FOREST BIOLOGY
PROJECT ADVISORY COMMITTEE
MEETING

October 15-16, 1997

FOREST BIOLOGY PROJECT ADVISORY COMMITTEE MEETING

October 15-16, 1997

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FOREST BIOLOGY FALL PAC REVIEW
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Thursday, October 16, 1997 (Seminar Room)

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12:00	Adjourn (Lunch will be available at 12:00)	

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MASS CLONAL PROPAGATION OF IMPROVED CONIFERS - ELEMENTAL ANALYSIS OF ZYGOTIC FEMALE GAMETOPHYTE AND EMBRYO TISSUES

Gerald Pullman
Mike Buchanan
Yolanda Powell
Greg Elay

Summary

Full term seeds from loblolly pine were dissected to remove the seed coat, integument, and nucellus and divided into the female gametophyte and embryo tissue. Tissue was analyzed in replicate for major and minor elements by use of Inductively Coupled Plasma Emission Spectroscopy (ICPES). Five sources of seed were analyzed in order to determine an approximate range of natural elemental composition. Variation between the five seed sources was minimal providing elemental composition targets for somatic embryos. Analyses of elemental composition of somatic embryos and comparison to zygotic targets show that somatic embryos contain 250 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 on target. Microelements such as nickel, cobalt, and molybdenum were below detection levels in somatic and zygotic embryos. Analyses of developing zygotic embryos and female gametophyte tissues show significant changes in elemental composition over time. Phosphorous, potassium, magnesium, calcium, boron, sodium, and zinc contents in the female gametophyte are highest during early seed development. Sulfur, iron, and copper contents are relatively constant during female gametophyte growth. Zygotic embryo tissue showed a manganese peak at stages 1-2. Peaks in boron and sodium occurred at zygotic embryo stage 3-4. Embryo tissue peaks in phosphorous, potassium, calcium, and zinc, occurred at stage 5-6. Iron showed an initial peak at stage 5-6 followed by a dip and then increased until embryos stopped growth. Magnesium rose slightly throughout embryo development and sulfur and copper were fairly constant through embryo development.

Introduction

The nutritional, osmotic, and hormonal environments surrounding an embryo are well known to control embryo growth. Optimization of these environments is critical for the growth and development of high quality, vigorous somatic embryos. In optimizing the nutritional environment for somatic embryo development, we propose that analysis of the elemental contents of the female gametophyte and the zygotic embryo will provide models and targets for the development medium and somatic embryo elemental compositions respectively.

Following is a tentative step by step plan of how we plan to use analysis information of zygotic and female gametophyte tissue to improve our somatic embryo protocol. So far, we have completed the steps 1,2 and have some data for step 4. Experiments are in progress for steps 3 and 5.

Step 1. The first phase of this research is to determine how wide or narrow the natural or acceptable range of elemental composition is for female gametophyte and embryo tissue. We decided to initially use full term loblolly pine seed due to its availability and ease in obtaining the necessary sample weights for analysis. This data provides elemental targets for the somatic embryo and potentially for the medium.

Step 2. Analyze somatic embryos for elemental composition. Use somatic embryos as close to maturation as we can currently grow. Compare to target range for each element in analysis.

Step 3. Modify development and maturation medium based on review of the elemental composition data. Grow new somatic embryos on modified medium, observe somatic embryos for morphological improvements, analyze somatic embryos for new elemental composition and fit to target range.

Step 4. Analyze female gametophyte and zygotic embryos for each developmental stage to provide a time course of elemental composition during embryo and female gametophyte developments. (Note, some methods development is necessary to analyze the small amounts available of very early stage zygotic embryos. Example, twenty stage one embryos have a dry weight of approximately 0.3 mg)

Step 5. Modify initiation, maintenance, and maturation medium based on the pattern of elemental change in developing female gametophyte and zygotic embryo tissues. Observe somatic embryos for morphological improvements, analyze somatic embryos grown in modified medium for new fit to elemental target range.

Materials and Methods

Step 1. (Presented in the 1997 Spring PAC Report.) Five sets of cones or seed lots of ½ sib materials from four locations and seed orchards were analyzed for embryo and female gametophyte elemental composition. In order to make seed and embryo dissection easier, cones were requested at the end of seed development just prior to the seed drying process. With seed collection just prior to drying any potential elemental loss due to imbibition was eliminated and seeds were easier to open and remove embryos. Seed collections of 50-100 mg dry weight were obtained as follows.

BC-1	Boisie Cascade 1995 seed produced in a seed orchard near Lake Charles, Louisiana.
UC5-1036	Union Camp 1995 seed produced in a seed orchard near Bellville, GA.
UC10-1003	Union Camp 1995 seed produced in a seed orchard near Rincon, GA.
UC10-14	Union Camp 1995 seed produced in a seed orchard near Rincon, GA.
7-56	Westvaco 1995 seed produced in a seed orchard near Summerville, SC

Collected embryos were dried overnight at 70 °C and stored in a freezer. Pre-dried specimens of Loblolly Pine embryos and gametophytes were submitted for elemental analysis in individually labeled polyethylene micro-centrifuge tubes. To enable these samples to be analyzed for trace metals by Inductively Coupled Plasma Emission Spectroscopy (ICPES), the constituent metals present in the solid sample material were dissolved in an aqueous acid solution. The preparation procedure followed is outlined below:

1. An aliquot (approximately 50 mg) of pre-dried sample, was weighed into a new, labeled, graduated, screw-cap, polyethylene centrifuge tube. The weight of the sample was recorded to the nearest 0.1 mg.

2. Five milliliters (5.0 ml) of high purity concentrated Nitric Acid (EM Science TracePur Plus Instrumental Grade) were added to each tube, then capped, and allowed to stand at room temperature for six hours in a fume hood.

3. The tube was uncapped and two milliliters (2.0 ml) of high purity 30% Hydrogen Peroxide (J.T. Baker Ultrex Ultrapure Reagent Grade) were added. The tube was capped, inverted twice to mix the contents, and vented to release any evolved oxygen. The cap was loosely screwed on the tube to prevent pressure buildup from evolved oxygen. Each sample was allowed to digest at room temperature for 24 hours in a fume hood.

4. At the end of the digestion period, ultrapure reagent grade deionized water (ASTM Type I water), was added to each tube to bring the total solution volume to 10.0 ml.

5. Prior to analysis, each sample was filtered through a 0.45 μm membrane syringe filter.

Analysis of the sample digests was conducted on a Perkin Elmer Optima 3000 DV ICP Emission Spectrometer. This instrument, equipped with an autosampler and integral computer workstation, is configured to detect up to 30 elements simultaneously in less than five milliliters (5 ml) of sample solution. The principle of analysis is the detection of characteristic ultraviolet and visible light emissions from metallic elements subjected to a high temperature argon plasma torch. To improve instrument performance, a Yttrium internal standard is added to each sample, standard and blank to compensate for small variations in sample flow rate, sample viscosity and acid concentration as well as to assist in the identification of potential interferences. Quantification of the metallic analytes in the sample is based on measuring specific wavelength intensities for each element and comparing these results to multi-point calibration standards analyzed in the same manner.

The instrument is calibrated daily with three multi-component standards and a blank. A series of verification standards, interference check solutions and blanks are analyzed and evaluated before any samples are analyzed. At a frequency of every ten samples, a calibration verification standard and blank are analyzed. Acceptance criteria for each standard, blank and sample measurement are defined and used to accept or reject results.

Step 2. Somatic embryos from genotype 195 (an initiation from immature seed from tree UC 10-1003) were produced in maintenance medium 16 and embryos grown on medium 240. Somatic embryos were collected at the end of the development period on medium 240; corresponding in appearance to zygotic stages 8-9.1. Somatic embryos were collected from three different batches of embryos over a period of three months, each batch was produced from the same genotype on the same maintenance and development medium. Each of these three replicates of somatic embryos contained 30-50 mg dry weight of embryo. Metals analysis were done as described for step 1.

Step 3. Experiments are ongoing for alteration of the maintenance and development media based on the analyses in steps 1 and 2.

Step 4. During 1994 loblolly pine cones were collected weekly throughout the sequence of embryo development from Union Camp tree UC5-1036, located in a seed orchard near Bellville, GA. Cones were shipped on ice to IPST and received within 24-48 hours of collection. Cones were opened and seeds collected for isolation of embryos. Seeds were cracked using a hemostat, pried open with the aid of a scalpel, and the integument and nucellus tissue removed from the ovule. The female gametophyte was slit, pried open and the dominant embryo or mass or embryos removed. Individual embryos were quickly observed through a dissecting microscope, evaluated for stage of development (Pullman & Webb, 1994), sorted by stage, and placed in vials partially immersed in liquid nitrogen. Stage 9 embryos were also categorized by the week they were collected; 9.1 (Stage 9, week 1) 9.2 (Stage 9, week 2) etc. Staged zygotic embryos were then placed in a glass vial partially immersed in liquid nitrogen. Frozen embryos were stored at -70 °C. Samples were dried overnight at 70 °C. Due to the small amounts of embryo tissue available available from stages 1-8, the analysis method was modified as follows.

1. An aliquot of pre-dried sample, was weighed into a new, labeled, graduated, screw-cap, polyethylene centrifuge tube. The weight of the sample was recorded to the nearest 0.1 mg. Weights ranged from a low of 0.5 mg for a mass of early stage embryos to 79.4 mg for later stage embryos or female gametophyte.

2. One half milliliter (0.5 ml) of high purity concentrated Nitric Acid (EM Science TracePur Plus Instrumental Grade) was added to each tube, then capped, and allowed to stand at room temperature for 18 hours in a fume hood.

3. The tube was uncapped and one milliliter (1.0 ml) each of high purity reagent water and 30% Hydrogen Peroxide (J.T. Baker Ultrex Ultrapure Reagent Grade) were added. The cap was loosely screwed on the tube to prevent pressure buildup from evolved oxygen. Each sample was allow to digest at room temperature for 24 hours in a fume hood.

4. The tube was uncapped and one milliliter (1.0) of high purity concentrated hydrochloric acid (J. T. Baker Instra-Analyzed Reagent Grade) was added. Each sample was allowed to digest an additional 24 hours. At the end of the digestion period, ultrapure reagent grade deionized

water (ASTM Type I water), was added to each tube to bring the total solution volume to 10.0 ml.

5. Prior to analysis, each sample was filtered through a 0.45 μm membrane syringe filter.

Operation of the Perkin Elmer Optima 3000 DV ICP Emission Spectrometer was described in step 1 above. Due to the low amounts of tissue available for analysis the standard operating mode was modified. To yield the lowest possible detection limits the instrument was operated in the "axial" view mode which effectively increases the path length of the spectroscopic measurement.

Results and Discussion

Step 1. The elemental analyses for zygotic embryo are very similar for the five seed sources tested. Summaries of the averages per seed source and for all replications are shown for zygotic embryos in Table 1. Analyses for female gametophyte tissue is also similar for the five seed sources and is shown in Table 2. Standard errors for zygotic embryo elemental variation between all of the replicates for Mn, Fe, Cu, Zn, P, S, Mg, and K are less than 4% of the mean values (Table 1). Nickel, B, Na, and Ca show greater variation with standard errors ranging from 5-16% of the mean (Table 1). Standard errors for female gametophyte elemental variation between all replicates for Fe, Ni, Cu, Zn, P, S, Mg, and K are less than 4% of the mean values (Table 2). Manganese, B, Na, and Ca show greater variation with standard errors ranging from 5-8%.

Individual replicate analyses for zygotic embryos per site are shown in Tables 3-7. Individual replicate analyses for female gametophyte tissue per site are shown in Tables 8-12. Elemental concentrations detected for Cobalt, Nickel, Molybdenum, and Sodium were sometimes below the accurate detection limits of the equipment and values for these replicates are shown as <.

Elemental concentrations of zygotic embryo and female gametophyte tissues were often different. Ratios of elemental compositions, on a dry weight basis, are shown in Table 13. Embryos contained low contents of Mn, B, and S, suggesting that these elements are selectively excluded from the embryo. Similar contents of Ca, Ni, Zn, and Cu were found in embryo and female gametophyte tissue suggesting that these are taken into the embryo by diffusion. Greater concentrations of P, K, Mg, Na, and Fe were found in the embryo compared to female gametophyte tissue suggesting that these elements are actively taken up by the embryo.

Overall, the similarity in analyses of zygotic embryo tissues suggest that the mean elemental compositions of zygotic embryos provide reasonable targets for the elemental composition of somatic embryos.

Step 2. The analyses for somatic embryos are shown in Table 14. Table 15 shows a comparison of average zygotic female gametophyte, average zygotic embryo, Clone 195 somatic

embryo and a ratio of somatic/zygotic. These comparisons clearly show that our somatic embryo is way off for certain elements and on target for others.

Somatic embryos show major differences in some elements compared to zygotic embryos. The greatest difference is for Boron. Somatic embryos contain 250 times as much sodium as zygotic embryos, 17 times as much boron, three times the target amount of calcium, and 1.8 times potassium. These observations suggest the hypothesis that decreasing sodium, boron, calcium, and potassium in the maintenance or development media will improve embryo growth and development. On the other side, some elements were low in the somatic embryos. Somatic embryos contained only 10% of the copper contained in zygotic embryos, 21% of the iron, 46% of the phosphorous, 50% of the magnesium, and 66% of the manganese. These observations suggest that these elements should be increased in the maintenance or development media. Other elements such as zinc, and sulfur appear to be on target. Microelements such as nickel, cobalt, and molybdenum were present in zygotic and somatic embryos at amounts below detection levels of the equipment used.

Step 4. Elemental analyses throughout the sequence of development for female gametophyte and embryo tissues are shown in Figures 1-8. The female gametophyte tissue feeds the embryo during development and during germination. Elemental contents of the female gametophyte change over time (Figures 1-4). Phosphorous, potassium, magnesium, calcium, boron, sodium, and zinc contents in the female gametophyte are highest during early seed development. Sulfur, iron, and copper contents are relatively constant during female gametophyte growth. Female gametophyte elemental concentrations were more closely related to the date of tissue collection than to the stage of embryo contained within.

Zygotic embryo elemental contents also change over time (Figures 5-8). Manganese showed a peak at stages 1-2. Peaks in boron and sodium occurred at stage 3-4. Since embryos were collected in sodium borosilicate glass vials during 1994, it is possible that some container contamination may be present. However, all samples were treated similarly yet distinct peaks in boron and sodium are clearly present. Embryo collections for later years have used plastic cryostorage vials. Peaks in phosphorous, potassium, calcium, and zinc, occurred at stage 5-6. Iron showed an initial peak at stage 5-6 followed by a dip and then increased until embryos stopped growth. Magnesium rose slightly throughout embryo development and sulfur and copper were fairly constant through embryo development. In evaluating these mineral changes it is important to consider that early embryo stages 1-4 contain decreasing masses of suspensor tissue. Analyses of stage l embryo would consist mostly of suspensor tissue.

Table 1. Summary of averages of replicated elemental analysis of zygotic embryos collected from loblolly pine seeds grown on different mother trees and in different locations.

Tree	Location	Reps	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
BC-1	Lake Charles, LA	5	89.4	258		3.4	28.7	146	6.0	17053	2747			7671	12678	173
UC5-1036	Bellville, GA	4	90.6	279		1.0	24.6	133	8.7	17432	2619			8122	13092	275
7-56	Summerville, SC	3	66.4	221		1.0	22.7	113	1.2	15440	2242		7.1	7352	11552	140
UC10-14	Rincon, GA	2	76.7	181			31.0	113	2.2	14883	2312		6.6	7283	11219	174
UC10-1003	Rincon, GA	1	82.1	215	<.40	<.59	30.0	147	<.36	16421	2410	<.52	<3.23	7618	11833	146
Mean		15	83.0	243	####	2.1	26.8	132	6.2	16500	2531	####	6.6	7672	12312	192
Std Error		15	3.0	9.7	####	0.3	0.8	4.3	1.0	282	57	####	0.3	99	213	28
Std Er/Mean			0.04	0.04	####	0.16	0.03	0.03	0.16	0.02	0.02	####	0.05	0.01	0.02	0.15

Table 2. Summary of averages of replicated elemental analysis of female gametophyte tissue embryos collected from loblolly pine seeds grown on different mother trees and in different locations.

Tree	Location	Reps	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
BC-1	Lake Charles, LA	5	243	74		3	20	127	28	12415	5867			5019	9410	203
UC5-1036	Bellville, GA	4	233	82			22	143	21	13525	5547			5453	9744	344
7-56	Summerville, SC	3	121	53			16	126	17	10804	4633		4	4621	7843	270
UC10-14	Rincon, GA	2	251	79			18	155	19	11981	5430		4	5435	9102	150
UC10-1003	Rincon, GA	1	287	66	<.35	<.55	21	193	12	12348	5465	<.45	<2.83	5129	9460	311
Mean		15	221	72	####	2.6	20	139	22	12327	5450	####	3.9	5118	9148	254
Std Error		15	14.8	3.1	####	0.1	0.6	4.9	1.5	256	120	####	0.2	88	199	21
Std Er/Mean			0.07	0.04	####	0.03	0.03	0.04	0.07	0.02	0.02	####	0.05	0.02	0.02	0.08

Table 3. Elemental analysis (mg/Kg) for zygotic embryos of BC-1 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
BC-1	89.3	283.4	<0.43	3.5	29.2	154.7	7.7	17302	2715	<0.55	<3.44	7740	12675	171.9
BC-1	87.9	249.8	<0.43	4.1	28.9	139.5	6.9	16806	2711	<0.55	<3.45	7617	12708	200.7
BC-1	89.1	271.3	<0.40	2.3	30.4	160.6	4.2	17543	2824	<0.52	<3.24	7924	13009	137.0
BC-1	97.8	266.3	<0.40	3.3	28.4	147.0	3.1	17525	2793	<0.51	<3.22	7823	13050	180.2
BC-1	83.2	218.3	<0.42	3.9	26.7	129.5	8.1	16091	2692	<0.54	<3.40	7253	11948	175.6
Mean	89.4	257.8		3.4	28.7	146.3	6.0	17053	2747			7671	12678	173.1
Std Error	2.4	11.3		0.3	0.6	5.5	1.0	274.9	25.8			116.1	197.8	10.3

Table 4. Elemental analysis (mg/Kg) for zygotic embryos of Union Camp 5-1036 from full term cones collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
UC5-1036	83.2	267.3	<0.42	1.4	24.6	129.4	8.0	16961	2539	<0.54	<3.35	7958	12791	150.6
UC5-1036	97.6	291.6	<0.42	0.9	24.6	126.6	3.1	17630	2652	<0.54	<3.39	8158	13196	188.9
UC5-1036	100.6	292.7	<0.38	0.7	25.2	143.7	14.3	17700	2595	<0.48	5.1	8273	13120	574.1
UC5-1036	81.2	265.5	<0.42	1.1	24.0	130.5	9.3	17435	2688	<0.54	<3.37	8098	13259	185.1
Mean	90.6	279.3		1.0	24.6	132.5	8.7	17432	2619			8122	13092	274.7
Std Er	4.9	7.4		0.2	0.2	3.8	2.3	167	33			66	104	100.2

Table 5. Elemental analysis (mg/Kg) for zygotic embryos of Westvaco 7-56 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
7-56	73.0	227.6	<0.40	1.4	24.8	118.3	1.2	16132	2340	<0.51	8.4	7707	12232	100.7
7-56	60.6	210.5	<0.38	0.7	21.5	108.9	<0.35	14617	2122	<0.49	7.2	6948	10732	115.4
7-56	65.8	223.8	<0.41	<0.61	21.9	112.5	<0.38	15572	2265	<0.53	5.8	7401	11691	203.9
Mean	66.4	220.7		1.0	22.7	113.2	1.2	15440	2242		7.1	7352	11552	140.0
Std Er	3.6	5.2		0.3	1.1	2.7	0.0	442	64		0.7	220	438	32.2

Table 6. Elemental analysis (mg/Kg) for zygotic embryos of Union Camp 10-14 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
UC10-14	73.5	179.6	<0.40	<0.58	31.2	115.3	<0.36	15063	2312	<0.51	<3.18	7338	11328	157.1
UC10-14	79.9	181.5	<0.41	<0.60	30.8	110.5	2.2	14703	2312	<0.53	6.6	7228	11109	191.8
Mean	76.7	180.6			31.0	112.9	2.2	14883	2312		6.6	7283	11219	174.5

Table 7. Elemental analysis (mg/Kg) for zygotic embryos of Union Camp 10-1003 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
UC10-1003	82.1	214.8	<0.40	<0.59	30.0	147.5	<0.36	16421	2410	<0.52	<3.23	7618	11833	146.5

Table 8. Elemental analysis (mg/Kg) for female gametophyte tissue of BC-1 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
BC-1	237.2	78.5	<0.37	2.2	21.2	129.2	28.3	12624	5774	<0.48	<3.00	4934	9503	240.8
BC-1	264.1	70.5	<0.38	2.8	19.5	131.8	23.3	12291	5883	<0.49	<3.06	4785	9343	198.6
BC-1	248.2	79.8	<0.38	2.3	19.8	129.4	31.2	12619	5775	<0.49	5.4	5128	9530	208.0
BC-1	257.3	77.3	<0.41	2.7	21.2	130.5	28.0	12636	5977	<0.52	<3.26	5199	9943	111.7
BC-1	205.8	65.6	<0.40	3.1	18.6	114.7	28.1	11907	5925	<0.51	<3.22	5050	8732	257.9
Mean	242.5	74.4		2.6	20.1	127.1	27.8	12415	5867			5019	9410	203.4
Std Er	10.2	2.7		0.2	0.5	3.1	1.3	143	41			73	197	25.3

Table 9. Elemental analysis (mg/Kg) for female gametophyte tissue from Union Camp 5-1036 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
UC5-1036	231.8	72.9	<0.40	<0.60	22.2	141.5	16.1	13448	5606	<0.52	<3.25	5455	9761	294.5
UC5-1036	241.5	88.4	<0.39	<0.58	22.2	151.9	20.8	13944	5600	<0.50	<3.14	5600	10024	402.2
UC5-1036	248.5	86.1	<0.39	<0.57	22.3	138.2	22.7	13218	5537	<0.50	<3.11	5278	9449	292.0
UC5-1036	211.7	79.2	<0.41	<0.60	21.2	141.2	22.9	13493	5444	<0.52	3.8	5477	9743	386.5
Mean	233.3	81.7			21.9	143.2	20.6	13525	5547			5453	9744	343.8
Std Er	8.0	3.5			0.3	3.0	1.6	152	38			66	118	29.4

Table 10. Elemental analysis (mg/Kg) for female gametophyte tissue of Westvaco 7-56 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
7-56	145.6	50.3	<0.34	<0.51	16.9	126.8	19.9	10913	4638	<0.44	3.9	4657	8150	253.5
7-56	100.0	51.1	<0.33	<0.48	15.4	120.5	16.1	10561	4451	<0.42	3.4	4570	7568	273.3
7-56	118.8	56.9	<0.33	<0.49	16.6	129.9	15.6	10936	4811	<0.42	3.3	4636	7811	284.3
Mean	121.4	52.7			16.3	125.7	17.2	10804	4633		3.5	4621	7843	270.3
Std Er	13.2	2.1			0.5	2.7	1.3	121	104		0.2	26	169	9.0

Table 11. Elemental analysis (mg/Kg) for female gametophyte tissue of Union Camp 10-14 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
UC10-14	210.5	80.3	<0.37	<0.54	17.4	157.8	17.7	11846	5472	<0.47	3.0	5381	8792	149.1
UC10-14	291.4	78.4	<0.39	<0.58	18.5	152.4	20.3	12116	5388	<0.50	4.3	5490	9412	150.6
Mean	251.0	79.4			17.9	155.1	19.0	11981	5430		3.6	5435	9102	149.9

Table 12. Elemental analysis (mg/Kg) for female gametophyte tissue of Union Camp 10-1003 from full term seed collected prior to drying in 1995.

Tree	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
UC10-1003	287.0	66.4	<0.35	<0.55	21.3	192.7	11.6	12348	5465	<0.45	<2.83	5129	9460	310.7

Table 13. Ratio of average elemental concentrations in dried zygotic embryo vs. female gametophyte tissues.

Ratio	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
Zyg/FG	0.38	3.37	#####	0.80	1.37	0.95	0.29	1.34	0.46	#####	1.72	1.50	1.35	0.75

Table 14. . Elemental analysis (mg/Kg) for oven-dried somatic embryos of clone 195.

Somatics	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
SE	61.7	53	<0.72	<1.07	3.2	123.0	55	8157	3239	<0.93	829	4028	21703	485.6
SE	56.7	48	<0.44	<0.66	3.1	131.9	88	7939	2659	<0.57	1539	4153	23262	576.5
SE	42.6	46	<0.41	<0.61	2.2	116.3	91	6251	2241	<0.53	1576	3280	21645	590.0
Mean	53.7	49			2.8	123.7	78	7449	2713		1315	3820	22204	550.7
Std Er	5.7	2.0			0.3	4.5	12	602	290		243	273	529	32.8

Table 15. Comparison of elemental compositions for zygotic female gametophyte, zygotic embryo, and somatic embryo tissues with along witht 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

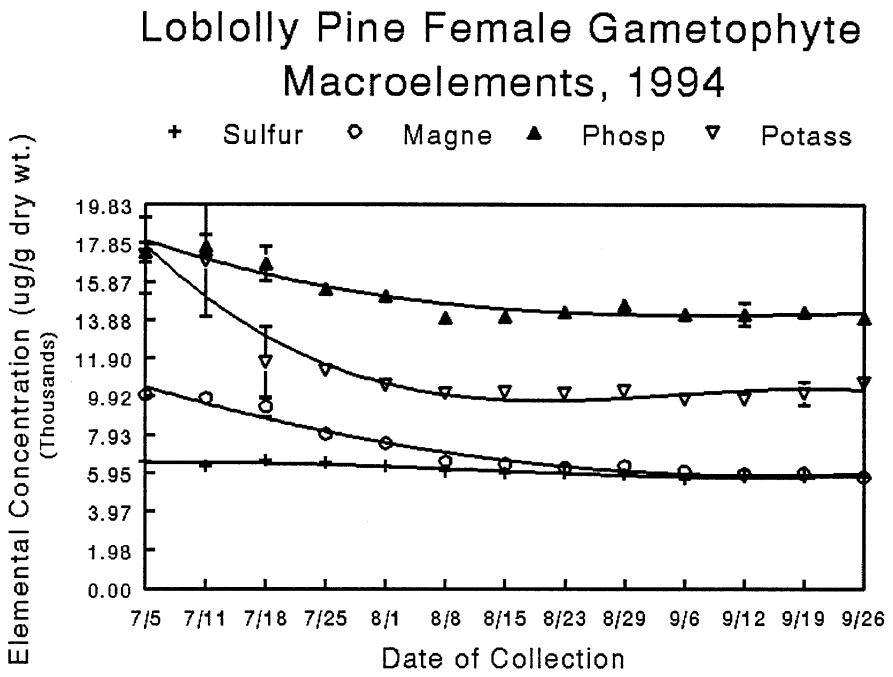


Figure 1. Elemental analysis (mg/Kg dry wt.) for female gametophyte tissue of Union Camp 10-1036 seed collected in Summer 1994. Sulfur, magnesium, phosphorous, and potassium shown by date of collection of female gametophyte tissue.

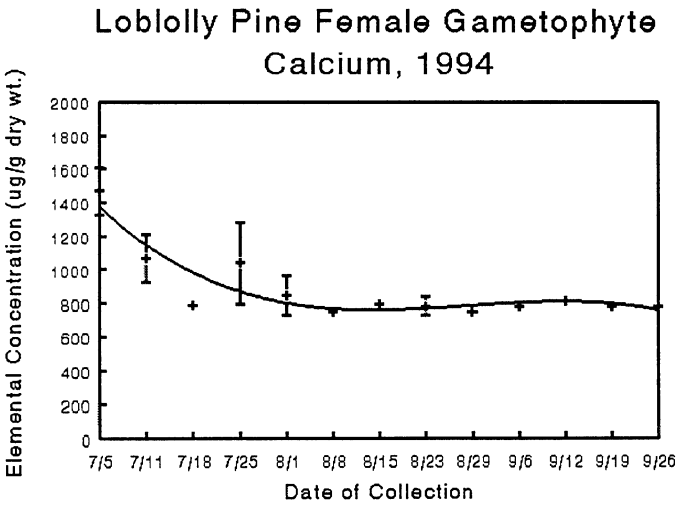


Figure 2. Elemental analysis (mg/Kg dry wt.) for female gametophyte tissue of Union Camp 10-1036 seed collected in Summer 1994. Calcium content shown by date of collection of female gametophyte tissue.

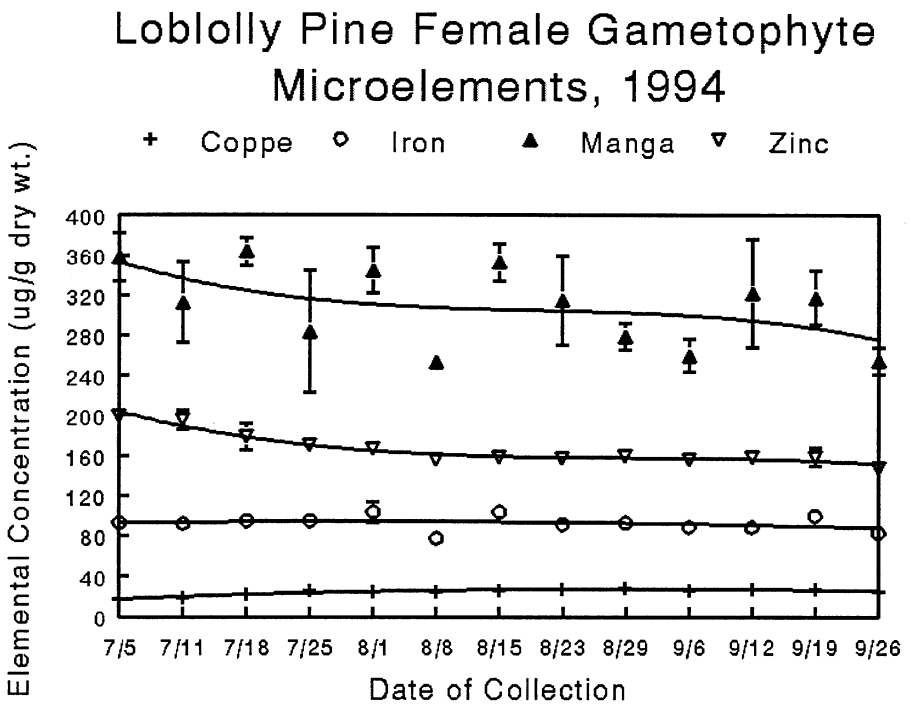


Figure 3. Elemental analysis (mg/Kg dry wt.) for female gametophyte tissue of Union Camp 10-1036 seed collected in Summer 1994. Copper, iron, manganese, and zinc content shown by date of collection of female gametophyte tissue.

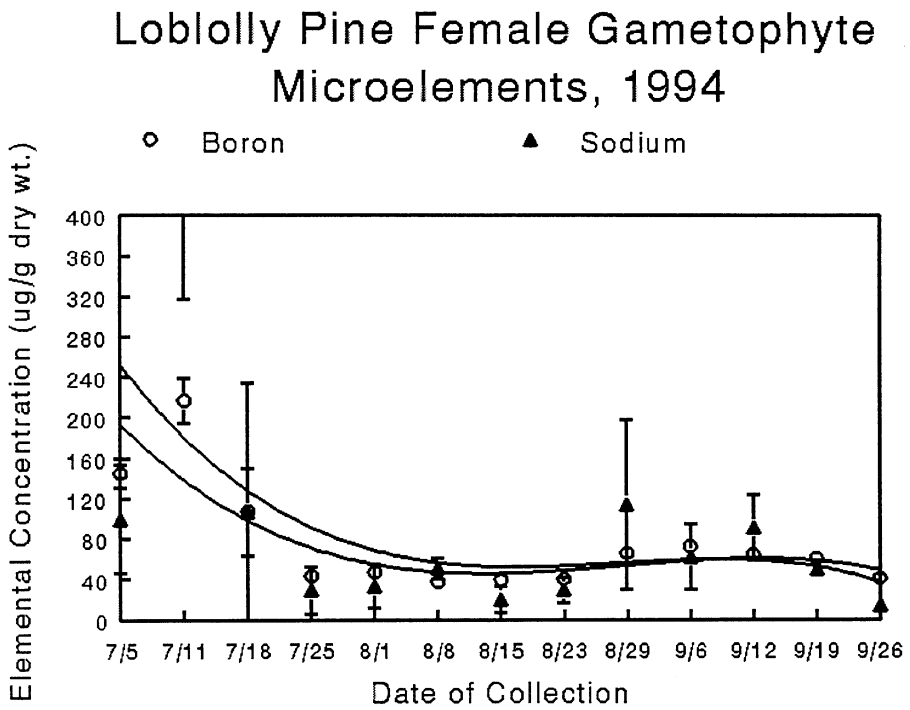


Figure 4. Elemental analysis (mg/Kg dry wt.) for female gametophyte tissue of Union Camp 10-1036 seed collected in Summer 1994. Boron and sodium content shown by date of collection of female gametophyte tissue.

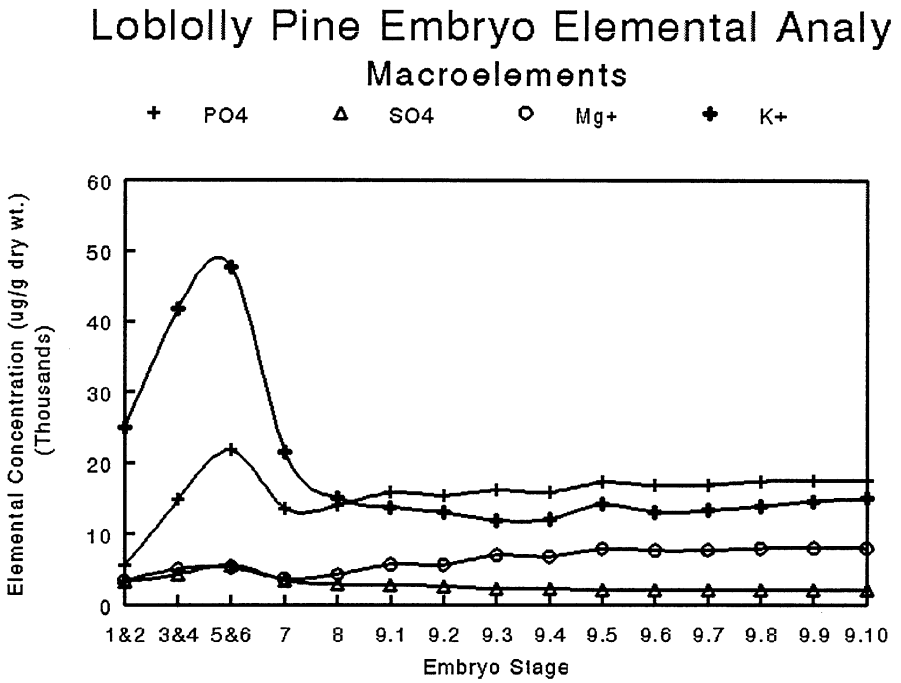


Figure 5. Elemental analysis (mg/Kg dry wt.) for zygotic embryo tissue of Union Camp 10-1036 seed collected in Summer 1994. Phosphorous, sulfate, magnesium, and potassium content shown by date of collection of embryo tissue.

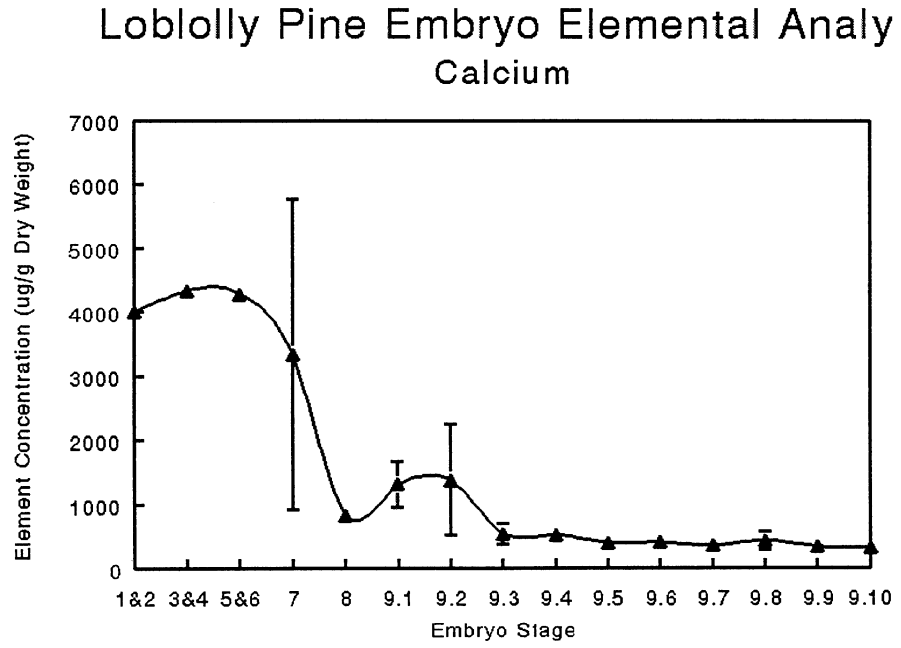


Figure 6. Elemental analysis (mg/Kg dry wt.) for zygotic embryo tissue of Union Camp 10-1036 seed collected in Summer 1994. Calcium content shown by date of collection of embryo tissue.

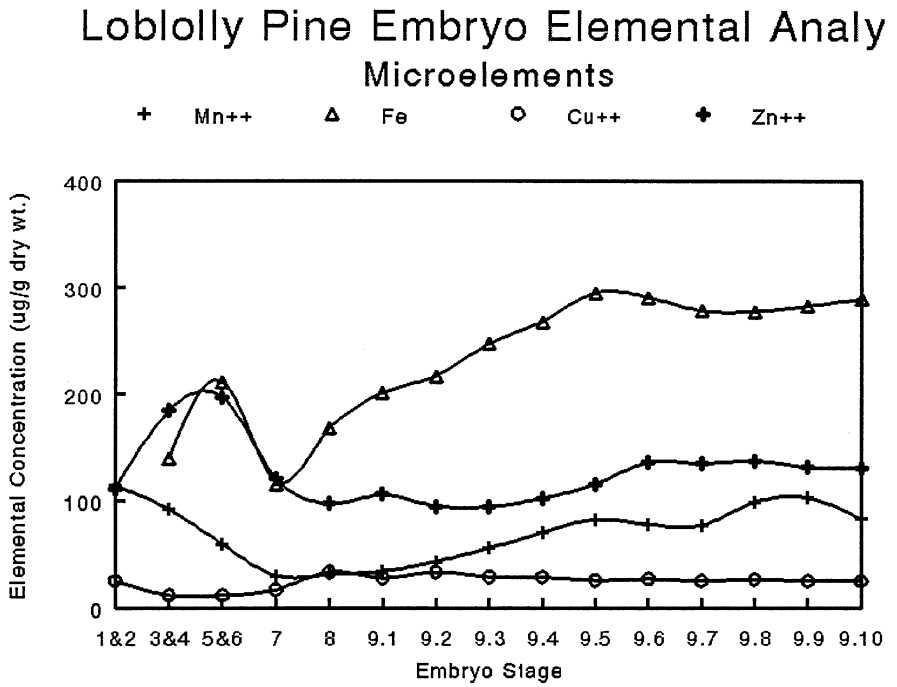


Figure 7. Elemental analysis (mg/Kg dry wt.) for zygotic embryo tissue of Union Camp 10-1036 seed collected in Summer 1994. Manganese, iron, copper, and zinc content shown by date of collection of embryo tissue.

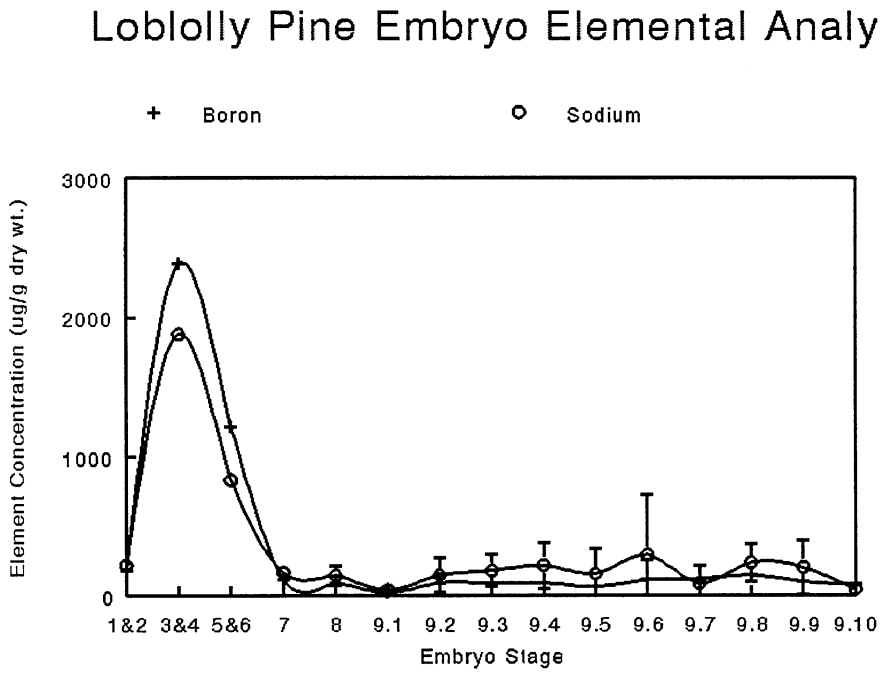


Figure 8. Elemental analysis (mg/Kg dry wt.) for zygotic embryo tissue of Union Camp 10-1036 seed collected in Summer 1994. Boron and sodium content shown by date of collection of embryo tissue.

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

Gerald Pullman
Xiaorong Feng
Paul Montello
Yolanda Powell

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 improvements in embryo yield have been documented due to reductions in boron and calcium in the development and maturation medium. Reductions in boron speeded embryo development and caused more embryos to develop. Calcium reductions also caused more embryos to be produced on development and maturation medium. A combination of ½ strength boron and ¼ strength calcium caused a repeatable statistically significant increase in yield with a marginal increase in visual embryo quality.

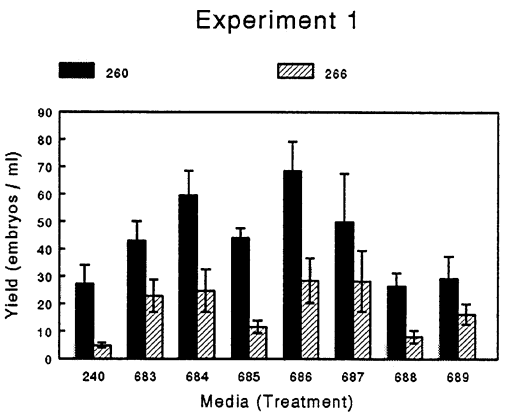
Introduction

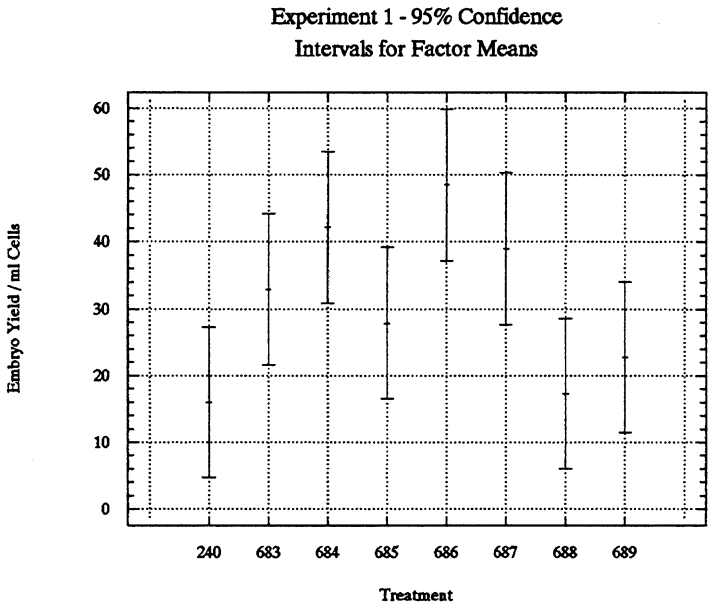
Table 1 shows a comparison of elemental analyses for zygotic and somatic tissues with the ratio of somatic / zygotics. Analyses of elemental composition of somatic embryos and comparison to zygotic targets show that somatic embryos contain 191 times the sodium, 17 times the boron, 3 times the calcium, 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 ongoing experiments was begun to tests modifications in the media to produce somatic embryos which better match zygotic embryo elemental compositions. The first experiments targeted the development and maturation step but experiments are also in progress or planned to modify the initiation, maintenance and germination media.

Materials and Methods

Experiment 1. Our standard maintenance medium 16 was used to produce early-stage embryo suspension cultures. One ml of settled cells was plated onto sterile black filter (not containing activated carbon) papers placed onto medium 240. Modifications of 240 were tested which contained altered levels of copper, iron, or boron. Results showed statistically significant increases in embryo yield with increasing copper and with lowering boron content. Lowering boron content of the development media also speeded embryo development. Embryos produced with 1/2 the boron content appeared normal while embryos produced with increased copper were often smaller in size.

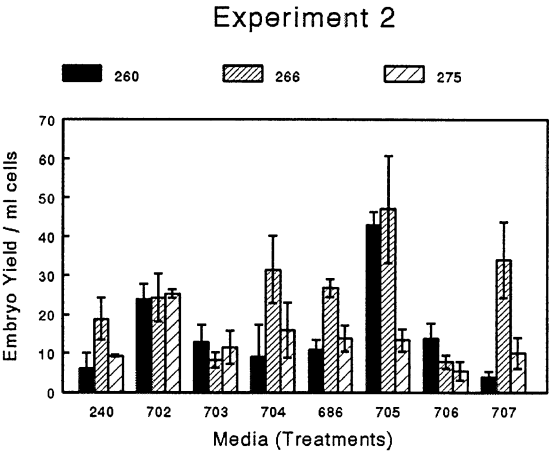
Media	Copper	Iron	Boron
240	1x	1x	1x
683	2x	1x	1x
684	5x	1x	1x
685	1x	2x	1x
686	1x	1x	1/2x
687	1x	1x	1/5x
688	1x	1x	1/10x
689	2x	2x	1/5x

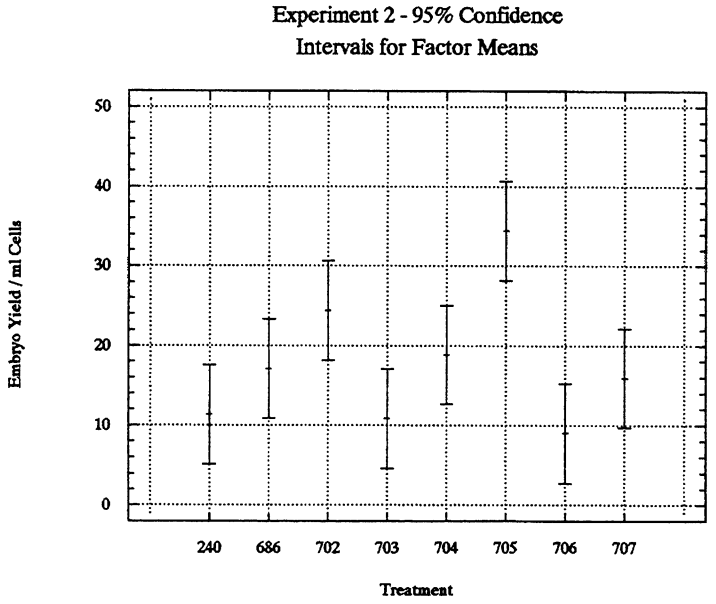




Experiment 2. With the results from experiment 1 suggesting that 1/2 boron was beneficial to speed embryo development a new experiment was set up to build upon this observation. Normal boron level or 1/2 boron were coupled with additional changes in calcium, magnesium, or phosphorous as suggested by the metal analyses. A statistically significant increase in yield over the control was seen with lowering calcium content or especially with lowering calcium combined with lowering boron. Although not significant, dry weight analyses of embryos showed that the lower boron / calcium treatment produced marginally heavier embryos.

Media	B	Ca^{++}	Mg^{++}	$P04^{-}$
240	1x	1x	1x	1x
702	1x	0.5x	1x	1x
703	1x	1x	1.5x	1x
704	1x	1x	1x	1.5x
686	0.5x	1x	1x	1x
705	0.5x	0.5x	1x	1x
706	0.5x	1x	1.5x	1x
707	0.5x	1x	1x	1.5x

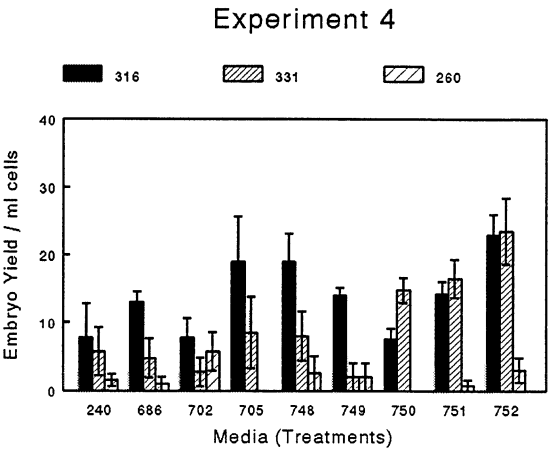




Experiment 3. This experiment followed up on the observation in experiment 1 that increasing the phosphorous increased embryo yield. Three genotypes were used to test three levels of boron (1x, 0.75x, 0.5x) combined in all combinations with three levels of phosphorous (1x, 1.5x, 2x). Embryo yield was not significantly increased above the control by any combination of boron or phosphorous.

Experiment 4. To confirm the statistically significant increases in yield by lowering boron and calcium together and to optimize the media composition for these elements, a experiment was designed to develop embryos using three levels of boron (1x, 0.75x, 0.5x) combined in all combinations with three levels of calcium (1x, 0.5x, 0.25x). Four genotypes were used in this experiment, one did not yield embryos.

Media	Boron	Calcium
240	1x	1x
702	1x	1/2x
748	1x	1/4x
749	3/4x	1x
750	3/4x	1/2x
751	3/4x	1/4x
686	1/2x	1x
705	1/2x	1/2x
752	1/2x	1/4x



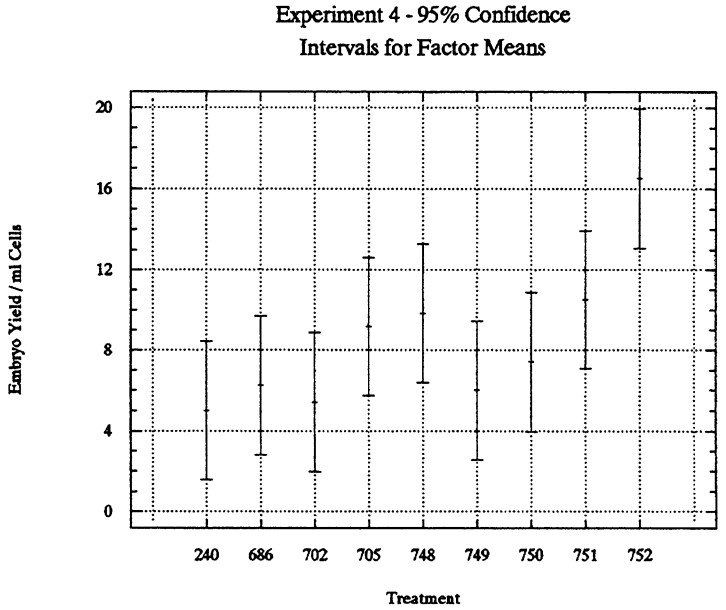


Table 1. Comparison of elemental compositions for zygotic female gametophyte, zygotic embryo, and somatic embryo tissues with along with the ratio for each element found in somatic / zygotic embryos.

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	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: 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.

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. This past winter 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.

As of October 2, 1997 the trees displayed 100% survival in the field. The average height of the 35 trees is 1.9 feet. The tallest tree is 2.6 feet and the shortest is 1.2 feet (photo taken late September 1997).

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

COMBINED EFFECTS OF ACTIVATED CARBON AND pH ON IONIC COMPOSITION AND 2,4-D AVAILABILITY IN A TISSUE CULTURE MEDIUM

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ABSTRACT

Activated carbon (AC) is added to tissue culture medium, often giving positive results. Two different activated carbons have been found to give different success rates in tissue cultures. The two carbons have been characterized using several different techniques with the goal of correlating character with impact on medium composition. Ash %, point of zero charge (PZC), and apparent surface area, showed the greatest differences. Medium pH was found to vary with AC type, and preparation technique. The combination of AC and medium pH (ranging from pH 4.8 to pH 6.8) could significantly alter the ionic composition of the medium, resulting in decreased Cu (-90%), Zn (-50%), Fe (-50%) and Mn (-60%) and increased Mg (+50%). The sorption of 2,4-D varied with apparent surface area (BET) and system pH: the combination of factors resulting in nearly 50% differences in sorption capacity. These findings may help to explain some of the contradiction in the literature regarding the benefits of including AC in tissue culture medium.

INTRODUCTION

The addition of activated carbon (AC) to plant tissue culture medium has been shown to benefit many different tissue culture systems, including algal cultures (1), anther cultures (2, 3), date palm cultures (4), and Douglas-fir and other conifer cultures (5). Several different hypotheses have been advanced to explain the observed benefits: adsorption of growth inhibitors (toxic metabolites) and prevention of unwanted callus growth (6), adsorption of breakdown components of sucrose (7), removal of excess hormone (2,4-D) (8) and release of substance (9) and the sorption of mineral nutrients (10, 11).

Anther culture research has shown that success may vary with the source of activated carbon (12). Work with Douglas-fir somatic embryogenesis at IPST (unpublished results) has revealed that acid-washed activated carbon performs differently from non-acid washed activated carbon. Further work at IPST using a Norway spruce somatic embryogenesis system has shown that success may also vary with AC production lots.

Research at IPST has shown that the presence of AC in media used for initiation of loblolly pine results in a 50% decrease in available zinc and a 90% decrease in available copper (13). The sorption of 2,4-D onto a single activated carbon was modeled, allowing predictions of available 2,4-D based on initial relative concentrations of 2,4-D and AC (13).

The physical and chemical properties of AC derive from its extensive surface area, which may include a high percentage of micropores (pores less than 2 nm) and its surface chemistry, which is primarily determined by functional groups containing oxygen. Activated carbon is produced through oxidation of pyrolyzed material, which is subsequently pulverized. This oxidation may be achieved with acids, typically nitric or phosphoric, or using high temperature treatments under an oxidizing atmosphere, commonly steam or CO_2. The surface may be either acidic or basic (14).

In this on-going study we seek to correlate the physical/chemical characteristics of activated carbon with the impact of AC on the medium composition, and subsequently, its impact on culture success. This paper reports results for two different activated carbons for the sorption of 2,4-dichlorophenoxy acetic acid (2,4-D) from aqueous solution as well as AC impact on cation concentrations in an initiation medium (Norway spruce).

EXPERIMENTAL

Characterization of Activated Carbon

Activated carbon was supplied by Sigma as untreated powder (C-5260), designated "N" type, and acid-washed tissue culture tested (C-9157), designated "T" type. Two production lots, designated "1" and "2", for each type were characterized. For each characterization, unless stated otherwise, at least two replications were performed.

Ash % was determined on dry AC (dried using a vacuum oven at 120°C overnight) through thermo-gravimetric analysis. The point of zero charge, PZC, was approximated through mass titration (15) which simply involved adding an increasing mass of AC to a given mass of water until some limiting pH value was approached. Apparent surface area (BET) was determined though nitrogen sorption (Micromeretics Flowsorb II 2300).

2,4-D Sorption

Experiments were performed using sealed 40mL glass vials or 200mL plastic bottles. To reach pH targets, acid solution (0.01N or 0.1N HCl) was first added to AC and allowed to equilibrate. The proper amount of acid had been previously determined through titrations and trial and error. Phosphate buffer, adjusted to the final pH target, was added such that the final diluted concentration was approximately 0.0125M. Stock solution of 2,4-D (1g/L) was added to give a final concentration of 200mg/L (200ppm). All aqueous phases were mixed using degassed, deionized water. All vessels were shaken for the duration of the experiment. Experiments were performed in duplicate.

Following sorption, typically longer than five days, samples (10mL) were taken from the vessels and passed through a syringe filter (Gelman Acrodisc 0.2 μm, HT Tuffryn® membrane). The first 1.5 mL were discarded. The 2,4-D concentration was measured at 284nm (Beckman DU 640 Spectrophotometer).

Cation Analysis

Ion analyses were performed on complete and partial media. Experiments were performed in duplicate. The medium consisted of a liquid version of the Institute of Paper Chemistry Norway spruce initiation medium (16), with 2,4-D (2ppm) substituted for NAA, and BAP (1ppm) as specified previously. When activated carbon was present (1.25 g/L), the hormone levels were elevated to 90ppm BAP and 100ppm 2,4-D. Media were typically allowed to equilibrate three days at room temperature (21°C) before being analyzed. Samples (approximately 7.5mL) were collected as per the 2,4-D procedure. Two drops of concentrated nitric acid were added to each sample. The samples were then analyzed for cations using inductively coupled plasma atomic emission spectroscopy (ICP-AES, Perkin Elmer Optima 3000 DV). Each ICP measurement was replicated three times.

RESULTS

Characterization of AC

Mean values for the characterization results are summarized in Table 1. The ash percentage varied significantly between AC types, with N1 and N2 containing more than twice as much ash percent as T1 or T2. Significant variation in ash % was evident between batches 1 and 2 for the N-type AC. Analysis for trace metals (SEM-EDS) failed to detect significant differences in atomic species present in the ash for the different AC samples: the release of toxic metals does not appear to be a concern when using these AC's.

Table 1. Summary AC Character

	Ash %	PZC, pH	BET m2/g
T1	2.8	8.5	1050
T2	2.6	10	940
N1	5.8	10.7	560
N2	8.7	11	710

The point of zero charge (PZC) characterization gives the pH at which the sum of the charges on a particle suspension is equal to zero. This measurement differs from the isoelectric point (IEP) in that the isoelectric point is determined from moving particles: the internal surface is not important to IEP determinations. The PZC data indicate that the T1 and T2 are less basic than N1 and N2. Since the pH scale is logarithmic, a pH unit of difference is quite significant at pH 10. It can be concluded that the carbon types are significantly different with respect to PZC. The difference between T1 and T2 indicates that production lots may vary significantly.

The apparent surface area ranged from 560 m^2/g to 1050 m^2/g. Comparing the average of T1 and T2 to that for N1 and N2, it may be seen that the difference due to carbon type was approximately 350m^2/g. The greatest difference was between T1 and N1. These were the two carbons which have been used in tissue culture media at IPST. The between-batch variation, i.e. T1 vs. T2, and N1 vs. N2, appears to be significant.

Culture Medium pH

As a result of the AC characterization, experiments were conducted in which media were mixed, allowed to equilibrate two days and pH measurements made. The two day period was representative of what might occur in actual lab practice.

According to our procedure at IPST, the medium pH was adjusted after adding AC, prior to autoclaving. In this case, the target pH was 5.8. As can be seen from Figure 1, the amount of time allowed for pH adjustment influenced the pH of the medium after two days. Referring to the figure, the x-axis is labeled according to the amount of time allowed to adjust pH after addition of AC to the medium. For the media labeled, "0 min", the pH was adjusted prior to AC addition with no further adjustment after AC addition. As more time was allowed for pH adjustment after AC addition, the closer was the final pH to the target. The tendency, however, was for the pH to drift in the basic direction when AC was present.

After two days, N1 gave higher pH for each treatment, approaching pH 6.8 when no time was allowed for pH adjustment. Past experience has shown that the pH of the

medium just after autoclaving is typically below 5.8, about 5.6 for T-type and above 6.5 for N-type. Titrations with acid have revealed that ten times as much volume is required to reach a given pH point for N-type vs. T-type carbons. Also, it has been found that pH drift after the titration end point has been reached is much more significant for the N-type carbons: it is much more difficult to obtain pH 5.8 with the N-type carbons.

For media exposed to a growing Norway spruce culture, the medium pH drops to about 4.8 over a period of days or weeks. This phenomenon has been observed for media with T-type AC and for media without AC. When N-type carbons have been used, this drop tends to be much more gradual and doesn't dip below pH 5.

Therefore, the pH range of interest for this research is from pH 4.8 to pH6.8.

Sorption of 2,4-D

pH effect.

Data were collected at room temperature (21°C) for sorption of 2,4-D (200mg/L, 200mL volume, phosphate buffer) onto AC (0.125g/L) over the pH range of 4.8 to 6.8. These relative concentrations were chosen after initial experiments revealed that 2,4-D was being depleted at ten to twenty times more than what had been expected based on previous literature (17). It was found that equilibrium was reached within two days but typically more than five days were allowed before measurements were taken. These data have been normalized to sorption capacity at pH 6.8 and are presented in Figure 2. It can be seen that there was a trend for both carbons towards greater sorption of 2,4-D as the pH declined from 6.8. In fact, at pH 4.8 vs. 6.8 the sorption capacity has increased by more than 40% for both carbons.

Apparent surface area.

Using the same relative concentrations of 2,4-D and AC as the previous experiments and adjusting pH to approximately 6.8 for each solution, sorption data were generated for each of the four activated carbons. These data were then plotted as a function of apparent surface area in Figure 3.

The two samples per AC type show a good deal of spread in their sorption capacity, indicating a high level of variability in the material. The mean trend is that sorption capacity for 2,4-D increases with apparent surface area. Comparing mean values for T1 and N1, the difference is about one third more sorption onto T1.

Ion experiments.

Equilibrium conditions were reached within a day but typically two days were allowed before samples were analyzed. Data were initially produced for thirty ions. However, with the exception of silica, neither N1 nor T1 were releasing measurable quantities of non-nutrient ions. The media at pH 4.8 were visibly clearer than media at higher pH. After autoclaving, a precipitate was visible in media with pH above 5.8. The precipitate could be avoided by omitting the macronutrients from the medium.

Figures 4 and 5 present data for copper and zinc, respectively. The concentration of each ion is depicted as a function of medium pH. Media without carbon are designated "Con" short for "control" and were otherwise identical to media which included AC. Across the top of each figure a line is drawn defining the concentration of ion calculated to be present based on the medium "recipe".

From the data in Figure 4 it can be seen that more than 90% of the copper was depleted compared to the control. The control levels were in good agreement with the calculated values. There appeared to be a slight increase in copper availability as pH increased. The data for the two different AC's showed no difference.

Figure 5 shows that the control responded to changes in pH. About 50% of the zinc was depleted in response to a pH increase from pH 5.8 to pH 6.75, without AC present. The presence of AC resulted in reduced availability of zinc compared to the control across the pH range of 4.8 to 6.75. The scatter in the data prevented conclusions as to whether there was an effect due to AC type.

Figure 6 presents data for iron. All of the media were low in iron relative to the calculated amount, across the pH range studied. This phenomenon has been observed previously and attributed to the precipitation of phosphate and Fe-EDTA (18). Relative to the amount of iron present at pH 4.8, about 50% was depleted as pH increased to 6.8. The control sample showed higher precipitation than the activated carbon samples.

Data for magnesium are depicted in Figure 7. Only the media with N1 showed significant deviation from the calculated value: N1 added about 50% more magnesium relative to the calculated value. A pH effect was not observed in the control or for media containing T1.

Manganese showed a very large pH-dependent drop (Figure 8): more than 60% was depleted as pH increased from 5.8 to 6.8. This drop was independent of the presence of AC.

Molybdate was reduced more than 50% across the pH range, independent of AC. Cobalt was reduced to levels near zero and its importance to the medium needs further investigation.

CONCLUSIONS

The T type activated carbons are different physically and chemically from the N-type, with at least 50% more apparent surface area, half as much ash % and a PZC which is 1 pH unit lower than the N-type carbons. There are also differences between the production lots within either type, most notably for BET surface area.

The pH of the medium may have a profound impact on the medium composition. Medium pH is influenced by AC type and how much time is allowed for pH adjustment prior to autoclaving the medium.

The sorption of 2,4-D onto AC appears to be a function of the apparent surface area and the pH of the medium. The combination of effects when comparing media with T1 to media with N1 results in about 40% more 2,4-D sorption capacity for T1 (over 40% more 2,4-D available in media with N-type AC).

Several ions showed increased availability as the pH of the medium declined from 6.8 to 4.8: P (+33%), Mn (+200%), Fe (+140%), Zn (+190%).

Including AC in the medium results in the increased availability of several ions. A greater effect was observed for N1, with increased Si (+30%), Mg (+50%), Ca (+15%), and Al (+300% approx.).

Only two ions appeared to be sorbed onto AC : Cu (-90%) and Zn (-50%). When AC was present, the availability of Cu increased as pH increased.

Figure 9 summarizes a few of the changes in medium composition which would result from substitution of N1 for T1. The data have been normalized against a medium containing T1 at pH 5.8.

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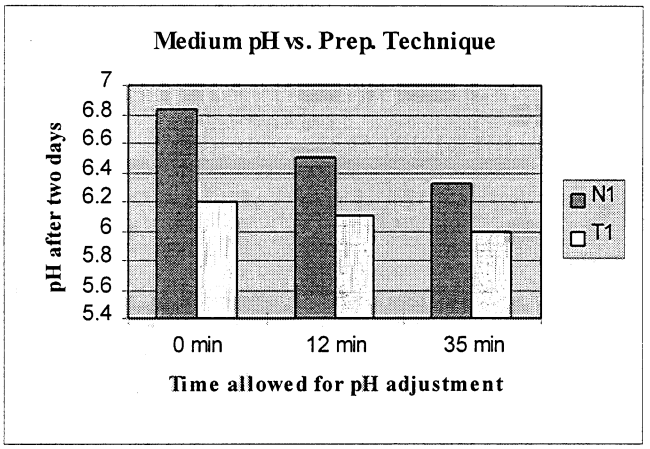

Figure 1. Medium pH as a function of the time allowed for pH adjustment after the addition of activated carbon (1.25 g/L). Media have been adjusted to pH 5.8 and then autoclaved and stored for two days prior to measurement.

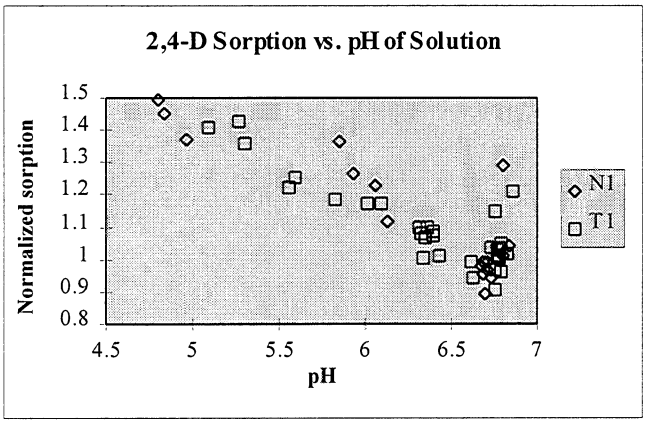

Figure 2. Sorption of 2,4-D (initial conc. 200ppm) onto activated carbon (0.125 g/L) at room temperature from buffered aqueous solution. The data for each carbon type were normalized to their respective mean values at pH 6.8.

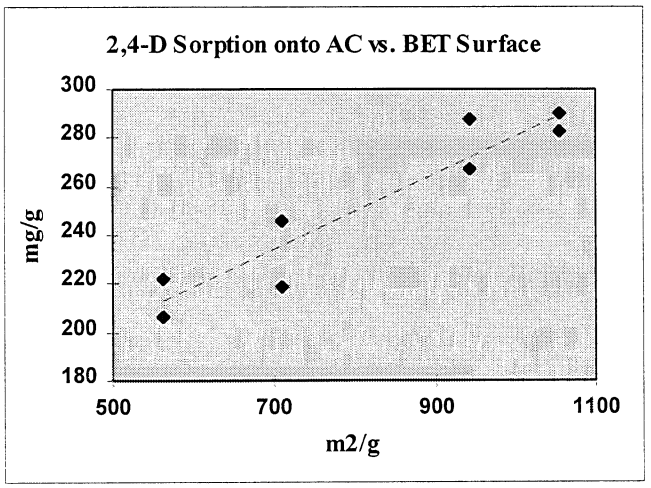

Figure 3. Sorption capacity for 2,4-D as a function of BET surface area of activated carbon. The experimental conditions were: 2,4-D, 200 ppm; AC, 0.125 g/L; pH 6.8; room temp. (21°C). The range in apparent surface area for the four different activated carbons is presented in Table 1.

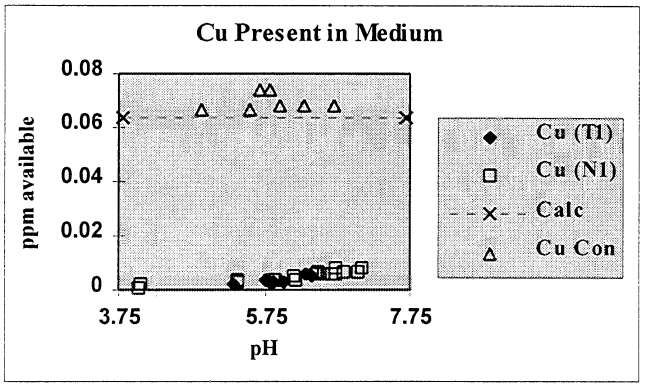

Figure 4. Copper availability vs. medium pH. Data were produced using ICP. Media were complete, including 2,4-D at 100ppm and BAP at 90ppm. Experiments were conducted with one production lot of each AC type, designated T1 and N1. "Calc" designates the amount of ion expected to be present. The control, designated "Con", contained no AC but was otherwise identical to media with AC.

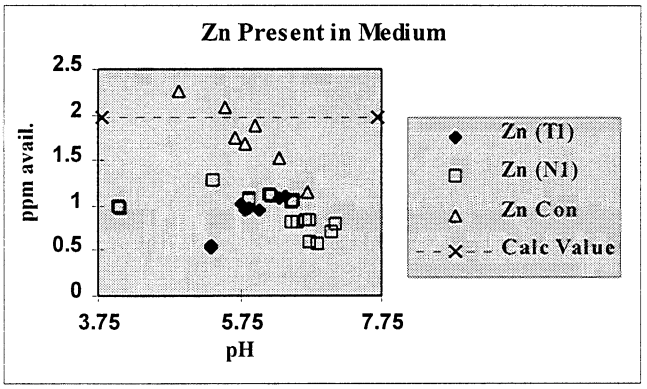

Figure 5. Zinc availability vs. medium pH. See caption for Figure 4.

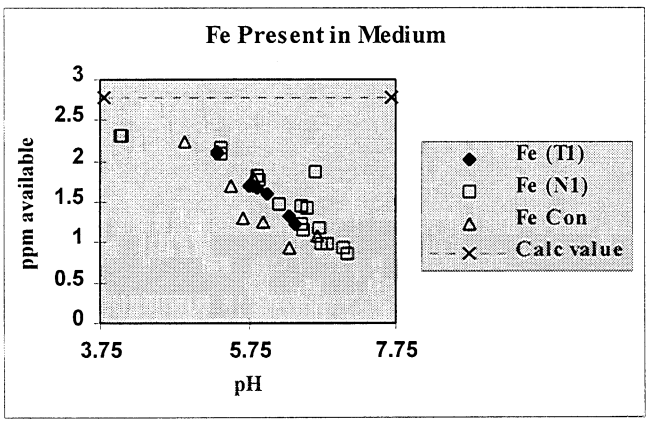

Figure 6. Iron availability vs. medium pH. See caption for Figure 4.

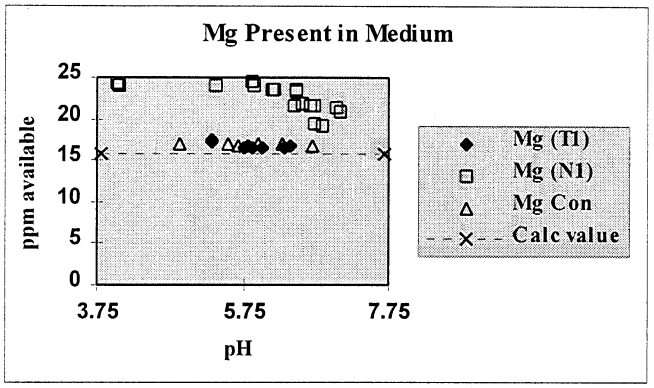

Figure 7. Magnesium availability vs. medium pH. Data were produced using ICP. Media were complete, including 2,4-D at 100ppm and BAP at 90ppm. Experiments were conducted with one production lot for each AC, designated T1 and N1. "Calc" designates the amount of ion expected to be present. The control, designated "Con", contained no AC but was otherwise identical to media without AC.

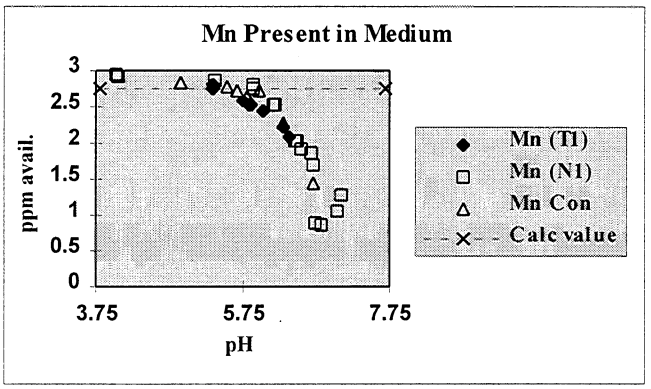

Figure 8. Manganese availability vs. medium pH. See caption for Figure 7.

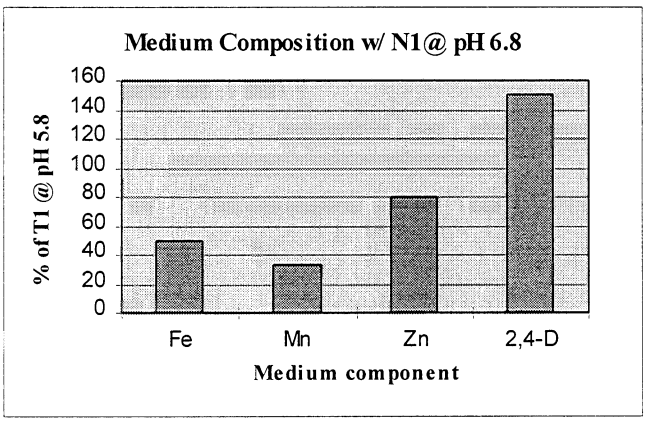

Figure 9. Impact on medium composition of substituting N1 for T1, including pH effects. Data have been normalized to expected medium composition when T1 is present at pH 5.8

F010: MASS CLONAL PROPAGATION OF SOFTWOODS

Functional Analysis of Zygotic Embryo Germination

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Chantal Murenzi
Jennifer Langstraat
Gerald Pullman

SUMMARY:

In previous studies at IPST comparisons of the fresh weight, dry weight, and patterns of gene expression between zygotic and somatic embryos, suggested that our somatic embryos develop to stages 8-9.1 In order to compare in a more functional way zygotic and somatic embryos, we tested the ability of immature zygotic embryos to germinate. The results show a parallel between the existence of a functional root meristem and germination. They also indicate that our somatic embryos develop only to stages 7-8. This suggests that early stages of somatic embryos development are occurring parallel to zygotic embryo development and that no major developmental step is missed. The results also suggests that our somatic embryos are not mature enough for normal germination, and that root growth appears to limit normal germination both in immature somatic and zygotic loblolly pine embryos.

INTRODUCTION:

In previous studies at IPST comparisons of the fresh weight, dry weight, and patterns of gene expression between zygotic and somatic embryos, suggested that our somatic embryos develop to stages 8-9.1. However, the somatic embryos that germinate do so in reverse sequence compared to 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. In order to compare the developmental potential zygotic and somatic embryos, we tested the ability of immature zygotic embryos to germinate. To determine when immature zygotic embryos acquire the capacity for normal germination we isolated embryos from stages 5-9.2 and placed them on germination media. From this functional study of zygotic germination capacity we can determine the developmental stage and the size and dry weights of immature zygotic embryos that are competent to germinate. This identifies a target for somatic embryo development. It also allows us to compare these results of immature zygotic embryos to the germination results from our somatic embryos that are also immature and obtain an estimate of somatic embryo quality. The results indicate that our somatic embryos develop to stages 7-8. The results also suggest that in our somatic embryos early stages of development probably occur normally and no major developmental step is missed.

EXPERIMENTAL APPROACH AND DESIGN:

Genotype UC 5-1036 was used this summer for mass embryo collections for initiation experiments. To perform germination tests, ovules were surface sterilized and the embryos were surgically isolated. Up to ten embryos from each stage were placed on one germination plate. This germination media

did not contain exogenous hormones. Following our normal germination protocol, the embryos were then cultured in the dark for 7 days and subsequently shifted to continuous white light. After 6-7 weeks in the light the embryos were scored for the presence of roots and shoots under the dissecting microscope. An embryo was considered to have germinated when it contained both a root and a shoot. The hypocotyl and root lengths were measured when possible.

RESULTS:

Sterilization and Isolation Techniques Are Not Lethal.

Most embryos survived the sterilization and isolation procedure. Seeds were sterilized as for initiation experiments. Figure 1 shows that the most sensitive embryos were from stage 5, but still 80% of the embryos survived and continued to grow on hormone free media. In addition, the more mature the embryos were before isolation the greater their rate of survival, with 100% of the stage 9.2 embryos surviving (Figure 1).

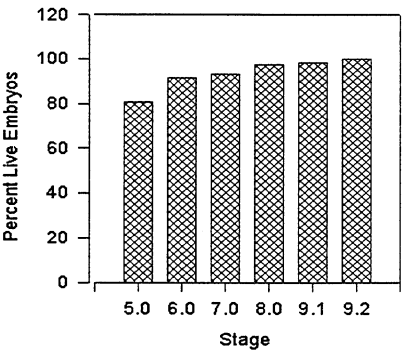


Figure 1

Cotyledon Development: The Number of Cultured Zygotic Embryos with Abnormal Cotyledons Decreases with Increasing Maturity

Zygotic embryos isolated at stage 5 showed the greatest percent (40) of embryos that formed abnormal cotyledons (Figure 2). The embryos that formed abnormal cotyledons were small and the axis was reduced. Cotyledons were abnormal either when the number of individual cotyledons was reduced or their morphology was altered. Mostly this was detected as fleshy and fused cotyledons (Figure 3, next page). By definition, stage 5 embryos have a shoot apical meristem or dome but no cotyledonary primordia are visible with the dissecting microscope. Thus, it was surprising that 60% of the zygotic embryos from this early stage of development when cultured on plates without hormone, developed normal cotyledons. One possible explanation for this is that microscopic cotyledonary primordia were present but not detectable with the dissecting microscope used. For stage 6 embryos, the earliest stage with cotyledonary primordia are visible, 84% formed normal cotyledons on our germination plates. About 85% of the stage 7-9.1 embryos formed normal cotyledons. Thus, once cotyledonary primordia become visible most are capable of normal growth and greening on our germination plates.

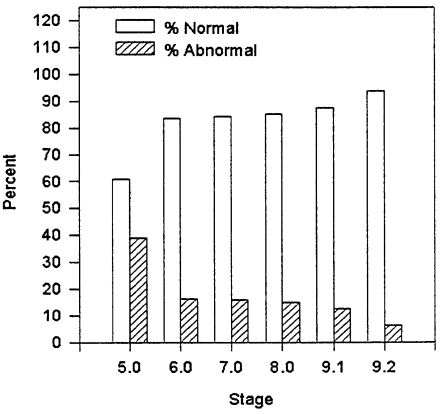


Figure 2

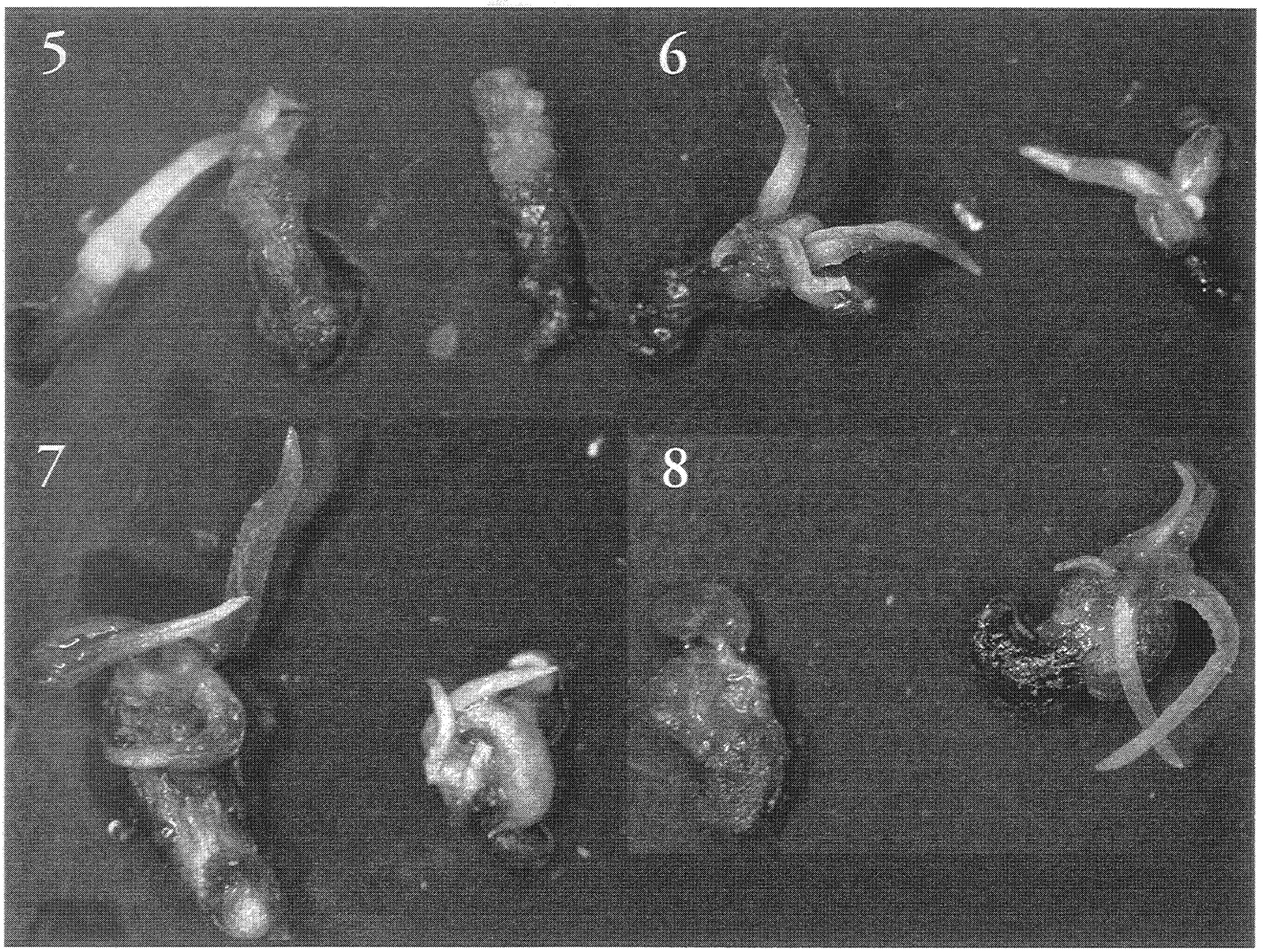
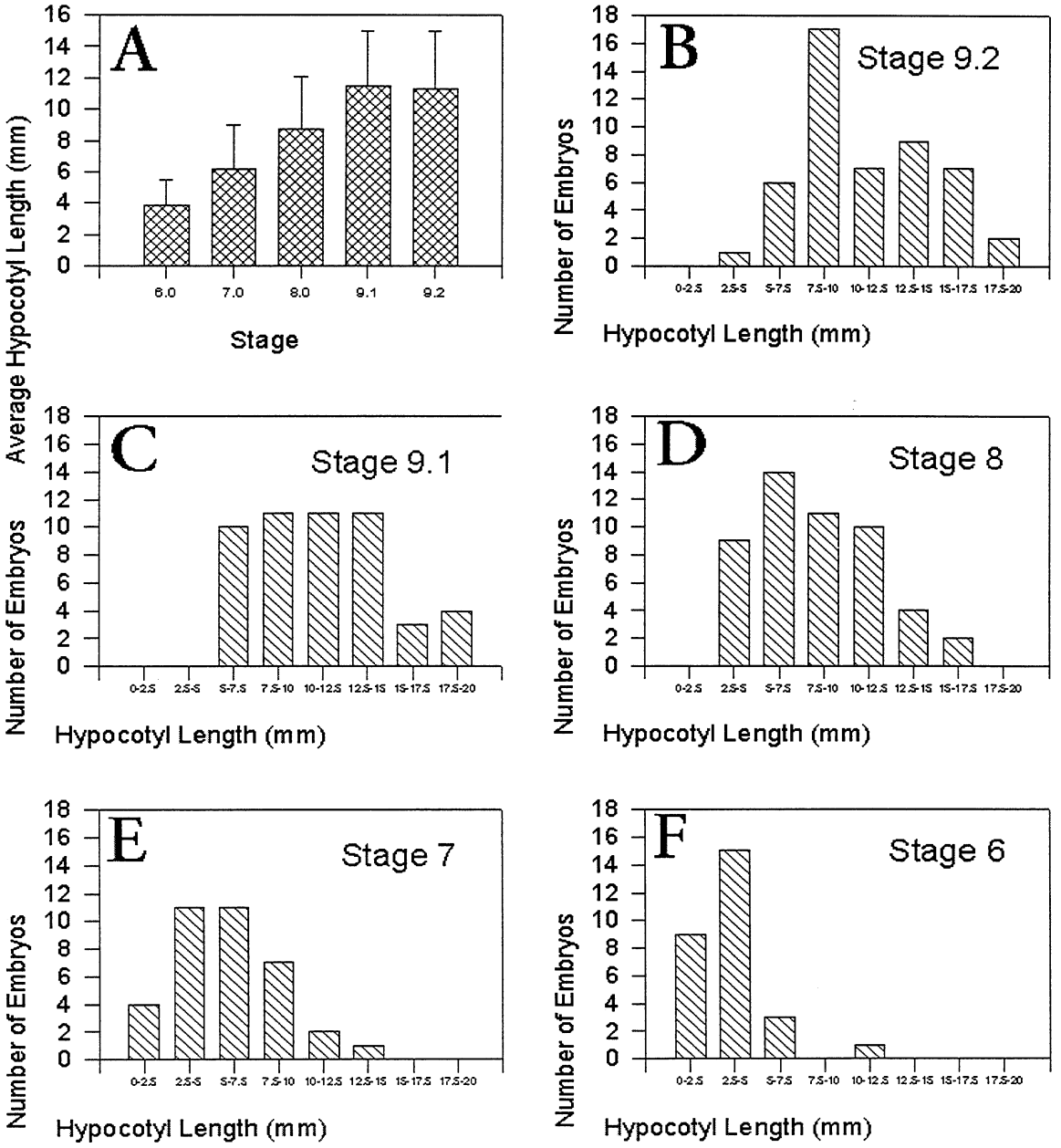


FIGURE 3

Hypocotyl Growth Increases with Maturity.

Zygotic embryos harvested later in development showed greater hypocotyl elongation than zygotic embryos harvested at earlier stages (Figure 4). For example, although shoots formed in ~50% of the stage 5 embryos (next page), the hypocotyls did not elongate. The hypocotyls began to elongate in stage 6 and 7 embryos (Figure 4F). From stages 6-9.2 the average hypocotyl lengths increased (Figure 4A) as did the distribution of longer hypocotyls (Figure 4B-E).

FIGURE 4



Development of a Functional Shoot Occurs as Early as Stage 5 and Possibly Before.

About 50% of the stage 5 embryos had formed a functional shoot meristem that developed after transfer to germination plates (Figure 5). This indicates that a functional shoot apical meristem is formed at or before stage 5. Our staging system defines a stage 5 embryo as one with an apical dome but without cotyledonary primordia; therefore shoot meristem development is probably complete when the dome is visible as evidenced by the autonomy of the shoot meristem to produce leaves without exogenous hormones. Why only ~50% of the embryos formed a functional shoot is unclear. However, 18% of the embryos formed abnormal cotyledons and none of these produced shoots. Of the stage 6 zygotic embryos, 63% formed functional shoots. This indicates that the apical meristem in at least half of the immature zygotic embryos does not require exogenous hormone treatment for it to be functional.

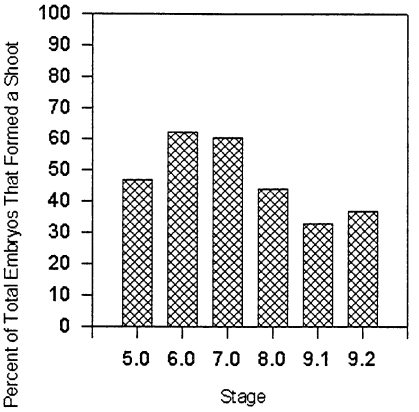


Figure 5

Development of a Functional Root Begins at Stage 7

No embryos from stage 5 and only 2 from stage 6 formed a root after harvest and transfer to germination plates. In contrast, at stage 7 ~18% of the zygotic embryos formed a functional root. The percent of embryos that formed a root increased with stage (Figure 6). Thus, functional roots are formed beginning at stage 7. Note this does not mean that the roots are not anatomically present at earlier stages. Roots may be present but have yet to acquire the ability or autonomy to germinate and grow.

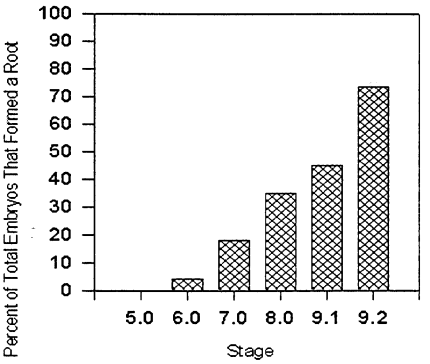


Figure 6

Root Growth Increases with Maturity

The average root length is relatively low for embryos harvested at stage 7 (Figure 7A, see next page). However, at stage 8-9.2 the average root length (Figure 7A) and the length distributions are similar for most roots (Figure 7B-D; see next page). One possible reason for the shorter roots at stage 7 is that the embryos took a longer time to germinate. Alternatively the roots might not be so competent for growth.

Zygotic Embryo Germination Begins at Stage 7 and Increases with Maturation.

The percent of embryos that germinate, i.e., form both a root and a shoot increases with stage (Figure 8). Figure 9 shows representative germinated embryos from each stage. Of the 48 stage 6 embryos only 1 germinated. Starting at stage 7 significant numbers of embryos germinated and the percent that germinated increased with stage. This confirms the straightforward notion that the stage to which a zygotic embryo develops is important for it's ability to germinate. More importantly it gives us atarget stage of maturity to aim for in developing maturation protocols.

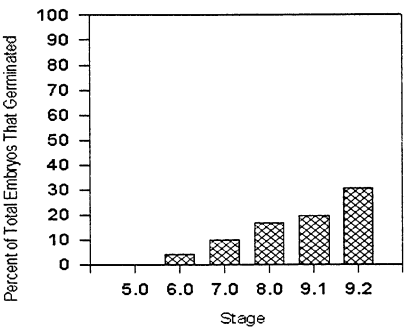


Figure 9 shows representative embryos that germinated (See next page).

FIGURE 7

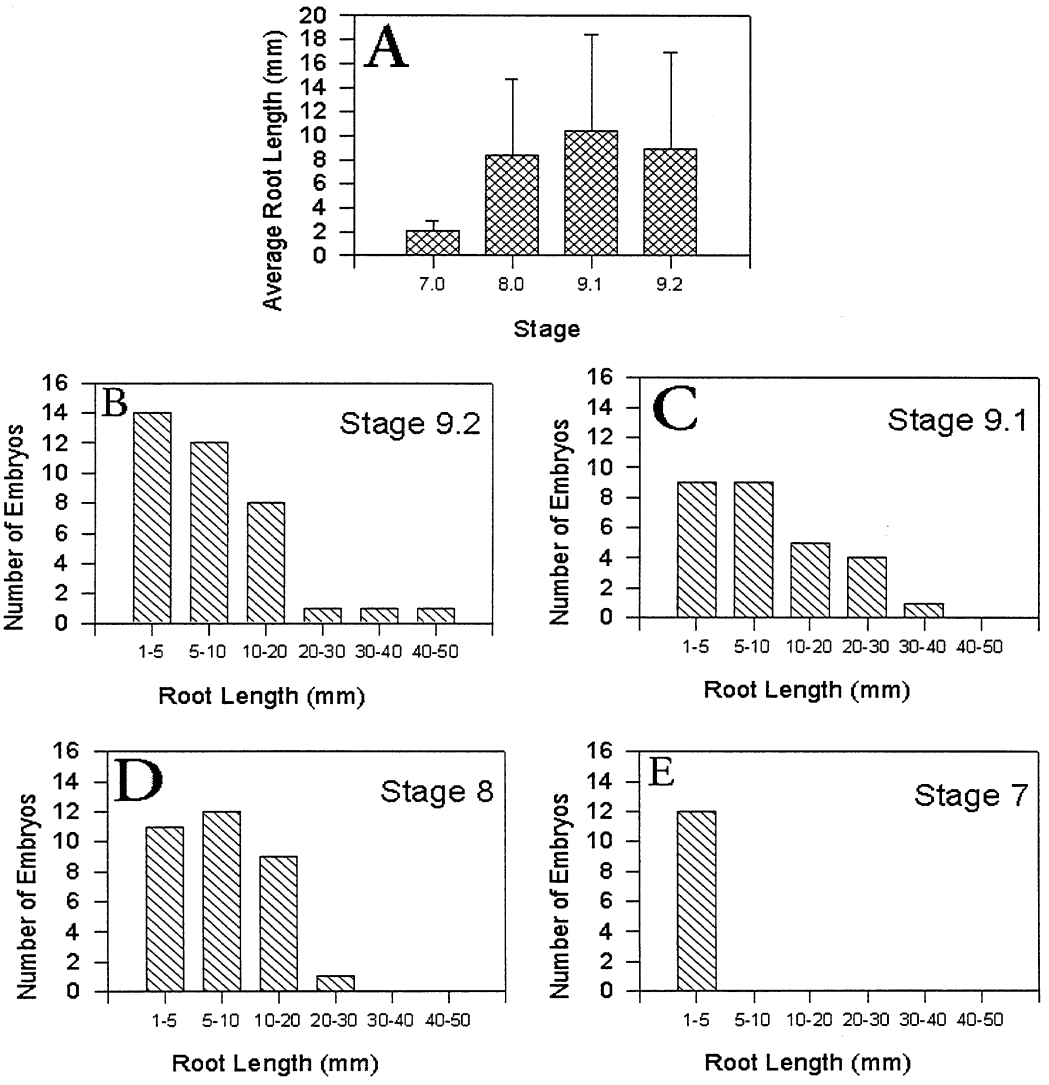


Table 1. Summary of averages of replicated elemental analysis of zygotic embryos collected from loblolly pine seeds grown on different mother trees and in different locations.

Tree	Location	Reps	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
BC-1	Lake Charles, LA	5	89.4	258		3.4	28.7	146	6.0	17053	2747			7671	12678	173
UC5-1036	Bellville, GA	4	90.6	279		1.0	24.6	133	8.7	17432	2619			8122	13092	275
7-56	Summerville, SC	3	66.4	221		1.0	22.7	113	1.2	15440	2242		7.1	7352	11552	140
UC10-14	Rincon, GA	2	76.7	181			31.0	113	2.2	14883	2312		6.6	7283	11219	174
UC10-1003	Rincon, GA	1	82.1	215	<.40	<.59	30.0	147	<.36	16421	2410	<.52	<3.23	7618	11833	146
Mean		15	83.0	243	####	2.1	26.8	132	6.2	16500	2531	####	6.6	7672	12312	192
Std Error		15	3.0	9.7	####	0.3	0.8	4.3	1.0	282	57	####	0.3	99	213	28
Std Er/Mean			0.04	0.04	####	0.16	0.03	0.03	0.16	0.02	0.02	####	0.05	0.01	0.02	0.15

Table 2. Summary of averages of replicated elemental analysis of female gametophyte tissue embryos collected from loblolly pine seeds grown on different mother trees and in different locations.

Tree	Location	Reps	Mn	Fe	Co	Ni	Cu	Zn	B	P	S	Mo	Na	Mg	K	Ca
BC-1	Lake Charles, LA	5	243	74		3	20	127	28	12415	5867			5019	9410	203
UC5-1036	Bellville, GA	4	233	82			22	143	21	13525	5547			5453	9744	344
7-56	Summerville, SC	3	121	53			16	126	17	10804	4633		4	4621	7843	270
UC10-14	Rincon, GA	2	251	79			18	155	19	11981	5430		4	5435	9102	150
UC10-1003	Rincon, GA	1	287	66	<.35	<.55	21	193	12	12348	5465	<.45	<2.83	5129	9460	311
Mean		15	221	72	####	2.6	20	139	22	12327	5450	####	3.9	5118	9148	254
Std Error		15	14.8	3.1	####	0.1	0.6	4.9	1.5	256	120	####	0.2	88	199	21
Std Er/Mean			0.07	0.04	####	0.03	0.03	0.04	0.07	0.02	0.02	####	0.05	0.02	0.02	0.08

Germination: A Fundamental Transition Occurs between Stages 8 and 9 of Zygotic Development.

At stage 9.1 and 9.2 more embryos that had a root only were observed than ones with a shoot only or that germinated (Table 1 and Figure 10). This is in striking contrast to embryos isolated at stages 6 and 7 in which only shoots grew and where very few roots were formed (Table 1 and Figure 10). Stage 8 had only slightly more embryos that formed only shoots versus ones that formed only roots or that germinated (Table 1 and Figure 10). This suggests that in embryos at stage 9.1 and beyond the root meristem is activated first upon germination. This is a the natural sequence to mature zygotic embryo germination from seed. It also shows that a developmental transition occurs in the ability of the shoot to form first during zygotic embryo germination.

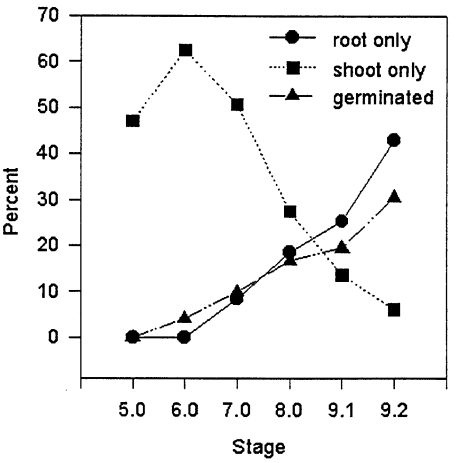


Figure 10

TABLE 1

Stage	Total # of Live Embryos	# of Embs with Roots Only	% of Embs with Roots Only	# of Embs with Shoots Only	% of Embs with Shoots Only	# of Embs Germinated	% of Embs Germinated
9.2	49	21	42.3	3	6.1	15	30.6
9.1	118	30	25.4	16	3.6	23	19.5
8	113	21	18.6	31	27.4	19	16.8
7	71	6	8.5	36	50.7	7	9.86
6	48	0	0	30	62.5	2	4.2
5	51	0	0	24	47	0	0

DISCUSSION:

A number of interesting observations were obtained from this study. One, root apical meristem autonomy develops after the shoot apical meristem autonomy during zygotic embryogenesis in loblolly pine. Two, when the root meristem does develop its autonomy it becomes the first organ to emerge from the embryo upon germination. Three, there is a parallel between the formation of a functional root and germination. Thus, root growth appears to limit normal germination both in immature somatic and zygotic loblolly pine embryos. Four, most of our somatic embryos form shoots first and then only a few much later form roots. This suggests that our somatic embryos are not mature enough for normal germination. Since we observe both shoot growth and hypocotyl growth in somatic embryos, these organs of the embryo develop normally. This suggests that early somatic embryo development, stages 1-6, are occurring normally and our block is in the later stages of embryo development. Autonomous root meristems are not forming in the somatic embryos and they are not going through the transition were the roots emerge first from the embryos.

Analysis of Stage Specific Bands from Differential Display of Loblolly Pine Somatic Embryos

John Cairney and Barbara Johns

Embryo development, be it for humans, or sheep or plants, is the result of a controlled program of gene activity. The products of these genes are usually proteins. These may be enzymes, which utilize resources for growth, or structural proteins, which go to form the cell walls or other constituents. Unfortunately, no sensitive method of monitoring the biochemical changes during the course of development has been available. Development is currently followed by viewing a few gross morphological changes. However two morphologically similar embryos may have vastly different biochemical constitutions. The goal of our research using differential display to determine gene activity in embryos has been to characterize these differences. An overview of gene expression is reported in the following sections (Xu et al). This current section focuses on cDNA cloning of mRNA which appears at single stage of embryo development. Through cloning , sequencing and identification of cDNAs, we hope to illuminate biochemical events which are characteristic of a stage and to generate markers whose presence will indicate that a given stage of development has been reached, or at least, an event normally correlated with a certain level of maturity, is occurring.

cDNA cloning of Stage-Specific mRNA

Total RNA has been isolated from stages four through nine of somatic embryos of Loblolly pine and used for Differential Display employing an oligo(dT) primer, $T_{12}MG$ and AP_5, a short 10 mer random primer to give distinctive banding patterns. Ten bands which appear to be possible markers for stage specific gene expression were cut from the Differential Display gels. Each of the stages four through nine are represented.

The cDNA was re-amplified and cloned into Stratagene pCR-Script Amp SK(+) Cloning kit (cat # 211186) and electroporated into XL1-Blue electroporation-competent cells (with modifications to the Stratagene protocol[1]). Colony PCR (Figure 1) confirmed the successful cloning of the fragments and restriction enzyme digestion with Mse I and Nla III classified the transformants into 66 clones. cDNA clones isolated from the same band, give rise to different restriction digestion patterns, indicating differences in sequence (Figure 2).

Each of the selected clones was labeled as a somatic embryogenic clone and also by excised Differential Display band number, stage, experiment number, and colony PCR number as in S5-6-17-51, a somatic clone from band 5; stage 6; experiment 4431-17; colony clone 51.

[1] a quality control test (Experiment 4431-29) revealed a flaw in the Stratagene transformation protocol for electroporation of the insert and the XL1-Blue competent cells. A significant increase in transformation results when the voltage is increased from 1.7 kV to 2.0 kV and the cells are pulsed 10 times instead of once. Also the cells should be placed on ice 15 minutes prior to electroporation and the SOC buffer added afterward at 37°C.

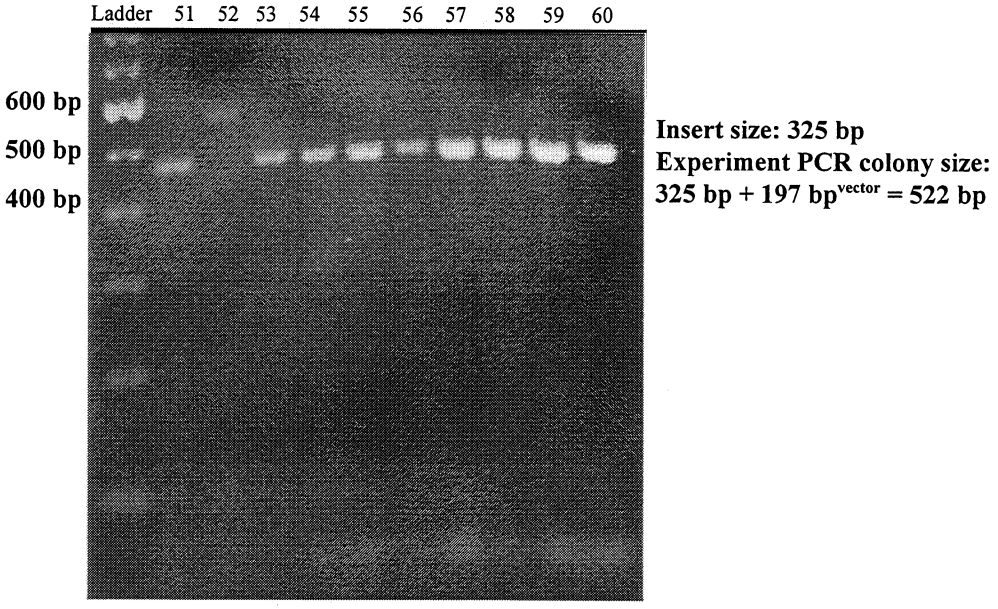


Figure 1: Colony PCR of S5-6-17; Clones 51-60.

The plasmid DNA from 22 clones representing each class of clones from each Differential Display band has been isolated and Southern analysis of the clones is underway. This sets the stage for generating stage specific markers to follow somatic embryogenic development in Loblolly pine with the possible application to other genotypes and species.

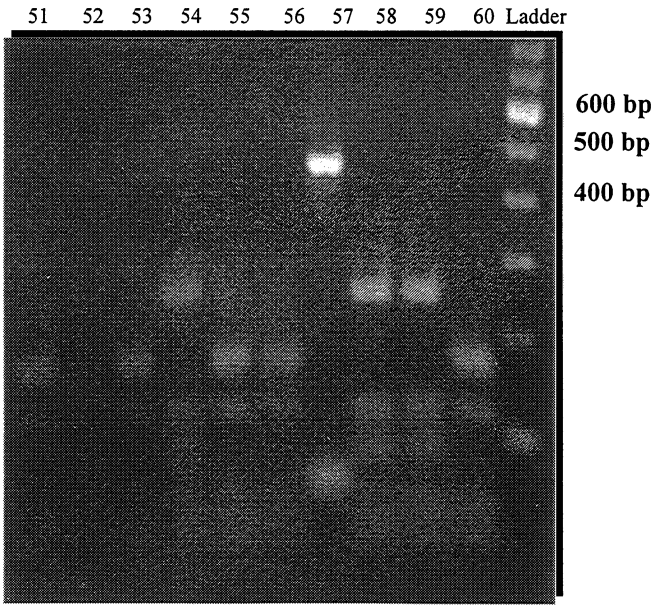


Figure 2: Restriction Digest of S5-6-17; Clones 51-60.
Note three classes of clones: Class 1; #51,53,60; Class 2; #5458,59; Class 3; #57

Analysis of Somatic Embryo Development Using Differential Display I: Advancements in Techniques

Nanfei Xu, Gerald Pullman and John Cairney

Summary:

The understanding of plant embryogenesis is fundamental to the improvement of the quantity and quality of somatic embryos which is a most promising approach for fast propagation of forest trees. However, little is know about the molecular events that are unique to the different stages of embryo development. In an effort to improve the quality of somatic embryos of loblolly pine, we are in the process of identifying genes that are expressed at all the stages of embryo development. Isolation and characterization of expressed genes in embryos are central to the study of embryogenesis, but few genes have been studied because of the difficulty in obtaining enough starting material. To overcome this limitation, we have made three major technological advancements which allow us to rapidly clone and study a massive number of genes expressed during embryogenesis using as little as a single embryo. A solid-phase RNA differential display technique has been improved to achieve high sensitivity, speedy, versatility and reproducibility. A highly sensitive and reliable Dot-Array Southern protocol has been developed that can be used to examine the expression of several hundred of cloned genes in a single embryo. We have also developed two computer programs that greatly speed up the processing of the sequencing data generated in cDNA cloning.

Introduction:

Somatic embryogenesis is one of the most promising approach for the rapid propagation of high quality forest trees. Although somatic embryos can now be produced in many species through plant tissue culture, the efficiency is still too low for commercial use. Currently, no molecular markers are available to follow embryo development in trees. Our goal is to examine the gene expression patterns at different developmental stages of somatic and zygotic embryos of loblolly pine.

RNA differential display (Liang and Pardee, 1992) could be employed to serve our purpose, but major modification is needed. This technique has been extensively used in comparing gene expressions between developmental stages or growth conditions. It has the advantages of technical simplicity, lower bias against rare messages and lower requirement for mRNA starting materials compared to cDNA library screening. However, the amount of RNA required in traditional differential display is still a limitation for studying embryogenesis because of the lack of large amount of embryo materials and inconsistent results in RNA extraction due to the presence of large amount of polysaccharides and other interfering substance in the pine embryos. Here we adopted a solid-phase approach for RNA differential display. Using this method, we were able to generate clear band patterns using as little as one embryo.

A problem with cloning differential display bands is that the PCR products derived from the sequencing gel may be heterogeneous (Welsh *et al.*, 1992) and a cloned cDNA may not represent the differentially expressed gene of interest. Usually, northern blotting using the cloned cDNA as probe is needed to confirm the pattern of expression. When large numbers of

bands are to be evaluated, this approach would be tedious and would require large amount of RNA, an impossible requirement for tissues such as early stages embryos. Dot-blots (Callard *et al.*, 1994; Corton and Gustafsson, 1997) or slot-blots (Liu and Raghothama, 1996) have been used to screen the cDNAs representing differentially expressed genes. These methods all require relatively large amount of mRNA for making probes. We have modified the dot blot strategies, reducing the requirement for RNA, by employing RT-PCR to amplify the messages. In this fashion we were able to verify the expression of hundreds of genes in a single embryo.

Currently available computer software for DNA sequencing analysis is usually single data set based. To deal with large number of data sets, laboriously repeated steps are required. To speedy up the DNA sequence data processing, we have written computer programs that can perform a variety of functions in an automated batch processing of unlimited number of data sets.

Results and Discussions:

Differential Display:

The success of RNA differential display relies on the availability and quality of mRNA. Although total RNA extracted from abundant tissues is suitable for differential display, this approach is time consuming and usually requires large amount of starting material. Magnetic separation of RNA has been extensively used in mRNA isolation and thus isolated mRNA has been used in differential display (McKendree *et al.*, 1994; Rosok *et al.*, 1996). This technique offers rapidity and sensitivity. However, to investigate the gene expression in early stage embryos, we need a more sensitive and reliable method. We have developed a procedure that is less time consuming and more sensitive. From starting material to the end of PCR, our new procedure takes less than 6 h., which is the fastest ever reported.

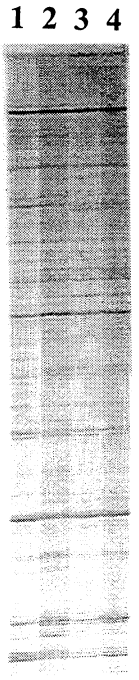


Figure 1: RNA differential display of a single embryo of loblolly pine. Poly(A) RNA was isolated from 1, 2, 5 or 10 stage 9.7 zygotic embryos (lanes 1, 2, 3, 4, respectively), and RT-PCR performed according to the procedures outlined in the Protocol. The embryos were from tree BC-1. T12VC and 5'-GGTACTCCAC-3' primers were used in the PCR.

ons of some genes across the developmental

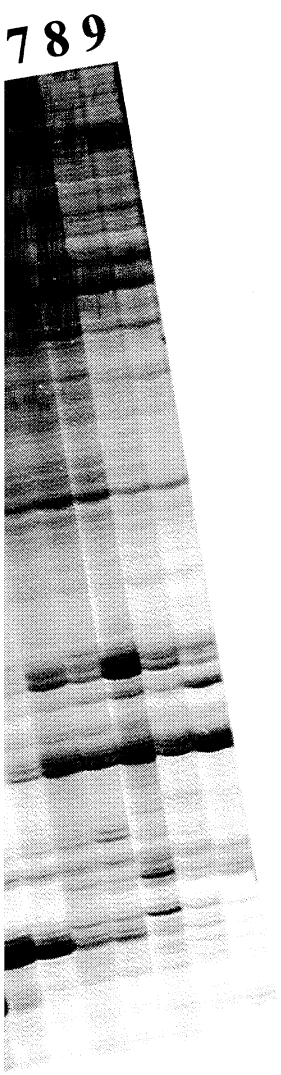


Figure 2: Differential display band patterns of somatic embryos of loblolly pine. The stage of the embryos are on the top, and S represent suspension stage which covers both stage 1 and stage 2. T12VC and 5'-GGTACTCCAC-3' primers were used in the PCR for the left panel, and T12VG and 5'-GGTACTCCAC-3' for the right panel.

viously reported solid-phase differential display (Rosok *et al.*, 1996), al additional advantages. The use of oligo(dT) beads in stead of with biotin labeled T12VN shortened mRNA isolation time by ed in preparing the streptavidin-biotin conjugates. The poly(T) on ger than that of the T12 as in T12VN, and this should increase mRNA se of enhanced binding. Most importantly, because d(T), in stead of riming in the RT reaction, any anchored primers can be used in the

Compared to the previously reported solid-phase differential display (Rosok *et al.*, 1996), the new method offers several additional advantages. The use of oligo(dT) beads in stead of streptavidin beads coupled with biotin labeled T12VN shortened mRNA isolation time by eliminating the steps involved in preparing the streptavidin-biotin conjugates. The poly(T) on the oligo$(dT)_{25}$ beads is longer than that of the T12 as in T12VN, and this should increase mRNA isolation efficiency because of enhanced binding. Most importantly, because d(T), in stead of TnVN, was used for the priming in the RT reaction, any anchored primers can be used in the subsequent differential display PCR reactions.

Less beads were used in our method. The Dynal oligo(dT) beads can bind to 2 μg mRNA/ml. When small amount of tissue is used, most of the oligo(dT) is wasted and pose potential interference in the subsequent PCR reaction. We reduced the amount from the manufacture recommended 50 μl/sample to 8 μl/sample, the amount that is barely seen in the tube during isolation. If a larger amount of tissue is used, the oligo(dT) beads could be over loaded. This overloading helps to even out the amount of mRNA isolated from different samples, which is beneficial for differential display.

Dot-Array Southern:

False positives are common problems in RNA differential display (Liang *et al.*, 1992; Sun *et al.*, 1994) and northern blotting is usually required to confirm the nature of differential expression of the cloned DNA fragments. This is impossible for us to carry out because our goal is to clone hundreds of the differentially expressed gene fragments and the amount of plant material we are working with is very small. To avoid these problems, we chosen high density dot-blotting reverse Southern analysis. The cloned DNAs were dot-blotted on membranes at a density of 2.5 dot/cm^2 and hybridized to cDNA probes made from the embryos at different stages. The probes are made by PCR in the presence of ^{32}P-dCTP using the first strand cDNA templates which were generated from one or a few embryos using the SMART PCR cDNA Synthesis Kit (Clontech, CA). Figure 4 shows the results of the Southern blots using probes made from Suspension stage and stage 9 somatic embryos respectively (Fig. 3). There were 96 cloned cDNA fragments blotted on each of the membranes, and 70 of them were cloned from differential display bands present either at suspension stage or at stage 9. The stage appearance of 49 of them correspond to that on the differential display.

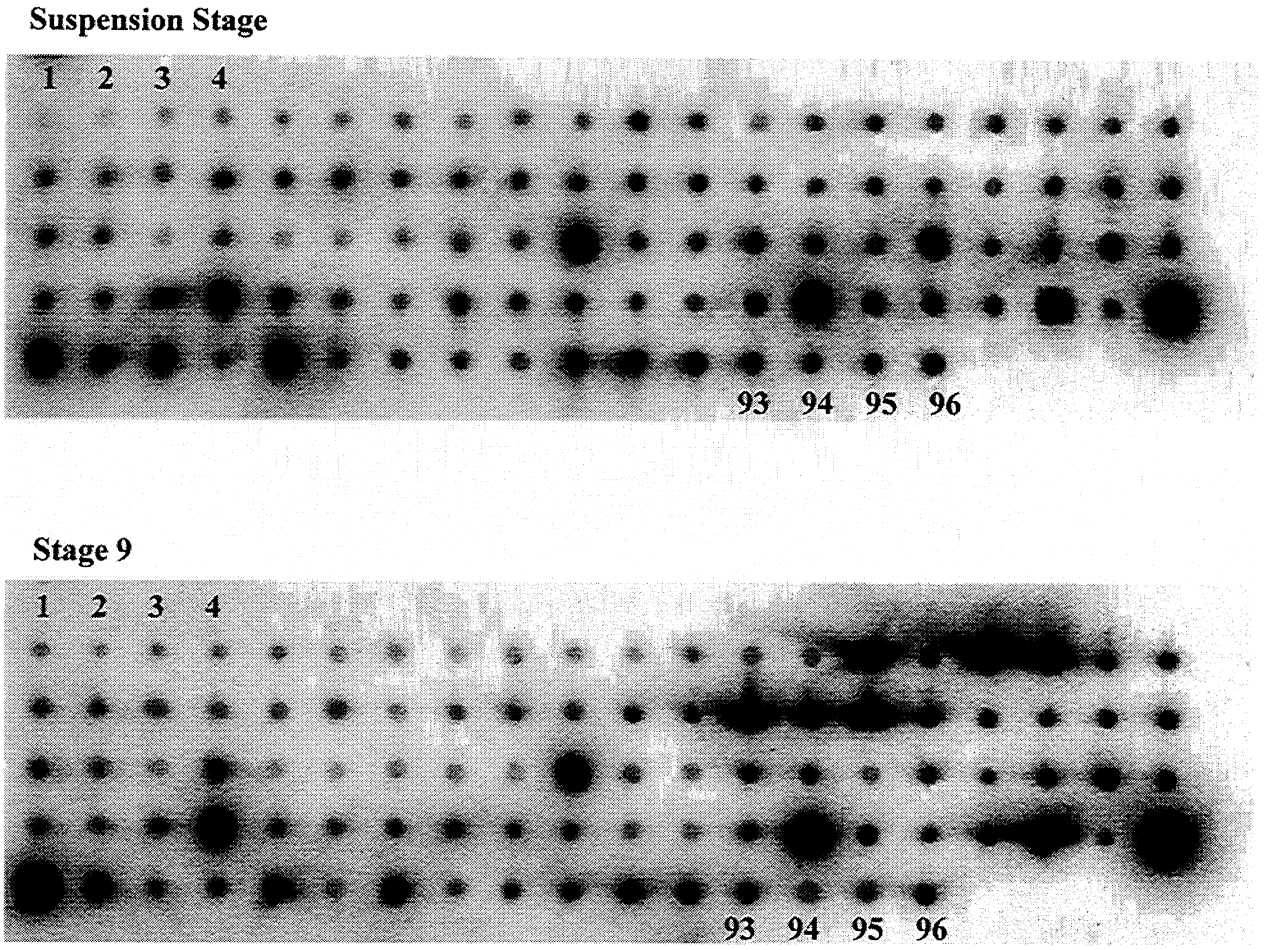


Figure 3: Dot-blot reverse Southern analysis. LPS001 to LPS096 cDNAs were cloned from differential display of somatic embryos of all stages. The insert sequences were amplified by PCR and blotted on the membrane. They were hybridized with probes made from suspension stage (top) and stage 9 (bottom) embryos.

We also performed northern analyses (Fig. 5) on a few selected cDNA fragments to check if the result of the Dot-Blot-Southern agree with that of northern (for suspension culture and Stage 9 embryos it is possible to isolate relatively large amounts of RNA, compared to intermediate stages). Our results showed that both methods give similar results for both the pattern and level of expression.

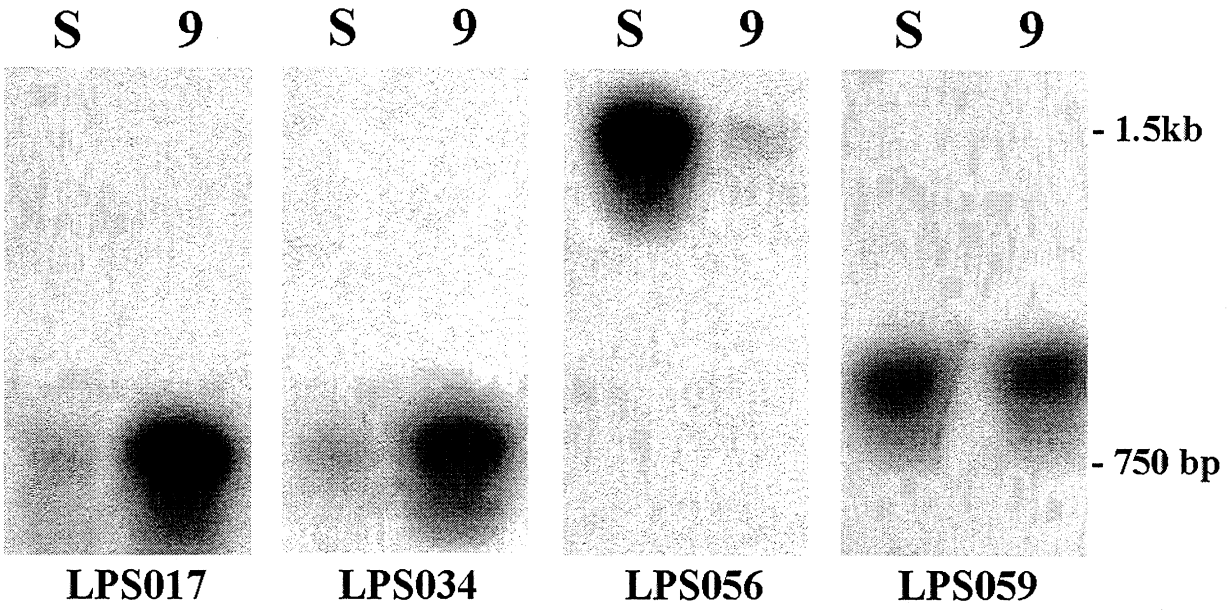


Figure 4: Northern blotting analysis of selected cDNAs. S and 9 are lanes for total RNA isolated from suspension stage and stage 9 somatic embryos. The probes were made from late stage expression cDNA LPS017, LPS034, early stage expression cDNA LPS056 and both stage expression (at a lower level) cDNA LPS059, respectively. See Figure 4 for the expression level detected by reverse Southern.

Computer Software:

Two programs have been written for PC. One is named CSE (**C**loned **S**equence **E**ditor). The functions of this software include, but are not limited to, form and format converting, cloned insert searching and processing, automated header info generating, peptide translation and ORF searching. Different options and combinations of these options are available to the user. A glance of the time saving power of this software is given in the following table, presuming that the insert sequence in the data is extracted, favorably orientated, annotated, all possible frames translated and ORF searched.

Work performed	Use GENEWORK	Use CSE
Process 1 DNA sequence	> 5 Minutes	< 30 seconds
Process 100 DNA sequences	> 8 hours	< 2 Minutes

Once a DNA sequence is available, it is easy to use BLAST to search the GeneBank database for possible homology to the new sequence. However, adequate homology search PC software for local database is not available. When hundreds of cDNAs are cloned, we needed to examine any possible homology among these sequences, to determine whether a newly isolated

cDNA is a duplicate of a previously isolated sequence. Therefore, we have developed another program called LDHS (**L**ocal **D**atabase **H**omology **S**earch). Like any other homology search software, LDHS demands large computer power and runs a little slow on our current PCs; however, it can batch process any number of sequences and can run in the background in the computer.

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Analysis of Somatic Embryo Development Using Differential Display II: Cloning, Sequencing and Characterization of Genes Differentially Expressed at Different Stages of Somatic Embryos

Nanfei Xu, Michelle Lane, Gerald Pullman and John Cairney

Summary:

As a prelude of cDNA cloning in the zygotic embryos, we have isolated 94 bands from the differentially display of somatic embryos. Cloning of these bands generated 96 cDNA clones. We have finished sequencing them and initially characterized their expression patterns during somatic embryogenesis. Sequence analysis of these 96 cDNAs showed that they belong to 86 groups of sequences that are different from each other. Comparison of their sequences to GenBank database indicated that most of them are novel sequences, while a few match regulatory proteins expressed during embryogenesis.

Introduction:

One major obstacle in the gene study of plant embryogenesis is the very limited availability of embryo material. The dissection and collection of early stage zygotic embryos are particularly difficult and laborious tasks yielding small amounts of tissue. In loblolly pine, the problem is compounded as the presence of large amount of polysaccharides in this tissue interferes with RNA extraction. To overcome these difficulties and facilitate our research of embryo development, we have developed sensitive methods that can be used in mass cloning and characterization of genes that are differentially expressed at different stages of embryo development. Since somatic embryos are more abundant than zygotic embryos, we developed the new methods using somatic embryos. To date our methods have enabled us to isolated 96 cDNA clones.

Results and Discussions:

Differential display:

Primers T12VA, T12VC, T12VG, T12VT, AP2 (5'-AGCCAGCGAA-3'), AP3 (5'-AGGTGACCGT-3'), AP4 (5'-GGTACTCCAC-3') and RAP4 (5'-GGTGCGATCC-3') have been used in 16 combinations in the PCR reactions using the RT templates from somatic embryos at suspension stage and stages 3, 4, 5, 6, 7, 8 and 9. Figure 1 shows typical band patterns in the differential display. Almost all PCR reactions resulted in informative display patterns which reflect the dynamic status in the gene expression of embryos at different stages.

***Cloning*:**

The Invitrogen TA cloning kit is convenient for PCR cloning, however, at least in our hands, gives variable results. Many white or light blue clones with no detectable insert were often produced when PCR product was directly used in the ligation as recommended by the manufacture. We routinely size purify the PCR product using CHROMA SPIN columns (Clontech, CA) and use the purified DNA for ligation. This greatly reduced the number of light

blue clones, and most of the white clones have inserts.

The DNA amplified from the differential display bands may be heterogeneous in sequence. This heterogeneity was examined by restriction enzyme digestion. After the DNA from a differential display band was cloned in the pCR2.1 vector and the insert was amplified by PCR, two 4-cutters, Mse I and Nla III were used to digest the insert DNA from different clones. Different band patterns on the gel of the digestion revealed the difference in sequence (Fig. 2).

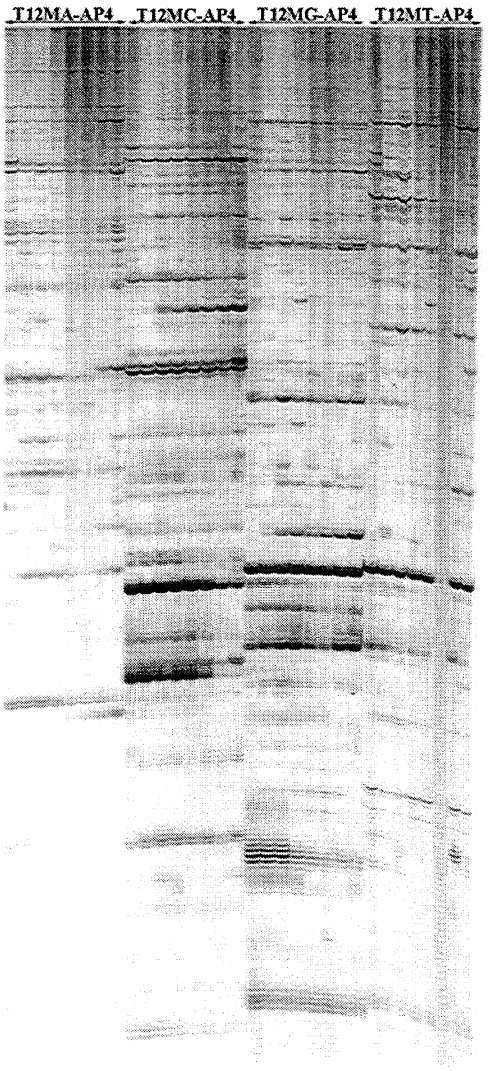

Figure 1: Differential display band patterns of somatic embryos of loblolly pine. The primer combinations are on the top of panels. Each panel consists of 8 lanes which are from suspension stage (left), stage 3, 4, 5, 6, 7, 8 and 9 (right) somatic embryos.

Currently, 96 unique cDNAs clones have been isolated. They are named LPS001 to LPS096, respectively. LPS stands for **L**oblolly **P**ine **S**omatic embryos. The insert DNA of all these clones have been sequenced and the sequence data analyzed (Table 1). While the majority of these sequences are novel, some of them match previously characterized genes. LPS094 has high degree of homology to a DC3 promoter-binding factor. DC3 gene is known to be activated during embryogenesis and it is ABA regulated (Lorenz *et al.*, 1995; Kim *et al.*, 1997).

Table 1. A list of the cDNAs cloned from different stage embryos of loblolly pine.

Clone name	Appearance at stages*								GeneBank match‡ (accession number)
	S	3	4	5	6	7	8	9	
LPS001						*	*	*	Cyt B6-F Fe-S subunit[M], *Cyanobacterium nostoc*, (P14698)
LPS002						*	*	*	Cyt B6-F Fe-S subunit[M], *Cyanobacterium nostoc*, (P14698)
LPS003							*	*	Novel
LPS004						*	*	*	Novel
LPS005						*	*	*	Novel
LPS006	*	*	*						Novel
LPS007	*	*	*						Novel
LPS008	*	*	*						Novel
LPS009	*	*	*						Novel
LPS010	*	*	*	*	*				T-cell receptor J[L], human, (S80858)
LPS011	*	*	*	*	*				Novel
LPS012		*	*	*	*				Novel
LPS013		*	*	*	*				Novel
LPS014		*	*	*	*				Novel
LPS015				*	*	*	*	*	Novel
LPS016				*	*	*	*	*	Novel
LPS017				*	*	*	*	*	Novel
LPS018					*	*	*	*	Novel
LPS019					*	*	*	*	Novel
LPS020				*	*	*	*	*	Novel
LPS021				*	*	*	*	*	Novel
LPS022				*	*	*	*	*	Novel
LPS023				*	*	*	*	*	Novel
LPS024	*	*	*	*	*	*	*		Novel
LPS025	*	*	*	*	*	*	*		Novel
LPS026	*	*	*	*	*	*			Novel
LPS027		*	*	*					Novel
LPS028	*	*	*						Novel
LPS029	*	*	*						Novel
LPS030	*	*	*	*	*	*			Novel
LPS031	*	*	*						Novel
LPS032	*	*	*	*	*	*	*	*	Novel
LPS033				*	*	*	*	*	Novel
LPS034					*	*	*	*	Novel
LPS035					*	*	*	*	Novel
LPS036		*	*	*	*	*	*		Novel
LPS037			*	*	*	*			Novel
LPS038				*	*	*			EF-2[H], *Chlorella kessleri*, (S32819)
LPS039				*	*	*			EF-2[H], *Chlorella kessleri*, (S32819)

LPS040				*	*	*			Novel
LPS041		*	*	*	*				Novel
LPS042	*	*	*	*	*				Novel
LPS043			*	*	*	*	*	*	Novel
LPS044	*	*	*	*	*				Novel
LPS045	*	*							Novel
LPS046	*	*							Novel
LPS047	*	*							Novel
LPS048	*	*							Novel
LPS049				*	*	*			rab7b mRNA^{L}, *N. tabacum*, (L29275)
LPS050				*	*	*			Novel
LPS051					*	*	*	*	Novel
LPS052	*	*	*	*	*				Novel
LPS053	*	*	*	*	*				rab7b mRNA^{M}, *N. tabacum*, (L29275)
LPS054	*	*	*	*	*				Novel
LPS055			*	*	*	*	*	*	yj19g05.r1 cDNA clone 149240^{L}, human, (R82572)
LPS056	*	*	*	*	*	*			Novel
LPS057		*	*	*					Novel
LPS058	*	*	*	*	*	*			Novel
LPS059	*	*	*	*	*	*			cDNA clone 2897s^{L}, *Pinus taeda*, (H75243)
LPS060	*	*	*	*	*	*	*	*	XET1^{H}, *Tropaeolum majus*, (L43094)
LPS061	*	*	*	*	*	*	*	*	Novel
LPS062	*	*	*	*	*	*			Novel
LPS063	*	*	*	*	*	*			Novel
LPS064	*	*	*	*	*	*			Novel
LPS065		*	*	*	*				Embryonic ectoderm development protein mRNA^{L}, *Mus musculus*, (U97675)
LPS066	*	*	*	*	*	*	*	*	Novel
LPS067		*	*	*	*	*	*		ξ-crystallin^{M}, *A. thaliana*, (S57612)
LPS068			*	*	*	*	*	*	Novel
LPS069			*	*	*				Novel
LPS070			*	*	*				Novel
LPS071					*	*	*	*	Novel
LPS072					*	*	*	*	Novel
LPS073			*	*	*	*	*	*	Expressed tag^{H}, *A. thaliana*, (ATEST958)
LPS074			*	*	*	*	*	*	Novel
LPS075	*								tXET-b1 mRNA^{H}, *L. esculentum*, (X82685)
LPS076	*								Novel
LPS077		*	*	*	*	*			Novel
LPS078		*							60S ribosomal protein L35^{H}, rat, (P17078)
LPS079		*	*						Novel
LPS080					*				Etr-3^{L}, *Xenopus laevis*, (1568645)
LPS081						*			Novel
LPS082	*	*	*	*	*	*			Novel
LPS083		*	*	*					Novel

LPS084	*	*	*						Novel
LPS085	*	*	*						mRNA for α-tubulin[H], *P. amygdalus*, (X67162)
LPS086			*	*	*				Novel
LPS087			*	*	*				Novel
LPS088			*						Novel
LPS089		*	*	*					Novel
LPS090		*	*	*					Novel
LPS091			*	*	*	*	*	*	Novel
LPS092			*	*	*	*	*	*	AR192[M], *A. thaliana*, (d1014382)
LPS093			*						Serine kinase mRNA[H], human, (U09564)
LPS094				*					Dc3 promoter-binding factor[H], *H. annuus*, (2228773)
LPS095			*						Novel
LPS096			*						Novel

*. Larger * indicate the most abundant stage.

‡. Match is labeled H if BLAST score is >400 for DNA and >200 for protein, M if score is 300-399 for DNA and 150-199 for protein, and L if score is 200-299 for DNA and 80-149 for protein.

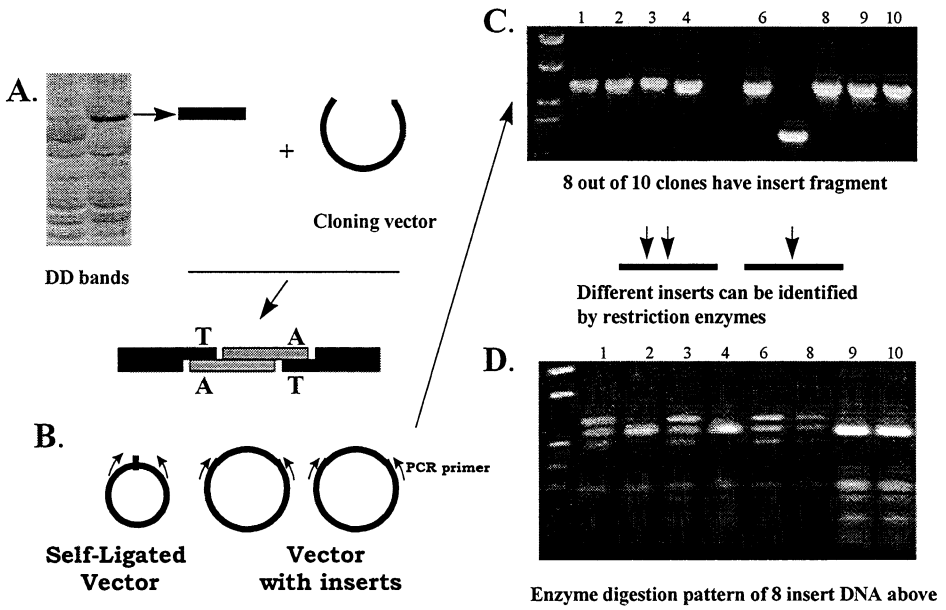

Figure 2: Graphic presentation of the cloning protocol for isolated differential display bands. A. A band is cut from differential display gel and the cDNA is amplified. B. The cDNA is ligated to pCR2.1 vector DNA and the ligation is used to transform *E. coli* cells. C. The bacterial clones are screened for insert by colony PCR. D. Identification of the inserts by restriction enzyme digestion.

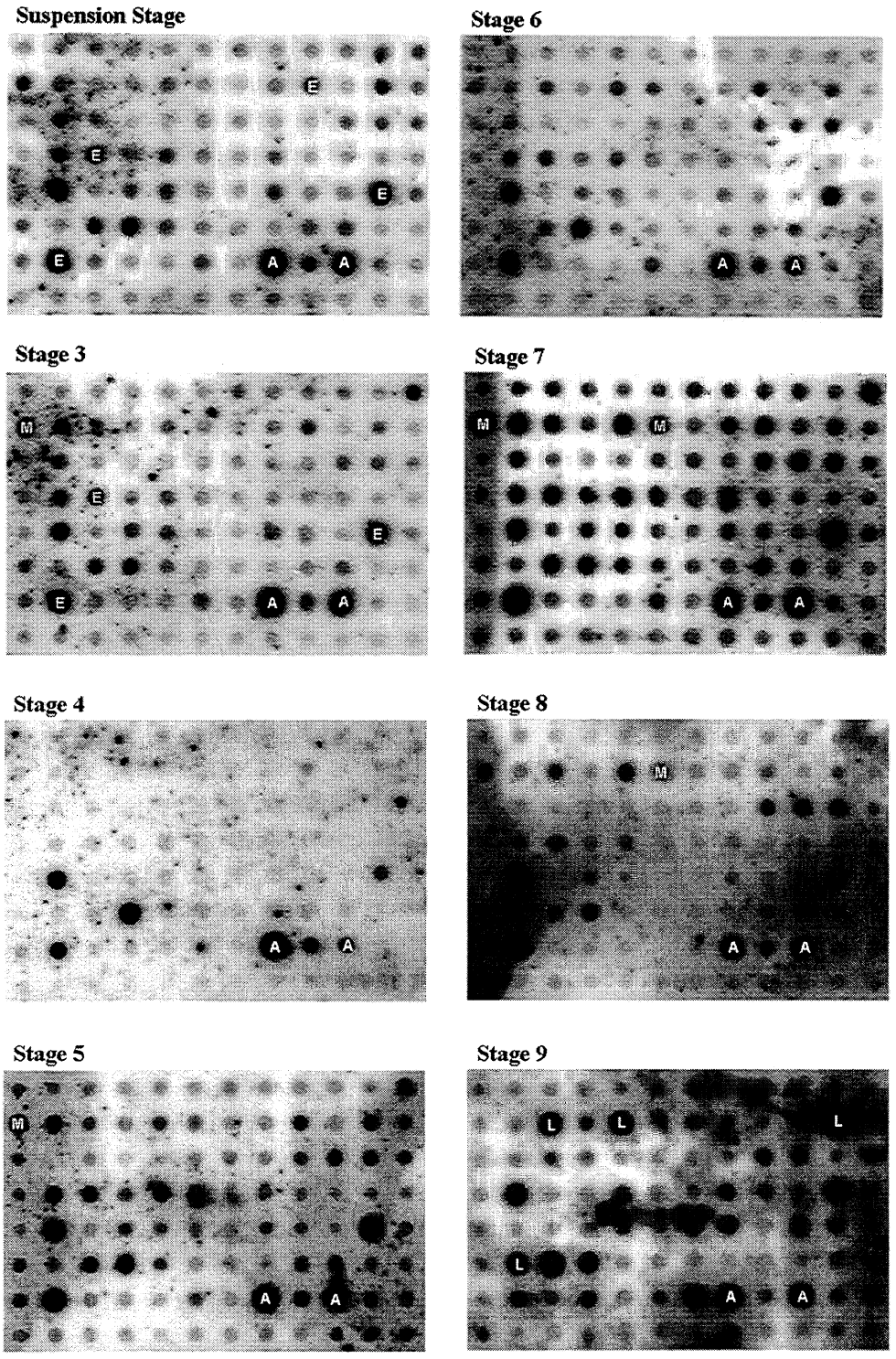

Figure 3: Dot-blot reverse Southern analysis. LPS001 to LPS096 cDNAs were cloned from differential display of somatic embryos of all stages. The insert sequences were amplified by PCR and blotted on the membrane. They were hybridized with probes made from suspension stage to stage 9 embryos. The probes were made by PCR using RT as templates and differential display primers.

Expression confirmation:

A important step in the cloning of cDNAs derived from differential display is the confirmation of the expression pattern of the cloned cDNA. To work with minute amount of plant material, a sensitive method has to be developed. It is very difficult to obtain enough RNA to perform northern analysis to confirm the expression of one cloned cDNA, and it is impossible to repeat this analysis for hundreds of cDNAs. A reverse Southern analysis is a better choice. Initially, we used PCR conditions similar to that used in differential display to amplify the reverse-transcribed mRNAs from embryos and used this RT-PCR amplified DNA as probes. As shown in Figure 3, this approach gave a pattern of different abundance of the amplified messages, and some of the changing in abundance correspond to the pattern seen in differential display. However, the overall hybridization signal level is variable at different stages, and the appearance of many cDNA signals do not correspond to the differential display pattern. Because the band pattern in differential display is usually reproducible using our newly developed method, the discrepancy here is probably due to the Southern technique we employed.

To search for a better method of examining the *in vivo* expression of the genes corresponding to the cloned cDNAs, we used the approach of amplifying full length cDNA libraries constructed from different stages of embryos. Hybridization using the full length probes made from suspension stage and stage 9 embryos gave excelent results (see Analysis of Somatic Embryo Development Using Differential Display I: Advancements in Techniques). Northern analysis using a few selected cDNAs showed the same pattern. We are in the process of performing this improved Dot-Southern for other stages of somatic embryos.

Reference:

Kim, S.Y., H.J. Chung, and T.L. Thomas (1997). Isolation of a novel class of bZIP transcription factors that interact with ABA-responsive and embryo-specification elements in the Dc3 promoter using yeast one-hybrid system. Plant J. **11**, 1237-1251.

Lorenz, K., S. Lienhard and A. Sturm (1995). Structural organization and differential expression of carrot beta-fructofuranosidase genes: identification of a gene coding for a flower bud-specific isozyme. Plant Mol. Biol. **28**, 189-194.

Analysis of Embryo Development Using Differential Display III: Cloning, Sequencing and Characterization of Genes Differentially Expressed at Different Stages of Zygotic Embryos.

Nanfei Xu, Michelle Lane, Gerald Pullman and John Cairney

Summary:

The quality of somatic embryos can usually be measured by their likeness to the zygotic embryos. Presumably, if the gene expressions in the somatic embryos at different stages matched that found in their zygotic counterparts, the quality of the somatic embryos would match that of the zygotic embryos. To this end, we plan to clone several hundred cDNAs that represent genes differentially expressed at different stages of zygotic embryo development. The expression pattern of this large set of cDNAs can be determined and used as blue-print for improving the quality of somatic embryos. To date, 238 cDNAs have been cloned and some of them are initially characterized.

Introduction:

High quality somatic embryos are valuable resources for quick propagation of desirable forest tree species. Although somatic embryos are been produced in several forest species including loblolly pine, the quality of these embryos must be improved for use at commercial scale. The quality of the somatic embryos is usually measured by their resemblance to their zygotic counterparts, both in germinability and morphology. Currently, research is focusing on the morphological, physiological and biochemical improvement of the somatic embryos through manipulation of the culture conditions. The changes in morphology, and physiology of the embryos in any experimental treatment is usually observed long after the treatment was applied. Also, the morphological and physiological effects are the combined results of the many genes expressed early during the culture. It would be more informative and precise if the effects of any treatment could be dissected into individual changes in gene expression and into different time points along stages of development. Numerous genes are expressed during embryogenesis; and many of them are so called "house-keeping" genes. It is impractical, currently, to characterize all these expressed genes. Our proposed approach is to characterize a set of genes that are expressed during different stages of embryogenesis. The first step in this approach is to clone these genes. This report list the genes we have cloned.

Results and Discussion:

Cloning:

Using our newly improved differential display technique, mRNAs were extracted from small amount zygotic embryos at all 18 stages of development, and RT-PCR was performed using many primer pairs. Hundreds of clear differential display patterns have been generated. Figure 1 shows a typical differential display band pattern. Bands that appeared at one or a few stages in the differential display of zygotic embryos were cut out of the gel. The cDNAs in the bands were amplified and cloned using pCR2.1 TA cloning vector. The cloned cDNAs are named with prefix LPZ (Loblolly Pine Zygotic embryo) and a sequential number. So far, 238

cDNAs have been cloned (LPZ001 to LPZ238). Nineteen-eight of them are sequenced and the GenBank homology searched. All the cDNAs cloned from zygotic embryos are listed in Table 1.

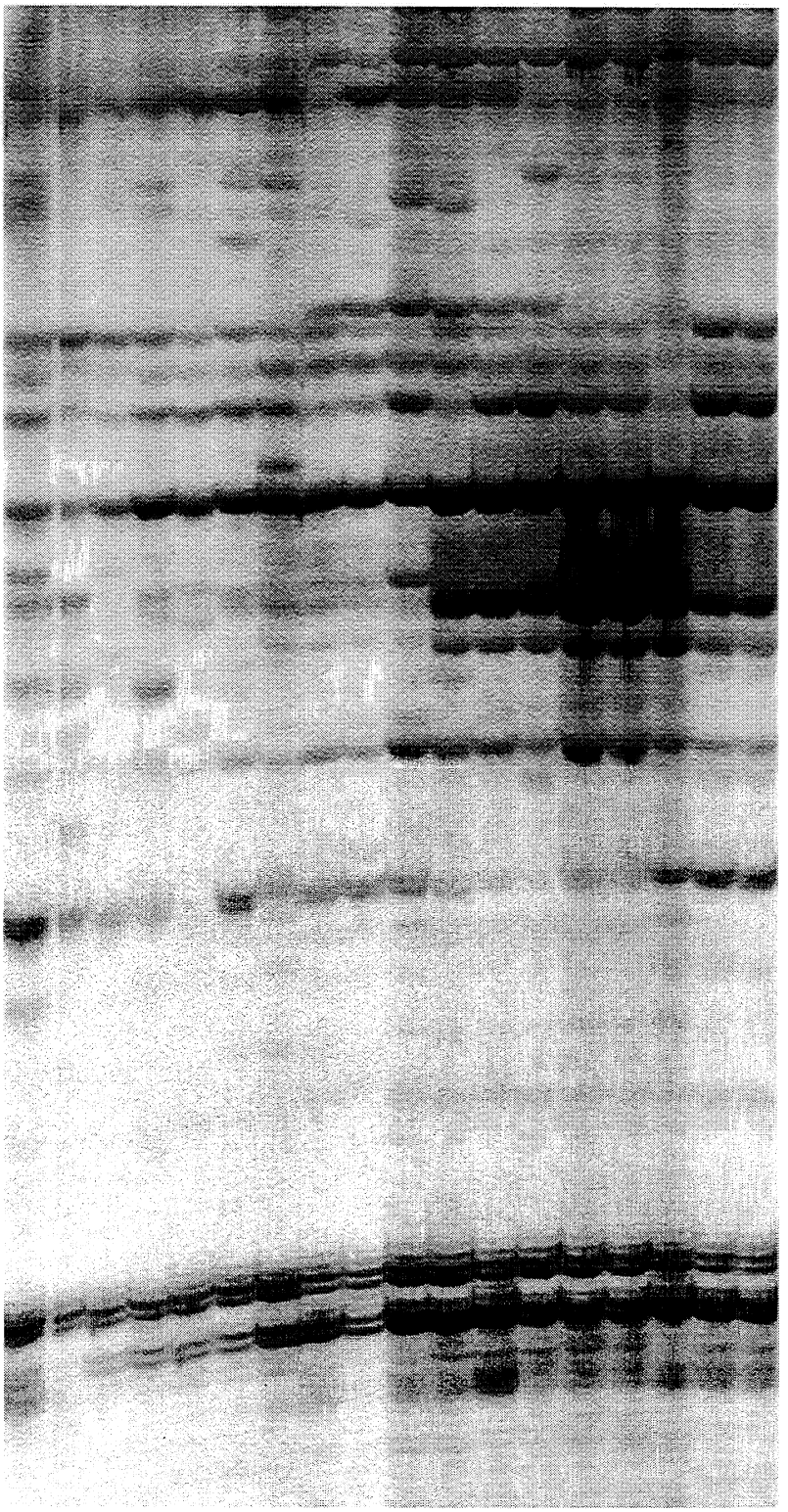

Figure 1: Differential display band patterns of zygotic embryos of loblolly pine. Message RNA was extracted using the bead method from stage 1 (left lane) to stage 9.10 (right-most lane) zygotic embryos obtained from tree BC-1. The mRNA was converted to cDNA and subsequent PCR was performed using T12VA and RAP4 primers.

Table 1. DNAs cloned from loblolly pine zygotic embryos at different stages of development.

Clone name	Appearance at stages according to differential display*																	GenBank match‡ (accession number)
	1	2	3	4	5	6	7	8	9.1	9.2	9.3	9.4	9.5	9.6	9.7	9.8	9.9	9.10	
LPZ001	*	*	*	*															
LPZ002	*	*	*	*	*	*	*	*	*	*	*								
LPZ003			*	*															
LPZ004				*	*	*	*	*											
LPZ005				*															
LPZ006										*									
LPZ007											*	*	*	*	*	*	*	*	ERECTA receptor protein kinase[L], (1345132)
LPZ008											*	*	*	*	*	*	*	*	Lily mRNA[H] in flowering buds, (D21823)
LPZ009											*	*	*	*	*	*	*	*	
LPZ010													*						Human aorta cDNA[L], (C16240)
LPZ011													*						
LPZ012															*	*	*	*	
LPZ013	*	*	*	*	*	*	*	*	*	*									
LPZ014	*	*	*	*	*	*	*	*	*	*									
LPZ015		*	*	*	*														
LPZ016		*	*																Cyclic P-diesterase[M], *A. thaliana*, (e311727)
LPZ017		*	*	*	*	*													
LPZ018			*	*	*														
LPZ019			*	*	*														Ribosomal protein L5[H], rice, (P49625)
LPZ020		*	*	*	*	*	*	*	*	*									
LPZ021		*	*	*	*	*	*	*	*	*									
LPZ022		*	*	*	*														Human EST sequence[L], (F18185)
LPZ023		*	*	*	*														Similar to insulin 1[H], (C06946)
LPZ024				*	*	*													
LPZ025				*	*	*													*A. thaliana* cDNA clone[H], (T43932)
LPZ026						*	*	*	*										
LPZ027						*	*	*	*										
LPZ028	*	*	*	*	*	*	*	*											
LPZ029				*	*	*	*	*	*	*	*	*							Photosynthetic OEC[L], *S. oleracea*, (X05512)
LPZ030													*	*	*				
LPZ031													*	*	*				
LPZ032													*	*	*				2S stroage protein[H], *P. strobus*, (X62433)
LPZ033														*	*	*			
LPZ034													*	*	*	*	*		
LPZ035															*	*	*	*	
LPZ036															*	*	*	*	
LPZ037															*	*	*	*	OEC 17kD protein, *Zea. mays*, (M87435)
LPZ038															*	*	*	*	
LPZ039															*	*	*	*	Actin[L], *S. lycopersicum*, (U60481)
LPZ040															*	*	*	*	
LPZ041															*	*	*	*	
LPZ042															*	*	*	*	
LPZ043				*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
LPZ044												*	*	*	*	*	*	*	
LPZ045												*	*	*	*	*	*	*	Rac-like protein, *A. thaliana*, (S79308)
LPZ046	*	*																	
LPZ047	*	*																	
LPZ048	*	*																	ZK792.1[L], *C. elegans*, (e219740)
LPZ049	*	*	*	*	*	*	*	*	*										Starch synthase[H], *M. esculenta*, (X74160)
LPZ050	*	*	*	*	*	*	*	*	*										Starch synthase[H], *M. esculenta*, (X74160)
LPZ051	*	*																	
LPZ052	*	*																	Dynamin[H], soybean, (S63667)
LPZ053		*	*	*	*														Rice cDNA[H], (D41438)

Clone name	Appearance at stages according to differential display*																	GenBank match‡ (accession number)
	1	2	3	4	5	6	7	8	9.1	9.2	9.3	9.4	9.5	9.6	9.7	9.8	9.9	9.10	
LPZ054		*	*	*	*														
LPZ055		*	*	*	*	*	*	*	*	*	*	*	*	*					
LPZ056		*	*	*	*	*	*	*	*	*	*	*	*	*					
LPZ057					*	*	*	*											ZK792.1[L], *C. elegans*, (e219740)
LPZ058					*	*	*	*											
LPZ059						*	*												Transposon[L], *Lilium henryli*, (X13886)
LPZ060						*	*												
LPZ061		*	*	*	*	*	*	*	*	*	*	*							
LPZ062		*	*	*	*	*	*	*	*	*	*	*							
LPZ063					*	*	*	*	*										
LPZ064					*	*	*	*	*										
LPZ065						*	*	*											
LPZ066						*	*	*											Acyl-CoA-binding protein[L], (e286172)
LPZ067				*	*	*	*	*	*										
LPZ068				*	*	*	*	*	*										
LPZ069					*	*	*	*											
LPZ070					*	*	*	*											
LPZ071				*	*	*	*	*	*	*	*	*							
LPZ072				*	*	*	*	*	*	*	*	*							
LPZ073				*	*	*	*	*	*	*									
LPZ074										*									
LPZ075										*									
LPZ076										*									
LPZ077										*									
LPZ078											*								
LPZ079											*								
LPZ080									*	*	*	*	*	*					Acyl-CoA-binding protein[M], (e286172)
LPZ081									*	*	*	*	*	*					
LPZ082											*	*	*	*	*				
LPZ083											*	*	*	*	*				
LPZ084											*	*	*						Acyl-CoA-binding protein[M], (e286172)
LPZ085											*	*	*						
LPZ086											*	*	*	*	*	*			Acyl-CoA-binding protein[M], (e286172)
LPZ087											*	*	*	*	*	*			
LPZ088													*						
LPZ089													*						
LPZ090													*	*	*	*			Acyl-CoA-binding protein[M], (e286172)
LPZ091											*	*	*	*	*	*			LMW hsp[L], *P. menziessii*, (X92983)
LPZ092														*					
LPZ093														*					
LPZ094													*	*	*	*			
LPZ095													*	*	*	*			
LPZ096											*	*	*	*	*	*			
LPZ097												*	*	*	*	*			
LPZ098				*	*	*	*	*	*	*	*	*	*	*	*	*			
LPZ099	*	*																	
LPZ100		*	*	*	*	*													
LPZ101				*															
LPZ102	*	*	*	*	*	*	*	*	*	*	*								
LPZ103			*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
LPZ104															*	*	*	*	
LPZ105				*	*	*	*	*											
LPZ106				*	*	*	*	*											
LPZ107						*													
LPZ108				*	*	*	*	*	*	*									

Clone name	Appearance at stages according to differential display*																		GenBank match‡ (accession number)
	1	2	3	4	5	6	7	8	9.1	9.2	9.3	9.4	9.5	9.6	9.7	9.8	9.9	9.10	
LPZ109									*	*	*	*	*	*	*				
LPZ110				*	*	*	*	*	*	*	*	*							
LPZ111											*	*	*	*	*	*	*	*	
LPZ112											*	*	*	*	*	*	*	*	
LPZ113												*							
LPZ114	*	*	*	*	*	*	*	*	*	*									
LPZ115	*	*	*	*	*	*													
LPZ116	*	*	*	*	*	*	*	*	*	*									
LPZ117	*	*	*	*	*	*	*	*	*	*	*	*							
LPZ118		*	*	*	*	*	*	*	*	*	*	*							
LPZ119		*	*	*	*	*													
LPZ120		*	*	*	*	*	*												
LPZ121		*																	
LPZ122			*																
LPZ123			*																
LPZ124							*	*	*										
LPZ125							*												
LPZ126							*	*	*	*	*	*							
LPZ127							*	*	*										
LPZ128							*	*	*										
LPZ129				*	*	*	*	*	*	*	*	*							
LPZ130		*	*	*	*	*	*	*	*	*	*	*							
LPZ131								*	*	*									
LPZ132										*									
LPZ133									*	*	*	*	*						
LPZ134								*	*	*	*	*	*	*					
LPZ135														*					
LPZ136											*	*	*	*	*	*	*	*	
LPZ137		*	*	*	*	*	*	*	*	*	*								
LPZ138		*	*	*	*	*	*	*	*	*	*								
LPZ139	*																		
LPZ140	*																		
LPZ141	*																		
LPZ142	*																		
LPZ143	*	*	*	*	*	*	*	*											
LPZ144			*																
LPZ145			*	*															
LPZ146			*	*															
LPZ147			*																
LPZ148			*																
LPZ149	*	*	*	*	*	*	*	*	*	*									
LPZ150				*	*	*	*	*	*	*	*	*	*						
LPZ151										*									
LPZ152								*	*	*	*	*	*	*					
LPZ153											*	*	*	*	*				
LPZ154											*	*	*	*	*				
LPZ155													*	*	*	*			
LPZ156													*	*	*	*			
LPZ157													*	*	*	*			
LPZ158												*	*	*	*	*	*	*	
LPZ159												*	*	*	*	*	*	*	
LPZ160												*	*	*	*	*	*	*	
LPZ161														*	*	*	*	*	
LPZ162															*	*	*	*	

Clone name	Appearance at stages according to differential display*																	GenBank match‡ (accession number)
	1	2	3	4	5	6	7	8	9.1	9.2	9.3	9.4	9.5	9.6	9.7	9.8	9.9	9.10	
LPZ163															*	*	*	*	
LPZ164												*	*	*	*	*	*	*	
LPZ165												*	*	*	*	*	*	*	
LPZ166												*	*	*	*	*	*	*	
LPZ167										*	*	*	*	*	*	*	*	*	
LPZ168										*	*	*	*	*	*	*	*	*	
LPZ169											*	*	*	*	*	*	*	*	
LPZ170													*	*	*	*	*	*	
LPZ171								*	*	*	*	*	*	*	*	*	*	*	
LPZ172								*	*	*	*	*	*	*	*	*	*	*	
LPZ173								*	*	*	*	*	*	*	*	*	*	*	
LPZ174			*																
LPZ175	*	*																	
LPZ176	*	*																	
LPZ177				*															
LPZ178				*															
LPZ179						*													
LPZ180						*	*												
LPZ181						*	*												
LPZ182							*												
LPZ183										*									
LPZ184										*									
LPZ185								*	*	*									
LPZ186								*	*	*									
LPZ187											*								
LPZ188													*	*	*				
LPZ189											*	*	*	*	*	*	*	*	
LPZ190											*	*	*	*	*	*	*	*	
LPZ191											*	*	*	*	*	*	*	*	
LPZ192											*	*	*	*	*	*	*	*	
LPZ193						*	*	*	*	*	*	*	*	*	*	*	*	*	
LPZ194	*																		
LPZ195		*	*																
LPZ196			*	*	*														
LPZ197			*	*															
LPZ198				*															
LPZ199				*															
LPZ200		*	*	*	*	*	*	*	*										
LPZ201								*											
LPZ202								*											
LPZ203								*											
LPZ204								*											
LPZ205				*	*	*	*	*	*										
LPZ206									*	*									
LPZ207										*	*								
LPZ208										*									
LPZ209											*								
LPZ210										*	*								
LPZ211											*								
LPZ212											*								
LPZ213											*								
LPZ214											*								
LPZ215											*	*	*						
LPZ216											*	*	*	*	*	*	*	*	

Clone name	Appearance at stages according to differential display*																	GenBank match‡ (accession number)
	1	2	3	4	5	6	7	8	9.1	9.2	9.3	9.4	9.5	9.6	9.7	9.8	9.9	9.10	
LPZ217												*	*	*					
LPZ218														*					
LPZ219														*					
LPZ220															*				
LPZ221										*	*	*	*	*	*	*	*	*	
LPZ222										*	*	*	*	*	*	*	*	*	
LPZ223		*	*	*	*														
LPZ224		*	*	*	*														
LPZ225			*																
LPZ226				*															
LPZ227			*	*	*	*													
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LPZ233										*	*	*	*						
LPZ234											*								
LPZ235												*	*	*	*	*	*		
LPZ236												*	*	*	*	*	*		
LPZ237															*	*	*		
LPZ238															*	*	*		

*. Larger * indicate the most abundant stage.

‡. Match is labelled H if BLAST score is >400 for DNA and >200 for protein, M if score is 300-399 for DNA and 150-199 for protein, and L if score is 200-299 for DNA and 80-149 for protein.

Perspective:

To provide an overview of gene expression during embryogenesis we must monitor a large number of genes. Our initial goal is to clone 400 cDNAs from both somatic and zygotic embryos. Currently, we have cloned a total of 336 (96 from somatic and 238 from zygotic embryos). We may reach 400 by the end of the year. All these 400 cDNAs will be dot-blotted on a piece of membrane (Fig. 2, next page) at high density and the expression of their corresponding genes at each stage of zygotic embryo development will be examined by hybridization of the membrane to labeled cDNA probes made from the embryos in a fashion similar to that shown in Part II of this report (Fig.3). The patterns obtained will be used as blue prints for improving the quality of somatic embryos. The membrane with all the 400 cDNAs can be used as a diagnostic kit, patterns will be recognized which are characteristic of a certain embryo stage. Because as little as one embryo is needed in the dot-Southern, the expression of the genes corresponding to these 400 cDNAs can be detected in a single embryo.

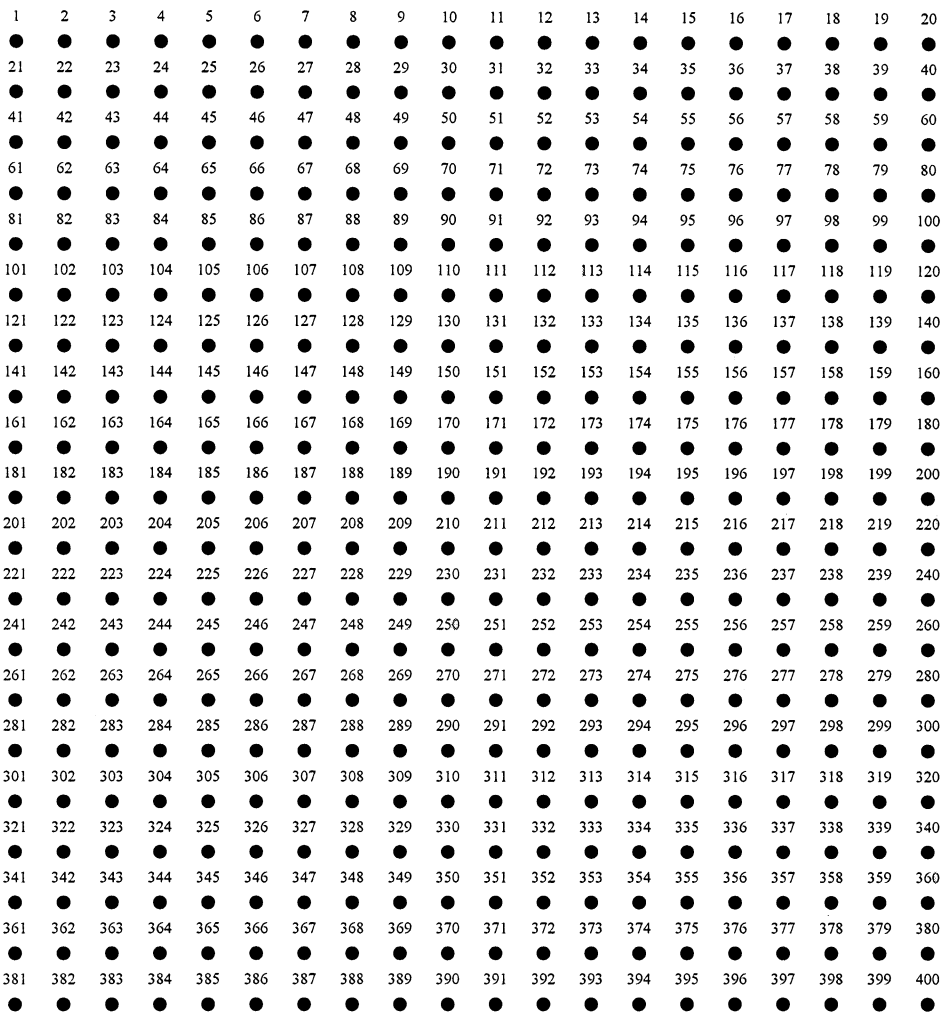


Figure 2. Schematic presentation of high density dot-Southern analysis. All 400 cDNAs can blotted on a 5"x5" membrane and their expression in embryos or any other tissues can be scanned by a simple hybridization.

Isolation and Characterization of Loblolly pine embryo specific vicilin-like storage protein gene; Use of this gene as a molecular marker for somatic embryo sorting

Ranjan Perera, Gary Peter, Gerald Pullman and John Cairney

Introduction

Seed storage protein accumulation is tissue specific. It occurs primarily in specialized storage cells in the embryonic axis, cotyledons, and the endosperm of developing seeds but never in mature vegetative organs (Thomas 1993). Seed storage protein genes are regulated temporally and spatially during seed development (Goldberg et al;). However behavior of these genes during embryogenesis has not been investigated extensively.

Storage proteins are classified into four groups according to their solubility properties. Albumins are water soluble; Globulins are salt soluble; Glutelins are soluble in acids, alkali ionic detergent, and urea-containing solutions; and the Prolamins are alcohol solubleHere we discuss the isolation and characterization of vicilin (7S-globulin) like seed storage protein gene pIPST-RP22, from gymnosperm *Pinus taeda.*

Results

Loblolly pine vicilin gene was isolated by Polymerase chain reaction from the stage 8 Zygotic embryo cDNA . Sequence comparison of the initial 380 bp cDNA fragment shows 90% Nucleotide sequence alignment with an already identified vicilin -like gene from *Picea glauca,* and 60% with *Zamia furfuraceae* (Cycad) *(figure 1).* Also, figure 2 shows the amino acid alignment. We have now cloned a 1400 bp, (almost full length cDNA) of the Loblolly pine vicilin gene and currently sequencing it.

Gene expression analysis showed that, vicilin gene is expressed in stage 8 and stage 9 pine Zygotic embryos, and not in stage 3 and stage 4 embryos. Also this gene is expressed in stage 9 somatic embryos and not in pine cell suspension culture. Currently, we are proceeding similar experiments to demonstrate vicilin gene expression in other somatic and zygotic embryos.

Copy number of this gene in loblolly pine and other plant species, southern data with PCR products and gene expression in northern analysis will be discussed

Somatic Embryo sorting

Here we demonstrate how vicilin gene can be used as a useful marker gene to detect the somatic embryo development process, using PCR base technique. This will allow the researcher to sort the somatic embryos more accurately according to their stage specificity. This technique is based on a competitive PCR approach using non-homologous internal standards called PCR MIMICs. These are DNA fragments

constructed for use in competitive PCR amplification for quantitation of target mRNA levels. Each PCR MIMIC consists of a heterologous DNA fragment with primer templates that are recognized by a pair of gene-specific primers. Thus, these templates "mimic" the target and are amplified during PCR.
By knowing the amount of PCR MIMIC added to the reaction, one can determine the amount of target template, thus the initial mRNA levels.

It is important to understand the somatic embryo specific transcriptional control and assess the effects of morphogenesis on vicilin like Loblolly pine storage gene expression in different stages in somatic embryogenesis.

Promoter of the Loblolly pine vicilin gene could be fused with Green Fluorescent protein GFP and then the resultant construct (chimeric gene), representative of the mid and late embryo maturation program (embryo detector) will be introduced into cell suspension or calli via an appropriate transformation technique. We will require only single independent trasformant (either calli or early embryo) as a starting material for further continuation.

According to the level of chimeric gene expression , one can sort the somatic embryos more accurately according to their development stages. This will provide more precise sorting system, compare to currently available phenotypic methods. One of the major advantages of using the Viclin promoter compare to other embryo specific promotes are, vicilin gene is expressed mainly in the mid and late stages of embryogenesis and this is where we see a major embryo development and germination problem in somatic embryos.

Literature Cited

Thomas T (1993) Gene expression during plant embryogenesis and germination: an overview. **Plant Cell 5: 1401-1410**

Goldberg RB, Baker SJ, Perez-Grau L (1989) Regulation of gene expression in plant embryogenesis. **Cell 56: 149-160**

Figure 1

Amino Acid Sequence alignment of *Pinus taeda* vicilin gene pIPST-RP 22 with *Picea glauca and Zamia furfurac*

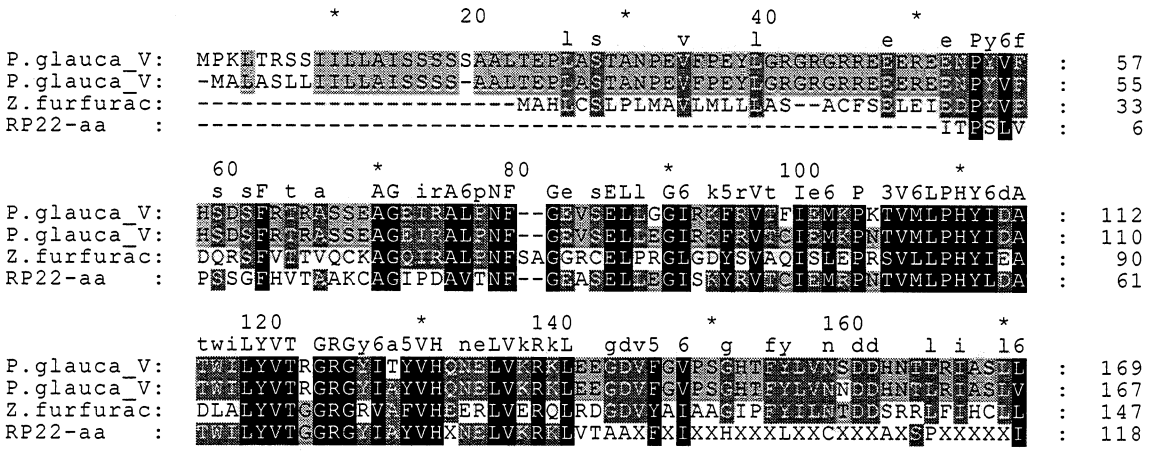
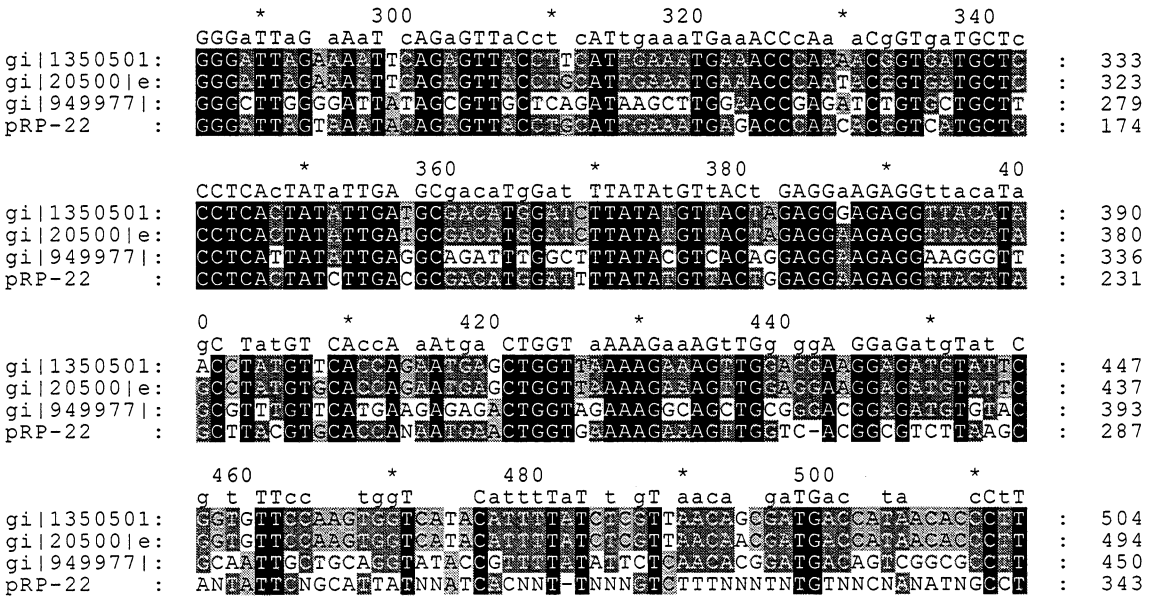


Figure 2

Nucleotide alignment with *P.glauca* and *Z.furfurac*



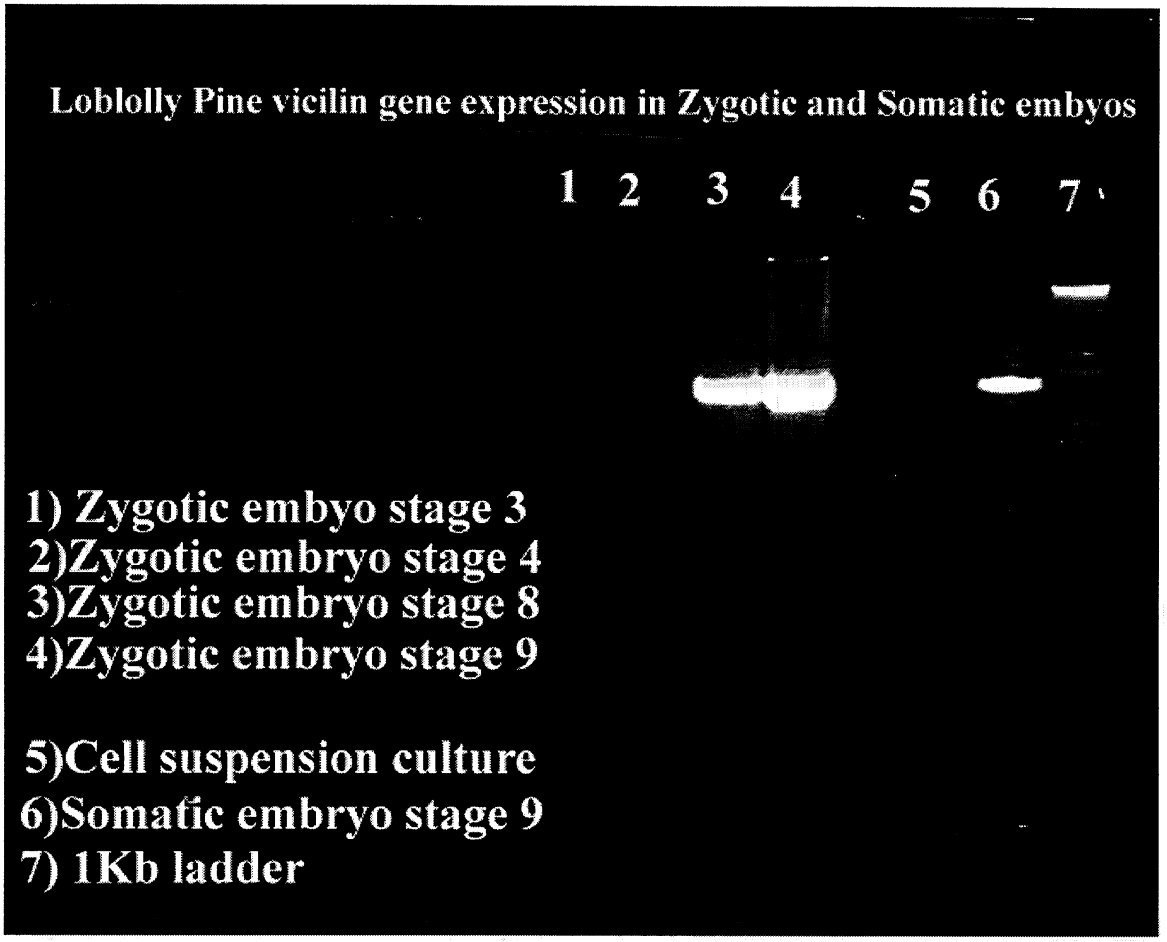


Figure 3

Literature Cited

Thomas T (1993) Gene expression during plant embryogenesis and germination: an overview. **Plant Cell 5: 1401-1410**

Goldberg RB, Baker SJ, Perez-Grau L (1989) Regulation of gene expression in plant embryogenesis. **Cell 56: 149-160**

Applying Expertise in Flowering Research to Embryogenesis I: Expression of Floral Homeotic Genes in Pine Embryos

Lin Ge, Ranjan Perera, Gerald Pullman, Gary Peter and John Cairney

Floral homeotic genes are important regulators of floral development. Their sequence is strongly conserved between species but their pattern of expression is variable. We have applied the expertise and insight gained through studying pine homeotic genes to the study of pine embryogenesis. We have demonstrated the expression of certain gene regulators during embryogenesis, a hitherto unreported and unsuspected finding. This work has important consequences for Somatic Embryogenesis and for genetic engineering of floral development.

The goal of the early flowering project is to generate early flowering plants for commercially important conifer (loblolly pine) by genetically manipulating the expression of floral meristem identity genes. Create controlled early flowering loblolly pine plants will greatly increase the breeding efficiency and dramatically shorten the breeding cycle. Accelerated breeding programs will allow us to rapidly characterize genetically engineered transgenic plants, evaluate the strategy of our sterility project, and fix valuable new traits introduced by gene transfer into the new genetic backgrounds.

Floral meristem identity genes are early-acting genes in flower development. They promote the formation of floral meristems and activate the expression of flower development intermediate genes and floral organ identity genes. LEAFY is one of the first known floral meristem identity genes. Constitutive expression of LEAFY leads to early flowering in Arabidopsis and hybrid aspen plants. LEAFY is also the candidate for the generating of early flowering loblolly pine plants. Here we report our preliminary results towards the isolation and expression study of Loblolly Pine LEAFY gene.

Isolation of LEAFY gene from Loblolly Pine

We have constructed a "Genomewalker Library" (Clontech, CA) in order to isolate fragments upstream of genes (eg. Promoters) or downstream of genes (eg. 3'regulatory regions). This technique involves enzymatic digestion of genomic DNA, ligation of linkers to the endz. PCR is then conducted using a primer specific to the gene of interest plus a primer specific to the end linker. The intervening sequence is amplified and in two rounds of PCR (using a second set of nested primers) and the amplified region is analyzed.

LEAFY and its homologs have been cloned from different plant species. According to the multiple alignment of amino acids sequences of LEAFY proteins, LEAFY gene specific degenerate primers were designed according to the conserved regions of LEAFY proteins. Using one of the LEAFY specific primer and conventional Genome Walk Library primers (from Clontech, CA), several putative loblolly pine LEAFY fragments were amplified from loblolly pine Genome Walk Library. One of the fragment, shown strong hybridization signals with

Arabidopsis LEAFY coding region were cloned and sequenced. This putative loblolly Pine LEAFY fragment contains two interesting regions, a LEAFY homologous domain and a kinase homologous domain. The LEAFY homologous domain could be the conserved DNA binding domain which exits in all LEAFY proteins. The kinase domain could be a novel domain regulating the cell cycles or the target of complicated signal transduct pathway. To confirm the extraordinary structure of this putative loblolly pine LEAFY fragment, new primers were designed and longer fragment will be amplified by using same strategy. In the meantime, other putative fragments are under cloning and sequence analysis.

Vegetative Expression of LEAFY in Loblolly Pine Embryos

The vegetative expression of loblolly pine LEAFY and its homologs was suggested by the hybridization of arabidopsis LEAFY cDNA coding sequence to cDNAs originated from mRNA of stage 4, 8, 9.2 and 9.8 zygotic embryos. And the expression was also investigated by RT-PCR. The RT-PCR results suggested that loblolly pine LEAFY is expressed in zygotic and late stage of somatic embryos.

In conclusion, putative loblolly pine LEAFY, AP1 and AP2 fragments were obtained and our preliminary experiments suggest loblolly pine LFY, AP1 and AP2 might also expressed in vegetative embryos other than reproductive organs. This might explain why LFY and AP1 transgenic plants intended to not survive at the early stage. If these homeotic genes are also involved in the development of other important plant organs other than reproductive organs, interrupting their normal expression will certainly result the death of plant. The vegetative expression of these homeotic genes also might interfere the regeneration of transgenic plants and propagation of elite plant through embryogenesis approach. This may present a severe challenge to the regeneration of sense suppression, antisense or Dominant Negative mutation floral homeotic transgenic plants.

Our future work will including cloning full length LEAFY, AP1, AP2 cDNA from embryos and flower primordia. The temporal and spatial expression of these genes will be studied using In Situ Hybridization and RNAse Protection Assay. Also new homeotic genes will be isolated using differential display and subtractive hybridization method. And only the proper unique homeotic genes and their promoters will be used for the generating of sterile and precocious early flowering loblolly pine plants.

Applying Expertise in Flowering Research to Embryogenesis II: Isolation and Characterization of Flower specific (homeotic) genes from Loblolly Pine, and expression of these genes in vegetative tissues.

Ranjan Perera, Lin Ge, Gerald Pullman, Gary Peter and John Cairney

Abstract:

Concern over the dispersal of genetically engineered 'transgenes', through pollen, has quickened interest in controlling flowering in plants. In addition to preventing escape of 'supergenes', the suppression of flowering in trees may result in an increase in biomass, diverting energy from the reproductive organs.

Homeotic genes are essential in floral development in herbaceous plants. Though mutational studies, the involvement of particular homeotic genes in the production of stamens, carpels, sepals, and petals have been demonstrated. Disrupting the expression of these can result in disruption of flower formation or failure of plants to produce certain floral organs. The recognition that a small number of genes can determine the production of floral structures has allowed a number of groups to create male-sterile plants by disrupting of aborting the cells destined to become floral organs. Several groups have already demonstrated that these genes in softwood and hardwood species have a high homology to already characterized homeotic genes in *Arabidopsis thaliana.*

In a number of model plants, reducing the expression of homeotic genes, by antisense or co-suppresion strategies, has been used to engineer sterility. However, when genes from one species were used to suppress flowering in a related species, the results were not always successful, due, presumably, to minor but significant differences in patterns of gene expression. The implication is that floral suppression will require genes from the plant under study or from a very near relative.

Currently we are focusing on the plant homeotic genes such as: LEAFY (LFY), AGAMOUS (AG1), APETALA (AP2) since these are ideal targets for engineering sterile plants, and seeking to isolate the Loblolly Pine homologs of these genes.

Project Objectives:

- Isolate genomic and cDNA clones of Homeotic genes from Loblolly Pine mainly by Polymerase chain reaction technology.
- Clone and sequence these gene fragments to confirm the identity.

- Investigate the expression of these genes in floral organs as well as in different stages of Loblolly Pine zygotic embryos.

- Isolate promoter fragments from homeotic genes in order to engineer constructs for cell ablation studies.

- Transfer above recombinant gene constructs (vectors) into Loblolly Pine and other gymnosperms by biolistics or Agrobacterium mediated transformation.

Results and discussion:

⇒ We have isolated partial copies of the **AP2** , **AP1,** and putative **Agamous** genes from Loblolly Pine. Several other additional genes with homology to MADS box genes have also been isolated. To our knowledge, this is the first time **AP2** and **AP1** genes has been isolated from a conifer.

⇒ A systematic approach to optimizing PCR reaction conditions was undertaken. By altering potassium and magnesium iron concentration and also the pH of the buffers, several PCR products (homeotic gene homologs to Arabidopsis) from Loblolly Pine were isolated. We have cloned these fragments into the Invitrogen pCR2.1 cloning vector and sequenced the fragments. Using the PCR primers based on the sequence of AP2 from *Arabidopsis thalians*, we isolated a partial Loblolly Pine apetala genomic fragment. The DNA sequence shows 88% nucleotide identity to *Arabidopsis thaliana* sequence.

⇒ We have also studied the expression of apetala homeotic genes in Loblolly Pine embyos. We prepared cDNA from mRNA extracted from Loblolly Pine zygotic embryos from stage 3, 4, 8 and 9. These cDNA pools were used as templates for several PCR reactions in order to observe the expression of homeotic genes at various stages of embryogenesis.

⇒ Southern hybridization and subsequent blotting and probing of PCR products using an AP2 probe, confirm the presence of an AP2-like mRNA in Loblolly Pine Zygotic embryos.

⇒ We have in addition, demonstrated the presence of floral (homeotic) genes *agamous*, *apetala*, and *leafy* in Loblolly Pine by genomic southern hybridization.

⇒ We have already prepared a promoter library to isolate promoters from the pine homeotic genes and also started to prepare a cDNA library from Loblolly Pine Zygotic embryos, to obtain full length of cDNA clones.

⇒ We will commence the *in situ* hybridization to demonstrate the expression of homeotic genes in Loblolly Pine embryos. Protein and RNA from different stages of Pine embryos will be used to confirm the above results by western blotting and Ribonuclease protecting assay.

⇒ A number of additional genes with homology to MADS box genes have been isolated and are being analyzed.

Conclusion:

- We have, demonstrated the presence of floral (homeotic) genes agamous, apetala2 and apetala1 in Loblolly Pine by genomic southern hybridization.

- We have isolated a partial copy of the AP2 gene and Ap1 gene and a putative copy of *Agamous* gene from Loblolly Pine . To our knowledge, this is the first time *Apetala* like gene has been isolated from a conifer.

- We have demonstrated that Homeotic genes *Apetala* and *Agamous* are expressed in Loblolly Pine embryos in stages 3, 4, 8 and 9.

F011: Mass Clonal Propagation of Hardwoods

Establishing and Improving the *Zinnia elegans* Tracheary Element Differentiation System at IPST

Gary Peter
Karen Floyd
Chris Ricker

Introduction:

Zinnia elegans is a model tissue culture system that can be used to study cellular and molecular mechanisms that control tracheary element (TE) differentiation. This culture system was developed in the early 1980's (H. Fukuda and A. Komamine, 1980 Plant Physiol. 65: 57-60). In this system primary leaves from 10-14 day old seedlings are harvested and surface sterilized. Individual cells are mechanically isolated by grinding and cultured in defined media. In the presence of auxin and cytokinin ~50% of the isolated mesophyll cells transdifferentiate into TEs within 72 hours. The properties that make this a valuable model system include its hormone inducibility, synchrony, and single celled nature. This system has been used to elucidate aspects of hormone signal transduction in TE differentiation, cytoskeletal control of secondary cell wall formation, biochemical analyses of lignin biosynthesis, identification of genes expressed in differentiating TEs, and programmed cell death (H. Fukuda, 1996 Annu. Rev. Plant Physiol. Plant Mol. Biol. 47: 299-325; H. Fukuda, 1997 Plant Cell 9 (7)).

One of the major technical challenges to improve this system for investigating regulatory mechanisms was to develop a robust transient genetic transformation method. Transformation can then be used to identify gene regulatory sequences, help elucidate the function of unknown genes expressed in TEs, and to rapidly test strategies for altering TE formation. Previously at University of California Berkeley, I developed a transient transformation assay for *Z. elegans* utilizing *Agrobacterium tumefacians* (G. Peter, unpublished).

We are now standardizing this transformation assay to the growth conditions used here at IPST. We are also designing, constructing, and testing gene expression vectors to use in transient assay experiments that are meant to elucidate the function of novel gene products in TE differentiation and secondary wall formation with the *Z. elegans* model system.

Critical Parameters for Establishing *In Vitro* Differentiation of TEs with *Z. elegans*

A number of parameters are important for establishing the *Z. elegans* differentiation system in a new lab. One of the most important aspects of making the *Z. elegans* tissue culture system work is the growth of the plants. Plants are grown in chambers with 16h of light, 8h of darkness at 27°C. After repair of the existing growth chambers we started growing our plants as close to the fluorescent and incandescent bulbs as possible but still maintaining good temperature control. Maximizing light intensity helps the plants to grow quickly. Primary leaves should be >4 cm within 12 days.

Other critical parameters for successfully getting TE differentiation *in vitro* with *Z. elegans* is the leaf sterilization and maceration techniques. We use low levels of bleach (0.05%) for 10 minutes. After sterilization, the epidermal surfaces of the leaves should still look glossy and not dark green. These low levels were used so as not to damage or kill leaf cells. The maceration process is necessarily damaging, but it is important to limit this damage and cell death. After maceration and washing > 50% of the cells must be viable for efficient differentiation to occur.

Optimization of TE Transdifferentiation of *Z. elegans* Mesophyll Cells

We have standardized the growth of *Z. elegans* plants to new soil mixes, fertilizers and growth chambers. Leaf sterilization and cell isolation were done successfully once good plant growth conditions were established. We are now getting normal rates of differentiation and normal timing for the onset of differentiation (Table 1).

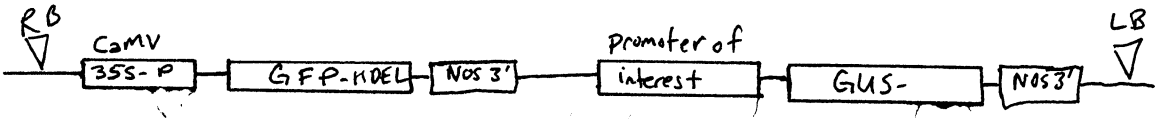
Table 1

Experiment #	% of Tracheary Elements at 72H
1	55
2	50
3	45
4	62
5	55

Expression Vector Construction:

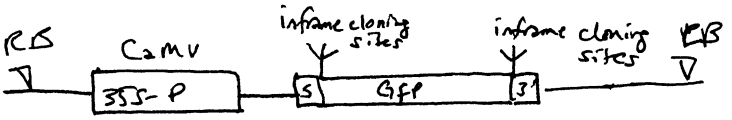
In order to determine gene regulatory elements, the subcellular locations of and to test for the functions of novel proteins by overexpression or antisense strategies we need to design and construct at least three separate expression vectors. To analyze for gene regulatory elements, the 5' and 3' sequences that flank the coding region of the gene are placed next to a reporter gene for example, β-glucuronidase (GUS) (Figure 1). In addition on this vector a control gene e.g., green fluorescent protein (GFP), is required to standardize the results from transformation efficiency. For subcellular localization studies a protein fusion vector to green fluorescent protein (Figure 2) is needed. This vector must contain a promoter that drives expression in differentiating TE's and cloning sites to fuse the protein of interest to either the amino terminus or the carboxyl terminus of GFP. A third vector is one where the gene of interested is placed downstream either in the sense or antisense direction of a promoter that is active in differentiating TE's (Figure 3). This vector also must have an control marker gene to identify which cells in the experiment were transformed.

Figure 1: Vector to Identify Gene Regulatory Sequences



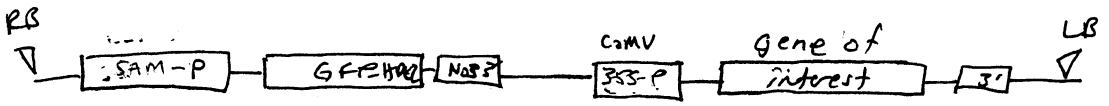
To identify gene regulatory sequences the vector we are constructing contains the SAM promoter driving the expression of GFP-HDEL (see below for a description) and the promoter of interest driving the expression of β-glucuronidase gene. The backbone vector is pBI 101 in which the NOS-NPT II gene has been deleted.

Figure 2: Vector for Subcellular Localization of Novel Proteins in Differentiating TEs



We are in the process of the constructing expression vectors for subcellular localization. For the subcellular localization experiments we propose to use the codon modified synthetic GFP, which enhances expression in humans and plant cells (Hass, Park, Seed 1996 Curr. Biol. 6: 315-324). This GFP will be driven by the CaMV 35S promoter and in frame cloning sites at both the 5' and 3' ends will be engineered into this vector. The backbone vector for these constructions will be pBIN 19 that have the NOS promoter and NPT II gene deleted from it.

Figure 3 Vector for Sense and Antisense Expression



For overexpression and antisense strategies to work a strong promoter needs to drive the expression of the gene of interest. We will use the CaMV 35S promoter, since I already know that this works well in differentiating TEs from *Z. elegans*. This vector also needs an internal reporter to mark cells that are transformed. The internal control for in our overexpression vector is a version of GFP with an HDEL sequence for localization to the endoplasmic reticulum (ER). This ER resident version of GFP is being used, because it removes the protein from the cytoplasm and minimizes any adverse or toxic effects that could occur due to its presence in the cytoplasm. For expression of this GFP we are testing whether the S-adenosyl methionine (SAM) synthase promoter is expressed in differentiating TEs from *Z. elegans*. The SAM promoter is highly expressed in the xylem tissues of *Arabidopsis thaliana*.

Testing of the Constructs in Transient Assays:

We are in the process of standardizing our transient assays so that we can test the SAM promoter and different versions of GFP. This work is just getting under way.

Gene Regulation in Woody Plants I: Promoter and 3'UTR effects

Regulation of Expression of a Stress-related Gene: Promoter and 3' UTR Analysis- An Update

Luis Destéfano-Beltrán and John Cairney

In spite of the repeated accomplishments of plant genetic engineering in the last ten years, constant levels of expression of the transgenes is not a foregone conclusion of a successful transformation. Some of the "rules" for obtaining good expression are lately becoming clear. For instance choice of a suitable promoter, a proper 5'-UTR or use of an intron in a gene that lacks one of its own can increase the level of expression obtained from a given gene. Notwithstanding, in addition to large variations in the expression levels due to the positional insertion site, a large number of other potential problems with detrimental effects on gene expression still subsist, especially when using a gene from a heterologous source. Improper polyadenylation, improper nuclear transport, instability of the resulting cytosolic mRNA, or an impaired translatability can result in accumulation of only a low level of both mRNA and the resulting protein. Altogether, these different levels of control of gene expression fall in the realm of what is known as **post-transcriptional** regulation. This swift and flexible form of gene regulation is relatively understudied, compared to, say **transcription**, but it has a central role in plant growth and development especially for generating a fast response to environmental and intracellular signals. Correspondingly, it has an important application in biotechnology for the development of plant expression vectors which ensure correct expression of transferred gene sequences. Our work focuses on the study of **two drought-responsive** genes which are regulated by distinctive and possibly overlapping post-transcriptional mechanisms. We are studying two examples of posttranscriptional control and probing the transcriptional control of one of the genes.

In mammalian and more recently in plant systems such features have been implicated in the translational control of their downstream messages. The second is exemplified by the AU-rich containing 3'-UTR of one of the members of a multigene family of proteinase inhibitors (PI) up-regulated by drought-stress from *Atriplex canescens*. Similar motifs have been implicated in the regulation of the stability and translatability of other RNA messages. We are conducting experiments to illuminate the function of the 5'- and 3'-UTR's by examining their effect upon adjacent sequences (reporter genes) and by following expression of endogenous sequences. We will also examine the structure of the cognate genes.

Characterization of Two PI Promoters:

Two genomic clones, 18-1 and 12-95, contain promoter sequences of ca. 1.3 and 2.4 kb respectively. Several constructs including full-length and deletion mutants of these promoters have been obtained and fused to the GUS reporter gene. In both cases the GUS

gene was fused in-frame a few codons downstream from the AUG initiator codon. Similarly, two extra constructs contain the full length promoters, the 5' untranslated region, the first exon, the first intron and a few codons of the second exon fused in-frame upstream of the reporter gene.

The promoter of the 18-1 gene contains several single restriction sites that have resulted in the production of the following deletion mutants expressed as the distance in base pairs from the transcription initiation: -1397 (full length), -949, -794, -558, -254, and -146. Additionally, there is an A-rich region (60/108), including a row of 30 A's, between -254 and -146 that has been deleted from a full length construct in order to evaluate its role as a putative novel regulatory element. Similarly, deletion mutants for the 12-95 promoter gene include: -2400 (full length), -1800, -990, -541, -266, -133, and -101. The borders of all constructs have been confirmed by sequencing.

Update: *All constructs based in the pBIN19 binary vector have been introduced to Agrobacterium tumefaciens following standard protocols. The integrity of these constructs has been confirmed by restriction analysis. Selected constructs are currently being introduced into tobacco plants. Tobacco is easy to regenerate and will act as a model plant giving us information quickly. Those constructs which look most promising can be in more long-term experiments in trees. Expression of the reporter GUS gene will evaluated in planta under normal and stressed conditions. Selected cassettes containing /Promoter fragment/GUS/NOS 3'/ based in pUC derivative vectors will be used in transient assays using tobacco and Atriplex cell suspensions.*

Characterization of a 3' UTR element:

Previous work in our lab showed that the PI gene family could be grouped into two classes according to the presence or absence of an AU-rich region in their 3'UTR end. One of the genomic clones, 18-1, contains such a motif in its 3'UTR region. A similar approach used for the analysis of the promoters has been used to analyze the role of such region in mRNA stability and/or translatability. Several 3' to 5' deletions of the region after the termination codon have been placed downstream of the cassette CaMV35S/GUS using single restriction sites. Standard cloning techniques have resulted in the production of the following deletion mutants expressed as the distance in base pairs after the termination codon: + 187, +361, +583, +766, +1005 and +1574. As before, the pBIN19-based constructs have been introduced into *Agrobacterium tumefaciens* and their integrity and stability upon transformation examined. Selected pUC-based constructs will be used in transient assays in either tobacco or Atriplex cell suspension cultures. Different effectors will be tested to monitor any effect in the chimeric GUS mRNA stability and/or translatability: ABA, PEG, MeJ, temperature and salt stress.

Update: Construction of a "sense" PI vector. In order to examine the role of these proteins during drought stress the coding sequence of one isoform, clone 8-3, has been placed under the control of the constitutive CaMV35S promoter followed by the nopaline synthase polyadenylation signal. The resulting expression cassette was transferred to the

pBIN19 binary vector and subsequently introduced into A. tumefaciens. We are developing a collaboration with Dr. Kevan Gartland, University of Abertay, Dundee, Scotland. We intend writing a NATO grant to support collaborative research. The construct will be introduced into selected transgenic plants via A. tumefaciens by a collaborator of ours in Scotland

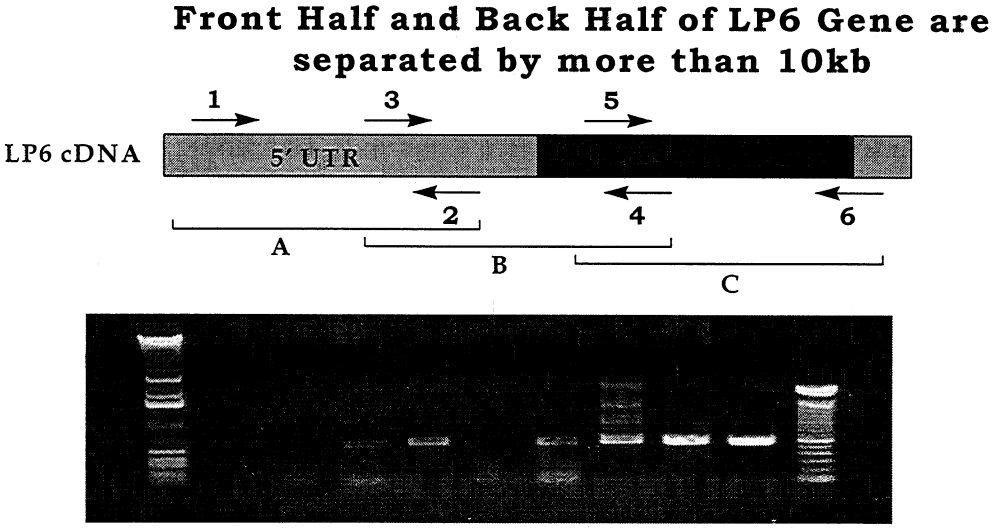
Gene Regulation in Woody Plants II: 5'UTR effects

Regulation of Expression and Gene Structure of a down-regulated gene during drought stress

Luis Destéfano-Beltrán and John Cairney

As part of a project to examine gene expression in response to water deficit in loblolly pine, a cDNA library was constructed from the roots of 5-month old seedlings which had been deprived of water for 11 days. The library was differentially screened and several examples of inducible and repressible clones were selected. One clone, LP6 (1,488 bp), was strongly expressed in the roots, stems and needles of well-watered plants, but mRNA levels declined rapidly as plants dehydrate. On Northerns, a single transcript of ca. 1.5 was detected suggesting that we had a full length clone. The longest ORF could encode a 216 amino acids polypeptide with a predicted MW of 24.2 kDa. and a pI of 5.05. This polypeptide showed strong homology to a number of class I chitinases from bean, tobacco and poplar.; however the similarity was only with the carboxy half of these proteins and none of the features typically found in the amino terminal end (signal peptide, cysteine-rich chitin binding region and glycine-proline-rich "hinge" region) of these proteins are present. Also, neither the putative catalytic site nor the carboxy terminal sequence involved in translocation to the vacuole was present. In addition, the LP6 protein has a carboxy extension of 69 residues not present in any of the chitinases. In contrast to chitinases, LP6 transcripts are not induced but they are greatly reduced 6 h after wounding. An examination of the long 5'UTR leader (721 bp) revealed six upstream sORF's, two in each reading frame, and four inverted repeat structures with secondary structure energies of moderate to high stability. A preliminary analysis of the secondary structure of this long leader suggested that it indeed can be folded into a large stem-loop structure.

The complex nature of the 5'UTR of LP6 suggests a possible role in the regulation of translation of the downstream message. In order to test this hypothesis the full length of this leader has been spliced upstream of the reporter GUS gene and its effect on in vitro translation of the construct will be compared with that of a leaderless GUS. In general, this kind of experiments with other similar systems have showed a negative effect in the translation of the downstream message when compared with a leaderless reporter gene. Several deletions in the 5'UTR spanning regions of possible secondary structure are being made and placed upstream of the GUS gene.



- **In "Long Distance PCR", primer pair 3 + 4 failed to give a band with genomic DNA. This reaction, which uses Polymerases Tth plus 'Vent',should amplify up to 10kb. Control reactions with the plasmid were sucessful and reactions with genomic DNA using the other primer pairs gave a band of expected size. Genomic 1 &2 are different DNA sources**

A recent look into the gene structure of LP6 has rendered some intriguing results. When primers designed to amplify the second third of the cDNA were used in genomic PCR's no products were observed even when Long Distance-PCR (LD-PCR) conditions were used (up to 10 kb). These results are consistent either with the presence of a rather long intron (very unusual in plants) or with the presence of trans-splicing. In order to gain a better understanding of the gene structure of LP6 five different promoter libraries of loblolly pine have been made using a commercial kit. The cloning of upstream and downstream sequences of LP6 will surely reveal its intriguing gene structure.

Also, when total RNA from different stages of development of loblolly pine somatic embryos are used in RT-PCR experiments only the presence of the first third of the message is detected suggesting that the long 5'UTR might be spliced upstream of different messages. 3' RACE and Northern analysis are being performed to test this hypotheses.

To extend the expression analysis of LP6 at the protein level, the longest coding sequence, i.e. the chitinase homologue protein, CHP, has been spliced into two E. coli expression vectors. The successful expression of CHP in E. coli will result in the generation of enough protein to obtain antibodies. The availability of CHP antibodies will allow us to monitor the expression of this putative product of LP6 in stressed and unstressed plants. Overexpression constructs containing full length LP6 and the CHP coding sequence respectively have been made using CaMV35S--NOS3' expression cassettes. The corresponding binary vectors are being introduced into tobacco via Agrobacterium.

Gene Regulation in Woody Plants III:

Identification of A Plant Enzyme Which Activates A Regulatory Peptide

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The continuation of a work initiated as an A190 project with Georgia Tech has resulted in the isolation of several putative genomic clones, from an Arabidopsis library, for a plant counterpart of a regulatory enzyme which in mammals systems regulates the activity of many neuropeptides.
This project seeks in the short term to isolate and characterize such a gene from Arabidopsis, the model plant par excellence, and subsequently from loblolly pine.

The mammal PAM-PGL enzyme system

In mammals, many regulatory peptides require a modification -an amidation- at their carboxy terminal in order to become physiologically active. **PAM** (Peptidylglycine a-amidating Monooxygenase) and **PGL** (Peptidoaminoglycolate Lyase) work in close collaboration to modify and activate certain enzymes. Such mammalian enzymes target include calcitonin, growth hormone releasing factor, LH-RH (luteinizing hormone releasing hormone), vasopressin, gastrin, a-MSH (a-melanotropin) and others. The production of C-terminally amidated peptides can be better described as a two-step process in which a non-amidated peptide produced in the first step, is thereafter amidated at its carboxy end.

The monooxygenase PAM forms the α-hydroxyglycine derivative of the target peptide and the PGL catalyzes the dealkylation step to form the amide and glyoxylate products (Fig. 1)

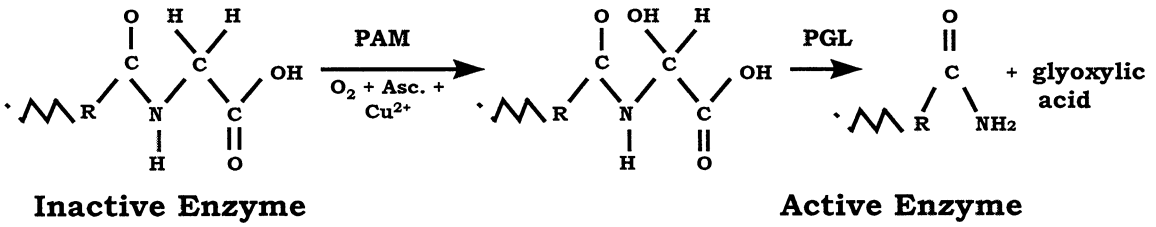

Figure 1. Mechanism of the PAM-PGL system.

In general the animal PAM and PGL enzymes are translated as a single peptide which is immediately self-cleaved into two active peptides. Several PAM-PGL cDNA and

genomic clones have been isolated from several mammalian sources and their gene regulation has been specially investigated for the rat and human forms.

This projects is jointly undertaken with GIT and provides an opportunity to determine novel forms of regulation, to establish an active collaboration with GIT, and to obtain extramural funding.

Early cell culture work showed that a highly specific inhibitor of PAM-PGL arrests growth of pine somatic embryos in a concentration dependent manner. More recently we have pursed this work following molecular approaches and conducted a number of Southern blot analysis to establish the presence of a PAM-PGL counterpart in plants. We did generate a 500 bp PAM fragment from a recombinant baculovirus clone from Dr. Mays lab and used it to probe genomic DNA from Loblolly Pine and Arabidopsis; although the loblolly pine gave a smeared signal the Arabidopsis gave somewhat more pronounced bands at low stringency.

This preliminary result supported the idea that a similar gene might indeed be present in plants and next we decided to screen a library. Since we had no idea as to when and where the gene might be expressed cDNA libraries were ruled out as a source for the clone. Our attention then focused on Arabidopsis because,

- Arabidopsis has a small genome with little repetitive DNA; genes are relatively easy to find
- Genomic libraries are available free of charge from the Arabidopsis Stock Center
- Genomic clones obtained from Arabidopsis will be a useful probe for isolating other plant copies e.g. from Loblolly Pine

Update: *We have now obtained a full-length bovine cDNA clone from Dr. Richard Means, Johns Hopkins University. We have screened 66 000 clones (equivalent to just over three genomes) from an Arabidopsis genomic library. Our initial screening yielded about twenty faint signals and the corresponding plugs were isolated for secondary re-screening. A second round of purification focused on six clones, the remaining clones will be checked in future. DNA from these lambda clones was isolated, and digested, in separate experiments, with EcoRI, HindIII, or XbaI, releasing fragments ranging in size from 6kb to a few hundred base pairs. Gels were blotted onto nylon membranes and Southerns were performed using the bovine PAM-PGL clone as a probe. Hybridization was performed under conditions similar to those used in plaque hybridization: hybridization at 50° C and four washes with 2X SSC, 0.1% SDS. All six clones showed one to several positive signals, confirming that the probe was detecting a specific sequence. To complete the identification of the clone, and to compare it to the animal genes, we must next subclone and sequence these fragments. The fragments have been 'shotgun cloned' i.e. the mixture of fragments from a given lambda clone have been ligated to a vector so that at least one copy of each fragment now resides in a more manageable plasmid vector. The subcloned are currently being analyzed to confirm their hybridization to bovine cDNA. Positive clones will be sequenced.*

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

Following is a list of 1996-1997 research proposals which have been awarded funds, were submitted are currently under review, or were rejected.

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

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. Department 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 to IPST, $21,000 to 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**

Title:	Methods to Assay and Identify Populations of Bacteria Associated with Recycled Containerboard.
Authors (Affiliation):	Gerald Pullman
Awarding Agency:	Containerboard and Kraft Paper Group (CKPG)
Amount Requested:	$42,644
Period of Proposal:	1997
Submitted:	September 17, 1996, Status: **Awarded, $42,644 to IPST**

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:	1996-1997
Submitted:	August 30, 1996
Status:	**Grant Awarded ($20 K to IPST Forest Biology, $20 K to GIT Dept. Chemistry & Biochemistry)**

Title:	The Role of Calcium Dependant Protein Kinases in Xylem Tracheary Element Differentiation and Zygotic Embryos of Loblolly Pine
Authors (Affiliation):	Gary Peter (IPST), and Jung Choi (GIT)
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:	1996-1997
Submitted:	August 30, 1996
Status:	**Grant Awarded ($20 K to IPST Forest Biology, $20 K to GIT Dept. of Biology)**

Title:	Molecular Methods for Induction of Early Flowering in Forest Trees.
Authors (Affiliation):	Jeffrey F.D.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:	$230,000 ($120,000 to IPST)
Period of Proposal:	Fiscal Year 1997 (July 1, 1996 - June 30, 1997)
Submitted:	February 1, 1996
Status:	**Awarded $72,000 to IPST**

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:	$94,075 (IPST), $20,000 (GIT)
Period of Proposal:	Fiscal Year 1997 (July 1, 1996 - June 30, 1997)
Submitted:	13th July 1995
Status:	**Awarded $98,779 to IPST**

Title:	Genetically Engineering Sterility in Commercially Important Southern Trees
Authors (Affiliation):	John Cairney (IPST), Gerald Pullman (IPST), Ronald Dinus (IPST)
Awarding Agency:	The Georgia Consortium for Technological Competitiveness in Pulp and Paper
Amount Requested:	$68,000 (IPST)
Period of Proposal:	Fiscal Year 1997 (July 1, 1996 - June 30, 1997)
Submitted:	13th July 1995 Status: **Awarded $73,500 to IPST**

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:	$68,000 (IPST)
Period of Proposal:	Fiscal Year 1997 (July 1, 1996 - June 30, 1997)
Submitted:	13th July 1995
Status:	**Awarded $68,000 to IPST**

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:	$94,075 (IPST), $20,000 (GIT)
Period of Proposal:	Fiscal Year 1996 (July 1, 1995 - June 30, 1996)
Submitted:	13th July 1995
Status:	**Awarded, $75,000 to IPST, Fiscal Year 1996**

Title:	Ensuring Fiber Supply Through Biotechnology: New Tissue Culture Tools from Gene Expression Studies (Matching Funds).
Authors (Affiliation):	G. Pullman (IPST), J. Cairney (IPST)
Awarding Agency:	Georgia Pacific Company
Amount Requested:	$ 10,000 ($10,000 to IPST)
Period of Proposal:	1996 (These Matching Funds are contingent on receipt of grant (same title) from Georgia Consortium in 1995.
Status:	**Awarded January, 1996 ($10 K to IPST Forest Biology)**

Title:	The Role of Peptide Processing in Plant Cell Growth and Development
Authors (Affiliation):	Ronald Dinus (IPST) and Sheldon W. May (GIT)
Awarding Agency:	Georgia Institute of Technology/Institute of Paper Science and Technology, Pulp and Paper Education Program
Amount Requested:	$ 38,990 ($ 19,681 to IPST)
Period of Proposal:	1995-1996
Submitted:	8th August, 1995
Status:	**Grant Awarded ($20 K to IPST Forest Biology, $20 K to GIT Dept. Chemistry & Biochemistry)**

Title:	Methods to Assay and Identify Populations of Bacteria Associated with Recycled Containerboard.
Authors (Affiliation):	Gerald Pullman
Awarding Agency:	Containerboard and Kraft Paper Group (CKPG)
Amount Requested:	$31,329
Period of Proposal:	1996
Submitted:	January 16, 1996
Status:	**Awarded, $31,329 to IPST**

Pending Proposals

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:	In review

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 $74.5 K (IPST), $64 K (NCSU), Year 2 $78,225 (IPST), $56 K (NCSU)
Period of Proposal:	Fiscal Years 1999 and 2000
Submitted:	August 26, 1997
Status:	In review

Title:	A Loblolly Pine Mutant for Simplified Lignin Removal
Authors (Affiliation):	J. MacKay, D. Dimmel, G. Pullman, and G. Peter
Awarding Agency:	Preproposal - 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:	In review

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:	In review

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 and 1998
Submitted:	April 25, 1997
Status:	In review

Rejected Proposals

Title:	Trees Containing Built-In Pulping Catalysts
Authors (Affiliation):	Gerald Pullman, Don Dimmel, John Cairney (all IPST)
Awarding Agency:	Preproposal - Agenda 2020 - Capital Effectiveness, U.S. Department of Energy.
Amount Requested:	$137,335.65
Period of Proposal:	1997 Fiscal Year
Submitted:	August 31, 1996
Status:	Not Funded

Title:	Signaling Mechanism that Coordinates Lignin Biosynthesis - A General Approach to Regulated Decreases of Lignin Content in Trees.
Authors (Affiliation):	Gary Peter, Gerald Pullman, John Cairney (all IPST)
Awarding Agency:	Preproposal - Agenda 2020 - Capital Effectiveness, U.S. Department of Energy.
Amount Requested:	$113,407
Period of Proposal:	1997 Fiscal Year
Submitted:	September 9, 1996
Status:	Not Funded

DROUGHT PROTECTION IN FOREST TREES: POST-TRANSCRIPTIONAL REGULATION OF STRESS-INDUCIBLE GENES

A Proposal Submitted to BARD

Submitted 8/30/97

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ABSTRACT

Gene transfer technologies may have an enormous impact on tree improvement programs, where long generation times frustrate accelerated breeding. The limited understanding of **post-transcriptional** regulation of gene expression, however, is restricting the potential of genetic transformation. Our studies of water stress-responsive genes in woody plants have highlighted novel features which regulate and may optimize gene expression under drought stress. A 700bp 5'UTR possessing much secondary structure and several upstream open reading frames precedes a 216 residue open reading frame in clone LP6. Further, this 5'UTR appears to be present in several transcripts at different stages of pine embryo development. Stem loops in the 3'UTR of a boiling-stable, drought-inducible protein (BspA) may be responsible for the high expression of this protein and its translational control. Under drought stress, a proteinase inhibitor gene is expressed through two classes of transcript, differing principally in the presence or absence of AU-rich sequences in the 3'UTR. These sequences, once understood, may be incorporated in plant transformation vectors to improve the reliability of gene expression in plants grown in the field where drought is a perennial problem. This proposal describes several experimental strategies which will be used to elucidate the mechanism of regulation of these genes.

Agenda 2020 Sustainable Forestry Program-United States Department of Energy: A Technology Vision and Research Agenda for America's Forest, Wood, and Paper Industry. Submitted 8/26/97

TITLE: Transformation of loblolly pine: *Agrobacterium tumefaciens* gene transfer and improved selection, evaluation, and plant regeneration using somatic embryogenesis.

PRIMARY INVESTIGATORS: **Dr. Allan Wenck**, Postdoctoral Research Associate, Forest Biotechnology Group, North Carolina State University, Box 8008, Raleigh, NC 27695, Phone (919) 515-7800, Fax (919) 515-7801, Wenck@unity.ncsu.edu. **Dr. Gerald Pullman**, Associate Professor, Forest Biology Group, Institute of Paper Science and Technology, 500 10th St. NW, Atlanta, GA., 30318-5794, Phone (404) 894-5307, Fax (404) 894-4778 Jerry.Pullman@ipst.edu. **Dr. Gary Peter**, Assistant Professor, Forest Biology Group, IPST, 500 10th St. NW, Atlanta, GA., 30318-5894, Phone (404) 894-1081, Fax (404) 894-4778 Gary.Peter@ipst.edu.

COLLABORATORS: **Dr. Ross Whetten**, Research Assistant Professor, Forest Biotechnology Group, NCSU. **Dr. Ron Sederoff**, Professor of Forestry, Genetics and Biochemistry, Forest Biotechnology Group, NCSU.

RESEARCH AREA(S) IN THE RFP TO WHICH THE WORK IS TARGETED: Biotechnology: c) genetic transformation methods for tree species, and d) mass propagation methods for tree species.

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 clonal propagation of high-value trees through somatic embryogenesis, along with tree improvement through breeding and genetic engineering, has the potential to help meet future industry needs by increasing forest yields and improving raw material uniformity and quality. The lack of a reliable and efficient procedure to genetically transform loblolly pine and to develop high quality somatic embryos are the major barriers for the commercial implementation of these technologies.

Genetic transformation of many crop species is now viable as shown by the commercial success of recent releases of vegetable and fiber crops with genetically engineered traits. For example, insect and herbicide resistance have been engineered into agriculturally important plants leading to increased yields while simultaneously decreasing the need for agrochemicals. Traits that enhance the quality of the final product have also been engineered. In addition, genetic engineering of plants has helped provide answers to basic biological questions concerning gene function and expression.

Genetic engineering for many important crop plants are now common research and commercial undertakings; however, conifers in general are less advanced and loblolly pine has proved recalcitrant. Two principle reasons account for this: 1) a lack of reliable and efficient regenerative tissue culture method and 2) the most commonly used method for gene transfer, *Agrobacterium,* has shown a low efficiency when wild type strains were used to transform stem tissues in initial experiments. For genetic transformation of recalcitrant species, including conifers, the most successful method so far has been biolistic transformation. However, this technique has several significant problems, including low transformation efficiencies and multi-copy insertions that contribute to a high probability of gene rearrangement, genetic instability, and gene silencing. This later problem is critical because the selectable marker gene must be active to select transformants. *Agrobacterium*-mediated gene transfer is the preferred method for transformation in all amenable crop species due to higher transformation efficiencies, higher frequency of single copy insertion events that are actively expressed, and lack of rearrangement of linked genes. Recent advances in *Agrobacterium*-mediated gene transfer has broadened the applicability of this bacteria to species that were at one time considered resistant to this method of transformation. Most noticeably, high efficiency transformation of rice and corn has recently been reported. In addition, a recent publication has documented transient expression following *Agrobacterium*-mediated transformation of sitka spruce (*Picea stichensis*: Drake *et al.,* (1997) J. Exp. Bot. **48**:151-155).

We also have recently reported high efficiency transformation of Norway spruce (*Picea abies*) using *Agrobacterium*-mediated gene transfer. Norway spruce (NS) was chosen for this work due to the availability of a well defined somatic embryo culture system which readily regenerates plants. All of these reports have used an *Agrobacterium* strain that overproduces the virulence G and B genes, encoded on the tumor inducing plasmid pTiBo542. High efficiency transformation of NS is successful not only because of the *Agrobacterium* strain used, but also because of the availability of rapidly dividing embryogenic suspension cultures.

Somatic embryogenesis is a type of plant tissue culture that starts with a piece of a donor plant and forms new embryos. In conifers, somatic embryogenesis currently involves the culture of zygotic embryos to start or

PROJECT TITLE: A Loblolly Pine Mutant For Simplified Lignin Removal. Submitted 7/1/97

PRIMARY INVESTIGATORS: Drs. John MacKay and Donald Dimmel, and collaborators Drs. Gerald Pullman and Gary Peter, Institute of Paper Science and Technology (IPST)

RESEARCH AREAS IN THE RFP TO WHICH THIS WORK IS TARGETED: Environmental Performance #6 [pulping processes capable of producing easily bleachable pulps, low-kappa pulps at acceptable yield and strength] and #7 [reducing emission of odorous gasses].

BACKGROUND: Several research groups are studying genetic manipulation of lignin in trees as a means to facilitate the pulping and bleaching of wood. Although lignin manipulation has been achieved in poplar, the potential benefits for pulping have not been adequately verified. In addition, it is currently difficult to obtain genetically transformed conifer trees on a sufficient scale to test the effect of genetic modifications of lignin. Therefore, it is unresolved whether improved pulp production in conifers can be achieved by genetic modification of lignin.

We have identified a loblolly pine mutant that synthesizes modified lignin which is comparable to the lignin produced in some genetically engineered poplars. The mutant pine is well defined genetically and is deficient in one enzyme normally required for lignin biosynthesis, cinnamyl alcohol dehydrogenase (CAD). This deficiency is due to a single naturally occurring mutant gene, the *cad-n1* allele, found in breeding stocks of loblolly pine. Mutant *cad-n1* trees can be obtained by well defined crosses, identified through relatively simple genetic selections, and have lignin properties of potential commercial value. The *cad-n1* allele can be used to produce: (a) trees almost completely deficient in CAD activity and having brown wood, and (b) trees partially deficient in CAD activity (~50% of normal) with normal wood color.

The lignins from *completely CAD-deficient trees* contain significantly more aldehyde subunits and two unusual subunits: dihydroconiferyl alcohol and 2-methoxy-substituted structures. Wood from such trees was more extensively delignified in preliminary pulping experiments using uncatalyzed soda cooks. Pulping studies need to be extended to standard kraft, extended kraft, soda/AQ, and polysulfide/kraft and AQ. In addition, the bleachability of the pulps from CAD-deficient trees should be conducted in order to establish that the residue lignin from the pulping step can be easily removed. It might be that the unusual lignin has an easily removed lignin, together with low amounts of hard to extract lignin.

Finally, *partially CAD-deficient trees* (*cad-n1* heterozygotes) are likely to have commercial interest. In genetically engineered poplar, a 30-50% reduction of CAD activity significantly improved delignification. Partial reduction of CAD appears to increase aldehyde content without incorporation of other unusual subunits in the lignin; thus, such wood could be easier to delignify. *Cad-n1* heterozygotes are easier to produce through crosses than trees completely deficient in CAD. In summary, the *cad-n1* allele offers unique opportunities to explore the effect of genetic modification of lignin on delignification during pulping and bleaching, in a conifer.

OBJECTIVES: The general objective is to improve delignification during pulping and bleaching by using trees that contain the *cad-n1* allele. Specifically we will:

1. Identify and characterize trees that have the *cad-n1* allele and are partially deficient in CAD. Analyze partial suppression of CAD in diverse genetic backgrounds.
2. Evaluate the potential benefits for pulping and bleaching of trees with complete and partial CAD-deficiency, emphasizing kraft, kraft/AQ, and soda/AQ pulping and standard DEDED bleaching.
3. Investigate the chemical basis of the modified extractability of lignin in *cad-n1* pines.
4. Determine the spatial distribution of condensed and uncondensed lignin in wild type and *cad-n1* wood chips during various stages of pulping.
5. Evaluate the potential for propagation of superior genotypes containing the *cad-n1* allele.

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PROJECT PROPOSAL APPLICATION

PROJECT TITLE: Molecular analysis of male and female cottonwood trees

Submitted 6/27/97

NAMES OF PRINCIPAL INVESTIGATORS:

David E. McMillin, Ph.D., Clark Atlanta University
John Cairney, Ph.D., Institute of Paper Science and Technology

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AMOUNT OF FUNDING REQUIRED FOR PROJECT 65,000

AMOUNT OF TAPPI GRANT REQUESTED 40,000

BALANCE FROM OTHER SOURCES Graduate stipend and partial salary for Dr. Cairney

TAPPI Research Grants are intended as "seed money" to support new investigations, not for continuation of those already under way. Does the proposed project qualify for such a grant?

YES [X] NO [ ]

SOURCE(S) OF ADDITIONAL FUNDS:

Do you have a firm commitment to fund? YES [X] NO [ ]

ANTICIPATED STARTING DATE October, 1997

PROPOSERS David E. McMillin, John Cairney

ANTICIPATED COMPLETION DATE November, 1998

AUTHORIZED INSTITUTIONAL REP. _____

STATEMENT AND EXPLANATION OF THE WORK PROPOSED:

The forest industry will change dramatically in the near future as the amount of land available decreases. Emphasis must be put on the development of new and innovative methods for tree improvement with the ultimate goal of producing faster growing trees for paper fiber. The hardwood trees are becoming more important to the industry because of their rapid maturation rate. Some of these species are dioecious with separate male and female trees. Tree improvement programs attempt to shorten the growing time and to improve the characteristics of the paper and pulp industry. Currently, it takes at least eight years before the trees can be classified as either male or female. The proposed research is designed to use molecular methods to uncover differences between male and female trees with the goal of sex determination of the tree prior to planting in the nursery saving considerable space, labor and money. The techniques used in the research could be used for the detection of other characteristics of interest.

RELEVANCE TO THE PULP, PAPER AND/OR RELATED INDUSTRIES:

Hardwood trees are becoming an increasingly important source of fiber. Hardwood improvement methods will seek to have a faster growth rate, cold tolerance, and to increase disease and pest resistance. For the breeding program, the research must be able to determine the sex of the tree if it is a dioecious species. Currently, it takes a number of years before the sex of the trees can be determined. We propose to use two molecular methods to determine if differences between male and female trees can be found. The methods that will be used for the study are differential display analysis and representational difference analysis. Both methods are based on subtractive hybridization and could be used for the detection of other traits of interest to the paper and pulp industry. The benefits of this research are:

1. Development of an assay for sex determination prior to planting the trees in the nursery.
2. Information could be used directly by breeding improvement programs.
3. The research serves as a model system for molecular genetic analysis of other traits of interest to the paper and pulp industry. Similar approaches could be used to isolate other genes.

ABSTRACT:

The importance of hardwood trees for the paper and pulp industry is increasing with hardwoods continuing to be a important source of fiber in the future. Some of these trees such as the cottonwood have separate male and female trees. Interest has increased in *Populus* because of the increased growth that has been observed for this tree. This means a faster source of fiber for the paper industry. Tree improvement programs could lead to even shorter growth times, increased disease resistance, cold tolerance, better fiber characteristics, and resistance to herbicides. However, it takes time, labor, space and money to plant trees and find out after eight or more years which trees are male and which are female. The proposed research is designed to isolate differences between male and female trees and to use these differences to develop a molecular genetic test that will be used for sex determination before planting the trees in a nursery breeding program. Finally, the molecular genetic approach being used in this study will serve as a model system to determine if these techniques can be used to isolate different genes coding for other traits useful to the paper and pulp industry, such as fiber characteristics. The two molecular genetic techniques that will be used in the research both are based on substractive hybridization. Differential display analysis is used to isolate expressed sequence differences (mRNA) between two groups. RT-PCR is used in this procedure to make a DNA copy of the RNA. Some dioecious plants have a X/Y chromosome system and *Populus* was one of the species reported as possibly having X/Y chromosomes. Y chromosomes often do not have a lot of expressed sequences. Therefore, the other technique uses DNA differences between the two groups whether the genes are expressed or not. Combining these two procedures, the proposed research has a increased chance of finding differences between the male and female trees.

Title: Fast Growing Trees Through Biotechnology: An Alternative High Value Crop

Co-Principal Investigators: Gary Peter, John Cairney, Gerald Pullman

Institution: Forest Biology Group, Institute of Paper Science and Technology

Rhural America Program - Dept of Agriculture Submitted 4/25/97

PROJECT SUMMARY

Several South American and Southeast Asian countries can export and sell pulp and paper in the US below the cost of production here in the US. The primary advantage of these countries is the high growth rate of trees, especially hardwoods. It is universally recognized that the US forest products industry, an important rural industry, needs a sustainable, low cost supply of raw materials in order to stay globally competitive. Farming of high value, fast-growing trees, in a crop-like setting, offers the potential of sustainably meeting future US needs for high quality forest raw materials. Currently, short rotation hardwoods, mainly *Populus* species, farmed on annual crop lands of marginal quality are optimally harvested in eight years. This delay before a return is the obstacle that keeps the majority of individual farmers from planting trees instead of annual crops. Increasing the growth rate of trees solves these problems. Individual farmers win by gaining profit from unused and marginal lands. Companies win by having a sustainable supply of low cost and high quality, raw materials. The US wins by keeping wood production domestic. We propose to create faster growing trees by genetically engineering elite *Populus* species for increased expression of cyclins in the cambial meristem. We expect increased cyclin activity to stimulate cambial cell divisions, doubling the rate of stem growth and increasing the uniformity of wood fibers used for paper production. This approach has already shown promise in *Arabidopsis thaliana* by increasing the rate of root growth five fold.

PUBLICATIONS - 1996-1997
(Issued, in press, or submitted, * = work done at IPST)

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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 with a * are specifically targeted at the Softwoods Project.

Levi Barclay (M. Sc.) * Second year student.
Title: Bioactive Characterization of Embryo Development Using Antibody Staining Techniques
Advisor: Gary Peter
Summary: Antibody staining techniques will be used to identify specific proteins that are activated during embryo development. The research will relate morphological development with the biochemical changes that occur during embryo development. The ultimate goal is to identify markers, or regulatory changes, which are predictive of the developmental potential of somatic embryos. These markers and rapid staining techniques will be used to determine the quality of somatic embryos propagated from various genotypes and grown in various culture conditions. It is expected that these antibody markers will accelerate research directed towards improving the quality of somatic embryos.

Karen Crews (M. Sc.) * Second year student.
Title: A survey of Anthraquinones in Commercial Trees and their Potential Release from Chips during Pulping
Advisor: Jerry Pullman and Don Dimmel
Summary: Anthraquinones (AQs) can be used at extremely small amounts to improve pulping productivity and environmental factors. AQ increases pulping rates and product yields, removing greater amounts of lignin during pulping. Presently an AQ extract has been found in teakwood as well as several other angiosperms. One goal of this project is to expand our knowledge of AQ content in other tree species. Knowing if commercial tree species contain AQ may give us the knowledge that the pathway for AQ production is present. This will allow us to perhaps "turn up" the particular gene for AQ production in major pulping tree species. Using the trees that contain AQs to facilitate pulping of loblolly pine or another major pulping softwood would be the next step. A tree containing AQ components could be added to a major softwood pulp source, such as loblolly pine. These could be pulped together to promote delignification of the latter. Alternatively, liquors from a hardwood cook that contained AQs could be added to a softwood cook. Such experiments will be done with teak and pine. Other comparisons that will be made will be between known woods that are easily pulped and woods that are hard to pulp. If AQ is determined to have an effect on the ability to pulp commercial trees using the above experiments, altering the gene that produces AQ to generate more would allow for faster and more productive cooks for both AQ containing trees as well as non-AQ containing trees. The discovery of new pulping techniques, as well as a broader knowledge of the content of tree extracts, are possible benefits from this research.

John Ceranski (M. Sc.) Second year student.

Title: Fluorescent Microscopy: A Tool for Determining the Spatial Distribution of Secondary Wall Components Remaining during Kraft Pulping of Loblolly Pine

Advisor: Earl Malcolm and Gary Peter

Summary: The goal of this study is to determine the sequence of lignin and hemicelluloses removal from specific cell types, regions of cells and secondary cell wall layers during Kraft pulping of *Pinus taeda.* The removal and spatial organization of the remaining components at various times during pulping will be characterized with fluorescent probes to specific cell wall components and fluorescent microscopy in thin sections of pulped pine.

Cristine Estes (M. Sc.) * Completed M.S. degree June, 1997. Now working for International Paper.

Title: Free amino acid levels in developing zygotic embryos of *Pinus taeda*

Advisor: Jerry Pullman

Summary: Plant tissue culture is the link between genetic advances made in the laboratory and improved trees and forest products. The goal in tissue culture is to copy high-value trees through somatic embryogenesis. The procedures involved in tissue culture are both time and labor intensive, so methods of improvement to increase embryo quality and yield are desirable.

One of the critical aspects of somatic embryogenesis development is the media on which embryos are grown. Because somatic embryos have been removed from their natural environment, they rely on media to provide them with all the nutrients, hormones, minerals, etc. required for proper development. The media is influential in two aspects of embryonic growth and development; first, it provides nutrients to feed the embryo, and second, it influences the water potential of the system through the solute potential.

Among the compounds present in the natural system are amino acids, or protein precursors. These amino acids are suspected to play crucial roles in both feeding the embryo by providing building blocks for growth and development and in determining the solute potential of the osmotic system. Identification of which free amino acids are present in the female gametophyte and the embryo itself at different developmental stages could provide information on which specific amino acids are essential at which stages.

In this project, frozen embryos and female gametophytes of two clones of *Pinus taeda* will be analyzed for free amino acid content. Embryos will be collected at precise stages of development, stored at -70 degrees Celsius, and analyzed for free amino acids in the system. Free amino acids are available to the embryo for growth and are able to influence osmotic potential in the seed; however, bound amino acids cannot affect embryos in the same ways and will not be measured. By knowing which free amino acids are present in the natural systems, tissue culture media for laboratory somatic embryos can be formulated with specific amino acids to better mimic the natural osmoticants and precursors for growth and development. If tissue culture procedures can come closer to matching the natural processes of embryo development, improve embryo quality and yield could result.

Dana Freeman (M. Sc.)* Completed M.S. degree June, 1997. Now continuing in the Ph.D. program at IPST.

Title: The Role of Peptide Processing in Plant Cell Growth and Development.

Advisor: Jerry Pullman and John Cairney

Summary: Bioactive peptides are involved in practically every cellular function in animal cells, but in the cells of plants, it is just now becoming apparent that peptides play a part in cell growth and development. Bioactive peptides are generated from precursors through post-translational modifications, in particular, carboxy-terminal amidation. The amide group is required for bioactivity and is speculated to play a role in receptor binding and regulation. Two "amidating enzymes", Peptidylglycine a-Monooxygenase (PAM), and Peptidylamidoglycolate Lyase (PGL), have been demonstrated to function in carboxy-terminal amidation of glycine-extended substrates. A number of substrates, inhibitors, and assays have been developed to detect the presence of PAM and PGL enzymes.

A recent discovery of a 37 amino acid peptide isolated from soybean was found to stimulate the phosphorylation activity of a soybean receptor protein, suggesting a role for the peptide in signal transduction. The peptide has a glycine at its C-terminus, and via mass spectral evidence it is revealed that a portion of the peptide is processed to delete this terminal glycine. Through this evidence, it is highly suggestive that amidative processing of bioactive peptides may occur in plant cells.

The objective of this research is to explore whether amidative processing of bioactive peptides occurs in plant cells of both angiosperms and gymnosperms. The research will be carried out with Eastern cottonwood (*Populus deltoides*) and Loblolly pine (*Pinus taeda*). Research with cottonwood seems ideal because the first finding of bioactive peptides in plants was reported for soybean, another dicotyledonous Angiosperm. Loblolly pine embryos exhibit several measurable variations (cell elongation, cell wall development, cell differentiation) that may possibly be altered in the presence of amidation inhibitors. The goal of the research is to obtain evidence for bioactive processing and to determine what consequences inhibition of such processing may have on plant cell development and growth. The experimental approach will be to make use of the selective and potent amidation inactivators and inhibitors by determining their effects on plant cells in culture.

Jeff Grass (M. Sc.)* Completed M.S. degree June, 1997. Now continuing in the Ph.D. program at University of Wisconsin, Madison..

Title: Molecular Characterization of Floral Homeotic Genes Expressed in *Pinus taeda* and *Populus deltoides.*

Advisor: John Cairney

Summary: The pulp and paper industry supports research in genetic engineering with the goal of developing an improved source of raw material. Traditional breeding techniques have been used to successfully achieved this goal in the past. However, the techniques developed by genetic engineering are proving to be more economical than traditional breeding techniques. State and federal agencies strictly regulate the release and use of genetically engineered organisms in the environment. The greatest concern of

the agencies is the transfer of selected genes from one organism. It is hoped that sterile organisms will express lower than normal rates of gene transfer.

The goal of the project is to identify regulatory genes expressed in reproductive tissue of loblolly pine and cottonwood trees. These genes will be used to genetically engineer sterile trees for use in the pulp and paper industry. Sterile organisms can be genetically engineered by disrupting the function of regulatory and structural genes responsible for the development of reproductive tissue. Introduction of modified copies of these genes can cause selective tissue death. The desired genes will be identified using information from previously discovered genes with similar known functions. Once the desired genes have been identified, they will be sequenced and isolated.

Michael Sullivan (M. Sc.) * Second year student.
Title: Assessing Gene Expression Changes During Culture Cycling
Advisor: John Cairney
Summary: Changes in the settled cell volume of liquid suspension cultures have been observed for many Loblolly Pine genotypes. The volume of settled cells follows a pattern, diminishing to a nadir then rising once more to peak at close to (but often below) the previous high. This cycling may be part of a trend of deterioration; maximum cell volumes never matching a previous high, minimum volumes being lower than the previous low. We wish to learn more about this phenomenon, both to follow the cultures through phases of a cycle and to determine similarities and differences between different peaks, different troughs and between peaks and troughs. Differential Display will be employed as a means of following biochemical changes, as manifest in changes in gene expression. Patterns of gene expression will be determined and potential 'marker' bands will be cloned and analysed.

Byron Waldrop (M. Sc.)* Completed M.S. degree June, 1997. Now working for Champion International.
Title: Assessing Somatic Embryo Quality Using Differential Display Techniques
Advisor: John Cairney
Summary: Somatic embryogenesis offers forest geneticists and planters opportunities to multiply superior plants, multiply clones of genetically altered plants, and store genetic information for future plantings. This technique promises to be a powerful tool for insuring an adequate supply of high quality raw material for the paper industry's future. However, the adaptation of somatic embryogenesis techniques to commercially valuable species, such as conifers, has been problematic. It has been difficult to identify high quality embryos, and rates of induction and maturation remain very low compared with those of non-woody plants. Analysis and comparison of gene expression in zygotic and somatic embryos are the keys to understanding these problems. If a gene, or series of genes, are found to be expressed by only a certain quality embryo or only during a certain stage of development, these genes can be used as markers for the classification of other embryos. Comparison of genes that are expressed by zygotic embryos but not by somatic embryo at the analogous developmental stage, may reveal nutritional or environmental imbalances present in the somatic embryogenesis protocol, thus allowing for corrections to be made. The proposed study will use differential display techniques to compare gene

expression during developmental stages of zygotic and somatic embryos. Genes whose expression is found to be specific to a growth condition or developmental stage will be investigated further by isolating, cloning, and sequencing cDNA fragments from gels. The information gathered will then be used to identify high quality embryos, and guide protocol adjustments directed at improving somatic embryo quality.

Vincent Ciavatta (Ph. D.) * Ph.D. 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 which this 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.

Stephen Van Winkle (Ph. D.)* Ph.D. 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.

Thomas Welt (Ph. D.) Matriculated with Ph.D. in June, 1997. Currently working for Procter & Gamble.
Title: Enzymatic de-inking - Effectiveness and mechanisms.
Advisor: Ron Dinus and John Cairney
Summary: Although several theories explaining enzymatic de-inking have been proposed few studies have focused on the mechanism(s) involved. Therefore, the overall

objective of the present study is to generate data which will yield a better understanding of the mechanisms involved in enzymatic de-inking. More specifically we will evaluate the effect of enzymatic action on ink and fibers, and how these actions affect ink release during paper disintegration. A well-defined paper material and highly purified enzymes will be used throughout the study. A technique for visualization of the enzymatic attack on pulp fibers will be developed. Scanning electron microscopy (SEM) will be used to study surface changes caused by enzymatic action. Colloidal gold coated antibodies directed against enzymes or gold-labeled enzymes and transmission electron microscopy (TEM) will be employed to help visualize enzymatic attack on and inside fiber walls. In addition, research efforts will focus on methods to immobilize enzymes. These methods will help to determine: 1) individual effects of a particular enzyme type; 2) if synergistic effects between enzymes are important in de-inking; 3) the spatial distribution of enzymatic attack; and 4) the effect of surface and/or internal action of enzymes on pulp fibers. A literature review based on this dissertation research proposal was published in the February issue of Progress in Paper Recycling.