DE-FC07-00ID13876 FINAL REPORT

Project Title: Accelerated Stem Growth Rates and Improved Fiber Properties of Loblolly Pine: Functional Analysis Of CyclinD from *Pinus taeda*

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Recipient: Georgia Tech Researach Corporation

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Subcontractors: Gary Peter, University of Florida.

Other Partners:

E.-M. Ulrika Egertsdotter, Ph.D.
Associate professor of Genetics and Biotechnology
Dept. of Forestry, College of Natural Resources
Virginia Tech
Blacksburg, VA 24061
Office +1 (540) 231 7267
Cell +1 (404) 663 6950
Fax +1 (540) 231 3330
Email: uegertsd@vt.edu

Dr. Armin Wagner
Research Scientist
Cellwall Biotechnology Centre (CBC)
New Zealand Forest Research Institute Ltd.
Private Bag 3020
Rotorua, New Zealand
Phone: 64 (07) 343 5449
Fax: 64 (07) 343 5444

Contact(s):

Dr. John Cairney,
School of Biology and
Institute of Paper Science and Technology @ Georgia Tech
500 10th Street
Atlanta, GA 30332-0620
Tel: (404) 894 1079
FAX: (404) 894 4778
John.Cairney@ipst.gatech.edu

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Project Team: Joe Springer, PE, PMP, DOE/GO Project Officer, Carrie Capps, Project Monitor, McNeil Technologies, US DOE Golden Field Office

DE-FC07-00ID13876 FINAL REPORT ABSTRACT:

A sustained supply of low-cost, high quality raw materials is essential for the future success of the U.S. forest products industry. To maximize stem (trunk) growth, a fundamental understanding of the molecular mechanisms that regulate cell divisions within the cambial meristem is essential. We hypothesize that auxin levels within the cambial meristem regulate cyclin gene expression and this in turn controls cell cycle progression as occurs in all eukaryotic cells. Work with model plant species has shown that ectopic overexpression of cyclins promotes cell division thereby increasing root growth > five times. We intended to test whether ectopic overexpression of cambial cyclins in the cambial zone of loblolly pine also promotes cell division rates that enhance stem growth rates. Results generated in model annual angiosperm systems cannot be reliably extrapolated to perennial gymnosperms, thus while the generation and development of transgenic pine is time consuming, this is the necessary approach for meaningful data.

We succeeded in isolating a cyclin D gene and Clustal analysis to the *Arabidopsis* cyclin D gene family indicates that it is more closely related to cyclin D2 than D1 or D3. Using this gene as a probe we observed a small stimulation of cyclin D expression in somatic embryo culture upon addition of auxin. We hypothesized that trees with more cells in the vascular cambial and expansion zones will have higher cyclin mRNA levels. We demonstrated that in trees under compressive stress where the rates of cambial divisions are increased on the underside of the stem relative to the top or opposite side, there was a 20 fold increase in the level of PtcyclinD1 mRNA on the compressed side of the stem relative to the opposite. This suggests that higher secondary growth rates correlate with PtcyclinD1 expression. We showed that larger diameter trees show more growth during each year and that the increased growth in loblolly pine trees correlates with more cell divisions in the cambial meristem as expected.

We isolated a promoter from a cambial specific gene and commenced development of transformation protocols for loblolly pine. Since our results show that cyclin D expression correlates with increased growth we continued with experiments to demonstrate the effect of cyclin overexpression upon tree growth. Vectors which constitutively express the cyclin D cDNA were constructed and transformed into a transgenic pine system through the collaboration with Forest Research, New Zealand. The transformation system for *Pinus radiata* is well established and we hoped to gain

phenotypic information in a closely related pine, rather than await development of a robust loblolly pine transformation method. Transformation experiments were conducted by a biolistic method developed at Forest Research, NZ.

A total of 78 transgenic embryogenic lines were generated and bulked up with a good representation of transgenic lines per construct. Transformed calli were originally identified by resistance to the antibiotic Geneticin contained in the medium. The transgenic nature of the selected lines was subsequently confirmed using histochemical GUS staining.

To date, 10 out of 13 selected transgenic lines have produced embryos and we are currently harvesting the first transgenic plantlets. At present time 22 of those plantlets have been moved to GMO facilities. We will soon develop a strategy for assessing potential phenotypic differences between the transclones and non-transformed controls. Transgenic plants are being grown to a stage (approx. 1 year) when meaningful phenotypic evaluation can be conducted. The recent availability of 10,000 element loblolly pine cDNA microarray (Cairney, NSF grant DBI-0217594,) will permit the evaluation of cyclinD overexpression upon gene expression in transgenic Pinus.

Patents: None

Publications/Presentations:

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"Cyclin D expression in cambial cells of developing and physically stressed loblolly pine" Peter et al (in preparation)

"Overexpressionof Cyclin D in Pinus radiata – phenotypic and genotypic responses" Wagner, Cairney, Egertsdotter, Peter (in preparation)

Presentation to Forest Products Industry Companies, March 2003, 2004

Milestone Status Table:

ID Number	Task / Milestone Description	Planned Completion	Actual Completion	Comments
	Collection of Plant Material	9/9/2001	9/9/2001	
Task 1	Isolation of full-length Loblolly Pine Cyclin D cDNA	10/1/2003	2/1/2004	
Task 2	Study the expression patterns of cyclins within cambial meristem cells	7/1/2003	7/1/2003	Estimated dates
Task 3	Isolate promoter(s) for cambial cell specific expression	6/1/2003	6/1/2003	
Task 4	Construct plant vector for expressing cyclin(s) in cambial cells	10/1/2003	12/1/2003	
Task 5	Generate 5-10 independent transgenic lines of P. taeda with these gene constructs	2/1/2004	Ongoing,	Transformed calli generated, optimization continuing
Task 6	Initiate analyses of cyclin expression, stem growth, vascular cell differentiation, and xylem cell properties	3/1/2004	Ongoing	Transgenic plants generated and are being grown to a stage (approx. 1 year) when meaningful phenotypic evaluation can be conducted

Collection of Plant Material

Year 1 collections June-August (Q2 & Q3)

We have focused much of our efforts on collecting material since this was our first season of growth during this award. We obtained tissues from *six* 17-year-old trees. For each tree, three stem sections were collected starting in the live crown and progressing downward (Table 1). For larger scale RNA isolations to prepare cDNA libraries and RNA gel blots (task 1 & 4) cambial regions were obtained by scraping the log and bark surfaces from isolated stem sections.

Table 1. Tree Collections Year 1

Tree #	Date	Log 1		Log 2		Log 3	
		S*	V^	S	V	S	V
1	6-15-00	+	+	+	+	+	+
2	6-16-00	+	+	+		+	
3	6-26-00	+	+	+	+	+	
4	6-27-00	+	+	+	+		
5	7-20-00	+	+	+	+		
6	7-24-00	+	+	+		+	

* scrapings; ^ tangential vibratome sections.

For more detailed expression studies (task 2 & 3), we collected with the vibratome a large number of individual 150uM thick tangential sections of phloem-cambium-xylem regions from disks obtained from the same five trees that scrapings were obtained. For future *in situ* hybridizations (tasks 2 & 3), small blocks of wood from log #1 were dissected, quick frozen in liquid N2 and freeze substituted in methanol/glutaraldehyde at –80^{o}C (Regan et al. 1999, Plant J. 19 (3) 363).

Year 2 collections April-September (Q6 & Q7)

Table 2. Summary of Samples Collected in 2001

DS = destructive scrapings, DP = destructive planeings, NS = nondestructive scrapings, NP= nondestructive planeings

Time	Day no.	Tree (D)	DS	DP	Cookie	Blocks (ht/ft)	Tree (N)	NS	NP
May 16	1						1-4	X	X
May 23	2	A	X	X	X	5			
May 30	3	B	X	X	X	5	5-8	X	X
June 6	4	C	X	X	X	5			
June 6	4	D				5,17,33			
June 8	5						9-24	X	X
June 20	6	E	X	X	X		1-4	X	X
June 20	6	F				5,17,33			
June 26	7	G	X	X	X				
June 26	7	H				5,17,33			
July 3	8	I	X	X	X		5-8	X	X
July 3	8	J				5,17,33			
July 16	9	K	X	X	X		9-15	X	X
July 20	10	L				5,17,33			
July 20	10	M	X	X	X				
July 20	10	N				5,17,33			
July 27	11	O				5,17,33	16-18	X	X
July 27	11	P	X	X	X	5,17,33			
Aug 3	12	Q	X	X	X	5	1-4, 19-21	X	X
Aug 15	13	R	X	X	X	5,17,33	22-24	X	X

Task 1 – Isolation of Loblolly Pine Cyclin D cDNA

We have also constructed, amplified and characterized cDNA libraries from the xylem and phloem side scrapings in lambda Triplex. A loblolly pine cyclin D has been obtained. Sequence analysis with BlastX shows that this cyclin D is most closely related to other plant cyclin D's obtained from tobacco and clustal analysis to the *Arabidopsis* cyclin D gene family indicates that it is more closely related to cyclin D2 than D1 or D3 (Figure 1).

To date, we have isolated several cDNA clones showing similarity to cyclin genes. The expression of these clones have been studied in different types of xylem tissue, e.g. compression wood, side wood, early wood and latewood (data shown in previous report).

We are currently seeking a reverse genetics approach to further characterize one of the cyclin genes. The full length cDNA of the Pt cyclin D2 gene was isolated and work is underway to test the function of this specific cyclin in a trangenic pine system through the collaboration with Forest Resarch, NZ. The transformation system for *Pinus radiata* is well established (Walter et al. 2002).

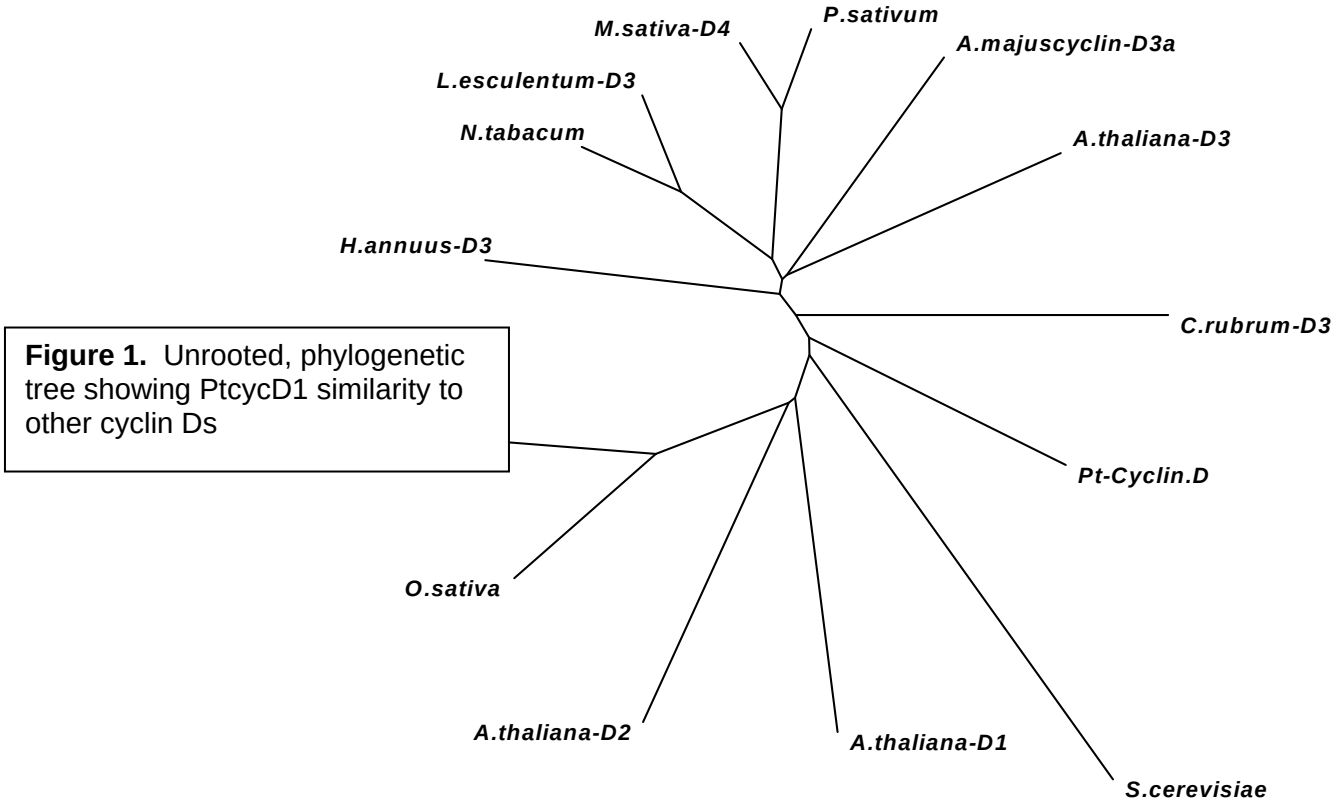

Figure 1. Unrooted, phylogenetic tree showing PtcycD1 similarity to other cyclin Ds

Isolation and characterization of PtCyclinD2 from xylem tissue of Pinus taeda.
Primers were designed based on previous sequence information on the consensus sequence for the cyclin D from *Pinus taeda* (Fig. 1). The full length cDNA of PtcyclinD2 cDNA was isolated. Sequencing from both ends revealed a full length cyclin sequence identical to the consensus sequence from cyclin D of Pt cDNA.The consensus sequence was previously constructed by aligning sequenced fragments encompassing partial sequences of the cyclin cDNA.

PtcyclinD>
ATGGCACCCAACTGCATAGACTGTGCCCCTAGTGATCTGTTTTGCGCGGAGGATGCTTTTG
GAGTTGTGGAATGGGGCGATGCAGAGACTGGAAGTTTGTATGGAGATGAGGATCAGCTGC
ATTATAATTTGGACATTTGTGACCAGCATGATGAGCATTTGTGGGATGACGGTGAACTTGTA
GCTTTTGCAGAAAAAGAGACCCTCTATGTTCCTAACCCAGTTGAGAAAAACAGTGCTGAAG
CTAAAGCTAGGCAGGATGCTGTGGATTGGATTTTGAAGGTTCATGCACATTATGGCTTTGG
TCCTGTGACTGCAGTGCTCTCAATAAACTATCTTGATCGGTTTTTGTCTGCAAATCAATTAC
AGCAAGATAAGCCATGGATGACTCAACTGGCAGCTGTGGCTTGCCTCTCCCTCGCTGCCA
AGATGGATGAGACAGAGGTTCCCCTTCTCCTGGACTTTCAGGTTGAGGAGGCTAAGTATAT
ATTCGAATCTCGCACCATTCAGAGAATGGAATTACTGGTGCTCAGTACCCTTGAATGGCGA
ATGAGTCCTGTGACACCTCTTTCCTACATTGATCATGCCAGTCGTATGATTGGGTTGGAGA
ATCACCATTGTTGGATTTTCACAATGCGCTGCAAGGAGATACTGTTGAATACACTCAGAGAT
GCAAAGTTTTTGGGCCTTCTGCCCTCTGTTGTAGCTGCTGCAATAATGCTGCATGTGATCA
AGGAAACAGAGCTTGTTAATCCATGTGAGTACGAGAATCGCCTGCTCAGTGCCATGAAAGT
TAATAAGGACATGTGTGAAAGATGCATAGGACTACTCATAGCCCCTGAATCATCATCCTTG
GGCAGTTTCTCTTTGGGTTTGAAAAGAAAGAGCAGCACCATCAATATTCCTGTTCCTGGCA
GCCCAGATGGAGTGCTAGACGCTACCTTTAGCTGCAGCAGCAGCAGCTGTGGTAGCGGAC
AGAGCACCCCAGGGTCATATGATTCCAATAACTCCAGCATTCTCTGCATCTCACCAGCGGT
GATAAAGAAGAGAAAGCTTAATTACGAGTTTTGTAGCGATCTTCATTGTTTGGAGGATTAGT
AG

Figure 2. The predicted full length consesnus sequence of PtcyclinD2.

Homology searches against BLASTX (NCBI)

The PtcyclinD2 sequence showed sequence similarity to several other cyclins (Fig. 2). The highest similarity (E value $2e^{-69}$) was to a poplar (*Populus alba*) cyclin.

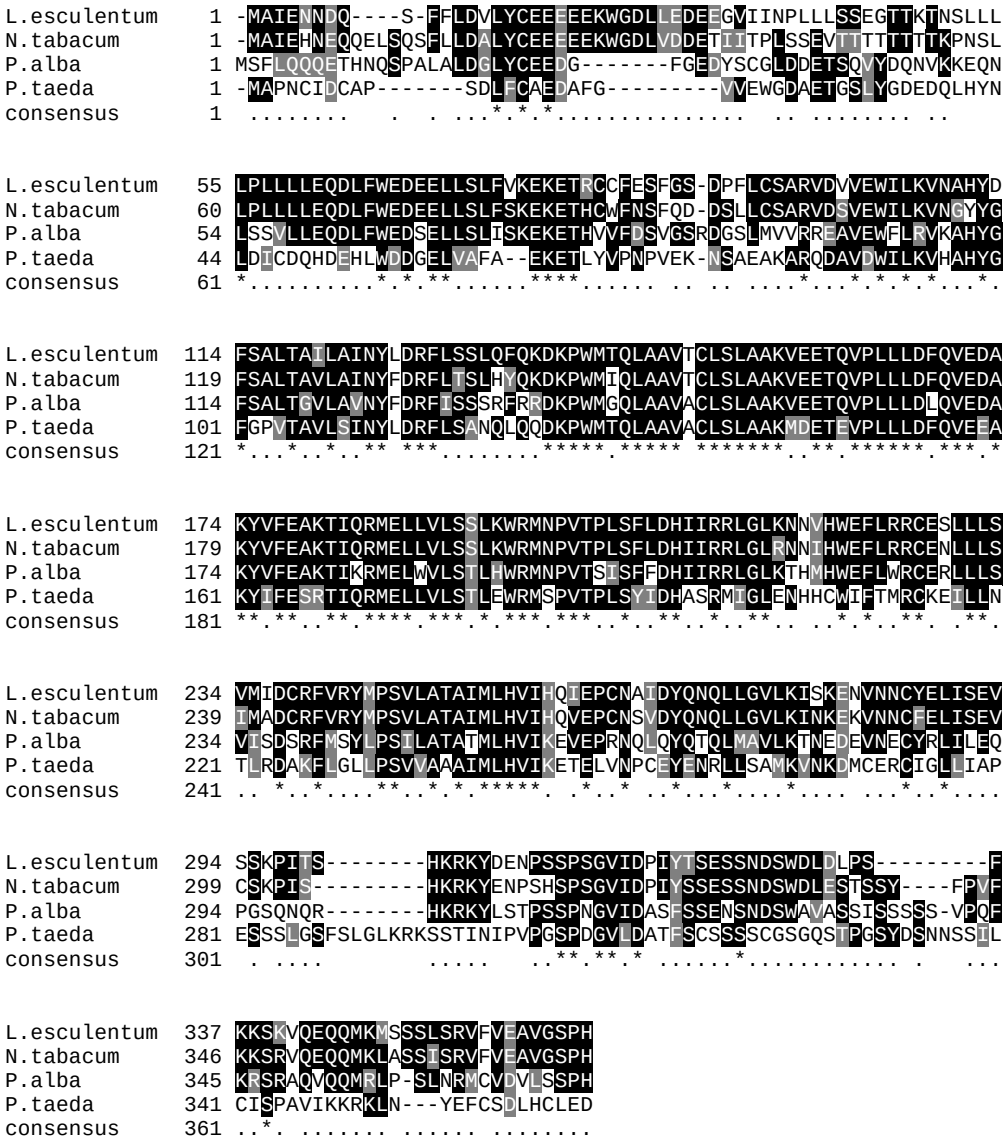

Figure 2. Similarity of the PtCyclin D to other cyclins in the NCBI database. The three most similar cyclins are poplar (*Populus alba*; E value $2e^{-69}$), tomato (*Lycopesricon esculentum*; E value $2e^{-67}$), and tobacco (*Nicotiana tabacum*; E value $2e^{-67}$).

Task 2- Study the expression patterns of cyclins within cambial meristem cells

8 trees were felled between early May and the end of June. Scraping and planning the 2 ft. log sections at 5 ft., 17ft. and 35 ft. up the trees was done to collect cambial and xylem tissue samples. Disks were also taken from the base of these logs at each height to determine the # of cells in the cambial zone, which includes the cambial meristem & xylem mother cells, tracheid expanding and nonlignifying zone. The data on # of cells in the cambial zone indicates that trees collected in the last week of May had 1/2—1/3 the number of cells when compared to June (Figure 1). In addition, the number of cells in the zone also increased with height on the tree as expected (Figure 3). These collections represent an excellent sample set for determining whether quantitative differences in mRNA and protein levels of the cyclin D relative correlate with cell division rates. Additional samples for each tree at all heights have been collected for dissection of the differentiating zone by tangential sectioning and in situ hybridization. Table II summarizes all of the samples collected during the summer of 2001.

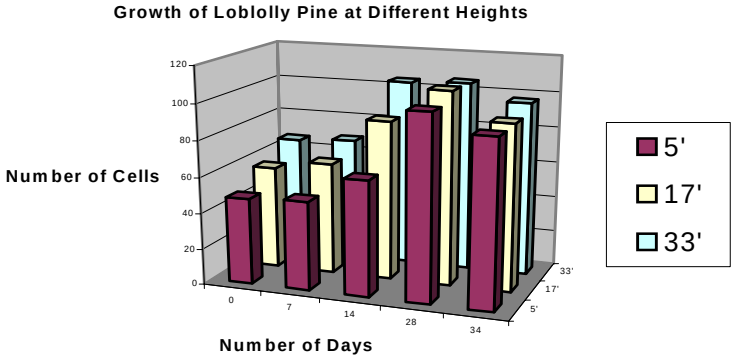

Figure 3. Number of cells in the cambial zone from 5 trees collected on successive weeks in May and June. The # of cells in the cambial zone was determined 5, 17, & 33 ft.

To relate changes in mRNA level of this cyclin to cell division rates we quantified the level of PtcyclinD1 in the nonlignified xylem from the trees characterized for cambial growth by quantitative RT-PCR. The hypothesis is that trees with more cells in the vascular cambial and expansion zones will have higher cyclin mRNA levels. At 5 ft tree E had twice the number of cells in the cambial and expansion zone as tree B. Figure 4 shows little difference in the amount of PtcyclinD1 mRNA between trees B and E at 5 ft. In addition a slight positive relationship

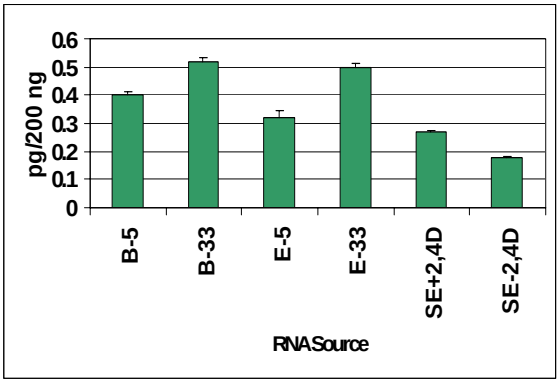

Figure 4. PtcyclinD1 mRNA levels detected by quantitative PCR in trees and somatic embryos. The results are of 3 replicate samples. Nonlignified xylem from tree B and tree E respectively at 5 ft and 35 ft. Early stage somatic embryo cultures with and without exogenous Auxin.

between the height of the tree and the mRNA was detected. In contrast, we did observe in the same experiment that the level of PtcyclinD1 mRNA is slightly decreased in rapidly dividing early stage after withdrawal of exogenous auxin from somatic embryo cultures for 4 weeks.

An additional test to correlate PtcyclinD1 mRNA levels with secondary growth in stems was carried out with compression wood. One of the features of trees under compressive stress is that the rates of cambial divisions are increased on the underside of the stem relative to the top or opposite side. The advantage of this approach is that it provides a test within the same tree and tree to tree variation is minimized. In our experiment we had one naturally bent tree that had been under compression for at least 4 years and one manually bent tree that had been under compression for only 5 days were used. The nonlignified xylem from both the compression side and the opposite side were harvested and quantitative PCR was used to measure the mRNA levels of PtcyclinD1 and COMT as a control. Figure 5 shows the ratio of mRNA these genes. The data show a 20 fold increase in the level of PtcyclinD1 mRNA on the compressed side of the stem relative to the opposite. This suggests that higher secondary growth rates correlate with PtcyclinD1 expression. Similarly for the tree bent for only 5 days showed a slight increase in PtcyclinD1 expression. The results for COMT were as expected, a strong increase in mRNA level on the compressed side of the stem.

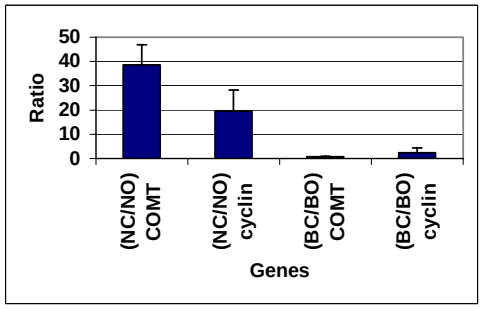

Figure 5. The ratio of PtcyclinD1 and COMT mRNA levels in nonlignified xylem from compressed and opposite sides of the stem from a naturally bent tree (NC/NO) and from a manually bent tree (BC/BO)

New trees have been identified for testing the hypothesis that increase cell divisions within the cambial meristem correlate with increased levels of PtcycD1. Figure 6 shows that the average ring width of 4 clonal replicates of 7 year old loblolly pine trees. Trees were selected based on stem diameter at year 4. Larger diameter trees show more growth during each year. Figure 5 shows that the increased growth in loblolly pine trees correlates with more cell divisions in the cambial meristem as expected. We are in the process of determining the levels of PtcycD1 mRNA levels in these fast and slow growing trees (Figure 7).

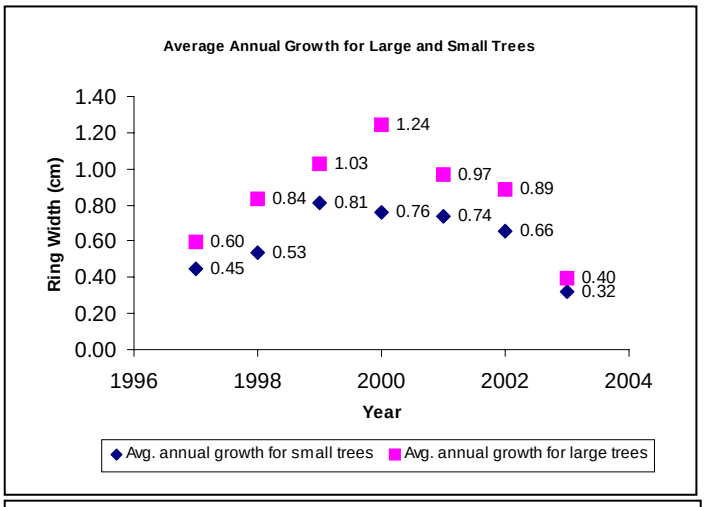

Figure 6. Seven year old loblolly pine clones that are larger in diameter grow faster in every season

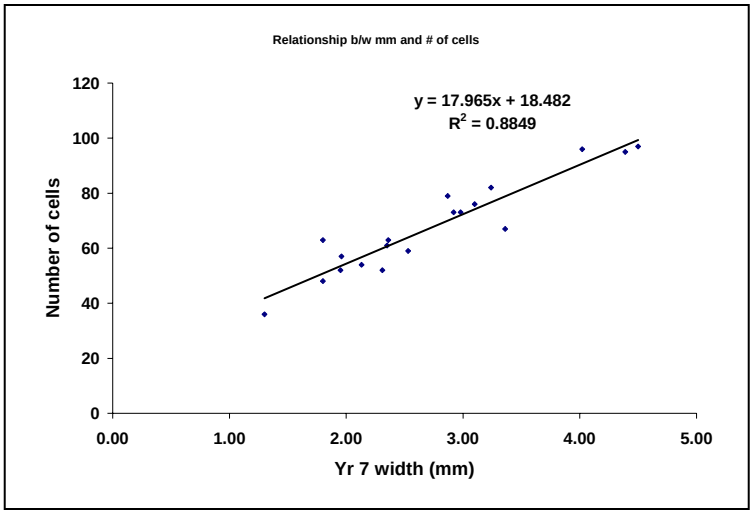

Figure 7. Faster loblolly pine stem growth rates are due to more xylem cells and higher rates of cell division in the cambial meristerm

To localize the cyclin mRNAs we are trying to develop tissue printing/in situ hybridization methods. In this method 15-30 um radial, tangential and cross-sections are cut using the cryomicrotome. For the tissue printing method, we have followed (Conley & Hanson 1997, Biotechniques 488: 491-496). Briefly, the sections are thawed unto nylon membranes impregnated with gel mount and the RNA UV fixed to the membrane. Hybridizations have used digoxigenin labeled DNA probes and alkaline phosphatase/chemiluminescence detection. To standardize this method in our lab we are using AGP as a probe since it is a very abundant message. Preliminary data with an AGP probe looks promising in terms of signal to noise and specificity (Figure 8).

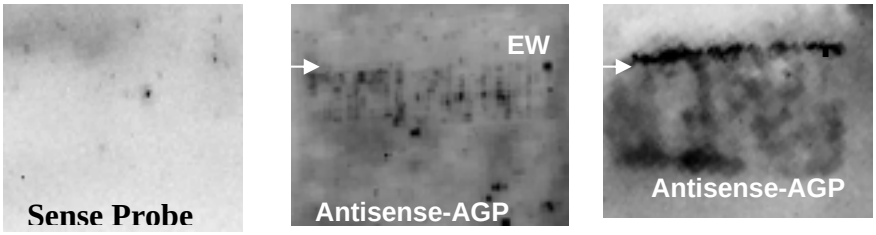

Figure 8. Cryotissue printing, cross sections from earlywood (EW) and latewood (LW) of 18 yr old trees were probed with sense and antisense AGP probes. Dark spots by arrows indicate AGP mRNA expression in cambial and expanding xylem zones.

With tissue printing approach described above it was difficult to localize the messages relative to cell types. Hence, we are now standardizing another approach using cryosectioning and in situ hybridization. In this method a CryoJane tape transfer system is used. With this system the soft cambial meristem and the cells in the expanding zone are maintained intact much better than with traditional cryosectioning. We are currently testing methods for improved freezing and fixation.

Sequential 30 uM tangential sections from earlywood (2 trees) and latewood (1 tree) have been collected. The RNA from these sections will be isolated and used for analysis of the zone that cyclins are expressed in. Preliminary tests show that using SMART-PCR we are able to amplify the mRNA from one section. Tests of SMART-PCR show that they tend to compress the differences in expression level between the abundant and not so abundant mRNAs. In contrast, aRNA amplification methods, which are linear and not exponential since they do not use PCR, do not compress the expression levels. aRNA amplification is suitable for amplifying RNA from one 30 uM tangential section.

Task 3- Isolate promoter(s) for cambial cell specific expression

We have isolated a promoter region whose mRNA is most abundantly expressed in secondary xylem. This mRNA is not detected in needles or early stage somatic embryo cultures (Figure 9). Our clone contains ~ 1 kb of the 5' promoter region of this fragment.

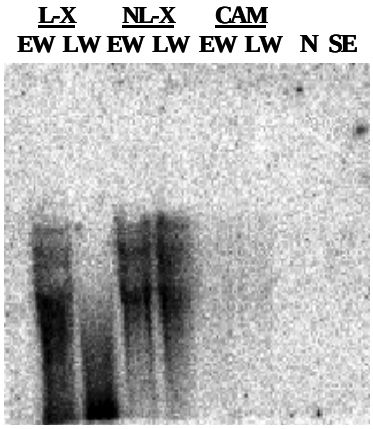

Figure 9. RNA gel blot with 10 ug of total RNA L-X is lignifying xylem, NL-X is nonlignifying xylem, EW is earlywood, LW is latewood, CAM is barkside scrapings, N is needles, SE is early stage somatic embryos

Task 4 – Construct plant vector for expressing cyclin(s) in cambial cells

We have cloned our full-length PtcycD1 into a 2X35SCaMV-TEVL- for preliminary overexpression experiments in pine.

Task 5 - Generate 5-10 independent transgenic lines of *P. taeda* with these gene constructs

Transformation efforts are on going with State of Georgia funds. We are optimizing selection procedures and DNA transfers with *Agrobacterium tumefacians*. Initial constructs for transformation procedure development include 2X-35S CaMV-TEVL-NPT II & 35S GFP-ER. We have made a T-DNA vector construct in pBIN19 that contains the maize ubi 1 promoter and intron regulating expression of the NPTII gene.

We now have putatively transformed early stage somatic embryo cultures growing on geneticin selection with the ubi1 promoter. These lines are growing whereas control untransformed lines are not growing on geneticin. Putatively transformed lines have been confirmed by PCR analysis. Sufficient tissue is being grown for seedling regeneration experiments. Unfortunately this tissue did not produce embryos.

We have found that growth of our best seedling producing lines was slow relative to other lines. Consequently we have gone back to optimize the tissue culture media to support faster growth of this line. Figure 10 shows media that improve the growth rate of early stage embryos from loblolly pine.

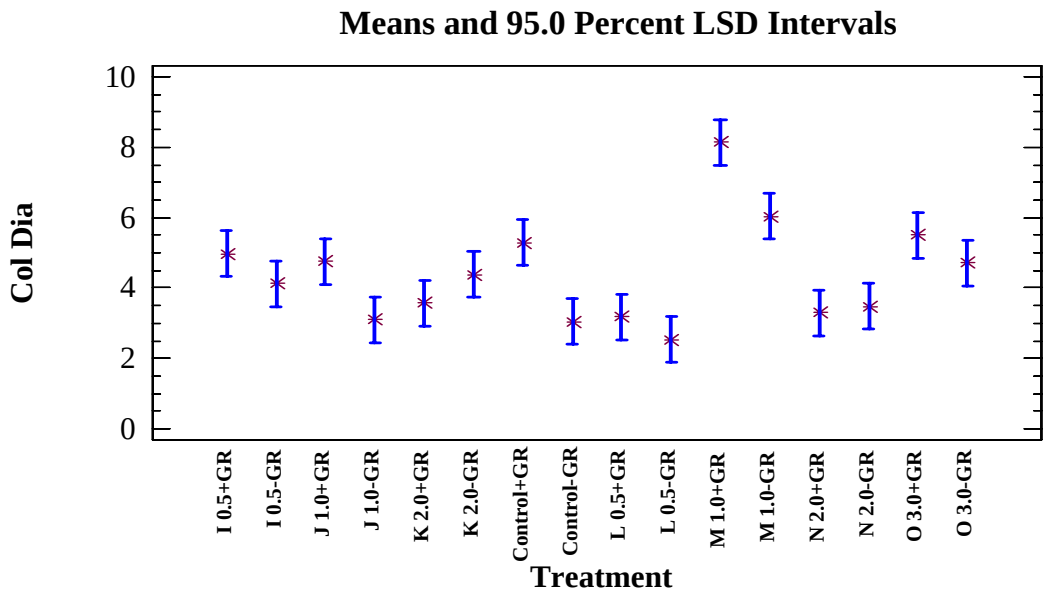

Figure 10. Optimization of growth media for selection of transformed early stage embryos. Media M promotes the highest rates of early embryo division

The transformation efforts in loblolly pine are ongoing at IPST as previously discussed. The system is however not yet reached a level of efficiency that allows for analyses of gene characterization in vivo. We have therefore established a collaboration with Dr. Armin Wagner at Forest research, Rotorua, New Zealand, to study the function of the cyclin D. Forest Research has developed a functional system for transformation of conifers (Walter et al. 2002, Plant Cell, Tiss Org Cult 70: 3-12) that has proven succesful for *Pinus radiata* and *Picea abies* (Walter et al. 1998, Plant Cell Rep. 17: 460-468), *Abies nordmanniana* (Find, in prep.), *Pinus taeda* (Grace, unpublished). We will supply the full length cDNA clone to Forest Research (non-disclosure agreement between Georgia Institute of Technology and Forest Research has been signed). The in house vector at Forest research will be used for making the construct (Walter et al. 1998, Plant Cell Rep. 17: 460-468). In addition, a newly characterized promoter with cambium specific activity will be added to the construct (Johansson et al. 2003, Plant Mol Biol. 52(2):317-29). This promoter is currently being tested at Forest Research for activity in conifers (Wagner. pers. comm.).

Task 6- Initiate analyses of cyclin expression, stem growth, vascular cell differentiation, and xylem cell properties

Ulrika Egertsdotter[1], John Cairney [1], Armin Wagner[2] , Gary Peter[3]

1. Institute of Paper Science and Technology, Georgia Institute of Technology, Atlanta, GA
2. Cellwall Biotechnology Centre (CBC), New Zealand Forest Research Institute Ltd., Rotorua, New Zealand
3. UFL, Gainesville, FL

Transformation of conifer species

The cloned PtcyclinD2 sequence showed sequence similarity to several other cyclins (Fig. 1). The highest similarity (E value $2e^{-69}$) was to a poplar (*Populus alba*) cyclin. (Fig. 2)

The full length PtCyclinD cDNA, was sent to Forest Research, NZ. Forest Research has a well established Constructs are being made with three different promoters: *Zea mays* ubi-1 promoter, an AGP promoter, and the *Pinus radiata* CCoAOMT promoter. The promoters come with introns, which are part of the original 5' UTR of the attached genes

Gymnosperms have proven recalcitrant to genetic transformation. We are currently developing protocols for the transformation of *P. taeda*. We have initiated a collaboration with Dr. Armin Wagner of the New Zealand Forest Research Institute. Dr. Wagner, who is working as a scientist at the Cellwall Biotechnology Centre (CBC), has contributed in the development of a robust transformation protocol for *Pinus radiata* and other conifer species (Walter et al., 1998). He is in process of transforming the isolated *P. taeda cyclinD* cDNA clone into this pine species. Through the collaboration IPST hopes to gain insight into improving transformation methods for *P. taeda* while establishing transformed lines expressing *PtcyclinD*, which may be evaluated.

Cloning *Pinus taeda* cyclin D cDNA into Plant Transformation Vectors

The *P. taeda cyclinD* cDNA clone was sent to CBC, where it was characterized using a restriction analysis based on the sequence information provided. It was noticed that the isolated cDNA clone has a very short 5'UTR, which has the potential to impact on translation efficiency. Consequently, our collaborators at CBC tried to allow for this in the design of the over-expression (OE) constructs by choosing promoters, which have an intron 3' of the TATA box (see Figure 1 & 2).

Two different promoters have been chosen for the *PtcyclinD* OE experiments: (i) The *Z. mays ubi1*-promoter, since histological investigations and quantitative ELISA measurements with *P. radiata* transclones at CBC have indicated that this promoter is fairly active in a broad range of different tissue types in this conifer (Wagner et al., unpublished results). A plasmid map illustrating the *ZmUbi1promoter-PtCyclinD* OE construct is given Figure 1. The intention of this experiment is to observe any phenotypic differences possible to get an idea, whether the isolated *P. taeda cyclinD* clone is capable of producing any kind of effect at all when over-expressed in *P. radiata*. The use of a constitutive promoter has also the potential to give an indication on whether it is necessary to use a tissue specific promoter to avoid undesired pleitropic effects in the produced transclones.

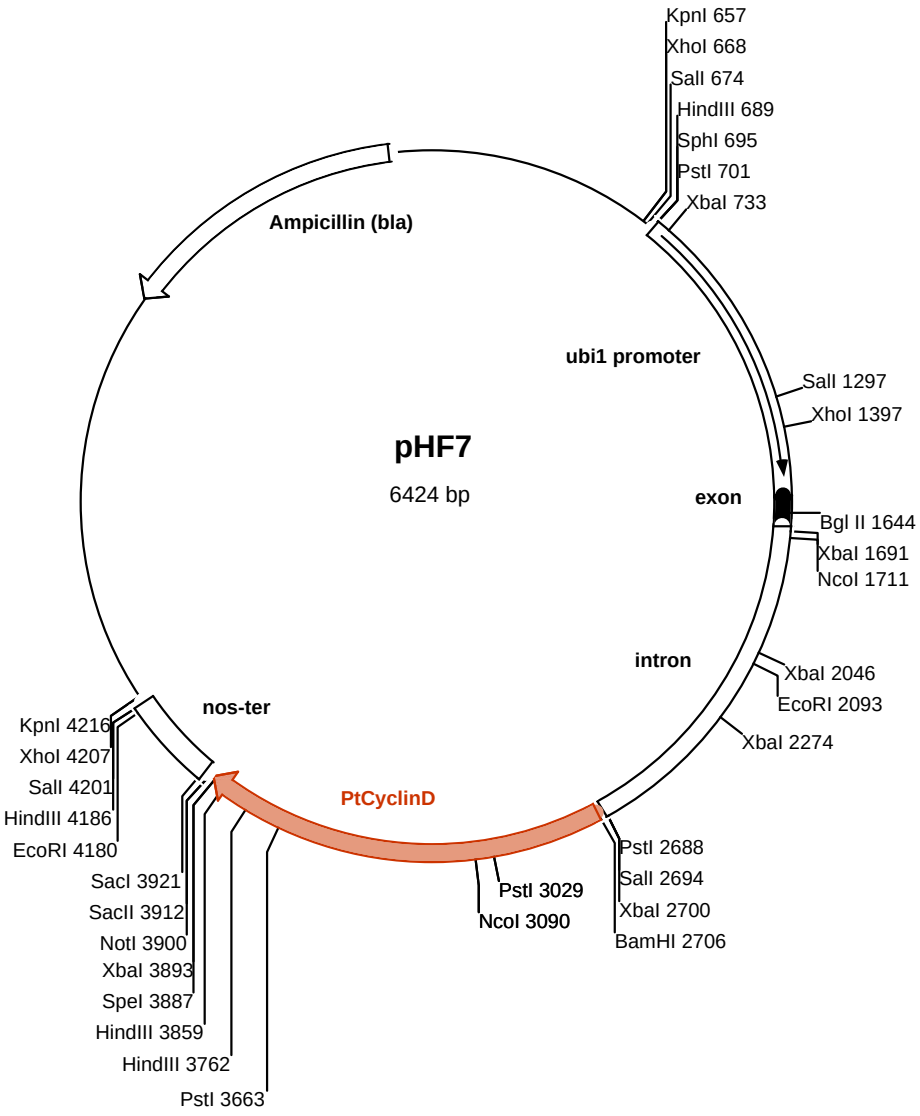

Figure 11: Schematic plasmid map containing the *ZmUbi1promoter-PtCyclinD* construct

(ii) The *P. radiata CCoOMT* promoter has also been used to drive *PtCyclinD* expression, since this promoter is preferentially active in differentiating xylem, which has been shown by histological studies in *N. tabacum* transformed with a *PrCCoAOMT-GUS* construct (Wagner et al., unpublished results). The intention of this experiment is to try and enhance cell division in a tissue specific manner during xylogenesis and therefore to increase biomass acquisition in the wood-forming tissue of the plant. Our collaborators at CBC have made the *PrCCoOMT-cyclinD* construct and analyzed it by restriction analysis. The restriction map of this construct is shown in Figure 2.

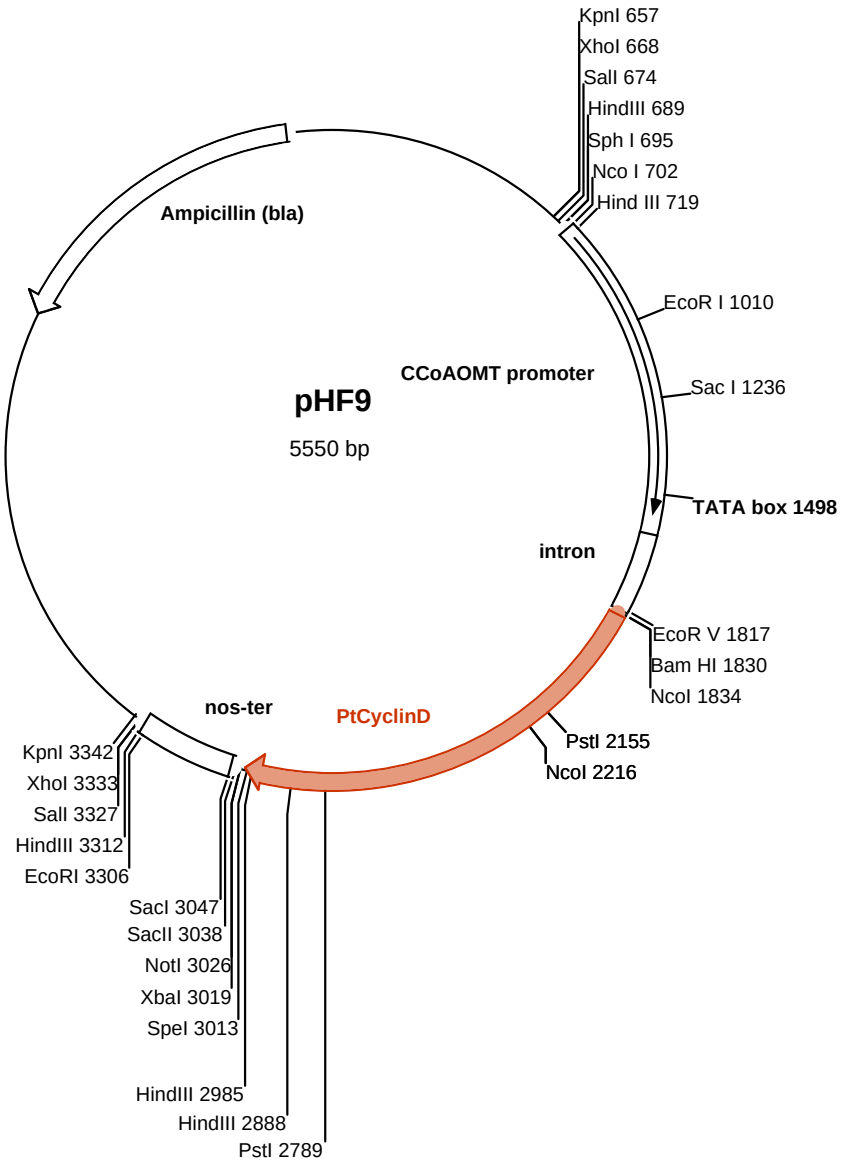

Figure 12: Schematic plasmid map containing the *PrCCoAOMTpromoter-PtCyclinD* construct

Transformation of embryogenic *Pinus radiata* tissue

Several plasmid DNA maxi-preps of the plant transformation vector constructs described earlier have been made and embryogenic tissue suitable as a target for transformation experiments was bulked up. Transformation experiments were conducted by a biolistic method developed at CBC earlier (Walter et al., 1998). All experiments were done in a co-transformation mode with the vector *pAW16* (Wagner et al., unpublished results) containing a *NPTII* selection and *GUS* reporter gene cassette. At CBC approximately 440 plates of embryogenic *P. radiata* tissue were bombarded using a biolistic device. At present 78 transgenic embryogenic lines have been identified and bulked up with a good representation of transgenic lines per construct (see Table 1). Transformed calli were originally identified by resistance to the antibiotic Geneticin contained in the medium. The transgenic nature of the selected lines was subsequently confirmed using histochemical GUS staining (see Figure 3).

Table 1: Overview of the *P. radiata* transformation experiments conducted at CBC

Construct	P. radiata genotype	Bombarded plates	Recovered transclones	Efficiency [transclone/plate]	GUS positive
pHF7	I99-76	220	44	0.2	84%
pHF9	I99-76	220	34	0.15	71%

Transclone HF7-29

Transclone HF7-30

Transclone HF7-31

Figure 13: Histochemical GUS staining in regenerated transgenic embryogenic *P. radiata* lines indicating different levels of reporter gene expression.

Preservation and molecular analysis of *Pinus radiata* transclones

All *P. radiata* transclones generated in this study are currently bulked up for cyropreservation and embryo maturation purposes. Cryopreservation is useful to maintain the juvenile character of the tissue and enables us to go back to generated transclones for potential reinvestigations.

A series of confirmatory experiments are currently in progress at CBC. Genomic PCR (gPCR) experiments with *P. radiata* transclones are in progress to confirm the integration of the *PtCyclinD* OE constructs in the *P. radiata* genome. First gPCR experiments with *P. radiata* lines transformed with the vector pHF7 containing the *ZmUbi1promoter-PtCyclinD* construct has confirmed the integration of the construct in approximately 91% of the tested transgenic lines.

Quantitative RT-PCR experiments are also currently designed at CBC to determine the degree to which the target gene is expressed in different transgenic lines. However, the lack of comprehensive sequence data covering all members of this gene family has the potential to complicate this experimental procedure.

Transformation of xylogenic *Pinus radiata* tissue

The vector pHF7 containing the *ZmUbi1promoter-PtCyclinD* construct has also been transformed in xylogenic *P. radiata* tissue. This tissue cultures system, which is capable of undergoing xylogenesis, was developed at CBC for functional gene testing in conifers and has the potential to generate phenotypic data much faster than plants. The effect of *PtCyclinD* over-expression on cell proliferation and cytokinin independent cell division can be investigated in such a model system.

The selection process of bombarded tissue is currently in progress at CBC Currently, 10 out of 13 transgenic lines have produced embryos so far and that we are currently harvesting the first
transgenic plantlets. At present time 22 of those plantlets have already gone to GMO facilities. We will soon develop a strategy on how to asses potential phenotypic differences between the transclones and non-transformed controls.

References

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