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FY 97 FINAL REPORT

1. PROJECT TITLE: "Genetic Engineering Sterility in Commercially Important Southern Trees" (FS-3B)

2. PRINCIPAL INVESTIGATOR: Dr. John Cairney

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3. EXECUTIVE SUMMARY OF WORK COMPLETED

- We have isolated a partial copy of the AP2 gene from Loblolly Pine. To our knowledge, this is the first time this gene has been isolated from a conifer.
- We have demonstrated the a number of floral homeotic genes are expressed during embryogenesis in Loblolly Pine. The activity of these genes during this stage of development was previously unknown in plants.
- We have identified several genes related to floral homeotic genes in Arabidopsis. We are in the final stages of confirming the isolation of the Agamous gene from Loblolly pine.
- cDNA libraries representing mRNA from different stages of embryo development are being prepared. These will be a resource for our colleagues at UGA and at other institutions.
- One Research Paper is in preparation, one Poster Abstract has been Published (see attached)
- Three Poster presentations will be made at International Meetings within the next three months
- A Post-Doctoral Scientist with several year experience in International Research laboratories has been recruited to work on the project and an MS student has been supported by IPST

Background and Introduction

The recent spectacular advances in plant molecular biology have made the genetic manipulation of plants a routine procedure. Advances in plant biochemistry and physiology have resulted in increased understanding of plant metabolism and through the techniques of gene cloning, gene transfer and plant regeneration from tissue culture, transgenic plants with altered characteristics have been produced. The reservation which limits the deployment of genetically altered food crops and forest trees alike, is concern over the widespread dispersal of "super genes" through pollen. Environmental Regulations may require that genetically manipulated trees be sterile to prevent dispersal of genes through pollen. There may be additional advantages to sterile trees: several lines of evidence suggest additional growth yields are obtained where metabolism is not directed towards producing reproductive structures. Further, the reduction in pollen is welcomed in urban areas by allergy sufferers.

Research on creating male-sterile trees which produce no pollen has been focused in the Northwest and has concentrated on species important to that area. Currently there is no research focusing on southeastern trees. If Georgia's Pulp and Paper is to remain competitive and avail itself of modern genetic advances, then research on sterility must be conducted with trees important to this region of the country. The following proposal describes a program of research with Loblolly Pine and Cottonwood, both important commercial species for the South.

'Homeotic genes' are very important in floral development in herbaceous species. Through 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 genes 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 or aborting the cells destined to become floral organs.

Once homeotic genes have been cloned, male sterility may be engineered by two approaches:

1. Anti-sense constructs may be used to diminish or abolish expression of the corresponding homeotic gene. This approach is similar to that used for the FLAVR-SAVR tomato.
2. The promoter (control region) of the homeotic gene may be used to direct synthesis of a cytotoxic gene whose product causes cell death. In such a way the cells involved in floral organ formation are destroyed without destruction of other plant structures.

Recently Strauss' group demonstrated that homeotic genes in softwood and hardwood species were very similar to the previously characterized homeotic genes in herbaceous plants. This similarity allowed the cloning of homeotic gene fragments from poplar, pine and Douglas-fir. Thus the materials and strategy for the engineering of sterility in trees seem readily available. However, while the genes involved in flower formation are very similar in even quite distantly related species their differences appear significant: genes from one species show different patterns of expression in another species. Genes specific to floral structure in Species A are also 'on' in other cell lines in species B - using genes from Species A to drive a cytotoxic gene may work well for that species but would likely kill another species. This finding implies that to make a species of tree sterile, the particular homeotic genes from that species must be isolated. Since at present, no-one is working with southeastern trees, Georgia runs the risk of being left out of Tree Biotechnology.

- To ensure that the needs of Georgia Pulp and Paper Industries are being addressed we propose to work on trees of importance to this State. Our approach is to employ both established approaches using genes characterized in other plants and to seek new types of genes. Such genes may have specific biochemical advantages and would of course be free of patents restraining their use.

Progress in FY 97

- We have isolated a partial copy of the AP2 gene from Loblolly Pine. To our knowledge, this is the first time this gene 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. cloned and sequenced. Using PCR primers based on the sequence of AP2 from Arabidopsis, we obtained a partial loblolly pine apetala genomic fragment. The DNA sequence shows 88% homology to *Arabidopsis thaliana* sequence (Figure 1 in Attachments)

- We have demonstrated the a number of floral homeotic genes are expressed during embryogenesis in Loblolly Pine. The activity of these genes during this stage of development was previously unknown in plants.

We have also studied the expression of apetala and leafy homeotic genes in loblolly pine embryos . We prepared cDNA from mRNA extracted from Loblolly pine embryos from stages 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 (apetala and leafy) at various stages of embryogenesis. Figure 2 shows the results of PCR using AP2 primers and subsequent blotting and probing of the PCR products using an AP2 probe. These results confirm the presence of an AP2-like mRNA in pine zygotic embryos.

Figure 3 shows the results of a similar experiment. This time the primers and probes were derived from LFY, indicating the presence of a LFY-like mRNA in loblolly pine embryos.

We have, in addition, demonstrated the presence of floral (homeotic) genes agamous, apetala, and leafy in loblolly pine by genomic southern hybridization (data not shown).

We have already started to prepare a cDNA library from loblolly pine zygotic embryos, to obtain full length of cDNA clones.

In situ hybridization is about to commence to demonstrate the expression of homeotic genes in loblolly pine embryos. These experiments will be conducted in collaboration with Dr. Gary Peter in the Forest Biology Group at IPST. Dr. Peters' group has already started fixing the embryos for *in situ* hybridization experiments.
We have also started to prepare RNA and protein from different stages of embryo genesis to confirm above results by ribonuclease protecting assay and western blotting.
A number of additional clones with homology to MADS box genes have been isolated and are being analyzed.

This project is now closely tied with the groups of Drs. Jeff Dean, Scott Merkle and Sarah Covert at UGA, Athens. Additional work is being communicated by Dr. Dean in a separate report which may reiterate some of these results.

4. DELIVERABLES

Major Milestones & Dates	Original Proposal	Actual
1. Isolate DNA fragments of Homeotic genes by PCR from Loblolly Pine and Cottonwood using degenerate oligonucleotides.	Mo. 1-3	COMPLETED FOR LFY & AG
2. Clone and sequence fragments to confirm identity.	Mo. 1-3	ONGOING FOR LFY & AG
3. Clone reverse orientation fragments into plant expression vectors	Mo. 3-6	ONGOING
6. Construct Genomic Library from Loblolly Pine	Mo. 6-12	ONGOING

Other Objectives contingent on successful completion of these earlier objectives

5. BUDGET

State Funds	Total FY 97	6-Month Expended
	$	$

Matching Funds	Original Proposal	Actual
	$	$

6. Additional Information/Results/Graphics can be attached if available

Project Objectives:

1. Isolate DNA fragments of Homeotic genes by PCR from Loblolly Pine and Cottonwood using degenerate oligonucleotides.
2. Clone and sequence fragments to confirm identity.
3. Clone reverse orientation fragments into plant expression vectors
4. Transfer vectors into Pine and Cottonwood cultures by biolistics (with UGA) and agrobacterial means
5. Regenerate antisense plantlets of loblolly pine and cottonwood.
6. Construct Genomic Library from Loblolly Pine
7. Begin Screening Library using loblolly Pine PCR fragments as probes
8. Isolate promoter fragments and where needed genomic clones for these genes with a view to use in ablation studies using cytotoxic genes.
9. Assay specificity of these promoter fragments by cloning into GUS vectors.
10. Clone promoter fragments into a vector expressing a cytotoxic gene.
11. Transfer vectors into Pine and Cottonwood cultures by biolistics (with UGA) and agrobacterial means
12. Regenerate plantlets of loblolly pine and cottonwood.
13. Begin growth of transgenic trees for subsequent assay.
14. Isolate Novel Genes Involved in Flower Development by Comparing Gene Expression (by Differentila Display) in Axillary Buds destined to form, Pollen Cone, Seed Cone and Vegetative Apices.

B. **FY 96 Milestones:**

Time Table for the Tasks Proposed for FY 1997 and Potential for Future Work

Title: Genetically Engineering Sterility in Commercially Important Southern Trees
Authors (Affiliation): John Cairney (IPST), Gerald S. Pullman (IPST)

Months 1 3 6 9 12 15 18 21 24 Estimated Cost in $

Tasks (See Objectives, p4-5)

1 xxxxxxx
2 xxxxxxxxxxx
3 xxxxxxxxxxxx
4 xxxxxxxxxxxx
5 xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx
6 xxxxxxxxxxxxx

Potential for Future Work

7 xxxxxxxxxxxxxxxxxxxxxxxxx
8 xxxxxxxxxxxxxxxxxxxxxxx
9 xxxxxxxxxxxxxxxxxxxxxxxxx
10 xxxxxxxxxxxxxxxxxxxxxxxxx
11 xxxxxxxxxxxxxxxxxx
12 xxxxxxxxxxxxxxxxxxxxxxxxx
13 xxxxxxxxxxxxxxxxxxxxxxxxxxxxx
14 xxxxxxxxxxxxxxxxxxxxxx

Figure Legends

Figure 1. "BLAST" computer search showing identities in DNA sequence between a DNA fragment from Loblolly Pine which we generated by PCR and the Apetala2 gene from Arabidopsis. This very strong similarity suggests that we have succeeded in cloning the Apetala2 gene from Loblolly Pine

Figure 2. Right: Gel showing products of PCR experiment using AP2 primers and cDNA from loblolly pine zygotic embryos stages 3,4,8 &9. Left: same gel blotted and probed with AP2 from Arabidopsis.

Figure 3. Right: Gel showing products of PCR experiment using AP2 primers and cDNA from loblolly pine zygotic embryos stages 3,4,8 &9. Left: same gel blotted and probed with LFY from Arabidopsis.

NCBI **BLAST Search Results** Entrez ?

BLASTN 1.4.9MP [26-March-1996] [Build 14:27:07 Apr 1 1996]

Reference: Altschul, Stephen F., Warren Gish, Webb Miller, Eugene W. Myers, and David J. Lipman (1990). Basic local alignment search tool. J. Mol. Biol. 215:403-10.

Notice: this program and its default parameter settings are optimized to find nearly identical sequences rapidly. To identify weak similarities encoded in nucleic acid, use BLASTX, TBLASTN or TBLASTX.

Query= tmpseq_1 = (Pine fragment generated by PCR)
(986 letters)

Database: Non-redundant GenBank+EMBL+DDBJ+PDB sequences
335,513 sequences; 516,533,051 total letters.

Sequences producing High-scoring Segment Pairs:		High Score	Smallest Sum Probability P(N)	N
gb|U12546|ATU12546	Arabidopsis thaliana Columbia homeo...	1006	3.9e-74	1
gb|AF003100|AF003100	Arabidopsis thaliana AP2 domain con...	196	8.9e-06	1
gb|U89257|LEU89257	Lycopersicon esculentum DNA-binding...	176	0.00043	1
emb|Z97343|ATFCA8	Arabidopsis thaliana DNA chromosome...	149	0.082	1
dbj|AB005239|AB005239	Arabidopsis thaliana genomic DNA, c...	127	0.998	1

gb|U12546|ATU12546 Arabidopsis thaliana Columbia homeotic APETALA2 protein (APETALA2) mRNA, complete cds.
Length = 1680

Minus Strand HSPs:

Score = 1006 (279.6 bits), Expect = 3.9e-74, P = 3.9e-74
Identities = 220/249 (88%), Positives = 220/249 (88%), Strand = Minus / Plus

Pine
```
Query:   284 TTGGANGTTGGTATGGGTCAATTCTTNGGCNNNANGTATGTTTATTTGGGTTTGTTGGAC 225
             | ||| | | |||||||||||||||| |||   | ||||||||||||||||||||| |||
Sbjct:   892 TGGGAAGCTCGTATGGGTCAATTCTTAGGCAAAAAGTATGTTTATTTGGGTTTGTTCGAC 951

Query:   224 ACCGAGGTCGAAGNTGCTAGAGCTTACGATAAAGCTGCAATCAAATGTAACGCCAAAGAC 165
             ||||||||||||| |||||||||||||||||||||||||||||||||||||| |||||||
Sbjct:   952 ACCGAGGTCGAAGCTGCTAGAGCTTACGATAAAGCTGCAATCAAATGTAACGGCAAAGAC 1011

Query:   164 TCCGTGACCAAGTTTGATCCGAGTATTTACGATGAGGAATTCAATGCCGAGTTATCAGGG 105
             |||||||||| |||||||||||||||||||||||||| |||||||||||| |||||||
Sbjct:  1012 GCCGTGACCAACTTTGATCCGAGTATTTACGATGAGGAACTCAATGCCGAGTCATCAGGG 1071

Query:   104 TATCCTNCNATTCCACCAGATCACNACCTCTATCTGATCTTGGGNAATTGTGTTAATTTG 45
             ||||| | | ||||| ||||||| ||||| |||||| |||||| ||||  | ||||| |
Sbjct:  1072 AATCCTACTACTCCACAAGATCACAACCTCGATCTGAGCTTGGGAAATTCGGCTAATTCG 1131

Query:    44 AAGCATAAA 36
             |||||||||
Sbjct:  1132 AAGCATAAA 1140
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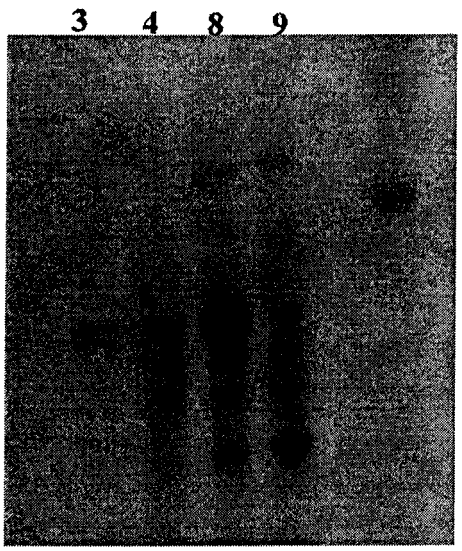
gb|AF003100|AF003100 Arabidopsis thaliana AP2 domain containing protein RAP2.7 mRNA, partial cds

Expression of homeotic gene Apetala
in different stages (3,4,8,9) of Loblolly Pine Embryos.



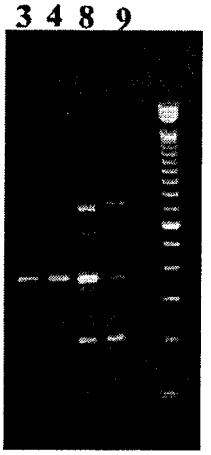


Figure 2

Expression of Leafy in Loblolly Pine Zygotic Embryos (Stage 4, 8, and 9)

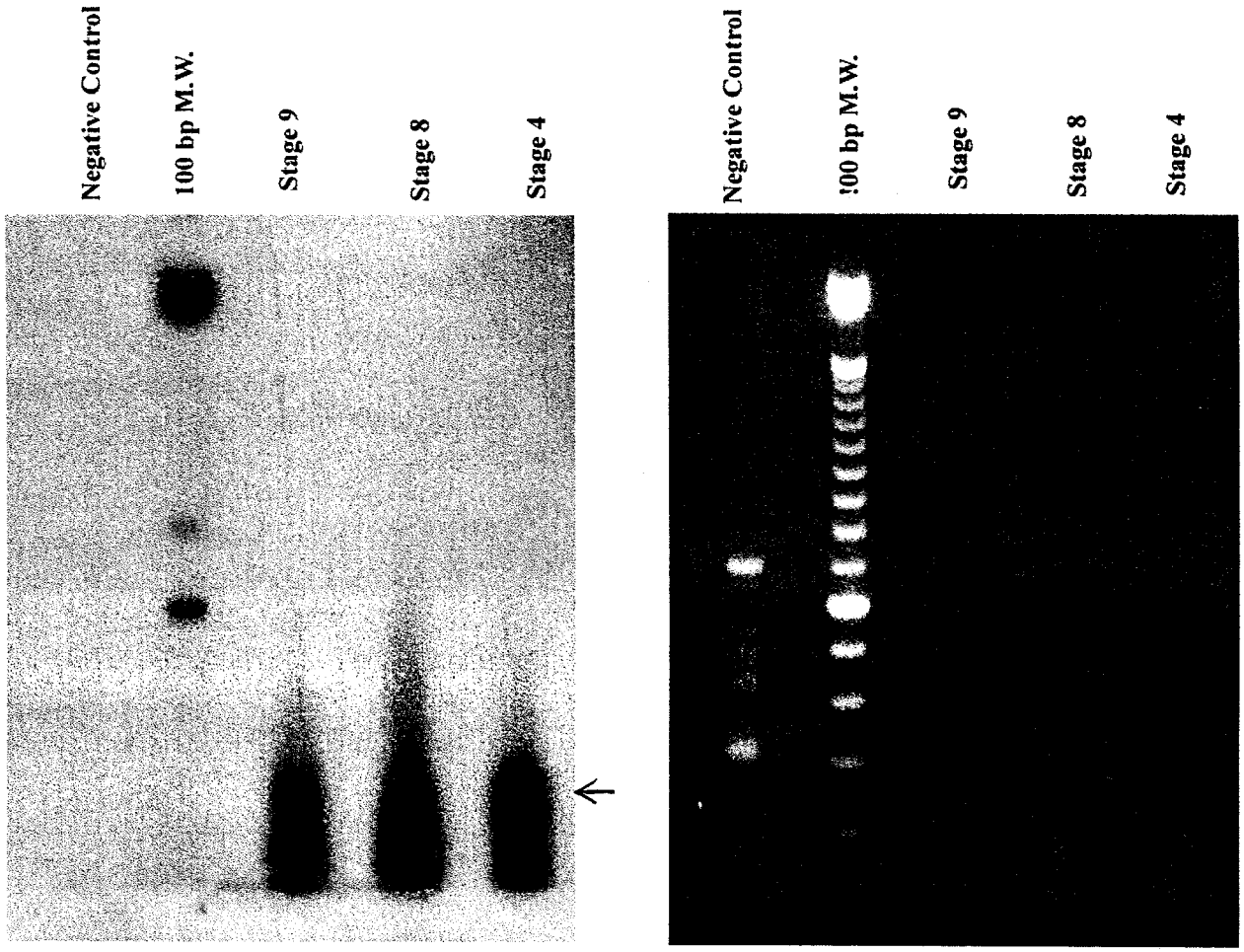


Figure 3