WOX Gene Phylogeny in Poaceae: A Comparative Approach Addressing Leaf and Embryo Development

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The phylogeny based on the homeodomain (HD) amino acid sequence of the WOX (WUSCHEL-related homeobox gene family) was established in the 3 major radiations of the Poaceae family: Pooidaeae (Brachypodium distachyon), Bambusoideae (Oryza sativa), and Panicoideae (Zea mays). The genomes of all 3 grasses contain an ancient duplication in the WOX3 branch, and the cellular expression patterns in maize and rice indicate subfunctionalization of paralogues during leaf development, which may relate to the architecture of the grass leaf and the encircling of the stem. The use of WOX gene family members as molecular markers in maize embryo development for the first time allowed us to visualize cellular decisions in the maize proembryo, including specification of the shoot/root axis at an oblique angle to the apical–basal polarity of the zygote. All molecular marker data are compatible with the conclusion that the embryonic shoot/root axis comprises a discrete domain from early proembryo stages onward. Novel cell fates of the shoot and the root are acquired within this distinct morphogenetic axis domain, which elongates and thus separates the shoot apical meristem and root apical meristem (RAM) anlagen in the maize embryo.

Introduction

Angiosperms, flowering plants, are the largest group of embryophytes with at least 260,000 living species. They consist of 2 classes—the monocotyledons and the dicotyledons—which diverged over 150 MYA (Wikstrom and Kenrick 2001). Despite their striking diversity, angiosperms share several synapomorphies (Doyle and Donoghue 1986). For example, sexual reproduction is characterized by a double fertilization event where one sperm cell fuses with the egg cell that will develop into the embryo and the second fuses with the binucleate central cell to give rise to the endosperm. Whereas endosperm development starts with a syncitial phase, the zygote follows a defined cell division program (Johansen 1950; Wardlaw 1955). The first division of the zygote is asymmetric and always perpendicular to the endosperm. Whereas endosperm development starts with a syncitial phase, the zygote follows a defined cell division program. The genomes of all 3 grasses contain an ancient duplication in the WOX3 branch, and the cellular expression patterns in maize and rice indicate subfunctionalization of paralogues during leaf development, which may relate to the architecture of the grass leaf and the encircling of the stem. The use of WOX gene family members as molecular markers in maize embryo development for the first time allowed us to visualize cellular decisions in the maize proembryo, including specification of the shoot/root axis at an oblique angle to the apical–basal polarity of the zygote. All molecular marker data are compatible with the conclusion that the embryonic shoot/root axis comprises a discrete domain from early proembryo stages onward. Novel cell fates of the shoot and the root are acquired within this distinct morphogenetic axis domain, which elongates and thus separates the shoot apical meristem and root apical meristem (RAM) anlagen in the maize embryo.

Key words: WOX gene family, Poaceae, embryo patterning, leaf development.

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Table 1
Primer Pairs to Amplify WOX HD of Brachypodium distachyon (Bd)

<table>
<thead>
<tr>
<th>Degenerate Forward Primer (5’–3’)</th>
<th>Degenerate Reverse Primer (5’–3’)</th>
<th>Resulting HD</th>
</tr>
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<tbody>
<tr>
<td>TGGAAYCCYACIGCARCARAT</td>
<td>GCTTTRGRTTYTGRAACCCARAA</td>
<td>BdWOX13</td>
</tr>
<tr>
<td>TGGTYGACICCIACIGCARCARAT</td>
<td>GCTTTRGRTTYTGRAACCCARAA</td>
<td>BdWOX12</td>
</tr>
<tr>
<td>GGGIACIACICTGGAAYCC</td>
<td>GCTTTRGRTTYTGRAACCCARAA</td>
<td>BdWOX11</td>
</tr>
<tr>
<td>TGGACICCIACIACICGARCARAT</td>
<td>GCTTTRGRTTYTGRAACCCARAA</td>
<td>BdWOX10</td>
</tr>
<tr>
<td>TGGAAYCCYACIGCARCARAT</td>
<td>GCTTTRGRTTYTGRAACCCARAA</td>
<td>BdWOX9</td>
</tr>
<tr>
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<td>GCTTTRGRTTYTGRAACCCARAA</td>
<td>BdWOX8</td>
</tr>
<tr>
<td>CCIAARCCICGITGGAAYCC</td>
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<td>GCTTTRGRTTYTGRAACCCARAA</td>
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</tr>
<tr>
<td>TGGACICCIACIACICGARCARAT</td>
<td>GCTTTRGRTTYTGRAACCCARAA</td>
<td>BdWOX3</td>
</tr>
</tbody>
</table>

During the transition stage of the maize embryo (Smith et al. 1995). The activation of KN1 when the maize embryo consists of several hundred cells is rather late compared with that of STM in Arabidopsis, which is activated at the 32-cell stage (Long and Barton 1998). Although later in development, the gene expression patterns appear to be conserved, there is no prepatterning of the prospective SAM domain in the maize embryo. A root endodermis marker is provided by ZmSCR, the maize orthologue of SCARECROW in Arabidopsis in the coleoptilar stage embryo (Lim et al. 2005). The SAM and RAM transcriptional markers show perception of adaxial and central positional information for establishment of the shoot/root axis in the maize embryo.

In Arabidopsis, members of the WUSCHEL homeobox (WOX) gene family comprise earliest cell fate markers from the first zygotic divisions on Haecker et al. (2004). Most members of the maize WOX gene family have been identified in the course of the phylogenetic identification of WUS orthologues from rice and maize (Nardmann and Werr 2006). Their analysis has revealed major adaptations in the WOX family phylogeny suggesting significant freedom for the specification of the shoot stem cell niche during angiosperm evolution. However, the WOX gene phylogeny also revealed that this gene family existed prior to the separation of monocot and dicot species. Based on pioneering work in Arabidopsis, members of the WOX gene family, therefore, were attractive candidates to be tested as molecular markers of embryonic patterning in maize. Here we describe the transcription patterns of patterning WOX gene family members in the maize embryo. They uncover a significant degree of pattern conservation between maize and Arabidopsis embryos and reveal that the shoot/root axis in maize comprises a rather discrete domain from the proembryo stage on, which is distinct from that of the prospective scutellum.

Materials and Methods

WOX Family Members from Brachypodium distachyon and Computational or Database Analysis

Homeobox-encoding amplicons of the Brachypodium distachyon WOX family members were obtained in polymerase chain reactions (PCRs) on genomic DNA with primers listed in table 1. Analysis of DNA and protein sequences was performed using the Wisconsin GCG software package version 7.0 (University of Wisconsin Genetics Computer Group). Homology searches used TBLastN at http://www.ncbi.nlm.nih.gov/blast/, http://www.ddbj.nig.ac.jp, or http://www.gov/blast.org with default parameters. Multiple sequence alignment was achieved using ClustalW (http://www.ebi.ac.uk/protovo/). PHYLIP (http://www.phylip.com), was used for phylogenetic and molecular evolutionary analyses based on the maximum likelihood method. MEGA version 3.1 (Kumar et al. 2001) was used for phylogenetic and molecular evolutionary analyses based on the Neighbor-Joining (NJ) and maximal parsimony (MP) methods. Sequences selected for the phylogenetic tree in figure 1 are as follows (accession numbers in parentheses): ZmNS1 (AJ536578), ZmNS2 (AJ472083), and OsWOX5 (Q8WOF1); AtWOX1—AtWOX14 as published recently (Haecker et al. 2004); and all other accession numbers are listed in table 2.

In Situ Hybridization

Nonradioactive in situ hybridization followed the protocol of Jackson (1991). For fixation, maize kernels were trimmed on both sides of the embryo axis; nuclellar explants for early embryos were obtained 45–48 h after pollination. Paraffin-embedded tissue was sectioned by the use of the Leica RM 2145 rotary microtome (7 μm). Probes for in situ hybridization comprising sequences downstream of the homeobox (table 3) were cloned in sense or antisense orientation to the T7 promoter, and digoxigenin-labeled RNA probes were obtained as described by Bradley et al. (1993). The KN1 probe corresponded to accession AY312169 (Vollbrecht et al. 1991).

Reverse Transcriptase–Polymerase Chain Reaction Analysis

For expression analysis of ZmWOX2A and ZmWOX9A-C, the μMACS One-step cDNA Kit was used for isolation of total RNA from egg cells and zygotes and for subsequent cDNA synthesis according to the manufacturer’s protocol (Qiagen, Hilden, Germany). Total RNA of later embryonic stages was isolated using the RNeasy plant mini kit according to the manufacturer’s protocol (Qiagen, Hilden, Germany). Two micrograms of DNase I-treated RNA was used as a template for the first-strand cDNA synthesis with SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). Subsequent PCRs were performed using standard procedures with 35 or 40 cycles of amplification. ZmWOX2A primers fw (5’-CCGCTGCCCCGTGCTTCCATG) and rev (5’-GATCGGCCACAGGGCTTCCTCCAG) (Tm = 59°C, 40 cycles);
FIG. 1.—Phylogeny of the WOX gene family in 3 grasses compared with dicots. Abbreviations are Arabidopsis thaliana (At), Populus trichocarpa (Pt), Brachypodium distachyon (Bd), Oryza sativa (Os), and Zea mays (Zm). ROA (ROSULATA) and TER (TERMINATOR) are WUS orthologues from Antirrhinum majus or Petunia hybrida, respectively. The complete dendrogram (right) was obtained by the maximum likelihood method; the blow ups of the WOX3 and WOX8/9 branches are based on the NJ method, which results in a different branching pattern in the PRS/NS group and in the positions of Arabidopsis WOX8 or WOX9 members. Bootstrap values are indicated at each branching.
ZmWOX9A fw (5'-GATCAACCATCAGCACCACAAGC) and rev (5'-AATCGGCACAAACACCTA) (T_m = 59 °C, 35 cycles); ZmWOX9B fw (5'-CGGCAAGAA-CAACAACACC) and rev (5'-GCCAGCTGATACCCTA-CAAC) (T_m = 59 °C, 35 cycles); ZmWOX9C fw (5'-GTCAGGCCTCCAGGAAACC) and rev (5'-CCCCACG-CTGACTTCTCTC) (T_m = 57 °C, 35 cycles). Reverse transcriptase–polymerase chain reaction (RT–PCR) on the GAPDH cDNA transcript with primers GAPDH fw (5'-CATTCTAGCAGCACC-GTG) and rev (5'-CAAGCAGCAACCATCCATGAG) served as a control.

Egg Cell and Zygote Isolation

All plant materials were isolated from line A 188. Unfertilized egg cells were isolated and selected as described in Kranz (1999). In vivo fertilized egg cells were isolated as described in Okamoto et al. (2005). It was estimated that fertilization occurred 17–20 h before zygote isolation. After washing in mannitol solution (0.650 osmolar), cells were transferred into tubes, snap frozen in liquid nitrogen, and stored at −80 °C until use.

Light Microscopy and Image Processing

Images were taken using an Axioskop microscope equipped with an Axiocam camera (Zeiss, Oberkochen, Germany). Pictures were processed using Adobe Photoshop version 7.0.

Results

Phylogeny of WOX HDs

The WOX phylogenetic tree based on the homeodomain (HD) amino acid sequence (fig. 1) contains members of the 3 major radiations of the Poaceae family: Pooideae (B. distachyon; Bd), Bambusoideae (Oryza sativa; Os), and Panicoideae (Zea mays; Zm). Arabidopsis thaliana, Populus trichocarpa, and O. sativa members were obtained from the established genome sequences. The isolation of maize WOX gene family members was described recently (Nardmann and Werr 2006). WOX gene sequences of B. distachyon were newly isolated by PCR approach with degenerate primers targeting conserved polypeptide sequences in WUS or WOX HDs. The informative HD sequences are 41 amino acid long in WOX proteins or 42 amino acid in WUS orthologues due to an extra Y residue. The phylogenetic tree (fig. 1A) was calculated by the maximum likelihood method (http://www.evolution.genetics.washington.edu/phylip.html), although branches in the WOX3 and WOX8/9 subgroup are more consistently resolved by the NJ or MP methods (Kumar et al. 2001). The duplication of the maize genome is evident as 2 maize paralogues are often accompanied by only a single rice or Brachypodium orthologue in individual subbranches (WUS, WOX2, WOX5, or WOX13).

The situation is different in the WOX branch; PRS in Arabidopsis and the ZmNS1/ZmNS2 paralogues represent homologous functions single orthologous genes exist in rice (OsNS) and in Brachypodium (BdNS). However, in contrast to Arabidopsis, the grass genomes contain further members, which root outside of the PRS/NS branch. This duplication existed prior to the allotetraploidization of the maize genome because 2 maize paralogues (ZmWOX3A/B) relate to a single rice (OsWOX3) or Brachypodium (BdWOX3) orthologue. Significant divergence also exists in the WOX8/9 branch; no close AtWOX8 relative exists in Populus or grasses, but all genomes contain A1WOX9 orthologues. Close relatives to A1WOX1 or A1WOX6 are absent in grasses, whereas orthologues are found in Populus. However, the Populus and the grass genomes lack orthologues to A1WOX10/A1WOX14, which indicates a branch unique to the Arabidopsis lineage. In contrast, A1WOX3/A1WOX7 or A1WOX11/A1WOX12 are close relatives in Arabidopsis but root with orthologues in Populus or grasses and therefore may occupy ancient branches in the WOX gene family.

ZmWOX2A Expression Prepatterns the SAM Position and Shifts from an Apical to a Lateral Position in the Embryo Proper

In Arabidopsis, AtWOX2 provides a molecular marker for the apical domain of the globular embryo, which gives
rise to the SAM (Haecker et al. 2004). Gene expression becomes restricted to the apical cell of the 2-cell stage embryo and is later confined to the upper cell tier in the 8-cell embryo. RNA in situ hybridization experiments on the maize embryo revealed that ZmWOX2A is transcribed in the apical domain of the early embryo proper (45–48 h after fertilization to 4 days after pollination (dap); fig. 2A). Slightly later, ZmWOX2A expression becomes confined to a few cells at a lateral position of the proembryo facing toward the axis of the ear (fig. 2C). At the late proembryo/early transition stage, the expression domain shifts more to the base of the embryo and transcripts become restricted to the L1 layer (fig. 2D). Transcriptional activity ceases thereafter, which coincides with the activation of KN1 transcription in a lateral domain of the transition stage embryo (Smith et al. 1995). ZmWOX2A transcripts are again detected when the SAM has been established in leaf stage embryos. Transcripts accumulate in the outer cell layers of the SAM predominantly lateral to the apical tip (fig. 2E). Such hybridization signals are only observed in a subtraction of tissue sections, indicating that ZmWOX2A transcriptional activity is transient, possibly relating to the initiation of new leaf primordia. In analogy to Arabidopsis, we also tested for transcription of ZmWOX2A in the egg cell and the zygote by performing RT–PCR experiments. ZmWOX2A transcripts were found in the zygote, but in contrast to AtWOX2 expression in Arabidopsis (Haecker et al. 2004), transcripts were not detected in the unfertilized egg cell (data not shown), therefore, ZmWOX2A expression is zygotic.

Expression Patterns of ZmWOX5 Paralogues and Root Development

The maize genome contains 2 WOX5 paralogues, ZmWOX5A and ZmWOX5B. Based on the transcriptional activity in the root QC, an orthologue to AtWOX5 is encoded by the ZmWOX5B gene (fig. 3A–C). Expression is first detected in a central domain of the embryo proper above the uppermost tier of vacuolized suspensor cells at the proembryo stage, when ZmWOX2A is already expressed in a lateral adaxial domain (fig. 3A). During later stages of embryo development, ZmWOX5B is transcribed in cells of the QC. However, in contrast to ZmSCR, which is transcribed in the endodermis and in a single row of cells through the maize root QC (Lim et al. 2005), the ZmWOX5B expression domain initially extends into the tissue above (fig. 3C). The position of this ZmWOX5B domain in the coleoptilar stage embryo is striking relative to the expression pattern of KN1. Side-by-side RNA in situ hybridizations show that the ZmWOX5B domain is located internally to an inverted cup-shaped KN1 expression domain (fig. 3C and D). This KN1/ZmWOX5B expression domain comprises the entire shoot/root axis of the maize embryo from the L2 layer of the SAM towards the root QC. Later in the elaborated root of the maturing embryo or the vegetative seedling, ZmWOX5B transcripts mark not only a single row of cells in the root QC but also the base of provascular bundles specified immediately above the QC (fig. 3B). ZmWOX5B in maize is expressed outside the root QC in the cambial cells of the basal vascular system, differently to AtWOX5 in Arabidopsis and QHB in rice (Kamiya et al. 2003; Haecker et al. 2004).

The ZmWOX5A paralogue has acquired a completely different expression pattern and is not detected in the embryo prior to early leaf stage 1. Expression starts in the suspensor region in 4–6 cell layers below the root QC (fig. 3E), the intervening cells express the coleorhiza/calyptrogon marker ZmNAC5 (Zimmermann and Werr 2005). Although the coleorhiza delaminates from the primary embryonic root laterally, it remains connected to the subtending embryonic suspensor at its basal end. The ZmWOX5A gene is transcribed in cells subtending the central coleorhiza domain, which forms a continuous cell array with the root (fig. 3F). Concerning the function of these cells in the suspensor, the transcriptional activity of ZmWOX5A in the early endosperm may provide a hint. Prior to the expression in the embryo, high levels of ZmWOX5A transcripts are transiently detected in the endosperm shortly after cellularization of the basal transfer layer (fig. 3G). This specialized endosperm domain is essential for nutrient supply from
the transverse sections compared in ZmWOX5B exclusive expression patterns of longitudinal sections (G are located 4–6 cell layers below the root QC (asterisk). (H) suspension of leaf stage 1 or leaf stage 4 embryos. Both expression domains (E and F) ZmWOX5A transcription in the suspensor of leaf stage 1 or leaf stage 4 embryos. Both expression domains are located 4–6 cell layers below the root QC (asterisk). (G) ZmWOX5A expression in the basal transfer layer of the 9 dap endosperm, and (H) ZmWOX5A expression in the rhizodermis. Frames in the schematic representations (E–G) indicate the depicted area of the photographs.

maternal into the filial tissue, that is, endosperm and embryo (Hueros et al. 1995). By analogy, ZmWOX5A transcriptional activity in the suspensor could indicate a cell type specialized for acropetal transport through the root into the embryonic plantlet. In the seedling, ZmWOX5A expression is detectable in the rhizodermis, which is involved in uptake of ions and water from the rhizosphere into the root (fig. 3H).

**ZmWOX4 Expression Marks Provascular Cells of the Hypocotyl**

Similar to ZmWOX5A in the embryo, ZmWOX4 expression is first detectable during leaf stage 1. Transcription is confined to the morphogenetic axis in the maize embryo and starts in a broad domain between the SAM and RAM (fig. 4A). During later leaf stages, transcripts are restricted to the vascular system, marking vascular strands at the height and above the scutellar node (fig. 4B). No expression was detected in the emerging vascular system at the base of the root, which is marked by ZmWOX5B expression or in the prominent vascular strand connecting the embryonic axis with the scutellum. ZmWOX4, therefore, provides a marker specific for the vascular system of the hypocotyl.

As the expression pattern of the Arabidopsis orthologue ArWOX4 has not been reported previously, we analyzed its transcriptional activity in the dicot embryo. The resulting expression pattern is similar to that described for ArWOX1, although both HDs, AtWOX4 and AtWOX1, group into distant subbranches of the WOX phylogenetic tree (fig. 1). ArWOX4 activity is detected at the apical tip of the cotyledons in heart stage Arabidopsis embryos (fig. 4C) for a short interval and ceases thereafter. The At-WOX4 and ZmWOX4 expression patterns exhibit no obvious similarities but ZmWOX4 marks provascular strands in the prospective hypocotyl domain of the shoot root axis.

**The WOX3 Branch and Grass Leaf Development**

All 3 grass genomes contain WOX3 relatives, which root outside of the PRS/NS branch (Matsumoto and Okada 2001; Nardmann et al. 2004; Nardmann and Werr 2006). Expression analyses here focus on the newly identified branch members. Gene-specific RT–PCR experiments revealed that both maize paralogues, ZmWOX3A and ZmWOX3B, are transcribed. However, the sequence similarity between both maize paralogues is so high that we used a cross-hybridizing ZmWOX3A probe to establish the cellular expression pattern. ZmWOX3A/B transcripts are first detected in the anlage of the coleoptile where the marginal cell file is marked (fig. 5A). This ZmWOX3A/B expression pattern is reminiscent of that of ZmNS1 expression (Nardmann et al. 2004) and may explain why ns1/ns2 double mutants exhibit no phenotype in the coleoptile other than in leaves and leaf-like floral organs.

During later stages of embryogenesis and during the vegetative phase, ZmWOX3A/B expression is detected in the margins of developing true foliage leaves, another aspect shared with ZmNS1/NS2 (fig. 5B). However, significant differences exist between the expression patterns of both gene pairs in the SAM. ZmWOX3A/B transcription marks a ring-shaped expression domain of cells recruited for the P₀/P₁ leaf primordium (fig. 5B–D). In addition, ZmWOX3A/B are transcribed at the base of developing
phytomers opposite to the midrib where the axillary meristem resides and intermingling leaf margins are attached to the node (compare side-by-side sections in fig. 5E). Similar expression patterns were obtained with the rice orthologue OsNS1. (A) In leaf stage 4 embryos, ZmWOX4 transcripts are restricted to the vascular system. (C) Expression of the Arabidopsis orthologue AtWOX4 is confined to the coteddons of heart stage embryos; the maize and Arabidopsis patterns show hardly any similarity.

**Discussion**

The WOX Gene Family Is Ancient in the Angiosperm Lineage

The grass part of the WOX phylogenetic tree (fig. 1) contains members of the 3 major radiations of the Poaceae family: Pooideae (B. distachyon), Bambusoideae (O. sativa), and Panicoideae (Z. mays). The tree is therefore representative for true grasses or Gramineae, which comprise 600 genera and between 9,000 and 10,000 species. Regarding dicots, the tree contains WOX family members from A. thaliana and P. trichocarpa and because both genomes have been sequenced should provide the whole WOX gene complement for 2 eudicot species, as is the case of WOX family members in rice.

The phylogeny reveals significant differences between the 3 grass and the 2 eudicot species. There is no evidence that grass genomes contain paralogues with WOX1, WOX6, WOX7, and WOX10. In contrast, orthologues of WOX1, 6, and 7 are found in the genome of P. trichocarpa. Populus and Arabidopsis both belong to the core eudicots but are members of the Salicaceae and Brassicaceae group, respectively, and fall into euroids I and II. The WOX1, 6, or 7 orthologues in both species possibly indicate a common ancestry, consistent with the assumption that rosids comprise a monophyletic group (Jansen et al. 2006). As euroids I and II are the major radiations among rosids, the differences concerning WOX10 and 14 family members may relate to the depth of branching between both clades but this would need substantiation from other clade members. In contrast, the homogeneity among grasses implies few changes between Pooideae, Bambusoideae, and Panicoideae. This is consistent with estimations that Poales represent a rather young monophyletic group dating back to the late Cretaceous (>65 Myr; Bremer 2002), whereas the first undisputed evidence of angiosperms appears in the fossil record of the early Cretaceous period with a rapid diversification in the mid-Cretaceous (approximately 100 MYA).

Despite a possible loss of individual WOX family members, all 3 grass genomes contain an interesting duplication in the WOX3 subbranch. The rice and Brachypodium genomes both contain 2 WOX3 members, whereas the maize genome encodes 2 pairs of paralogues. The duplication in the WOX3 branch, therefore, existed prior to the
duplication of the maize genome. For maize and rice, it has been shown that\(\text{NS}\) orthologues and additional\(\text{WOX3}\) relatives both relate to leaf development but are expressed in different domains. These expression patterns provide evidence that the\(\text{WOX3}\) duplication could be preserved by subfunctionalization (Lynch and Force 2000), possibly relating to the peculiar architecture of the grass leaf, with a basal sheath enclosing the stem. Suggestively,\(\text{PRS}\) and\(\text{ZmNS1/2, OsNS, and BdNS}\) group in a unique branch of the phylogenetic tree, whereas the grass-specific\(\text{WOX3}\) paralogues do not find an obvious counterpart in eudicots.

**Early Embryonic Patterning in Maize**

The isolation of maize\(\text{WOX}\) genes was performed to raise molecular markers for patterning of the grass embryo. The RNA in situ hybridization analyses presented here show that\(\text{WOX}\) family members provide such markers on the transcriptional level and allow the comparison to cellular decisions in the\(\text{Arabidopsis}\) embryo (fig. 7).\(\text{ZmWOX}\) transcriptional gene activity was absent in the egg cell but starts in the zygote with\(\text{ZmWOX2A}\). From the few-celled stage onward, transcripts are exclusively detected in the apical embryo proper domain. This expression domain is maintained throughout most of the proembryo stage before the transcriptional activity becomes confined to the adaxial face of the embryo proper, where the SAM will later emerge. Ultimately, at the late proembryo/early transition stage,\(\text{ZmWOX2A}\) transcription is restricted to the L1 layer at the adaxial side of the embryo. In relation to\(\text{Arabidopsis}\), it is striking that both\(\text{ZmWOX2A}\) and\(\text{AtWOX2}\) are active in the zygote, mark the apical cell fate and prepattern the position of the SAM (fig. 7). This similarity of expression patterns makes it tempting to consider that\(\text{ZmWOX2A}\) and\(\text{AtWOX2}\) represent orthologous gene functions. However, this conclusion implies that\(\text{WOX2}\) provides an ancient angiosperm function associated with the embryo proper cell
fate and SAM anlage prior to the separation of monocots and dicots. The confinement of transcription from an early pattern throughout the embryo proper to the adaxial face might be interpreted that dorso/ventral polarity does not exist in the early proembryo but is established later.

The second marker, which is informative in a temporal and spatial pattern, is ZmWOX5B; it is activated in central cells at the base of the embryo proper in the 6 dap proembryo. The expression pattern is dynamic during subsequent stages of embryo development. However, restriction of expression to the root QC implies that ZmWOX5B expression marks the anlage of the root meristem. ZmWOX5B expression appears later than that of ZmWOX2A, and this activation coincides with the start of the restriction of the ZmWOX2A transcription domain to the adaxial face of the embryo proper. Whereas ZmWOX2A interprets adaxial positional information, the early pattern of ZmWOX5B expression indicates the perception of centro/radial information. Based on 2 ZmWOX markers and on ZmSCR (Lim et al. 2005), the RAM and SAM anlagen seem to be established coordinately in time and at an oblique angle relative to the initial apical/basal polarity of the zygote.

The Root/Shoot Axis, a Discrete Embryo Domain

The adaxial ZmWOX2A and the central ZmWOX5B expression domains comprise a distinct centrolateral domain of the embryo proper, whereas the prospective scutellum fate at the abaxial face is marked by ZmDRN transcription (Zimmermann and Werr 2007). The view that already in the proembryo the root/shoot axis is set apart from the rest of the embryo, for example, suspensor and scutellum, gains support from the KN1 expression pattern. KN1 is activated at a late proembryo/early transition stage at the adaxial face of the embryo, when ZmWOX2A transcription ceases in the L1 layer (Smith et al. 1995). In contrast to STM, which is considered the orthologous gene function in Arabidopsis (Kerstetter et al. 1997), KN1 expression is not only confined to the SAM of the maize embryo but also transcription extends centrally and basally toward the boundary between embryo proper and suspensor (fig. 7). At the coleoptilar stage, the KN1 expression domain comprises an inverted cup-shaped domain, enclosing the ZmWOX5B expression domain, which becomes confined to the QC and basal provascular strands during subsequent developmental stages. In the basal cell layer of the ZmWOX5B transcription domain, ZmSCR is coexpressed and overlaps with the KN1 expression domain in the periphery (data not shown).

At this intermediate stage of primary embryonic axis development, all cells of the prospective root/shoot axis—from SAM (except the L1 layer) to QC—express either KN1 or ZmWOX5B and thus based on transcriptional molecular markers are different from adjacent scutellum cells. Starting with the restriction of ZmWOX2A expression to the...
adaxial embryo proper domain and the activation of ZmWOX5B, the root/shoot axis of the maize embryo comprises a discrete domain, which elongates and where KN1 activity may indicate a meristematic ground state. From an initially broad transcription pattern inside this KN1 domain, expression of ZmWOX4 focuses to provascular strands, similar to ZmWOX5B in subtending cells above the root QC. The ZmWOX4 and ZmWOX5B patterns in the embryo are nonoverlapping and according to the position of the scutellar node may correspond to the hypocotyl and primary root of the maize seedling.

The first evidence for further differentiation of the SAM is provided by ZmWOX3 paralogues. Together with ZmNS1 (Nardmann et al. 2004), the grass-specific ZmWOX3A/B paralogues are expressed in marginal cells in the emerging coleoptile above the prospective SAM. This coexpression of NS1/ZmWOX3A/B may indicate functional redundancy and may explain why ns1/ns2 double mutants are aphytotic in the coleoptile. However, the expression patterns differ largely during leaf development. Whereas ZmNS1/2 activity in the shoot apex is restricted to small foci where lateral leaf domains are initiated and will detach from the apex (Nardmann et al. 2004), ZmWOX3A/B expression marks a ring of SAM cells recruited for the P0 primordium. Expression starts and ceases first at the midrib position of a new leaf primordium and extends to the opposite face of the SAM, where the axillary meristem is initiated. In median longitudinal sections through the maize seedling, consequently, 1 or 2 foci of expression become evident at the flank of the SAM (fig. 5B), which correspond to different stages during P0 development. In contrast to ZmNS1/2 transcripts, which are restricted to basal lateral margins, transcription of ZmWOX3A/B is also detected in the apical domain of the P1 leaf (fig. 5D). Although we have not analyzed the pattern of OsNS and OsWOX3 in the rice embryo, all the seedling patterns consistently agree with the maize data, except that maize has 2 paralogues, whereas rice has a single orthologue. It should be noted that in addition to ZmNS1/2 and ZmWOX3A/B also ZmWOX9B is transiently expressed at the flank of the embryonic SAM. The anlage of the complex maize leaf around the SAM circumference, therefore, involves the activity of at least 5 ZmWOX genes, not taking into consideration the 2 maize WUS paralogues ZmWUS1 and ZmWUS2 which may contribute to leaf development (Nardmann and Werr 2006). Only ZmWUS1 is transiently expressed in the embryo, at the end of coleoptilar stage, when the SAM starts to initiate leaves.

Presently there is no evidence for ZmWOX gene activity in the scutellum, whereas 12 ZmWOX genes are transiently expressed in the embryonic shoot/root axis or the subtending suspensor. This is in contrast to the development of the cotyledons in Arabidopsis, where several WOX family members are expressed (Haeker et al. 2004, fig. 4). Beside ZmNS1 or ZmWOX3A/B and AtWOX3/PR5, which only share marginal expression patterns in coleoptile and cotyledons (Nardmann et al. 2004), also AtWOX1 and 4 are expressed in the cotyledons. Whereas the expression patterns established here demonstrate that ZmWOX genes may contribute to various aspects of shoot/root axis development pattern in the maize embryo, there seems no contribution in the scutellum. This relates to the controversial and long-standing discussion whether the cotyledons and the scutellum represent orthologous organs (Weatherwax 1920).

However, the alternative hypothesis that the scutellum may be an invention of grasses and consequently may find no counterpart in dicot embryos has also been raised (Reeder 1953). The ZmWOX expression data support this view as the WOX patterns shared between the Arabidopsis and the maize embryo involve only the shoot/root axis including the coleoptile of the grass embryo. Strikingly, the WOX2/5 phylogeny and the patterns of ZmWOX2A and ZmWOX5B relative to those in Arabidopsis suggest that the embryonic axis is specified early, in an adaxial–central domain of the embryo proper. Further, cell-type specifications in this shoot/root axis involve WOX family members in maize as is observed during globular–heart stage transition in the Arabidopsis embryo including the cotyledons (see fig. 7). The ZmWOX data, therefore, suggest that during the early proembryo stage, the maize embryonic proper is regionalized into 2 domains; the adaxial shoot/root axis and an abaxial domain determined to be the scutellum. Both the early discrete scutellum domain and the absence of WOX gene activity, which is detected in the cotyledons or the coleoptile of Arabidopsis and maize embryos, respectively, support the initial assumption that the scutellum may be an organ without counterpart in dicot embryos and thus a new acquisition of grasses.

In conclusion, analysis of the WOX gene family reveal interesting phylogenetic differences between monocots and dicots and confirm that individual family members provide sophisticated transcriptional markers for plant development. The use of maize WOX gene family members as molecular markers in maize embryo development allowed us to visualize cellular decisions in the maize proembryo for the first time, including specification of the shoot/root axis at an oblique angle to the apical–basal polarity of the zygote. All molecular marker data are compatible with the conclusion that the embryonic shoot/root axis comprises a discrete domain from the early proembryo stage on. All cell fates of the shoot and the root are established within this distinct morphogenetic axis domain, which elongates and separates the anlagen of SAM and RAM in the course of embryo development.

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Literature Cited


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