Cell fate specification in the cereal endosperm

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While superficially simple, endosperm development is a complex, dynamic process. Cereal endosperms contain three major cell types: starchy endosperm, transfer cells and aleurone. The localized accumulation of the END1 transcript in the syncitial endosperm suggests that signals from the maternal placental tissue specify transfer cell type early. Aleurone fate is plastic and requires the continual input of positional cues to maintain cell identity. Starchy endosperm appears to be the default cell type. Mutant patterns suggest that a regulatory hierarchy integrates endosperm development. Requirements for gametic imprinting, maternal:paternal genome ratios and putative chromatin modeling factors indicate the importance of genomic control.

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Introduction

The endosperm is well recognized for its importance as food and feed, yet the many fascinating features of endosperm biology are often overlooked. The perception of endosperm as uninteresting is perhaps in part because of its familiarity as a foodstuff and part because of the ‘dead end’, often transitory, nature of the endosperm. However, these impressions belie the dynamism and many unique features of endosperm development. Positional cues, cell cycle modifications, genomic imprinting and programmed cell death figure prominently in endosperm development.

In double fertilization, two different cells of the megagametophyte are fertilized. One pollen sperm fertilizes the egg cell to form the zygote and the other sperm nucleus fuses with the two polar nuclei in the central cell to yield the triploid endosperm. The evolutionary origin of the endosperm is an interesting problem with two competing century-old hypotheses to explain its derivation (reviewed by Friedman). One suggests that the endosperm is derived from an altruistic second embryo produced by double fertilization, while the alternative hypothesis proposes that the endosperm is derived from an extension of megagametophyte development. This issue is pertinent to understanding endosperm development because the developmental program is built on the genetic platform inherited from the ancestral predecessor of the endosperm. Thus, understanding evolution will help us understand development and vice versa.

The endosperm is a transitory tissue in many species, particularly dicots such as Arabidopsis where the cotyledons represent the major site of food storage. In cereals, the endosperm is persistent, contains several specialized cell types and is a prominent feature of the mature seed. Despite the very different fates of the endosperm in species such as Arabidopsis and maize, early endosperm development shows remarkable conservation.

The cereal endosperm contains three major cell types. The bulk of the endosperm is composed of the starchy endosperm or internal endosperm cells, which accumulate starch and storage proteins. Adjacent to the pedicel is a transfer layer, specialized for nutrient uptake and transport from the maternal tissues to the developing endosperm. An epidermal layer, called the aleurone, covers the endosperm and during seed germination, secretes hydrolytic enzymes to digest the storage products in the starchy endosperm.

Understanding how the identity of these different cell types is specified during development has a number of potential practical applications. For
example, manipulating the transfer layer might allow enhanced solute uptake efficiency, leading to increased yield. The aleurone is relatively lipid rich and so altering aleurone development could allow seed composition to be manipulated. In addition, the aleurone layer is rich in phytic acid, which is detrimental in uncooked corn products because of its ability to bind dietary Ca\(^{2+}\) and Zn\(^{2+}\). It is also a leading source of phosphorus in livestock wastes, posing a serious water pollution problem. Finally, the aleurone is the major source of amylase in the malting process and is therefore of great interest to the malt beverage industry. This paper will highlight recent advances in our understanding of how cell fates are decided during cereal endosperm development.

**Overview of endosperm development**

There are three main types of endosperm development, nuclear, cellular and helobial. Only nuclear endosperm development will be considered here as it occurs in most familiar plants including cereals and *Arabidopsis*. Following fertilization of the central cell, the primary endosperm nucleus enters a period of free nuclear division. Although the early endosperm is syncitial, the daughter nuclei of early divisions migrate to relatively invariant positions. Clonal analysis in maize showed that following the first division, daughter nuclei occupy lateral positions and give rise to the left and right halves of the endosperm.\(^2\) Products of subsequent divisions give rise to predictable quarters and eighths and so forth. These results demonstrate that positional information is present within the early syncitial endosperm, possibly preexisting within the megagametophyte.

After a period of free nuclear division, the nuclei occupy the peripheral cytoplasm surrounding a large central vacuole and cytoskeletal arrays define nucleocytoplasmic domains (NCDs).\(^3\)–\(^5\) In cereals, syncitial divisions appear synchronous\(^3\) while in *Arabidopsis* there appear to be mitotic domains.\(^6\) Cellularization ensues through a process of free cell wall formation whereby unusual microtubule structures called adventitious phragmoplasts form at the boundaries between NCDs. The phragmoplasts direct membrane vesicles containing cell wall constituents to the growing points of the cell walls. Beginning at the periphery and growing centripetally, cell walls grow between non-daughter nuclei, separating the nuclei into tube-like alveoli that remain open at their inner face. The next cell division occurs periclinally and is accompanied by cytokinesis to yield a fully cellular peripheral layer and an alveolar internal layer. This process repeats until the entire volume of the endosperm is cellularized. With minor differences, this early development is remarkably conserved in plants as divergent as *Arabidopsis*, with a transitory endosperm, and cereals.

Shortly after cellularization, the *Arabidopsis* endosperm begins degenerating as the growing embryo crushes surrounding cells. Only an epidermal layer of endosperm, reminiscent of the cereal aleurone, remains in the mature seed.\(^5\) The function of this cell layer is unknown. In cereals, the persistent endosperm grows to a substantial size. Cell division initially occurs throughout the cellular endosperm and then becomes restricted to a cambial zone at the periphery. More interior cells continue to grow by cell enlargement as they accumulate starch and protein bodies. This growth is accompanied by an increase in genomic DNA content through endoreduplication. As growth continues, programmed cell death (PCD) initiates and progresses throughout the starchy endosperm until only the aleurone cells remain alive in the mature endosperm (reviewed by Young\(^7\)). Castor bean contains a living endosperm that remains attached to the cotyledons and serves as a storage site for lipids. This endosperm undergoes PCD after germination,\(^8\) similar to the aleurone of cereals.\(^9\)

**Cell fate acquisition in cereal endosperm**

As mentioned, the cereal endosperm has three major cell types. In addition, there are regions or sub-types of cells within the starchy endosperm that are histologically distinct or show specialized patterns of gene expression. The organization of these cells in the maize endosperm is illustrated in Figure 1.

**Transfer layer**

The transfer layer, also called ‘modified aleurone’, is the first cell type to become histologically differentiated during endosperm development, becoming visible at approximately 6 days after pollination (DAP) in maize.\(^10\) These cells develop extensive inward cell wall projections that increase the area of plasma membrane by approximately 20-fold [Figure 1(c); see Thompson in Reference 11 for review]. Molecular evidence suggests that positional
whether these genes have a developmental function is also unknown. BETL1 was originally proposed to have a structural function because of its tight association with the cell wall but the small cysteine-rich BETL1 and BETL3 peptides also show similarity with defensins, suggesting possible antimicrobial functions. However, SCR, the pollen determinant of sporophytic self-incompatibility in *Brassica*, and likely ligand for the S receptor kinase (SRK), shows a similarity to defensins. Thus it is also possible that the BETL1 and 3 peptides function as signaling molecules. BETL2 has recently been shown to be a member of a small family of related peptides that possess antifungal activity. These peptides are called BAP1-3 (basal layer antifungal proteins) and BETL2 has been renamed BAP2.

Interestingly, a proper 2:1 maternal to paternal genomic ratio is critical for transfer cell differentiation in maize. The basal cells of tetraploid (2m : 2p) endosperms fail to initiate cell wall ingrowths. In addition, they show cytoplasmic disorganization and abnormal inclusions in the cell wall matrix. Defects in other parts of the endosperm, including necrosis and failure of aleurone cell differentiation were considered potential secondary effects of impaired nutrient uptake. These effects of paternal genomic excess suggest that a regulatory factor, critical for transfer cell fate or differentiation, is differentially imprinted in the pollen and the egg.

Consistent with the role of the basal transfer cells in solute uptake, a cell wall acid invertase gene, INCW1, is specifically expressed in these cells. In fact, sugar sensing, and possibly flux, appear to influence the development of maternal and endosperm tissues. Endosperms mutant in *mn1*, which codes for INCW2, cause degeneration of the maternal placentals tissues. Since hydrolysis of sucrose to hexose is a key step in sugar import to the endosperm, the placental defects might result from reduced flux. The *reduced grain filling1* (*rgf1*) mutant has a similar phenotype to *mn1* but has apparently normal sugar levels, suggesting the lesion may be impaired sugar sensing. Both *mn1* and *rgf1* showed morphologically normal basal transfer cells but in the *rgf1* mutant the levels of BETL1 and BETL2 (BAP2) proteins were decreased. This did not occur in *mn1* or several starch biosynthetic mutants. The basal transfer cell-specific INCW1 transcript shows sugar dependent post-transcriptional regulation. Thus, sugars influence gene expression in the basal transfer cells. The differentiation of transfer cells in other systems has been shown to be dependent on solute transport (reviewed by Thompson), hence it would

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**Figure 1.** Cell types of the cereal endosperm. (a) A diagrammatic representation of a developing maize kernel in longitudinal section. The pericarp is maternally derived tissue that covers the kernel and the embryo is the other product of double fertilization. Everything else is endosperm. (b) A histological section through the peripheral crown region of a mature maize kernel, showing starchy endosperm (se), subaleurone (sa), aleurone (a) and the pericarp (p). (c) A histological section of the basal transfer layer cells showing the extensive cell wall projections (wp) associated with this cell type. The cells to the lower right part of the figure belong to the maternal placental tissue, which unloads the solutes taken up into the endosperm (upper left) by the transfer cells. (d) *In situ* hybridization showing the expression of the BETL1 transcript specifically in the basal transfer layer.
not be surprising if source–sink relationships were important for inducing the formation of endosperm transfer cells.

**Aleurone**

During cellularization, the periclinal division that first sets aside a cellular peripheral cell layer gives rise to two daughter cell lineages with different fates; the internal daughter has the invariant fate of producing only starchy endosperm cells while the peripheral cell layer will produce aleurone cell initials. Because of the different fates of the daughter cells, this early periclinal division has been called a formative division and it was commonly believed that the aleurone formed a separate cell lineage from the starchy endosperm. However it is clear, at least in maize, that this initial cell division is not a determinative event but the daughter cell fates remain plastic through late stages of endosperm development. This conclusion is based on endosperm cell lineage analysis and developmental genetic analysis of the *DEK1* gene function, required for aleurone cell fate.

Cell lineage analysis was conducted in maize endosperm using a technique similar to that of Barbara McClintock. A chromosome breaking *Ds* transposon was used to simultaneously mark starchy endosperm and aleurone by uncovering the *wx* and *C1* markers, respectively. The size of a sector indicates the relative time during development when an event occurred; small sectors occurred late in development because there were few cell divisions following the event. Small sectors containing both aleurone and starchy endosperm were observed, indicating that both cell types could be derived from a common precursor at late stages of development. These results show that the aleurone cell lineage is not distinct but that the peripheral cell layer contributes cells internally to differentiate as starchy endosperm throughout development. This is consistent with a report from wheat that describes the redifferentiation of aleurone cells to starchy endosperm following an aleurone cell division that contributed a daughter cell internally. That starchy endosperm cells can be derived from the aleurone cell lineage implies that position, not lineage, determines endosperm cell fate.

The question of aleurone cell fate determination was further addressed by examining unstable *dek1* alleles. *dek1* is a recessive mutation in which aleurone differentiation fails to initiate and the peripheral cell layer adapts a starchy endosperm fate. Therefore the wild-type *DEK1* gene product is required for the perception or response to the positional cues that specify aleurone cell fate. In a transposon-induced *dek1* mutant, revertant sectors as small as a single cell allowed aleurone cell development. Because the revertant cell’s ability to perceive or respond to the positional cues was not restored until near the end of active cell division, the positional cues that specify aleurone identity must still be present late in development. Furthermore, starchy endosperm cells in a peripheral position remain competent to respond to those signals.

In a reciprocal experiment, *Ds* induced chromosome breakage was used to cause the loss of the wild-type *DEK1* gene in a *dek1/DEK1* heterozygote, uncovering the mutant allele in sectors. Cells within mutant sectors assumed starchy endosperm identity, even in late sectors as small as two cells. Because aleurone cells were clearly recognizable at earlier stages of development, the fate loss of *DEK1* gene function caused peripheral cells to lose aleurone identity and switch fate to starchy endosperm. This demonstrates that the positional cues that specify aleurone identity are also required to maintain it. Such transdifferentiation events demonstrate that cell fate specification is a continual process and highlight the developmental plasticity of the endosperm.

The maize *crinkly4* mutant causes mosaic aleurone development. In regions that lack aleurone, the peripheral cells develop as starchy endosperm. This fate switch of peripheral cells indicates that *CR4* is involved in the aleurone cell fate decision. The identification of *CR4* as a receptor-like kinase suggested the exciting possibility that *CR4* may be the receptor for the positional cues that specify aleurone identity. *CR4* is not specific to the aleurone but rather regulates a wide variety of cellular developmental processes in the plant and endosperm, suggesting that the function is analogous to a growth factor receptor, where a widespread signal regulates various responses in a context-dependent manner. A region in the extracellular domain shows similarity to the ligand-binding domain of the mammalian tumor necrosis factor receptor (TNFR) family suggesting that the ligand for *CR4* (i.e. the putative positional cue for aleurone identity) may be related to TNF. Unfortunately, the TNF family shows poor primary sequence conservation, creating difficulty for sequence based strategies for identifying TNF-related molecules as potential *CR4*...
ligands. Genetic analysis suggests that a hierarchy of genetic functions is involved in the specification and differentiation of aleurone cells.\textsuperscript{28} Genetics may represent the most viable approach to identifying the CR4 ligand and will help identify other components of the CR4 signal transduction system.

Maternal: paternal genomic ratio is also important for aleurone development because tetraploid endosperm fails to differentiate an aleurone layer.\textsuperscript{17} It was not reported whether tetraploidy caused the peripheral endosperm cells to switch fate to starchy endosperm or simply to differentiate incompletely. Whether this particular effect was due to a regulatory factor that controls aleurone development being differentially imprinted in male and female gametes or was a secondary consequence of disrupted transfer cell differentiation cannot be determined at present, but there is ample evidence that imprinting is critical for aleurone development. Imprinting of anthocyanin factors in the aleurone is a well-known phenomenon\textsuperscript{32,33} and \textit{Dappled} mutants that disrupt aleurone development only show phenotypes when inherited through the female, suggesting they are imprinted.\textsuperscript{34}

Among the molecular markers that have been identified for the aleurone are lipid transporters,\textsuperscript{35–37} \textit{Myo-inositol-1-phosphate synthase},\textsuperscript{38} a defense-related gene,\textsuperscript{39} and in certain genotypes of maize, genes of the anthocyanin biosynthetic pathway.\textsuperscript{34,40}

\textbf{Starchy endosperm}

Starchy endosperm cells constitute the bulk of the cereal endosperm and represent the major storage site for starch and protein. Despite intensive study, it is surprising that no genetically defined regulatory factors for starchy endosperm development have been isolated. Even among the various metabolic pathways to storage product accumulation, surprisingly few regulators have been isolated. Mutants are known in many of the enzymes of the starch biosynthetic pathway but no genetic regulators have been identified. \textit{OPAQUE2} is the only genetically defined regulator of storage proteins that has been isolated.\textsuperscript{41,42} The paucity of identified genetic regulators in these systems suggests that there is either a high degree of redundancy or that such mutants are lethal, perhaps present in the many non-descript \textit{defective kernel (dek)} mutants.\textsuperscript{43,44}

Available evidence suggests that starchy endosperm represents the default cell type because loss-of-function mutants cause peripheral cells to adapt a starchy endosperm fate instead of aleurone. In a sense then, the question of how starchy endosperm cell fate is specified comes down to the question of how the endosperm, with the same genetic makeup as the embryo except for the 2:1 maternal:paternal genomic ratio, is specified. This, in turn returns to the question of the evolutionary origins of the endosperm. More work is required before these questions can be answered.

\textbf{Specialized regions within the starchy endosperm}

Within the starchy endosperm are several specialized regions of note. These include the subaleurone, the ‘embryo surrounding region’ (ESR) and conducting zone (Figure 1). The subaleurone is a cambial zone of active cell division. Cells are small and generally contain small amyloplasts in the early stages of starch accumulation.\textsuperscript{29,45} The specific expression of the basic leucine zipper transcriptional factor, RISBZ1, in the aleurone and subaleurone\textsuperscript{46} suggests that the subaleurone might in fact possess a distinct identity. However, this might also merely reflect a phase in starchy endosperm cell development because there is a progression of cells passing from the subaleurone to mature regions of the endosperm as new cells are added to the periphery during endosperm growth.

Cells surrounding the embryo have a distinct morphology, with compact size and dense cytoplasm containing extensive rough endoplasmic reticulum.\textsuperscript{47} This morphology suggests a synthetic function, possibly producing substances to be taken up by the suspensor of the developing embryo. Three genes called \textit{ESR1-3} are specifically expressed in this region.\textsuperscript{48,49} The function of these genes is unknown but the promoters of all three drive GUS expression in the ESR indicating that regulators function specifically within this region.\textsuperscript{48} The pattern is fairly static in the endosperm, initially surrounding the whole embryo but as the embryo proper grows up and out of this region, the ESR is left surrounding just the suspensor.

It has been reported that developing maize endosperm contains a column of elongated conducting cells extending through the central core of the endosperm from the transfer layer to near the apex.\textsuperscript{17} These cells are called prismatic cells in barley,\textsuperscript{50} and are believed to transport solutes throughout the kernel. Like the transfer cells and aleurone, this conducting tissue failed to differentiate in tetraploid endosperms, suggesting either that a determinant is differentially imprinted in male and
female gametes or that the activity of the transfer layer is required for the differentiation of this tissue as well. No molecular markers have yet been reported for this tissue but two barley mutants specifically eliminate this tissue from the endosperm.

**Pattern formation in the endosperm**

From the array of specialized cell types and molecular markers, each with their characteristic spatial patterns within the endosperm, it is apparent that pattern formation is an essential component of endosperm development. Analysis of mosaic aleurone mutants of maize (Figure 2) suggested that endosperm development is integrated by at least two systems. Mosaic patterns of mutants that affect early steps of the aleurone development have a strong tendency for showing defective aleurone differentiation preferentially in the abgerminal crown region, reflecting a gradient from the silk scar region of the kernel. The source of the positional information for this gradient is not the embryo because a mutant that altered embryo position did not alter the distribution of the mosaic pattern. This pattern is reminiscent of the anterior–posterior pattern shown by the MEA, FIS2 and FIE genes of Arabidopsis. These putative chromatin-modeling factors are required in the megagametophyte to inhibit endosperm development until fertilization and are involved in establishing axial pattern in the developing endosperm (see accompanying article by Chaudhury). It will be interesting to see whether the homologs of these genes are involved in establishing the germinal–abgerminal pattern in maize.

Mutants that affect later stages of aleurone differentiation show blocky patterns of mosaicism, first in large patterns, then in smaller patterns, suggesting that development is somehow integrated in progressively smaller fields. What these fields represent, whether the limits of a diffusible factor, symplastic fields, or something else, and the functional meaning of these fields in endosperm development are questions that will be answered as the genes that show these mutant patterns are isolated.

**Summary**

The picture that emerges of endosperm development is of a dynamic system, containing several novel features. In early stages of development, the cell cycle is highly modified, first to allow nuclear division without cytokinesis, then to allow an unusual cytokinesis involving free cell wall growth in the absence of nuclear division. Later the cell cycle is modified again to allow endoreduplication in the starchy endosperm.

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**Figure 2.** Representative mutants showing the characteristic patterns for each class of mosaic aleurone phenotype. The dark regions of the kernels contain anthocyanin specifically in the aleurone. The light regions show areas that fail to accumulate anthocyanin because aleurone differentiation is perturbed. The highly characteristic patterns of mutants in multiple genes suggest they reflect an underlying developmental pattern. (a) The bareback mutant showing the germinal–abgerminal pattern also observed in crinkly4 and dek1 mutants. (b) A collapsed2-o12 kernel showing a large blocky pattern of mosaicism, also typical of several Dap and Msc mutants. (c) The white2-dek21 pattern consisting of small patches of aleurone.
cells before they undergo programmed cell death. The establishment of pattern and the specification of cell fates are also very complex, involving positional cues, signal transduction, chromatin modeling factors, genomic imprinting, probable interactions with maternal tissues and metabolic signaling. Understanding how all these processes are integrated to allow the development of a functional endosperm will require much more research but the potential rewards of being able to improve seed composition, not to mention the intellectual gratification, are ample.

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