

Transcriptional and Hormonal Regulation of Gravitropism of Woody Stems in *Populus*^{OPEN}

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Angiosperm trees reorient their woody stems by asymmetrically producing a specialized xylem tissue, tension wood, which exerts a strong contractile force resulting in negative gravitropism of the stem. Here, we show, in *Populus* trees, that initial gravity perception and response occurs in specialized cells through sedimentation of starch-filled amyloplasts and relocalization of the auxin transport protein, PIN3. Gibberellic acid treatment stimulates the rate of tension wood formation and gravibending and enhances tissue-specific expression of an auxin-responsive reporter. Gravibending, maturation of contractile fibers, and gibberellic acid (GA) stimulation of tension wood formation are all sensitive to transcript levels of the Class I KNOX homeodomain transcription factor-encoding gene *ARBORKNOX2* (*ARK2*). We generated genome-wide transcriptomes for trees in which gene expression was perturbed by gravistimulation, GA treatment, and modulation of *ARK2* expression. These data were employed in computational analyses to model the transcriptional networks underlying wood formation, including identification and dissection of gene coexpression modules associated with wood phenotypes, GA response, and *ARK2* binding to genes within modules. We propose a model for gravitropism in the woody stem in which the peripheral location of PIN3-expressing cells relative to the cambium results in auxin transport toward the cambium in the top of the stem, triggering tension wood formation, while transport away from the cambium in the bottom of the stem triggers opposite wood formation.

INTRODUCTION

Gravity is a universal input that modulates plant growth and development, and various plant lineages and organs have evolved mechanisms to regulate growth and orientation relative to the force of gravity. Much of what is known about plant responses to gravity comes from studies of herbaceous annual plants wherein gravitropic responses rely on differential elongation. By contrast, lignified woody stems can no longer undergo elongation, necessitating a different solution. Instead, gravistimulated woody branches and stems undergo asymmetric radial growth to produce “reaction wood” (Sinnott, 1952; Wilson and Archer, 1977; Ruelle, 2014). In gymnosperms, reaction wood is termed compression wood and forms on the bottom side of the stem where it generates compressive force to push the stem upward (Timell,

1986; Ruelle, 2014). In angiosperms, reaction wood is termed tension wood and forms on the upper side of gravistimulated stems where it generates a tensile force that pulls the stem upward (Mellerowicz and Gorshkova, 2012). Tension wood is produced via an increased rate of cell division in the vascular cambium and is characterized by a reduced number of water conducting vessel elements and specialized tension wood fibers containing a gelatinous cell wall layer (G-layer), which is believed to be central to force generation (Mellerowicz and Gorshkova, 2012). Tension wood fibers are capable of generating a strong contractile force, which results in negative gravitropism of the stem. The term “opposite wood” describes the wood that forms on the lower side of gravistimulated stems, but, because it is anatomically similar to “normal wood” formed by upright stems, opposite wood has received little research attention.

To comprehensively describe the gravitropism of woody angiosperm stems, four questions must be addressed: (1) What are the cells responsible for sensing gravity (i.e., graviperception), (2) what are the signals produced by gravity-sensing cells and how are they perceived by wood-forming and cambial cells, (3) how is force generated by tension wood fibers, and (4) how are the developmental processes leading to tension wood production

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regulated? Currently, which cells are responsible for gravity perception in woody stems is unclear. One possibility is that graviperception occurs in the shoot apex of the stem, and a signal is propagated down the stem. Alternatively, graviperception could occur within the woody stem itself. In *Populus*, starch-filled plastids that could serve as statoliths were observed in some cells of the cortex of young stems (Leach and Wareing, 1967), but no function could be ascribed to these cells in graviperception. Likewise, the mechanisms responsible for the initial graviresponse of woody stems have not been established. Similar to the graviresponses of roots and herbaceous shoots, auxin has been implicated in the graviresponse of woody stems, but conflicting conclusions from experimental ectopic application of auxin versus measurements of auxin concentrations within stems are difficult to reconcile (Hellgren et al., 2004). Other hormones, including gibberellic acid (GA) (Israelsson et al., 2005) and ethylene (Andersson-Gunnerås et al., 2003), have also been implicated in tension wood formation and are potentially involved in initial graviresponse signaling, but a comprehensive understanding of how these hormones interact and affect development of secondary vascular tissues is still incomplete.

Several competing hypotheses for force generation by tension wood formation have been proposed (Clair et al., 2006; Goswami et al., 2008; Mellerowicz and Gorshkova, 2012) but all involve the development of fibers with specialized wall properties. Notably, the G-layer is a tertiary cell wall layer in tension wood fibers characterized by low lignin, high cellulose, and a low cellulose microfibril angle (Norberg and Meier, 1996; Clair et al., 2011) that may be fundamental to force generation. Fasciclin-like arabinogalactan-encoding genes (*FLAs*) are highly upregulated during tension wood formation (Lafarguette et al., 2004; Andersson-Gunnerås et al., 2006; Azri et al., 2014), and the *FLA* protein products have been shown to affect stem biomechanics through changes in cellulose deposition and/or cell wall structural properties (MacMillan et al., 2010). Thus, in addition to serving as molecular markers of tension wood development, *FLA* proteins could be directly involved in contributing to the altered mechanical properties of tension wood. Additionally, xyloglucan endo-transglycosylase (XET)-dependent linkages between the G-layer and secondary cell walls have been identified and have been implicated in the transmission of tensile stress between the G-layer and adjacent cell wall layers (Mellerowicz et al., 2008; Mellerowicz and Gorshkova, 2012).

At the regulatory level, large numbers of genes are differentially expressed in tension wood, including large suites of cell wall- and hormone-related genes (Déjardin et al., 2004; Andersson-Gunnerås et al., 2006). Individual transcription factors have been characterized that affect wood development (Zhong and Ye, 2013), including the *Populus* Class I KNOX homeodomain protein ARBORKNOX2 (*ARK2*; Potri.002G113300), which is orthologous to *Arabidopsis thaliana* BREVIPEDECELLUS/*KNAT1* (At4g08150) (Chuck et al., 1996; Venglat et al., 2002). *ARK2* is expressed broadly in the cambial zone and lignifying cells and affects cell differentiation and expression of tension wood-related genes (Du et al., 2009). However, to date there are no transcription factors identified that specifically affect tension wood development, and the transcriptional networks underlying tension wood development remain poorly defined.

Here, we present experiments in *Populus* that identify the cells responsible for graviperception, describe changes in radial auxin transport during graviresponse, and describe the *ARK2* and GA-sensitive transcriptional networks underlying tension wood development. We suggest that the early graviresponse of auxin transport toward the cambium on the tension wood side of the stem and toward the outer cortex on the lower side of the stem sets in place a developmental switch leading to the different transcriptional and developmental outcomes of these tissues.

RESULTS

ARK2 Transcript Levels Influence Fiber Development and Gravitropism

To develop a comprehensive model of tension wood formation, we first focused on characterizing tension wood formation and gravitropism in *Populus* with altered *ARK2* expression. Using previously characterized genotypes (Du et al., 2009), we assayed gravibending using time-lapse videos for wild-type, *ARK2*-downregulated (*miRNA-ARK2*), and *ARK2*-overexpressing (*OE-ARK2*) *Populus* trees for a 2-week period after the trees were subjected to 90° gravistimulation (Supplemental Movies 1 to 3). *miRNA-ARK2* plants had a reduced gravibending response in comparison to the wild type, whereas *OE-ARK2* plants displayed dramatically enhanced gravibending (Figures 1A to 1C; quantified in Supplemental Figures 1A to 1C and Supplemental Movies 1 to 3). Measurement of elongation growth in upright versus gravistimulated plants revealed an additional difference among the genotypes in response to gravistimulation. The wild type and *OE-ARK2* both reduced elongation growth during gravistimulation, while *miRNA-ARK2* plants did not. Surprisingly, histological comparison of stem sections showed that after 2 weeks of gravibending *miRNA-ARK2* stems had produced more tension wood fibers in comparison to the wild type, whereas the exceptional gravibending response of *OE-ARK2* was associated with fewer tension wood fibers formed (Figures 1D to 1F; Supplemental Figure 2 shows matched opposite wood sections). This difference was also reflected in stem diameters, with *miRNA-ARK2* and the wild type having similar diameters nearly twice that of *OE-ARK2* (Supplemental Figure 1E).

The negative correlation between number of tension wood fibers produced and gravibending among *ARK2* genotypes could reflect differences in the competency of tension wood fibers to generate force in the different genotypes. To test the hypothesis that *ARK2* might also influence the production of functional cell walls capable of generating force, each genotype was assayed for two markers that are known to be associated with maturation of functional tension wood fibers and G-layers, *FLAs* (Lafarguette et al., 2004; Andersson-Gunnerås et al., 2006; Azri et al., 2014), and XET (Mellerowicz et al., 2008; Mellerowicz and Gorshkova, 2012). Immunolocalizations using an antibody (JIM14) recognizing arabinogalactan protein epitopes (Knox et al., 1991) (presumably including *FLAs*) strongly labeled the developing G-layer of tension wood fibers (Figure 2) as well as rays in all wood types (Supplemental Figure 3). JIM14 labeling revealed distinct differences in the maturation schedule of tension wood fibers among

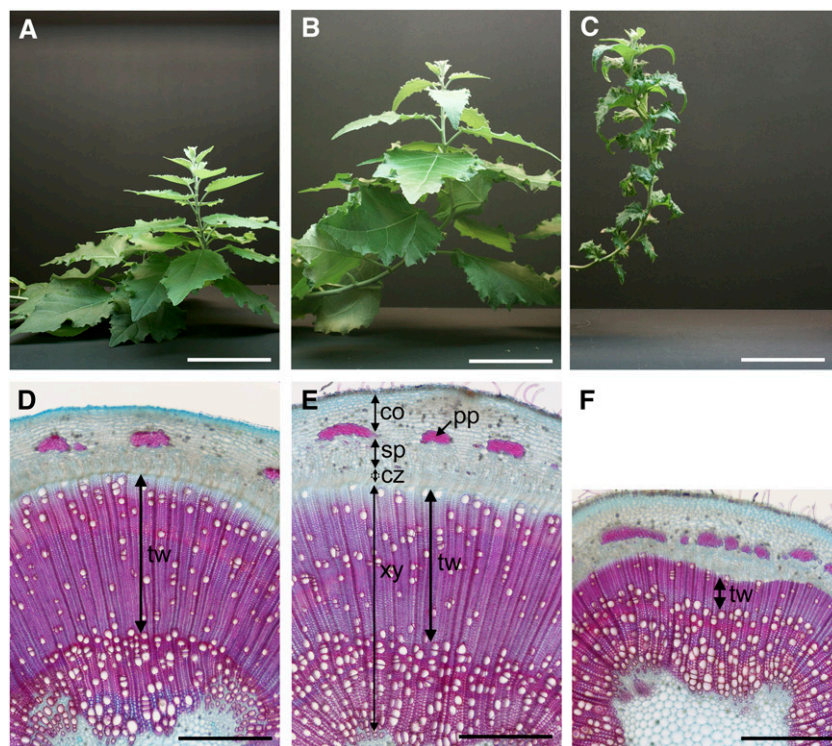


Figure 1. *ARK2* Expression Correlates with Gravibending and Tension Wood Fiber Production.

(A) End point of 2-week time-lapse gravibending trial with miRNA-*ARK2* (see Supplemental Movies 1 to 3; quantified in Supplemental Figure 1).

(B) End point of 2-week time-lapse gravibending trial with the wild type (see Supplemental Movies 1 to 3; quantified in Supplemental Figure 1).

(C) End point of 2-week time-lapse gravibending trial with OE-*ARK2* (see Supplemental Movies 1 to 3; quantified in Supplemental Figure 1).

(D) Histology of tension wood production in miRNA-*ARK2*.

(E) Histology of tension wood production in wild type.

(F) Histology of tension wood production in OE-*ARK2*.

Histological staining in (D) to (F) is with phloroglucinol and astra blue (see Methods). co, cortex; cz, cambial zone; pp, phloem fibers; sp, secondary phloem; tw, tension wood; xy, secondary xylem. Bars = 10 cm in (A) to (C) and 500 μ m in (D) to (F).

the genotypes. OE-*ARK2* trees displayed a distinct G-layer with JIM14 labeling within a few cell divisions of the cambium, while JIM14-labeled G-layers appeared much later and more erratically in miRNA-*ARK2* tension wood (Figures 2A to 2C). For each genotype, the average number of tension wood fibers produced per day and the average number of tension wood fibers between the cambium and the first JIM14-labeled G-layer fiber was determined and used to calculate the average number of days required for G-layers to mature. As shown in Table 1, G-layer maturation required an average of 6.6, 4.9, and 3.6 d for miRNA-*ARK2*, the wild type, and OE-*ARK2*, respectively. Thus, faster maturation of G-layers was associated with enhanced gravibending.

Similarly, in situ imaging of XET activity using the fluorescent substrate XXXG-SR (Nishikubo et al., 2007) labeled cells of the cortex and secondary phloem in all wood types and specifically labeled G-layers in developing tension wood (Figure 3; Supplemental Figure 4). Similar to JIM14 labeling, OE-*ARK2* G-layers were labeled within a few cells of the cambium, while miRNA-*ARK2* showed variable and significantly greater numbers of cells between the cambium and fibers with labeled G-layers (Figure 3). Thus, altering *ARK2* expression manifests in dramatic differences in fiber production, fiber maturation, and stem

gravibending, with gravibending performance positively correlated with the rate of G-layer maturation.

Graviperception and Response Involves Changes in Auxin Transport

We next asked whether changes in tension wood and gravibending associated with altered *ARK2* transcript abundance could reflect differences in auxin transport. While the interpretations have varied (Hellgren et al., 2004), application of exogenous auxin to the upper surface of a *Populus* branch typically inhibits tension wood formation (Wilson and Archer, 1977; Little and Savidge, 1987), raising the possibility that changes in auxin transport down and/or around the stem could influence developmental outcomes. We first measured polar (longitudinal) auxin transport from the shoot apex toward the base (basipetal) in woody stems. The greatest amount of transport was found for miRNA-*ARK2* trees, while OE-*ARK2* trees transported less auxin than wild-type controls (Figure 4A).

To understand how the lateral transport of auxin might be changed during graviresponse, antibodies were raised against *Populus* orthologs of the auxin polar transport protein *Arabidopsis*

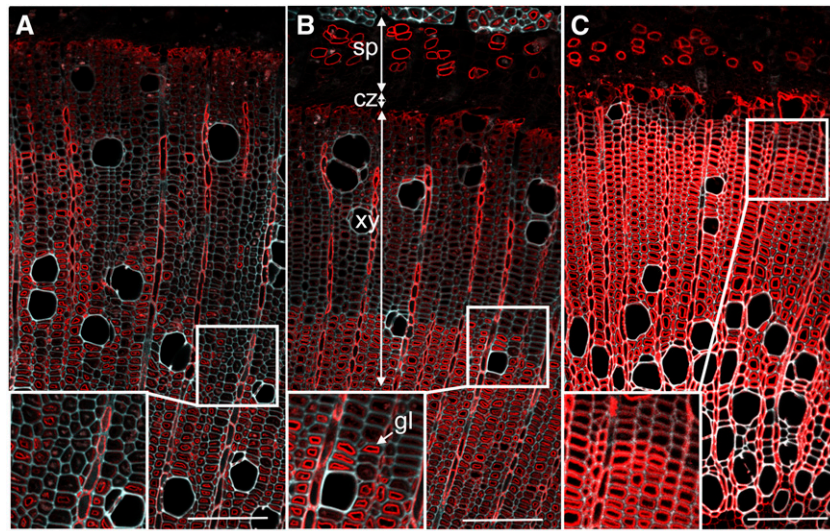


Figure 2. Immunolocalizations with JIM14 Antibody to Reveal Differences in Timing of Arabinogalactan Protein Epitope Development in Developing G-Layers among *ARK2* Genotypes.

(A) miRNA-*ARK2*.

(B) The wild type.

(C) OE-*ARK2*.

In each panel, red signal originates from JIM14 labeling, while blue signal is UV autofluorescence from lignified cell walls. Inset boxes show magnified view of position where first JIM14-labeled G-layer occurs for each genotype, which is quantified in Supplemental Figure 1D. cz, cambial zone; gl, G-layer; sp, secondary phloem; tw, tension wood. Bars = 200 μ m.

PIN-FORMED3, tested for specificity in immunoblots and control immunolocalizations (see Methods; Supplemental Figure 5), and used in immunolocalization experiments to visualize PIN3 protein. In younger, upright stems that had recently initiated secondary growth, PIN3 showed a distinct uniform localization to the plasma membranes of endodermal cells in the inner cortex (Figure 4B), consistent with previous description of this gene's expression in the cortex in hybrid aspen (*Populus tremula* \times *tremuloides*) (Schrader et al., 2003). The endodermal cells contained starch-filled amyloplasts (Figure 4C), which sediment in response to gravity, strongly suggesting these are gravity-perceiving cells. In older stems with more extensive secondary growth, PIN3 expression could be found both in the endodermis as well as cells within the secondary phloem (Figures 4D to 4I). Importantly, acquisition of PIN3 expression by cells in secondary phloem identifies a mechanism by which the woody stems can continue to perceive and respond to gravity after the loss of the endodermal layer during long-term secondary growth. Following 4 d of gravistimulation, PIN3 preferentially localized toward the ground in both the tension wood (Figures 4D to 4F; quantified for all genotypes in Supplemental Figure 6) and opposite wood sides of stems (Figures 4G to 4I). Because the PIN3-expressing cells are peripheral to the cambium in the radial organization of the stem, auxin would thus be predicted to be transported toward the cambial zone on the tension wood side of the stem, but toward the cortex (away from the cambium) on the opposite wood side of the stem. Thus, lateral auxin transport toward the ground by PIN3-expressing cells could elicit differential growth responses of the cambium to gravistimulation on the upper (tension wood) versus lower (opposite wood) sides of the stem.

GA Potentiates Auxin Response and *ARK2*-Dependent Fiber Production

Previous reports have identified GA metabolism genes as direct targets of KNOX transcription factors (Bolduc and Hake, 2009; Hay and Tsiantis, 2010), and GA has been shown to affect the differentiation of fibers during wood development (Eriksson et al., 2000; Mauriat and Moritz, 2009). The effect of GA treatment on auxin responses during graviresponse and tension wood formation was assayed through expression of a DR5:GUS construct

Table 1. Tension Wood Fiber Production and Maturation in *ARK2* Genotypes

Genotype	Fibers Produced ^a	Fibers per Day ^b	Fibers to G-Layer Maturation ^c	Days for G-Layer Maturation ^d
miRNA- <i>ARK2</i>	76.3 (SE 6.2)	5.5	36.4 (SE 6.3)	6.6
Wild type	74.4 (SE 4.7)	5.3	26.0 (SE 1.8)	4.9
OE- <i>ARK2</i>	33.8 (SE 2.3)	2.4	8.7 (SE 1.8)	3.6

Measurements were made for a minimum of three plants per genotype after 14 d of gravistimulation.

^aAverage number of tension wood fibers within a cell file.

^bAverage number of tension wood fibers produced per day.

^cAverage number of tension wood fibers between the cambium and the first fiber with G-layer with JIM14 immunofluorescence signal, a proxy for G-layer maturation.

^dAverage number of days for tension wood fiber G-layer to mature, as assayed by JIM14 immunofluorescence.

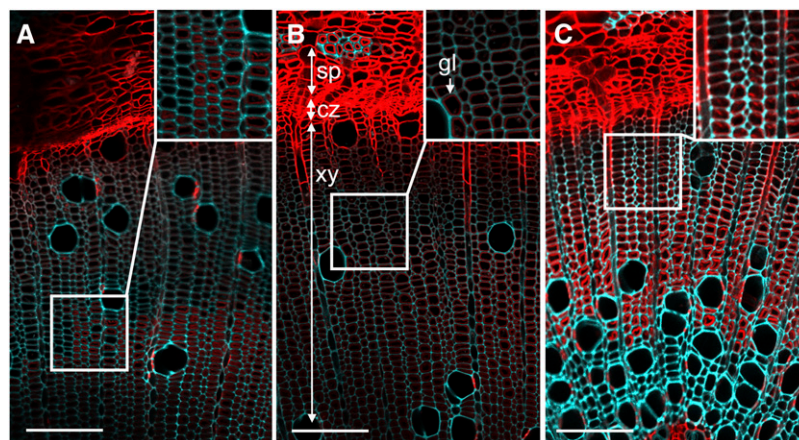


Figure 3. In Situ Imaging of XET Activity in G-Layers to Reveal Differences in Timing of Developing G-Layers among *ARK2* Genotypes.

(A) XET activity in miRNA-*ARK2*.

(B) XET activity in the wild type.

(C) XET activity in OE-*ARK2*.

In each panel, red signal originates from fluorescent XET substrate XXXG-SR incorporation, while blue signal is UV autofluorescence from cell walls. Inset boxes show magnified view of position where XET labeling first occurs in G-layers for each *ARK2* genotype. cz, cambial zone; sp, secondary phloem; xy, secondary xylem. Bars = 100 μ m.

that reports auxin response (Spicer et al., 2013) in trees subjected to a soil drench GA treatment (29 μ M in soil; see Methods). In upright trees, DR5:GUS expression was detected in the cambial zone of trees after GA treatment but not in control trees (Supplemental Figure 7). In untreated trees gravistimulated for 4 d, the GUS reporter staining was weakly apparent in the cambial zone of the tension wood side of the stem, while stronger staining was apparent in the cortex in the opposite wood side of the stem (Figures 5A and 5C). This staining pattern was enhanced in response to GA treatment (Figures 5B and 5D). Notably, the endodermis appeared to be a boundary for GUS staining in the opposite wood side of the stem (Figure 5E), consistent with endodermal PIN3 directing auxin flow toward the cambium on the tension wood side and toward the cortex on the opposite wood side of the stem after gravistimulation. These results also show that increased GA levels potentiate the auxin responsiveness of these same tissues and are reminiscent of GA regulation of PIN protein stability and localization that has been described for root gravitropism in *Arabidopsis* (Willige et al., 2011; Löffke et al., 2013).

The effect of GA on tension wood fiber development was tested by treating wild-type, OE-*ARK2*, and miRNA-*ARK2* trees with a soil drench of either water (control) or 29 μ M GA. Trees treated with GA showed an increase in gravibending response in comparison to controls (Supplemental Figure 7C). GA modestly increased the number of tension wood fibers formed after 2 weeks gravibending for wild-type and OE-*ARK2* trees (Supplemental Figure 7D). By contrast, the number of fibers produced by control and GA-treated miRNA-*ARK2* trees did not vary and was similar to that found in GA-treated wild-type trees (Supplemental Figure 7D). The maturation of G layers as assayed by JIM14 labeling (Supplemental Figure 8) and XET labeling (Supplemental Figure 9) showed similar trends, with GA treatment modestly increasing the rate of maturation in the wild type and OE-*ARK2* but having no significant effect in miRNA-*ARK2*. Together, these observations

suggest that GA stimulates tension wood fiber production and that this effect is sensitive to *ARK2* levels, consistent with negative regulation of GA levels by *ARK2* and elevated endogenous levels of GA in miRNA-*ARK2*.

Transcriptional Networks Associate Gene Expression with Wood Development

In a final set of experiments, computational approaches were used to model the transcriptional regulation of tension wood development using genome-wide transcriptome data from trees in which gene expression had been perturbed by gravistimulation, GA treatment, and modulation of *ARK2* expression. A full factorial experiment was performed with trees of wild-type, miRNA-*ARK2*, and OE-*ARK2* genotypes subjected to either GA or a control (mock) treatment and then either gravistimulated or allowed to remain upright. After 2 d, developing xylem was lightly scraped from both tension wood and opposite wood from gravistimulated trees after debarking or from two opposing sides of the upright trees and subjected to RNA sequencing (see Methods). Differential transcript abundance was calculated for comparisons of wood types, genotypes, and GA treatment, with relatively large numbers of genes showing differential expression in each comparison (Supplemental Figure 10). Notably, comparison of transcript levels across wood types revealed a massive reprogramming of gene expression in opposite wood, with the majority of differentially expressed genes being upregulated in that tissue (Supplemental Figure 10).

Next, weighted gene correlation network analyses (Langfelder and Horvath, 2008) were performed to define modules (clusters) of coexpressed genes whose transcript levels covaried across samples. Modules were initially characterized for robustness by repeated random sampling and recalculation of modules (Supplemental Figure 11A) and by calculating coexpression

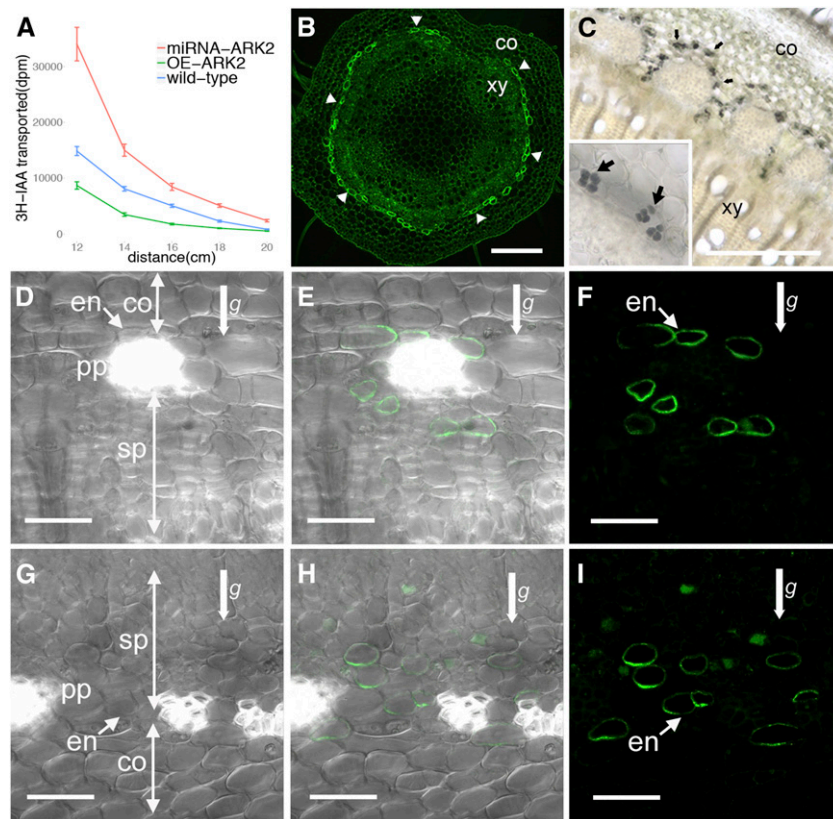


Figure 4. Polar Auxin Transport Differences among *ARK2* Genotypes and Primary Graviresponse through Relocalization of PIN3 Auxin Transport Proteins. **(A)** Quantification of apical to basal auxin transport in stems of *ARK2* genotypes, showing faster transport in miRNA-*ARK2* and slower transport in OE-*ARK2* in comparison to wild-type controls.

(B) Immunolocalization with PIN3 antibody, showing labeling of the endodermis layer in the cortex of this younger, upright stem.

(C) Lugol staining of starch showing staining in the endodermis and secondary phloem (arrows) of cells containing starch-filled amyloplasts (inset).

(D) to (F) Immunolocalization of PIN3 in endodermal and secondary phloem cells on the tension wood side of a wild-type stem gravistimulated for 4 d. *g* indicates the gravity vector. Bright-field **(D)**, PIN3 signal **(F)**, and merged **(E)**.

(G) to (I) Immunolocalization of PIN3 in endodermal and secondary phloem cells on the opposite wood side of a wild-type stem gravistimulated for 4 d. *g* indicates the gravity vector. Bright-field **(G)**, PIN3 signal **(I)**, and merged **(H)**.

co, cortex; en, endodermis; sp, secondary phloem; xy, xylem. Bars = 500 μ m in **(B)** and **(C)** and 50 μ m in **(D)** to **(I)**.

relationships among all combinations of modules (Supplemental Figure 11B). Modules were next annotated to describe the expression and functional properties of genes within modules and their correlations to traits of interest (Supplemental Data Set 1). As visualized by eigengene values (the first principal component of transcript profiles) for each module (Figure 6A), variation in transcript levels across wood types was the primary factor in defining gene coexpression modules, followed by GA treatment and to a lesser extent *ARK2* genotype. Coexpression relationships across modules (Supplemental Figure 11B) were evident based on eigengene values; for example, the sister brown, black, pink, and yellow modules were characterized by tension wood expression, while the sister magenta, blue, green-yellow, purple, and red modules were characterized by opposite wood expression (Figure 6A). Correlations were calculated between each gene module and phenotypic traits, including *ARK2* genotype, wood type, GA treatment, and wood phenotypes (microfibril angle, cellulose crystallinity, fiber morphology, lignin, and carbohydrates;

Supplemental Figure 12). Interestingly, significant correlations were found between each trait and at least one gene module (Figure 6A). All five gene modules with significant correlations to GA treatment (brown, yellow, magenta, blue, and red) were also correlated with *ARK2* genotype, and four were also correlated with wood type. However, of the three modules (brown, black, and pink) showing significant correlations with microfibril angle and cellulose crystallinity traits associated with functional tension wood fibers, only brown showed significant correlation with GA, suggesting that the role of GA may be more closely tied to stimulation of tension wood fiber production than regulation of cell wall properties. Modules were also identified that had significant enrichment for specific Gene Ontology (GO) categories (Supplemental Figure 11C and Supplemental Data Set 2), allowing further insight into the functional properties of modules. For example, each of the modules characterized by tension wood expression had distinct GO enrichment profiles, with the brown module characterized by enrichment for "localization," the black module enrichment for

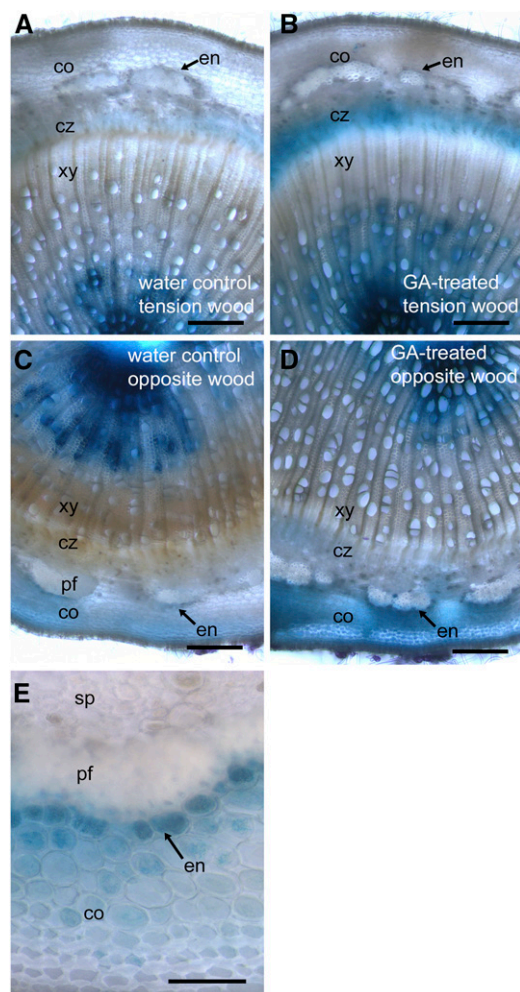


Figure 5. Effects of GA on Auxin Response Measured by DR5:GUS.

Auxin response assayed by the DR5:GUS reporter for tension wood and opposite wood for water control or GA-treated trees. co, cortex; cz, cambial zone; en, endodermis; pf, phloem fibers; xy, secondary xylem. Bars = 200 μ m in (A) to (D) and 50 μ m in (E).

(A) Water control, tension wood.

(B) GA-treated, tension wood.

(C) Water control, opposite wood.

(D) GA-treated, opposite wood.

(E) DR5:GUS expression in the endodermis of opposite wood for a water control tree.

replication, the pink module for hormone, auxin, and cell wall, and yellow module for cell wall, meristem, and localization GO terms. Together, these annotations can be used to develop and test new hypotheses regarding the function of *ARK2*, response to GA, or tension wood development through the identification and further dissection of gene modules of interest, as illustrated below.

ARK2 was placed in the blue module and showed modest connectivity within that as well as several other modules (Supplemental Data Set 1). *ARK2* binding was recently evaluated, genome-wide, using chromatin immunoprecipitation sequencing (ChIP-seq) (Liu et al., 2015). Here, four modules (brown, black,

yellow, and magenta) showed enrichment for genes bound by *ARK2* (Figure 6A). All of the *ARK2* binding enriched modules also showed correlations with wood type and three showed correlations with GA treatment (brown, yellow, and magenta) and/or genotype (brown, yellow, and magenta), reflecting our experimental data indicating that *ARK2* and GA interact and have major effects on wood development. Together, the modest connectivity of *ARK2* and binding to large numbers of genes of diverse function suggest that this transcription factor does not act as a “master regulator.” Instead, these data are more consistent with *ARK2* acting as a transcription factor that influences and potentially integrates multiple development-related processes, including cell wall development and hormone-related functions.

As an example of how coexpression modules can be further dissected to understand tension wood development, we focused on the pink module, which is characterized by expression in tension wood across all genotypes (Figure 6A) and has correlations with cellulose crystallinity and microfibril angle that are correlated with tension wood development and function (Figure 6A; Supplemental Figure 12). When genes within this module were plotted relative to their correlation with wood type and module membership (a measure of connectivity within the module), several FLA-like genes that are markers of tension wood development (Lafarguette et al., 2004; Andersson-Gunneras et al., 2006; Azri et al., 2014) and reflected in our JIM14 labeling of tension wood G-layers (Figure 2) were identified in the upper quartile of both traits (black data points in Figure 6B; Supplemental Data Set 1), suggesting this module contains genes directly relevant to differentiating tension wood. Strikingly, the transcription factors within the module (red data points in Figure 6B) include 11 MYB, 6 NAC, and 2 KNOX transcription factors incorporating orthologs of characterized genes known to regulate meristematic growth (Zhou et al., 2009), fiber development, and/or secondary cell wall biosynthesis in *Arabidopsis* (e.g., MYB52 and MYB85, KNAT6 and KNAT7, BLH4 and VND1; Belles-Boix et al., 2006; Ragni et al., 2008; Zhong et al., 2008; Liu et al., 2014; Taylor-Teeples et al., 2015). These serve as excellent candidates for regulating the expression of other genes within the module (Supplemental Data Set 1), which include genes central to gravi-response and tension wood formation, including auxin (e.g., *PIN1* and *PIN3* orthologs), GA biosynthesis (*GA20-Oxidase*), and cell wall biosynthesis (e.g., *IRREGULAR XYLEM* genes and 19 *FLAs*). Finally, potential radial signaling-related genes with high wood type and module membership also reside in the pink module, including CLE13-like and SCARECROW-like 8.

FLA Expression Reveals Differential GA Response across Wood Types and *ARK* Genotypes

Looking in detail at the expression of the *FLAs* within the pink module, three trends are apparent (Figure 6C): (1) *FLA* transcript levels are highest in tension wood and lowest in opposite wood in all genotypes ($t = 15.304$, $df = 18$, P value = 4.603×10^{-12}), (2) *FLA* transcript levels are lower in the tension wood of miRNA-*ARK2* than the wild type or OE-*ARK2* ($t = -12.764$, $df = 18$, P value = 9.284×10^{-11}), and (3) *FLA* transcript levels show *ARK2*-dependent differential expression in response to GA treatment in tension wood and opposite wood (P values < 0.001). Specifically, *FLA*

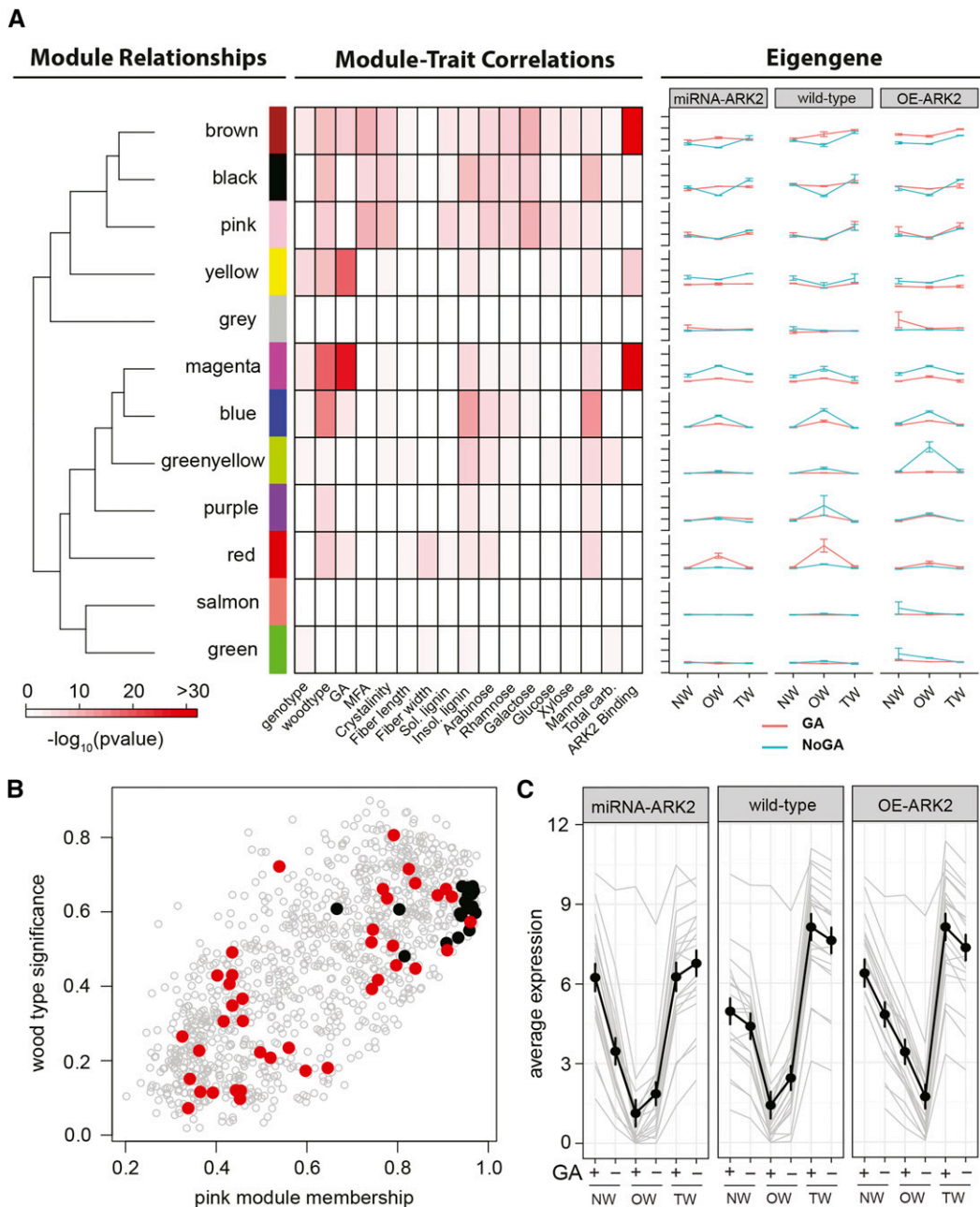


Figure 6. Transcriptional Network Regulating Wood Formation and Associations with Phenotypes.

(A) Dendrogram showing relationships of gene modules (colors), correlations of individual gene modules with phenotypes, and module eigengene expression across *ARK2* genotypes (miRNA, miRNA-*ARK2*; OE, OE-*ARK2*) and wood types (NW, normal wood; OW, opposite wood; TW, tension wood) in response to GA treatment.

(B) Plot of genes in the pink module for differential expression across wood types (y axis) and module membership/connectivity (x axis). Black data points are *FLA* genes. Red data points are transcription factor-encoding genes.

(C) Line graphs showing the average (bold line) and individual transcript levels (gray lines) of the 19 *FLA* genes in the pink module across *ARK2* genotypes and wood types in response to GA treatment.

levels are induced by GA treatment in tension wood for OE-*ARK2* and the wild type, but miRNA-*ARK2* shows repression of *FLA* transcript levels by GA. These results are reminiscent of the responses of these same genotypes to GA treatment with regards to maturation of G-layers as assayed by JIM14 immunolocalization

(Supplemental Figure 8). Additionally, *FLA* levels are repressed by GA in opposite wood of wild-type and miRNA-*ARK2* trees, but increased by GA in opposite wood of OE-*ARK2*. Thus, the response to GA is different across both wood types and *ARK2* genotypes, potentially revealing a developmental switch set in

place by the initial graviresponse and radial auxin transport that is sensitive to *ARK2* transcript levels.

DISCUSSION

The negative gravitropism of woody angiosperm stems is fundamentally different from that of herbaceous stems. The herbaceous stem achieves negative gravitropism through differential growth on the bottom of the stem that pushes the stem upward. By contrast, the woody stem undergoes differential growth on the top of the stem to produce tension wood that pulls the stem upward with contractile forces (Sinnott, 1952; Wilson and Archer, 1977; Ruelle, 2014). In this study, we identified key regulatory mechanisms involved in negative gravitropic responses in woody *Populus* stems, including the cells responsible for graviperception, the initial graviresponse via relocalization of the PIN3 auxin transporter, and interacting roles for auxin, GA, and the *ARK2* transcription factor in regulating fiber development and gravibending.

Using *Populus* as a model species, we found that graviperception likely occurs directly in the woody stem in specialized cells containing starch-filled amyloplasts that sediment toward the ground. These cells are further defined by expression of the auxin transport protein, PIN3, which relocalizes toward the ground during gravistimulation. The PIN3-expressing cells are initially found in the endodermis, similar to what has been found in *Arabidopsis* (Friml et al., 2002) and other herbaceous stems, but later in development cells within the secondary phloem acquire PIN3 expression and possess amyloplasts. Thus, even though the endodermis is eventually lost during the outward expansion of the cortex during secondary growth, older woody stems can continue to sense gravity as new cells within the secondary phloem acquire PIN3 expression. Importantly, the reorientation of PIN3 toward the ground in gravistimulated woody stems would be predicted to have different outcomes on auxin transport in the top versus bottom of the stem. Because of the radial organization of the stem, on the top (tension wood side) of a gravistimulated stem PIN3 orientation is toward the cambium and center of the stem, whereas in the bottom (opposite wood side) of the stem PIN3 orientation is toward the cortex and periphery of the stem. These orientations are reflected in expression of a *DR5::GUS* reporter in our experiments, which shows preferential cambial expression on the top and cortical expression in the bottom of gravistimulated stems. This lateral auxin transport and response represents the earliest known graviresponse of woody stems and provides an obvious initial mechanism triggering the different developmental fates of xylem tissues that develop on the top (tension wood) versus bottom (opposite wood) of the gravistimulated stem.

Our results concerning the location of graviperceptive cells and radial auxin transport in the tension wood versus opposite wood side of gravistimulated stems may also help in understanding previous, apparently conflicting, research results. Exogenous application of auxin to the upper surface of a leaning stem resulted in inhibition of tension wood formation (Cronshaw and Morey, 1968), while application of antiauxins induces tension wood (Cronshaw and Morey, 1965). These results led to the proposition that low auxin levels in the cambium induce tension wood

development. However, direct measurement of auxin in gravi-stimulated *Populus* stems did not detect lower levels in cambia producing tension wood in comparison to cambia in upright trees; in fact, significantly lower auxin levels were found for cambia producing opposite wood (Hellgren et al., 2004). Our results would suggest that exogenous auxin application could likely mimic the conditions in the opposite wood side of the stem, where auxin transport would result in higher auxin and auxin response in the cortex than the cambial zone. The lower levels of auxin reported for the cambial region on the bottom of the stem (producing opposite wood) are consistent with auxin transport away from the cambium, as suggested by PIN3 localization and the *DR5::GUS* reporter in our experiments. However, the previous observation that levels of auxin in cambia producing tension wood are not higher than those for cambia producing normal wood (Hellgren et al., 2004) may indicate that it is not necessarily the absolute concentration of auxin in the cambial zone that determines the type of wood produced by the cambium. Instead, the relative amounts of auxin across the radially arranged secondary vascular tissues or differential sensitivity of auxin across these tissues may be more important. It is also possible that auxin itself is not directly inducing the different developmental outcomes at the cambial zone, but rather is involved in generating the signals that more directly elicit the developmental changes.

Auxin is not the only hormone influencing wood development, and we found GA to be an important hormonal regulator of tension wood, capable of stimulating fiber production and gravibending. Interestingly, previous authors have also attributed positive regulation of tension wood development to GA and speculated, based on gene expression and direct measurement of GA, that GA precursors may be synthesized in the phloem and transported to the xylem, potentially through rays (Israelsson et al., 2005). The rays interconnect the secondary xylem (wood), cambium, and secondary phloem (Lev-Yudan and Aloni, 1995) and, although their role in development is poorly understood, could be important in radial signaling in woody stems. For example, radial movement of auxin from the primary vascular bundles to the cambial zone has been demonstrated in *Populus* and likely involves the rays (Spicer et al., 2013). Strikingly, ectopic application of GA can induce tension wood formation (Nakamura et al., 1994), making it a good candidate for a more direct signal controlling differentiation downstream of auxin. We found that transcriptional responses to GA treatment were sensitive to *ARK2* transcript levels and that, in contrast with wild-type or *ARK2* overexpressing trees, trees with reduced *ARK2* transcript abundance failed to produce additional tension wood fibers in response to GA treatment. These results would be consistent with the idea that decreased levels of *ARK2* transcript are associated with elevated GA levels, similar to previous work in herbaceous species that have demonstrated a direct positive regulation of GA catabolism-related and negative regulation of GA biosynthesis-related genes by Class I KNOX transcription factors (Sakamoto et al., 2001; Jasinski et al., 2005; Bolduc and Hake, 2009). In addition, we found that GA potentiated the expression of the auxin-responsive *DR5::GUS* reporter in gravistimulated stems. This result is reminiscent of previous studies in trees showing GA stimulation of polar auxin transport during wood development (Bjorklund et al., 2007) and of reports of

GA regulation of PIN protein stability and localization during root gravitropism in *Arabidopsis* (Willige et al., 2011; Löffke et al., 2013).

Additionally, we found evidence that fiber production can be genetically decoupled from the maturation of cell walls competent of generating contractile force. We imaged two molecular markers of functional tension wood G-layers: JIM14 immunolocalization of arabinogalactan proteins (including FLAs) and XET in gravistimulated stems of wild-type trees and trees with increased or decreased *ARK2* transcripts. Stems with decreased *ARK2* transcript abundance produce more fibers but are delayed in fiber maturation (as assayed by JIM14 and XET labeling) and display reduced gravibending. Stems with increased *ARK2* transcripts produce fewer fibers, but fiber maturation and gravibending are enhanced. Thus, force generation and gravibending can be genetically decoupled from fiber differentiation, per se. While we expect that the dramatic differences in fiber maturation among *ARK2* genotypes is a primary factor, undoubtedly other differences such as stem stiffness also contribute to the observed differences in gravibending among these genotypes.

We used GA treatment, *ARK2* transcript levels, and gravistimulation as perturbations of gene expression to model gene coexpression networks underlying tension wood formation. An unexpected finding using comparisons of differentially expressed gene transcripts across all treatments was that opposite wood undergoes a massive reprogramming of gene expression. While typically assumed to play a passive role in gravibending, these results suggest the possibility of an active role, perhaps through production of signals or metabolic contributions to gravitropism. To more fully extract information from the transcriptome data, gene modules were defined based on gene coexpression across wood types, genotypes, and GA treatments. Each module was annotated and characterized based on correlations with wood phenotypes, GA response, *ARK2* binding to genes within specific modules, correlations with wood properties, and enrichment of member genes in specific gene ontology categories. These annotations allowed us to not only identify gene modules of interest for further study, but also define relationships among modules and make inferences about mechanisms regulating xylem development. For example, of the three modules showing significant correlations with microfibril angle and cellulose crystallinity (traits strongly associated with tension wood), only two were correlated with GA treatment, potentially reflecting our experimental data showing the primary effect of GA was in promoting fiber development but not cell wall development. We were also able to dissect modules based on annotations, module membership (a measure of connectivity of a gene with other genes in the module), and differential expression across treatments. For example, *ARK2* showed only modest connectivity and differential expression across wood types. Along with the observation that *ARK2* binds to large numbers of genes of diverse function, these properties suggest that *ARK2* is not a “master regulator” but rather influences the expression of genes of different function, potentially to integrate different developmental processes. This would be consistent with reports of *KNOX* genes regulating both hormone-related as well as cell wall-related genes in other model species (Sakamoto et al., 2001; Mele et al., 2003; Jasinski et al., 2005; Bolduc and Hake, 2009; Bolduc et al., 2012).

The interaction of GA and *ARK2* in regulating tension wood development was evident not only in experimental data, but also in coexpression analyses. A coexpression module was selected for dissection that showed significant correlations with tension wood properties and contained a large number of FLAs implicated in the tension wood development and function (Lafarguette et al., 2004; Andersson-Gunneras et al., 2006; Azri et al., 2014). Interestingly, the expression of these *FLA* genes was differential across both wood types and *ARK2* genotypes in response to GA, similar to what we observed in response to GA treatment of fiber production and maturation across *ARK2* genotypes. Taken together, these results are consistent with *ARK2* negatively regulating GA levels, as has been reported in other species (Sakamoto et al., 2001; Hay et al., 2002; Bolduc and Hake, 2009), but also having an additional role in regulating cell differentiation and cell wall biosynthesis.

In conclusion, the results from these studies not only provide insights into the regulation of gravitropism and wood formation in angiosperm trees, but also demonstrate that the combination of experimental and computational approaches can be used to dissect complex developmental traits in these long-lived organisms. Challenges for future studies include determining the evolutionary relationships of graviperception and graviresponse mechanisms in woody versus herbaceous stems and roots and whether the mechanisms described here for *Populus* also function across the amazing diversity of angiosperm tree species.

METHODS

Replication

In all experiments, a minimum of three biological replicates were included for each treatment.

Plant Growth

The genetic background of all trees utilized is hybrid aspen (*Populus alba* × *Populus tremula*) clone INRA 717-IB4. Representative *ARK2* transgenic genotypes were selected from those previously described and characterized (Du et al., 2009). The creation and characterization of the DR5:GUS reporter line was characterized and reported previously (Spicer et al., 2013). Plants were grown at ~22°C under continuous light provided by Philips TL830 and TL850 fluorescent bulbs. Plants used for time-lapse movies, immunolocalizations, XET labeling, GUS expression assays, and histology were transferred to soil (Sunshine Mix 4; SunGro Horticulture) in 0.5-liter pots, covered by a transparent lid (Super Sprouter), and adjusted to ambient humidity over the course of 2 weeks. Plants were used for experiments after 2 months growth in soil with water supplemented with Miracle Grow fertilizer using the concentration recommended by the manufacturer. For GA soil drench treatments, 50 mL of 0.01% GA (Sigma-Aldrich G-7645) in water was added to each pot to give an approximate concentration of 29 µmol GA in the soil. Plants were grown for four additional days after GA treatment prior to gravistimulation.

Histology, Imaging, and Auxin Transport Measurement

Time-lapse movies were captured using Wingscape time-lapse digital cameras (Wingscapes; EBSCO Industries). Plants were positioned horizontally and imaged once every 10 min on the first day and then once per hour for 2 weeks. Quantification of stem lift and curvature was analyzed using custom R scripts, using data derived from digitized tracings of stems from individual frames taken from time-lapse movies. Tracings analyzed

were from the base of the stem to the apex of curvature (defined as the basal point of tropism of the primary, herbaceous portion of the stem).

Stems were sectioned for histology using a Vibratome Series 1000 (Heath Company) to a thickness of 50 to 100 μm . Sections for anatomical assays were stained with astra blue and phloroglucinol (Ruzin, 1999) and visualized with a Leica DMB compound microscope using a Leica DFC450C digital camera with live image stitching using Leica Live Image Builder software.

For XET activity visualization, XXXG-SR was synthesized as previously described (Nishikubo et al., 2007). The previously described labeling protocol (Nishikubo et al., 2007) was modified by utilizing Vibratome sections rather than hand-sections and by blocking sections (25 mM MES, pH 5.5, 1% BSA, and 2% fish gelatin) prior to incubation with 4 $\mu\text{g}/\text{mL}$ XXXG-SR in block. For estimation of background signal, sections were boiled for 10 min prior to blocking and incubation with XXXG-SR. After overnight incubation at room temperature, sections were rinsed four times with 25 mM MES and then stepped gradually into a wash solution of 15:1:4 ethanol:formic acid:water. After multiple changes of wash solutions, sections were stepped back to 25 mM MES prior to imaging on a Zeiss LSM 710 confocal microscope.

The Pt-PIN3 antibody was raised against a conjugated peptide (DQREKEGPTGLNKLGS-C) unique to paralogous *Populus* PIN3-like proteins Potri.008G129400 and Potri.010G112800 in rabbits and used at a dilution of 1:250. To assay specificity of the antibody, membrane proteins were extracted from both leaf and stem tissues of *P. alba* \times *P. tremula* clone INRA 717-IB4. Tissues were ground in liquid nitrogen and suspended in an extraction buffer before extraction as previously described (Abas and Luschnig, 2010). Resulting pellets were frozen at -80°C and then thawed and subjected to a second extraction using transmembrane protein extraction reagent (FIVEphoton Biochemicals) according to the manufacturer's protocol.

Immunolocalizations were performed using Vibratome stem sections that were fixed at room temperature in formalin, acetic acid, and alcohol for 45 min under vacuum. Sections were then stepped to PBT (1 \times PBS with 0.2% Tween 20) and washed four times 15 min each. Sections were then blocked (1 \times PBS, 2% fish gelatin, 1% BSA, and 0.2% Tween 20) for 1 h prior to addition of the primary antibody. The JIM14 antibody was obtained from the Complex Carbohydrate Research Center (University of Georgia, Athens, GA) and used at a dilution of 1:500. After overnight incubation at 4°C , sections were washed four times in 1 \times PBT, blocked for 1 h, and probed with biotin-labeled antibodies (for JIM14, biotin-xx-goat-anti-rat IgG, Life Technologies A10517; for Pt-PIN3 biotin-xx-goat-anti-rabbit IgG, Life Technologies B2770) at 1:400 dilution. After overnight incubation at 4°C , sections were washed four times with PBT, blocked for 1 h, and 4 μL Streptavidin Alexa Flour-488 (Life Technologies S11223) added per milliliter of block. After 0.5 h incubation at room temperature, sections were washed four times with PBT prior to visualization on a Zeiss LSM 710 confocal microscope.

GUS staining was performed on 100- to 150- μm thick sections by vacuum infiltration of staining solution (100 mM sodium phosphate, pH 7, 10 mM EDTA, 0.1% Triton X-100, 0.5 mg/mL X-glucuronide, and 100 $\mu\text{g}/\text{mL}$ chloramphenicol) followed by overnight incubation at 37° . Sections were cleared in 70% ethanol prior to imaging on a Leica DMLB compound microscope with Leica DFC450C digital camera and live image stitching using Leica Live Image Builder software.

Basipetal (toward the root) auxin transport was measured in stems of poplar seedlings in tissue culture (Groover et al., 2006) using a radio-tracer auxin transport assay adapted from *Arabidopsis thaliana* inflorescences (Lewis and Muday, 2009). Developmentally matched seedlings with consistent stem thickness were stripped of leaves and cut 20 and 40 mm from the shoot apex, forming a 20-mm section. These samples were inverted and placed into an open 0.5-mL microcentrifuge tube containing 200 μL 100 nM ^3H -IAA (24 Ci/mmol; American Radiochemical) such that

the apical cut end was submerged. Assay tubes were placed in a sealed box filled with water to prevent evaporation. After 18 h, the seedlings were removed and the apical 10 mm from the site of auxin application was removed and discarded. The next remaining 10-mm basal section was then divided into 2-mm segments and assayed for radioactivity in a Beckman LS6500 scintillation counter. The average and SE of ^3H -IAA in each sample is reported relative to the distance to the apical end.

RNA-seq and ChIP-seq

Plants grown as described previously were transferred to 8-liter pots in Sunshine Mix 4 (SunGro Horticulture) and grown in a greenhouse in Placerville, CA without supplemental light for 3 months. For GA soil drench treatments, 0.01% GA in water was added to each pot to give an approximate concentration of 29 μM GA in the soil. Trees subjected to gravistimulation were placed horizontally 4 d after GA treatments were applied, and differentiating xylem for RNA-seq was harvested from all plants 2 d after gravistimulation commenced. For gravistimulated trees, bark was removed from the top (for tension wood) or bottom (for opposite wood) quarter of the stem and differentiating xylem harvested by light scraping with a sterile double-edged razor blade. For upright trees, the stem was randomly divided in half, and two xylem samples were independently harvested from opposing sides. For all trees, xylem was harvested from the twentieth leaf to the midpoint of the total length of the stem and flash-frozen in liquid nitrogen. Total RNA was isolated by grinding the tissue to a fine powder in liquid nitrogen, followed by extraction using Trizol (Life Technologies) using the manufacturer's suggested protocol. RNA samples were then treated with RNase-free DNase (Qiagen) prior to secondary isolation using the Qiagen RNeasy kit following the manufacturer's protocol. Quality and quantity of RNA was assessed using Qubit (Life Technologies) and Bioanalyzer (Agilent Technologies), respectively, prior to generation of RNA-seq libraries using the Illumina TruSeq Library Preparation Kit V2. Six samples were multiplexed into each lane of an Illumina HiSeq run to produce 50-bp single-end reads. ARK2 ChIP-seq data were produced and analyzed as previously described (Liu et al., 2015) and are available as NCBI SRA accession SRP0533 or through popGENIE (<http://popgenie.org/>).

Bioinformatics

For each RNA-seq library, contamination from adaptor sequences was removed using scythe V0.95 (<https://github.com/vsbuffalo/scythe>), and sequenced reads were trimmed using sickle (Joshi and Fass, 2011) with default settings. Sequenced reads were then mapped to the *Populus* genome assembly V3.0 (<http://www.phytozome.net/poplar.php>) using TopHat v2.0.6 (Trapnell et al., 2009), and reads for *Populus* V3.0 gene models were counted using HTseq (Trapnell et al., 2009) version 0.5.4p1. Gene expression levels were calculated as reads per kilobase per million reads using the TMM normalization method in the package edgeR V3.6.8 (Robinson et al., 2010). All statistical analyses were implemented in R (R Development Core Team, 2014) unless stated otherwise.

Coexpression and module analyses were performed using functions from the R package WGCNA V1.41-1 (Langfelder and Horvath, 2008) and publically available scripts (<http://labs.genetics.ucla.edu/horvath/CoexpressionNetwork/Rpackages/WGCNA/>). Briefly, coexpression relationships between genes were summarized as an adjacency matrix and raised to the soft threshold power of eight. The soft threshold power was determined based on a greater 80% model fit to scale-free topology and low mean connectivity. Coexpression modules were defined as clusters of highly interconnected genes and calculated using hierarchical clustering of dissimilarity of the topological overlap measure. Hierarchical clustering was performed using dynamic tree cutting with a minimum module size of 500 and a cut height of 0.998, and closely related modules (0.3) based on module eigengene values were collapsed. Module stability was assessed

by resampling 75% of the RNA-seq libraries and rerunning the coexpression analysis 20 times (Langfelder and Horvath, 2012).

Traits were related to modules using module eigengene values. Module correlations for experimental treatments such as *ARK2* genotype, wood type, and GA treatments were tested using ANOVA, and wood chemistry phenotypes were tested using Fisher's exact tests. Enrichment of *ARK2* ChIP-seq binding in coexpression modules was determined based on a one-tailed hypergeometric distribution. Hub genes in the pink module were identified as genes that had high gene significance with wood type and high module membership in the pink module. Gene significance was calculated as the correlation between gene expression (reads per kilobase per million reads) and wood type. Module membership was calculated as the correlation between gene expression and module eigengene values for the pink module.

Functional enrichment of coexpression modules was based on GO enrichment and calculated using Arabidopsis best BLAST hit of *Populus* gene models. GO analysis was performed using Arabidopsis GO annotations because categories related to relevant categories (including meristem, shoot development, hormones, cell wall and cellulose) have limited annotation in *Populus*. Analyses were performed using the Bioconductor package GOSTats V2.30.0 (Falcon and Gentleman, 2007) with $P < 0.01$.

Wood Chemistry and Cell Wall Phenotyping

A micro-Klason method was applied to quantify lignin and cell wall carbohydrates (Coleman et al., 2007). Approximately 200 mg of extracted, oven-dried wood was reacted in a 125-mL serum bottle with 3 mL of 20°C 72% (w/w) sulfuric acid (H_2SO_4), with thorough mixing with a glass rod for 1 min every 10 min, followed by mixing for 15 s every 10 min thereafter. After 2 h, 112 mL deionized water was added to the reaction. The serum bottles were then sealed and immediately autoclaved at 121°C for 60 min. Sugar standards were subjected to the same treatment. Samples were allowed to cool and settle at 4°C and were vacuum-filtered through preweighed medium coarseness (M) sintered-glass crucibles. Each sample was washed (and the serum bottle also rinsed) with 200 mL warm (~50°C) deionized water to remove residual acid and sugars and dried overnight at 105°C. The dry crucibles were reweighed to determine Klason lignin (acid-insoluble lignin) gravimetrically. The filtrate was then analyzed for acid-soluble lignin by absorbance at 205 nm using a UV/Vis spectrometer. Samples had to be diluted with 4% H_2SO_4 to reach an absorbance value between 0.2 and 0.7. The concentrations for neutral cell wall-associated carbohydrates (glucose, xylose, mannose, galactose, rhamnose, and arabinose) in the hydrolyzate were determined using HPLC. Prior to injection, samples were filtered through 0.45- μ m HV filters (Millipore). The samples for HPLC runs were prepared by exact weights of ~950 mg hydrolyzate + 50 mg fucose as the internal standard. The concentrations of sugar monosaccharides were determined using a Dionex HPLC system (DX-600) equipped with an ion exchange PA1 column (Dionex), a pulsed amperometric detector with a gold electrode, and a SpectraAS3500 auto injector (Spectra-Physics). The column was equilibrated with 250 mM NaOH and eluted with deionized water at flow rate of 1 mL/min. Total lignin content was calculated as the sum of the Klason lignin and soluble lignin fractions. Lignin and cell wall sugar content was determined as a percentage of dry wood, based on the initial weight of the extract-free wood sample analyzed.

Microfibril angle and cell wall crystallinity were determined by x-ray diffraction using a Bruker D8 Discover x-ray diffraction unit equipped with an area array detector (GADDS) on the radial face of the wood section precision cut (1.68 mm) from the growing stem isolated from the tension wood and opposite wood sections isolated from 10 cm above the root collar. Wide-angle diffraction was used in transmission mode, and the measurements were performed with $CuK\alpha 1$ radiation ($\lambda = 1.54 \text{ \AA}$), the x-ray source fit with a 0.5-mm collimator, and the scattered photon collected by a GADDS detector. Both the x-ray source and the detector were set to $\theta = 0^\circ$

for microfibril angle determination, while the 2θ (source) was set to 17° for wood crystallinity determination. The average T-value of the two 002 diffraction arc peaks was used for microfibril angle calculations, as per the method of Ukrainetz et al. (2008), while crystallinity was determined by mathematically fitting the data using the method of Coleman et al. (2007). Crystallinity measures were precalibrated by capturing diffractograms of pure *Acetobacter xylinum* bacterial cellulose (known to be 87% crystalline).

Fiber length was analyzed using the woody material isolated from the tension wood and opposite wood side of each specimen, according to Park et al. (2008). In brief, each sample was macerated in a solution consisting of 30% hydrogen peroxide and glacial acetic acid in a 1:1 ratio (Franklin solution) at 70°C for 48 h. Following the reaction, the remaining fibrous material was washed with deionized water until the samples were neutralized. Subsamples were resuspended in 10 mL deionized, distilled water and analyzed on a fiber quality analyzer (FQA; Optest Equipment). Fiber length was recorded in millimeters.

Accession Numbers

The sequences associated with RNA-seq experiments are deposited in NCBI SRA under BioProject PRJNA284622. The sequences associated with ChIP-seq of *ARK2* are deposited in NCBI SRA under accession SRP0533. Phytozome accession numbers for *Populus* orthologs of Arabidopsis genes discussed in reference to gene module dissection are as follows: *MYB52* (Potri.012G039400), *MYB85* (Potri.015G129100), *KNAT6* (Potri.010G043500), *KNAT7* (Potri.001G112200), *BLH4* (Potri.007G032700), *VND1* (Potri.007G014400), *PIN1* (Potri.015G038700), *PIN3* (Potri.015G038700 and Potri.008G129400), *GA20-Oxidase* (Potri.015G134600), *IRREGULAR XYLEM* genes (Potri.004G117200, Potri.009G042500, and Potri.001G248700), *CLE13-like* (Potri.008G115600), and *SCARECROW-like 8* (Potri.004G078800).

Supplemental Data

Supplemental Figure 1. Quantification of gravitropic bending and longitudinal growth in *ARK2* genotypes during a 2-week gravistimulus trial.

Supplemental Figure 2. Opposite wood histology.

Supplemental Figure 3. JIM14 immunolocalization of arabinogalactan protein epitopes in opposite wood of *ARK2* genotypes and negative control showing background labeling.

Supplemental Figure 4. Imaging of XET activity in opposite wood of *ARK2* genotypes.

Supplemental Figure 5. PIN3 and control immunolocalization.

Supplemental Figure 6. PIN3 quantification in tension wood and localization in opposite wood.

Supplemental Figure 7. GA effects on DR5:GUS expression in upright stems and on gravibending in *ARK2* genotypes.

Supplemental Figure 8. JIM14 immunolocalization for stem sections of trees treated with GA.

Supplemental Figure 9. In situ imaging of XET activity in stems of trees treated with GA.

Supplemental Figure 10. Differential expression of genes in opposite wood versus tension wood and normal wood.

Supplemental Figure 11. Characterization of gene coexpression network modules.

Supplemental Figure 12. Quantification of wood property and chemistry traits for wood types within each *ARK2* genotype.

Supplemental Movie 1. Time-lapse movie of wild-type poplar undergoing gravitropic bending.

Supplemental Movie 2. Time-lapse movie of OE-ARK2 poplar undergoing gravitropic bending.

Supplemental Movie 3. Time-lapse movie of miRNA-ARK2 poplar undergoing gravitropic bending.

Supplemental Data Set 1. Gene module memberships and statistics for poplar genes expressed during wood formation.

Supplemental Data Set 2. Gene Ontology enrichment analysis for gene modules.

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AUTHOR CONTRIBUTIONS

A.G., S.G., and V.F. conceived of this research program. S.G. performed time-lapse videos, immunolocalizations, Lugol staining, GUS staining, histology, and fiber counts. M.Z. performed bioinformatics analyses with guidance from V.F., A.G., S.G., and M.Z. performed RNA-seq experiments. S.D.M. and F.H. contributed wood chemistry, ultrastructure, and fiber data. G.K.M. and D.R.L. contributed auxin transport assays. F.M.I. and H.B. synthesized the XET substrate. A.G. performed XET imaging and wrote the article.

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