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Cross-species PCR and field studies on *Paulownia elongata*: A potential bioenergy crop

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Abstract: *Paulownia elongata* is a short-rotation fast growing tree and is known for high biomass accumulation and carbon sequestration potential. Optimization of protocols for nucleic acid extraction, PCR, RT-PCR, and other molecular biology techniques are required for better understanding of cellulose synthesis and to assess the potential of *Paulownia* as a biofuel tree. The main objective of this work was to study a putative cellulose synthase amplicon expression under various environmental conditions and evaluate the potentials of *Paulownia* as a biofuel tree. Using cross-species PCR an amplicon representative of a putative cellulose synthase gene from *Paulownia was identified*. This 177-bp long DNA sequence was 46% similar with cellulose synthase genes from *Arabidopsis* as expected. Gene specific primers for this particular *Paulownia* cellulose synthase gene were designed and reverse transcription PCR was performed to confirm its transcription. We report an inexpensive cDNA dot-blot method to study expression of this gene under various environmental conditions. We observed that cold and, to a lesser extent, heat stress downregulated its expression. This information will help to understand cellulose deposition in plant cell wall under stressful conditions. To the best of our knowledge this is the first characterization of a cDNA sequence from *Paulownia elongata*.

Keywords: biomass, carbon sequestration, cDNA, cellulose synthase, cross-species PCR, *Paulownia elongata*

HIGHLIGHTS

- *Paulownia elongata* is a fast growing tree and has the potential to be used as source of biofuel
- A putative cellulose synthase gene, which plays an important role in biomass formation, was identified in *Paulownia elongata*
- We report about cDNA dot blot is an inexpensive method used to study the differential expression of this cellulose synthase gene at various abiotic stressful environments
- Cold and, to a lesser extent, heat stress dowregulated this gene expression.
- Biomass and carbon sequestration studies suggest *Paulownia elongata* is an excellent candidate for lignocellulosic biofuel production

1 Introduction

The genus *Paulownia* (family Paulowniaceae) is comprised of nine species of fast growing trees native to China [1]. Some of these species have spread around the world, both through cultivation as ornamental tree as well as inadvertently, being released through seeds, which were used as packaging material, to the USA, Europe, and Australia. All species are extremely adaptive to the environment, both in terms of climatic changes as well as soil variations, and have been found to be sustainable on marginal lands. The elevation range for this genus includes plains to low mountainous regions up to 2000 feet [1]. China has historically been the largest grower of *Paulownia*. In China, the wood is used for making furniture, construction, toys, plywood, musical instruments and

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for packaging. For decades, Japanese craftsmen have utilized *Paulownia* wood in ceremonial furniture, musical instruments, decorative moldings, laminated structural beams and shipping containers. The tree made its way to the USA in the mid-1800s. *Paulownia* seeds were used as packaging material for delicate porcelain dishes on their journey across the Pacific. Once unpacked, the tiny wind-blown seeds dispersed and the germinated plants were naturalized throughout the eastern USA [2]. Despite having great commercial value for the pulp, paper, textile, and chemical industries that use cellulose to produce commercially important polymers, cellulose biosynthesis in trees is still not well understood [3]. Such trees offer an inexpensive source of woody biomass for the energy and lumber industries [4,5]. Summative analysis of *Paulownia* wood reveals that it could be a good source of cellulose and hemicellulose [5]. In addition, wood shavings and waste biomass could also be employed as natural fibers for incorporation into thermoplastic resins. Recent studies indicated that biocomposites composed of smaller particles exhibited tensile strength values similar to neat poly lactic acid (PLA) but had Young's modulus values that were 25% higher than neat PLA with a reduction in elongation factor [5]. Further, hot water extraction studies of *Paulownia* wood chips indicated that it could be a good substrate to release chemicals of high value [6].

Cellulose obtained from wood tissues of trees such as *Paulownia*, could be a major source of biofuel [7] including bioethanol production. Plant biomass used for cellulosic biofuel production is primarily from vegetative tissues. Therefore, an increase in the vegetative growth of plants will lead to a higher production of plant biomass. A number of factors that take part in vegetative meristem activities, cell elongation, photosynthetic efficiency, and secondary wall biosynthesis are crucial for plant biomass production [8]. The regulators of these processes could potentially be applied to improve the yield of biomass crops in the near future. Hence, a sound knowledge of genes involved in cellulose synthesis and their regulation in different climatic conditions would help selection of desirable tree stocks for future tree breeding and improvement programs. Comparative studies indicate that components necessary for cellulose synthesis are conserved across plant species, potentially allowing information from *Arabidopsis* to be transferred to more commercially important species [9]. Collectively the cellulose synthesizing genes are also known as cellulose superfamily genes, which includes cellulose synthase genes (cesA) and cellulose synthase like genes (csl) [10]. The size of the *Arabidopsis thaliana* cesA genes is 3.5-5.5 kb. These genes have 9-13 introns, which encode proteins with 985-1088 amino acid residues that share 53-98% sequence identity [11,12]. Since the report of the first characterization of a cellulose synthase gene from cotton (*Gossypium hirsutum*) [12], cellulose synthase genes from various wood producing trees are well characterized [11] and their role in biofuel development have been emphasized [13].

Cellulose synthase genes have been reported to be differentially expressed at various plant tissues in aspen (*Populus tremuloides*) [14]. These genes are also differentially expressed in mechanical stress responsive elements in wood forming tissues in *Eucalyptus grandis* [15]. CesA2 was reported to be more abundant than CesA4 in various potato (*Solanum tuberosum*) tissues[16]. RNA expression profiling from cold induced sugarcane (*Saccharum* sp. cv SP80-3280) revealed multiple cellulose synthase ESTs (Expressed Sequence Tags) [17]. It was reported that heat stressed plants can synthesize more cellulose to protect the plant from heat stress [18].

The main goal was to understand cellulose synthase gene expression pattern under various environmental conditions (namely heat and cold stress). Understanding of cellulose synthase gene expression in *Paulownia* will help understand the molecular basis of plant-based cellulosic or lignocellulosic biofuel production.

Two of the most common methods to document gene expression are real-time quantitative Polymerase Chain Reaction (qPCR) and by northern blot. qPCR is very sensitive, however has its own limitations, for example, variations in nucleotide extraction methods from various environmental samples could affect qPCR results [19]. PCR inhibitors in environmental samples could also affect qPCR [20] and the qPCR thermal cycler is expensive and could itself be a limitation. Another potential issue with qPCR is selection of housekeeping genes. Some of the widely used housekeeping genes (beta-tubulin, actin, 18S rRNA) were found to be differentially expressed under various environmental conditions in potato (*Solanum tuberosum*) [21]. Hence, proper selection of housekeeping genes to normalize qPCR data can be sometimes challenging. Besides qPCR, northern blot is also a traditional method of gene expression studies. However, northern blot requires a complete RNAse free environment, which requires extra precaution on every step. Also, RNA itself is susceptible to degradation and can often generate suboptimal results.

Here we report an alternative approach to study gene expression- cDNA dot blot. cDNA dot blot does not require an RNAse-free environment, or use of hazardous radioactivity. A putative cellulose synthase gene from *Paulownia* was succesfully amplified by a cross-species PCR method [22], cloned the gene and studied its expression patterns using cDNA dot bot.

2 Materials and Methods

The overall experimental approaches described in this paper are shown in Fig. 1. Throughout the paper the terminology 'putative cellulose synthase gene' refers to an amplicon representing a putative cellulose synthase gene cloned from *Paulownia elongata*.

2.1 Polymerase chain reaction of putative cellulose synthase gene from Paulownia using Arabidopsis gene specific primers

One year-old *Paulownia* plants maintained in the greenhouse at the California State University, Northridge, USA were used to harvest leaves for genomic DNA extraction. Genomic DNA was extracted from *Paulownia* leaves using GenJet™ Plant Genomic DNA Extraction kit (Fermentas, USA), following the manufacturer's instruction as described below. One hundred mg of *Paulownia* leaves were placed in liquid nitrogen and ground using a mortar and pestle. After addition of lysis buffer and RNAse A (to degrade any residual RNA) to the sample, it was incubated at 65^{0}C for 10 minutes. Then a precipitation solution was added and the sample was washed with wash solution. Finally DNA was eluted with sterile nuclease free water. DNA concentration and quality were checked using a NanoDrop 2000C UV-VIS spectrophotometer (Thermo, USA).

Since the *Paulownia* genome is not sequenced, cellulose synthase gene sequences from *Arabidopsis thaliana* and poplar (*Populus sp.*) were used to design 10 sets of PCR primers (Table 1). Primer design was performed by PrimerQuest software available on www.idtdna.com.

The PCR conditions were followed as specified in the 2 X PCR Master Mix kit (Fermentas, USA) using Multigene Optimax thermal cycler (Labnet International Inc., USA) and a gradient of annealing temperatures from 55^{0}C - 63^{0}C (Fig. 2).

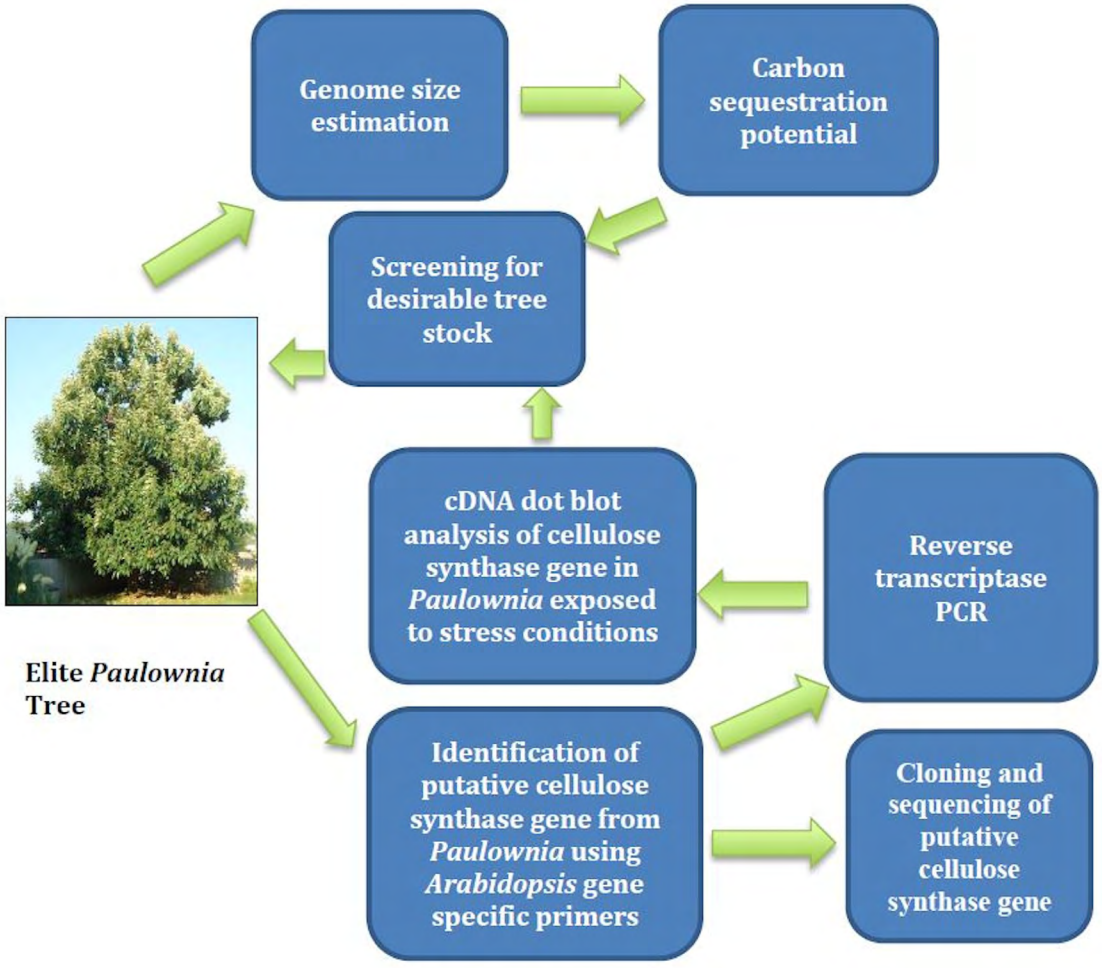

Fig. 1. Schematic representaion of overall experiemnts.

Table 1. List of cellulose synthase gene specific primers used for cross-species PCR of *Paulownia elongata.*

Source species	GenBank accession number	Primer sequences
Poplar	XM_002302133.2	Forward: 5'-GTC GTG GTT CAC AAG CCC ATG TTT-3' Reverse: 5'-TTC TGG AGC TCG AGG TTC AAT GCT-3'
Poplar	AY 162181.1	Forward: 5'- GAT TTG GTT TGC CTT GTC CTG GCT-3' Reverse: 5'- TTT CAT TGC TCG GCG CTC TTT CAC-3'
Poplar	AY 162184	Forward: 5'- AGA CAG CAC AAG GAC GAT GAA CCT-3' Reverse: 5'- GCA AAG CAG CAG CTA CAG CAA TCT-3'
Poplar	XM_002318966.2	Forward: 5'- AAA CCA CCA ACG AGG ACA TGC AAC-3' Reverse: 5'- TGT TCG TTT CTC CAC CAC TCG TCA-3'
Poplar	JX986629.1	Forward: 5'- AGT CCT TTC CAT CCT TGC TGT GGA-3' Reverse: 5'- TGC CAT CAA ACC TCT GAG GGA ACT-3'
Arabidopsis thaliana	NM_123770	Forward: 5'- CAG TAA GCA GCA ATG GCG TCC AAA-3' Reverse: 5'- AGC AGC TCA CCT TAT TCA CTG GGT-3'
Arabidopsis thaliana	NM_120095	Forward: 5'- AAC GCC GAT GAG AGT GCC AGA ATA-3' Reverse: 5'- TCG CCT TGT CGT CTC TTC CAA ACT-3'
Arabidopsis thaliana	NM_102254	Forward: 5'- ATG GCT ACA AAG CCG GAG CTC TTA-3' Reverse: 5'- ATT CTC CAT ACA CCC GCA GTT CCA-3'
Arabidopsis thaliana	NM_127746	Forward: 5'-TGG GAC ACA GCG GAG TTT GTG ATA-3' Reverse: 5'-TGG TGG TTG CTT CTT CTT TGG TGC-3'
Arabidopsis thaliana (PCR amplification was successful)	NM_125870	Forward: 5'- TCC AAT GAT GGA TGA GGG AAG GCA-3' Reverse: 5'- TCC AAC CAT CCT CAG GCA CTT TCT-3'

Note: Only one set of primer was able to amplify a putative cellulose synthase gene.

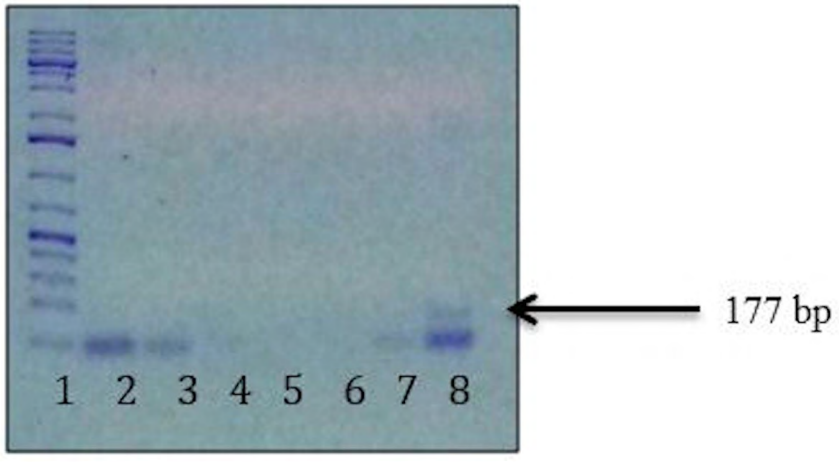

Fig. 2. Cross-species PCR amplification of *Paulownia* genomic DNA with *Arabidopsis* primers. PCR amplification (by gradient PCR) of a putative cellulose synthase gene with DNA using *Arabidopsis* cellulose synthase gene (GenBank accession no. NM-127746) specific primers. Lane 1: GeneRuler 1 kb Ladder (Fermentas), Lane 2: Negative control (water). Lane 3 to 8: Annealing temperatures 55°C, 56°C, 58°C, 60°C, 61°C, and 63°C respectively. The putative *Paulownia* cellulose synthase gene was amplified at annealing temperature 63°C (The top band, shown with arrow in lane 8). Image color was inverted from original. Brightness and contrast were adjusted to -40% and +40% respectively.

2.2 Cloning of Paulownia cellulose synthase gene

The PCR amplified product/putative cellulose synthase gene (Fig. 2) was gel purified using Nucleospin ® PCR and Gel Cleanup Kit (Macherey- Nagel, Germany). The putative *Paulownia* cellulose synthase gene was then cloned into a pJET TA cloning vector (Fermentas, USA) following manufacturer's instruction. Restriction digest with XhoI and XbaI of pJET vector was used to confirm the presence of the cloned putative cellulose synthase gene. The cloned gene was sequenced at Laragen, Culver City, USA (Supplementary data, Fig. 1). After sequencing, the VecScreen program (www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html) was used to remove the contaminating vector sequences to find 177 bp sequence (Supplementary data, Fig. 2) without any vector contamination.

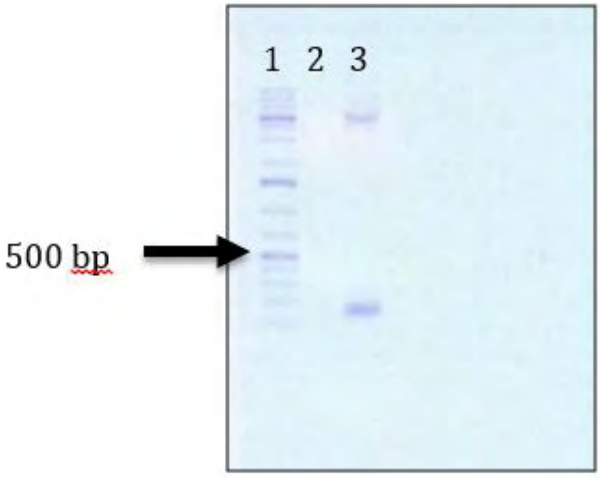

Fig. 3. Confirmation of cloned *Paulownia* cellulose synthase gene. Lane 1: GeneRuler 1 kb plus ladder (Fermentas, USA), Lane 2: Empty, Lane 3: Digested pJET vector with XhoI and XbaI restriction enzymes. The 177 bp band is visible at the bottom of the gel. Image color was inverted from original.

Lineage Report

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Spermatophyta   [seed plants]
. Mesangiospermae [flowering plants]
. . Pentapetalae    [eudicots]
. . . rosids        [eudicots]
. . . . malvids       [eudicots]
. . . . . Brassicales     [eudicots]
. . . . . . Tarenaya hassleriana (spider flower) ------   53  2 hits [eudicots]     PREDICTED: Tarenaya hassleriana uncharacterized LOC10481777
. . . . . . Arabidopsis thaliana (thale-cress) ........   46 15 hits [eudicots]     Arabidopsis thaliana chromosome 2, complete sequence
. . . . . . Eutrema salsugineum .......................   40  2 hits [eudicots]     Eutrema salsugineum hypothetical protein (EUTSA_v10024277mg
. . . . . . Arabidopsis lyrata subsp. lyrata ..........   40  4 hits [eudicots]     Arabidopsis lyrata subsp. lyrata hypothetical protein, mRNA
. . . . . . Brassica rapa subsp. pekinensis (pe-tsai) .   40  3 hits [eudicots]     Brassica rapa subsp. pekinensis clone KBrH004J18, complete
. . . . . . Camelina sativa (gold-of-pleasure) ........   37  3 hits [eudicots]     PREDICTED: Camelina sativa cellulose synthase A catalytic s
. . . . . . Capsella rubella ..........................   37  1 hit  [eudicots]     Capsella rubella hypothetical protein (CARUB_v10004034mg) m
. . . . . Citrus sinensis (navel orange) --------------   39  3 hits [eudicots]     PREDICTED: Citrus sinensis peptidyl-prolyl cis-trans isomer
. . . . . Citrus clementina ...........................   39  6 hits [eudicots]     Citrus clementina hypothetical protein (CICLE_v10008911mg)
. . . . Populus trichocarpa (western balsam poplar) ---   46  1 hit  [eudicots]     Populus trichocarpa hypothetical protein (POPTR_0006s07590g
. . . . Ricinus communis ..............................   46  1 hit  [eudicots]     Ricinus communis conserved hypothetical protein, mRNA
. . . . Prunus persica ................................   44  1 hit  [eudicots]     Prunus persica hypothetical protein (PRUPE_ppa012757mg) mRN
. . . . Prunus mume (ume) .............................   42  1 hit  [eudicots]     PREDICTED: Prunus mume uncharacterized LOC103323662 (LOC103
. . . . Medicago truncatula ...........................   42  1 hit  [eudicots]     Medicago truncatula clone JCVI-FLMt-7I18 unknown mRNA
. . . . Fragaria vesca subsp. vesca ...................   40  1 hit  [eudicots]     PREDICTED: Fragaria vesca subsp. vesca uncharacterized LOC1
. . . . Glycine max (soybeans) ........................   40  6 hits [eudicots]     Glycine max uncharacterized LOC100305658 (LOC100305658), mR
. . . . Vitis vinifera ................................   40  4 hits [eudicots]     Vitis vinifera, whole genome shotgun sequence, contig VV78X
. . . Beta vulgaris subsp. vulgaris (spinach chard) ---   40  2 hits [eudicots]     PREDICTED: Beta vulgaris subsp. vulgaris mechanosensitive i
. . . Solanum pennellii ...............................   37  2 hits [eudicots]     Solanum pennellii chromosome ch05, complete genome
. . Brachypodium distachyon (purple false brome) ------   49  1 hit  [monocots]     PREDICTED: Brachypodium distachyon uncharacterized LOC10083
. . Oryza brachyantha .................................   49  2 hits [monocots]     PREDICTED: Oryza brachyantha uncharacterized LOC102722500 (
. . Sorghum bicolor (milo) ............................   37  1 hit  [monocots]     Sorghum bicolor hypothetical protein, mRNA
. Picea glauca ----------------------------------------   37  1 hit  [seed plants]  Picea glauca clone GQ04105_B12 mRNA sequence
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Organism Report

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 Tarenaya hassleriana (spider flower) [eudicots] taxid 28532
ref|XM_010547091.1| PREDICTED: Tarenaya hassleriana unchar...     53  1e-04
ref|XM_010547090.1| PREDICTED: Tarenaya hassleriana unchar...     53  1e-04

 Brachypodium distachyon (purple false brome, ...) [monocots] taxid 15368
ref|XM_003564007.2| PREDICTED: Brachypodium distachyon unc...     49  0.001

 Oryza brachyantha [monocots] taxid 4533
ref|XM_006656029.1| PREDICTED: Oryza brachyantha uncharact...     49  0.001
ref|XM_006656028.1| PREDICTED: Oryza brachyantha uncharact...     49  0.001

 Populus trichocarpa (western balsam poplar) [eudicots] taxid 3694
ref|XM_002308084.2| Populus trichocarpa hypothetical prote...     46  0.017

 Arabidopsis thaliana (thale-cress, ...) [eudicots] taxid 3702
gb|CP002685.1| Arabidopsis thaliana chromosome 2, complete...     46  0.017
ref|NM_127746.1| Arabidopsis thaliana cellulose synthase A...     46  0.017
gb|AC007019.5| Arabidopsis thaliana chromosome 2 clone F7D...     46  0.017
gb|KJ139038.1| Arabidopsis thaliana glycosyltransferase (A...     40  0.73
gb|CP002687.1| Arabidopsis thaliana chromosome 4 sequence         40  0.73
gb|FJ687279.2| Arabidopsis thaliana AthA (CESA2) gene, com...     40  0.73
ref|NM_120095.3| Arabidopsis thaliana cellulose synthase A...     40  0.73
gb|AY093308.1| Arabidopsis thaliana cellulose synthase cat...     40  0.73
gb|AY059858.1| Arabidopsis thaliana cellulose synthase cat...     40  0.73
emb|AL161395.2| Arabidopsis thaliana DNA chromosome 4, con...     40  0.73
emb|AL050351.1| Arabidopsis thaliana DNA chromosome 4, BAC...     40  0.73
gb|AF027173.1|AF027173 Arabidopsis thaliana cellulose synt...     40  0.73
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Fig. 4. A BLAST search showing simiarities of the identified putative cellulose synthase gene with other species.

2.3 Abiotic stress assay of Paulownia

One-year old *Paulownia* plants were stressed for 24 hours at 42 ^{0}C (heat stress), 4 ^{0}C (cold stress) and 25 ^{0}C (control) in plant growth chambers. Total RNA was extracted from leaves harvested from these plants using the GenJet™ Plant RNA Extraction kit (Fermentas, USA), following manufacturer's instruction. Five hundred ng of total RNA were used to synthesize cDNAs using the Superscript III cDNA synthesis kit (Invitrogen, USA) and random primers

from the kit. cDNA synthesis was performed from RNA samples from all three conditions (heat, cold and control conditions).

2.4 Reverse transcription polymerase chain reaction (RT-PCR) of putative cellulose synthase gene from Paulownia using Paulownia gene specific primers

The 177 bp putative *Paulownia* cellulose synthase sequence (Supplementary data, Fig. 2) was used to design primers and RT-PCR was performed to confirm the transcription of this gene in *Paulownia* (Fig. 5). cDNA obtained from the control condition (25°C), as described above, was used as template for the PCR. The sequences of PCR primers (synthesized by IDT DNA, USA) are as follows:
Forward: 5'-GCA AGA TTG GTG GTT GCT TC-3'
Reverse: 5'-TTT GGA TCA ACA GCC TTT CC-3'
The PCR conditions were followed as specified in the 2X PCR Master Mix kit (Fermentas, USA) and an annealing temperature of 50 ^{0}C was used.

2.5 cDNA hybridization

The cDNA hybridization was performed as previously described [23] and the instructions supplied with the DIG High Prime DNA Labeling and Starter Kit (Roche Applied Sciences, USA). Fifteen µL of total plant cDNAs obtained from heat, cold and control conditions were spotted onto positively charged nylon membrane (Roche Applied Sciences, USA). The putative cellulose synthase cDNA of the control condition (25°C) amplified as described in the previous section was used as a probe in this experiment. The probe was PCR amplified again as described in section 2.3, the PCR product was purified using Nucleospin ® PCR and Gel Cleanup Kit (Macherey- Nagel, Germany) and labeled with digoxigenin as described in the DIG High Prime DNA Labeling and Starter Kit (Roche Applied Sciences, USA). The membrane with total cDNAs from stressed plants and 1.3 µL of labeled probe (cellulose synthase cDNA) were hybridized in a hybridization chamber at 38 ^{0}C overnight. The membrane was incubated overnight in NBT/BCIP (nitro blue tetrazolium/ 5-bromo-4-chloro-3-indolyl-phosphate) color substrate and then the blot was documented with a standard computer scanner (Fig. 6).

2.6 Nuclear DNA determination by flow cytometry

The procedure used to analyze nuclear DNA content (Table 2) in plant cells was modified from previous report [24]. Though the research reported here is on *Paulownia elongata*, nuclear DNA determination was carried out on three species that we have in our collection to get an idea of genome size diversity within the genus. Briefly, the procedure consists of preparing suspensions of intact nuclei by chopping plant tissues in $MgSO_4$ buffer mixed with DNA standards and stained with propidium iodide in a solution containing DNAse-free RNAse. Fluorescence

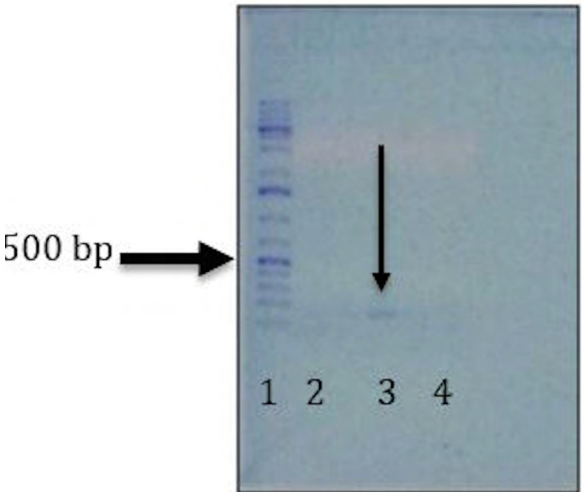

Fig. 5. RT-PCR of *Paulownia* cDNA (plant was grown at 25 ^{0}C) with *Paulownia* gene specific primers. Lane 1: GeneRuler 1 kb plus ladder (Fermentas, USA), Lane 2: Negative control (water), Lane 3: 300 ng of amplified cDNA, Lane 4: PCR failed. Image color was inverted from original color and brightness and contrast were adjusted to -40% and +40% respectively. The arrow pointing downward is showing amplified *Paulownia* cDNA.

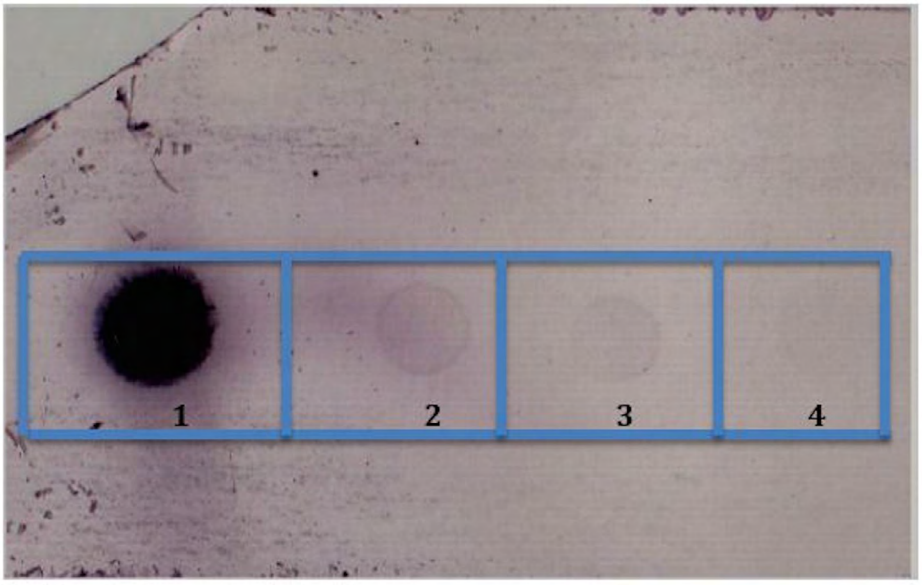

Fig. 6. cDNA dot blot analysis for gene expression using *Paulownia* cDNAs. Spot 1: Labeled probe (putative cellulose synthase PCR amplified cDNA) as positive control; Spot 2: cDNA from control plant; Spot 3: cDNA from heat stressed plant; Spot 4: cDNA from cold stressed plant. Image brightness was adjusted to -25%.

Table 2: DNA content and genome size analysis of three *Paulownia species*

Paulownia spp.	Sample ID (Rep)	DNA (pg/2C)	1C Genome Size (Mbp)
P. catalpifolia	1 (4)	1.16	567
P. elongata	2 (4)	1.17	572
P. fortunei	3 (4)	1.21	592

intensities of the stained nuclei are measured by a flow cytometer. Values for nuclear DNA content were estimated by comparing fluorescence intensities of the nuclei of the test population with those of an appropriate internal DNA standard that is included with the tissue being tested. Chicken red blood cell nuclei (2.33 pg / 2C) were used as the internal standard. Specifically for flow cytometric analysis, 50 mg of fresh leaf tissue was placed on ice in a sterile 35 x 10 mm plastic petri dish. The tissue was sliced into 0.25 mm to 1 mm segments in a solution containing 10 mM $MgSO_4.7H_2O$, 50mM KCl, 5 mM HEPES, pH 8.0, 3 mM dithiothreitol, 0.1 mg / mL propidium iodide, 1.5 mg / mL DNAse-free RNAse (Roche, Indianapolis, IN) and 0.25% Triton X-100. The suspended nuclei were withdrawn using a pipettor, filtered through 30-µm nylon mesh, and incubated at 37°C for 30 min before flow cytometric analysis. Suspensions of sample nuclei were spiked with a suspension of standard nuclei (prepared in above mentioned solution) and analyzed with a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA). For each measurement, the propidium iodide fluorescence area signals (FL2-A) from 1000 nuclei were collected and analyzed by CellQuest software (Becton-Dickinson, San Jose, CA) on a Macintosh computer. The mean position of the G0/G1 (Nuclei) peak of the sample and the internal standard were determined using the CellQuest software. The mean nuclear DNA content of each plant sample, measured in picogram (pg), was based on 1000 scanned nuclei.

2.7 Carbon sequestration estimation and biomass production

The rate of carbon sequestration depends on the growth characteristics of the tree species, climatic conditions where the tree is planted, moisture content, and the density of the tree's wood. Using the method developed by 'The Shodor Education Foundation' (http://www.shodor.org/succeedhi/succeedhi/weightree/teacher/activities.html), and http://www.broward.org/NaturalResources/ClimateChange/Documents/Calculating%20CO2%20Sequestration%20by%20Trees.pdf, rough estimate of the amount of CO_2 sequestered in a given tree can be calculated. The original formula $W= 0.25D_2H$ was used to estimate carbon sequestration in the *Paulownia* stand.

The only modification was to use moisture content at 45% instead of 27.5%. This adjustment in moisture content changes the multiplication factor of 72.5 to 55 (Table 3).

3 Results and Discussion

3.1 Genome size

Flow cytometry is a convenient and rapid method that has been used extensively for estimation of nuclear genome size in plants [24,25]. In diploid organisms, genome size is used interchangeably with the term C-value. As evident from Table 2, three *Paulownia* species exhibited the genome size as 567, 576, and 592 Mbp for *Paulownia catalpifolia*, *P. elongata*, and *P. fotunei*, respectively. A comparison of literature dealing with genome size of tree species indicates that it is on the small side, close to poplar genome size (580 Mbp; [26]).

3.2 Identification of a putative cellulose synthase gene from Paulownia using Arabidopsis gene specific primers

In this paper a putative *Paulownia* cellulose synthase gene (amplicon) was amplified by cross-species PCR (Fig. 2), cloned into the pJET TA cloning vector (Fig. 3)

Table 3. Twenty-five, three year old *Paulownia elongata* trees at random were used from 'Bioenergy Plot' to estimate carbon sequestration and biomass production potential.

Tree #	Diameter (in) (D)	Height (ft) (H)	Above ground weight (lb) (W)	Weight with roots (WR)	Dry Weight (DW)	Carbon Weight (CW) (lb)	Total CO_2 Weight (TCO_2) (lb)	Sequestered CO_2 /Year (lb)
	D	H	$W=025*D^2*H$	WR=W*1.2	DW= (WR *0.55)	CW= DW*0.5	CW* 3.6663	$TCO_2/3$
1	5.81	27.25	229.96	275.96	151.78	75.89	278.23	92.74
2	4.3	23.5	108.63	130.35	71.69	35.85	131.43	43.81
3	4.25	21.7	97.99	117.59	64.67	32.34	118.55	39.52
4	4.14	23.7	101.55	121.86	67.02	33.51	122.87	40.96
5	5.65	27.1	216.27	259.53	142.74	71.37	261.67	87.22
6	3.98	19.7	78.01	93.62	51.49	25.74	94.39	31.46
7	4.38	23.3	111.75	134.10	73.75	36.88	135.20	45.07
8	4.34	20.5	96.53	115.84	63.71	31.86	116.79	38.93
9	4.3	20.4	94.30	113.16	62.24	31.12	114.09	38.03
10	4.14	20	85.70	102.84	56.56	28.28	103.68	34.56
11	4.06	18	74.18	89.01	48.96	24.48	89.74	29.91
12	5.65	26.75	213.48	256.18	140.90	70.45	258.29	86.10
13	4.62	21	112.06	134.47	73.96	36.98	135.58	45.19
14	4.62	18.85	100.59	120.70	66.39	33.19	121.70	40.57
15	4.06	21.75	89.63	107.56	59.16	29.58	108.44	36.15
16	4.54	20.55	105.89	127.07	69.89	34.94	128.12	42.71
17	5.57	20.2	156.68	188.01	103.41	51.70	189.56	63.19
18	5.81	25.15	212.24	254.69	140.08	70.04	256.79	85.60
19	4.77	20.9	118.88	142.66	78.46	39.23	143.84	47.95
20	4.22	18.7	83.25	99.91	54.95	27.47	100.73	33.58
21	4.77	22.2	126.28	151.53	83.34	41.67	152.78	50.93
22	4.7	22.8	125.91	151.10	83.10	41.55	152.34	50.78
23	5.01	24.6	154.37	185.24	101.88	50.94	186.76	62.25
24	4.06	19.9	82.01	98.41	54.12	27.06	99.22	33.07
25	4.54	25.3	130.37	156.44	86.04	43.02	157.73	52.58
Avg	**4.65**	**22.15**	**124.26**	**149.11**	**82.01**	**41.01**	**150.34**	**50.11**

and sequenced (Supplementary data, Fig. 2). A BLAST search with this sequence showed 40-46% similarity with cellulose synthase genes from *Arabidopsis* (Fig. 4). This cellulose synthase gene expression was confirmed by RT-PCR in plants grown at 25 ^{0}C (Fig. 5) and by cDNA dot blot (Fig. 6). We designed 10 sets of primers from *Arabidopsis* and poplar cellulose synthase gene sequences (Table 1) and were able to amplify a putative cellulose synthase gene from *Paulownia* with only one set of primers from *Arabidopsis* (Table 1).

Cross-species PCR has been widely used but with moderate success [27]. However, it is a powerful tool, especially when working with a species where limited or no genomic sequence is publicly available [27]. Despite inherent difficulty in performing a cross-species PCR, it has been successfully used in a wide range of species. Cross-species PCR has been successfully used to identify microsatellites in birds [28], cetacean populations [29], and various metazoan taxa [30]. One of the approaches of designing primers for cross-species PCR is identification of conserved regions between genomes and design primers on the basis of the conserved nucleotide sequences [27]. In this case above mentioned approach could not be employed, as the *Paulownia* genome has not been sequenced yet. It is common for cross-species PCR to obtain suboptimal results and optimization may often be necessary. Success of cross-species PCR also been shown to negatively correlate with the genome size [30]. An optimization of a range of microsatellite markers identification in African Cichlids were reported earlier and the researchers scored their success rate of PCR amplifications [31]. A low success rate of microsatellite marker identification in Ranid frogs was also previously reported [28]. It has been proposed that sequence analysis should be performed before any cross-species PCR [32]. With the methodology used in this work, cross-species PCR successfully identify a putative cellulose synthase gene in the unsequenced genome of *Paulownia elongata*.

3.3 Differential expression analysis of a previously unreported putative cellulose synthase gene using cDNA dot blot

We analyzed the expression of the newly identified putative cellulose synthase gene from *Paulownia* at various environmental conditions (control, heat, and cold stresses) (Fig. 5). In aspen (*Populus tremuloides*), cellulose synthase gene expression can be tissue specific due to mechanical stress [33]. Cellulose synthase gene can be silenced by virus induced gene silencing (VIGS) in *Nicotiana benthamiana*, as previously reported [34]. Naturally occurring small interfering RNAs (siRNA) were identified in barley (*Hordeum vulgare*) and it was reported that these siRNAs can regulate cellulose synthase gene expression in various tissues and in various developmental stages [35]. Cao et al (2013) reported upregulation of cellulose synthase proteins in rice (*Oryza sativa*) following heat stress [18]. In our case we saw slight downregulation of cellulose synthase gene (Fig. 5). More research is needed to explain the reasons for slight downregulation of this putative cellulose synthase gene.

3.4 cDNA dot blot: An alternative approach to detect gene expression

We propose that cDNA dot blot is an inexpensive and quick method to study gene expression. In our method, we have extracted total RNA from plants, reverse transcribed it, and labeled putative cellulose synthase cDNA with digoxigenin. We then hybridized the labeled cDNA (probe) onto cDNAs spotted on membrane. This is a simple method and does not require any hazardous radioisotopes. Our method also does not require special treatments of glasswares to denature any residual RNAse. In that respect, cDNA dot blot is an economical alternative to traditional northern blot. A similar type of cDNA gel blot (but not the type of dot blot we reported) was previously used for gene expression studies in bilberry fruit, where high levels of RNAse were a limiting factor in performing northern blot analysis [2].

3.5 Carbon sequestration and biomass production.

This study included assessment of carbon sequestration and biomass production from 25 *Paulownia elongata* trees after their third growing season (Table 3). A three year old *Paulownia* tree was estimated to sequester, on an average, 50.11 lb CO_2 /year (29.91- 92.74 lb CO_2 /year). These studies conducted at Fort Valley State University, Fort Valley, GA, USA indicate that three-year-old trees can reach a diameter of 14.8 cm (5.81 in) and achieve height of 8.31 m (27.25 feet) (Table 3). New shoots sprout from a stump (coppicing) after harvesting and grow as much as 20 feet in the first growing season (Fig. 7).

The carbon sequestration and fast growth make *Paulownia* an ideal candidate for biofuel production. It is clear from the field studies that these trees are fast growing but there is large variability in biomass accumulated and

Fig. 7. Biomass potential of *Paulownia elongata*. A. After harvesting the tree, many new shoots sprout from the stump next spring reducing replanting cost. B. New shoots grow fast, acquiring 20 ft or more in the first growing season.

height gained (Table 3). On an average, 38 green tons/acre (13 tons/year) biomass was produced in a soil that did not receive fertilizer and irrigation. To maximize the growers' returns, selection of elite trees on the basis of desirable traits (growth, cellulose content, tolerance to abiotic stresses, insect and pest resistance, and other uses) is imperative and can be achieved with the help of molecular biology tools by understanding genetic make up.

As ethanol production through conversion of biomass becomes commercially viable in the future, growing short rotation woody crops (SRWC) for substituting fossil fuels may become a cost-effective strategy to combat climate change [36]. Another advantage of carbon sequestered through afforestation projects may help earn carbon credits to meet carbon reduction targets as outlined in Kyoto protocol. Eventually a sustainable forest management strategy aimed at maintaining or increasing forest carbon stocks, while producing sustained yield of timber, fiber or energy, will generate the largest sustained mitigation benefit.

4 Conclusion

To the best of our knowledge this is the first report on the analysis of a putative cellulose synthase cDNA from *Paulownia elongata*. The sequence identified may not be a full-length gene, but the goal was to identify a putative cellulose synthase gene in *Paulownia* and we consider our approach as a 'proof of concept'. We studied the expression pattern of the cellulose synthase gene using an inexpensive and fast cDNA dot blot method, and found that cold and, to a lesser extent, heat stress downregulated its expression. Our future goal is the construction and analysis of a cDNA library from *Paulownia* and identification of other economically important genes by cDNA library screening.

Preliminary study to assess the biomass generation potential of *Paulownia* indicates a huge range (22.93 – 71 tons/acre) at 8 ft x 8 ft spacing in the Middle Georgia conditions. This range indicates possibilities to improve *Paulownia* as a bioenergy crop by identifying better performing trees (growth, height, trunk circumference) and develop cloning protocols using plant tissue culture techniques. Secondly, as these numbers have been obtained from trees that were devoid of fertilizer application and irrigation, there is room to further enhance biomass yield by adding proper management practices (fertilizer, irrigation, spacing, cover crop etc.).

We are suggesting *Paulownia* as a short rotation tree crop for sustainable bioenergy production in Southern USA that has proven attributes to qualify as a 'multipurpose tree' [37]. This can assist in improving rural economy tremendously and provide a good source of timber, biomass, honey, leaf with high protein content for fodder, and wood pellets for heating.

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