Title: Analysis of the *WUSCHEL-RELATED HOMEOBOX* gene family in *Pinus pinaster*: new insights into the gene family evolution

José M. Alvarez[1*], Natalia Bueno[1], Rafael A. Cañas[2], Concepción Avila[2], Francisco M. Cánovas[2], Ricardo J. Ordás[1]

[1] Departamento de Biología de Organismos y Sistemas, Universidad de Oviedo, Spain

[2] Departamento de Biología Molecular y Bioquímica, Facultad de Ciencias, Universidad de Málaga, Spain.

* Corresponding author: alvarezmanuel@uniovi.es

ABSTRACT

WUSCHEL-RELATED HOMEOBOX (*WOX*) genes are key players controlling stem cells in plants and can be divided into three clades according to the time of their appearance during plant evolution. Our knowledge of stem cell function in vascular plants other than angiosperms is limited, they separated from gymnosperms ca 300 million years ago and their patterning during embryogenesis differs significantly. For this reason, we have used the model gymnosperm *Pinus pinaster* to identify *WOX* genes and perform a thorough analysis of their gene expression patterns. Using transcriptomic data from a comprehensive range of tissues and stages of development we have shown three major outcomes: that the *P. pinaster* genome encodes at least fourteen members of the *WOX* family spanning all the major clades, that the genome of gymnosperms contains a *WOX* gene with no homologues in angiosperms representing a transitional stage between intermediate- and WUS-clade proteins, and that we can detect discrete *WUS* and *WOX5* transcripts for the first time in a gymnosperm.

Highlights

- *Pinus pinaster* genome encodes at least fourteen members of the *WOX* family spanning all the major clades
- Discrete *WUS* and *WOX5* transcripts were detected for the first time in a gymnosperm
- The genome of gymnosperms contains a *WOX* gene, *WOXX*, with no homologues in angiosperms representing a transitional evolutionary stage from intermediate- to WUS-clade proteins

Keywords: embryo development, FISH, meristem, plant evolution, root, seedlings, somatic embryogenesis, shoot apex, WOX phylogeny, *WUSCHEL-RELATED HOMEOBOX*

1. INTRODUCTION

Homeobox (HB) proteins are a superfamily of transcription factors containing a DNA-binding homeodomain (HD), which is a conserved 60-amino acid motif. Evolutionary studies indicate that the different families of HB transcription factors have diverged prior to the separation of the branches leading to animals, plants and fungi [1]. In plants, they have been recently classified into 14 classes: homeodomain-leucine zipper (HD-ZIP) classes I to IV, BEL-like (BEL), KNOTTED1-like homeobox (KNOX), plant zinc finger (PLINC), WUSCHEL-related homeobox (WOX), Plant homeodomain (PHD), DDT, Nodulin Homeobox genes (NDX), Luminidependens (LD), SAWADEE and Plant Interactor Homeobox (PINTOX) [2]. HB transcription factors participate in a great variety of processes during plant growth and development, such as determination of cell fate, cell differentiation, morphogenesis or responses to stress among others. Members of the WOX family play important roles in key developmental processes, such as embryonic patterning, stem-cell maintenance and organ formation [3-5]. Some members of the plant-specific WOX protein family can act both as activators and repressors depending on tissue type or developmental stage [6, 7].

The genome of *Arabidopsis* (*Arabidopsis thaliana*) contains 15 *WOX* genes. The *WOX* gene family has been divided into three major clades: the WUSCHEL (WUS) clade (*AtWUS* and *AtWOX1-7*), specific to ferns and seed plants; the intermediate clade (*AtWOX8*, *9*, *11* and *12*), present in vascular plants; and the ancient clade (*AtWOX10*, *13*, and *14*), with representatives in the earliest diverging green plants and therefore probably derived from an ancestral *WOX* gene [4]. The role of the *WOX* genes during plant development has been studied in some angiosperms, such as *Arabidopsis*, *Petunia hybrida*, *Zea mays*, *Oryza sativa* and *Populus tomentosa* [3, 8-11]. However, little information is available in conifers. All *WOX* genes examined show very specific expression patterns, both spatially and temporally, which are important for their functions. Members of the ancient clade are expressed all over (roots, shoots and reproductive organs) and developmental stages [12]. *WOX* genes belonging to the intermediate clade, as well as *WOX2* belonging to the WUS clade, are preferentially expressed during embryo development [13]. Some members of the WUS clade are involved in stem-cell regulation. In *Arabidopsis*, *AtWUS* is expressed in the organizing center (OC) and is involved in the maintenance of the shoot apical meristem (SAM) by a regulatory loop with *CLAVATA*, while *AtWOX5* is involved in the maintenance of the root apical meristem (RAM) [14, 15]. *AtWOX4* is involved in the cambial meristem differentiation [16], while *AtWOX3/PRS1* is involved in lateral organ development through recruiting organ founder cells forming the lateral domain [17, 18]. This functional divergence appears to have resulted primarily from the

evolution of divergent expression patterns, as many studies have shown that most of the WUS-clade members are interchangeable in the *Arabidopsis* SAM [11, 15, 18, 19].

Arabidopsis has been widely used as a model organism for studies in plants [20]. Gymnosperm and angiosperm species, which have a common ancestor ca 300 million years ago [21, 22], share many morphological and physiological features. However, there are key differences, such as the patterning during embryogenesis, which may alter the underlying genetic programs. Therefore, it is not known whether the model of genic expression during angiosperm development may be applicable to conifers. Several studies suggest that the *WOX* gene family may be involved in the evolution of developmental processes [8, 12, 23]. Thus, analysis of the tissue-specific expression of *WOX* genes using other model species outside the angiosperms are needed to elucidate similarities and differences in the regulatory mechanisms of plant development.

Recent works in conifers have shown functional conservation for some *WOX* genes. *AtWOX8*, *AtWOX9* and *AtWOX2* play important roles during the patterning and morphogenesis of the early embryo in *Arabidopsis* [13, 24]. Their orthologues in the conifer *Picea abies PaWOX8/9* and *PaWOX2* have similar functions [25, 26]. *PaWOX3*, the orthologue of *AtWOX3*, has been shown to play an important role in lateral organ outgrowth [27]. Despite the functional conservation of some *WOX* genes between angiosperms and gymnosperms, previous reports in gymnosperms suggested that the shoot-specific expression of *WUS* and root-specific expression of *WOX5* is restricted to angiosperms. Only single homologues of *WUS*/*WOX5* were identified in three gymnosperms (*Pinus sylvestris*, *Ginkgo biloba*, and *Gnetum gnemon*), which were expressed in both the shoot and the root, suggesting that a single *WUS/WOX5* functional gene performs its role both in the shoot and root meristems [28]. Basing on these results, it was proposed the hypothesis that the last common ancestor of seed plants contained a single *WUS/WOX5* precursor gene, and *WUS* and *WOX5* probably arose as a consequence of a gene duplication event followed by a neofunctionalization that took place at the base of angiosperms. Recent studies in *P. abies* found differentiate *WUS* and *WOX5* genes in its genome. *PaWOX5* was preferentially in roots tips, but also in shoot tips. However, no *PaWUS* expression was detected in any of the plant parts studied. Based on that, it was proposed that both genes originated before the split between gymnosperms and angiosperms, but the functional specialization took place only in the angiosperms lineage [29].

In the present work, the analysis of the *WOX* gene family in the conifer maritime pine (*Pinus pinaster* Aiton) is presented. Fourteen *WOX* genes have been identified and the phylogenetic relationships of these genes compared to other known *WOX* genes in green alga, bryophyte, lycophyte, pteridophyte, gymnosperm, and angiosperm representative species have been

analysed. The phylogenetic analyses have identified three members of the ancient clade, five members in the intermediate clade, and six members in the WUS clade including five clear orthologues of the angiosperm WUS-clade genes and a new member, *PpWOXX*, with no homologues in angiosperms. Furthermore, the expression pattern for each of the 14 *WOX* genes was analysed in different developmental stages during somatic embryo development, and in different germination stages and tissues in seedlings from zygotic embryos. The detection of discrete *PpWUS* and *PpWOX5* transcripts for the first time in a gymnosperm and their differentiated expression patterns, which was thought to be exclusive from angiosperms, might indicate that these genes perform similar roles to those described for their *Arabidopsis.* These results suggest that the functional specialization might have taken place before the split between angiosperms and gymnosperms. The identification of *WOX* genes in *P. pinaster* provides new insights into the *WOX* family evolution in plants and will facilitate molecular studies to characterize the function of stem cells in gymnosperms.

2. MATERIALS AND METHODS

2.1. Identification and phylogenetic analysis of the *Pinus pinaster WOX* gene family

2.1.1. Identification

The identification of the *WOX* gene family members in *P. pinaster* was carried out by combining PCR-based detection and the screening of *P. pinaster* transcriptome data obtained in the frame of the European projects ProCoGen [30] and SustainPine [31]. Genome data, when available, were used to identify exon-intron pattern.

WUSCHEL (*WUS*) sequences from different species were found through the search in the public databases GenBank (http://www.ncbi.nlm.nih.gov/), Dendrome (http://dendrome.ucdavis.edu/), and Congenie (http://congenie.org/). After determining conserved domains and motifs through ClustalW alignments, a fragment of the coding sequence was amplified using cDNA obtained from *P. pinaster* embryos as template. The full *WUS* mRNA sequence was obtained by Rapid Amplification of cDNA Ends (RACE) using the FirstChoice RLM-RACE Kit (Ambion, Applied Biosystems Inc., Foster City, CA, USA) according to the manufacturer's instructions. The TBLASTN and BLASTP algorithms [32], and HMM profile via HMMER (http://hmmer.org/) with default settings were used for the screening of *P. pinaster* transcriptome and proteome data searching for sequences containing the characteristic *WOX* homeodomain. The full-length cDNAs were cloned using CloneJET PCR Cloning Kit (Thermo Scientific, Waltham, MA, USA) and sequenced (at least three clones per band) at the Oviedo

University DNA Analysis Facility (Spain). *WOX* sequences obtained in *P. pinaster* were also used as queries to identify new *WOX* sequences in the genomes of *Pinus taeda* and *Picea abies*.

2.1.2. Phylogenetic analysis

Sequences for WOX proteins from green alga (*Ostreococcus tauri*), moss (*Physcomitrella patens*), lycophyte (*Selaginella moellendorffii*), fern (*Ceratopteris richardii* and *Cyathea australis*), gymnosperm (*Ginkgo biloba, Gnetum gnemon*, *Picea abies*, *Pinus pinaster*, *Pinus sylvestris*, and *Pinus taeda*), and angiosperm (basal angiosperm: *Amborella trichopoda*, monocots: *Oryza sativa* and *Zea mays*, and dicots: *Arabidopsis thaliana*, *Populus euphratica*, *Populus trichocarpa* and *Vitis vinifera*) representatives were identified through the search in public databases (accession numbers for all sequences are listed in Supplementary Table S1).

Protein sequences were aligned using the MAFFT plug-in in Geneious (Biomatters Ltd., New Zealand) and edited manually. Non-conserved parts of the sequences were excluded from the analyses to reduce noise. The unrooted amino acid sequence similarity trees were generated using the Geneious software by the Neighbour-Joining method and the Jukes-Cantor genetic distance model. The green alga OtWOX sequence was used as outgroup for the trees.

Non-synonymous (Ka) and synonymous (Ks) nucleotide substitution rates for the *WOX* gene family in *P. pinaster* were also calculated using the Computational Biology Unit (CBU) Ka/Ks Calculation tool (http://services.cbu.uib.no/tools/kaks). The resulting phylogenetic tree was obtained by the parsimony method.

2.2. Characterization of the *Pinus pinaster WOX* gene family

To characterize the *WOX* gene family we carried out expression and localization studies in a comprehensive range of tissues and stages of development by mean of quantitative real-time PCR (RT-qPCR), laser capture microdissection (LCM) and RNA sequencing (RNA-Seq), and fluorescent *in situ* hybridization (FISH).

2.2.1. Quantitative real-time PCR (RT-qPCR)

Gene expression analysis was performed by RT-qPCR in a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). *P. pinaster* ubiquitin gene (Acc. AF461687) was used as endogenous reference gene [33-35]. Specific primers for each gene were designed with Primer3 software [36] following the parameters recommended by Udvardi et al. [37] (Primers used in this work are listed in Supplementary Table S2). Individual reactions were assembled in triplicate with 5 µl of Fast SYBR Green Master Mix (Applied Biosystems Inc., Foster City, CA, USA), oligonucleotide primers (0.20 µM each) and 100 ng of cDNA in a final

volume of 10 µl. The following protocol was used for amplification: 95 °C 20 s; 45 cycles of 95 °C 3 s and 60 °C 30 s, with a final melting curve to assess for non-specific products. For this purpose, negative controls (no template) and RT- controls (non-retrotranscribed RNA) were also included, and PCR amplicons from selected wells were cloned and sequenced.

2.2.1.1. Plant material

The *P. pinaster* embryogenic line P5LV4.1, which had been obtained and cryopreserved as described by Alvarez et al. [38, 39], was used to study the expression of the *WOX* genes during somatic embryogenesis. After thawing the cryopreserved tissues, proembryogenic masses (PEMs) were cultured on proliferation medium, which consisted on WV5 salts and vitamins (Duchefa, Haarlem, The Netherlands) [40], 1 g l^{-1} casein hydrolysate, 2.2 µM benzyladenine (BA), 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g l^{-1} sucrose, 4 g l^{-1} Gelrite (Duchefa), and 0.5 g l^{-1} L-glutamine (Duchefa). The pH was adjusted to 5.8 before autoclaving and the glutamine was filter-sterilized and added after autoclaving. Cultures were maintained at 23 °C in darkness and subcultured to fresh medium every three weeks.

To promote maturation PEMs were disaggregated and cultured onto a piece of sterile filter paper Whatman #2 70 mm diameter placed on maturation medium (150 mg per plate). This medium consisted on VW5 salts (Duchefa), 60 g l^{-1} sucrose, and 9 g l^{-1} Gelrite (Duchefa). The pH was adjusted to 5.8 before autoclaving. The medium was supplemented with 80 µM abscisic acid (ABA) (Duchefa) and the amino acid mixture from embryo development medium (EDM) [41], which was composed of 525 mg l^{-1} L-asparagine, 500 mg l^{-1} L-glutamine, 175 mg l^{-1} L-arginine, 19.75 mg l^{-1} L-citrulline, 19 mg l^{-1} L-ornithine, 13.75 mg l^{-1} L-lysine, 10 mg l^{-1} L-alanine, and 8.75 mg l^{-1} L-proline. All amino acids were supplied by Duchefa, except L-citrulline (Alfa Aesar, Karlsruhe, Germany). Both ABA solution and EDM amino acid mixture were filter-sterilized and added after autoclaving. Cultures were maintained in darkness at 23 °C and subcultured to fresh medium every four weeks.

Material from four different developmental stages was collected along the maturation process (Figure 1A-D), based on the classification established by Hakman and von Arnold [42] and adapted by Tereso et al. [43]: proembryogenic masses proliferating in the presence of the plant growth regulators auxins and cytokinins (PEM); early embryos (EE), with a translucent embryo proper and a long suspensor; late embryos (LE), with a prominent and opaque embryo proper; and mature embryos (ME), which have well-defined apical meristem and cotyledons. Samples were immediately frozen in liquid nitrogen and stored at -80 °C until analysis was carried out.

WOX gene family expression dynamics were also analysed during seed germination and in different parts of three-week-old seedlings. For that purpose, mature seeds from open-pollinated

P. pinaster trees growing in a natural stand (ES08 Meseta Castellana provenance, Spain) and provided by 'Servicio de Material Genético del Ministerio de Medio Ambiente' (Spain) were imbibed in water with aeration for 48 hours, transferred to wet vermiculite and maintained at 23 °C under a 16 h photoperiod.

As shown in Figure 1E-H, the analyses were carried out with germinated embryos with a radicle length inferior to 1 cm (G1), germinated embryos with a radicle between 1 and 2 cm (G2), germinated embryos with a radicle between 2 and 3 cm (G3), as well as in four different tissues from three-week-old seedlings: root tip (5 mm of the apical part of the root), shoot apex (3 mm of the emerging epicotyl including the shoot apical meristem and needle primordia), hypocotyl (a portion of 5 mm situated right under the shoot apex), and cotyledons. All tissues were immediately frozen in liquid nitrogen after isolation and stored at -80 °C until use.

2.2.1.2. RNA extraction and cDNA synthesis

RNA was extracted using the GeneMATRIX Universal RNA Purification Kit (EURx, Gdańsk, Poland). The RNA was quantified spectophotometrically (260 nm) and its integrity was checked by agarose gel electrophoresis. For each sample, one microgram of total RNA (0.5 µg for somatic embryogenesis samples) was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA, USA) following the manufacturer's instructions.

2.2.1.3. Data analysis

Analysis of the RT-qPCR data was performed with the qpcR package for R software (www.dr-spiess.de/qpcR.html), which allows the fitting of the RT-qPCR fluorescence raw data to a five-parameter sigmoidal model for obtaining essential PCR parameters such as efficiency, threshold cycle and transcript abundance [44]. Relative abundance of each transcript was calculated as the mean of the three technical replicates and normalized to the expression value of the reference gene in each sample. Results are expressed as mean normalized expression values ± standard error of three biological replicates. Significant differences in mRNA levels were determined by t-test analysis or ANOVA using the Student–Newman–Keuls test for post hoc comparisons (SIGMA-PLOT v11 software, Chicago, IL, USA).

2.2.2. Laser Capture Microdissection (LCM) and RNA sequencing (RNA-Seq)

To complement the information obtained by RT-qPCR and to gain insight into the expression pattern of the *WOX* gene family, different tissues of one-month-old seedlings were isolated by LCM and transcripts were studied by RNA-Seq.

2.2.2.1. Plant material

P. pinaster seeds (Sierra de Oria provenance, Spain) were imbibed in water with aeration for 48 hours, transferred to wet vermiculite and maintained at 23 ºC under a 16 h photoperiod. Fourteen days after imbibition, seedlings were individually transferred to a 0.2-l pot with soil and watered twice a week with distilled water. The seedlings were harvested 1 month after the emergence of the shoots over the vermiculite. The seedlings were cut and tissue sections of 5 mm were mounted in a specimen holder with optimal cutting temperature (OCT) embedding medium (Tissue-Tek, USA) and snap-frozen in liquid nitrogen for cryostat sectioning. The frozen samples were stored at -80 ºC until use.

2.2.2.2. Laser capture microdissection

The Laser Capture Microdissection (LCM) procedure was carried out as previously described in Cañas et al. [45]. Twenty-µm-thick sections were made using a cryostat (HM 525, Thermo, USA) at -20 ºC and mounted on PET-membrane 1.4 µm steel frames (Leica Microsystems, Wetzla, Germany) with the help of a plexiglass Frame Support (Leica Microsystems, Wetzla, Germany). Before the microdissection, samples were fixed in cold 100% ethanol for 10 s, the OCT was removed in DEPC-treated water for 2 minutes and refixed in 100% ethanol for 1 minute. Subsequently the samples were air-dried and used for microdissection. The microdissection was made in a laser microdissector (LMD700, Leica Microsystems, Wetzla, Germany). The microdissection samples were placed into the caps of 0.5 ml tubes containing 10 µl lysis buffer from the RNAqueous-Micro RNA Isolation Kit (Ambion, USA). Before RNA extraction, all the samples were placed at -80 ºC. All the RNA extractions were performed using the standard-volume protocol (non-LCM) with the RNAqueous-Micro RNA Isolation Kit (Ambion, USA).

Samples from 14 different tissues were isolated by LCM: Apical Meristem (AM), Emerging Needles (EN), Young Needles Mesophyll (YNM), Young Needles Vascular (YNV), Cotyledon Mesophyll (CM), Cotyledon Vascular (CV), Hypocotyl Cortex (HC), Hypocotyl Vascular (HV), Hypocotyl Pith (HP), Root Cortex (RC), Root Vascular (RV), Developing Root Cortex (DRC), Developing Root Vascular (DRV) and Root Meristem (RM).

2.2.2.3. cDNA amplification

As the amounts of RNA isolated form LCM samples were not enough for 454 pyrosequencing, a previous cDNA synthesis and amplification were made using the Conifer RNA Amplification (CRA+) protocol described in Cañas et al. [45].

The quantity of the amplified ds cDNA was determined using the Quant-iT™ PicoGreen® dsDNA Kit (Invitrogen, Paisley, UK). The quality of the amplified ds cDNA was determined using the Agilent 7500 DNA Kit in the 2100 Bioanalyzer (Agilent, CA, USA).

2.2.2.4. 454 pyrosequencing

RNA-Seq was performed at the University of Málaga ultrasequencing facility using the GS-FLX+ platform with a GS FLX Titanium kit (Roche Applied Sciences, Indianapolis, IN, USA). Each sample was run in one half of a 454 PicoTiterPlate following the manufacturer's sequencing protocol.

The quantity of the cDNA libraries was determined using the Quant-iT™ PicoGreen® dsDNA Kit (Invitrogen, Paisley, UK). The quality of the cDNA libraries was determined using the Agilent High Sensitivity DNA Kit in the 2100 Bioanalyzer (Agilent, CA, USA). The runs were analysed using the Roche GS-FLX+ software. The high throughput sequencing data have been deposited in NCBI's Gene Expression Omnibus [46] and are accessible through GEO Series accession number GSE78263 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE78263).

2.2.2.5. 454 pyrosequencing reads pre-processing, assembling and mapping

The raw data were first checked for quality using the *fastqc* software. Based on examination of the output quality plots it was decided to clip off the first 35 bp of each read using the FastX toolkit (*fastx_trimmer*). Next, the reads were filtered for overall quality using fastq_quality_filter with a minimum Q-score of 30 and minimum remaining length of 60%. The remaining reads were subsequently used for read mapping with the BWA software (MEM option) [47] against the transcript sequences of the 14 *P. pinaster WOX* genes.

2.2.3. Fluorescent in situ *hybridization (FISH)*

The visualization of *PpWUS*, *PpWOXX* and *PpWOX3* gene expression domains in the shoot apex was carried out through mRNA fluorescent *in situ* hybridization (FISH). For that purpose, shoot apexes inferior to 5 mm long were harvested from three-week-old seedlings obtained as mentioned in 2.2.1.1. Tissues were immediately fixed with freshly prepared FAA solution (3.7% formaldehyde, 5% glacial acetic acid, 50% ethanol) at 4 °C under vacuum overnight. Then, they were dehydrated in an ascendant ethanol series (50, 75, 90, and 100%) and maintained in 100% ethanol at 4 °C until next step.

Tissues were infiltrated and embedded with Technovit® 8100 (Heraeus kulzer GmbH, Wehrheim, Germany) according to manufacturer's instructions. Embedded shoot tips were

sectioned longitudinally in a microtome at 10 µm thickness and sections were mounted on Menzel-Gläser Superfrost Ultra Plus slides (Thermo-Scientific, Waltham, Massachusetts, USA). Air-dried sections were directly used for *in situ* hybridization following the protocol described by Valledor et al. [48] with some modifications. Shoot apex sections were initially incubated in 0.5% (v/v) Triton X-100 in PBS for 10 minutes and washed with PBS for 5 minutes. Subsequently, sections were treated with 2% (w/v) cellulase in PBS for 2 hours at room temperature and, after a wash step with PBS for 5 minutes, were also treated with HCl 0.2N for 30 minutes. After a new wash step with PBS, sections were equilibrated with 2X SSC buffer for 5 minutes and prehybridized with hybridization buffer HB50 (50% (v/v) formamide in 2X SSC + 50 mM phosphate buffer pH 7.0) for 2 hours at 37 °C. After denaturing samples at 78 °C for 5 minutes, 8 µl of hybridization mix were applied onto each section and maintained at 78 °C for 5 minutes. Hybridization mix was composed by 20% (w/v) dextran sulfate, hybridization buffer HB50, and 100 µM solution of the corresponding labelled probe in a proportion 2:1:1. Single-stranded antisense probes of 33 nucleotides long labelled with Cyanine 5 (Cy5) in their 3' end were designed to hybridize in a specific region of each mRNA (Probes are listed in Supplementary Table S2). Tissue sections were also incubated without any probe as a negative control, in order to evaluate the presence of autofluorescence. Then, sections were maintained overnight at 55 °C in a moist chamber.

After hybridization, sections were washed with 2X SSC and 1X SSC at 40 °C for 15 and 10 minutes, respectively. Subsequently, two additional wash steps with 0.5X SSC and autoclaved ddH_2O for 10 and 2 minutes, respectively, were performed at room temperature. Sections were then incubated with 4,6-diamidino-2-phenylindole (DAPI; AppliChem GmbH, Darmstadt, Germany) 1 µg ml^{-1} for 15 minutes. Finally, sections were washed with autoclaved ddH_2O for 5 minutes, air-dried and mounted with Mowiol® 4-88 (AppliChem GmbH, Darmstadt, Germany). Sections were observed and photographed with a Leica DMRXA fluorescence microscope (Leica Microsystems, Wetzlar, Germany) and images were processed with ConfocalUniovi ImageJ software (http://spi03.sct.uniovi.es/confocaluniovi/).

3. RESULTS

3.1. Identification and phylogenetic analysis of the *Pinus pinaster WOX* gene family

The *P. pinaster* genome has been sequenced in the frame of the European project ProCoGen but a draft of the genome has not been released yet. We were able to identify 14 *WOX* genes in the *P. pinaster* genome by combining PCR-based detection and the screening of *P. pinaster* transcriptome data (SustainPine DB, http://www.scbi.uma.es/sustainpinedb/). All these genes showed the characteristic highly conserved HD and motifs of *WOX* genes. The gene structures of 11 out of 14 *WOX* genes were obtained by comparing the DNA and RNA sequences (Figure

2). We found clear orthologues for the *Arabidopsis WOX2*, *WOX3*, *WOX4*, *WOX5*, *WUS* and *WOX13* that were designated as *PpWOX2*, *PpWOX3*, *PpWOX4*, *PpWOX5*, *PpWUS*, and *PpWOX13*, respectively. The rest of *WOX* genes did not have clear orthologues with other angiosperm *WOX* genes and were named *PpWOXA*, *PpWOXB*, *PpWOXC*, *PpWOXD*, *PpWOXE*, *PpWOXF*, *PpWOXG*, and *PpWOXX*. Our screening also allowed the identification of 11 *WOX* genes in *P. taeda* (*PtWOX2*, *PtWOX3*, *PtWOX4*, *PtWOX5*, *PtWUS*, *PtWOXX*, *PtWOX13*, *PtWOXA*, *PtWOXB*, *PtWOXE* and *PtWOXG*) and two new *WOX* genes in *P. abies* (*PaWOXG* and *PaWOXX*). Accession numbers for all sequences identified in this work can be found in Supplementary Table S1.

Phylogenetically, *PpWOX* genes were distributed throughout the three clades established by van der Graaff et al. [4] (Figure 3). *PpWOX13*, *PpWOXA* and *PpWOXG* are members of the ancient clade, which includes *WOX* genes present in green algae and all land plants. *PpWOXB*, *PpWOXC*, *PpWOXD*, *PpWOXE* and *PpWOXF* belong to the intermediate clade. The WUS clade, originally defined as specific from seed plants, included the genes *PpWUS*, *PpWOX2*, *PpWOX3*, *PpWOX4*, *PpWOX5* and *PpWOXX* (Figure 4). *PpWOXX* is a new gene that had not been described in any other conifer species until this moment. A thoroughly screening in public databases allowed us to identify orthologues of this gene present in the genome of the conifers *P. abies* and *P. taeda*, but it was not found in any angiosperm species. The phylogenetic analysis showed that the *WOXX* pine orthologues constitute a monophyletic group that occupies the basal position of the WUS clade and group together with *G. gnemon GgWOXY* gene reported by Nardmann and Werr [49].

The analysis of the 14 WOX polypeptide sequences showed that all *P. pinaster* WOX proteins contained the characteristic WOX HD motif. All WUS-clade members contained the characteristic WUS box motif T-L-X-L-F-P. In addition, the EAR motif (L-X-L-X-L) is present in PpWUS and PpWOX5 proteins after the HD and the WUS box (Figure 5A). Furthermore, similarly to *Arabidopsis* and other species, PpWUS deduced protein has a 66 amino acid homeodomain with a Y residue at position 21 that is absent in the rest of PpWOX proteins (Figure 5B).

Regarding substitution rates, all Ka/Ks values obtained for each node of the *P. pinaster WOX* phylogenetic tree were inferior to 1, which indicates that the evolution of this family in *P. pinaster* was under negative or purifying selection. That is, there was pressure to conserve protein sequences as changes in their sequence might cause a disruption of their function (Figure S1).

3.2. Characterization of the *Pinus pinaster WOX* gene family

In order to gain insight into the specific role of each *PpWOX* gene in plant development, their expression during somatic embryogenesis and seed germination was analysed by quantitative real time PCR (RT-qPCR). Transcripts from each isolated gene were detected at least in some of the stages or tissues included in this study, which indicated that they are not pseudogenes. Members of the ancient clade, *PpWOX13*, *PpWOXA* and *PpWOXG*, were shown to be constitutively expressed, and their expression was higher during embryogenesis (Figures 6 and 7). However, the intermediate-clade members *PpWOXB*, *PpWOXC*, *PpWOXD* and *PpWOXE* were expressed exclusively during embryogenesis, reaching a peak in the early embryo phase (Figure 6). Their levels were very low in the mature embryo and no expression was detected after the beginning of germination. *PpWOX2*, member of the WUS clade, showed a similar expression pattern. However, the expression of the last intermediate-clade member *PpWOXF* increased gradually during embryogenesis and reached its maximum level at the mature-embryo stage. During germination its levels dramatically decreased. No transcripts were detected in seedlings for *PpWOX2*, *PpWOXB*, *PpWOXC*, *PpWOXD*, *PpWOXE* and *PpWOXF* genes.

PpWOXX, *PpWUS* and *PpWOX5* showed a similar expression pattern during embryogenesis, as their expression reached their maximum at mature-embryo stage (Figure 6). However, in seedlings, while *PpWUS* expression was only detected in the shoot apex, *PpWOX5* transcripts were preferentially located in the root tip of seedlings, with low levels of expression in other seedling tissues (Figure 7). Similar to *PpWUS*, *PpWOX3* and *PpWOXX* expression was restricted to the shoot apex of seedlings, although transcripts from both genes were also found at a very low level in the hypocotyl (Figure 7).

PpWOX4 expression levels were quite constant during embryogenesis and increased progressively during germination. In seedlings, this gene was mainly expressed in the hypocotyl. Lower levels were detected in the shoot apex and cotyledons, and very little expression was found in the root apex (Figures 6 and 7).

To gain insight into the expression pattern of the *WOX* gene family, different tissues of one-month-old seedlings were isolated by LCM and transcripts were studied by RNA-Seq. RNA-Seq results confirmed that only members of the ancient and WUS clades were expressed in seedlings. Members of the ancient clade (*PpWOX13*, *PpWOXA* and *PpWOXG*) were expressed in most tissues, while *PpWOX* members of the WUS clade had specific expression patterns suggesting a neofuncionalization phenomenon for these genes (Figure 8).

Fluorescent *in situ* hybridization (FISH) was performed in order to determine the precise expression domain of *PpWUS*, *PpWOXX* and *PpWOX3* genes in the shoot apex of three-week-old seedlings (Figure 9, Supplementary Figures S2 and S3). Both *PpWUS* and *PpWOXX* were shown to be expressed in a few cells located in the central region of the meristem. In particular, *PpWOXX* expression was detected in cells situated in the third cell layer (Figure 9C-D), whereas *PpWUS*-expressing cells were situated slightly deeper in the meristem (Figure 9A-B). On the other side, the gene *PpWOX3* was expressed in the peripheral zone of the meristem, where lateral organs initiate. Specifically, signal was detected in the putative founder cells of lateral organs, and in leaf primordia (Figure 9E-F).

In order to complement all the information obtained in this work, we have summarized the data concerning the expression domains and putative functions of WOX proteins in the literature relative to angiosperms and gymnosperms in Table 1.

4. DISCUSSION

4.1. Identification and phylogenetic analysis of the *Pinus pinaster WOX* gene family

Increasing availability of data on the genome and transcriptome of several gymnosperms is facilitating the study of the evolution of their gene families [50] (Table 1). In this work, we report the identification of 14 *WOX* genes in *P. pinaster*, including three *WOX* genes not previously described in any other conifer species (*PpWOXA*, *PpWOXG* and *PpWOXX*). Our screening also allowed the identification of 11 *WOX* genes in *P. taeda* and two new *WOX* gene in *P. abies*. Recently, Hedman et al. [29] had characterized the *WOX* gene family in *P. abies*, which comprised a total of 11 *WOX* genes. In accordance with their results, our phylogenetic analyses showed that *PpWOX* genes are distributed along the three clades established in WOX phylogeny [4] supporting the hypothesis that a major diversification in *WOX* gene family took place before the split between angiosperms and gymnosperms.

Basing on phylogenetic analysis, it has been proposed that the *WOX* gene family has a monophyletic origin and that the last common ancestor of green algae and land plants contained at least one *WOX* member [51]. The ancient clade is present in all major plant lineages, including green algae, and lower plants only contain members from this clade. Basing on these observations, the ancient clade is thought to represent the oldest and most conserved clade of the *WOX* gene family [12, 52]. In *P. pinaster*, ancient clade includes three members, *PpWOX13*, *PpWOXA*, and *PpWOXG*. We found that these three genes are present in other *Pinus* species like *P. taeda*, while only *PpWOX13* and *PpWOXG* orthologues were detected in other gymnosperm genera like *Picea* and only *PpWOX13* orthologues in *Gnetum* or *Gingko*. These

ancient-clade genes appear to have a monophyletic origin and they might have arisen after duplication events that took place before the diversification of the *Pinus* genus, although this hypothesis should be confirmed in the future.

The intermediate clade, present in all vascular plants, and the WUS clade, exclusive from ferns, gymnosperms and angiosperms, originated subsequently by gene duplication and diversification from ancient members in the course of plant evolution [51, 53]. *WOX* gene family underwent a great expansion after the separation of mosses from other land plants, as the number of *WOX* genes increased substantially with the emergence of the vascular plant lineages [12, 54]. Furthermore, modifications in the homeodomain 3D structure and the apparition of specific motifs in the protein sequence might have contributed to the functional change of *WOX* family during evolution [51].

Five *PpWOX* genes belong to the intermediate clade in *P. pinaster*. The phylogenetic analyses showed that these genes group very closely, which is consistent with the available data from other conifer species. This supports the hypothesis that there was a gene expansion of the intermediate clade within the *Pinaceae* family [29].

Phylogenetic analyses revealed that WUS-clade members emerged in the last common ancestor of leptosporangiate ferns and seed plants [55]. Whereas only one member has been reported in ferns [55], the WUS clade have expanded and evolved in seed plants. Similarly to that observed in *P. abies* [29], we found orthologues for most *Arabidopsis* WUS-clade members in *P. pinaster* except for *AtWOX1*, *AtWOX6* and *AtWOX7*. This suggests that *WOX2*, *WOX3*, *WOX4*, *WOX5* and *WUS* were present in the last common ancestor of angiosperms and gymnosperms, and a gene diversification happened after the bifurcation of both seed plant groups. Several members of the WUS clade have been involved in stem cell maintenance in the shoot meristem (*WUS*), in the root meristem (*WOX5*), in leaf marginal meristems (*WOX3*) and in vascular meristems (*WOX4*) in angiosperms [15, 18, 19, 56]. The fact that gymnosperms also contain orthologues for these genes could indicate that their functional specialization and association with discrete stem cell niches had been stablished prior to the divergence of gymnosperms and angiosperms, in concordance with the assumption made by Nardmann and Werr [49]. Recent interspecies complementation experiments have shown that gymnosperm WUS/WOX5 proteins rescue *Arabidopsis wus-1* defects in both shoot apical stem-cell maintenance and flower organ formation [54]. This could suggest a conservation of their function all along seed plants, although more functional studies are needed in order to confirm this hypothesis.

Interestingly, a WUS-clade member, *PpWOXX*, not previously described in angiosperms was identified in *P. pinaster* in this work. We also identified orthologues of *PpWOXX* in the genome of other conifers such as *P. taeda* and *P. abies*. These sequences constitute a monophyletic group at the base of the WUS clade. A more detailed study of the HD peptide sequence showed that all the conifer WOXX proteins as well as the proteins GgWOXY, GgWOX2A and

GgWOX2B from the gymnosperm *Gnetum gnemom*, also included in the basal positions of the WUS clade, contain the particular sequence FYWF(QK)NR. The particular sequences FYWFQNR and FYWFQNH are characteristics of the intermediate- and WUS-clade WOX proteins, respectively [53, 55, 57]. This fact coupled to the recent identification of WUS-clade *WOX* members in the leptosporangiate ferns *Ceratopteris richardii* and *Cyathea australis* (*CrWUL* and *CaWUL*, respectively) [55] might indicate that, although these proteins are usually included in the WUS clade, they originated from a *WOX* gene present in the last common ancestor of all vascular plants, representing a transitional stage between intermediate- and WUS-clade proteins lost in the angiosperm lineage. Alternatively, they could have arisen as a consequence of a gene expansion in the gymnosperm lineage.

4.2. Characterization of the *Pinus pinaster WOX* gene family

Available information about gene function in gymnosperms is limited, partly owing to the lack of a characterized mutant collection and the difficulty to carry out genetic transformation in many species. To characterize the *WOX* gene family in *P. pinaster* the expression pattern of each gene was analysed in different developmental stages and tissues by RT-qPCR and RNA-Seq. In addition, the gene expression of *PpWUS*, *PpWOXX* and *PpWOX3* in the shoot apex was analysed by FISH.

Transcripts of the ancient-clade members *PpWOX13*, *PpWOXA* and *PpWOXG* were detected in all the stages and tissues studied. In green algae and mosses, which only contain ancient-clade members, it was reported that the single *WOX* gene identified in *Ostreococcus tauri* participated in its maintenance in an undifferentiated state [12], whereas ancient-clade genes played an important role in stem cell formation and regeneration in *Physcomitrella patens* [58]. Previous reports in *Arabidopsis* [12, 59] and *P. abies* [29] showed that members of the ancient clade are expressed in most tissues and developmental stages and their function could be related to root initiation, embryo development, floral transition, and replum formation.

All the intermediate-clade members and the WUS-clade member *PpWOX2* were expressed during embryo development and no expression was detected once the embryos germinated. All these genes (except *PpWOXF*) are expressed mainly during the first stages of embryo development. This pattern is similar to that observed for *PaWOX8/9* and *PaWOX2* genes in *P. abies* [25, 26, 60]. *PaWOX8/9* RNA interference (RNAi) lines showed an altered orientation of the cell division planes at the basal part of embryonal masses during early embryo development, giving rise to aberrant embryos. *PaWOX2* RNAi affected the establishment of the border between the embryo proper and the suspensor. Besides, suspensor cells do not elongate as usual and the protoderm was not established properly. No effects were found when *PaWOX2* interference happened after late embryos were formed. The high homology and the similar expression pattern between *PpWOX2* and *PaWOX2* suggest that its function could be conserved

in conifers. Similarly, intermediate-clade genes *PpWOXB*, *PpWOXC*, *PpWOXD* and *PpWOXE* might be involved in apical-basal polarity establishment during early embryogenesis. Their orthologues in *Arabidopsis AtWOX8*, *AtWOX9* and *AtWOX2* are also involved in early embryonic pattern formation [13, 24]. The intermediate-clade gene *PpWOXF* showed a different expression pattern from other intermediate members. The highest transcript abundance was found in mature embryos, detecting very little expression during the initial stages of both embryogenesis and germination. According to the phylogenetic analyses, this gene is closely related to *WOX11* and *WOX12* genes from different angiosperm species. The expression and function of *AtWOX11* and *AtWOX12* has so far not been described in detail in *Arabidopsis* but recent studies showed that both genes are involved in *de novo* root organogenesis [61]. Based on the available information, the role of *PpWOXF* remains unknown and more studies will be needed to clarify its function.

Some members of the WUS clade are involved in stem-cell regulation. *PpWOX3* is homologue to *WOX3* genes from various angiosperm species [17, 62] and the conifer *P. abies* [27, 29]. In *Arabidopsis*, *AtWOX3/PRS* is expressed in embryos defining a border between the ad- and abaxial sides of the cotyledons [13, 63], and in lateral regions of young primordia [17]. Alvarez et al. [27] reported that *PaWOX3* has an important role in the lateral outgrowth of lateral organs in *P. abies* similar to the function of its angiosperm orthologues. By *in situ* hybridization, these authors also found *PaWOX3* expression in the lateral parts of the shoot meristem from adult vegetative buds during the shoot elongation period, but not during the dormant phase. Previously, it had been reported *WOX3* expression in the periphery of the SAM in other gymnosperm species such as *G. gnemon* and *P. sylvestris* [49]. In particular, *GgWOX3* initially presented a ring-shaped expression domain in the periphery of the SAM, being subsequently restricted to the leaflet precursors. *PsWOX3* was first detected in cells situated in the surface of the peripheral zone (PZ) of the SAM, where leaf initiation begins, and in the apical cells of leaf primordia. Expression of *PpWOX3* in *P. pinaster* was detected during embryo development and later preferentially in the shoot apex, being also slightly expressed in the emerging needles. Furthermore, analysis of its expression in the shoot apex by FISH showed that this gene is expressed in cells from the PZ of the meristem that will give rise to new lateral organs and in needle primordia. This expression pattern suggests a conserved function in expansion and development of lateral organs.

PpWOX4 grouped together with other *WOX4* genes described in angiosperms and gymnosperms, constituting a monophyletic group. *WOX4* is transcribed in the developing vasculature of multiple tissues and was predominantly expressed in hypocotyls of *Solanum lycopersicum* [16]. Recent studies in *Arabidopsis* and *S. lycopersicum* showed that WOX4 is closely related to vascular cambium promoting differentiation and/or maintenance of the vascular procambium, the initial cells of the developing vasculature. Our analysis showed that

PpWOX4 expression increases during embryo germination and is higher in the hypocotyl than in other plant parts analysed. Transcripts of *PpWOX4* were detected specifically in the vascular tissue of hypocotyl and young needles by RNA-Seq analysis. This expression pattern could fit well with a function in the vascular procambium. However, we have not found functional studies of this gene in any gymnosperm species.

In *Arabidopsis*, the WUS-clade members *AtWUS* and *AtWOX5* are expressed in the organizing center (OC) of the SAM, and the quiescent center (QC) of the RAM, respectively. Both genes carry out a similar function maintaining a pool of undifferentiated cells in the SAM and RAM, respectively [14, 15]. In this work, we report the identification of orthologues for both *WOX5* and *WUS* in *P. pinaster* and *P. taeda*, which is in concordance with the results of Hedman et al. [29] in *P. abies*. However, whereas only *WOX5* transcripts were detected in conifers to date, one of the main breakthroughs of this work is the detection of discrete *PpWUS* and *PpWOX5* transcripts, suggesting that both genes are functional in *P. pinaster*. This is the first time that expression of both *WUS* and *WOX5* is detected in a gymnosperm species to our knowledge. *PpWOX5* is expressed during embryogenesis, especially in mature embryos, and preferentially in the seedling´s root meristem. *PpWUS* is also expressed during embryogenesis, especially in mature embryos, and in the shoot apex of the seedlings. Furthermore, *PpWUS* expression was localized by FISH in the central zone (CZ) of the SAM. According to the model that describes the molecular basis of the SAM maintenance in angiosperms, WUS together with CLAVATA1 (CLV1) and its ligand CLAVATA3 would form a regulatory loop that controls the balance between cell proliferation and differentiation in the SAM [3, 64]. Previous studies in our lab found a putative *CLV1* orthologue (*PpCLV1L*) that is overexpressed during *de novo* shoot organogenesis in *P. pinaster* [34]. These results suggest that at least some of the mechanisms involved in SAM homeostasis regulation might be conserved in both seed plant groups. However, a total comparison of these data is not possible due to the morphological differences, the different growth habits and the evolutionary distance between angiosperms and gymnosperms. While a zonation model of the SAM that divides the SAM into a CZ comprising stem cells and a surrounding PZ recruiting cells for differentiation has been claimed for all seed plants, a tunica-corpus model characteristic from most angiosperms cannot be applied to all gymnosperms. A recent study [65] showed that in most conifer species there is no a ‘proper’ tunica but a small number cells from the superficial layer situated in the summit of the SAM, which are designated apical initials, directly contribute to the superficial PZ and to the CZ. In *Pinus* genus, this central summit zone is composed by only three cells, which may have originated from the same initial cell. These cells divide anticlinally and perpendicular to each other giving rise to the surface cells from the PZ, and some of them (in some species, only one of the apical initials) also divide periclinally, giving rise to the stem cell population situated in the CZ. Despite the progress made in the understanding of the SAM architecture in conifers,

little information is available by the moment about the underlying molecular mechanisms of SAM formation and maintenance in these organisms. In summary, although the detection of discrete *WUS* and *WOX5* transcripts for the first time in a gymnosperm and their differentiated expression patterns might indicate that these genes perform similar roles to those described for their *Arabidopsis* orthologues, a deeper functional analysis of both genes is needed in order to validate this hypothesis. Interestingly, the expression domain of *PpWOXX*, the new WUS-clade member identified in conifers, during somatic embryogenesis and in the shoot apex is similar to that described for *PpWUS*. These results could indicate that this gene may play a role in the SAM maintenance. However, the information available at this moment is not enough to infer which the specific role of this gene is.

In conclusion, our results suggest that an expansion of the intermediate clade took place within the *Pinaceae* family, that the last common ancestor of all seed plants contained a *WOX* gene representing a transitional stage between intermediate- and WUS-clade proteins, and that the last common ancestor of the extant gymnosperms and angiosperms contained both *WUS* and *WOX5* genes probably functionally specialized.

AUTHOR CONTRIBUTIONS

The design of the study was made by JMA, RAC, CA, FMC and RJO and the experimental setup was planned by JMA, NB, RAC and RJO. JMA and NB performed most of the experimental work. JMA, RAC and NB analysed and interpreted the data. JMA drafted the manuscript. RAC, NB, CA, FMC and RJO revised the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

This work was supported by the EU FP7 (ProCoGen 289841), INIA and FEDER cofunding (RTA2013-00048-C03-02), University of Oviedo (PAPI-17-PEMERG-3), a predoctoral grant from the 'Plan de Ciencia Tecnología e Innovación del Principado de Asturias', FICYT, Spain (BP10-098) to NB, and a grant from the 'Spanish Ministerio de Economía y Competitividad' (BIO2015-73512-JIN; MINECO/AEI/FEDER, UE) to RAC. We thank Dr. Eduardo Cires for the helpful comments and Dr. Rubén Alvarez for critical reading of the manuscript.

REFERENCES

[1] R.L. Chan, G.M. Gago, C.M. Palena, D.H. Gonzalez, Homeoboxes in plant development, Biochim. Biophys. Acta 1442 (1998) 1-19.

[2] K. Mukherjee, L. Brocchieri, T.R. Bürglin, A comprehensive classification and evolutionary analysis of plant homeobox genes, Mol. Biol. Evol. 26 (2009) 2775-2794.

[3] H. Schoof, M. Lenhard, A. Haecker, K.F.X. Mayer, G. Jürgens, T. Laux, The stem cell population of *Arabidopsis* shoot meristems is maintained by a regulatory loop between the *CLAVATA* and *WUSCHEL* genes, Cell 100 (2000) 635-644.

[4] E. van der Graaff, T. Laux, S.A. Rensing, The WUS homeobox-containing (WOX) protein family, Genome Biol. 10 (2009) 248.

[5] M. Ueda, Z. Zhang, T. Laux, Transcriptional activation of *Arabidopsis* axis patterning genes *WOX8/9* links zygote polarity to embryo development, Dev. Cell 20 (2011) 264-270.

[6] M. Ikeda, N. Mitsuda, M. Ohme-Takagi, *Arabidopsis* WUSCHEL is a bifunctional transcription factor that acts as a repressor in stem cell regulation and as an activator in floral patterning, Plant Cell 21 (2009) 3493-3505.

[7] H. Lin, L. Niu, N.A. McHale, M. Ohme-Takagi, K.S. Mysore, M. Tadege, Evolutionarily conserved repressive activity of WOX proteins mediates leaf blade outgrowth and floral organ development in plants, Proc. Natl. Acad. Sci. 110 (2013) 366-371.

[8] A.B. Rebocho, M. Bliek, E. Kusters, R. Castel, A. Procissi, I. Roobeek, E. Souer, R. Koes, Role of EVERGREEN in the development of the cymose petunia inflorescence, Dev. Cell 15 (2008) 437-447.

[9] X. Zhang, J. Zong, J. Liu, J. Yin, D. Zhang, Genome-wide analysis of *WOX* gene family in rice, sorghum, maize, *Arabidopsis* and poplar, J. Integr. Plant Biol. 52 (2010) 1016-1026.

[10] B. Liu, L. Wang, J. Zhang, J. Li, H. Zheng, J. Chen, M. Lu, *WUSCHEL-related Homeobox* genes in *Populus tomentosa*: diversified expression patterns and a functional similarity in adventitious root formation, BMC Genomics 15 (2014) 296.

[11] A. Dolzblasz, J. Nardmann, E. Clerici, B. Causier, E. van der Graaff, J. Chen, B. Davies, W. Werr, T. Laux, Stem cell regulation by *Arabidopsis WOX* genes, Mol. Plant 9 (2016) 1028-1039.

[12] Y. Deveaux, C. Toffano-Nioche, G. Claisse, V. Thareau, H. Morin, P. Laufs, H. Moreau, M. Kreis, A. Lecharny, Genes of the most conserved *WOX* clade in plants affect root and flower development in *Arabidopsis*, BMC Evol. Biol. 8 (2008) 1-19.

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

[14] K.F.X. Mayer, H. Schoof, A. Haecker, M. Lenhard, G. Jürgens, T. Laux, Role of *WUSCHEL* in regulating stem cell fate in the *Arabidopsis* shoot meristem, Cell 95 (1998) 805-815.

[15] A.K. Sarkar, M. Luijten, S. Miyashima, M. Lenhard, T. Hashimoto, K. Nakajima, B. Scheres, R. Heidstra, T. Laux, Conserved factors regulate signalling in *Arabidopsis thaliana* shoot and root stem cell organizers, Nature 446 (2007) 811-814.

[16] J. Ji, J. Strable, R. Shimizu, D. Koenig, N. Sinha, M.J. Scanlon, WOX4 promotes procambial development, Plant Physiol. 152 (2010) 1346-1356.

[17] N. Matsumoto, K. Okada, A homeobox gene, *PRESSED FLOWER*, regulates lateral axis-dependent development of *Arabidopsis* flowers, Genes Dev. 15 (2001) 3355-3364.

[18] R. Shimizu, J. Ji, E. Kelsey, K. Ohtsu, P.S. Schnable, M.J. Scanlon, Tissue specificity and evolution of meristematic WOX3 function, Plant Physiol. 149 (2009) 841-850.

[19] J. Ji, R. Shimizu, N. Sinha, M.J. Scanlon, Analyses of *WOX4* transgenics provide further evidence for the evolution of the *WOX* gene family during the regulation of diverse stem cell functions, Plant Signal. Behav. 5 (2010) 916-920.

[20] T. Laux, T. Würschum, H. Breuninger, Genetic regulation of embryonic pattern formation, Plant Cell 16 (2004) S190-S202.

[21] L. Savard, P. Li, S.H. Strauss, M.W. Chase, M. Michaud, J. Bousquet, Chloroplast and nuclear gene sequences indicate late Pennsylvanian time for the last common ancestor of extant seed plants, Proc. Natl. Acad. Sci. 91 (1994) 5163-5167.

[22] S.A. Smith, J.M. Beaulieu, M.J. Donoghue, An uncorrelated relaxed-clock analysis suggests an earlier origin for flowering plants, Proc. Natl. Acad. Sci. 107 (2010) 5897-5902.

[23] M. Vandenbussche, A. Horstman, J. Zethof, R. Koes, A.S. Rijpkema, T. Gerats, Differential recruitment of *WOX* transcription factors for lateral development and organ fusion in Petunia and *Arabidopsis*, Plant Cell 21 (2009) 2269-2283.

[24] H. Breuninger, E. Rikirsch, M. Hermann, M. Ueda, T. Laux, Differential expression of *WOX* genes mediates apical-basal axis formation in the *Arabidopsis* embryo, Dev. Cell 14 (2008) 867-876.

[25] T. Zhu, P.N. Moschou, J.M. Alvarez, J.J. Sohlberg, S. von Arnold, *WUSCHEL-RELATED HOMEOBOX 8/9* is important for proper embryo patterning in the gymnosperm Norway spruce, J. Exp. Bot. 65 (2014) 6543-6552.

[26] T. Zhu, P.N. Moschou, J.M. Alvarez, J.J. Sohlberg, S. von Arnold, *WUSCHEL-RELATED HOMEOBOX 2* is important for protoderm and suspensor development in the gymnosperm Norway spruce, BMC Plant Biol. 16 (2016).

[27] J.M. Alvarez, J. Sohlberg, P. Engström, T. Zhu, M. Englund, P.N. Moschou, S. von Arnold, The *WUSCHEL-RELATED HOMEOBOX 3* gene *PaWOX3* regulates lateral organ formation in Norway spruce, New Phytol. 208 (2015) 1078-1088.

[28] J. Nardmann, P. Reisewitz, W. Werr, Discrete shoot and root stem cell-promoting *WUS/WOX5* functions are an evolutionary innovation of angiosperms, Mol. Biol. Evol. 26 (2009) 1745-1755.

[29] H. Hedman, T. Zhu, S. von Arnold, J.J. Sohlberg, Analysis of the *WUSCHEL-RELATED HOMEOBOX* gene family in the conifer *Picea abies* reveals extensive conservation as well as dynamic patterns, BMC Plant Biol. 13 (2013) 89.

[30] R.A. Cañas, Z. Li, M.B. Pascual, V. Castro-Rodríguez, C. Ávila, L. Sterck, Y. Van de Peer, F.M. Cánovas, The gene expression landscape of pine seedling tissues, Plant J. 91 (2017) 1064-1087.

[31] J. Canales, R. Bautista, P. Label, J. Gómez-Maldonado, I. Lesur, N. Fernández-Pozo, M. Rueda-López, D. Guerrero-Fernández, V. Castro-Rodríguez, H. Benzekri, R.A. Cañas, M.-A. Guevara, A. Rodrigues, P. Seoane, C. Teyssier, A. Morel, F. Ehrenmann, G. Le Provost, C. Lalanne, C. Noirot, C. Klopp, I. Reymond, A. García-Gutiérrez, J.-F. Trontin, M.-A. Lelu-Walter, C. Miguel, M.T. Cervera, F.R. Cantón, C. Plomion, L. Harvengt, C. Avila, M. Gonzalo Claros, F.M. Cánovas, *De novo* assembly of maritime pine transcriptome: implications for forest breeding and biotechnology, Plant Biotechnol. J. 12 (2014) 286-299.

[32] S.F. Altschul, T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (1997) 3389-3402.

[33] P. Alonso, M. Cortizo, F.R. Cantón, B. Fernández, A. Rodríguez, M.L. Centeno, F.M. Cánovas, R.J. Ordás, Identification of genes differentially expressed during adventitious shoot induction in *Pinus pinea* cotyledons by subtractive hybridization and quantitative PCR, Tree Physiol. 27 (2007) 1721-1730.

[34] J.M. Alvarez, M. Cortizo, N. Bueno, A. Rodríguez, R.J. Ordás, *CLAVATA1-LIKE*, a leucine-rich-repeat protein receptor kinase gene differentially expressed during adventitious caulogenesis in *Pinus pinaster* and *Pinus pinea*, Plant Cell Tissue Organ Cult. 112 (2013) 331-342.

[35] J.M. Alvarez, M. Cortizo, R.J. Ordás, Characterization of a type-A response regulator differentially expressed during adventitious caulogenesis in *Pinus pinaster*, J. Plant Physiol. 169 (2012) 1807-1814.

[36] S. Rozen, H. Skaletsky, Primer3 on the WWW for general users and for biologist programmers, Methods Mol. Biol. 132 (2000) 365-386.

[37] M.K. Udvardi, T. Czechowski, W.R. Scheible, Eleven Golden Rules of Quantitative RT-PCR, Plant Cell 20 (2008) 1736-1737.

[38] J.M. Alvarez, N. Bueno, M. Cortizo, R.J. Ordás, Improving plantlet yield in *Pinus pinaster* somatic embryogenesis, Scand J. For. Res. 28 (2013) 613-620.

[39] J.M. Alvarez, M. Cortizo, R. Ordás, Cryopreservation of somatic embryogenic cultures of *Pinus pinaster*: effects on regrowth and embryo maturation, Cryo. Lett. 33 (2012) 476-484.

[40] J.E. Coke, Basal nutrient medium for *in vitro* cultures of loblolly pines, in: U.S. Patent (Ed.), Westvaco Corporation, New York, NY, 1996.

[41] C. Walter, J. Find, L. Grace, Somatic embryogenesis and genetic transformation in *Pinus radiata*, in: M. Jain, P. Gupta (Eds.) Protocol for Somatic Embryogenesis in Woody Plants, Springer, Dordrecht, The Netherlands, 2005, pp. 11-24.

[42] I. Hakman, S. von Arnold, Somatic embryogenesis and plant regeneration from suspension cultures of *Picea glauca* (white spruce), Physiol. Plant 72 (1988) 579–587.

[43] S. Tereso, K. Zoglauer, A. Milhinhos, C. Miguel, M.M. Oliveira, Zygotic and somatic embryo morphogenesis in *Pinus pinaster*: comparative histological and histochemical study, Tree Physiol. 27 (2007) 661-669.

[44] C. Ritz, A.-N. Spiess, qpcR: an R package for sigmoidal model selection in quantitative real-time polymerase chain reaction analysis, Bioinformatics 24 (2008) 1549-1551.

[45] R.A. Cañas, J. Canales, J. Gómez-Maldonado, C. Ávila, F.M. Cánovas, Transcriptome analysis in maritime pine using laser capture microdissection and 454 pyrosequencing, Tree Physiol. 34 (2014) 1278-1288.

[46] R. Edgar, M. Domrachev, A.E. Lash, Gene Expression Omnibus: NCBI gene expression and hybridization array data repository, Nucleic Acids Res. 30 (2002) 207-210.

[47] H. Li, Toward better understanding of artifacts in variant calling from high-coverage samples, Bioinformatics 30 (2014) 2843-2851.

[48] L. Valledor, M.J. Cañal, J. Pascual, R. Rodríguez, M. Meijón, Early induced protein 1 (PrELIP1) and other photosynthetic, stress and epigenetic regulation genes are involved in *Pinus radiata* D. don UV-B radiation response, Physiol. Plant 146 (2012) 308-320.

[49] J. Nardmann, W. Werr, Symplesiomorphies in the *WUSCHEL* clade suggest that the last common ancestor of seed plants contained at least four independent stem cell niches, New Phytol. 199 (2013) 1081-1092.

[50] D. Uddenberg, S. Akhter, P. Ramachandran, J.F. Sundström, A. Carlsbecker, Sequenced genomes and rapidly emerging technologies pave the way for conifer evolutionary developmental biology, Front. Plant Sci. 6 (2015) 970.

[51] G. Lian, Z. Ding, Q. Wang, D. Zhang, J. Xu, Origins and Evolution of WUSCHEL-Related Homeobox Protein Family in Plant Kingdom, The Scientific World Journal 2014 (2014) 12.

[52] M.K. Barton, R.S. Poethig, Formation of the shoot apical meristem in *Arabidopsis thaliana*: an analysis of development in the wild type and in the shoot meristemless mutant, Development 119 (1993) 823-831.

[53] Y. Ge, J. Liu, M. Zeng, J. He, P. Qin, H. Huang, L. Xu, Identification of *WOX* family genes in *Selaginella kraussiana* for studies on stem cells and regeneration in lycophytes, Front. Plant Sci. 7 (2016) 93.

[54] Y. Zhang, Y. Jiao, H. Jiao, H. Zhao, Y.-X. Zhu, Two-Step Functional Innovation of the Stem-Cell Factors WUS/WOX5 during Plant Evolution, Mol. Biol. Evol. 34 (2017) 640-653.

[55] J. Nardmann, W. Werr, The invention of WUS-like stem cell-promoting functions in plants predates leptosporangiate ferns, Plant Mol. Biol. 78 (2012) 123-134.

[56] T. Laux, K.F. Mayer, J. Berger, G. Jurgens, The *WUSCHEL* gene is required for shoot and floral meristem integrity in *Arabidopsis*, Development 122 (1996) 87-96.

[57] M. Zeng, B. Hu, J. Li, G. Zhang, Y. Ruan, H. Huang, H. Wang, L. Xu, Stem cell lineage in body layer specialization and vascular patterning of rice root and leaf, Sci. Bull. (2015) 1-12.

[58] K. Sakakibara, P. Reisewitz, T. Aoyama, T. Friedrich, S. Ando, Y. Sato, Y. Tamada, T. Nishiyama, Y. Hiwatashi, T. Kurata, M. Ishikawa, H. Deguchi, S.A. Rensing, W. Werr, T. Murata, M. Hasebe, T. Laux, *WOX13-like* genes are required for reprogramming of leaf and protoplast cells into stem cells in the moss *Physcomitrella patens*, Development 141 (2014) 1660-1670.

[59] M. Romera-Branchat, J.J. Ripoll, M.F. Yanofsky, S. Pelaz, The *WOX13* homeobox gene promotes replum formation in the *Arabidopsis thaliana* fruit, Plant J. 73 (2013) 37-49.

[60] J. Palovaara, H. Hallberg, C. Stasolla, I. Hakman, 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 Phytol. 188 (2010) 122-135.

[61] J. Liu, L. Sheng, Y. Xu, J. Li, Z. Yang, H. Huang, L. Xu, *WOX11* and *12* are involved in the first-step cell fate transition during de novo root organogenesis in *Arabidopsis*, Plant Cell 26 (2014) 1081-1093.

[62] S.-H. Cho, S.-C. Yoo, H. Zhang, D. Pandeya, H.-J. Koh, J.-Y. Hwang, G.-T. Kim, N.-C. Paek, The rice *narrow leaf2* and *narrow leaf3* loci encode WUSCHEL-related homeobox 3A (OsWOX3A) and function in leaf, spikelet, tiller and lateral root development, New Phytol. 198 (2013) 1071-1084.

[63] J. Nardmann, J. Ji, W. Werr, M.J. Scanlon, The maize duplicate genes *narrow sheath1* and *narrow sheath2* encode a conserved homeobox gene function in a lateral domain of shoot apical meristems, Development 131 (2004) 2827-2839.

[64] J.C. Fletcher, U. Brand, M.P. Running, R. Simon, E.M. Meyerowitz, Signaling of cell fate decisions by *CLAVATA3* in *Arabidopsis* shoot meristems, Science 283 (1999) 1911-1914.

[65] S. Conway, A. Drinnan, Analysis of surface growth in the conifer shoot apical meristem, Int. J. Plant Sci. 178 (2017) 273-287.

[66] U. Brand, M. Grünewald, M. Hobe, R. Simon, Regulation of *CLV3* expression by two homeobox genes in *Arabidopsis*, Plant Physiol. 129 (2002) 565-575.

[67] R. Groß-Hardt, T. Laux, Stem cell regulation in the shoot meristem, J. Cell Sci. 116 (2003) 1659-1666.

[68] F. Deyhle, A.K. Sarkar, E.J. Tucker, T. Laux, *WUSCHEL* regulates cell differentiation during anther development, Dev. Biol. 302 (2007) 154-159.

[69] Y. Zhang, R. Wu, G. Qin, Z. Chen, H. Gu, L.-J. Qu, Over-expression of *WOX1* leads to defects in meristem development and polyamine homeostasis in *Arabidopsis*, J. Integr. Plant Biol. 53 (2011) 493-506.

[70] M. Nakata, N. Matsumoto, R. Tsugeki, E. Rikirsch, T. Laux, K. Okada, Roles of the middle domain–specific *WUSCHEL-RELATED HOMEOBOX* genes in early development of leaves in *Arabidopsis*, Plant Cell 24 (2012) 519-535.

[71] X. Wu, J. Chory, D. Weigel, Combinations of *WOX* activities regulate tissue proliferation during *Arabidopsis* embryonic development, Dev. Biol. 309 (2007) 306-316.

[72] J. Palovaara, I. Hakman, Conifer *WOX*-related homeodomain transcription factors, developmental consideration and expression dynamic of *WOX2* during *Picea abies* somatic embryogenesis, Plant Mol. Biol. 66 (2008) 533-549.

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

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

[75] S.O. Park, Z. Zheng, D.G. Oppenheimer, B.A. Hauser, The *PRETTY FEW SEEDS2* gene encodes an *Arabidopsis* homeodomain protein that regulates ovule development, Development 132 (2005) 841-849.

[76] D. Kong, Y. Hao, H. Cui, The WUSCHEL related homeobox protein WOX7 regulates the sugar response of lateral root development in *Arabidopsis thaliana*, Mol. Plant 9 (2016) 261-270.

[77] M. Brinker, L. Van Zyl, W. Liu, D. Craig, R.R. Sederoff, Microarray Analyses of Gene Expression during Adventitious Root Development in *Pinus contorta*, Plant Physiol. 135 (2004) 1526-1539.

SUPPLEMENTARY DATA

Supplementary Table S1. Accession numbers (GenBank) for all proteins used in phylogenetic analyses

Supplementary Table S2. Primer and probe list

Supplementary Figure S1. Values of non-synonymous (Ka) and synonymous (Ks) substitution rates and Ka/Ks ratio for *P. pinaster WOX* gene family calculated through the Computational Biology Unit (CBU) Ka/Ks Calculation tool (http://services.cbu.uib.no/tools/kaks).

Supplementary Figure S2. Schematic representation of the shoot apex and *WOX* genes expression domain. SAM: shoot apical meristem. NP: needle primordia. CZ: central zone. PZ: peripheral zone. *PpWUS* (orange), *PpWOXX* (blue) and *PpWOX3* (red).

Supplementary Figure S3. *PpWUS* and *PpWOXX* transcript detection by fluorescent *in situ* hybridization (FISH) in longitudinal sections of shoot apex excised from three-week-old *Pinus pinaster* seedlings. Specific anti-mRNA probes labelled with Cyanine 5 (Cy5; red signal) were used. Blue signal represents nuclei staining with 4,6-diamidino-2-phenylindole (DAPI). Transcripts of both *PpWUS* (A) and *PpWOXX* (B) genes were detected in the central zone of the meristem. Needle primordia (np). Shoot apical meristem (sam) including the central zone (cz) and the peripheral zone (pz). All images were obtained with a Leica DMRXA fluorescence microscope (Leica Microsystems, Wetzlar, Germany) and processed with ConfocalUniovi ImageJ software (http://spi03.sct.uniovi.es/confocaluniovi/). Bar, 50 µm.

TABLES (submitted as a separated document)

Table 1. Summary of WOX protein expression domains and function. A comparison between angiosperms and gymnosperms. (*) Genes described in this work.

FIGURES

Figure 1. Material used for quantitative real-time PCR (RT-qPCR) analysis of the *WOX* family mRNA abundance in *Pinus pinaster* during the development of somatic embryos (A-D), germination (E-G), and in different parts of young plants (H). (A) Proliferating proembryogenic mass (PEM) in the presence of the plant growth regulators (PGRs) auxin and cytokinin; (B) early embryo (EE) 1 wk after withdrawal of PGRs; (C) late embryo (LE) before the emergence of cotyledons; (D) mature embryo (ME) after 12 wk on the maturation medium. (E) Germinated embryos with a radicle length inferior to 1 cm (G1); (F) germinated embryos with a radicle length between 1 and 2 cm (G2); (G) germinated embryos with a radicle length between 2 and 3 cm (G3). (H) Three-week-old seedling. Sa, shoot apex; Co, cotyledon; Hy, hypocotyl; Rt, root tip. Note that the number of cotyledons in *P. pinaster* varies between 3 and 8. Bar, 1 mm (A-D); 1 cm (E-H).

Figure 2. The *Pinus pinaster WOX* gene family. The structure of full-length (black line) cDNAs is depicted. Exons (grey), when possible, were obtained by comparison between the cDNA and genomic sequences; Conserved Homeodomain (red; HD). Open reading frames for *PpWOXD*, *PpWOXF* and *PpWOXG* genes (yellow).

Figure 3. Phylogenetic tree of the WOX proteins. The tree contains sequences from green alga (*Ostreococcus tauri*, Ot), moss (*Physcomitrella patens*, Ph), lycophyte (*Selaginella moellendorffii*, Sm), fern (*Ceratopteris richardii*, Cr; and *Cyathea australis*, Ca), gymnosperm (*Ginkgo biloba*, Gb; *Gnetum gnemon*, Gg; *Picea abies*, Pa; *Pinus sylvestris*, Ps; *Pinus pinaster*, Pp; and *Pinus taeda*, Pt), and angiosperm (basal angiosperm: *Amborella trichopoda*, Amt; monocots: *Oryza sativa*, Os; and *Zea mays*, Zm; and dicots: *Arabidopsis thaliana*, At; *Populus euphratica*, Poe; *Populus trichocarpa*, Pot; and *Vitis vinifera*, Vv) representative species. The unrooted amino acid sequence similarity trees were generated using the Geneious software by the Neighbour-Joining method and the Jukes-Cantor genetic distance model. WUS clade (red; W); intermediate clade (blue; I); ancient clade (black; A). The green alga OtWOX sequence was used as outgroup for the tree (orange). *Pinus pinaster* sequences are highlighted with an asterisk.

Figure 4. Phylogenetic tree of the WUS-clade WOX proteins. Species abbreviations are indicated as in Figure 3. The unrooted amino acid sequence similarity trees were generated using the Geneious software by the Neighbour-Joining method and the Jukes-Cantor genetic distance model. The green alga OtWOX sequence was used as outgroup for the tree. Each group

of orthologous genes is highlighted in a different colour. *Pinus pinaster* sequences are highlighted with an asterisk.

Figure 5. WOX protein domains. (A) Schematic representation of the conserved domains in all WOX family proteins in *Pinus pinaster*. Homeodomain (red; HD), WUS-box (orange; W), and EAR domain (purple). (B) Alignment of the 14 *P. pinaster* WOX homeodomain sequences. Note the extra Y residue in PpWUS sequence (red box).

Figure 6. Quantitative real-time PCR (RT-qPCR) analysis of the relative mRNA abundance of the *WOX* gene family in *Pinus pinaster* during embryo development and germination. PEM (proembryogenic mass); EE (early embryo); LE (late embryo); ME (mature embryo); G1, G2, and G3 (germinated embryo with radicle length <1 cm, 1-2 cm, and 2-3 cm, respectively). Different letters indicate significant differences in the relative mRNA abundance (Student–Newman–Keuls test, $\alpha = 0.05$).

Figure 7. Quantitative real-time PCR (RT-qPCR) analysis of the relative mRNA abundance of the *WOX* gene family in different parts of three-week-old seedlings of *Pinus pinaster*. Root tip (Rt); Hypocotyl (Hy); Shoot apex (Sa); Cotyledon (Co). Different letters indicate significant differences in the relative mRNA abundance (Student–Newman–Keuls test, $\alpha = 0.05$).

Figure 8. *PpWOX* gene expression obtained by RNA-Seq from tissues isolated through laser capture microdissection in one-month-old seedlings of *Pinus pinaster*. Apical Meristem (AM), Emerging Needles (EN), Young Needles Mesophyll (YNM), Young Needles Vascular (YNV), Cotyledon Mesophyll (CM), Cotyledon Vascular (CV), Hypocotyl Cortex (HC), Hypocotyl Vascular (HV), Hypocotyl Pith (HP), Root Cortex (RC), Root Vascular (RV), Developing Root Cortex (DRC), Developing Root Vascular (DRV) and Root Meristem (RM).

Figure 9. *PpWUS*, *PpWOXX* and *PpWOX3* transcript detection by fluorescent *in situ* hybridization (FISH) in longitudinal sections of shoot apex excised from three-week-old *Pinus pinaster* seedlings. Specific anti-mRNA probes labelled with Cyanine 5 (Cy5; red signal) were used. Blue signal represents nuclei staining with 4,6-diamidino-2-phenylindole (DAPI). (A) *PpWUS* transcripts were exclusively detected (arrows) in the central zone of the meristem. (B) Close up view. (C) Transcripts from *PpWOXX* also showed a restricted expression domain (arrows) in cells situated in the central zone of the meristem. (D) Close up view. (E) *PpWOX3* was shown to be expressed in the flanks of the meristem and in needle primordia. (F) Close up view. Needle primordia (np). Shoot apical meristem (sam) including the central zone (cz) and the peripheral zone (pz). All images were obtained with a Leica DMRXA fluorescence

microscope (Leica Microsystems, Wetzlar, Germany) and processed with ConfocalUniovi ImageJ software (http://spi03.sct.uniovi.es/confocaluniovi/). Bar, 50 µm.

AUTHOR CONTRIBUTIONS

The design of the study was made by JMA, RAC, CA, FMC and RJO and the experimental setup was planned by JMA, NB, RAC and RJO. JMA and NB performed most of the experimental work. JMA, RAC and NB analysed and interpreted the data. JMA drafted the manuscript. RAC, NB, CA, FMC and RJO revised the manuscript. All authors read and approved the final manuscript.

Table 1

Table 1. Summary of WOX protein expression domains and function. A comparison between angiosperms and gymnosperms. (*) Genes described in this work.

	Angiosperms				**Gymnosperms**			
Clade	**Gene**	**Expression domain**	**Function**	**References**	**Orthologue**	**Expression domain**	**Function**	**References**
WUS	***WUS***	SAM, floral meristem, ovule, anther	Stem-cell maintenance, anther and ovule development	[6, 14, 56, 66-68]	***PpWUS*(*), *GbWUS*, *GgWUS***	Embryo, shoot tip	Unknown	[28]
	WOX1	Lateral organ primordia	Lateral organ formation	[13, 23, 69, 70]				
	WOX2	Apical embryo domain	Embryo patterning	[13, 24, 71]	***PpWOX2*(*), *PaWOX2***	Embryo, Apical embryo domain	Embryo patterning	[26, 60, 72]
	WOX3 (*PRS1*, *NS1*, *NS2*)	SAM, peripheral zone	Promotes cell proliferation, lateral organ formation	[13, 17, 23, 70]	***PpWOX3*(*), *PaWOX3*, *GgWOX3*, *GbWOX3A*, *GbWOX3B*, *PsWOX3***	Embryo, SAM, peripheral zone, cotyledons, needles	Lateral organ outgrowth	[27, 49]
	WOX4	Vascular cambium	Procambial development	[16, 73, 74]	***PpWOX4*(*), *PaWOX4*, *GgWOX4*, *GbWOX4***	Germinating embryo, Vascular cambium	Unknown	[29, 49]
	WOX5	RAM	Stem-cell maintenance	[13, 15]	***PpWOX5*(*), *PaWOX5* *PsWOX5***	Embryo, RAM, SAM	Unknown	[28, 29]
	WOX6 **(***PFS2*, *hos9*)	Female gametophyte	Prevents differentiation, cold-stress response	[75]				
	WOX7	Root	Lateral root development	[76]				
					***PpWOXX*(*)**	Embryo, SAM, needles	Unknown	

Intermediate	***WOX8*** **(***STPL*)	Basal embryo domain	Embryo patterning	[13, 24, 71]	***PpWOXB*$^{(*)}$**, ***PpWOXC*$^{(*)}$**, ***PpWOXD*$^{(*)}$**, ***PpWOXE*$^{(*)}$**, ***PaWOX8A*, *PaWOX8/9***	Basal embryo domain	Embryo patterning	[25, 60]
	WOX9 **(***STIMPY*)	Basal embryo domain	Embryo patterning, promote cell proliferation	[13, 71]				
	WOX11	Root	Adventitious root formation	[61]	***PpWOXF*$^{(*)}$**, ***PaWOX8B*, *PaWOX8C*, *PaWOX8D***	Embryo	Unknown	[29]
	WOX12	Root	*De novo* root organogenesis	[77]				
Ancient	***WOX10***	Unknown	Unknown					
	WOX13	Root, inflorescence	Floral transition, root development	[12, 59]	***PpWOX13*$^{(*)}$**, ***PpWOXA*$^{(*)}$**, ***PpWOXG*$^{(*)}$**, ***PaWOX13***	All studied tissues	Unknown	[29]
	WOX14	Root, inflorescence	Floral transition, root development	[12]				

Figure 1

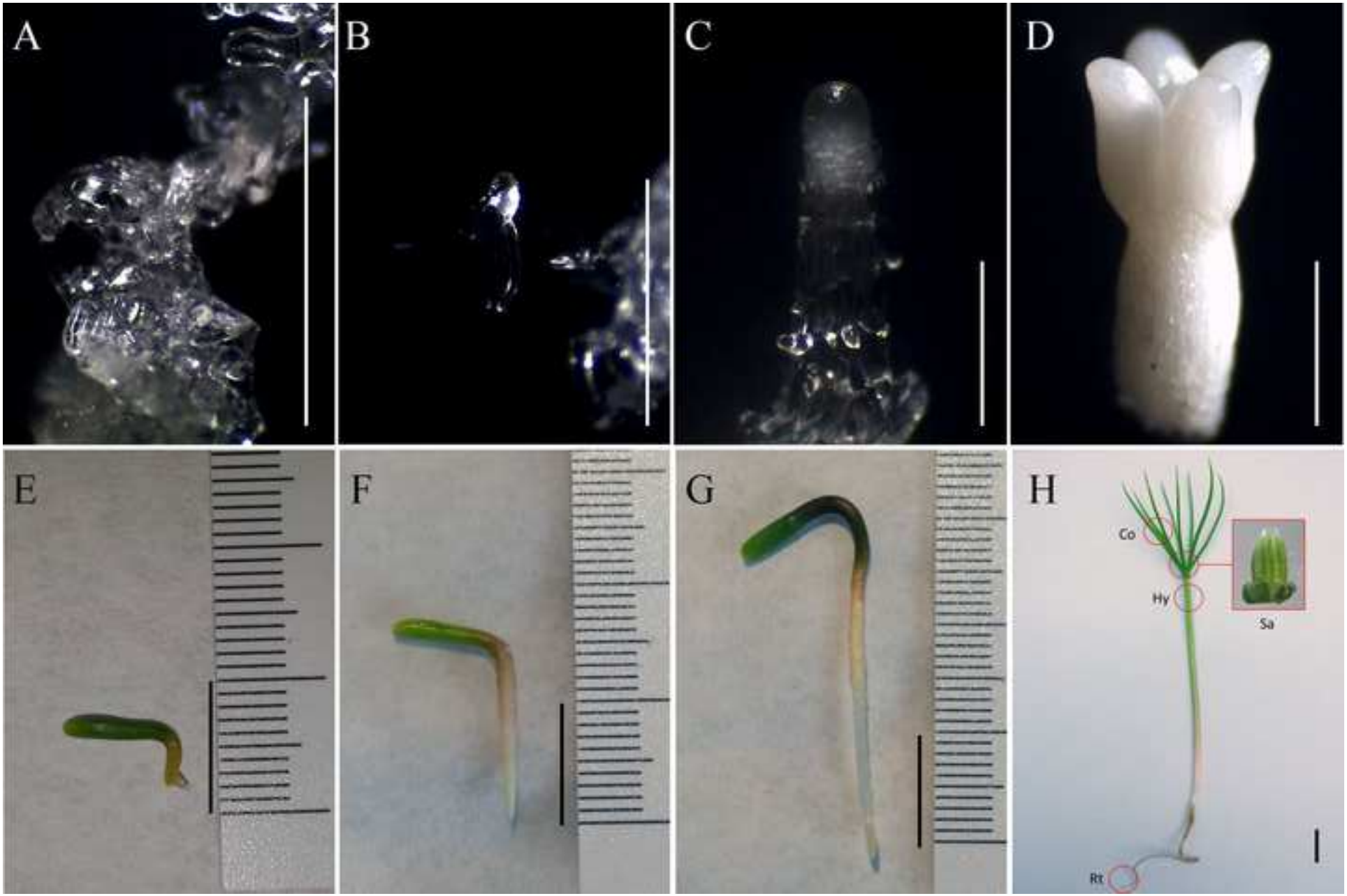


Figure 4

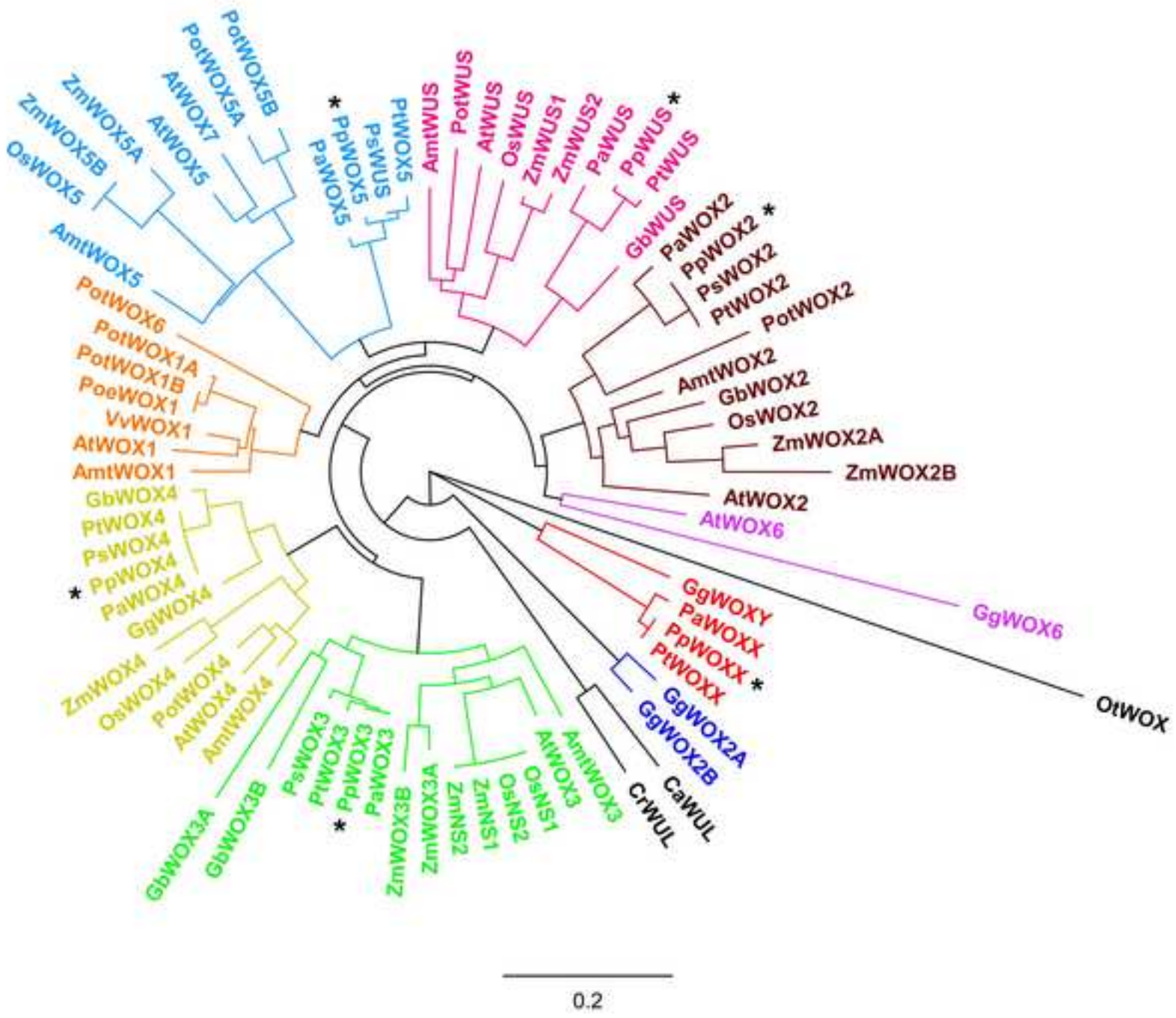

PotWOX5B
PotWOX5A
AtWOX7
AtWOX5
ZmWOX5A
ZmWOX5B
OsWOX5
AmtWOX5
PaWOX5
* PpWOX5
PsWOX5
PtWOX5
AmtWUS
PotWUS
AtWUS
OsWUS
ZmWUS1
ZmWUS2
PaWUS
PpWUS *
PtWUS
GbWUS
PaWOX2
PpWOX2 *
PsWOX2
PtWOX2
PotWOX2
AmtWOX2
GbWOX2
OsWOX2
ZmWOX2A
ZmWOX2B
AtWOX2
AtWOX6
GgWOX6
OtWOX
GgWOXY
PaWOXX
PpWOXX
PtWOXX *
GgWOX2A
GgWOX2B
CaWUL
CrWUL
PotWOX6
PotWOX1A
PotWOX1B
PoeWOX1
VvWOX1
AtWOX1
AmtWOX1
GbWOX4
PtWOX4
PsWOX4
* PpWOX4
PaWOX4
GgWOX4
ZmWOX4
OsWOX4
PotWOX4
AtWOX4
AmtWOX4
GbWOX3A
GbWOX3B
PsWOX3
PtWOX3
PpWOX3 *
PaWOX3
ZmWOX3B
ZmWOX3A
ZmNS2
ZmNS1
OsNS2
OsNS1
AtWOX3
AmtWOX3
0.2

Figure 2

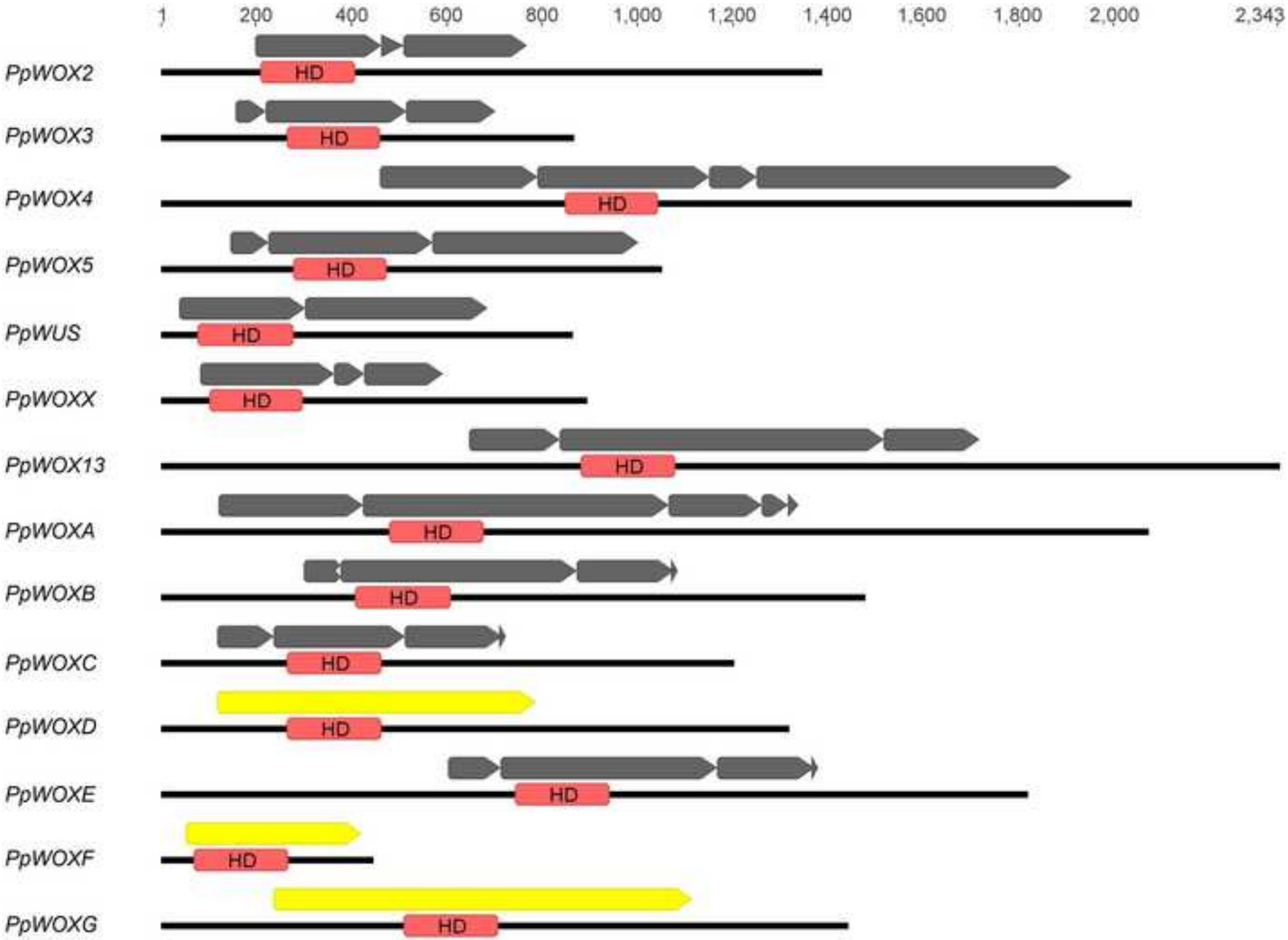

1
200
400
600
800
1,000
1,200
1,400
1,600
1,800
2,000
2,343
PpWOX2
HD
PpWOX3
HD
PpWOX4
HD
PpWOX5
HD
PpWUS
HD
PpWOXX
HD
PpWOX13
HD
PpWOXA
HD
PpWOXB
HD
PpWOXC
HD
PpWOXD
HD
PpWOXE
HD
PpWOXF
HD
PpWOXG
HD

Figure 3

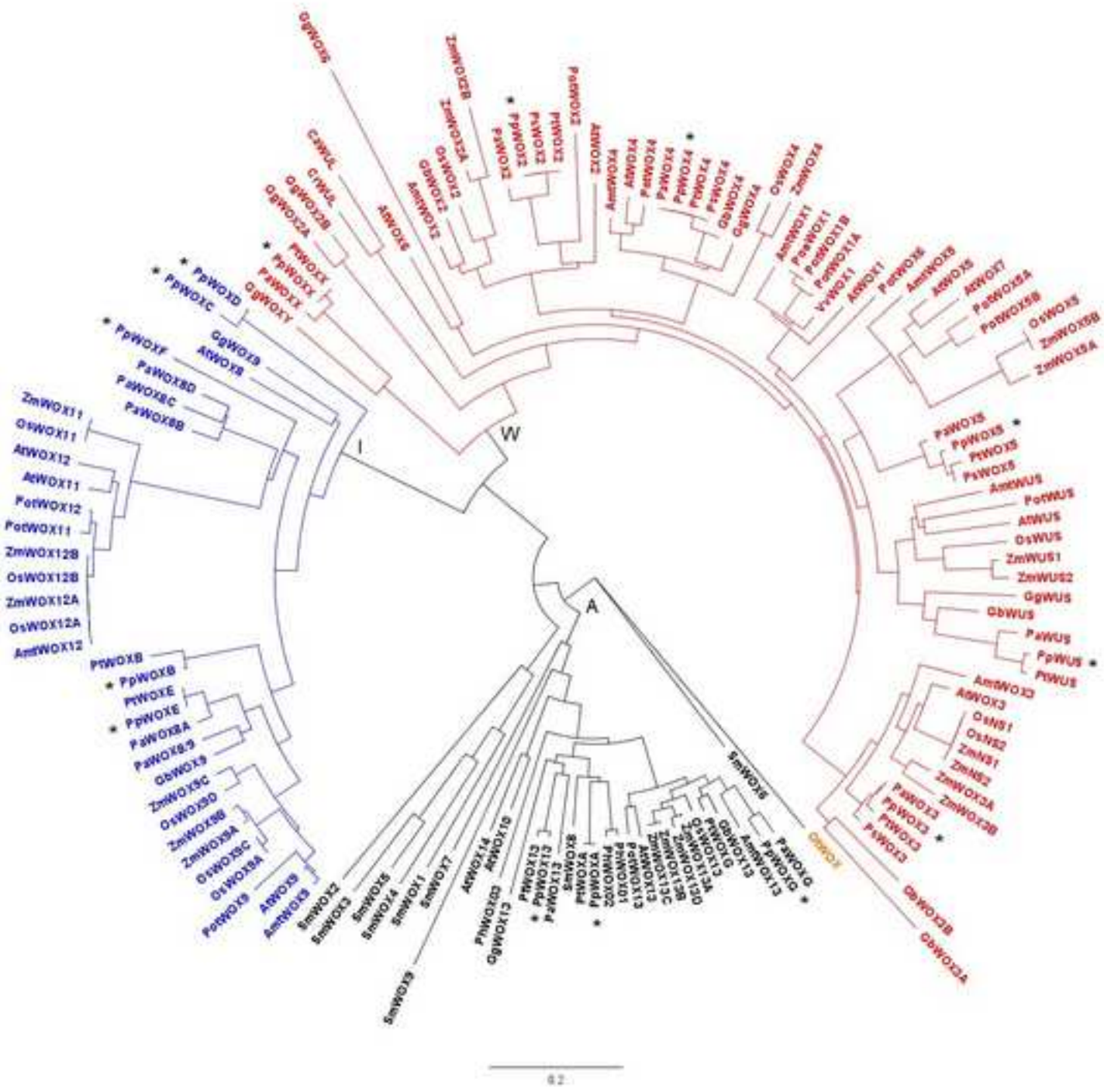


Figure 5

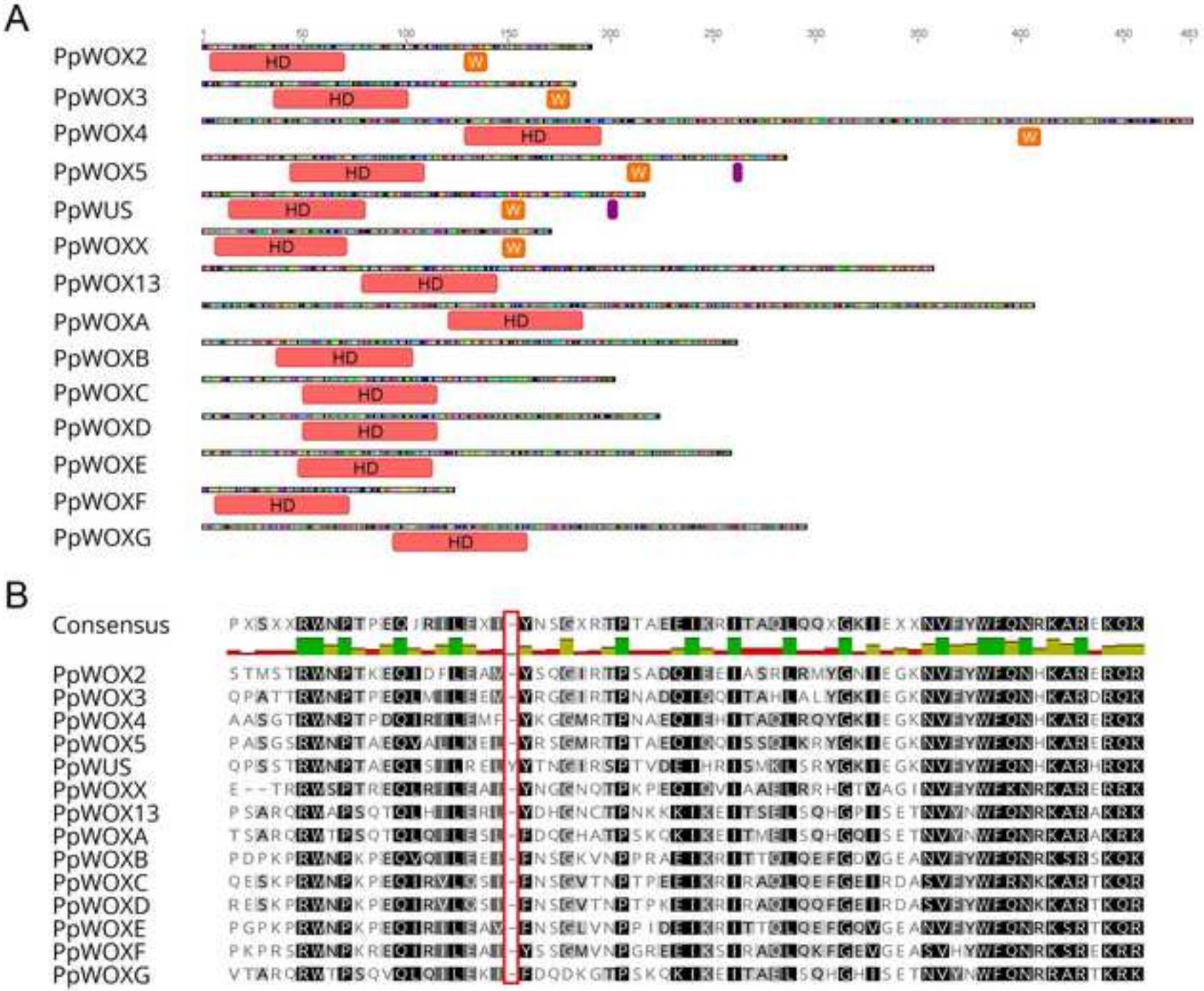

A
PpWOX2
PpWOX3
PpWOX4
PpWOX5
PpWUS
PpWOXX
PpWOX13
PpWOXA
PpWOXB
PpWOXC
PpWOXD
PpWOXE
PpWOXF
PpWOXG
HD
W
B
Consensus
PpWOX2
PpWOX3
PpWOX4
PpWOX5
PpWUS
PpWOXX
PpWOX13
PpWOXA
PpWOXB
PpWOXC
PpWOXD
PpWOXE
PpWOXF
PpWOXG

Figure 6

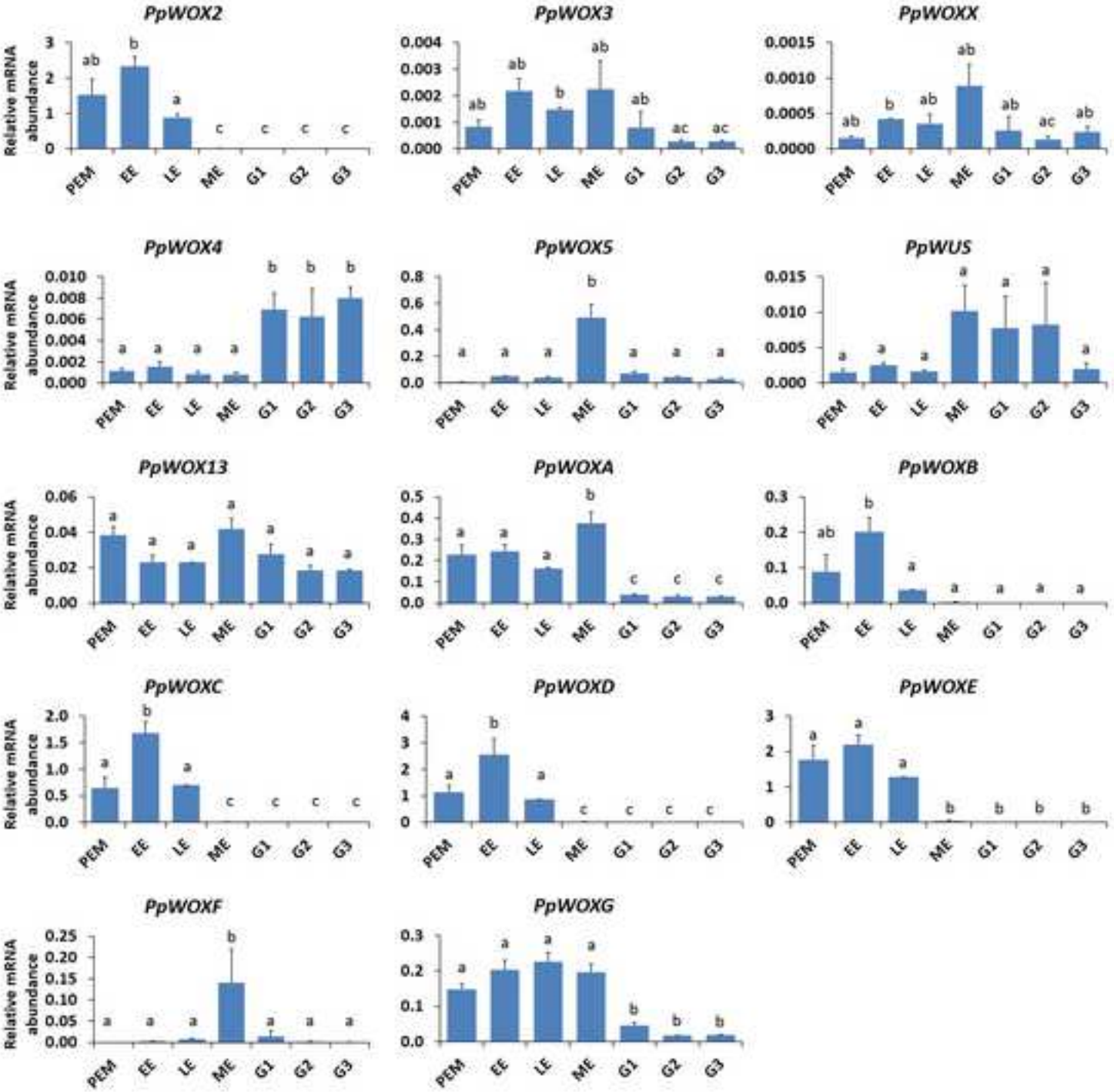

PpWOX2
PpWOX3
PpWOXX
PpWOX4
PpWOX5
PpWUS
PpWOX13
PpWOXA
PpWOXB
PpWOXC
PpWOXD
PpWOXE
PpWOXF
PpWOXG
Relative mRNA abundance
PEM
EE
LE
ME
G1
G2
G3

Figure 7

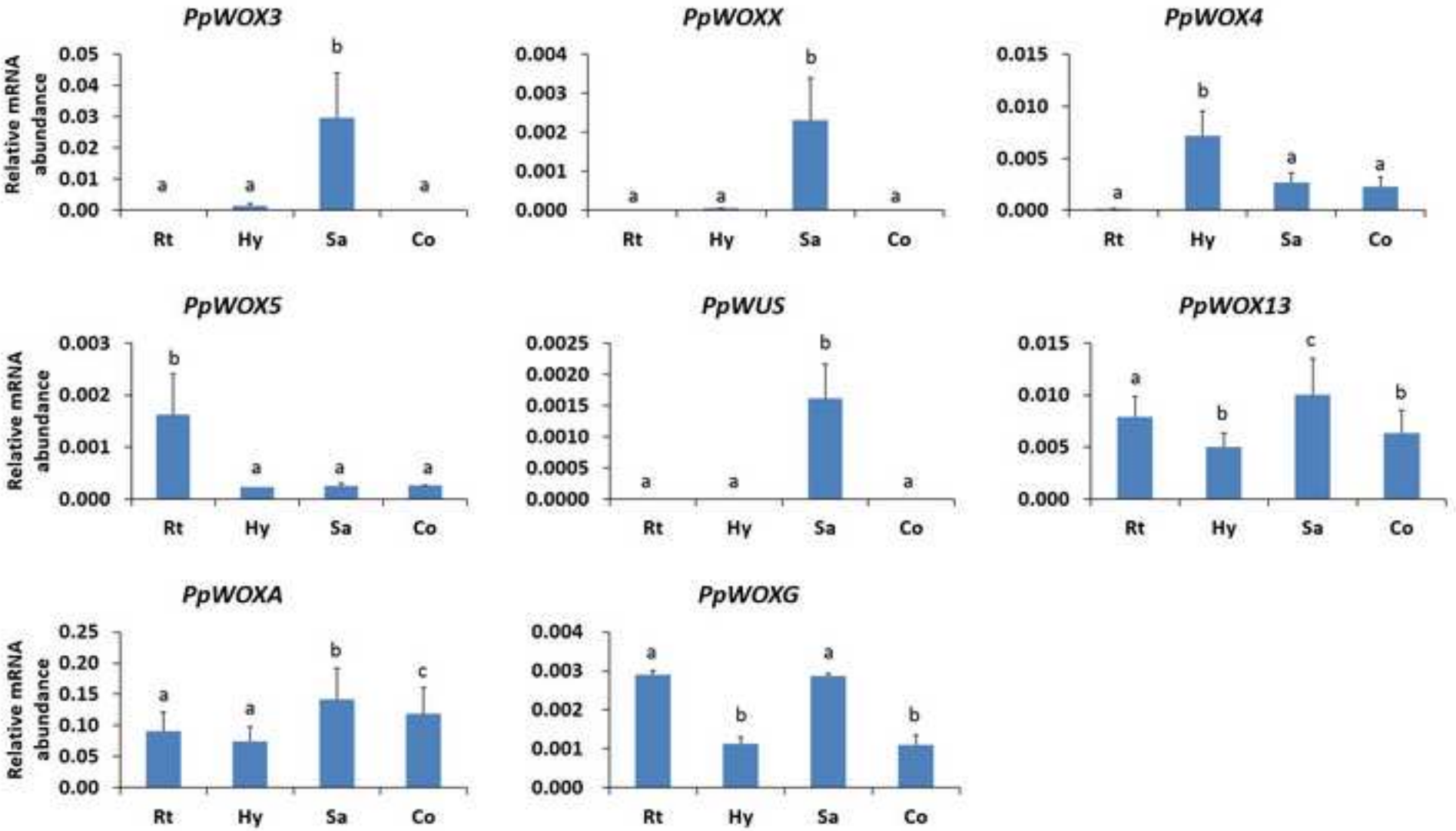

PpWOX3
PpWOXX
PpWOX4
PpWOX5
PpWUS
PpWOX13
PpWOXA
PpWOXG
Relative mRNA abundance
Rt
Hy
Sa
Co

Figure 8

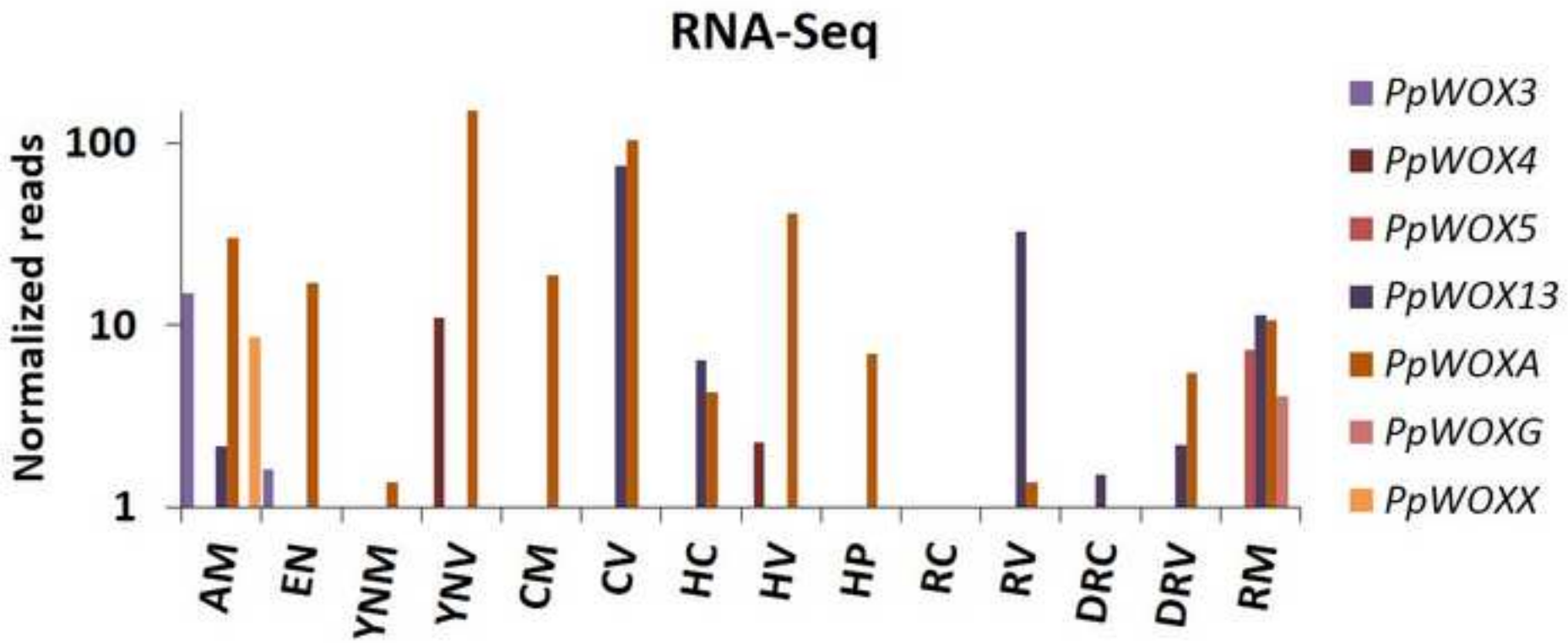
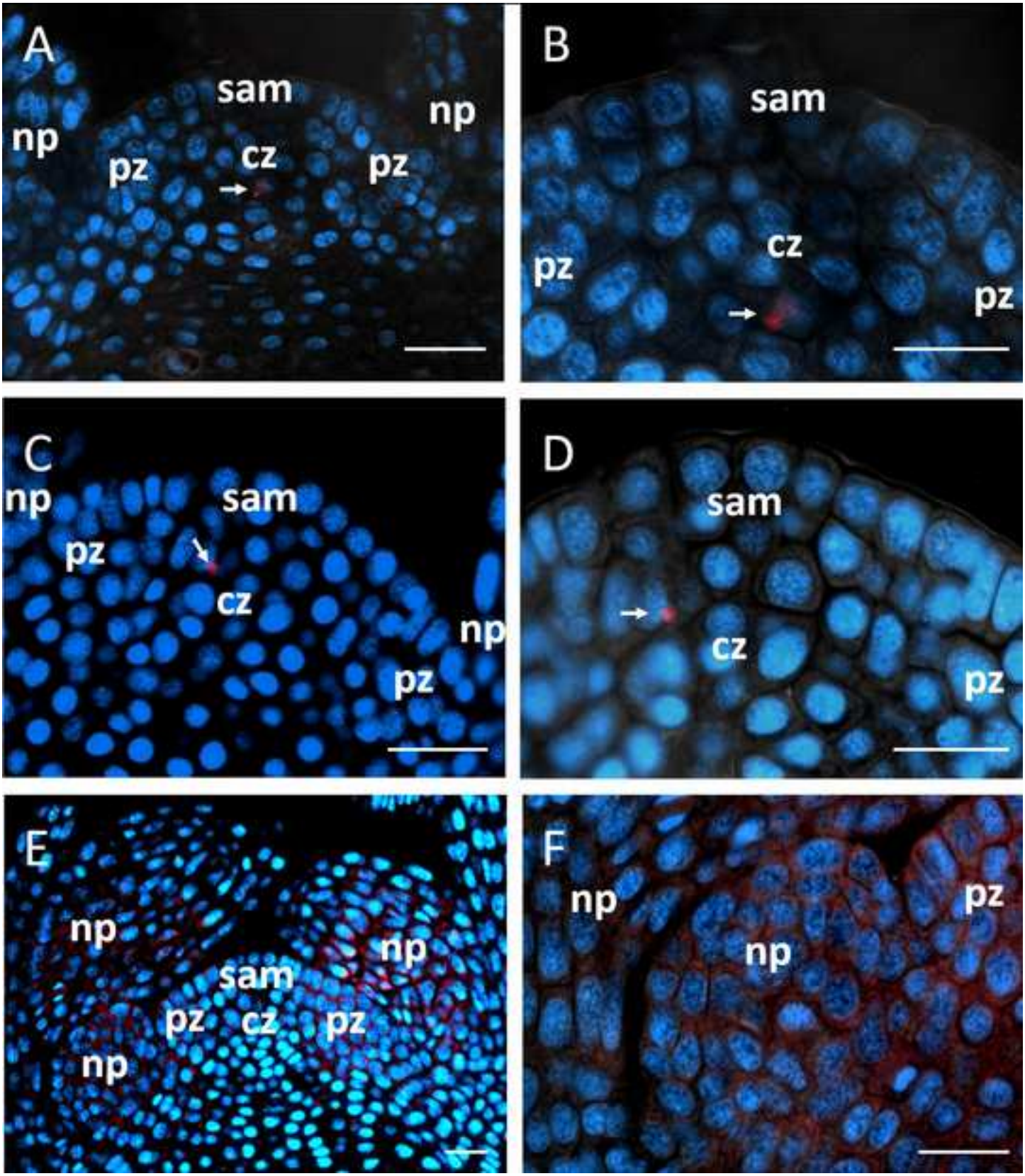

RNA-Seq
Normalized reads
100
10
1
AM
EN
YNM
YNV
CM
CV
HC
HV
HP
RC
RV
DRC
DRV
RM
PpWOX3
PpWOX4
PpWOX5
PpWOX13
PpWOXA
PpWOXG
PpWOXX

Figure 9


A
sam
np
np
pz
cz
pz
B
sam
cz
pz
pz
C
np
sam
pz
cz
np
pz
D
sam
cz
pz
E
np
np
sam
pz
cz
pz
np
F
np
pz
np