Developmental Cell
Short Article

Transcriptional Activation of *Arabidopsis* Axis Patterning Genes *WOX8/9* Links Zygote Polarity to Embryo Development

Minako Ueda,[1,3] Zhongjuan Zhang,[1] and Thomas Laux[1,2,*]
[1]BIOSS Centre for Biological Signalling Studies, Faculty of Biology, Albert-Ludwigs University Freiburg, Schaenzlestrasse 1, 79104 Freiburg, Germany
[2]Freiburg Institute of Advanced Studies, University of Freiburg, Albertstrasse 19, 79104 Freiburg, Germany
[3]Present address: Graduate School of Biological Sciences, Nara Institute of Science and Technology (NAIST), Takayama-cho 8916-5, Ikoma, Nara 630-0192, Japan
*Correspondence: laux@biologie.uni-freiburg.de
DOI 10.1016/j.devcel.2011.01.009

SUMMARY

In most flowering plants, the apical-basal body axis is established by an asymmetric division of the polarized zygote. In *Arabidopsis*, early embryo patterning is regulated by *WOX* homeobox genes, which are coexpressed in the zygote but become restricted to apical (*WOX2*) and basal (*WOX8/9*) cells. How the asymmetry of zygote division is regulated and connected to the daughter cell fates is largely unknown. Here, we show that expression of *WOX8* is independent of the axis patterning signal auxin, but, together with the redundant gene *WOX9*, is activated in the zygote, its basal daughter cell, and the hypophysis by the zinc-finger transcription factor WRKY2. In *wrky2* mutants, egg cells polarize normally but zygotes fail to reestablish polar organelle positioning from a transient symmetric state, resulting in equal cell division and distorted embryo development. Both defects are rescued by overexpressing *WOX8*, indicating that WRKY2-dependent *WOX8* transcription links zygote polarization with embryo patterning.

INTRODUCTION

Initiation of the main body axis is one of the first patterning steps in the development of an organism from a unicellular zygote. In most flowering plants, the apical-basal (shoot-root) axis of the embryo aligns with the longitudinal axis of unfertilized egg cell and zygote, which have the nucleus in the apical half and vacuoles at the basal end (Jürgens and Mayer, 1994; Mansfield and Briarty, 1991), but whether both processes are mechanistically linked is unknown. In *Arabidopsis thaliana*, the zygote divides asymmetrically into a small, cytoplasmic apical daughter cell and a large vacuolated basal daughter cell. The apical daughter cell gives rise to the apical embryo lineage forming the spherical proembryo by divisions in different orientations. By contrast, the vacuolated basal daughter cell of the zygote continues to divide horizontally giving the basal lineage, which produces a filamentous suspensor of vacuolated cells that connects the proembryo to maternal tissue (Jürgens and Mayer, 1994; Mansfield and Briarty, 1991). Only the uppermost cell of the basal lineage, the hypophysis, becomes incorporated into the embryo giving rise to a part of the root meristem (Dolan et al., 1993).

Recent studies have begun to identify components of the molecular network involved in early embryo patterning. Numerous findings indicate an important role for the plant hormone auxin at different stages of embryo development (for review, see Moller and Weijers, 2009). After the division of the zygote, auxin response is first observed in the apical daughter cell where it is required for normal embryo axis formation (Friml et al., 2003). *WUSCHEL RELATED HOMEOBOX* (*WOX*) genes encode essential regulators of early embryo patterning (Breuninger et al., 2008; Haecker et al., 2004; Wu et al., 2007). *WOX2* and *WOX8* mRNAs have been detected in the egg cell and the zygote before they become restricted to the apical (*WOX2*) and basal (*WOX8*) daughter cell by the zygotic division (Haecker et al., 2004). It is unclear whether both genes are transcribed in the zygote or whether their mRNA is inherited from the egg cell, similar to the situation in animals where transcription is largely inhibited in the zygote (Schier, 2007). After the zygote division, *WOX8* (also named *STIMPY-LIKE*, Wu et al., 2007) and its close homolog *WOX9* (also called *STIMPY*, Wu et al., 2005) regulate development of the basal embryo lineage and also of the apical embryo lineage (the embryo proper) via non-cell autonomous activation of *WOX2* (Breuninger et al., 2008). *WOX2* triggers expression of the auxin transport promoting protein PINFORMED1 (PIN1) and localization of auxin to the root pole and the cotyledonary tips of the growing embryo (Breuninger et al., 2008). Recent reports also demonstrate an important role of *WOX9/STIMPY* in maintenance and growth of the shoot meristem (Wu et al., 2005; Skylar et al., 2010). In contrast to these later developmental stages, the role of WOX mRNAs in the zygote has been obscure since neither single or multiple mutants display any abnormalities in the zygote or its asymmetric cell division (Breuninger et al., 2008). Mutations affecting the MAPKK kinase YODA (YDA) signaling pathway suppress elongation and asymmetric division of the zygote and development of the suspensor, suggesting that MAP kinase signaling promotes extraembryonic fate in the basal lineage (Bayer et al., 2009; Lukowitz et al., 2004; Wang et al., 2007). Genetic

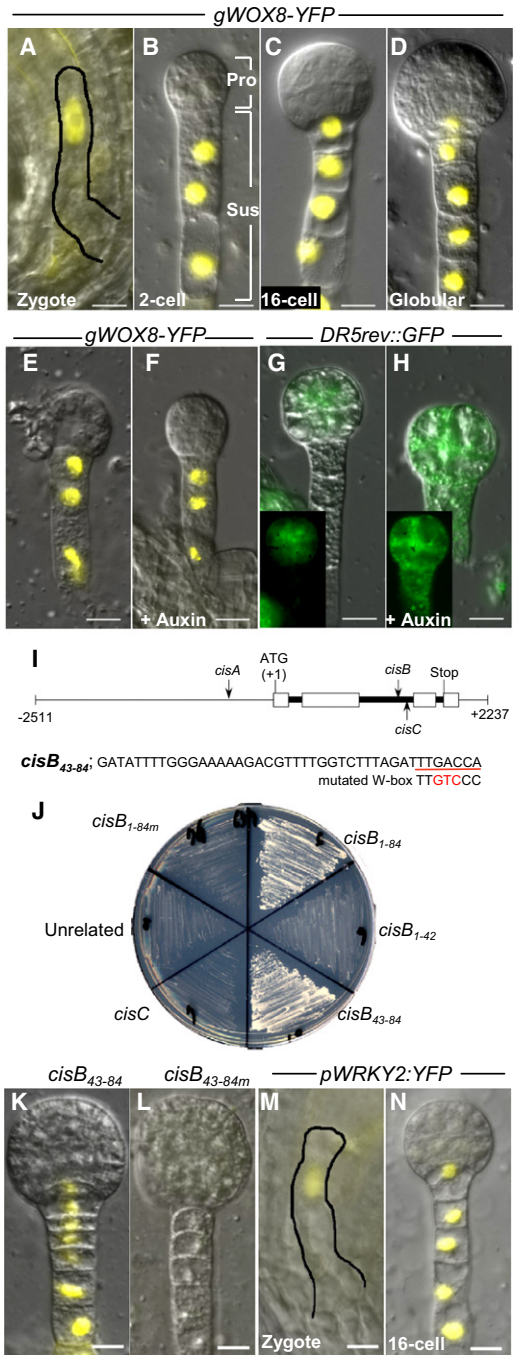

Figure 1. Regulation of *WOX8* Expression

(A–D) *gWOX8-YFP* expression in wild-type. The zygote is outlined (A). The proembryo (pro) and the suspensor (sus) are marked (B), and the cell numbers of the proembryo are indicated (B and C).

(E–H) 8-cell embryos from in vitro cultured wild-type ovules treated without (E and G) and with (F and H; + Auxin) 2,4-D. The control *DR5rev::GFP* signal marks the cells responding auxin. The insets show the GFP signal without DIC images.

(I) *WOX8* genomic structure and the position of the identified *cis* elements. Exons, introns, and nontranscribed regions are shown as boxes, thick and thin lines, respectively. The $cisB_{43-84}$ sequence is shown underneath. The conserved W-box is underlined, and the introduced TGA to GTC mutation is indicated.

(J) Growth on histidine free media of yeast containing the histidine synthetase gene driven by the indicated *WOX8* fragments and WRKY2-AD.

(K and L) *WOX8*-like YFP signal of $cisB_{43-84}$*:YFP* (K) and no expression of mutated $cisB_{43-84m}$*:YFP* (L).

(M and N) *pWRKY2:YFP* expression in the zygote (M; outlined) and 16-cell embryo (N).

Scale bars, 10 μm. See also Figure S1.

interaction studies suggested that WOX and YDA pathways converge in early embryo patterning (Breuninger et al., 2008).

Here, we address how the WOX pathway is activated during early *Arabidopsis* patterning. We show that the *WOX8* expression pattern is not affected by auxin but requires the plant specific zinc-finger transcription factor WRKY2. WRKY2 directly activates *WOX8* transcription in the zygote to regulate polar organelle localization and asymmetric division of the zygote, thus linking zygote polarity to regulators of asymmetric embryo lineage development.

RESULTS

WRKY2 Directly Activates Transcription of *WOX8* via Its W-box Promoter Motif

In order to reveal mechanisms governing the asymmetry of the zygote division, we searched for factors regulating the asymmetric expression of *WOX8*, the most upstream factor of the *WOX* pathway in embryo patterning by using a *gWOX8-YFP* reporter gene that mimicked *WOX8* mRNA expression (Breuninger et al., 2008). Auxin distribution is affected by WOX2 at later embryo stages (Breuninger et al., 2008), but since auxin function in patterning frequently involves reinforcing feedback loops (Smith and Bayer, 2009) and auxin response is observed after the zygotic division in the apical descendants in a complementary pattern to *WOX8* mRNA (Friml et al., 2003; Haecker et al., 2004), we addressed whether auxin might repress *WOX8* expression in the apical embryo lineage. However, application of the synthetic auxin 2,4-D did not noticeably affect *gWOX8-YFP* expression at embryo stages when endogenous auxin accumulates apically (8-cell) or basally (globular) (Figures 1E and 1F; see Figure S1 available online), whereas expression of the auxin response gene *DR5rev::GFP* as a control was induced (Figures 1G and 1H). Furthermore, *gWOX8-YFP* expression pattern was not affected in *gnom* (*gn*) embryos that are defective in vesicle trafficking disturbing embryo division patterns and polar auxin transport (Mayer et al., 1993; Steinmann et al., 1999), or in *monopteros* (*mp*) embryos that are compromised in auxin signal response (Hardtke and Berleth, 1998) (Figure S1). Application of the cytokinin 6-benzylaminopurine (BAP), which antagonizes auxin function in embryonic root formation (Figure S1) (Muller and Sheen, 2008) also did not affect *gWOX8-YFP* expression. Thus, *WOX8* expression appears not to be patterned by auxin distribution.

In order to isolate direct regulators of *WOX8* transcription during early embryo patterning, we first analyzed a series of promoter deletions, which indicated the presence of multiple redundant *cis* elements (Figure S1). In a second series of experiments, we expressed NLS-YFP from tetramers of progressively smaller *WOX8* promoter fragments (Figure S1). This resulted in the identification of three separate promoter elements, named

cisA, *cisB*, and *cisC* (Figure 1I; Figure S1), each of which was sufficient to direct NLS-YFP expression in a *WOX8*-like pattern (Figure 1K; Figure S1). No common sequence motif is present within these three elements, suggesting that *WOX8* expression is redundantly regulated by factors with different DNA binding specificities. Based on a yeast-one-hybrid screen using an expression library constructed from ovule cDNA, we isolated *WRKY2* (At5g56270) cDNA as a potential interactor of *cisB* (Figure 1J), but no other interactors with specific binding to *cisB*, *cisA*, or *cisC* could be isolated.

WRKY proteins are zinc-finger domain transcription factors specific to plants, which have been implicated in environmental and developmental interactions (Eulgem et al., 2000), but not in embryo patterning. WRKY proteins bind with high affinity to the canonical W-box TTGACC/T (Eulgem et al., 2000). The activation of the *cisB* construct by WRKY2 in yeast was restricted to the 3′ half ($cisB_{43\text{-}84}$) of *cisB*, which contains a W-box (Figure 1I). Furthermore, point mutations in the W-box of *cisB* ($cisB_{1\text{-}84\ m}$) abolished WRKY2 dependent transcription (Figures 1I and 1J). Interaction of WRKY2 and $cisB_{1\text{-}84}$ was independently confirmed using a transactivation assay in *Arabidopsis* protoplasts (Figure S1). Taken together, these results show that WRKY2 specifically activated transcription from the W-box of the *WOX8* intron fragment *cisB*.

WRKY2 Regulates *WOX8* and *WOX9* Expression and Basal Cell Division Patterns during Early Embryogenesis

Consistent with a function of *WRKY2* in regulating *WOX8* expression, *pWRKY2:YFP* and *WOX8* expression patterns overlapped in the zygote and the basal embryo lineage (Figures 1M and 1N, compare to Figures 1A and 1C). We analyzed five independent lines with sufficient expression levels, all of which displayed the same expression pattern as shown in Figures 1M and 1N. Furthermore, the 3.4 kb promoter fragment used in *pWRKY2:YFP* was sufficient to completely rescue the *wrky2-1* mutant (see below) when driving *WRKY2* cDNA (99% wild-type-like zygotes, n = 119; four out of four transformants). Together, these results suggest that *WRKY2* is expressed in a similar pattern as *WOX8*. Furthermore, the same point mutations in the W-box that abolished WRKY2 dependent activation in yeast also abolished $cisB_{43\text{-}84}$*:YFP* expression in *Arabidopsis* embryos (Figure 1L). We further found that the *WOX8* cDNA driven from *cisB* significantly restored the defect of *wox8 wox9* mutant as early as two to eight-cell stage (Table S2), supporting the role of *cisB* in *WOX8* expression. The incomplete rescue is consistent with the importance of the promoter fragments other than *cisB* for normal *WOX8* expression levels.

To address whether WRKY2 activity is required for *WOX8* expression during embryogenesis, we characterized three loss-of-function mutants from T-DNA mutant collections (Figure S2). Among them, *wrky2-1* showed the strongest defects in embryo development (Figure S2; see below) that were complemented by transformation with a wild-type *WRKY2* copy (Figure 3I; Table S1), and did not contain detectable amounts of *WRKY2* transcript (Figure S2), suggesting that *wrky2-1* is a strong loss of function or a null allele. *wrky2-1* displayed an almost complete reduction of $cisB_{43\text{-}84}$*:YFP* expression in the zygote and the basal daughter cell compared to wild-type (Table S3). YFP expression from the complete *WOX8* promoter

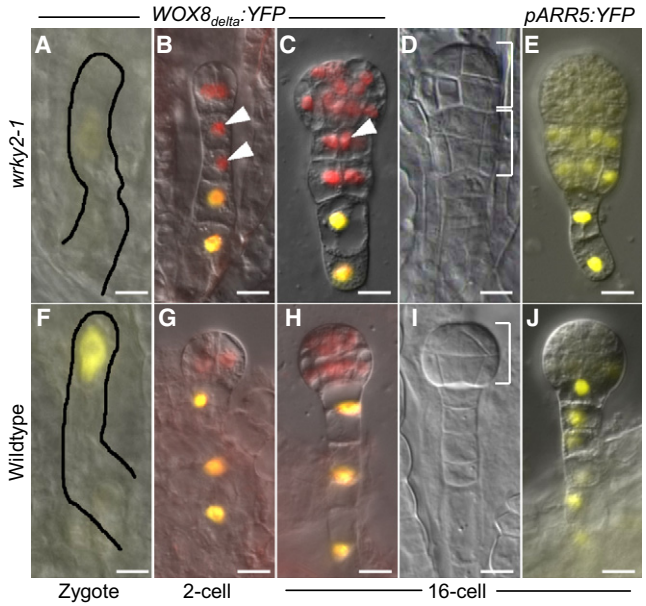

Figure 2. *WRKY2* Is Required for *WOX8* Expression in the Early Embryo

(A–C and F–H) $WOX8_{delta}$*:YFP* expression in *wrky2-1* and wild-type as indicated. Arrowheads show suspensor cells lacking *WOX8* reporter expression in *wrky2-1* (B) that at later stages divide abnormally (C). *pWOX2:DsRed2* expression (red) marks all embryo nuclei.

(D and I) DIC images. The proembryo-like structures are marked by parenthesis.

(E and J) *pARR5:YFP* expression. Cell numbers refer to wild-type stages.

Scale bars, 10 μm. See also Figure S2 and Tables S1–S3.

($WOX8_{delta}$*:YFP*; Breuninger et al., 2008) was also significantly reduced at these stages compared to wild-type (Figures 2A and 2F; Table S3), but was not completely absent, likely due to weak expression provided by *cisA* and *cisC* elements in the *WOX8* promoter (Figure 1I). At later stages, $WOX8_{delta}$*:YFP* is expressed in wild-type in all basal lineage cells. By contrast, $WOX8_{delta}$*:YFP* signal was undetectable in the hypophysis and often also in the directly subtending suspensor cell of *wrky2-1*, but remained expressed at near wild-type levels in the residual suspensor cells (Figures 2B and 2G). The *WOX9* gene, which is the closest homolog of *WOX8*, is coexpressed with and acts redundantly to *WOX8* in early embryo patterning (Breuninger et al., 2008; Wu et al., 2007). The *WOX9* promoter also carries a W-box and we found that expression of a *pWOX9:YFP* reporter gene was also significantly reduced in *wrky2-1* zygotes, 1-cell embryos (Table S3), and at later stages in the uppermost suspensor cells (Figure S2). Together, this indicates that *WRKY2* is an essential transcriptional activator of the two redundant embryo patterning genes, *WOX8* and *WOX9* in the zygote, basal daughter cell, and uppermost suspensor cells.

The uppermost cells of the basal lineage that lacked detectable *WOX8/9* expression in *wrky2-1* divided abnormally and gave rise to a second proembryo-like structure (Figures 2C, 2D, 2H, and 2I; Table S1), suggesting a transition from basal toward apical division patterns. Nevertheless, these cells still expressed the basal lineage marker *ARABIDOPSIS RESPONSE REGULATOR5* (D'Agostino et al., 2000; *pARR5:YFP*; Figures 2E

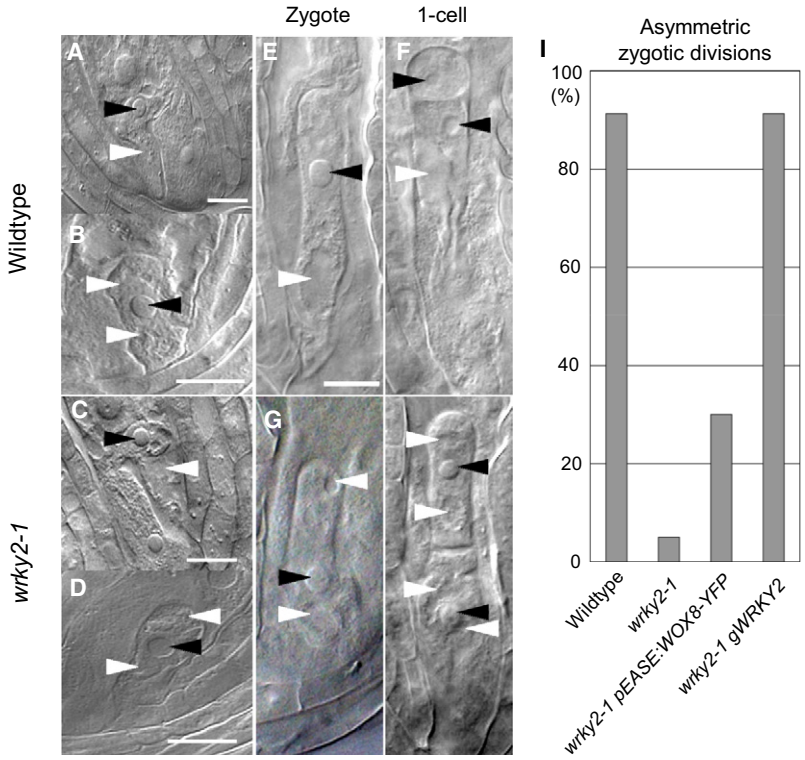

Figure 3. *WRKY2* Regulates the Cell Polarity and Asymmetric Division of the Zygote

(A–D) Nucleus (black arrowheads) and vacuoles (white arrowheads) positions in *wrky2-1* egg cells (C) and early zygotes (D) are not different from wild-type (A and B).

(E–H) In *wrky2-1* late zygotes, the nucleus and the vacuoles are mislocalized, and the division occurs more symmetrically than in wild-type.

(I) Frequency of wild-type-like asymmetric zygotic divisions where the apical cell typically is less than one third of the basal cell in length. The genotypes are indicated. Differences between *wrky2-1* and wild-type, and between *wrky2-1* and *wrky2-1 pEASE:WOX8-YFP* are significant ($p < 0.01$ by two-tailed Fisher's exact test).

Scale bars, 10 μm. See also Figure S3 and Table S1.

and 2J) and not the proembryo marker *ZWILLE* (Tucker et al., 2008; not shown). Thus, *WRKY2* is required at this stage for some but not all aspects of basal lineage identity. At the heart stage, *wrky2-1* embryos started to recover (Figure S2) and eventually gave rise to viable seeds, suggesting that early defects were compensated by alternative factors, as suggested by the high expression levels of *WOX8/9* in the suspensor (Figures 2B and 2C), and similarly to recoveries of previously characterized early embryo patterning mutants (Bayer et al., 2009; Friml et al., 2003).

WRKY2 Is Required to Repolarize the Zygote from a Transient Symmetric State

Since *WRKY2* was required for *WOX8/9* expression already in the zygote, we analyzed its potential function there. In wild-type, the unfertilized egg cell is polarized with the nucleus at the apical and the vacuoles at the basal position (Figure 3A). After fertilization, we found that the nucleus is initially in a central position and the vacuoles are evenly distributed (Figure 3B) before the nucleus becomes localized to the apical part and a large vacuole is formed at the basal end of the zygote (Figure 3E). Subsequently, only the large basal daughter cell inherits large vacuoles (Figure 3F). Thus, the polar distribution of organelles is not contiguous between the egg cell and the zygote, but the zygote is repolarized from a transient symmetric stage. *wrky2-1* was indistinguishable from wild-type at the egg cell and the symmetric zygote stage (Figures 3C and 3D). However, during the repolarization stage, *wrky2-1* zygotes did not position the nucleus apically (Figure 3G) and the vacuoles were not localized at the basal pole as in wild-type, but remained dispersed throughout the cell (23.1%, n = 117 versus 2.2%, n = 228 in wild-type; $p < 0.01$ by two-tailed Fisher's exact test). Subsequently, *wrky2-1* zygotes divided more symmetrically (Figures 3H and 3I) than wild-type. Only 5.0% of *wrky2-1* zygotes showed a wild-type-like division where the apical cell is less than one third of the basal cell in length (Figure 3I), and 21.3% of *wrky2-1* zygotes displayed a completely equal division (Figure 3H; n = 80 in all cases, $p < 0.01$ by two-tailed Fisher's exact test). After the division, prominent vacuoles were detected not only in the basal daughter cell of *wrky2-1*, like wild-type, but also in the apical daughter cell (Figure 3H). These results were confirmed using optical sectioning of embedded whole-mount ovules (Figure S3, compare to Figure 3). Together, *WRKY2* function is specifically required to reestablish the polar organization of the zygote and to break the symmetry of its division.

WRKY2-Dependent Repolarization Is Mediated via *WOX8* Transcription in the Zygote Itself

To test whether *WRKY2* function in zygote polarity is mediated by its target *WOX8*, we expressed a fully functional WOX8-YFP protein from the *pEASE* (*Egg Apparatus-Specific Enhancer*) promoter, which is active from the late stage of the female gametophyte until the globular embryo (Yang et al., 2005). In all 38 independent transformants, *pEASE:WOX8-YFP* expression in *wrky2-1* mutants significantly restored wild-type-like zygotes as shown in Figure 3I and globular embryos as given in Table S1. Together, WRKY2 is required for zygotic asymmetry and hypophysis development partially via its target gene *WOX8*.

Our finding that WRKY2 is required for establishment of polarity in the zygote but not in the egg cell raised the question whether WRKY2 activity after fertilization is sufficient to set up zygotic polarity. To test this idea, we fertilized *wrky2-1* mutant egg cell with wild-type pollen. This cross resulted in zygotic division (Figure 4A) and embryo development (Table S1) that were indistinguishable from wild-type, showing that *WRKY2* (or its downstream activities) is fully sufficient to establish zygote polarity after fertilization. In control reciprocal crosses, normal zygote divisions were also restored, indicating that the male or female *WRKY2* copies are equally sufficient for wild-type development.

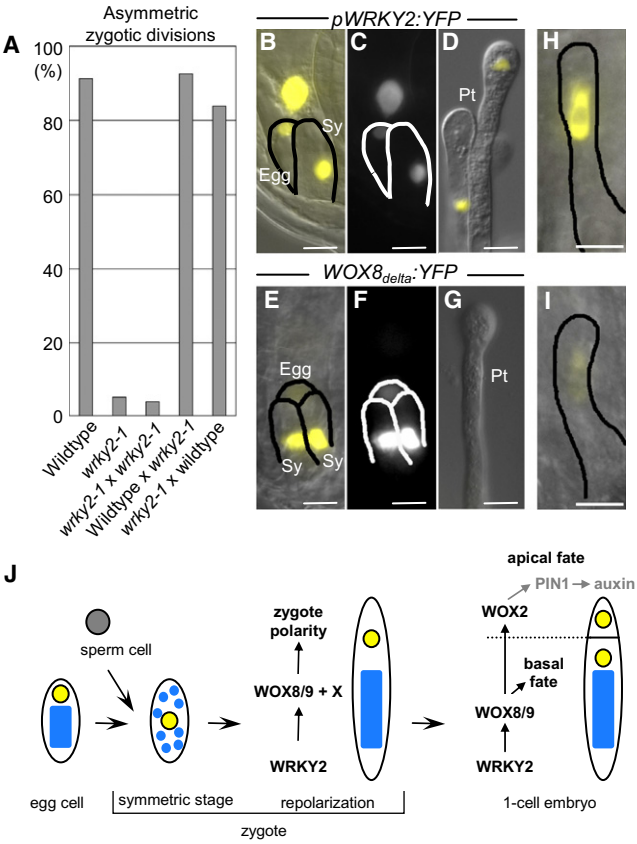

Figure 4. WRKY2-Mediated Transcriptional Activation in the Zygote Regulates Polarity

(A) Frequency of wild-type-like asymmetric divisions. The genotypes are indicated, and the cross is denoted as female x male. The differences between the reciprocal crosses and the selfed mutant are significant ($p < 0.01$ by two-tailed Fisher's exact test).

(B–G) *pWRKY2:YFP* and *WOX8$_{delta}$:YFP* expression as indicated in egg cells (egg), synergids (sy) and pollen tubes (pt). (B and E) Overlays of DIC images and the YFP images from (C and F).

(H and I) *WOX8$_{delta}$:YFP* expression in zygotes generated by the cross of wild-type (H) or *wrky2-1* (I) females to *wrky2-1* males possessing *WOX8$_{delta}$:YFP*. Cell structures are outlined.

(J) Model for the role of the WRKY2-WOX8/9 transcription cascade. In the zygote WRKY2 is the major transcriptional activator of *WOX8/9* and a redundant target (X) to rebuild the polar organization of the nucleus (yellow) and vacuole (blue). In the resulting asymmetric embryo, this pathway promotes development of the basal lineage and noncell autonomously also of the embryo proper via regulating *WOX2* expression and auxin distribution. Yet unidentified activators other than WRKY2 that provide only a small contribution to *WOX8/WOX9* transcription in the zygote, but full expression after the 1-cell stage in most basal descendents, are not shown for clarity. Gray indicates that the precise stage when WOX2 affects auxin transport (via PIN1) and distribution is yet to be determined.

Scale bars, 10 μm. See also Tables S1 and S3.

Expression of *pWRKY2:YFP* was detected in both the unfertilized egg cell and the pollen tube, which delivers the sperm cell to the egg cell (Figures 4B–4D), raising the possibility that WRKY2 mRNA or protein is delivered by one of the gametes to the zygote. By contrast, *WOX8$_{delta}$:YFP* expression was detectable only in the egg cell but was absent from pollen tubes (Figures 4E–4G), consistent with published transcriptome data (Borges et al., 2008). This situation enabled us to directly address whether WRKY2 is able to activate *WOX8* transcription in the zygote or whether the *WOX8* transcript might be solely inherited from the unfertilized egg cell. Crossing with pollen from a *wrky2-1* plant carrying the *WOX8$_{delta}$:YFP* reporter resulted in significantly higher YFP signal in zygotes when the mother plant was wild-type than when it was *wrky2-1* (Figures 4H and 4I; Table S3). This indicates that *WOX8* transcription is activated by WRKY2 after fertilization in the zygote.

DISCUSSION

One of the first patterning events in the life of plants and animals is the initiation of the major embryo axis. In several well-studied animal model organisms, including *Xenopus* and *Drosophila*, polarity is already established in the unfertilized egg by maternal factors and subsequently translated into the embryo axis (Riechmann and Ephrussi, 2001; Weaver and Kimelman, 2004). In higher plants, the unfertilized egg cell and the zygote are both polarized with the nucleus at the apical and vacuoles at the basal end. Notably, the axis of polarity of the zygote aligns with the apical-basal axis of the developing embryo, but it has been unclear whether the cellular polarity of the zygote is connected to the regulation of embryo development. Here, we identified WRKY2 as a direct transcriptional regulator of the axis patterning genes *WOX8* and *WOX9* and show that this pathway not only regulates asymmetric embryo lineage development after the division of the zygote as previously reported (Breuninger et al., 2008), but also the polarity of the zygote itself (Figure 4J).

We found that the polar organization of nucleus and vacuoles is not contiguous between egg cell and zygote in *Arabidopsis*, but is rebuilt from a transient symmetric state and that this repolarization step requires WRKY2. By contrast, egg cell polarity is normal in *wrky2* mutants, indicating different genetic requirements between the polarization processes of the unfertilized egg cell and the zygote. We realize that the transient symmetric stage of the zygote is at odds with previous findings from sectioned material which suggested that the *Arabidopsis* zygote is always polar (Mansfield and Briarty, 1991), but it is in agreement with recent confocal microscopy studies (Christensen et al., 2002; Faure et al., 2002). While it is possible that unidentified polar marks underlying the position of cell organelles are preserved between the egg cell and zygote, active repositioning of nucleus and vacuoles regulated by WRKY2 takes place before the asymmetric division of the *Arabidopsis* zygote. Disruption of this process conceivably caused symmetry of the zygotic division and abnormal vacuolization of the apical daughter cell as observed in *wrky2* mutants, underlining the significance of zygote polarity for setting up the properties of the daughter cells. Interestingly, the strongest division defect in *wrky2* zygotes is an equal division, as opposed to a reversal of asymmetry, suggesting that WRKY2 activity primarily breaks symmetry of the zygote, rather than shifting the position of the nucleus and the cell division plane in a graded way.

WRKY genes have been reported to be involved in biotic and abiotic stress responses (Eulgem et al., 2000), and developmental programs, such as seed size regulation (Luo et al., 2005) and trichome formation (Johnson et al., 2002). In seedlings, *WRKY2* expression is upregulated by the plant hormone

abscisic acid (ABA) and in turn suppresses ABA response by downregulating *ABI3* and *ABI5* genes (Jiang and Yu, 2009). However, our preliminary studies did not reveal any effect of ABA on early embryogenesis (J. Bellis and T.L. unpublished data). Rather, our results reported here show that WRKY2 regulates zygote repolarization in part through activation of *WOX8* transcription, and by inference of its functional homolog *WOX9*. We postulate additional targets for WRKY2 in the zygote that act redundantly to *WOX8/9* (Figure 4J), since the *wox8 wox9* double mutant did not give a clear zygote phenotype (Breuninger et al., 2008). During embryogenesis in animals, de novo transcription in the zygote is generally suppressed by various mechanisms, such as the exclusion of transcription machinery from nuclei, and thus early embryo patterning solely depends on maternal transcripts stored in the egg cell (Guven-Ozkan et al., 2008; Liang et al., 2008; Schier, 2007). Thus, the establishment of zygote polarity by transcriptional activation of *WOX8/9* in the zygote itself reported here may point toward a major difference in axis formation between plants and animals.

Taken together, our results suggest a model in which WRKY2-WOX8/9 transcription cascade establishes zygote polarity by initiating a shift in organelle positions in the zygote and thus enables asymmetric cell division (Figure 4J). Subsequently, this pathway regulates development of the basal and apical descendants including non-cell autonomous activation of *WOX2* in the embryo proper (Figure 4J). We did not observe polar localization of functional WOX8-YFP protein in the zygote (Figure 1A). However, since we cannot exclude a short transient polar localization, the mechanism by which WOX8 expression becomes confined to the basal daughter remains to be resolved. Our results suggest that auxin, which has previously been shown to be required for embryo axis formation (Friml et al., 2003), does not appear to pattern *WOX8* expression. This, together with the finding that *WOX2* is required to localize auxin to the hypophysis and the cotyledonary tips of developing embryos (Breuninger et al., 2008), suggests that the WRKY2-WOX pathway acts prior to and upstream of auxin pattern in the embryo, and that auxin acts to interpret axis information after the division of the zygote. Consistent with this hypothesis, developmental defects in auxin-related mutants have been detected only after the zygote division (Moller and Weijers, 2009). Since auxin was found to pattern the female gametophyte (Pagnussat et al., 2009) and the *gn* mutant has been reported to cause occasional asymmetric division (Mayer et al., 1993), additional yet uncovered functions of auxin in the zygote are possible. Another important regulator of the asymmetric division of the zygote is the YDA MAPKK pathway (Lukowitz et al., 2004; Bayer et al., 2009). *yda* zygotes like *wrky2* divide symmetrically, and double mutant zygotes resemble the ones of both single mutants (n = 97; not shown). Future work will address how key polarity components are connected in regulating the asymmetric division of the zygote, and how WRKY2-WOX activity in the zygote is translated into cellular polarity.

EXPERIMENTAL PROCEDURES

Strains

Arabidopsis lines used were in Columbia (Col) background, except for *gn* (*gn 4-13*) (Steinmann et al., 1999) and *wrky2-3* in Landsberg *erecta* (*Ler*). The *mp* mutant (*mpB4149*) and *DR5rev::GFP* were described previously (Friml et al., 2003; Hardtke and Berleth, 1998). The *wrky2-1* (SALK_020399), *wrky2-3* (GT_5_107439), and *wrky2-4* (GK_024B05) mutants were identified in the SALK, FGT (John Innes Center), and GABI-Kat databases, respectively.

Growth Conditions and Microscopy

Plant growth condition and microscopy were described previously (Breuninger et al., 2008). In vitro ovule culture was done as previously reported with 50 μM of 2,4-D or BAP (Sauer and Friml, 2004). For in vitro pollen germination, pollinated pistils were excised and incubated overnight on MS plates at room temperature. Optical sectioning of ovules by confocal microscopy was performed as described (Christensen et al., 2002; Faure et al., 2002).

Yeast Assays

The yeast-one-hybrid screen was performed as described (Lopato et al., 2006). The cDNA was constructed from ovules containing embryos at 8-cell to 16-cell stages. Full-length *WRKY2* cDNA was used for construction of WRKY2-AD.

RT-PCR

RT-PCR was performed as previously described (Breuninger et al., 2008) using RNA from inflorescences and *WRKY2* primers (GGAGAATTCATGGCTGGT TTTGATGAAAATGTTG and GAGCTCGAGTCAAATCTGAGGTAATCTACTCA TG), or *PP2AA3* primers (CGTTACTGCCAGCCATTGTAGAA and CCGCAGG TAAGAGTTTGGAACAT).

Protoplast Transactivation

Protoplast transformation method was described previously (Yoo et al., 2007). NLS-CFP and WRKY2-AD, which had been used for yeast assay, both were expressed under the control of the 35S promoter.

Plasmid Construction and Transgenic Plants

gWOX8-YFP includes a 4.7 kb genomic *WOX8* fragment with a triple NLS-venusYFP (NLS-YFPx3; (Nagai et al., 2002). The homeodomain was deleted in $WOX8_{delta}$*:YFP*. The YFP reporters of *cis* elements consist of a quadruple repeat of each element, CaMV 35S minimal promoter, and NLS-YFPx3 and 35S terminator. *gWRKY2* spans a 6.3 kb genomic *WRKY2* fragment including a 3.4 kb upstream region, which was fused to NLS-YFPx3 in *pWRKY2:YFP*. The previously described *pWOX9:YFP*, *pARR5:YFP* or *gZLL:YFP* was combined with *pWOX2:DsRed2*, which includes a 3.5 kb WOX2 promoter driving a triple NLS-DsRed2 (Tucker et al., 2008). *pEASE:WOX8-YFP* consists of a quadruple *EASE* fragment and *WOX8* gene fused to single YFP (Yang et al., 2005). Details of cloning procedures are available in Supplemental Experimental Procedures.

All of the PCR-derived clones were confirmed by sequencing. Plants were transformed using the floral dip method (Clough and Bent, 1998), and T1 plants were selected on soil with BASTA or on plates with hygromycin.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and three tables and can be found with this article online at doi:10.1016/j.devcel.2011.01.009.

ACKNOWLEDGMENTS

We thank members of the Laux laboratory for helpful comments on the manuscript. We thank T. Higashiyama for experimental support, P.B.F. Ouwerkerk for the pINT-HIS3 vector, D. Weijers for the *mpB4149* allele, and J. Friml and G. Jürgens for the *DR5rev::GFP* reporter line. We gratefully acknowledge financial support from the Deutsche Forschungsgemeinschaft (Arabidopsis Functional Genomic Network) and the EU (SIREN) to T.L. and by a JSPS fellowship to M.U.

Received: April 26, 2010
Revised: January 5, 2011
Accepted: January 24, 2011
Published: February 14, 2011

REFERENCES

Bayer, M., Nawy, T., Giglione, C., Galli, M., Meinnel, T., and Lukowitz, W. (2009). Paternal control of embryonic patterning in Arabidopsis thaliana. Science *323*, 1485–1488.

Borges, F., Gomes, G., Gardner, R., Moreno, N., McCormick, S., Feijo, J.A., and Becker, J.D. (2008). Comparative transcriptomics of Arabidopsis sperm cells. Plant Physiol. *148*, 1168–1181.

Breuninger, H., Rikirsch, E., Hermann, M., Ueda, M., and Laux, T. (2008). Differential expression of WOX genes mediates apical-basal axis formation in the Arabidopsis embryo. Dev. Cell *14*, 867–876.

Christensen, C.A., Gorsich, S.W., Brown, R.H., Jones, L.G., Brown, J., Shaw, J.M., and Drews, G.N. (2002). Mitochondrial GFA2 is required for synergid cell death in Arabidopsis. Plant Cell *14*, 2215–2232.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. *16*, 735–743.

D'Agostino, I.B., Deruere, J., and Kieber, J.J. (2000). Characterization of the response of the Arabidopsis response regulator gene family to cytokinin. Plant Physiol. *124*, 1706–1717.

Dolan, L., Janmaat, K., Willemsen, V., Linstead, P., Poethig, S., Roberts, K., and Scheres, B. (1993). Cellular organisation of the Arabidopsis thaliana root. Development *119*, 71–84.

Eulgem, T., Rushton, P.J., Robatzek, S., and Somssich, I.E. (2000). The WRKY superfamily of plant transcription factors. Trends Plant Sci. *5*, 199–206.

Faure, J.E., Rotman, N., Fortune, P., and Dumas, C. (2002). Fertilization in Arabidopsis thaliana wild type: developmental stages and time course. Plant J. *30*, 481–488.

Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa, R., and Jürgens, G. (2003). Efflux-dependent auxin gradients establish the apical-basal axis of Arabidopsis. Nature *426*, 147–153.

Guven-Ozkan, T., Nishi, Y., Robertson, S.M., and Lin, R. (2008). Global transcriptional repression in C. elegans germline precursors by regulated sequestration of TAF-4. Cell *135*, 149–160.

Haecker, A., Gross-Hardt, R., Geiges, B., Sarkar, A., Breuninger, H., Herrmann, M., and Laux, T. (2004). Expression dynamics of WOX genes mark cell fate decisions during early embryonic patterning in Arabidopsis thaliana. Development *131*, 657–668.

Hardtke, C.S., and Berleth, T. (1998). The Arabidopsis gene MONOPTEROS encodes a transcription factor mediating embryo axis formation and vascular development. EMBO J. *17*, 1405–1411.

Jiang, W., and Yu, D. (2009). Arabidopsis WRKY2 transcription factor mediates seed germination and postgermination arrest of development by abscisic acid. BMC Plant Biol. *9*, 96.

Johnson, C.S., Kolevski, B., and Smyth, D.R. (2002). TRANSPARENT TESTA GLABRA2, a trichome and seed coat development gene of Arabidopsis, encodes a WRKY transcription factor. Plant Cell *14*, 1359–1375.

Jürgens, G., and Mayer, U. (1994). Arabidopsis. In A Colour Atlas of Developing Embryos, J. Bard, ed. (London: Wolfe Publishing), pp. 7–21.

Liang, H.L., Nien, C.Y., Liu, H.Y., Metzstein, M.M., Kirov, N., and Rushlow, C. (2008). The zinc-finger protein Zelda is a key activator of the early zygotic genome in Drosophila. Nature *456*, 400–403.

Lopato, S., Bazanova, N., Morran, S., Milligan, A.S., Shirley, N., and Langridge, P. (2006). Isolation of plant transcription factors using a modified yeast one-hybrid system. Plant Methods *2*, 3.

Lukowitz, W., Roeder, A., Parmenter, D., and Somerville, C. (2004). A MAPKK kinase gene regulates extra-embryonic cell fate in Arabidopsis. Cell *116*, 109–119.

Luo, M., Dennis, E.S., Berger, F., Peacock, W.J., and Chaudhury, A. (2005). MINISEED3 (MINI3), a WRKY family gene, and HAIKU2 (IKU2), a leucine-rich repeat (LRR) KINASE gene, are regulators of seed size in Arabidopsis. Proc. Natl. Acad. Sci. USA *102*, 17531–17536.

Mansfield, S.G., and Briarty, L.G. (1991). Early embryogenesis in Arabidopsis thaliana. II. The developing embryo. Can. J. Bot. *69*, 461–476.

Mayer, U., Büttner, G., and Jürgens, G. (1993). Apical-basal pattern formation in the Arabidopsis embryo: Studies on the role of the gnom gene. Development *117*, 149–162.

Moller, B., and Weijers, D. (2009). Auxin control of embryo patterning. Cold Spring Harb Perspect Biol *1*, a001545.

Muller, B., and Sheen, J. (2008). Cytokinin and auxin interaction in root stem-cell specification during early embryogenesis. Nature *453*, 1094–1097.

Nagai, T., Ibata, K., Park, E.S., Kubota, M., Mikoshiba, K., and Miyawaki, A. (2002). A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. Nat. Biotechnol. *20*, 87–90.

Pagnussat, G.C., Alandete-Saez, M., Bowman, J.L., and Sundaresan, V. (2009). Auxin-dependent patterning and gamete specification in the Arabidopsis female gametophyte. Science *324*, 1684–1689.

Riechmann, V., and Ephrussi, A. (2001). Axis formation during Drosophila oogenesis. Curr. Opin. Genet. Dev. *11*, 374–383.

Sauer, M., and Friml, J. (2004). In vitro culture of Arabidopsis embryos within their ovules. Plant J. *40*, 835–843.

Schier, A.F. (2007). The maternal-zygotic transition: death and birth of RNAs. Science *316*, 406–407.

Skylar, A., Hong, F., Chory, J., Weigel, D., and Wu, X. (2010). STIMPY mediates cytokinin signaling during shoot meristem establishment in Arabidopsis seedlings. Development *137*, 541–549.

Smith, R.S., and Bayer, E.M. (2009). Auxin transport-feedback models of patterning in plants. Plant Cell Environ. *32*, 1258–1271.

Steinmann, T., Geldner, N., Grebe, M., Mangold, S., Jackson, C.L., Paris, S., Galweiler, L., Palme, K., and Jürgens, G. (1999). Coordinated polar localization of auxin efflux carrier PIN1 by GNOM ARF GEF. Science *286*, 316–318.

Tucker, M.R., Hinze, A., Tucker, E.J., Takada, S., Jurgens, G., and Laux, T. (2008). Vascular signalling mediated by ZWILLE potentiates WUSCHEL function during shoot meristem stem cell development in the Arabidopsis embryo. Development *135*, 2839–2843.

Wang, H., Ngwenyama, N., Liu, Y., Walker, J.C., and Zhang, S. (2007). Stomatal development and patterning are regulated by environmentally responsive mitogen-activated protein kinases in Arabidopsis. Plant Cell *19*, 63–73.

Weaver, C., and Kimelman, D. (2004). Move it or lose it: axis specification in Xenopus. Development *131*, 3491–3499.

Wu, X., Chory, J., and Weigel, D. (2007). Combinations of WOX activities regulate tissue proliferation during Arabidopsis embryonic development. Dev. Biol. *309*, 306–316.

Wu, X., Dabi, T., and Weigel, D. (2005). Requirement of Homeobox Gene STIMPY/WOX9 for Arabidopsis Meristem Growth and Maintenance. Curr. Biol. *15*, 436–440.

Yang, W., Jefferson, R.A., Huttner, E., Moore, J.M., Gagliano, W.B., and Grossniklaus, U. (2005). An egg apparatus-specific enhancer of Arabidopsis, identified by enhancer detection. Plant Physiol. *139*, 1421–1432.

Yoo, S.D., Cho, Y.H., and Sheen, J. (2007). Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat. Protoc. *2*, 1565–1572.