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 embryo 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 (catgcaaaccatcgtcttaaaacc) and *WOX2*-AS (cataaaatttataatttcattaaaccttcg), a 613 bp *PRS/WOX3* cDNA fragment was amplified with *PRS*-S1 (atgagtcctgtggcttcaacg) and *PRS*-AS1 (atgagggatatgatggtcgtgg), a 1027 bp *WOX8* cDNA fragment was amplified with primer *WOX8*-S (tacaccatcatcatgtcctcct) and *WOX8*-AS (tatccatagcaccataacatttgc) and a 590 bp *WOX5* cDNA fragment was amplified with F11-S (cgtaaaacagttgaggactttacatctg) and F11-As (ggtcaatgacttaaagaaagcttaatcg). 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 (tcccacgtctcacttcttgc) and *WOX1*-AS (cctggaggatctttcttgtcg), and genomic *WOX9* DNA with primers *WOX9*-S (ccaagatggaatccaaagccag) and *WOX9*-AS (ggtccgaagttgatgggacagtag).

All DNA fragments were subcloned into the pGEM-T® vector from Promega or the pBluescript II SK– vector from Stratagene. The WUS homeodomain sequence was used to search for related sequences with the NCBI Blast program. Sequences were aligned using Vector NTI and searched for protein domains using the ExPASy algorithms (http://us.expasy.org/tools/scanprosite/). A dendrogram was established using ClustalW from the DDBJ Homology Search System (http://crick.genes.nig.ac.jp/homology/clustalw-e.shtml). The dendrogram was drawn with help of the Treestar program (Page, 1996).

In situ hybridization

In situ hybridization was performed as previously described (Mayer et al., 1998). All plant material was of the L*er* background. *mp*, *bdl* and *gn* seeds were kindly provided by Dr Gerd Jürgens (Tübingen). For every gene, sense and antisense probes were analyzed and sense controls never showed specific signals. In all experiments, we confirmed expression patterns in more than 30 embryos for each stage, except for the expression analysis in zygotes (approx. 10 zygotes) and the infrequently occurring abnormal wild-type embryos (3 embryos). All hybridization probes lacked a poly(A) tail. The *WOX1* probe consisted of the cDNA fragment from position 114 to 645. For *PRS/WOX3*, *WOX5* and *WOX8* the complete cDNA fragments (see above) were used as probes. To exclude cross hybridization between genes with overlapping expression patterns, we generated additional probes for *WOX2* and *WOX9* excluding the homeobox as the most conserved domain within these genes. Both *WOX2* probes, 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 (aagtaaacgcaggaacagcaagcagttca) and m-as (cgaaacgagtagaagtagaaccaccagaa) 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 (gcttcctattatatcttcccaaattaccaataca). The boundaries of both insertions were sequenced to confirm the position of the insertion.

For the complementation experiment, a 9.7 kb *Hin*dIII 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* (*WUS HOMEOBOX*) 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 (TLPLFPMH) 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 Zinc 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

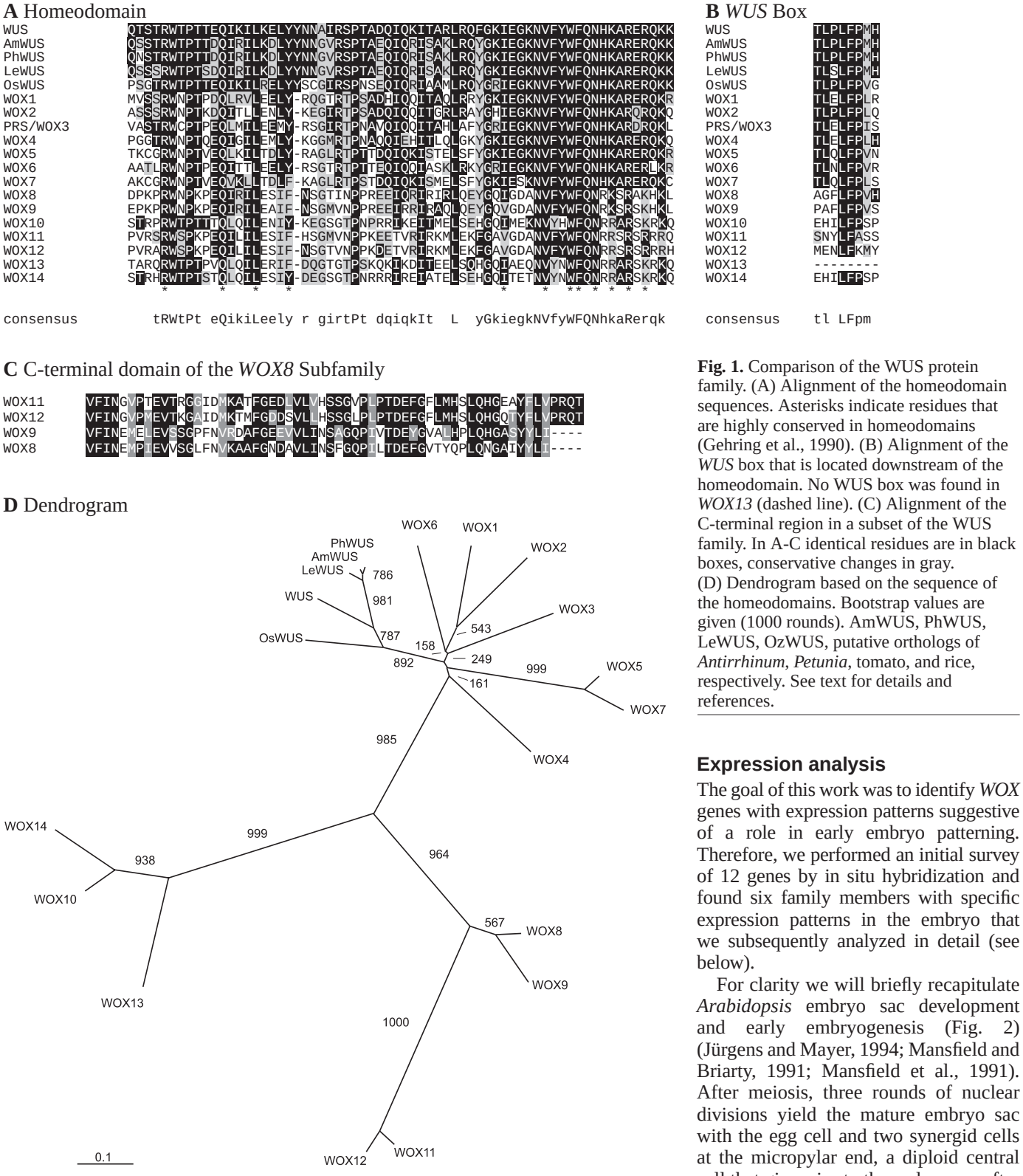

Fig. 1. Comparison of the WUS protein family. (A) Alignment of the homeodomain sequences. Asterisks indicate residues that are highly conserved in homeodomains (Gehring et al., 1990). (B) Alignment of the *WUS* box that is located downstream of the homeodomain. No WUS box was found in *WOX13* (dashed line). (C) Alignment of the C-terminal region in a subset of the WUS family. In A-C identical residues are in black boxes, conservative changes in gray. (D) Dendrogram based on the sequence of the homeodomains. Bootstrap values are given (1000 rounds). AmWUS, PhWUS, LeWUS, OzWUS, putative orthologs of *Antirrhinum*, *Petunia*, tomato, and rice, respectively. See text for details and references.

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

Gene name	Accession no.	Similarity*	cDNA	aa
WUS			(Mayer et al., 1998)	292
WOX1	AY251394	67 (87)	This study	349
WOX2	AY251392	66 (81)	This study	260
PRS	AY251397	68 (82)	(Matsumoto et al., 2001)	244
WOX4	AY251396	66 (78)	This study	251
WOX5	AY251398	66 (78)	This study	182
WOX6	AY251399	64 (81)	This study	295
WOX7	–	63 (77)	n.a.	122
WOX8	AY251400	45 (69)	This study	325
WOX9	AY251401	44 (71)	This study	378
WOX10	–	43 (69)	n.a.	197
WOX11	AY251402	63 (64)	This study	268
WOX12	AY251403	40 (62)	This study	268
WOX13	AY251404	40 (67)	This study	268
WOX14	–	38 (64)	This study	195

*Percentage of identical and similar (in parentheses) amino acid residues between the predicted homeodomains of WUS and WOX proteins.

daughter 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 (Jürgens, 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

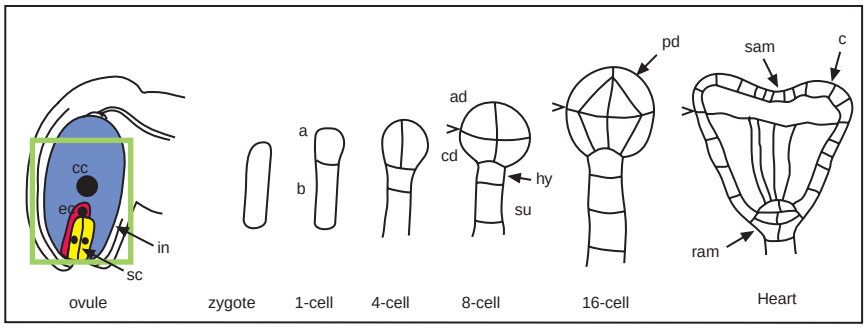

Fig. 2. *Arabidopsis* ovule organization and early embryo development. The green rectangle indicates the part of the ovule shown in Fig. 3. See text for details. a, apical daughter of the zygote; ad, apical embryo domain; b, basal daughter of the zygote; cd, central embryo domain; cc, central cell (blue); c, cotyledons; ec, egg cell (red); hy, hypophysis; in, integuments; pd, protoderm; ram, root apical meristem; sc, synergid cells (yellow); sam, shoot apical meristem. The demarcation line between the apical and central domain of the early embryo is indicated (open arrowheads).

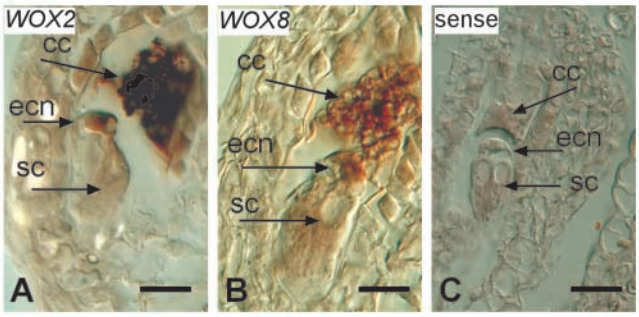

Fig. 3. *WOX2* and *WOX8* mRNA expression patterns (brown) in the female gametophyte. In situ hybridization of unfertilized ovules. Sections showing the mature embryo sac as outlined in Fig. 2. (A) Hybridization with a *WOX2* antisense probe. *WOX2* mRNA is detected in the egg cell and the central cell, but not in the synergids. (B) Hybridization with a *WOX8* antisense probe. *WOX8* mRNA is also detected in the egg cell and the central cell, but not in the synergids. (C) Hybridization with a *WOX8* sense probe. No staining was detected in any of the cells of the embryo sac. cc, central cell; ecn, egg cell nucleus; sc, synergid cells. Scale bars: 10 μm.

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. 4I). 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

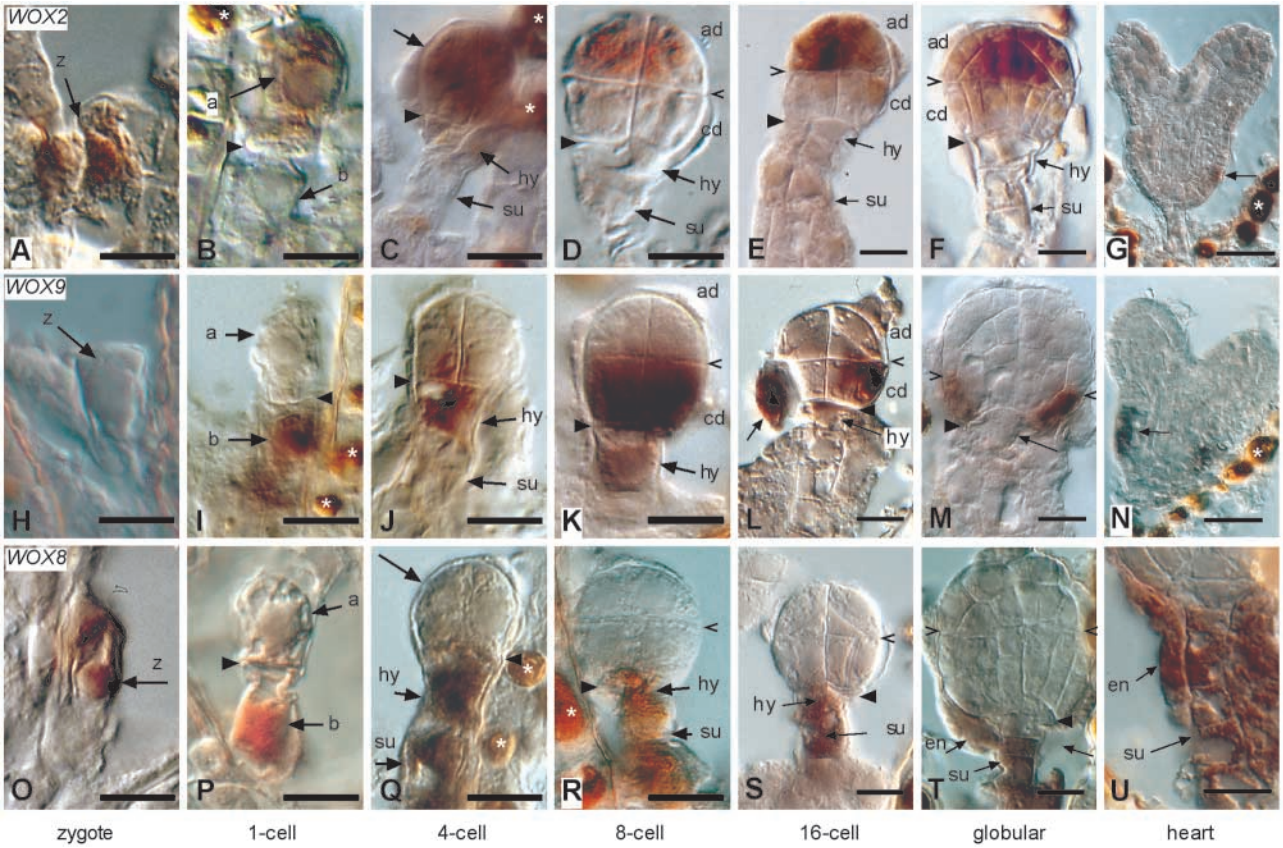

Fig. 4. mRNA expression patterns during early embryogenesis. (A-U) Longitudinal median sections of developing wild-type seeds. Bound probe is stained brown. The clonal boundary resulting from the division of the zygote is marked (black arrowheads). The demarcation line between the apical and central 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).

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

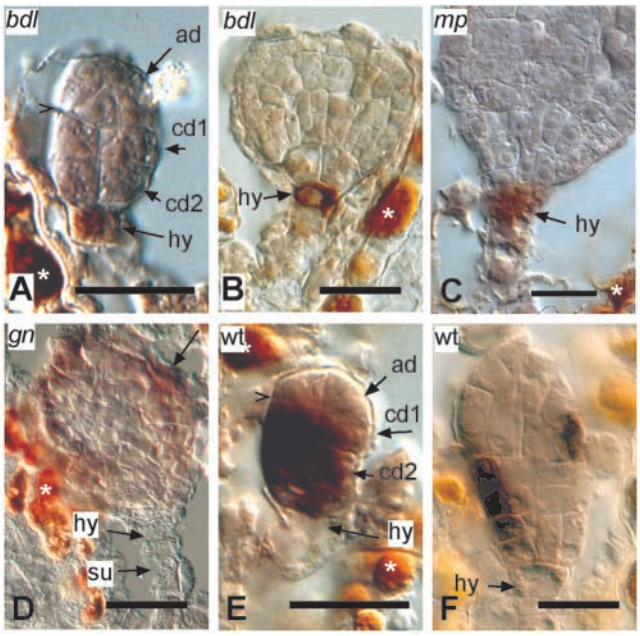

Fig. 5. *WOX9* mRNA expression in mutant embryos. (A-F) In situ hybridizations with a *WOX9* antisense probe (brown staining). Longitudinal sections of developing seeds. The demarcation line between the apical and central domain of the early embryo is indicated (open arrow heads). White asterisks indicate brown color of the seed coat independent of the staining reaction. (A,B) *bdl* embryos. *WOX9* mRNA is detected in the hypophysis, but not in the central domain of double octant embryos corresponding to the 8-cell stage wild type (A, compare to Fig. 4K) and early heart stage (B, compare to Fig. 4M,N) of wild-type siblings. (C) *mp* embryo. *WOX9* mRNA is detected in the hypophysis, but not in the central domain of embryos corresponding to the heart stage of wild-type siblings (compare to Fig. 4M,N). (D) *WOX9* expression in a *gn* embryo; the stage shown corresponds to the heart stage of wild-type siblings (compare to Fig. 4N). *WOX9* mRNA is detected in several epidermal cells throughout the embryo (arrow), but not in the hypophysis. (E,F) *WOX9* expression in wild-type embryos, with abnormal cell division patterns. (E) A double octant embryo resembling that of *bdl*. *WOX9* mRNA is detected in the central domain. (F) An abnormal embryo, corresponding to the late globular stage. Expression of *WOX9* is roughly similar to that of normally developed wild-type embryos (compare to Fig. 4M). ad, apical embryo domain; cd1 and cd2, doubled central domain of double octant embryos; hy, hypophysis; su, suspensor. Scale bars: 20 μm.

patterns of other genes in *gn* embryos (Vroemen et al., 1996). Thus, although *gn* embryos cells are severely perturbed in cellular polarity, this does not appear to affect the apical shift of *WOX9* expression from the hypophysis into the embryo proper.

WOX5 expression dynamics reveal early specification of quiescent center identity

After the basic apical-basal pattern is evident at the 8-cell stage, the body plan of the embryo is further elaborated by the initiation of shoot and root apical meristems and the cotyledons. We found that the quiescent center (QC) of the root meristem expressed the *WOX5* gene (Fig. 6E) and this allowed us to analyze early events during QC initiation. We detected specific expression of *WOX5* in the hypophysis of the majority 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 insertional 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 on, *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

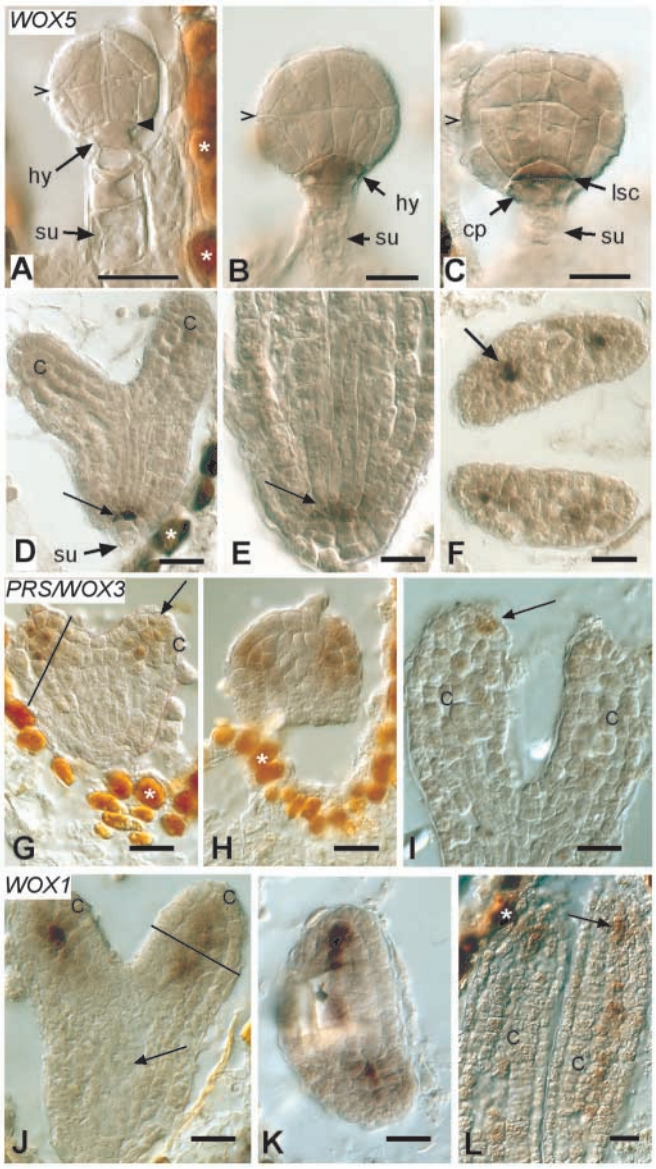

Fig. 6. *WOX5*, *PRS/WOX3* and *WOX1* mRNA localization (brown staining) during embryonic development. (A-L) Longitudinal median sections of developing wild-type seeds are shown, if not indicated otherwise. White asterisks indicate brown color of the seed coat independent of the staining reaction. (A-F) In situ hybridization with a *WOX5* antisense probe. (A) No expression is detected in 16-cell embryos. (B) In early globular embryos, expression is detected exclusively in the hypophysis. (C) After the division of the hypophyseal cell, *WOX5* expression becomes restricted to the upper daughter, the lens shaped cell (lsc), and is not detectable in the lower daughter, the columella precursor (cp). (D,E) In heart stage (D) and bent cotyledon stage (E) embryos, *WOX5* mRNA is detected in the derivatives of the lens shaped cell, the developing quiescent center of the embryonic root (arrows). (F) Cross section through cotyledon primordia of a torpedo stage embryo (plane of section shown in J). *WOX5* expression is detected in patches at about the position where lateral veins will form (arrow). (G-I) In situ hybridization with a *PRS/WOX3* antisense probe. (G,H) Early heart stage embryos. (G) Expression is detected in a subset of cotyledonary cells (arrow). (H) Section through a cotyledon as indicated by a line in G). *PRS/WOX3* mRNA is detected in two lateral stripes. (I) A torpedo stage embryo. *PRS/WOX3* mRNA is restricted to a single cell at the tip of the cotyledon (arrow). (J-L) In situ hybridization with a *WOX1* antisense probe. (J) A heart stage embryo. *WOX1* mRNA is detected in the provascular tissue of the cotyledons, but not in the vasculature of the hypocotyl (arrow). (K) Cross section through a torpedo stage cotyledon as indicated by the line in J. *WOX1* expression is detected in a stripe across the cotyledon. (L) Bent cotyledon stage. *WOX1* signal is weaker, but still detectable in the provascular tissue of the cotyledons (arrow). c, cotyledon, cp, columella primordium; hy, hypophysis; lsc, lens shaped cell; su, suspensor. Scale bars: 20 μm.

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 *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 2. *wox2* mutations result in defective embryo development

Genotype		Embryo phenotypes		
Genomic	Transgene	Normal	Abnormal	%Abnormal
wox2-1$^{-/-}$	None	587	289	33
wox2-1$^{-/-}$	*WOX2*$^{+/+}$	1352	2	0.1
wox2-2$^{-/-}$	None	295	64	17.8

The phenotypes of embryos up to the early globular stage from selfed *wox2* 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 WOX2*$^{+/+}$ were homozygously mutant for the genomic *WOX2* gene, but carried the 9.7 kb long *WOX2* containing transgene in a homozygous situation.

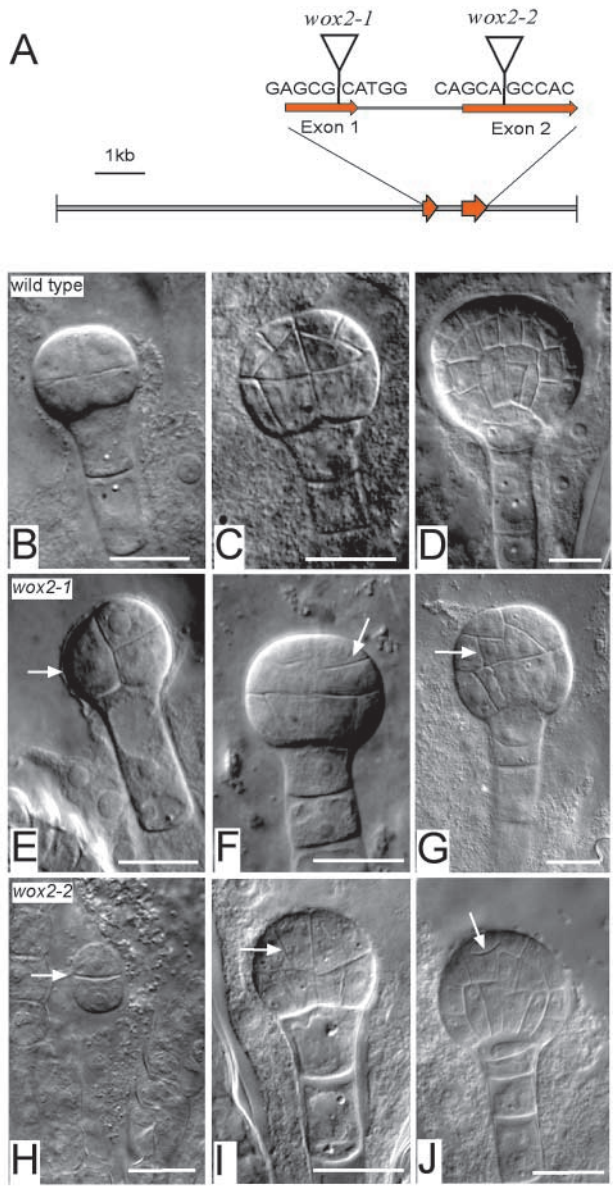

Fig. 7. *wox2* embryos show specific defects in early development. (A) The gray bar indicates the *WOX2* genomic DNA fragment used in rescue experiments. Above it, exon 1 and exon 2 have been enlarged to illustrate the positions of insertions within *WOX2*. (B-D) Wild-type embryos display a regular cell division pattern. (B) An 8-cell embryo. (C) An early globular stage embryo. (D) A mid-globular stage embryo. (E-G) By contrast, about 30-50% of *wox2-1* embryos display defective apical development. (E) A *wox2-1* embryo at the same stage as the one shown in B, where the left cell failed to divide (arrow). (F) A *wox2-1* embryo at the same stage as the ones shown in C, displaying aberrant oblique cell divisions in the apical embryo domain (arrow). (G) A *wox2-1* mid globular stage embryo. A protoderm has formed despite aberrant oblique cell divisions in the apical embryo domain (arrow). (H-J) About 15-30% of *wox2-2* embryos have similar defects to those of *wox2-1* embryos. (H) 2-cell embryo, showing a horizontal division (arrow) instead of a typical vertical one. (I) Early globular stage embryo, displaying an oblique cell division (arrow). (J) Mid stage embryo, displaying a misplaced tangential cell division (arrow) (B-G) DIC microscopy. Scale bars: 20 μm.

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). First, both the egg cell and the zygote express a mixture of mRNAs encoding *WOX* transcription factors specific for apical and basal early embryo development. Second, the asymmetric division of the zygote separates these mRNAs, thereby establishing two cells with different identities and thus setting up the apical-basal axis of the embryo. Third, new pattern elements expressing specific *WOX* genes are added and region boundaries are refined by interregional communication, resulting in the progressive elaboration of the embryo body plan.

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).

Table 3. Effects of the *wox2* mutation are restricted to zygotic development

Genotype *WOX2* locus			Embryo phenotypes		
Mother	Egg	Pollen	Normal	Abnormal	% Abnormal
++	+	+	250	1	0.4
−−	–	–	115	70	38
++	+	–	162	1	0.6
−−	–	+	162	1	0.6

The phenotypes of embryos up to early globular stages from selfed *wox2* and wild-type plants and from reciprocal crosses 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.

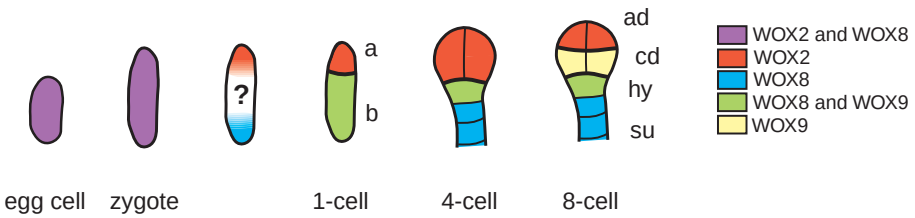

Fig. 8. *WOX* gene expression dynamics during early embryo patterning. First, the egg cell and the zygote express *WOX2* and *WOX8*. A hypothetical polar distribution of the respective RNAs, or factors regulating their expression, is shown. After division of the zygote, *WOX2* expression is restricted to the apical daughter cell, which will give rise to the embryo proper, whereas *WOX8* expression, together with *WOX9*, is restricted to the basal daughter cell, which will form the suspensor and the hypophysis. At the 8-cell stage, the basic domains of the embryo can be distinguished by different *WOX* gene expression profiles: (1) the apical domain that will give rise to most of the shoot expresses only *WOX2*, (2) the central domain that will form the hypocotyl and part of the root expresses *WOX9* (and briefly low levels of *WOX2* not illustrated here), (3) the hypophysis from which part of the root meristem will derive expresses *WOX8* and *WOX9*, and (4) the suspensor expresses only *WOX8*. a, apical daughter cell of the zygote; ad, apical embryo domain; b, basal daughter cell of the zygote; cd, central embryo domain, hy, hypophyseal cell; su, suspensor.

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 invariable 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).

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