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The *Arabidopsis ACR4* gene plays a role in cell layer organisation during ovule integument and sepal margin development

Miriam L. Gifford, Samuel Dean and Gwyneth C. Ingram*

Institute of Cell and Molecular Biology, Kings Buildings, University of Edinburgh, Edinburgh EH9 3JR, UK
*Author for correspondence (e-mail: Gwyneth.Ingram@ed.ac.uk)

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SUMMARY

The mechanisms regulating cell layer organisation in developing plant organs are fundamental to plant growth, but remain largely uninvestigated. We have studied the receptor kinase-encoding *ARABIDOPSIS CRINKLY4* gene and shown that its expression is restricted to the L1 cell layer of most meristems and organ primordia, including those of the ovule integuments. Insertion mutations show that *ARABIDOPSIS CRINKLY4* is required for regulation of cellular organisation during the development of sepal margins and ovule integument outgrowth. We show that *ARABIDOPSIS CRINKLY4* encodes a functional kinase that, in ovules and possibly other tissues, is abundant in anticlinal and the inner periclinal plasma membrane of 'outside' cells. We propose that ARABIDOPSIS CRINKLY4 may be involved in maintaining L1 cell layer integrity by receiving and transmitting signals from neighbouring L1 cells and/or from underlying cell layers.

Key words: L1, Cell layer, Integuments, Signalling, Receptor kinase, Ovule, *Arabidopsis thaliana*

INTRODUCTION

Plant meristems are composed of organised layers (files or plates) of cells arranged parallel to the 'outside' of the meristem. Each layer undergoes cell divisions in a defined plane or planes pushing cells to the periphery of the meristem where they are either incorporated into new meristems or become differentiated. In *Arabidopsis* the shoot apical meristem (SAM) has two outer tunica layers, the L1 and the L2, which undergo regulated divisions in the anticlinal plane. The inner cell layer or corpus is designated L3 and undergoes both anticlinal and periclinal divisions. As organ primordia arise on meristem flanks, changes in the regulation of cell division patterns occur. In dicotyledon leaf primordia, the epidermal cell layer is exclusively L1-derived and L1-derived cells continue to divide largely anticlinally until late in development. In contrast the L2 layer undergoes both anticlinal and periclinal divisions to contribute the leaf mesophyll, while the L3 contributes to both leaf mesophyll and the vasculature (Stewart and Burk, 1970). The contributions of meristematic cell layers to organ primordia vary. Whilst the *Arabidopsis* leaf is usually formed from L1-, L2- and L3-derived cells, petal primordia have been shown to contain cells of only L1 and L2 origin and ovule integuments are entirely L1 derived (Jenik and Irish, 2000). Integument cells undergo carefully regulated divisions, mainly in the anticlinal plane, so that the completed organ is a tubular plate of cells only 2-3 cells thick and effectively entirely epidermal (Schneitz et al., 1995; Robinson-Beers et al., 1992).

Experiments and observations in many plant species have shown that the developmental behaviour of cells in meristems and developing organs is largely dictated by their position rather than by lineage. Thus if the progeny of cells from one layer invade another layer during development, the displaced cells differentiate according to their new position (Stewart and Derman, 1975; van den Berg et al., 1995; Kidner et al., 2000). For this developmental plasticity to be achieved, cells must constantly receive and interpret information from their neighbours. Our understanding of how plant cell layers communicate is currently limited to a few specific examples. In *Arabidopsis* roots, an inside to outside movement of transcription factors (notably the SCARECROW (SCR) protein) is required for normal differentiation of ground cell layers (Nakajima and Benfey, 2002). In contrast, inter layer communication in shoot meristems appears to require the interaction of a diffusible ligand with a cell-autonomous receptor kinase complex (Fletcher et al., 1999). A similar interaction is invoked in the development of maize leaves and endosperm, where the receptor kinase-encoding *CRINKLY4* (*CR4*) and the calpain-encoding *DEFECTIVE KERNEL 1* (*DEK1*) genes are required for specification and maintenance of 'outer' cell layer identity during endosperm and leaf development (Becraft et al., 1996; Becraft et al., 2002; Lid et al., 2002). The maize *EXTRA CELL LAYERS 1* (*XCL1*) gene seems to be involved in pathways regulating division behaviour in L1 cells during organ formation. The *Xcl1* mutant provides intriguing evidence that cell identity can be uncoupled from positional cues at least late in development. (Kessler et al., 2002).

In a search to identify genes involved in inter-cell layer communication in *Arabidopsis*, a study of *ACR4,* an

Arabidopsis CR4 homologue, was carried out. *ACR4* was found to be required for normal cell organisation during ovule integument development and the formation of sepal margins. Both these tissues are formed exclusively from plates of L1 cells arranged back to back. By isolating the functional *ACR4* promoter, *ACR4* was shown to be expressed in L1 cells in all apical meristems and young organ primordia, including those of the developing ovule integuments. In addition, *ACR4* is expressed in an intriguing pattern in root meristems. The kinase activity of *ACR4* was demonstrated and, using fusion proteins expressed under the *ACR4* promoter, ACR4 protein localisation was visualised in vivo in the plasma membranes of L1-derived cells. The wide expression pattern of *ACR4* compared to its associated mutant phenotype may be a result of functional redundancy with other related proteins or functionally related pathways. Taken together, the data presented indicate a role for *ACR4* in the cellular signalling pathways required for correct cell organisation in ovule integuments and sepal boundaries, and may provide important clues as to the types of signalling involved in cell layer maintenance and specification in the wider context of plant development.

MATERIALS AND METHODS

Expression and complementation analysis of *ACR4*

The *ACR4* open reading frame (ORF) was PCR amplified from *Arabidopsis thaliana* genomic DNA ecotype Columbia (Col0) with CR5 (5′-TGGTACCTTTGAAAAGAATGAGAATGTTCG) and 5′-GAGCTCAGAAATTATGATGCAAGAACAAGC. The *ACR4* promoter was amplified with 5′-TGTCGACATAGTCAAGAAATGGCCTTTCC and 5′-TTCTAGACAAAGTCAACACACACGCTT. Products were cloned into pGEMT-easy (Promega) (pL92 and pL93 respectively). Probes (antisense and sense) for in situ hybridisation were made by linearising pL92 with *Nco*I or *Sal*I, respectively, and transcribing with Sp6 or T7 RNA polymerase, respectively. In situ hybridisations were carried out using a standard protocol (Jackson, 1991). For promoter expression analysis, the GAL4::VP16-encoding sequence and terminator were isolated from an enhancer trap vector (http://www.plantsci.cam.ac.uk/Haseloff/Home.html) (Haseloff, 1999) and transferred to the binary vector pSPTV20 (Becker et al., 1992). The *ACR4* promoter was inserted upstream of the GAL4::VP16 coding sequence (pL143). The *ACR4* promoter was placed upstream of H2B::YFP, by cloning the H2B::YFP-coding sequence from pBI121 (Boisnard-Lorig et al., 2001) into the binary vector pBIBHyg (pMD4) (Becker, 1990). The *ACR4* promoter was inserted upstream of H2B::YFP (pMD6). For deletion –1026 an *Xho*I-*Xba*I fragment from pL93 was cloned into pMD4 (pL227). For deletions –857 and –405, L93 was fully digested with *Xba*I and partially digested with *Hin*dIII. Appropriate fragments were cloned into MD4 (pL226 and pL225 respectively). To place the *ACR4* promoter upstream of mGFP6, an mGFP6-encoding fragment was cloned from pBSmGFP6 to pBIBHyg and the *ACR4* promoter was placed upstream (pL228). The mGFP6 variant is identical to mGFP5 (Haseloff, 1999) with two amino acid changes; F64-L and S65-T (J. Haseloff, personal communication). For protein localisation studies the full-length *ACR4* ORF was amplified with CR5 and 5′-GAGCTCGAGAAATTATGATGCAAGAACAAG, and mGFP6 was amplified from pBSmGFP6 with 5′-CTCGAGAAT-GAGTAAAGGAGAAGAAC and 5′-TCTAGTGTTTGTATAGTTC-ATCCATG so as to remove the ER retention signal. Green fluorescent protein (GFP) was cloned downstream of *ACR4* and the fusion protein-encoding fragment was then cloned into pBIBHyg. The *ACR4* promoter was then added (pMD11). For complementation studies the *ACR4* ORF was cloned into pBIBHyg. The *ACR4* promoter was added (pMD5). Plant transformations were carried out using *Agrobacterium* GV3101 (Koncz and Schell, 1986) and a floral dipping technique (Clough and Bent, 1998). Fluorescence studies were carried out using an Olympus Fluoview confocal microscope.

Expression of recombinant proteins in bacteria and kinase assays

To express recombinant GST fusion proteins in bacteria, the *ACR4* kinase domain was amplified using 5′-AGGATCCGTCCGGA-TCTTGATGAG and 5′-GAGCTCGAGTTTCCCATTAGCTGTGC, and cloned as an in-frame fusion with GST coding sequences in pGEX-3x (Amersham Pharmacia Biotech). Protein expression and purification using GST-sepharose (Amersham Pharmacia Biotech) was carried out according to the manufacturer's guidelines. Site directed mutagenesis was carried out using the QuikChange site-directed mutagenesis kit (Stratagene) with primer 5′- GGAACCACTGTTGCAGTGATGA-GAGCGATAATGTC and its reverse complement. GST fusion proteins were assayed for kinase activity by incubation in 30 μl (final volume) with 20 mM Tris (pH 7.5), 100 mM NaCl, 12 mM $MgCl_2$ with 10 μCi of [γ-^{32}P]ATP for 1 hour at room temperature. Samples were boiled in loading buffer and analysed by SDS-PAGE. Coomassie Blue-stained gels were dried and exposed to film.

Isolation and phenotypic characterisation of mutant alleles

To isolate *acr4-1* the Wisconsin collection was screened with oligos 5′-TGCCATCTCAGTACTTCATGACTCTCTCT and 5′- CTCTCTGC-CTCTTTGTTACTTTCCTGCCT as described previously (Krysan et al., 1999). The mutants *acr4-2*, *acr4-3*, *acr4-4* were identified on the Syngenta website (Sessions et al., 2002). To estimate insertion number, probes against the GUS marker gene or BAR selection gene were made by amplifying the GUS ORF with primers 5′-GTGGGAAAGCGCGT-TACAAGAAAGC and 5′-CACCATTGGCCACCACCTGCCAGTC or the BAR ORF with 5′-CGTACCGAGCCGCAGGAAC and 5′-ATCTCGGTGACGGGCAGGAC. For histological analysis, tissue was submerged overnight in 84 mM Pipes (pH 6.8) solution containing 4% acrolein, 1.5% glutaraldehyde 1% paraformaldehyde and 0.5% Tween 20. Tissue was rinsed several times in 100 mM Pipes and dehydrated using an ethanol series. JB4 resin was infiltrated into the tissue over a period of 2 weeks before embedding. 4.5 μm sections were stained in Toluidine Blue and visualised using a Leica standard light microscope. For creation of the *ATML1* marker line, the *ATML1* ORF was amplified by reverse transcription PCR and cloned into pGEMT-easy using oligos ATML1A and ATML1B (Abe et al., 2001). GFP was amplified using 5′-AGCTAGCATGAGTAAAGGAGAA-GAAC and 5′-AGCTAGCGTGTTTGTATAGTTCATC, and cloned pGEM-9z (Promega). The *ATML1* ORF was fused downstream of GFP and the fused construction was cloned downstream of the pAS99 *Hin*dIII insert [containing the full *ATML1* promoter (Sessions et al., 1999)] in pBIBHyg (pL178).

Brefeldin A experiments

Roots were incubated for 2 hours in 100 μM brefeldin A (BFA) (B7651, Sigma-Aldrich). The working BFA solution was made by diluting a 10 mM DMSO stock 1:100 in water. Control roots were incubated for the same period of time in a 1:100 dilution of DMSO in water.

RESULTS

ACR4 RNA is distributed in an outer cell layer specific pattern

Similarity searches were carried out using the maize CR4 (Becraft et al., 1996) protein against the annotated *Arabidopsis* genome. Five genes encoding predicted products showing sequence and structural similarity to CR4 were identified. One

predicted protein, encoded by *ACR4* (Tanaka et al., 2002), was considerably more similar to CR4 than the other sequences identified, both within the extracellular domain and the kinase domain. RNA in situ hybridisations were carried out to determine the distribution of *ACR4* transcripts in developing *Arabidopsis* tissues (Fig. 1). Embryonic *ACR4* expression was first observed at the eight-cell stage, throughout the eight cells of the embryo proper (Fig. 1A-D) and then became restricted to the outer cell layer (protoderm) of the developing embryo soon after the dermatogen stage. Expression was maintained at high levels in all protoderm cells until the early torpedo stage, when it diminished in non-meristematic cells. Cells of the embryonic root and shoot meristems continued to express *ACR4* at high levels until embryo maturity. No *ACR4* mRNA could be detected in the developing endosperm at any stage. Post-germination, *ACR4* transcripts were detected in the L1 cell layers of seedling apical meristems, inflorescence meristems (Fig. 1F), floral meristems and young leaf and floral organ primordia but decreased rapidly in older organs before cell expansion had initiated. Expression was also detected in ovule primordia, where it was initially limited to external cell layers as in other organs, and then detected in integument primordia. At maturity, expression in the ovule was most strongly maintained in the internal layer of the inner integument, the endothelium, although it was detectable throughout the integuments. In main and lateral root primordia, results were unclear although expression was observed in the outer (epidermal) cell layer of young roots in some transverse sections, and diminished as roots expanded. Transcript distribution at the root tip appeared strong in root-cap cells near the quiescent centre. In summary, *ACR4* transcripts were detected in all meristematic tissues tested and were, with the exception of roots, specifically localised to outer cell layers.

The *ACR4* promoter drives marker gene expression in patterns similar to RNA distribution

Because *ACR4* RNA expression levels were low, a two-component transactivation approach was used for promoter analysis. A 1.9 kb genomic fragment finishing at the presumptive ATG of the *ACR4* gene was placed upstream of a sequence encoding the chimaeric GAL4::VP16 transcriptional activator (Haseloff, 1999). Homozygous single-insertion transformants were crossed to plants containing a HISTONE 2B::YFP protein fusion encoding gene under control of a 35S minimal promoter and the GAL4-UAS (Boisnard-Lorig et al., 2001). In the immediate products of these crosses, nuclear-localised YFP was detected in embryos as early as 48 hours after pollination. Embryonic p*ACR4*-driven marker gene expression was protoderm localised, mirroring exactly *ACR4* mRNA distribution (Fig. 1E). The observation that *ACR4* is not expressed in the developing endosperm was confirmed. Post-germination expression patterns correlated with in situ hybridisation results in root, vegetative, inflorescence (Fig. 1G) and floral meristems as well as in leaf and floral organ primordia (Fig. 1H). In ovules all integument cells showed marker expression although expression was stronger in the ovule epidermis, the 'outer' layer of the inner integument, and the endothelium (Fig. 1I). *H2B::YFP* placed directly under control of the 1.9 kb *ACR4* promoter gave expression that was identical to, but weaker than trans-activated marker expression,

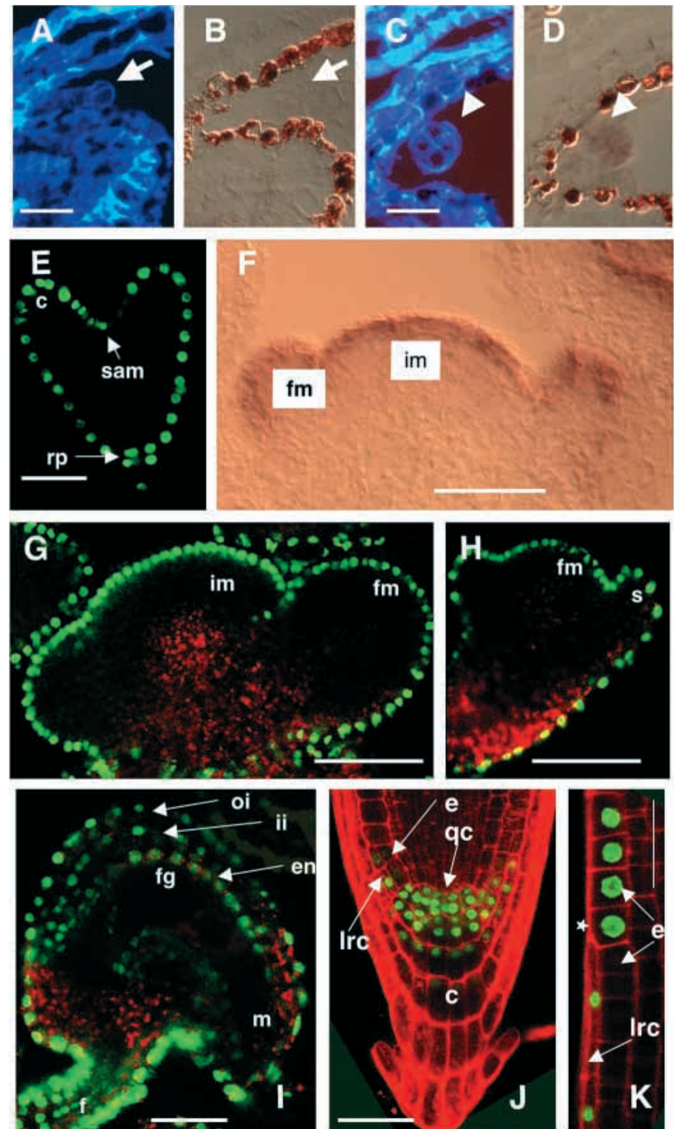

Fig. 1. Expression of *ACR4* during development. (A,C) Fluorescence images of two- to four-cell (A) and eight-cell (C) embryos (arrows). (B,D) In situ hybridisations of the same sections with *ACR4* antisense probe. Expression is detected as light brown coloration in the eight-cell embryos but not in two- to four-cell embryos. (E-H) *ACR4* expression in the L1 (outer) cell layer of developing embryo, inflorescence (im) and floral meristems (fm). In situ hybridisation (F) and confocal images (E,G,H) of H2B::YFP expression (green) in p*ACR4* transactivation lines. (I) In mature ovules expression is detected in the outer cell layer of the funiculus (f), the outer integument (oi) inner integument (ii) and endothelium (en). (J) In the root tip expression occurs in at least four columella (c) cells layers, the lateral root cap (LRC) and the quiescent centre (QC) but not in the epithelial cell file (e). (K) Expression in the root epithelial cell file (e) initiates as epithelial cells emerge from the LRC. c, cotyledon primordia; rp, embryonic root pole; sam, embryonic shoot apical meristem; fg, female gametophyte; m, micropyle. Scale bars: 25 μm except for K (10 μm).

confirming that the transactivation system amplified promoter activity without distorting expression patterns.

In the roots of plants transactivating H2B::YFP, marker

expression was observed in the quiescent centre (QC) central cells, columella initials and cells below the QC, the lateral root cap (LRC) and the initial cells destined to give rise to the root epidermal cell file and the LRC (Fig. 1J). However, expression was not observed in epidermal cells until the point where they emerged from under the LRC (Fig. 1K). This transition was sharp, with cells initiating expression as soon as they started to lose contact with the LRC. Expression in the root epidermis was maintained into the elongation zone, where it diminished. In more distal positions on the root, initiating lateral root primordia could be identified on the basis of their expression of H2B::YFP. Expression initiated in lateral root primordia at the four- to eight-cell stage, usually in a double file of cells (not shown). Expression in lateral roots resembled that observed in apical root meristems. The expression pattern of *ACR4* in roots differed from that in apical regions, firstly, in that a population of meristematic L1 cells (epidermal cell file under LRC) did not express *ACR4*, and secondly in that populations of 'internal' cells (QC, and lateral root primordium initials) expressed *ACR4*.

In contrast to in situ hybridisation results, H2B::YFP remained visible in developing organs until relatively late in development. To investigate this phenomenon, a sequence encoding a cytoplasmically localised version of mGFP6 was placed under the control of the 1.9 kb *ACR4* promoter. Lines expressing this construction showed expression in the same meristematic zones observed for lines expressing H2B::YFP, although fluorescent protein 'leaked' from outer cell layers into internal cell layers, especially in young embryos and floral/inflorescence meristems. GFP expression was not maintained in mature organs indicating that in some tissues H2B::YFP may persist in nuclei after gene expression has terminated.

The *ACR4* promoter is restricted to an 857 bp region upstream of the ATG

To determine the extent of the functional *ACR4* promoter, the 1.9 kb full-length promoter was reduced distally from –1849 (where –1 is the base before the ATG) to give a –1026, a –857 and a –405 deletion. These fragments were placed directly upstream of the *H2B::YFP* reporter gene previously described, and transformed into plants. Their ability to drive L1-specific expression was assessed in young roots, developing seeds and inflorescence meristems, and compared to that of the full-length promoter. Δ-1026 and Δ-857 both gave expression patterns identical to that shown by the full-length promoter in roots, embryonic and meristematic tissues (verified in 20 independent transformants). Δ-405 gave no detectable H2B::YFP expression (40 independent transformants screened). Thus all sequences required for normal *ACR4* expression were located in the first 857 bases of the promoter.

ACR4 is necessary for normal seed development

To gain material for functional analysis of *ACR4*, collections of T-DNA insertion lines were screened. One insertional mutant in *ACR4* was identified in the Wisconsin population (Krysan et al., 1999) and shown to be heterozygous for a double (back to back) T-DNA between bases 1066 and 1100 of the ORF. This allele was designated *acr4-1*. Three mutant lines were uncovered in the Syngenta collection (Sessions et al., 2002): the *acr4-2* allele contained a T-DNA insertion at base 249 of the *ACR4* ORF, *acr4-3* contained an insertion 570 bp downstream of the *ACR4* ORF and *acr4-4* housed two insertions in the *ACR4* promoter, one 1.6 kb and one 810 bp upstream of the start of transcription. PCR and subsequent Southern blot analysis confirmed that the progeny of heterozygous *acr4-1*, *-2*, *-3* and *-4* plants segregated wild-type, heterozygous and homozygous individuals in a 1:2:1 ratio. Southern blot analysis also showed that the *acr4-1* and *acr4-2* and backgrounds contained no other T-DNA insertions than those at the *ACR4* locus, but that both the *acr4-3* and *acr4-4* backgrounds contained multiple independently segregating T-DNAs. The positions of the insertions in *acr4-1* and *acr4-2* would be predicted to give strong mutant alleles and were therefore of particular interest for functional studies.

Segregating populations carrying *acr4-1*, *acr4-2*, *acr4-3* and *acr4-4* were analysed to identify potential mutant phenotypes associated with disruption of the *ACR4* gene. No differences in gross plant morphology between homozygous mutants and wild-type plants were noted in any of the four populations. However, all *acr4-1* and *acr4-2* homozygotes showed abnormalities in both the shape and texture of developing seeds. Instead of being elliptical and smooth, the developing seeds were rounded and rough in appearance. In addition, seeds were heterogeneous in their development compared to wild type, and siliques contained unfertilised ovules and aborted seeds at a rate of 40-85% (Fig. 2A,B). The developmental stage of seed abortion varied from just after pollination to just prior to maturity. When selfed heterozygous plants were analysed, no seed abnormalities were found, indicating that the phenotypes described were due to the maternal genotype. No seed defects were observed in the siliques of homozygous *acr4-3* and *acr4-4* plants.

To confirm that seed morphology and abortion phenotypes were entirely under maternal control, flowers from homozygous *acr4-2* plants were emasculated and pollinated either with self pollen, or pollen from heterozygous or wild-type siblings. Control flowers from heterozygous and wild-type siblings were either self pollinated or cross pollinated with pollen from the homozygous plant. Siliques from crosses onto heterozygous or wild-type plants were full of morphologically normal seed, independent of the genotype of the male parent (5 crosses of each). Self-pollinated siliques from homozygous plants were only 15-60% full, and contained seeds exhibiting the mutant phenotypes previously described. Siliques from crosses of wild-type or heterozygous pollen to a homozygous female presented identical phenotypes to self-pollinated homozygotes (10 crosses of each). In all cases mature seed germinated successfully and segregated homozygous, heterozygous or wild-type seedlings in the proportions expected, confirming that the embryo sac genotype plays no role in the seed phenotype observed.

To understand the developmental basis of the observed seed phenotype, ovule morphology in mutant plants was analysed. Mutant ovules displayed phenotypes of varying severity (Fig. 3B-D). All ovules showed epidermal irregularities, including abnormal cell size and shape, callus-like outgrowths, and occasional inappropriate cell types such as stomata. Ovules sometimes fused together (Fig. 3D). In most (>90%) of mutant ovules the abaxial zone of the integuments failed to elongate sufficiently to give the curvature seen in wild-type ovules. In some cases the embryo sac/nucellus protruded from the shortened integuments (Fig. 4H,J). In addition to disruption in

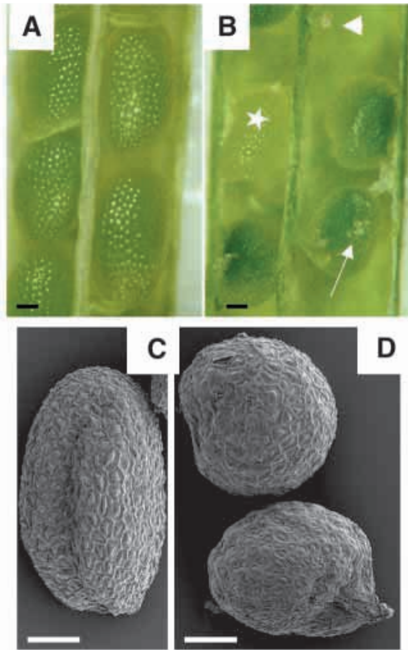

Fig. 2. *acr4* mutant seed phenotype. (A,B) Opened siliques showing differences in seed size and texture between a wild-type (A) and homozygous (B) plant at comparable stages (12 days after pollination). Aborted ovules (arrowhead), retarded seeds (star) and seeds with epidermal outgrowths (arrow) are frequent in mutant siliques. (C,D) Comparison between mature wild-type (C) and mutant (D) seeds observed by SEM. Scale bars: 100 μm.

ovule epidermal organisation, lack of organisation of integument cell layers was observed, with some ovules showing loss of cell layers, and others showing sporadic over-proliferation of integument cells. A varying proportion (20-50%) of ovules lacked a recognisable embryo sac (Fig. 3C,D). In extreme cases the endothelium was absent or reduced to a few disorganised cells. In other cases the endothelium cells enclosed differentiated/divided cells, or an empty space. In 30-50% of mutant ovules the egg apparatus (synergids, egg cell and polar nucleus) could be distinguished (Fig. 3B).

In order to ascertain at what stage ovule developmental defects first occurred, scanning electron microscopy (SEM) of developing ovules was undertaken. Wild-type development was as previously described (Schnietz et al., 1995; Robinson-Beers et al., 1992). Ovule primordia arose as bulges along the placenta, and developed into finger-like protrusions (Fig. 4A). Subsequently the inner and outer integuments initiated as two ring-shaped growths encircling the megasporocyte-containing ovule tip (nucellus), with the inner integument initiating just before the outer integument (Fig. 4C). Both integuments then elongated as sleeves of cells engulfing the nucellus, with the outer-integument growing faster than, and eventually overgrowing the inner integument (Fig. 4E,G). In *acr4* mutant ovules, development was normal until the point of integument initiation (Fig. 4B). However, instead of initiating as smooth ring-like bulges, the integuments of mutant ovules initiated unevenly, with some cell files bulging out, and others remaining flat. In many cases more than two sets of bulging cells could be seen in the proximodistal axis, and integuments did not initiate as coherent rings, suggesting that the points of integument initiation were not well defined (Fig. 4D). After initiation, mutant integuments appeared thicker than wild-type, and their more rounded cells gave developing ovules a rough texture (Fig. 4F). Integuments grew more slowly in mutant than in wild-type plants, and the leading edge of the integument, instead of being smooth, appeared disorganised. At maturity, even in the most ‘normal’ mutant ovules, integuments failed to fully enclose the nucellus (compare Fig. 4G with 4H). In some cases integument elongation either of one (Fig. 4I) or both integuments was severely compromised (Fig. 4J). Abnormal protruding cells were often observed on the surface of mutant ovules (Fig. 4H)

Defects observed in ovules were maintained in developing seeds when fertilisation had been possible. In particular, the texture of the seed coat was abnormal, with outgrowths observed, particularly in retarded seeds. A lack of proximodistal elongation of the mutant embryo sac after fertilisation caused the mutant endosperm to develop in a reduced volume giving seeds a round rather than elliptical shape (Fig. 2D). Although defects in embryo organisation were not observed, seeds with more severe defects in integument organisation were also retarded in embryo and endosperm development.

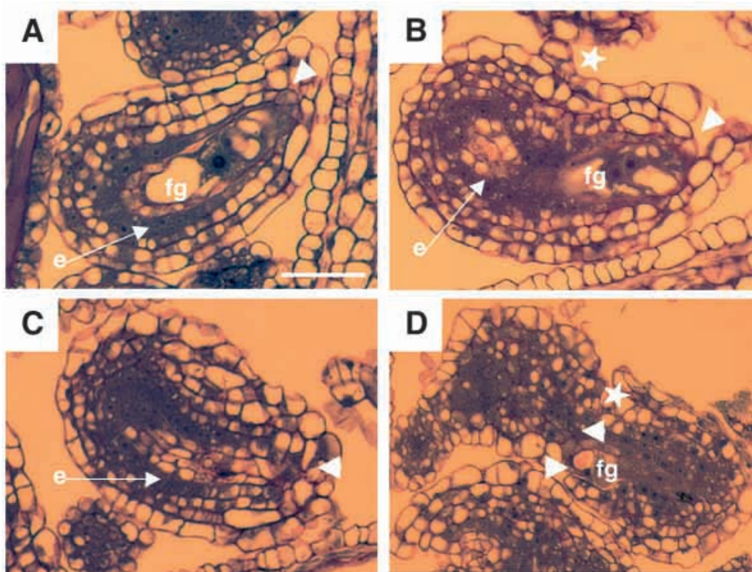

Fig. 3. The internal structure of *acr4* mutant ovules. Wild-type (A) compared with mutant (B,C,D) ovules. Micropyles are indicated by arrowheads. Female gametophytes (fg) and densely staining endothelial cells (e) are labelled where present. (A) In wild type neatly organised cell layers are visible, with the female gametophyte (fg) surrounded by an orderly endothelium. (B) Mutant ovule with weak phenotype. Fg is visible but outer integument is disrupted (to left of star). Endothelial layer is visible. (C) Mutant ovule with intermediate phenotype showing disorganised cell layers and replacement of fg with divided cells. (D) Two fused (star) mutant ovules with extreme phenotypes. Both show cell layer disorganisation but one has distinguishable (probably abnormal) fg. Scale bar: 25 μm.

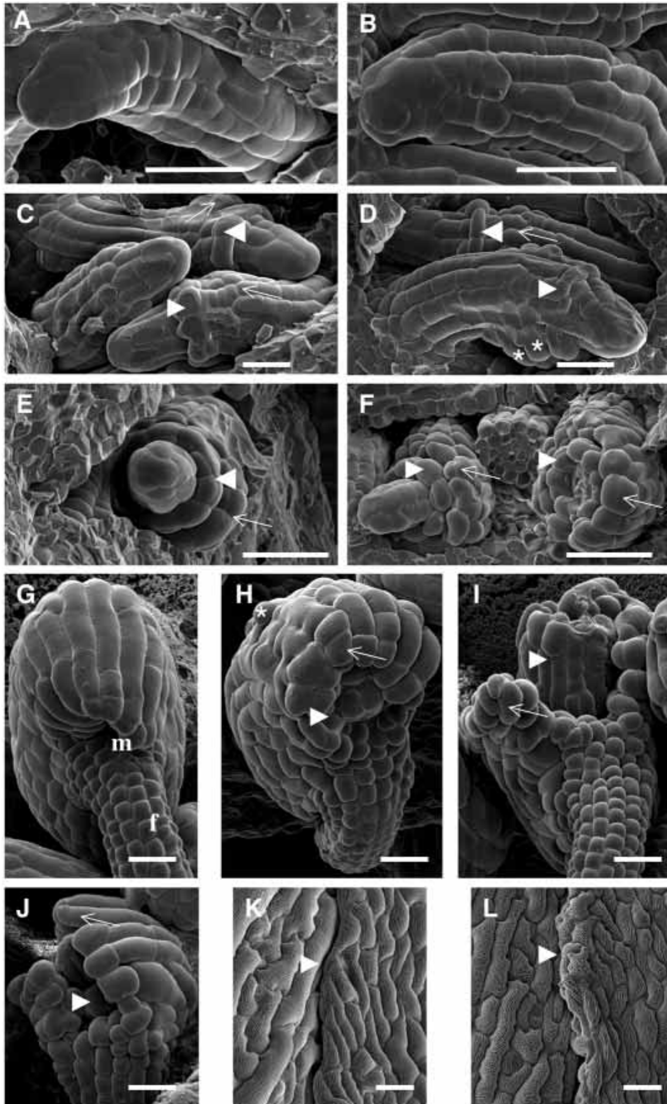

Fig. 4. Phenotypic analysis of wild-type and *acr4* mutant plants. (A,B) Ovule primordia immediately prior to integument initiation in wild-type (A) and mutant (B) plants. (C,D) The initiation of the inner (arrowheads) and outer (arrows) integuments in wild-type and mutant ovules respectively. In the mutant, the irregular initiation of integument outgrowth is visible, with at least two outgrowths observed in one region of outer integument initiation (asterisks), whereas other regions have no outgrowths. (E,F) Ongoing integument outgrowth. In wild-type ovules (E) the leading edges of the integuments are smooth, whereas in mutants (F) they are ragged and often retarded. (The nucellus has snapped off in the right hand ovule of F.) (G) A mature wild-type ovule at anthesis. The outer integument has overgrown the inner integument and nucellus to give a narrow micropyle (m) facing the funiculus (f). (H-J) A weak, a medium and a severe mature mutant ovule phenotype, respectively. In H, the retardation of outer integument (arrow) growth has left an open micropyle within which the inner integument (arrowhead) and nucellus are visible. In I the inner integument (arrowhead) looks relatively normal whereas the outer integument (arrow) has failed to elongate correctly. (J) The inner integument (arrowhead) has failed to grow out leaving the nucellus almost completely exposed. (K) Wild-type sepal margin (arrowhead) showing well organised border cells covered in cuticular decoration. (L) Mutant sepal margin (arrowhead) showing typical irregularities in cell organisation, 'lumpy' appearance and regions devoid of cuticular decoration. Scale bars: 20 μm.

Histological analysis supported the hypothesis that these seeds were those observed to abort.

To study the epidermal abnormalities observed in developing seeds, SEM analysis of mature mutant seeds was carried out. Although seed coat abnormalities were observed, particularly at the funiculus abscission scar and at the micropylar region, the majority of seed coat cells had a similar structure to those observed in wild-type seeds (Fig. 2C,D). Because homozygous seeds still differentiated appropriate epidermal cell types, and even in ovules, mis-specification of cell types (for example the presence of stomata) involved epidermal-specific identities, the expression of an L1 marker in mutant ovules was investigated. Homozygous *acr4-2* and *acr4-1* plants were crossed to marker lines expressing an N-terminal GFP::ATML1 fusion protein (unpublished results) under the *ATML1* promoter (Sessions et al., 1999). These lines expressed nuclear localised fusion protein in the L1-specific pattern previously reported for *ATML1* expression in embryos and meristems (Lu et al., 1996; Sessions et al., 1999). ATML1 fusion protein expression was observed in the outer cell layer and endothelium of mature ovules in wild-type plants, with weak expression occasionally observed in the inner cell layer of the inner integument. In *acr4* mutant ovules *ATML1* expression was similar to or more widespread than in wild type. In excrescences on the ovule surface, both protruding callus-like cells and underlying cells showed expression. Strong expression was sporadically seen in cells situated between the ovule epidermis and the endothelium. In several cases, the egg sac space was filled with expressing cells. This analysis suggests that although mutant ovule integument cells showed abnormalities in organisation, they did not loose their L1 identity.

Because *acr4* mutants showed abnormalities in ovule integuments, sepal margins, which have a similar structure (appressed layers of L1 cells) were examined in more detail. Although no major defects in sepal morphology were observed in *acr4* mutants, it was noted that the cells at sepal boundaries appeared less well organised than in wild-type plants, giving a somewhat ragged appearance (Fig. 4K,L). In general the border region was thicker (contained more cells) in the abaxial/adaxial dimension than in wild type, suggesting that outgrowth of sepal margins could be affected. Mutant margin cells were irregularly shaped and showed abnormal 'lumpy' areas and regions devoid of the cuticular decoration seen in wild-type cells. No defects at the margins of leaves or petals could be discerned.

Although two independent mutant alleles in two different backgrounds both gave identical phenotypes, a further confirmation that the observed phenotype was due to loss of *ACR4* function was obtained by genetic complementation of *acr4-2*. Homozygous mutants were crossed to hygromycin-resistant transformants carrying a full-length *ACR4* promoter driving the *ACR4* ORF. Four F_2 families corresponding to four independent transformants were selected on hygromycin and PCR-genotyped for homo- or heterozygosity of *acr4-2*. The phenotypes of homozygous plants were compared with those of heterozygous and wild-type plants in each case. For two families homozygosity of *acr4-2* plants was verified by Southern blot. For all four families full phenotypic complementation was apparent in immature and mature seeds of homozygous mutant plants, confirming that the observed mutant phenotypes were due to loss of *ACR4* function.

ACR4 encodes an active kinase domain

To establish whether ACR4 protein encodes a functional kinase, as predicted from its sequence, a GST fusion protein construct was engineered to express the *ACR4* kinase domain in bacteria. A 61 kDa protein encoding the GST-kinase was expressed and purified (Fig. 5). To act as a control in kinase assays, Lys 540 (a crucial amino acid in the kinase activation loop) was mutated to methionine. GST-kinase and GST-kinase-null proteins were subjected to in vitro kinase assays. The kinase domain showed phosphorylation that was absent in the kinase-null variant (Fig. 5). Incubation of the kinase domain with GST protein alone did not result in phosphorylation of GST, indicating that the kinase domain could autophosphorylate inter or intramolecularly in vitro (results not shown).

ACR4 fusion proteins localise to the plasma membrane and to intracellular bodies

Structural predictions indicated a plasma membrane localisation for ACR4. To test this prediction the entire *ACR4* ORF was fused at the C terminus in frame with GFP, placed under control of the complete *ACR4* promoter and introduced into plants. In order to test whether the fusion protein was being correctly localised, plants from two different expressing lines were crossed to homozygous *acr4-2* mutants, and F_2 plants were genotyped for the *acr4-2* allele. Full complementation of the *acr4-2* mutant phenotype was observed for one line, and partial complementation for the other line tested. Partially complementing plants showed reduced seed death, and a more normal seed shape, although seed texture was still abnormal. Expression of fusion proteins was detected in regions where H2B::YFP reporter expression had previously been observed (Fig. 6), and was identical in wild-type and in complemented homozygous mutant plants. Cellular localisation of fusion proteins varied from tissue to tissue. In some cells, for example those on the surface of ovules, most fluorescence appeared to be associated with plasma membranes (Fig. 6A). In the L1 cells of embryos, inflorescence and floral meristems and roots, plasma membrane localisation was observed, but fluorescence also localised to multiple small intensely staining bodies within cells (Fig. 6B,C,D,G). These bodies did not co-localise with red-fluorescing chloroplasts, but were the same size or smaller. To confirm that fluorescent protein was localised to plasma membranes rather than cell walls, roots were treated with 0.8 M mannitol to induce plasmolysis. Under these conditions fluorescence was pulled away from cell-cell boundaries, indicating that fluorescent proteins were indeed associated with the plasma membrane, rather than the cell wall (Fig. 6F). In order to further address ACR4::GFP localisation, the effect of brefeldin A (BFA) on protein localisation in roots was examined. BFA targets and inhibits the action of proteins involved in vesicle formation, thereby inhibiting vesicle trafficking within cellular membrane compartments and to and from the plasma membrane (Nebenführ et al., 2002). After treatment with BFA, ACR4::GFP localisation was compromised (Fig. 6G,H). The relative intensity of plasma membrane-associated fluorescence decreased, and instead of multiple small cytoplasmic bodies, one or two large fluorescent bodies were observed in each cell. An identical phenomenon has been observed using immunolocalisation of the auxin efflux carrier PIN1 in BFA-treated roots (Geldner et al., 2001; Geldner et al., 2003). The described result of BFA treatment on ACR4::GFP localisation supports the hypothesis that ACR4 is usually exported to the plasma membrane via the ER and Golgi, and that this export, or possibly some form of recycling, is inhibited by BFA. It seems likely that the cytoplasmic bodies observed in cells not treated with BFA correspond to elements of the endomembrane system, such as excretory vesicles or endosomes.

In all tissues studied, fusion protein was present in plasma membranes adjacent to both anticlinal and periclinal cell walls, although the degree of localisation adjacent to periclinal cell walls was variable. In root meristems (QC and root cap initials) localisation was observed uniformly in both anticlinal and periclinal plasma membranes (Fig. 6E). In cells situated on the surface of the plant, the amount of protein visible in plasma membranes adjacent to the outer periclinal cell wall appeared lower than that on anticlinal and inner periclinal cell plasma membranes (Fig. 6D,I). This phenomenon was particularly noticeable in the outer cells of ovule outer integuments where all cells expressed fusion protein, although this could in part be due to the additive signal from two appressed internal membranes (Fig. 6I).

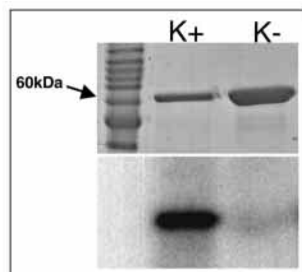

Fig. 5. ACR4 kinase activity. Coomassie Blue-stained gel (top) showing products of kinase assay on the wild-type ACR4 kinase domain (K+) and kinase null variant (negative control, K–). Autoradiography of this gel (below) shows that the native kinase domain has kinase activity whereas the kinase null variant does not.

DISCUSSION

ACR4 regulates the organisation of L1-derived ovule integuments and sepal margins

Despite the wide ranging expression pattern observed for *ACR4*, probable null mutants only show defects in two tissues; ovule integuments and sepal boundaries. Characterisation of mutants in several genes affecting integument development including *INNER NO OUTER, SHORT INTEGUMENTS 1, SHORT INTEGUMENTS 2, BELL, AINTEGUMENTA, ABERRANT TESTA SHAPE* and *NOZZLE*, has shown that integuments play an important role in female gametophyte development and maturation (Reiser and Fischer, 1993; Villanueva et al., 1999; Robinson-Beers et al., 1992; Broadhvest et al., 1999; Baker et al., 1997; Schneitz et al., 1998; Balasubramanian et al., 2002). In particular the presence of an intact endothelial cell layer is crucial, possibly because nutritionally and developmentally important substances are channelled to the gametophyte through this specialised cell layer (Kapil and Tiwari, 1978). We observed no defects in ovule development until integument initiation, when megaspores usually initiate meiosis, suggesting that the

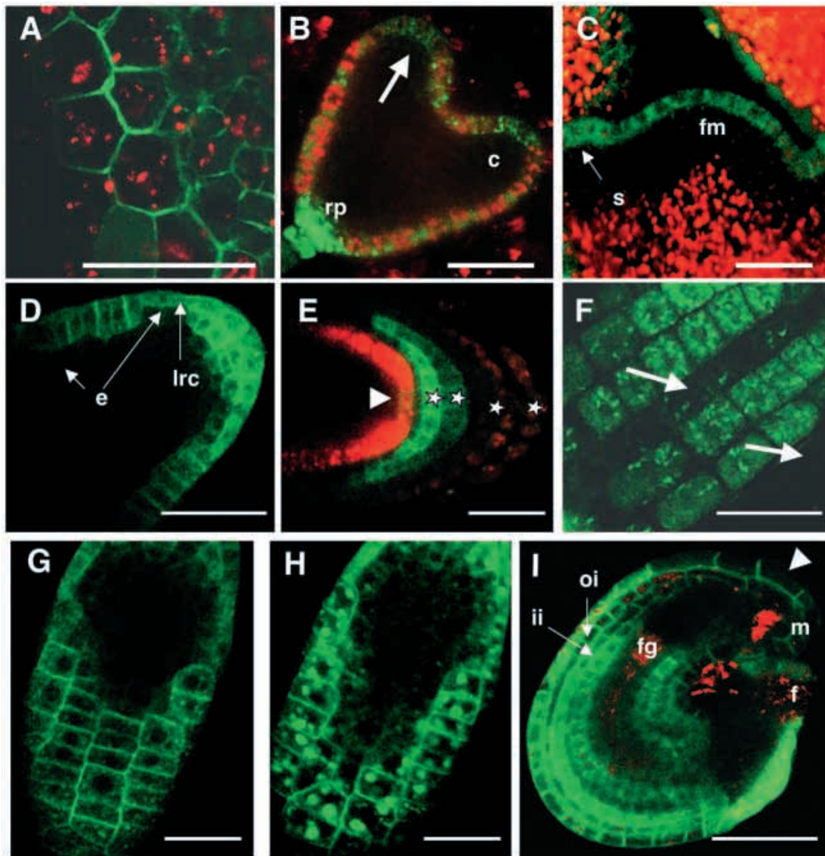

Fig. 6. Protein localisation in lines expressing an ACR4::GFP protein fusion. (A) localisation of fusion proteins (gren) in the plasma membranes of the ovule epidermis. (B,C) Expression patterns of fusion proteins (green) in embryos (B) and meristems (floral meristem, fm) (C). Plasma membrane localisation can be observed (arrow in B). (D-F) Protein localisation in root meristems. (D) Lateral root tip, columella and LRC expression are visible. No protein is seen in the epidermis (e) before emergence from the LRC. (E) Main root tip with columella cell layers indicated by stars. (F) surface view when mounted in 0.8 M mannitol. Cell wall area is clear of fluorescence. (G,H) Protein localisation in comparable untreated and 2-hour BFA-treated root samples, respectively, showing relative decrease in plasma membrane localisation and the appearance of bright perinuclear bodies. (I) mature ovule with fluorescence seen in outer integument (oi) (where little protein is detected in the outermost cell plasma membrane; arrowhead), inner integument (ii) and outer cell layer of funiculus (f). rp, embryonic root pole; c, cotyledon primordia; s, sepal primordia; m, micropyle; fg, female gametophyte.Scale bars: 25 μm.

observed lack of a female gametophyte in some mature *acr4* ovules may be due to degradation or de-differentiation rather than to lack of initiation of gametophyte development.

Many *acr4* mutant ovules are never fertilised because of severe morphological abnormalities, but of the ones that are, those with more severe organisational defects abort as developing seeds. Abortion is independent of zygotic genotype and is, moreover not due to developmental defects in embryo and endosperm development, although both tissues are retarded at the time of seed death. Retardation and abortion probably occur because defective seeds provide insufficient maternal support, in terms of nutrients, for embryo sac development. Similar retardation and death of embryo/ endosperm was observed when reduced expression of the genes *FBP7* and *FBP11* led to developmental abnormalities and degeneration in the endothelium and seed coat of *Petunia* (Colombo et al., 1997). The total lack of zygotically derived embryo development defects and the observation of seed coat abnormalities in our study contradicts results obtained using antisense experiments to reduce *ACR4* expression (Tanaka et al., 2002).

ACR4, as a membrane-localised receptor-like kinase, probably acts by perceiving extracellular ligands. Several genes encoding possible ligands, or ligand processing molecules for CR4 and related proteins have been proposed. These include the subtilase encoded by the *ABNORMAL LEAF SHAPE 1* (*ALE1*) gene (Tanaka et al., 2001). During embryo and endosperm development, signals from surrounding tissues (as could be provided by the action of genes such as *ALE1*) might be important in signalling required for 'outside' cell layer specification. However, it seems more likely that in organ primordia, as has been shown in root cell layer differentiation, an 'inside to outside' signalling process is involved in regulating cell layer behaviour, combined with a role for signals from neighbouring cells in the same cell layer (Nakajima and Benfey, 2002). Our observation that ACR4 protein is localised on 'internal' plasma membranes of 'outside' cells supports the hypothesis that *ACR4* may perceive signals from underlying cells and/or same-layer neighbours. If this is the case, the restriction of the *acr4* phenotype to ovule integuments and sepal margins could be attributable to the fact that these tissues are unique in the *Arabidopsis* plant, in being composed of two appressed layers of L1 cells. If normal L1 behaviour (i.e. anticlinal divisions giving rise to a monolayer of L1 cells) were dependent on perception of positional information both from underlying cells, and from same-layer neighbours, then a loss in signalling between same-layer neighbours could be compensated for by signals from underlying cells in most tissues. However, in the case of ovule integuments and sepal margins, positional information would be effectively limited to that exchanged between same-layer neighbours. The cells in these organs would thus be particularly sensitive to disruption of this signalling pathway, which would be expected to lead to a loss of cellular organisation and thus abnormalities in organ outgrowth, similar to the phenotype observed in *acr4* mutants.

Other pieces of the puzzle

The restricted mutant phenotype of *ACR4* compared to maize

CR4 mutants is surprising since *ACR4* appears to be unique in *Arabidopsis* in its degree of similarity to maize *CR4*. Unlike studies of *cr4* in maize, we find no evidence for a loss of epidermal identity in *acr4* mutants, but rather solely a loss of cell organisation. The cell disorganisation observed in *acr4* ovule integuments and sepal margins is, however, reminiscent of aspects of the epidermal defects observed in the leaves of maize *cr4* mutants. Notably, both phenotypes involve deregulation of the planes of division, and organisation of populations of L1 cells. Striking differences in expression also exist between *ACR4* and *CR4*. In maize, *CR4* is expressed in the aleurone cell layer and one of the major phenotypes associated with *cr4* mutants is a defect in aleurone differentiation (Becraft et al., 1996). *ACR4* shows no endosperm expression, although it is arguable whether *Arabidopsis* can be considered to differentiate a structure analogous to the cereal aleurone layer (Berger, 1999). In addition, unlike *ACR4*, *CR4* appears to be expressed throughout apical meristems, without restriction to the L1 layer until late in leaf development (Becraft et al., 1996), and no *CR4* expression has been reported in maize root tissue.

Functional redundancy between *ACR4* and four other *Arabidopsis* genes showing weaker similarity to *CR4* cannot be ruled out as an explanation for some of the differences in phenotypic severity between *cr4* and *acr4* mutants. The two most closely related genes encode proteins lacking a conserved kinase catalytic domain required for kinase activity (domain 8) (Hanks et al., 1998). *ACR4* encodes a functional kinase, and kinase activity is probably required for at least some of its functions. However, kinase-inactive receptors can retain partial function, possibly by interaction with other unrelated kinases. The kinase-null *clv1-6* allele, which causes part of the kinase domain of the CLAVATA1 protein to be deleted, causes only a weak mutant phenotype. The mutant protein thus retains functions that are independent of its ability to auto/transphosphorylate itself and other proteins (Torii and Clark, 2000). Of the two less similar genes, one encodes a protein closely related to tobacco CRK1, which has recently be implicated in cytokinin responses (Schafer et al., 2002). The other shares many more residues with CRK1 than with ACR4 and CR4, especially in the extracellular domain adjacent to the trans-plasma membrane domain, where *ACR4* and *CR4* encode putative TNFR-like repeats.

An alternative explanation for the weak *acr4* phenotype could be that although several independent mechanisms regulate L1 behaviour in both *Arabidopsis* and maize, mechanistic differences in organ primordium development in monocotyledonous and dicotyledonous species have led to less functional overlap in maize than in *Arabidopsis*. Considering the relatively large numbers of genes expressed in L1 cell layers from early in development in both species, this possibility seems realistic, and will be investigated using ongoing mutagenesis and double mutant analysis approaches in the near future.

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