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Research article

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

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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.

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 (Chan et al., 1998). 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) (Mukherjee et al., 2009). 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 (Schoof et al., 2000; van der Graaff et al., 2009; Ueda et al., 2011). Some members of the plant-specific WOX protein family can act both as activators and repressors depending on tissue type or developmental stage (Ikeda et al., 2009; Lin et al., 2013).

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 (van der Graaff et al., 2009). 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* (Schoof et al., 2000; Rebocho et al., 2008; Zhang et al., 2010; Liu et al., 2014a; Dolzblasz et al., 2016). 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 (Deveaux et al., 2008). *WOX* genes belonging to the intermediate clade, as well as *WOX2* belonging to the WUS clade, are preferentially expressed during embryo development (Haecker et al., 2004). 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) (Mayer et al., 1998; Sarkar et al., 2007). *AtWOX4* is involved in

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the cambial meristem differentiation (Ji et al., 2010a), while *AtWOX3/PRS1* is involved in lateral organ development through recruiting organ founder cells forming the lateral domain (Matsumoto and Okada, 2001; Shimizu et al., 2009). 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 (Dolzblasz et al., 2016; Sarkar et al., 2007; Shimizu et al., 2009; Ji et al., 2010b).

Arabidopsis has been widely used as a model organism for studies in plants (Laux et al., 2004). Gymnosperm and angiosperm species, which have a common ancestor ca 300 million years ago (Savard et al., 1994; Smith et al., 2010), 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 (Rebocho et al., 2008; Deveaux et al., 2008; Vandenbussche et al., 2009). 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* (Haecker et al., 2004; Breuninger et al., 2008). Their orthologues in the conifer *Picea abies PaWOX8/9* and *PaWOX2* have similar functions (Zhu et al., 2014, 2016). *PaWOX3*, the orthologue of *AtWOX3*, has been shown to play an important role in lateral organ outgrowth (Alvarez et al., 2015). 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 (Nardmann et al., 2009). Based 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 (Hedman et al., 2013).

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* counterparts. 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 (Cañas et al., 2017) and SustainPine (Canales et al., 2014). 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 (Altschul et al., 1997), 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