THE ANALYSIS OF TWO RECEPTOR-LIKE KINASES REDUNDANTLY REQUIRED FOR PATTERN FORMATION DURING ARABIDOPSIS EMBRYOGENESIS

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The coordination of various cellular differentiation and morphogenetic programs during plant embryogenesis is required to establish the basic adult body plan. The molecular basis of these patterning events remains to be fully understood. In particular, little is known about the roles of cell-cell signaling during embryonic pattern formation. I identified two receptor-like kinases, RECEPTOR-LIKE PROTEIN KINASE1 (RPK1) and TOADSTOOL2 (TOAD2), redundantly required for Arabidopsis thaliana embryonic pattern formation. Genetic analysis indicates that RPK1 and TOAD2 have overlapping embryonic functions. The zygotic gene dosage of TOAD2 in an rpk1 background is of critical importance, suggesting that signaling mediated by RPK1 and TOAD2 must be above a threshold level for proper embryo development. The localization of RPK1 and TOAD2 translational fusions to GFP coupled with the analysis of cell-type specific markers indicate that RPK1 and TOAD2 are redundantly required for both pattern formation along the radial axis and differentiation of the basal pole during early embryogenesis. I found that RPK1 and TOAD2 also have overlapping functions required for cotyledon primordia initiation during Arabidopsis embryogenesis. Genetic analyses indicate that cotyledon initiation is sensitive to TOAD2 gene dosage in an rpk1 background. Analysis of cell-specific markers suggest that RPK1 and TOAD2 are primarily required for the
differentiation of cell types (i.e. the central domain protoderm) subjacent to the cotyledon primordia, and that the cotyledon initiation defects are caused by defects in the central domain protoderm. In addition, RPK1-GFP and TOAD2-GFP translational fusions had overlapping localization patterns in the central domain protodermal cells when cotyledon primordia were first recognizable. I propose that \textit{RPK1} and \textit{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 throughout the embryo including the failure to initiate cotyledon primordia.

This work has identified two putative receptors for cell-cell signals that mediate key patterning events during plant embryogenesis. The future identification of components in the RPK1 and/or TOAD2 signaling pathways will yield further insight into the molecular basis of the generation and assembly of diverse embryonic cell types.
CHAPTER 1
INTRODUCTION

A fundamental objective of developmental biology is to understand how single cells generate multicellular organisms with differentiated cell types arranged in appropriate patterns. There are many different ways in which this complexity is achieved, as is reflected by the diverse morphologies displayed by multicellular organisms. Thus, it is essential to study the mechanistic basis of pattern formation in various species in order to obtain a more complete understanding of development.

Animals and plants are thought to have independently evolved multicellularity from a common unicellular ancestor. Many metazoan and vascular plant species establish their basic body plans during embryogenesis. The comparison of animal and plant embryonic pattern formation therefore enables the identification of similar and different mechanisms used to assemble complex organisms (Meyerowitz, 2002). Both animals and plants utilize cell-cell signaling during patterning. Whereas the role of cell-cell signaling during animal embryonic development has been well documented, surprisingly little is known about the role of cell-cell signaling during plant embryogenesis. However, recent results indicate that cell-cell signaling plays a prominent role during plant embryonic pattern formation, and this topic will be discussed below. Since most of these studies utilized the model plant system Arabidopsis thaliana, I will first give a brief background on Arabidopsis
embryogenesis. This will be followed by an overview of possible cell-cell interactions, as well as a description of the signaling components potentially involved in these processes.
Figure 1.1. Arabidopsis embryogenesis. Illustrations of frontal longitudinal sections through embryos at early stages of embryogenesis and a seedling 7 days after germination are shown. The apical-basal and radial axes are depicted as lines with arrowheads indicating polarity. Expression patterns for several cell- and stage-specific genes are color coded (Elliott et al., 1996; Haecker et al., 2004; Helariutta et al., 2000; Long and Barton, 1998; Long et al., 1996; Lu et al., 1996; Lynn et al., 1999; Mayer et al., 1998; Moussian et al., 1998; Wysocka-Diller et al., 2000). Note: WOX2 is expressed through the heart stage and ATML1 is both expressed prior to the dermatogen stage and in the hypophysis, but only their expression patterns through the octant stage and after the octant stage are illustrated for clarity.
Arabidopsis embryogenesis

Pattern formation during *Arabidopsis thaliana* embryogenesis occurs along two orthogonal axes: an apical-basal axis and a radial axis. The shoot and root meristem precursors are formed at the apical and basal poles of the embryo, respectively (Jurgens et al., 1994; Mansfield and Briarty, 1991). The body of the adult plant is derived from the post-embryonic activity of these meristems. Patterning along the radial axis coordinates the formation of the outer, middle and internal embryonic cell layers that generate the primary tissue layers of the plant body.

Apical-basal polarity is evident in the Arabidopsis zygote, which undergoes an asymmetric transverse division to generate a smaller apical cell and a larger basal cell (Fig. 1.1). Gene expression analyses are consistent with the idea that the asymmetric cell division of the zygote differentially segregates transcription factor mRNAs to its daughters. More specifically, transcripts corresponding to two *WUSCHEL*-related homeobox gene family members, *WOX2* and *WOX8*, are both present in the zygote, but are subsequently localized to either the apical cell (WOX2) or basal cell (WOX8) (Haecker et al., 2004). After the first zygotic division, a series of two bisecting longitudinal divisions form a quadrant stage embryo proper from the apical cell (Fig. 1.1). A set of transverse cell divisions then generate an octant stage embryo proper (Fig. 1.1). These transverse cell divisions partition the embryo proper into the apical and central domains (Fig. 1.1). RNA *in situ* analyses indicate that the apical and central
domains have characteristic RNA profiles (Elliott et al., 1996; Haecker et al., 2004; Kerstetter et al., 2001; Long and Barton, 1998) (Fig. 1.1) suggesting that they initiate unique differentiation programs. Furthermore, the apical and central domain derivatives produce characteristic seedling structures. The apical domain derivatives generate the cotyledons and the shoot meristem while the central domain derivatives produce portions of the cotyledons, the hypocotyl and the proximal root meristem initials.

Most of the basal cell derivatives produce a single file of cells (i.e. the suspensor) that supports the embryo proper during early development. The uppermost derivative of the basal cell lineage is referred to as the hypophysis or basal domain of the embryo at the octant stage (Fig. 1.1). The hypophysis is clearly distinct from the apical cell derivatives and its basal cell siblings by the early globular stage (i.e. ~32-cell stage) when it initiates the expression of the \textit{WOX5} and \textit{SCARECROW (SCR)} transcription factors (Haecker et al., 2004; Wysocka-Diller et al., 2000) (Fig. 1.1). The hypophysis undergoes an asymmetric cell division to generate an upper lens-shaped cell which will produce the quiescent center (QC) and a lower cell that will produce the columella initials at the mid-globular stage (~64-cell stage) (Fig. 1.1). The QC and columella initials make-up the distal portions of the root meristem and are responsible for maintaining root meristem activity and producing the root cap, respectively.

In contrast to apical-basal pattern formation, which is already apparent upon the asymmetric cell division of the zygote, radial pattern formation is not recognizable until
the octant-to-dermatogen stage transition. Formative tangential cell divisions in the apical and central domains partition the Arabidopsis embryo into the outer protoderm (epidermal precursors) and inner sub-protoderm cell types during this transition (Fig. 1.1). The apical and central domains of the octant stage embryo are composed of cells that simultaneously express genes that are characteristic of both the future protoderm and sub-protoderm cell types, and these transcripts are restricted to the appropriate cell layers upon the formation of the protoderm and sub-protoderm (Abe et al., 2003; Aida et al., 2004; Lu et al., 1996; Lynn et al., 1999) (Fig. 1.1). The protodermal cells divide along the surface of the embryo and produce a sheet of cells that surrounds the sub-protoderm (Fig. 1.1). During the globular stages of embryogenesis, the central domain sub-protodermal cells divide along the radial axis to form the vascular primordium at the core of the embryo and the ground tissue initials situated between the vascular primordium and protoderm (Fig. 1.1). By the late globular stage, the protoderm, ground tissue initials, and vascular primordium express different genes (Helariutta et al., 2000; Lu et al., 1996; Wysocka-Diller et al., 2000) (Fig. 1.1). This indicates that these three distinct cell layers are specified by the end of the late globular stage.

During the globular stages, the apical domain sub-protodermal cells divide in a more random manner relative to the central domain sub-protoderm, but still produce cell types with distinct cell fates. The cylinder of cells at the core of the apical domain expresses a characteristic set of genes and is referred to as the central region or inner region (Long and Barton, 1998) (Fig. 1.2). The concentric layers of cells (including the protoderm) that
surround the inner region comprise the peripheral region of the apical domain (Long and Barton, 1998) (Fig. 1.2).

After the late globular stage, the embryo transforms from a radially symmetric globular structure into a bilaterally symmetric heart-shaped structure. During this transition, the apical domain is subdivided into a medial region that extends through the middle part of the apical domain, and two lateral regions that flank the medial region (Aida et al., 1999; Long and Barton, 1998; Prigge et al., 2005) (Fig. 1.2). Each lateral region produces a cotyledon primordium, while the medial region is further partitioned into the presumptive shoot meristem precursors at the core of the apical domain and a group of cells that form boundaries between the developing cotyledon primordia (Fig. 1.2). The superimposition of the medial/lateral regional identities onto the radial identities appears to facilitate the generation of a properly patterned apical domain (Aida et al., 1999; Long and Barton, 1998; Prigge et al., 2005). Cell types that have both inner and medial characteristics are, for instance, the presumptive shoot meristem precursors, cells that have both peripheral and lateral characteristics will generate the cotyledon primordia, and derivatives of cells that have both peripheral and medial region characteristics will become the boundaries between the cotyledon primordia and the shoot meristem precursors. Therefore, different combinations of medial/lateral and radial differentiation programs generate diverse cell types in the apical domain during the transition/heart stages of embryogenesis. However, it remains to be fully determined how differentiation events within and between cells along the apical-basal and radial axes are coordinated.
**Figure 1.2. Pattern formation in the Arabidopsis apical domain.** Schematic of pattern formation during the 8-cell stage (left), late globular stage (middle) and heart stage (right) of Arabidopsis embryogenesis. Top: illustrations of frontal longitudinal sections; Bottom: illustrations of cross sections through the apical domains indicated by bold horizontal lines in the top illustrations. AD, apical domain; AD PD, apical domain protoderm (red); BD, basal domain; CD, central domain; CD PD, central domain protoderm (red); CP, cotyledon primordia (light blue); GTI, ground tissue initials (yellow); IR, inner region (pink); HS, hypophysis (brown); LR, lateral region (light blue); MR, medial region; PR, peripheral region (dark blue); SMP, presumptive shoot meristem precursors (green); VP, vascular primordium (orange).
Role of cell-cell signaling during embryonic pattern formation

Cell-intrinsic and cell-extrinsic positional information could both contribute to the coordination of cellular differentiation during plant embryogenesis. Asymmetric localization of cell fate determinants in animal embryos plays an important role in establishing diverse cell types (Betschinger and Knoblich, 2004). Although a direct role for the asymmetric inheritance of cell fate determinants has not been demonstrated, a few observations are consistent with this mechanism occurring in plants. As mentioned above, Haecker and colleagues demonstrated that the transcripts corresponding to two putative transcription factors (i.e. WOX2 and WOX8) are localized throughout the zygote, but localized to either the apical cell (WOX2) or basal cell (WOX8) subsequent to the asymmetric zygotic division (Haecker et al., 2004). Another asymmetric cell division during embryogenesis is the formation of the lens-shaped cell and columella initial precursors from the hypophysis. Both WOX5 and SCR transcripts are present in the hypophysis, and subsequently localized to the lens-shaped cell, but not the columella initial precursors (Haecker et al., 2004; Wysocka-Diller et al., 2000). However, signals from surrounding cells (i.e. maternal and/or embryonic) may ultimately regulate the polarization of the zygote and hypophysis. Therefore, only indirect evidence supports the model that cell-intrinsic positional information coordinates pattern formation. It should be recognized however that cell-intrinsic and cell-extrinsic positional information are not necessarily separate entities. For instance, signaling molecules could be secreted into the
extracellular matrix, and function as positional information for both the secreting cell and surrounding cells during later stages of development.

Several studies support a role for cell-extrinsic positional information or intercellular signaling during embryonic pattern formation. For example, embryonic fate mapping experiments indicate that embryonic cells do not have fully restricted fates prior to the octant stage (Saulsberry et al., 2002) suggesting that intercellular signaling contributes to cell fate decisions. In addition, although Arabidopsis embryos have cell divisions that follow a predictable pattern (Jurgens et al., 1994; Mansfield and Briarty, 1991), most plant embryos have more random cell division patterns (Johri, 1984). Moreover, fass mutants have irregular cell divisions throughout the early embryo prior to the initiation of many differentiation programs, but still form seedlings with proper body patterns (Torres-Ruiz and Jurgens, 1994). These results suggest that the position of the cell is a major contributing factor to cell fate specification.

Interactions between cells with different fates appear to coordinate plant embryonic pattern formation. Such interactions should involve an inducer cell that produces a signal and a responder cell that receives and interprets that signal. Identification of inducer and responder cell types is therefore a key step towards understanding how embryonic pattern is formed. Relatively little is known about the cells and inductive molecules involved intercellular communication during plant embryogenesis in comparison to animal embryogenesis. This is in part due to the inaccessibility of and difficulty in manipulating
plant embryos. However, results from several studies suggest that a number of cell-cell interactions take place during plant embryogenesis. For instance, intercellular signaling events involving the plant hormone auxin are perhaps the best characterized, and I will give a brief overview of the roles of auxin during embryonic patterning before discussing other potential cell-cell signaling events.

Role of auxin during embryonic patterning

Auxin is an intercellular signal required for pattern formation along the apical-basal axis (Friml et al., 2003; Weijers et al., 2006). The movement of auxin throughout the embryo is controlled by the PIN family of auxin efflux carriers (Benkova et al., 2003; Friml et al., 2003). Differential localization and activities of PIN proteins create regions of increased auxin signaling, which are referred to as auxin maxima (Benkova et al., 2003; Friml et al., 2003). Auxin maxima appear in the basal pole of the embryo at the early globular stage and the presence of the auxin maxima is correlated with hypophysis specification (Friml et al., 2003; Haecker et al., 2004; Wysocka-Diller et al., 2000). Furthermore, mutants defective in either auxin transport or response often exhibit abnormal cell divisions in the hypophysis (Friml et al., 2003; Friml et al., 2004; Weijers et al., 2006). Together, these results are consistent with auxin being involved in hypophysis specification.
However, auxin may not be directly required for hypophysis specification based on the following observations. First, hypophysis morphogenesis can be uncoupled from its specification (Jenik et al., 2005). Hypophysis division defects in auxin transport/response mutants may therefore not indicate hypophysis specification defects. Second, a recent study suggest that auxin may also have an indirect role in hypophysis specification by promoting the accumulation of a secondary signal in the overlying vascular primordium, which then moves to hypophysis precursor and in conjunction with auxin specifies the hypophysis (Weijers et al., 2006).

Auxin is also required for the establishment of bilateral symmetry. As seen for the hypophysis, auxin maxima are observed in the cotyledon primordia apices of Arabidopsis embryos (Benkova et al., 2003; Friml et al., 2003). Furthermore, *Brassica juncea* embryos cultured with auxin transport inhibitors generate collar-like cotyledon structures that surround the presumptive shoot apical meristem precursors (Hadfi et al., 1998; Liu et al., 1993). In addition, *pin1* hypomorphs produce variable defects in cotyledon separation (Aida et al., 2002; Liu et al., 1993; Vernoux et al., 2000) and *pin1 pin3 pin4 pin7* quadruple mutants often form fused or improperly arranged cotyledons (Friml et al., 2003). Similarly, mutations in the AUXIN RESPONSE FACTOR (ARF) transcription factor, *MONOPTEROS/ARF5*, result in embryos that produce variably fused cotyledons (Berleth, 1993) and embryos carrying mutations in both *mp* and the closely-related *nph4/arf7* frequently are “club-shaped” and fail to initiate cotyledon outgrowth or separation (Hardtke et al., 2004). These results indicate that auxin transport to cotyledon
primordia and auxin responses within cotyledon primordia are required for cotyledon outgrowth and for the separation of developing cotyledons. Several studies indicate that the establishment of auxin maxima are necessary to prevent the ectopic expression of medial region differentiation programs in the lateral regions of the apical domain (Aida et al., 2002; Furutani et al., 2004). Furthermore, auxin regulates organ primordium initiation during post-embryonic development and may have analogous functions during cotyledon initiation (Reinhardt et al., 2000; Reinhardt et al., 2003; Vernoux et al., 2000). Collectively, these results indicate that the establishment of auxin maxima in cotyledon primordia precursors is required to properly pattern the apical embryonic domain.

*Role of hypophysis and its derivatives in pattern formation*

The hypophysis and its derivatives have been proposed to regulate the cell fate of the adjacent central domain root meristem initial precursors during embryogenesis (Hamann et al., 1999; Mayer and Jurgens, 1998b; Willemsen et al., 1998). This model is mostly based on the role of the hypophysis-derived QC during post-embryonic development. Each type of root meristem initial divides to produce another initial and a daughter cell. These divisions occur reiteratively and increase the distance between the stem cell and its daughters. The daughter cell will undergo further differentiation and maturation as this distance increases. Thus, the root meristem initials function as stem cells and their activity ultimately produces a post-embryonic root with distinct cell layers. Presumptive signals emanating from the QC maintain the undifferentiated state of the surrounding
post-embryonic root meristem initials (Sabatini et al., 1999; van den Berg et al., 1997). Conversely, the more mature cells of each root meristem initial lineage appear to contribute to initial daughter cell differentiation (van den Berg et al., 1995). By analogy to the post-embryonic root meristem, the hypophysis-derived lens-shaped cell may therefore maintain the preformed root meristem initials during later stages of embryogenesis. However, it remains to be determined how the root meristem initial precursors are established during embryogenesis.

Analysis of embryos with lesions in the HOBBIT (HBT) gene suggested that the hypophysis regulates embryonic root meristem initial cell fates. hbt embryos have abnormal divisions in the presumptive hypophysis, and do not form morphologically recognizable lens-shaped cells (i.e. QC precursor) or columella initials (Willemsen et al., 1998). Furthermore, hbt embryos exhibit abnormal cell divisions in the embryonic root meristem and do not form a lateral root cap or properly differentiate columella initial daughters during later stages of embryogenesis (Willemsen et al., 1998). These results are consistent with HBT being required for hypophysis specification and indirectly required for the specification of the subjacent initials (Willemsen et al., 1998). However, subsequent studies suggest that HBT may not be required for hypophysis specification. First, HBT encodes a homolog of the CDC27 subunit of the anaphase promoting complex and is ubiquitously expressed during early embryogenesis (Blilou et al., 2002). Second, hbt embryos appropriately expressed reporter genes characteristic of the hypophysis, ground tissue and lateral root cap (Blilou et al., 2002). Thus, division defects in hbt
mutants are not necessarily indicative of differentiation defects and *HBT* may be cell-autonomously required for appropriate root meristem initial mitotic activity during embryogenesis.

Results from other studies also suggest that the hypophysis does not play a role in specifying the overlying root meristem initial precursors. Mutations in *TILTED1 (TIL1)*, which encodes the catalytic subunit of DNA polymerase ε, resulted in aberrant hypophysis cell divisions (Jenik et al., 2005). Despite these defective cell divisions, *til1* embryos expressed reporter genes characteristic of the hypophysis and ground tissue initials (Jenik et al., 2005). Hypophysis division and specification can therefore be uncoupled in *til1* embryos, and a properly divided hypophysis is not required for proper differentiation of overlying ground tissue initials. Furthermore, double mutations in the related AP2-like *PLETHORA1 (PLT1)* and *PLT2* transcription factor genes resulted in embryos that failed to express QC-specific reporter genes, but correctly expressed reporter genes characteristic of the ground tissue and vascular primordia (Aida et al., 2004). These results suggest that QC identity is not required for the initiation of ground tissue initial and vascular primordium differentiation programs.

Analysis of protoderm-specific markers in mutants with defective hypophysis development also suggests that signals from the hypophysis are not required for protoderm differentiation. For example, mutations in *MONOPTEROS (MP)*, which encodes a member of the auxin responsive transcription factor (ARF) gene family
(Hardtke and Berleth, 1998), result in defective hypophysis development (Berleth, 1993), but *mp* embryos appropriately express a protoderm-specific reporter gene (Takada and Jurgens, 2007). Mutations in *GNOM*, which encodes a GDP/GTP exchange factor required for intracellular trafficking and positioning of auxin efflux carriers (Geldner et al., 2003; Steinmann et al., 1999), result in severe apical-basal patterning defects (Mayer et al., 1993), but still appropriately express a protoderm-specific marker (Vroemen et al., 1996).

Collectively, these results suggest that signals from the hypophysis are not initiating pattern formation along the radial axis. These results, however, do not rule out the possibility that the hypophysis-derived QC has similar roles during embryonic and post-embryonic development. Thus, the hypophysis may not direct radial pattern formation per se, but instead may repress embryonic root meristem initial differentiation, and thus establish their stem cell-like characteristics.

*Role of vascular primordium in embryonic pattern formation*

It has been proposed that the inner-most cell layer is specified early in embryogenesis, and functions as an inducer of ground tissue initial and hypophysis differentiation programs in adjacent cell types (Berleth, 2001). Supportive evidence for this model comes from the functional analysis of the SHORT-ROOT (SHR) transcription factor. *SHR* is required for both the proper division of the ground tissue initial and the
differentiation of the ground tissue initial-derived endodermis (Helariutta et al., 2000; Scheres et al., 1995). Experiments done in embryonic and post-embryonic roots indicate that $SHR$ is expressed in the vascular primordium and that SHR protein moves from the vascular primordium to the overlying ground tissue initials and distal QC to positively regulate the expression of a related transcription factor, $SCARECROW$ ($SCR$) (Aida et al., 2004; Di Laurenzio et al., 1996; Nakajima et al., 2001). $SCR$ is required for the ground tissue initial cell division necessary for the formation of the endodermal and cortical cell layers, but not the initiation of endodermis-specific differentiation programs (Di Laurenzio et al., 1996; Scheres et al., 1995). SHR therefore regulates other factors involved in endodermis development. $SCR$ is expressed in the hypophysis prior to the onset of $SHR$ expression, and $SCR$ expression in early $shr$ embryos has not been examined. Thus, it remains to be determined whether SHR initiates $SCR$ expression during early embryogenesis.

Studies regarding the roles of the vascular primordium in auxin transport and response support the model that the vascular primordium regulates hypophysis development. PIN1 protein is predominately localized to the basal plasma membranes of vascular primordium cells, and mediates the formation of an auxin maximum in the hypophysis (Friml et al., 2003; Steinmann et al., 1999). Furthermore, Weijers and colleagues demonstrated that in addition to auxin, other factors originating from the vascular primordium likely contribute to hypophysis differentiation (Weijers et al., 2006). Therefore the vascular primordium appears to contribute to the differentiation of the
surrounding ground tissue initials through the movement of the SHR transcription factor, and to hypophysis development both by focusing auxin flow towards the hypophysis and by producing signals that contribute to hypophysis specification.

How then is the vascular primordium formed? The characterization of *WOODY LEG/CYTOKININ RECEPTOR1* (*WOL/CRE1*) has yielded some insight into this question. *WOL/CRE1* encodes a two-component hybrid signaling molecule that is expressed in the vascular primordium beginning at the globular stages and is required for the production of the correct number of cell layers in the vascular primordium, but not vascular primordium differentiation (Mahonen et al., 2000; Scheres et al., 1995). Cytokinin signaling in protoplasts and in a heterologous yeast system requires both the histidine kinase and phosphorelay activities of plasma membrane-localized *WOL/CRE1* (Hwang and Sheen, 2001; Inoue et al., 2001). This suggests that cytokinins can activate *WOL/CRE1*. Thus, cytokinin-mediated activation of *WOL/CRE1* is potentially involved in vascular primordium morphogenesis.

*Role of protoderm in embryonic pattern formation*

The protoderm is perhaps the most likely cell layer to induce differentiation programs of surrounding cell types. Several studies have demonstrated that the protoderm-derived epidermis has important roles during post-embryonic development. Microsurgical experiments in *Lycopersicon esculentum* and analysis of periclinal chimeras in
Antirrhinum majus indicate that the epidermal layer of the shoot apical meristem and floral meristem have critical roles in organ differentiation (Hantke et al., 1995; Reinhardt et al., 2003). Furthermore, a recent study in Arabidopsis shoots demonstrated that brassinosteroi

d signaling in the epidermis was both necessary and sufficient for proper cell growth (Savaldi-Goldstein et al., 2007). Collectively, these results suggest that the epidermal layer is regulating the post-embryonic development of sub-epidermal layers through non-autonomous signals. It is also possible that the protoderm has similar functions during embryogenesis.

The formation of the protoderm at the octant-to-dermatogen stage transition is the first indication of embryonic radial pattern. Thus, the protoderm could direct the subsequent differentiation of surrounding cell types at later stages of embryogenesis. Chemical ablation of protodermal cells at the dermatogen stage can result in hypophysis cell division defects at the globular stages (Baroux et al., 2001; Weijers et al., 2003). These results are consistent with the model that signals from the protoderm are required for hypophysis development. However, it remains to be determined if the protoderm can directly regulate the differentiation of the hypophysis, as well as other embryonic cell types.

Several studies suggest that signaling molecules secreted into and/or derived from the extracellular matrix (ECM) surrounding the protoderm play an important role during embryonic pattern formation. Bruck and Walker (1985) found that Citrus jambhiri
zygotes had a cuticle layer that was subsequently present exclusively on the protoderm surface upon its formation later in embryogenesis. Based on this observation, they proposed that the outer cells maintain an epidermal-specific differentiation program, while the inner cells repress an epidermal-specific differentiation program upon the formation of protoderm and sub-protoderm cell types. Furthermore, they also proposed that the outer most ECM of the embryo may confer protodermal characteristics to the outer most cell layer.

Results from experiments performed on somatic embryos are consistent with the protoderm ECM serving as a source of embryonic differentiation signals. For example, a 32 kD glycosylated acidic endochitinase rescued carrot somatic embryo ts11 mutants apparently by restoring protoderm formation (De Jong et al., 1992). Since the substrate of this 32-kD glycoprotein must have been accessible to the cultured somatic embryos, these results suggest that this endochitinase acts on a substrate localized to the protodermal ECM. Endochitinases are primarily thought to degrade the cell walls of pathogenic fungi by cleaving β-(1,4)-linked polymers of N-acetylglucosamine, which are not thought to be endogenous to higher plant cell walls. In a subsequent study, N-acetylglucosamine-containing lipooligosaccharides, which are produced by symbiotic Rhizobium bacteria and involved in signaling between bacteria and their host plants, were found to rescue ts11 mutants (De Jong et al., 1993). Perhaps endochitinases can therefore cleave endogenous ECM-localized substrates that are similar to N-acetylglucosamine-containing lipooligosaccharides produced by Rhizobium (De Jong et al., 1993). Another class of
ECM associated molecules that may be involved in zygotic embryogenesis is the arabinogalactan proteins (AGPs). The presence of a particular AGP was associated with carrot somatic embryogenesis (Pennell et al., 1992) and specific AGPs were demonstrated to be sufficient to direct the development of carrot somatic embryonic cells (Kreuger and van Holst, 1993).

Several studies done with zygotic embryos are also consistent with the ECM acting as a source of cell differentiation information. An elegant cell ablation study performed on brown algae *Fucus* embryos suggested that cell fate information is localized to the ECM (Berger et al., 1994). Furthermore, mutations in *Arabidopsis thaliana* Defective kernel 1 (*AtDEK1*), which encodes an integral membrane calpain cysteine protease, result in protoderm differentiation defects and abnormal divisions throughout the embryo proper (Johnson et al., 2005; Lid et al., 2005). The membrane-localized *AtDEK1* protease potentially receives and/or processes signals that mediate protoderm differentiation, which in turn may direct the development of sub-protodermal cells. A cell autonomous role of *AtDEK1* in sub-protodermal cells cannot be excluded however because *AtDEK1* transcripts are detected throughout the embryo proper (Johnson et al., 2005; Lid et al., 2005). Another example of such an ECM-localized protoderm cell differentiation factor is PROTODERM FACTOR1 (PDF1). *PDF1* encodes a putatively secreted protein with proline-rich motifs that are characteristic of proteins localized to the plant cell wall. *PDF1* mRNA is localized throughout octant stage Arabidopsis embryos, but predominately localized to the protoderm upon its formation (Abe et al., 1999).
Collectively, these results indicate that the ECM is involved in protoderm cell differentiation. It has been proposed that signaling molecules restricted to the outer face of early embryos allow for the stable segregation of protoderm-specific differentiation factors to the outer (i.e. protoderm), but not inner (i.e. sub-protoderm) cell types (Bruck and Walker, 1985; Laux et al., 2004). Perhaps early plant embryos secrete protoderm cell fate differentiation factors into the ECM, and these are released during later stages of embryogenesis to maintain protoderm cell fate. Since the sub-protodermal cells would not be exposed to such signals they would take on a non-protoderm differentiation program or a default differentiation program. Subsequent inductive interactions between the protoderm and surrounding cell types may then lead to the formation of various cell types arranged in coherent patterns.

Although the results discussed above are consistent with a model whereby the protoderm plays a fundamental role in directing embryonic pattern formation, conclusive evidence for such proposed inductive interactions and in particular the mechanistic basis for such signaling events is lacking. However, recent studies have generated insight into the molecular mechanisms involved in protoderm differentiation and the general role of the protoderm during embryonic patterning.

*CRINKLY4* encodes a member of the maize receptor-like kinase (RLK) gene family and is required for proper epidermal development during post-embryonic development.
The Arabidopsis homolog of CR4, *Arabidopsis CR4 (ACR4)*, is initially expressed throughout the embryo proper but is predominately expressed in the protoderm upon its formation (Gifford et al., 2003; Tanaka et al., 2002). Embryos with null mutations in *ACR4* displayed normal morphology and expressed appropriate markers (Gifford et al., 2003; Tanaka et al., 2007) suggesting that other closely-related gene-family members may have overlapping functions required for protoderm development. Consistent with this idea, embryos expressing ACR4 antisense RNA had morphological defects indicating that transcripts from both *ACR4* and genes with similar sequences may be necessary for embryo morphogenesis (Tanaka et al., 2002). *ACR4* was found to genetically interact with *ABNORMAL LEAF SHAPE1 (ALE1)* (Tanaka et al., 2007; Watanabe et al., 2004), which encodes a subtilisin-like serine protease potentially involved in the production of peptide ligands (Tanaka et al., 2001). Moreover, *ale1 acr4* double mutants exhibited inappropriate localization of protoderm-specific transcripts in the apical domain protoderm (Tanaka et al., 2007). These results suggest that *ALE1* and *ACR4* are members of parallel signaling pathways required for protoderm differentiation.

A recent study identified a previously uncharacterized RLK gene (i.e. *ALE2*) that is involved in protoderm development (Tanaka et al., 2007). Single *ale2* mutants did not exhibit morphogenesis or differentiation defects, while *ale1 ale2* double mutants frequently had both morphological and differentiation defects in some, but not all apical domain protoderm cells. Genetic analyses also suggest that *ALE2* and *ACR4* are in the same pathway. Furthermore, in vitro kinase assays revealed that ALE2 and ACR4 can
both transphosphorylate each other (Tanaka et al., 2007). These results support a model whereby ALE1 and ALE2/ACR4 receptor complexes function in parallel signaling pathways to promote apical domain protoderm differentiation.

Cotyledon development was also defective in ale1 ale2 and ale1 acr4 double mutants (Tanaka et al., 2007). Furthermore, mutations in ATML1 and PDF2, which encode two closely-related homeodomain transcription factors that are expressed in the protoderm, also result in cotyledon formation defects (Abe et al., 2003). These results are consistent with the protoderm playing an important role during cotyledon development.

A few studies indicate that the signaling events that regulate apical domain protoderm (AD-PD) differentiation are different than those that regulate central domain protoderm (CD-PD) differentiation. First, the protoderm differentiation defects of ale1 acr4, ale1 ale2 and atml1 pdf2 embryos were limited to the apical domain protoderm (Abe et al., 2003; Tanaka et al., 2007). Second, the KANADI1 (KAN1) and WOX9 transcription factor mRNAs are localized to the CD-PD, but not the AD-PD, while the AINTEGUMENTA (ANT) and WOX2 transcription factors are expressed in the AD-PD of globular-staged embryos (Elliott et al., 1996; Haecker et al., 2004; Kerstetter et al., 2001; Long and Barton, 1998). Third, different regions of the ATML1 promoter are active in the AD-PD, the CD-PD and/or the basal cell derivatives at the globular stage (Takada and Jurgens, 2007).
Since mutants that have defects in CD-PD differentiation (e.g. *dek1* mutants) also result in defects throughout the embryo and ultimately lead to embryonic lethality, it is reasonable to propose that the CD-PD may be a critical inducer of surrounding cell types. The lack of such mutants identified in forward genetic screens may be due to genetic redundancy of key differentiation factors. Thus, although several lines of evidence suggest that the protoderm has a fundamental role in embryonic pattern formation, direct data indicating that this cell layer is an important inducer is still not available. The signaling pathways involved in protoderm differentiation are beginning to be characterized and novel insights into the functions of the protoderm during embryonic patterning are likely to be generated from these analyses.

*Other signaling molecules potentially involved in embryonic patterning*

As discussed above, several cell-cell signaling events are involved in plant embryonic pattern formation. Accordingly, several signaling components have been identified, but with the exception of auxin signaling, relatively little is known about the molecular basis of intercellular signaling events required for embryonic pattern formation. Recent genome-wide studies have however identified many signaling molecules that may be involved in these key developmental events.

Approximately 25 genes encoding small secreted polypeptides with sequences similar to CLAVATA3 (CLV3) and EMBRYO SURROUNDING REGION (ESR) proteins, called
CLEs (for CLV3/ESR-related genes), were identified by analyzing EST and Arabidopsis genomic sequences (Cock and McCormick, 2001; Sharma et al., 2003). Since CLV3 functions upstream of the CLV1, which encodes an LRR-RLK, to regulate shoot apical meristem activity (Fletcher et al., 1999), it was proposed that other CLEs may also regulate developmental processes by activating other LRR-RLKs. Consistent with this proposal, a few CLEs with roles during post-embryonic development have been identified (Casamitjana-Martinez et al., 2003; Fiers et al., 2005; Hobe et al., 2003; Ito et al., 2006). The Brassica napus CLE19 promoter is active in the protoderm of globular and heart stage Brassica embryos (Fiers et al., 2004) suggesting that CLE19 may be an extracellular signal involved in plant embryo development.

CLEs are likely to interact with plasma membrane-localized receptors and the largest group of such receptors in Arabidopsis is the RLK gene family. Over 400 RLKs are predicted to have extracellular, transmembrane and kinase domains (Shiu and Bleecker, 2001). Furthermore, recent reports describe several examples of closely related RLKs with overlapping functions during post-embryonic pattern formation (Albrecht et al., 2005; Cano-Delgado et al., 2004; Shpak et al., 2004). Many RLK genes are expressed in young siliques (fruits) that contain embryos at early developmental stages (Hennig et al., 2004). Notably, CLV1, EXTRA SPOROGENOUS CELLS/EXTRA MICROSPOROCYTES (EXS/EMS) and SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1 (SERK1) encode LRR-RLKs that are expressed in zygotic embryos (Canales et al., 2002; Hecht et al., 2001; Long and Barton, 1998; Schmidt et al., 1997).
While mutant phenotypes for *clv1* and *serk1* zygotic embryos have not been observed, *exs/ems* mutants exhibit delayed embryo development (Canales et al., 2002). SERK1 protein, however, is specifically enriched in embryogenic competent carrot cell cultures (Schmidt et al., 1997) and over-/ectopically-expressed *SERK1* results in increased efficiency of somatic embryogenesis initiation from post-embryonic cell culture (Hecht et al., 2001). However, out of all of the RLKs, only *ACR4* and *ALE2* have been shown to contribute to the differentiation of embryonic cell types (Takada and Jurgens, 2007). These observations suggest that RLKs have overlapping functions during Arabidopsis embryogenesis. The characterization of signaling molecules, including CLEs and RLKs, offers a promising avenue of research towards a better understanding of the molecular mechanisms involved in plant embryonic pattern formation.
Perspective

Our understanding of how the basic plant body pattern is established during embryogenesis has increased at an unprecedented rate over the past decade. In particular, the ever-increasing amount of genomic resources available to the plant research community has facilitated our understanding of cell-cell signaling events during embryonic pattern formation. The further identification and functional characterization of the molecules involved in cell-cell signaling will generate insight into the mechanistic basis of how diverse cell types are generated and assembled into proper arrangements.
An explanation of the dissertation format

I present my dissertation in two chapters and two appendices. Chapter 1 provides an explanation of the problem and its context, as well as a review of the literature. Chapter 2 summarizes the methods, results and conclusions of the research. The two appendices include a published manuscript (Appendix A) and a manuscript that is in preparation (Appendix B). I designed the majority of experiments, performed all of the experiments and interpreted the majority of results described in both manuscripts. Although I was the primary author for both manuscripts, I did receive helpful advice and edits from my thesis advisor (Dr. Frans Tax) on both manuscripts. I am also grateful to Dr. Ramin Yadegari for both helping me think critically about the literature pertinent to the first manuscript (Appendix A) and in writing the manuscript. Dr. Karen Schumaker and Dr. John Walker both gave helpful comments on the first manuscript (Appendix A).
CHAPTER 2: PRESENT STUDY

The methods, results and conclusions of the present study are described in the manuscripts appended to this dissertation. The following is a summary of the most important findings of this study.

**RPK1 and TOAD2 are two receptor-like kinases redundantly required for Arabidopsis embryonic pattern formation (Appendix A)**

Although the basic plant body plan is established during embryogenesis, the molecular basis of embryonic patterning remains to be fully understood. We have identified two receptor-like kinases (RLKs), *RECEPTOR-LIKE PROTEIN KINASE1* (*RPK1*) and *TOADSTOOL2* (*TOAD2*), required for *Arabidopsis* embryonic pattern formation. Genetic analysis indicates that *RPK1* and *TOAD2* have overlapping embryonic functions. The zygotic gene dosage of *TOAD2* in an *rpk1* background is of critical importance, suggesting that signaling mediated by *RPK1* and *TOAD2* must be above a threshold level for proper embryo development. The localization of *RPK1* and *TOAD2* translational fusions to GFP coupled with the analysis of cell-type specific markers indicate that *RPK1* and *TOAD2* are redundantly required for both pattern formation along the radial axis and differentiation of the basal pole during early embryogenesis. We propose that *RPK1* and *TOAD2* receive intercellular signals and mediate intracellular responses that are necessary for embryonic pattern formation.
The establishment of Arabidopsis cotyledon primordia requires the overlapping functions of two receptor-like kinases (Appendix B)

Signaling between different regions of Arabidopsis thaliana embryos has important roles in coordinating embryonic pattern formation. 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-RLK), RECEPTOR-LIKE PROTEIN KINASE1 (RPK1) and TOADSTOOL2 (TOAD2), with overlapping functions required 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 addition, RPK1-GFP and TOAD2-GFP translational fusions were both localized to the central domain protodermal cells when cotyledon primordia were initiating. 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 throughout the embryo including the failure to initiate cotyledon primordia.
REFERENCES


regulates an asymmetric cell division that is essential for generating the radial organization of the Arabidopsis root. Cell 86, 423-433.


APPENDIX A

*RPK1* AND *TOAD2* ARE TWO RECEPTOR-LIKE KINASES
REDUNDANTLY REQUIRED FOR ARABIDOPSIS EMBRYONIC
PATTERN FORMATION
RPK1 and TOAD2 Are Two Receptor-like Kinases Redundantly Required for Arabidopsis Embryonic Pattern Formation

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SUMMARY

Although the basic plant body plan is established during embryogenesis, the molecular basis of embryonic patterning remains to be fully understood. We have identified two receptor-like kinases, RECEPTOR-LIKE PROTEIN KINASE1 (RPK1) and TOADSTOOL2 (TOAD2), required for Arabidopsis embryonic pattern formation. Genetic analysis indicates that RPK1 and TOAD2 have overlapping embryonic functions. The zygotic gene dosage of TOAD2 in an rpk1 background is of critical importance, suggesting that signaling mediated by RPK1 and TOAD2 must be above a threshold level for proper embryo development. The localization of RPK1 and TOAD2 translational fusions to GFP coupled with the analysis of cell-type-specific markers indicate that RPK1 and TOAD2 are redundantly required for both pattern formation along the radial axis and differentiation of the basal pole during early embryogenesis. We propose that RPK1 and TOAD2 receive intercellular signals and mediate intracellular responses that are necessary for embryonic pattern formation.

INTRODUCTION

The basic body plan of vascular plants is established during embryo development. Pattern formation during plant embryogenesis occurs along apical-basal and radial axes. Patterning along the apical-basal axis of the embryo forms the shoot meristem precursors at the apical pole and root meristem precursors at the basal pole (Jurcynski et al., 1994; Mansfield and Briarty, 1991). The body of the adult plant is docked entirely from the postembryonic activity of these meristems. Patterning along the radial axis coordinates the formation of the outer, middle, and internal embryonic cell layers that generate the primary tissue layers of the plant body. The molecular basis of these early patterning events remains to be fully understo.
Figure 1. RPK1 and TOAD2 Encode LRR RLKs Required for Proper Embryo Morphogenesis

(A) Schematic of radial pattern formation during Antirrhinum embryogenesis.
(B) Gene models of RPK1 and TOAD2. Colored boxes indicate predicted domains. Triangles, insertion sites of T-DNA mutants; numbers in parentheses, position of insertion in base pair downstream of start of translation.
(C–H) Representative Nomarski images of normal sowing embryos from gpk1-1 foad2-2/+ self-pollinated plants at the early globular (C), late globular (D), and heart (E) stages. The hypophysis and lens-shaped cotyledon are indicated by an arrowhead and arrow, respectively.
Developmental Cell
Two RLKs Required for Embryonic Pattern Formation

hormone auxin is an intercellular signal required for pattern formation along the apical-basal axis (Friend et al., 2003; Weijers et al., 2005). However, the signals and receptors required for pattern formation along the radial axis of the early embryo are unknown.

In animals, many cell-signaling processes, including those required for embryonic pattern formation, are mediated through membrane-localized receptors (Ollson et al., 2005; Vinken et al., 2004). The largest group of receptors in Arabidopsis is the receptor-like kinase (RLK) gene family, which has over 400 RLKs predicted to have extracellular and transmembrane domains (Shiu and Bleecker, 2001). Recent reports describe several examples of closely related RLKs with overlapping functions during postembryonic pattern formation (Albrecht et al., 2004; Caro-Delgado et al., 2004; Shpak et al., 2004). However, whereas many RLK genes are expressed in young siliques (fruit) containing embryos at early developmental stages (Hennen et al., 2004), no RLK genes required for embryonic pattern formation have been identified. These observations suggest that RLKs have overlapping functions during Arabidopsis embryonic pattern formation, and that this functional overlap might prevent their identification by forward genetic screens.

To identify RLKs involved in embryogenesis, we used phylogenetic relationships (Shiu and Bleecker, 2003) to guide reverse genetic studies of members of the leucine-rich repeat (LRR) RLK subfamily. Levels of mRNA and protein encoded by the LRR RLK RKPK1 were previously found to be increased in plants treated with auxin (ABA) (Hong et al., 1997; Osakabe et al., 2004). We initially named RKPK1 as TAO2/1 based on its mutant phenotype, but will refer to this gene as RKPK1 because of its prior characterization. To test whether RKPK1 and an uncharacterized LRR RLK with the most similar kinase domain to RKPK1 in the Arabidopsis genome (Shiu and Bleecker, 2003), TAO2/2, have overlapping functions, we attempted to create rpk1 tao2/2 double mutant plants. We found that RKPK1 and TAO2/2 are redundantly required for proper morphogenesis and differentiation of cells along the radial axis and in the basal pole of the early Arabidopsis embryo. In addition, RKPK1 and TAO2/2 transcriptional fusions to GFP had overlapping localization patterns within cell types that exhibited defects in the mutant embryos during early embryogenesis. Collectively, our results indicate that RKPK1 and TAO2/2 are redundantly required for radial pattern formation in early embryos.

RESULTS
 RKPK1 and TAO2/2 Are Redundantly Required for Seed Development
 To identify developmental roles of RKPK1 (At1g62700) and TAO2/2 (At5g21300), we examined plants with single mutations in each gene (Figure 1B). The T-DNA insertion alleles rpk1-1, tao2-1, and tao2-2 are likely null because no full-length transcripts were found, and the nonsense mutation in rpk1-5 is predicted to result in an early premature stop codon (Figure 1C; see Figure S1 in the Supplemental Data available with this article online). Developmental defects were not observed in rpk1-1 or rpk1-5 homozygous mutant plants. However, tao2-1 and tao2-2 plants exhibited developmental defects including the production of smaller rosette leaves, increased branching, and primary and lateral shoots, and sterility (data not shown). Because the predicted RKPK1 and TAO2/2 kinase domains are 76% identical, we next tested whether they have overlapping functions by attempting to generate seedlings with null mutations in both genes. However, rpk1 tao2/2 seedlings were never recovered. We then examined siliques from self-pollinated rpk1-1/ rpk1-1, tao2-1/− and rpk1-1/+, tao2-1/− plants and observed no viable seeds that became brown and shrunken upon maturation (Figure S2). Self-pollinated rpk1-1/−, tao2-1/− and rpk1-1/+, tao2-1/− plants produced 19.5% and 47.1% abnormal seeds, respectively (Table 1). Self-pollinated rpk1-1/−, tao2-1/− and rpk1-1/+, tao2-1/− plants yielded 14% and 44.5% abnormal seeds (Table 1). These results indicate that self-pollinated rpk1-1/−, tao2-1/− and rpk1-1/+, tao2-1/− plants produced normal seeds that segregate in an atypical Mendelian fashion. A PCR-based assay was used to test whether there was a reduction in the frequency of specific genotypes among the progeny from self-pollinated rpk1-1/−, tao2-1/− and rpk1-1/+, tao2-1/− plants. As shown in Table S3, rpk1-1, tao2-1 seedlings were never recovered from rpk1-1/−, tao2-1/− or rpk1-1/+, tao2-1/− self-pollinated plants. In addition, the frequencies of rpk1-1 tao2-1/− seedlings from rpk1-1/−, tao2-1/− and rpk1-1/+, tao2-1/− self-pollinated plants were reduced by approximately one-half of their expected values (Table S3). Similar results were also observed from rpk1-1/+, tao2-2/− and rpk1-1/+, tao2-2/− self-pollinated plants (Table S3). Therefore, the reduction in frequencies of the rpk1 tao2-1 and rpk1 tao2-2 genotypic classes was correlated with the frequency of the abnormal seed phenotype. These results suggest that the absence of rpk1-1 tao2-1 double homozygous plants and the reduction in the frequency of rpk1-1 tao2-1/− individuals are due to a defect during seed development.

Several genetic tests were performed to determine whether the abnormal seed phenotype, as well as the absence of the rpk1 tao2-2 and reduction of the rpk1 tao2-2 genotypic classes, were due to defects during embryo development. As shown in Table S4, reductions in the frequency of the rpk1-1 tao2-2 and rpk1-1 tao2-2 genotypes, and the associated mutant seed phenotype, were due to zygotic effects of the mutant alleles. Taken together, our genetic data indicate that RKPK1 and TAO2/2 are required for proper embryo development.
Table 1. Frequencies of Abnormal Seeds and Embryos

<table>
<thead>
<tr>
<th>Parent Genotype (Self-Pollinated)</th>
<th>Frequency of Abnormal Seeds (Number Observed/Total)</th>
<th>Frequency of Embryos with toast2-like Phenotype (Number Observed/Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpk1-1</td>
<td>3.2% (6/192)</td>
<td>2.2% (4/183)</td>
</tr>
<tr>
<td>rpk1-5</td>
<td>1.6% (4/266)</td>
<td>1.1% (5/568)</td>
</tr>
<tr>
<td>toast2-1/2/4</td>
<td>0.5% (7/1298)</td>
<td>0% (0/191)</td>
</tr>
<tr>
<td>rpk1-1; toast2-1/4</td>
<td>0.5% (5/1076)</td>
<td>0% (0/173)</td>
</tr>
<tr>
<td>rpk1-1; toast2-2/4</td>
<td>15.5% (156/1018)</td>
<td>15.4% (77/502)</td>
</tr>
<tr>
<td>rpk1-1; toast2-2/4 (male)</td>
<td>47.1% (424/901)</td>
<td>47.4% (117/247)</td>
</tr>
<tr>
<td>rpk1-1; toast2-2/4 (female) X rpk1-1 (male)</td>
<td>46.1% (234/477)</td>
<td>45.9% (133/290)</td>
</tr>
<tr>
<td>rpk1-1; toast2-2/4 (male) X rpk1-1 (female) X toast2-2/4 (male)</td>
<td>24.3% (35/144)</td>
<td>21.4% (29/137)</td>
</tr>
<tr>
<td>oot-0 (wild-type)</td>
<td>0.9% (6/690)</td>
<td>0% (0/63)</td>
</tr>
</tbody>
</table>

Embryo phenotypes were determined when the normal siblings were at the late globular stage.

Morogenesis is Abnormal in rpk1; toast2-2/+ and rpk1; toast2-1/+ Embryos

To determine the nature of the embryonic defect and the stage at which RPK1 and TOAD2 are initially required, we fixed, cleared, and examined whole seeds containing embryos at different developmental stages from self-pollinated rpk1-1; toast2-2/4 plants. As shown in Figure 1F, abnormal embryos were first observed at the early globular stage. Nearly half (42%: 38/91) of the embryos analyzed in individual siliquecs produced enlarged cells in the position of the central domain protoderm and frequently displayed abnormal planes of cell divisions in the presumptive hypophysis (Figure 1F), whereas the rest of the embryos (53/91) were indistinguishable from wild-type embryos (Figure 1G). At the late globular stage, nearly half (47%: 117/237) of the embryos showed a further increase in the size of the central domain protoderm cells, which also exhibited aberrant planes of cell division at a low frequency (compare Figures 1G and 1H). In the same embryos, the presumptive hypophysis frequently failed to produce a lens-shaped cell (compare Figures 1G and 1J), which is required for the proper development of the root apical meristem. By the heart stage, approximately half (47%: 127/235) of the embryos had undergone abnormal morphogenesis throughout both the embryonic proper and suspensor, resulting in a mushroom-shaped nonviable embryo (compare Figures 1I and 1K). Therefore, we named the mutant phenotype toast2 after the peculiar class of mushrooms. Embryos from self-pollinated rpk1-1; toast2-2/4 plants displayed identical phenotypes at similar frequencies to those observed for rpk1-1; toast2-2/4 (Table 1 and data not shown). These results indicate that self-pollinated rpk1-1; toast2-2/4 plants generate abnormal and normal sibling embryos in an approximately one-to-one ratio.

As described above, nearly 50% of the embryos from self-pollinated rpk1-1; toast2-2/4 plants exhibited the toast2-2/4 phenotype, whereas 25% are expected to exhibit this phenotype if only rpk1-1; toast2 embryos are defective during embryogenesis (Table 1). Together with our observation that the expected frequency of the rpk1-1; toast2-2/4 genotypic class was reduced by nearly one half (Tables S3 and S6), these results demonstrate that rpk1-1; toast2-2/4 embryos are ~50% penetrant for the toast2-like phenotype. Therefore, approximately one half of rpk1-1; toast2-2/4 embryos are indistinguishable from rpk1-1; toast2-2/4 embryos and undergo abnormal morphogenesis, while the other half of rpk1-1; toast2-2/4 embryos are indistinguishable from rpk1-1; toast2-4/4 embryos and undergo normal morphogenesis. Reciprocal crosses between rpk1-1 and rpk1-1; toast2-2/4 plants confirmed that rpk1-1; toast2-2/4 embryos are indeed haplosufficient for proper morphogenesis approximately 50% of the time (Table 1; Table S4).

To compare the embryo phenotypes from different parental genotypes, we examined embryos from self-pollinated rpk1-1; toast2-2/4 and rpk1-1; toast2-4/4 plants (Table 1). Abnormal sibling embryos from self-pollinated rpk1-1; toast2-2/4 plants were indistinguishable from abnormal sibling embryos that resulted from self-pollinated rpk1-1; toast2-2/4 and rpk1-1; toast2-2/4 plants, indicating that neither the expressivity nor the penetrance of the toast2-2/4 phenotype requires an rpk1 maternal genotype (data not shown). None of the progeny from self-pollinated rpk1-1; toast2-2/4 plants exhibited the toast2-2/4 phenotype. However, 2.5% (4/160) and 1.1% (6/568) of the progeny from self-pollinated rpk1-1; and rpk1-1; toast2-2/4 plants did exhibit this phenotype (Table 1). Therefore, the vast majority of abnormal sibling embryos that result from the self-pollination of rpk1-1; toast2-2/4 and rpk1-1; toast2-2/4 plants are due to mutations in both RPK1 and TOAD2.

RPK1-GFP and TOAD2-GFP Have Overlapping Localization Patterns during Embryogenesis

Because the RLKs encoded by RPK1 and TOAD2 are required for proper central domain protoderm and hypophysis morphogenesis during the globular stages (Figure 1), we predicted that RPK1 and TOAD2 have overlapping localization patterns before and/or during these stages.
of embryo development. To examine the localization of the RPK1 and TOA02 proteins, we generated RPK1 and TOA02 translational GFP fusions under the control of their respective upstream sequences (RPK1:p::RPK1-GFP and TOA02:p::TOA02-GFP) and introduced these into Arabidopsis plants. RPK1:p::RPK1-GFP and TOA02:p::TOA02-GFP both complemented the lost plasmid phenotype (Table S3), indicating that the translational fusions are functional.

As shown in Figure 2A, RPK1-GFP was first detected in the suspensor cells of cotyledon-stage embryos. At this stage, a strong signal was always observed in the presumptive basal plasma membrane of the basal most cell of the suspensor, which is in direct contact with maternal tissue (Figure 2A). A signal corresponding to RPK1-GFP was present throughout the central domain and basal domain of the embryo proper, as well as the suspensor at the cotyledon (Figure 2B) and globular stages (Figures 2C and 2D).
Furthermore, a proportion of RP11-GFP appeared to be localized to the plasma membrane of the suspensor cells; the basal domain, the central domain protoderm (Figures 2B-2D), and occasionally the ground tissue initial precursors (Figure 2C) at the dermatogen and globular stages.

TOAD2-GFP fusion protein was first detected in the presumptive plasma membrane of the central domain protoderm at the early globular stage (Figure 2G), although at a lower level than observed for RP11-GFP. TOAD2-GFP signal was not detectable in the suspensor cells at the early globular stage (data not shown). However, similar to RP11-GFP, TOAD2-GFP was detected throughout the central and basal domains at the late globular stage (Figure 2H, I, J). Thus, RP11-GFP and TOAD2-GFP had overlapping localization patterns within cell types that exhibit morphogenetic defects in the abnormal siblings during early embryogenesis.

**Protoderm Markers Are Not Expressed Properly in Abnormal Sibling Embryos**

To determine whether the cell types that have morphogenetic defects in the abnormal siblings also have cell differentiation defects along the radial axis, we analyzed the expression of radial cell-type markers in embryos obtained from self-pollinated *rpk1-1* *load2-1+/+* plants. First, we analyzed the localization of two protoderm marker mRNAs, *Arabidopsis thaliana* meristem L1 layer (ATML1) and KANADI (KAN). The ATML1 gene is expressed uniformly throughout the embryo proper until the cotyledon-to-dermatogen stage transition, at which time it becomes exclusively expressed in the protoderm of dermatogen- and globular-stage embryos (Lu et al., 1999). The KAN (KAN) gene is first expressed in the central domain protoderm at the late globular stage (Kerstetter et al., 2001). As shown in Figures 3A and 3B, ATML1 mRNA was present in the protoderm of dermatogen-stage embryos from self-pollinated wild-type (100%; 17/17) and *rpk1-1* *load2-1+/+* (85%; 33/39) plants. However, no early or late globular abnormal siblings (0/1) expressed ATML1 in cells in the position of the central domain protoderm (Figure 3E and data not shown). In addition, most early globular (64%; 16/25) and late globular abnormal siblings (94%; 34/36) also lacked ATML1 expression in the apical domain protoderm (Figure 3E and data not shown). In contrast, all early...
and late globular normal siblings (71/71) expressed ATML1 in the protoderm (Figure SD and data not shown). Kan mRNA was not detected in the cells in the position of the central domain in late globular abnormal siblings (9/18) (data not shown), but was detected in the central domain protoderm in late globular wild-type (30/30) and late globular normal sibling embryos (16/30) (data not shown). Therefore, cells in the position of the protoderm in abnormal sibling embryos initially express ATML1, but fail to maintain ATML1 expression during the globular stage. In addition, late globular abnormal siblings fail to initiate KAN expression. These results indicate that protoderm cell fate maintenance, rather than specification, is defective in the abnormal sibling embryos.

Subprotoderm and Vascular Primordium Markers Are Ecotopically Expressed in the Protoderm and Ground Tissue Initials of Abnormal Sibling Embryos

To determine whether vascular primordium cells are specified correctly in abnormal siblings, we examined the expression of two vascular primordium markers, PIN HEAD/PLZ3 (PINH/ZLL) and SHORT ROOT (SHR), in embryos obtained from self-pollinated pxl-1/pxl-1/pxl-1 plants. Expression of PINH/ZLL was detectable throughout the embryo proper as early as the quadrant stage and becomes increasingly restricted to subprotodermal cells during the dermagon (Figure 4A) and early globular stages (Figure 4C). At the late globular stage, PINH/ZLL is expressed predominantly in the inner-most vascular primordium (Jenn et al., 1999). Moussian et al. (1999) showed that SHR is detectable in all cells of the late globular stage (Helariutta et al., 2000). As shown in Figures 4A and 4B, the majority of dermagon-stage embryos from both wild-type plants (96/124) and self-pollinated pxl-1/pxl-1/pxl-1 plants (98/187) expressed PINH/ZLL in both the protoderm and subprotoderm (Figure 4E). During the late globular stage, abnormal sibling embryos either had unrestricted accumulation of PINH/ZLL mRNA in both the central domain (including the vascular primordium, ground tissue initials, and protoderm) (33/33) (Figure 4F) or had an expanded pattern of accumulation in the vascular primordium and the ground tissue initials (7/7) (1/43) data not shown). PINH/ZLL mRNA was also frequently detected in the apical domain, presumptive hypophysis at the late globular and transition stages (Figure 4H). In contrast, the normal siblings (Figures 4D and 4G) exhibited patterns of PINH/ZLL mRNA accumulation similar to wild-type embryos (Figures 4F and 4I).

An SHR::GFP reporter gene was also ectopically expressed in abnormal sibling embryos. During the late globular stage, abnormal sibling embryos exhibited SHR::GFP expression either in all cell layers of the central domain including the protoderm (84/100) or solely in the vascular primordium and the ground tissue initials (16/100) (data not shown). During the transition stage, which is directly after the late globular stage, all (9/9) abnormal sibling embryos examined exhibited a fully expanded pattern of SHR::GFP expression in all cell layers of the central domain, the presumptive hypophysis, and frequently in the apical domain (Figure 4I). Normal sibling embryos from the same plants exhibited expression patterns similar to those observed in wild-type embryos (Figures 4J and 4K). Taken together, these data indicate that subprotoderm and vascular primordium markers are ectopically expressed in cells in the position of the protoderm and ground tissue initials of the abnormal sibling embryos during the globular stages.

Ground Tissue Initial Markers Are Not Expressed in Abnormal Sibling Embryos

As discussed above, the ground tissue initials are specified during the globular stages, and are situated in the central domain between the protoderm and vascular primordium (Figure 1A). Because SCANEROW (SCR) is expressed in the lens-shaped cell and ground tissue initials at the late globular stage (Wysocka-Diller et al., 2000), we examined SCR expression in embryos obtained from self-pollinated pxl-1/pxl-1/pxl-1 plants to determine whether the ground tissue initials are specified correctly in the abnormal sibling embryos. SCR mRNA was detected in the ground tissue initials of all late globular wild-type (189/200) and normal sibling (6/6) embryos examined (Figures 5E and 5F). However, SCR mRNA was not detected in cells in the position of the ground tissue initials in any (0/30) abnormal sibling embryos during the late globular stage (Figure 5G). Together, the ectopic expression of PINH/ZLL and SHR::GFP, as well as the absence of SCR expression, in cells in the position of the ground tissue initials indicate that the ground tissue initials are misspecified in abnormal sibling embryos.

The morphological defects observed in the abnormal sibling embryos coupled with our analysis of protoderm, vascular primordium, and ground tissue initial markers in the abnormal sibling embryos indicate that radial patterning is defective in the abnormal sibling. More specifically, abnormal sibling embryos fail to maintain protoderm cell fate, fail to restrict vascular primordium cell fate to the inner-most cells, and fail to specify the ground tissue initials.

Abnormal Sibling Embryos Are Defective in Hypophysis Marker Expression

To determine whether hypophysis differentiation is defective in abnormal siblings, we first examined the expression of GEP, which is initially expressed in the hypophysis at the early globular stage (Wysocka-Diller et al., 2000). A nuclear-localized version of GFP under the control of the SCR upstream regulatory sequences was expressed in the presumptive hypophysis in all early globular normal siblings (14/14) and abnormal siblings (10/10) examined (data not shown). Furthermore, SCR mRNA was detected...
Figure 4. Sibpresoterm and Vascular Primordium Markers Are Ectopically Expressed in the Pretoderms and Ground Tissue Initiates of Abnormal Sibling Embryos

(A-H) In situ hybridizations with PNH/ZLL antisense probe. Representative embryos from wild-type (A, C, and E) and prog1-1 toad2-1/+ (B, D, E, G, and H) and normal sibling (I) and abnormal sibling (J) embryos.

(I-N) Representative confocal images of SHR::GFP expression in wild-type (L), normal sibling (M), and abnormal sibling (N) embryos. Developmental stages of embryos are indicated on the left-hand side of the figure and include dermatogen (A and B), early globular (D-K), and late globular and transition (I-N) stages.

The scale bars represent 20 μm.

In the presumptive hypophysis and its derivatives in the abnormal sibling embryos during the globular stages (Figures 1C and 1E) in expression patterns similar to those observed in wild-type (Figures 5A and 5C) and normal sibling embryos (Figures 5B and 5D). Therefore, SCA is appropriately expressed in the presumptive hypophysis.
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of abnormal siblings despite the division defects observed in these cell types.

Similar to SCR, WUSCHEL Related Homeobox 5 (WOX5) is expressed in the hypophysis at the early globular stage (Hecker et al., 2004). We also examined the expression of a nuclear-localized version of GFP under the control of the WOX5 upstream regulatory sequences (WOX5::GFP-NLS) in abnormal sibling embryos to test for hypophysis differentiation defects. WOX5::GFP-NLS was expressed in the hypophysis and its derivatives in all early and late globular wild-type embryos examined (Figures 5H and 5I). In contrast, WOX5::GFP-NLS was not expressed in the hypophysis in most (71%; 15/17) early globular abnormal siblings (Figure 5J), but was expressed in approximately half (48%; 11/23) of the late globular abnormal siblings examined (Figure 5K).

Therefore, WOX5::GFP-NLS expression is perturbed in a large proportion of abnormal sibling embryos, suggesting that hypophysis differentiation is defective.

Avin signaling has been shown to contribute to hypophysis development (Kita et al., 2004; Pfuhl et al., 2003; Wiegens et al., 2003). Because the avin-responsive DSR promoter fused to GFP (DSR::GFP) is expressed in the hypophysis and its derivatives during the whole stages (Pfuhl et al., 2003), we examined DSR::GFP expression in the hypophysis and its derivatives in all early and late globular wild-type embryos examined (Figures 5O and 5P). Based on these results, the response to avin in the hypophysis of abnormal siblings appears to be delayed.

DSR::GFP expression was also observed in the distal suspensor cells in most (86%; 22/26) of the late globular abnormal siblings that expressed WOX5::GFP (Figure 5U) and in all (17/17) of the abnormal siblings whose normal siblings were at the transition stage (Figure 5X). Similarly, late globular abnormal sibling embryos occasionally (26%; 2/8) exhibited ectopic expression of WOX5::GFP-NLS in distal suspensor cells (data not shown). Therefore, the expression of both DSR::GFP and WOX5::GFP-NLS reporter genes show a proximal-to-distal shift in a large proportion of the abnormal sibling embryos examined. In addition, cells in the position of the hypophysis derivatives frequently ectopically expressed vascular primordium markers (PNA222L, SHC::GFP, and PNH1::GFP) in abnormal siblings whose normal siblings were at the transition stage (Figures 4H and 4N, Figure 5Q).

Taken together, our results suggest that abnormal sibling embryos have differentiation defects during hypophysis development. Our marker analyses also suggest that cells in the position of the hypophysis derivatives have undergone at least a partial hypophysis-to-vascular primordium cell-fate transformation in abnormal sibling embryos whose normal siblings are at the transition stage.

Normal Sibling Embryos Frequently Exhibit Aberrant Marker Expression at the Early Globular Stage

During our analysis of cell-type-specific markers in abnormal sibling embryos obtained from self-pollinated pk1-1 fo202-1/+ plants, we observed that a significant proportion of the normal sibling embryos did not show proper expression of the SCR, WOX5::GFP-NLS, and DSR::GFP markers when compared to wild-type embryos. For example, in addition to SCR expression in the hypophysis, we also frequently detected expression in the central domain protoderm of both early globular normal siblings (57%; 17/36) (Figure 5B) and abnormal siblings (33%; 9/27) (data not shown). However, at the late globular stage, SCR was rarely expressed in the protoderm of normal (3%; 2/69) or abnormal siblings (5%; 2/42) (data not shown). Because both the pk1 and pk1 fo202+ genotypic classes make up the normal sibling phenotypic class, we then examined SCR expression in pk1-1 embryos to determine whether pk1-1 embryos were just as likely as the normal pk1-1 fo202-1/+ embryos to ectopically express SCR in the central domain protoderm. We found that the majority (86%; 19/22) of early globular pk1-1 embryos did not express SCR in the central domain protoderm (Figure 5D), suggesting that most of the early globular normal siblings that ectopically expressed SCR in the central domain protoderm were of the pk1-1 fo202-1/+ genotypic class.

Hypophysis differentiation defects were also frequently observed in early globular normal sibling embryos. For instance, WOX5::GFP-NLS and DSR::GFP were not expressed in the hypophysis in most (62%; 12/23) and 71%; 15/21, respectively) early globular normal siblings (Figures 5I and 5P). However, WOX5::GFP-NLS and DSR::GFP were expressed in the hypophysis in the majority (77%; 24/31 and 88%; 56/65, respectively) of late globular normal siblings (Figures 5M and 5T). To determine whether the pk1 fo202+ genotypic class of normal sibling embryos was more likely to have defects in WOX5::GFP-NLS and DSR::GFP expression than the pk1-1 genotypic class of normal siblings, we examined WOX5::GFP-NLS and DSR::GFP expression in pk1-1 embryos. WOX5::GFP-NLS and DSR::GFP were expressed in the hypophysis in nearly all (93%; 14/15 and 95%; 20/21, respectively) early globular pk1-1 embryos (Figures 5K and 5L). These results suggest that the majority of normal sibling embryos that were delayed in the onset of WOX5::GFP-NLS expression or avin response were of the pk1-1 fo202-1/+ genotypic class.

These results suggest that both normal siblings and abnormal siblings frequently ectopically express SCR in the central domain protoderm at the early globular stage, but are able to repress this ectopic SCR expression by the late globular stage. Furthermore, most early globular normal siblings and abnormal siblings are delayed in
Figure 5. Germband Tissue Initiation and Hypophysis Marker Expression in Normal and Abnormal Sibling Embryos

(A-D) In situ hybridizations with SCR antisense probes. Representative wild type (A) and (B), normal sibling (C) and (D), abnormal sibling (C) and (D), and rpkt-1 (D) embryos.

(E-H) Representative confocal images of WOX5p::GFP-NLS expression in wild-type (H) and (J), normal sibling (I) and (K), abnormal sibling (J) and (K), and rpkt-1 (K) embryos.

(L-R) Representative confocal images of DR5rev::GFP expression in wild-type (Q) and (R), normal sibling (P) and (Q), abnormal sibling (P) and (Q), and rpkt-1 (Q) embryos.
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DR5rev::GFP and WOX5::GFP-NLS expression, but are able to initiate the expression of these promoters by the late globular stage. Therefore, normal sibling embryos are able to repress improper differentiation programs and subsequently initiate the appropriate programs. Additionally, rpk1-1 toad2-1/+ normal sibling embryos were more likely than rpk1-1 normal sibling embryos to ectopically express SCR, and not express DR5rev::GFP and WOX5::GFP-NLS at the early globular stage. Similar to our observation that embryo morphogenesis is sensitive to TOAD2 gene dosage in an rpk1 background, these results suggest that activation of embryonic differentiation programs is also sensitive to TOAD2 gene dosage in an rpk1 background.

DISCUSSION

RPK1 and TOAD2 Are Redundantly Required for Embryonic Pattern Formation

Our results indicate that RPK1 and TOAD2 have overlapping functions required for embryonic pattern formation in Arabidopsis. The toad1/2 phenotype was rarely observed in rpk1 single mutant embryos and never observed in toad2 single mutant embryos (Table 1). However, our data indicate that rpk1 toad2 double mutant embryos always exhibit the toad1/2 phenotype. This strongly suggests that both RPK1 and TOAD2 are redundantly required for proper embryo development. Furthermore, the frequency of the toad1/2 phenotype is especially sensitive to zygotic TOAD2 gene dosage in an rpk1 background. More specifically, our results indicate that rpk1 toad2+ embryos are either indistinguishable from rpk1 embryos (normal phenotype) or rpk1 toad2 embryos (toad1/2 phenotype). Together with the observation that rpk1 embryos exhibit the toad1/2 phenotype at a low frequency, this suggests that signaling mediated by RPK1 and TOAD2 must be above a critical threshold. Additional evidence that RPK1 and TOAD2 are redundantly required during embryogenesis is provided by the observation that RPK1-GFP and TOAD2-GFP proteins have overlapping localization patterns in wild-type cell types that exhibit defects in the toad1/2 embryos (Figure 2). Finally, expression analyses of cell-specific markers in toad1/2 embryos indicate that RPK1 and TOAD2 are redundantly required for differentiation of particular cell types along the radial axis and in the basal embryonic pole (Figures 5-9).

A Potential Role for ABA Signaling in the Early Embryo

Previously, Hong et al. (1997) found that RPK1 expression is increased upon treatment with ABA and proposed that RPK1 may be involved in ABA signaling. A recent report provided evidence that the level of RPK1 protein is also increased upon treatment with ABA (Osakabe et al., 2005). Furthermore, rpk1 mutants are partially insensitive to the effects of ABA on several developmental processes (Osakabe et al., 2005). Based on these and other results, the authors proposed that the plasma membrane-localized RPK1 protein positively regulates ABA signal transduction (Osakabe et al., 2005). However, it remains to be determined whether ABA binds directly to RPK1 to induce intracellular ABA signaling events. As discussed below, RPK1 may have an indirect role in ABA signaling.

Whereas ABA is primarily known for its role in stress responses and embryo maturation and desiccation (Finkelstein et al., 2002), there is increasing evidence that ABA also plays a role in early embryonic development. For example, maternal ABA affects the rate of embryo growth in Nicotiana plumbaginifolia (Frey et al., 2004). As discussed above, ABA treatment of wild-type plants increases the amount of RPK1 mRNA and protein (Osakabe et al., 2005). Our results indicate that RPK1 protein is present in the suspensor and protoderm during early embryogenesis (Figures 2A–2D). These two embryonic cell types are potential entry points for maternal ABA. Together, these observations offer the intriguing possibility that ABA may influence cell differentiation during early embryogenesis by increasing the amount of RPK1. This increased level of RPK1 may facilitate ABA signaling by increasing the amount of RPK1 available for ABA binding. Alternatively, increased levels of RPK1 may mediate cell differentiation independently of ABA binding. This differentiation may in turn establish a cellular state that is competent to respond to ABA during later stages of embryogenesis.

RPK1 and TOAD2 Are Redundantly Required for Radial Pattern Formation in Arabidopsis Embryos

Our results indicate that RPK1 and TOAD2 are redundantly required for pattern formation along the radial axis of Arabidopsis embryos. In toad1/2 embryos, the cells in the position of the protoderm do not express protoderm markers, but do express subprotoderm markers. Furthermore, toad1/2 embryonic cells in the position of the outer tissue initials do not express ground tissue initials markers, but do express vascular primordium markers. Based on these results, there are outer-to-inner cell fate transformations along the radial axis of toad1/2 embryos.

Pattern formation along the radial axis of the Arabidopsis embryo is first evident when cell divisions during the octant-to-dermatogen stage transition separate the outer protoderm from the inner subprotoderm (Mansfield and Briarty, 1991). Our results indicate that RPK1 and TOAD2 are not required for the establishment of the protoderm at the dermatogen stage, but rather that RPK1 and TOAD2 are redundantly required for the maintenance of ABA signaling.

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![Diagram of cell differentiation](image)

**Figure 6. A Model for the Role of RPK1/TOAD2-Mediated Signalling in Radial Pattern Formation during Arabidopsis Embryogenesis**

Left: schematic of radial pattern formation during embryogenesis and role of RPK1 and TOAD2 in this process.

Middle: illustrations of wild-type embryos from the octant to late globular stages.

Right: illustrations of px1/ px2 and px1 px2 px3-abnormal embryos from the octant to late globular stages.

Cells and tissues are color-coded to indicate differentiation status. Blue: potential to become specified as either protoderm or subprotoderm; red: protoderm; orange: subprotoderm and vascular primordium; yellow: ground tissue initials.

See text for description of model.

of protoderm differentiation programs, as well as the repression of subprotoderm differentiation programs in the protoderm during the early globular stage.

Based on our observations that no toadstool early globular embryos expressed a protoderm marker and that only half of toadstool early globular embryos ectopically expressed a subprotoderm marker in cells that are in the position of the central domain protoderm, the ectopic activity of the subprotoderm differentiation program does not appear to be a prerequisite for the absence of the protoderm differentiation program. Instead, the derepression of subprotoderm differentiation programs in toadstool embryos may be an indirect consequence of the loss of protoderm identity (Figure 6). We propose that the RPK1 and TOAD2 receptors are directly required in the protoderm for cell-late maintenance subsequent to protoderm specification at the dermatochrome stage (Figure 6).

Similar to the requirement of RPK1 and TOAD2 for maintenance of protoderm differentiation and repression of subprotoderm differentiation in the outermost cell layer, RPK1 and TOAD2 are also redundantly required to both specify the ground tissue initials and to repress vascular primordium differentiation programs in the cell layer subjacent to the protoderm (Figure 6). Our results suggest that RPK1 and TOAD2 may be cell-autonomously required for ground tissue initial specification. Alternatively, the ground tissue initial differentiation defects observed in toadstool embryos could be indirectly caused by differentiation defects in other cell types.

**RPK1 and TOAD2 Are Redundantly Required for Hypophysis Differentiation during Embryogenesis**

Our results indicate that RPK1 and TOAD2 are also redundantly required for hypophysis differentiation. The
observations that SGR is expressed in the presumptive hypophysis in early globular toadstool embryos (Figures SC and data not shown), but WOX5p-GFP-NLS and DReve-GFP (an auxin response marker) expression are defective in the presumptive hypophysis in early globular toadstool embryos (Figures SJ and SK), suggest that at least two independent pathways regulate hypophysis differentiation at the early globular stage. These pathways would include an RPK1- and TOAD2-independent pathway that initiates SGR expression, and an RPK1- and TOAD2-dependent pathway that enables proper auxin response and the onset of WOX5 expression. Independent pathways that regulate SGR expression and auxin response have also been proposed to specify the apical derivative of the hypophysis at the late globular/transition stages (Aida et al., 2004).

In addition to being delayed in the onset of WOX5p-GFP-NLS and DReve-GFP expression, the cells in the position of the hypophysis derivatives express genes characteristic of the vascular primordium at the late globular and transition stages (Figures 4H and 4N; Figure S9). Furthermore, toadstool embryos at these stages frequently express markers that are characteristic of the hypophysis in their distal suspensor cells. Based on these observations, as well as a recent report that suggests that factors originating from the vascular primordium contribute to hypophysis differentiation (Weijers et al., 2008), the apparent hypophysis-to-vascular primordium cell-fate transformation observed in toadstool embryos may result in the proximal-to-distal movement of hypophysis differentiation factors, which may ultimately lead to the ectopic expression of hypophysis markers in distal suspensor cells.

Because RPK1-GFP and TOAD2-GFP are localized in the hypophysis derivatives, RPK1 and TOAD2 could be directly required in these cells for their proper differentiation. However, previous reports have demonstrated that chemical ablation of the protoderm can result in defective hypophysis cell divisions (Barzou et al., 2001; Weijers et al., 2003), suggesting that signals from the protoderm may contribute to hypophysis development. Because RPK1 and TOAD2 are redundantly required for protoderm development, the hypophysis defects in the toadstool embryos could be due to the lack of protoderm cell-fate maintenance.

We have identified two RLKs that are redundantly required for radial pattern formation and basal pole differentiation during Arabidopsis embryogenesis. We propose that the RPK1 and TOAD2 RLKs receive intercellular signals and mediate intracellular responses that are required for embryonic pattern formation. Identification of ligands for RPK1 and TOAD2, signaling components downstream of these receptors, and their relationship with ABA will yield further insight into the molecular basis of early patterning events in Arabidopsis embryos.

EXPERIMENTAL PROCEDURES

Growth Conditions and Genetic Analysis

The sra1-1 and sra-5 alleles were generated by the Wisconsin Arabidopsis Knockout facility and the Arabidopsis TILLING Project (ATTP) facility (TH et al., 2003), respectively. The toad1-1 and toad2-2 alleles were identified by the SALK Insertion Stock Center (Wu et al., 2003). P14p-PH3-GFP and DReve-GFP were obtained from the Nottingham Arabidopsis Stock Center (Schell et al., 2000).

All mutant lines were backcrossed at least four times into the Col-0 ecotype and were grown at 23°C in a Cool 1 grow chamber with a 16h light/8h dark cycle. To generate progeny from crosses, three primer polymerase chain reactions were performed using ex-Ata polymerase (TaKaRa). All primers used in this report are described in Table S7.

RPK1 and TOAD2 Translational Fusions

Genomic regions corresponding to 2.9 kb upstream of the RPK1 ATG to just downstream of the RPK1 coding sequences and 1.3 kb upstream of the TOAD2 ATG to just downstream of the TOAD2 coding sequence were cloned into PCR2.1-topo using the topoTA cloning kit (Invitrogen), subcloned into pBI-GFP(S65T) (Yadegari et al., 2000), and transformed into Col-0 plants via Agrobacterium-mediated transformation (Clough and Bent, 1998). At least 20 embryos from more than seven independently transformed lines were examined for every stage of embryogenesis described.

Microscopy

Ovules were fixed and cleared as previously reported (Oduma et al., 1996), and viewed using Nomarski optics on a Zeiss Axioptip equippd with a digital camera. Images were collected with PicturFrame 1.6 software. Embryos were dissected in water, stained with 1 μg/ml FM4-64 (Molecular Probes), and mounted in 10% glycerol. A Nikon 102/1.35M (Bio-Rad) equipped with a 488 nm laser and a 20% neutral density filter was used to excite GFP and FM4-64 fluorescence. GFP and FM4-64 fluorescence was captured with a 522/30 and 680/32 filter sets, respectively, and images were collected with LaserSharp 2000 software. All images were processed using Adobe Photoshop.

RNA in Situ Hybridizations

Fixing and sectioning of embryos was performed as previously described (Shinozaki et al., 1991). Riboprobes labeled with digoxigenin were made according to the manufacturer’s recommendations (Roche). AT1, KAN, PH3, and SGR probes correspond to those used in previous reports (Kretzschmer et al., 2001; Liu et al., 1995; Lynn et al., 1999; Wysocka-Diller et al., 2003). Hybridizations, washes, and immunological detection were performed as previously described (Ville-Cauchard et al., 1999). Images were captured with OCapturePro 5.0 software. Unless noted otherwise, at least 20 wild-type, normal sitting, and abnormal sitting embryos were examined for each stage of embryogenesis and marker described.

Supplemental Data

Supplemental Data include three figures and four tables and are available at http://www.developmentalcell.com/cgi/content/full/12/6/940/DCl.

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Supplemental Data

**RPK1 and TOAD2 Are Two Receptor-like Kinases**

**Redundantly Required for Arabidopsis**

**Embryonic Pattern Formation**

Michael D. Nodine, Ramin Yadegari, and Frans E. Tax

![Figure S1. Expression of RPK1 and TOAD2 in mutants by RT-PCR](image)

1. RPK1 expression in Col-0; 2. RPK1 expression in rpk1-1; 3. RPK1 expression in rpk1-5; 4. TOAD2 expression in Col-0; 5. TOAD2 expression in toad2-1; 6. TOAD2 expression in toad2-2; 7. ACTIN7 expression in Col-0; 8. ACTIN7 expression in rpk1-1; 9. ACTIN7 expression in rpk1-5; 10. ACTIN7 expression in toad2-1; 11. ACTIN7 expression in toad2-2; 12. ACTIN7 PCR product from genomic DNA template. Marker sizes in base pairs are indicated to the left of the panel.

RPK1 and TOAD2 PCR primers (RPK1F3, RPK1R3, TOAD2Fa and TOAD2R1) were based on sequences downstream of insertions and are listed in Table S7. Since RPK1 and TOAD2 do not have introns, ACTIN7 primers (ACT7F and ACT7R) flanking an intron serve as a control for genomic DNA contamination. RNA isolation and RT-PCR experiments were performed at least three separate times with similar results.
S2. Example of Abnormal Seed Phenotype

A silique from a rpk1-1 toed2-1/+ self-pollinated is shown in (A). A higher magnification image of this silique showing a normal seed (middle) flanked by brown and shrunked abnormal seeds (top and bottom) is shown in (B).
Table S3. Frequencies of \textit{rpki toad2} and \textit{rpki toad2} progeny from self-pollinated \textit{rpki/ toad2/+} and \textit{rpki toad2/+} plants

<table>
<thead>
<tr>
<th>Parent Genotype (self-pollinated)</th>
<th>Frequency of \textit{rpki toad2} plants (n, expected frequency; P)</th>
<th>Frequency of \textit{rpki toad2/+} plants (n, expected frequency; P)</th>
</tr>
</thead>
<tbody>
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<td>\textit{rpki-1/- toad2-1/-}</td>
<td>0% (131; 6.25 %; 0.004)</td>
<td>4.58% (131; 12.5 %; 0.010)</td>
</tr>
<tr>
<td>\textit{rpki-1/- toad2-2/-}</td>
<td>0% (152; 6.25 %; 0.002)</td>
<td>5.92% (152; 12.5 %; 0.022)</td>
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<tr>
<td>\textit{rpki-5/- toad2-1/-}</td>
<td>0% (170; 6.25 %; 1.11e-03)</td>
<td>6.47% (170; 12.5 %; 0.026)</td>
</tr>
<tr>
<td>\textit{rpki-1 toad2-1/-}</td>
<td>0% (142; 25 %; 2.55e-09)</td>
<td>35.92% (142; 66.6 %;*, 7.45e-06)</td>
</tr>
</tbody>
</table>

Chi-square tests were used for statistical analyses.

* expected frequency calculated with the frequency of the \textit{rpki toad2} genotypic class equal to zero.
Table S4. Frequencies of rpk1/+/toad2/+ and rpk1 toad2/+ progeny from crosses to test for rpk1 toad2 gametophyte defects and rpk1/rpk1/rpk1 toad2/toad2/+ haploinsufficient endosperm.

<table>
<thead>
<tr>
<th>Parent Genotype</th>
<th>Frequency of rpk1/+/toad2/+ plants (n; expected frequency; P)</th>
<th>Frequency of rpk1 toad2/+ plants (n; expected frequency; P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpk1-1/+ toad2-1/+ (female) X Wild-type (Col-0) (male)</td>
<td>52% (42; 50 %; 0.827)</td>
<td>-</td>
</tr>
<tr>
<td>Wild-type (Col-0) (female) X rpk1-1 toad2-1/+ (male)</td>
<td>52% (33; 50 %; 0.902)</td>
<td>-</td>
</tr>
<tr>
<td>rpk1-1 toad2-1/+ (female) X rpk1-1 (male)</td>
<td>-</td>
<td>25% (118; 50 %; 1.59e-04)</td>
</tr>
<tr>
<td>rpk1-1 (female) X rpk1-1 toad2-1/+ (male)</td>
<td>-</td>
<td>27% (81; 50 %; 0.004)</td>
</tr>
</tbody>
</table>

Chi-square tests were used for statistical analyses.

This table shows the results from several genetic tests that were done to determine whether the mutant seed phenotype, and the absence of the rpk1 toad2 and reduction of rpk1 toad2/+ genotypic classes from self-pollinated rpk1/+/toad2/+ and rpk1 toad2/+ plants were due to defects during gametophyte, endosperm or embryo development.
(Top two rows) Reciprocal crosses between *rpk1 toad2/+* and wild-type plants were performed and reduced frequencies of *rpk1/+ toad2/+* individuals were not observed indicating that haploid *rpk1 toad2* female and male gametophytes are not lethal.

(Bottom two rows) Reciprocal crosses between *rpk1* and *rpk1 toad2/+* plants were done to test whether triploid *rpk1/rpk1/rpk1 toad2/toad2/+* endosperm is haploinsufficient and causes the mutant seed phenotype and the approximately 50% reduction of *rpk1 toad2/+* individuals. Regardless of the direction of this cross, the frequency of *rpk1 toad2/+* progeny was reduced by approximately 50% indicating that seeds with *rpk1/rpk1/rpk1 toad2/toad2/+* and *rpk1/rpk1/rpk1 toad2/+/+* endosperm genotypes are equally likely to give rise to reduced frequencies of *rpk1 toad2/+* plants.
Table S5. *RPK1p:* RPK1-GFP and *TOAD2p:* TOAD2-GFP Complement the Abnormal Sibling Embryo Phenotype

<table>
<thead>
<tr>
<th>Parent Genotype (self-pollinated)</th>
<th>Frequency of abnormal embryos (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>rpk1-1 toad2-1/+ RPK1p::RPK1-GFP</em></td>
<td>0% (142)</td>
</tr>
<tr>
<td><em>rpk1-1 toad2-1 TOAD2p::TOAD2-GFP</em></td>
<td>0% (87)</td>
</tr>
</tbody>
</table>
Figure S6. Localization of PIN1-GFP

(A-F) Confocal images of PIN1-GFP in Col-0 (A and D), normal sibling (B and E) and abnormal sibling (C and F) embryos at the early globular (A - C) and late globular (D - F) stages.

Scale bars equal 25 μm.
Table S7. Sequences of PCR primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>RPK1F2</td>
<td>AATGACGATGATACACAGAGGATACACG</td>
</tr>
<tr>
<td>RPK1F3</td>
<td>AGCTGGGTTTCGACAGCTCATCA</td>
</tr>
<tr>
<td>RPK1F4</td>
<td>CGAAAAGCTTACCCCGAAGTTTCTTGTCTGTA</td>
</tr>
<tr>
<td>RPK1R3</td>
<td>GACACGGCGCTACATCAAGAGCAA</td>
</tr>
<tr>
<td>RPK1R5</td>
<td>AAAAGCTTTCTTTAGCTTTGTATAAAATCTAGAGGTG</td>
</tr>
<tr>
<td>TOAD2F</td>
<td>TGACTTCTTTGCTTCTCTGAGTCAAA</td>
</tr>
<tr>
<td>TOAD2F2</td>
<td>TTGAAGGGATCCCTCTCTTATGGTAAATGTAC</td>
</tr>
<tr>
<td>TOAD2Fa</td>
<td>AGAAGGCTCAAGTTGGAACCTCCCTG</td>
</tr>
<tr>
<td>TOAD2R1</td>
<td>TAAACAGCTTTGAGTGTCGAGTTAT</td>
</tr>
<tr>
<td>TOAD2R2</td>
<td>GGGGATCCCCAACACGAGCTTCTGAGCTGCTTCA</td>
</tr>
<tr>
<td>SALK LB</td>
<td>TGGTTCACGTAGGGGCCCATCG</td>
</tr>
<tr>
<td>JL202</td>
<td>CATTTTATAATAACGCTGCGGACATCTAC</td>
</tr>
<tr>
<td>ACT7F</td>
<td>GGTGAGGATATTCCAGCCAAGCTGCTG</td>
</tr>
<tr>
<td>ACT7R</td>
<td>TGGTAGATCCCGACCCGCAAGATC</td>
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</table>
APPENDIX B

THE ESTABLISHMENT OF ARABIDOPSIS COTYLEDON PRIMORDIA REQUIRES THE OVERLAPPING FUNCTIONS OF TWO RECEPTOR-LIKE KINASES
TITe

The establishment of Arabidopsis cotyledon primordia requires the overlapping functions of two receptor-like kinases

Short title: Two RLKs required for cotyledon intiation

Keywords: embryogenesis, Arabidopsis, receptor-like kinases, signaling, protoderm
AUTHORS AND AFFILIATIONS

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SUMMARY

Signaling between different regions of *Arabidopsis thaliana* embryos has important roles in coordinating embryonic pattern formation. 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-RLK), *RECEPTOR-LIKE PROTEIN KINASE1 (RPK1)* and *TOADSTOOL2 (TOAD2)*, with overlapping functions required 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 addition, RPK1-GFP and TOAD2-GFP translational fusions were both localized to the central domain protodermal cells when cotyledon primordia were initiating. 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 throughout the embryo including the failure to initiate cotyledon primordia.
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-unrelated apical, central and basal domain derivatives is required for embryonic pattern formation (Laux et al., 2004; Mayer and Jurgens, 1998a; 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, middle ground tissue initial and 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, 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). Cells within each lateral region will generate a cotyledon primordium (Fig. 1A). The medial
region is further partitioned into the presumptive shoot meristem precursors and two
groups of cells that will form boundaries between the margins of the developing
cotyledon primordia (Fig. 1A).

The plant hormone auxin plays an important role in patterning the apical embryonic
domain. Polar auxin transport 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, and several studies indicate that auxin response is required to
properly pattern the apical domain (Aida et al., 2002; Benkova et al., 2003; Berleth,
1993; Friml et al., 2003; 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 sub-cellular 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 demonstrate that RPK1 and TOAD2 also have
overlapping functions required for cotyledon initiation. 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
Seedlings were grown on 1% agar plates with 0.5X Murashige and Skoog (MS) media and 0.5% 2-(N-Morpholino)ethane sulfonic acid (MES) with a 16 hour light/8 hour dark cycle. Plants were grown at 22°C in a Conviron growth chamber with a 16 hour light/8 hour 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 \textit{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. \textit{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 \textit{in situ} hybridizations
RNA \textit{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’-AGCAGGATCTATTGTTTCGA-3’ and PIN1 R1: 5’-
ACCACCTCCTCCAGATTGAT-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’-CGAGCTTGATTACCAGATAATAGAAA-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 toad2* 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 a cotyledon (Fig. 1C; Table 1).

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 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/+* 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. 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 embryos with defective cotyledon primordia were composed of two distinct halves at these stages (Fig. 1E). One half of these embryos had a properly developing cotyledon primordium and organized central domain cell layers. The other half 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 \textit{rpk1-5 toad2-2/+} plants (Table 1). \textit{Defective half} embryos were also observed in approximately 5\% of \textit{rpk1-1} and \textit{rpk1-5} embryos, but not in the progeny of self-pollinated \textit{toad2-1/+} and \textit{toad2-2/+} plants (Table 1). The frequencies of \textit{defective half} embryos and seedlings with single cotyledons were nearly identical (Table 1). This indicates that the missing cotyledon phenotype of \textit{rpk1} and \textit{rpk1 toad2/+} seedlings is due to cotyledon initiation defects in \textit{rpk1} and \textit{rpk1 toad2/+} embryos.

**Expression of markers characteristic of different apical domain regions in \textit{defective half} embryos**

To test whether the failure to initiate cotyledon primordia were due to differentiation defects, we examined the expression of markers characteristic of different apical domain cell types in wild-type (Col-0) embryos and embryos from self-pollinated \textit{rpk1-1 toad2-1/+} plants. Self-pollinated \textit{rpk1-1 toad2-1/+} plants produce normal sibling embryos (including \textit{rpk1-1} and \textit{rpk1-1 toad2-1/+}) that resemble wild-type at the transition stage, as well as, \textit{toadstool} and \textit{defective half} embryos. \textit{ANT} encodes an \textit{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). \textit{ANT} transcripts were not detected in either the apical domain of \textit{toadstool} embryos or the malformed cotyledon primordium of all \textit{defective half} embryos (13/13) examined (Fig. 2B,C). All \textit{toadstool} and \textit{defective half
embryonic cells expressed *RIBOSOMAL PROTEIN 5A (RPS5A)* indicating that the lack of *ANT* transcripts are not due to a general loss of transcription (Fig. 2J,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, 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 (\textit{DR5rev::GFP}) (Friml et al., 2003). \textit{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). \textit{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 (0/23) observed in the apical domains of \textit{toadstool} embryos (Fig. 2M,N and data not shown). Furthermore, a signal corresponding to \textit{DR5rev::GFP} was not observed in the \textit{defective half} malformed cotyledon primordia (Fig. 2O). Based on these results, we suggest that auxin response is perturbed in \textit{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 \textit{PIN1} expression in \textit{defective half} embryos may result in the observed failure to establish auxin maxima in \textit{defective half} malformed cotyledon primordia. To test this idea, we examined \textit{PIN1} transcripts in \textit{defective half} embryos. \textit{PIN1} was expressed throughout the apical domain of late globular wild-type embryos (data not shown). Consistent with previous findings (Aida et al., 2002), \textit{PIN1} transcripts were detected exclusively in the cotyledon primordia and vascular primordium of all transition/early heart stage wild-type embryos examined (Fig. 2P). Therefore, \textit{PIN1} transcripts become localized in a bilateral arrangement at the transition stage. This bilateral \textit{PIN1} expression pattern was not
observed in *toadstool* or *defective half* embryos. *PIN1* was expressed throughout *toadstool* embryos, but strongest signals were observed in the central and basal domains (Fig. 2Q). *PIN1* transcripts were either not detectable or much reduced in the *defective half* malformed cotyledon primordia (Fig. 2R). 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* have overlapping functions required for peripheral region identity and auxin responses in the apical domain of transition/early heart stage embryos.

**Localization patterns of transcripts characteristic of different central domain cell layers in *defective half* embryos**

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 *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 had protoderm-specific transcripts. *ATML1* transcripts were observed in the protoderm of all wild-type embryos (29/29) examined, but were never detected in the central domain protoderm of similarly staged toadstool embryos (0/45) (Fig. 3A,B). *ATML1* transcripts were also never (0/18) detected in the central domain protoderm of the defective half malformed cotyledon primordium (Fig. 3C). However, *ATML1* was frequently (78%; 14/18) expressed in the apical domain protoderm of the malformed cotyledon primordium (Fig. 3C). 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, but were not observed in the cells in the position of
the ground tissue initials in any similarly staged toadstool embryos (0/39) examined (Fig. 3E,F). 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. 3G). 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. 3G). 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 indicate that cells in the position of the ground tissue initials below the malformed cotyledon primordia often, but not always, lack SCR transcripts.

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. 3I). Similar to our previous observations (Nodine et al., 2007), all toadstool embryos (48/48) whose normal siblings were at the transition/early heart stage expressed PNH/ZLL ectopically in the ground tissue initials. Most toadstool embryos (69%; 33/48) also expressed PNH/ZLL ectopically in the protoderm (Fig. 3J). 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. 3K). 

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. 2R). Therefore, 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 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) during the transition stage of embryogenesis. 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 (Fig. 4A). 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 initials and hypophyseal cells (Fig. 4A). TOAD2-GFP signals were weaker than RPK1-GFP signals, but were detected in the presumptive plasma membranes of protodermal cells (Fig. 4B). 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

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

Our genetic and phenotypic analyses indicate that *RPKI* and *TOAD2* have overlapping functions required for cotyledon initiation. Based on the following results, we suggest that *RPKI* and *TOAD2* are primarily required in the central domain protoderm for its differentiation, and that the cotyledon initiation defects observed in *defective half* embryos are caused by these 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 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. Third, transcripts characteristic of the ground tissue initials (SCR) and vascular primordia (PNH and PIN1) were occasionally localized appropriately in *defective half* embryos. This 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.

**Apical embryonic domain patterning**

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 region 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, 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 with 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; Tanaka et al., 2002). Embryos with mutations in both ALE1 and another RLK called ABNORMAL LEAF SHAPE2 (ALE2) also exhibit 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 had cotyledon formation defects, 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 maintain 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. Our model therefore proposes that the central domain protoderm is functioning as an organizer of Arabidopsis embryonic pattern.

The identification and characterization of RPK1 and TOAD2 extracellular ligands and downstream signaling components, as well as central domain protoderm-derived signals, will enable testing of the model described above. Investigating how the integration of signaling events mediated by ACR4, ALE1, ALE2, RPK1 and TOAD2 contributes to protoderm differentiation should yield significant insight into embryonic pattern formation.
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REFERENCES


**FIGURE LEGENDS**

**Fig. 1.** Seedlings and embryos from self-pollinated *rpk1-1 toad2-1/+* plants have cotyledon development defects.

(A) Schematic of pattern formation during the 8-cell (left), 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 (pink); 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.
**Fig. 2.** Expression of markers characteristic of different apical domain regions 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* hybridizations with STM antisense probe. Representative transition/early heart stage wild-type (A), *toadstool* (B) and *defective half* (C) 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 (E), *toadstool* (F) and *defective half* (G) embryos.

(L) RNA *in situ* hybridization of transition/early heart stage *toadstool* embryo with RPS5A sense probe.

(M, N and O) Representative confocal images of transition/early heart stage wild-type (M), *toadstool* (N) and *defective half* (O) embryos expressing DR5rev::GFP. Note: transition/early heart stage *toadstool* embryos predominately express DR5rev::GFP in the distal suspensor cells. Green, GFP; red, FM4-64.

(P-R) RNA *in situ* hybridization with PIN1 antisense probe. Representative transition/early heart stage wild-type (P), *toadstool* (Q) and *defective half* (R) embryos.
(S) RNA \textit{in situ} hybridization of transition/early heart stage \textit{toadstool} embryo with PIN1 sense probe. Arrowheads indicate ectopic transcript localization patterns. Scale bars represent 25 µm.

\textbf{Fig. 3.} Localization patterns of transcripts characteristic of different central domain cell layers in \textit{defective half} embryos

(A-C) RNA \textit{in situ} hybridizations with ATML1 antisense probe. Representative transition/early heart stage wild-type (A), \textit{toadstool} (B) and \textit{defective half} (C) embryos.

(D) RNA \textit{in situ} hybridization of transition/early heart stage \textit{toadstool} with ATML1 sense probe.

(E-G) RNA \textit{in situ} hybridizations with SCR antisense probe. Representative transition/early heart stage wild-type (E), \textit{toadstool} (F) and \textit{defective half} (G) embryos.

(H) RNA \textit{in situ} hybridization of transition/early heart stage \textit{toadstool} with SCR sense probe.

(I-K) RNA \textit{in situ} hybridizations with PNH antisense probe. Representative transition/early heart stage wild-type (I), \textit{toadstool} (J) and \textit{defective half} (K) embryos.

(L) RNA \textit{in situ} hybridization of transition/early heart stage \textit{toadstool} with PNH sense probe. Arrowheads indicate ectopic transcript localization patterns. Scale bars represent 25 µm.
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.
Figure 2

| Col-0 | root
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<td><strong>PIN1</strong></td>
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Figure 4
Table 1. Frequencies of seedlings and embryos with one cotyledon

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<tr>
<th>Parent Genotype (self-pollinated)</th>
<th>Frequency of Seedlings with One Cotyledon (^A) (total)</th>
<th>Frequency of Embryos with One Cotyledon Primordia (^B) (total (^C))</th>
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<tr>
<td>rpk1-1</td>
<td>4.6% (1018)</td>
<td>5.1% (336)</td>
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<tr>
<td>rpk1-5</td>
<td>4.8% (500)</td>
<td>4.8% (252)</td>
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<tr>
<td>toad2-1/+</td>
<td>0% (512)</td>
<td>0% (190)</td>
</tr>
<tr>
<td>toad2-2/+</td>
<td>0% (511)</td>
<td>0% (173)</td>
</tr>
<tr>
<td>rpk1-1 toad2-1/+</td>
<td>16.4% (^D) (935)</td>
<td>16.6% (^D) (223)</td>
</tr>
<tr>
<td>rpk1-5 toad2-2/+</td>
<td>15.7% (^D) (460)</td>
<td>17.9% (^D) (67)</td>
</tr>
<tr>
<td>wild-type (Col-0)</td>
<td>0% (494)</td>
<td>0% (151)</td>
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\(^A\) 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.