Developmental biology of the cereal endosperm

Odd-Arne Olsen, Casper Linnestad and Scott E. Nichols

The recent application of immunohistochemistry and molecular techniques has revealed that endosperm development depends on a genetic program that combines an ancient process for cellularization (similar to that seen in late Paleozoic seed ferns) with a mechanism for specifying asymmetric cell fates that has parallels to signaling processes in mammals. Progress has been further accelerated by the recent realization that the conserved nature of nuclear endosperm development extends beyond the grass species, to include dicots, such as *Arabidopsis*. It is anticipated that these ongoing studies will provide invaluable tools for the improvement of yield and grain quality in cereal crops.

Cereal grains are the most important renewable resource for food, fodder and industrial raw material. As such, cereal research is receiving increased attention from the commercial sector and this has augmented a revival in basic research in endosperm biology. Here, we attempt to integrate current insight into the mechanisms of nuclear endosperm cellularization and development in maize, barley, rice and wheat, and propose mechanisms that might be involved in the differentiation of the five major cell types of the endosperm. In doing so, we discuss possible roles for the maternal tissues in providing developmental signals for endosperm development.

At maturity, the cereal endosperm is composed of five tissues or cell-types (Fig. 1): the central starchy endosperm (CSE), the sub-aleurone layer (SAL), the aleurone layer (AL), the basal endosperm transfer layer (BETL) and the embryo-surrounding region (ESR).

Central cell and endosperm coenocyte

The endosperm develops from the fertilized triploid central cell, the largest structure of the megagametophyte[1] (Fig. 2). The polarity of the central cell, which is probably important for axis-determination in the developing endosperm, is clearly shown by the proximal positioning of the polar nuclei and by the positioning of the bulk of the cytoplasm and organelles (such as starch grains) near the micropylar end of the embryo sac. The precise mechanisms of megagametophyte differentiation are not well understood, but genetic data demonstrate that sporophytic as well as female gametophytic gene products are involved in this process[2]. Possible sporophytic influences on the megagametophyte, and later on the endosperm, might come from the body of nucellar parenchyma and the nucellar projection (small cereal grains) or the chalazal pad (maize), which serves as the main route for solutes into the endosperm (Fig. 1). Although it is only speculative at this point in time, the nucellain protease[3], or similar proteases located in the nucellar wall, might be involved in the processing of molecules influencing endosperm development. Cross-talk between the chalazal pad and the endosperm is indicated by observations from the homozygous *minature1* mutant of maize, where a lack of endosperm invertase activity leads to defects in maternal tissue[4].

Insight into the mechanisms of early endosperm differentiation might come from studies of *in vitro* fertilized, isolated maize central cells, which result in a bipartite endosperm-like structure[5]. After fertilization, the *in vitro* endosperm expands rapidly, passing from a coenocytic to a cellular stage. It is not known whether the cellularization process and aleurone cell differentiation *in vitro*, are identical to that of the *in vivo* endosperm. One interesting application of the *in vitro* endosperm system is that it might be used in studies aimed at determining whether or not signals from the sporophyte are involved in early endosperm differentiation.

An appreciation of the early events during endosperm development came from several clonal analyses of maize[6,7]. These studies examined the sectoring in the anthocyanin coloration of the aleurone layer and of the iodine staining of the starchy endosperm resulting from an induced or spontaneous change of marker gene function during the early divisions of the endosperm nuclei. Such data, supplemented by cytological observations, revealed that the triploid endosperm nucleus divides three times in predictable planes (Fig. 2b–e). Nuclear migration or repositioning then follows according to a fixed pattern (Fig. 2f,g). The effect, if any, of this fixed pattern of nuclear division and migration on gene expression patterns during endosperm development is unknown.

Recently, it was demonstrated that early endosperm development in *Arabidopsis* follows the same general pattern seen in cereals[8]. The whole-mount clearing technique, used to screen for embryo mutants of *Arabidopsis*, is equally applicable to similar studies on the endosperm[9]. Examples of *Arabidopsis* mutants that have been identified in this way include *pilz* (Ref. 10) and the *titan1* mutant[11].

In barley, and presumably also in other species, the cell cycle is arrested after the initial phase of endosperm mitosis without cytokinesis[12]. At this stage, the nuclei are evenly distributed throughout the peripheral central cell cytoplasm (Fig. 2h). The only report of a transcript that is preferentially expressed in endosperm coenocytes is *END1* in barley, which is initially expressed in the nuclei positioned above the nucellar projection[13] (Fig. 3a and b). The function of the END1 gene product is unknown. What restricts the distribution of the transcript in the coenocyte, and what the source of the signal is that drives this restricted pattern of *END1* expression in a selected subset of nuclei in the endosperm coenocyte, is also unknown. However, the anchoring of mRNA molecules, by RNA-binding proteins, to the cytoskeleton at the poles of the syncytial blastoderm stage of *Drosophila* larvae might provide clues to this puzzle[14]. Based on the cellular structure of the maternal nucellar projection or the chalazal pad, the transfer of maternal mRNAs to the endosperm coenocyte appears unlikely. Thus, if a signal(s) from the sporophyte acts as an activator of *END1* transcription, one possible source of a protein factor(s) is the nucellar projection region. Alternatively, the source of this polarity might be a maternal signal, deposited in the central cell lying above the developing nucellar projection.

Endosperm cellularization

Preparation for cellularization occurs during a mitotic hiatus, which lasts for two days in barley. During this phase, the multinucleate cytoplasm becomes reorganized into nuclear cytoplasmic domains (NCDs) defined by radial systems of nuclear-based

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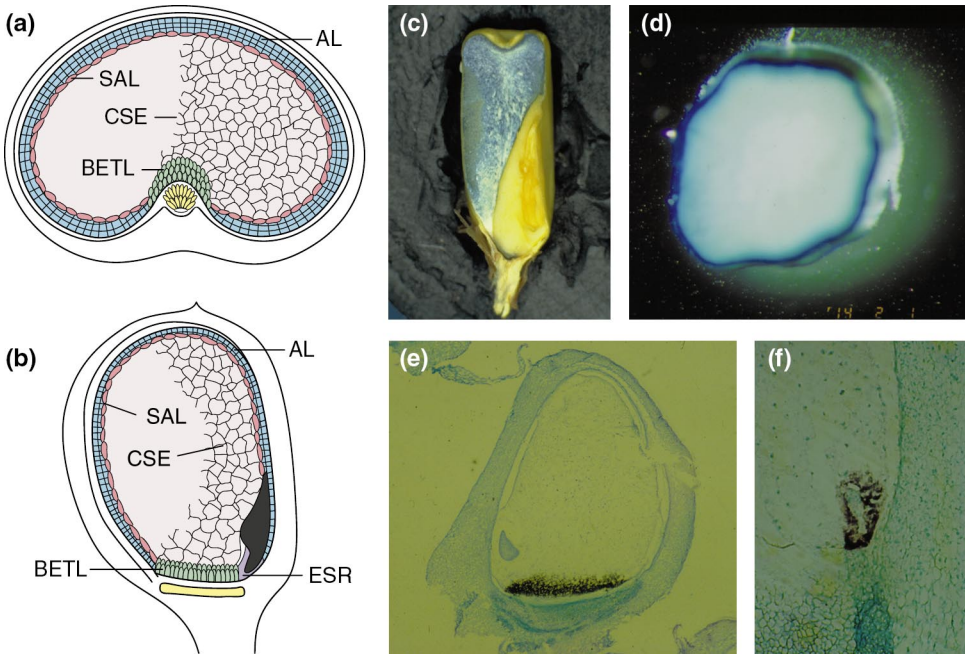

Fig. 1. Endosperm cell types and tissues. (a) Barley grain at 15 days after pollination (DAP). Nucellar projection is shown in yellow. (b) Maize grain at 15 DAP. The embryo is shown in black, the chalazal pad in yellow. (c) Transgenic maize grain with GUS-marker gene activity driven by the starchy endosperm-specific γ-zein promoter[41] (photograph kindly provided by J. Ranch, Pioneer Hi-Bred International, Johnston, IA, USA). (d) Transgenic rice grain with GUS-expression driven by the aleurone-specific *Ltp2* promoter from barley (photograph kindly provided by H-G. Opsahl-Ferstad, Agricultural University of Norway, Ås, Norway). (e) Micrograph of young maize grain showing *in situ* hybridization of the *Bet1* probe to BETL cells (micrograph kindly provided by R. Thompson, Max Planck Institute, Cologne, Germany). (f) Micrograph of young maize grain showing *in situ* hybridization of the *Esr-1* probe to ESR cells (micrograph kindly provided by P. Rogowsky, Ecole Normale Supèrieure de Lyon, France). Abbreviations: AL, aleurone layer; BETL, basal endosperm transfer layer; CSE, central starchy endosperm; SAL, sub-aleurone layer.

microtubules[12,15,16] (Fig. 2i). Next, an unusual form of anticlinal wall deposition, termed 'free-growing', occurs in the thin layers of common cytoplasm among adjacent NCDs. The NCDs elongate along axes that are perpendicular to the central cell wall and the radial microtubules are rearranged into axial systems emanating from the tips of the nuclei (Fig. 2j). Following this distinctive polarization, adventitious phragmoplasts develop at the interfaces of microtubule systems emanating from adjacent NCDs, and serve to guide continued centripetal growth of the anticlinal walls[17]. This results in compartmentalization of the cytoplasm into tube-like structures (alveoli), with an overlying layer, composed of the remaining syncytial cytoplasm, adjacent to the central vacuole (Fig. 2j). In barley, this process is first initiated over the nucellar projection, but the factors driving this highly directional process are unknown.

Two lines of evidence suggest that endosperm cellularization occurs via an ancient genetic program. Firstly, endosperm cellularization occurs in the same way as in the megagametophyte (i.e. via nucleo-cytoplasmic domains[18]). Secondly, the process of forming alveoli in nuclear endosperm development and in the female gametophyte development of gymnosperms, is remarkably similar. In both cases, the transition from the initial coenocytic stage to a cellular stage begins with the formation of open-ended alveoli by centripetal growth of anticlinal walls. This process appears to have occurred in late-Paleozoic seed ferns some 300 million years ago (Ref. 19, B.E. Lemmon and R.C. Brown, pers. commun.). Unfortunately, we have no comparable knowledge of the role of the cytoskeleton in fern alveolation.

Interestingly, the mode of nuclear endosperm formation described here does not take place in the proximal part of the endosperm surrounding the embryo of barley[20] and maize (R.C. Brown *et al.*, unpublished), where cytokinesis by cell plate formation results in cellularization, whereas the remainder of the endosperm is syncytial.

Following the formation of alveoli, the mitotic block is released and the nuclei divide synchronously, in what has been termed the formative division of endosperm development[17] (Fig. 2k). Periclinal division in the alveoli results in a layer of peripheral cells and a layer of alveoli displaced towards the center. The outer daughter cells give rise to aleurone initials (except for the BETL), whereas the inner alveoli give rise to the starchy endosperm. This is the first stage in endosperm development (with the exception of the ESR) where cell plate formation is mediated by interzonal phragmoplasts[12].

Aleurone cell differentiation

In barley, the different fates of the daughter nuclei resulting from the formative division are observable soon after completion of the division. The inner cell, part of a cell file extending centripetally, goes on to divide in the same way as the cells of the first layer of alveoli (Fig. 2l and m). The outer cell, now an aleurone initial, divides according to the pattern commonly seen in plant meristems (i.e. in a cell cycle with hoop-like cortical arrays of microtubules, pre-prophase bands and phragmoplasts; Fig. 2n). In maize, the peripheral layer undergoes rounds of periclinal divisions, which results in radial rows of cells before the appearance of cells with typical aleurone morphology. In all cereals investigated, typical aleurone cells are first visible at the basal surface near the nucellar projection and these then spread laterally. The strict spatial control of aleurone cell fate is demonstrated by the observation that in the mature barley grain the aleurone layer is three cells thick, whereas in maize it is only one cell thick (Fig. 1).

The recent cloning of the maize mutant gene *crinkly4* (*cr4*)[21], which results in patches of missing aleurone cells in homozygous mutant seeds (Fig. 4), has shed some light on the molecular basis of aleurone cell specification. One possible role of the *cr4* gene, which encodes a protein receptor-like kinase with similarity to the tumor-necrosis-factor receptor of mammals, is that it specifies aleurone cell fate in the formative endosperm division[22]. If this is the case, then what is the source of the ligand that activates the CR4 receptor? One possibility is that the signal is of maternal origin, and is either deposited in the central cell or provided by the surrounding maternal tissue. Support for maternal control of zygotic tissues comes from *Petunia*, where ovule-specific MADS-box

genes have an effect on seed development[23]. Alternatively, the ligand might be zygotic, possibly encoded by the recessive defective kernal mutant (*dek1*) gene[24], for which the homozygous mutant endosperm lacks an aleurone layer altogether[25] (Fig. 4b). A maize mutant with three layers of aleurone cells (similar to barley) has also been reported[26], suggesting that there might be a simple genetic basis for determining the number of aleurone cell layers. This mutant, as well as a novel collection of maize mutants affecting aleurone cell differentiation (O-A. Olsen and S.E. Nichols, unpublished) represent valuable tools for dissecting the signal transduction pathway for aleurone cell differentiation. In maize, large collections of recessive *dek* mutants[27], affecting the endosperm alone, or both the endosperm and the embryo, are also valuable resources for this work.

In maize, studies of genes differentially expressed in cereal aleurone cells have revealed a regulatory hierarchy including the transcription factor Vp1, which activates *C1* at the Sph-box (an RY-motif containing sequence) and the *bz1* gene[28]. The MYB transcription factor, C1, together with the basic helix–loop–helix transcription factor R, activates the promoter of the *bz* gene, a structural gene in the anthocyanin pathway[29]. In barley, aleurone cell marker genes include *Ltp2* (Ref. 30; Fig. 1d) and *Chi26*, a chitinase gene for which transient-expression analysis has identified promoter upstream elements implicated in aleurone-specific gene transcription[31]. Dissection of the barley *Ltp2* promoter, which is preferentially expressed in aleurone cells right after the onset of aleurone cell differentiation, is currently under way in transgenic cereals, and will hopefully lead to the identification of early activators in the aleurone genetic program.

Subaleurone cells

In the bulk of the starchy endosperm of developing seeds, the cells closest to the aleurone layer, often referred to as subaleurone cells, are smaller than those in the deeper layers (Fig. 1). This is also the position in which cell divisions remain active the longest – until about the grain mid-stage. In wheat, the loss of storage vacuoles that are typical of aleurone cells suggests a redifferentiation of aleurone initials to the starchy endosperm cell fate[32].

Basal endosperm transfer cell layer

The BETL forms over the nucellar projection in barley and wheat, and over the chalazal pad in corn (Fig. 1). Molecular markers for BETL include *BET1* in maize[33] (Fig. 1) and *END1* in barley[13] (Fig. 3). The discovery of the presence of the *END1* molecular marker in the endosperm coenocyte nuclei that give rise to the BETL cells and, subsequently, in the BETL cells themselves, suggests a model for BETL cell differentiation (Fig. 3). Assuming that *END1* expression is representative of the BETL gene program, the pattern of *END1* gene expression suggests that BETL cell differentiation occurs earlier than aleurone cell differentiation, which is initiated after the first periclinal division. Considering the role of the BETL region in the transfer of nutrient to the developing endosperm, this timing of developmental events appears reasonable. In the model shown in Fig. 3d, *END1* is expressed in the two basal cells of cell files developing from the green nuclei shown in Fig. 3b. Whether or not the starchy endosperm cells above the BETL region in the cellular endosperm are derived from the distal portion of these cell files or from the opposing cell files is unknown. The factor(s) initiating *END1* and *BETL* gene transcription are unknown and mutants lacking BETL cells have not been reported.

Central starchy endosperm cells

The inner daughter cells of the formative division represent starchy endosperm cell initials (Fig. 2k). In barley, wheat and maize,

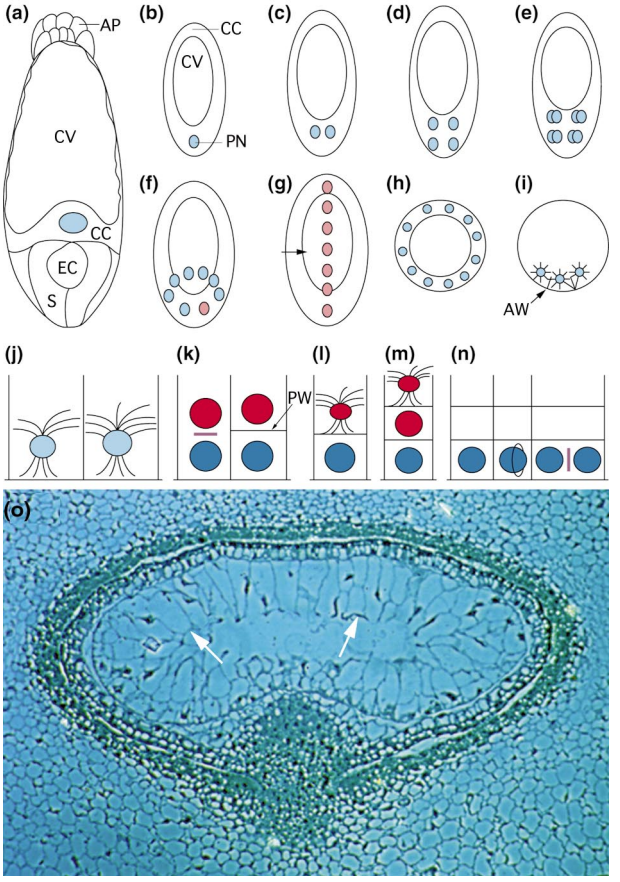

Fig. 2. Endosperm cellularization and differentiation. (a) *Polygonum*-type of megagametophyte consisting of a central cell (CC) with central vacuole (CV), egg cell (EC), two synergids (S) at the micropylar end and antipodals (AP) at the chalazal end. (b–g) Division planes and pattern of nuclear migration in the endosperm coenocyte. The pink nucleus in (f) and (g) illustrates compartmentalized nuclear migration. (h) A transverse section of endosperm coenocyte showing the distribution of nuclei in the peripheral cytoplasm of the coenocyte. The section plane is indicated by the arrow in (g). (i) Radial microtubules organized at nuclear membranes form nucleo-cytoplasmic domains that guide the formation of the first anticlinal wall (AW). (j) Endosperm alveoli (central cell wall is the horizontal base line). (k) Formative division (for the basal transfer cell layer see Fig. 3d also). The periclinal phragmoplast is shown in purple; the aleurone initial is dark blue; the starchy endosperm nucleus is red; PW, periclinal cell wall. (j) and (m) Centripetal extension of cell file towards the center of the endosperm cavity. (n) Pattern of division of aleurone cells. The ring around the second nucleus represents a pre-prophase band. The anticlinal phragmoplast is shown in purple. (o) Transverse section of barley grain at six days after pollination showing fusing cell files from opposite sides (closure), the fusion line is marked by arrows. Micrograph (o) kindly provided by M. Bosnes, Agricultural University of Norway, Ås, Norway.

continued extension of anticlinal walls, and one or two repetitions of the process seen in the original layer of alveoli, lead to the development of cell files that meet in the center of the endosperm cavity (Fig. 2o). In rice, several concentric layers form as a repetition of the original alveolation process, leaving an irregular open space in the center of the endosperm cavity that is rapidly filled with starch granules[15]. In contrast to aleurone cells, divisions in the starchy endosperm occur without pre-prophase band formation. After closure, divisions in each cell file occur in random planes, and the

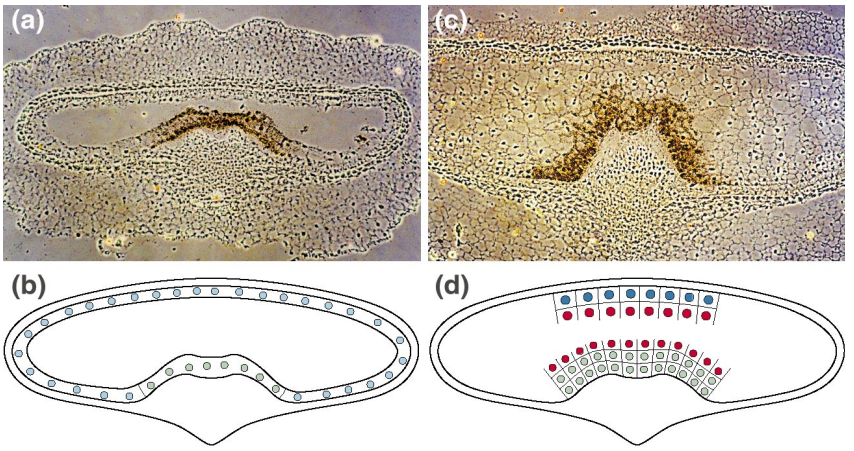

Fig. 3. Establishing domains of gene expression in the endosperm. (a) *In situ* hybridization micrograph showing *END1* transcript in the barley endosperm coenocyte over the nucellar projection. (b) Diagrammatic representation of *END1* gene expression (green nuclei) shown in (a). (c) *In situ* hybridization micrograph showing *END1* transcript in basal transfer cell layer of young cellular barley endosperm. (d) Diagrammatic representation of the developing cell files in the region over the ventral crease. Cells expressing the END1 transcript are shown in green, starchy endosperm cells in red. For orientation of cell files, see Fig. 2o.

cell file pattern is soon lost. Closure occurs four days after pollination in rice[15] and maize (R.C. Brown, B.E. Lemmon and O-A. Olsen, unpublished), and during day six in barley[12]. In maize, analysis of clonal sectors places the origin of the starchy endosperm sectors to one side of the crown of the seed[6].

One feature of endosperm cells is that they undergo endoreduplication, resulting in highly polyploid starchy endosperm cells in maize[34] and aleurone cells in barley[35]. Interestingly, in maize, endoreduplication of the endosperm correlates positively with phosphorylation of the retinoblastoma homolog[34], and high levels of expression of the ZmWee1 protein kinase in mid-stage maize endosperms suggests that it might play a role in endoreduplication[36]. In contrast to the aleurone cells, which survive seed desiccation, the starchy endosperm cells of maturing grains die, possibly as a result of a process similar to programmed cell death[37].

The most intensively studied genes, expressed specifically in CSE cells, are those encoding prolamin storage proteins (Fig. 1). It appears that the specificity of expression requires the binding of at least two factors to the region upstream of the promoter in the −300 prolamin endosperm box[38]. These include one factor at the GCN4 box and a second factor called the prolamin box-binding factor. In addition, a third Myb-class protein also appears to be important for cereal endosperm-specific gene expression[39]. Factors that bind to the GCN4-motif include opaque-2 in maize and bZIP factors (that closely resemble opaque-2) in barley and wheat, all of which appear to be endosperm-specific. However, because the *opaque-2* transcript is first observable ten days after pollination in maize, which is about five days after the appearance of starchy endosperm cells, a major part of the genetic program of the early starchy endosperm cells remains unknown.

The embryo surrounding region

The embryo develops in a pocket within the starchy endosperm, which at the proximal end is lined with cells with a dense cytoplasm (Fig. 1b). The expression of the *Esr 1* transcript in the area surrounding the embryo at early developmental stages (Fig. 1f), demonstrates that these cells represent a separate domain of gene expression in the endosperm[40]. Little is known about the mechanisms involved in the formation of the endosperm cavity, including whether or not the ability to form the cavity is an intrinsic property of the endosperm, or whether it requires interactions with the embryo. One possibility is that the cellularization that occurs in the proximal part of the central cell after fertilization around the embryo in barley[20] and maize represents the initiation of embryo cavity formation. To our knowledge, mutants lacking the ESR have not been reported.

Future perspectives

Several events are expected to contribute to an increased understanding of nuclear endosperm development in the years ahead. First, screens for *Arabidopsis* mutants should help to throw light on the early phases of endosperm coenocyte development, including operation of the cell cycle and mitotic machinery, nuclear positioning and alveoli formation. In the cereals, identification of Mu-transposon-induced mutants perturbed at each step of the pathway of endosperm development should facilitate the identification of genes controlling development and those effecting grain traits of economic importance. Finally, output from genome projects will provide invaluable tools in the ongoing hunt for the genetic basis of mankind's most important renewable resource.

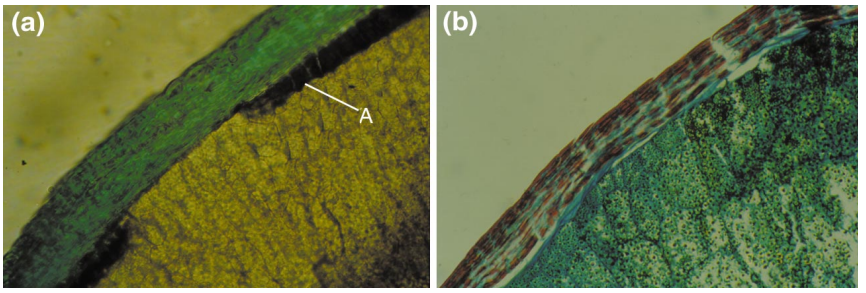

Fig. 4. Mutants in aleurone cell differentiation. (a) Light micrograph showing the peripheral part of a transverse grain section of the *crinkly4* maize mutant, which is missing some aleurone cells (A indicates the aleurone cells; seeds provided by the Maize Genetics Cooperation Stock Center). (b) Light micrograph showing the peripheral part of the transverse grain section of a *dek1* maize mutant that has no aleurone cells. Grain section in (b) kindly provided by S. Dolfini and G. Gavazzi, University of Milan, Italy.

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- **Raymond Alan Dixon** John Innes Centre, UK
- **Anthony James Trewavas** University of Edinburgh, UK

Foreign member

- **George Ledyard Stebbins** University of California, Davis, USA