FOREST BIOLOGY PROJECT ADVISORY COMMITTEE MEETING

Slide Material

October 15-16, 1997

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Institute of Paper Science and Technology
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FOREST BIOLOGY FALL PAC REVIEW
AGENDA

Wednesday, October 15, 1997 (Seminar Room)

10:00 A.M.	Welcome, introduction, antitrust statement	Lazar
10:15	IPST Update	Baum, Malcolm

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

11:00	F-010 Mass Clonal Propagation of Improved Conifers	Pullman
	Summary of Accomplishments Since Last Meeting	
	Goals	
	Personnel	
	Grants	
	Research Findings	
11:15	Softwood Embryogenesis	
	Initiation (F010 - Goal 1)	Pullman
	Metal analysis (F010 - Goal 2)	Pullman
	Embryo Development (F010 - Goal 5)	Pullman
	Conversion Update (F010 - Goal 6)	Montello
12:00	Lunch	
1:00	Zygotic Embryogenesis	
	Zygotic vs. Somatic Germination (F010 - Goal 5)	Peter
	cDNAs, library, antibody marker updates (F010-8,9)	Peter
1:20	Molecular Biology - Softwoods	
	Differential Display -Introduction (F010 - Goal 10)	Cairney
	Somatic Embryos - Stage specific markers (F010 - Goal 10a)	Johns

EXTERNALLY FUNDED & STUDENT PROJECTS RELATED TO F010

1:40	Gene expression during embryogenesis	
	Zygotic (F010 - Goals 10a,b,c)	Xu
	Somatic (F010 - Goal 10d)	Xu
2:20	Vicilin Storage Protein Gene in LP (F010 - 10a)	Perera

2:40	Activated Carbon/Media Interactions (F010- Goals 1,4,5)	Van Winkle
3:00	Break	
3:15	Vegetative Expression of Floral Genes in Loblolly Pine (State of GA goals)	Perera, Ge
	Summary	Cairney

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

3:40	F-011 Mass Clonal Propagation of Genetically Improved & Engineered Hardwoods Program and Goals (F011 - Goals 4,5,7,8)	Peter
	Cottonwood Regeneration & Transformation Update (F011 - Goals 1,2)	Peter
	Model Systems for Fiber Formation (F011 - Goal 6)	Peter

EXTERNALLY FUNDED PROJECTS RELATED TO F011

	Gene Regulation Studies (F011 - Goal 3)	Cairney
	Stress gene regulation (F011 - Goal 3)	Destefano
	Activation of Regulatory Peptides PAM / PGL (F011 - Goal 3)	Cairney
5:30	Dinner	
6:30	Speaker - Information Services	Bob Patterson

Thursday, October 16, 1997 (Seminar Room)

8:00 A.M. Coffee and Donuts

NON-DUES FUNDED CONSORTIUM PROJECTS RELATED TO F011 CONTINUED

8:15	Gene Regulation Continued	
	LP 6 (F011 - Goal 3)	Destefano
	Gene Regulation Summary	Cairney

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

9:00	John MacKay (New Associate Scientist) Past and Future Research	MacKay
9:45	Grant Proposal Activity, Student Research, Publications, IPST meeting host	Pullman, Peter Cairney
10:15	Break	
10:30	Comments on Research Programs, Questions, Discussion, Issues	PAC
12:00	Adjourn (Lunch will be available at 12:00)	

F-010 GOALS FOR FY 1997-1998

1. Improve initiation protocol to meet target of 35%.
   - Run Summer Expts.
   - Evaluate Summer Expts.
   - Run & Evaluate Winter Expts.
2. Metals analysis of somatic and zygotic embryos.
   - Analyze somatic embryos for metals content.
   - Analyze female gametophyte tissue over embryo development cycle.
   - Repeat zygotic embryo & gametophyte analysis, more trees.
3. Evaluate new cultures for performance in current Protocols.
4. Improve quality of early-stage embryos in liquid media.
5. Improve maturation protocol .
6. Establish 50+ seedlings from each of 5 genotypes.
7. Continue work on analysis of zygotic embryos for amino acids.
   - Extraction, Purification, Derivitization Method for Small Amounts of Embryos.
   - HPLC Analysis Equipment, Repeatable Analysis
8. Evaluate concept of antibody staining for predictive developmental markers.
9. Develop high quality cDNA libraries representing staged loblolly pine zygotic embryo development.
10. Gene expression during conifer embryogenesis.
    - Identify marker genes which are active at specific stages of zygotic embryo development.
    - Clone and sequence gene fragments.
    - Use information to expand usefulness of these markers, applying them to somatic genotypes.
    - Identify marker genes which are active in somatic embryos during development.
    - Begin to determine where (anatomically) in embryo specific genes are expressed.

ID	Task Name	4/97	5/97	6/97	7/97	8/97	9/97	10/97	11/97	12/97	1/98	2/98	3/98	4/98	5/98	6/98	7/98	8/98
1	**INITIATION AT 35%**																	
2	Run Summer Expts.																	
3	Evaluate Summer Expts																	
4	Run & Evaluate Winter Expts.																	
5	**METALS ANALYSIS OF Somatic and Z**																	
6	Analyze somatic embryos for metals conten																	
7	Analyze zygotic embryo & female gametop																	
8	Repeat zygotic embryo & gametophyte ana																	
9	**Evaluate Cultures in New Protocols**																	
10	**Improve Quality of early stage embryos**																	
11	**Improve Maturation Protocol**																	
12	**Establish 50+ Seedlings for 5 Genotype**																	
13	**Continue Work on Analysis of Zygotic**																	
14	Extraction, Purification, Derivitization Metho																	
15	HPLC Analysis Equipment, Repeatable An																	
16	**Evaluate Antibody Staining for Predicti**																	
17	**Develop High Quality cDNA libraries Re**																	
18	**Gene Expression During Conifer Embr**																	
19	Identify marker genes which are active at s																	
20	Clone and sequence gene fragments.																	
21	Use information to expand usefulness of th																	
22	Identify marker genes which are active in s																	
23	Clone and sequence somatic embryo gene																	

Pullman
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JP,GP
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JP,GP
JP,GP
Pullman
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Peter
Peter,MacKay
CAIRNEY,MacKay
CAIRNEY,MacKay
CAIRNEY
CAIRNEY
CAIRNEY

Project: F010 Goals Gant Chart
Date: Mon 10/13/97

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

ID	Task Name	4/97	5/97	6/97	7/97	8/97	9/97	10/97	11/97	12/97	1/98	2/98	3/98	4/98	5/98	6/98	7/98	8/98
24	Begin to determine where (anatomically) in																CAIRNEY	

3

Project: F010 Goals Gant Chart
Date: Mon 10/13/97

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

Germination of Immature Zygotic Embryos

- To compare and evaluate somatic embryo quality and germination potential, we tested for the ability of immature zygotic embryos to germinate.
- Identify the relative timing of critical developmental events, e.g. shoot and root apical meristem autonomy, and correlate these to our staging system.
- Establish target for somatic embryo maturity

Experimental Procedure

- Sterilize seed
- Surgically remove embryos
- Place embryos on germination media
  - < 10 embryos/plate
- Grow in the dark for 1 week
- Grow in continuous light for ~6 weeks
- Evaluate embryos for germination with dissecting microscope

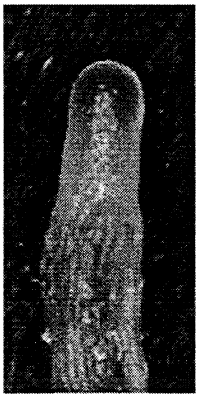
Experimental Design

- Isolate (≥ 50) immature embryos from stages *5, 6, 7, 8, 9.1, 9.2*
- Follow our normal protocol for germinating somatic embryos
  - no hormone germination media
  - 1 week dark
  - ~6 weeks continuous light
- Analyze for growth and germination

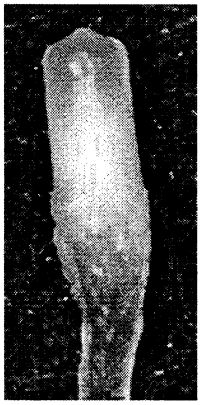
Scoring Growth & Germination

- Germinated embryos were defined as having both a growing shoot and root.
- Embryos with only a growing root or only a growing shoot were also scored.
- Hypocotyl and root lengths were measured.
- Cotyledon greening and morphology were assessed.

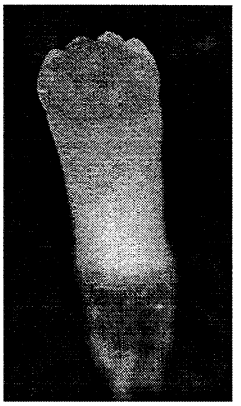
Zygotic Embryos: Stages 4-6



Stage 4

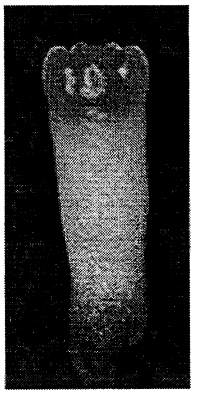


Stage 5

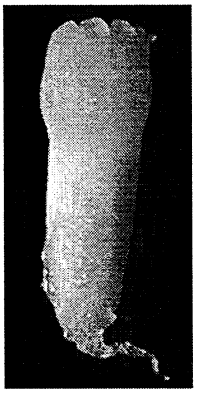


Stage 6

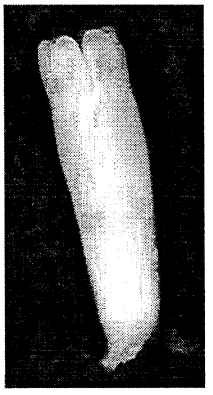
Zygotic Embryos: Stages 7-9.2



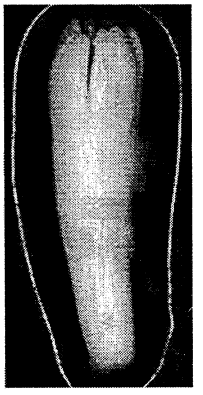
Stage 7



Stage 8



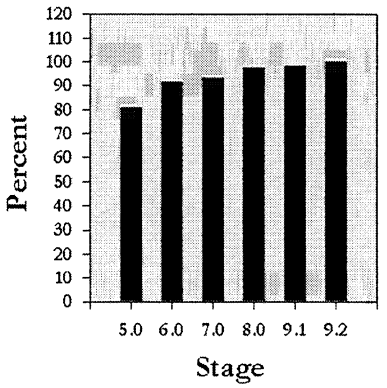
Stage 9.1



Stage 9.2

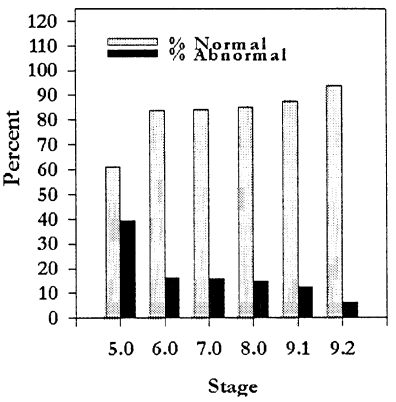
Embryo Viability

- To validate that our dissections were not too damaging, we quantitated the percent live embryos from each stage.
- Live embryos had green cotyledons and hypocotyls that were growing.

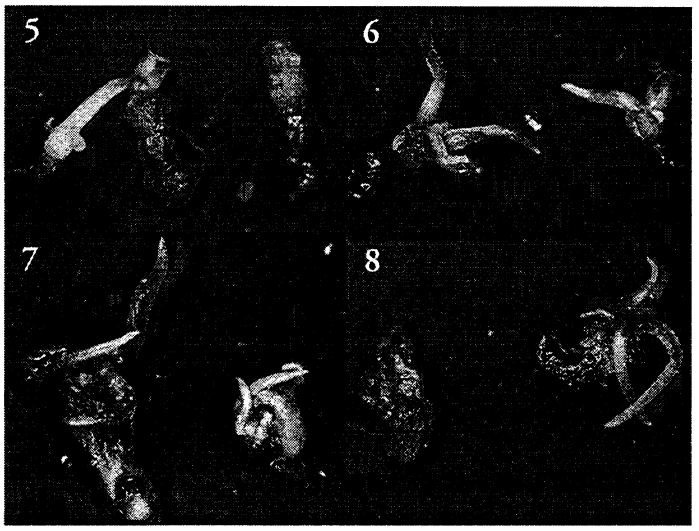


Cotyledon Development Occurs Normally

- To investigate cotyledon development we quantitated the percent that were normal vs. abnormal.
- The younger the embryos, the more that formed abnormal cotyledons.

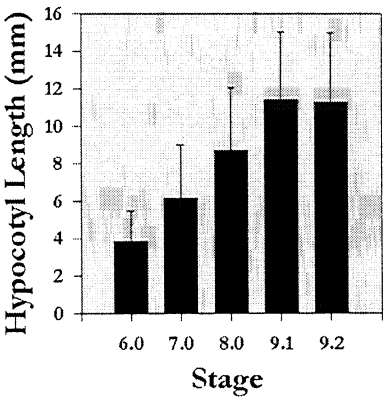


Abnormal Cotyledons from Stages 5-8



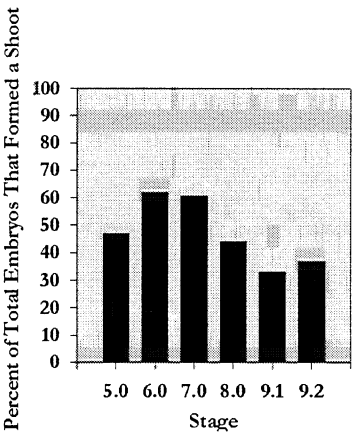
Hypocotyl Growth

- **Average hypocotyl length increases with maturity**
- **Stage 5 embryos - no hypocotyl growth**



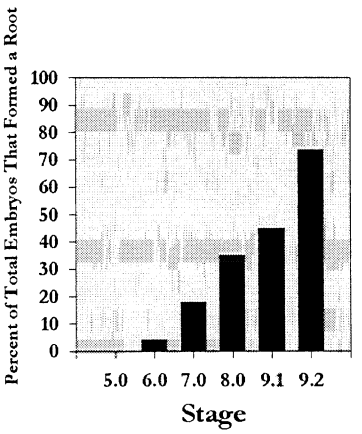
Shoot Development & Meristem Autonomy

- Shoots form on ~40% of stage 5 embryos
- Shoot apical meristem autonomy established at or before stage 5
- % of shoots decreases (delayed?) as roots are formed



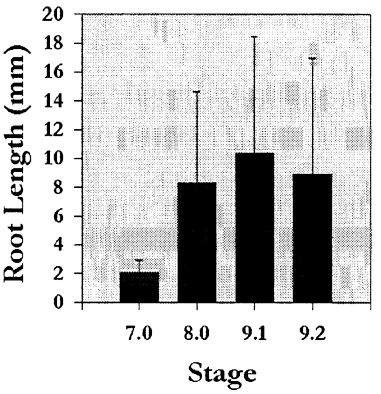
Root Development & Mersitem Autonomy

- Root emergence or autonomy of the root apical meristem occurs at stage 7
- Root emergence increases with embryo maturity



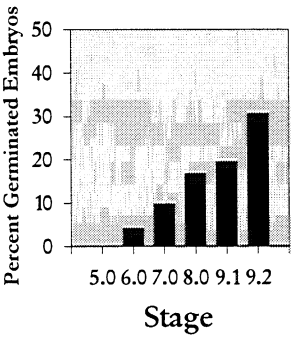
Average Root Length

- Average root length increases with embryo maturity
- Roots grow similarly from stage 8 on; however, there were longer roots as stage increased

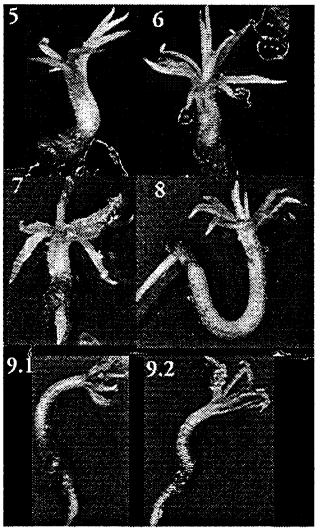


Immature Zygotic Embryo Germination

- Germination frequency increases with embryo maturity

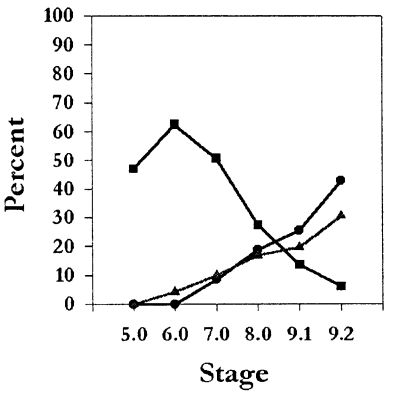


Germinated Embryos



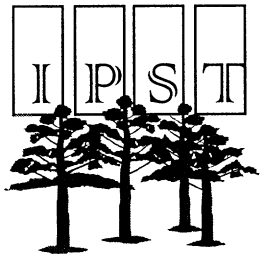
Transition from Shoot to Root Emergence

- When roots emerge before shoots they possibly delay shoot emergence
- Root emergence parallels germination capacity



Conclusions

- **Shoots emerge first in stage 8 embryos; whereas, in stage 9.1 embryos roots emerge first.**
- **Root meristem autonomy, possibly formation, limits germination both in immature zygotic and somatic embryos**
- **Somatic embryos develop to stages 7-8**
- **Early stages (up to stage 6-7) of somatic embryo development probably occur normally**



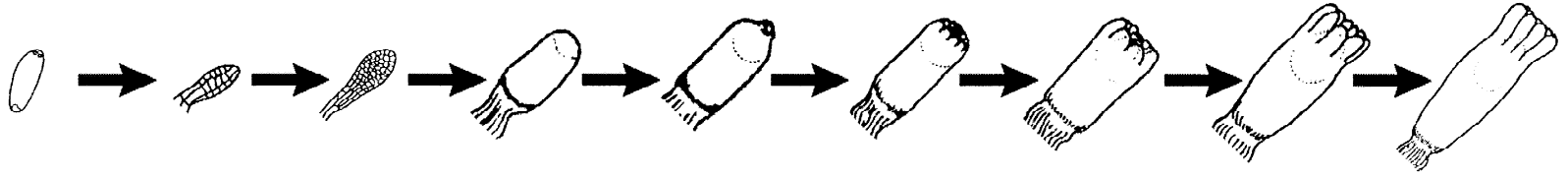
Analysis of Stage Specific Bands from Differential Display of Loblolly Pine Somatic Embryos

John Cairney and Barbara Johns

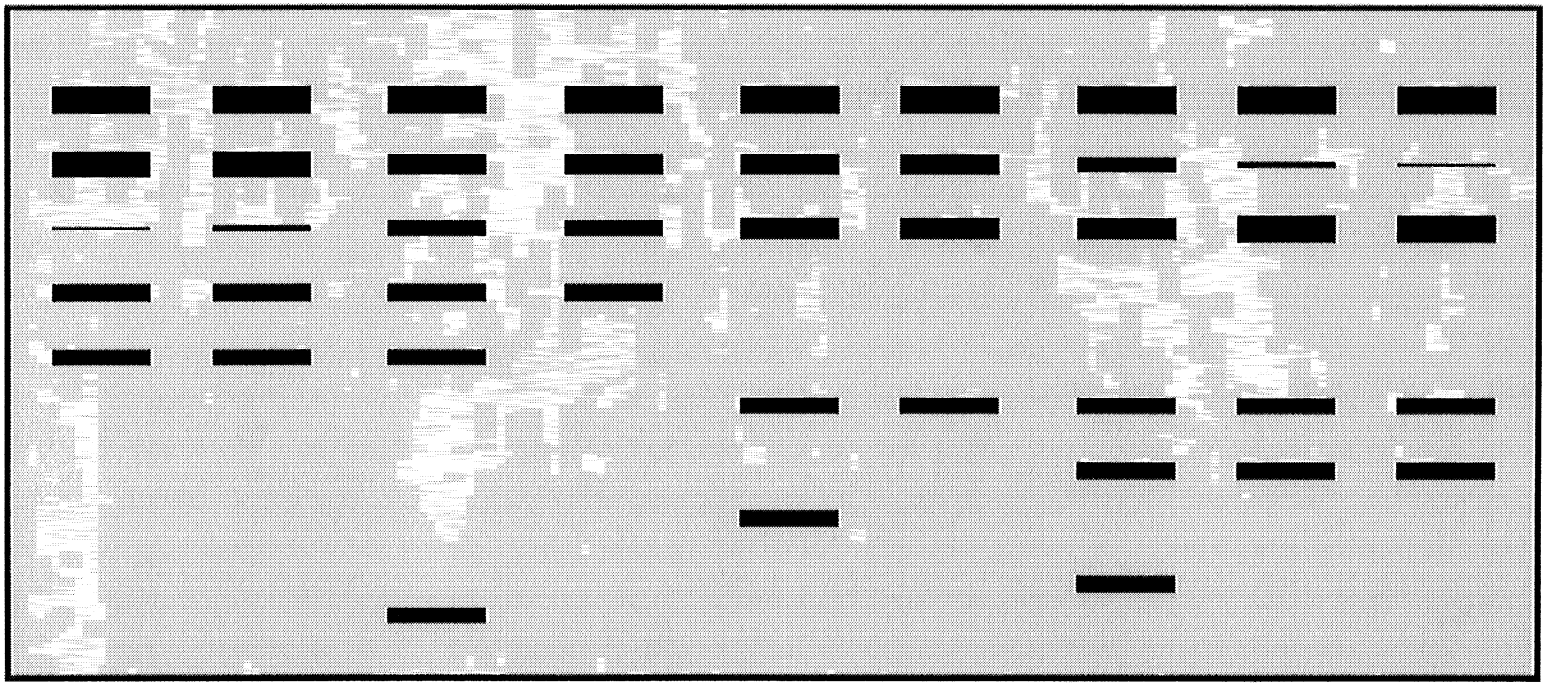
Hypothesis

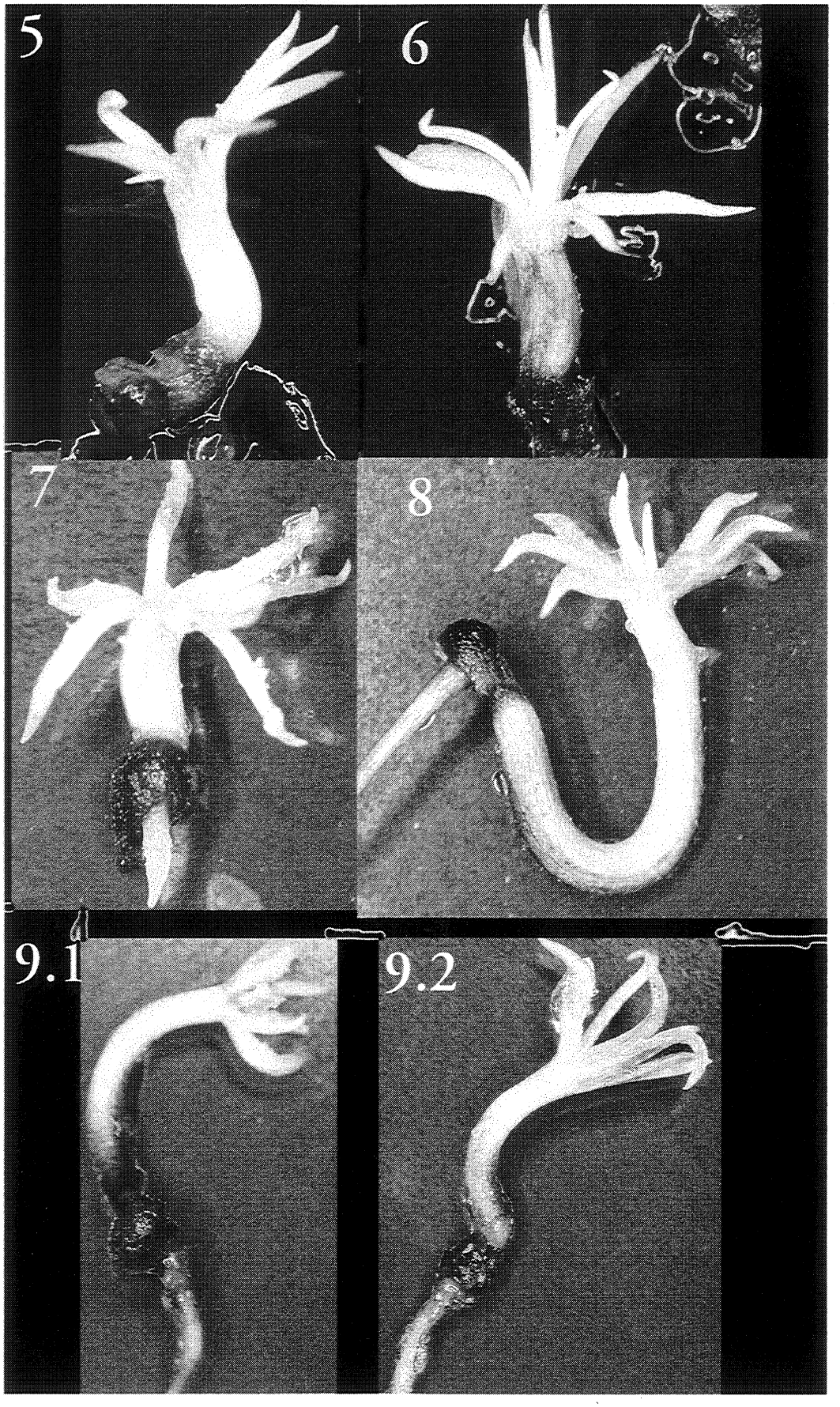
- Different genes are active at different stages of embryo development
- Transcripts of these genes (mRNAs) can be detected
- Expression markers for individual stages of pine development can be identified from Differential Display technology

Stage Specific Gene Expression (Marker Bands) Can Be Reavealed by Differential Display



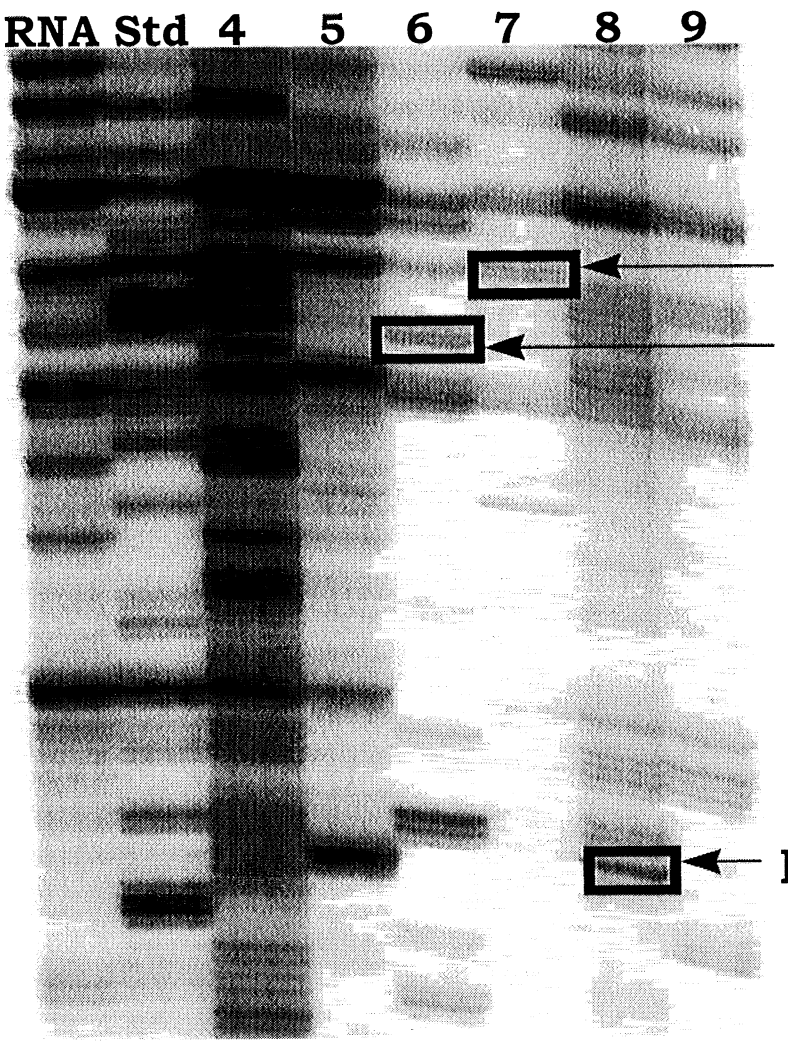
1 2 3 4 5 6 7 8 9





FIGURE]0

Differential Display 4431-17: Excised Bands 5, 6, and 7

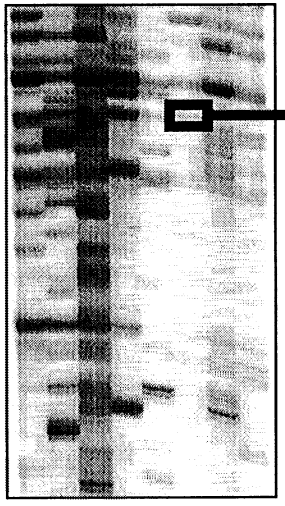


Band 5: stage 7, 0.25 ug/ul

Band 6: stage 6, 0.25 ug/ul

Band 7: stage 8, 0.25 ug/ul

Cloning Differential Display Bands



Excise band 5

Amplify

YOUR GENE

through PCR

YOUR GENE

+

Cloning Vector

pCR-Script Amp SK(+)

Plate cells

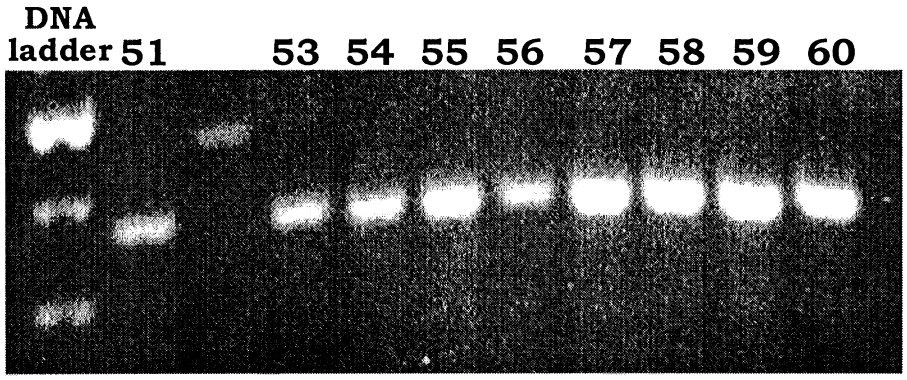
Self-ligated Vector

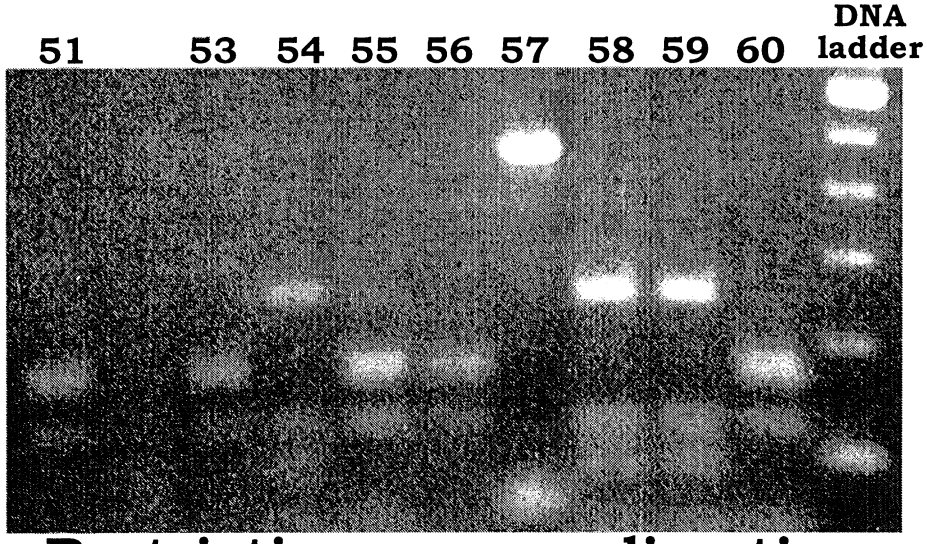
Blue Colonies

Vector with Inserts

White Colonies

Identifying DNA Insert Fragments

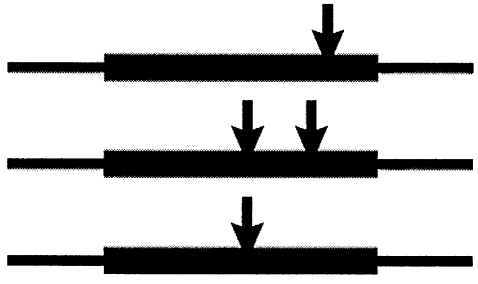




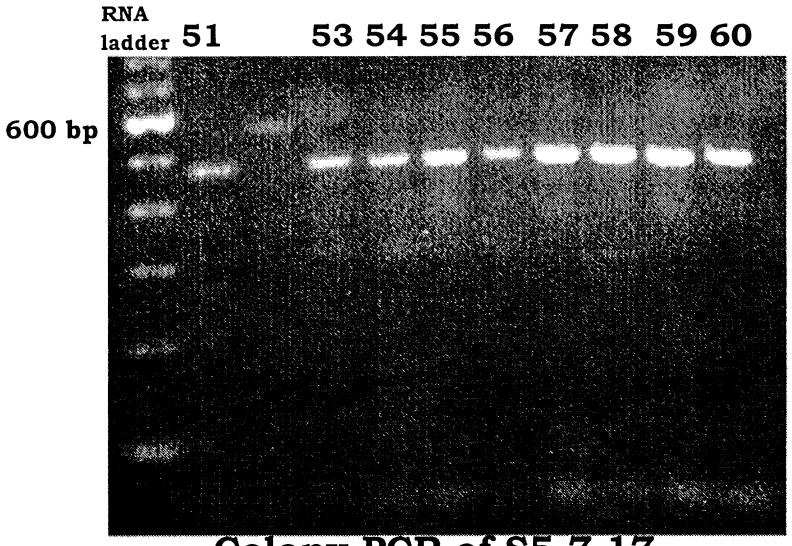
Restriction enzyme digestion pattern of 9 insert DNA above

Colony PCR: 9 out of 10 clones have the insert fragment

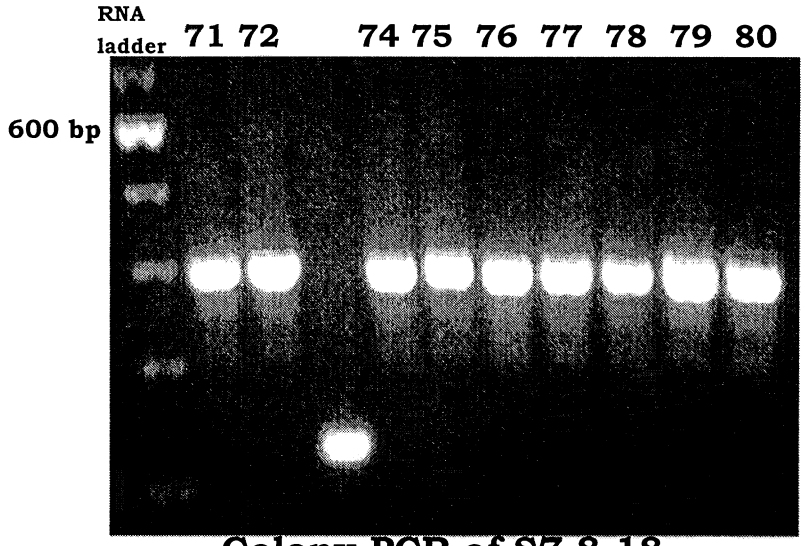
Different inserts can be identified by restriction enzymes:



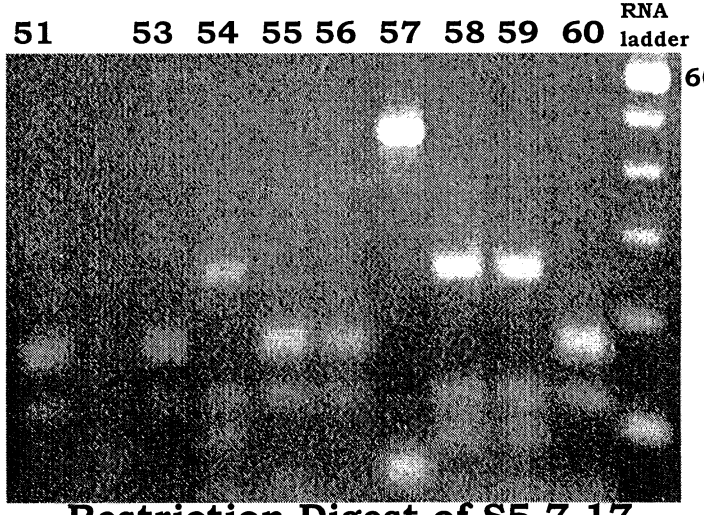
Colony PCR and Digests of Two Differential Display Bands



Colony PCR of S5-7-17



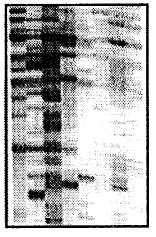
Colony PCR of S7-8-18

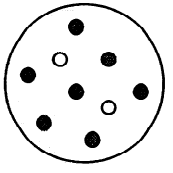
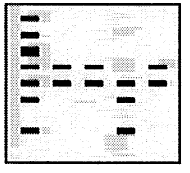


Restriction Digest of S5-7-17

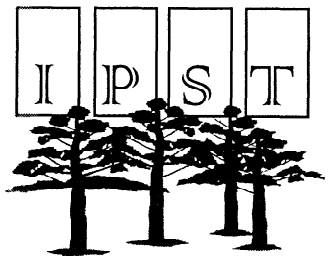
Restriction Digest of S7-8-17

Ten Bands from Differential Display Gels May Give Rise to Possible Stage Specific Markers



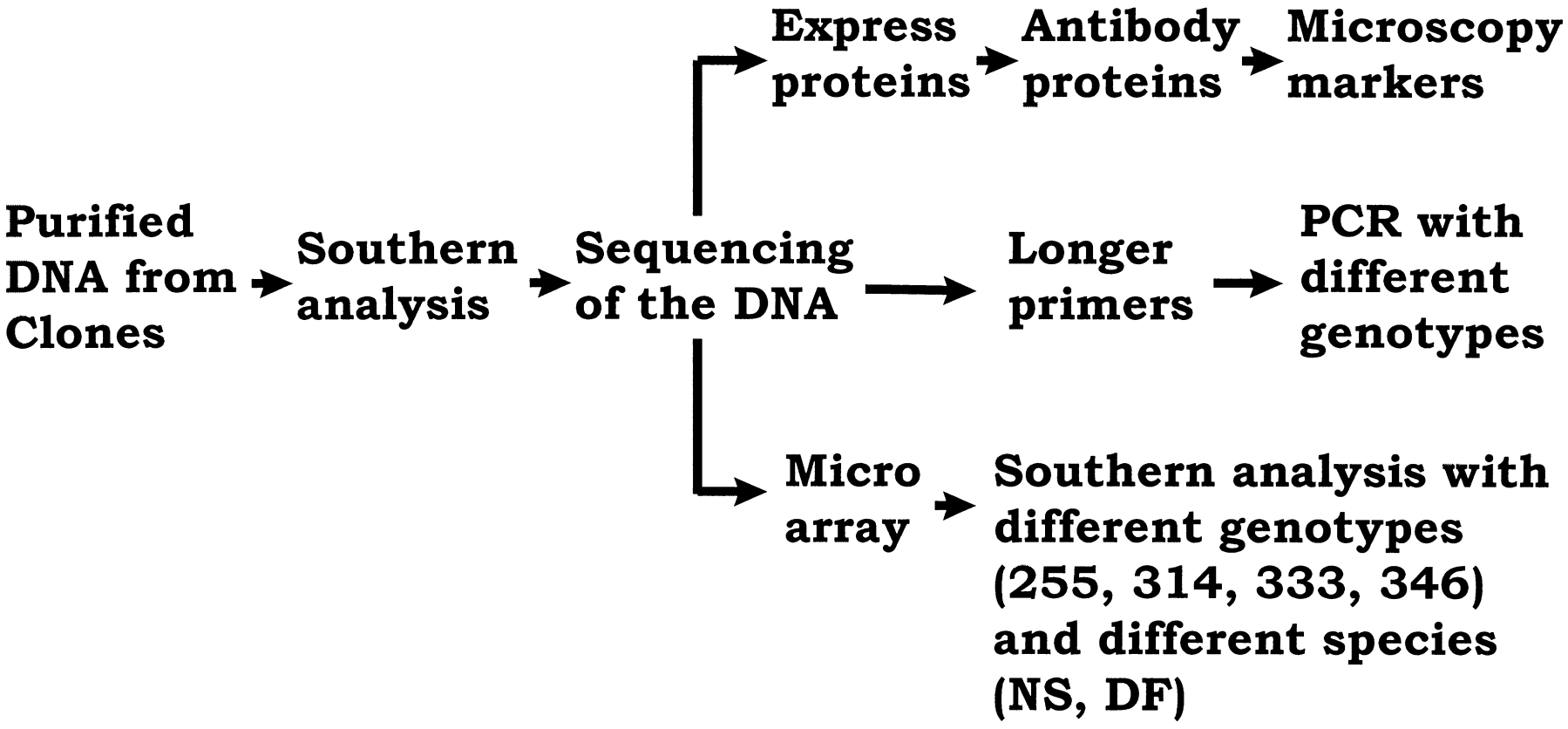

DD Band	Embryo Stage	Number of Clones	Number of Classes	Plasmid Isolation	Southern
1	6	5	2	YES	in progress
2	4	5	2	YES	in progress
3	5	4	1	YES	in progress
4	8	7	1	YES	in progress
5	7	9	3	YES	in progress
6	6	4	2	YES	in progress
7	8	9	2	YES	in progress
8	8	9	1	YES	in progress
9	7	6	2	YES	in progress
10	9	7	4	YES	in progress

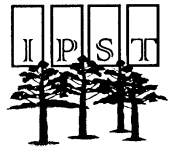


Conclusions

- Apparent marker bands for genotype 260 are recognizable and consistently produced
- cDNA fragments can be cloned and different inserts can be identified by restriction enzyme digest
- The plasmid with the insert can be purified for sequencing and further analysis

Next Steps in Identifying Useful Marker Genes





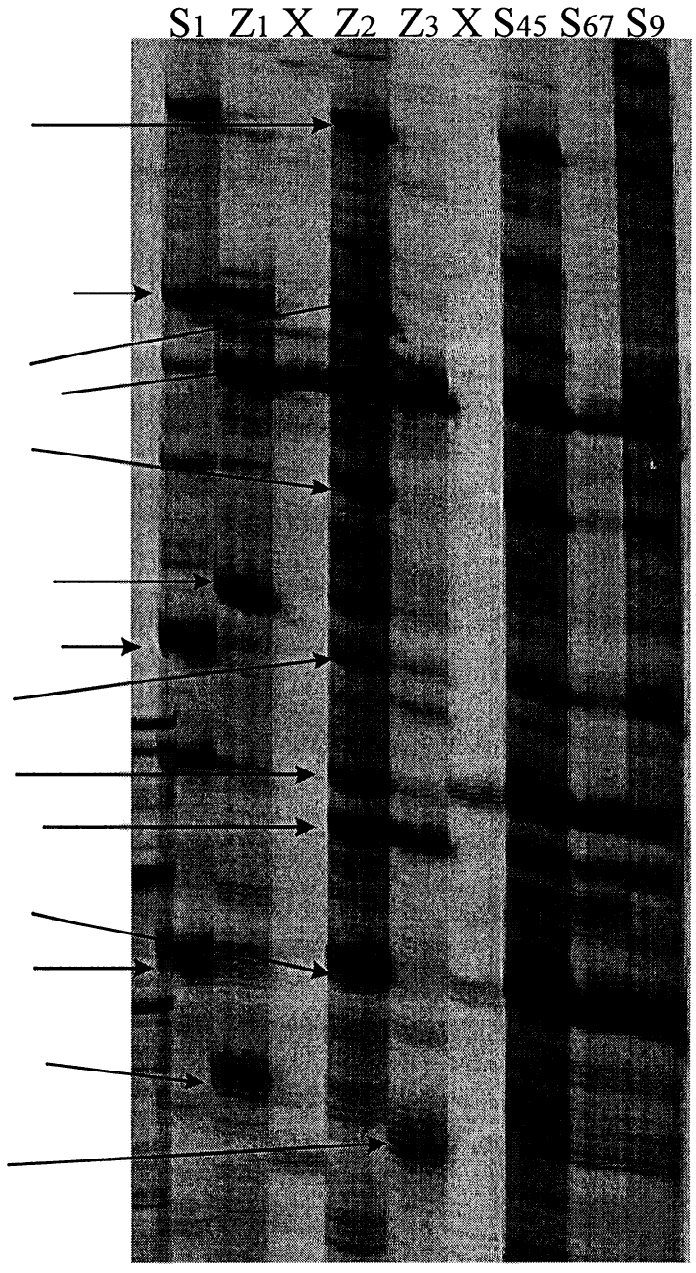
cDNA cloning of mRNAs present in Early Embryo Stages

Vincent Ciavatta, Jerry Pullman and John Cairney

Reasons For Cloning Early Stage Markers

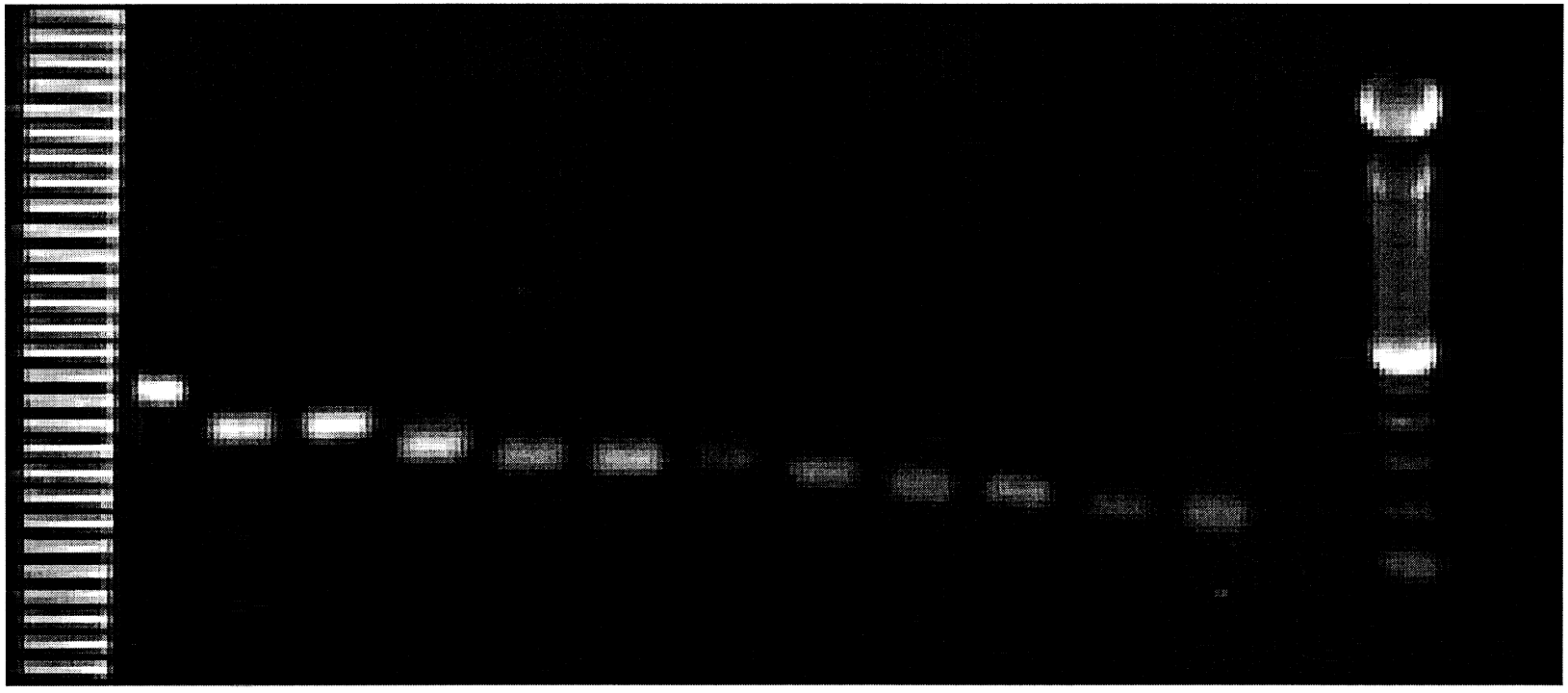
- **Early Stage embryos are very similar morphologically and very few markers are available to discriminate between stages**
- **Markers will allow us to follow more closely the development of early-stage cultures, how they respond to conditions and the the effect of plating different stages**

Bands (cDNAs) spefic to Early Stages of Embryo Development Were Identified and Cloned

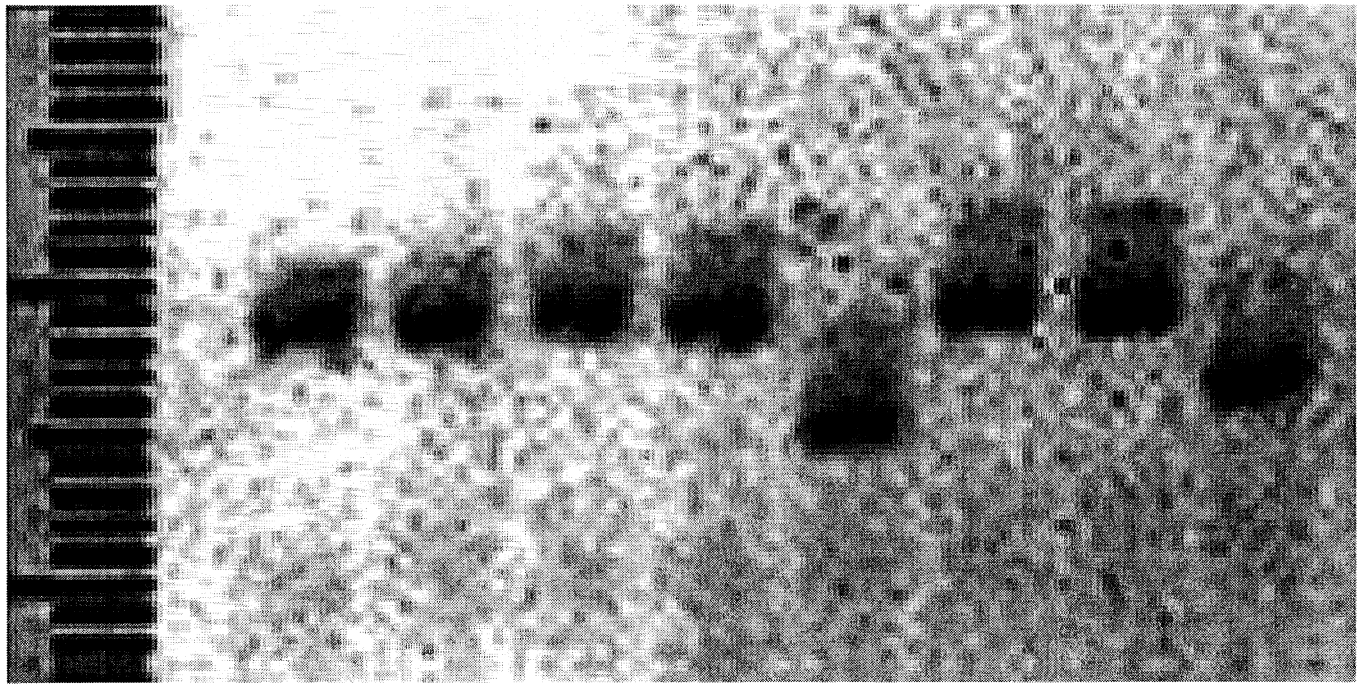


- $Z_{1,2}$ etc = Zygotic Embryo stages 1or 2 (different genotypes)
- $S_{1,67}$ etc = Somatic EmbryoStage 1, 6+7

Bands Were Isolated From Gel and Amplified

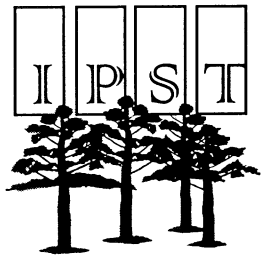


Bands Were Classified By Restriction Digestion



Future Work

- Nothern Analysis Will Confirm Expression Patterns
- cDNA clones will be sequenced
- Full length clones will be isolated in some cases
- Protein will be produced in vitro
- Antibodies will be generated for future work



Differential Display in Somatic Embryos:

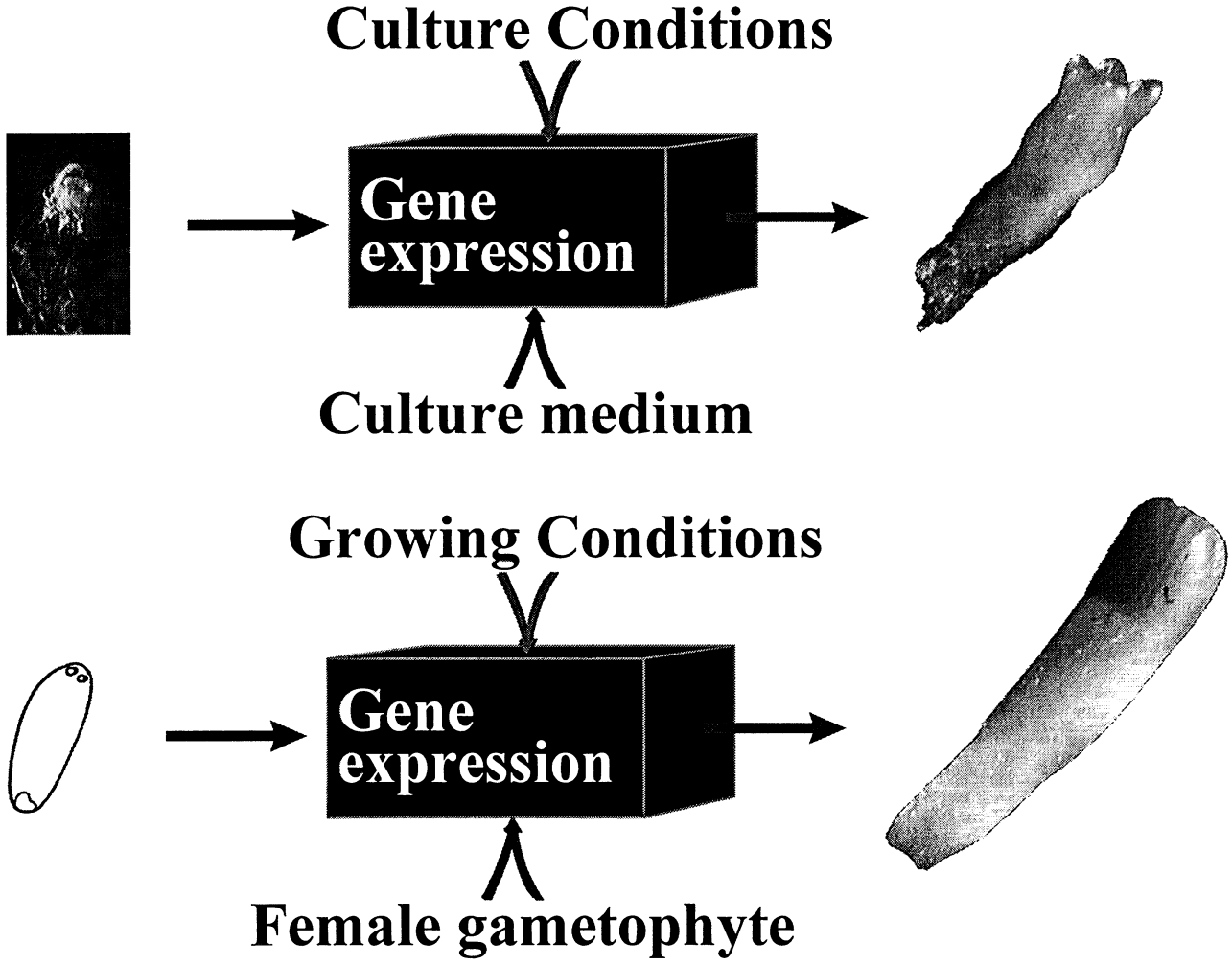
Project Design and Technical development

Nanfei Xu

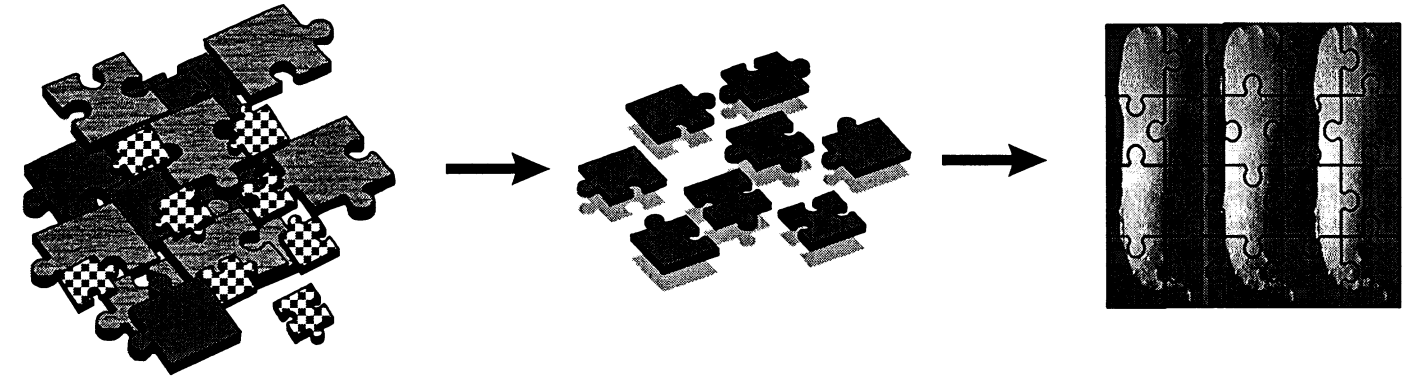
Gerald Pullman

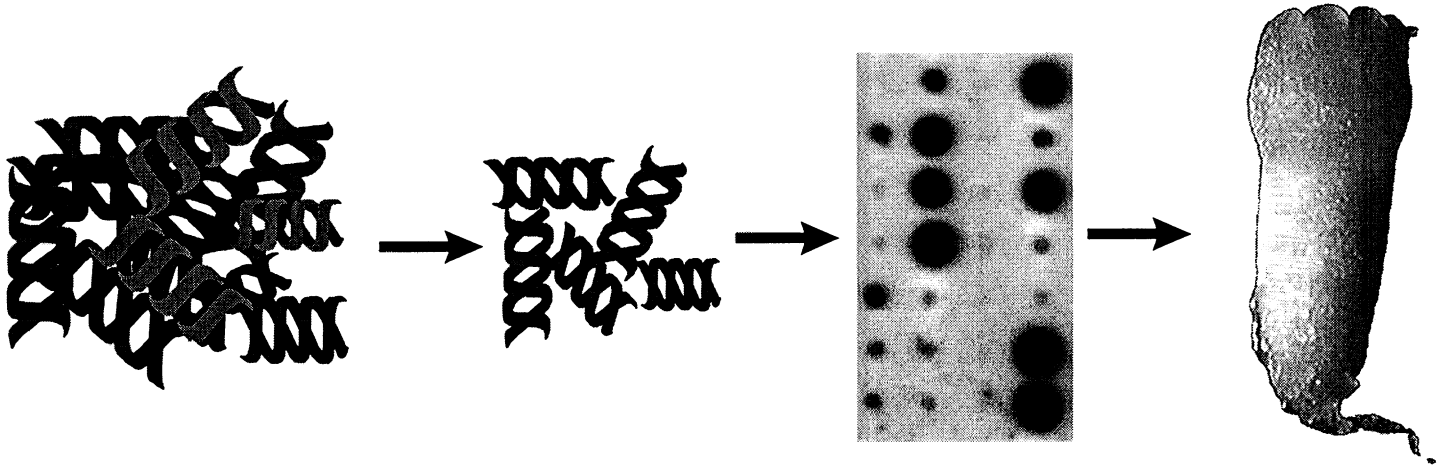
John Cairney

The Difference between SE and ZE Lies in Gene Expression

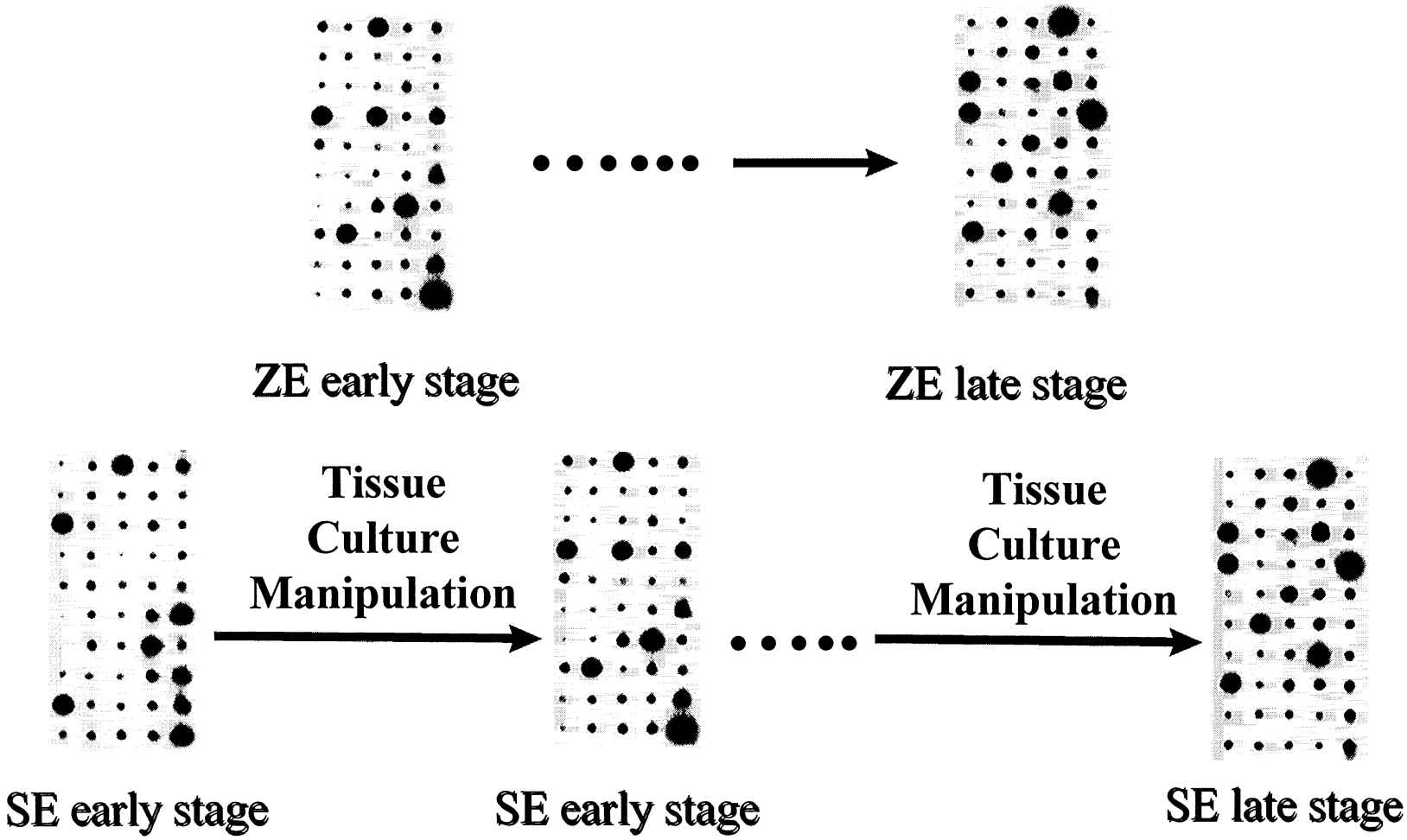


Focus on The Genes Expressed during Embryogenesis





Match Gene Expression in SE to ZE Through Tissue Culture



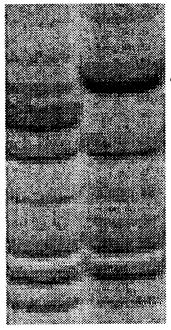
Tasks and Technical Requirements

- Differential display
- cDNA cloning

} Gene function and regulation studies

- Confirmation of expression
- Gene expression diagnosis

Reliable Cloning of DD Bands



DD bands

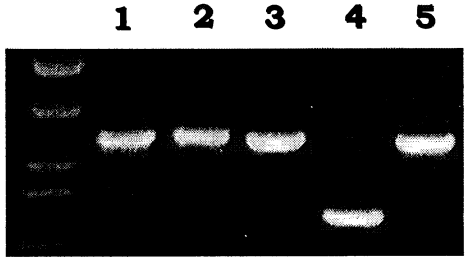
Column purification

Cloning vector

+

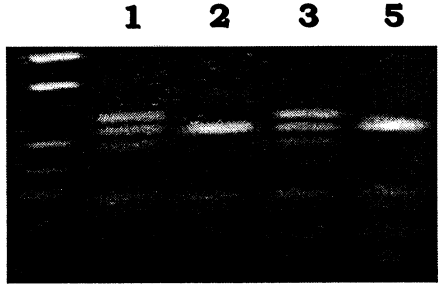
PCR primer

Vector with inserts



4 out of 5 clones have insert fragment

Different inserts can be identified by restriction enzymes



Enzyme digestion pattern of 4 insert DNA above

DNA sequencing

High Speed Sequencing Data Processing

- **CSE: Cloned Sequence Editor**

  Sequence form and format converting, cloned insert searching and processing, automated header info generating, peptide translation and ORF searching

Sequencing data processed	Use GENEWORK	Use CSE
1	>5 minutes	<30 seconds
100	>8 hours	<2 minutes

- **LDHS: Local Database Homology Search**

  Perform homology search among cloned cDNAs

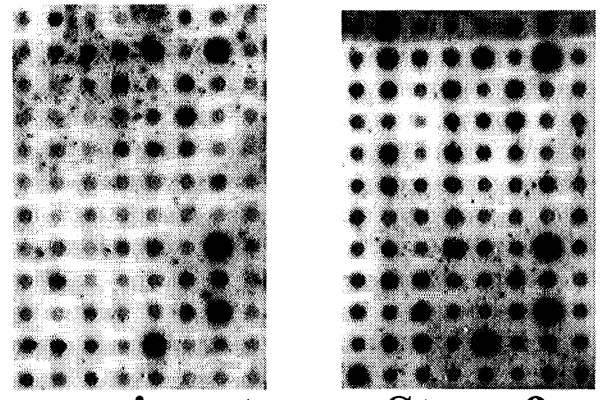
Expression Detection

- Traditionally northern, not suitable for our purpose

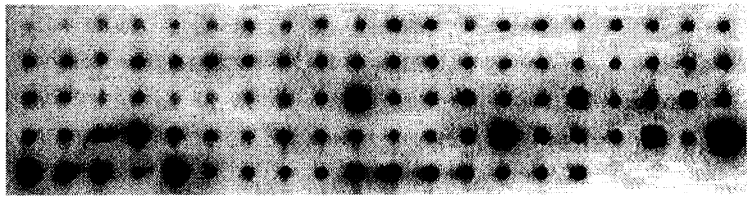
- DAS: Dot Array Southern

  - RT-DD Probe approach
    High sensitivity, easy, low cost, low reproducibility

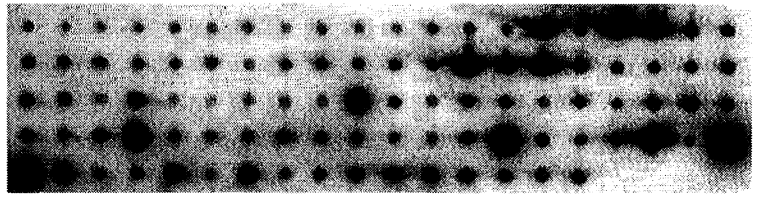
  - Full length cDNA probe approach
    High sensitivity, high reproducibility



Suspension stage Stage 9



Suspension stage



Stage 9

CONCLUSIONS

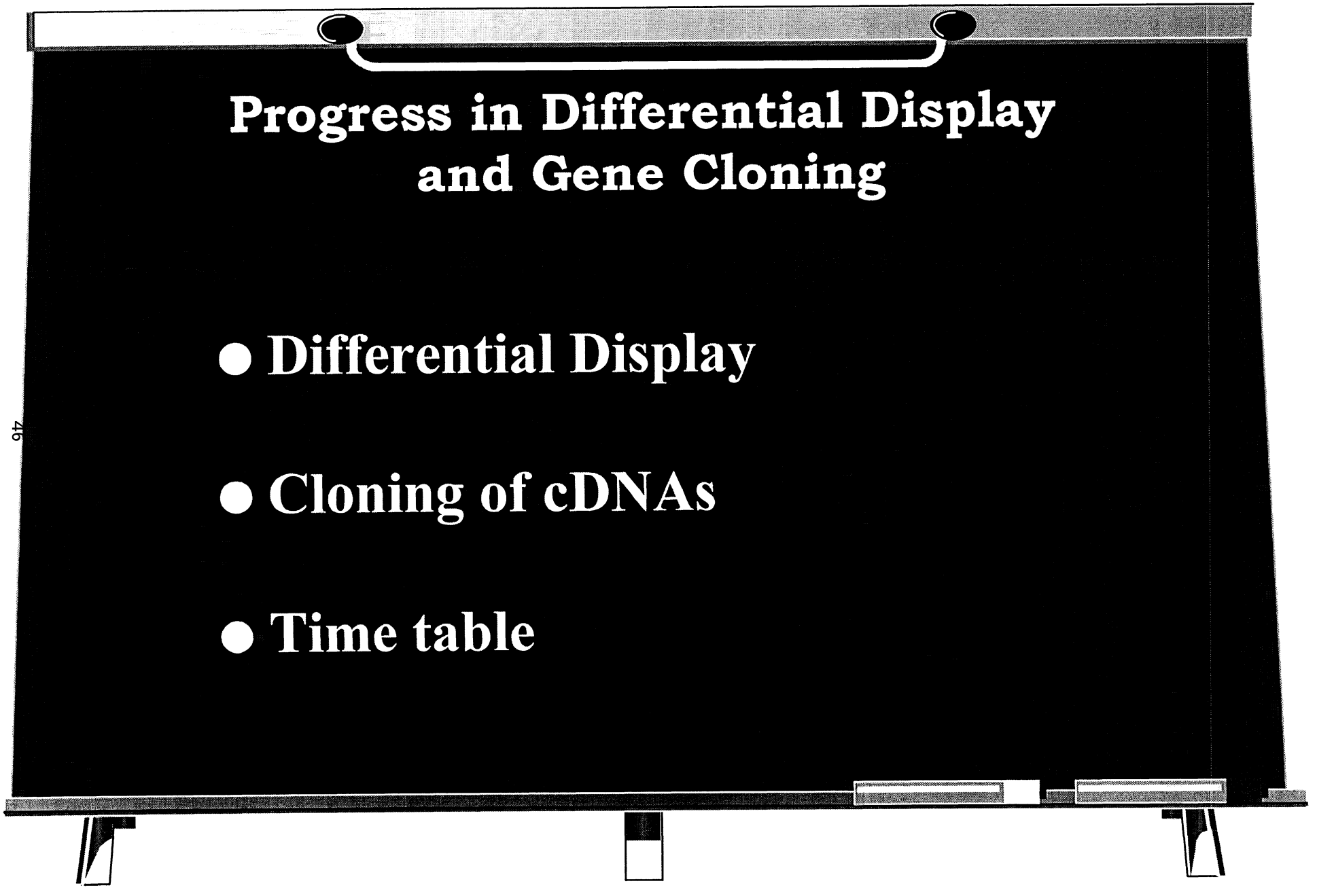
- We plan to clone 400-500 cDNAs from loblolly pine embryos and use these cDNAs in a diagnostic kit for detecting gene expression during embryogenesis .

- We have developed and optimized all the techniques required for cDNA cloning and gene expression detection

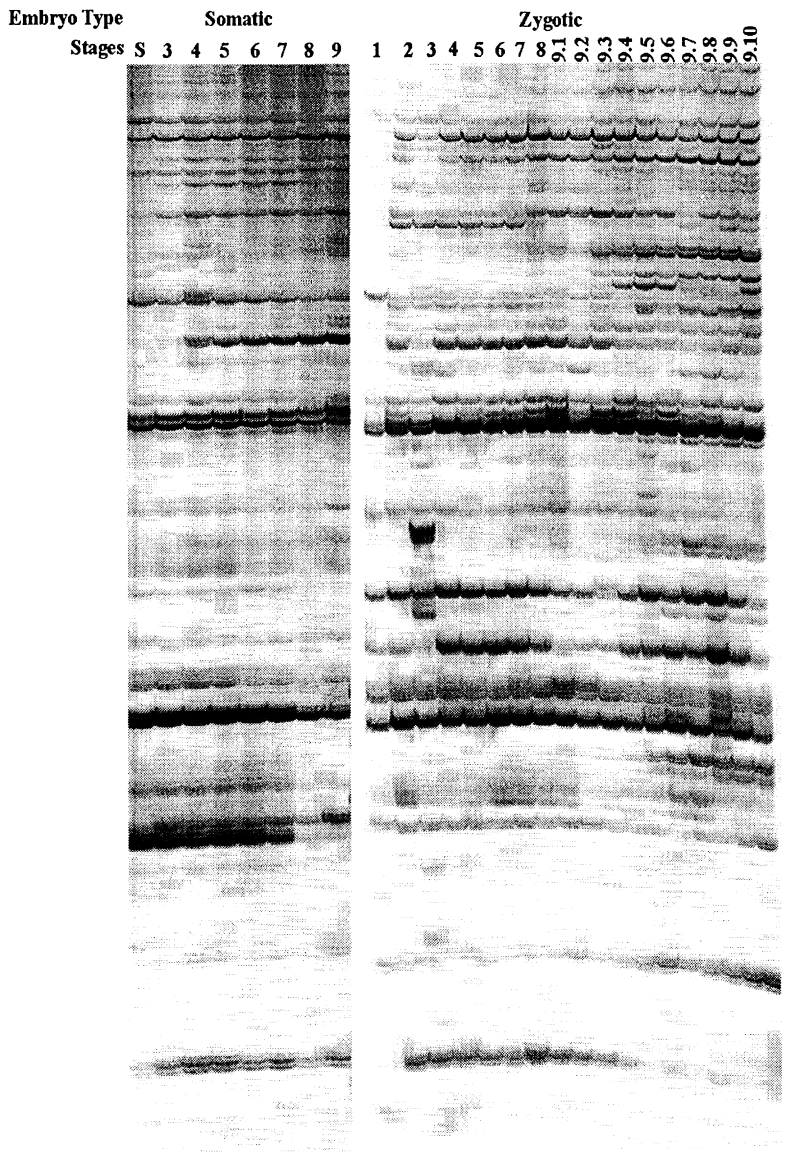
Differential Display in Somatic Embryos:

Progress in Differential Display and Gene Cloning

Nanfei Xu
Michelle Lane
Gerald Pullman
John Cairney

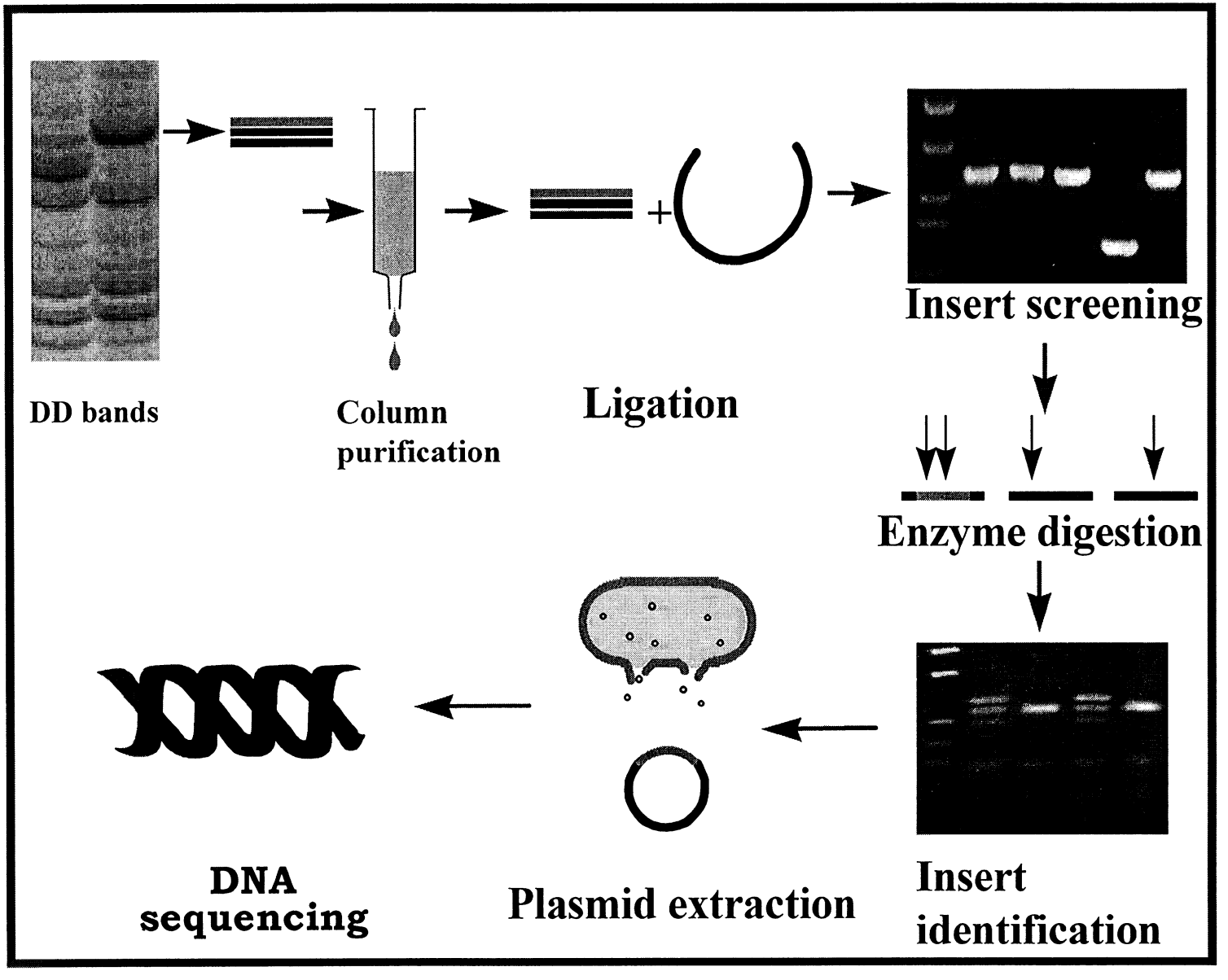

Progress in Differential Display
and Gene Cloning
● Differential Display
● Cloning of cDNAs
● Time table

Differential Display



	Somatic Embryos	Zygotic Embryos
DD primer sets used	16	20
Lanes generated	128	360
DD bands identified	94	412

Reliable Cloning of DD Bands



X 350

DNA cloned

	Somatic Embryos	Zygotic Embryos
DD bands identified	94	412
Bands Cloned	94	351
cDNA clones generated	96	239
Cloning rate:	14 clones/week	

Statistics of Cloned cDNAs

	Somatic Embryos	Zygotic Embryos
Clones sequenced	96	98
Unique groups	86	76
Novel groups	68	54
GenBank hits	18	22
Example interesting hits	DC3 promoter binding factor, Serine Kinase	2S storage protein, Dynamin, Starch synthase

Future plans

- Finish DD and band cloning by the end of this year
- Finish expression confirmation before next PAC meeting
- Diagnosis of somatic gene expression
- Improving somatic embryogenesis by monitor gene expression
- Full length cDNA cloning and characterization

Seed Storage proteins are classified according to their solubility properties

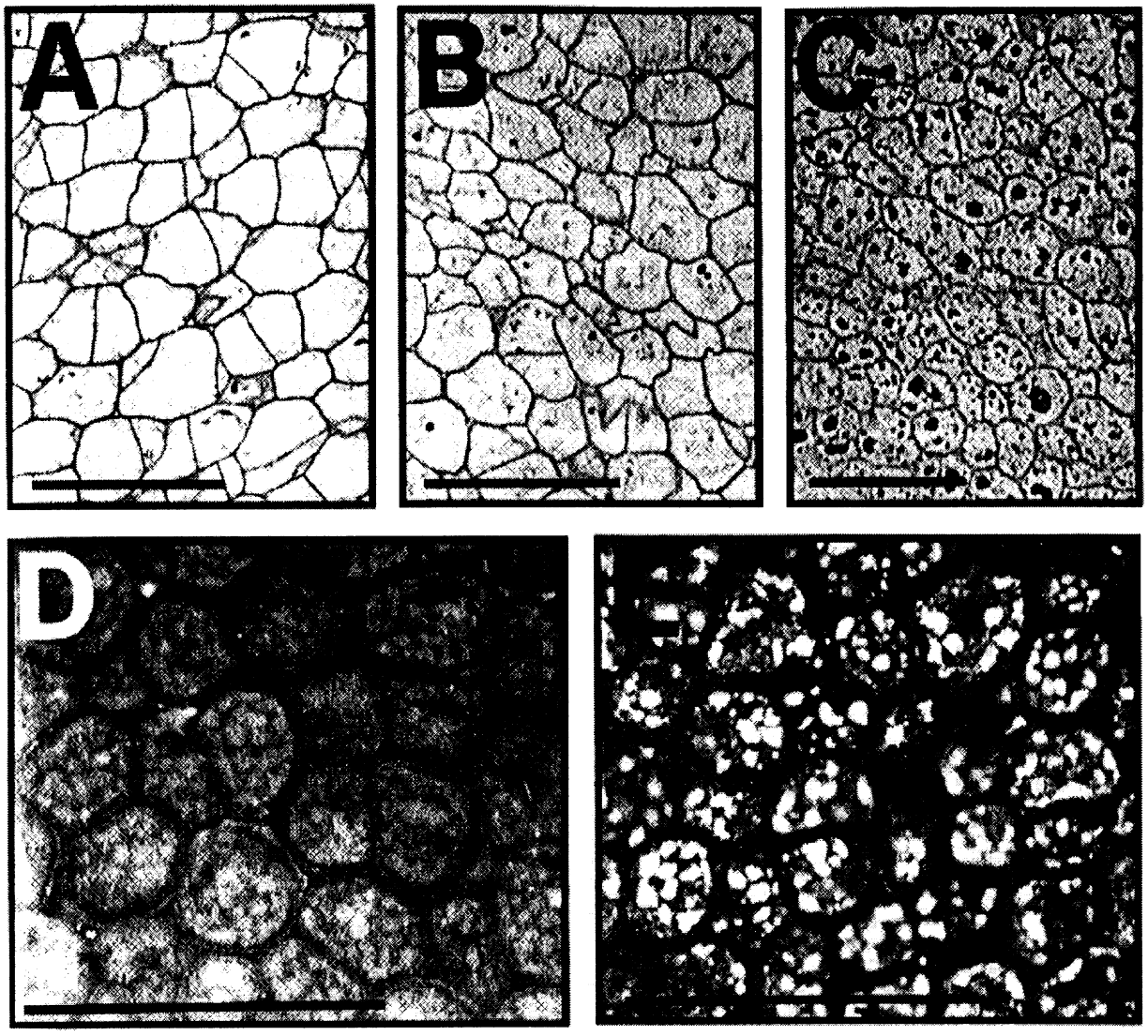
Albumin - water soluble

Globulin - Salt soluble

Glutelin - acid, alkali urea soluble

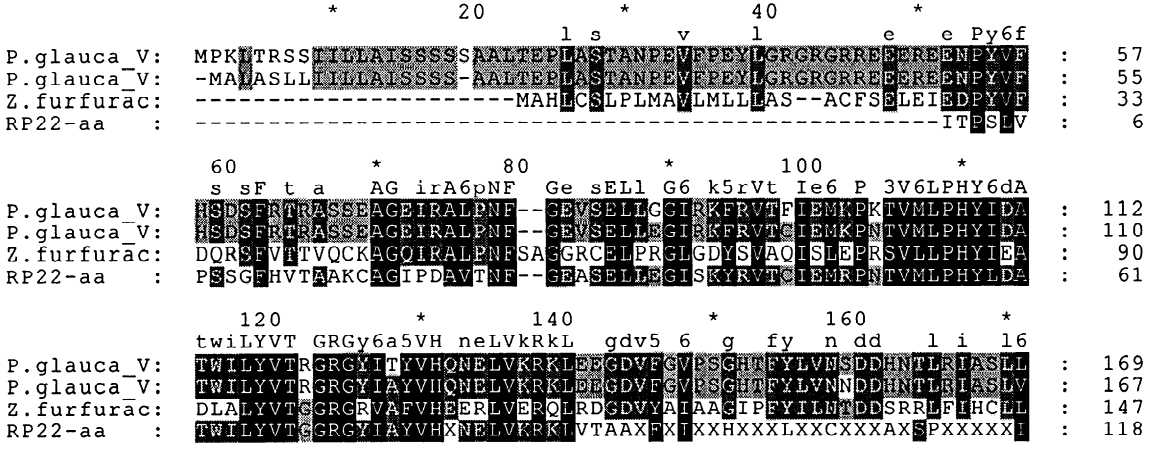
Prolamins - alcohol soluble

Histological observation of seed storage protein shows accumulation in late stages embryos



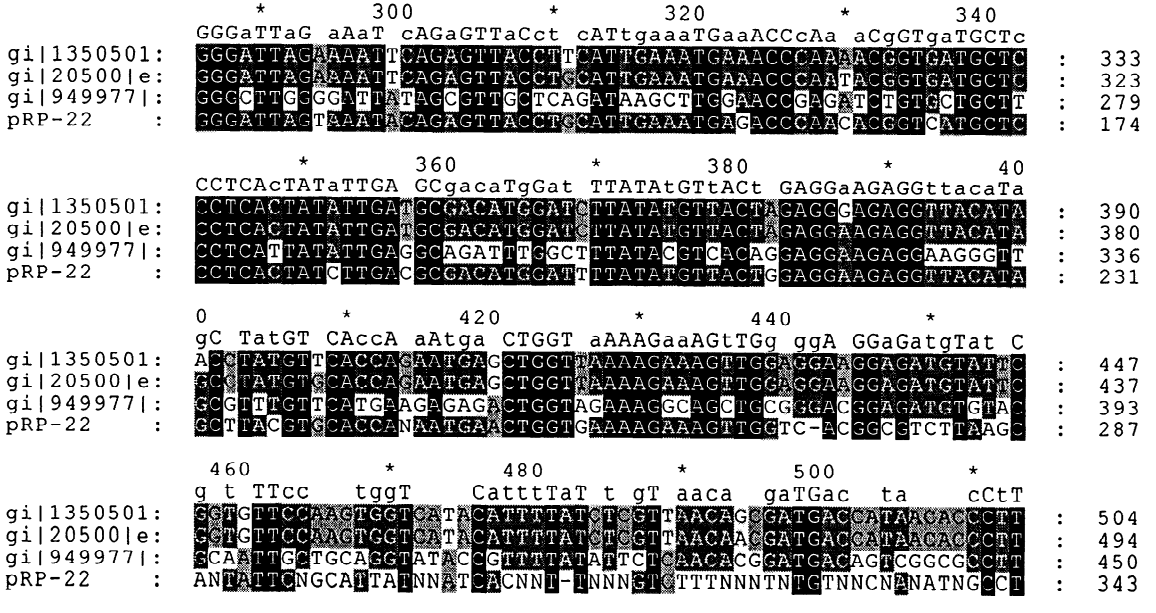
Loblolly pine storage protein is very similar to white spruce

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



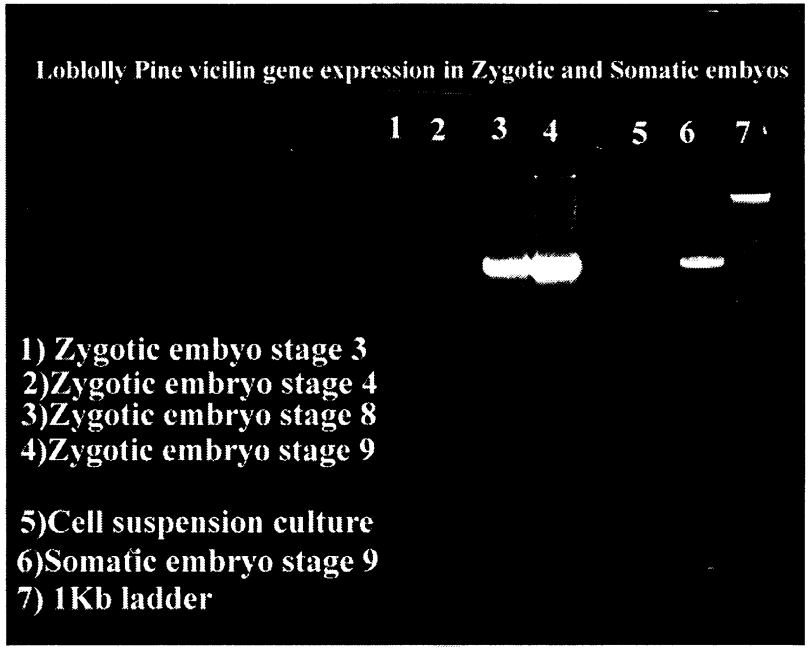
Loblolly pine DNA sequence is very similar to white spruce

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



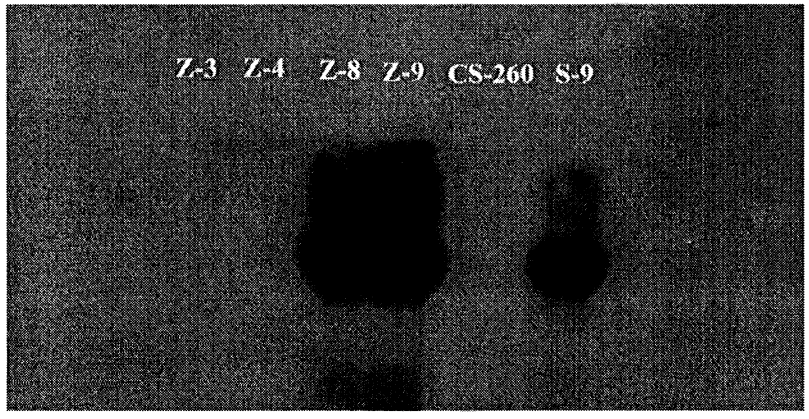
Vicillin mRNA accumulate late in development: Expression of vicillin gene in zygotic and somatic embryos.

- Polymerase chain reaction generates 1400bp fragment (almost the full length) of vicillin gene.



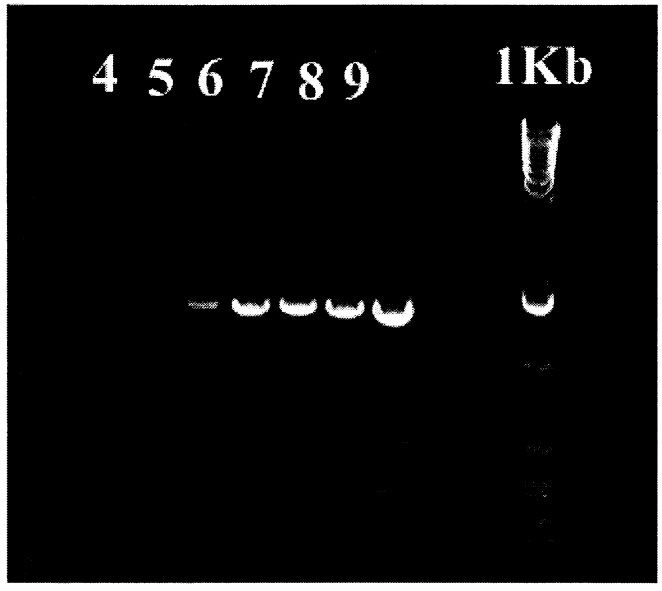
Southern hybridization of PCR products confirms that these are homologous to vicillin mRNA

- Vicilin gene is not expressed in stage 3 and stage 4 of <u>zygotic embryos</u>.
- Vicilin gene is expressed in stage 9 <u>somatic embryos,</u> but not in cell suspension.



Vicillin mRNA increases in quantity gradually over development

- Expression of vicillin gene starts in stage 5 somatic embryos.
- Equal amount of template was used for each reaction. (1 Micro gram of total RNA was used for each RT reaction)



Differences of seed storage protein accumulation in somatic and zygotic embryos in other plant species

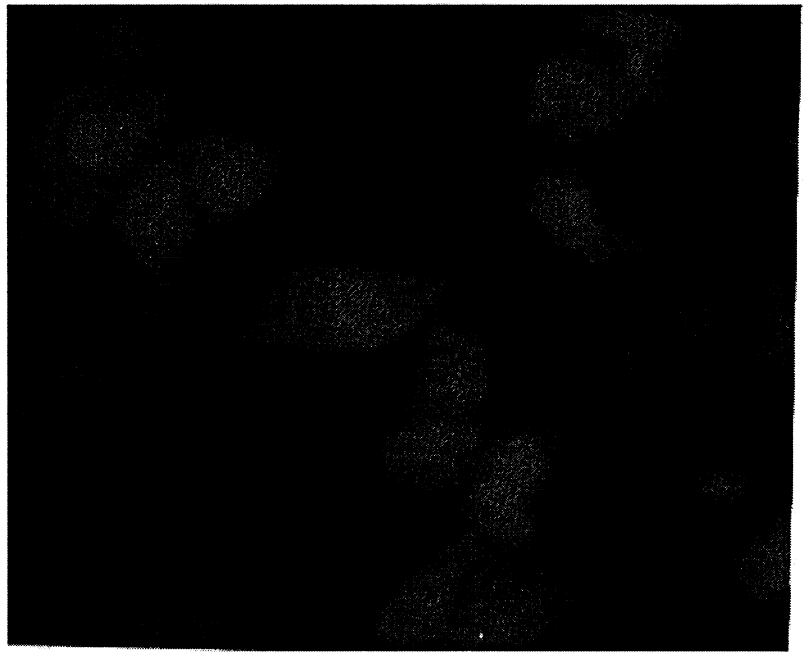
- Generally somatic embryos produce 20% less seed storage proteins compared to natural zygotic embryos.
- Mature alfalfa somatic embryo resemble stage 5 zygotic embryo in seed storage protein production.

Though somatic embryos mimic the developmental stages of natural zygotic embryos:

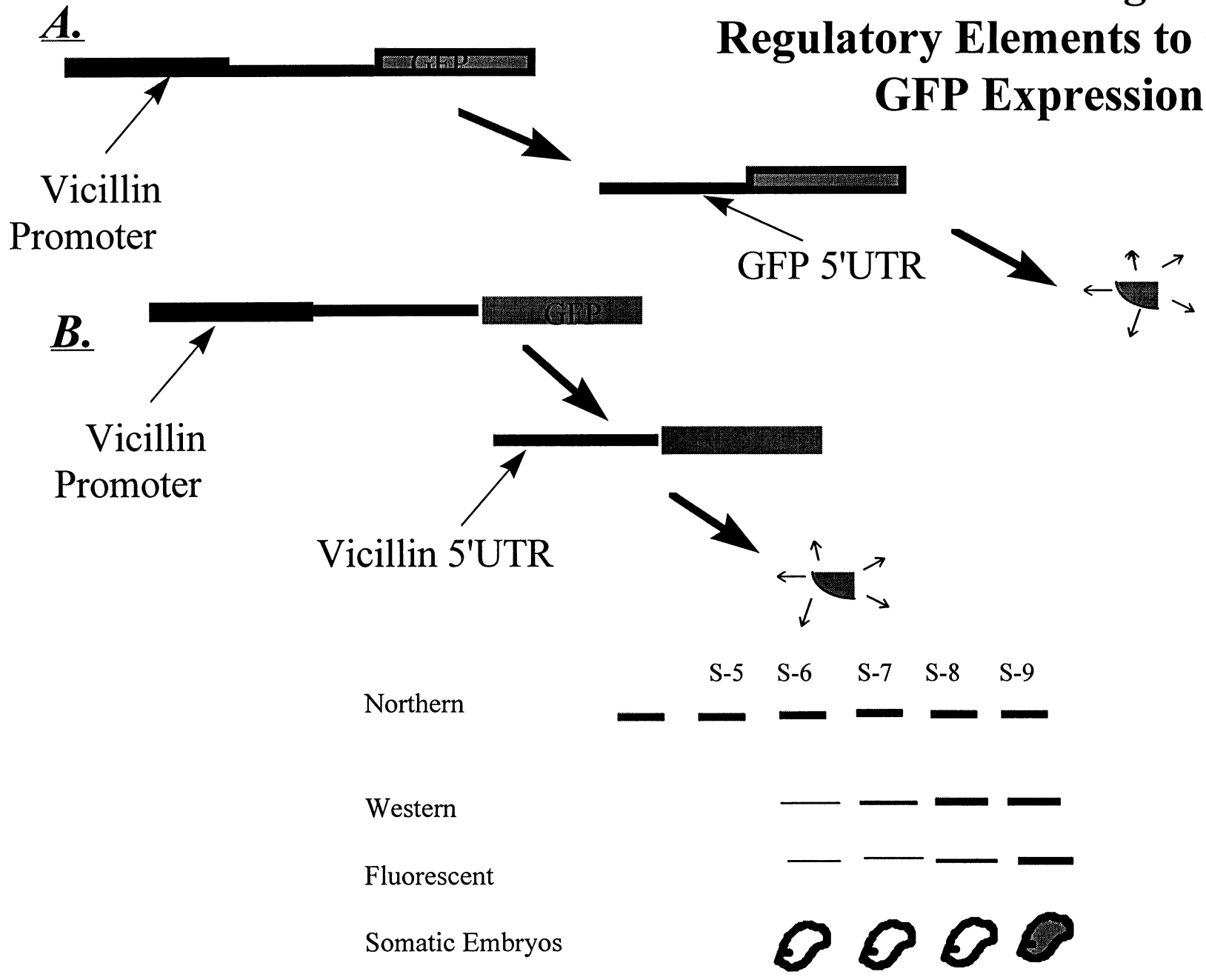
- Somatic embryos sometimes exhibit
- a) Truncated cotyledonary development
- b) Precocious germination
- c) Recallusing
- d) Fused cotyledons
- e) Inability to germinate

Green Fluorescent Proteins Markers "GFP"

- **GFP reporter gene from jellyfish can monitor gene expression and protein localization *invivo* and *insitu.***
- **GFP protein is stable, species independent, and can monitor in living cells <u>noninvasively.</u>**

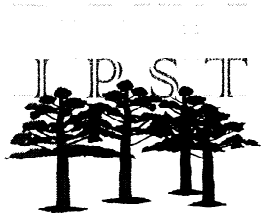


Schemes for Using Vicillin Regulatory Elements to Control GFP Expression



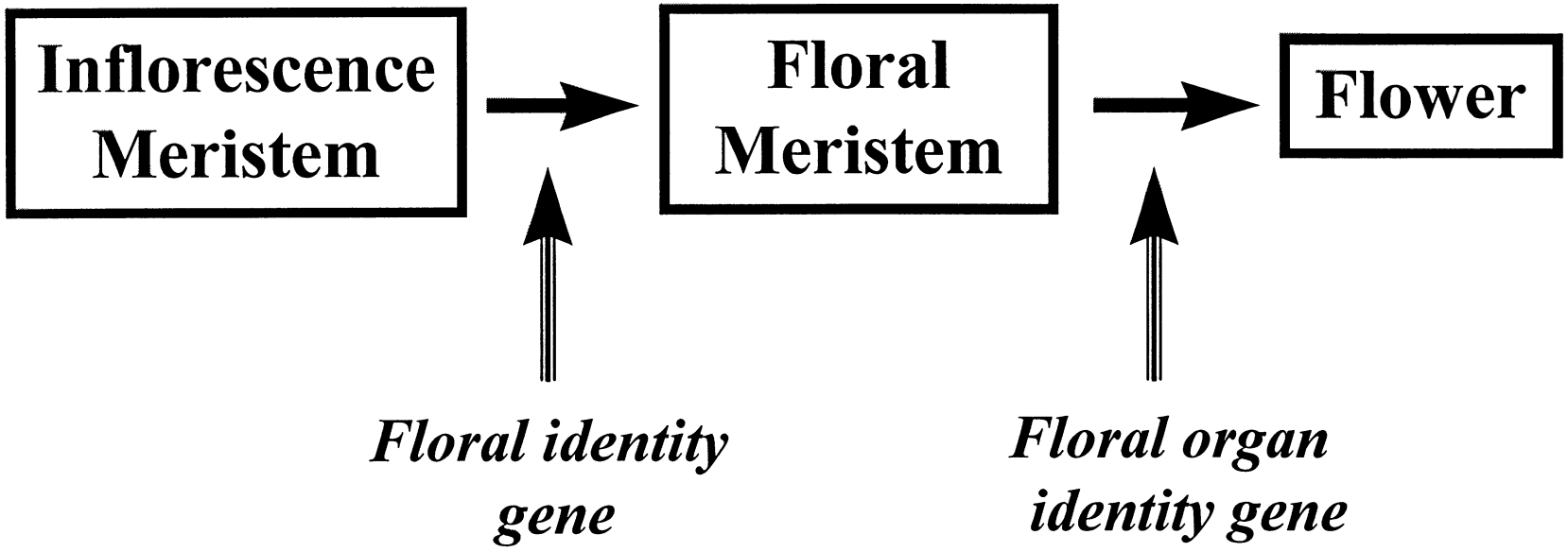
Summary

- We have isolated Vicillin like embryo storage protein from Loblolly pine.
- Sequence analysis revealed that this gene has high homology with white spruce vicillin like storage protein.
- This gene is expressed in mid and late stages of somatic embryos.
- We propose a precise system to sort somatic embryos using Loblolly pine vicillin promoter and GFP gene.
- We will commence doing insitu hybridization and western blotting to investigate correlation between transcription and translation.



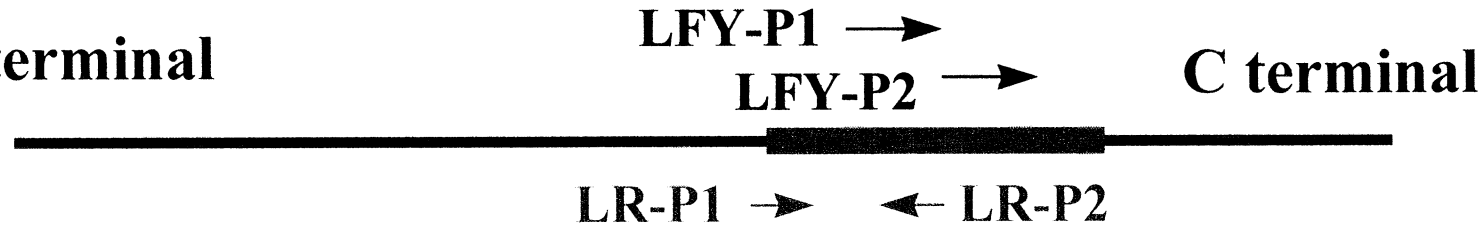
Isolation of Homeotic LEAFY-Like Gene and Its Expression in Loblolly Pine Embryos

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



- LEAFY is important floral identity gene.
- LEAFY is expressed in floral tissues and tobacco vegetative shoots.
- LEAFY overexpression can induce early flowering

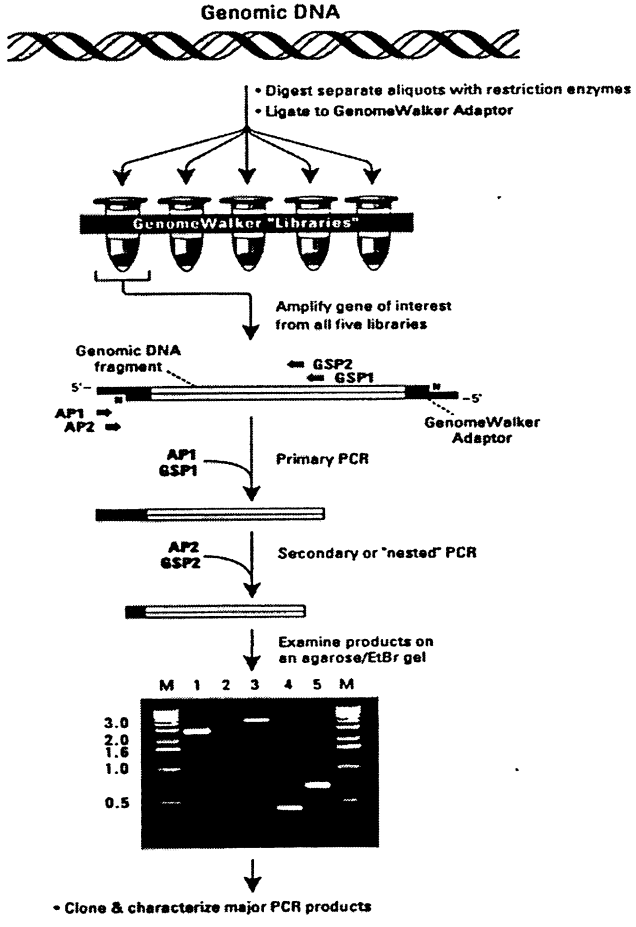
LEAFY-Like Gene Cloning Primer Design Strategy

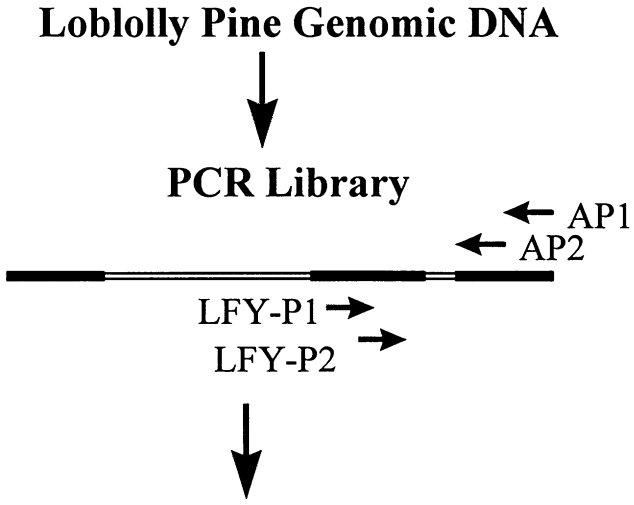


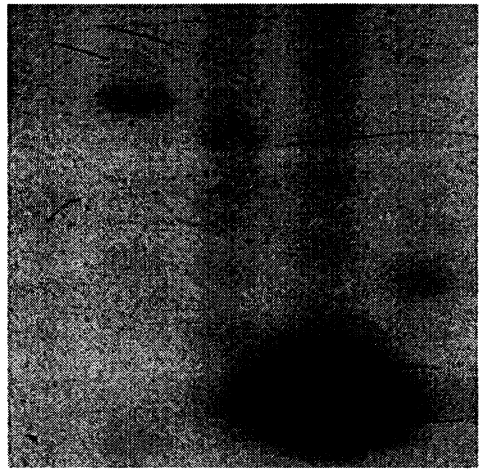
Genomewalk Primers: LFY-P1, LFY-P2

RT-PCR Primers: LR-P1, LR-P2

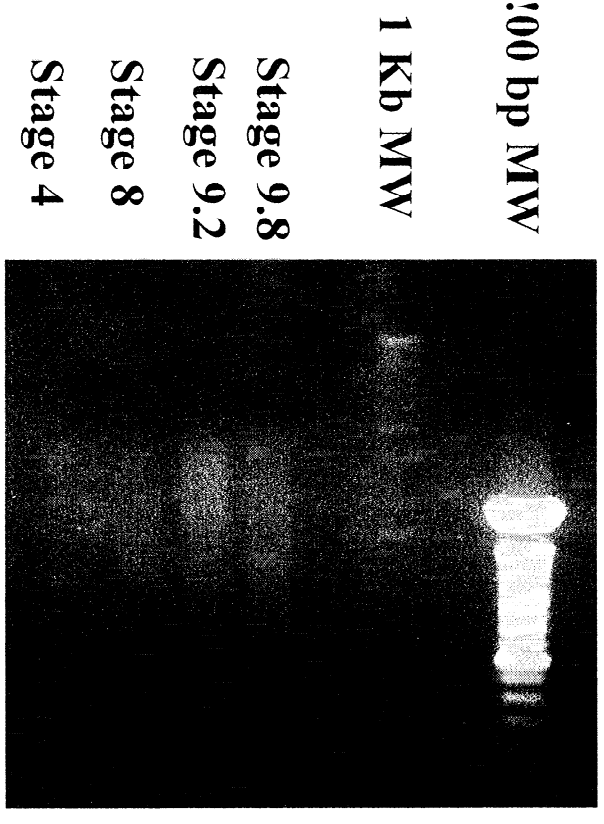
A LEAFY Fragment Has Been Cloned From a Loblolly Pine PCR Library

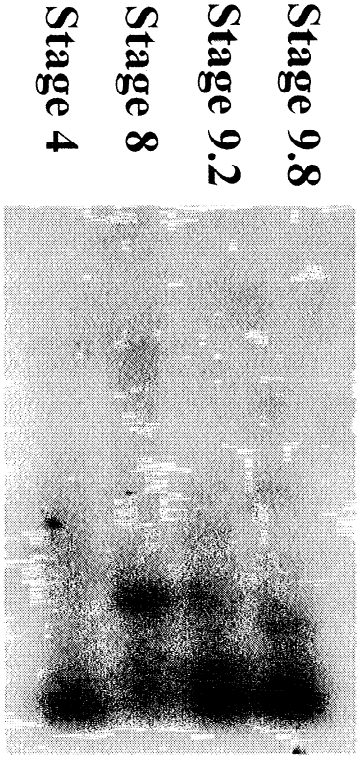




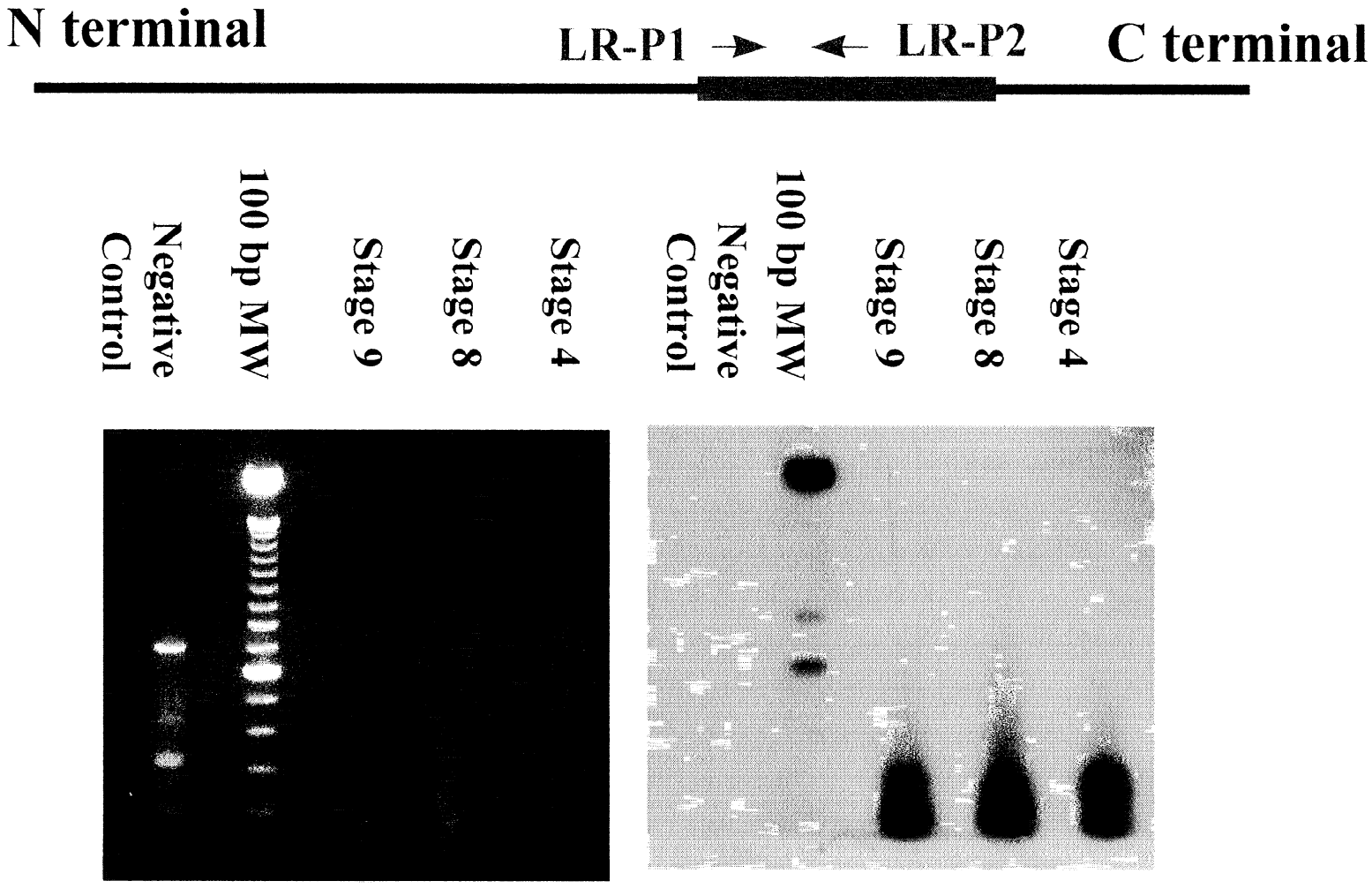


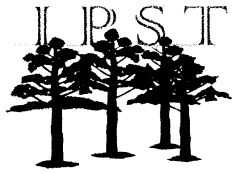
The Expression of LEAFY-Like Gene in Loblolly Pine Zygotic Embryos was Suggested by Pseudo-Northern Blot.





Expression of LEAFY-Like Gene in Loblolly Pine Embryos





Conclusion:

- **Loblolly Pine LEAFY-Like Gene Fragment was Cloned.**
- **The Vegetative Expression of LEAFY-Like Gene in Loblolly Pine Embryos was studied.**

Future Work:

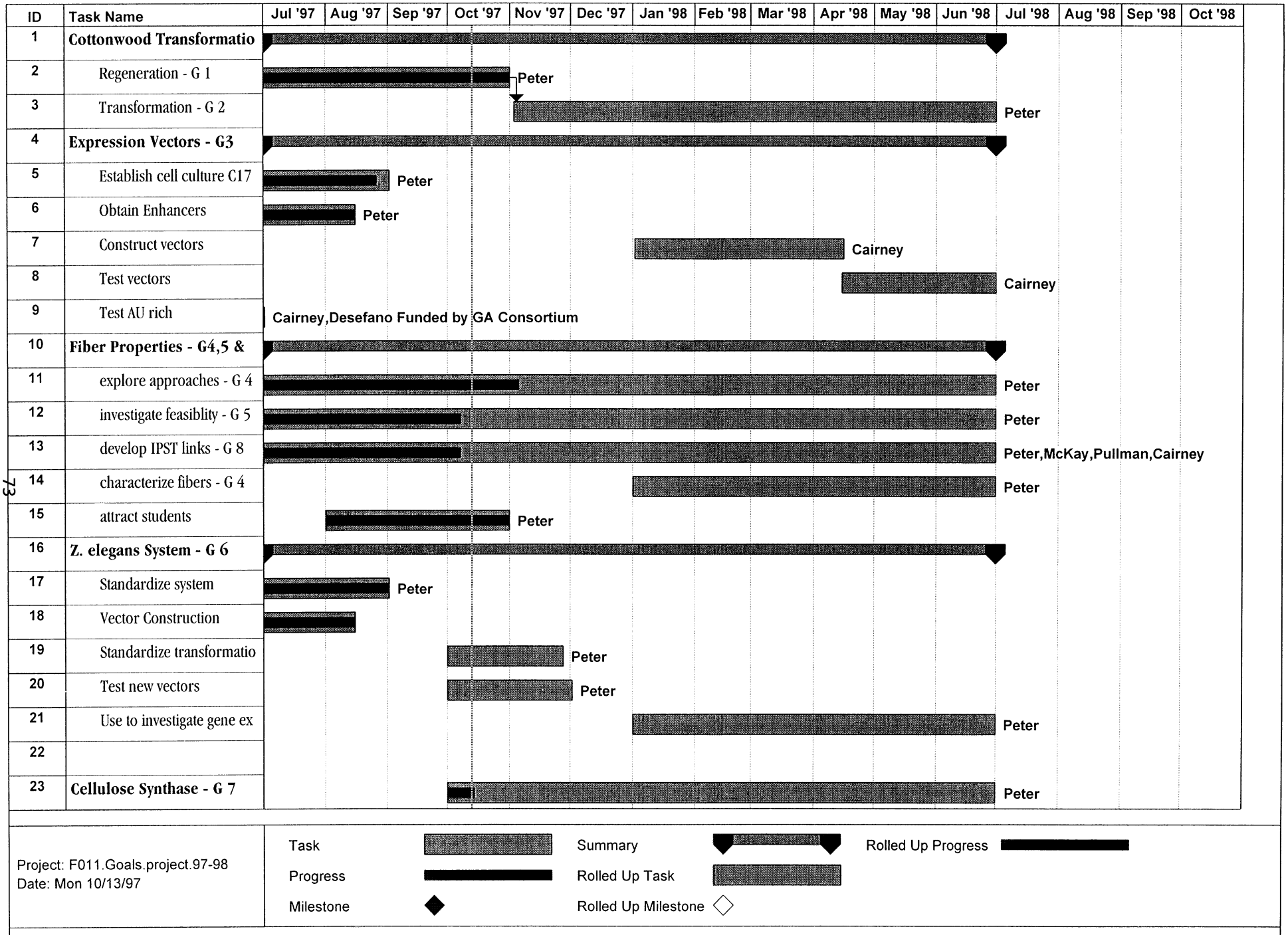
- **Cloning Full Length LEAFY-Like Gene from Loblolly Pine.**
- **Study the Expression of the Loblolly Pine LEAFY-Like Gene by *In situ* Hybridization and RNase Protection Assay.**

F-011 GOALS FOR FY 1997-1998

1. Initiate *in vitro* regeneration experiments with commercially relevant *Populus* clones from the northwest and southeast.
2. Initiate transformation of *Populus* hybrids and *Populus deltoides*
3. Construct nonproprietary sequences for stable, high level expression of transgenes useful for transformation studies.

   Evaluate the effect of an AU rich sequence on the stability / translation of a mRNA.

   Test usefulness of viral translational enhancers in transient assays.
4. Explore approaches for creating transgenic cottonwood with improved cambial growth and fiber properties and characterize fiber properties of short rotation cottonwood.
5. Investigate the feasibility of using conserved sets of genes that when overexpressed in cottonwood are predicted to stimulate the rate of cambial cell divisions, promote xylem tracheary elements or fiber differentiation, stimulate xylem cell elongation...
6. Continue development and use of *Z. elegans* as a model system for isolating and elucidating the mechanisms that regulate tracheary element differentiation and fiber properties.
7. Isolate cellulose synthase gene(s) from cottonwood cambium and differentiating xylem cells.
8. Develop links with experts at IPST to measure wood and fiber characteristics of short rotation cottonwood.


ID
Task Name
Jul '97
Aug '97
Sep '97
Oct '97
Nov '97
Dec '97
Jan '98
Feb '98
Mar '98
Apr '98
May '98
Jun '98
Jul '98
Aug '98
Sep '98
Oct '98
1 Cottonwood Transformatio
2 Regeneration - G 1
3 Transformation - G 2
4 Expression Vectors - G3
5 Establish cell culture C17
6 Obtain Enhancers
7 Construct vectors
8 Test vectors
9 Test AU rich
10 Fiber Properties - G4,5 &
11 explore approaches - G 4
12 investigate feasiblity - G 5
13 develop IPST links - G 8
14 characterize fibers - G 4
15 attract students
16 Z. elegans System - G 6
17 Standardize system
18 Vector Construction
19 Standardize transformatio
20 Test new vectors
21 Use to investigate gene ex
22
23 Cellulose Synthase - G 7
Peter
Peter
Peter
Peter
Cairney
Cairney
Cairney,Desefano Funded by GA Consortium
Peter
Peter
Peter
Peter,McKay,Pullman,Cairney
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Date: Mon 10/13/97
Task
Progress
Milestone
Summary
Rolled Up Task
Rolled Up Milestone
Rolled Up Progress

97-'98 Goals F-011: Mass Clonal Propagation of Genetically Improved Hardwoods

- Improve Fiber Properties
  - Explore Approaches
  - Investigate Feasibilities
  - Develop IPST Links
  - Characterize Fibers
- *Z. elegans* Model System
- Cellulose Synthase
- Transformation
  - Regeneration
  - Transformation
- Gene Regulation
  - Expression Vectors

Transformation: Standardizing Cottonwood C175

- Goal 1 - Regeneration
  - Propagated sterile shoots; to bulk up material for sterile leaves
  - Standardized shoot regeneration system from leaves
- Goal 2 - Transformation
  - Obtained pBI121 with an ER resident Green Fluorescent Protein and transformed into *Agrobacterium tumefacians*
  - Ready to standardize *Agrobacterium* transformation procedure

Gene Expression/Fiber Properties

- Goal 3 - Gene Expression
  - Established liquid suspension cultures of C175 for transient assays
  - Obtained DNA that encdoes the Omega and TEV enhancers
  - Need to construct & test vectors with translational enhancers
- Goal 4 - Approaches
  - Grant to stimulate cambial cell divisions submitted to USDA and DOE
- Goal 4 - Characterize Fibers from Plantation Grown Hardwoods
  - Methods development
  - Recruited MS student

Fiber Property Improvement

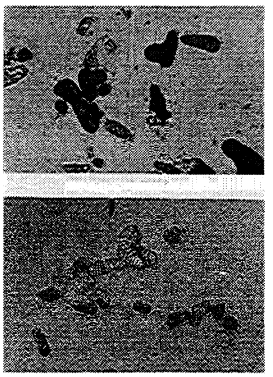
- Goal 5 - Feasibility
  - Attracting graduate students
  - Consulting with other faculty
  - Giving A390 problems; literature research
- Goal 6 - *Z. elegans*
  - Standardized *in vitro* differentiation system
  - Vector construction
  - Standardizing transformation assays
  - Testing new vector constructions

Fiber Properties

- **Goal 7 -Cellulose Synthase**
  - **Created alignment of all known Cel A like sequences**
  - **Designed degenerate primers**
  - **Designed specific primers**
  - **Obtained cDNA library from cambial region of Aspen from Lin Ge**
- **Goal 8 - IPST Links**
  - **Hiroki Nanko**
  - **Don Dimmel**
  - **Art Ragauskas**

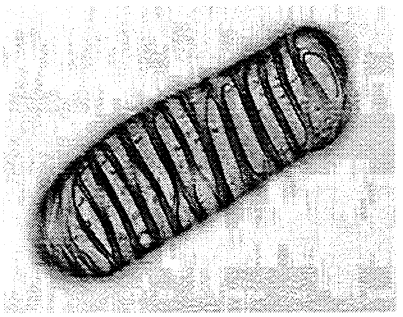
Goal: Modify Fiber Properties to Improve Pulping Efficiency and Paper Quality

- Use model system to dissect the cellular and molecular mechanisms that control cellulose angle, wall thickness, fiber cell length, lignin quantity and quality
- Isolate critical genes that regulate fiber cell wall properties
- Develop and test strategies to modify fiber cell wall properties



Zinnia elegans a Model System to Study Xylogenesis

- Developmental Characteristics
  - Inducible
  - Synchronous
  - Single celled
  - No cell division
  - 50% Tracheary Elements in 3 days



Uses of *Z. elegans* Model System

- Rapid identification of DNA sequences that regulate gene expression in differentiating tracheary elements
- Rapid tests of novel gene function
  - subcellular localization
  - overexpression
  - dominant negative mutations
  - antisense
- Rapid tests of strategies for altering xylem cell properties

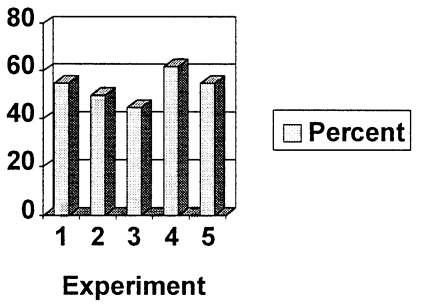
Uses of *Z. elegans* Model System

- Signal transduction of hormone responses in xylem differentiation
- Mechanisms of cytoskeletal organization and control of secondary wall formation
  - pattern of cellulose synthesis
  - assembly of secondary cell wall components
- *In vivo* dynamics of cellular processes

Establishing *Z. elegans In vitro* Differentiation and Transformation

- **Differentiation**
  - **Plant growth**
  - **Leaf sterilization**
  - **Maceration**
  - **Cell culture conditions**
  - **Media preparation & composition**

Percent TEs at 72H



Current Goals for *Z. elegans*

- **Standardize transformation procedure**
- **Construct three vectors to identify**
  - **1. Gene regulatory sequences**
  - **2. Subcellular location of novel proteins**
  - **3. Overexpression and antisense expression to establish function of proteins**
- **Test newly constructed vectors**

Current Goals: Vector Construction

- Standardizing Transformation
  - Testing with ER-GFP
- Gene Regulatory Seqences
  - 1. Quantitative marker (GUS) to assay expression level from 5' & 3' sequences of interest
  - 2. Internal control (GFP-HDEL) to quantitate transformation efficiency

Current Goals: Vector Construction

- Subcellular localization
  - In frame cloning sites to make amino and carboxy terminal fusions to GFP
- Test with Rac proteins
- Overexpression & antisense expression
  - Gene of interest expressed from strong promoter
  - Internal control (GFP-HDEL) to identify cells that are transformed
- Test with modified CDPK's

Summary of Goals

- **Test SAM promoter and GFP-HDEL**
- **Test CDPK for role in xylem differentiation (Initiated with seed grant funding with Jung Choi Dept. of Biology Georgia Tech)**
- **Test for subcellular location and functions of Rac GTP binding proteins in secondary wall formation**

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

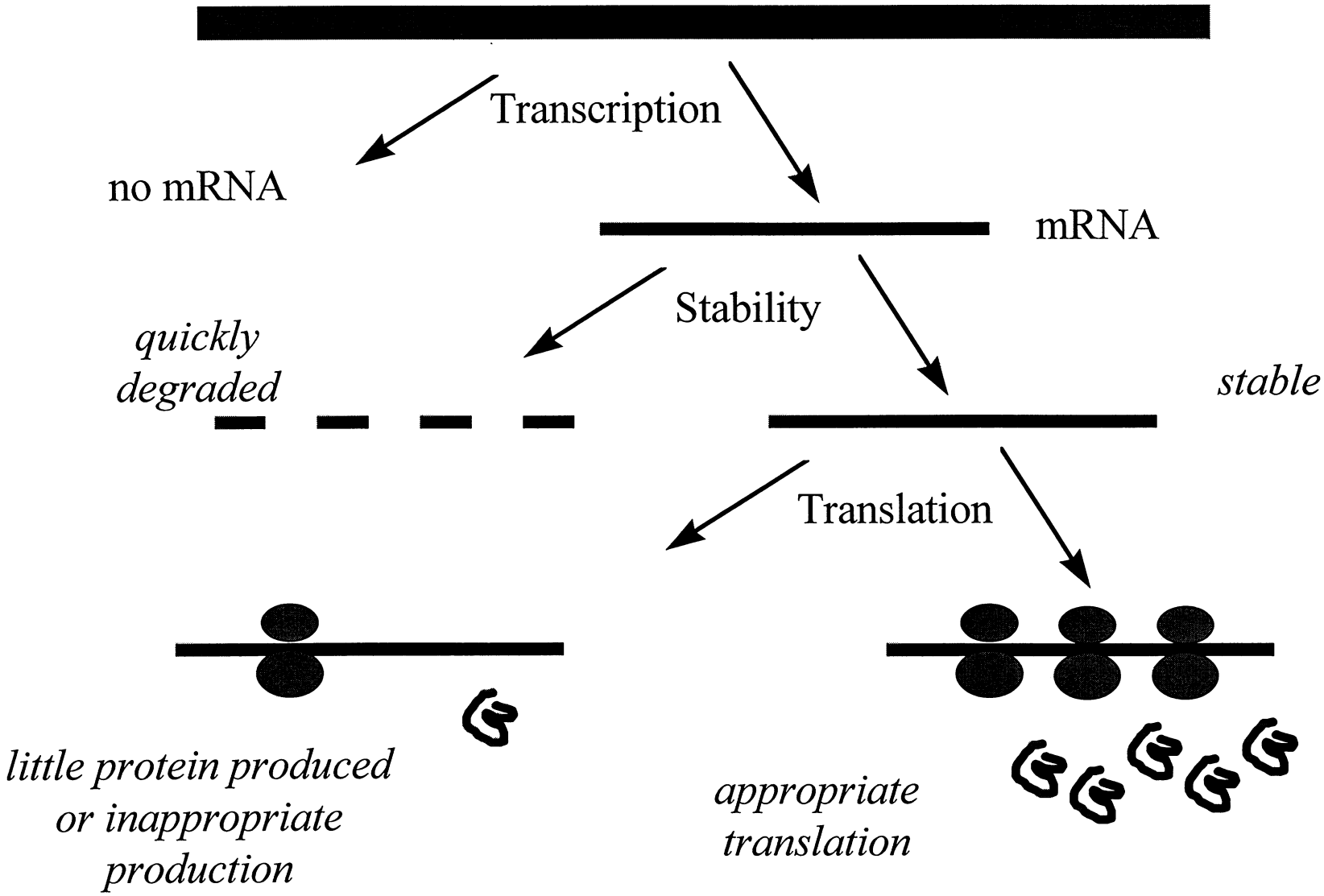
Regulation of Expression of a Stress-related Gene: Promoter and 3' UTR Analysis- An Update

Luis Destéfano-Beltrán

and

John Cairney

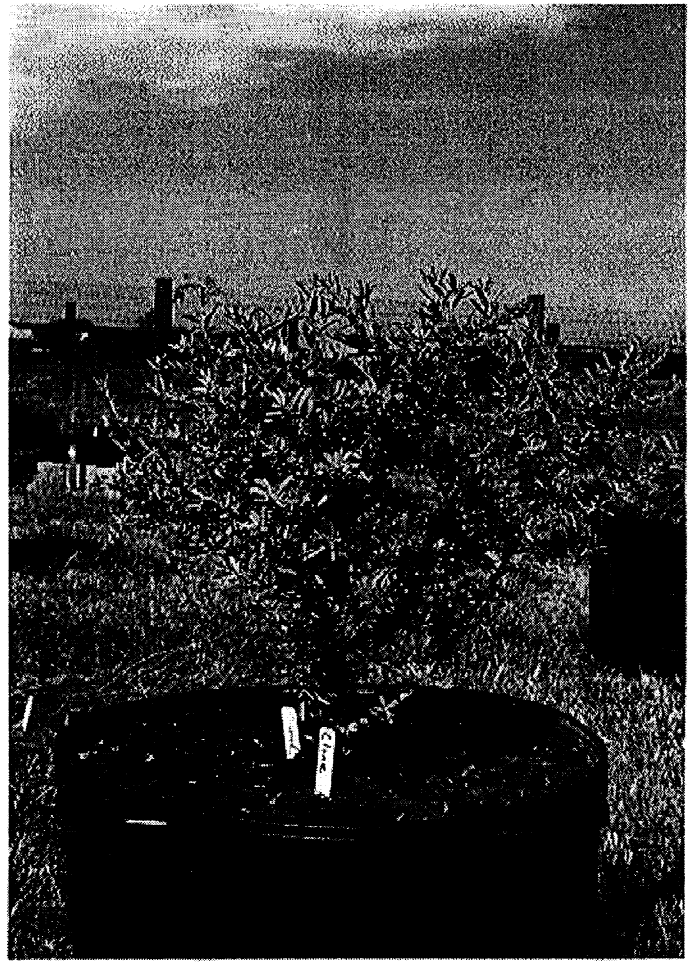
Factors Affecting Gene Expression



Atriplex canescens (Saltlbush)

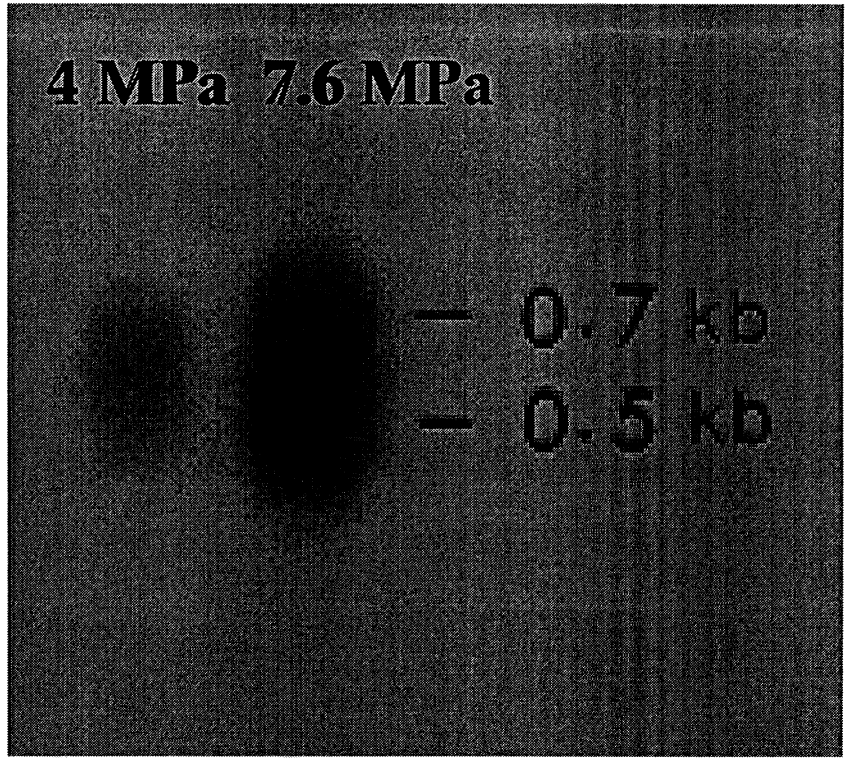
-Atriplex is a woody desert shrub, found in arid lands where few other plants can strive

-In the experiments described, water was witheld from greenhouse grown plants in a staggered fashion. Water potentials were measured at pre-dawn



PI Clone Hybridizes to Several mRNAs

Poly-A mRNA was isolated from the leaves of water-stressed *Atriplex* plants and used to construct a cDNA library. This was differentially screened and several clones were isolated.
A clone, 23-3 hybridized to several mRNA species.



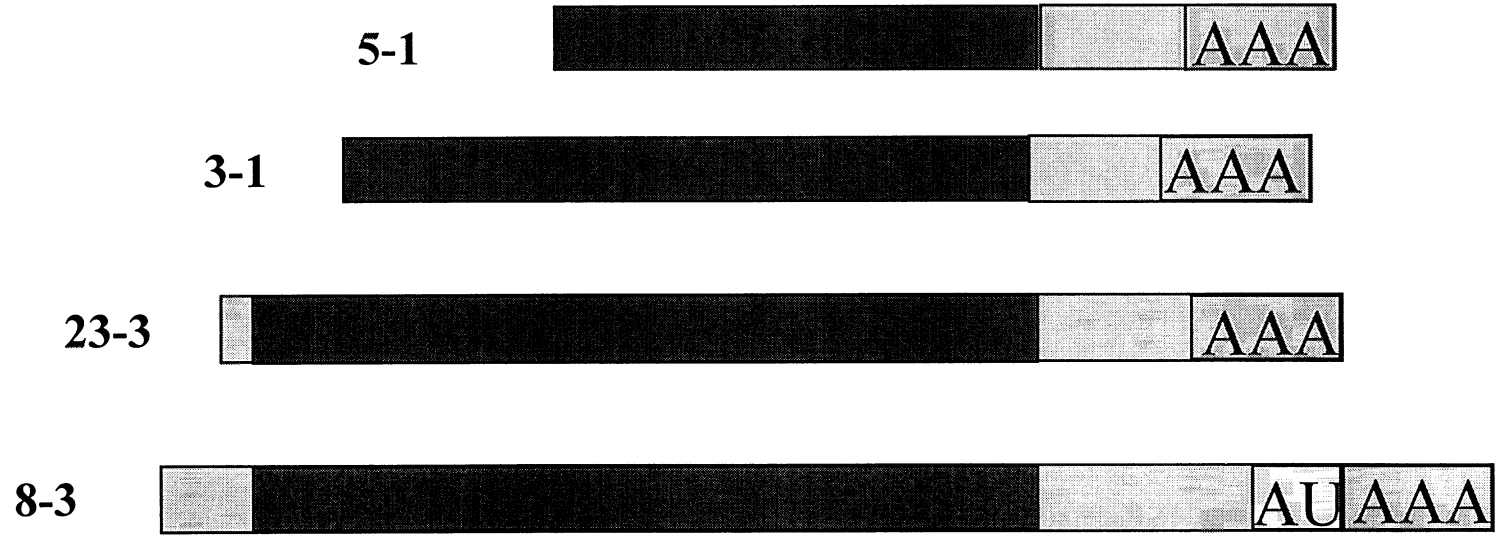
Several cDNA Clones Were Isolated Using the 23-3 clone: All were Homologous to Proteinase Inhibitors

Untranslated Region

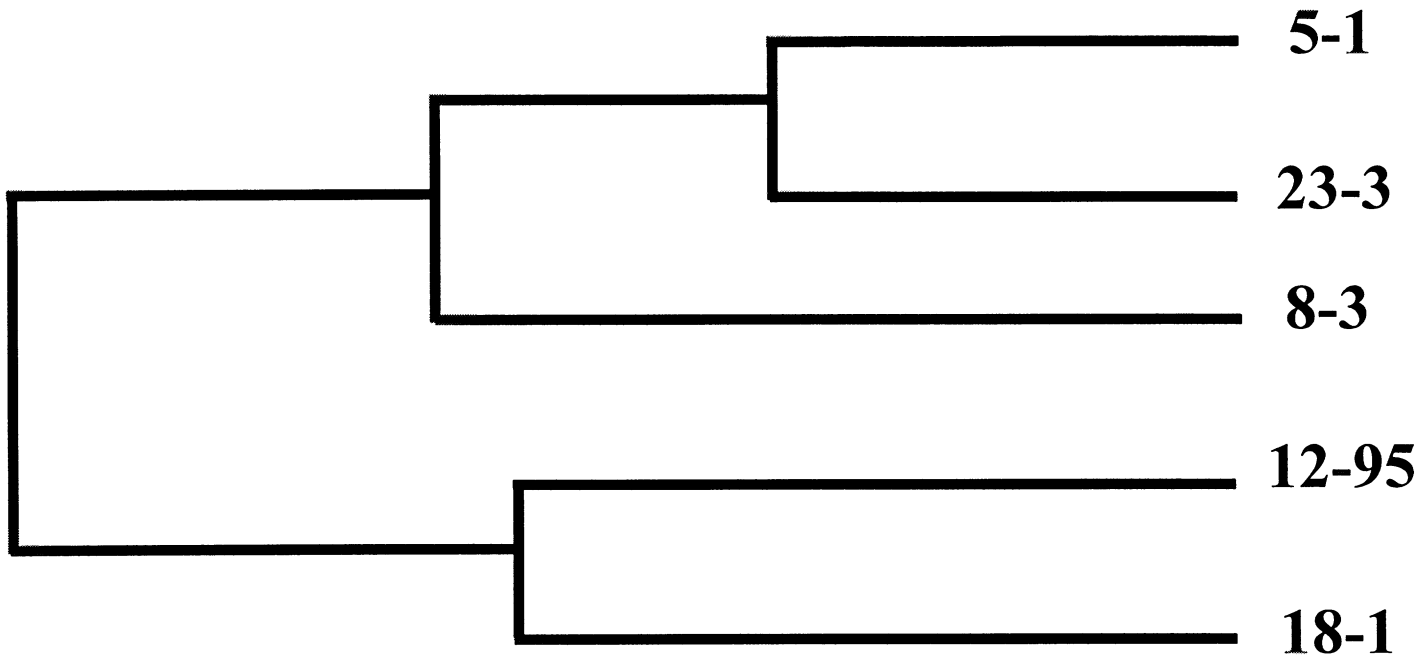
Translated Region

AU-Rich Region

1. Induced by drought stress
2. Share 95% sequence identity
3. Most likely represent a multigene family
4. Vary at 3' end
5. Encode for a proteinase inhibitor



UPGMA Tree of Five Proteinase Inhibitors from *Atriplex*

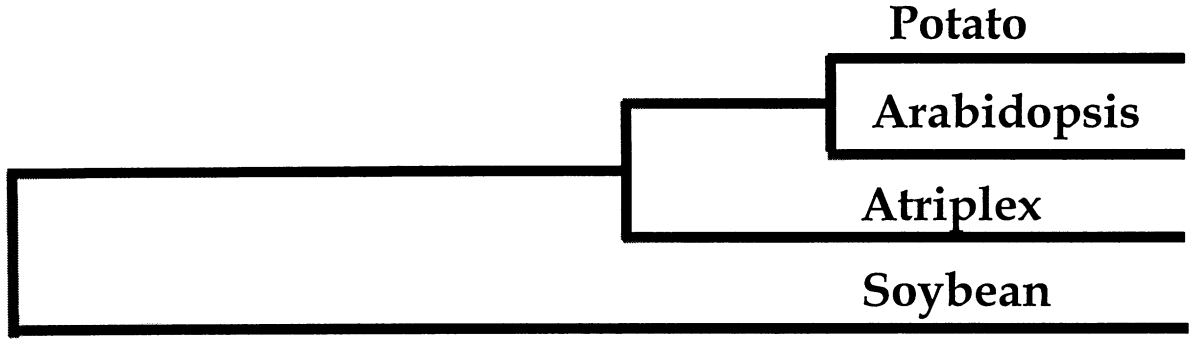


Amino Acid comparison of cDNA clones using the Geneworks Software Package

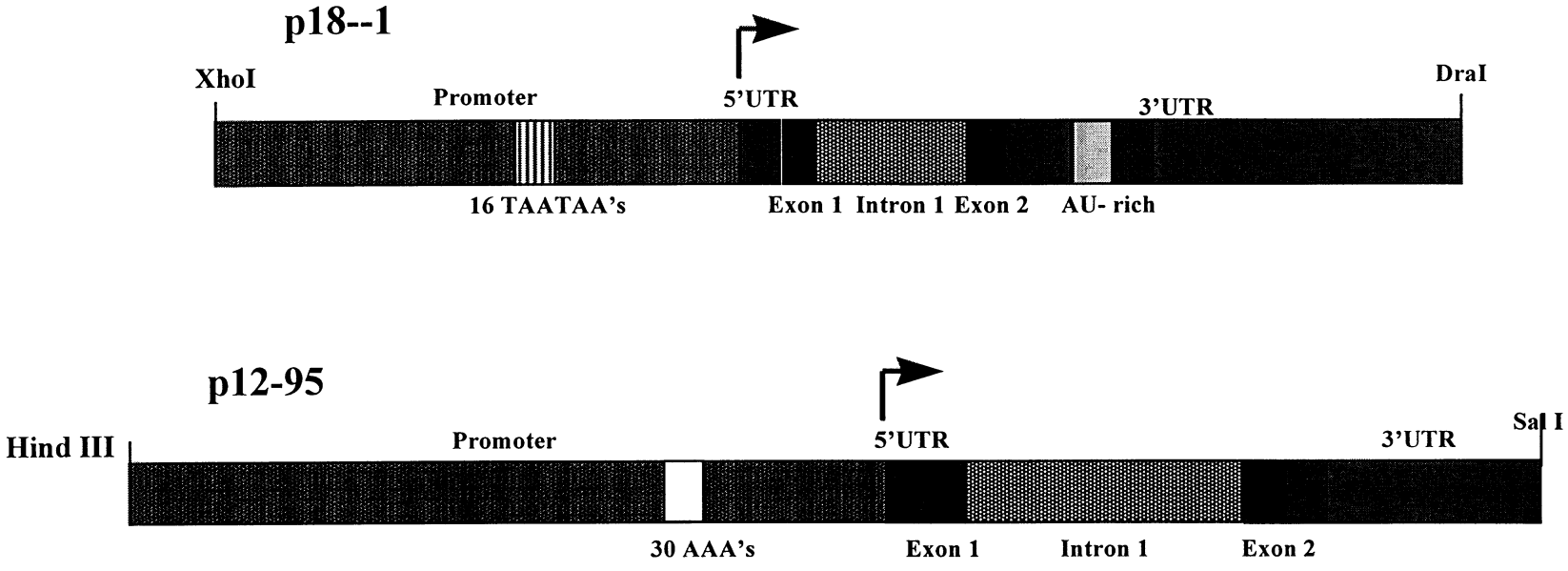
Comparison of Other Proteinase Inhibitors with Atriplex clone 23-3

```
SOYBEAN      SDQ-SSSYDD DEYSK----P CCDLCTRSM- -PPQCSCEDI RLN-SC--NS   40
ATRIPLEX     MRPIAVFFLV FLLALTTEEV GPRVAEGFLG IGKKCSIPSK TKKGPCFSDT   50
POTATO       LS--MRFFAT FFLAMLV--V ATKMGPMRIA EARHCESLSH RFKGPC-TRS   45
ARABIDOPSIS  MKLSMRLISA VLIMFMI-FV ATGMGPVTV- EARTCESQSH RFKGTCVSAS   48

SOYBEAN      DCKSCMCTRS QCRCLDTNDF CYKPCKSRDD                        70
ATRIPLEX     NCDS-ICRAE RMRAGICHGV RRRCLCCR--                        77
POTATO       NCAS-VCETE RFSGGNCHGF RRRCFCTKPC                        74
ARABIDOPSIS  NCAN-VCHNE GFVGGNCRGF RRRCFCTRHC                        77
```

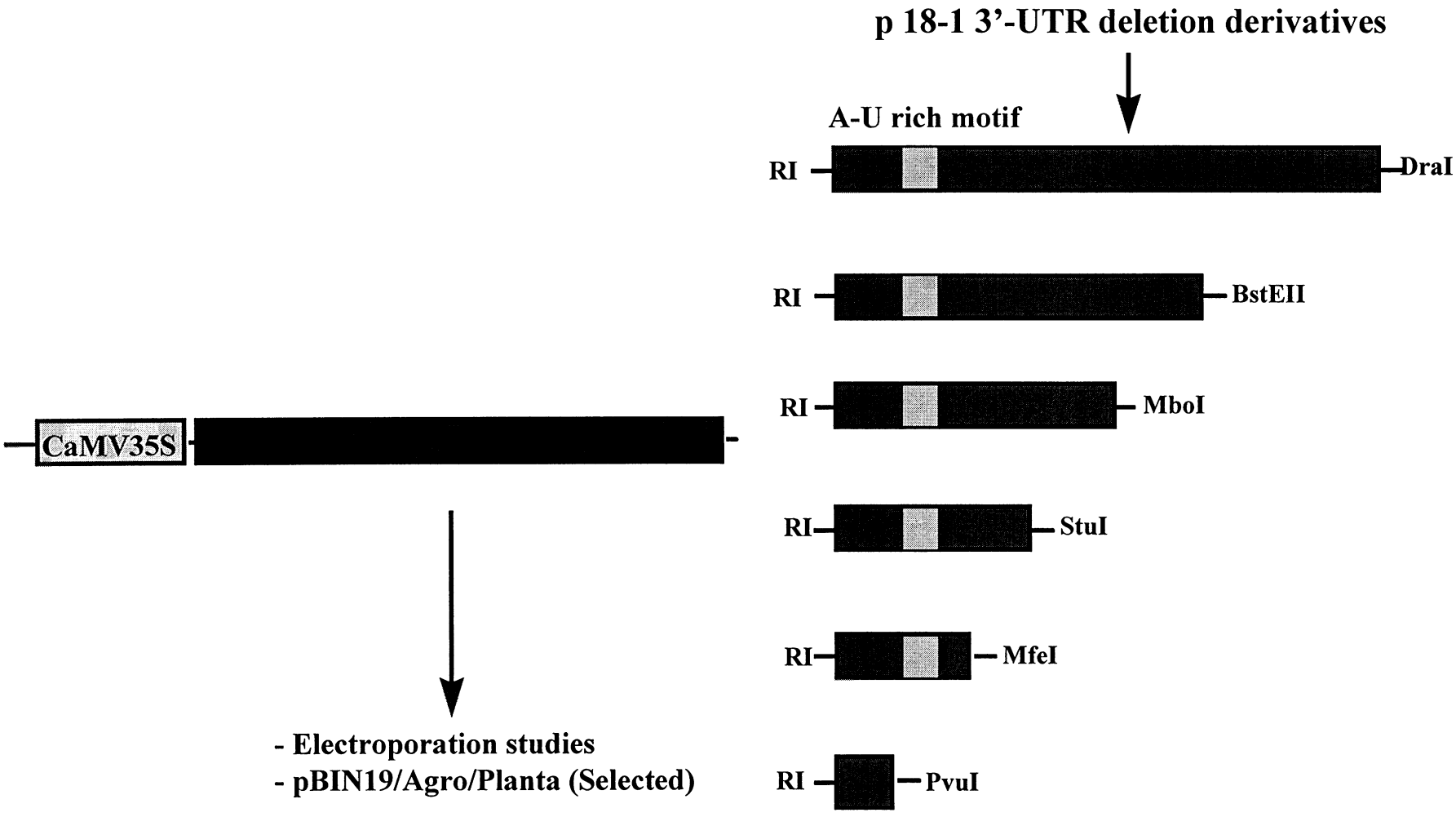


Schematic of *Atriplex* PI genomic clones

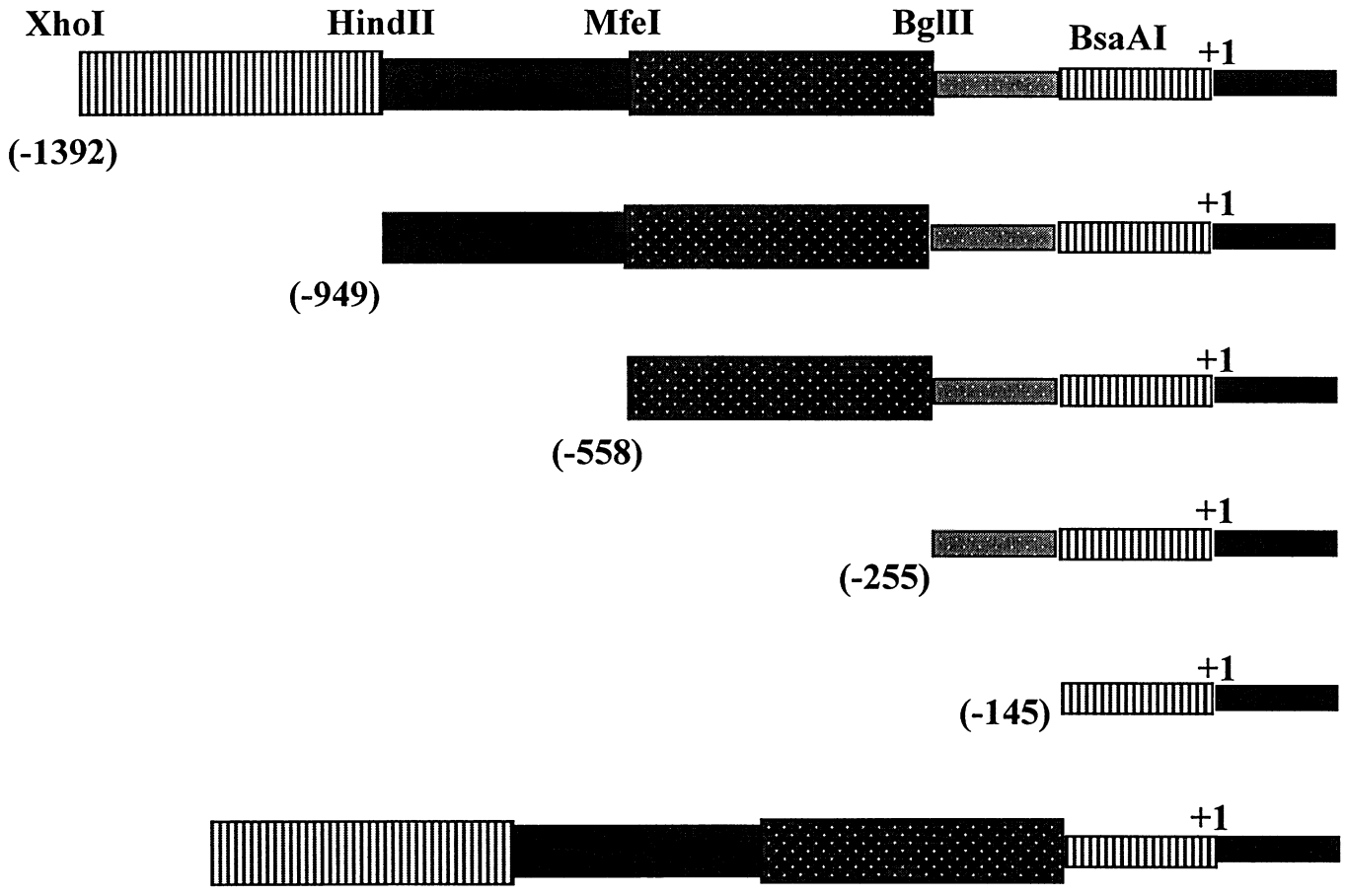


An *Atriplex* genomic library was screened with clone 23-3. Two genomic clones were isolated which differed principally in the length of their introns and in the presence of a 3'-AU-rich region

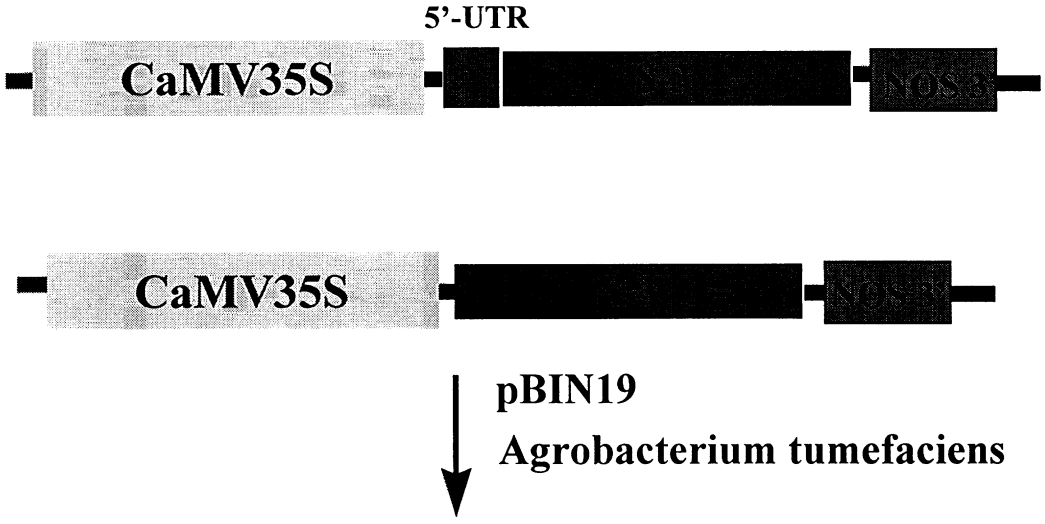
3' Fusions to 35S-GUS: Strategy for replacement of 3'-NOS with 3'UTR from PI clone 18-1



18-1 Promoter Deletion Derivatives Have Been Cloned into a GUS Expression Vector



Sense Vectors for PI Overexpression



Scotland (Dr. Kevan Gartland
University of Abertay, Dundee)
Selected Plants

Update of PI Project

1.- All constructs based in the pBIN19 vector have been introduced into the *Agrobacterium tumefaciens* GV3101. Integrity of the constructs has been checked by restriction analysis.

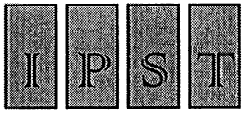
2.- Selected cassettes of PI Promoter/sGUS/NOS3' will be evaluated in transient assays. Some promoter constructs are being introduced into tobacco plants. Expression of the GUS reporter gene will be evaluated in planta under normal and stressed conditions.

3.- Analysis of 35S/GUS/3'PI-UTR will be evaluated in transient assays Selected constructs can be in more long-term experiments in trees.

4.- Sense vectors for PI overexpression have been constructed and will be introduced in plants by a collaborator in Scotland

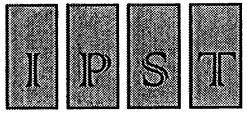
Conclusions and Future Work

- Two genes for a drought-inducible Proteinase Inhibitor (PI) possess unusual features
- These features may be involved in RNA stability or translation efficiency
- We have cloned regulatory sequences into plant transformation vectors for transformation into plants
- We will clarify the nature of changes which occur to PI mRNA in response to growth stress (drought, heat etc.) by assesing mRNA size, polysome isolation, polyA tail length



Identification of A Plant Enzyme Which Activates A Regulatory Peptide

IPST: John Cairney, Luis Destefano, Cody Cain,Jerry Pullman,
GIT: Sheldon May, Charlie Oldham, Allison Moore

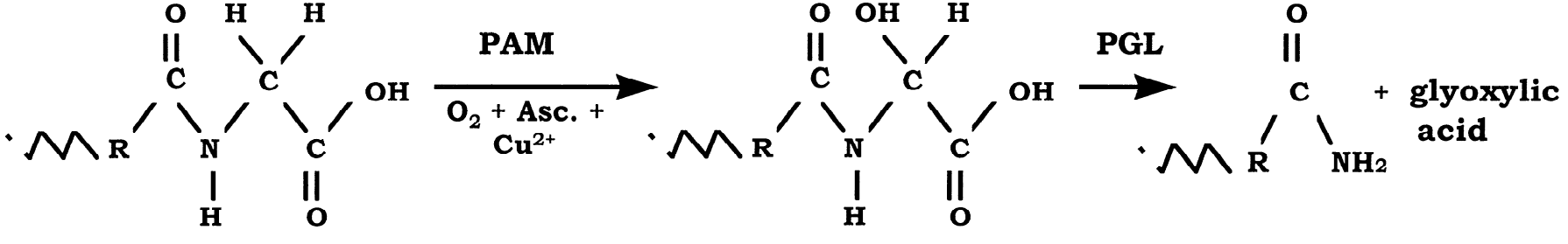


The PAM-PGL Enzyme System

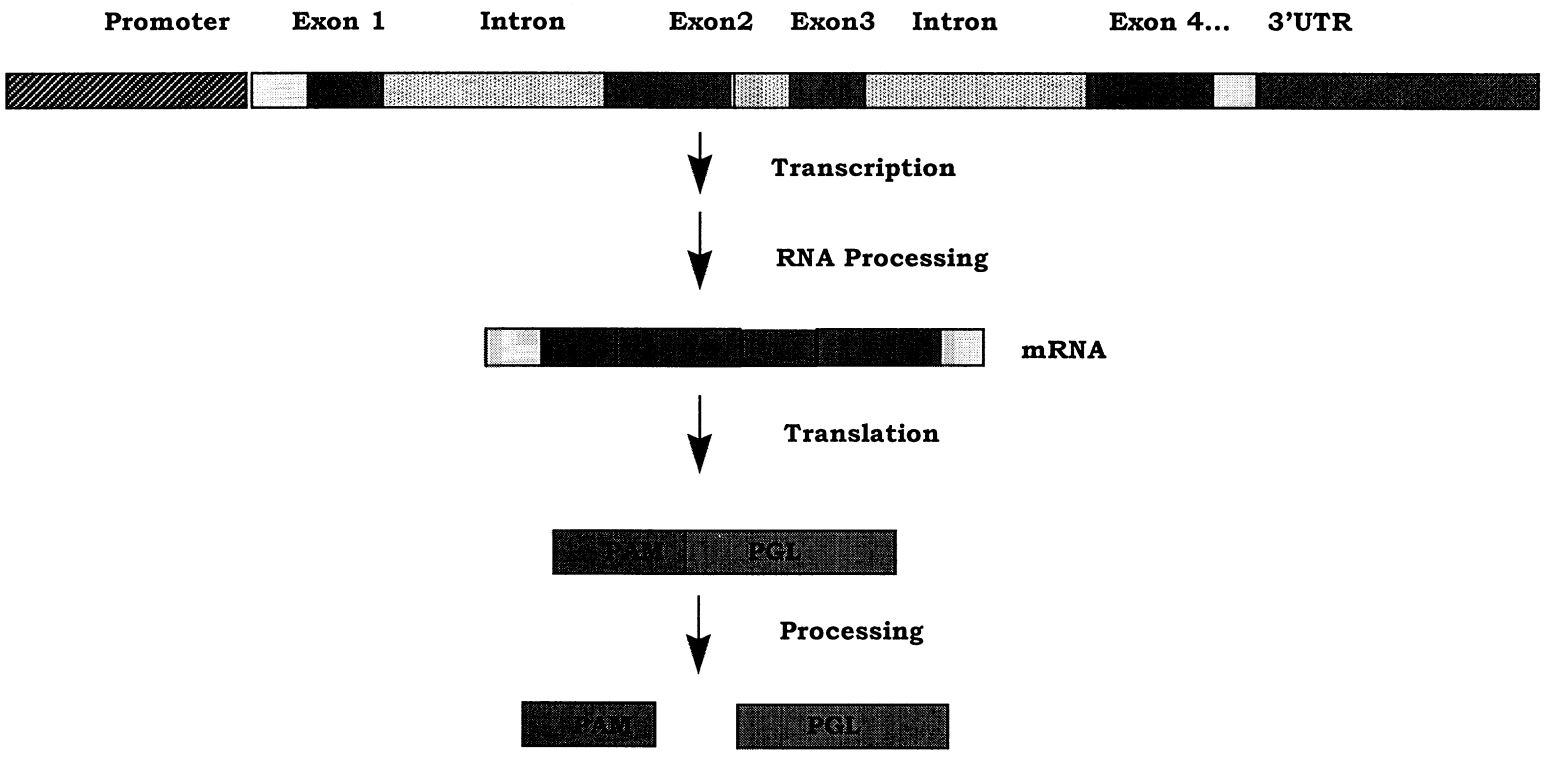
- Many regulatory peptides require modification (amidation) at their carboxy terminal in order to become physiologically active
- PAM (Peptidylglycine a-amidating Monooxygenase) and
- PGL (Peptidoaminoglycolate Lyase) work in concert to modify and activate certain enzymes

Enzyme Mechanism

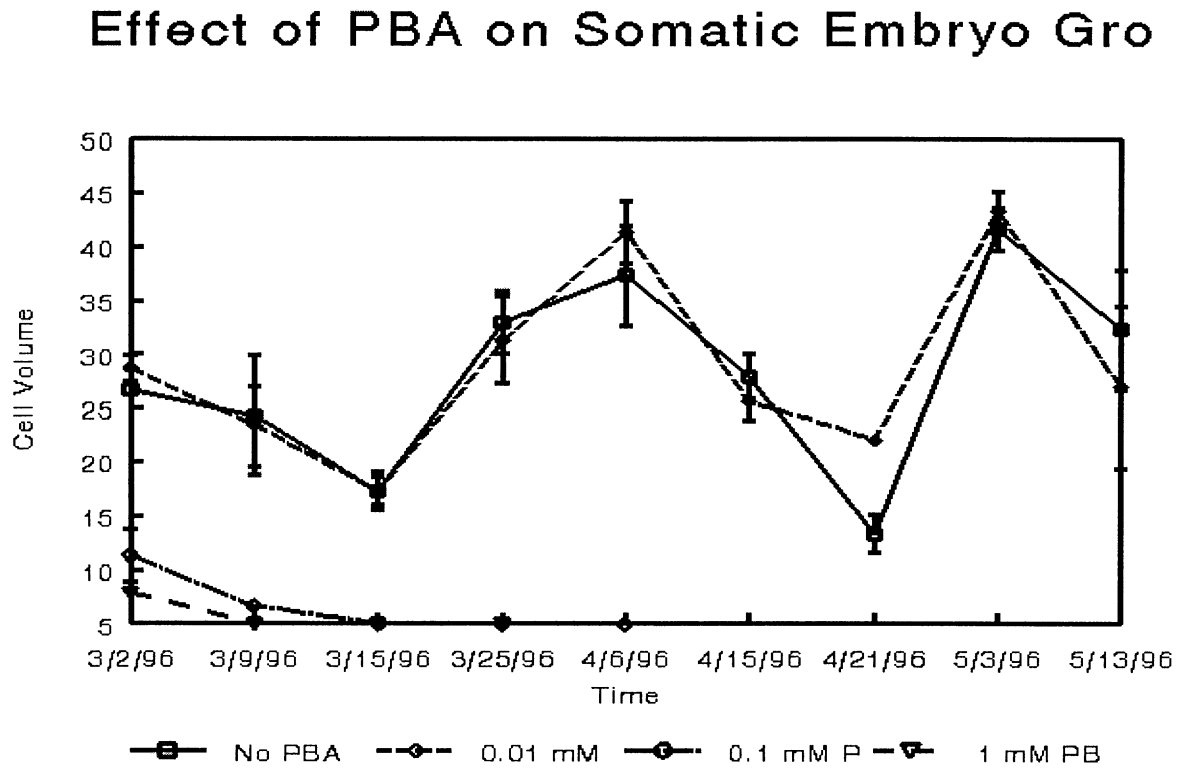
- **The monooxygenase, PAM, forms the a-hydroxyglycine derivative of the substrate peptide, and the PGL catalyzes the dealkylation step to form the amide product and glyoxylate**



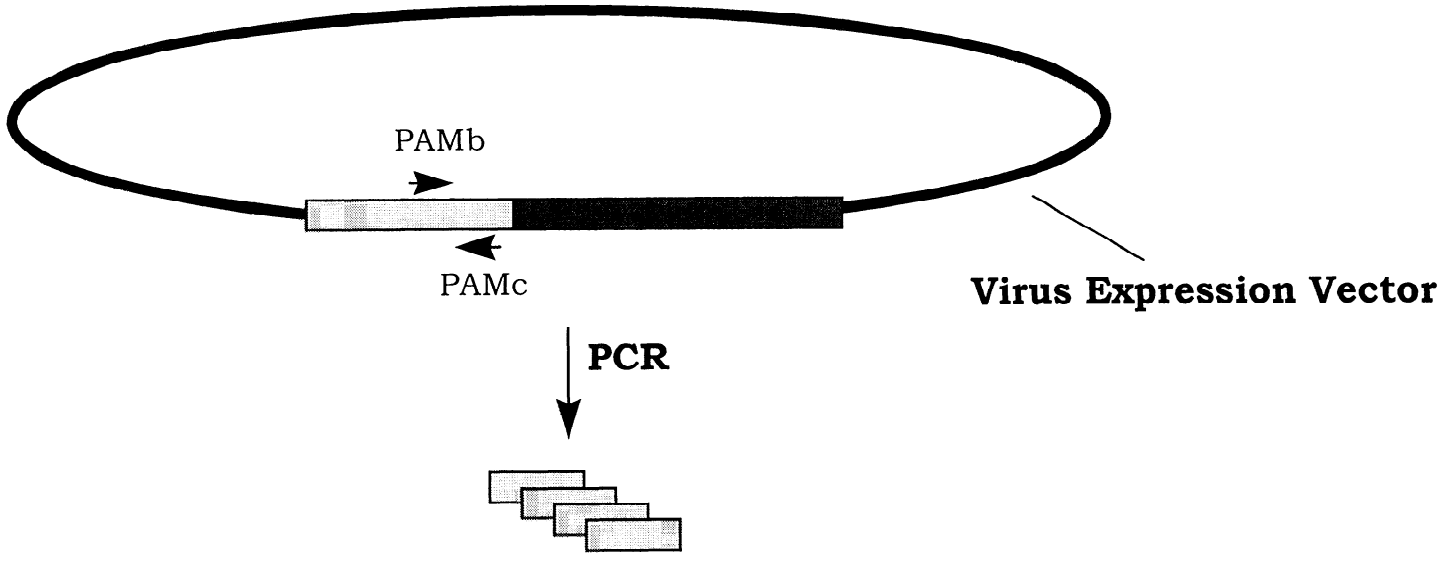
PAM-PGL is Produced As a Single Peptide Which is Then Cleaved Into the Two Activities



An Inhibitor of PAM-PGL Inhibits Growth of Pine Somatic Embryos



DNA Evidence for Plant PAM-PGL Enzyme



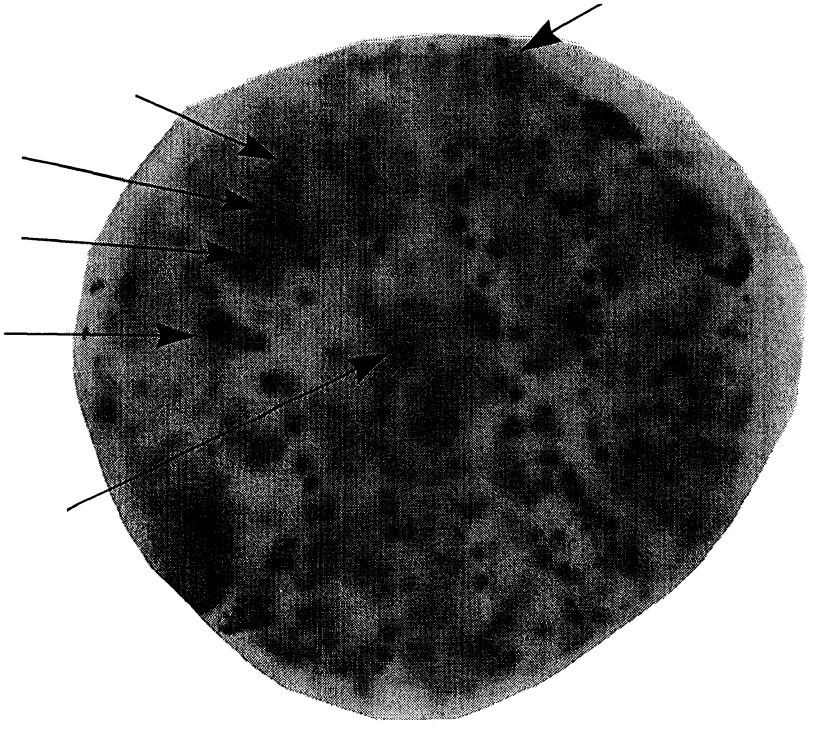
- **PCR used to isolate subfragments of bovine gene, these cloned and used for probing**

Arabidopsis Genomic Library Screening

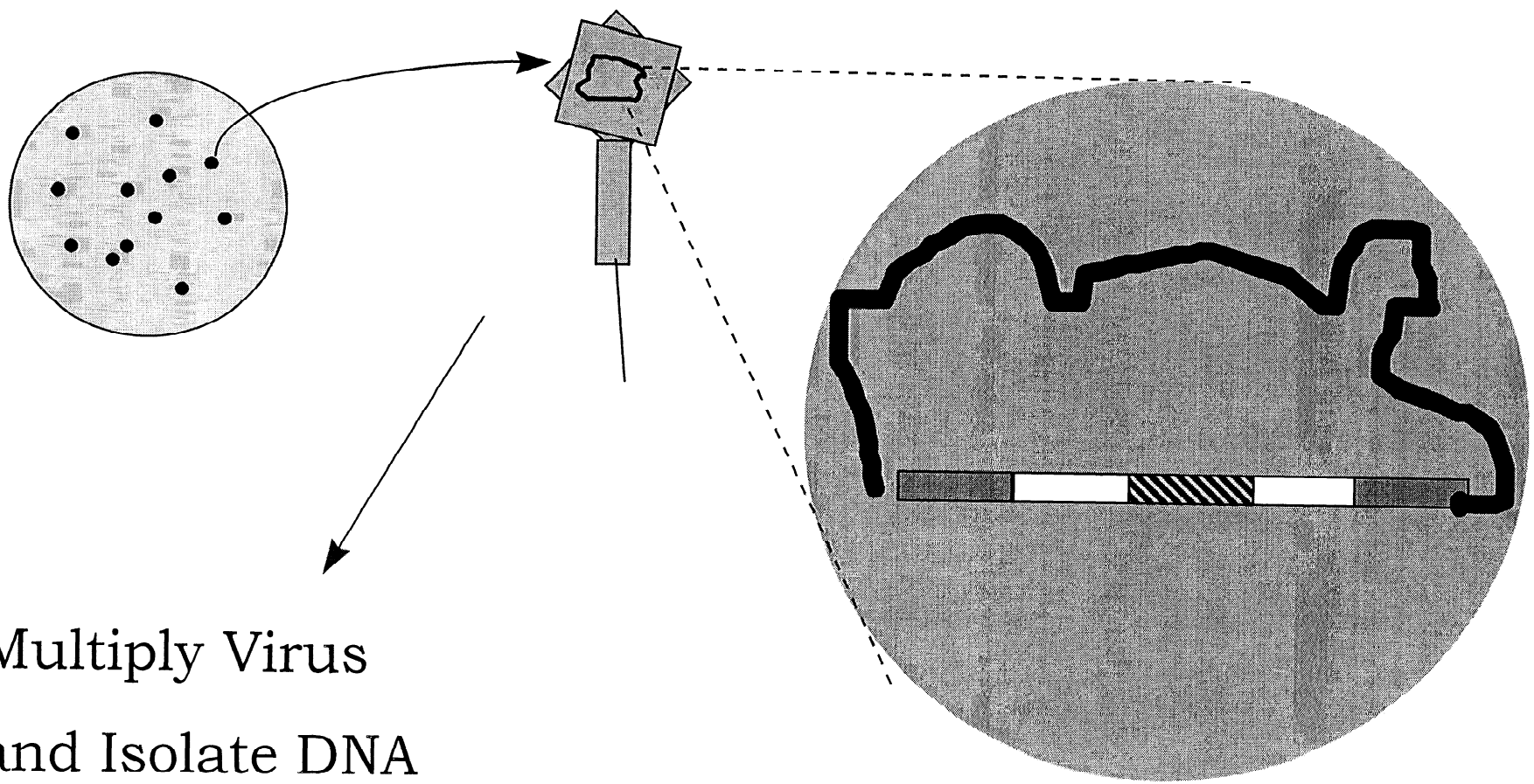
- 60 000 clones of an Arabidopsis genomic library were screened (ca. 3 genomes) using a 500bp fragment of the bovine cDNA and 6 putative positive clones were isolated
- Further screening will be performed with a full length bovine clone recently acquired

Positive Arabidopsis Plaques were Identified with the Bovine PAM-PGL cDNA Clone

- Several Hybridization Signals, Cleary Above Background, Were Identified and Clones Were Picked



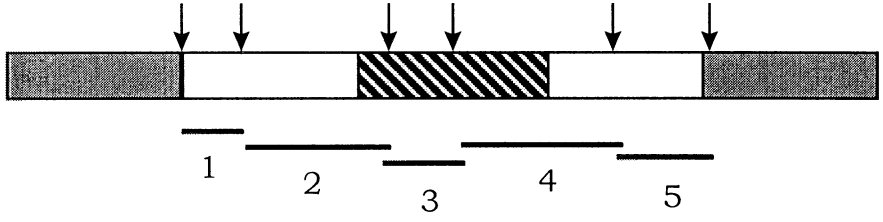
Positive Plaques are Picked and DNA is Isolated



Multiply Virus

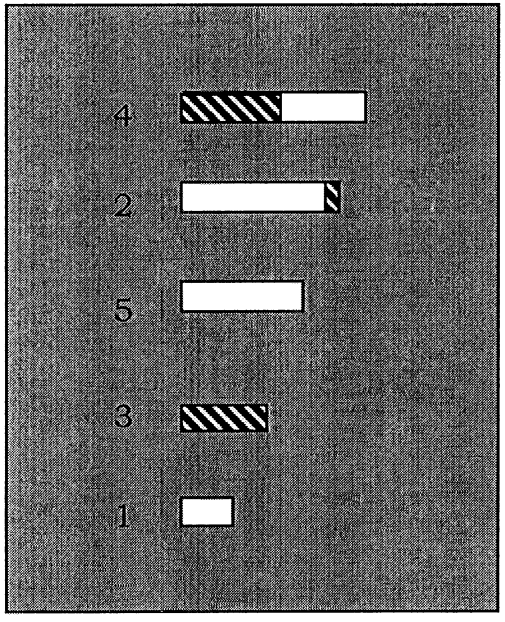
and Isolate DNA

Southern Blotting of DNA from Recombinant Virus Can Reveal Fragments Containing Gene of Interest



Digest

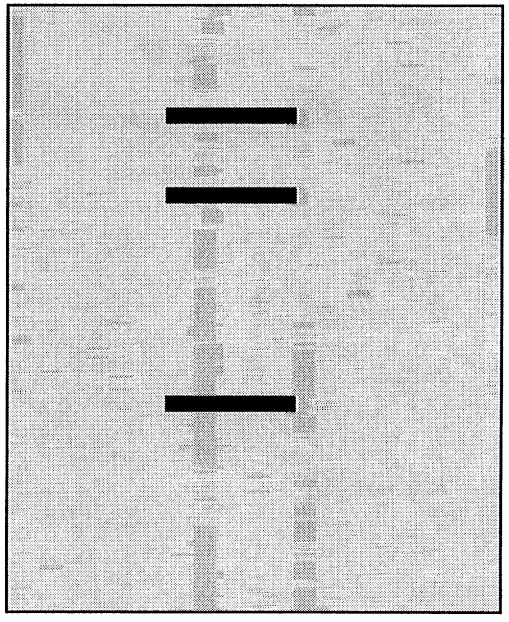
Gel



Blot and Probe

(Probe)

Autorad

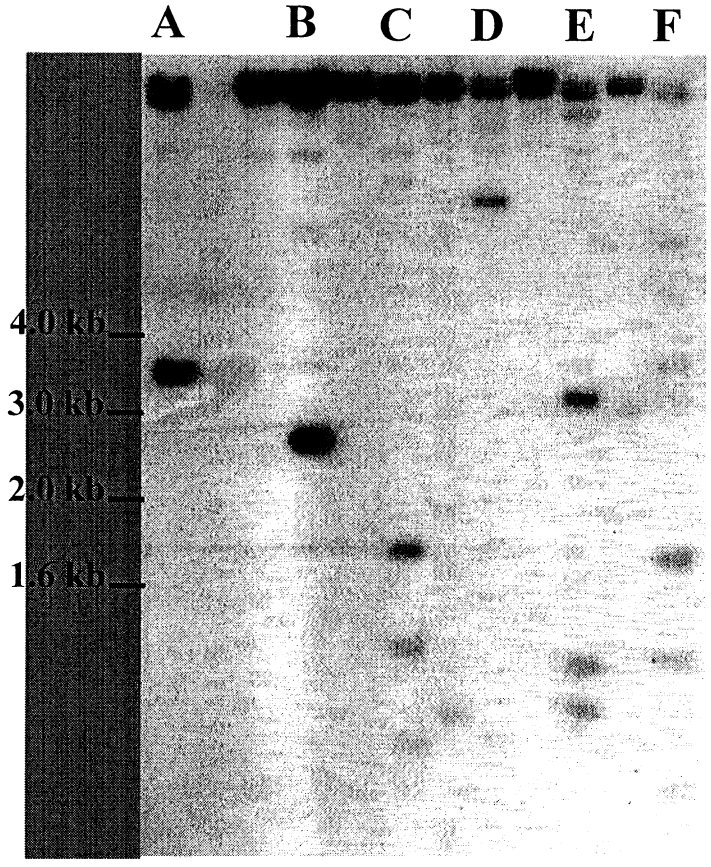


Southern Blotting of Enzyme Digested Lambda Clones Identified Hybridizing Fragments

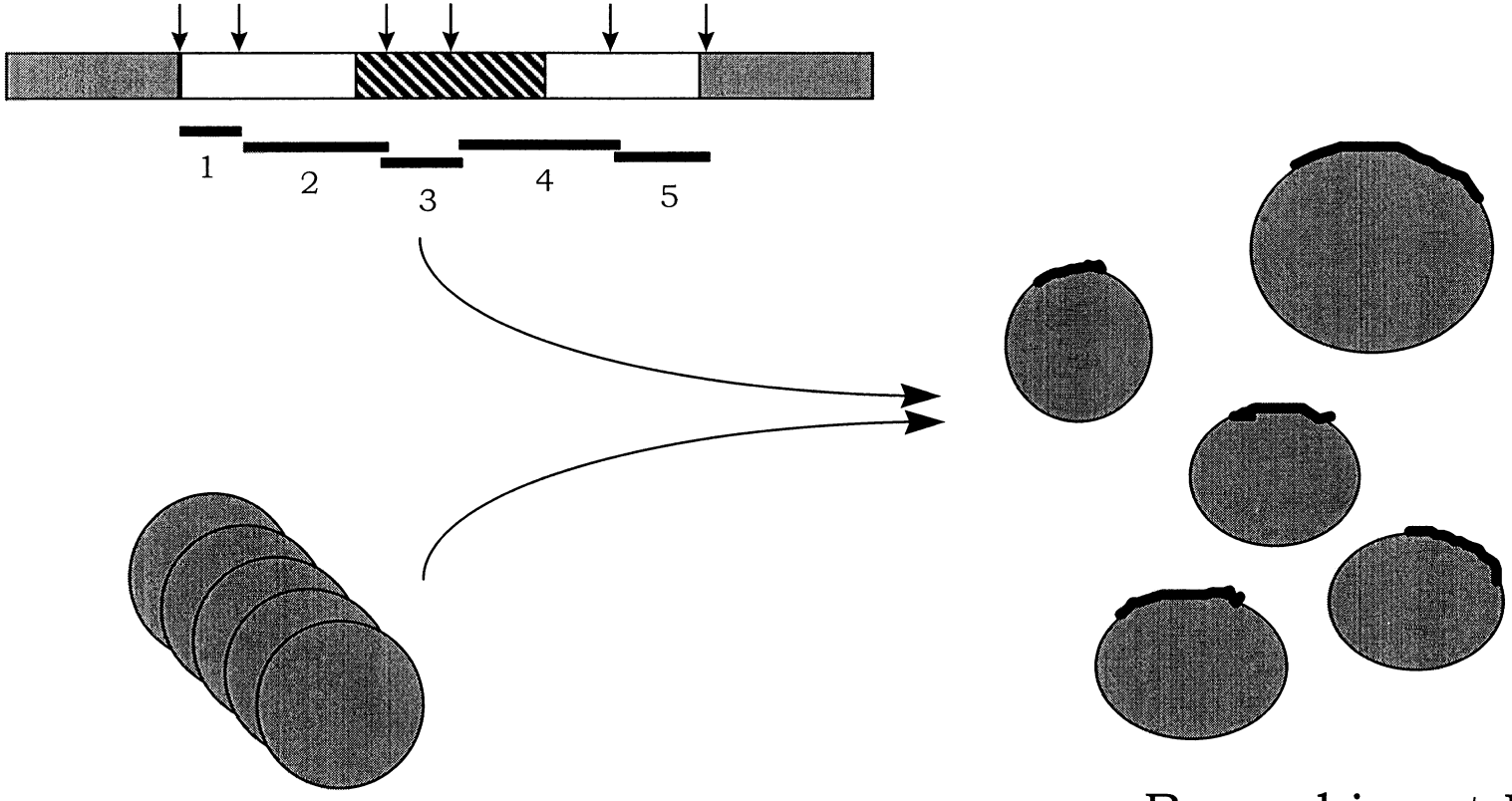
Lambda DNA from several clones was digested with EcoRI (A) or with XbaI (B-F), separated on a 1% agarose gel and blotted onto N+ membrane.

Membrane was probed with the full length bovine PAM/PGL cDNA , washed 4 times with 2XSCC, 0.1% SDS and exposed to a film for 30 min at room temperature.

DNA from the positive clones has been shot-gun cloned into pBluescript. Several clones have the same size as the positive signals, they will be sequenced.

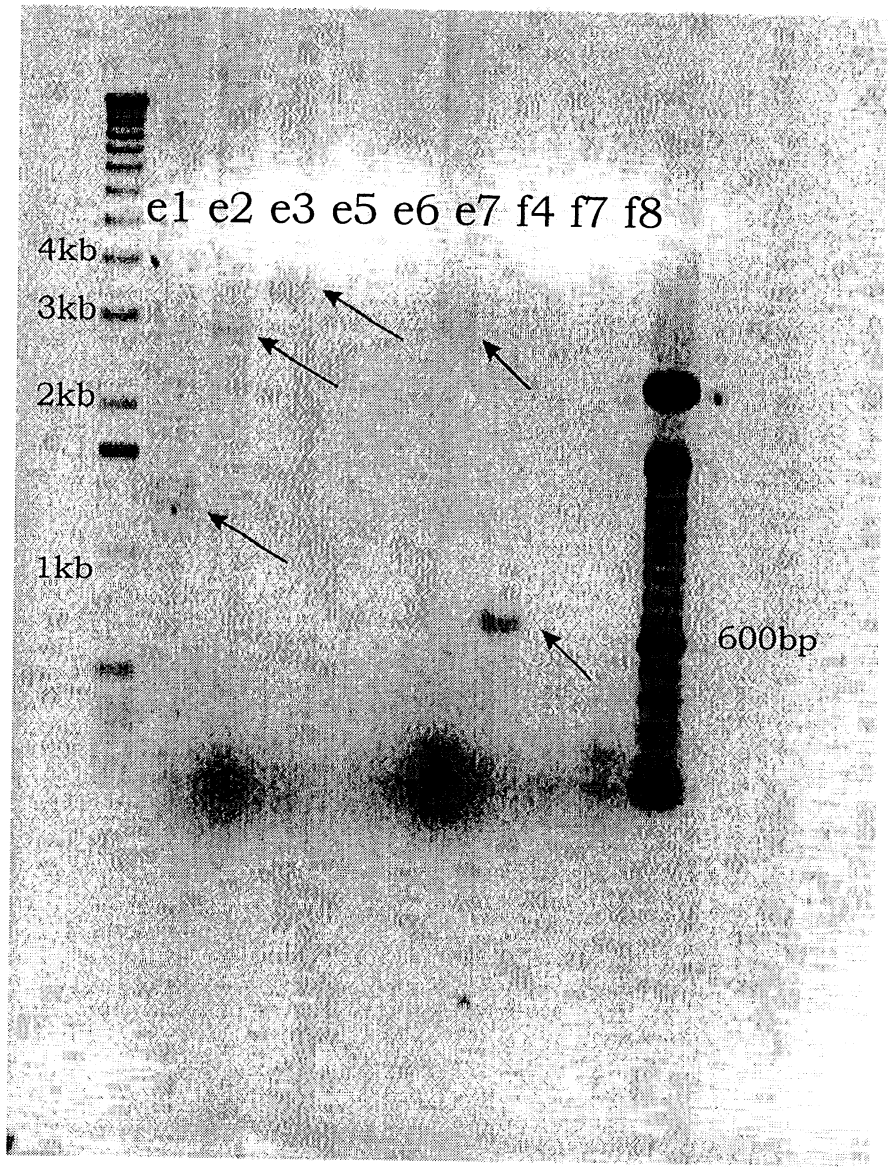


Fragments of the Positive Genomic Clones Were Subcloned



Several Hybridizing Fragments Have Been Subcloned

- Colony PCR Results Show Fragments of Expected size, now in plasmid vectors



Conclusions

- **Biochemical Evidence of a PAM/PGL-like system has been obtained in Pine Cell Culture**
- **Molecular evidence for the existence of PAM/PGL-like in Arabidopsis has been obtained**
- **Preliminary screening of an Arabidopsis library has yeilded Six clones which are being characterized**
- **These Lines of Evidence Suggest a Gene Similar To PAM-PGL is present in Plants**

Future Goals

- Sequence fragments
- Continue with subcloning
- Isolate Pine or Arabidoposis PAM-PGL cDNA (designing primers based on new sequence data)
- Further characterize enigmatic activities

Gene Regulation in Woody Plants II:

5' UTR effects

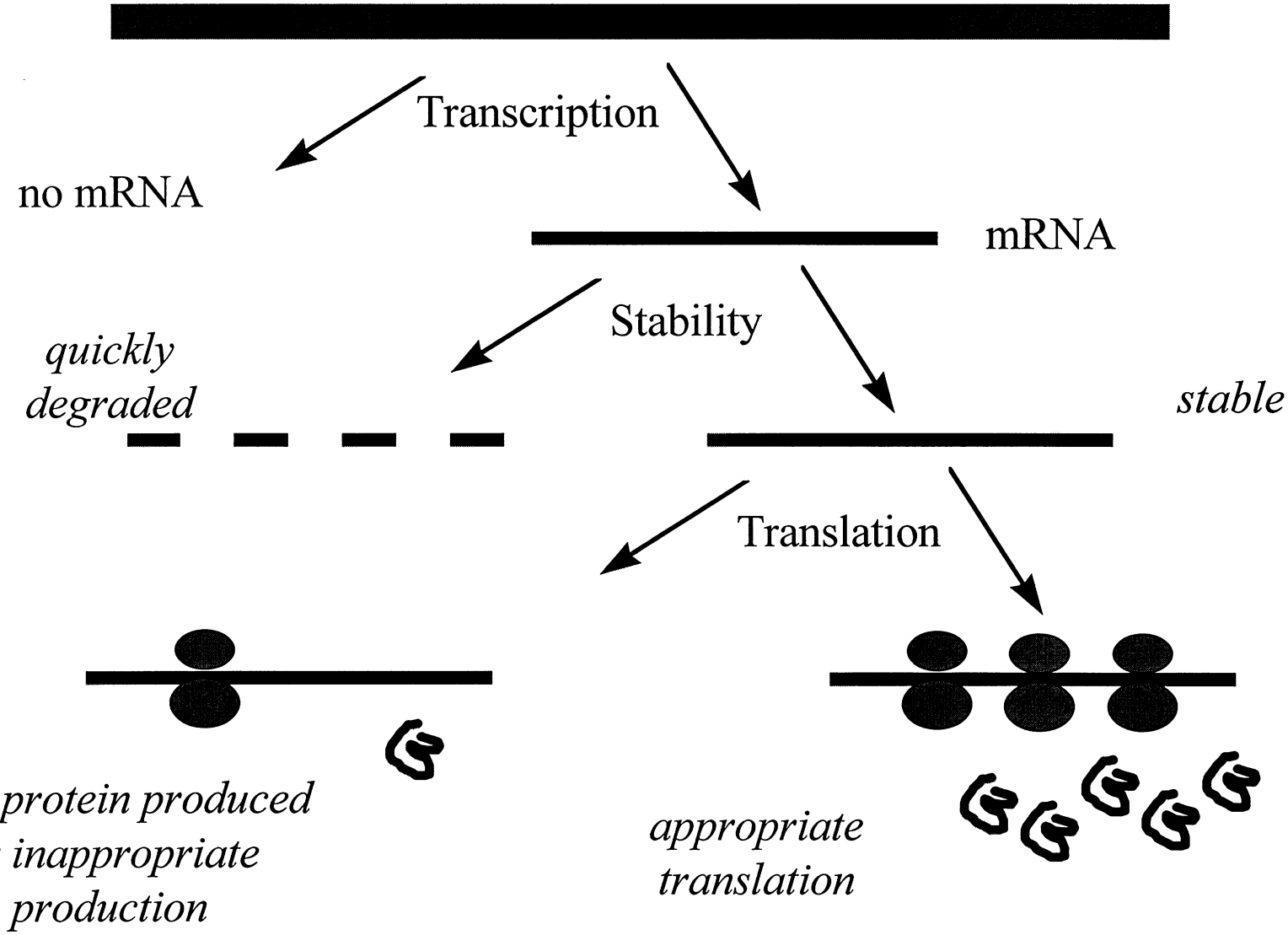
Regulation of Expression and Gene Structure of a Down-Regulated Gene during Drought-Stress

Luis Destéfano-Beltrán

and

John Cairney

Factors Affecting Gene Expression

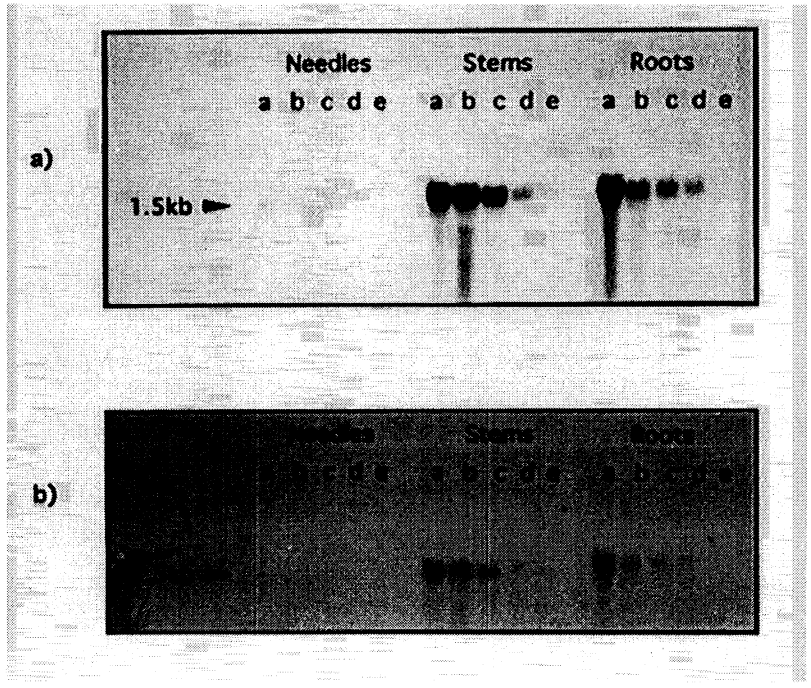


Northern Analysis of LP6 gene expression

- LP6 is strongly expressed in the roots and stems of well watered seedlings, but mRNA levels decline rapidly as plants dehydrate.

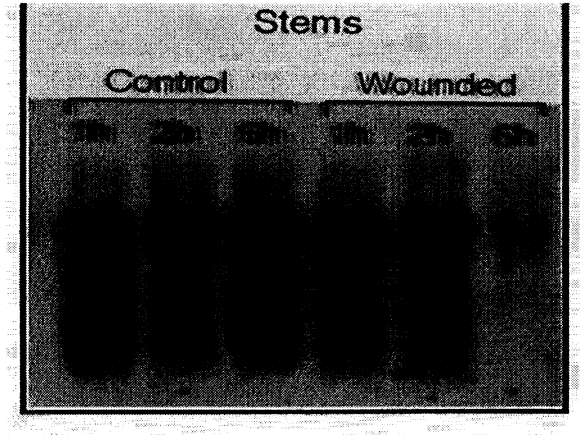
- Same pattern observed in needles but absolute levels are much lower

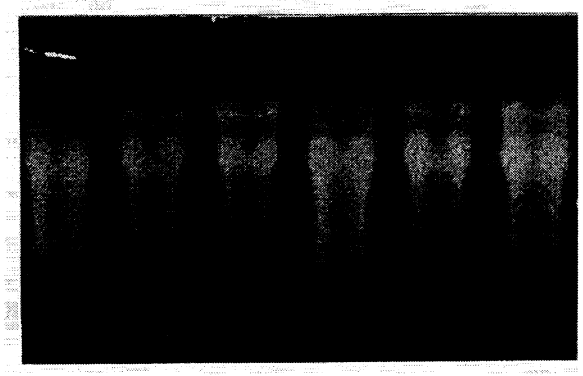
- Membrane reprobed with 5' UTR fragment gave same expression pattern



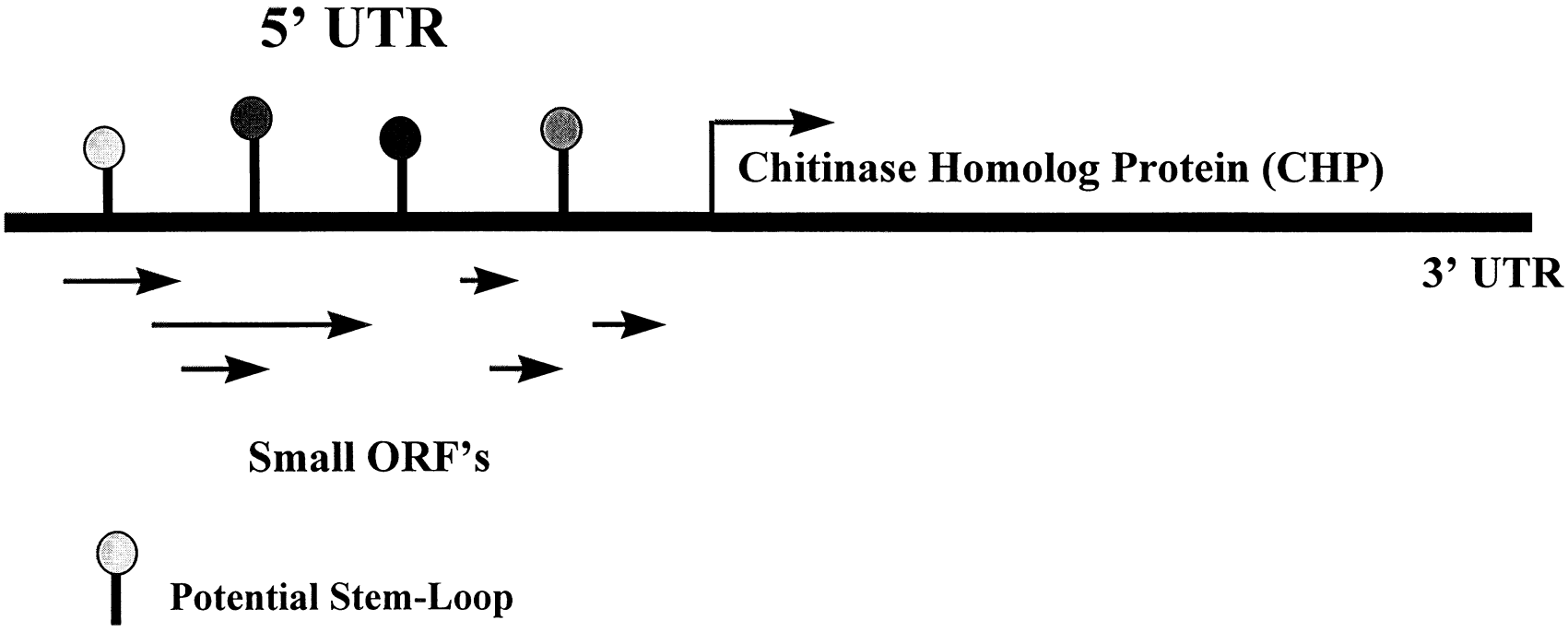
Northern Analysis of Stem Tissue after wounding

- In general chitinases are induced by wounding.
  However, LP6 mRNA levels decline in stems, roots and needles after 6 hr of wounding .

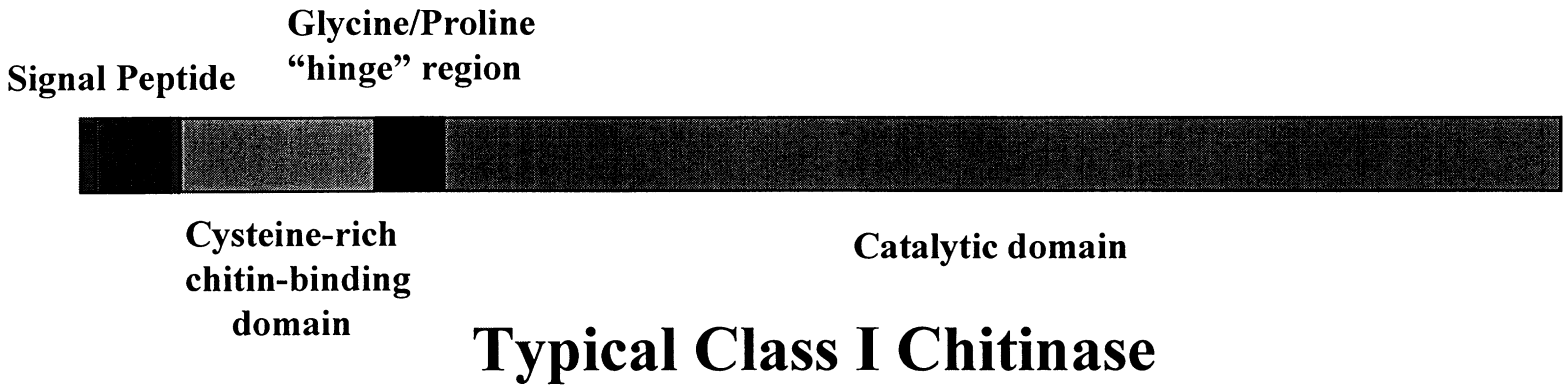




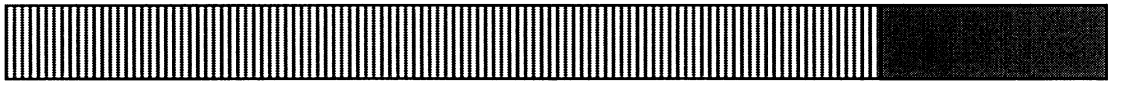
LP6 5'UTR contains much secondary structure and six ORF's



LP6 Chitinase-Homolog Protein differs from Typical Class I chitinases



Typical Class I Chitinase



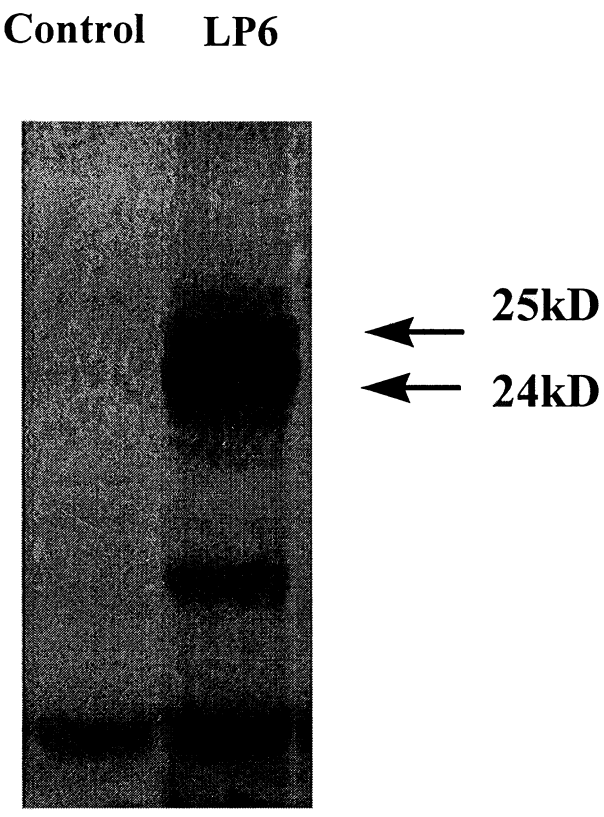
Chitinase Homolog Protein

In vitro translation of LP6 mRNA

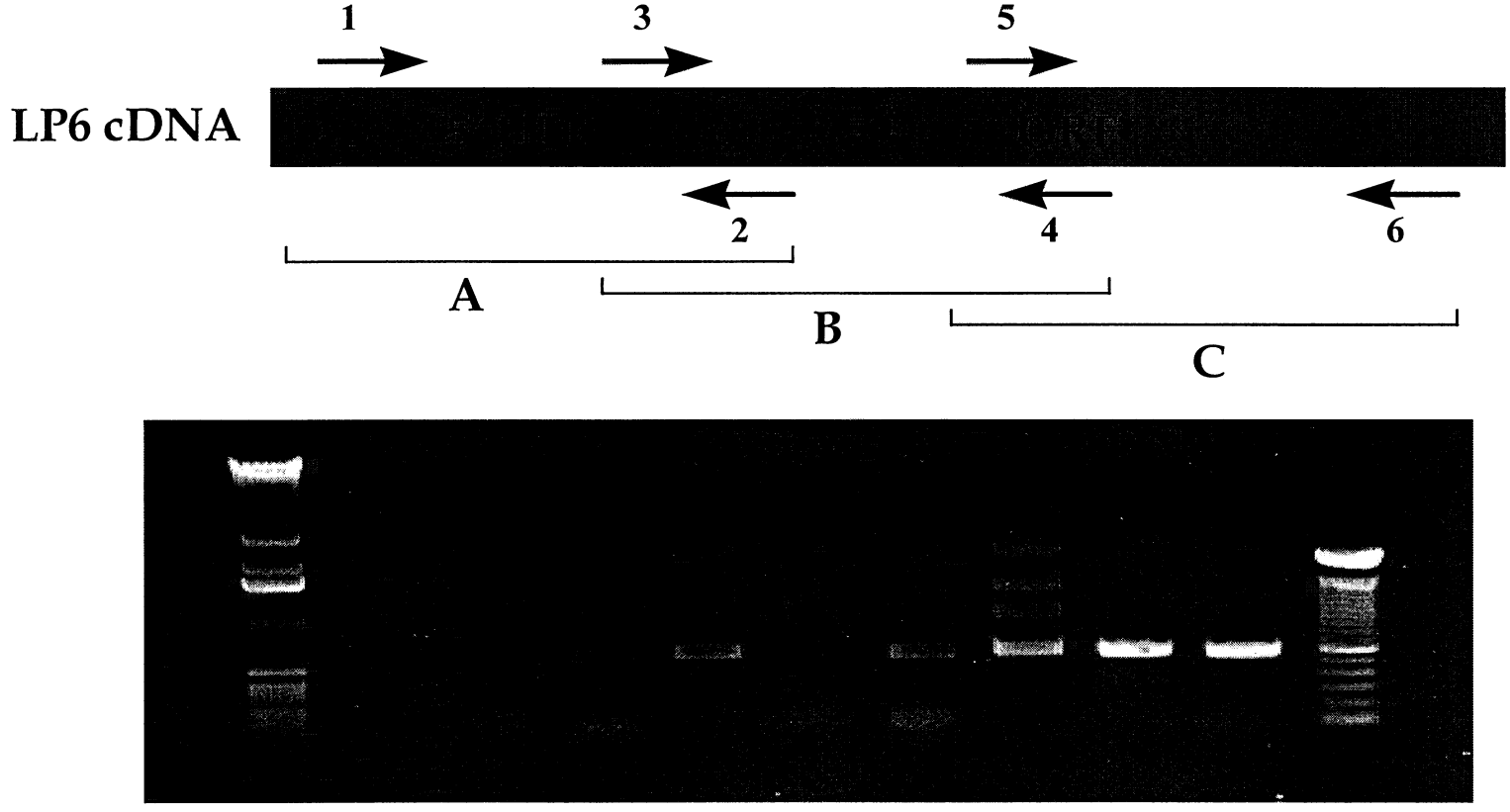
- pLP6 was transcribed using an in vitro T3 polymerase transcription kit.

- Transcribed RNA was then translated in an in vitro wheat-germ lysate translation system.

- [^{35}S]-Methionine labeled products were separated on SDS gels.

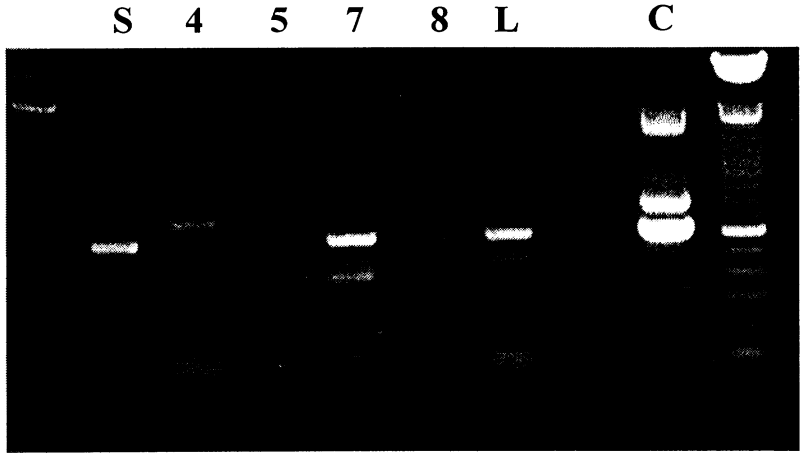


Front Half and Back Half of LP6 Gene are separated by more than 10kb (?)

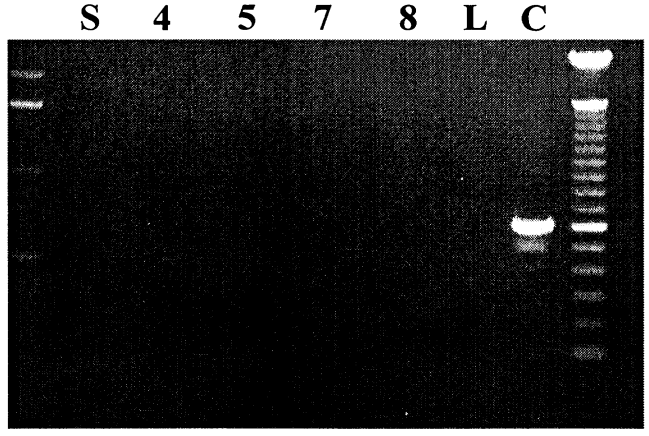


In "Long Distance PCR", primer pair 3 + 4 failed to give a band with genomic DNA. This reaction, which uses Polymerases Tth plus 'Vent',should amplify up to 10kb. Control reactions with the plasmid were sucessful and reactions with genomic DNA using the other primer pairs gave a band of expected size.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

LP6 may be expressed in some stages of somatic embryos



A

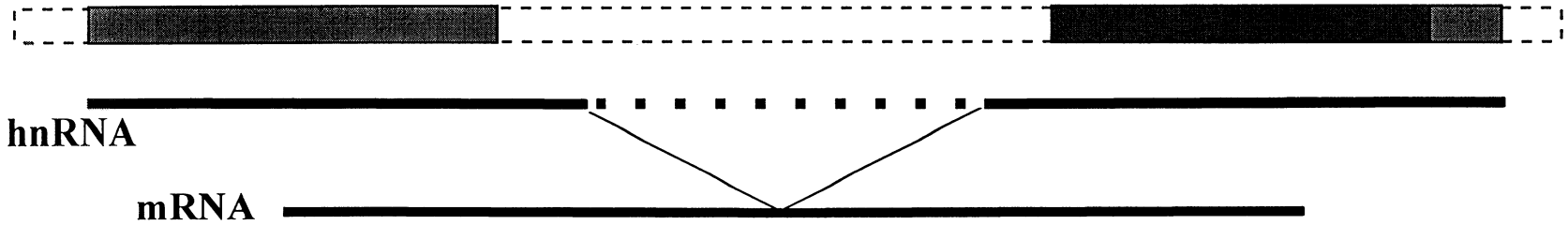


B

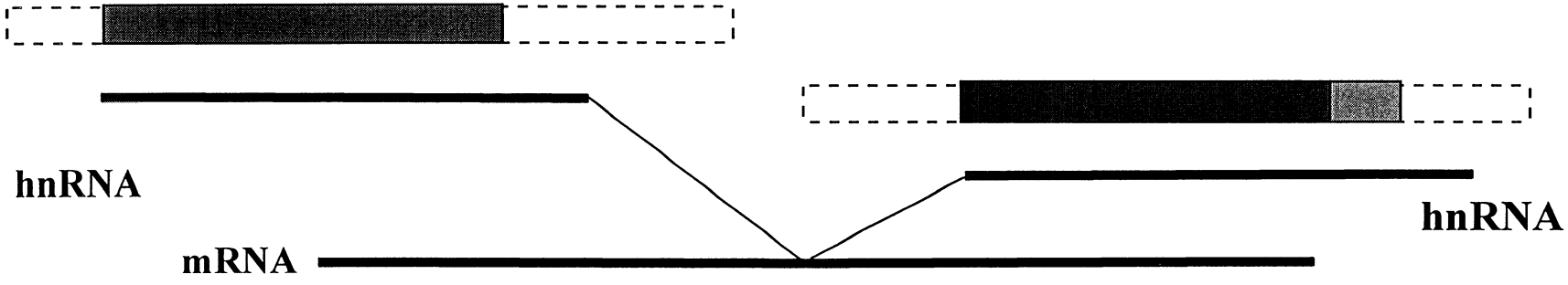
Poly mRNA isolated from different stages were reversed transcribed and amplified with 2 different pair of primers. A) . Primers LDB1 and LDB2 amplify most of the LP6 5'UTR. B) . Primers LDB5 and LDB6 amplify most of the chitinase homologue protein. S, liquuid cell suspension; 4-8, stages 4-8; L, late embryo stage; C, control reaction with LP6. The pictures show the results of secondary PCR reactions.

Possible Structures for LP6 gene

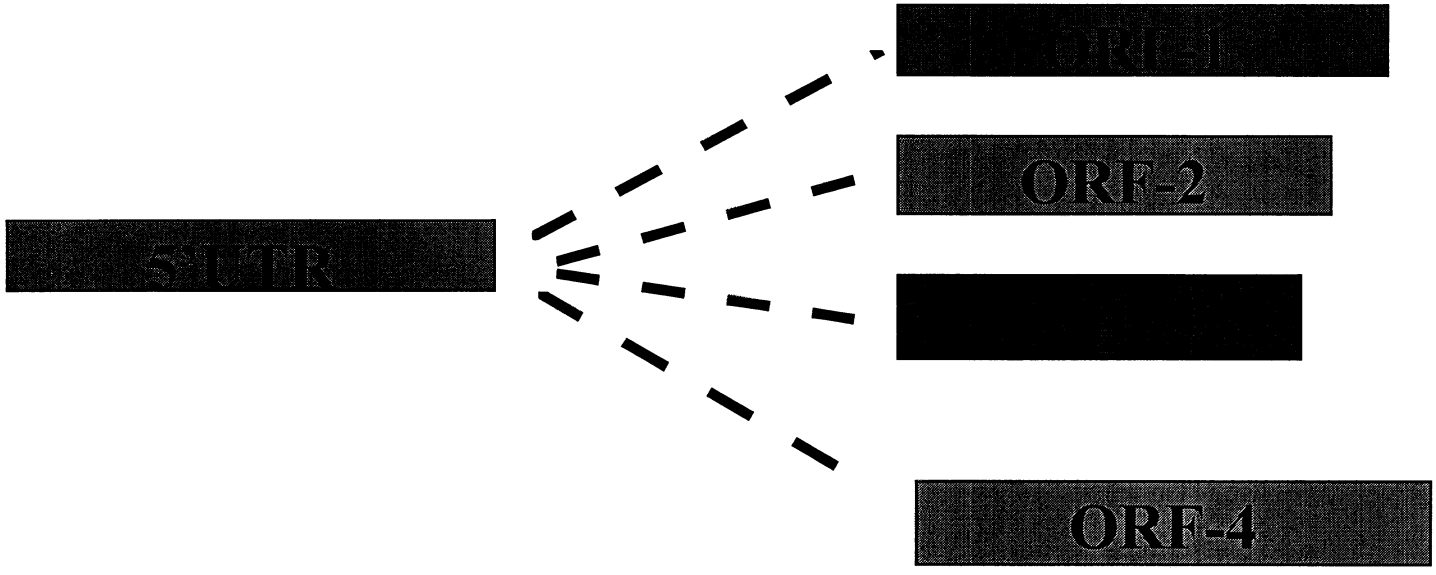
a) One Gene, large intron



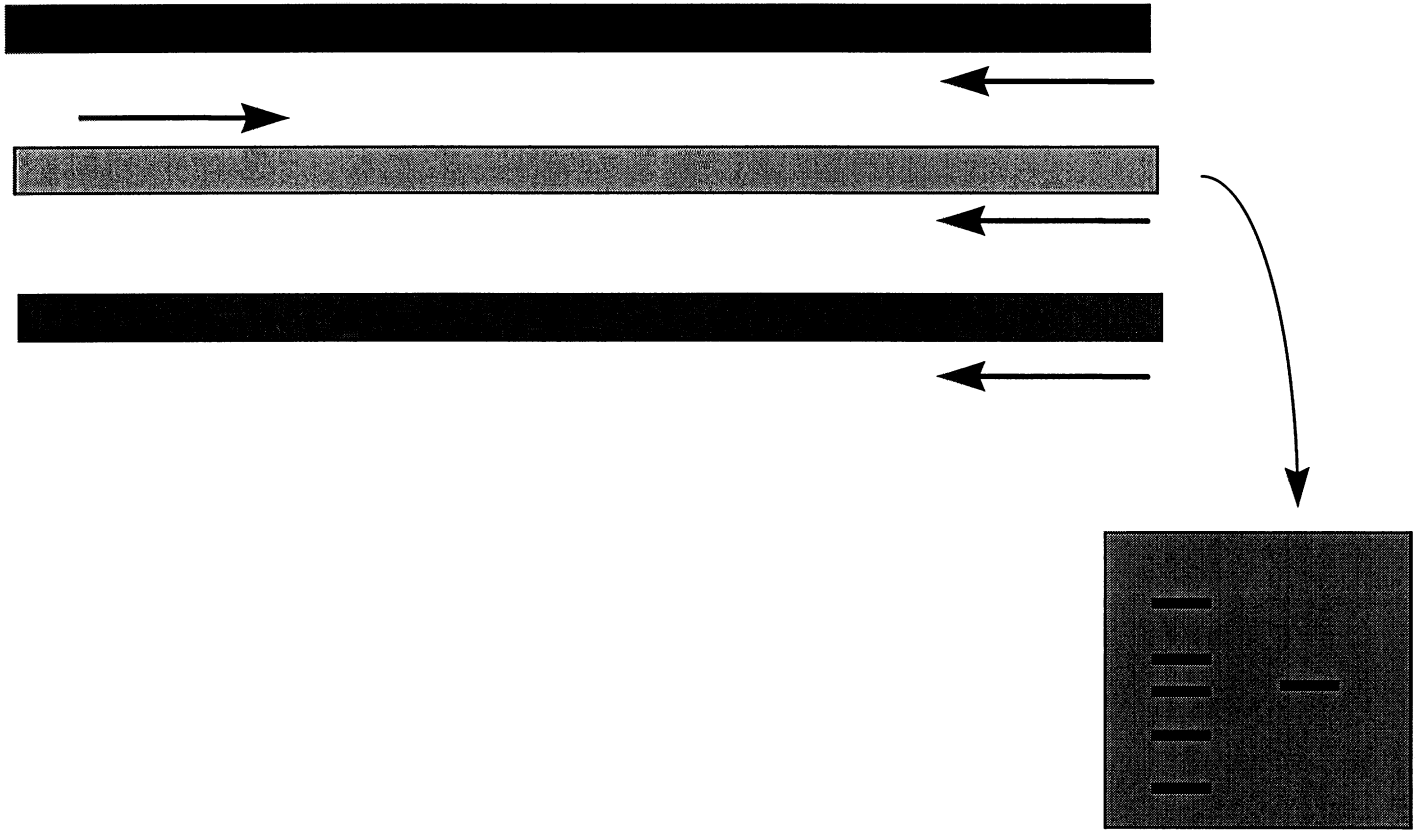
b) Two Genes, trans-spliced



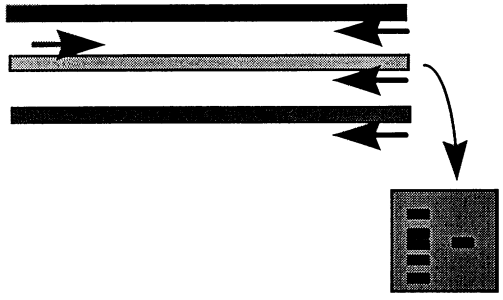
Developmentally Regulated Trans-Splicing to several acceptor messages?



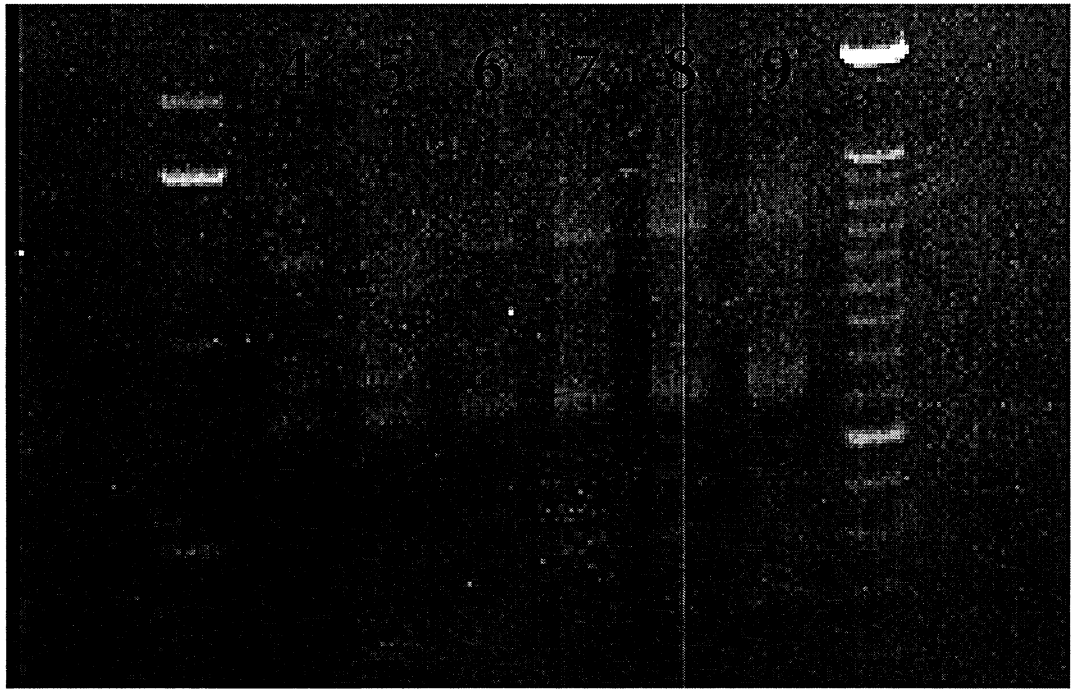
3'-RACE - principle



Several mRNA species are revealed by 3' RACE of Somatic Embryo RNA



SE Stages



- In 3' RACE a 5' UTR specific Primer and a poly A primer form an amplification pair (see diagram above)
- The results shown right, suggest that the 5'UTR primer binds to several transcripts

Questions to be answered regarding LP6

1) .- Does this long and rather complex 5'-UTR has any role in the translation regulation of its downstream gene?

2).- If that is the case, is this the same in all tissues ?

3).- Is the LP6 message translated under normal conditions?

4).- Is the message translated under drought stress?

5).- Are there any trans-acting factors "sitting" on the 5'-UTR?

Questions to be answered regarding LP6 (cont..)

6).- What is the role, if any, of the small open reading frames?

7).- Are these translated?

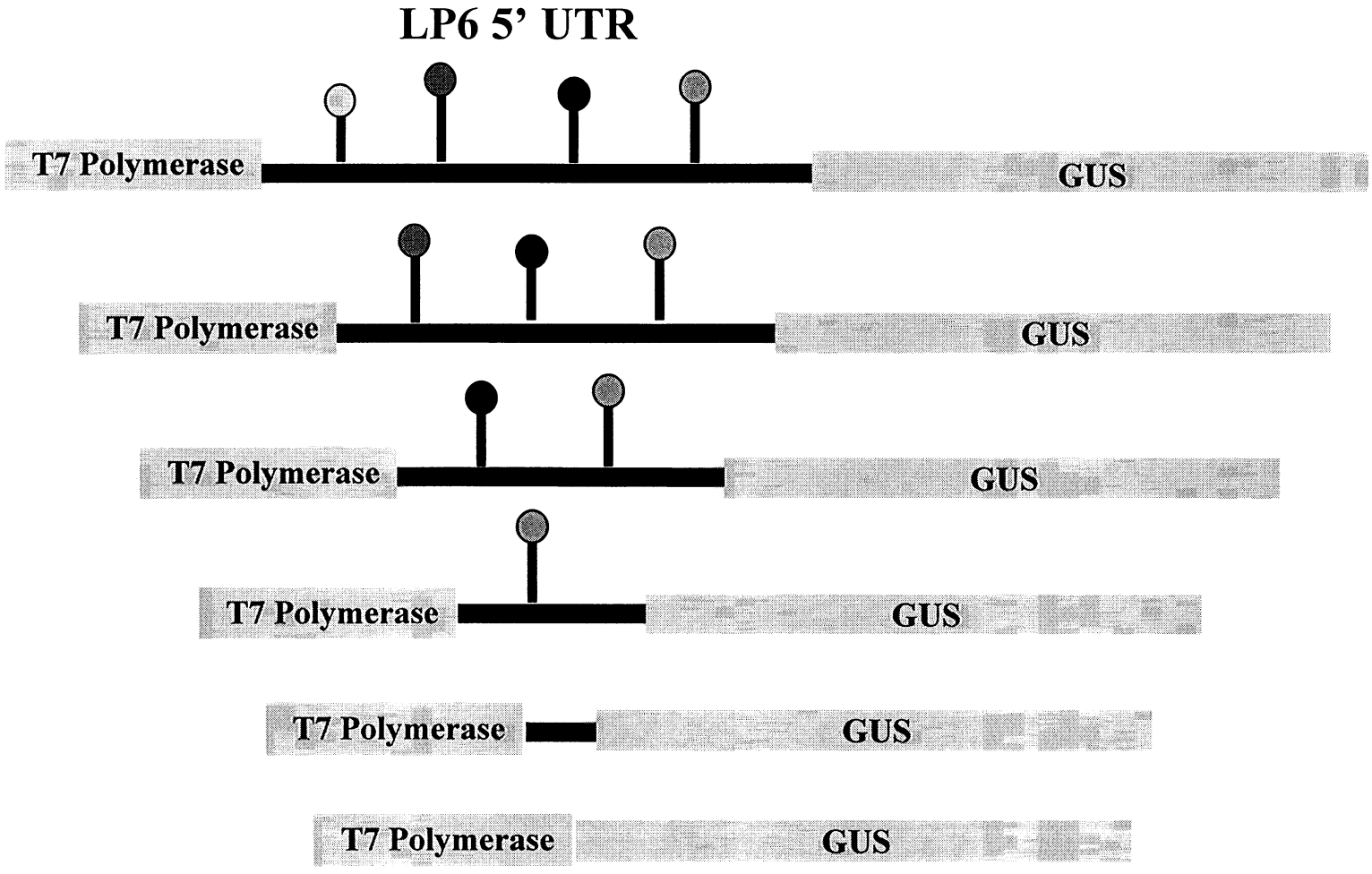
8).- Is LP6 expressed in somatic (and zygotic) embryos?

9).- What is the structure of the LP6 gene?

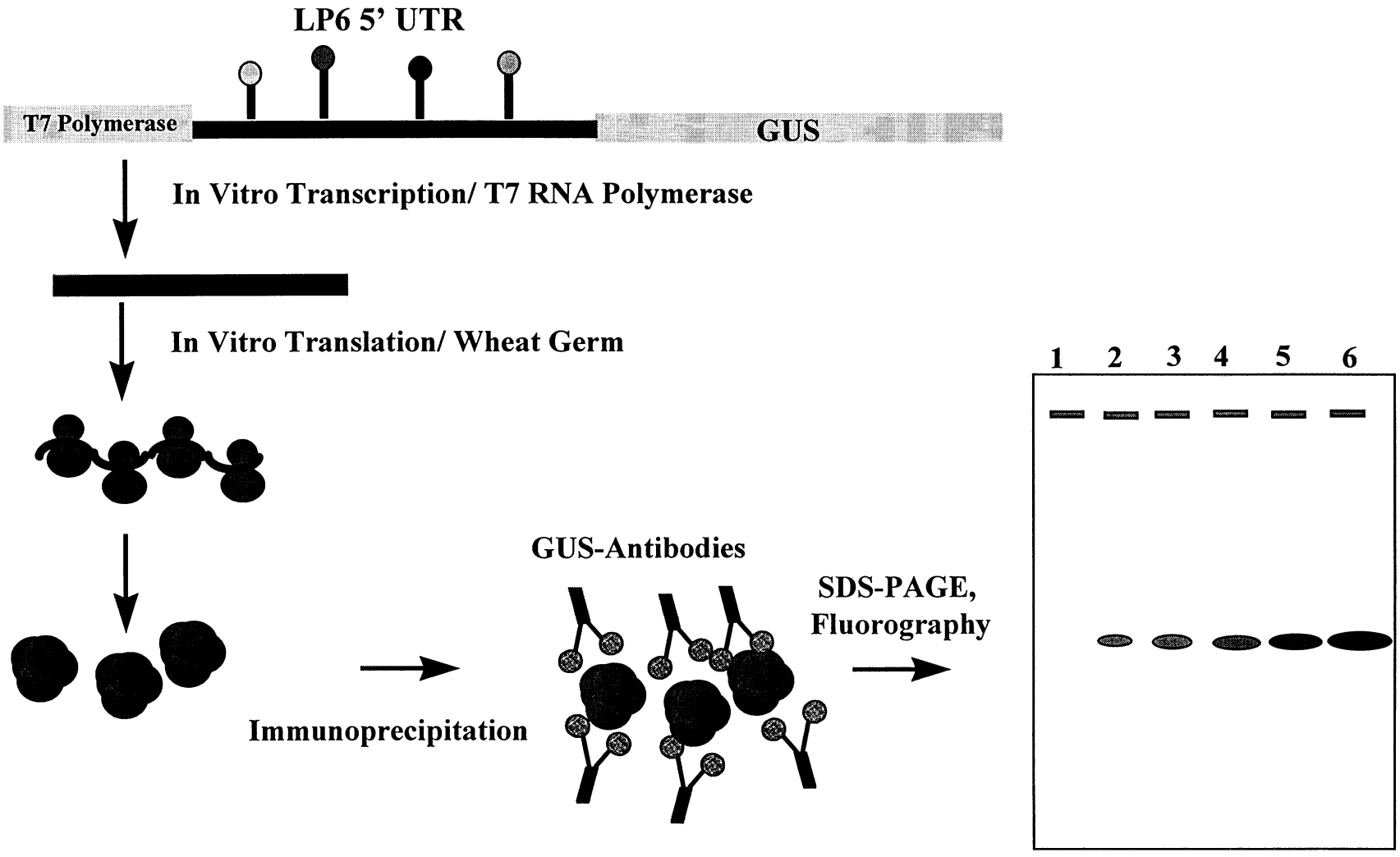
10).- Are there other genes controlled by this unusual 5'-UTR?

11).- Finally, what is the function of the CHP?

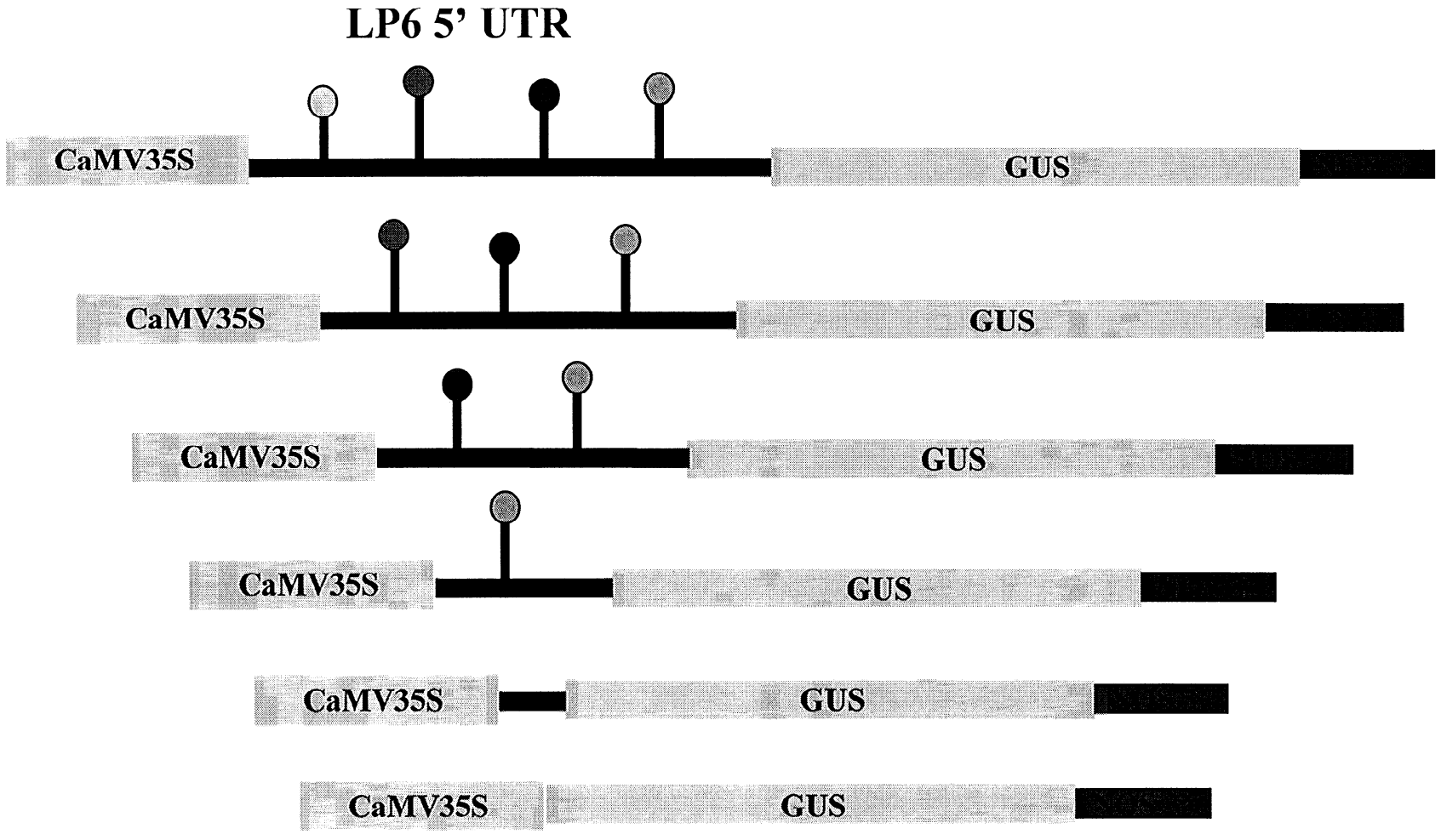
Effect of leader on in vitro translation of reporter gene (I)



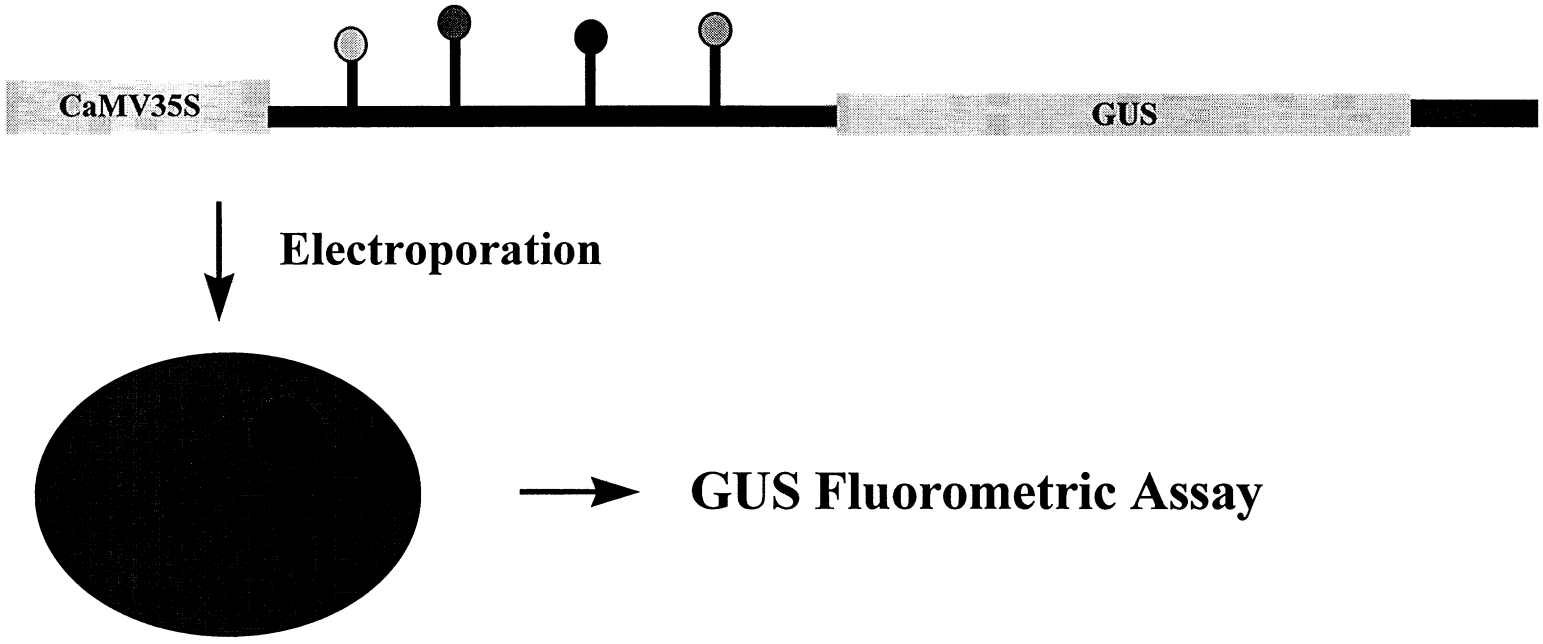
Effect of leader on in vitro translation of reporter gene (II)



Constructs to be tested in Transient Assays (I)

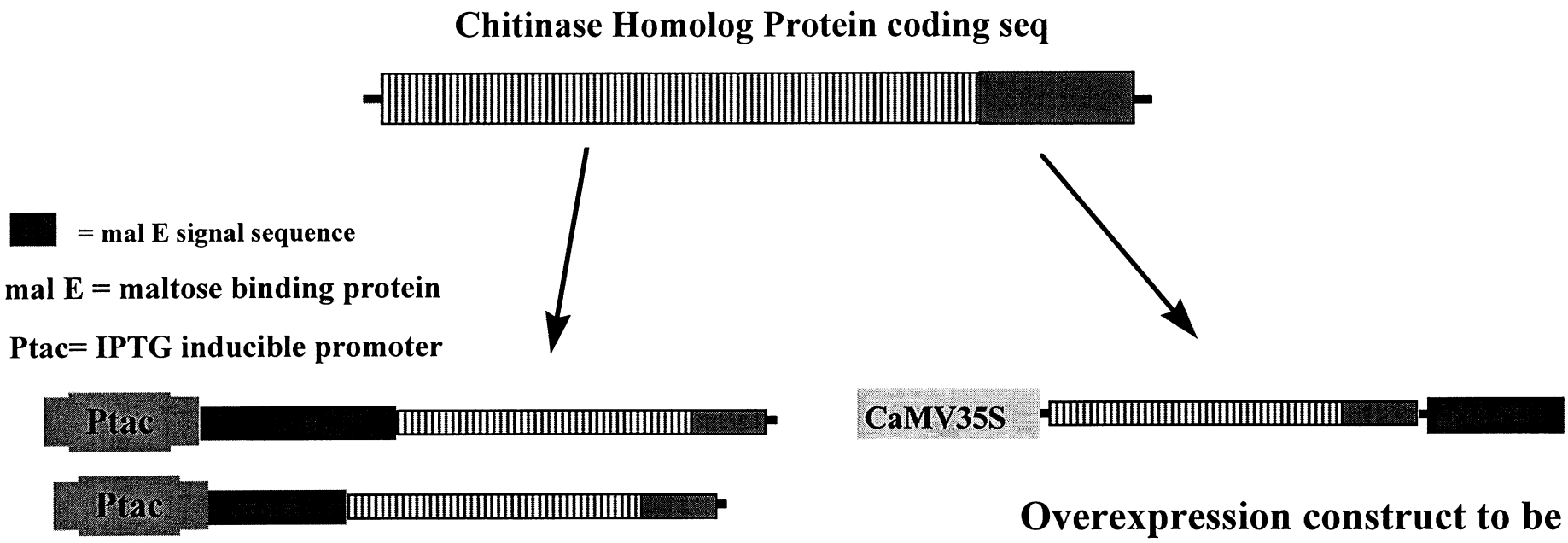


Constructs to be tested in Transient Assays (I)



Selected constructs will be introduced into plants via Agrobacterium GUS activity will be determined before and after drought stress in different organs.

Other Constructs



Overexpression construct to be introduced in planta

These constructs will allow us to express CHP in E. coli and generate CHP-antibodies (Biofarm)

Conclusions and Future Work

1.- LP6 gene expression is repressed under water deficit and wounding in roots, stems and to a much lower extent in needles. Expression in some stages of somatic embryos has to be confirmed.

2.- Role of 5'-UTR is being studied by progressive deletion derivatives spliced upstream of the GUS reporter gene. Selected constructs are being introduced in planta

3.- Lp6 gene structure is intriguing. Failure to amplify second third of the sequence in genomic PCR suggest either the presence of an unusual long intron (>10kb) or independent transcriptional units -transplicing (?)

4.- Upstream and intermediate sequences are being cloned using LD-PCR and "promoter libraries"

5.- LP6 5'-UTR has an unusual structure that sugest role in post-trancriptional regulation (six small ORF's and 4 putative stem-loop structures)

Role of the Suspensor in Early Embryo Development

Role of the Suspensor: Background

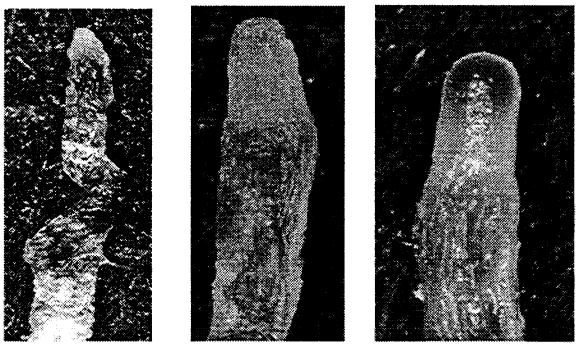
- Multiplication Embryo Quality
- Required for early zygotic embryo development
- Believed to regulate development

Zygotic Embryo
(Stage 3)

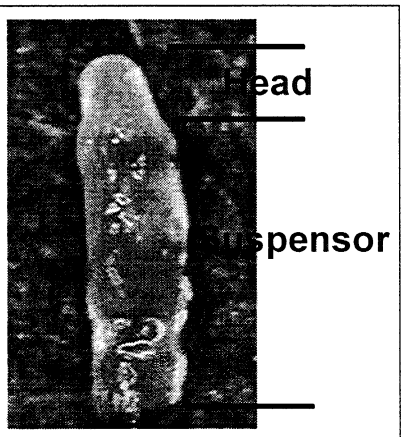
Role of the Suspensor: Background

- Zygotic conifer suspensor: little is known
- Suspensor-like cells in somatic cultures

In early stage zygotic embryos, the suspensor represents much of the mass of the embryo



Role of the Suspensor: Strategies



Embryo Dissection

- **Differentially Expressed Genes**
- Survey expression of specific target genes during suspensor development
- Compare Zygotic and Somatic "Suspensor" Cells

Role of the Suspensor: Objectives

Isolate Suspensors from Zygotic Embryos

↓

Isolate and characterized Genes expressed in the Suspensor → Biological Role

↓

Markers of Suspensor Development → Monitor Development

↓

Analyse Embryogenic Cultures → Improve Multiplication & Quality

Assess the effect of culture media

Role of the Suspensor: Progress

- PCR amplified cDNAs from Suspensors and Embryo Heads
- Differential Display: Compare cDNAs
- Next: Substractive Hybridization to obtain Genes that may be more highly expressed in the Suspensor

A lignin mutant in pine

NCSU, Forest Biotechnology Group

A lignin mutant in pine

- A GENETIC APPROACH TO A BIOCHEMICAL QUESTION

A lignin mutant in pine: Overview

A CAD allozyme with very little activity

↓ Gene inheritance, Genetic mapping

Mutant form of the *cad* gene: Cad-n1, a "null allele"

↓ Molecular analysis
↓ Crosses, pedigree analyses

Grow and analyse plants with mutant gene

↓ Gene expression
↓ Chemical analyses &Pulping experiments

Plants with modified lignin and with less lignin

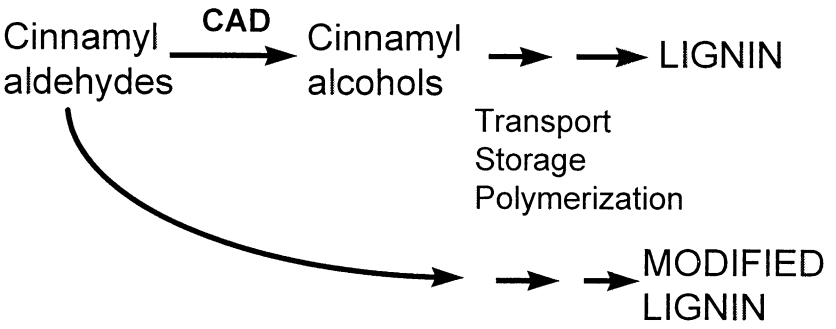
-Extensively altered lignin composition

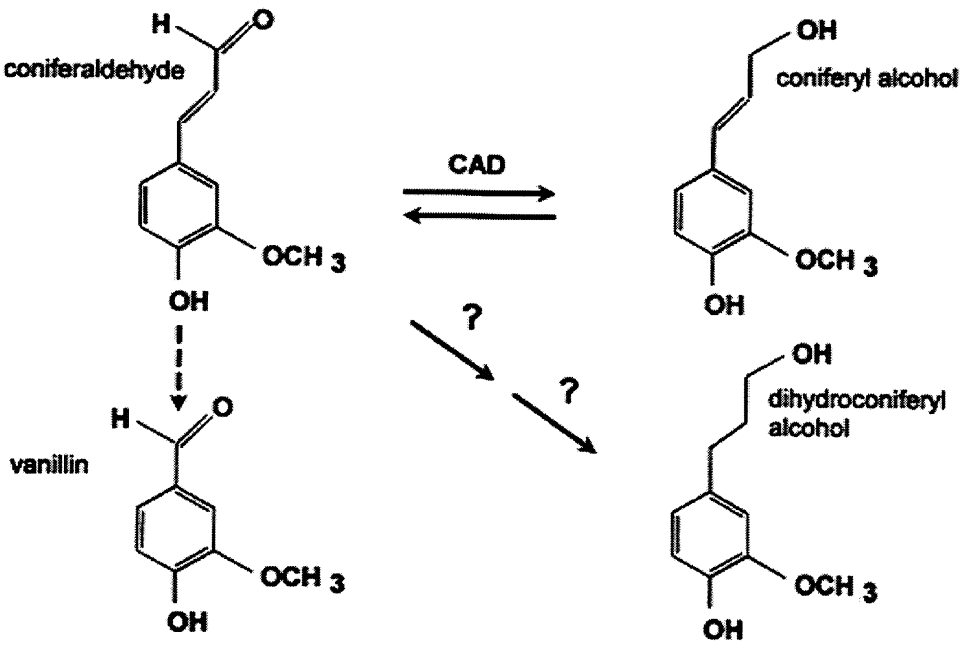
-Lignin more easily removed

A lignin mutant in pine: Findings

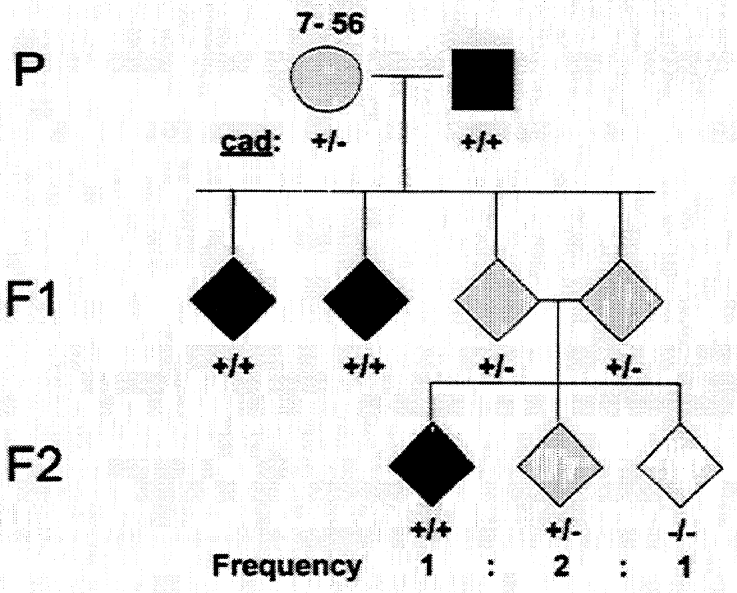
- Very low Residual Gene Expression and CAD enzyme activity activity
- Single Mutant Gene:
  - Recessive
  - Maps to the same Genomic Region as the *Cad* Gene
- Lignin Content of Mutant Seedlings is 86-91% of Normal
- Lignin Polymer Incorporates the Substrate of CAD and Unexpected Monomer

Hypothesis


Cinnamyl aldehydes
CAD
Cinnamyl alcohols
LIGNIN
Transport
Storage
Polymerization
MODIFIED LIGNIN

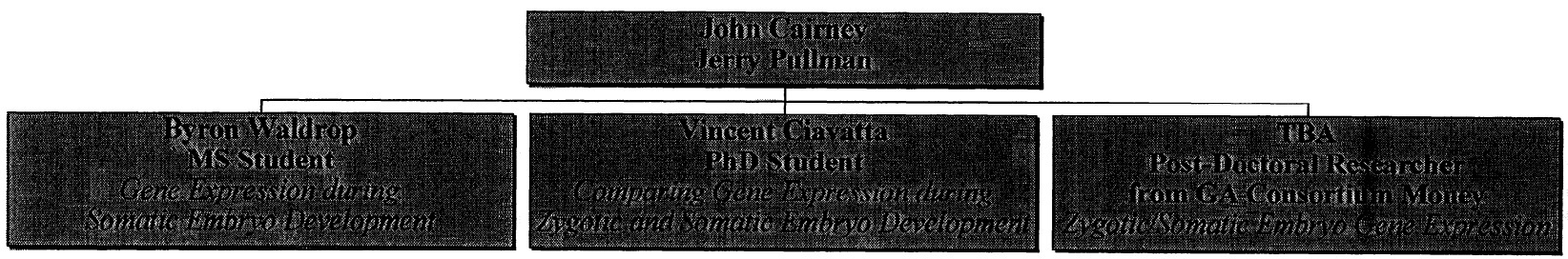

coniferaldehyde
OCH 3
OH
CAD
coniferyl alcohol
OCH 3
OH
?
?
vanillin
OCH 3
OH
dihydroconiferyl alcohol
OCH 3
OH

Inbred Pedigree of Loblolly Pine



Gene Expression During Conifer Embryogenesis: People and Projects

PROJECT ORGANIZATION



A lignin mutant in pine: Genetics

- Mutant discovered by analyzing allelic variation
- Mutant gene lead to mutant plants

A lignin mutant in pine: Genetics

Tools to Investigate Genetic Architecture in Pine

- Haploid Megagametophyte
  - Segregation and Inheritance Analyses
  - Genetic Mapping
- Pedigrees of loblolly pine
  - Inheritance over 3 Generations
  - Identify existing Mutant Plants and Seeds

A lignin mutant in pine: Future Work

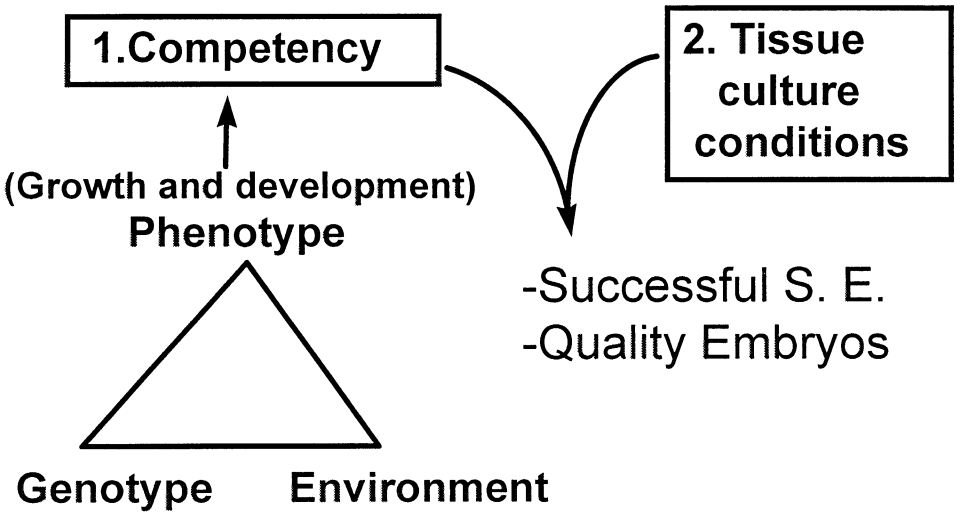
- Lignin structure and properties (Collaboration with NCSU)
  - USDA grant
  - Lignin Removal and Reactivity (D. Dimmell, Wood Chemistry Group)
- Preproposal to Agenda 2020
  - MacKay, Dimmell, Peter, Pullman
  - Pulping, Wood Chemistry, Genetics

Genetic dissection of Factors Controlling Somatic Embryogenesis

Genetic dissection of S. E.: Background

- Variation between genotypes or lines is very significant
- Variation within genotypes or lines
- Genetic vs Developmental vs Environmental variation are confounded

Genetic dissection of S. E.: Background



Genetic dissection of S.E.: Why?

- Identify factors that determine success rates
  - Determine relative importance of genotype and other factors in the response to tissue culture
  - Manipulate conditions or adapt strategies
- Help capture a greater number of genotypes, including recalcitrant genotypes

Genetic dissection of S.E.: How?

- **Goal**: Undestrand the Architecture of the Genetic Control of S.E.
- Initiation Rate: First Target
  - Major Bottle Neck for Success
- Use well defined genotypes and pedigrees
  - Loblolly pine has advanced breeding
  - Intensify collaboration with breeding programs

Genetic dissection of S.E.: How?

- Estimate Heritability to establish the Magnitude of Genetic Control
- Determine type of Genetic Effects: Additive vs Dominance
- Determine importance of Maternal, Paternal and Embryo genotypes using Controlled Crosses