plant biology

Plant Biology ISSN 1435-8603

RESEARCH PAPER

Wood development regulators involved in apical growth in *Pinus canariensis*

V. Chano, J. Sobrino-Plata, C. Collada & A. Soto

GENFOR, Grupo de Investigación en Genética y Fisiología Forestal, ETSI Montes, Universidad Politécnica de Madrid. Ciudad Universitaria s/n, 28040, Madrid, Spain

Keywords
primary growth; secondary growth; shoot development; transcription factors.

Correspondence
V Chano, GENFOR, Grupo de Investigación en Genética y Fisiología Forestal. ETSI Montes. Universidad Politécnica de Madrid. Ciudad Universitaria s/n 28040 Madrid, Spain.
E-mail: vm.chano@upm.es

Present address
Department of Forest Genetics and Forest Tree Breeding, University of Göttingen, Büsgenweg 2, 37077, Göttingen, Germany

Editor
B. Hause

Received: 23 November 2020; Accepted: 30 November 2020

doi:10.1111/plb.13228

ABSTRACT

- The shoot apical meristem is responsible of seasonal length increase in plants. In woody plants transition from primary to secondary growth is also produced during seasonal apical growth. These processes are controlled by different families of transcription factors.
- Levels of transcriptomic activity during apical growth were measured by means of a cDNA microarray designed from sequences related to meristematic activity in *Pinus canariensis*. The identification of differentially expressed genes was performed using a time-course analysis.
- A total of 7170 genes were differentially expressed and grouped in six clusters according to their expression profiles. We identified master regulators, such as WUSCHEL-like HOMEOBOX (WOX), to be involved in the first stages of apical development, *i.e.* growth of primary tissues, while other transcription factors, such as Class III HOMEODOMAIN-LEUCINE ZIPPER (HD-ZIP III) and KNOTTED-like (KNOX) and BEL1-like (BELL) HOMEODOMAIN proteins, were found to be induced during last stages of apical seasonal development, already with secondary growth.
- Our results reveal the main expression patterns of these genes during apical development and the transition from primary to secondary stem growth. In particular, the regulatory factors identified play key roles in controlling stem architecture and constitute candidate genes for the study of other development processes in conifers.

INTRODUCTION

The shoot apical meristem (SAM) is a stem cell niche placed at the tip of the apex and new buds, responsible for shoot apical growth and which gives rise to three meristematic tissues, protoderm, ground meristem and procambium (Murray *et al.* 2012). Primary vascular tissues are derived from the last, arranged into the vascular bundles. Most commonly, vascular bundles show xylem towards the inner part of the organ and phloem on the outside, eventually separated by the remainder of the meristematic tissue (fascicular cambium) (Ha *et al.* 2010). In those plants capable of secondary growth, *i.e.* gymnosperms and woody eudicots, these vascular bundles are arranged surrounding the pith, forming a so-called eustele. Periclinal divisions of the initial cells in the fascicular cambium will give rise to a new series of radially disposed cells, thus increasing the size of vascular bundles. Additionally, the parenchyma cells between bundles become meristematic and form the interfascicular cambium, and the merger of both fascicular and interfascicular cambium forms the vascular cambium, a continuous lateral meristem that originates from secondary xylem to the interior and secondary phloem to the exterior. This secondary growth results in the seasonal increase in stem diameter (Beck 2005).

Master regulators, *i.e.* transcription factors that control the expression of downstream genes, are involved in SAM maintenance and organization, as well as in the transition from dormancy to primary and to secondary growth (George *et al.* 2008). Some of the transcription factors reported to be involved in apical development include the WUSCHEL (WUS) homeobox transcription factor, a positive regulator of undifferentiated meristematic cell maintenance (Sparks *et al.* 2013; Miyashima *et al.* 2013; Jha *et al.* 2020), and proliferation from the stem cell niches in apical meristems after embryogenesis (Haecker *et al.* 2004), together with CLAVATA (CLV) proteins (Somssich *et al.* 2016). Expression of the WUSCHEL-RELATED HOMEOBOX (WOX4) transcription factor is detected in the procambium and cambium (Hirakawa *et al.* 2010) and acts redundantly in *Arabidopsis* with another WUSCHEL homologue, WOX14 (Etchells *et al.* 2013). Furthermore, the HOMEODOMAIN-LEUCINE ZIPPER (HD-ZIP) class III transcription factors are mainly associated with vascular growth regulating development from the early globular embryo to secondary growth (McConnell *et al.* 2001; Emery *et al.* 2003; Ramachandran *et al.* 2016) and have emerged as important regulators of vasculature organization and shoot polarity (Sanchez *et al.* 2012). This master regulator family has five HD-ZIP III members in *Arabidopsis*: REVOLUTA/INTERFASCICULAR FIBERLESS1 (REV/IFL1), PHABULOSA (PHB/ATHB14), PHAVOLUTA (PHV/ATHB9), CORONA/INCURVATA4 (CNA/ICU4/ATHB15) and ARABIDOPSIS

Plant Biology **23** (2021) 438–444 © 2020 The Authors. *Plant Biology* published by John Wiley & Sons Ltd on behalf of German Society for Plant Sciences, Royal Botanical Society of the Netherlands

THALIANA HOMEOBOX8 (ATHB8), and their expression is controlled epigenetically though microRNA165/166 activity (Ramachandran *et al.* 2016). Conversely, genes from the KANADI family were shown to act in opposition to HD-ZIP III in radial patterning, abaxially on the phloem side (Emery *et al.* 2003). In addition, the plant TALE (*i.e* three-amino-acid-loop-extension) superfamily, which in *Arabidopsis* comprises the KNAT (for KNOTTED-like from *Arabidopsis thaliana*) members and the BEL1-like (BELL) members. The KNAT family is formed by two clades that include KNAT class I (KNOX1), with SHOOT MERISTEMLESS (STM), BREVIPEDICELLUS (BP)/KNAT1, KNAT2 and KNAT6, KNAT class II (KNOX2), with KNAT3, KNAT4, KNAT5 and KNAT7. On the other hand, the BELL family comprises 13 genes (*e.g.* BELL1 (BEL1) or PENNYWISE (PNY); Furumizu *et al.* 2015). Both KNOX and BEL1-like proteins, which interact and form heterodimers, are highly conserved in structure and regulate cell wall biosynthesis during secondary growth (Du *et al.* 2009). Moreover, while KNOX1 clade expression is restricted in shoot and inflorescence tissue (Byrne *et al.* 2000), KNOX2 is expressed in different tissues which may play specific roles depending on the process (Furumizu *et al.* 2015).

Pinus canariensis is one of the few conifers with the ability to re-sprout and give rise to new vegetative shoots after losing its canopy due to mechanical injury and forest fires (Climent *et al.* 2004), making this species a suitable model for studies on transcription factors regulating meristem activity and apical growth in conifers. In a previous study, we analysed and reported the transcription profiles of these regulators during cambial activity in *P. canariensis* (Chano et al. 2917b). Here, we aim to comprehensively extend this study by analysing the activation and transcription patterns of these transcription factors during the transition from primary to secondary growth in apical growth. For this purpose, we used a cDNA microarray designed with sequences from these cambial samples and complemented with contigs from other apical cDNA libraries. Our study reports genes coding for putative regulatory transcription factors involved in development processes and provides insights in their specific roles in apical growth and the initiation of secondary growth.

MATERIAL AND METHODS

Plant material

For this work we used three 5-year-old *P. canariensis* trees grown in the experimental garden at UPM facilities under environmental conditions with regular watering. At the beginning of this experiment, trees were approximately 2.5-m high and 5–7 cm in diameter at breast height. Apical shoot harvesting was performed at five sampling times during the growing season: I, represents the beginning of the season (15 April), when most buds started to swell due to increasing temperatures; II samples collected 2 weeks later (30 April 30), when apical shoot elongation starts; III and IV (22 May and 3 July, respectively) represent two intermediate states of shoot elongation; and V is samples harvested on 11 September, when apical shoots were at the end of their seasonal development. From each sampling date, four replicates were collected, three for microarrays analysis, which were frozen in liquid nitrogen and stored at −80 °C until RNA extraction, and one for the anatomical study.

Anatomical analysis

Using a Leica SM2400 microtome (Leica Biosystems, Nussloch, Germany), 20-μm thick cross-sections were obtained from the base of the apical samples. Slices were treated with sodium hypochlorite, washed with distilled water two to three times, then stained for 2 min with 1% safranin (v/v) and for 1 min with 1% Alcian blue (w/v), washed with distilled water and dehydrated in an ethanol series. Finally, sections were mounted on slides for bright-field microscopy.

Extraction of RNA and microarray analysis

Total RNA was isolated from each sample stored at −80 °C using the CTAB-LiCl precipitation method (Chang *et al.* 1993) and purified with the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Quantity of total RNA for each sample was measured with a Nanodrop model ND-1000 (Thermo Scientific, Waltham, MA, USA), and RNA quality was checked using Experion Bioanalyzer (Bio-Rad, Hercules, CA, USA).

A one-colour 60K microarray (Agilent, Santa Clara, CA, USA) was designed using a previously constructed transcriptome of meristematic activity in *P. canariensis* plus a set of contigs from *Pinus pinea* apical tissue cDNA library and hybridized as described in Chano *et al.* (2017b). Differentially Expressed Genes (DEGs) were detected using a time-course analysis of microarray normalized data. For this purpose, the software *maSigPro* for R (Conesa *et al.* 2006) was used with the following options: (1) polynomial degree 4 for regression complexity; (2) FDR adjustment; (3) significance level of 0.05; (4) hierarchical clustering method; and (5) number of clusters = 6.

Validation with qRT-PCR of gene expression

The expression patterns of seven DEGs obtained from microarrays experiments of apical growth were confirmed by qRT-PCR using the same RNA employed for microarray hybridizations. First strand cDNA synthesis was performed using SuperScript™ III reverse transcriptase (Invitrogen, Waltham, MA, USA) following the manufacturer's instructions and using 4 μg total RNA. Gene specific primers were designed for selected DEGs (Table 1) with a melting temperature between 60 and 65 °C and producing amplicons between 80 and 120 bp. qRT-PCR was performed in a CFX96™ Real-Time PCR Detection System (Bio-Rad), using the SsoFast™ EVAgreen. Supermix (Bio-Rad), according to the manufacturer's protocol, and following the standard thermal profile: 95 °C for 3 min, 40 cycles of 95 °C for 10 s and 60 °C for 10 s. In order to compare data from different qRT-PCR runs, the CT values were normalized using Ri18S as housekeeping gene. The expression ratios were then obtained using the Δ-Δ-CT method corrected for the PCR efficiency for each DEG (Pfaffl 2001).

Distribution of qRT-PCR data was assessed in R by means of the Shapiro-Wilk test, and homoscedasticity was analysed by using the Bartlett formula when data were normally distributed; otherwise, a Levene test was used. For homoscedastic data, differences between growth stages were tested by means of ANOVA or the Kruskal-Wallis test, depending on whether data were Gaussian or non-Gaussian, respectively. For heteroscedastic data, Welch's ANOVA test was used. Finally, concordance between microarray and qRT-PCR expression

Table 1. Primers used for qRT-PCR

Contig name	Oligo name	Description	Fwd/Rev	bp	Tm	GC	Sequence (5′-3′)
Contig00787	Pc_00787_NAC_F1	NAC2-like protein	Forward	20	62.5	45	CTAAATGGCCCTGGGTAAAA
	Pc_00787_NAC_R1		Reverse	20	62.8	50	CCCCTTCTTCTTACCAACCA
Contig03225	Pc_03225_EXPANSIN_F1	α-expansin-like protein	Forward	20	62.8	45	AAGCGGAGCTGATTCTTGAT
	Pc_03225_EXPANSIN_R1		Reverse	20	63.1	60	CTCAGAGCCACAGAGACGAG
Contig05551	Pc_05551_WRKY_F1	WRKY51-like protein	Forward	20	62.5	45	ACGCAGAGGGGAATAAGAAA
	Pc_05551_WRKY_R1		Reverse	20	63.2	50	CAGAAAACGTTCACCCACAG
Contig06476	Pc_06476_CCoAOMT_F1	CCoAOMT-like protein	Forward	20	64.0	50	GATTGAACAACCGAGGTGCT
	Pc_06476_CCoAOMT_R1		Reverse	20	63.6	45	TGCAACACCTGAATTCCAAC
Contig05410	Pc_05410_PECTINESTERASE_F1	Pectin esterase 2-like	Forward	20	63.1	55	GTACTCTCGCACGGTCTTCA
	Pc_05410_PECTINESTERASE_R1		Reverse	20	62.5	45	ATAATAAAGCGTCCCCAACG
Contig13239	Pc_13239_YABBY_F1	axial regulator yabby 5-like	Forward	20	62.4	45	AGAGGATCAAAGCCCACAAT
	Pc_13239_YABBY_R1		Reverse	20	63.4	45	TGTCCATCATCATCCCAAAG
Contig04961	Pc_04961_BEL1_F1	BEL1-like protein	Forward	21	64.2	47.6	TGAAACTTCAGCACCTCATCC
	Pc_04961_BEL1_R1		Reverse	20	63.9	50	AGCTCTGGCTTAGGCAACAA
Housekeeping	Ri18S_FW	18S ribosomal	Forward	19	62.4	53	GCGAAAGCATTTGCCAAGG
	Ri18S_RV		Reverse	21	62.4	48	ATTCCTGGTCGGCATCGTTTA

Tm, Melting temperature; GC, guanine–cytosine content; bp, base pair.

data was calculated using Pearson's correlation test for normally distributed data and Spearman's *rho* correlation for not normally distributed data.

RESULTS AND DISCUSSION

Anatomical observations

To determine anatomical changes during primary and secondary growth, we analysed cross-sections from the base of sequentially collected samples. As shown in Fig. 1, stage I corresponded to the beginning of apical shoot development, as assumed during sample harvesting. Primary phloem and primary xylem were formed from early differentiating fascicular cambium within individual vascular bundles. In stage II samples, vascular bundles established contact and a complete vascular cambium could be observed. These samples, harvested just 2 weeks after the initiation of the experiment, indicate a fast transition from primary to secondary growth. From stage III onwards, vascular cambium drives secondary vascular growth, with the formation and lignification of new tracheids. Finally, stage V highlighted the differentiation between early- and latewood, based on cell lumen diameter and secondary cell wall thickness and lignification.

Transcriptomic profiling

A time-course analysis was performed to determine transcriptomic changes during seasonal apical growth. We identified 7,170 genes using an adjusted *P*-value and False Discovery Rate of 0.05, showing significant differences in their transcription levels, being considered as DEGs (Table S1). Six clusters were identified after hierarchical clustering, summarized into three main profiles and their opposites (Fig. 2): (i) while Cluster A showed a continuous increase in transcription levels of 1538 DEGs, Cluster B included 1399 DEGs whose transcription levels decreased steadily throughout the analysed period; (ii) Cluster C showed a strong increase in transcription from stage I to II for 659 genes, followed by a progressive decrease in gene expression until almost reaching null expression in stage V, while, conversely, Cluster D displayed a strong gene repression from stage I to II; (iii) and finally, Cluster E showed repression from stage I to II but followed by induction of gene expression in stages IV and V, while Cluster F exhibit an opposed profile – induced to stage II and then repressed to stages IV and V. Interestingly, variability in shoot growth in selected trees do not differ greatly, especially during the initiation of apical growth (stages I and II), and for those genes included in Cluster D. In contrast, these differences are higher for the last stage (V), as seen in Clusters A, B and F.

We designed gene-specific primers for seven DEGs in order to validate cDNA microarray data using qRT-PCR (Fig. 3). Overall, high Pearson's or Spearman's correlation coefficients (depending on data normality) were found between microarray and qRT-PCR expression values, ranging from $r = 0.62$ (Contig04961 for BEL1-like protein) to $r = 1$ (Contig00787 for NAC2-like protein). The highest values were found for rank-based Spearman's correlation analysis, *i.e.* NAC2-like protein (Contig00787), pectin esterase-like protein (Contig05410) and WRKY51-like protein (Contig05551). Moreover, significant differences ($P < 0.05$) among samples were found for all analysed DEGs, with the exception of Contig05410, which was significant only at $P = 0.65$.

As expected, genes involved in cell growth and primary and secondary cell wall formation were found among these clusters. DEGs coding for expansin-like and endoglucanase-like proteins, mainly expressed during earlywood formation in the Canary Island pine (Chano *et al.* 2017b), were found to be mostly involved in the first stages of apical growth (Cluster B and Cluster D). Conversely, some transcripts expressed during latewood development in the Canary Island pine, including transcription factors such as MYB-like and NAC78-like proteins (Chano *et al.* 2017b), were found to be highly expressed at stage V (Cluster A), when latewood is already forming (Fig. 1). In addition, in this work we found that several DEGs with the same annotation presented different behaviours and were clustered separately, suggesting that different members of one genic family may play different roles. This is the case of

Plant Biology **23** (2021) 438–444 © 2020 The Authors. *Plant Biology* published by John Wiley & Sons Ltd on behalf of German Society for Plant Sciences, Royal Botanical Society of the Netherlands

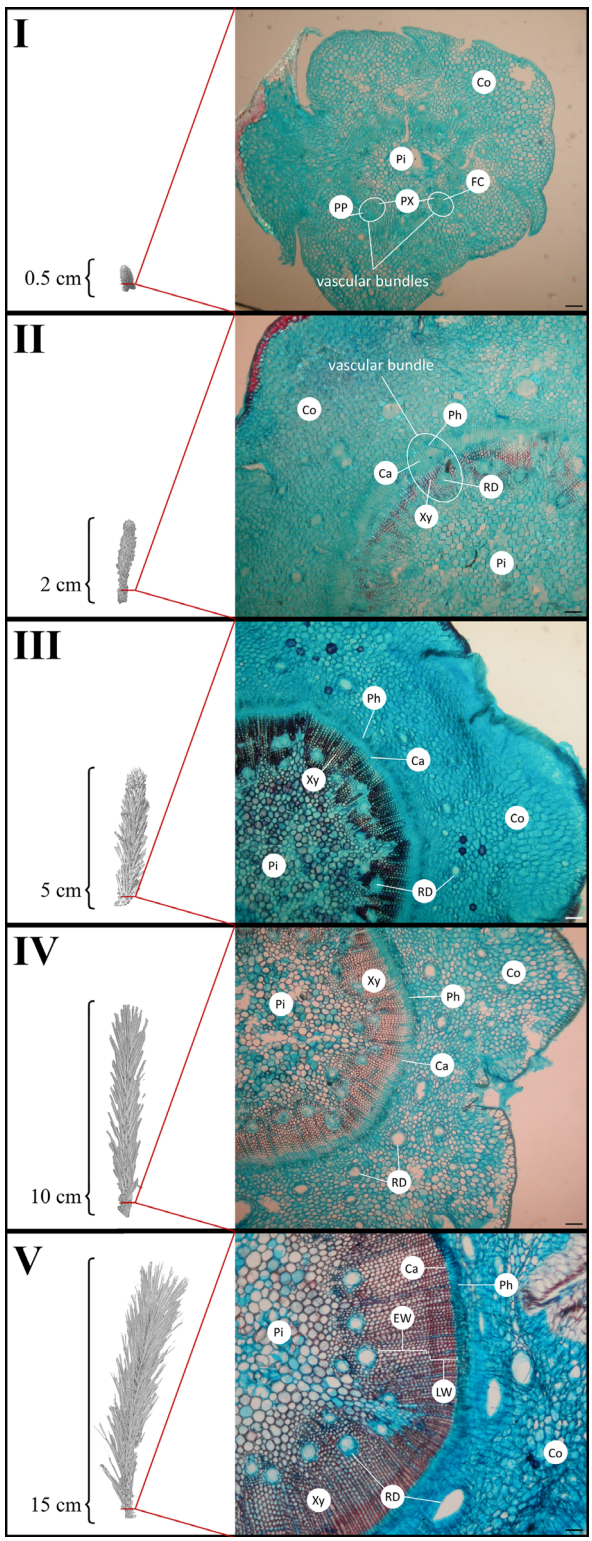

Fig. 1. Cross-sections through five development stages in *Pinus canariensis* apical shoots. 20-μm-thick cross-sections were obtained from the base of five sequentially collected apical shoots and stained with safranin and Alcian blue for bright-field microscopy observation. Co: cortex; Pi: pith; FC: fascicular cambium; Ca: vascular cambium; PP: primary phloem; PX: primary xylem; Ph: secondary phloem; Xy: secondary xylem; RD: resin ducts; EW: earlywood; LW: latewood; Scale bars: I = 200 μm; II–IV: 100 μm; V: 50 μm.

CAZyme superfamily members, cellulose synthases or sucrose synthases, as well as DEGs coding for transcription factors belonging to the NAC, MYB or WRKY families, which are involved in multiple biological processes (Hu *et al.* 2010; Ambawat *et al.* 2013; Sun *et al.* 2018).

Major transcription factor families involved in SAM development showed different expression patterns (Table 2), suggesting specific roles during the overall apical growth period. Thus, the WOX family showed early expression during primary growth (Cluster D), whereas the HD-ZIP III and TALE families were found during secondary growth at advanced stages (Cluster A). Three transcripts (Ppnisotig01375, −07657 and −08931) annotated to the WOX family were found to only be overexpressed at stage I, with a sharp decline at the very beginning of secondary growth (II) onwards (Cluster D). According to the original annotation of these transcripts to the Viridiplantae section of the RefSeq protein database (NCBI; Chano *et al.* 2017b), Ppnisotig01375, −07657 and −08931 are homologous to WOX9, WOX2 and WOX4, respectively. In *Arabidopsis*, AtWOX2 is expressed during apical patterning in embryo development under regulation of AtWOX8/9 (Breuninger *et al.* 2008). In conifers, PcWOX2 is mainly expressed in embryo mass derived from buds, but not in callus derived from seedling needles (Park *et al.* 2010). Similarly, PaWOX2 and PaWOX8/9 are highly expressed at early stages of embryonic development, although only PaWOX8/9 is also expressed in procambium during later embryo stages (Palovaara *et al.* 2010). Moreover, WOX is also implicated in *in vitro* caulogenesis for micropropagation of *P. pinea* genotypes (Alvarez *et al.* 2020). Furthermore, WOX4 has been found preferentially expressed in procambium and cambium regulating the maintenance of vascular stem cells (Ji *et al.* 2010; Hirakawa *et al.* 2010; Suer *et al.* 2011). In *Arabidopsis*, this gene has been shown to induce xylem differentiation (Zhang *et al.* 2019), whereas many other WUS genes have been described as negative regulators of cell differentiation by repression of transcription factors, such as KANADI1/KAN1 and KANADI2/KAN2, or YABBY3/YAB3, and in the

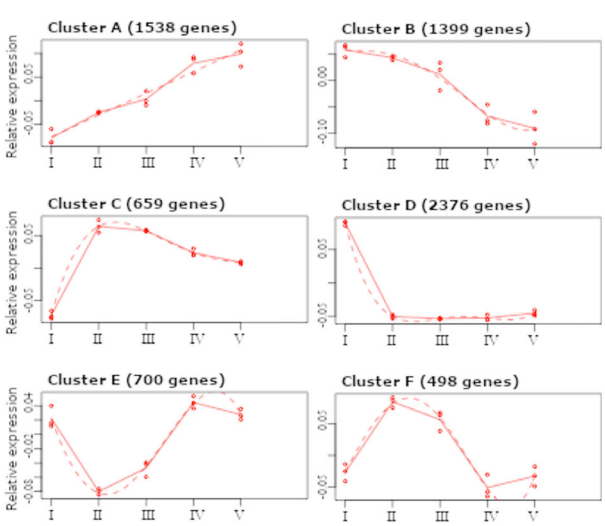

Fig. 2. K-means clustering (k = 6) of all differentially expressed genes during apical growth in *Pinus canariensis*. *x*-axis: development stages of apical growth; *y*-axis: relative expression values from microarray experiments. In parentheses: number of genes included in each cluster.

specification of cell fate, such as KNAT1 and BLH5 (Yadav *et al.* 2013). This suggests that the sharp decline produced in WOX transcription levels at stage II may be required for the overexpression of vascular growth-related genes, such as those grouped in Cluster A, and the transition to secondary growth, which is produced just 2 weeks after the activation of apical development. Conversely, the expression pattern observed for the putative WOX4-like coding transcript (Ppnisotig08931) contrasts with that described for another WOX-4 paralogue (Contig06813), expressed during earlywood development in *P. canariensis* (Chano *et al.* 2017b) and in later stages of wound-wood formation in response to mechanical damage (Chano *et al.* 2017a).

Control of meristem size by repression of WUS genes is produced by three parallel pathways, CLV, ERECTA (ER) and HD-ZIP III (Mandel *et al.* 2016). While transcripts coding for CLV and ER were not differentially expressed, we found two members of the HD-ZIP III family (Contig01739 and −32787) coding for different isoforms of PHABULOSA (PHB)/ ATHB14, gradually overexpressed to stages IV and V (Cluster

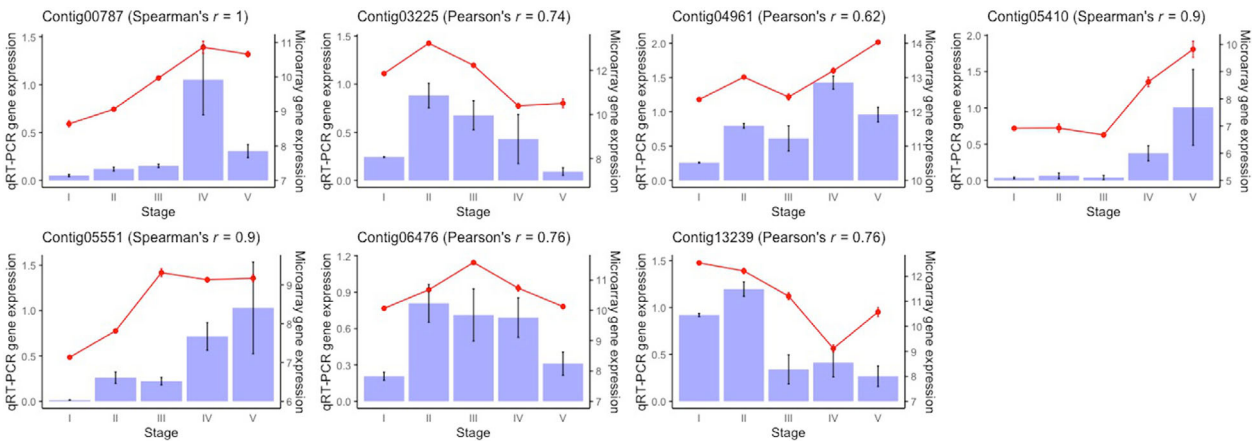

Fig. 3. Real-time PCR validation of microarray expression values in *Pinus canariensis* of seven genes coding for NAC2-like (Contig00787), expansin-like (Contig03225), BLH1-like (Contig04961), pectinesterase-like (Contig05410), WRKY51-like (Contig05551), CCoAOMT-like (Contig06476) and YAB5-like (Contig13239). *x*-axis: development stages of apical growth; *y*-axis left: normalized gene expression values for qRT-PCR (bars); *y*-axis right: absolute expression values for microarray experiments (continuous line). Pearson's/Spearman's correlation coefficients between qRT-PCR and microarray expression values are also indicated for each gene.

Table 2. Selected differentially expressed Transcription Factors during apical growth in *Pinus canariensis*

Cluster	Seq. Name	Top-Hit Description	e-value	Seq. Length
HD-ZIP III				
A	Contig01739	homeobox-leucine zipper protein athb-14-like	0	1696
A	Contig32787	homeobox-leucine zipper protein athb-14-like isoform x2	3.92E-06	638
HD-ZIP IV				
B	Ppnisotig05462	homeobox-leucine zipper protein meristem l1-like	1.99E-177	1217
B	Ppnisotig07853	homeobox-leucine zipper protein hdg11-like	7.47E-21	932
D	Ppnisotig07553	homeobox-leucine zipper protein meristem l1-like	2.30E-25	952
KANADI				
D	Contig22823	probable transcription factor kan2-like	1.23E-32	651
WUSCHEL-like				
D	Ppnisotig01375	wuschel-related homeobox 9	6.54E-40	1405
D	Ppnisotig07657	wuschel-related homeobox 2	1.41E-10	935
D	Ppnisotig08931	wuschel-related homeobox 4-like	2.26E-10	844
BELL and KNOX				
A	Contig04961	bel1-like homeodomain protein 1	6.39E-128	1843
A	Contig01956	homeobox protein knotted-1-like 2-like	2.75E-91	1510
A	Contig05803	homeobox protein knotted-1-like 3-like	3.28E-116	1713
A	Contig11838	homeobox protein knotted-1-like 2-like	5.26E-99	2495
A	Contig20487	homeobox protein knotted-1-like 2-like	1.79E-96	1376
OTHER FACTORS				
B	Ppnisotig04954	growth-regulating factor 1-like	4.61E-22	1336
B	Contig03423	floricaula leafy homologue	7.09E-69	681
B	Ppnisotig08430	protodermal factor 1-like	6.06E-23	878
B	Contig13239	axial regulator yabby 5-like	1.84E-56	894

Plant Biology **23** (2021) 438–444 © 2020 The Authors. *Plant Biology* published by John Wiley & Sons Ltd on behalf of German Society for Plant Sciences, Royal Botanical Society of the Netherlands

A). PHB/ATHB14 is involved in vascular development and acts redundantly with PHAVOLUTA (PHV)/ATHB9 (McConnell *et al.* 2001). However, this gene was not found to be differentially expressed during lateral growth in the Canary Island pine (Chano *et al.* 2017b). A close homologue of ATHB14 in *Populus trichocarpa* x *P. deltoides* is mainly expressed in the apical shoot (Côté *et al.* 2010), while PmHB11, homologue of ATHB14 in *Prunus mume*, is expressed preferentially in xylem and cambium (Li *et al.* 2019). We also found a putative KAN2 coding transcript (Contig22823) in Cluster D, overexpressed at stage I. The members of the HD-ZIP III and KANADI families play complementary roles in the establishment and maintenance of radial patterning in stems, with xylem developing inwards and phloem outwards (McConnell *et al.* 2001; Emery *et al.* 2003; Hawker & Bowman 2004). These results may indicate that ATHB14 is closely associated with advanced stages of secondary xylem development, while KAN2 acts during both primary and secondary phloem formation. Moreover, the HD-ZIP IV is also related to development processes but expressed specifically in the outer cell layer of plant organs related to protodermal cells (Nakamura *et al.* 2006). We found transcripts coding for ATML1 (Ppnisotig05462 and −07553 in Clusters B and D, respectively) and HDG11 (Ppnisotig07853 in Cluster B) overexpressed at the beginning of apical growth. Similarly, other DEGs involved in bud development and organogenesis initiation were also found in Cluster B, namely GROWTH-REGULATING FACTOR1-like (GRF1; Ppnisotig04954), LEAFY (LFY; Contig03423); PROTODERMAL FACTOR1-like (PDF1; Ppnisotig08430) and YABBY5-like (YAB5; Contig13239) (Abe *et al.* 2003; Moyroud *et al.* 2011; Omidbakhshfard *et al.* 2015; Finet *et al.* 2016).

Finally, members of the TALE family were also found to be mainly expressed during late stages of apical growth (Cluster A). Transcript Contig04961 coding for a BLH1 (BEL1-like HOMEODOMAIN 1) was co-expressed with three KNAT2 (HOMEOBOX KNOTTED1-like 2) paralogues (Contig01956, −11838 and −20487) and one KNAT3 (HOMEOBOX KNOTTED1-like 3) transcript (Contig05803). Some TALE members found in this work (*i.e.* BLH1, KNAT3 and one KNAT2 paralogue) were also found during latewood development in the Canary Island pine (Chano *et al.* 2017b). This is consistent with the presence of a growth ring in the stage V micrograph. In *Arabidopsis,* KNAT2 is involved in developmental regulation of the shoot apical meristem (Smith & Hake 2003) and acts redundantly with KNAT6 during inflorescence growth (Ragni *et al.* 2008). This gene is also expressed in the root tip in *Pachyareus pringlei* (Reyes-Rivera *et al.* 2017) and in shoot and root tissues of *Lophosphora williamsii* (Ibarra-Laclette *et al.* 2015), together with KNAT3. The latter regulates ABA responses in germination and early seedling development (Kim *et al.* 2013) and acts together with BLH1 during embryo sac development (Pagnussat *et al.* 2007). The BEL1-like is required for SAM development (Rutjens *et al.* 2009). Although we have found KNAT2 and KNAT3 are co-expressed in Cluster A, these genes have been found to act antagonistically in *Arabidopsis* during aerial organ differentiation (Furumizu *et al.* 2015).

CONCLUSIONS

In this work we showed that transcriptional activity during apical shoot development includes paralogues of transcription factors involved in secondary xylem development in *P. canariensis*. We identified three transcripts coding for WOX family members (*i.e.* WOX2-, WOX4- and WOX9-like transcription factors) controlling primary growth at early stages of this process, and whose repression might be needed for the transition to secondary growth. In addition, two ATHB14-like paralogues were co-clustered with several members of the KNOTTED-like and BEL1-like families, suggesting major roles during secondary growth. These results provide a better understanding of primary growth and the transition to secondary growth, and these genes are potential candidates to learn more about the developmental processes in conifers, but further research in functional genomics is needed in order to resolve the roles of genes and proteins in apical development.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

AS and CC designed the experiments. VC realized the anatomical approach, RNA extraction, gene expression analysis and wrote the manuscript. JSP performed validation of gene expression values using qRT-PCR. All authors contributed for the final version of the manuscript.

ACKNOWLEDGEMENTS

This work was supported by the projects AGL2009-10606 (Spanish Ministry of Science and Innovation), SPIP2014-01093 (Spanish National Parks Agency, Ministry of Agriculture) and FP7-KBBE-2011-5 289841 (European Commission, Seventh Framework Programme).

Data Availability Statement

Microarray data were deposited in the NCBI Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo) under the accession number GSE154508.

SUPPORTING INFORMATION

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

Table S1. Differentially expressed genes during apical shoot development in *Pinus canariensis*.

REFERENCES

Abe M., Katsumata H., Komeda Y., Takahashi T. (2003) Regulation of shoot epidermal cell differentiation by a pair of homeodomain proteins in *Arabidopsis*. *Development*, **130**, 635–643.

Alvarez J.M., Bueno N., Cuesta C., Feito I., Ordás R.J. (2020) Hormonal and gene dynamics in de novo shoot meristem formation during adventitious caulogenesis in cotyledons of *Pinus pinea*. *Plant Cell Reports*, **39**, 527–541.

Ambawat S., Sharma P., Yadav N.R., Yadav R.C. (2013) MYB transcription factor genes as regulators for plant responses: an overview. *Physiology and Molecular Biology of Plants*, **19**, 307–321.

Beck C.B. (2005) *An introduction to plant structure and development*, 2nd edn. Cambridge University Press, New York, USA.

Breuninger H., Rikirsch E., Hermann M., Ueda M., Laux T. (2008) Differential Expression of WOX Genes Mediates Apical-Basal Axis Formation in the *Arabidopsis* Embryo. *Developmental Cell*, **14**, 867–876.

Byrne M.E., Barley R., Curtis M., Arroyo J.M., Dunham M., Hudson A., Martienssen R.A. (2000) *Asymmetric leaves1* mediates leaf patterning and stem cell function in *Arabidopsis*. *Nature*, **408**, 967–971.

Chang S., Puryear J., Cairney J. (1993) A simple and efficient method for isolating RNA from pine trees. *Plant Molecular Biology Reporter*, **11**, 113–116.

Chano V., Collada C., Soto Á. (2017) Transcriptomic analysis of wound xylem formation in *Pinus canariensis*. *BMC Plant Biology*, **17**, 234.

Chano V., López de Heredia U., Collada C., Soto Á., Soto A. (2017) Transcriptomic analysis of juvenile wood formation during the growing season in *Pinus canariensis*. *Holzforschung*, **71**, 919–937.

Climent J., Tapias R., Pardos J.A., Gil L. (2004) Fire adaptations in the Canary Islands pine (*Pinus canariensis*). *Plant Ecology*, **171**, 185–196.

Conesa A., Nueda M.J., Ferrer A., Talón M. (2006) maSigPro: a method to identify significantly differential expression profiles in time-course microarray experiments. *Bioinformatics*, **22**, 1096–1102.

Côté C.L., Boileau F., Roy V., Ouellet M., Levasseur C., Morency M.-J., Cooke J.E.K., Séguin A., MacKay J.J. (2010) Gene family structure, expression and functional analysis of HD-Zip III genes in angiosperm and gymnosperm forest trees. *BMC Plant Biology*, **10**, 273.

Du J., Mansfield S.D., Groover A.T. (2009) The *Populus* homeobox gene ARBORKNOX2 regulates cell differentiation during secondary growth. *The Plant Journal*, **60**, 1000–1014.

Emery J.F., Floyd S.K., Alvarez J., Eshed Y., Hawker N.P., Izhaki A., Baum S.F., Bowman J.L. (2003) Radial Patterning of *Arabidopsis* Shoots by Class III HD-ZIP and KANADI Genes. *Current Biology*, **13**, 1768–1774.

Etchells J.P., Provost C.M., Mishr L., Turner S.R. (2013) WOX4 and WOX14 act downstream of the PXY receptor kinase to regulate plant vascular proliferation independently of any role in vascular organisation. *Development*, **140**, 2224–2234.

Finet C., Floyd S.K., Conway S.J., Zhong B., Scutt C.P., Bowman J.L. (2016) Evolution of the YABBY gene family in seed plants. *Evolution and Development*, **18**, 116–126.

Furumizu C., Alvarez J.P., Sakakibara K., Bowman J.L. (2015) Antagonistic roles for KNOX1 and KNOX2 genes in patterning the land plant body plan following an ancient gene duplication. *PLoS Genetics*, **11**, e1004980.

George E.F., Hall M.A., Klerk G.-J.D, (2008) Developmental biology. In: George E.F., Hall M.A., De K.-J. (Eds), *Plant Propagation by Tissue Culture*, 3rd edn. Springer, Dordrecht, the Netherlands, pp 283–333.

Ha C.M., Jun J.H., Fletcher J.C. (2010) Shoot apical meristem form and function. *Current Topics in Developmental Biology*, **91**, 103–140.

Haecker A., Gross-Hardt R., Geiges B., Sarkar A., Breuninger H., Herrmann M., Laux T. (2004) Expression dynamics of WOX genes mark cell fate decisions during early embryonic patterning in *Arabidopsis thaliana*. *Development*, **131**, 657–668.

Hawker N.P., Bowman J.L. (2004) Roles for Class III HD-Zip and KANADI Genes in *Arabidopsis* Root Development. *Plant Physiology*, **135**, 2261–2270.

Hirakawa Y., Kondo Y., Fukuda H. (2010) TDIF peptide signaling regulates vascular stem cell proliferation via the WOX4 homeobox gene in *Arabidopsis*. *The Plant Cell*, **22**, 2618–2629.

Hu R., Qi G., Kong Y., Kong D., Gao Q., Zhou G. (2010) Comprehensive analysis of NAC domain transcription factor gene family in *Populus trichocarpa*. *BMC Plant Biology*, **10**, 145.

Ibarra-Laclette E., Zamudio-Hernández F., Pérez-Torres C.A., Albert V.A., Ramírez-Chávez E., Molina-Torres J., Fernández-Cortes A., Calderón-Vázquez C., Olivares-Romero J.L., Herrera-Estrella A., Herrera-Estrella L. (2015) *De novo* sequencing and analysis of *Lophophora williamsii* transcriptome, and searching for putative genes involved in mescaline biosynthesis. *BMC Genomics*, **16**, 657.

Jha P., Ochatt S.J., Kumar V. (2020) WUSCHEL: a master regulator in plant growth signaling. *Plant Cell Reports*, **39**, 431–444.

Ji J., Strable J., Shimizu R., Koenig D., Sinha N., Scanlon M.J. (2010) WOX4 promotes procambial development. *Plant Physiology*, **152**, 1346–1356.

Kim D., Cho Y.H., Ryu H., Kim Y., Kim T.H., Hwang I. (2013) BLH1 and KNAT3 modulate ABA responses during germination and early seedling development in *Arabidopsis*. *The Plant Journal*, **75**, 755–766.

Li L., Zheng T., Zhuo X., Li S., Qiu L., Wang J., Cheng T., Zhang Q. (2019) Genome-wide identification, characterization and expression analysis of the HD-Zip gene family in the stem development of the woody plant *Prunus mume*. *PeerJ*, **7**, e7499.

Mandel T., Candela H., Landau U., Asis L., Zelinger E., Carles C.C., Williams L.E. (2016) Differential regulation of meristem size, morphology and organization by the ERECTA, CLAVATA and class III HD-ZIP pathways. *Development*, **143**, 1612–1622.

McConnell J.R., Emery J., Eshed Y., Bao N., Bowman J., Barton M.K. (2001) Role of PHABULOSA and PHAVOLUTA in determining radial patterning in shoots. *Nature*, **411**, 709–713.

Miyashima S., Sebastian J., Lee J.-Y.-Y., Helariutta Y. (2013) Stem cell function during plant vascular development. *The EMBO Journal*, **32**, 178–193.

Moyroud E., Minguet E.G., Ott F., Yant L., Posé D., Monniaux M., Blanchet S., Bastien O., Thévenon E., Weigel D., Schmid M., Parcy F. (2011) Prediction of regulatory interactions from genome sequences using a biophysical model for the *Arabidopsis* LEAFY transcription factor. *The Plant Cell*, **23**, 1293–1306.

Murray J.A.H.H., Jones A., Godin C., Traas J. (2012) Systems analysis of shoot apical meristem growth and development: Integrating hormonal and mechanical signaling. *The Plant Cell*, **24**, 3907–3919.

Nakamura M., Katsumata H., Abe M., Yabe N., Komeda Y., Yamamoto K.T., Takahashi T. (2006) Characterization of the Class IV Homeodomain-Leucine zipper gene family in *Arabidopsis*. *Plant Physiology*, **141**, 1363–1375.

Omidbakhshfard M.A., Proost S., Fujikura U., Mueller-Roeber B. (2015) Growth-Regulating Factors (GRFs): A small transcription factor family with important functions in plant biology. *Molecular Plant*, **8**, 998–1010.

Pagnussat G.C., Yu H.-J., Sundaresan V. (2007) Cell-fate switch of synergid to egg cell in *Arabidopsis eostre* mutant embryo sacs arises from misexpression of the BEL1-like homeodomain gene *BLH1*. *The Plant Cell*, **19**, 3578–3592.

Palovaara J., Hallberg H., Stasolla C., Hakman I. (2010) Comparative expression pattern analysis of WUSCHEL-related homeobox 2 (WOX2) and WOX8/9 in developing seeds and somatic embryos of the gymnosperm *Picea abies*. *New Phytologist*, **188**, 122–135.

Park S.Y., Klimaszewska K., Park J.Y., Mansfield S.D. (2010) Lodgepole pine: the first evidence of seed-based somatic embryogenesis and the expression of embryogenesis marker genes in shoot bud cultures of adult trees. *Tree Physiology*, **30**, 1469–1478.

Pfaffl M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, **29**, e45.

Ragni L., Belles-Boix E., Günl M., Pautot V. (2008) Interaction of KNAT6 and KNAT2 with BREVIPEDICELLUS and PENNYWISE in *Arabidopsis* inflorescences. *The Plant Cell*, **20**, 888–900.

Ramachandran P., Carlsbecker A., Etchells J.P., Turner S. (2016) Class III HD-ZIPs govern vascular cell fate: An HD view on patterning and differentiation. *Journal of Experimental Botany*, **69**, 55–69.

Reyes-Rivera J., Rodríguez-Alonso G., Petrone E., Vasco A., Vergara-Silva F., Shishkova S., Terrazas T. (2017) Expression of the KNOTTED HOMEOBOX genes in the Cactaceae cambial zone suggests their involvement in wood development. *Frontiers in Plant Science*, **8**, 218.

Rutjens B., Bao D., Van Eck-Stouten E., Brand M., Smeekens S., Proveniers M. (2009) Shoot apical meristem function in *Arabidopsis* requires the combined activities of three BEL1-like homeodomain proteins. *The Plant Journal*, **58**, 641–654.

Sanchez P., Nehlin L., Greb T. (2012) From thin to thick: Major transitions during stem development. *Trends in Plant Science*, **17**, 113–121.

Smith H.M.S., Hake S. (2003) The interaction of two homeobox genes, BREVIPEDICELLUS and PENNYWISE, regulates internode patterning in the *Arabidopsis* inflorescence. *The Plant Cell*, **15**, 1717–1727.

Somssich M., Je B., Il S.R., Jackson D. (2016) CLAVATA-WUSCHEL signaling in the shoot meristem. *Development*, **143**, 3238–3248.

Sparks E., Wachsman G., Benfey P.N. (2013) Spatiotemporal signalling in plant development. *Nature Reviews Genetics*, **14**, 631–644.

Suer S., Agusti J., Sanchez P., Schwarz M., Greb T., Agustí J., Sanchez P., Schwarz M., Greb T. (2011) WOX4 imparts auxin responsiveness to cambium cells in *Arabidopsis*. *The Plant Cell*, **23**, 3247–3259.

Sun H., Hu M., Li J., Chen L., Li M., Zhang S., Zhang X., Yang X. (2018) Comprehensive analysis of NAC transcription factors uncovers their roles during fiber development and stress response in cotton. *BMC Plant Biology*, **18**, 150.

Yadav R.K., Perales M., Gruel J., Ohno C., Heisler M., Girke T., Jönsson H., Venugopala R.G. (2013) Plant stem cell maintenance involves direct transcriptional repression of differentiation program. *Molecular Systems Biology*, **9**, 654.

Zhang J., Eswaran G., Alonso-Serra J., Kucukoglu M., Xiang J., Yang W., Elo A., Nieminen K., Damén T., Joung J.G., Yun J.Y., Lee J.H., Ragni L., Barbier de Reuille P., Ahnert S.E., Lee J.Y., Mähönen A.P., Helariutta Y. (2019) Transcriptional regulatory framework for vascular cambium development in *Arabidopsis* roots. *Nature Plants*, **5**, 1033–1042.