Early transcriptional response to gravistimulation in poplar without phototropic confounding factors
David Lopez, Jérôme Franchel, Jean-Stéphane Venisse, Joël Drevet, Philippe Label, Catherine Coutand, Patricia Roeckel-Drevet

To cite this version:
David Lopez, Jérôme Franchel, Jean-Stéphane Venisse, Joël Drevet, Philippe Label, et al.. Early transcriptional response to gravistimulation in poplar without phototropic confounding factors. AoB Plants, Oxford University Press 2021, 13 (1), 10.1093/aobpla/plaa071. hal-03149688

HAL Id: hal-03149688
https://hal.inrae.fr/hal-03149688
Submitted on 23 Feb 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution 4.0 International License
STUDIES

Early transcriptional response to gravistimulation in poplar without phototropic confounding factors

David Lopez1,2, Jérôme Franchel1, Jean-Stéphane Venisse3, Joël R. Drevet4, Philippe Label1, Catherine Coutand3 and Patricia Roeckel-Drevet1**

1CIRAD, UMR AGAP, F-34958 Montpellier, France, 2AGAP, Univ Montpellier, CIRAD, INRAE, Institut Agro, Montpellier, France, 3Université Clermont Auvergne, INRAE, PIAF, Campus Universitaire des Cézeaux, 1 Impasse Amélie Murat, TSA 60026, 63178 Aubière Cedex, France, 4Université Clermont Auvergne, GR6 INSERM U1103-CNRS UMR 6293, Faculté de Médecine, CRBC (Centre de Recherche Bio-Clinique), 28 Place Henri Dunant, 63000 Clermont-Ferrand, France, 5INRAE, UR 115 PSH, Centre de recherche PACA, 228, route de l’aérodrome, CS 40509, 84914 Avignon Cedex 9, France

*Corresponding author’s e-mail address: patricia.drevet@oca.fr

Form & Function. Chief Editor: Kate McCulloh

Associate Editor: Brian Atwell

Abstract

In response to gravstimulation under anisotropic light, tree stems showing an active cambium produce reaction wood that redirects the axis of the trees. Several studies have described transcriptional or proteomic models of reaction wood relative to the opposite wood. However, the mechanisms leading to the formation of reaction wood are difficult to decipher because so many environmental factors can induce various signalling pathways leading to this developmental reprogramming. Using an innovative isotropic device where the phototropic response does not interfere with gravistimulation we characterized the early molecular responses occurring in the stem of poplar after gravistimulation in an isotropic environment, without deformation of the stem. After 30 min tilting at 35° under anisotropic light, we collected the upper and lower xylems from the inclined stems. Controls were collected from vertical stems. We used a microarray approach to identify differentially expressed transcripts. High-throughput real-time PCR allowed a kinetic experiment at 0, 30, 120 and 180 min after tilting at 35°, with candidate genes. We identified 668 differentially expressed transcripts, from which we selected 153 candidates for additional Fluidigm qPCR assessment. Five candidate co-expression gene clusters have been identified after the kinetic monitoring of the expression of candidate genes. Gene ontology analyses indicate that molecular reprogramming of processes such as ‘wood cell expansion’, ‘cell wall reorganization’ and ‘programmed cell death’ occur as early as 30 min after gravistimulation. Of note is that the change in the expression of different genes involves a fine regulation of gibberellin and brassinosteroid pathways as well as flavonoid and phosphoinositide pathways. Our experimental set-up allowed the identification of genes regulated in early gravitropic response without the bias introduced by phototropic and stem bending responses.

Keywords: Cell wall; Fluidigm; gravitropism; gravity; isotropic light; phototropism; Populus alba; Populus tremula; qPCR; tension wood; transcriptome; xylem poplar.

Introduction

The perception of Earth’s gravity leads to a gravity-driven growth process called gravitropism. In higher plants, shoots grow upwards (negative gravitropism), while roots grow downwards (positive gravitropism). Gravitropism includes the perception of gravity, signal transmission and growth response. Many studies were conducted on roots in which the key gravity detection site
(columella cells at the root tip) is separated from the bending zone (extension zone) for a review of the topic, see Baldwin et al. 2013. Several plant species were also used to study the response to gravistimulation of aerial parts including maize coleoptiles, pulvini barley, Pisum hypocotyls, Arabidopsis inflorescence stems and hypocotyls (for a review, see Hashiguchi et al. 2013). In these herbaceous species, vertical root or shoot movements are caused by differential elongation between the upper and lower parts of the gravistimulated organs. In trees, two different motors allow the curvature of the stem as reorientation is obtained by differential growth in areas of primary elongation, while it is obtained by differential cellular differentiation between the two sides of the stem in areas with active cambium (for a review, see Groover 2016). Consequently, the cambium produces an asymmetric ‘reaction wood’ (Côté and Day 1964). In gymnosperms, ‘reaction wood’ is called ‘compression wood’ and develops on the underside of the inclined rods where it generates a compressive force to push the stem up (Timell 1956). In angiosperms, the ‘reaction wood’ is called ‘tension wood’ (TW) and forms on the upper face of the inclined stem when the tree begins the reorientation process (for a review, see Du and Yamamamoto 2007). The ‘opposite wood’ forms on the underside and is anatomically similar to the ‘normal wood’ formed by straight stems. Tension wood is a particular type of secondary xylem that generates high internal tension forces, mainly due to the longitudinal shrinkage of mature wood cells (Kübler 1959). Ruelle (2014) reviewed the variations in the anatomy and ultrastructure of TW compared to the ‘opposite wood’ (opposite side of the stem) and ‘normal wood’ (in a non-tilted tree). In many angiosperms, TW is characterized by the presence of gelatinous fibres (G-layer) of almost pure cellulose.

The molecular basis of the early events that followed gravistimulation and led to the formation of TW is still fragmented. The literature review showed that most studies using comprehensive approaches such as proteomics and transcriptomics have examined the formation of ‘reaction wood’ at developmental stages when it is already histologically observable and/or when the tree undergoes recovery movement (for a review, see Tocquard et al. 2014). For example, Andersson-Gunnarsson et al. (2006) pioneering work reported a complete quantification of gene expression and related metabolites occurring during TW formation in poplar. In this study, xylem samples from plants that had been tilted for 3 weeks were used, focusing the analysis on the stage of the secondary wall formation. Chen et al. (2015) characterized gene expression patterns in TW opposite and normal wood in 30-year-old Populus tremuloides. Gerthwa et al. (2015) produced genomic transcriptomes of 3-month-old Populus tremula x alba, stimulated either by gravistimulation (placed horizontally for 2 days), gibberellin acid treatment or ARBORKNOX2 expression. In the present work, we have focussed on deciphering transcriptomic events caused by stem tilting before the beginning of the righting movement. For that reason, xylem samples were collected after 30 min of tilting, before the appearance of gravitational motion and TW formation. In addition, to our knowledge, previous experimental designs did not distinguish gavisting from mechanosensing signalling pathways (Lopez et al. 2014; Groover 2016). For example, although Azzi et al. (2009, 2013) have characterized the protome of young poplars inclined at 35° for 45 min. In that particular set-up, the inclined organ induces flexion under its own weight, which can be considered as a unigomorphogenetic stimulus (Couand 2010). To avoid such situation, we limited our analyses to the part of the stem showing secondary growth. The trees were planted a week before use, so there was no bending due to the weight of the tree after tipping. Finally, and most importantly, phototropic and gravitropic responses as well as the autotropnic response, which is essentially the straightening of a curved organ, are processes in constant interaction under natural conditions that were not dissociated in the experimental situations reported so far (Basile et al. 2015).

In a recent study, we succeeded in dissociating these complex stimuli thanks to an original device that allowed us to combine isotropic lightening and tilting of young poplars (Couand et al. 2019), thus avoiding the phototropic response. In such a device, we showed that the gravitropic response was partly distinct when comparing an anisotropic light condition to an isotropic light condition (Couand et al. 2019). To better understand the molecular basis of these responses, we used here this device that allows us to distinguish between gravitropic, mechanosenstive and phototropic responses in plants in order to study the molecular adjustments due to gravitisation on stems in the early phases of ‘reaction wood’ formation.

Materials and Methods

Plant material

The study was conducted on young poplars (Populus tremula × Populus alba cv 717 184) produced by in vitro propagation (Azzi et al. 2009). They were placed in growth chambers after acclimation (photoperiod: 16 h/8 h; day: 24 °C, night: 18 °C). For transcriptome studies, plants with straight stem, aged 3 months, with a height of between 40 and 50 cm and an average diameter at half height of 10 mm were carefully and loosely staked avoiding any mechanical stress 1 week before use. The side of the stem to the stake was marked with dots using a black marker.

Isotropic light device and tilting of the plants

The spheres used for gravitisation were 150 cm wide and uniformly bright (i.e. isotropic light; Couand et al. 2019) (Fig. 1). Growing conditions were 16 h/8 h photoperiod; 24 °C (day)/18 °C (night). The temperature was maintained by a controlled cooling system (Bliss, WAP 267, E). One week after staking (staking took place in the growth chamber), the plants reached 35 cm (SD = 3.7 cm, n = 76). Staked trees were placed in the sphere (one plant per sphere). First of all, the plants were left in an upright position for 24 h. Then, 7 h after the beginning of the photoperiod, the plants were tilted 35° from the vertical. The control plants were kept straight in the sphere. When tilted, the stake was oriented beside the upper face of the stem to prevent the stem from bending due to its own weight (Fig. 2A).

Tissue sampling

After treatment, for each plant, we selected a stem segment (15 cm long, 5 cm above ground level, i.e. internodes 4–11) that did not contain any TW according to Astra-blue/SAframine staining (Azzi et al. 2009). In this part of the stem, the cambium is active and a secondary xylem is formed. Stem segments 0.5 mm long were taken from internodes 4, 7 and 11 for further microscopic checks. The rest of the stem was divided longitudinally into two parts using a scalpel (Fig. 2B). The lower (opposite the stake) and upper (towards the stake) sides of the stems were collected; the bark was peeled and discarded (Fig. 2C). Lower xylem (XL) and upper xylem (UX) were instantly frozen in liquid nitrogen and
The hybridization, scanning and analysis of the biochips were performed by the Plant Genomics Research Unit (Evry, France) according to their standard procedures. From 200 ng total RNA, amplification and labelling of cDNA with Cy3-dUTP or Cy5-dUTP (Perkin-Elmer-NEN Life Science Products) was performed with the TransFlair Complete Whole Transcriptome Amplification Kit (WT2A; Sigma-Aldrich, Inc.) as recommended by the manufacturer. The Populus microarray based on Roche-NimbleGen technology (Roche, Boulogne-Billancourt, France) was used. A single high-density Populus microarray slide contains 12 chambers, each containing 135 029 primers representing 49 474 gene models based on the annotation of the Populus trichocarpa v1.1 genome. Hybridization and washing were carried out in accordance with the recommendations of NimbleGen (Roche NimbleGen Technologies, Inc.). A two-microscan was performed with the InnoScan300 scanner (InnopsysR, Caronne, France) and the raw data were extracted with the MapxR software (InnopsyR, Caronne, France). The measured fold variation was corrected according to Benferroni, followed by a Benjamin–Hochberg P-value adjustment (Benjamini and Hochberg 2000). Biochip data from this study have been deposited at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/, access number GSE86951) and CATdb (http://urgv.evy.inra. fr/CATdb/; Project: 12plex-poplar-2012_01) according to MIAME standards.

Statistical analysis of microarray data
For each array, the raw data included the logarithm of the median pixel intensity of the characteristics at 635 nm (red) and 532 nm (green) wavelengths. For each array, global normalization according to intensity was performed using the LOESS procedure (Yang et al. 2002) to correct for dye bias. The differential analysis was based on the average of log-ratios on duplicate probes and technical replications. Therefore, the number of data available for each gene is equal to the number of biological replicates and was used to calculate the moderate t-test (Smyth 2004). Under the null hypothesis, Limma (version 3.34.8) found no evidence that specific variances varied from one probe to another and, therefore, the moderate t-statistics was assumed to follow a standard normal distribution. To control the rate of false discoveries, the corrected P-values found were calculated using the optimized FDR approach (Storey 2003). We considered the probes with an adjusted P-value < 0.05 to be differentially expressed.

The analysis was done with the R software (version 3.4.3, R Core Team 2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/). The SqueezeVar function of the Limma library was used to smooth specific variances by calculating empirical Bayesian posterior averages. The library Kaffir (version 2.0.1) was used to calculate the adjusted P-values.

Data management, statistical analysis and hierarchical grouping were performed using R. Hierarchical grouping was calculated using the hclust function (set of statistics) using Euclidean distances and the Ward method. The false discovery rate was calculated using the p.adjust function (set of statistics) using the Benjamini and Yekutieli (2001) method.

Validation of microarray results
For the qPCR validation, we selected 153 genes amongst the most differentially expressed genes from biochip analyses. Before proceeding with the qPCR analysis, the cDNA was synthesized from 0.5 μg total RNA using SuperScript III (Invitrogen, USA) with

Figure 1. Experimental device of isotropic light to disentangle gravitropic and phototropics plant responses. The isotropic light is obtained from circular neon tubes arranged in a turtle pattern at the periphery of the white plastic hemisphere. The light is concentrated towards the inside of the hemisphere by metallic plates covering the device. The poplar plant is placed in the centre of the device, and the hemispheres are tightly connected (Costand et al. 2013) for the full description of the isotropic device. (A) Straight young poplar plant. (B) Plant subjected to tilting.
a 1:1 mixture of oligo-dT and random hexamers. The primer pairs were designed using the Quant primer online service [www.quantprime.de] [see Supporting Information—Table S1]. The specificity of the primers was ensured by checking the melting curve and sequencing the PCR products on the cDNA. The primers were validated when the efficiency was close to 100% on the slopes of the standard curve. Relative quantification with respect to vertical controls was performed using the 2^(-ΔΔCt) method developed by Pfaffl (2001). The expression levels of the 153 genes obtained by qPCR and microarray analysis were compared using an analysis of variance followed by a Tukey Honest Significance Difference (HSD) post hoc test.

**Kinetics study of candidate gene expression**

The plants were either straight or inclined at 35° for 30, 60, 120 or 180 min in the isotropic device. Eight independent biological replicates (individual plants) were produced for each condition. XU and XL cDNA samples were used to monitor the expression of the 153 candidate genes by real-time qPCR at high-throughput. The experiments were performed according to the Fluidigm gene expression protocol without modification using the Biomark platform (Fluidigm, USA) and the 96x96 IFC gene expression (integrated fluidic circuit). Relative quantification was performed using the 2^(-ΔΔCt) method developed by Pfaffl (2001). Expression levels were related to the geometric mean of the 153 expression levels of the candidate genes proposed by the Bestkeeper reference research software [Vandesompele et al. 2002, Pfaffl et al. 2004], but using a larger subset of genes. No significant Tukey post hoc HSD was found in the change in gene expression (P < 0.05, data not shown) between qPCR and microarray, which validated the microarray results.

**Bioinformatics analyses**

The functional annotation was retrieved using a standalone version of the BioMart software [http://biomart.org] and the genomic annotation P. trichocarpa v3.0 [http://phytozome.jgi.doe.gov]. The singular AgriGO enrichment analysis [http://bioinfo.cau.edu.cn/agriGO/, Zhou et al. 2010] was used for the analysis of gene set enrichment (GSEA) using the genetic ontology terms (GO) annotated for genes identified on the DNA chip and the default AgriGO parameters, adapted to large data sets (Fisher test, Yekutieli multiple test adjustment method and significance level at 0.05). We evaluated the possible bias of the GO term enrichment procedure when using the Nimblegen poplar network by comparing the whole gene models, based on P. trichocarpa V1.1, present on the Nimblegene array with the P. trichocarpa V3 genome gene models. No significant representativeness bias was detected. Thus, we considered that the enrichment of the term GO based on Nimblegene data was valid when using GO annotations of the poplar genome V3. Further analyses were carried out using PopGenIE [http://popp genie.org, Sjödin et al. 2009, Sundell et al. 2013] for the analysis of qPCR clusters and the discovery of miRNA targets.
Results

Neutral discovery of early modulated genes

In the isotropic device, the phototropic response cannot occur. This device was used to identify genes regulated by gravistimulation in forming 'reaction wood' using DNA chip technology (Nimblegene microarray). Analyses of xylem samples were carried out by comparing the expression levels of vertical (control) and inclined plants in the lower part of the xylem (XL, i.e. the face facing the ground when it is inclined) and in the upper part of the xylem (XU). Array hybridizations resulted in a subset of 2355 gene models (poplar genome version 3) expressed differentially after Benjamin and Hochberg as well as Bonferroni multiple test corrections were made (P-value < 0.05; see Supporting Information—Table S2). Our experimental set-up provided well-controlled conditions, as evidenced by the reproducibility of the microarray experiments [see Supporting Information—Fig. S1].

From the list of 2355 genes, 668 showed significant differential expression between the two conditions (annotation was performed using the poplar genome version 3; see Supporting Information—Table S3). Of these, 214 and 250 genes were modulated exclusively in XU or XL, respectively, while 204 were modulated on both sides of the xylem in response to gravity (Fig. 3A). For this last set of genes, there is no tendency for 'side-specific' expression, as confirmed by the hierarchical grouping of biological repetitions showing that the XL and XU samples are mixed. A high-resolution version of the heatmaps containing the names of the gene models is available in Supporting Information—Fig. S2. As shown in the expression maps and density histogram (Figs 3A and B), the most differentially expressed genes are within a range of 2-fold variation between inclined and control trees. Figure 3C shows, a total of 379 genes were always regulated upward (133 + 121 + 125) and 262 genes (81 + 56 + 125) were always regulated downward.

Functional exploration

In order to clarify the role of modulated genes in response to gravistimulation, the OSEA was performed using GO term enrichment with the three different ontologies available (Biological Process (BP), Molecular Function (MF), Cell Component (CC)) and calculated with AgriGO. Considering the

![Image](https://example.com/image1.png)  
![Image](https://example.com/image2.png)  
![Image](https://example.com/image3.png)

Figure 3: Identification of poplar genes with expression significantly regulated in response to a 30 min, 35° gravi-stimulation in xylem tissues. (A) Heatmaps of differentially expressed genes found in Upper Xylem (XU), Lower Xylem (XL) and in both XU and XL. Gene models are hierarchically clustered on the X-axis and the four biological repeats are hierarchically clustered on the Y-axis. (B) Key of the heatmaps, fold changes are represented on the X-axis and frequencies on the Y-axis. (C) Detail by tissue and trend of the gene expression patterns. Note that 27 genes showed a significant regulation in both XU and XL but with opposite trends. However, none of these “opposite” trends were statistically significant hence, they are not accounted in the "XU and XL" column.
Table 1. GO term enrichment analysis of the 668 differentially expressed xylem genes (35° tilted vs. control).

| GO term | Ontology | Description | Number in input list | Number in BG/Ref | P-value | FDR | GO term | Ontology | Description | Number in input list | Number in BG/Ref | P-value | FDR |
|---------|----------|-------------|----------------------|------------------|---------|-----|---------|----------|-------------|----------------------|------------------|---------|-----|
| GO:0005973 P | | Carbohydrate metabolism | 71 | 2018 | 5.10E-10 | 1.50E-06 | GO:00046271 P | | Phenylpropanoid catabolic process | 5 | 37 | 0.00046 | 0.025 |
| GO:0036461 P | | Cellular nitrogen compound metabolism | 45 | 1031 | 2.40E-09 | 2.30E-06 | GO:0042180 P | | Cellular ketone metabolic process | 49 | 1886 | 0.00056 | 0.03 |
| GO:0005976 P | | Polysaccharide metabolism | 34 | 639 | 2.40E-09 | 2.30E-06 | GO:0070297 P | | Regulation of two-component signal transduction system (phosphorelay) | 5 | 40 | 0.00064 | 0.032 |
| GO:0044264 P | | Cellular polysaccharide metabolism | 26 | 457 | 5.30E-08 | 3.80E-05 | GO:0010104 P | | Regulation of ethylene-mediated signaling pathway | 5 | 40 | 0.00064 | 0.032 |
| GO:0016051 P | | Carbohydrate biosynthetic process | 31 | 641 | 9.40E-08 | 5.40E-05 | GO:0010105 P | | Negative regulation of ethylene-mediated signaling pathway | 5 | 40 | 0.00064 | 0.032 |
| GO:0006519 P | | Cellular amino acid and derivative metabolic process | 57 | 1688 | 1.10E-07 | 5.40E-05 | GO:0070298 P | | Negative regulation of two-component signal transduction system (phosphorelay) | 5 | 40 | 0.00064 | 0.032 |
| GO:0006073 P | | Cellular glucon metabolism | 20 | 298 | 1.60E-07 | 6.50E-05 | GO:0009063 P | | Cellular amino acid catabolic process | 9 | 144 | 0.00067 | 0.033 |
| GO:0044262 P | | Cellular carbohydrate metabolism | 41 | 1093 | 6.10E-07 | 0.00022 | GO:0009266 P | | Response to temperature stimulus | 35 | 1229 | 0.00077 | 0.037 |
| GO:0044042 P | | Glucan metabolic process | 20 | 329 | 7.00E-07 | 0.00022 | GO:0009310 P | | Amine catabolic process | 9 | 149 | 0.00084 | 0.04 |
| GO:0019748 P | | Secondary metabolic process | 44 | 1235 | 8.90E-07 | 0.00026 | GO:0022611 P | | Dormancy process | 5 | 43 | 0.00087 | 0.04 |
| GO:0009066 P | | Aspartate family amino acid metabolism | 13 | 145 | 1.20E-06 | 0.00032 | GO:0046395 P | | Carboxylic acid catabolic process | 12 | 253 | 0.00097 | 0.043 |
| GO:0034637 P | | Cellular carbohydrate biosynthetic process | 24 | 483 | 1.70E-06 | 0.00037 | GO:0000097 P | | Sulfur amino acid biosynthetic process | 6 | 67 | 0.00095 | 0.043 |
| GO:0007018 P | | Microtubule-based movement | 14 | 174 | 1.60E-06 | 0.00037 | GO:0016054 P | | Organic acid catabolic process | 12 | 253 | 0.00097 | 0.043 |
| GO:0009308 P | | Amine metabolic process | 41 | 1171 | 3.20E-06 | 0.00061 | GO:0009873 P | | Ethylene-mediated signaling pathway | 10 | 185 | 0.00099 | 0.043 |
| GO:0006555 P | | Methionine metabolic process | 10 | 88 | 3.00E-06 | 0.00061 | GO:0009312 P | | Oligosaccharide biosynthetic process | 7 | 95 | 0.0011 | 0.046 |
| GO:0033692 P | | Cellular polysaccharide biosynthetic process | 18 | 311 | 4.70E-06 | 0.00085 | GO:0016491 P | | Oxidoreductase activity | 97 | 3249 | 1.30E-09 | 1.30E-06 |
| GO term     | Ontology | Description                                      | Number in input list | Number in BG/Ref | P-value | FDR  | GO term     | Ontology | Description                                      | Number in input list | Number in BG/Ref | P-value | FDR  |
|-------------|----------|--------------------------------------------------|----------------------|-------------------|---------|------|-------------|----------|--------------------------------------------------|----------------------|-------------------|---------|------|
| GO:0005978  | P        | Glycogen biosynthetic process                    | 6                    | 24                | 5.80E-06 | 0.00098| GO:00005506 | F        | Iron ion binding                                 | 49                   | 1268              | 1.90E-08 | 9.30E-06 |
| GO:000271   | P        | Polysaccharide biosynthetic process              | 19                   | 354               | 7.20E-06 | 0.0012| GO:0046906 | F        | Tetrapyrrole binding                             | 34                   | 819               | 6.80E-07 | 0.00022 |
| GO:004106   | P        | Cellular amine metabolic process                | 33                   | 890               | 9.70E-06 | 0.0015| GO:0020037 | F        | Heme binding                                     | 31                   | 744               | 2.00E-06 | 0.00047 |
| GO:0006575  | P        | Cellular amine acid derivative metabolic process | 33                   | 908               | 1.40E-05 | 0.0021| GO:0016638 | F        | Oxidoreductase activity, acting on the CH-NH₂ group of donors | 10                   | 94                | 5.20E-06 | 0.001 |
| GO:0042221  | P        | Response to chemical stimulus                   | 114                  | 4909              | 1.50E-05 | 0.0021| GO:0009055 | F        | Electron carrier activity                        | 43                   | 1346              | 1.60E-05 | 0.0026 |
| GO:0042546  | P        | Cell wall biogenesis                             | 17                   | 313               | 1.90E-05 | 0.0025| GO:0004091 | F        | Carboxylesterase activity                        | 24                   | 564               | 2.00E-05 | 0.0028 |
| GO:0009409  | P        | Response to cold                                 | 31                   | 855               | 2.70E-05 | 0.0034| GO:0004553 | F        | Hydrolyase activity, hydrolyzing O-glycosyl compounds | 29                   | 773               | 2.70E-05 | 0.0033 |
| GO:0009415  | P        | Response to water                                | 24                   | 584               | 3.40E-05 | 0.0004| GO:0016758 | F        | Transferase activity, transferring hexosyl groups | 27                   | 707               | 3.80E-05 | 0.0037 |
| GO:0008577  | P        | Glycogen metabolic process                       | 7                    | 52                | 3.50E-05 | 0.0004| GO:0046914 | F        | Transition metal ion binding                     | 101                  | 4311              | 3.80E-05 | 0.0037 |
| GO:0006112  | P        | Energy reserve metabolic process                 | 7                    | 54                | 4.40E-05 | 0.0006| GO:0004497 | F        | Monoxygenase activity                             | 25                   | 638               | 5.00E-05 | 0.004 |
| GO:0000906  | P        | Sulfur amino acid metabolic process              | 10                   | 122               | 4.20E-05 | 0.0006| GO:0019825 | F        | Oxygen binding                                   | 19                   | 409               | 4.80E-05 | 0.004 |
| GO:0009067  | P        | Aspartate family amino acid biosynthetic process | 8                    | 75                | 4.50E-05 | 0.0006| GO:0008509 | F        | Anion transmembrane transporter activity          | 14                   | 251               | 7.70E-05 | 0.0057 |
| GO:0009698  | P        | Phenylpropanoid metabolic process                | 25                   | 639               | 5.10E-05 | 0.0005| GO:0016762 | F        | Xyloglucan-xylglucosyl transferase activity       | 6                    | 44                | 0.00012 | 0.0082 |
| GO:0006520  | P        | Cellular amino acid metabolic process            | 31                   | 890               | 5.60E-05 | 0.0053| GO:0016757 | F        | Transferase activity, transferring glycosyl groups | 31                   | 954               | 0.00019 | 0.011 |
| GO:0009250  | P        | Glucan biosynthetic process                      | 12                   | 184               | 6.20E-05 | 0.0057| GO:0016798 | F        | Hydrolyase activity, acting on glycosyl bonds    | 29                   | 864               | 0.00018 | 0.011 |
| GO:0010871  | P        | Regulation of hormone levels                     | 18                   | 387               | 7.40E-05 | 0.0065| GO:0003824 | F        | Catalytic activity                                | 344                  | 19164             | 0.00023 | 0.013 |
| GO:0019439  | P        | Aromatic compound catabolic process              | 7                    | 59                | 7.30E-05 | 0.0065| GO:0005200 | F        | Structural constituent of cytoskeleton           | 7                    | 73                | 0.00025 | 0.013 |
| GO:0009832  | P        | Plant-type cell wall biogenesis                  | 14                   | 252               | 8.00E-05 | 0.0068| GO:0015549 | F        | Hexose transmembrane transporter activity         | 6                    | 59                | 0.00051 | 0.026 |
| GO term   | Ontology | Description                                      | Number in input list | Number in Bg/Ref | P-value | FDR | GO term   | Ontology | Description                                      | Number in input list | Number in Bg/Ref | P-value | FDR |
|-----------|----------|--------------------------------------------------|----------------------|------------------|---------|-----|-----------|----------|--------------------------------------------------|----------------------|------------------|---------|-----|
| GO:0009719 P | Response to endogenous stimulus                  | 69                   | 2734               | 9.60E-05         | 0.0079  |     | GO:0022891 F | Substrate-specific transmembrane transporter activity | 48                   | 1842             | 0.0006  | 0.027 |
| GO:0009834 P | Secondary cell wall biogenesis                    | 9                    | 112                | 0.00012          | 0.0093  |     | GO:0008471 F | Laccase activity                              | 5                    | 39               | 0.00058 | 0.027 |
| GO:0009414 P | Response to water deprivation                     | 22                   | 550                | 0.00013          | 0.01    |     | GO:0016641 F | Oxidoreductase activity, acting on the CH-NH₃⁺ / group of donors, oxygen as acceptor | 6                    | 63               | 0.00071 | 0.031 |
| GO:0042445 P | Hormone metabolic process                         | 13                   | 232                | 0.00013          | 0.01    |     | GO:0043169 F | Cation binding                                 | 132                  | 6449             | 0.0008  | 0.033 |
| GO:0051258 P | Protein polymerization                            | 10                   | 147                | 0.00018          | 0.013   |     | GO:0043167 F | Ion binding                                     | 132                  | 6472             | 0.00091 | 0.037 |
| GO:0009086 P | Methionine biosynthetic process                   | 5                    | 30                 | 0.00019          | 0.014   |     | GO:0015145 F | Monosaccharide transmembrane transporter activity | 6                    | 67               | 0.00095 | 0.037 |
| GO:0009628 P | Response to abiotic stimulus                      | 82                   | 3504               | 0.00023          | 0.016   |     | GO:0016705 F | Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen | 16                   | 415              | 0.0013  | 0.047 |
| GO:0010114 P | Response to red light                             | 10                   | 154                | 0.00026          | 0.017   |     | GO:0012505 C | Endomembrane system                             | 98                   | 3339             | 2.50E-09 | 1.10E-06 |
| GO:0010876 P | Lipid localization                               | 5                    | 32                 | 0.00025          | 0.017   |     | GO:0009501 C | Amyloplast                                      | 7                    | 25               | 5.00E-07 | 0.00011 |
| GO:0019252 P | Starch biosynthetic process                       | 5                    | 35                 | 0.00037          | 0.022   |     | GO:0045298 C | Tubulin complex                                 | 7                    | 33               | 2.50E-06 | 0.00035 |
| GO:0009741 P | Response to brassinosteroid stimulus             | 13                   | 258                | 0.00035          | 0.022   |     | GO:0005618 C | Cell wall                                       | 37                   | 1051             | 8.60E-06 | 0.00082 |
| GO:0043436 P | Oxocarboxylic acid metabolic process              | 49                   | 1849               | 0.00037          | 0.022   |     | GO:0031225 C | Anchored to membrane                            | 23                   | 501              | 9.50E-06 | 0.00082 |
| GO:0019752 P | Carboxylic acid metabolic process                | 49                   | 1849               | 0.00037          | 0.022   |     | GO:0048046 C | Aoplast                                         | 15                   | 285              | 8.00E-05 | 0.0057 |
| GO:0006082 P | Organic acid metabolic process                    | 49                   | 1852               | 0.00038          | 0.023   |     | GO:0030312 C | External encapsulating structure                | 37                   | 1207             | 0.00014 | 0.0076 |
| GO:0046274 P | Lignin catabolic process                          | 5                    | 36                 | 0.00041          | 0.032   |     | GO:0009505 C | Plant-type cell wall                            | 30                   | 896              | 0.00014 | 0.0076 |
| GO:0010033 P | Response to organic substance                    | 76                   | 3252               | 0.00042          | 0.024   |     | GO:0031224 C | Intrinsic to membrane                           | 96                   | 4289             | 0.0003 | 0.014 |
| GO:0009725 P | Response to hormone stimulus                     | 62                   | 2522               | 0.00043          | 0.024   |     | GO:0009705 C | Plant-type vacuole membrane                     | 8                    | 113              | 0.00062 | 0.024 |
| GO:0050896 P | Response to stimulus                              | 192                  | 9863               | 0.00045          | 0.025   |     | GO:0005576 C | Extracellular region                            | 32                   | 1072             | 0.00061 | 0.024 |

Ontology "P" indicates Biological Process, ontology "F" indicates Molecular Function, and ontology "C" indicates Cellular Component. "Number in input list" is the number of occurrence of the Gene Ontology term in the input list (948 genes). "Number in Bg/Ref" is the observed occurrence of the Gene Ontology term in the Populus trichocarpa genome. P-value represents the statistical significance of the enrichment test and FDR is the false discovery rate set to 0.05.
668 genes significantly differentially expressed for GSEA, 104 GO terms had a significantly different frequency of occurrence and a wide range of BPs were affected (Table 1; see Supporting Information—Fig. S3). Among the 67 significant BP terms brought forward, ‘hormone (ethylene and brassinosteroids) level response and regulation’, ‘cell wall biogenesis’, ‘amino acid metabolism’, ‘phenylpropanoid metabolism’, ‘lignin catabolic process’, ‘carbohydrate metabolism’ and ‘microtubule-based movements’ were the most prominent. For the MF terms, ‘ion binding’ was the most over-represented term, followed by ‘oxidoreductase activity’ and ‘transmembrane transport’. Several CC terms of interest were affected including ‘membrane and endomembrane systems’, ‘cell wall’, ‘apoplast’, ‘amyloplast’, ‘vacuole’ and ‘tubulin complex’ [see Supporting Information—Fig. S3].

Table 2 and 3 list the significant GO terms for the 379 genes regulated upwards and the 262 genes regulated downwards, respectively. It is interesting to note that most

| GO term          | Ontology     | Description                                      | Number in input list | Number in BG/Ref | P-value | FDR  |
|------------------|--------------|--------------------------------------------------|----------------------|-------------------|--------|------|
| GO:0005977       | P            | Glycogen metabolic process                       | 5                    | 52                | 0.00016| 0.014|
| GO:0007017       | P            | Microtubule-based process                        | 16                   | 605               | 0.00018| 0.014|
| GO:0006112       | P            | Energy reserve metabolic process                 | 5                    | 54                | 0.00019| 0.015|
| GO:0009250       | P            | Glucan biosynthetic process                      | 8                    | 184               | 0.00036| 0.026|
| GO:0009873       | P            | Ethylene-mediated signalling pathway             | 8                    | 185               | 0.00037| 0.026|
| GO:0009461       | P            | Response to water                                | 13                   | 584               | 0.00038| 0.026|
| GO:0009741       | P            | Response to brassinosteroid stimulus             | 9                    | 258               | 0.00074| 0.043|
| GO:0009834       | P            | Secondary cell wall biogenesis                   | 8                    | 112               | 1.3E-05| 0.0016|
| GO:0044262       | P            | Cellular carbohydrate metabolic process          | 28                   | 1093              | 1.3E-06| 0.0033|
| GO:0009409       | P            | Response to cold                                 | 26                   | 855               | 1.4E-07| 3.8E-05|
| GO:0004042       | P            | Glucan metabolic process                         | 13                   | 329               | 1.5E-05| 0.0016|
| GO:0005975       | P            | Carbohydrate metabolic process                   | 48                   | 2018              | 1.5E-09| 2.8E-06|
| GO:0006121       | P            | Cellular amino acid and derivative metabolic process | 34               | 1688              | 1.6E-05| 0.0016|
| GO:0051258       | P            | Protein polymeization                            | 10                   | 147               | 1.7E-06| 0.0038|
| GO:0007018       | P            | Microtubule-based movement                       | 13                   | 174               | 1.7E-08| 1.5E-05|
| GO:0009266       | P            | Response to temperature stimulus                 | 27                   | 1229              | 2.9E-05| 0.0029|
| GO:0034637       | P            | Cellular carbohydrate biosynthetic process       | 17                   | 483               | 3.4E-06| 0.0068|
| GO:0044264       | P            | Cellular polysaccharide metabolic process         | 18                   | 457               | 3.7E-07| 0.0017|
| GO:0006575       | P            | Cellular amino acid derivative metabolic process | 22                   | 908               | 4.1E-05| 0.0038|
| GO:0034641       | P            | Cellular nitrogen compound metabolic process      | 26                   | 1031              | 4.1E-06| 0.0073|
| GO:0042546       | P            | Cell wall biogenesis                             | 12                   | 313               | 4.3E-05| 0.0018|
| GO:0009832       | P            | Plant-type cell wall biogenesis                   | 12                   | 252               | 5.4E-06| 0.0083|
| GO:0006073       | P            | Cellular glucan metabolic process                | 13                   | 298               | 5.5E-06| 0.0083|
| GO:0005976       | P            | Polysaccharide metabolic process                 | 21                   | 639               | 6.9E-07| 0.0022|
| GO:0002071       | P            | Polysaccharide biosynthetic process              | 14                   | 354               | 7.1E-06| 0.0098|
| GO:0016051       | P            | Carbohydrate biosynthetic process                | 21                   | 641               | 7.3E-07| 0.0022|
| GO:0033692       | P            | Cellular polysaccharide biosynthetic process      | 13                   | 311               | 8.6E-06| 0.0011|
| GO:0016758       | F            | Transferase activity, transferring hexosyl groups | 18                 | 707               | 0.00011| 0.022|
| GO:0004553       | F            | Hydrolyase activity, hydrolyzing O-glycosyl compounds | 18               | 773               | 0.00032| 0.043|
| GO:0016641       | F            | Oxidoreductase activity, acting on the CH-NH2 group of donors, oxygen as acceptor | 5                    | 63                | 0.00037| 0.043|
| GO:0005507       | F            | Copper ion binding                               | 10                   | 301               | 0.00055| 0.048|
| GO:0016491       | F            | Oxidoreductase activity                          | 48                   | 3249              | 0.00057| 0.048|
| GO:0005506       | F            | Iron ion binding                                 | 24                   | 1268              | 0.00068| 0.05  |
| GO:0016638       | F            | Oxidoreductase activity, acting on the CH-NH2 group of donors | 7                    | 94                | 3.6E-05| 0.011|
| GO:0005200       | F            | Structural constituent of cytoskeleton           | 7                    | 73                | 7.9E-06| 0.0046|
| GO:0030312       | C            | External encapsulating structure                 | 24                   | 1207              | 0.00034| 0.014|
| GO:0016020       | C            | Membrane                                         | 111                  | 9319              | 0.00065| 0.024|
| GO:0032224       | C            | Intrinsic to membrane                            | 59                   | 4289              | 0.00076| 0.025|
| GO:0044255       | C            | Membrane part                                    | 71                   | 5411              | 0.00087| 0.025|
| GO:0044430       | C            | Cytoskeletal part                                 | 17                   | 790               | 0.0011| 0.029|
| GO:0033225       | C            | Anchored to membrane                             | 21                   | 501               | 1.4E-08| 3.4E-06|
| GO:0012505       | C            | Endomembrane system                              | 63                   | 3339              | 3.2E-08| 3.4E-06|
| GO:0005418       | C            | Cell wall                                        | 24                   | 1051              | 4.5E-05| 0.0013|
| GO:0042798       | C            | Tubulin complex                                  | 7                    | 33                | 6.1E-08| 6.1E-06|
| GO:0048046       | C            | Apoplast                                         | 11                   | 285               | 8.4E-05| 0.0047|
| GO:0005505       | C            | Plant-type cell wall                             | 21                   | 896               | 9.6E-05| 0.0047|

Ontology “P” indicates Biological Process, ontology “F” indicates Molecular Function, and ontology “C” indicates Cellular Component. “Number in input list” is the number of occurrence of the Gene Ontology term in the input list (668 genes). “Number in BG/Ref” is the observed occurrence of the Gene Ontology term in the Populus trichocarpa genome. P-value represent the statistical significance of the enrichment test and FDR is the false discovery rate set to 0.05.
Table 3. GO term enrichment analysis of the 262 downregulated xylem genes (35° tilted vs. control).

| GO term          | Ontology | Description                              | Number in input list | Number in BG/Ref | P-value | FDR  |
|------------------|----------|------------------------------------------|----------------------|------------------|--------|------|
| GO:0010876       | P        | Lipid localization                       | 5                    | 32               | 2.80E-06 | 0.0037 |
| GO:0009067       | P        | Aspartate family amino acid biosynthetic process | 6                    | 75               | 9.70E-06 | 0.0065 |
| GO:0034641       | P        | Cellular nitrogen compound metabolic process | 19                   | 1031             | 2.20E-05 | 0.0088 |
| GO:0042221       | P        | Response to chemical stimulus            | 52                   | 4909             | 3.40E-05 | 0.0088 |
| GO:0009066       | P        | Aspartate family amino acid metabolic process | 7                    | 145              | 3.90E-05 | 0.0094 |
| GO:0015498       | P        | Inorganic anion transport                | 7                    | 146              | 3.80E-05 | 0.0088 |
| GO:0000097       | P        | Sulfur amino acid biosynthetic process   | 5                    | 67               | 7.50E-05 | 0.013  |
| GO:0019748       | P        | Secondary metabolic process              | 20                   | 1235             | 7.60E-05 | 0.013  |
| GO:0009308       | P        | Amine metabolic process                 | 19                   | 1171             | 0.00011 | 0.017 |
| GO:0050896       | P        | Response to stimulus                    | 85                   | 9663             | 0.00013 | 0.017 |
| GO:0006555       | P        | Methionine metabolic process             | 5                    | 88               | 0.00225 | 0.031 |
| GO:0041106       | P        | Cellular amino acid metabolic process    | 15                   | 890              | 0.00041 | 0.042 |
| GO:0006520       | P        | Cellular amino acid metabolic process    | 15                   | 890              | 0.00041 | 0.042 |
| GO:0097555       | P        | Hormone-mediated signalling pathway      | 16                   | 1003             | 0.00047 | 0.046 |
| GO:0006820       | P        | Anion transport                          | 8                    | 297              | 0.00055 | 0.049 |
| GO:0016491       | F        | Oxidoeductase activity                   | 43                   | 3249             | 9.10E-07 | 0.00041 |
| GO:0009055       | F        | Electron carrier activity                | 24                   | 1346             | 2.90E-06 | 0.00066 |
| GO:0046306       | F        | Tetrapyrrole binding                    | 17                   | 819              | 1.40E-05 | 0.0018 |
| GO:0020037       | F        | Heme binding                            | 16                   | 744              | 1.60E-05 | 0.0018 |
| GO:0015103       | F        | Inorganic anion transmembrane transporter activity | 7                   | 154              | 5.70E-05 | 0.0051 |
| GO:0005506       | F        | Iron ion binding                        | 20                   | 1268             | 0.00011 | 0.008 |
| GO:0006390       | F        | Anion transmembrane transporter activity  | 8                    | 251              | 0.00018 | 0.012 |
| GO:0016209       | F        | Antioxidant activity                     | 6                    | 297              | 0.00055 | 0.03  |
| GO:0022491       | F        | Substrate-specific transmembrane transporter activity | 23                   | 1842             | 0.00089 | 0.044 |
| GO:0019825       | F        | Oxygen binding                          | 9                    | 409              | 0.001   | 0.045 |
| GO:0016848       | F        | Oxidoeductase activity, acting on peroxide as acceptor | 7                   | 262              | 0.0013  | 0.046 |
| GO:0042603       | F        | Protein homodimerization activity        | 7                    | 268              | 0.0014  | 0.046 |
| GO:0009289       | F        | Lipid binding                           | 11                   | 606              | 0.0014  | 0.046 |
| GO:0046401       | F        | Peroxidase activity                     | 7                    | 262              | 0.0013  | 0.046 |

Ontology "P" indicates Biological Process, ontology "T" indicates Molecular Function, and ontology "C" indicates Cellular Component. "Number in input list" is the number of occurrence of the Gene Ontology term in the input list (262 genes). "Number in BG/Ref" is the observed occurrence of the Gene Ontology term in the Populus trichocarpa genome. P-value represents the statistical significance of the enrichment test and FDR is the false discovery rate set to 0.05.

Enriched GO terms are exclusive to genes regulated upwards or downwards. Since 27 genes with opposite tendencies were included in either group, they were analysed separately (Table 4).

By looking more precisely at CC terms impacted by the gravitational stimulation treatment, we discovered how the cells integrate the stimulus. Figure 4 is a graphical analysis of the CC term enrichment related to modulated genes. XU-specific genes appeared to be significantly linked to the ‘cell wall’ while the ‘plasma membrane’ was the only significant XL-specific CC. XU- and XL-modulated genes covered the ‘apoplast’ and the ‘endomembrane system’. When considering genes according to their expression tendency, no significant CC was identified for the 262 genes regulated downstream. The greatest variety of CC was found in upregulated genes including: ‘apoplast’, ‘cell wall’, ‘tubulin complex’, ‘amyloplast’, ‘endomembrane and membrane anchored’. For the 27 genes with upwards or downwards regulation on the xylem side, the ‘cell wall’ compartment was significantly impacted, in the same way as the specific XU genes (Fig. 4). Finally, it is interesting to note that among the genes expressed differently after gravistimulation, 36 were annotated as putative transcription factor (TF) [see Supporting Information—Table S4].

Exploration of candidate genes

The experiments on DNA chips identified 668 genes with significant differential expression after 30 min of inclination at 35°. A subset (153 genes) was used to further investigate their involvement in gravitropic response by high-throughput quantitative FCR in a kinetic study (0, 30, 60, 120 and 180 min at 35°). As a prerequisite, we ensured the results of the bioships by reanalyzing the expression of candidate genes found in the bioships experiment on five independent biological replicates. We found significant pairwise correlations between the two approaches (i.e. 0.67, 0.50, 0.71, 0.72, 0.67) allowing for a reliable interpretation of the data produced subsequently. We then performed a hierarchical grouping within the inclination expression data that led to the identification of five co-expression groups identified thereafter as Cluster #1 to Cluster #5 (Fig. 5).

Cluster #1 identified 59 genes mainly downregulated in XU after gravistimulation. A large bulk (40/59) concerned the metabolism of sugars (glycolys groups and/or carbohydrates), whereas another group of five genes had something to do with auxin and/or ethylene responses and/or metabolism. Lastly, four genes were involved in transmembrane transport. Cluster #2 comprised the highest number of genes (81) and a wide panel of metabolic processes. Using a KEGG enrichment analysis, three
major pathways were brought forward including photosystem II oxygen-evolving enhancer protein 1 (i.e. OEE), the SAUR protein family (i.e. auxin responsive genes) and β-tubulin (i.e. cytoskeleton). Briefly, cell redox players, putative mechanical sensors, various kinases, several brassinoids, ethylene- and auxin-related genes and TFs were well-represented in that cluster. It is also in Cluster #2 that we found genes already known to be involved in lignin synthesis and cell wall modification. Cluster #3 identified three fasciclina-like arabinogalactan genes, expression of which was bimodal in XU and XL reaching two maxima at 60 and 180 min after gravistimulation. Cluster #4 was the second largest cluster with 42 genes over-expressed in XL and whose expression was not modified in XU. Functional annotations highlighted phenylpropanoids, flavonoids and terpenoid biosynthesis. Of note within that cluster was the significant BF enrichment in ‘transmembrane transport’ especially concerning sugars. ‘Lipid metabolic process’ was the second biological process pinpointed. Finally, Cluster #5 was the smallest cluster identified with only three co-expressed genes upregulated in both XL and XU. Functional annotations suggested that oxidative stress and oligopeptide mobilization were the processes concerned.

**Discussion**

The experimental device allowed to identify genes regulated by inclination

This study aimed to better understand the molecular processes that occur in the secondary xylem of the woody stem after short-term tilting while separating the phototropic effect from gravitropic stimulation. To do so, we used an innovative experimental design (Coutand et al. 2019) to identify a list of differentially expressed genes specifically responding to phototropic without interference with the phototropic response. Stem staking in our condition was shown to be effective in preventing stem deformation as assessed by the absence of induction of \( \text{PtaZFP2} \), a well-characterized reporter gene for stem bending in poplar (Martin et al. 2009). It is interesting to note that we were able to record very early molecular responses, whereas previous studies, which did not use our innovative device and are therefore difficult to compare, found significant differential gene expression only after 8 h of gravistimulation (Zinkgraf et al. 2018). It is difficult to say whether this difference is due to the distinctive statistical power of the studies or whether it is due to other factors. The idea that an early molecular adjustment could occur in the gravitropic response is however supported by our earlier observation that changes in the poplar stem proteome were recorded as early as 45 min after tilting (Azri et al. 2009).

Genes supporting cell wall reorganization are modulated in ‘opposite and tension wood’ forming zones

Among the many gene classes identified in this study, we have chosen to first focus on the genes involved in cell wall formation and modification. The cell component ‘cell wall’ was indeed one of the CCs of interest and this choice was strongly supported by the biological process terms that were significantly brought forward in our analysis including ‘cell wall biogenesis’, ‘carbohydrate metabolic process’ and ‘catabolic lignin process’. Within that compartment, our experimental set-up allowed the identification of genes involved in wood formation specifically regulated by inclination independently of the phototropic stimulation response. It was interesting to note that as early as after 30 min of gravistimulation modulation of gene expression was recorded on both sides of the inclined stem (Fig. 3), thus concerning the whole secondary xylem. From the analysis of the GO enrichment (Fig. 4), it appears that the changes mainly affect the cell wall compartment on the upper surface, which corresponds to the installation of G-layer fibres (Jeurus et al. 2001). For the lower xylem, the plasma membrane is the significant cellular component affected, which could indicate the importance of control between adjacent compartments. Some genes, encoding mainly TFs involved in the formation of the secondary cell wall, appear to be regulated in both XU and XL. This is the case with Petri.001G112200, a KNAT7 homologue (class II KNOX gene) that has been shown elsewhere to be a key negative regulator of secondary cell wall formation (Li et al. 2012). Bhargava et al. (2013) suggested that KNAT7 could be involved in the suppression of lignin biosynthesis in stems. The analysis of promoters of genes encoding such TF sensitive to gravitational stimulation could provide a good opportunity to decipher molecular events upstream.

Rose et al. (2004) suggested that cell wall remodelling involves a complex interplay of enzymatic and non-enzymatic oxidative and hydrolytic changes. In agreement with this we found in Cluster #2 several redox-regulating players including: four oxygen-evolving enhancer proteins, a glutathione S-transferase, thioredoxin b, three 2OG-Fe(II) oxygenases and a peroxidase.
We also found within that same cluster eight carbohydrate active enzymes (CAZymes), four glycosyltransferases (GTs) and four glycoside hydrolases (GHs) that could participate in modulating the composition and structure of gravistimulated xylem cell wall. The fact that they were all clustered together supports a synergistic effect on cell wall properties as suggested
earlier by Tenhaken (2015). In addition, the observation that these genes also clustered with genes involved in regulation of cell expansion (namely Potri.009G067100, Potri.009G067200 and Potri.008G162260) suggests that they all may be mobilized during cell wall modifications accompanying cell expansion.

A well-established process is that of the reprogramming of sugar metabolism when normal wood is compared to TW (Mizrachi et al. 2014). Several reports (Lafargue et al. 2004; Paux et al. 2005; Andersson-Gunneras et al. 2006) showed that in TW (upper side), the upregulation of cellulose synthase activity was responsible for the production of the so-called gelatinous layer (G-layer). In our experimental setting, reprogramming of sugar metabolism appears to occur on both sides of the stem shortly after gravistimulation as evidenced by the increased expression of two genes encoding cellulose synthases in XL and XU (CESAs Potri.004G059600, Potri.002G257900). In addition,
the expression of two cellulose synthase-like genes (CSL, β-1,4-mannan synthases) was downregulated in the two xylem sides. In poplar it was shown that Fasciculin-like arabinogalactan proteins (FLA) were induced after gravitropism and thought to participate in TW G-layer synthesis (Lafargue et al. 2004; Fagerstedt et al. 2014; MacMillan et al. 2015; Wang et al. 2015). We show here that a bulk of FLA were upregulated after 30 min of gravitropism. Cluster #1 also brought forward genes involved in sugar transport/removal/hydrolisis (Potri.005G260300, and the CAZymes: Potri.001G028100, Potri.004G064100, Potri.004G113500, Potri.002G80700, Potri.003G126000) suggesting that sugar repartition is modified in xylem after inclination.

In agreement with the observation made by Villalobos et al. (2012) that ‘opposite wood’ (lower side) has a greater lignin content than TW we found four upregulated LIM TFs (Potri.002G157300, Potri.002G118000, Potri.009G087200, Potri.014G080900) that were suspected to play a role in lignin biosynthesis (Chen et al. 2015). Our analysis also brought forward genes involved in lignins (production such as phenylalanine ammonia-lyase (PAL1L2, Potri.006G126800) expression of which was repressed in XL and 4-coumarate:CoA ligase (ACL, Potri.003G188500) repressed in XU along with caffeoyl-CoA O-methyltransferase 1 (CCoAMT1, Potri.009G099800). In addition, Potri.016G112100, a laccase 1 homolog was found upregulated in XU although it is not yet consensually admitted that it participates in the lignification process (Cavanholt and Larsen 2002; Berthet et al. 2002). Consistent with the idea that TW has a lower lignin content we found that PtSAM51 (Potri.013G004100), a S-adenosylmethionine synthetase (SAM-S) suspected to play a central role in lignin biosynthesis (Vander Mijnsbrugge et al. 2000; Shen et al. 2002), was inhibited in XU.

Finally, we also found that two sequences (Potri.011G006800 and Potri.004G096900) encoding two β-1,4-glycan deacetylase complex (GDC) participating in serine biosynthesis were downregulated in XU. This is in line with the suggestion that GDC may allow woody perennial to cope with G1 demands of lignification (Rajinikanth et al. 2007). It is also consistent with the report of GDC involvement in the context of compression wood formation (Villalobos et al. 2012).

Impact of gravitropism on wood cell expansion

Some of the significant B7, Mf, and CC terms brought forward by the present study strongly suggest that wood cell expansion is a process impacted by gravitropism. This is in agreement with a growing number of studies linking giberellins and TW (Eriksen et al. 2000; Maurat and Maritz 2009; Nguro et al. 2012, 2013; for a review, see Felten and Sundberg 2013). The prevailing view, as reviewed recently by Ruele (2014), is that in poplar TW fibres are longer and thinner when compared to that in ‘opposite wood’, suggesting complex regulation of cell expansion processes. In agreement with these observations, we found that Potri.012G132400 and Potri.015G134600, two active gibberellins (GA20OX) coding genes that have been linked elsewhere with cell expansion were modulated (Erassimov et al. 2005). Besides gibberellins (GA), other phytohormones are known to play a role in cell expansion. In particular, brassinosteroids (BR) were shown to be involved in stem reorientation and cell elongation (Bienert et al. 2012; Vandenbussche et al. 2013). Interestingly, we found that a homolog to BR1 (brassinosteroid-insensitive1, an A. thaliana cell surface LRR-S/T kinase receptor for BR) (here identified as Potri.007G078100), and a serine carboxypeptidase (SCP) homolog to brassinosteroid insensitive1 suppressor (BRI1) (here identified as Potri.011G046600) were found modulated within Cluster #2 in response to gravitropism. Of course, we cannot rule out the hypothesis that the listed genes involved in detection/response to GA and BR have a role in the regulation of cell differentiation and secondary cell wall biogenesis as suggested by Du et al. (2019). In addition, GA may regulate key genes involved in BR response (Gertula et al. 2013).

As cell expansion is regulated by the plasticity of the primary cell wall (Cosgrove 2005) it was consistent to see that after our stimuli several genes encoding proteins involved in loosening tension between primary cell wall constituents were found modulated, including expansin, expansin-like and pectin xylanuclosases modifier genes (Potri.007G083400, Potri.010G167700, Potri.013G134300, Potri.002G060500, Potri.013G005700, Potri.014G115000, Potri.018G084300).

At last, supporting the involvement of cell expansion in response to gravitropism we recorded the upregulation of Potri.004G117200 in XL. This sequence is a homolog of the A. thaliana COBRA protein, an extracellular glycosyl-phosphatidyl inositol-anchored protein required for polarized cell expansion through cellulose microfibril orientation (Schindelman et al. 2001; Roudier et al. 2005).

Could concerted regulations of flavonoid and phosphonositides pathways occur after gravitropism?

Following gravitropism, signal transduction leads to complex biochemical and genomic responses at the subcellular level. In Cluster #4, we observed the co-expression of genes involved in phenylpropanoid biosynthesis (PAL1—Potri.006G126800) and flavonoid (Potri.002G036600, Potri.003G102000) pathways. The TF LIM1 (Potri.002G118000) is also found in this cluster which agrees with Chen et al. (2015) who have found co-expression in ‘opposite wood’ of several LIM TFs with PAL genes. Several studies suggest that the synthesis or deposition of flavonoids regulates auxin transport during gravitropic responses (Geisler et al. 2005; Buer et al. 2007, 2013; Santelia et al. 2008; Lewis et al. 2011). Gayomba et al. (2016) demonstrated their role in auxin transport inhibition and antioxidant activity in Arabidopsis.

Furthermore, it is known that phosphonositides regulate vesicle trafficking (for a review, see Krishnamoorthy et al. 2014) and auxin-dependent development (Tejera et al. 2014). Phosphatidylinositol transfer protein SEC14 (Potri.006G157600) is co-expressed in Cluster #4 with CLATHRIN assembly protein (Potri.006G066900) indicating the regulation of vesicle trafficking. Lipid metabolism is also regulated (Potri.006G166800, Potri.012G12900).

In this context we hypothesize that concerted regulations of the flavonoid and phosphonositides pathways led to auxin modulation after gravitropism, probably through vesicle trafficking.

In the research areas of nutrition and dietetics, it has been shown that flavonoids exert modulating actions on the cellular system through direct action on various signalling pathways such as those of phosphoinositide 3-kinase, and protein kinase C (Mansuri et al. 2014).

What about cytoskeleton involvement in xylem following gravitropism?

On the understanding that the involvement of the cytoskeleton following shoot gravitropism is still an unsolved question (Song et al. 2019), we noticed that GC terms such as ‘membrane and endomembrane systems’, ‘cell wall’, ‘apo plast’, ‘amyloplast’, ‘vacuole’ and ‘tubulin complex’ were affected in our gravitropism experiment. The observation that the expression
of tubulin genes, as well as that of an actin-depolymerizing factor (Potri.010G106200) and a β-actin capping protein alpha subunit (Potri.013G017000) is modulated suggests that modification of the cell cytoskeleton is partly a response to gravistimulation as suggested earlier by Nick (2011). It was shown already in Arabidopsis stem that the actin microfilaments may be considered as negative regulators of the gravitropic response considering their interaction with the amyloplasts through the link to SGR9 (Kolesnikov et al. 2016). Song et al. (2019) highlighted the role of the actin-bonding protein Rice Morphology Determinant (RMD) in the regulation of the gravitropic response of the light-grown shoots. In different studies, microtubules were also shown to participate in gravitropism mainly via cellulose deposition in xylem (Fagerstedt et al. 2014; Araniti et al. 2016).

To conclude, our experimental isotopic light experimental set-up allowing the strict analysis of gravitropic stimulation of trees not polluted by the phototropic response highlighted a list of 668 xylem-regulated genes involved in early gravitropic response. Some of the genes pinpointed here were already reported to participate in gravity response confirming the pertinence of our approach and attesting that they were indeed related to the gravity stimuli and not to another confounding factor (phototropic response, stem bending response). Interestingly, more than a third of these genes are still not annotated for gene function. We consider that this particular gene subset, especially those still not annotated, constitutes a good starting point to expand our current knowledge on gravitropism. This work opens new research prospects towards the understanding of events triggering ‘reaction wood’ formation.

**Supporting Information**

The following additional information is available in the online version of this article—

- **Table S1.** List of qPCR primers.
- **Table S2.** List of the 2355 differentially expressed genes based on poplar annotation V1.
- **Table S3.** List of the 668 differentially expressed genes based on poplar annotation V3.
- **Table S4.** List of transcription factors.
- **Figure S1.** Correlation analysis of microarray data.
- **Figure S2.** High-resolution version of Fig. 3 with gene model names.
- **Figure S3.** GO term enrichment of Biological Processes (BP), Molecular Functions (MF) and Cellular Components (CC) of the 668 differentially expressed genes in XU and XL.

**Sources of Funding**

This work was supported by the ‘Agence Nationale de la Recherche’ (ANR-11-BSV7-0012).

**Conflict of Interest**

None declared.

**Contributions by the Authors**

D.L. and J.F. performed the experiments and collected data. DL and PRD designed the study, analysed the data and wrote the first draft. J.S.V., P.L., C.C. and J.R.D. critically reviewed the manuscript before final approval.

**Acknowledgements**

We thank Brigitte Girard, Christelle Boisselet and Sylvaine Blatetron for the production and care of the plants, RNA extraction and their help during the measurements. We would like to thank Stéphane Ploquin for his technical skills in improving the spheres. We thank the Plant Genomics Research Unit (URGV) platform for their advice.

**Literature Cited**

Andersson-Gunnarsson S, Mellerowicz EJ, Love J, Segerman B, Ohmiya Y, Coutsio P, Nilsson P, Henri salt B, Marita T, Sundberg B. 2006. Biosynthesis of cellulose-enriched tension wood in Populus: global analysis of transcripts and metabolites identifies biochemical and developmental regulators in secondary wall biosynthesis. The Plant Journal 45:144–165.

Araniti F, Graña E, Krauska U, Bogatek R, Reigors MJ, Abensvoldi MR, Sánchez-Moreiras AM. 2016. Loss of gravitropism in farenese-treated Arabidopsis is due to microtubule malformations related to hormonal and ROS imbalance. PLoS One 11:e0160202.

Aziz W, Brunel N, Tranchel J, Ben Rejeb J, Jacquet JP, Julien JL, Herbette S, Rochele-Dreyvet P. 2013. Putative involvement of Thioredoxin H in early response to gravitropic stimulation of poplar stems. Journal of Plant Physiology 170:707–711.

Aziz W, Chambon C, Herbette S, Brunel N, Coutaud C, Lepiè RC, Ben Rejeb J, Ammar S, Julien JL, Rochele-Dreyvet P. 2009. Proteome analysis of apical and basal regions of poplar stems under gravitropic stimulation. Physiologia Plantarum 136:193–208.

Baldwin KL, Strohm AK, Masson PH. 2013. Gravity sensing and signal transduction in vascular plant primary roots. American Journal of Botany 100:162–172.

Batienne R, Devaux S, Meulia B. 2015. A unified model of shoot tropism in plants: photo-, grav- and proprio-reception. PLoS Computational Biology 11:e1004037.

Benjamin Y, Hochberg Y. 2000. On the adaptive control of false discovery rate in multiple testing with independent statistics. Journal of Educational and Behavioral Statistics 25:40–63.

Benjamin Y, Yekutieli D. 2001. The control of the false discovery rate in multiple testing under dependency. Annals of Statistics 29:1165–1188.

Berthelot S, Thévenin J, Baratay D, Demoust-Coulet N, Debesoujien I, Bédziniski P, Lepiè RC, Huds S, Hawkins S, Gomez LD, Lapierre C, Jouanin L. 2012. Role of plant laccases in lignin polymerization. Chapter 5 in: Jacquet J-P, Gadial F, Jouanin L, Lapierre C, eds. Lignins—biosynthesis, bioconversion and biotechnology. Advances in botanical research. London: Academic Press; Elsevier, 145–172.

Bhargava A, Ahd A, Wang S, Mannsfeld SD, Hoagland GW, Dougless GJ, Ellis RE. 2013. The interacting MYB75 and KNAT7 transcription factors modulate secondary cell wall deposition both in stems and seed coat in Arabidopsis. Plant Cell 23:1179–1191.

Bienert MD, Delanoy M, Navacore C, Boutry M. 2012. NISCP1 from tobacco is an extracellular serine carboxypeptidase III that has an impact on cell elongation. Plant Physiology 158:1220–1229.

Buer CS, Koibachirhe V, Truong TT, Horvat CH, Djordjevic MA. 2013. Alteration of flavonoid accumulation patterns in transparent testa mutants disturbs auxin transport, gravity responses, and imparts long-term effects on root and shoot architecture. Plant Cell 23:173–189.

Buer CS, Muday GK, Djordjevic MA. 2007. Flavonoids are differentially taken up and transported long distances in Arabidopsis. Plant Physiology 145:477–490.

Chen J, Chen B, Zhang D. 2015. Transcript profiling of Populus tremuloides genes in normal, tension, and opposite wood by RNA-seq. BMC Genomics 16:164.

Cognoove DJ. 2005. Growth of the plant cell wall. Nature Reviews Molecular Cell Biology 6:855–861.

Côté WAJ, Day AC. 1964. Anatomy and ultrastructure of reaction wood. New York: Syracuse University Press.

Coutaud C. 2010. Mechnoengineering and chimeronomorphogenesis, a physiological and biomechanical point of view. Plant Science 179:164–162.
Coutard C, Adam B, Floquin S, Moulin B. 2019. A method for the quantification of phototropin and gravitropic sensitivities of plants combining an original experimental device with model-assisted phenotyping: exploratory test of the method on three hardwood tree species. Planta 246:2000973.

Du J, Geerts S, Li Z, Zhao ST, Liu YL, Liu Y, Lu MZ, Groover AT. 2013. Brassinosteroid regulation of wood formation in poplar. The New Phytologist 205:1516-1530.

Du S, Yamamoto F. 2007. An overview of the biology of reaction wood formation. Journal of Integrative Plant Biology 49:131-143.

Eriksson MS, Israelsson M, Olson G, Moritz T. 2003. Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production, and xylem fiber length. Nature Biotechnology 18:784-788.

Fagerstedt KV, Mellerowicz E, Gershikova T, Riel K, Jauhiainen J-P. 2016. Cell wall polymers in reaction wood. In: Gardiner B, Barnett J, Saranpää P, Joseph G, eds. The biology of reaction wood. Montpellier: Springer (Springer series in wood science), 37-106.

Felten J, Sundberg B. 2013. Biology, chemistry and structure of tension wood. In: Promm J, ed. Cellular aspects of wood formation. Plant Cell Monographs 20. Berlin, Heidelberg: Springer-Verlag, 203-226.

Garnoth R, Larsen K. 2002. Molecular biology of plant laccases in relation to lignin formation. Physiologia Plantarum 116:273-282.

Gayomar SB, Watkins JM, Muddy GK. 2016. Flavonoids regulate plant growth and development through regulation of auxin transport and cellular redox status. Recent Advances in Polyphenol Research 3:143-170.

Geiser M, Blakelock J, Bouchard R, Lee OR, Vincenzetti V, Vandivopper B, A, Titapawatanakum R, Peer WA, Baille A, Richards JJ, Jendela KT, Smith AP, Barouz C, Grossniklaus U, Müller A, Hrncica CA, Diederul R, Murphy AS, Martineau E. 2005. Cellular efflux of auxin catalyzed by the Arabidopsis MDR/PGP transporter AHA2/3. The Plant Journal 44:174-194.

Gerthsen S, Zinnkgraf M, Muday GK, Lewis DR, Ibarbullin FM, Brunner H, Hart F, Mansfield SD, Filev V, Groover A. 2015. Transcriptional and hormonal regulation of gravitropism of wood in Poplar. The Plant Cell 27:2800-2813.

Groover A. 2016. Gravitropism and reaction wood: a role of forest trees - evolution, functions and mechanisms. The New Phytologist 212:790-802.

Hashiguchi Y, Tazaka M, Marita MT. 2013. Mechanism of higher plant gravity sensing. American Journal of Botany 90:1-100.

Israelsson M, Sundberg B, Moritz T. 2005. Tissue-specific localization of gibberellins and expression of gibberellin-biosynthetic and signaling genes in wood-forming tissues in aspen. The Plant Journal 44:496-504.

Jouve B, Riboux A, Leclercq A. 2001. Anatomical characteristics of tension wood and opposite wood in young inclined stems of poplar (Populus Eucameria cv "Ghey"). IAWA Journal 22:13-157.

Kolesnikov YS, Kretynin SV, Volofovsky ID, Kordoym EL, Rueland E, Kravets VS. 2016. Molecular mechanisms of gravity perception and signal transduction in plants. Proteomics 16:1647-1658.

Krishnamorthy P, Sanchez-Rodriguez C, Heilmann I, Persson S. 2014. Regulatory roles of phosphoinositides in membrane trafficking and their potential impact on cell-wall synthesis and re-modelling. Annales Botanica 114:1049-1057.

Kubitz K. 1939. Studies on growth stresses in trees. 1. The origin of growth stresses and the stresses in transverse direction. Holz als Roh- und Werkstoff 17:1-9.

Lafraguette F, Lepel JC, Dejardin A, Laurans F, Costa G, Lescage-Descarreaux MC, Plaute G. 2004. Poplar genes encoding fasciclin-like arabinogalactan proteins are highly expressed in tension wood. The New Phytologist 164:107-121.

Lewis DR, Ramirez MV, Miller ND, Vallabhaneni P, Ray WK, Helm RJ, Winkel BS, Muddy GK. 2011. Auxin and ethylene induce flavonol accumulation through distinct transcriptional networks. Plant Physiology 154:164-166.

Li E, Bhargava A, Jiang W, Friedmann MC, Forneris N, Savidge RA, Johnson LA, Mansfield SD, Ellis BE, Douglas CJ. 2012. The Class II KNOX gene KNAT7 negatively regulates secondary wall formation in Arabidopsis and is functionally conserved in Populus. The New Phytologist 194:102-114.

Lopes D, Tholouard K, Venisse JS, Legué Y, Roelck-Drevet P. 2014. Gravity sensing, a largely misunderstood trigger of plant orientated growth. Frontiers in Plant Science 5:610.
Song Y, Li G, Nowak J, Zhang X, Xu D, Yang X, Huang G, Liang W, Yang L, Wang G, Bulone V, Nikolski Z, Hu J, Persson S, Zhang D. 2019. The rice actin-binding protein RMD regulates light-dependent shoot gravitropism. Plant Physiology 181:630–644.

Starkey JD. 2003. The positive false discovery rate: a Bayesian interpretation and the q-value. Annals of Statistics 31:2011–2015.

Sundell D, Mannapperuma C, Neto A, Debieux N, Lin YC, Sjödin A, Van de Peer Y, Jansen S, Jividen TR, Street NR. 2015. The plant genome integrative explorer resource: PlantGinIE.org. The New Phytologist 208:1149–1156.

Tejos R, Sauvè M, Vanneste S, Palacios-Gomez M, Li H, Heilmann M, van Wijk R, Vermeer JE, Heilmann I, Munnek T, Friml J. 2014. Bipolar plasma membrane distribution of phosphoinositides and their requirement for auxin-mediated cell polarity and patterning in Arabidopsis. The Plant Cell 26:2114–2126.

Tenhaken R. 2015. Cell wall remodeling under abiotic stress. Frontiers in Plant Science 5:771.

Timell TE. 1986. Compression wood in gymnosperms. Berlin: Springer.

Tocquaud K, Lopez D, Decourteix M, Thibaut B, Julien JL, Label P, Leblanc-Fournier N, Roeske-Kreyer P. 2014. The molecular mechanisms of reaction wood induction. In: Gardiner B, Barnett J, Sananpaa P, Cell J, eds. The biology of reaction wood. Berlin, Heidelberg: Springer-Verlag, 107–138.

Vandenbussche F, Callebert F, Zadnikova P, Benkova E, Van Der Straeten D. 2013. brassinosteroid control of shoot gravitropism interacts with ethylene and depends on auxin signaling components. American Journal of Botany 100:215–225.

Vander Mijnsbrugge K, Meyermans H, Van Montagu M, Bauw G, Boerjan W. 2000. Wood formation in poplar: identification, characterization, and seasonal variation of xylem proteins. Planta 210:589–598.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3:RESEARCH0034.

Villalobos DP, Diaz-Moreno SM, Said el-SS, Cañas RA, Osuna D, Van Kerckhove SH, Bautista R, Clarot MG, Cánovas FM, Cantón FR. 2012. Reprogramming of gene expression during compression wood formation in pine: coordinated modulation of 5′-adenosylmethionine, lignin and lignin related genes. BMC Plant Biology 12:100.

Wang H, Jiang C, Wang C, Yang Y, Yang L, Guo X, Zhang H. 2015. Antizein expression of the fasciclin-like arabinogalactan protein FLA6 gene in Populus inhibits expression of its homologous genes and alters stem biomechanics and cell wall composition in transgenic trees. Journal of Experimental Botany 66:1291–1302.

Yang YH, Dudoit S, Luu P, Lin DM, Peng V, Ngai J, Speed TP. 2002. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Research 30:e45.

Zhou D, Zhao X, Ling Y, Zhang Z, Su Z. 2010. AgriGO: a GO analysis toolkit for the agricultural community. Nucleic Acids Research 38. doi:10.1093/nar/gkq310.

Zinkgraf M, Gertula S, Zhao S, Filkov V, Groover A. 2018. Transcriptional and temporal response of Populus stems to gravit-stimulation. Journal of Integrative Plant Biology 60:178–190.