Expression dynamics of *WOX* genes mark cell fate decisions during early embryonic patterning in *Arabidopsis thaliana*

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Summary

During embryonic pattern formation, the main body axes are established and cells of different developmental fates are specified from a single-cell zygote. Despite the fundamental importance of this process, in plants, the underlying mechanisms are largely unknown. We show that expression dynamics of novel *WOX* (WUSCHEL related homeobox) gene family members reveal early embryonic patterning events in *Arabidopsis*. *WOX2* and *WOX8* are co-expressed in the egg cell and zygote and become confined to the apical and basal daughter cells of the zygote, respectively, by its asymmetric division. *WOX2* not only marks apical descendants of the zygote, but is also functionally required for their correct development, suggesting that the asymmetric division of the plant zygote separates determinants of apical and basal cell fates. *WOX9* expression is initiated in the basal daughter cell of the zygote and subsequently shifts into the descendants of the apical daughter apparently in response to signaling from the embryo proper. Expression of *WOX5* shows that identity of the quiescent center is initiated very early in the hypophyseal cell, and highlights molecular and developmental similarities between the stem cell niches of root and shoot meristems. Together, our data suggest that during plant embryogenesis region-specific transcription programs are initiated very early in single precursor cells and that *WOX* genes play an important role in this process.

Key words: *WOX* genes, *Arabidopsis* embryogenesis, Pattern formation

Introduction

During embryo development in plants a relatively simple seedling is formed consisting of a shoot apical meristem, cotyledons, hypocotyl, embryonic root and root meristem along its apical-basal axis. In *Arabidopsis*, the origin of these pattern elements can be traced back to early embryo stages because of an almost invariant cell division pattern. Hence the fate of single cells in the early embryo can be followed to determine their specific contribution to the seedling body.

How do different cell fates originate from a single celled zygote and how are they integrated into a meaningful context? Expression studies indicated that molecular differences between cells are established relatively early in embryo development (Lu et al., 1996; Weterings et al., 2001). However, it is clear from a large body of evidence that plant cells acquire their fate irrespective of their clonal origin. Rather they are specified by signals at their current position. In this regard, auxin signaling appears to play an important role during early embryonic patterning (Jürgens, 2001). The distribution of auxin within the embryo appears to require directional transport involving putative auxin transporters of the PINFORMED (PIN) family (Friml et al., 2002; Gälweiler et al., 1998; Hadfi et al., 1998; Steinmann et al., 1999). Polar localization of PIN1 at one side of the cell is established by intracellular vesicle transport mediated by GNOM/EMB30 (GN) activity and predicts the directionality of auxin flow (Busch et al., 1996; Geldner et al., 2001; Shevell et al., 1994; Steinmann et al., 1999). Mutations in the *GN* gene result in cellular mislocalization of PIN1 and embryos that variably have lost various aspects of apical-basal patterning (Mayer et al., 1993). In addition to its regulated transport, local auxin response is also important for embryo patterning. For example, normal root development requires specific auxin response in the embryo proper, mediated by *MONOPTEROS* (MP) and *BODENLOS* (BDL) functions, and subsequent signaling to the hypophyseal cell (Hamann et al., 2002; Hardtke and Berleth, 1998).

Despite these findings, the question of when and how cells become different and which mechanisms govern embryonic pattern formation remains largely elusive. Further important genes regulating early embryonic pattern formation might have been overlooked genetic mutant screens because of genetic redundancy. This is exemplified by findings that specification of epidermal cells and patterning of the shoot apex are controlled by redundant pairs of genes and only double mutants display informative phenotypes, but single mutants do not (Abe et al., 2003; Aida et al., 1997).

We have therefore used a genomic approach to analyze early events in embryonic patterning and searched for homeodomain transcription factor genes that are expressed in a manner...
suggestive of a specific role in this process. This approach was based on the observation that members of homeobox gene families in diverse organisms are involved in the regulation of similar developmental processes. For example, members of the HOX gene family are expressed in specific regions of animal embryos and play a major regulatory role during early pattern formation (Krumlauf, 1994).

We have chosen WUSCHEL (WUS) related genes as an entry point, since WUS is expressed very early in precursor cells of the shoot meristem primordium and plays an important role in regulating cell fates during embryonic shoot meristem formation (Brand et al., 2002; Laux et al., 1996; Mayer et al., 1998). We describe the WUS gene family and show that the expression dynamics of several family members reveal events in early embryonic patterning. We also show that one member, WOX2, is functionally required for correct development of the apical domain of the embryo.

Materials and methods

DNA isolation

RNA harvested from inflorescence and silique material was reverse-transcribed using the Superscript Kit (BRL). Using specific primers, an 850 bp WOX2 cDNA fragment was amplified with WOX2-S (ctcggatccgatggctgga) and WOX2-AS (ctaaatctgattcattgaaatatcctctc), a 613 bp PRS/WOX3 cDNA fragment was amplified with PRS-S1 (atgatcctggtggctgga) and PRS-AS1 (atgggagatgtgatcctctc) and a 1027 bp WOX8 cDNA fragment was amplified with primer WOX8-S (tatcaccatcatgtttgcc) and WOX8-AS (tacaggagctctctgtcattg). A 590 bp WOX5 cDNA fragment was amplified with F11-S (gataaagcttggagactattcctg) and F11-AS (gtgaagctattcattgctctac). Additional cDNAs were isolated by screening a phage cDNA library generated from inflorescence material (Weigel et al., 1992) with genomic probes.

Genomic WOX1 DNA was amplified with primers WOX1-S (ctcggatccgatggctgga) and WOX1-AS (ctcggatccgatggctgga), and genomic WOX9 DNA with primers WOX9-S (cgaattcgattcattgaaatatcctctc) and WOX9-AS (ctcggatccgatggctgga). All DNA fragments were subcloned into the PGEM-T® vector from Promega or the pBluescript II SK– vector from Stratagene. The WUS gene in Arabidopsis was sequenced to confirm the position of the insertion.

All hybridization probes, the WUS probe (taacggattcattcattcattgaatcattcattg), the complete 850 bp probe and the homeodomain-deleted 442 bp probe from position 408-850, gave the same expression pattern. A 1461 bp WOX9 DNA fragment (1-1461) and a 717 bp probe excluding the homeodomain (position 744 to 1461) gave the same expression pattern.

Plant work

Plant growth and phenotypic embryo analysis by DIC microscopy were performed as described previously (Laux et al., 1996). The wox2-1 line was obtained from the Arabidopsis Knockout Facility of the University of Wisconsin and the insertion was detected by PCR using the primer m-s (aattacaggtttgagcagtaga) and m-as (gtaaaagagttgagcagtaga). Following the protocol of the provider, the wox2-2 line was obtained from the Torrey Mesa Research Institute (San Diego) and the insertion was detected by PCR using the primer m-as and the T-DNA specific primer LB2 (gtgctttattattcattcattcattg). The boundaries of both insertions were sequenced to confirm the position of the insertion.

For the complementation experiment, a 9.7 kb HindIII genomic DNA fragment was cloned into a pBAR-A vector [a derivative of pGPTV-BAR (Becker et al., 1992)] and transformed into wox2-1 plants by the floral dipping method (Clough and Bent, 1998).

Results

Identification of the WUS gene family

The WUS gene encodes a homeodomain subtype with 66 amino acid residues (Mayer et al., 1998), compared to 60 of typical homeodomains (Gehring et al., 1994). By searching the Arabidopsis database for WUS-related sequences, we identified 14 open reading frames that encode conceptual proteins with homeodomains sharing 38% to 67% identity and 62% to 87% similarity to the WUS homeodomain (Fig. 1, Table 1) and named them WOX (WOX HOMEBOX) genes. In contrast to WUS, their homeodomains comprise 65 amino acid residues. Residue Y at position 21 in WUS and its putative orthologs in Antirrhinum majus (M. Kieffer, H. Cook, Y. Stern, I. Weir, M. Wilkinson, C. Maulbetsch, T.L. and B. Davies unpublished), Petunia (Stuurman et al., 2002) and rice (Feng et al., 2002) is not present in other family members. One WOX gene in Arabidopsis, WOX3, has previously been described as PRESSED FLOWER (PRS) (Matsumoto and Okada, 2001). We have not found any WUS-related protein sequence outside the plant kingdom.

Sequence comparison of WUS and its putative orthologs from other species revealed a sequence of eight identical residues (TLPFLPWMH) downstream of the homeodomain that we named the WUS Box (Fig. 1B). A similar motif was found in all but one of the WUS family members in Arabidopsis. In addition, an acidic domain was identified approximately ten residues upstream of the WUS box in WUS and three additional members, namely WOX1, WOX4, and WOX5. One member, WOX2, has a putative Cys Finger domain downstream of the homeodomain (data not shown). A subgroup of four members of the WUS family (WOX8, WOX9, WOX11, and WOX12) shows additional sequence similarities and a conserved exon/intron structure in the C-terminal region (Fig. 1C). Interestingly, the last exon of these genes codes for only 1 (WOX8, WOX9) or 5 (WOX11, WOX12) amino acids. The two closely related genes WOX10 and WOX14 are located next to each other in tandem on chromosome 2, suggestive of a duplication event. We did not find any further clustering of WOX genes. We isolated cDNAs
for 12 genes, indicating that they are expressed. For two reading frames (WOX7, WOX10) we were unable to isolate cDNA clones despite several attempts. Comparison of cDNA clones with the annotation data in the database revealed deviations in several genes.

Expression analysis

The goal of this work was to identify WOX genes with expression patterns suggestive of a role in early embryo patterning. Therefore, we performed an initial survey of 12 genes by in situ hybridization and found six family members with specific expression patterns in the embryo that we subsequently analyzed in detail (see below).

For clarity we will briefly recapitulate Arabidopsis embryo sac development and early embryogenesis (Fig. 2) (Jürgens and Mayer, 1994; Mansfield and Briarty, 1991; Mansfield et al., 1991). After meiosis, three rounds of nuclear divisions yield the mature embryo sac with the egg cell and two synergid cells at the micropylar end, a diploid central cell that gives rise to the endosperm after fusion with one of the sperm nuclei, and three antipodal cells at the chalazal end. Following fertilization of the egg cell, the pattern of cell division is almost invariant during the early stages of embryogenesis; first, the elongated zygote divides asymmetrically, producing two daughter cells that differ in size and developmental fates. The smaller apical
Table 1. The WUS gene family

<table>
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<th>Accession no.</th>
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<td>(Matsumoto et al., 2001)</td>
<td>244</td>
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<td>38 (64)</td>
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*Percentage of identical and similar (in parentheses) amino acid residues between the predicted homeodomains of WUS and WOX proteins.

dughter cell will give rise to the so-called embryo proper and finally to most of the seedling body. It first divides twice longitudinally to give the 4-cell embryo, and then horizontally to produce the 8-cell embryo proper with an apical and a central domain. Subsequently, after a round of periclinal divisions, the protoderm is separated from the inner cells, creating a radial axis of tissues in the 16-cell embryo. The larger basal daughter cell of the zygote undergoes a series of transverse cell divisions to give the extra-embryonic suspensor, a file of seven to nine cells that pushes the embryo into the lumen of the developing seed but whose uppermost cell forms the hypophysis and contributes to the root meristem.

Thus, at the 8-cell stage, four basic domains with different developmental perspectives can be distinguished along the apical-basal body axis: (1) the apical embryo domain consisting of the four apical cells of the embryo proper that will give rise to the shoot meristem and most of the cotyledons, (2) the central embryo domain consisting of the lower four cells of the embryo proper that will produce hypocotyl and most of the embryonic root, (3) the basal embryo domain consisting of the hypophyseal cell that will form the quiescent center and columella of the root meristem, and (4) the remaining extra-embryonic suspensor (Jurgen, 2001; Scheres et al., 1994). In an initial set of in situ hybridization experiments we found that these four basic domains are marked by specific expression of three WOX genes, namely WOX2, WOX8 and WOX9 (see below). In order to elucidate early events in embryonic pattern formation we analyzed the respective gene expression patterns from the egg cell stage onwards. Our focus was on the development of the embryo, but for completeness we will also include postembryonic tissues where they were also analyzed.

**WOX2 and WOX8 mRNAs co-exist in the egg cell and zygote**

WOX2 mRNA was detected in the egg cell and the central cell of the embryo sac, but not in the synergids (Fig. 3A), the antipodals or the male gametophyte (not shown). After fertilization, WOX2 was expressed in the zygote (Fig. 4A). At these stages, the WOX8 expression pattern was indistinguishable from the WOX2 pattern: expression was found in the egg cell and the central cell of the embryo sac (Fig. 3B) and thereafter in the zygote (Fig. 4O). Thus, mRNAs encoding both apical and basal cell-specific transcription factors (see below) are already present in the egg cell.

**WOX2 mRNA becomes restricted to apical embryo cells**

After the first division of the zygote, WOX2 expression was detected exclusively in the apical daughter cell (Fig. 4B). Subsequently, WOX2 mRNA was detected in all cells of the 4-cell embryo proper (Fig. 4C) and predominantly in the apical domain of the 8-cell embryo (Fig. 4D). However, in some 8-cell embryos we also detected faint staining in the central domain suggesting that expression shifted to the most apical cells during this stage. WOX2 expression remained restricted to the apical domain in the 16-cell embryo (Fig. 4E) and the early-globular stage (Fig. 4F). No expression was found in the apical domain thereafter. However, in heart stage embryos we detected weak expression in a ring of epidermal cells approximately at the junction of hypocotyl and root (Fig. 4G). No expression was found in mature embryos, endosperm or postembryonically in inflorescences (not shown).

**WOX8 mRNA becomes restricted to basal derivatives of the zygote**

After the division of the zygote, WOX8 expression was complementary to that of WOX2 and restricted to the basal daughter cell (Fig. 4P). Through the 16-cell stage, WOX8 expression was found in all descendants of the basal daughter, the developing suspensor and the hypophyseal cell (Fig. 4Q-S). After the hypophysis had divided, WOX8 expression ceased in its descendants (Fig. 4T), but remained present in the extra embryonic suspensor (Fig. 4T-U). Additional WOX8 expression was found in the cellularized endosperm of the micropylar region during the globular and heart stages of...
embryogenesis (Fig. 4T,U). Similar to WOX2, we did not detect WOX8 later in embryogenesis or in postembryonic stages, suggesting a specific role for these genes very early in embryonic development.

The asymmetric division of the zygote results in separation of WOX2 and WOX8 mRNA expression domains

Given that WOX2 and WOX8 are co-expressed in the zygote, their asymmetric expression in its daughter cells could be achieved in two ways. First, asymmetry could be already established in the zygote itself, if either mRNA species were localized specifically at the apical or basal pole of the zygote. In this case, each daughter cell would obtain only one mRNA species. Alternatively, each daughter cell could initially contain both mRNA species, but subsequently establish asymmetric mRNA expression.

To distinguish between these possibilities we performed a series of in situ hybridizations. Since the experimental procedure used does not provide subcellular resolution, we could not directly assess whether WOX2 and WOX8 mRNAs were localized in a polar fashion in the zygote. Instead, we examined over 100 embryos after the division of the zygote. We exclusively found asymmetric expression of WOX2 (Fig. 4B) and WOX8 (Fig. 4P) mRNAs, but not a single case where both daughter cells expressed the same gene. This suggests that asymmetry of the mRNA distribution is established either before cytokinesis in the zygote or rapidly thereafter in the daughter cells.

WOX9 expression dynamics reflect the initiation of the central embryo domain

WOX9 expression was first detected in the basal daughter cell of the zygote (Fig. 4J). Unlike WOX2 and WOX8, we never detected WOX9 expression in the egg cell (not shown) or the zygote (Fig. 4H). During two subsequent rounds of transverse cell divisions, WOX9 expression became restricted to the hypophysis (Fig. 4J). At the 8-cell stage, WOX9 expression expanded into the central domain of the embryo, in addition to weakening in the hypophysis (Fig. 4K). After protoderm formation, WOX9 expression in the central embryo domain became restricted to the protodermal cells and also disappeared from the hypophyseal cell (Fig. 4L). In subsequent stages, a ring of WOX9 expression remained at the presumptive boundary between root and hypocotyl (Fig. 4M,N), at about the same position as WOX2 expression in heart stage embryos (compare to Fig. 4G). In addition to its embryonic expression, WOX9 expression was found postembryonically in the epidermal cells of the placenta during gynoecium development, but not in the developing ovules (not shown). The placental expression disappeared soon after fertilization.

In summary, we established that in the 8-cell embryo the basic domains along the apical-basal axis are distinguished by the expression patterns of three WOX genes: (1) the apical domain expresses WOX2, (2) the central domain expresses WOX9 and for a limited time low levels of WOX2, (3) the basal domain (hypophysis) expresses WOX8 and WOX9, and (4) the suspensor expresses WOX8. Expression of these genes is initiated in single precursor cells as early as the egg cell stage and subsequently becomes dynamically confined to the respective embryo domains.

WOX9 expression dynamics require MP and BDL, but not GN activity

Since the WOX9 expression domain shifted across the clonal boundary between derivatives of the basal and apical daughter cells of the zygote, i.e. hypophysis and central embryo region, we considered how this process might be regulated. MP and BDL encode proteins presumably involved in auxin-dependent development of the embryo proper and signaling from the embryo proper to the hypophysis for its correct specification (Hamann et al., 2002; Hardtke and Berleth, 1998). The earliest defect detected in each mutant is an abnormal division of the apical daughter cell of the zygote, eventually leading to a double octant embryo proper (Fig. 5A). To address whether WOX9 expression dynamics requires MP/BDL-dependent signaling from the embryo proper, we analyzed WOX9 expression in mp and bdl mutants. We found that in contrast to wild type, WOX9 expression was not shifted into the embryo proper in the mutants (Fig. 5A-C) but rather persisted in the hypophysis (compare to Fig. 4K-N).

To determine whether the altered expression dynamics of WOX9 in mp and bdl embryos could be a secondary effect of the abnormal cell divisions in these mutants, we analyzed rarely occurring wild-type embryos with similar abnormal divisions. WOX9 expression was detected in the enlarged central domain of these misshapen wild-type embryos (Fig. 5E, compare with Fig. 4K) and later became restricted to epidermal cells in the central domain (Fig. 5F, compare with Fig. 4L), but was absent from the hypophysis. Thus, in contrast to mp and bdl, the cells of misshapen wild-type embryos are correctly specified with respect to the WOX9 expression, suggesting that the cell division pattern per se is not essential for the correct WOX9 expression dynamics. To address whether gene expression per se was disturbed in mp and bdl embryos or whether the effects were more specific for WOX9, we analyzed WUS expression in mp embryos. WUS is tightly regulated and restricted to a few cells in the apical domain of wild-type embryos (Mayer et al., 1998). In mp embryos, we found that...
WUS was expressed at its correct position from an early stage, even though embryo morphology was severely compromised (not shown), suggesting that cells in the apical domain were properly specified.

Taken together, our results strongly suggest that MP- and BDL-dependent signaling from the embryo proper is required for the shift of WOX9 expression from the hypophysis to the lower cells of the embryo proper.

We also considered whether the apical shift of WOX9 expression requires correct apical-basal polarization of embryo cells by analyzing WOX9 expression in gn mutant embryos. In the strongest manifestation of the gn defect, a ball-shaped embryo with severely disturbed cellular polarity is formed (Steinmann et al., 1999). We found WOX9 to be expressed in the gn embryo proper, but not in the hypophysis, similar to wild-type embryos (Fig. 5D, compare with the wild-type embryo in Fig. 4N). In contrast to wild type, however, expression was scattered in epidermal cells throughout the embryo proper rather than being restricted to the central domain, consistent with observations made on the expression

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**Fig. 4.** mRNA expression patterns during early embryogenesis. (A-U) Longitudinal median sections of developing wild-type seeds. Bound probe is stained brown. The clonal boundary resulting from the division of the zygote is marked (black arrowheads). The demarcation line between the apical and central domain of the early embryo is indicated (open arrowheads). The white asterisks indicate brown color of the seed coat independent of the staining reaction. (A-G) In situ hybridization with a WOX2 antisense probe. (A) WOX2 mRNA is detected in the zygote. (B) After the first zygotic division, WOX2 expression is restricted to the apical daughter cell, and absent from the basal one. (C) WOX2 mRNA is expressed in all cells of the 4-cell embryo proper (unspecified arrow), but not in the suspensor. (D) WOX2 mRNA is expressed in the apical domain of the 8-cell embryo, but not in the central domain, the suspensor or the hypophysis. (E-F) At the 16-cell stage (E) and in early globular embryos (F), WOX2 mRNA remains restricted to the apical embryo domain. (G) In heart stage embryos, WOX2 expression is only weakly detected in epidermal cells of the central domain (arrow). (H-N) Hybridization with a WOX9 antisense probe. (H) No expression is detectable in the zygote. (I) WOX9 mRNA is detected only in the basal daughter cell of the zygote. (J) At the 4-cell stage, WOX9 expression is restricted to the hypophysis. (K) In 8-cell embryos, WOX9 mRNA is expressed in the central embryo domain and in the hypophysis. (L) In 16-cell embryos, WOX9 mRNA expression is restricted to epidermal cells of the central domain and to the hypophysis (arrows). (M) In mid globular embryos, WOX9 expression is detected in epidermal cells of the central domain, but not in the hypophysis (arrow). (N) In heart stage embryos, WOX9 mRNA is only detected in a ring of epidermal cells (arrow). (O-U) Hybridization with a WOX8 antisense probe. (O) WOX8 mRNA is detected in the zygote. (P) After the first division of the zygote WOX8 mRNA is exclusively found in the basal daughter cell but not in the apical one. (Q,R) WOX8 expression marks all suspensor cells of the 4- and 8-cell embryo, but is not expressed in the embryo proper (unspecified arrow in Q). (S) At the 16-cell stage, WOX8 mRNA is detected in all suspensor cells, including the hypophysis. (T) In mid globular embryos, continuous strong WOX8 expression is detected in the suspensor, but there is no expression in the derivatives of the hypophysis. (U) In heart stage embryos, WOX8 is expressed in the suspensor and surrounding endosperm cells. a, apical daughter cell of the zygote; ad, apical domain of the early embryo; b, basal daughter cell of the zygote; cd, central domain of the early embryo; en, endosperm; hy, hypophysis; su, suspensor; z, zygote. Scale bars: 10 μm (A-T); 20 μm (U).
of early globular embryos, approximately one round of cell division after the 16-cell stage (Fig. 6B), but never at the 16-cell stage itself (Fig. 6A). In rare cases early globular embryos did not show expression (not shown), suggesting that WOX5 expression was initiated at some time during this stage. After the division of the hypophysis, WOX5 mRNA was detected in the upper lens-shaped cell that gives rise to the QC (Fig. 6C), but not in the lower daughter cell that gives rise to the central root cap (Scheres et al., 1994). Subsequently, in heart stage (Fig. 6D) and bent cotyledon stage embryos (Fig. 6E), WOX5 mRNA was detected in the four cells of the QC, which are the direct descendants of the lens-shaped cell. In addition to the expression in the QC and its precursor cells, we found expression in patches of cells that appeared associated with the vascular primordium of the cotyledons (Fig. 6F). This expression was strongest in late heart stage embryos and then gradually decreased.

Expression dynamics of WOX1 and PRS/WOX3 genes reflect initiation of tissue primordia

During initiation of the shoot apical meristem, cotyledonary primordia arise at the flanks of the apical embryo domain. We found two members of the WOX gene family, namely WOX1 and PRS/WOX3, which were specifically expressed during this phase. PRS/WOX3 expression was detected at the margins of cotyledonary primordia of heart stage embryos (Fig. 6G,H), but was not detectable during torpedo stage except for a single cell at the very tip of the cotyledons (Fig. 6I). Postembryonically, PRS/WOX3 was expressed at the margins of floral organ primordia where it is required for cell fate specification (Matsumoto and Okada, 2001). WOX1 expression was confined to the initiating vascular primordium of the cotyledons during heart and torpedo stages (Fig. 6J,K), but only a weak signal was detected during bent cotyledon stage (Fig. 6L). Taken together, patterning of cotyledonary primordia involves initiation of tissue-specific WOX transcription programs.

WOX2 function regulates establishment of the apical embryo domain

In order to obtain initial insight into the function of WOX genes during embryo development, we analyzed insertion mutants for WOX1, 2, 5, 8 and 9. We found that embryonic patterning was specifically perturbed in wox2 mutants but not in any other mutant.

We identified two independent insertion mutants, wox2-1, and wox2-2. By sequencing the wox2-1 allele, we detected an insertion after nucleotide 233 in the first exon that disrupts the predicted homeodomain, indicating that this allele probably represents a complete loss of WOX2 function (Fig. 7A). After backcrossing three times to wild-type plants, we were able to identify fertile plants homozygous for the wox2 mutation by PCR based insert analysis. Self-fertilized homozygous wox2-1 plants gave rise to 30-50% of embryos with abnormal apical development (Fig. 7, Table 2). Between the 4-cell and 16-cell stage, some cells in the mutant embryo failed to divide (Fig. 7E) From the 16-cell stage onwards, wox2 embryos additionally showed aberrant oblique cell divisions not observed in the wild type (Fig. 7F,G). At the mid-globular stage, wox2-1 embryos started to recover by forming a protoderm (Fig. 7G) and eventually gave rise to fertile plants. The wox2-2 allele contains
an insertion after nucleotide 477 in the second exon (Fig. 7A).
Homozygous wox2-2 plants produced embryos with defects similar to those observed for wox2-1 (Fig. 7, Table 2) and did not complement the wox2-1 mutation.

To unambiguously confirm that the insertions in the WOX2 gene caused the embryo phenotype, we transformed homozygous wox2-1 plants with a 9.7 kb wild-type genomic DNA fragment containing the WOX2 gene and approximately 6.9 kb of upstream sequence (Fig. 7A). All the embryos produced by transformed wox2-1 plants that were homozygous for the genomic WOX2 transgene, as determined by BASTA selection and PCR, were indistinguishable from wild-type embryos, whereas all untransformed wox2-1 plants produced mutant embryos (Table 2). This verifies that the phenotype observed in the wox2-1 line is caused by the insertion in the WOX2 gene.

Since WOX2 is expressed in the female gametophyte from an early stage, we considered whether WOX2 function is essential during female gametophytic and/or during embryonic development. For this purpose, we compared the progeny of selfed wox2-1 mutants with those obtained from reciprocal back-crosses between a homozygous wox2-1 plant and wild type. We found embryos with abnormal apical cells exclusively in the progeny of selfed wox2-1 plants. Division patterns of apical cells were unperturbed in heterozygous wox2-1 embryos, even when these developed in a homozygous wox2-1 mother (Table 3). Thus, even though WOX2 is expressed in the embryo sac, it is only required for apical embryo development after fertilization.

### Discussion

While the stereotypic cell division pattern allows seedling structures to be traced back to regions of the early Arabidopsis embryo, the mechanisms governing the initiation of body axes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Embryo phenotypes</th>
<th>Normal</th>
<th>Abnormal</th>
<th>% Abnormal</th>
</tr>
</thead>
<tbody>
<tr>
<td>wox2-1/+</td>
<td>None</td>
<td>587</td>
<td>289</td>
<td>33</td>
</tr>
<tr>
<td>wox2-1/+</td>
<td>WOX21/+</td>
<td>1352</td>
<td>2</td>
<td>0.1</td>
</tr>
<tr>
<td>wox2-2/+</td>
<td>None</td>
<td>295</td>
<td>64</td>
<td>17.8</td>
</tr>
</tbody>
</table>

The phenotypes of embryos up to the early globular stage from selfed wox2-1 plants were scored as ‘normal’ if indistinguishable from Arabidopsis wild type and ‘abnormal’ if cell division patterns in the apical embryo cell lineage were different from wild type. wox2-1 WOX21/+ were homozygously mutant for the genomic WOX2 gene, but carried the 9.7 kb long WOX2 containing transgene in a homozygous situation.
and the establishment of specific domains during early embryogenesis have yet to be elucidated. We have identified a new family of plant-specific WUS-related homeobox (WOX) genes, allowing us to address early steps in embryonic patterning. Our results suggest a model in which the basic body plan is derived from precursor cells that are specified at the earliest stages of embryogenesis. These cells initiate region-specific transcription programs that subsequently become progressively confined to different domains of the embryo (Fig. 8).

The asymmetric division of the zygote separates apical and basal specific mRNAs

In most plants, the first division of the zygote results in two daughter cells of different size, cytoplasmic density, and developmental perspectives. Our results show that this division also establishes an asymmetric distribution of mRNAs encoding potential developmental regulators involved in the initiation of the apical-basal body axis.

How is this asymmetry established? Two mechanisms can be envisioned. In the first, WOX2 and WOX8 mRNAs, or factors that regulate their expression such as RNA degrading enzymes or transcriptional regulators, could be localized in the zygote in a polar fashion and be inherited asymmetrically by the daughter cells (Fig. 8). This could be accomplished by interactions of molecules with the cytoskeleton and subsequent localization to one pole of the zygote. Consistent with this hypothesis, both egg cell and zygote display a highly polar organization; the nucleus and most of the cytoplasm are located at the apical pole whereas the vacuole is located at the basal pole (Mansfield and Briarty, 1991; Mansfield et al., 1991). In an alternative mechanism, the daughter cells would initially inherit the same molecules and asymmetric mRNA expression would be established afterwards in response to differential positional cues by specific RNA degradation and/or gene transcription. One example of such a mechanism is the gradual restriction of ATML1 gene expression to the apical protoderm of globular embryos (Lu et al., 1996).
Our results argue in favor of the model in which apical- and basal-specific molecules, either WOX2 and WOX8 mRNAs or factors regulating them, are already laid down in a polar fashion within the zygote (Fig. 8). In every embryo we examined we found asymmetric distribution of WOX2 and WOX8 mRNAs in the apical and basal daughter cells. It is possible that both daughter cells contain the same transcript for a very brief period after division of the zygote, and that we have missed observing this situation because of its transient nature. However, this scenario would nevertheless require that factors regulating mRNA degradation and/or specific gene expression be asymmetrically distributed upon the division of the zygote. The proposed mechanism would be similar to, for example, separation of developmental determinants by the asymmetric cell divisions of the Caenorhabditis elegans zygote (Lyczak et al., 2002) suggesting that related strategies are employed during initiation of the main body axis in plants and animals. It is noteworthy that such a mechanism does not imply autonomous specification of cell fates, since polarization of the plant zygote might conceivably be regulated by positional cues from the surrounding micropylar and/or chalazal tissues.

**Dynamic establishment of central embryo domain identity**

Once apical-basal polarity is established, progressive refinement along the apical-basal axis is evident by WOX gene expression dynamics: WOX2 and WOX8 expression become gradually confined to the most apical and basal descendants of the zygote respectively and the precursors of hypocotyl and root are established between them. This is reflected by the shift of WOX9 expression across the clonal boundary from the hypophysis into the basal cells of the embryo proper, concurrent with the downregulation of WOX2 expression, indicating progressive confinement of ‘apicalness’ to the most apical cells and specification of central embryo domain identity in the cells underneath.

Our results indicate that the shift of WOX9 expression requires auxin response in the embryo proper mediated by MP and BDL activities and signaling from the embryo proper to the hypophysis. The failure to initiate WOX9 expression in the central domain and the inability to repress it in the hypophysis do not appear to be due to the aberrant morphology of mp and bdl embryos. This suggests WOX9 as a potential target of MP/BDL-dependent signaling.

**A common mechanism during initiation of apical and basal stem cell niches**

After embryogenesis, the cells required for continuous plant growth are ultimately derived from stem cell niches within the root and shoot meristems. In the shoot meristem, WUS expression in the organizing center (OC) provides signals to maintain adjacent undifferentiated stem cells (Mayer et al., 1998). Likewise, in the root meristem, signaling from the QC is required to maintain neighboring stem cells in an undifferentiated state (van den Berg et al., 1997), suggesting that OC and QC are functionally equivalent signaling centers that constitute stem cell maintaining microenvironments.

Our results suggest that during formation of the root pole in the embryo, QC identity is established in the hypophyseal cell soon after the 16-cell stage and subsequently becomes restricted to the lens-shaped upper daughter cell by an asymmetric division. Similarly, at the future shoot pole, WUS expression specifies precursor cells of the OC from the 16-cell stage onwards and subsequently becomes restricted to its appropriate position within the shoot meristem by asymmetric cell divisions (Mayer et al., 1998). Therefore, both signaling centers are not only functionally equivalent but also share striking developmental and molecular similarities.

**A potential role for the WOX family in embryonic pattern formation**

Several lines of evidence suggest that WOX genes function in early embryonic patterning. First, it is plausible that WOX homeodomain proteins confer specific transcriptional programs upon the cells expressing them. Second, these programs are initiated in precursor cells as early as in the egg cell, suggesting a function early in the regulatory hierarchy. Third, WOX gene expression is restricted to stages in embryogenesis during which developmental decisions conceivably take place.

Finally, our mutant analysis demonstrates that WOX2 is functionally required to regulate the timing and the orientation of divisions in the cells expressing it, the descendants of the apical daughter cell of the zygote. At this stage of embryonic development the cell division pattern is essentially invariant in Arabidopsis, indicating that the information about when and how to divide is an integral part of the identity of a cell. We therefore suggest that WOX2 is involved in specifying apical
cell identity during early embryogenesis. In this view, the separation of WOX2 and WOX8 expression domains during the asymmetric division of the zygote appears to be a very early event in establishing different cell fates along the apical-basal body axis of the Arabidopsis embryo. We do not know why embryos mutant for WOX genes other than WOX2 and WUS, which exhibit intriguing expression dynamics in early embryogenesis, did not show any detectable developmental defects. However, since several of the respective genes, e.g. WOX8 and WOX9 represent pairs or triplets of highly related WOX family members (compare Fig. 1D), the lack of phenotypic defects in these mutants could be due to genetic redundancy.

In several animal species, cell fate decisions during early embryonic development are regulated by members of a homeobox gene family (HOX) which are expressed in specific domains of the embryo (Krumlauf, 1994). Although a detailed functional analysis is the subject of further studies, our results suggest that members of the plant-specific WOX homeodomain family could fulfill similar functions in plant embryonic patterning.

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Note added in proof

While this paper was under review, a putative rice homolog of WOX5 was described (Kamiya et al., 2003).

References


