

ARTICLE

The Maize CR4 Receptor-Like Kinase Mediates a Growth Factor-Like Differentiation Response

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Summary: The maize *crinkly4* (*cr4*) gene encodes a predicted receptor kinase that is required for the normal differentiation of leaf epidermis (Beecraft *et al.*, 1996). Here we confirm that the presumptive cytoplasmic domain is capable of autophosphorylation on serine and threonine residues. The *cr4* transcript is most abundant in young developing tissues of the shoot but was not detected in the root. Phenotypic analysis of strong mutant alleles indicates that *cr4* functions throughout shoot development to control the differentiation of internal as well as epidermal tissues. However, epidermal cells appear most sensitive to perturbations in CR4 signaling because epidermal defects are more pronounced than internal cell defects. Epidermal cells show irregularities in shape, cell wall thickness and structure, cuticle formation, and vesicle trafficking. Some plants show tumor-like proliferations of cells on the leaves. Thus CR4 regulates an array of developmental responses including cell proliferation, fate, pattern, and differentiation suggesting a function analogous to growth factor responses in animals. *genesis* 27:104–116, 2000.

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INTRODUCTION

Determination of cell fate and the regulation of cell differentiation are complex processes that involve both specific and general signals that can occur globally or be highly localized. The interplay among general and specific regulatory signals controls the coordinated differentiation of the various cell types that comprise a given tissue. In animal systems, contact dependent signaling, short-range signaling and hormonal signaling can all influence cell fate decisions and control differentiation.

Growth factors are critical for controlling cell differentiation. They can function as either general or specific regulators. Growth factor receptors often regulate differentiation in a variety of cell types and the specificity of the response depends on the cellular context. For example, the *Drosophila* epidermal growth factor receptor (EGFR) regulates a wide variety of cell fate decisions, cell

differentiation, and patterning events throughout embryonic and larval development (see Schweitzer and Shilo, 1997, for review). Several different ligands interact with the receptor, but a common RAS/MAP kinase pathway appears to be activated in all cells. The RAS/MAP kinase signal transduction pathway regulates different molecular responses in different cell types, thus EGFR activation acts as a general trigger for a differentiation response that is specifically programmed in the responding cells.

Plant hormones such as auxin regulate many aspects of cell differentiation and have been intensively studied. These are small molecules that function combinatorially to regulate various cell differentiation responses in a tissue specific manner. For example, high auxin to cytokinin ratios inhibit lateral branch growth in the shoot but promote lateral root formation (Davies, 1995). For the most part, it is not yet clear how perception and transduction of these hormonal signals is achieved. Recently, several other classes of molecules, including peptides (Pearce *et al.*, 1991; Watanabe *et al.*, 1994; van de Sande *et al.*, 1996; Billington *et al.*, 1997) have been implicated in regulating plant cell differentiation.

Receptor protein kinases (RPKs) represent an important class of molecules that function in many signaling events, including growth factor responses. For example, both EGF and TGF- β receptors are RPKs (Schweitzer and Shilo, 1997; Massague, 1998). Several RPKs (often referred to as RLKs, for 'receptor-like kinases') are known to be important for plant development (Beecraft, 1998). BRI1 of *Arabidopsis* is necessary for perception of brassinosteroid hormones, which regulate cell expansion and light-regulated development (Li and Chory, 1997). Mutants in *bri1* have a light-grown habit when grown in the dark, and are severely stunted when grown in the light. CLV1 is an RLK necessary to maintain the

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proper balance of cell proliferation and differentiation in the *Arabidopsis* shoot apical meristem (Clark *et al.*, 1997). Mutants in *clv1* have abnormally large shoot apical meristems, resulting in altered phyllotaxy and extra organs in floral whorls (Clark *et al.*, 1993). The extracellular domains of most plant RLKs contain leucine-rich repeats, which are commonly involved in protein-protein interactions (Kobe and Deisenhofer, 1994). Several other motifs similarly suggest peptide signal ligands. The recently cloned *CLV3* gene represents the likely signal ligand for CLV1; genetic evidence places *CLV3* in the same developmental pathway as *CLV1* (Clark *et al.*, 1995), the 96 amino acid CLV3 protein is predicted to be secreted and processed to 78 residues (Fletcher *et al.*, 1999), and *CLV3* is required for formation of active CLV1 complexes (Trotochaud *et al.*, 1999). Similar evidence suggests that the *S-locus cysteine-rich* (SCR) gene encodes the ligand for the S-locus receptor kinase (SRK) involved in pollen self-incompatibility in *Brassica* (Schopfer *et al.*, 1999; Takayama *et al.*, 2000).

The maize *cr4* gene encodes a unique class of RLK. The extracellular domain contains a cysteine rich region similar to the cysteine rich repeats of tumor necrosis factor receptors (TNFRs) in mammals (Becraft *et al.*, 1996). A second motif consists of a 39 amino acid repeat present in seven copies, which has unknown function and no detectable similarity to known protein sequences. CR4 is necessary for proper differentiation of the leaf epidermis and the aleurone layer (Becraft *et al.*, 1996; Becraft and Asuncion-Crabb, 2000; Becraft *et al.*, 2000). Here we show that CR4 contains a functional serine/threonine kinase domain. Analysis of strong mutant alleles shows that CR4 also regulates the differentiation of internal cells in the both the plant and endosperm. Ultrastructural defects include abnormal cell walls and lack of vacuoles and altered vesicle trafficking. The array of processes controlled by CR4, including cell proliferation, differentiation, and patterning, indicates that CR4 functions in a signaling system analogous to growth factor signaling in animals.

RESULTS

CR4 Controls an Array of Developmental Processes

CR4 was reported to effect the differentiation of leaf epidermis (Becraft *et al.*, 1996). This analysis was based on the phenotype of the original *cr4-R* allele, which turned out to be a medium-strength allele. To further analyze the developmental function of the CR4 receptor-like kinase, additional alleles were examined (Table 1). Four additional insertional mutants were obtained by directed *Mu* transposon tagging (Becraft *et al.*, 1996) and six EMS alleles were obtained from M.G. Neuffer, Laurie Smith, and Lisa Harper, and from a mutagenesis in this lab. Although these mutants had similar phenotypes to the *cr4-R* allele, the severity of the phenotypes showed a range that included more severe. The assigned strength is based on the relative strength of the pheno-

Table 1
Mutant Alleles of *cr4*

Allele	Mutagen	Phenotypic severity	Source
<i>cr4-R</i>	<i>Mu</i>	medium	Don Robertson/Phil Stinard
<i>cr4-25</i>	EMS	medium-strong	Becraft lab
<i>cr4-30</i>	<i>Mu</i>	medium	Becraft lab
<i>cr4-58</i>	<i>Mu</i>	medium	Becraft lab
<i>cr4-60</i>	<i>Mu</i>	strong	Becraft lab
<i>cr4-98</i>	<i>Mu</i>	medium	Becraft lab
<i>cr4-590</i>	EMS	mild	M. G. Neuffer
<i>cr4-624</i>	EMS	medium	Lisa Harper/Laurie Smith
<i>cr4-647</i>	EMS	medium	M. G. Neuffer
<i>cr4-651</i>	EMS	strong	Lisa Harper/Laurie Smith
<i>cr4-1231</i>	EMS	mild	Lisa Harper/Laurie Smith

types of each allele after a minimum of four generations of backcrossing to the W22 inbred background.

As previously described, *cr4* mutants show characteristic leaf phenotypes consisting of enlarged, irregularly shaped epidermal cells (Fig. 1; Becraft *et al.*, 1996). This defect shows that CR4 is involved in regulating the normal morphogenesis and differentiation of epidermal cells. Further examination revealed a variety of defects in *cr4* mutants that indicate the gene is involved in the regulation of diverse developmental processes, other than epidermal differentiation. The occurrence of these defects was not allele specific; it is possible to observe any of the defects described below in any but the weak alleles. Thus, these defects are indicators of the overall function of the CR4 signaling system.

Mutants often show wartlike outgrowths on the leaf surface. In section these outgrowths have a tumor-like appearance; cells are large, undifferentiated, and disorganized (Fig. 1D). Although these are not true tumors in that they do not grow indefinitely, the overproliferation of cells in these areas indicates that growth is not properly controlled. Thus, CR4 regulates cell proliferation.

Occasionally, epidermal cell types are observed below the surface layer. Figure 1E shows an example of trichome and bulliform cells, which differentiated in the tissue layer below the epidermis. Multiple layers of cells with epidermal characteristics are found in a number of naturally occurring species. In such species, a multiple epidermis is distinguished from a subepidermis by cell lineage relationships. If all layers of epidermis-like cells are derived from the surface layer (L1) during leaf development, the plant is considered to contain a multiple epidermis. If the internal layer(s) of epidermis-like cells are derived from subsurface cells (L2) during leaf development, the plant is considered to contain a subepidermis (Esau, 1977). A lineage analysis has not been conducted to ascertain whether the *cr4* mutant should be considered to have a multiple epidermis or whether these epidermal cell types are forming subepidermally.

Strongly mutant leaves are sometimes distorted and may show internal deformations. In the original analysis, it was unclear whether the internal defects observed were a primary phenotypic effect of the *cr4* mutation or

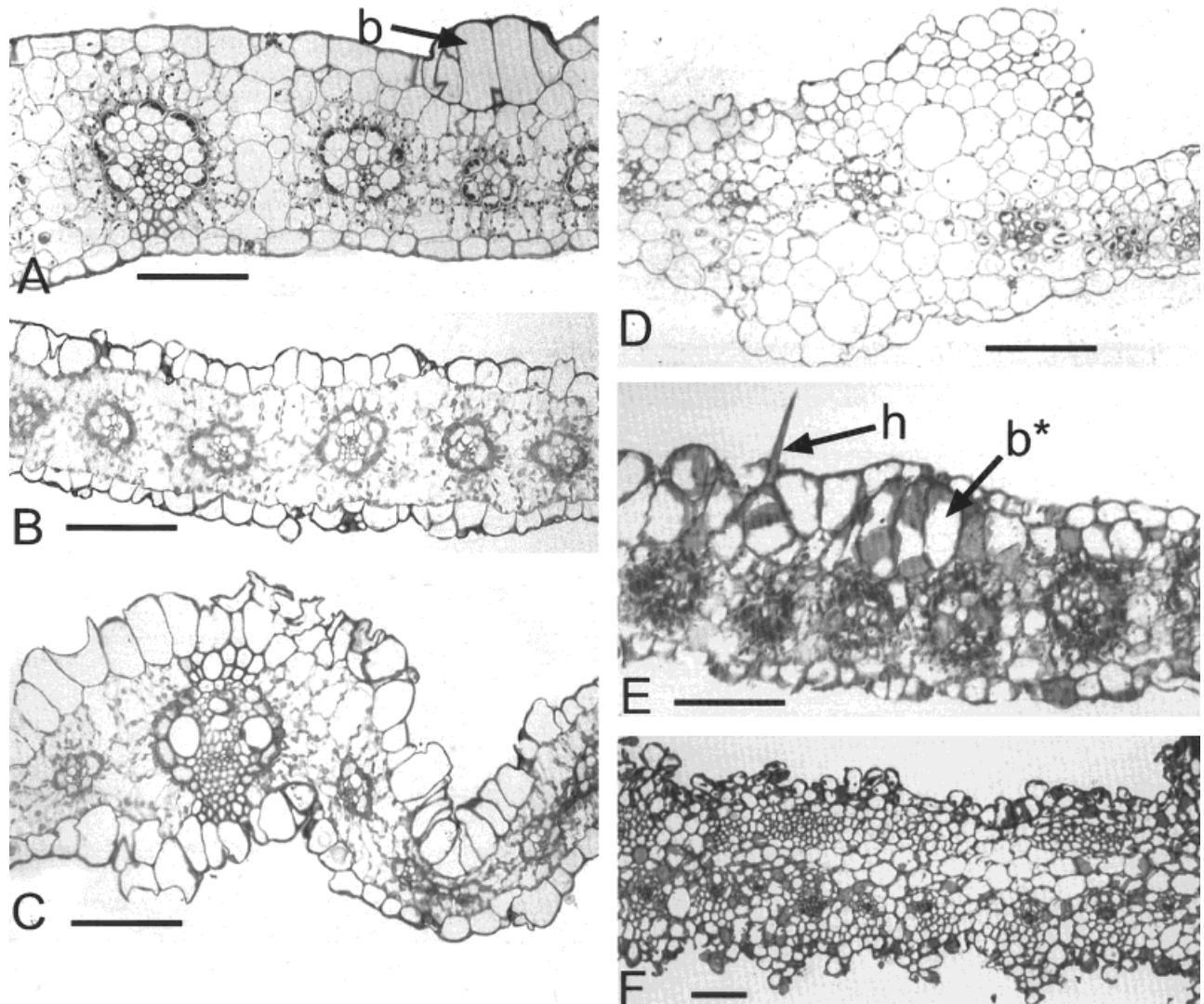


FIG. 1. Transverse sections of normal and *cr4* mutant leaves. **(A)** Wild type. **(B)** A mild mutant phenotype in a *cr4-60* leaf. **(C)** A strong mutant phenotype in a *cr4-60* leaf. **(D)** A tumor-like outgrowth on a *cr4-98* mutant leaf. **(E)** Epidermal cell types in subepidermal layers of a *cr4-98* mutant leaf. **(F)** A leaf lacking mesophyll and bundle sheath differentiation in a *cr4-624* mutant. There is no clear demarcation between the epidermis and internal cells. **(A–D)** are plastic embedded, **(E–F)** are paraffin embedded sections. b, bulliform cells; b*, putative subepidermal bulliform cells; h, hair (prickle hair). Scale bars, 100 μ m.

were secondary effects of physical distortions caused by the severe epidermal abnormalities (Becraft *et al.*, 1996). Further analysis makes it clear that CR4 is required for proper differentiation of internal cells. Severely mutant leaves lack several anatomical features including bundle sheaths (Fig. 1F). Other than the vascular traces, there is little organized structure in the transverse section of such leaves. These defects can be observed in regions that are not grossly distorted, indicating that they are not caused by physical stresses imposed by the epidermal defects. These data do not rule out the possibility that signaling from the epidermis could influence internal cell differentiation.

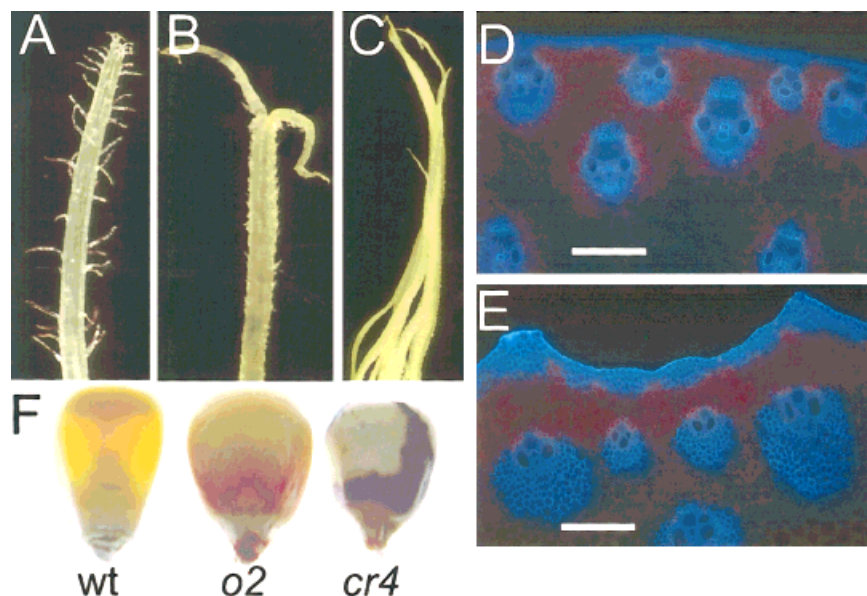
The epidermis appears to be more sensitive to perturbations in CR4 signaling than internal tissues. In *cr4*

mutants showing weak phenotypes, the leaf epidermis shows obvious abnormalities while the mesophyll appears nearly normal (Fig. 1B). In strong phenotypes, epidermal defects are generally more severe than internal defects (Fig. 1C). When examining the range of expressivity of any given allele, the same trend is noted with epidermal defects more pronounced than internal defects at any level of phenotypic severity (Fig. 1B,C).

CR4 Functions Throughout Shoot Development

All aerial organs examined showed phenotypic effects of the mutation. This included leaves, stems, tassel glumes, anthers and silks. Occasional epidermal fusions occurred which could either be between two like organs or different. For example, fusions are common among tassel

FIG. 2. Mutant phenotype of organs other than leaves. Individual silks of (A) wild type and (B) *cr4-651* mutant ears. (C) A cluster of adherent silks from a mutant ear. Cross sections through a wild type (D) and *cr4-58* mutant (E) stem, viewed by autofluorescence. (F) Transmitted light through wild type, *opaque2* (*o2*) mutant and *cr4-R* mutant kernels. The normal kernel is translucent while both mutants do not transmit light.



glumes but also can occur between a glume and leaf or between a glume and stem (not shown). Plant height is reduced because of shortened internodes. Mutant defects were also observed in internal tissues of the stalk. As shown in Figure 2, the fiber caps of the vascular bundles are missing at the phloem pole and are greatly enlarged at the xylem pole. The bundles also often show a greater prominence of xylem vessel elements in the mutant.

Silks each consist of two congenitally fused styles. Normal silks appear completely fused along their entirety except for a subtle indication of a suture at the tip (Fig. 2) but different silks do not fuse with one another. Papillar hairs appear periodically along the length of the silk. In *cr4* mutants, fusion between styles of an individual silk is reduced such that there is an unfused region at the tip and a suture is apparent along much of the silk. Papillar hairs are short and deformed and in severe cases, different silks are fused together.

The function of *cr4* is also required for proper development of subsurface cells in the endosperm. Strong expression of the mutant phenotype in kernels results in an opaque endosperm phenotype in addition to the mosaic aleurone (Fig. 2F). Opaque phenotypes commonly occur because of defective zein storage protein accumulation in the starchy endosperm cells and our preliminary results are consistent with this hypothesis (M.P. Scott and P.W. Becraft, unpublished).

The timing of *cr4* action during development was monitored by examining the phenotype of developing wild type and mutant leaves with SEM (Fig. 3). Subtle alterations in cellular morphology are evident in young leaf primordia as early as plastochron 2. Cells display irregular shapes and patterns of surface bulges indicating an improper pattern of cell expansion in the growing organ. These defects are particularly evident at the leaf

tip and margins. As the leaf primordium grows, the defects become more pronounced and by plastochron 4, are evident in all regions of the organ. The apical dome usually appears normal, although in very severe cases, the size can be reduced. Occasionally, the leaf primordia will fail to wrap completely around the dome (Fig. 3G) and this is due to a failure of the primordium to completely encircle the apex (not shown).

The cellular organization of the apical dome appears fairly normal in the mutant but defects are apparent in the subjacent tissues of the developing stem (Fig. 3F). Cells appear larger, more spherical, and loosely organized. The differentiation of procambial strands is delayed. In wild type, vascular differentiation is evident in a late plastochron 2 primordium whereas in the mutant shown in Figure 3, procambial strands are first evident in the plastochron 4 primordium. The extent of these defects was variable, but they have been observed repeatedly in several dozen sectioned mutant meristems.

Ultrastructural Aspects of the *cr4* Mutant Phenotype

Mature epidermal ground cells of adult wild type leaves are rectangular in transverse section, highly vacuolate and have a thick outer cell wall covered by a cuticle (Fig. 4). The outer epidermal wall is very uniform and under our growing conditions, averaged approximately 0.8 μm thick. The electron-dense cuticle is also very uniform. The wall proper has a laminar structure with several alternating light and dark staining layers. The wall separating adjoining epidermal cells is also laminar with a well-defined middle lamella between adjoining walls. The wall tapers in thickness from the thick outer wall to approximately 250 nm around the rest of the circumference of the epidermal cell. This thickness can be sub-

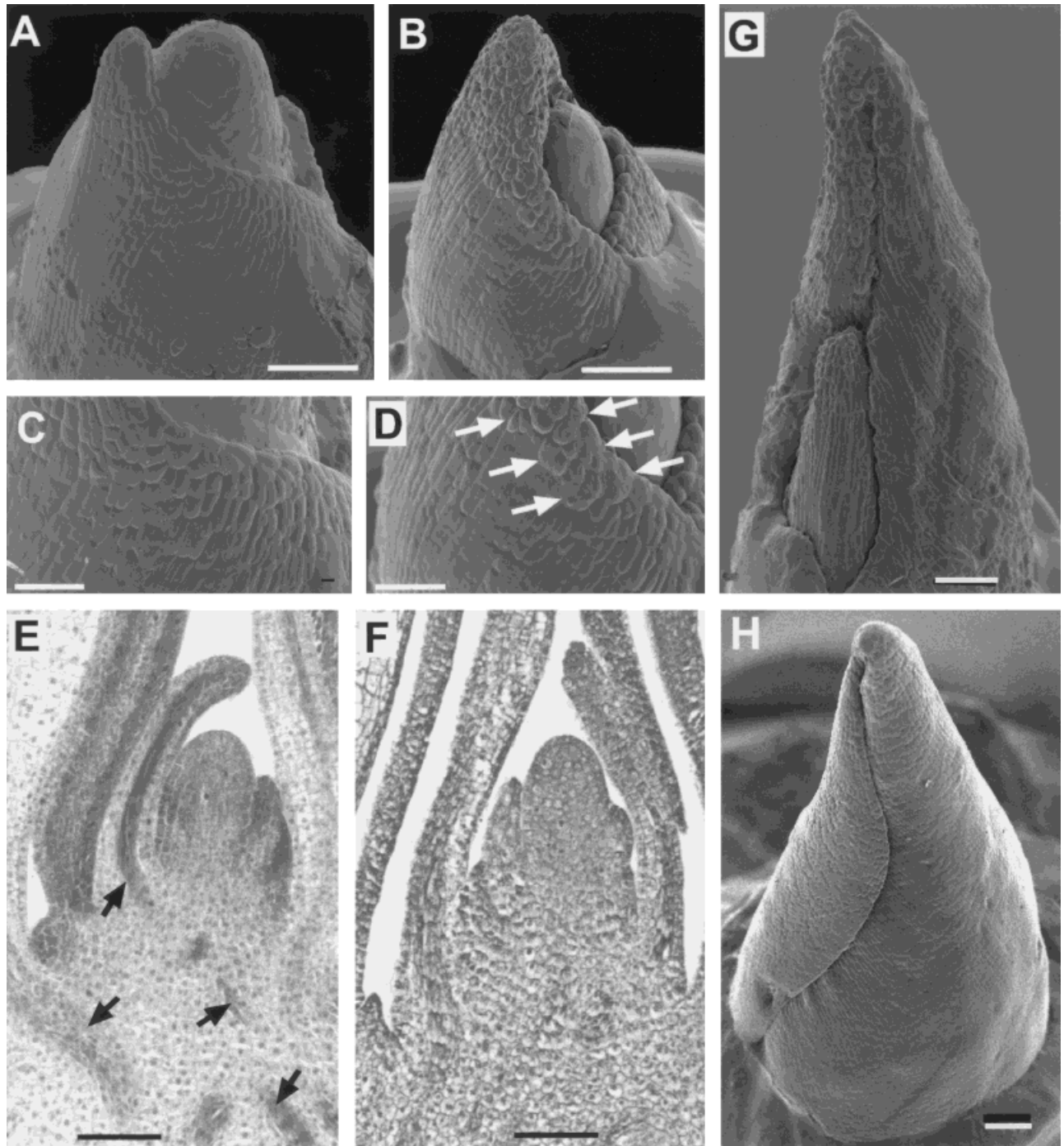


FIG. 3. The onset of cellular defects in *cr4* mutant shoot development. **(A,C,E,H)** Wild type shoot apices. **(B,D,F,G)** *cr4-651* mutant shoot apices. **(A–D,G,H)** scanning electron microscope analysis of shoot apical meristems and developing leaves. **(E, F)** paraffin sections of shoot apical meristems. **(A)** and **(C)** show a wild type shoot apical meristem and early plastochron 2 leaf primordium, while **(B)** and **(D)** show a *cr4-651* mutant. No defects are apparent on the surface of the apical dome but the leaf primordia show subtle defects in cell shape and cell surface morphology, particularly at the leaf tip and margins (region between arrows). The longitudinal section of a *cr4-651* mutant apex shown in **(F)** has no obvious abnormalities in the apical dome but the differentiating tissues in the developing leaf primordia and subjacent stem show evidence of disorganization and delayed onset of differentiation. Procambial strands (arrows) are not as prevalent in the mutant as in the wild type **(E)**. In **(G)** the plastochron 4 leaf primordium does not completely enclose the subsequent plastochron 3 leaf and both primordia show cells with obvious irregularities. A wild type plastochron 4 leaf primordium is shown in **(H)**. Scale bars 100 μm **(A,B,E–H)**; 50 μm **(C,D)**.

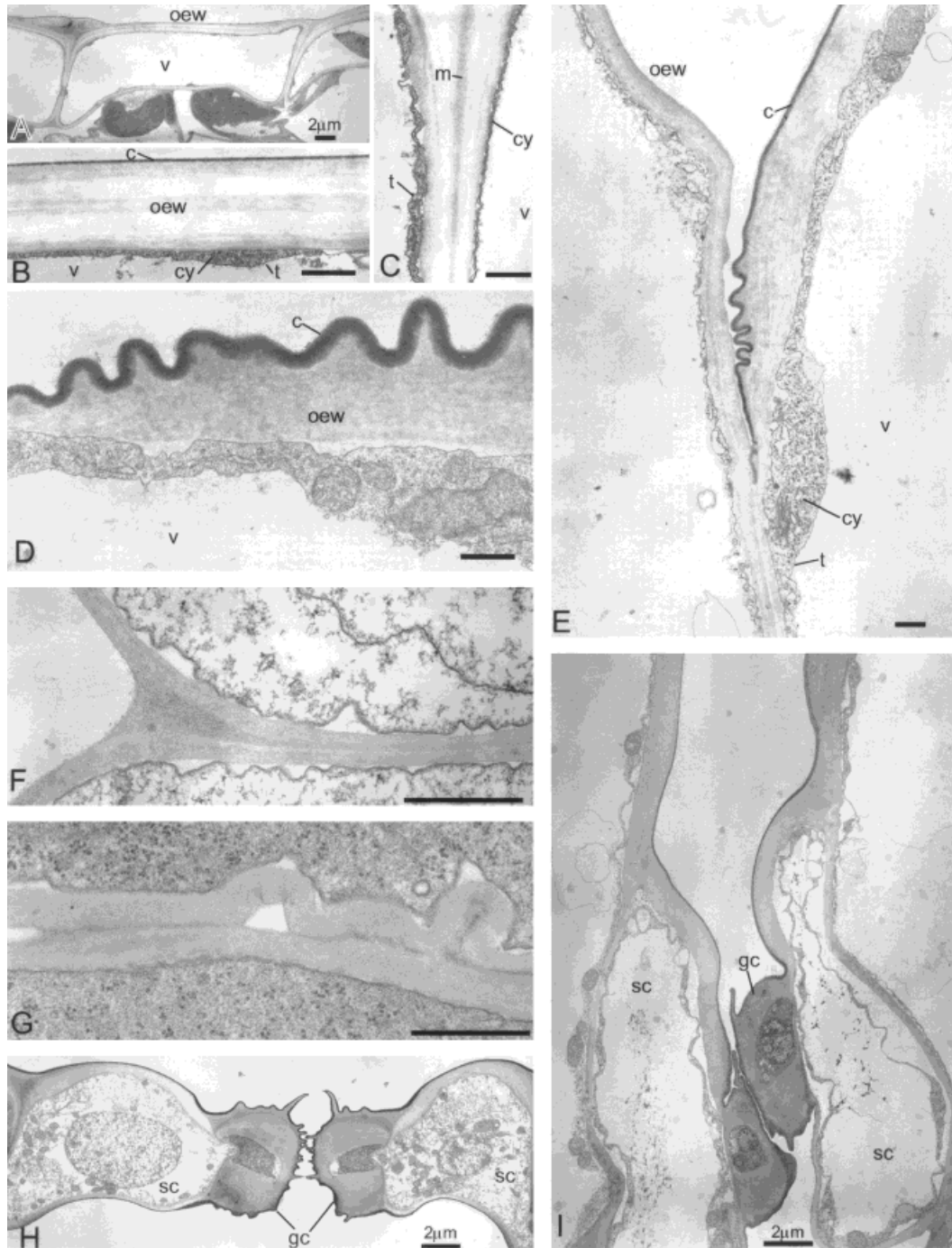


FIG. 4. Ultrastructure of wild type and *cr4-651* mutant cells. **(A)** Normal epidermal cell. **(B)** High magnification of the outer epidermal cell wall of the cell in **(A)**. **(C)** Cell wall separating the two wild type epidermal cells from the left side of the cell in **(A)**. **(D)** High magnification of a mutant epidermal outer cell wall showing unusual ridges and thickened cuticle. **(E)** Junction between adjacent mutant epidermal cells. **(F)** Cell wall separating two wild type mesophyll cells. **(G)** Cell wall between mutant mesophyll cells. **(H)** Wild type stomatal complex. **(I)** Mutant stomatal complex. c, cuticle; cw, cell wall; cy, cytoplasm; gc, guard cell; m, middle lamella; oew, outer epidermal wall; sc, subsidiary cell; t, tonoplast; v, vacuole. Size bars, 500 nm except where noted.

stantially reduced in specialized pit regions containing concentrations of plasmodesmata. The cytoplasm occupies a narrow region between the vacuole and cell wall.

The thickness was variable but typically was in the range of 50–200 nm. Little or no secretory activity or endocytosis was evident.

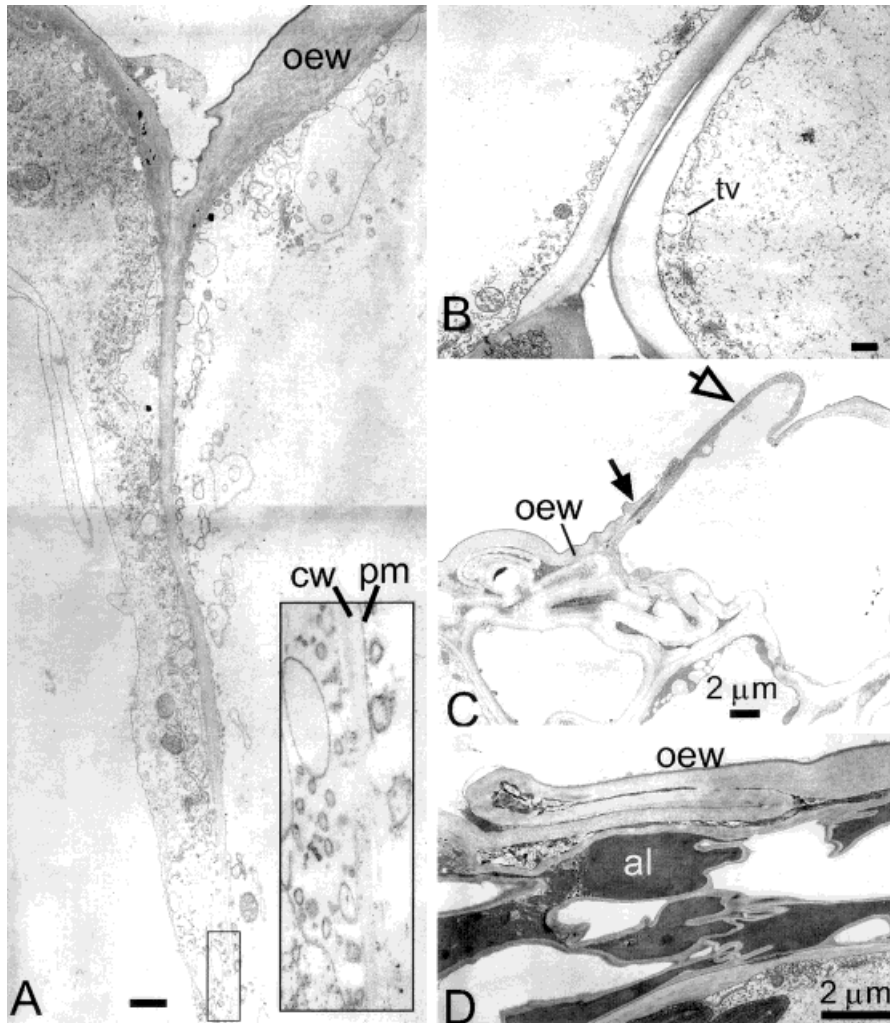


FIG. 5. Mutant defects in cell wall formation and vesicle trafficking. **(A)** The junction between two adjoining *cr4-651* mutant epidermal cells. In the lower portion of the figure, the wall becomes thin and structurally diffuse. The inset is a high magnification of the boxed region. **(B)** Junction between a pair of *cr4-60* mutant epidermal cells where the walls are separated. The cell on the right contains no tonoplast or vacuole. Both cells contain an abundance of transport vesicles. **(C)** A region of extremely distorted epidermis from a *cr4-60* mutant. The large cell in the upper right displays abnormal characteristics in the outer epidermal wall. The open arrow denotes a region where the wall is extremely thin and lacks a cuticle and the normal laminar structure. The black arrow highlights the spot where a relatively normal appearing wall terminates. The adjacent cell appears collapsed. There is a convoluted cell wall present, with characteristics of an outer epidermal wall, but the cytoplasm shows no intact subcellular structures. **(D)** A region of a *cr4-651* mutant leaf showing collapsed epidermal cells. The outer epidermal wall is folded back on itself and in places appears to have fused together. The cytoplasm of the collapsed epidermal cell and the subjacent cell show evidence of autolysis while the internal-most cell remains intact. al, autolysed cell; cw, cell wall; oew, outer epidermal cell wall; pm, plasma membrane; tv, transport vesicle. Size bars 500 nm unless otherwise noted.

Several striking features are apparent in mature epidermal cells of *cr4* mutant leaves. The epidermal cells possess highly variable and irregular cell walls. The walls may vary from cell to cell or in different regions within a cell. Figure 4E shows the junction between adjoining epidermal cells of the mutant. The cell on the right has an outer wall of nearly normal thickness and with an abnormally thick cuticular layer. The outer wall of the cell on the left is approximately half the normal thickness and has no apparent cuticle. In the cell in Figure 5C, part of the wall (black arrow) has a seminormal structure with a laminar wall and cuticle, while within the same cell other regions of the outer wall are very thin, have a highly abnormal structure, and lack a cuticle. The outer wall of a given cell may vary several-fold in thickness over the space of a few microns and some cells possess cell wall deposits in wavelike patterns of peaks and troughs (Fig. 4D). Every epidermal cell type, including stomatal cells, appears to be susceptible to these mutant defects (Fig. 4H,D). Walls between adjoining epidermal cells and between mesophyll cells also showed variable defects. These walls also often lacked the clear

laminar structure of normal walls. Whereas the walls adjoining wild type mesophyll cells were tightly cemented by the middle lamella, mutant cells sometimes show areas of separation between adjacent cell walls (Fig. 4G, 5B). Other cells showed what appeared to be incompletely deposited cell walls (Fig. 5A); the wall in certain regions was indistinct and in some cases it was not clear whether a plasma membrane was present. These might represent regions where adjacent cells were incompletely separated during cytokinesis.

A number of defects were apparent in the endomembrane systems of mutant cells. Many mutant epidermal cells showed evidence of transport vesicle activity. It was not distinguished whether these represent secretory vesicles or endocytic vesicles. Figure 5B shows a clear example of vesicles fused with the plasma membrane. Other cells show additional membrane bound vesicles, which may be secretory in nature (Fig. 4E, 5A). Additionally, some cells have no obvious central vacuole or tonoplast; the entire cytoplasm is filled with a diffuse granular material (Fig. 4E, 5A,B).

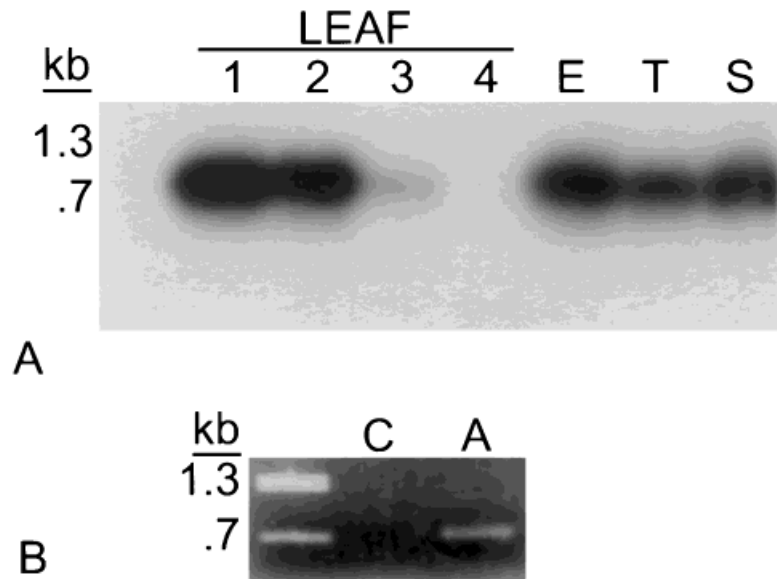


FIG. 6. Analysis of *cr4* RNA expression by RT-PCR in normal plants. **(A)** Analysis of aerial portions of the plant. Leaf samples were taken from regions of different maturity along the length of a single developing leaf blade. Lane 1 is from the actively dividing basal region, Lane 2 is a slightly distal region where cell differentiation was commencing as evidenced by the presence of macrohair primordia, Lane 3 is a differentiated region that was nearing the end of growth, and Lane 4 is a fully mature area. Lanes E, T, and S represent samples taken from developing ear, tassel and stalk, respectively. After 20 cycles, the amplification products were electrophoresed and the gel was blotted and probed with a labeled *cr4* cDNA fragment. **(B)** RT-PCR analysis of root RNA after 35 cycles of amplification. Lane C is amplification using the same *cr4* primers as in **(A)** and lane A is a positive control using actin primers to demonstrate that the reaction conditions were suitable for RT-PCR.

Irregular folds and involutions are evident in some cells and evidence of autolysis is also found in some mutants (Fig. 5C,D). This does not appear to be an artifact of fixation because the cytoplasm of neighboring cells in the same sample is well preserved and we have never seen similar effects in normal leaves that were processed simultaneously.

The *cr4* Gene Is Expressed in Developing Shoot Tissues

RNA was isolated from various plant tissues, including leaf, stem, tassel, ear, and root. A *cr4* transcript was detected by RT-PCR in all the shoot tissues but not in roots (Fig. 6). To verify the lack of message in the root, the RT-PCR sample was Southern blotted and probed with a radiolabeled *cr4* probe and still no signal was detected. The same oligo-dT primed cDNA that was used for the *cr4* RT-PCR was used to amplify *actin1*, verifying that conditions were suitable for RT-PCR.

RT-PCR was also performed on RNA isolated from various portions of developing leaves comprising different stages of differentiation. The youngest tissue consisted of the first 1 cm of leaf blade out from the base and showed no visible signs of epidermal cell differentiation. In the next stage, we sampled tissue where developing marginal hairs were just visible, followed by samples containing nearly fully expanded marginal hairs and fully mature tissue. The strongest amplification occurred in the youngest tissue and decreased with maturity until in

fully differentiated tissue no amplification product was detected. Thus, expression in leaves is highest during the early stages of cell division and differentiation, decreasing thereafter.

The *cr4* Gene Encodes a Functional Serine/Threonine Kinase

The function of the predicted kinase domain was tested biochemically by *in vitro* autophosphorylation. The CR4 kinase domain was expressed in bacteria as a GST fusion, GST-CR4K. The purified GST-CR4K fusion protein was phosphorylated in a kinase assay (Fig. 7). A substitution of Asp⁶⁵² to Ala⁶⁵² aborted this activity, indicating that the detected kinase activity was due to CR4 autophosphorylation and not a contaminant. This position corresponds to the essential Asp¹⁸⁴ in subdomain VII of the type α cAMP-dependent protein kinase catalytic subunit (PKA-C α) (Gibbs and Zoller, 1991). It is one of the 15 invariant amino acid residues in all functional protein kinases and forms part of the active site (Taylor *et al.*, 1992; Hanks and Hunter, 1995; Hanks *et al.*, 1988; Gibbs and Zoller, 1991).

Sequence analysis predicted that CR4 contained a serine/threonine protein kinase domain (Becraft *et al.*, 1996). Phosphoamino acid analysis verified that autophosphorylation occurred on serine and threonine residues (Fig. 7). These results indicate that CR4 contains a functional serine/threonine protein kinase.

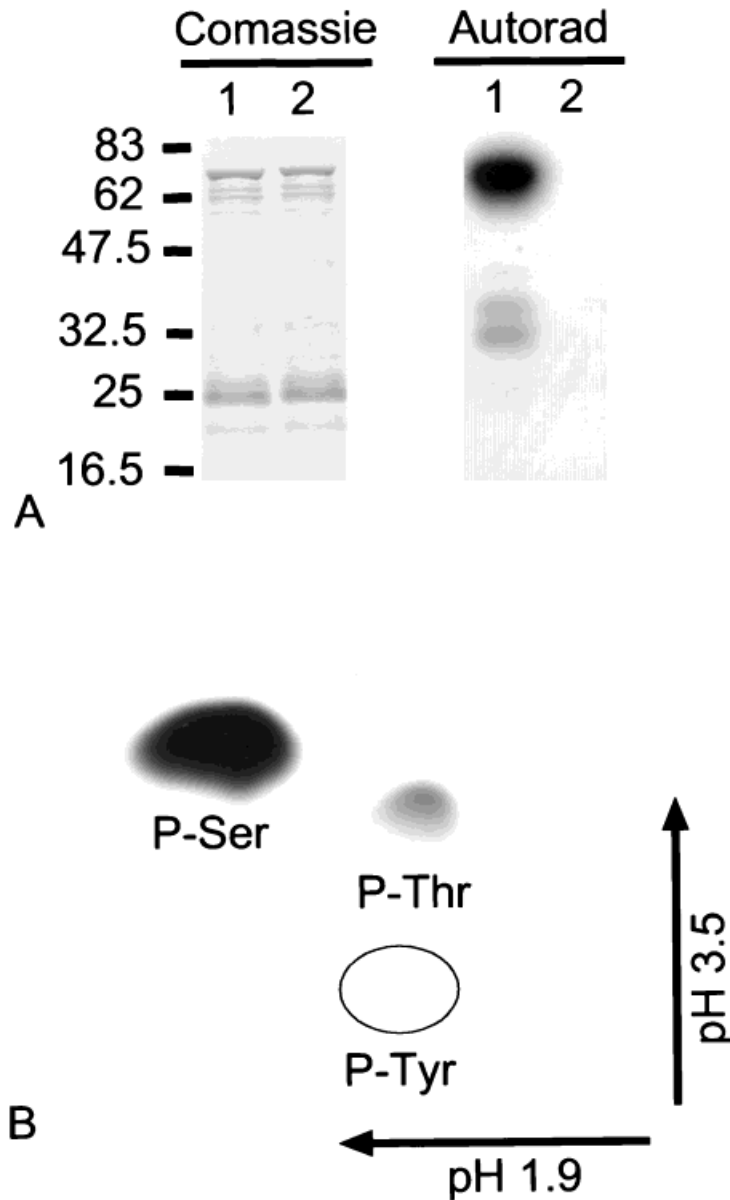


FIG. 7. Biochemical analysis of the CR4 protein kinase catalytic domain. **(A)** Autophosphorylation of the bacterially expressed kinase domain translationally fused to GST. Lane 1 is the wild type kinase domain and lane 2 contains a substitution of alanine for the essential aspartic acid residue 652. The purified protein was incubated with γ -[32 P]ATP and electrophoresed. The Coomassie stained gel and autoradiograph of the same gel are shown. **(B)** Phosphoamino acid analysis of the autophosphorylated CR4 kinase domain. The protein was acid hydrolyzed and subjected to two-dimensional thin layer electrophoresis. The autoradiograph revealed labeling of serine and threonine but not tyrosine.

DISCUSSION

CR4 Functions Throughout Shoot Development

Mutants in *cr4* have striking effects on the leaf epidermis and because of this it was unclear whether defects of internal tissues were secondary effects of physical distortions caused by the epidermal deformations. It is now clear that *cr4* is required for the proper differentiation of tissues other than the epidermis. Patterning of internal

cells of the stem is altered and in some cases, the tissues of the leaf blade show a dramatic lack of cell differentiation throughout, except possibly in vascular traces. These defects can be observed in regions that do not suffer from undue tissue distortions indicating that they are not a secondary consequence of epidermal deformations. These data do not discount the possibility that internal cell differentiation is governed by CR4-regulated signaling from the epidermis.

Although tissues other than the epidermis are affected by the mutant, the epidermis appears the most sensitive to perturbations in CR4 signaling. Every allele examined to date affects the epidermis more than the mesophyll. Weak alleles have obvious effects on the epidermis but not the mesophyll. Similarly, when considering the phenotypic variability of a given allele, weak expression always affects the epidermis while strong expression has greater effects on the epidermis than internal tissues.

Implications of CR4 Signaling in Development

The *cr4* mutant phenotype is complicated, but the overriding principle appears to be a failure of cells to properly differentiate. The mutant phenotype affects nearly all cell types of the shoot that were examined. In leaves, the differentiation of epidermis, mesophyll, and bundle sheaths are inhibited. In the stem, the cellular pattern of internal tissues is altered. CR4 action in the endosperm is not limited to the aleurone as first reported (Becraft *et al.*, 1996) but the differentiation of starchy endosperm cells is also affected as evidenced by a reduction in zein content in some mutant kernels (P.W. Becraft and M.P. Scott, unpublished). Since zeins accumulate in protein storage vesicles, a type of vacuole, the zein defect in the mutant might be a secondary consequence of a vacuole defect in endosperm cells, similar to that observed in the leaf epidermis. The array of phenotypic defects in *cr4* mutants indicates that the CR4 signal transduction pathway has a wide-ranging role in regulating cell differentiation.

One possibility is that CR4 signaling is involved in a general aspect of differentiation such as temporal regulation. In such a case, failure of cells to perceive the necessary cues would result in failure of cells to undergo the proper differentiation. Another possibility is that CR4 signaling regulates different responses that are specific to each cell type. The altered cell patterning of *cr4* mutants suggests a role in establishing spatial relationships although it is conceivable that mistiming of differentiation could leave cells in an inappropriate state to interpret spatial cues at discrete times in development. Such a temporal model has been proposed for specifying cell fate in maize leaves (Freeling, 1992; Sylvester *et al.*, 1996; Muehlbauer *et al.*, 1997).

The *cr4* gene encodes a functional serine/threonine kinase, as was predicted from the sequence (Becraft *et al.*, 1996). The proven functionality of the protein kinase domain and the similarity to a known ligand-binding domain strongly suggest that CR4 functions as a receptor kinase. As such, CR4 probably mediates a cellular response to an extracellular signal. Other components of this signaling pathway are not yet known. KAPP, a type 2C protein phosphatase that interacts with a number of plant receptor-like kinases of several classes, including CLAVATA1 from *Arabidopsis* (Stone *et al.*, 1998; Williams *et al.*, 1997) was unable to bind to the kinase domain of CR4 (Braun *et al.*, 1997). Experiments to test interactions with factors known to interact with other

plant receptor-like kinases and to identify unknown factors are currently underway.

The phenotype provides clues as to some possible targets of CR4 regulation, either direct or indirect. The failure of leaf primordia to fully encircle subsequent primordia suggests a defect in founder cell recruitment. Similar defects have been reported for *narrow sheath* (*ns*) and *rough sheath2* (*rs2*) mutants (Scanlon *et al.*, 1996; Schneeberger *et al.*, 1998). The *rs2* gene encodes a myb-like protein presumed to be a transcription factor (Timmermans *et al.*, 1999; Tsiantis *et al.*, 1999). The occurrence of subepidermal trichomes and bulliform cells in *cr4* mutants is reminiscent of the phenotype observed in *Arabidopsis* when *GL1* is overexpressed in a *try* mutant background (Schnittger *et al.*, 1998; Szymanski and Marks, 1998). *GL1* also encodes a myb-related transcription factor (Oppenheimer *et al.*, 1991). The cytological defects suggest additional possible downstream processes under CR4 regulation. Defects were noted in cell wall formation, cell adhesion and vesicle trafficking. One recurring theme to the *cr4* mutant phenotype is the disruption of cell adhesion; cells such as epidermal cells, which normally form very strong junctions, are incompletely joined, while ectopic adhesions form on cells such as in the epidermis of separate leaves. The autolytic cells observed in TEM suggest the possibility of misregulated cell death programs, although it has not been determined whether these cells resulted from apoptotic death or simply died as a consequence of other cellular defects.

The epidermis possesses properties that allow it to meet the unique demands of a surface tissue (see Becraft, 1999, for review). The thick outer wall and cuticle provide protection against biotic and abiotic stresses. The epidermis also serves critical developmental functions, including delineating individual organs during development by preventing organ fusions. Recent reports suggest that the cuticle has a key role in determining the consequence of epidermal cell contact. While epidermal cells are normally inert to contact-mediated fusion (Sinha, 2000), plants with mutations in genes for cuticular enzymes, or overexpressing cutinase, a cuticle degrading enzyme, often show postgenital organ fusion (Lolle *et al.*, 1997; Lolle *et al.*, 1998; Pruitt *et al.*, 2000; Yephremov *et al.*, 1999). The organ fusions observed in *cr4* could be related to the defective cuticle deposition.

CR4 Signaling Mediates a Growth Factor-Like Response

The function of CR4 is best understood by analogy to growth factor receptors in animal systems. Many mammalian growth factors were identified because of a specific effect on a particular tissue but were later found to participate in the regulation of many developmental processes. For example, TGF- β controls the differentiation of many different cell types throughout the development of most metazoans (Padgett *et al.*, 1998). Mutations in *punt*, the *Drosophila* TGF- β type II receptor, cause an array of defects in cell patterning and differentiation

including loss of dorsality in dorso-ventral patterning, a homeotic transformation of parasegment 7 to 6, and abnormal wing and leg patterning and eye morphogenesis (Ruberte *et al.*, 1995; Letsou *et al.*, 1995). Similarly, the *Drosophila* EGFR controls the fate of many different cell types at various stages of development (Price *et al.*, 1989). Such growth factors play central roles in regulating the coordinated proliferation, specification, differentiation, and programmed death of many different cell types during development.

One can also draw analogy to the tumor suppressor function of many growth factor receptors in maintaining a differentiated state and preventing cell proliferation. Mutations in the TGF- β receptor or other molecules in the TGF- β signal transduction chain are associated with several human cancers, including pancreatic cancer and colorectal cancer (Markowitz and Roberts, 1996; Padgett *et al.*, 1998). Cell proliferations are often observed in *cr4* mutants, sometimes forming localized warty outgrowths (Fig. 1). Although not tumors in the true sense, these outgrowths share several features with tumors; cells are abnormally large, irregularly shaped, and lack cytological characteristics of differentiated cells, and cell division is misregulated (Glover *et al.*, 1994).

In plants, the only factors known to regulate such general differentiation responses are plant growth regulators such as auxins (Creelman and Mullet, 1997; Kende and Zeevaert, 1997). Although aspects of the *cr4* mutant phenotype resemble hormone effects, none of the many reported hormone manipulations have produced the same array of effects seen in the *cr4* mutants. It is possible that *cr4* mutants disrupt a response to a known hormone that is too specific to have been identified physiologically but the very general defects in cell differentiation argue against this. An alternative hypothesis, which we favor, is that the signal molecule recognized by CR4 is an unknown class of plant growth factor, likely to be a protein. These issues will be resolved when the CR4 signal transduction system is better understood.

METHODS

Histology

All leaves examined were fully expanded adult leaves (leaf 10–12) of greenhouse-grown plants. Samples were fixed in FAA (10% formalin, 10% acetic acid, 45% ethanol) for 1 h, dehydrated in an ethanol/TBA series, and embedded in Paraplast Plus according to standard procedures (Berlyn and Miksche, 1976). Ten μm sections were stained with Toluidine Blue O. Alternatively, samples were fixed in 2% paraformaldehyde, 3% glutaraldehyde, 0.1 mM cacodylate, pH 7.2 overnight at 4°C, dehydrated in an ethanol/acetone series, and embedded in Spurr's resin. One μm sections were stained with Toluidine Blue O. Photomicrography was done with an Olympus BX-60 microscope.

Scanning Electron Microscopy

Samples were fixed in 2% paraformaldehyde, 3% glutaraldehyde, 0.1 mM cacodylate, pH 7.2 overnight at 4°C, dehydrated in an ethanol series, and critical point dried. Alternatively, replicas were made according to (Sylvester *et al.*, 1990). Perform Light low-viscosity dental impression medium was used to make molds of plant surfaces. A cast was made by filling the mold with Spurr's resin, placing under vacuum for 1 h and then heating to 70°C overnight. Replicas and critical point dried specimens were mounted on stubs and gold coated to 25 nm. Samples were viewed with a Jeol 5800LV SEM operating at 10 kV accelerating voltage. Images were digitally recorded.

Transmission Electron Microscopy

Samples were fixed overnight in a solution of 2% paraformaldehyde, 3% glutaraldehyde, 100 mM cacodylate pH 7.2. Samples were postfixed in osmium tetroxide, embedded in Spurr's resin, and sectioned to approximately 60 nm. Sections were stained with uranyl acetate and viewed with a Jeol 1200EX STEM at an accelerating voltage of 80 kV.

RT-PCR

Total RNA was isolated by grinding tissue in liquid nitrogen followed by extraction with Trizol reagent (BRL Life Technologies). First-strand cDNA was synthesized using 2.5 pmoles oligo-dT primer and 5 μg total RNA. Reverse transcription was performed with a RT-PCR kit (BRL Life Technologies) according to the manufacturer's instructions. PCR was performed using the pair of gene-specific primers. For *cr4*, 5 pmoles each of the following primers were used: DM277 GAGGAGATAAGACTGGATCAAG and DM265 GCACAAGCTTCGACATTG. For maize *actin1*, the primers TGGCATTGTCAACAACCTGG and CTCCTTGCTCATACGATCGG were used. Figure 6A was produced after 20 cycles of amplification while for figure 6B, 35 cycles of amplification were performed in a 25 μl reaction volume using a Biometra thermocycler. The products were gel blotted, hybridized with a ^{32}P labeled *cr4* cDNA probe and autoradiographed.

Leaf material was collected from greenhouse-grown W22 inbred maize plants. RNA was isolated from four regions of developing leaves, representing four stages of development, at the time of tip emergence. First was the proximal 1 cm of the leaf where no differentiation of specialized epidermal cell types are yet evident. Second is from 1–3 cm of the same leaf, where macrohair primordia and stomate initials are apparent. Next was 3–5 cm and most epidermal cell types have differentiated but the tissue is not yet fully green. Fourth was near the leaf tip where all cells are fully mature. Roots were isolated from germinating seedlings. Young tassels and ear primordia were obtained while spikelet primordia were still being formed near the tip. Growing stem tissue was taken from the first several nodes below the vegetative shoot apical meristem.

Kinase Analysis

The presumptive cytoplasmic domain (Leu⁴⁵¹-Phe⁹⁰¹) was expressed in *E. coli* as a GST fusion in the pGEX-4T3 (Pharmacia) vector. Expression of this fusion protein, GST-CR4K, was induced in a 75 ml culture with 0.5 mM IPTG (Sigma), and was purified from the cell lysate by binding to glutathione Sepharose[®] 4B (Pharmacia Biotech). While still bound to the column, the protein was incubated at room temperature for 30 min in kinase buffer (50 mM Tris-Cl, pH 7.5, 10 mM MgCl₂, 10 mM MnCl₂, and 0.2 mM [³²P]γ-ATP (Amersham)). The phosphorylated GST-CR4K was then eluted with 10 mM glutathione, pH 8.0 to a final volume of 1 ml. Twenty μl was subjected to SDS-PAGE and Coomassie stained followed by autoradiography.

As a control, Asp⁶⁵² was substituted with alanine. This substitution in the corresponding Asp¹⁸⁴ in the kinase subdomain VII of type α cAMP-dependent protein kinase catalytic subunit (PKA-Cα) has been shown to abolish protein kinase activity (Gibbs and Zoller, 1991). The mutation was introduced by PCR where a GAC to GCC codon change was incorporated by primer mismatch. This mutant kinase was also expressed as a GST fusion protein, GST-CR4Kmu and subjected to the kinase assay in parallel with GST-CR4K.

The autophosphorylated GST-CR4K was subjected to phosphoamino acid analysis. Using a modified protocol (Boyle *et al.*, 1991), 500 μl of the eluate was TCA precipitated, hydrolyzed in 6 N HCl for 1 h at 110°C, lyophilized, and loaded onto a thin-layer cellulose plate. The amino acids in the hydrolyte were separated by two-dimensional thin layer electrophoresis. Phospho-serine, phospho-o-threonine, and phospho-o-tyrosine standards (Sigma) were visualized by ninhydrin staining and radiolabeled amino acids resulting from CR4K autophosphorylation were detected by autoradiography.

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