**ENDOSPERM DEVELOPMENT: Cellularization and Cell Fate Specification**

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**Abstract** The endosperm develops from the central cell of the megagametophyte after introduction of the second male gamete into the diploid central cell. Of the three forms of endosperm in angiosperms, the nuclear type is prevalent in economically important species, including the cereals. Landmarks in nuclear endosperm development are the coenocytic, cellularization, differentiation, and maturation stages. The differentiated endosperm contains four major cell types: starchy endosperm, aleurone, transfer cells, and the cells of the embryo surrounding region. Recent research has demonstrated that the first two phases of endosperm occur via mechanisms that are conserved among all groups of angiosperms, involving directed nuclear migration during the coenocytic stage and anticlinal cell wall deposition by cytoplasmic phragmoplasts formed in interzones between radial microtubular systems emanating from nuclear membranes. Complete cellularization of the endosperm coenocyte is achieved through centripetal growth of cell files, extending to the center of the endosperm cavity. Key points in cell cycle control and control of the MT (microtubular) cytoskeletal apparatus central to endosperm development are discussed. Specification of cell fates in the cereal endosperm appears to occur via positional signaling; cells in peripheral positions, except over the main vascular tissues, assume aleurone cell fate. Cells over the main vascular tissue become transfer cells and all interior cells become starchy endosperm cells. Studies in maize have implicated Crinkly4, a protein receptor kinase-like molecule, in aleurone cell fate specification.

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INTRODUCTION

A little over a century ago, endosperm was recognized as an independent genetic entity, the result of a second fertilization event that is typical for angiosperms (67, 127). Following the discovery of double fertilization, two hypotheses, which still persist today, were proposed for the evolutionary origin of the angiosperm endosperm [reviewed in (59, 60)]. In one, the endosperm was hypothesized to originate as an altruistic twin embryo, which evolved into a nourishing endosperm for the surviving embryo, whereas in the second, the endosperm was viewed as a continued development of the megagametophyte triggered by fertilization. In early accounts of endosperm development, dating back to the late nineteenth century (19, 82, 96, 175) and in subsequent work (92, 114, 149), the processes involved in subdivision of the multinucleate endosperm into cellular compartments were unclear. These processes have remained elusive until recently. Endosperm cellularization is the topic of the first part of this review. Based on studies using immunohistochemical methods, it is now known that cellularization of the endosperm coenocyte is mediated through a tightly orchestrated interplay between the machinery controlling progression through the cell cycle and the microtubular cytoskeleton, integrating all known plant cytoskeletal components, and four mechanisms of cell wall deposition, into the sequence of events leading to endosperm cellularization. Far from being an oddity in the plant kingdom, this mode of cellularization is an integral part of the cell life cycle, occurring during pollen, megagametophyte, and endosperm development (23). In the meristematic cell, the far better understood part of the plant cell life cycle, cytokinesis is mediated by interzonal phragmoplasts (71, 163, 166). Although endosperm appears to use the same basic machinery for cytokinesis and progression through the cell cycle, the controls of these processes differ from those in meristematic cells.

The second topic of this review is the specification of the developmental fate of the four endosperm cell types, the embryo surrounding region, the transfer cells, the aleurone layer, and the starchy endosperm. Each of these cell types has a characteristic temporal and spatial developmental profile. For aleurone cells, recent data strongly suggest that cell fate specification occurs via positional signaling. Because of the simple overall organization of nuclear endosperm, this system provides a unique opportunity to study the integration and coordination of genetic subprograms into a unit specifying the body plan of an “organism.” This review discusses steps in endosperm development that may account for its unique
developmental pathway, and describes how these controlling steps are integrated temporally as well as spatially. Several approaches to improve our understanding of these controls are also discussed. For earlier reviews of endosperm development, the reader is referred to (7, 11, 47, 110, 138, 140).

OVERVIEW OF ENDOSPERM CELLULARIZATION

The model presented in this section is based on data from dicot and monocot species, and represents a highly conserved mode of nuclear endosperm cellularization. The data underlying the model are discussed in the following sections. The fate of the endosperm varies in different species. In the grasses, including the cereals, the endosperm is persistent and contains four major cell types (see below). In some dicots, e.g. Arabidopsis, the endosperm is nonpersistent, and only one cell type is retained in mature seeds.

The endosperm develops from the fertilized central cell of the megagametophyte or embryo sac, which is positioned within the mass of nucellar parenchyma cells (Figure 1a). At the time of fertilization, the embryo sac, which contains both the central cell and the egg cell, constitutes only a small portion of the grain. The central cell is an ellipsoid structure that fills up most of the embryo sac, with its diploid nucleus positioned proximally in a cytoplasm surrounding a large central vacuole (Figure 2a). Shortly after fertilization, repeated rounds

Figure 1 Overview of endosperm development in maize. (a) The endosperm develops from the fertilized triploid central cell which is positioned within the megagametophyte or embryo sac. The embryo sac is embedded in a large body of nucellar parenchyma cells, which is enclosed in the integuments of the grain. For details of the central cell, please see Figure 2. (b) In maize, expansion of the endosperm occurs rapidly after fertilization, as demonstrated by the relative size of the dissected endosperm from the interval between 4 and 15 DAP (days after pollination). (c) Diagram of longitudinal section of 15 DAP maize grain consisting of the aleurone layer (AL), the starchy endosperm (SE), the transfer cell region (TC), and the embryo surrounding region (ESR) around the basal part of the embryo (EMB).
of mitosis, but no cell wall formation or cytokinesis, take place, leading to the endosperm coenocyte (Figure 2b). Cellularization of the coenocyte is initiated by the formation of a radial microtubular system (RMS) from the envelope of all nuclei (Figure 2c). After the formation of cytoplasmic phragmoplasts in the interzones between opposing RMSs, cell walls are deposited around each nucleus forming alveoli, which are tube-like structures (Figure 2d). Mitotic divisions of nuclei within alveoli, followed by cell wall formation, lead to one peripheral layer of cells, and a new layer of alveoli (Figure 2e). Repeated rounds of the same cycle of events lead to cell files that eventually completely invade the central vacuole (Figure 2f). The cellularization process occurs during the first few days after fertilization. In maize, the cellularization process is completed at the end of day 4 after pollination (Figure 1b). The rapid expansion of the endosperm during the interval 4 to 15 days after pollination (DAP) (Figure 1b) is attributable to both cell division and cell expansion. The fully developed endosperm consists of four major cell types, the starchy endosperm, the aleurone layer, the transfer cells, and the embryo surrounding region (Figure 1c). Details of the formation of the endosperm coenocyte are shown in Figures 3 and 4, and the cellularization process is shown in Figures 5 to 7. A model for endosperm cell fate specification in cereals is presented in Figure 8 (Figure 8). The pattern of cell division in starchy endosperm and aleurone cells is presented in Figures 7 and 9, respectively. Table 1 summarizes the main events in endosperm development.

THE ENDOSPERM COENOCYTE

Mitotic Divisions and Suppression of Phragmoplast Function

Shortly after fertilization, the central cell nucleus (Figure 2a) divides without the formation of cell walls. The microtubular (MT) cytoskeleton typical for endosperm coenocyte mitosis in barley (27) is illustrated in Figure 3, which shows two neighboring endosperm coenocyte nuclei (Figure 3a). The dividing nuclei display metaphase/anaphase spindles (Figure 3b): telephase spindles at the poles and interzonal MT connecting the two poles (Figure 3c), assembly of interzonal phragmoplast (Figure 3d), and structurally complete phragmoplasts between condensing complements of chromosomes (Figure 3e). Up to this stage, the mitosis of the endosperm coenocyte is identical to that of meristematic cells. In contrast to meristematic cells, however, where the phragmoplast facilitates the assembly of the cell wall in the interzone between its two parallel MT discs (arrow, Figure 3e), no cell plate forms in the barley endosperm coenocyte mitosis. After completion of the division cycle, the phragmoplast remains only as traces of condensed MT material on the nuclear membranes (Figure 3f). In a similar study in wheat, deposition of a short-lived cell plate has been reported, but it fails to extend to a complete cell wall, suggesting that the transient phragmoplast structure is partly functional (172). At least for the small grains, therefore, formation of the
TABLE 1  Summary of events in endosperm cellularization and cell differentiation

<table>
<thead>
<tr>
<th>Stage/event</th>
<th>Cell cycle</th>
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<tr>
<td>Coenocytic stage</td>
<td>Repeated rounds of mitosis;</td>
<td>Suppression of interzonal phragmoplast formation;</td>
<td>Specification of T-cell fate; Figure 8a (green cytoplasm)</td>
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<td></td>
<td>Figure 2b</td>
<td>Nuclear migration;</td>
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<td>Alveolar stage</td>
<td>Cell cycle arrest, mitotic hiatus</td>
<td>RMS formation; Figure 2c and 5a-c; Cell wall deposition by cytoplasmic phragmoplasts leading to alveolus formation; Figure 2d, 5d and e</td>
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<tr>
<td>Completion of endosperm cellularization</td>
<td>Release of cell cycle arrest and synchronous mitosis of alveolar nuclei; Figure 6</td>
<td>Deposition of periclinal cell walls by interzonal phragmoplasts; Figure 2e and 6e-g</td>
<td>Specification of aleurone and starchy endosperm cell fates; Figure 8c (blue and red cells)</td>
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<td></td>
<td>Continued mitosis in the inner cell layers; Figure 2f</td>
<td>Repeated rounds of RMS and alveolus formation leads to the cellular endosperm; Figure 2f and 7b</td>
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<tr>
<td>Formation of Starchy endosperm cells</td>
<td>Continued mitosis in inner cell; Figure 8c (red cells)</td>
<td>Interzonal phragmoplast deposit cell walls at random planes; Figure 7c, d</td>
<td></td>
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<tr>
<td>Formation of aleurone cells</td>
<td>Release of mitotic arrest in peripheral cell layer; Figure 8c (blue cells)</td>
<td>PPBs and interzonal phragmoplasts deposit cell walls at anticlinal (or periclinal) planes; Figure 9</td>
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endosperm coenocyte is mediated through an inhibition of phragmoplast function, but not phragmoplast assembly.

A second example of a nonfunctional phragmoplast, supporting the presence of a checkpoint for overriding cell plate formation, is evident in orchids that produce pollen with no set arrangement of tetrads. Following meiosis I, a phragmoplast develops in the interzone, but no wall is deposited (20). Interestingly, a coenocytic stage also occurs in the development of embryo sacs, from which the central cell develops (5). Thus, suppression of cytokinesis is an important phenomenon in the
Figure 3  Mitosis without cytokinesis in the endosperm coenocyte. (a) Two interphase endosperm coenocyte nuclei from a stage between those depicted in Figure 2a and b. The line below the nuclei represents the central cell wall, and the central vacuole is oriented on top of the nuclei. (b) Mitotic spindles separating the two diploid sets of chromosomes. The two nuclei are at slightly different stages; the nucleus to the left is in late metaphase, the one to the right in anaphase. For simplicity, the chromosomes (the asterisks indicates centromere attachment points) are not drawn. (c) Both dividing nuclei have MT arrays typical of telophase. Note the MTs connecting the two poles. (d) Initiation of phragmoplast assembly in the interzone between separating groups of chromosomes, and (e) phragmoplasts between the two sets of condensing complements of chromosome sets. In contrast to phragmoplasts in meristematic cells, no cell plate is formed between the separating nuclei. Instead, the phragmoplasts degenerate, leaving traces of condensed MTs on the nuclear membranes of the four daughter nuclei (f).

plant life cycle, and insight into the stage at which cytokinesis is blocked in the different systems may also yield valuable information about the control points in the meristematic mitotic cycle.

What is the mechanism controlling the suppression of phragmoplast function in nuclear endosperm? Rapidly accumulating evidence points to a link between the dynamics of MT formation and regulators of the cell cycle. Thus, Cdc-2-like kinases and mitotic cyclins, associating to form the complexes that regulate cell cycle progression, have been localized in phragmoplasts as well as in spindles and preprophase bands, suggesting a role for Cdc-cyclins in the regulation or function of these structures [reviewed in (163)]. In maize cells, cyclins II and III are associated with the postmitotic spindle, such as those depicted in Figure 3c and d, possibly reflecting kinase-mediated regulation [reviewed in (145)]. Recent work also suggests a role for MAP kinases in cytokinesis. The two closely related MAP kinases NTF6 (32) and MMK3 (14) from tobacco and alfalfa, respectively, are specifically activated during M-phase. Here they reach peak activity during cytokinesis, when they are present in the cell plate, suggesting that they regulate the activity of protein(s) involved in cell plate formation. Further evidence for the
involvement of MAP kinases in the regulation of cytokinesis comes from a study of MAP-KKK, which is presumed to act upstream of MAPK in kinase cascades (4). MAP-KKK kinases were also found to be associated with cytokinesis (4). Finally, the most direct evidence for a link between MAP kinases and phragmoplast formation was seen in tobacco, where NPK1 seems to interact directly with a phragmoplast-localized kinesin-like protein (111).

Based on the observation that phragmoplast initiation, and even partial cell plate deposition, occurs in syncytial endosperm, regulation of one or more steps of phragmoplast function downstream of phragmoplast initiations seem likely control points to regulate endosperm coenocyte formation. Systematic probing of molecules associated with phragmoplasts in meristematic cells in syncytial endosperm is one way to search for mechanisms involved in phragmoplast suppression. Genetically, this problem may be addressed by a search for mutants that lack a coenocytic stage because of a defect in phragmoplast suppression.

In addition to specific control points discussed here, recent studies suggest the existence of an overriding control of endosperm development acting on endosperm chromatin structure. This conclusion is based on the Arabidopsis “maternal-effect” mutants fie (fertilization independent) (137), fis (fertilization-independent seeds) (37), and Medea (66); reviewed in (147). The products of two of these genes have similarity to Polycomb group proteins, which regulate gene expression through epigenetic silencing (146). In Drosophila, these genes play a role in maintaining established patterns of gene expression. Establishment or maintenance of patterns of endosperm gene expression, including suppression of phragmoplast formation, may therefore also be the function of these genes in plants. Interestingly, polycomb-like genes may provide insight into the mechanisms underlying the requirement for a 1:2 ratio between maternally and paternally derived genomes for normal endosperm development (106, 158).

Nuclear Migration

In maize, the initial endosperm coenocyte mitoses occur at predictable planes, followed by migration of the daughter nuclei to restricted domains of the coenocyte cytoplasm. After fertilization, the first mitosis occurs in a plane perpendicular to the longitudinal axis of the embryo sac (Figure 4a and b), and the next two at alternate planes (Figure 4c and d). At the eight-nuclear stage, the nuclei form a ring in the proximal cytoplasm around the embryo (Figure 4e), before migrating to discrete and separate longitudinal sectors of the peripheral cytoplasm of the central cell (Figure 4f). This interpretation of the orientation of mitotic planes and the pattern of nuclear migration is based on direct observations of the early division planes and on revertant sectors of mutants with different marker genes (45, 119, 180). In Arabidopsis, nuclear migration occurs in the direction from the micropylar to to the chalazal chambers (24). A second example of a plant system where positioning of nuclei in a syncytium precedes differentiation is the eight-celled Polygonum-type megagametophyte of cereals and
Arabidopsis (151), in which the two mitotic nuclei of the functional megaspore migrate to opposite poles of the cell. The fate of the cells derived from these two nuclei are widely different, and are possibly determined by developmental cues deposited in a polarized cytoplasm. In this system, the antipodals develop in the chalazal end, the synergids in the micropylar end, and the egg and the central cell between these two extreme positions.

Attention to improving our understanding of the underlying controls of this stage of endosperm development should focus on the mechanism involved in nuclear migration, and its functional significance, if any. In Drosophila, compartmentalization (i.e. a common genetic control of cells in a parasegment that prevents cells from mixing with their neighbors and controls their subsequent development) plays an important role in the development of segments in the fly body [(150) and references therein]. Although cell-lineage studies demonstrate a predictable spatial order in the migration of the endosperm nuclei in the coenocyte, the borders between domains do not reflect obvious developmental patterns in the mature endosperm. A more likely possibility, therefore, is that nuclear positioning plays a role in the differentiation of the endosperm coenocyte (23). As described below, the nuclei in the proximal end of the endosperm coenocyte already assume transfer cell fate at the coenocytic stage, indicating the presence of particular developmental cues in this portion of the central cell cytoplasm. Also, in the young Arabidopsis endosperm, a developmental gradient exists. The structure referred to as the chalazal cyst, which is located in the opposite (proximal) end of the horseshoe-shaped central cell, possibly plays a role in nutrient uptake (24). Deposition of nuclei in the distal cytoplasm, through nuclear migration, may therefore be a way to ensure differentiation of the chalazal cyst, and hence provide access to maternal nutrients.

In the cereals, possible approaches to dissect these processes include studies of the maize indeterminate1 mutant, in which abnormal microtubular behavior results in irregular positioning of megametophyte nuclei (75), as well studies of mutants with an uneven distribution of endosperm cells in the endosperm cavity (O-A Olsen, unpublished). A more direct approach is the study of Arabidopsis mutants in which disruption of cytoskeletal organization appears to cause a failure of endosperm nuclei to migrate to the chalazal end of the seed. Such mutants include pilz (118) and titan-1 (109).

Cell Cycle Arrest at the End of the Coenocytic Stage

After the period of mitotic divisions and nuclear migration leading to formation of the endosperm coenocyte, as depicted in Figure 2b, mitosis in barley ceases at 3 DAP and resumes 2 days later. During this mitotic hiatus, the endosperm coenocyte prepares for cellularization by a series of remarkable rearrangements of the MT cytoskeleton. Direct observations of a similar mitotic hiatus have not been made in other species, most likely because the cellularization process is much more rapid in species such as wheat, maize, and Arabidopsis. In these species, cellularization takes only 3 to 4 days, compared with 6 days in barley. It is therefore assumed that a similar mitotic hiatus occurs in other species as well. The variation in the final
number of nuclei in different species also suggests that the mitotic arrest point is under genetic control. In maize, cellularization is initiated after eight or nine rounds of nuclear divisions (180); Astelaria and many legume species produce many nuclei before cellularization, whereas Helianthus cellularization begins when fewer than ten free nuclei are present in the endosperm coenocyte (57).

In plants, arrest after mitosis can occur in either G1 or in G2 [for an overview see (48, 79, 83)]. Of these, the best studied checkpoint is the G1, which has been documented in yeast, flies, and mammals as well as in plants. In mammalian cells, retinoblastoma protein (Rb) and its relatives prevent cells from progressing to S phase by inactivating E2F transcription factors that activate the transcription of S phase–specific genes. Inactivation of Rb by phosphorylation in late G1 releases genes under E2F control from repression and the cells become ready for S-phase entry (102). In plants, homologues of most of the key players in the Rb pathway, including Rb itself, have been identified, and most show structural and functional similarities to their animal counterparts (65). Similarly, in animal cells, control of Rb activity may occur through CycDs, the transcription of some types of CycD being inducible during cell-cycle entry by mitogens. Recently, E2F transcription factors were also identified in wheat and tobacco, both of which were shown to interact with maize Rb1 and tobacco Rb, respectively, in yeast two-hybrid assays (148). CDK activity requires activating phosphorylation by CDK-activating kinase (176). In addition, four CDK inhibitor (CKI) protein genes are reported in plants, but only one, ICK1, has been characterized biochemically (181). ICK1, which interacts with both CDK-a (cdc2a) and cyclin CycD3, is an inhibitor of plant cdc2-like kinases in vitro. Its C-terminal consensus sequence resembles part of the CDK2-binding domain of the mammalian CKI p27Kip1 (181).

It is not known whether the nuclei of the endosperm coenocyte are arrested in the G1 or in G2 stages of the cell cycle. In light of the cell cycle research summarized above, and assuming that cell cycle arrest occurs in G1, one possibility is that control involves Rb. This would be similar to maize leaf development, where lateral cells in the shoot apical meristem exit the cell cycle in G1 (69). This event is correlated with Rb expression and loss of cell division activity. Analysis of DNA contents as well as expression and activity analysis of Rb at the early phase of endosperm development is one approach to studying the mechanisms underlying this step in endosperm development. Analyses of mutants arrested at the syncytial stage in Arabidopsis and barley (16) also represent attractive material for such studies.

ENDOSPERM CELLULARIZATION

Radial Microtubular Systems (RMS) and Alveolus Formation

The process leading to subdivision of the endosperm coenocyte into cellular compartments is initiated by the formation of RMS emanating from the membrane of each endosperm nucleus (Figures 2c and 5a). RMS in developing nuclear endosperm are well documented, and have been observed in the endosperm
Figure 5  Formation of RMS (radial microtubular systems) and initiation of anticlinal cell wall formation. Illustrations of the process leading to the formation of the partly cellularized endosperm in Figure 2d from the endosperm coenocyte in Figure 2b. (a) RMS forms at the nuclear membranes. Orientation is the same as in Figure 3. (b) RMSs extend and initially overlap, but soon form cytoplasmic phragmoplasts that deposit anticlinal cell walls (dotted line) (c). Partial anticlinal cell wall are formed around each nucleus (d). (e) Walls extend centripetally (toward the central vacuole) aided by adventitious phragmoplasts formed by a canopy of MT arrays extending from the upper pole of endosperm nuclei.

coenocyte in a number of species, including wheat (172, 177), Arabidopsis (24, 144, 183), Ranunculus sceleratus (186), barley (27), rice (26), Phaseolus vulgaris (185), Brassica napus (178), and Myrsine laetevirens (143). In addition, extensive studies of extruded Haemanthus endosperm have contributed to a greater understanding of the mitotic apparatus as well as MT arrays similar to those of the early cereal endosperm [see for instance (2, 161) and references therein].

Initially, in barley, the MTs of neighboring RMSs overlap (Figure 5b), but interzones soon form between individual RMSs (Figure 5c), marking out a nucleocytoplasmic domain for each nucleus (22). Arrays of microtubuli, assumed to have the same polarity (plus ends), were termed cytoplasmic phragmoplasts by Brown and coworkers (27), based on the observation that cellularization of the endosperm coenocyte is initiated by deposition of anticlinal walls in these RMS interzones. For a reason not yet fully understood, the anticlinal walls are first visible in the light microscope close to the former central cell wall. Soon these walls surround
each nucleus, forming an alveolus, or a tube-like wall structure (Figures 2d and 5d). The pattern of cytoplasmic phragmoplast development seen in the cereals closely resembles those of the cytoplasmic phragmoplast that form in isolated bits of coenocytic endosperm of Haemanthus (2). As the alveolus extends centripetally (toward the central vacuole), the MT arrays in barley and rice undergo dramatic reshaping, assuming a tree-like shape with MTs anchoring the elongated nucleus to the central cell wall, and sending a canopy of microtubules up toward the central vacuole near the upper end of the alveolus (Figure 5e). MTs in the canopy from opposing NCDs function to deposit the walls of the leading edge as the alveolus extends toward the center of the central vacuole. These structures were termed adventitious phragmoplasts in barley (27).

The mechanisms involved in cell wall initiation between non-sister nuclei in nuclear endosperm have been debated extensively [reviewed in (47, 87, 138)]. These walls represent an exception to the default mechanism for cell wall formation in meristematic cells of somatic tissue, where interzonal phragmoplasts facilitate formation of the wall leading to cytokinesis or cell division. In the early literature addressing this problem in the cereals, the initial anticlinal walls were described as extensions of wall pegs protruding from the embryo sac or central cell wall (116, 117, 124, 125). Similar descriptions of so-called free-growing walls in the absence of interzonal phragmoplasts were also given for nuclear endosperm of dicot plants, for instance in soybean (35, 51), Ranunculus sceleratus L (39), and Papaver somniferum L (13). This mode of cellularization may resemble the cellularization process in Drosophila embryos, where membrane material is deposited in a process involving syntaxin, leading to the centripetally growing furrows that result in the cellular blastoderm (30). In 1982, Fineran and co-workers proposed a solution to the problem of initial cell wall formation in nuclear endosperm, namely, that these walls are formed by normal cytokinesis (56). Although a similar mode of anticlinal cell wall formation was also reported for Ranunculus sceleratus L (186), few other reports support this mode of cellularization in early nuclear endosperm.

Observations by light and transmission electron microscopy of the MT arrays in early wheat endosperm led van Lammeren to conclude: “It is likely that the radiating microtubular arrays function in the formation of phragmoplasts, independent of nuclear divisions” (177). The dynamics of these structures was clarified in 1994 by Brown et al using confocal microscopy in three-dimensional reconstructions of the nuclei and the cytoskeletal arrays in developing barley endosperm (27). These reconstructions form the basis for the model shown in Figure 5, in which the initial anticlinal walls form in RMS interzones, termed cytoplasmic phragmoplasts (27). Using similar techniques, cytoplasmic phragmoplasts have been implicated in the cellularization process in rice (25, 28), maize (RC Brown, BE Lemmon & O-A Olsen, unpublished), and Arabidopsis (24, 115). Recently, high-pressure freezing/freeze-substitution techniques were used to study initial cell formation in Arabidopsis endosperm, confirming that initial cell plates form in RMS interzones (144).
Is the cytoplasmic phragmoplast in endosperm cell wall formation unique to nuclear endosperm? As reviewed in (23), several observations suggest that the cytoplasmic phragmoplast represents an integral part of the plant life cycle. In addition to endosperm, RMS plays a central role in sporogenesis in lower plants, microsporogenesis of heterosporous plants, male and female gametophyte development in gymnosperms and angiosperms, and embryogenesis of gymnosperms. One example of NCD-mediated wall formation and secondary phragmoplast is found in orchids that have no fixed arrangement of tetrads, mentioned above (20). Following meiosis I, a phragmoplast develops in the division interzone, but no wall is deposited. After meiosis II, two interzonal phragmoplasts form between pairs of sister nuclei simultaneous with the formation of radial microtubules on the surface of all nuclei, interacting to form a secondary (cytoplasmic) phragmoplast among non-sister nuclei in the same way as in the coenocytic endosperm. Confirming the similar nature of the two types of structures, the interzonal and the secondary phragmoplasts become indistinguishable, depositing cell walls that subdivide the four spores into approximately equal volumes at the end of meiosis. A second example of formation of MT structures with phragmoplast function occurs during meiosis of Selaginella and Marattiales. Here, a raft of vesicles accumulates at the interface of the opposing microtubule arrays resulting in a phragmoplast-like structure known as the procytokinetic plate (PCP) (21).

The role and occurrence of cytoplasmic phragmoplasts support the earlier suggestion by Bajer & Mole-Bajer (3) that plant MTs possess an inherent property that causes opposing arrays of MTs to function in cell wall assembly. Other lines of evidence also support the functional similarity between interzonal and cytoplasmic phragmoplasts, including the data of Endle et al (52), who demonstrated that a novel population of short actin filaments continuously assembles in the phragmoplast at the growing cell plate in both interzonal phragmoplasts and in cytoplasmic phragmoplasts in endosperm syncytia. Also, plant polypeptides that share antigenic epitopes with human vinculin are revealed within the forming cell plate of both systems, suggesting their recruitment during cytokinesis-associated actin assembly. These vinculin-like antigens may participate in membrane/F-actin anchorage of protein complexes involved in cell wall assembly. Further evidence for the functional similarity between cytoplasmic phragmoplasts and interzonal phragmoplasts is that the Knolle protein, a cytokinesis-specific syntaxin functioning in vesicle fusion leading to cell plate growth in Arabidopsis, is also expressed in both types of phragmoplast (118). Although functional similarities exist between interzonal and cytoplasmic or phragmoplasts, structural as well as compositional differences can also be found. The differences involve the mechanisms of fusion of the cell plate–forming vesicles, the lack of terminal fucose residues on the trisaccharide side chains of xyloglucans, as well as the persistence of callose in endosperm cell walls (28, 143).

In plants, the mechanisms involved in MT polymerization have remained elusive, in part because of the lack of centrosomes, which function as microtubule
organizing centers (MTOC) in animal cells. Currently, two alternative scenarios are envisioned [reviewed in (33)]. First, multiple nucleation sites are suggested to be responsible for the assembly and organization of the various microtubule arrays. Second, microtubule nucleation and organization may take place at one site, the nuclear surface, and the MTs are translocated from here to the different sites where they function. Several lines of experimentation have established that plant nuclei can assemble microtubules, including microinjection of fluorescent brain tubulin into living higher plant cells (99, 160, 168, 169), and the incorporation of reporter tubulin into lysed endosperm cells (179). Interestingly, Mizuno incubated nuclear particles from tobacco BY-2 cells with bovine brain tubulin and obtained aster-figures similar to the RMS of endosperm nuclei (121). In addition, antigenic determinants of the pericentriolar material of isolated animal centrosomes have been detected on the plant nuclear surface at the site where MTs are anchored (38). γ-tubulin, a universal component of MTOCs and an MT minus end marker (136), is detectable at plant nuclear surfaces in preparations with high numbers of nuclear-associated MTs (108). A close association between the MTOCs of nuclear surfaces and the nuclear matrix was recently demonstrated using an antibody directed against calf thymus centrosomes that recognized an antigen on plant nuclear surfaces (64, 168, 169). Electron micrographs of nuclear pore complexes have shown cytoplasmic as well as nuclear fibrils connected to several pore complexes, and these peripheral structures are thought to connect nucleo-cytoplasmic and cytoplasmic cytoskeletal elements (72).

What causes the remarkable MTOC activity on nuclear membranes in RMS formation? Assuming that nuclear surfaces represent the principal MTOC activity in plant cells, one possibility is that RMS formation results from a block in the translocation of MT to other locations in the cells, a view that is compatible with the lack of other MT cytoskeletal components in endosperm coenocytes during RMS formation. Alternatively, the RMS assembly could be caused by a change in the rate of MT initiation and/or depolymerization.

The machinery involved in regulation of plant MT polymerization is unknown, but it is generally assumed to be similar to that in animal cells, where centrosome activity is thought to be cell cycle–regulated by cyclin-dependent protein kinases [see for instance (29)]. In tobacco BY-2 cells, DNA replication must be completed before the pre-prophase band (PPB) is formed (145), again suggesting a link between the machinery that controls progression through the cell cycle and the dynamics of the MT cytoskeleton. Mutants perturbed in the assembly and function of the MT cytoskeleton in endosperm alveolation should yield valuable insights into the components involved in this process. Indications that at least some components of the MTOC apparatus function in more than one context come from a study of the Arabidopsis PILZ mutants, which affect the MT cytoskeleton (118). Defects are seen in these mutants in both the formation of mitotic spindles and the radial MT systems of endosperm nuclei. These studies also demonstrate the existence of context-specific components, because development of the gametophyte is
unaffected in these mutants (118). If sufficient material of in vitro fertilized central cells can be produced, this system should yield valuable insight into the early steps in endosperm cellularization (95).

**Release of Mitotic Block and Cell File Development**

Following the completion of alveolus formation in barley, a synchronous round of mitosis is initiated in all alveolar nuclei (Figures 2d and 6), wherein the plane of division is periclinal (i.e. oriented in parallel to the former central cell wall) (Figure 6b). The cytoskeletal arrays are the same as those in the endosperm coenocyte, except that the phragmoplast is functional, depositing a cell wall perpendicular to the former central cell wall (Figure 6g). The formation of these periclinal cell walls results in a layer of complete cells in the periphery of the developing endosperm, and an inner layer of alveoli with their openings toward the central vacuole (Figures 2e and 7a). The same process is subsequently reiterated several times, leading to cell files converging toward the center of the central vacuole from

![Figure 6](image) **Figure 6** Periclinal division in endosperm alveoli. Mitotic division of a nucleus in the endosperm alveoli depicted in Figure 2d. This mitotic division gives rise to the two endosperm cell layers shown in Figure 2e. (a) Endosperm alveolus. (b and c) Mitotic spindles in metaphase and anaphase, in periclinal orientation, respectively. Chromosomes are not shown. (d–f) A functional interzonal phragmoplast is formed in the mitotic interzone between the separating daughter nuclei, depositing a periclinal endosperm cell wall that separates the outer cell layer from the second layer of alveoli (g).
Figure 7  Endosperm cell file development. Development of three cell files extending from the periphery of the endosperm (down) towards the central vacuole (up) as depicted in Figure 2e and f. (a) Development of the second layer of alveoli from the upper cell of Figure 6g. (b) Continued centripetal growth toward the central vacuole after three rounds of mitotic divisions. (c) Mitosis in starchy endosperm cells lacking PPBs are at random division planes. (d) Starchy endosperm cells resulting from the random division planes in (c). After two rounds of divisions, the cell file pattern is lost.
all sides (Figures 2f and 7b), eventually completely invading it. This event marks the end of the endosperm cellularization phase.

What is the mechanism involved in the release of cell cycle arrest following the mitotic hiatus? Although direct experimental data are lacking, one possibility is that hormones play a role at this checkpoint, in particular cytokinin, which peaks early in endosperm development. In many species, cytokinin levels start to rise after fertilization when rapid cell divisions are occurring [for a review, see (122)]. For instance, in barley, cytokinin activity starts to rise around the time of cell divisions in the alveoli, lasting for a few days and coinciding with the most rapid period of mitotic activity in the endosperm. In plants, the link between auxin and cytokinin and cell division is now well established (48), and auxin alone has been shown to increase the level of cyclin dependent kinase (CDK) protein in cultured tobacco cells and stem pith explants. However, the addition of cytokinin is required to activate this kinase (84). Somewhat confusingly, both hormones have been associated with progression through the G1-S and the G2-M control points. One example of this is seen in root pericycle cells, which are arrested in G2 phase and normally assume divisions at an appropriate distance from the apical meristem at a critical ratio of auxin to cytokinin. Arrest of pericycle cells at the hormonal checkpoint is indicated by a rapid increase in p34cdc2 activity in root tissue during the induction of lateral root meristems by auxin (84), consistent with the presence of p34cdc2 awaiting activation by tyrosine dephosphorylation (189). Recently, CycD3 has been shown to be induced by cytokinin in both cultured cells and intact plants, a finding that supports the proposed role for this hormone in the G1-S transition. Evidence came from overexpression of CycD3 in stable Arabidopsis transformants, where it was found to remove the requirement for exogenous cytokinin during callus initiation and growth from leaf pieces (152). A model for the action of cytokinin in the G1-S transition has been proposed in which cytokinin- and sucrose-induced D-type cyclins bind to CDK-a to form inactive heterodimers. Phosphorylation of Rb by CDK-a complexes releases the transcription factor E2F, which is the active molecule required to enter S phase. The phosphorylation of plant CDK-a by CAK and the presence of Rb-E2F complexes on the promoters of S-phase genes remain to be demonstrated for plants, and are inferred from the mammalian G1-S model (48). The existence of several plant-specific CDKs, which are unique among CDKs in showing cell-cycle regulation of expression, suggests control mechanisms yet to be explored (48). If cytokinin plays a role in stimulating mitotic activity after the mitotic hiatus, the response may be mediated by cycD3. One way to test this hypothesis is to overexpress or transcriptionally silence the cydD3 in endosperm at the syncytial stage. If this hypothesis is correct, inactivation could block progression into mitosis. Conversely, ectopic expression of cycD3 could eliminate the mitotic hiatus.

What determines the periclinal plane of cell division in the first alveolar mitosis? One likely possibility is that the axis is set by space constraints in combination with
the lack of PPBs in this round of mitosis. Barley mutants displaying alternative division planes in alveolar mitosis (138) should be suitable objects for the study of this step in endosperm cellularization.

ENDOSPERM CELL FATE SPECIFICATION AND DIFFERENTIATION

The differentiated endosperm contains four tissues or cell types: the embryo surrounding region, transfer cells, the starchy endosperm, and the aleurone layer (Figure 1c). Based on current insight, the underlying genetic programs for endosperm cellularization (described above) and cell fate specification (described below) probably originated as independent genetic programs, which have been integrated during the course of evolution. For simplicity, endosperm cellularization has been described first, serving as a structural basis for the discussion of endosperm cell fate specification to follow. However, cellularization and differentiation are closely integrated processes, both temporally and spatially. In the following text, cell fate specifications of the four endosperm cell types are described in the order in which they are believed to occur.

The Embryo Surrounding Region

The embryo surrounding region represents the cells that line the cavity of the endosperm in which the embryo develops (Figure 1c). In maize, these cells are identifiable by their dense cytoplasmic contents (94, 157) as well as by the cell-specific expression of three different *Esr* (embryo surrounding region) transcripts (142). The function of the embryo surrounding region is unknown, but may include a role in embryo nutrition or in establishing a physical barrier between the embryo and the endosperm during seed development. In spontaneously occurring embryoless endosperms, *Esr* expression is lacking, suggesting a dependence of *Esr* on signaling from the embryo (142). On the other hand, the observation that the endosperm of embryoless mutants form a normal size embryo cavity suggests that the endosperm has an intrinsic program for formation of this structure (70).

The mechanism underlying cell fate specification of the embryo surrounding region is unknown. Based on the observation in maize that cell walls appear to be forming in the endosperm coenocyte around the embryo during the coenocytic stage (RC Brown, BE Lemmon & O-A Olsen, unpublished), the embryo surrounding region may form through a mechanism that permits a functional phragmoplast to form near the embryo. Also in barley, cellularization occurs early in the immediate vicinity of the embryo (53). Further studies are needed to confirm whether these cells represent precursors of the embryo surrounding region. Studies involving transgenic maize lines expressing the GUS reporter in
this region under the control of Esr promoters (15) should contribute insight into the molecular mechanisms underlying differentiation of this region of the endosperm.

Transfer Cells

Transfer cells develop over the main vascular tissue of the maternal plant (Figure 1c), where they facilitate transport of photosynthate (e.g. sucrose) into the endosperm. In cereals, endosperm transfer cells are characterized by prominent secondary wall ingrowths. At a young stage of the grain, these cells possess an extensive and complex endomembrane system (43, 182). In maize, two to three cell layers of endosperm cells have cell wall ingrowths in a gradient decreasing toward the interior of the endosperm (62, 157). In barley, the transfer cell-specific transcript END1 appears in the endosperm coenocyte over the maternal vascular tissue (49) (Figure 8a; green cells). The nuclei in this region give rise to the transfer cells (Figure 8b and c). END1 transcript is also present in fully developed grains (49). A second group of transfer cell-specific transcripts, consisting of four Bet genes, are found in maize (76, 78). The function of these two groups of genes are yet to be identified, but a weak similarity between BETL1 and 3 and plant defensins suggests a role in plant defense (76). The soluble acid invertase Incw2 from maize also represents a molecular marker for the endosperm transfer cell layer (171).

What determines transfer cell fate? The restricted expression of the END1 transcript in endosperm coenocyte nuclei over the vascular tissue (Figure 8a) hints at the presence of a localized factor that activates END1 transcription. Polarized transcription in a plant coenocyte has not been reported to date, but is known to occur at the syncytial stage during Drosophila embryogenesis and could suggest a mechanism for END1 transcriptional control. In this system, at the syncytial blastoderm stage, the maternally derived anterior or head-specific transcript bicoid is anchored to the MT cytoskeleton at the anterior end (135). From this site, the bicoid protein diffuses in a gradient toward the posterior end, with high concentrations activating head-specific genes by binding to the promoter upstream region of head-specific genes. Alternatively, END1 transcription could be activated through a mechanism similar to the localized nuclear import of DORSAL protein in response to localized activation of the TOLL receptor in the specification of ventral cell fate in the syncytial blastoderm in Drosophila (9). Recently, the promoter upstream regions of the BETL-2, 3, and 4 genes were used in electrophoretic mobility shift assays to detect common DNA binding activity from endosperm extracts (76, 77). This approach, in combination with studies of maize mutants such as rg f1 (reduced grain filling) (113) and a maize mutant recently isolated in our laboratories that lacks differentiated transfer cells (O-A Olsen, V Meeley & SE Nichols, unpublished), should lead to improved insight into the mechanism underlying transfer function and differentiation.
Aleurone Cells

The aleurone layer covers the entire perimeter of the endosperm except for the transfer cell region (Figure 1c). In maize (180) and wheat (31), there is one cell layer; in rice, one to several layers (74); and in barley, three layers (85). In maize, the aleurone layer consists of an estimated 250,000 cells (180), whereas barley grains have around 100,000 aleurone cells (97). The aleurone cell cytoplasm is dense and granular owing to the presence of many aleurone grains, small vacuoles with inclusion bodies (31). Aleurone cells contain lytic vacuoles and protein storage vacuoles (170), both of which may contain two major types of inclusion bodies (80)—the globoid bodies, which contain a crystalline matrix of phytin, protein, and lipid, and protein-carbohydrate bodies. Lipid droplets surround the aleurone grains (85, 98, 123). The endoplasmic reticulum of these cells is well developed, and a large number of mitochondria are also observable (85). Mature aleurone cells appear cuboidal in section and contain anthocyanins, which are responsible for the colorful grains of corn (131). In barley, the aleurone cells are highly polyploid (90).

The earliest molecular marker for aleurone cells is the barley Ltp2 transcript, which is present shortly after the onset of visible aleurone cell differentiation (88). Other aleurone marker genes include Ltp1 (107, 126a, 159a), B22E (93), pZE40 (164), ole-1 and ole-2 (1), and per-1 (165). Using a transient assay, Leah and colleagues identified putative enhancer elements in the barley chi33 gene involved in aleurone cell-specific gene expression (103). In maize, C1 represents an aleurone-specific marker (131). Future studies of the mechanisms involved in the cell specific regulation of these genes should yield valuable information about aleurone cell differentiation.

Toward the end of seed maturation, a specialized developmental program confers desiccation tolerance to the aleurone cells, allowing them to survive the maturation process (73, 89, 100, 128, 153). After imbibition, the function of the aleurone cells is to produce cell wall degrading, proteolytic, and hydrolytic enzymes to convert the storage proteins and starch granules of the starchy endosperm into sugars and amino acids for the growing embryo. Production of these enzymes is initiated by de novo transcription upon gibberellic acid stimulation from the imbibed embryo (86). At the end of the germination phase, aleurone cells undergo programmed cell death (12).

When is the fate of aleurone cells specified during endosperm development? The first sign of aleurone cell differentiation using immunohistochemical methods is observable in the peripheral layer of cells after completion of the cellularization process (Figure 8c; blue cells). These cells are derived from the peripheral daughters of the first periclinal division of the alveolar nuclei. The exact time for the onset of aleurone cell fate specification is unclear, but the first manifestation of a difference in the fate of this cell layer comes from barley, in which the MT cytoskeletal cycle is regulated differently from interior starchy endosperm cell
layers (Figure 8c; red cells). In these peripheral cells, for the first time during endosperm development, the full complement of cytoskeletal arrays of meristematic cells is displayed, including hoop-like cortical arrays (Figure 9a), PPB (120), which guides the division plane for cell wall deposition by the phragmoplast (Figure 9b), the mitotic spindle apparatus (Figure 9c), and functional phragmoplasts (Figure 9d), which deposit cell walls in the anticlinal plane predetermined by the PPB (Figure 9e, anticlinal division plane). One argument for considering this MT cytoskeletal cycle of arrays as part of the aleurone cell program is the importance of the cell division plane in the onotogeny of the aleurone layer. As the grain grows because of the expansion of the starchy endosperm cells (Figure 1b), anticlinal divisions such as those in Figure 9 are essential for surface area of the aleurone to expand. In maize, an estimated 17 rounds of anticlinal divisions are needed to create the surface of the mature aleurone layer of mature grains (104), which could be achieved through a control mechanism involving the PPB (145). It is therefore tempting to suggest that the rate of anticlinal (and periclinal) cell divisions in the aleurone layer is directly linked to the expansion of the grain via a mechanism that is yet to be determined. The distribution between anticlinal and periclinal divisions in the aleurone layer is unknown, but Kiesselbach reported that in maize, a shift to exclusive anticlinal divisions occurs about 20 days after pollination (92). Genetic evidence for the existence of a control of periclinal division

![Figure 9](image)

**Figure 9** The MT cytoskeletal cycle of aleurone initials. (a) Two neighboring aleurone cell initials with hoop-like cortical arrays. (b) The future division plane is determined by anticlinally oriented PPBs. (c) Mitotic spindle axis. (d) Interzonal phragmoplast oriented in the anticlinal plane predicted by the PPBs. Note that the steps involving phragmoplast formation have been omitted from this figure. (e) The resulting four aleurone daughter cells. Anticlinal divisions in the aleurone expand the surface of the aleurone layer.
rates comes from the maize mutant xcl1 (extra cell layers), which possesses extra leaf epidermis cell layers as well as aleurone layers in the endosperm because of aberrant periclinal divisions (91).

Using light or electron microscopy, typical features of aleurone cell differentiation such as numerous small vacuoles and a dense cytoplasm can be observed in the outermost cell layer in barley at 8 DAP (17), only 2 days after introduction of the meristematic division cycle. Subsequently, the second and third layers differentiate into aleurone cells. In maize, wheat, and rice, which complete the cellularization process 2 days earlier than barley, the morphology of the peripheral cell layer is at first indistinguishable from internal cells (27, 98, 123, 149, 157). In maize, Randolph reported that the first sign of aleurone cell differentiation occurs as a reorientation of cell division planes from random to mostly anticlinal and as the appearance of cuboidal cells (149). Similarly, in wheat, the first indication of aleurone differentiation is the appearance of cuboidal cells in the periphery (123). Following a change in cell shape around 10 DAP, vacuoles accumulate and the cytoplasm becomes dense (98, 123, 157), similar to barley and rice (17, 25). Confocal microscopy analysis is needed to determine whether the meristematic MT cycle is introduced in these species as well.

What determines aleurone cell fate? Based on current insight, aleurone cell fate is thought to be determined by positional information deposited in the periphery of the endosperm close to the former central cell wall (139). This is in contrast to the view of aleurone cells as a separate cell lineage from the starchy endosperm (8, 45, 104, 149). Recently, this model was supported and expanded by the Becraft laboratory in a lineage analysis in maize using Ds-induced chromosome breakage to uncover the waxy (wx) starchy endosperm marker and the aleurone anthocyanin marker C1 (6, 7). The result of this analysis showed that the aleurone does not form a separate lineage from the starchy endosperm. Furthermore, studies of revertant sectors from a mutable allele of dek1 in maize, which entirely lacks aleurone cells (130), suggest that positional cues are required throughout endosperm development to maintain aleurone cell fate. In these studies, following a somatic mutation in the dek1 gene, aleurone cells converted to starchy endosperm cells. Conversely, somatic reversion of a mutant dek1 gene to wild type converted aleurone cells to starchy endosperm cells even late in grain development. These data show that neither aleurone nor starchy endosperm cell fates are terminally determined states of differentiation, and are in agreement with earlier descriptions of periclinal divisions in wheat aleurone where the interior daughter cells dedifferentiated and converted to starchy endosperm (123).

What is the molecular basis for the interpretation of the positional information? In the proposed model (139), the positional information is “read” by a signal transduction pathway that starts with the Crinkly4 protein kinase-like receptor on the surface of endosperm cells (8). The Crinkly4 gene encodes a protein receptor kinase-like molecule with similarity to tumor necrosis factor receptors (TNFRs), prototypes of a large family of cell surface receptors that are critical for lymphocyte development and function in mammals [(36) and references therein]. The ligand
for TNFRs, tumor necrosis factor (TNF-α), is an important effector cytokine for immune responses and inflammation. Homotrimeric TNF-α is thought to recruit three receptor chains into a complex that juxtaposes the cytoplasmic domains. The extracellular domain of both TNFRs contain three cysteine-rich domains that characterize the TNFR superfamily and a less conserved, membrane proximal, fourth cysteine-rich domain (81). The ligand binding pocket for TNF-α is mainly formed by the second and the third cysteine-rich domains, whereas the first cysteine-rich domain mediates ligand-independent assembly of the receptor trimers interacting with the ligand (36). Interestingly, Crinkly4 bears resemblance to TNFR in the second and the third cysteine-rich domains.

The positional information believed to define aleurone cell fate is likely to represent a ligand binding to and activating the Crinkly4 receptor, and could exist at threshold concentration only in the periphery of the endosperm. In maize and other species with only one layer of aleurone cells, the gradient of the ligand is believed to decline more rapidly toward the interior than in barley, which has three layers of aleurone cells. Alternatively, three layers of aleurone cells could be achieved through a higher sensitivity of the receptor to ligand binding (139). The \textit{crinkly4} grain phenotype is most easily seen as patches of white on the surface of purple kernels (167). These white patches come from the lack of aleurone cells, as starchy endosperm cells are positioned in the periphery of the endosperm. In addition to aleurone cell differentiation, homozygous \textit{crinkly4} plants have crinkly leaves, demonstrating that this receptor-like kinase also functions in leaf differentiation. In addition to studies of Crinkly4 and its proposed ligand, ongoing efforts in several laboratories to clone the \textit{dek-1} gene, the second gene in maize known to abolish aleurone cell identity (134), should help to elucidate the mechanism establishing and maintaining aleurone cell identity. Mutants with multiple aleurone layers may also be expected to reveal gene functions involved in interpreting the positional information leading to aleurone cell fate specification (129, 184).

In addition to maize mutants that either lack or have multiple layers of aleurone, mutants in which aleurone cell differentiation is disrupted have also been reported. \textit{cp2} (collapsed) -\textit{o12} mutants have thin-walled, flattened aleurone cells with numerous vacuoles; \textit{pfc} (paleface) mutants have unusually rounded cells and sporadically more than one cell layer (6), and \textit{Dap} (dappled) mutants show abnormalities in aleurone cell morphology (63, 167). Also, mutants in the \textit{etched} loci are affected in aleurone cell development (132, 154). Several maize mutant lines have also been reported that show color mosaicism, but no effect on aleurone cell morphology. The coloration patterns of these mutants are interpreted to reflect processes that function to organize and coordinate endosperm development (6). Interestingly, these patterns differ from the clonal patterns of endosperm development, which originates from the directed migration of endosperm coenocyte nuclei (Figure 4; see above). Recently, our laboratory carried out a microscopy screen of the Pioneer TUSC collection (10), identifying many maize mutants perturbed
in aleurone cell differentiation, revealing mutants that entirely lack aleurone cells, contain only patches of aleurone cells, defective aleurone cell morphology, multiple layers of aleurone cells that are either well organized or completely unorganized with respect to plane of cell division (O-A Olsen, SE Lid, B Meeley & SE Nichols, unpublished). Allelism tests are under way to establish whether this collection represents new mutant variation in aleurone cell fate determination or differentiation. Endosperm mutants affected in aleurone cell differentiation have also been reported in barley (16), but the progress of these studies has been hampered by the lack of a facility to clone the underlying mutant genes.

The aleurone system represents an interesting system to advance our knowledge of fate determination in plants. The study of signal transduction pathways in plants is still in its infancy, and except for the brassinosteriod system (68) and the clavata1 receptor and its proposed ligand clavata3 (18, 174), there are few concrete examples of such signal transductions pathways. The maize aleurone, with its richness of mutants and repertoire of molecular techniques, could potentially expand our knowledge in this area.

Starchy Endosperm Cells

The starchy endosperm represents the largest body of cell mass in the endosperm and consists of an estimated 80,000 to 90,000 cells in barley (44, 97), and 60,000 in wheat (40). The bulk of the starchy endosperm is starch, synthesized within amyloplasts by the four enzymes, ADP-glucose pyrophosphorylase (AGPase), starch synthases (SS), branching enzymes (BE), and debranching enzymes [reviewed in (162)]. Of these, the wheat SS1 gene (105) and the major isoform of barley AGPase (50) are preferentially expressed in early starchy endosperm cells, representing the earliest known molecular markers for this tissue. The second major cell-specific component of starchy endosperm cells are prolamin storage proteins (126, 159). The basis for the tissue-specific expression of prolamin storage proteins rests on two common promoter motifs: the P-box (prolamin-box) (58), for which protein factors have been identified (179a); and a binding site 20 bp downstream of the P-box in the 22-kDa zein gene promoter, which binds the basic leucine zipper protein Opaque2, an endosperm specific transcriptional activator of zein gene expression (179a). In barley, the starchy endosperm-specific barley leucine Zipper1 (BLZ2) protein, dimerizing in vitro with the ubiquitously expressed Blz1 gene, binds specifically to the GCN4-like motif in a 43-bp oligonucleotide derived from the promoter region of a Hor-2 gene, which also includes the P-box (141). Similar in vitro studies in wheat of the bi-factorial endosperm box region of the wheat prolamin LMWG-1D1 gene demonstrated that transcriptional activation by bZIP transcription factor SPA is dependent on the binding activity of the nuclear protein ESBFI (46). These transcriptional activators, which lead to prolamin gene expression around 10 DAP, are valuable tools in understanding what underlies starchy endosperm cell differentiation. Following the phase of cellularization and cell fate specification, starchy endosperm cells undergo endoreduplication [reviewed in
Toward the end of the grain-filling period, the starchy endosperm cells die, involving a process that resembles programmed cell death in animal cells (188). The first starchy endosperm cells are the inner part of the cell files at endosperm closure, which are all derived from the interior daughter cells of the first periclinal division in the endosperm alveoli (Figure 8c; red cells). In contrast to the aleurone cell initials, which display the full MT cytoskeletal arrays of meristematic cells, the starchy endosperm cells develop cortical microtubules, but lack PPBs (27, 42). Presumably, as a result of a lack of PPBs combined with less spatial constraint than endosperm alveoli, starchy endosperm cell walls mediated by interzonal phragmoplast are deposited in random planes (Figure 7c). Consequently, after one or two rounds of cell divisions, the cell file pattern is lost (Figure 7d). An additional source of starchy endosperm cells is the inner daughter cells of aleurone cells, which divide periclinally and subsequently redifferentiate to become starchy endosperm cells (see discussion above). The outer layer of the starchy endosperm has smaller cells and a different concentration of storage proteins (110); most, if not all, of these cells may be derived from periclinal division of aleurone cells.

Several mutants that lack PPB have been described. Similar to starchy endosperm cells, these mutants orient division planes randomly, and cell plate deposition is mediated by interzonal phragmoplasts in the default plane, which is perpendicular to the axis of the mitotic spindle [reviewed in (145)]. Such mutants include the Arabidopsis fass and toneau (173) and the maize mutants dsc1 and dsc2 (discordia) (61) and tan1 (tangled) (41). One interpretation of the tan1 phenotype is that the so-called actin depleted zone, which forms in the area surrounding the narrowing MT in the phragmoplast, is defective, permitting phragmoplast fusion to occur anywhere on the inner surface of the cells (166).

Little is known about the steps involved in PPB formation or its regulation. However, the fact that the progenitors of daughter cells of the first periclinal division in the endosperm turns on the full meristematic MT cytoskeletal cycle, whereas the other daughter cells suppress PPB formation, demonstrates that plant cells can regulate PPB formation. Whether the control mechanism in endosperm involves the same genes that are affected in the mutants listed above remains to be determined. Probing for the expression of these mutant genes in aleurone and starchy endosperm cells should shed light on this question and possibly identify the mechanisms involved in the regulation of PPB formation in plants.

Several collections of mutants with defects in the starchy endosperm have been described, and are broadly referred to as decks (defective kernel) mutants (132, 155). In most cases, the developmental lesion in these mutants has not been identified. One exception is the Dsc1 (discolored) gene, which has been cloned (156). The Dsc1 mRNA is detected specifically in kernels 5–7 DAP, but its function is yet to be determined. In addition to these maize collections, barley shrunken endosperm mutants similar to the maize decks as well as barley mutants with defective or reduced endosperm that are maternally inherited have been reported (16, 54, 55).

Little is known about the mechanisms involved in starchy endosperm cell fate specification, in part because of the lack of cereal mutants that directly affect
starchy endosperm fate specification. In particular, mutants with aleurone cells, but no starchy endosperm, would be useful in addressing the question of starchy endosperm cell fate specification. Potentially, such mutants could be present in extreme deks such as the emp (empty pericarp) mutants (155). Alternative strategies, currently under way in many laboratories, include identification of early starchy endosperm markers through functional genomics and proteomics based on tissues from young, dissected endosperm.

Does Arabidopsis represent a useful model system to elucidate endosperm cell fate specification? More data on Arabidopsis endosperm development are needed to fully answer this question. Clearly, the cell contents differ markedly between the persistent peripheral cell layer of the Arabidopsis endosperm and the cereal aleurone. Also, the contents of the nonpersistent inner cell mass of Arabidopsis are different from the persistent cereal starchy endosperm. However, based on the high degree of conservation of the cellularization process between Arabidopsis and the cereal endosperm, as well as the similarity in the ontogeny of the two main endosperm cell types, studies of Arabidopsis may well contribute valuable information on starchy endosperm as well as aleurone cell fate specification, provided effective screens for relevant mutants can be designed.

SUMMARY AND FUTURE DIRECTIONS

Nuclear endosperm offers the opportunity to study the identity and integration of components of a developmental program specifying an entire plant body plan. There appear to be few regulatory steps required for endosperm formation, and these consist of control points in highly conserved plant cell processes (Table 1). The first step in endosperm development is coenocyte formation, which results from a suppression of interzonal phragmoplast function. During the ensuing mitotic arrest, cellularization of the coenocyte is initiated by RMS formation and cell wall deposition by cytoplasmic phragmoplasts in a cycle of events that is also applied in microsporogenesis and megagametophyte cellularization. The underlying basis for these events is the ability of nuclear surfaces to act as MTOCs, which in endosperm coenocytes are regulated in a way that leads to an accumulation of MTs forming the RMS. The second feature is cell wall deposition by cytoplasmic phragmoplasts in a cycle of events that is also applied in microsporogenesis and megagametophyte cellularization. The underlying basis for these events is the ability of nuclear surfaces to act as MTOCs, which in endosperm coenocytes are regulated in a way that leads to an accumulation of MTs forming the RMS. The second feature is cell wall deposition by cytoplasmic phragmoplasts, which appear to be functionally similar to interzonal phragmoplasts. Positional information in the basal part of the endosperm coenocyte may be involved in the specification of transfer cell fate. Following the formation of the first layer of alveoli, mitosis resumes, and periclinal cell wall deposition is mediated for the first time in endosperm development by a functional interzonal phragmoplast. The MT cytoskeletal cycle is different in the two daughter nuclei resulting from this periclinal division; the peripheral (aleurone) daughter cells use a cycle typical of meristematic cells, including PPBs and interzonal phragmoplast, whereas the inner (starchy endosperm) daughters lacks PPBs. As a result, the cell division plane is strictly controlled in aleurone cells (predominantly anticlinal),
whereas division planes in starchy endosperm cells are randomly oriented. Aleurone cell fate specification occurs via positional signaling, and involves the Crinkly4 receptor-like kinase.

From the data summarized above, the endosperm body plan likely results from the regulation of conserved plant processes rather than involving a process not applied by meristematic cells. The study of the key regulatory mechanisms in endosperm development outlined above should therefore yield valuable insight into processes such as cell cycle regulation, the regulation of MT cytoskeletal structure and function, as well as mechanisms in cell fate specification. Important progress in endosperm research has been achieved by the use of the two most potent experimental plant systems available, Arabidopsis and maize. Much excitement lies ahead in endosperm research.

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Figure 2 Diagrammatic overview of the endosperm cellularization process. (a) In the central cell, the fertilized triploid nucleus (orange) is situated in the proximal end of a cytoplasm which surrounds a large central vacuole (yellow). (b) Mitotic divisions occur without cell wall formation and leads to a multinucleate cell with a large central vacuole; the endosperm coenocyte. (c) Endosperm cellularization is initiated by the formation of RMS (radial microtubular systems) at the surface of the endosperm nuclei. (d) Cell wall formation is facilitated by interaction between RMSs from each nucleus forming the cytoplasmic phragmoplast, and each nucleus becomes surrounded by a tube-like wall structure (alveolus) with its open end towards the central vacuole. (e) Continued growth of alveoli towards the central vacuole, and periclinal cell divisions with wall formation between the daughter nuclei within the alveoli, leads to two cell layers, one complete layer in the periphery, and a new layer of alveoli internally. (f) After one repetition of the alveolation process and a mitotic division, two layers of peripheral cells are formed, the new layer of alveoli extending almost to the center of the central vacuole. After further centripetal growth of the cell files, the central vacuole is completely closed. Please note that the figure has not been drawn to scale, and only one layer of endosperm cells is shown.
Figure 4  The first mitotic divisions in the endosperm coenocyte in maize occur at predictable planes. Diagrammatic representation of the first mitotic division in the cytoplasm of the central cell, the endosperm coenocyte. Only the nuclei and the cytoplasm of the central cell and the endosperm coenocyte of Figure 2 a and b are shown. (a and b) The first division. (c and d) Second and third divisions. (e) The eight nuclei are positioned in the periphery of the basal cytoplasm. (f) Each of the eight nuclei gives rise to a population of cells that migrate to a proximal-distal sector of the endosperm cytoplasm corresponding to one eighth of the surface of the coenocyte. After cellularization in the centripetal direction (toward the center), these nuclei will form a part of the endosperm with a shape similar to a segment of an orange.
Figure 8  A model for endosperm cell fate specification. The endosperm in (a) and (b) correspond to those of Figure 2b and d, respectively, (c) to a stage later than Figure 2f. (a) A transfer cell specific transcript (green) is detectable in the basal endosperm at the coenocytic stage, indicating that cell fate specification has already taken place. (b) Nuclei in the basal region of the endosperm coenocyte give rise to alveoli with transfer cell identity. (c) After completion of endosperm cellularization, the peripheral cell layer (blue) assumes aleurone cell fate, the interior cells assume starchy endosperm cell fate (red). In the cell files of the basal endosperm, the lower cells assume transfer cell fate (green), and the upper cells starchy endosperm cell fate (red).