The maize (Zea mays) CRINKLY4 (cr4) gene encodes a receptor-like kinase that controls a variety of cell differentiation responses, particularly in the leaf epidermis and in the aleurone of the endosperm. In situ hybridization indicated that the cr4 transcript is present throughout the shoot apical meristem and young leaf primordia. A genetic mosaic analysis was conducted to test whether CR4 signal transduction directly regulated the cellular processes associated with differentiation or whether differentiation was controlled through the production of a secondary signal. Genetic mosaics were created using γ-rays to induce chromosome breakage in a cr4/Cr4+ heterozygote. The mutant cr4 allele was marked with the albino phenotype, Oy-700. Breakage and loss of the chromosome arm carrying the wild-type alleles created a sector of albino, cr4 mutant tissue in an otherwise normal leaf. Analysis of such sectors indicated that cr4 functions cell autonomously to regulate cell morphogenesis, implying that CR4 signal transduction regulates cell differentiation through strictly intracellular functions and not the production of secondary intercellular signals. However, several sectors altered cell patterning in wild-type tissue adjacent to the sectors, suggesting that cr4 mutant cells are defective in the production of other lateral signals.

Cell interactions are important in the development of all multicellular organisms. Proper cell patterning and coordinated cell differentiation necessitate communication among cells. Receptor protein kinases mediate cellular responses to many extracellular stimuli, including developmental signals and several receptor-like kinases (RLKs) are known to be important for plant development (Becraft, 1998).

The maize (Zea mays) CRINKLY4 (CR4) RLK mediates cellular differentiation responses in tissues of the shoot and endosperm (Becraft et al., 1996; Jin et al., 2000). Kernels mutant for cr4 often display mosaic aleurone, where portions of the endosperm fail to differentiate aleurone. Cells on the surface of the endosperm have attributes of starchy endosperm cells indicating that cell fate was not properly specified in these cells. This suggests that CR4 may function in the perception of positional cues that specify aleurone cell fate throughout endosperm development (Becraft and Asuncion-Crabb, 2000). In leaves and other organs of the shoot, the most pronounced mutant effects are on the epidermis (Becraft et al., 1996; Jin et al., 2000). Epidermal cells are abnormally large, irregularly shaped, and cell pattern is disorganized. Abnormal proliferation is evident in localized regions and sometimes leaves form graft-like fusions. Defects in cell differentiation are also evident internally in strong mutant phenotypes. This array of differentiation defects indicates that CR4 regulates a diverse set of cellular functions during development, analogous to the function of growth factor receptors in animals. Growth factor receptors are receptor protein kinases. Binding of the growth factor to the extracellular domain activates the cytoplasmic kinase domain, triggering a phosphorylation cascade that leads to a change in cell activity.

When studying the developmental function of a gene, it is of interest to determine whether it functions cell autonomously. Nonautonomy would indicate either that the gene of interest encodes a product that can be transmitted from cell to cell, or that it regulates the production of such a product. Testing whether a gene product functions cell autonomously is accomplished by a genetic mosaic analysis: the analysis of individuals composed of both mutant and wild-type cells. If the mutant and wild-type cellular phenotypes correspond strictly to the cellular genotypes, the gene acts cell autonomously. If genetically wild-type cells are able to rescue the phenotype of neighboring mutant cells (or vice versa, depending on the nature of the gene product and the mutation), the gene acts nonautonomously.

In the case of receptors, although the direct response to receptor activation is intracellular, the phenotypic consequences of growth factor reception are...
often non-cell-autonomous. Such an example is the specification of dorsal cell fate in the *Drosophila melanogaster* embryo. The oocyte is surrounded by a layer of maternal cells called follicle cells. The *D. melanogaster* sp. epidermal growth factor receptor, a receptor Tyr kinase, is expressed in the follicle cells and is activated in the dorsal region by a signal from the oocyte (Schüpbach, 1987; Price et al., 1989; Neuman-Silberberg and Schüpbach, 1993). The activated receptor promotes dorsal follicle cell fate, which entails the repression of another signal back to the embryo. If not repressed, this second signal specifies ventral cell fate in the embryo (Stein et al., 1991; Stein and Nüsslein-Volhard, 1992). Thus, specification of dorsal cell fate in the embryo requires the activation of a receptor kinase located in the maternal follicle cells.

In der genetic mosaics, it is the genotype of the follicle cells, not the oocyte or embryo, that determines whether the dorsal-ventral axis of the embryo is properly specified (Schüpbach, 1987).

To better understand the role of CR4 signaling in coordinating cell differentiation in maize leaves, we examined the cellular expression pattern of *cr4* by in situ hybridization and performed a genetic mosaic analysis. The results indicate that CR4 functions cell autonomously and is required in both the mesophyll and epidermis. Wild-type tissues bordering mutant sectors occasionally showed altered cell patterns, suggesting that lateral signals were disrupted in mutant cells.

**RESULTS**

**The cr4 Gene Is Expressed throughout Developing Leaf Tissues**

In situ hybridization was performed to examine the cellular pattern of *cr4* gene expression. Previous analysis demonstrated that *cr4* expression was highest in developing tissues of the shoot, decreasing with tissue maturation (Jin et al., 2000). As shown in Figure 1, *cr4* is expressed throughout the vegetative shoot apical meristem and young leaf primordia. The *cr4* transcript is distributed evenly in cells of the apical dome and the several youngest leaf primordia. As cells begin to mature, as evidenced by increased vacuolation, the signal intensity diminishes. This is first evident in the midrib region of the plastochron 3 leaf and progresses toward the margins. In plastochron 5, signal levels remain high in the leaf margins, vascular traces, and epidermes, whereas the signal is weaker in the mesophyll. By plastochron 6, only the epidermes show consistently strong signal with the parenchyma cells of some vascular traces also retaining high levels. Expression in the bundle sheaths was similar to the mesophyll. In the stem, strong signal becomes localized to the vasculature and epidermis within four nodes of the apex, with weaker signal throughout the ground tissues.

Two *cr4* mutants were examined. The *cr4-60* allele contains a *Mu* transposon in the region coding for the extracellular domain and shows no detectable transcript on RNA gel blots probed with 3’ regions of the *cr4* gene (data not shown). Consistent with this, no detectable signal was produced from in situ hybridization to *cr4-60* tissues (Fig. 1E), confirming that the probe was specific to the *cr4* transcript. The *cr4-651* allele contains a point mutation producing a premature stop codon in subdomain IX of the protein kinase domain. This mutation is predicted to eliminate kinase function and to delete the carboxy terminal domain from the protein but shows near-normal transcript levels on RNA gel blots (data not shown). In this mutant, the tissue-specific diminution of *cr4* transcript levels seen in wild type was less evident. The transcript levels remained relatively high and evenly distributed at least through plastochron 6, diminishing with no preferential expression in the epidermis thereafter (Fig. 1D).
Generation of cr4 Mutant Sectors

Previous analyses indicated that cr4 mutants could show defects in both the epidermis and the mesophyll, with the epidermis showing more pronounced effects. (Becraft et al., 1996; Jin et al., 2000). These studies showed that cr4 is required for the normal development of both tissues but left open the possibility that the site of action for cr4 was in one tissue and that differentiation of the other was controlled by secondary signaling. A genetic mosaic analysis was conducted to test this possibility. The strategy for generating cr4 mutant sectors is depicted in Figure 2. Seedlings heterozygous for cr4 linked to the chlorophyll deficient marker, oy, were treated with $\gamma$-rays to induce chromosome breakage. Breakage and loss of the chromosome 1OS arm carrying the wild-type alleles uncovers the recessive cr4 allele and the oy marker, resulting in sectors of cr4 mutant tissue, marked by chlorophyll deficiency, in an otherwise normal plant. Wild-type cells can be identified with fluorescence microscopy by the red fluorescence of chlorophyll, which is absent in albino mutant cells (Fig. 3). The epidermal genotype is inferred from the guard cells, the only epidermal cells to contain chloroplasts.

Experiments were conducted using two different oy alleles, oy and Oy-700. Sectors that were hemizygous for the recessive oy mutation are yellow because of a defect in chlorophyll biosynthesis; however, by fluorescence microscopy, mutant cells were difficult to discern because the chloroplasts showed only subtle changes in fluorescence color. Figure 3A shows an example of a sector containing mutant mesophyll and epidermis marked by the recessive oy allele. To avoid ambiguity, these sectors were not considered further and the experiment was repeated using the dominant Oy-700 allele as a marker. In the heterozygous state, Oy-700 confers a yellow-green phenotype but in the homozygous or hemizygous state, Oy-700 produces an albino phenotype that was readily distinguishable by fluorescence microscopy (Fig. 3, B–F).

Mutant Sectors Display a Typical cr4 Phenotype

The phenotypes displayed by mutant sectors showed all the attributes associated with cr4 mutants (Becraft et al., 1996; Jin et al., 2000). Cells were abnormally expanded, irregularly shaped, and disorganized (Figs. 2–4). Crenulations of neighboring epidermal cells may not match up or interlock (Fig. 4E) and the cell wall corrugations visible in Figure 4C probably correspond to the ridges noted in transmission electron microscopy (Jin et al., 2000). Regions of over-abundant cell proliferation were evident on some sectors (Figs. 2B and 3E). The epidermis showed the most striking effects of cr4 loss (Fig. 3, A,B, and E). As shown in Table 1, roughly one-half the sectors with a mutant phenotype only showed discernable defects in the epidermis. Of the sectors that included all cell layers, none showed mesophyll defects that did not also show epidermal defects.

The cr4 mutant epidermis also appears more prone to over-proliferation than the mesophyll. The arrows in Figure 3E denote cell walls that appear continuous with the inner epidermal walls of the adjacent wild-type tissues. This suggests that the cells to the outside of these walls are derived from the proliferation of epidermal cells. The proliferation appeared much more extensive in the epidermis than the mesophyll.

The phenotypic variability of cr4 mutants was evident in this study, both among and within sectors. Some sectors showed phenotypes that would have likely been lethal had the entire shoot been so effected, whereas almost one-third of the sectors that carried the hemizygous Oy-700 marker showed no discernible defects. Variability along the length of a single sector was common and one sector that extended for three nodes showed a strong mutant phenotype in two leaves but appeared normal in the third leaf. Figure 4, D through F, shows examples of
sectors that show phenotypic variation across the width of the sector, with some regions showing a strong phenotype and other areas with a weak phenotype. Each of these sectors was mutant throughout the thickness of the leaf, so the strong and mild regions did not correlate with different constellations of cell layer genotypes.

Cr4 Functions Cell Autonomously in Both Mesophyll and Epidermis

Mutant sectors displayed a cr4 mutant phenotype, regardless of sector width, and the phenotype was confined to mutant cells. Control sectors in plants that were heterozygous for the Oy-700 marker but homozygous wild type for Cr4 showed no morphological defects, indicating that the effects were not caused by hemizygosity for chromosome 10S or Oy-700. The results from sectors that did not include all tissue layers of the leaf further indicated that cr4 function is required autonomously in both epidermal and mesophyll tissues. Figure 3B shows an example of a sector where mutant epidermis overlies wild-type mesophyll. The epidermal cells show mutant morphology, indicating that the underlying mesophyll cells are not able to rescue their phenotype.

Although the mutant phenotypes of mesophyll cells are not typically as dramatic as epidermal cells (Fig. 3A), several examples clearly showed that cr4 function is also required in the mesophyll. Figure 3C shows a sector that had wild-type epidermis on both surfaces but the mesophyll was mutant throughout. The sectored area shows a noticeably reduced leaf thickness indicating that the mesophyll cell development was not completely normal in the absence of cr4 function, even though the overlying epidermis was wild type. Figure 3D shows an example where the mutant sector encompassed only the adaxial one-half of the mesophyll, and the mutant cells were smaller than normal despite their juxtaposition to normal epidermal cells. Thus, wild-type epidermal cells are not able to confer a normal phenotype on mutant mesophyll cells.

Our results were also consistent with a cell autonomous function of cr4 within the epidermal layer or the mesophyll, although this point cannot be stated definitively. The sector borders in the epidermis had sharp borders, with one cell appearing normal and

![Figure 3.](image-url)
the adjacent cell showing a mutant phenotype (Fig. 4A-C). However, in the epidermis, only the stomatal guard cells contain chlorophyll and can therefore be scored for genotype. No sectors were observed with a marked stomatal file directly adjacent to a wild-type stomatal file. Sectors were observed with a cell file containing marked guard cells and showing the cr4 mutant phenotype adjacent to a cell file with a normal phenotype, giving the appearance of cell autonomy. However, it is formally possible that the cell file with the normal phenotype could have been genetically mutant and been phenotypically rescued by nonautonomous functions in neighboring wild-type cells. Thus, although the sectors had the appearance of cell autonomy within the epidermis, this cannot be proved because of limitations in the marker system.

Within the mesophyll, mutant cells were readily identified by the albino marker but the cr4 mutant phenotype is difficult to recognize because the effect on cell morphology is subtle. Nonetheless, the results suggest that CR4 is likely to act cell autonomously within the mesophyll as well. Twelve sectors were observed that contained mutant cells through only part of the thickness of the leaf and yet still showed a mutant phenotype. Figure 3D shows a sector where the adaxial side of the mesophyll was mutant and the

Figure 4. Scanning electron microscope (SEM) images of sectors. A through C, Mutant sector showing a sharp delineation between the normal and mutant phenotypes. The area in the white box is enlarged in B, whereas the area within the black box is enlarged in C. The black arrowheads denote the first file of mutant cells with normal cells to the left and the mutant sector to the right. The sector boundary in B is offset by several cell files. The mutant cells in B show several different morphologies, including nearly spherical (e.g. to the right of the arrowhead) to irregularly shaped with scalloping caused by noninterlocking crenulations (white arrow). Some cells in C show additional defects including cell wall corrugations and several cells are collapsed. D, Sector where part of the sector showed a strong mutant phenotype, whereas some showed a mild phenotype. The arrowheads identify the genotypic sector boundary, with mutant cells to the left and normal on the right. The far left part of the sector showed a typical strong mutant phenotype, whereas the right-hand part of the sector appears nearly normal. The mild versus strong phenotypes did not correlate with the mesophyll genotype because both areas contained mutant underlying cells. E, The sector shows a gradation from severely mutant to wild type (on the right). The exact sector boundary is not readily distinguishable. F, Another sector showing two distinct severities of the mutant phenotype. There is an abrupt change from strong to mild at the cleft (white arrow) but a gradual change from mild to wild type (somewhere between the black arrows). The severity of the epidermal phenotype did not correlate with the mesophyll genotype. Size bars: A = 500 μm; B, E, and F = 100 μm; C = 50 μm; and D = 200 μm.
abaxial side was wild type. The mutant cells show a clearly decreased cell size, indicating that a partially wild-type mesophyll is not sufficient to confer a normal phenotype through the entire thickness of the leaf. Thus, if there are any non-cell-autonomous effects of CR4 on mesophyll cell phenotype, they occur over a very short range.

One impetus for this study was the possibility that defects noted in mesophyll tissues of cr4 mutants could have been secondary effects caused by physical stresses imposed by the deformed epidermis (Becraft et al., 1996). Although it is now clear that CR4 has a direct function in mesophyll development, it is also clear that a deformed epidermis resulting from a cr4 mutation can have indirect effects on the morphology of mesophyll cells. Figure 3B shows an example where one-half the sector is mutant in only the adaxial epidermis and yet the whole leaf is bent in that region. In addition, as shown in Table I, one individual that was mutant only in the epidermis showed cellular defects in the subtending wild-type epidermis. Therefore, in cr4 mutant plants, the cellular abnormalities observed in the mesophyll likely arise from a combination of direct and indirect effects.

### Table I. Summary of the sectors in the major genotypic and phenotypic classes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
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<tr>
<td>cr4</td>
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<td>cr4</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
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<td>n.a.</td>
<td>cr4/+</td>
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<tr>
<td>Controls</td>
<td>Oy/-</td>
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<td>+</td>
<td>Oy/-</td>
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*Epidermis (genotype ascertained by guard cell chlorophyll fluorescence).

b Mesophyll.

c cr4/+ represents sectors where the mesophyll was mutant partway through the thickness of the leaf and wild type the rest of the way.

d Sectors mutant only for the Oy-700 marker but not cr4.

**Table 1.** Summary of the sectors in the major genotypic and phenotypic classes

Mutant Sectors Show Enhanced Anthocyanin Pigmentation

We hypothesized that CR4 was involved in promoting epidermal cell fate. As such, we predicted that cr4 mutant epidermal cells might show reduced expression of an epidermal marker. Anthocyanins accumulate preferentially in epidermal cells of maize lines carrying dominant alleles of the B gene (Poethig et al., 1986); thus, we expected that cr4 mutant sectors would express less anthocyanin than wild-type tissues. To test this, three families were generated for the mosaic analysis in a B, pl/ background. pl makes anthocyanin accumulation sunlight dependent (Cone et al., 1993). Five sectors were generated in these lines and contrary to our prediction, four of the mutant sectors showed elevated anthocyanin accumulation (Fig. 5A), whereas one had no effect. Therefore, either our hypothesis that cr4 mutant cells do not acquire epidermal identity is incorrect, or B is not a marker of epidermal identity per se.

There was variability for the enhanced anthocyanin accumulation within sectors. One sector extended for six nodes and entered five leaf blades. The sector showed elevated pigmentation throughout the entire length in the culm and sheaths but only showed this effect in one of the five leaf blades. Another showed the effect in all but one of the five leaf blades it entered, whereas a third had enhanced anthocyanin in two of three leaf blades.

**Mutant Sectors Disrupt Spatial Patterns of Adjacent Cells**

Two types of sectors were observed that suggested that cr4 mutant cells were defective in communicating spatial cues with neighboring cells. The first type of sector produced a high density of macrohair trichomes along the sector border. Figure 6, A and B shows an example of such a sector on the abaxial surface of a leaf and Figure 6, C through E shows an example on the adaxial surface. The abaxial surface does not normally produce macrorhairs (Becraft, 1999). The hairs appeared to be produced by wild-type cells adjacent to mutant cells along the sector border. Three examples of hairy sector boundaries were observed, two in the abaxial and one in the adaxial leaf surface. The sectors included all cell layers in every case.

Two sectors were observed that showed ligule displacement from one side to the other (Fig. 5B). No ligule or auricle structures differentiated within the cr4 mutant sector. Plants with strong mutant phenotypes often show disrupted ligular regions with sporadic or discontinuous ligule formation (not shown).

![Figure 5](image_url) Leaves with mutant sectors showing unusual properties. A, Sector showing elevated anthocyanin accumulation in the cr4 mutant cells. B, Sector showing a displaced ligule. The ligule and auricles do not align normally across the sector; the ligule is displaced basally on the marginal side of the sector relative to the midrib side.
One sector occurred in a tiller and extended for six leaves. The sector was about 7 mm wide and located adjacent to the midrib in the blade of the first leaf. The lamina on the outside portion of the leaf was deleted (not shown). This suggests that the sector disrupted the ability of the leaf to coordinate the development of tissues from one side of the sector to the other. The sector was mutant in all cell layers and showed a \textit{cr4} mutant phenotype in every leaf. The other leaves in this sector were shaped normally and this effect was not seen with any other sector. Thus, although the \textit{cr4} mutant phenotype per se (i.e. abnormal cell morphology) appears cell autonomous, there are non-cell-autonomous effects on the development of neighboring wild-type cells.

**DISCUSSION**

The **Cellular Expression Pattern of \textit{cr4}**

Expression of the \textit{cr4} gene was observed evenly throughout the apical dome and early leaf primordia. By plastochron 3, the hybridization signal in the mesophyll and bundle sheaths declined in the midrib region and decreased progressively toward the margins as maturation, as evidenced by cell vacuolation, proceeded. Signal remained prominent in the epidermis near the leaf base through plastochron 7. We believe this pattern to be indicative of gene expression and not merely a consequence of vacuolation because in the \textit{cr4-651} mutant, there is no such preferential signal either in the epidermis or in the vascular traces. This expression pattern is also consistent with a role of CR4 in directing cell differentiation with a preferential requirement in the epidermis (Becraft et al., 1996; Jin et al., 2000).

In the \textit{cr4-651} mutant, which contains a point mutation but produces a strong mutant phenotype, transcript levels remained nearly uniform in primordial leaf tissues through plastochron 6. This suggests that expression of the \textit{cr4} gene might be regulated by a feedback mechanism. The wild-type expression pattern suggests that \textit{cr4} expression is regulated by, or at least correlates with, the maturation state of the cell. One possibility is that as cells differentiate and mature, under the direction of CR4 signaling, the level of \textit{cr4} expression declines. In the mutant with defective CR4 signaling, differentiation may be impeded, allowing prolonged expression of the \textit{cr4} transcript. A delayed rate of maturation was suggested by a developmental analysis of \textit{cr4} mutants (Jin et al., 2000). Another possibility is that the expression of the \textit{cr4} transcript could be directly regulated as a target of the CR4 signal transduction system. These possibilities await further examination.

The **Developmental Function of CR4**

Analysis of \textit{cr4} mutant sectors showed that the function of \textit{cr4} is required throughout the tissues of the shoot, and that the phenotype appeared to be cell autonomous. That is, wild-type cells could not rescue the phenotype of neighboring mutant cells. Thus, the differentiation response does not appear to be controlled by a secondary signal produced in response to CR4 signal transduction in neighboring cells. The fact that the mutant phenotype was confined to mutant cells also indicates that CR4 signaling does not negatively regulate a non-cell-autonomous signal that inhibits normal differentiation.

Although it is not surprising that a receptor would function cell autonomously, there are known examples where one signaling system regulates another, leading to a non-cell-autonomous phenotype. For example, activation of the \textit{D. melanogaster} EGF receptor (a Tyr receptor kinase), in dorsal follicle cells represses another signaling system that would otherwise specify ventral fate in the embryo (Nilson and Schüpbach, 1999). Thus, loss of Der function in the follicle cells leads to ventralization of the embryo. The mutual regulation of the Wingless and Hedge-
The CRINKLY4 Receptor Kinase Acts Cell Autonomously

This analysis reaffirmed the preferential function of cr4 in the epidermis. Of the 82 sectors that included both epidermis and mesophyll and that showed a mutant phenotype, 43 showed phenotypic defects only in the epidermis (Table I). Several sectors produced wart-like cell masses. In most cases, the tissue in these masses was too disorganized to determine the cellular origin but in several, the outgrowth could be ascribed to the overproliferation of epidermal cells (Fig. 3E).

Sixty-five sectors were obtained that displayed the Oy-700 marker but that did not show a cr4 mutant phenotype. Several factors probably contributed to this. The cr4 and oy loci are located 10 cM apart; therefore, some of these sectors could have arisen in recombinant individuals that no longer had the cr4 mutant allele linked to the Oy-700 marker. A more significant factor is likely to be the variability inherent in the phenotype of cr4 mutants (Jin et al., 2000). Within a mutant leaf, it is not uncommon to find areas that appear relatively normal. The cr4-R allele used in this analysis is a moderate-strength allele, caused by insertion of a Mu transposon, and is prone to this type of variation. That we observed large sectors showing no mutant phenotype and very narrow sectors that showed a strong phenotype argues against nonautonomous rescue of mutant cells by neighboring wild-type cells as the explanation for the normal phenotyped sectors. Furthermore, one sector that extended for three leaves showed a mutant phenotype in two leaves but looked normal in the third. This particular sector showed ligule displacement in one leaf.

cr4 Mutant Cells Are Defective in Lateral Signaling

Although the cr4 mutant phenotype per se appeared to be cell autonomous, several mutant sectors caused perturbations in patterning outside the sectors. Two sectors caused ligule displacement. A similar phenomenon was seen with liguleless1 (lg1) mutant sectors and it was concluded that the lg1 gene product was required to propagate an inductive signal that organizes the ligular region of maize leaves (Becraft et al., 1990; Becraft and Freeling, 1991). Thus, it appears that cr4 mutant sectors also occasionally disrupt this signal. One possibility is that CR4 is directly involved in propagating this signal. Another possibility, which we prefer, is that ligule displacement could be an indirect effect of the cr4 mutation disrupting the differentiation of the cellular machinery needed to propagate this signal. We favor this possibility because of the widespread cellular defects seen in cr4 mutants and because of the rarity of this occurrence.

Several sectors were lined along the borders with macrohair trichomes. Two of these sectors occurred on the abaxial face of the leaf where macrohairs do not normally form. The hairs appeared to originate from the wild-type cells bordering the mutant sector. Therefore, the mutant sectors either induced the neighboring cells to form hairs, or failed to inhibit macrohair formation. The phenotype is reminiscent of the phenotype of D. melanogaster mosaic for components of the notch signaling pathway. Notch signaling is important for the lateral inhibition that establishes the spacing pattern of neural precursors within the equivalent cells of the neural ectoderm. Disruption of the Notch signaling pathway causes the entire neurogenic ectoderm to adopt neural precursor cell fates at the expense of epidermal cells. Mutant sectors of delta, a ligand for the Notch receptor, contain dense clusters of misshapen sensory bristles (Heitzler and Simpson, 1991). Unlike the situation observed here, the extra bristles were confined to the mutant sector; normal cells rescued the mutant
cells along the sector boundary but mutant sectors had no effect on neighboring wild-type cells. Lateral inhibition is thought to be important for generating the spacing pattern of Arabidopsis trichomes (Larkin et al., 1996; Schnitter et al., 1999).

The maize leaf has at least two types of macrohairs. On the adaxial surface, associated with bulliform rows, are macrohairs that possess a multicellular base. On the leaf margin, macrohairs have no multicellular base. The hairs lining the sectors did not have a multicellular base. One intriguing idea is that the cells bordering the sector cannot sense the abnormally differentiated cr4 mutant cells. They would only detect cells on one side and thus perceive their position as at the leaf margin. This is speculative because the sector borders did not form fiber bundles like those found at true margins. In any case, it is clear that lateral signaling is important for specifying epidermal cell patterns and that this signaling is disrupted in the cr4 mutant tissue. Disruption of this signaling on one side of normal epidermal cells has the potential to cause an abnormally high proportion of epidermal cells to adapt a trichome cell fate. These results suggest that the disrupted cell patterning seen in mutant leaves might be an indirect consequence of the cr4 mutation rather than a direct function of CR4 signal transduction (Becraft et al., 1996; Jin et al., 2000).

Enhanced Anthocyanin Accumulation in cr4 Sectors

An interesting effect was observed when cr4 mutant sectors were generated in plants carrying a B allele that confers anthocyanin accumulation to leaves. The mutant sectors showed elevated levels of anthocyanin (Fig. 5A). This was the opposite of the expected result; we hypothesized that cr4 interferes with epidermal differentiation and because anthocyanin accumulates preferentially in the epidermis of B plants, we expected to see reduced pigmentation in cr4 sectors. Either our hypothesis was incorrect or B expression does not strictly mark epidermal cell identity.

The enhanced anthocyanin accumulation could result from several factors. First, anthocyanins typically accumulate in the vacuole (Marrs et al., 1995). Epidermal cells of cr4 mutants have altered vesicle trafficking, leading to or resulting from disturbed vacuole formation (Jin et al., 2000). It is not clear whether vacuoles are missing or are present as many small bodies instead of a single large central vacuole. If vacuoles were completely absent, then the cytosolic anthocyanin would be expected to be oxidized and have a brownish color similar to bronze2 mutants (Marrs et al., 1995). The light responsiveness of anthocyanin accumulation in a pl genetic background provides another possible explanation. It has not been reported what light wavelengths pl responds to but because anthocyanins are often cited as being UV protectants, it would make sense that they might accumulate in response to UV light. The cuticle normally forms barrier to UV and the cuticle is partially disrupted in cr4 mutant epidermal cells. Therefore, cr4 mutant cells could receive greater UV exposure, which might increase anthocyanin accumulation.

It was not possible to conduct a genetic mosaic analysis of Cr4 function in the endosperm. There are no suitable endosperm markers linked to the cr4 locus and the incomplete penetrance of cr4 in the endosperm results in sporadic phenotypes (Becraft et al., 1996; Jin et al., 2000). Therefore, genetic mosaic experiments would be uninterpretable. Whether the developmental function of CR4 is similar in endosperm and leaf cells will be determined when targets of CR4 signaling are determined in the two tissues.

MATERIALS AND METHODS

In Situ Hybridization

Shoot apices and young leaf primordia were fixed in 2% (v/v) formaldehyde, 5% (v/v) glacial acetic acid, and 45% (v/v) ethanol under vacuum for 2 h, stored for 2 d at 4°C, dehydrated with a graded ethanol/tertiary butyl alcohol series, and embedded in ParaPlast Plus (Fisher, Pittsburgh). Eight-micrometer sections were floated on poly-L-Lys-coated glass slides (Sigma, St. Louis) flooded with 1% (v/v) formaldehyde in water, drained, and dried overnight at 43°C. Sections were dewaxed in xylene, transferred to 100% (v/v) ethanol, and rehydrated in a graded ethanol-water series. Sections were then treated for 30 min at 37°C with proteinase K (Sigma) at 100 μg mL⁻¹ in 0.01 M Tris and 0.005 M EDTA (pH 8.0).

RNA probes for in situ hybridization were labeled with DIG-11-UTP using a nucleic acid labeling kit (Boehringer, Indianapolis). The plasmid cr4c-XES20, containing the unique carboxy terminal domain and the 3′-untranslated region of the cr4 cDNA inserted into pBluescript KS (Stratagene, La Jolla, CA), was linearized with XbaI or EcoRI and 1 μg used as a template to synthesize DIG-labeled RNA using T3 (antisense probe) or T7 (sense probe) polymerase, respectively. The RNA probes were subjected to mild alkali hydrolysis by heating at 60°C for 40 min in 100 mM carbonate buffer, and 4% of each labeling reaction was used as a probe in 40 μL of hybridization buffer (Ingham et al., 1985).

Slides were hybridized with the probes overnight at 50°C. Slides were washed in 2× SSC, 50% (v/v) formamide, 0.2× SSC, 50% (v/v) formamide, and 2× SSC at 60°C for 1 h each, followed by two rinses with 0.5 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and treated with 20 μg mL⁻¹ RNase A in this buffer at 37°C for 30 min. The slides were then washed in 2× SSC, 50% (v/v) formamide, and 0.2× SSC, 50% (v/v) formamide at room temperature for 1 h each. Immunological detection was as described (Jackson et al., 1994). The slides were rinsed in 130 mM NaCl and 10 mM sodium phosphate (pH 7.0). Slides were incubated with gentle agitation for 45 min in 0.5% (v/v)
Genetic Mosaic Induction

Ionizing radiation-induced chromosome breakage was used to uncover the recessive cr4-R mutant allele in heterozygous individuals. The cr4-R allele was marked with either oy or Oy-700. The oy locus is located approximately 10 CM proximal to cr4 on chromosome 10S (Stinard, 1992). The oil yellow (oy) gene is involved in chlorophyll biosynthesis (Mascia and Robertson, 1980; Polacco and Walden, 1987); oy1 is a recessive mutant allele that confers a yellow phenotype when homozygous or hemizygous. Oy-700 is a dominant mutant allele that produces a yellow phenotype when heterozygous and an albino phenotype when homozygous or hemizygous. Philip Stinard (U.S. Department of Agriculture-Agricultural Research Service Maize Genetics Cooperation Stock Center, Urbana, IL) and Tony Pryor (Commonwealth Scientific and Industrial Research Organization, Canberra, Australia) provided genetic stocks carrying the oy and Oy-700 markers, respectively. Each mutant oy allele was linked in coupling to cr4-R (Cr4+ Oy+ /cr4-R oy or Cr4+ Oy+/cr4-R Oy-700) and heterozygous seeds were γ-irradiated to induce chromosome breakage. Breakage and loss of the chromosome arm carrying the dominant wild type Cr4+ allele generated a sector of hemizygous cr4 mutant cells marked with the chlorophyll deficiency conferred by oy or Oy-700, in otherwise normal plants. As a control, Cr4+ Oy+/Cr4+ oy and Cr4+ Oy+ /Cr4+ Oy-700 seeds were used to generate hemizygous oy or Oy-700 sectors that were not mutant for cr4. Two sets of irradiations were conducted in consecutive years. The 1st year, seed was imbibed for 24 h at room temperature, placed in plastic bags, and put in a box that was 26 cm square by 4 cm thick. The box was centered 46 cm from a 137Cs point source and exposed to gamma rays for 15.5 h. The kernels were immediately hand planted in the field.

The second year, only the Oy-700 marker was used. Seeds were imbibed 40 h prior to irradiation and placed in plastic bags. Seeds were irradiated at the University of Iowa Radiation Laboratory (Iowa City) with a 137Cs rod source. Bags were laid flat beneath the source so that the seed formed a single layer. The seed received a dose of 10 gray over a period of 10 min. Approximately 3,000 test seeds and 1,000 control seeds were irradiated.

Microscopy

Sectors were examined by observing fresh hand sections with a BX-60 fluorescent microscope (Olympus, Melville, NY) equipped with a narrow violet filter (excitation 400–410 nm, dichroic mirror and barrier filter, 455 nm). In situ-hybridized slides were observed and photographed with bright-field and DIC optics. All microphotography was performed with an Olympus PM-20 photography system using Ektachrome 160T film (Kodak, Rochester, NY).

Scanning Electron Microscopy

Tissue was fixed in 2% (v/v) formaldehyde, 5% (v/v) glacial acetic acid, and 45% (v/v) ethanol, dehydrated through an ethanol series, and critical point dried. Samples were sputter coated with palladium in a Denton sputter coater and examined with a JEOLO580LV SEM operating at 10-kV accelerating voltage. Images were digitally recorded.

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LITERATURE CITED


The CRINKLY4 Receptor Kinase Acts Cell Autonomously


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