Research

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Brassinosteroid regulation of wood formation in poplar

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Summary

- Brassinosteroids have been implicated in the differentiation of vascular cell types in herbaceous plants, but their roles during secondary growth and wood formation are not well defined.
- Here we pharmacologically and genetically manipulated brassinosteroid levels in poplar trees and assayed the effects on secondary growth and wood formation, and on gene expression within stems.
- Elevated brassinosteroid levels resulted in increases in secondary growth and tension wood formation, while inhibition of brassinosteroid synthesis resulted in decreased growth and secondary vascular differentiation. Analysis of gene expression showed that brassinosteroid action is positively associated with genes involved in cell differentiation and cell-wall biosynthesis.
- The results presented here show that brassinosteroids play a foundational role in the regulation of secondary growth and wood formation, in part through the regulation of cell differentiation and secondary cell wall biosynthesis.

Introduction

Secondary growth is the radial expansion of stems from a vascular cambium, and ultimately produces secondary xylem (wood) and secondary phloem (inner bark) (Larson, 1994). This development requires a complex coordination of cell division, cell differentiation, tissue patterning and coordinated growth across tissues and organs. The secondary vascular tissues that are produced during secondary growth are responsible not only for mechanical properties of the stem, but also conduction of water through secondary xylem and photoassimilates through the phloem. At the same time, secondary growth must integrate environmental cues, such as water stress and gravity, to produce secondary vascular tissues with functional properties that help the plant survive under diverse environmental conditions.

The gravitropic response of woody angiosperm stems is a striking example of how environmental cues influence wood development. When inclined, angiosperm tree stems produce 'tension wood' on the upper side of the leaning stem (Groover, 2016). In contrast with 'normal wood' produced by upright stems, tension wood has fewer vessel elements, and is dominated by specialised fibres that have a tertiary cell wall, the gelatinous layer (G layer). These specialised gelatinous fibres produce a large contractile force during their maturation (Mellerowicz & Gorshkova, 2012), resulting in the tension wood 'pulling' a leaning stem upwards. The gravitropic response and tension wood have been used as a model system for wood development, as a dramatic change in wood anatomy and biochemistry can be experimentally manipulated through a simple and easily applied stimulus. The tension wood response is accompanied by a large change in gene expression, both in the tension wood side of the stem but also in the 'opposite wood' side of the stem facing the ground (Gerttula *et al.*, 2015; Zinkgraff *et al.*, 2018), supporting the notion that transcription is a key point of regulation for wood development.

Key questions for wood development include how environmental cues are integrated into developmental programs, and how development is coordinated in a stem, both longitudinally and radially. Hormones have been identified as playing key roles in both processes. For example during the gravitropic response in poplar stems, the first known response is the reorientation of PIN auxin efflux carrier proteins towards the ground in the plasma membranes of cells within the endodermis and secondary phloem (Gerttula *et al.*, 2015). Because of the radial organisation of the stem, this reorientation results in auxin transport away from the PIN-expressing cells and towards the cambium on the tension wood-forming side of the stem, but on the opposite wood-forming side of the stem the same reorientation results in auxin being transported away from the cambium and, instead, towards the

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cortex of the stem. These results illustrate how hormone production can both incorporate environmental cues and coordinate wood development. Interestingly auxin, gibberellic acid (GA), and cytokinin have been shown to have distinct concentration gradients across secondary vascular tissues, and that these gradients correspond to gene expression and developmental stages of developing secondary vascular tissues (Immanen *et al.*, 2016). GA stimulates tension wood formation (Funada *et al.*, 2008; Gerttula *et al.*, 2015), enhances auxin response during gravitropism, and upregulates key genes involved in brassinosteroid response (Gerttula *et al.*, 2015). Interestingly GA precursors are found predominantly in secondary phloem, whereas bioactive GA as well as GA-responsive gene expression peak in the region of cell expansion within secondary xylem (Israelsson *et al.*, 2005), suggesting a process of transport of GA precursors radially across the cambium. Collectively, these observations underscore that the movement and distributions of hormones in vascular tissues is highly coordinated, interacting and a key regulatory feature of secondary growth.

Brassinosteroids (BRs) are plant-specific steroid hormones that are involved in many aspects of growth and development (Li & Jin, 2007; Divi & Krishna, 2009), including the regulation of cell elongation and division (Azpiroz *et al.*, 1998; Cheon *et al.*, 2010), vascular differentiation (Yamamoto *et al.*, 1997; Hiroo, 2004), photomorphogenesis (Neff *et al.*, 1999; Song *et al.*, 2009), leaf angle inclination (Wada *et al.*, 1981, 1984), seed germination (Sasse *et al.*, 1995; Steber & McCourt, 2001), stomatal development (Tae-Wuk *et al.*, 2012), and suppression of leaf senescence and abscission (Iwahori *et al.*, 1990). Primary mechanisms for the biosynthesis, perception, signal transduction and response for BRs have been described in Arabidopsis. Key steps of BR biosynthesis are mediated by cytochrome P450 monooxygenases (Fujioka & Yokota, 2003; Clouse, 2011), and specific BR biosynthesis inhibitors have proven useful tools to study the synthesis and action of BRs. For example the triazole compound propiconazole (PCZ; 1-[[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl]1H-1,2,4-triazole), is a potent and specific inhibitor of BR biosynthesis (Katsuhiko *et al.*, 2002; Thomas *et al.*, 2012). BRs are perceived by a receptor-like kinase, BRASSINOSTEROID INSENSITIVE 1 (BRI1) and co-receptor kinase BRI1-ASSOCIATED KINASE (BAK1) at the plasma membrane (Chory & Li, 1997; Wang *et al.*, 2001). BR binding results in the dissociation of a negative regulator, BRI1 KINASE INHIBITOR 1 (BKI1) (Xuelu & Joanne, 2006), and initiates a phosphorylation cascade starting with transphosphorylation of BRI1 and BAK1, and terminating with the BRASSINOSTEROID INSENSITIVE 2 (BIN2) kinase. BIN2 phosphorylation regulates a class of plant-specific transcription factors, BRASSINAZOLE-RESISTANT1 (BRZ1) and BR-INSENSTIVE-EMS-SUPPRESSOR 1 (BES1/BZR2), which effect the many downstream consequences of BR by binding specific cis-element motifs in the promoter region of a large repertoire of target genes (Yin *et al.*, 2002; He *et al.*, 2005).

Previous experiments have linked BRs to specific aspects of primary vascular development. During the *in vitro* differentiation of tracheary elements in the Zinnia system, BR can promote the formation of tracheary elements by triggering secondary cell-wall formation and cell death (Yamamoto *et al.*, 1997), and BR levels increase while expression of genes involved in BR action change during differentiation (Yamamoto *et al.*, 2001, 2007). Zinnia genes encoding key enzymes of BR biosynthesis (ZeSTE1, ZeDIM, ZeDWF4, ZeCPD1) increase in transcript levels during differentiation, and *in situ* hybridisation experiments of ZeDWF4 and ZeCPD1 mRNAs revealed their preferential accumulation in procambium cells, immature xylem cells and xylem parenchyma cells (Yamamoto *et al.*, 2007). In Arabidopsis, *BRL1* is expressed in the vascular tissues, and *brl1* knockouts display expanded phloem development at the expense of xylem (Caño-Delgado *et al.*, 2004). Analysis of mutants and transgenics with impaired or enhanced BR signaling showed that BR affects the numbers and patterning of vascular bundles in Arabidopsis (Marta *et al.*, 2009).

The role of BR during secondary growth and wood formation is not as well explored. Exogenously applied BR can reduce lignification and alter cell-wall carbohydrate biosynthesis in the secondary xylem of *Liriodendron tulipifera* trees (Hyunjung *et al.*, 2014). In poplar, the gene encoding the BRASSINOSTEROID ENHANCED EXPRESSION 3 (BEE3) basic helix−loop−helix transcription factor is induced by BR, and when overexpressed results in enhanced secondary xylem formation (Noh *et al.*, 2015). Over expression of BR biosynthesis gene BR6OX in poplar showed increased secondary xylem formation (Jin *et al.*, 2017). By contrast, results from exogenous application of BR and the inhibitor brassinazole (BRZ) to debarked poplar stems led to the conclusion that inhibition of BR biosynthesis results in increased tension wood fibre maturation and negative gravitropism (Gao *et al.*, 2019).

Here we report experiments showing a positive role of BR in promoting tension wood formation during gravitropic response in poplar stems. BR levels were experimentally manipulated through pharmacological and genetic means, which resulted in corresponding changes in secondary growth. Overall, BR promoted cell differentiation and accelerated secondary growth. Downstream changes in molecular mechanisms and gene expression were detailed using a gene co-expression approach. Together our results suggest that BR is a key regulator of secondary growth, by acting to stimulate cell differentiation.

Materials and Methods

Replication

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

Plant materials and growth conditions

The *Populus alba* × *P. glandulosa* clone '84K' was used in all experiments. Trees were transferred to soil in 0.25-l pots and grown at *c.* 22°C under long-day conditions (14 h : 10 h, light : dark). Trees were grown for 1 month in soil with water

supplemented with Gatit fertiliser (Gat Fertilizers Ltd, Afula, Israel) using concentrations recommended by the manufacturer, before experimental treatments.

For soil drench treatments, 144 µl of a stock solution of 10 mg ml^{-1} 24-epibrassinolide (BL; Phyto Technology Laboratories, Lenexa, KS, USA) in ethanol was added to 50 ml water to make a working solution. Here, 50 ml of the working solution was added to each pot to give an approximate concentration of 10 µM BL in the soil. Plants were grown for an additional 10 d after BL treatment before gravi-stimulation. For propiconazole (PCZ) soil drench treatments, 250 mg PCZ (Sigma) was dissolved in 5 ml ethanol to make a 50 mg ml^{-1} stock solution, then 2.04 ml stock solution was added to 50 ml water to make a working solution. Fifty millitres of the working solution was added to each pot to give an approximate concentration of 1 mM PCZ in the soil. Plants were grown for an additional 4 d after PCZ treatment before gravi-stimulation. Lower concentration of BL and PCZ were applied using water to replace lost volumes. In all cases, a single treatment was applied (that is plants were not exposed to multiple rounds of BL or PCZ treatment).

DET2 gene cloning and DET2 overexpression in transgenic poplar lines

The full-length coding sequence of *PagDET2* gene was cloned (GenBank accession no. MK317884) from cDNA of 84K poplar using sequence-specific primers (Table S5) was cloned into the overexpression vector pMDC32 driven by 2X35s promoter and transformed into poplar 84K. 15 independent transgenic lines were obtained and identified through quantitative reverse transcription polymerase chain reaction (qRT-PCR). Three lines were selected for further study based on overexpression levels, and were named as DET2-OE1, DET2-OE2, DET2-OE3 and selected for further analysis.

Stem lift, histology and imaging

Graviresponse and stem lift were measured as previously described (Gerttula *et al.*, 2015). Briefly, after 3 wk horizontal gravi-stimulation, stem lift was measured as the vertical distance from the base of the stem to the point deemed the 'apex of curvature,' defined by the point at which primary stem bending occurred initially during gravi-stimulation. Fresh stems were sectioned for histology using a Leica VT1000 S to a thickness of 50 µm. Sections for anatomical assays were stained with 0.05% toluidine blue (TBO) for 60 s, washed three times in water, and then visualised with an Olympus BX51 compound microscope using an Olympus DP74 digital camera with live image stitching using CellSens Standard software (Olympus Corp., Tokyo, Japan).

RNA extraction and qRT-PCR qualification of gene transcripts

Developing leaf tissues were harvested from the top three elongating internodes at 0, 4, 8, 12, 24, 36, 48 and 72 h after BL treatment and flash frozen in liquid nitrogen. Total RNA was isolated by and their quality and quantity were checked using the NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). First-strand cDNA synthesis was carried out with *c.* 2 µg RNA using the Superscript III reverse transcription kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction. Real-time qRT-PCR was performed as described by Shu *et al.* (2019) using PagActin gene as an internal reference. Amplified fragments were confirmed using agarose gel electrophoresis. Primers are used for real-time PCR are listed in the Table S5.

RNA-seq

One-month old soil grown poplar trees were treated with 10 µM BL, 1 mM PCZ (method as above), and mock water treated poplar trees used as control plants. Plants were grown for an additional 10 d after BL, PCZ and water control treatment before gravi-stimulation. All plants were placed horizontally to induce gravi-stimulation for 48 h before being harvested. Tissue harvest and total RNA isolation was as described in (Gerttula *et al.*, 2015).

Total RNA was isolated using RNeasy mini kit (Qiagen, Germany). Sequencing libraries were synthesized using the TruSeq® RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) following the TruSeq® RNA Sample Preparation Guide. The products were then purified and enriched by PCR to create the final cDNA libraries. Purified libraries were quantified by Qubit® 2.0 Fluorometer (Life Technologies) and validated using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) to confirm the insert size and calculate concentrations. The libraries were sequenced on the Illumina HiSeq 150-bp paired-end sequencing (Illumina). The library construction and sequencing were performed at Shanghai Biotechnology Corp.

Sequencing, data processing and analysis

Sequencing reads were preprocessed, trimmed and filtered using Seqtk with parameters: -q 0.01 -l 1 (https://github.com/lh3/seqtk), and then mapped to the using Hisat2 v.2.0.4 with parameters: -t –dta -k 1 (Kim *et al.*, 2015) to the JGI P. trichocarpa_444_v3.1 reference genome. After genome mapping. Stringtie v.1.3.0 with default parameter (Pertea *et al.*, 2015) was run with a reference annotation to generate FPKM values for known gene models. Differentially expressed genes were identified using edgeR with default parameters (Robinson *et al.*, 2010). The *P*-value significance threshold in multiple tests was set by the false discovery rate (FDR) (Dudoit *et al.*, 2008). The fold-changes were also estimated according to the FPKM in each sample. The differentially expressed genes were selected using the following filter criteria: FDR ≤ 0.05 and fold-change ≥ 2. In order to examine the biological significance of the differentially expressed genes, the Gene Ontology (GO) enrichment analysis using AgriGO v.2 (Tian *et al.*, 2017) were performed using R scripts based on a hypergeometric test. All the R code for analysis is presented in Table S4.

Analysis of BR level in plants by enzyme-linked immunosorbent assay (ELISA)

For the BR concentration measurement in normal wood formation, 1-month old soil grown 84K plants were treated with 10 μM BL and allowed to continue growing for an additional 10 d. The first three elongating internodes from 10 μM BL and water treated mock control were harvested for ELISA analysis. The first three elongating internodes of 1-month-old soil DET2-OE lines were used for ELISA analysis. The internode samples were collected and rapidly frozen with liquid nitrogen.

For the BR concentration measurement in tension wood bark and xylem tissue three 1-month-old soil grown poplar trees were treated with 10 μM BL and allowed to grow for an additional 10 d, and three DET2-OE lines and mock water treated 84K poplar trees used as control. All plants were placed horizontally for gravi-stimulation 48 h before being harvested for BR concentration measurements. Bark and xylem tissues were harvested separately from both tension wood and opposite wood sides.

For the BR concentration measurements methods, 200 mg samples of tissue powder were transferred into a 2 ml centrifuge tube and containing 200 μl methanol and 1.6 ml PBS (pH 7.2–7.4, 0.1 M). Samples were homogenised and centrifuged 20 min at 700 ***g***. The supernatants were collected for ELISA analysis. BR levels were assayed by Plant Brassinosteroid ELISA kit (Beijing Chenlin Biotechnology Co., Beijing, China). The ELISA procedure was conducted according to Khripach *et al.* (2008); Pradko *et al.* (2015).

Statistical analysis

The statistical analysis of the BL, PCZ-treated normal wood and tension wood phenotypes and qRT-PCR data were analyzed using SPSS 10 (SPSS Inc., Chicago, IL, USA). Analyses of variance (ANOVA) for sets of data groups were performed with *post hoc* testing. For variables with a significant effect ($P < 0.05$), *post hoc* tests using the Games-Howell algorithm were performed to test which of the possible multiple comparisons between the data groups were significant.

Results

Manipulation of BR levels and response in poplar stems

Soil application of 24-epibrassinolide (BL) results in elevated BR levels and response in poplar stems. To test if soil-applied BL could be taken up and transported through the stem, BL was applied to soil of potted poplar trees at a final soil concentration of 10 μM (see 'Materials and Methods'). The transcript levels of poplar genes homologous with genes shown to be BR responsive in other systems (He *et al.*, 2005; Spartz *et al.*, 2012; Frederikke Gro *et al.*, 2014) were assayed by quantitative RT-PCR (see 'Materials and Methods'). Poplar genes homologous to *BZR1, EXPA8* and *SAUR-AC1* were induced in transcript levels in response to the BL treatment in a complex, time-dependent manner suggestive of a bi-phasic response peaking at 12 and 48 h after treatment (Supporting Information Fig. S1).

BL treatment influences stem growth and xylem formation during secondary growth

Soil-applied BL can enhance poplar stem growth, but only within a concentration-dependent range. Upright potted poplar trees were soil treated with BL for 1 month, testing a concentration gradient of 1, 10 and 50 μM soil BL (see the 'Materials and Methods' section). The 10 μM BL treatment showed significantly increased stem height (Fig. 1a,f), while 1 and 50 μM BL-treated trees were not significantly different from the mock control trees (Fig. 1a,f). Histological comparison of 'normal wood' from stem sections showed that 10 μM treated trees showed an increase in secondary xylem width (Fig. 1d,g) while 1 μM (Fig. 1c,g) and 50 μM BL (Fig. 1e,g) treated trees were not significantly different from the mock control trees. The number of cells per cell file was increased in 1 and 10 μM BL-treated trees, but not in 50 μM BL-treated trees (Fig. 1h).

BL treatment affects tension wood formation in response to gravibending

Soil-applied BL also enhances the formation of tension wood and gravibending response, but only within a concentration-dependent range. Trees were treated with soil-applied BL for 10 d using 1, 10 and 50 μM BL, and then subjected to 90° gravi-stimulation for a period of 21 d (see 'Materials and Methods'). Each tree was then measured for stem lift and anatomical features. The 10 μM BL treatment resulted in significant increase in gravibending stem lift (Fig. 2a,f), while 1 and 50 μM BL treatments did not significantly differ from the mock-treated control. Similarly, the 10 μM BL treatment resulted in an increased width of tension wood produced (Fig. 2d,g) and tension wood fibres formed (Fig. 2d,h), compared with 1 and 50 μM BL treatments and mock controls (Fig. 2c,e,g,h).

BR biosynthesis inhibitor propiconazole inhibits stem growth and secondary xylem formation

The BR biosynthesis inhibitor, propiconazole (PCZ), was used to examine the effect of lowered BR levels on poplar stem growth. Trees were treated for a 1-month growth period with PCZ soil concentrations of 750 μM, 850 μM and 1 mM (see 'Materials and Methods'). As shown in Fig. 3(a), in comparison with mock-treated controls, PCZ-inhibited growth in a dose-dependent fashion, with 1 mM PCZ having the largest effect. Reduced height growth in response to PCZ treatment (Fig. 3a,f) resulted from significantly shorter internodes, while the internode number did not vary significantly among PCZ treatments and controls (data not shown). Histological comparison of stem sections (Fig. 3b–e) showed a negative impact of increasing PCZ levels on xylem width (Fig. 3g) and number of cells within xylem cell files (Fig. 3h), suggesting that decreased xylem width resulted from reduced cell division vs smaller diameter cells.

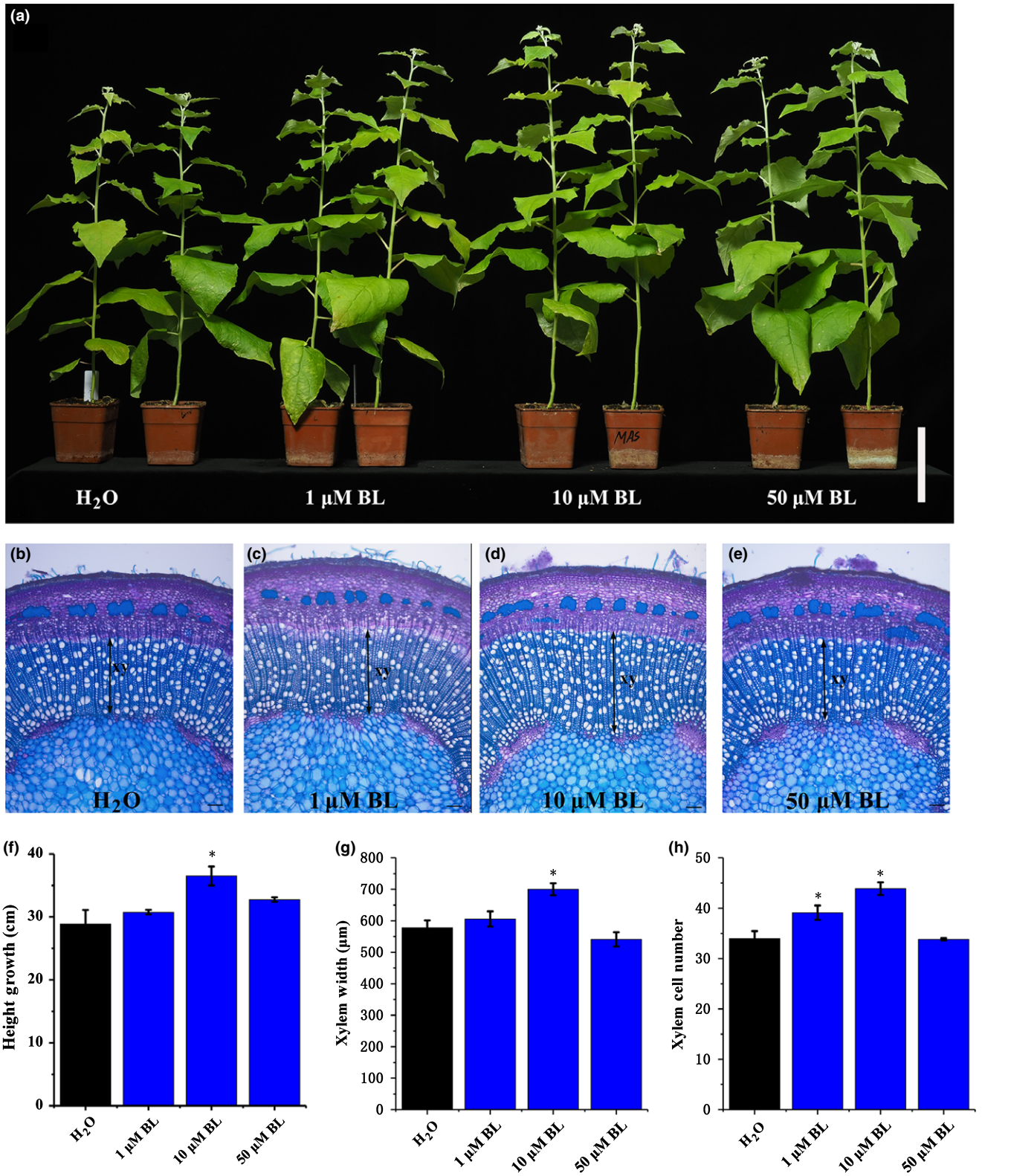

Fig. 1 Effects of BR on stem growth and development. (a) Whole plant phenotype after 30 d 24-epibrassinolide (BL) treatment. Bar, 10 cm. (b–e) Normal wood histology of stem sections. Bars,100 μm. xy, xylem. Quantification of elongated growth for upright trees after 4 wk growth at series BL treatment (f). Secondary xylem width (g) and secondary xylem cell number (h). Error bars ± SE. *, $P < 0.05$. NW, normal wood.

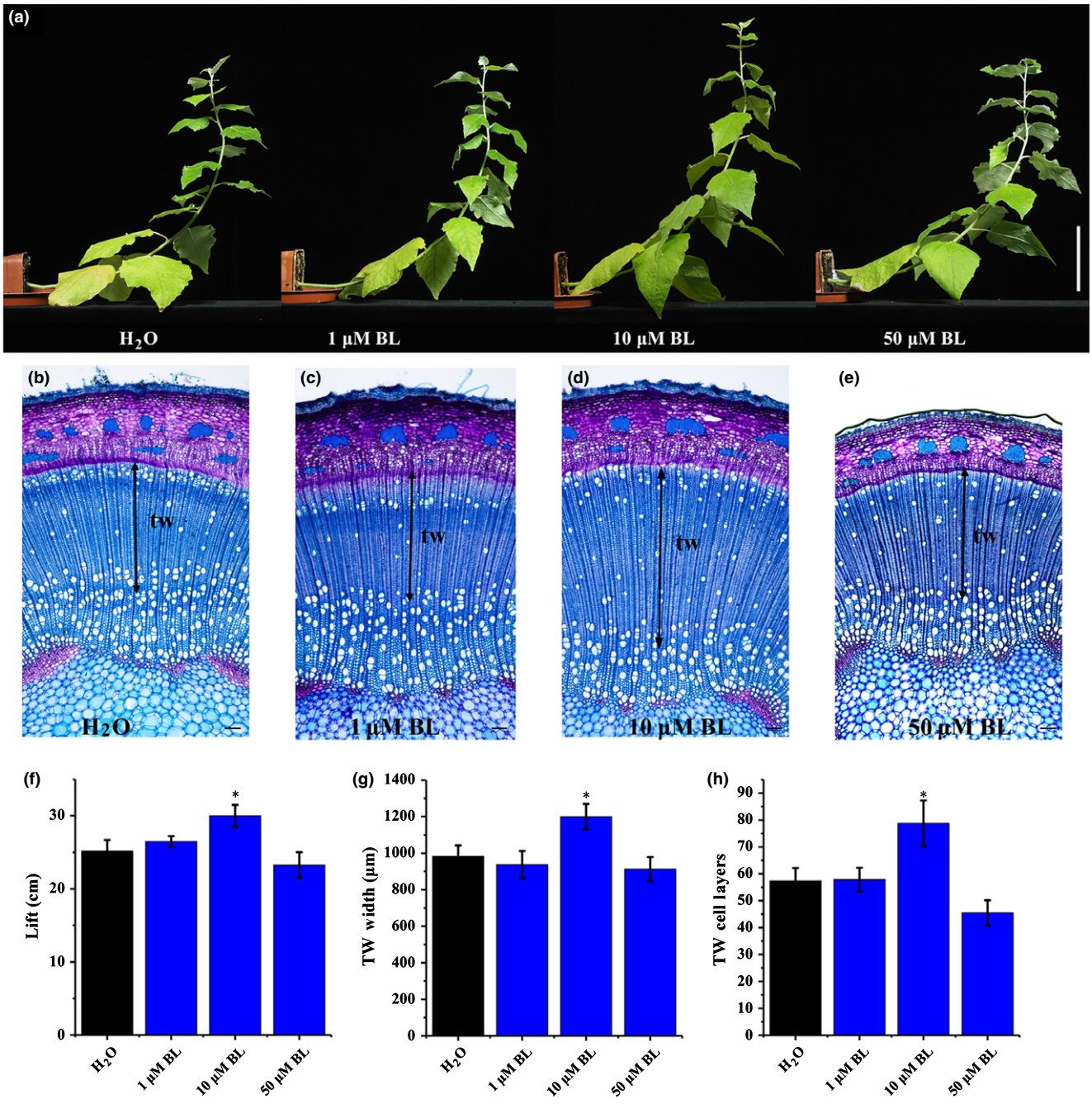

Fig. 2 Effects of brassinosteroids (BRs) on gravi-stimulated stem growth and development. (a) Whole plant phenotype after 24-epibrassinolide (BL) treatment. Bar, 10 cm. (b–e) Tension wood histology of stem sections. Bars, 100 μm. xy, xylem; tw, tension wood. Tension wood stem lift (f), tension wood width (g) and tension wood cell layers (h) at series BL treatment. Error bars ± SE. *, $P < 0.05$. TW, tension wood.

Propiconazole inhibits tension wood formation and gravibending

We next assayed the effect of PCZ treatment on tension wood formation and gravibending. Trees were treated with a PCZ soil concentrations ranging from 750 μM, 850 μM to 1 mM for 4 d, and then gravi-stimulated for 21 d (see 'Materials and Methods'). In comparison with mock-treated controls, PCZ-inhibited gravibending stem lift in a dose-dependent fashion (Fig. 4a,f). Histological comparison of stem sections (Fig. 4b–e) showed a similar dose-dependent inhibition of tension wood width (Fig. 4g) and number of tension wood fibres formed (Fig. 4h) in response to PCZ treatment.

BL and propiconazole treatments affect cambial zone cell divisions during gravibending

To further study the mechanism of how BL and PCZ affect tension wood formation and gravibending, a time course histology analysis was performed for control, 10 μM BL, and 1 mM

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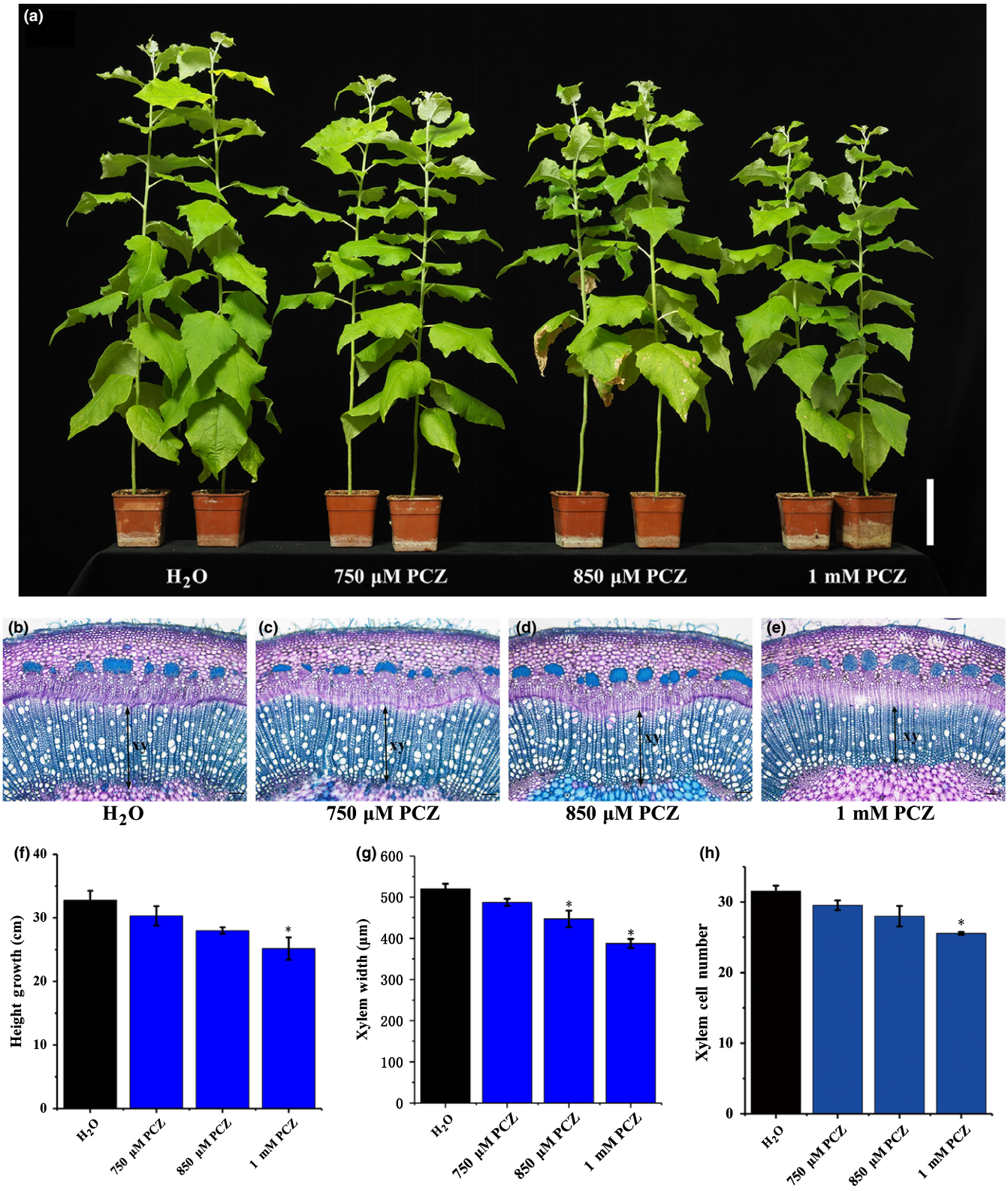

Fig. 3 Effects of propiconazole (PCZ) on stem growth and development. (a) Whole plant phenotype after PCZ treatment. Bar, 10 cm. (b–e) Normal wood histology of stem sections. Bars,100 μm. xy, xylem. Mean normal wood delta stem height (f), stem xylem width (g) and stem xylem cell number (h). Error bars, ± SE. *, $P < 0.05$. NW, normal wood.

PCZ soil-treated trees after 0 h, 48 h, or 21 d of gravi-stimulation (see 'Materials and Methods'). As shown in Fig. 5(a–i) and quantified in Fig. 5(j), in comparison with the control or PCZ-treated trees, BL-treated trees showed a significant increase in the number of xylem mother cell layers in the tension wood cambium zone 48 h after gravibending (Fig. 5e), typical of stems

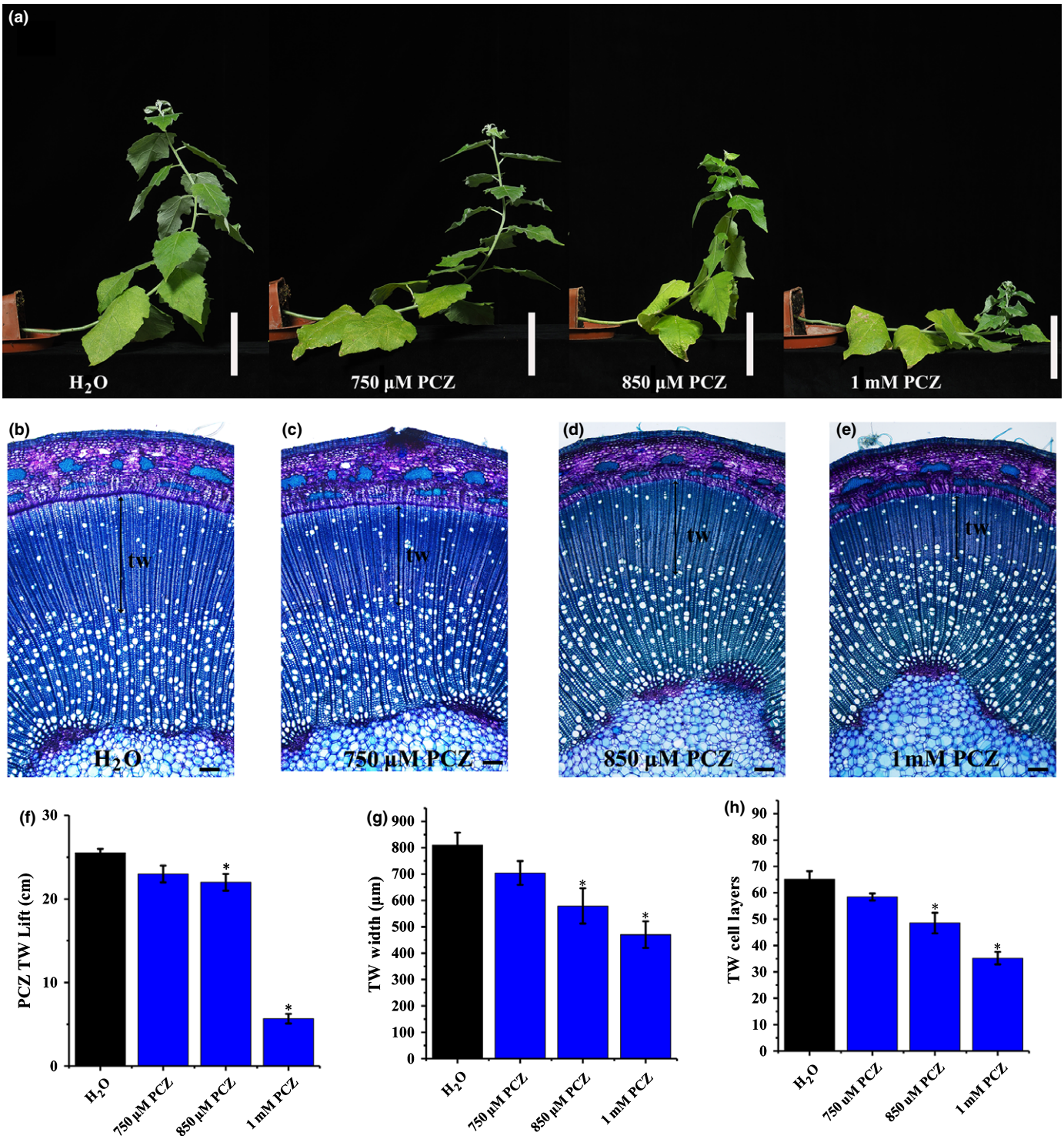

Fig. 4 Effects of propiconazole (PCZ) on gravi-stimulated stem growth and development. (a) Whole plant phenotype after PCZ treatment. Bars, 10 cm. (b–e) Tension wood histology of stem sections. Bars, 100 μm. xy, xylem; TW, tension wood. Mean tension wood stem lift (f), tension wood width (g) and tension wood cell layers (h). Error bars, ± SE.*, $P < 0.05$. TW, tension wood.

undergoing rapid rates of cell division. In the 21 d time point, control, BL and PCZ-treated trees all showed similarly elevated numbers of cambial xylem mother cell layers (Fig. 5j). At the end of the trial, BL-treated trees had significantly more, and PCZ significantly less numbers of tension wood fibres produced per cell files in comparison to mock control plants (Fig. 5k). Together these results suggested that the initial response to BL treatment is an elevation in cell division within the cambial zone, and that response is attenuated over time in our experimental system.

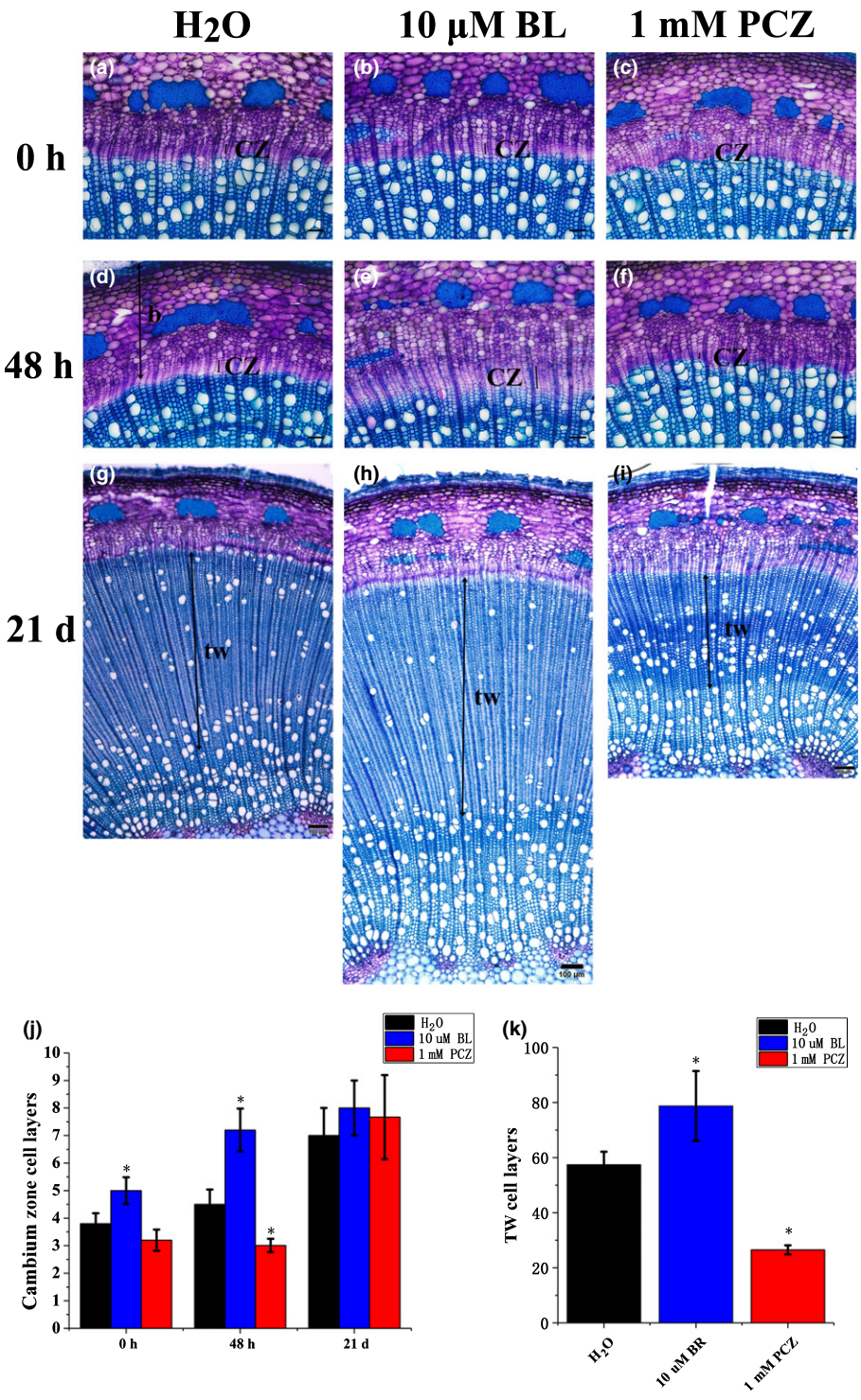

Fig. 5 Effects of BR, PCZ on gravi-stimulated stem growth and development. Tension wood histology of stem sections at 0 h gravi-stimulated (0 h), 48 h gravi-stimulated (48 h) showing cambium zone cell layers (a–i). Bars, 100 μm. CZ, cambium zone; TW, tension wood. Mean tension wood stem cambium zone cell layers (j) and cambium zone width (k). Error bars ± SE. *, $P < 0.05$.

Elevated endogenous BR levels in DET2 Overexpression poplar enhance normal and tension wood formation

A gene encoding a BR biosynthesis enzyme, DET2 (a steroid 5α-reductase), was shown to be upregulated during tension wood induction in previous experiments (Gerttula *et al.*, 2015). To explore the role of *PagDET2* in tension wood formation in poplar 84K, a poplar *DET2* orthologue was overexpressed in 84K poplar. Three independent transgenic lines (DET2-OE1, DET2-OE2, DET2-OE3) with high *PagDET2* abundance (Fig. S3a) were chosen for functional study and showed significantly enhanced whole plant growth including enhanced plant stem height (Fig. 6a). Histological comparison of 'normal wood' from stem sections showed that *PagDET2* transgenic plants showed an increase in secondary xylem width and the number of cells per cell file was increased (Fig. 6b,c). *PagDET2* transgenic

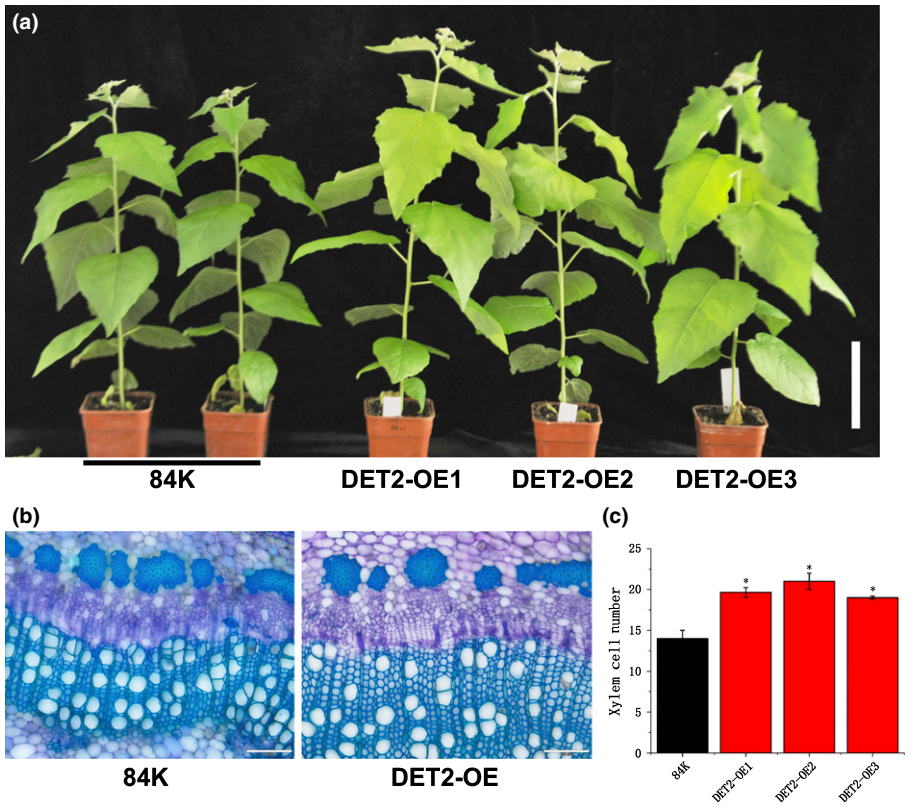

Fig. 6 DET2 overexpression lines enhance the normal wood and tension wood formation. (a). Phenotype of brassinosteroid (BR) biosynthesis gene *DET2* overexpression poplar lines and control. (b) Normal wood anatomy analysis of same developmental internode of DET2 overexpressed poplar lines and control. Bars, 100 μm, (c). Statistical analysis of xylem cell layers; Error bars ± SE. *, $P < 0.05$.

plants also showed significant increase in gravibending stem lift (Fig. S2a,c), tension wood production (Fig. S2b,c) and tension wood fibres formed (Fig. S2b,c) compared with the wild-type.

Endogenous BR levels are elevated in stems of BL-treated and DET2-OE trees

Endogenous BR levels were assayed in BL-treated samples, DET2-OE lines and matched control samples by HRP-linked immunosorbent assay (ELISA) with an anti-BR antibody (see 'Materials and Methods'). BR levels were significantly elevated in the normal wood tissue of 10 μM BL-treated trees and DET2 overexpression transgenic poplars (Fig. S3b). BR levels were also significantly elevated in the normal wood tissues of DET2 overexpression transgenic poplars, compared with controls (Fig. S3b). During tension wood induction, BR levels were significantly elevated in tension wood bark tissue, compared with tension wood xylem as well as opposite wood xylem and bark tissues (Fig. S3c); DET2 overexpression transgenic poplars also show significantly elevated BR concentration in tension wood bark tissue, compared with controls (Fig. S3c).

Transcript levels for genes associated with cell division and xylem differentiation respond to BR treatment

To determine biological processes linked to BR and PCZ action in woody stems, RNA-sequencing was used to quantify transcripts for expressed genes in both the secondary xylem and bark tissue (including secondary phloem and cambium zone cells) from upright trees (normal wood), and the upper (tension wood) side and lower (opposite wood) side of gravi-stimulated stems. Upright and gravi-stimulated trees were assigned to either a 10 μM BL treatment, 1 mM PCZ treatment or mock control, and xylem and bark tissues harvested at 48 h after induction of gravibending (see 'Materials and Methods'). Three biological replicates were included for each treatment. Gene expression in FPKM for each treatment and differentially expressed genes for treatment comparisons are presented in Tables S1–S9. The RNA-sequencing data are available at the Sequence Read Archive of the National Center for Biotechnology Information (accession no. PRJNA533743).

Gene transcript abundance data were used in a gene co-expression analysis to establish groups of co-expressed genes associated with BR-related treatments in stem bark and secondary xylem (see 'Materials and Methods'). Co-expressed gene modules were identified whose expression significantly correlated with BR, PCZ-treated normal wood, tension wood and opposite wood tissues (Fig. 7a; Table S2). For the most part, module correlations were dominated by tissue effects, followed by wood type effects, and lastly by BR and PCZ effects. Eigengene values of each module are shown in Fig. 7(b) and reveal finer detail of how genes within each module vary across tissues and in response to treatments. While tissue-level effects predominate, significant effects of BR and PCZ treatments are evident on expression of genes within specific modules, including differences in response to treatments seen across different tissues (for example modules 'grey60' and 'midnightblue'). GO analysis was used to further describe the function of genes

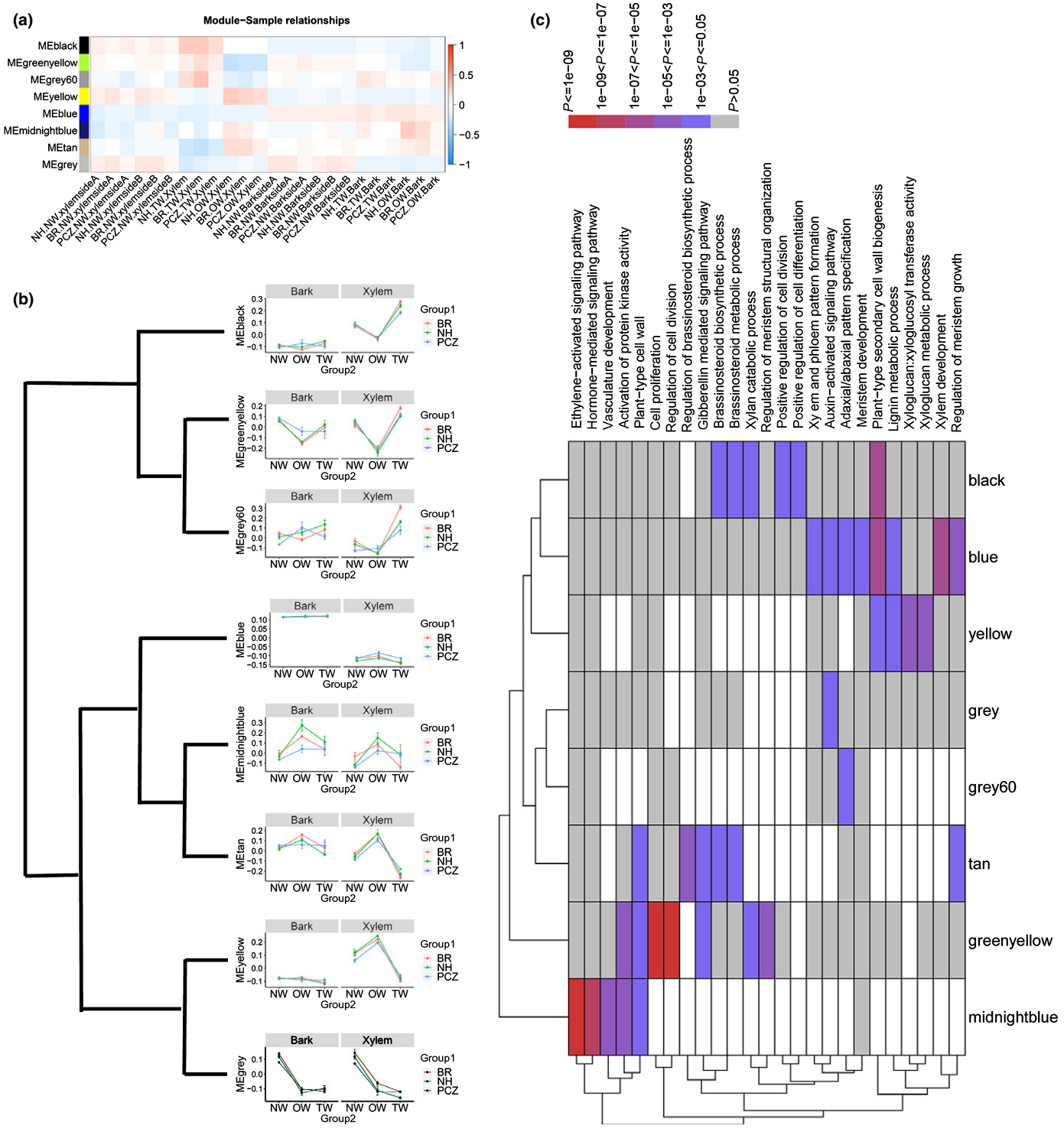

Fig. 7 Gene co-expression analysis. (a) The colour of each cell at the row−column intersection indicates the correlation coefficient between the module and the tissue type. Scale is shown to right of plot. (b) Dendrogram showing module eigengene expression across bark and xylem tissues (bark, xylem) and wood types (NW, normal wood; OW, opposite wood; TW, tension wood) in response to brassinosteroid (BR), propiconazole (PCZ), or no hormone (NH) treatment. Error bars ± SE. (c) Functional GO enrichment analysis of all modules. Scale is shown above the plot.

within each module (Fig. 7c; Table S3), providing additional insight into the functional nature of the correlations of each module with tissues and treatments. Two modules, 'black' and 'tan' both showed enrichment for genes involved in BR biosynthesis and metabolism. Interestingly, these modules showed divergent eigengene patterns, with 'black' having highest expression in tension wood and low expression in bark, while 'tan' had the lowest expression in tension wood and

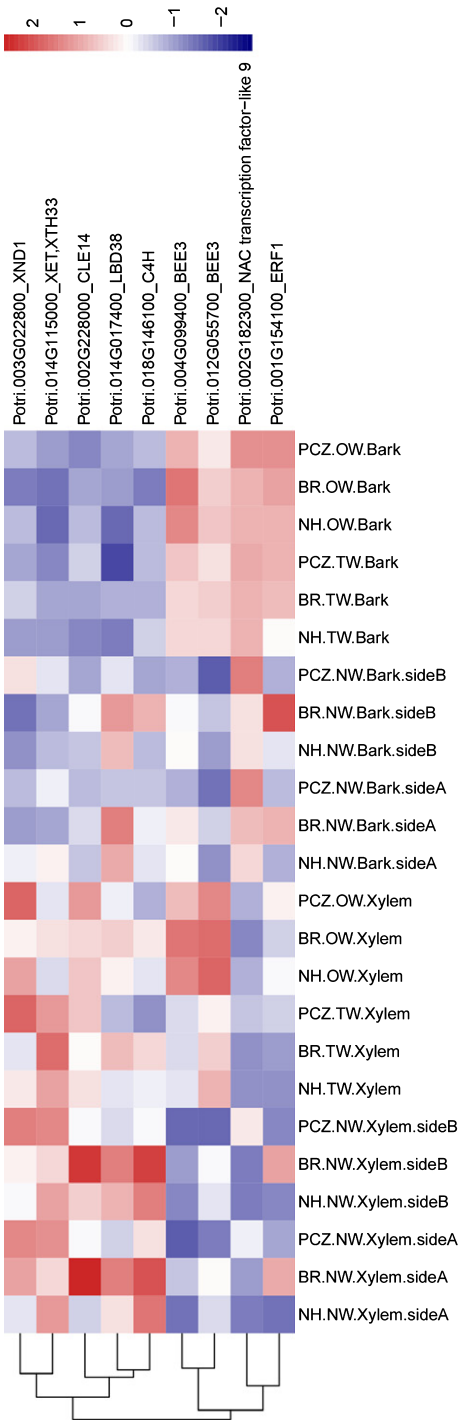

Fig. 8 Heatmap analysis of selected function genes expression. Heatmap analysis show genes expression across all the 24 samples, including transcription factors of brassinosteroid (BR) signaling BEE3; cell-wall biosynthesis-related genes C4H, XET; vascular tissue differentiation related genes expression CLAVATA3/ESR-RELATED 14 (CLE14), LBD38, XND1.

higher expression in bark tissues. Additional modules were identified that have strong enrichment for genes involved in hormones previously demonstrated to underlie gravitropism and tension wood formation, including ethylene (module 'midnight blue'), and GA (modules 'tan' and 'green-yellow'). Multiple modules showed enrichment for genes involved in secondary cell-wall biosynthesis.

In a final analysis, expression was detailed for candidate genes orthologous to previously defined BR-related genes from other species (Figs 8, S4). A poplar orthologue of the gene encoding the BR signaling pathway transcription factor, BR ENHANCED EXPRESSION 3 (BEE3) was significantly upregulated in the BR-treated wood types and downregulated in the PCZ-inhibited wood types. Secondary cell-wall biosynthesis-related genes encoding C4H and xyloglucan endotransglucosylase (XET) showed significant misregulation in the BR-treated and PCZ-treated wood tissues. The gene expression of orthologues encoding xylem differentiation regulators XND1, LBD38 and CLE14's was altered in response to BR and PCZ treatment. XND1 is a negative regulator of xylem differentiation, which is downregulated in the BR-treated tissues, and upregulated in PCZ-treated wood tissues.

Discussion

In this study, we examined the role of BR on wood formation in both upright and gravi-stimulated plants. We took two approaches to alter BR levels in poplar stems. In the first, we treated trees in pots by applying BL or a BR biosynthesis inhibitor (PCZ) as a soil drench, relying on root uptake and subsequent transport of the compounds through the xylem in the transpiration stream. In the second approach, we overexpressed a poplar orthologue of a gene encoding an enzyme shown to be a rate-limiting step in BR biosynthesis in other species, DET2. Both the BL treatment and *DET2* overexpression lines were associated with modest increases in endogenous BR levels in developing wood (Fig. S3). A more detailed analysis of BR levels across tissues showed that, in both wild-type and *DET2* overexpression lines BR levels are elevated in both bark and xylem tissue on the upper, tension wood side of the stem in comparison to the downward facing, opposite wood side of the stem during gravi-stimulation. This asymmetric distribution suggests that during gravi-stimulation BR may be asymmetrically produced, degraded or transported around the stem.

Our results overall are consistent with a positive role for BR in stimulating wood formation and gravibending response. BR has been negatively associated with gravibending Arabidopsis seedlings (Vandenbussche *et al.*, 2011, 2013), but this type of bending is accomplished by elongation of existing cells on the bottom of the stem, where in the case of woody poplar stems bending is accomplished by the production of new cells that differentiate into contractile fibres (Groover, 2016). In this regard our results are perhaps more closely related to and are generally consistent with previous reports in herbaceous systems that point to BRs promoting the differentiation of vascular cells, specifically tracheary element differentiation (Yamamoto *et al.*, 2001, 2007). Somewhat conflicting conclusions are given by a recent report examining the role of BRs in secondary growth (Gao *et al.*, 2019), but may be attributed to differences in the experimental treatments employed. In contrast to our approaches using intact plants, bark was dissected away from the stem at points of application of BL or an inhibitor of BR biosynthesis, brassinazole

(BRZ) in a lanolin paste. BRZ was positively associated with tension wood formation and gravibending, while BL had no effect (Gao *et al.*, 2019). Unfortunately, the effect of bark removal was not assayed, and it is unclear how the results reported relate to our results or the role of BR during normal development. More research will be needed to understand the interactions of bark and xylem tissues regarding to BR transport and signaling during secondary growth.

Our results revealed important details of BR importance during secondary growth and casts highlights over remaining questions for future research. Perhaps not surprisingly, the results of our pharmacological and genetic treatments suggest that BR levels are highly regulated. We found that BL applied as a soil drench can be taken up and transported in stems, providing a convenient method for perturbing BR levels in stems. Although the mechanisms of uptake and transport were not specifically investigated, long distance transport in the xylem is the most likely route. While useful as an experimental treatment, it is not known if BR is normally transported over long distances in poplar stems, and would be in contrast to views that BRs act locally (Saini *et al.*, 2015). Based on plant growth phenotypes, a relatively narrow range of BR concentration appears optimal for promoting growth, similar to other plant hormones that show pleiotropic or toxic effects at higher concentrations. Interestingly, time course analysis after BL treatments (Fig. S1) suggested a cyclic response of BR-responsive genes' gene expression, similar to that seen in Ca^{2+}-dependent gene expression responses to BR in other systems (Liu *et al.*, 2015), and that may suggest mechanisms for attenuation of response over time. Our estimates of BR concentrations in stems indicates that BR is found in both stem and bark, and that BR concentrations are asymmetrically distributed around the stem during gravi-stimulation, with higher concentrations found in the bark and xylem of the upwards facing (tension wood) side of the stem (Fig. S3). Together these dynamics point to a need for future research to detail the sites of synthesis of BRs in the stem, and how BRs are transported longitudinally and radially in the stem.

Through our analysis of gene transcript levels we were able to assign genes to functional co-expression modules that showed a diversity of eigengene expression patterns and enrichment of genes for specific biological function. This analysis shows that the effects of experimentally modulating BR levels were modest compared with differences attributable to tissue type and gravi-stimulation. One interpretation of this result is that our experimental treatments were buffered or compensated for by endogenous mechanisms for BR inactivation or catabolism, or by attenuation of BR response. However, specific modules were identified that showed clear response to the BL and PCZ treatments in both bark and wood tissues (Fig. 7). Among those was the 'midnight blue' module that showed strong overrepresentation of genes associated with ethylene signaling, which has been shown to promote tension wood formation in poplar (Love *et al.*, 2009), and the 'grey60' module enriched in genes associated with adaxial/abaxial patterning that is reflected by the extreme eccentric radial growth associated with tension wood formation. Two modules were enriched in BR-related genes: the 'black' module showed enrichment for cell wall and cell division-related genes, while the 'tan' module was enriched for GA and cell-wall-related genes. GA has been shown to stimulate tension wood formation (Gerttula *et al.*, 2015), and cell-wall modifications are a primary feature of tension wood formation, making these modules excellent targets for future study.

Some fundamental issues need to be addressed to fully integrate BR into our understanding of wood formation. Our study suggests that transport of BR both radially and longitudinally should be specifically examined in trees that, in contrast with herbaceous annuals, have large stature and radial growth. The interaction of BR with other key hormones has been shown to be critical for BR function in herbaceous plant development, and must be explored for wood formation. Critically, sites of synthesis and response of BR to environmental conditions must be established for woody growth.

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Author contributions

SG, JD and AG performed the BR concentration optimisation in poplar trees. JD performed the PCZ concentration optimisation in poplar trees. ZL performed the histology, ELISA and RNA minipreps. S-TZ, Y-LL performed the DET2 overexpression lines transformation. JD, ZL performed the phenotype analysis of DET2 lines in normal wood and tension wood. JD, YL carried out the RNA-seq data analysis. JD, SG and M-ZL, ATG designed the experiments. ATG and JD wrote the paper. JD and SG contributed equally to this work.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Expression of BR-related genes in wild-type plants after treatment with 10 μM exogenous BL.

Fig. S2 BR concentration measurement in DET2 overexpression lines and 10 μM exogenous BL-treated wild-type plants.

Fig. S3 DET2 overexpression enhance the gravibending and Tension wood formation.

Fig. S4 BR concentration measurement in DET2 overexpression lines and 10 μM exogenous BL-treated wild-type plants.

Table S1 Summary of RNA-Seq Read counts and FPKM.

Table S2 Differentially expressed genes (DEGs) identified by comparing BR-NW bark with NH-NW bark.

Table S3 Differentially expressed genes (DEGs) identified by comparing BR-NW xylem with NH-NW xylem.

Table S4 Differentially expressed genes (DEGs) identified by comparing BR-OW bark with NH-OW bark.

Table S5 Differentially expressed genes (DEGs) identified by comparing BR-OW xylem with NH-OW xylem.

Table S6 Differentially expressed genes (DEGs) identified by comparing BR-TW bark with NH-TW bark.

Table S7 Differentially expressed genes (DEGs) identified by comparing BR-TW xylem with NH-TW xylem.

Table S8 Module_gene_anno.

Table S9 GO enrichment analysis of each module genes.

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