The maize *dek1* gene functions in embryonic pattern formation and cell fate specification

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SUMMARY

Mutants in the maize *defective kernel1* (*dek1*) gene are blocked in embryogenesis and the endosperm is chalky and lacks an aleurone layer. Here we show that intermediate alleles result in embryos that lack a shoot axis while weak alleles result in endosperms with mosaic aleurone and deformed plants with epidermal cells that resemble bulliform cells, a specialized epidermal cell type. This indicates that *dek1* functions in embryonic pattern formation, cell fate specification and pattern formation in the leaf epidermis, and cell fate specification in the endosperm. Thus, the *dek1* gene product appears to control different cellular-developmental processes depending on cellular context. The phenotype of the weak *dek1-Dooner* allele resembles the *crinkly4* (*cr4*) mutant phenotype.

Double mutants between *dek1* and *cr4* showed elements of epistasis, additivity and synergy, suggesting that the gene products may function in overlapping developmental processes. *cr4* transcript was detectable in *dek1* mutant kernels indicating that DEK1 was not required for *Cr4* transcript accumulation. To test whether DEK1 regulated the ligand for the CR4 receptor kinase, a genetic mosaic analysis was performed. The dek1 phenotype appeared to be generally cell-autonomous, leading to the conclusion that it was not likely to produce a diffusible signal molecule, and therefore was not likely to regulate the CR4 ligand.

Key words: Maize, Endosperm, Aleurone, Embryo, Epidermis, Cell fate

INTRODUCTION

The majority of nutritional storage in cereal grains occurs in the extraembryonic tissue of the endosperm. The endosperm is an organizationally simple structure containing 3 major cell types. The central starchy endosperm tissue constitutes the bulk of the endosperm and serves as the major site of starch and storage protein deposition. The basal endosperm transfer layer is adjacent to the pedicel and transports maternal nutrients into the endosperm. The aleurone layer occurs in the surface 1-3 cells of the endosperm. While serving as a storage site for some compounds, the major function of the aleurone is as a digestive tissue. At germination, the aleurone secretes hydrolytic enzymes that break down the storage products of the starchy endosperm into free sugars and amino acids that are then available for the growing seedling. Several other regions of the endosperm can be recognized by localized patterns of gene expression (reviewed by Becraft, 2001; Becraft et al., 2001a; Olsen, 2001; Olsen et al., 1999).

Despite the apparent simplicity of the mature tissue, endosperm development is complex and shows several unique innovations in the regulation of cell cycle, cytokinesis and cytoskeletal functions (reviewed by Becraft, 2001; Becraft et al., 2001a; Olsen, 2001; Olsen et al., 1999). Endosperm development is surprisingly plastic, with aleurone cell fate decisions occurring dynamically throughout the course of

kernel development (Becraft and Asuncion-Crabb, 2000). Positional cues are required throughout endosperm development to specify and maintain aleurone cell identity, and starchy endosperm cells in the peripheral region remain competent to respond to these cues.

The *crinkly4* (*cr4*) gene is important for the aleurone cell fate decision because mutations in this gene disrupt aleurone development. In regions where aleurone fails to differentiate, the peripheral cell layer of the endosperm is composed of starchy endosperm cells. This cell fate switch indicates that $Cr4^+$ is important for the aleurone cell fate decision (Becraft and Asuncion-Crabb, 2000; Becraft et al., 1996; Jin et al., 2000). The *cr4* gene encodes a receptor-like kinase (Becraft et al., 1996) suggesting that CR4 might function in the perception of the positional cues that specify aleurone cell identity. As such, understanding the CR4 signal transduction system will be essential for understanding cell fate acquisition in endosperm development.

Maize *dek1* mutants lack aleurone cells, demonstrating that $Dek1^+$ is also critical for aleurone development (Becraft and Asuncion-Crabb, 2000; Lid et al., 2002). Late reversion of the transposon-induced *dek1-PIA* mutant allele allows peripheral starchy endosperm cells to switch fate to aleurone, while late loss of $Dek1^+$ gene function causes aleurone cells to transdifferentiate to starchy endosperm (Becraft and Asuncion-Crabb, 2000). Thus, the $Dek1^+$ gene product is required in

endosperm cells for the perception and/or response to the positional cues that specify aleurone identity. The weak *dek1-Dooner* (*dek1-D*) allele shows a mosaic aleurone, with the pattern of mosaicism reflecting the germinal-abgerminal polarity that is typical of mutants, including *cr4*, that act early in aleurone differentiation (Fig. 1) (Becraft and Asuncion-Crabb, 2000; Becraft et al., 1996).

Dek1 encodes a large protein of 2,159 amino acid residues (Lid et al., 2002). It is predicted to contain an N-terminal integral membrane domain with 21 membrane-spanning regions. The cytoplasmic carboxyl terminus is similar to the calcium-dependent cysteine protease, calpain. The phenotypic similarities between *cr4* and *dek1* mutants suggest that DEK1 might act in the CR4 signal transduction pathway. How these molecules may function within the context of a pathway is not yet clear. The *dek1* mutant phenotype was suggested to be non cell-autonomous (Neuffer, 1994; Neuffer, 1995) which is the expected property for a diffusible signal. The molecular identity of DEK1 makes it unlikely to function as a diffusible ligand for the CR4 receptor (i.e. the positional cue for aleurone cell fate), however, the possibility that DEK1 regulates production of the CR4 ligand remained open. Here we present a genetic analysis of *dek1*, testing this hypothesis. The phenotypes of *dek1* mutants were examined in detail, cell-autonomy was tested with genetic mosaics, and double mutants with *cr4* were analyzed. We conclude that DEK1 is a good candidate for a component of the CR4 signal transduction system but that it is not likely to function in the production of a diffusible ligand.

MATERIALS AND METHODS

Histology

Standard histological processing was followed as described previously (Berlyn and Miksche, 1976). Briefly, tissue was fixed for 1 hour under vacuum in 100 mM cacodylate buffer, pH 7.2, 2% paraformaldehyde, 3% glutaraldehyde, dehydrated through an ethanol series, embedded in LR white resin and 1 µm thick sections cut with a glass knife. Sections were affixed to glass microscope slides, stained with Toluidine Blue O and mounted with Permount™. Alternatively, tissue was fixed in FAA (5% formalin, 5% acetic acid, 45% ethanol), embedded in Paraplast Plus and 10 µm sections cut. Sections were mounted on slides, stained with Toluidine Blue and examined. Slides were observed with bright-field microscopy using an Olympus BX-60 microscope.

Scanning electron microscopy (SEM)

Tissue was fixed overnight in FAA, dehydrated through an ethanol series and critical point dried. Samples were sputter coated with palladium in a Denton sputter coater and examined with a Jeol 5800LV SEM operating at 10 kV accelerating voltage. Images were digitally recorded.

Genetic mosaic analysis

Fig. 3A shows the general strategy for generating genetic mosaics. The mutant *dek1-792* allele was marked by the linked *vp5* mutation, which confers carotenoid deficiency, causing a white endosperm and albino leaves because of chlorophyll photobleaching. Chromosome breakage results in the loss of the homologous arm carrying the wild-type alleles of both loci. This uncovers the mutant alleles and the cells derived from such an event form an albino, *dek1* mutant sector in an otherwise normal plant. Two methods were used to induce chromosome breakage. In the presence of an *Ac* element, the chromosome-breaking *Ds* element, present on chromosome *1S* in the Ds1S4 stocks, causes chromosome breakage through an aberrant transposition (Weil and Wessler, 1993). Using the *Ds*-induced breakage, every plant showed sectors. Leaves from approximately 70 plants were examined for infrequent sectoring. Leaves from 23 plants were used for the analysis. Alternatively, γ-rays were used to induce chromosome breakage in double heterozygotes. Seeds were irradiated at the University of Iowa Radiation Laboratory (Iowa City, IA) with a ^{137}Cs rod source. Bags were laid flat beneath the source so the seed formed a single layer. The seed received a dose of 20 gray over a period of 20 minutes. Approximately 800 seeds were irradiated and 14 sectors obtained.

Sectors were examined by observing fresh hand-cut sections with an Olympus BX-60 fluorescence microscope equipped with a narrow violet filter (excitation 400-410 nm, dichroic mirror and barrier filter, 455 nm). Wild-type cells can be identified by the red fluorescence of chlorophyll, which is absent in albino mutant cells. The epidermal genotype is inferred from the guard cells, the only epidermal cells to contain chloroplasts. All photomicrography was performed with an Olympus PM-20 photography system using Ektachrome 160T film (Kodak).

Reverse transcription-polymerase chain reaction (RT-PCR)

Endosperm tissues of W22 wild-type and *dek1* mutant kernels were collected 12 to 24 days after pollination (DAP). Samples were pooled for each genotype and total RNA extracted with the 'hot-phenol' method. Briefly, tissue was ground in liquid nitrogen and suspended at 1 weight:2 volumes in 1:1 phenol:buffer (0.1 M LiCl, 0.01 M EDTA, 1% SDS, 0.1 M Tris pH 9.0) at 80°C. After 30 minutes incubation at room temperature, the mixture was centrifuged and the RNA precipitated from the supernatant with 2 M LiCl. The pellet was washed in 2 M LiCl, then ethanol, air dried and dissolved in water. mRNA was purified from the total RNA using PolyATract mRNA isolation systems (Promega, Madison, WI, USA). First strand cDNA was synthesized from 2.0 µg mRNA with AMV reverse transcriptase and Oligo(dT) (T-17) primers at 42°C for 1.5 hours. PCR was performed on 1 µl of first strand reaction products using Taq DNA polymerase (Promega), and *cr4* gene-specific primers CR2007 (5′-GGGAATTGAGTACTTGCATGG-3′) and CR2792 (5′-AGTCCG-TCACCTATGCTGCT-3′). Twenty-six cycles of PCR were conducted with denaturation at 92°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 2 minutes. Seven µl of each PCR reaction was analyzed on 1% agarose gel.

RESULTS

Dek1⁺ is required for aleurone cell fate in the endosperm

Mutations in the *dek1* gene behave as single recessive Mendelian factors. As shown in Table 1, 12 mutant alleles were examined. All but the *dek1-Dooner* (*dek1-D*) and *dek1-928A* alleles showed very similar endosperm phenotypes. Mutant kernels are small, white from apparent carotenoid deficiency and opaque with a floury texture. In a genetic background that confers anthocyanin pigmentation to the aleurone, *dek1* kernels are unpigmented because of a lack of an aleurone cell layer (Fig. 1) (Becraft and Asuncion-Crabb, 2000; Lid et al., 2002). The aleurone becomes histologically distinguishable at approximately 10-12 DAP in wild-type kernels (Becraft and Asuncion-Crabb, 2000). In mutant kernels, the peripheral cell layer of the endosperm shows characteristics of starchy endosperm cells from this stage onward (Fig. 1D,E). Thus *dek1* mutations cause a cell fate switch, indicating that the normal DEK1 gene product is required for aleurone cell fate

Table 1. Mutant *dek1* alleles

Allele	Strength	Mutagen	Source
34	Strong	*Mu*	Becraft
*DR85	Strong	*Mu*	Robertson[†]
*DR86	Strong	*Mu*	Robertson
PIA	Strong	*Mu*	(Scanlon et al., 1994)
1348	Strong	EMS	Neuffer[‡]
1394	Strong	EMS	Neuffer
1401	Strong	EMS	Neuffer
792	Medium	EMS	Neuffer
928A	Weak	EMS	Neuffer
971	Strong	EMS	Neuffer
388	Strong	EMS	Becraft
D	Weak	Spontaneous	Dooner[§]

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[‡]M. Gerald Neuffer, University of Missouri.
[§]Hugo Dooner, Waksman Institute, Rutgers University.

Fig. 1. Phenotypes of *dek1* mutant kernels. (A) *dek1-388* mutant kernel. (B) *dek1-D* mutant kernel. (C) Medial cut through a wild-type (left) and *dek1-1394* mutant (right) kernel. (D) Wild-type endosperm and aleurone. (E) *dek1-792* mutant endosperm. (F,G) Section of a wild-type embryo and endosperm at (F)7 DAP and (G) 12 DAP. (H,I) Section of a *dek1-792* embryo and endosperm at (H) 7 DAP and (I) at 12 DAP. Black arrows indicate embryos; white arrows designate transfer cells. Scale bars: 100 μm (D,E); 200 μm (F,H); 500 μm (G,I).

specification. Occasionally an endosperm cell with partial aleurone morphology was observed on the periphery of the endosperm but rarely is anthocyanin pigmentation observed in any of the stronger alleles. The effect on endosperm cell fate appears specific to aleurone cells because the basal transfer layer develops in mutant kernels (Fig. 1F,H).

The *dek1-D* and *dek1-928A* alleles are leaky, sometimes causing complete loss of the aleurone layer but often causing only partial loss of the aleurone layer. Partial loss causes mosaic aleurone development, with some regions showing a nearly normal aleurone layer and others completely lacking aleurone cells (Fig. 1B). The pattern of mosaicism is non-random with the region surrounding the silk scar being most likely to form an aleurone layer and the abgerminal crown region most likely to lack aleurone (Becraft and Asuncion-Crabb, 2000). There is also variability in other aspects of the endosperm phenotype with color ranging from normal yellow to nearly white and texture ranging from normal vitreous to opaque and floury. The severities of these different aspects of the endosperm phenotype are correlated.

Dek1 is required for axial pattern formation in embryogenesis

Mutations in *dek1* have dramatic effects on embryogenesis. Mutant kernels often appear germless as a result of the early arrest of embryo development. Early embryo development up to the globular (proembryo) stage appears normal. During transition, when normal embryos begin to elongate, growth of strong *dek1* mutants becomes disorganized. In strong *dek1* mutants (Table 1), only a limited amount of growth continues beyond the globular stage and mature kernels contain a small necrotic mass. Embryos carrying the intermediate *dek1-792* allele develop into spherical bodies, often with a root primordium (Fig. 1) (Lid et al., 2002; Neuffer et al., 1997). Sometimes the root can grow in germinated seeds (not shown). Shoot primordia do not form and there is no scutellar structure. The mutant phenotype in these seeds also first appears during the transition stage of embryogenesis. In our greenhouse grown material, this occurred at approximately 6DAP, which is considerably earlier than in most published descriptions (Abbe and Stein, 1954). When normal embryos elongate from the globular (proembryo) stage, mutant embryos undergo symmetrical growth. By 7 DAP, normal embryos had reached
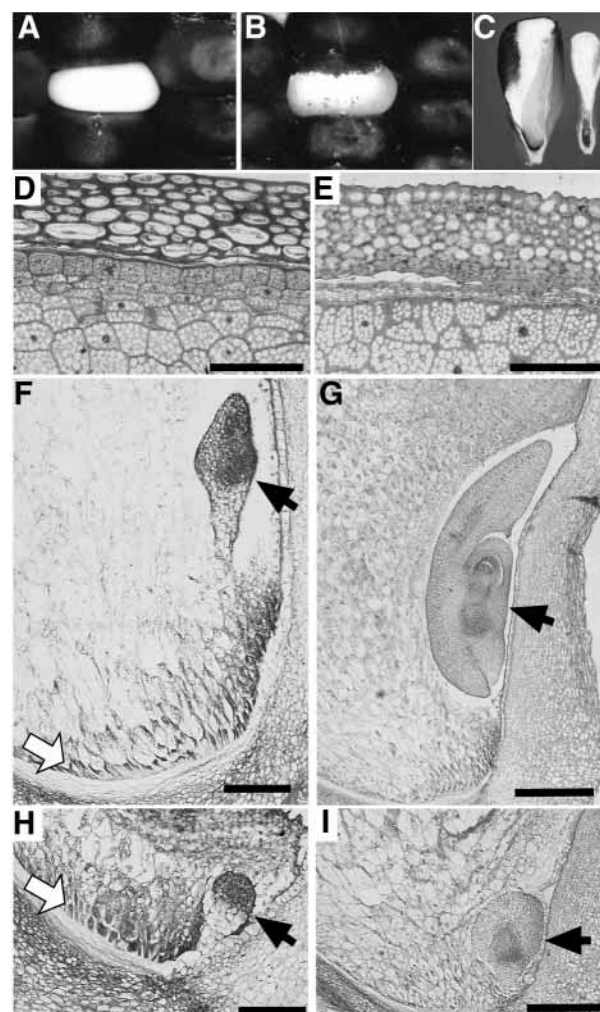
coleoptilar stage (Fig. 1F), while mutant embryos from the same ear were much smaller and the embryo proper was spherical in shape (Fig. 1H). No evidence of cell death was observed in mutant embryos, suggesting that the defects are caused by disrupted pattern formation rather than death of apical cells. By 12 DAP, normal embryos had established a shoot apical meristem and initiated 2-3 leaves (Fig. 1G). A scutellum was present and the root primordium well organized. Mutant embryos showed a center of meristematic activity at the site of root primordium initiation but no indication of a shoot or scutellum (Fig. 1I). These characters persisted throughout seed maturation (not shown).

The weak *dek1-D* allele alters cell fate in the leaf epidermis

In *dek1-D* mutants there is a range of effects on embryogenesis,

from the small spherical clusters of cells seen in stronger alleles, to nearly normal embryos. Kernels with well-formed embryos can produce a viable, albeit abnormal, plant. *dek1-D* plants have crinkled leaves, shortened internodes and nodes that bend alternately back and forth (Fig. 2A). Inflorescences commonly show a barren region of rachis midway along their length, with florets formed at the proximal and distal ends (Fig. 2B).

Normal maize leaves contain a variety of epidermal cell types. Bulliform cells form distinct files approximately 4 cells wide on the adaxial surface of adult leaves (Fig. 2) (reviewed by Becraft, 1999). Bulliform rows are typically bordered by prickle hairs and contain periodic macrohairs within the rows. Ground cells of the adult leaf epidermis are rectangular with a smooth surface and crenulations that interlock adjoining cells along the lateral walls (Fig. 2I). In transverse section, they appear cuboidal with a thick outer wall and stain turquoise with Toluidine Blue (Evans et al., 1994). Bulliform cells show a reticulate pattern of ridges on their surfaces (Fig. 2G). In section, they appear bulbous and are approximately 2.5 to 4 times as thick as most epidermal ground cells. Bulliform cells stain deep purple-blue with Toluidine Blue. Occasionally, angular interlocking walls among neighboring bulliform cells are observed (Fig. 2C).

In the *dek1-D* mutants, epidermal cells on both sides of the leaf are intermediate in size and shape between epidermal ground cells and bulliform cells, when viewed in section. The cells stain dark purple-blue with Toluidine Blue, and show the angular junctions between cells similar to bulliform cells. Cells of the *dek1-D* epidermis have the reticulate ridges on their surfaces, like bulliform cells, when viewed by SEM (Fig. 2H,J). Macrohairs and prickle hairs do not always occur in discrete files (Fig. 2F). Stomata are misshapen and smaller than normal (Fig. 2I,J) but usually occur in files, similar to normal.

The effects of the *dek1-D* mutant are most pronounced in the epidermis but there are clear effects on internal cells too. Vascular bundles are often flattened or oblong in section (Fig. 2D). Mesophyll cells sometimes show an unusual degree of lobing and often appear elongated radially around the vascular bundles (not shown). Sometimes, multiple cell layers with epidermal cell characteristics are observed (not shown).

There appears to be an environmental influence on the phenotype of *dek1-D* mutant plants. In winter crops grown in Juana Diaz, Puerto Rico or in the greenhouse, the phenotype was ameliorated and performing pollinations with homozygotes was feasible. When the same lines were grown during the summer in Ames, Iowa, the phenotype was strong and it was rare to find a plant that produced functional anthers. The basis of these effects is unknown.

Genetic mosaic analysis of *Dek1* function

Aspects of the phenotype of *dek1* mutant sectors in endosperm tissue appeared non cell-autonomous (Neuffer, 1994; Neuffer, 1995). A genetic mosaic analysis was conducted to rigorously test the cell autonomy of *Dek1+* function. This also afforded the opportunity to examine the phenotype of strong *dek1* mutant leaf cells. *dek1* mutant sectors were generated in otherwise normal plants, as shown in Fig. 3 and described in Materials and Methods. The *dek1-792* allele was marked with
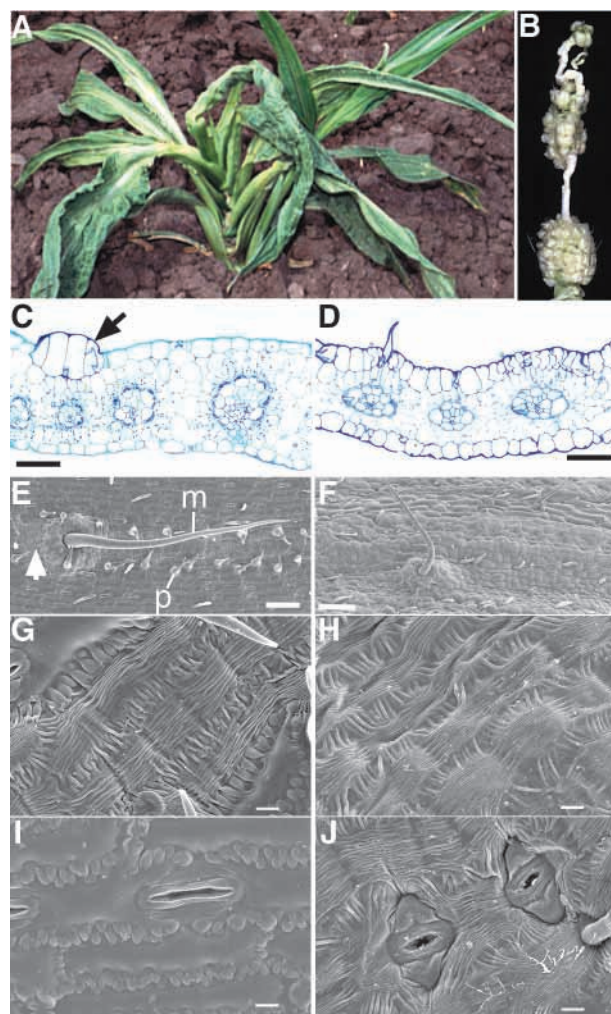

Fig. 2. The *dek1-D* mutant transforms leaf epidermal cells to bulliform cells. (A) *dek1-D* mutant plant. (B) Ear from a *dek1-D* mutant, showing a barren region in the middle of the inflorescence. (C,D) Cross sections of (C) wild-type leaf with bulliform cells (arrow) and (D) *dek1-D* mutant leaf. (E) SEM of a wild-type leaf showing a bulliform row (arrows). (F) SEM of *dek1-D*. (H) High magnification of bulliform cells on a wild-type leaf. (H) High magnification of *dek1-D* epidermal cells showing characteristics of bulliform cells. (I,J) High magnification of epidermal ground cells and a stoma in wild type (I) and *dek1-D* (J). Scale bars: 100 μm (C-F); 10 μm (G-J).

the linked *viviparous5* (*vp5*) mutation, which confers albinism due to carotenoid deficiency. Loss of the chromosome *1S* arm carrying the wild-type *Dek1+* and *Vp5+* alleles simultaneously uncovers the recessive mutant *dek1-792* and *vp5* alleles, resulting in a clone of albino *dek1* mutant cells in an otherwise normal leaf or endosperm. Endosperm sectors were not considered because the exact cellular boundaries were equivocal. In leaves, mesophyll sectors were albino and the cellular boundaries were ascertained by examining hand-sectioned fresh tissue by fluorescence microscopy. Albino cells lack red-fluorescing chloroplasts (Fig. 3B-D). It was also possible to determine sector boundaries by the presence or absence of chloroplasts in stained sections of fixed and embedded tissue (Fig. 3F). The epidermal genotype was

determined by examining guard cells, which are the only epidermal cell type to contain chloroplasts.

Sectored plants contained small protruding ridges in the epidermis (Neuffer, 1995). These are formed by files of distended cells (Fig. 3F-I) that are not seen on normal leaves. These cells have some of the attributes of bulliform cells: in transverse section they are large, bulbous cells with angular junctions between neighbors and stain deep purple-blue with Toluidine Blue. They have reticulate ridge-work on their surfaces, however the ridges are less pronounced than on normal bulliform cells. Fig. 3H shows a direct comparison of mutant cells (lower part of figure) and normal bulliform cells in the same leaf. Several sectors produced unusual cup-shaped structures on epidermal hairs (Fig. 3I).

The mutant phenotype of internal cells was less dramatic than that of the epidermis. The morphology of vascular elements and bundle sheath cells was indistinguishable from normal. Some mesophyll cells also had nearly normal morphology but in other cases the cells were highly lobed (Fig. 3E). The lobing was more extensive than that observed in *dek1-D* leaves.

In most cases, the mutant phenotype appeared cell-autonomous (Table 2); that is, genetically wild-type cells showed a normal phenotype and neighboring mutant cells showed a mutant phenotype. Fig. 3B shows a sector where only epidermal cells were mutant. The wild-type internal cells could not rescue the mutant epidermis. Fig. 3C,D shows a sector with a large area of mutant adaxial epidermis (upper surface), overlying wild-type internal cells, marked by red fluorescence. Again, the wild-type mesophyll did not rescue the mutant epidermis. It also appeared that wild-type epidermal cells cannot rescue adjacent mutant cells. The enlarged view in Fig. 3D shows the boundary between the wild-type and mutant epidermis on the abaxial side. A wild-type guard cell was directly adjacent to a cell showing the mutant phenotype. Fourteen such examples were observed. Fig. 3G shows a mutant cell surrounded on three sides by cells with normal phenotypes and Fig. 3H shows a normal cell surrounded on 3 sides by abnormal cells. In both cases the phenotypes show sharp cellular boundaries. In Fig. 3E, mutant mesophyll cells directly under a wild-type epidermis show a mutant phenotype indicating that the epidermis could not rescue mesophyll cells. Mutant mesophyll cells showed a mutant phenotype even when in direct contact with wild-type mesophyll or bundle sheath cells (Fig. 3F).
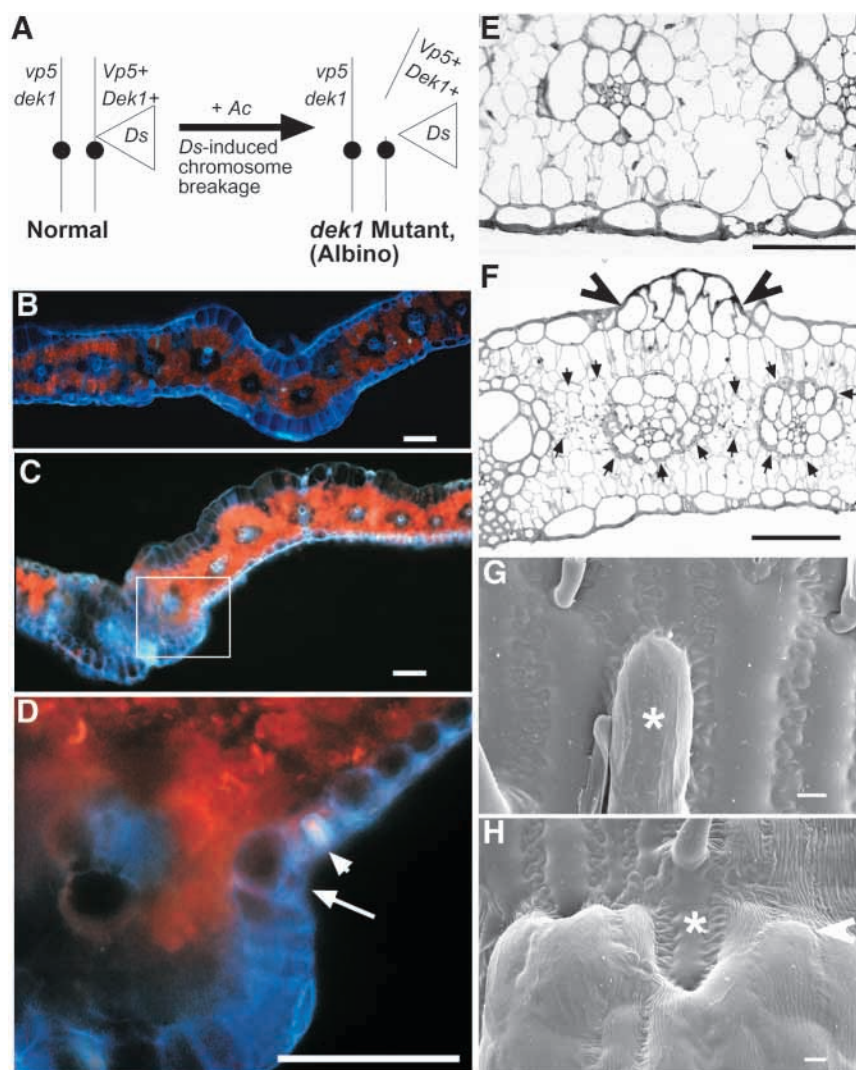
Several instances showed subtle evidence of

Fig. 3. Generation of *dek1* genetic mosaics and examples of an autonomous phenotype. (A) Diagram depicting the strategy for generating plants genetically mosaic for the *dek1-792* allele. (B) Fluorescence micrograph of a leaf with mutant epidermal cells over wild-type internal cells. The normal mesophyll cannot rescue the mutant phenotype in the epidermis. The sector caused the leaf to bend, highlighting the influence of the epidermis on leaf morphology. (C) Another fluorescence micrograph of a sector. In the mesophyll, lack of red-fluorescing chloroplasts indicates that the tissue is genetically mutant for *dek1*. The guard cells are the only epidermal cells to contain chloroplasts. (D) Enlargement of the boxed region shown in C. The red-fluorescing wild-type guard cell (arrowhead) is directly adjacent to an epidermal cell showing a clear mutant phenotype (arrow). (E) Mutant mesophyll cells in a section of leaf with wild-type epidermis. The cells show unusual lobing. (F) Section where the internal mesophyll and bundle sheath cells are wild type (arrows) and the outer mesophyll layer is mutant and shows a mutant phenotype. The epidermis is wild type except for the bulged region between the arrowheads on the adaxial (upper) surface. (G) A single epidermal cell with mutant phenotype (asterisk) surrounded on 3 sides by normal looking cells. The phenotype appears cell-autonomous. (H) A cell with wild-type phenotype (asterisk) surrounded on 3 sides by cells with mutant phenotypes. The arrowhead designates the boundary between abnormal and normal bulliform cells. (I) An example of a cupped hair. Scale bars 100 μm (B-D,F,I); 50 μm (E); 10 μm (G,H).

Table 2. Mosaic analysis of *dek1*

Sector	Autonomous	Non autonomous
Epidermal	108	8*; 4†
Mesophyll	52	0
Epidermis and mesophyll	44	1

*Non autonomy within the epidermis.
†Non autonomous effects of the epidermis on the mesophyll.

non cell-autonomy and in all cases it appeared that mutant cells influenced the phenotype of wild-type neighbors (Fig. 4). Eight examples were found where the surface ridge-work typical of mutant cells affected only part of a cell's surface. Fig. 4A,B shows an example where partially affected cells occur neighboring files of mutant cells. Fig. 4C,D shows an isolated wild-type mesophyll cell surrounded by mutant epidermis and mesophyll cells. The wild-type cell is larger than normal. In Fig. 4E, the wild-type mesophyll cells beneath a mutant epidermal sector show an elongated appearance similar to that described for *dek1-D* mutant leaves. One leaf formed an ectopic margin in the middle of the lamina (Fig. 5). It occurred in wild-type tissue just proximal (toward the midrib) to the mutant sector.

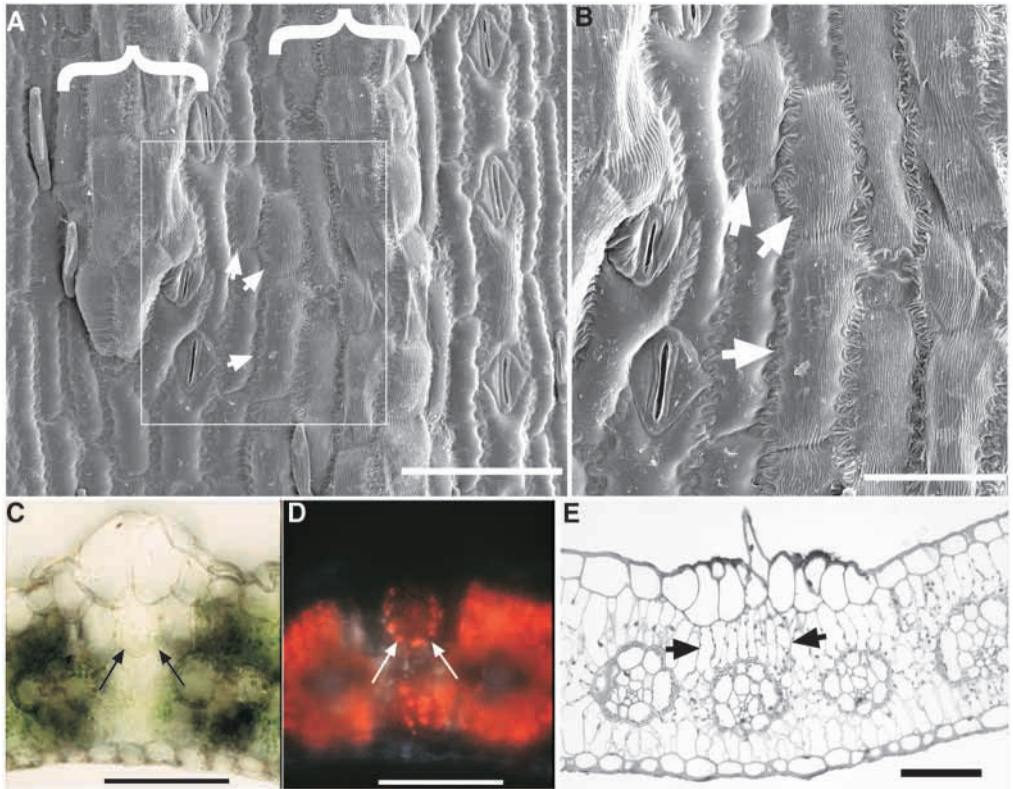### Genetic interactions between *dek1* and *cr4*

The phenotypic similarities between *dek1* and *cr4* mutants suggested that they might function in the same developmental process or pathway. To test this, double mutants were produced between *dek1* and *cr4* using all the EMS-induced *dek1* alleles, *dek1-D* and 5 EMS induced *cr4* alleles (*cr4-1231, -624, -647, -25, -651,*). These *cr4* mutants range from mild (*cr4-1231*) to strong (*cr4-651*) (Jin et al., 2000). All strong alleles of *dek1* were completely epistatic to *cr4*. The endosperm was white, chalky and lacked an aleurone layer, and the embryo developed according to the *dek1* allele.

The double mutants between

dek1-D and *cr4* were more complex. *cr4* mutants did not have any consistent effect on the *dek1-D* endosperm phenotype. No viable double mutant seedlings were produced with the stronger *cr4* alleles when seeds were germinated in sand; double mutant plants were only obtained between *dek1-D* and the weak *cr4-1231* allele. In the segregating family, both *cr4-1231* and *dek1-D* single mutants showed relatively mild phenotypes (Fig. 6A-C). Double mutant plants were highly contorted, similar to strong *cr4* mutants, although leaves were less adherent. Most epidermal cells showed the bulliform-like surface features of *dek1-D* (Fig. 6D) but were enlarged similar to strong *dek1-792* mutant cells in genetic mosaics (Fig. 6H,I). These cells did not show the irregular morphology of *cr4* mutants. A novel cellular phenotype of straight sidewalls lacking the interlocking crenulations between epidermal cells was observed in some areas (Fig. 6E). The surfaces on these cells showed less prominent ridges than on *dek1-D* single mutants or wild-type bulliform cells, being more similar to the surfaces of *dek1-792* epidermal cells. Scattered groups of epidermal cells showed a highly irregular morphology that was stronger than *cr4-1231* single mutants (Fig. 6F) and even unusual for strong *cr4* alleles (Jin et al., 2000). Double mutants also showed a greater propensity for apparent multiple-cell-layered epidermis than either single mutant in this line.

The epistasis of *dek1* to *cr4* suggested that *Dek1* might function upstream of *Cr4*. To test whether *Dek1+* is required for *Cr4* transcription, *dek1-792* mutant kernels were assayed for *Cr4* transcripts by RT-PCR. Fig. 6J shows that *Cr4*-specific primers amplify a fragment of the expected 785 base pair size from both wild-type and mutant endosperm, indicating that *Cr4* transcript is present in *dek1* mutant kernels. Therefore, *Dek1+* is not required for *Cr4* gene transcription.

Fig. 4. The mutant phenotype sometimes appears non cell-autonomous. (A) A pair of mutant sectors (brackets) with some cells between showing a mild mutant phenotype. The boxed region is shown in higher magnification in B. The arrows indicate cells partially affected by surface ridges typical of mutant cells. (C) Light and (D) fluorescence micrographs of a sector where a wild-type mesophyll cell is surrounded on 3 sides by mutant cells. The wild-type cell has a large spherical morphology not typical of normal mesophyll cells. (E) Wild-type mesophyll cells (between arrows) subjacent to a mutant epidermal sector showed a mutant phenotype. Size bars 100 μm (A,C,D); 50 μm (B,E).
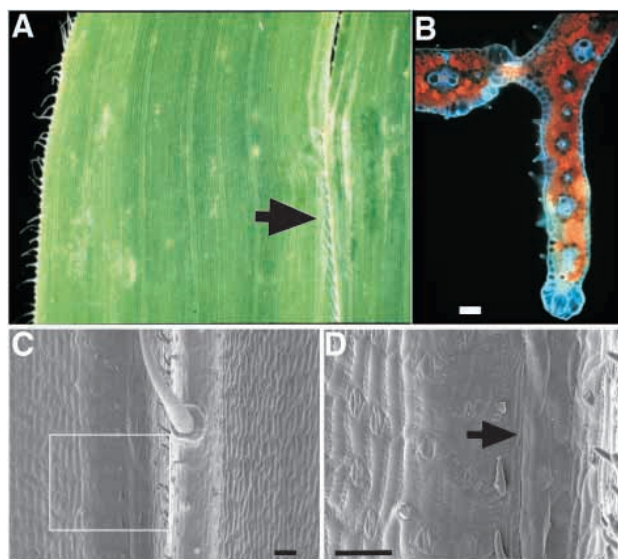
DISCUSSION

Dek1 is required for embryonic pattern formation and cell fate specification

The phenotypic examination of a series of *dek1* mutant alleles revealed that the gene is important for multiple developmental processes. There is a specific loss of aleurone cell fate in the endosperm. In the embryo, *Dek1* is essential for the establishment of axial pattern. *dek1* mutant embryos show disorganized growth, sometimes forming a root primordium but not a shoot. Elements of the embryo phenotype are reminiscent of several *Arabidopsis* mutants. The *gurke* mutant lacks an apical domain but forms a root (Torres-Ruiz et al., 1996). Several mutants involved in auxin transport or response are disrupted in axialization (Galweiler et al., 1998; Hardtke and Berleth, 1998; Steinmann et al., 1999). Whether the function of *dek1* is related to any of these genes remains to be determined.

The weak *dek1-D* mutants are capable of completing embryogenesis and producing a viable, albeit highly abnormal, plant. Most ground cells of the leaf epidermis possess characteristics of bulliform cells suggesting their fate may be altered. The patterns of other cell types such as trichomes are irregular. Mutant leaf epidermal cells in genetic mosaics of the stronger *dek1-792* allele appear highly distended but also show features of bulliform cells. Mutant mesophyll cells develop an unusual lobed morphology with no evidence for cell fate

Fig. 5. Ectopic margin associated with a mutant sector. (A) The margin formed on the proximal side of the sector. (B) Fluorescence micrograph of the ectopic margin. The midrib is toward the right of the figure and the normal margin toward the left. (C,D), Scanning electron micrographs of the ectopic margin and the neighboring mutant cells. The boxed region in C is shown magnified in D. The arrow indicates epidermal cells with a mutant phenotype on the distal side of the ectopic margin. Scale bars 50 μm (B), 100 μm (C,D).

Fig. 6. *dek1-D, crinkly4-1231* double mutant analysis. (A,B) Scanning electron micrographs of a typical *cr4-1231* single mutant. Arrows in A denote a bulliform row. The boxed area is enlarged in B. The arrows in B indicate cells with mild bulliform-like surface ridges. Note the aberrant cell shapes and the mismatched crenulations of neighboring cell sidewalls. (C) A *dek1-D* single mutant. Some cells appear normal, reflecting the leaky allele and mild expression. (D) A double mutant showing primarily the *dek1-D* phenotype. Most epidermal cells show the surface features of bulliform cells and lack the irregular morphology of *cr4*. (E) Over-expanded rectangular cells that lack interlocking crenulations along their sidewalls. (F) Highly irregular cells on a double mutant with a strong phenotype. (G) Cross section of a *cr4-1231* single mutant. (H) A typical cross section through a double mutant. (I) A cross section through a strong double mutant. The arrow highlights an example of apparent multiple epidermal cell layers. (J) RT-dependent PCR amplification of *Cr4* transcripts from wild-type and *dek1-792* mutant endosperm. Lane 1, *dek1-792* mutant endosperm, with reverse transcriptase (+RT); lane 2, wild-type endosperm (+RT); lane 3, *Cr4* cDNA plasmid template; lane 4, *dek1-792* RNA (–RT); lane 5, wild-type RNA (–RT). Scale bars 100 μm (A-D,G-I); 10 μm (E,F).
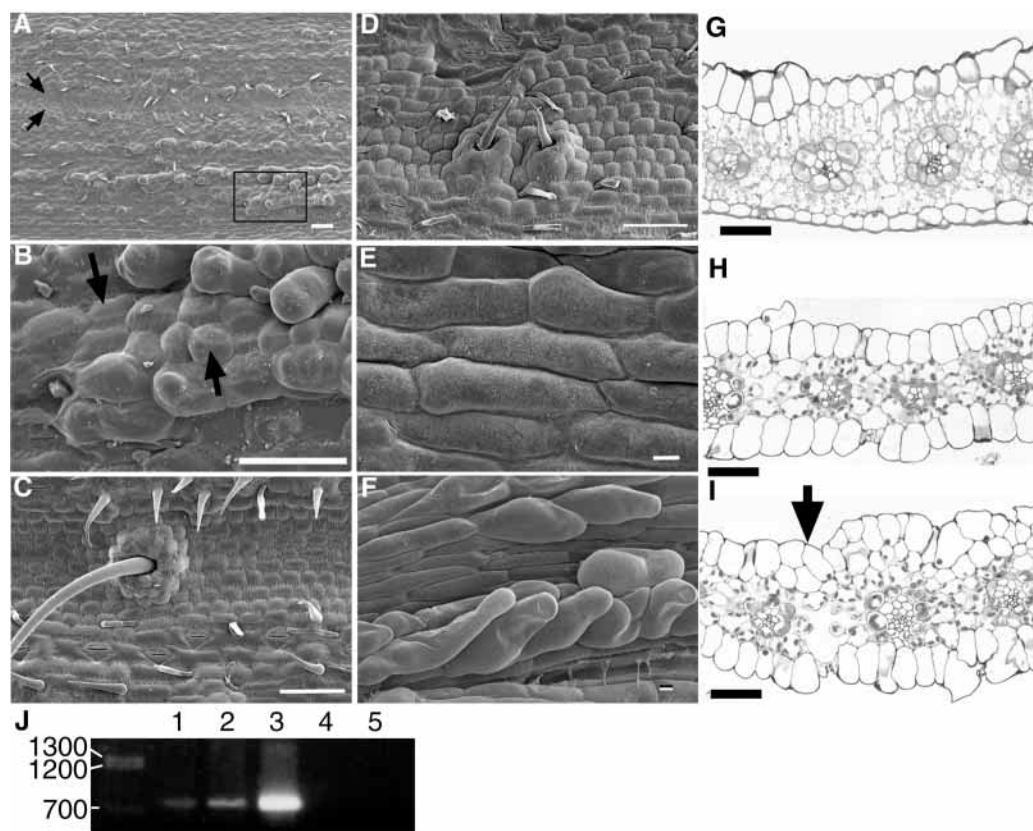
changes. Thus, *Dek1* appears to participate in different developmental processes in different contexts.

dek1 functions overlap with *cr4*

The phenotype of the weak *dek1-D* allele is similar to *cr4* mutants. Both change the fate of peripheral endosperm cells from aleurone to starchy endosperm (Becraft and Asuncion-Crabb, 2000; Becraft et al., 1996). In both cases, this effect is seen in a mosaic pattern with the germinal face of the kernel most likely to produce aleurone. The prevalence of this pattern in several mutant genes suggests that it reflects pattern-forming information in endosperm development (Becraft and Asuncion-Crabb, 2000). *dek1-D* and *cr4* mutant plants also have similar but distinct phenotypes with shortened internodes and crinkled leaves (Fig. 1) (Becraft et al., 1996; Jin et al., 2000). Both affect the epidermis more strongly than mesophyll (Figs 3, 4) (Becraft et al., 1996; Becraft et al., 2001b; Jin et al., 2000). Epidermal cells are enlarged and sometimes the epidermis appears more than one cell thick. *cr4* mutant epidermal cells also often show characteristics including surface ridges (Fig. 6A,B) (Jin et al., 2000) that could indicate a partial transformation to bulliform cell fate.

dek1 was completely epistatic to *cr4* in double mutant endosperms, consistent with both genes functioning in the same developmental process. In plants, the double mutant phenotype was more difficult to interpret. Only plants double mutant for *dek1-D* and the weak *cr4-1231* allele were recovered, suggesting that stronger alleles of either gene confer synthetic lethality. At the cellular level, some aspects of the phenotype appeared epistatic, while others appeared additive or synergistic.

Both *dek1* and *cr4* appear to perform different developmental functions, depending on cellular context (this study) (Jin et al., 2000). Considering the similar, but distinct mutant phenotypes and the results of the double mutant analysis, it is likely that the *Dek1* and *Cr4* gene products function in overlapping regulatory systems. How the membrane-localized calpain-like DEK1 protein (Lid et al., 2002) and the CR4 receptor kinase (Becraft et al., 1996) molecules might fit into a pathway is not yet known. *Dek1* is not required for *Cr4* transcript accumulation. CR4 could regulate DEK1 because in animal systems, calpain activity can be regulated by phosphorylation of either calpain or its substrate (Nicolas et al., 2002; Shiraha et al., 2002). Alternatively, DEK1 could regulate CR4 or a component of CR4 signal transduction system through proteolytic processing. Proteolytic steps appear important for other RLK signaling systems. The BRS1 carboxy peptidase seems to function upstream of the *Arabidopsis* BRI1 brassinolide receptor kinase through an unknown mechanism (Li et al., 2001).

Dek1 function is primarily cell-autonomous

With the similarity in mutant phenotypes, a report that *Dek1* appeared non cell-autonomous (Neuffer, 1994; Neuffer, 1995) suggested that DEK1 could be a diffusible signal ligand for the CR4 receptor kinase, or could regulate the production of such a signal. The recent identification of DEK1 as a large membrane protein decreases the likelihood that it would act as a ligand, although such mechanisms exist in animal systems. For example, in *Drosophila* eye development, Boss is a 7-transmembrane

protein that functions non cell-autonomously in the R8 cell to activate the Sevenless receptor tyrosine kinase in the neighboring R7 precursor cell, triggering differentiation of the R7 photoreceptor (Cagan et al., 1992; Hart et al., 1993). Presumably cell walls would prevent such a mechanism in plants. Non autonomous function could also have occurred if DEK1 regulated the production of a diffusible signal. The calpain protease domain had potential to provide non autonomy, because peptide signals are often produced as proproteins that are proteolytically processed to release the active signal molecules. However, such processing proteases are generally extracellular and the calpain domain is predicted to be cytoplasmic (Lid et al., 2002).

With the carotenoid-deficient *vp5* mutation as a marker for *dek1* mutant cells, we were unable to discern the exact cellular boundary of endosperm sectors and so could not analyze cell-autonomy in the endosperm. However, the presence of revertant or mutant sectors as small as a single cell connotes cell-autonomy (Becraft and Asuncion-Crabb, 2000). In leaves, the marker was unequivocal in chlorophyll-containing cells. The vast majority of leaf sectors showed a mutant phenotype that appeared strictly cell-autonomous in all tissues (Fig. 3, Table 2). Some sectors showed non cell-autonomous effects that were probably due to secondary effects. The formation of an ectopic margin alongside a mutant sector suggests that *Dek1*-regulated cellular functions may be required to propagate spatial information across the leaf.

Several sectors showed hints of non cell-autonomy that appeared directly related to *Dek1* function. Because all known *dek1* mutant alleles are recessive loss-of-function mutations, it would be predicted from the hypothesis that *Dek1* promoted the production of a diffusible signaling ligand, that wild-type cells should rescue the phenotype of neighboring mutant cells. Contrary to this prediction, it appeared that mutant cells could sometimes impose a mutant phenotype on neighboring wild-type cells (Fig. 4). One model that would account for this would be if DEK1 were a negative regulator of an inhibitory signal that blocked cell differentiation (perhaps by inhibiting CR4). Thus DEK1 would promote cell differentiation by blocking the inhibitor, and loss-of-function *dek1* mutants would lack normal differentiation because the inhibitor was unchecked. Why such a signal would usually appear cell-autonomous but occasionally act non-autonomously is not known.

REFERENCES

Abbe, E. C. and Stein, O. L. (1954). The growth of the shoot apex in maize: embryogeny. *Am. J. Bot.* **41**, 285-293.

Becraft, P. W. (1999). Development of the leaf epidermis. *Curr. Top. Dev. Biol.* **45**, 1-40.

Becraft, P. W. (2001). Cell fate specification in the cereal endosperm. *Semin Cell. Dev. Biol.* **12**, 387-394.

Becraft, P. W. and Asuncion-Crabb, Y. T. (2000). Positional cues specify and maintain aleurone cell fate during maize endosperm development. *Development* **127**, 4039-4048.

Becraft, P. W., Brown, R. C., Lemmon, B. E., Opsahl-Ferstad, H. G. and Olsen, O.-A. (2001a). Endosperm Development. In *Current Trends in the Embryology of Angiosperms* (ed. S. S. Bhojwani), pp. 353-374. Dordrecht: Kluwer.

Becraft, P. W., Kang, S.-H. and Suh, S.-G. (2001b). The maize CRINKLY4 receptor kinase controls a cell-autonomous differentiation response. *Plant Physiol.* **127**, 486-496.

Becraft, P. W., Stinard, P. S. and McCarty, D. R. (1996). CRINKLY4: a TNFR-like receptor kinase involved in maize epidermal differentiation. *Science* **273**, 1406-1409.

Berlyn, G. P. and Miksche, J. P. (1976). Botanical Microtechnique and Cytochemistry. Ames, IA: Iowa State University Press.

Cagan, R. L., Kramer, H., Hart, A. C. and Zipursky, S. L. (1992). The bride of sevenless and sevenless interaction: internalization of a transmembrane ligand. *Cell* **69**, 393-399.

Evans, M. M., Passas, H. J. and Poethig, R. S. (1994). Heterochronic effects of *glossy15* mutations on epidermal cell identity in maize. *Development* **120**, 1971-1981.

Galweiler, L., Guan, C., Müller, A., Wisman, E., Mendgen, K., Yephremov, A. and Palme, K. (1998). Regulation of polar auxin transport by AtPIN1 in Arabidopsis vascular tissue. *Science* **282**, 2226-2230.

Hardtke, C. S. and Berleth, T. (1998). The Arabidopsis gene MONOPTEROS encodes a transcription factor mediating embryo axis formation and vascular development. *EMBO J.* **17**, 1405-1411.

Hart, A. C., Kramer, H. and Zipursky, S. L. (1993). Extracellular domain of the boss transmembrane ligand acts as an antagonist of the sev receptor. *Nature* **361**, 732-736.

Jin, P., Guo, T. and Becraft, P. W. (2000). The maize CR4 receptor-like kinase mediates a growth factor-like differentiation response. *Genesis* **27**, 104-116.

Li, J., Lease, K. A., Tax, F. E. and Walker, J. C. (2001). BRS1, a serine carboxypeptidase, regulates BRI1 signaling in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA.* **98**, 5916-5921.

Lid, S. E., Gruis, D., Jung, R., Lorentzen, J. A., Ananiev, E., Chamberlin, M., Niu, X., Meeley, R., Nichols, S. and Olsen, O.-A. (2002). The defective kernel 1 (dek1) gene required for aleurone cell development in the endosperm of maize grains encodes a membrane protein of the calpain gene superfamily. *Proc. Natl. Acad. Sci. USA* **99**, 5460-5465.

Neuffer, M. G. (1994). Chimeras for genetic analysis. In *The Maize Handbook* (ed. M. Freeling and V. Walbot), pp. 258-262. New York: Springer-Verlag.

Neuffer, M. G. (1995). Chromosome breaking sites for genetic analysis in maize. *Maydica* **40**, 99-116.

Neuffer, M. G., Coe, E. H. and Wessler, S. R. (1997). *Mutants of Maize.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Nicolas, G., Fournier, C. M., Galand, C., Malbert-Colas, L., Bournier, O., Kroviarski, Y., Bourgeois, M., Camonis, J. H., Dhermy, D., Grandchamp, B. et al. (2002). Tyrosine phosphorylation regulates alpha II spectrin cleavage by calpain. *Mol. Cell Biol.* **22**, 3527-3536.

Olsen, O. A. (2001). Endosperm development: cellularization and cell fate specification. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**, 233-267.

Olsen, O.-A., Linnestad, C. and Nichols, S. E. (1999). Developmental biology of the cereal endosperm. *Trends Plant Sci.* **4**, 253-257.

Scanlon, M. J., Stinard, P. S., James, M. G., Myers, A. M. and Robertson, D. S. (1994). Genetic analysis of 63 mutations affecting maize kernel development isolated from *Mutator* stocks. *Genetics* **136**, 281-294.

Shiraha, H., Glading, A., Chou, J., Jia, Z. and Wells, A. (2002). Activation of m-calpain (calpain II) by epidermal growth factor is limited by protein kinase A phosphorylation of m-calpain. *Mol. Cell Biol.* **22**, 2716-2727.

Steinmann, T., Geldner, N., Grebe, M., Mangold, S., Jackson, C. L., Paris, S., Galweiler, L., Palme, K. and Jurgens, G. (1999). Coordinated polar localization of auxin efflux carrier PIN1 by GNOM ARF GEF. *Science* **286**, 316-318.

Torres-Ruiz, R. A., Lohner, A. and Jurgens, G. (1996). The GURKE gene is required for normal organization of the apical region in the Arabidopsis embryo. *Plant J.* **10**, 1005-1016.

Weil, C. F. and Wessler, S. R. (1993). Molecular evidence that chromosome breakage by Ds elements is caused by aberrant transposition. *Plant Cell* **5**, 515-522.