* (GenBank AF072131) and *Pinus taeda*, (GenBank AA556640) (See Figure 1) (Allona, *et. al.*, 1998). Interestingly, the *Pinus taeda* nucleic acid sequence is more similar to *celA1* than to *celA2* from cotton and shares considerable similarity to three *Arabidopsis* and one rice *celA* gene. To date there is little information available about the expression of these genes in differentiating xylem or the rest of the tree.

BLAST Search Results with Pinus taeda cDNA:

```
Sequences producing significant alignments:                          (bits)  Value

gb|AF062485|AF062485  Arabidopsis thaliana cellulose synthase mR...    74  3e-11
gb|U58283|GHU58283  Gossypium hirsutum cellulose synthase (celA1...    72  1e-10
gb|AF027172|AF027172  Arabidopsis thaliana cellulose synthase ca...    70  4e-10
gb|AF030052|AF030052  Oryza sativa subsp. japonica RSW1-like cel...    60  4e-07
gb|U48693|TAU48693  Triticum aestivum calmodulin TaCaM3-1 mRNA, ...    56  6e-06
gb|AF081534|AF081534  Populus alba x Populus tremula cellulose s...    50  4e-04
dbj|AB016893|AB016893  Arabidopsis thaliana genomic DNA, chromos...    48  0.001
gb|AF027174|AF027174  Arabidopsis thaliana cellulose synthase ca...    44  0.022
gb|AF027173|AF027173  Arabidopsis thaliana cellulose synthase ca...    42  0.089
```

Alignment of partial cDNA sequences of *Pinus taeda* and *Populus alba* x *Populus tremula* cellulose synthases (cel1) isolated from secondary xylem.

Score = 50.1 bits (25), Expect = 4e-04
Identities = 124/158 (78%), Positives = 124/158 (78%)

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Query: 195  tttgccagaaaatgggtaccattctgcaagaaattcgacattgagcctcgcgctcccgaa 254
|||||  |||||  |||||  |||||  ||||||||      ||||||||||   |  |||||  ||
tttgctagaaagtgggttccattttgcaagaagcataacattgagccaagggctcctgag 1157
Sbjct: 1098

Query: 255  atctatttctctcagaaaattgactatctgaaggacaaatttcaacccacctttgtcaaa 314
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ttctacttcactcagaagattgactacttgaaagacaaagtgcatcccaactttgtgaag 1217
Sbjct: 1158

Query: 315  gancgccgggccatgaaganaaatatgaagaattcaa 352
             ||  |||  |  ||  |||||  |  |  ||||||||||||||||
Sbjct: 1218 gagcgcagagctatgaaaagagaatatgaagaattcaa 1255
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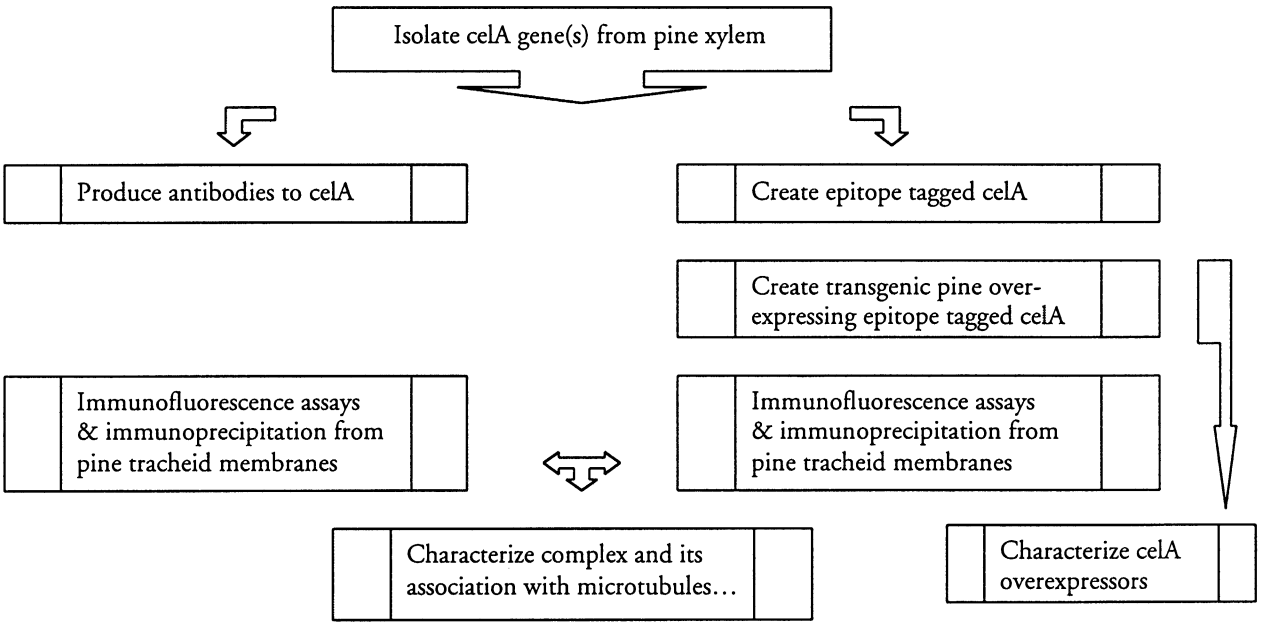
Fundamental Unanswered Questions for Cellulose Synthesis in Plants

There are many important unanswered questions about cellulose synthesis. Questions related to the biochemical mechanisms of chain elongation, chain termination, and crystallization; biochemical composition of the complex -what additional subunits are in the functional complex, how are they organized. Cellular questions include the mechanisms that orient cellulose synthase during microfibril formation, what limits the rate of cellulose synthesis. How is cellulose synthase gene expression regulated during xylem tracheary element differentiation?

Focus of Cellulose Synthesis Work for F011

We proposed to focus our research of cellulose synthesis on the cellular questions: 1) the mechanisms that regulate the orientation of the cellulose synthase complex and its subunit composition and 2) what limits cellulose synthesis.

EXPERIMENTAL STRATEGY



PROGRESS/RESULTS

Secondary xylem in the early stages of differentiation and cambial meristem/phloem was previously collected from loblolly pine. The RNA from this material has been isolated and reverse transcribed to make cDNA. cDNA library construction is in progress. Primers have been designed and obtained for PCR synthesis of a full-length *celA* cDNA from *Pinus taeda*. (GenBank #3365655).

Alignments of the protein coding regions identified the most highly conserved region of the three *Arabidopsis* and two cotton genes. This region was synthesized for the Ath-A from *Arabidopsis* genomic DNA to use this as a probe if necessary.

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EXTERNALLY FUNDED RESEARCH
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Elizabeth Althan
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Karen Crews
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October 14-15, 1998

TOWARDS GENETIC ENGINEERING OF FOREST TREES WITH ANTHRAQUINONES AS BUILT-IN PULPING CATALYST

Endogenous anthraquinones in pulpwood trees and the cloning of a key gene for anthraquinone biosynthesis

Huabin Meng
Karen Crews
Don Dimmel
Gerald Pullman
Gary Peter

SUMMARY

A survey has revealed that anthraquinones (AQs) naturally exist in many commercially important angiosperm trees. Kraft pulping studies showed that when present, endogenous AQs enhance delignification. These studies show that it should be possible to increase the endogenous AQ levels by genetically manipulating the key genes for AQ biosynthesis. As the first step toward this objective, we isolated an essential gene(s) involved with AQ biosynthesis, isochorismate synthase from Arabidopsis thaliana. *This is the first AQ related gene cloned from plants. A binary expression vector with this isochorismate synthase coding region regulated by the CaMV 35S promoter has been constructed and is being used to transform model plants as well as* Populus deltoides.

INTRODUCTION

Kraft pulping uses NaSH and NaOH to degrade lignin and is the dominant chemical pulping process in the world. While the process obviously has advantages, several shortcomings are also associated with this process: low yield, malodorous emissions and the dark color of the resulting pulp. Catalysts based on anthraquinone (AQ) chemistry offer a way to overcome many of these problems. Anthraquinone can be used at <0.1% levels to improve pulping efficiency by increasing delignification rates and increasing yields (1). In addition, AQ offers a route to eliminate sulfur from the pulping process, thus solving the perennial odor problem that haunts kraft pulp mills.

Anthraquinones are natural products that are present in many plants including some tree species (2). Previously, we found AQs in the wood of teak and showed that teak extracts containing AQs catalyze delignification reactions better than chemically synthesized AQ giving pulp with lower kappa numbers (3). We proposed that if we can increase AQ level in pulpwood trees through genetic engineering, we will provide a cost-effective alternative to the use of chemically synthesized AQ and these trees will be a significant benefit to the pulp & paper industry.

A key step leading to the success of this research is to isolate the gene(s) that are rate limiting for anthraquinone biosynthesis. We are focusing on isochorismate synthase, which

converts chorismic acid to isochorismic acid and catalyzes the first committed step in the biosynthesis of anthraquinones (4-6). In higher plants, chorismic acid plays a central role for the synthesis of a number of aromatic compounds including lignin precursors (Figure 1) (7). We propose to isolate the gene for isochorismate synthase and increase its expression in trees to enhance anthraquinone production. The higher level of isochorismate synthase will increase its competition for chorismic acid and therefore increase the flux of chorismic acid into anthraquinone biosynthesis while at the same time decreasing the flux of chorismic acid into lignin precursor biosynthesis.

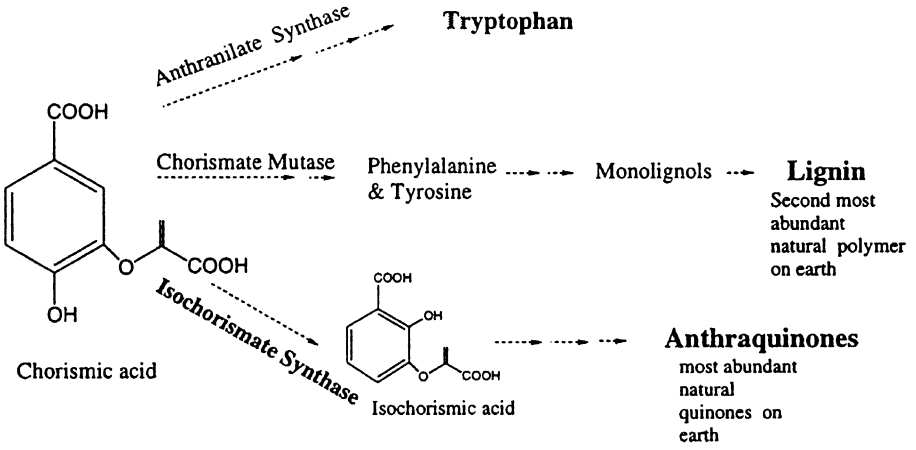


Fig. 1. The competition of anthranilate synthase, chorismate mutase and isochorismate synthase on chorismic acid for the synthesis of aromatic acids and a number of secondary metabolites.

In this report, we first summarize our survey of AQs in commercial trees and their potential release from chips during pulping, we then present our progress towards the isolation of the gene for isochorismate synthase and the transferring of the gene into model plants.

RESULTS

I. Survey of AQs in ten commercial hardwoods and one softwood

Wood samples from teak, red oak (2 sources), red maple, elm, chestnut, walnut, hickory, blackjack oak, eastern cottonwood, and aspen were extracted and analyzed by GC/MS. Six of the ten commercial hardwood trees were shown to contain significant levels of anthraquinone or anthraquinone derivatives (Table 1) (8). This finding, that AQs naturally exist in these trees, indicates that trees have the biosynthetic enzymes for AQ production. Thus, as long as we find the key genes for AQ biosynthesis, we might be able to manipulate its production. The survey also showed that levels of naturally occurring AQs varied in trees from 0.007 to 0.33 percentage on wood weight basis (Table 1). This indicates that we can at least increase AQ levels to ~0.3% of the weight of wood without harming normal growth patterns. Eastern cottonwood is especially valuable and contained three

forms of AQs (AQ, MMAQ, DMAQ) useful for wood pulping. This might explain why eastern cottonwood is such an easy wood to pulp. In addition, cottonwood has the distinct advantage that it is easily manipulated in tissue culture for gene transfer experiments and it is of commercial interest due to its rapid growth rates in plantation settings. Cottonwood will therefore serve as a good model system for transgenic studies to increase anthraquinone levels.

Table 1. Percentage of Anthraquinones present in wood.

Tree	AQ types present	Percentage of AQ Components in Wood
Eastern Cottonwood	AQ	0.016
	DMAQ	0.003
	Mono-MAQ	0.035
Red Maple	AQ	0.011
Red Oak (wet)	AQ	0.020
Red Oak (Dry)	AQ	0.005
Walnut	AQ	0.010
Elm	AQ	0.007
Teak (prior results)	Mono-MAQ	0.33
	DMAQ	Present, not quantified
Chestnut	AQs	None detected
Hickory	AQs	None detected
Blackjack Oak	AQs	None detected
Aspen	AQs	None detected
White Spruce	AQs	None detected

II. Anthraquinones are released from wood chips during Kraft pulping

To demonstrate that our approach of elevating endogenous AQ levels in trees will be of value to the industry, we designed two experiments to show that the endogenous AQs in wood can be released from wood chips during pulping and can catalyze delignification reactions. In one experiment pine and teak chips were pulped simultaneously. Figure 2 shows that when a relatively small amount of teak chips were added to the pine chips, a greater amount of delignification of pine was achieved. This suggests that the AQ was released from the teak chips during normal pulping conditions.

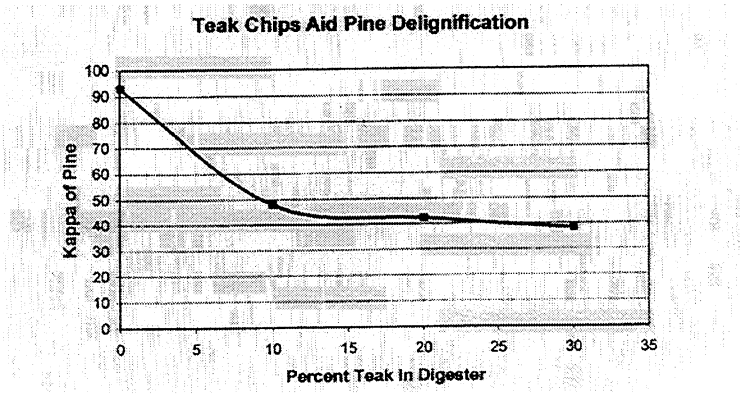
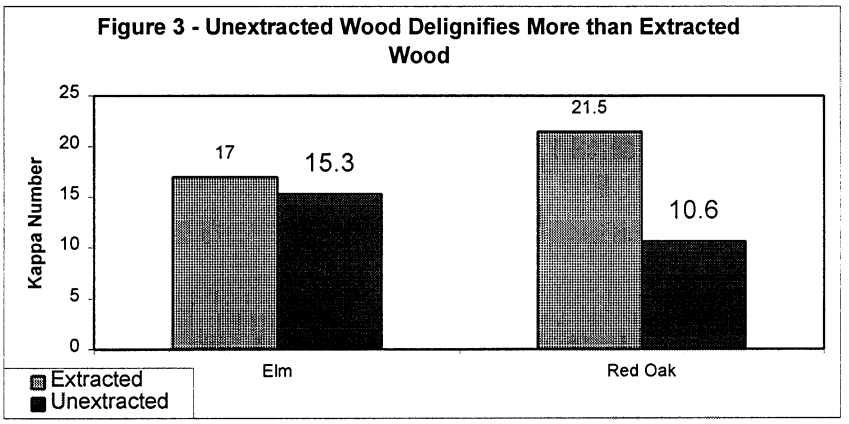


Figure 2. The addition of teak chips to a pine cook can aid the delignification of pine

In another experiment, we compared the kraft pulping of extracted vs unextracted wood (Figure 3). When AQs are removed via extraction, both Elm and Red Oak woods do not delignify as much under identical pulping conditions as unextracted wood.



III. Molecular Cloning of Isochorismate Synthase

To achieve the goal of increasing AQ levels in trees by genetic engineering, an essential step is to isolate the genes that encode enzymes that catalyze AQ synthesis. We focused on isochorismate synthase because it catalyzes the first committed step in the biosynthesis of anthraquinones in plants, and it competes for its substrate chorismic acid with chorismate mutase that leads to the synthesis lignin precursors.

Isochorismate synthase has been well studied and cloned from many prokaryotic organisms. The study of this enzyme in plants lags behind prokaryotic organisms. A partial sequence of about 400 base pairs from an *Arabidopsis thaliana* EST (GenBank T42189) was found to be similar to the C-terminal region of bacterial isochorismate synthase. From this sequence information, we designed nested PCR primers and successfully isolated a full-length isochorismate synthase cDNA from an *Arabidopsis* cDNA library.

This cDNA is 1973 base pairs long and encodes a protein of 503 amino acids with a predicted molecular weight of 55,368 Daltons. It represents the first full-length isochorismate synthase cDNA isolated from an eukaryotic system. This eukaryotic isochorismate synthase was aligned with several prokaryotic isochorismate synthases at the amino acid level (Figure 4). These isochorismate synthases vary in size ranging from 372 to 503 amino acids with the *Arabidopsis* isochorismate synthase being the largest. They all share considerable similarity in the C-terminal regions. A gel blot with *Arabidopsis* genomic DNA (Figure 5) showed a single or at most two isochorismate synthase genes in *Arabidopsis*.

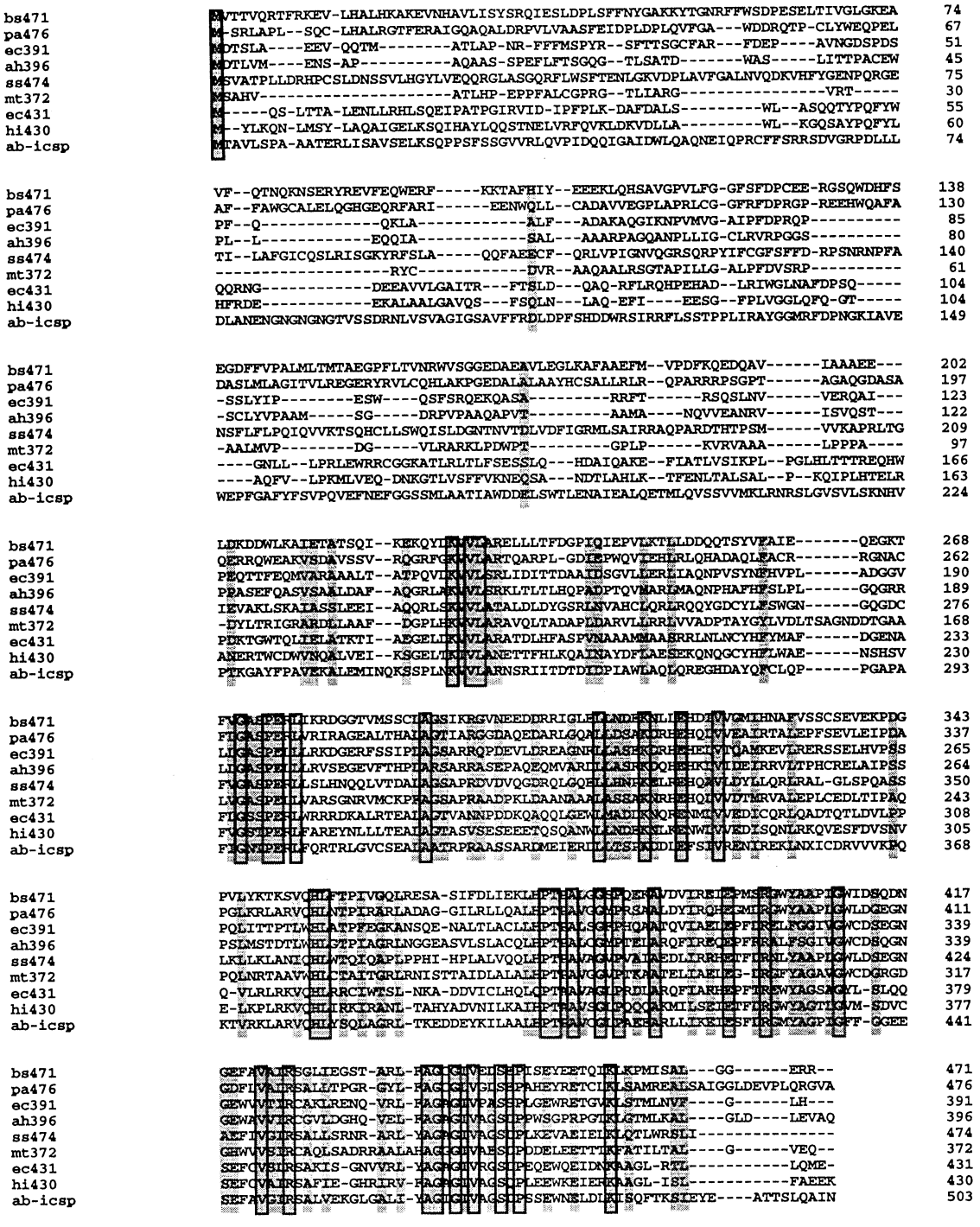


Fig 4. Arabidopsis isochorismate synthase (ab-icsp) was aligned at amino acid level with bacterial isochorismate synthases: Bacillus subtilis (bs471), Pseudomonas aeruginosa (pa476), Synechocystis sp (ss474), E.coli (ec391, and ec431), Mycobacterium tuberculosis (mt372), Haemophilus influenzae Rd (hi430). Despite the variation of the size of these enzymes, they all share high similarity at the C-end.

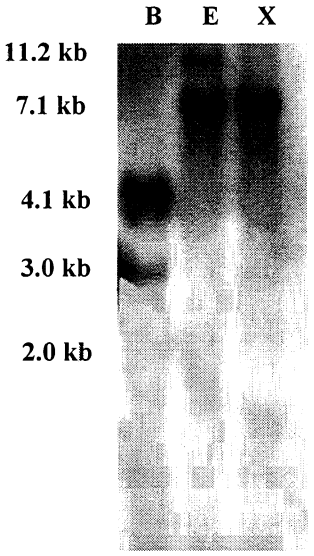


Figure 5. DNA gel blot of *Arabidopsis* genomic DNA with full-length ^{32}P labeled isochorismate synthase cDNA probe. Each lane contains 10 ug of *Arabidopsis* DNA digested with BamHI (B), EcoRI (E) and XbaI (X).

IV. Towards Overexpression of Isochorismate Synthase in Plants

Our successful isolation of the full length isochorismate synthase cDNA makes it possible for transfer of this gene into model plants to test the hypothesis that it is the rate limiting enzyme in anthraquinone biosynthesis. A binary expression vector with isochorismate synthase gene (pBIN-ICS) driven by Cauliflower Mosaic Virus 35S promotor has been constructed (Figure 6). Transformation of this expression construct into *Agrobacteria tumefacians* was achieved by electroporation and confirmed by colony PCR. We are currently progressing towards delivering isochorismate synthase gene into our model plants (*Arabidopsis* and cottonwood) via *Agrobacterium* infection.

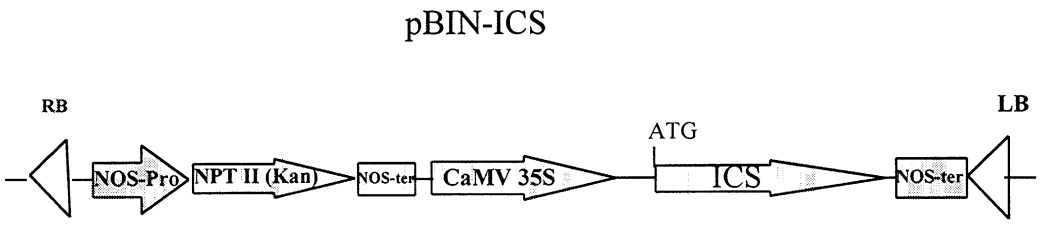


Figure. 6. Construct of binary expression vector with isochorismate synthase driven by the CaMV 35S promoter.

CONCLUSIONS

1. A survey of many commercially important pulp trees shows that AQs naturally exist in many of them. This indicates that a biosynthetic pathway for AQ exists in these trees and that overexpressing the rate-limiting enzyme in the pathway should lead to elevated levels of AQs in the wood.

2. Pulping studies show that endogenous AQ is released from the wood chips during pulping and catalyzes delignification reactions leading to pulp with decreased lignin levels. This means that genetically manipulating AQ levels in wood should be valuable to the pulp and paper industry.

3. An essential gene for AQ biosynthesis has been isolated. It represents the first AQ gene to be isolated from plants. Enhancing the expression of this gene in plants will potentially increase AQ levels while at the same time reduce the level of lignin precursors. To test the hypothesis delivery of the gene into cottonwood via *Agrobacterium* infection is ongoing.

ACKNOWLEDGMENTS

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ENVIRONMENTAL AND GENETIC REGULATION OF MICROFIBRIL ANGLE IN SOUTHERN PINE

Determination of the Microfibril Angle in the S2 Layer of *Pinus taeda* Using Differential Interference Contrast Microscopy: Validation of the Method

Douglas Benton
Keith Bennett (Weyerhaeuser Company)
Gary Peter

SUMMARY

Previously it was discovered that the microfibril angle (MFA) in the S2 layer could be observed by differential interference contrast microscopy (DIC) in pulped fibers. To use this rapid and simple method to measure MFA required validating this method relative to other accepted methods. Comparisons of DIC with X-ray analyses with earlywood and latewood samples with a range of MFAs shows an excellent correspondence between the two methods, R^2 0.9-1.0 The results validate the DIC method on a population basis but not at a single fiber level, since X-ray measurements were done on wood samples.

INTRODUCTION

The microfibril angle (MFA) of the S2 layer in plant cell walls has been shown in many studies to be an important parameter in determining fiber and paper's strength, stiffness, and shrinkage characteristics. Unfortunately, wood samples are not always available for study, leaving X-Ray analysis out of the question. In addition, it is sometimes desirable to know the MFA distribution of a particular sample in order to more completely characterize a given population. X-Ray analysis cannot always distinguish between two samples varying only in the broadness of their normal curve. And current methods used for measuring the MFAs from fibers are often time-consuming, arduous, and inaccurate.

Recently published work in the Journal of Pulp and Paper Science (1), and Appita (2) has shown that Polarized Confocal Scanning Laser Microscopy is one viable technique for accurately measuring MFAs in pulped samples. However, this instrument is capital intensive, costing hundreds of thousands of dollars to install for fibril angle measurement studies.

A more cost-effective method is the soft rot technique. This method developed by ESPRI (3), inoculates fibers with a soft rot fungus which selectively degrades fibril bundles along their longitudinal axis allowing the MFA to become visible under microscopic examination. The downside to this technique, however, is that sample preparation requires a sterile environment, biohazard hoods, and 6-14 weeks for incubation.

Due to the short-comings of the two aforementioned techniques, the purpose of this project is to develop Differential Interference Contrast Microscopy (DIC) as a relatively inexpensive, reliable and less time consuming method which can accurately determine the MFA in pulped fiber samples.

METHODS

Sample Preparation

Loblolly pine wood samples were visually screened for compression wood and uniform MFA using X-Ray analysis. From these samples, three earlywood samples (E3, E7, & 7E3) and three latewood samples (L4, 20L2, & L5) of low (0-10°), medium (15-30°), and high (31-50°) MFA where selected and again measured in multiple positions using x-ray analysis. The samples were then gently pulped at 70° C overnight, using peracetic acid made up of 50% hydrogen peroxide (30%) and 50% glacial acetic acid. The samples were then carefully rinsed (H_2O) and neutralized (1M NaOH). The samples were then allowed to air dry overnight. Due to a communication mix-up, only the pulped earlywood samples were re-examined using x-ray analysis to determine their final MFA. Differences in MFA from pulped and unpulped samples appear to be negligible (Table 1). However, some differences may exist. One would expect that pulping would have a tendency to increase the MFA of a wood by releasing the strain of from the composite structure (lignin and fibers). In order to verify this, however, a more in depth study would be required.

Table 1: X-Ray analysis of pulped and unpulped chips MFA

	Unpulped							Pulped	
sample	Tangential	Tangential	tangential	tangential	radial	radial	radial	tangential	radial
Late1	6	7	6	6	5				
Late2	51				50	50			
Late3	33				32	32			
Early1	6	6	4		3			4	3
Early2	29	29	31		37			30	37
Early3	42				45	46	46	44	47

After the samples were re-examined, the pulped chips were then re-hydrated in distilled H_2O and left over night. The hydrated samples were then dispersed by vigorously manually shaking the samples in their containers.

Imaging

Temporary slides were made from the dispersed fibers using a pipette to collect fibers and move them onto a glass slide. A needle was used to further spread the fibers so overlapping was minimized. Approximately 40 different fiber images of microfibril angles were captured using a digital camera, image analysis program and saved onto a hard drive using either JPEG, BMP, or TIF formats. In early DIC work, the measurement of 40 fibers appeared to give similar standard deviations compared to 80 measurements. Thus, time could be saved by reducing the amount of fibers collected. Optimization of sample size would require more study.

If visualization of the microfibril angle was difficult or infrequent, fiber treatment with $CoCl_2$ and ultrasonic energy was used. To prepare both earlywood and latewood samples, about 200-300 dispersed fibers were put into a plastic weighing boat. Any excess water was removed simply by decanting. Next, a 5-10% solution of $CoCl_2$ (in water) was poured into the weighing boat with the sample. Enough solution was used so that all the fibers were either floating or immersed in the solution. After applying the $CoCl_2$ solution, the samples were either left out overnight so that the $CoCl_2$ could diffuse into the fiber walls. Another option is to place the boat and its contents into a

$CoCl_2$ could diffuse into the fiber walls. Another option is to place the boat and its contents into a oven at about 50-60° C for 3-4 hours. Caution must be used not to evaporate all the solution and dry out the fibers. Fibers must be hydrated for DIC to work.

After the fibers have had time to absorb the $CoCl_2$, the boats were placed in an ultrasonic bath. Earlywood fibers were exposed to the ultrasound for 15-20 minutes, while latewood were exposed for 10-12 minutes. Time may vary between different samples. Excessive fibrillation is possible using this treatment, and it is better to under fibrillate than over fibrillate. Earlywood fibers were more difficult to fibrillate than latewood, thus more time was required for earlywood. During the summer two other treatments showed potential for improved fibril visualization. The treatments are: slow freezing and thawing (in freezer); and refining in a PFI mill for 2000-3000 revolutions.

Familiarity with the DIC microscope is important for rapid MFA visualization. Filter use, axial position of the fiber on the rotating stage, polarization orientation, amount of filtering from the DIC condenser, condenser position (I for 16x lens, II for 40x lens), and magnification all significantly affect resolution and MFA visualization. If one is not familiar with polarizing microscopes, set aside time for practice in order to become familiar with subtleties of the DIC microscope. See Zeiss operating manuals for a brief discussion of DIC and its operation.

In addition, familiarity with fiber morphology will aid in DIC MFA visualization. Often, both the S1 and S2 layers are visible. Although it is usually obvious which layer is S2 (lower MFA), in mixed samples of wide ranging MFAs it becomes important to verify that one is indeed looking at the S2 layer.

Indications of S2 MFA can be obtained from tears, rips or cut ends in fibers of earlywood and latewood. These can apparently be induced by vigorously shaking pulped chips. Latewood cross-field pits also aided in MFA determination in these fibers. Earlywood crossfield pits, however, were too sporadic and variable to be used as a reference in MFA determination.

In this study, oil immersed lenses were not used. Although it was found that these lenses perform better than non-oil immersion lenses for MFA visualization, oil is difficult to clean from the slides and also tends to slow the over all image sampling procedure when many samples of different types are used.

MFA Determination

Once the images have been captured onto a hard-drive, NIH's Scion Image analysis program was used to determine the average MFA of each fiber. Scion Image works on both NT and Window's 95 systems and the beta version can be downloaded from the Internet at no cost. Scion Image has a protractor tool that can measure to the nearest fraction of a degree (two decimal places). Data from Scion Image was then manually recorded in an Excel spreadsheet and analyzed. Approximately four angle measurements were used per fiber to obtain the average MFA for individual fibers.

RESULTS

Tabulated data sets of all six samples are included in theappendix. This section provides a summary of the results.

Regression Analysis

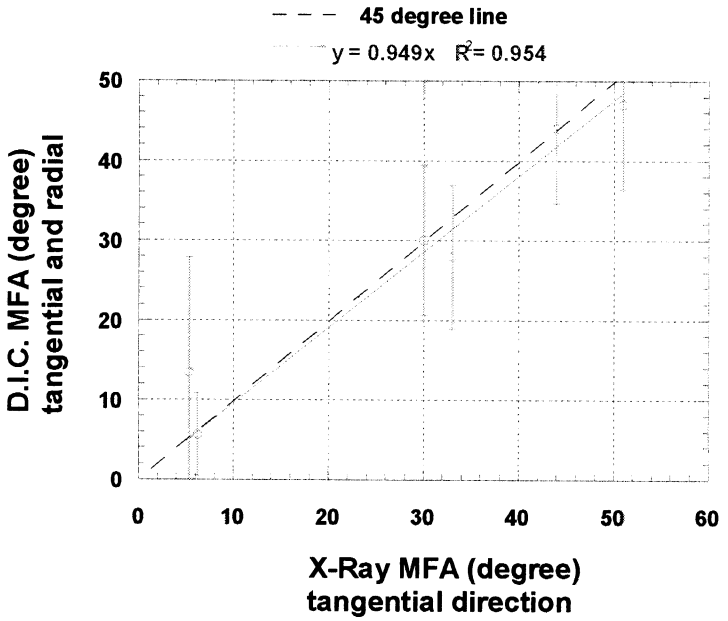
As can be seen from Table 2, nearly all sets of data have a high degree of correlation. X-Ray tangential and D.I.C. combined, however, show the highest degree of correlation between the two methods. This result should not be surprising because the technique of X-Ray analysis was developed and calibrated only in the tangential direction at Weyerhaeuser. The fact that the best correlation between X-Ray analysis and D.I.C. results when the X-Ray was used on its calibrated face is a strong indication that D.I.C. strongly mimics the hand sectioning technique used to calibrate the X-Ray machine.

Table 2: Regression Analysis

Regression	X-Ray tan	X-Ray rad	X-ray comb	D.I.C. Comb	D.I.C. radial	D.I.C. tan
X-Ray tan		1.04, 0.973	1.03, 0.977	0.949, 0.954	0.95, 0.953	0.946, 0.975
X-Ray rad			0.99, 1.0	0.909, 0.94	0.911, 0.943	0.906, 0.951
X-ray comb				0.911, 0.939	0.913, 0.942	0.908, 0.955
D.I.C. Comb					1.0, 0.999	1.0, 0.992
D.I.C. radial						1.0, 0.987
D.I.C. tan						

It is important to note that in looking at tangential DIC and tangential X-Ray, the data show nearly a unity slope and an excellent fit. The data is misleading, however. No tangential measurements were obtained from the E3 sample. The E3 sample gave the most deviant MFA results in this study, and by eliminating this sample set, the data appears to fit much better. In addition, tangential measurements may not be obtainable in some earlywood samples, like E3 for instance.

Figure 2: Regression Analysis X-Ray (tangential) vs. DIC (combined)



From the regression line in **Figure** 2, it appears DIC has a tendency to underestimate the MFA. A likely cause of this reduced angle estimation is in measuring fibrils too close to the sides of the fiber. This problem is likely to be less frequently found in earlywood measurements because thinner walled fibers tend to lay flatter on a slide (see **Figures** 3 and 4). However, thick-walled latewood samples have a greater ability to resist collapsing, making DIC fibril measurements near the sides more likely to be biased towards a lower MFA average. The slope of the regression line derived from only the latewood regression analysis is in agreement with this rationale. In addition, looking at the data points in **Figure** 2, latewood is more frequently below the regression line and the 45° line in two of the three samples. Therefore, greater care seems to be necessary when deciding where to take fibril measurements in latewood.

Figure 3: Earlywood MFA, DIC vs. X-Ray

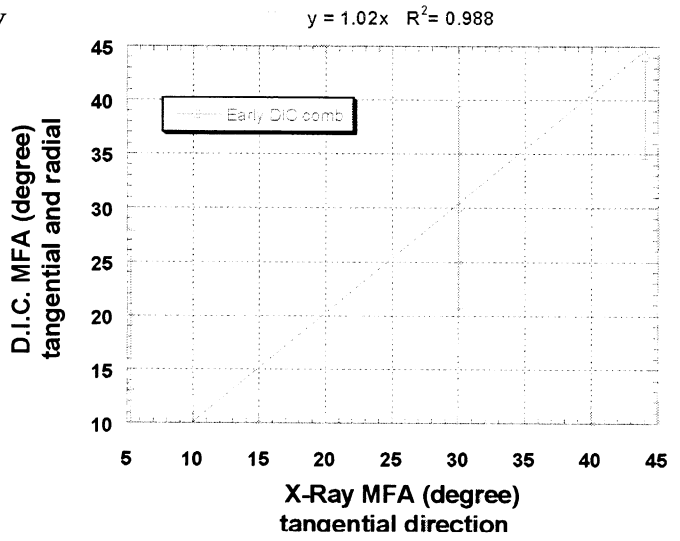
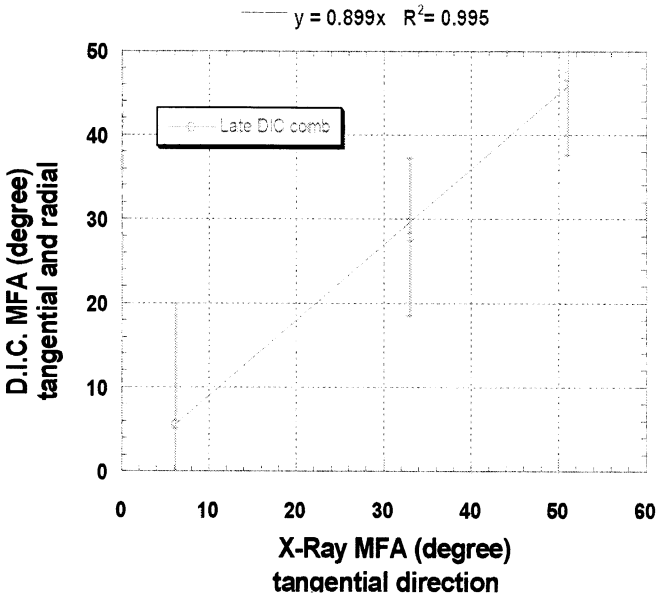


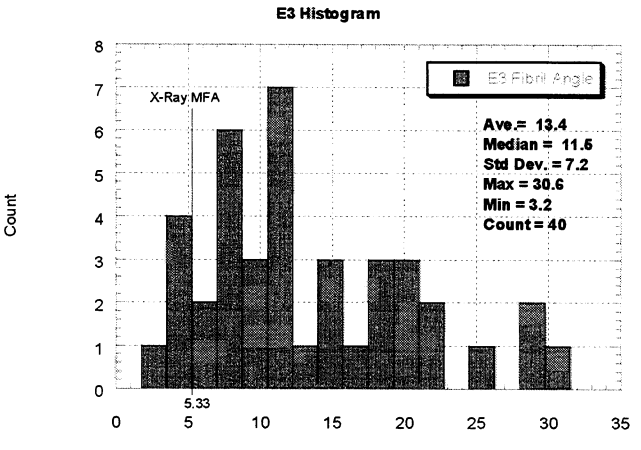
Figure 4: Latewood MFA, DIC vs. X-Ray

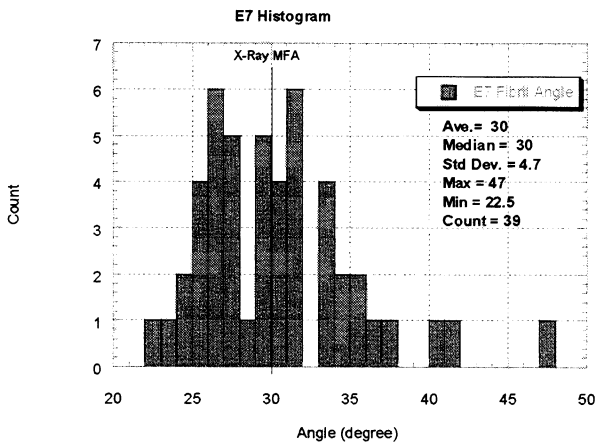


Another possible explanation for the tendency of latewood to underestimate the MFA of a sample may be the fact that the MFA initially taken by X-Ray may have decreased due to pulping treatment. As mentioned earlier, latewood samples were not re-measured for their MFAs after being pulped. However, this explanation does not fit well with what would be expected from pulping treatment: strain release should lead to higher MFAs not lower MFAs. One cannot rule out the possibility that the MFA may have changed upon pulping.

Distribution Analysis

Figure 5: Earlywood DIC MFA distributions





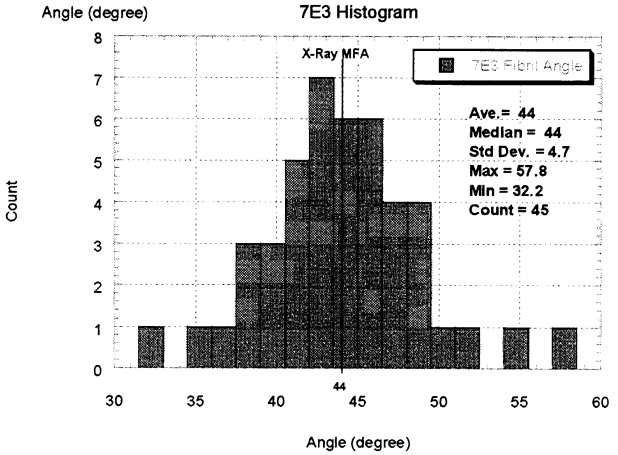
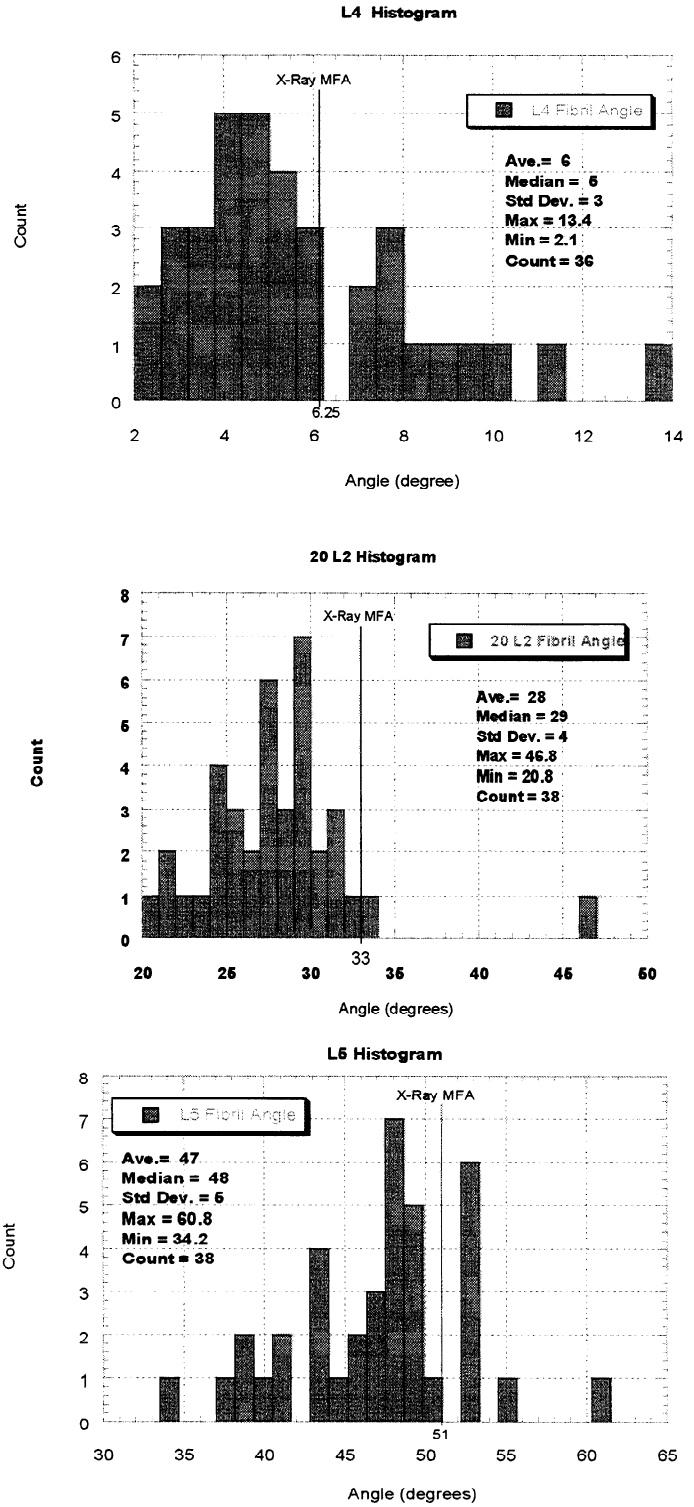


Figure 6: Latewood DIC MFA distributions



From Figures 5 and 6, it appears normalized distributions where obtained from all the samples. The most deviant distribution appears to be the E3 which was the earlywood low fibril angle sample. Perhaps the S2 and S1 layers were confused in a few measurements of this sample. This theory could explain why the MFA was overestimated in comparison to X-Ray analysis. Furthermore, the E3 sample had, by far, the greatest range of MFAs of any of the other five samples.

Besides the fact that the MFA averages taken by DIC compared well with the averages obtained by X-Ray analysis, there is no way to prove that the MFA distributions are really found within the sample population. Some other accepted MFA technique that can measure individual fibers would have to be performed on the same samples in order to verify the distributions obtained by DIC. Until then, little can be concluded about the DIC distributions except that they appear to be normal gaussian distribution.

Acknowledgements

I would like to thank all those who made this work possible by providing assistance, guidance, and advice throughout the summer. I would like to especially thank: Ron Zarges, Mark Young, Joanne Doolittle, and Pedro Armenta for their technical support, resources, and expertise in microscopy. David Bremer, Kari Frostad, Jonas Winbolt, Jennifer Roers, and Betsy (Elizabeth) Erhardt for their help in obtaining samples and performing the X-Ray analysis portion of the study. Greg Leaf, Chih-Lin Huang, Jeff Mathews, David Bremer, Keith Bennett and Bob Megraw for their helpful discussions, trouble shooting, and expertise in microfibril angle techniques. Heide Nutwell, Shawna Brown, and Nancy Draper for general assistance and expertise with software, documentation, and presentation. Finally, I would like to thank the entire Paper Physics and Mechanics team for providing the support and encouragement throughout the summer.

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Appendix DIC MFA DATA

E1	MFA Measurements	
Earlywood	Average	13.4
Low MFA	Median	11.5
(E3)	Std Dev	7.2
	Count	40
	max	30.6
	min	3.2

	MFA Measurements	
E2	Average	30.0
Earlywood	Median	30.0
Medium	Std Dev	4.7
MFA		
(E7)	Count	39
	max	47.0
	min	22.5

	MFA Measurements	
E3	Average	44.0
Earlywood	Median	44.0
High MFA	Std Dev	4.7
(7E3)	Count	45.0
	max	57.8
	min	32.2

	MFA MEASUREMENTS	
L1	Average	5.6
Latewood	Median	5.0
Low MFA	Std Dev	2.6
(L4)	Count	36.0
	max	13.4
	min	2.1

	MFA MEASUREMENTS	
	Average	27.9
L2	Median	27.5
Latewood	Std Dev	4.5
Medium MFA	Count	38.0
(20L2)	max	46.8
	min	20.8

	MFA MEASUREMENTS	
	Average	47.0
L3	Median	47.5
Latewood	Stnd Dev	5.3
High MFA	Count	38.0
(L5)	max	60.8
	min	34.2

Fiber I.D. Key (ie. E31*r16x)

E3 = Earlywood low MFA
E7 = Earlywood medium MFA
7E3 = Earlywood high MFA
L4 = Latewood low MFA
L5 = Latewood high MFA
20L2 = Latewood medium MFA
*sample number

r = Radial direction
t = Tangential direction
s = Sonicated fiber
p = Pit measurement
16x = 16x lens
40x = 40x lens

GENETICALLY MODIFIED LIGNIN IN PINE: STRUCTURE AND PROPERTIES

Lignin Reactivity in CAD-deficient Trees During Pulping and Bleaching.

Donald Dimmel **John MacKay**
Elizabeth Althen
Christy Parks

PROJECT OBJECTIVE

The aim of this project is to understand the structure and reactivity of the lignin in a loblolly pine tree that is deficient in cinnamyl alcohol dehydrogenase (CAD), an enzyme involved in lignin precursor biosynthesis. The general objective is to evaluate whether such trees or similar trees that could be produced with genetic engineering will be superior to natural trees as the raw material for producing paper products. Environmental impacts of chemical pulping and bleaching of wood will be minimized by developing forests of healthy trees that contain modified lignins that are easily degraded under mild conditions, consuming less chemicals and/or less energy. A similar report is being presented to the Chemical Pulping and Bleaching PAC.

BACKGROUND

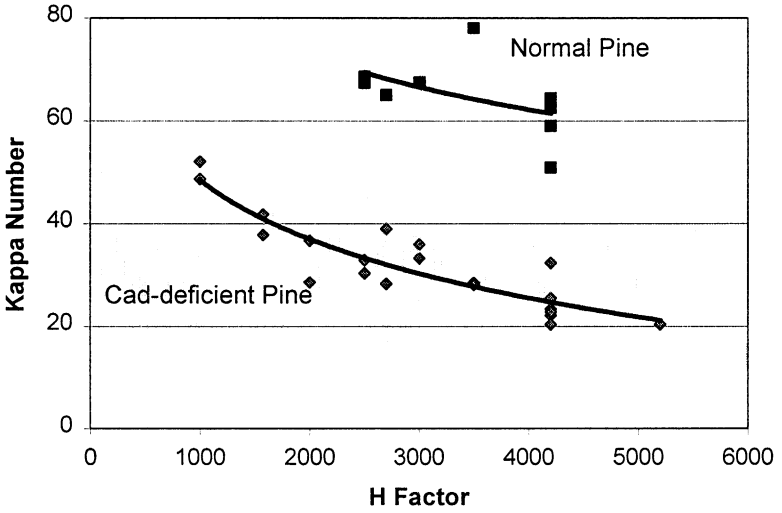
A joint research effort between IPST and North Carolina State University is being conducted on ways to improve delignification rates by altering the nature of the lignin in the tree. Funds come from a USDA grant to NC State the primary contractor; IPST is a subcontractor. Controlled crosses using a genotype that contains one copy of the mutant allele of the *cad* gene has lead to the production of totally CAD-deficient (CAD^-) loblolly pine trees with substantially altered lignins and to partially CAD-deficient trees (MacKay et al., 1997; Ralph et al., 1997). Recenlty it was shown that partially CAD-deficient trees had higher juvenile volume growth than completely normal trees, suggesting that these trees may be interesting not only for their altered lignin but also because of potential growth gains. For our studies, 400 grams of wood chips from a 12-year old totally CAD^- tree has been made available. We have a similar quantity of wood from a 12-year old normal loblolly pine tree grown on the same site.

We are studying the pulping efficiency, yield, and bleachability of these woods using kraft, soda and other pulping systems. To better understand the chemical basis for the delignification of CAD^- pines, we will examine the rate of lignin removal with different pulping additives and the molecular weight of dissolved and residual lignins. Hopefully, such information will help determine (a) the commercial viability of CAD-deficient pines and (b) the best strategies toward genetic modification of lignin.

SUMMARY OF RESULTS

The initial months of our study demonstrated that (a) small scale (0.5 g) cooks and mini-kappa number determinations gave results similar to 1 kg cooks and regular kappas and (b) reliable brightness values can be obtained from mini-handsheets. Five cook sets have been performed using 4-mL pressure vessels in a fluidized sand bath with automated temperature control. Our research has shown that CAD^- pines are easily pulped under soda conditions and that (in limited tests) the addition of NaSH or AQ to the cooking liquor has little impact on the delignification rate. We have examined different H-factors (cook severity) for both CAD^- and control pines (see figure). We observe that CAD^- wood is much easier to delignify. There is a lower pulp yield in the CAD^- case,

Relationship between H Factor and Kappa number in soda cooks of Cad-deficient and normal pine



which we attribute at least partly to juvenile state of the specific CAD$^-$ tree in these experiments.

Lignins isolated from the CAD$^-$ and control pine cooking liquors have nearly the same molecular weights as normal lignin, regardless of the length of the cooks. At the same H-factor, there was ~50% more dissolved lignin in the CAD$^-$ case. The molecular weight of an isolated milled wood CAD$^-$ lignin was ~ 20% lower than that of a normal lignin.

FUTURE STUDIES

Our immediate activities will concern:

1. Determining if kappa number is a good measure of lignin to compare CAD$^-$ and control pulps.
2. Performing a more quantitative analysis of the affects of NaOH, NaSH, and AQ.
3. Producing a CAD$^-$ and control pulp of similar kappa number in order to compare effluent lignin molecular weights.
4. Determining the bleachability of CAD$^-$ and control pulps.
5. Conducting sodium sulfite pulping of CAD$^-$ and control wood at different pH values to help define the reactivity of the CAD$^-$ wood under different conditions.
6. Determining molecular weights and structural differences for residual lignins from CAD$^-$ and control pulps for select pulping systems. This will be the last area of study since we will consume large amounts of our precious wood supply.

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Ralph, J., **MacKay, J. J.,** Hatfield, R. D., O'Malley, D. M., Whetten, R. W. and Sederoff, R. R. (1997) Abnormal Lignin in a Loblolly Pine Mutant. *Science*, 277: 235-239

IPST FOREST BIOLOGY
RESEARCH PROPOSALS
(Awarded, Submitted and in Review or Rejected)

Following is a list of 1997-1998 research proposals which have been awarded funds, were submitted and are currently under review, or were rejected.

Awarded to IPST **1998 - Approximately $ 406,222**
1997 - Approximately $ 504,037
1996 - Approximately $ 312,279
1995 - Approximately $ 142,329
1994 - Approximately $ 78,789

Awarded 1997-1998

Title:	Environmental Influences and Genetic Regulation of Microfibril Angle in Southern Pine
Authors (Affiliation):	Gary Peter (IPST) and Timothy Faust (UGA)
Awarding Agency:	Traditional Industry Program in Pulp and Paper (The Georgia Consortium for Technological Competitiveness in Pulp and Paper)
Amount Requested:	$83,823 ($70,323 to IPST and $13,500 to UGA)
Period of Proposal:	Fiscal Year 1999 (July 1, 1998 - June 30, 1999)
Submitted:	1st January, 1998
Status:	**Awarded $31000 to IPST, $13000 to UGA**

Title:	Genetic Transformation Methods for Southern Pine
Authors (Affiliation):	Gerald Pullman (IPST), Gary Peter (IPST), John Cairney (IPST), Scott Merkle, (UGA), Jeffery Dean (UGA), Sarah Covert (UGA)
Awarding Agency:	The Georgia Consortium for Technological Competitiveness in Pulp and Paper
Amount Requested:	$ 105000 ($ 55000 to IPST)
Period of Proposal:	July 1, 1998 – June 30, 1999
Submitted:	June 6, 1998
Status:	**Awarded $55000 to IPST, $50000 to UGA**

Title:	Ensuring Fiber Supply Through Biotechnology: New Tissue Culture Tools from Gene Expression Studies.
Authors (Affiliation):	Nanfei Xu (IPST), G. Pullman (IPST), J. Cairney (IPST)
Awarding Agency:	Traditional Industry Program in Pulp and Paper (The Georgia Consortium for Technological Competitiveness in Pulp and Paper)
Amount Requested:	$144,377
Period of Proposal:	Fiscal Year 1999 (July 1, 1998 - June 30, 1999)
Submitted:	1st January, 1998
Status:	**Awarded $144,377**

Title:	Trees Containing Built-In Pulping Catalysts
Authors (Affiliation):	Gerald Pullman, Don Dimmel, John Cairney, Gary Peter (all IPST)
Awarding Agency:	Preproposal - Agenda 2020 - Environmental Group, U.S. Department of Energy.
Amount Requested:	Year 1 $168,658 and **Year 2 $178,590**
Period of Proposal:	Fiscal Years 1998-1999
Submitted:	Requested 2nd year funding February 1998
Status:	**Awarded, $175,845 for the second year (8/19/1997 - 8/18/1999)**

Title:	The Role of the Mechanical Environment in Cambial Meristem Identity, Xylem Secondary Cell Wall Biosynthesis and Loblolly Pine Somatic Embryo Development.
Agency:	Georgia Institute of Technology / IPST, Pulp & Paper Education Program.
Authors (Affiliation):	G. Peter (IPST), C. Zhu (GIT)
Amount requested:	$40,000 ($20 K to IPST, $20 K to GIT)
Period of proposal:	1997 / 1998
Submitted:	August, 1997
Status:	**Awarded $20 K to IPST**, $20 K to GIT

Title:	The Role of Peptide Processing in Plant Cell Growth and Development
Authors (Affiliation):	Sheldon W. May (GIT), Gerald Pullman (IPST), and John Cairney (IPST)
Awarding Agency:	Georgia Institute of Technology/Institute of Paper Science and Technology, Pulp and Paper Education Program
Amount Requested:	$ 40,000 ($ 20,000 to IPST)
Period of Proposal:	1997-1998
Submitted:	August 29, 1997
Status:	**Grant Awarded ($20 K to IPST Forest Biology, $20 K to GIT Dept. Chemistry & Biochemistry)**

Title:	Genetically modified lignin in pine: structure and properties
Agency:	USDA (NRI) Improved utilization of wood and wood fiber
Authors (Affiliation):	Ronald R. Sederoff (NCSU) John J. MacKay (IPST)
Amount requested:	$220, 000 over three years
Period of proposal:	Requested for 3 years, awarded for 2 years: 10/1/97- 9/30/99
Submitted:	January 14th, 1997
Status:	**Awarded $55,000 total for Year 1, $ 24,000 K to IPST (subcontract) and $ 31,000 K to NCSU**

Title:	Trees Containing Built-In Pulping Catalysts
Authors (Affiliation):	Gerald Pullman, Don Dimmel, John Cairney, Gary Peter (all IPST)
Awarding Agency:	Preproposal - Agenda 2020 - Environmental Group, U.S. Depart. of Energy.
Amount Requested:	Year 1 $168,658 and Year 2 $178,590
Period of Proposal:	Fiscal Years 1997-1998
Submitted:	Requested expanded proposal submitted April 15, 1997
Status:	**Awarded, $161,003 for the first year beginning September 1997**

Title: Ensuring Fiber Supply Through Biotechnology: New Tissue Culture Tools from Gene Expression Studies.
Authors (Affiliation): G. Pullman (IPST), J. Cairney (IPST), Jung H. Choi (GIT), Art Ragauskas (IPST)
Awarding Agency: The Georgia Consortium for Technological Competitiveness in Pulp and Paper
Amount Requested: $142,249 ($121,249 to IPST, and $21,000 to GIT)
Period of Proposal: Fiscal Year 1998 (July 1, 1997 - June 30, 1998)
Submitted: 12th July 1996 Status: **Awarded $ 109,249 (IPST), $21,000 (GIT)**

Title: Protecting the Fiber Supply Through Genetic Engineering of Drought Tolerance
Authors (Affiliation): John Cairney (IPST), Gerald Pullman (IPST)
Awarding Agency: The Georgia Consortium for Technological Competitiveness in Pulp and Paper
Amount Requested: $84,622 (IPST)
Period of Proposal: Fiscal Year 1998 (July 1, 1997 - June 30, 1998)
Submitted: 12th July 1996 Status: **Awarded $ 73,622**

Title: Molecular Manipulation of Reproduction in Southeastern Tree Species of Commercial Importance
Authors (Affiliation): Jeffrey F. Dean (UGA), Scott A. Merkle (UGA), Sarah Covert (UGA), Gerald Pullman (IPST), and John Cairney (IPST)
Awarding Agency: The Georgia Consortium for Technological Competitiveness in Pulp and Paper
Amount Requested: $355,448 ($180,119 to IPST and $175,329 to UGA)
Period of Proposal: Fiscal Year 1998 (July 1, 1997 - June 30, 1998)
Submitted: July 15, 1996
Status: **Awarded $136,163 to IPST, $160,620 to UGA**

Pending Proposals

Title: A Loblolly Pine Mutant for Simplified Lignin Removal
Authors (Affiliation): J. MacKay, D. Dimmel, G. Pullman, G. Peter, and J. Cairney
Awarding Agency: Preproposal - Agenda 2020 - Environmental Group, U.S. DOE
Amount Requested: Year 1 $185,000 and Year 2 $207,000
Period of Proposal: Fiscal Years 1999 and 2000
Submitted: July 31, 1998 Status: in review

Title: Low VOC and Pitch Containing Loblolly Pine Trees
Authors (Affiliation): G. Peter, G. Pullman, J. MacKay, and J. Cairney
Awarding Agency: Preproposal - Agenda 2020 - Environmental Group, U.S. DOE
Amount Requested: Year 1 $120,000 , Year 2 $ $125,000 and Year 3 $130,000
Period of Proposal: Fiscal Years 1999 -2001
Submitted: July 31, 1998 Status: in review

Title:	Molecular Basis of Wood Formation in the Pine Megagenome
Authors (Affiliation):	Multiple locations and PIs – R. Sederoff, D. Neale, C. Kinlew, C. Loopstra, R. Newton, E. Retzel, (IPST - John MacKay, Gary Peter, Gerald Pullman
Awarding Agency:	NSF
Amount Requested:	$500000
Period of Proposal:	2000-2004
Submitted:	April 6, 1998
Status:	in review

Title:	Capital Equipment Requested: Storm® Gel and Blot Imaging System for the Clonal Propagation and Genetic Engineering of Forest Trees
Authors (Affiliation):	Gerald Pullman, John Cairney, Gary Peter, John MacKay
Awarding Agency:	TIP3 - The Georgia Consortium for Technological Competitiveness in Pulp and Paper
Amount Requested:	$85200
Period of Proposal:	1998/1999
Submitted:	August 5, 1998
Status:	in review

Title:	Image Analyzer and 3D Optical Microscope for Wood Fiber Analysis, Genetic Engineering, & Clonal Propagation of Forest Trees
Authors (Affiliation):	Gerald Pullman, John Cairney, Gary Peter, John MacKay, Martin Ostoja
Awarding Agency:	NSF
Amount Requested:	$191300
Period of Proposal:	1999
Submitted:	July 27, 1998 Status: in review

Rejected Proposals

Title:	Loblolly Pine Embryogenesis - A Study of Early Development Genes
Authors (Affiliation):	Nanfei Xu, J. Cairney, and Gerald Pullman
Awarding Agency:	USDA - National Research Initiative Competitive Grants Program
Amount Requested:	Years 1 $70,001, Year 2: $72636.
Period of Proposal:	Fiscal Years 1999, and 2000
Submitted:	December 15, 1997 Status: Not Funded

Title:	Transformation of loblolly pie: *Agrobacterium tumefaciens* gene transfer and improved selection, evaluation, and plant regeneration using somatic embryogenesis.
Authors (Affiliation):	A. Wenck (NCSU), G. Pullman (IPST), and G. Peter (IPST)
Awarding Agency:	Preproposal - Agenda 2020 - Sustainable Forestry Program, U.S. DOE
Amount Requested:	Year 1 $123,830 ($59,830 to IPST, $64,000 to NCSU), Year 2 $118,820 ($62,820 to IPST, $56,000 to NCSU)
Period of Proposal:	Fiscal Years 1999 and 2000
Submitted:	Submitted 8/26/1997, 2nd submission 1/21/1998 Status: Not Funded

Title:	Fast Growing Trees Through Biotechnology: An Alternative High Value Crop.
Authors (Affiliation):	G. Peter, J. Cairney and G. Pullman
Awarding Agency:	Agenda 2020 Sustainable Forestry Program
Amount Requested:	Year 1 $117,315, Year 2 $118,906, Year 3 $117,726
Period of Proposal:	Fiscal Years 1998 - 2000
Submitted:	Original submission August 26, 1997, Second submission 21 January, 1998
Status:	Not Funded

Title:	A Loblolly Pine Mutant for Simplified Lignin Removal
Authors (Affiliation):	J. MacKay, D. Dimmel, G. Pullman, and G. Peter
Awarding Agency:	Agenda 2020 - Environmental Group, U.S. DOE
Amount Requested:	Year 1 $158,087 and Year 2 $165,992
Period of Proposal:	Fiscal Years 1998 and 1999
Submitted:	July1, 1997
Status:	Not Funded

Title:	Drought Protection in Forest Trees: Post-Transcriptional Regulation of Stress-Responsive Genes.
Authors (Affiliation):	J. Cairney (IPST), L. Destefano (IPST), A. Altman (HUJ), O. Shoseyov (HUJ)
Awarding Agency:	BARD, USDA-ARS-OIRP (U.S. - Israel)
Amount Requested:	IPST Years 1-3: $69.1K, 67.9 K, 71.4 K; HUJ Years 1-3: $54.6 K, 55.8 K, 58.8 K.
Period of Proposal:	Fiscal Years 1998, 1999, and 2000
Submitted:	August 30, 1997 Status: Not Funded

Title:	Molecular Manipulation of Reproduction in Southeastern Tree Species of Commercial Importance
Authors (Affiliation):	Jeffrey F. Dean (UGA), Scott A. Merkle (UGA), Sarah Covert (UGA), Gerald Pullman (IPST), and John Cairney (IPST)
Awarding Agency:	Traditional Industry Program in Pulp and Paper (The Georgia Consortium for Technological Competitiveness in Pulp and Paper)
Amount Requested:	$278,681 ($164,251 to IPST and $114,430 to UGA)
Period of Proposal:	Fiscal Year 1999 (July 1, 1998 - June 30, 1999)
Submitted:	1st January, 1998
Status:	Not Funded

Title:	Drought Protection in Forest Trees: Post-Transcriptional Regulation of Two Stress-Responsive Genes
Authors (Affiliation):	John Cairney (IPST), Gerald Pullman (IPST), Luis Destéfano-Beltrán
Awarding Agency:	Traditional Industry Program in Pulp and Paper (The Georgia Consortium for Technological Competitiveness in Pulp and Paper)
Amount Requested:	$90,065 (IPST)
Period of Proposal:	Fiscal Year 1999 (July 1, 1998 - June 30, 1999)
Submitted:	1st January, 1998 Status: Not Funded

Title:	Molecular analysis of male and female cottonwood trees.
Authors (Affiliation):	David E. McMillin (Clark Atlanta Univ.) and John Cairney (IPST)
Awarding Agency:	TAPPI
Amount Requested:	$40,000 ($20,000 IPST and $20,000 CAU)
Period of Proposal:	October, 1997-November 1998
Submitted:	June 27, 1997
Status:	Not funded

Title:	Fast Growing Trees Through Biotechnology: An Alternative High Value Crop
Authors (Affiliation):	G. Peter, J. Cairney and G. Pullman
Awarding Agency:	Rural America Fund - Department of Agriculture
Amount Requested:	Year 1 $168,658 and Year 2 $178,590
Period of Proposal:	Fiscal Years 1997 - 1999
Submitted:	April 25, 1997
Status:	Not Funded

AF & PA United States Department of Energy - Agenda 2020 Environmental Program
A Technology Vision and Research Agenda for America's Forest, Wood, and Paper Industry

TITLE *Low VOC and Pitch Containing Loblolly Pine Trees*

PRIMARY INVESTIGATOR

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TARGETED RESEARCH AREAS

Wood Products - Prevention of volatile organic compounds and hazardous pollutants released from lumber and wood panel production facilities.

BACKGROUND

Destroying volatile organic compounds (VOCs) released during drying of wood requires expensive equipment and increased energy. Regenerative thermal oxidation units are expensive, use natural gas and emit CO_2. The problem is especially acute for softwoods, which have high terpene amounts: 3-8% of the dry weight of wood. In addition, with the use of more trees from rapidly growing plantations that are enriched in juvenile wood which has higher extractive contents than mature wood the foam, pitch and VOC problems are expected to increase.

A long-term solution to reducing VOCs from wood is to decrease the amount of terpenes present. In the wood of pines, terpenes are synthesized and accumulate principally within the resin canals. Terpenes are a complex set of related organic molecules for which the biosynthetic pathway is not fully elucidated. The first committed and rate-limiting step in the biosynthetic pathway is catalyzed by terpene synthases. Terpene synthases catalyze the cyclization of C10, C-15 and C-20 prenyl diphosphates to generate a diverse set of natural products. The availability of thirty-three terpene synthase cDNAs from seventeen plant species, and three classes of terpene synthase cDNAs from Grand fir greatly enhance the chances for success of this project. We propose to isolate from loblolly pine terpene synthase or other genes that are expressed selectively in resin canal cells. These resin canal specific genes will be used to down regulate terpene biosynthesis and/or inhibit the formation of resin canal cells.

OBJECTIVES

- *Create loblolly pine trees with dramatically reduced terpene and triglyceride contents*

Specific Goals 1) Isolate cDNAs selectively expressed in resin canals, 2) Isolate the corresponding gene regulatory regions for resin canal selectively expressed genes, 3) Construct vectors with a cytotoxic protein and antisense terpene synthase regulated by resin canal selective elements, 4) Transform loblolly pine with ablation and antisense constructs, 5) Analyze transgenic trees for resin canal development, terpene and triglyceride levels

METHODS & EXPERIMENTAL PLAN

1) **Isolate cDNAs selectively expressed in resin canals.** Two approaches will be used. In the first approach cell specific cDNA libraries will be constructed with mRNA obtained from isolated resin canal epithelial cells. This library, enriched in resin canal selective cDNAs, will be differentially screened with subtracted cDNA probes to identify those mRNAs that are selectively expressed in the resin canal cells. The second approach relies on isolating gene(s) that are involved with terpene biosynthesis. In this approach, targeted cloning of terpene synthases by PCR will be carried out with degenerate oligonucleotide primers and cDNA isolated from resin canal cells as well as whole stems. In both approaches *in situ* hybridization will be used to identify those cDNAs that are selectively expressed in resin canal cells.
2) **Isolate the corresponding gene regulatory regions for resin canal specific expression.** Once cDNAs that are selectively expressed in resin canal cells are isolated the 5' and 3' regions from one of these genes will be isolated by gene walking. In this method, PCR with a genomic DNA template is used to synthesize the 5' and 3' regulatory regions immediately surrounding the cDNA. 5' and 3' mapping studies will be done to establish the 5' and 3' ends of the message.
3) **Construct vectors that express antisense and cytotoxic proteins selectively within resin canals.** We will use the Barnase RNase as the cytotoxic gene. This cytotoxin cDNA or an antisense terpene synthase will be inserted between the 5' and 3' regulatory regions of the resin canal selective gene as both transcriptional and translation fusions.
4) **Transform loblolly pine trees with cytotoxic gene expressed specifically in resin canal cells.** Transformation of somatic embryo cultures of loblolly pine is successfully being carried out by a number of labs. We will use *Agrobacterium tumefacians* to generate at least 10 independent transformants from 2 genotypes with both the transcriptional and translational fusions will be generated.
5) **Analyze transgenic trees for resin canal development, terpene and triglyceride accumulation.** Seedlings will be analyzed for resin canal development. After extraction terpenes will be measured by gas chromatography and triglycerides will be measure by liquid chromatography.

BENEFITS TO INDUSTRY

- *Reduced VOCs and CO_2 emissions from wood drying facilities by existing processing methods*
- *Reduced energy and capital costs*
- *No pitch and foam problems for pulps made with loblolly pine; especially mechanical pulps*
- *Faster tree growth or higher cellulose contents due to 2-4% more carbon availability*
- *Cell selective gene regulatory elements*

SCHEDULE, MILESTONES, MEASURES OF SUCCESS

- *Success will be measured first by isolating cDNAs for resin canal selective genes and second by creating transgenic pine trees with greatly reduced terpenes and triglycerides*

Year 1 - Isolate and characterize cDNAs selectively expressed in resin canal cells
Year 2 – Isolate gene regulatory regions, create fusions and initiate transformations
Year 3 – Complete transformations and begin analyses of transgenic pines

INVESTIGATOR'S QUALIFICATIONS

IPST Forest Biology Group is applying integrated biochemical, cellular, molecular and genetic approaches to elucidate the molecular mechanisms that regulate embryogenesis and wood formation in loblolly pine. Major projects target efficiency improvements of somatic embryogenesis, genetic transformation and understanding wood formation of loblolly pine. We have developed and are improving our reliable protocol for somatic embryogenesis for loblolly pine and using it to develop efficient transformation methods for loblolly pine.

BUDGET & PROPOSED FUNDING SOURCES

Year 1 120,00 Year 2 125,000 Year 3 130,000
20% matching funds from IPST consortium

Capital Equipment Requested: Storm® Gel and Blot Imaging System for the Clonal Propagation and Genetic Engineering of Forest Trees

Molecular Dynamics Sorm 860 Imaging System	**$ 74,500**
Storm 860 Image Analyzer Work Station	**$ 5,000**
Phosphor Screens and Cassettes	**$ 5,700**
	$ 85,200

Current TIP3 Projects that this Equipment will Support:

1) **FS-8** Ensuring Fiber Supply Through Biotechnology: New Tissue Culture Tools from Gene Expression Studies.
2) **FS-18** Environmental Influences and Genetic Regulation of Microfibril Angle in Southern Pine
3) **FS-19** Development of Transformation Systems for Southern Pine

PIs: G. Pullman (IPST), J. Cairney (IPST), G. Peter (IPST), J. MacKay (IPST)

Project Summary: It is expected that the U.S. Forest Products Industry may soon face a shortage of low cost fiber. If the productivity of U.S. forests cannot be increased within the next decade it is likely that much of the industry will turn to importation of raw materials or relocation. This lends urgency to research efforts to boost forest productivity domestically. We are requesting **a *Storm® Gel and Blot Imaging System*** to continue to develop state of the art molecular biology and analytical capabilities that are needed in order for biotechnology to deliver timely gains in improved fiber productivity and quality.

Our program is oriented to the production of conifers with improved fiber quality and the mass clonal propagation of these trees, through somatic embryogenesis. A program to improve somatic embryos has been supported for a decade by internal funds. In the past three years, novel molecular approaches have been employed to study gene expression during embryo development.

A Storm® Gel and Blot Imaging System is needed to support our *functional genomics* approach to pine embryogenesis, in which we are using gene arrays to monitor the activity of hundreds of genes in pine zygotic and somatic embryos (Cairney et al. 1997, Xu et al. 1997, Cairney et al. 1998, Pullman et al 1998). Gene arrays could be used to address many fundamental and applied questions of embryo develop, its regulation and the control of somatic embryogenesis *in vitro*. However, high sensitivity and the ability to quantify large numbers of hybridization signals accurately are necessary for these experiments to yield reliable data upon which hypotheses may be based. Gene array systems will lead to development of powerful methods to assess somatic embryo development and quality. High quality embryos will in turn accelerate the deployment of superior genotypes and the implementation of clonal forestry resulting in the deployment of improved varieties of fast growing trees.

Our program is also aimed at understanding the cellular and molecular mechanisms of wood formation, with a long term objective to modify wood quality (Peter, 1998). This research will also benefit from the flexibility of the *Storm® Gel and Blot Imaging System* as means of imaging and quantifying gene expression data in Northern and Western analysis and of reporter gene expression in transgenic plants.

Detailed Hypotheses, Experimental Plans, and References are available Upon Request.

NSF Equipment Grant Proposal - $191,300

2) Project Summary Form 1358 - 3D Optical Microscope for Wood Fiber Analysis, Genetic Engineering & Clonal Propagation of Forest Trees

It is expected that the U.S. Forest Products Industry may soon face a shortage of low cost fiber. If the productivity of U.S. forests cannot be increased within the next decade it is likely that much of the industry will turn to importation of raw materials or relocation. This lends urgency to research efforts to boost forest productivity domestically. We are requesting a ***Storm® Gel and Blot Imaging System*** and a ***3D Optical Microscope*** to continue to develop state of the art molecular biology and analytical capabilities that are needed in order for biotechnology to deliver timely gains in improved fiber productivity and quality.

Our program is oriented to the production of conifers with improved fiber quality and the mass clonal propagation of these trees, through somatic embryogenesis. A program to improve somatic embryos has been supported for a decade by internal funds. In the past three years, novel molecular approaches have been employed to study gene expression during embryo development. *A Storm® Gel and Blot Imaging System* is needed to support our *functional genomics* approach to pine embryogenesis, in which we are using gene arrays to monitor the activity of hundreds of genes in pine zygotic and somatic embryos (Cairney et al 1997; Cairney et al. 1998). Gene arrays could be used to address many fundamental and applied questions of embryo develop, its regulation and the control of somatic embryogenesis *in vitro*. However, high sensitivity and the ability to quantify large numbers of hybridization signals accurately are necessary for these experiments to yield reliable data upon which hypotheses may be based. Gene array systems will lead to development of powerful methods to assess somatic embryo development and quality. High quality embryos will in turn accelerate the deployment of superior genotypes and the implementation of clonal forestry resulting in the deployment of improved varieties of fast growing trees.

Our program is also aimed at understanding the cellular and molecular mechanisms of wood formation, with a long term objective to modify wood quality. This research will also benefit from the flexibility of the *Storm® Gel and Blot Imaging System* as means of imaging and quantifying gene expression data in Northern and Western analysis and of reporter gene expression in transgenic plants.

A *3D Optical Microscope* is also requested. Our long term goal is to genetically engineer trees as raw materials for improved and lower cost pulp and paper products. This requires an integrated program in biology chemistry and physics. The equipment is essential to develop a dynamic 3D understanding of the fiber as it develops and to follow the interactions among fibers in paper structure. Specifically this equipment will be useful in understanding assembly of the secondary cell wall, wood microstructure, effects of delignification on secondary cell wall structure, and fiber-fiber bonding during paper sheet formation. The *3D Optical Microscope* will also be needed to further develop studies of Multiscale Mechanics of Paper and the 3D Geometry of Cellulose Fiber Microstructures.

STUDENT RESEARCH - COMPREHENSIVE LIST

Following is a list of students in the Forest Biology Group along with their project or thesis titles and a summary of the work proposed or in progress. Projects marked with an @ are targeted at the fundamentals mechanisms of tree growth and fiber properties. Projects marked with an * are targeted at somatic embryogenesis.

FIRST YEAR M. SC. STUDENTS

Currently recruiting students into biology program
Student decisions due by October 23, 1998.

SECOND YEAR M. SC. STUDENTS (2)

Douglas Benton
@Title: **Determination of the Microfibril Angle in the S2 Layer of Commercially Important Trees Using Differential Interference Contrast Microscopy**
Advisor: Gary Peter
Summary: The microfibril angle (MFA) of the S2 layer in plant cell walls has been shown in many studies to be an important parameter in determining both fiber and paper's strength, stiffness, and shrinkage characteristics. Unfortunately, current methods used for measuring the MFAs from core samples are often time consuming, arduous and require expensive equipment. The purpose of this project is to develop Differential Interference Contrast Microscopy (DIC) as a reliable and less time consuming method to accurately determine the MFA.

Validation of DIC will be done by measuring the MFAs in standard fiber samples using DIC, then comparing these results to those obtained from two proven techniques: polarizing light microscopy and confocal laser scanning microscopy. If DIC proves to be a valid technique for measuring the MFA, it will then be used to investigate the range MFAs in commercially important angiosperm and gymnosperm genotypes obtained from short rotation plantations under various growing conditions. Data obtained from this study will help to address quality issues pertaining to short rotation fiber supply.

Douglas Mancosky
* Title: **Temporal and Spatial Analysis of Gene Expression During Pine Zygotic Embryogenesis**
Advisor: John Cairney
Summary: The proposed study will select a known clone and study gene expression at the mRNA and protein level for different stages of development. This will be accomplished using Northern blotting, RT-PCR and antibody probing. We will study gene expression *in situ*, determining where within the embryo genes are expressed, as well as at what stages of development expression occurs. Three clones, previously isolated and sequenced and shown to bear homology with well characterized regulatory genes from other organisms have been selected for this study. These clones will serve as markers for embryo development and their

detailed study will reveal many of the regulatory features, which determine the timing, and localization of gene expression during embryo development.

Ph.D. STUDENTS (3)

Vincent Ciavatta Candidate.
*Title: **Analysis of Gene Expression During Development of Somatic and Zygotic Embryos.**
Advisor: John Cairney
Summary: As a tool to follow Embryogenesis in vitro and in vivo, gene expression during equivalent stages of development will be compared. Differential Display will be used and patterns diagnostic of a particular stage of development for a particular genotype will be generated for somatic embryo. Bands which appear characteristic of early or late development or of a particular stage will be cloned and characterized. Equipped with the technical expertise and physiological insight that will give us, similar experiments will be conducted with zygotic embryos. Comparing and contrasting gene expression in somatic and zygotic embryos will allow us to evaluate their state of health and permit informed modifications to media which should improve quality and quantity of embryos generated in vitro.

Michael Sullivan Completed pre-Ph.D. A390 problems
@Title: **Isolation of b- 1,4-xylan Synthase**
Advisor: Gary Peter
Summary: Project will use biochemical and molecular approaches to isolate and characterize b- 1,4-xylan synthase(s).

Stephen Van Winkle Candidate.
*Title: **An investigation into an unsuccessful tissue culture medium: Determining the role of activated charcoal.**
Advisor: Jerry Pullman
Summary: Previous tissue culture experiments with two different activated carbons supplied by Sigma revealed that one carbon promoted embryogenesis of Douglas-fir while the other did not. The goal of this project is to discover why one carbon was ineffective. Research will be directed towards physically and chemically characterizing many (~20+) different carbons with the goal of correlating these characteristics with carbon performance in tissue culture medium. Activated carbon is known to be a versatile sorbent: performance will be measured in terms of sorption of tissue culture medium components (particularly hormone and mineral nutrients). Performance will also be measured using a bioassay model for the Douglas-fir initiation system. This bioassay will be developed using Norway Spruce zygotic and somatic embryos. Initial results indicate that the two carbons have different porosity, different surface charge, and different ionic content. A liquid Norway Spruce initiation system has been successfully demonstrated for use as a bioassay.

GRADUATES 1998 (4) – Theses Abstracts /Current Positions

Levi Barclay (M. Sc.) Completed Spring – ***Entered Ph.D. Program Summer 98***
*Title: **Using Microscopy to Determine the Division Rate of Cells in A Loblolly Pine Suspension Culture**
Advisor: Gary Peter

Summary: It appears that, in the future, somatic embryogenesis will supply the paper and forest products industries with their softwood seedlings. However, the process is currently used only for specialty purposes because it does not produce seedlings as cheaply as conventional forestry programs. In order to better understand and evaluate the multiplication of somatic embryos, an antibody staining technique was developed and optimized. This technique was applied to measure the rate of cell division in a culture of early stage somatic embryos of loblolly pine.

The technique revealed that embryonic cells are replicating about twice as fast than non-embryonic cells. However, because of the size difference (as measured by the number of cells per cluster) between the embryo clusters and the non-embryonic clusters, this higher division rate did not translate into a higher volumetric growth rate.

A model for dilution based culture was proposed. This model was checked by counting embryos and non-embryonic tissue in a sample from a suspension culture and calculating their volume fractions. The data showed that the concentration of embryos in a culture of 346 decreased 10% during the course of a week, which corresponded to the embryos multiplying 85% as fast as non-embryonic material on a volumetric basis. However, the volumetric fraction of embryos decreased only 50% over the course of a year. This is consistent with a model of dilution based decline in which the embryos are growing 3% slower than non-embryonic tissues. If the model holds true, then the difference between the weekly and yearly rates is probably due to the concentrating effect that occurs when the suspension is settled during subculturing and the heavy embryo rich fraction is drawn off to start the new culture.

This concentrating effect due to the gravity fractionation caused by settling should be exploited to improve the durability of suspension cultures and slow culture decline. It would be beneficial to investigate other possible fractionation techniques in order to improve culture contents and slow decline.

John Ceranski (M. Sc.) Completed Spring - ***Employed Consolidated Papers***
@Title: **The Study of Delignification in *Pinus Taeda* via Light Microscopy and Immunological Staining Techniques**
Advisor: Earl Malcolm and Gary Peter

Summary: Immunocytochemical labeling was used for the study of lignin localization in *Pinus taeda*. Within the earlywood fraction of a tissue sample from the 5 to 7 year growth rings of *Pinus taeda*, lignification is fixed with the highest concentration of lignin found in the middle lamella and the greatest total amount of lignin being present in the secondary cell wall. Softwoods contain lignin of the guaiacyl type with the assumption the middle lamella and cell corners fractions being mainly of the condensed type and the secondary cell wall lignin consisting of the uncondensed type. The delignification of these regions was investigated by evaluating antibodies generated against guaiacyl and guaiacyl/syringyl lignin for specificity and

using the antibodies to map lignin distribution. The specificity of the probes appears to be effective, and the tissue areas mentioned above were stained and imaged. Possibly for the first time, the distinction of condensed/uncondensed lignin has been shown in wood in relation to their location within the tissue. Both types of antibodies claimed to be specific for condensed lignin structure by Joseleau and Ruel (Joseleau and Ruel, 1997) appear to confirm the lignin deposition in the middle lamella and cell corners was of the condensed type and the cell corners delignify at a slower rate than the rest of the middle lamella. Both types of antibodies also showed a lack of staining in the secondary cell wall, suggesting a lack of condensed structure in that region.

Karen Crews (M. Sc.) Completed Spring - ***Employed International Paper***
@Title: **Anthraquinones in Commercial Trees and Their Potential Release From Chips During Pulping**
Advisor: Jerry Pullman and Don Dimmel
Summary: Anthraquinones can be used at extremely small amounts to improve pulping productivity and environmental factors. Anthraquinone (AQ) increases pulping rates and product yields, removing greater amounts of lignin during pulping. Presently AQ has been extracted from teakwood as well as several other angiosperms. One goal of this project was to expand our knowledge of AQ content in other hardwood tree species. Knowing which commercial tree species contain AQ tells us that a pathway for AQ production is present. This information will allow us to attempt to 'turn up' the particular gene for AQ production in major pulping tree species.

Eleven hardwood species and one softwood species were extracted with chloroform. Both a GC-FID and a GC-MS were used to analyze the extracts for AQ components. Six of the hardwood species were shown to contain AQ, mono-methyl-AQ (MMAQ), or dimethyl-AQ (DMAQ). Elm, walnut, red oak, red maple, and eastern cottonwood were found to have AQ contents of .007wt %, .010wt%, .020wt%, .011wt %, and .054wt% respectively. E. cottonwood contained three forms of anthraquinone useful for pulping, AQ, MMAQ, and DMAQ. The other species contained AQ in detectable amounts. It has been shown that AQ components in teak chips can be released and can effectively delignify pine chips cooked in the same pressure vessel. Chips from AQ containing species were extracted to remove the naturally occurring anthraquinones and then were kraft pulped. The amount of delignification was compared to unextracted chips that were kraft pulped. Unextracted chips showed greater amounts of delignification than extracted chips.

Michael Sullivan (M. Sc.) Completed Spring - ***Entered Ph.D. program summer 98***
*Title: **pH Cycling in Loblolly Pine Suspension Cultures and Associated Gene Expression Changes**
Advisor: John Cairney
Summary: Liquid suspension cultures of *Pinus taeda* somatic embryos have been observed to undergo a cycling phenomena, during which time fluctuations in settled cell volume and growth media pH occur. The cycling phenomena has been described as a symptom of tissue culture decline; however, a relationship between changes in growth rate, growth media pH and the cultures ability to yield cotyledonary embryos has not been established. The inability to draw a relationship between the cycling phenomena and culture decline does not detract from the

significance of the cycling phenomena however, as the observed fluctuations are related to embryonic growth mechanisms that may potentially be under genetic control.

Analysis of the gene expression of embryonic material isolated from pH peaks and troughs of Pinus taeda genotype 266A using differential display, revealed that several genes were differentially expressed under each growth condition. Further analysis of cDNA believed to be differentially expressed between pH peaks and troughs by nonradioactive Northern blotting indicated that two trough specific cDNA s were indeed differentially expressed under low pH. The nucleotide and amino acid sequence of clone T52 showed high homology to several members of the *Arabidopsis thaliana* multigene family encoding for plasma membrane H+-ATPase.

IPST FOREST BIOLOGY TEAM PUBLICATIONS - 1997-1998
(Published, in press, or submitted, * = work done at IPST)

1998

Wu R., D. M. O'Malley, D.L. Remington, J. J. MacKay and S.E. McKeand, Average effect of a mutation in lignin biosynthesis in loblolly pine. Submitted to Molecular Breeding

*Meng, Huabin, G. Pullman, and G. Peter. 1998. Cloning of a Plant Isochorismate Synthase. Plant Physiol. Gene Register (submitted).

Stoop, J., M. H., Williamson, J. D., Conkling, M. A., MacKay, J. J., and Pharr, D. M., 1998 Characterization of NAD-dependent mannitol dehydrogenase from celery as affected by ions, chelators, reducing agents and metabolites. Plant Science 131: 43-51

*Cairney, J., N. Xu, G. S. Pullman, V. Ciavatta and B. Johns. 1998. Large-Scale Gene Discovery and Expression Analysis - Embryo Development. Abstract presented at the 1998 IEG-40 Meeting, Institute of Paper Science and Technology, Atlanta, GA, 19 - 22 July 1998.

MacKay, John, J., John Ralph, David M. O'Malley, Rongling Wu, Timothy Presnell, Donald Dimmell, Brigitte Pollet, Catherine Lapierre, Jaap Boon, Ronald Sederoff. 1998. Variation at the *cad* locus in pine modifies lignin and growth. Abstract presented at the 1998 IEG-40 Meeting, Institute of Paper Science and Technology, Atlanta, GA, 19 - 22 July 1998.

*Van Winkle, S. and G. S Pullman. 1998. Modeling Sorption of 2,4-D and BAP onto Activated Carbon. Poster presented at the 1998 IEG-40 Meeting, Institute of Paper Science and Technology, Atlanta, GA, 19 -22 July 1998.

*Montello, P. and G. S. Pullman. 1998. Cryopreservation of Loblolly Pine Embryogenic Cultures. Poster presented at the 1998 IEG-40 Meeting, Institute of Paper Science and Technology, Atlanta, GA, 19 -22 July 1998.

*Meng, Huabin, J. Pullman, and G. Peter. 1998. Genetic Engineering of Forest Trees for the Pulping Catalyst Anthraquinone – Molecular Cloning Isochorismate Synthase. Poster presented at the 1998 IEG-40 Meeting, Institute of Paper Science and Technology, Atlanta, GA, 19 -22 July 1998.

*Douglas M. Benton and Gary F. Peter (1998) A Rapid Method for Measuring Microfibril Angle in the S2 Layer of Southern Pine. IEG-40 Workshop Wood and Wood Fibers: Properties and Genetic Improvement. Abstract presented at the 1998 IEG-40 Meeting, Institute of Paper Science and Technology, Atlanta, GA, 19 -22 July 1998.

*Pullman, G.S., J. Cairney, N. Xu, and X. Feng. 1998. Gene Expression Differences Between Zygotic and Somatic Embryos Monitored by Differential Display and cDNA Array: A Potential Tool to Improve Loblolly Pine Embryo Quality. Proceedings Ninth International Congress on Plant Tissue and Cell Culture. Plant Biotechnology and In Vitro Biology in the 21st Century. Jerusalem, Israel, June 14-19, 1998 (in press).

*Cairney, J., N. Xu, G. S. Pullman, V. T. Ciavatta, and B. Johns. 1998. Natural and Somatic Embryo Development in Loblolly Pine: Gene Expression Studies Using Differential Display. Applied Biochemistry and Biotechnology. (in press)

*Wenk., M. Quinn, R. Whetten, G. S. Pullman, R. Sederoff. 1998. High efficiency Agrobacterium mediated transformation of Norway spruce (*Picea abies*) and loblolly pine (*Pinus taeda*). Molecular Biology Reporter, (in press). (Work done jointly at NCSU and IPST)

*MacKay, John, D. O'Malley, R. Sederoff, J. McFall, J. Boon, T. Presnell, R. Wu, D Dimmel, and J. Ralph. 1998. Modification of lignin structure in pine with *Cad-n1* allele. 8th Conifer Biotechnology Working Group, June 7-11. Rutgers University, New Brunswick, New Jersey.

*Van Winkle, Stephen and G. S. Pullman. 1998. Modeling sorption of 2,4-D and BAP onto activated carbon. 8th Conifer Biotechnology Working Group, June 7-11. Rutgers University, New Brunswick, New Jersey.

*Cairney, John, N. Xu, G. Pullman, V. Ciavatta, and B. Johns. 1998. Mass gene cloning, high-density cDNA array and somatic embryogenesis in loblolly pine: tools for monitoring embryogenesis. 8th Conifer Biotechnology Working Group, June 7-11. Rutgers University, New Brunswick, New Jersey.

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