The role of the ARABIDOPSIS CRINKLY4 receptor-like kinase in regulating L1 cell-layer organisation

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Declaration

I declare that this is my own work and that any contribution made by other parties is clearly cited.

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Contents

Acknowledgements I

Publication II

Abbreviations III

Abstract IV

Chapter I. Introduction: literature review and aims 1

I.1. Introduction 2

I.2. The functional importance of a layered structure in the plant body 3
- I.2.1. Plant cell-layer patterning during development 3
- I.2.2. Cell layer communication is required for proper coordinate development 6
- I.2.3. Cell layer specification and organisation: the basic pattern elements 8
- I.2.4. Meristem maintenance 11
- I.2.5. Internal layer patterning 14
- I.2.6. Cell-cell communication channels 16

I.3. L1 cell layer specification and organisation in *Arabidopsis*: the origin, maintenance and elaboration of the pattern 18
- I.3.1. L1 layer specialisation 18
- I.3.2. Setting up the pattern 20
- I.3.3. Differentiation and patterning of the L1 layer 22
- I.3.4. Epidermal cell layer specification and organisation 29

I.4. Signalling mechanisms in plants: the role of receptor-like kinases 35
- I.4.1. The plant receptor-like kinase gene family 35
- I.4.2. Receptor-like kinases: localisation and ligand binding 40
  - I.4.2.A. Localisation 40
  - I.4.2.B. Ligand binding 41
  - I.4.2.C. Oligomerisation: ligand-independent and ligand-induced 43
- I.4.3. Receptor-like kinases: signalling dynamics 46
  - I.4.3.A. Downstream signalling targets 46
  - I.4.3.B. Damping down the signal: endocytosis and protein processing 48

I.5. Outside cell layer organisation in *Arabidopsis*: how is this achieved? 53
- I.5.1. *ACR4* as a candidate regulator of epidermal specification in *Arabidopsis* 53

Chapter II. Materials and methods 56

II.1. Plant culture and plant material 57
- II.1.1. Cultivation of plant lines 57
- II.1.2. Tissue culture 58
- II.1.3. Mutagenesis of the *acr4-2* seed line 58
- II.1.4. Ovule development mutants 58
- II.1.5. Hormone signalling pathway mutants 59
- II.1.6. Additional mutants and marker lines 59

II.2. DNA techniques 60
- II.2.1. Genomic DNA extraction 60
- II.2.2. PCR reaction 60
- II.2.3. Agarose gel electrophoresis of DNA 61
- II.2.4. Digestion and ligation of DNA 62
- II.2.5. Southern blotting 63
- II.2.6. Cloning and transformation of plasmids into *E.coli* and *Agrobacterium* cells 64
- II.2.7. Preparation of plasmid DNA from bacterial cultures: *E.coli* and *Agrobacteria* 65
- II.2.8. Sequencing of plasmid DNA 67
- II.2.9. *Agrobacterium* mediated plant transformation 68

II.3. Construct production 69
- II.3.1. Expression pattern analysis 70
- II.3.2. Complementation analysis 71
- II.3.3. Protein localisation and functional analysis 71
- II.3.4. Genetic analysis: *Pressed Flower* constructs 73
- II.3.5. Additional constructs 74

II.4. Isolation of t-DNA insertion and TILLING generated mutant alleles 75
- II.4.1. *acr4-1* – *acr4-6*: t-DNA insertion alleles 75
- II.4.2. *acr4-7* – *acr4-22*: TILLING alleles 77

II.5. Phenotypic and expression pattern analysis 78
- II.5.1. Light microscopy techniques 78
- II.5.2. Confocal and fluorescence microscopy 78
- II.5.3. Scanning electron microscopy (SEM) 79

II.6. Protein localisation and functional analysis 80
- II.6.1. Protein extraction 80
- II.6.2. Western blotting 80
- II.6.3. Brefeldin A treatment 82

Chapter III. *ACR4* plays a role in maintaining L1 layer integrity 83

III.1. Introduction 84

III.2. *acr4* alleles have defects specifically in ovule integument outgrowth and sepal margin organisation **86**
III.2.1. Isolation of loss of function *acr4* alleles **86**
III.2.2. Phenotypic analysis of *acr4* lines **90**
III.2.2.A. *ACR4* is required for normal seed development **90**
III.2.2.B. *ACR4* is required for proper ovule integument outgrowth **92**
III.2.2.C. *ACR4* is required for proper sepal margin cell organisation **95**
III.2.3. The *acr4* phenotype is maternal sporophytic **96**
III.2.4. Single base pair mutations in the 39aa repeat region render *ACR4* non-functional **97**
III.2.5. *ACR4* sequence analysis **97**
III.2.6. *acr4-1* carries a *sirene*-like mutation **98**

III.3. Isolated *acr4* null mutants are complemented by wild-type *ACR4* gene expression **100**

III.4. *ACR4* is expressed in the outside cell layer of embryos, meristems and in ovule integuments **101**
III.4.1. Construction and optimisation of *ACR4* marker lines **101**
III.4.1.A. Two-component (transactivation) marker line **101**
III.4.1.B. Single and enhanced-single marker lines **102**
III.4.2. *ACR4* expression pattern analysis **103**
III.4.2.A. *ACR4* is expressed in the outside layers of the embryo, shoot apical, inflorescence and floral meristems **103**
III.4.2.B. *ACR4* is expressed in ovule integuments, the root meristem and in outside layer cells at the lateral root cap boundary **104**

III.5. *ACR4* appears to be involved in signalling between cells in order to maintain outside (L1) layer integrity **107**
III.5.1. *ACR4* function: a hypothetical model **107**
III.5.2. Opposing opinions and a comparison of *ACR4* with *CR4* **108**
III.5.3. A wider context: what signal is *ACR4* responding to? **111**

Chapter IV. ACR4 protein is localised to the L1 plasma membrane may be endocytosed and cleaved in the cell 114

IV.1. Introduction 115

IV.2. Functional ACR4 protein is localised to the plasma membranes of L1 cells and to intracellular bodies **117**

IV.3. ACR4 functional analysis **120**
IV.3.1. An ACR4 deletion protein lacking the transmembrane, kinase and C-terminal regions is exported to the cell wall **121**
IV.3.2. The 39aa repeat region of ACR4 is vital for function whilst kinase activity is not **123**

IV.4. The dynamics of ACR4 localisation 126
IV.4.1. BFA-sensitive vesicle trafficking is required for some aspect of correct ACR4 localisation or protein turnover 126
IV.4.2. ACR4 protein is subject to rapid turnover and may be cleaved in the cell 129

IV.5. The 39aa repeat domain is required for protein turnover: a ligand binding domain? 134

IV.6. The molecular mechanism of ACR4 action: homo-/hetero-oligomers with other ACR4-like proteins? 137

Chapter V. *ACR4* genetic analysis: elaboration of the signalling network 143

V.1. Introduction 144

V.2. *ACR4* does not interact with genes involved in ovule morphogenesis 146

V.3. How does *ACR4* fit into the wider signalling network? 153
V.3.1. *ACR4* does not interact specifically with either *GAI* or *SPY* 155
V.3.2. *ACR4* does not interact specifically with *CRE1* 157

V.4. New γ-mutagenesis-generated mutants are candidate enhancers of the *acr4* mutant phenotype 158
V.4.1. A γ-mutagenesis enhancer screen based on the *acr4-2* mutant 158
V.4.2. Identified mutants have an enhancement in seed abortion or defects in cell layer organisation; are they dependent on *acr4*? 161
V.4.2.A. Description of mutants identified in the γ-mutagenesis enhancer screen 161
V.4.2.B. Clean-up of lines and test of dependency on the *acr4-2* background 164
V.4.3. *hulk* mutants have a loss of epidermal integrity 166

V.5. Other aspects of the *ACR4* signalling pathway 173

Chapter VI. General discussion and perspectives 180

VI.1. *ACR4* plays a role in maintaining the integrity of the L1 layer: a conserved function across plant species? 181

VI.2. ACR4 appears to undergo ligand-binding mediated endocytosis and seems to be rapidly turned over in the cell: defining a concept in plant RLK behaviour 185

VI.3. Expanding the *ACR4* signalling pathway 189

Appendix 1 191
References 193

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Publication

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Development **130**: 4249-58
(See bound copy inside back cover)

Abbreviations

APS	ammonium persulphate
bp	base pair
BFA	Brefeldin A
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dH_2O	sterile distilled water
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dTTP	2'-deoxythymidine 5'-triphosphate
ETDA	ethylene-diaminetetraacetic acid
HEPES	*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid
Kb	kilobase
LB	Luria broth
M	molar
mg	milligram
ml	microlitre
mM	millimolar
MS	Murashige and Skoog medium
ng	nanogram
Nonidet	(octyl)polyethoxyethanol
PCR	polymerase chain reaction
pM	picomolar
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl-sulphate
SSC	standard saline citrate
TE	Tris·HCl, ethylene-diaminetetraacetic acid
TEMED	tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
Triton	iso-ocytlphenoxypolyethoxylethanol
Tween	polyethylenesorbitan monolaurate
μg	microgram
μl	microlitre
μM	micromolar
UTR	untranslated region
UV	ultra violet
v	volts
X-gal	4-bromo-4-chloro-3-inodyl-β-D-galactopyranoside
YEP	yeast extract peptone

Abstract

The mechanisms regulating cell-layer organisation in developing plant organs are fundamental to plant growth, but remain largely un-investigated. In order to understand the signalling pathways potentially involved in this process, the receptor kinase-encoding *ARABIDOPSIS CRINKLY 4* (*ACR4*) gene was studied. *ACR4* expression is restricted to the Ll/outside cell-layer of most meristems and organ primordia, including those of the ovule integuments. Mutant analysis shows that *ACR4* is required for regulation of cellular organisation during the development of sepal margins and ovule integument outgrowth.

ACR4 encodes a protein that in ovules, and possibly other tissues, is abundant in anticlinal and the inner periclinal plasma membrane of "outside" cells. It is proposed that ACR4 may be involved in maintaining L1 cell-layer integrity in aerial organs by receiving and transmitting signals from neighbouring L1 cells and/or from underlying cell layers. In order to discover additional components involved in this process a mutagenesis screen based on the *acr4* line was carried out. Potential enhancers of the *acr4* phenotype were identified and selected for future analysis.

In order to further investigate the molecular mechanism of ACR4 function a comprehensive functional dissection of the ACR4 protein was carried out, based on the ability of deletion derivatives to complement the mutant phenotype. This has permitted identification of functionally important domains of ACR4 and the formulation of a functional model for ACR4 as a partially redundant component of a developmentally crucial signalling pathway involved in the maintenance of L1-layer integrity throughout *Arabidopsis* development.

Chapter I. Introduction: literature review and aims

I.1. Introduction

I.2. The functional importance of a layered structure in the plant body

I.3. L1 cell layer specification and organisation in *Arabidopsis*: the origin, maintenance and elaboration of the pattern

I.4. Signalling mechanisms in plants: the role of receptor-like kinases

I.5. Outside cell layer organisation in *Arabidopsis*: how is this achieved?

I.1. Introduction

The question here is the way in which the outside cell layer is specified and organised in *Arabidopsis thaliana.* In order to address the question the current field of knowledge will be explored. Firstly a general introduction will be given as to the layered structure of *Arabidopsis* to show how this body plan is set up and maintained (Section I.2). Next the outside cell layer will be investigated in more detail, highlighting the specifications which must be developed for proper functioning. The importance of cell layer specification and maintenance of layer integrity will be shown (Section I.3). Thirdly the mechanisms involved in signalling between cells will be addressed. Proper cell-cell communication is important for the specification and organisation of cell layers. One important means of signalling in many biological systems is through the action of receptor kinases. The characteristics and behaviour of these molecules will be presented (Section I.4). Finally the lessons learned above and parallels drawn from other organisms will be applied to the specific question of outside layer specification and organisation in *Arabidopsis* (Section I.5). The focus and questions raised in this thesis will thereby be set out.

I.2. The functional importance of a layered structure in the plant body

I.2.1. Plant cell-layer patterning during development

Most angiosperm development occurs post-embryonically, however the basic patterning elements of the plant body, an apical/basal pattern and a radial layered pattern, are set down during embryogenesis (reviewed in Jurgens, 1992; Jurgens, *et al.*, 1995; Souter and Lindsey, 2000; Jurgens, 2001; Paquette and Benfey, 2001) (Fig.I.1). In this introduction the development of *Arabidopsis thaliana* will be the focus, although applicable examples and important paradigms from research in other species will also be discussed.

The *Arabidopsis* embryo develops from a fertilised egg (the zygote) which is situated within the embryo sac contained in the ovule (see Chapter III, Fig. III.3 for a schematic of ovule development). Embryogenesis starts with elongation of the zygote followed by an asymmetric apical-basal cell division (Mansfield and Briarty, 1991). The smaller basal cell then divides and develops into the suspensor which attaches the developing embryo to the plant. The apical cell divides and develops into the embryo proper through a sequence of divisions which is highly reproducible in its early stages. This is the first manifestation of the apical/basal patterning of the embryo; during mature plant growth this apical/basal axis is also particularly important for pattern formation. During the subsequent growth of the embryo a radial pattern of organisation is established (Fig.I.2). This pattern first becomes evident at the 16 cell stage when cell division generates an embryo with an inside cell layer and an outside cell layer. Interestingly, as will be discussed in Section I.2.3, differential expression of certain genes can be detected between the inside and outside layers at this stage. Prior to this stage these genes were either expressed in all eight cells of the embryo or not expressed in any. This inside/outside differentiation is thought to be one of the first cell specification events during embryogenesis (Jurgens, *et al.*, 1991). The distinction is elaborated on during development and thus early events are critical for proper plant patterning.

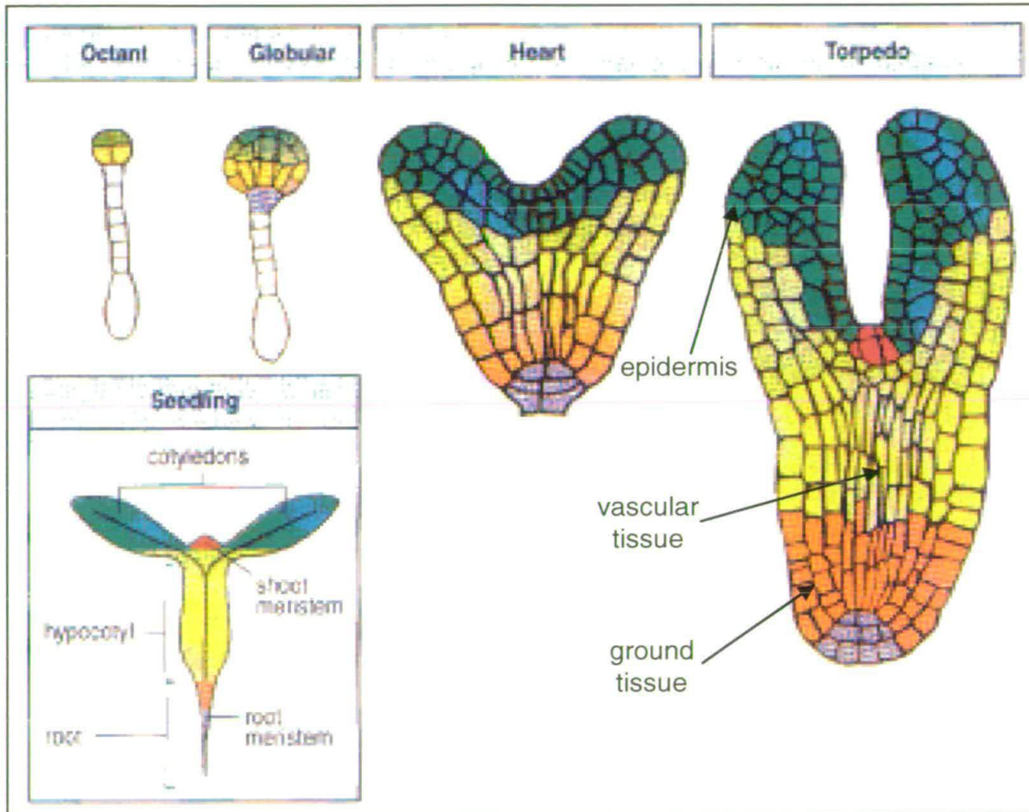

Figure I.1. The major patterning elements in *Arabidopsis* development are set down during embryogenesis. This schematic shows the corresponding patterned regions of the embryo at intermittent stages of embryo development and a germinated seedling (denoted by coloured sections). The octant (eight cell), globular, heart and torpedo embryo stages are shown. Through development during these stages the main regions of the plant are defined. The shoot meristem (red) and the root meristem (lilac) are visible at the apex (between the cotyledons) and the base respectively in both later stage embryos and the seedling. The epidermis, ground tissue and vascular tissue of the embryo are indicated. Schematic taken and adapted from Scheres (1993).

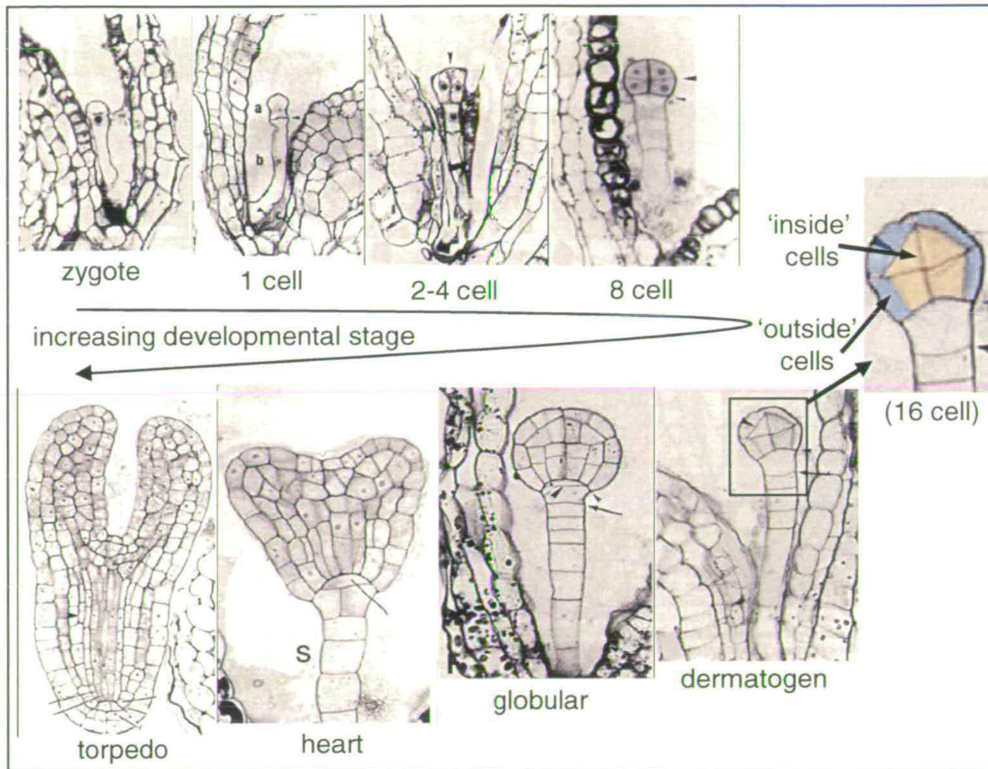

Figure I.2. Cell layer specification during *Arabidopsis* embryogenesis. The embryo develops from a fertilised egg cell (zygote) in a series of well characterised stages. The zygote divides asymmetrically to give an apical cell (a) and a basal cell (b) at the 1 cell stage. The apical cell develops into the embryo proper, with the basal cell developing into the suspensor ((s) in heart stage embryo). The stages of development up to the torpedo stage of embryogenesis are shown here. Cell layer specification occurs early during embryo development. At the dermatogen (16 cell) stage of embryo development there are two physical cell layers: an 'inside' layer and an 'outside' layer (see coloured inset) which have been shown to be defined by differential gene expression. Schematic taken and modified from Mayer, *et al.* (1993).

Following the early events outlined above the outer layer of the embryo divides predominantly anticlinally. This results in contribution of cells to the outside layer only. The outer cells make up the protodermal layer, which later gives rise to the epidermis of the plant. During the 'globular' stage of development the inner cells of the embryo at first divide uniformly along the apical-basal axis. Gradually though, through successive rounds of oriented division in the centre of the lower part of the embryo, a section of narrower cells is formed. This is a further reflection of the apical/basal pattern. This manifestation of cell division also radially subdivides the inner cells into central vascular and surrounding ground tissue. The three major tissues of epidermis, ground and vasculature are thus specified early in development as shown in Fig.I.1. Differential cell division in the embryo through subsequent stages gradually alters the shape of the embryo and refines the initial plan. These stages are defined sequentially as 'heart', 'torpedo' and then 'bent cotyledon'. Meristems, which are regions of undifferentiated cells, subsequently arise as terminal elements of the apical/basal pattern along the axis of polarity during embryogenesis. At the heart stage of embryo development the apical meristem, located at the apex of the embryo, becomes discernable. In the later torpedo and bent cotyledon stages the provascular tissues are obvious and the root primordium organisation is completed. Meristems generate all differentiated cells and the organs which they compose. The layered pattern of shoot and root meristems will now be considered.

The shoot apical meristem (SAM) of angiosperms consists of two superimposed 'histogenic' cell compartments which are arranged parallel to the surface of the plant: these are periclinal cell layers (Satina, *et al.*, 1940) (Fig.I.3A). The outer compartment is termed the tunica. In dicotyledonous species such as *Arabidopsis* there are two tunica layers; there is only one in maize (*Zea mays*). These layers generally divide only anticlinally, resulting in a contribution of cells to the same cell layer only. The tunica layers are therefore mono-layers (one cell thick). In *Arabidopsis* the outer tunica layer is designated the L1 layer, with the underlying layer of cells the L2. The inner compartment or corpus divides randomly, both anticlinally and periclinally to increase the bulk of the plant (Tilney-Bassett, 1986;

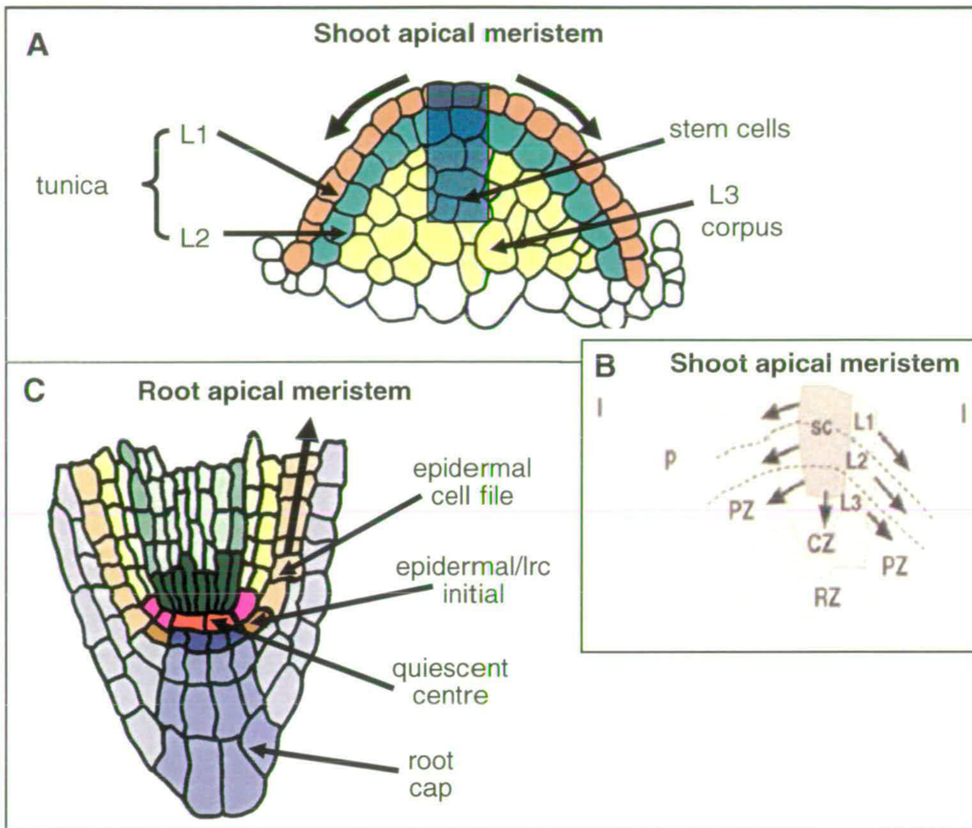

Figure I.3. Schematics showing the layered structure of *Arabidopsis* meristems. (A) There are two tunica layers (L1, L2) in the *Arabidopsis* shoot apical meristem and one corpus layer (L3). The L1 and L2 are one cell thick due to a restricted plane of division (anticlinal only) whereas the L3 divides both anti- and peri-clinally. The stem cells of the shoot apical meristem are shaded in a blue box. (B) The shoot meristem is organised into a central zone (CZ) which contains the stem cells (sc), a peripheral zone (PZ) where new cells differentiate and are incorporated into organ primordia, and a rib zone (RZ) where vasculature is generated. I, leaf primordium; P, meristem periphery. (C) The root apical meristem consists of cylindrical periclinal layers. The cells in each layer are derived from the quiescent centre of stem cells and the root initials. For example the epidermal/lateral root cap (lrc) initial and resultant epidermal cell file are labelled. The root apical meristem is covered by root cap cells. See Fig.III.10 for a more detailed version of this diagram. Arrows in A-C without labelling indicate the direction of cell division. Schematics taken from (A) taken from Ingram (2004), (B) Berleth and Chatfield (2002), (C) Van Den Berg, *et al.* (1997) originally, and adapted in Ingram (2004).

Wegner, 2003). In *Arabidopsis* this is designated the L3 layer; it is the L2 layer in maize. In accordance with the planes of division, cortical microtubules are orientated and arranged anticlinally in the tunica and randomly in the corpus (Traas and Vernoux, 2002). The molecular basis for the establishment and maintenance of the tunica-corpus structure remains unknown, however it seems that a layered organisation is vital for meristem function (see Section I.2.4). Cells from all three layers usually contribute to plant organ formation and the L1/L2/L3 layered structure is generally maintained after floral induction. The resulting inflorescence and floral meristems tend to derive each layer from the corresponding one in the SAM (Vaughan, 1955; Tilney-Bassett, 1986).

The shoot meristem is also divided into a central zone (CZ), a peripheral zone (PZ) and a rib zone (RZ) (Steeves and Sussex, 1989) (Fig.I.3B). The CZ and the PZ consist of L1, L2 and L3 cells. The CZ of the meristem consists of a population of pluripotent slowly dividing cells from which all mature plant organs are generated. These cells appear to be generally equivalent to stem cells in animals (Laux, 2003) and will be referred to as such. Below the stem cells there is an 'organising centre' (OC) which maintains the activity of the overlying stem cells. The stem cells and OC are located in the upper and lower region of the CZ respectively. In the PZ cells generated from the CZ are incorporated into organ primordia or new meristems. The RZ consists of L3 cells only and consists of cell files where the internal regions of the stem, including the vasculature, are generated.

The *Arabidopsis* root apical meristem (RAM) generates all subterranean parts of the plant and is also arranged in a radial pattern. It is a well ordered structure composed of cylindrical periclinal layers of cells (Fig.I.3C) (Dolan, *et al.*, 1993). These layers are, from outside to inside: the protective root cap, epidermis, cortex, endodermis, pericycle and then the stele (vasculature) in the centre. The meristem consists of four central cells known as the quiescent centre, surrounded by initial cells. The initial cells are mitotically active and act as stem cells from which all root cell files (epidermal, cortical etc.) are produced by anticlinal divisions (again maintaining single cell thick files). There is no L1/L2/L3 distinction in the root. The

number of cells and their position as they are generated during development has been extensively studied and is fairly invariant (Dolan, *et al.*, 1993; Scheres, *et al.*, 1994; Scheres, *et al.*, 1996). Cell fate in the RAM is controlled by short range signalling (van den Berg, *et al.*, 1995; Helariutta, *et al.*, 2000, Nakajima, *et al.*, 2001). From the primary root both new root primordia which give rise to lateral roots, and root hairs are derived. Lateral root formation is initiated by a well defined sequence of periclinal and anticlinal divisions in the primary root pericycle. Subsequent cell divisions in a subset of primordial cells result in formation of the lateral root meristem; this structure is fairly similar to that of the RAM (Dubrovsky, *et al.*, 2000; Dubrovsky, *et al.*, 2001). Root hair development has also been well characterised and an elegant model developed for the control of this aspect of cell patterning (reviewed in Larkin, *et al.*, 2003). In the epidermal layer, cells either become root hairs or non root hairs in a position-dependent fashion. Positional information is generated by cells in the underlying cortical layer (Masucci, *et al.*, 1996; Lin and Schiefelbein, 2001; Webb, *et al.*, 2002; Bernhardt, *et al.*, 2003).

I.2.2. Cell layer communication is required for proper co-ordinate development

The L1, L2 and L3 shoot apical layers are cytologically and largely clonally distinct populations of cells due to the restriction of cell division within each layer (Traas and Vernoux, 2002). In leaves of *Arabidopsis*, division in the L1 layer results in contribution of cells to the epidermis of the plant (Irish and Sussex, 1992). The L2 layer contributes cells to the mesophyll and also the plant germ line. The ground tissue and vasculature are composed of L3 cells. Although these layers are distinct they communicate during organ formation in order to coordinate developmental processes. Studies indicate that defects in one cell layer can affect the morphology and development of other layers. Therefore cross-talk and signalling between cells is critical for correct plant patterning as a whole (Irish and Jenik, 2001).

The layered structure of the meristem is maintained to some extent in organs, for example in leaves. It is possible to examine the contribution of meristematic layers to the cell layers of organs such as leaves using periclinal chimeras. Such chimeras, either naturally occurring or artificially constructed, comprise cell layers with differing expression of markers or differing phenotypic traits. Periclinal chimeras have been used for many years in both classical and more recent molecular-based studies (Tilney-Bassett, 1963; Stewart and Burk, 1970; Stewart and Dermen, 1970; Stewart and Dermen, 1975; Irish and Sussex, 1992; Szymkowiak and Sussex, 1992; Jenik and Irish, 2000; Irish and Jenik, 2001). Through such study it was found that meristem layer separation is not always absolute, as there are examples of cells invading other layers by inappropriate periclinal division. However if an L1 cell invades the L2 cell layer due to an abnormal cell division, it differentiates as an L2 cell. Therefore plant cells differentiate according to position rather than lineage. This fact has also been shown for cells of the root using clonal analysis (van den Berg, *et al.*, 1995; Kidner, *et al.*, 2000) and is one of the key concepts in plant development. This indicates that cells continuously receive positional information which is fundamental for specification and maintenance of cell identity, organisation and patterning. This information is conferred both by cells in adjacent cell layers (inter-cell layer communication), and also from cells in the same layer (intra-cell layer communication). The molecular basis of cell layer communication has been investigated through a multitude of methods (Szymkowiak and Sussex, 1996; Jenik and Irish, 2000; reviewed in Ingram, 2004). The cell-cell signalling involved in specifying and organising cell layers will be the focus of this thesis. Naturally occurring cell layer invasion seems to be fairly frequent in many plants including ivy and maize. It appears to be relatively rare in *Arabidopsis*. This lessened complication makes this an ideal plant for the study of cell layer communication.

I.2.3. Cell layer specification and organisation: the basic pattern elements

Plant cells differentiate according to position rather than lineage. The next point to be addressed is where the information that specifies position comes from, and how plants perceive it. As mentioned in Section I.2.1. the key patterning elements are set up during embryogenesis. In the dermatogen embryo there is already differential gene expression between the inside and outside cell layers. This suggests that positional information is perceived by cells from the earliest stages. The way in which a radial and an apical/basal pattern develop in *Arabidopsis* has been extensively studied (reviewed in Souter and Lindsey, 2000; Jurgens, 2001; Berleth and Chatfield, 2002). Here the differentiation between the inside layer and the outside layer (L1) which is derived from the protoderm of the developing embryo, will be discussed. Research in the last ten years has led to a basic understanding of some of the elements of this pattern. The specialisation of the L1 layer will be covered in more detail in Section I.3. The initial patterning events will be discussed here.

One particularly important gene which is expressed differentially between the inside and outside cell layers is *ARABIDOPSIS MERISTEM LAYER1* (*AtML1*). *AtML1* is a member of the plant specific class of HD-Zip GL2 homeobox transcription factors (Lu, *et al.*, 1996; Sessions, 1999). Families of homeobox genes in both plants and animals are thought to be important in pattern formation and cell fate specification of specific layers (Williams, 1998). It is thought that their expression is involved in defining morphogenetic boundaries of positional information in the embryo and in the shoot apical meristem of plants. *AtML1* expression begins in the apical cell of the embryo after the first division. It is expressed in all cells of the embryo proper at the eight-cell stage but becomes restricted to the outside cell layer at the dermatogen stage. *AtML1* is then expressed in the L1 layer of the embryo, in the protoderm and the SAM. *AtML1* expression is maintained throughout plant development in the L1 layer of shoot, inflorescence and floral meristems as well as in the L1 of young organ primordia including ovule integuments; *AtML1* is not expressed in root meristems. Another outside-specific

gene, *PROTODERMAL FACTOR2* (*PDF2*), is closely related and functionally redundant with *AtML1* (Abe, *et al.*, 2003).

AtML1/PDF2 play a crucial role in maintaining L1 cell identity by regulating the transcription of downstream target-gene promoters. Through this they play a critical role in pattern formation (Lu, *et al.*, 1996). AtML1/PDF2 have been shown to bind specific motifs (L1 boxes) present in the promoters of genes expressed in the L1 (Abe, *et al.*, 2003). For example an L1 box in the promoter of the L1-specific *PROTODERMAL FACTOR 1* (*PDF1*) gene has been shown to be bound by AtML1 *in vitro* (Abe, *et al.*, 2001). *PDF1* encodes a putative extracellular proline-rich protein (Abe, *et al.*, 2001). The promoters of several genes expressed post-embryonically in the meristematic or organ L1 layer have also been found to contain L1 boxes. For example the promoter of *FIDDLEHEAD* (*FDH*), a gene involved in cuticle formation (see Section I.3.3) contains an L1 box (Abe, *et al.*, 2001). As well as being expressed in the mature plant L1, *FDH* is expressed strongly in the L1 layer of the embryo (Gwyneth C. Ingram, The University of Edinburgh, UK, pers. comm.). The promoter of *PRESSED FLOWER* (*PRS*) which plays a role in the regulation of cell recruitment in the L1 cell layer also contains an L1 box (Pruitt, *et al.*, 2000; Matsumoto and Okada, 2001). Inside-specific genes are also important in patterning. *MONOPTEROS* (*MN*) encodes a transcriptional regulator and is expressed in the inside cells of the embryo from the dermatogen stage onwards. *MN* is involved in establishing vascular and body patterns in embryonic and post-embryonic development, by mediating auxin directional cues along the apical/basal axis (Berleth and Jurgens, 1993; Przemeck, *et al.*, 1996; Hardtke and Berleth, 1998; Hardtke, *et al.*, 2004).

During maize embryogenesis, genes related to *AtML1* may play a similar role in specifying cell layers. Another HD-Zip homeobox gene *Zea mays OUTER CELL LAYER1* (*ZmOCL1*) and related *ZmOCL* genes are expressed in a cell-layer specific fashion (Ingram, *et al.*, 2000). A similar mechanism may also be conserved in rice which contains homologous genes (Ito, *et al.*, 2002).

How the pattern of regulatory genes itself is first set up is not yet fully known. Some clues as to genes that may act upstream of *AtML1/PDF2* to specify and organise the L1 cell layer will be addressed in Section I.3.4. The investigation of such genes is the subject of this thesis.

There are suggestions that the hormone auxin might be important in the early stages of pattern formation. This is partially due to the fact that MN is an auxin response factor (ARF) (reviewed in Ingram, 2004). ARFs respond to auxin by binding to auxin response elements (AREs) in the promoters of auxin-regulated genes. Auxin is central to the regulation of all aspects of early and later patterning and development, and is thought to be involved in the first asymmetric cell division of the zygote (Mayer, 1993). It regulates a multitude of developmental processes including embryo polarity determination, apical dominance and vascular patterning (reviewed in Leyser, 2001).

Auxin is transported through cells and organs in a polar fashion (Friml and Palme, 2002). This polar transport is mediated by the asymmetric distribution of a family of auxin efflux carriers encoded by the *PIN-FORMED* (*PIN*) genes at the basal end of cells (Galweiler, *et al.*, 1998; Geldner, *et al.*, 2001). Polar auxin transport facilitates the formation of a gradient of auxin through tissue. Auxin signalling is mediated by regulated protein degradation via a ubiquitin-mediated proteolysis pathway (Kepinski and Leyser, 2004). This auxin-mediated pathway is thought to be involved in negatively regulating a group of transcriptional repressors, the Aux/IAA family, which usually repress the function of the ARFs. Auxin thereby regulates gene transcription and patterning.

The seemingly static polar localisation of PIN1 protein is in fact the result of rapid cycling by an actin-dependent process between the plasma membrane and endosomal compartments (Geldner, *et al.*, 2001). This is mediated by GNOM (Steinmann, *et al.*, 1999). *GNOM* encodes a member of the ARF GTP exchange factors (ARF-GEFs) which regulate vesicle trafficking in a variety of organisms (Geldner, *et al.*, 2003; Muday, *et al.*, 2003; Geldner, *et al.*, 2004). Mutations in *GNOM* result in loss of proper PIN1 localisation, loss of polar auxin transport, and therefore disrupted

auxin-dependent apical/basal patterning (Mayer, 1993; Shevell, *et al.*, 1994, Vroemen, *et al.*, 1996; Vroemen, *et al.*, 1996).

In the *Arabidopsis* RAM (as in the SAM) the main pattern of cellular organisation is apparent in the mature embryo. It is then maintained in the primary root of the seedling after germination (Dolan, *et al.*, 1993). There is good evidence for the role of auxin in patterning the root apex during embryo development (Grebe, 2004). Auxin is transported, mediated by polar PIN localisation, from the tips of the embryo cotyledons through the developing vasculature, reaching a maximum at the embryonic root pole (Benkova, *et al.*, 2003; Friml, *et al.*, 2003). Perception of this maximum results in specification of stem cells and the QC. This specification is required for maintenance of the root meristem (van den Berg, *et al.*, 1997). The role of auxin is evident if for example, the function of the putative auxin efflux carrier *PIN4* is lost - the organisation of the root apical meristem is disrupted (Friml, *et al.*, 2002). Similarly mutations in the *MN* gene result in loss of the embryo root pole (Hardtke and Berleth, 1998; Abe, *et al.*, 2003).

If differential auxin localisation and differential gene expression are involved in early patterning events, this leads to the question of how these elements are themselves set up. Some possibilities are reviewed in Section I.3.2.

I.2.4. Meristem maintenance

In order to produce well organised organ cell layers it is important that the activity of the root and shoot meristems is properly regulated. The SAM is a dynamic region of cell proliferation, cell expansion, and cell differentiation yet displays a stable organisation; the stem cells themselves divide slowly (reviewed in Gross-Hardt and Laux, 2003). The production of new cells must be balanced with their incorporation into organ primordia and subsequent differentiation. In addition the pluripotent fate and undifferentiated state of the stem cell population must be maintained (Grandjean, *et al.*, 2004). The mechanism of stem cell specification and maintenance in

Arabidopsis shoot and root meristems has been the subject of intensive research (reviewed in Laux and Mayer, 1998; Lenhard and Laux, 1999; Fletcher, 2002; Doerner, 2003). The underlying SAM pattern develops during embryogenesis (Long and Barton, 1998). The OC of the SAM is thought to confer stem cell identity on the cells above it, while the stem cells restrict the size of the OC below (Mayer, *et al.*, 1998). The genetic basis of this relationship has now been elucidated. A model has been developed in *Arabidopsis* which consists of antagonistic regulation between 'stem cell genes' and 'differentiation' genes (Schoof, *et al.*, 2000; Gross-Hardt and Laux, 2003). This allows maintenance of the organisation of the shoot meristem despite a changing cellular context. The model highlights the importance of cell to cell signalling between meristematic cells for proper development (Haecker and Laux, 2001). Although many genes are involved in this process, only the central genes will be discussed here.

WUSCHEL (*WUS*) encodes a homeodomain protein which is expressed in the OC and promotes a meristem cell fate of the stem cells above (Laux, *et al.*, 1996; Mayer, *et al.*, 1998). Mutations in *WUS* cause a reduction in SAM size due to a loss of stem cell identity and loss of SAM activity (Gallois, *et al.*, 2004). This results in seedlings which terminate development after producing a few primordia. Mutations in either of the three *CLAVATA* genes confer an opposite phenotype to that of *wus* (Clark, *et al.*, 1993; Clark, *et al.*, 1996; Jeong, *et al.*, 1999; Jeong, *et al.*, 1999; Rojo, *et al.*, 2002). Rather than reduction in size, shoot and floral meristems are enlarged and disorganised. The three *CLV* genes are expressed in overlapping domains in the SAM, and are thought to form a functional complex which consists of a ligand and a receptor hetero-oligomer (Jeong, *et al.*, 1999). CLV3 is a small peptide molecule and a stem cell marker which is thought to function as a mobile intercellular signal (Fletcher, *et al.*, 1999). The movement of CLV3 is restricted by binding to its receptor which is thought to be a complex of CLV1 and CLV2 (Trotochaud, *et al.*, 1999; Lenhard and Laux, 2003). *CLV1* encodes a plasma membrane-localised receptor-like kinase (Jeong, *et al.*, 1999). Receptor-like kinases have been shown to function in a wide variety of cellular processes in the plant (see Section I.4). *CLV2* encodes a receptor-like protein which is thought to act as a partner for CLV1 (Jeong,

et al., 1999). The CLV complex provides a good example of the regulation of development by a receptor-ligand pair (Matsubayashi, 2003).

Expression of *WUS* in the OC induces stem cell identity and the expression of the stem cell marker *CLV3*. In turn the CLV3 signal is received by the CLV1/CLV2 receptor kinase complex. The activity of this complex promotes organ initiation by restricting the expression of *WUS* both in stem cells and their lateral neighbours (Brand, *et al.*, 2000; Lenhard and Laux, 2003). Together this interaction between *WUS* and *CLV* establishes a negative feedback loop between the stem cells and the underlying organising centre (Brand, *et al.*, 2000; Schoof, *et al.*, 2000). This regulated spread of a secreted signalling molecule (CLV3) therefore enables cell differentiation in the meristem periphery, but at the same time maintains the stable stem cell niche in the centre. This genetic interaction network has been extensively studied and is now being incorporated into a mathematical developmental model which is being used to further test and investigate these interactions (Jonsson, *et al.*, 2003).

Pattern formation in the *Arabidopsis* root meristem is also controlled by a balance between short-range signals that inhibit differentiation, and signals that reinforce cell fate decisions (van den Berg, *et al.*, 1997). One well characterised specification pathway involves the *SHORTROOT* (*SHR*) and *SCARECROW* (*SCR*) genes. *SHR* and *SCR* both encode *Arabidopsis* GRAS family transcription factors that are important in the regulation of endodermal cell fate (Di Laurenzio, *et al.*, 1996; Pysh, *et al.*, 1999; Helariutta, *et al.*, 2000; Heidstra, *et al.*, 2004). *SHR* is expressed solely in cells of the stele and controls a non-cell autonomous response (Nakajima and Benfey, 2002). It is able to act in adjacent cells however by means of SHR protein export via the symplast (see Section I.2.6). There it acts upstream to regulate expression of the *SCR* gene in the endodermal/cortical initials and the endodermal layer. Through *SCR*, *SHR* mediates specification of the endodermis and the longitudinal division of the cortical/endodermal initial daughter cell (a process important for cell layer patterning).

Patterning of the shoot and root are also controlled by common factors. *HALTED ROOT* (*HLR*) is a recently cloned gene which is important in maintaining the cellular organisation of the RAM and the SAM (Ueda, *et al.*, 2004). Loss of *HLR* results in loss of root quiescent centre identity and disruption of the OC in the shoot meristem. *HLR* encodes a proteasome component, and it is thought that proteasome-dependent proteolysis is important for maintenance of both shoot and root meristem integrity.

I.2.5. Internal layer patterning

The most internal layers of the plant, consisting of the vasculature, are also patterned and organised via a tightly controlled genetic network. The vasculature is a ramified network of continuous cell files, each made up of elongated and interconnected cells involved in transporting water and nutrients. The vasculature consists of xylem and phloem cells, their related loading companion cells and elements as well as procambium and vascular cambium. The latter are vascular stem cells from which vascular cells are continuously generated. Xylem strands, which are composed of tracheary elements and fibres conduct water basipetally from the point of uptake (roots) to the shoot of the plant. Phloem fibres which are composed of sieve elements and sieve tubes transport sugars from the point of production (source) such as mature leaves, to regions of carbon need (sinks) such as roots and developing organs (reviewed in Fukuda, 2004).

In the plant stem, vascular cells are collectively organised into vascular bundles. In *Arabidopsis* collateral vascular bundles are formed in which the arrangement of xylem and phloem cells within the bundles is orientated in a radial pattern. Xylem develops on the adaxial (internal) pole of the bundle, with phloem on the abaxial (peripheral) pole. Patterning of the internal provascular system is thus connected to organ polarity in both embryonic and post-embryonic development (Zhong and Ye, 2004). Shoots and lateral organs are also patterned with an adaxial/abaxial polarity which is thought to be connected to vascular patterning (Dinneny and Yanofsky, 2004). In the root the vasculature is again specified and organised in a radial pattern

but rather than collateral bundles there is a central axis of xylem with two phloem poles arranged opposite one another at the edge of the stele. Vascular patterning and organ morphogenesis are tightly linked. However the vascular pattern must also be flexible due to the need for adaptive responses to wounding or abnormal growth conditions.

The vascular pattern is established during embryogenesis when the vascular initials are specified. Vascular patterning is thought to depend on signals directing the routes of vascular strands as well as the oriented differentiation of each cell within the vascular system (into phloem/xylem etc.). Auxin has been implicated in correct vasculature development (reviewed in Berleth and Mattsson, 2000). It is thought that the polar flow of auxin acts early in development to induce the differentiation of continuous tracheary elements and fibres (Fukuda, 2004). As mentioned earlier, mutations in the ARF-encoding gene *MN* interfere with proper formation of the vascular strands (Hardtke and Berleth, 1998). Downstream of this, auxin has been found to regulate members of a family of homeodomain leucine zipper transcription factors that are expressed in vascular tissue, and regulate the differentiation of procambial and cambial cells (Baima, *et al.*, 2001).

There is also thought to be an aspect of patterning that is independent of auxin flow, potentially an autonomous pattern formation mechanism (Parker, *et al.*, 2003). Cytokinins and brassinosteroids (BR) (which have widespread roles during plant growth and development) have also been implicated in vascular formation and differentiation. Through characterisation of the *CYTOKININ RESISTANT* (*CRE*) cytokinin receptor, it was found that cytokinins are important for the formation of vascular initials during embryogenesis and for the maintenance of procambial activity (Mahonen, *et al.*, 2000). Brassinosteroid application promotes xylem formation and suppresses phloem formation. BR is secreted from cells and it is thought that there is a vascular-cell-specific BR-perception system (reviewed in Fukuda, 2004). This is thought to involve reception of BR by plasma membrane bound receptor-like kinases such as that encoded by the recently cloned *VASCULAR HIGHWAY1* which is involved in regulating leaf venation patterns (Clay and Nelson, 2002).

Correct vascular patterning and downstream differentiation events are then mediated by members of several families of transcription factors. Some members of the MYB-like, HD-ZIP III and KANADI families of transcription factors, which are involved in regulating the adaxial/abaxial patterning of lateral organs, have been found to be directly involved in vascular patterning in shoots (Emery, *et al.*, 2003). Together vascular cell development is controlled and organised primarily by plant hormones which act as intercellular signals, and are received by molecules such as receptor-like kinases. Downstream events include the direction of cell differentiation by regulatory transcription factors.

I.2.6. Cell-cell communication channels

The way in which signalling molecules move from cell to cell in the plant is of major importance. There are two possible routes for signal movement through the plant: through the apoplast or through the symplast. The CLV3 signalling molecule is thought to travel via the extracellular matrix, the apoplast (Fletcher, *et al.*, 1999; Brand, *et al.*, 2000; Rojo, *et al.*, 2002). Auxin travels through both the apoplast and the symplast (Delbarre, *et al.*, 1996). The symplastic pathway consists of connections through plasmodesmata between the cytoplasm and endoplasmic reticulum of adjacent cells. Recent research has shown that these connections are important for the movement of regulatory signalling molecules such as protein and RNA (reviewed in Heinlein, 2002). For example the symplastic movement of the transcription factor LEAFY is important for its role in the regulation of floral development (Sessions, *et al.*, 2000). In the root, movement of the transcription factor SHR through the symplastic pathway is critical for the specification of root cell types, as mentioned previously (Nakajima and Benfey, 2002). The type of plasmodesmata differs between cells depending on their location and can change throughout development. The behaviour of plasmodesmata also differs between plant species. It is thought that symplastic connections mediate the unification of cells into communication compartments. This allows maintenance of morphogenetic

gradients which are involved in directing growth (Rinne and van der Schoot, 1998). It is thought that the symplastic connections between cells allow a coordinated response in a group of cells (Hake, 2001).

It is clear from the research outlined that cell-cell communication provides the basis for proper patterning of plant cell layers and their subsequent development, both during embryogenesis and post-embryonically. As the position of a cell - the environment which it is in - determines the cellular fate, such signalling is vital for co-ordination of developmental processes. Key receptors receive molecular signals i.e. hormones or proteinaceous ligands, which are involved both in setting up the pattern between and within cell layers, and the later elaboration and specification events. In parallel, the movement of molecules between neighbouring cells could operate to allow a co-ordinated response and uniform development within cell populations.

I.3. L1 cell layer specification and organisation in *Arabidopsis*: the origin, maintenance and elaboration of the pattern

I.3.1. L1 layer specialisation

The outside or L1 cell layer comprises epidermal cells that cover the aerial and subterranean parts of the plant. In addition certain organs are composed entirely of L1 derived cells, such as the ovule integuments which are formed due to L1 outgrowth (Fig.III.3). This epidermal layer is highly specialised and fulfils multiple important roles during the development of the plant (reviewed in Lolle and Pruitt, 1999) (see Fig.I.4 for a basic *Arabidopsis* leaf schematic). It is responsible for contact between the plant and the outside environment. It protects the underlying cells of the plant from desiccation and pathogen invasion, while allowing gas exchange via stomata and efficient light penetration. In this section *Arabidopsis* is again the focus of attention although some points from study in maize will also be addressed.

The epidermis is able to play these multiple roles due to its differentiation into a wide range of cell types. Some form unicellular structures – such as *Arabidopsis* trichomes or root hairs. Others develop into guard cells in order to form stomata. During floral development epidermal cells differentiate into stigmatic papillar cells which are involved in contact with pollen grains at pollination. All epidermal cells aside from those of the root are covered by a cuticle. The cuticle is located on the surface of the external cell wall. The cuticle is a complex mixture of three constituents: cutin (esterified fatty acids), polysaccharide microfibrils and waxes. The waxes are localised both within the cuticular matrix (intracuticular waxes) and on the plant surface (epicuticular waxes). This composition varies between organs and through development to allow the cells that it covers to carry out different roles. The cuticle fulfils various functions including as a selective barrier against water loss, and as a mechanical barrier against damage and invasion. It is a dynamic milieu that is involved in the response to internal and environmental factors

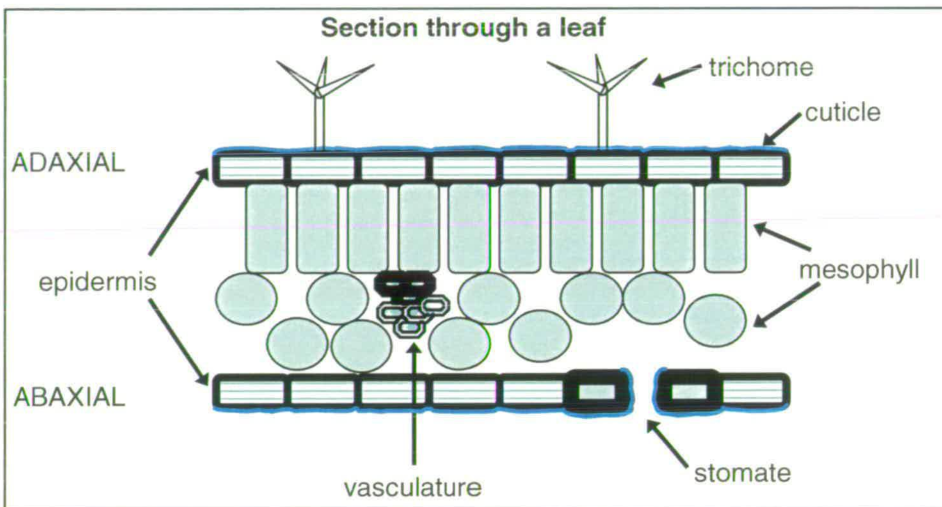

Figure I.4. Schematic showing the layered structure of an *Arabidopsis* leaf. The figure shows the outside epidermal layer, the mesophyll below and the underlying vasculature cells. In the epidermal layer trichomes (on the adaxial side only) and stomata (mainly on the abaxial side) differentiate. In addition the epidermal cells exude a layer of cuticle containing cutin and waxes (not drawn to scale). Schematic adapted from figure by Gwyneth C. Ingram

(Kerstiens, 1996). It is also a developmentally important conduit for regulatory signals as shall be shown later (Section I.3.3).

Epidermal cells are specialised so that when in contact with other epidermal cells, for example during the early stages of leaf development at the shoot apical meristem, they remain unresponsive and do not fuse. Loss of, or defects in the cuticle can result in organ fusion (see Section I.3.3). The only exceptions to the unresponsive state in *Arabidopsis* are firstly during floral development when cell-cell fusion between carpel primordia (syncarpy) is necessary for proper reproductive development, and secondly when the cells of the stigmatic papillae are responsive and fuse to pollen grains during pollen hydration and growth. Altogether this shows that the epidermal layer must be dynamic and selective in order to allow certain interactions but not others (Lolle, *et al.*, 1998). Signals can be exchanged across the cuticle and cell wall. The extracellular matrix itself contains a mass of developmentally important signalling molecules (Fowler and Quatrano, 1997). For example it has been shown that the control of stomatal patterning is regulated by a lipid-soluble signal (von Groll and Altmann, 2001) (see Section I.3.3).

The epidermis is a well-studied model for cell differentiation and cell patterning in plants because it is readily accessible and consists of few cell types. Also due to strict controls on cell division it is the most stable lineage in the plant body (Szymkowiak and Sussex, 1996). The specifics of the downstream genetic networks that regulate the formation of these L1 cell types is not the focus here, although some of these aspects will be covered in Section I.3.3. The specification of L1 identity, L1 organisation and maintenance of cell layer continuity and integrity, rather than specialisation of cell types, will be discussed. These earlier events, including maintenance of cell layer polarity and continuity of signalling channels within the cell layer, are vital for specification of positional information and so proper later signalling events. L1 mediators such as the *AtML1/PDF2* homeobox transcription factors act to specify L1 cell fate. Such genes then mediate the regulation of downstream effectors including genes involved in epidermal differentiation, in a cell layer specific way.

I.3.2. Setting up the pattern

As mentioned in Section I.2.2, the outside layer of the plant is specified early during embryogenesis. This is reflected by differential expression patterns of genes such as *AtML1* and *PDF2*. However AtML1 is unlikely to be the initial signal itself as it is a transcription factor (Lu, *et al.*, 1996), and so more likely acts downstream of other signalling events. How such specific expression is set up and maintained will now be addressed. Here two possible mechanisms that could be involved in separating inner and outer cell fate are proposed (reviewed in Berleth and Chatfield, 2002). The first is an 'inside-out' model of specification and the second is an 'outside-in' model (Fig.I.5). The 'inside-out' model suggests that there is a morphogen gradient across the embryo. The concentration maxima is in the centre of the embryo and this decreases towards the outside (Fig.I.5A). A certain threshold value of the morphogen could therefore direct internal cell fate separately to that of outside cell fate. A morphogen that could play this role has not however been identified. The second 'outside-in' hypothesis suggests that there is a signal present either in the region surrounding the embryo or stored in the outer cell wall (Fig.I.5B). A diffusible signal prepattern could be provided by the tissue in which the embryo develops. Cells in inner cell layers would be physically isolated from such a signal whereas outside cells would not. Alternatively storage of a signal in the external cell wall could be set up during the course of egg formation, or in the zygote. Such a zygotic or maternally inherited signal would remain in the outer cell wall. The outer epidermal cell layer would still be in contact with the original outer cell wall but the first tangential division (that results in an inner cell layer being formed) would separate the inner cells from the signal. Either type of signal could therefore be involved in setting up the differential gene expression pattern as mentioned above. As will be discussed later the second model seems more likely, however there is as yet no definitive evidence for either hypothesis.

In order for such models to be operational, intact cell boundaries are required. For example, mutations in two genes involved in embryo patterning, *KNOLLE* and *KEULE* result in defective cell division (Waizenegger, *et al.*, 2000). In *knolle* and

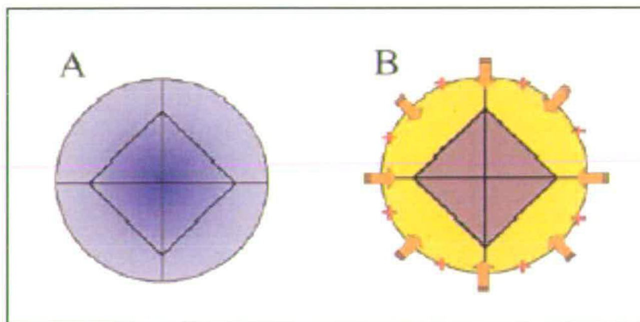

Figure I.5. Pattern formation in the 16-cell stage *Arabidopsis* embryo. Two hypothetical mechanisms that could explain the inside/outside differentiation seen at the dermatogen stage of embryogenesis are proposed. (A) A stable morphogen gradient with the morphogen concentration decreasing from the centre of the embryo to the outside. This could lead to concentration-dependent specification of inside and outside cell layers. (B) Deposition of a substance in the cell wall (red crosses) or reception of a signal from the endosperm (orange arrows) are examples of signals from the outside, directing cell fate. Isolation from such a signal results in differential cell fate of inside (purple) and outside (yellow) cell layers. Taken from Berleth and Chatfield (2002).

keule incomplete cell division results in abnormal cytoplasmic continuity, connecting cells between which there are normally boundaries. This results in inappropriate expression of the outside L1 cell layer marker *ARABIDOPSIS LIPID TRANSFER PROTEIN* (*AtLTP1*) (Assaad, *et al.*, 1996, Lukowitz, *et al.*, 1996; Waizenegger, *et al.*, 2000).

It is probable that several related processes such as those described above act concertedly to specify the primary planes of division and early cell fate distinctions. Later in development there are likely to be additional signals between cells of the L1 cell layer, or from underlying cells, that maintain an outside-layer cell fate.

There are some clues as to early patterning events during embryogenesis from studies in maize which suggest the presence of a signal located on the outside of the embryo. Although the structure of the environment in which *Arabidopsis* embryos develop differs from maize, these studies could help in the search for such signals in *Arabidopsis*. The endosperm of both develops after the double fertilisation event characteristic of angiosperms. However, the endosperm of maize is a more complex structure in which four distinct compartments are specified and differentiated (reviewed in Olsen, *et al.*, 1999). These regions are the aleurone, starchy endosperm, basal transfer cells and the embryo surrounding region (Fig.I.6). The aleurone layer is an epidermal-like layer of isodiametric cells. It is important in the mobilisation of reserve substances stored in the starchy endosperm cells to provide embryo nutrition. The aleurone layer is replaced as the outermost layer at the chalazal pole of the endosperm by the basal transfer layer, which is involved in the transfer of nutrients from the maternal vasculature to the endosperm. The embryo surrounding region is a small region of densely cytoplasmic cells at the base of the suspensor of the embryo which could be involved in embryo nutrition; this has not yet been shown.

The *EMBRYO SURROUNDING* (*ESR*) genes are a group of three maize genes which are expressed in the embryo surrounding region (Bonello, *et al.*, 2000; Bonello, *et al.*, 2002). They are associated with the cell wall in the extracellular region (Bonello, *et al.*, 2002). *ESR*s encode small hydrophilic proteins which are thought to play a role either in the nutrition of the developing embryo or in the

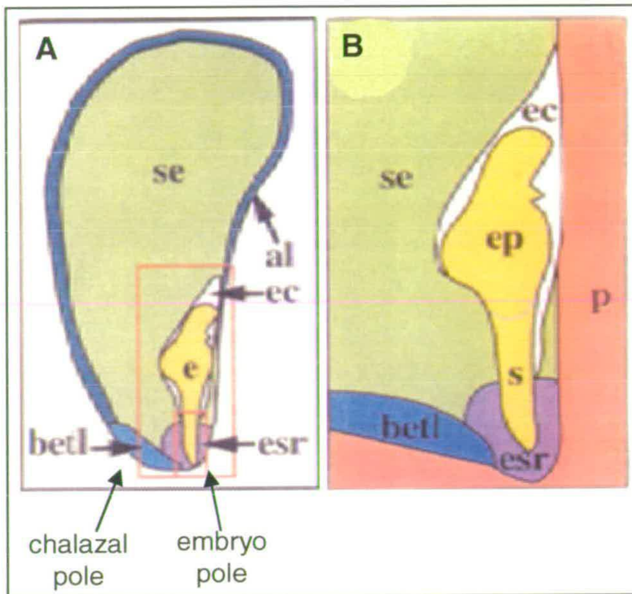

Figure I.6. Schematic of a maize cereal grain containing a developing embryo nine days after pollination (9DAP). (A) Whole grain view showing the mature endosperm regions: the starchy endosperm (se), the single cell thick aleurone layer (al), the basal endosperm transfer layer (betl) and the embryo-surrounding region (esr). ec, embryonic cavern; e, embryo. The chalazal and embryo poles of the grain are indicated. (B) Close-up of embryo (red box in A). The maize embryo consists of the embryo proper (ep) and suspensor (s), as in the *Arabidopsis* embryo. p, pericarp. Taken and adapted from Opsahl-Ferstad, *et al.* (1997).

establishment of a physical barrier between the embryo and endosperm. They could also have a signalling role in embryo-endosperm interactions (Opsahl-Ferstad, *et al.*, 1997). ESRs resemble CLV3 in size and share a conserved motif with CLV3 which gives them the capacity to form protein-protein interactions (Bonello, *et al.*, 2002). In *Arabidopsis* there is a large family of *CLV3/ESR*-like genes (*CLE*s) which are secreted proteins thought to be involved in the regulation of diverse developmental processes (Sharma, *et al.*, 2003). As yet no receptors have been characterised for these molecules (CLEs), although the evidence so far suggests that they could be ligands for receptor-like kinases (see Section I.4). The existence of these molecules in maize suggests one possible mechanism for conferring positional information to the embryo. Several *Arabidopsis* genes which could play similar roles embryonically and post-embryonically will be discussed in Section I.3.4.

I.3.3. Differentiation and patterning of the L1 layer

The downstream specification events associated with L1 cell fate will now be addressed. As mentioned the epidermal layer of aerial organs is characterised by secretion of a cuticle. Cuticle mutants fall into several categories. Mutations in most of the genes described result in reduced levels of cuticular wax deposition and give plants a 'glossy' appearance. One large group are encoded by the *ECERIFERUM* (*CER*) genes of which there are now 24 identified members (Rashotte, *et al.*, 2004). *CER* genes encode various catalytic components of the epicuticular wax biosynthetic pathway (Jenks, *et al.*, 1995; Hannoufa, *et al.*, 1996; Negruk, *et al.*, 1996; Fiebig, *et al.*, 2000). Mutations in the *cer* genes affect the composition of epicuticular wax. WIN1 is a transcription factor involved in the activation of the biosynthesis of epicuticular wax (Broun, *et al.*, 2004). Overexpression of *WIN1* results in upregulation of genes such as *CER1* and *CER2*, and increased wax deposition. *ACYL-CoA BINDING PROTEIN1* (*ACBP1*) encodes a novel membrane localised acyl CoA binding protein (ACBP) which is thought to be involved in the regulation of cutin formation in the cuticle. It is thought to be involved in intermembrane lipid

transport from the ER via vesicles to the plasma membrane (Chye, *et al.*, 1999). Normal epicuticular wax deposition also seems to be important for proper trichome growth. For example double mutants in the *cer1* and closely related *yore yore* genes result in abnormal wax formation and trichome deformities (Kurata, *et al.*, 2003).

AtLTP1 is involved in the secretion or deposition of lipophilic substances in the cell wall (Thoma, *et al.*, 1994). *FIDDLEHEAD* (*FDH*) encodes a member of the Fatty Acid Elongation protein family which is thought to be involved in regulating the permeability barrier of the epidermal cell wall and the cuticle (Yephremov, *et al.*, 1999). *fdh* mutants display organ fusion as a result of an abnormal cuticle (Lolle, *et al.*, 1992). In addition, the mutant shoot epidermal cells are competent to interact with pollen grains - there is an effect on an epidermis-specific developmental program that normally only occurs during floral gynoecium development (Lolle and Cheung, 1993; Lolle, *et al.*, 1997). Such defects then result in ectopic organ fusion and abnormal pollen-stigma interactions due to loss of proper epidermal surface functions (Aarts, *et al.*, 1995; Lolle, *et al.*, 1998). There are also deleterious effects on trichome differentiation, suggesting that these two processes are connected.

The *Arabidopsis ABNORMAL LEAF SHAPE1* (*ALE1*) gene also plays a role in cuticle formation (Tanaka, *et al.*, 2001). *ALE1* encodes a subtilisin-like serine protease (subtilase). In eukaryotes subtilases are thought to be involved in processing peptidic signal molecules to generate biologically active ligands (Siezen and Leunissen, 1997). *ALE1* is expressed in endosperm cells adjacent to the embryo, and within the young embryo. Mutations in *ALE1* result in loss of a proper cuticle on the embryo and juvenile plants, resulting in associated sensitivity to decreasing humidity and organ fusion. The expression pattern of *ALE1* makes it a good candidate for processing of a ligand that affects cuticle development and possibly early embryo development such as described in Section I.3.2.

Recent work on the *SHINE* gene highlights the importance of the cuticle in providing a conduit for cell-cell signals (Aharoni, *et al.*, 2004). The *SHINE* gene seems to be involved in the transcriptional regulation of genes involved in wax biosynthesis. Over-expression of the *SHINE* gene results in plants with shiny leaves due to increased cuticular wax, increased cuticle permeability and a decreased

number of trichomes. It is thought that cuticle permeability influences cell-cell communication by augmenting or attenuating the movement of signals from cell to cell (Pruitt, *et al.*, 2000). It seems that the movement of signals involved in regulating trichome density (see later) is affected by the increased *SHINE* expression. Another piece of evidence for the role of the cuticle in providing a conduit for the movement of patterning signals comes from work on the *Arabidopsis cer* mutants. Corresponding to a decrease in trichome number with more wax in the *shine* mutants, there is an increase in stomatal density in some *cer* mutants which produce less cuticular material (Gray, *et al.*, 2000). This decrease is thought to allow signals which promote stomatal formation to move more freely, and thus the number of stomata specified increases. This response is not seen in all *cer* mutants - it depends on the composition of wax produced as well as the amount of it (Bergmann, *et al.*, 2004).

Stomata themselves are specified by a combination of mechanisms, based primarily on control of the orientation of cell divisions (Nadeau and Sack, 2002; Bergmann, 2004). Both this and the distribution and density of stomata in the epidermis is determined by a combination of factors relating to cell lineage, cell-cell interactions and long distance signalling. Stomatal density is also regulated by exogenous environmental factors (Bergmann, 2004). Another subtilase, encoded by *STOMATAL DENSITY AND DISTRIBUTION (SDD1)* is involved in stomatal patterning (Berger and Altmann, 2000; Von Groll, *et al.*, 2002). In *sdd1-1* mutants the establishment of the stomatal pattern is disrupted. This results in increased stomatal density and formation of abnormal stomatal clusters. It is thought that SDD1 is exported to the apoplast, and there it might process a ligand that controls the development of cell lineages that lead to guard cell formation. Downstream of a postulated receptor for the SDD-1 processed ligand might be encoded by the recently isolated *YODA* gene (Bergmann, *et al.*, 2004; Lukowitz, *et al.*, 2004). *YODA* encodes a MAPKK kinase and could act as a molecular switch to control downstream events.

Root hair patterning and trichome development are controlled by a common position-dependent patterning process (reviewed in Ryan, *et al.*, 2001; Larkin, *et al.*, 2003). This consists of closely related transcription factors which act in similar lateral inhibition signalling pathways (Schnittger, *et al.*, 1999; Schellmann, *et al.*, 2002). Both root hairs and trichomes must be formed at discrete distances between one another for correct functioning. The final pattern produced differs due to the underlying prepattern that is interpreted. Positioning of root hairs begins early in development, just after specification of the protoderm itself (Lin and Schiefelbein, 2001). Therefore correct protoderm specification at early stages is vital for these later processes. There are many genes which are involved in proper root hair patterning. These include *GLABRA2* (*GL2*) which is an *AtML1*-related *Arabidopsis* gene involved in specifying the outside cell layer in both the shoot and the root (Rerie, *et al.*, 1994). Together with the *TRANSPARENT TESTA GLABROUS* (*TTG*) gene (and through interactions with other genes) it is thought to inhibit new root hair formation (Hung, *et al.*, 1998).

In order to properly orientate root hairs and trichomes within epidermal cells – to make sure that outgrowth is towards the outside of the plant – an axis directing cell polarity must be set up and maintained. This specifies the directions to which the interior and exterior of the plant lie. It is also required for proper cell patterning i.e. the cuticle-secretion components must be located on the external side of the cell where the cuticle is secreted. In addition it is required for proper cell-cell communication and to maintain both the direction and site of cell division. The mechanisms controlling the polarity of organs including that of the adaxial/abaxial pattern have been studied in depth (reviewed in Hudson, 2001). There is good evidence for the role of auxin in setting up and maintaining an apical/basal axis in the plant from early embryogenesis (Friml, *et al.*, 2003). The question here is how the apical/basal polarity of each cell is organised. The linked mechanism of planar polarity will be covered later.

As mentioned in Section I.2.1, actin microfilaments are arranged perpendicular to the surface in line with the plane of normal division (Traas and Vernoux, 2002).

Actin microfilaments constitute a three dimensional cytoskeletal network within each cell. Actin filaments provide an essential network for the stable positioning and orientation of organelles, organ motility and vesicle transport within the cell. Their orientation in the cell is a consequence of cell polarity as well as contributing to the maintenance of it. The orientation of microtubules and actin microfilaments also regulates cell wall deposition which impacts upon cell shape, as well as the location of new cell walls (and therefore the direction of cell growth) (Mathur and Hulskamp, 2002). The delivery of cell wall materials to determined sites also affects signal transduction (Vantard and Blanchoin, 2002). Cytoskeletal orientation is thus critical for proper cell layer function.

Due to the complex orientation and shape attained, trichome morphogenesis includes a distinct requirement for the microtubule and actin filament network. (Szymanski, *et al.*, 1999). For example *ATARP3/DISTORTED1* (*ARP3/DIS1*), *ATARP2/WURM* (*ARP2/WRM*) and *DISTORTED2* (*DIS2*) encode components of the evolutionarily conserved ARP2/3 complex that nucleates actin filament polymerization (Le, *et al.*, 2003; El-Din El-Assal, *et al.*, 2004; Saedler, *et al.*, 2004). *dis1*, *dis2* and *wrm* mutations cause severe trichome growth deformities. This is due to defects in cytoplasmic actin bundle organization and abnormally clustered microtubules (which are important in maintaining growth polarity). They also result in defects in cell-cell adhesion, with unusual small gaps being visible between epidermal pavement cells. Several genes involved in regulating actin polymerisation have also been shown to be required to maintain root hair polarity and cell shape. For example the *deformed root hairs* (*der1*) mutant alleles of the *ACTIN2* gene exhibit defects in root hair morphogenesis (Ringli, *et al.*, 2002). Recently a gene which regulates actin polarisation specifically to control epidermal cell morphogenesis in maize leaves was isolated. *brick1* (*brk1*) mutant epidermal pavement cells are unusually shaped and there is also abnormal stomatal formation (Frank and Smith, 2002). *BRK1* is conserved across plants and animals and thus a common process could be involved in regulating actin polarisation.

In order to regulate the cytoskeleton organisation itself, members of the RHO family of small GTPases (ROPs) have been postulated to mediate an auxin/ethylene

signal (Grebe, 2004). ROPs bind GTP and act as molecular switches which regulate diverse cellular activities. Several *ROP* family members have been shown to be expressed in epidermal cells and affect the planar outgrowth of root hairs (Molendijk, *et al.*, 2001).

As mentioned, regulation of the actin cytoskeleton affects the plane of cell division. In maize the *DISORGANISED ALEURONE1* and *2* (*DIL1* and *2*) genes have found to be important for maintenance of the division plane of both the maize endosperm aleurone layer and also the epidermis (Lid, *et al.*, 2004). Regulation of proper aleurone layer division patterns is vital for aleurone function (Mineyuki, 1999). In *dil1* and *dil2* mutants the mitotic division plane is not properly controlled, which results in disorganisation of aleurone layers in mature maize grains. It is thought that the *DIL* genes are involved in regulating the dynamics of the actin cytoskeleton to control the site of cell division. Due to improper embryo nutrition in the maize grain the embryo arrests. Rescued embryos have an irregular leaf epidermis, abnormal roots and aberrant root hair morphology. It seems therefore that in maize the division plane of the aleurone and the epidermis are regulated by common factors (Lid, *et al.*, 2004). This is also evident in the *extra cell layer1* (*xcl1*) mutant which has extra cell layers in both the aleurone and the leaf epidermis (Kessler, *et al.*, 2002). Whether or not endosperm and embryo development in *Arabidopsis* are also commonly controlled is yet to be determined. However as there is no structure comparable to the maize aleurone layer in *Arabidopsis*, this does not seem likely. Studies of the *xcl1* mutant also highlight the importance of maintenance of correct cell division planes in the outside cell layer to maintain correct cellular identity (Kessler, *et al.*, 2002).

In addition to maintaining apical/basal polarity within each cell, the polarity of the outside/L1 layer as a whole is maintained. Planar polarity is the common polar arrangement of cells within the plane of an epithelium. Maintenance of the correct orientation of cells within a tissue is vital when cell layers and organs are developing. The *Arabidopsis* root is a useful system for studying the mechanism of

planar polarity as the common orientation of root hairs in the root epidermis is a visible consequence of its maintenance.

As mentioned in Section I.2.3, auxin is transported in a planar fashion, down from the shoot to the root tip, through the vasculature. It is then transported back up, basipetally, through the epidermal layer due to the polar localisation of auxin efflux and influx carriers. This provides a mechanism to orientate the plane of polarity and is also important for root gravitropism (Rashotte, *et al.*, 2000; Grebe, 2004). The auxin influx carrier AUXIN-RESISTANT1 (AUX1) has been shown to be involved in maintaining the planar polarity of root hairs (Grebe, *et al.*, 2002). It is thought that AUX1-mediated auxin influx from the auxin maxima at the root tip is important in setting up the planar polarity of the root epidermis (Grebe, 2004). A second pathway important for planar polarity but independent of *AUX1* involves sterol action. For example a recent study shows that proper PIN1 localisation and cell polarity require the normal synthesis and a balanced composition of membrane sterols (Willemsen, *et al.*, 2003). Loss of *STEROL METHYLTRANSFERASE1* function resulted in various cell polarity defects including in the polar initiation of root hairs. PIN1 and PIN3 localisation were found to be disrupted in this mutant and it seems that the correct recycling of the PIN1 protein, which is vital for auxin flow function, might be affected by sterols. There is also some evidence for the role of the hormone ethylene in maintaining planar polarity (Masucci and Schiefelbein, 1994). Downstream of an auxin/ethylene response it seems likely that cytoskeletal elements are regulated.

Complex and interlinked signalling pathways are involved in differentiation of the epidermal layer. These pathways are dependent on the continuity of the epidermal layer as well as the initial epidermal specification event. Cell differentiation is also controlled by an abundance of regulatory transcription factors which are controlled by upstream factors such as *AtML1/PDF2*. Recent research points to the existence of extracellular signals (such as those processed by *ALE1* and *SDD1*) with roles in controlling differentiation and patterning. The reception of such signals therefore seems key for controlling epidermal development. The examples described above

highlight the importance of cell-cell communication for proper cell patterning and co-ordinated cell differentiation.

I.3.4. Epidermal cell layer specification and organisation

As already mentioned, *AtML1/PDF2* plays a critical role in L1 layer specification and in the regulation of the genes and pathways discussed above. The subject of this thesis is to investigate epidermal specification and organisation further, and to characterise other genes involved in this process. In maize one pathway involved in the specification of the epidermal layer has been partially dissected. This will now be outlined.

In maize the *CRINKLY4* (*CR4*) gene seems to be involved in mediating cellular differentiation responses in the epidermis, and specification of the aleurone layer (Becraft, *et al.*, 1996; Jin, *et al.*, 2000; Becraft, *et al.*, 2001). The *CR4* gene was originally isolated from a population of Mutator (Mu)-transposable element-mutagenised maize plants (Becraft, *et al.*, 1996). *crinkly4* plants are short in stature, late flowering and have small, crinkly leaves of rough texture. Histological analysis revealed that defects which occurred were predominantly in the epidermal cell layer. There were also some defects during floral development but no effect on roots. The mutant epidermal surface functions were found to be compromised, allowing graft-like fusions to occur between organs. Adherence between leaves therefore resulted in contortion of the *cr4* plant. There were also abnormal L1-cell division patterns in *cr4* mutants. The important functions of the epidermis in restricting cell division patterns to anticlinal planes, and in preventing surface de-differentiation, were severely compromised.

The internal anatomy of epidermal cells was found to be relatively normal although there were various defects in cell morphology including an effect on cell shape, cell wall thickness and structure, cuticle formation and vesicle trafficking. Certain cell types were also found to be placed in inappropriate positions on *cr4* leaves. In

addition there were also tumour-like outgrowths on the surface of some *cr4* mutant leaves. This indicated a partial transformation to bulliform cell fate (specialised epidermal cells involved in water storage and regulation of leaf shape) (Jin, *et al.*, 2000). However the epidermis of *cr4* mutant leaves did generally contain normal epidermal cell types, which suggested that there was not a specific effect on epidermal identity *per se*. There were also effects on the underlying cell layer although this appeared to be a secondary response due to the physical stress of malformed epidermal cells (Becraft, *et al.*, 1996). In summary, mutations in the *CRINKLY4* gene result in defective epidermal differentiation and organ fusion. The latter effect is however the result of disruption of a different mechanism to that affected in *fdh* mutants (Lolle, *et al.*, 1998).

In addition to epidermal defects *cr4* seeds were found to have defects in the specification of aleurone layer cells (Becraft, *et al.*, 1996). Portions of the endosperm failed to differentiate aleurone and instead the cells on the surface of the endosperm had attributes of starchy endosperm cells, indicating improper cell specification. This suggested that *CR4* might function in the perception of positional cues that specify aleurone cell fate during endosperm development.

CR4 therefore seems to be involved in specification of aleurone cell fate and epidermal differentiation. As mentioned earlier several genes appear to be involved in regulating the development of both the epidermis and the aleurone layer. The aleurone layer and the epidermal layer share some similar characteristics. They both grow by anticlinal division on the surface of their respective organs and contain morphologically similar cells with a cuboidal shape and thick cell walls. It is likely that cell-cell interactions are involved in differentiation of both structures. It also seems likely that *CR4* has such a function, playing a single role that varies with the cellular context (Becraft, *et al.*, 1996).

This array of defects suggested that CR4 regulated a diverse set of cellular functions during development, analogous to growth factor receptors in animals (Jin, *et al.*, 2000). In animals growth factor receptors are receptor protein kinases involved in receiving growth factor signals and transducing them with the result of changes in cell activity. For example, the *Drosophila* epidermal growth factor

receptor (EGFR) regulates an extensive range of processes involved in cell fate decisions, cell differentiation and patterning during embryo development (Schweitzer and Shilo, 1997).

CR4 was cloned and found to encode a putative membrane-localised receptor-like kinase of classical structure (Becraft, *et al.*, 1996). Receptor-like kinases will be reviewed in Section I.4. CR4 has a predicted extracellular region which contains a cysteine-rich domain similar to the ligand binding domain in the mammalian TUMOUR NECROSIS FACTOR RECEPTOR (TNFR) (Idriss and Naismith, 2000). Identification of this extracellular region at the time defined a new class of receptor-like kinases. In addition, proximal to this, are seven repeats of a novel motif. The predicted intracellular region consists of a predicted kinase domain capable of auto-phosphorylation, and an unusually long C-terminal region (Walker, 1994; Jin, *et al.*, 2000).

As will be discussed in Section I.4, motifs in the extracellular region of receptor-like kinases are important in ligand binding. What these consist of in the CR4 protein therefore may provide clues as to the nature of the ligand(s) bound. TNFRs are membrane localised proteins which are involved in numerous biological processes including mammalian immune responses (Dempsey, *et al.*, 2003). TNFRs possess an extracellular canonical motif of three cysteine-rich repeats. The second and third repeats are thought to be involved in binding the TNF small peptide ligand. Crystallographic study shows that 80% of the interaction between the TNFR and its ligand occurs within the second repeat (Banner, *et al.*, 1993). This is where the highest degree of similarity between CR4 and TNFR exists. If this motif functions similarly in plants then a CR4 ligand could be a peptide. There is a fascinating possibility that CR4 binds TNF-like peptide hormones which are yet to be found in plants. The first cysteine-rich repeat is thought to mediate interactions involved in forming TNFR trimers (see Section I.4.2.C for more detail on multimerisation of receptor molecules) (Banner, *et al.*, 1993; Idriss and Naismith, 2000). TNFR activation is thought to result in recruitment of various intracellular adaptors to the cytoplasmic domain of the protein. These adaptors include the TNFR-associated

factors (TRAFs) and are involved in signal transduction (Rothe, *et al.*, 1994). Depending on the adaptor recruited there is activation of differential downstream signal transduction pathways such as apoptosis, or the activation of transcription factors involved in immune and inflammatory responses (Dempsey, *et al.*, 2003).

An alternative ligand binding site to the TNFR-like repeat domain, is the domain containing seven repeats of a novel motif. The putative function of this domain will be discussed in Chapter IV of this thesis.

CR4 is expressed in the outer layer of the embryo and all aerial organs, particularly in younger tissues of the shoot and leaves, and is absent from roots (Kang, *et al.*, 2002). From the work described above it seems that it has two related functions. The first is an involvement in the reception of positional cues that are involved in regulating the differentiation of the epidermis in the embryo. The second is a role in the specification of aleurone cell fate through endosperm development. What the ligand(s) for CR4 could be is as yet unknown. Due to the presence of a TNFR-like repeat domain it has been hypothesised that CR4 receives a peptide ligand. The fact that CR4 is putatively membrane localised, with part of the protein in the extracellular domain makes a small peptide ligand (potentially processed by ALE1; Tanaka, *et al.*, 2001) located in the apoplast a good candidate. Reception of such a ligand could direct the fate of the outside cell layer. It has been shown that *CR4* controls a cell autonomous response (Becraft, *et al.*, 2001). It regulates cell differentiation through strict intracellular functions, consistent with its molecular identity as a transmembrane receptor.

Two other maize mutants have similar phenotypes to that of *cr4*. It has been suggested that they act in overlapping pathways. The first is the *DEFECTIVE KERNEL1* (*DEK1*) gene (Becraft, *et al.*, 2002; Lid, *et al.*, 2002). Weak *dek1* alleles show similar endosperm and vegetative phenotypes to *cr4* mutants. Aleurone formation is blocked at an early stage and similarly to *cr4* mutants the peripheral endosperm cells develop as starchy endosperm (Becraft and Asuncion-Crabb, 2000). Aleurone cell fate is specified early in maize endosperm development (Morrison, *et al.*, 1975; Brown, *et al.*, 1994). The fact that *dek1* mutants have an altered aleurone

cell identity suggests that *DEK1* plays a role in specifying and maintaining aleurone cell fate and differentiation. It was at first thought that DEK1 might be a ligand for CR4 (Becraft and Asuncion-Crabb, 2000). Later research however showed that this was not the case, and that it was instead involved in early events that direct aleurone cell fate in another way (Becraft, *et al.*, 2002).

Weak *dek1* alleles have defective epidermal layers and increased numbers of bulliform cells, similar to *cr4*. Strong *dek1* alleles however show more pronounced epidermal defects in the embryo than *cr4* alleles. In addition the defects in strong *dek1* mutants are more suggestive of a mis-specification event, rather than differentiation defects as seen in the *cr4* mutant epidermal layer. *dek1* has severe defects in cell-layer organisation of the developing embryo which fails to form a proper embryo axis and instead arrests (Becraft, *et al.*, 2002). *DEK1* is therefore required for the establishment of an embryonic axial pattern and seems to have a more fundamental effect on epidermal specification than *CR4* (Becraft, *et al.*, 2002). In double mutants of a weak *dek1* and a strong *cr4* allele, the embryo phenotype is exacerbated (Becraft, *et al.*, 2002). Double mutants show elements of epistasis, additivity and synergy. This suggests that the two genes might act in the same pathway (Becraft, *et al.*, 2002). Like *CR4*, *DEK1* is expressed in most plant tissues (Lid, *et al.*, 2002). There are also similar genes in *Arabidopsis*, rice and loblolly pine (a gymnosperm) which suggests that *DEK1* plays a conserved role in plant development.

The *DEK1* gene was cloned and found to encode a predicted membrane-anchored calpain-like cysteine proteinase (Lid, *et al.*, 2002; Wang, *et al.*, 2003). The DEK1 protein contains 21 predicted membrane spanning domains, a loop region predicted to be located in the extracellular region, and a cysteine protease domain inside the cell. Cysteine proteases are thought to be involved in selected cleavage of proteins to regulate their function. DEK1 defines the only family of calpains in plants (Becraft, *et al.*, 2002; Lid, *et al.*, 2002). It seems unlikely that DEK1 is involved in cleaving a precursor ligand for reception by CR4 as the protease domain of DEK1 is intracellular. However as DEK1 also functions cell autonomously it might instead negatively regulate an inhibitor of CR4, as suggested in Becraft, *et al.* (2002). A

model was therefore proposed for the specification and regulation of aleurone cell fate and epidermal differentiation, involving an interaction between DEK1 and CR4.

The third hypothetical member of this pathway is encoded by *SUPERNUMARY ALEURONE LAYER1* (*SAL1*) and is involved in regulating the aleurone cell layer (Shen, *et al.*, 2003). The *sal1* mutant was isolated from a screen on a Mu-mutagenised population of maize plants for mutants with multiple aleurone layers. *sal1* mutants carry up to seven layers of aleurone in the endosperm of defective kernels (rather than one layer as in wild-type kernels). *SAL1* encodes a homolog of a human Class E vacuolar sorting protein. In animals, similar proteins appear to be involved in the vesicle trafficking and turnover of plasma-membrane localised receptor proteins. As will be discussed in Section I.4.3.B, this sort of turnover is often important for correct signalling or damping down of receptor-kinase activity. As *CR4* encodes a putative membrane-localised receptor-like kinase, it is possible that SAL1 is involved in regulating CR4 turnover or the turnover of *CR4*-pathway elements. In *sal1* mutant plants there are additional aleurone layers, rather than fewer layers as in *dek1* mutants. This could suggest that loss of turnover of CR4 signalling results in constitutive signalling of aleurone cell fate, due to CR4 protein build-up at the membrane. Alternatively SAL1 might be involved in targeting components involved in aleurone cell fate via an independent pathway to *CR4/DEK1*. SAL1 has a bipartite nuclear localisation signal and it could be that it functions in the control of other cell-fate regulators in the nucleus (Shen, *et al.*, 2003). However, involvement of *SAL1* in a pathway with *CR4* and *DEK1* certainly seems possible. Whether a similar mechanism is involved in *Arabidopsis* will be discussed in Section I.5. Firstly though the receptor-like kinase gene family (of which *CR4* is a member) will be discussed.

I.4. Signalling mechanisms in plants: the role of receptor-like kinases

I.4.1. The plant receptor-like kinase gene family

The importance of inter-cell communication in order for the plant to properly control development has been discussed. But how are cell-cell signals transmitted? A common way for cells to convey signals to other cells is through reversible phosphorylative activity - phosphorylation of target proteins. Protein kinases generally add phosphate ions to a protein, and phosphatases remove them. These mechanisms can be used to activate or inactivate target proteins. Receptor kinases are membrane-localised proteins that are involved in receiving signals or ligands from the exterior of the cell. When activated by ligand-binding, receptor kinases phosphorylate themselves and downstream targets, thus mediating and transducing extracellular stimuli and developmental signals into the cell. The eventual downstream target is usually a change in gene regulation.

In animals, protein receptor kinases make up a large family, being involved in signalling to regulate a wide range of responses (Fantl, *et al.*, 1993). There are two catalytic categories of kinases with the distinction based on substrate specificity. The first are receptor tyrosine kinases which specifically phosphorylate tyrosine residues. These are the more common than the second type, which phosphorylate serine or threonine residues. In animals there are many receptor tyrosine kinases (RTKs) which are generally receptors for growth factors (Fantl, *et al.*, 1993). They have key roles in cellular processes and are involved in co-ordinating the development of multicellular organs (van der Geer, *et al.*, 1994). Animal receptor kinases have been extensively studied and many of the ligands and downstream molecules have been elucidated. The ligands found for receptors include various hormones such as the epidermal growth factor (EGF) (Schweitzer and Shilo, 1997). Downstream targets include protein phosphatases and the evolutionarily conserved MAP kinase phosphorylation cascade (Feng, *et al.*, 1993; Neiman, 1993).

The idea that similar proteins to RTKs were involved in the recognition of polypeptide ligands by transmembrane receptors at the cell surface in order to direct

plant development, was at first thought unlikely. It was thought that the cell wall that surrounds the plant cell would inhibit such a mechanism of plant cell-cell communication. However in recent years similar proteins have in fact been found in plants – these are designated plant receptor-like kinases (RLKs). RLKs are now widely known with around 615 putative members in *Arabidopsis* (Shiu and Bleecker, 2003). Those characterised have been found to play roles in many disparate signalling processes (reviewed in Torii and Clark, 2000; Shiu and Bleecker, 2001; Morris and Walker, 2003). Transmembrane RLKs constitute 60% of the kinases in *Arabidopsis* and represent a sizeable 2.5% of all protein-coding genes (Shiu and Bleecker, 2001; Shiu and Bleecker, 2003). They are involved in the control of a wide range of developmental processes including organ patterning (Perez-Perez, *et al.*, 2002; Shpak, *et al.*, 2004), cell differentiation (Matsubayashi, *et al.*, 2002; Higuchi, *et al.*, 2004) and self incompatibility (Cabrillac, *et al.*, 2001; Murase, *et al.*, 2004). Many RLKs are important in response to the environment including roles in disease resistance (He, *et al.*, 1998), symbiont nodulation (Endre, *et al.*, 2002) and response to insect attack (Morris and Walker, 2003). One particularly well characterised RLK is CLAVATA1 (CLV1) which is involved in maintaining the shoot apical meristem (as mentioned in Section I.2.4) (Shiu and Bleecker, 2001). The diverse range of biological processes that RLKs affect is underpinned by common signalling elements. Although performing distinct roles, plant RLKs are thought to function in an analogous way to protein kinases in animals. The study of plant RLKs is however still in its early stages relative to that of animal receptor kinases.

The dynamics of plant receptor-like kinases including the mechanism of localisation, signalling and turnover in the cell will be addressed in this section. Parallels and paradigms in the animal protein kinase family will also be discussed. The RLK family as a whole will be briefly introduced but the specifics of the roles that disparate RLKs play will not be looked at in detail.

Plant RLKs have a separate origin to that of animal receptor like kinases. They form a distinct monophyletic gene family and are descended from an ancestor similar to the animal Pelle/IRAK cytoplasmic kinases (Shiu and Bleecker, 2001). The downstream components regulated by RTKs and RLKs are clearly different which confirms that they have evolved independently in plants and animals. However RTKs and RLKs do share common features and have behavioural similarities, suggesting that their evolution has likely been in parallel (Cock, *et al.*, 2002). Convergent evolution has also generated similar receptor kinase motifs in both families - they share structural similarities (Walker, 1994; Shiu and Bleecker, 2003).

The characterised RLKs in plants possess all of the structural characteristics of receptors. This includes an amino-terminal signal peptide, followed by a predicted extracellular region of variable structure where the ligand is predicted to bind. Adjacent to this region is a hydrophobic domain of variable length predicted to span the plasma membrane. At the carboxy-terminal end of the protein is the kinase domain (which is intracellular) and a C-terminal end of variable length; a long C-terminal region is uncommon in plants. In plants almost all known RLKs are serine-threonine kinases – this is unlike the situation in animals where most are tyrosine specific (van der Geer, *et al.*, 1994). Almost all RLKs phosphorylate either serine or threonine residues, although some have been reported that phosphorylate both; POLLEN RECEPTOR-LIKE KINASE1 (PRK1) which is a pollen-specific RLK in *Petunia inflata* has been shown to autophosphorylate on both serine and tyrosine residues (Mu, *et al.*, 1994). The RLK kinase domain has itself been extensively studied in order to define the conserved catalytic residues and to determine residue specificity. Despite their structural diversity and differing substrate specificity, the primary sequence and secondary structure of the catalytic domains of all eukaryotic protein kinases are highly conserved (Hanks, *et al.*, 1988; Hanks and Quinn, 1991). This information is particularly useful for characterising putative receptor kinases and can also be used for phylogenetic analysis of kinase families (Walker, 1994). The kinase domain of many RLKs contains a small structural feature known as the activation loop. These RLKs appear to be positively regulated by phosphorylation on a key reside in this loop, although it is not known what purpose this plays in RLK

function (Johnson, *et al.*, 1996). Mutation of residues in the activation loop can inactivate a protein kinase.

The receptor kinase family in plants is a large and diverse one. It includes receptor-like kinases (RLKs) and also non-receptor kinases - receptor-like cytoplasmic kinases (RLCK). Based on sequence identity between the extracellular domain and kinase phylogeny, the receptor kinase family can be divided into 44 subfamilies (Shiu and Bleecker, 2001). This consists of 12 subfamilies of RLCKs and 32 subfamilies of RLKs. The extracellular domains of RLKs share some motifs with animal RTKs, some with other animal proteins, and some with plant proteins (reviewed in Torii and Clark, 2000). The variety of extracellular motifs probably reflects a diverse range of ligands. This, and the wide range of processes regulated, suggest that the RLK family is a highly evolved group of signalling proteins designed to control multiple processes.

The majority of RLKs contain leucine-rich repeat (LRR) motifs, which are implicated in protein-protein interactions (Kobe and Deisenhofer, 1994). There are about 220 LRR-class members grouped into 14 subfamilies that play a wide range of roles (reviewed in Dievart and Clark, 2004). They include proteins which are receptors for hormones. For example BRASSINOSTEROID INSENSITIVE1 (BRI1) is a key component of a membrane brassinosteroid/brassinolide receptor. BRI1 is involved in growth promotion in response to brassinosteroids (Li and Chory, 1997; Friedrichsen, *et al.*, 2000). The LRR class also includes ERECTA which is involved in the regulation of organ shape, and CLV1 (Torii, *et al.*, 1996; Shpak, *et al.*, 2003; Shpak, *et al.*, 2004). The *Arabidopsis* variant Landsberg erecta has a mutation in the *ERECTA* locus which alters the form of the plant.

The epidermal growth factor (EGF) class contains proteins with EGF-like repeats. These repeats are found in various animal extracellular receptor domains and are thought to play a role in protein-protein interactions (Rebay, *et al.*, 1991). EGFR is a member of the well characterised EGFR/ErbB family of animal RTKs which bind EGF-related peptide growth factors. They are involved in cell differentiation and proliferation (Olayioye, *et al.*, 2000). Plant EGF-repeat-containing RLKs consist of

one subfamily and are represented by the cell wall-associated receptor kinases (WAKs) which have been shown to be involved in pathogenic responses (He, *et al.*, 1998).

There are three S-locus domain subfamilies of RLKs. S-domain RLKs, including S RECEPTOR KINASE (SRK), are involved in the self-incompatibility recognition between pollen and stigma, and some play roles in defense signalling (Stein, *et al.*, 1991; Cabrillac, *et al.*, 2001). The tumour necrosis factor receptor (TNFR)-class of RLKs share a TNFR-like motif which consists of three cysteine rich domains. There is one TNFR-class subfamily which contains the previously mentioned maize gene *CRINKLY4* (Section I.3.4) and its homologs in other plant species. There are two subfamilies of lectin-domain RLKs which are thought to be involved in sugar-mediated signal transduction (Harve, *et al.*, 1996). The pathogenesis-related protein 5 (PR5) class has one member, PR5-like receptor kinase (PR5K), which is involved in the pathogenesis response and in pollen development (Wang, *et al.*, 1996).

There are also various small subfamilies which contain recently characterised RLKs with novel extracellular motifs. For example the *PROLINE EXTENSIN-LIKE RECEPTOR KINASE 1 (PERK1)* gene encodes a novel plant RLK from *Brassica napus* (Silva and Goring, 2002). It contains a proline-rich extracellular domain with sequence similarity to extensins. It is thought to be involved in perception and response to wounding. The function of some other identified subfamilies of RLKs with novel motifs are as yet unknown (Shiu and Bleecker, 2001).

Understanding the molecular mechanism of plant RLK action, including defining kinase activity, identifying ligands and dissecting the downstream signalling pathways, are major challenges which are gradually being solved. This is particularly aided by the advent and availability of new techniques and resources (Lease, *et al.*, 1998; de Wildt, *et al.*, 2002; Dievart and Clark, 2003).

I.4.2. Receptor-like kinases: localisation and ligand binding

I.4.2.A. Receptor-like kinase localisation

Plant RLKs are generally plasma-membrane localised proteins. The mechanism of transport to the membrane is often facilitated by an actin-dependent vesicular transport system which traffics the protein from the endoplasmic reticulum (ER)/golgi. This is directed by the hydrophobic amino terminus of the protein which acts as a signal peptide. The transmembrane domain itself is a hydrophobic region which is situated within the phospholipid bi-layer. At the carboxy terminal side of the transmembrane domain are basic residues such as arginines and lysines which stop the protein moving completely through the membrane. This is a typical Type I integral membrane protein structure (Singer, 1990). The basic amino acids act as a 'stop-transfer' signal which situates the amino terminal domain in the extracellular region, while the carboxy terminus resides on the cytoplasmic face of the plasma membrane (Walker, 1994).

After the protein arrives in the plasma membrane it must be retained there. In some cases this has been shown to involve interaction to subcellular scaffold proteins. The animal ErbB2 RTK in the ErbB family interacts with members of the PDZ-domain-containing protein family via carboxy-terminal motifs in the kinase domain (Carraway and Sweeney, 2001). These PDZ membrane proteins act as scaffolds to restrict receptor kinases to certain sites of regions of the cell. For example the PDZ-domain-containing protein Erbin binds to ErbB2 to mediate a basolateral localisation of the RLK in epidermal cells (Carraway and Sweeney, 2001). PDZ-domain proteins are also involved in targeted delivery of proteins to the membrane. It seems that the location of receptors in the membrane can alter their signalling properties. For example, whether the ErbB2 RTK is localised on the basolateral surface or on the apical surface of the epidermal cell modulates which downstream targets are bound, and so which signals are sent to the cell (Carraway and Sweeney, 2001). This provides a mechanism by which the relative location of

the ligand bound can be perceived. It also suggests that there is compartmentalisation of downstream components along an apical-basal axis.

One structure thought to be important in modulating animal cell signalling is the lipid raft. Lipid rafts are subdomains of the plasma membrane which are rich in cholesterol and glycosphingolipids (reviewed in Pike, 2003). Lipid rafts vary in the proteins and lipids that they contain and appear to involved in partitioning signalling components within the cell. In doing this they are involved in regulating signalling pathways. For example they may contain incomplete signalling pathways that are activated by recruitment of a certain molecule into the lipid raft. Alternatively they could physically isolate signalling components to block certain interactions within the cell. They also provide an alternative route of endocytosis into the cell. Lipid rafts are important in modulating the signalling of receptor tyrosine kinases such as members of the EGFR and Ephrin receptor (Eph) families in animals (Gauthier and Robbins, 2003; Pike, 2003). For example EGFRs rapidly move out of the lipid rafts in which they are situated upon ligand binding (Mineo, *et al.*, 1999). It is thought that different classes of the ligands for Eph receptors, ephrins, associate with distinct populations of lipid rafts in the cell. This is proposed to allow specificity of ephrin signalling (Gauthier and Robbins, 2003). Whether or not similar structures exist in plant cells is not yet known.

I.4.2.B. Ligand binding

Ligand binding occurs on a specialised receptive domain of the protein, which is located in the extracellular region of the cell. The similarity of plant and animal extracellular motifs suggests that similar ligands might be bound by both. By comparison to the animal equivalents, the LRR, TNFR and EGF-repeat like ligand binding domains and similar protein-protein interaction motifs of plant RLKs are suggestive of proteinaceous ligands such as peptides or polypeptides (Walker, 1994). Whether TNF-like peptides will be found in plants to bind TNFR-motif containing proteins such as CR4 is yet to be seen. The TNF family of ligands is large and

diverse which would make identifying such molecules difficult (Orlinick and Chao, 1998). Plant RLK ligands are likely to be secreted rather than membrane-bound, due to the presence of the cell wall which separates adjacent cell membranes. The only exception to this would be in the case of the RLK and ligand being produced by the same cell.

So far very few ligands have been conclusively proven to bind plant RLKs. Many of these are peptides such as CLV3 (Trotochaud, *et al.*, 1999). This is like the situation in animals where ligands are mostly polypeptides. One of the best characterised ligand molecules is the S locus cysteine-rich protein (SCR) which has been shown to interact directly with SRK *in vitro* (Kachroo, *et al.*, 2001). In tomato the small peptide systemin has been found to bind the LRR RLK systemin cell-surface receptor SR160 (Scheer, *et al.*, 2003). SR160/systemin is an important receptor/ligand pair involved in the wounding response in tomato (Scheer and Ryan, 2002). The *Arabidopsis* WAK1 was found to bind a glycine rich extracellular peptide, GRP (Park, *et al.*, 2001). In addition modified peptides such as the bacterial elicitor flagellin are bound. Flagellin is a glycoprotein which has been shown to be bound by the *Arabidopsis* LRR RLK FLAGELLIN SENSITIVE 2 (FLS2) (Gomez-Gomez and Boller, 2000; Gomez-Gomez, *et al.*, 2001). As well as peptide ligands, plant steroid hormones also act as ligands. For example the steroid brassinolide/brassinosteroid is the ligand for BRI1 (Wang, *et al.*, 2001). The LRR-domain of BRI1 contains a 'loop-out' island which is postulated to be the ligand binding domain for the protein (Li and Chory, 1997). Bacterial Nod factors provide another example of this sort of ligand. Lipochitin oligosaccharides, which are involved in nodule development in legumes, appear to be sensed by another LRR RLK, NODULATION RECEPTOR KINASE (NORK) (Endre, *et al.*, 2002).

There are also various small peptide growth factors in plants for which no receptor has yet been identified (Torii and Clark, 2000). For example there is a large family of ligands related to SCR which could turn out to be ligands for the other S-locus RLKs (Vanoosthuyse, *et al.*, 2001). There is also a large family of 'orphan' ligands related to the CLV3 peptide – the widely expressed CLV3/ESR-like (CLE) family (Sharma, *et al.*, 2003). It is possible that these proteins will turn out to be ligands for

important RLKs involved in regulating development. Improved knowledge of receptor-ligand dynamics will be key to understanding RLK signalling.

I.4.2.C. Oligomerisation: ligand-independent and ligand-induced

Receptor like kinases commonly form complexes with each other (dimers/trimers) or with other similar proteins (di-oligomers/tri-oligomers) (Olayioye, *et al.*, 2000). Multimerism is a functionally important process and is a critical event in the activation of almost all known receptor protein kinases (Walker, 1994). Multimerisation allows transphosphorylation of the kinase domains of two monomeric units by bringing them closer together to activate the receptor complex. There are some animal RTKs in the epidermal ErbB-family that act as monomers, but these are catalytically less active than those that act as dimers (Waterman and Yarden, 2001). There are various examples of both homo- and hetero-oligomers in plants. For example the BRI1 LRR-RLK interacts with the LRR-RLK BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1) forming a ligand-dependent hetero-oligomer to transduce the brassinosteroid/brassinolide signal (Li, *et al.*, 2002).

The kinase catalytic domain itself is absolutely required for function (Hunter and Lindberg, 1994). However, a lack of kinase activity can be provided by another kinase, even one of divergent sequence. For example the activity of CLV1 can be functionally replaced by that of cytoplasmic kinases (Trotochaud, *et al.*, 1999). This is also seen in animals where the kinase activity of 'dead' or 'fractured' animal RTKs which lack kinase activity can be compensated for by cytoplasmic kinases (Kroiher, *et al.*, 2001). So far the kinases found to provide such activity seem to be cytoplasmic kinases, however hetero-oligomeric partners could also be proposed to provide kinase activity.

There are two ways by which receptor kinases form multimers and are activated (Fig.I.7A-C). The first is via activation-mediated oligomerisation. This means that binding of a ligand to one or two closely situated monomers can cause a conformational change which results in the formation of a multimeric complex

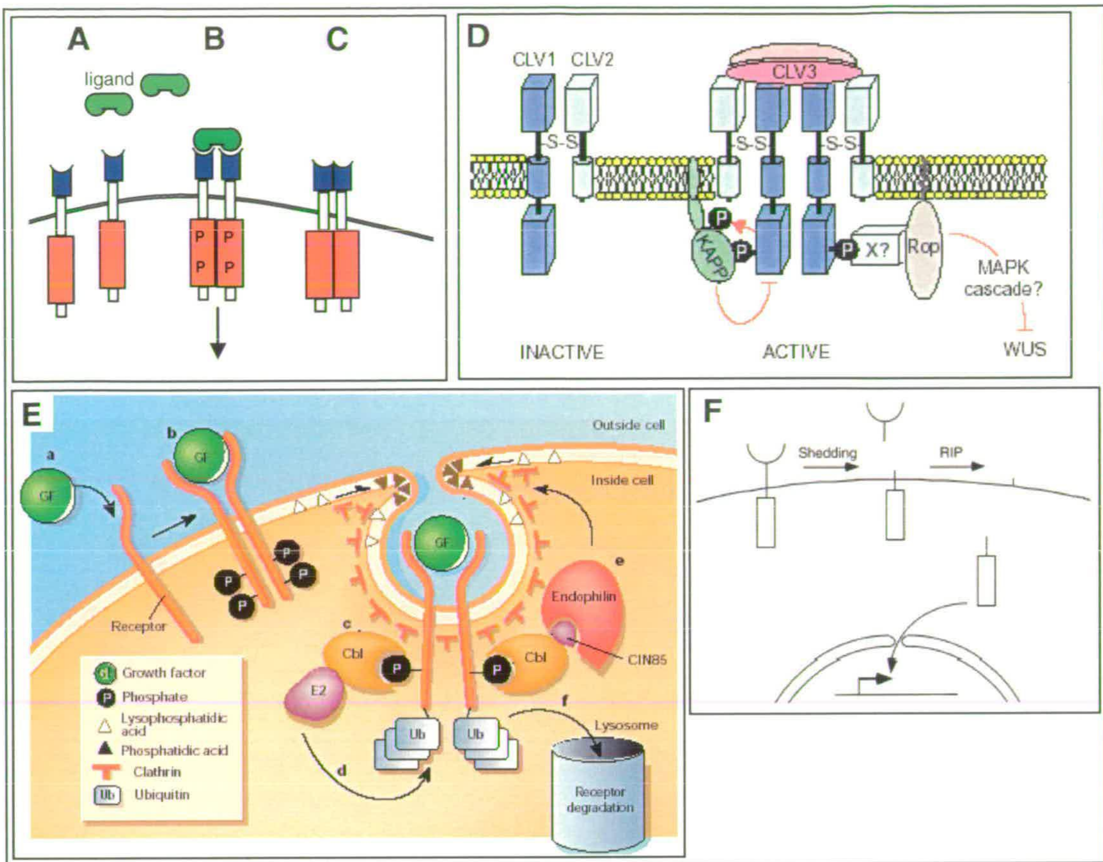

Figure I.7. Schematics of RLK behaviour. (A) RLK molecules situated in the plasma membrane (blue box, ligand-binding domain; white box, transmembrane domain; orange box, kinase domain). Ligand molecules (green) are present in the extracellular matrix. (B) Ligand-dependent dimerisation. Two RLK molecules bind one ligand molecule to form a homodimer complex. After binding transphosphorylation (P) occurs on the kinase domain. This is followed by downstream signalling (arrow). (C) Ligand-independent dimerisation. Two RLK molecules come together in the absence of a ligand molecule. Dimerisation brings the two RLK monomers closer together and alters their conformation. This 'active' complex conformation is ready to receive a ligand. (D) CLV1 binds to CLV2 at the plasma membrane to form a hetero-oligomer. Two of these hetero-oligomers come together to form a hetero-multimer. After binding the CLV3 ligand, transphosphorylation occurs between the two CLV1 kinase domains. This phosphorylation results in the binding of cytoplasmic targets including ROP, which might activate a MAPK kinase cascade. The final target of this signal transduction is likely to be repression of the *WUS* gene. In addition to downstream signalling it seems that there is phosphorylation-dependent KAPP binding of CLV1. KAPP binding is involved in RLK complex downregulation. (E) Ligand-binding dependent endocytosis of the ErbB1 dimer. After ligand (green) binding-induced dimerisation of the ErbB1 receptor (orange), Cbl proteins bind to the complex and induce endocytosis. This occurs via recruitment of scaffold molecules such as CIN85, and endophilins which are regulatory components of clathrin-coated vesicles. In addition Cbl binding targets ErbB1 for ubiquitylation (Ub) and subsequent lysosomal degradation. (F) Ectodomain shedding of the extracellular domain of a membrane-localised protein may release part of the protein to act as a ligand. Regulated intramembranous cleavage (RIP) processing, which results in cleavage at an intracellular site on the protein, can result in release of an active signal into the cell which could regulate transcription in the nucleus. (D) Taken from Cock, *et al.* (2002). (E) Taken from Oved and Yarden, (2002). (F) Taken from Arribas and Borroto, (2002).

(Fig.I.7B). This then facilitates transphosphorylation and allows the signal to be transmitted downstream. This is a ligand-dependent process. The second is via oligomerisation which then results in activation of the protein complex. This is the formation of multimers in the absence of ligand, which results in an active receptor competent to receive a ligand molecule (Fig.I.7C). This is not a ligand-dependent process (but the later signalling events themselves are dependent on ligand binding). In either case it could be that the multimers are composed of identical units, forming dimers or trimers. Alternatively hetero-oligomers can be formed of different units if they are functionally competent to form a complex. For example as mentioned in Section I.2.4, CLV1 interacts with CLV2 to form a hetero-oligomer. CLV2 is a receptor-like protein, a truncated RLK which lacks a kinase domain (Jeong, *et al.*, 1999) (Fig.1.7D).

Via genetic approaches it might be possible to determine whether certain proteins are likely to form multimeric complexes, either with each other or with related proteins. It is harder to dissect exactly the mechanism of multimerism, although progress towards this is now being made. Some clues come from structural observations. As well as containing protein-protein interaction domains for ligand binding, receptor kinases also contain putative dimerisation motifs. For example many LRR-class RLKs have putative dimerisation modules which consist of closely situated pairs of cysteines (Torii and Clark, 2000). These paired cysteines are thought to form disulfide bonds involved in intermolecular assembly of hetero-multimers. Genetic and molecular evidence suggests that the active CLV receptor complex consists of two CLV1/CLV2 hetero-oligomers, which associate together as a hetero-multimer (Fig.I.7D) (Clark, *et al.*, 1995; Jeong, *et al.*, 1999; Trotochaud, *et al.*, 1999). The S-domain in the plant S-locus RLK consists of an array of ten cysteine residues which are thought to be involved in homodimerisation of RLK molecules via formation of disulfide bridges. These dimers form *in planta*. It seems that subsequent ligand binding then brings the kinase domains of the proteins closer together to allow autophosphorylation and recruitment of intracellular substrates (Giranton, *et al.*, 2000). As mentioned in Section I.3.4, the mammalian TNFR receptor contains one cysteine rich domain which is thought to be involved in

trimerisation (Chan, 2000). Although not a RTK this protein is worthy of discussion and shows some parallels to RTKs. Similarly to SRK it seems that receptor assembly occurs prior to ligand binding. It had been previously thought that trimerisation was induced by binding of a trimeric TNF molecule to three monomeric TNFR subunits. However more recently it was found that the TNFR protein contains a domain that mediates receptor self-assembly - the pre-ligand binding assembly domain (PLAD) (Chan, *et al.*, 2000). Therefore it seems that trimerisation begins with interactions between TNFR monomers, allowing the TNFR complex to be receptive to ligand binding (Chan, 2000). In contrast the mammalian epidermal growth factor receptor (EGFR) undergoes ligand binding-dependent dimerisation which is mediated by interactions between specific residues in the 'dimerisation loop' (Schlessinger, 2002). Within the ErbB family of RTKs there are examples of both hetero- and homo-dimerisation. It seems that hetero-dimerisation of ErbB RTKs combined with multiple ligand types allows an expansion of ErbB signalling potential. This therefore enlarges the number of possible downstream outcomes (Olayioye, *et al.*, 2000). Mutation of certain critical residues involved in receptor kinase multimerisation can result in altered complex conformation. This can then result in ligand-independent dimerisation and autophosphorylation. For example insertion of an extra cysteine residue in the extracellular juxta-membrane region of EGFR results in a constitutively active dimeric receptor (Sorokin, *et al.*, 1994).

In order to understand the mechanism of receptor multimerisation various genetic approaches can be taken. These include investigation of truncated proteins which can have dominant positive or negative effects. In animals co-expression of a functional RTK and a mutant RTK lacking the kinase domain has been shown to have a dominant negative effect (Kroiher, *et al.*, 2001). This is due to the formation of heterodimers which are unable to signal downstream. The ligand for the receptor is however still received and retained so that it is effectively sequestered. It is generally thought that the supply of ligand in a receptor/ligand pairing is the limiting factor for signalling, thus sequestering the ligand will effectively block signalling (Freeman and Gurdon, 2002). In plants too such an effect has also been seen. For

example recent research shows dominant negative effects were associated with both ERECTA which lacked the kinase domain (Shpak, *et al.*, 2003), and CLV1 (Dievart, *et al.*, 2003) where the cytoplasmic region of CLV1 was replaced by the cytoplasmic region of BRI1.

In a similar fashion it has been shown that expression of an RTK lacking an extracellular region can confer a dominant positive effect (Basler, *et al.*, 1991). Due to the mechanistic similarity between plant and animal receptor kinases such an outcome might also be expected to occur with plant RLKs.

I.4.3. Receptor-like kinases: signalling dynamics

I.4.3.A. Downstream signalling targets

In animals, binding of a ligand molecule to the extracellular domain of the RTK complex usually stimulates receptor autophosphorylation. Autophosphorylation occurs at multiple sites and each site functions as a high-affinity binding site for a diverse array of downstream targets which contain a *src* homology-2 domain (SH2) (Heldin, 1991). These downstream signalling components mediate the RTK signal and include proteins with catalytic activity, and molecular adaptors which have a *src* homology-3 (SH3) domain (Pawson and Gish, 1992). These molecular adaptors include molecules that facilitate the reversible formation of protein complexes by recognising only active forms of the RTKs. Small GTP binding proteins such as Ras, serine/threonine kinases such as Raf and the MAPK kinase phosphorylation cascade are all thought to play a downstream role in relaying the signal (Ma, 1993; Neiman, 1993; Moodie and Wolfman, 1994). The final targets of this cascade are likely to be transcription factors involved in regulating gene expression in order to modulate development according to the signal transduced. Activated ErbB family receptors can also act as docking sites for other cytoplasmic molecules. Downstream signalling pathways such as the MAPK cascade can be activated by catalytically inactive receptors, such as cytokine receptors, through ErbB. For example, binding

of a ligand to the mammalian growth hormone receptor (GHR) results in binding and activation of the Janus tyrosine kinase (Jak) to GHR (Yamauchi, *et al.*, 1997). This leads to recruitment and phosphorylation of cytoplasmic signalling molecules which bind to ErbB1. This increases the level of ErbB1 activation and so increases activation of the downstream MAPK cascade. This allows ErbBs to act as signal integrators and therefore to expand their roles in development (Hynes, *et al.*, 2001). The C-terminal end of the protein can also function in protein-protein interactions after kinase activation (Torii and Clark, 2000). The intercellular targets for animal serine/threonine receptor kinases - which are β-transforming growth factor receptors (β-TGFRs) - have not been clearly defined although both heterotrimeric G-proteins and the *Ras/Raf/MAPK* cascade have been implicated (Massague, 1992; Kolodziejczyk and Hall, 1996).

Plants do not seem to have proteins with SH2- and SH3-domains, suggesting that the RLK motifs which interact with downstream targets are distinct from animal RTKs (Torii and Clark, 2000). In plants there is evidence for downstream components that effectively act in a similar fashion, however the interacting components themselves are not homologous (reviewed in Cock, *et al.*, 2002). Again the fact that there are similar components suggests parallel evolution of plant and animal receptor kinases. For example the Rho-type GTPases (ROPs), which are similar to Ras proteins are one possible downstream target. ROPs have been shown to be involved in polarised plant cell growth (as mentioned in Section I.3.3). Interestingly CLV1 has been found to associate with a ROP through which it could activate a MAPK cascade (Fig.I.7D). This then might be involved in targeting repression of *WUS* as described in Section I.2.4 (Clark, 2001). The eventual downstream targets for the *BRI1* pathway are also being uncovered, although no direct substrate for BRI1/BAK1 has yet been found (reviewed in Dievart and Clark, 2004). Downstream of BRI1/BAK1 binding of BR, the GSK3-like kinase BRASSINOSTEROID INSENSITVE2 (BIN2) is inactivated (Li, *et al.*, 2001). This allows two novel proteins, BRI1-EMS-SUPRESSOR1 (BES1) and BRASSINAZOLE RESISTANT1 (BRZ1), which are normally phosphorylated and degraded due to BIN2 interaction, to be translocated the nucleus. There they can

activate their specific BR responsive targets (Yin, *et al.*, 2002; Zhao, *et al.*, 2002). Whether other plant RLKs activate similar components to those found for CLV1, and how the dynamic array of plant signalling pathways interact is a question yet to be answered.

I.4.3.B. Damping down the signal: endocytosis and protein processing

After ligand binding and phosphorylation on the kinase domain the receptor-like kinase is usually active and can signal to downstream proteins. If signalling gives an indication of ligand availability and in some cases ligand position, it is important that catalytic activity is only transitory and so truly responsive. Damping down of the kinase activity is a vital mechanism to ensure this and to regulate the kinetics of response (Waterman and Yarden, 2001). In animals there are examples of both transitory and definitive inhibition of signalling (Dikic and Giordano, 2003). Transitory inhibition is concerned with fine-tuning RTK signalling. It involves reversible dephosphorylation of activation loop sites which inactivate the kinase domain, or phosphate removal from docking tyrosines which blocks the activation of specific signalling pathways (Hunter, 1995). In plants, constitutive activation of SRK in the absence of its ligand is blocked by interaction with the thioredoxin-h-like THRL1 protein, which binds to a site on the cytosolic site of the transmembrane domain and prevents phosphorylation (Cabrillac, *et al.*, 2001).

In order to definitively deactivate the protein and inhibit signalling, the most common mechanism is (ligand-binding) activation-dependent protein degradation. The major process that regulates the amplitude and kinetics of signal transduction is endocytosis. Endocytosis removes ligand-receptor complexes from the plasma membrane, which are then sorted for degradation or recycling (Waterman and Yarden, 2001). Many steps involved in endocytosis are thought to be regulated by ubiquitylation (Hicke, 2001). In animals RTKs are commonly removed from the cell surface via clathrin-dependent endocytosis, and are then degraded in lysosomes (Oved and Yarden, 2002).

In order for internalisation to occur there must be interaction with downstream signalling components within the cell. Various motifs have found to be involved in internalisation. Most of these are in the transmembrane or kinase domains. For example there is an adhesion motif in the transmembrane domain of the EGF-receptor ErbB1; deletions in this domain affect internalisation. ErbB1 endocytosis itself is a phosphorylation-dependent process which is regulated by Cbl proteins (Oved and Yarden, 2002; Soubeyran, *et al.*, 2002) (Fig.I.7E). Cbl is a multi-adaptor protein involved in ligand-induced ubiquitylation and down-regulation of RTKs (Waterman and Yarden, 2001). Cbl rapidly recruits the scaffold molecule CIN85 (Cbl-interacting protein) and endophilins (regulatory components of clathrin-coated vesicles) to form a complex with activated ErbB1 receptors (Petrelli, *et al.*, 2002; Kowanetz, *et al.*, 2004). These interactions induce actin-dependent endocytosis of the complex into the cell. After internalisation Cbl is involved in the targeting of receptors for lysosomal degradation (Waterman and Yarden, 2001). Plants do not have homologs of these internalisation-regulating genes, although there are some proteins which carry out a similar function. These include an arm repeat-containing protein (ARC1) which interacts specifically with the autophosphorylated form of the SRK cytoplasmic domain (Gu, *et al.*, 1998). ARC1 has been shown to have ubiquitin ligase activity and its binding to SRK is thought to trigger ubiquitin-degradation of SRK (Stone, *et al.*, 2003).

In plants one possible pathway leading to endocytosis is through association with kinase-associated protein phosphatase (KAPP). Binding of KAPP to active receptor kinases results in dephosphorylation. KAPP binding then acts as a target for subsequent endocytosis and intracellular vesicle trafficking. It seems to associate *in vitro* with several plant RLKs in a phosphorylation dependent manner, and might be involved in their downregulation. For example CLV1 activity has been shown to be modulated by KAPP *in vitro* (Braun, *et al.*, 1997; Stone, *et al.*, 1998) (Fig.I.7D). KAPP-associating RLKs also include SRK and the *Arabidopsis* LRR-RLK Somatic Embryogenesis Receptor-like Kinase (AtSERK). *AtSERK* is thought to be involved in the switch from somatic to embryogenic development in carrot cell cultures and its

expression marks embryonic competence (Schmidt, *et al.*, 1997). It was shown that when fluorescent protein-tagged AtSERK1 and KAPP were expressed in cowpea protoplasts the two proteins co-localised at the membrane and also in intracellular vesicles (Shah, *et al.*, 2002). Fluorescence-resonance energy transfer (FRET) experiments were carried out in order to examine the interaction between KAPP and AtSERK. FRET was observed in intracellular vesicles (Shah, *et al.*, 2002). These findings do not however show definitively that ligand-binding mediated endocytosis is taking place. Firstly, FRET was only observed in intracellular vesicles. It was not actually observed at the membrane where an interaction between ligand-bound AtSERK and KAPP would be expected to occur. Secondly, the experiments were carried out in cowpea protoplasts and it may be questioned whether the AtSERK ligand is actually present in such a system. Further work is therefore required to conclusively prove that plant RLKs are subject to ligand-binding mediated turnover. Interestingly KAPP has been shown not to associate with CR4 or BRI1 (Braun, *et al.*, 1997).

Research into the mammalian family of low-density lipoprotein receptors (LDLR) gives an insight into the possibilities of receptor-ligand dynamics. LDLRs are involved in removal of cholesterol-carrying lipoproteins from plasma membrane circulation by receptor-mediated endocytosis (Jeon and Blacklow, 2003). The LDL ligand associates with the LDLR at the plasma membrane. This association triggers endocytosis of the LDLR-LDL complex into clathrin-coated pits which form endosomes. This is thought to be mediated by an internalisation motif in the LDLR cytoplasmic tail (Kurten, 2003). Inside the endosome the differing pH of the environment precipitates a conformational change within the receptor. This causes the β-propeller domain of LDLR to act as an alternative ligand at the ligand binding domain. The LDL ligand is then released and degraded whist the receptor is recycled to the membrane (Innerarity, 2002; Rudenko, *et al.*, 2002). Recent evidence suggests that the released intracellular domain of LDLR-related protein1 (LRP1) is involved directly in signal transduction (May, *et al.*, 2003). Whether similar mechanisms are linked to receptor endocytosis in RTKs or RLKs is not yet known.

This example is one elegant mechanism for ligand release, and highlights the possibility that one part of a protein receptor could act as the ligand for itself in order to regulate a developmental mechanism.

Another mechanism which is involved in regulating the behaviour of receptor kinases is cleavage at the membrane. So far all known examples of this occur in animal RTKs – there are no known plant RLKs which undergo this mechanism. Cleavage comes in two forms: firstly ectodomain shedding where the extracellular domain of the receptor kinase is released, and secondly regulated intramembranous cleavage (RIP) by which the cytoplasmic end of the receptor kinase is cleaved into the cell (Arribas and Borroto, 2002) (Fig.I.7F). These mechanisms are not just involved in degradation of the protein however - they are also of great functional significance in the regulation of downstream signalling components. Both ectodomain shedding and RIP are regulated by an array of proteases (Arribas and Borroto, 2002). These proteases are recruited to the protein in what is thought to be a ligand-dependent mechanism. Ectodomain shedding results in release of a soluble protein which can then travel between cells to act as a ligand and regulate the activity of other transmembrane bound proteins (Blobel, 2000). For example in the case of TGF-α, shedding releases the soluble growth factor from the cell surface (Fan and Derynck, 1999). RIP processing can produce a modified receptor fragment that can either act as an intracellular signal or regulate transcription in the nucleus (Carpenter, 2003). Although not a receptor kinase, the *Drosophila* plasma membrane-localised protein NOTCH provides a good example of signalling via RIP processing. NOTCH is an animal receptor that specifies cell fate during embryogenesis (Artavanis-Tsakonas, *et al.*, 1999). RIP results in release of the Notch-intra-cellular domain (NCID). NCID is the activated form of the receptor and it is NCID that is translocated to the nucleus where it acts as a transcriptional regulator (Fortini, 2001; Schweisguth, 2004). This is an example of cleavage which results in protein activation, rather than the more common cleavage resulting from activation. In the case of the EGFR-related ErbB-4 receptor, binding of ligand induces cleavage of both an ectodomain fragment and then a smaller cytoplasmic fragment containing a

tyrosine kinase domain (Vecchi, *et al.*, 1996). This at first appeared to be purely a mechanism for protein degradation. However more recent work showed that the intracellular fragment was translocated to the nucleus where it could play a role in the phosphorylation of nuclear targets. Whether such similar processes to these will be found in plants is yet to be seen.

I.5. Outside cell layer organisation in *Arabidopsis*: how is this achieved?

Receptor-like kinases in plants play an important role in the regulation of development. As mentioned earlier the inside and outside cell layers in *Arabidopsis* are differentiated very early on during development. The question of how the outside layer in *Arabidopsis* is specified and organised is the focus here. It is known that *AtML1* plays an important role in the specification of the outside cell layer. It is not however known which genes are upstream of *AtML1* and involved in signalling to direct this specification. Here the focus is to identity such potential upstream signalling components.

I.5.1. *ACR4* as a candidate regulator of epidermal specification in *Arabidopsis*

As discussed earlier the CR4 RLK plays an important role, possibly with DEK1 and SAL1 in the control of epidermal specification/differentiation in maize. The prospect of a *CR4*-like gene in *Arabidopsis* playing a similar role to *CR4* in maize was investigated in the lab (Gifford, *et al.*, 2003; initial analysis by Gwyneth C. Ingram). By homology comparison using the *CR4* sequence against the *Arabidopsis* genome it was found that there were five genes showing structural similarity to *CR4* (see Fig.I.8). Only one however showed a high degree of sequence similarity (57.5%) at the amino acid level to the predicted maize *CR4* gene. This gene was named *ACR4* (*Arabidopsis thaliana CRINKLY4*) (gene number At3g59420). *ACR4* was found to have no introns. The predicted proteins of ACR4 and CR4 share the classical membrane localised receptor-like kinase structure and have a very high degree of similarity between their kinase domains at the amino acid level (81.5%). Both ACR4 and CR4 share all of the conserved residues in the kinase domains that are necessary for predicted kinase activity for serine-threonine kinases (Hanks, *et al.*, 1988). *ACR4* has been shown to encode an active kinase domain (Gifford, *et al.*, 2003). *ACR4* is the only similar gene which encodes a protein with a long C-

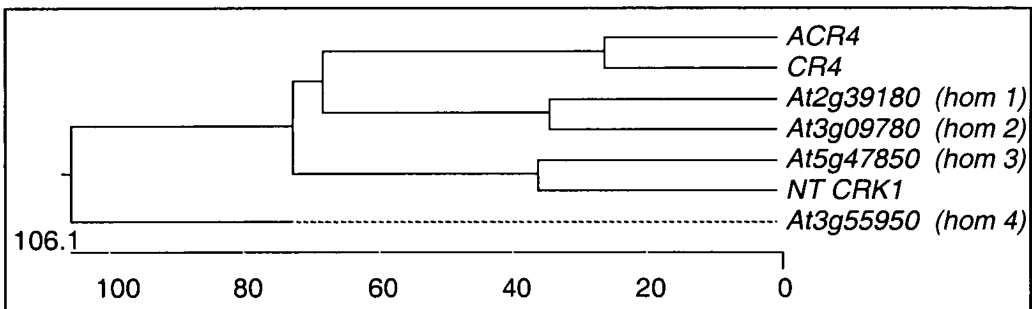

Figure I.8. Phylogenetic tree showing *CR4*, *ACR4*, the four other *CR4*-like genes in *Arabidopsis* and the *Nicotiana tabacum CRK1* gene which was included for comparison. This tree shows a comparison of the amino acid sequences of the protein kinase domains. Alignments were carried out by Gwyneth C. Ingram using T-Coffee (Notredame, *et al.* 2000). Unrooted trees were constructed using the MEGA2 minimum evolution algorithm (Kumar, *et al.* 2001).

terminal region like that of CR4. The C-terminal region shares a high degree of similarity (57% at the amino acid level). The extracellular domain is also conserved with 52% similarity between the seven 39aa repeats, and 35% between the TNFR-like repeat domain. The second and third TNFR cysteine-rich repeats however share 57% similarity. As mentioned in Section I.3.4. these two TNFR-repeats are thought to be the site of TNF ligand binding on the TNFR receptor.

As mentioned, four other similar genes were also found (Fig.I.9). These shared 23.6% (*AtHOM1*, At2g39180) 25.2% (*AtHOM2*, At3g09780), 23.1% (*AtHOM3*, At5g47850) and 23.7% (*AtHOM4*, At3g55950) similarity at the amino acid level with *ACR4*. AtHOM1 and AtHOM2 were not predicted to have kinase activity, due to missing critical resides in the kinase domain known to be important for activation (Hanks, *et al.*, 1988). They did however share a high degree of similarity within their seven 39aa repeat domains and TNFR-like repeat domains. All of the cysteines which were conserved between ACR4 and CR4 were also conserved in AtHOM1 and AtHOM2. AtHOM3 and AtHOM4 were instead predicted to have kinase activity but the extracellular domains of these genes were less similar to that of ACR4. They both shared the seven 39aa repeat domain and the first TNFR-like repeat, but were missing the second two TNFR-like repeats. Instead of the third TNFR-like repeat, AtHOM4 has a proline-rich domain which could function in protein-protein interactions (Kay, *et al.*, 2000). AtHOM3 groups closely to the *Nicotiana tabacum* CYTOKININ-REGULATED KINASE1 (CRK1) RLK (Schafer and Schmulling, 2002). *CRK1* is negatively regulated by cytokinin and might be involved in an early step in cytokinin signal transduction. In summary, only *ACR4* conserves all sequence motifs to the maize *CR4* gene and so is more likely to be functionally analogous. The *ACR4* gene was therefore of importance as a candidate regulator of epidermal specification in *Arabidopsis*. The other *CR4*-like genes are however also the subject of investigation in the lab.

In the lab preliminary *in situ* hybridisations were carried out in order to determine the pattern of *ACR4* RNA distribution. In addition preliminary fluorescent marker lines were developed. *ACR4* was found to be first expressed embryonically at the 8-

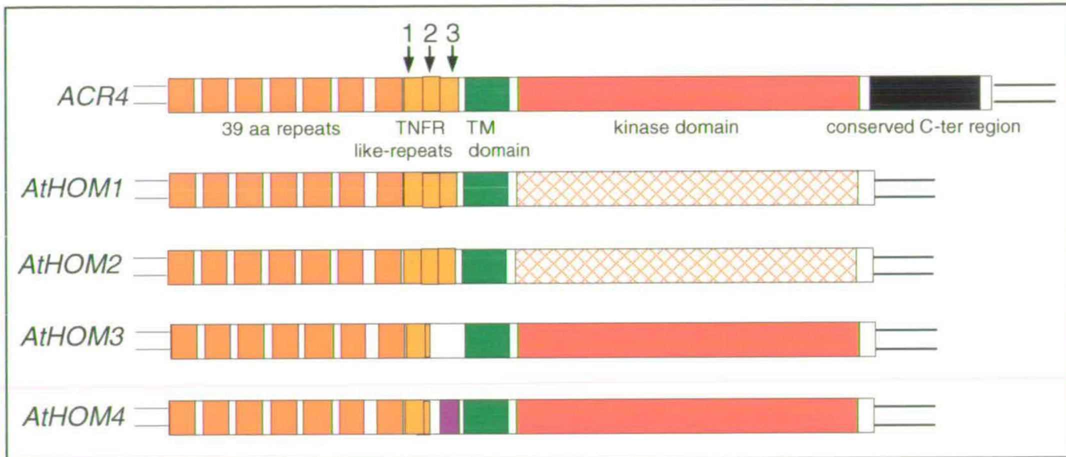

Figure I.9. Schematic of the proteins encoded by the *ACR4* and *ACR4*-like genes in *Arabidopsis*. The predicted domains of the *ACR4*-encoded protein are shown as follows: seven 39aa repeats (orange), three TNFR-like cysteine rich repeats (yellow), transmembrane domain (green), kinase domain (red) and conserved (to maize *CR4*) C-ter region (black); see Appendix 1 for more detail. The first (1) cysteine rich TNFR-like repeat is thought to be involved in trimerisation, while the second and third (2,3) are thought to be involved in ligand binding. None of the other proteins encoded by *CR4*-like genes share the conserved C-terminal region. The extracellular domains of AtHOM1 and AtHOM2 have a high degree of similarity with respect to ACR4. All seven 39aa repeats and all three TNFR-like repeats are present, including the conserved cysteines. Both AtHOM1 and AtHOM2 however are missing critical residues in the kinase domain (Hanks, 1988). This renders them kinase null (denoted as cross-filled kinase domains). *AtHOM3* and *AtHOM4* are predicted to encode proteins with active kinase domains, however the extracellular domains are less similar to ACR4. Both AtHOM3 and AtHOM4 contain the seven 39aa repeats, although these share a lower degree of similarity to ACR4 than the other ACR4-like proteins. The first TNFR-like repeat is conserved in both AtHOM3 and AtHOM4, but the second two are not. Instead of the third TNFR-like repeat the AtHOM4 protein has a proline-rich domain (purple).

cell stage. Its expression was found to be restricted to the outside cell layer at the dermatogen stage and then maintained in the outside layer of organs and meristems throughout development. *ACR4* was expressed in roots and was not found to be expressed in endosperm cells, suggesting that *ACR4* and *CR4* might not have identical functions. Also curious was the fact that, unusually for an L1-specific gene, the *ACR4* promoter did not contain an L1 box. This suggests that *ACR4* may be expressed independently of *AtML1/PDF2*.

There are also *DEK1*-like and *SAL1*-like genes in the *Arabidopsis* genome (*AtDEK1*, *AtSAL1,2*). The prospect that *AtDEK1* is involved in a pathway with *ACR4* in the specification of epidermal cell identity is therefore worthy of investigation. Exactly if and how these two genes interact is the subject of investigation in the lab by Kim Johnson and Gwyneth C. Ingram. The *AtSAL1* and *2* genes are also being studied in the lab.

Altogether this initial work gave a good indication that *ACR4* might be involved in receiving and transducing postulated signals (as mentioned in Section I.3.2) involved in epidermal specification in *Arabidopsis*. The gene was selected for further analysis, which is the primary subject of this thesis. Some preliminary data has been published by Tanaka, *et al.* (2002) and more recently by Watanabe, *et al.* (2004) which suggested that *ACR4* might be involved in embryo morphogenesis. This data will be examined in Chapter III. The specific role that *ACR4* plays during *Arabidopsis* development will be discussed in the chapters that follow. Part of the work described in this thesis is published in Gifford, *et al.* (2003) (see bound copy inside back cover).

Chapter II. Materials and methods

II.1. Plant culture and plant material

II.2. DNA techniques

II.3. Construct production

II.4. Isolation of t-DNA insertion and TILLING generated mutant alleles

II.5. Phenotypic and expression pattern analysis

II.6. Protein localisation and functional analysis

II.1. Plant culture and plant material

II.1.1. Cultivation of plant lines

Seeds were sterilised by rinsing in 70% ethanol (EtOH), 0.05% Triton-X-100 for a period of 12 minutes (mins). 95% EtOH was then used to rinse the seeds, for two washes of two mins. Seeds were pipetted on to 3MM filter paper in a sterile tissue hood, the ethanol allowed to evaporate and seeds sprinkled evenly on to Murashige and Skoog (MS) nutrient medium agar plates (0.5X MS salt and vitamin mix (Gibco/BRL, Gaithersburg, MD, USA), 0.6% sucrose, 1% microagar (Detriot, MI, USA); pH5.7, containing appropriate selection antibiotics). Plates were stratified at 4°C for three days before transferring them to a Percival incubator (Percival Scientific, Perry, Iowa, USA). Plates were then incubated at 22°C with a photoperiod of 16 hours.

After two weeks growth in the incubator, seedlings were transferred to a soil mixture (3 parts Fison's F_2 compost:1 part sand:1 part perlite, with 'Intercept' fungicide (Clydeside Trading Society Ltd, Strathclyde, UK)) and placed in conditions with an 18 hour (long day) or 10 hour (short day) photoperiod at 22°C, 50% humidity. The humidity level was increased for early stages by keeping plants under clear plastic domes for the first four days.

Crosses between plant lines were made by first emasculating three flowers that had not yet dehisced on a lateral shoot of the acceptor plant. Carpels were then hand pollinated one day later with pollen from two different flowers from the donor plant. Tweezers used for emasculating and transferring pollen were sterilised in 70% EtOH between flowers, to avoid contamination. Mature seed was collected and stored in air-permeable envelopes.

II.1.2. Tissue culture

In order to obtain root tissue, plants were grown in conical culture flasks. Once sterile, seed was suspended in 0.1% agarose powder (Bioline, London, UK) solution

and the seeds stratified for 3 days at 4°C. Seeds were then sown into sterile flasks containing 10ml 0.5X MS, 0.3% sucrose solution. Flasks were incubated under long day conditions (as for seedlings on soil), on a rotating platform at 40rpm. The MS solution was changed after six days, then every two days following that. After two weeks of growth root tissue was harvested in a sterile environment using a scalpel and tweezers. Tissue was dried on paper towel and stored at -80°C for protein/RNA extraction.

II.1.3. Mutagenesis of the *acr4-2* seed line

In order to mutagenise seeds from the homozygous *acr4-2* line, approximately 3,000 seeds were exposed to gamma (γ) rays emanating from a cobalt source. In order to achieve an absorbed dose of 300 Grays (recommended for such mutagenesis: Frédéric Berger, Ecole Normale Supérieure, Lyon, France, pers. comm.). This equated to an exposure time of 11 minutes for the cobalt source used (Andrew Sanderson, University of Edinburgh, UK, pers. comm.).

All M_0 seeds were sown immediately after mutagenesis. The ratio of seed death was estimated, with 2500 individual plants then transplanted to soil. M_1 seeds were collected from individual lateral branches on individual plants, to create single segregating lines.

II.1.4. Ovule development mutants

The following lines were ordered as heterozygous segregating lines from the Nottingham *Arabidopsis* Stock Centre (NASC), UK. *bell-1*, *1-3* (N3030 and N8545, in L *er*), *ino-1* (N3881, in L *er*), *sin-1* (N3089, in L *er*) and *ats* (N154, in L *er*). A heterozygous line of *ant* (in L *er*) was kindly provided by David Smyth (Monash University, Australia).

II.1.5. Hormone signalling pathway mutants

The following homozygous lines were obtained from the NASC: *gai1-1*, *1-3* (NW58, N3104, both in L *er*), *spy1*, *3*, *5* (N6266, N6268 in Col0; N8094 in L *er*). *cre1-1*, *1-2*, *1-4* were kindly supplied by T Kakimoto (Osaka University, Toyonaka, Japan). Homozygous *gai* seed and the resultant plants were supplied with 100μM gibberellic acid (GA) in order for germination to occur and to rescue the flowering phenotype.

II.1.6. Additional mutants and marker lines

A heterozygous *wus-1* line (in L *er*) was kindly provided by Dr. Rüdiger Simon (Universiton of Kooln, Germany). A heterozygous *gnom* line (CS8146, in Col0) was obtained from the *Arabidopsis* Biological Research Centre (ABRC) stock centre, UK. The *pLAT52::EGFP* line was kindly provided by Richard Parton and Masaki Watahiki (The University of Edinburgh, UK).

II.2. DNA techniques

II.2.1. Genomic DNA extraction

Leaf samples were collected and stored in Eppendorf tubes (Eppendorf, Hamburg, Germany) at -80°C prior to DNA extraction. Frozen leaves were crushed to a fine powder using a mini-pestle inside the tube. 500μl of extraction buffer (50mM EDTA, 0.1M NaCl, 0.1M Tris·HCl, 1% SDS) was then added and the tissue mixed to create a paste. Samples were then placed in N_2(l) before thawing them, first on the bench at room temperature (room temp. ~25°C), and then at 65°C for five minutes. A phenol/chloroform extraction was then performed to remove contaminants from the extraction. 500μl phenol/chloroform (equilibrated phenol:chloroform:isoamylalcohol 25:24:1) was added, the sample vortexed to mix and then left to stand for five minutes. The upper (aqueous) phase was removed and the phenol/chloroform extraction repeated. To the second aqueous extract (400μl), 50μl 3M NaAc (pH5.2) and 350μl isopropanol were added. The sample was inverted gently to precipitate DNA, then centrifuged at 14,000rpm for five minutes. The pellet was rinsed with 70% EtOH, then air-dried before being dissolved in 50μl R40 [TE (10mM Tris·HCl pH8, 1mM EDTA) containing 5μg/ml ribonuclease A (pre-boiled (100°C) for five minutes to remove DNAase activity and stored as a 1mg/ml stock)], and stored at -20°C.

II.2.2. PCR reaction

The following PCR constituents were made as a master-mix, pipetted into a PCR tube, then 1μl of plasmid/genomic DNA was added. The master-mix consisted of 2μl 10X PCR buffer (500mM KCl, 100mM Tris·HCl pH9, 1% Triton-X-100), 2μl 25mM $MgCl_2$, 1μl 10μM forward primer, 1μl 10μM reverse primer, 0.4μl 10mM dNTPs (10mM of each: dATP, dCTP, dGTP, dTTP), 0.5 units of Taq polymerase enzyme (Promega, Madison, USA) and 12.5μl dH_2O. Oligonucleotide stocks were

either already available in the lab, or ordered from Qiagen Operon (Cologne, Germany). Primers were designed by eye, making sure that no secondary structures would form, with an optimal ratio of 30-50% dGTP/dCTP and a G-clamp at the 3' end. For primer pairs, the melting temperatures were chosen to be compatible. PCR tubes were placed in a T3 Thermocycler (Biometra, Goettingen, Germany) and the following program run: 94°C for two minutes, followed by 30 cycles of [94°C for 30 seconds (denaturing), 55°C/60°C (depending on primer set) for 30 seconds (annealing), 72°C for 30 seconds per expected 500 base pairs (bp) (extension)], followed by 72°C for five minutes to complete extension.

Alternatively, for high fidelity PCR reaction, PfU Turbo (Stratagene, La Jolla, CA, USA) was used for amplification. 1μl of plasmid/genomic DNA, 2μl 10X PfU Turbo PCR buffer, 1μM forward primer, 1μl 10μM reverse primer, 0.4μl dNTPs (10mM), 0.4μl PfU polymerase enzyme, 14.2μl dH_2O, was used. An extension temperature of 68°C was used, with an extension time of one minute per 500bp. In order to clone resultant products into the pGEMT-easy vector (Promega), the reaction was treated with 2 units of standard Taq polymerase for one hour at 37°C.

II.2.3. Agarose gel electrophoresis of DNA

DNA fragments were separated and quantified by using electrophoresis. 1% (3% for distinguishing between TILLING PCR products, 0.8% for a Southern blot) agarose gel was made by dissolving agarose powder in 1X TAE buffer (0.04M Tris acetate, 0.001M EDTA pH8, with 0.0005μg/ml EtBr). Gels with a thickness of 8-12mm were prepared in Owl moulds (Autogen bioclear, Santa Cruz, CA, USA), and run in the corresponding tanks.

DNA samples were loaded after addition of 1/10 volume of loading buffer (40% w/v sucrose, 0.25% bromophenol blue). 0.5μg of either 1Kb or 100bp ladder (NEB, Beverly, MA, USA) was used as a size and concentration marker (according to manufacturer's guidelines) for DNA or RNA. Gels were run at room temp. at 100volts for about 40 minutes for diagnostic tests, 70v for isolation of cleaved DNA

by gel-extraction (~90 minutes), or at 30v when run overnight for a Southern blot (see Section II.2.5). After electrophoresis, separated DNA fragments were viewed on a transilluminator.

II.2.4. Digestion and ligation of DNA

Restriction digestions were carried out according to manufacturer's guidelines (Promega/NEB) at suggested temperatures using the restriction enzyme, appropriate buffer and bovine serum albumin (BSA) where required. Digests were incubated for between one and three hours (five hours for a Southern blot). For diagnostic digestions, 1-5μl DNA was digested (~100ng). For preparatory digestions 10-15μl DNA was used (~200ng); 25μl genomic DNA (~500ng) was digested for a Southern blot. In double digests the two enzymes were added at the same time if requiring the same buffer. If not, the DNA was first cut with the enzyme requiring a lower salt concentration, then this adjusted for optimal cutting of the second. Digested DNA was separated on an agarose gel and extracted from excised bands using the QIAEX II Gel Extraction kit (Qiagen, Cologne, Germany). Calf intestinal alkaline phosphatase (dCIP) (Promega) was used to dephosphorylate the restricted ends of fragments when recommended for the enzyme used, in order to prevent re-ligation at the same position.

Ligation of insertions into the pGEMT-easy vector were made according to manufacturer's guidelines. The following were mixed: (1.0μl PCR product (insertion), 0.5μl pGEMT-easy vector, 0.5μl DNA ligase, 5.0μl DNA ligase buffer, 3.0μl dH_2O), and the reaction incubated for one hour at room temp., before being transformed into bacterial cells. Other ligations were made using T4 DNA ligase (NEB) according to manufacturers guidelines, using 10-20ng plasmid DNA, 50-60ng insert DNA, in a total reaction volume of 10μl. Ligations were incubated overnight at 16°C prior to transformation into bacterial cells.

II.2.5. Southern blotting

DNA was digested, fractionated on an agarose gel, transferred to membrane and hybridised to radioactively labelled DNA probes similarly to that described by Southern (1975). DNA samples were fractionated on an 0.8% agarose gel, in an Owl Scientific gel system (overnight at 30v). The gel was then photographed under a trans-illuminator with a ruler, in order to record the positions of the size ladder fragments. The gel was then rinsed consecutively in: depurination solution (0.2M HCl) for ten minutes, dH_2O for 30 seconds, twice in denaturation solution (1.5M NaCl, 0.5M NaOH) for 30 minutes, then twice in neutralization solution (0.5M Tris, 1.5M NaCl; pH8) for 30 minutes. DNA was then transferred to Hybond membrane (Amersham Biosciences, Uppsala, Sweden) which had been pre-wetted in 2X SSC (20X SSC stock = 3M NaCl, 0.3M Sodium citrate; pH7). Capillary transfer was carried out in 10X SSC buffer overnight at room temp. After marking the position of the gel wells (for orientation) and air-drying the membrane, DNA was cross-linked to the membrane using a UV light. The UV-treated membrane was then baked at 65°C between Whatman 3MM blotting paper (Whatman Inc., Clifton, NJ, USA) for two hours before being dampened in 2XSSC and rolled into a hybridisation tube. Pre-hybridisation treatment was made by incubating the membrane in 25ml Church buffer (1% w/v BSA, 0.001M EDTA, 0.5M $NaPO_4$ pH7.2, 7% SDS) for at least five hours at 65°C in a rotary incubator oven.

Radioactively labelled probe was prepared using either random hexamers or specific primers as follows. For random hexamers, 50ng template DNA in 10μl dH_2O was boiled for five minutes then placed on ice for one minute. The following was added: 1μl (1.85μg/μl) random hexamers (Boehringer, Ingelheim, Germany), 5μl 5X buffer/dNTP mix (100mM of: dATP, dTTP, dGTP, 50mM $MgCl_2$, 450mM HEPES solution (0.5g HEPES, 0.8g NaCl, 0.037g KCl, 0.0135g $Na_2HPO4 \cdot 2H_2O$, 0.1g dextrose); pH6.6), 5 units of DNA polymerase I ((Klenow) Gibco/BRL), 5μl $\alpha^{32}P$ dCTP (Amersham Biosciences), and then the tube incubated at 37°C for one hour. For specific primers, 25ng DNA, 100ng forward primer and 100ng reverse primer together in 14μl dH_2O was boiled for three minutes. The tube was placed on

ice for one minute before adding 5μl 5X buffer/dNTPs, 5μl $\alpha^{32}P$ dCTP and 1μl Klenow, then incubated at 37°C for one hour.

After incubation at 37°C, 30μl dye (1% Dextran blue, 0.1% Orange G in TE) was added to the labelling reaction. This was then passed over a Sephadex G50 (Sigma-Aldrich, St. Louis, MO, USA) column, in order to remove unincorporated radioisotopes. Radiolabelled probe (which migrates with the blue dye through the column) was collected after fractionation. The percentage of incorporation was estimated by comparing the radioactivity in the collected probe fraction in comparison to the remainder, using a Geiger counter.

Labelled DNA probe fragments were then denatured by heating at 102°C for ten minutes before adding to the pre-hybridised membrane in 10ml Church buffer. Hybridisation was carried out at 65°C for at least five hours. The membrane was then rinsed at high stringency twice in 2X SSC/1% SDS (pH7.2) at 65°C, then twice in 0.2X SSC/1% SDS at 65°C. The first rinses for each were for 30 minutes, with the second for 15 minutes. The membrane was air-dried and autoradiography performed using Kodak X-OMAT AR film (Sigma-Aldrich) and intensifying screens, exposed overnight at -80°C inside a cassette. Film was then developed using a Konica developing machine (Konica, Langenhagen, Germany).

II.2.6. Cloning and transformation of plasmids into *E.coli* and *Agrobacterium* cells

In order to clone a vector sequence, the vector was transformed into competent DH5α *Escherichia coli* by heat-shock induced transformation. Competent cells were available in the lab, made according to Inoue (1990). 100μl *E. coli* cells were thawed on ice for ten minutes before 5μl of ligation, or <10ng of already circular DNA, was added, then mixed gently by flicking the tube. The tube was incubated on ice for 30 minutes before heat-shocking the cells at 42°C for one minute. One ml of Luria broth (LB) (1% tryptone, 0.5% yeast extract, 0.5% NaCl; pH7) was then added before incubating the tubes at 37°C for 45 minutes. Meanwhile, plates containing

solid LB (LB containing 1% bactoagar (Difco, Haarlem, The Netherlands), were prepared containing appropriate concentrations of antibiotics used to select for transformed bacteria. Ampicillin (Sigma-Aldrich) at a concentration of 100μg/ml, or kanamycin (Duchefa Biochemie, The Netherlands) at 50μg/ml was added. After incubation, 100μl of the bacterial culture was plated, using a moulded glass Pasteur pipette, on to one plate. The remaining cells were harvested by centrifugation at 7,000rpm, then resuspended in 100μl LB and plated on to a second plate. Plates were then incubated at 37°C upside-down and overnight, to allow multiplication of the bacteria. For selection of transformed bacteria which carry a vector containing an insertion which disrupts the LacZ gene, allowing blue/white colony selection (e.g. for pGEMT-easy), plates were spread with 100μl of X-gal solution (5-bromo-4-chloro-3-indoyl-β-D-galactosidase) (20mg/ml in dimethylformamide (DMF)). Colonies were picked using a sterile toothpick and inoculated into culture tubes containing three ml of liquid LB (containing antibiotics). Tubes were incubated overnight at 37°C on a shaking incubator.

In order to transform binary vectors into *Agrobacterium tumefaciens* cells, a cold-shock method was used. 100μl of competent *Agrobacterium* cells (available in the lab, prepared according to Cui (1995) were thawed from -80°C on ice for 90 mins. 1μg plasmid DNA was added to the cells and mixed gently by flicking. Cells were then incubated on ice for 30 minutes, before they were snap frozen in N_2(l) for one minute, then thawed at 37°C for a few minutes. One ml of YEP (1.0% bactopeptone, 1.0% yeast extract, 0.5% NaCl; pH7.5) was added, and the cells incubated at 28°C on a shaking incubator for three hours. 10μl of the sample was then plated on to a solid YEP plate (YEP containing 1% bactoagar), containing appropriate selection antibiotics (gentamycin (Sigma-Aldrich), to select *Agrobacterium* cells was added at 80μg/ml, with kanamycin, to select colonies containing the binary vector, at 50μg/ml). The remainder was centrifuged at 7000rpm for one minute to harvest cells, resuspended in 100μl YEP and plated on to a second YEP plate. Plates were incubated upside down at 28°C for three days. Resultant colonies were inoculated into three ml of liquid YEP (containing antibiotics), and incubated overnight at 28°C on a shaking incubator in the dark.

II.2.7. Preparation of plasmid DNA from bacterial cultures: *E.coli* and *Agrobacteria*

Vector DNA was mini-preped from *E. coli* bacteria in the following way: 1.5ml fresh overnight bacterial culture was centrifuged at 7,000rpm for three minutes. Harvested cells were resuspended using a pipette in 350µl of boiling buffer (8% (w/v) sucrose, 0.5% Triton-X-100, 50mM EDTA, 10mM Tris·HCl pH8) with 0.01% w/v lysozyme (to lyse cells), and then boiled for one minute. Tubes were then placed immediately on ice for two minutes, then centrifuged at 14,000rpm for 20 minutes at 4°C. A pellet of bacterial genomic DNA and protein was removed using a sterile tooth-pick, then 40µl 3M NaAc (pH 5.2) and 400µl isopropanol (propan-2-ol) added to precipitate DNA. After inverting the tube several times it was centrifuged at 14,000rpm for five minutes, the pellet washed with 70% EtOH, left to air-dry, then resuspended in 50µl R40.

DNA was cleaned up using a phenol/chloroform extraction and ethanol precipitation as follows. Samples were made up to 200µl with dH_2O, and a phenol/chloroform clean-up was made as for genomic DNA extraction (Section II.2.1), this time using 200µl phenol/chloroform. To the resultant 180µl sample, 20µl NaAc (pH5.2) and 550µl 100% EtOH was added, and the sample placed at -20°C for four hours to precipitate DNA. The tube was centrifuged at 14,000rpm for 30 minutes at 4°C, the DNA pellet washed with 70% EtOH, then resuspended in 40µl elution buffer (10mM Tris·Cl pH8.5).

In order to increase the amount of DNA obtained from *E. coli*, a QIAfilter Plasmid Midi Kit (Qiagen) protocol was followed; this gave a yield of ~ 0.5µg/µl DNA.

In order to extract DNA from *Agrobacterium* cells, 1.5ml of fresh overnight culture was centrifuged at 14,000rpm for three minutes to harvest the cells. Cells were resuspended in 100µl ice-cold lysis buffer P1 (50mM Tris·HCl, 10mM EDTA) containing 1/5 volume lysozyme. This was incubated at room temp. for 30 minutes, before 200µl buffer P2 (0.2M NaOH, 1% SDS) was added. This was mixed by inverting several times and the tube stored on ice for 5 minutes. 150µl ice-cold buffer P3 (KAc, pH4.8) was added, the tube vortexed upside down for 10 seconds,

stored on ice for five minutes, then centrifuged at 14,000rpm for five minutes at 4°C. The supernatant was removed to a sterile tube, 315μl isopropanol added, mixed by inverting, and then centrifuged at 14,000rpm for ten minutes at 4°C. The DNA pellet was then rinsed in 70% EtOH, air-dried and dissolved in 10μl R40 to give a concentration of ~0.5μg/μl.

For long term storage of bacterial lines, glycerol stocks containing equal volumes of 80% glycerol:bacterial solution were made, snap frozen in N_2(l) and stored at -80°C.

II.2.8. Sequencing of plasmid DNA

The QIAEX II Gel Extraction kit (Qiagen) was used to clean mini-preped vector DNA from *E. coli* in order to remove contaminants. This was carried out according to the manufacturer's guidelines by treating the sample, made up to 100μl with dH_2O, as a 100mg gel slice. DNA was then quantified on an agarose gel, with 100ng used for a 0.25X sequencing reaction. The following was added to a PCR tube: 2μl DNA/dH_2O, 2μl BigDye Version 3.1 sequencing mix (Applied Biosystems, Foster City, CA, USA), 1μl 0.8pM oligo. This reaction was overlaid with mineral oil to minimise evaporation and the following PCR program used: 96°C for two minutes, followed by 30 cycles of [96°C for 30 seconds, 50°C for 15 seconds, 60°C for four minutes]. Mineral oil was removed from samples by rolling across Parafilm M film (Sigma-Aldrich). Samples were then made up to 20μl with dH_2O and processed in-house, within the ICMB (Institute of Cell and Molecular Biology, The University of Edinburgh). Prior to sequencing, contaminants were removed by clean-up by a MWG robot, using the Millipore Montage clean up kit. Samples were then analysed using ABI Prism 3100 (Abgene, Epsom, UK). Sequences were viewed and analysed using Sequence Navigator (Abgene).

II.2.9. *Agrobacterium* mediated plant transformation

Plants were transformed with the appropriate constructs by using an *Agrobacterium*-mediated floral dipping technique (Clough and Bent, 1998) using *Agrobacterium* strain GV3101. Transformed *Agrobacterium* lines were grown on YEP plates in the dark for two days at 28°C, and then inoculated stepwise into three ml, 50ml and finally into 500ml of YEP (containing appropriate selection antibiotics), over a period of three days. 500ml cultures were spun down at 4,200rpm for 20 minutes at 4°C and the bacterial pellet resuspended in 500ml 0.5X MS with 200µl 'Silwet' detergent (Lehle Seeds, Round Rock, USA). Plants were grown in short day conditions for five weeks to enhance vegetative rosette leaf size, and increase the number of axillary buds that form during flowering. Plants were then shaved to stimulate axillary inflorescence formation, then dipped in the bacterial solution two weeks later. After one week the same plants were re-transformed with the same bacterial lines.

Transformed plants (germinating from generally 5% of seeds collected), carrying the appropriate vector were selected on 0.5X MS plates containing 200mg/l Timentin (GlaxoSmithKline, Brentford, UK) for selection against *Agrobacteria*, with 15mg/ml hygromycin B (Calbiochem, Darmstadt, Germany) or 50µg/ml kanamycin for vector selection, according to the original plasmid used. Transformants were visible as plants able to develop true leaves (resistant to the antibiotic). Resultant T_1 lines harbouring a single insertion were selected on antibiotic-containing plates for a ratio of 3 resistant plants:1 susceptible plant. Resultant T_2 lines homozygous for the insertion were selected for a ratio of 100% resistant plants.

II.3. Construct production

Sequences of all primers used are listed in Table II.1. All constructs containing insertions generated by PCR amplification from either Col0 DNA or from available plasmids were sequenced using internal primers prior to transformation into plants. In addition, diagnostic restriction digestions were made on vector DNA from both *E. coli* and *Agrobacterium* colonies to confirm the insertion size and orientation.

Primer name	Primer sequence	Description
35Ssx3	5'- TCTCGAGCACTGATAGTTTCGGATCTAG -3'	*35s* promoter oligo
35Ssx5	5'- ACTCGAGTCGATTCGACTCACTATAGG -3'	*35s* promoter oligo
BAR139	5'- CGTACCGAGCCGCAGGAAC -3'	*BAR* selection ORF oligo
BAR559	5'- ATCTCGGTGACGGGCAGGAC -3'	*BAR* selection ORF oligo
CAPS1	5'- TTTAACGGGTGGAGATGGG -3'	TILLING oligo
CAPS2	5'- TTCGAACTCTAAGCTCTTTCC -3'	TILLING oligo
CAPS3	5'- ACTCTTAGCCGTGGTAGGTG -3'	TILLING oligo
CAPS4	5'- TTTGCAAGGCGAGTTTTCTTGG -3'	TILLING oligo
CAPS5	5'- AGTTCCTCAACCAATGACC -3'	TILLING oligo
CR-155	5'- AACCACAAGATGAGACCC -3'	*ACR4* ORF oligo
CR2520	5'- ACATCATCGCAGAGGAG -3'	*ACR4* ORF oligo
CR1860	5'- GCAAGATTCGATTCCTC -3'	*ACR4* ORF oligo
CR-370	5'- CTTCTAAATACTCAGCTCC -3'	*ACR4* ORF oligo
CR4-3	5'- GAGCTCAGAAATTATGATGCAAGAACAAGC -3'	*ACR4* ORF oligo
CR4-5	5'- TTTGAAAAGAATGAGAATGTTCG -3'	*ACR4* ORF oligo
CRCT3	5'- AGATCTAGAATGAGTAAAGGAGAAGAAC -3'	Deletion construct oligo
CRCT5	5'- GGATCCCATTAGCTGTGCAAGC -3'	Deletion construct oligo
CRCT-stop	5'- GAGCTCTATAGCTGTGCAAGCGCTCG -3'	Deletion construct oligo
CREC3	5'- GGATCCCAGGTATCGGCTTTTATGATC -3'	Deletion construct oligo
CREC5	5'- AGATCTGACCAGGAGTCCCATCG -3'	Deletion construct oligo
CRFUS1	5'- CTCGAGGAGCACCTACAATTCCTCAATC -3'	*ACR4* ORF oligo
CRNM5	5'- GGATCCTTTTTCCTTGCCTCCACTGG -3'	Deletion construct oligo
CRNOT	5'- AGCGGCCGCTATGATCATCGTGCG -3'	*ACR4* ORF oligo
CRTN3	5'- GGATCCCAGTGGAGGCAAGGAAAAAG -3'	Deletion construct oligo
CRTN5	5'- AGATCTATACAGAGTCCTGGTG -3'	Deletion construct oligo
dC383C	5'- TTCTGAAACTTTCCTTCCTTGG -3'	TILLING oligo
dC59T	5'- GCAGTGTTTCCCCACAATAAG -3'	TILLING oligo
dC758T	5'- GACAGCTAAAGGGACCGAAGCAG -3'	TILLING oligo
dC771T	5'- GAGTCCTGGTGAGCCAGCTAAA	TILLING oligo
dG235A	5'- CCTCTTCTCCAGTTGATTGTTG -3'	TILLING oligo

Primer name	Primer sequence	Description
dG350A	5'- GATTACTTGACTACGATTCTCAT -3'	TILLING oligo
dG72A	5'- CCATTTGAATAAATGAACTGTTT -3'	TILLING oligo
GFP-3-stop	5'- TCTAGTGTTTGTATAGTTCATCCATG -3'	*GFP* ORF oligo
GFP-Kpn3	5'- AGGTACCAGGTGTTTGTATAGTTCATCC -3'	*GFP* ORF oligo
GFP-Xba5	5'- ATCTAGAATGAGTAAAGGAGAAGAAC -3'	*GFP* ORF oligo
GIW1	5'- TGCCATCTCAGTACTTCATGACTCTCTCT -3'	*ACR4*-specific oligo (Wisconsin screen)
GIW2	5'- CTCTCTGCCTCTTTGTTACTTTCCTGCCT -3'	*ACR4*-specific oligo (Wisconsin screen)
GUS6T	5'- CACATTGGCCACCACCTGCCACTC -3'	*GUS* marker ORF oligo
GUS7B	5'- GTGGGAAAGCGCGTTACAAGAAAGC -3'	*GUS* marker ORF oligo
JL202	5'- CATTTTATAATAACGCTGCGGACATCTAC -3'	Wisconsin t-DNA left border oligo
LB2	5'- CCTATTATATCTTCCCAAATTACC -3'	SAIL t-DNA left border oligo
pCR4-3	5'- TTCTAGACAAAGTCAACACACACGCTTC -3'	*ACR4* promoter oligo
pCR4-5	5'- TGTCGACATAGTCAAGAAATGGCCTTTCC -3'	*ACR4* promoter oligo
PRS3	5'- AGAGCTCCTTTGACACATCATACAGTG -3'	*PRS* ORF oligo
PRS5	5'- AGTCGACTCTGAACGGAGAATGAGTCC -3'	*PRS* ORF oligo
QRB3	5'- CGCCATGGCATATGCTAGCATGCATAATTC -3'	SAIL t-DNA right border oligo
SALK LB	5'- GCTGTTGCCCGTCTCACTGGTG -3'	SALK t-DNA left border oligo

Table II.1. Primers used for DNA amplification by PCR reaction (alphabetically listed).

II.3.1. Expression pattern analysis

For promoter expression analysis the *ACR4* promoter was placed upstream of *H2B::YFP* in order to drive its expression. The *H2B::YFP*-coding sequence[1] was digested from pL99[2] (which contained the *H2B::YFP* sequence) using BamHI and SacI, cloned into Bluescript KS (Stratagene) and removed as a KpnI/SacI fragment. This was cloned into KpnI/SacI-cut pL3 binary vector pBIBHyg (Becker, 1990) which contained the *Nos* terminator sequence[3] downstream of a *GFP* sequence; the *GFP* sequence was removed from the vector via the KpnI/SacI digestion. Excess poly-linker sequences were then removed by digestion using SmaI (pMD4). The *ACR4* promoter was then cloned in as an SalI/XbaI fragment from vector pL93 (which contained the p*ACR4* sequence; Gifford, *et al.*, 2003), creating construct pMD6.

[1] The *H2B::YFP* coding sequence was originally isolated from pBI121 (Boisnard-Lorig, *et al.*, 2001).
[2] Constructs labelled L. were constructed by Gwyneth C. Ingram and were already available in the lab.
[3] The *Nos* terminator sequence was used to terminate transcription in all constructs generated.

To enhance expression of this construct, four *35s* enhancer elements were placed upstream of the *ACR4* promoter. The elements were amplified from the pSKI015 vector (Weigel, *et al.*, 2000) using 35sx5 and 35sx3 and then cloned into pGEMT-easy (Promega). Vectors carrying the insertion in the appropriate orientation were digested with XhoI, and the resultant fragment was inserted into the dCIP-treated SalI site upstream of *pACR4* in pMD6 to create construct pMD24. A second variant with a reduced yet functional promoter size (as Gifford, *et al.*, 2003), was created in the same way by inserting the enhancer elements into the pBIBHyg vector pL226 (Gifford, *et al.*, 2003); this created construct pMD63.

In addition to this approach a trans-activation system, available in the lab as transformed plant lines, was optimised. Homozygous driving lines (*pACR4*::*GAL4*::*VP16* (pL143))[4] and target lines (*UAS*::*H2B*::*YFP* (pL2)) were selected to give the highest intensity of fluorescence when crossed.

II.3.2. Complementation analysis

For complementation studies the *ACR4* open reading frame (ORF) was removed as a KpnI/SacI fragment from pL92 (which contained the *ACR4* ORF sequence; Gifford, *et al.*, 2003) and inserted into KpnI/SacI cut binary vector pL3. The *ACR4* promoter was then added as a SalI/XbaI fragment from pL93 upstream of the ORF to create pMD5.

II.3.3. Protein localisation and functional analysis

For protein localisation studies the full length *ACR4* ORF::*GFP* was amplified with CR4-5 and GFP-3-stop from pL205 (containing the *ACR4* ORF cloned in-frame with m*GFP6* from pBSMGFP6; Gifford, *et al.*, 2003), and cloned into pGEMT-easy (pMD9). It was then removed with KpnI/SacI, and cloned into KpnI/SacI-cut pMD5

[4] The *GAL4*::*VP16* coding sequence and terminator were originally isolated from an enhancer-trap vector (http://www.plantsci.cam.ac.uk/Haseloff/Home.html) (Haselhoff, 1999)

to make pMD11. To make construct pΔK/C-ter::GFP the *ACR4* ORF lacking both kinase and C-terminal regions, with a C-terminal *GFP* was amplified from pL180 (containing the *ACR4* ORF missing the kinase and C-terminal regions, cloned in-frame with m*GFP6* from pBSMGFP6) using CR4-5 and GFP-3-stop, and cloned into pGEMT-easy. This fragment was then removed with KpnI/SacI, and cloned into KpnI/SacI-cut pMD5, creating pMD12.

To create variants containing N-terminal *GFP* sequences, the *GFP* ORF was amplified from pBSmGFP6 using primers GFP-Xba5 and GFP-Kpn3, cloned into pGEMT-easy, then removed using KpnI and SacI. This was inserted in-frame, upstream of the *ACR4* ORF in pMD6 to create pMD58. The *GFP* ORF was also cloned into pMD11 to create a line with *GFP* tags at both termini of the *ACR4* gene (pMD59). In order to ensure correct targeting of ACR4, the *ACR4* putative signal peptide was placed upstream of the N-terminal *GFP* sequences. The putative signal peptide was removed from construct pL218a (which contained the *ACR4* putative signal peptide) by digesting with EcoRI. The fragment was then cloned into EcoRI-cut Bluescript KS vector, and clones in the appropriate orientation digested with XbaI to remove the putative signal peptide. This was then inserted into dCIP-treated, XbaI-digested pMD58 and pMD59, creating pMD61 and pMD60 respectively. pMD61 is referred to as pGFP::ACR4, and pMD60 as pGFP::ACR4::GFP.

In order to create a construct where five of the seven 39aa repeats had been removed, CREC5 and CR4-5 were used to amplify the N-terminal end of *ACR4* from pL92 up to the start of the second 39aa repeat; this product was cloned into pGEMT-easy (pMD30). CREC3 and CR4-3 were used to amplify the C-ter end, from the start of the seventh repeat to the end of the ORF. This (N-ter end) product was cloned into pGEMT-easy, removed by digesting with BamHI and SacI, and ligated in frame into BglII/SacI-cut pMD30 (containing the N-ter end). This *ACR4* Δ39aaORF was then removed using KpnI/SacI and cloned into KpnI/SacI-cut pMD5 downstream of the *ACR4* promoter, creating pΔ39aa.

In a similar fashion pΔTNFR, pΔTM/K/C-ter and pΔC-ter constructs were made by amplifying with the primer combinations listed in Table II.2. The N-ter and C-ter ends (where required) were then ligated and cloned into pMD5 as above.

To create *GFP*-tagged variants of these deletion constructs, the required C-ter end of the *ACR4* ORF fused to *GFP* was amplified from pMD9 with primers as listed in Table II.2, ligated to the N-ter end and cloned into pMD5.

Construct	N-ter end amplified with	C-ter end amplified with:
pΔ39aa	CR4-5/CREC5	CREC3/CR4-3
pΔ39aa::GFP	CR4-5/CREC5	CREC3/GFP-3-stop
pΔTNFR	CR4-5/CRTN5	CRTN3/CR4-3
pΔTNFR::GFP	CR4-5/CRTN5	CRTN/ GFP-3-stop
pΔTM/K/C-ter	CR4-5/CRNM5	-
pΔTM/K/C-ter::GFP	CR4-5/CRNM5	CRCT3/GFP-3-stop
pΔC-ter	CR4-5/CRCT-stop	-
pΔC-ter::GFP	CR4-5/CRCT5	CRCT3/GFP-3-stop

Table II.2. Primer pair combinations used for construction of deletion constructs.

Similar constructs in which the conserved lysine (at amino acid position 540) in the ACR4-encoding ORF (see Appendix 1) was mutated to a methionine (Gifford, *et al.*, 2003) were constructed in order to create kinase null *ACR4* versions. KpnI/SacI-cut insertions from pL256 (which contained the mutated *ACR4* ORF, for pK-null) or from pL257 (which contained the mutated *ACR4* ORF fused in frame to *GFP*, for pK-null::GFP) were cloned into KpnI/SacI-cut pMD5. In addition a construct was made in which *ACR4* missing both the TNFR-like repeat and 39aa repeat domains was placed under the control of the *ACR4* promoter. This was made by digesting vector pL220 (which contained the *ACR4* ORF missing the 39aa repeat domain and the TNFR-like repeat domain) to remove a fragment which contained only the *ACR4* putative signal peptide, transmembrane and cytoplasmic domains, and then cloning it into pMD5. This made pΔ39aa/TNFR.

Predicted protein molecular weights were calculated in EditSeq (Abgene).

II.3.4. Genetic analysis: *Pressed Flower* constructs

The *PRESSED FLOWER* (*PRS*) ORF was amplified from both wild-type (WT) Col0 genomic DNA (gDNA) extracted as Section II.2.1, and from WT Col0 cDNA

(available in the lab), using PRS5 and PRS3 and then cloned into pGEMT-easy. The ORFs were then removed by digestion with SalI and SacI, and each inserted into two different SalI/SacI digested vectors containing the *35s* promoter sequence which were available in the lab: pL260 (*p35s* in pBIBHyg) and pL261 (*p35s* in pSTV20Kan). This therefore resulted in the creation of four constructs: pMD66 (*35s*::gDNA *PRS* in pBIBHyg), pMD67 (*35s*::gDNA *PRS* in pSTV20Kan), pMD68 (*35s*::cDNA *PRS* in pBIBHyg), and pMD69 (*35s*::cDNA *PRS* in pSTV20Kan).

II.3.5. Additional constructs

To confirm the second t-DNA insertion site in *acr4-3*, the 3' end of the promoter region was PCR amplified with LB2 and pCR4-3, cloned into pGEMT-easy (pMD14) and sequenced.

II.4. Isolation of t-DNA insertion and TILLING-generated mutant alleles

II.4.1. *acr4-1 – acr4-6*: t-DNA insertion alleles

The *acr4-1* allele was obtained through a screen of the Wisconsin knockout facility using primers GIW1 and GIW2 (as Krysan, *et al.*, 1999) in the lab. The insertion line had been narrowed down to being in one of 25 pools (each containing nine plants), of which seed was available for further screening. Seed carrying the *acr4-2, -3* and *-4* alleles was screened for and obtained through the Syngenta SAIL/GARLIC online collection of t-DNA insertion lines (Sessions, 2002). Seed carrying the *acr4-5* and *-6* alleles was obtained by screening the SALK online collection of t-DNA insertion lines (Alonso, *et al.*, 2003), and ordering lines from the Arabidopsis Biological Resource Centre (ABRC). Table II.3 gives details of the *acr4-1* to *-6* alleles, and Fig.II.1 shows the positions of the t-DNA insertions in *ACR4*; see Appendix 1 for more detailed positional information regarding the sites of t-DNA insertion.

Plants were PCR genotyped as listed in Table II.4. In order to confirm the genotyping, DNA from plants was digested and Southern blotted with an *ACR4*-specific fragment that allowed homozygous, heterozygous and wild type plants to be distinguished. The number of insertions was determined by probing with a t-DNA-specific fragment.

***acr4* allele**	**Knockout collection**	**Seed stock No**	**Ecotype**	**t-DNA insertion vector**	**Insertion site**
acr4-1	Wisconsin	*Collection screened with oligos GIW-1 and GIW-2*	Wassilewskija	pD991 (Krysan, *et al.*, 1999)	1078bp downstream of ATG
acr4-2	SAIL	Garlic_240.B04.b.1a.Lb3Fa	Col0	pCSA110 (McElver J, 2001)	249bp downstream of ATG
acr4-3	SAIL	Garlic_442.C01.b.1a.Lb3Fa	Col0	pCSA110	1587bp upstream of ATG
acr4-4	SAIL	Garlic_599.C01.b.1a.Lb3Fa	Col0	pDAP101 (Baulcombe, *et al.*, 1986)	574bp downstream of ORF
acr4-5	SALK	CLONSALK_043641.23.10.x	Col0	pROK2 (Baulcombe, *et al.*, 1986)	100bp downstream of ATG
acr4-6	SALK	CLONSALK_064665..56.00.x	Col0	pROK2	657bp upstream of ATG

Table II.3. t-DNA insertion lines used for analysis of *ACR4* knockout.

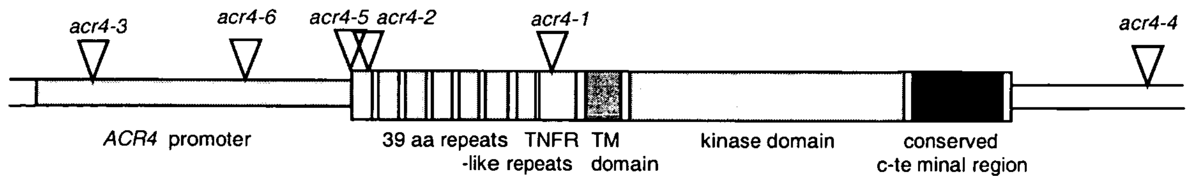

Figure II.1. Predicted sites of t-DNA insertions in the *ACR4* gene; See Appendix 1 for a more detailed schematic showing the positions of the t-DNAs.

***acr4* allele**	**Genotyping: WT band**	**Genotyping: mutant band**	**Southern digest enzyme**	***ACR4* probe fragment and amplification primers**	***ACR4* probed Southern band sizes**	**t-DNA probe fragment and amplification primers**
acr4-1	CR1860 and CRNOT (1000bp)	JL202 and CRNOT (400bp)	EcoRI	ORF fragment from HindIII-cut pL92 (random hexamers)	mutant: 0.8Kb, 1.6Kb, 5.46 Kb WT: 0.8Kb, 5.2Kb	*GUS* marker gene from reporter line FA4C (Colon-Carmona and Doerner, 1999) (GUS7B and GUS6T)
acr4-2	CR4-5 and CR-930 (1050bp)	CR4-5 and LB2 (300bp)	HindIII	ORF fragment from HindIII-cut pL92 (random hexamers)	mutant: 7.8Kb, 1.9Kb, two 0.45 Kb WT: two 0.45Kb, 3.3Kb	*GUS* marker gene (GUS7B and GUS6T)
acr4-3	pCR4-5 and CR-370 (380bp)	LB2 and pCR4-3 (1600bp)	EcoRI	ORF fragment from HindIII-cut pL92 (random hexamers)	mutant: 13Kb, 0.85 Kb WT: 5.46Kb, 0.85Kb	*GUS* marker gene (GUS7B and GUS6T)
acr4-4	-	LB2 and CR2520 (700bp)	PstI	ORF fragment from HindIII-cut pL92 (random hexamers)	mutant: 3.26Kb, 3.31Kb, 0.02Kb WT: 3.26Kb, 2.62Kb, 0.02Kb	*BAR-G* selection gene from the pSKI015 vector (Weigel, *et al.*, 2000) (BAR159 and BAR559)
acr4-5	CR4-5 and CR-155 (170bp)	CR4-5 and SALK LB (100bp)	HindIII	ORF fragment from HindIII-cut pL92 (random hexamers)	mutant: 1.84Kb, 0.46Kb, 1.34Kb, 4.86Kb WT: 2.3Kb, 1.34Kb, 0.46Kb	*35s* promoter from HindIII/XbaI-cut pL259 (containing the *35s* promoter) (random hexamers)
acr4-6	pCR4-5 and pCR4-3 (1800bp)	SALK-LB and pCR4-3 (650bp)	HindIII	Promoter fragment from XbaI/SalI-cut pL93 (random hexamers)	mutant: 1.44Kb, 2Kb, 0.46Kb, 2.86Kb WT: two 0.46Kb, 1.435Kb	*35s* promoter (random hexamers)

Table II.4. Genotyping primers, Southern digest enzymes, expected band sizes, and probes used for Southern blots carried out during investigation of *acr4* knockout alleles.

II.4.2. *acr4-7 – acr4-22*: TILLING alleles

In order to isolate single base pair mutations in the extracellular region of *ACR4*, this region was subject to TILLING (Till, *et al.*, 2003a; Neff, *et al.*, 1998). Seed from lines of interest was ordered through the TAIR website from the ABRC. See Appendix 1 for positions of TILLING changes. Plants were genotyped using cleaved amplified polymorphic sequence (CAPS) analysis or dCAPS analysis (Neff, *et al.*, 1998). These methods utilise the property of differential restriction sites between mutated and WT lines, which are either inherent in the sequence due to the induced mutations (CAPS), or created using mismatches in PCR primers used for genotyping (dCAPS). The TILLING alleles generated, primers and restriction site polymorphisms used to genotype with dCAPS/CAPS are listed in Table II.5. PCR products were amplified, cleft and then products run on 1-3% agarose gels for separation of fragments to allow genotyping of lines.

TILLING allele	ABRC Stock No	Nucleotide change	Effect	Genotyping primers	RE Polymorphism gained/lost with mutation
acr4-7	CS89759	G309A	C17OY	CAPS5/dG309A	CAPS: loss of HhaI site
acr4-8	CS94128	G235A	W155*	CAPS5/dG309A	CAPS: gain of MnlI site
acr4-9	CS93212	G625A	W285*	CAPS3/dC758T	CAPS: gain of MnlI site
acr4-10	CS92031	G745A	W325*	CAPS3/CAPS4	CAPS: gain of MnlI site
acr4-11	CS92277	C59T	P97S	CAPS1/dC59T	dCAPS: loss of BslI site
acr4-12	CS89742	G72A	G101E	CAPS1/dG72A	dCAPS: loss of XmnI site
acr4-13	CS90771	G22A	D153N	CAPS1/CAPS2	CAPS: gain of MseI site
acr4-14	CS91694	G350A	D194N	d235A/dG309A	dCAPS: loss of BslI site
acr4-15	CS86843	C383T	P205S	d383T/dG235A	dCAPS: loss of BslI site
acr4-16	CS91144	G420A	G217E	CAPS5/CAPS2	CAPS: gain of HpHI site
acr4-17	CS86834	C680T	L304F	CAPS3/dC758T	CAPS: loss of MboI site
acr4-18	CS86715	C689A	G307R	CAPS3/CAPS4	CAPS: gain of MnlI site
acr4-19	CS89891	C735T	P322L	CAPS3/CAPS4	CAPS: loss of HpHI site
acr4-20	CS91018	G891A	P330S	CAPS3/CRFUS1	CAPS: gain of MwoI site
acr4-21	CS87697	C758T	P334L	CAPS3/dC758T	dCAPS: loss of BslI site
acr4-22	CS93965	C771T	S374N	CAPS3/ dC771T	dCAPS: loss of BslI site

Table II.5. TILLING allele effects and the genotyping methods used; (*) indicates a stop codon. See Appendix 1 for positions of TILLING changes in the *ACR4* gene.

II.5. Phenotypic and expression pattern analysis: microscopy techniques

In order to characterise the *acr4-2* phenotype, seedlings and plants at a range of developmental stages during embryogenesis and floral development were dissected and analysed using a variety of methods. Photographs taken were processed in Adobe Image Ready 7.0 (Adobe Systems, San Jose, CA, USA).

II.5.1. Light microscopy techniques

Seeds from a single silique were removed by dissecting and removing the septum to which the seeds were attached. Seeds were then mounted in chloral hydrate solution (8g chloral hydrate in 3ml of 33% glycerol) under a glass coverslip. Chloral hydrate was used as a clearing agent to aid visualisation of developing ovules and seeds. Tissue was then viewed using a compound microscope, using Differential Interference Contrast (DIC) microscopy.

II.5.2. Confocal and fluorescence microscopy

In order to study ovule development in greater detail, ovules were fixed and their auto-fluorescence exploited to enable their structure to be viewed using Confocal LASER Scanning microscopy (as Christensen, *et al.*, 1997). Floral buds, prior to opening, were dissected to remove the unpollinated carpels. These were slit down each side using a needle to allow fixative to penetrate. Carpels were placed in tubes containing fixative solution (12.5mM cacodylate, 4% glutaraldehyde, 0.02% Tween, 0.02% Nonidet P40; pH6.9) and vacuum infiltrated for two hours, with a change of fixative at one hour. Tissue was then dehydrated using an ethanol series: 20% EtOH for 10 mins, 40% EtOH for 10 mins, 60% EtOH for 10 mins, 80% EtOH for 15 mins, then three changes into 100% EtOH for 15 minutes each. Cleared carpels were then

mounted in immersion oil (518N; Carl Zeiss, Jena, Germany) under a No.0 glass coverslip (Chance Propper, Smethwich, UK) and sealed with nail polish. Tissue was viewed using confocal microscopy using an Olympus Fluoview FV300 microscope (Olympus, Tokyo, Japan). Excitation light at 543nm was provided by a helium-neon laser. Fluorescence light was selected using a dichromatic beamsplitter and a band pass BP560-615 emission filter. In general, optical sections of 2μm were taken with a pinhole diameter of 200μm. Images were acquired using the Olympus Fluoview camera and software.

The Olympus Fluoview microscope was also used to view tissue from plant lines expressing GFP or YFP. Tissue was dissected and mounted in dH_2O under a No.0 glass coverslip. Excitation light of 388nm was provided by an argon laser light. Fluorescence light was selected using a BP460-490 emission filter. To visualise root cell walls of YFP-expressing plants, two week old seedling roots were stained in 10μg/ml propidium iodide. Excitation light of 388nm was provided by an argon-ion laser light, then the resulting fluorescence light was selected using a BP510-550 emission filter.

II.5.3. Scanning electron microscopy (SEM)

Mature seeds were prepared for SEM as follows with the assistance of Chris Jeffree at the SEM facility, The University of Edinburgh. To make seeds conductive, they were stuck to double-sided carbon tape (DiA, No 63347, Agar Scientific, Stansted, UK). Samples were gold-sputter coated in an argon atmosphere, the seeds unstuck and rotated, then re-coated to ensure all surfaces were completely covered. Samples were then viewed using the SEM microscope.

In order to view developing ovules, dissected carpels were first cryo-fixed in $N_2(l)$, then freeze-fractured in a manipulation chamber. Floral buds and seedlings were also fixed for SEM viewing by cryo-fixation. Ice crystals were removed from samples by partial thawing, then the samples were gold-sputter coated as above and viewed.

II.6. Protein localisation and functional analysis

II.6.1. Protein extraction

Inflorescence material was collected and stored at -80°C prior to protein extraction. A mortar and pestle was pre-chilled using N_2(l), then used to grind tissue to a fine powder. To the ground tissue 200µl extraction buffer (0.05M Tris pH7.5, 0.15M NaCl, 0.1M filter-sterile dithiothreitol (DTT) pH8, one tablet of protease inhibitor cocktail (Roche, Basel, Switzerland), in 10ml dH_2O) was added and mixed to form a paste. At this point one of two routes were followed. For total (single fraction) protein extraction, 1/5 volume of 6X protein loading buffer (0.35M Tris•HCl pH6.8, 10.28% w/v SDS, 36% w/v glycerol, 0.6M DTT, 0.012% w/v bromophenol blue) was added and samples stored at -80°C until required.

Alternatively, to separate cytoplasmic- and membrane- enriched fractions, an additional 200µl extraction buffer was added before centrifuging samples at 14,000rpm for 30 mins at 4°C. To precipitate cytoplasmic-enriched protein, one ml of ice cold 100% acetone was added to the supernatant, the solution placed at -20°C for two hours and then centrifuged at 14,000rpm for ten minutes at 4°C. The protein pellet was washed in ice-cold 80% acetone and re-centrifuged before air-drying. It was resuspended in 25% extraction buffer with 2/5 volume protein loading buffer, and then stored at -80°C. Meanwhile, one ml of extraction buffer was used to resuspend the pellet containing membrane-enriched protein, and the solution was centrifuged at 14,000rpm for ten mins at 4°C. This was repeated, then the final pellet resuspended in 20µl extraction buffer with ½ volume loading buffer and stored at -80°C.

II.6.2. Western blotting

Protein gels were prepared and run as follows using the BIORAD mini-protean 3 system. To make the gel, 'resolving' gel (10% w/v acryl/bis-acrylamide 37:5:1

(Amresco, Ohio, USA), 0.375M Tris pH8.8, 0.1% w/v SDS, 0.1% w/v APS, 0.01% v/v TEMED) was overlaid with 1/5 depth of 'stacking' gel (6% w/v bis/acrylamide, 0.125M Tris pH6.8, 0.1% w/v SDS, 0.1% w/v APS, 0.01% v/v TEMED). 15μl protein samples were denatured by boiling for five minutes, then were centrifuged for five minutes at 14,000rpm prior to loading on to the gel. A protein size marker ladder was also loaded. The gel was run for one hour at a fixed voltage of 120v at room temp. in running buffer (0.6% TRIS, 2.28% glycine, 1.0% w/v SDS; pH8.3). Separated protein was then transferred to Hybond Nitrocellulose membrane in a BIORAD (Hercules, CA, USA) mini-protean 3 transfer cassette and tank; buffer containing 1.44% glycine, 0.3% TRIS, 20% v/v methanol, was used to soak the membrane and gel prior to transfer.

Transfer was carried out at 4°C in transfer buffer (1.44% glycine, 0.3% TRIS, 0.1% SDS, 20% v/v methanol), at 65v for one hour and 40 minutes. Protein transfer and the ladder position was monitored by rinsing the membrane briefly in Ponceau stain (0.1 w/v Ponceau, 5% v/v acetic acid). The membrane was blocked by rinsing for two hours at room temp. in PBST milk (5% Marvel milk powder (Premier International Foods (UK) Ltd, Spalding, UK), 1% phosphate buffered saline solution (PBST) with Tween: 0.8% NaCl, 0.02g KCl, 0.144% Na_2HPO_4, 0.024% KH_2PO_4; pH7.2; 0.2% Tween).

The membrane was incubated in 1/1000 primary (1°) antibody, (anti-GFP rabbit polyclonal antibody (Molecular Probes, Netherlands)) in PBST milk, overnight at 4°C on a rolling incubator. The membrane was rinsed for one hour with several changes of PBST, and then incubated in a 1/4000 dilution of secondary (2°) antibody in PBST milk, (anti rabbit horseradish peroxidase (Amersham Biosciences)) at room temp. for one hour on a rolling incubator. The membrane was again rinsed in PBST with four changes of PBST over one hour, and then the position of antibody attachment detected using an ECL reaction. The membrane was incubated at room temp. in equal volumes of ECL detection reagents 1 and 2 (Amersham Biosciences) for two minutes, then exposed to photographic film (Kodak X-OMAT AR, Sigma-Aldrich) in the dark for periods of five minutes or one hour (to completion of light emanation). The film was developed as for a Southern blot (Section II.2.5).

II.6.3. Brefeldin A treatment

Two week old seedling roots were incubated in a solution of 100μM Brefeldin A (BFA) (B7651, Sigma-Aldrich) for two hours. The working BFA stock was made by diluting a 10mM DMSO stock 1:100 in dH_2O. Control seedling roots were incubated in a 1:100 dilution of DMSO in dH_2O for two hours.

Chapter III. *ACR4* plays a role in maintaining L1 layer integrity

III.1. Introduction

III.2. *ACR4* alleles have defects specifically in ovule integument outgrowth and sepal margin organisation

III.3. Isolated *acr4* null mutants are complemented by wild-type *ACR4* gene expression

III.4. *ACR4* is expressed in the outside cell layer of embryos, meristems and in ovule integuments

III.5. *ACR4* appears to be involved in signalling between cells in order to maintain outside (L1) layer integrity

III.1. Introduction

As discussed in Chapter I, the *Arabidopsis ACR4* gene encodes a receptor-like kinase which shares a high degree of sequence similarity with the maize *CR4* gene. In maize *CR4* appears to be involved in a process which results in specification of aleurone cells in the endosperm of the maize grain. It is also involved in proper epidermal cell layer development. Homozygous *cr4* allele plants show defects in the way that the epidermal cell layer is specified and organised. These defects result in malformation of maize leaves, giving them a 'crinkly' appearance (Becraft, *et al.*, 1996). Defects are also apparent in the underlying cell layers of *cr4* mutant leaves.

The *Arabidopsis ACR4* gene was investigated to determine whether it played a similar role in *Arabidopsis* to that of *CR4* in maize. An initial study of the function of *ACR4* had shown that it was expressed specifically in the protoderm during embryogenesis and in the outside cell layer in the plant. As *ACR4* encodes a receptor-like kinase, the possibility that *ACR4* played a role in receiving and transducing a signal between cells, in order to specify and maintain cell identity in the protoderm and epidermis of *Arabidopsis* was particularly exciting.

The question of whether *ACR4* was important in protodermal specification in the embryo was addressed in this study and the results are presented in this chapter. In order to approach this question a range of mutant alleles were isolated to study the effect of loss of *ACR4* function. It was hypothesised that if *ACR4* were to be critical for correct specification of the protodermal cells in the embryo, then loss of *ACR4* function would result in aberrant development of the protoderm, which would be likely to cause major problems during embryogenesis. This would mean that homozygous null individuals could be embryo lethal or show severe developmental defects. In order to determine whether *ACR4* did play a role in protoderm specification the phenotype of *acr4* mutants, which were not actually embryo lethal, was investigated in great detail. The phenotype observed was confirmed to be the result of loss of *ACR4* function by carrying out a complementation analysis.

An in depth analysis of the expression pattern of *ACR4* was carried out by constructing and using a range of fluorescent reporter-gene-expressing marker lines.

This data was used to confirm and elaborate on previously available *in situ* data obtained in the lab. Knowledge of the expression pattern of the gene is critical to complement mutant analysis in such a functional study. Together the data was used to synthesise a functional model for the role of *ACR4* in *Arabidopsis thaliana*. This model is assessed in the context of wider research into cell layer specification and organisation. Questions are posed and discussed in order to aid the elucidation of the precise mechanism of *ACR4* signalling.

III.2. *acr4* alleles have defects specifically in ovule integument outgrowth and sepal margin organisation

III.2.1 Isolation of loss of function *acr4* alleles

In order to investigate the effect of loss of *ACR4* function, a range of *acr4* alleles were isolated (Chapter II, Table II.3). The *acr4-1* allele was predicted to contain a t-DNA insertion near the start of the TNFR-like repeat region of *ACR4* and was isolated after screening the Wisconsin population of knockout lines. This was carried out in a series of stages. The initial steps had been carried out in the lab prior to this study and are as follows. In the first instance PCR reactions were performed by the Wisconsin knockout facility using combinations of *ACR4*-specific and t-DNA specific primers (Chapter II, Table II.4). PCR amplification was used to identify plant families in the collection that contained an insertion in the *ACR4* gene; positive identification was indicated by the presence of an amplified band in a reaction. In the first step PCR amplification was carried out from DNA extracted from super-pools of plants. There were 30 super-pools that each contained DNA from 2025 individual families. The products from the PCR reactions were probed in the lab to identify whether any of the 30 super-pools contained an *ACR4*-specific band. After a positive hit was found in one super-pool, the same procedure was repeated at the Wisconsin facility on nine pools that each contained DNA from 225 families. Again one positive hit was found. Seed from 25 sub-pools, each containing seed from nine families was received by the lab. Seedlings germinating from this seed were grown and DNA for each sub-pool extracted. It was at this stage that the work described in this chapter began. PCR reactions on sub-pool DNA were carried out and one positive hit again found. The remaining seed for that sub-pool was sown out, germinated and plants grown. Individual plants which harboured the t-DNA insertion could then be identified using the same PCR procedure. This combination of primers could only be used to determine whether a t-DNA insertion was there or not, and so a second round of genotyping on the same plants was carried out using

primers as described in Chapter II, Table II.4, to identify homozygous, heterozygous and wild-type plants.

Alleles *acr4-2,-3* and *-4* were isolated from the Syngenta SAIL/GARLIC collection (Sessions, 2002). Alleles *acr4-5* and *-6* were isolated from the SALK collection. *acr4-2* was predicted to contain a t-DNA insertion 249bp after the start of the ORF in the first of the seven 39aa repeats. *acr4-3* was predicted to contain a t-DNA 1587bp upstream of the *ACR4* ORF, in the promoter region (p*ACR4*). *acr4-4* was predicted to contain a t-DNA insertion 574bp downstream of the *ACR4* gene in the 3' UTR. Line *acr4-5* was predicted to contain a t-DNA insertion 100bp after the start of the ORF (in the ORF) just prior to the first 39aa repeat, and *acr4-6* was predicted to contain an insertion 657bp upstream of the ORF in the *ACR4* promoter. The positions of the insertions in *acr4-1*, *acr4-2* and *acr4-5* were predicted to give strong mutant alleles and were therefore of particular interest. Alleles from the SAIL/GARLIC and SALK collections were initially identified on the web-based catalogues. Seed from heterozygous plants for each line had been deposited to seed stock centres by Syngenta and SALK. Seed for all SAIL/GARLIC and SALK lines described above was germinated and DNA extracted from the resulting plants. PCR genotyping reactions were carried out on the extracted DNA to identify homozygous, heterozygous and wild-type plants (see Chapter II, Table II.4 for primer combinations).

In order to confirm the initial genotyping results of all t-DNA insertion lines, Southern blotting of digested DNA was carried out. A restriction enzyme site cleavage polymorphism was present within each t-DNA insertion line due to the presence (or not) of additional restriction recognition sites carried on the t-DNA vector. DNA was digested with specific enzymes in order to generate different sized DNA bands in plants within each insertion line. The DNA bands were identified by probing with *ACR4*-specific probes which had been designed to identify the differential bands of interest (see Table II.4 for details of the enzymes and probes used for each line together with the banding patterns expected). This method also allowed identification of whether each plant had t-DNA insertions in both, one, or

neither copies of the genome: whether plants were homozygous, heterozygous or wild-type was confirmed.

Southern blotting of digested DNA from all t-DNA insertion lines was also used to determine number of t-DNAs present for each line. This was predicted to be one per line in the locations as described above by the databases of the t-DNA collections (Krysan, *et al.*, 1999; Sessions, 2002; Alonso, *et al.*, 2003), however that information had to be confirmed in the lab. To determine the number of t-DNAs, a t-DNA specific probe was designed for each t-DNA vector that had been used for insertional mutagenesis (see Table II.4). After Southern blotting, the genotype of plants in each line was compared to the number and segregation of bands seen using the t-DNA specific probe. This was used to determine both the number of insertions in each line, and where more than one insertion was present whether the t-DNAs segregated separately (un-linked) or together (linked).

Both *acr4-2* and *acr4-6* lines contained a single t-DNA which segregated specifically with homo- or hetero-zygous plants. *acr4-4* was found to contain several independently segregating t-DNAs. *acr4-5* was found to contain no t-DNAs and therefore was not of use for analysis. This was likely either due to poor annotation of t-DNA insertion lines, or excision of the expected t-DNA due to chromosomal rearrangement in the stock prior to the analysis here. During Southern blot analysis and PCR amplification from the right t-DNA border in the *acr4-2* line, it was found that the sizes of the bands identified were not the same as those expected (as in Chapter II, Table II.4). The bands amplified or cut were found to be roughly 1Kb larger than expected. The region of DNA between the predicted right border of the t-DNA vector and the insertion site in *ACR4* was sequenced and compared to the sequence expected. An additional 1.2Kb of DNA vector fragment was found to be adjoining the right border of the t-DNA insertion vector (not shown). The presence of the extra fragment accounted for the larger band sizes and was likely present due to some rearrangement during the mutagenesis used to create the Syngenta SAIL/GARLIC lines.

During the genotyping of *acr4-1*, it was found that a t-DNA specific band could unusually be detected when amplifying DNA using combinations of the t-DNA

specific primer both with *ACR4*-specific primers to the left, and with primers to the right of the t-DNA. Usually only one combination results in amplification of a band as the t-DNA specific primer primes from the left or right t-DNA border only. Through Southern blotting it was found that the *acr4-1* line contained two back to back t-DNA insertions, which therefore accounted for the abnormal genotyping results. By sequencing the insertion sites of the two back-to-back t-DNAs, it was found that they were between bases 1066 and 1100 in the *ACR4* ORF (within the TNFR-like repeat region of *ACR4* near the site that had been initially predicted: See Chapter II, Table II.3 for predicted sites). Nested PCR between the two t-DNAs was carried out, and the t-DNAs were found to be only 5bp apart.

acr4-3 was found to contain two linked t-DNAs. In order to determine the site of the second t-DNA in *acr4-3*, the flanking t-DNA sequence and adjacent bases of the *ACR4* gene were amplified and sequenced. By comparison to the known *ACR4* promoter sequence, the second t-DNA was found to be 824bp upstream of the *ACR4* ORF. The first t-DNA was found to be 1587bp upstream of the *ACR4* ORF as predicted.

In addition to lines containing t-DNA insertions, 16 *acr4* alleles containing single base pair mutations were generated by TILLING. TILLING (targeting-induced local lesions in genomes) is a process carried out by the *Arabidopsis* Tilling Project (ATP) in which induced point mutations are identified in a target region of interest (Till, *et al.*, 2003a; Banner, *et al.*, 1993). 15 *acr4* alleles were isolated in the seven 39aa repeat region of *ACR4* and one *acr4* allele was isolated in the TNFR-like repeat region (see Appendix 1 for TILLING allele positions). The 39aa repeat region/TNFR-like repeat region of *ACR4* was selected for TILLING analysis for two main reasons. Firstly, single base pair mutations in *ACR4* could provide weaker alleles than those generated using t-DNA knockouts. Weaker alleles are helpful for phenotypic investigation when t-DNA insertions completely knock out the gene of interest, resulting in a severe phenotype. Secondly, the 39aa repeat domain was of particular interest as it is part of the extracellular region of the protein where a potential ligand could be bound (as discussed in Chapter I). Disrupting the 39aa

repeat region could interrupt ligand-binding and would therefore be helpful for elucidation of ACR4 function. This disruption could also potentially provide clues as to the nature of the ACR4 ligand. Thirdly TILLING alleles could be used to confirm the null phenotype of *acr4*.

After the TILLING process 33 alleles containing single base pair mutations were generated. Any alleles where the single base pair mutation resulted in a change in an amino acid that was not conserved either between the *ACR4* repeats or between *ACR4* and the maize *CR4* repeats were discarded. From the remaining alleles a range throughout the 39aa region were selected for analysis, as well as the single change generated in an amino acid at the start of the TNFR-like repeat region; the change in the TNFR-like repeat region was not in one of the conserved cysteines (as Banner, *et al.*, 1993). *acr4* alleles where there were changes in particularly conserved amino acids were of particular interest. These included the highly conserved cysteines in the 39aa repeat region (see Chapter IV for a discussion of the 39aa domain structure), and also any alleles where coding amino acids had been substituted for stop codons (which would definitely be null *acr4* alleles due to lack of full length ACR4 protein translation). Altogether 16 alleles were isolated: *acr4-7* to *-22*.

III.2.2. Phenotypic analysis of *acr4* lines

Plants for each *acr4* line were grown and genotyped for the disrupting t-DNA insertion. Genotyped plants were observed during growth and all aspects of plant morphology and development were studied. In particular though, protoderm formation in the embryo and epidermal development was carefully analysed. This was due to the predicted phenotype of epidermal defects and embryo lethality in loss of function null *acr4* mutants (as mentioned in Section III.1). Surprisingly however, *acr4* homozygous mature plants were isolated. Indeed all lines excepting *acr4-5* (which did not contain a t-DNA insertion) segregated wild-type, heterozygous and homozygous plants in a 1:2:1 ratio which had not been expected.

Homozygous and heterozygous plants of *acr4-3*, *-4*, and *-6* lines were all found to be aphenotypic. *acr4-3*, *-4* and *-6* all had insertions either far upstream or downstream of the *ACR4* ORF and were thus not expected to completely disrupt function. *acr4-5* was found not to carry a t-DNA insertion, therefore only wild-type plants could be isolated.

It was predicted that homozygous plants of *acr4-1* and *-2* lines would disrupt *ACR4* function due to the presence of t-DNA insertions in the coding region of *ACR4*. As mentioned this was found not to result in embryo lethality as mature and viable homozygous *acr4* seed was generated. Embryo development was found to be normal in *acr4* homozygous plants and in general homozygous individuals appeared similar to wild-type siblings. After thorough investigation however, a defect in silique filling was noted in homozygous *acr4* plants. Heterozygous *acr4-1* and *-2* plants were aphenotypic.

III.2.2.A. *ACR4* is required for normal seed development

Siliques from *acr4-2* homozygous plants (as well as *acr4-1* homozygotes) were found to be shorter than those of wild-type siblings (Fig.III.1A). After detailed analysis of silique formation it was found that there were variable levels of abortion during seed development, consequentially resulting in a shorter silique length. Aborted seeds were visible as small pale or yellowing structures which eventually shrivelled up while other seeds continued to mature (Fig.III.1B,C). Abortion occurred at a frequency of around 25% and was noted at a range of time points throughout development. Embryo development inside aborting seeds was investigated and this was found to be normal. The stage in embryogenesis at which seeds aborted was scored to determine whether abortion occurred at a specific point. Most seeds appeared to abort in early to mid embryo development - from the globular to the early torpedo stage – but this was variable both within siliques and between siliques on the same plant. Fig.III.1C shows an aborting seed containing a torpedo embryo inside an *acr4-2* silique, indicated by an asterisk. The *acr4-2* silique

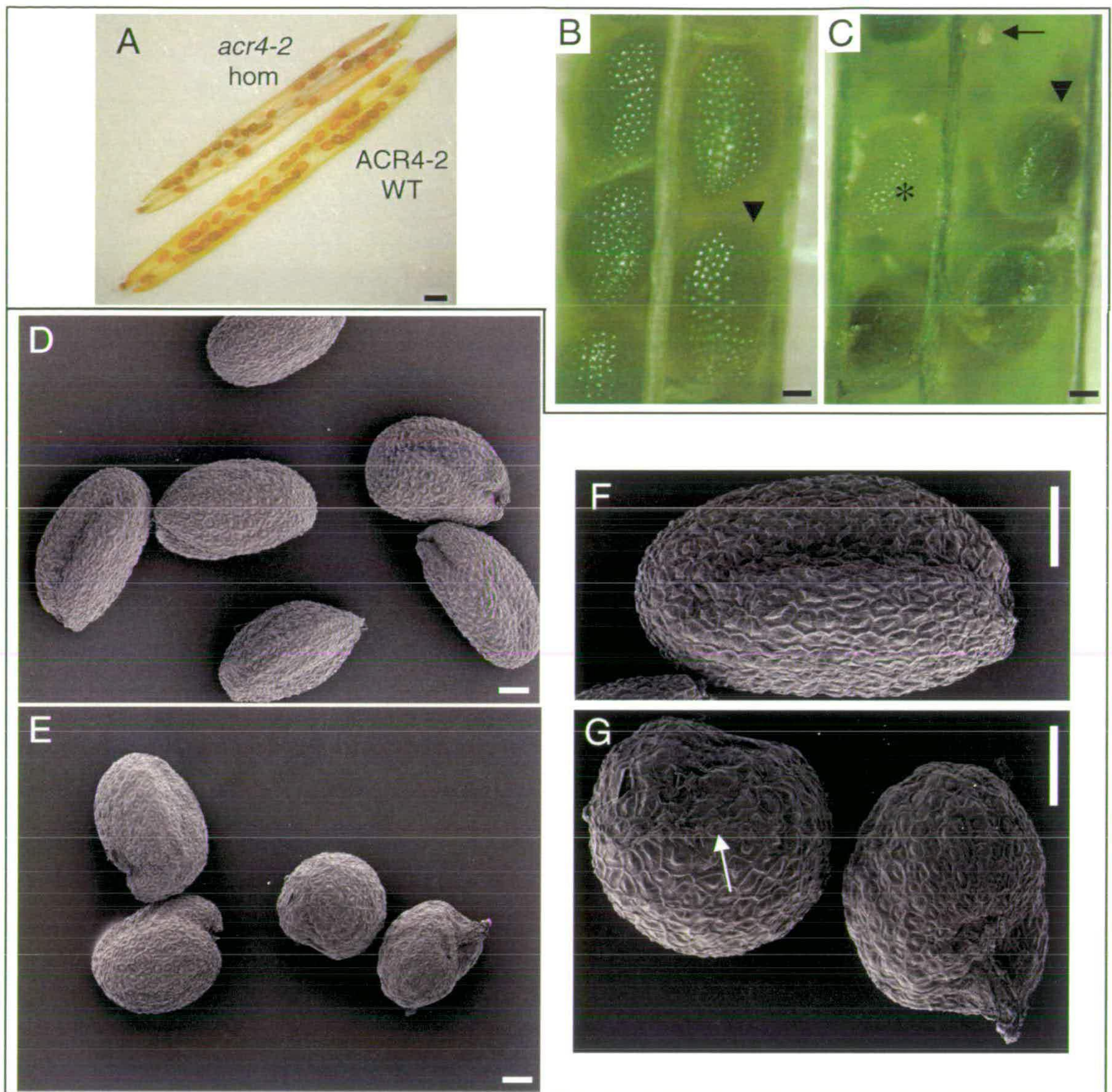

Figure III.1. *acr4-2* seed morphology phenotype. (A) Opened mature siliques showing reduced seed set in *acr4-2* homozygote mutants (hom) in comparison to *ACR4-2* wild-type (WT) plants. (B,C) Maturing seeds at comparative stages of development inside siliques. In comparison to an *ACR4-2* wild-type sibling (B) there is variable seed (asterisk) and ovule (arrow) abortion during development in *acr4-2* homozygotes (C). *acr4-2* maturing seeds are smaller and rounder than wild-type siblings, and have a 'rougher' appearance (compare seed marked by arrowheads). (D-G) SEM images of mature seed. In comparison to wild-type seed (D,F) *acr4-2* seed (E,G) size and shape abnormalities are prominent, with defects in surface structure due to callus-like outgrowths on the seed surface (arrowhead). (G) Outside cell layer abnormalities are present, with variable patches of cells which appear to have lost organisation with respect to each other (arrow). Scale bars: 100μm, except for A (600μm).

in Fig.III.8A (see later) carries aborting seeds both at the early globular (small yellow seed) and heart (browning seed) stages of embryogenesis. Therefore abortion of seeds in *acr4-2* homozygous plants was unrelated to the stage of embryo development.

As previously mentioned *acr4-1* and *-2* could both be maintained as homozygous seed lines. This was due to the fact that 40-85% of seeds were still able to develop to maturity and contained viable embryos. However these mature seeds appeared to be morphologically abnormal. In order to characterise the phenotype, mature *acr4-2* mutant and wild-type sibling seeds were analysed with the aid of scanning electron microscopy (SEM) (Fig.III.1D-G). Rather than being elliptical with a smooth surface as in wild-type, *acr4-2* homozygous seeds were rounder with a 'chick-pea' shape, and were also smaller. In addition seeds had a 'rougher' outer surface which appeared to be due to the presence of callus-like outgrowths (Fig.III.1G, arrowhead). Epidermal cells on the surface of seeds appeared to be improperly organised in comparison to the wild-type, with some parts of the seed coat losing their regularly spaced cell morphology (Fig III.1G, arrow). Defects in the seed outer cell layer morphology were particularly noticeable in cleared developing seeds when viewed using DIC microscopy (Fig.III.2). Abnormalities were also seen in the organisation of underlying integument and endothelium (most inner layer of the inner integument) cell layers in *acr4-2* mutants (Fig.III.2B) in comparison to wild-type (Fig.III.2A).

Interestingly, during fixation or staining of developing seeds, stains or fixatives used appeared to penetrate tissue more quickly and to a greater depth (data not shown), which suggested that seeds were compromised in their surface integrity.

III.2.2.B. *ACR4* is required for proper ovule integument outgrowth

The reduced size and round shape of seeds was noticeable and was postulated to be due to a lack of proper ovule integument growth. During the course of normal ovule development in *Arabidopsis* two layers of tissue - the ovule integuments – are initiated and elongate. These then go on to form the testa (seed coat) which expands

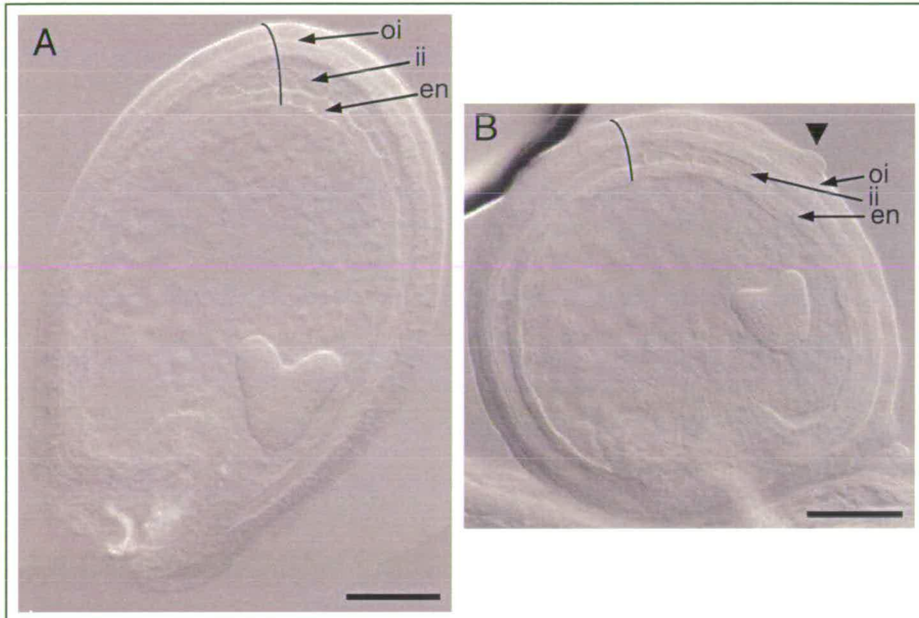

Figure III.2. *acr4-2* phenotype in seed layers. In comparison to a wild-type sibling (A) *acr4-2* mutant cleared seed (B) has reduced elongation of integument layers, which results in a rounder overall seed shape. Cell extrusions are apparent on the surface (arrowhead). In addition, integuments and the endothelium layers appear to be thinner and aberrantly defined (compare width of bracketed areas) in *acr4-2* mutants. oi, outer integument; ii, inner integument; en, endothelium. Scale bars: 50μm.

as the embryo and endosperm develop, giving seeds their elliptical shape. Therefore it seemed likely that there was a defect in ovule morphology. In addition, the number of aborted seeds did not entirely account for the reduced seed set, which suggested that some ovules remained un-fertilised. In order to investigate both the underlying basis of the seed abortion phenotype and the abnormal seed morphology, ovule development was analysed. Developing carpels, prior to fertilisation, were cryo-fixed and fractured in order to break open the outer carpel wall tissue. Scanning electron microscopy was then used to visualise the uncovered developing ovules (Figs.III.4,5).

Ovule development in wild-type plants was as previously described (Robinson-Beers, *et al.*, 1992; Schneitz, *et al.*, 1995); see schematic of a fully mature ovule just prior to fertilisation (Fig.III.3). In the wild-type carpel, ovules are first initiated as bulges of cells which originate from the placental tissue and develop into finger like protrusions (Fig.III.4A). Each protrusion, known as a nucellus, contains a female megasporophyte (see diagram beside SEM image) which through the course of ovule development eventually gives rise to the haploid female gametoyphyte. Next, at defined positions near the nucellus tip two bulging rings of L1-derived cells grow out. These two rings are the ovule integuments. The inner integument grows out closest to the nucellus apex, with the outer integument initiating further down (Fig.III.4C). Development then proceeds with the integuments growing out over the nucellus (Fig.III.4E). The outer integument grows over both the inner integument and the nucellus, to give a narrow micropyle where the pollen tube can grow into the ovule to deliver the sperm nuclei. This course of development forms a mature ovule in which the female gametophyte is almost completely enclosed by the integuments (Fig.III.4G).

Abnormalities in ovule integument outgrowth and organisation were found in *acr4* mutants. These defects account for the variable degree of ovule and seed death visible at later stages. During the earliest stages of ovule formation *acr4-2* mutants develop as wild-type, with a normal nucellus being initiated (Fig.III.4B). At the point of integument initiation, problems in *acr4-2* mutant ovules start to be visible (Fig.III.4D). The positions of both inner and outer integument initiation on the *acr4-*

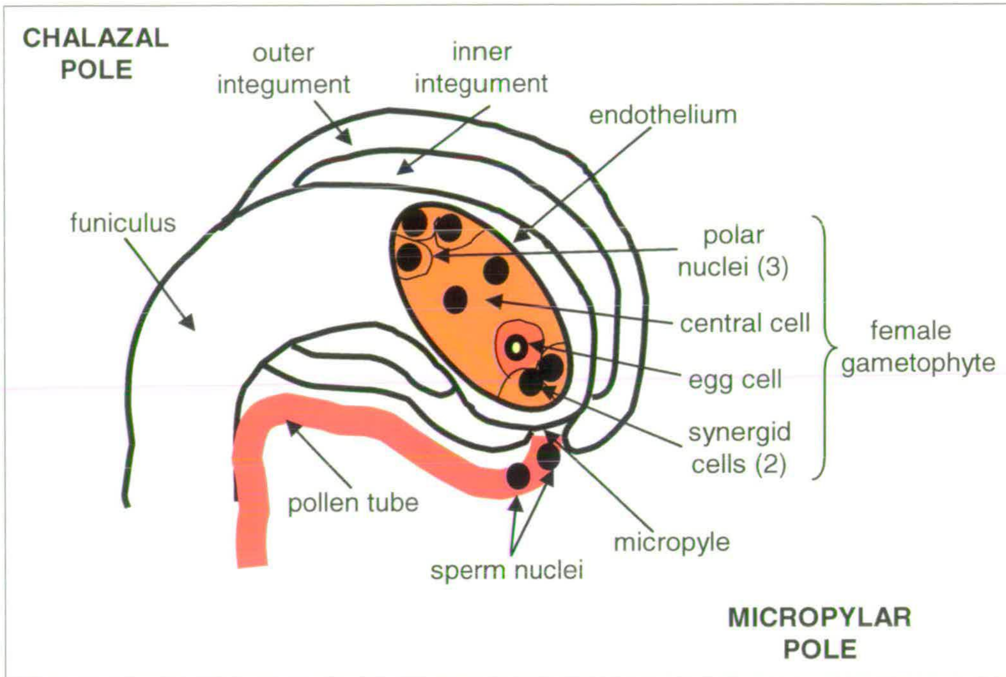

Figure III.3. Schematic of a mature ovule just prior to fertilisation. The main part of the ovule is connected to the carpel by the funiculus. The female gametophyte is surrounded by the endothelium layer through which nutrients are transported to the developing gametophyte. The endothelium is the most inner cell layer of the inner integument. It is surrounded by the outer layer of the inner integument, and then by the outer integument. A pollen tube which has grown down through the carpel tissue towards the ovule is indicated (blue). The pollen tube reaches the female gametophyte (purple) through the micropyle. After contacting the synergid cells and after a recognition event the pollen tube bursts to release the two sperm nuclei. One nucleus fertilises the egg cell resulting in development of the embryo. The second fertilises one of the central cell nuclei which results in development of the endosperm tissue. Schematic adapted from figure by Gwyneth C. Ingram.

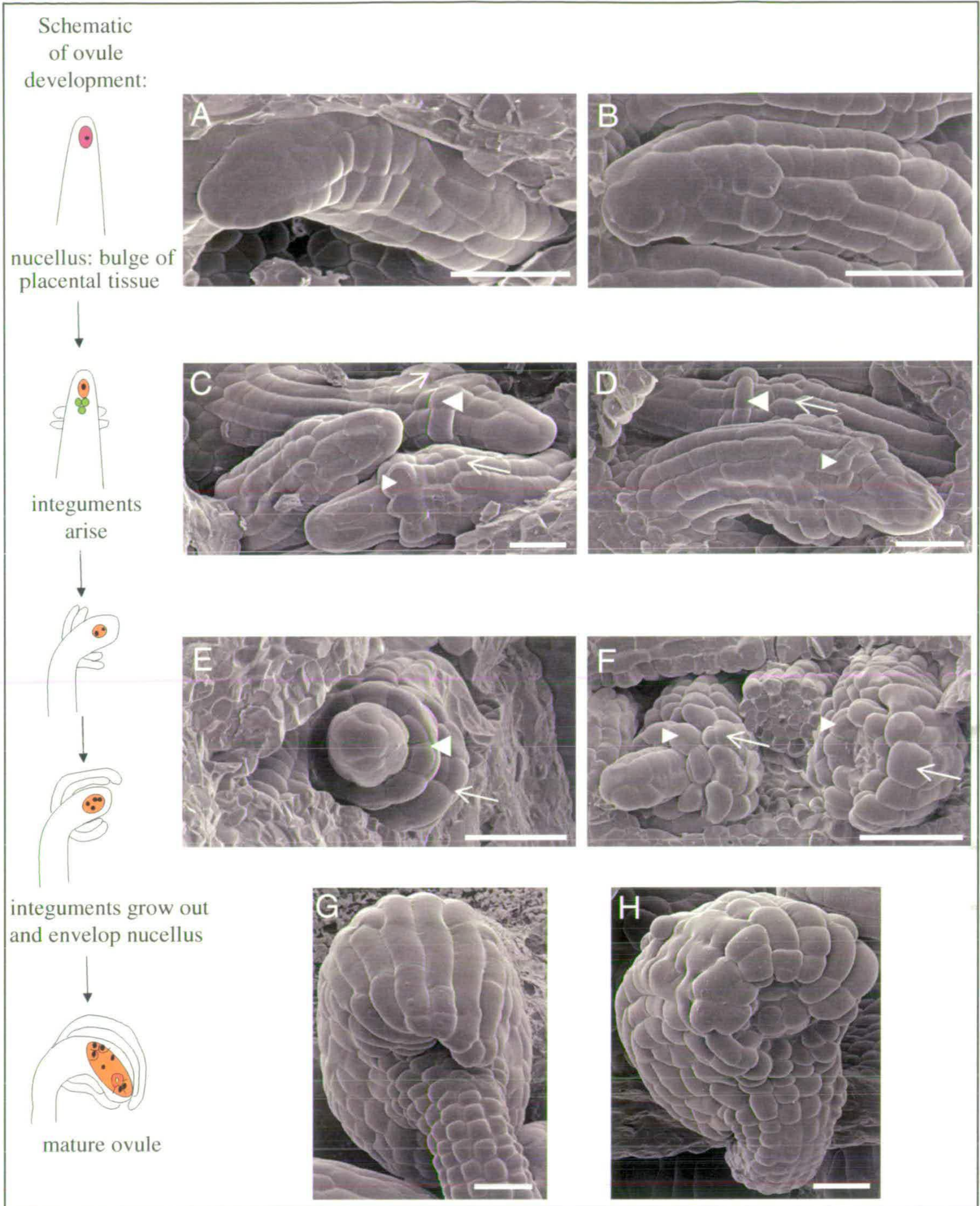

Figure III.4. Phenotypic analysis of wild-type and *acr4-2* ovules. (A,C,E,G) SEM images of cryo-fixed wild-type ovules, with the stage of ovule developmental illustrated to the left. (B,D,F,H) *acr4-2* mutant ovules. (A,B) Ovule primordia immediately prior to integument initiation, showing comparable development. (C,D) Initiation of inner (arrowhead) and outer (arrow) integument growth. In wild-type, single integuments are initiated, whilst in *acr4-2* integuments are initiated irregularly. Two regions of outgrowth are visible in the region of outer integument initiation (asterisks), while other regions lack outgrowths. (E,F) Integument outgrowth is retarded in *acr4-2* ovules (F). In comparison to wild-type (E) where the leading edges of ovule integuments are smooth, those of *acr4-2* are ragged with disorganised cells; the nucellus has snapped off the right-hand ovule in (F) during sample preparation. (G,H) Mature ovules, just prior to fertilisation. (H) Weakly affected *acr4-2* ovule in comparison to wild-type ovule (G), see Fig.III.5 for details. Scale bars: 20μm.

2 mutant nucellus, rather than being distinct, were variable and not well defined. Rather than two integuments being initiated, between one and three bulging and often incomplete rings of cells appeared at varying points on the nucellus (Fig.III.4D). It was not clear whether (in Fig.III.4D) each of the three bulges of cells would actually then develop, forming an ovule with three integuments, as the development of a single ovule over time could not be monitored. It is however unlikely that such growth would have been maintained as this would have caused severe morphological problems.

Integument outgrowth itself was also severely affected in many ovules. Instead of smooth rings of outgrowing cells which are visible in ovules of wild-type plants (Fig.III.4E), those of *acr4-2* mutant ovules were instead disorganised (Fig.III.4F). Outgrowth of integument cells in *acr4-2* mutant ovules did not occur at the same rate across the integument as a whole. At certain places the integument expanded whereas at others it did not. This produced uneven, disordered and retarded integument outgrowth. The result of this was that in *acr4-2* mutants the ovule integuments failed to grow out properly, leaving the nucellus and inner integument visible (Fig.III.4H). This aspect of the phenotype was variable, being dependent on the degree to which integument development was disorganised. Mature *acr4-2* ovules thus exhibited a wide range of defects, with more weakly affected ovules exhibiting the same features seen in *acr4-2* mature seeds: they were rounder and smaller than wild-type, with cell outgrowths on the surface (Fig.III.5B) (compare to wild-type Fig.III.5A). More severely affected ovules showed an acute lack of, in particular, outer integument outgrowth which resulted in 'ragged' looking ovules (Fig.III.5C). If fertilised, such ovules would have been unlikely to maintain proper development. This would therefore have resulted in abortion during seed development. Ovules with even greater abnormalities show little elongation of either integument (Fig.III.5D). These ovules would have been likely to abort during development and certainly would never have been fertilised due to serious abnormalities. In some particularly severely affected ovules, no functional female gametophyte was present. However, this was likely to be an indirect effect of abnormal cell layer formation (see below) which resulted in gametophyte abortion or

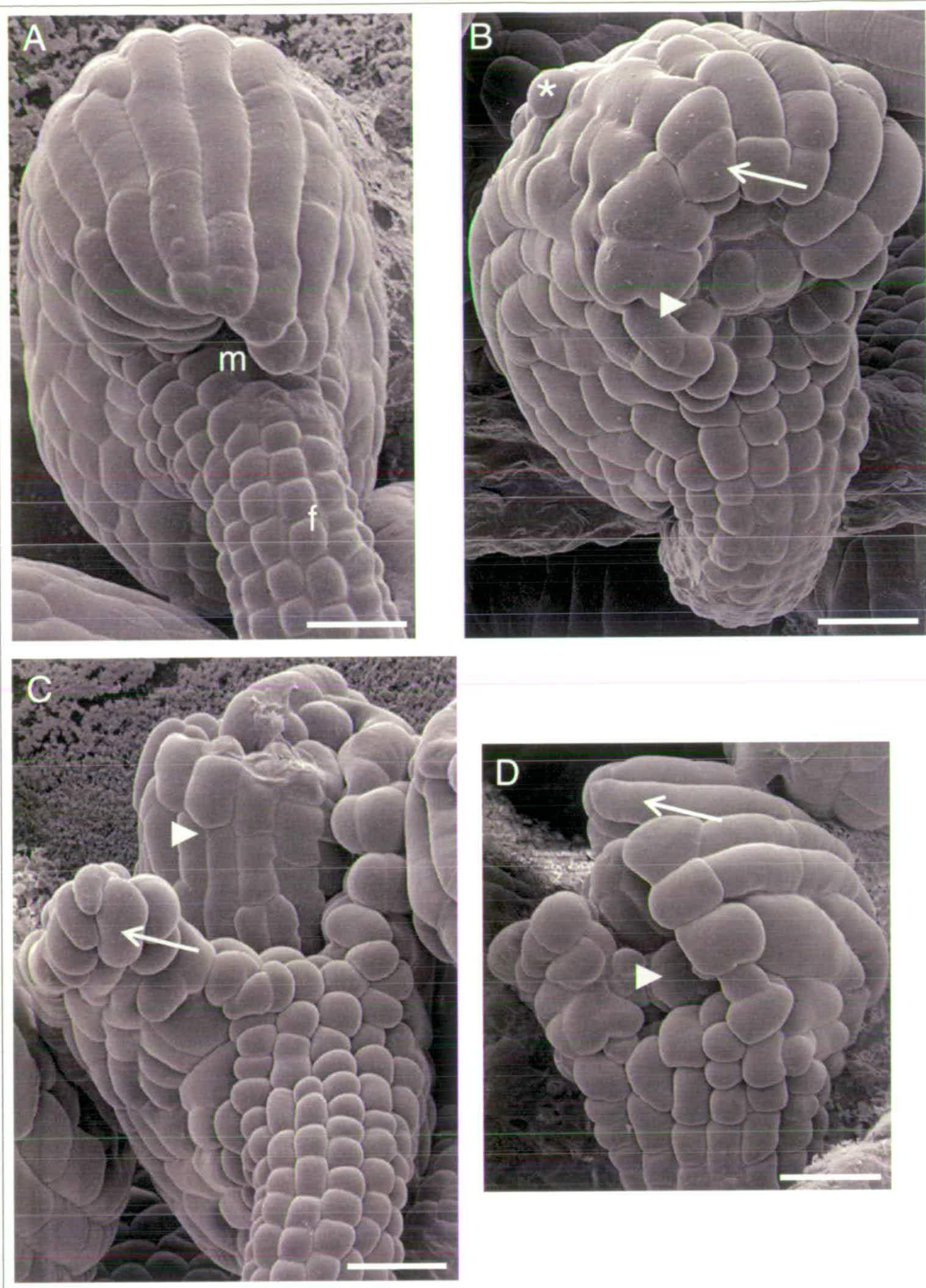

Figure III.5. Mature *acr4-2* ovule phenotype. (A-D) SEM images of cryo-fixed mature stage ovules; inner (arrowhead) and outer (arrow) integuments are labelled. In wild-type (A) the outer integument has grown over the inner integument and nucellus to give a narrow micropyle (m), facing the funiculus (f). (B-D) Weak, medium and severely affected *acr4-2* ovules respectively. (B) The outer integument has retarded growth, leaving the inner integument and nucellus exposed. Surface abnormalities (asterisk) where cells appear to be growing in the wrong direction are visible. (C) Both the inner integument appears fairly normal, whereas the outer integument has failed to elongate. (D) The inner integument, and the outer integument have failed to elongate, which leaves the nucellus exposed. Scale bars: 20μm.

degradation, rather than lack of initiation of gametophyte development. Irregularities in *acr4-2* mutants were only first seen at a point (integument initiation) where the female gametophyte has already started to develop.

In summary, loss of *ACR4* function results in abnormal ovule integument outgrowth with defects in integument cell layer organisation apparent. Isolation of a wide range of mutants defective in ovule formation including *BELL1*, *SHORT INTEGUMENTS1*, *AINTEGUMENTA*, and *INNER NO OUTER* show that proper integument formation is important for normal development of the female gametophyte of the ovule (Reiser and Fischer, 1993; Baker, *et al.*, 1997; Broadhvest, *et al.*, 1999; Villanueva, *et al.*, 1999). An intact endothelial layer is also crucial for proper development, it being important in transfer of nutrients to the developing gametophyte from the plant (Kapil and Tiwari, 1978). Abnormal integument outgrowth in *acr4* mutants has an affect on the underlying cell layers (see Fig.III.2B). This would be likely to adversely affect normal nutrition, which is the likely cause of seed and ovule abortion of development. In more weakly affected ovules, normal development can be maintained.

In order to determine whether there was any genetic relationship between *ACR4* and genes known to be important in ovule development including those mentioned above, double mutants were generated by crossing. The resultant plants were analysed to determine whether any enhancement or alteration of phenotype occurred (see Chapter V).

III.2.2.C. *ACR4* is required for proper sepal margin cell organisation

Additional defects in *acr4-2* were searched for but the only other defect seen was one occurring at the margins of the sepals (calyx) in the flower. Sepal margins had a rougher appearance when viewed under a light microscope, in comparison to those of wild-type flowers. In order to investigate this defect floral buds were partially dissected to expose the margins of developing sepals and then cryo-fixed. *acr4-2*

mutant and wild-type sibling sepals were viewed using SEM. *acr4-2* mutant marginal cells appeared to have a defect in cell organisation. Rather than a smooth monolayer of cells as seen in the wild-type sepal margin (Fig.III.6A), *acr4-2* mutant margins appear thicker (Fig.III.6B). This appeared to occur due to the fact that *acr4-2* marginal cells were not properly orientated with respect to each other, giving them a 'lumpy' appearance. Wild-type marginal cells were completely covered in cuticular decoration, visible as if the surface was covered with veins when viewed using SEM (Fig.III.6A). In contrast *acr4-2* mutant marginal sepal cells had regions where the cuticular decoration was absent (Fig.III.6B). This suggested that some element of cuticle formation had been affected in *acr4-2* mutants. This aspect will be further explored in later chapters (Chapters V and VI).

III.2.3. *acr4* has a maternal sporophytic effect

In order to confirm that the seed morphology and abortion phenotype was under female sporophytic control, *acr4-2* homozygous carpels were self-pollinated or cross pollinated with pollen from wild-type or heterozygous siblings. Control flowers on heterozygous or wild-type siblings were either self pollinated, or pollinated using pollen from an *acr4-2* homozygous plant. Siliques from crosses using either wild-type or heterozygous carpels were both full of normal elliptical seed (five crosses each). Siliques from self-pollinated *acr4-2* individuals contained only 15-60% mature seed, with a phenotype as described above (Section III.2.2A). Siliques resulting from crosses of wild-type or heterozygous pollen onto homozygous *acr4-2* females showed the same phenotype to that of self-pollinated homozygotes (10 crosses of each). Seed from each cross was sown out and all mature seeds germinated successfully. Mature plants of each line were genotyped, and the expected ratios of homozygous, heterozygous and wild-type plants found. If the genotype of the fertilised embryo sac had an affect on the seed phenotype, a lower proportion of mature homozygous plants would occur due to an enhancement of

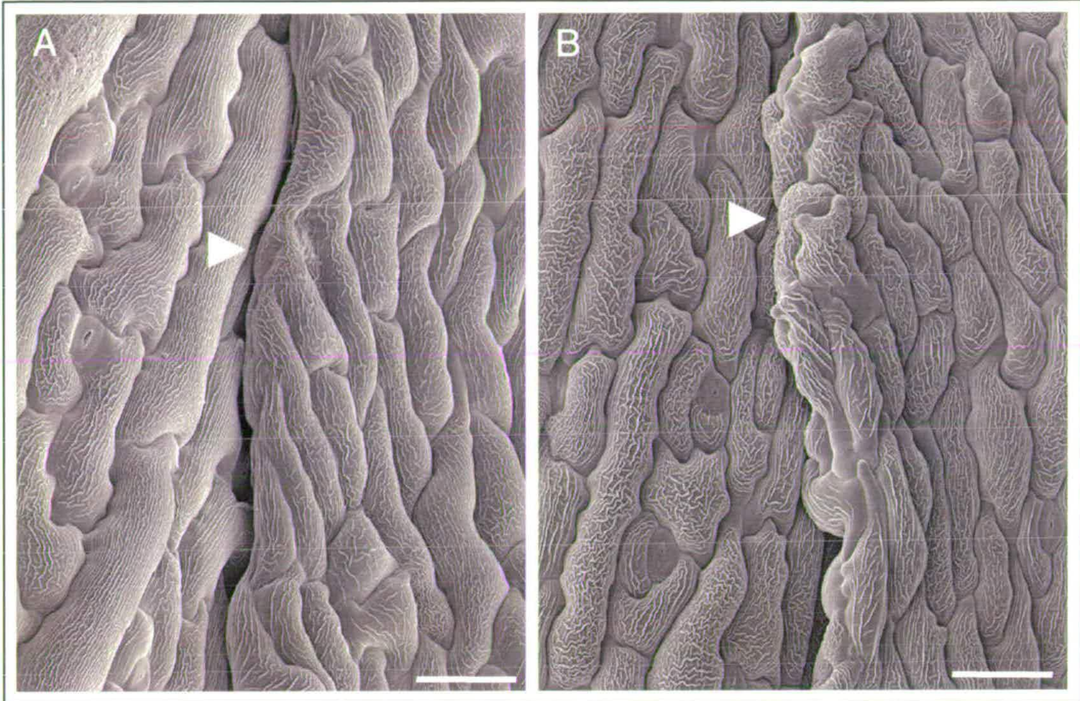

Figure III.6. *acr4-2* mutant sepal margin phenotype. (A-B) SEM images of cryo-fixed sepals. (A) Sepal margins (indicated by arrowhead) in wild-type are smooth and covered in cuticular decoration. (B) Marginal cells in *acr4-2* mutant sepals in comparison are improperly organised, resulting in a thick ridge of cells which gives sepal margins a rough appearance. In addition some parts of *acr4-2* marginal cells are bald, with no embellishment of cuticle. Scale bars: 20μm.

defective seed development. However, as the expected numbers of each genotype were found, *acr4* carries no such gametophytic effect.

III.2.4. Single base pair mutations in the 39aa repeat region render *ACR4* non-functional

In addition to analysing t-DNA knockout lines, the phenotype of alleles generated by TILLING was analysed. Lines *acr4-11* to *-21* were found to be aphenotypic, but lines *acr4-7* to *-10* all exhibited an identical phenotype to that of the two independent t-DNA insertion alleles *acr4-1* and *acr4-2* (see above). The severity of defects in both ovule development and sepal margin organisation was found to be the same, thus no alleles exhibiting weaker phenotypes were isolated. *acr4-8*, *9* and *10* all contained base changes in the *ACR4* 39aa repeat region which caused coding amino acids to be substituted for stop codons, presumably resulting in a truncated and non-functional ACR4 product being translated. The fact that these three isolated TILLING alleles exhibit the same phenotype as the two t-DNA insertion alleles is good evidence that the defects seen are truly the result of complete *ACR4* loss of function. This therefore confirms that the *acr4-1* and *acr4-2* alleles are null.

Interestingly, line *acr4-7* contained a base change substituting a cysteine amino acid for a tyrosine in the fourth 39aa repeat. The importance of this change will be discussed in detail later (see Chapter IV).

III.2.5. *ACR4* sequence analysis

As well as in elucidating the function of *ACR4*, t-DNA insertion lines can also be of use in clarifying the boundaries of the *ACR4* promoter and coding regions. Promoter deletion studies carried out in the lab showed that a 857bp promoter region upstream of the *ACR4* ORF is sufficient to drive *ACR4* expression, whilst a 405bp region is not (Gifford, *et al.*, 2003). As *acr4-6* is aphenotypic, it suggests that the functional promoter region can be restricted to 657bp. In addition, as the *acr4-4* allele confers

no phenotype it confirms that the 3' UTR of *ACR4*, at least beyond 574bp downstream of the ORF, is not required for function.

III.2.6. *acr4-1* carries a *sirene*-like mutation

The Wisconsin collection *acr4-1* allele carries an additional, un-linked mutation which segregates separately from the t-DNA insertion in *ACR4*. It is not linked to a detectable t-DNA insertion. Reciprocal crosses from the *acr4-1* line to Col0 wild-type plants were used to separate the two mutations. The additional mutation caused half of the ovules in each developing silique to remain unfertilised. In order to investigate this lack of fertilisation ovules were fixed and analysed, using tissue auto-fluorescence to view ovules using confocal microscopy (not shown).

The phenotype of mutant ovules was found to be very similar to that seen when the *SIRENE* gene is knocked out (Rotman, *et al.*, 2003). The mutation was named *carmen*. In *sirene* a signalling defect results in a lack of recognition between the female gametophyte of the ovule and a pollen tube which has entered the synergid cell. In wild-type when normal recognition between pollen tube and female gametophyte occurs the pollen tube bursts upon entering the synergid. This results in release of the two male gametes which go on to fertilise the egg cell and the central cell of the female gametophyte (Christensen, *et al.*, 1997; Faure, *et al.*, 2002) (see Fig.III.3). In *sirene*, pollen tubes do not burst and instead continue to grow around the inside of the female gametophyte before eventually stopping. As male gametes are not released ovules remain unfertilised. The defect in *sirene* is female-gametophytic lethal.

In fixed homozygous *carmen* ovules, a large mass of tissue with high refractive index was observed at the micropylar end of the ovule (not shown). With reference to and knowledge of the *sirene* phenotype, the tissue was postulated to be a clump of pollen tube. To investigate this theory further, pollen tubes growing inside *carmen* carpels were examined. In order to visualise pollen tubes, carpels of homozygous *carmen* mutant plants and control wild-type plants were pollinated using pollen from

the *pLAT52::EGFP* marker line (kindly provided by Richard Parton and Masaki Watahiki, The University of Edinburgh, UK).

LAT52 is a tomato gene which is specifically expressed in the cytoplasm of the pollen vegetative cell (Muschietti, *et al.*, 1994). When plants carrying *pLAT52::EGFP* are viewed using confocal microscopy pollen tubes appear green. Normal pollen tube growth and bursting response was observed in the wild-type ovule (Fig.III.7A), but in the *carmen* ovule this was not evident. The pollen tube did not burst and instead continued to grow at the micropylar end of the ovule (Fig.III.7B), as was visualised in cleared ovules. This mutant does not represent an additional allele of *sirene* (Jean-Emmanuel Faure, Ecole Normale Supérieure, Lyon, France, pers. comm.) but could be potentially caused by a mutation in a related gene.

In order to ensure that even when this second mutation was removed from the *acr4-1* line, any additional background effects did not modify the *acr4* phenotype, the *acr4-2* line was used primarily for all phenotypic analysis. In addition, as the *acr4-2* allele was in the Col0 background it was more suitable for the subsequent work carried out here.

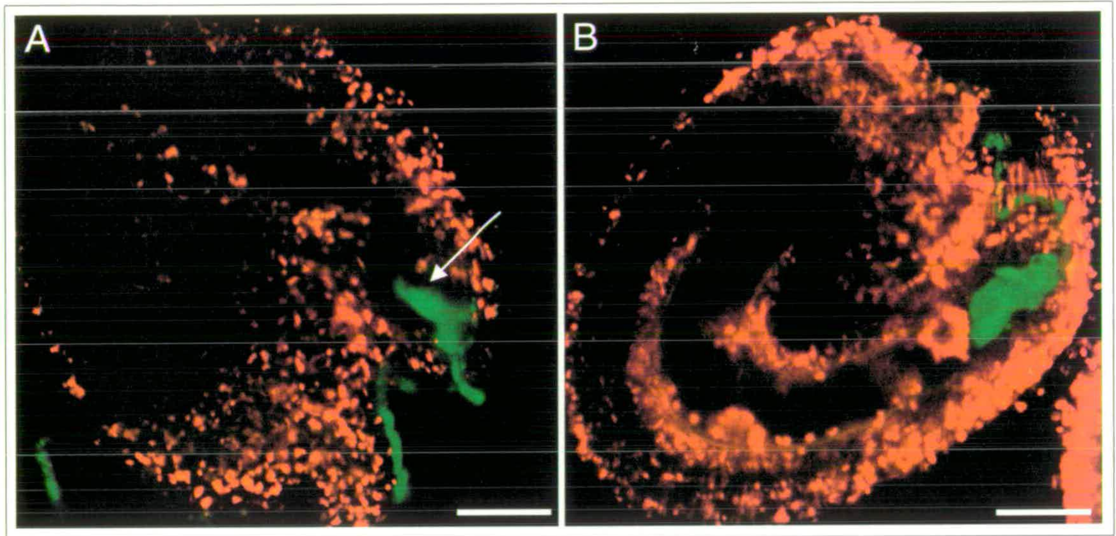

Figure III.7. *sirene*-like mutation carried by the *acr4-1* line. (A-B) Confocal images of pLAT52::EGFP expression (green) in ovules one day after pollination (1DAP). (A) WT ovule containing a normal bursting pollen tube (arrow). (B) *acr4-1* ovule: due to a lack of a bursting response, the pollen tube continues to grow into a ball at the micropylar end of the ovule, visible when viewed under light microscopy as a mass of tissue (not shown). Scale bars: 30 µm. Photos taken by Gwyneth C. Ingram and included with kind permission.

III.3. Isolated *acr4* null mutants are complemented by wild-type *ACR4* gene expression

Although two independent mutant alleles of *acr4* showed identical defects, an additional conformation that the phenotype seen was really due to a loss of *ACR4* expression was made. In order to do this a complementation analysis was carried out to determine whether an introduced copy of *ACR4* could rescue the seed death and morphology phenotype.

Homozygous *acr4-2* plants were crossed in both directions to a transgenic line carrying a full length *ACR4* ORF under the control of the *ACR4* promoter (pMD5: see Chapter II.3.2 for details of line construction); the transgene conferred hygromycin antibiotic resistance. In addition, control homozygous carpels were self pollinated. Four F2 generation families were selected on media containing hygromycin for plants either homo- or hetero-zygous for the *ACR4* transgene. Plants were then genotyped by PCR to ascertain *acr4-2* homo- or heterozygosity, with a Southern blot used to confirm homozygosity of plants in two families. The phenotype of *acr4-2* homozygous, heterozygous and wild-type plants was compared.

In all four families, full complementation of the *acr4-2* homozygous phenotype was evident. In comparison to selfed *acr4-2* homozygous where seed death and morphology abnormalities are apparent (Fig.III.7A), siliques of *acr4-2* homozygotes carrying the *pACR4::ACR4* transgene are full of normal wild-type seed (Fig.III.7B). Sepal margins of these plants were also as wild-type (not shown). In conclusion, defects seen in isolated *acr4-2* alleles are complemented by expression of an introduced copy of the *ACR4* gene, thus the plants have a phenotype which is directly associated with loss of *ACR4* function.

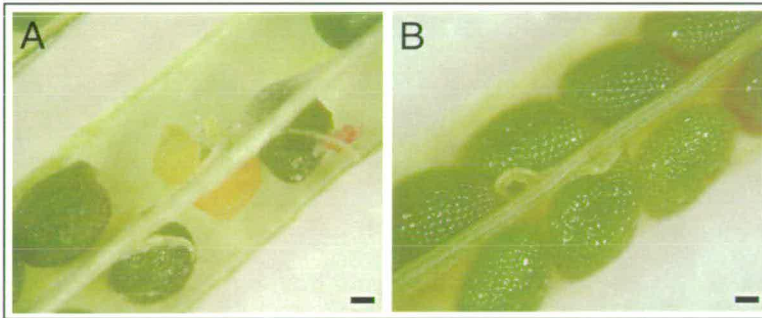

Figure III.8. Complementation of *acr4* mutants. (A,B) Opened siliques showing *acr4-2* lines either harbouring a transgene expressing p*ACR4*::*ACR4* (B), or not (A). (A) *acr4-2* siliques contain small, round seeds with a varying degree of seed abortion. In (B), expression of *ACR4* has restored normal seed development. Siliques are full of elliptical, smooth seed. Scale bars: 100μm.

III.4. *ACR4* is expressed in the outside cell layer of embryos, meristems and in ovule integuments, defining the L1 layer

A detailed study of the *ACR4* expression pattern was undertaken in order to both confirm, and elaborate on *ACR4* RNA distribution results obtained from *in situ* hybridisation experiments carried out in the lab (see Gifford, *et al.*, 2003). In order to do this, various *ACR4* promoter-driving marker line tools were used or constructed. Firstly, a two-component marker line already available in the lab was optimised. Secondly, a single marker line with the *ACR4* promoter directly driving expression of the yellow fluorescent protein HISTONE2B::YFP (H2B::YFP) marker gene was constructed. Thirdly, an enhanced version of the single marker line was made.

III.4.1. Marker line construction and optimisation

III.4.1.A. Two-component (transactivation) marker line

In the lab a two-component marker line had already been constructed, with plant lines available for use. The *ACR4* promoter had been placed upstream of the chimaeric *GAL4::VP16* transcriptional activator (Haseloff, 1999) and transformed into plants (driving line). In a second plant line the *HISTONE2B::YFP* protein fusion encoding marker gene had been placed under the control of a *35s* minimal promoter and the *Upstream Activating Sequence* (*UAS*) (Boisnard-Lorig, *et al.*, 2001) (transactivation line). Homozygous single-insertion driving and transactivation lines were crossed (in either direction). When both transgenes were present in the same plant, the GAL4:VP16 DNA binding domain bound and activated the UAS. This resulted in expression of *H2B::YFP* wherever the *ACR4* promoter was activated and thus where *ACR4* was expressed. H2B::YFP is a stable marker and a nuclear-localised protein. The dual nature of the system results in an intrinsic amplification

of expression levels, allowing visualisation of promoter expression pattern where the intrinsic promoter activity is weak.

Initial experiments were underway in the lab to confirm that in the two-component marker line the *ACR4* promoter was driving expression of *H2B::YFP* in the same pattern of distribution found for *ACR4* RNA. These tests were continued and completed. The two-component marker line system was optimised by selecting the strongest driving and transactivation lines. In order to do this, crosses were performed between individuals from 12 families of each line; the driving line carried kanamycin antibiotic resistance and the transactivation line carried hygromycin resistance. F1 generation plants carrying both transgenes (and thus expressing *H2B::YFP* where *ACR4* is expressed) were selected using double antibiotic resistance. The relative fluorescence of roots for individuals from each cross was assessed, and optimum combinations of families determined (not shown). Three lines for each construct in the two-component marker system were thereby selected, seed for each line bulked up and used in expression pattern analysis.

III.4.1.B. Single and enhanced-single marker lines

The transactivation approach, although resulting in strong marker gene expression levels, carries several innate problems in application. Firstly, if the two-component line is being used to analyse *ACR4* gene expression in mutants it requires the crossing into and maintenance of two individual transgenes in a single plant. This is likely to pose a technical difficulty. Secondly, the presence of two transgenes which carry similar terminator sequences (as used here) could result in some degree of silencing of the transgene sequences. This could reduce the level of marker gene expression in later generations, which could potentially cancel out the inherent enhanced expression expected. After initial tests with the two-component line, transgene silencing did not appear to be taking place, but a single marker line was still constructed for ease of use.

A single marker line was constructed by placing the *ACR4* promoter directly upstream of the *H2B::YFP* marker gene, in order to directly drive expression. As predicted, *H2B::YFP* was found to be expressed at a lower level to that of the two-component marker line, thus an enhanced marker line was constructed. To do this four *35s* enhancers were placed upstream of the full length (2Kb) *ACR4* promoter (see Chapter II.3.1 for details). In addition the enhancers were placed upstream of a truncated *ACR4* promoter, driving *H2B::YFP* (857bp truncated promoter as Gifford, *et al.*, 2003). This alternative line was made in order to reduce the size of the promoting region.

H2B::YFP was found to be expressed to a higher level in the 35s enhanced lines as predicted. This level of expression was found to be comparable to that of the two-component marker line and thus provides a more useful tool for examining the expression pattern of the *ACR4* gene. No difference in expression pattern or intensity of fluorescence was seen between the two 35s lines. The expression of *H2B::YFP* in the enhanced single marker lines was found to be identical to that in the single marker line in all organs analysed. This confirmed that the addition of enhancer elements had not distorted the observed pattern of *ACR4* promoter expression.

III.4.2. *ACR4* expression pattern analysis

III.4.2.A. *ACR4* is expressed in the outside layers of the embryo, shoot apical, inflorescence and floral meristems

Wild-type tissue expressing the above marker lines was dissected, mounted in water under a coverslip, and the fluorescence of tissue examined using confocal laser scanning microscopy. The expression pattern of *H2B::YFP* in the two-component, single- and single enhanced- marker lines was found to be identical to that of the RNA distribution of *ACR4* in all organs examined. Protoderm-specific expression throughout embryo development was seen from the 8-16 cell stage of embryo

development and is maintained throughout embryogenesis (Fig.III.9A). *H2B::YFP* expression is also detected in the embryonic root pole (Fig.III.9A). No expression of *H2B::YFP* was detected in the endosperm tissues of the seed: *H2B::YFP* fluorescence in the embryo when viewed using confocal microscopy is clearly visible despite the embryo being surrounded by endosperm tissue. L1-specific expression in the shoot apical, inflorescence (Fig.III.9B) and floral meristems (Fig.III.8C) was confirmed. L1-specific expression in all organs derived from these meristems was also confirmed (not shown).

III.4.2.B. *ACR4* is expressed in ovule integuments, the root meristem and in outside layer cells at the lateral root cap boundary

In previous *in situ* hybridisation experiments carried out in the lab the distribution of *ACR4* in both root and ovule tissue had proven difficult to clarify. *ACR4* expression in these two organs, determined using the constructed marker lines, will now be discussed. Schematics of the root apical meristem (Fig.III.10) and a mature ovule (Fig.III.3) are provided for reference.

In ovules *ACR4* expression was observed in all integument cells and in the outer layer of the funiculus (Fig.III.9D). Expression was strongest in the most outer layer of the ovule (effectively the ovule epidermis), the 'outer' layer of the inner integument and the endothelium. These ovule cell layers are derived from outgrowths of the epidermal L1 cell layer and thus *ACR4* expression in ovules is L1-specific.

At the lateral root cap boundary of the apical root meristem *ACR4* expression was detected in 'outside' cells, and also the epidermis/lateral root cap initial (Fig.III.9H,I). Expression was maintained in the cells of the lateral root cap, but not in cells of the epidermis. Epidermal cells only started to express *H2B::YFP* when they were directly on the outside of the root after they had emerged from under the lateral root cap (Fig.III.9I). Epidermal cells below the lateral root cap did not

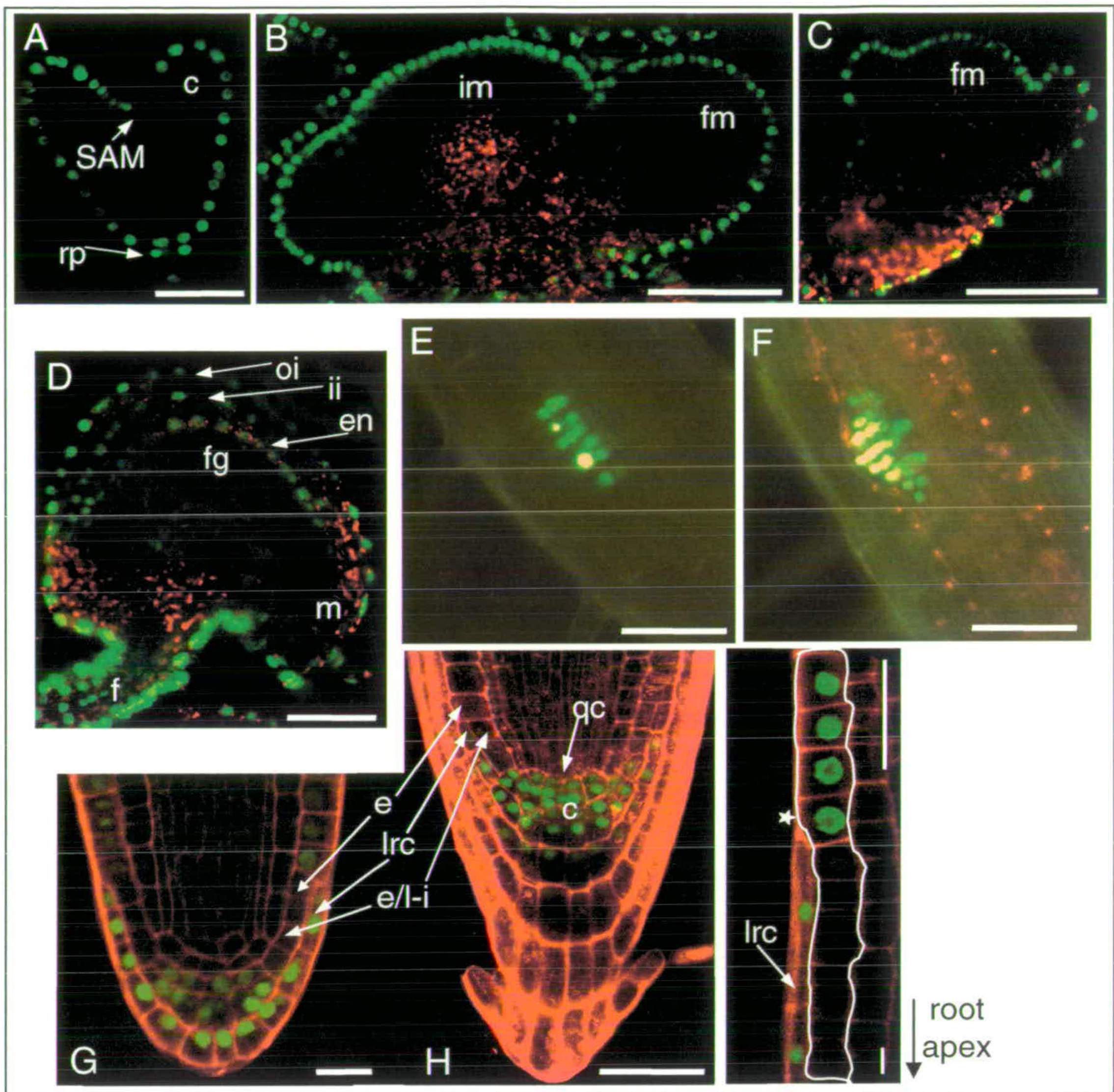

Figure III.9. *ACR4* promoter activity throughout development. (A-I) Confocal images of lines expressing *H2B::YFP* (green) under the control of the *ACR4* promoter. (A) Early heart stage embryo where *H2B::YFP* expression is detected in the protodermal cells on the outside of the embryo and in the root pole (rp). No expression is detected in the surrounding endosperm. (B,C) Inflorescence (im) and floral meristems (fm) respectively, where expression of *ACR4* is detected specifically in the outside (L1) cell layer. (D) Mature ovule: *ACR4* expression is detected in the outer layer of the funiculus (f), outer integument (oi), inner integument (ii) and endothelium (en). (E,F) Confocal images of emerging lateral roots from the pericycle at the 16 cell stage (E) and further in development (F). (G-I) Propidium iodide staining of the cell wall (red) is used to mark root cells. (H) Apical root meristem: *ACR4* is expressed in the columella initials (c), the epidermal/lateral root cap initial (e/l-i), the lateral root cap (lrc) and the quiescent centre (qc), but not in the epidermal cell file (e). *ACR4* is similarly expressed in the corresponding regions of the lateral root meristem (G). (I) Upper lateral root cap boundary: *ACR4* expression is detected in the epidermal cell file only after cells emerge from under the lateral root cap (at star). This is also visible at the lateral root cap boundary in (G). c, cotyledon primordium; SAM, shoot apical meristem; fg, female gametophyte; m, micropyle. Scale bars: 25μm, expect for I (10μm). Photos A-F taken by Gwyneth C. Ingram and included with kind permission.

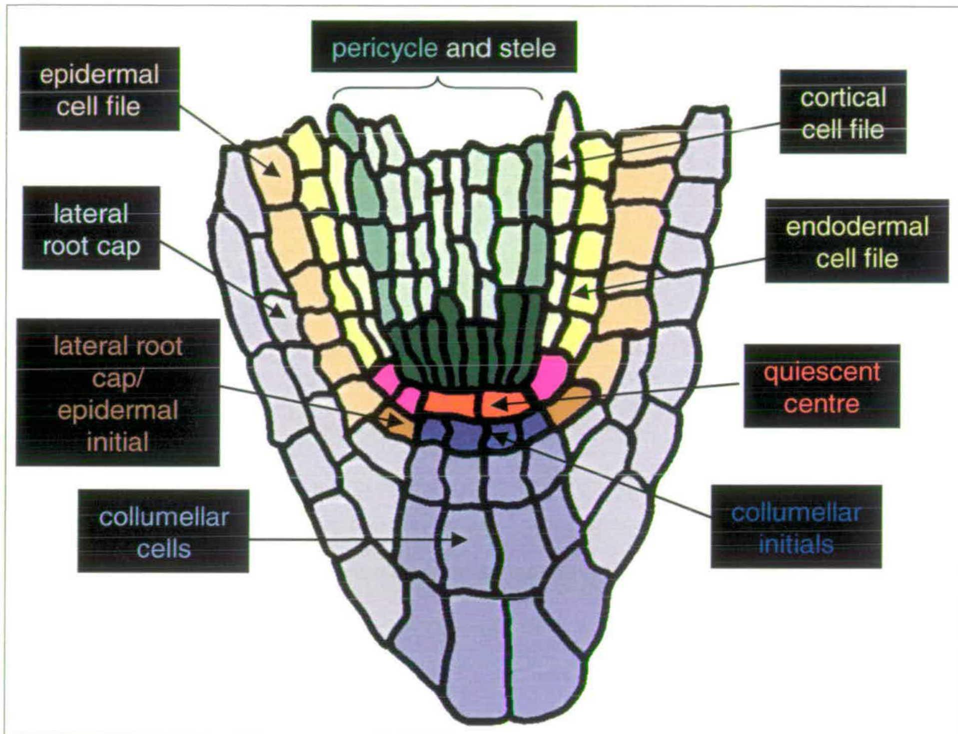

Figure III.10. Schematic of the root apical meristem. The meristematic zone quiescent centre, the cell files and the cell file initials as well as the lateral root cap are indicated with colour-coded labels. The epidermal/lateral root cap initial (brown) divides anticlinally to contribute cells to the epidermal cell file. This initial also divides periclinally to contribute cells to the lateral root cap which covers the apical tip of the meristem. Schematic taken from Van Den Berg, *et al.* (1997) and adapted in Ingram (2004) .

express *H2B::YFP*. Outside-layer specific expression in epidermal cells of the root was maintained, becoming weaker in more mature roots beyond the elongation zone. *ACR4* therefore is expressed not specifically in epidermal cells, but rather in the 'outside' cells at the lateral root cap boundary. This finding is interesting as it suggests that root cells are able to perceive the outside environment that they are situated in, or at least recognise a lack of some sort of signalling at one cell edge that the surrounding cells usually provide.

However, as well as being expressed in outside tissues of the root, *ACR4* was expressed in some non-outside tissues (Fig.III.9G,H). This is quite distinct to the case of *ACR4* expression in aerial meristems where *H2B::YFP* is only detected in outside/L1 cells (Fig.III.9B,C). *ACR4* expression was detected in the epidermis/lateral root cap and columellar initials, and the quiescent centre cells. In addition *ACR4* expression was detected in the pericycle cells of initiating lateral root primordia (Fig.III.9E,F). *H2B::YFP* expression was first detected at the four to eight cell stage of lateral root primordia development. Lateral roots are initiated from founder cells in the pericycle which maintain their competence for division throughout the elongation and differentiation zones. Lateral roots are initiated at positions defined by a combination of developmental and environmental cues (Dubrovsky, *et al.*, 2000; Dubrovsky, *et al.*, 2001). One or two longitudinally adjacent pericycle cells (founder cells) per cell file undergo one round of asymmetric cell division to create adjacent shorter cells. These shorter cells form the centre of the future lateral root. It has been found that a minimum of three founder cells from adjacent pericycle cell files are required for lateral root primordia to be initiated (Dubrovsky, *et al.*, 2001). The shorter cells subsequently divide anticlinally in a strict series of morphogenetic events, which together with differential gene expression form a proper lateral root. After being expressed early in lateral root development in these dividing cells, *H2B::YFP* expression (under the control of the *ACR4* promoter) was maintained in the outside and meristematic cells throughout lateral root outgrowth. Expression in fully mature lateral root meristems was subsequently detected in the same pattern as described for the apical root meristem (Fig.III.9G).

In conclusion *ACR4* expression is detected in 'outside' cells of the root in the lateral root cap and most epidermal cells, but also in cells of the meristem which are not on the outside. This will be discussed in more detail in Section III.5.

As *H2B::YFP* is a fairly stable marker gene it was important to confirm that the expression pattern seen (particularly the transition at the lateral root cap) was a true spatial indication of *ACR4* expression, and not an artefact of earlier expression which had been maintained through time. This was confirmed firstly by analysis in the lab of plants expressing *GFP* (green fluorescent protein) under the control of the *ACR4* promoter. *GFP* was found to be localised to the same cells as mentioned above for *H2B::YFP* (Ingram, 2004). However one caveat that had to borne in mind was that GFP moves between cell layers, potentially distorting the expression pattern seen (Ingram, 2004). This is the reason why GFP was not used as a marker for the *ACR4* expression pattern here. In order to further verify the expression pattern a second construct was analysed. The same expression pattern was seen in plants expressing an *ACR4::GFP* fusion under the control of the *ACR4* promoter (see Chapter IV), although *H2B::YFP* expression was maintained for slightly longer in the root cap than *ACR4::GFP* was. Together this data suggests that the marker expression pattern described here is a faithful representation of where *ACR4* is expressed.

III.5. *ACR4* appears to be involved in signalling between cells in order to maintain outside L1 layer integrity

ACR4 is expressed in the outside layer of embryos and meristems, and is involved in regulating proper ovule integument outgrowth and sepal margin organisation. As a receptor-like kinase it could act by perceiving extracellular signals, transmitting the signal into the cell via phosphorylation; *ACR4* has been shown to encode a protein with an active kinase domain (Gifford, *et al.*, 2003). Gene expression studies indicate that *ACR4* is not exclusively expressed in ovule integuments and sepal margins. Thus, why is a phenotype seen only in these two organs? Why is such an apparently weak phenotype noted for such a widely expressed gene? There is only one likely explanation for these findings, as the possibility that the *acr4* alleles are not null has already been discounted (see Chapter III.2.4). There could be redundancy with one or more of the four other members of the *Arabidopsis CR4*-like gene family. In this study the results obtained cannot eliminate this possibility, and indeed a role for one or more genes with similarity to *ACR4* is proposed in Chapter IV as part of a molecular model for ACR4 function. A comprehensive study of the four other *CR4*-like genes in *Arabidopsis* which includes a dissection of their expression patterns and functions is currently taking place in the lab.

III.5.1. *ACR4* function: a hypothetical model

In order to explain the phenotype seen in *acr4* mutants a mechanistic model is proposed. Interestingly, marginal sepal cells and outside-layer derived ovule integuments are unique in *Arabidopsis* in being composed of two single appressed L1 cell layers (Fig. III.11A,B). All other organs, including leaves, roots and embryos (all of which *ACR4* is expressed in) have an underlying cell layer. In order to regulate correct outside/L1 cell layer development, so that cells form as a well organised mono-layer (which is developmentally important), it could be hypothesised that two possibly related signalling operations are involved. The first

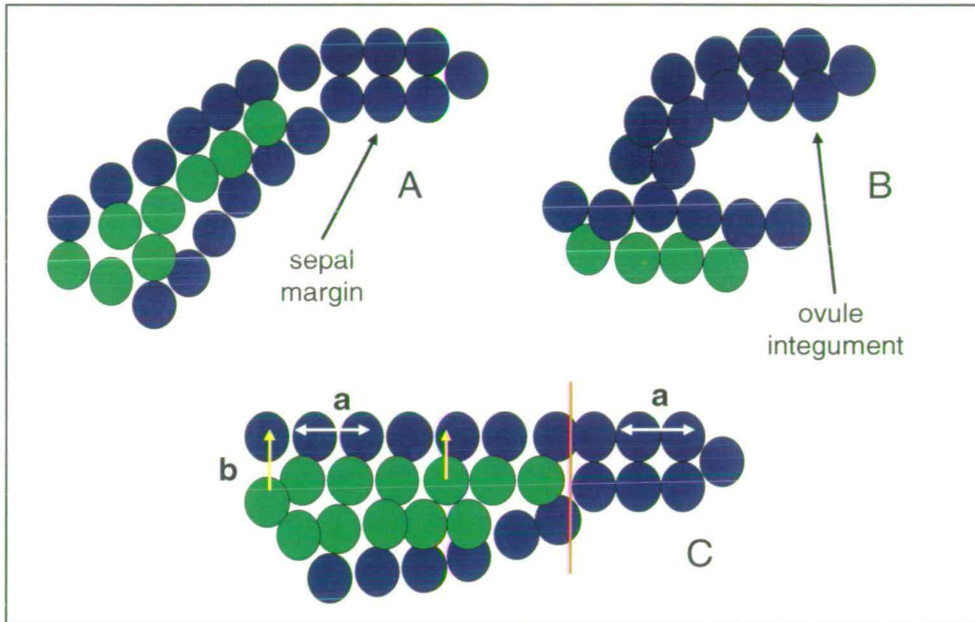

Figure III.11. *ACR4* may receive positional information to maintain outside-layer organisation. (A-B) Schematic of cell layer organisation in sepal margins (A) and ovule integuments (B). The L1 cell layer is indicated by blue coloured cells, with underlying cells coloured green. (C) Hypothetical signalling between outside cells (a) and from underlying cells (b), together providing positional information required to maintain proper outside (L1) cell layer organisation in aerial organs.

would be between cells of the outside/L1 layer, and the second would be from underlying cells (Fig.III.11C). Together these would provide positional information to maintain proper outside/L1 layer organisation. If *ACR4* is implicated as a molecule involved in signalling between outside-layer cells (process (a) in Fig. III.11C), then this signalling would be compromised in *acr4* mutant L1 cells. A signal from underlying cells (process (b) in Fig. III.11C) could compensate for loss of *ACR4* activity in most organs, ensuring normal development. However as sepal margins and ovule integuments have no underlying cell layer, this compensation could not occur, and thus defects would be manifest in these two organs. This is exactly what is observed in *acr4* mutants. The effect of loss of *ACR4* function in L1-derived stipules was not determined in this study. However, as stipules are composed of a single layer of L1 cells - not two single appressed L1 layers - a defect in cell layer organisation similar to that seen in sepal margins and ovule integuments would have been unlikely to occur.

Thus *ACR4* is likely to be involved in a process in aerial organs by which L1 cells recognise their position with respect to each other. Were ACR4 to be localised at the plasma membrane of L1 cells, such a signalling process would be feasible. The localisation of ACR4 protein will be discussed in Chapter IV.

III.5.2. Opposing opinions and a comparison of *ACR4* with *CR4*

In contrast to the results presented in this chapter and in Gifford, *et al.* (2003), several authors find defects in the protoderm and epidermis of *acr4* mutants. These opposing findings will be examined here. Also the role of *ACR4* in *Arabidopsis* in comparison to the role of *CR4* in maize will be discussed. Tanaka, *et al.* (2002) find defects during embryo development when using an antisense approach to reduce the level of *ACR4* expression. In a second paper by the same group, Watanabe, *et al.* (2004) report defects in epidermal cells and the cuticle of the leaf in an *acr4* allele generated by t-DNA insertion. In maize (as discussed earlier), loss of *CR4* function

results in defects in epidermal specification as well as defects in the aleurone layer (Becraft, *et al.*, 1996). Considering the high degree of similarity between maize *CR4* and *ACR4*, it would be expected that *ACR4* would play a similar role in *Arabidopsis*. However, despite careful examination of all t-DNA insertion and TILLING-generated alleles, no defects in protodermal or epidermal specification were found here. Indeed *acr4* mutants express epidermal cell markers in a normal fashion, which suggests that epidermal cells are correctly specified (Gifford, *et al.*, 2003).

In Tanaka, *et al.* (2002), antisense expression of *ACR4* appears to result in malformation of embryos during development. However it is likely that Tanaka, *et al.* are seeing an effect not directly associated with loss of *ACR4* expression for a number of reasons. Firstly, the use of antisense to reduce gene expression levels is unreliable due to its lack of specificity. Secondly, the expression pattern of antisense *ACR4* shown in the publication appears to be patchy, which could affect results. Finally, antisense *ACR4* seeds shown appear to exhibit some similar features to those found in this study: seeds appear to be rounder with abnormal outer layers. Defects in embryo development could therefore occur indirectly as a result of abnormal seed development (although no such effects are seen here). The possibility that antisense expression is reducing the expression levels of the other *ACR4*-like genes, producing an attenuated and less restricted phenotype to that seen when only *ACR4* expression is lost, is a prospect worth considering. However due to the low similarity between *ACR4* and the other *CR4*-like genes in *Arabidopsis*, this seems unlikely.

Watanabe, *et al.* (2004) confirm the importance of *ACR4* in integument and seed development, but also report defects on the leaf surface including apparent extrusions of epidermal cells. The authors suggest that *ACR4* is important in epidermal differentiation, but do not hypothesise why a phenotype is not seen in the epidermis of the root. The *acr4* allele used in their study (named *acr4-1*) appears to correspond to the *acr4-1* line used here. It is possible that the *acr4* mutation is more penetrant in a Wassilewskija (Ws) background (which *acr4-1* is in) than in a Columbia 0 (Col0) background. Ecotype differences were not investigated in detail between *acr4-1* (Ws) and *acr4-2* (Col0) in this study. As mentioned above (Section III.2.6), the *acr4-1* line was found to carry an additional mutation, not associated

with a t-DNA insertion. Alternatively then it is possible that additional background effects are seen in the line used by the authors. The leaf cuticle of *acr4* mutant alleles was not investigated in detail in this study but no major defects, as suggested by Watanabe, *et al.* were obvious. In particular no cell to cell fusion which is often seen in mutants where the cuticle is defective, was observed. In conclusion therefore, data from the studies discussed above may have been incorrectly interpreted, and does not impinge significantly on the conclusions drawn here.

As to a comparison between *ACR4* and *CR4*, one interesting point worth discussing is that the phenotypes seen in *Arabidopsis* and maize may not be as unrelated as first they appear. Although cell layer abnormalities are seen in different organs to those of maize, the types of cell disorganisation defects observed in *acr4* ovule integuments and sepal margins are similar to the epidermal defects observed in the leaves of maize *cr4* mutants. In addition, due to the differing expression patterns of *ACR4* and *CR4*, a similar phenotype would be unlikely to occur in mutants. For example *ACR4* is not expressed in the endosperm of developing seeds (Gifford, *et al.*, 2003), whereas *CR4* is expressed in the aleurone layer of maize (Becraft, *et al.*, 1996). *ACR4* is expressed in roots (Gifford, *et al.*, 2003) whereas *CR4* is not (Jin, *et al.*, 2000). Also work on epidermal specification in maize suggests that the abnormalities in *cr4* mutants are not directly the result of incorrect epidermal specification (as discussed in Chapter I). Becraft, *et al.* (2001) analysed the expression of epidermal cell markers in *CR4* and *cr4* leaf sectors of genetic mosaics. One maize epidermal cell marker is anthocyanin accumulation. Rather than reduced levels of anthocyanin pigmentation as would be expected if epidermal cell fate was lost, elevated levels were in fact observed. In addition, *cr4* mutant sectors had an effect on the spatial patterning of adjacent *CR4* wild-type sectors cells: leaf sectors showed a displacement of ligules (Becraft, *et al.*, 2001). This work suggests that *cr4* mutants have defects in epidermal signalling, rather than in epidermal specification itself. Therefore *CR4* and *ACR4* seem to be playing more similar roles than first thought, both involved in regulating the organisation of cells rather than acting to specify cell fate.

A better candidate for a gene involved in epidermal specification in maize is the *DEK1* gene. *DEK1* is expressed in most maize tissues and is important in pattern formation in the embryo and leaf epidermis, as well as cell fate specification in the leaf epidermis and aleurone cells of the endosperm (Becraft, *et al.*, 2002; Lid, *et al.*, 2002). The defects in *dek1* mutants are more suggestive of a role in epidermal specification than those in *cr4* mutants, as misspecification of cell identity rather than a lack of organisation is seen. Interestingly recent unpublished work suggests that the *Arabidopsis* homolog of *DEK1*, *AtDEK1* is important in the specification of epidermal cells in *Arabidopsis* (Gwyneth C. Ingram, pers. comm.).

The underlying basis of, and evolutionary implications for the differences in expression pattern and phenotype seen between *Arabidopsis ACR4* and maize *CR4* will be addressed in more detail in Chapter VI.

III.5.3. A wider context: what signal is *ACR4* responding to?

So, if *ACR4* is involved in receiving and transducing some sort of signal which helps to maintain cell layer organisation, what is the identity of the signal? In order to identify the ligand(s) bound by *ACR4* several experimental approaches have been taken, both here and within the lab (see Chapter IV). As the potential ligand-binding domain of *ACR4* is located in the extracellular matrix, a secreted ligand would seem likely. It is possible that several *ACR4* ligands exist, differing with the stage of development (embryo or mature plant), or perhaps with organ type (ovule, shoot or root). One insight into a possible ligand that could be received by *ACR4* during embryo development comes from investigation of the *ALE1* (*ABNORMAL LEAF SHAPE 1*) gene by Tanaka, *et al.* (2001). *ALE1* is strongly expressed in endosperm cells which surround the embryo and is required for proper cuticle formation. *ALE1* encodes a subtilisin-like serine protease (subtilase) which could cleave and thus activate a potential ligand peptide. The ligand would then be present on the exterior of the embryo. A zygotically controlled signal such as a potential ligand processed by ALE1, or a maternally produced signal could be important in specifying

positional information in cell layers of the embryo. This would then also affect gene expression and developmental patterning in the resultant plant. Interestingly it has been shown that *acr4/ale1* double mutants exhibit an exacerbated phenotype in the seedling not seen in either single mutant (Nakajima and Benfey, 2002). Whether ALE1 does in fact process a ligand directly received by ACR4 though is yet to be determined.

In organ primordia (such as in the root) rather than a ligand being located on the exterior of the plant, it is more likely that differentiation is maintained by an inside to outside signalling process or by signalling within the outside layer (Nakajima and Benfey, 2002). This might act in combination with signals from neighbouring cells. Interestingly *ACR4* expression is detected in the meristematic cells of the root meristem which are not L1-derived. This suggests that there could be two factors, potentially two independent ligands, which activate and control the expression of *ACR4*. What role *ACR4* might play in the root meristem is unclear. It could be involved in controlling proper outside layer organisation in the root by maintaining proper cell organisation patterns in the root meristem itself. The fact that *ACR4* is being expressed in non-outside tissues of the root indicates that some inside-outside patterning could be taking place. Again such a process in organ primordia involving ACR4 is supported by the localisation of ACR4 protein (see Chapter IV).

Interestingly there are some similarities between the expression pattern of ACR4 and that of AUX1, an auxin influx, carrier in the root meristem. A potential link between ACR4 and auxin is of particular interest due to auxin being a regulator of pattern organisation. AUX1 expression follows the pattern that auxin is transported in, which goes down the centre of the root and then after reaching the meristem back up through the epidermal layer (Colon-Carmona, *et al.*, 2000). ACR4 is expressed in a similar fashion in the meristem and in most cells of the epidermal layer. There are differences between the patterns of gene expression: ACR4 it is not expressed in the central tissues of the whole root. However the similarities suggest that auxin could in some part be influential in regulating the pattern of ACR4 expression. Interestingly the ACR4 promoter contains six putative auxin response element

(ARE) binding boxes (ARE sequence reviewed in Ulmasov, *et al.* (1995)). More detailed work would be required to investigate this possible link.

In summary, the *ACR4* gene is expressed in the outside cell layer of embryos, meristems and ovules, defining the L1 layer. *ACR4* appears to be involved in a signalling process which helps to maintain L1 cell layer organisation, with gene expression being required for proper development of ovule integuments and sepal margins.

Chapter IV. ACR4 protein is localised to the L1 plasma membrane and may be endocytosed and cleaved in the cell

IV.1. Introduction

IV.2. Functional ACR4 protein is localised to the plasma membranes of L1 cells and to intracellular bodies

IV.3. ACR4 functional analysis

IV.4. The dynamics of ACR4 localisation

IV.5. The 39aa repeat domain is required for protein turnover: a ligand binding domain?

IV.6. The molecular mechanism of ACR4 action: homo-/hetero-oligomers with other ACR4-like proteins?

IV.1. Introduction

The *ACR4* gene encodes a receptor-like kinase which plays a role in maintaining L1 cell layer integrity. The ACR4 protein contains a putative transmembrane domain with predicted extracellular and intracellular regions (see Appendix 1). The predicted extracellular (N-terminal) region of *ACR4* consists of two domains. The first domain comprises seven novel 39aa repeats, and the second is a tumour-necrosis factor receptor (TNFR)-like region which contains three cysteine rich repeats (as discussed in Chapter I). In addition there is a signal peptide at the N-terminus of the *ACR4* ORF. The predicted cytoplasmic region also consists of two recognisable domains. Downstream of the transmembrane domain there is firstly a kinase domain which has been shown to be active (Gifford, *et al.*, 2003). Secondly there is a C-terminal region which shows a particularly high degree of conservation with respect to the maize *CR4* gene.

The ACR4 receptor-like kinase seems to be involved in a process by which L1 cells are organised with respect to each other. It was hypothesised at the end of Chapter I that ACR4 protein acts by receiving signals from neighbouring cells in the outside (L1) cell layer. A signal could then be transmitted by activation of downstream signalling components, via phosphorylation by the kinase domain of the ACR4 protein. Each L1 cell would then be receiving and transmitting signals in a pathway involving *ACR4*. The concerted action of a downstream signalling pathway could then be involved in regulating L1 cell layer organisation as a whole, ensuring that the L1 cell layer is sustained as a well organised mono-layer. Such signalling is probably required for normal L1 layer characteristics, such as the formation of a protective cuticle, to be maintained (as discussed in Chapter I).

For such a signalling process to take place the ACR4 protein must be localised in such a way that cell-cell signalling is possible. Localisation at the cell plasma membrane between L1 cells would support the above hypothesis, as the ACR4 protein would be situated in a way such that it could receive a signal or ligand from a neighbouring L1 cell.

In order to investigate the ACR4 protein in detail several approaches were taken. Firstly the localisation of wild-type protein was investigated in order to determine where in the cell it could be receiving a signal. Secondly a functional dissection of the protein was carried out. The purpose of this approach was two-fold: (i) to determine which domains of ACR4 were required for function of the protein, and (ii) to ascertain which domains were necessary for correct localisation. Thirdly the dynamics of ACR4 localisation were analysed in order to examine the connection between protein localisation and function. Together this data was used to synthesise a model for the molecular action of ACR4.

IV.2. Functional ACR4 protein is localised to the plasma membranes of L1 cells and to intracellular bodies

The sequence of *ACR4* contained a predicted trans-membrane domain and thus ACR4 protein was expected to be localised to the plasma membrane. In order to test this a green fluorescent protein (GFP) tag was added to ACR4 and the localisation of the fusion protein determined. To create tagged ACR4 an *ACR4*::*GFP* in frame gene fusion was placed under the control of the *ACR4* promoter (see Chapter II.3.3 for details of construction). When transformed into plants, the translated ACR4::GFP was visualised by exciting the fluorescence of GFP using confocal laser scanning microscopy. The localisation of ACR4::GFP was examined in all organs of the plant in which *ACR4* is expressed. As well as examining protein localisation within the cell itself, the intensity of fluorescence as a comparable measure of protein concentration between individual cell membranes was examined.

ACR4::GFP was observed to localise to the plasma membrane of L1/outside layer cells in embryos (Fig.IV.1A), meristems (Fig.IV.1B,F), lateral and apical roots (Fig.IV.1E,G) and ovules (Fig.IV.1C,D). This confirms the *ACR4* sequence predictions and supports a role for ACR4 in signalling between cells. ACR4 protein was seen in exactly the same cells in which the *H2B*::*YFP* expression in *pACR4*::*H2B*::*YFP* lines was observed. This correlation acts as a good confirmation that the expression pattern of *H2B*::*YFP* really does mark the cells in which wild-type *ACR4* is being expressed.

ACR4::GFP was localised to the plasma membranes of cells in the two ovule integuments, the endothelium and the L1 of the funiculus (Fig.IV.1D). ACR4::GFP fluorescence was present at all membranes in each of these cells, but it was weaker in the most outer membrane of the outer integument (which is directly on the outside of the ovule). It was stronger in membranes adjacent to the anticlinal cell wall (perpendicular to the surface of the ovule) and in membranes facing towards the inside of the ovule. It is possible that the fluorescence only appears to be of a higher intensity in these cases (inner and 'anticlinal' membranes of the outer integument)

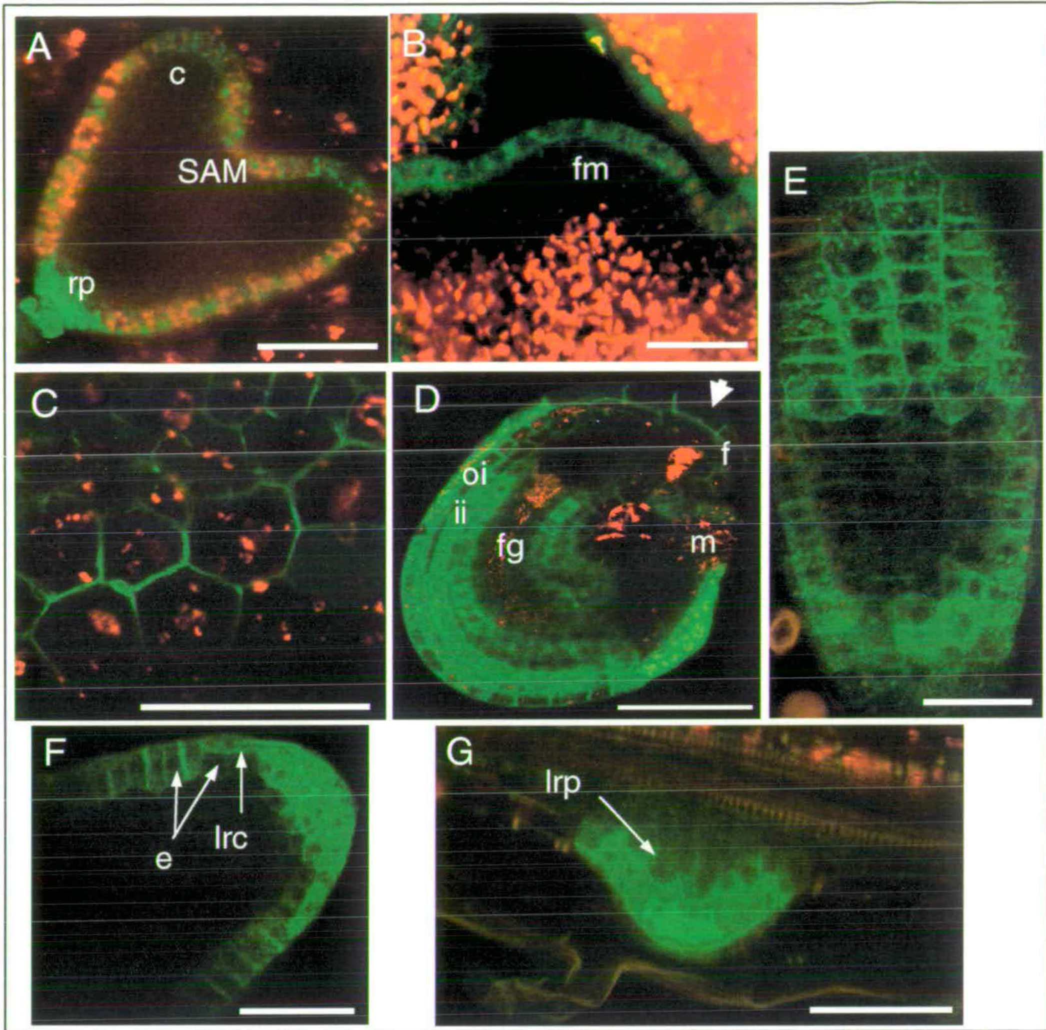

Figure IV.1. ACR4::GFP protein localisation. (A-G) Confocal images of plants expressing an ACR4::GFP protein fusion (green). (A) Early heart stage embryo and (B) floral meristem showing membrane localised and outside-layer specific ACR4::GFP localisation. (C) Surface view of an ovule, ACR4 protein is localised to the plasma membrane of epidermal cells. (D) Longitudinal section through a mature ovule. Fluorescence is seen in the outer integument (oi), the inner integument (ii) and the outer layer of the funiculus (f) Little protein is detected in the most outer cell membrane (arrowhead). (E) Surface view of a root showing plasma membrane ACR4::GFP localisation in epidermal cells. (F) Localisation of ACR4::GFP specifically to the plasma membrane of 'outside' cells in the lateral root cap (lrc), and in epidermal cells (e) of the lateral root, only when not underneath the lateral root cap. (G) ACR4::GFP protein localisation in an emerging lateral root primordium (lrp). Scale bars: 25μm. c, cotyledon primordium; SAM, shoot apical meristem; rp, root pole; fm, floral meristem; m, micryopyle; fg, female gametopyhte. Photos A-D, F taken by Gwyneth C. Ingram and included with kind permission.

simply due to an additive fluorescence from the membranes of two neighbouring cells.

In the root epidermis and root meristem ACR4::GFP was found to be localised to membranes adjacent to both anticlinal and periclinal walls of cells (Fig.IV.1E,F). The levels of fluorescence in both anticlinal and periclinal membranes was found to be similar in the internal cells of root meristems (the quiescent centre, collumellar initials, and the epidermis/lateral root cap initials). However in outside cells ACR4::GFP was preferably localised to the inner periclinal membrane and anticlinal membranes (Fig.IV.1F), as was the case in ovules (Fig.IV.1D). This preferential localisation will be discussed in Section IV.6.

As well as being localised to the plasma membrane ACR4::GFP was observed to be localised to multiple small bodies which were present at varying positions throughout the cell. These bodies did not co-localise with chloroplasts, and appeared to be of a smaller size. The size and number indicated that they could be vesicles. ACR4::GFP was seen in these bodies in all of the same cells that plasma membrane localisation was observed in, although they were particularly noticeable in outside cells of the root (Fig.IV.1E). This is likely due to the fact that epidermal cells of the root are older and more cytoplasmically dense than, for example, integument cells which are younger and highly vacuolated. The localisation of ACR4::GFP to these bodies will be discussed in detail in Section IV.4.

In order to confirm that ACR4 was specifically associated with the plasma membrane and not just secreted to the cell wall, the localisation of ACR4::GFP was examined in plasmolysed cells. Roots of seedlings carrying the ACR4::GFP protein fusion were treated with 0.8M mannitol to induce cell plasmolysis: the plasma membrane tears away from the cell wall (Fig.IV.2). When treated roots were viewed using confocal microscopy the ACR4::GFP fluorescence was observed to move away from cell-cell boundaries as the membrane contracted (Fig.IV.2B). The cell wall and extracellular space were observed to be clear of ACR4::GFP, confirming that ACR4::GFP is not localised to the cell wall. In untreated roots the ACR4::GFP fluorescence remained at the plasma membrane (Fig.IV.2A).

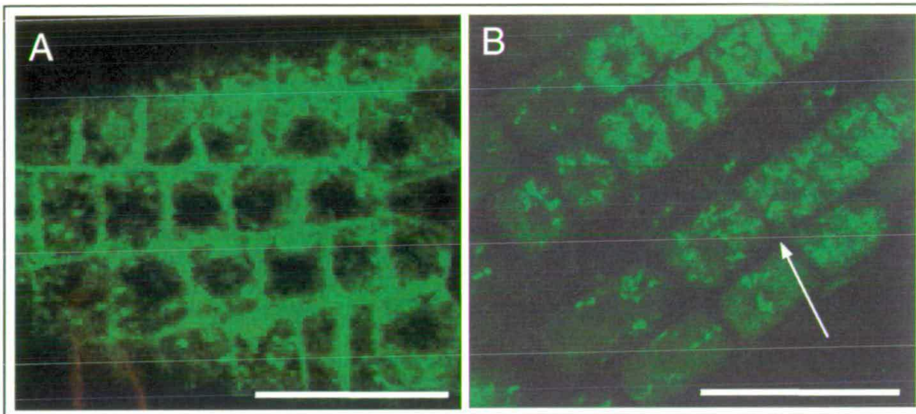

Figure IV.2. Treatment of ACR4::GFP fusion lines with mannitol. (A,B) Confocal images of roots expressing ACR4::GFP. (A) Control roots as Fig.IV.1E, mounted in water. (B) Roots mounted in 0.8M mannitol to induce plasmolysis of cells. The cell wall region is cleared of fluorescence (arrow) which is seen to pull away from cell-cell boundaries as the plasma membrane contracts. Scale bars: 25μm. Photo B taken by Gwyneth C. Ingram and included with kind permission.

In order to confirm that the introduced ACR4::GFP protein was functional and thus that the correct localisation of ACR4 activity was being indicated by fluorescence, a complementation analysis was carried out. Plants from two families which carried and were expressing the *pACR4::ACR4::GFP* transgene were crossed to the *acr4-2* homozygous mutant. The ability to complement the *acr4* defects in homozygous *acr4-2* mutants was assayed (as carried out for the line carrying non-GFP-tagged ACR4 in Chapter III.3). In the F2 progeny of one family cross, full complementation was observed (as Fig.III.8). For the second family partial complementation was seen, i.e. the level of seed and ovule abortion was decreased and seeds exhibited a more normal morphology with most seeds being elliptical. Seeds were generally smooth although some still appeared to have surface irregularities. Overall the presence of ACR4::GFP was able to complement the *acr4* phenotype which proves that the ACR4::GFP protein is functional. The pattern of ACR4::GFP localisation was compared in *ACR4-2* wild-type and *acr4-2* mutant plants, and was found to be identical.

In summary, functional ACR4 protein is localised to the plasma membrane of L1/outside cells in such a fashion that cell to cell signalling in the L1/outside layer could be possible.

IV.3. ACR4 functional analysis

In order to study the ACR4 protein in more depth a functional analysis was undertaken. The purpose of this was twofold. Firstly, the requirement of each ACR4 domain for function was determined using complementation analysis. If ACR4 missing a certain domain was not able to complement *acr4* it was asked whether this was due to a lack of correct protein localisation. Therefore secondly, an assay to ascertain which domains were necessary for proper plasma membrane localisation was carried out. In order to address both of these points a range of deletion derivatives of the ACR4 protein were constructed and tested (see Chapter II.3.3 for details of construction).

ACR4 gene sequences were first amplified and ligated in order that one or more of the ACR4 domains would be removed. Sequences where translated proteins would be missing the following domain(s) were constructed: five of the seven 39aa repeats ('Δ39aa'), the TNFR-like repeat region ('ΔTNFR'), the C-terminal region ('ΔC-ter') and the kinase domain and C-terminal regions ('ΔK/C-ter'). In addition a variant of *ACR4* where the kinase domain had been inactivated was used: 'K-null'. The amino acid change in this sequence has been shown to result in inactivation of the ACR4 protein kinase *in vitro* (Gifford, *et al.*, 2003); see Appendix 1. All Δ*ACR4* sequences were placed under the control of the *ACR4* promoter and constructs were transformed into plants to create plant lines as named above. These lines were used for functional analysis.

In order to determine the localisation of each protein derivative, a *GFP* sequence was fused in-frame to the C-terminus of all deletion *ACR4* sequences and the *ACR4* K-null. An *ACR4* transgene missing both the transmembrane and intercellular domains (kinase domain and C-terminal regions) ('ΔTM/K/C-ter') was also fused to *GFP*. This was made in order to confirm that the extracellular part of the protein is targeted to the extracellular space, in other words to confirm the predicted orientation of the protein. In addition an N-terminal GFP-tagged variant of the wild-type ACR4 protein, as well as a variant with GFP tags at both ends of ACR4 were generated. In order to ensure that the N-terminal GFP-tagged proteins were correctly targeted to

the plasma membrane a second (the first being the endogenous signal peptide) *ACR4* signal peptide was added to the N-terminus of *GFP*(N-ter)::*ACR4* sequences. See Fig.IV.3 for schematic of proteins in each line.

All Δ*ACR4*::*GFP*, both *GFP*::*ACR4*, and the K-null *ACR4*::*GFP* transgenes were transformed into wild-type Col0 plants. All Δ*ACR4*, the K-null and some Δ*ACR4*::*GFP* (Δ*TM/K/C-ter ACR4*::*GFP*, K-null *ACR4*::*GFP*) transgenes were transformed into both the *acr4-1* and *acr4-2* mutant lines; no difference in results was seen between the *acr4-1* and *acr4-2* lines. Plant lines carrying the remaining Δ*ACR4* and the *GFP*::*ACR4* transgenes were crossed to *acr4-2* mutants. The transformants or F2 *acr4-2* homozygous progeny, resulting from the transformations and crosses respectively, were subject to complementation analysis (as Chapter III.3). This allowed determination of which ΔACR4 proteins were functional, and thus which ACR4 domains were required for function and which were not. Complementation analysis was also used to compare the function of N-terminal and C-terminal tagged ACR4 proteins. In addition, in all *acr4-2* and wild-type lines transformed with all of the above transgenes, any enhancement or alteration of the phenotype which might be associated with dominant negative or positive effects was investigated.

IV.3.1. An ACR4 deletion protein lacking the transmembrane, kinase and C-terminal regions is exported to the cell wall

As mentioned above, the localisation of ACR4 variants was determined. Both N-terminal GFP-tagged and all ΔACR4 proteins analysed were localised (correctly or not as will be discussed) to cells of the L1/outside cell layer as for C-terminal tagged GFP. This was an important confirmation that the introduced transgenes are being correctly transcribed and translated. In addition, identical localisation in both Col0 wild-type and *acr4* mutant plants was observed.

Full length ACR4 proteins with N-terminal, and both N- and C-terminal GFP tags were correctly localised to the plasma membrane as the C-terminal tagged ACR4

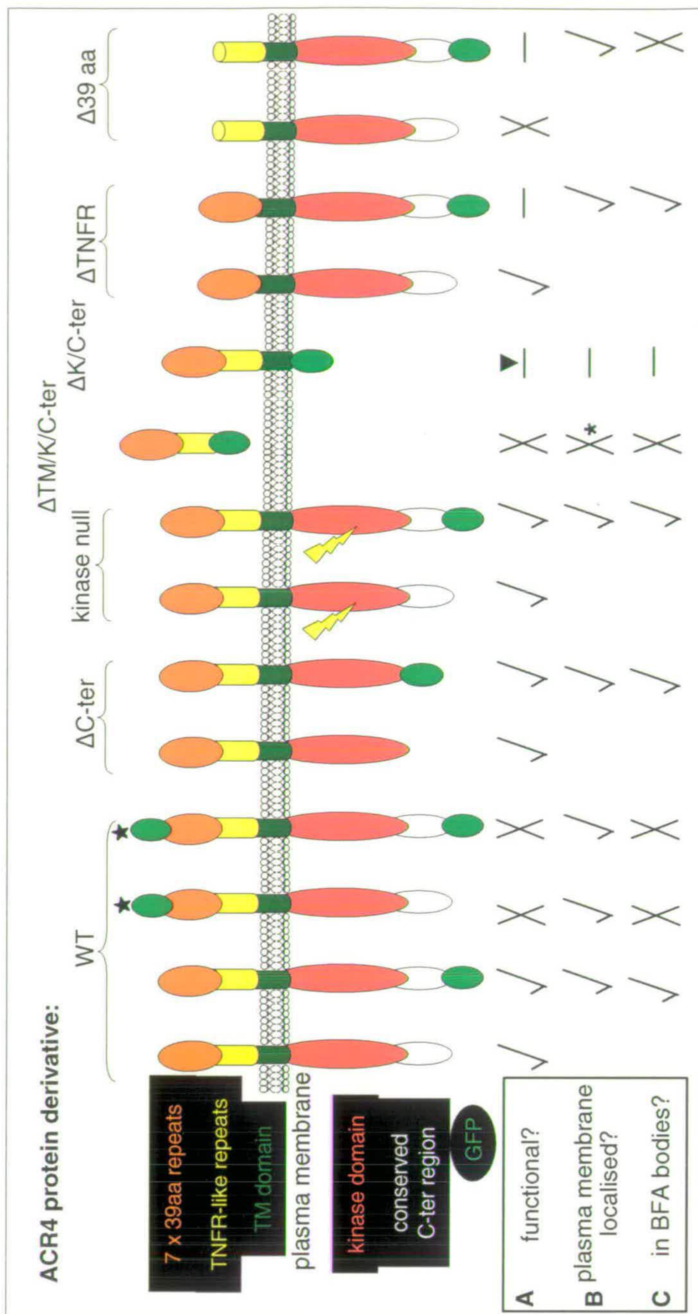

Figure IV.3. ACR4 functional and localisation analysis. Schematic showing deletion derivatives of ACR4 and the full length wild-type tagged proteins (WT) and a summary of results. (A) The functionality of each derivative was tested by the ability of the protein to complement the *acr4* phenotype. Whether or not ACR4 variants were localised to the plasma membrane (B) and BFA-compartments (C) is indicated. (-) indicates result was not obtained. (A) Removal of the 39aa repeat domain of ACR4 (Δ39aa) or addition of an N-terminal GFP tag (star-marked proteins) rendered ACR4 non-functional. A kinase-null derivative was functional. A protein lacking all but the extracellular protein domains was non-functional (ΔTM/K/C-ter). (B) The transmembrane domain was required for localisation of ACR4 to the plasma membrane: (*) as predicted ΔTM/K/C-ter was localised to the cell wall. (▼) a derivative lacking the kinase domain and C-terminal regions (ΔK/C-ter) was unstable; data from this construct was not usable. All other variants were localised to the plasma membrane as wild-type ACR4::GFP. (C) Removal of the 39aa repeat region (Δ39aa) or addition of an N-terminal GFP tag (stars) resulted in no ACR4::GFP being detected in BFA compartments. These three proteins were visible only at the plasma membrane. ΔTM/K/C-ter ACR4::GFP was also not detected in BFA compartments, it remained at the cell wall.

(Section IV.2). Deleted ACR4 proteins ΔTNFR and ΔC-ter were both correctly localised (Fig.IV.4C and D respectively). It does not appear therefore that either the TNFR-like repeat domain or the C-terminal region are required for normal plasma membrane localisation of ACR4. The ΔTNFR ACR4::GFP fluorescence was overall slightly brighter than that of ΔC-ter ACR4::GFP. Δ39aa ACR4::GFP and K-null ACR4::GFP were also correctly localised to the plasma membrane which suggests that neither the 39aa repeat domain nor kinase activity are necessary for correct targeting and localisation (Fig.IV.4A and E respectively). The relative fluorescence of plants between lines carrying these two proteins in comparison to the wild-type ACR4::GFP did appear to vary however. There was a stronger level of ACR4::GFP fluorescence in the line carrying Δ39aa ACR4::GFP which is consistent between many families. Fluorescence of plants carrying K-null ACR4::GFP is consistently lower. These differences could be due to positional effects related to the insertion site of the introduced transgene. However the range of variation is smaller within families transformed with one construct, than between lines transformed with different constructs, which suggests that this is not the case. It is more likely that there are differences in ACR4 protein behaviour. ΔK/C-ter ACR4::GFP was undetectable in transformed plants. It is likely that this protein derivative is unstable and thus no meaningful results can be gained from this construct. It is possible that the kinase domain is involved in maintaining correct localisation at the membrane. Interaction between the kinase domain of a protein with sub-cellular scaffolding proteins has been shown for members of the epidermal growth factor receptor (EGFR/ERBIN) (ErbB) family in animals, as discussed in Chapter I (Carraway and Sweeney, 2001). However it is not possible to conclude anything from the results here.

ΔTM/K/C-ter ACR4::GFP was localised to the extracellular matrix/cell wall region (Fig.IV.4B). ACR4::GFP fluorescence in plants carrying this protein was quite distinct from membrane localised ACR4::GFP protein fluorescence. Large areas of fluorescence were seen at cell-cell boundaries where the width of cell wall is thicker. This suggested that ΔTM/K/C-ter ACR4::GFP was mobile in the apoplast. When roots carrying ΔTM/K/C-ter ACR4::GFP were treated with mannitol (as carried out

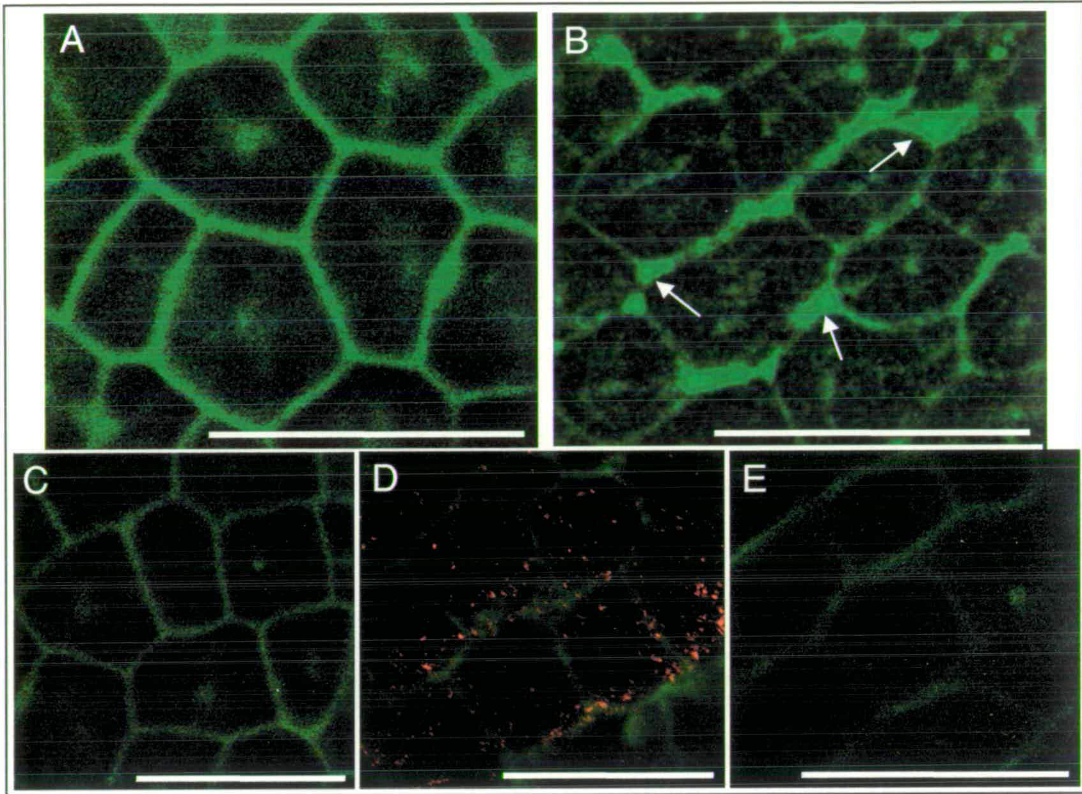

Figure IV.4. ΔACR4::GFP fusion protein localisation. (A-E) Confocal images of ovule epidermal cells of lines carrying deletion derivatives of ACR4. (A) Bright fluorescence localised at the membrane of Δ39aa ACR4::GFP-expressing ovules. (B) Extracellular localisation of ΔTM/K/C-ter ACR4::GFP. Fluorescence is brighter at cell-cell boundaries (arrows). (C-E) Membrane localisation in ovules of plants expressing (C) ΔTNFR ACR4::GFP, (D) ΔC-ter ACR4::GFP and (E) kinase null ACR4::GFP. Scale bars: 25μm. Photos B-E taken by Fiona Robertson and included with kind permission.

in Section IV.2) fluorescence was seen to remain at the cell wall rather than enter cells (not shown). The fact that ΔTM/K/C-ter ACR4::GFP was localised to the extracellular space confirms the predicted orientation of ACR4 in the plasma membrane.

IV.3.2. The 39aa repeat region of ACR4 is vital for function whilst kinase activity is not

The ability of ACR4 variants to complement the defects seen in *acr4* mutants was assayed in at least 20 T_1 transformants or in four independent crosses (depending on the complementation test method). Identical results were obtained using either ACR4 or GFP-tagged ACR4 deletion derivatives when tested. The results are presented in Fig.IV.3. Lines carrying either ΔTNFR or ΔC-ter ACR4 protein were able to rescue the *acr4* phenotype. Introduction of these proteins into the *acr4* mutant restored a wild-type seed shape and normal seed development. Complemented siliques were completely full of seeds. This suggests that neither the TNFR-like repeat region nor the C-terminal region are required for normal function.

Plants carrying ΔTM/K/C-ter ACR4::GFP were not able to complement *acr4* and thus this protein is non-functional. This is to be expected as ΔTM/K/C-ter ACR4::GFP is incorrectly localised. The ΔK/C-ter construct was not tested although is not likely to be able to complement *acr4* defects due to protein instability (as mentioned in Section IV.3.1). In *acr4-2* plants that carried ΔTM/K/C-ter ACR4::GFP there was no exacerbation of the *acr4* defects. This might have been expected if the construct had a dominant negative effect (as discussed in Chapter I, (Shpak, *et al.*, 2003)). The lack of such an effect will be discussed in Section IV.6. Interestingly, kinase activity itself was not required for function: the K-null ACR4 construct was able to complement *acr4* mutant defects.

A deletion of *ACR4* where the entire extracellular region had been removed (*Δ39aa/TNFR ACR4*) was transformed into Col0, as carried out for the other deletion derivatives. It had been hypothesised that this transgene would confer a dominant

positive phenotype by comparison to studies in mammal cells (as discussed in Chapter I, (Basler, *et al.*, 1991)). However no altered phenotypes were seen and all organs of the transformed plants looked wild-type. The reasons why a dominant positive effect might not have been seen will be discussed in Section IV.6.

When the 39aa repeat region is removed, the ACR4 protein is rendered non-functional. Introduction of the *Δ39aa ACR4::GFP* transgene was not able to rescue the *acr4* defects. As mentioned in Chapter III, the 39aa repeat region is of particular importance as a potential ligand binding domain. The fact that the domain is vital for function supports a potential important role in receptor function. The 39aa repeat region is located at the N-terminal end of ACR4 and has been shown (see Section IV.3.1 above) to be localised to the extracellular matrix. When the seven repeats are aligned it is clear that the domain has a highly conserved and almost invariant consensus sequence of: cysteine, around ten variable amino acids, cysteine, tryptophan, glycine [C (X~10) CWG] (Fig.IV.5).

Modelling studies were carried out in collaboration with Dinesh Soares and Dietlind Gerloff (The University of Edinburgh). The 39aa repeat domain gene sequence shows similarity to the sequence of beta-lactamase inhibitor II. The conformation of this protein has been solved by crystallography and been shown to fold up as a seven-bladed β-propeller (Lim, *et al.*, 2001). A β-propeller structure could be stabilised in ACR4 by the cysteine residues in the seven repeats. These cysteine residues, although absent in the beta-lactamase inhibitor are positioned in such a way that disulfide bridges could form between the seven blades. The modelled structure of the 39aa repeats β-propeller is shown in Fig.IV.6. The ACR4 β-propeller has a highly electronegative pore as well as pockets of positively and negatively charged amino acids around the sides of the doughnut-shaped ring. If the 39aa repeat region is in fact the ACR4 ligand binding domain it is possible that a ligand could bind at one or more of these charged regions.

The phenotype of *acr4* mutants could also not be rescued when lines carrying either GFP::ACR4::GFP or GFP::ACR4 were crossed to *acr4* homozygotes. This suggests that blocking the 39aa repeat region (here with a GFP tag), renders the

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ISYGEGGSVF-CGLKS--DGSHLVVCYGSNSAI--LYGTPG
FIGLTGGDGFMCGLLM--LS-HQPYCWGNSAFI--QMGVPQ
LCGLRKPIVGRRKNSNIIS-SSLVDCWGYNMTR--NFVFDK
LHSLSAGSEFNCALSS---KDKSVFCWG-DENSS---QVIS
FQKIAAGGYHVCGILD--GLESRVLCWGKSLEF---EEEVT
LLAVVGGKFYACGIKR--YD-HSAVCWGFFVNR---STPAP
FYDLAAGNYFTCGVLT--GTSMSPVCWGLGFPASIPLAVSP
```

Figure IV.5. Pileup of the seven *ACR4* 39aa repeats, aligned by eye. Amino acids that are highly conserved between repeats are coloured red, whilst conservation to the repeats in maize *CR4* is indicated with underlining. The repeats have an almost invariant consensus sequence motif of C (X~10) CWG.

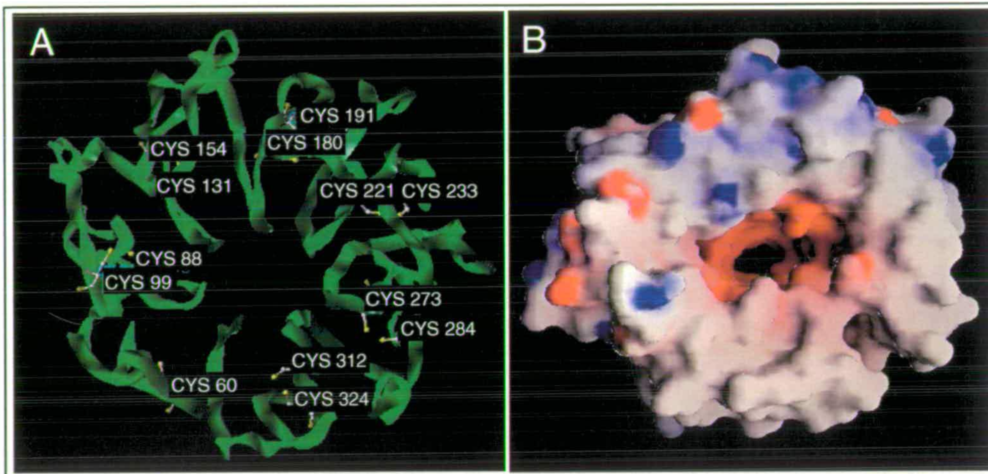


Figure IV.6. The predicted conformation of the ACR4 39aa repeat domain. (A) Conformation of amino acids with the folded structure indicated as a green string. Disulfide bridges between conserved cysteines are highlighted (numbering coincides with the numbering of ACR4 amino acids shown in Appendix 1. In the *acr4-7* allele the cysteine at position 180 marked on the schematic is changed to a tyrosine. (B) Surface view of the folded protein with electrostatic charges indicated. White = neutral, red = negatively charged, blue = positively charged. Images obtained through collaborative work carried out with Dinesh Soares and Dietlind Gerloff (The University of Edinburgh).

ACR4 protein non-functional. The lack of function was not due to protein instability as both of these N-terminal GFP-tagged proteins were properly translated: fluorescence was visible at the plasma membrane (see Section IV.3.1). An alternative explanation for this result will be discussed in Section IV.6.

Interestingly the *acr4-7* allele, where one of the conserved cysteines in the fourth ACR4 39aa repeat has been substituted (by TILLING) for a tyrosine residue (see Fig.IV.6A for TILLING allele position), confers a loss of function phenotype as described in Chapter III.2.4. It seems likely that this change at a conserved cysteine in some way disrupts the β-propeller conformation, and the result highlights the functional importance of the 39aa repeat domain. In order to confirm that the loss of *ACR4* function is not merely due to the fact that stable ACR4 protein is not being translated, experiments are currently being carried out by Ross Walker in the lab. The modified *acr4-7* ORF will be introduced as a transgene tagged with GFP into Col0 wild-type and homozygous *acr4-2* plants (as for the ACR4 localisation experiments in Section IV.3.1). Whether stable ACR4(2-7)::GFP protein is able to be translated will be evident as ACR4(2-7)::GFP protein fluorescence being visible and being localised to L1 cell plasma membranes.

IV.4. The dynamics of ACR4 localisation

IV.4.1. BFA-sensitive vesicle trafficking is required for some aspect of correct ACR4 localisation or protein turnover

As mentioned in Section IV.2, small intercellular bodies were visible in the cytoplasm of cells of plants expressing ACR4::GFP. Similar bodies were also noted in lines carrying ΔACR4::GFP, K-null ACR4::GFP and N-ter GFP::ACR4 proteins (not shown). These small bodies were hypothesised to be vesicles which were trafficking ACR4::GFP within the cell. This trafficking could potentially be transporting ACR4 protein to the membrane, and/or could perhaps be part of some sort of endocytosis of ACR4 after ligand binding. As mentioned in Chapter I, endocytosis of active animal receptor kinases is thought to be an important method of damping down the signalling activity, and maintaining sensitivity to incoming signals (Waterman and Yarden, 2001). In order to address these issues, the potential vesicle trafficking of ACR4 was examined.

In order to investigate vesicle trafficking, the effect of Brefeldin A (BFA) on ACR4::GFP protein localisation was examined. BFA is a commonly used inhibitor of vesicle movement in both animals and plants which acts by targeting and inhibiting the action of proteins involved in vesicle formation. BFA inhibits of a subset of GDP/GTP guanine-nucleotide exchange factors, ARF-GEFs, which catalyse activation of ADP ribosylation factors. ARF-GEFs are involved in formation and coating of vesicles which are vital in the correct functioning and transport between the endoplasmic reticulum (ER) and golgi stacks (Nebenfuhr, *et al.*, 2002). Despite being a widely used drug for study of protein trafficking in cells, it is not known precisely what resultant effect this BFA-inhibition has in either animal or plant cells. However by using organelle specific markers some of the consequences on vesicle transport within the endomembrane system, and between the endomembrane system and the plasma membrane can be dissected. After BFA treatment, vesicle movement comes to a stop and vesicle aggregates form. Vesiculation at the golgi is inhibited, with the result that golgi cisternae fuse directly

with the ER stacks or seem to disappear. In addition, large intercellular membranous aggregates appear inside the cell. These aggregates are thought to consist of two structures. Firstly BFA-induced ER-golgi hybrid compartments which are rapidly formed (apparent within 30 minutes) as a result of breakdown of the physical separation between the two organelles. Secondly 'BFA compartments/bodies' which comprise a conglomerate of tubules, vesicles, the trans-most golgi cisternae which split from the main stack, as well as endosomes (Nebenfuhr, *et al.*, 2002; Samaj, *et al.*, 2004).

Roots of plants carrying the ACR4::GFP protein fusion were bathed in a 100μM BFA solution for two hours. After treatment with BFA the number of small sized ACR4::GFP-staining bodies decreased. At the same time there was an increase in signal in what appeared to be larger organelles that rapidly coalesced into one or two large brightly fluorescing perinuclear aggregates per cell (Fig.IV.7). The size and number of aggregates in each cell was consistent with the structures being 'BFA bodies'. As ACR4::GFP is localised to BFA bodies it seems therefore that ACR4::GFP is being trafficked via a BFA-sensitive pathway. ACR4::GFP was however still strongly localised to the plasma membrane of the cell. Membrane-associated fluorescence remained bright or became brighter. This is evident in a comparison of the membranes in roots of Fig.IV.7A and B. The roots used for both control and BFA treatment came from seedlings of the same homozygous pACR4::ACR4::GFP line in which the level of fluorescence between plants was uniform. This suggests that ACR4::GFP transport to the membrane is not the direct target of BFA inhibition. The target could therefore be some other aspect of ACR4 protein localisation.

One possible explanation for the BFA-sensitivity of ACR4 is that the protein is trafficked via a GNOM-mediated pathway. *GNOM* encodes a BFA-sensitive endosome-localised ARF-GEF; there are many other ARF-GEFs including several closely related to GNOM (Steinmann, *et al.*, 1999), but not all are BFA sensitive (Donaldson and Jackson, 2000). *GNOM* is ubiquitously expressed and mediates auxin transport through correct localisation of the auxin efflux carrier PIN1

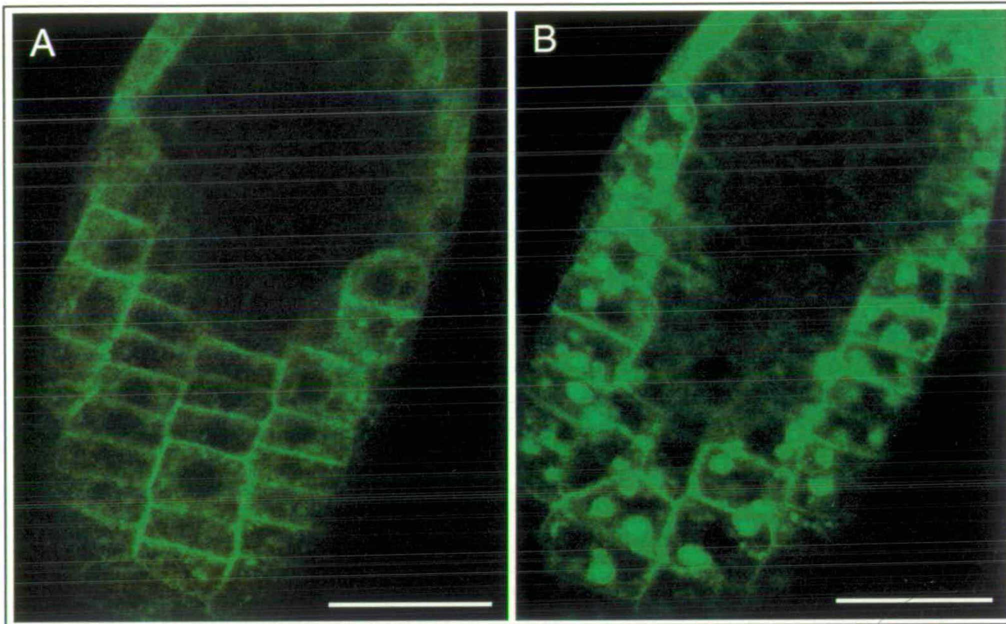


Figure IV.7. The effect of Brefeldin A treatment on ACR4::GFP protein localisation. (A-B) Confocal images of root cells expressing an ACR4::GFP protein fusion (green). (A) Surface view of a root (untreated; incubation for 2hr in DMSO solution). Normal plasma membrane localisation of ACR4 is seen. (B) Surface view of a root treated with Brefeldin A (BFA) in DMSO solution for two hours. The relative plasma membrane fluorescence decreases with respect to that in the cell, with ACR4::GFP instead being localised internally to large endomembrane compartments or perinuclear bodies termed 'BFA compartments'. Scale bars: 25μm. Photos taken by Gwyneth C. Ingram and included with kind permission.

(Geldner, *et al.*, 2003). GNOM is essential for correct PIN1 localisation, mediating shuttling between the basal domain of the plasma membrane and intracellular compartments (Steinmann, *et al.*, 1999; Geldner, *et al.*, 2001). In *gnom* embryos the characteristic polar PIN1 protein localisation of wild-type cells is disorganised. When treated with BFA, PIN1::GFP is localised to BFA bodies. This is consistent with the idea that GNOM regulates vesicle trafficking required for the polar localisation of PIN1. Loss of GNOM activity results in loss of the auxin gradient across organs which is vital for proper organisation and development. In order to determine whether ACR4 trafficking was mediated by GNOM, the localisation of ACR4::GFP protein in *gnom* embryos was analysed.

Plants carrying the ACR4::GFP protein fusion were crossed to a heterozygous *gnom* null mutant line. In the F2 generation pACR4::ACR4::GFP-expressing plants which were segregating *gnom* homozygous seed were selected. The localisation of ACR4::GFP in *gnom* embryos was examined using confocal microscopy. If GNOM was to be required for proper ACR4 trafficking, abnormal ACR4::GFP localisation would be expected. However normal ACR4::GFP plasma membrane localisation was seen as in wild-type embryos; this can not be shown due to a technical difficulty in image capture. Thus ACR4 is trafficked via a BFA-sensitive ARF-GEF vesicular transport system, but not one that is GNOM-mediated.

So, normal plasma membrane localisation of ACR4::GFP is maintained after BFA treatment, but some other aspect of ACR4::GFP localisation is affected. It seems therefore that BFA-sensitive vesicle trafficking is not required for membrane loading *per se*. Alternatively an effect on protein turnover might be occurring. In order to further investigate BFA-sensitive vesicle trafficking of ACR4, lines containing all ΔACR4::GFP and GFP::ACR4 constructs were subject to BFA treatment as above. GFP-tagged ACR4 was seen to be localised to BFA-bodies for all but four constructs in a similar fashion to that described earlier. GFP fluorescence was not seen in BFA bodies in lines carrying ΔTM/K/C-ter ACR4::GFP, Δ39aa ACR4::GFP, nor in the lines which carried N-terminal GFP::ACR4 fusion constructs. Fluorescence from

ACR4::GFP constructs in these lines was seen to remain at the cell plasma membrane (or at the cell wall for ΔTM/K/C-ter ACR4::GFP).

The fact that the cell wall localised ΔTM/K/C-ter ACR4::GFP is not present in BFA bodies but is still present in the extracellular compartment again suggests that normal movement of ACR4::GFP to (and across) the membrane is taking place, but that some sort of processing of ACR4::GFP is inhibited by BFA. This also holds true for the membrane-localised Δ39aa ACR4::GFP and N-ter GFP::ACR4 proteins, which are all still present at the membrane after BFA treatment. The level of membrane fluorescence remained similar throughout BFA treatment for these proteins. Processing could involve endocytosis into the cell from the plasma membrane. As ΔTM/K/C-ter ACR4::GFP is not membrane localised it would have been unlikely for it to have been present in endosomes, and thus this is why it is not seen in BFA bodies. The potential reasons for, and implications resulting from the fact that neither Δ39aa ACR4::GFP nor the N-terminal GFP::ACR4 proteins were present in BFA bodies will be discussed in Section IV.5.

Recent work in both the lab, and elsewhere (reviewed in Samaj, *et al.*, 2004), is starting to decipher the mechanism of BFA inhibition (which was poorly understood). It seems that BFA inhibits trafficking of endocytic vesicles from the plasma membrane into the cell. It does not however inhibit the trafficking of outgoing vesicles, which are likely to contain newly synthesised protein, from the golgi to the plasma membrane (Gwyneth C. Ingram, pers. comm.). Recent work in the lab suggests that ACR4 is present in endosomes within the cell. This work is part of ongoing research to elucidate the potential processing of ACR4 in the cell and will be discussed in Section IV.6.

IV.4.2. ACR4 protein is subject to rapid turnover and may be cleaved in the cell

In order to investigate the ACR4 protein in more detail and to address the possibility that ACR4 could be subject to processing within the cell, protein from lines carrying

all ACR4::GFP variants was extracted and subject to western blotting. Protein was either extracted from inflorescence material as a total cell fraction, or as two fractions: one membrane protein enriched and one cytoplasmic protein enriched fraction (depending on the method used). The presence of some membrane-bound proteins, for example those attached to or trapped in vesicles, cannot be discounted in the cytoplasmic enriched fraction. In order to detect ACR4::GFP proteins on the western blot an anti-GFP polyclonal antibody was used. Table IV.1 lists the predicted molecular weight of each ACR4::GFP derivative or variant used in this study.

Protein	Predicted molecular weight	Protein	Predicted molecular weight
ACR4::GFP	124.2KDa	ΔTM/K/C-ter:: ACR4::GFP	72.6 KDa
GFP::ACR4	129.2 KDa	ΔK/C-ter:: ACR4::GFP	74.9 KDa
GFP::ACR4::GFP	155.2 KDa	K-null:: ACR4::GFP	124.2 KDa
Δ39aa::ACR4::GFP	101.0 KDa	ΔC-ter:: ACR4::GFP	114.2 KDa
ΔTNFR:: ACR4::GFP	117.9 KDa		

Table IV.1. Table to show the predicted molecular weights of all ACR4::GFP protein variants used in functional and localisation analysis.

An initial western blot was carried out on protein extracted from the plants carrying a full length ACR4::GFP C-terminal protein fusion. To act as controls for this western blot, protein was also extracted from plants expressing GFP under the control of the *ACR4* promoter, as well as wild-type Col0 plants. Neither of these two lines contained GFP-tagged ACR4 protein. A method to extract two fractions of protein was used and the extracted protein western blotted. ACR4::GFP was expected to be present as a band at about 125KDa (see Table IV.1). Unexpectedly rather than a single band of this size being seen, two smaller bands with sizes of roughly 77KDa and 55KDa were present on the western blot (Fig.IV.8). These two bands were seen specifically in the line expressing ACR4::GFP, being absent in the two control protein extracts. The bands are present in both membrane- and cytoplasmic-enriched fractions. This result was observed in two independent experiments using independent ACR4::GFP lines, and thus the smaller sized bands

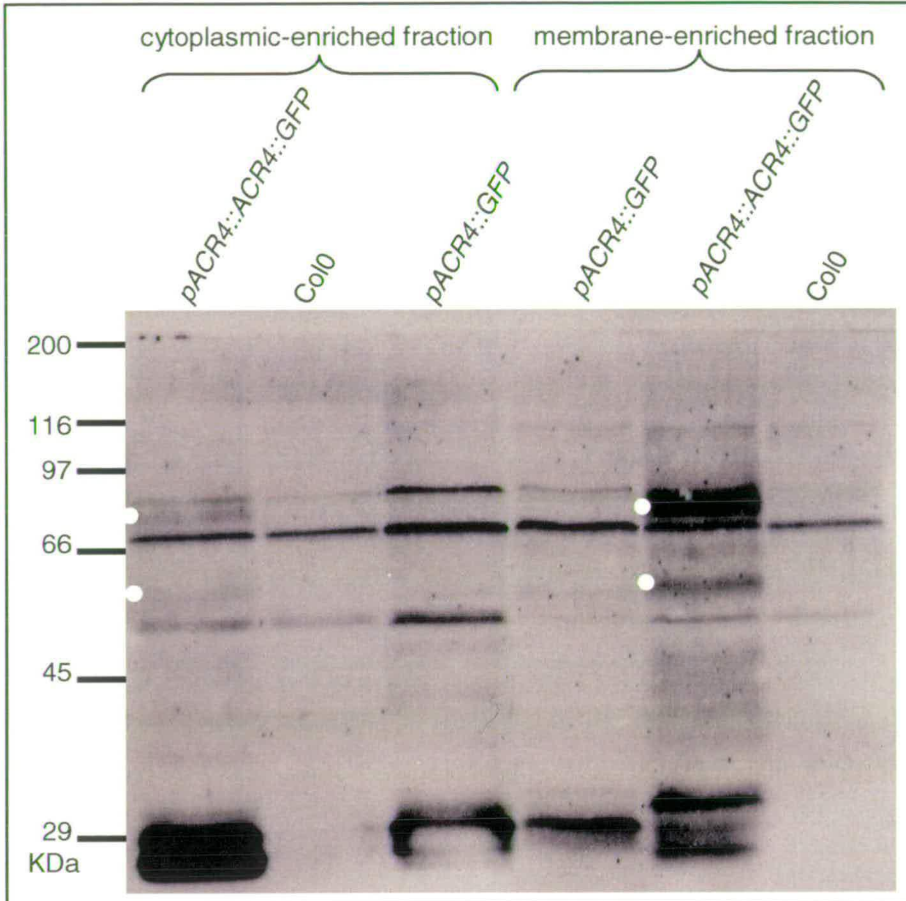


Figure IV.8. Western blot of extracted ACR4::GFP protein using an anti-GFP polyclonal antibody. Protein was extracted from three types of plants: one carrying the ACR4::GFP protein fusion (*pACR4::ACR4::GFP*), one expressing GFP under the control of the ACR4 promoter (*pACR4::GFP*) and also wild-type Col0 plants. Two fractions were obtained using the protein extraction method chosen, one cytoplasmic-enriched and one membrane-enriched. The two fractions contain ACR4 bands of the same size. Rather than a band of 125KDa as expected for full length ACR4::GFP, two smaller bands were seen (marked by white dots). One band ran at ~77KDa and a second ran at ~55KDa. These bands are specific to the ACR4::GFP line. In addition two or three bands of cleaved off GFP are also present at 27-29KDa in both GFP expressing lines.

do not appear to be the result of random ACR4 protein degradation. It is possible therefore that ACR4::GFP is being cleaved at the membrane. The larger fragment corresponds to the size expected were the kinase and C-terminal domain regions to be cleaved internal to the transmembrane domain. The smaller sized band could correspond to cleavage within the kinase domain. Both of these bands on the western blot do not appear to be sharply defined. This can suggests that the protein fragments are phosphorylated or otherwise modified. This is consistent with the hypothesis that they could be cleavage products containing the ACR4 kinase domain, however further experiments would be required to ascertain exactly what protein fragments these bands correspond to. It is also possible that rather than resulting from protein cleavage, the lack of full length ACR4::GFP indicates that the protein is being rapidly turned over in the cell. Rapid turnover is consistent with the results obtained during the BFA experiments discussed above (Section IV.4.1). Several small bands are visible at the size of 27-29KDa on the western blot of ACR4::GFP. This corresponds to GFP which may be cleaved off during processing. Similar sized bands are also visible in large quantities (as would be expected) in the *pACR4::GFP* line.

A second western blot was carried out on protein extracted from plants containing all N-terminal or C-terminal tagged GFP and derivative ACR4::GFP proteins in the Col0 background. This time a method to extract whole cell protein was used and a anti-GFP polyclonal antibody used to detect ACR4::GFP protein fragments (Fig.IV.9). In the lab a third set of protein extractions from the same material was carried out using immuno-precipitation affinity purification. This was carried out with anti-GFP antibody using Protein A-anti GFP-coupled Dynabeads (as manufacturer's instructions: Dynal (UK) Ltd, Merseyside, UK) by Gwyneth C. Ingram. Immuno-precipitation (IP) was used to enrich for ACR4::GFP protein. The same anti-GFP antibody was used to detect ACR4::GFP protein fragments on the western blot. Some of the results from this experiment are included here due to technical difficulties arising in earlier experiments (Fig.IV.9).

The method of protein extraction seems to affect the results seen on the western blot. When protein was extracted using the single fraction or IP single fraction

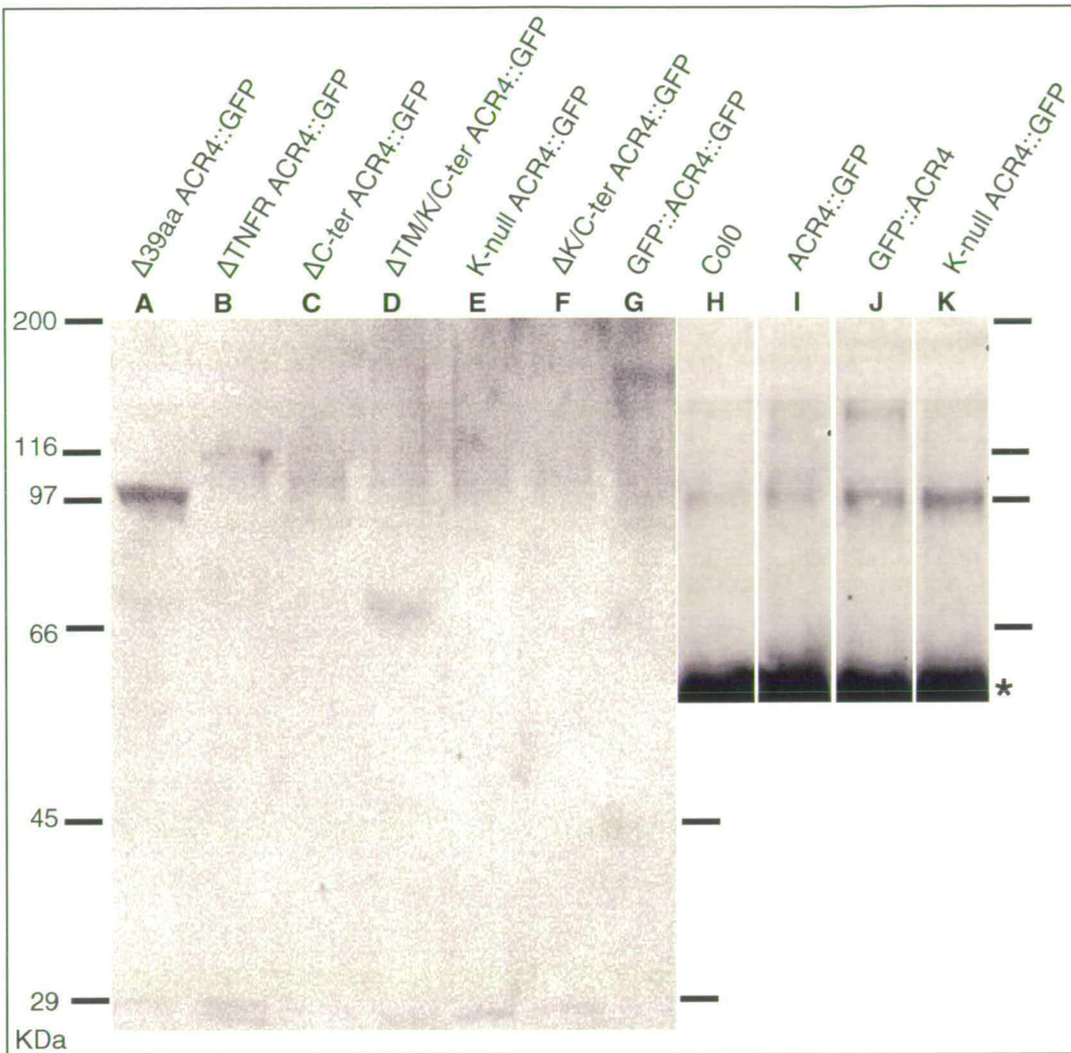


Figure IV.9. Western blot of extracted protein from plants carrying ACR4::GFP, GFP::ACR4 and ΔACR4::GFP proteins using an anti-GFP antibody. (A-G) Western blot of protein extracted as a single fraction as in Chapter II.6.1. (H-K) Western blot carried out by Gwyneth C. Ingram using immuno-precipitation (IP) to enrich for ACR4::GFP protein; the Protein A-anti GFP band is labelled with an asterisk (*). When using either the single fraction or the IP method (rather than the duel-fraction method used for the western blot in Fig.IV.8), the two ACR4 cleavage products could not be detected. (A) In Δ39aa::GFP-expressing plants a single (ACR4-specific) band of ~105KDa is seen. (B) In ΔTNFR::GFP plants a band at ~115 kDa was seen. (C) In ΔC-ter::GFP plants a predicted full length band (~114KDa) could not be detected. (D) In ΔTM/K/C-ter::GFP plants a single band at ~70KDa is visible. (E,K) In K-null::GFP plants a predicted full length band (~125KDa) could not be detected. (F) No ACR4-specific protein bands could be detected in plants carrying ΔK/C-ter::GFP. (G) In protein from plants carrying GFP::ACR4::GFP a single (ACR4-specific) band of ~155KDa was seen. (H) In protein extracted from Col0 plants no ACR4-specific bands could be detected. (I) In ACR4::GFP plants a predicted full length band (~125KDa) could not be detected. (J) In GFP::ACR4 plants a single band at ~130KDa is visible. Two or three bands of cleaved off GFP are also present at 27-29KDa in all lines (not shown for lanes H-K).

method, the smaller sized (potential cleavage product) bands of ~77KDa and ~55KDa could not be detected in the extracts from ACR4::GFP-expressing plants (Fig.IV.9I). It may be that using the single fraction extraction methods that potential cleavage products are not properly extracted. As the smaller bands were not present in protein extracted from plants expressing ACR4::GFP, their absence in other extractions was not informative. As in the first western blot full length ACR4::GFP was impossible to detect (Fig.IV.9I). It seems likely that some full length ACR4::GFP would be present in the cell/at the plasma membrane, even if the protein was cleaved there. However as mentioned for the first western blot, if ACR4::GFP is rapidly turned over in the cell it may be hard to detect.

Unlike for ACR4::GFP, predicted full length protein fragments were observed for the lines carrying Δ39aa ACR4::GFP (Fig.IV.9A), ΔTM/K/C-ter ACR4::GFP (Fig.IV.9D), GFP::ACR4::GFP (Fig.IV.9G), GFP::ACR4 (Fig.IV.9J) and ΔTNFR ACR4::GFP (Fig.IV.9B). Protein bands were detected at roughly 105, 70, 155, 135 and 118 KDa respectively. As in the case of ACR4::GFP, full length protein was impossible to detect for K-null ACR4::GFP (Fig.IV.9K) or ΔC-ter ACR4::GFP (Fig.IV.9C), predicted to be 125 and 114KDa respectively. No ACR4::GFP specific bands were seen in protein extracted from Col0 plants (control) (Fig.IV.9H), or in ΔK/C-ter ACR4::GFP (Fig.IV.9F); as mentioned earlier in Section IV.3.1, the ΔK/C-ter ACR4::GFP results are not informative. Although cleavage products could not be detected for ACR4::GFP or any of the other lines, the fact that predicted full length protein could be detected for Δ39aa ACR4::GFP, ΔTM/K/C-ter ACR4::GFP, GFP::ACR4, GFP::ACR4::GFP and ΔTNFR ACR4::GFP, but not for ACR4::GFP, K-null ACR4::GFP or ΔC-ter ACR4::GFP was interesting. This may suggest that the ACR4 protein in plants carrying the former five proteins has been stabilised and is not turned over or cleaved; this must however be confirmed as part of future work. For extractions where the ACR4::GFP-specific bands were not detectable it might suggest that rapid protein turnover, or possibly protein cleavage is taking place. The differences seen are not merely due to differences in the intensity of ACR4::GFP expression: plants carrying the N-ter GFP-tagged ACR4 proteins showed weak expression in comparison to ΔC-ter ACR4::GFP and K-null ACR4::GFP. The

increased stability tends to coincide with loss of protein function. Neither Δ39aa ACR4::GFP, ΔTM/K/C-ter ACR4::GFP, GFP::ACR4 nor GFP::ACR4::GFP are functional proteins. ACR4::GFP, K-null ACR4::GFP and ΔC-ter ACR4::GFP on the other hand are all functional proteins. The only protein for which this does not hold true is ΔTNFR::GFP. This could suggest that the TNFR domain plays a role that has not been uncovered in the complementation analysis. This matter will be discussed in Section IV.6. The results from plants carrying N-ter GFP::ACR4 proteins are intriguing. These lines were initially made in order to follow the N-terminal end of ACR4 after cleavage, however it was found that addition of an N-ter GFP actually resulted in loss of ACR4 function, and potentially also protein stabilisation.

IV.5. The 39aa repeat domain is required for protein turnover: a ligand binding domain?

The importance of the seven x 39aa repeat domain has been indicated and highlighted by results gained from *acr4* mutant analysis and ACR4 protein functional analysis. This has also been seen at the protein level with western blotting and Brefeldin A drug treatment of ACR4::GFP fusion lines. A summary of the results from functional, localisation and BFA-treatment analysis is presented in Fig.IV.3.

Complementation analysis using Δ39aa ACR4 indicates that the 39aa repeat region of ACR4 is vital for function. This is not due to incorrect protein localisation as Δ39aa ACR4::GFP is localised to the plasma membrane as for full length ACR4::GFP. Therefore the 39aa repeat domain does not appear to be required for localisation of ACR4. Indeed the level of fluorescence for Δ39aa ACR4::GFP suggests that the protein is present in high quantities at the membrane. Δ39aa ACR4::GFP is also visible in what appear to be vesicles in the cell. When roots carrying Δ39aa::GFP line are treated with Brefeldin A, Δ39aa ACR4::GFP is not present in BFA bodies. The mechanism of BFA action is now starting to be understood in plants (reviewed in Samaj, *et al.*, 2004). Recent work in the lab suggests that BFA targets and inhibits the movement of endocytotic vesicles but not outgoing vesicles to the membrane (Gwyneth C. Ingram, pers. comm.). It therefore seems likely that if Δ39aa ACR4::GFP is not present in BFA compartments, then it is not present in endocytotic vesicles within the cell and is therefore not endocytosed. Western blot results shown suggest that turnover and/or cleavage of ACR4 also might be dependent on the presence of the 39aa repeat region.

The same results for Δ39aa ACR4::GFP are obtained when a GFP tag is added to the N-terminal end of ACR4. Even though the protein is membrane-localised it is non-functional. GFP::ACR4 is not found in BFA bodies and the protein does not seem to be turned over. An alternative explanation is that the N-ter GFP::ACR4 proteins are not orientated properly at the plasma membrane. Both *N-ter GFP::ACR4* constructs include two signal peptides. It is possible that after the first

signal peptide has targeted the protein to the extracellular matrix the second signal peptide itself acts as the transmembrane domain. This would mean that the GFP::ACR4 protein would be membrane localised, but only the GFP would be outside the cell – the 39aa repeat domain would be inside the cell. The protein would therefore likely be non-functional. However the amino acid residues adjacent to the second signal peptide are not sufficiently hydrophilic to maintain anchoring of the protein at that point. As mentioned in Chapter I, the juxtamembrane domain of plasma membrane localised proteins is distinct in being rich in highly hydrophilic lysine (K) and arginine (R) residues. This is designated the 'stop-transfer' signal and prevents the protein from passing further into the membrane (Walker, 1994). There is such a K/R-rich region between the ACR4 transmembrane domain and the kinase domain, but not immediately after the signal peptide next to the 39aa repeat region. Therefore it is highly unlikely that the second signal peptide acts as a transmembrane domain and thus that the N-ter ACR4::GFP proteins are incorrectly orientated.

As well as evidence from functional and localisation studies the predicted orientation of ACR4 has been confirmed (Section IV.3.1). The 39aa repeat domain of ACR4 is definitely outside the cell, at a site where it could receive ligands from neighbouring cells. The extracellular region is the classical ligand-binding domain of protein kinases (Walker, 1994; Torii and Clark, 2000). Put together this evidence supports a hypothesis that the seven x 39aa repeat region is the ligand binding domain for the ACR4 receptor-like kinase. When the 39aa repeat region is not part of the ACR4 protein, ligand binding cannot therefore occur. It is likely that addition of a GFP on to the N-terminus of ACR4 structurally inhibits binding by blocking the ligand attachment site. Either way no ligand can be bound to ACR4. The probable consequence of this is that the kinase domain of ACR4 is not activated and that downstream signalling can not occur. The protein is thus rendered non-functional. Results from BFA treatment and the presence of ACR4::GFP in intercellular vesicles suggest that wild-type ACR4 could be endocytosed. Western blot results suggest that ACR4 is turned over in the cell. Due to the fact that there is both lack of localisation to BFA bodies, and potentially increased protein stability of full length

Δ39aa ACR4::GFP, GFP::ACR4 and GFP::ACR4::GFP, it can be postulated that endocytosis and turnover are both directly or indirectly dependent on ligand binding. As discussed in Chapter I, endocytosis of the animal ErbB1 receptor tyrosine kinase and the later degradation steps following internalisation are phosphorylation-dependent (Oved and Yarden, 2002; Soubeyran, *et al.*, 2002). It seems possible therefore that ACR4 is subject to ligand-mediated endocytosis and processing within the cell. As mentioned previously this is a good way to dampen down signalling from receptor kinases in order to avoid a constitutive signal being produced (Waterman and Yarden, 2001). It makes sense then that if ligand binding is not able to occur on Δ39aa ACR4::GFP, there will be no signal produced, and subsequently no damping down. This could be why bright fluorescence is seen in L1 cell membranes of the plants carrying Δ39aa ACR4::GFP: the protein builds up at the membrane and is not processed in the cell. It also explains why such bright BFA bodies are normally seen in full length ACR4::GFP fluorescing cells – ACR4::GFP is being processed in the cell and thus is present in endocytotic vesicles which are incorporated into BFA bodies. Therefore the hypothesis that there is ligand binding (on the 39aa repeat domain) mediated endocytosis fits all of the available data. However, further work will be needed to confirm this prediction. When potential ligand candidate molecules have been identified, an interaction with the 39aa repeat domain could be tested *in vitro* or *in vivo* using a binding assay. For example various assays have been used to investigate ligand binding to the animal ErbB family of receptor tyrosine kinases (such as Zhang, *et al.*, 1997; Lemmon, *et al.*, 1997). Here the isolated TILLING alleles will be of use. The amino acid changes could be modelling in order to investigate how the conformation of the 39aa repeat region might be altered, and therefore which changes might affect ligand binding.

IV.6. The molecular mechanism of ACR4 action: homo-/hetero-oligomers with other ACR4-like proteins?

In order to explain the relatively weak phenotype of *acr4* mutants and to take into account the data gained from functional and protein analysis we have developed a model for ACR4 action. It proposes the formation of hetero- and/or homo-oligomers with one or more of the four other ACR4-like *Arabidopsis* proteins (Fig.IV.10A). Redundancy with such hetero-oligomeric partners is hypothesised to occur and indeed the other *ACR4*-like genes are the subject of detailed investigation in the lab in order to elucidate their relationship to *ACR4*.

The model proposes that a hetero- or homo-oligomer is formed at the plasma membrane between ACR4 and an ACR4-like protein. As mentioned in Chapter I, multimerism is a common mechanism for plant and animal receptor-like kinases (Olayioye, *et al.*, 2000). Ligand binding is proposed to occur on the extracellular 39aa repeat domains of the oligomer, with proper conformation and presence of the ACR4 39aa repeat domain vital for binding. When the 39aa repeat domain is missing it is likely that the ligand cannot bind the hetero- or homo-oligomer; this is represented in Fig.IV.10B.

After normal ligand binding, auto- and/or trans-phosphorylation between the kinase domains of the oligomer partners is proposed (as for other RLKs as discussed in Chapter I, and reviewed in Walker, 1994). This phosphorylation would act as the first step in a downstream signalling cascade. A lack of ACR4 kinase activity could be compensated for through trans-phosphorylation of ACR4 by the active kinase domain of a hetero-oligomeric partner. As discussed in Chapter I, research into another *Arabidopsis* receptor-like kinase, CLAVATA1 (CLV1), has found such an effect (Trotochaud, *et al.*, 1999). The *clv1-6* allele of the *CLV1* gene is effectively kinase null, yet the CLV1-6 protein encoded retains some of its function. As introduced in Chapter I this is because the kinase activity can be provided by another protein kinase (Torii and Clark, 2000). A similar explanation could be used to understand the behaviour of the kinase null variant of ACR4. The model is therefore consistent with the functional analysis of the kinase null ACR4 protein; this aspect of

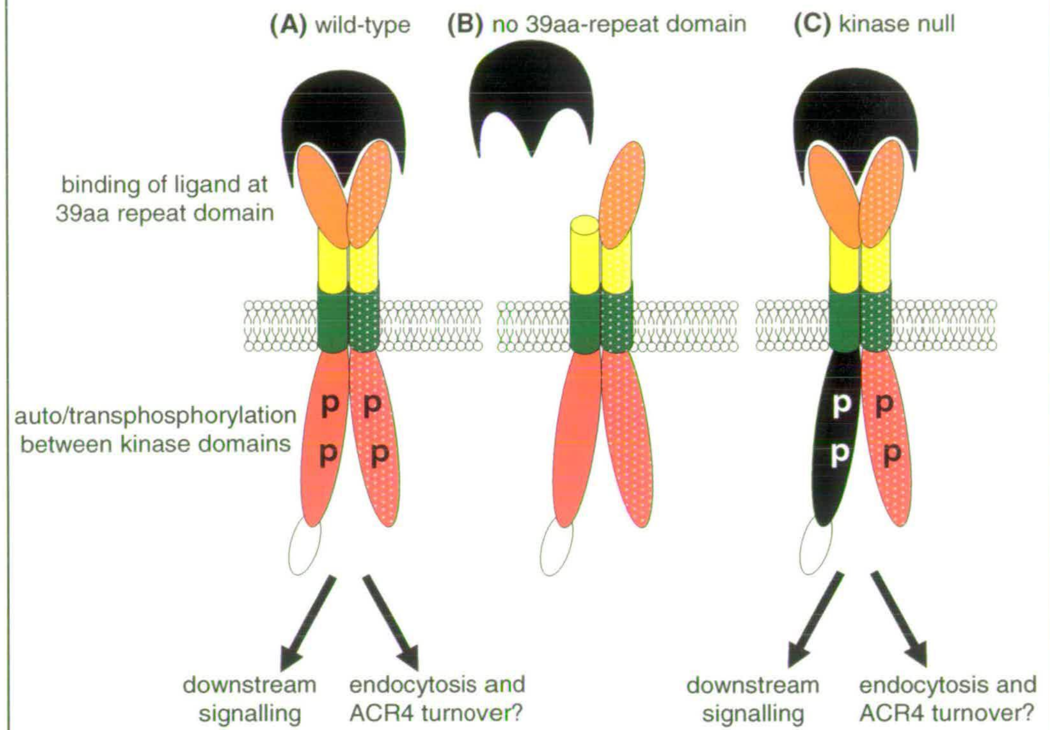


Figure IV.10. Schematic illustrating the predicted molecular mechanism of ACR4 action; protein domain labelling as Fig.IV.3. (A) Wild-type protein. It is hypothesised that ACR4 (on the left) forms homo-oligomers or hetero-oligomers with one or more of the other CR4-like proteins in *Arabidopsis*. The ACR4/ACR4-like homo/hetero-oligomer receives a ligand at the predicted ligand-binding domain (39aa repeat region). This results in kinase activity and downstream signalling via phosphorylation. Western blot and BFA-treatment results suggest that ACR4 is endocytosed and turned over, either to allow proper signalling and function, or as a result of it. (B and C) The model is consistent with results obtained from functional analysis: (B) When the 39aa region is removed from ACR4, this is likely to alter the ligand-binding site conformation. As no ligand can bind the complex there is no downstream signalling (ACR4 is not functional) and the protein is not endocytosed or turned over. (C) A kinase null ACR4 variant can still bind the ligand. An active kinase domain (of an ACR4 homo-/hetero-oligomer partner) could then transactivate the inactive ACR4 kinase domain. Normal function and turnover are thereby maintained.

the model is represented in Fig.IV.10C. An alternative explanation of the behaviour of the kinase null ACR4 protein is that the kinase domain is simply not required for function. However by analogy to other plant and animal RLKs discussed earlier this seems unlikely. The importance of the kinase domain could be confirmed by repeating construction and testing of the ΔK/C-ter construct. At present the most likely hypothesis is that presence of a kinase domain is required for ACR4 function, but that loss of kinase activity can be compensated for. Two of the four *ACR4*-like genes have predicted kinase activity and thus are the best candidates for formation of ACR4/ACR4-like oligomers. The results obtained here differ to those published by Watanabe, *et al.* (2004). The authors found that introduction of a kinase null ACR4 variant was unable to complement the *acr4* mutant. In addition they found that introduction of an ACR4 derivative missing the C-terminal region was unable to complement *acr4*. These results however are unreliable, firstly because the *35s* rather than the endogenous *ACR4* promoter was used to express the deletion constructs. The *35s* promoter is not very efficient in developing seeds and thus this may have led to poor expression of the constructs introduced. Secondly the localisation of these proteins was not determined. Although a full length ACR4 protein expressed under the same conditions was able to complement *acr4* it may be that the ACR4 derivatives were not correctly localised. An ACR4 derivative lacking the whole extracellular domain (including the 39aa repeat region) was unable to complement as found here, but again the membrane-localisation of this protein was not confirmed.

It seems likely that as part of ACR4 function, the ACR4 protein is endocytosed and turned over in the cell. Turnover/cleavage of a receptor-like kinase is thought to be a biologically sound method of damping down kinase activity and maintaining sensitivity to the presence of a ligand (Waterman and Yarden, 2001). This ensures that downstream signalling is not switched on constitutively, rather only when the appropriate signalling molecule is present. This is the first example of RLK endocytosis in response to ligand binding to be shown *in planta* in *Arabidopsis* (as opposed to the apparent endocytosis of AtSERK shown in cow-pea protoplasts by Shah, *et al.* (2002). Whether this endocytosis is mediated by the KAPP protein as

seems to be the case for plant RLKs such as CLV1 and AtSERK as discussed in Chapter I (Braun, *et al.*, 1997; Stone, *et al.*, 1998; Shah, *et al.*, 2002) is not known. As mentioned, the maize CR4 protein has not been shown to associate with KAPP (Braun, *et al.*, 1997). In the lab work is being carried out by Gwyneth C. Ingram in order to determine whether ACR4 associates with KAPP. If it is found not to, then ACR4 endocytosis may define a novel endocytic mechanism. Whether ACR4 activation by ligand binding and phosphorylation results in protein turnover/cleavage (as is the case for the animal RTK ErbB1, Waterman and Yarden, 2001), or turnover/cleavage itself is required for activation (as demonstrated for proteins such as NOTCH, Fortini, 2001; Schweisguth, 2004), is yet to be determined. It is also not clear through which protein domains the two oligomeric partners might interact. It is possible that ligand binding is necessary for stabilisation of a hetero-oligomer. For example the mammalian EGFR receptor undergoes ligand-dependent dimerisation (Schlessinger, 2002).

As mentioned in Section IV.4.2, deletion of the TNFR-like repeat domain of ACR4 seems to result in the protein being less susceptible to the turnover/processing seen with ACR4::GFP; this result however must be confirmed. It is possible that this domain is involved somehow in oligomer-stabilisation and perhaps correct orientation of ACR4 for phosphorylation. Removal of the TNFR-like repeat domain could potentially cause a conformational change in the ACR4 protein which results in inefficient phosphorylation or processing. That could either result in ACR4 not being efficiently removed from the membrane, or perhaps being less susceptible to degradation and cleavage as part of protein turnover once removed. Alternatively the ΔTNFR ACR4::GFP protein might bind ligand less efficiently. However as the presence of the TNFR-like repeat domain is not required for *acr4* mutant complementation, it seems that the domain is not strictly necessary for function. Thus ΔTNFR ACR4::GFP is still found in BFA bodies and is likely only partially stabilised, suggesting that some function is maintained. If the function of the oligomer partner for ACR4 were to be disrupted in some way, and the TNFR-like domain has such a function as suggested, it is unlikely that ΔTNFR::GFP would be able to compensate for such defects and thus might not complement the *acr4/acr4-*

like gene double mutant phenotype. Through investigation of the other *ACR4*-like genes in the lab this prediction will be tested. As mentioned in Chapter I, the TNFR-like repeat region consists of three cysteine rich repeats. In mammals the second two repeats are thought to act as ligand binding domains whilst the first is proposed to be involved in trimerisation of protein molecules (Chan, 2000). It is possible then that the TNFR-like repeat region of ACR4 plays some minor role in one or both of these processes; it might be that tri-oligomers of ACR4 and ACR4-like proteins are formed. Whatever the exact function of the TNFR-like repeat region is, it seems likely that it does play some part in regulating the molecular mechanism of ACR4 action. It would be interesting to generate more TILLING alleles throughout the TNFR-like repeat region in order to determine the effect of such changes on the stability of the ACR4 protein.

A mode of action for ACR4 involving hetero- as well as homo-oligomers can also help to explain why no dominant positive or negative effects were observed during expression of ACR4 protein variants. For example a dominant negative effect could have been postulated when only the N-terminal end of the protein was expressed (ΔTM/K/C-ter ACR4::GFP). As discussed in Chapter I such effects have been seen with other plant RLKs (Shpak, *et al.*, 2003). It is possible that ligand binding at the 39aa repeat domain might have occurred but this would not have activated proper kinase activity and signalling (due to no kinase domain being present). This would have resulted in a dominant negative effect as the ligand would have been sequestered by the protein derivative. However no such effect was observed. In the proposed homo-/hetero-oligomer it is likely that both the presence as well as proper conformation and orientation (ACR4 being membrane bound) of the 39aa repeat domain of ACR4, and of the extracellular regions of the partner protein, are required for ligand binding. Therefore the ACR4 extracellular domain on its own, as in ΔTM/K/C-ter ACR4::GFP, would not be able to bind a ligand. In order to investigate the behaviour of a membrane bound version of ΔTM/K/C-ter ACR4::GFP, a line that carried an appropriate construct was made in the lab. However again no effect was observed (Gwyneth C. Ingram, pers. comm.). This

might suggest that proper interactions between ACR4 and an ACR4 oligomeric partner, at the cytoplasmic domain, are also necessary for ligand binding.

It was also initially hypothesised that expression of a protein lacking the extracellular regions (Δ39aa/TNFR::ACR4) might result in a dominant positive effect by comparison to previously mentioned studies in mammal cells. Removal of the extracellular region of the SEVENLESS receptor tyrosine kinase, which functions in photoreceptor cell fate in the *Drosophila* eye results in ligand-independent receptor dimerisation (Basler, *et al.*, 1991). Induction of a dominant positive effect could allow the consequences of ACR4 activity in places where it is not normally switched on by ligand binding to be determined. This would aid further elucidation of *ACR4* function. However no alteration of phenotype which might occur as a consequence was seen in this experiment. As with the potentially dominant negative protein, a dominant positive effect with this approach might not occur due to the unique nature of hetero-oligomers. One alternative explanation is that the deletion protein was either not properly translated or correctly localised. The localisation of this protein was not determined and so this question cannot be answered here. Another alternative explanation is that as the phenotypic effect of gain of ACR4 function or a dominant negative effect is not known, the defects that are associated with these are present but have not been found. This could be addressed as part of further work.

As mentioned in Section IV.2, ACR4::GFP fluorescence is localised preferentially to the cell inner and anticlinal membranes. This could be due to the additive effects of two appressed membranes. However there could in fact be a functional basis for such preferential localisation. If ACR4 is involved in receiving a signal from a neighbouring cell, the consequence being to ensure proper organisation of the L1 cell layer, then such signals are likely to be received at cell-cell boundaries. There might therefore be a greater amount of ACR4 protein at such sites. As mentioned in Chapter III a signal from inside the organ could provide positional information. Put together this would mean that ACR4 would be preferentially localised to L1

membranes adjacent to underlying cells and to neighbouring L1 cells. This is exactly what is observed.

In order to elucidate a potential pathway involving cleavage of ACR4, there are some other clues from maize genes as discussed in Chapter I. Both *dek1* and *sal1* mutants in maize have a very similar phenotype to *cr4* when their function is knocked out (Lid, *et al.*, 2002; Shen, *et al.*, 2003). It is thought that *DEK1* and *SAL1* may therefore be involved in the same regulatory pathway as *CR4*. *DEK1* encodes a cysteine protease and *SAL1* encodes a vacuolar sorting protein. *DEK1*- and *SAL1*-like genes are present in the *Arabidopsis* genome. It is possible that they interact with *ACR4* and are potentially involved in the cleavage and processing of the ACR4 protein due to their predicted functions. In the lab the function of both *AtDEK* and *AtSAL1/2* are being elucidated and any relationship to *ACR4* investigated.

Therefore in summary ACR4 protein is localised to the plasma membranes of L1/outside cells in such a fashion that cell-cell signalling is possible. Formation of homo-/hetero-oligomers between ACR4 and ACR4-like proteins at the membrane is proposed as a molecular model of ACR4 action. A ligand is postulated to be bound at the 39aa repeat domain of the ACR4 protein homo-/hetero-oligomer with the result of transphosphorylation between oligomer kinase domains, and then downstream signalling. This ligand binding could mediate endocytosis of the protein and turnover within the cell.

Chapter V. *ACR4* genetic analysis: elaboration of the signalling network

V.1. Introduction

V.2. *ACR4* does not interact with genes involved in ovule morphogenesis

V.3. How does *ACR4* fit into the wider signalling network?

V.4. New γ-mutagenesis-generated mutants are candidate enhancers of the *acr4* mutant phenotype

V.5. Other aspects of the *ACR4* signalling pathway

V.1. Introduction

The *ACR4* gene plays a role in the maintenance of Ll/outside cell layer integrity. In Chapter III the expression pattern and function of the gene was investigated. In Chapter IV it was shown that ACR4 protein localises to the plasma membrane of Ll/outside cells. The protein is positioned and orientated in such a way that it could receive signals which are located in the extracellular matrix. These signals could be secreted from adjacent cells, therefore allowing cell-cell signalling in the outside cell layer. ACR4 localisation is dynamic, the protein is endocytosed and cleaved in the cell. This intercellular processing seems to be mediated by ligand binding on the 39aa repeat domain of the protein. In addition it is likely that ACR4 forms homo- and/or hetero- oligomers with other ACR4-like proteins. After a ligand signal is received, trans-/auto-phosphorylation could occur between the kinase domains of the protein complex. Phosphorylation of downstream targets in a signalling cascade is then probable. This signalling may ensure that the Ll/outside layer divides and develops as a monolayer and that proper organisation is maintained. Precisely what the ACR4 ligand(s) is/are, and what proteins act as downstream signalling targets has yet to be determined.

In order to address these two points a range of genetic approaches have been taken. One approach taken was to identify genes that might be hypothesised to play a role with *ACR4* in controlling L1 cell layer integrity in aerial organs. The *acr4-2* homozygous mutant was then crossed to plants which carried mutations in the genes of interest. Double mutants were isolated and the phenotype examined for any enhancement or alteration from the single mutants. This analysis was used to determine whether the genes could be in the same genetic pathway.

The genes chosen can be divided into two groups. Firstly various genes involved in regulating ovule morphogenesis were selected (Section V.2). Although *acr4* mutants have defects in ovule integument outgrowth, *ACR4* does not seem to play a role specific to ovule development. This is evidenced both by the more widespread expression pattern of the gene and by the fact that defects are not specific to ovules (the sepal marginal cells are also disorganised). However it was still important to

analyse the relationship between *ACR4* and such genes in order to confirm this hypothesis. In addition, it might be that some poorly characterised mutants which have defects in ovule development are in fact additional components in the *ACR4* signalling pathway. The second group of genes analysed were chosen with reference to a previous yeast-two-hybrid screen carried out in the lab by Samuel Dean and Gwyneth C. Ingram. Two proteins involved in the response to the hormone gibberellic acid were identified as potential interactors at the C-terminal end of ACR4. Genes involved in gibberellic acid signalling were therefore chosen as candidate genes (Section V.3). As well as investigating an interaction with the gibberellic acid signalling pathway, the cytokinin hormone receptor was of interest. Cytokinins regulate cell division patterns as a whole (Hutchison and Kieber, 2002) and thus it was postulated that there could be a link to *ACR4* which regulates cell layer organisation (also Section V.3).

Another approach taken was to carry out an enhancer screen on a mutagenised population of *acr4-2* homozygous mutant seed (Section V.4). The mutagenesis screen was designed to identify genes which might act in parallel to *ACR4*. Any enhancement of the seed abortion phenotype of *acr4-2* in new mutants was analysed. In addition, mutants with potential defects which might suggest improper cell layer organisation were of interest. These included seedlings with apparent disorganisation of cells in the developing organs. The dependency of new mutations on the *acr4-2* background was tested in order to identify specific interactors.

Finally the hypothetical model describing the role of *ACR4* in L1 cell layer signalling in aerial organs was investigated in more detail using information gained from published research in the literature (Section V.5). An experiment was recapitulated that generated a mutant plant in which signalling from the L2 to the L1 cell layer was postulated to have been disrupted. The effect of loss of L2 to L1 signalling on the *acr4* mutant which seems to have a loss of L1-L1 signalling was analysed. In addition other possible *ACR4* interactors are hypothesised and discussed.

Together through these three approaches the aim was to elucidate where *ACR4* fitted into the wider signalling network.

V.2. *ACR4* does not interact with genes involved in ovule morphogenesis

In order to investigate potential interactions with other genes involved in ovule morphogenesis, crosses to a number of known ovule development mutants were carried out. These included crosses to plants containing mutations in the following genes. *BELL1* (*BEL1*) (Robinson-Beers, *et al.*, 1992, Reiser, *et al.*, 1995), *SHORT INTEGUMENTS 1* (*SIN1*) (Robinson-Beers, *et al.*, 1992; Ray, *et al.*, 1996), *INNER NO OUTER* (*INO*) (Villanueva, *et al.*, 1999; Meister, *et al.*, 2004), and *AINTEGUMENTA* (*ANT*) (Klucher, *et al.*, 1996; Krizek, 2003) encode proteins which are required for various different aspects of proper ovule integument morphogenesis. *bel1*, *sin1*, *ino* and *ant* all exhibit sporophytic female sterility as the result of aberrant ovule development. The *ABBERRANT TESTA SHAPE* (*ATS*) (Leon-Kloosterziel, *et al.*, 1994) gene was also of interest due to its role in patterning the ovule. The *ats* mutant was therefore used in this study. Some of these genes have additional roles during floral development, however their specific role in ovule development is the focus here. The phenotype in ovules of each of these mutants, as well as the wild-type role of each gene affected will be discussed. See Figure III.3 for a schematic of a wild-type ovule.

The *BEL1* gene encodes a homeodomain transcription factor (Reiser, *et al.*, 1995). Wild-type *BEL1* gene function is required for morphogenesis of the ovule integuments and is responsible for early development of the central or chalazal region of the ovule (Robinson-Beers, *et al.*, 1992; Reiser, *et al.*, 1995). *BEL1* also plays a separate role in regulating normal embryo sac development (Western and Haughn, 1999). In *bel1* homozygous mutant ovules rather than two integuments being initiated, a single integument-like structure forms. This structure develops aberrantly into a collar-like outgrowth. This gives *bel1* mutant ovules a bell-shaped appearance; the nucellus is the 'clapper' of the bell. In addition a normal embryo sac is not formed. *bel1-1* and *bel1-3* are two alleles which have similar phenotypes, the former being a stronger allele.

SIN1 encodes a gene with similarity to the *Drosophila melanogaster* gene *DICER* (Golden, *et al.*, 2002); *DICER* encodes a multidomain ribonuclease which is involved in RNA silencing (Schauer, *et al.*, 2002). *SIN1* plays a wide range of roles including in maternal sporophytic control of embryo pattern formation, meristem fate determination, flowering time control and in during ovule morphogenesis (Robinson-Beers, *et al.*, 1992; Ray, *et al.*, 1996). The role of *SIN1* during ovule morphogenesis in particular has been well characterised. *sin1-1* mutant ovules initiate two integuments, but the outgrowth of these integuments is disrupted. Normal cell division occurs in the integuments but there is a lack of cell elongation. Due to this retarded growth *sin1* mutant ovules are overall rounder and also narrower at the micropylar end than ovules of wild-type. Ovules of *sin1* mutants like those of *bel1* mutants also fail to form an embryo sac.

The *INO* gene encodes a YABBY family transcription factor which is a positive regulator of ovule integument growth and is required for abaxial-adaxial patterning of the ovule (Villanueva, *et al.*, 1999; Meister, *et al.*, 2004). *INO* plays a role in the regulation of the formation and asymmetry of the outer integument. It is expressed specifically in cells on one side of the ovule primordium which give rise to the outer integument. Ovules homozygous for the *ino-1* allele initiate and develop a normal inner integument, however the outer integument fails to initiate on the abaxial side of the ovule. Instead a protuberance is initiated on the adaxial side which undergoes only limited development.

The *ANT* gene encodes a member of a plant specific family of transcription factors (Krizek, 2003). *ANT* is required for proper formation of the ovule integuments and also for regulation of floral organ number (Klucher, *et al.*, 1996). Strong *ant* allele mutant ovules fail to form either integuments or a female gametophyte. Weaker *ant* mutant ovules develop normal inner and outer integuments but fail to form a functional female gametophyte. *ANT* has overlapping roles with the *HUELLENLOS* gene which regulates the initiation and maintenance of integument and embryo sac development (Schneitz, *et al.*, 1998). The strong *ant-1* allele was obtained for use here.

ATS is a gene which plays a role in proximal-distal patterning of ovule development (Leon-Kloosterziel, *et al.*, 1994). Unlike the above mutants the *ats* mutant can form viable seed. The seeds however are heart-shaped rather than oval due to abnormal integument morphogenesis. The inner and outer integuments are not clearly defined in *ats* mutants. This is due to deviant cell divisions and abnormal cell expansion during integument initiation and outgrowth. The *ats* ovule embryo sac is enclosed by three rather than five integument cell layers. It is thought that this is either due to initiation of only one integument or fusion between the two integuments. *ATS* encodes a gene which has not been characterised, its precise function is unknown. As *ats* mutant seeds, like those of *acr4* are of an unusual shape due to problems in integument development, it was of particular interest as a possible player in the *ACR4* pathway.

The genes discussed above have distinct but also interlinked roles during ovule and floral development. For example the zone of *INO* expression is expanded in *bell* and *ant* mutants. It is thought therefore that *BEL1* and *ANT* might be involved in down-regulating *INO* in order to maintain its abaxial-specific expression pattern in wild-type ovules. (Villanueva, *et al.*, 1999). In addition the *bell* phenotype is dependent on function of the floral meristem identity gene *AGAMOUS* (*AG*). *BEL1* acts in a partially redundant manner with *AG* to specify proper ovule identity (Western and Haughn, 1999).

If *ACR4* was specifically involved in regulating ovule development then it might interact genetically with one or more of the ovule developmental genes discussed above. In order to test whether there were any genetic interactions a double mutant analysis was carried out. The phenotype of double mutants was analysed to see if there was any exacerbation or alteration of the defects seen in single mutants, which might be associated with such an effect.

Crosses of *bell-1*, *bell-3*, *sinl-1*, *ino-1* and *ant-1* mutants to the *acr4-2* homozygous mutant were made in both directions for at least three heterozygous

individuals. The heterozygosity of the ovule mutant individuals was confirmed by checking for segregation of female sterile mutants in the progeny of the plants crossed. The *acr4-2* homozygous phenotype is selectable for by screening siliques of plants for seed abortion and round-shaped seeds (as discussed in Chapter III, Section 2.2). The ovule mutant homozygous phenotype is selectable for by screening for individuals with female sterility: no seeds are formed and thus siliques are very short. In order to isolate double mutants there were two stages of screening. Firstly plants homozygous for the *acr4-2* mutation were selected by means of their seed phenotype in the F2 generation. Segregation ratios were monitored: 4/16 plants were expected to be *acr4-2* homozygotes in the F2. If *ACR4* and the ovule gene under investigation were unlinked ¼ of these (i.e. 1/16 of the total) would have been double mutants. However these double mutants would have been female sterile and would thus have looked similar to the single ovule mutants (3/16 of the total). In order to specifically select double mutants, all F2 individuals homozygous for *acr4-2* were sown out. The resultant F3 generation lines were screened for those segregating female sterile individuals. Such individuals were therefore definitely double mutants. For the *ino-1* cross a different approach was taken. All F2 individuals with female sterility (as a result of *ino-1* homozygosity) were genotyped by PCR in order to identify *acr4-2* homozygotes and thus double mutants.

In the crosses to and from the *ant-1* lines, no *ant-1* homozygotes could be isolated. When the *ant-1* line individuals crossed were resown it was found that none were heterozygous for the *ant-1* mutation. There seemed to be a problem with the *ant-1* line used here and thus results from this cross could not be obtained and are not included.

As well as selecting for homozygous *acr4-2* mutant plants in the F2 generation then double mutants in the F3 generation, the phenotype of all plants was examined. The *bell-1*, *bell-3*, *sin1-1*, *ino-1* and *ats* mutations were all in the Landsberg erecta (L *er*) background while the *acr4-2* mutation was in the Col0 background. Because the *erecta* mutation was therefore segregating in the background of the F2 plants it was important to check for any modifier effects of the ovule phenotypes. However no differences were seen between plants.

The numbers of plants showing these phenotypes were recorded for both the F2 and (where screened) the F3 generations in several families for each cross carried out. The results from one representative F2 family for each mutant cross are shown in Table V.1.

Several crosses between the *ats-1* homozygous mutant and the *acr4-2* homozygous mutant were made in both directions. In the F2 generation double mutants were selected as those carrying both heart shaped seeds (indicating homozygosity for the *ats* mutation) and variable levels of seed abortion (indicating homozygosity for the *acr4-2* mutation). Again the numbers of plants of each possible phenotype were noted (Table V.1) and the double mutants analysed.

Cross performed	No plants	wild-type -like	single *acr4-2* mutant	single ovule dev. mutant	Double mutant
acr4-2 x *bell-1*					
F2 (2)	15	11	2	2*	*
F3 (14•)	16		13		3
acr4-2 x *sin-1-1*					
F2 (1)	13	11	1	1*	*
F3 (2•)	18		13		5
acr4-2 x *ino-1*					
F2 (2)	22	15	3	3	1
ats-1 x *acr4-2*					
F2 (2)	23	13	5	4	1

Table V.1. Phenotypic analysis of the F2 and F3 plants generated from crosses between *bell-1*, *sin1-1*, *ino-1* and *ats-1* with *acr4-2*. The number of plants of each phenotype are listed for each F2 or F3 family number (in brackets). *plants of these two genotypes would have looked indistinguishable. •Each of these F3 families originated from one F2 plant that was homozygous for the *acr4-2* mutation.

The segregation ratios of plants in each family were analysed. If the two genes involved in each cross were unlinked there would predicted to be 9/16 plants with a wild-type phenotype, 3/16 plants with a homozygous ovule mutant phenotype, 3/16 with a homozygous *acr4-2* phenotype and one double mutant. Genetic linkage was not expected to cause a problem as all of the ovule development genes reside on different chromosomes to *ACR4* (which is on chromosome 3): *BEL1* and *ATS* are on

chromosome 5 and *SIN1* and *INO1* are on chromosome 1. The ratios for each phenotype seen were in accordance with no linkage (see Table V.1).

The phenotype of *bel1-1*, *sin1-1* and *ino-1* single mutants was compared with that of the double mutants for each cross. For each comparison ovules at stage 13 of development (developmental stages as Robinson-Beers, *et al.*, 1992) just prior to when fertilisation would occur were dissected from floral buds. The ovules were cleared in chloral hydrate, mounted and viewed using DIC microscopy. The seeds of the *ats-1* single mutant and the *ats-1 acr4-2* double mutant were compared by viewing opened siliques of each plant. For the double mutants generated in each of these crosses an additive phenotype was seen and no specific exacerbation of single mutant defects was visible.

acr4-2 sin1-1 double mutant ovules were similar to those of *sin1-1* (Fig.V.1A-F). Integument outgrowth was retarded in ovules of both the single and double mutants. It was particularly evident that the outer integument had not fully elongated. This meant that the inner integument and the nucellus were visible in cleared ovules. This aspect of the *sin1-1* phenotype was identical in ovules of the *acr4-2 sin1-1* double mutant. However, ovule integuments of the double mutant ovules were more disorganised (Fig.V.1B,D,F). There were outgrowths on the surface of the outer integument, consistent with the defects seen in *acr4-2* single mutants (as Chapter III, Figs.III.4,5).

acr4-2 bel1-1 double mutant ovules were indistinguishable from those in the *bel1-1* single mutant line (Fig.V.1G-H). Both the single and double mutant ovules had 'bell' shaped ovules, with a nucellus and an integument-like structure. Unlike on the *acr4-2 sin1-1* double mutant ovules, no clear additional bulges were seen on the *acr4-2 bel1-1* double mutant ovules (Fig.V.1H). However the presence of such bulges occur only variably on *acr4-2* mutant ovules. The bulges might only be noticeable on integuments that are more fully developed than those of *bel1* mutant ovules. *acr4-2 bel1-3* double mutants ovules in comparison with *bel1-3* single mutants ovules at first glance also appeared to be indistinguishable, although they were not analysed in detail.

Figure V.1. Phenotype of double mutants of *acr4-2* with sin*1-1* (A-F), *bel1-1* (G-H) and *ino-1* (I-K). Light microscopy images of ovules in (A,C,E,G,I) single mutants and (B,D,F,H,J,K) double mutants. All ovules were dissected from carpels at floral stage 13. (A-F) *acr4-2 sin1-1* double mutants exhibit an additive phenotype. In *sin1-1* mutants (A,C,E) the outer integument (oi) in particular shows a lack of complete outgrowth. This results in the micropylar end of the ovule (arrowhead in E) being narrower than the chalazal end (star in E). *acr4-2 sin1-1* double mutant ovules (B,D,F) look very similar to those of *sin1-1*. The integuments in the double mutant are however less organised than those of *sin1-1* single mutants. This disorganisation is evident as apparent bulges on the ovule surface (arrows in B, D and F). In (B) the nucellus (nuc) is visible due to reduced inner integument growth. (G,H) Single *bel1-1* (G) and double *acr4-2 bel1-1* (H) mutant ovules are indistinguishable. Both show the characteristic 'bell' shape due to improper integument development. A single integument-like structure (ils) is seen in place of two integuments. The nucellus (nuc) is clearly visible. (I,J,K) Single *ino-1* and double *acr4-2 ino-1* mutant ovules are almost indistinguishable. Both *ino-1* (I) and double *acr4-2 ino-1* (J,K) ovules are characteristically narrow due to lack of the outer integument. The nucellus (nuc) and inner integument (ii) form fairly normally. In *acr4-2 ino-1* double mutants the inner integument of the ovule is however slightly less organised, as evidenced by the appearance of bulges on the inner integument tissue (arrow in J). Scale bars: 50μm.

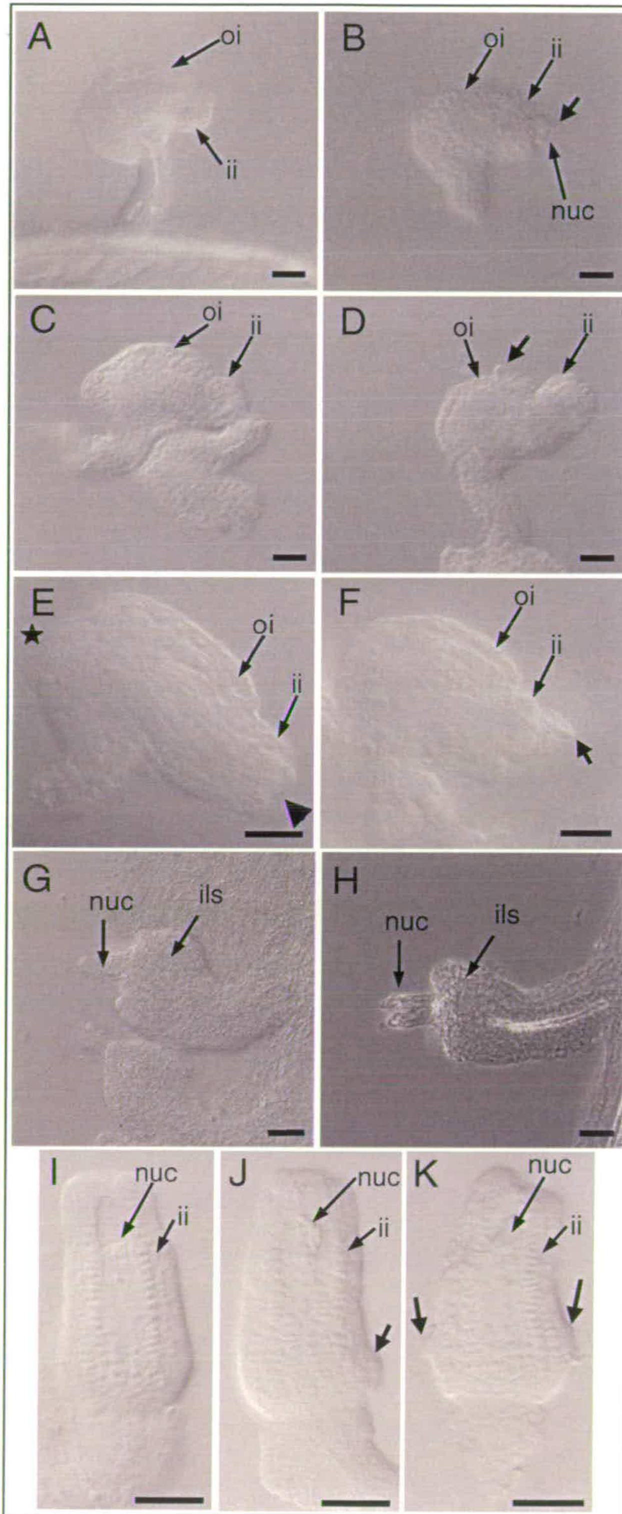


Figure V.1. Phenotype of double mutants of *acr4-2* with sin*1-1* (A-F), *bel1-1* (G-H) and *ino-1* (I-K).

No exacerbation of the *ino-1* phenotype was seen in *acr4-2 ino-1* double mutant ovules in comparison to ovules in the *ino-1* single mutant line (Fig.V.1I-K). Ovules in both the double and single mutant plants were similar. Both *ino-1* (Fig.V.1I) and double *acr4-2 ino-1* (Fig V.1J,K) ovules consisted of a nucellus and an inner integument, but no outer integument. This gave them a characteristically narrow appearance in comparison to wild-type ovules. No outer-integument-like protuberance was obvious in homozygous ovules of this strong *ino-1* allele line. Although similar, the inner integument of *acr4-2 ino-1* double mutant ovules was more disorganised than that of the *ino-1* single mutant ovule inner integument. There were outgrowths on the inner integument surface, similar to those seen in *acr4-2* single mutants.

In siliques of the *acr4-2 ats-1* double mutant an additive phenotype was seen (Fig.V.2A). All mature seeds in the double mutant were heart-shaped, as in seen in the *ats-1* single mutant silique. The seeds were slightly smaller in the siliques of *acr4-2 ats-1* double mutants, consistent with the smaller size of seeds in *acr4-2*. *acr4-2 ats-1* double mutant siliques are also shorter than those of *ats-1* single mutants and there was a variable degree of seed abortion. No new defects which do not normally occur as a result of loss of each single gene were seen.

In conclusion no exacerbation of the phenotype in a range of mutants defective in aspects of ovule development was found when *ACR4* function was lost. The defects seen in double mutants were additive. This shows that *ACR4* does not interact specifically with either *BEL1*, *SIN1*, *INO* or *ATS*. It suggests that as expected *ACR4* does not play a role specific to ovule development. Rather as previously discussed (in Chapter III) it seems that *ACR4* is involved in maintaining L1 integrity in the whole plant.

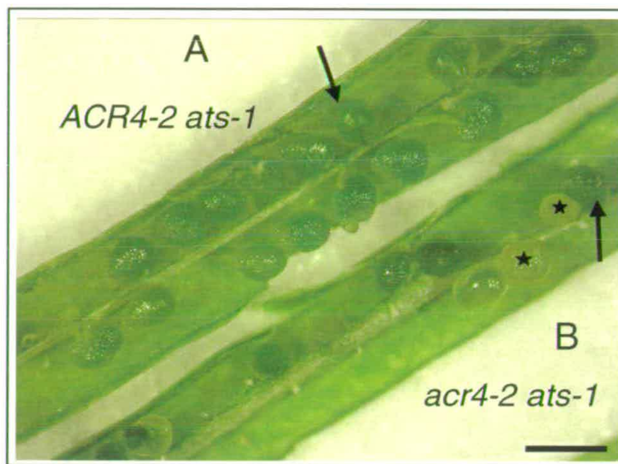


Figure V.2. Phenotype of *acr4-2 ats-1* double mutant. Photograph of an *ats-1* mutant opened silique (A) and an *acr4-2 ats-1* double mutant opened silique (B). The characteristic heart-shaped seeds of the *ats-1* homozygote are evident in both the single and double mutant siliques (arrows). In the *acr4-2 ats-1* double mutant the reduced seed set and seed abortion (starred aborting seeds) are particularly visible and seeds are marginally smaller than those of *ats-1*, as seen in *acr4-2*. No additional or attenuated defects are seen. Scale bar: 1mm.

V.3. How does *ACR4* fit into the wider signalling network?

ACR4 does not interact specifically with genes involved in regulating ovule development. It seems to be regulating a more general process. So how is the regulation of L1 cell layer integrity related to the more widespread control of development? Also, which other genes might *ACR4* be interacting with? In order to address these questions one approach utilised data gained from a yeast-two-hybrid (Y2H) screen carried out by Samuel Dean and Gwyneth C. Ingram in the lab. In the Y2H screen the C-terminal region of the ACR4 protein was used as bait against protein in a seedling library. The screen was designed to identify proteins which interact with the cytoplasmic C-terminal domain of ACR4 and thus those that could be potential downstream targets of ACR4. Several potential interactors were identified in the screen of which two are proteins involved in the gibberellic signalling pathway: *GIBBERELLIC ACID INSENSITIVE* (*GAI*) (Peng, *et al.*, 1997) and *REGULATOR OF GIBERELLIC ACID* (*RGA*) (Dill and Sun, 2001). The hormone gibberellic acid (GA) regulates multiple aspects of plant development including the break of seed dormancy, the rate of growth and the transition to flowering. A link from *ACR4* to the GA signalling pathway would be interesting due to the multiplicity of developmental processes that are regulated by GA. In order to investigate this potential genetic link, double mutants between *acr4-2* and two genes important in the GA response pathway were generated. Any genetic interaction was screened for by comparing the phenotypes of single and double mutants.

GIBBERELLIC ACID INSENSITIVE (*GAI*) encodes a transcription factor which is a negative regulator of the gibberellic acid signalling pathway (Peng, *et al.*, 1997). The *gai* mutation affects the reception of GA or the resultant signal transduction. Loss of function *gai1-1* and *gai1-3* dominant mutants are late flowering, dwarfed due to reduced stem elongation and dark green in colour. In addition *gai* mutant seed requires external application of GA for germination. *GAI* has partially redundant functions with *RGA* (Dill and Sun, 2001).

Another component involved in a signalling pathway that negatively regulates GA responses is the *SPINDLY* (*SPY*) gene (Jacobsen, *et al.*, 1996). *SPY* encodes a tetratricopeptide repeat protein which has a protein-protein interaction domain. *spy* alleles are compromised in gibberellin signal transduction and resemble plants that have been constitutively treated with gibberellic acid. This results in early flowering plants that are lighter green and exhibit increased stem elongation. Together this gives *spy* mutants a characteristically 'spindly' appearance. *spy* mutants also have reduced fertility due to an effect on male sterility. Loss of *spy* function can suppress the *gai* phenotype. Three *spy* mutants exist: *spy-1*, *spy-3* and *spy-5*; these are listed in descending order of severity.

Another class of plant hormones of interest in the context of *ACR4* function are cytokinins. Cytokinins regulate patterns of cell division in plants by triggering division of cells in the meristem and controlling their meristematic competence (Higuchi, *et al.*, 2004, Nishimura, *et al.*, 2004; Nishimura, *et al.*, 2004). A link to *ACR4* which is involved in maintaining the integrity of the L1 layer might be possible. As part of this *ACR4*-regulated process it is important that the outside cell layer is maintained as a monolayer. In order for it to be so, the proper pattern of cell division must be controlled. In addition a possible link to cytokinin was a possibility as one of the other *CR4*-like genes in *Arabidopsis* (*AtHOM3*) groups closely in terms of sequence similarity to the cytokinin-regulated *CRK1* gene of *Nicotiana* as mentioned in Chapter I, Section I.5.1.

The *CRE1* (*CYTOKININ RESPONSE 1*) gene encodes a histidine kinase which has been identified as cytokinin receptor (Franco-Zorrilla JM, 2002). *cre1* mutants exhibit reduced responses to cytokinins. Three alleles of *cre1-1* were obtained and used in this study. *cre1-1* (Harrar, *et al.*, 2003), *cre1-2* and *cre1-4* (Harrar, *et al.*, 2003). The *cre1-4* allele contains a t-DNA insertion near the N-terminal end of *CRE1* and confers the strongest phenotype. In the same way as for the GA signalling pathway mutants above, a possible genetic link between *acr4-2* and *cre1* was investigated by generating double mutants.

V.3.1. *ACR4* does not interact specifically with either *GAI* or *SPY*

Crosses to *acr4-2* were made in both directions for at least three homozygous individuals of *gai1-1*, *gai1-3*, *spy-1*, *spy-3* and *spy-5*. *rga* was not crossed to *acr4-2* as it was not possible to germinate the seed obtained for the line (Gwyneth C. Ingram, pers. comm.). Homozygous *gai* seed, and seed generated during crossing to/from the *gai1-1*, and *gai1-3* lines was supplied with 100μM gibberellic acid (GA) in order to allow germination to occur. Two weeks after transfer to soil the plants which were homozygous for the *gai* mutation were visible as being stunted and dark in colour. In order to rescue these mutants to flowering, plants were sprayed with a 100μM solution of GA at weekly intervals. This was necessary in order to generate flowers and pollen for crosses of the homozygous *gai* lines, and also so that the seeds produced by the *gai* plants could be screened in the F2 generation for homozygosity of the *acr4-2* allele. The results for a representative F2 family from each cross are listed in Table V.2. Again, as with the ovule development mutants in Section V.2, the *gai1-1*, *1-3* and *spy-5* mutants were all in the L *er* background while *acr4-2* was in the Col0 background. As before no differences between the phenotypes of plants with or without the *erecta* mutation were seen.

Cross performed	No plants	wild-type-like	single *acr4-2* mutant	single *gai/spy* mutant	Double mutant
gai1-1 x *acr4-2* F2 (1)	27	15	3	7	2
gai1-3 x *acr4-2* F2 (1)	19	11	5	2	1
spy-1 x *acr4-2* F2 (2)	28	19	3	3	3
spy-3 x *acr4-2* F2 (2)	16	9	2	3	2
acr4-2 x *spy-5* F2 (1)	26	19	4	2	1

Table V.2. Phenotypic analysis of the F2 plants generated from crosses between *spy-1*, *-3*, *-5* and *gai1-1*, *-3* with *acr4-2*. The number of plants of each phenotype are listed for each F2 family number (in brackets).

Genetic linkage was not expected to occur between *GAI1* (on chromosome 1) and *ACR4* (on chromosome 3). *SPY* is on chromosome 3, but it is on the opposite chromosome arm to *ACR4* and thus any linkage is likely to be very weak. The ratios for each phenotype seen were in accordance with no linkage between the genes (see Table V.2).

The phenotype of double mutants in all five crosses was compared with that of the corresponding single mutants. The defects associated with the individual single mutants were found to be additive in the double mutants for both *gai* and *spy*. No alteration or exacerbation of either phenotype was seen. All *acr4-2 spy* double mutant plants exhibited the same phenotype to those of the corresponding *spy* single mutants (Fig.V.3A-C). In each case both the double and single mutants were lighter green and spindly as previously described. The only difference between the two was that double mutant siliques were shorter and contained abnormal seeds as seen in *acr4-2* homozygotes. Both single *gai1-1* and double *acr4-2 gai1-1* mutants were stunted, dark green in colour and late flowering (Fig.V.4A). *acr4-2 gai1-3* double mutant plants were also indistinguishable from *gai1-3* single mutants in their growth form (Fig.V.4B). The only difference was that the double mutant plants had shorter siliques due to being homozygous for the *acr4-2* mutation. During analysis of the *gai* crosses it was noted that the ratios of *acr4-2 gai* double, and *gai* single mutants, were not in accordance with the dominance of the *gai* mutation - there were too few plants in each category. As the presumed double mutants were not test-crossed to confirm their genotype, it is possible that the genotyping of this cross progeny was incorrect. The crosses of *acr4-2* to *gai1-1* and *gai*1-3 should therefore be repeated. However as no unusual phenotype was seen whatsoever during this experiment, there is still good evidence for a lack of interaction between *GAI* and *ACR4*.

Therefore it seems that there is no direct interaction between the *ACR4* gene and either the *GAI* or *SPY* genes. This result does not however invalidate the findings of the yeast-two-hybrid study. It could be that the effect of this interaction was not uncovered in this study, for example there might have been some subtle differences at the protein level.

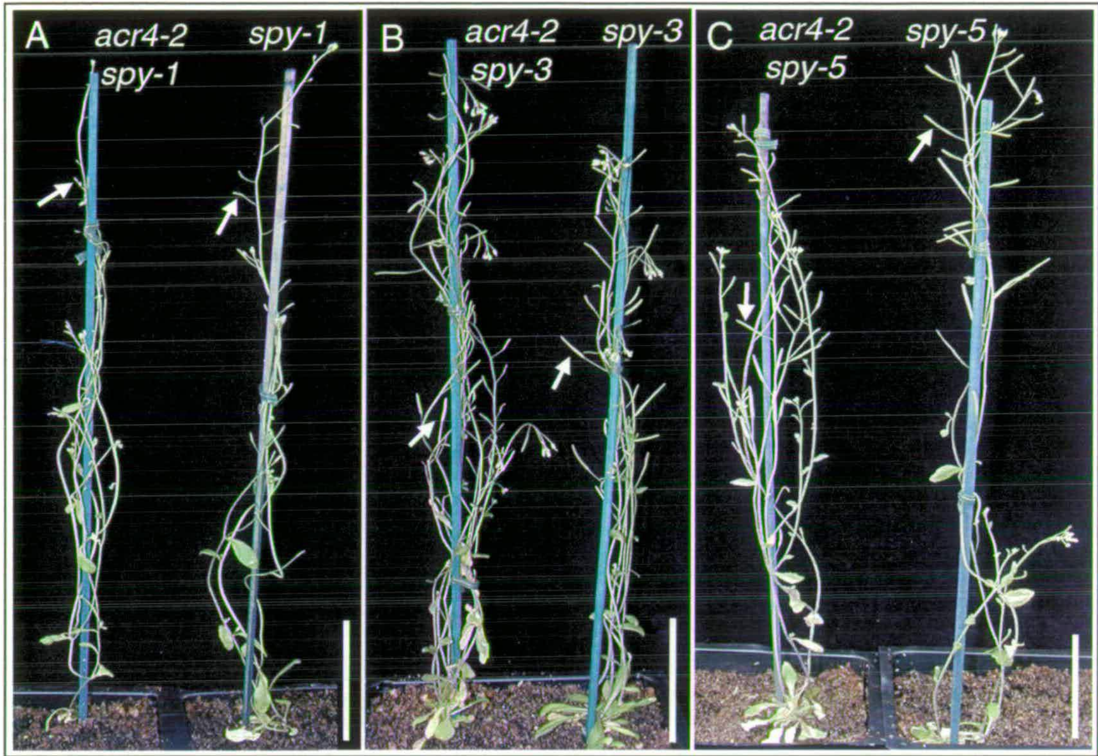


Figure V.3. Double mutant analysis of *acr4-2* with *spy-1* (A), *spy-3* (B) and *spy-5* (C). (A-C) Photographs of plants after five weeks growth on soil. Comparative double and single mutants are all phenotypically similar in terms of their vegetative development. *acr4-2* double mutants differ only from the single *spy* mutants in carrying *acr4* mutant seed. This results in the double mutants having shorter siliques than the single *spy* mutants: compare arrowed siliques of similar ages in each photograph (A) *acr4-2 spy-1* double mutant plant (left) and *spy-1* single mutant plant (right). Both plants are characteristically 'spindly': they are early flowering, pale in colour and show an increase in stem elongation. (B) *acr4-2 spy-3* double mutant (left) and *spy-3* single mutant (right). (C) *acr4-2 spy-5* double mutant (left) and *spy-5* single mutant (right). Plants in B and C are all 'spindly' although have a less severe phenotype to that of *spy-1*. Scale bars: 5cm.

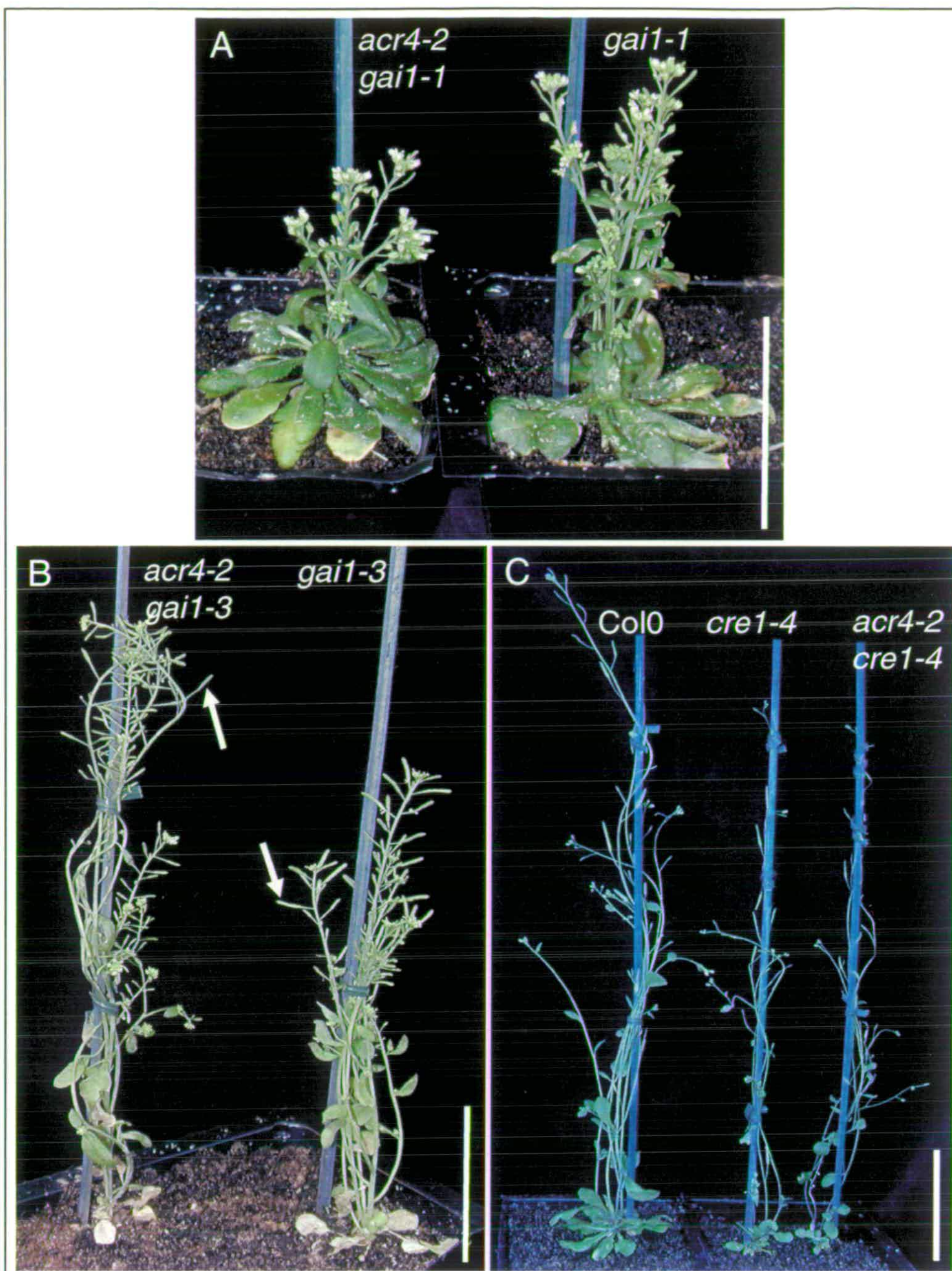


Figure V.4. Double mutant analysis of *acr4-2* with *gai1-1* (A), *gai1-3* (B) and *cre1-4* (C). Photographs of plants after (A) five weeks (B) six weeks or (C) four weeks on soil. (A) The *acr4-2 gai1-1* double mutant (left) has a similar phenotype to the *gai1-1* single mutant (right), although again with *acr4* mutant seeds (both in Laer background). Plants were sprayed once a week for three weeks with 100μM GA to rescue the plants to flowering. (B) The *acr4-2 gai1-3* double mutant (left) is of a similar size and form to the *gai1-3* single mutant (right) (both in Laer background). *acr4-2 gai1-3* double mutants contain *acr4* mutant seed which results in a shorter silique length: compare arrowed siliques of similar age. Plants were sprayed once a week for four weeks with 100μM GA to rescue the plants to flowering. (C) Col0 plant on left; both *acr4-2* and *cre1-4* are in the Col0 background. The *cre1-4* single mutant (in the middle) is indistinguishable from the *acr4-2 cre1-4* double mutant (on the right), apart from that the *acr4-2 cre1-4* double mutant has *acr4* mutant seed. Scale bars: 5cm.

V.3.2. *ACR4* does not interact specifically with *CRE1*

Crosses to *acr4-2* were made in both directions for at least three homozygous individuals of *cre1-1*, *cre1-2* and *cre1-4*. To investigate whether loss of *CRE1-1* function had any affect on the pattern of *ACR4* gene expression, the *cre1-1* mutant was also crossed to homozygous individuals of the single *ACR4* marker line (*pACR4::H2B::YFP*); the *cre1-2* and *-4* alleles were not available for crossing at the time. In order to isolate double *acr4-2 cre1* mutants, plants homozygous for the *acr4-2* mutation were selected by means of their seed phenotype in the F2 generation. Under the conditions in which plants were grown in, *cre1* homozygous mutant plants appeared to be spindlier than wild-type Col0 plants. Plants homozygous for the *cre1* mutation could therefore be selected due to their growth phenotype. Genetic linkage was not expected between *CRE1* (on chromosome 2) and *ACR4* (on chromosome 3).

In double mutants of *acr4-2* with *cre1-1*, *-2* and *-4* the defects associated with each allele were found to be additive as in the *gai* and *spy* crosses. The *cre1-4* allele is the strongest of the three and the phenotypic ratio results for a representative F2 family generated in a cross are presented in Table V.3. The results for the *acr4-2 cre1-1* and *acr4-2 cre1-2* double mutants are not presented as the phenotypic results were the same. The ratios for each phenotype in the F2 generation were in accordance with no genetic linkage between *ACR4* and *CRE1*.

Cross performed	No plants	wild-type like	single *acr4* mutant	single *cre1* mutant	Double mutant
cre1-4 x *acr4-2* F2 (2)	19	10	3	5	1

Table V.3. Phenotypic analysis of the F2 plants generated from a cross between *cre-1-4* with *acr4-2*.

acr4-2 cre1-4 double mutants were phenotypically identical to *cre1-4* single mutants, apart from the presence of *acr4-2* seed (Fig.V.4C); no enhancement of *acr4-2* seed defects was seen. Therefore it does not seem that the *ACR4* and *CRE1* genes interact directly. In addition there was no difference in the pattern and intensity of *ACR4* gene expression between plants wild-type for the *CRE1* gene and those homozygous for the *cre1-1* mutation.

V.4. New γ-mutagenesis-generated mutants are candidate enhancers of the *acr4* mutant phenotype

V.4.1. A γ-mutagenesis enhancer screen based on the *acr4-2* mutant

In order to identify additional components in the *ACR4* signalling pathway a mutagenesis enhancer screen was carried out. *acr4-2* homozygous seed was mutagenised and the resultant lines were screened for enhancement or alteration of the *acr4-2* phenotype. The screen was designed to uncover pathway components or elements which act in parallel or redundantly with *ACR4* in order to maintain L1 cell layer integrity. Components directly up- or down-stream of *ACR4* might not be identified using this approach as the *ACR4* receptor is already completely null. Therefore mutations in such epistatic components could confer the same phenotype as loss of *ACR4* function, and thus might be indistinguishable from the background. However if the downstream components are targeted by both homo- and hetero-oligomers of ACR4 and ACR4-like proteins (see Chapter IV), or also by a parallel pathway then they might be detected. γ-rays were chosen as the mutagen for this screen rather than the commonly used EMS (ethyl-methanesufonate) due to the defects in the seed coat of *acr4-2* homozygous seed. It was thought that the EMS mutagen, which is applied in a liquid solution, might penetrate the *acr4-2* seeds more quickly than it would wild-type seed (as was seen when using dyes and fixatives in Chapter III). Determining the correct conditions for the altered substrate would therefore have been very time and material consuming.

About 3000 *acr4-2* homozygous seeds were measured out by weighing 100 *acr4-2* seeds and multiplying up to calculate the weight of 3000 seeds. The seeds were then placed next to a γ-ray source for a period of time which would give an absorbed dose of radiation of 300 Grays. This dosage had been recommended as being optimum for mutagenesis for saturation of the whole genome of *Arabidopsis* (Frédéric Berger, Ecole Normale Supérieure, Lyon, France, pers. comm.). This original population of mutagenised seed was designated the M_0 generation.

All M_0 seeds were sown immediately after mutagenesis on to MS agar plates to allow germination to take place. After two weeks growth in an incubator the number of seeds that did not germinate was compared to the number that germinated to give viable plants. The ratio of seed death of M_0 seeds was found to be high but was not accurately enumerated. Together with the ratio of albino-type mutations and the ratio of sterility found in M_2 plants this can be used to calculate the level of genome saturation of mutagenesis events (Jurgens, *et al.*, 1991; Guitton, *et al.*, 2004). However it was not possible to use the ratio of seed death for calculation as the ratio included seeds that do not germinate simply due to abnormalities associated with the *acr4-2* phenotype.

From this initial M_1 population, 2500 individual M_1 seedlings were transplanted to soil. After eight weeks on soil, seed from M_1 plants was collected. In order to create single segregating lines, seed was collected from individual lateral branches on individual M_1 plants. This ensured that all seeds in each line were either homozygous, heterozygous or wild-type for the same mutation(s). Plants in the M_2 generation will therefore be segregating this mutation event as homozygous, heterozygous and wild-type plants in a ratio of 1:2:1. Sowing of mutagenised seed, transplanting of seedlings and collection of seed was carried out with technical assistance from Kathryn Degnan and Ross Walker.

About 50 seeds from each M_1 seed line were sown on to individual MS agar plates and the resultant M_2 seedlings of each line screened on the plate. This screen was carried out for 1000 out of the 2500 M_1 lines. The phenotype of seedlings in each line was compared with that of the background *acr4-2* line. Any abnormalities which segregated in a ratio suggestive of either heterozygous or homozygous mutations were examined. These abnormalities included alterations in the rate of germination as well as defects in seedling formation. Defects in the rate of germination could indicate a sporophytic mutation which results in defective embryogenesis. Seedlings that terminate growth very early on during development or those which have abnormal cotyledons might be the due to irregular embryo morphogenesis, the effects of which are manifest after germination. For example the defects in *gnom* homozygous seedlings are due to incorrect embryo development but

are visible in germinated embryos (Jurgens, *et al.*, 1991, Shevell, *et al.*, 1994; Shevell, *et al.*, 1994). Abnormal seedling shape or size was also investigated. Mutations resulting in improper seedling cell layer organisation were of particular interest. Mutations occurring in components that act redundantly with *ACR4* could result in enhanced outside cell layer disorganisation. The function of such redundant components would normally restrict cell layer integrity defects to the ovule integuments and to sepal margins in *acr4* homozygous mutants.

After screening on plates, 15 plants for every line (regardless of whether a defect on the plate was seen or not) were transplanted to soil. A second stage of screening was then carried out. Here a range of abnormalities were again searched for. These included defects in mature plant growth and form, such as smaller or spindlier plants. An enhancement of the *acr4* mutant phenotype could be expected to adversely affect general plant size or shape. In addition the length of siliques was analysed: shortened siliques could indicate a lack of fertilisation or early seed abortion. Of particular interest was any enhancement or suppression of the ovule and seed abortion seen in the *acr4-2* line. In order to be able to identify defects both during early and late seed development several siliques of various ages were screened for each plant. Seed development was observed by opening siliques and viewing them using a dissecting microscope. Early stages of seed development were viewed by choosing the fourth or fifth silique down from the last open flower. Later stages were analysed by choosing the tenth or eleventh silique down. A mutation with a gametophytic effect would be likely to result in early seed abortion and thus would be particularly visible at early stages. In older siliques any seeds that aborted early in development would be obscured by larger maturing seed. Older siliques were however required for analysis of seed abortion at late stages due to aberrant embryo development: such abnormalities would not be obvious at early stages. Any seeds appearing white or yellow, rather than green, were dissected to determine whether they had a normal morphology yet were albinos (loss of proper chlorophyll pigmentation), or whether they harboured a mutation altering embryo development itself. Out of the 1000 lines screened, 4.8% were found to carry mutations resulting in albino seeds and/or seedlings. 2.2% of the screened lines were found to carry a

mutation resulting in male sterility and thus very short siliques. Reduced male sterility was identified as lower amounts of pollen on the stamens of open flowers in comparison to wild-type or *acr4-2* plant flowers. Male sterility was not of interest here and so any lines carrying such a phenotype were noted but abandoned. In general the number of lines carrying any sort of mutant phenotype seemed to be low (~20%), which suggests that the screen was not at saturation (Jurgens, *et al.*, 1991).

After the initial screen of all 1000 M_2 lines, a second round of screening on the M_3 generation of all lines with interesting defects was carried out. If after screening the lines were not found to carry the expected defect, they were abandoned. As well as identifying mutant phenotypes in the lines of interest, the ratios of mutant to background *acr4-2* seedlings was determined in order to understand how the mutations were segregating.

V.4.2. Identified mutants have an enhancement in seed abortion or defects in cell layer organisation: are they dependent on *acr4*?

V.4.2.A. Description of mutants identified in the γ-mutagenesis enhancer screen

After screening 1000 M_2 lines a total of 130 candidate mutant lines were identified. After two rounds of re-screening on these lines this was narrowed down to 37 lines. This reduction was due to either false positive identification of defects, for example some apparent defects in seedling development were due to the fact that seeds had been collected too early and thus had not properly matured. In other lines it seemed to be that the M_2 generation carried more than one mutation, the sum total of which had an effect on plant growth but when separated (in the M_3 generation) did not.

A brief description of the 37 mutants identified is given in Table V.4. Isolated mutants were generally divided into three categories depending on the phenotype. These include where a phenotype was visible in the vegetative phase of development, where defects occurred in seed development, or when defective

Table V.4. Table showing the 37 γ-mutagenesis lines of interest with a brief description of each phenotype (see text of Section V.4.2 for details). The phenotype of each line is divided into 'plate' (B) or 'plant' (C) phenotype which indicates at what screening stage the defects were noted. The 37 lines are colour coded by phenotype (A). Red indicates lines with a phenotype generally related to the vegetative stage of development i.e. abnormal seedlings or stunted plants (18 in total). Green indicates lines with defects in seed development i.e. increased seed abortion (seed ab.) or reduced germination rate (xgerm) (14 in total). Blue indicates lines in which defective embryo development is observed when siliques are screened; seeds containing these defective embryos germinate to produce abnormal seedlings (5 lines in total). An asterisk indicates that some lines carried mutant seedlings that could survive on soil. Therefore the phenotype recorded for 'plate' and 'plant' refers to the defects seen in the same individual (e.g. line 18). The average ratios of the mutants in each line are indicated below the phenotype. This includes the average % of seed abortion for lines carrying such a defect.

The table also shows the results from screening the F2 generation resulting from crosses carried out to determine whether the new mutations were dependent on the *acr4-2* mutant background (D) and crosses carried out to clean up the new lines (E). Not all results were obtained due to time restrictions; some lines have been crossed but not screened. (F) For each test the cross carried out and the F2 family number (in brackets) is included. (G,H) All F2 families were screened on plates (G) and most were then also screened at the mature plant stage (H). At the mature plant stage the phenotypes of F2 family members were divided into four categories. In the F2 families resulting from crosses to clean up lines there were only two categories: plants homozygous for the *acr4-2* allele (thus carrying *acr4* mutant seed) with or without the new mutation (*acr4* and new, or *acr4*). In crosses to check for dependency there were also *ACR4-2*-like plants with or without the new mutation (*ACR4* and new, or *ACR4* -like). The ratios of interest for each cross are highlighted in red. A dash indicates that no plants of the phenotype indicated could be present (e.g. no Col0 plants in the crosses to *acr4-2*). A blank box indicates that screening for plants of the phenotype listed was not carried out (i.e. for some of the crosses to mutants carrying a phenotype visible on the plate, the mature plants were not screened). See Section V.4.3 for details about line 852. cots, cotyledons; sm, small; wh, white; xger, not germinated.

Table V.4.

(A) γ-line	(B) Plate phenotype	(C) Plant phenotype	(D) Dependent on *acr4-2* ?	(E) Cleaned up line?	(F) Crosses performed	(G) Plate phenotype	(H) Plant phenotype			
							ACR4 -like	*acr4*	*acr4* and new	*ACR4* and new
18	*as-1* like* ¼	*small, sterility	crossed	-						
57	lumpy short roots ¼	some late seed ab. ⅓, 25%	crossed	crossed						
60	small blobby/xgerm ½	globular seed ab. ⅔, 50%	no	yes	Col0 x 60-3-4 (1) *acr4-2* x 60-3-4 (1)	5/50 xger 6/44 xger	8/19 -	1/19 4/6	0/19 2/6	10/19 -
70	-	stunted, sterility ¼	no	-	Col0 x 70-2-5 (1)	-	14/31	10/31	1/31	6/31
136	-	late seed ab. ¼, 25%	crossed	-						
145	-	late seed ab. ¼, 50%	no	-	Col0 x 145-1/2 (1)	-	7/9	0/9	1/9	1/9
159	-	late seed ab. ½, 50%	no	yes	Col0 x 159-1-2-2 (1) *acr4-2* x 159-1-2-1 (1)	- -	15/25 -	3/25 12/19	1/25 7/19	6/25 -
160	small, lumpy, yellow ¼	-	no	yes	Col0 x 160-1-2-1 (3) *acr4-2* x 160-1-2-1 (2)	12/53 small 7/24 small				
166	-	small, stumpy ¼	no	yes	Col0 x 166-1-3-1 (2) *acr4-2* x 166-1-3-1 (1)	- -	17/25 -	4/25 30/31	2/25 1/31	2/25 -
248	small, blobby ¼	*fusca* -like embryos ½, 25%	no	yes	Col0 x 248-1 (2) *acr4-2* x 248-1 (3)	- 11/45 *fusca* -like	3/8 -	2/8 3/6	0/8 3/6	3/8 -
271	-	early seed ab. ½, 25%	no	yes	Col0 x 271-1 (1) *acr4-2* x 271-1 (2)	- -	4/11 -	2/11 4/6	0/11 2/6	5/11 -
443	-	spindly ¼	no	-	Col0 x 443-5-5 (2)	-	19/32	9/32	1/32	3/32
468	-	late seed ab. ¼ to ⅓, 50%	no	yes	Col0 x 468-4-2 (1) *acr4-2* x 468-2 (2)	- 3/49 xger	10/22 -	7/22 14/15	3/22 1/15	2/22 -

Table V.4. cont.

(A) γ-line	(B) Plate phenotype	(C) Plant phenotype	(D) Dependent on *acr4-2* ?	(E) Cleaned up line?	(F) Crosses performed	(G) Plate phenotype	(H) Plant phenotype			
							ACR4 -like	*acr4*	*acr4* and new	*ACR4* and new
470	-	late seed ab. ¼, 50%	to cross	-						
498	xgerm ½	late seed ab. ¼, 50%	no	yes	498-1-2-1 x Col0 (2) 498-1 x *acr4-2* (2)	5/31 xger -	7/12 -	3/12 18/23	1/12 5/23	1/12 -
514	small white cots ¼	late seed ab. ½, 25%	no	-	Col0 x 514-2-4 (2)	5/79 sm wh cots	19/34	5/34	2/34	8/34
527	small white cots ¼	-	no	yes	Col0 x 527-1-2 (1) 527-2-1-2 x *acr4-2* (1)	6/28 sm wh cots 3/32 sm wh cots				
537	squat, *as-1* like* ¼	*small, sterility	no	yes	537-5-2-1 x Col0 (2) 537-5-3-1 x *acr4-2* (2)	6/23 squat 7/34 squat	8/11	3/11	-	-
540	white/brown, blobby ¼	brown embryos ½, 25%	no	-	Col0 x 540-1-3 (1)	-	4/11	0/11	0/11	7/11
546	-	sterility, wh stems, serrated leaves ¼	no	-	Col0 x 546-2-2 (1)	-	20/42	7/42	2/42	3/42
552	-	late seed ab. ¼, 25%	no	yes	552-4 x Col0 (1) 552-2 x *acr4-2* (2)	- -	18/26 -	4/26 20/26	2/26 4/26	2/26 -
561	*emf* -like ¼	-	no	-	Col0 x 561-3-2 (1)	14/67 *emf* -like				
619	-	small serrated leaves ¼	no	-	Col0 x 619-4 (3)	-	19/33	6/33	5/33	3/33
645	-	late seed ab. ¼, 25%	no	yes	645-1-2-2 x Col0 (3) 645-1-2-1 x *acr4-2* (2)	- -	5/17 -	2/17 7/12	2/17 5/12	8/17 -
662	xgerm ¼ to ½	late seed ab. ¼ to ½, 50%	no	yes	662-2-1 x Col0 (1) 662-2-1 x *acr4-2* (2)	- -	8/13 -	2/13 12/15	1/13 3/15	2/13 -
685	xgerm ¼ to ½	late seed ab. ½, 25%	no	yes	685-2-2-2 x Col0 (2) 685-2-2-1 x *acr4-2* (1)	4/38 xger 2/33 xger	15/27 -	2/27 8/10	2/27 2/10	8/27 -

Table V.4. cont

(A) γ-line	(B) Plate phenotype	(C) Plant phenotype	(D) Dependent on *acr4-2* ?	(E) Cleaned up line?	(F) Crosses performed	(G) Plate phenotype	(H) Plant phenotype			
							ACR4 -like	*acr4*	*acr4* and new	*ACR4* and new
724	curved leaves* ¼	* serrated leaves	crossed	- crossed						
778	-	spindly, sterility ¼	no	-	Col0 x 778-2 (2)	-	18/25	5/25	0/25	2/25
784	xgerm ¼	late seed ab. ¼, 25%	no	yes	784-3 x Col0 (2) 784-1 x *acr4-2* (1)	2/22 xger 6/30 xger	2/18 -	0/18 3/17	3/18 14/17	13/18 -
841	-	late seed ab. ½, 25%	no	yes	841-1-1 Col0 (1) 841-1-5 x *acr4-2* (1)	- -	5/20 -	2/20 11/14	1/20 3/14	12/20 -
851	small, blobby ¼	-	crossed	- crossed						
852	'HULK' ¼	-	no	- crossed	852-1-1-2 x Col0 (4)	24/129 hulk 5/129 sulk	18/24	4/24	-	-
895	pale cots and leaves ¼	-	no	-	Col0 x 895-3-2 (2)	12/50 pale				
899	-	late seed ab. ¼, 25%	crossed	yes	*acr4-2* x 899-1-1 (2)	-	-	10/12	2/12	-
933	blobby cots ¼	-	crossed	- crossed						
977	-	white embryos ¼ to ½, 25%	crossed	-						
980	pointed leaves* ¼	*small leaves, sterility	crossed	- crossed						

embryo development was manifest and apparent in both seeds in the silique and in the resultant germinated seedlings. Not all mutant phenotypes and the ratios of segregating mutations in the lines isolated were investigated in depth due to time constraints. Only lines which were dependent on the *acr4-2* background were of real interest. A brief summary of the phenotype in each line and some preliminary conclusions will however now be given.

Some mutants found were very similar or identical to known mutants. The mutants isolated here might therefore represent the same alleles or new mutations in those genes. Lines 18 and 537 carried *asymmetric leaves1*-like seedlings which are small and squat due to abnormal leaf patterning (Byrne, *et al.*, 2000; Xu, *et al.*, 2003). Line 248 carried *fusca*-like embryos and dark brown abnormal seedlings. *fusca* seedlings are defective in responses to various endogenous and environmental factors (Castle and Meinke, 1994; Misera, *et al.*, 1994). This causes a build up of anthocyanins in *fusca* embryos and the resultant seedlings, which gives them a dark purple/brown colouration. Line 561 mutant seedlings were similar to *embryonic flower*-like seedlings (Yang, *et al.*, 1995; Chen, *et al.*, 1997) (Fig.V.5E). *EMF* polycomb-group genes mediate shoot development and flowering (Moon, *et al.*, 2003). The other mutants of interest seemed to be novel, or at least were not clearly identifiable as known mutants.

Around a third of the 37 mutants isolated carried defects resulting in increased seed abortion at a late stage in development. This increase was obvious above the normal levels of seed abortion seen in *acr4-2* mutants. It was also visible as the aborting seeds which carried the new mutation were all of the same developmental age, unlike the aborting seed in *acr4-2* mutant siliques. Lines with increased late stage seed abortion included lines numbered 136, 145, 159, 468, 470, 498, 552, 645, 662, 685, 784, 841 and 899. In the siliques of these lines the aborting seeds were yellow or brown, whilst those at the same developmental stage in wild-type were green. The stage at which abortion occurred varied between the different lines. In general it ranged between the late heart and bent cotyledon stages of embryo development. As all aborting seeds in these lines were of roughly the same phenotype it was likely that the abortion was directly due to abnormal embryo development (unlike in *acr4-2*).

The ratio of seed abortion within siliques varied between the lines listed above. Some lines had roughly 50% late seed abortion which suggested a female gametophytic mutation (lines 145, 159, 468, 470, 498, 662, 685). Others had roughly 25% seed abortion which suggested a sporophytic defect (lines 136, 552, 645, 784, 841, 899). In some lines the stage at which seeds were aborting was much later than others and there was a reduced germination rate. In these cases the ratio of seed death was equivalent to the ratio of seeds not germinating, which suggested that the aborting seeds could be harvested, but then were not viable (lines 498, 662, 685, 784).

One line, 271, carried a putative sporophytic mutation that caused early seed abortion, around the globular stage of embryo development. Another line, 977, carried seeds which contained embryos that looked white. These were not albinos but the reason for the colour alteration was not apparent. The germination rate for line 977 was normal and there were no visibly abnormal seedlings in the line.

In five lines there were both seed abnormalities in the silique and malformed seedlings. It is possible that in these lines the abnormal seeds do not abort totally and thus can be harvested and can germinate. However as the seeds contain defective embryos, the resultant seedlings will be defective and so do not continue growth after cotyledon and/or root emergence. This group of mutants include the *fusca*-like defective mutants seen in line 248. In addition it includes lines 57 (Fig.V.5A), 60 (Fig.V.5B), 514 and 540 (Fig.V.5D) in which mutants had late stage (or globular stage for 60) seed abortion as well as small and defective seedlings.

Seven lines carried mutations resulting in defective seedling development. The *emf*-like line 561 also falls into this category. Lines 160 (Fig.V.5C), 527, 851 (Fig.V.5F,G) and 933 (Fig.V.5J) had small and malformed seedlings with deformities. Line 895 mutant seedling cotyledons and leaves were pale in colour. The apparent outgrowths on seedlings in line 852 (Fig.V.5H,I) were of particular interest and will be discussed in detail in Section V.4.3.

Four lines contained mutants which had abnormal seedling and mature plant morphology. The abnormal seedlings in these lines could be grown to maturity although there were associated defects in fertility in the mutant plants. These lines

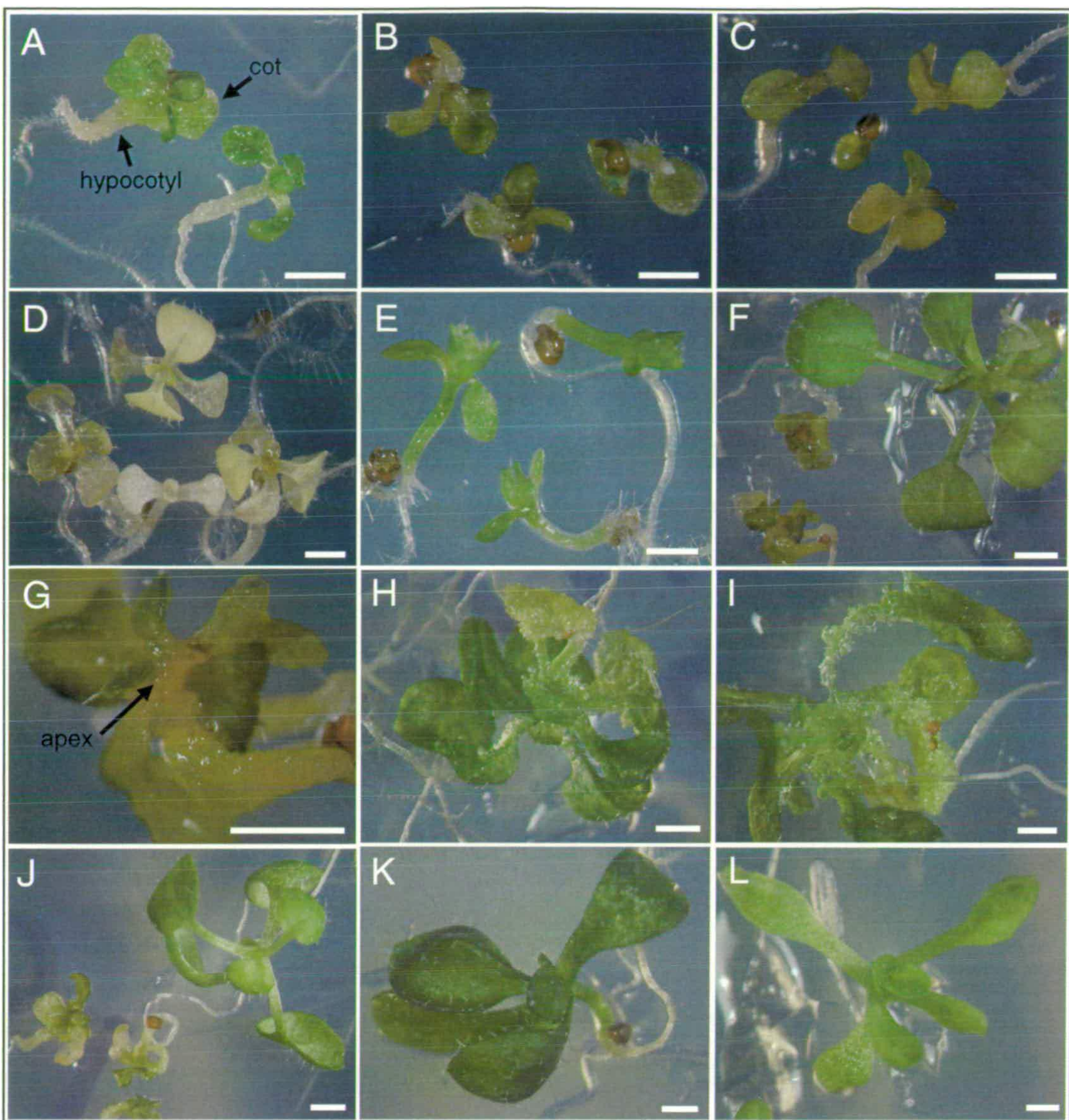


Figure V.5. Phenotype of lines generated from a γ-mutagenesis screen. (A-L) Photographs of two-week old seedlings in γ-mutagenesis lines exhibiting a range of phenotypes. All seedlings shown are in the *acr4-2* homozygous background. (F,J) Photographs also show *acr4-2* sibling seedlings in the top right of the picture for comparison. (A) Line 57 mutant seedlings are stunted and lumpy. The hypocotyl in particular is thicker. (B) Line 60 mutant seedling development is stunted. Seedlings have very small cotyledons and leaves and if present a shortened root. (C) Line 160 mutant seedlings are similar to those of line 60, although with a more normal root. (D) Line 540 mutant seedlings are about half the size of wild-type. The leave are yellow or almost transparent. (E) Line 561 mutant seedlings are *emf*-like as discussed. (F,G) Line 851 mutant seedlings are very small and have a wet appearance. The mutant seedlings show a lack of proper development with the apex appearing disorganised. (H,I) Line 852 mutant seedlings appear to have outgrowths of cells over the entire surface of green parts. See Fig.VI.6 for more details. (J) Line 933 mutant seedlings are similar to those in line 57 (A). (K) Line 724 mutant seedlings have leaves which curve upwards at the edges. The leaves are also dark in colour. (L) Line 980 mutant seedling leaves are pointed at the tip and pale in colour. Unlike all other mutant seedlings shown here, those of lines 724 and 980 can grow to maturity on soil, producing a limited number of seeds. N.B. The number of mutant seedlings in pictures also containing wild-type siblings is not representative of the ratios actually seen within each line. cot, cotyledon; Scale bars: 1mm except (G) 500μm.

included the two *as-1* like lines 18 and 537. Line 724 mutant seedlings had leaves which curled upwards at the edges (Fig.V.5K). As the mutant plants in line 724 matured, the leaf edges became serrated. Mutant seedlings in line 980 had pale coloured leaves which were pointed at the tips (Fig.V.5L).

The final group of mutants had normal seed and seedling development but had abnormal plant architecture or form. Mutant plants in lines 70 and 166 were stunted in size (about 1/3 of the height of the *acr4-2* background) and had spindly stems. Line 70 mutants were also early flowering and fairly sterile. Mutant plants in lines 443 and 778 were both spindly with phenotypes that had some similarity to that of the *spy* mutant alleles. There was a reduction in fertility in line 778 but not in line 443. Line 546 mutant plants had several defects: white stems, serrated leaves and sterility. These three defects were all linked, but it seems likely that more than one gene is affected as such a combination of abnormalities is unusual. Mutant plants in line 619 were of normal height but had smaller serrated leaves.

Out of all the mutants with defective seedling development, mutants in lines 57, 851, 852 and 933 were of particular interest. The seedlings of these lines showed abnormalities resulting in lumpy or disorganised cotyledons and leaves which were suggestive of some sort of cell-layer organisation defect.

V.4.2.B. Clean-up of lines and test of dependency on the *acr4-2* background

In order to test whether the phenotypes caused by the new mutations of interest were dependent on the original *acr4-2* background mutation, crosses were made to Col0 (the original ecotype in which *ACR4* was mutated). These crosses were used to see whether the new mutations would be apparent in a Col0 background (not dependent), or whether they were only visible in the *acr4-2* background or were exacerbated in it (and thus were dependent). In order to do this, plants either heterozygous or homozygous (when possible) for the new mutation (in the *acr4-2* background) were crossed to Col0 plants. Several crosses for each line were performed and the heterozygosity of the new mutation in the F2 generation for each cross was

confirmed. The F2 generation was analysed by screening for the new phenotype of interest and examining the segregation ratios of expected phenotypes in comparison to the actual numbers. If a new mutation was dependent on the *acr4-2* background then the mutant phenotype would only be visible in *acr4-2* plants. The ratio of plants carrying the new mutation in the *ACR4-2*/Col0 background would thus be much lower than expected for two independent and unlinked mutations. The dependency of mutants which had defects in seed development or vegetative development could easily be determined by screening the plant siliques to identify *acr4-2* homozygotes or *ACR4-2*-like plants, and then screening for the new mutation.

In crosses to lines carrying mutations which caused a phenotype visible at the seedling stage, the F2 families were generally only screened on plates to test the dependency of the mutation. However a problem with this approach arises if a mutated gene which causes seedling death is linked to *ACR4*. If it is, then the same ratio of mutant seedlings would be expected even if the mutation was not dependent. This is because due to reduced recombination, a higher number of double mutants would be seen in comparison to the number carrying the new mutation in the *ACR4-2*/Col0 background. This would therefore make it appear as if the mutation was not dependent. Whether or not the gene in which the new mutation occurs is linked to *ACR4* can however be determined. All F2 seedlings can be transplanted and then the number of *acr4-2* homozygous plants compared to the number of wild-type plants (determined by examining the siliques). If there is a much lower ratio of *acr4-2* homozygotes than expected, it is likely that the two genes are linked: the majority of *acr4-2* homozygotes would have died at the seedling stage due to carrying two copies of the new mutation (because they were double mutants). This test has been carried out for two of the six lines in question (537 and 852) and will be carried out for the remaining seedling mutants in the future.

Even although not all isolated mutants were likely to be dependent on *acr4-2* (for example the *emf*-like allele), all lines were crossed to Col0. The results from the dependency check are presented in Table V.4. Due to time constraints not all dependency test results have been obtained (27/37).

Out of all the mutants tested thus far, none of the mutant phenotypes have been found to be dependent on the *acr4-2* background; see Section V.4.3 for discussion of the result for line 852. In the test crosses it was possible to isolate all of the new mutations in a Col0 background. In addition the ratios as presented appear to be generally in agreement with the expected ratios (compared to how the mutations normally segregate). This suggests that none of the isolated and tested mutations are in genes that interact directly with *ACR4*. Some mutants which have apparent defects in seedling layer organisation such as those in line 852 (see Section V.4.3) however are still of interest as they could potentially be part of separate pathways which are involved in regulating cell layer organisation, independent of the function of *ACR4*. These mutants might therefore be of use in future research. In addition some of the mutants not tested, such as 57, 851 or 933 could turn out to be dependent and so be of special interest in further work.

For such particularly promising new mutants, and most other mutants, back-crosses to the original *ac4-2* background line were performed in order to remove any additional mutations caused by the mutagenesis. These crosses were therefore used to 'clean-up' the lines for future analysis (see Table V.4). Where mutations were not dependent on the *acr4-2* background such crosses were not always performed due to time restrictions, and also because the new mutations had already been selected in the clean Col0 background.

V.4.3. *hulk* mutants have a loss of epidermal integrity

One mutant of particular interest was that in line 852. This is a novel mutant, although a mutant with a phenotype which bears some similarities to it has been reported recently (Basu, *et al.*, 2004); see later for discussion of this research. One quarter of seedlings in this line seemed to have outgrowths of epidermal cells on the surfaces of leaves, cotyledons and petioles. The ratio (¼) is consistent with these abnormal seedlings being homozygous for a new mutation. These mutant seedlings were not able to survive when transplanted to soil: a high humidity environment was

required. In order to investigate the apparent surface outgrowths, seedlings were examined using scanning electron microscopy (SEM).

Seedlings were cryo-fixed, gold-vapour sputter coated and viewed under the SEM (Fig.V.6). A representative example of a mutant in line 852 is shown in Fig.V.6A. The apparent outgrowths over the surfaces of the seedlings turned out to be tears in the epidermal cell layer (Fig.V.6B). In addition there were holes in the surface of the cotyledons (Fig.V.6B). In comparison the surface of an *acr4-2* sibling seedling is free of epidermal tears or holes (Fig. V.6C).

The tears in the epidermal layer of mutants were the result of the fact that the epidermal cells were not properly attached to one another. Unlike the epidermal cells of an *acr4-2* homozygous mutant plant cotyledon which are properly connected (Fig. V.6I: leaf), the epidermal cells of cotyledons and leaves in line 852 mutants have small gaps between them (Fig.V.6G: leaf). As the organs in line 852 mutants expand, the epidermal cells do not maintain their connections and are pulled apart (Fig.V.6H: cotyledon). This results in large holes appearing in the surface of cotyledons and leaves (Fig.V.D,E: cotyledons). Such holes are not present in the *acr4-2* mutant (Fig.V.6F: cotyledon). Where large regions of cells in the line 852 mutant pull apart, curls of epidermal cells are apparent (as in Fig.V.6B). These tears are particularly obvious on the petioles of seedling leaves (Fig.V.6J,K). The petiole of an *acr4-2* mutant in contrast is smooth with no holes or rips in the epidermal cell layer (Fig.V.6L). It is the presence of these holes and tears in the epidermal cell layer that means that line 852 mutants cannot survive in the air. Due to these holes the surface of the 852 mutants is often covered in liquid which has been exuded from the exposed underlying cells. An intact epidermal layer normally stops this from occurring. The mutant in line 852 was named *hulk*. At the leaf margins the cells of *hulk* mutants are also disorganised (Fig.V6M). The defects seen there are reminiscent of the loss of cell organisation seen in sepal margins. The defects in cell layer integrity seen in *hulk* mutants only affect the aerial parts of the seedlings, the roots appear to be normal. As seen in *acr4-2* mutants, *hulk* mutants range in their severity of defects, dependent on the degree of loss of cell adhesion (compare Figs.V.D and E). Overall though the seedlings are fairly similar.

Figure V.6. Phenotype of the *hulk* mutant. (A) Light microscopy image of a representative two week old *hulk* mutant seedling. (B,D,E,G,H,J,K,M,N) SEM images of *hulk* mutants. (C,F,I,L,O) SEM images of an *acr4-2* sibling. (A) *hulk* homozygous mutant seedlings appear to have outgrowths of tissue on the surfaces of cotyledons, petioles and leaves. The *hulk* leaves look 'wet' to the eye. These seedlings only survive in a highly humid environment. *hulk* mutant roots appear to be normal. (B) Under SEM the apparent outgrowths are actually rips of tissue (arrowhead). There are also holes in the cotyledon surface (arrows); holes are also present on the surface of leaves. (C) Such rips and holes are not present on the surface of an *acr4-2* mutant seedling. (D,E) At higher magnification the holes in the cotyledons (arrows) are particularly obvious in *hulk* mutants; the defects in (E) are more severe than those in (D). (F) No holes are visible in *acr4-2* mutant cotyledons. (G,H) The holes occur as a result of loss of epidermal cell connection. (I) In an *acr4-2* leaf the epidermal cells are properly connected and the surface is smooth. (G) In a young *hulk* leaf small gaps between the edges of epidermal cells are visible (arrow). (H) As the *hulk* leaf (or cotyledon as shown here) expands, the epidermal cells are pulled apart and the underlying mesophyll cells are visible where holes appear (arrows). (J,K) Rips in the epidermal layer of the *hulk* leaf petiole are evident as areas where the epidermal layer has 'pealed' away from the underlying cells (arrows). (K) Mesophyll cells beneath the epidermal layer in *hulk* petioles seem to be forming a cuticle (star). (L) *acr4-2* mutant leaf petiole cells in contrast are well organised and the surface integrity of the outside layer is maintained. (M) The margins of *hulk* leaves are disorganised and irregular. Lumps of cells are visible (arrowhead) as well as gaps in the epidermis, as were seen in *hulk* cotyledons (arrow). (H,J,M) Epidermal cell identity in *hulk* mutants appears to be correctly specified, but there are differences in the numbers of stomata. The stomatal density is increased in many area (stars). In addition stomata are not properly formed and guard cells often protrude above the leaf surface. (N) Two guard cells that have not properly developed appear to be pushed out between two epidermal cells of the *hulk* leaf. (O) SEM showing stomata in an *acr4-2* mutant leaf which are flush with the epidermal cell layer. In (G) a stoma is abnormally covered by cuticle (star). Scale bars: (A-F) 500μm, (G-I) 50μm, (J-M) 100μm (N-O) 20μm.

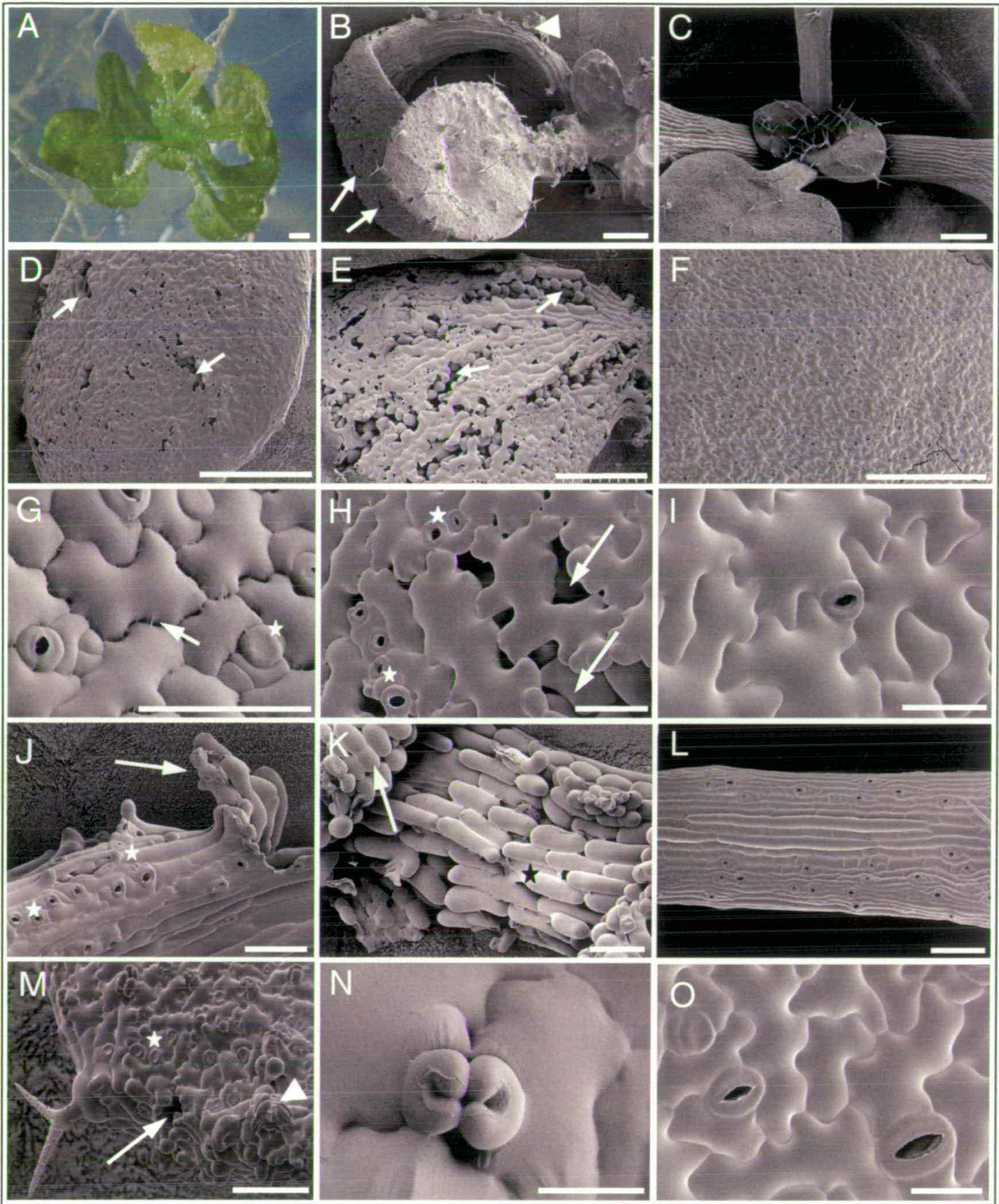


Figure V.6. Phenotype of the *hulk* mutant.

It is clear that *hulk* mutants only have a single epidermal cell layer, just as *acr4-2* mutants (there were in fact no epidermal outgrowths as it was initially thought). The mesophyll cells are visible directly through the holes in the epidermal layer (Fig. V.6.H,K). In addition the epidermal cells themselves seem to be relatively normal: they form proper trichomes (on the leaves) and stomates at the epidermal surface (compare Fig.V.6G and I; J and L). The epidermal cells however do appear to be distended, suggesting that there may be an effect on cell morphology due to the *hulk* mutation. It seems therefore that an epidermal identity is being properly specified. It seems that there is rather a loss of epidermal layer integrity in *hulk* mutants, just as is seen in *acr4* mutants. There do however appear to be consequences on the behaviour of the exposed mesophyll cells of *hulk* mutants. The mesophyll cells seem to be forming a cuticle which is visible as a 'skin' between adjacent cells (Fig.V.6K).

Interestingly *hulk* mutant seedlings have a higher density of stomata. In addition the stomata are incorrectly spaced, often being clustered together (Fig.V.6H,J,M). The *acr4-2* mutant seedling in comparison has regularly spaced stomata (see Fig.V.6O cotyledon and Fig.V.6L petiole). The morphology of many stomata in *hulk* mutants is also abnormal. Stoma in *acr4-2* mutants are composed of properly developed and connecting guard cells which appear flush with the surface of the epidermis (Fig.V.6O). In comparison the guard cells of some stomata on the leaves and cotyledons of the *hulk* mutant are pushed up on top of the epidermis surface, which gives these stomata an unusual shape (Fig.V.6N). The guard cells themselves seem to have a normal morphology but the shape of the stomatal pore seems to be abnormal. In a stoma the stomatal pore is formed between two guard cells which are connected by a ventral wall. In wild-type during stomatal development a lens-shaped thickening occurs in the middle of the ventral wall. After the wall thickening reaches a critical stage, the anticlinal walls separate in the region of the thickening to create the stomatal pore (Zhao and Sack, 1999; Bergmann, 2004). In *hulk* mutants the connections between cells are not properly formed or maintained. This is likely to impact upon the shape of the stomata, as proper connections between the two guard cells must be maintained in order to create the correct forces required for pore formation. The stomatal defects here can therefore be explained by the lack of

connection between the guard cells. As well as defects in stomatal morphology there is also a higher density of stomata on *hulk* leaf surfaces as mentioned above. The regulation of stomatal density is tightly controlled and is thought to be controlled by a diffusible lipid-soluble signal which regulates the asymmetric cell divisions involved in stomatal formation (von Groll and Altmann, 2001). If the surface integrity of the epidermal layer is not maintained, as is seen in *hulk* mutants, then signals which travel between cells to regulate cell layer patterning would likely be compromised. Such an effect on the development and distribution of stomata seems to be occurring here.

The *hulk* mutation is not dependent on the *acr4-2* background. The results of this dependency test will be discussed shortly. From the phenotypic evidence *HULK* is still a good candidate to be involved in some aspect of maintaining outside layer integrity. However it is possible that other processes are affected in *hulk* mutants which would cause the phenotype seen. One possibility is that *HULK* encodes a protein which is simply involved in maintaining correct cell adhesion as a component of the cell wall. In order to address this point a comparative analysis of the composition of the cell wall in the *hulk* mutant and the background *acr4-2* mutant was carried out in the lab by Kim Johnson. Both the aerial parts of the plant and the roots were compared. It was found that the pectin types and levels in both roots and aerial parts were normal. Apart from cellulose, pectins constitute a major part of the cell wall. They constitute most of the middle lamella which 'glues' neighbouring cells together (Brett and Waldron, 1996). As these are normal it provides some evidence that *hulk* mutants are not compromised in cell wall structure. Interestingly it seems that there are effects on cuticle formation in *hulk* mutants. The *hulk* mutants appear to be producing more cuticle than *acr4-2* mutants. Many stomata appear to be covered in cuticle (Fig.V.6G) and the underlying mesophyll layer was seen to be producing a cuticle when left exposed (Fig. V.6K). At present the possibility that *HULK* is involved in maintaining cell layer integrity by regulating the production of the cuticle is being investigated in the lab by Gwyneth C. Ingram and Kim Johnson.

When line 852 was under investigation it was found that an additional phenotype was visible in some heterozygous *hulk* lines. As well as *hulk* seedlings as described above (Fig.V.7A) there were some seedlings with similar but much less pronounced defects (Fig.V.7B). In these seedlings the *hulk*-like surface defects were visible on the cotyledons and on some leaf petioles but the leaves were almost normal. See Fig. V.7C for a comparative *acr4-2* seedling. There were obviously still some defects in the leaves of this mutant however as the seedlings were much more susceptible to drying out than *acr4-2* mutant seedlings were. When these mutant seedlings were viewed using SEM the cotyledons were seen to be as defective (full of holes) as those of *hulk* (not shown). The leaves of this mutant still had some holes in the epidermal layer but there were far fewer holes than in the epidermis of *hulk* seedling leaves. This variant of *hulk* was named *suppressor of hulk* (*sulk*). In line 852 families which carried this *hulk* variant there was roughly one *sulk* seedling for every three *hulk* seedlings. This ratio is consistent with the likely effects of a suppressing mutation. When seedlings are homozygous for *sulk* the *hulk* phenotype is slightly alleviated. Not all line 852 families carried *sulk* seedlings and thus the *hulk* and *sulk* mutations do not seem to be linked. The effects of the *HULK* and *SULK* genes perhaps counteract each other in wild-type plants.

The dependency of *hulk* on the *acr4-2* background was tested as for the other isolated mutants. Unbeknown at the time, a line carrying both the *hulk* and *sulk* mutations was crossed to Col0. In the F2 of this cross 24 *hulk* mutants, 5 *sulk* mutants and 100 seedlings which were *acr4-2* homozygous, heterozygous or ACR4-2 wild-type were seen. This ratio is accordance with the *hulk* mutant not being dependent on the *acr4-2* background. In order to confirm that the mutations were not dependent but linked (as discussed earlier), 24 mature F2 plants were screened. Four of these were found to be homozygous for the *acr4-2* mutation, consistent with no dependency and no linkage.

In summary then, various new mutants of interest were isolated by carrying out an enhancer screen on a mutagenised population of *acr4-2* mutants. The most promising candidate mutant enhancer of the *acr4-2* phenotype was found in line 852

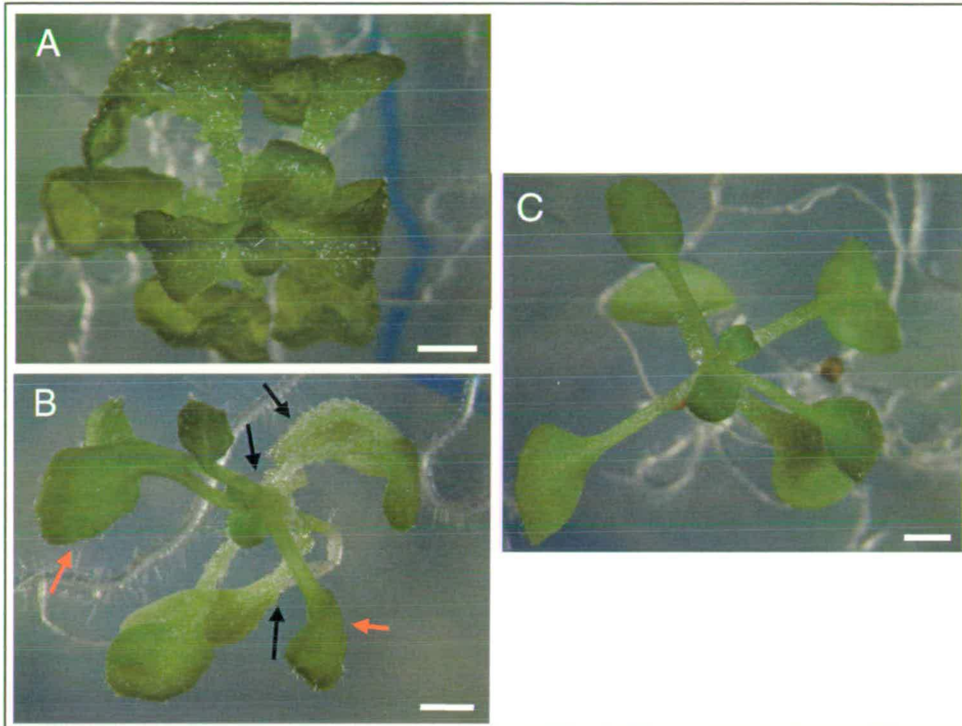


Figure V.7. Phenotypes of the *hulk* and *sulk* mutants. (A-D) Photographs of two-week old seedlings. (A) *hulk* homozygous mutant exhibiting characteristic lumpy cotyledons, petioles and leaves. (B) The *sulk* mutant has lumpy cotyledons and cotyledon petioles (black arrows) which appear white and rough in texture. The true leaves and leaf petioles in contrast appear almost as wild-type (red arrows). They are however more susceptible to desiccation than an *acr4-2* homozygous or wild-type seedlings, which gives *sulk* seedlings a limp appearance if exposed to the air for a short period of time (five minutes). (C) Wild-type sibling seedling (in an *acr4-2* mutant background). Scale bars: 1mm.

and was named *hulk*. Although *hulk* was later found not to be dependent on the *acr4-2* mutant background, the defects in epidermal cell layer integrity in *hulk* plants are still of interest. In addition a potential suppressor of the *hulk* phenotype, *sulk*, was also identified. The *hulk* and *sulk* mutants are currently being investigated in the lab as mentioned above. This work will involve further characterisation of the *hulk* and *sulk* phenotypes as well as identification of the *HULK* and *SULK* genes by mapping.

Interestingly recent research has uncovered a gene which when mutated has some similarities in phenotype to *hulk*. In a new paper by Basu, *et al.* (2004) the function of the *PIROGI* (*PIR*) gene was investigated. The *PIR* gene is part of a complex (the 'distorted group') of genes in *Arabidopsis* which is involved in remodelling the actin cytoskeleton. *PIR* function is required for maintenance of cell shape and in particular the polarised growth of trichomes. When *PIROGI* gene function is lost a range of cell shape and actin-cytoskeleton related phenotypes are seen. These include partial loss of cell adhesion between leaf cotyledon pavement cells, a phenotype which is similar to the reduced adhesion seen in *hulk* mutants. The defects are not however identical, the cell adhesion defects are of a much lower severity in *pir* mutants in comparison to those of *hulk* and the *pir* seedlings can survive on soil. In addition there are other defects such as aberrant trichome shape which are not seen in *hulk* mutants. The similarity is however interesting because a link between maintenance of cell layer integrity (as regulated by *ACR4*) and actin cytoskeleton dynamics is certainly not unlikely. As discussed in Chapter I, the actin cytoskeleton is an important factor in the regulation of cell shape, organelle positioning and intracellular transport in plant cells (Mathur and Hulskamp, 2002). It can be postulated that the regulation of actin cytoskeleton dynamics is therefore important when maintaining correct cell organisation, and *vice versa*. Similar small defects in epidermal adherence as well as trichome abnormalities were seen in *atarp* mutants which also have defects in actin polymerisation (Le, *et al.*, 2003). It is possible that there could be a direct interaction between genes such as *ATARP*, *PIROGI* and *HULK*. Upstream of these genes *ACR4* might indirectly influence their

expression or regulation. In addition there is a possibility that genes involved in regulating the actin cytoskeleton are important for the correct localisation and/or endocytosis of ACR4 (See Chapter IV). The actin cytoskeletal framework is important for transport of proteins within the cell (Mathur and Hulskamp, 2002) and for proper regulation of endocytosis (Samaj, *et al.*, 2004). These lines of investigation would be interesting to follow up as part of future research.

One problem with the use of a γ-mutagenesis screen is that the identification of the site of mutagenesis in a mutant line is a particularly time consuming process. It involves a series of crosses between the mutant line (which is in one ecotype background) and a second ecotype, followed by the tracking of genetic markers which differentiate between the two ecotypes. A second type of mutagenesis makes use of random t-DNA insertions into the background of interest. TAIL PCR can then be used to map insertion sites with more ease (Singer and Burke, 2003). One particularly useful variant of this is activation tagging which can be used to produce dominant mutations (Weigel, *et al.*, 2000; Memelink, 2003). This approach was not taken here however, partly due to time constraints resulting from the fact that t-DNA is a less penetrant mutagen than γ-rays or chemical alternatives such as EMS. In addition it was not because the starting background line (*acr4-2*) already contained a t-DNA insertion. The presence of the original t-DNA could have led to silencing of the newly inserted t-DNA. Such silencing would inhibit the activation tagging and thus gain of function mutants would not be generated. This approach can however now be taken by using the isolated TILLING lines as the starting basis. Loss of *acr4* function TILLING alleles do not contain t-DNA insertions and thus would be a useful basis for an activation tagging enhancer screen.

V.5. Other aspects of the *ACR4* signalling pathway

It has been shown here that *ACR4* does not directly interact either with genes which are specifically involved in regulating ovule development, or with elements of the cytokinin or gibberellic signalling pathways. In addition it had been shown previously that the expression pattern of *ACR4* was not affected by mutations in a number of genes involved in embryo development: *gurke*, *gnom*, *monopteros* and *ettin* (Gwyneth C. Ingram, pers. comm.) (the roles that these genes play is reviewed in Jurgens, 2001). Also in previous work the *acr4-2* mutant was crossed to an *AtML1* protein fusion line; *AtML1* is a gene which is involved in specifying the L1 layer of plants (Lu, *et al.*, 1996). *AtML1* was found to be localised to L1 cells in a normal fashion in *acr4-2* mutants (Gifford, *et al.*, 2003). These results together confirmed that the *ACR4* gene was not involved in specifying L1 cell layer identity *per se*, or involved in regulating the development of specific organs. Rather it seems that *ACR4* plays a role in maintaining the integrity of the L1 cell layer.

So far no genes have been found to interact directly with *ACR4*. The other members in an *ACR4*-directed pathway which is involved in controlling L1 integrity are therefore as yet unknown. Some of the newly isolated mutants from the γ-mutagenesis enhancer screen described in Section V.4 could be involved in this pathway. In addition, although the *hulk* mutation was not found to be dependent on the *acr4-2* background, the *HULK* gene could be involved in regulating a related process to that controlled by *ACR4*. As discussed, *hulk* is now the subject of investigation by other members of the lab. As mentioned in Chapter IV the other *ACR4*-like genes in *Arabidopsis* are also being studied. In addition a second yeast-two-hybrid screen using the extracellular domain of ACR4 is being carried out by Ross Walker in the lab to search for possible ACR4-ligands.

Recent research elsewhere has brought to light some other possible genes that might be involved with *ACR4* directly, or might be involved in other aspects of cell layer organisation. Firstly a potential link between *WUSCHEL* and *ACR4* was addressed. It was found recently that the *WUSCHEL* (*WUS*) gene which plays a role

in stem cell regulation in shoot and floral meristems, is also involved in regulating ovule development (Gross-Hardt, *et al.*, 2002). *wus* homozygous mutant seedlings terminate development prematurely, just after a few leaves are formed. As discussed in Chapter I, this termination is due to the fact that the *wus* meristem population of stem cells is not properly maintained. A role for *WUS* in controlling ovule morphogenesis was uncovered through a combination of expression pattern analysis and by rescuing *wus* seedlings to the reproductive stage (Gross-Hardt, *et al.*, 2002). *WUS* is expressed in the nucellus of ovules and the intensity of gene expression is greatest in the region where the integuments are initiated. *wus* plants were rescued to the stages of ovule development to determine whether *WUS* played a role in the nucellus. In rescued plants abnormalities in ovule morphogenesis were indeed found - *wus* mutant ovules were found to lack integuments. These defects were found to be due to compromised inter-regional signalling between the nucellus and the chalazal region during ovule formation. *WUS* activity seems to generate a signal in the nucellus which controls integument initiation in the chalaza. Therefore *WUS*, like *ACR4* plays a role in regulating ovule integument development. Interestingly the *ACR4* promoter contains three perfect putative *WUS* transcription factor binding sites (WUS binding site analysis in Lohmann, *et al.*, 2001; Hong, *et al.*, 2003). Together these two pieces of evidence suggested that there might be a genetic link between *ACR4* and *WUS*. In order to investigate this possibility double mutants between the two genes were generated and screened for any enhancement of phenotype (as for the crosses in Sections V.2 and 3).

Crosses to *acr4-2* were made in both directions for at least three heterozygous *wus-1* individuals. *wus-1* heterozygotes were also crossed to homozygous individuals carrying the single *ACR4* marker transgene (*pACR4::H2B::YFP*). This was carried out in order to determine whether loss of *wus* function had any affect on *ACR4* expression. The heterozygosity of plants in the *wus-1* mutant line was confirmed by checking for segregation of *wus-1* homozygous seedlings in the progeny of the plants crossed. In order to isolate double mutants, plants homozygous for the *acr4-2* mutation were selected by means of their seed phenotype in the F2 generation. Seed from these *acr4-2* homozygotes was collected and sown out. Any *wus-1* seedlings

that segregated out in the F3 generation were therefore double mutants. The number of seedlings and plants of each expected phenotype in the F2 and F3 generations were counted and the results for one representative cross are presented in Table V.5. Again, as in Section V.2. with the ovule development mutants, the *wus-1* mutant was in the L *er* background while *acr4-2* was in the Col0 background. However as before no differences between the phenotypes of plants with or without the *erecta* mutation were seen.

Genetic linkage was not expected between *WUS* which is on chromosome 2, and *ACR4* which is on chromosome 3. The ratios for each phenotype seen were in accordance with no genetic linkage between the two genes.

Cross performed	No plants	wild-type-like	single *acr4* mutant	single *wus* mutant	Double mutant
wus-1 x *acr4-2*					
F2 (1)	10	6	1	3*	*
F3 (6•)	17	-	9	-	8

Table V.5. Phenotypic analysis of the F2 and F3 plants generated from a cross between *wus-1* and *acr4-2*. •This F3 family originated from the one F2 plant that was homozygous for the *acr4-2* mutation. *Plants of these two genotypes would have looked indistinguishable.

The phenotype of mutant seedlings that were homozygous for both the *acr4-2* and *wus-1* alleles was found to be identical to that of *wus-1* mutant seedlings (Fig.V.8). Both the single and double mutant seedlings both terminated development prior to full leaf emergence. In addition no difference in the expression pattern of *ACR4* was seen in the root or meristem in *wus-1* homozygous seedlings in comparison to that seen in wild-type. Therefore at early stages it seems that there is no direct interaction between *ACR4-2* and *WUS*. However this does not exclude the possibility that there is an interaction later on, during ovule development. *acr4-2 wus-1* plants were not rescued to flowering as in Gross-Hardt, *et al.* (2002). It is possible that if they were, a genetic interaction between the two genes would be uncovered. This experiment would be useful to carry out as part of future work.

Another gene of interest is *PRESSED FLOWER* (*PRS*) which encodes a homeobox transcription factor that is related to *WUS* (Matsumoto and Okada, 2001). *PRS* is

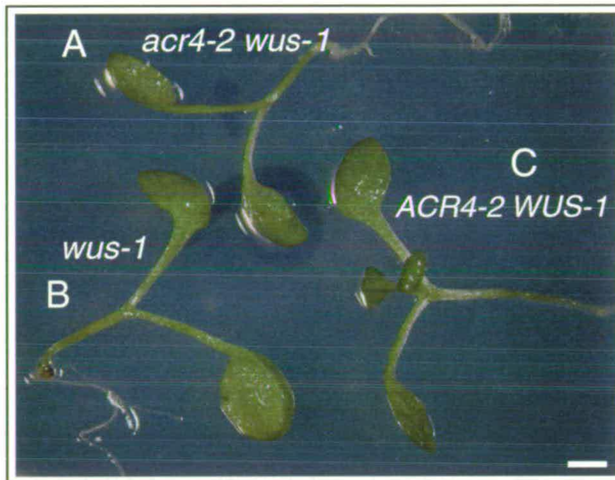


Figure V.8. Phenotype of *acr4-2 wus-1* double mutant. Photograph of 10 day old *acr4-2 wus-1* double mutant (A), *wus-1* single mutant (B) and wild-type sibling (C) seedlings. The *acr4-2 wus-1* double mutant is indistinguishable from the *wus-1* single mutant. Both lack activity at the apical meristem which normally results in production of leaf primordia and leaves (as seen in the wild-type). Scale bar: 1mm.

involved in regulating lateral axis-dependent organ development. *PRS* is expressed in L1 cells at the lateral regions of various organs including those of the floral sepals, at the sepal margins. It is the role of *PRS* in sepal margins which is of interest here. Loss of *PRS* expression results in loss of the marginal cells of the sepal. In contrast when the gene was over-expressed using *35s* enhancers sepal margin-like structures were formed over the whole sepal surface, rather than just at the margin. This results in sepals with white wrinkly structures on the edges (see Fig.V.9D). This was thought to be due to over-proliferation of cells. In addition an over-proliferation of cells was observed in the form of multicellular bulges on the stems and peduncles of *35s::PRS* plants.

Together this data led to the hypothesis that *PRS* could be involved in a process by which the proliferation of marginal cells is activated. The timing and location of *PRS* expression at the leaf margins of leaf primordia correlates with the temporal activity of the marginal meristem. *PRS* is related to the *NARROW SHEATH* (*NS*) genes in maize which are also required for the development of leaf margins (Gross-Hardt, *et al.*, 2002). Recent research into the *NS* genes has further uncovered the role of *PRS*. It seems that the *NS* genes in maize and the *PRS* gene in *Arabidopsis* are both involved in a process by which organ founder cells are recruited from a lateral domain of plant meristems.

Interestingly there are parallels between the role that *PRS* plays in sepal marginal cells and the role that *WUS* plays in ovule integument outgrowth. As mentioned *PRS* is expressed in marginal sepal cells and when there is loss of *PRS* function there is a loss of marginal sepal cells. Interestingly *WUS* is strongly expressed in the outer layer of nucellus, in cells which will form the integuments (Gross-Hardt, *et al.*, 2002). When *WUS* function is lost those integument cells do not form: the integument cells are lost. This parallel is intriguing and suggests that these two related genes have related functions in different parts of the plant. Whether over-expression of *WUS* would result in over-proliferation of integument cells is yet to be investigated.

In order to explain the role of *PRS* it can be hypothesised that there is a signal from the L2 cell layer to the L1 cell layer which is important in maintaining a single cell

thick (monolayer) outside layer. This would inhibit the division of L1 cells away from underlying cells. It could be that in tissues overexpressing *PRS* this signal is not recognised or responded to. This would therefore result in L1 cells being able to divide away from underlying cells in lateral regions where *PRS* is normally expressed. *35s::PRS* therefore would render all L1 cells blind to such an L2-L1 signal. However the outgrowths in *35s::PRS* still form properly organised back-to-back (appressed) monolayers of cells, which suggests that the communication between L1 cells is normal. The effect of *acr4* on *35s::PRS* plants would be interesting. The sepal marginal cells in *acr4* mutants lack proper organisation as a hypothetical result of loss of L1-L1 signalling. These cells are still in theory competent to respond to an L2-derived signal (as hypothesised in Chapter III). If this competence was removed, and the model for *ACR4* function holds true, then there would be loss of organisation in *35s::PRS* associated L1 outgrowths. Such an effect might result in the formation of sepals which are covered in a thick mass of disorganised L1 cells. See Figure V.9. for schematic representation of this hypothesis.

In order to test this hypothesis and therefore to examine the model of *ACR4* action, *35s::PRS* expressing Col0 and *acr4-2* plants were generated. In order to do this, firstly the *PRS* ORF was amplified from both cDNA and from genomic DNA. Gross-Hardt, *et al.* (2002) amplified *PRS* from cDNA only, but it was decided here to use *PRS* from both sources for completion. The *PRS* ORFs were cloned downstream of a promoter sequence consisting of four *35s* enhancer sequences. Two different binary vectors containing the *35s* enhancers were used to contain the *PRS* ORF from each DNA source. This made four over-expressing *PRS* vectors in total. All four vectors were transformed into both Col0 wild-type and *acr4-2* homozygous plants. Transformants were selected using antibiotic resistance in the T_1 generation. These primary transformants were then analysed as both seedlings and as mature plants. The phenotype of plants was compared between the four types of *35s::PRS* lines both within and between Col0 and *acr4-2* transformants.

The phenotype of *35s::PRS* plants in each background was compared and defects as seen in Matsumoto and Okada (2001) searched for. The type of *35s::PRS*

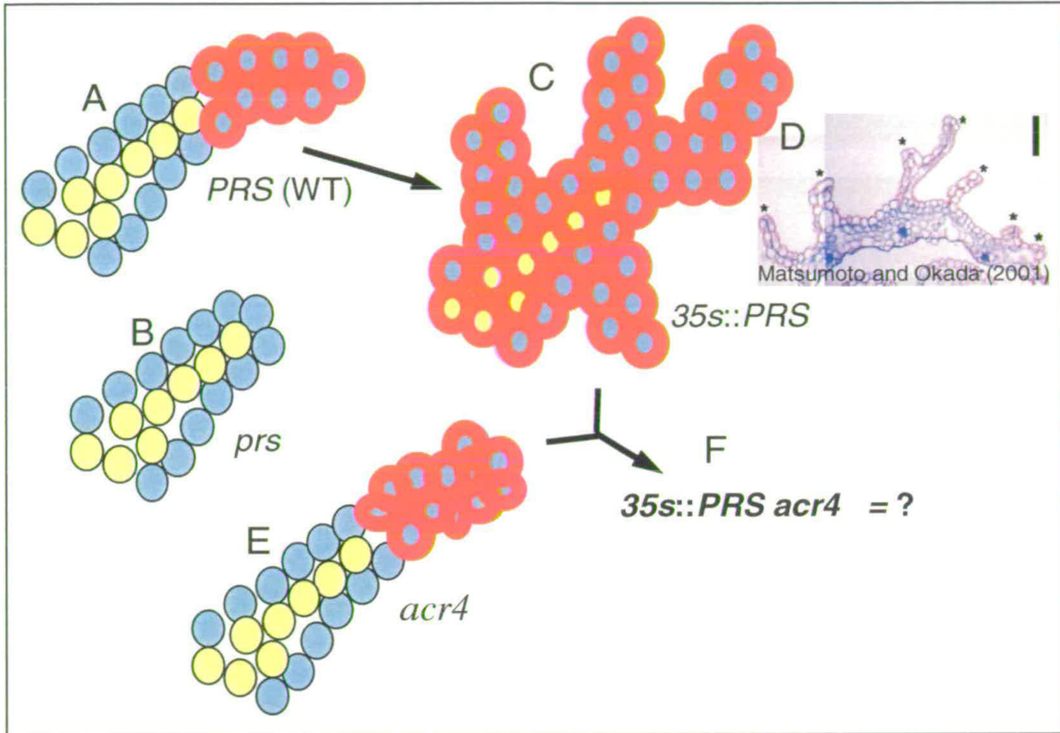


Figure V.9. Testing the hypothetical model of *ACR4* function using *PRS* over-expression. (A) *PRS* is expressed in cells of lateral organs including sepal margins as shown in this schematic. *PRS* expression is indicated by red colour. (B) Schematic showing the result of loss of *PRS* expression: loss of marginal sepal cells. (C,D) Over-expression of *PRS* using *35s* enhancers results in sepal margin-like outgrowths of L1 cells over the sepal surface. (C) Schematic of extra sepal margins. (D) Light microscopy image of extra sepal margins (asterisks) in a sectioned sepal, taken from Matsumoto and Okada (2001). This unusual outgrowth of L1 cells may be due to the L1 cells being rendered blind to a signal which emanates from the underlying L2, and usually controls L1 proliferation. (E) The expected expression pattern of *PRS* in a disorganised *acr4* mutant sepal margin. (F) It was hypothesised that *ACR4* is involved in an signalling process between cells of the L1 cell layer, helping to maintain L1 cell layer integrity. In *acr4* mutants there seems to be a lack of L1-L1 cell signalling. If *ACR4* expression is lost in *35s*::*PRS* it is hypothesised that the extra sepal margins will lose their integrity. This may result in a mass of disorganised cells being seen on the sepal surface. Scale bar: 25µm.

construct used was not found to alter the phenotype seen. Some similar phenotypes were seen in this recapitulation experiment, such as outgrowths on flower pedicels (Fig.V.10F, compare with those seen in the literature: Fig.V.10E). However these outgrowths were only seen in a very small proportion (<1%) of *35s::PRS* expressing plants. No multicellular bulges on the stems or sepals were obvious. In contrast to the literature, defects were primarily observed in a subset of seedlings in both Col0 and *acr4-2* backgrounds which carried the *35s::PRS* constructs (as Fig.V.10A-D). Mutant seedlings were stunted: the cotyledons and a few small leaf primordia emerged and then seedlings terminated development. The cotyledons and the hypocotyl of seedlings in particular had a 'lumpy' appearance with apparent outgrowths of cells. Roughly 10% of seedlings exhibited these defects although the severity varied. This lumpiness is likely to be due to some sort of over-proliferation and is therefore consistent with the effect of *35s::PRS*, albeit at a different stage in development. The *35s::PRS* transformed Col0 and *acr4-2* abnormal seedlings looked very similar, they were both small and lumpy. All other *35s::PRS* transformed seedlings in both the *acr4-2* and Col0 backgrounds appeared wild-type throughout development. Therefore after initial observations there did not seem to be any enhancement or alteration of *35s::PRS* defects in plants in the *acr4-2* background.

The effect of over-expression of *PRS* is still however of interest and to investigate this further it would be useful to express it specifically in the outside cell layer. This could be achieved by expressing *PRS* under the control of an epidermal-layer specific gene such as *FIDDLEHEAD* (*FDH*) (Lolle, *et al.*, 1997). In this more specific system i.e. *pFDH::PRS*, the effect of potential loss of an L2-L1 signalling response in *acr4* mutants could be investigated again. It would be likely that loss of such a response would result in down-regulation of factors responding to a signal from the L2 layer, which could also potentially include *ACR4*. In addition to this work the 'lumpy' seedlings which were observed in the above experiment could be examined in more detail, in order to see if any subtle alterations in cell layer organisation occur between Col0 and *acr4-2* background seedlings. Over-expressing

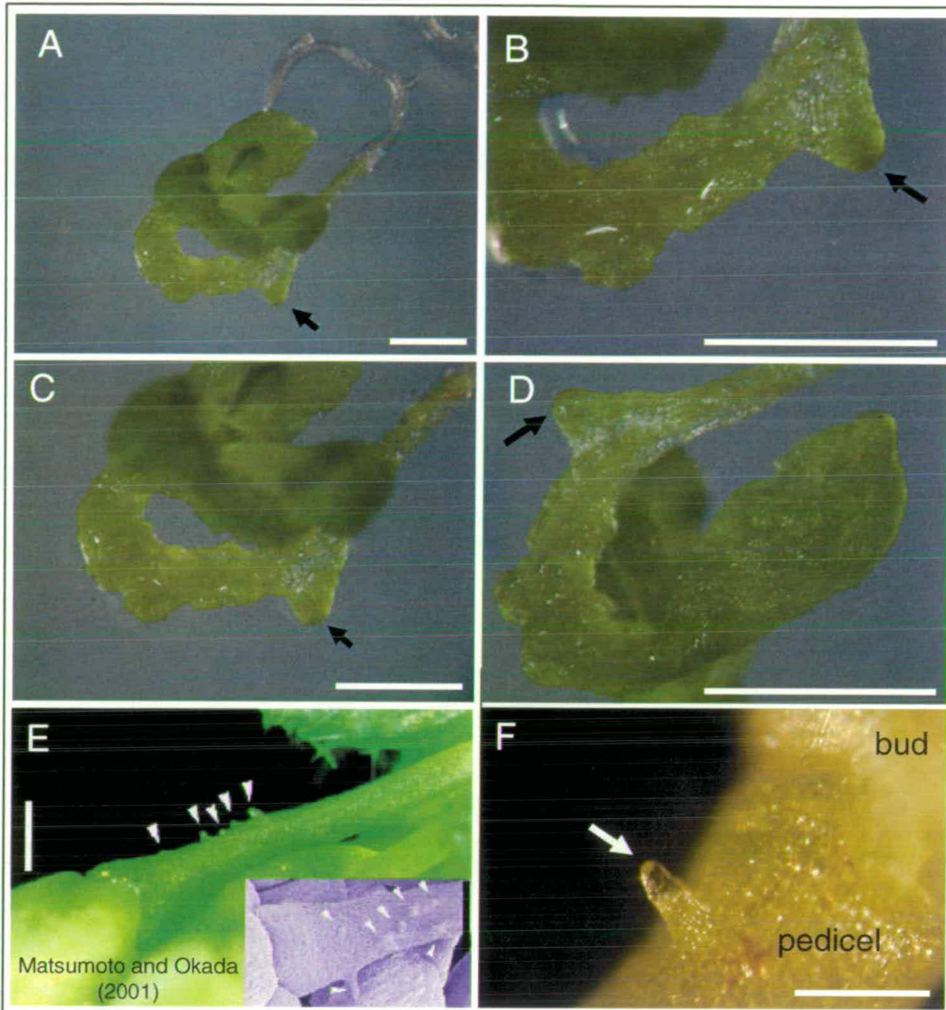


Figure V.10. Recapitulation of the over-expression of *PRS* phenotype. Photographs of the defects in a two week old seedling (A-D) and a pedicel (F) of Col0 plants expressing a *35s*::*PRS* construct. (A-D) The hypocotyl of a *35s*::*PRS* seedling is lumpy with areas of apparent over-proliferation of cells. The surface of *35s*::*PRS* seedlings is also rough in texture. One lump on the hypocotyl is labelled with an arrow for orientation in each picture. The root is shortened although fairly normal. See Fig.V.7C for comparative wild-type seedling. (E) Pedicel outgrowths of cells (arrows), taken from Matsumoto and Okada (2001). (F) Pedicel outgrowths in a recapitulated *35s*::*PRS* Col0 plant. The pedicel is normal apart from a single outgrowth (arrow) which is visible just at the base of a floral bud (bud). Scale bars: (A-D) 2mm (E-F) 500μm.

PRS seedlings in both Col0 and *acr4-2* backgrounds have been fixed for such examination.

In summary then potential genetic interactions between *ACR4* and a number of genetic pathways have been investigated. It was found that *ACR4* does not interact specifically with genes involved in ovule morphogenesis, or with several genes involved in hormone signalling pathways. An interaction between ACR4 and proteins in the gibberellic signalling pathway is still however of interest due to results from a yeast-2-hybrid study. Using an mutagenesis screen based on the *acr4-2* homozygous mutant, several new mutants which might interact with *ACR4* in maintaining the integrity of the L1 layer have been isolated. These include four mutants of special interest which appear to have defects in cell layer organisation. Whether or not the phenotype associated with new mutations was dependent on the *acr4-2* background was tested; some results from these tests are pending. One mutant, named *hulk*, with putative loss of epidermal adhesion has been the subject of preliminary phenotypic investigation. In addition an analysis of the mechanistic aspect of the role of *ACR4* was carried out. The relationship of *ACR4* to the *WUS* and *PRS* genes which are both involved in the regulation of cell outgrowth was investigated. This aspect is of particular interest and although no conclusive results were obtained here, the work has provided some clues for future approaches. A continuation of the research as mentioned above will help to better understand what part *ACR4* plays during plant development. This will be carried out as part of ongoing research into the function of *ACR4*, currently taking place in the lab.

Chapter VI. General discussion and perspectives

VI.1. *ACR4* plays a role in maintaining the integrity of the L1 layer: a conserved function across plant species?

VI.2. ACR4 appears to undergo ligand-binding mediated endocytosis and seems to be rapidly turned over in the cell: defining a concept in plant RLK behaviour

VI.3. Expanding the *ACR4* signalling pathway

VI.1. *ACR4* plays a role in maintaining the integrity of the L1 layer: a conserved function across plant species?

ACR4 had been identified as a candidate gene with a role in *Arabidopsis* epidermal specification by means of comparison with the *CR4* gene in maize. The expression pattern of *ACR4* was investigated and a loss of function analysis carried out in order to characterise the actual role of *ACR4*. *ACR4* was found to be expressed in all Ll/outside cells of the plant. These included the 'outside' or epidermal cells of the embryo, all aerial organs and meristems as well as the L1-derived ovule integuments. *ACR4* was also expressed in the lateral root cap and the epidermal layer of the root. However it was only expressed in those epidermal cells that had emerged from under the lateral root cap - only in the 'outside' epidermal cells. Expression was also detected in some 'inside' cells of the root. The role, if any, that *ACR4* plays in the inner cells of the root is unknown, although a potential link to expression of the *AUX1* influx carrier, and the auxin maximum there (Colon-Carmona, *et al.*, 2000; Benkova, *et al.*, 2003) has been postulated.

When *ACR4* function was lost, organisational defects were found in the integuments of the ovules and at sepal margins. The *acr4* phenotype was found to be maternal sporophytic. The defects seen were complemented by introduction of full length *ACR4* under the control of its own promoter. The phenotype was not consistent with an initially proposed role for *ACR4* in specifying the identity of Ll cells. However as the gene was expressed in all Ll cells it was still postulated to play a role there. Rather than being involved in specifying an outside layer identity it was postulated that *ACR4* plays a role in the organisation of Ll cells in aerial organs. A model was suggested by which the loss of *ACR4* function in Ll cells can be compensated for by a signal from the L2 cell layer. In organs or regions which have no L2 cell layer the loss of *ACR4* can not be compensated for and organisational defects are manifest. This is the case only in ovule integuments and sepal margins which are regions formed from back-to-back epidermal cell mono-layers. This model provides an explanation of why such minor defects are seen when a gene with a widespread expression pattern is knocked out. As discussed in

Chapter III it seems unlikely that *ACR4* plays a specific role in epidermal specification as suggested by Tanaka, *et al.* (2002) and Watanabe, *et al.* (2004). The evidence presented in these two papers has been discussed and in several details it may have been incorrectly interpreted.

As well as being specified through the action of genes such as *AtML1/PDF2*, the organisation of the L1 layer must also be properly maintained. In most tissues the epidermis forms a planar mono-layer, allowing it to carry out defensive functions as well as efficiently allowing gas exchange, regulation of water balance and allowing light – the energy source of the plant – to pass (reviewed in Lolle and Pruitt, 1999). In addition as mentioned in Chapter I, there are many developmentally important signals that travel through the cuticle covering the outside of the plant (Kerstiens, 1996). If the integrity of the outside layer is disrupted, this will have an affect on the function of such signals. Therefore *ACR4* defines an important function during *Arabidopsis* development. This work has also contributed to the dissection of cell layer signalling mechanisms during *Arabidopsis* development. It seems to show that there are several connected mechanisms involved in organising the outside cell layer. By removing *acr4* function an intracellular mechanism involved in this is disrupted in L1 cells. It seems however that it can be compensated for by an intercellular mechanism operating from the underlying cell layer.

ACR4 was originally postulated to be involved in epidermal specification in *Arabidopsis* due to its similarity to *CR4*, which at the time appeared to play that role in maize (Becraft, *et al.*, 1996). The research here has shown that *ACR4* may not be primarily involved in this process. In addition, as discussed in Chapter III, later research into the function of *CR4* showed that it plays a role in the differentiation of the epidermal layer, rather than the specification of epidermal cells (Jin, *et al.*, 2000; Becraft, *et al.*, 2001, Jin, *et al.*, 2000). The specification of the epidermis in maize seems to be more dependent on the function of *DEK* than *CR4* (Becraft, *et al.*, 2002). Therefore *ACR4* and *CR4* do in fact seem to play a similar role during plant development and are more functionally analogous than was originally thought. One example of this similarity is that the epidermis of *cr4* plants shows some areas of abnormal cells interspersed with normal areas (Becraft, *et al.*, 1996). This is similar

to what is seen in the integuments and sepal margins of *acr4* mutants – there is a mosaic pattern of organised and disorganised cells.

As discussed in Chapter III, *CR4* is expressed in the aleurone layer of maize and plays a role there (Becraft, *et al.*, 1996). As mentioned earlier the epidermal layer and the aleurone layer in maize have similar characteristics and so it is reasonable that they are under common control. *ACR4* does not play a role in *Arabidopsis* endosperm development. However there is no evidence of a comparable structure to the aleurone layer in *Arabidopsis*, and so this aspect of *CR4* function in maize cannot be directly compared to the function of *ACR4*. The fact that similar genes are employed in a similar process to control outside layer cell behaviour across both dicots and monocots suggests an evolutionary conserved function. There are also five *CR4*-like genes in the rice sequence database, although it remains to be seen what role they may play. In which plant species these genes originated is unknown. No similar genes can be found in the available sequence database of the bryophyte model species *Physcomitrella patens*. *Physcomitrella*, although being a multi-cellular organism has a very simple body plan in comparison to angiosperms. Most *Physcomitrella* organs, including the leaves are composed of only one layer of cells. Due to this difference it is likely that, at least in part, different genes govern cell layer morphogenesis in *Physcomitrella* compared to species such as maize and *Arabidopsis*. A functional origin for *ACR4/CR4* in higher plants seems most likely. As plant morphology became more complex in higher plants, the function of genes such as *CR4* would allow the developing layered organisation of the plant body to be organised.

The relatively weak phenotype seen in loss of function *acr4* alleles can be accounted for by functional overlap between *ACR4* and related *ACR4*-like genes, as introduced in Chapter I. The expression patterns of these genes are being investigated in the lab and functional analysis carried out in order to see if any interact with *ACR4*. Why *CR4* is more strongly responsible for control of L1 behaviour in maize than *ACR4* in *Arabidopsis* is a good question. It may be that mechanistic differences between the development of organs in monocots such as

maize and dicots such as *Arabidopsis* result in less functional overlap in maize than in *Arabidopsis*. Alternatively it may be that the degree of redundancy with other genes is changed in maize, compared to *Arabidopsis*.

As mentioned in Chapter I, the promoter of *ACR4* unusually does not contain an L1 box. It suggests that *ACR4* might be expressed in an independent pathway to *AtML1/PDF2*. This is highlighted by the fact that *ACR4* is expressed in roots whereas *AtML1/PDF2* is not (Lid, *et al.*, 2002). It seems likely that several independent pathways operate to control the development of the L1 cell layer, as evidenced by the fact that many potentially developmentally important genes are expressed in the L1 layer from early on.

VI.2. ACR4 appears to undergo ligand-binding mediated endocytosis and seems to be rapidly turned over in the cell: defining a concept in plant RLK behaviour

In order to understand more about *ACR4*, the behaviour of the ACR4 protein was investigated. Full length functional ACR4::GFP was found to be localised to the plasma membrane of cells, as predicted by the structure and as expected for RLKs (Torii and Clark, 2000). ACR4::GFP was also found in bodies within the cell which appeared to be intracellular vesicles. When roots carrying an ACR4::GFP protein fusion were treated with BFA which disrupts vesicle trafficking within the cell, the intracellular ACR4::GFP localised to BFA bodies in the cell. Later experiments carried out in the lab by Gwyneth C. Ingram showed that ACR4::GFP localises to endocytic vesicles within the cell, suggesting that ACR4 was subject to endocytosis. In addition, analysis of extracted protein suggested that ACR4 was rapidly turned over and possibly cleaved in the cell.

In order to investigate the behaviour of ACR4 in more detail a series of deletion derivatives of ACR4 were constructed and studied. Of prime significance in the results obtained was that loss of the novel 39aa repeat domain, or blocking of it by addition of an N-terminally positioned GFP, rendered ACR4 non-functional. In addition these ACR4 variants were not present in BFA-induced bodies, and seemed to be stabilised rather than turned over in the cell. The novel seven 39aa-repeat domain is predicted to adopt a β-propeller conformation and be stabilised by the formation of disulphide bridges between conserved cysteines in each repeat. TILLING analysis indicated that the conformation of this extracellular ACR4 region is vital for function.

The extracellular region of an RLK is generally proposed to be the ligand-binding domain (Torii and Clark, 2000). The results summarised above suggest a mechanism by which the 39aa repeat domain acts as a ligand binding domain for ACR4 – localisation analysis showed that was situated in the extracellular matrix, where likely ligands would be present. It seems likely that ligand binding mediates ACR4 endocytosis, as loss/obstruction of the ligand binding domain seems to result

in protein building up at the membrane, rather than being endocytosed into the cell. As discussed in Chapter I for animal receptor kinases, as in the example given of the EGF-binding ErbB1 RTK, endocytosis is one biological method by which the activity of receptor kinases can be modulated (Waterman and Yarden, 2001). It seems therefore that this process is involved in modulating the behaviour of ACR4. As mentioned in Chapter I for ErbB1, endocytosis is phosphorylation dependent – a consequence associated with ligand binding (Oved and Yarden, 2002). In addition it seems that after ligand binding, ACR4 activity is regulated by turnover/degradation in the cell; this seems to be inhibited by loss of the 39aa repeat domain. It is likely that this is involved in deactivating ACR4 after ligand binding. It is possible that the postulated cleavage products have downstream regulatory roles in the cell, although there is no evidence for this so far. The biological relevance of ACR4 endocytosis and cleavage is yet to be uncovered.

This is the first demonstration of potential ligand-binding mediated endocytosis in *Arabidopsis*. The research has therefore uncovered an important regulatory process in plants. It furthers the parallels that can be drawn between animal and plant receptor kinases. Which components are actually involved in regulating ACR4 endocytosis is one further piece of research being addressed in the lab at present. From what is postulated regarding endocytotic mechanisms which regulate RLKs in plants, a role for the KAPP protein phosphatase is suggested (Braun, *et al.*, 1997; Stone, *et al.*, 1998; Shah, *et al.*, 2002). As mentioned, KAPP has not been shown to bind CR4. Due to this, and the fact that degradation of ACR4 appears to play a role in the regulation of its function (not seen for CLV1), a different mechanism may be operating here. Whether or not KAPP binds ACR4, and the mechanism of endocytosis are currently being investigated in the lab.

It was found that loss of ACR4 kinase activity was able to be compensated for *in planta*, as had been found for CLV1 (Trotochaud, *et al.*, 1999). This result in particular pointed to a possible mechanism of action involving hetero-oligomers of ACR4 and a second protein with kinase activity. It is possible that this second protein is one of the two ACR4-like proteins that are predicted to have functional kinase domains (AtHOM3 or AtHOM4). This also fits in with the idea that there is

redundancy in the *ACR4* pathway. As mentioned in Chapter I homo-or hetero-oligomerisation is thought to be a crucial aspect of receptor kinase function in both plants and animals (Walker, 1994; Olayioye, *et al.*, 2000). Here the formation of both homo-oligomers and hetero-oligomers is proposed. The former is proposed to account for the redundancy seen when *ACR4* function is lost. The latter is proposed to account for the function of the kinase null ACR4 variant. Whether formation of oligomers is ligand-dependent or ligand-independent is yet to be seen. If the TNFR repeat-domain is involved in multimerisation by analogy to the TNFR receptor, then oligomers might be formed before ligand binding (Chan, *et al.*, 2000). In either case ligand binding appears to be required for *ACR4* signalling, transmission of a signal and downstream ACR4 turnover/processing.

What exactly the roles of the TNFR-like repeat domain and the C-terminal region of ACR4 are is as yet unclear. The TNFR-like repeat domain could be involved in some aspect of multimerism, or perhaps contribute to ligand binding. The seemingly increased stability of the ΔTNFR ACR4::GFP protein, compared to the wild-type ACR4::GFP protein, points to this domain having some importance. The answer may be uncovered through investigation and study of possible RLK partners for ACR4.

What roles *AtDEK1* and *AtSAL1,2* play in *Arabidopsis* and what relationship, if any, they have to *ACR4* is under investigation in the lab. By analogy to the *CR4* pathway in maize, *AtDEK1* might be involved in cleaving a negative regulator of *ACR4* (Becraft, *et al.*, 2002). As in maize it might play a specific role in the specification of epidermal cells. *AtSAL1/2* might be involved in regulating ACR4 trafficking or turnover in the cell.

Another important angle at which to approach the study of RLK behaviour is by the creation of dominant negative and dominant positive alleles. As shown in Chapter IV none of the proteins expressed that could have such effects, by comparison to other studies of plant RLKs (Basler, *et al.*, 1991; Dievart, *et al.*, 2003; Shpak, *et al.*, 2003), did so. They did however suggest that ACR4 may associate with other factors that could compensate for the effects of the introduced derivatives. In the lab several new approaches are underway to knock out the function of ACR4

and potential oligomeric partners by engineering new derivative proteins. If the potential partner of ACR4 as well as ACR4 itself is blocked from signalling, and the ligand sequestered, then a stronger phenotype might be seen than that of the *acr4* alleles. Combinations of deletions, such as ΔTNFR-repeats with loss of kinase activity might help to elucidate the function of all of the ACR4 domains. These approaches will also contribute to an understanding of the precise molecular mechanism of ACR4 action.

VI.3. Expanding the *ACR4* signalling pathway

It was found that *ACR4* does not play a specific role in ovule development and also does not specifically interact with elements of the gibberellic acid or cytokinin signalling pathways. It may be that the major components of the *ACR4* pathway have already been identified by comparison to *CR4*: *AtDEK* and *AtSAL1/2*. However other analysis presented here suggests some possible indirect interactions between *ACR4* and the regulation of both L1-specific processes and wider developmental controls.

Through an enhancer screen of mutagenised *acr4-2* seed several interesting mutants which could potentially be involved with *ACR4* in maintaining L1 layer integrity, were isolated. These newly isolated mutants included lines where there was defective embryo/seed development, and also lines containing putative cell-layer organisation defects. The latter grouping included the *hulk* mutation which is currently postulated to be involved in controlling cuticle deposition. Although none of these newly isolated mutants tested so far seem to be directly dependent on loss of *ACR4* function, it may be that they play a related role. As mentioned in Chapter I, the control of epidermal differentiation (cuticle formation, stomatal development etc.) is dependent on maintenance of outside layer integrity. A potential link between *ACR4* and cuticular formation is worthy of further investigation. As shown in Chapter III, *acr4* mutant sepals have an absence of cuticular decoration. This may be a downstream result of loss of L1 layer integrity. *hulk* mutants in contrast seem to have increased cuticular deposition and also disorganised L1 layer cells, partially due to a loss of cell adhesion. This is the opposite effect to that seen in *fdh* mutants which have abnormal cuticular material and associated organ fusion (Lolle, *et al.*, 1997).

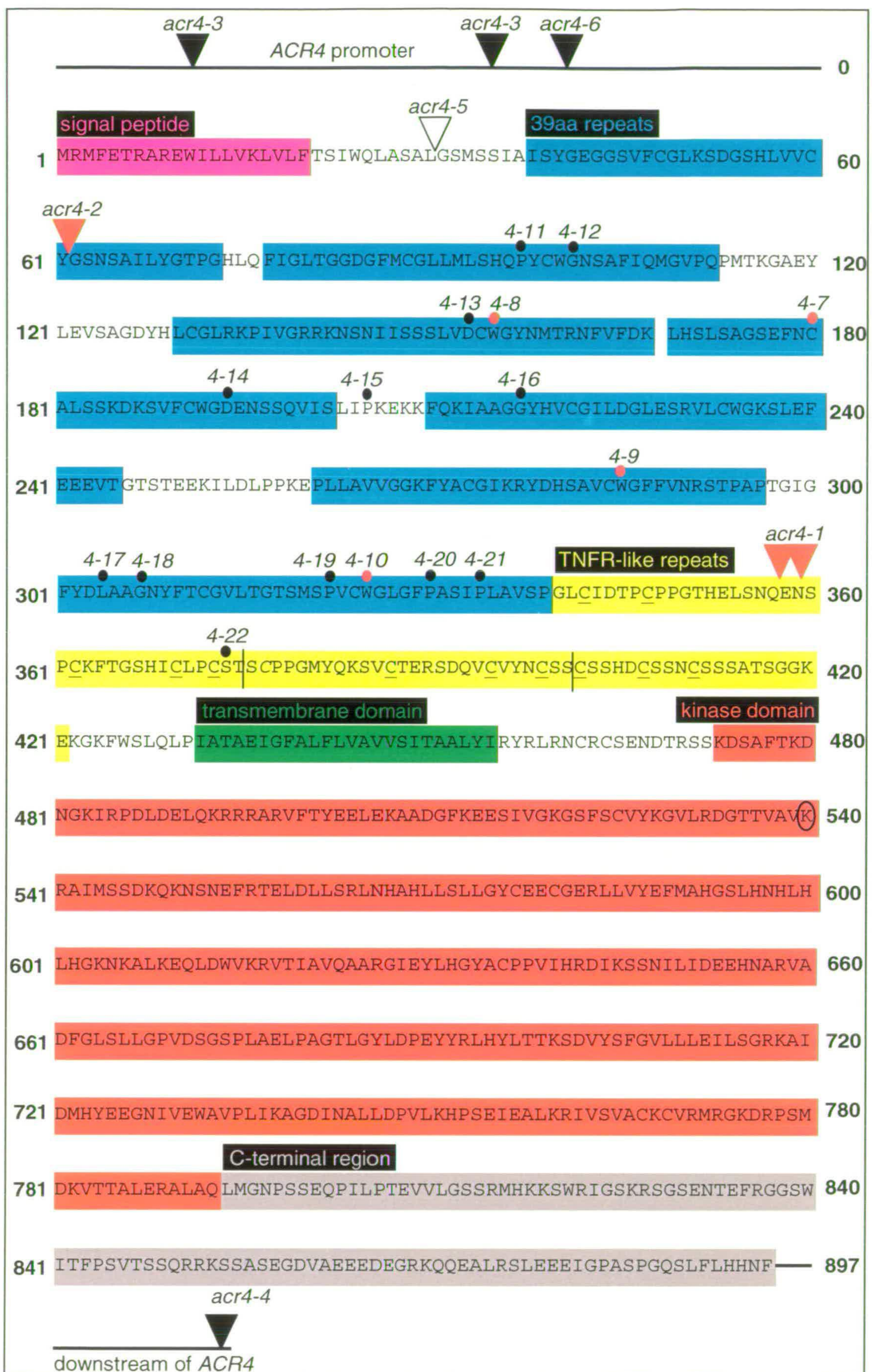
As discussed in Chapter I, a properly polarised and orientated actin cytoskeleton is important for maintaining a correctly organised outside cell layer (as well as all other cell layers) (Mathur and Hulskamp, 2002). It is likely that downstream of *ACR4* signalling the elements of the cytoskeleton are regulated. The *PIROGI* gene as discussed in Chapter V plays a role in regulation of the actin cytoskeleton (Basu, *et*

al., 2004). Interestingly *pirogi* mutants also have defects in cell-cell adhesion as seen in *hulk* mutants. It is a reasonable possibility that *ACR4* regulates genes such as *HULK* and *PIROGI.* In addition to this the cytoskeleton itself might be important for proper ACR4 localisation. It could act as a conduit for outward- and inward-going vesicles which transport ACR4 to the membrane, and are involved in its endocytosis into the cell.

A potential interaction between *ACR4* and genes such as *PRS* would provide a link between cell recruitment to the L1 layer and layer organisation. As mentioned in Chapter V, *PRS* seems to be involved in maintaining lateral regions of L1 cells (Matsumoto and Okada, 2001). It is not specifically involved in specifying sepal identity, but rather like the *NS* genes of maize seems to play an evolutionary conserved role in establishing the competence of meristem cells to become lateral founder cells (Nardmann, *et al.*, 2004). As proposed in Chapter V it seems that over-expression of *PRS* renders L1 cells blind to a signal from the L2 layer which is involved in preventing the L1 from dividing away from the L2. The interaction between this effect, and the loss of L1 layer integrity associated with the *acr4* mutation was initially investigated, but no conclusions could be drawn so far. The analysis of *ACR4* and *PRS* function started here will be continued in the lab in order to determine whether the roles played by these two genes are interlinked.

In conclusion then, the *ACR4* gene plays an important role in maintaining the integrity of the outside (L1) cell layer in aerial organs. After ligand binding, ACR4 seems to be endocytosed and rapidly turned over in the cell, consistent with its function as a receptor-like kinase. What the ligand(s) received by ACR4 are, now remains one of the major questions to be answered.

Appendix 1. Schematic of the translated amino acid sequence of the *ACR4* ORF, highlighting the ACR4 receptor-like kinase functional domains. The amino acid numbers (starting from the ATG of *ACR4*) are indicated. The promoter region (upstream of *ACR4*) and the downstream region are included, but are not drawn to scale. The three TNFR-like repeats (yellow shading) are divided by black lines. The cysteines conserved (to the TNFR receptor) within each repeat are underlined. See Fig. IV.5. for a pileup of the seven 39aa repeats showing the conserved consensus sequence. The circled lysine (K) at position 540 is altered to a methionine (M) in the kinase-null *ACR4* version. Filled triangles represent the sites of t-DNA insertions. There are two back-to-back insertions in the *acr4-1* line, two insertions in the *acr4-3* line and one insertion in each of the *acr4-2* and *acr4-6* lines; *acr4-4* contained one insertion in the *ACR4* UTR region but additional t-DNAs in the genome. The unfilled *acr4-5* triangle indicates that the line obtained did not in fact contain a t-DNA. Filled circles represent sites of amino acid substitutions in isolated TILLING lines. The TILLING sites are labelled with shorthand i.e. *4-7* corresponds to allele *acr4-7*. Red filled triangles/circles represent the t-DNA insertions or substitutions that result in loss of *ACR4* function. Black filling indicates that no visible phenotype is associated with the change.



Appendix 1. Schematic highlighting the *ACR4* ORF regions that code for the ACR4 receptor-like kinase functional domains.

References

Aarts, M. G., Keijzer, C. J., Stiekema, W. J. and Pereira, A. (1995)
Molecular characterization of the *CER1* gene of *Arabidopsis* involved in epicuticular wax biosynthesis and pollen fertility.
Plant Cell **7**: 2115-27

Abe, M., Katsumata, H., Komeda, Y. and Takahashi, T. (2003)
Regulation of shoot epidermal cell differentiation by a pair of homeodomain proteins in *Arabidopsis*.
Development **130**: 635-43

Abe, M., Takahashi, T. and Komeda, Y. (2001)
Identification of a cis-regulatory element for L1 layer-specific gene expression, which is targeted by an L1-specific homeodomain protein.
Plant J. **26**: 487-94

Aharoni, A., Dixit, S., Jetter, R., Thoenes, E., Van Arkel, G. and Pereira, A. (2004)
The SHINE clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle properties, and confers drought tolerance when overexpressed in *Arabidopsis*.
Plant Cell **16**: 2453-80

Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C. C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D. E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W. L., Berry, C. C. and Ecker, J. R. (2003)
Genome-wide insertional mutagenesis of *Arabidopsis thaliana*.
Science **301**: 653-7

Arribas, J. and Borroto, A. (2002)
Protein ectodomain shedding.
Chem. Rev. **102**: 4627-38

Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999)
Notch signaling: cell fate control and signal integration in development.
Science **284**: 770-6

Assaad, F. F., Mayer, U., Wanner, G. and Jurgens, G. (1996)
The *KEULE* gene is involved in cytokinesis in *Arabidopsis*.
Mol. Gen. Genet. **253**: 267-77

Baima, S., Possenti, M., Matteucci, A., Wisman, E., Altamura, M. M., Ruberti, I. and Morelli, G. (2001)
The *Arabidopsis* ATHB-8 HD-zip protein acts as a differentiation-promoting transcription factor of the vascular meristem.
Plant Physiol. **126**: 643-55

Baker, S. C., Robinson-Beers, K., Villanueva, J. M., Gaiser, J. C. and Gasser, C. S. (1997)
Interactions among genes regulating ovule development in *Arabidopsis thaliana.*
Genetics **145**: 1109-24

Banner, D. W., D'arcy, A., Janes, W., Gentz, R., Schoenfeld, H. J., Broger, C., Loetscher, H. and Lesslauer, W. (1993)
Crystal structure of the soluble human 55 kd TNF receptor-human TNF beta complex: implications for TNF receptor activation.
Cell **73**: 431-45

Basler, K., Christen, B. and Hafen, E. (1991)
Ligand-independent activation of the sevenless receptor tyrosine kinase changes the fate of cells in the developing *Drosophila* eye.
Cell **64**: 1069-81

Basu, D., El-Assal, S. E., Le, J., Mallery, E. L. and Szymanski, D. B. (2004)
Interchangeable functions of *Arabidopsis* PIROGI and the human WAVE complex subunit SRA1 during leaf epidermal development.
Development **131**: 4345-55

Baulcombe, D. C., Saunders, G. R., Bevan, M. W., Mayo, M. A. and Harrison, B. D. (1986)
Expression of biologically-active viral satellite RNA from the nuclear genome of transformed plants.
Nature **321**: 446-9

Becker, D. (1990)
Binary vectors which allow the exchange of plant selectable markers and reporter genes.
Nuc. Ac. Res. **18**: 203

Becraft, P. W. and Asuncion-Crabb, Y. (2000)
Positional cues specify and maintain aleurone cell fate in maize endosperm development.
Development **127**: 4039-48

Becraft, P. W., Kang, S. H. and Suh, S. G. (2001)
The maize *CRINKLY4* receptor kinase controls a cell-autonomous differentiation response.
Plant Physiol. **127**: 486-96

Becraft, P. W., Li, K., Dey, N. and Asuncion-Crabb, Y. (2002)
The maize *DEK1* gene functions in embryonic pattern formation and cell fate specification.
Development **129**: 5217-25

Becraft, P. W., Stinard, P. S. and Mccarty, D. R. (1996)
CRINKLY4: A TNFR-like receptor kinase involved in maize epidermal differentiation.
Science **273**: 1406-9

Benkova, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertova, D., Jurgens, G. and Friml, J. (2003)
Local, efflux-dependent auxin gradients as a common module for plant organ formation.
Cell **115**: 591-602

Berger, D. and Altmann, T. (2000)
A subtilisin-like serine protease involved in the regulation of stomatal density and distribution in *Arabidopsis thaliana.*
Genes Dev. **14**: 1119-31

Bergmann, D. C. (2004)
Integrating signals in stomatal development.
Curr. Opin. Plant Biol. **7**: 26-32

Bergmann, D. C., Lukowitz, W. and Somerville, C. R. (2004)
Stomatal development and pattern controlled by a MAPKK kinase.
Science **304**: 1494-7

Berleth, T. and Chatfield, S. (2002)
Embryogenesis: pattern formation from a single cell.
in *The Arabidopsis Book* American Society of Plant Biologists

Berleth, T. and Jurgens, G. (1993)
The role of the *MONOPTEROS* gene in organising the basal body region of the *Arabidopsis* embryo.
Development **118**: 575-87

Berleth, T. and Mattsson, J. (2000)
Vascular development: tracing signals along veins.
Curr. Opin. Plant Biol. **3**: 406-11

Bernhardt, C., Lee, M. M., Gonzalez, A., Zhang, F., Lloyd, A. and Schiefelbein, J. (2003)
The bHLH genes *GLABRA3* (*GL3*) and *ENHANCER OF GLABRA3* (*EGL3*) specify epidermal cell fate in the *Arabidopsis* root.
Development **130**: 6431-9

Blobel, C. P. (2000)
Remarkable roles of proteolysis on and beyond the cell surface.
Curr. Opin. Cell Biol. **12**: 606-12

Boisnard-Lorig, C., Colon-Carmona, A., Bauch, M., Hodge, S., Doerner, P., Bancharel, E., Dumas, C., Haseloff, J. and Berger, F. (2001)
Dynamic analyses of the expression of the HISTONE::YFP fusion protein in *Arabidopsis* show that syncytial endosperm is divided in mitotic domains.
Plant Cell **13**: 495-509

Bonello, J. F., Opsahl-Ferstad, H. G., Perez, P., Dumas, C. and Rogowsky, P. M. (2000)
Esr genes show different levels of expression in the same region of maize endosperm.
Gene **246**: 219-27

Bonello, J. F., Sevilla-Lecoq, S., Berne, A., Risueno, M. C., Dumas, C. and Rogowsky, P. M. (2002)
Esr proteins are secreted by the cells of the embryo surrounding region.
J. Exp. Bot. **53**: 1559-68

Brand, U., Fletcher, J. C., Hobe, M., Meyerowitz, E. M. and Simon, R. (2000)
Dependence of stem cell fate in *Arabidopsis* on a feedback loop regulated by CLV3 activity.
Science **289**: 617-9

Braun, D. M., Stone, J. M. and Walker, J. C. (1997)
Interaction of the maize and *Arabidopsis* kinase interaction domains with a subset of receptor-like protein kinases: implications for transmembrane signaling in plants.
Plant J. **12**: 83-95

Brett, C. T. and Waldron, K. W. (1996)
Physiology and Biochemistry of Plant Cell Walls.
Chapman & Hall

Broadhvest, J., Baker, S. C. and Gasser, C. S. (1999)
SHORT INTEGUMENTS 2 promotes growth during *Arabidopsis* reproductive development.
Genetics **155**: 899-907

Broun, P., Poindexter, P., Osborne, E., Jiang, C. Z. and Riechmann, J. L. (2004)
WIN1, a transcriptional activator of epidermal wax accumulation in *Arabidopsis*.
Proc. Natl. Acad. Sci. USA **101**: 4706-11

Brown, R. C., Lemmon, B. E. and Olsen, O. A. (1994)
Endosperm development in barley: microtubule involvement in the morphogenetic pathway.
Plant Cell **6**: 1241-52

Byrne, M. E., Barley, R., Curtis, M., Arroyo, J. M., Dunham, M., Hudson, A. and Martienssen, R. A. (2000)
Asymmetric leaves1 mediates leaf patterning and stem cell function in *Arabidopsis*.
Nature **408**: 967-71

Cabrillac, D., Cock, J. M., Dumas, C. and Gaude, T. (2001)
The S-locus receptor kinase is inhibited by thioredoxins and activated by pollen coat proteins.
Nature **410**: 220-3

Carpenter, G. (2003)
Nuclear localization and possible functions of receptor tyrosine kinases.
Curr. Opin. Cell. Biol. **15**: 143-8

Carraway, K. L. R. and Sweeney, C. (2001)
Localization and modulation of ErbB receptor tyrosine kinases.
Curr. Opin. Cell Biol. **13**: 125-30

Castle, L. A. and Meinke, D. W. (1994)
A *FUSCA* gene of *Arabidopsis* encodes a novel protein essential for plant development.
Plant Cell **6**: 25-41

Chan, F. K. (2000)
The pre-ligand binding assembly domain: a potential target of inhibition of tumour necrosis factor receptor function.
Ann. Rheum. Dis. **59 Suppl 1**: i50-3

Chan, F. K., Chun, H. J., Zheng, L., Siegel, R. M., Bui, K. L. and Lenardo, M. J. (2000)
A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling.
Science **288**: 2351-4

Chen, L., Cheng, J. C., Castle, L. and Sung, Z. R. (1997)
EMF genes regulate *Arabidopsis* inflorescence development.
Plant Cell **9**: 2011-24

Christensen, C. A., King, E. J., Jordan, J. R. and Drews, G. N. (1997)
Megagametogenesis in *Arabidopsis* wild type and the *Gf* mutant.
Sexual Plant Rep. **10**: 49-64

Chye, M. L., Huang, B. Q. and Zee, S. Y. (1999)
Isolation of a gene encoding *Arabidopsis* membrane-associated acyl-CoA binding protein and immunolocalization of its gene product.
Plant J. **18**: 205-14

Clark, S., Running, M. P. and Meyerowitz, E. M. (1995)
CLAVATA3 is a specific regulator of shoot and floral meristem development affecting the same processes as *CLAVATA1*.
Development **121**: 2057-67

Clark, S. E. (2001)
Cell signalling at the shoot meristem.
Nat. Rev. Mol. Cell Biol. **2**: 276-84

Clark, S. E., Jacobsen, S. E., Levin, J. Z. and Meyerowitz, E. M. (1996)
The *CLAVATA* and *SHOOT MERISTEMLESS* loci competitively regulate meristem activity in *Arabidopsis*.
Development **122**: 1567-75

Clark, S. E., Running, M. P. and Meyerowitz, E. M. (1993)
CLAVATA1, a regulator of meristem and flower development in *Arabidopsis*.
Development **119**: 397-418

Clay, N. K. and Nelson, T. (2002)
VH1, a provascular cell-specific receptor kinase that influences leaf cell patterns in *Arabidopsis*.
Plant Cell **14**: 2707-22

Clough, S. J. and Bent, A. F. (1998)
Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*.
Plant J. **16**: 735-43

Cock, J. M., Vanoosthuyse, V. and Gaude, T. (2002)
Receptor kinase signalling in plants and animals: distinct molecular systems with mechanistic similarities.
Curr. Opin. Cell Biol. **14**: 230-6

Colon-Carmona, A., Chen, D. L., Yeh, K. C. and Abel, S. (2000)
Aux/IAA proteins are phosphorylated by phytochrome *in vitro*.
Plant Physiol. **124**: 1728-38

Colon-Carmona, A. and Doerner, P. (1999)
Technical advance: spatio-temporal analysis of mitotic activity with a labile cyclin-GUS fusion protein.
Plant J. **20**: 503-8

Cui W, L. W. A. W. G. (1995)
A simple method for the transformation of Agrobacterium tumefaciens by foreign DNA.
Chin J Biotechnol. **11**: 267-74

De Wildt, R. M., Tomlinson, I. M., Ong, J. L. and Holliger, P. (2002)
Isolation of receptor-ligand pairs by capture of long-lived multivalent interaction complexes.
Proc. Natl. Acad. Sci. USA **99**: 8530-5

Delbarre, A., Muller, P., Imhof, V. and Guern, J. (1996)
Comparison of mechanisms controlling uptake and accumulation of 2,4-dichlorophenoxycetic acid, naphthalene-1-acetic acid, and indole-3-acetic acid in suspension cultured tobacco cells.
Planta **198**: 532-41

Dempsey, P. W., Doyle, S. E., He, J. Q. and Cheng, G. (2003)
The signaling adaptors and pathways activated by TNF superfamily.
Cytokine Growth Factor Rev. **14**: 193-209

Di Laurenzio, L., Wysocka-Diller, J., Malamy, J. E., Pysh, L., Helariutta, Y., Freshour, G., Hahn, M. G., Feldmann, K. A. and Benfey, P. N. (1996)
The *SCARECROW* gene regulates an asymmetric cell division that is essential for generating the radial organization of the *Arabidopsis* root.
Cell **86**: 423-33

Dievart, A. and Clark, S. E. (2003)
Using mutant alleles to determine the structure and function of leucine-rich repeat receptor-like kinases.
Curr. Opin. Plant Biol. **6**: 507-16

Dievart, A. and Clark, S. E. (2004)
LRR-containing receptors regulating plant development and defense.
Development **131**: 251-61

Dievart, A., Dalal, M., Tax, F. E., Lacey, A. D., Huttly, A., Li, J. and Clark, S. E. (2003)
CLAVATA1 dominant-negative alleles reveal functional overlap between multiple receptor kinases that regulate meristem and organ development.
Plant Cell **15**: 1198-211

Dikic, I. and Giordano, S. (2003)
Negative receptor signalling.
Curr. Opin. Cell Biol. **15**: 128-35

Dill, A. and Sun, T. (2001)
Synergistic derepression of gibberellin signaling by removing *RGA* and *GAI* function in *Arabidopsis thaliana.*
Genetics **159**: 777-85

Dinneny, J. R. and Yanofsky, M. F. (2004)
Vascular patterning: xylem or phloem?
Curr. Biol. **14**: R112-4

Doerner, P. (2003)
Plant meristems: a merry-go-round of signals.
Curr. Biol. **13**: R368-74

Dolan, L., Janmaat, K., Willemsen, V., Linstead, P., Poethig, S., Roberts, K. and Scheres, B. (1993)
Cellular organisation of the *Arabidopsis thaliana* root.
Development **119**: 71-84

Donaldson, J. G. and Jackson, C. L. (2000)
Regulators and effectors of the ARF GTPases.
Curr. Opin. Cell Biol. **12**: 475-82

Dubrovsky, J. G., Doerner, P. W., Colon-Carmona, A. and Rost, T. L. (2000)
Pericycle cell proliferation and lateral root initiation in *Arabidopsis.*
Plant Physiol. **124**: 1648-57

Dubrovsky, J. G., Rost, T. L., Colon-Carmona, A. and Doerner, P. (2001)
Early primordium morphogenesis during lateral root initiation in *Arabidopsis thaliana.*
Planta **214**: 30-6

El-Din El-Assal, S., Le, J., Basu, D., Mallery, E. L. and Szymanski, D. B. (2004)
DISTORTED2 encodes an ARPC2 subunit of the putative Arabidopsis ARP2/3 complex.
Plant J. **38**: 526-38

Emery, J. F., Floyd, S. K., Alvarez, J., Eshed, Y., Hawker, N. P., Izhaki, A., Baum, S. F. and Bowman, J. L. (2003)
Radial patterning of *Arabidopsis* shoots by class III HD-ZIP and KANADI genes.
Curr. Biol. **13**: 1768-74

Endre, G., Kereszt, A., Kevei, Z., Mihacea, S., Kalo, P. and Kiss, G. B. (2002)
A receptor kinase gene regulating symbiotic nodule development.
Nature **417**: 962-6

Fan, H. and Derynck, R. (1999)
Ectodomain shedding of TGF-alpha and other transmembrane proteins is induced by receptor tyrosine kinase activation and MAP kinase signaling cascades.
EMBO J. **18**: 6962-72

Fantl, W. J., Johnson, D. E. and Williams, L. T. (1993)
Signalling by receptor tyrosine kinases.
Annu. Rev. Biochem. **62**: 453-81

Faure, J. E., Rotman, N., Fortune, P. and Dumas, C. (2002)
Fertilization in *Arabidopsis thaliana* wild type: developmental stages and time course.
Plant J. **30**: 481-8

Feng, G. S., Hui, C. C. and Pawson, T. (1993)
SH2-containing phosphotyrosine phosphatase as a target of protein-tyrosine kinases.
Science **259**: 1607-11

Fiebig, A., Mayfield, J. A., Miley, N. L., Chau, S., Fischer, R. L. and Preuss, D. (2000)
Alterations in *CER6*, a gene identical to *CUT1*, differentially affect long-chain lipid content on the surface of pollen and stems.
Plant Cell **12**: 2001-8

Fletcher, J. C. (2002)
Shoot and floral meristem maintenance in *Arabidopsis.*
Annu. Rev. Plant Biol. **53**: 45-66

Fletcher, J. C., Brand, U., Running, M. P., Simon, R. and Meyerowitz, E. M. (1999)
Signalling of cell fate decisions by *CLAVATA3* in *Arabidopsis* shoot meristems.
Science **283**: 1911-4

Fortini, M. E. (2001)
Notch and presenilin: a proteolytic mechanism emerges.
Curr. Opin. Cell Biol. **13**: 627-34

Fowler, J. E. and Quatrano, R. S. (1997)
Plant cell morphogenesis: plasma membrane interactions with the cytoskeleton and cell wall.
Annu. Rev. Cell. Dev. Biol. **13**: 697-743

Franco-Zorrilla Jm, M. A., Solano R, Rubio V, Leyva a, Paz-Ares J. (2002)
Mutations at CRE1 impair cytokinin-induced repression of phosphate starvation responses in Arabidopsis.
Plant Journal **32**: 353-60

Frank, M. J. and Smith, L. G. (2002)
A small, novel protein highly conserved in plants and animals promotes the polarized growth and division of maize leaf epidermal cells.
Curr. Biol. **12**: 849-53

Freeman, M. and Gurdon, J. B. (2002)
Regulatory principles of developmental signaling.
Annu. Rev. Cell Dev. Biol. **18**: 515-39

Friedrichsen, D. M., Joazeiro, C. A., Li, J., Hunter, T. and Chory, J. (2000)
Brassinosteroid-insensitive-1 is a ubiquitously expressed leucine-rich repeat receptor serine/threonine kinase.
Plant Physiol. **123**: 1247-56

Friml, J., Benkova, E., Blilou, I., Wisniewska, J., Hamann, T., Ljung, K., Woody, S., Sandberg, G., Scheres, B., Jurgens, G. and Palme, K. (2002)
AtPIN4 mediates sink-driven auxin gradients and root patterning in *Arabidopsis.*
Cell **108**: 661-73

Friml, J. and Palme, K. (2002)
Polar auxin transport--old questions and new concepts?
Plant Mol. Biol. **49**: 273-84

Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa, R. and Jurgens, G. (2003)
Efflux-dependent auxin gradients establish the apical-basal axis of *Arabidopsis.*
Nature **426**: 147-53

Fukuda, H. (2004)
Signals that control plant vascular cell differentiation.
Nat. Rev. Mol. Cell Biol. **5**: 379-91

Gallois, J. L., Nora, F. R., Mizukami, Y. and Sablowski, R. (2004)
WUSCHEL induces shoot stem cell activity and developmental plasticity in the root meristem.
Genes Dev. **18**: 375-80

Galweiler, L., Guan, C., Muller, A., Wisman, E., Mendgen, K., Yephremov, A. and Palme, K. (1998)
Regulation of polar auxin transport by *AtPIN1* in *Arabidopsis* vascular tissue.
Science **282**: 2226-30

Gauthier, L. R. and Robbins, S. M. (2003)
Ephrin signaling: One raft to rule them all? One raft to sort them? One raft to spread their call and in signaling bind them?
Life Sci. **74**: 207-16

Geldner, N., Anders, N., Wolters, H., Keicher, J., Kornberger, W., Muller, P., Delbarre, A., Ueda, T., Nakano, A. and Jurgens, G. (2003)
The *Arabidopsis* GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth.
Cell **112**: 219-30

Geldner, N., Friml, J., Stierhof, Y. D., Jurgens, G. and Palme, K. (2001)
Auxin transport inhibitors block PIN1 cycling and vesicle trafficking.
Nature **413**: 425-8

Geldner, N., Richter, S., Vieten, A., Marquardt, S., Torres-Ruiz, R. A., Mayer, U. and Jurgens, G. (2004)
Partial loss-of-function alleles reveal a role for GNOM in auxin transport-related, post-embryonic development of *Arabidopsis*.
Development **131**: 389-400

Gifford, M. L., Dean, S. and Ingram, G. C. (2003)
The *Arabidopsis ACR4* gene plays a role in cell layer organisation during ovule integument and sepal margin development.
Development **130**: 4249-58

Giranton, J. L., Dumas, C., Cock, J. M. and Gaude, T. (2000)
The integral membrane S-locus receptor kinase of *Brassica* has serine/threonine kinase activity in a membranous environment and spontaneously forms oligomers in planta.
Proc. Natl. Acad. Sci. USA **97**: 3759-64

Golden, T. A., Schauer, S. E., Lang, J. D., Pien, S., Mushegian, A. R., Grossniklaus, U., Meinke, D. W. and Ray, A. (2002)
SHORT INTEGUMENTS1/SUSPENSOR1/CARPEL FACTORY, a *Dicer* homolog, is a maternal effect gene required for embryo development in *Arabidopsis*.
Plant Physiol. **130**: 808-22

Gomez-Gomez, L., Bauer, Z. and Boller, T. (2001)
Both the extracellular leucine-rich repeat domain and the kinase activity of FSL2 are required for flagellin binding and signaling in *Arabidopsis*.
Plant Cell **13**: 1155-63

Gomez-Gomez, L. and Boller, T. (2000)
FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*.
Mol. Cell **5**: 1003-11

Grandjean, O., Vernoux, T., Laufs, P., Belcram, K., Mizukami, Y. and Traas, J. (2004)
In vivo analysis of cell division, cell growth, and differentiation at the shoot apical meristem in *Arabidopsis*.
Plant Cell **16**: 74-87

Gray, J. E., Holroyd, G. H., Van Der Lee, F. M., Bahrami, A. R., Sijmons, P. C., Woodward, F. I., Schuch, W. and Hetherington, A. M. (2000)
The HIC signalling pathway links CO_2 perception to stomatal development.
Nature **408**: 713-6

Grebe, M. (2004)
Ups and downs of tissue and planar polarity in plants.
Bioessays **26**: 719-29

Grebe, M., Friml, J., Swarup, R., Ljung, K., Sandberg, G., Terlou, M., Palme, K., Bennett, M. J. and Scheres, B. (2002)
Cell polarity signaling in *Arabidopsis* involves a BFA-sensitive auxin influx pathway.
Curr. Biol. **12**: 329-34

Gross-Hardt, R. and Laux, T. (2003)
Stem cell regulation in the shoot meristem.
J. Cell Sci. **116**: 1659-66

Gross-Hardt, R., Lenhard, M. and Laux, T. (2002)
WUSCHEL signaling functions in interregional communication during *Arabidopsis* ovule development.
Genes Dev. **16**: 1129-38

Gu, T., Mazzurco, M., Sulaman, W., Matias, D. D. and Goring, D. R. (1998)
Binding of an arm repeat protein to the kinase domain of the S-locus receptor kinase.
Proc. Natl. Acad. Sci. USA **95**: 382-7

Guitton, A. E., Page, D. R., Chambrier, P., Lionnet, C., Faure, J. E., Grossniklaus, U. and Berger, F. (2004)
Identification of new members of Fertilisation Independent Seed Polycomb Group pathway involved in the control of seed development in *Arabidopsis thaliana.*
Development **131**: 2971-81

Haecker, A. and Laux, T. (2001)
Cell-cell signaling in the shoot meristem.
Curr. Opin. Plant Biol. **4**: 441-6

Hake, S. (2001)
Transcription factors on the move.
Trends Genet. **17**: 2-3

Hanks, S. K. and Quinn, A. M. (1991)
Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members.
Methods Enzymol. **200**: 38-62

Hanks, S. K., Quinn, A. M. and Hunter, T. (1988)
The protein kinase family: conserved features and deduced phylogeny of the catalytic domains.
Science **241**: 42-52

Hannoufa, A., Negruk, V., Eisner, G. and Lemieux, B. (1996)
The *CER3* gene of *Arabidopsis thaliana* is expressed in leaves, stems, roots, flowers and apical meristems.
Plant J. **10**: 459-67

Hardtke, C. S. and Berleth, T. (1998)
The *Arabidopsis* gene *MONOPTEROS* encodes a transcription factor mediating embryo axis formation and vascular development.
EMBO J. **17**: 1405-11

Hardtke, C. S., Ckurshumova, W., Vidaurre, D. P., Singh, S. A., Stamatiou, G., Tiwari, S. B., Hagen, G., Guilfoyle, T. J. and Berleth, T. (2004)
Overlapping and non-redundant functions of the *Arabidopsis* auxin response factors MONOPTEROS and NONPHOTOTROPIC HYPOCOTYL4.
Development **131**: 1089-100

Harrar, Y., Bellec, Y., Bellini, C. and Faure, J. D. (2003)
Hormonal control of cell proliferation requires *PASTICCINO* genes.
Plant Physiol. **132**: 1217-27

Harve, C., Dabos, P., Galaud, J.-P., Rouge, P. and Lescure, B. (1996)
Charactierization of an *Arabidopsis thaliana* gene that defines a new class of putative plant receptor kinases with an extracellular lectin-like domain.
J. Mol. Biol. **258**: 778-88

Haseloff, J. (1999)
GFP variants for multispectral imaging of living cells.
Methods Cell Biol. **58**: 139-51

He, Z. H., He, D. and Kohorn, B. D. (1998)
Requirement for the induced expression of a cell wall associated receptor kinase for survival during the pathogen response.
Plant J. **14**: 55-63

Heidstra, R., Welch, D. and Scheres, B. (2004)
Mosaic analyses using marked activation and deletion clones dissect *Arabidopsis SCARECROW* action in asymmetric cell division.
Genes Dev. **18**: 1964-9

Heinlein, M. (2002)
Plasmodesmata: dynamic regulation and role in macromolecular cell-to-cell signaling.
Curr. Opin. Plant Biol. **5**: 543-52

Helariutta, Y., Fukaki, H., Wysocka-Diller, J., Nakajima, K., Jung, J., Sena, G., Hauser, M. T. and Benfey, P. N. (2000)
The *SHORT-ROOT* gene controls radial patterning of the *Arabidopsis* root through radial signaling.
Cell **101**: 555-67

Heldin, C. H. (1991)
SH2 domains: elements that control protein interactions during signal transduction.
Trends Biochem. Sci. **16**: 450-2

Hicke, L. (2001)
Protein regulation by monoubiquitin.
Nat. Rev. Mol. Cell Biol. **2**: 195-201

Higuchi, M., Pischke, M. S., Mahonen, A. P., Miyawaki, K., Hashimoto, Y., Seki, M., Kobayashi, M., Shinozaki, K., Kato, T., Tabata, S., Helariutta, Y., Sussman, M. R. and Kakimoto, T. (2004)
In planta functions of the *Arabidopsis* cytokinin receptor family.
Proc. Natl. Acad. Sci. USA **101**: 8821-6

Hong, R. L., Hamaguchi, L., Busch, M. A. and Weigel, D. (2003)
Regulatory elements of the floral homeotic gene *AGAMOUS* identified by phylogenetic footprinting and shadowing.
Plant Cell **15**: 1296-309

Hudson, A. (2001)
Plant development: two sides to organ asymmetry.
Curr. Biol. **11**: R756-8

Hung, C. Y., Lin, Y., Zhang, M., Pollock, S., Marks, M. D. and Schiefelbein, J. (1998)
A common position-dependent mechanism controls cell-type patterning and *GLABRA2* regulation in the root and hypocotyl epidermis of *Arabidopsis.*
Plant Physiol. **117**: 73-84

Hunter, T. (1995)
Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling.
Cell **80**: 225-36

Hunter, T. and Lindberg, R. A. (1994)
Receptor protein-tyrosine kinases.
in *Protein kinases* IRL Press

Hutchison, C. E. and Kieber, J. J. (2002)
Cytokinin signaling in *Arabidopsis.*
Plant Cell **14 Suppl**: S47-59

Hynes, N. E., Horsch, K., Olayioye, M. A. and Badache, A. (2001)
The ErbB receptor tyrosine family as signal integrators.
Endocr. Relat. Cancer **8**: 151-9

Idriss, H. T. and Naismith, J. H. (2000)
TNF alpha and the TNF receptor superfamily: structure-function relationship(s).
Microsc. Res. Tech. **50**: 184-95

Ingram, G. C. (2004)
Between the sheets: inter-cell-layer commuication in plant development.
Philos. Trans. R. Soc. Lond. Ser. B. Biol. Sci. **359**: 891-906

Ingram, G. C., Boisnard-Lorig, C., Dumas, C. and Rogowsky, P. M. (2000)
Expression patterns of genes encoding HD-ZipIV homeo domain proteins define specific domains in maize embryos and meristems.
Plant J. **22**: 401-14

Innerarity, T. L. (2002)
Structural biology. LDL receptor's beta-propeller displaces LDL.
Science **298**: 2337-9

Irish, V. F. and Jenik, P. D. (2001)
Cell lineage, cell signaling and the control of plant morphogenesis.
Curr. Opin. Genet. Dev. **11**: 424-30

Irish, V. F. and Sussex, I. M. (1992)
A fate map of the *Arabidopsis* embryonic shoot apical meristem.
Development **115**: 745-53

Ito, M., Sentoku, N., Nishimura, A., Hong, S. K., Sato, Y. and Matsuoka, M. (2002)
Position dependent expression of GL2-type homeobox gene, *Roc1*: significance for protoderm differentiation and radial pattern formation in early rice embryogenesis.
Plant J. **29**: 497-507

Jacobsen, S. E., Binkowski, K. A. and Olszewski, N. E. (1996)
SPINDLY, a tetratricopeptide repeat protein involved in gibberellin signal transduction in *Arabidopsis*.
Proc. Natl. Acad. Sci. USA **93**: 9292-6

Jenik, P. D. and Irish, V. F. (2000)
Regulation of cell proliferation patterns by homeotic genes during *Arabidopsis* floral development.
Development **127**: 1267-76

Jenks, M. A., Tuttle, H. A., Eigenbrode, S. D. and Feldmann, K. A. (1995)
Leaf epicuticular waxes of the *eceriferum* mutants in *Arabidopsis*.
Plant Physiol. **108**: 369-77

Jeon, H. and Blacklow, S. C. (2003)
An intramolecular spin of the LDL receptor beta propeller.
Structure (Camb) **11**: 133-6

Jeong, S., Trotochaud, A. E. and Clark, S. E. (1999)
The *Arabidopsis CLAVATA2* gene encodes a receptor-like protein required for the stability of the CLAVATA1 receptor-like kinase.
Plant Cell **11**: 1925-34

Jin, P., Guo, T. and Becraft, P. W. (2000)
The maize CR4 receptor-like kinase mediates a growth factor-like differentiation response.
Genesis **27**: 104-16

Johnson, L. N., Noble, M. E. and Owen, D. J. (1996)
Active and inactive protein kinases: structural basis for regulation.
Cell **85**: 149-58

Jonsson, H., Shapiro, B. E., Meyerowitz, E. M. and Mjolsness, E. (2003)
Signaling in multicellular models of plant development.
in *On Growth, Form, and Computers* 156-61
Academic Press

Jurgens, G. (1992)
Pattern formation in the flowering plant embryo.
Curr. Opin. Genet. Dev. **2**: 567-70

Jurgens, G. (2001)
Apical-basal pattern formation in *Arabidopsis* embryogenesis.
EMBO J. **20**: 3609-16

Jurgens, G., Mayer, U., Busch, M., Lukowitz, W. and Laux, T. (1995)
Pattern formation in the *Arabidopsis* embryo: a genetic perspective.
Philos. Trans. R. Soc. Lond. Ser. B. Biol. Sci. **350**: 19-25

Jurgens, G., Mayer, U., Ramon, A., Torres Ruiz, T., Berleth, T. and Misera, S. (1991)
Genetic analysis of pattern formation in the *Arabidopsis* embryo.
Development Supp. **1**: 27-38

Kachroo, A., Schopfer, C. R., Nasrallah, M. E. and Nasrallah, J. B. (2001)
Allele-specific receptor-ligand interactions in *Brassica* self-incompatibility.
Science **293**: 1824-6

Kang, S.-G., Lee, H. J. and Suh, S.-G. (2002)
The maize *Crinkly4* gene is expressed spatially in vegetative and floral organs.
J. Pl. Biol. **45**: 219-24

Kapil, R. N. and Tiwari, S. C. (1978)
The integumentary tapetum.
Bot. Rev. **44**: 457-90

Kay, B. K., Williamson, M. P. and Sudol, M. (2000)
The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains.
Faseb J. **14**: 231-41

Kepinski, S. and Leyser, O. (2004)
Auxin-induced SCFTIR1-Aux/IAA interaction involves stable modification of the SCFTIR1 complex.
Proc. Natl. Acad. Sci. USA **101**: 12381-6

Kerstiens, G. (1996)
Signalling across the divide: a wider perspective of cuticular structure-function relationships.
Trends Plant Sci. **1**: 125-9

Kessler, S., Seiki, S. and Sinha, N. (2002)
xcl1 causes delayed oblique periclinal cell divisions in developing maize leaves, leading to cellular differentiation by lineage instead of position.
Development **129**: 1859-69

Kidner, C., Sundaresan, V., Roberts, K. and Dolan, L. (2000)
Clonal analysis of the *Arabidopsis* root confirms that position, not lineage, determines cell fate.
Planta **221**: 191-9

Klucher, K. M., Chow, H., Reiser, L. and Fischer, R. L. (1996)
The *AINTEGUMENTA* gene of *Arabidopsis* required for ovule and female gametophyte development is related to the floral homeotic gene *APETALA2*.
Plant Cell **8**: 137-53

Kobe, B. and Deisenhofer, J. (1994)
The leucine-rich repeat: a versatile binding motif.
Trends Biochem. Sci. **19**: 415-21

Kolodziejczyk, S. M. and Hall, B. K. (1996)
Signal transduction and TGF-beta superfamily receptors.
Biochem. Cell Biol. **74**: 299-314

Kowanetz, K., Husnjak, K., Holler, D., Kowanetz, M., Soubeyran, P., Hirsch, D., Schmidt, M. H., Pavelic, K., De Camilli, P., Randazzo, P. A. and Dikic, I. (2004)
CIN85 associates with multiple effectors controlling intracellular trafficking of epidermal growth factor receptors.
Mol. Biol. Cell **15**: 3155-66

Krizek, B. A. (2003)
AINTEGUMENTA utilizes a mode of DNA recognition distinct from that used by proteins containing a single AP2 domain.
Nucleic Acids Res. **31**: 1859-68

Kroiher, M., Miller, M. A. and Steele, R. E. (2001)
Deceiving appearances: signaling by "dead" and "fractured" receptor protein-tyrosine kinases.
Bioessays **23**: 69-76

Krysan, P. J., Young, J. K. and Sussman, M. R. (1999)
T-DNA as an insertional mutagen in *Arabidopsis.*
Plant Cell **11**: 2283-90

Kumar, S., Tamura, K., Jakobsen, I. B. and Nei, M. (2001)
MEGA2: molecular evolutionary genetics analysis software.
Bioinformatics **17**: 1244-5

Kurata, T., Kawabata-Awai, C., Sakuradani, E., Shimizu, S., Okada, K. and Wada, T. (2003)
The *YORE-YORE* gene regulates multiple aspects of epidermal cell differentiation in *Arabidopsis.*
Plant J. **36**: 55-66

Kurten, R. C. (2003)
Sorting motifs in receptor trafficking.
Adv. Drug Deliv. Rev. **55**: 1405-19

Larkin, J. C., Brown, M. L. and Schiefelbein, J. (2003)
How do cells know what they want to be when they grow up? Lessons from epidermal patterning in *Arabidopsis.*
Annu. Rev. Plant Biol. **54**: 403-30

Laux, T. (2003)
The stem cell concept in plants: a matter of debate.
Cell **113**: 281-3

Laux, T. and Mayer, K. F. (1998)
Cell fate regulation in the shoot meristem.
Semin. Cell Dev. Biol. **9**: 195-200

Laux, T., Mayer, K. F., Berger, J. and Jurgens, G. (1996)
The *WUSCHEL* gene is required for shoot and floral meristem integrity in *Arabidopsis.*
Development **122**: 87-96

Le, J., El-Assal Sel, D., Basu, D., Saad, M. E. and Szymanski, D. B. (2003)
Requirements for *Arabidopsis ATARP2* and *ATARP3* during epidermal development.
Curr. Biol. **13**: 1341-7

Lease, K., Ingham, E. and Walker, J. C. (1998)
Challenges in understanding RLK function.
Curr. Opin. Plant Biol. **1**: 388-92

Lemmon, M. A., Bu, Z., Ladbury, J. E., Zhou, M., Pinchasi, D., Lax, I., Engelman, D. M. and Schlessinger, J. (1997)
Two EGF molecules contribute additively to stabilization of the EGFR dimer.
Embo J. **16**: 281-94

Lenhard, M. and Laux, T. (1999)
Shoot meristem formation and maintenance.
Curr. Opin. Plant Biol. **2**: 44-50

Lenhard, M. and Laux, T. (2003)
Stem cell homeostasis in the Arabidopsis shoot meristem is regulated by intercellular movement of CLAVATA3 and its sequestration by CLAVATA1.
Development **130**: 3163-73

Leon-Kloosterziel, K. M., Keijzer, C. J. and Koornneef, M. (1994)
A seed shape mutant of *Arabidopsis* that is affected in integument development.
Plant Cell **6**: 385-92

Leyser, O. (2001)
Auxin signalling: the beginning, the middle and the end.
Curr. Opin. Plant Biol. **4**: 382-6

Li, J. and Chory, J. (1997)
A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction.
Cell **90**: 929-38

Li, J., Nam, K. H., Vafeados, D. and Chory, J. (2001)
BIN2, a new brassinosteroid-insensitive locus in Arabidopsis.
Plant Physiol. **127**: 14-22

Li, J., Wen, J., Lease, K. A., Doke, J. T., Tax, F. E. and Walker, J. C. (2002)
BAK1, an Arabidopsis LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling.
Cell **110**: 213-22

Lid, S. E., Al, R. H., Krekling, T., Meeley, R. B., Ranch, J., Opsahl-Ferstad, H. G. and Olsen, O. A. (2004)
The maize *disorganized aleurone layer 1* and *2* (*dil1*, *dil2*) mutants lack control of the mitotic division plane in the aleurone layer of developing endosperm.
Planta **218**: 370-8

Lid, S. E., Gruis, D., Jung, R., Lorentzen, J. A., Ananiev, E., Chamberlin, M., Niu, X., Meeley, R., Nichols, S. and Olsen, O. A. (2002)
The *defective kernel 1* (*dek1*) gene required for aleurone cell development in the endosperm of maize grains encodes a membrane protein of the calpain gene superfamily.
Proc. Natl. Acad. Sci. USA **99**: 5460-5

Lim, D., Park, H. U., De Castro, L., Kang, S. G., Lee, H. S., Jensen, S., Lee, K. J. and Strynadka, N. C. (2001)
Crystal structure and kinetic analysis of beta-lactamase inhibitor protein-II in complex with TEM-1 beta-lactamase.
Nat. Struct. Biol. **8**: 848-52

Lin, Y. and Schiefelbein, J. (2001)
Embryonic control of epidermal cell patterning in the root and hypocotyl of *Arabidopsis*.
Development **128**: 3697-705

Lohmann, J. U., Hong, R. L., Hobe, M., Busch, M. A., Parcy, F., Simon, R. and Weigel, D. (2001)
A molecular link between stem cell regulation and floral patterning in *Arabidopsis*.
Cell **105**: 793-803

Lolle, S. J., Berlyn, G. P., Engstrom, E. M., Krolikowski, K. A., Reiter, W. D. and Pruitt, R. E. (1997)
Developmental regulation of cell interactions in the *Arabidopsis fiddlehead-1* mutant: a role for the epidermal cell wall and cuticle.
Dev. Biol. **189**: 311-21

Lolle, S. J. and Cheung, A. Y. (1993)
Promiscuous germination and growth of wildtype pollen from *Arabidopsis* and related species on the shoot of the *Arabidopsis* mutant, *fiddlehead*.
Dev. Biol. **155**: 250-8

Lolle, S. J., Cheung, A. Y. and Sussex, I. M. (1992)
fiddlehead: an *Arabidopsis* mutant constitutively expressing an organ fusion program that involves interactions between epidermal cells.
Dev. Biol. **152**: 383-92

Lolle, S. J., Hsu, W. and Pruitt, R. E. (1998)
Genetic analysis of organ fusion in *Arabidopsis thaliana*.
Genetics **149**: 607-19

Lolle, S. J. and Pruitt, R. E. (1999)
Epidermal cell interactions: a case for local talk.
Trends Plant Sci. **4**: 14-20

Long, J. A. and Barton, M. K. (1998)
The development of apical embryonic pattern in *Arabidopsis*.
Development **125**: 3027-35

Lu, P., Porat, R., Nadeau, J. A. and O'neill, S. D. (1996)
Identification of a meristem L1 layer-specific gene in *Arabidopsis* that is expressed during embryonic pattern formation and defines a new class of homeobox genes.
Plant Cell **8**: 2155-68

Lukowitz, W., Mayer, U. and Jurgens, G. (1996)
Cytokinesis in the *Arabidopsis* embryo involves the syntaxin-related *KNOLLE* gene product.
Cell **84**: 61-71

Lukowitz, W., Roeder, A., Parmenter, D. and Somerville, C. (2004)
A MAPKK kinase gene regulates extra-embryonic cell fate in *Arabidopsis*.
Cell **116**: 109-19

Ma, H. (1993)
Protein phosphorylation in plants: enzymes, substrates and regulators.
Trends Genet. **9**: 228-30

Mahonen, A. P., Bonke, M., Kauppinen, L., Riikonen, M., Benfey, P. N. and Helariutta, Y. (2000)
A novel two-component hybrid molecule regulates vascular morphogenesis of the *Arabidopsis* root.
Genes Dev. **14**: 2938-43

Mansfield, S. G. and Briarty, L. G. (1991)
Early embryogenesis in *Arabidopsis thaliana*: I. The developing embryo.
Can. J. Bot. **69**: 461-76

Massague, J. (1992)
Receptors for the TGF-beta family.
Cell **69**: 1067-70

Masucci, J. D., Rerie, W. G., Foreman, D. R., Zhang, M., Galway, M. E., Marks, M. D. and Schiefelbein, J. W. (1996)
The homeobox gene *GLABRA2* is required for position-dependent cell differentiation in the root epidermis of *Arabidopsis thaliana*.
Development **122**: 1253-60

Masucci, J. D. and Schiefelbein, J. W. (1994)
The *rhd6* mutation of *Arabidopsis thaliana* alters root-hair initiation through an auxin- and ethylene-associated process.
Plant Physiol. **106**: 1335-46

Mathur, J. and Hulskamp, M. (2002)
Microtubules and microfilaments in cell morphogenesis in higher plants.
Curr. Biol. **12**: R669-76

Matsubayashi, Y. (2003)
Ligand-receptor pairs in plant peptide signaling.
J. Cell. Sci. **116**: 3863-70

Matsubayashi, Y., Ogawa, M., Morita, A. and Sakagami, Y. (2002)
An LRR receptor kinase involved in perception of a peptide plant hormone, phytosulfokine.
Science **296**: 1470-2

Matsumoto, N. and Okada, K. (2001)
A homeobox gene, *PRESSED FLOWER*, regulates lateral axis-dependent development of *Arabidopsis* flowers.
Genes. Dev. **15**: 3355-64

May, P., Bock, H. H. and Herz, J. (2003)
Integration of endocytosis and signal transduction by lipoprotein receptors.
Sci. STKE **2003**: PE12

Mayer, K. F., Schoof, H., Haecker, A., Lenhard, M., Jurgens, G. and Laux, T. (1998)
Role of *WUSCHEL* in regulating stem cell fate in the *Arabidopsis* shoot meristem.
Cell **95**: 805-15

Mayer, U. B., G. Jurgens, G. (1993)
Apical-basal pattern formation in the *Arabidopsis* embryo: studies on the role of the *gnom* gene.
Development **117**: 149-162

Mcelver J, T. I., Aux G, Rogers R, Ashby C, Smith K, Thomas C, Schetter a, Zhou Q, Cushman Ma, Tossberg J, Nickle T, Levin Jz, Law M, Meinke D, and Patton D (2001)
Insertional mutagenesis of genes required for seed development in Arabidopsis thaliana.
Genetics **159**: 1751-63

Meister, R. J., Williams, L. A., Monfared, M. M., Gallagher, T. L., Kraft, E. A., Nelson, C. G. and Gasser, C. S. (2004)
Definition and interactions of a positive regulatory element of the *Arabidopsis INNER NO OUTER* promoter.
Plant J. **37**: 426-38

Memelink, J. (2003)
T-DNA activation tagging.
Methods Mol. Biol. **236**: 345-62

Mineo, C., Gill, G. N. and Anderson, R. G. W. (1999)
Regulated migration of epidermal growth factor receptor from caveolae.
J. Biol. Chem. **274**: 30636-43

Mineyuki, Y. (1999)
The pre-prophase band of microtubules: its function as a cytokinetic apparatus in higher plants.
Int. Rev. Cytol. **187**: 1-49

Misera, S., Muller, A. J., Weiland-Heidecker, U. and Jurgens, G. (1994)
The *FUSCA* genes of *Arabidopsis*: negative regulators of light responses.
Mol. Gen. Genet. **244**: 242-52

Molendijk, A. J., Bischoff, F., Rajendrakumar, C. S., Friml, J., Braun, M., Gilroy, S. and Palme, K. (2001)
Arabidopsis thaliana Rop GTPases are localized to tips of root hairs and control polar growth.
EMBO J. **20**: 2779-88

Moodie, S. A. and Wolfman, A. (1994)
The 3Rs of life: Ras, Raf and growth regulation.
Trends Genet. **10**: 44-8

Moon, Y. H., Chen, L., Pan, R. L., Chang, H. S., Zhu, T., Maffeo, D. M. and Sung, Z. R. (2003)
EMF genes maintain vegetative development by repressing the flower program in *Arabidopsis*.
Plant Cell **15**: 681-93

Morris, E. R. and Walker, J. C. (2003)
Receptor-like protein kinases: the keys to response.
Curr. Opin. Plant Biol. **6**: 339-42

Morrison, I. N., Kuo, J. and O'Brien, T. P. (1975)
Histochemistry and fine structure of developing wheat aleurone cells.
Planta **123**: 105-16

Mu, J. H., Lee, H. S. and Kao, T. H. (1994)
Characterization of a pollen-expressed receptor-like kinase gene of *Petunia inflata* and the activity of its encoded kinase.
Plant Cell **6**: 709-21

Muday, G. K., Peer, W. A. and Murphy, A. S. (2003)
Vesicular cycling mechanisms that control auxin transport polarity.
Trends Plant Sci. **8**: 301-4

Murase, K., Shiba, H., Iwano, M., Che, F. S., Watanabe, M., Isogai, A. and Takayama, S. (2004)
A membrane-anchored protein kinase involved in *Brassica* self-incompatibility signaling.
Science **303**: 1516-9

Muschietti, J., Dircks, L., Vancanneyt, G. and Mccormick, S. (1994)
LAT52 protein is essential for tomato pollen development: pollen expressing antisense *LAT52* RNA hydrates and germinates abnormally and cannot achieve fertilization.
Plant J. **6**: 321-38

Nadeau, J. A. and Sack, F. D. (2002)
Stomatal development in *Arabidopsis*.
in *The Arabidopsis Book* American Society of Plant Biologists

Nakajima, K. and Benfey, P. N. (2002)
Signalling in and out: control of cell division and differentiation in the shoot and root.
Plant Cell **14**: S265-76

Nakajima, K., Sena, G., Nawy, T. and Benfey, P. N. (2001)
Intercellular movement of the putative transcription factor SHR in root patterning.
Nature **413**: 307-11

Nardmann, J., Ji, J., Werr, W. and Scanlon, M. J. (2004)
The maize duplicate genes *narrow sheath1* and *narrow sheath2* encode a conserved homeobox gene function in a lateral domain of shoot apical meristems.
Development **131**: 2827-39

Nebenfuhr, A., Ritzenthaler, C. and Robinson, D. G. (2002)
Brefeldin A: deciphering an enigmatic inhibitor of secretion.
Plant Physiol. **130**: 1102-8

Neff, M. M., Neff, J. D., Chory, J. and Pepper, A. E. (1998)
dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms: experimental applications in *Arabidopsis thaliana* genetics.
Plant J. **14**: 387-92

Negruk, V., Yang, P., Subramanian, M., Mcnevin, J. P. and Lemieux, B. (1996)
Molecular cloning and characterization of the *CER2* gene of *Arabidopsis thaliana.*
Plant J. **9**: 137-45

Neiman, A. M. (1993)
Conservation and reiteration of a kinase cascade.
Trends Genet. **9**: 390-4

Nishimura, C., Ohashi, Y., Sato, S., Kato, T., Tabata, S. and Ueguchi, C. (2004)
Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in *Arabidopsis.*
Plant Cell **16**: 1365-77

Notredame, C., Higgins, D. G. and Heringa, J. (2000)
T-Coffee: a novel method for fast and accurate multiple sequence alignment.
J. Mol. Biol. **302**: 205-17

Olayioye, M. A., Neve, R. M., Lane, H. A. and Hynes, N. E. (2000)
The ErbB signaling network: receptor heterodimerization in development and cancer.
EMBO J. **19**: 3159-67

Olsen, O. A., Linnestad, C. and Nichols, S. E. (1999)
Developmental biology of the cereal endosperm.
Trends Plant Sci. **4**: 253-7

Opsahl-Ferstad, H. G., Le Deunff, E., Dumas, C. and Rogowsky, P. M. (1997)
ZmEsr, a novel endosperm-specific gene expressed in a restricted region around the maize embryo.
Plant J. **12**: 235-46

Orlinick, J. R. and Chao, M. V. (1998)
TNF-related ligands and their receptors.
Cell Signal **10**: 543-51

Oved, S. and Yarden, Y. (2002)
Signal transduction: molecular ticket to enter cells.
Nature **416**: 133-6

Paquette, A. J. and Benfey, P. N. (2001)
Axis formation and polarity in plants.
Curr. Opin. Genet. Dev. **11**: 405-9

Park, A. R., Cho, S. K., Yun, U. J., Jin, M. Y., Lee, S. H., Sachetto-Martins, G. and Park, O. K. (2001)
Interaction of the *Arabidopsis* receptor protein kinase Wak1 with a glycine-rich protein, AtGRP-3.
J. Biol. Chem. **276**: 26688-93

Parker, G., Schofield, R., Sundberg, B. and Turner, S. (2003)
Isolation of *COV1*, a gene involved in the regulation of vascular patterning in the stem of *Arabidopsis*.
Development **130**: 2139-48

Pawson, T. and Gish, G. D. (1992)
SH2 and SH3 domains: from structure to function.
Cell **71**: 359-62

Peng, J., Carol, P., Richards, D. E., King, K. E., Cowling, R. J., Murphy, G. P. and Harberd, N. P. (1997)
The *Arabidopsis GAI* gene defines a signaling pathway that negatively regulates gibberellin responses.
Genes Dev. **11**: 3194-205

Perez-Perez, J. M., Ponce, M. R. and Micol, J. L. (2002)
The *UCU1 Arabidopsis* gene encodes a SHAGGY/GSK3-like kinase required for cell expansion along the proximodistal axis.
Dev. Biol. **242**: 161-73

Petrelli, A., Gilestro, G. F., Lanzardo, S., Comoglio, P. M., Migone, N. and Giordano, S. (2002)
The endophilin-CIN85-Cbl complex mediates ligand-dependent downregulation of c-Met.
Nature **416**: 187-90

Pike, L. J. (2003)
Lipid rafts: bringing order to chaos.
J. Lipid Res. **44**: 655-67

Pruitt, R. E., Vielle-Calzada, J. P., Ploense, S. E., Grossniklaus, U. and Lolle, S. J. (2000)
FIDDLEHEAD, a gene required to suppress epidermal cell interactions in *Arabidopsis*, encodes a putative lipid biosynthetic enzyme.
Proc. Natl. Acad. Sci. USA **97**: 1311-6

Przemeck, G. K., Mattsson, J., Hardtke, C. S., Sung, Z. R. and Berleth, T. (1996)
Studies on the role of the *Arabidopsis* gene *MONOPTEROS* in vascular development and plant cell axialization.
Planta **200**: 229-37

Pysh, L. D., Wysocka-Diller, J. W., Camilleri, C., Bouchez, D. and Benfey, P. N. (1999)
The GRAS gene family in *Arabidopsis*: sequence characterization and basic expression analysis of the *SCARECROW-LIKE* genes.
Plant J. **18**: 111-9

Rashotte, A. M., Brady, S. R., Reed, R. C., Ante, S. J. and Muday, G. K. (2000)
Basipetal auxin transport is required for gravitropism in roots of *Arabidopsis.*
Plant Physiol. **122**: 481-90

Rashotte, A. M., Jenks, M. A., Ross, A. S. and Feldmann, K. A. (2004)
Novel *eceriferum* mutants in *Arabidopsis thaliana.*
Planta **219**: 5-13

Ray, A., Lang, J. D., Golden, T. and Ray, S. (1996)
SHORT INTEGUMENT (*SIN1*), a gene required for ovule development in *Arabidopsis*, also controls flowering time.
Development **122**: 2631-8

Rebay, I., Fleming, R. J., Fehon, R. G., Cherbas, L., Cherbas, P. and Artavanis-Tsakonas, S. (1991)
Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor.
Cell **67**: 687-99

Reiser, L. and Fischer, R. L. (1993)
The ovule and embryo sac.
Plant Cell **5**: 1291-1301

Reiser, L., Modrusan, Z., Margossian, L., Samach, A., Ohad, N., Haughn, G. W. and Fischer, R. L. (1995)
The *BELL1* gene encodes a homeodomain protein involved in pattern formation in the *Arabidopsis* ovule primordium.
Cell **83**: 735-42

Rerie, W. G., Feldmann, K. A. and Marks, M. D. (1994)
The *GLABRA2* gene encodes a homeo domain protein required for normal trichome development in *Arabidopsis.*
Genes Dev. **8**: 1388-99

Ringli, C., Baumberger, N., Diet, A., Frey, B. and Keller, B. (2002)
ACTIN2 is essential for bulge site selection and tip growth during root hair development of *Arabidopsis.*
Plant Physiol. **129**: 1464-72

Rinne, P. L. and Van Der Schoot, C. (1998)
Symplasmic fields in the tunica of the shoot apical meristem coordinate morphogenetic events.
Development **125**: 1477-85

Robinson-Beers, K., Pruitt, R. E. and Gasser, C. S. (1992)
Ovule development in wild-type *Arabidopsis* and two female-sterile mutants.
Plant Cell **4**: 1237-49

Rojo, E., Sharma, V. K., Kovaleva, V., Raikhel, N. V. and Fletcher, J. C. (2002)
CLV3 is localized to the extracellular space, where it activates the *Arabidopsis* CLAVATA stem cell signaling pathway.
Plant Cell **14**: 969-77

Rothe, M., Wong, S. C., Henzel, W. J. and Goeddel, D. V. (1994)
A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor.
Cell **78**: 681-92

Rotman, N., Rozier, F., Boavida, L., Dumas, C., Berger, F. and Faure, J. E. (2003)
Female control of male gamete delivery during fertilization in *Arabidopsis thaliana.*
Curr. Biol. **13**: 432-6

Rudenko, G., Henry, L., Henderson, K., Ichtchenko, K., Brown, M. S., Goldstein, J. L. and Deisenhofer, J. (2002)
Structure of the LDL receptor extracellular domain at endosomal pH.
Science **298**: 2353-8

Ryan, E., Steer, M. and Dolan, L. (2001)
Cell biology and genetics of root hair formation in *Arabidopsis thaliana.*
Protoplasma **215**: 140-9

Saedler, R., Mathur, N., Srinivas, B. P., Kernebeck, B., Hulskamp, M. and Mathur, J. (2004)
Actin control over microtubules suggested by *DISTORTED2* encoding the Arabidopsis ARPC2 subunit homolog.
Plant. Cell. Physiol. **45**: 813-22

Samaj, J., Baluska, F., Voigt, B., Schlicht, M., Volkmann, D. and Menzel, D. (2004)
Endocytosis, actin cytoskeleton, and signaling.
Plant Physiol. **135**: 1150-61

Satina, S., Blakeslee, A. and Avery, A. (1940)
Demonstration of the three germ layers in the shoot apex of *Datura* by means of induced polyploidy in periclinal chimeras.
Amer. J. Bot. **27**: 895-905

Schafer, S. and Schmulling, T. (2002)
The *CRK1* receptor-like kinase gene of tobacco is negatively regulated by cytokinin.
Plant Mol. Biol. **50**: 155-66

Schauer, S. E., Jacobsen, S. E., Meinke, D. W. and Ray, A. (2002)
DICER-LIKE1: blind men and elephants in *Arabidopsis* development.
Trends Plant Sci. **7**: 487-91

Scheer, J. M., Pearce, G. and Ryan, C. A. (2003)
Generation of systemin signaling in tobacco by transformation with the tomato systemin receptor kinase gene.
Proc. Natl. Acad. Sci. USA **100**: 10114-7

Scheer, J. M. and Ryan, C. A., Jr. (2002)
The systemin receptor SR160 from *Lycopersicon peruvianum* is a member of the LRR receptor kinase family.
Proc. Natl. Acad. Sci. USA **99**: 9585-90

Schellmann, S., Schnittger, A., Kirik, V., Wada, T., Okada, K., Beermann, A., Thumfahrt, J., Jurgens, G. and Hulskamp, M. (2002)
TRIPTYCHON and *CAPRICE* mediate lateral inhibition during trichome and root hair patterning in *Arabidopsis.*
EMBO J. **21**: 5036-46

Scheres, B. (1993)
Cellular organisation of the *Arabidopsis thaliana* root.
Development **119**: 71-84

Scheres, B., Mckhann, H. I. and Van Den Berg, C. (1996)
Roots redefined: anatomical and genetic analysis of root development.
Plant Physiol. **111**: 959-64

Scheres, B., Wolkenfelt, H., Willemsen, V., Terlouw, M., Lawson, E., Dean, C. and Weisbeek, P. (1994)
Embryonic origin of the *Arabidopsis* primary root and root meristem initials.
Development **120**: 2475-87

Schlessinger, J. (2002)
Ligand-induced, receptor-mediated dimerization and activation of EGF receptor.
Cell **110**: 669-72

Schmidt, E. D., Guzzo, F., Toonen, M. A. and De Vries, S. C. (1997)
A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos.
Development **124**: 2049-62

Schneitz, K., Baker, S. C., Gasser, C. S. and Redweik, A. (1998)
Pattern formation and growth during floral organogenesis: *HUELLENLOS* and *AINTEGUMENTA* are required for the formation of the proximal region of the ovule primordium in *Arabidopsis thaliana.*
Development **125**: 2555-63

Schneitz, K., Hulskamp, M. and Pruitt, R. E. (1995)
Wild-type ovule development in *Arabidopsis thaliana*: a light microscope study of cleared whole-mount tissue.
Plant J. **7**: 731-49

Schnittger, A., Folkers, U., Schwab, B., Jurgens, G. and Hulskamp, M. (1999)
Generation of a spacing pattern: the role of triptychon in trichome patterning in *Arabidopsis*.
Plant Cell **11**: 1105-16

Schoof, H., Lenhard, M., Haecker, A., Mayer, K. F., Jurgens, G. and Laux, T. (2000)
The stem cell population of *Arabidopsis* shoot meristems is maintained by a regulatory loop between the *CLAVATA* and *WUSCHEL* genes.
Cell **100**: 635-44

Schweisguth, F. (2004)
Notch signaling activity.
Curr. Biol. **14**: R129-38

Schweitzer, R. and Shilo, B. Z. (1997)
A thousand and one roles for the Drosophila EGF receptor.
Trends Genet. **13**: 191-6

Sessions, A., Yanofsky, M. F. and Weigel, D. (2000)
Cell-cell signaling and movement by the floral transcription factors LEAFY and APETALA1.
Science **289**: 779-82

Sessions, A. B., E. Presting, G. Aux, G. Mcelver, J. Patton, D. Dietrich, B. Ho, P. Bacwaden, J. Ko, C. Clarke, J. D. Cotton, D. Bullis, D. Snell, J. Miguel, T. Hutchison, D. Kimmerly, B. Mitzel, T. Katagiri, F. Glazebrook, J. Law, M. Goff, S. A. (2002)
A high-throughput *Arabidopsis* reverse genetics system.
Plant Cell **14**: 2985-94

Sessions, A. W., D. Yanofsky, M. F. (1999)
The *Arabidopsis thaliana MERISTEM LAYER 1* promoter specifies epidermal expression in meristems and young primordia.
Plant J. **20**: 259-63

Shah, K., Russinova, E., Gadella, T. W., Jr., Willemse, J. and De Vries, S. C. (2002)
The *Arabidopsis* kinase-associated protein phosphatase controls internalization of the somatic embryogenesis receptor kinase 1.
Genes Dev. **16**: 1707-20

Sharma, V. K., Ramirez, J. and Fletcher, J. C. (2003)
The *Arabidopsis CLV3*-like (*CLE*) genes are expressed in diverse tissues and encode secreted proteins.
Plant Mol Biol **51**: 415-25

Shen, B., Li, C., Min, Z., Meeley, R. B., Tarczynski, M. C. and Olsen, O. A. (2003)
SAL1 determines the number of aleurone cell layers in maize endosperm and encodes a class E vacuolar sorting protein.
Proc. Natl. Acad. Sci. USA **100**: 6552-7

Shevell, D. E., Leu, W. M., Gillmor, C. S., Xia, G., Feldmann, K. A. and Chua, N. H. (1994)
EMB30 is essential for normal cell division, cell expansion, and cell adhesion in *Arabidopsis* and encodes a protein that has similarity to Sec7.
Cell **77**: 1051-62

Shiu, S. H. and Bleecker, A. B. (2001)
Plant receptor-like kinase gene family: diversity, function, and signaling.
Sci. STKE **2001**: RE22

Shiu, S. H. and Bleecker, A. B. (2001)
Receptor-like kinases from *Arabidopsis* form a monophyletic gene family related to animal receptor kinases.
Proc. Natl. Acad. Sci. USA **98**: 10763-8

Shiu, S. H. and Bleecker, A. B. (2003)
Expansion of the receptor-like kinase/Pelle gene family and receptor-like proteins in *Arabidopsis*.
Plant Physiol. **132**: 530-43

Shpak, E. D., Berthiaume, C. T., Hill, E. J. and Torii, K. U. (2004)
Synergistic interaction of three ERECTA-family receptor-like kinases controls *Arabidopsis* organ growth and flower development by promoting cell proliferation.
Development **131**: 1491-501

Shpak, E. D., Lakeman, M. B. and Torii, K. U. (2003)
Dominant-negative receptor uncovers redundancy in the Arabidopsis ERECTA Leucine-rich repeat receptor-like kinase signaling pathway that regulates organ shape.
Plant Cell **15**: 1095-110

Siezen, R. J. and Leunissen, J. A. (1997)
Subtilases: the superfamily of subtilisin-like serine proteases.
Protein Sci. **6**: 501-23

Silva, N. F. and Goring, D. R. (2002)
The *proline-rich, extensin-like receptor kinase-1* (*PERK1*) gene is rapidly induced by wounding.
Plant Mol. Biol. **50**: 667-85

Singer, S. J. (1990)
The structure and insertion of integral proteins in membranes.
Annu. Rev. Cell Biol. **6**: 247-96

Singer, T. and Burke, E. (2003)
High-throughput TAIL-PCR as a tool to identify DNA flanking insertions.
Methods Mol. Biol. **236**: 241-72
Sorokin, A., Lemmon, M. A., Ullrich, A. and Schlessinger, J. (1994)
Stabilization of an active dimeric form of the epidermal growth factor receptor by introduction of an inter-receptor disulfide bond.
J. Biol. Chem. **269**: 9752-9

Soubeyran, P., Kowanetz, K., Szymkiewicz, I., Langdon, W. Y. and Dikic, I. (2002)
Cbl-CIN85-endophilin complex mediates ligand-induced downregulation of EGF receptors.
Nature **416**: 183-7

Souter, M. and Lindsey, K. (2000)
Polarity and signalling in plant embryogenesis.
J. Exp. Bot. **51**: 971-83

Southern, E. M. (1975)
Detection of specific sequences among DNA fragments separated by gel electrophoresis.
J. Mol. Biol. **98**: 503-17

Steeves, T. A. and Sussex, I. A. (1989)
Patterns in plant development.
Cambridge University Press

Stein, J. C., Howlett, B., Boyes, D. C., Nasrallah, M. E. and Nasrallah, J. B. (1991)
Molecular cloning of a putative receptor protein kinase gene encoded at the self-incompatibility locus of *Brassica oleracea.*
Proc. Natl. Acad. Sci. USA **88**: 8816-20

Steinmann, T., Geldner, N., Grebe, M., Mangold, S., Jackson, C. L., Paris, S., Galweiler, L., Palme, K. and Jurgens, G. (1999)
Coordinated polar localization of auxin efflux carrier PIN1 by GNOM ARF GEF.
Science **286**: 316-8

Stewart, R. and Dermen, H. (1970)
Determination of number and mitotic activity of shoot apical initial cells by analysis of mericlinal chimeras.
Am. J. Bot. **57**: 816-26

Stewart, R. N. and Burk, L. G. (1970)
Independence of tissues derived from apical layers in ontegeny of the tobacco leaf and ovary.
Am. J. Bot. **57**: 1010-6

Stewart, R. N. and Dermen, H. (1975)
Flexibility in ontogeny as shown by the contribution of the shoot apical layers to the leaves of periclinal chimaeras.
Am. J. Bot. **62**: 935-47

Stone, J. M., Trotochaud, A. E., Walker, J. C. and Clark, S. E. (1998)
Control of meristem development by CLAVATA1 receptor kinases and kinase-associated protein phosphatase interactions.
Plant Physiol. **117**: 1217-25

Stone, S. L., Anderson, E. M., Mullen, R. T. and Goring, D. R. (2003)
ARC1 is an E3 ubiquitin ligase and promotes the ubiquitination of proteins during the rejection of self-incompatible *Brassica* pollen.
Plant Cell **15**: 885-98

Szymanski, D. B., Marks, M. D. and Wick, S. M. (1999)
Organized F-actin is essential for normal trichome morphogenesis in *Arabidopsis.*
Plant Cell **11**: 2331-47

Szymkowiak, E. J. and Sussex, I. M. (1992)
The internal meristem layer (L3) determines floral meristem size and carpel number in tomato periclinal chimeras.
Plant Cell **4**: 1089-100

Szymkowiak, E. J. and Sussex, I. M. (1996)
What chimeras can tell us about plant development.
Annu. Rev. Plant Physiol. Plant Mol. Biol. **47**: 351-76

Tanaka, H., Onouchi, H., Kondo, M., Hara-Nishimura, I., Nishimura, M., Machida, C. and Machida, Y. (2001)
A subtilisin-like serine protease is required for epidermal surface formation in *Arabidopsis* embryos and juvenile plants.
Development **128**: 4681-9

Tanaka, H., Watanabe, M., Watanabe, D., Tanaka, T., Machida, C. and Machida, Y. (2002)
ACR4, a putative receptor kinase gene of *Arabidopsis thaliana*, that is expressed in the outer cell layers of embryos and plants, is involved in proper embryogenesis.
Plant Cell Physiol. **43**: 419-28

Thoma, S., Hecht, U., Kippers, A., Botella, J., De Vries, S. and Somerville, C. (1994)
Tissue-specific expression of a gene encoding a cell wall-localized lipid transfer protein from *Arabidopsis.*
Plant Physiol. **105**: 35-45

Till, B. J., Colbert, T., Tompa, R., Enns, L. C., Codomo, C. A., Johnson, J. E., Reynolds, S. H., Henikoff, J. G., Greene, E. A., Steine, M. N., Comai, L. and Henikoff, S. (2003a)
High-throughput TILLING for functional genomics.
Methods Mol. Biol. **236**: 205-20

Tilney-Bassett, R. A. E. (1963)
The structure of pericinal chimeras.
Heredity **18**: 265-85

Tilney-Bassett, R. A. E. (1986)
Plant Chimeras.
Edward Arnold

Torii, K. and Clark, S. (2000)
Receptor-like kinases in plant development.
Adv. Bot. Res. **32**: 225-67

Torii, K. U., Mitsukawa, N., Oosumi, T., Matsuura, Y., Yokoyama, R., Whittier, R. F. and Komeda, Y. (1996)
The *Arabidopsis ERECTA* gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats.
Plant Cell **8**: 735-46

Traas, J. and Vernoux, T. (2002)
The shoot apical meristem: the dynamics of a stable structure.
Philos. Trans. R. Soc. Lond. Ser. B. Biol. Sci. **357**: 737-47

Trotochaud, A. E., Hao, T., Wu, G., Yang, Z. and Clark, S. E. (1999)
The CLAVATA1 receptor-like kinase requires CLAVATA3 for its assembly into a signaling complex that includes KAPP and a Rho-related protein.
Plant Cell **11**: 393-406

Ueda, M., Matsui, K., Ishiguro, S., Sano, R., Wada, T., Paponov, I., Palme, K. and Okada, K. (2004)
The *HALTED ROOT* gene encoding the 26S proteasome subunit RPT2a is essential for the maintenance of *Arabidopsis* meristems.
Development **131**: 2101-11

Ulmasov, T., Liu, Z. B., Hagen, G. and Guilfoyle, T. J. (1995)
Composite structure of auxin response elements.
Plant Cell **7**: 1611-23

Van Den Berg, C., Willemsen, V., Hage, W., Weisbeek, P. and Scheres, B. (1995)
Cell fate in the *Arabidopsis* root meristem determined by directional signalling.
Nature **378**: 62-5

Van Den Berg, C., Willemsen, V., Hendriks, G., Weisbeek, P. and Scheres, B. (1997)
Short-range control of cell differentiation in the *Arabidopsis* root meristem.
Nature **390**: 287-9
Van Den Berg, C. W., V. Hage, W. , Weisbeek, P. and Scheres, B. (1995)
Cell fate in the Arabidopsis root meristem determined by directional signalling.
Nature **378**: 62-65

Van Der Geer, P., Hunter, T. and Lindberg, R. A. (1994)
Receptor protein-tyrosine kinases and their signal transduction pathways.
Annu. Rev. Cell Biol. **10**: 251-337

Vanoosthuyse, V., Miege, C., Dumas, C. and Cock, J. M. (2001)
Two large *Arabidopsis thaliana* gene families are homologous to the *Brassica* gene superfamily that encodes pollen coat proteins and the male component of the self-incompatibility response.
Plant Mol. Biol. **46**: 17-34

Vantard, M. and Blanchoin, L. (2002)
Actin polymerization processes in plant cells.
Curr. Opin. Plant Biol. **5**: 502-6

Vaughan, J. (1955)
The morphology and growth of the vegetative and reproductive apices of *Arabidopsis thaliana* (L.) Heynh., *Capsella bursapastoris* (L.) Medic. and *Anagallis arvensis*.
L. J. Linn. Soc. Bot. **55**: 279-300

Vecchi, M., Baulida, J. and Carpenter, G. (1996)
Selective cleavage of the heregulin receptor ErbB-4 by protein kinase C activation.
J. Biol. Chem. **271**: 18989-95

Villanueva, J. M., Broadhvest, J., Hauser, B. A., Meister, R. J., Schneitz, K. and Gasser, C. S. (1999)
INNER NO OUTER regulates abaxial-adaxial patterning in *Arabidopsis* ovules.
Genes Dev. **13**: 3160-9

Von Groll, U. and Altmann, T. (2001)
Stomatal cell biology.
Curr. Opin. Plant. Biol. **4**: 555-60

Von Groll, U., Berger, D. and Altmann, T. (2002)
The subtilisin-like serine protease SDD1 mediates cell-to-cell signaling during Arabidopsis stomatal development.
Plant Cell **14**: 1527-39

Vroemen, C. W., Langeveld, S., Mayer, U., Ripper, G., Jurgens, G., Van Kammen, A. and De Vries, S. C. (1996)
Pattern formation in the *Arabidopsis* embryo revealed by position-specific lipid transfer protein gene expression.
Plant Cell **8**: 783-91

Waizenegger, I., Lukowitz, W., Assaad, F., Schwarz, H., Jurgens, G. and Mayer, U. (2000)
The *Arabidopsis KNOLLE* and *KEULE* genes interact to promote vesicle fusion during cytokinesis.
Curr. Biol. **10**: 1371-4

Walker, J. C. (1994)
Structure and function of the receptor-like protein kinases of higher plants.
Plant Mol. Biol. **26**: 1599-609

Wang, C., Barry, J. K., Min, Z., Tordsen, G., Rao, A. G. and Olsen, O. A. (2003)
The calpain domain of the maize DEK1 protein contains the conserved catalytic triad and functions as a cysteine proteinase.
J. Biol. Chem. **278**: 34467-74

Wang, X., Zafian, P., Choudhary, M. and Lawton, M. (1996)
The PR5K receptor protein kinase from *Arabidopsis thaliana* is structurally related to a family of plant defense proteins.
Proc. Natl. Acad. Sci. USA **93**: 2598-602

Wang, Z. Y., Seto, H., Fujioka, S., Yoshida, S. and Chory, J. (2001)
BRI1 is a critical component of a plasma-membrane receptor for plant steroids.
Nature **410**: 380-3

Watanabe, M., Tanaka, H., Watanabe, D., Machida, C. and Machida, Y. (2004)
The ACR4 receptor-like kinase is required for surface formation of epidermis-related tissues in *Arabidopsis thaliana.*
Plant J. **39**: 298-308

Waterman, H. and Yarden, Y. (2001)
Molecular mechanisms underlying endocytosis and sorting of ErbB receptor tyrosine kinases.
FEBS (Fed Eur Biochem Soc) Lett. **490**: 142-52

Webb, M., Jouannic, S., Foreman, J., Linstead, P. and Dolan, L. (2002)
Cell specification in the *Arabidopsis* root epidermis requires the activity of ECTOPIC ROOT HAIR 3--a katanin-p60 protein.
Development **129**: 123-31

Wegner, J. (2003)
On the developmental analogy of the shoot apex and the leaf margin and the genesis of L1-derived mesophyll areas in the leaf margin.
Plant Sci. **164**: 565-9

Weigel, D., Ahn, J. H., Blazquez, M. A., Borevitz, J. O., Christensen, S. K., Fankhauser, C., Ferrandiz, C., Kardailsky, I., Malancharuvil, E. J., Neff, M. M., Nguyen, J. T., Sato, S., Wang, Z. Y., Xia, Y., Dixon, R. A., Harrison, M. J., Lamb, C. J., Yanofsky, M. F. and Chory, J. (2000)
Activation tagging in *Arabidopsis.*
Plant Physiol. **122**: 1003-13

Western, T. L. and Haughn, G. W. (1999)
BELL1 and *AGAMOUS* genes promote ovule identity in *Arabidopsis thaliana.*
Plant J. **18**: 329-36

Willemsen, V., Friml, J., Grebe, M., Van Den Toorn, A., Palme, K. and Scheres, B. (2003)
Cell polarity and PIN protein positioning in *Arabidopsis* require *STEROL METHYLTRANSFERASE1* function.
Plant Cell **15**: 612-25

Williams, R. W. (1998)
Plant homeobox genes: many functions stem from a common motif.
Bioessays **20**: 280-2

Xu, L., Xu, Y., Dong, A., Sun, Y., Pi, L. and Huang, H. (2003)
Novel *as1* and *as2* defects in leaf adaxial-abaxial polarity reveal the requirement for *ASYMMETRIC LEAVES1* and *2* and *ERECTA* functions in specifying leaf adaxial identity.
Development **130**: 4097-107

Yamauchi, T., Ueki, K., Tobe, K., Tamemoto, H., Sekine, N., Wada, M., Honjo, M., Takahashi, M., Takahashi, T., Hirai, H., Tushima, T., Akanuma, Y., Fujita, T., Komuro, I., Yazaki, Y. and Kadowaki, T. (1997)
Tyrosine phosphorylation of the EGF receptor by the kinase Jak2 is induced by growth hormone.
Nature **390**: 91-6

Yang, C. H., Chen, L. J. and Sung, Z. R. (1995)
Genetic regulation of shoot development in *Arabidopsis*: role of the *EMF* genes.
Dev. Biol. **169**: 421-35

Yephremov, A., Wisman, E., Huijser, P., Huijser, C., Wellesen, K. and Saedler, H. (1999)
Characterization of the *FIDDLEHEAD* gene of *Arabidopsis* reveals a link between adhesion response and cell differentiation in the epidermis.
Plant Cell **11**: 2187-201

Yin, Y., Wang, Z. Y., Mora-Garcia, S., Li, J., Yoshida, S., Asami, T. and Chory, J. (2002)
BES1 accumulates in the nucleus in response to brassinosteroids to regulate gene expression and promote stem elongation.
Cell **109**: 181-91

Zhang, D., Sliwkowski, M. X., Mark, M., Frantz, G., Akita, R., Sun, Y., Hillan, K., Crowley, C., Brush, J. and Godowski, P. J. (1997)
Neuregulin-3 (NRG3): a novel neural tissue-enriched protein that binds and activates ErbB4.
Proc. Natl. Acad. Sci. USA **94**: 9562-7

Zhao, J., Peng, P., Schmitz, R. J., Decker, A. D., Tax, F. E. and Li, J. (2002)
Two putative BIN2 substrates are nuclear components of brassinosteroid signaling.
Plant Physiol. **130**: 1221-9

Zhao, L. and Sack, F. D. (1999)
Ultrastructure of stomatal development in *Arabidopsis* (*Brassicaceae*) leaves.
Am. J. Bot. **86**: 929-39

Zhong, R. and Ye, Z. H. (2004)
Amphivasal vascular bundle 1, a gain-of-function mutation of the *IFL1/REV* gene, is associated with alterations in the polarity of leaves, stems and carpels.
Plant Cell Physiol. **45**: 369-85

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

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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′-CTCGAGAATGAGTAAAGGAGAAGAAC and 5′-TCTAGTGTTTGTATAGTTCATCCATG 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′-AGGATCCGTCCGGATCTTGATGAG 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′- GGAACCACTGTTGCAGTGATGAGAGCGATAATGTC 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′- CTCTCTGCCTCTTTGTTACTTTCCTGCCT 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′-GTGGGAAAGCGCGTTACAAGAAAGC 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′-AGCTAGCATGAGTAAAGGAGAAGAAC 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,

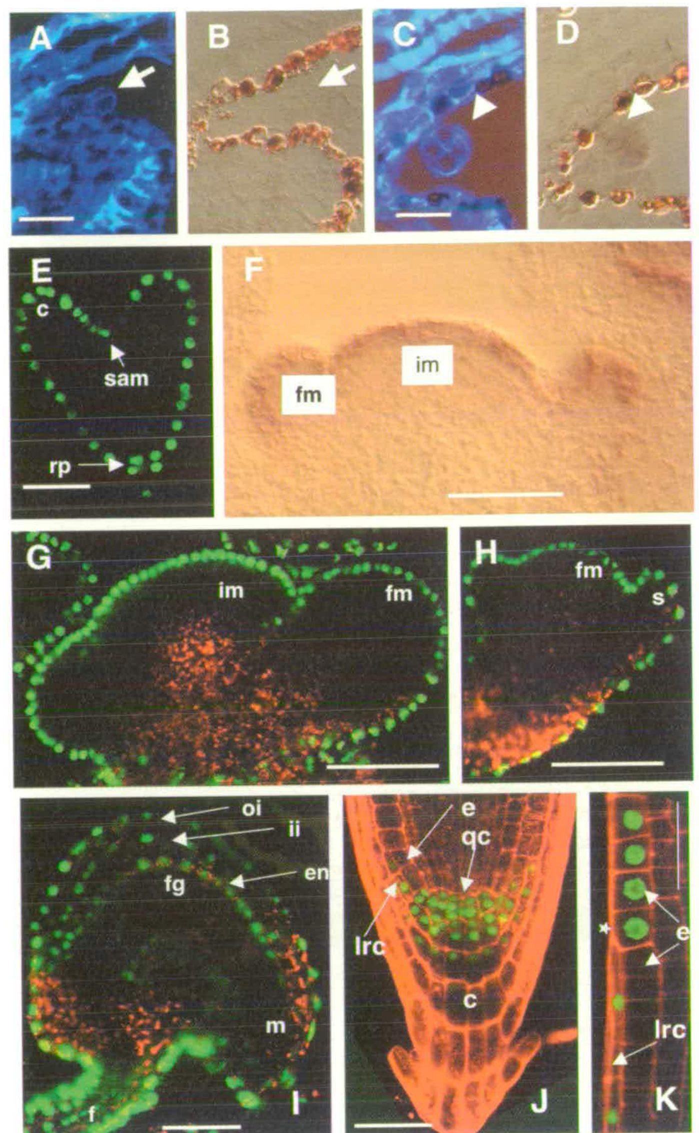

Fig. 1. Expression of *ACR4* during development. (A,C) Fluorescence images of two- to four-cell (A) and eight-cell (C) embryos (arrows). (B,D) In situ hybridisations of the same sections with *ACR4* antisense probe. Expression is detected as light brown coloration in the eight-cell embryos but not in 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

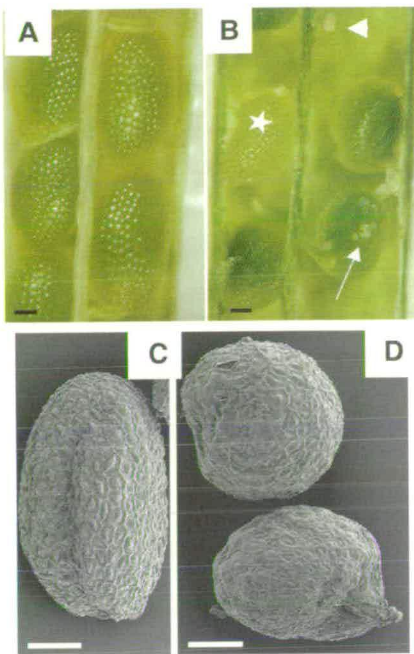

Fig. 2. *acr4* mutant seed phenotype. (A,B) Opened siliques showing differences in seed size and texture between a wild-type (A) and homozygous (B) plant at comparable stages (12 days after pollination). Aborted ovules (arrowhead), retarded seeds (star) and seeds with epidermal outgrowths (arrow) are 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.

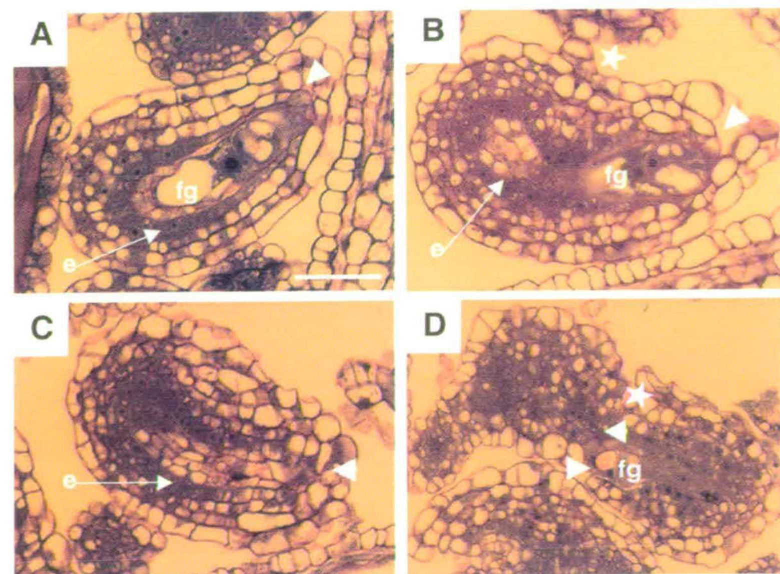

Fig. 3. The internal structure of *acr4* mutant ovules. Wild-type (A) compared with mutant (B,C,D) ovules. Micropyles are indicated by arrowheads. Female gametophytes (fg) and densely staining endothelial cells (e) are labelled where present. (A) In wild type neatly organised cell layers are visible, 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.

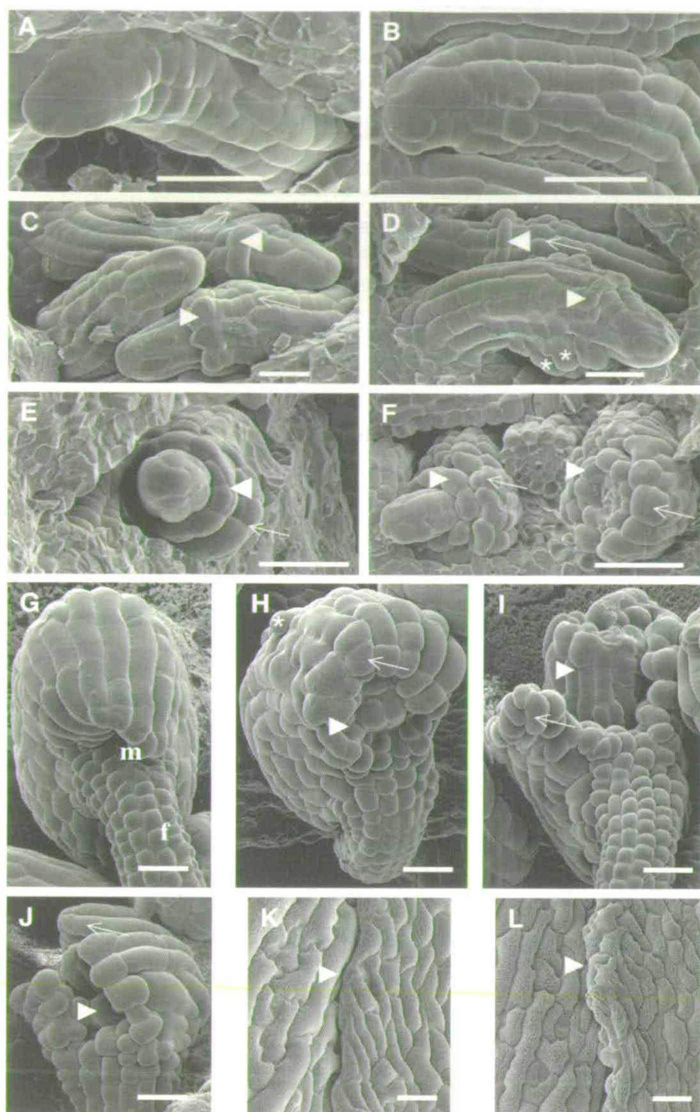

Fig. 4. Phenotypic analysis of wild-type and *acr4* mutant plants. (A,B) Ovule primordia immediately prior to integument initiation in wild-type (A) and mutant (B) plants. (C,D) The initiation of the inner (arrowheads) and outer (arrows) integuments in wild-type and mutant ovules respectively. In the 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).

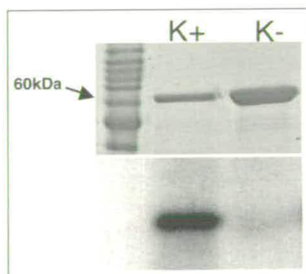

Fig. 5. ACR4 kinase activity. Coomassie Blue-stained gel (top) showing products of kinase assay on the wild-type ACR4 kinase domain (K+) and kinase null variant (negative control, K–). Autoradiography of this gel (below) shows that the native kinase domain has kinase activity whereas the kinase null 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

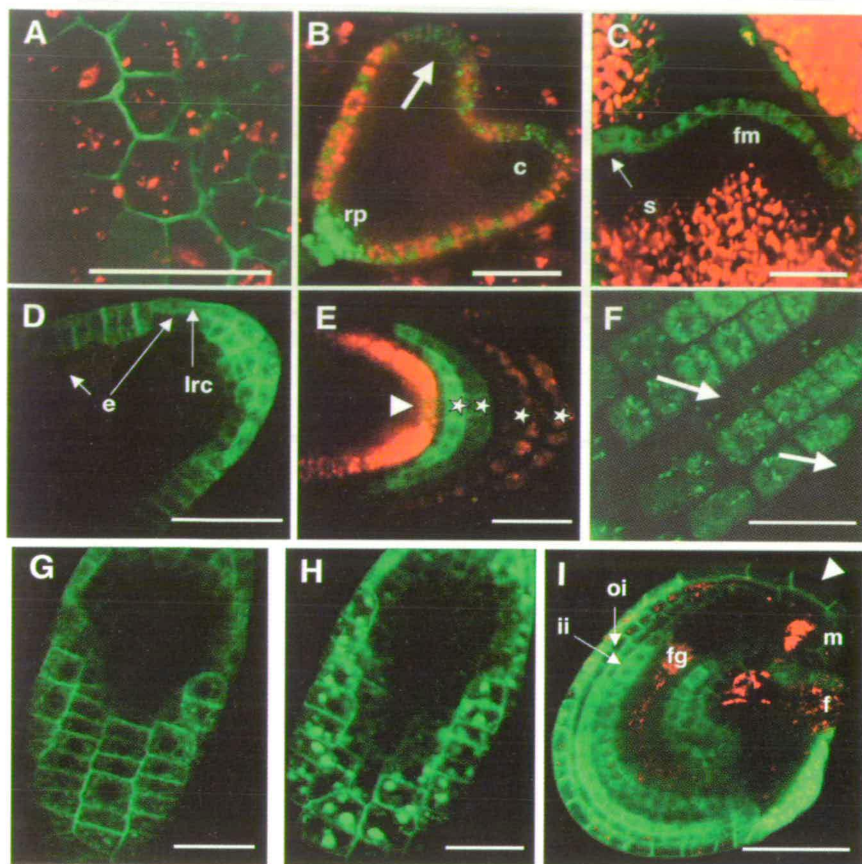

Fig. 6. Protein localisation in lines expressing an ACR4::GFP protein fusion. (A) localisation of fusion proteins (gren) in the plasma membranes of the ovule epidermis. (B,C) Expression patterns of fusion proteins (green) in embryos (B) and meristems (floral meristem, fm) (C). Plasma membrane localisation 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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REFERENCES

Abe, M., Takahashi, T. and Komeda, Y. (2001). Identification of a cis-regulatory element for L1 layer-specific gene expression, which is targeted by an L1-specific homeodomain protein. *Plant J.* **26**, 487-494.

Baker, S. C., Robinson-Beers, K., Villanueva, J. M., Gaiser, J. C. and Gasser, C. S. (1997). Interactions among genes regulating ovule development in *Arabidopsis thaliana*. *Genetics* **145**, 1109-1124.

Balasubramanian, S. and Schneitz, K. (2002). NOZZLE links proximal-distal and adaxial-abaxial pattern formation during ovule development in *Arabidopsis*. *Development* **129**, 4291-4300.

Becker, D. (1990). Binary vectors which allow the exchange of plant selectable markers and reporter genes. *Nucleic Acids Res.* **18**, 203.

Becker, D., Kemper, E., Schell, J. and Masterson, R. (1992). New plant binary vectors with selectable markers located proximal to the left T-DNA border. *Plant Mol. Biol.* **20**, 1195-1197.

Becraft, P. W., Li, K., Dey, N. and Asuncion-Crabb, Y. (2002). The maize dek1 gene functions in embryonic pattern formation and cell fate specification. *Development* **129**, 5217-5225.

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

Berger, F. (1999). Endosperm development. *Curr. Opin. Plant Biol.* **2**, 28-32.

Boisnard-Lorig, C., Colon-Carmona, A., Bauch, M., Hodge, S., Doerner, P., Bancharel, E., Dumas, C., Haseloff, J. and Berger, F. (2001). Dynamic analyses of the expression of the HISTONE::YFP fusion protein in *Arabidopsis* show that syncytial endosperm is divided in mitotic domains. *Plant Cell* **13**, 495-509.

Broadhvest, J., Baker, S. C. and Gasser, C. S. (1999). *SHORT INTEGUMENTS 2* promotes growth during *Arabidopsis* reproductive development. *Genetics* **155**, 899-907.

Clough, S. J. and Bent, A. F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735-743.

Colombo, L., Franken, J., van der Krol, A. R., Wittich, P. E., Dons, H. J. M. and Angenent, G. C. (1997). Downregulation of ovule-specific MADS box genes from *Petunia* results in maternally controlled defects in seed development. *Plant Cell* **9**, 703-715.

Fletcher, J. C., Brand, U., Running, M. P., Simon, R. and Meyerowitz, E. M. (1999). Signalling of cell fate decisions by CLAVATA3 in *Arabidopsis* shoot meristems. *Science* **283**, 1911-1914.

Geldner, N., Anders, N., Wolters, H., Keicher, J., Kornberger, W., Muller, P., Delbarre, A., Ueda, T., Nakana, A. and Jürgens, G. (2003). The *Arabidopsis* GNOM ARF-GEF mediates endosomal recycling, auxin transport and auxin-dependent plant growth. *Cell* **112**, 219-230.

Geldner, N., Friml, J., Stierhof, Y.-D., Jürgens, G. and Palme, K. (2001). Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature* **413**, 425-428.

Hanks, S. K., Quinn, A. M. and Hunter, T. (1998). The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* **241**, 42-52.

Haseloff, J. (1999). GFP variants for multispectral imaging of living cells. *Methods Cell Biol.* **58**, 139-151.

Jackson, D. (1991). In situ hybridisation in plants. In *Molecular Plant Pathology: A Practical Approach* (ed. D. J. Bowles, S. J. Gurr and M. McPherson). Oxford: Oxford University Press.

Jenik, P. D. and Irish, V. F. (2000). Regulation of cell proliferation patterns by homeotic genes during *Arabidopsis* floral development. *Development* **127**, 1267-1276.

Kapil, R. N. and Tiwari, S. C. (1978). The integumentary tapetum. *Bot. Rev.* **44**, 457-490.
Kessler, S., Seiki, S. and Sinha, N. (2002). *Xcl1* causes delayed oblique periclinal cell divisions in developing maize leaves, leading to cellular differentiation by lineage instead of position. *Development* **129**, 1859-1869.
Kidner, C., Sundaresan, V., Roberts, K. and Dolan, L. (2000). Clonal analysis of the *Arabidopsis* root confirms that position, not lineage, determines cell fate. *Planta* **221**, 191-199.
Koncz, C. and Schell, J. (1986). The promoter of T_L-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. *Mol. Gen. Genet.* **204**, 383-396.
Krysan, P. J., Young, J. K. and Sussman, M. R. (1999). T-DNA as an insertional mutagen in *Arabidopsis*. *Plant Cell* **11**, 2283-2290.
Lid, S. E., Gruis, D., Jung, R., Lorentzen, J. A., Ananiev, E., Chamberlin, M., Niu, X., Meeley, R., Nichols, S. and Olsen, O. A. (2002). The defective kernel 1 (dek1) gene required for aleurone cell development in the endosperm of maize grains encodes a plasma membrane protein of the calpain gene superfamily. *Proc. Natl. Acad. Sci. USA* **99**, 5460-5465.
Lu, P., Porat, R., Nadeau, J. A. and O'Neill, S. D. (1996). Identification of a meristem L1 layer-specific gene in *Arabidopsis* that is expressed during embryonic pattern formation and defines a class of homeobox genes. *Plant Cell* **8**, 2155-2168.
Nakajima, K. and Benfey, P. N. (2002). Signalling in and out: control of cell division and differentiation in the shoot and root. *Plant Cell* **14**, S265-S276.
Nebenführ, A., Ritzenthaler, C. and Robinson, D. G. (2002). Brefeldin A: Deciphering an enigmatic inhibitor of secretion. *Plant Physiol.* **130**, 1102-1108.
Notredame, C., Higgins, D. G. and Heringa, J. (2000). T-Coffee: A novel method for fast and accurate multiple sequence alignment. *J. Mol. Biol.* **302**, 205-217.
Reiser, L. and Fischer, R. L. (1993). The ovule and embryo sac. *Plant Cell* **5**, 1291-1301.
Robinson-Beers, K., Pruitt, R. E. and Gasser, C. S. (1992). Ovule development in wild-type *Arabidopsis* and two female-sterile mutants. *Plant Cell* **4**, 1237-1249.
Schafer, S. and Schmulling, T. (2002). The CRK1 receptor-like kinase of tobacco is negatively regulated by cytokinin. *Plant Mol. Biol.* **50**, 155-166.
Schneitz, K., Baker, S. C., Gasser, C. S. and Redweik, A. (1998). Pattern formation and growth during floral organogenesis: HUELLENLOS and AINTEGUMENTA are required for the formation of the proximal region of the ovule primordium in *Arabidopsis thaliana*. *Development* **125**, 2555-2563.
Schneitz, K., Hulskamp, M. and Pruitt, R. E. (1995). Wild-type ovule development in *Arabidopsis thaliana*: a light microscope study of cleared whole-mount tissue. *Plant J.* **7**, 731-749.
Sessions, A., Burke, E., Presting, G., Aux, G., McElver, J., Patton, D., Dietrich, B., Ho, P., Bacwaden, J., Ko, C. et al. (2002). A high-throughput *Arabidopsis* reverse genetics system. *Plant Cell* **14**, 2985-2994.
Sessions, A., Weigel, D. and Yanofsky, M. F. (1999). The *Arabidopsis thaliana* MERISTEM LAYER 1 promoter specifies epidermal expression in meristems and young primordia. *Plant J.* **20**, 259-263.
Stewart, R. N. and Burk, L. G. (1970). Independence of tissues derived from apical layers in ontegeny of the tobacco leaf and ovary. *Am. J. Bot.* **57**, 1010-1016.
Stewart, R. N. and Dermen, H. (1975). Flexibility in ontogeny as shown by the contribution of the shoot apical layers to the leaves of periclinal chimaeras. *Am. J. Bot.* **62**, 935-947.
Tanaka, H., Onouchi, H., Kondo, M., Hara-Nishimura, I., Nishimura, M., Machida, C. and Machida, Y. (2001). A subtilisin-like serine protease is required for epidermal surface formation in *Arabidopsis* embryos and juvenile plants. *Development* **128**, 4681-4689.
Tanaka, H., Watanabe, M., Watanabe, D., Tanaka, T., Machida, C. and Machida, Y. (2002). ACR4, a putative receptor kinase gene of *Arabidopsis thaliana*, that is expressed in the outer cell layers of embryos and plants, is involved in proper embryogenesis. *Plant Cell Physiol.* **43**, 419-428.
Torii, K. and Clark, S. (2000). Receptor-like kinases in plant development. *Adv. Bot. Res.* **32**, 225-267.
van den Berg, C., Willemsen, V., Hage, W., Weisbeek, P. and Scheres, B. (1995). Cell fate in the *Arabidopsis* root meristem determined by directional signalling. *Nature* **378**, 62-65.
Villanueva, J. M., Broadhvest, J., Hauser, B. A., Meister, R. J., Schneitz, K. and Gasser, C. S. (1999). INNER NO OUTER regulates abaxial-adaxial patterning in *Arabidopsis* ovules. *Genes Dev.* **13**, 3160-3169.