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Transcriptome profiling the basal region of poplar stems during the early gravitropic response

W. AZRI[1]*, A. ENNAJAH[2], Z. NASR[2], S.-Y. WOO[3], and A. KHALDI[2]

Laboratoire de Biologie et Physiologie Végétales, Département de Biologie, Faculté des Sciences de Tunis, Campus Universitaire, 1060, Tunis, Tunisia[1]
Institut National de Recherches en Génie Rural, Eaux et Forêts, Ariana 2080, Tunisia[2]
College of Natural Sciences, University of Seoul, Dongdaemungu, Seoul 130-743, Korea[3]

Abstract

Stems of angiosperm trees are affected by gravity. Young poplar stems show primary growth in the top internodes and differential cambium activity in the basal internodes after inclination with some tension wood formed after 45 min. This study was conducted in order to characterize the early changes in poplar gene expression during the early stages of the gravitropic response. Using microarray technology, the expression of 15 000 genes was examined. Approximately 3.1 % of these genes exhibited significant expression changes within the first 45 min of gravity stimulation. Approximately 73 % of the identified genes were found to have a known or predicted molecular function. Many of these genes were involved in secondary wall formation (cellulose synthesis and lignification), cytoskeleton development, signaling, and stress response. Some identified genes seem to play a role in sensing gravitational signals during tension wood formation. A large number of the identified *arabinogalactan* (*AGP*) and transcription factor genes were involved in cell wall biosynthesis suggesting that these genes are particularly active in the first phases of signal transduction during tension wood formation.

Additional key words: arabinogalactan, microarrays, *Populus tremula* × *Populus alba*, tension wood, transcription factors.

Introduction

For their survival, all plants must perceive and respond to many different environmental stimuli, and gravity is one of them. The growth of plants and plant organs in response to gravity is known as gravitropism (Kiss 2000). Two different plant mechanisms enabling axes curvature have been reported: *1*) the first axis exists in zones of primary growth, where the gravitropic curvature results from differential cell elongation on opposite sides of the displaced organ; *2*) the second axis exists in secondary growth zones of complete elongation, where the gravitropic response includes the formation of tension wood in angiosperms and compression wood in gymnosperms (Coutand *et al*. 2007).

The process of gravitropism can be divided into three steps: sensing, transduction, and differential growth response (Haswell 2003). Analyses of mutants suggest that the endodermis is essential for shoot gravitropism and is most likely the site of gravity sensing in the shoot (Fukaki *et al*. 1998). The results of studies with herbaceous plants provide evidence that the perception of gravity in younger stems is mediated by sedimentable amyloplasts located in the endodermal starch sheath cell along the stem axis (Nakamura *et al*. 2012) and thus support the starch-statolith theory for gravity perception (reviewed in Sack 1997). Within the sensing cells, a gravitational signal perceived through the relocalization of amyloplasts is converted into biochemical signals and transmitted to adjacent cells, leading to the observed alteration in growth of the targeted organ (Blancaflor *et al*. 1998).

Though it is clear that gravitational force has a crucial influence on higher plant growth and development, little

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Abbreviations: AGP - arabinogalactan; CTAB - cetyltrimethylammonium bromide; L.R. - London resin.
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* Corresponding author; fax: (+216) 97 243 698, e-mail: azwassim@yahoo.fr

is known about the molecular mechanisms underlying gravity perception and early signaling. Such information is particularly lacking for woody species since the majority of the prior molecular studies have been conducted on herbaceous plants. One explanation for this gap in the literature could be that the process of straightening is particularly complex in trees involving both primary and secondary growth.

Although genetic approaches have been successful at identifying gravity signal transducers, they have limitations due to the functional redundancy associated with frequent gene duplications as well as pleiotropy, both of which mask function in gravitropism (Harrison *et al*. 2008). Thus, transcriptomic and proteomic techniques have been used to identify genes or proteins whose expression varies early in response to gravi-stimulation. Using cDNA array technology, the transcripts of 231 genes preferentially expressed in the xylem of artificially bent *Eucalyptus* trees were profiled for 6 h to 1 week in a tension time course (Paux *et al*. 2005). Some genes encoding enzymes from the lignin biosynthesis pathway appeared down-regulated during tension wood formation in poplar trees (Pilate *et al*. 2004). Moseyko *et al*. (2002) reported an 8 300 gene microarray analysis of *Arabidopsis* seedlings submitted to 30 min of gravistimulation. The whole-genome *Affymetrix ATH1* microarray was used by Kimbrough *et al*. (2004) to analyze gene expression in primary root tips during the first hour of gravity exposure and/or mechano-stimulation. The majority of the gravity-regulated genes were reported to belong to well-known categories of important genes involved in transcription, metabolism, protein fate, signal transduction, defense, and stress. The review by Harrison *et al*. (2008) of these two studies reported that only three genes were found to be regulated by gravity and/or touch (Moseyko *et al*. 2002, Kimbrough *et al*. 2004). The first proteomic analysis on gravitropism was conducted on *Arabidopsis* roots (Kamada *et al*. 2005).

The precise transcript arrays and proteomic analyses of tension wood *versus* normal wood have been well reported by different research teams interested in understanding wood formation (Lafarguette *et al*. 2004, Pilate *et al*. 2004, Paux *et al*. 2005). In the present paper, we undertook a global approach to analyze the modifications of gene expression in chosen internodes as a parallel to studies classically performed in herbaceous species.

Materials and methods

Plants and culture conditions: Hybrid poplar (*Populus tremula* × *Populus alba*), clone INRA No. 717-1-B4, was multiplied clonally *in vitro* on Murashige and Skoog (1962; MS) medium. When poplar plantlets were approximately 4 cm in height, they were gradually acclimatized in hydroponic solution (Herbette *et al*. 2004) in a growth chamber with a 16-h photoperiod, photon flux density of 60 µmol m^{-2} s^{-1}, day/night temperatures of 22/18 °C, and relative humidity of 70 ± 10 %. At the 14 internodes stage, poplars were transferred to a new device where they were attached a few centimeters above the collar, and some basal leaves were removed. This device allowed the inclination of the stem with 20 developed internodes. The mineral solution and the environmental conditions of the growth chamber were not modified.

Stress treatment: After being kept for one week in straight position, the poplars were inclined at 35° from the vertical axis, as previously described by Azri *et al*. (2009). This angle caused a gravitropic response in the stems without the initiation of secondary branching (data not shown). The plants were inclined for 1 h before the end of the ptotoperiod, and the harvest was performed under green radiation. At the end of the week, the each plant had 26 developed internodes. Starting from the base, the internodes 1 - 5 (zone of preponderant secondary growth) and 13 - 20 (zone of preponderant primary growth) were harvested from the inclined and non-inclined plants, frozen in liquid nitrogen, and stored at -80 °C.

Histochemical analysis: Stem portions were fixed in solution containing 3.7 % (v/v) formaldehyde, 5 % (v/v) acetic acid, and 50 % (v/v) ethanol at 4 °C for 4 h. The samples were then dehydrated and gradually infiltrated with medium grade London resin (L.R.) The samples were treated with solutions of white resin (*Sigma*, St. Louis, USA) and ethanol in different ratios (25:75, 50:50, and 75:25, v/v) L.R for 1 h each. Infiltration was completed in 100 % L.R, and then the plants were treated with white resin at 4 °C overnight. The samples were then placed in gelatin-embedding capsules, filled with fresh resin, and heat polymerized at 55 °C for at least 15 h before semi-thin sectioning. In order to visualize the reaction wood generated by stem inclination, semi-thin sections were stained with astra-blue (visualizing cellulose) and safranine (visualizing lignin) (both from *Sigma Aldrich*). The stained sections were dried, mounted in *Eukitt*, and examined under a *Axioplan 2* (*Zeiss*, Jena, Germany) microscope. Data were recorded using a digital camera (*AxioCam HR*, *Zeiss*) with *AxioVision* imaging software.

mRNA isolation and cDNA synthesis: The frozen stems were ground to fine powder in liquid nitrogen. Total RNA was extracted from 200 mg of the stems using cetyltrimethylammonium bromide (CTAB) extraction

buffer as described by Chang *et al*. (2003) and then treated with RNase-free *RQ1* DNase (*Promega*, Charbonnières les Bains, France). RNA was quantified by a spectrophotometer *ND-1000 v 3.3* (*NanoDrop*, Wilmington, DE, USA), and the quality was verified using 1.2 % (m/v) agarose gel electrophoresis. For first-strand cDNA synthesis, 1 µg of the total RNAs was reverse-transcribed using *Oligo(dT)* and *SuperScript III* (*Invitrogen*, Groningen, The Netherlands) according to the protocol recommended by the manufacturer.

RNA amplification for hybridization: RNA amplification was performed using the *Amino Allyl Message-Amp II* aRNA kit (*Ambion*, Austin, TX, USA**)** which was used and validated by Lafarguette *et al*. (2004). Total RNA (1 µg) from each sample was reverse-transcribed at 42 °C for 2 h using 1 mm^3 of *T7 Oligo(dT)*, 2 mm^3 of first strand buffer (10×), 4 mm^3 of dNTP mix, 1 mm^3 of RNase inhibitor, and 1 mm^3 of *ArrayScript*. The second strand was synthesized using 10 mm^3 of second strand buffer (10×), 4 mm^3 of dNTP mix, 2 mm^3 of DNA polymerase, 1 mm^3 of RNase H, and 63 mm^3 of nuclease-free water at 16 °C for 2 h. Double-stranded cDNA products were purified using the *Amino Allyl Message-Amp II* aRNA kit according to the manufacturer's protocol. *In vitro* transcription of each sample was realized using 3 mm^3 of UTP (50 mM), 12 mm^3 of ATP, CTP, and GTP mix (25 mM), 3 mm^3 of UTP (50 mM), 4 mm^3 of reaction buffer (10×), and 4 mm^3 of *T7* enzyme polymerase mix. The reaction proceeded at 37 °C for 16 h and was terminated using 60 mm^3 of nuclease-free water. Amplified RNA (aRNA) was purified using the *Amino Allyl Message-Amp II* aRNA kit and quantified by the spectrophotometer *Nano-Drop v 3.3*.

Dye coupling and labeled aRNA for hybridization: aRNA coupling to Cy3 and Cy5 dyes was performed according to the recommendations of the manufacturer (*CyDy* post labeling reactive dye kit; *Amersham Biosciences*, Orsay, France). Amplified RNA (10 µg) was incubated with 11 mm^3 of *Cy3* or *Cy5* at room temperature in the dark for 30 min. Next, 4.5 mm^3 of 4 M hydroxylamine was added and incubated in the dark at room temperature for 15 min, and then 5.5 mm^3 of nuclease-free water was added to a volume of 30 mm^3. Labeled RNA was purified according to the recommendation of the manufacturer and quantified by the *Nano-Drop v 3.3* spectrophotometer. The incorporation rates of the dyes were evaluated by measurements of absorbance at 550 and 650 nm for *Cy3* and *Cy5*, respectively, in order to estimate the quantity of labeled aRNA.

Hybridizations and microarray analyses: The *PICME* (http://www.picme.at) *Poplar 30K* array Series 2 (GEO accession GPL6038) was used. The entire procedure (labeling, hybridization, data acquisition) was carried out as previously described by Lafarguette *et al*. (2004).

Hybridizations were performed to compare gravi-stimulated poplar stems after 45 min of inclination and untreated control stems. Two biological replicates were carried out, with two hybridizations with a dye swap for each tree pair, resulting in a total of four hybridizations.

The labeled RNA with dyes *Cy3* and *Cy5* was denatured at 95 °C for 1 min and collected in a 0.06 cm^3 hybridization buffer containing 25 % (m/v) formamide, 5× SCC, and 0.1 % (m/v) sodium dodecylsulphate (SDS). RNA was then incubated at 65 °C for 2 min. The samples were loaded on the slides, placed in a hybridization chamber, and incubated in a water bath at 42 °C for 18 h. After hybridization, the slides were washed in 1× SSC and 0.2 % SDS for 10 min, then in 0.1× SSC and 0.2 % SDS for 10 min, and finally in 0.1× SSC for 10 min. All washing steps were carried out at 55 °C. The slides were dried by centrifuging at 800 *g* for 10 s prior to scanning.

The array slides were scanned by a *GenePix 4000B* microarray reader (*Axon Instrument*, *Molecular Devices Corporation*, Union City, CA, USA). Spot intensities were quantified using *GenePix Pro 5.0* (http:// www.axon.com/gn_GenePixSoftware.html). Spots were excluded from the analysis if the signal-to-noise ratio of SNR 635 and SNR 532 was greater than three, the F 635 sat. and F 532 sat. were greater or equal to two, and the spots were flagged as "bad" (flags = -100). After the quantification of the signal intensities, the data were normalized to compensate for the nonlinearity of the intensity distributions using the normalization method (Yang *et al*. 2002). A "lowess" normalization was applied. The determination of differentially expressed genes was carried out using the *Anapuce* package developed at the *URGV* by Aubert *et al*. (2004), which is available at the following website: (http://www. inapg.inra.fr/ens_rech/mathinfo/recherche/mathematique/ outil.html). The raw *P* values were adjusted by the Bonferroni method. Only genes showing significant transcript level changes in both biological replicates were considered (significance threshold 0.05).

Real-time quantitative PCR: The real-time quantitative PCR amplifications were conducted according to Mai *et al*. (2009) with the specific primers (Table 1). The oligonucleotide primers were constructed based on a *Populus* database [http://genome.jgi-psf.org] with expressed sequence tags (ESTs) detected by the *BLAST* program. The reference genes 18S RNA and ubiquitin transcripts (POPTR_0012s01250, *Phytozome* http://www. phytozome. net) were amplified using the primers 18S and Ubi. The reference genes were combined into an index using the *BestKeeper* software tool (http:// www.wzw.tum.de/gene-quantification/bestkeeper.html) (Pfaffl *et al*. 2004). Target gene abundance was conventionally normalized using this *BestKeeper* index (I) and the delta-delta method (McMaugh *et al*. 2003).

Table 1. Specific primers used for real-time quantitative PCR.

Gene	Primer forward	Primer reverse	Length [bp]
PopFLA4	5'-CTTTGCTCAGACGTCACCAG-3'	3'-CACCAGAGCTTGAATCATTA-5'	255
PopFLA5	5'-CTGGAACCGTATACACAGAC-3'	3'-ATATCCACAGAAGCTGCAGG-5'	181
PopFLA7	5'-CTCGCCGCCCGTTATGGTTC-3'	3'-ACCAACTCAATCTTCTGATG-5'	256
PopFLA15	5'-AAGGTGGTCAGTTCAGCGTT-3'	3'-ATTCACAGAGTTTCCTGTGG-5'	320
18S	5'-CTTCGGGATCGGAGTAATGA-3'	5'-GCGGAGTCCTAGAAGCAACA-3'	
Ubi	5'-CCCGGCTCTAACCATATCCA-3'	5'-GGG TCCAGCTTCTTGCAGTC-3'	

Results

In order to characterize the plant material used for the transcriptomic studies, microscopy was used to analyze the stem sections from the internode 4 (basal part) and the internode 15 of the stems inclined for 45 min. Dual staining with safranine and astra blue show tension wood in the basal region of the stems inclined for 45 min. This tension wood was limited to the upper face of the stem (Fig. 1*B*). On the lower face, the opposite wood fibers were produced at a slower rate, causing a pith eccentricity. No tension wood was observed in the top internodes even when the stem was inclined for one week (data not shown).

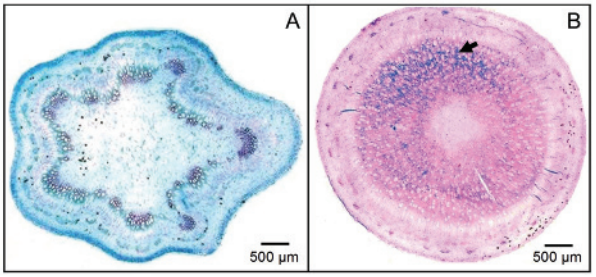

Fig. 1. Localization of tension wood in young poplar stems by astra-blue + safranine double staining. Cross-sections of stems inclined for 45 min were collected from the top (*A*) and basal (*B*) internodes. The *arrow* indicates the tension wood on the upper face of the stem.

Transcriptomic analysis of the stems inclined for 45 min were performed in the basal internodes showing differential cambium activity with some tension wood formed after the inclination (Fig. 1*B*). Comparison of the transcripts present in the control and in the gravistimulation experiments revealed that 474 of the 15 000 genes (approximately 3.1 %) represented on the array exhibited significant expression changes after the inclination of the plantsby 35 ° for 45 min (raw datasets of microarray gene expression published in *GEO* and can be viewed at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE44981). Among these 474 genes, 240 were up-regulated and 234 were down-regulated. We found that many more transcripts of genes with unknown functions were down-regulated (71) rather than up-regulated (56). Genes regulated by gravistimulation were classified based on functional categories using the annotation "Gene Ontology" for *Populus trichocarpa* (http://genome.jgi-psf.org/annotator/servlet/jgi.annotation). The differentially expressed genes identified are listed in the supplementary Table 1). These genes with differential expression suggest that early tension wood formation in the stem transcriptome was affected by gravitational stimulation. The gravitropic response was accompanied by a greater accumulation of transcripts involved in transcription factors, signaling, the cell wall, the cytoskeleton, water transport, stress response, and lignification, suggesting that these transcripts might play roles during tension wood formation.

All of these functional groups contained genes with different ratios of increased and decreased transcript amounts (Fig. 2). Overall, the ratio of up-regulated *versus* down-regulated genes in a given category of biological function could indicate its relative activation and contribution to the biological response. For the transcriptional activities associated with the gravitropic response, the data implied the up-regulation of genes involved in lignification (*phenylalanine ammonia-lyase, S-adenosylmethionine decarboxylase*, *caffeic acid 3-O-methyltransferase*, and *glyceraldehyde 3-phosphate dehydrogenase)*, cellulose synthesis and microfibril orientation (*tubulin*), energy (H^+*-ATP synthase* and *F0F1 ATP synthase*), sugar metabolism (*dTDP-glucose 4-6 dehydratase*, *UDP-glucose 4-epimerase*, *glycine hydroxymethyltransferase*, *phosphoglucomutase*, and *glycoside hydrolase*), water transport (*aquaporin*), stress response (*annexin* and *14-3-3 protein*), and transcription factors (*eIF-1/SUI1*, *eIF4-gamma/eIF5/eIF2-epsilon*, *elongation factor 1 beta/delta chain, elongation factor 1-alpha*). At the same time, there was a down-regulation of genes associated with the cell wall formation (*arabinogalactan*, *glycosyl transferase*), stress response (*dehydrin*), the cytoskeleton (*profilin*), transport (*Ran-binding protein RANBP1*), and other transcription factors (*eIF-5A*, *Zn-finger protein, elongation factor G,*

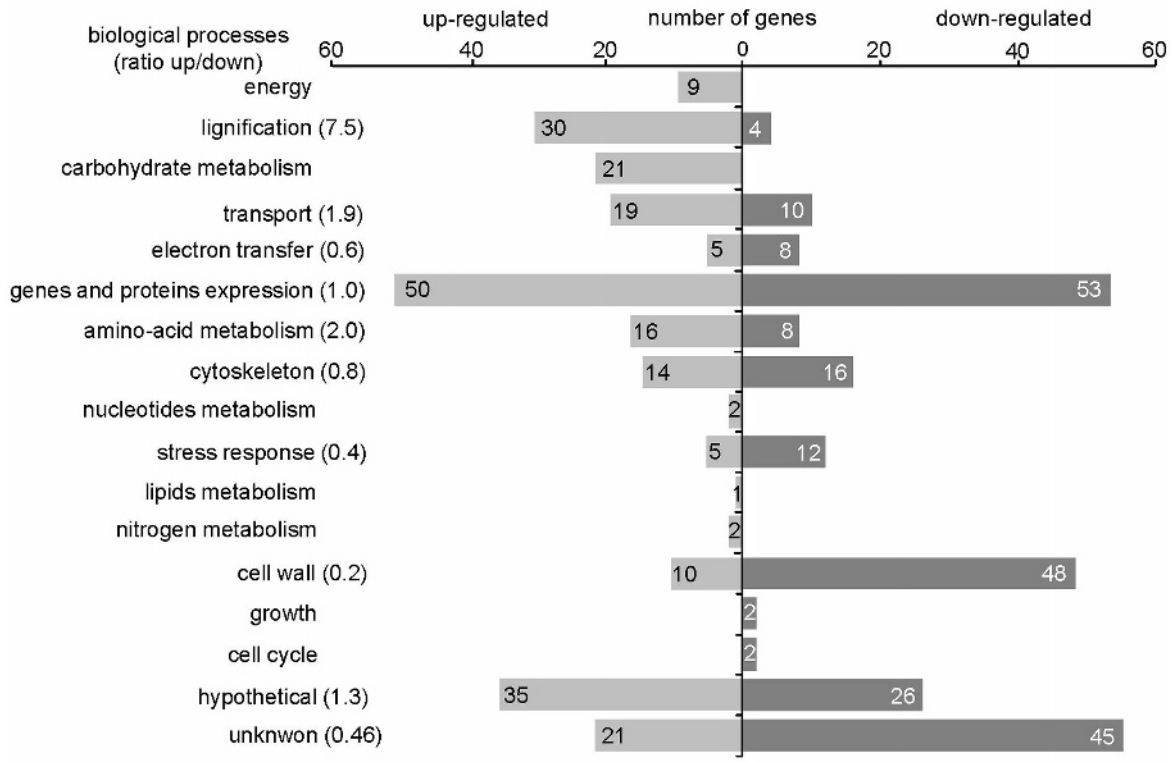

Fig. 2. Comparison of genes according to functional classification regulated by gravitational stimulation.

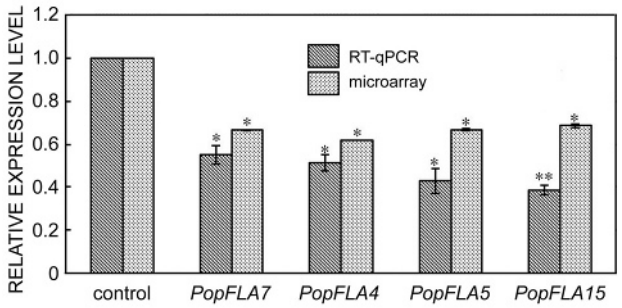

Fig. 3. Relative expression levels of the *PopFLA7*, *PopFLA4*, *PopFLA5,* and *PopFLA15* measured by real-time quantitative PCR and by microarray in basal internodes of plants inclined for 45 min compared to non-inclined controls. Values that significantly differ between the inclined and control samples are indicated with (*) ($P<0.05$) or with (**) ($P<0.01$).

LIM TF family, and *C2H2 TF family*). A larger number (48) of differentially-expressed candidates belonging to *arabinogalactan* genes expressed in the cell wall were identified. Microarray data for four of them (*PopFLA4*, *5*, *7*, and *15*) were validated by quantitative RT-PCR (Fig. 3), suggesting the reliability of the cDNA array results. These results suggest that the *arabinogalactan* genes were particularly active during tension wood formation.

Genes associated with auxin (*AUX-IAA*), water transport (*aquaporin*), heat shock (*Hsp70*), cold resistance (*serine/theronine protein kinase*), guanine nucleotide binding protein (*G-protein*), and ABA signaling (*14-3-3 protein*) were also identified. These genes might be involved in the early stages of the gravitropic response. Tension wood formation was accompanied by a greater accumulation of these transcripts (Andersson-Gunneras *et al*. 2006).

Discussion

During the upright reorientation of trees, tension wood is established through a coordinated expression of a number of genes involved in different functional categories. Genes transcribed in the early stages of the gravitropic response were mostly associated with transcription factors, the cell wall, cytoskeleton development, signaling, water transport, and stress response (Table 1 Suppl.). These classes of genes play important roles in the synthesis and development of new tracheids. Many secondary cell wall genes were up-regulated, such as the *arabinogalactan* (*AGP*) and cellulose synthase (*PttCes A2*) genes involved in secondary wall formation (Lafarguette *et al*. 2004, Samuga and Joshi 2004). These genes can contribute to the thicker walls and stronger mechanical properties of tension wood. In contrast, the current study found that *AGPs* were down-regulated (Table 2), the findings validated by quantitative RT-PCR. These genes were also identified during the first hour of

Table 2. Cell wall-related genes differentially expressed by a gravitational stimulation. Each value for the ratio and *P*-value is the average of the identified genes with the same accession number. The ratio represents relative transcript abundance between inclined and control conditions (I/C).

Accession No.	Annotation	Ratio	*P*-value
grail3.0094006801	*PopFLA5 (Beta-Ig-H3/fasciclin)*	-0.587	0.007
estExt_Genewise1_v1.C_LG_IX4802	*PopFLA8 (Beta-Ig-H3/fasciclin)*	-0.587	0.003
eugene3.00151077	*PopFLA15 (Beta-Ig-H3/fasciclin)*	-0.543	0.008
eugene3.00131208	*PopFLA6 (Beta-Ig-H3/fasciclin)*	-0.591	0.002
grail3.0031016901	*PopFLA7 (Beta-Ig-H3/fasciclin)*	-0.590	0.001
eugene3.00130132	*PopFLA4 (Beta-Ig-H3/fasciclin)*	-0.698	0.000
eugene3.00131210	*PopFLA4 (Beta-Ig-H3/fasciclin)*	-0.606	0.000
estExt_fgenesh4_pg.C_1480042	*Glycosyl transferase. family 48*	1.056	0.000
estExt_fgenesh4_pg.C_LG_I0109	*Glycosyl transferase. family 48*	0.931	0.000
gw1.V.3193.1	*Cellulose synthase*	0.831	0.000
gw1.XVIII.3152.1	*Cellulose synthase*	0.585	0.001
estExt_fgenesh4_pg.C_LG_VII0650	*Cellulose synthase*	0.592	0.000
gw1.123.86.1	*S-adenosylmethionine decarboxylase*	0.680	0.001
estExt_Genewise1_v1.C_LG_IX2359	*CAD - cinnamyl alcohol dehydrogenase*	-0.540	0.003
fgenesh4_pg.C_LG_XIX000854	*putative orcinol O-methyltransferase*	0.963	0.000
estExt_fgenesh4_pm.C_LG_XII0129	*Caffeic acid 3-O-methyltransferase 1*	0.590	0.001
estExt_fgenesh4_pm.C_LG_I1023	*Caffeoyl-CoA O-methyltransferase 2*	-0.489	0.043
grail3.0021004701	*Glutathione-dependent formaldehyde-activating*	-0.669	0.000
estExt_Genewise1_v1.C_280658	*PAL - Phenylalanine and histidine ammonia-lyase*	0.607	0.002
estExt_Genewise1_v1.C_LG_XII1463	*Glyceraldehyde 3-phosphate dehydrogenase*	0.569	0.003
eugene3.00180906	*UDP-glucose 4-epimerase/UDP-sulfoquinovose synthase*	0.555	0.002
grail3.0039009101	*Glycoside hydrolase. family 28*	0.685	0.000
eugene3.00700070	*dTDP-glucose 4-6-dehydratase/UDP-glucuronic acid decarboxylase*	0.504	0.021
gw1.IX.2621.1	*Alpha tubulin*	0.548	0.010
estExt_fgenesh4_pg.C_LG_II1020	*Alpha tubulin*	-0.939	0.000
estExt_fgenesh4_pm.C_LG_IX0458	*Beta tubulin*	0.586	0.001
grail3.0068008502	*Beta tubulin*	0.545	0.003
grail3.0013025102	*Profilin*	-0.556	0.009
grail3.0007002601	*Profilin*	-0.519	0.011
estExt_Genewise1_v1.C_LG_IX4297	*Actin depolymerizing factor*	-0.652	0.000

Table 3. Transcription factors-related genes differentially expressed by gravitational stimulation. Each value for the ratio and *P*-value is the average of the identified genes with the same accession number. The ratio represents relative transcript abundance between inclined and control conditions (I/C).

Accession No.	Annotation	Ratio	*P*-value
eugene3.00111103	*Translation initiation factor 1 (eIF-1/SUI1)*	0.598	0.007
estExt_fgenesh4_pm.C_LG_VII0088	*Translation initiation factor 1 (eIF-1/SUI1)*	0.549	0.011
estExt_fgenesh4_kg.C_870004	*eIF4-gamma/eIF5/eIF2-epsilon*	0.520	0.020
eugene3.00091463	*Elongation factor 1 beta/delta chain*	0.576	0.001
grail3.0049009101	*Elongation factor 1-alpha (EF-1-alpha)*	0.514	0.013
estExt_fgenesh4_pm.C_LG_XVIII0351	*Eukaryotic initiation factor 5A hypusine (eIF-5A)*	-0.584	0.012
gw1.70.171.1	*AUX-IAA TF family (DPTF annotation) AUX/IAA protein*	-0.533	0.005
eugene3.00070822	*Elongation factor G. domain IV*	-0.741	0.000
gw1.XVIII.1819.1	*PHD TF family (DPTF annotation)*	-0.647	0.000
grail3.0095002101	*Zn-finger. C2H2 type containing protein - IPR007087*	-0.656	0.000
estExt_Genewise1_v1.C_1520011	*Predicted Zn-finger protein*	-0.622	0.000
grail3.0001021601	*Predicted Zn-finger protein*	-0.732	0.000
estExt_fgenesh4_pg.C_LG_II1071	*LIM TF family (DPTF annotation) Regulatory protein MLP and related LIM proteins*	-0.668	0.000
estExt_Genewise1_v1.C_LG_I1393	*C2H2 TF family (DPTF annotation)*	-0.559	0.002

Table 4. Stress response and transport-related genes differentially expressed by a gravitational stimulation. Each value for the ratio and *P*-value is the average of the identified genes with the same accession number. The ratio represents relative transcript abundance between inclined and control conditions (I/C).

Accession No.	Annotation	Ratio	*P*-value
estExt_fgenesh4_pg.C_LG_V0157	*Annexin*	0.516	0.015
eugene3.01230072	*14-3-3 protein*	0.500	0.026
estExt_fgenesh4_pg.C_LG_V1612	*Dehydrin Dhn1*	-0.606	0.005
estExt_fgenesh4_pm.C_280080	*Mn2+ and Fe2+ transporters of the NRAMP family*	0.752	0.000
estExt_Genewise1_v1.C_LG_XV1399	*Xanthine/uracil transporters*	0.635	0.000
eugene3.00102165	*Aquaporin (major intrinsic protein family)*	0.604	0.004
estExt_Genewise1_v1.C_LG_I5715	*Aquaporin (major intrinsic protein family)*	0.524	0.008
estExt_Genewise1_v1.C_LG_III0271	*Aquaporin (major intrinsic protein family)*	0.494	0.033
grail3.0049017002	*Aquaporin (major intrinsic protein family)*	0.491	0.039
grail3.0038018602	*Endosomal membrane proteins. EMP70*	-0.496	0.031
eugene3.00090923	*Ran-binding protein RANBP1 and related RanBD domain proteins*	-0.580	0.002
grail3.0034007101	*Phosphatidylinositol transfer protein SEC14 and related proteins*	-0.679	0.000
eugene3.00031404	*Nuclear transport factor 2*	-0.526	0.007

gravitropic stimulation in the *Arabidopsis* root apex and show a decrease in abundance (Kimbrough *et al*. 2004). In previous studies on the responses of poplars to gravistimulation, *AGP* genes were up-regulated (Lafarguette *et al*. 2004). Differences between the observations of the current study and previous research findings with regard to the *AGPs* genes may be explained by the different application of gravistimulation and the different stages of development. In this study, the poplars were inclined 35° from the vertical axis for 45 min compared with poplars inclined 45° for three weeks in previous studies. The differences in the expression pattern of AGP proteins probably reflect different cellular functions that remain to be elucidated. Based on their predicted structure, the role of these proteins during tension wood formation may be related to adhesion properties and signaling.

Although gravity influences the formation of secondary cell walls, the regulatory mechanism of their formation influenced by gravity is not yet understood. Many secondary cell wall genes involved in the synthesis of lignin and cellulose, and controlling the orientation of cellulose microfibril deposition were up-regulated during the gravitropic response (Table 2). The expression of these genes underlining the thicker walls, lower microfibril content, higher wood density, and larger tracheids in mature wood likely reflects the adaptation of trees to increased tensile stress due to increased gravitational forces (Li *et al*. 2011). Candidate genes likely playing a specific role in cell signaling include *β-tubulins* and *α-tubulins*, transcripts which were observed to accumulate (Table 2). Tubulins have been observed to guide microfibril orientation and deposition (Baskin 2001, Paredez *et al*. 2006), and the up-regulation of a *β-tubulin* in eucalypt xylem has been observed to affect microfibrils (Spokevicius *et al*. 2007). Changes in the expression of *α-tubulin* and several other genes (*aquaporin-like and lipid transfer protein*) also correlate with variation in similar traits in the wood properties from the base to the crown of *Pinus sylvestris* (Kumar *et al*. 2009).

Some candidate genes responsive to stress or involved in signaling were identified (Table 3), including genes involved in hormone signaling (*AUX/IAA* and *14-3-3 protein*), transcription factors (*eIF-1/SUI1*, *eIF4-gamma/eIF5/eIF2-epsilon*, *eIF-5A*, and *Zn-finger protein*), heat shock (*Hsp70*), and stress responses (*annexin*, *dehydrin*) (Table 4). These signaling genes may be involved in the early events leading to wood formation. Among the genes related to hormonal signaling that were affected by gravitational stimulation, auxin response factors (*AUX/IAA*) were decreased. Several *AUX/IAA* genes that have been suggested to respond to gravitational stimuli (Andersson-Gunneras *et al*. 2006) were down-regulated during tension wood formation. Auxin is a major signal involved in the formation of the vascular system (for review see, Sachs 2000) and is considered to be a key signal for secondary xylem formation (Oh *et al*. 2003, Ko *et al*. 2004). Auxin may exert its influence *via* components of its signaling pathway, as suggested by changes in the expression of some members of the aspen *Aux/IAA* gene family during tension wood formation (Moyle *et al*. 2002, Hellgren *et al*. 2004). The perception is that the gravitational stimulus affecting tension wood formation must be transmitted by signals such as transcription factors which are likely to trigger transcriptional regulators (Andersson-Gunneras *et al*. 2006, Demura and Fukuda 2007). Recent molecular studies of various trees as well as the non-tree species *Arabidopsis thaliana* and *Zinnia elegans* have revealed coordinated gene expression during the differentiation of these cells in wood and the presence of several transcription factors that may govern the complex networks of transcriptional regulation involved in wood

formation (Demura and Fukuda 2006). Indeed, a rather large set of transcription factors are differentially regulated in developing tension wood (Andersson-Gunneras *et al*. 2006). The up-regulation of the *annexin* gene, which has been implicated in stress response, can be involved in signaling during tension wood formation. After gravitational stimulation, the annexins are redistributed all around the cell, most distinctly at the cellular periphery (Clark *et al*. 2000). Accumulation of transcripts involved in water transport (*aquaporin*) (Table 4) could affect the influx of water and likely contributed to the curvature of the organ and reaction wood formation. Expression of a plasma membrane aquaporin (*OsRWC3*) is differentially induced in the abaxial (lower) side of rice leaf sheath bases during gravitropism (Hu *et al*. 2006).

Some of the genes expressed during the early stages of the gravitropic response may be related to changes in secondary cell-wall structure and composition. They therefore represent candidate genes controlling tension wood formation.

Conclusion

Microarray analysis provides a powerful approach to identify the changing patterns of gene expression in response to gravity. The present transcriptomic profiling analysis supports the hypothesis that gravity enhances the formation of lignified secondary cell walls by changing the expression pattern of genes involved in the lignification of cell wall components. Several candidate genes identified in this study may be involved in sensing gravitational signals and cell wall biosynthesis. A much larger number of the transcription factors identified in this study, which are likely to trigger transcriptional regulators genes, are likely to play a major role in signaling and in controlling the formation of tension wood. Using inducible systems to identify the genes directly targeted by transcription factors should expand our knowledge of the transcriptional network during tension wood formation.

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