

Positional cues specify and maintain aleurone cell fate in maize endosperm development

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SUMMARY

A genetic analysis of maize aleurone development was conducted. Cell lineage was examined by simultaneously marking cells with *Cl* for anthocyanin pigmentation in the aleurone and *wx1* for amylose synthesis in the starchy endosperm. The aleurone and starchy endosperm share a common lineage throughout development indicating that positional cues specify aleurone fate. Mutants in *dek1* block aleurone formation at an early stage and cause peripheral endosperm cells to develop as starchy endosperm. Revertant sectors of a transposon-induced *dek1* allele showed that peripheral endosperm cells remain competent to differentiate as aleurone cells until late in development. *Ds*-induced chromosome breakage was used to generate *Dek1* loss-of-function sectors. Events

occurring until late development caused aleurone cells to switch fate to starchy endosperm indicating that cell fate is not fixed. Thus, positional cues are required to specify and maintain aleurone fate and *Dek1* function is required to respond to these cues. An analysis of additional mutants that disrupt aleurone differentiation suggests a hierarchy of gene functions to specify aleurone cell fate and then control aleurone differentiation. These mutants disrupt aleurone differentiation in reproducible patterns suggesting a relationship to endosperm pattern formation.

Key words: Endosperm, Maize, Cereal, Pattern formation, Aleurone

INTRODUCTION

The cereal endosperm is a structurally simple tissue consisting of three major cell types: the starchy endosperm, aleurone and basal endosperm transfer cells (Olsen et al., 1999; Becraft et al., 2000). The starchy endosperm occupies the central region and constitutes the bulk of the endosperm tissue. These cells are packed with amyloplasts, which contain prominent starch grains, and with protein bodies, which are specialized vacuoles filled with seed storage proteins. Two specialized zones are visible within the starchy endosperm. The subaleurone is the region just internal to the aleurone and is a region of cell proliferation during later stages of endosperm development. The 'embryo-surrounding region' consists of small cells with denser cytoplasm than other starchy endosperm cells. The basal endosperm transfer cells reside over the pedicel and transfer nutrients from the maternal vasculature into the developing endosperm. They are elongate cells with many finger-like cell wall involutions that maximize membrane surface area, thought to aid the transfer function. The aleurone is a layer of densely cytoplasmic cells covering the surface of the endosperm, just beneath the maternal pericarp tissue. Upon germination, the aleurone cells are stimulated by gibberellic acid to secrete hydrolytic enzymes that break down the storage compounds

in the starchy endosperm, making free amino acids and simple sugars available for uptake by the growing seedling.

Despite the apparent structural simplicity, endosperm development is complex and involves several unique processes. In double fertilization, one sperm nucleus fertilizes the egg cell to form the zygote and the second sperm nucleus fertilizes the central cell to give rise to the endosperm. Following karyogamy of the two polar nuclei with the sperm nucleus, the triploid primary endosperm nucleus of cereals undergoes a series of free nuclear divisions to form a syncytium. After several rounds of division, the nuclei migrate to the periphery of the endosperm. Here, networks of microtubules radiate from the nuclei, defining cytoplasmic domains around each nucleus (Brown et al., 1994). After approximately 8 rounds of free nuclear division (Randolf, 1936), a unique form of cytokinesis ensues; vesicles begin to accumulate and fuse in the boundaries between the nucleocytoplasmic domains to form free growing cell walls (Brown et al., 1996a,b). These walls form independent of mitosis and occur between non-daughter nuclei. Free cell wall formation initiates at the periphery of the endosperm and progresses centripetally, with new vesicles added to the growing ends of the walls through the action of adventitious phragmoplasts. This process results in the formation of a tube-like wall structure, called an alveolus, surrounding each nucleus. At this point the nuclei divide

periclinally and form a typical cell plate between daughter nuclei. The result of this division is that one daughter remains at the periphery of the endosperm and the other is displaced toward the interior. The peripheral layer is now completely cellularized while the internal layer retains the alveolar structure. This process repeats in the central-most layer until the entire endosperm cavity becomes cellularized.

As the endosperm grows, cell division becomes localized to a cambium-like zone at the periphery. At some point, the outermost cell(s) take on the characteristics of aleurone, including small columnar shape, dense, granular cytoplasm and thick, autofluorescent cell walls. The timing of the transition to aleurone cells varies among cereal species, occurring almost immediately upon cellularization in barley (Bosnes et al., 1992), but later in rice, wheat and maize (Brown et al., 1996a; Kyle and Styles, 1977; Morrison et al., 1975; Randolph, 1936; Schel et al., 1984).

Several molecular markers have been identified that are expressed in specific domains of the endosperm during development. *END1* message is found in the coenocytic embryo, localized in the nuclei above the nucellar projection (Doan et al., 1996). *Esr-1* is expressed in endosperm cells surrounding the embryo beginning at 4 days after pollination (DAP), just as the endosperm is cellularizing (Opsahl-Ferstad et al., 1997). A gene encoding a lipid transfer protein is expressed in barley aleurone cells beginning at 10 days after fertilisation (DAP) (Olsen et al., 1990). The *BET1* gene is transcriptionally active only in the basal endosperm transfer layer cells (Hueros et al., 1995, 1999). These markers provide indications that positional information exists within the developing endosperm.

Despite the detailed descriptions of endosperm development, several fundamental questions remain. In particular, the mechanisms of cell fate determination and pattern formation are not well understood. One gene involved in these processes has been isolated. Mutants in the *crinkly4* (*cr4*) gene cause aleurone mosaicism, where patches of aleurone fail to differentiate and peripheral cells develop as starchy endosperm. The *cr4* gene encodes a receptor-like kinase (Becraft et al., 1996), invoking a model that cell signaling is important for determining endosperm cell fate (Olsen et al., 1998). According to this model, the mitosis that results in a cellular periphery and an alveolar internal endosperm is a formative division in that the peripheral cells will become aleurone precursors while the internal cells give rise to starchy endosperm. A gradient of signal from the periphery would specify aleurone cell fate. Variations in the concentration of the signal, the slope of the gradient or sensitivity of endosperm cells to the signal would result in different numbers of aleurone cell layers in different species. Genetic tools are available in maize to test aspects of this model and to address several other fundamental questions of aleurone development. In this paper, we show by lineage analysis that the aleurone and starchy endosperm are not derived from separate lineages. We also show that aleurone cell fate is not fixed in the initial cellularization of the endosperm, but remains flexible until late in development. Finally we describe a collection of mosaic aleurone mutants that appear to define a genetic hierarchy regulating aleurone development and reveal further complexity in endosperm development.

MATERIALS AND METHODS

Genetic stocks

Genetic stocks were generously provided for this study as follows. The *Ds*, *CI-I*, *Wx* and *CI, wx*; *Ac11* stocks used in the cell lineage study were provided by Kelly Dawe, University of Georgia. The *Ds1S4*, *collapsed2-o12* (*cp2-o12*), *white2* (*w2*) and *Dappled1* (*Dap1*) lines were obtained from the Maize Genetics Cooperation Stock Center, USDA-ARS, Urbana, IL. *Ds1S1*, *defective kernel1* (*dek1-792*), and *Mosaic1* (*Msc1*) stocks were received from M. G. Neuffer, University of Missouri. The *dek1-PIA* mutant was obtained from Martha James, Iowa State University. Hugo Dooner, Waksman Institute, donated the *dek1-Dooner* (*dek1-D*) allele. The *wandering embryo* (*wem*) line was provided by Erin Irish, University of Iowa.

Histology

Kernels were dissected at 22 days after pollination, when aleurone pigmentation was obvious but when the kernels were not yet fully desiccated. The crown region of the kernel was fixed overnight in 3% glutaraldehyde, 2% paraformaldehyde in 100 mM cacodylate buffer, pH 7.2. The tissue was embedded in LR White resin (Electron Microscopy Sciences), sectioned at 1 μ m on an LKB ultramicrotome and stained with Toluidine Blue. Slides were viewed and photographed on an Olympus BX60 microscope.

Lineage and cell fate analysis

Surface views of aleurone cells were obtained by peeling the pericarp and viewing with an Olympus SZH-10 stereomicroscope with a polarizing filter. Sectors of chromosome 9S breakage, 1S breakage or *dek1-PIA* reversion were observed by hand sectioning dry, mature kernels. Starch staining was conducted as described previously (Burnham, 1982). The hand sectioned kernels were observed either with an Olympus SZH-10 stereo microscope or an Olympus BX-60 compound microscope using either epi-illumination or epi-fluorescence with a narrow violet filter (excitation 400-410 nm, emission 455 nm).

RESULTS

The aleurone and the starchy endosperm are not separate lineages

An early account of maize endosperm development states that cell division in the aleurone layer is restricted almost exclusively to the anticlinal plane (Randolph, 1936). Because anticlinal divisions produce a cell plate perpendicular to the surface, daughter cells remain within the same cell layer. This has led to the view that the aleurone and starchy endosperm represent independent lineages (Becraft et al., 1996; Walbot, 1994). However periclinal cell divisions have been reported in wheat aleurone cells (Morrison et al., 1975) and in our studies of aleurone development, it became apparent that periclinal divisions also occurred in maize aleurone (see Fig. 3A). We therefore decided to examine lineage relationships between the aleurone and starchy endosperm using a similar approach to McClintock for studying clonal relationships in early endosperm development (McClintock, 1978).

A line homozygous for a chromosome breaking *Ds* element, *CI-I* and *Wx* on chromosome 9S, and carrying *Ac11* (Dawe and Freeling, 1990) was crossed to a stock homozygous for *CI, wx*. The resultant plants had the following constitution: *CI wx/Ds CI-I Wx* (Fig. 1A). *CI* controls anthocyanin production in the aleurone and *CI-I* is a dominant negative allele that inhibits aleurone pigmentation in the heterozygous state. *Ds*-induced

chromosome breakage causes loss of the *C1-I* allele, allowing expression of *C1* and resulting in purple aleurone sectors on an unpigmented background. The *Wx* gene encodes a starch-granule-bound nucleotide diphosphate-starch glucosyl transferase that catalyzes the production of amylose (Nelson and Rines, 1962). In recessive *wx* mutant kernels, starch is in the form of amylopectin. Amylose stains deep blue with iodine while amylopectin stains red. *Ds*-induced chromosome breakage results in the loss of *Wx*, thus producing *wx* sectors

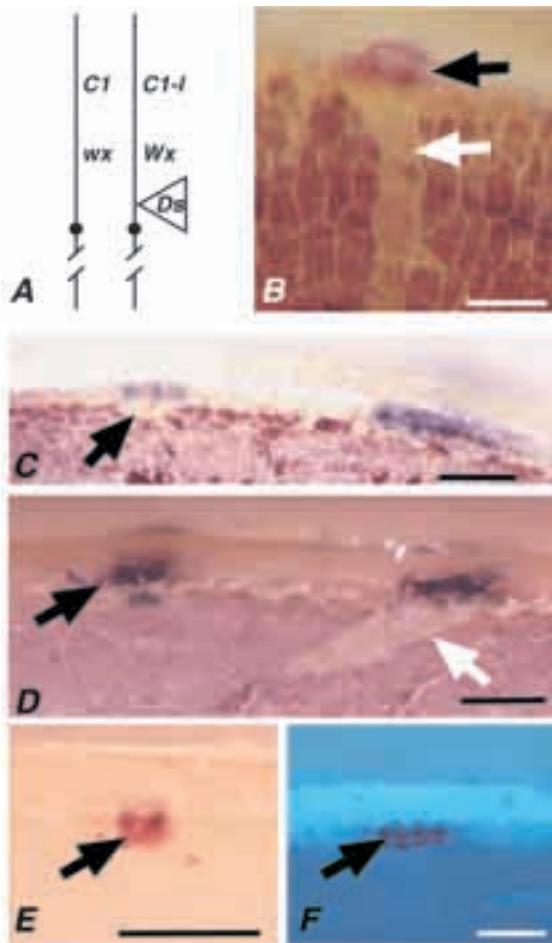


Fig. 1. Lineage analysis of aleurone development. (A) Chromosome constitution used for aleurone cell lineage analysis. *Ac* induced chromosome breakage at *Ds* uncovers the *C1* and *wx* genes, resulting in a clone of cells that contain anthocyanin in the aleurone and do not stain darkly with iodine in the starchy endosperm. (B-D) Examples of sectors that show a common lineage between aleurone and starchy endosperm. (B) A sector containing 32 aleurone cells (black arrow) which was marked by *wx1* deep into the starchy endosperm (white arrow). (C) The arrow shows a sector with 12 aleurone cells and with the immediately adjacent starchy endosperm cells marked. The sector on the right contained 18 aleurone cells and the underlying starchy endosperm was not marked. (D) The sector on the left (black arrow) contained 9 aleurone cells and no marked starchy endosperm, while the sector on the right (white arrow) contained 14 aleurone cells and extended obliquely several layers into the starchy endosperm. (E) A sector containing 4 marked aleurone cells in the surface layer and 2 marked aleurone cells in the subsurface layer. (F) A sector that contained 10 marked surface aleurone cells and at least 4 marked subsurface aleurone cells. Scale bars, 100 μ m.

that can be followed in the starchy endosperm by iodine staining. Because this single chromosome breakage event uncovers markers for both aleurone and starchy endosperm, it is possible to examine lineage relationships in the two tissues.

To ensure that the sectors examined were derived from single events and not multiple independent events in adjacent lineages, only kernels showing infrequent sectoring were examined. Sectors were examined by hand sectioning mature, dry kernels and staining with IKI solution to determine the genotype of the underlying starchy endosperm cells. The results of scoring 73 sectors containing between 2 and 22 surface aleurone cells are shown in Figs 1 and 2. The majority of sectors with 6 marked aleurone cells or greater contained underlying *wx* cells indicating that the sector included both aleurone and starchy endosperm. Five sectors of 4 or fewer aleurone cells contained marked subtending cells. The results indicate that the aleurone and underlying starchy endosperm cells can share the same lineage at least to within the last two divisions from the cessation of endosperm development. The results are conservative because as it was only feasible to score any given sector in a single section plane, some sectors recorded as not including starchy endosperm might have included internal cells in other regions of the sector. Many more sectors were sampled but most small sectors were discarded due to ambiguity; small sectors were difficult to examine and subaleurone cells are often devoid of starch grains making it impossible to ascertain their *wx* genotype. A few larger sectors were also scored; of 18 sectors containing 25-204 aleurone cells, marked internal cells were observed in all but the 25-cell sector (not shown).

Eight examples were observed where marked aleurone cells occurred in the second layer of endosperm (Fig. 1E,F). This indicates that periclinal divisions had occurred in the aleurone but that the internal daughter cells had retained aleurone identity at the cessation of endosperm development. Two examples were in very late sectors, one containing 4 surface cells and 2 in the subsurface layer and another containing 2 surface cells and 2 subsurface aleurone cells. The latter sector demonstrates that a periclinal division occurred in one of the two last rounds of cell division in the aleurone.

The lineage analysis cannot distinguish unambiguously between contributions from the aleurone layer to the internal

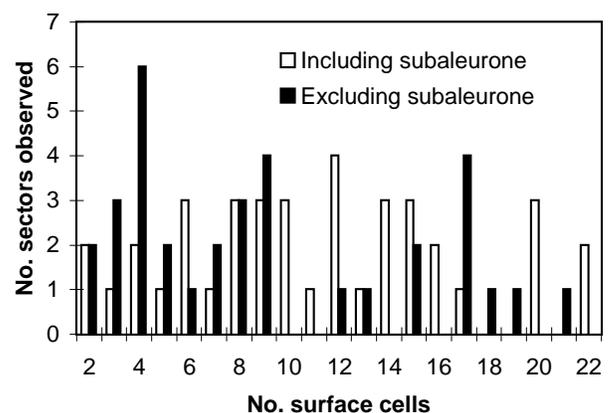


Fig. 2. Graph showing the relative frequency of sectors with concordance between the aleurone and subaleurone.

endosperm and vice versa. Several observations suggest that cells are contributed internally: the late sectors we focused on in this study were always more extensive in the aleurone than in the internal endosperm. This would be expected if one or more anticlinal divisions occurred in the aleurone following a chromosome breakage event, prior to a periclinal division. Second, the presence of marked aleurone cells internal to the peripheral layer is consistent with the daughter of an aleurone cell division being arrested in the process of redifferentiation to a starchy endosperm cell (Morrison et al., 1975). Starchy endosperm cells would not be expected to redifferentiate as aleurone prior to invading the peripheral layer. However, cell layer invasions are a common feature in plant development (Stewart et al., 1974) and we acknowledge the possibility of their occurrence in the endosperm.

Endosperm cells remain competent to acquire aleurone fate until late in development

Strong *dek1* mutant alleles completely lack aleurone cells as shown in Figs 3 and 4. We used two transposable element systems to address questions concerning aleurone cell fate determination. In the first, a mutable *Mutator* induced allele, *dek1-PIA*, produces small revertant sectors in the fine spotting pattern characteristic of *Mu*. It is possible to observe sectors as small as one cell (Fig. 3). This indicates that it is possible for endosperm cells to differentiate into aleurone late in development. Because histological examination of *dek1* mutant kernels shows that the surface cells of the endosperm display a starchy endosperm phenotype at an earlier stage in development (Fig. 3B), this implies that the gain of *Dek1+*

function can switch the fate of cells, already differentiated as starchy endosperm, into aleurone cells if they occupy the peripheral position. Therefore, peripheral endosperm cells retain the capacity to acquire an aleurone cell fate throughout endosperm development.

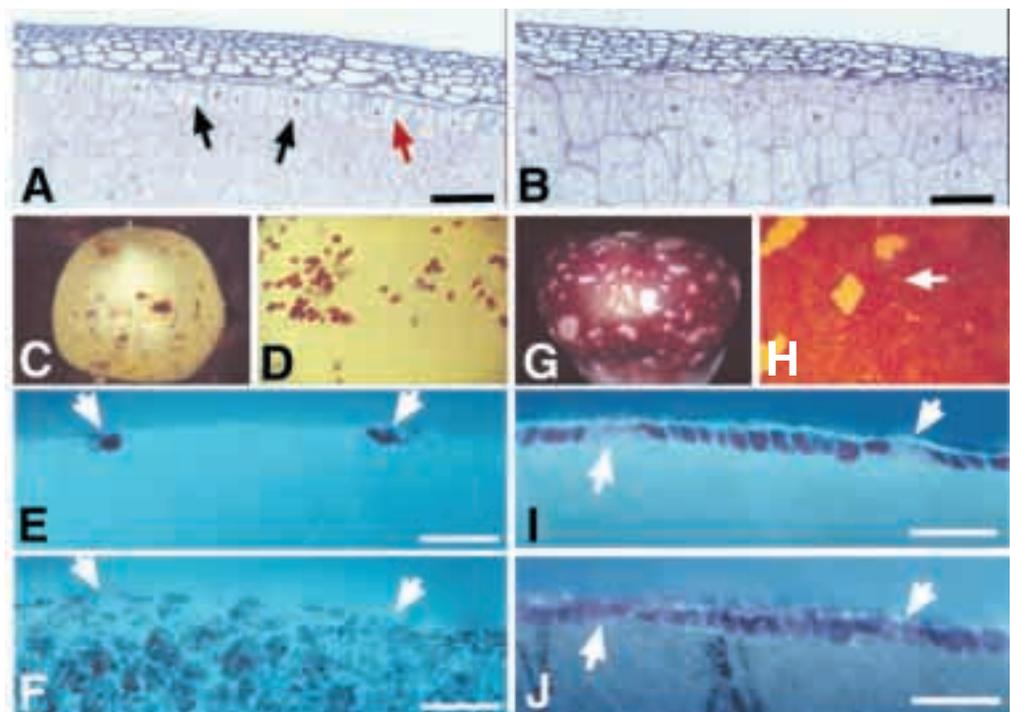
Aleurone cell fate is not fixed

To test whether aleurone fate represents a stably determined state, the chromosome breaking *Ds* transposable element, *Ds1S4* was utilized to generate sectors of *dek1* loss. As shown in Fig. 3, very small sectors of *dek1* mutant tissue are readily observable and peripheral cells within those sectors possess starchy endosperm identity. Because an aleurone cell phenotype is recognizable at an earlier stage in normal endosperm development, cells within those sectors have switched fate from aleurone to starchy endosperm upon loss of *Dek1+* function. Thus, aleurone cell fate is not stably determined, and the action of the *Dek1+* gene is required to maintain aleurone cell identity.

Mutants disrupting aleurone differentiation

Anthocyanin pigmentation in the maize endosperm is limited to aleurone cells. We took advantage of this marker to identify mutants that disrupt aleurone differentiation. Analyses of mutants that showed mosaic pigmentation phenotypes are presented below. Note that names of mutants marked with an asterisk are provisional until map location can be determined (as per maize genetics nomenclature conventions). All mutants presented here represent independent loci as determined by map position or complementation.

Fig. 3. Sectors of *dek1* mutants indicate that endosperm cell fate is flexible through late developmental stages and that *Dek1* is required for aleurone cell fate acquisition and maintenance. (A) Section of a wild-type kernel at 12 DAP. The red arrow points to aleurone cells. Also note aleurone cells containing periclinal cell walls (black arrows), indicating the internal contribution of aleurone daughter cells. (B) Section of a *dek1* mutant kernel at 12 DAP. The peripheral endosperm cells have the attributes of starchy endosperm cells. (C,D) Late revertant sectors of *dek1-PIA*. (E) Fluorescent image of a hand-sectioned *dek1-PIA* kernel showing individual aleurone cells surrounded by starchy endosperm. (F) The section in E following iodine staining for starch. Arrows in E and F denote individual aleurone cells. All cells except the aleurone cells stained positively for starch indicating starchy endosperm identity. The water soluble anthocyanin was washed out of the aleurone cells in F during the iodine staining. (G,H) Sectors of *Dek1* loss from *Ds1S4* induced chromosome breakage. The arrow in H points to an example of a late sector showing one or two starchy endosperm cells in a field of aleurone. (I) Fluorescent image of sectioned *Ds1S4/dek1* kernel. (J) The same section as in I following iodine staining for starch. Arrows in I and J denote cells that lacked anthocyanin and stained for starch indicating they have acquired a starchy endosperm cell fate. Scale bars, 100 μ m.



crinkly4 (cr4)

The phenotype of recessive *cr4* mutant kernels (not shown) has been previously reported (Becraft et al., 1996). Mutant kernels show mosaic aleurone with the abgerminal region of the crown most prone to showing defects. The unpigmented regions of the kernel contain peripheral endosperm cells with a starchy endosperm phenotype. Penetrance of this phenotype is variable, but typically 5-10%. The *cr4* gene maps to chromosome 10S.

defective kernel1 (dek1)

Strong *dek1* alleles produce kernels that are devoid of aleurone, with peripheral endosperm cells showing the histological

attributes of starchy endosperm (Figs 3, 4B,C). Mutants also possess a white, floury endosperm and have embryos arrested at variable stages of embryogenesis (not shown). Sometimes embryos contain a root primordium but lack shoot structures (Neuffer et al., 1997). Weaker alleles, such as *dek1-D*, produce a mosaic aleurone reminiscent of *cr4*, with the abgerminal region of the crown most prone to defects. *dek1* mutants are recessive and the gene maps to chromosome 1S (MaizeDB, <http://www.agron.missouri.edu/>).

bareback (bbk*)*

The *bbk** mutant (Fig. 4E,F) shows a similar kernel phenotype to strong *cr4* mutants, with the abgerminal region of the kernel

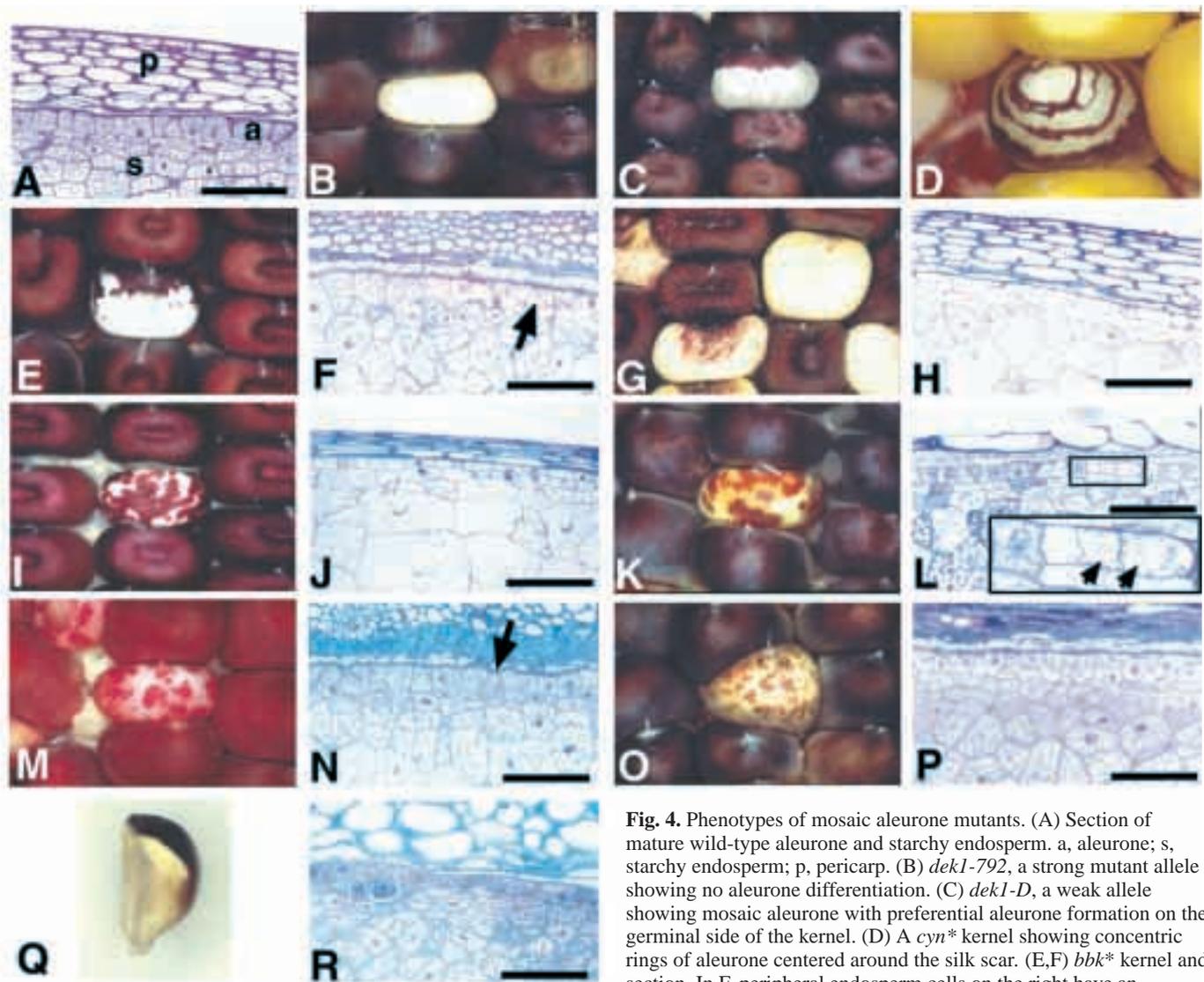


Fig. 4. Phenotypes of mosaic aleurone mutants. (A) Section of mature wild-type aleurone and starchy endosperm. a, aleurone; s, starchy endosperm; p, pericarp. (B) *dek1-792*, a strong mutant allele showing no aleurone differentiation. (C) *dek1-D*, a weak allele showing mosaic aleurone with preferential aleurone formation on the germinal side of the kernel. (D) A *cyn** kernel showing concentric rings of aleurone centered around the silk scar. (E,F) *bbk** kernel and section. In F, peripheral endosperm cells on the right have an aleurone phenotype (arrow) while on the left, peripheral cells have

differentiated as starchy endosperm. (G,H) *nkd** kernel and section. Peripheral cells show defects in aleurone differentiation including lack of aleurone grains and abnormally thin walls. However, the peripheral cells have not differentiated as starchy endosperm indicating the phenotype does not involve a cell fate switch. (I,J) *cp2-o12* kernel and section. Peripheral endosperm cells show defective aleurone differentiation. Starch grains appear abnormally large in the starchy endosperm cells. (K,L) *Msc1* kernel and section. Peripheral endosperm cells have the characteristic shape of aleurone cells but cells show variable cytoplasmic defects. The arrows in the high magnification inset of the boxed region in L denote starch grains in aleurone cells. (M,N) *Dap1* kernel and section. Aleurone cells are fairly well formed. The arrow in (N) shows a starchy endosperm cell located in the peripheral layer. (O,P) *w2-dek21* kernel and section. Aleurone organization and cell morphology are mildly disrupted. (Q,R) A *pfc** kernel and section of the unpigmented region. Aleurone defects are relatively mild. Scale bars, 100 μ m.

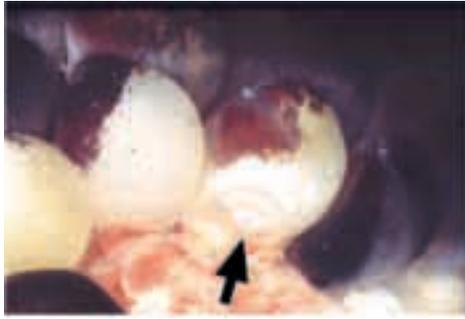


Fig. 5. The pattern of mosaicism in *dekl-D* does not change with altered embryo position in *wem* mutants. The arrow denotes an ectopic embryo. The pattern of mosaicism is the same as in the *dekl-D* kernel to the left with the embryo in the normal position. The pattern still centers around the position of the silk scar which is not altered in *wem*.

most prone to being devoid of aleurone. The endosperm is usually opaque and often white. This mutant was generated in an EMS screen for enhancers of *cr4*. Mutant kernels often produce normal looking plants, which has never been observed with *cr4* alone. The map location of *bbk** is not yet known.

*naked** (*nkd**)

*nkd** mutants show variable defects in the aleurone. Strong expression leads to a complete absence of aleurone and a floury-opaque endosperm (Fig. 4G,H). Weak expression causes a rough-textured aleurone while intermediate expression causes mosaicism. In mosaic kernels the distribution of differentiated aleurone is similar to *cr4* and *dekl-D*, with the region surrounding the silk scar most likely to contain aleurone cells while the abgerminal face is most prone to failure of aleurone differentiation. In section, surface cells of unpigmented kernels are irregular in form and devoid of the dense cytoplasm typical of aleurone cells. No starch grains were evident in the surface cells suggesting that the cells had not switched fate to starchy endosperm. Segregation ratios indicate that *nkd** either is a duplicate factor or has incomplete penetrance. Genetic mapping efforts are underway.

collapsed2-o12 (*cp2-o12*)

cp2-o12 is a weak allele of the *cp2* gene. Strong alleles show shriveled endosperms, presumably due to defects in starch accumulation. *cp2-o12* has nearly normal sized kernels that show a blocky pattern of mosaicism evenly distributed on the kernel (Fig. 4I,J). In unpigmented regions, peripheral endosperm cells lack the thick cell walls, cuboidal cell shape and dense cytoplasm of normal aleurone cells. Instead the cells are flattened, with thin walls and many small vacuoles. These cells do not resemble starchy endosperm and do not contain starch grains but have not properly differentiated as aleurone. Internal starchy endosperm cells contain fewer than normal amyloplasts but the starch grains are abnormally large. *cp2* mutants are recessive and map to chromosome 7S (MaizeDB, <http://www.agron.missouri.edu/>).

Dappled1 (*Dap1*)

Dap1 mutants show blocky patches of pigmented aleurone interspersed by unpigmented regions (Fig. 4M,N).

Occasionally the unpigmented regions appear white and opaque. The mosaicism is evenly distributed over the surface of the endosperm. A differentiated aleurone layer is apparent histologically throughout most kernels, including unpigmented regions. Most cells appear normal, but in some regions there are irregularities in cell shape and arrangement. *Dap1* mutants are dominant when inherited through the female but not when inherited through the male (Stinard and Robertson, 1987). The gene maps to chromosome 5L (MaizeDB, <http://www.agron.missouri.edu/>).

Mosaic1 (*Msc1*)

Mutants are very similar to *Dap1* in both phenotype and inheritance, although the *Msc1* aleurone defects were slightly stronger at the histological level. In one sample, starch grains were observed in peripheral endosperm cells that had the general shape of aleurone cells (Fig. 4K,L). *Msc1* maps to chromosome 1L (MaizeDB, <http://www.agron.missouri.edu/>).

white2 (*w2*)

Mutants have albino seedlings and mosaic aleurone pigmentation, however the aleurone cells appear nearly normal, histologically (Fig. 4O,P). Cellular defects include a slight disorganization in the aleurone layer and mild defects in aleurone cell shape. *w2* maps to chromosome 10L (MaizeDB, <http://www.agron.missouri.edu/>).

*paleface** (*pfc**)

*pfc** mutants lack anthocyanin pigmentation on the germinal face of the kernel and often lack embryos (Fig. 4Q,R). The kernels possess a well differentiated aleurone layer, however, the cells appear unusually rounded and the aleurone is sporadically more than one cell thick. This mutant shows poor penetrance and the genetic map location is unknown.

*cyclone** (*cyn**)

Kernels of this mutant have a variable phenotype with very poor penetrance. Most common is a floury endosperm with a dull, discolored appearance. Rarely, kernels show aleurone mosaicism in a concentric ringed pattern, centered around the silk scar (Fig. 4D). The poor penetrance of this mutant has thus far interfered with genetic mapping attempts.

Patterns of mosaicism

The mosaic phenotypes of the mutants discussed above show distinctive distributions on the kernel. The aleurone defects for *cr4*, *dekl-D*, *bbk** and *nkd** mutants occur more prominently on the abgerminal face of the kernel while aleurone is most likely to develop on the germinal face, particularly in the silk scar region. *pfc** mutants show the converse pattern where defects are more prominent on the germ face. *Dap1*, *Msc1*, *cp2-o12* and *w2* all produce mosaicism distributed evenly over the kernel. The *w2* phenotype contains small patches of pigmentation while the other mutants produce larger blocks of pigmentation.

Because mutations in multiple genes show similar patterns of mosaicism, these patterns are likely to reflect pattern forming events in endosperm development. That several patterns reflect the location of the embryo suggested that the embryo could be a reference point for the positional cues that determine these patterns. The *wandering embryo* (*wem*)

mutant, in which the embryo forms on random faces of the kernel (Erin Irish, personal communication), was used to test this hypothesis. Double mutants were created between *wem* and both *cr4* and *dek1-D*. The patterns of mosaicism coincided with the position of the silk scar and were independent of the embryo position (Fig. 5). This indicates that the embryo does not provide the positional cues that determine the pattern of aleurone mosaicism for these mutants.

DISCUSSION

Establishing and maintaining aleurone cell fate

Previous results of aleurone differentiation result in two models for the establishment of aleurone cell identity. Some accounts consider the acquisition of aleurone identity as occurring when the aleurone layer is histologically recognizable (Kyle and Styles, 1977; Morrison et al., 1975; Randolph, 1936). In maize, this typically occurs at 10–15 DAP, depending on genetic and environmental factors. Other accounts interpret the first periclinal cytokinesis, which forms a surface layer of cells during cellularization of the syncytial endosperm, as the key event (Brown et al., 1994, 1996b; Olsen et al., 1998; Walbot, 1994). This initial cytokinesis has been called a formative division because the surface cells give rise to aleurone initials whereas the internal cells produce only starchy endosperm cells (Brown et al., 1996b; Olsen et al., 1998). A formative division implies that the division per se specifies the fate of the daughter cells, perhaps through the asymmetric inheritance of some factor.

The results presented here do not support the concept of a formative division leading to aleurone cell fate. First, the lineage analysis shows that the aleurone and underlying starchy endosperm are clonally related indicating that the surface layer gives rise to both aleurone and starchy endosperm cells. Second, cell fate remains flexible until late in endosperm development, requiring the constant action of the *dek1* gene. Wild-type kernels have a histologically recognizable aleurone layer at 12 DAP. It has been estimated that this stage corresponds to the 10th round of cell divisions and that approximately 17 rounds of anticlinal divisions contribute to the surface layer (Levy and Walbot, 1990). Single-celled revertant sectors of a *dek1* mutant show that it is possible for individual cells to acquire aleurone fate after the last cell division. Conversely, sectors of *Dek1* loss show that *Dek1* is necessary to maintain aleurone cell identity because surface cells in very late sectors differentiate as starchy endosperm. Because at 12 DAP aleurone cells are apparent in normal kernels while *dek1* mutant endosperms contain cells with starchy endosperm identity in the surface layer, cells in both types of sectors have switched fates (undergone transdetermination). These results indicate that the initial cytokinesis per se is not the determining event in aleurone cell fate acquisition, but that cell identity is continually specified and maintained by positional cues throughout late endosperm development.

The aleurone layer has also been shown to contribute to internal endosperm cells in wheat (Morrison et al., 1975). Following a periclinal division, the internal daughter loses aleurone grains and cell wall autofluorescence and begins to acquire characteristics of a starchy endosperm cell. Thus, the

mechanisms that specify and maintain aleurone cell identity do so in a position-dependent manner, acting only on the surface cell layer. This gradual dedifferentiation and switching of cell identity is likely to account for the internal aleurone cells observed in the lineage study presented here. Endosperm development probably arrested shortly after the periclinal aleurone division, before the internal daughter cell had the opportunity to fully dedifferentiate and redifferentiate as a starchy endosperm cell. A sector comprising 4 cells, 2 on the surface and 2 subsurface, demonstrated that a periclinal division occurred within the last 2 divisions. The retention of aleurone cell characteristics at the time of developmental arrest suggests the periclinal division might have occurred in the last cell division.

Sectors greater than 25 cells all contained marked subjacent endosperm. Casual observations indicate that the sectors penetrated various depths into the internal endosperm suggesting that periclinal divisions are not restricted to particular times in endosperm development. Rather our data and observations, combined with previous studies (McClintock, 1978; Morrison et al., 1975) are consistent with periclinal divisions occurring stochastically throughout the cellular phase of endosperm development.

Several factors are likely to have contributed to the misconception that the aleurone only divided anticlinally. A preponderance of anticlinal divisions in the aleurone is necessary due to the physical constraints of covering a volume of large cells with a surface layer of small cells. Periclinal divisions contribute to the radial dimension of the endosperm while anticlinal divisions contribute to increased cell number on the surface. Thus, anticlinal divisions must accommodate a surface area that increases as the square of the radial increase produced by periclinal divisions. In addition, periclinal divisions can occur in either the peripheral starchy endosperm or the aleurone, while surface increases occur only by anticlinal divisions in the aleurone. Furthermore, starchy endosperm cells are relatively large, (>50 µm in diameter), while aleurone cells are small (approximately 25 µm in diameter), compounding the relative number of anticlinal divisions required to keep pace with periclinal divisions. Our observation that smaller aleurone sectors often are independent of starchy endosperm cells is consistent with this notion.

The continual monitoring of positional cues throughout endosperm development is consistent with aspects of a model of aleurone differentiation that has been presented by Olsen et al. (1998). According to this model, the CR4 receptor kinase is involved in perceiving a signal at the endosperm surface. A gradient in the signal would occur at the surface and cells receiving a high concentration of this morphogen would respond by forming aleurone. Differences in the slope of the concentration gradient or in the sensitivity of the endosperm cells to the signal would account for the variable number of aleurone layers in different cereal species. The differentiation of starchy endosperm cells that are derived from periclinal divisions in the aleurone would occur because the cells displaced internally would move out of the critical range of morphogen concentration.

A genetic hierarchy controlling aleurone differentiation

Examination of mutants that cause aleurone mosaicism

revealed that aleurone differentiation was disrupted at different characteristic stages of development. Thus aleurone differentiation appears to be genetically regulated at several levels, suggesting that a regulatory hierarchy controls this process. We have assigned tentative positions to the mutants within this putative hierarchy, however, further genetic analysis is required to test epistatic relationships among these genes.

The first event is the perception of position. Perception of surface position leads to the decision to acquire aleurone cell identity. The *dek1* and *cr4* mutants alter the fate of aleurone cells to starchy endosperm indicating that these genes are required for either the perception of surface position or for the interpretation of this information. It seems unlikely that either of these genes acts to specify position. As a receptor-like kinase, the function of CR4 would more likely be in the perception of positional cues. Both loss and reversion of *dek1* can produce individual cells of one cell type in a field of the opposite cell type suggesting that *Dek1* acts cell autonomously to regulate aleurone fate. Positional information seems more likely to be provided by a diffusible signal, perhaps from the pericarp or degenerating nucellus, which could serve as a reference point for surface position.

Following the perception of position and the specification of aleurone cell fate, a set of genes function in the differentiation of the cellular characteristics of aleurone. Cells on the surface of *nkd** mutant endosperms do not show the phenotype of starchy endosperm cells indicating that the cells have acquired a different fate. However, the cells show few attributes of aleurone demonstrating that the gene(s) act at an early point in aleurone cell differentiation. The defects in surface cells of *cp2-o12* mutants generally show a greater degree of aleurone differentiation suggesting that this gene acts at a later step than *nkd**. Some aleurone cells appear to have degenerated (not shown) suggesting that *cp2* may be needed to maintain the integrity of aleurone cells. *cp2* mutants also show defects in starchy endosperm cells, including oversized starch grains, indicating that the gene functions in a general aspect of endosperm development or in more than one process.

Msc1 and *Dap1* produce aleurone layers with relatively

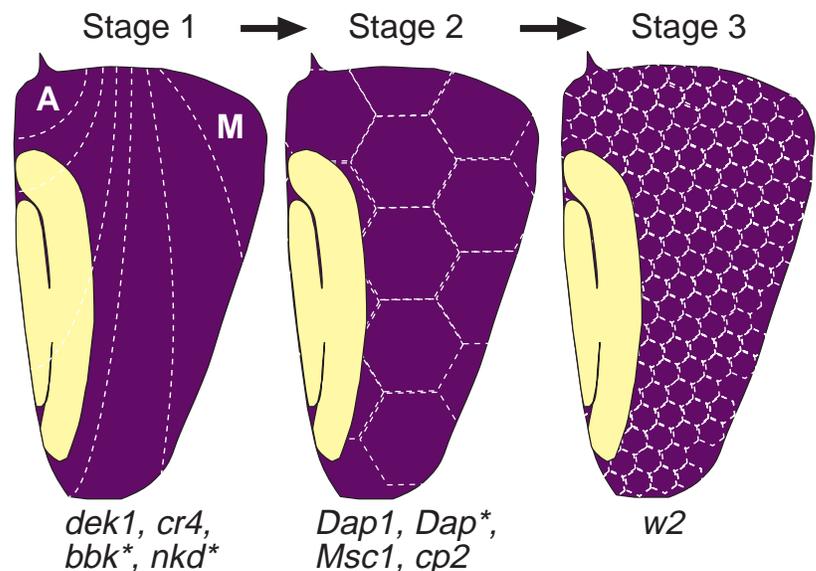
minor defects in aleurone cell morphology and organization suggesting that a late step in aleurone differentiation is blocked. Occasional starch-containing cells in the aleurone layer indicate that one aspect of aleurone differentiation that is compromised in these mutants is the repression of starch biosynthesis. These mutants appear similar to other *Dap* mutants that alter aleurone cell morphology and inhibit anthocyanin gene expression (Gavazzi et al., 1997). The genetic map location indicates that these mutants represent different loci. Because these mutants are dominant, it is difficult to know whether the wild-type alleles of these genes represent normal components of aleurone differentiation. However, even if they are neomorphic, the mutants identify a genetically controlled step because they disrupt aleurone development at a particular stage. The *w2* mutant shows only mild defects. Histologically, the aleurone looks nearly normal suggesting an even later step is affected. Similarly, the aleurone in *pfc** mutants shows only minor defects. Thus these mutants appear to act late in aleurone differentiation.

Pattern elements revealed by mosaic aleurone mutants

The mosaic aleurone mutants described above each display characteristic patterns of mosaicism that fall into a small number of discrete classes. That mutations in several genes produce similar patterns suggests that these patterns reflect pattern-forming events in endosperm development. We propose a model where these genes fit into a genetic hierarchy and either control or respond to pattern forming events in endosperm development (Fig. 6).

Strong expression of the *dek1*, *bbk** or *nkd** mutants can completely block aleurone differentiation over the entire endosperm surface. Weaker expression of these mutants produce kernels with a distinct pattern of aleurone mosaicism similar to that reported for *cr4* (Becraft et al., 1996); the abgerminal region of the crown shows a strong tendency for showing disrupted aleurone, while surrounding the silk scar, aleurone differentiation tends to remain intact. In intermediate phenotypes, aleurone tissue spreads to cover the germinal face

Fig. 6. Model of endosperm pattern formation. Mutants affecting early steps in aleurone development, including *cr4*, *dek1* and *nkd*, show patterns of aleurone mosaicism that reflect the germinal-abgerminal organization of the kernel. The region surrounding the silk scar region of the kernel, denoted A, is the area most likely to form aleurone cells in strongly mutant kernels. The abgerminal region of the crown, denoted M, is the region most likely to show mosaicism due to aleurone defects in weakly mutant kernels. The dotted lines represent a progression from strongly to weakly mosaic phenotypes. In the second stage, cells are organized into relatively large fields where developmental activity is coordinated. These fields are distributed evenly around the endosperm. Mutations in *Dap1*, *Msc1* and *cp2* reveal this level of organization. In later stages, the fields have diminished in size, or the larger fields from stage 2 have been subdivided into smaller fields, as illustrated by *w2* mutants.



and then progresses to the abgerminal face (Fig. 6). One mutant showed a phenotype that was nearly the reciprocal of the *cr4* class; the aleurone on the abgerminal face of the kernel was normal while the region surrounding the embryo showed defects. Yet another mutant, *cyn**, occasionally shows a concentric pattern of mosaicism, centered around the silk scar.

Other mutants show phenotypes that are evenly distributed on the kernel. *Dap1*, *Msc1* and *cp2-ol2* all show similar phenotypes of large, block-like patches of pigmented aleurone separated by fissure-like regions of defective aleurone. The overall appearance is reminiscent of a mud crack pattern. The *w2* mutant shows much smaller patches of pigmentation.

These patterns do not correspond to clonal patterns of endosperm development which first tend to divide the kernel into right and left halves, then into quarters, finally ending up in cone shaped sectors emanating from the center of the endosperm out to the surface (McClintock, 1978). Therefore these patterns are likely to reflect processes that function to organize and coordinate endosperm development. Other non-clonal patterns have been reported that support the idea that the endosperm contains several regulatory domains. *R-nj* specifically controls anthocyanin pigmentation in the crown of the kernel (Coe et al., 1988), while several modifiers of *opaque2* specifically affect the crown or subcrown regions (Lopes and Larkins, 1991). A concentric pattern, similar to *cyn** was reported for clonal sectors of *Ac* activity (McClintock, 1978), thus it is possible that this particular case has a clonal basis.

It is significant that mutants that appear to act early in aleurone development show the pattern reflecting the germ-abgerminal organization while genes that act later show the evenly distributed blocky patterns. This suggests that genes acting early respond to or regulate pattern formation events that determine the regional organization of the endosperm. In double mutants of *cr4* or *dek1-D* with *wem*, where the embryo formed on ectopic faces of the kernel, the pattern of mosaicism did not reflect the altered embryo position. This indicates that the embryo does not provide the positional cues to establish this pattern. Because the patterns appear to center around the silk scar, it is possible that the maternal tissues surrounding the endosperm provide the positional information. The precise tissue(s) that might be the source of this positional information cannot be ascertained at this time.

An exception to the rule that early genes reflect the germ-abgerminal pattern is the *pf^c** mutant which affects late aleurone differentiation, only on the germ face of the kernel. Perhaps this phenotype does not respond to the same cues as the *cr4* class of mutants; double mutants with *wem* have not yet been tested. The patterns of later genes such as *Dap1* and *cp2* suggest that at later stages, the endosperm is a mosaic of developmental fields. These genes may control or respond to mechanisms that coordinate the activity of these developmental fields. The *w2* mutant shows a smaller mosaic pattern and acts later in development suggesting that either the size of the developmental fields shrinks late in development or that another tier of sub-fields functions at these later stages.

Keying in on anthocyanin production as a visible marker for aleurone differentiation allows ready screening for mutants and has revealed several key aspects of aleurone differentiation. However, this approach monitors only one aspect of aleurone cells and it is likely that regulatory pathways branch to control

various steps in aleurone differentiation. Furthermore, mutants with increased numbers of aleurone layers would not be identified and such variants are known in maize (Wolf et al., 1972). To identify every gene involved in aleurone development will require more laborious mutant screens or genomics approaches.

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