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Two receptor-like kinases required together for the establishment of Arabidopsis cotyledon primordia

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Abstract

Inter-regional signaling coordinates pattern formation in *Arabidopsis thaliana* embryos. However, little is known regarding the cells and molecules involved in inter-regional communication. We have characterized two related leucine-rich repeat receptor-like kinases (LRR-RLKs), *RECEPTOR-LIKE PROTEIN KINASE1* (*RPK1*) and *TOADSTOOL2* (*TOAD2*), which are required together for patterning the apical embryonic domain cell types that generate cotyledon primordia. Central domain protoderm patterning defects were always observed subjacent to the defective cotyledon primordia cell types in mutant embryos. In addition, RPK1-GFP and TOAD2-GFP translational fusions were both localized to the central domain protodermal cells when cotyledon primordia were first recognizable. We propose that *RPK1* and *TOAD2* are primarily required to maintain central domain protoderm cell fate and that the loss of this key embryonic cell type in mutant embryos results in patterning defects in other regions of the embryo including the failure to initiate cotyledon primordia.

Keywords: Embryogenesis; Arabidopsis; Receptor-like kinases; Signaling; Protoderm

Introduction

The coordination of cellular differentiation along the apical–basal and radial axes during Arabidopsis embryogenesis establishes the basic adult body plan. At the octant stage, the Arabidopsis embryo is partitioned along its apical–basal axis into the apical, central and basal domains (Fig. 1A). The derivatives of these domains will produce characteristic seedling structures (Jurgens et al., 1994) (Fig. 1A). Although communication between the clonally-distinct apical, central and basal domain derivatives is required for embryonic pattern formation (Laux et al., 2004; Mayer and Jurgens, 1998; Weijers et al., 2006), the cells and molecules involved in inter-regional signaling remain to be fully characterized.

Patterning along the central domain radial axis generates the outer-most protoderm, the middle ground tissue and the inner vascular primordium layers during early embryogenesis (Mansfield and Briarty, 1991) (Fig. 1A). Radial patterning in the apical domain of globular embryos establishes a cylinder of cells at the core of the apical domain (the central or inner region) and a surrounding group of cells referred to as the peripheral region (Long and Barton, 1998) (Fig. 1A). After the late globular stage, the embryo transforms from a radially symmetric structure into a bilaterally symmetric heart-shaped structure with two developing cotyledon primordia (Fig. 1A). During this transition stage, the apical domain is subdivided into a medial region and two flanking lateral regions (Aida et al., 1999; Long and Barton, 1998; Prigge et al., 2005) (Fig. 1A). One model proposes that medial/lateral regional identities are superimposed onto radial identities of late globular/transition stage embryos to pattern the apical embryonic domain (Aida et al., 1999; Long and Barton, 1998; Prigge et al., 2005). For instance, cells in the peripheral/lateral, peripheral/medial and central/medial regions will generate cotyledon primordia, boundaries between the cotyledon primordia margins and the presumptive shoot meristem precursors, respectively (Fig. 1A).

The plant hormone auxin plays an important role in patterning the apical embryonic domain. Polar auxin transport

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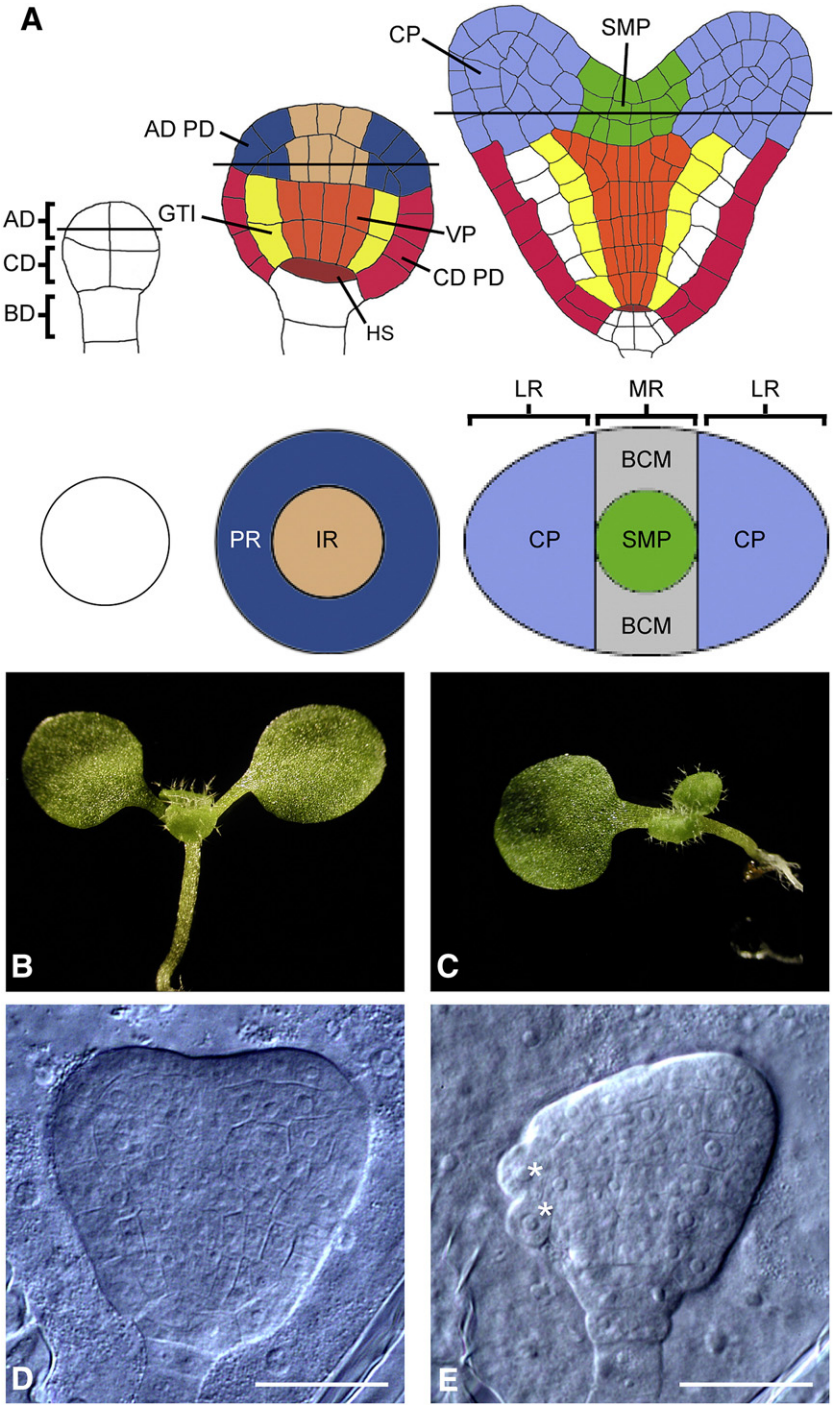

Fig. 1. Seedlings and embryos from self-pollinated *rpk1-1 toad2-1/+* plants have cotyledon development defects. (A) Schematic of pattern formation from the 8-cell (left) to the late globular (middle) and heart (right) stages of Arabidopsis embryogenesis. Top: Schematics of frontal longitudinal sections; Bottom: Schematics of cross sections through the apical domains indicated by bold horizontal lines in the top illustrations. AD, apical domain; AD PD, apical domain protoderm; BD, basal domain; BCM, boundary between cotyledon primordia margins (gray); CD, central domain; CD PD, central domain protoderm (red); CP, cotyledon primordia (light blue); GTI, ground tissue initials (yellow); HS, hypophysis (brown); IR, inner region (light brown); LR, lateral region; MR, medial region; PR, peripheral region (dark blue); SMP, presumptive shoot meristem precursors (green); VP, vascular primordium (orange). (B) Wild-type (Col-0) seedling 7 days after germination (dag). (C) Seedling from self-pollinated *rpk1-1 toad2-1/+* plant with missing cotyledon 7 dag. (D) Representative early heart stage embryo from self-pollinated *rpk1-1 toad2-1/+* plant with correctly formed cotyledon primordia. (E) Representative transition/early heart stage Defective half embryo from self-pollinated *rpk1-1 toad2-1/+* plant with a malformed cotyledon primordium. Enlarged central domain protodermal cells are indicated by asterisks. Scale bars represent 25 μm.

through the PIN1 putative auxin efflux carrier is thought to establish groups of cells with increased auxin signaling, or auxin maxima, at the apices of cotyledon primordia (Benkova et al., 2003; Friml et al., 2003), and many studies indicate that auxin response is required to properly pattern the apical domain (Aida et al., 2002; Berleth and Juergens, 1993; Furutani et al.,

2004; Hadfi et al., 1998; Hardtke et al., 2004; Liu et al., 1993; Vernoux et al., 2000). Furthermore, the juxtaposition of inner and peripheral region cell types in the apical domain is required for the correct subcellular localization of PIN1 protein and the establishment of cotyledon primordia auxin maxima (Izhaki and Bowman, 2007). The establishment of the inner and peripheral apical domain regions therefore precedes and is required to generate bilateral symmetry.

In a previous report, we demonstrated that two genes encoding leucine-rich repeat receptor-like kinases (LRR-RLKs), *RECEPTOR-LIKE PROTEIN KINASE1* (*RPK1*) and *TOADSTOOL2* (*TOAD2*), are redundantly required for central domain radial pattern formation and basal pole differentiation during the globular stages of embryogenesis (Nodine et al., 2007). In this report, we show that *RPK1* and *TOAD2* are also required together for cotyledon initiation during later embryonic stages. Furthermore, apical domain patterning defects correlate with central domain radial patterning defects in mutant embryos. Our data indicate that *RPK1* and *TOAD2* are required to maintain central domain protoderm cell fate through the late globular stage. We propose that the failure to maintain central domain protoderm differentiation in the mutant embryos results in the loss of non-cell autonomous signals that are required for the establishment of apical domain peripheral region cell fates. Coupled with previous analyses, our results implicate the central domain protoderm as a key source of positional information required for embryonic pattern formation.

Materials and methods

Genetic and phenotypic analyses

The *rpk1-1*, *toad2-1* and *toad2-2* alleles have exonic T-DNA insertions and do not produce full-length transcripts, and *rpk1-5* contains a premature stop codon (Nodine et al., 2007). Seedlings were grown on 1% agar plates with 0.5× Murashige and Skoog (MS) media and 0.5% 2-(*N*-Morpholino)ethane sulfonic acid (MES) with a 16 h light/8 h dark cycle. Plants were grown at 22 °C in a Conviron growth chamber with a 16 h light/8 h dark cycle. PCR reactions were performed using Ex-Taq polymerase (TaKaRa) with previously described primers (Nodine et al., 2007) to genotype seedlings from self-pollinated *rpk1-1 toad2-1/+* plants.

Ovules were fixed and cleared as previously reported (Ohad et al., 1996), and viewed using Nomarski optics on a Zeiss Axiophot equipped with a digital camera. Images were collected with PictureFrame 1.0 software. *DR5rev*::*GFP* lines were obtained from the Nottingham Arabidopsis Stock Center (Scholl et al., 2000). Embryo dissections and confocal imaging were performed as previously described (Nodine et al., 2007). All images were processed using Adobe Photoshop.

RNA in situ hybridizations

RNA *in situ* hybridization and probe preparation were performed as previously described (Nodine et al., 2007). ANT, ATML1, PNH, SCR and STM probes correspond to those used in previous reports (Long and Barton, 1998; Lu et al., 1996; Lynn et al., 1999; Wysocka-Diller et al., 2000). For PIN1 antisense and sense probes, a 460 base pair fragment corresponding to nucleotides 602 to 1062 of the cDNA were amplified with PIN1 F1: 5′-AGCAGGATCTATTG-TTTCGA-3′ and PIN1 R1: 5′-ACCACTTCCTCCAGATTGAT-3′. For RPS5A antisense and sense probes, a 324 base pair fragment corresponding to 51 nucleotides of coding region and 273 nucleotides of 3′ UTR were amplified with RPS5AF2: 5′-GCCATCAAGAAGAAGGATGA-3′ and RPS5AR2: 5′-CGAG-CTTGATTACCAGATAATAGAAAC-3′.

Results

Seedlings and embryos from self-pollinated rpk1 toad2/+ plants have defects in cotyledon development

We previously found that all embryos homozygous for both *rpk1* and *toad2* null alleles (*rpk1-1 toad2-1* and *rpk1-5 toad2-2* embryos) and approximately half of *rpk1 toad2/+* embryos exhibit distinct patterning defects collectively referred to as the Toadstool phenotype (Nodine et al., 2007). Upon further examination of the progeny from *rpk1 toad2/+* self-pollinated plants, we found that ~16% of seedlings derived from *rpk1-1 toad2-1/+* and *rpk1-5 toad2-2/+* self-pollinated plants were missing one cotyledon (Fig. 1C; Table 1). These seedlings were viable and gave rise to adult plants without any noticeable defects (data not shown).

Since early embryogenesis is sensitive to *TOAD2* gene dosage in an *rpk1* background (Nodine et al., 2007), we reasoned that cotyledon development may also be sensitive to *TOAD2* gene dosage in an *rpk1* background. To test this idea, we examined *rpk1-1* and *rpk1-5* seedlings, as well as seedlings

Table 1
Frequencies of seedling and embryo phenotypes

Parent genotype (self-pollinated)	Frequency of seedlings with two cotyledons[a] (total)	Frequency of seedlings with one cotyledon[a] (total)	Frequency of seedlings with incompletely separated cotyledons[a] (total)	Frequency of embryos with one cotyledon primordia[b] (total[c])
rpk1-1	93.2% (1018)	4.6% (1018)	2.2% (1018)	5.1% (336)
rpk1-5	94.6% (500)	4.8% (500)	0.6% (500)	4.8% (252)
toad2-1/+	100% (512)	0% (512)	0% (512)	0% (190)
toad2-2/+	100% (511)	0% (511)	0% (511)	0% (173)
rpk1-1 toad2-1/+	82.4% (935)	16.4%[d] (935)	1.2% (935)	16.6%[d] (223)
rpk1-5 toad2-2/+	84.3% (460)	15.7%[d] (460)	0% (460)	17.9%[d] (67)
Wild-type (Col-0)	100% (494)	0% (494)	0% (494)	0% (151)

[a] Seedling phenotypes were determined 7 days after germination.
[b] Embryo phenotypes were determined at the transition and early heart stages.
[c] Totals do not include *toadstool* embryos.
[d] The probability that the difference between the observed frequency of mutants from *rpk1 toad2/+* self-pollinated parents and the expected frequency of mutants due to the *rpk1* single mutant alone is not due to chance is less than 10e−5. The probability was calculated using the Yates' chi-square test.

from self-pollinated *toad2-1/+* and *toad2-2/+* plants. Progeny from *toad2/+* plants were examined because *toad2* plants are sterile (Mizuno et al., 2007). Approximately 5% of *rpk1-1* and *rpk1-5* seedlings had single cotyledons, while no seedlings from *toad2-1/+* and *toad2-2/+* self-pollinated plants had single cotyledons (Table 1). In addition, a small percentage of *rpk1-1* (2.2%; 22/1018), *rpk1-5* (0.6%; 3/500) and *rpk1-1 toad2-1/+* (1.2%; 11/935) seedlings had two incompletely separated cotyledons (Table 1). To test whether *rpk1-1 toad2-1/+* seedlings were more likely to have cotyledon formation defects than *rpk1-1* seedlings, we used a PCR-based assay to genotype seedlings with single cotyledons from self-pollinated *rpk1-1 toad2-1/+* plants. The majority (92%; 88/96) of these seedlings were *rpk1-1 toad2-1/+*. These results indicate that cotyledon development is sensitive to *TOAD2* gene dosage in an *rpk1* background.

To determine whether the missing cotyledon phenotype of *rpk1 toad2/+* seedlings was reflective of cotyledon formation defects during embryogenesis, seeds from self-pollinated *rpk1 toad2/+* plants containing transition/early heart stage embryos were fixed, cleared and examined using Nomarski optics. In order to accurately calculate the frequency of embryos with cotyledon defects, we did not include the number of Toadstool embryos in the totals since they arrest at stages prior to cotyledon formation (Nodine et al., 2007). Approximately 17% of transition/early heart stage embryos from self-pollinated *rpk1-1 toad2-1/+* plants were missing one of the two developing cotyledon primordia (Table 1; Fig. 1E). In contrast to wild-type embryos that are bilaterally symmetric at the transition/early heart stage, the mutant embryos with cotyledon primordia defects were composed of two distinct halves at these stages (Fig. 1E). One side of these embryos had a properly developing cotyledon primordium and organized central domain cell layers. The other side of these embryos lacked cotyledon primordium outgrowth, always had enlarged central domain protoderm cells, and often had abnormal planes of cell division in the basal embryonic domain (Fig. 1E and data not shown). We will refer to embryos that exhibit this phenotype as Defective half embryos. Defective half embryos were detected at a similar frequency in the progeny from self-pollinated *rpk1-5 toad2-2/+* plants (Table 1). Defective half embryos were also observed in approximately 5% of *rpk1-1* and *rpk1-5* embryos, but not in the progeny of self-pollinated *toad2-1/+* and *toad2-2/+* plants (Table 1). The frequencies of Defective half embryos and seedlings with single cotyledons were nearly identical (Table 1). This indicates that the missing cotyledon phenotype of *rpk1* and *rpk1 toad2/+* seedlings is due to cotyledon initiation defects in *rpk1* and *rpk1 toad2/+* embryos.

Apical domain markers exhibit inappropriate expression in Defective half embryos

To test whether the failure to initiate cotyledon primordia is due to defects in cotyledon differentiation programs, we examined the expression of markers characteristic of different apical domain cell types in wild-type (Col-0) embryos and embryos from self-pollinated *rpk1-1 toad2-1/+* plants. Self-pollinated *rpk1-1 toad2-1/+* plants produce normal sibling embryos (including *rpk1-1* and *rpk1-1 toad2-1/+*) that resemble wild-type embryos at the transition stage, as well as, Toadstool and Defective half embryos. *ANT* encodes an *APETALA2*-like transcription factor (Elliott et al., 1996; Klucher et al., 1996) and is expressed in the peripheral region of the apical domain including the cotyledon primordia (Elliott et al., 1996; Long and Barton, 1998) (Fig. 2A). *ANT* transcripts were not detected in either the apical domain of Toadstool embryos or the malformed cotyledon primordium of all Defective half embryos (13/13) examined (Figs. 2B, C). All Col-0, Toadstool and Defective half embryonic cells expressed *RIBOSOMAL PROTEIN 5A* (*RPS5A*) indicating that the lack of *ANT* transcripts is not due to a general loss of transcription (Figs. 2I–K). Cells in the position of the Defective half malformed cotyledon primordium therefore lack transcripts characteristic of the peripheral region and cotyledon primordia.

We examined *SHOOTMERISTEMLESS* (*STM*) transcripts to test whether medial region-specific transcripts were present in the apical domain cell types adjacent to the malformed cotyledon primordia. *STM* encodes a homeodomain protein required for the initiation of the shoot meristem, and is expressed in the medial region of the apical embryonic domain (Barton and Poethig, 1993; Long and Barton, 1998; Long et al., 1996) (Fig. 2E). Similarly staged Toadstool embryos expressed *STM* throughout the apex of the embryo (Fig. 2F). *STM* was expressed in the presumptive medial region of all (17/17) Defective half embryos examined (Fig. 2G). In addition, Defective half embryos frequently (76%; 13/17) expressed *STM* in the malformed cotyledon primordia (Fig. 2G). These results suggest that medial region identity is established in the Defective half embryos. The frequent ectopic *STM* expression together with the absence of *ANT* transcripts in cells in the position of the improperly formed cotyledon primordia indicate that peripheral region identity is not established and/or maintained in the mutant embryos.

Since auxin-mediated signaling is required for cotyledon outgrowth (Aida et al., 2002; Berleth and Juergens, 1993; Friml et al., 2003; Hadfi et al., 1998; Hardtke et al., 2004; Liu et al., 1993), we tested whether auxin response was perturbed in the Defective half malformed cotyledon primordia. Auxin response was assessed by examining the expression of a synthetic auxin-responsive promoter fused to GFP (*DR5rev*::*GFP*) (Friml et al., 2003). *DR5rev*::*GFP* expression marks groups of cells with increased auxin signaling, or auxin maxima, in the embryonic basal domain beginning at the early globular stage and in the cotyledon primordia at the transition/early heart stages (Benkova et al., 2003; Friml et al., 2003). *DR5rev*::*GFP* was expressed in the cotyledon primordia in most wild-type (86%; 19/22) and normal sibling (94%; 16/17) transition/early heart stage embryos, but never observed in either the apical domains of Toadstool embryos or in the Defective half malformed cotyledon primordia (0/14) (Nodine et al., 2007) (Figs. 2M, N). Based on these results, we suggest that auxin response is perturbed in the malformed cotyledon primordia of Defective half embryos.

PIN1-mediated auxin transport is thought to contribute to auxin maxima formation in cotyledon primordia (Benkova et al., 2003). We therefore proposed that inappropriate *PIN1*

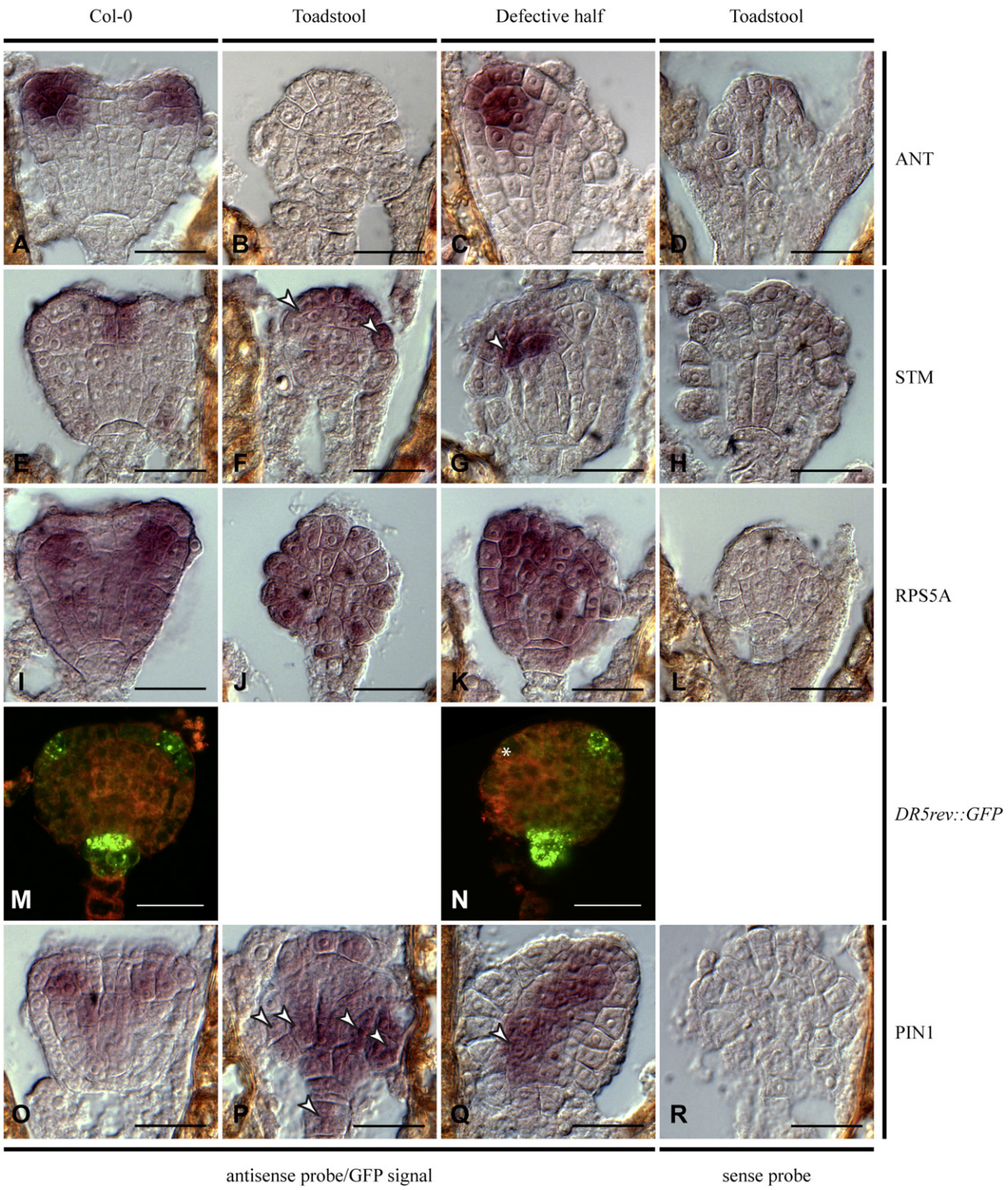

Fig. 2. Apical domain markers exhibit inappropriate expression in Defective half embryos. (A–C) RNA *in situ* hybridization with ANT antisense probe. Representative transition/early heart stage wild-type (Col-0) (A), Toadstool (B) and Defective half (C) embryos. (D) RNA *in situ* hybridization of transition/early heart stage Toadstool embryo with ANT sense probe. (E–G) RNA *in situ* hybridization with STM antisense probe. Representative transition/early heart stage wild-type (E), Toadstool (F) and Defective half (G) embryos. (H) RNA *in situ* hybridization of transition/early heart stage Toadstool embryo with STM sense probe. (I–K) RNA *in situ* hybridization with RPS5A antisense probe. Representative transition/early heart stage wild-type (I), Toadstool (J) and Defective half (K) embryos. (L) RNA *in situ* hybridization of transition/early heart stage Toadstool embryo with RPS5A sense probe. (M and N) Representative confocal images of transition/early heart stage wild-type (M) and Defective half (N) embryos expressing *DR5rev*::*GFP*. The malformed cotyledon primordia of the Defective half embryo shown in panel N is indicated by an asterisk. Green, GFP; red, FM4-64. (O–Q) RNA *in situ* hybridization with PIN1 antisense probe. Representative transition/early heart stage wild-type (O), Toadstool (P) and Defective half (Q) embryos. (R) RNA *in situ* hybridization of transition/early heart stage Toadstool embryo with PIN1 sense probe. Arrowheads indicate ectopic transcript localization patterns. Scale bars represent 25 μm.

expression in Defective half embryos may result in the observed failure to establish auxin maxima in Defective half malformed cotyledon primordia. To test this idea, we examined *PIN1* transcripts in Defective half embryos. *PIN1* was expressed throughout the apical domain of late globular wild-type embryos (data not shown). Consistent with previous findings (Aida et al., 2002), *PIN1* transcripts were detected exclusively in the cotyledon primordia and vascular primordia of all transition/early heart stage wild-type embryos examined (Fig. 2O). Therefore, *PIN1* transcripts become localized in a bilateral arrangement at the transition stage. This bilateral *PIN1* expression pattern was not observed in Toadstool or Defective half embryos. *PIN1* was expressed throughout Toadstool embryos, but the strongest signals were observed in the central and basal domains (Fig. 2P). *PIN1* transcripts were either not detectable or much reduced in the Defective half malformed cotyledon primordia (Fig. 2Q). The absence/reduction of *PIN1* transcripts in the Defective half malformed cotyledon primordium therefore correlates with the lack of *DR5rev*::*GFP* expression in these cell types.

Collectively, our genetic and phenotypic analyses indicate that *RPK1* and *TOAD2* are redundantly required for cotyledon primordia initiation. More specifically, our results suggest that *RPK1* and *TOAD2* are required together for peripheral region identity and auxin response in the apical domain of transition/early heart stage embryos.

Central domain markers are inappropriately expressed in cell types subjacent to Defective half malformed cotyledon primordia

In terms of both morphology and expression of cell-specific markers, the apical domain region that generated the properly formed cotyledon primordia of Defective half embryos resembled wild-type embryos, whereas the apical domain region that generated the malformed cotyledon primordia of Defective half embryos resembled Toadstool embryos. Since central domain radial patterning defects were previously observed in Toadstool embryos (Nodine et al., 2007), we tested whether the apical domain patterning defects of Defective half embryos were correlated with radial patterning defects in the subjacent central domain cell types. The localization patterns of cell-type specific transcripts in transition/early heart stage wild-type, normal sibling, Toadstool and Defective half embryos were examined and compared using *in situ* hybridizations.

Arabidopsis thaliana MERISTEM LAYER1 (*ATML1*) encodes a homeobox protein that is expressed in the protoderm beginning at the dermatogen stage of embryogenesis (Lu et al., 1996). We examined *ATML1* transcripts to test whether the outermost cell layer of the Defective half malformed cotyledon primordia produced protoderm-specific transcripts. *ATML1* transcripts were observed in the protoderm of all wild-type embryos (29/29) and normal siblings (41/41) examined, but were never (0/18) detected in the central domain protoderm of the Defective half malformed cotyledon primordium (Figs. 3A, B and data not shown). However, *ATML1* was frequently (78%; 14/18) expressed in the apical domain protoderm of the malformed cotyledon primordium (Fig. 3B). These results demonstrate that the absence of *ATML1* transcripts in the central domain protoderm is correlated with the Defective half cotyledon primordia initiation defects.

Since *SCARECROW* (*SCR*) is expressed in the ground tissue initials beginning at the late globular stage (Wysocka-Diller et al., 2000), we examined *SCR* transcripts to test whether transcripts characteristic of the ground tissue initials are appropriately localized in Defective half embryos. *SCR* transcripts were detected in all transition/early heart stage wild-type embryos (29/29) examined (Fig. 3D). The central domain cells of Defective half embryos subtending the malformed cotyledon primordium, but not the properly formed cotyledon primordium, frequently had inappropriate *SCR* transcript localization patterns (Fig. 3E). While *SCR* was expressed in the ground tissue initials below the malformed cotyledon primordium of some Defective half embryos (25%; 5/20), most Defective half embryos (75%; 15/20) lacked *SCR* transcripts in these cells (Fig. 3E). *SCR* transcripts were also occasionally detected in the central domain protoderm subjacent to the malformed cotyledon primordia of Defective half embryos (10%; 2/20). These results suggest that cells in the position of the ground tissue initials below the malformed cotyledon primordia often, but not always, have differentiation defects.

Since *PINHEAD*/*ZWILLE* (*PNH*/*ZLL*) transcripts are present at high levels in the vascular primordium of transition/early heart embryos (Lynn et al., 1999; Moussian et al., 1998), we examined *PNH*/*ZLL* transcripts in Defective half embryos to test whether transcripts characteristic of vascular primordium differentiation programs are properly localized. During the transition/early heart stage, *PNH/ZLL* was expressed exclusively in the vascular primordium of all wild-type embryos examined (Fig. 3G). *PNH/ZLL* transcripts were frequently (83%; 19/23) detected in both the vascular primordium and the cells in the positions of the ground tissue initials subjacent to the Defective half malformed cotyledon primordia (Fig. 3H). *PNH/ZLL* transcript localization patterns were however occasionally (17%; 4/23) appropriately localized in the Defective half vascular primordium only (data not shown).

PIN1 is also expressed in the vascular primordium of transition/early heart stage embryos (Aida et al., 2002). Similar to the *PNH/ZLL* expression patterns, *PIN1* was ectopically expressed in the cells in the position of the ground tissue initials subjacent to the malformed cotyledon primordia in most (70%; 14/20), but not all, Defective half embryos examined (Fig. 2Q). Therefore, our results suggest that both *PNH/ZLL* and *PIN1* are frequently, but not always, inappropriately expressed in cells in the position of the ground tissue initials below the Defective half malformed cotyledon primordia.

Together, the localization patterns of cell-type specific transcripts in Defective half embryos at the transition/early heart stages of embryogenesis indicate that central domain radial patterning defects are correlated with the apical domain patterning defects observed in Defective half embryos. That is, we never detected protoderm-specific transcripts, and only occasionally detected appropriately localized ground tissue initial and vascular primordia-specific transcripts, in the central

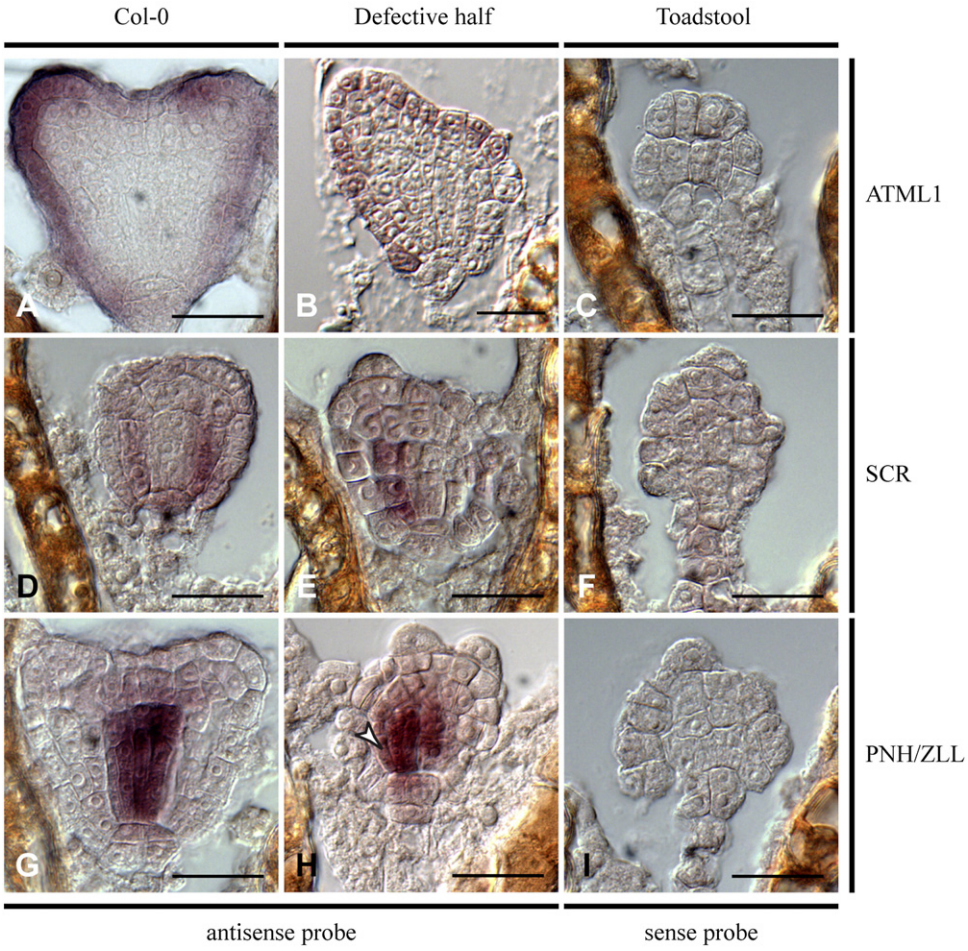

Fig. 3. Central domain markers are inappropriately expressed in cell types subjacent to Defective half malformed cotyledon primordia. (A and B) RNA *in situ* hybridizations with ATML1 antisense probe. Representative transition/early heart stage wild-type (A) and Defective half (B) embryos. (C) RNA *in situ* hybridization of transition/early heart stage Toadstool with ATML1 sense probe. (D and E) RNA *in situ* hybridizations with SCR antisense probe. Representative transition/early heart stage wild-type (D) and Defective half (E) embryos. (F) RNA *in situ* hybridization of transition/early heart stage Toadstool with SCR sense probe. (G and H) RNA *in situ* hybridizations with PNH antisense probe. Representative transition/early heart stage wild-type (G) and Defective half (H) embryos. (I) RNA *in situ* hybridization of transition/early heart stage Toadstool with PNH sense probe. Arrowheads indicate ectopic transcript localization patterns. Scale bars represent 25 μm.

domain cell types subtending the malformed cotyledon primordia of Defective half embryos.

RPK1-GFP and TOAD2-GFP translational fusions are both present in the central domain protoderm of transition-staged embryos

The genetic and phenotypic analyses described above suggest that *RPK1* and *TOAD2* have overlapping functions during cotyledon initiation. To test this further, we examined the localization of RPK1-GFP and TOAD2-GFP translational fusions (Nodine et al., 2007) in transition stage embryos, which have recognizable cotyledon primordia. RPK1-GFP was detected predominantly in the central domain cell types, but was also present at low levels in the apical domain cells that surround the presumptive shoot apical meristem precursors (compare Fig. 4A with Fig. 4C). In addition to the presence of cytoplasmic RPK1-GFP signals in the cell types described above, RPK1-GFP appeared to be localized to the plasma membranes of the central domain protodermal, ground tissue initial and hypophyseal cells (compare Fig. 4A with Fig. 4C). TOAD2-GFP signals were weaker than RPK1-GFP signals, but were detected in the presumptive plasma membranes of protodermal cells (compare Fig. 4B with Fig. 4C). Therefore, RPK1-GFP and TOAD2-GFP are both localized to the central domain protodermal cells of transition stage embryos. This result, together with those from the genetic and phenotypic analyses described above, indicates that RPK1 and TOAD2 have overlapping functions in the central domain protoderm at the stage of embryogenesis when cotyledon primordia are being established.

Discussion

RPK1 and TOAD2 have overlapping functions in the central domain protoderm required for cotyledon initiation

Our genetic and phenotypic analyses indicate that *RPK1* and *TOAD2* are redundantly required for cotyledon initiation. We

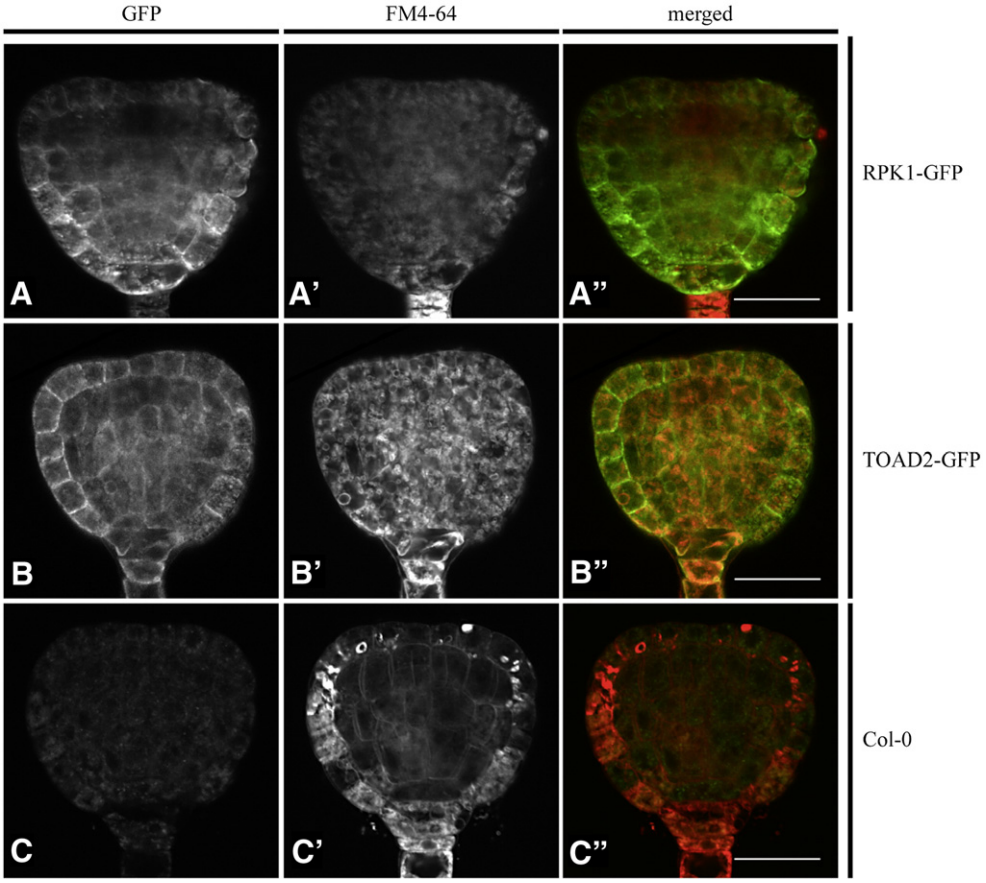

Fig. 4. RPK1-GFP and TOAD2-GFP localization patterns in transition stage embryos. (A, B and C) GFP signals. (A′, B′ and C′) FM4-64 (lipophilic dye) signals. (A″, B″ and C″) Merged GFP and FM4-64 signals. (A–A″) Representative transition stage embryo expressing RPK1-GFP. (B–B″) Representative transition stage embryo expressing TOAD2-GFP. (C–C″) Representative transition stage Col-0 embryo not expressing GFP. Scale bars represent 25 μm.

cannot presently rule out the possibility that *RPK1* and *TOAD2* function directly within apical domain cell types to promote cotyledon initiation and that the central domain protoderm defects observed in Defective half embryos are a consequence of apical domain patterning defects. However, the following observations support an alternative model proposing that *RPK1* and *TOAD2* are required in the central domain protoderm for its differentiation, and that the cotyledon initiation defects observed in Defective half embryos are caused by these central domain protoderm defects. First, we always observed morphological defects in the cells in the position of the central domain protoderm below the Defective half malformed cotyledon primordia. Transcripts characteristic of the protoderm layer (ATML1) were also undetectable in these cell types suggesting that there were defects in protoderm differentiation. In contrast, *ATML1* transcripts were frequently (14/18) detected in the apical domain protoderm suggesting that the apical domain protoderm does not always have differentiation defects in Defective half embryos. Second, transcripts characteristic of the peripheral region/cotyledon primordia (ANT) were never observed in the malformed cotyledon primordia of Defective half embryos. Therefore, central domain protoderm patterning defects were always observed subjacent to the defective cotyledon primordia of mutant embryos. However, all embryos from self-pollinated *rpk1-1 toad2-1/+* plants with two cotyledons did express *ATML1* in the central domain protoderm. Third, transcripts characteristic of the ground tissue initials (SCR) and vascular primordia (PNH and PIN1) were occasionally localized appropriately in Defective half embryos. These results suggest that defects in central domain sub-protodermal cell types are not required for cotyledon initiation defects. Finally, RPK1-GFP and TOAD2-GFP were both detected in central domain protodermal cells of transition-stage embryos. This is consistent with *RPK1* and *TOAD2* having overlapping functions in the central domain protoderm at the stage when cotyledon primordia are being specified in the apical domain.

Basis of abnormal apical domain patterning in Defective half embryos

Results from several studies support a model whereby medial/lateral regional identities are superimposed onto radial identities of late globular/transition stage embryos to pattern the apical embryonic domain (Aida et al., 1999; Long and Barton, 1998; Prigge et al., 2005). Recent results suggest that the juxtaposition of inner and peripheral region apical domain cell types is required for the correct polar localization of PIN1 auxin efflux carriers and presumably the establishment of auxin maxima in cotyledon primordia (Izhaki and Bowman, 2007). Auxin transport to, and auxin responses within, cotyledon

primordia are then required for their outgrowth and separation (Berleth and Juergens, 1993; Friml et al., 2003; Hadfi et al., 1998; Hardtke et al., 2004; Liu et al., 1993; Vernoux et al., 2000). Auxin signaling is also apparently required to repress the expression of medial region-specific genes (including *STM*) in the cotyledon primordia (Aida et al., 2002; Furutani et al., 2004). The loss of auxin response and frequent ectopic *STM* expression observed in Defective half malformed cotyledon primordia are consistent with the idea that signaling downstream of auxin helps define the medial region boundaries.

The absence of peripheral region-specific transcripts (ANT) and the frequent ectopic *STM* expression indicate that peripheral region identity is either not established and/or maintained correctly in one-half of the Defective half embryos. The malformed cotyledon primordia fail to express *PIN1* appropriately and the lack of *DR5rev*::*GFP* reporter gene activity indicates that auxin responses are also perturbed in these cell types. We propose that the failure to specify and/or maintain peripheral region cell fate in Defective half embryos results in the loss of *PIN1* expression, which in turn leads to the loss of auxin responses required for cotyledon outgrowth.

Role of the protoderm in the formation of cotyledon primordia

Results from a number of studies suggest that proper protoderm differentiation is required for cotyledon primordia initiation. For instance, embryos with mutations in both *ATML1* and *PROTODERMAL FACTOR2*, which encode two homeodomain transcription factors expressed in the protoderm, have cotyledon formation defects (Abe et al., 2003). Furthermore, embryos homozygous for mutations in both Arabidopsis *CRINKLY4* (*ACR4*) and *ABNORMAL LEAF SHAPE1* (*ALE1*) exhibit defects in both apical domain protoderm differentiation and cotyledon formation (Tanaka et al., 2007; Watanabe et al., 2004). *ACR4* and *ALE1* encode an RLK and a subtilisin-like serine protease, respectively (Tanaka et al., 2001, 2002). Embryos with mutations in both *ALE1* and another RLK called *ABNORMAL LEAF SHAPE2* (*ALE2*) also exhibit defects in both apical domain protoderm differentiation and cotyledon formation (Tanaka et al., 2007). Based on these and other results, Tanaka et al. proposed that the ALE1 protease and the ACR4/ALE2 receptor complex function in parallel signaling pathways to promote protoderm differentiation (Tanaka et al., 2007).

The protoderm differentiation defects in the *ale1 acr4*, *ale1 ale2* and *atml1 pdf2* double mutant embryos were limited to the apical domain protoderm (Abe et al., 2003; Tanaka et al., 2007). Since these double mutant embryos also formed defective cotyledons, these results suggest that the apical domain protoderm is required for proper cotyledon development. As discussed above, our results indicate that *RPK1* and *TOAD2* have overlapping functions required for maintaining central domain protoderm cell fate, which in turn appears to be required for apical domain patterning and cotyledon primordium initiation. We propose that the apical domain and central domain protoderm are both required for proper cotyledon development. Since the central domain protoderm is not incorporated into the developing cotyledon primordia, non-cell autonomous signals from the central domain protoderm may be involved in apical domain patterning and the ensuing cotyledon primordia initiation.

We previously demonstrated that *RPK1* and *TOAD2* are redundantly required to maintain central domain protoderm cell fate in early globular embryos (Nodine et al., 2007). Therefore, RPK1/TOAD2-mediated signaling presumably maintains central domain protoderm differentiation throughout the globular stages of embryogenesis. Furthermore, Toadstool and Defective half embryos have differentiation defects in cell types that are adjacent to the central domain protoderm (Nodine et al., 2007) (this study). However, the initial morphogenetic and differentiation defects observed in Toadstool and Defective half embryos are in the central domain protoderm, and RPK1-GFP and TOAD2-GFP are both localized to the central domain protoderm when these defects are first recognizable (Nodine et al., 2007) (this study). We speculate that the loss of central domain protoderm cell fates in both Toadstool and Defective half embryos results in the loss of central domain protoderm-derived signals that coordinate the differentiation of surrounding embryonic cell types. More specifically, we propose that the loss of central domain protoderm cell fate in early globular Toadstool embryos results in patterning defects throughout the radial axis and embryo lethality. In contrast, the loss of central domain protoderm cell fate in late globular/transition Defective half embryos results in patterning defects that are limited to one side of viable, yet inappropriately patterned, embryos. Our model therefore proposes that the central domain protoderm is functioning as an organizer of Arabidopsis pattern at multiple stages of embryogenesis.

It is interesting that the patterning defects observed in Defective half embryos are limited to one side of the embryo, while the defects observed in Toadstool embryos are present throughout the embryo. To explain this observation, we speculate that the central domain protoderm may function as one self-maintaining unit during the dermatogen/early globular stage and as two self-maintaining units at the late globular/transition stage. Therefore, when a critical number of central domain protodermal cells are defective in dermatogen/early globular *rpk1 toad2*/+ embryos, then differentiation throughout the central domain protoderm is abnormal. This would result in patterning defects throughout the embryo and the Toadstool phenotype. Approximately 50% of *rpk1 toad2*/+ embryos are able to develop normally past the early globular stage and these have been referred to as *rpk1 toad2*/+ normal siblings (Nodine et al., 2007). The model above predicts that when a critical number of central domain protodermal cells are defective in late globular/transition-staged *rpk1 toad2*/+ normal siblings, only one of the two central domain protoderm self-regulating units is defective. This would result in patterning defects limited to one side of the embryo and the Defective half phenotype. Furthermore, a fraction (<2%) of *rpk1 toad2*/+ late globular/transition embryos are predicted, by chance, to have defects in both central domain protoderm units. This hypothetical class of embryos might be difficult to distinguish from Toadstool embryos.

The identification and characterization of RPK1 and TOAD2 extracellular ligands and downstream signaling components, as well as the signals transmitted by the central domain protoderm, will enable testing of the models described above. Investigating the integration of signaling events mediated by ACR4, ALE1, ALE2, RPK1 and TOAD2 required for protoderm differentiation should yield significant insight into the coordination of embryonic pattern formation in Arabidopsis.

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