Genome **Biology**

Protein family review

The WUS homeobox-containing (WOX) protein family

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Abstract

The WOX genes form a plant-specific subclade of the eukaryotic homeobox transcription factor superfamily, which is characterized by the presence of a conserved DNA-binding homeodomain. The analysis of WOX gene expression and function shows that WOX family members fulfill specialized functions in key developmental processes in plants, such as embryonic patterning, stem-cell maintenance and organ formation. These functions can be related to either promotion of cell division activity and/or prevention of premature cell differentiation. The phylogenetic tree of the plant WOX proteins can be divided into three clades, termed the WUS, intermediate and ancient clade. WOX proteins of the WUS clade appear to some extent able to functionally complement other members. The specific function of individual WOX-family proteins is most probably determined by their spatiotemporal expression pattern and probably also by their interaction with other proteins, which may repress their transcriptional activity. The prototypic WOX-family member WUS has recently been shown to act as a bifunctional transcription factor, functioning as repressor in stem-cell regulation and as activator in floral patterning. Past research has mainly focused on part of the WOX protein family in some model flowering plants, such as *Arabidopsis thaliana* (thale cress) or *Oryza sativa* (rice). Future research, including so-far neglected clades and non-flowering plants, is expected to reveal how these master switches of plant differentiation and embryonic patterning evolved and how they fulfill their function.

Gene organization and evolutionary history

The eukaryotic superfamily of homeobox (HB) transcription factors is characterized by the presence of a short stretch of amino acids (60-66 residues) that folds into a DNA-binding domain termed the homeodomain, which is encoded by the HB DNA sequence [1,2]. HB transcription factors are important regulators of developmental decisions in eukaryotes, as exemplified by the prototypic HB transcription factors, the animal HOX proteins. HOX genes were initially identified in *Drosophila melanogaster* by homeotic mutations that transform one body segment into another, which indicated the involvement of HOX proteins in patterning along the main body axis [3]. HB transcription factors also occur in plants, where they have a wide variety of roles. The WUSCHEL (WUS) homeobox transcription factor is the prototypic member of the plant-specific WUS homeobox (WOX) protein family, one of a number of plant HB transcription factor families. WUS itself is expressed in the organizing-center cells of the shoot apical meristem and regulates shoot stem-cell maintenance. Families of HB transcription factors are generally distinguished by the phylogenetic relatedness of their homeodomains, and by the presence or absence of additional domains. The WOX family is distinguished by the phylogenetic relatedness of its homeodomains [4], as is the plant HB family Knotted related homeobox (KNOX). Other plant HB protein families are distinguished by the possession of additional domains, for example, the HD-Zip family have leucine zippers and the Zf-HD family zinc finger domains [5-7].

Phylogenetic reconstruction of protein sequences that contain the homeodomain as defined by the PFAM database [8,9] (Figure 1a) reveals that this DNA-binding motif probably originated before the divergence of the eukaryotes [5]. (The PFAM-defined homeodomain is the one referred to throughout this article.) The last common ancestor of all extant eukaryotes probably already harbored several HB proteins (see Figure 1a). These were subsequently subject to loss as well as expansion among different lineages and diversified in function. However, because of the short length of the homeodomain, convergent evolution (evolution leading to similar sequences that lack a common ancestor) due to structural constraints imposed by a requirement for DNA binding, for example, cannot be excluded. This might explain some surprising appearances of HB proteins from different taxonomic groups within families that otherwise are apparently specific to a certain lineage (see Figure 1a).

The phylogenetic tree of the plant WOX proteins (see Figure 1b) can be naturally divided into three clades. *Arabidopsis thaliana* WUS, as well as its orthologs from other flowering plant species, is located in a clade that also harbors the root apical meristem regulator WOX5 and the remainder of the WOX proteins 1-7 (Table 1); we will refer

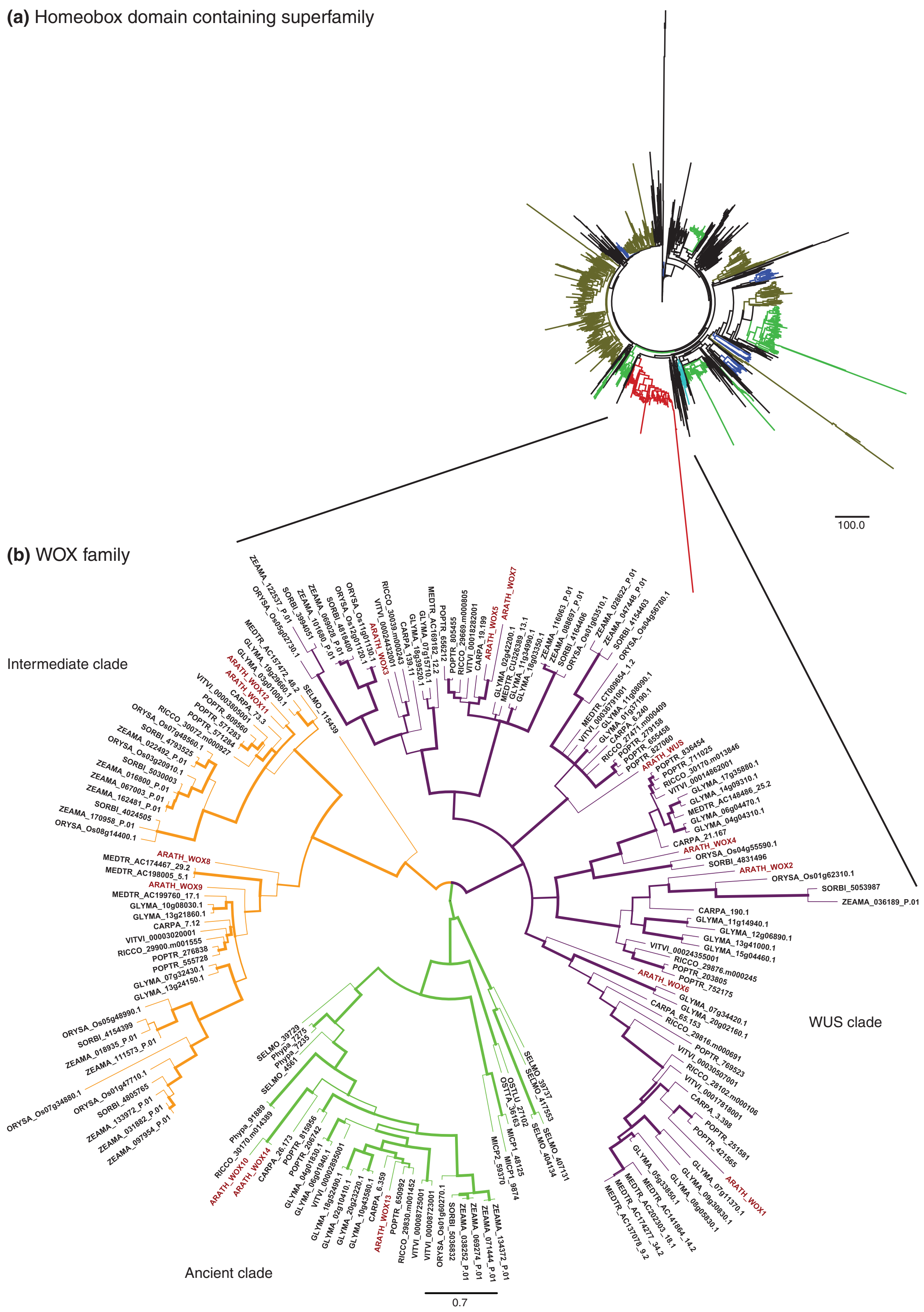

Figure 1

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Figure 1 continued
Phylogenetic relationships of WOX family proteins. **(a)** Relationship of the WOX family to the other members of the HB transcription factor superfamily. Proteins matching the PFAM Homeodomain hidden Markov model (PF00046) [9] were retrieved from completely sequenced genomes of plants, animals, algae and non-photosynthetic protists and subjected to multiple sequence alignment using MAFFT [50]. After manual removal of nonconserved regions, essentially resulting in an alignment of homeodomains, phylogenetic inference was conducted using quicktree_sd [51,52]. Subsequent midpoint-rooting and visualization was performed using FigTree v1.2.2 [53]. Branch width corresponds to bootstrap support. The WOX family is in red. Other HB protein families consisting exclusively of proteins from one of the three kingdoms are colored in green (plants), blue (animals) and cyan (fungi), respectively. Families specific to opisthokonts (animals and fungi) are colored in brown (these clusters contain occasional protist sequences, for example, from Mycetozoa and Amoebozoa). Families indicated in black consist of members from both plants and opisthokonts or contain significant amounts of protist (algal, protozoan) sequences. **(b)** The WOX protein family. Proteins from genomes of completely sequenced plant and algal species were used to generate this phylogeny, which is essentially a representation of the red clade from (a). After manual removal of regions of low alignment quality, phylogenetic inference was conducted using MrBayes [54]. Branch width corresponds to support values; the *A. thaliana* proteins are shown in red. The three subclades are color-coded, WUS/WOX1-7 (WUS) in purple, WOX8, 9, 11, 12 (intermediate) in orange and WOX10, 13, 14 (ancient) in green.

Table 1

Summary of WOX protein expression domains and function

Protein	Alternative name (if any) in species listed	Clade	Expression domain	Function	Species
WUS		WUS clade	SAM, ovule, anther	Stem-cell maintenance, anther and ovule development	*A. thaliana*, snapdragon, petunia
WOX1		WUS clade	Lateral organ primordia	Lateral organ formation	*A. thaliana*, petunia
WOX2		WUS clade	Apical embryo domain	Embryo patterning	*A. thaliana*
WOX3	PRS1 (in maize NS1 and NS2)	WUS clade	SAM, peripheral zone	Promotes cell proliferation, lateral organ formation	*A. thaliana*, maize, petunia, rice
WOX4		WUS clade	Unknown	Unknown	
WOX5		WUS clade	RAM	Stem-cell maintenance	*A. thaliana*, rice
WOX6	PFS2, hos9	WUS clade	Female gametophyte	Prevents differentiation, cold-stress response	*A. thaliana*
WOX7		WUS clade			*A. thaliana*
WOX8		Intermediate clade	Basal embryo domain	Embryo patterning	*A. thaliana*
WOX9	STIMPY	Intermediate clade	Basal embryo domain	Embryo patterning, promote cell proliferation	*A. thaliana*, tomato, petunia
WOX10		Ancient clade	Unknown	Unknown	*A. thaliana*
WOX11		Intermediate clade	SAM and RAM	Crown root development	Rice
WOX12		Intermediate clade	Unknown	Unknown	*A. thaliana*
WOX13		Ancient clade	Root, inflorescence	Floral transition, root development	*A. thaliana*
WOX14		Ancient clade	Root, inflorescence	Floral transition, root development	*A. thaliana*

SAM, shoot apical meristem; RAM, root apical meristem.

to this clade as the WUS clade. The sister clade of the WUS clade contains the *A. thaliana* WOX8, 9, 11 and 12 proteins; we will refer to this clade as the intermediate clade, as it is interspersed between the other two clades. Separated by the midpoint root from the two other clades is the ancient clade (probably representing the earliest diverging WOX genes), which harbors the *A. thaliana* WOX10, 13 and 14 proteins. It is noteworthy that only the ancient clade contains WOX sequences from green algae and from the non-vascular moss *Physcomitrella patens* (see Figure 1b). Therefore, at least one WOX gene must already have been present in the last common ancestor of the 'green' lineage (the lineage consisting of land plants and green algae). The longest internal branch separating the ancient clade from

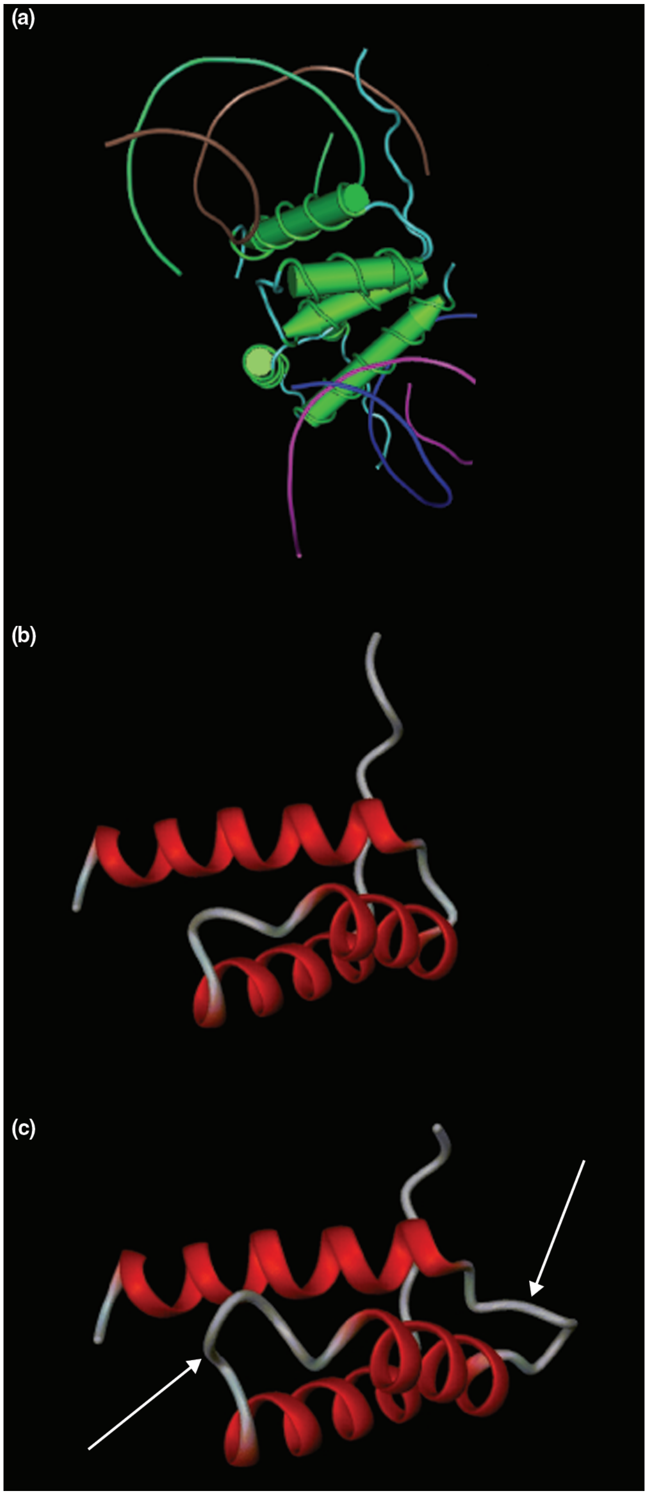

Figure 2

Three-dimensional structure of homeodomains from different groups. **(a)** Crystal structure of the homeodomain from the mammalian ParaHox protein Pdx1 in complex with DNA [55] (PDB 2h1kB, visualized with NCBI MMDB [56] using Cn3D 4.1). **(b)** Visualization of the homeodomain shown in (a) without DNA, visualized with the Protein Picture Generator [57] using DINO [58]. **(c)** Visualization (carried out as in (b)) of the *A. thaliana* WUS homeodomain. Template search was conducted using HHSearch (identifying 2h1kB) and subsequent homology modeling using the alignment mode as implemented in SWISS-MODEL [59]. Note the two loop extensions (arrowed) in the WOX homeodomain as compared with the animal protein.

the remainder of the WOX family [7,10], and the positioning of the root at this branch [7,11], has been established before.

Whether the last common ancestor of the green lineage already possessed two WOX genes, and the gene that gave rise to the intermediate clade was subsequently lost from parts of the lineage, or whether a paralog that gave rise to these clades was established later, in the last common ancestor of vascular plants, cannot be resolved at present [7,11,12]. The intermediate clade contains, besides members from flowering plants, sequences from the vascular lycophyte *Selaginella moellendorffii*. The paralogs giving rise to this clade must therefore have already been present in the last common ancestor of vascular plants. The WUS clade contains protein sequences from flowering plants only. Analyses of organisms for which we have no genome sequence at present have demonstrated that the WUS clade is specific to seed plants and that *WUS* and *WOX5* arose after the divergence of gymnosperms (plants bearing naked seeds) and angiosperms (plants bearing enclosed seeds) [12].

Characteristic structural features

The homeodomain binds DNA through a helix-turn-helix (HTH) structure. The HTH motif is characterized by two α-helices, which make intimate contacts with the DNA and are joined by a short turn. The second helix binds to DNA via a number of hydrogen bonds and hydrophobic interactions, which occur between specific side chains and the exposed bases and thymine methyl groups within the major groove of the DNA [9]. The recognized DNA core motifs differ. Homology modeling of the plant WOX homeodomain reveals two extended loops within a generally highly conserved structure as compared with the animal HOX homeodomain (Figure 2). Such extensions are also known from other HB families, for example the ancient TALE class homeodomain family [13], which has a three-amino-acid loop extension between helices 1 and 2 and has important roles in plant, animal and fungal development, for example as cofactors of the HOX proteins. The position of the homeodomain within the protein varies in different members of the WOX family (Figure 3).

As well as the homeodomain, the WOX proteins contain the distinctive WUS-box motif (essentially of the form T-L-X-L-F-P-X-X, where X can be any amino acid) [4] that

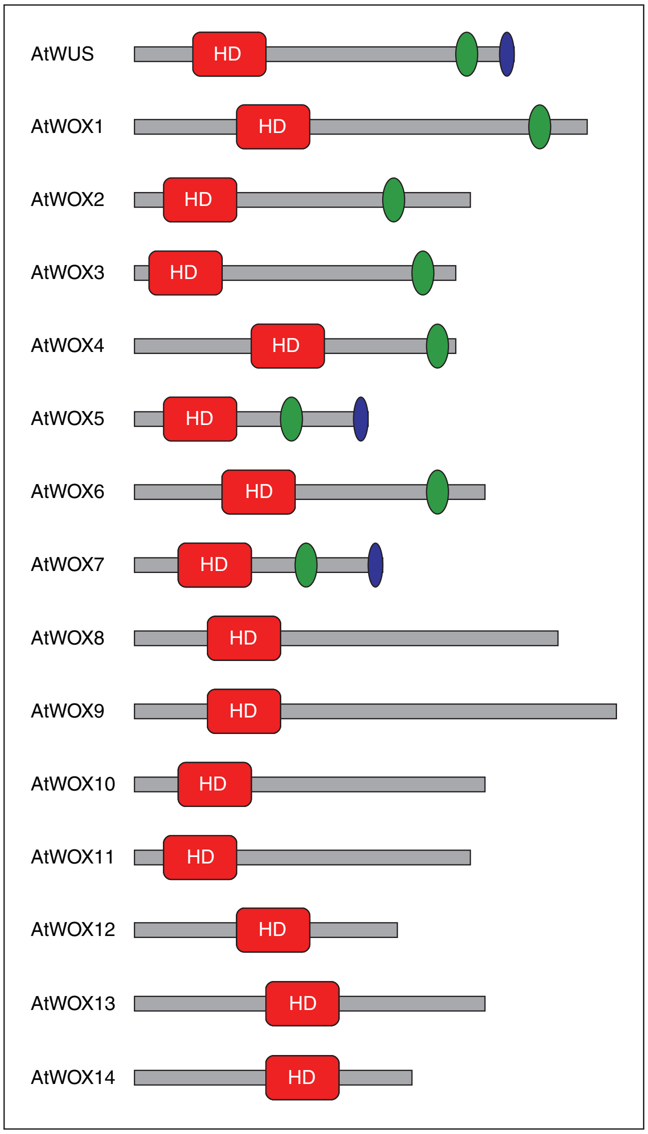

Figure 3

Schematic domain structure of WOX family proteins. Domains were defined using manual annotation of a multiple sequence alignment and subsequent generation and matching of hidden Markov models using HMMER [60]. The homeodomain (HD) (red) is the most prominent and defining feature of the family. The WUS-box motif (green) was defined in a strict sense, as T-L-[DEQP]-L-F-P-[GITVL]-[GSKNTCV], consensus TLELFPLH. The ERF-associated amphiphilic repression (EAR) motif (blue) was also defined in a strict sense, as L-[ED]-L-[RST]-L, in which form it can be detected at the carboxy-terminal end of the WUS, WOX5 and WOX7 proteins.

Box 1

Flowering plant apical stem cells and their function in development

Flowering plants can repeatedly form organs during post-embryonic development from stem cells contained in apical meristems located at the tips of shoots and roots [30].

The shoot apical meristem (SAM) is located at the tip of the shoot and gives rise to all the above-ground tissues after germination [18,24]. Studies in *A. thaliana* showed that the pool of undifferentiated stem cells in the outermost cell layers of the central zone is maintained through a negative feedback loop between stem cells and an underlying organizing center. Expression of the WUS transcription factor in the organizing center represses stem-cell differentiation and induces expression of the secreted peptide signal CLV3. Binding of CLV3 to its receptor CLV1 leads to repression of *WUS* transcription [40]. Cells that exit this stem-cell niche become recruited into differentiation pathways, which, for example, form leaves.

After the transition from vegetative to reproductive development, the SAM turns into the inflorescence meristem, which produces lateral flower meristems, each of which produces an individual flower. The determinate flower meristem is organized in a similar way to the indeterminate shoot meristem, but *WUS* expression is terminated by *WUS-AGAMOUS* negative feedback interaction during the later stages of floral development, resulting in the differentiation of all the stem cells and termination of meristem activity [41,42].

The root apical meristem (RAM) is located at the tip of the root and provides the cells for root growth. Every stem cell in the RAM is in direct contact with the root quiescent center, which expresses the WUS-family member WOX5 [21]. The quiescent center is analogous with the shoot organizing center and provides signals for stem-cell maintenance. The daughter cells of the distal columella stem cells directly differentiate into the gravity-sensing root-cap cells, whereas the progeny of the proximal and lateral stem cells undergo subsequent rounds of cell division and produce the main body of the primary root. Lateral roots are initiated in the pericycle cell layer of the primary root. The stem-cell niche of a lateral root functions similarly to that of the primary root [23].

distinguishes them from other HB transcription factors. In its strict definition, this motif is present in the members of the WUS clade and is located carboxy-terminal to the homeodomain (see Figure 3). The WUS-box motif was shown to be essential for WUS function in both the regulation of the shoot stem-cell population (Box 1) and in floral patterning [14]. The members of the WUS clade all possess the two-amino-acid motif T-L at the start of the WUS box, whereas the non-WUS WOX-family members show variation at this position. The WUS-box motif is found in the same relative position in all other members of the *A. thaliana* WOX family.

Several WOX proteins contain a stretch of acidic amino acids between the homeodomain and the WUS box that could potentially function as an activator domain, and/or contain a carboxy-terminal ERF-associated amphiphilic repression (EAR) domain that has been shown to be involved in transcriptional repression ([15] and references therein). As the EAR domain can mediate interaction with the co-repressor protein TOPLESS [15] (see Figure 3), the repressor activity of proteins containing EAR domains might depend on protein interaction. The recently described expanded Aux/IAA EAR domain [LI]-X-[LI]-[AG]-[LP]-[PGST] [15] has not been detected in any *A. thaliana* WOX protein. Only a relaxed form of the EAR motif, namely [LVI]-X-[LVI]-X-[LVI], can be detected in members of all three WOX subclades. This motif is not always present at the carboxyl terminus and in some cases overlaps with other domains and is present in multiple copies (for example, two in *A. thaliana* WOX4, 9 and WUS and three in WOX8). Simple L-X-L motifs are present in all *A. thaliana* WOX proteins except WOX8 and 10. Only WUS, WOX5 and WOX7 contain the carboxy-terminal EAR motif in its strict sense (see Figure 3).

Localization and function

Despite their function as transcription factors, no clear nuclear localization signal (NLS) can be predicted for any of the WOX family members (using PSORT [16] and PredictNLS [17]). Subcellular localization has been investigated so far only for some WOX-family members. WUS [18], WOX6/PFS2 (WOX6 is named PRETTY FEW SEEDS 2 in *A. thaliana*) [19] and WOX11 [20] are localized to the nucleus. This nuclear localization might involve cryptic NLS motifs not detected by prediction algorithms and/or interactions with other proteins that themselves contain a NLS. The positively charged amino acids present as stretches of two or three residues throughout the homeodomain might represent such a cryptic NLS. Table 1 lists the expression domains and putative functions of WOX proteins in several plant species.

The WUS clade

The *WUS* and *WOX5* genes are expressed in the organizing-center cells of the shoot and root apical meristem, respectively, where they are involved in the maintenance of stem-cell function (see Box 1) [17,21,22]. In addition to its expression in the quiescent center cells (see Box 1), WOX5 is expressed early during the initiation and outgrowth of lateral root primordia (which produce the lateral roots post-embryonically) and in the cotyledon primordia (which produce the cotyledons, flanking the shoot apical meristem) [23], suggesting that WOX5 also functions in these tissues. Interestingly, WOX5 and WUS were shown to be exchangeable in regulating stem-cell maintenance in shoot and root [21]. The function of WUS and WOX5 in stem-cell maintenance was demonstrated by loss-of-function mutations. In *wus* loss-of-function mutants the stem cells that are maintained by signaling from the organizing center undergo differentiation, both in *A. thaliana* and *Antirrhinum majus* (snapdragon) [18,24], Besides its role in stem-cell maintenance, *WUS* is involved in ovule and anther development in *A. thaliana* [25,26] and fulfills a similar role in grasses [27]. In the *A. thaliana wox5* loss-of-function mutant, the root columella stem cells, which normally produce the gravity-sensing root cap cells, undergo differentiation [21].

WOX5 and WUS function is conserved in angiosperms, but only a single WOX5/WUS homolog is present in gymnosperms [12]. The *WOX3/PRS1* (*PRESSED FLOWER 1*) gene and the *Zea mays* (maize) orthologs *NS1* and *NS2* (*NARROW SHEATH*) regulate the recruitment of organ founder cells from the lateral domains of plant meristems and promote cell proliferation [28]. *A. thaliana WOX6/PFS2* prevents premature differentiation during formation of the integument (the structure enclosing the embryo sac) and the egg cell [19]. An additional role for *WOX6* in the response to cold stress was identified by the isolation of a mutant allele of *WOX6* named *hos9-1* [29]. *hos* (high expression of osmotically responsive genes) mutants grow more slowly, flower later, and are more sensitive to freezing.

The WOX2 protein was shown to be required for apical patterning during *A. thaliana* embryo development (see Box 1) [30,31] and is regulated by WOX8 and WOX9 (also known as STIMPY in *A. thaliana*). In *Picea abies* (Norway spruce) *WOX2* expression is correlated with somatic embryogenesis [10,32]. Interestingly, *A. thaliana* WOX1, WOX3 and WOX5 act redundantly with WOX2 during apical patterning [30]. In *Petunia hybrida* (petunia), the WOX family member MAW (MAEWEST) combines the separate functions of *A. thaliana* WOX1 and WOX3 proteins in lateral organ development and prevention of organ fusion [33].

The ancient and the intermediate clades

The *WOX8* and *WOX9* genes are redundantly required for development of the basal lineage (giving rise to the hypophysis and suspensor) in the *A. thaliana* embryo and for regulation of *WOX2* expression in the apical domain (the embryo proper) [30,31]. Initially, *WOX2* and *WOX8* are coexpressed in the zygote, but during embryo development, *WOX2* expression and *WOX8* and *WOX9* expression become restricted to the apical and basal domains, respectively [34]. The identified WOX cascade sets up the main body axis in the embryo and regulates the localized auxin response through the auxin-transporter protein PIN1; PIN family members function as auxin-efflux carriers and are crucially involved in the establishment of directed auxin transport.

In addition to its role in embryonic patterning, *WOX9* was shown to be required for shoot apical meristem maintenance [35] and for maintaining cell division activity during embryonic and post-embryonic development in *A. thaliana* [31], *Solanum esculentum* (tomato) [36] and *P. hybrida* [37]. Transcript profiling in *Brassica napus* (rape) identified *WOX2* and *WOX9* as markers of embryogenesis

[38], indicating that these genes serve as robust markers for (somatic) embryogenesis. The *O. sativa WOX11* ortholog is expressed in the proliferating regions of both shoot and root meristems and functions in crown root development [20]. In *A. thaliana*, the basal WOX-family members *WOX13* and *WOX14* are expressed in primary and lateral roots and floral organs, where they appear to prevent premature differentiation [39].

Mechanism of action

The function of most of the *WOX* genes studied so far can be related to either promotion of cell division and/or prevention of premature differentiation. In a number of cases, mutations in WOX genes cause non-cell-autonomous effects, suggesting that they trigger the production of intercellular signals. In *A. thaliana*, WUS and WOX5 function non-cell-autonomously in stem-cell maintenance [21], and WOX3 in organ initiation [28]. In addition, WOX8 and WOX9 function non-cell-autonomously to regulate the apical domain during embryonic patterning [30]. To explain the non-cell-autonomous functions, the transcriptional activity of the WOX proteins in the nucleus could trigger the production of mobile signals.

WUS activity in the apical meristem is regulated through a regulatory negative feedback loop between the *WUS* and *CLAVATA* (*CLV)* genes [40]. The CLV3 peptide (which belongs to the CLE family) functions as a mobile signal and upon binding to its receptor CLV1, a receptor protein kinase, results in the repression of *WUS* transcription. A *WUS-AGAMOUS* negative feedback loop is involved in floral stem-cell maintenance. WUS activates transcription of the floral organ identity gene *AGAMOUS*, and in turn AGAMOUS (a MADS-box HB transcription factor) is able to repress *WUS* transcription [41,42]. Recently, a putative negative feedback loop involving another member of the CLE family of peptide signals, CLE40, and the ACR4 receptor-like kinase was identified as regulating *WOX5* activity in the *A. thaliana* root in a similar way to the regulation of *WUS* by CLV3 and CLV1 [43], suggesting that similar regulatory mechanisms are responsible for stem-cell maintenance in the shoot and root.

WUS has been shown to directly repress the transcription of several *ARR-A* genes, which encode negative regulators of signaling by the plant hormone cytokinin. AAR-A proteins probably act by competing for phosphorylation with the ARR-B positive regulators of cytokinin signaling - phosphorylation is required for activation of ARR-B transcription factors [44]. Interestingly, WUS can act as both a repressor and an activator of gene expression, and the WUS-box motif is essential for both functions [14]. In contrast, the carboxy-terminal EAR domain, found in WUS, WOX5 and WOX7 (as noted previously), is not essential for transcriptional repression of *WUS* and probably only enhances the repressor activity [14]. The repressor activity of WUS could be partly mediated by the co-repressor protein TOPLESS [45], which interacts with WUS via the carboxy-terminal EAR motif [24]. The EAR domain of WOX5 [12], however, was shown to act as a repressor *in vitro* [14]. Despite not possessing the EAR repressor motif, WOX11 and WOX3 seem to function as repressors; *O. sativa* WOX11 directly represses transcription of *RR2*, which encodes an ARR-A-type negative regulator of the cytokinin response [20], and WOX3 was shown to repress the gene for the YABBY transcription factor (*YAB3*) during leaf development in *O. sativa* [46] and functions as repressor *in vitro* [14]. Therefore, transcriptional repression appears to be a common mode of action for WOX proteins, but both the type of target gene and the functional domain(s) involved in repression appear to differ. Future research will need to unravel which of these motifs have a regulatory function.

Frontiers

The WOX proteins regulate key developmental processes in plants. However, only a subset of the family members has yet been characterized in detail in a small set of seed plants. It will be important to investigate the function of all family members, including the little studied ancient WOX clade, in a broad range of plant species to understand how the distinct functions have evolved. To understand the mechanism by which the WOX family members regulate the expression of their target genes, comprehensive expression analyses are required. While the WOX proteins seem to be able to directly repress transcription of their target genes, the role of the EAR repressor motif and/or interaction with TOPLESS-like co-repressors is still unclear.

In flowering plants, often only a small number of cells express a given *WOX* gene and these cells are relatively inaccessible inside surrounding tissue. Technological developments that enable ‘omics’ approaches with a limited amount of starting material should therefore prove beneficial. Current developments in fluorescence-activated cell sorting enable the isolation of cells expressing specific *WOX* genes [47] for transcriptome studies. The dominant gametophytic generation and the less complex morphology and easier accessibility of stem cells in non-seed plants will also enable future insights, especially into the function of members of the ancient and intermediate clade [48,49].

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