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The *thick aleurone1* Mutant Defines a Negative Regulation of Maize Aleurone Cell Fate that Functions Downstream of *dek1*

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Abstract

The maize (Zea mays) aleurone layer occupies the single outermost layer of the endosperm. The dek1 gene is a central regulator required for aleurone cell fate specification. dek1 mutants have pleiotropic phenotypes including lack of aleurone cells, aborted embryos, carotenoid deficiency and a soft, floury endosperm deficient in zeins. Here we describe the thick aleurone1 (thk1) mutant which defines a novel negative function in the regulation of aleurone differentiation. Mutants possess multiple layers of aleurone cells as well as aborted embryos. Clonal sectors of thk1 mutant tissue in otherwise normal endosperm showed localized expression of the phenotype with sharp boundaries indicating a localized cellular function for the gene. Sectors in leaves showed expanded epidermal cell morphology but the mutant epidermis generally remained in a single cell layer. Double mutant analysis indicated that the thk1 mutant is epistatic to dek1 for several aspects of the pleiotropic dek1 phenotype. dek1 mutant endosperm that was mosaic for thk1 mutant sectors showed localized patches of multilayered aleurone. Localized sectors were surrounded by halos of carotenoid pigments and double mutant kernels had restored zein profiles. In sum, loss of thk1 function restored the ability of dek1 mutant endosperm to accumulate carotenoids and zeins and to differentiate aleurone. Therefore the thk1 mutation defines a negative regulator that functions downstream of dek1 in the signaling system that controls aleurone specification and other aspects of endosperm development. The thk1 mutation was found to be caused by a deletion of approximately 2 megabases.
Introduction

The importance of cereal grain is increasing due to demands for food, feed, energy and other industrial applications. Different seed components contribute various biological functions as well as properties important for these diverse grain uses. The aleurone is important for mineral storage and the remobilization of storage compounds during germination. In *Arabidopsis*, the aleurone controls seed dormancy (Bethke et al., 2007). Aleurone is also the major source of amylase enzymes needed for the malting process and is thought to be responsible for many of the dietary benefits of cereal bran (Becraft and Yi, 2011).

Most cereal grains including maize have a single cell layer of aleurone that forms at the periphery of the endosperm. Barley (*Hordeum vulgare*) and some varieties of rice (*Oryza sativa*) have multilayer aleurones (reviewed in Becraft et al., 2001). In maize (*Zea mays*), the Coroico landrace was reported to have a multilayer aleurone (Wolf et al., 1972). In barley, aleurone layer number is under genetic control and is inherited as a quantitative trait (Jestin et al., 2008).

Aleurone cell fate specification and differentiation has been recently reviewed (Becraft and Yi, 2011). Plants such as cereals that undergo nuclear type endosperm development show distinct cellular behaviors in the peripheral cell layer compared to internal cells from the onset of cellularization (Brown et al., 1994; Brown et al., 1996; Brown et al., 1999). The peripheral cells assume a typical plant cell division cycle, including the formation of a preprophase band of microtubules that predicts the plane of mitotic cell division. In contrast, the internal cells do not form preprophase bands. This demonstrates a differential response to cellular position at the earliest cellular stages of endosperm development. Nonetheless, aleurone cell fate remains plastic throughout development. The *defective kernel1* (*dek1*) gene is required for aleurone cell identity indicating it is required for the response of endosperm cells to peripheral position. In
*dek1* mutant kernels, the peripheral cell layer assumes starchy endosperm cell fate instead of aleurone (Becraft and Asuncion-Crabb, 2000; Becraft et al., 2002; Lid et al., 2002; Wisniewski and Rogowsky, 2004). Reversion of an unstable *dek1* mutant late in development results in the formation of somatic sectors of aleurone cells in a *dek1* mutant background lacking aleurone (Becraft and Asuncion-Crabb, 2000). These represent transdifferentiation of starchy endosperm cells to aleurone. Conversely, somatic loss of *dek1* function late in development results in the transdifferentiation of aleurone cells to starchy endosperm. These results demonstrate that the positional cues which specify aleurone cell identity are present throughout endosperm development, that the cells retain the ability to respond to those cues throughout development, and that the cues are required to maintain as well as specify aleurone cell identity (Becraft and Asuncion-Crabb, 2000).

Mutant analyses suggest that aleurone cell differentiation is under a hierarchical genetic control (Becraft and Asuncion-Crabb, 2000; Wisniewski and Rogowsky, 2004). As described, the *dek1* gene is one of the key components in this regulatory system (Becraft and Asuncion-Crabb, 2000; Becraft et al., 2002; Lid et al., 2002; Wisniewski and Rogowsky, 2004). The *dek1* gene encodes a plasmamembrane protein with 21 predicted trans-membrane domains, an extracellular loop region, and a cytoplasmic domain containing a calpain protease (Lid et al., 2002; Wang et al., 2003). Mutants of *crinkly4* (*cr4*) show similar phenotypes to *dek1* suggesting they function in the same regulatory system (Becraft et al., 1996; Becraft and Asuncion-Crabb, 2000; Becraft et al., 2002). CR4 is a receptor-like kinase (Becraft et al., 1996; Jin et al., 2000), and as such, both CR4 and DEK1 could potentially function as receptors for the positional cues that induce and maintain aleurone cell fate.
The *supernumerary aleurone1* (*sal1*) mutant produces multiple aleurone layers, indicating it is a negative regulator of aleurone cell fate. The gene encodes a Class E vacuolar sorting protein suggesting it is involved in membrane vesicle trafficking (Shen et al., 2003). SAL1 is proposed to negatively regulate CR4 and/or DEK1 by directing their retrograde cycling from the plasma membrane and thereby dampening their levels of signaling. Thus, the *sal1* mutant would have increased DEK1 or CR4 signaling, inducing extra layers of aleurone cells. SAL1, DEK1 and CR4 proteins all co-localize in endocytic vesicles which is consistent with this hypothesis (Tian et al., 2007). As such, it appears that SAL1 functions upstream of DEK1 and CR4.

Here we report a novel multilayer aleurone mutant, *thick aleurone1* (*thk1*). Genetic mosaic analysis indicates the gene functions locally to inhibit aleurone cell fate in sub-aleurone cells. Double mutant analysis demonstrates that the *thk1* mutant is epistatic to *dek1*, suggesting that *Thk1*+ functions downstream of *Dek1*+ to regulate cell layer number. We propose a model where *Dek1*+ functions as a negative regulator of *Thk1*+.

**Results**

*The thk1 mutant causes specification of extra aleurone layers*

In genetic backgrounds that confer anthocyanin pigmentation to the aleurone, recessive *thk1* mutant kernels can be recognized on a segregating ear by their dark pigmentation (Fig. 1, Supplemental Fig. S1). The *thk1* mutant is inherited as a recessive trait that shows complete penetrance and is fully transmissible through both male and female. Sectioning revealed that mutant kernels contain an abnormally thick aleurone layer, typically 4-6 cells thick, in contrast to the single cell layer of wild type (WT). The conclusion that these cells possess aleurone identity
is based on their small cuboidal geometry, thick autofluorescent cell walls, accumulation of anthocyanin, lack of starch accumulation, and expression of a *Vp1-GUS* transgene, all markers of aleurone identity (Fig. 1, Supplemental Fig. S1).

The *thkl* gene is required for embryo development

Mutant kernels lack well developed embryos and fail to germinate when sewn. At the macroscopic level, normal kernels have a large embryo that occupies most of the adaxial face of the kernel. The embryo is greatly reduced or absent in *thkl* mutant kernels (Fig. 1B, C, Supplemental Fig. S1). Microscopic examination showed that mutant embryos are sometimes able to initiate basic embryonic structures, including shoot and root apical meristems and a scutellum. Other times variable morphological abnormalities manifest. Development always lags behind WT and arrests shortly after the transition stage. A novel aspect of the *thkl* mutant phenotype is the lack of a well developed embryo cavity in the endosperm. *Embryoless (emb)* mutants typically display a cavity in the endosperm at the site vacated by the aborted embryo (Clark and Sheridan, 1991). In *thkl* mutant kernels, the cavity is much less pronounced.

The *thkl* locus maps to chromosome 1S

Recessive mutants can be mapped to chromosome arm using B-A translocations, which undergo non-disjunction in the second pollen mitosis (Beckett, 1978). The *thkl* locus was localized to chromosome 1S when the mutant phenotype was revealed in segmental monosomic endosperms generated by nondisjunction of the B-A translocation, TB-1Sb. Bulk segregant SNP assays using Sequenom Mass Array™ (Liu et al., 2010) confirmed the chromosomal location and suggested the locus mapped toward the distal end.
Further mapping was conducted in an F2 population generated by crossing *thk1/+*, in a W22 inbred background, to the B73 inbred. A summary of markers and recombination frequencies is presented in Table S1 in the supplemental material. Tight linkage was detected with markers in the subdistal region of 1S, however nine markers to the distal most region did not amplify from the mutant, even though they amplified from W22, the background in which *thk1* arose, and B73 (Supplemental Tables S1 and S2). Among the markers tested, the distalmost to amplify from mutant DNA was 363D20-3,4 located at position 2.09 megabase pairs from the chromosome end, according to B73 genome assembly version 2.0. We interpret this to mean that the *thkl* mutant is likely caused by a deletion encompassing approximately 2 megabases. It is therefore formally possible that the phenotype is caused by loss of multiple genes.

*The thkl gene functions locally to regulate aleurone fate*

Several potential mechanisms for the action of the *thkl* gene in aleurone cell fate specification give different predictions for the behavior of mutant sectors in genetic mosaics. For example, it is possible that the *thkl* mutant alters the concentration of a hormone or other morphogen that forms a gradient at the endosperm periphery. In such a case, then the gene would be expected to act cell non-autonomously and mutant sectors would show diffuse boundaries. On the other hand, if the *thkl* gene were involved in the interpretation or response to positional information, gene action would more likely be cell-autonomous and sector boundaries would be sharp.

The *Ds1S4* chromosome-breaking *Ds* transposable element was used to generate *thkl* mutant sectors in a background of WT tissue (Neuffer, 1995; Becraft and Asuncion-Crabb, 2000). As shown in Figure 2, aberrant transposition of the *Ds* element leads to chromosome breakage followed by loss of the acentric chromosome fragment carrying the WT *Thkl*+ allele.
Daughter cells from such an event do not inherit the WT allele and therefore form a clonal sector of hemizygous \textit{thkl} mutant cells. The \textit{Ds1S4} line carrying \textit{Ac} at the \textit{P1} locus was crossed as a male to heterozygous \textit{thkl}/+ mutant females. Among the F1 were kernels containing darkly pigmented sectors in an otherwise normally pigmented endosperm (Fig. 2, Table 1). Sectioning of these dark sectors revealed that they displayed the multilayered aleurone phenotype typical of \textit{thkl} mutants.

Sector borders were sharp as would be expected for cell-autonomous gene action. A stringent test of cell-autonomy requires that sectors be marked with an independent marker. Sectors were generated that contained \textit{thkl} marked with the linked \textit{vp5} carotenoid-deficient mutant but unfortunately \textit{vp5} sectors were not discernable in the endosperm. Therefore, it can be concluded that the \textit{thkl} gene functions locally in a manner consistent with cell-autonomy but because sector boundaries could not be assessed independent of the \textit{thkl} mutant phenotype, cell-autonomy remains equivocal.

It was of interest to test whether the \textit{thkl} mutation could uncouple aleurone cell fate from surface position. Transpositions that occur late in development result in the formation of small sectors and the \textit{Ds1S4} line has been shown to generate single-celled sectors (Becraft and Asuncion-Crabb, 2000). Therefore, if \textit{thkl} uncoupled aleurone fate from surface position, it should be possible to detect internally isolated aleurone cells. To explore this, 60 kernels were sectioned and a total of 421 sectors examined. Late \textit{thkl} sectors were readily observed in most mosaic kernels but none were observed where there was not a continuity of aleurone cell contacts to the surface of the endosperm (Fig. 2 and Supplemental Fig. S2). That is, no internal aleurone cells were observed where all their surrounding neighbors were starchy endosperm cells.
The *thk1* deletion affects leaf epidermal cell size

These genetic stocks also afforded the opportunity to examine the phenotype of *thk1* mutant cells in sporophyte tissues, which cannot otherwise be studied because *thk1* mutants are embryo lethal. For these experiments, the *thk1* mutant allele was linked in coupling to *vp5*, which confers cell-autonomous albino sectors in leaves due to carotenoid deficiency (Becraft et al., 2002). As a control, the *vp5* mutant linked to the normal *Thk1*+ allele was used. These chromosomes were made heterozygous with the Ds1S4 chromosome-breaker and leaves were examined for *thk1* mutant sectors. In particular, we were interested in testing the hypothesis that the *thk1* mutant might confer multiple leaf epidermal layers because many genes such as *Extra Cell Layers (Xcl)* similarly affect the aleurone and leaf epidermis (Kessler et al., 2002; Becraft and Yi, 2011). Ds1S4 leaves carrying the *thk1* mutant allele showed prominent ridges that often caused the leaves to crease (Supplemental Fig. S3). These were not present in control *vp5*-sectored leaves, nor did they form in *dek1* sectors (Becraft et al., 2002). Examination of these sectors revealed irregular epidermal cells that often appeared enlarged in section. In most instances, mutant epidermis was clearly a single cell layer, even when the sector covered an extensive area of the epidermis (e.g. Fig 3B). Several instances were observed where internal cells displayed characteristics consistent with epidermal cell identities. Figure 3C shows an example where internal cells either showed features of sclerenchyma or bulliform-like cells. Sclerenchyma cells occur internally in localized foci associated with major and intermediate veins (Fig. 3A). In *thk1* mutant sectors, internal sclerenchyma cells were observed interveinally. Other internal cells were observed that did not have characteristics of mesophyll or other normal internal cell types but resembled bulliform cells.
The thk1 mutant is epistatic to dek1

Of the various mutants that disrupt aleurone cell fate specification, dek1 is the strongest and most consistent. The weak dek1-D allele causes a partial and somewhat variable loss of aleurone identity while the severe dek1-1394 allele causes a complete loss of aleurone (Becraft and Asuncion-Crabb, 2000; Becraft et al., 2002). To test the genetic relationship between the two genes, thk1/+ was crossed to dek1-D/+ and the resultant F1 plants were self pollinated to generate ears segregating F2 kernels. Six full ears were obtained that segregated both mutants.

A summary of the totals of each phenotypic class are presented in Table 2. Kernels with dek1-D phenotypes were examined for the presence of thick aleurone but none was found. Ears contained three major phenotypic classes, WT, dek1-D and thk, with a few malformed and aborted kernels of questionable phenotype with respect to dek1 and thk. Notably, there was an excess of thk mutant kernels relative to dek1-D. A \( \chi^2 \) test for goodness of fit of the data to a 9:3:3:1 ratio, expected for independent assortment, produced a value of 229.89 indicating that this hypothesis should be rejected. When tested for fit to a 9:4:3 ratio, a \( \chi^2 \) value of 9.68 indicated that the data fit this hypothesis. The thk1 mutant was then crossed to dek1-1394 to test whether the same segregation ratios would hold for a strong dek1 allele. Three ears were obtained that segregated both mutants, and again thk1 mutants were in excess relative to dek1 and the data fit a 9:4:3 ratio (\( \chi^2 = 3.31 \)) but not 9:3:3:1 (\( \chi^2 = 95.74 \)). Thus, segregation ratios suggested that the excess thk1 mutants were in fact thk1, dek1 double mutants and that thk1 is epistatic to dek1.

Two alternative explanations other than epistasis were considered to explain the data. The first was linkage; both thk1 and dek1 loci are on chromosome 1S. However, dek1 maps proximally and thk1 maps distally, and in a cross of thk1, dek1-1394/+ to B73, the mutants
were transmitted to progeny independently, indicating that the loci are genetically unlinked. The second possibility considered was inefficient transmission of the *dek1* mutant alleles. The cross that generated the ears segregating both *thkl* and *dek1-1394* also generated ears segregating for each mutant individually. As summarized in Table 2, ears segregating only *dek1-1394* contained mutant kernels in a ratio that did not differ significantly from 3:1 ($\chi^2=1.91$), indicating the mutant allele was transmitted with full efficiency.

Finally, kernels with a *thkl* mutant phenotype were selected from an F2 ear segregating for both the *thkl* and *dek1* mutants. These kernels were genotyped using a DNA marker, IDP112, located approximately 2 cM from the *dek1* locus (Fig. 4A). Among 18 kernels genotyped, 5 were homozygous *Dek1+*, 8 were heterozygous *Dek1+/dek1-1394* and 5 were homozygous mutant *dek1-1394* (barring recombination). Therefore, double homozygous mutants have a *thkl* phenotype and *thkl* is epistatic to *dek1-D* and *dek1-1394*.

*The epistatic action of thkl revealed in genetic mosaics*

To further test the epistatic relationship of *thkl* to *dek1*, we generated genetic mosaics that contained *thkl* mutant sectors in a *dek1-1394* background (Fig. 5, Supplemental Fig. S4). More precisely, *thkl, dek1-1394/-, -* hemizygous sectors were generated in a *dek1-1394/dek1-1394* background. The epistatic action of *thkl* predicted that such mosaics should contain sectors of thick aleurone on kernels otherwise devoid of aleurone. The strategy for generating these mosaics is shown in Figure 5A and the cross used to generate the appropriate genotypes is described in Supplemental Fig. S4. Data are summarized in Table 1.

Figure 5 shows examples of clonal sectors of *thkl, dek1-1394/-, -* double mutant cells in otherwise *dek1-1394* single mutant kernels. Whereas *dek1-1394* kernels are devoid of aleurone
cells, the loss of Thk1+ function restored the ability of dek1 mutant endosperm cells to form aleurone, and conferred the multilayered aleurone phenotype of thk1 mutants.

In addition to the sector types described above, kernels were frequently observed that contained separate dek1 and thk1 sectors (Supplemental Fig. S4). Such kernels most likely result from interstitial chromosome deletions or rearrangements as has been described for chromosome-breaking double Ds elements (English et al., 1995). Because the exact chromosomal position of the Ds1S4 is not known, the experiment was repeated with two well characterized fAc derivatives of P1-ww. P1-wwB54 has been shown to cause various chromosome rearrangements, including interstitial deletions and rearrangements, while P1-vv9D9A generates primarily distal deletions (Zhang and Peterson, 2004; Yu et al., 2011). Both are located at the P1 locus, 1 cM proximal to dek1. Crosses with P1-wwB54 generated the same array of kernel types as Ds1S4 (data not shown). In contrast, p1-vv9D9A generated all the expected sector types but very few kernels with mixed thk1 and dek1 sectors. Among 5 ears from crosses to dek1-1394/+ females, 320 dek1 mutant kernels were observed and none contained any aleurone cells. From 18 crosses to thk1,dek1-1394/+,+ females, 14 kernels were recovered that had thk1 phenotypic sectors in dek1 mutant kernels. Thus, for all three chromosome breakers, crosses to thk1,dek1-1394/+,+ females produced dek1 mutant kernels with thk1 sectors, while when crossed to dek1-1394/+ alone, no instance of a pigmented sector or aleurone cell was observed among 1586 dek1 mutant kernels examined, validating the interpretation of sector types.

thk1 epistasis is pleiotropic

Mutant dek1 kernels show a variety of phenotypic defects in addition to the lack of aleurone. These include a deficiency of carotenoid pigments as well as opaque endosperm with a
soft floury texture. Mosaic kernels with isolated thk1 sectors in a dek1 mutant background showed yellow halos of carotenoid pigments surrounding the thk1 sectors (Fig. 5C) suggesting that thk1 is epistatic to dek1 for this trait as well.

Floury endosperm can result from a deficiency in zein storage protein accumulation. The double mutant kernels genotyped above did not show a floury texture. To test the hypotheses that the floury texture of dek1 endosperm reflected a zein deficiency and that the thk1 mutant suppressed this deficiency in double mutants, zein profiles were analyzed by HPLC (Fig. 4). Compared to wild type, dek1 mutants showed dramatic reductions in gamma zeins including the 27kD gamma zeins. In contrast, the thk1 mutant endosperms showed zein profiles very similar to wild type. The dek1, thk1 double mutant endosperms also showed zein profiles remarkably similar to wild type. Thus, the thk1 mutant is epistatic to dek1 for the regulation of aleurone formation, carotenoid accumulation and gamma zein accumulation.

Discussion

We described a new recessive loss-of-function mutant that causes an increased number of aleurone layers in maize endosperm. In addition the mutant results in embryo lethality. Other than the effect on the aleurone layer, the overall morphology of the endosperm is generally normal. One aspect of the thk1 mutant kernels that is novel compared to most emb mutants is that they do not form a prominent cavity in the region of the endosperm vacated by the aborted embryo (Clark and Sheridan, 1991).

The thk1 mutant is likely caused by a deletion encompassing approximately 2 megabases. It is therefore formally possible that the phenotype is caused by loss of multiple genes. Regardless of whether the thk1 mutant phenotype results from loss of one or multiple genes, the
analysis presented here clearly demonstrates a function in the aleurone signaling system encoded by the distal region of chromosome 1S.

*The thk1 gene functions locally in the cellular response to position*

The *thk1* gene functions locally within the endosperm as evident by the distinct sector boundaries. Similarly clear boundary delineations were observed both for *thk1* mutant sectors in fields of normal endosperm cells and for double mutant sectors in fields of *dek1* mutant cells. Because mutant sectors were not marked with an independent cell-autonomous marker, it cannot be definitely concluded that *thk1* function is cell-autonomous but this is likely the case.

The *dek1* gene produces similarly sharp sector boundaries and has been proposed to function in the cellular response to positional cues (Becraft and Asuncion-Crabb, 2000; Becraft et al., 2002; Lid et al., 2002; Tian et al., 2007). The results reported here strengthen that model. The sharp boundaries of *thk1,dek1* double mutant sectors in *dek1* mutant endosperm indicate that the epistatic relationship of *thk1* to *dek1* is likely to function at the cell-autonomous level. As such, the signaling system defined by these two genes would appear to function in the response of endosperm cells to position rather than in the generation of those positional cues.

Aleurone forms on the surface of endosperm that is isolated from contacts with surrounding kernel tissues either by in vitro culture or as a consequence of a mutant phenotype (Olsen, 2004; Gruis et al., 2006; Reyes et al., 2010). These observations led to the proposal of the ‘surface rule’ which posits that the source of the positional information that specifies aleurone identity is intrinsic to the endosperm. The *Ds1S4* line generates late-occurring transposition events that produce sectors as small as a single cell (Becraft and Asuncion-Crabb, 2000). Late *thk1* sectors were readily observed but interestingly, none were observed where there was not a continuity of aleurone cell contacts to the surface layer of the endosperm (Fig. 2 and
Supplemental Fig. S2). That is, no internal aleurone cells were observed that were completely surrounded on all sides by starchy endosperm cell neighbors. In contrast, isolated single cells at the surface can be completely surrounded by starchy endosperm and still assume aleurone identity (Supplemental Fig. S2; Becraft and Asuncion-Crabb, 2000). So while the thk1 mutant produces aleurone cells in sub-peripheral cell layers, there still appears to be adherence to the surface rule. It may be that determinants of aleurone fate are transmitted from the surface only via aleurone cells. This transmission could potentially be through plasmodesmatal connections, which are distinct between aleurone cells compared to other endosperm cell types (Tian et al., 2007), via lineage, or by an aleurone-specific signaling mechanism. These results imply that Thk1+ function is normally required for the transdifferentiation event that occurs in the internal daughters of periclinal aleurone cell divisions. Such internal cells normally redifferentiate to starchy endosperm cells (Becraft and Asuncion-Crabb, 2000) and this might be the primary site of action for Thk1+. The formation of small isolated thk1 sectors in dek1 kernels indicates there is no requirement for aleurone continuity in the lateral dimension (Supplemental Fig. S2).

Mutant sectors in leaves produced enlarged epidermal cells that resembled bulliform cells in morphology and histological staining characteristics. In most sectors observed, these cells occupied a single epidermal layer. In cr4 mutants, cells with unmistakable epidermal identities, such as epidermal hair cells, were observed internally (Jin et al., 2000). A few examples were observed in thk1 sectors where internal cells had characteristics consistent with epidermal identity, but not unequivocal. Thus, it seems possible that thk1 may function to regulate leaf epidermal cell layer number, but this remains tenuous. It is clear that a thk1 deficiency in epidermal cells does not directly cause them to form a multi-layered epidermis. Furthermore, because multiple genes are deficient due to the deletion, it is not possible to formally conclude
that the gene(s) regulating leaf epidermal cells are the same as those regulating aleurone
development, although this seems likely given the similar functions of other genes in aleurone
and leaf epidermis (Kessler et al., 2002; Becraft and Yi, 2011).

The regulation of aleurone layer number

The genetic regulation of aleurone cell layer number does not appear to be simple.
Typical barley cultivars contain 3-4 aleurone layers, with layer number inherited as a quantitative
trait (Jestin et al., 2008). The recessive barley des5 mutant possesses a single aleurone layer
indicating this gene normally functions to increase layer number, opposite Thk1+ (Olsen et al.,
2008). The Multiple Aleurone Layer trait present in Coroico maize landraces is inherited as a
partially dominant, but complex, trait (Wolf et al., 1972, our unpublished observations). Xcl is
another dominant maize mutant that produces double aleurone cell layers (Kessler et al., 2002).
Transgenic knockdown of two rice transcription factors also affected aleurone layer number.
RISBZ1 and RPBF are bZIP and DOF zinc finger transcription factors, respectively, that
function together to regulate endosperm storage protein gene expression (Yamamoto et al.,
2006). Knockdown of either factor singly had mild or no effects on the aleurone but the double
knockdown of both genes produced a multilayered, disordered aleurone (Kawakatsu et al., 2009).

Maize, sal1 mutants also produce extra layers of aleurone cells. The sal1 gene maps to
chromosome 9 and encodes a CHMP Class E vacuolar sorting protein. It appears to act upstream
as a negative regulator of dek1 (Shen et al., 2003; Tian et al., 2007). In contrast, the epistatic
relationship of thk1 over dek1 suggests that thk1 functions downstream of dek1. dek1 encodes a
plasmamembrane localized protein with a predicted extracellular domain and a cytoplasmic
calpain protease domain (Lid et al., 2002; Wang et al., 2003). It functions in the signaling system
that specifies aleurone fate (Becraft and Asuncion-Crabb, 2000; Becraft et al., 2002; Lid et al.,
As such, the \textit{thkl} gene product represents a likely target of regulation by DEK1, potentially a direct target.

The calpain protease function of DEK1 suggests that targets of regulation would likely be proteolytic substrates, which could provide a clue to the identity of the \textit{thkl} gene. However, substrate recognition by calpains is complex and involves three dimensional structures that are not easily recognized in primary sequences (reviewed in Goll et al., 2003; Croall and Ersfeld, 2007). Known calpain substrates in mammalian systems include many membrane proteins including receptors, but also diverse other proteins such as cytoskeletal proteins and transcription factors. Therefore, it is not feasible to predict a candidate \textit{thkl} gene from within the deleted region based on potential calpain substrates.

\textit{A model for the regulation of aleurone cell fate}

The loss of function \textit{thkl} mutant results in the specification of extra layers of aleurone cells indicating that the normal gene function is to negatively regulate aleurone fate in the sub-peripheral layers. The function of the normal \textit{dekl} gene is to promote aleurone identity in the peripheral cell layer. The epistatic relationship of \textit{thkl} over \textit{dekl} suggests that the \textit{thkl} gene functions downstream of \textit{dekl} in the specification of aleurone fate (and in the control of other endosperm traits). As such, a model where \textit{Dekl}+ promotes aleurone identity by negatively regulating \textit{Thkl}+ accounts for all the available data. As shown in Figure 6, \textit{Dekl}+ could normally function in the peripheral layer to inhibit \textit{Thkl}+ function and thereby permit aleurone formation. In \textit{dekl} mutants, \textit{Thkl}+ function would be derepressed in the peripheral cell layer and block aleurone differentiation. In \textit{thkl} mutants, there would be no repressive function regardless of whether \textit{dekl} were functional. As discussed below, additional factors must be involved that limit aleurone fate to the peripheral regions of the endosperm.
Pleiotropic epistasis

DEK1 function is not restricted to the regulation of aleurone cell fate as is evident from the pleiotropy of the dek1 mutant phenotype. Mutants are carotenoid deficient, have floury, opaque endosperms and are embryo lethal. The opaque endosperm phenotype likely relates to a zein storage protein deficiency (Fig. 4). Carotenoid and zein content are important grain quality traits. Interestingly, thk1 mutants appear to rescue these aspects of the dek1 mutant phenotype indicating that Thk1+ has functions in addition to aleurone regulation.

That dek1 and thk1 appear to function in a regulatory pathway that controls zein and carotenoid accumulation in internal starchy endosperm cells indicates that the function of this pathway to regulate aleurone specification is context dependent and only occurs in the peripheral few cell layers of the endosperm. It is therefore evident that additional factors are ultimately involved in patterning the endosperm. One possible source of positional information could be auxin, which was recently reported to be at highest concentrations in the periphery of maize endosperm and to possibly influence aleurone fate specification (Forestan et al., 2010).

The relationship between protein content and aleurone cell layer number is not currently understood but several previous reports have hinted at it. The Coroico landrace with the Multiple Aleurone Layer trait is also high in protein content (Wolf et al., 1972). While it was recently shown that zeins are expressed in aleurone cells (Reyes et al., 2011), such a minor fraction of the endosperm is unlikely to have a major impact on the overall endosperm protein content, and it was concluded that the aleurone layer and protein content traits of Coroico were likely controlled separately (Nelson and Chang, 1974). As mentioned, the rice RISBZ1 and RPBF transcription factors function to promote prolamine storage protein gene expression in the endosperm as well as restrict aleurone layer number (Yamamoto et al., 2006; Kawakatsu et al., 2009). This
relationship is different from \textit{thkl} which appears to negatively regulate both aleurone layers and zein accumulation.

Signaling has previously been implicated in the regulation of zein accumulation. Protein levels and activity, as well as transcript levels of maize OPAQUE2 (O2), a bZIP transcription factor that promotes alpha-zein gene expression, are regulated diurnally (Ciceri et al., 1997; Ciceri et al., 1999). The protein undergoes cyclic phosphorylation and dephosphorylation, with unphosphorylated forms having higher DNA binding activity. Highest levels are present during the daytime phase of the diurnal cycles, but phosphorylation ratios do not appear to change during development. Genetic and cell biological evidence suggest that DEK1 functions in the same regulatory system as the CR4 receptor kinase to regulate aleurone cell fate as well as other aspects of development (Becraft and Asuncion-Crabb, 2000; Becraft et al., 2002; Tian et al., 2007). Mutants of \textit{cr4} show an opaque kernel phenotype, similar to \textit{dekl}, consistent with the hypothesis that both genes may also function together to regulate storage proteins (Jin et al., 2000). The current study now places \textit{thkl} in that system. It is not yet evident why the same signaling system responsible for aleurone cell fate specification would also be used to regulate zein accumulation. This will become clear when the regulatory networks of endosperm development are more completely understood.

Materials and Methods

\textit{Genetic stocks}

The \textit{thkl} mutant arose in a \textit{Mutator} transposon line in a maize (\textit{Zea mays}) W22 inbred background and was a kind gift from Donald McCarty, University of Florida. The \textit{dekl}-1394 and \textit{vp5} mutants and the Ds1S4 line were obtained from the Maize Genetics Cooperation Stock
The Center, Urbana, IL. The dkl-D mutant was a gift from Hugo Dooner, Waksman Institute. The
P1-WWB54 and p1-yy9D9A lines were gifts from Thomas Peterson, Iowa State University. The
thkl and dkl mutants were backcrossed 5 generations into a B73 inbred background.

Microscopy and histology

For Figure 1 C-F, developing kernels were dissected from young ears, fixed in FAA
(formaldehyde, alcohol and acetic acid) and embedded in Paraplast Plus according to standard
procedures (Berlyn and Miksche, 1976). 10μm sections were affixed to glass slides,
deparaffinized, stained with fast green and mounted. Figure 1 G and H were 28 DAP kernels
fixed in 2% paraformaldehyde and 3% gluteraldehyde buffered in 0.1M cacodylate (pH 7.2) and
embedded in LR White resin. Samples were sectioned to 1 μm on a Leica EM UC6
ultramicrotome and stained with Periodic Acid Schiff’s (PAS) and Toluidine blue. Leaf sections
(Fig. 3) were prepared the same way except no PAS stain was applied. For Figure 1I and J, 24
DAP kernels were hand sectioned and incubated in GUS staining solution (50 mM sodium
phosphate [pH 7.0], 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM
EDTA, 0.05% [v/v] Triton X-100, 0.35 mg/ml 5-bromo-4-chloro-3-indolyl-b-D-glucuronide) for
24 h at 37°C for several hours until blue precipitate was apparent (Jefferson et al., 1987). Figures
1K and L were hand sectioned mature kernels examined under epifluorescence using a narrow-
violet filter (excitation 400–410 nm, dichroic mirror and barrier filter, 455 nm). Mosaic kernel
sectors were examined using epi-illumination of hand sections. All micrography was performed
with an Olympus BX-60 microscope equipped with a Jenoptik C-5 camera.
Genetic mapping and genotyping

Genomic DNA was extracted from mature dried kernels with modifications to a described protocol (Edwards et al., 1991). Briefly, kernels were cut with a razor blade and a half kernel was extracted for mutants and a quarter kernel for wild type (~100mg). Kernels were soaked in 200 µl extraction buffer for 3 to 12 hours at room temperature. Samples were then ground with a plastic pestle and a hand-held drill. Additional buffer was added to a total volume of 500 µl, followed by the addition of 400 µl chloroform. Samples were vortexed and then centrifuged 10 min at 13,000 rpm with a tabletop centrifuge. After transferring 250 µl of supernatant to a new tube, DNA was precipitated with an equal volume of isopropyl alcohol. Samples were centrifuged and the pellet was washed with 70% ethanol and air-dried. The pellet was then dissolved in 500 µl distilled water. 1 µl (20-50 ng genomic DNA) was used for PCR. All PCR was performed with Go Tag Green Mastermix (Promega) in 10 µl reactions containing 5 pmol of each primer. Cycling conditions were 94°C for 4 min followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min. After an additional 10 min at 72°C, reactions were kept at 4°C until visualized by agarose gel electrophoresis.

HPLC analysis of zein proteins

Alcohol-soluble proteins were extracted from 25 mg of flour using 1000 µL extraction buffer consisting of 70% EtOH, 61 mM NaOAc, and 5% β-mercaptoethanol. The mixture was vortexed briefly, shaken for 1 h at room temperature, and then centrifuged for 10 min at 13,000 rpm. An aliquot of 25 µL of each extract was injected into a C18 protein and peptide column heated to 55°C in a Waters 2695 Separation Module, and absorbance at 200 nm was measured with a Waters 2487 Dual Absorbance Detector. Separation of distinct proteins based on hydrophobicity was achieved with a gradient of ultrapure water and acetonitrile, both
containing 0.01% trifluoroacetic acid. The complex gradient ranged from 20 to 60% acetonitrile for a total of 75 min excluding equilibration steps before and after elution. Gradient slopes were optimized for separation of peaks. A flow rate of 1 mL/min was used.

Acknowledgements

The authors thank Donald R. McCarty for the gift of the \textit{thkl} mutant and Thomas A. Peterson for the \textit{fAc} lines. Amanda Ripley provided excellent technical assistance. The Iowa State University Microscopy and NanoImaging Facility assisted with some of the microscopic preparations. The Iowa State University Genomic Technologies Facility provided the mass array SNP genotyping service.

Literature Cited


Wolf MJ, Cutler HC, Zuber MS, Khoo U (1972) Maize with multilayer aleurone of high protein content. Crop Sci. 12: 440-442

Figure Legends
Figure 1. Analysis of the *thkl* mutant phenotype. A, Mutant kernel (right) shows increased anthocyanin pigmentation and lack of a well-developed embryo. B, C, Longitudinal sections of WT (B) and *thkl* (C) embryos at 12 DAP. D-G, microscopic sections showing WT (D, F) and *thkl* (E, G) aleurone. D, E, Histologically stained sections. Starch grains stained pink with PAS and protein-dense aleurone cells (arrow) stain intensely with Toluidine blue. F, G, Expression of the *Vp1-GUS* transgene as determined by X-gluc histochemical stain. Scale bars = 100 μm

Figure 2. Genetically mosaic kernels with *thkl* sectors. A, The chromosomal basis of *thkl* sector generation. Endosperm cells were heterozygous for the recessive *thkl* mutant and therefore phenotypically normal. The chromosome breaking *Ds1S4* element is located proximal to the wild type *Thk1*+ allele. In the presence of an *Ac* element, aberrant *Ds1S4* transposition results in chromosome breakage and loss of the distal chromosome 1S arm containing the *Thk1*+ allele, uncovering the *thkl* mutant allele. Such events occur in individual cells occasionally during kernel development, and subsequent mitotic divisions generate clonal sectors of hemizygous *thkl* mutant cells in a background of otherwise normal endosperm. B, Example of a kernel containing darkly pigmented *thkl* mutant sectors (lower kernel, arrow) compared to a normal unsectored kernel (top). C, D, Kernel cross-sections showing examples of *thkl/-* mutant sectors with adjacent normal endosperm. Sector boundaries appear sharp, consistent with a laterally cell-autonomous function. E, Several small sectors producing one or a few sub-peripheral aleurone cells (arrows). All are in direct contact with the peripheral layer of aleurone cells. Scale bars (B-D)= 200 μm; (E)=50 μm.
Figure 3. Effect of the thk1 mutant on leaf cells. A, Control leaf with vp5 sector. The arrow designates a vascular bundle where the right half of the bundle sheath is devoid of well developed chloroplasts due to the carotenoid deficiency conferred by the vp5 mutation. No morphological defects were observed. The brackets highlight an intermediate vascular bundle with foci of sclerenchyma cells (small thick-walled cells, turquoise stained online) at the adaxial and abaxial poles. B, A thk1/- mutant sector covering an extensive area of upper leaf epidermis, including the entire area shown. The cells are enlarged, but only occupy a single cell layer. C, Sector where internal cells show possible epidermal identities. The internal cells have attributes distinct from normal mesophyll or bundle sheath, but which resemble bulliform cells (denoted with asterisks). Also, the lower (abaxial) epidermal cells resemble bulliform cells, which are normally restricted to the upper surface. Cells with clearly recognizable mesophyll or vascular bundle identities are outlined. Scale bars A, B, = 100 μm; C, = 50 μm.

Figure 4. thk1 is epistatic for multiple pleiotropic dek1 phenotypes. A, Marker genotyping demonstrated the epistasis of thk1 over dek1. Kernels from an F2 ear segregating for the thk1 and dek1-1394 mutants were genotyped for the marker IDP112 (Fu et al., 2006). DNA from five pooled kernels with thk1 mutant phenotypes showed two polymorphic alleles. DNA from 12 pooled dek1 kernels only showed the upper band indicating all were homozygous for the upper allele and that this marker allele is tightly linked to the dek1-1394 mutant allele. Among 18 individual kernels with thk1 mutant phenotypes, five were homozygous for the marker allele linked to dek1-1394 (arrows), indicating that these individuals were likely homozygous for the dek1 mutation. M=molecular weight marker. B, Zein profiles were examined by HPLC analysis. The dek1 single mutant (bottom trace) is deficient in zein proteins compared to WT (upper
trace), particularly for the gamma zein peaks. Both the thkl single mutant and thkl, dekl double traces (second and third from the top, respectively) are similar to WT.

**Figure 5.** Genetic mosaics with thkl sectors in dekl mutant kernels. A, The chromosomal basis of these mosaics. Kernels were homozygous for the recessive dekl-1394 mutant allele and heterozygous thkl/Thkl+. Breakage and loss of the chromosome arm carrying the Thkl+ allele resulted in thkl, dekl/-, - double mutant sectors in a background of dekl single mutant endosperm cells. B, An example of a dekl mutant kernel with thkl mutant sectors. The purple sectors are thkl, dekl/- - double mutant cells. C, Yellow halos surrounding isolated thkl sectors in a dekl mutant kernel suggest carotenoids are produced by the thkl, dekl/- - double mutant cells, while the surrounding dekl single mutant tissue is carotenoid deficient. D, E, Sectioned kernels showing the multiple aleurone phenotype of thkl sectors even though cells are also mutant for dekl. The red pigment is anthocyanin. Scale bars = 200 μm

**Figure 6.** Model for thkl function in dekl regulation of aleurone cell fate. Thkl+ functions as a negative regulator of aleurone identity. In the outer layer, DEK1 inhibits THK1 function to derepress aleurone fate. In internal layers, THK1 is not inhibited and therefore represses aleurone fate. In dekl mutants, THK1 function would not be inhibited and aleurone fate would be repressed in all cell layers. In thkl mutants, there is no repression of aleurone identity in any of the cell layers, permitting the formation of aleurone, independent of DEK1 function.
Table 1. Summary of genetic mosaic analysis.

<table>
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<tr>
<th>Female(^a)</th>
<th># Ears</th>
<th># WT K</th>
<th># dek1 K</th>
<th># sectored K</th>
<th># thk sectored K</th>
<th># dek1 K w/ thk sectors</th>
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<td>thk1, dek1/+,+</td>
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<td>1684</td>
<td>947</td>
<td>386</td>
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<td>2642</td>
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\(^a\) All males in these crosses were of the genotype Thk1+, dek1-1394 / Thk1+, Dek1+, Ds1S4.
Table 2. F2 segregation of *dek1* and *thk1* mutant kernel phenotypes

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Obs.</th>
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<td>b</td>
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<td></td>
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<td>WT</td>
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<td>1635</td>
<td>1635</td>
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<td>545</td>
<td>545</td>
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<tr>
<td><em>thk1</em></td>
<td>714</td>
<td>545</td>
<td>727</td>
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<td><em>dek1</em>-D; <em>thk1</em></td>
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<td>280</td>
<td>280</td>
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<tr>
<td><em>thk1</em></td>
<td>364</td>
<td>280</td>
<td>373</td>
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<tr>
<td><em>dek1</em>-1394; <em>thk1</em></td>
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<td><em>dek1</em>-1394</td>
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<td>239</td>
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---
a Observed number of kernels for each phenotype.
b Expected number of kernels for the given phenotypic segregation ratio (parentheses).
c Kernels displayed aborted kernel phenotypes indistinguishable as to their *thk1* or *dek1* phenotypes.
A

Pooled Kernels

| thk1 | dek1 |

Individual thk1 Mutant Kernels

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 M

B

27 kDa gamma zein

Absorbance @ 200 nm

Elution Time (Min)

+/-, +/-

thk1/thk1, +/-

thk1/thk1, dek1/dek1

+/-, dek1/dek1

Alpha zeins