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Transcriptional and temporal response of *Populus* stems to gravi-stimulation[FA]

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High-Impact Article

Abstract Plants modify development in response to external stimuli, to produce new growth that is appropriate for environmental conditions. For example, gravi-stimulation of leaning branches in angiosperm trees results in modifications of wood development, to produce tension wood that pulls leaning stems upright. Here, we use gravi-stimulation and tension wood response to dissect the temporal changes in gene expression underlying wood formation in *Populus* stems. Using time-series analysis of seven time points over a 14-d experiment, we identified 8,919 genes that were differentially expressed between tension wood (upper) and opposite wood (lower) sides of leaning stems. Clustering of differentially expressed genes showed four major transcriptional responses, including gene clusters whose transcript levels were associated with two types of tissue-specific impulse responses that peaked at about 24–48 h, and gene clusters with sustained changes in transcript levels that persisted until the end of the 14-d experiment. Functional enrichment analysis of those clusters suggests they reflect temporal changes in pathways associated with hormone regulation, protein localization, cell wall biosynthesis and epigenetic processes. Time-series analysis of gene expression is an underutilized approach for dissecting complex developmental responses in plants, and can reveal gene clusters and mechanisms influencing development.

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INTRODUCTION

Plants modify developmental processes in response to external stimuli. Responses to external stimuli are highly dynamic and involve coordinated changes in gene expression to elicit context-specific developmental outcomes (Bar-Joseph et al. 2012). This interplay between environmental cues and development is seen in long-lived forest trees, where the development of wood tissues is modified in response to environmental conditions. For example, in angiosperm trees like poplar, the relative number and morphology of water conducting vessel elements within wood is changed in response to drought (Janz et al. 2012). These changes are reflected in familiar tree rings, showing the change in the amount and type of wood produced in transition from favorable conditions in spring, to water stress in the heat of late summer (Larson 1962; Zobel and van Buijtenen 1989). Trees modify the formation of wood to optimize mechanical support, water conduction, and nutrient storage and transport functions performed by wood tissues (Badel et al. 2015). However, the regulatory mechanisms responsible for integrating environmental cues and wood developmental pathways is poorly understood.

Most angiosperm trees respond to external forces including gravity, wind, and snow loading through the production of specialized tension wood fibers to reinforce and reorient the lignified portion of stems and branches (Ruelle et al. 2009; Groover 2016). For example, *Populus* trees subjected to a 90° gravi-stimulation treatment reorient the lignified portion of

the stem against the gravity vector (gravibending) (Figure 1A). Temporally, after being placed horizontal, trees stems initiate molecular genetic and cell biology responses before initiating gravibending, with a lag (1–2 d) between initial gravi-stimulation and initiation of gravibending response to a more vertical orientation. Further analysis of stem lift shows that the change (Δ) in normalized stem lift per day is not continuous through time, and the rate of bending positively increases between 2 and 7 d and followed by a steady or slight decreased rate of bending by the end of the 14-d treatment (Figure 1B). This suggests that gravibending is a dynamic process that is composed of multiple steps, such as sensing, integration, response and maintenance of a new developmental trajectory.

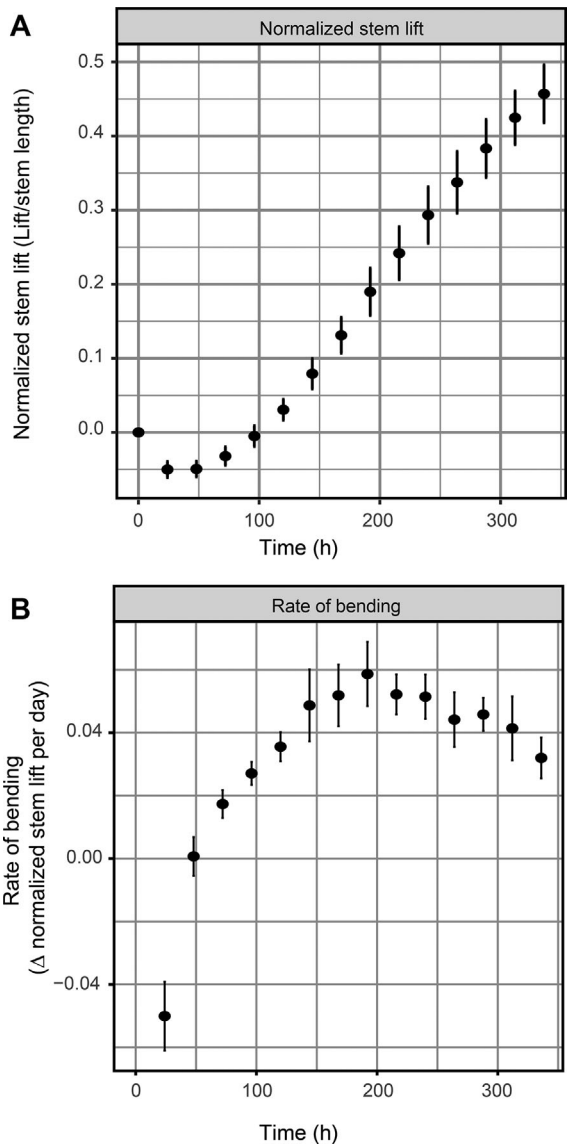

Figure 1. Quantification of stem bending for hybrid aspen during a 14-d gravistimulation experiment (Gerttula et al. 2015)

(A) Normalized stem lift and **(B)** rate of bending of wild type plants after being placed horizontal (0 h).

The initial sensing of woody stems to gravi-stimulation includes the sedimentation of starch filled amyloplasts and the re-localization of PIN-like auxin transporter proteins in the endodermal cells of the inner cortex and secondary phloem tissues towards the direction of the gravity vector (Toyota et al. 2013; Gerttula et al. 2015). This re-localization creates unique auxin gradients between the tissues (cortex, secondary phloem, cambium and secondary xylem) of a leaning stem and may represent the initial integration of signals that elicit differential growth of the vascular cambium, which leads to development of tension wood (upper surface) and opposite wood (lower surface). Following these initial responses, the vascular cambium undergoes faster growth on the upper surface to produce tension wood, which contains proportionately more fibers and fewer water conducting vessels when compared to opposite wood or normal wood from upright grown trees (Jourez et al. 2001). In addition, the newly formed fibers in tension wood contain a gelatinous layer highly enriched in cellulose, which is thought to be responsible for generating the tensile force used to pull the stem upright (Mellerowicz and Gorshkova 2012).

Previous studies have investigated the transcriptional response to gravi-stimulation primarily at individual time points and showed that trees undergo major transcriptional remodeling in developing xylem in both the tension wood and opposite wood sides of a leaning stem (Paux and Carocha 2005; Andersson-Gunneråsa et al. 2006; Jin et al. 2011; Chen et al. 2015; Gerttula et al. 2015). In addition, the integration of molecular and genomics datasets have shown that transcriptional changes are highly correlated with functional characteristics (e.g., cellulose crystallinity and microfibril angle) and secondary cell-wall components associated with tension wood fibers (Gerttula et al. 2015). These advances notwithstanding, the timing and duration of transcriptome-wide changes involved in regulating the formation of tension wood and response to gravi-stimulation in woody stems is largely unknown.

Analysis of gene expression changes at multiple time points in response to external stimuli has the potential to refine transcriptional networks by inferring rates of

temporal change in gene expression and the order in which specific genes are either activated or repressed (Bar-Joseph et al. 2012). Such patterns are difficult to identify from static experiments that are conducted at a single time point, as the information on continuity of cellular responses is lost. Studies ranging from prokaryotes to eukaryotes have used time-series experiments with both microarray and RNA-sequencing technologies and described two major types of transcriptional responses. The first type is an impulse response and is defined by an upregulation or downregulation of transcript levels in a single pulse, followed by decay to pre-treatment levels or a new steady state (Yosef and Regev 2011; Bar-Joseph et al. 2012; Levine et al. 2013; Lin et al. 2015). Impulse responses have been implicated as a major regulatory process that can trigger shifts in developmental states and response to pathogens (Oliveri et al. 2008; Davidson 2010). For example, pulses of gene expression following initial pathogen infection in *Arabidopsis* can lead to systemic acquired resistance through the upregulation of hormonal responses associated with the salicylic acid, jasmonic acid, and ethylene pathways (Moore et al. 2011; Lewis et al. 2015). A second type of transcriptional response is a long-term sustained change in gene expression, which is defined as a change in expression (increase or decrease) from time zero to a new steady state. Sustained expression responses often precede impulse responses and have been associated with shifts in developmental processes (Davidson 2010; Klepikova et al. 2015; Ryan et al. 2015).

In this paper, we investigate the transcriptional response of developing xylem in *Populus* stems subjected to gravi-stimulation using seven time points over a 14-d time-series experiment. Sampling gene expression (RNA-seq) from opposite wood and tension wood tissues across multiple time points, we address the following questions: (i) How does gene expression respond temporally to gravi-stimulation and are there specific types of responses? (ii) Do specific functional groups of genes or molecular pathways respond temporally to gravi-stimulation? (iii) How do these processes compare in opposite wood versus tension wood? (iv) How do the results from this time-series experiment compare to a previous gravitropic co-expression network in *Populus*, and do co-expression modules contain sub-networks of genes that exhibit temporal responses to gravi-stimulation?

RESULTS

Potted, clonal propagules of a hybrid aspen genotype (*Populus alba* × *P. tremula* INRA 717-1B4) were subjected to gravi-stimulation by placing the trees horizontally in a controlled greenhouse environment. Over a 14-d time-series experiment, individual trees were harvested and opposite wood and tension wood tissues sampled at seven time points (0, 2, 8, 24, 48, 96 and 336 h). Messenger RNA sequencing libraries (RNA-seq) were prepared for biological replicates of tension wood and opposite wood from each tree and subjected to 50 base pair single end Illumina sequencing (Materials and Methods). Sequence read processing and read counts were determined, as previously described (Zinkgraf et al. 2017).

The differential expression between tension wood and opposite wood transcripts showed dynamic changes over the 14-d experiment. A general linear model was used to compare tissue-specific expression at individual time points (Materials and Methods), and indicated that the number of genes that displayed transcript level differences between opposite wood and tension wood tissues changed dramatically over the course of the experiment (Figure 2). Early in the experiment (2–8 h), relatively few genes (<200 genes) displayed significant differences in transcript levels between tissues. Transcript level differences peaked at 48 h, with 7,844 genes showing differential transcript levels between the two wood types. After 48 h, the number of genes showing differential transcript levels between the wood types steadily

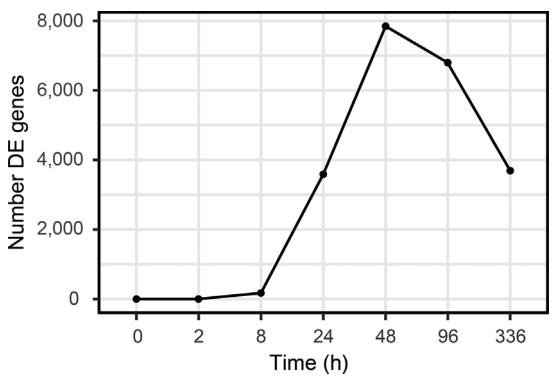

Figure 2. Summary of the number of differentially expressed (DE) genes at each time point during the 14-d gravi-stimulation experiment
The number of genes is based on a false discovery rate $< 1.0 \times 10^{-5}$.

decreased until the termination of the 14-d experiment (3,701 genes).

In a second analysis, genes were identified that were differentially expressed (DE) at some point during the time-series experiment. Using natural cubic spline regression, 8,919 genes were identified that displayed significant (FDR $< 1.0 \times 10^{-5}$) differential expression between opposite and tension wood tissues (Table 1). Of the DE genes, 4,816 showed relatively higher expression in tension wood tissues, and 4,103 genes showed higher expression in opposite wood tissues.

To reduce the dimensionality of transcriptional response, fuzzy c-means clustering was used to assign the DE genes to clusters based on the mean transcript level of the biological replicates, for each tissue, at each time point. A total of 16 clusters were identified that ranged in size from 330 to 1,019 genes (Figure 3, Table S1). As described below, these clusters showed three major transcriptional responses (impulse responses, long-term sustained expression changes and linear expression changes). Functional annotation of each cluster was performed using gene ontology (GO) enrichment analysis (Materials and Methods) and the full results associated with biological processes are presented in the supplemental information (Table S2).

A summary of the GO enrichment results (Figure 4) shows that the gene clusters were enriched for numerous individual GO terms (red bars in heatmap) associated with general categories likely to be involved in wood formation (plant hormones, peroxisome, protein localization, saccharides, cell wall, meristematic processes, epigenetics and development). For example, cluster 6 appears to be generally involved in meristematic processes and was enriched for 12 GO terms that ranged from initiation, regulation, growth, maintenance and structural organization of the meristem.

Table 1. Identification of differentially expressed (DE) genes across opposite and tension wood time-series experiment using natural cubic spline regression

	Number DE genes
Up-regulated in:	
Tension wood	4,816
Opposite wood	4,103
Total	8,919

Significance of DE genes is based on a FDR $< 1.0 \times 10^{-5}$

Tissue-specific impulse response of gene expression

Seven clusters of DE genes displayed tissue-specific impulse responses (Figure 3). Expression impulse responses are defined by a sharp increase in expression (pulse), sustained for a period of time and followed by decay back to steady state or pre-treatment expression level (Yosef and Regev 2011; Levine et al. 2013; Lin et al. 2015).

Three of the gene clusters (clusters 4, 14, 15) exhibited pulses of increased transcript levels in opposite wood, with peaks in transcript levels between 24 and 96 h (Figure 3). The transcript levels of genes from cluster 14 peaked at 24 h, and functional annotation of this cluster, using GO enrichment analysis, suggested strong over-representation of genes associated with hormone regulation, including abscisic acid, auxin, brassinosteroids, ethylene, gibberellic acid, jasmonic acid and salicylic acid GO terms (Figure 4). The transcript levels of genes from clusters 4 and 15 peaked at 48 and 96 h, respectively, and these clusters were primarily enriched for genes associated with the peroxisome, and to a lesser extent hormone regulation and protein localization.

There were four gene clusters (clusters 1, 2, 7, 12) that showed a pulse of increased transcript levels between 24 and 48 h in tension wood (Figure 3). These clusters were enriched for genes associated with protein localization, saccharides (mono- and polysaccharides), development and epigenetic processes such as histone modification, chromatin and methylation (Figure 4). Thus, while both opposite wood and tension wood showed clusters of genes with pulsed expression, the function and biological processes associated with the genes was different between wood types.

Long-term sustained expression changes

Seven clusters of DE genes displayed long-term sustained changes in transcript levels (Figure 3). Sustained changes in gene expression are similar to a logistic growth curve, where expression changes (increases or decreases) from time zero and then reaches an expression plateau for the remainder of the experiment. These clusters can be further classified into two groups: (i) clusters that displayed a sustained increase in opposite wood transcript levels (clusters 8, 3, 16), and (ii) clusters that displayed a sustained increase in tension wood transcript levels (clusters 13, 5, 11, 10).

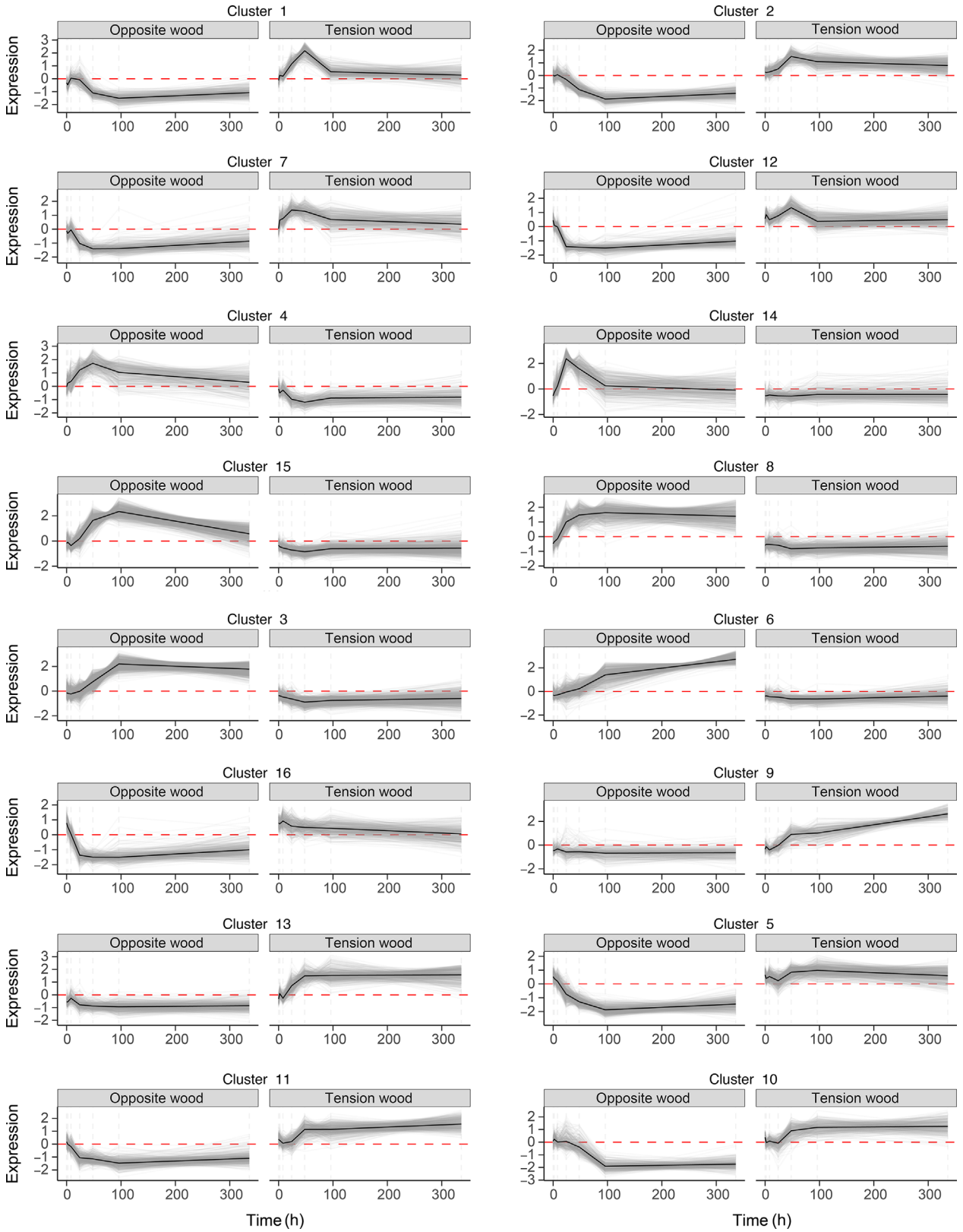

Figure 3. Clustering of differentially expressed genes show substantial variation in how opposite wood and tension wood tissues respond to gravi-stimulation treatment over the course of a 14-d experiment (0–336 h)
Grey lines represent standardized expression for individual genes, black lines represent cluster means, and red lines represents zero change in expression between opposite wood and tension wood.

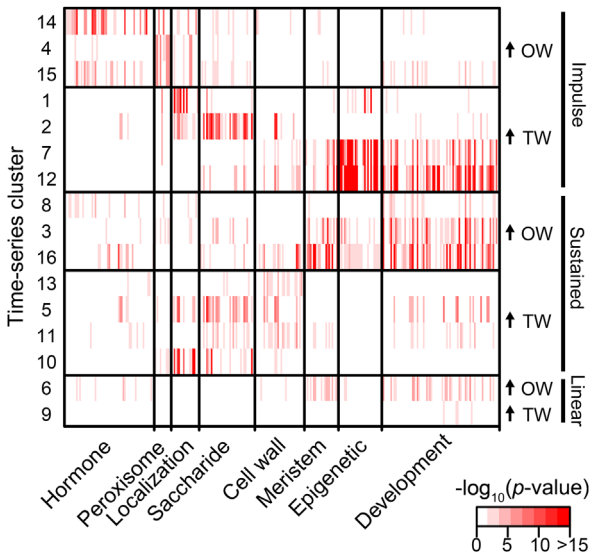

Figure 4. Functional annotation using GO enrichment analysis shows that time-series clusters represent distinct biological processes

Right column labels highlight the clusters that display tissue-specific impulse, sustained or linear expression response. Color intensity represents the significance ($-\log_{10}$ *P*-value) of individual GO terms and terms were grouped into categories involved in hormone, peroxisome, protein localization, saccharides, cell wall, meristem, epigenetics and developmental processes. Description of analyses is found in Materials and Methods. OW, opposite wood; TW, tension wood.

Functional annotation of the sustained expression clusters showed an over-representation of pathways that were specific to opposite wood or tension wood, similar to the results for impulse response clusters (Figure 4). Clusters with a sustained increase in opposite wood expression were enriched for genes associated with meristem, epigenetic and developmental processes. Clusters with a sustained increase in tension wood expression were enriched with genes associated with protein localization, (mono- and poly-) saccharides and cell wall biosynthesis.

Linear expression changes

Two clusters (clusters 6, 9) displayed linear increases in transcript levels for the duration of the time-series experiment (Figure 3). Cluster 6 showed increasing transcript levels in opposite wood and marginal changes in tension wood. Conversely, cluster 9 showed increasing transcript levels in tension wood and minimal changes in opposite wood expression. Functional annotation of these clusters indicated that cluster 6 was enriched with genes associated with meristem processes and development (Figure 4), while cluster 9 was enriched with genes involved in pollen tube development and extracellular matrix organization (Table S2).

Comparison of expression to gravibending response

To understand how changes in expression contribute to the gravitropic responses, we correlated mean cluster expression with two measurements (Figure 1) associated with gravibending from previously published gravistimulation experiments in hybrid aspen (Gerttula et al. 2015). Using Pearson's correlations, we identified wood type-specific expression of four clusters (clusters 10, 11, 13, 14) that were highly associated with the rate of bending ($R^2 = 0.91$–0.98; *P*-value < 0.05; Table S3; Figure S1). For example, the tension wood expression of cluster 13 was strongly correlation with rate of bending ($R^2 = 0.91$; *P*-value $=$ 0.0476; Figure S1C) and GO enrichment analysis suggests that genes in this cluster maybe involved in regulation (GO:2000652; *P*-value $=$ 0.002) and biogenesis (GO:0009834; *P*-value $= 2.33 \times 10^{-5}$) of the secondary cell wall. In addition, cluster 13 contained KNAT7 (Potri.001G112200) and numerous (16) FASCICLIN-like arabinogalactan proteins (FLA), including PtFLA6 (Potri.013G151300, Potri.013G151400, Potri.013G151500) and FLA11, which have previously been shown to be involved in tension wood formation (Lafarguette and Leple 2004; Andersson-Gunneras et al. 2006; MacMillan et al. 2010; Wang et al. 2015).

Comparison to a gravitropic co-expression network

Lastly, we sought to use the results of this time-series experiment to validate a previously published gravitropic co-expression network from Gerttula et al. (2015), by providing additional experiments that can be used to test the robustness of the network, as well as determine if co-expression modules contained potential sub-networks that exhibited temporal responses.

The 16 clusters of differentially expressed genes from the time-series experiment were compared to the 11 co-expression modules (Gerttula et al. 2015) using a hypergeometric test to determine if genes from the time-series clusters were over-represented in specific co-expression modules. We found that genes from each

time course cluster were significantly (*P*-value $< 1.0 \times 10^{-14}$) over-represented in at least one co-expressed module (Figure 5). In addition, multiple time course clusters of differentially expressed genes mapped to individual co-expression modules. For example, the two largest co-expression modules (magenta and blue) were enriched with genes from five and six time-series clusters, respectively. The magenta module was over-represented with genes from clusters associated with both impulse (cluster 2) and sustained (clusters 5, 10, 11) expression in tension wood, and sustained expression in opposite wood (cluster 3). The blue module was enriched for genes associated with impulse (cluster 4, 14, 15) and sustained (clusters 8, 16) expression in opposite wood, and sustained expression in tension wood (cluster 5). Together, this shows that the time-series analysis was able to identify the general module structure from Gerttula et al. (2015) but some co-expression modules contain potential sub-networks of genes that exhibit unique temporal responses under well-watered wild type conditions.

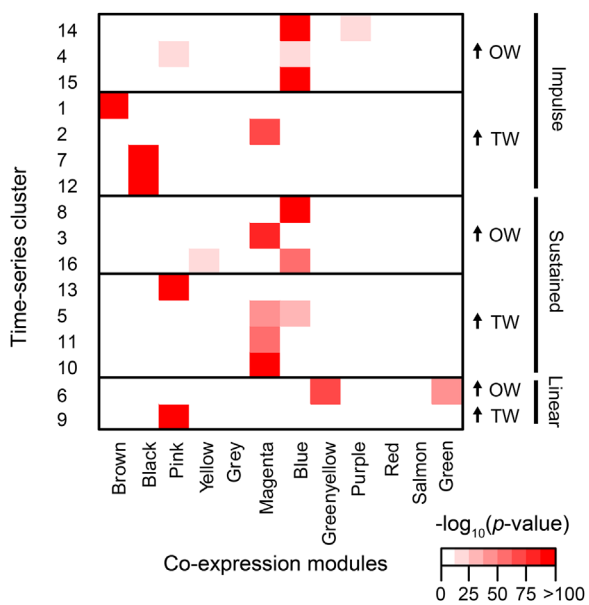

Figure 5. Preservation of time-series clusters in gravitropism modules from the Gerttula et al. (2015) co-expression network

Color intensity represents the probability ($-\log_{10}$ *P*-value) that differentially expressed genes from time-series clusters were over-represented in each co-expressed gene module. Right column labels highlight the clusters that display tissue-specific impulse, sustained or linear expression response.

DISCUSSION

The complex biological processes that regulate development and responses to environmental stimuli in plants and animals do not happen simultaneously. They unfold over a time frame that can range from seconds, in the case of signaling cascades within a cell, to days or even years in the case of the long-term development in forest trees (Forterre 2013). For example, during wood development in trees, tree rings reflect changes in rates of cell division and changes in cell differentiation taking place within and across growing seasons (Larson 1994). In this study, we considered the dynamic changes in the expression of genes involved in gravitropic response and tension wood formation in *Populus* trees over the course of 14 d. Tension wood formation is an excellent subject for a time-series approach, as it can be precisely and uniformly induced, and results in predictable changes in development that unfold over the course of many days. Our results provide some basic new insights into the temporal changes in gene expression and development during tension wood formation and gravitropism, as described below.

In the current study, we identified dynamic changes in gene expression at seven time points across the 14-d experiment that illustrates the importance of examining more than a single time point. Previous studies have primarily examined single time points of gene expression during tension wood formation, but do not provide a full account of changes in development over time (Andersson-Gunneras et al. 2006; Jin et al. 2011; Chen et al. 2015; Gerttula et al. 2015). For example, it would have been a reasonable guess that gene expression would change relatively soon after gravi-stimulation commenced because experiments in *Eucalyptus* showed significant changes in expression at 6 and 24 h (Paux and Carocha 2005). However, our results from developing xylem in *Populus* showed very little gene expression changes in the first 8 h of treatment and most changes in expression peaked at 48 h (Figure 2). Had we sampled one or two early time points, we might have erroneously concluded that gene expression does not change significantly during gravitropism or tension wood formation in *Populus*. Similarly, our results reveal a dynamic shift in the biological processes underlying changes in development across the time points of the experiment and can guide future studies into the genetic basis of tension

wood formation. Again, had we sampled only one time point, we might have biased our interpretation and emphasize the importance of the biological processes that are most pronounced at that time point. Thus, the information gained about the dynamic biology through the use of time-series data was extremely important for us to more fully understand the changes in development and associated biological processes over time.

Our time-series experiment also revealed fundamental features of gene expression over time, which in turn provided new insights into both the gravitropic response and overall wood formation in *Populus*. By assigning DE genes into clusters, based on temporal changes in expression between wood types, it was clear that the dynamics of change in expression were quite different among the clusters, but could be grouped into three main types of responses: impulse, sustained and linear.

The genes within impulse clusters show a relatively rapid increase in expression, followed by rapid decline. Previous work in model systems have shown that impulse response in gene expression may be involved in regulatory processes that contribute to shifts in developmental stats or integration of environmental signals (The FANTOM Consortium and Riken Omics Science Center 2009; Sivriver et al. 2011; Yosef and Regev 2011; Bar-Joseph et al. 2012). Interestingly, two of the tension wood-related impulse clusters (clusters 7 and 12) showed strong enrichment for genes that have been assigned to numerous GO terms involved in developmental and epigenetic processes (including methylation, chromatin and histone processes). While epigenetic processes influencing wood formation are not well characterized, our results are consistent with an initial role for epigenetic reprogramming of gene expression in transition to tension wood formation. This unexpected result could explain in part how the expression of many thousands of genes can be coordinately changed in transition from normal wood to tension wood. Interestingly, impulse clusters associated with opposite wood (clusters 4, 14, 15) are all enriched for genes with peroxisome function (whose diverse functions include hormone biosynthesis), and two (clusters 14 and 15) are enriched for genes associated with hormone-related functions. The role of opposite wood in gravitropic response is poorly understood but involves massive changes in gene expression (Gerttula et al. 2015). These impulse clusters may thus point to important roles in early changes in hormones in opposite wood that drive later changes in expression of large numbers of genes with diverse functions.

Gene clusters showing sustained expression were characterized by an increase or decrease in gene expression early, and then maintenance at level throughout the experiment. Gene clusters showing sustained expression in tension wood (clusters 5, 10, 11, 13) all showed significant enrichment for genes involved in localization, saccharide, and cell wall-related processes. Tension wood is characterized by reprogramming of cell wall synthesis, including production of a unique gelatinous layer in tension wood fibers and an overall reduction in the percentage of cells that differentiate as water conducting vessel elements. How these cell wall modifications are achieved are reflected in functional categories overrepresented by genes within these gene clusters, including functions associated with modification of what cell wall precursors are targeted to the cell wall, shifts in production of cell wall precursors (e.g., increase in the glucose-based cellulose content of the gelatinous layer) and ultimately how the cell wall precursors are assembled. Of the two gene clusters showing linear expression, one (cluster 9) was defined by tension wood expression but only showed enrichment for a limited number of development-related functional categories. The other linear gene cluster (6) was defined by opposite wood expression and was primarily associated with enrichment for genes involved with hormone, meristem, and developmental functions.

To further understand how changes in expression may influence gravibending outcomes, we correlated mean cluster expression with two gravibending metrics (normalized stem lift and rate of bending). Results from this analysis show that tension wood expression from clusters 10, 11 and 13 were highly correlated with the rate of gravibending but not normalized lift (Table S3) and suggests that a subset of genes (385, 511, 529 genes respectively) may be directly involved in tension wood formation. These clusters contain numerous genes that have previously been implicated in wood formation processes (Zhong et al. 2010; Hussey et al. 2013; Nakano et al. 2015; Ye and Zhong 2015; Groover 2016) and provide insight into genes may be directly involved in tension wood specific processes such as increase rate of cell division, cell differentiation, and the formation and maturation of the gelatinous cell wall layer

(Andersson-Gunneråas et al. 2006; Mellerowicz and Gorshkova 2012; Gerttula et al. 2015). For example, these clusters contained genes that have been characterized as major regulators of wood formation (ARK1 (Potri.004G004700), BEL1 (Potri.003G131300), GATA12 (Potri.006G237700), KNAT7 (Potri.001G112200), MYB4 (Potri.004G174400, Potri.009G134000), MYB42 (Potri.001G118800), MYB85 (Potri.015G129100), MYB103 (Potri.001G099800, Potri.003G132000), WOX13 (Potri.005G101800, Potri.005G252800)), structural genes involved in secondary cell wall formation (CESA (Potri.001G266400, Potri.002G066600, Potri.004G059600, Potri.005G087500, Potri.006G052600, Potri.007G076500, Potri.009G060800, Potri.011G069600, Potri.016G054900, Potri.018G029400), COBL4 (Potri.004G117200), IRX10 (Potri.001G068100), LAC17 (Potri.006G087100, Potri.006G087500) and tension wood formation (PtFLA6, FLA11-like, FLA12-like, xyloglucan endotransglycosylase). Furthermore, many of these gene families have been implicated in tension wood formation in other forest tree species such as *Eucalyptus* (Paux and Carocha 2005) and *Liriodendron* (Jin et al. 2011), and the temporal work in *Populus* may provide an additional level of resolution for identifying orthologous genes involved in tension wood formation in more distantly related species.

To validate our results here, we compared the time-series DE gene clusters to co-expression gene modules previously defined (Gerttula et al. 2015) by an extensive RNA-seq data set from a fully factorial experiment including samples from three *Populus* genotypes subjected to gravitropic treatment and gibberellic acid treatment. As shown in Figure 5, significant overlap was found among the gene clusters/modules independently defined by the two experiments. Additionally, several of the large gene co-expression modules previously identified in the single time point experiment could be further decomposed into sub-modules using the time-series data. More specifically, these large modules were comprised of smaller groups of DE genes that exhibited different temporal responses to the gravi-stimulation treatment such as impulse and sustained expression. This approach not only provides an illustration of cross-validation, but also an advantage of the integrating gene expression data from different experiments addressing the same biological process. While extremely laborious in trees, it would of course be desirable in the future to further validate and extend the results of these experiments to include more treatments or perturbations, for example, through the study of CRISPR-CAS9 deletion mutants for key regulatory genes identified in gene network models.

Computational approaches such as the ones taken here can be used to develop new hypotheses and provide new insights into complex biological processes, including by revealing emergent properties that are not otherwise apparent or expected. Our experiments here show that time-series analyses can be applied to dissect a set of relatively poorly understood, complex biological responses in an undomesticated forest tree. This is especially relevant, as the process of selecting individual genes for study, and creating appropriate genetic materials for studying gene function (e.g., mutants) is challenging and laborious in trees. The potential for further extension of time-series approaches in trees and other plant species is illustrated by experiments in more advanced systems, including prokaryote, yeast, sea urchin and mammalian systems. Examples of analyses performed in these systems that could be potentially extended to plants include using the temporal nature of time-series gene expression data sets to infer causal relationships of transcriptional regulators up- or downregulated in previous time points to changes in expression of putative target genes in later time points (The FANTOM Consortium and Riken Omics Science Center 2009). Such analyses are supported by knowledge of target genes regulated by transcription factors, but that knowledge is quickly expanding for *Arabidopsis* and a handful of model plants, including some modest data for *Populus* (Liu et al. 2015). Future time-series experiments integrating gene expression data with ChIP-seq and other genomic data types associated with gene expression could prove very powerful in advancing the ability to comprehensively describe and dissect complex biological processes in plants.

MATERIALS AND METHODS

Plant material and sample collection

The greenhouse time-series experiment was conducted at the Institute of Forest Genetics in Placerville, CA, USA during August of 2015 and performed using 6-month old clonally propagated hybrid aspen (*Populus alba* × *P. tremula* INRA 717-1B4). Three biological replicates

for each gravi-stimulated time point were placed horizontally for 2, 8, 24, 48 and 96 h, and two replicates for the 336-h time point.

Woody tissues were harvested from the middle third (internodes 20 through 40) of the plant and collected by lightly scrapping the xylem side of a debarked stem using a double-edged razor blade. For gravi-stimulated plants, developing xylem was separately harvested from the upper surface (tension wood) and from the lower surface (opposite wood) of a leaning stem. For time zero samples, two upright grown trees were randomly divided in half and independent xylem samples were assigned to either opposite or tension wood. All tissue was immediately flash frozen in liquid nitrogen and stored at −80°C.

RNA library construction and sequencing

Frozen tissue was ground to a fine powder in liquid nitrogen and total RNA was extracted using TRIzol reagent (Invitrogen, USA) following the manufacturer's protocol. RNA samples were subsequently cleaned using a Qiagen RNeasy on-column DNase treatment (Qiagen, USA) following the manufacturer's protocol. The RNA quantity was measured using a Qubit V2 (Invitrogen, USA) and quality assessed using a Bioanalyzer (Agilent Technologies, USA). RNA sequencing libraries were generated using KAPA mRNA Hyper Prep Kit (KAPA BioSystems, USA) following the manufacturer's instructions and multiplexed using 12 unique adapter sequences (KAPA BioSystems, USA). Multiplexed samples were sequenced using 50 bp single-end runs on an Illumina HiSeq 4000 system (Illumina, USA) at the QB3 Vincent J. Coates Genomics Sequencing Laboratory in June of 2016.

Bioinformatics

Illumina sequencing reads for each RNA library were uniformly processed using the following steps. First, adaptor contaminations were removed using scythe V0.950 (https://github.com/vsbuffalo/scythe) and reads were trimmed using sickle V1.200 using single-end mode with default settings (https://github.com/najoshi/sickle). Second, cleaned sequencing reads were mapped to the *Populus* genome V3.0 (http://www.phytozome.net/poplar.php) using HISAT2 v2.0.4 with default parameters (Kim et al. 2015), and uniquely mapped reads were counted for each gene model using HTseq V0.6.1p1 (Anders et al. 2015) with default settings. Third, gene expression was calculated using the TMM normalization method in edgeR V3.16.5 (Robinson et al. 2010) and expression for each *Populus* gene model was outputted as reads per kilobase per million reads (rpkm). We used *P. trichocarpa* as the reference genome in this study because previous work from our group (Liu et al. 2014) found similar mapping efficiencies between *P. trichocarpa* and hybrid aspen for RNA-seq datasets, and to make the time series results comparable with findings from previous research in our groups and the greater *Populus* community, which have largely aligned to *P. trichocarpa*.

The transcriptional response to gravi-stimulation was evaluated using two approaches. First, transcriptional activity at individual time points was determined using a general linear model in edgeR, and the number of genes was determined using statistical contrasts (FDR $< 1.0 \times 10^{-5}$) between tension wood and opposite wood expression at individual time points. Second, identification of differentially expressed (DE) genes across the entire time-series experiment was determined using natural cubic splines regression with the splineTimeR V1.2.0 package (Michna et al. 2016). This approach compared expression between tension wood and opposite wood tissues from biological replicates across all time points, and significance was determined using a FDR threshold of 1.0×10^{-5}. To reduce the dimensionality of the DE genes, we clustered significant genes from the spline regression results into 16 groups based on mean expression using fuzzy c-means with the Mfuzz V2.34.0 package (Kumar and Futschik 2007) and a fuzzifier value of 1.25.

Functional annotation of DE gene clusters was performed using Gene Ontology (GO) enrichment analysis. The analysis used the *Arabidopsis* best BLAST hits of *Populus* gene models and significant ($P < 0.01$) enrichment of GO terms was calculated using GOstats V2.40.0 (Falcon and Gentleman 2007). We used the *Arabidopsis* annotations for the enrichment analysis because important GO categories associated with hormone regulation and meristem functions have not been annotated in the *Populus* V3.0. The TAIR10 GO annotations were downloaded from agriGO (http://bioinfo.cau.edu.cn/agriGO/). In addition, we present a summary of the GO results because specific functions associated with individual GO terms may not be preserved in more distantly related species due to

evolutionary processes (such as whole genome duplications) that shape functional divergence between orthologous and paralogous genes (Studer and Robinson-Rechavi 2009). Importantly, *Populus* contains a recent whole genome duplication that is not present in *Arabidopsis* (Tuskan et al. 2006). The GO results were summarized by grouping individual GO terms from biological processes and into eight categories that represent key aspects to wood formation: (i) plant hormones (including auxin, GA, brassinosteroid, cytokinin, ethylene, and abscisic, jasmonic and salicylic acids), (ii) peroxisome, (iii) protein localization, (iv) saccharides (including mono- and poly-saccharides), (v) cell wall (including cellulose, xylan, xylose and lignin biosynthesis), (vi) meristem (including shoot development, xylem/phloem patterning), (vii) epigenetics (including histone, methylation and chromatin processes) and (viii) development.

To further characterize the time-series clusters, we tested if changes in expression were correlated with changes in stem response to gravi-stimulation treatments. Using previously published bending results from 55 independent gravi-stimulation experiments using wild type genotypes from the same hybrid aspen system (Figure 1; Gerttula et al. 2015), we correlated mean cluster expression from each wood type with two measurement that summarize gravibending in trees: (i) normalized stem lift (at 0, 24, 48, 96, 336 h) and (ii) rate of bending (at 24, 48, 96, 336 h). Briefly, normalized stem lift was calculated by dividing lift by the length of the stem (measured in points), where lift represents the vertical distance (points) between the position of the stem at a given time point and the horizontal starting position at time zero. The normalized stem lift values are unitless because this metric represents the ratio between two length measurements of the same units. The rate of bending was calculated as the change (Δ) in normalized stem lift per day. The measurements were obtained from digitized traces of images that were captured once a day for 14 d for each of the 55 experiments and calculated using customized scripts (see Gerttula et al. (2015) and https://github.com/mzinkgraf/StemCurve for details). The correlations between expression and bending measurements were calculated using Pearson's correlation. All statistical analyses were implemented in R (R Development Core Team 2017) unless stated otherwise.

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AUTHOR CONTRIBUTIONS

A.G. and V.F. conceived the project. S.G., M.Z., V.F., and A.G. designed the project. S.G., S.Z., M.Z., and A.G. performed the raw data generation. M.Z. performed the data analyses. M.Z., A.G. and V.F. wrote the manuscript. All authors read and approved the final manuscript.

AVAILABILITY OF MATERIALS

The Illumina sequence data associated with the time-series RNA-seq experiment have been deposited in the NCBI sequence reads archive (SRA) under project SRP115696.

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SUPPORTING INFORMATION

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Figure S1. Wood type specific expression of four clusters (clusters 10, 11, 13, 14) that were significantly (*P*-value < 0.05) correlated with the rate of bending

Black points and line depict the mean wood type expression of a cluster through time (h). Red points and line depict the rate of bending in hybrid aspen through time (h) and obtained from Gerttula et al. (2015). The statistics represent Pearson's correlation coefficient (r) and significance of correlation (*P*-value).

Table S1. Time-series clusters, co-expression module (Gerttula et al. 2015), and functional annotations for expressed *Populus* genes

Table S2. Gene ontology enrichment results of biological processes (BP) for time-series clusters

Table S3. Correlation of mean cluster expression and stem bending in hybrid aspen

Bending measurements obtained from Gerttula et al. (2015) and bold text designates a significant correlation (*P*-value < 0.05)

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