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The *Arabidopsis* RWP-RK protein RKD4 triggers gene expression and pattern formation in early embryogenesis

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Arabidopsis RWP-RK protein triggers embryogenesis

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Summary

Morphogenesis of seed plants commences with highly stereotypical cell division sequences in early embryogenesis [1, 2]. Although a small number of transcription factors and a mitogen-activated protein kinase (MAP) cascade have been implicated in this process [3-8], pattern formation in early embryogenesis remains poorly understood. We here show that the *Arabidopsis* RKD4, a member of the RWP-RK motif-containing putative transcription factors [9], is required for this process. Loss-of-function *rkd4* mutants were defective in zygotic cell elongation, as well as subsequent cell division patterns. As expected from this mutant phenotype, *RKD4* was transcribed preferentially in early embryos. RKD4 possessed functional characteristics of transcription factors, and was able to ectopically induce early embryo-specific genes when overexpressed in seedlings. Strikingly, induced overexpression of *RKD4* primed somatic cells for embryogenesis independently of external growth regulators. These results reveal that RKD4 is a novel key regulator of the earliest stage of plant development.

Results

Loss-of-function *rkd4* mutants show embryo-specific developmental defects

As a part of a reverse-genetic study of *Arabidopsis* RWP-RK genes, we analyzed two mutant alleles of *RKD4* (At5g53040), designated *rkd4-1* and *rkd4-2* (Figure 1A). We initially found germination defects in both alleles; five days after transferring the seeds to conditions that normally allow synchronous germination, only 33 % and 79 % of the *rkd4-1* and *rkd4-2* seeds germinated, respectively, while the rest did not germinate at all, or terminated their growth after extending a small root-like structure from the seed coat. Most of germinated *rkd4* seedlings had short primary roots or lacked primary roots (Figure 1B). In addition, the cellular architecture of the *rkd4* primary roots were severely disturbed (compare Figures S1A and S1B). *RKD4* transcripts were barely detectable in both *rkd4* alleles by reverse transcription-polymerase chain reaction (RT-PCR) analysis (data not shown). All phenotypic defects were rescued by the introduction of a wild-type genomic fragment spanning from 1.5 kb 5' upstream of the ATG codon to 0.7 kb 3' downstream of the stop codon of *RKD4* (Figures 1C and S1G), indicating that the observed defects were caused by the loss of RKD4 function. *rkd4* seedlings later developed lateral roots with essentially normal cellular patterns (Figure S1C), and mature *rkd4* plants were indistinguishable from wild type (Figure S1D).

The restriction of mutant seedling phenotypes to germination and primary root growth suggested that the loss of *RKD4* primarily affected embryogenesis, and this was confirmed by comparing wild-type and *rkd4* embryogenesis (see below). Because the

embryonic defects of both *rkd4* alleles were very similar, except that a larger proportion of the embryos was affected in *rkd4-1*, we here mainly describe *rkd4-1*. All phenotypic descriptions below are for the embryos from homozygous *rkd4* parents.

After fertilization, wild-type zygotes elongate anisotropically and their nuclei typically become localized to the apical (chalazal) end (Figure 1D and S1E). *rkd4-1* zygotes elongated less than the wild type, but nuclear positioning was maintained at the apical end (Figures 1I and S1E). After the first division, the basal cells of *rkd4-1* embryos were significantly shorter than those of wild-type embryos, whereas the apical cells of *rkd4-1* and wild-type embryos were of comparable size (Figures 1E, 1J and S1F). These observations suggest that the loss of *RKD4* affects zygotic cell elongation and subsequent asymmetric division, though *rkd4-1* zygotes retain cell polarity at least partially.

At the time corresponding to the two- to four-cell stages (Figure 1F), 65 % (n = 34) of the *rkd4-1* embryos exhibited an abnormal arrangement of cells, and their suspensors were consistently shorter than those of wild-type embryos (Figure 1K). Both these phenotypes persisted throughout the subsequent stages (Figures 1L, 1M and S1G). At the time corresponding to the heart stage, the lens-shaped cell (LSC), a progenitor of the root quiescent center [10], appeared only in 33 % (n = 67) and 49 % (n = 78) of *rkd4-1* and *rkd4-2* embryos, respectively (Figures 1M and S1G). Expression of the *WUSCHEL-RELATED HOMEOBOX5 (WOX5)* gene, which normally starts in LSC and later functions in root stem cell maintenance [4, 11], was either lost completely or occurred irregularly (Figure S1H).

The lack of LSC and the root growth defects suggested that auxin distribution

and/or response are impaired in *rkd4* embryos [4, 12-14]. In support of this view, *rkd4* embryos lacked clear polar localization of an auxin efflux facilitator PIN-FORMED1 (PIN1) to the basal side of provascular cells as well as a focused auxin response at the site of root initiation [13-14] normally observed in wild-type (Figures 1N-1Q). These observations indicate that loss of *RKD4* impairs formation of the auxin-mediated embryonic axis and the initiation of organ primodia.

A reciprocal cross between *rkd4-1* and wild-type plants indicated no parent-of-origin effects; F1 embryos developed normally, regardless of whether the female gametophyte (100 %, *n*=28) or pollen (100 %, *n*=44) was wild-type, indicating that RKD4 functions are required after fertilization.

RKD4 is preferentially expressed in early embryos

The severe embryonic defects observed in *rkd4* mutants, as opposed to their normal post-embryonic growth, suggested specific requirement of *RKD4* during early embryogenesis. In support of this view, RT-PCR analysis revealed that *RKD4* transcripts preferentially accumulated in developing seeds (Figure 2A).

To analyze the spatiotemporal expression pattern of *RKD4* during embryogenesis, we prepared *RKD4* reporter constructs. Direct transcriptional fusion of the 0.65-kb *RKD4* promoter to an endoplasmic reticulum (ER)-targeted GFP (*GFPer*) reporter did not yield detectable fluorescence in transgenic plants, probably reflecting the relatively low level of *RKD4* transcription (data not shown). We therefore created a two-component reporter construct, in which the *RKD4* promoter drove the GAL4:VP16 (GV) transcriptional

activator, which in turn activated a *GFPer* reporter included in the same T-DNA (Figure 2B). GFP fluorescence was detected already in fertilized zygotes (Figure 2C) and persisted throughout the embryo proper and suspensor until early globular stage (Figures 2D and 2E). At the late globular stage, GFP fluorescence became confined to the basal part of the embryo (Figure 2F). This pattern was maintained through the triangle stage, after which GFP became restricted to the suspensor (Figure 2G). Expression of an RKD4:GFP fusion protein in the same two-component configuration (Figure S2A) completely rescued *rkd4-1* (Figures S2B-S2D), indicating that the observed expression pattern included all functionally relevant aspects. Fluorescence from the RKD4:GFP protein, however, could not be detected in the rescued embryos possibly because of reduced stability compared to GFPer (Figure S2E).

Post-embryonic RKD4 expression activates early embryo-specific genes

Based on regional similarities to bZIP and bHLH proteins, RWP-RK proteins have been proposed to act as transcription factors [9, 15]. On the basis of the preferential expression of *RKD4* in early embryos and the embryo-specific defects of *rkd4*, we hypothesized that RKD4 regulates early embryogenesis at the level of gene expression. In order to test this possibility, we first examined whether RKD4 possesses functional characteristics of transcription factors. In a transient expression assay with onion epidermis, both RKD4:GFP and GFP:RKD4 fusion proteins localized to the nucleus (Figure S3A). In addition, either the entire RKD4 polypeptide or fragments including an amino-terminal Ser-rich region when fused with the yeast GAL4 DNA-binding domain significantly activated transcription

of a *UAS-luciferase* reporter in tobacco BY-2 cells (Figures S3B and S3C). These results support the proposed role of RKD4 as a transcription factor.

We next generated a transgenic line, designated *indRKD4ox*, which allows overexpression of *RKD4* upon induction by the synthetic steroid hormone dexametason (DEX). We compared transcript profiles between *indRKD4ox* and the control (*p35S-GVG)* seedlings 24 hours after DEX treatment using Affymetrix ATH1 microarrays. A scatter plot indicated that a number of genes were up-regulated by ectopic *RKD4* expression in seedlings (Figure 3A). Among the 112 probe sets that were induced more than 10-fold (q-value < 0.05), 76 were assigned an 'absent' call in both replicates of control samples, indicating that a majority of highly induced genes are normally not expressed in seedlings (plotted as red dots in Figure 3A).

A clustering analysis of the 76 probe sets using publically available expression data for wild-type seed or seed compartments (Harada-Goldberg Arabidopsis Laser Capture Micro-dissection (LCM) GeneChip Data Set [16]) as well as numerous organs and tissues [17-21] indicated that 27 of the 76 probe sets (hereafter called *RKD4*-responsive genes, listed in Table S1) are preferentially expressed in the "*RKD4* expression domain", i.e. the pre-globular/globular embryo proper and the suspensors at the globular stage (Figure 3B; see Figure 3C for a magnified view of the suspensor and embryo proper expression data). We confirmed up-regulation of all 27 *RKD4*-responsive genes in *indRKD4ox* by real-time RT-PCR (Table S1). These results indicate that short-term expression of RKD4 in the seedlings is sufficient to induce the expression of a number of early embryo-specific genes.

Overexpression of RKD4 primes somatic cells for embryogenesis

Prolonged DEX treatment of *indRKD4ox* seedlings enhanced cell proliferation in the regions normally rich in cycling cells, such as the root meristem (Figures 4C and 4D, compare with 4A and 4B) and young leaf primordia (Figure S4A). In the root, proliferation in response to *RKD4*-overexpression occurs in most cell types and thus differs from the proliferation response to hormones, which is confined to the pericycle (Figure S4B) [22].

When *indRKD4ox* seedlings were treated with DEX for 8 days and then transferred to DEX-free medium, globular structures composed of cytoplasm-rich cells were formed from the proliferating cells (arrowhead in Figures 4F), which then grew to embryo-like structures in the following 2-4 days (Figures 4G and 4H). Similar to *Arabidopsis* zygotic embryos, the embryo-like structures were intensely stained by Sudan Red 7B, suggesting that they are somatic embryos (Figure 4G, inset) [23]. In support of this conclusion, a time course study revealed that early embryo-specific genes were induced by DEX-dependent *RKD4* overexpression and down-regulated after transfer to DEX-free medium, whereas marker genes normally expressed in more mature zygotic embryos were induced only after the transfer to DEX-free medium (Figure S4C). In contrast, a mock transfer to fresh DEX-medium failed to trigger the formation of globular structures (Figures 4I and 4J). Instead, it resulted in sustained cell proliferation as well as expression of early embryo-specific genes and a slight induction of genes expressed in mid stage embryos, but not of those transcripts specific to mature embryos (Figure. S4C). These results suggest that induced overexpression of *RKD4* allows somatic cells to acquire embryogenic potential, which enables them to produce somatic embryos independently of external growth

regulators as soon as RKD4 activity is removed.

Discussion

RKD proteins, one of the two subfamilies of RWP-RK motif proteins, are widely spread in plants, but their biological function has only been inferred from the preferential expression of some family members in the female gametophyte [24, 25]. Here, we show that RKD4 acts as a novel key regulator of early embryogenesis.

In zygotic embryos, *RKD4* is required for pattern formation from the first division onward. Loss of *RKD4* resulted in reduced elongation of the zygote and abnormal early cell division patterns, and an accompanying paper [26] places RKD4 downstream of the MAP kinase module that is activated in the zygote upon fertilization [6-8]. Later, *rkd4* embryos were defective in auxin-mediated axis formation and organogenesis. Since *rkd4* mutants deviate from normal development well before apical-to-basal auxin transport is established, these later effects are probably indirect. *RKD4* expression is confined to the early embryo proper and the suspensor; *RKD4* transcripts were barely detectable in postembryonic tissues, and *rkd4* embryos that developed to produce seedlings had no obvious defect as adults. Strikingly, transient *RKD4* overexpression was sufficient to induce early embryo-specific genes and somatic embryogenesis. While several *Arabidopsis* genes are known to induce somatic embryos when overexpressed [27], *RKD4* appears to act differently in that progression of somatic embryogenesis only occurred once transient *RKD4* overexpression had been released, while continuous overexpression locks the cells in

an early embryonic state.

Based on these observations, we propose that the function of RKD4 is to directly or indirectly promote the expression of genes required for initiating the patterning process in the zygote and early embryo. Among the 27 genes identified here that were highly responsive to RKD4, several are predicted to encode proteins with regulatory functions, such as transcription factors, epigenetic regulators, and mediators of protein turnover. Thus, a functional analysis of these *RKD4*-responsive genes presents a rare opportunity for expanding our mechanistic understanding of early embryonic patterning, which remains one of the least explored processes in plant development.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, Supplemental References, one table and four figures and can be found with this article online.

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Legends to figures

Figure 1. Post-embryonic and embryonic defects of loss-of-function *rkd4* mutants

(A) A diagram showing exon/intron organization of *RKD4* and the sites of T-DNA insertions in *rkd4-1* and *rkd4-2*. A schematic representation of RKD4 polypeptide is shown below the gene structure, with regions having characteristic structural motifs colored differently.

(B-C) Seedlings of *rkd4-1* (B) and *rkd4-1* complemented with a wild-type copy of *RKD4* (C), five days after germination. Inset in B shows a rootless *rkd4-1* seedling.

(D-M) Comparison of wild-type (D-H) and *rkd4-1* (I-M) embryogenesis. (D, I) Zygotes 6-8 hours after flowering (HAF). (E, J) One-cell stage embryos 12-14 HAF. Arrowheads indicate first cell division planes. (F, K) 2- to 4-cell stage embryos 1 day after flowering (DAF). (G, L) Early globular stage embryos 2 DAF. (H, M) Heart stage embryos (3 DAF). Asterisk in (H) indicates LSC derivatives. Brackets in (F-H and K-M) indicate suspensors.

(N-Q) Expression of *PIN1-GFP* (N, P) and *DR5rev-GFP* (O, Q) in wild-type (N, O) and *rkd4-1* (P, Q) embryos. Closed and open arrowheads indicate the presence and absence of strong GFP signal, respectively.

Scale bars represent 1 cm (B, C) and 10 μm (D-Q). See also Figure S1.

Figure 2. Expression pattern of *RKD4*

(A) RT-PCR analysis of *RKD4* in various organs. Stages of developing seeds were roughly separated as follows; early, zygote/pre-globular; mid, globular/heart, late, torpedo/mature.

(B) Configuration of the *pRKD4>>GFPer* two-component reporter vector.

(C-G) Expression patterns of the *pRKD4>>GFPer* reporter in wild-type embryos. Asterisks in E-G indicate LSC and its derivatives.

Scale bars represent 20 μm. See also Figure S2.

Figure 3. Overexpression of *RKD4* activates early embryo-specific genes in seedlings

(A) A scatter plot comparing the signal intensity between *indRKD4ox* and control *p35S-GVG* seedlings treated for 24 hours with DEX. Red symbols represent 76 probe sets corresponding to the genes ectopically induced by *RKD4* overexpression.

(B-C) A diagram showing the clustering result for the expression patterns of the 76 probe sets induced by *RKD4* in wild-type plant (B), and the magnification of the region surrounded by a blue box in B, corresponding to the expression profiles of the suspensor and embryo proper (C). Two regions surrounded by green boxes indicate 27 probe sets that are induced by *RKD4* and normally expressed in the "*RKD4* expression domain", and hence named "*RKD4*-responsive genes". See also Figure S3 and Table S1.

Figure 4. Overexpression of *RKD4* primes somatic cells for embryogenesis.

(A-J) Primary root tips (upper panels) and their cross sections (bottom panels) of control (A, B) and *indRKD4ox* (C-J) seedlings grown under indicated conditions. All seedlings were grown on DEX-free medium (MS) for the first two days after germination, and then transferred to DEX-containing medium. Inset in G shows a root stained with Sudan Red 7B. Arrowhead in F points to a globular structure composed of cytoplasm-rich cells formed

among the proliferated cells. Scale bars represent 200 μm (upper panels) and 40 μm (lower panels).