



## Research article

# In seedlings of *Pinus radiata*, jasmonic acid and auxin are differentially distributed on opposite sides of tilted stems affecting lignin monomer biosynthesis and composition

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## ABSTRACT

Plants respond to the loss of vertical growth re-orientating their affected organs. In trees, this phenomenon has received the scientific attention due to its importance for the forestry industry. Nowadays it is accepted that auxin distribution is involved in the modulation of the tilting response, but how this distribution is controlled is not fully clear. Auxin transporters that determine the spatio-temporal auxin distribution in radiate pine seedlings exposed to 45° of tilting were identified. Additionally, based on indications for an intimate plant hormone crosstalk in this process, IAA and JA contents were evaluated. The experiments revealed that expression of the auxin transporters was down-regulated in the upper half of the tilted stem, while being induced in the lower half. Moreover, transporter-coding genes were first induced at the apical zone of the stem. IAA was consistently redistributed toward the lower half, which is in accordance with the expression profile of the auxin transporters. In contrast, JA was mainly accumulated in the upper half of tilted stems. Finally, lignin content and monomeric composition were analyzed in both sides of stem and along the time course of tilting. As expected, lignin accumulation was higher at the lower half of stem at longer times of tilting. However, the most marked difference was the accumulation of the H-lignin monomer in the lower half, while the G-lignin unit was more dominant in the upper half. Here, we provide detailed insight in the distribution of IAA and JA, affecting the lignin composition during the tilting response in *Pinus radiata* seedlings.

## 1. Introduction

Plants have the ability to modify their growth in response to numerous environmental stimuli. This developmental plasticity is, in part, key for their successful survival. Gravity plays an important role as one of those environmental stimuli, and trees must respond to gravity effects as in nature stems or trunks can be displaced from normal vertical growth, triggering differential cellular responses (Timell, 1986). Molecular events that promote differential stem growth have been reported in response to tilting, such as calcium signaling, synthesis of hormones like auxin and ethylene, and regulation of transcription factors (Li et al., 2009; Muday and DeLong, 2001; Qiu et al., 2008). This differential growth in trees induces the formation of reaction wood,

which in gymnosperms is called compression wood (CW) and is formed in the lower side of stem, displaying a specific composition of walls of tracheids (Zhang et al., 2016, 2017, 2018).

The plant hormone indole-3-acetic acid (IAA) is well known as a master signaling molecule involved in plant embryogenesis, organ development and plant body formation (Zazimalova and Napier, 2003), and is the ‘morphogen’ or ‘positional signaling factor’ as proposed by (Wolpert, 1969). Loss-of-function mutation in the auxin regulated transcriptional activator ARF7 disrupts tropic responses in Arabidopsis hypocotyls (Harper et al., 2000). Characterization of these mutants led to the hypothesis that changes in gene expression represent a critical molecular response to the redistribution of IAA that occurs in response to tropic stimuli (Guilfoyle and Hagen, 2007; Harper et al., 2000).

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The signal transduction cascade involved in the differential cell elongation that causes gravitropic responses, however, remains largely unclear. Nevertheless, a role for auxin in tropic bending has been proposed some time ago by the Cholodny–Went hypothesis (Geisler et al., 2014; Went, 1974). This theory proposes that unequal distribution of auxin between the opposite sides of an organ provokes the differential cell elongation. The mechanism responsible for auxin distribution from sites of synthesis and their relationship to auxin-mediated development have been studied since, which led to the discovery of polar auxin transport (PAT) in plants (Leyser, 2006; Muday and DeLong, 2001). Many genetic and functional approaches indicated that members of AUX1/LAX (AUXIN-INSENSITIVE/LIKE AUX1), ABCB and PIN families are involved in PAT in root gravitropic response (Geisler et al., 2014). Recently, genes that respond to differential auxin concentrations in Arabidopsis inflorescence stems were identified as gravitropic response indicators. Results demonstrated that auxin is differentially distributed between the upper and lower sides of inflorescence stems (Taniguchi et al., 2014). Two R2R3-MYB transcription factors, Arabidopsis FOUR LIPS (FLP; MYB124) and MYB88, have been identified to be involved in the roots gravitropic response modulating PIN3 y PIN7 auxin transporters expression (Wang et al., 2015).

On the other hand, the phytohormone jasmonic acid (JA), ubiquitous in the plant kingdom (Farmer et al., 2003; Wasternack, 2007), regulates several biological processes and metabolite pathways in plants, including flavonoid biosynthesis (Shan et al., 2009; Shimizu et al., 2010). JA was indirectly associated with the auxin distribution during the gravitropic response in rice coleoptiles, in which JA displays a pattern opposite to the prevalent auxin gradient (Gutjahr et al., 2005). The flavonols quercetin and kaempferol inhibit auxin efflux (Jacobs and Rubery, 1988) and, more recently, studies showed that polar auxin transport (PAT) is blocked by kaempferol 3-O-rhamnoside-7-O-rhamnoside in Arabidopsis shoots (Yin et al., 2014).

Lignin is synthesized by the phenylpropanoid pathway, from which the three main hydroxycinnamyl alcohols (lignin monomers or monolignols), *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol forming *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) lignin subunits, respectively, emerge (Boerjan et al., 2003). Recently, it has been shown that the lignin polymer also contains a flavonolignin unit derived from triclin (Lan et al., 2015). In addition, it has been shown that the lignin biosynthesis can be regulated by a JA-dependent regulatory complex (Vélez-Bermúdez et al., 2015).

In this work, we investigate the time-course of the differential accumulation of JA and IAA in young seedlings of *P. radiata* exposed to tilting stress. In addition, different members of AUX, PIN and ABCB transporter families were found differentially expressed in stems of *P. radiata*. Alongside, JA contents were evaluated in the same time-course experiment with tilted *P. radiata* pine seedlings. In order to associate evidences of differential auxin distribution with transcriptional control of genes involved in monolignol biosynthesis, determination of the lignin composition in response to tilting stress was assessed. These

results suggest that auxin transporters could be directing the auxin flux to the lower half of tilted stem, where auxin could be controlling the specific lignin composition in response to this abiotic stress. The presented results suggest that flavonols biosynthesis in the upper side of the tilted stem could be controlled by the spatial and temporal distribution of JA.

## 2. Materials and methods

### 2.1. Plant material

One-year-old half sib seedlings of *Pinus radiata* D. Don (radiata pine) grown in pots and around 30 cm in height were obtained from a nursery in Talca, Chile. Seedlings in pots were tilted to 45° in a growth chamber and harvested after 2.5, 10 and 24 h of tilting employing 25 seedlings for each sampling time (Ramos et al., 2012a). Twenty-five seedlings were kept upright (vertical growth) and used as control plants. Seedlings were maintained under LD conditions (16 h light/8 h dark) and illuminated with halogen lamps (314–494 μmol m<sup>-2</sup>s<sup>-1</sup>) at 25 °C and watered daily by a drip irrigation system. At each sampling time, the 25 seedlings were collected and stems dissected by a longitudinal cut, separating upper from lower half and immediately frozen in liquid nitrogen and stored at –80 °C until RNA extraction and/or hormone content determination was realized. In addition, a group of “non-tilted” seedlings was sampled at each sampling time-point as appropriate controls to evaluate changes in transcript abundance during the day. Moreover, based on previous reports (Ramos et al., 2012a, 2012b), samples from different heights of stems were obtained by transversal cuts of the tilted and non-tilted stems and separated into 3 equal sections designated as apical, medial and basal zone designated as A, M and B, respectively.

### 2.2. Identification of AUX1, PIN and ABCB transcripts

The full-length sequences of the two *PrABCBs*, three *PrAUX1s* and one *PrPIN1* were obtained from the *Pinus radiata* D. Don transcriptome database, which was obtained from sequencing of seven RNA libraries from non-tilted and tilted stems through Illumina HiSeq 2000 platform (Macrogen Inc., Seoul, Korea) (data unpublished). Primers of full-length cDNA sequences are listed in (Table 1).

### 2.3. Total RNA extraction and cDNA synthesis

Total RNA was extracted based on a previous work (Ramos et al., 2012a). Remaining genomic DNA was removed using TURBO DNA-free™ Kit (Ambion, Life Technologies) according to the manufacturer's procedure. Integrity of RNAs was checked on agarose gels and concentration determined in a ND-1000 UV spectrophotometer (Nanodrop Technologies, Montchanin, DE, USA). Three independent RNA extractions were carried out from each frozen pool of samples. First-strand

**Table 1**  
List of primers used in qPCR analysis.

Target gene	Accession number	Primer forward/reverse	Efficiency %	Product size (bp)
<i>PrABCB1</i>	KY807679	5'-CCTCTGCTTCAGTTCCTCACT-3' 5'-TTTTGAGAGAGGGGGTGATG-3'	100	175
<i>PrABCB2</i>	KY807680	5'-ACTGCAATCTCTAGTCAGGGC-3' 5'-TGCTTTCAAACGACTTATCCCAT-3'	100	90
<i>PrAUX1-1</i>	KY807681	5'-CACCTGTGTCTCCCAAT-3' 5'-AGTTGCACAACAGGCAACAG-3'	100	164
<i>PrAUX1-2</i>	KY807682	5'-GCGGAAAATGAAGCTTTGTGC-3' 5'-ATTTCCTCAAGAGCCAGTCT-3'	100	200
<i>PrAUX1-3</i>	KY807683	5'-GCCCATGGCAGACTCATTAT-3' 5'-ACTTGGGCTTCTGCTCTCAA-3'	97	204
<i>PrPIN1</i>	KY807684	5'-CTACGTTGTGTGGGCCTCT-3' 5'-TGCATCTAGGTGCTGACACA-3'	100	160

complementary DNA (cDNA) synthesis was performed using a First Strand cDNA Synthesis Kit (Fermentas Life Science, Glen Burnie, MD, USA) following the manufacturer's instructions.

#### 2.4. Transcripts abundance analysis by real time quantitative PCR (qRT-PCR)

The mRNA abundance of *PrABCBs*, *PrAUX1s* and *PrPIN1* were measured by qRT-PCR analysis. Reaction and quantification were performed following the procedure described previously (Ramos et al., 2012a). Specific primers from UTR-3' region were designed based on a unique full-length sequence (accession numbers in Table 1). Primers used for qPCR analysis are listed in Table 1. Amplification reactions were performed using Maxima SYBR Green/ROX qPCR Master Mix (2X) (ThermoFisher Scientific) in a Stratagene Mx3000P thermocycler (Agilent Technologies) according to the manufacturer's instructions. The protocol used was reported in a prior work (Ramos et al., 2012a), including that for normalizing genes (Ramos et al., 2012b). Data were analyzed using the Excel (Microsoft) macro GENEX v1.10 (gene expression analysis for iCycle iQ<sup>®</sup> real-time PCR detection system, v1.10, 2004; Bio-Rad Laboratories), using the methods derived from the algorithm of Vandesompele (Vandesompele et al., 2002). Data obtained from three individual experiments were analyzed by ANOVA test.

#### 2.5. Sequence analysis

Deduced amino acid sequences were analyzed using the ExPASy Translate Tool available on the ExPASy website (<http://ca.expasy.org>). The multi-alignment of amino acid sequences was performed using ClustalW and BioEdit Sequence Alignment Editor v7.0 software. Molecular mass and isoelectric point prediction were performed using Compute pI/Mw tool ([http://web.expasy.org/compute\\_pi/](http://web.expasy.org/compute_pi/)). The sub-cellular location prediction was performed using the WoLF PSORT II software (Horton et al., 2007). Phylogenetic analyses were conducted using MEGA version 4 software and Bootstrap N-J Tree (with 1000 bootstrap trials) (Tamura et al., 2007). The secondary structure was deduced by PSIPRED software (Jones, 1999).

#### 2.6. Lignin analysis

Whole stems of control and upper and lower halves of radiata pine exposed to tilting were dried and ground to a fine powder, extracted with 20 vol of methanol, and filtered through Whatman GF/A micro-fiber filters to obtain an alcohol insoluble residue (AIR) that was dried at 60 °C for 4 days. 20 mg of AIR was used to determine the lignin composition by thioacidolysis and subsequent GC-MS analysis following the standard procedure (Lapierre et al., 1986). Thioacidolysis liberates the lignin subunits by cleaving the  $\beta$ -ether bonds. 100 mg of AIR was used to determine total lignin content using the gravimetrically Klason Lignin method (Lin and Dence, 1992).

#### 2.7. Hormone profile analysis

Plants material previously frozen was lyophilized. Plant hormone extraction was carried out as previously described (Loba et al., 2017; Loba and Pollmann, 2017). In brief, to approximately 50 mg of each lyophilized plant sample 1 ml of pre-warmed (65 °C) methanol was added and the extraction proceeded for another 60 min at room temperature under gentle shaking. Each sample was supplemented with 50 pmol [<sup>2</sup>H<sub>2</sub>]-IAA, 50 pmol and 50 pmol [<sup>2</sup>H<sub>5</sub>]-JA (stable isotope-labelled internal standards). Cell-free supernatants were dried under vacuum and pre-purified for subsequent gas chromatography-mass spectrometry analysis. For this, the dry extracts were fully dissolved in 50  $\mu$ l methanol and 200  $\mu$ l of diethyl ether. Thereafter, they were loaded onto aminopropyl solid-phase extraction cartridges. Each cartridge was washed twice with 250  $\mu$ l of CHCl<sub>3</sub>:2-propanol (2:1, v/v) before the

hormone containing fraction was eluted with 400  $\mu$ l of acidified diethyl ether (2% acetic acid, v/v). The eluates were transferred into 0.8 ml autosampler vials and again taken to dryness in a gentle stream of nitrogen. Prior to mass spectrometric assessment, samples were derivatized by adding 20  $\mu$ l of a mix consisting of 220  $\mu$ l of acetone:methanol (9:1, v/v), 27  $\mu$ l of diethyl ether and 3  $\mu$ l of a (trimethylsilyl)diazomethane solution (2.0 M in diethyl ether) and letting them rest for 30 min at room temperature. The setting for the gas chromatograph and the mass spectrometer were as described previously (Loba et al., 2017). The following transitions were recorded: MeJA, m/z 151 to m/z 108 (quantifier ion) and m/z 224 to m/z 151 (qualifier ion); [<sup>2</sup>H<sub>5</sub>]-MeJA, m/z 154 to m/z 111 (quantifier ion) and m/z 229 to m/z 154 (qualifier ion); MeIAA, m/z 189 to m/z 130 (quantifier ion) and m/z 130 to m/z 103 (qualifier ion); [<sup>2</sup>H<sub>2</sub>]-MeIAA, m/z 191 to m/z 132 (quantifier ion) and m/z 132 to m/z 103 (qualifier ion). The amount of endogenous hormone contents was calculated from the signal ratio of the unlabelled over the stable isotope-containing mass fragment observed in the parallel measurements.

### 3. Results

#### 3.1. Identification of members of AUX1, ABCB and PIN family transcripts in pine stems

Three sequences encoding for AUX1-like, two for ABCB-like and one for PIN-like transporter proteins were obtained from tilted pine stem. These full-length transcripts were all highly represented in the RNAseq libraries built from stem samples obtained at different times of tilting (unpublished data) and showed high similarity to AUX1, ABCB and to PIN family transporters, respectively. The deduced proteins from their respective genes display an open reading frame (ORF) with a number of amino acids, molecular weight (Mw) and isoelectric point (pI) (Table 2).

Multiple alignment analysis and phylogenetic classification of the presumably full-length deduced protein sequences (Fig. 1A, B and C) showed a high sequence similarity to other characterized ABCB, AUX1 and PIN proteins, respectively.

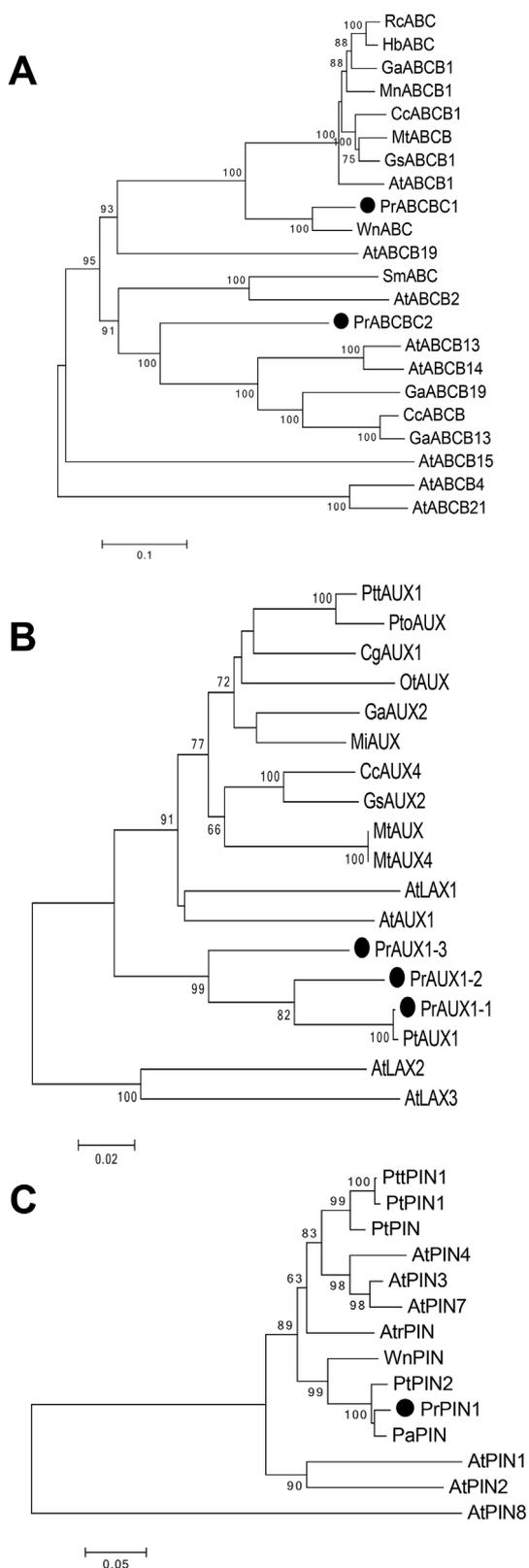
The ATP-binding cassette (ABC) superfamily comprises more than 100 members in plants (Kang et al., 2011) of which members of the superfamily B (ABCB) are involved in auxin transport (Cho and Cho, 2013). In Arabidopsis, AtABCB1, -4, -14, -15, -19 and -21 have been associated with auxin transport, specifically AtABCB1 and AtABCB19 that participate in long distance transport of auxin and in the regulation of root and cotyledon development (Christie et al., 2011; Kamimoto et al., 2012; Kaneda et al., 2011; Lin and Wang, 2005). *AtABCB14* is expressed in vascular tissues of primary stem and the *atabcb14* mutant displays anatomical alterations and also reduced IAA transport along the inflorescence, suggesting that AtABCB14 participates in auxin transport (Kaneda et al., 2011). PrABCB1 shares an identity of 67% and 51% with AtABCB1 and AtABCB19, respectively. In the case of PrABCB2, the deduced protein shares 58% of identity with AtABCB14. These results may underline a putative role of these proteins in auxin transport in the stem of pine.

AUX/LAX is a superfamily, which is recognized as auxin input

**Table 2**

List of genes and their deduced proteins with the respectively biochemical characteristics.

Gene name	ORF (bp)	Amino acids number	Mw (kDa)	pI
<i>PrABCB1</i>	4023	1340	146.18	8.79
<i>PrABCB2</i>	3795	1264	137.29	6.38
<i>PrAUX1-1</i>	1449	482	54.29	8.61
<i>PrAUX1-2</i>	1482	493	55.33	8.85
<i>PrAUX1-3</i>	1485	494	55.55	8.58
<i>PrPIN1</i>	2019	702	76.71	9.13



**Fig. 1.** Phylogenetic analysis of deduced ABCB, AUX/LAX and PIN proteins from radiata pine. **A)** Analysis was performed using following plant ABCB proteins: *Arabidopsis thaliana* AtABCBC1 (AAM98246), AtABCBC13 (Q9C7F8), AtABCBC19 (Q9LJX0), *Cajanus cajan* CcABCBC (KYP70901), CcABCBC1 (KYP38572), *Glycine Soja* GsABCBC1 (KHN09525), *Gossypium arboreum* GaABCBC1 (KHG30028), GaABCBC13 (KHG13479), GaABCBC19 (KHG20565), *Hevea brasiliensis* HbABCBC (AIU41628), *Medicago truncatula* MtABCBC (AES81895), *Morus notabilis* MnABCBC1 (EXB89000), *Ricinus communis* RcABCBC (EEF42902), *Selaginella moellendorffii* SmABCBC (EFJ36032), *Wollemia nobilis* WnABCBC (JAG88297). **B)** Analysis was performed using following plant AUX proteins: *Arabidopsis thaliana* AtAUX1 (CAA67308), AtLAX1 (NP\_195744), AtLAX2 (NP\_179701), AtLAX3 (NP\_177892), *Cajanus cajan* CcAUX4 (KYP53943), *Casuarina glauca* CgAUX1 (ABN81349), *Glycine soja* GsAUX2 (KHM99981), *Gossypium arboreum* GaAUX2 (KHG06629), *Mangifera indica* MiAUX (AFG18185), *Medicago truncatula* MtAUX (CAC12996), MtAUX4 (AAM55305), *Populus tomentosa* PtoAUX (AAW57318), *Populus trichocarpa* PtAUX1 (EEE86686), *Populus tremula x Populus tremuloides* PttAUX1 (AAF21982), *Ochetophila trinervis* OtAUX (AII98157). **C)** Analysis was performed using following plant PIN proteins: *Amborella trichopoda* AtrPIN (ERN11331), *Arabidopsis thaliana* AtPIN1 (AAD04376), AtPIN2 (AAC39513), AtPIN3 (AAD52695), AtPIN4 (AAF36769), AtPIN7 (AAD52697), AtPIN8 (CAC01829), *Picea abies* PaPIN (ACH91613), *Pinus tabuliformis* Ptpin2 (AJP06341), *Populus trichocarpa* Ptpin (EEE89803), Ptpin1 (EEF00981), *Populus tremula x Populus tremuloides* PttPIN1 (AAM54033), *Wollemia nobilis* WnPIN (JAG87809). Deduced proteins of radiata pine are indicated with a black dot.

76% of identity with AtAUX1, respectively. Apart of the *Arabidopsis* AUX transporter, *MtAUX4* encodes a putative auxin import carrier involved in lateral root and nodule development in *Medicago truncatula* (de Billy et al., 2001). The PrAUX proteins share 79% of identity with MtAUX4. Primary sequence alignments and phylogenetic analyses suggest that the identified PrAUXs are likely to encode putative auxin transporters that are possibly involved in the tilting response of pine seedlings.

The PIN protein family encodes for auxin carrier widely studied in *Arabidopsis*. The PIN efflux transporter locates to the plasma membrane and drives the polar flow of auxin (Wisniewska et al., 2006). PIN transporters are key players in important plant processes that include root meristem patterning, root hair growth and lateral root development, vascular differentiation and embryo development among others (Friml et al., 2002; Robert et al., 2013). The identified PrPIN1 protein displays an identity ranging from 54% to 64% with plasma membrane localized *Arabidopsis* AtPIN1, -2, -3, -4 and 7 proteins (Mravec et al., 2009).

An *in-silico* analysis to predict the subcellular localization was performed using the WoLF PSORT II server (Horton et al., 2007) and results suggest a plasma membrane location for all of the radiata pine deduced-proteins. This is in accordance with a putative function as auxin transporter and with their predicted secondary structure showing membrane intrinsic domains for each transporter identified (Suppl. Fig. 1, 2 and 3).

### 3.2. Differential transcript accumulation along the stem exposed to tilting

To investigate the global relative expression profile of the different auxin transporters in response to tilting in whole stem, stress-treated radiata pine seedlings were evaluated at different time points at three heights along the stem of tilted seedlings denominated as apical, medial and basal zones. In this analysis, two controls were considered. The first corresponds to a control of whole stem of non-tilted seedling sampled after 24 h, and the others correspond to control of whole stem non-tilted seedlings at each sampling time to discard the daily fluctuation of gene transcription (Fig. 2).

In the case of ABCB transporters, both *PrABC1* and *PrABC2* transcript profiles were analyzed at different heights of stem. As can be observed in Fig. 2, *PrABC1* has the highest transcript abundance about

carrier. AtAUX1 encodes a protein containing 11 transmembrane helices (Swarup et al., 2004) and the *Ataux1* mutant displays an agravitropic phenotype and selective resistant to auxin (Bennett et al., 1996). The deduced proteins for the identified open reading frames for *PrAUX1-1*, *PrAUX1-2* and *PrAUX1-3* from pine share 77%, 76% and

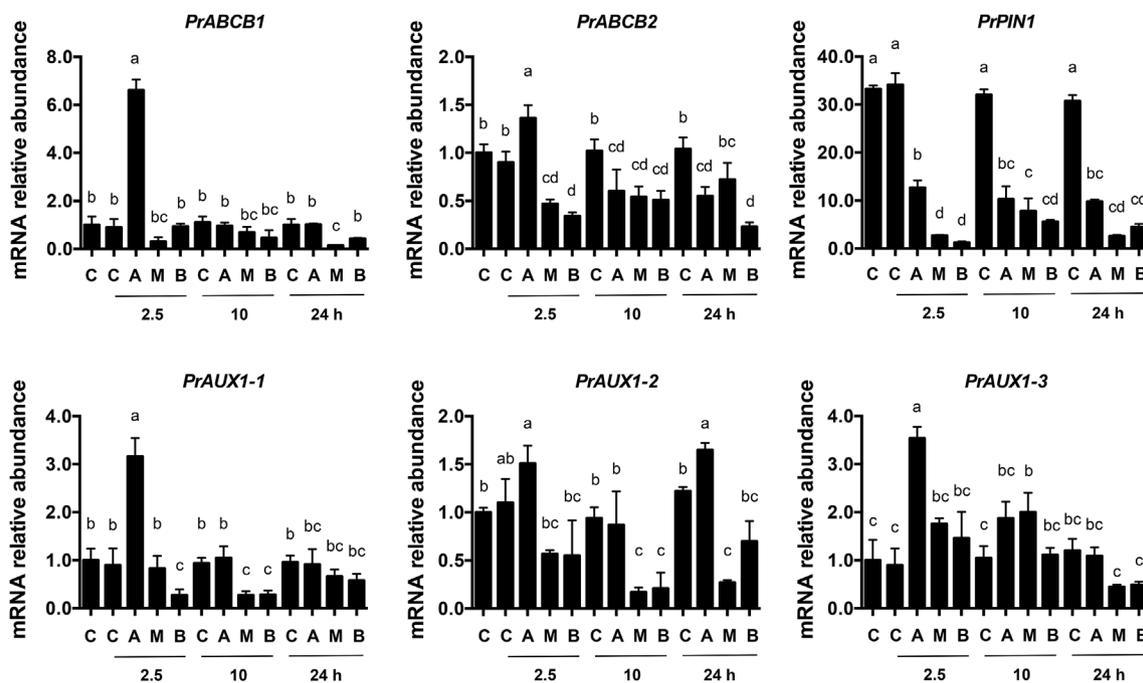


Fig. 2. Transcript levels of *PrABC1*, *PrABC2*, *PrAUX1-1*, *PrAUX1-2*, *PrAUX1-3* and *PrPIN1* transporters after tilting stimuli in radiata pine stem at different heights of tilted stem. Sampling of stems was performed after different tilting times. C means whole stem of non-tilted seedling as control. The first C corresponds to non-tilted seedling sampled after 24 h, and the others correspond to non-tilted seedlings at each sampling time to discard the daily fluctuation of gene transcription. Stems from tilted seedlings were divided into different heights, apical (A), medial (M) and basal (B) section. Data correspond to mean ± SE of three biological replicates. Different letters indicate significant differences with control and samples at each sampling time ( $P < 0.05$ ; ANOVA).

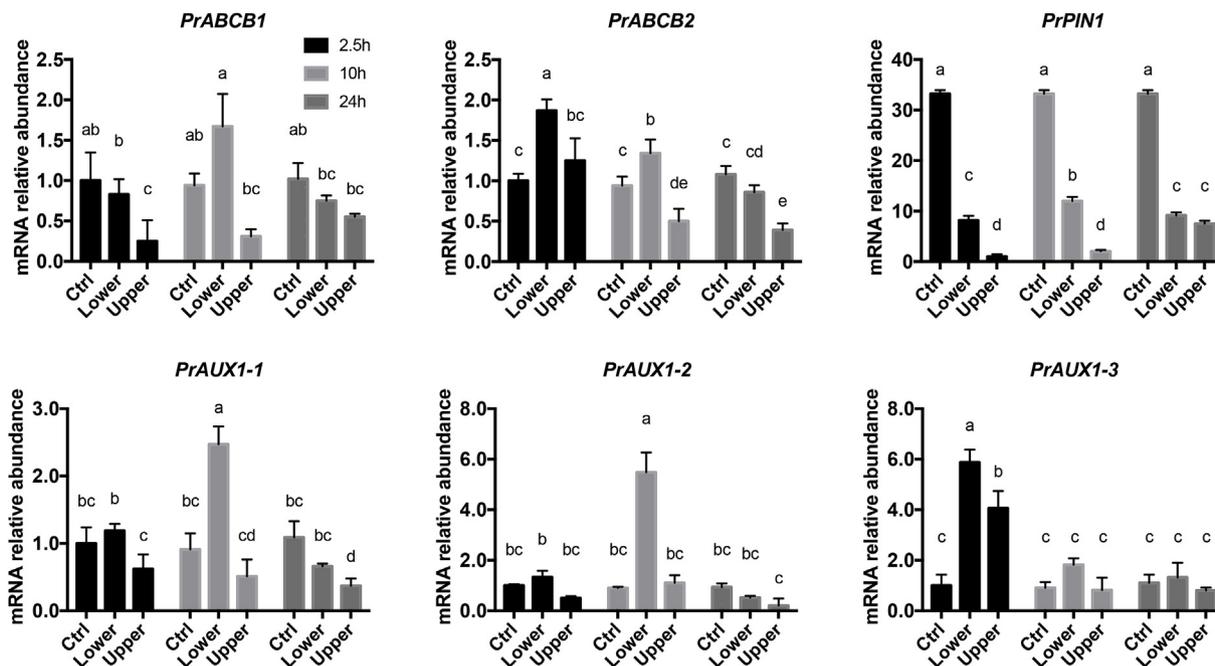


Fig. 3. Transcript levels of *PrABC1*, *PrABC2*, *PrAUX1-1*, *PrAUX1-2*, *PrAUX1-3* and *PrPIN1* transporters after tilting stimuli in radiata pine stem at both sides of tilted stem. Sampling of stems was performed after different tilting times. Ctrl means non-tilted seedling as control. Stems from tilted seedlings were divided into upper and lower halves. Data correspond to mean ± SE of three biological replicates. Different letters indicate significant differences with control and samples at each sampling time ( $P < 0.05$ ; ANOVA).

seven-fold compared to non-tilted control seedlings in the apical section after 2.5 h of tilting and a more discrete induction was observed in the case of *PrABC2*. Moreover, the expression of the two ABCB transporter-like genes was always higher in the apical section compared to the medial and basal parts after different times of tilting, with a strong induction of *PrABC1*. The three transcripts encoding for AUX1/LAX-

like transporter proteins were also evaluated with respect to their time-resolved response to the imposed tilting stress. In the case of *PrAUX1-1*, transcript distribution along the stem showed that the highest abundance was detected in the apical section at all tested time points, compared to the medial and basal sections. For *PrAUX1-2*, the abundance of transcripts was higher at the apical zone of tilted stems along

the time course experiment. Finally, the expression profile of *PrAUX1-3* showed that transcript abundance was significantly higher in the apical zone after 2.5 h compared to the medial and basal zones, as well as to the control (Fig. 2).

In the case of the identified PIN-like transporter transcript, the expression profile analysis of *PrPIN1* at different heights of tilted stem showed the same down-regulation pattern compared to the non-tilted control stem and display an accumulation in the apical zone compared to the medial and basal in all times of tilting stress (Fig. 2).

### 3.3. Differential transcript accumulation between the upper and lower section of stem exposed to tilting

To have deeper sight of the auxin transporters expression profile, we have also investigated whether the *Pinus radiata* ABCB, PIN and AUX genes are differentially expressed depending on the site of the tilting of the stem. Control corresponds to whole stem of non-tilted seedlings after 24 h, considering that controls at each time of tilting did not show changes in transcripts abundance. The mRNA of *PrABCB1* mainly accumulates in the lower half of the stem after 10 h of tilting, while in the case of *PrABCB2*, transcript abundance was higher in the upper half of stem after 2.5 h compared to the lower half, but no differences were observed compared to non-tilted control stem. This difference between the two stem sides was sustained over all time points (Fig. 3).

Additionally, similar to the observed for *PrAUX1-1* that increase the expression in lower half of stem about three-fold compared to non-tilted control stem, *PrAUX1-2* was showed an induction of six-fold after 10 h of tilting at the lower half of stem (Fig. 3). *PrAUX1-3* showed a differential transcript profile between upper and lower half of stem at 2.5 h of tilting. This induction was also observed compared to the non-tilted control stem (Fig. 3).

In addition, analysis of transcript abundance of *PrPIN1* revealed a strong decrease in the relative transcript abundance at both sides of the stem compared to the non-tilted control stem. Interestingly, the transcript abundance analyses in the tilted stem showed significant differences between lower and upper side at 2.5 and 10 h after tilting (Fig. 3).

### 3.4. Tilting exposure induce hormonal differential distribution

Plant hormone contents in upper and lower halves of tilted radiata pine stems were evaluated. JA accumulation analysis showed a statistically significant difference between both sides of tilted stem, increasing in the upper half compared to the lower half at 2.5 and 10 h after tilting. The JA content in the upper half was almost 2-fold higher compared to the non-tilted control stem and to the lower half of stem at 10 h post tilting (Fig. 4).

When IAA levels were evaluated, an early accumulation was recorded in the lower half of the tilted stems after 2.5 h of treatment, relative to the upper half and also to the non-tilted control stem and sustained during 10 and 24 h post tilting (Fig. 4).

### 3.5. Tilting stress response in pine stems affects lignin content and composition

Based on the results presented here and in previous report, auxin appears to be differentially distributed across the tilted stem, suggesting a contribution of the plant hormone in the transcriptional control of phenylpropanoid-related genes (Ramos et al., 2016). Thus, we decided to evaluate compositional changes in the lignin biopolymer in stems of tilted radiata pine seedlings in a time-resolved fashion. Quantitative analysis of lignin indicated that the total lignin content is significantly increased at the lower half of tilted stems after 1 month compared to all other times in upper and lower half (Table 3). The determination of the monomeric composition indicates that changes in the lignin polymer are mostly due to an increase of H monomers, while G monomers

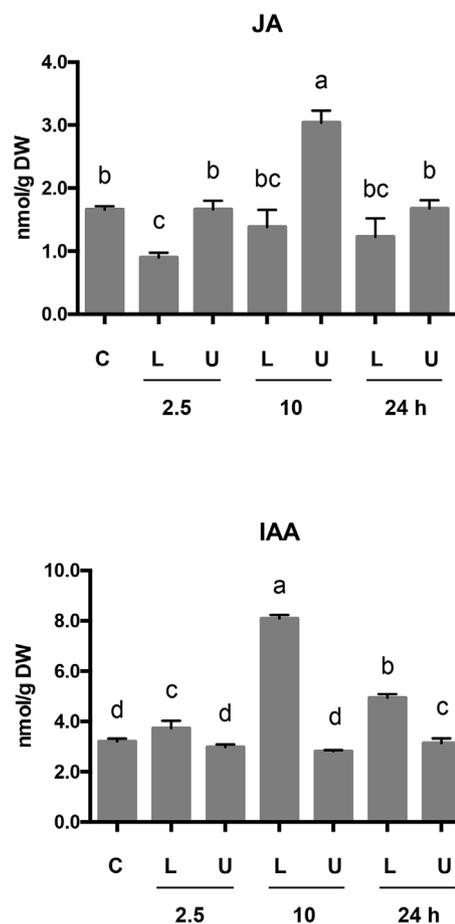


Fig. 4. Content of hormones in plants exposed to tilting stimuli. Sampling of stems was performed after different tilting times. C means non-tilted seedling as control. Stems from tilted seedlings were divided into upper (U) and lower (L) halves. Different letters indicate significant differences ( $P < 0.05$ ; two-way ANOVA). Bars represent means  $\pm$  S.E.

showed a significant decrease at the lower half of tilted stem after 1 month of tilting, while both monomers remain invariable at the upper half (Table 3). The S monomer was not detected in all samples at different times of tilting and also in non-tilted control stem of radiata pine seedlings.

## 4. Discussion

Auxin plays a crucial role in the spatio-temporal control of plant growth and development (Vanneste and Friml, 2009). The key-role and distribution of auxin in the gravitropic response in plant roots has been widely studied (Geisler et al., 2014), but in the aerial part of plants the response it yet not well-understood and even less in stems of trees. Here, we evaluated the pattern of IAA and JA at both sides of tilted stems of radiata pine seedlings. Previous studies in rice reveal a relation between IAA and JA, where JA is accumulated at the opposite part to which auxin is distributed (Gutjahr et al., 2005). In this work we observed that the distribution of JA in gymnosperm trees is in accordance with this previous study in rice.

In plants, the gradients of auxin are generated by polar auxin transport (PAT) (Vanneste and Friml, 2009). PAT is finely coordinated by auxin efflux transporters, i.e. PIN and ABCB plasma membrane proteins, and influx transporters, such as AUX1 (Blakeslee et al., 2007; Zazimalová et al., 2010). For this reason and to generate a transcriptional panorama, we performed a comprehensive spatial and temporal transcript profile analysis of putative auxin transporters from *P. radiata*. The study disclosed that the mRNA abundance of auxin transporters

**Table 3**

Analysis of lignin content and monomeric composition in young seedlings of radiata pine exposed to different times of tilting.

	24 h		1 month	
	Lower	Upper	Lower	Upper
KL (mg/gAIR)	391.0 ± 22.6 <sup>ab</sup>	334 ± 18.4 <sup>b</sup>	417.5 ± 18.4 <sup>a</sup>	367.4 ± 5.7 <sup>b</sup>
%H	14.2 ± 1.3 <sup>b</sup>	13.5 ± 0.9 <sup>b</sup>	47.9 ± 5.1 <sup>a</sup>	16.9 ± 0.3 <sup>b</sup>
%G	85.8 ± 1.3 <sup>a</sup>	86.5 ± 0.9 <sup>a</sup>	52.1 ± 5.1 <sup>b</sup>	83.1 ± 0.3 <sup>b</sup>
G/H	6.0	6.4	1.1	4.9

Stems from tilted seedlings were divided into upper and lower halves. Data correspond to mean ± SE of three biological replicates. Different letters indicate significant differences with control and samples at each sampling time ( $P < 0.05$ ; ANOVA).

was consistently increased in the lower part of the stems, as well as in the apical zone in which auxin is presumably synthesized (Ljung et al., 2001). These results seemingly likely suggest that auxin moves basipetal from the apex via the lower half of the stem, which is in agreement with the relative transcript abundance of the identified putative transporter proteins, and with previous results demonstrating that auxin is distributed to the lower half of tilted stem (Ramos et al., 2016). In addition, the results are consistent with the previously proposed function of ABCBs that are thought to be involved in minimizing apoplastic reflux in apical tissues with high auxin content and in the long-range auxin transport, due to their multilateral expression (Wang et al., 2013). We also identified tree *PrAUXs* that showed an interesting expression profile (Figs. 2 and 3), which is in line with the function of AUX1 transporter observed in the *aux1* mutant in Arabidopsis that displays a strong agravitropic phenotype (Pernisova et al., 2016). Finally, *PrPIN1* displays a strong differential expression between upper and lower half and also along the heights of tilted stem, which highlights the participation of this PIN transporter in gravitropic response in roots (Kleine-Vehn et al., 2010). Recently, Kuhn et al. (2017) observed that flavonols are able to affect the PAT by induction of the movement of AtPIN2 to apical instead to basal localization through a mechanism involving phosphatase/kinase equilibrium. This is in accordance with the observations of differential accumulation of flavonols in the upper half of tilted stem, which suggest that flavonols promotes the auxin redistribution to the lower half of tilted stem and also agreed with expression analysis of auxin-repressed protein gene (ARP) and an immunoassay confirming the distribution at the lower part of tilted stem exposed to tilting (Ramos et al., 2016). Additionally, genes encoding to key enzymes related to the monolignols biosynthesis are up regulated by auxin treatment, which suggest this hormone as commander of lignin deposition in lower half of tilted seedlings (Ramos et al., 2016).

Based on the concomitant temporal and spatial accumulation of phytohormones and the transcriptional profiles of genes assessed, it may be concluded that JA regulates auxin distribution towards the lower half of tilted stem through, at least, two complementary mechanisms; one related to a negative transcriptional regulation of auxin transporters (Sun et al., 2009) and another modulating the gene expression of the flavonol biosynthesis pathway-related genes in the upper half of tilted stem (Shan et al., 2009; Shimizu et al., 2010). To probe this hypothesis, future approaches employing the isolation of promoter regions of auxin transporters and flavonoid biosynthetic pathway genes and their subsequent analysis in transactivation assays, in order to test their interaction and functionality.

The hormonal distribution observed, suggested that the flux of auxin is redirected toward the lower half, where it presumably contributes to the control the lignin monomer biosynthesis (Ramos et al., 2016). In this work, the authors suggested that auxin treatment repress caffeic acid O-methyl transferase (COMT) more than cinnamoyl-CoA reductase (CCR), which is evidenced by differential transcript levels at the lower half of tilted stem (Ramos et al., 2016). Here, we analyzed the composition of the lignin biopolymer at both side of the stem. The obtained results are consistent with those findings, observing that the *p*-hydroxyphenyl unit (H) is the main monomer synthesized in response

to tilting at the lower half of tilted stem, suggesting that the lower half of the tilted stem is producing a lignin polymer that is more condensed than the one produced in the upper half (Welker et al., 2015). This result is in accordance with a study performed in radiata pine trees exposed to longer times of tilting to generate compression wood (Zhang et al., 2017). Authors report that compression wood cell walls accumulate more lignin with higher proportions of H units than lignin accumulated in normal and opposite wood. The increase in lignin concentration and the proportion of H units depends on the extent of compression wood development, also referred to as compression wood severity (Zhang et al., 2017). A more condensed lignin in the lower side of the tilted stem suggest that *Pinus radiata* is producing to some extent a lignin polymer that is more enriched in carbon-carbon linkages and therefore that it is more resistant lignin than the one produced in the upper side of the tilted stem. This result is in line with a previous report, in which transcripts of several genes involved in lignin biosynthesis are overexpressed in compression wood of loblolly pine (Villalobos et al., 2012) and with higher *p*-hydroxyphenyl units reported by Dey (1990). This behavior underlines the fact that lignin composition is altered by different stresses in plants (Moura et al., 2010). We also observed that lignin in young radiata pine seedlings analyzed contains a high proportion of H units relative to G units, which could be explained based on observations that percentage of different lignin monomers change with the age of the plant, the percentage of H units decrease conforming trees get older (Rencoret et al., 2011). This happens because the monomers do not deposit on the wall at the same time. First, the H units are synthesized, then the synthesis of H is stopped and the G synthesis is initiated (and then the S, if any). Therefore, as the plant gets older, the percentage of H decreases and in an adult tree it is only detected in percentages smaller than what we observed in young seedlings. Consequently, adult plants have been more time to accumulate G units (and S) and, therefore, a smaller percentage of H units are detected.

#### CRediT authorship contribution statement

**Romina Salazar:** Formal analysis, Formal analysis.  
**Stephan Pollmann:** Formal analysis, Writing - original draft.  
**Luis Morales-Quintana:** Formal analysis, Writing - original draft.  
**Raul Herrera:** Formal analysis. **David Caparrós-Ruiz:** Formal analysis, Writing - original draft. **Patricio Ramos:** Formal analysis, Formal analysis, Formal analysis, Writing - original draft.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2018.12.008>.

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