Characterisation of morphogenesis mutants in Arabidopsis

Horne, Kirsty L.

CHARACTERISATION OF MORPHOGENESIS MUTANTS IN *ARABIDOPSIS*

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by

Kirsty L. Horne BSc (York)
Department of Biological Sciences
University of Durham

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ABSTRACT

In this thesis is described the identification and characterisation of two morphogenesis mutants of *Arabidopsis thaliana*. One, *vertically challenged* (*vch1*) exhibits much reduced cellular elongation. The second, *altered suspensor fate* (*asf1*) is embryonic-lethal. A thorough phenotypic analysis of both mutants is presented, as are the results of genetic analysis.

vch1 exhibits a severe reduction in cellular elongation throughout the plant, resulting in a dwarfed phenotype. Despite its stunted morphology, *vch1* exhibits normal cellular patterning, demonstrating that cell morphogenesis can be uncoupled from correct cellular pattern formation. *vch1* follows a normal life history by all parameters examined demonstrating that the timing of developmental events can be uncoupled from correct morphogenesis. The phenotype of *vch1* cannot be rescued by the exogenous supply of a range of hormones, signalling inhibitors or growth conditions, although it can respond to each, in a proportionately similar manner to wild type seedlings. No defects in cell wall architecture nor in cytoskeletal organisation were detected during this study. Speculative models for the role of the *VCH1* gene are proposed.

In *asf1*, the embryo proper arrests at the transition stage of embryogenesis. The wild type suspensor is a single file of cells which serves to anchor the embryo proper to the maternal tissue and acts as a conduit for, and source of, nutrients to the developing embryo. In *asf1*, suspensor cells undergo inappropriate proliferation following the arrest of the embryo proper. Evidence is presented from cytological and ultrastructural examination, and expression of spatially restricted *gus*-fusion marker genes, that the ectopically divided suspensor cells take on aspects of embryo proper-like character. Models for the role of the *ASF1* gene are proposed. It is likely that the mutant phenotype results from disruption of intercellular communication between the embryo proper and suspensor.

DECLARATION

The material contained in this thesis has not been submitted for a degree in this or any other university.

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Miss Bainbridge

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ABBREVIATIONS

asf1	*altered suspensor fate* mutant
AGP	arabinogalactan protein
bp	base pair (length of a single nucleotide)
BSA	bovine serum albumin
CaMV35S	cauliflower mosaic virus 35S RNA gene
cDNA	complementary DNA
cv.	cultivar
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA	deoxyribonucleic acid
dNTPs	2'-deoxynucleoside 5'-triphosphates
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid, ~~disodium salt~~
GA3	gibberellic acid (A3)
GUS	β-glucuronidase
gusA (*uidA*)	gene encoding β-glucuronidase
HCl	hydrochloric acid
IAA	indole-3-acetic acid
IPCR	inverse PCR
kb	kilobase (length of 1000 nucleotides)
KCl	potassium chloride

KOH	potassium hydroxide
LiCl	lithium chloride
$MgCl_2$	magnesium chloride
mRNA	messenger RNA
NAA	napthaleneacetic acid
NaCl	sodium chloride
Na_2HPO_4	disodium hydrogen orthophosphate
NaH_2PO_4	sodium dihydrogen orthophosphate
NaOH	sodium hydroxide
NPTII	neomycin phosphotransferase
nptII	gene encoding neomycin phosphotransferase
OD	optical density
PCR	polymerase chain reaction
PEG	polyethylene glycol
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SAM	shoot apical meristem
SE	standard error of the mean
SEM	scanning electron microscopy
TEM	transmission electron microscopy
T-DNA	transferred DNA
TIBA	triiodobenzoic acid
Ti plasmid	tumour-inducing plasmid
Tris	tris(hydroxymethyl)aminomethane

vch1	*vertically challenged* mutant
X-Gluc	5-bromo-4-chloro-3-indoyl β-D-glucuronic acid

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INTRODUCTION

1.0 - Introduction

Plants are essential to the life of man. It is of considerable importance that we should study how they grow and develop so that we may better manipulate their habits for our own benefit. For centuries crop species have been selected for desirable phenotypic traits. We are now able to understand and manipulate plant growth, development and response to external factors at a molecular genetic level. It is important that we aim towards a detailed understanding of genetic control mechanisms and that we clone genes, ultimately enabling us to perform such manipulations.

1.0 Plant development

Development in plants, from the first zygotic division onwards, occurs by the co-ordination of cell division and cell expansion. The beginning of the development of a plant is with the formation of the single-celled zygote following the fertilisation of the egg cell. Subsequent tightly regulated cell division and cell expansion and biochemical changes leading to seed formation comprise the process of embryogenesis, during which the zygote becomes a mature embryo ready to germinate into a seedling following seed maturation and a period of seed dormancy. Whereas in animals cell migration events are important in determining the final body plan, in plants this is determined solely by the control of planes of cell division and the direction and extent of cell expansion. During embryogenesis root and shoot meristems are specified and the basic body plan of the seedling established. The establishment of the relative positions of the components of the embryo (root and shoot meristems, axis and cotyledons) is termed pattern formation. The mature embryo often contains high levels of storage molecules such as proteins and lipids. Such embryonic events prepare the embryo for both dormancy and germination. (Bewley & Black, 1994; Yeung & Meinke, 1993; West & Harada, 1993).

Unlike the situation in animals, the establishment of organ systems in plants is largely post-embryonic. All post-embryonic growth is initiated from the root and shoot meristems and the cambium.

1.0.1 Fertilization

Fertilization in angiosperms involves so called *double fertilization*. This refers to the fact that two distinct nuclear fusions occur: one sperm cell with the egg nucleus to produce the zygote and a second sperm cell with the two *polar nuclei* of the female gametophyte to produce a triploid nucleus, which through subsequent divisions forms the nutritive triploid endosperm. It has been suggested that double fertilization may be instrumental in epigenetic control of gene expression, through creating different gene dosage in embryo and endosperm (Lopes & Larkins, 1993).

This contrasts with the single fertilization of gymnosperms, in which one female gametophyte is fertilized by one pollen grain. This single diploid cell thus produced represents the progenitor of both embryo and storage reserve tissues.

1.0.2 Embryogenesis

In most angiosperms, the initial division of the zygote is transverse and asymmetric, generating a small, densely cytoplasmic apical cell and a larger, more vacuolate basal cell (Schulz & Jensen, 1968; Mansfield & Briarty, 1990). In most dicotyledenous species, including the *Brassicaceae*, the apical cell, through subsequent divisions, leads to the formation of the majority of the embryo proper, whilst the basal cell goes to form the suspensor and a part of the root apex (root cortex initials and columella root cap) (reviewed in Yeung & Meinke, 1993). In monocotyledenous species, the apical cell from the initial zygotic division forms both the embryo and most of the cells of the suspensor, whilst the basal cell does not enter any further divisions and forms only the terminal cell of the suspensor. The suspensor is a group of cells distinct from the embryo proper which serves to anchor the embryo proper to the maternal tissue of the seed and serves as both a source of, and conduit for, nutrients and growth regulators for the developing embryo proper. In most species it undergoes

programmed cell death (*apoptosis*) at a point during embryogenesis after which it is no longer necessary.

The first zygotic division is not always asymmetrical and transverse; in some plants it may be symmetrical, and may be longitudinal or oblique in orientation (Sivaramakrishna, 1978). The details of subsequent steps in embryogenesis also vary widely amongst different plant species, not only in the overall shape of the developing embryo, but also in the extent to which the embryo, suspensor and endosperm develop within the seed and the details of the cell division processes. For example, in maize (*Zea mays*), after the initial asymmetric zygotic cell division, subsequent divisions are predominantly asymmetric (Sheridan & Clarke, 1987). This contrasts with the case in *Arabidopsis thaliana*, for example, in which the majority of embryonic cell divisions, following the initial asymmetric division of the zygote, are symmetrical (Mansfield & Briarty, 1991). The details of embryogenesis vary between different plant species. The common end result, however, is the generation of a developmentally arrested mature embryo comprising cotyledon(s) plus root and shoot apices at either end of an embryonic axis, often containing high levels of storage products (West & Harada, 1993).

Embryogenesis occurs within a maternal structure which may take a variety of forms, from large fruit bodies to the small pods or *siliques* of *Arabidopsis.*

The details of embryogenesis in *Arabidopsis* will be discussed in more detail (Section 1.1.2) as an example of the process. *Arabidopsis* is an excellent model system for the study of dicot embryogenesis as it is both morphologically relatively simple and highly predictable (Meinke & Sussex, 1979), presumably due to tight control at the genetic level.

1.1 *Arabidopsis* as a model plant

1.1.1 Why study *Arabidopsis*?

Arabidopsis thaliana enjoys widespread use as a model dicotyledonous plant species for the molecular and physiological studies of many facets of plant development. *Arabidopsis* is a small ~~cruciferous~~ weed of the *Brassicaceae* (mustard family), and is increasingly the model plant species of choice for genetic studies of developmental processes. Its main attributes which make it so suitable for such studies are its small genome size, relative to that of other plant species, ease of transformation, short generation time (approximately 6 to 8 weeks under standard growth conditions), simple morphogenesis, and a relatively well characterized genome. Its genome is diploid and contains only a small proportion of repetitive DNA sequences (only 10-14%); total genome size is estimated at 7×10^7 bp (Pruitt & Meyerowitz, 1986), this being at least one order of magnitude smaller than most crop plant species, and less than half that of the genetic model animal species, *Drosophila*. An international initiative is currently underway sequencing the entire *Arabidopsis* genome: to date approximately 30% of the genome is sequenced, representing over 4000-4500 genes, of which around half have been assigned a cellular role (K. Lindsey, pers. comm.). Another important feature is that *Arabidopsis* is normally self-fertile: this being advantageous in that large numbers of seeds of a particular genotype can rapidly be generated from a single plant. It is, however, also able to outcross, and this is exploited in genetic experiments where cross-fertilization may be performed manually, with the aid of a microscope, allowing the introduction of genes of interest from different transgenic lines into the same genetic background without the need for transformation.

Arabidopsis is the species under study in this thesis. A number of examples of physiological studies, mutants generated and genes cloned in *Arabidopsis* will be described in subsequent sections of this chapter.

1.1.2 Embryogenesis in *Arabidopsis*

Although the details of embryogenesis do vary amongst dicot species, similar overall events occur, making the use of a model feasible. Also, once genes have been identified in one species, their counterparts in other species can be more readily isolated (Section 1.7).

Embryogenesis in *Arabidopsis* is rapid, concluding approximately 11-12 days post-fertilization (Lindsey & Topping, 1993). The first zygotic division in *Arabidopsis* is asymmetric, producing a small, cytoplasmically dense apical cell and larger, more vacuolate basal cell (Mansfield & Briarty, 1991). What determines the asymmetry of this initial division is an intriguing question and is briefly discussed in Section 1.3.1. Through subsequent divisions, the derivatives of the apical cell form the majority of the cells of the embryo proper. The basal cell enters a more limited programme of cell division to form the 7-9 cells of the suspensor (discussed in Section 1.1.3) and a small region of the embryonic root: the columella root cap. Differences in gene expression between the apical and basal cell are apparent, for example, the apical cell has been shown to accumulate the *ARABIDOPSIS THALIANA MERISTEM LAYER 1* (*ATML1*) gene transcript, which is not detected in the basal cell (Lu *et al.*, 1996).

The apical cell then undergoes two symmetrical vertical divisions at right angles to one another, followed by division of each of these four cells in the transverse plane, to produce an isodiametric eight-cell (octant) embryo. The boundary between the upper and lower tiers of four cells each respectively is termed the "O' boundary" (Tykarska, 1976). The cells of the upper tier will, through subsequent divisions, form the cotyledons and shoot meristem, whilst those of the lower tier will form the hypocotyl and much of the root. The basal cell from the first division by this stage has undergone divisions to produce the file of 7-9 cells comprising the suspensor, which is by now already functionally differentiated (Section 1.1.3). The next round of cell divisions in the embryo proper leads, by the 16-cell stage, to the appearance of the protoderm, an outer layer of cells which form the basis of what will become the

epidermis. Cells of the protoderm continue to divide anticlinally, whilst those in the centre of the embryo divide both periclinally and anticlinally, whilst maintaining regular tiers within the embryo. This globular stage is concluded when the embryo reaches approximately 30 cells in total and approximately 40μm in diameter (Lindsey & Topping, 1993). Beyond this stage the pattern of cell divisions is such that the embryo begins to develop bilateral symmetry, with the appearance of two lobes which form the precursors of the cotyledons. By this heart-stage (approximately 250 cells, 3-4 days post-fertilization) the three basic tissues (epidermis, ground tissue and vascular tissue) of the embryo, and later seedling, have been established in a rudimentary form. Accumulation of chlorophyll begins to occur at this stage, resulting in "greening" of the embryo.

Elongation of the embryonic axis continues as the embryo passes through the torpedo- and into the cotyledonary-stage. The suspensor begins to degenerate at the torpedo stage and is absent by the mid-cotyledonary stage. In the mature cotyledonary-stage the embryo comprises approximately 20,000 cells and is approximately 500μm in length (Lindsey & Topping, 1993), although it is in a curled up state within the seed, and is effectively longer than this in its long axis. The hypocotyl and cotyledons are now well defined, with clear patterning of the embryonic root meristem (Scheres *et al.*, 1994a). The shoot meristem is still quite undeveloped and will remain so until after germination and early seedling growth. The embryo then undergoes maturation and desiccation. (Section 1.3.2). By maturity, the chlorophyll which has been accumulating from the heart stage through to the mid-cotyledonary stage disappears and the embryo again becomes colourless.

1.1.3 The *Arabidopsis* suspensor

In *Arabidopsis* the suspensor is a fully functionally differentiated file of cells by the time the embryo proper is still at the globular stage. The suspensor comprises an enlarged basal cell which is partially embedded in maternal tissue, a single file of 6-8 similarly sized cells, except for the basal cell which is larger, and the hypophysis, which is the cell directly below the embryo proper.

It has been argued that the hypophysis should not be considered part of the suspensor (Mansfield & Briarty, 1991) since it does become functionally incorporated into the basal region of the embryo proper and is structurally different from the "suspensor proper" cells from an early stage. This argument is a semantic one since, whilst this is true, the hypophysis is clonally related to the rest of the suspensor and it does exhibit some ultrastructural features more akin to suspensor than embryo proper cells. It is perhaps most useful to consider the hypophysis an entity in itself, distinct from both suspensor and embryo proper.

Unlike other species in which radiolabelling of nutrients has been used in suspensor functional analyses, no such direct evidence has been obtained for the role of the *Arabidopsis* suspensor. Indirectly, however, ultrastructural features suggest that the suspensor plays a role in absorption and transport of nutrients to the developing embryo proper. The whole suspensor is closely associated with nutritive endosperm and the basal cell in particular has a crenelated basal cell wall and many mitochondria, ribosomes and dense Golgi apparatus; these are strongly suggestive of a role in absorption (Schulz & Jensen, 1969). A symplastic pathway is formed by plasmodesmata from the basal cell, through suspensor cells and the hypophysis to the embryo proper (Schwartz *et al.*, 1997), again suggesting translocation is an important process in these cells. Unlike the suspensors of some other plant species, the *Arabidopsis* suspensor is probably not very metabolically active; the suspensor cells contain large vacuoles surrounded by a relatively small amount of cytoplasm in which there is a low density of ribosomes and mitochondria. Plastids in the suspensor are not well developed and are, therefore, not photosynthetically active.

1.2 Embryo proper-suspensor interaction

The interaction between embryo proper and suspensor is an excellent system in which to study intercellular communication. That the suspensor is important for normal development of the embryo proper has long been believed. Early experiments

involving irradiation or chemical treatment of dicot supensors (*e.g.* Haccius, 1963) led to developmental arrest of the embryo proper. Experiments in which *Phaseolus* embryos were cultured *in vitro* demonstrated that when the suspensor cells are mechanically removed, the embryo proper grows poorly. These embryos can, however, be rescued by the exogenous application of hormones, including cytokinins and gibberellins (Yeung & Sussex, 1979). They can also be partially rescued by placing the excised suspensor in close proximity. This strongly suggests that the suspensor normally acts to synthesize, and not just transport, these important development-regulating hormones.

Evidence for communication from embryo proper to suspensor has been gleaned from recent studies of *Arabidopsis* embryonic mutants, most notably the abnormal *sus*pensor (Schwartz *et al.*, 1994) and *raspberry* (Yadegari *et al.*, 1994) mutants. In both of these classes of mutants, the embryo proper arrests and the suspensor subsequently enters into a series of inappropriate divisions. It has been suggested that the normally developing embryo proper somehow signals to the suspensor to maintain its differentiated state, and in the case of some developmentally arrested embryos, including these mutants, this signal is blocked or not produced, this having a knock-on effect on the fate of the suspensor (Schwartz *et al.*, 1994).

1.3 Important events during embryogenesis

1.3.1 Asymmetry of the first zygotic division

It has been argued that the asymmetric nature of the first zygotic division is determined by maternal influences, perhaps through determination of asymmetry in some cytoplasmic factor(s). However, the fact that isolated somatic embryos are able to develop normal asymmetry in the absence of maternal tissues argues against this being the sole determinant (West & Harada, 1993). There may be some maternal influence since, in the seed, the apical-basal axis of the embryo consistently aligns along the chalaza-micropyle axis of the ovule (Laux & Jürgens, 1997). In *Drosophila* it is well established that the maternally expressed *bicoid* gene, which encodes a transcription factor, is essential to polarity establishment in the embryo (reviewed in

Wilkins & Gubb, 1991). The nature of the genetic control of this first division is interesting since the mechanism of establishment of polarity is generally little understood. Studies on the mechanisms of polarity establishment are being carried out on single cell spherical zygotes of the brown algae *Fucus* (Goodner & Quatrano, 1993; Berger *et al.*, 1994; Kropf, 1994, 1997). These zygotes are easier to study than higher plant zygotes since they are free-living and not surrounded by maternal tissue. The zygote is produced by fertilization away from the parent plant, and apparently has no inherent asymmetry. Polarity develops, though, within a few hours of fertilization, with asymmetry in the distribution of organelles, cell wall components, cytoskeletal filaments and electrical fluxes within the cell. It seems that the stimulus leading to polar development could be the site of penetration of the egg cell by the sperm, acting through signalling pathways which are as yet little understood. It is also the case, however, that environmental cues, such as directional light, ion and temperature gradients and gravity, may over-ride the sperm penetration site as the primary determinant (Kropf, 1997). It will be interesting to see whether similar mechanisms of axis fixation operate in the zygote of higher plants. Recent studies of *Arabidopsis* mutants such as *fass* (Torres-Ruiz & Jürgens, 1994; Fisher *et al.*, 1996), which undergoes a symmetrical first zygotic division, yet still goes on to develop an embryo with apical-basal polarity, albeit morphologically abnormal, suggest some mechanism whereby stable asymmetry of the embryo is established, even in the absence of an asymmetrical first division. This and other mutants in which this first division asymmetry is abolished, such as the *gnom/emb30* mutant (Mayer *et al.*, 1993), will prove interesting in dissecting such crucial early events. The *gnom* mutant does give rise to an embryo proper and suspensor which, although abnormal, do arise from the apical and basal cell respectively, suggesting that the asymmetry of the first division is not entirely determinative of subsequent polarity establishment. Other mutants have been identified in which the asymmetry of later division events is affected, such as *scarecrow*, in which root cell organisation is disrupted as a result of the abolition of the asymmetry of the division of the cells which divide to give rise to the cortical and endodermal cells, resulting in a single layer of ground tissue with

molecular characteristics of both normal cortical and endodermal layers (Di Laurenzio *et al.*, 1996).

This abolition of the asymmetry of divisions in such mutants is analogous to the situation in *Fucus* embryos in experiments in which Golgi-mediated secretion is chemically inhibited, blocking the secretion required for the establisment of cell wall asymmetry (Quatrano & Shaw, 1997). The *gnom/emb30* protein shows sequence similarity to yeast proteins involved in vesicle transport between the endoplasmic reticulum and Golgi complex, suggesting a role in directional vesicle transport of proteins which may help establish apical-basal polarity (Laux & Jürgens, 1997).

1.3.2 Seed maturation

The details of embryonic and seed maturation, dessication, dormancy and later germination vary widely amongst plant species. Following a period of seed dormancy, germination is initiated by imbibition and is metabolically driven by the hydrolysis of protein and lipid reserves stored during seed maturation. A controlled period of dormancy is important both to prevent seeds germinating whilst still on the parent plant and to promote germination under favourable environmental conditions. These processes are comprehensively reviewed in Bewley and Black (1994).

1.4 Genetic control of embryogenesis

It has been estimated from DNA-RNA hybridisation experiments that as many as 20,000 diverse genes are expressed at any stage during seed development (in soybean, tobacco) (Goldberg *et al.*, 1989).

The role of embryonically-expressed genes controlling pattern formation and morphogenesis (the establishment of body shape) are being investigated through the identification and characterization of recessive mutants disrupted in specific aspects of these processes. Such studies have previously been largely carried out in the monocotyledonous plant species maize (*Zea mays e.g.* Clarke & Sheridan, 1991), and

are now increasingly being carried out in the model dicotyledonous species *Arabidopsis thaliana.*

One conclusion emerging from such studies is that embryogenesis can be conceptualised as comprising three fundamental elements of development, namely morphogenesis, pattern formation and cytodifferentiation. Morphogenesis refers to the establishment of the overall shape of the embryo, pattern formation to the establishment of the precise spatial organisation of cells and organs within the embryo and cytodifferentiation to the generation of cellular diversity, or division of labour between component cells in both biochemical and structural properties leading to their functional specialisation (Lindsey *et al.*, 1996). Analysis of a number of mutant phenotypes has led to the suggestion that these components may be regulated independently of one another. This will be clarified by some examples.

Several lines of evidence suggest that plant cells have little developmental memory and that their differentiation is largely determined by molecular signals from surrounding cells. Cells which have become de-differentiated into callus can re-differentiate as a variety of different cell/organ types dependent on the hormonal composition of the medium. Also see Section 1.6.4 for further examples of plasticity in plant development. It is likely that, for example, shoot and root meristems differentiate as a result of the activity of locally expressed genes. Differentiation of floral organs has been causally linked to the expression of genes encoding transcription factors in a spatially restricted manner (Coen & Meyerowitz, 1991). Cytodifferentiation may also be a result of a gene(s) whose expression is expressed in a cell-autonomous manner within a tissue, for example the *glabrous1* gene is essential for trichome development . It encodes a *MYB*-like transcription factor and is expressed in the leaf epidermis in isolated cells which become trichome cells (Larkin *et al.*, 1993).

That morphogenesis can be regulated independently of biochemical differentiation is suggested by the phenotype of the *fusca* mutants, which accumulate anthocyanins

inappropriately during embryogenesis, lack chlorophyll and storage products, and in some alleles fail to initiate trichomes. The overall morphology of the embryo, however, is unaffected by the mutation. Conversely, the *sus* (Schwartz *et al.*, 1994) and *raspberry* (Yadegari *et al.*, 1994) mutants fail to undergo normal morphogenesis, arresting as an enlarged globular embryo. They have been shown, however, to accumulate storage proteins and organelles in a radial pattern similar to that in wild type maturation stage embryos, suggesting that biochemical cytodifferentiation can proceed chronologically in the absence of correct morphogenesis.

A number of mutants are obviously defective in both overall shape and cell shape, including *emb30* (Mayer *et al.*, 1993; Schevell *et al.*, 1994) (described in Section 1.8.5), *hydra1* (Section 1.8.5; Topping *et al.*, 1997) and *fass* (Torres-Ruiz & Jürgens, 1994; Fisher *et al.*, 1996). These mutants are all to some extent disrupted in tissue differentiation insofar as, whilst they all differentiate at least some vascular cell types, the relative positioning of these is disrupted. It seems then that in this case the establishment of the relative positions of cells (pattern formation) is disrupted by aberrant cellular patterning in the embryo. It might be expected that the precise coordination of rates and planes of cell division and expansion would be absolutely necessary for normal pattern formation and morphogenesis. In the wild type *Arabidopsis* embryo, rates and planes of cell division and expansion are highly predictable and are therefore assumed to be tightly regulated at the genetic level. This is not the case in the embryos of all species. In maize, for example, cell divisions are much less predictable and cells are not organised into discrete files as they are in the *Arabidopsis* embryo.

There are mutant phenotypes which do suggest an important role for controlled cell division and expansion in pattern formation and morphogenesis. These include *hydra1* in which the direction of cell expansion appears to be disturbed, resulting in a reduction in cell filing, leading to abnormal morphogenesis and to disrupted radial pattern formation, as suggested above (Lindsey *et al.*, 1996; Topping *et al.*, 1997). A number of mutants have been described from the laboratory of Jürgens which

apparently lack a whole segment of the apical-basal axis of the seedling. These include *monopteros* (Berleth & Jürgens, 1993), which lacks a root and hypocotyl. The defect has been traced back to the octant stage of embryogenesis when there is excessive division of the hypophysis, disrupting the organisation of cells in the basal region; *gurke* (Mayer *et al.*, 1991; Torrez-Ruiz *et al.*, 1996) exhibits an apical deletion of the shoot meristem and cotyledons. This defect arises at the heart-stage. *fackel* (Mayer *et al.*, 1991) lacks a hypocotyl, with the cotyledons being directly attached to the root, this representing a central deletion. These pattern element deletion mutants originally led to the suggestion that embryogenesis is modular, with discrete domains being established at an early stage, but this is now considered too simplistic a view (Mayer & Jürgens, 1998).

The basic body plan of the embryo is established in rudimentary form within the first 3-4 days. Morphogenetic organisation itself can be viewed as the integration of three aspects of patterning: apical-basal patterning along the main axis of the embryo, radial patterning across the axis of the root, hypocotyl and stem, and the development of bilateral symmetry, manifest as the formation of cotyledons. Patterning involves the development of an array of morphologically distinct elements along these axes: in the case of apical-basal patterning this begins with the asymmetry of the zygotic division and results in the establishment of shoot meristem, cotyledon(s), hypocotyl, embryonic root and root meristem. Radial patterning establishes the three major tissue types of epidermis, ground tissue and vascular tissue. Mutants with defective radial patterning include *knolle* (Mayer *et al.*, 1991) which has an enlarged and morphologically indistinct epidermis. This arises in the 8-cell embryo as a result of failure of the periclinal divisions which normally serve to separate the epidermis from the inner cell mass. *short-root* (Benfey *et al.*, 1993) lacks an endodermal cell layer, with knock-on effects on overall morphogenesis including reduced root length, *wooden leg* (Scheres *et al.*, 1995) has fewer vascular cells than normal and those which are present all differentiate into xylem.

It seems from these mutant phenotypes that the basic plan of the plant is laid down in the early embryo in *Arabidopsis*, providing the basis for later embryonic and post-embryonic development through elaboration of this basic pattern. Control of rates and planes of cell division obviously is important at early stages of embryogenesis to establish files of cells from which the seedling root develops in a highly predictable manner (Dolan *et al.*, 1993). A number of genes seem to be expressed in response to positional information rather than lineage, for example *STM* (Barton & Poethig, 1993). Also, the *POLARIS* gene (Topping & Lindsey, 1997) is expressed in the basal part of the embryo from the heart-stage, in cells which are spatially but not clonally related.

1.5 Post-embryonic organogenesis

Post-embryonic events establish the architecture of the mature plant by elaboration of the basic pattern laid down during embryogenesis. All leaves and flowers originate from primordia produced by cell divisions in the shoot meristematic region, and similarly root growth occurs from the root meristem. All somatic cells are generated by cell division at the meristematic apices or vascular cambium and subsequently attain their final shape by controlled cell expansion/directional elongation.

1.5.1 The shoot meristem

Growth from the shoot apical meristem (SAM) comprises two distinct phases: firstly a vegetative phase, during which stems and leaves are produced and elaborated, and secondly, following a defined transition and major changes in the patterns of differentiation at the SAM, a reproductive phase, leading to the production and proliferation of floral organs (Sung *et al.*, 1992).

Genes have been identified which are important in the specification of the position of the SAM during embryogenesis: these include the *KNOTTED* gene of maize (Hake *et al.*, 1989) and the *STM* gene of *Arabidopsis* (Barton & Poethig, 1993; Long *et al.*, 1996). When the function of the *STM* gene is disrupted by mutation, then the plant is no longer able to produce a SAM, either anatomically or functionally. When the

spatial and temporal expression pattern of this gene was investigated by *in situ* hybridisation, it was found that the *STM* transcript is produced in a spatially restricted manner in the embryo at what will later become the site of SAM initiation. This expression is observed well in advance of morphogenetic differentiation of the SAM, suggesting a determinative role of this gene. Further evidence for such a role for genes of this family in SAM initiation came from the constitutive expression of the wild type maize *KNOTTED-1* gene in tobacco, which resulted in the formation of many ectopic SAMs. A similar phenotype was observed when an *Arabidopsis KNOTTED*-like homologue was overexpressed in transgenic plants (Lincoln *et al.*, 1994). Other genes have been identified as playing a role in SAM identity, including the *WUSCHEL* gene, which is essential for SAM maintenance once it is established (Laux *et al.*, 1996).

The transition of the SAM from the vegetative to the reproductive phase is an important stage in the plant's life-cycle, and leads to the formation of the floral organs (sepals, petals, stamens and carpels). The stamens and carpels, respectively, subsequently give rise to the microspores and megaspores which generate the gametes which, in turn, upon fusion will give rise to the embryos of the next generation (Section 1.0.1). The timing of the transition to flowering seems to be determined by both internal and external influences: the age, or size, of the plant being an important internal factor, and the time of year, through external environmental parameters, such as daylength and temperature, being external (Taiz & Zeiger, 1991). Genes have been identified which are important in the maintenance of the vegetative phase (Sung *et al.*, 1992), and in the transition to the reproductive phase and formation of the floral structures (Bradley *et al.*, 1993; Coen & Meyerowitz, 1991). In *Antirrhinum*, for example, the vegetative growth phase involves the production of opposing pairs of leaves; the leaves at each successive node being at right-angles to those at the previous node. After a period of vegetative growth, the meristem proceeds into an inflorescence phase, producing small leaves (bracts) in a spiral arrangement at each node. The internodes elongate (this is known as *bolting*), unlike in the rosette of the vegetative phase where nodes are closely

spaced (Schultz & Haughn, 1993). In the axils of these bracts (in the acute angle where they join the stem), floral meristems are initiated: these determinate meristems produce four consecutive whorls of floral organs, separated by very short internodes (Bradley *et al.*, 1993).

All of these processes have been dissected at a genetic level by the study of mutants defective in specific aspects of both the transition to flowering and of the elaboration of floral structures (Carpenter & Coen, 1990; Coen *et al.*, 1990; Haughn & Somerville, 1988; Schultz & Haughn, 1991; Bradley *et al.*, 1993; Shannon & Meeks-Wagner, 1991; Irish & Sussex, 1990; Komaki, 1988).

1.5.2 Floral organogenesis

Following the transition, each floral meristem develops into a single flower by producing a concentric arrangement of whorls of the four types of floral organs: sepals, petals, stamens and carpels. Through mutant analysis a number of genes which specify and maintain the identity of floral organs in particular whorls have been identified (Coen & Meyerowitz, 1991; Bradley *et al.*, 1993; Schultz & Haughn, 1993; Mizukami & Ma, 1992; Ma *et al.*, 1991).

1.5.3 Environmental control of flowering

Whilst genetic control of flowering is obviously of major importance, environmental controls are also significant. It is generally accepted that the hormone gibberellin is an important regulator of timing of flowering and floral development in higher plants (reviewed in Okamuro *et al.*, 1993; Wilson *et al.*, 1992) and is affected by changes in both photoperiod and temperature. Other external factors which may play a part in regulating flowering time include water and nutrient availability, indirectly through influencing the growth rate of vegetative tissues.

1.5.4 Root development

Whilst a number of homeotic genes which specify organ identity, particularly in flowers, have been characterized, relatively little is known about the genes which

actually regulate organ formation at the cellular level. The root has increasingly been seen as a useful system in which to study organ development (Aeschbacher *et al.*, 1994) due to its relative simplicity as an organ: growth is quite uniform and continuous, roots have a relatively small number of differentiated cell types, and they exhibit radial symmetry. Identification and characterization of mutant plants with abnormal root development is consequently relatively simple. Many recent studies have been carried out in *Arabidopsis*, partly because of its suitability for genetic and molecular analyses (see Section 1.1.1), and partly because of its small physical size which allows many plants to be grown and screened in a small space. With regard to the relative simplicity, small size, ease of genetic manipulation, and visual tranparency, the *Arabidopsis* root has been described as the "*Caenorhabditis elegans* of the plant world" (Aeschbacher *et al.*, 1994).

1.5.5 The root meristem

Root development occurs from the root meristem, as does the shoot from the SAM; the meristem again being specified during embryogenesis. Development of the root is simpler to dissect than that of the aerial parts insofar as differentiated cells are arranged in discrete files, which can be traced back to a small number of cells near the apex, termed *initials*, from which they derive. Cellular patterning in the root is highly predictable, unlike in the shoot, where chance events are more significant in patterning at the apex and fate of cells can only be predicted in a probabilistic fashion (Furner & Pumfrey, 1992; Irish & Sussex, 1992). Not all cells at the root apex, however, actively divide to form new cells; so-called *quiescent centre* cells are located near to the root apex but divide only infrequently; they may serve to replace the rapidly dividing initial cells to which they are adjacent (Scheres *et al.*, 1994). The embryonic origin of root meritem initials has been studied in the cruciferous species *Brassica napus* and *Arabidopsis* (Dolan *et al.*, 1993; Scheres *et al.*, 1994). Highly specified cell division patterns lead to the formation of a set of initial cells surrounding a quiescent centre. The mitotically inactive quiescent centre, in *Arabidopsis* comprising four cells which are surrounded by the initial cells, has recently been demonstrated, by laser ablation experiments, to serve to maintain initial

cells in an initial-specific undifferentiated state by a mechanism requiring cell-cell contact (van den Berg *et al.*, 1997). This shows that, as well as long range positional signals, short-range signals between adjacent cells are also important in root meristem patterning. The set of initials has been termed the *promeristem* and is formed at the heart stage of embryogenesis. Dolan *et al.* (1993) suggested that post-germinative growth of the seedling root is simply by the perpetuation of this pattern of cell divisions initiated in the promeristem.

Histological analysis of the promeristem formed at the heart stage of embryogenesis suggests a lineage pattern of initial cells and their derivatives. Such analysis, however, does not provide conclusive proof of the details of the ontogeny of the root and it was necessary to carry out an experimental lineage analysis (Scheres *et al.*, 1994). Such lineage, or clonal, analysis relies on the study of the distribution and extent of genetically marked somatic sectors of cells. Traditional lineage analyses relied on the use of irradiation at defined developmental stages (*e.g.* Poethig *et al.*, 1987) to produce easily scorable genetic markers in a single cell and in resulting sectors of cells derived from it. This, combined with histological analysis can provide information regarding the participation of specific cells in the development of an organ. Such a study in the formation of leaves in *Nicotiana tabacum* (Poethig & Sussex, 1985) suggested a lack of predictability of cell lineage in the formation of leaves: it appears that the aquisition of cell fate and cell identity seem to be quite lineage-independent. Similar clonal analyses, through irradiation of specific cells, have also been performed in roots. One major limitation of this irradiation approach, however, particularly in roots, is that X-rays may actually activate normally quiescent cells to replace initials which have been damaged. Recent analyses in *Arabidopsis* (Dolan *et al.*, 1994; Scheres *et al.*, 1994) have exploited the phenomenon of excisability of transposable elements, in this case of the autonomously transposable maize *Ac* element, from within a marker gene, here the β-glucuronidase (*gusA*) gene under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter. This construct was stably introduced into the *Arabidopsis* genome. The presence of the *Ac* element within the *gusA* gene blocks its transcription. When the *Ac* element

excises, however, the 35S-*GUS* gene is then able to be transcribed and produce a functional *GUS* enzyme product which can be detected histochemically (Section 2.4). All derivatives of the cell in which this excision event occurred will be thus detectable and the lineage of such cells can be followed. Unlike irradiation, excision of the element out of the *gusA* transgene is unlikely to perturb normal root development. This analysis largely supported histological analyses, insofar as defined sectors are derived from the small set of initials produced at the heart stage of embryogenesis. It also supported the observation that the *hypophysis*, the top cell of the suspensor, contributes to form part of the root: derivatives of hypophyseal cell divisions adjacent to the epidermis initial, formed by the heart stage, through subsequent divisions go on to form the columella initials and ultimately the columella. These effectively merge with the clonally distinct embryo proper-derived cells comprising the lateral root cap (Dolan *et al.*, 1994).

1.5.6 Establishment of secondary meristems

In both shoot and root development an initial period of development from the primary meristem is followed by the establishment of secondary meristems, leading to the formation of lateral branches and lateral roots. The control of secondary meristem formation is both directly genetic, since genes have been identified which are essential for lateral root production (Celenza *et al.*, 1995) and indirectly, through the action of hormones, maybe partly in response to external stimuli. Production of auxin at the shoot meristem, for example, and its transport down the plant is important in the supression of secondary meristem activity. Secondary meristems in the root are formed *de novo* from the pericycle (Taylor, 1997), whereas in shoots they arise in the bracts of leaves.

1.6 How do plants grow and develop?

1.6.1 The plant cell wall

The plant cell wall may represent the single most important constraint on post-embryonic growth. At the simplest level it may be said that plant cell growth results from turgor-driven yielding of the cell wall (Cosgrove, 1986). This mechanical explanation obviously only relates to the growth of individual cells and does not attempt to explain the myriad other genetically controlled processes which influence rates, planes and coordination of cell divisions.

At the mechanistic level it is generally believed that cell expansion is as a direct result of enzymatic modification of the cell wall, which allows osmotic water uptake. Under normal growth conditions, in the absence of drought stress, cells are turgid and the wall is elastically stretched by the protoplast. Higher plant cell walls typically comprise crystalline cellulose microfibrils embedded in a gel-like matrix of mixed-linked polysaccharides and proteins (McQueen-Mason & Cosgrove, 1994). It is generally accepted that a group of enzymes serve to promote wall "loosening" through hydrolysis or tranglycosylation of tension-bearing polymers. These include *xyloglucan endotransglycosylase* enzymes (de Silva *et al.*, 1994; Wu *et al.*, 1996) and other enzymes with hydrolytic activity. Another apparently important group of "cell wall loosening enzymes" recently identified are the *expansins*, which seem to exert their effect through reversibly disrupting non-covalent interactions within the cell wall thus allowing cellular expansion (McQueen-Mason & Cosgrove, 1994). The relative importance of the roles played by each of these groups of enzymes in causation of wall loosening and concomitantly on cell expansion/elongation is not yet clear. Regulation of rates of cell expansion by hormones or other agents is partly through their influence on cell wall properties (Cosgrove, 1997).

1.6.2 Role of hormones

The effects of hormones on many diverse aspects of plant growth, development and responses to external stimuli have been studied for many decades. Their production, transport, perception and effects are now increasingly well understood at a molecular level. There is considerable interaction between different hormones in specific aspects of their action. For example, it is the ratio of auxin to cytokinin which is critical in the control of root and shoot formation, rather than their absolute concentrations (Garbers & Simmons, 1994).

The multitudinous effects of the plant hormones are too complex to discuss fully here. A review can be found in Kende & Zeervaart (1997). In brief, the main effects of auxins are on cell division and elongation and they affect processes as diverse as root formation and development, maintenance of apical dominance, control of flowering time and tropisms. Cytokinins largely promote cell division activity where appropriate and again are active in a wide variety of processes. Gibberellins are also promoters of both cell division and expansion and are important signals in germination and desiccation tolerance, along with abscisic acid. Abscisic acid is generally an inhibitor of growth and germination. Ethylene is important in the control of root elongation and differentiation and in the control of fruit ripening.

A number of hormone receptors and signalling pathway components have recently been identified through analysis of *Arabidopsis* hormone response mutants. One example is the *AXR1* gene, mutation of which results in lack of response to auxin in terms of root growth and gravitropism (Leyser *et al.*, 1993). Ethylene response mutants, *e.g. etr1* (*ethylene-resistant-1*), have also been identified (Ecker, 1995); they fail to exhibit the classic "triple response" to exogenous ethylene of stunted growth, swelling of root and hypocotyl, and increased apical hook formation. Such *ETR* genes have been found to encode membrane-bound receptor proteins. Genes which act downstream of the *ETR* genes, mutation of which cause a *constitutive triple response*(*ctr*) phenotype have also been identified (Kieber *et al.*, 1993): These act as

negative regulators of the ethylene response and *CTR1* has been found to encode a putative serine/threonine protein kinase.

Future dissection of the role of hormones will be gleaned from transgenic studies. Such studies could be refined by placing hormone-related genes under the control of cell-, tissue- or organ-specific promoters (Kende & Zeervaart, 1997).

Other "non-traditional" hormone-like compounds have been identified recently. These include the oligosaccharins, brassinosteroids and jasmonates, all of which appear to play major roles in plant growth and development. For a recent review see Creelman & Mullet (1997).

1.6.3 Intercellular signalling

How plant cells receive and respond to positional information from neighbouring cells is still little understood. It has been suggested that the cytoskeleton of cells and the *extracellular matrix* (the complex networks of polysaccharides, proteoglycans and proteins of the cell wall) form an interactive scaffold for perception and transduction of such information (Wyatt & Carpita, 1993).

In addition to the extracellular matrix, plants have another major route for cell-cell communication: the plasmodesmata. Plasmodesmata are complex channels which span the extracellular matrix to connect the cytoplasm of adjoining cells (McLean *et al.*, 1997). Initially, in the embryo of higher plants, all cells are interconnected by plasmodesmata (Mansfield & Briarty, 1991) forming a single symplast, which is effectively a single cytoplasm separated by the membranes of individual cells. Later, as the plant differentiates, individual cells or groups of cells become separated into distinct symplastic domains, maybe by loss of functional plasmodesmata (McLean *et al.*, 1997). Separation into these domains facilitates their structural and functional specialization. One example of this is in the root epidermis of *Arabidopsis*, in which the mature epidermis becomes symplastically isolated from underlying cells, and epidermal root hair-bearing cells become isolated from non-hair epidermal cells

(Duckett *et al.*, 1994). Movement of molecules through the plasmodesmata has been investigated by various means, including the use of fluorescent tracer molecules. Plant hormones, such as auxin, are thought to move through the plasmodesmata, in addition to their active transport around the plant. Plasmodesmata can alter their permeability and therefore rates of transport of molecules of different sizes (so-called "gating"), thus affecting symplastic intercellular communication and ultimately plant development. They may respond to external stimuli such as light conditions and, for example, to pathogenesis, through direct targeting of specific pathogenesis-related (PR) proteins to the plasmodesmata and concomitant effects on the intercellular transport of signalling molecules involved in eliciting resistance responses. There have been studies demonstrating that developmental regulators can move between cells. These include the maize *KNOTTED* transcription factor and two *Antirrhinum* MADS-box proteins (reviewed in McLean *et al.*, 1997). How plasmodesmal transport of these transcription factors interplays with the control of plant development remains unclear at present.

Signal transduction in plants has only been begun to be understood at a molecular level in recent years. A number of signalling molecules have now been identified. These include the protein kinases, calmodulin, calcium-dependent protein kinases, cGMP, cAMP and specific receptors for light and hormonal signals (Trewavas & Malhó, 1997). Signals are first perceived by receptors located in the cell membrane, nucleus, cytoplasm or cell wall. A major group of such receptors are the protein kinases which, upon ligand binding, exert their kinase activity on other proteins - the signal transduction occurring through a protein kinase cascade.

The effects elicited through signal transduction pathways are many and variable. The complex interplay between intrinsic genetic programming, environmental stimuli and plant morphogenesis are mediated by such pathways and are extremely difficult to dissect, since pathways are highly branched and a similar end result may be attained via different signalling routes.

1.6.4 Plasticity in plant development

One phenomenon evident in a number of facets of plant growth and development is that termed *plasticity*. This refers to the ability of plants to reverse or change the identity or size/shape of cells in response to phenotypic perturbation. It specifically refers to the potential for cells to undergo genetic re-programming to take on a different fate to that which they were apparently previously already committed. This occurs in response to the cellular environment in which individual cells find themselves.

This phenomenon accounts for the ability of plants to regenerate themselves following even quite extensive mechanical damage and removal of whole organs. Often, when the apical meristem is removed from a plant, compensatory growth occurs from lateral meristems. Apical dominance (the suppression of lateral meristems by the apical meristem) is mediated by the production of auxin from the apical meristem. Mechanical removal of the apical meristem removes this inhibition and allows lateral meristems to become proliferative. Plasticity is also essential in allowing plants to change their form in response to environmental changes: this is necessary as they cannot move towards more favourable environmental conditions.

In plants, because of the way they undergo extensive post-embryonic morphogenesis through the differentiation of cells behind the proliferative meristems, cell identity is determined almost entirely by the position of a cell relative to neighbouring cells. Where this cellular environment is altered, either by mutation or by other means (see cell ablation studies in Section 1.6.4) a given cell may receive altered spatial information from surrounding cells and respond by dividing or by changing its identity according to this information. This is in sharp contrast to the situation in animals where the identity of a cell is generally much more dependent on its lineage (Doonan & Hunt, 1996) than its position. In animals studies on the determination, or otherwise, of cell fate have been carried out by cell and tissue transplantations; this is much more difficult in plants due to the inaccesibility of tissues and rigidity imposed by the plant cell wall (Laux & Jurgens, 1994).

There are various examples of plasticity occurring as a result of genetic mutation. A number of mutants have been identified which exhibit either an alteration of fate of specific cells, tissues or of whole organs, or in cell size or shape. The floral homeotic mutations described in Section 1.5.2 provide examples of alteration at the organ level. When there is a failure of floral organ whorl identity specification due to mutation of homeotic genes, whole whorls may be replaced by whorls of another type of organ.

At the cellular level there are examples of alteration in cell size and shape without alteration of the overall morphology of the plant. These include the *tangled-1* mutant of maize (Smith *et al.*, 1996) in which leaf cells undergo cell divisions in abnormal orientations without altering overall leaf shape. A second example of this type of phenotype was produced by the deliberate over-expression in *Arabidopsis* of B-cyclin, an enzyme important in controlling the rate of cell cycling (Doerner *et al.*, 1996). The result of this was that the number of cells in the roots was significantly increased, without seriously disrupting the body plan of the plant. Roots did grow longer, but cells within the root grew to a similar size to those in wild type plants and were simply incorporated into the body plan of the plant. This is in stark contrast to the situation in animals, in which the inappropriate division of cells has seriously deleterious consequences, manifested as the formation of tumours. Cell number has also been decreased by genetic modification in plants. Hemerley *et al.* (1995) affected cyclin activity by generating transgenic plants expressing a CDK (cyclin-dependent kinase) protein which generates inactive complexes with cyclins. The result of this is that cell division activity is reduced. This, however, does not result in much reduction in the overall size and growth rate of the transgenic plants, despite their containing less than half the normal number of cells. Individual cells then have undergone an increase in size. This phenomenon suggests that there is some mechanism by which plants, in determining their overall size, must control events at a level above that of the control of cell number alone.

A class of *Arabidopsis* mutants, called the *dov* (*differential development of vascular-associated cells*) mutants (Kinsman & Pyke, 1997) exhibit a marked reduction in the number of spongy mesophyll cells and so increased airspace in the leaf without affecting overall leaf form or function.

Even more dramatic mutants exist in which not simply cell size, but overall cell identity have been altered from one differentiated type to another. These include the *twin* mutant of *Arabidopsis* (Vernon & Meinke, 1994) in which, during embryogenesis, a morphologically and functionally differentiated suspensor cell enters a new developmental programme of embryo-proper character, to eventually give rise to a fully functional second embryo within the same seed as the original embryo proper. Such genetic re-programming is also seen in the embryonic root cell ablation studies in which specific cells were laser-ablated. Cells adjacent were induced to divide and produce a daughter cell which took on the cellular identity of that destroyed, effectively replacing the lost cell (Scheres *et al*, 1994; Dolan *et al*, 1993; Van den Berg *et al.*, 1995, 1997).

Such genetic re-programmability is also seen in the establishment of somatic embryos from root-derived callus tissue, *in vitro*. Cells previously differentiated as root cells are obviously not irreversibly committed to this fate with loss of the potential function of genes encoding aspects of a completely different developmental fate.

It is suggested that the tolerance of plants to variation in cell number could be exploited to increase the biomass of either whole plants or individual organs, for example to produce larger potatoes (Doonan & Hunt, 1996). The molecular mechanisms integrating rates of cell division, growth and development at present remain obscure.

1.6.5 Organismal versus cell theory

Morphogenesis in plants, as has already been discussed, is more dependent on positional determination of cell fate than on lineage-dependent determination of the

fate of individual cells. The organismal theory of plant development (Kaplan, 1992; Jacobs, 1997) suggests a model whereby plant form is determined by the differential expansion of organs and the simple partitioning of these organs by mitoses of their constituent cells. This, then, suggests control at a "supracellular" level. Obviously there is not a standard size at which cells undergo division, and mutants have been identified in which cell size is much reduced without a correlative change in the rate of cell division (Baskin *et al.*, 1995; Wilson *et al.*, 1996). Cell sizes do vary according to tissue and organ type: it could possibly be spatial differences in the amount of cell division promoters or inhibitors which control the point at which cells divide following expansion. Some candidate proteins involved in controlling the rate of cell cycling have been identified including cyclin proteins, mentioned in the previous section. The signalling pathways involved in the coordination of cell division and cell expansion remain obscure at present. They are likely to be complex and to be regulated differentially according to local cellular environment.

The phenotypes of some mutants support this idea of supracellular control of morphogenesis, including the *Arabidopsis* embryonic mutants *fass* (Smith *et al.*, 1996) and *hydra1* (Topping *et al.*, 1997), which do produce a seedling with correct positioning and recognisable structure of the major components, despite gross disorganisation at the cellular level. Similarly, the *tangled* mutant of maize exhibits normal overall leaf morphology despite gross disruption of cellular patterning within the leaf (Smith *et al.*, 1996). The manipulation of cyclin expression by the transgenic approaches described in the previous section also lend support to the theory.
Such phenotypes suggest that control of morphogenesis and patterning can, at least in some circumstances, be uncoupled from the control of rates and planes of cell division.

1.7 Gene cloning

Obviously, one of the main goals in biology today is to understand the details of the genetic processes which control the many aspects of the development and growth of

plants and their responses to external factors in their environment. Identification and characterisation of numbers of these genes is not only interesting but may also lead to long-term targeted genetic improvement of crop species.

A number of approaches are at our disposal to identify and isolate genes, including: differential screening of cDNA libraries, screening of libraries with heterologous probes either from different species or related genes within a species, and screening of expression libraries with antibodies against known proteins (Topping & Lindsey, 1995). The major problem with such approaches, however, is that the transcripts of many developmentally interesting genes in particular are likely to be in low abundance and often restricted to a small number of cells of a specific type. This is an especially large hurdle to overcome for some stages of development. During embryogenesis, for example, a large number of genes are required for the complex process of laying down the basic body plan of the plant. Due to the small physical size, especially of early stage embryos, it would be extremely difficult technically to isolate sufficient amounts of mRNA for library construction.

The major way in which developmentally interesting genes are being isolated, therefore, is through the generation and characterisation of mutants with specific defects in developmental processes, each caused by a single genetic lesion.

Once genes have been cloned in one species, then their cloning in other species is facilitated by the use of PCR-based techniques, or by screening libraries with heterologous probes (reviewed in Foster & Twell, 1996).

1.8 Mutagenesis strategies

Mutant lines described in such studies are generated by three main approaches: chemical, physical, and insertional mutagenesis.

1.8.1 Chemical and physical mutagenesis

Chemical mutagenesis has generally involved soaking *Arabidopsis* seeds in a solution of ethyl methane sulphonic acid ester (EMS), a powerful mutagen which chemically modifies guanine residues, causing base substitions during DNA replication (King & Stansfield, 1990; Meinke & Sussex, 1979). Physical approaches typically use X-rays to irradiate dry or imbibed seeds.

The advantage of both chemical and physical mutagenesis strategies is that large numbers of mutagenized plants can be produced and screened for the mutant phenotype of choice. Many mutants have been identified, many of which have morphological abnormalities, such as abnormal flower or leaf morphology or colour; there are also those with altered resistance to certain chemicals, altered nutritional requirements, altered response to environmental stimuli, altered hormone production or response, and those with abnormal organ development, including embryo defective or lethal mutations (Pang & Meyerowitz, 1987). Large numbers of alleles of a given mutant gene can be idenified by such an approach. For example, it seems that probably all possible *fusca* genes have been identified by such mutageneses, with 280 identified mutants falling into 14 complementation groups, each representing on average 18 alleles of the gene. Such saturation screening yields information regarding the number of genes involved in a given process. The major disadvantage of both chemical and physical mutageneses is that whilst they may produce a wide range of mutant phenotypes, it is still not simple to clone the mutated genes. Cloning is becoming a little easier, however, with the construction of increasingly detailed RFLP (restriction fragment length polymorphism) maps of the *Arabidopsis* genome (Hauge *et al.*, 1993; Lister & Dean, 1993). These easily detectable sequences are being mapped to increasingly closely spaced locations in the genome. Co-segregation of the mutant phenotype with given RFLP markers provides a starting point from which to clone the gene, by succesive identification of overlapping DNA fragments - so called *Chromosome Walking* (Leyser & Chang, 1996).

1.8.2 Insertional mutagenesis

An alternative mutagenesis approach now recognised as facilitating the most effective means of cloning the mutated genes is that of *Gene Tagging* or *Insertional Mutagenesis.* The basic principle of this strategy is that DNA of known sequence is introduced into the genome of the species under study, causing mutation by physical disruption of genes. Cloning of the disrupted genes is facilitated by the presence within the gene of the "molecular tag" of known sequence.

There are a number of approaches to the cloning of genes disrupted by insertional mutagenesis. One method is to use Inverse PCR (IPCR), in which genomic DNA flanking the known insert is circularized and amplified with primers designed to amplify outwards from the borders of the insert (Thomas, 1996). In a second approach, probes to known sequences within the insert can be used to screen genomic or cDNA libraries produced from the mutant of interest. A third method, plasmid rescue, relies on the presence of a bacterial origin of replication within the T-DNA to permit cloning of excised and recircularizes genomic DNA (Behringer & Medford, 1992).

The two most useful types of molecular tag used in this way are tranposons (naturally occuring "jumping genes") found in the genomes of some species (reviewed in Walbot, 1992; Section 1.8.3) and T-DNA from *Agrobacterium tumefaciens* (Section 1.8.4).

1.8.3 Transposon tagging

Endogenous transposons in maize have been used to clone a number of developmentally interesting genes such as the *C1* gene involved in anthocyanin biosynthesis (Paz-Ares *et al.*, 1986), the *KNOTTED* gene involved in shoot meristem morphogenesis (Hake *et al.*, 1989) and a number of others. Similarly in *Antirrhinum*, an endogenous transposon (*Tam3)* has been used to isolate some genes, including the *floricaula* gene (Coen *et al.*, 1990).

Endogenous transposons have now been identified in *Arabidopsis* (*e.g.* Peleman *et al.*, 1991; Tsay *et al.*, 1993). However, past efforts have used heterologous transposons from other species, especially the maize *Ac/Ds* transposon system (*e.g.* van Sluys *et al.*, 1987; Dean *et al.*, 1992), which essentially produces random, stable mutations. Transposon systems which allow more targeted disruption of genes of interest have been developed in recent years (Coupland, 1992).

One useful feature of the transposon tagging system is that confirmation that the mutant phenotype is caused by disruption of the cloned gene may be obtained since reversion of the mutant phenotype, by excision of the element, is likely to occur in later generations. Correlation of the loss of the mutant phenotype with the timing of this excision event provides evidence of linkage and strongly suggests causation. Another useful feature is that the number of different mutant lines will increase through sucessive generations, due to transposition: this reduces the number of initial transformants needed in establishing a mutant screen.

One possible shortcoming of transposon tagging is that transposition can occur over limited distances in the genome, limiting random mutagenesis over large regions, though this is useful for targeted transposon mutagenesis of defined loci. These problems are being overcome by various modifications to increase transposition frequencies. Another potential problem is that transposon excision from a given locus may leave behind a molecular footprint, which may complicate subsequent cloning efforts of mutated genes. *Arabidopsis* genes have been sucessfully cloned using transposon tagging, for example, *drl1* - a gene essential in a number of morphogenetic processes (Bancroft *et al.*, 1993).

1.8.4 T-DNA tagging

Agrobacterium tumefaciens is a soil-borne plant pathogen which, as part of its normal life cycle, effectively acts to genetically engineer plant genomes, in that it integrates a portion of DNA (transfer DNA or 'T-DNA'), derived from its Ti plasmid, into the plant genome. Wounded tissues of dicotyledonous plants release phenolic compounds

which induce the bacteria to become attached to the wounded tissue. The virulence region of the Ti plasmid is activated, encoding the production of endonucleases which cleave at the borders of the T-DNA and of other proteins which mediate transfer and integration of the T-DNA into the plant genome (reviewed by Zambryski *et al.*, 1989). Wild-type *A. tumefaciens* causes crown gall disease in the plants it infects, inducing the formation of tumours at the infection site.

Use of this bacterium to genetically modify plants in a controlled manner has involved the genetic modification of the Ti plasmid, primarily to disarm the plasmid of sequences involved in causing disease symptoms. These sequences can be physically removed by use of restriction enzymes, whilst virulence sequences, which mediate the T-DNA transfer, are left intact. Genes of interest can be inserted in place of the removed oncogenic genes; a wide variety of such disarmed vectors are now routinely used in *Agrobacterium*-mediated plant transformation.

Transgene integration in lower eukaryotes, such as yeast, is largely by homologous recombination. In plants, however, it is largely by illegitimate (non-homologous) recombination (Gheysen *et al.*, 1991), although homologous recombination does in fact occur but only at low frequency (Lee *et al.*, 1990). This illegitimacy of recombination is important in considering the use of T-DNA for gene tagging, since the essentially random nature of integration means that all genes in the plant genome are potentially susceptible to mutagenesis by integration of the T-DNA.

Gene identification and cloning over the past few years has been widely carried out by T-DNA insertional mutagenesis of *Arabidopsis* by *A. tumefaciens* transformation. The strategy involves the generation of a large population of transgenic plants with *Agrobacterium* containing T-DNA bearing selectable marker genes, by one of a variety of available transformation methods. This population of transformants (following growth on selective medium) is then screened for mutant phenotypes of interest. Segregation analysis is carried out on subsequent generations of the selected plants to determine whether the mutant phenotype co-segregates with the presence of

the T-DNA. If such genetic analysis suggests that the mutant phenotype is linked to, and therefore likely to be caused by, the T-DNA then the gene may potentially be cloned by several techniques: IPCR, screening of a genomic DNA library made from the mutant line, or by plasmid rescue (Topping & Lindsey, 1995).

Because the number of genes tagged does not increase in succesive generations, unlike the case with transposons, it is necessary initially to produce large numbers of T-DNA transformants. This is becoming less of a problem as more efficient transformation methods are developed (*e.g.* Bechtold *et al.*, 1993).

1.8.5 T-DNA tagged mutants

T-DNA tagging has been used to clone a large number of *Arabidopsis* genes, involved in a wide spectrum of processes (Feldmann, 1991). These include, for example: *GLABROUS* (*GL1* and *GL2*), involved in trichome development (Herman & Marks, 1989; Larkin, *et al.*, 1993); *AGAMOUS* (*AG2*), a homeotic gene of which mutation causes sterility (Yanofsky *et al.*, 1990); *FLOWER* (*FL1*), a floral homeotic gene (den Boer *et al.*, 1991); *DIMINUTO* (*DIM*), a mutant defective in brassinosteroid signalling, resulting in a dwarfed phenotype (Takahashi *et al.*, 1995).

A number of embryonic mutants are amongst those for which genes have been cloned (Castle *et al.*, 1993). These include the *emb30* gene essential for regulation of cell size and shape in the embryo (Shevell *et al.*, 1994) and for post-embryonic root initiation, and a number of *fusca* mutants (Castle & Meinke, 1994).

Other T-DNA tagged embryo-defective mutants include *leafy cotyledon* (Meinke, 1992; West *et al.*, 1994) and *fusca* mutants (Castle & Meinke, 1994). A number identified in this laboratory include *golftee*, which has been found to be allelic to the *emb30* mutant, *hydra1* (Topping *et al.*, 1997), which develops too many cotyledons, is dwarfed and is disorganised at a cellular level, and *fusca* mutants, which inappropriately accumulate anthocyanins during embryogenesis. A further embryonic

mutant will be described in subsequent chapters of this thesis as well as a post-embryonic seedling-defective mutant.

1.9 Gene entrapment techniques

There are limitations in the approach of T-DNA insertional mutagenesis: tagging of functionally redundant genes will produce no detectable phenotype; multiple copies of T-DNA may integrate, thus complicating gene cloning; it may be difficult to demonstrate linkage between the mutant phenotype and the presence of the T-DNA as reversion of the phenotype does not occur, due to the stability of T-DNA integration (Lindsey & Topping, 1995). Final proof of causation of a mutant phenotype by an integrated T-DNA can only be obtained when the wild type copy of the mutated gene has been cloned and used to introduce into the mutant background to complement the mutation (André *et al.*, 1986).

One way of overcoming some of these difficulties is by the use of entrapment vectors. This involves creating T-DNA constructs which contain a reporter gene lacking regulatory elements; the reporter gene can only be expressed when it integrates into the plant genome within, or in such a position as to come under the control of, plant regulatory elements. The reporter genes are dominant, and so can be detected even in the absence of a mutant phenotype, including in the tagging of functionally redundant genes. Mutated genes which cause lethality in homozygous mutant plants can be studied in hemizygotes, their expression pattern potentially yielding information regarding their normal role. Screening for subtle mutant phenotypes can also be aided, since active gene fusions in cells of interest can focus screening on a smaller proportion of the total population of transformants.

1.9.1 Promoter trapping

This specific type of trapping involves the use of T-DNA containing a detectable marker gene lacking a promoter; its expression depending on *in vivo* fusion downstream of a native plant promoter. The earliest examples of such promoter trap vectors used in plants used promoterless selectable marker genes. The first example,

described by André *et al.* (1986), used a T-DNA construct containing a promoterless aminoglycoside phosphotransferase II (*aph*(3')II) gene, which confers resistance to the antibiotic kanamycin. *Nicotiana plumbaginifolia* protoplasts were transformed with this construct and callus regenerated in the presence of kanamycin, to select for those clones derived from a single cell in which an active gene fusion had occured.

The active APH(3')II gene product could be produced by either transcriptional or translational fusions, depending on where the T-DNA integrated relative to the native promoter sequences. When transcriptional fusion occurs, protein synthesis starts at the initiation codon of the *aph(3')II* gene transcript, producing the wild type APH(3')II protein. In translational fusion, translation starts st the initiation codon of a native plant gene transcript, producing a chimeric protein with APH(3')II activity; this relies on the *aph(3')II* sequences integrating in the correct reading frame. Both types of fusion were found to have occurred in different transformed lines.

Problems occurred when using this early promoterless selectable marker technique: particularly that selection pressure tended to lead to identification of lines with multiple T-DNA copies (Koncz *et al.*, 1989), making cloning more difficult. Subsequent studies then used constructs containing a selectable marker gene with a linked promoter, preventing the problem of selecting for transformants with multiple T-DNA inserts; the promoter trapping aspect depending on expression of a promoterless dominant screenable marker gene, such as the *gusA* gene (Jefferson *et al.*, 1987). The *gusA* gene encodes β-glucuronidase (GUS), an enzyme which can be localized histochemically by its ability to cleave a supplied chromogenic substrate. This detection is highly sensitive, allowing detection of low abundancy transcripts, even in the case of very spatially restricted expression patterns. There are several advantages to using such a marker: genes can be isolated even in the absence of a mutant phenotype, where there is gene redundancy, or in polyploid species; mutated genes lethal in mutant homozygotes can be studied in the hemizygous state, yielding information about their possible role; correlation of the expression pattern of the screenable marker with the nature of the mutant phenotype may support evidence of

linkage with, and causation of, the mutant phenotype by the T-DNA insert (Topping & Lindsey, 1995).

Many transgenic lines which exhibit *gus*-fusion activity do not apparently exhibit a mutant phenotype. There are several possible reasons for this (Lindsey *et al.*, 1996). The mutated gene may be functionally redundant, with its role fulfilled by another similar gene; if T-DNA inserts within an intron it may be spliced out during RNA processing; and a mutant phenotype may only be apparent under certain environmental conditions. For example, mutants defective in phytochrome A only exhibit growth defects when grown in far-red light enriched growth conditions (Whitelam *et al.*, 1993).

1.9.2 Secondary mutagenesis

A further use of such marker gene fusion lines is to study the regulation of the expression of the tagged genes. "Secondary mutagenesis" aims to identify regulatory genes or those of associated signal transduction pathways by mutagenising the tagged line and screening for up- or down-regulation of marker gene expression (Lindsey & Topping, 1993).

1.9.3 Enhancer trapping

The level of expression of an integrated transgene can vary widely, depending on its site of integration into the host genome. This phenomenon, termed *position effect* (e.g. Bellen *et al.*, 1989; Wilson *et al.*, 1989), is due to the presence in the genome of silencer and enhancer elements, which can affect gene expression over distances of several kilobases (e.g. Ptashne, 1986). The effect of these sequences has been investigated by use of a reporter gene attached to a weak promoter. Higher than expected levels of expression are presumed to reflect the effects of enhancer sequences; certainly expression levels vary over a wide range (Topping *et al.*, 1991). The limitation of this approach is that enhancer sequence isolation is still potentially difficult, since they may be located several kilobases up- or downstream of the reporter gene.

1.9.4 Frequency of reporter gene activation

It has been demonstrated that promoterless reporter gene activation occurs in a wide range of cell types in all organs examined. The frequency of activation in different organs, however, is not random and is differentially biased in different species (Lindsey *et al.*, 1993; Koncz *et al.*, 1989; Kerbundit *et al.*, 1991). In the study by Lindsey *et al.*, (1993) the binary vector *pΔgusBin19* containing the promoterless *gusA* gene (Topping *et al.*, 1991) was introduced, by *Agrobacterium*-mediated transformation, into populations of tobacco, *Arabidopsis* and potato. Individual organs of the T_1 generation of transformants were subject to GUS fluorimetric assays and histochemical localisation analysis. Activation was at high frequency in all three species. Approximately 20-30% of positive transformants (selected on the basis of kanamycin resistance) exhibited *gusA* activation in leaf in all three species. Frequencies of activation were more variable in other organs, being as high as 75% of all transformants in the roots of tobacco and as low as 9% in the roots of potato.

Despite the widely different genome sizes and amounts of repetitive DNA of *Arabidopsis*, potato and tobacco (Bennett & Smith, 1976) frequencies of *gusA* activation overall are similarly high in all three species. This strongly supports the hypothesis that T-DNA integration is preferentially into transcriptionally active, or potentially active, regions of chromatin (Koncz *et al.*, 1989, 1992; Topping *et al.*, 1991; Lindsey *et al.*, 1993).

The transcription promoting properties of tagged sequences isolated by IPCR have been investigated in re-transformation experiments. Some such sequences potentially may not be associated with native genes, but may be acting as "pseudo-promoters". Identification of such sequences may still be informative, potentially yielding information on the evolution of gene structure and regulation. It has been demonstrated in *Drosophila* (Wilson *et al.*, 1989) that at least some reporter fusions are with native genes and, due to the low frequency of repetitive DNA in the

Arabidopsis genome (Meyerowitz, 1987), insertion into genes might be expected to occur at high frequency.

Insertional mutagenesis, then, is a powerful tool, both for generating mutant phenotypes, the disrupted genes of which may be cloned relatively easily by virtue of the presence of integrated DNA of known sequence. It can also be used for studying the expression patterns and the potential cloning of regulatory sequences within the genome through the analysis of gene fusion lines.

1.10 *gus*-fusion marker genes

A variety of specific marker gene fusions have been generated in a number of species, particularly in *Drosophila* (Bellen *et al.*, 1989), *Caenorhabditis elegans* (Hope, 1991) and *Arabidopsis* (Lindsey & Topping, 1995).

A large number of *Arabidopsis gus*-fusion lines have been generated in this laboratory, including the *POLARIS* and *EXORDIUM* genes (Topping & Lindsey, 1997), which will be described more fully in Section 4.8. Both of these genes are expressed in a spatially restricted manner in the embryo and in the mature plant. There are examples of expression in a wide variety of organ and tissue types, including a number in the vascular system. The native gene relating to one of these vascular-specific markers, *VT-1* has been cloned in this laboratory and found to encode a novel helicase protein (Wei *et al.*, 1997).

1.11 The new generation of reporter genes

Although *GUS* as a reporter gene is easily assayed and detectable even when expressed at low levels, it has a limitation in that the histochemical assay for its activity is destructive and so can only be used to look at individual plants at a single developmental stage. It cannot, therefore, be used to study the precise course of gene expression over time in an individual plant, nor can it be used to rapidly screen a population of primary transformant seedlings for activity. A new type of reporter gene overcomes these limitations: the *green fluorescent protein* (GFP) gene from the

jellyfish *Aequorea victoria* can be directly visualized in living tissue (Chalfie *et al.*, 1994; Siemering *et al.*, 1996; Hassleoff *et al.*, 1997) by scanning-laser confocal microscopy.

1.12 Use of marker genes to study cellular organisation of mutants

One application of marker gene fusion lines, such as the *gus*-fusion lines described above, involves introducing such genes into mutant lines as a tool by which to study their cellular organisation. The principle behind this is that if a fusion gene is expressed only in defined cell types, then if cells of that specific type are disrupted in size, shape, number, or spatial context within the mutant plant then this will be more easily detected than by simple anatomical observation of the mutant phenotype.

For example, a *gus*-fusion marker gene, *VS-1,* is expressed exclusively in all living, differentiated cells of the vascular system of *Arabidopsis* (Muskett, 1998). When introduced into the *hydra1* mutant background (described in Section 1.8.5), this marker yielded information regarding the nature of the cellular defect in *hydra1* roots: it confirmed the physiological evidence that in this mutant vascular cells are present in a differentiated state much closer behind the root tip than in wild type roots (Topping *et al.*, 1997).

Another possible type of information to be gleaned from such crosses is regarding the nature of the control of the expression of the native gene tagged by the *gus*-fusion marker. Whilst a certain gene may be expressed only in specific regions or cell types, the switching on of that gene may be due not to the identity of that cell type, but in response to the spatial context of that cell with respect to other cells (See Section 1.6.3). When cellular organisation is disrupted in mutants, cell patterning may be disrupted and sometimes the marker genes expression pattern differs from that predicted from the morphological identity of the cells present. This rationale may be clarified by an example of such an experiment. The *POLARIS* gene (Topping & Lindsey, 1997), is expressed in the basal part of embryos and progressively spatially restricted to the root meristem. When introduced into the *emb30* mutant (described in

Section 1.8.5), it was surprisingly found that the gene was expressed in the correct spatial context: in the basal part of the embryo. Prior to this experiment it had been assumed that *POLARIS* was a gene involved in root meristem formation or function as this is where it is expressed. It seems from this result, however, that whilst *POLARIS* marks meristem position, it is not switched on specifically in cells morphologically organized as root meristem, since this mutant lacks, both anatomically and functionally, such a meristem. It seems to be expressed either as a determinant, or in response to other determining factors, of root meristem *position*, rather than the cellular *identity* of the cells of the root meristem *per se*.

1.13 Production of the population of T-DNA transformed *Arabidopsis* used in this study

Plants can be tranformed with DNA by two broad approaches: direct gene transfer, particularly protoplast transfection methods, and the use of vector-based systems for DNA transfer, the most commonly used vector being *Agrobacterium tumefaciens* (Topping *et al.*, 1993). *Agrobacterium* has been used to transform various tissues of *Arabidopsis*: intact seeds (Feldmann & Marks, 1987), cotyledons (Schmidt &Willmitzer, 1991), roots (Valvekens *et al.*, 1992) and recently flowers (Bechtold *et al.*, 1993).

The method used to produce the population of transformed plants from which the mutants described here were isolated is as previously described (Clarke *et al.*, 1992; Topping *et al.*, 1993). Plants were previously transformed with the vector, *pΔgusBin19*, which contains a promoterless *gusA* reporter gene (Fig. 1.1; Topping *et al.*, 1991).

The transformation method involves co-cultivation of roots with *Agrobacterium* and is based on the method of Valvekens *et al.*, (1992), with modifications to increase the frequency of transformation up to 100-fold (Clarke *et al.*, 1992).

1.14 Project Aims and Objectives

The overall aim of this work was to further our understanding of the genetic control of aspects of plant development through the identification of seedling and/or embryonic defective mutants. There is a longer term objective of cloning and functionally analysing the disrupted genes.

Specifically, the objectives of the work described in this thesis were to identify and carry out a phenotypic and genetic characterisation of such mutants, defective in morphogenic aspects of development, and to gain an insight into the possible nature of the roles of the disrupted genes.

The two mutants described were identified from a population of *pΔgusBin19* transformants.

One mutant, *vertically challenged1* (*vch1*), was identified in a screen for morphogenic mutants at the seedling stage. It is characterised by its very short stature and much reduced root system.

The second mutant, *altered suspensor fate1* (*asf1*), was identified within the same transformed line when it was examined at the embryonic stage. This mutant appeared as an enlarged, arrested globular embryo with an aberrantly developed suspensor. The two mutations had segregated apart in the next generation of the line examined.

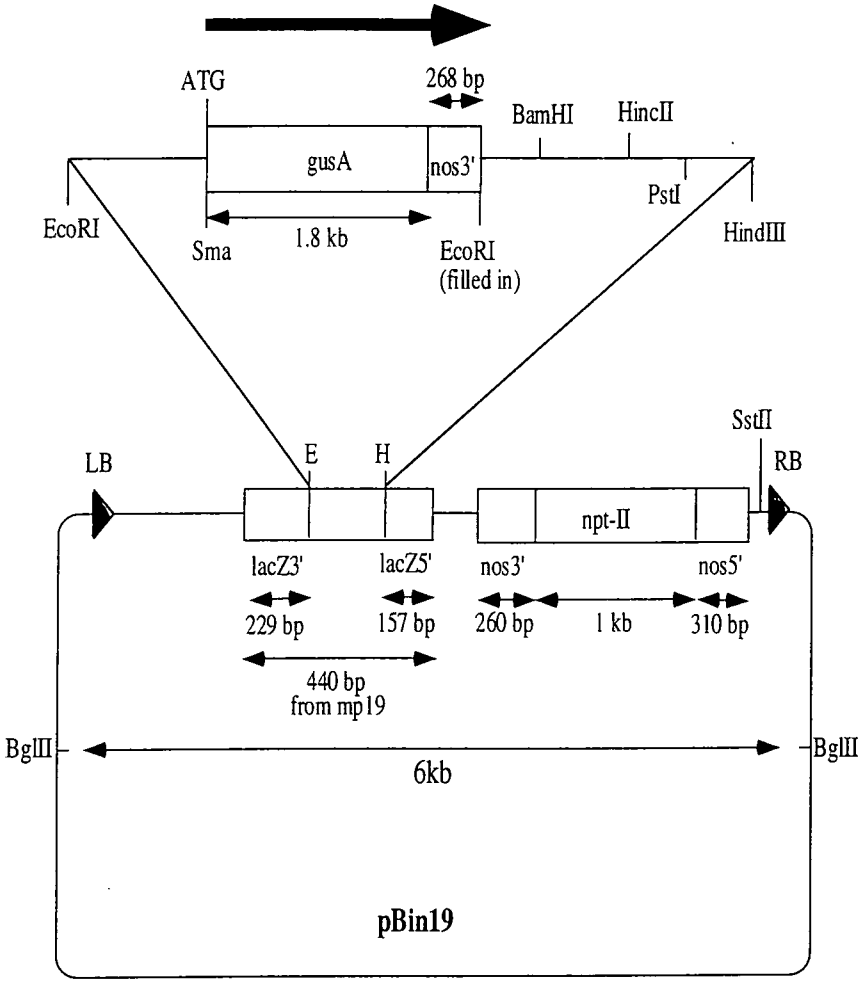
1.15 Summary

This chapter has proposed the importance of studying the genetic control of plant development. Various aspects of development have been discussed, with particular reference to the model dicot plant species, *Arabidopsis thaliana*, under study here. Methods of cloning developmentally interesting genes have been described including different mutagenesis strategies, with particular reference to T-DNA tagging as used in this study.

Subsequent chapters will describe the methods used (Chapter 2) and the results of the genetic, phenotypic and preliminary molecular characterisation of the two mutants under study (Chapters 3-6). Chapter 7 will discuss the results obtained and assess their biological significance.

Fig. 1.1

pΔgusBin19



from Topping *et al.* (1991)

MATERIALS AND METHODS

2.0 - Materials and Methods

All chemical reagents were analytical reagent grade and were obtained from Sigma (Poole, UK) or BDH (Lutterworth, UK), unless otherwise stated.

2.1 Plant growth conditions

Arabidopsis plants for crossing, routine bulking up of seed stocks and *in vivo* physiological studies were grown in 4:1 Levingtons multipurpose compost: silver sand (to enhance drainage) at 22°C and a light regime of 16 hr light : 8 hr dark, with daily watering from above, using a watering can with a fine nozzle. Aracon tubes (BetaTech, Belgium) were used to aid bulk seed collection, and stems bearing siliques containing seeds from first generation crosses were supported by wooden stakes and plant ties.

Crossing was routinely carried out using fine watchmakers forceps to dissect floral buds under a stereomicroscope, emasculation using a fine wire loop to remove immature anthers, and physical transfer of mature pollen from the male parent to the stigma using forceps.

Brassica napus plants were grown in 3:1 Levingtons multi-purpose compost:coarse gravel in 15cm plantpots. Following 5 days stratification at 4°C seeds were germinated and grown in a growth room under a diurnal 16h light (150 μmol m^{-2} s^{-1}) at 20°C: 8h dark at 15°C cycle, at 60% humidity.

2.1.1 Germination and seedling growth

Arabidopsis seedlings for physiological studies, and for nucleic acid extractions, were grown on 1/2MS10 medium (Appendix 1) in sterile Petri dishes, at 22 ± 2°C under continuous light at 150 μmol m^{-2} s^{-1}.

For measurements of rates of root growth, seeds were germinated along a line marked on a square Petri dish containing 1/2MS10 medium and, upon germination, the plates were supported at an angle of approximately 70°.

Seedlings to be grown in the dark were treated exactly as those in the light except the Petri dishes were covered in aluminium foil prior to placement in the growth chamber.

Prior to germination, seeds, on the medium in plates, were stratified at 4°C in the dark for 4-7 days, to increase germination frequency and ensure relative uniformity of germination timing.

2.1.2 Hormones and signalling inhibitors

For the hormone and signalling inhibitor experiments described in Sections 4.9 and 5.7 the hormones/signalling inhibitor compounds were filter-sterilized through 0.2µm pore Acrodiscs™ (Gelman, Northampton, UK) then added to molten 1/2MS10 medium cooled to below 60°C. All were first dissolved in high quality water, except kinetin which was dissolved in dilute HCl, and IAA and 2,4-D, which were dissolved in 70% ethanol.

2.1.3 Injection of hormones and inhibitors into developing siliques

It has been previously demonstrated that the exogenous application of certain hormones and inhibitors to developing embryos can perturb their development (*e.g.* Liu *et al.*, 1993). Such experiments have been carried out on *in vitro* cultured embryos.

The aim of the experiments described in Section 5.7 was to introduce various hormone or signalling pathway inhibitors into the locule (the hollow core) of developing siliques in an attempt to interfere with the development of embryos *in vivo*. Fine glass needles were produced by heating, stretching, and breaking when cooled, fine glass pipettes. These were then used, with a rubber pipette bulb, to inject into the locule various solutions of hormone or signalling inhibitors. In each inhibitor solution was included 0.1% (w/v) phenosafranin, as a tracking dye.

2.1.4 Seed sterilization

It is necessary to sterilize seeds prior to germination on medium in sterile Petri dishes to remove any contaminating fungi or bacteria which would otherwise thrive on the nutrient-rich culture medium. Exposure of the seeds to ethanol prior to the sterilizing bleach solution serves to partially de-wax the seeds, thus enhancing penetration of the bleach, and may also kill some microorganisms. Addition of a detergent, such as Tween 20, aids this. It is important to wash the seeds thoroughly to remove bleach following sterilization, since failure to do so will result in a reduction in seed viability.

A maximum of approximately 150 seeds were placed in a 7 ml plastic Bijou bottle (BDH) and the bottle filled with 70 % v/v ethanol for 20 seconds before removal and replacement with 10 % v/v Chloros commercial bleach solution plus 1-2 drops of Tween 20 detergent. These were gently mixed by drawing in and out of a transfer pipette and the seeds were left to sterilize in this solution for 10-15 min. This solution was washed off thoroughly with 5-6 changes of sterile distilled water, and the seeds plated out onto germination medium (1/2MS10) immediately.

Sterile transfer pipettes were used for each change of solution, and all sterilization was carried out in a laminar flow cabinet.

2.2 Root and shoot culture of *Arabidopsis*

2.2.1 Shoot culture

The method employed was as described by Topping and Lindsey (1991b) for the sustained culture of tobacco shoots *in vitro*.

Shoot cultures comprise rootless aerial parts (in this case hypocotyl, cotyledons and leaves) growing on a defined medium supplemented with agar. They are initiated from explant tissue containing meristems, in this case apical buds, and are subcultured by the transplantation of excised buds onto fresh medium at intervals of 3-4 weeks.

The shoot apex, cotyledons and emerging first leaves of *Arabidopsis* were excised at the hypocotyl from seedlings at 1-2 weeks post-germination and transferred to MS20 (Appendix 1) in a sterile Petri dish, embedding the cut surface in the agar medium. The cultures were maintained at 22°C under continuous light, and were routinely sub-cultured at intervals of 3-4 weeks. Sub-culture was carried out by excision of the apical bud, along with the newest pair of leaves, and transfer to fresh MS20 medium.

2.2.2 Root culture

Arabidopsis root explants can be indefinitely maintained and propagated in liquid culture, where thay maintain their morphogenic potential. The method for root culture employed here was developed as a system for the mass clonal propagation of genetically stable *Arabidopsis* material by Czakó *et al.* (1993). Addition of indoleacetic acid (IAA) to the liquid medium at the time of initiation of the root culture promotes adventitious root branching whereas in the absence of IAA root elongation and branching cease soon after excision. Once the culture is established, the IAA is removed in order to avoid excessive branching and to allow elongation.

The apical 5-7 mm of the roots of seedlings of 2-3 weeks were excised and placed in a sterile Petri dish containing Root Culture Medium (Appendix 1) and were grown on an orbital shaker shaking at 150 rpm at room temperature with continuous illumination. The initial medium containing IAA (0.05 mg/l) was removed after 2 days, and replaced with the same medium lacking IAA. Subculture was routinely carried out at 2-3 week intervals by excision of young roots from the periphery of the inoculum and transfer to fresh medium, repeating the 2 day IAA treatment each time.

2.2.3 Growth of callus

Callus was initiated and maintained according to Topping *et al.* (1993).

Seeds were sterilized and germinated on 1/2MS10 medium (Section 2.1.1). After 14 days of seedling growth, roots were excised, transferred to plates containing Callus Induction Medium (CIM; Appendix 1) and replaced in the growth room. The callus

formed was maintained by excision of pieces of callus from the periphery of the explant and transfer to fresh CIM at intervals of 7-10d.

2.3 Dry weight and chlorophyll content

2.3.1 Determination of percentage dry weight of tissues

Dry weight measurements were obtained by weighing large numbers of whole seedlings or plants, drying the samples thoroughly at 60°C for 24 h, and re-weighing.

2.3.2 Determination of chlorophyll content

The chlorophyll content of the aerial portion of 14 d.o. seedlings was assayed spectrophotometrically, according to the method of Harbourne (1973). Assays were performed for pooled samples of *vch1* and WT seedlings Values are expressed as µg chlorophyll/g fresh weight of tissue±SE.

Brifly, the aerial portions of seedlings were excised and ground in 80% (v/v) acetone with a pestle and mortar. The supernatant was transferred into tubes and briefly centrifuged to pellet out cellular debris and the resulting supernatant was immediately spectrophotometrically assayed. Absorbance was measured at 645nm and 663nm. Chlorophyll content was calculated as follows:

Chl. A (g/l) = (0.0127 x A_{663}) - (0.00269 x A_{645})

Chl. B (g/l) = (0.0027 x A_{645}) - (0.00468 x A_{663})

2.4 Microscopy

2.4.1 Light microscopy

An Olympus SZH10 stereomicroscope was used for all low magnification observations, manipulations (including genetic crossing) and photography. Photographs were taken using an Olympus SC35 35mm camera on tungsten-balanced ISO-160 film.

High magnification examinations (100-400x) were carried out using a Zeiss Axioskop compound microscope fitted with a Yashica 108 camera, again using tungsten-balanced ISO-160 film.

Sample preparation

Samples for observation on the stereomicroscope were generally placed directly on the microscope stage and observed. For photography of samples, particularly seedlings, it was found to be best to partially embed the samples in the surface of a gel matrix (*e.g.* Phytagel) in a Petri dish, add 2-3 drops of water and cover with a large (28x50 mm) coverslip, carefully avoiding trapping any air bubbles. This served to not only flatten the samples and so reduce the depth of field, but also to keep the samples hydrated, thus maintaining the structural integrity of root hairs and other delicate structures.

Samples for observation of the compound microscope were prepared in a number of ways.

(1) Embryos

Embryos at a range of developmental stages can be observed by chemical clearing of the seed testa. This was achieved by dissecting seeds out from siliques, adding a drop of 0.5M KOH and covering with a cover-slip. By pressing down gently on the cover-slip embryos could sometimes be extruded from the seed. Others could be clearly seen through the testa since KOH treatment causes it to become semi-transparent.

(2) Histological sections

For screening large numbers, sections were observed without treatment on the glass slides on which they were collected during sectioning. For photography, however, chosen sections were mounted in DPX mounting medium (Fisons, Loughborough, UK) under a cover-slip. The DPX was allowed to harden overnight at room temperature.

(3) Whole mount preparations of root explants

Roots were mounted in a drop of water under a cover-slip.

Chlorophyll visualisation

Autofluorescence of chlorophyll was visualised in tissues mounted in a drop of water under a coverslip. Using a Zeiss Axioskop microscope, samples were excited at a wavelength of 490nm and fluorescence detected at 515nm. Photography was with an Olympus OM10 camera on Kodak Ektachrome 400 film.

2.4.2 Histological sectioning

The method followed for preparation of material for histological sectioning was largely as described in the Historesin^{R} embedding kit (Leica Instruments GmbH, Heidelberger, Germany) with additional infiltration steps added to ensure complete infiltration of larger samples. It was used for the sectioning of embryos, seedlings and mature roots.

Material was fixed in 4% *para*-formaldehyde solution (pH 7.0), vacuum infiltrated for 5 min, replaced with fresh solution and incubated overnight at 4°C. The fixative was replaced and the material dehydrated through an ethanol series: first incubated in 70% v/v ethanol for 2-4 hr at 4°C, then in 95% v/v ethanol overnight. Infiltration was carried out through a series of 95% v/v ethanol:infiltration solution (Historesin^{R} kit) in the following ratios: 3:1, 2:1, 1:1, 1:2, 1:3. The material was vacuum infiltrated for 20 min in each solution, and incubated for a minimum of 4 hr at 4°C.

Embedding resin was made up according to the manufacturer's instructions. Once the resin is mixed it is important to work quickly as it polymerises rapidly. Samples were embedded in the resin in BEEM capsules with pointed tips (BEEM, UK), by first half-filling the capsule with resin, then positioning the sample and filling the remaining volume with resin. Any air bubbles can be removed by the use of a fine needle. The resin is left to polymerize at least overnight or until it becomes hardened.

It is preferable, in the case of embryos, to embed a number in each resin block and, in the case of roots, to embed a whole seedling root system per block. This ensures that

sections will be cut from material lying in a variety of orientations, thus increasing the probability of obtaining some sections of the type required.

A razor blade was used to remove the embedded samples from the *BEEM* capsule and to flatten off the base of the resin block, which is then mounted in Paraplast wax onto a stub to fit the microtome to be used, with the pointed end of the resin block protruding.

Sections of 5-10μm were cut using disposable steel blades (TAAB, UK) on a Leitz 1512 rotary microtome, and transferred, using a fine brush, into drops of water on a subbed microscope slide (see Section 2.4.3), and fixed by drying on a heating block on a low temperature setting. Sections were stained by immersion in 0.01% (w/v) toludine blue for at least 1 min. Excess stain was washed off with distilled water and sections dried as above.

Slides could be made permanent by mounting in DPX mounting medium, covered with a coverslip and left to harden for at least overnight.

Sections were observed and photographed as described in Section 2.4.1.

2.4.3 Subbing of slides for histological sectioning

Standard glass microscope slides were coated with a mixture of gelatin and chromium potassium sulphate prior to use, to ensure the adherence of sections during the staining procedure.

A solution of 0.1% gelatin and 0.01% chromium potassium sulphate was vacuum filtered through a filter of pore size 0.45μm (Sartorius, UK), and clean microscope slides were submerged briefly in this solution before being removed and left to drain and dry, at an angle, overnight. The subbed slides can be stored indefinitely before use.

Alternatively, ready-coated Superfrost Plus^{TM} slides (BDH) were used.

An alternative fixation and embedding procedure was later found to yield superior sections with better morphological preservation than those obtained using this method. This involved the use of LR White resin as the embedding medium. The protocol for this method is described in Section 2.4.4.

2.4.4 LR White embedding and sectioning

Seeds containing immature embryos at various stages were removed from siliques and placed in a solution of 3% *para*-formaldehyde in 0.1M phosphate buffer (0.1M Na_2HPO_4, NaH_2PO_4, pH 7.0). Following vacuum infiltration for 5 min twice, samples were incubated at 4°C overnight. Samples were then washed for 30 min twice in 0.1M phosphate buffer alone, prior to dehydration through a graded ethanol series. Solutions of 10%, 20%, 30%, 50%, 70%, 90% and 100% (v/v) ethanol in water were added, removed and replaced at 10 min intervals, at room temperature. It was found to be necessary to use this gradual change in ethanol concentration in order to obtain best morphological preservation of tissue when embedded. Samples were finally washed once more in 100% ethanol for 20 min, prior to a gradual infiltration with the embedding medium LR White resin (London Resin Co., UK). The 100% ethanol was removed and replaced succesively by 3:1 and then 1:1 ethanol:LR White (v/v) for 30 min each at room temperature. It was important to mix the ethanol:LR white solutions thoroughly prior to use, to maximise tissue infiltration. 100% LR White was then added and removed and replaced a total of 6 times at regular intervals over a period of 3 days. This long incubation is necessary to ensure complete infiltration of all tissue by the resin.

The resin was then polymerised by incubation at 50°C for 24 hours. Prior to polymerization the samples were carefully transferred into gelatin pharmaceutical capsules (size 2; Parke-Davis and Co., Hounslow, UK). Approximately 10-15 seeds were placed in each capsule. To optimise LR White polymerization it is necessary to exclude atmospheric oxygen during the polymerization process. This was achieved by placing the rounded lid of the gelatin capsule upside-down on the resin filled base. Following polymerization the capsules were left at room temperature overnight to

harden prior to careful removal of the gelatin capsule using a clean razor blade. Blocks can be stored at this stage for several weeks at room temperature if necessary.

Sections of 2μM thickness were cut on an Ultracut OM30 microtome using glass knives cut on a Jeol 7800 Knifemaker. Sections were collected onto the surface of de-ionised water in a home-made "boat" on the knife. They were transferred using a clean glass rod into drops of PBS on Superfrost Plus^{TM} slides (BDH). These slides are coated with a positively charged ionic layer, which aids adhesion of sections to the slide.

2.4.5 Confocal microscopy

Samples were examined using a Zeiss Axiovert microscope to which was attached a BioRad MRC600 confocal scanning laser system (BioRad, Hemel Hempsted, UK). Cell measurements were made using the MRC Comos imaging software. Digitized images were printed on a Sony thermal printer.

Sample preparation

Samples (embryos within seeds dissected out from siliques, or excised pieces of root or hypocotyl) were placed on a clean microscope slide. One drop of 0.5M KOH plus one drop of 0.001% (w/v) ethidium bromide were layered over the samples and covered with a large (32x24mm) zero-thickness cover-slip. The edges of the cover-slip were sealed with nail varnish. Samples could be observed from approximately 20 min after preparation, however, it was found that if the slides were left at this stage at 4°C for 2-3 days, the samples became more thoroughly infiltrated with the EtBr and so more evenly stained and cellular organisation was found to be preserved.

2.4.6 Scanning electron microscopy

Method based on that of Vasil and Vasil (1984).

This method was used to study both excised embryos of *Arabidopsis*, and seedlings of 1-3 weeks.

The material is fixed in gluteraldehyde and osmium tetroxide, both of which are extremely toxic. Osmium should only be handled with extreme care in a designated area and any contaminated glassware soaked overnight in milk protein solution before disposal.

Fixation

For preparation of mature embryos for observation, dried mature seeds were imbibed overnight in water. The embryos were excised by placing the imbibed seeds on filter paper and teasing the embryos out with syringe needles. The embryos were placed directly into distilled water in glass vials to prevent dehydration. When all embryos had been collected, the water was carefully removed and immediately replaced with 2.5% v/v gluteraldehyde in 0.1M sodium cacodylate buffer (pH 7.2-7.5, pH adjusted with dilute HCl), and incubated overnight at room temperature.

Samples were washed three times, for 30 minutes each time, with 0.1M sodium cacodylate. After the third wash the buffer was replaced with 1% osmium tetroxide in 0.1M sodium cacodylate, and incubated at least overnight at 4°C. The material is fixed when it becomes black; it is then washed three times in distilled water for 30 min each time.

Dehydration

The samples were dehydrated through an acetone in water (v/v) series of: 25%, 50%, 75% and 100%, at room temperature, and were finally rinsed twice in 100% acetone.

The samples could be left at the 75% acetone stage, at room temperature, for up to four weeks before completing the dehydration process. It is important to ensure that the samples remain immersed in the solution during this period.

The method was modified for seedlings to include vacuum infiltration for 20 min at all stages where solution changes were made, except where osmium tetroxide was added

and the first wash thereafter. The infiltration is necessary to ensure complete fixation and dehydration of internal tissues. Seedlings were left in osmium tetroxide for a minimum of 24 hrs, to ensure adequate fixation.

Critical point drying

Drying of the samples is carried out very rapidly by exploiting the ability of carbon dioxide to lyophilize, or change from a liquid to a gasous phase, almost instantaneously under defined critical temperature and pressure conditions. The 100% acetone is replaced with liquid carbon dioxide under high pressure, and the temperature raised until the liquid carbon dioxide reaches its critical point, and lyophilyses. This ensures that the tissues are dried with minimum structural disruption.

Critical pont drying was carried out on a home-made machine.

The dried samples were mounted onto metal stubs (Agar Scientific, Stansted, UK) to fit the microscope stub carrier with double-sided adhesive discs and a fine brush to transfer the samples. Embryos and small seedlings, at this stage, tend to become highly electrostatically charged and hence difficult to manipulate. An air de-ioniser was employed to alleviate this problem.

Sputter coating

Samples were coated with an electron-dense layer of gold-palladium of 5-50 Å, using a Polaron sputter coating machine.

The prepared samples could then be stored for several months under vacuum conditions, before observation.

Observation and imaging

A JEOL IC848 SEM was used to observe the samples and images were digitally captured.

2.4.7 Transmission electron microscopy

The fixation procedure employed was essentially that of Schwartz *et al.* (1994).

Seeds at various developmental stages were dissected out from siliques using fine dissecting needles, and were immediately placed into a 2% gluteraldehyde in 0.1M phosphate buffer solution (0.1M Na_2HPO_4, 0.1M NaH_2PO_4, pH 6.8), on ice for 4-6 hours. Roots and hypocotyls were treated in the same manner, but were incubated in the gluteraldehyde solution on ice overnight to ensure penetration of the fixative into all internal tissues. All traces of gluteraldehyde were removed by rinsing the samples three times, for a minimum of 30 min each time, in 0.1M phosphate buffer. Samples were post-fixed in 1% osmium tetroxide in 0.1M phosphate buffer (pH 6.8) overnight at 4°C, and were then rinsed three times, for a minimum of 30 min each time, with distilled water, before dehydration through a graded ethanol in water series: 25%, 50%, 75%, 100% (v/v). Samples were incubated, at room temperature, for a minimum of 2 hours at each ethanol concentration, before removal and replacement with the next solution.

Embedding

Samples were embedded in epoxy resin (Araldite) prior to sectioning according to the method of Karnovsky (1965).

The Araldite solution, which is polymerised to embed the samples into a solid resin support, is not miscible with ethanol, therefore, it is necessary to first replace the ethanol in the dehydrated tissues with propylene oxide, which acts as an intermediate solution, being miscible with both ethanol and Araldite solution. All Araldite components were obtained from Agar Scientific (Stansted, UK).

Samples were incubated in a 1:1 (v/v) solution of ethanol:propylene oxide at room temperature, for 3 periods of 10 minutes, with the solution being removed and replaced with fresh solution at each stage. This procedure was repeated three times, with 100% propylene oxide, with similar incubation periods of three times 10 min. Araldite resin

solution was made as follows: 10ml Araldite CY212 and 10ml D.D.S.A were warmed to 45C before use. To these was added 1ml dibutyl phthalate and 0.4ml B.D.M.A. All components were mixed thoroughly to ensure even polymerisation. Araldite resin solution was initially added to the samples as a 1:1 v/v propylene oxide:Araldite solution with incubation at 45°C for 30 min, followed by 100% Araldite, for a further 30 min at 45°C. It is important to leave lids off the sample containers once Araldite has been added, to prevent possible explosion. The samples are finally embedded into fresh Araldite, in disposable flat aluminium containers, and were carefully aligned in the correct orientation using dissecting instruments. Extreme care is needed at this stage, as the dehydrated samples are very brittle and easily damaged. The Araldite containing the samples was allowed to polymerise at 60°C for a minimum of 48 hours before proceeding to cutting out blocks with a hacksaw, from the hardened resin containing samples of a shape suitable for sectioning.

Sectioning

Sections were cut on an Ultracut OM30 ultramicrotome. Semi-thin sections of 2μm were cut first to locate areas of interest. Once a small region of such a section was chosen for TEM observation, the block was razor trimmed to produce a small trapezoid block face of approximately 1-2mm square around this area, and semi-thin sections were then cut from this face.

Sections, collected on copper grids, were stained with the heavy metal compounds uranyl acetate and lead citrate, according to a standard technique.

Onto a piece of clean parafilm was placed drops of 1% uranyl acetate (w/v in 70%v/v ethanol). Grids, with sections on, were placed carefully section side-down on the drops. After 10 min grids were removed and were washed by dipping rapidly and repeatedly through the surface of distilled water for 1 min. Grids were then placed on 1% w/v lead citrate drops, on parafilm, stained for 1 minute, and the washing procedure repeated. It was important to avoid breathing near the lead citrate, as this would lead to precipitation of lead carbonate residue on samples.

2.5 Histochemical localisation of GUS activity

Direct visualisation of β-glucuronidase (GUS) enzyme activity and indirectly, therefore, of *gusA* reporter gene activity within specific cells or tissues of transformed plants can be achieved histochemically using a chromogenic substrate (Jefferson, 1987).

The substrate used was 5-bromo-4-chloro-3-indoyl β-D-glucuronic acid (X-Gluc) (Melford Labs, Ipswich, UK). The effect of GUS activity on this substrate is to cause oxidative dimerisation to form an insoluble dye which is easily visualized as blue colouration at the site of GUS enzyme activity. Potassium ferricyanide and ferrocyanide were including in the reaction buffer since they act as oxidation catalysts and reduce diffusion of a soluble reaction intermediate into surrouding tissues (Mascarenhas, 1992).

Samples were placed in X-Gluc which had been dissolved in a small volume of dimethylformamide and then diluted to a final concentration of 1mM in histochemical buffer (100mM phosphate buffer, pH 7.0, 10mM EDTA, 0.1% v/v Triton X-100, 1mM potassium ferricyanide, 1mM potassium ferrocyanide).

Samples were vacuum infiltrated for 5 min in this solution and were then incubated at 37°C for a period ranging from 3h to 3 days, depending on the GUS activity level expected in the given sample. Tissue was then cleared of chlorophyll by replacing the X-Gluc in histochemical buffer with 70% (v/v) ethanol, to aid visualisation of the blue precipitate.

2.6 Immunolabelling

Immunolabelling is a useful technique in studying developmental processes since it allows the study of the temporal and spatial accumulation of specific proteins during cellular differentiation and organogenesis (Harris, 1993). This, along with localization of mRNAs by *in situ* hybridisation (Section Jackson 1992), allows us to elucidate

patterns of gene expression and, with the aid of molecular analysis, to understand the regulation of such gene expression. The technique can be used at an ultrastructural level to investigate the subcellular localization of proteins, particularly using electron microscopy to detect gold particles conjugated to a secondary antibody.

The general methodology of immunolocalization involves: antibody preparation, specimen preparation, removal of non-specifically bound antibody and finally the visualisation of specifically bound antibody.

2.6.1 Whole mount immunolabelling

Immunological detection of cytoskeletal components was performed on whole mounts of seedling roots, essentially according to McClinton & Sung (1997).

Whole seedlings were fixed in 4% formaldehyde in PEM (50mM 1,4-piperazinediethane- sulphonic acid, pH 6.9; 5mM $MgSO_4$; 1mM EGTA) for 45 min and then washed in PEM twice. Cellulose was then digested by placing the seedlings in 1% cellulase in PEMS (PEM plus 0.25M sorbitol) containing protease inhibitors (20µg/ml leupeptin, 50µg/ml phenylmethylsulphonylfluoride, 0.5µg/ml aprotinin) for 1 hour at room temperature. Roots were dissected from the rest of the seedling and were placed on a microcope slide in PEMS, covered with a coverslip and the root tips squashed by gentle tapping. Slides were rapidly frozen on dry ice and the coverslips removed without disturbing the samples. Samples were treated with 0.5% Triton-X in PBS (137mM NaCl, 2.68mM KCl, 8.0mM Na_2HPO_4, 1.76mM KH_2PO_4) for 30 min and washed in PBS. Methanol was layered over the samples which were then placed at -20°C for 10 min and again washed in PBS.

Labelling was carried out overnight in a moist chamber at room temperature using each antibody (anti-α-tubulin, anti-β-tubulin (ICN, Oxfordshire, UK), anti-actin (Amersham, Buckinghamshire, England) diluted 1:1000 in 3% BSA, 0.02% Tween 20. Following 2 x 10 min washes in PBS the secondary antibody, fluorescein

isothiocyanate-conjugated goat anti-mouse IgG (Sigma) diluted 1:40 in PBS was added and incubated for 1 h at room temperature. Samples were washed for 2 x 10 min in PBS and mounted in 50% PBS: 50% glycerol and were examined under a UV microscope and by confocal scanning laser microscopy.

2.7 DNA extraction from plant tissue

To extract DNA from plant cells it is necessary to rupture the cells by grinding, and to disrupt the cell membranes by the addition of a detergent. Such treatments must be carried out with care, since they can lead to shearing of the DNA. To prevent endonuclease digestion of the DNA during extraction, the magnesium-chelating agent EDTA was included in the extraction buffer; the EDTA binds to magnesium ions, which could otherwise act as co-factors for the endogenous endonucleases. Addition of phenol to the extraction mixture acts to denature contaminating proteins and facilitates their separation from the nucleic acids extracted. Material from seedlings or young rosette leaves was found to provide the highest yields of DNA.

2.7.1 Small scale extraction method

Method based on that of Lindsey and Jones (1989).
This method can be scaled up if higher yields are required.

Prior to extraction, plants were placed in the dark overnight to reduce the amount of carbohydrate present in the tissue, since this may form a major contaminant in nucleic acid extractions.

Approximately 0.5 g of leaf material, either fresh or previously frozen at -80°C, was ground in liquid nitrogen using a pre-chilled pestle and mortar. The frozen, ground material was then transferrred to a microfuge tube containing 0.5ml of TES (100 mM Tris-HCl, 150 mM EDTA, pH 8.8, 0.1% w/v SDS), 10mM dithiothreitol and 100 mM phenanthroline hydrate and mixed by gentle inversion of the tube. Phenol was added in equal volume to the reaction mix, and following emulsification by gentle mixing, the phases were separated by centrifugation in a microfuge at 13krpm for 10 min. The

upper aqueous layer was transferred to a fresh tube, avoiding transfer of material from the interface. The lower layer was re-extracted with TES, as above.

Nucleic acids were precipitated by the addition of 0.6 volumes of cold isopropanol and 0.1 volumes of 3M sodium acetate (pH 5.2), with incubation at -20°c for 30 min, and collected by centrifugation at 13 krpm in a microfuge for 10 min. The resultant pellet was resuspended in 0.5 ml of TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.6) and extracted with an equal volume of phenol:chloroform: isoamyl alcohol (25:24:1) by vortexing and centrifugation at 13 krpm for 5 min. The upper aqueous layer was removed and re-extracted in this manner until protein was no longer visible at the interface after centrifugation. Extraction was repeated with chloroform:isoamyl alcohol (24:1) twice, to remove any traces of phenol.

RNA was removed by digestion at 37°C for 30 min, with 5 µl of RNase A (10mg/ml, heat treated to denature DNases), followed by extraction with an equal volume of phenol:chloroform:isoamyl alcohol, followed by chloroform:isoamyl alcohol, as above. The purified DNA was precipitated, by addition of 2 volumes of ethanol and 0.1 volumes of 3 M sodium acetate (pH 5.2), with incubation at -20°c for 30 min, and precipitated by centrifugation at 13 krpm for 15 min. The pellet was washed twice with cold 70% v/v ethanol, allowed to air dry, and resuspended in either sterile distilled water or TE (50-100 µl).

2.7.2 Plant genomic DNA extraction using PhytoPure kit

DNA was extracted from *Arabidopsis* rosette leaves or seedlings using the Nucleon^{R} PhytoPure Genomic DNA Extraction Kit (Scotlab, Lanarckshire, UK) essentially as according to the manufacturer's instructions, with some modifications to increase removal of contaminants. This method is rapid and simple, allowing for preparation of a number of samples at once. The PhytoPure resin binds and thus removes polysaccharides efficiently.

Again, plants were placed in the dark overnight to reduce the amount of carbohydrate present in the tissue.

4.6ml of *Reagent 1* plus 184µl of a 10mg/ml DNase-free RNase (previously boiled for 30 min to destroy any contaminating RNases) were added to a 50ml centrifuge tube. 1g of plant tissue previously ground to a fine powder in liquid nitrogen was added to this solution and the tubes incubated at 37°C for 30 min. To each tube was added 1.5 ml of *Reagent 2* and the contents were then thoroughly mixed by inverting the tube several times. Tubes were incubated at 65°C in a shaking water bath for 10 min, and then on ice for 20 min. Tubes were removed from the ice and 2ml of cold chloroform plus 200µl of PhytoPure *resin* were added. Tubes were shaken at room temperature for 10 min, centrifuged at 1300xG for 10 min and the upper aqueous layer removed to a new tube. An equal volume of isopropanol, previously stored at -20°C, was added to precipitate the DNA and left for 2-3 min at room temperature. The DNA was not visible at this stage. DNA, along with contaminants still present, were pelleted by centrifugation at 4000xG for 5 min, the pellet washed in 200µl 70% (v/v) ethanol and re-pelleted by a further centrifugation at 4000xG for 5 min. Ethanol was thoroughly drained from the pellet, which was then resuspended in 0.5ml overnight at room temperature. The DNA solution was then transferred to an eppendorf tube and centrifuged for 1 min at 13krpm in a microfuge, to pellet contaminants, and the supernatant containing the DNA transferred to a fresh tube. DNA was then precipitated by the addition of 3M sodium acetate (pH 5.5) and 1ml 100% ethanol: the DNA was visible as a cloudy precipitate at this stage. The DNA was then pelleted by centrifugation at 13krpm in a microfuge for 10 min, the pellet washed briefly with 70% (v/v) ethanol and the ethanol thoroughly drained off. The DNA pellet was resuspended in 200-300 µl TE (pH 8) (depending on estimated yield) overnight at room temperature. Tubes were centrifuged at 13krpm in a microfuge for 1 min to remove any remaining contaminants, and the DNA concentration was then quantified by electrophoresis alongside DNA standards of known concentration. DNA was stored at 4°C.

2.7.3 Rapid DNA preparation method for PCR

This method was used to isolate genomic DNA rapidly. It yields DNA which is of lower purity than than that from the methods described above, but is adequate for the purpose of genomic PCR. Based on Edwards *et al.* (1991).

One small leaf was homogenised by grinding in a 1.5ml microfuge tube containing 400µl of PCR Extraction Buffer (100mM Tris, pH8; 50mM EDTA, pH8; 500mM NaCl; 1.4% SDS; 10mM β-mercaptoethanol). Grinders for homogenisation were made by heating the end of a 1ml Gilson pipette tip and sealing by moulding the cooling tip in the bottom of a microfuge tube. The homogenate was centrifuged at 13krpm in a microfuge for 5 min and 300µl of supernatant transferred to a fresh tube. To this was added an equal volume of propan-2-ol, followed by a further 5 min centrifugation at 13krpm in a microfuge and removal of the supernatant. The resultant pellet was drained, washed in 70% (v/v) ethanol, drained and re-suspended in 20µl high quality water.

2.8 Gel electrophoresis of DNA

DNA can be separated, purified and quantified by electrophoresis through an agarose gel matrix. The range of size of DNA molecules separated can be altered by altering the agarose concentration. The migration of linear nucleic acid molecules through the gel is directly proportional to the length of the molecule and so, by loading markers of known length alongside samples it was possible to determine their size.

The position of DNA within the gel following electrophoresis was visualised through the addition of ethidium bromide to the gel. This dye intercalates between bases of DNA and dye:DNA complexes can be visualised due to their fluorescence under UV light.

Agarose concentrations ranging from 0.7% (for Southern blotting of genomic DNA) to 2% (for separation of low molecular weight PCR products) were used.

Agarose (IGI, Sunderland, UK) was dissolved in TAE buffer (40mM Tris-acetate, 1mM EDTA, pH 8.0) by heating in a microwave oven. Ethidium bromide was added to a final concentration of approximately 0.5µg/ml. The molten agarose was allowed to cool to approximately 60°C and then poured into an appropriately sized gel tray with comb and allowed to set for 30 min at room temperature.

DNA samples were mixed with loading buffer (50% glycerol, 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol) to aid loading and to allow the extent of the migration of the DNA to be estimated during electrophoresis. The gel was immersed in TAE buffer in a gel tank, the DNA samples and DNA markers added and was subject to a constant electrical voltage of 1.0-5.0 V/cm.

Following electrophoresis the DNA was visualized on a UV transilluminator using a Molecular Analyst^{R} software package (Bio-Rad, Hemel Hempstead, UK) and digitally recorded or printed on Mitsubishi K61B thermal printer paper.

2.9 DNA manipulation

2.9.1 Restriction enzyme digestion

Restriction enzymes (Restriction Endonucleases) are isolated from a variety of bacteria and are widely used to cleave DNA molecules. They recognise a short palindromic DNA sequence, normally of 4, 5 or 6 nucleotides at which they cleave the molecule.

Restriction enzymes have specific requirements for optimal reaction conditions, being particularly sensitive to the concentration of certain ions. The enzymes used here were used with the optimised reaction buffers supplied by the manufacturer (Boehringer-Mannheim, Lewes, UK).

DNA was pipetted into a microfuge tube along with the appropriate volume of 10x reaction buffer, the required restriction enzyme (2-3 units/ μg DNA) and the volume made up to 10-40μl, depending on DNA amount and concentration, with distilled water. The tube was incubated at 37°C for 1-2 hr, in the case of plasmid DNA, or overnight, in the case of plant genomic DNA for Southern analysis.

2.10 Extraction of plasmid DNA

2.10.1 Large scale plasmid preparation

This method was used to isolate a high yield of plasmid DNA fragments required for labelling as a probe in Southern hybridisations.

Bacterial cells were collected from a 500 ml overnight culture of *E.coli* , grown at 37°C in LB broth (10 g/l bacto-tryptone; 5 g/l bacto-yeast extract; 10 g/l NaCl; pH 7; 15% w/v bacto-agar) with agitation, by centrifugation at 2500 x G for 10 min at 4°C. The bacterial pellets were resuspended in 3 ml of lysozyme solution (10 mg/ml) in lysis solution (50 mM glucose, 25 mM Tris, pH 7.5, 10 mM EDTA, pH 8.0), per 250 ml of culture, transferred to 50 ml tubes, and left at room temperature for 5 min. 6 ml of alkaline lysis buffer (0.2 % sodium hydroxide, 1 % w/v SDS) was added to each tube, mixed by inversion and the tube placed on ice for 7 min. 4.5 ml of cold 3 M sodium acetate (pH 5.2) was added to each tube, and mixed by inversion, followed by incubation on ice for 30 min. Bacterial cell debris and chromosomal DNA were pelleted out by centrifugation at 1300 x G for 30 min at 4°C, and the supernatant transferred to fresh tubes containing 2 volumes of cold ethanol and incubated at -20°C for 30 min to precipitate nucleic acids. These were collected by centrifugation at 9.5 x G for 10 min at 4°C, and were resuspended, by vortexing, in 3 ml cold TE (10 mM Tris-HCl, 1mM EDTA, pH 7.6).

The differential precipitation of large RNAs was accomplished by addition of 1ml 5M cold lithium chloride, with incubation on ice for 30 min, followed by centrifugation at 9500 x G for 10 min at 4°C. The pellet was discarded and the supernatant transferred to a fresh tube, and the nucleic acids precipitated as above. The resultant pellet

was dissolved in 0.5-1.0ml of sterile distilled water, and transferred to a 1.5ml microfuge tube, and treated with 5µl RNase A (10mg/ml, heat treated) for 2-3hr at 37°C.

Extraction with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) mixed by vortexing, followed by centrifugation in a microfuge at 13krpm for 5 min at 4°C, facilitated the removal of protein contamination. This was repeated 3-4 times, until no protein was visible at the interface after centrifugation. The aqueous layer was extracted twice with chloroform:isoamyl alcohol to remove any traces of phenol, and the DNA precipitated by addition of 2 volumes of cold ethanol and 0.1 volumes of 3M sodium acetate (pH 5.2) and incubation at -20°c for 30 min. The DNA was collected by centrifugation at 14 krpm for 15 min, washed twice with 70% v/v ethanol, air (or vacuum) dried, and dissoved in 250-500µl of sterile distilled water or TE (pH 8.0).

The concentration and purity of the DNA was determined by electrophoresis alongside known standards, and the concentration adjusted to 1mg/ml.

2.11 Purification of DNA fragments from agarose gels

DNA fractionated by electrophoresis can be isolated and purified in various ways such as electroelution, organic solvent extraction, isolation from low melting temperature agarose, and capture on DEAE-cellulose membranes. The method employed here relies on the mechanical isolation of DNA fragments of interest from an agarose gel, its capture on a silica matrix (*e.g.* GeneClean^{R}), and washing whilst bound to the matrix. This method is rapid and relatively efficient (Vogelstein & Gillespie, 1979). Washing removes any impurities, and the purified DNA is subsequently eluted from the matrix into a low salt buffer.

2.11.1 GeneClean procedure

GeneClean^{R} II kit was supplied by Anachem (Luton, UK).

Following electrophoresis, the band containing the DNA fragment of interest was excised from the gel using a clean scalpel blade, transferred to a pre-weighed

microfuge tube and weighed; 1 g of gel was assumed to equal 1 ml. 6M sodium iodide was added to the tube, at a minimum of 3 volumes per volume of gel slice, and the tube placed in a water bath at 45-55°C until the agarose had dissolved (approximately 3-5 min). *Glassmilk* (silica matrix) was added, the volume depending on the mass of DNA present in the sodium iodide; for 5 µg of DNA or less, 5 µl was added, and this volume increased by 1 µl for each additional 0.5 µg of DNA. The contents of the tube were mixed by briefly vortexing, and the tube incubated on ice for 5 min, with mixing every 1-2 min to keep the silica suspended. The silica with bound DNA was pelleted by centrifugation at 13 krpm in a microfuge for 5 sec and the supernatant removed and re-extracted with fresh glassmilk before being pelleted. The pellets were resuspended in 50 volumes of cold *New Wash* (containing 50 % v/v ethanol, NaCl, Tris and EDTA) to remove impurities, repelleted by centrifugation for 5 sec and the supernatant removed. This wash procedure was carried out a total of 3 times, before resuspension of the pellet in either distilled water or TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.6), and the DNA was then eluted by incubation at 45-55°C for 3 min. The supernatant, containing DNA, was removed following centrifugation at 13 krpm for 30 sec, the pellet eluted for a second time and the supernatants pooled. The purified DNA was then quantified by electrophoresis alongside DNA samples of known quantity.

2.12 PCR

The polymerase chain reaction (PCR) results in the amplification of a segment of DNA between two regions of known sequence, catalysed by *Taq* DNA Polymerase purified from the bacterium *Thermus aquaticus*. Oligonucleotide primers are designed which are complementary to the regions of known sequence, and the reaction allowed to proceed through several cycles of denaturation, primer annealing and polymerisation. DNA template plus primers, *Taq* Polymerase (Promega, UK), the supplied 10 x reaction buffer, dNTPs and distilled water to make up to the desired final volume (approx. 40µl) were placed in a 0.5ml microfuge tube and mixed thoroughly. The *Taq* Polymerase was always added last. The components were mixed and overlaid with a drop of mineral oil to prevent evaporation during incubation. Reactions were carried out in a Perkin-Elmer thermal cycling machine.

In the first amplification cycle the reaction mixture was incubated at 95°C for 3 min to ensure complete denaturation of the DNA template, followed by approximately 30 cycles comprising: 1 min denaturation at 95°C, 2 min annealing at approximately 60°C (optimum annealing temperature was calculated for each primer combination) and 2 min primer extension at 72°C (this was increased to 10 min in the final cycle).

PCR products were sized by electrophoresis alongside known size DNA standards.

2.13 Southern blotting

Southern blot analysis facilitates confirmation of the presence of specific DNA sequences within genomic DNA, and allows for the determination, or confirmation, of the size of the DNA fragment in which they are contained.

2.13.1 Genomic DNA digestion

Genomic DNA was digested with appropriate restriction enzymes (Section 2.9.1) and fractionated by gel electrophoresis (Section 2.8). This fractionated DNA was denatured *in situ* and transferred to a nylon membrane using the capillary transfer technique devised by Southern (1975). Since the relative positions of the DNA fragments are maintained during transfer, it is possible to locate, and determine the size of, fragments containing sequences complementary to a radiolabelled probe which is hybridized to the fractionated DNA. Determination of size is by comparison of the migration of the labelled fragments with that of DNA markers of known molecular weight.

2.13.2 Electrophoresis and DNA transfer

Digested DNA samples were loaded on a gel and marker DNA was loaded in the outer wells. Following electrophoresis, the gel was photographed alongside a ruler: this facilitated the determination of the size of bands detected by autoradiography, by comparison with that of the marker DNA fragments.

In order to enable the rapid transfer of genomic DNA from gel to membrane, it is necessary to reduce the sizes of the fragments. This was done by soaking the agarose gel in depurination solution (0.25M HCl) for 15 min, which partially depurinates the DNA at sites which are hydrolysed in the following denaturation step. Following depurination, the gel was briefly rinsed in distilled water and then bathed in denaturation solution (0.5M NaOH, 1.5M NaCl) for 30 min, with gentle agitation. It was then rinsed again in distilled water and soaked in neutralizing solution (0.5M Tris-HCl pH7.4, 1.5M NaCl) for 45 min with gentle agitation, renewing the solution after 20 min.

The Southern blotting apparatus was assembled in a reservoir of transfer buffer (10xSSC: 1.5M NaCl, 0.15M sodium citrate) by placing the gel onto filter paper on a sponge support of 3-4cm thick. The transfer of the DNA from the gel to the nylon membranes is by capillary flow of transfer buffer, through the gel and membrane, and into a stack of towels. To eliminate the possibility that the buffer should pass from the reservoir to the stack of towels without passing throught the gel and membrane, the gel was surrounded by strips of old autoradiography film. Nylon membrane (Zeta-Probe GT from Bio-Rad), cut to the size of the gel and pre-wet for 5 min in distilled water, was laid on the gel surface, and any air bubbles carefully removed. 3 layers of filter paper, pre-wet with transfer buffer, were laid on top of the membrane, followed by a stack of paper towels, approximately 10 cm thick, and the whole stack was weighed down with a medical flat bottled filled with water and loosely taped down to ensure stability. DNA transfer was allowed to occur over 16-20hr, during which time the reservoir of buffer was replenished as necessary.

Following transfer, the membrane was washed briefly in 2xSSC (0.3M NaCl, 30mM sodium citrate), and the DNA UV cross-linked to the membrane by placing the membrane in a UV stratalinker (Bio-Rad, Hemel Hempstead, UK). The denatured DNA immobilized on the membrane was subsequently hybridized with a radiolabelled probe (Section 2.13.4).

2.13.3 Preparation of radiolabelled probes by oligolabelling

High specific activity probes can be produced by the use of random sequence oligonucleotides as primers in the labelling of DNA. Random hexanucleotides ~~are able to~~ anneal at multiple sites along the length of a DNA template, the resulting primer-template complexes being a substrate for the Klenow fragment of *Escherichia coli* DNA Polymerase I. New DNA strands are synthesized by the enzyme by the incorporation of nucleotide monophosphates at the free 3' -OH group of the primer. By substituting a ^{32}P-radiolabelled nucleotide, dCTP, for the non-radioactive version in the reaction mixture, the DNA probe synthesized is radiolabelled along its entire length, producing probes with high specific activities (10^8-10^9cpm/μg), as is necessary for detecting single copy sequences in eukaryotic genomes.

As labelling an entire vector containing the DNA sequence to act as a template leads to the production of only a low signal to background ratio during hybridization, the DNA template fragment was digested from the vector and isolated by gel electrophoresis, followed by purification using the GeneClean^R procedure (Section 2.11.1).

The labelling reaction was carried out using a Prime-It^R II Random Primer Labelling Kit (Stratagene, La Jolla, California) according to the manufacturers instructions.

25ng (1-23μl) of the DNA template to be labelled were added to a clean microfuge tube, with 1-23μl of high quality water, to make a total volume of 23μl, boiled for 5 min, snap-cooled on ice, and briefly centrifuged to collect the liquid in the bottom of the tube. 10μl of random oligonucleotide 9-mer primers (27 OD units/ml) were added, along with 10μl of primer buffer (dATP, dGTP, dTTP, each at 0.1mM in buffered aqueous solution) and 1μl of Exo(-) Klenow (5 U/μl in buffered glycerol solution). All the contents of the above tube were then transferred to another microfuge tube containing a 5μl aliquot of ^{32}P dCTP, and carefully mixed by drawing in and out of a Gilson pipette tip, and briefly centrifuged to collect all components at the bottom of the tube. The reaction mix was incubated at 37°C for 2-10 min, and the reaction then

quenched by the addition of 2µl of Stop Mixture (0.5M EDTA, pH 8.0); the EDTA chelates the magnesium ions which are an essential co-factor for the enzyme.

Before using the probe, it was boiled for 5 min, to ensure its denaturation, and then snap-cooled on ice.

Probes were purified using NucTrap Push^{R} Columns (Stratagene, La Jolla, California), which separate unincorporated nucleotides from radiolabeled DNA probes upon passage of the probe solution through a resin column, to which the unincorporated nucleotides are bound.

2.13.4 Hybridisation of a radiolabelled DNA probe with immobilised genomic DNA

The method employed was that recommended by the manufacturers of the nylon membrane used, Zeta-Probe GT (Bio-Rad), with hybridisation being carried out in Techne (Cambridge, UK) bottles in a Techne hybridisation oven. In order to prevent non-specific binding of the DNA probe to the filter, it is necessary to include, in the prehybridisation and hybridisation buffers, a blocking agent - in this case, SDS.

The nylon membrane, with bound nucleic acids, was placed in a Techne bottle with 20-30ml prehybridisation/hybridisation buffer (0.25M Na_2HPO_4, pH 7.2, 7%w/v SDS) and incubated for 0.5-1 hr at 65°C. The prehybridisation/hybridisation solution was replaced with 10-15 ml of fresh prehybridisation/hybridisation solution, along with the DNA probe, previously denatured by incubation at 100°C for 10 min and snap cooled on ice. Hybridisation was allowed to occur over 12-16 hr at 65°C.

Following hybridisation the membrane was washed to remove any non-specifically bound probe. The prehybridisation/hybridisation solution containing unbound probe was removed, and replaced with 30 ml of wash solution 1 (20mM Na_2HPO_4, pH 7.2, 5 %w/v SDS) and incubated for 30 min at 65°C. This was repeated with wash solution 1, and then twice with wash solution 2 (20mM Na_2HPO_4 pH 7.2, 1 %w/v SDS).

Background radioactive counts were detected, with a hand-held Geiger counter, on areas of the membrane which were not expected to emit a significant signal. If background counts were high (above 10cpm), then the membrane was further washed with wash solution 2.

2.13.5 Detection and visualisation

The detection of sites of hybridisation of the radiolabelled probe to DNA was by autoradiography with exposure of the membrane to X-ray film (HyperFilm^{TM}, Amersham, UK), in a light-tight cassette with intensifying screens at -70°C, at least overnight depending on the strength of the signal expected.

Films were developed using an automated developing machine.

2.14 Genetic mapping

Genetic mapping of the chromosomal location of mutant genes was carried out by the introduction of scorable phenotypic markers of known genetic map position into the mutant backgrounds. The distance of the mutant genes from a given marker gene could be calculated from the frequency of co-segregation of the mutant and marker phenotype in the progeny of plants heterozygous for each of the genes.

This linkage analysis was performed using the DP23, DP24 and DP28 multiple tester lines (Franzmann *et al.*, 1995) obtained from the Nottingham Arabidopsis Seed Centre. Each of these lines exhibits 4/5 specific phenotypic traits associated with mutation at genes of known genetic map location. These three lines were chosen as the marker genes represent loci on each of the arms of the five *Arabidopsis* chromosomes (Appendix 3).

Crossing was carried out as described in Section 2.1. Crosses were made between each of the three multiple tester lines and plants heterozgous for each of the two mutations. In the case of the *asf1* mutant, heterozgous plants were easily identified due to the

embryonic nature of the mutant phenotype; siliques were removed and scored for the presence of 25% *asf1* embryos.

Because *vch1* does not exhibit an embryonic phenotype and of the difficulty of identifying heterozygous seedlings on the basis of kanamycin selection, it was necessary to use for crosses a number of the progeny of a known heterozygous parent. Of the 75% of the progeny of such a parent which did not exhibit the mutant phenotype, it was to be expected that two-thirds would be heterozgous for the mutation. It was necessary, therefore, to use a sufficient number of these phenotypically wild type individuals to ensure that some of the progeny of the crosses would be heterozygous for the *vch1* mutation.

Seeds collected from both sets of crosses were germinated and grown to maturity. In the case of *asf1* the presence of the mutant phenotype (F_2 embryos) could be detected in the siliques of this F_1 generation of plants. In the case of *vch1*, it was necessary to check the seedlings of the F_2 generation for the presence of the mutant phenotype. All plants were grown under standard greenhouse conditions. Seeds from the F_1 and F_2 generations were collected in Aracon tubes (Beta-Tech, Belgium).

Over 300 F_2 siblings were analyzed for each cross, being scored for the presence of each of the marker phenotypes. In the case of *asf1*, siliques were removed and scored for the presence of 25% mutant embryos. In the case of *vch1*, seeds were harvested from this F_2 generation and germinated to score for the presence of the *vch1* phenotype in the seedlings of the F_3 generation.

Linkage analysis on this data is performed using MapMaker/Exp. 3.0 (Lander *et al.*, 1997; Lincoln *et al.*, 1992).

RESULTS

3.0 - Preliminary Analysis of Mutant Lines

3.1 Objectives

The objective of this thesis was to identify and characterise morphogenesis mutants of *Arabidopsis*. Such mutants were identified from a population of plants transformed with the T-DNA of the binary vector *pΔgusBin19* containing a promoterless *gusA* reporter gene (Fig. 1.1; Topping *et al.*, 1991).

In this chapter is described the identification and preliminary genetic analysis of the two mutant lines under study.

3.2 Mutants identified

A mutant, now named *vch11* (*vertically challenged1*) had already been identified in a previous screen of the transformed lines. It was a T_3 family of plants thought to contain this mutation which was re-screened. The *vch1* mutant was indeed present and the next generation (T_4), produced by selfing of this T_3 generation, and found to segregate 1:3 (187 *vch1*:538 WT) for the mutant phenotype. *vch1* individuals were identified by their small stature and very much reduced root system compared to wild type seedlings (Fig 3.1a). Whilst screening at the embryonic stage for the *vch1* mutant (which was originally thought to possibly be an embryonic mutation), another mutant, called *asf11* (*altered suspensor fate1*) was also identified in one of the T_3 plants. This was also found to segregate out at a ratio of 1:3 (102 *asf1*:331 WT) in subsequent generations. *asf1* individuals were easily distinguished from normal embryos within a heterozygous silique by their much smaller size, lack of bilateral symmetry (no cotyledons) and what appeared to be an aberrantly-developed suspensor (Fig. 3.1b).

This preliminary genetic analysis showed that two recessive mutants had been identified.

3.3 Determination of T-DNA presence

It was considered important to determine the presence of the T-DNA in the genomes of the two mutants. The most rapid method of determining the presence of a given DNA sequence in a heterologous mixture of nucleic acid sequences is the technique of PCR. As the nucleic acid sequence of the *gusA* gene is known (Jefferson *et al.*, 1987) oligonucleotide primers designed to specifically amplify the gene were used in order to confirm its presence in the genomes of the mutant lines. Genomic DNA was isolated from a pool of seedlings from each of the two mutant lines, *asf1* and *vch1*, and from untransformed seedlings as a control and was analysed for the presence of the *gusA* gene by PCR. The 610 bp product expected was indeed detected for both mutant lines, and not when untransformed DNA or a control reaction mixture lacking DNA template were subject to the same PCR conditions (Fig. 3.2).

3.4 Detection of the *nptII* gene in the transgenic lines

Because the T-DNA also contains the *nptII* gene, which confers resistance to the antibiotic kanamycin, transgenic plants could be selected by their ability to grow on kanamycin-containing selective medium. The ratio of sensitive:resistant seedlings could be used as an indication of the number of loci at which T-DNA had integrated into the genome of transformants. Co-segregation of kanamycin resistance with the mutant phenotypes would confirm whether the mutant phenotypes were linked to the T-DNA in their genomes.

The results of this analysis proved to be complex. No kanamycin-sensitive individuals could be identified in either line (from over 500 seedlings examined), even when kanamycin concentrations were increased to a level where it was generally cytotoxic to all seedlings (300mg/l), suggesting the presence of at least 3 or 4 T-DNA copies at multiple loci in both lines.

Fig. 3.2

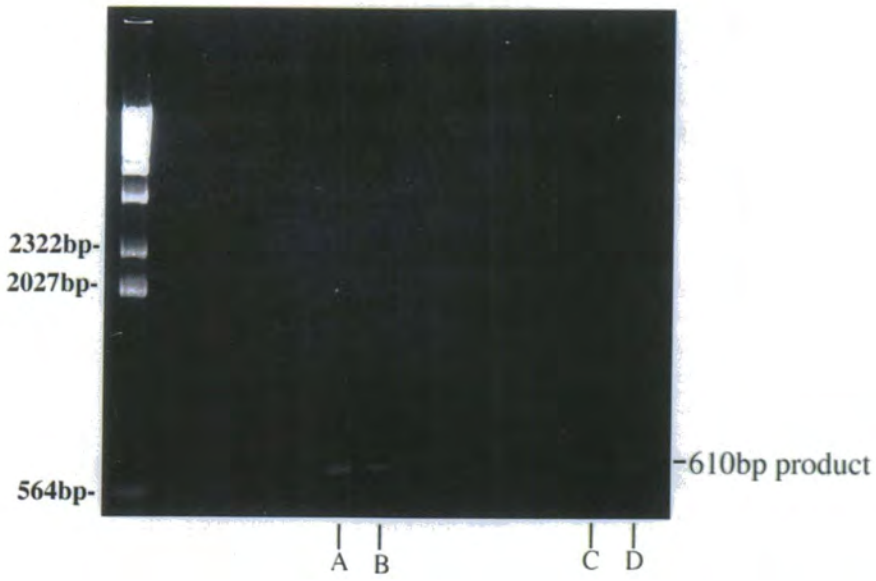
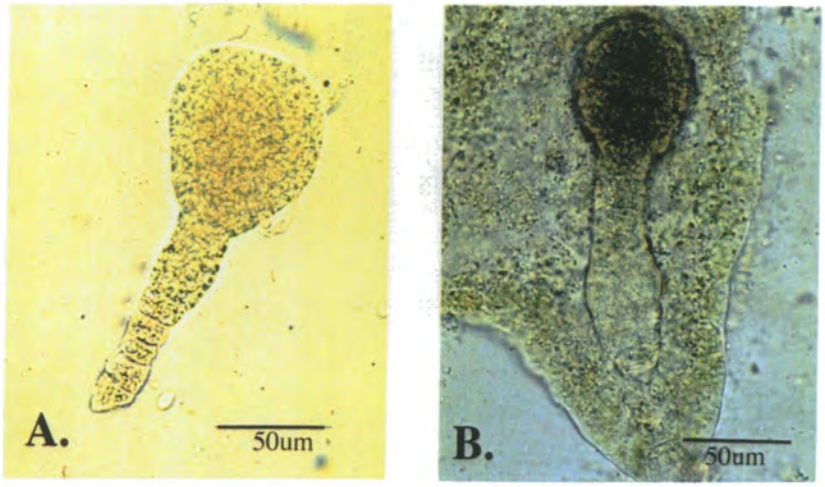


Fig. 3.3



3.5 Analysis of GUS expression in the mutant lines

The integration of our T-DNA construct into the plant genome can have two possible effects. Firstly, it may cause a mutant phenotype by inserting within a gene or its regulatory sequences, thus causing physical disruption and affecting the production of the gene transcript. Secondly, either in addition to or instead of causing a mutant phenotype, when this T-DNA inserts downstream of native plant promoter sequences it may be activated by these promoter sequences to produce a GUS transcript. The enzymatic product of the GUS gene can be detected histochemically by providing the chromogenic substrate X-gluc, which produces a blue enzymatic product in tissues in the presence of the active GUS enzyme. The detection method is described in Section 2.5. Both the *vch1* and *asf1* lines were assayed in this way for evidence of GUS-activity in tissues at a range of developmental stages. No blue staining was detected in either line, indicating that a GUS fusion transcript had not been produced.

3.6 Complementation analysis

Once the *asf1* mutant had been identified it was compared to previously described mutants with similar phenotypes, such as the abnormal *sus*pensor mutants (Schwartz & Meinke, 1994) and the *raspberry* mutants (Yadegari *et al.*, 1994) and others (Marsden & Meinke, 1985). It appeared similar to these classes of mutants insofar as it arrests as an enlarged globular embryo, with a mass of cells at its basal end apparently derived from ectopic suspensor cell division. Because *asf1* appeared morphologically very similar to one of these mutants, *sus1* (Schwartz & Meinke, 1994), it was necessary to determine whether this putative suspensor mutant in fact represented another mutant allele of *sus1* at the same genetic locus. *sus1-2* seeds were kindly provided by Dr. D. Meinke (Oklahoma State University, USA) for complementation analysis. If these two mutant phenotypes were indeed genetically allelic (see Fig. 3.3 for a comparison of their phenotypes), then crossing plants heterozygous for each of the mutations respectively would be expected to yield the mutant phenotype in F_1 embryos. If the mutants were not allelic, however, then the mutant phenotype would not be expected to be apparent in the F_1 embryos as the

genetic defect in each line would be complemented by the wild type copy of the gene from the other line.

Four reciprocal crosses were made between 4 separate plants of each line heterozygous for the mutations. The resultant seeds were harvested, germinated and allowed to self-pollinate to produce F_1 embryos. Over 20 embryos were examined within each of 10 siliques of each of the 8 F_1 plants, and were scored for the presence of the *asf1/ sus1-2* phenotype. No such phenotype was detected in any of the siliques examined, indicating that complementation had indeed occurred and that the *asf1* mutant represented a mutation at a different genetic locus.

In the case of *vch1*, no other mutants had been previously identified with a similar phenotype and it was, therefore, assumed that this also represented a mutation at a novel genetic locus.

Recently another allele of the *vch1* gene, named *vch1-2*, has been identified in a screen of an EMS mutagenised population for seedling-defective mutants carried out on this laboratory (P. Chilley & K. Lindsey, unpublished data). Complementation analysis confirmed that this phenotypically indistinguishable second mutant was indeed allelic with *vch1* (*vch1-1*). This strongly suggests that *vch1* does indeed represent a single gene mutation and that both mutations are likely to be complete loss of function since their phenotypes are indistinguishable.

3.7 Summary

This chapter describes the identification of two morphological mutants of *Arabidopsis*. Preliminary genetic analysis indicated that both were recessive single-gene mutations, as they both segregated out at 25% in successive generations of selfed heterozygous plants. Both lines appeared to contain at least 3-4 copies of the T-DNA insert, as indicated by the kanamycin selection segregation data (where there any fewer copies than this, some kanamycin sensitive individuals would have been

observed amongst the 500 seedlings examined). The presence of the T-DNA in each line was also confirmed by the detection of the *gusA* gene by PCR. No GUS activity was detected in either line, suggesting that a functional *gus*-fusion gene was not produced in either case. Complementation analysis of the *asf1* line with a similar previously described mutant confirmed that this mutation was, in fact, at a different genetic locus. It seems likely that two novel genetic loci have been identified in this study.

The next stage was to carry out detailed phenotypic analyses of the two mutant lines to determine the precise physiological effects of the genetic alterations. These analyses will be described in the following two chapters: *vch1* in Chapter 4 and *asf1* in Chapter 5. Chapter 6 will then deal with the subsequent steps of mapping the chromosomal location of the two mutations, and their preliminary molecular characterisation. The results presented here and in these following chapters will be discussed in Chapter 7.

4.0 - The *vch1* mutant

4.1 Objectives

The preliminary observations of the *vch1* phenotype led to a more detailed study of the nature and possible causes of the failure of normal organ elongation in this mutant. The *vch1* mutant was identified at the seedling stage. *vch1* seedlings are much smaller than wild type and had very short roots, small rounded cotyledons and first leaves, a shortened hypocotyl and an epinastic growth habit (Fig.3.1a).

The objective of the work described in this chapter was to establish at which stage of development the *vch1* phenotype becomes evident; which, and to what extent, individual organs are affected; and the nature of the defect at the whole plant, organ, tissue, cellular and sub-cellular levels.

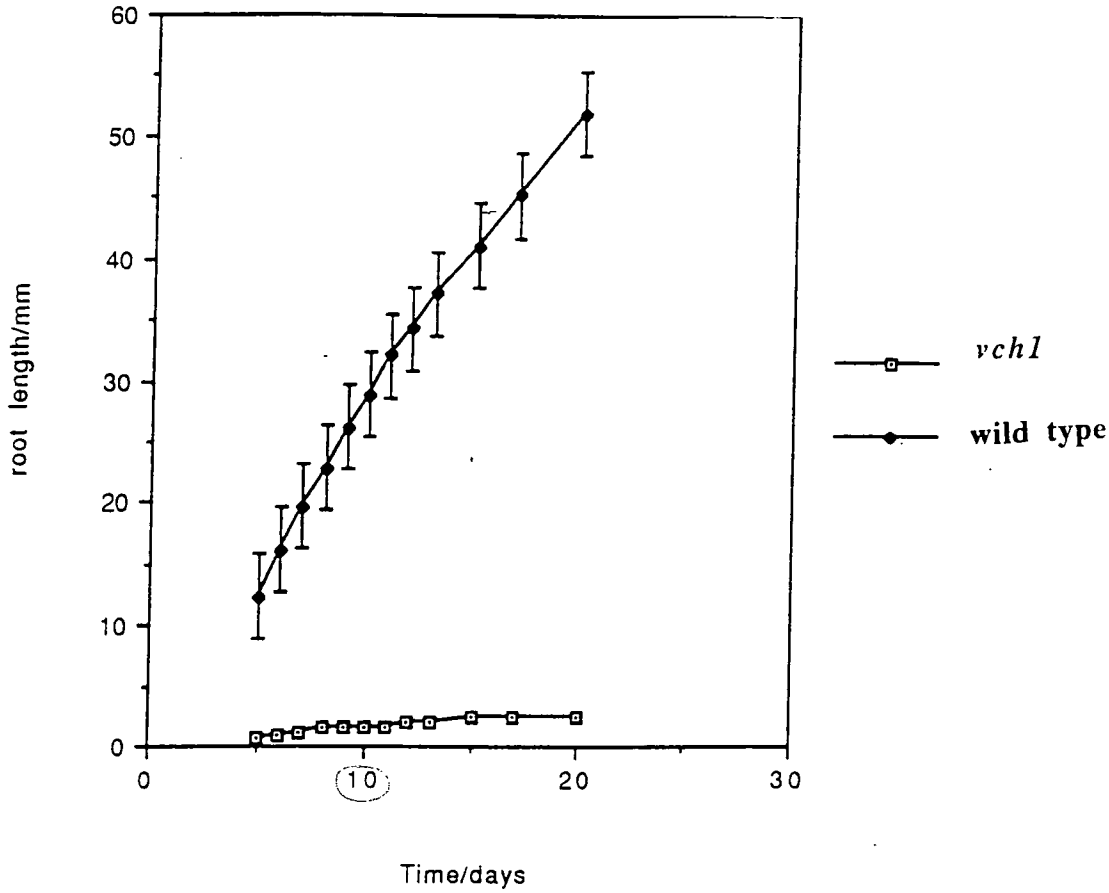
4.2 Growth characteristics of *vch1*

The rates of growth of *vch1* and wild type seedlings were determined by germinating seedlings of both types on 1/2MS10 germination medium on large square Petri-dishes and placing them at 20° from the vertical, such that the roots grew vertically down the surface of the medium, as described in Section 2.1.1. The total length of the primary root and length of hypocotyl were measured at two day intervals following germination for 30 seedlings each of *vch1* and wild type. The results of these measurements are presented in Fig. 4.1. By 5 days post-germination, mean primary root length and hypocotyl length were shown to both be significantly different (WT root = 5.93 cm ± 0.52SE, *vch1* root = 0.65 cm ± 0.13SE, WT hypocotyl = 2.10 cm ± 0.18SE, *vch1* hypocotyl = 1.55 cm ± 0.13SE; $p<0.001$) and by 7 days, the total mean length of the *vch1* primary root was less than 10% of that of the wild type roots.

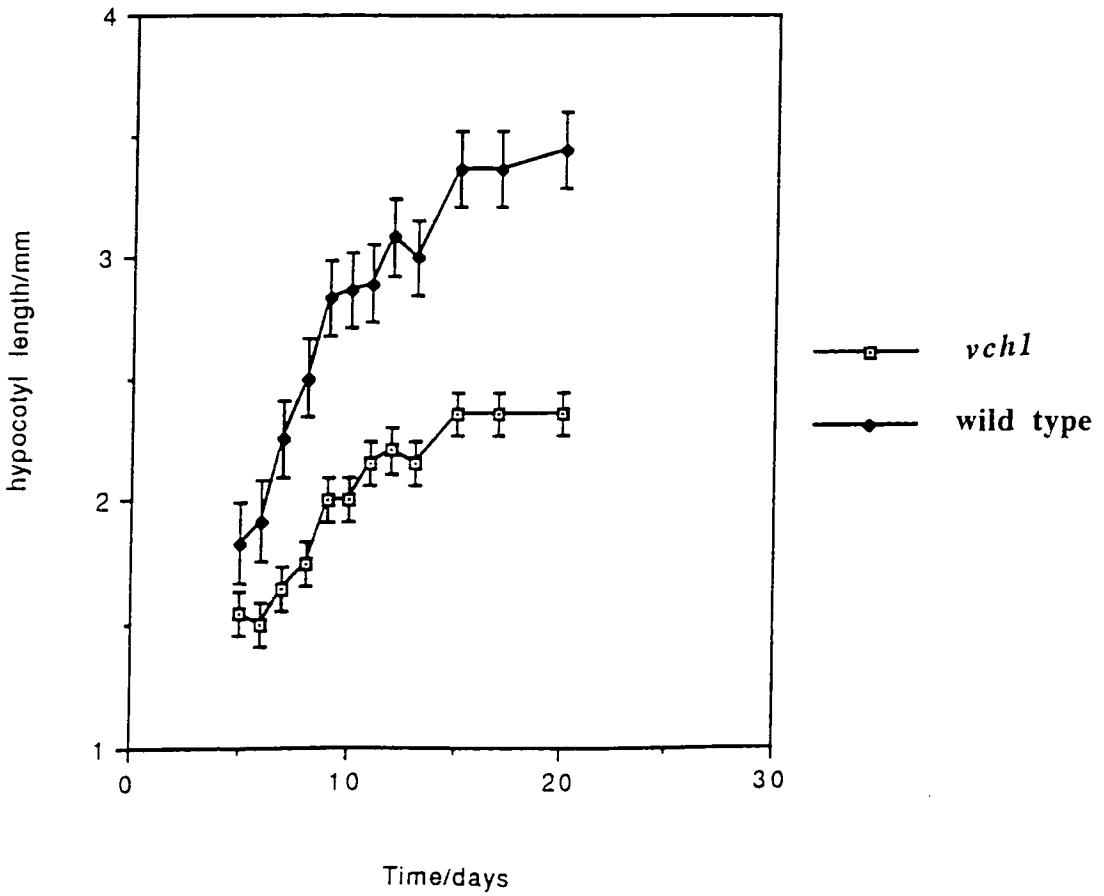
The ratio of leaf lamina length:width was calculated as an indication of leaf "roundness", resulting from the lack of elongation growth for 15 *vch1* and wild type seedlings at 14d post-germination. This was significantly less for *vch1* than wild type seedlings (WT = 1.58±0.21, *vch1* = 0.88±0.17; $p<0.001$).

Fig. 4.1

A. Root growth rates of *vch1* and wild type seedlings



B. Hypocotyl growth rates of *vch1* and wild type seedlings



The number of lateral roots formed was determined for 10 individuals each of *vch1* and wild type at 12d and 20d post-germination (Table 4.1). The mean number counted was significantly less for *vch1* than wild type seedlings at both time points (at 12d: *vch1*= 1.7 cm±0.4SE, WT = 12.0 cm ± 1.47SE; $p<0.01$: at 20 d *vch1* = 3.8 ± 0.47 cmSE, WT = 8.56 cm±1.34SE; $p<0.01$). However, the mean number of lateral roots per length of primary root was not statistically significantly different between the two groups (at 12d *vch1* = 0.86 ± 0.24SE, WT = 0.35 ± 0.29SE; $p>0.1$: at 20d *vch* = 1.54 ± 0.21SE, 0.70 ± 0.82SE; $p>0.1$).

The number of rosette leaves was counted for 10 of each of *vch1* and wild type individuals at 12 and 20d post-germination. The number was significantly less for *vch1* than wild type (at 12d *vch1* = 2.5 ± 0.17SE, WT = 4.67 ± 1.24SE; $p<0.01$: at 20d *vch1* = 7.0 ± 0.26SE, 8.56 ± 0.34SE; $p<0.01$).

Flowers of *vch1* are abnormal in shape (Fig. 4.3E), whilst retaining normal relative positioning of floral organs. All floral organs are axially compressed. Both sepals and petals are much reduced in length: this results in the carpels protruding further than from wild type flowers. Siliques were stunted and statistically significantly reduced in length at maturity in *vch1* compared to those of wild type plants (Fig 4.3 F-G; *vch1* = 3.05 ± 0.22SE, WT = 16.1 ± 0.74SE; $p<0.01$).

4.3 Life history of *vch1*

4.3.1 Fertility of *vch1* plants

Examination of large numbers of embryos from *vch1* heterozygous plants suggests that embryogenesis in *vch1* is normal. The number of seeds released by *vch1* plants on senescence was observed to be very low.

This was quantified by growing 150 each of *vch1* and wild type plants to maturity and collecting and counting seeds. 89% (134/150) of *vch1* homozygous plants set no seed at all.

Table 4.1
Leaf and lateral root numbers in *vch1* and wild type seedlings at 12 days and 20 days.

	12 days		20 days	
	vch1	WT	*vch1*	WT
number of leaves in rosette (±SE)	2.5 ± 0.17	4.67 ± 1.24	7.0 ± 0.26	8.56 ± 0.34
number of lateral roots (±SE)	1.7 ± 0.40	8.56 ± 1.34	3.8 ± 0.47	12.0 ± 1.47
1° root length (mm ±SE)	1.9 ± 0.18	34.44 ± 3.29	2.5 ± 0.22	52.0 ± 0.22
number of laterals / 1° root length (±SE)	0.86 ± 0.24	0.35 ± 0.29	1.54 ± 0.21	0.70 ± 0.82

Of the 11% which did, these typically only produced 1 or 2 seeds, with the maximum observed number being 5 (Table 4.2). All wild type plants produced at least 400 seeds per plant. When the very small siliques of *vch1* mutant plants were dissected, approximately 40% contained seeds bearing embryos at a range of developmental stages (mostly globular- to heart-stage; Table 4.3). These embryos must generally fail to complete embryogenesis and arrest at the globular/heart-stage, as very few later stage embryos were observed.

That this was not due to inviability of the pollen of *vch1* plants was demonstrated by using the pollen from *vch1* anthers to manually pollinate wild type flowers. 10 such crosses were performed and all were successful, producing siliques containing at least 15 embryos. This demonstrates that *vch1* pollen is viable. This, and that fact that *vch1* heterozygote plants produce 1:3 *vch1*:WT seeds (Section 3.2) suggests that there is no gametophytic effect of the *vch1* mutation.

Meinke (1982) described an experiment to determine whether the pollen of mutant plants were at a competitive disadvantage in the fertilization of ovules. The rationale was that by looking at the distribution of seeds of different genotypes within a given silique it could be elucidated whether the mutant pollen were able to penetrate the ovule with the same or lesser efficiency than wild type pollen. This was investigated for *vch1* by using a *vch1* heterozygote as the male parent and a wild type female parent. Siliques were collected from such crosses and were cut in half transversely and the seeds collected in two separate batches. The frequency of *vch1* heterozygotes arising in the F_2 progeny of these two groups was recorded and compared statistically. The frequency was not significantly different between the two groups ($vch1_{TOP}$=10.0±2.76SE, WT_{TOP}=10.9±2.69SE, $vch1_{BTM}$=9.0±0.45SE, WT_{BTM}=8.4±0.99SE;$p<0.01$), suggesting that *vch1* pollen is as effective as wild type or *vch1* heterozygous pollen in fertilisation.

Observation of pollen grains under the light microscope revealed no apparent difference in shape nor size between that from WT and *vch1* individuals (Fig 4.8E, F).

Fig. 4.3
The *vch1* mutant

a. *vch1* (left) and wild type (right) seedlings at 10 d.o.
b. *vch1* (left) and wild type (right) plants at 5 weeks
c. *vch1* (left) and wild type (right) seedlings at 20 d.o.
d *vch1* (left) and wild type (right) rosette at 20d post-germination
e. *vch1* (left) and wild type (right) flowers
f. *vch* mature siliques
g. wild type mature siliques
h. 3 *vch1* seedlings 14d post-germination

bars = approx. 1cm

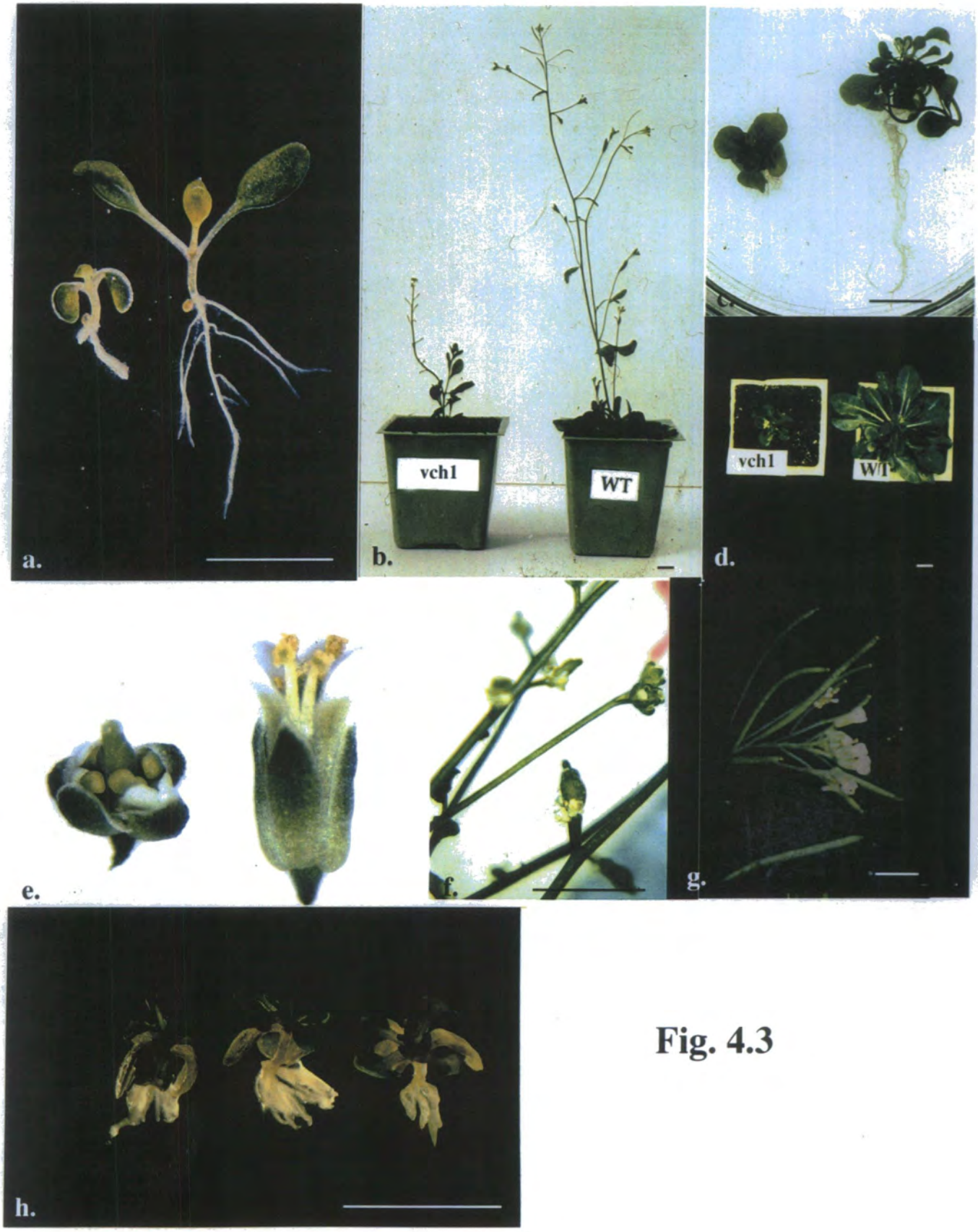


Fig. 4.3

Table 4.2
Number of seeds released from *vch1* homozygous plants.

Number of seeds released	Number of individuals
0	134
1	6
2	5
3	3
4	1
5	1

Table 4.3
Developmental stage of embryos in mature siliques of *vch1* homozygous plants.

Stage	Individual				
	1	2	3	4	5
globular	16	23	8	14	12
heart	6	8	9	4	3
torpedo	3	0	2	3	0
early cotyledonary	0	0	0	1	0
late cotyledonary	0	0	0	0	0

type plants was distorted, with fewer than expected *vch1* plants at maturity. Following germination and two weeks growth of seedlings under standard greenhouse conditions (Section 2.1), 200 of each of *vch1* and phenotypically wild type 2 week old seedlings were transferred from the dense growth conditions under which they were sown into individual 2" pots. At this stage the ratio did not differ significantly from the expected 1:3 (268:732=1:2.73), showing that there was no gametophytic nor seedling lethal effect of the mutation. These seedlings continued growth under the same conditions. By 6 weeks post-germination, the number of plants surviving in each group was scored. 93.5% of the phenotypically wild type plants (187/200) survived to this stage, whilst only 67.0% of *vch1* mutant plants (134/200) had survived.

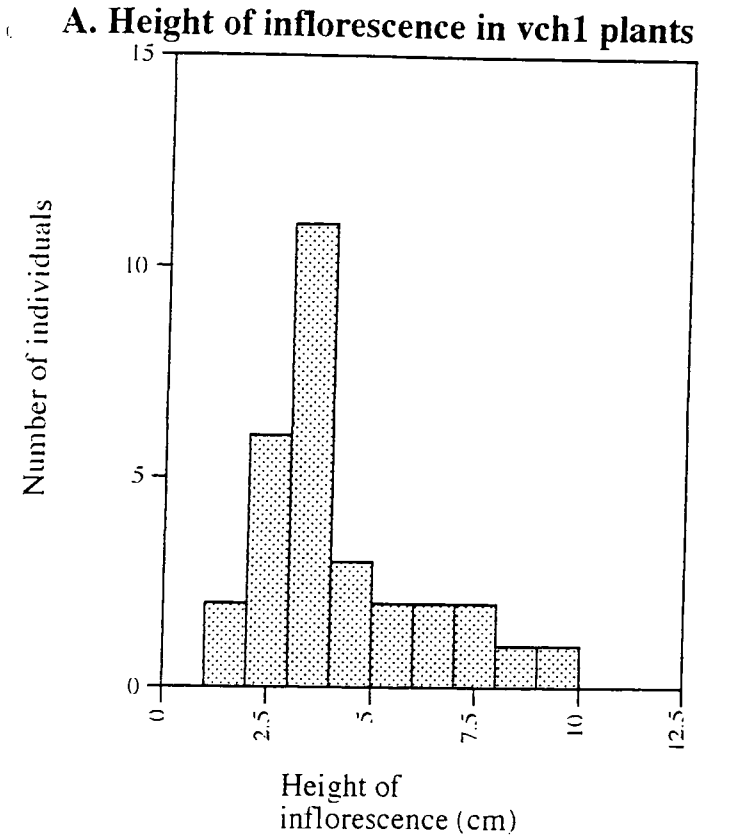
It was further observed that those *vch1* plants which did survive to this 6 week old stage continued to grow for longer than the wild type plants before undergoing senescence. The time at which approximately 50% of siliques had become brown was recorded for the two groups of plants and was found to be statistically significantly different (WT = 57.26d±0.38SE, *vch1* = 53.86d±0.26SE; $p<0.001$).

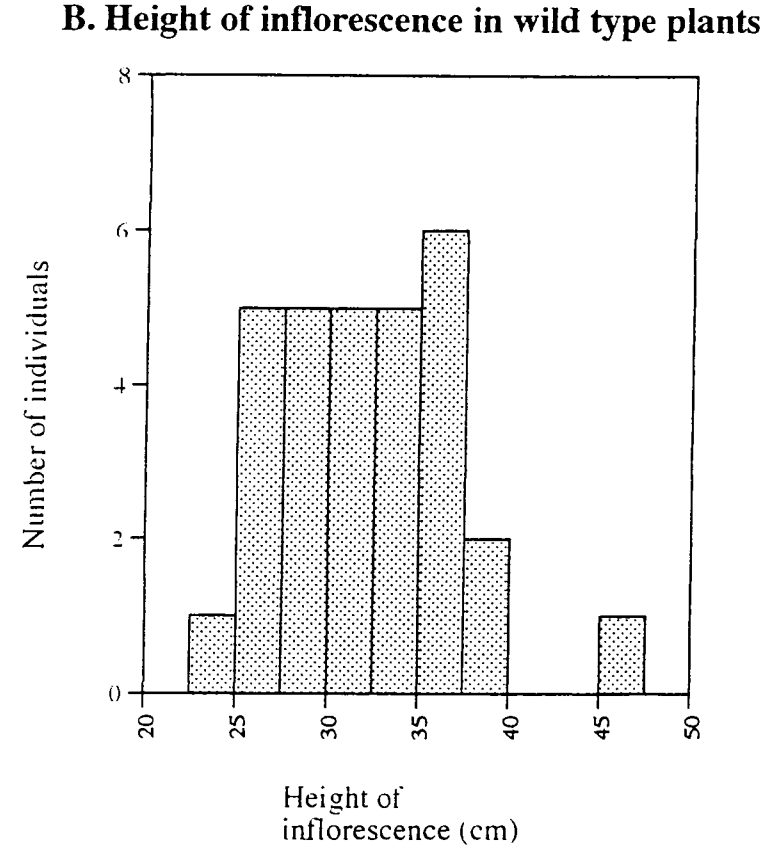
4.3.4 Variability of severity of mature plant phenotype

The aerial phenotype of mature *vch1* plants was observed to be quite variable. In order to quantify this observation, the total length of the longest inflorescence axis was measured at 5 weeks post-germination for 20 individuals each of *vch1* and wild type plants.

Whilst some *vch1* plants grew to a maximum height of 9cm, some failed to grow beyond approximately 1.5cm in total height (Fig. 4.4). No individuals which survived to the 6 week stage were ever observed to fail to make the transition from vegetative to inflorescence phase, since all individuals produced a bolt, albeit sometimes as short as 1.5cm in height. Wild type plants were less variable in height, ranging from 24cm to 46cm. This is a variation of less than 2 fold, as compared to the 4.5 fold variation in height of *vch1* individuals.

Fig. 4.4 Comparison of inflorescence heights





4.4 Cellular organisation of *vch1*

A range of microscopic techniques were employed to investigate ~~further~~ the nature of the phenotypic defect in the *vch1* mutant. Initial observations at the seedling stage were that the mutant appeared to exhibit a general lack of elongation in all major organs, appearing compressed along the apical-basal axis. A primary objective was to determine at what stage of development the defect became apparent.

Crude observation of mature embryos dissected ~~out~~ from mature imbibed seeds suggested that the embryos were normal, as was the case for earlier embryos observed through chemical clearing of the seed testa (Section 2.4.1).

4.4.1 Scanning electron microscopy

In order to investigate the morphology of mature embryos further, scanning electron microscopy (SEM) was employed to look in more detail at both isolated mature embryos and post-germination early seedlings. SEM is used to observe the surface morphology of specimens and, in this case, allowed for the elucidation of the patterning, size and shape of epidermal cells.

Because of the scarcity of embryos produced in the siliques of *vch1* homozygote plants, it was necessary to look at a mixed genetic population of embryos (*vch1* homozygotes, heterozygotes and segregating wild types) from a known *vch1* heterozygous parent. Consequently, it was necessary to observe a large number of embryos and seedlings, to ensure that a proportion were statistically likely to be mutant homozygotes. At least 15 embryos or seedlings were screened at each developmental stage observed.

For SEM, embryos and seedlings were fixed, dehydrated and metal coated as described in Section 2.4.6. Seedlings were fixed and imaged at 0, 2, 4, 6 and 8 days post-germination. By 8 days the abnormal phenotype was apparent even without the aid of microscopy. A detailed developmental series of both mutant and normal embryos and seedlings is presented in Fig.4.5. No abnormalities were evident in either mature

Fig. 4.5
vch1 and wild type SEM images

A. embryo from mature seed - *vch1* and wild type indistinguishable
B. 2 d.o. seedling -*vch1* and wild type indistinguishable
C. WT root at 6d post-germination
D. WT root at 8d post-germination
E. *vch1* root at 6d post-germination
F. *vch1* root at 8d post-germination
bars = approx. 50μm

G. WT stigma surface
H. *vch1* stigma surface
I. WT trichome
J. *vch1* trichome

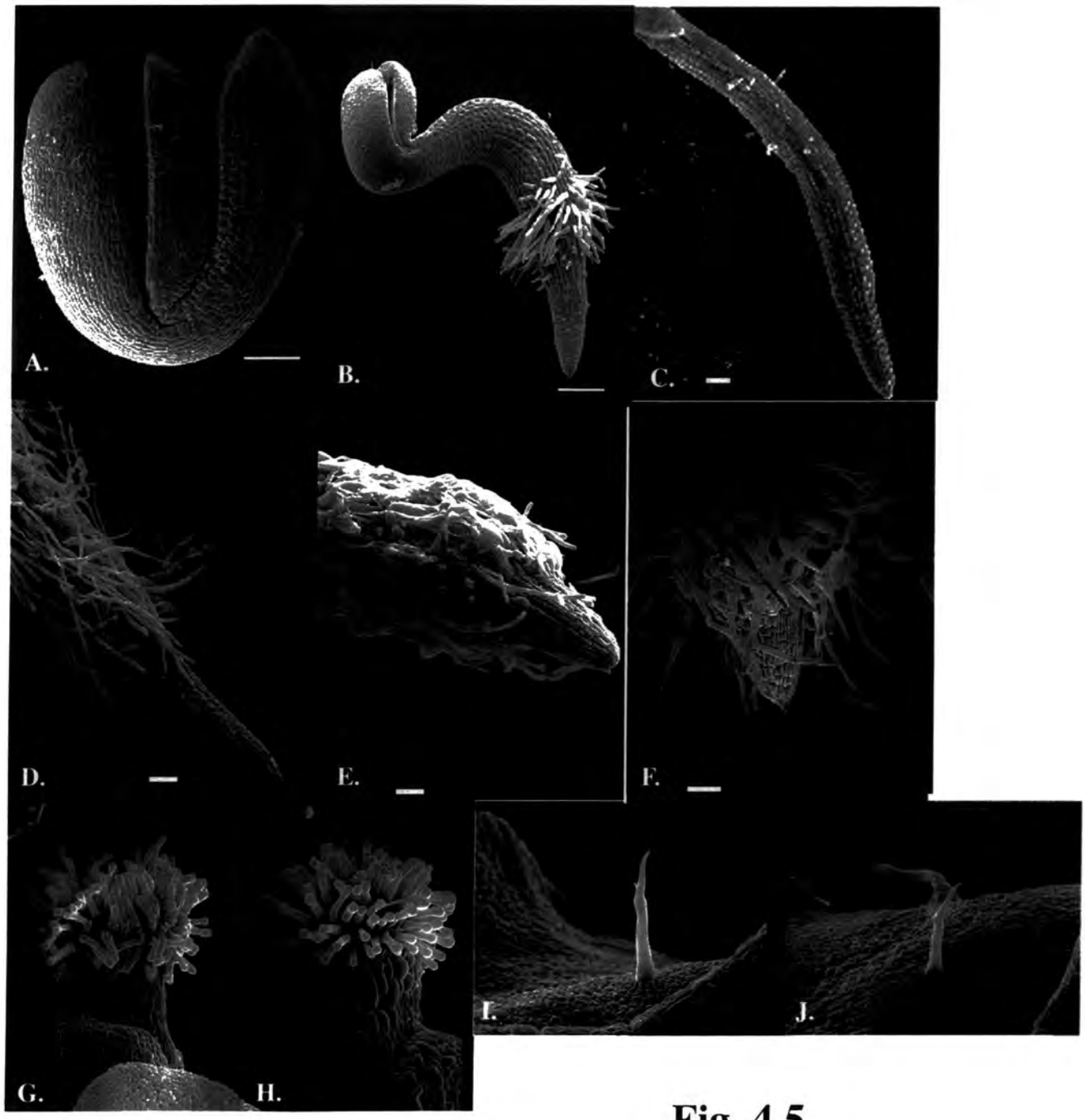


Fig. 4.5

embryos nor in early seedlings up to 4 days post-germination. It appears that the abnormal phenotype becomes evident at between 4 and 6 days post-germination, initially manifest as a slight reduction in the length of the elongation zone in roots, shorter hypocotyl and smaller leaves.

Epidermal cells of roots, hypocotyls, siliques, leaves, petals and sepals were all shorter in *vch1* than wild type plants, but were normally arranged. See Section 4.4.3 for cell length measurements.

Roots appeared more hairy on *vch1* (Fig. 4.5 E-F) than on wild type seedlings (Fig. 4.5C-D), but it is suggested that this may be not due to an increased number of root hairs but to increased density of hairs per unit length of root, due to the smaller size of root epidermal cells.

Whilst the petals, sepals, anthers and style of *vch1* flowers were compressed in the vertical plane (Fig. 4.3 E), the stigmatic papillae were of normal shape and size (Fig. 4.5 G-H). Leaf trichomes were also normal (Fig. 4.5 I-J). Similarly were root hairs (Fig 4.5 E-F and 4.11 A-D), despite the reduced length of the trichoblasts from which they emanate.

4.4.2 Histological analysis

SEM is limited to the observation of surface morphology only. To examine cellular organisation within embryos and seedlings it was necessary to section tissues. Tissues were fixed, embedded and sectioned on a rotary microtome, as described in Section 2.4.2., stained with toludine blue (Section 2.4.2) and observed under a compound microscope.

A large number of sections were cut to ensure that all orientations of specimens and all tissue types were represented in the sections observed. Those sections which as closely as possible represented longitutinal and transverse sections relative to the apical-basal axis of the plant were chosen for observation. This approach allows the detailed

observation of the distribution, size and shape of the various cell types present and, also, in the case of transverse sections of roots and hypocotyls, allows the rapid generation of large numbers of sections for comparison. Fig. 4.6 shows typical examples of longitudinal and transverse sections from roots of *vch1* and wild type individuals. It appears that all the cell types are present in *vch1* and are in their correct spatial context, except that all cell types appear to be more numerous in transverse section in *vch1* than in wild type seedlings.

To quantify this observation, the number of both epidermal and cortical cells per section were counted in transverse sections cut from the primary root of a total of 50 individual 10 d.o. seedlings of each of *vch1* and wild type. Epidermal and cortical cells were chosen because they are large, easily identified in section and are quite invariant in size within a given region of the root. Measurements of the lengths of these two cell types were made within the elongation zone of young roots. In wild type roots, this was defined as the region 700-1000μm behind the root tip and, in *vch1* as 300-550μm behind the tip. These regions were chosen on the basis of identification of the region in the WT root in which cell elongation was maximal and consistent but in which vascular differentiation was not yet complete. The region chosen for comparison in *vch1* took account of the difference in total length of *vch1* and wild type roots.

The mean number of epidermal cells per section in *vch1* roots (23.15 ± 0.88SE) was significantly greater ($p<0.01$) than in wild type roots (16.35 ± 0.31SE) as was the mean number of cortical cells per section (8.22 ± 0.46SE in wild type roots, 9.54 ± 0.57SE in *vch1*; $p<0.01$). Possible explanations for this observation are discussed in Section 7.5; also *cf.* Fig. 4.7). An unquantified observation was that, in the short region behind the meristem in *vch1* in which cells were wild type in size, the number of cells per transverse section appeared similar to that in wild type roots.

In longitudinal section it also appears that all cell types are present and located in the correct relative position (Figs. 4.6 B-C). However, it was observed that all elongated cells appeared significantly shorter in the apical-basal dimension and wider in the

Fig. 4.6
vch1 and wild type roots

A. wild type seedling root tip LS
B. *vch1* seedling root tip LS
C. *vch1* seedling root tip LS
D. wild type seedling root TS
E. *vch1* seedling root TS
F. *vch1* seedling root TS
G. *vch1* root tip confocal image
H. wild type root LS confocal image
I. *vch1* root LS confocal image
J. wild type root whole mount

bars = approx. 50μm

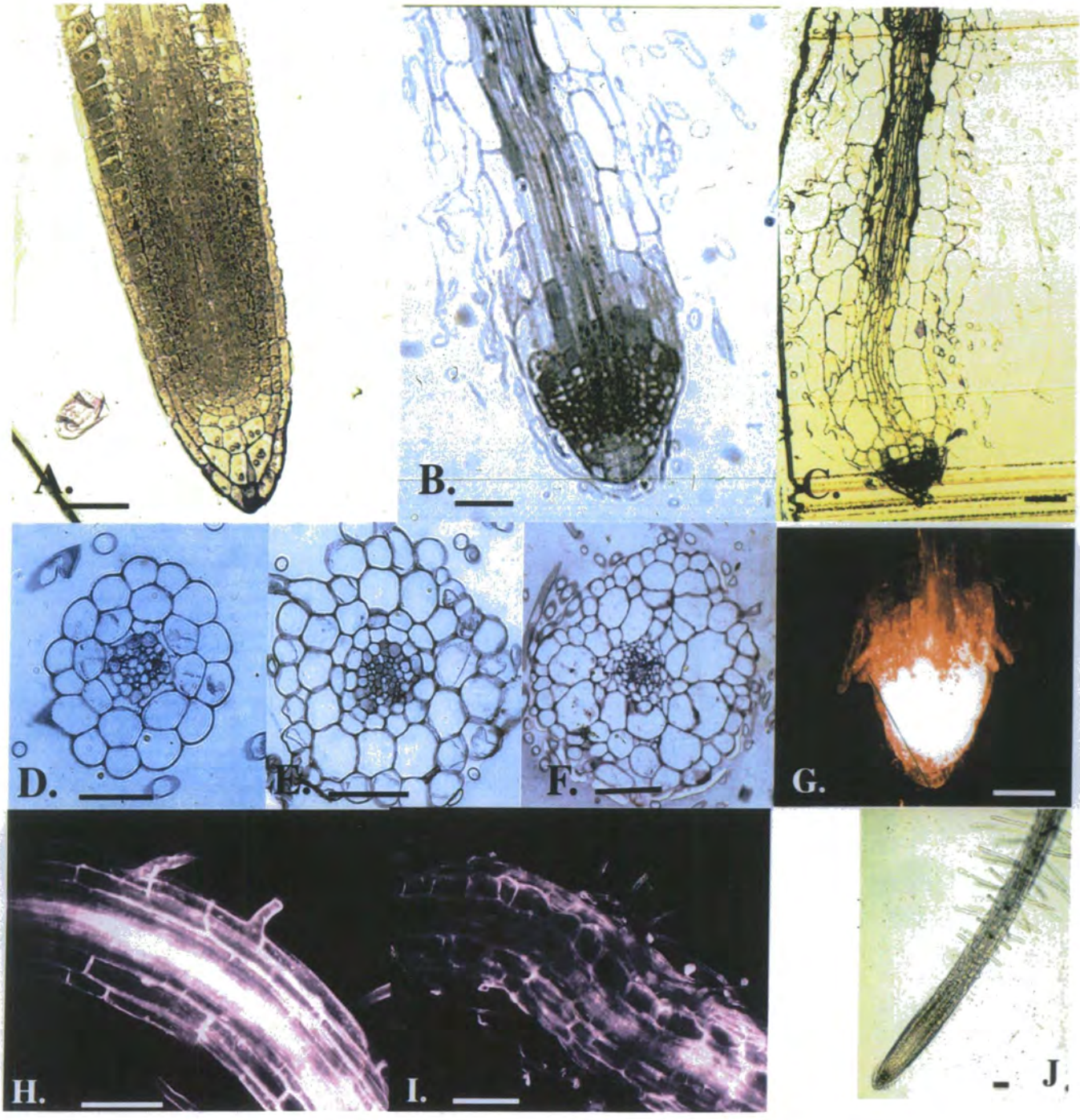
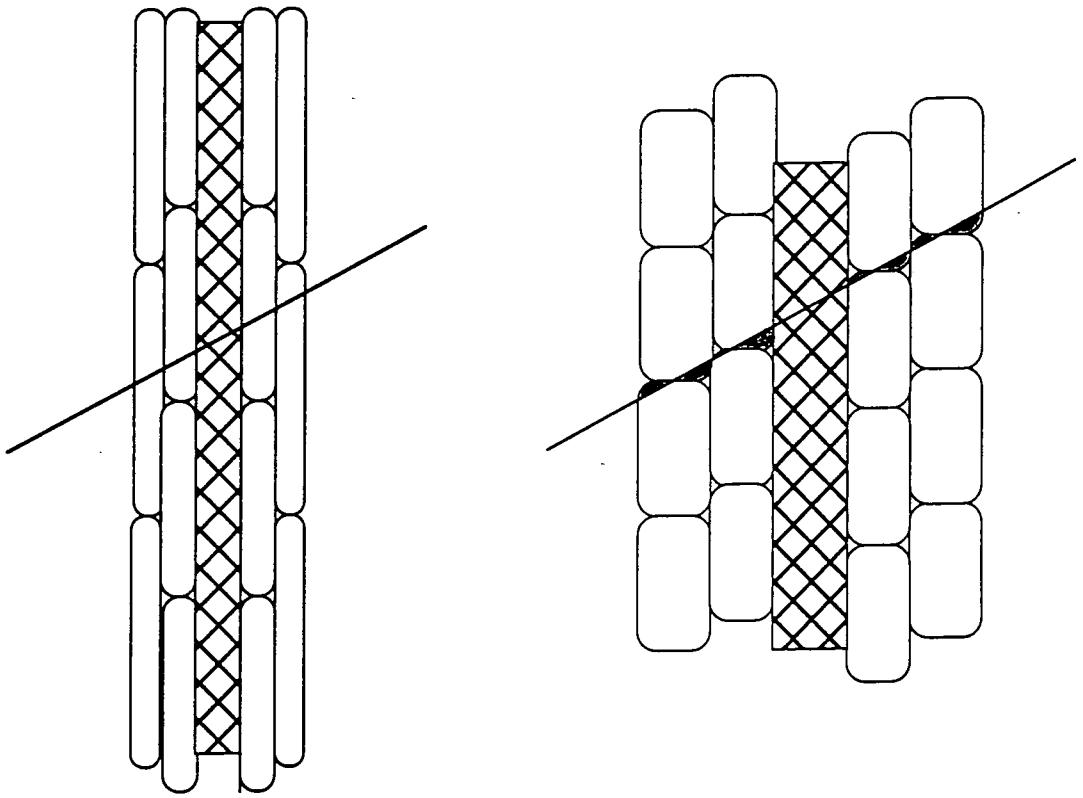

A.
B.
C.
D.
E.
F.
G.
H.
I.
J.

Fig. 4.7

Diagram to show how it might be possible to cut transverse sections with increased cell number at an angle in *vch1* roots



WT ***vch1***

radial dimension, in agreement with the SEM studies described above. The meristematic zone comprising small densely packed cells was much shorter in *vch1* than in wild type roots (Figs. 4.6 A-C) and became restricted to a small number of cells at the root apex in older *vch1* roots (Fig. 4.6C).

4.4.3 Confocal microscopy

Because of the difficulty in orienting roots flat for the cutting of longitudinal sections, another technique, confocal laser-scanning microscopy, was used to obtain cell size measurements for a large number of roots. The advantage of this technique is that roots are simply laid flat on a glass slide and stained *in situ* prior to observation (Section 2.4.5), ensuring correct orientation of the specimen flat - along the apical-basal axis of the root. The technique, however, is not suitable for studying details of cellular arrangements, particularly in the root tip, as resolution is limited particularly where cells are small and densely packed. The image analysis software used allows the accurate measurement of cell sizes further back in the root where cells are larger and less intensely stained.

Fig. 4.6 H-I shows images obtained from 10 d.o.*vch1* and wild type roots. The longest axes of 200 epidermal and cortical cells (10 of each cell type from actively elongating roots of each of 20 individuals of *vch1* and WT respectively) were recorded and compared statistically. Both were found to be significantly reduced in *vch1* roots (cortical cells -*vch1* = 62 μm ± 3.28SE; WT = 120 μm ± 8.23SE; $p<0.001$; epidermal cells - *vch1* = 56 ± 11.16SE; WT = 108 μm ± 11.47SE; $p<0.001$). *vch1* root epidermal and cortical cells were also wider in transverse section, to the order of approximately 1.2-1.3 times that in wild type roots (Fig.4.6 D-F). This was reflected in the increased diameter of roots (Figs. 4.5 C-F and 4.6 A-C).

The diameter of vch1 hypocotylswas greater than wild type. A meaningful measurement of root thickness is difficult to obtain, since roots show considerable variation in thickness along their length. That of hypocotyls is more consistent along their length and this was measured using the Comos software.

Measurements were taken of the diameter of the middle of the hypocotyl for 10 *vch1* and wild type 10 d.o. seedlings. This was significantly greater in *vch1* than in wild type seedlings (*vch1*=147.07μm±2.803SE, WT=106.36μm±2.125SE).

The distance from the root tip of the closest (and therefore youngest) root hair was also measured for each root. Comparison of this measurement was made in 20 roots of each of *vch1* and wild type individual seedlings, using the Comos imaging software (Section 2.4.5). The result confirmed the observation from the SEM that this length is significantly less in *vch1* than in wild type roots (*vch1* = 115.05μm±16.79SE; WT = 303.95μm±65.73SE; $p<0.001$).

The confocal microscope was also used to investigate the development of early embryos. The advantage of this technique is that early embryos can be easily located within the seed due to their intense fluorescence following ethidium bromide staining. They can then be observed in detail at high magnification and resolution.

Large numbers of embryos at all stages were examined both in histological section and by scanning-laser confocal microscopy (over 250 in total). No abnormalities were discernable in *vch1* embryos at any stage of their development.

4.4.4 Transmission electron microscopy

Since the cell wall imposes a significant constraint on cell expansion and elongation (*e.g.* Cosgrove, 1986; 1997), it was postulated that in *vch1* there may be some defect in cell wall architecture or mechanics. One direct way to investigate this was to look at cell wall ultrastructure using transmission electron microscopy (TEM).

Ultra-thin sections were cut from root epidermal and cortical cells of both wild type and *vch1* seedlings at 10 days post-germination (Section 2.4.7). By this 10 day old stage the morphological defect in *vch1* is clearly apparent and the roots exhibited a rapid rate of growth and thus also of cell wall extension. The results of this study are presented in Figs. 4.9a and 4.9b.

There appeared to be no obvious difference in the arrangement of cell wall material between the mutant and wild type roots (Figs. 4.9b A-F) nor in the overall thickness of the walls. Up to around 300-400μm back from the tip, cell wall material appeared to be tightly aligned along the long axis of the root. Further back in the root some walls appeared less organised (Figs. 4.9b C), with a more random array of cellulose microfibrils. However, this was also seen in wild type roots in regions behind the zone of elongation, although such walls were observed more frequently in *vch1* (unquantified observation). The size of intercellular spaces at the "corners" of adjoining cells appeared, on average, larger in area in *vch1* than wild type roots (unquantified observation; Fig 4.9b D), however, again this was frequently observed in wild type roots in cells which were no longer actively elongating. Further experiments would be required to quantify and confirm these observations.

The distribution, size and number of vesicles observed associated with newly forming end walls of cells appeared similar in *vch1* and wild type roots (Figs. 4.9b E-F), but again further quantitative experiments would be required to quantify this observation.

4.4.5 Comparison with other cell expansion-defective mutants under TEM

Two other mutants were also examined in a similar way: *hydra1* (Topping *et al.*, 1997) and *emb30* (Mayer *et al.*, 1993; Shevell *et al.*, 1994). The *emb30* mutant has been previously shown to exhibit much variation in cell size, shape and cell wall thickness in its hypocotyl region (Mayer *et al.*, 1993) and was used here as a control specimen. The *hydra1* mutant had previously been examined by histological sectioning (Topping *et al.*, 1997) and was also found to exhibit abnormal variation in cell size and shape in the thickened hypocotyl.

Fig. 4.9a
TEM ultrastructure of *vch1* and other cell shape mutants

A. WT root cortical cells x 1,200
B. WT root cortical cells x 1,500
C. *vch1* root cortical cells x 1,800
D. *gnom* hypocotyl x 950
E. *hydra1* hypocotyl x 1,200
F. *hydra1* hypocotyl x 1,200
G. *hydra1* hypocotyl x 1,200

W=wall
V=vesicle
Va=vacuole
P=plastids
PB=plastid

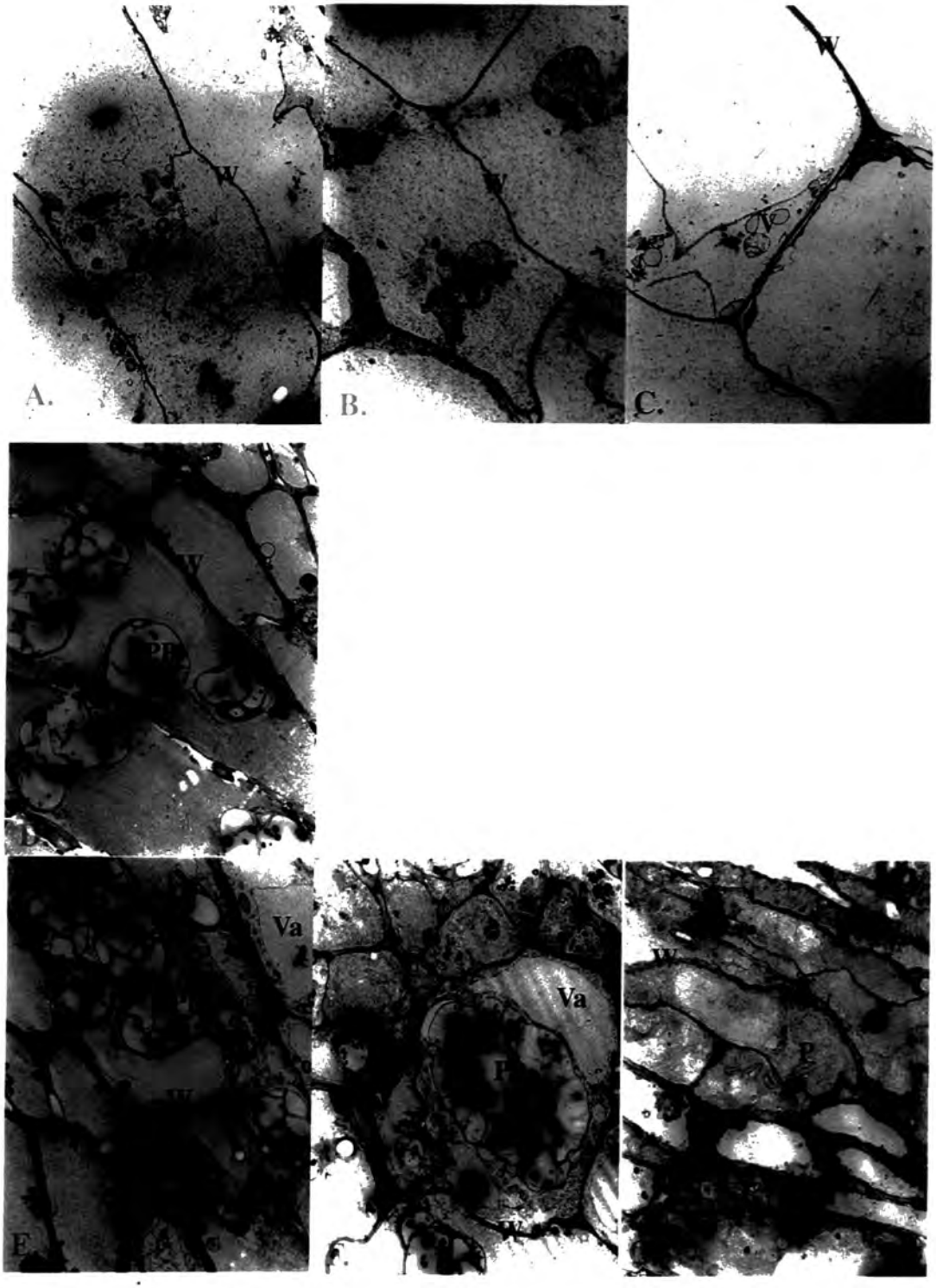

A.
B.
C.
D.
E.
Va
Va

Fig. 4.9b.
TEMs of vch1 and wild type cell walls

A. WT cell wall - epidermal-cortical cell x 22,500
B. *vch1* cell wall - epidermal-cortical cell x 17,500
C. *vch1* cell wall - epidermal-cortical cell x 10,500
D. *vch1* cell wall - epidermal-cortical cell x 22,500
E. Plastids at wall in *vch1* root - cortical end-wall x 22,500
F. Plastids at wall in WT root - cortical end-wall x 17,500

W=wall
V=plastids
E=epidermal cell
C=cortical cell

Fig. 4.10
Immunolabelling of cytoskeletal components

A. WT root labelled with anti-α-tubulin
B. *vch1* root labelled with anti-α-tubulin
C. WT root labelled with anti-α-actin
D. *vch1* root labelled with anti-α-actin

Fig. 4.9b

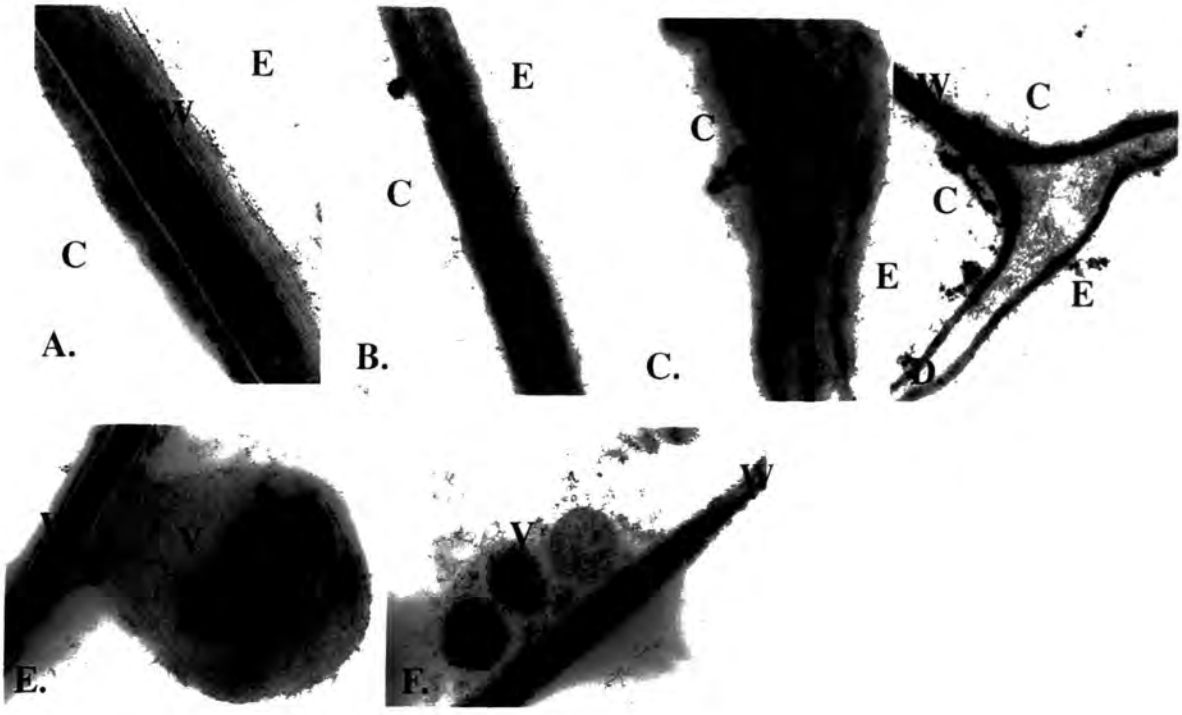


Fig. 4.10

Ultra-thin sections were cut from the root and hypocotyl regions of mutant and wild type seedlings (as described in Section 2.4.7) and observed on the TEM.

It was found here that the cells of the hypocotyl of the *hydra1* mutant, as the *emb30* mutant, also exhibited greater than normal variation in cell wall thickness (Fig. 4.9a E-G). The roots, however, which do appear normal in histological sections, also appeared normal at the ultrastructural level (data not shown). Examination of cells of the hypocotyl of the *hydra1* mutant confirmed the observation from sectioning that differentiated cells are arranged inappropriately in this mutant. For example, some cells obviously differentiated as tracheids, with cell wall projections and substantial wall thickening, sometimes occurred in inappropriate locations within the hypocotyl (Fig. 4.9a G), often as single cells in isolation from other vascular type. This also revealed that the *hydra1* defect is not one of failure to form cross walls, as was the case in *knolle* (Lukowitz *et al.*, 1996), a cell shape mutant which is defective in the formation of the cell plate at cytokinesis.

Such cell patterning disorganisation was not observed in either the roots or hypocotyls of *vch1*, and nor was there observed any great degree of variation in wall thickness between cells.

4.4.6 Percentage dry weight

The percentage dry weight of *vch1* and wild type plants at 2 weeks and 6 weeks post-germination was determined by weighing pooled samples of at least 50 seedlings of *vch1* and wild type, drying them in an oven at 80°C overnight and re-weighing.

The percentage dry weight of 2 week old plants did not differ significantly between the two groups (WT mean = 7.75%; *vch1* mean = 7.13%). That of 6 week old plants, however, was found to be greater for *vch1* than for wild type (WT mean = 9.83%; *vch1* mean = 11.85%).

4.4.7 Chlorophyll content

An increase in chlorophyll content has previously been observed in some dwarf mutants, including *hydra1* (Topping *et al.*, 1997) and *diminuto* (Takahashi *et al.*, 1995). In *diminuto*, the number of chloroplasts per cell was not significantly reduced compared to wild type cells, however, cell size was reduced and the increase was in cellular chloroplast density. *vch1* did appear slightly greener than wild type seedlings and so chlorophyll content was assayed.

Chlorophyll content was determined spectrophotometrically for pooled samples of over 50 seedlings of each of *vch1* and wild type, as described in Section 2.3.4.

There was no marked difference between chlorophyll content of 14 d.o. seedlings of *vch1* and WT, in either chlorophyll A (236±41μg/g fresh weight for *vch1*, 202±47μg/g fresh weight for WT) or chlorophyll B content (136±49μg/g fresh weight for *vch1*, 120±34μg/g fresh weight for WT). The slightly greener appearance of *vch* leaves is interpreted to be likely to be due to the adaxial-abaxial expansion of leaf cells, resulting in increased lamina thickness.

4.5 Examination of the cytoskeleton

Both α- and β-tubulin are major components of microtubules (MTs) and α-actin of microfilaments (MFs). MTs are assembled into different configurations during the cell cycle. Prior to cell division MTs form an array across the cell at right angles to its long axis - the Pre-Prophase Band (PPB) (Webb & Gunning, 1991). This seems to be essential for defining the plane of division. In *fass* (Fisher *et al.*, 1996) the microtubules are disorganised and do not form the PPB, resulting in loss of control of the plane of cell divisions. During interphase, when cell elongation occurs, MTs are arranged into the Cortical Array. This comprises parallel microtubular strands wrapped around the cell, perpendicular to the primary axis of cell expansion (Goddard *et al.*, 1994; Baskin *et al.*, 1994). Actin MFs are arranged along the longitudinal axis of the elongating cell. Both are thought to guide cellulose deposition (Baskin *et al.*, 1994).

That the arrangement of the cytoskeleton is important in mediating changes in cell shape during growth and in response to hormonal stimuli (Shibaoka, 1994) led to the hypothesis that, because cell shape is altered in *vch1*, this might reflect a defect in cytoskeletal organization.

Cytoskeletal components were examined through immunolabelling. Primary antibodies against α-tubulin, β-tubulin and actin were used to label whole mount root preparations of *vch1* and wild type seedlings, as described in Section 2.6.1. An FITC-labelled secondary antibody was used and was visualised by UV epifluorescence microscopy or scanning-laser confocal microscopy (Section 2.4.5).

Fluorescence was visualised in roots labelled with anti-α-tubulin (Fig. 4.10 A-B). Labelling in *vch1* roots was less clear, possibly due to reduced penetration of the antibodies due to root tips being covered with a dense layer of root hairs. However, in individual cells in which labelling is clear (one example is marked on Fig. 4.10 B), it can be seen that cortical MTs are arranged in the characteristic transverse band (PPB), as in cells of the wild type root (Fig. 4.10 A).

Anti-β-tubulin labelled in a similar pattern, as expected, though less clearly. Results demonstrated that the pattern of MTs in the cortical array was similar in *vch1* and wild type root epidermal cells.

Resolution of MFs was less good (Fig 4.10 C-D). The only meaningful conclusion which could be drawn regarding their subcellular localisation was that the intensity of labelling appeared similar in wild type and *vch1* roots. No conclusions could be drawn regarding their orientation and arrangement. Intense labelling of α-actin was restricted to a much smaller region at the tips of *vch1* roots than in wild type roots (Fig 4.10 C-D).

4.6 Organ specificity of defect

Previous sections have described how mutation in the *VCH1* gene affects the morphology of all major organ types. It was thought possible that this did not necessarily reflect the fact that the gene is normally expressed in all tissues. It was possible that alteration of expression of *VCH1* in one organ type might indirectly affect other organs, through the action of an inhibitory substance produced in the organ in which *vch1* expression is altered on the morphology of other organs, or the failure of transport of water or nutrients to the aerial parts from the poor root system.

4.6.1 Root and shoot cultures

To investigate this possibility, shoot and root cultures were set up from material excised from *vch1* and wild type seedlings, as described in Sections 2.2.1; 2.2.2. The cultures were grown for 10 days following initiation.

The defect was found to persist in both excised shoots and roots grown under these conditions (data not shown). There was no detectable difference between the growth rate of excised shoots and those of control intact seedlings. New leaves continued to be formed and these were of the same shape as those of *vch1* leaves of intact seedlings. *vch1* and wild type roots proliferted in culture. Roots were examined under the compound microscope. The *vch1* defect persisted, as evidenced by the short length of such roots, the formation of root hairs close to the root tip and the short length of individual cells. Roots from culture and those excised from intact seedlings, of both *vch1* and wild type, were indistinguishable. It is concluded that the *VCH1* gene is necessary for normal growth of both shoots and roots.

4.6.2 Generation of callus from *vch1* and wild type roots

To detect whether the defect in axial cell elongation in *vch1* cells as seen in the plant is also seen in cultured cells, which are characterized by disorganised growth, callus cultures were established from *vch1* and wild type roots. When callus was initiated from the *stunted plant1* mutant (Baskin *et al.*, 1995), which exhibits reduced

Fig 4.8
vch1 and wild type callus and pollen

A. and B. = callus from *vch1* root tissue, x250
C. and D. = callus from WT root tissue, x250

E. *vch1* mature pollen, x120
F. WT mature pollen, x120

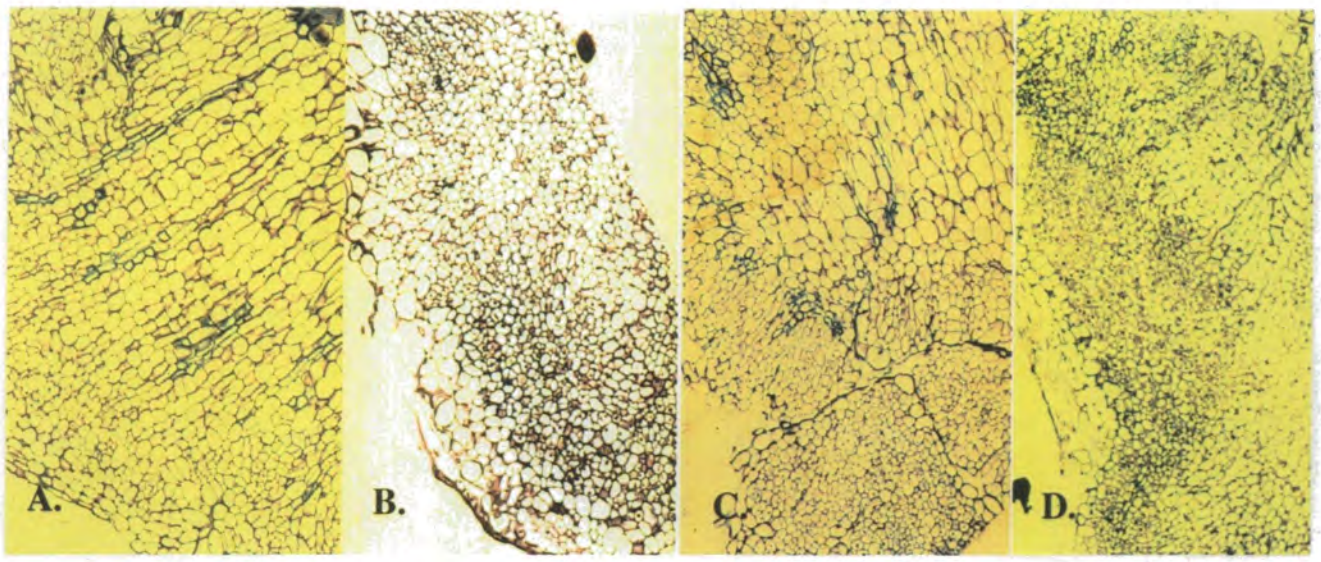
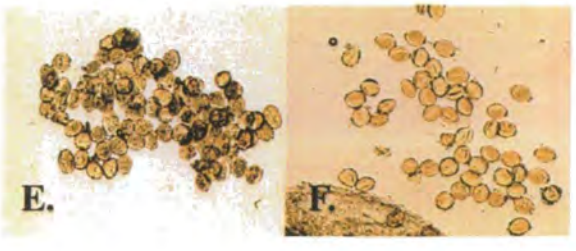


Fig 4.8



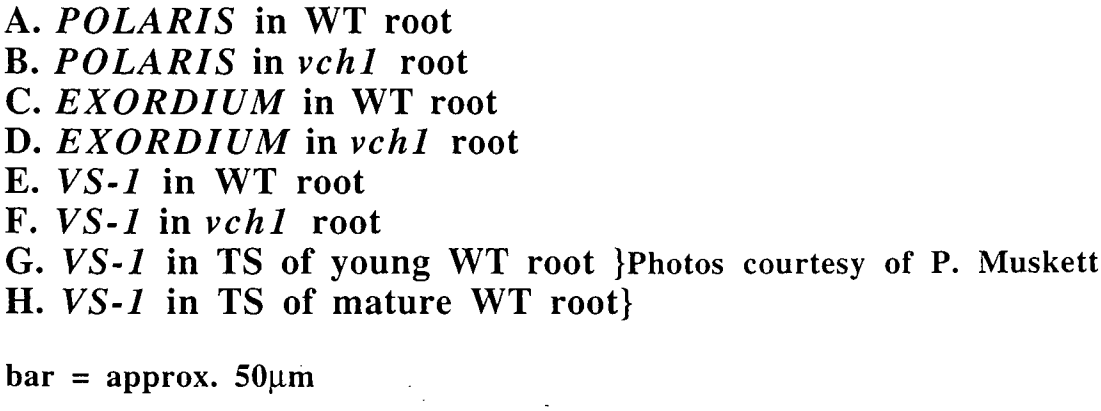
cell sizes in the elongation zone of roots but not the meristematic zone, cell sizes in the callus were the same as those in wild type callus.

vch1 and wild type seeds were germinated and grown on 1/2MS10, as described in Section 2.1.1. At 14 days post-germination, roots were excised from the seedlings and transferred to Callus Induction Medium (CIM; Section 2.1.3) and replaced in the growth room. Following transfer to fresh CIM twice, at 7d intervals, pieces of callus were fixed, embedded and sectioned, as described in Section 2.4.2). Sections were stained with toludine blue (Section 2.4.2) and observed under a compound microscope. Photographs of some regions of *vch1* and wild type callus are presented in Fig 4.8A.-D. Measurements of individual cell sizes were not made as callus is highly heterogeneous and results would be subject to sampling bias. However, from crude observation it was found that the range of cell sizes observed in the *vch1*- and wild type-derived callus was not noticeably different. Cell sizes within the callus in regions of maximum and minimum cell size respectively, appeared similar in that derived from *vch1* and wild type tissue.

4.7 Genetic crosses with *gus*-fusion marker lines

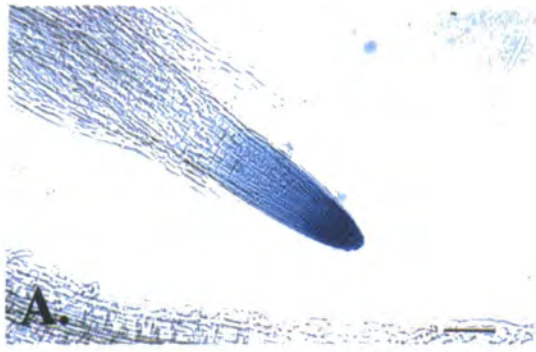
In order to obtain information regarding the cellular organisation of the mutant embryos (*cf.* Section 1.12) a number of *gus*-fusion genes were introduced into the mutant background by genetic crossing. These tagged genes were selected for their spatially restricted GUS expression patterns. Three such *gus*-fusion lines were used here, all of which have been identified and characterized in this laboratory. These were introduced in Section 1.12. *EXORDIUM* (Fig. 4.11 C) is a line which exhibits GUS expression throughout the embryo. Post-germination it is restricted to meristematic regions of the plants roots and the shoot meristem. The second line, *POLARIS* (Topping & Lindsey, 1997; Fig. 4.11 A) shows GUS expression from the heart stage in the basal part of the developing embryo and, post-germination, in root tips. Thirdly, *VS-1* (Fig. 4.11 E, G, H; Muskett, 1998) is a vascular tissue expressed *gus*-fusion marker, expressed in all differentiated living cells of the vascular system and in the endodermis of the root.

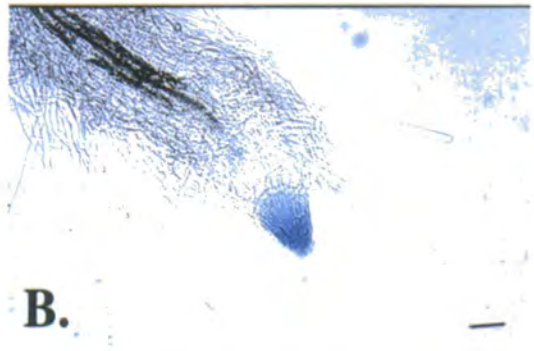
Fig. 4.11
Expression of *gus*-fusion markers in WT and *vch1* roots

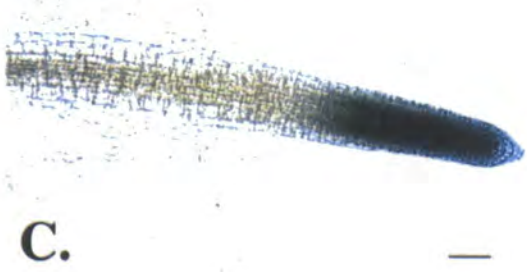


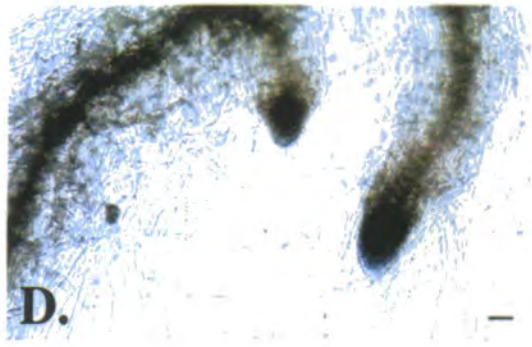
A. *POLARIS* in WT root
B. *POLARIS* in *vch1* root
C. *EXORDIUM* in WT root
D. *EXORDIUM* in *vch1* root
E. *VS-1* in WT root
F. *VS-1* in *vch1* root
G. *VS-1* in TS of young WT root }Photos courtesy of P. Muskett
H. *VS-1* in TS of mature WT root}

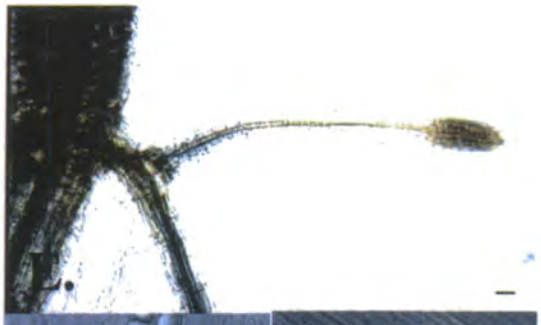
bar = approx. 50μm


A.

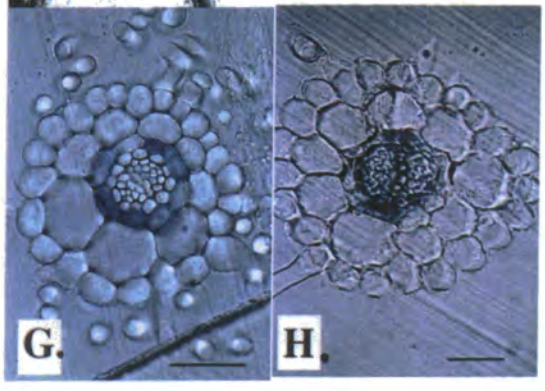

B.


C.


D.


E.


F.


G.
H.

The results of these crosses are presented in Fig. 4.11 B, D, F.

Both *EXORDIUM* and *POLARIS*, when introduced into the *vch1* mutant exhibited an embryonic expression pattern exactly as that in normal embryos (data not shown). Similarly, in phenotypically abnormal *vch1* roots, both of these *gus*-fusion markers are expressed in their correct cellular and spatial context (Fig. 4.11 D & B). The pattern of both, however, differs from that in normal root meristems insofar as the expression in the meristem is more spatially restricted - presumably due to the smaller physical extent of the meristematic zone in the mutant roots.

The *VS-1* gene is also expressed in the correct cell types when introduced into the *vch1* mutant. The distance back from the root tip to the start of the region in which *VS-1* is expressed is reduced in *vch1* as compared to that in the normal root (WT≈650-700μm; *vch1*≈400-450μm; Fig. 4.11F.

4.8 Effect of growth medium on *vch1* phenotype

4.8.1 Effect of carbohydrate source on seedling growth

Some mutant phenotypes vary in severity depending on growth medium composition. For example, the radial swelling observed in root cells of *lions tail* and *cobra* is abolished when they are grown on low sucrose medium (Benfey *et al.*, 1993).

To investigate whether the carbohydrate source and concentration differentially affected the phenotype of *vch1* and wild type seedlings, seeds were germinated and seedlings grown on various media and organ shape and size observed and compared to those on standard (1/2MS10) medium.

vch1 and wild type seedlings were germinated and grown for 7 days on half-strength MS medium containing sucrose concentrations ranging from 0.5-4 % w/v. This was duplicated using other carbohydrate sources (mannitol, glucose, fructose) and PEG as a non-carbohydrate osmoticum, each at 1 % and 2 % w/v.

The effect of each on seedling phenotypes was investigated. Both *vch1* and wild type seedlings on mannitol, glucose and fructose grew poorly, especially at 2%. Those on PEG at 1% and 2% showed only a slight reduction in growth compared to those on the same concentration of sucrose. There was not, however, any differential effect on the phenotype of wild type and *vch1* seedlings (data not shown).

4.8.2 Effect of growth medium pH on root elongation

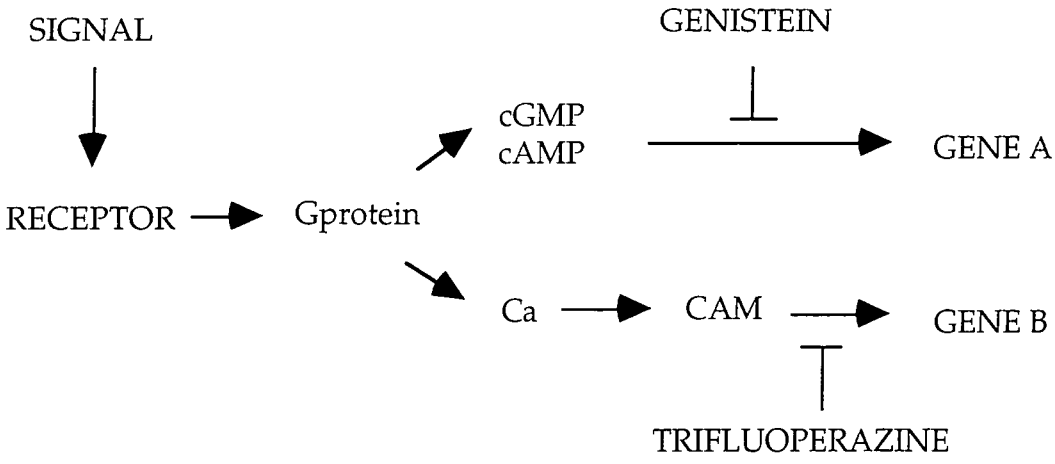
Cell expansion can be influenced by wall pH (acid growth hypothesis; Taiz & Zeiger, 1991) perhaps through the effects of pH on the activity of enzymes such as expansins and XETs. Expansins exert their effect on cell wall extension by loosening of cell wall components. McQueen-Mason & Cosgrove (1994) demonstrated that elongation of hypocotyls of cucmber (*Cucumis sativus* L. cv. Burpee Pickler) was sensitive to growth medium pH, with increased elongation under low pH conditions. Similarly, isolated and purified expansin proteins, when applied to cellulose paper (as a crude model of cellulose cell walls), of which they were capable of inducing extension under load-bearing conditions, showed a similar sensitivity to pH.

The effect of growth medium pH on the extension of hypocotyls of wild type and *vch1* seedlings was investigated here. It was postulated that, if the activity of such enzymes is reduced in *vch1*, it might exhibit a lesser response to pH alteration than wild type seedlings.

10 seedlings of each of *vch1* and wild type were germinated and grown on 1/2MS10 medium (Appendix 1) adjusted to each of pH 4.0, 4.5, 5.0, 5.5 and 6.0 respectively. Seedlings were grown on these media in square Petri dishes at 20° from the vertical, as described in Section 2.1.1 and total primary root length was measured at 14 days post-germination.

No significant difference in root growth were observed (Table 4.4) for either *vch1* or wild type seedlings under any of the pH conditions tested.

Diag. 4.1
G-protein signalling pathways



Modified after Barnes *et al.*(1995)

4.9 Hormone and inhibitor experiments

4.9.1 Hormones

The role and importance of hormones and other signalling molecules in the control and co-ordination of numerous aspects of plant growth and development were outlined in Section 1.6.2. Some of the many mutants which show a lack of, inappropriate or excessive production, perception or response to such signals were also described.

The effects of the exogenous application of a range of hormones in the growth medium on the the *vch1* phenotype were investigated here. The rationale behind this approach is that if the mutant were defective in production of a hormone, then exogenous application of that hormone may rescue the phenotype. Also, the nature of the mutants response to each hormone provides information about whether it can perceive and respond to the hormone in a normal manner.

Auxin

A number of mutants have been identified which are defective in auxin production, perception or response. For example, in *fass*, which is dwarfed, levels of free auxin are 3 times as high as in wild type embryos (Fisher *et al.*, 1996) and it was hypothesized that excess free auxin is inhibitory to elongation in *fass*, perhaps through increasing ethylene production. Both auxin and ethylene have been shown to be capable of causing the reorientation of microtubules (Shiboaka, 1994). Auxin-resistant mutants such as *axr1* (Lincoln *et al.*, 1990) and *axr2* (Wilson *et al.*, 1990) have reduced auxin-mediated responses, including plant height, root gravitropism and vascular bundle differentiation. *axr2* (Wilson *et al.*, 1990; Timpte *et al.*, 1992) is a severe dwarf and exhibits abnormal gravitropism and a lack of root hairs and is resistant to the exogenous supply of auxin.

It was hypothesized that the dwarfed *vch1* phenotype might result from increased auxin production or sensitivity.

Following germination and 7 days growth on 1/2MS10 medium (Section 2.1.1) 10 of each of *vch1* and wild type seedlings were transferred to 1/2MS10 containing an auxin (Indole-3-acetic acid or 2,4-D) at a concentration of 10mg/l. Following transfer to this medium the seedlings were grown for a further 7 days under standard growth conditions before examination. The effects of IAA treatment on the phenotypes of *vch1* and wild type seedlings are shown in Fig. 4.12B. The response to 2,4-D was indistinguishable from that to IAA. It appears that *vch1* responds to the application of auxin qualitatively in the same way as wild type seedlings, showing a large increase in the number of lateral roots formed (Fig. 4.12 B; Table 4.5). When seedlings were grown on IAA concentrations of 5, 1, and 0.1 mg/l respectively, the magnitude of the phenotypic response was dose dependent in terms of number of lateral roots counted in both *vch1* and wild type seedlings (Table 4.5). It is concluded that *vch1* can perceive and respond to auxins in a proportionately similar manner to wild type seedlings. It is concluded that *vch1* exhibits neither ~~lack of~~ sensitivity, nor hypersensitivity to the hormone.

Cytokinin

The experiment was also performed with the exogenous provision of kinetin, a synthetic cytokinin.

That cytokinin can play a role in cell elongation is evidenced by the *amp1* mutant (Chaudhury *et al.*, 1993), which is dwarfed and exhibits increased accumulation of cytokinin. Transgenic overexpression of cytokinin upregulators (Medford *et al.*, 1989) results in plants exhibiting a similar phenotype. It was hypothesized that *vch1* might be defective in the regulation of cytokinin response. Seedlings were grown on kinetin to investigate the response of *vch1* to this hormone.

Following germination and 7 days growth on 1/2MS10 medium (Section 2.1.1), 10 of each of *vch1* and wild type seedlings were transferred to 1/2MS10 containing 5mg/l kinetin. Following transfer, seedlings were grown for a further 7 days before examination.

Kinetin elicited a normal response by *vch1* and wild type seedlings. The effects of this treatment on the phenotypes of *vch1* and wild type seedlings are shown in Fig. 4.12 C. Both responded by showing a reduction in root growth, hypocotyl thickening and accumulation of purple pigmentation (presumably anthocyanin). (Table 4.5; Fig. 4.12 C). It is concluded that *vch1* can perceive and respond to kinetin in a proportionately similar manner to wild type seedlings.

Gibberellin

When applied to some classes of dwarf mutants, gibberellins have been able to rescue their phenotype (Koornneef & van der Veen, 1980; Feldmann *et al.*, 1989). Gibberellic acid, when applied to wild type seedlings, produces no discernable phenotypic alteration.

10 of each of *vch1* and wild type seedlings were germinated and grown on 1/2MS10 medium for 7 days (Section 2.1.1) and then transferred to medium containing GA_3 (a gibberellin) at concentrations of 1µg/ml, 10µg/ml, 100 µg/ml and 1mg/ml, and grown for 7 days: no phenotypic difference was identified from control *vch1* nor wild type seedlings transferred at the same time to 1/2MS10 with no hormones added (Table 4.5). The *vch1* phenotype is, therefore, not rescued by the exogenous application of gibberellin. It cannot be discounted that the defect could be in perception of GA3.

Ethylene

Ethylene is a plant hormone with a wide range of phenotypic effects. Studies on the physiological effects of ethylene have largely been carried out in mutants with altered ethylene production, transport or perception (Kieber *et al.*, 1993; Ecker, 1995); by adding the ethylene precursor, ACC; or by using silver ions in the growth medium to block ethylene perception. Silver ions bind ethylene in competition with its receptors (Tanimoto *et al.*, 1995). Ethylene response mutants, such as *etr-1* (Kieber *et al.*, 1993) also exhibit reduced hypocotyl and root elongation. Ethylene has been shown to be capable of causing the reorientation of microtubules and cell (Mayumi *et al.*, 1995). In

order to investigate whether *vch1* responds to the blocking of ethylene perception by the exogenous application of silver ions, *vch1* and wild type seedlings were grown in the presence of silver thiosulphate.

10 of each of 7 day old *vch1* and wild type seedlings germinated and grown on 1/2MS10 (Section 2.1.1) were transferred to 1/2MS10 containing silver thiosulphate at 1mg/ml and grown on this medium for 7 days.

This elicited a similar response (Fig. 4.11 D) in both *vch1* and wild type seedlings. Roots grew significantly longer than controls (Table 4.5) transferred to medium with no hormones, and also showed a reduction in root hair production. *vch1* primary roots showed an average of approximately 50% greater root length on this medium than on 1/2MS10 medium alone, as compared to a difference of approximately 60% for wild type roots. It is concluded that *vch1* can perceive and respond to ethylene.

Brassinosteroids

Brassinosteroids are a group of plant hormones which show a high degree of structural similarity to animal steroid hormones. This group of compounds have been shown to play a role in the control of cell elongation. A number of dwarfed mutants of *Arabidopsis* have been able to be phenotypically rescued by the exogenous application of brassinosteroids (Kauschmann *et al.*, 1996). Brassinosteroids have also been demonstrated to be capable of causing the reorientation of microtubules (Mayumi *et al.*, 1995).

Following the methods of Kauschmann *et al.*, *vch1* and wild type seeds were germinated directly on 1/2MS10 medium containing epibrassinolide (a brassinosteroid which occurs naturally in *Arabidopsis*) at 0.1 and 1.0μm. After 7 days growth post-germination on this medium, 10 of each of *vch1* and wild type seedlings were compared to those germinated and grown on 1/2MS10 medium alone.

Table 4.5
Effect of exogenous supply of hormones on phenotype of *vch* 1 and wild type seedlings

Hormone	Concn. mg/ml	Effect on *vch* 1 seedlings	Effect on WT seedlings	Fig.
Indole-3-acetic acid	10 5 1 0.5 0	Reduced primary root length Increased lateral root formation 1°root length (mm ±SE) / no. of lats. (±SE) 0.45 ±0.089 / 32.6 ±0.312 0.85 ±0.107 / 29.2 ±0.50 1.35 ±0.167 / 13.0 ±0.702 1.95 ±0.189 / 11.6 ±0.301 1.85 ±0.15 / 8.8 ±0.106	Reduced primary root length Increased lateral root formation 1°root length (mm ±SE) / no. of lats. (±SE) 7.4 ±0.323 / 58 ±0.38 7.35 ±0.2 / 61 ±0.12 16.0 ±0.856 / 49 ±0.269 16.2 ±0.601 / 40 ±0.344 23.7 ±1.017 / 17 ±0.250	4.12B
Kinetin	10 5 1 0.5 0	Reduced primary root length hypocotyl thickening increased anthocyanin increased leaf growth 1°root length (mm ±SE) 0.55 ±0.115 0.50 ±0.167 1.25 ±0.189 1.35 ±0.167 2.45 ±0.155	Reduced primary root length hypocotyl thickening increased anthocyanin increased leaf growth 1°root length (mm ±SE) 8.4 ±0.355 7.1 ±0.245 16.9 ±0.456 15.0 ±0.112 21.8 ±0.478	4.12C
Gibberellin	1 0.1 0.01 0.001	no effect apparent at any concentraion tested	no effect apparent at any concentraion tested	
2,4-epibrassinolide	1	Paling and slight enlargement of leaves	Paling and slight enlargement of leaves	4.12E
Silver thiosulphate (blocks ethylene perception)	1.25 0	Increased root growth Reduced root hair density 1°root length (mm ±SE) 3.1 ±0.145 1.9 ±0.140	Increased root growth Reduced root hair density 1°root length (mm ±SE) 32.5 ±0.233 20.0 ±0.168	4.12D

Fig. 4.12
Effect of hormones and dark on seedling growth

A. *vch1* (left) and WT seedling at 14 d.o. - controls
B. *vch1* (left) and WT seedling at 14 d.o. - plated on 5mg/l IAA for 7d
C. *vch1* (left) and WT seedling at 14 d.o. - plated on 5mg/l kinetin for 7d
D. *vch1* (left) and WT seedling at 14 d.o. - plated on 1mg/l Silver thiosulphate for 7d
E. *vch1* seedlings at 10 d.o. - on 1 μm epibrassinolide (left) and untreated control (right)
F. 2 *vch1* (left) and 2 WT seedlings (right) germinated and grown in dark for 7d

bars = approx. 1cm

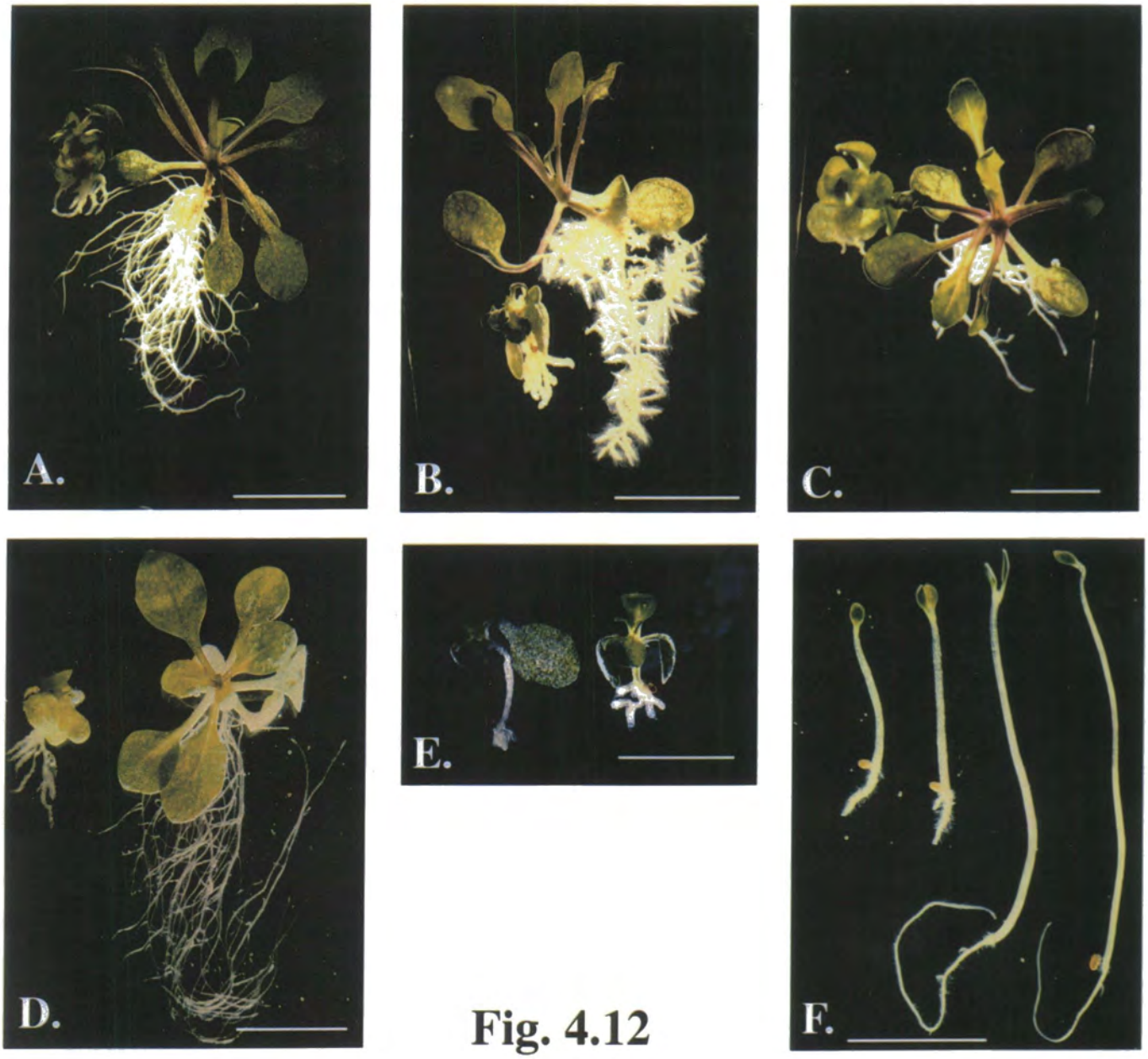


Fig. 4.12

There was a difference between the brassinosteroid treated seedlings and controls. This was a slight paling of leaves, some leaf expansion and reduced root length (Table 4.5; Fig. 4.12 E). It is concluded, however, that epibrassinolide is not sufficient to rescue the phenotype of *vch1*. It cannot be discounted that the defect could be a reduction in ability to perceive the compound.

4.9.2 Signalling inhibitors

It could be postulated that disruption of the production of the normal product of the *VCH1* gene directly or indirectly results in the failure of suppression of signalling cascades which somehow restrict normal elongation processes. The following experiments were designed to investigate several possiblities: 1) whether the phenotype of *vch1* could be rescued by exogenous supply of components of signalling pathways, which may be reduced in endogenous concentration by the mutation; 2) whether *vch1* could respond to the application of each in a similar manner to wild type seedlings; 3) whether any phenocopy of *vch1* might be elicited in wild type seedlings treated with inhibitors of signalling pathways.

Genistein is an inhibitor of cyclic-GMP-dependent protein kinase activity (Diag. 4.1 presents a summary of possible G-protein signalling pathways; Barnes *et* al., 1995; Bowler *et al.*, 1994). If c-GMP-dependent protein kinase activity is upregulated as a result of the *vch1* mutation, then blocking of this pathway by exogenous application of this inhibitor might rescue the *vch1* phenotype. If c-GMP-dependent protein kinase activity is downregulated as a result of the *vch1* mutation, then exogenous application of the inhibitor might be sufficient to phenocopy *vch1* in wild type seedlings. As there is "crosstalk" between the branches of the G-protein signallin pathway, downregulation of one branch can result in increased acivity of the other branch (Bowler *et al.*, 1994). For example, downregulation of cGMP-dependent signalling may result in upregulation of calcium-dependent signalling pathways. This might have concommitant effects on the phenotype of *vch1* or wild type seedlings.

10 of each of *vch1* and wild type seedlings were germinated on 1/2MS10 medium (Section 2.1.1). Genistein was included in this germination medium, at concentrations of 10 and 50μM. Seeds were stratified and germinated (Section 2.1.1) and were grown for 14d on this medium.

No effect was seen on the phenotypes of either wild type or *vch1* seedlings, examined at 7 and 14 days post-germination (Table 4.6).

Trifluoperizine inhibits calcium-dependent protein kinase activity (Neuhaus *et al.*, 1993; Bowler *et al.*, 1994). Similarly, if activity of this signalling pathway is upregulated by the *vch1* mutation, then blocking of it by the addition of the inhibitor might rescue the *vch1* phenotype. If it is downregulated, then exogenous application of the inhibitor might phenocopy *vch1* in wild type seedlings.

10 of each of *vch1* and wild type seedlings were grown for 7d on 1/2MS10 and then transferred to 1/2MS10 containing trifluoperizine at 10 and 50μM for 7d.

No phenotypic alteration was seen in either *vch1* or wild type seedlings (Table 4.6) grown on this medium.

8-Br-cGMP is a membrane-soluble, biologically active, cyclic nucleotide analogue of cGMP which promotes cGMP-dependent signalling (Neuhaus *et al.*, 1993; Bowler *et al.*, 1994). "Flooding" of the system with this analogue can cause upregulation of genes regulated by cAMP-dependent signalling and downregulation of calcium-dependent signalling-regulated genes. Depending on the signals which regulate or mediate the effects of the *VCH1* gene, addition of this analogue may cause phenocopy or rescue of *vch1*.

10 of each of seedlings of *vch1* and wild type were germninated and grown on 1/2MS10 for 7d, prior to transfer to 1/2MS10 containing 8-Br-cGMP at 10 and 50μM.

8-Br-cGMP caused a reduction in primary root length to approximately 60% of that of untreated controls for both *vch1* and wild type seedlings at both 10 and 50μM (Table 4.6). There was no significant suppression of lateral root formation (Table 4.6).

TIBA is an inhibitor of polar auxin transport. It has been shown to be capable of inhibiting the development of bilateral symmetry in cultured *Brassica juncea* somatic embryos (Liu *et al.,* 1993). That auxin is important in elongation growth is evidenced by the dwarfed phenotype of auxin-response mutants, such as *axr1* (Lincoln,*et al.* 1990). The effects of blocking polar auxin transport on the phenotype of *vch1* and wild type seedlings was investigated by germinating and growing these on medium containing TIBA.

10 of each of seeds of *vch1* and wild type were stratified and germinated on 1/2MS10 plates (Section 2.1.1) containing TIBA at 10μM and 50μM. Seedlings were grown for 14d on this medium before examination.

At 10μM, TIBA was able to almost completely suppress the formation of lateral roots in both wild type and *vch1* seedlings. By 14 days, one or two were seen on a few seedlings of both groups, but formation was significantly reduced in both (2.2±0.179 in *vch1*, 5.0±0.056 in untreated *vch1* controls, 3.4±0.116 in WT, 13.3±0.210 in untreated WT controls; $p < 0.001$). Total length of the primary root was also significantly reduced in both groups, in each case to approximately 50% of that of control seedlings (Table 4.6). This effect did not appear to be dose dependent over the range of concentrations tested, with similar root lengths observed on concentrations from 10-50μM (Table 4.6). *vch1* exhibits a quantitatively and qualitatively similar response to that of wild type plants to this compound.

Hydroxyurea is a cell cycle inhibitor, blocking DNA polymerase activity and S phase progression (Topping & Lindsey, 1997). It may thus inhibit primary root growth and lateral root initiation. It was hypothesized that the defect in cell expansion in *vch1*

Table 4.6
Effect of exogenous supply of signalling inhibitors and analogues on phenotype of *vch1* and wild type seedlings

Compound	Concn. mg/ml	Effect on *vch1* seedlings		Effect on WT seedlings	
Genistein	10 50	no effect		no effect	
Trifluorperizine	10 50	no effect		no effect	
8-Br-cGMP		no. of laterals (±SE)	1°root length (mm ±SE)	no. of laterals (±SE)	1°root length (mm ±SE)
	0	3.2±0.651	2.3±0.104	11.4±0.222	31.2±0.204
	10	4.4±0.116	1.9±0.155	10.1±0.234	19.3±0.550
	50	3.8±0.215	1.6±0.214	8.0±0.145	18.3±0.258
TIBA		no. of laterals (±SE)	1°root length (mm ±SE)	no. of laterals (±SE)	1°root length (mm ±SE)
	0	5.0±0.056	2.1±0.117	13.3±0.210	26.8±0.320
	10	2.2±0.179	1.2±0.132	3.4±0.116	12.4±0.179
	50	0.15±0.016	1.1±0.089	3.0±0.150	14.0±0.145

might be related to cell division activity. Blocking of cell division activity by the addition of hydroxyurea might be able to phenocopy or rescue *vch1*.

10 of each of *vch1* and wild type seedlings were stratified, germinated and grown on 1/MS10 for 7d. They were then transferred to 1/2MS10 containing 10 μM or 50 μM hydroxyurea (HU) and grown for 5 d on this medium before examination.

No reduction in growth of the primary root was detected. Both wild type and *vch1* roots roots exhibited a reduction in lateral root number formed compared to that in untreated controls (*vch1* untreated = 4.1 ± 0.032, *vch1* on 10 μM HU = 3.3 ± 0.051, WT untreated = 14.6 ± 0.102, WT on 10 μM HU = 10.4 ± 0.114; $p<0.001$).

As for the hormone experiments described above, *vch1* did not show any inappropriate response to the inhibitor substances supplied.

4.10 Detection of AGPs with Yariv reagent

β-glucosyl Yariv reagent (β-GlyY), a synthetic phenyl glycoside, reacts specifically with arabinogalactan-proteins (AGPs) (Willats & Knox, 1996), a class of proteoglycans associated with the plasma membrane and cell wall (Knox *et al.*, 1991). The reagent is red-coloured, allowing detection of binding sites. AGPs are able to cause cell elongation (Willats & Knox, 1996). The *diminuto* mutant, a dwarf, has a severe reduction in cell length in many tissues and also has a marked reduction in β-GlyY-reactive AGP (Takahashi *et al.*, 1995). It was hypothesized that the defect in expansion in *vch1* might be due to a reduction in cell wall AGPs. Detection of AGPs with Yariv reagent was performed to investigate this.

When *vch1* and wild type seedlings were transferred to medium containing 30μM β-GlyY at 7 days post-germination and grown on this medium for 3 days, red colouration of the roots was observed (Fig. 4.13). There was no reduction in the intensity of staining with Yariv reagent and it was concluded from this that there was no reduction in AGPs on the cell surfaces of *vch1* roots. There was a slight enhancement

Fig. 4.13
Detection of AGPs with Yariv's reagent

vch1 (left) and WT (right) seedlings grown for 3d on 30 μm Yariv's reagent

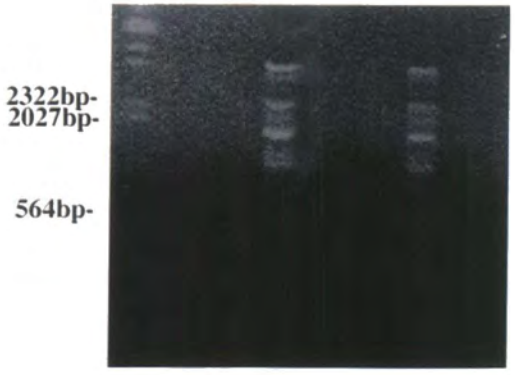
Fig. 4.15
Expansin PCR products

DNA sequences were amplified with degenerate expansin primers (Section 4.12).

Lane 1 = 1kb marker DNA
Lane 2 = DNA from wild type seedling
Lane 3 = DNA from *vch1* mutant seedling

Fig. 4.13

Fig. 4.15



in red coloration compared to wild type roots. This may possibly be due to an increase in density of AGPs on the surface of *vch1* cells, due to reduced cell size.

4.11 Dark response

Wild type seedlings, when grown in the dark exhibit a specific set of growth characteristics (skotomorphogenesis). These include hypocotyl elongation, development of an apical hook, reduced root growth and pale coloration (Chory, 1993).

At least 15 of each of *vch1* and wild type seeds were sterilized and transferred to 1/2MS10 germination medium in Petri dishes (Section (2.1.1). They were stratified at 4°C for 5 days prior to placement in a growth room with lights (Section 2.1.1) to initiate germination. After 24h, the Petri dishes were wrapped in aluminium foil, to exlude light, and replaced in the growth room. Control plates with no foil were included. At 7 days post-germination hypocotyl lengths were measured for each group of seedlings.

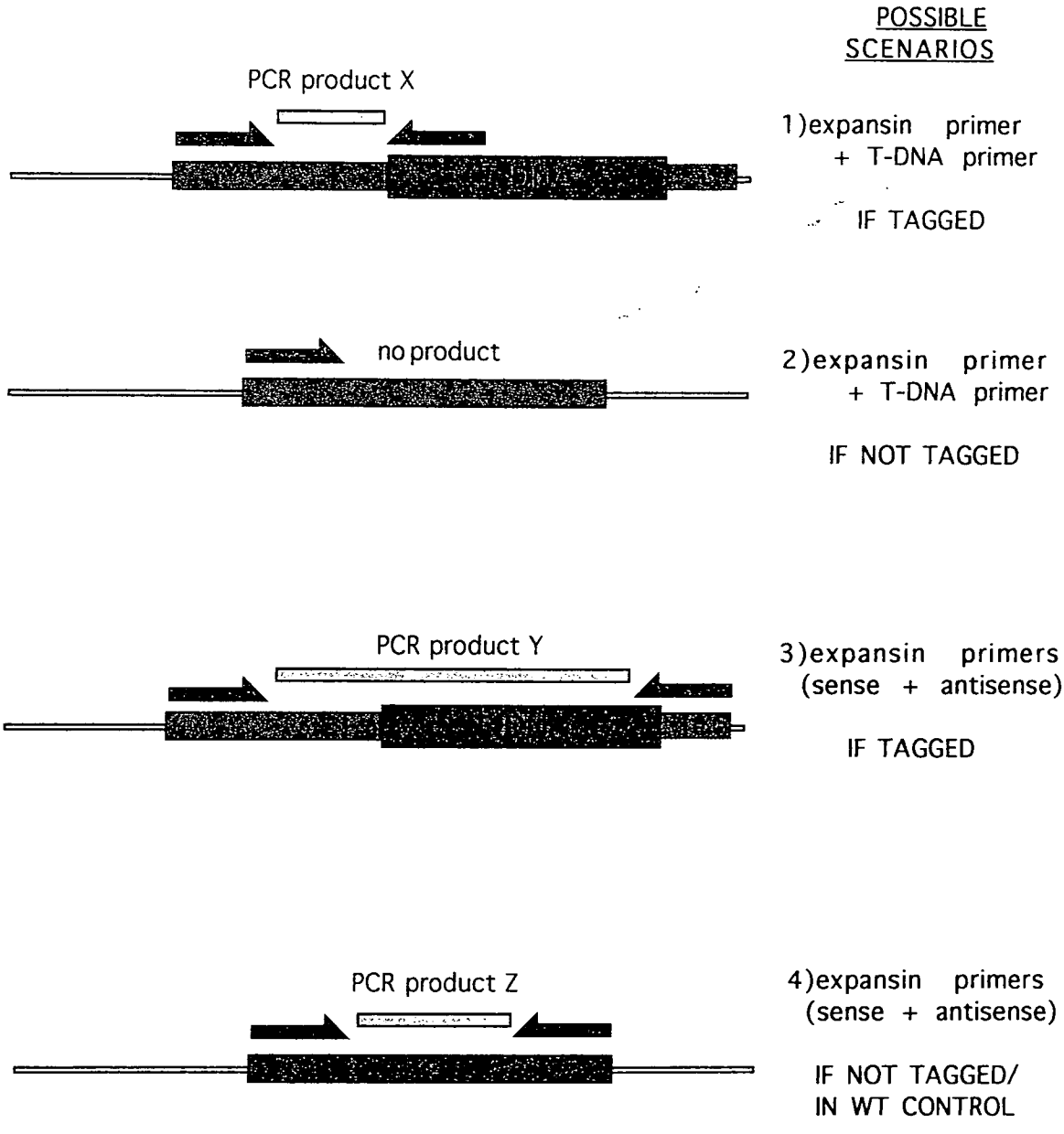
The wild type hypocotyl, when grown in the dark elongates to a mean of 501% of its length in the light and that of *vch1* to 362% of its mean light-grown length (WT_L = 3.86 ± 0.48, WT_D = 19.33 ± 1.75, *vch1*$_L$ = 2.312 ± 0.40, *vch1*$_D$ = 8.37 ± 1.71; $p<0.001$). *vch1* and control seedlings also showed the other chacteristics associated with dark growth (Chory, 1993), including, development of an apical hook, reduced root growth and pale colouration (Fig 4.12 F). It was concluded that *vch1* can perceive and respond to dark-growth in the same manner as wild type seedlings. However, the magnitude of the response is quantitatively limited .

4.12 Detection of expansin genes in *vch1*

A number of groups of enzymes are known to be involved in the control of cell elongation through their effects on the arrangement of cell wall structural components. This is discussed more fully in Section 7.11. One important group of such enzymes are the *expansins,* described in Section 1.6.1 (McQueen-Mason & Cosgrove, 1994).

Fig. 4.14

Possible products from expansin PCR experiment



This experiment was designed to test the hypothesis that the *vch1* defect is due to the knockout of expansin genes.

A PCR-based approach was used in an attempt to determine whether any of the expansin genes in *vch1* had been physically disrupted by the integration of T-DNA. Whilst the expansin genes both within the *Arabidopsis* genome and between a range of plant species are quite divergent at the protein sequence level, they are highly conserved at the genetic level. Because of this conservation it has been possible to design degenerate primers which are able to specifically amplify all expansin genes (kindly supplied by Dr. S. McQueen-Mason, Univ. of York, UK).

The rationale of the approach used here was firstly to use primers facing outwards from the left and right borders respectively of the T-DNA, in a range of combinations with the sense and antisense expansin primers. If the T-DNA had integrated either within or closely upstream or downstream of one of the expansin genes, then a PCR product (X) should be produced (Scenario 1, Fig 4.14).

Genomic DNA from individual *vch1* homozygous and wild type seedlings was extracted by the quick extraction method described in Section 2.7.3. Pairwise combinations of expansin sense or antisense primers, each with each of 3 T-DNA left border and 2 right border outward-facing primers were used for amplification. PCR was carried out as described in Section 2.12.

No product was produced with any pairwise combination of primers. At the time of carrying out this experiment it was postulated that *vch1* was T-DNA tagged. Although it turned out not to co-segregate with the presence of a *gusA* gene (Section 6.3), it still may be that fragments of the T-DNA did cause the mutation and so this approach is not completely invalid.

In a second approach, the degenerate sense and antisense expansin primers were used to amplify expansin genes from the genomic DNA of both *vch1* and wild type plants.

In a second approach, the degenerate sense and antisense expansin primers were used to amplify expansin genes from the genomic DNA of both *vch1* and wild type plants.

Genomic DNA was extracted (Section 2.7.1, 2.7.2) from individual *vch1* and wild type seedlings. PCR was carried out as described in Section 2.12.

The rationale was that a difference in the size of one of the PCR products (products Y and Z) obtained would indicate that the T-DNA had integrated within an expansin gene (Scenario 3 & 4, Fig 4.14). One advantage of this approach is that it potentially could detect any integration of T-DNA into the coding region of an expansin gene, or gene re-arrangements caused by its integration, irrespective of possible truncation at right- and/or left-border ends of the T-DNA which could prevent T-DNA primer binding.

No difference was observed in the size of the range of products obtained from such reaction (see Fig. 4.15) suggesting that there was no T-DNA integration within an expansin gene.

It is likely that not all expansin genes were amplified in this experiment. At least 11 expansin cDNAs have been isolated to date in *Arabidopsis* (Cosgrove, 1997), and there may be more as yet unidentified. 8 differently sized products were detected (Fig. 4.15). It may be that there are some expansin genes are of the same size, and so not detectable as separate bands following electrophoresis. However, if one expansin gene did contain a T-DNA insert, this might have then been detected as an extra band compared to those in the wild type track.

In order to confirm the identity of the PCR products as sequences amplified from expansin genes, it would be necessary to clone and sequence the products. This experiment, therefore, was considered to be very preliminary.

4.13 Summary

This chapter has described the phenotypic and physiological characterisation of the *vch1* mutant. The *vch1* mutant phenotype was first evident at approximately 4-6 days post-germination. *vch1* mutant plants exhibit a severe reduction in cell elongation of all major organs and this was evident at the cellular level. Cells were found to be much reduced in length along the apical-basal axis of the plant and were increased in diameter in the radial dimension. Cellular patterning within the mutant appeared normal: cell types were present in their correct spatial context despite their altered shape, although there was an increase in the number of cortical and epidermal cells in cross section. *vch1* is able to respond in the correct quantitative manner to the exogenous application of hormones within the constraints imposed by the mutation. In addition, *vch1* plants elicited a hypocotyl elongation response when grown in the dark. Such responses were not, however, sufficient to rescue the mutant phenotype.

It is possible that the failure of cell elongation might be due to a defect in cell wall expansion mechanisms. However, no major difference was observed between the cell walls of *vch1* and wild type seedlings when examined at the ultrastructural level.

It was not possible to show a difference between the structure of the genes encoding the wall loosening enzymes, expansins.

The phenotypic characterisation of the *asf1* mutant will be presented in the next chapter. The preliminary molecular characterisation and progress towards the genetic mapping of the *VCH1* locus will be presented in Chapter 6. The results presented here and in subsequent chapters will be discussed in Chapter 7.

5.0 - The *asf1* mutant

5.1 Objectives

As for the *vch1* mutant in Chapter 4, the primary objective of this chapter was to elucidate the details of the aberrant morphogenesis of the *asf1* mutant, and to obtain information regarding the likely nature of the genetic change. The *asf1* phenotype was initially identified at a stage when heterozygous and wild type embryos in the same silique of an *asf1* heterozygote parent plant were at the cotyledonary stage of development. The *asf1* phenotype observed at this stage is shown in Fig. 3.1b. As for *vch1*, it was essential to determine the earliest stage at which the mutant phenotype becomes manifest, and to examine in detail the progression of the aberration through embryogenesis.

5.2 Developmental profile of *asf1* embryos

Two main approaches were used to observe the early embryos. Histological sectioning was initially useful to trace the defect back to an earlier point than that at which the initial observations were made. The ease of producing large numbers of sections allowed for the observation of large numbers of mutant and wild type embryos and facilitated a thorough investigation of the predictability of the organisation of the mutant at these later stages. This approach, however, was more technically limited for studying embryos at earlier stages due to the extremely small size of such early embryos and the consequent difficulty of locating and sectioning large numbers. The complementary approach employed, therefore, was confocal scanning laser microscopy. The advantage of this approach is that, using the sample preparation and imaging methods (described in Section 2.4.5), the small embryos are relatively easily identified as brightly fluorescing structures either within, or following removal from, the testa, and can then be more closely examined. Large numbers of embryos could thus be examined. This approach was more limited for later stage embryos due to difficulty in penetrating internal cell layers with the ethidium bromide stain (Section 2.4.5).

Fig. 5.1 presents a developmental profile of the *asf1* mutant embryos and the equivalent wild type developmental stages.

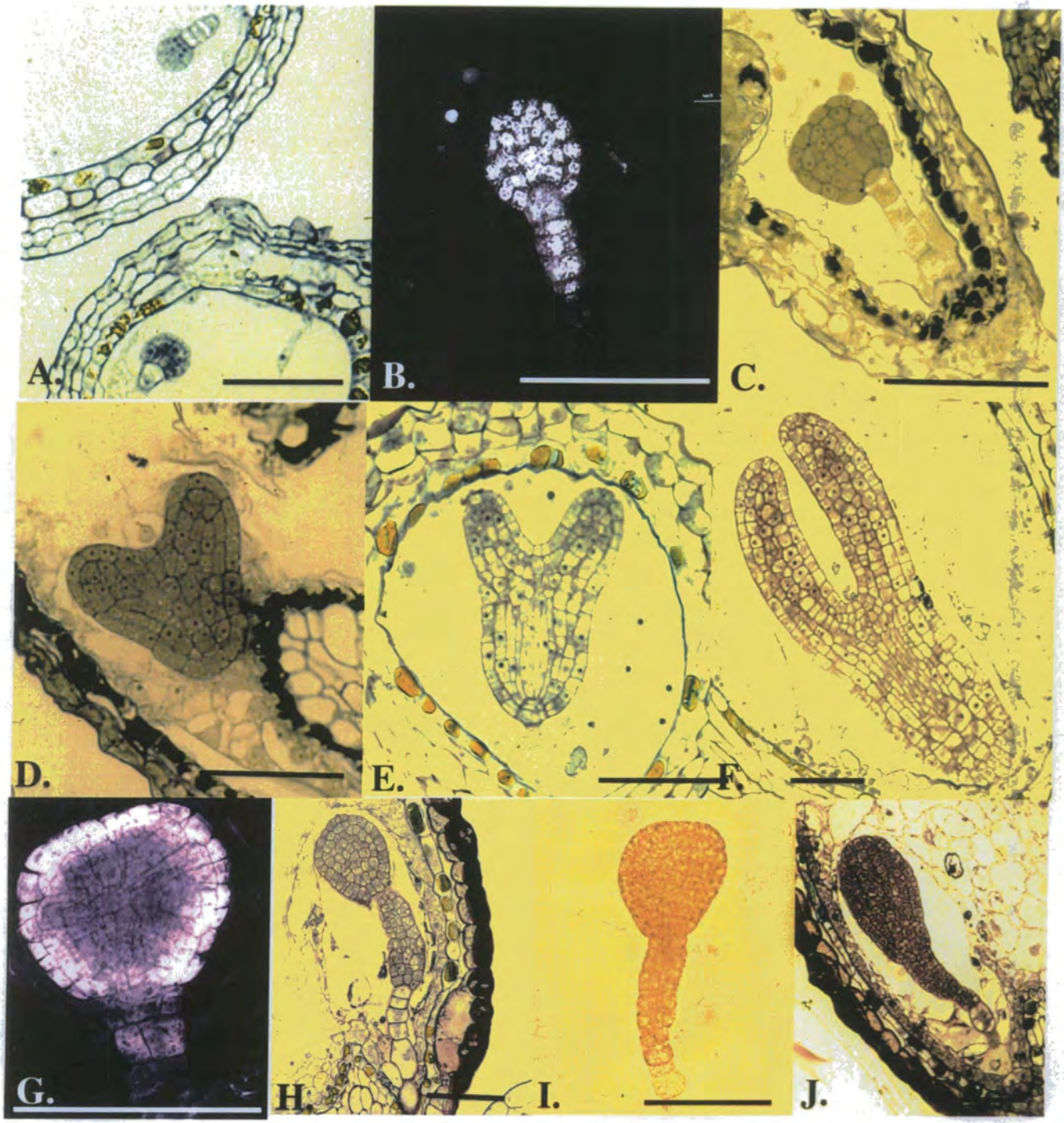
The first evidence of aberrant cellular organisation (Fig. 5.1 G) was at the stage when normal embryos undergo the transition from the globular to the heart-stage of embryogenesis. All embryos at earlier stages appeared normal. At the point of this transition, mutant embryos failed to establish bilateral symmetry and instead underwent misoriented cell divisions within the still globular shaped embryo proper, leading to an increase in size of the embryo to a diameter of approximately 45-60μm. The wild type globular-stage embryo was approximately 30-35μm in diameter. The deliniation of the O' boundary, evident at the octant stage, became obscured. This continued until the normal embryos reached approximately the heart/early torpedo stage (see Fig. 5.1 D-E), at which point the suspensor of the mutant was observed to undergo inappropriate cell divisions. This ectopic division began with the longitudinal division of the hypophysis (the uppermost cell of the suspensor) (Fig. 5.1 G). This then underwent further longitudinal and transverse divisions as the next cell down in the suspensor concomitantly began to divide. This pattern was repeated as the suspensor cells begin and continue to divide progressively from the 'embryo-proper end' towards the basal cell of the suspensor. The result was a mass of cells emanating from the base of the steadily growing, but still globular-shaped, embryo proper. The boundary between the embryo-proper and suspensor-derived cells in the growing mass was found to become obscured, as did the O' boundary between upper and lower tier cells apparent in both *asf1* and wild type octant-stage embryos. The embryo proper was observed to elongate slightly along its apical-basal axis (Fig. 5.1 J).

The size of cells comprising the wild type suspensor, the *asf1* embryo proper and *asf1* suspensor-derived structure were measured using the scanning-laser confocal microscope (Section 2.4.5) and the Comos imaging software. 10 cells were measured at random within each structure and the length of their longest axis recorded. Cell measurements were not made within the wild type embryo proper since such cells

Fig. 5.1
asf1 and wild type embryogenesis

A. early globular WT embryos
B. globular WT embryo
C. transition-stage WT embryo
D. heart-stage WT embryo
E. early torpedo-stage WT embryo
F. late torpedo-stage WT embryo
G. *asf1* embryo at transition stage
H. *asf1* embryo at heart/early torpedo-stage
I. *asf1* embryo at torpedo-stage
J. *asf1* embryo at late torpedo-stage

bars = approx. 50μm



bars = approx. 50um

differentiate and elongate from the globular stage making comparison with the homogeneous cells of the *asf1* embryo proper and suspensor more difficult.

The cells produced from suspensor cell division were significantly smaller (8.05 ± 0.369) than wild type suspensor cells (15.55 ± 0.320; $p<0.001$). They were more cytoplasmically dense (Fig. 5.3 C), being more similar in size and appearance to cells of the embryo proper (Fig. 5.3 A) than those of the normal suspensor (Fig. 5.3 C). The size of these suspensor-derived cells was not significantly different (9.3 ± 0.484; $p<0.001$) to that of the cells comprising the embryo proper region of the *asf1* embryo.

Morphologically, *asf1* embryos did appear to structurally differentiate a rudimentary protoderm, insofar as a discrete layer of elongate cells was observed at the periphery of the embryo.

5.3 Stage of arrest of mutant embryos

The phenotypically similar *abnormal suspensor* (Schwartz *et al.*, 1994) and *raspberry* (Yadegari *et al.*, 1994) mutants are characterized by seed abortion prior to the start of seed desiccation. This is indicated by the appearance of 25% shrivelled, brown, dead seeds within the siliques of plants heterozygous for the mutant gene.

Mature seeds harvested from a plant heterozygous for the *asf1* mutation were cleared with KOH (Section 2.3.1) to reveal a proportion containing *asf1* mutant embryos. This proportion (24/200=3.0%) was less than the 25% expected if the mutant seeds were always able to develop to this stage: this represents 12% of the total number of *asf1* seeds. A number of mutant seeds (42/250=16.8%) were observed to abort prior to desiccation (13/250=5.2% in a wild type control).

In the case of *asf1*, however, some mutant embryo-bearing seeds appeared to persist and undergo maturation and desiccation events to produce an externally normal mature seed. Prior to dessication the seeds bearing mutant embryos were easily distinguished

from their wild type counterparts, due to their pale green appearance (normal dark green mature embryos can be seen through the immature testa).

Despite this ability of a small percentage (12%) of *asf1* mutant embryo-bearing seeds to mature and withstand dessication, the mutant can be said to be predominantly embryo-lethal.

asf1 homozygous seed could be distinguished from wild type seed on the basis of a paler brown colouration of the testa. 350 *asf1* seeds were sterilised, plated on 1/2MS10 medium in Petri dishes (Section 2.1.1), stratified and placed in the growth room (Section 2.1.1). Plated seeds were examined after 5 days for germinated individuals.

More than 98% of such seeds (344 of 350) failed to germinate under normal culture conditions. A very small number, however, were observed to germinate and undergo some, albeit very abnormal, post-germinative development when maintained *in vitro*. Fig. 5.2 A-E shows the phenotype of most of the germinated individuals observed. The 'seedling' is a green, globular structure, which failed to grow significantly and became necrotic within 2-3 weeks. Just two individuals were ever identified which underwent the post-germinative series of events seen in Figs. 5.2 A-E. In this case the globular-shaped 'seedling' emerged from the seed with the aberrant suspensor-derived structure attached and intact. The whole 'seedling' was green and appeared to be living. At 3 weeks post-germination the main body of the 'seedling' had not increased in size, the only change being that the suspensor-derived structure had begun to appear brown and necrotic, and by 5 weeks had died and shrivelled. By 6 weeks the 'seedling proper' had begun to grow and develop villus-like projections over its surface (Fig. 5.2 C). This continued to grow and develop more such projections over the following 3 weeks before finally becoming necrotic at 9 weeks post-germination (Fig. 5.2 E).

Fig. 5.2
Germinated *asf1* individuals

A. Germinated *asf1* at 3 d post-germination
B. Germinated *asf1* at 25d post-germination
C. Germinated *asf1* at 6 weeks post-germination
D. Germinated *asf1* at 8 weeks post-germination
E. Germinated *asf1* at 9 weeks post-germination

F. Germinated *asf1* at 3 d post-germination
(different individual)

bars = approx. 100μm

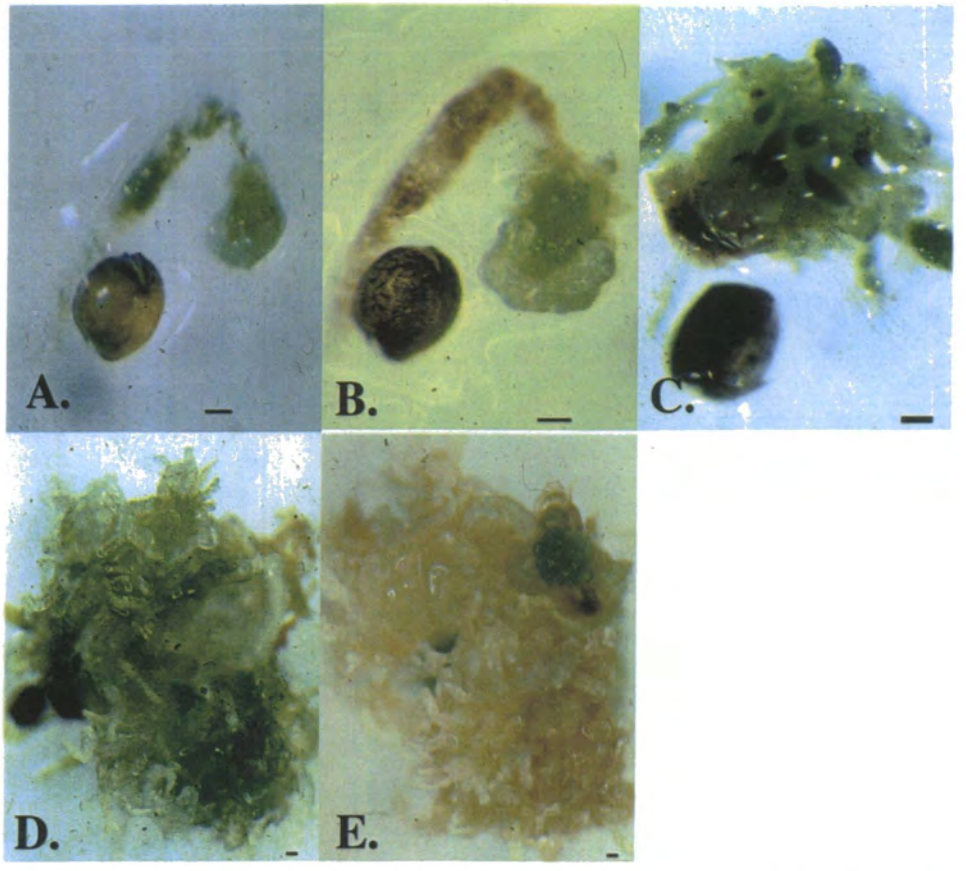
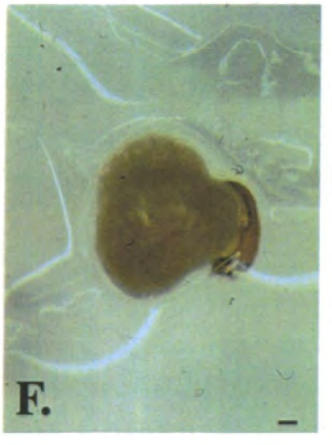


Fig. 5.2



5.4 Characteristics of suspensor-derived cells

5.4.1 TEM analysis

The *sus* (Schwartz *et al.* 1994) and *raspberry* (Yadegari *et al.*, 1994) mutants were subjected to ultrastuctural analysis by TEM. Such analysis revealed that, whilst the mutant embryos arrested at a globular state, they developed ultrastructual features more characteristic of wild-type maturation stage embryos. This will be discussed in some detail in Section 7.2.1.

Such ultrastructural analysis was carried out on the *asf1* mutant.

Ultra-thin sections of *asf1* and wild type embryos and suspensors were cut,and heavy metal stained as described in Section 2.4.7. These sections were then observed by TEM.

This analysis revealed that the inappropriately proliferated suspensor cells exhibited an organelle distribution pattern (Fig. 5.3 B) more characteristic of the wild type embryo proper (Fig. 5.3 A) than of the wild type suspensor (Fig. 5.3 B). The *asf1* suspensor-derived cells were observed to contain both large protein bodies and lipid bodies, which are not detected in cells of the wild type suspensor. Also, the *asf1* suspensor-derived cells are noticeably more cytoplasmically dense and less vacuolate than wild type suspensor cells. In both of these respects they appear more like cells of the wild type embryo proper.

5.4.2 Accumulation of chloroplasts in *asf1*

Another observation of the mutant suspensor-derived structure of *asf1* which differed from that of the *sus* and *raspberry* mutants is that it appears dark-green in colour. In the *sus* and *raspberry* mutants the embryo proper only undergoes greening. This observation suggested the accumulation of chlorophyll in the cells, and indeed when examined by UV microscopy, red autofluorescence of chlorophyll was apparent in

Fig. 5.3
TEM analysis of *asf1*

A. WT embryo proper x3000
B. WT suspensor x6000
C. *asf1* suspensor-derived cells x4000

W=wall
PB=protein body
L=lipid body
V=vacuole

Fig. 5.4 Chlorophyll autofluorescence in *asf1*

Fig. 5.3

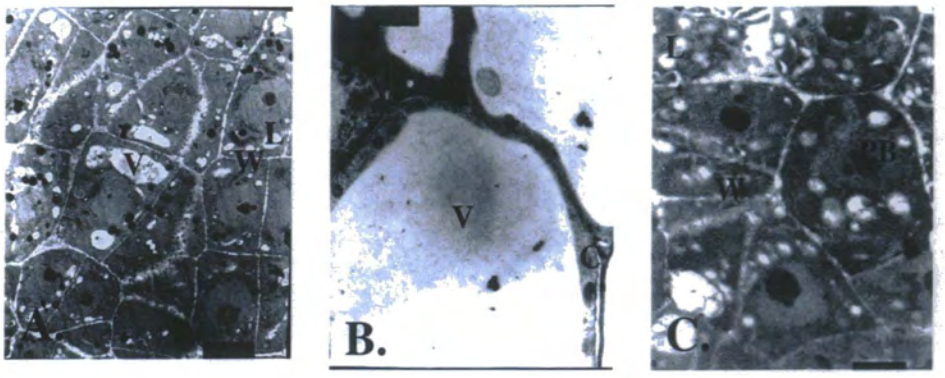


Fig. 5.4

these cells (Fig. 5.4). This represents ectopic accumulation of chlorophyll in the suspensor-derived cells. The chlorophyll was absent in maturation stage *asf1* embryos, as in wild type.

5.5 Genetic crosses with *gus*-fusion marker lines

In order to obtain information regarding the cellular organisation of the mutant embryos, a number of *gus*-fusion marker genes, which exhibit spatially restricted expression patterns in wild type embryos, were introduced into the mutant background by genetic crossing. Three such *gus*-fusion genes were introduced into this mutant (*cf.* Sections 1.12, 4.7).

EXORDIUM and *POLARIS* were described in Sections 1.12, 4.7.
During embryogenesis, *POLARIS* is expressed in the basal part of the embryo proper from the heart-stage onwards (Topping & Lindsey, 1997) and not in cells of the suspensor. *EXORDIUM* is expressed throughout the embryo proper and not in cells of the suspensor (Topping & Lindsey, 1997).

Line *276-1* was kindly provided by Prof. P. Gallois (University of Perpignan, France). The GUS expression in this line is throughout the cells of the suspensor, with no expression in the embryo proper (Fig 5.5 I).

Seeds containing F_2 embryos at a range of developmental stages were dissected out from siliques and were histochemically stained by incubation in X-Gluc at 37°C, for the times indicated, to detect the GUS enzyme product (Section 2.5). Seeds were cleared in 0.5M KOH (Section 2.4.1) and were mounted under a coverslip and examined under a compound microscope.

5.5.1 *POLARIS* expression patterns in *asf1* embryos

Expression of *POLARIS* in both wild type and *asf1* mutant embryos was found to be at a very high level. It was found ~~to be~~ necessary to incubate in X-Gluc at 37°C (Section 2.4) for only 3h to detect the GUS expression pattern.

Fig. 5.5 D shows the pattern of expression of the *POLARIS* gene in *asf1* at the most advanced developmental stage observed in *asf1*, when all cells of the suspensor have undergone ectopic divisions (as described in Section 5.2). In phenotypically normal embryos *POLARIS* is, from the heart-stage onwards, only ever expressed in the basal part of the embryo proper, and never in the suspensor cells. In *asf1*, however, it can be seen that *POLARIS* was found to be expressed in those cells at the basal end of the mass of cells derived from inappropriate suspensor cell divisions. When the GUS localization pattern was observed at an earlier stage of the development of the aberrant phenotype, then the expression pattern is shown in Fig. 5.5 C was observed. The GUS expression was always restricted to those cells at the basal end of the suspensor-derived structure, effectively moving down immediately in the wake of the wave of cell divisions passing down the suspensor. No expression was seen in those suspensor cells which had not yet undergone inappropriate divisions (Fig. 5.5 C). Once abnormal suspensor cell divisions had produced a mass off approximately 12-16 cells, then *POLARIS* expression was observed only in these suspensor-derived cells, and no longer in cells of the embryo proper.

5.5.2 *EXORDIUM* expression patterns in *asf1* embryos

EXORDIUM x *asf1* seeds were incubated in X-Gluc (Section 2.5) at 37°C overnight prior to examination of the staining pattern.

Figs 5.5 G-H show the progressive change in the expression pattern of *EXORDIUM* in *asf1* over time. At all stages of development of *asf1*, *EXORDIUM* was expressed throughout the abnormal embryo proper and also throughout all of the inapproriately

divided cells of the suspensor. *EXORDIUM* was expressed throughout the wild type embryo, but was never detected in any cells of the suspensor (Figs. 5.5 E-F). As for *POLARIS*, expression of *EXORDIUM* in the suspensor-derived cells was switched on directly behind the margin between apparently normal suspensor cells and the ectopically divided cells. In this case, however, the gene was expressed in all cells in the mass of cells derived from this ectopic division, in continuum with its continued expression throughout the embryo proper.

5.5.3 *276-1* expression patterns in *asf1* embryos

Expression of *276-1-gus* was found to be at a very low level in both *asf1* and wild type suspensors. It was necessary to incubate *276-1* x *asf1* embryos for 3 days in X-Gluc at 37°C (Section 2.5) in order to detect the GUS localization pattern. After this time there was some structural disintegration and embryos had to be handled with extreme care and large numbers examined to find some with sufficient morphological preservation to elucidate the expression pattern.

276-1 in wild type and *asf1* suspensors was expressed as shown in Figs. 5.5 I-J. As in wild type embryos it was expressed throughout all cells of the *asf1* suspensor from the globular stage of embryogenesis onwards. As the suspensor cells started to divide, however, *276-1* was switched off in those cells resulting from these divisions, and was only expressed in the morphologically normal suspensor cells which persisted as a single file at the basal end.

5.5.4 Summary of *gus*-fusion marker expression patterns in *asf1*

In summary, the patterns of expression of these three *gus*-fusion were found not to be related to the lineage of the cells in *asf1*. *POLARIS*, normally expressed in the basal part of the embryo only, is expressed instead in the basal part of the abnormal region of cells derived from the suspensor. *EXORDIUM*, was expressed throughout the embryo proper, but also throughout the abnormal part of the suspensor. In direct apposition to the expression pattern of *EXORDIUM* in suspensor cells, *276-1* was expressed only in

Fig. 5.5
Expression of *gus*-fusion genes in *asf1* and wild type embryos

A. *POLARIS* in WT torpedo-stage embryo
B. *POLARIS* in WT late torpedo-stage embryo
C. *POLARIS* in *asf1* torpedo-stage embryo
D. *POLARIS* in *asf1* cotyledonary-stage embryo
E. *EXORDIUM* in WT globular-stage embryo
F. *EXORDIUM* in WT heart-stage embryo
G. *EXORDIUM* in *asf1* torpedo-stage embryo
H. *EXORDIUM* in *asf1* cotyledonary-stage embryo
I. *276-1* in *asf1* transition-stage embryo
J. *276-1* in WT transition-stage embryo

bars = approx. 50μm

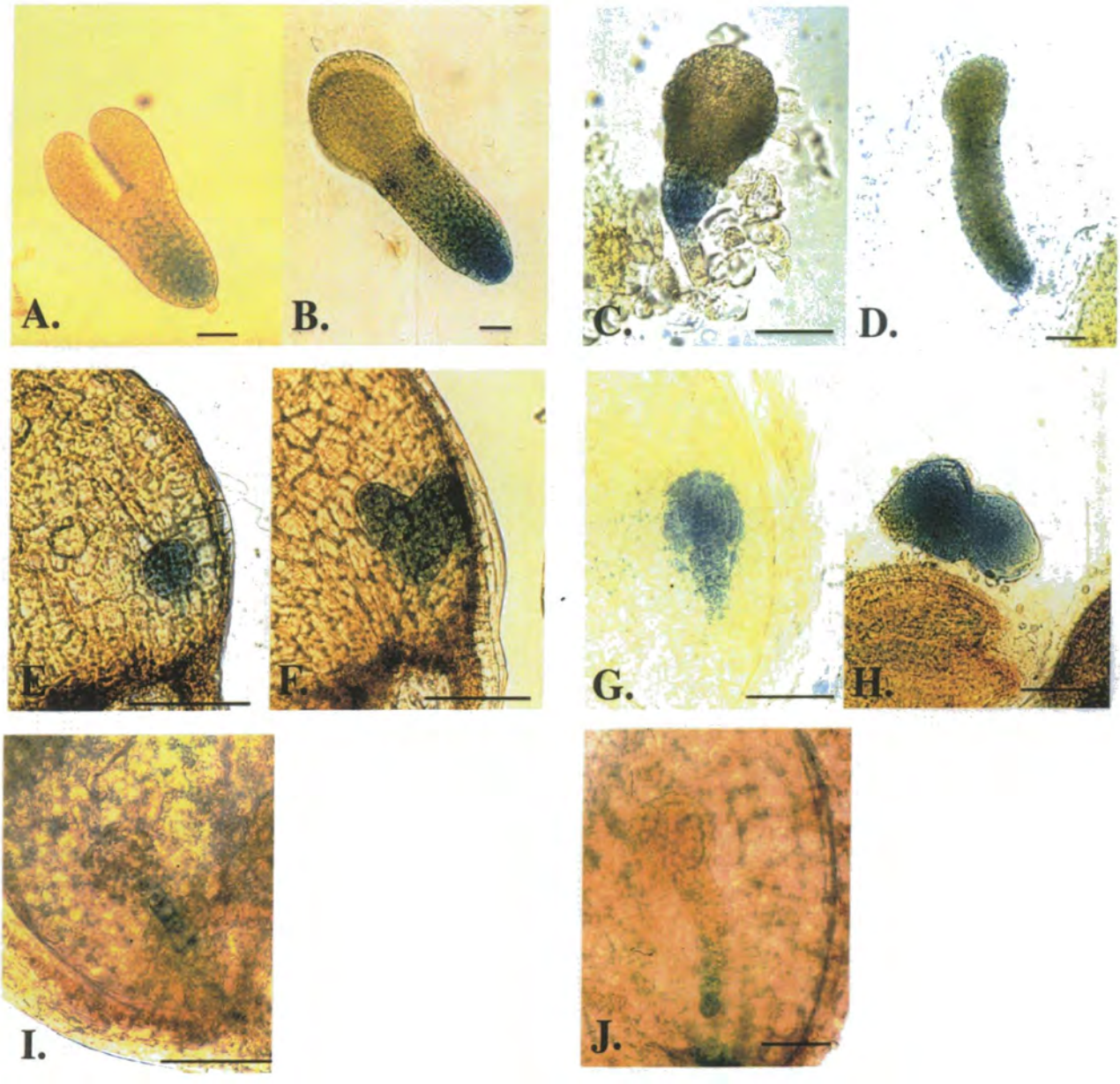

A.
B.
C.
D.
E.
F.
G.
H.
I.
J.

Fig. 5.7
Embryos from same F_1 silique of *asf1* x *hydra1* cross

A. wild type embryo, x250
B. *hydra1* embryo, x250
C. *asf1* embryo, x250
D. putative *asf1* x *hydra1* double mutant embryo, x250

Fig. 5.8
B. napus embryogenesis

A. 2 cell embryo
B. 4 cell embryo
C. globular embryo
D. torpedo-stage embryo

bar = approx. 25μm

Fig. 5.7

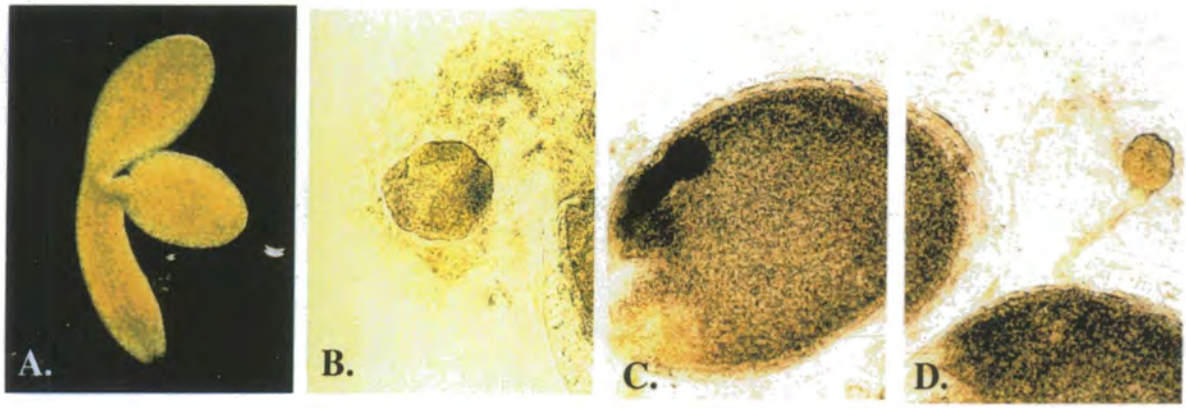
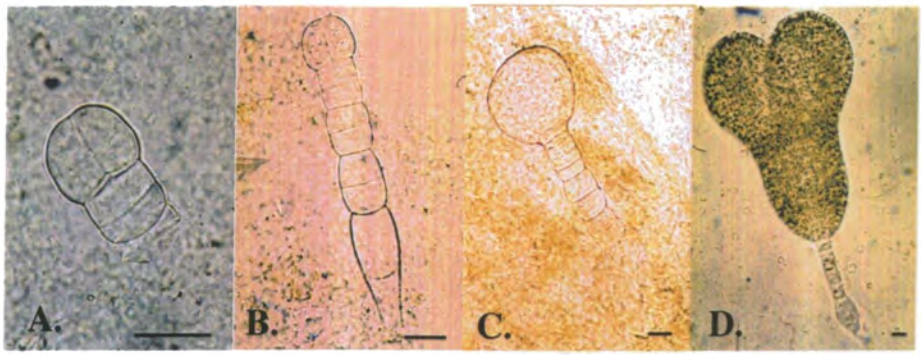


Fig. 5.8



silique (Fig 5.7 C). The effect of both mutations was, therefore, concluded to be additive in that aspects of both mutant phenotypes were evident.

5.7 Hormone and inhibitor experiments

As with the hormone and inhibitor experiments on *vch1* described in Section 4.9, the aim of the analogous experiments on *asf1* described in this section was to either 1) phenocopy the *asf1* phenotype in genetically wild type embryos by disrupting normal development-regulating signals; 2) rescue the mutant phenotype of *asf1* by suppying a hormone which is reduced in concentration by the mutation; or 3) to inhibit a signal which may be transmitted inappropriately as a result of the mutation.

In view of the embryonic nature of the *asf1* mutant, the approach used here was not to simply grow the parent plant on media supplemented with the supplied substance, but to supply it in the immediate environment of the developing embryos. This was considered more likely to have an effect since it reduces the problem of transporting the substance throughout the plant which may be inefficient and may also have a range of other effects on the whole plant. Also, it allows the solutions to be supplied at a concentration which would be unlikely to be achieved by transport from the roots. It has previously been demonstrated in *Brassica juncea* (Liu *et al.*, 1993) that the exogenous supply of polar auxin transport inhibitors, such as TIBA, can dramatically affect embryo morphogenesis in cultured zygotic embryos, in this case suppressing the establishment of bilateral symmetry, resulting in a fused ring of cotyledonary tissue around the top of the embryos. *Arabidopsis* zygotic embryos have not yet been successfully cultured *in vitro* and so an *in vivo* approach was employed here.

Each of a number of hormones or signalling inhibitors was directly injected into the locule (the "hollow" core) of young siliques, as described below. The following substances were injected: the hormones IAA and kinetin; the polar auxin transport inhibitor, TIBA; genistein, trifluoperizine, 8-Br-cGMP and okadaic acid; and

hydroxyurea, a cell division inhibitor. The action of each of these compounds is outlined below.

IAA is an auxin. As described above, auxins are known be be important in the establishment of embryonic bilateral symmety. It could be that, if the defect in *asf1* were due to a reduced concentration of auxin, then exogenous supply of it might rescue the phenotype. Alternatively, if what is important to normal morphogenesis is not the total concentration of auxin, but its spatially restricted distribution in the embryo, then exogenous application of high concentrations of auxin could potentially disrupt this gradient, resulting in phenocopy of *asf1* in genetically wild type embryos.

Kinetin is a cytokinin. Cytokinins have also been shown to be important in embryonic morphogenesis. Transgenic overexpression of cytokinin upregulators (Medford *et al.*, 1989) produces embryos which fail to elongate normally. Also, the *amp1* mutant (Chaudhury *et al.* 1993) is dwarfed, and this correlates with a six fold increase in cytokinin levels. Again, it was possible that exogenous application of this hormone could either phenocopy *asf1* in genetically wild type embryos, or rescue *asf1* mutant embryos.

TIBA (triiodobenzoic acid) is a polar auxin transport inhibitor (Topping & Lindsey, 1997). As described above, the phenotype of *asf1* bears some similarity to that of embryos treated with PAT inhibitors insofar as it exhibits a reduction in axial elongation and failure to establish bilateral symmetry. Exogenous application of TIBA to wild type locules then might phenocopy the *asf1* phenotype, if the defect in *asf1* did indeed involve disruption of a polar auxin gradient.

Genistein is an inhibitor of cAMP-dependent G-protein signalling. Diag. 4.1 presents a summary of G-protein signalling pathways (Barnes *et al.*, 1995; Bowler *et al.*, 1994). If such a signalling pathway is involved in the activation of *ASF1*, or is upregulated in the

asf1 mutant, then inhibition of this pathway by the exogenous application of genistein might phenocopy or rescue, respectively, *asf1*.

Trifluoperizine is an inhibitor of calcium-dependent G-protein signalling (Diag. 4.1; Barnes *et al.*, 1995; Bowler *et al.*, 1994). If such a signalling pathway is involved in the activation of *ASF1*, or is upregulated in the *asf1* mutant, then inhibition of this pathway by the exogenous application of genistein might phenocopy or rescue, respectively, *asf1*.

8-Br-cGMP is a membrane-soluble synthetic analogue of cAMP which has the same physiological effect,. cAMP is involved in cAMP-dependent G-protein signalling (Diag. 4.1; Barnes *et al.*, 1995; Bowler *et al.*, 1994). "Flooding" of the system with this analogue can cause upregulation of genes regulated by this pathway and can also downregulate calcium-dependent G-protein signalling, as there is "crosstalk" between the two branches of the G-protein signalling pathway. Exogenous application of this compound could mediate phenocopy or rescue of *asf1*, depending on the signalling pathways involved in regulation of its expression or mediating its effects.

Okadaic acid is an inhibitor of serine-threonine protein phosphatases (Cohen *et al.*, 1989). Protein phosphatases are important in signal transduction pathways in plants (Scutt *et al.*, 1993) and again it was possible that this pathway might be involved in activation, or mediation of the effects of the *asf1* gene. The objective of this experiment was to supply okadaic acid to the locule of wild type siliques to investigate whether blocking of this signalling pathway could phenocopy *asf1*.

Each compound was injected into the locule of siliques at approximately 2, 4 and 6 days after siliques started to develop, at concentrations of 10μM and 25μM, along with a physiologically inert tracking dye, phenosafranin, at 0.1% w/v. Solutions were injected with very fine capillary needles produced by heating and stretching glass pipettes, as described in Section 2.1.3. Siliques were dissected 3 days after injection,

and the embryos mounted in 0.5M KOH and examined under a compound microcope. A trial run of the experiment was performed first as a control to eliminate the possibility that mutant embryos might be produced by the mechanical damage caused by the glass needle with which the solutions were injected, or by the phenosafranin tracking dye. The trial demonstrated that over 70% of the more than 150 examined arrested and later aborted. This was presumably as a result of mechanical damage. No abnormal phenotypes *per se* were observed. The siliques themselves stopped growing in approximately 80% of those injected and in many cases shrivelled and became necrotic within 2-3 days of injection. To eliminate the possibility that the phenosafranin was causing these problems the injections were repeated with sterile water alone, however, the problem persisted. It was concluded that mechanical damage of the silique at the site of injection elicited a wound response which caused the embryos to abort.

In an attempt to overcome these technical limitations imposed by the small size of the *Arabidopsis* silique, it was decided to carry out the experiments in *Brassica napus*, a species with a very similar embryonic developmental pattern to that of *Arabidopsis* (Fig. 5.8), but on a larger scale, the siliques growing to up to 5-6cm in length as compared to 0.5-1cm for *Arabidopsis,* with the embryos also being significantly larger. Obviously, in the absence of the *asf1* mutation in the *Brassica napus* embryos, the ability of these compounds to rescue the mutant phenotype could not be tested. The aim was simply to phenocopy *asf1* by perturbing the development of early wild type embryos.

The trial run with this species was more successful, with good detection of the tracking dye in the locule and relatively little mechanical damage. Over 80% of siliques continued to grow and the embryos therein to develop.

In all of the siliques injected and examined there was no evidence of phenotypic perturbation of the embryos therein. A developmental series of *Brassica napus*

embryos is presented in Fig. 5.8. Although a number of embryos did abort, as in the trial run, this was not at any greater frequency than that caused by the physical process of injection (approx. 20%). No abnormalities in embryo morphology were observed.

5.8 Summary

This chapter has described a phenotypic analysis of the *asf1* mutant. A developmental profile of the mutant is presented. The terminal phenotype is of an enlarged globular embryo and a mass of suspensor-derived cells. Various lines of evidence suggest that the inappropriately divided suspensor cells take on some embryo proper characteristics.

Chapter 6 will present the results of the preliminary molecular analysis of the *asf1*. Progress towards the genetic mapping of the chromosomal location of the *ASF1* gene is reported. The results presented in this chapter will be discussed in Chapter 7.

6.0 - Mapping and Molecular Characterisation

6.1 Objectives

In this chapter results will be presented of the molecular characterisation of both lines with regard to T-DNA locus number, copy number and linkage with the mutant phenotypes, as ascertained by Southern analysis and segregation data. It will also describe the strategy for the mapping of the chromosomal locations of the *vch1* and *asf1* loci.

6.2 T-DNA copy number

Germination and growth of both *vch1* and *asf1* lines on kanamycin-containing selective medium suggested the presence of multiple T-DNA copies in the genomes of both mutant lines (see Section 3.4).

To determine whether a single T-DNA copy co-segregated with, and was therefore likely to be causative of, each of the two mutant phenotypes, outcrosses of both lines with untransformed C24 plants were performed.

The purpose of this was to produce subsequent generations of plants that were heterozygous for the mutant phenotype, but in which T-DNA copies had segregated apart such that copy number in some heterozygous lines was reduced. The progeny of the F_1 individuals produced from such crosses was screened for the presence of the *vch1* phenotype and those lines still exhibiting the phenotype were plated on kanamycin selection to determine numbers of individuals still carrying the *nptII* kanamycin resistance gene. Since there was still a high level of kanamycin resistance in this generation, outcrossing was repeated and the next generation of seedlings was screened in the same way. For *vch1*, this was performed through a total of four generations of outcrossing until the kanamycin segregation ratios suggested the presence of just two T-DNAs in the genome. Following one further generation of outcrossing, Southern blot analysis, using the *gusA* gene as a probe for the presence of

T-DNA, was performed. Southern blot analysis was carried out on *asf1* plants following three generations of outcrossing and two of selfing.

6.3 Southern analysis of *vch1*

Southern analysis was carried out to determine the copy number of integrated T-DNA in the *vch1* line.

Rosette leaves of individual phenotypically wild type plants grown from the seed of a *vch1* heterozygous parent were snap-frozen in liquid nitrogen and stored at -70°C. Seeds were collected from these individual plants, germinated and scored for the presence of the *vch1* phenotype in the next generation. Genomic DNA was extracted (Section 2.7.2) from the leaves of one individual which proved to be heterozgous for the *vch1* mutation; one which did not; and one untransformed C24 plant. Each DNA sample was separately digested with 4 restriction enzymes: *EcoRI, BamHI, HindIII and PstI*. Restriction enzymes were chosen which would cleave once within the inserted T-DNA. It is expected that wherever each enzyme cleaves in the plant DNA outside the T-DNA, a specific single fragment will be produced with each enzyme, containing known sequences within the T-DNA. The size of these so-called *border fragments* was determined by Southern blot analysis using the *gusA* gene as a probe.

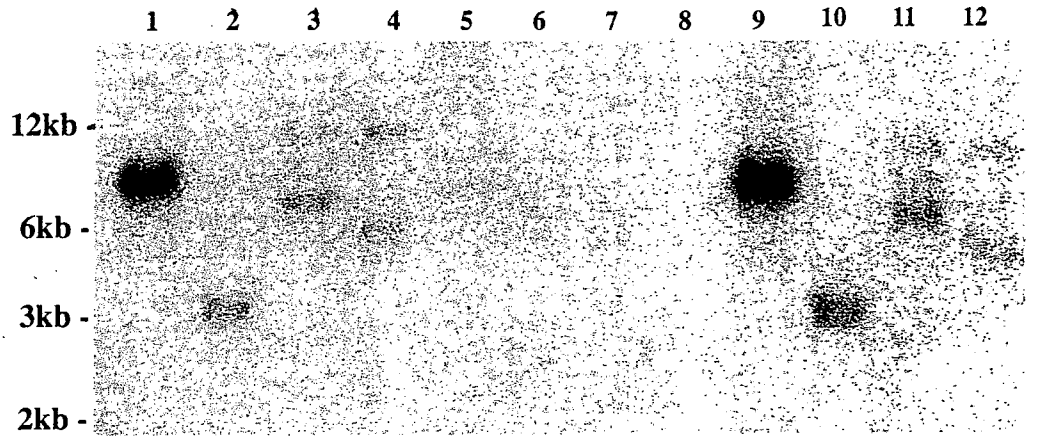
1 μg of DNA was digested with the appropriate restriction enzyme, was separated by electrophoresis as described in Section 2.8 and was transferred by Southern blotting to a nylon membrane, as described in Section 2.13.2. The presence of the *gusA* gene within the digested DNA on the membrane was detected by hybridisation of a radiolabelled *gusA* probe (Section 2.13.4), and the size of the bands to which this hybridised was determined by autoradiography (Section 2.13.5).

The results of this analysis are presented in Fig. 6.1. The single hybridising bands generated by digestion with *EcoRI* and *HindIII* respectively (Lanes 1 and 2) indicate that there is a single copy of the *gusA* gene present in the *vch1* heterozygous plants analysed. The presence of more than one band in samples digested with *PstI* and

Fig. 6.1 - Southern analysis of *vch1*

Lanes 1-4 = DNA from a *vch1* heterozygous plant
Lanes 5-8 = DNA from a C24 wild type plant
Lanes 9-12 = DNA from a wild type segregant in the progeny of a selfed *vch1* heterozygote

DNA samples (1 µg) in lanes 1,5 and 9 were digested with *EcoRI*
DNA samples (1 µg) in lanes 2,6 and 10 were digested with *HindIII*
DNA samples (1 µg) in lanes 3,7 and 11 were digested with *PstI*
DNA samples (1 µg) in lanes 4,8 and 12 were digested with *BamHI*



BamHI respectively (Lanes 3 and 4) and the non-stoichiometric relationship between the instensity of the bands is suggestive of a partial digestion of the DNA samples. This is consistent with the view that a single T-DNA copy is present. No hybridisation was observed with DNA extracted from the untransformed control plants (Lanes 5-8). In DNA samples extracted from wild-type plants which had been demonstrated to have segregated within the selfed *vch1* progeny, an identical hybridization signal was seen for each of the treatments with the respective restriction enzymes (Lanes 9-12). It was concluded that the *vch1* mutation did not co-segregate with a *gusA* gene. This analysis was repeated a further three times, using different individual heterozygous and wild-type plants, and an identical result was obtained each time (data not shown). It was therefore concluded that the *vch1* mutation was not caused by an insertion of a *gusA* gene from the T-DNA.

6.4 Southern analysis of *asf1*

Southern analysis was carried out to determine the copy number of integrated T-DNA in the *asf1* line.

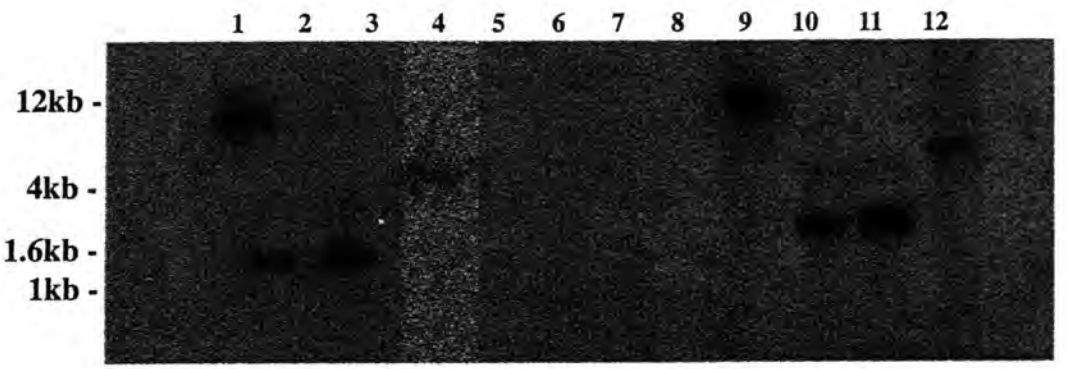
Individual segregants in the progeny of an *asf1* heterozygote were examined for the presence of *asf1* mutant embryos in their siliques. Genomic DNA was extracted (Section 2.7.2) from the rosette leaves of one *asf1* heterozygous plant, one segregating wild type plant and one untransformed C24 plant. Each DNA sample was separately digested with 4 restriction enzymes: *EcoRI, BamHI, HindIII and PstI*. Restriction enzymes were chosen which would cleave once within the inserted T-DNA. It is expected that wherever each enzyme cleaves in the plant DNA outside the T-DNA, a specific single fragment will be produced with each enzyme, containing known sequences within the T-DNA. The size of these so-called *border fragments* was determined by Southern blot analysis using the *gusA* gene as a probe.

1 μg of DNA was digested with the appropriate restriction enzyme, was separated by electrophoresis as described in Section 2.8 and was transferred by Southern blotting to

Fig. 6.2 - Southern analysis of *asf1*

Lanes 1-4 = DNA (1 μg) from an *asf1* heterozygous plant
Lanes 5-8 = DNA (1 μg) from a C24 wild type plant
Lanes 9-12 = DNA (1 μg) from a wild type segregant in the progeny of a selfed *asf1* heterozygote

DNA samples (1 μg) in lanes 1,5 and 9 were digested with *EcoRI*
DNA samples (1 μg) in lanes 2,6 and 10 were digested with *HindIII*
DNA samples (1 μg) in lanes 3,7 and 11 were digested with *PstI*
DNA samples (1 μg) in lanes 4,8 and 12 were digested with *BamHI*



a nylon membrane, as described in Section 2.13.2. The presence of the *gusA* gene within the digested DNA on the membrane was detected by hybridisation of a radiolabelled *gusA* probe (Section 2.13.4), and the size of the bands to which this hybridised was determined by autoradiography (Section 2.13.5).

The results of this analysis are presented in Fig. 6.1. The single hybridising bands generated by digestion each of the 4 restriction enzymes, *EcoRI*, *HindIII*, *PstI* and *BamHI* (Lanes 1-4) indicate that there is a single copy of the *gusA* gene present in the *asf1* heterozygous plants analysed. No hybridisation was observed with DNA extracted from the untransformed control plants (Lanes 5-8). In DNA samples extracted from wild-type plants which had been demonstrated to have segregated within the selfed *asf1* progeny, an identical hybridization signal was seen for each of the treatments with the respective restriction enzymes (Lanes 9-12). It was concluded that the *asf1* mutation did not co-segregate with a *gusA* gene. This analysis was repeated a further four times, using different individual heterozygous and wild-type plants, and an identical result was obtained each time (data not shown). It was therefore concluded that the *asf1* mutation was not caused by an insertion of a *gusA* gene from the T-DNA.

6.5 Genetic mapping of the *vch1* and *asf1* loci

It was important to map the chromosomal location of the *vch1* and *asf1* loci, firstly to confirm the identification of novel genetic loci; secondly, as a step towards the cloning of both genes by chromosome walking (Leyser & Chang, 1996), or targeted transposon tagging (Coupland, 1992).

Genetic mapping of the mutant genes was carried out by the introduction of scorable phenotypic markers of known genetic map position into the mutant backgrounds. The distance of the mutant genes from a given marker gene could be calculated from the frequency of co-segregation of the mutant and marker phenotype in the progeny of plants heterozygous for each of the genes.

This linkage analysis was performed using the DP23, DP24 and DP28 multiple tester lines (Franzmann *et al.*, 1995) obtained from the Nottingham Arabidopsis Seed Centre. Each of these lines exhibits 4 or 5 specific phenotypic traits associated with mutation in genes of known genetic map location. These three lines were chosen because the marker genes represent loci on each of the arms of the five *Arabidopsis* chromosomes. The marker genes and their phenotypes are listed in Appendix 3.

Crossing was carried out as described in Section 2.1. Crosses were made between each of the three multiple tester lines and plants heterozygous for each of the two mutations. In the case of the *asf1* mutant, heterozgous plants were easily identified due to the embryonic nature of the mutant phenotype; siliques were removed and scored for the presence of 25% *asf1* embryos.

Because *vch1* does not exhibit an embryonic phenotype and of the difficulty of identifying heterozygous seedlings on the basis of kanamycin selection, it was necessary to use a number of the progeny of a known heterozygous parent for crosses. Of 75% of the progeny of such a parent which did not exhibit the mutant phenotype, it was to be expected that two-thirds would be heterozgous for the mutation. It was necessary, therefore, to use a sufficient number of these phenotypically wild type individuals to ensure that some of the progeny of the crosses would be heterozygous for the *vch1* mutation.

Seeds collected from both sets of crosses were germinated and grown to maturity. In the case of *asf1* the presence of the mutant phenotype (F_2 embryos) could be checked for in the siliques of this F_1 generation of plants. In the case of *vch1*, it was necessary to check the seedlings of the F_2 generation for the presence of the mutant phenotype. All plants were grown under standard greenhouse conditions. Seeds from the F_1 and F_2 generations were collected in Aracon tubes (Beta-Tech, Belgium).

Over 300 F_2 siblings were analysed for each cross, being scored for the presence of each of the marker phenotypes. In the case of *asf1*, siliques were removed and scored for the presence of 25% mutant embryos. In the case of *vch1*, seeds were harvested from this F_2 generation and germinated to score for the presence of the *vch1* phenotype in the seedlings of the F_3 generation.

Linkage analysis on this data was performed using MapMaker/Exp. 3.0 (Lander *et al.*, 1997; Lincoln *et al.*, 1992).

Results of this analysis are not yet available.

6.6 Summary

This chapter has presented the results of the preliminary molecular characterisation of the *vch1* and *asf1* mutants. It is concluded that neither mutation was caused by T-DNA integration. Genetic mapping of the loci of both genes has been initiated as a first step towards cloning the disrupted genes. These results will be discussed in Chapter 7.

DISCUSSION

7.0 - Discussion

7.1 Objectives

As stated in Chapter 1, the objective of this thesis was to identify and characterise morphological mutants of *Arabidopsis* as a means by which to study aspects of the genetic control of morphogenesis. Through the characterisation of such mutants we may eludidate the normal role of the mutated gene and thus gain an insight into developmental control mechanisms. This thesis has described the characterisation of two such mutants.

One, *vch1*, is defective in elongation in all organs and under all growth conditions examined. The second, *asf1*, is an embryonic-defective mutant in which the embryo proper arrests and suspensor cells divide inappropriately and take on embryo proper-like characteristics.

The results of the genetic, phenotypic and preliminary molecular analyses of these two mutants have been described in the preceding chapters and will be discussed here. Models for the possible nature of their genetic defects will be presented.

7.2 The *vch1* mutant is defective in axial cell elongation

Whilst cell expansion is quite well understood at the level of the mechanics of turgor-driven wall yielding (Cosgrove, 1997), relatively little is known about the genes which control the establishment of cell shape by regulating directional cell expansion. We are beginning to gain insights into such genetic mechanisms by the analysis of mutants defective in cell shape, such as *fass* (Torres-Ruiz & Jürgens, 1994; Fisher *et al.*, 1996) and *hydra1* (Topping *et al.*, 1997).

The *vch1* mutant had been identified in a previous screen of a T3 generation of *pΔgusBin19* transformants. *vch1* appeared as a small, stunted plant with a very short root system, the cotyledons were small and rounded and the hypocotyl shortened. It was thought that this mutant might prove to be defective in embryonic morphogenesis.

The screen of early seedlings was designed to detect embryonic patterning/morphogenesis mutants since such defects are likely to be reflected in an aberrant seedling phenotype. This approach has advantages over screening for mutants during embryogenesis. First, it is technically easier and second, although some embryonic phenotypes will be due to defective morphogenesis, many are expected to be due to more general "housekeeping gene" defects, often being embryo lethal. The *vch1* mutant, however, proved to not be an embryonic mutant (Section 4.4), with the aberrant phenotype not becoming apparent until approximately 4-6 days post-germination (Section 4.4.1). It was considered an interesting phenotype nonetheless since it exhibited a uniquely severe retardation of axial elongation in all major organs. Analysis of the *vch1* phenotype might yield valuable insights into global mechanisms regulating axial cell expansion.

Such a mutant phenotype has not been previously described. Mutants have been described which fail to elongate a specific organ or group of organs. These include several classes of dwarf mutants (Feldmann *et al.*, 1989; Takahashi *et al.*, 1995) and the *cabbage* (*cbb*) mutants of Kauschmann *et al.*. (1996), some of which can be rescued by the exogenous application of brassinosteroids. Both of these groups of mutants exhibit a failure of hypocotyl and stem elongation. An auxin signalling mutant (Wilson *et al.*, 1990) exhibits a reduced hypocotyl and roots. Ethylene response mutants, such as *ctr-1* (Kieber *et al.*, 1993) also exhibit reduced hypocotyl and root elongation. There are condition-specific mutants which, for example, fail to respond normally to growth in the dark, such as *det/cop* (Deng, 1994; Chory *et al.*, 1992). In all of the above classes of dwarfs, some organs, for example flowers, are normal. Some mutants show a highly organ-specific reduction in cell expansion, such as *pfl* (*pointed first leaves*; van Lijsebettens *et al*, 1991; 1994).

The cell elongation defect in *vch1* is ubiquitous and has a drastic effect on the shape of all organs. *stunted plant1* (Baskin *et al.*, 1995) exhibits a reduced rate of growth, growing as dwarfs with all organs reduced in size but not obviously changed in shape. The *tiny* mutant (Wilson *et al.*, 1996) has very small cells and organs, especially in the

hypocotyl. Leaf epidermal cells are also shorter than in wild type, as are cells of floral organs. However, in overall morphology of these organs, with the exception of the hypocotyl, they are much less axially compressed than those of *vch1*.

vch1 segregated out at a ratio of 1:3 mutant:wild type in subsequent generations (Section 3.2), suggesting that it represented a homozygous recessive loss-of-function mutation at a single genetic locus.

7.3 The *VCH1* gene is not T-DNA tagged

Although T-DNA was present in the genome of the first generation of *vch1* examined, it was later proven that the mutation was not linked to the presence of T-DNA (Section 6.3).

Because of the presence of the promoterless *gusA* reporter gene within the T-DNA it was possible that GUS enzyme could be produced in each line. When embryos of *asf1* and seedlings and embryos of *vch1* were subjected to histochemical analysis for GUS, however, no activity was detected (Section 3.5) suggesting no promoter-GUS fusion had occurred in either case. This could potentially be due to the integration of the T-DNA in a reverse orientation relative to a gene promoter, or in a non-coding region of the genome. It may reflect integration into transcriptionally silent regions or within genes expressed only under certain environmental conditions. Alternatively it might reflect structural re-arrangements within the T-DNA which have eliminated its coding potential: such rearrangements are not uncommon (Castle *et al.*, 1993).

Whether the *vch1* mutation does, in fact, represent partial T-DNA construct integration is unknown. It could be that small fragments of the construct are causative of the mutations and these were not detected by the probe used. Such problems associated with T-DNA tagging are not unknown. Chromosomal rearrangements are known to occur following transformation (Castle *et al.*, 1993) and complex and partial inserts have previously been found. Southern analysis of the *dwarf* mutant, for example,

revealed that the causative insert was a concatamer of four to six T-DNAs in direct and inverted repeats (Feldmann *et al.*, 1989).

Because the gene was not T-DNA tagged it was necessary to adopt an alternative stategy towards cloning the *vch1* gene. Progress towards mapping of the chromosomal location of the *vch1* gene has been made (Section 6.5) as a first step towards cloning the gene (Section 1.8.1).

7.4 The *vch1* mutation is at a novel genetic locus

vch1 looked unlike any mutants previously described in the literature and this suggested that a novel genetic locus had been identified. Recently another allele of the *vch1* gene, named *vch1-2*, has been identified in a screen of an EMS mutagenised population for seedling-defective mutants carried out in this laboratory (P. Chilley & K. Lindsey, unpublished data). The fact that the alleles are phenotypically indistinguishable suggests that *vch1* represents a single gene null mutation.

7.5 *vch1* is defective in morphogenesis but shows normal cellular patterning

The mutant phenotype, initially identified at the seedling stage, was examined at a wide range of developmental stages and at a hierarchy of levels from whole plant to ultrastructural. The broad conclusions were that *vch1* is not an embryo-defective mutant, and that the mutant phenotype only becomes apparent from approximately 5 days post-germination. The defect is apparent in all organs examined. Whilst patterning and cytodifferentiation appear normal in *vch1*, overall morphology of the plant is abnormal due to a failure in cellular, and therefore organ, elongation. Roots and hypocotyls of *vch1* grow much more slowly than those of wild type seedlings (Section 4.2). The ratio of leaf lamina length:width was significantly reduced (Section 4.2).

Histological sectioning of roots and hypocotyls and observation of the surface morphology of all organs by SEM demonstrated that, whilst *vch1* is morphologically abnormal at the gross phenotypic level, it appears that all cells are present in their

correct spatial context. However, the shape of the cells is abnormal. Whilst they remain fairly regular in shape, insofar they are consistently longer in their apical-basal than transverse axis, and the ratio of cell length to width does not exhibit abnormal variation, in absolute terms this ratio is reduced compared to that in the wild type. This is exemplified by comparing cell measurements of the cortical and epidermal cells of roots in the elongation zone. Both types of cell are very significantly reduced in length, to of the order of approximately 50% of the length of their wild type equivalents (Section 4.4.3). They are also slightly increased in width to the order of approximately 1.2-1.3 times the width of their wild type equivalents (Section 4.4.3).

The mean number of root epidermal cells per section in *vch1* roots (23.15±0.88SE) was significantly greater than in wild type roots (16.35±0.31SE) as was the mean number of cortical cells per section (8.22±0.46SE) in wild type roots (9.54±0.57SE in *vch1*). This could imply a loss of control over radial patterning. Radial control of cell number could be altered in each cell layer. Alternatively, excessive radial swelling of specific layers might indirectly stimulate cells in further external layers to expand and/or divide. This could be tested by the use of transposon excision markers to study cell lineages within the developing root (*e.g.* Dolan *et al.*, 1994; Scheres *et al.*, 1994). However, it is also possible that the reduced length of cells (Section 4.4.3) and the increased total cross sectional area of roots and of individual cells (Section 4.4.1; 4.4.2), led to an increase in the probability that a given transverse section will intersect the ends of longitudinally juxtaposed cells (Fig 4.7). That radial patterning *per se* is not disrupted is supported by the observation that in sections immediately behind the root tip the number of epidermal and cortical cells per section, and cell sizes, are similar to those in wild type roots.

The diameter of cortical and epidermal cells was increased in *vch1* roots. This phenomenon of radial swelling has been previously described in mutants with altered root morphogenesis, including *short-root, cobra, lions tail* and *sabre* (Benfey *et al.*, 1993; Benfey & Schiefelbein, 1994). In each of these, radial swelling was primarily in a specific cell layer, suggesting a lack of control of cellular morphogenesis in that

layer. They suggest that this is due to some lack of control over the interplay between cytoskeleton and cell wall material deposition, although no information on the specific nature of the defects at a mechanistic level are yet available.

Radial swelling of cells appears to be more ubiquitous in *vch1* than in the above mutants, being not only in the root, but also in the hypocotyl and in aerial organs (Section 4.2). One might ask the following questions: First, is the primary defect in *vch1* is one of cells not receiving a signal determining directionality of expansion, perhaps in the form of an axial gradient of a hormone or other signalling molecule? Alternatively, we can ask whether the defect is a failure to mechanically respond to such a signal, for example by cytoskeletal organization or assembly/yielding of the cell wall. The former scenario implies control of morphogenesis at a supercellular level (Section 1.6.5; Kaplan, 1992; Jacobs, 1997). Possible mechanisms for determination and establishment of the axial direction of elongation growth will be discussed in Section 7.9-7.14.

Although the majority of cells fail to elongate in *vch1*, there is a sub-set of cells which elongate normally. These are pollen tubes, root hairs, trichomes and stigmatic papillae (Section 4.4.1). What differentiates these cell types from all others is that they grow by *tip growth*, as opposed to the *diffuse growth* of all the other cell types. Tip growth is by deposition of new wall material in a spatially restricted manner at the leading tip of an outgrowing cell (McClinton & Sung, 1997; Wymer *et al.*, 1997). Deposition of material in diffuse growing cells is more uniform around the periphery of the cell. This observation indicates that *VCH1* is required for normal elongation in diffuse growing, but not in tip growing, cells.

Introduction of *gus*-fusion marker lines into *vch1* confirmed the anatomical observations regarding its normal cellular patterning (Section 4.7). Patterns of expression of both *POLARIS* and *EXORDIUM* were the same in wild type and in *vch1* embryos and seedlings up to 5 days post-germination, supporting the observation that the *vch1* defect is not apparent at these stages. Once the mutant phenotype does

become apparent, however, the expression patterns are each more spatially restricted than in wild type plants (Section 4.7; Fig. 4.11). Both of these marker genes are expressed in root apices and, supporting anatomical evidence, these apical regions are shorter in length in *vch1* than in wild type roots. It can be concluded that there is no perturbation in the signals which determine the expression patterns of these marker genes.

VS-1 is similarly expressed in the correct spatial pattern in *vch1* roots. *VS-1* expression is in structurally differentiated cells of the vascular system. At the point nearest the tip its expression is restricted to the endodermis but further back is in all living cells of the vascular system (Muskett, 1998). Expression of *VS-1* in *hydra1* (Section 1.1.2; Topping *et al.*, 1997), was that in *hydra1*, which ceases root meristematic activity after a period of initial root growth, expression of *VS-1* was in vascular cells immediately behind the root tip, unlike in the wild type root where it is expressed only from approximately 650μm behind the tip. It was argued that this reflected the fact that in *hydra1* roots, cells of the vascular tissues differentiate very close behind the tip, since the root has ceased meristematic and elongation activity. This was not the case in *vch1*, which did not express *VS-1* immediately behind the root tip. The gene was, in fact, expressed from approximately 400-450μm back from the tip. Considering the shorter length of cells in *vch1*, this is at a proportionately similar position to that in wild type roots (650-700μm).

7.6 Different aspects of cell differentiation in the root are regulated independently

Another observation on *vch1* roots was that root hairs initiated much closer behind the root tip than on wild type roots. The distance back from the root tip of the first root hair was very much reduced in *vch1* roots (*vch1*=115.05μm±3.75SE; WT=303.95μm±14.70SE; $p<0.01$).

However, whilst root hair formation occurs much closer behind the root tip in *vch1* than in wild type roots (*vch1* = 115.05μm ± 16.79; WT = 303.95μm ± 65.73; $p<0.001$),

the distance at which the differentiated vascular tissue marker *VS-1* expression is first apparent is not so drastically reduced (WT≈650-700μm; *vch1*≈400-450μm) and considering the reduction of cell lengths in *vch1* is approximately the same number of cells back from the tip.

In the *vch1* root, the distance between epidermal cells structurally differentiating to produce root hairs and vascular tissues switching on the expression of the *VS-1* gene was approximately similar (300-350μm) to that in WT roots. Considering the reduction in cell lengths in *vch1*, this difference is greater in *vch1* in terms of number of cells.

This observation suggests that hair formation and the switching on of *VS-1* expression are not co-ordinately controlled, since they can be uncoupled in mutants such as *vch1*. In *hydra1*, both hair formation and *VS-1* expression were observed to occur close behind the root tip. This might suggest the existence of a gradient of "*VS-1* expression inhibitor" which is maintained in *vch1*, but which is reduced or abolished in *hydra1*, or might imply that there is usually coordinate control of hair formation and *VS-1* expression, and that this is maintained in *hydra1*, but not in *vch1*. Alternatively, it might be that the *VCH1* gene product is more important in maintaining trichoblasts and atricoblasts in their undifferentiated state than in repressing *VS-1* expression in vascular cells or in vascular cell differentiation in general.

The appearance of root hairs represents a differentiation of epidermal cells (Schiefelbein & Somerville, 1990; Dolan *et al.*, 1994; Dolan & Roberts, 1995). The root is traditionally conceptualized as comprising discrete zones of (1) a meristem, in which new cells are produced by cell division, (2) an elongation zone, in which cells elongate but remain morphologically and functionally simple, and (3) a differentiation zone, in which cells become structurally and functionally specialized (Section 1.5.4 and refs. therein). Cells in wild type roots enter a differentiation phase after they have elongated. How cells make the decision to differentiate is not well understood. There are two broad hypotheses. Firstly, cells could switch on genes involved in differentiation in a cell-autonomous manner once they have attained a certain size.

Secondly, differentiation may be in response to intercellular signalling. This certainly seems to be the case in the differentiation of trichoblasts in the formation of root hairs. The ethylene precuror ACC acts as a positive regulator of trichoblast differentiation (Tanimoto *et al.*, 1995) whilst the *CTR1* gene has been shown to be a negative regulator, through inhibition of the ethylene response signal cascade (Kieber *et al.*, 1993). It has been shown that the ACC synthase gene is highly expressed at the root tip. The point at which trichoblasts differentiate to produce root hairs then might represent a threshold at which ACC inactivates *CTR1*, thus allowing root hair differentiation (Tanimoto *et al.*, 1995). Vascular cell differentiation might involve a similar mechanism.

7.7 *vch1* ~~follows~~ has a normal life history

The reduced survival to maturity (defined as 6 weeks post-germination) of *vch1* (Section 4.4.3) may be simply due to the small, abnormal roots being unable to supply sufficient water and/or nutrients from the soil, or perhaps that aspects of transport, metabolism or intercellular signalling are hindered either as a direct or indirect effect of the genetic defect. This could be investigated by dye-loading experiments.

The height of the inflorescence of *vch1* is more variable than that of wild type plants in that the total height of the plant ranges from approximately 2 to 9 cm (Section 4.3.4; Fig. 4.4). This 4.5-fold difference is much greater than the maximum of approximately 0.5 fold variability in the height of wild type plants under the growth conditions used. Although *vch1* is morphologically very abnormal at a gross phenotypic level, at the cellular level there is normal patterning, despite the reduction in cell lengths. This variability in height may be not due to disrupted morphogenesis *per se*, but may be due to limiting effects of a reduction in water and nutrient uptake or transport, or disrupted intercellular communication (as discussed in Section 7.10; 7.14). Water and nutrients should not be limiting to wild type plants under standard greenhouse conditions, but *vch1* may be much closer to a "threshold level of survival" than wild type plants under these conditions because of their reduced root system, and any small reduction in local nutrient or water availability may have more drastic consequences, further retarding the

growth of the plant. Some mutants, such as *hydra1* and *emb30* exhibit inter-sibling variability in the severity of the mutant phenotype (Topping *et al.*, 1997; Mayer *et al.*, 1993). This may be due either to the mutation not representing a complete loss of function of the disrupted gene, or may be simply a product of chance events in early cell division and expansion as a result of the reduction in predictability of extent and planes of cell expansion in such mutants. There is little evidence of inter-sibling variability in other aspect of the phenotype of *vch1*. Cell elongation appears to be reduced to a similar degree in comparable organs, other than the inflorescence axis of all individuals examined and, at the organ level, root and hypocotyl growth, are quite uniformly retarded: the seedling phenotype is quite invariable (Fig. 4.3 H). Inflorescence elongation, on the other hand, is quite variable, as mentioned above. The reason for this variability in the height of the inflorescence unclear, although one hypothesis might be that organs vary in their sensitivity to the lack of the *VCH* protein, or its effects on hormone levels and that small fluctuations in hormone levels have a more extreme effect on elongation of the inflorescence axis. This hypothesis could be tested by assaying for the levels of different hormones, either directly, or indirectly through the analysis of the activity of hormone-inducible genes, such as *SAUR-AC1*, expression of which correlates with auxin levels (Timpte *et al.*, 1992).

Some embryos do develop within the siliques of *vch1* homozygotes, however, most (>95%) arrest (Section 4.3.1) and only rarely is any mature seed released from the plant. That all embryos of *vch1* heterozygous parents complete embryogenesis normally, suggests that the arrest of embryos in *vch1* homozygotes must represent a maternal effect. Since no abnormal phenotype was ever observed in the embryos of *vch1* plants (Section 4.4.1), it is concluded that embryos arrest without developmental perturbation *per se*. *vch1* siliques exhibit a gross failure in elongation, being typically less than 10% the length of wild type siliques. This may be directly a result of the failure of ovule fertilization: this normally triggers an increase in level of gibberellin and subsequently in auxin in the ovary (Gillaspy *et al.*, 1993). The accumulation of these hormones correlates with fruit set and fruit body elongation: exogenous application has been found to be sufficient to induce these developmental

events. The act of fertilization, therefore, stimulates silique elongation and where fertilization fails, as in the case of male sterile mutants (*e.g.* Worrall *et al.*, 1992), the style remains short. *vch1* can be considered largely sterile, since, although pollen is apparently viable in outcrossing, fertilization is probably mechanically constrained in *vch1* flowers. Alternatively, it is possible that seeds arrest due to an insufficient supply of nutrients from the maternal tissues inhibiting either embryogenesis directly or ovule development. Finally, seed expansion may be mechanically inhibited by the constraining silique walls.

The fact that pollen from *vch1* plants is able to fertilise wild type flowers (Section 4.3.1), and *vch1* and wild type pollen from a *vch1* heterozygote is able to do so at the same frequency argues against male sterility *per se* of *vch1*. Interestingly, when the stigmatic surface of *vch1* flowers was examined by SEM, it appeared normal in both structure and size, with normal stigmatic papillae (Fig. 4.5 G-H). These observations lend support to the hypothesis that the low fertility of *vch1* is due to either mechanical constraint on embryo or ovule development, or to mechanistic inhibition of fertilisation. The latter may arise since the anthers are short and so more distant from the stigma surface than in wild type flowers. The stigma of *vch1* flowers is exposed, due to the shortness of the sepals, petals and anthers (Section 4.2; Fig. 4.3 E): pollen tube growth may be inhibited as a result of this disturbing the microenvironmental conditions necessary for pollen germination. Release of pollen in *vch1* might be reduced due to constrained anther dehiscence (Park *et al.*, 1996), but this was not observed.

Senescence is not simply a passive, mechanistic process, but is a metabolically active phase change involving major gene switching (Bleeker & Patterson, 1997). The time at which 50% of siliques of *vch1* and wild type plants had senesced was significantly different (Section 4.4.3). An important unquantified observation is that *vch1* individuals do, in fact, begin to undergo senescence at approximately the same time as wild type plants. It could be that, although the genetic control of the onset of senescence is normal, subsequent stages of the process are impeded simply by

hormonal or structural differences. Delay of senescence has been observed in some other dwarfed mutants. *dwf1* described by Feldmann *et al.* (1989) exhibits a delay in senescence of about one month relative to wild type plants; some cytokinin over-expressing lines also exhibit a delay (Chaudhury *et al.*, 1993): the effect in *vch1* could similarly be caused by hormonal differences to wild type plants.

One physiological difference between *vch1* and wild type plants is in their mean percentage dry weight. There is no difference in this at 2 weeks post-germination, however, by 6 weeks there is (Section 4.4.6).

The reason for this is unclear. It could be that either the rate of water uptake or of water loss are reduced or increased, respectively, due to structural or transport problems in the mutant. It is possible also that, because of the reduced cell length in *vch1*, there are effectively more cell walls per unit volume. Why this might be more significant in older plants is that as plants age, secondary thickening occurs, vascular tissue accumulates, and the relative contribution of turgor pressure to maintaining cell architecture diminishes. Percentage dry weight, then, increases as plants mature and it may be that it increases proportionately more in *vch1* due to the increased ratio of cell wall per unit volume of cells.

vch1, then, despite its grossly stunted morphology, follows a normal life history as defined by all parameters examined, including germination rate, time of bolting, flowering (Section 4.3) and onset of senescence. This suggests the importance of an "internal molecular clock" as a mechanism for determining the timing of these events, each of which involves genetic regulation. The control of timing must be independent of correct morphogenesis *per se* and is not dependent on parameters such as amount of vegetative development achieved, which has been suggested to be a factor in determining the timing of the transition to flowering (Taiz & Zeiger, 1991). This phenomenon of sequential expression of genes in the absence of normal preceding events has been termed heterochrony (*e.g.* Keith *et al.*, 1994; West *et al.*, 1994; Section 7.3.1) and must itself involve the expression of genes at a higher level in the regulatory

hierarchy than those which initiate these developmental transitions. *fass* (Fisher *et al.*, 1996) is similarly unaffected in the timing of the initiation, positioning and identity of organs suggesting that these features can in some instances be uncoupled from, not only normal morphogenesis as in *vch1*, but also from normal cellular patterning.

7.8 *vch1* can respond to hormones, signalling inhibitors and dark growth

From the battery of observations on the response of *vch1* to exogenous supply of a range of hormones and signalling pathway inhibitors (Section 4.9; Fig. 4.12; Tables 4.5-4.6) it was found that *vch1* responds to each of these in a similar quantitative manner as wild type seedlings. Those which elicited a response include the hormones auxin, which caused increased lateral root proliferation, and cytokinin, which inhibited primary root growth and lateral root initiation and promoted shoot growth. TIBA (a polar auxin transport inhibitor) reduced lateral root formation and total root growth. 8-Br-cGMP (which promotes cGMP-dependent signalling pathways), also reduced root growth. Silver ions, which bind the hormone ethylene preventing it binding its receptors (Tanimoto *et al.*, 1995), promoted root elongation and this also occurred normally in *vch1*. Gibberellins have previously been shown to be sufficient to rescue the phenotype of some dwarfs (Koornneef & van der Veen, 1980; Winkler & Helentjaris, 1995) but when applied to both wild type and *vch1* seedlings, had no effect. Brassinosteroids have similarly been shown to be sufficient to rescue some members of another class of dwarfs, the *cabbage* (*cbb*) mutants (Kauschmann *et al.*, 1996). They had little effect when wild type seedlings were germinated on them at concentrations which are able to rescue this class of mutant, apart from a paling and slight enlargement of cotyledons. Similarly, *vch1*, was little affected by their application (Fig. 4.12 E, Table 4.5).

vch1 was also grown in the presence of a range of signalling pathway inhibitors, to investigate the possibility that the wild type *VCH1* gene might act as a positive regulator of cell expansion. Rescue of the mutant phenotype by one or more of these inhibitors would suggest that *vch1* is a negative regulator of a negative regulator of cell

expansion, dependent, in turn, upon that signalling pathway. If the *VCH1* gene is positively involved in cell expansion, then growth of wild type seedlings in the presence of these signalling inhibitors might produce seedlings which phenocopy *vch1*. It seems, from these results, that the regulation or the effects of *VCH1* expression are not mediated by these pathways.

No phenocopy or rescue was seen in these experiments, however, these models are not discounted because it could be that there are alternative parallel signalling pathways.

That *vch1* responds in a qualitatively and quantitatively normal manner to exogenous hormones and signalling inhibitors does suggest that signalling pathways are unhindered. It lends support to the hypothesis that *VCH1* acts, not upstream of, or as a component of a signalling pathway to promote cell elongation, but more directly in mediating cell elongation through encoding a component of the cell expansion machinery. Alternatively, the defect could be upstream of hormonal or other signalling pathways, maybe in the perception of environmental stimuli to elongate, for example, the perception of light. This model will be discussed further in Section 7.14.

The *cbb* mutants have a stunted apical-basal axis, reduced root and a compact rosette (Kauschmann *et al.*, 1996). They show no elongation response when grown in the dark, and in this respect are similar to the *de-etiolated* (*det*) (Chory, 1993) or *constitutive photomorphogenesis* (*cop*) (Deng, 1994) mutants.

vch1 does show a hypocotyl elongation response in the dark. It elongates to 362% of its mean light-grown length (compared to 501% for wild type seedlings; Section 4.11), indicating constraints imposed by the genetic defect. It differs, therefore, from the above described mutants in that it can both perceive and respond to darkness, albeit within these constraints.

Some other dwarfs, including some which can be rescued by exogenous gibberellin (Koornneef & van der Veen, 1980) appear almost normal when grown in the dark,

suggesting that different pathways control aspects of hypocotyl elongation in the light and dark. The *VCH1* gene is obviously necessary for both light and dark growth and so must be involved in a different pathway to these genes.

Northern analysis showed reduced expression of *TCH4* and *meri5* genes in the *cbb* mutants (Kauschmann *et al.*, 1996). Both of these genes are thought to encode XETs, which directly links this lack of cell expansion phenotype to gene expression changes. Brassinosteroids normally stimulate growth through enhanced cell division and elongation, probably through the induction of expression of these genes.

Experiments in which aerial parts and roots of *vch1* and wild type seedlings were cultured independently *in vitro* (Section 4.6.1) revealed that the phenotypic defect persisted in these isolated organs. This suggested that the wild type *VCH1* gene is expressed ubiquitously. If the phenotype were less severe in isolated organs it might have been inferred that the inhibition of growth of that organ is inhibited by a hormonal or other signal from other parts of the intact plant. This was found to be the case in *fass* (Fisher *et al.*, 1996), the roots of which, when separated from the aerial parts of the seedling, elongate much faster than those on intact plants. Alternatively, inhibition of growth of the aerial parts in *vch1* could have been due to an insufficient supply of water, nutrients or other growth factors from the small, stunted roots.

When callus was initiated from *vch1* and wild type root explants, there was no detectable difference in the distribution of cell sizes in the callus (Section 4.6.2). There is some evidence that expansion of rapidly dividing cells and that of cells in a rapid elongation phase may be differentially regulated by some genes, such as *STP1* (Baskin *et al.*, 1995). Mutation of this gene does not affect size of root meristematic cells but does that of cells in the expansion zone. Callus from *stp1* explants similarly did not exhibit a reduction in cell sizes compared to that from wild type plants, as was the case for *vch1*.

7.9 There are several possible models for the role of the *VCH1* gene

Although *vch1* seedlings responded in the correct qualitative manner to auxins, cytokinins, silver ions and growth in the dark, the extent of the response was quantitatively limited by the constraint on cellular elongation inherent in the phenotype. This is best understood by considering the elongation responses of the roots to silver ions, and of hypocotyls to dark growth. Although these organs do elongate in *vch1*, the final length attained is still significantly less than that of wild type seedlings. In fact, the percentage increase in total root length of seedlings grown in the presence of silver ions, over that of seedlings grown under standard growth conditions, is not greatly different between *vch1* and wild type roots, with their elongating by approximately 150% and 160% respectively (Section 4.9.1).

The hypocotyl of *vch1* elongates to a lesser extent, in percentage terms (363%), than the wild type hypocotyl when grown in the dark (503%; Section 4.11). This could be due to the fact that under standard growth conditions in the light hypocotyls are in a very de-etiolated state. Under these conditions the *vch1* hypocotyl at 7 days post-germination is closer to the length of the wild type hypocotyl (60%) than is its root to the wild type root (approx. 8-10%). Individual cells in the wild type hypocotyl in the light are significantly shorter than their equivalent cell types in the root and this might suggest less directional "pressure to elongate", until grown in low-light or dark conditions. It could be postulated that the *vch1* phenotype is, in effect, less severe in the hypocotyl than in the root because under light grown conditions the "pressure to elongate" is less, and the *VCH1* gene product contributes less under these conditions. If *VCH1* does encode a cell wall or cytoskeletal component, then this protein may normally be upregulated in rapidly elongating cells, but be expressed only at a relatively low level in less rapidly elongating cells.

Whatever is limiting cell elongation in *vch1* is still limiting to a similar degree under all growth conditions examined. It does reveal that the constraint is not an inviolable

limitation on elongation *per se*, in that cells can exceed the lengths of those in untreated seedlings, given a sufficient hormonal or environmental stimulus.

There are several broad possible explanations of the *vch1* phenotype. Firstly, it may be that cell wall expansion is inhibited through a structural defect, either in cell wall extensibility or in the assembly or functioning of the cytoskeleton. Secondly, the defect could be disruption of intercellular signalling at the whole plant level, maybe in the gradient of a factor essential for causing directional cell expansion along the apical-basal axis of the plant. This could be as a result of phytochrome detection of light, gravity sensing, or another mechanism by which the appropriate "directionality" of expansion is perceived and elicits a growth response: this could be in primary sensing of such stimuli, or in downstream signalling, perhaps in hormone production or translocation. Finally, a structural or biochemical defect may be responsible for reducing water uptake or transport and thus turgor-driven cell expansion.

These proposed broad models are discussed below.

7.10 The defect in *vch1* might be in hormone production, transport, perception or response

The basic defect in *vch1* is a failure to correctly axial cell elongation. A number of hormones have been implicated in control of cell elongation, mediated through effects on microtubule organization (Shibaoka, 1994). Whilst *vch1* is not rescued by the exogenous application of any of the hormones tested (Section 4.9.1), the possibility exists that the defect lays, not in the production of a hormone, but in the polar transport of, or specific response to a hormone.

Auxin, is believed to be distributed in a concentration gradient: abolition of this gradient, either by mutation or by chemical inhibition, severely disrupts embryonic axis elongation (Liu *et al.*, 1993; Neuhaus *et al.*, 1997) and some post-embryonic developmental processes including floral organogenesis (Okada *et al.*, 1991). The observation that embryogenesis is normal in *vch1* suggests that auxin transport is not

defective. It cannot be discounted that different pathways of polar auxin transport might operate embryonically and post-embryonically, although there is no existing evidence to suggest this. Further evidence against a defect directly in auxin signalling is that *vch1* responds normally to exogenous auxin, suggesting that transport and perception are unaffected; again parallel signalling pathways cannot be discounted. Altered gradients in other intercellular signalling molecules is another possibility, although the hormone and signalling inhibitor experiments (Section 4.9) did not provide any supporting evidence for this either. It has been found that in *fass*, levels of free auxin are three times as high as in wild type embryos (Fisher *et al.*, 1996). Application of auxin to *in vitro* cultured heart-stage embryos phenocopied *fass*, supporting the hypothesis that excess free auxin is inhibitory to elongation in *fass*, perhaps through increasing ethylene production. It is suggested that *FASS* might encode an auxin-conjugating enzyme, or positive regulator theoreof. Both auxin and ethylene have been shown to be capable of causing the reorientation of microtubules, perhaps resulting in the isodiametricity of cell expansion in *fass,* as have brassinosteroids (Mayumi *et al.*, 1995). Auxin-resistant mutants such as *axr1* (Lincoln *et al.*, 1990) and *axr2* (Wilson *et al.*, 1990) have reduced auxin-mediated responses, including plant height, root gravitropism and vascular bundle differentiation. *axr2* (Wilson *et al.*, 1990; Timpte *et al.*, 1992) is a severe dwarf and exhibits abnormal gravitropism and a lack of root hairs. It is resistant to the exogenous supply of auxin, ethylene and ABA and it is suggested that the mutated gene probably encodes a protein which is a common messenger in the elicitation of response to all of these hormones. *rooty* (King *et al.*, 1995) shows excessive root growth and all types of auxin are at higher levels than in wild type seedlings (conjugated and unconjugated) which may represent a defect in auxin-degradation. Transgenic overexpression of *A. tumefaciens tryptophan monooxygenase* (*iaaM*) results in a four fold increase in free IAA and increases hypocotyl elongation in the light (Romano *et al.*, 1995). *amp1* (Chaudhury *et al.*) is dwarfed and this correlates with a six fold increase in cytokinin levels. Transgenic overexpression of cytokinin upregulators (Medford *et al.*, 1989) produces a similar phenotype. Mutation in a brassinosteroid biosynthesis enzyme, a cytochrome

P450, results in a dwarfed phenotype, *dwf4* (Choe *et al.*, 1998), with a shortened hypocotyl.

In none of the hormone mutants described has there been such a severe and ubiquitous effect on cell elongation in the mature plant but not embryonically, as in *vch1*. It is possible that *vch1* does encode a component of one or more hormone signalling mechanisms. However, the putative mechanism would have to be essential only to the post-embryonic development of the plant.

7.11 The defect in *vch1* might be in cell wall extensibility

It is generally accepted that the expansion or elongation of plant cells represents an equilibrium between the outward pressure of turgor and constraint by the cell wall. The cell wall yields in a controlled manner during development through the action of enzymes which catalyse loosening of bonds between wall components and the deposition of new wall material. Wall yielding is the rate limiting step in cell elongation (Timpte *et al.*, 1992; Fry, 1994; Thomos & Pritchard, 1994).

In *vch1* the ability of the wall to yield is reduced but not eliminated. Walls can extend, for example, those of hypocotyl cells in response to dark growth and those of root cells in the presence of silver ions (Section 4.11 and 4.9.1).

The failure of *vch1* to elongate normally may be due to a reduction in the expression of enzymes which catalyze wall yielding, such as expansins (McQueen-Mason & Cosgrove, 1994; 1995) or XETs (Xu *et al.*, 1996; de Silva *et al.*, 1994). Expansins are proteins that catalyse acid-induced wall extension by binding at the interface between cellulose microfibrils and matrix polysaccharides, inducing extension by reversibly disrupting non-covalent bonds (Fry; 1994; McQueen-Mason & Cosgrove, 1995; Cosgrove, 1997). Expansin genes comprise a multi-gene family and it is unknown whether isoforms are functionally interconvertible. If they are functionally interconvertible, then it might be predicted that mutation of one expansin gene might have little or no phenotypic effect. It may be that different isoforms are expressed in a

tissue-specific manner, or that *VCH1* could encode not an expansin directly, but a positive regulator of expansin gene expression or an expansin-interacting protein. That expansin genes in *vch1* have not been disrupted by insertional mutagenesis was suggested by the PCR of expansin genes with degenerate primers (Section 4.12), although these results were preliminary.

Other cell wall enzymes include extensins and other PR proteins, such as chitinases (Patil & Widholm, 1997). Also, arabinogalactan proteins (AGPs) are associated with the plasma membrane and cell wall (Knox *et al.*, 1991; Willats & Knox, 1996). Their function is not well understood and may be diverse: it is known that they are highly developmentally regulated (Pennell *et al.*, 1991). *diminuto* shows a severe reduction in cell length in many tissues and also has a marked reduction in AGPs detected with Yariv's reagent (Takahashi *et al.*, 1995). Other examples include peroxidase, which catalyses cross-linking of wall components (Goldberg *et al.*, 1987) probably aiding "tightening" of side walls. Also, endotransferases such as XETs catalyse molecular grafting between cellulose microfibrils and are upregulated during cell growth (Xu *et al.*, 1996; de Silva *et al.*, 1994).

It would be possible to perform Northern blot analysis to detect the transcripts of genes encoding enzymes of each of these classes. However, this might still not yield information regarding the primary genetic defect in *vch1*. *diminuto*, for example (a brassinosteroid signalling mutant) shows reduced expression of genes including a tubulin gene, *TUB1* (Takahashi *et al.*, 1996). Such results need to be interpreted with caution in the absence of cloning the mutant gene.

It is possible that the failure of elongation in *vch1* represents a failure of deposition of new wall material during growth, especially of the major component cellulose. The structure of root epidermal and cortical cell walls was examined by TEM (Section 4.4.4). There appeared to be no obvious difference in the arrangement of cellulose. Also, there was no detectable difference in the overall thickness of the walls, arguing against the defect being in the deposition of new cellulose in the expanding wall. Close

behind the root meristem (approx. 200-300μm back from the tip), there was no noticable difference between *vch1* and wild type roots in the alignment of cellulose microfibrils. Further back in the root some walls appeared less organised, with a more random array of cellulose microfibrils. However, this was also seen in wild type roots in regions behind the zone of elongation and although such walls were observed more frequently (unquantified observation) this may simply be a secondary consequence of the reduced cell elongation in *vch1*. When cells cease to be actively elongating, there is some relaxation in the cell wall, resulting in reduced structural organization. The fact that cells are able to organise their walls initially in the short region in *vch1* in which they are actively elongating suggests that the primary defect does not lie in cellulose microfibril organization *per se*. Similarly, intercellular spaces at the "corners" of juxtaposed cells appeared to be generally larger in area in *vch1*, however, again this was also seen in regions of the wild type root in which elongation had slowed and cell walls were less stretched; this also may be a secondary consequence of the *vch1* mutation. Another unquantified observation was that the distribution, size and number of secretory vesicles observed associated with newly forming end walls of cells appeared similar in *vch1* and wild type roots, suggesting that delivery of material to the wall is not impaired.

7.12 The defect in *vch1* might be in the assembly or organization of the cytoskeleton

That the arrangement of the cytoskeleton is important in mediating changes in cell shape during growth and in response to hormonal stimuli (Shibaoka, 1994) led to the hypothesis that, because cell shape is altered in *vch1*, this might reflect a defect in cytoskeletal organization.

The cytoskeleton plays a pivotal role in determining the plane of cell division and the direction and degree of expansion. Prior to cell division microtubules form an array across the cell at right angles to its long axis, the Pre-Prophase Band (PPB) (Webb and Gunning, 1991); this seems to be essential for defining the plane of division. In *fass* (Fisher *et al.*, 1996) the microtubules are disorganised and do not form the PPB,

resulting in loss of control of the plane of cell divisions. During cell elongation tubulin microtubules are oriented across the cell at right angles to its longitudinal axis (Cortical Array), and actin microfilaments along its longitudinal axis: both are thought to guide cellulose deposition (Baskin *et al.*, 1994). How they interact with the membrane and wall is little understood, although increasing numbers of microtubule associated proteins (MAPs) (Goddard *et al.*, 1994) are being identified, some of which may play a role in such interactions. The response of individual cells to hormonal stimuli is mediated through microtubule re-orientation (Shibaoka, 1994), although again this is little understood at a mechanistic level. Microtubules are also necessary for the delivery of vesicles containing membrane and cell wall components to the cell plate (Goddard *et al.*, 1994).

Several lines of evidence argue against the primary defect in *vch1* being in microtubule organization. Firstly, cell patterning is unaffected by the mutation (Section 4.4.1-4.4.3), suggesting the correct organization of the PPB. Secondly, the normal qualitative and quantitative response of *vch1* to exogenous hormones suggests correct microtubule re-organization in elicitation of this response (Section 4.9.1). Thirdly, although the elongation of *vch1* cells is severely reduced in magnitude, the direction of elongation is always correct, unlike in *fass* (Torrez-Ruiz & Jürgens, 1994; Fisher *et al.*, 1996) and *diminuto* (Takahashi *et al.*, 1994), both of which exhibit a reduction in number and organization of transverse microtubules and diminished control over the direction of cell expansion, with some cells being radially expanded or even isodiametric. Fourthly, the distribution, size and number of vesicles in epidermal cells of *vch1* did not appear to differ significantly from that in wild type cells of the same type (Section 4.4.4): vesicle trafficking is via microfilaments and this observation may be interpreted to argue against a defect in microfilament assembly or behaviour. The *EMB30* gene was found to show homology with the yeast *SEC7* gene, which is involved in vesicle trafficking (Shevell *et al.*, 1994). This correlates with the *emb30* phenotype, with loss of control over the plane of cell division and expansion and impaired intercellular adhesion. Finally, the cortical array of microtubules appeared normal in *vch1*, when examined by immunolabelling techniques. Both α- and β-tubulin are major

components of microtubules, and α-actin of microfilaments. Microtubules are assembled into different configurations during the cell cycle. During interphase, when cell elongation is occurring, microtubules are arranged into the Cortical Array. This comprises parallel microtubular strands wrapped around the cell, perpendicular to the primary axis of expansion (Goddard *et al.*, 1994). This was visualised in roots labelled with anti-α-tubulin (Section 4.5; Fig. 4.10). The pattern of microtubules in the cortical array looked similar in *vch1* and wild type root epidermal cells, suggesting that at least this particular microtubule array can be organised normally in *vch1*.

The observation that tip growth is unaffected by the *vch1* mutation argues against some possible defects which could otherwise explain the *vch1* phenotype. Wall deposition involves intense secretory activity of vesicles at the growing tip, which are delivered along a dense network of microfilaments (Taylor, 1997). There are also significant numbers of microtubules present (Bibikova *et al.*, 1997). These features argue against defects in secretory processes, microfilament and microtubule behaviour in *vch1*, although it is possible that those disrupted in diffuse-growing cells represent a diffuse growth-specific sub-set of one of these.

It cannot be discounted that a specific sub-set/organization of microtubules is disrupted in *vch1*. In *fass*, for example, MTs failed to organise into a cortical array or PPB, but they were present and did form mitotic spindles and the phragmoplast (McClinton & Sung, 1997). It was suggested that *fass* is impaired in the functioning of the microtubule-organising centre(s).

7.13 Insufficient turgor is unlikely to be the cause of the *vch1* phenotype

The observation that cells which grow by tip growth elongate normally in *vch1* (Section 4.4.1) could help us to distinguish between alternative possible explanations of the *vch1* defect. One difference between diffuse and tip growing cells is that turgor pressure is not necessary for tip growth. Tip growth is driven by actin polymerisation along a calcium gradient (Wymer *et al.*, 1997). However, turgor pressure is generally

not limiting to cell expansion under normal growth conditions (Timpte *et al.*, 1992; Fry, 1994; Thomos & Pritchard, 1994). Also, the defect in the *vch1* phenotype is manifest at a stage where there is no significant difference in percentage dry weight between *vch1* and wild type seedlings. The possibility that reduced turgor causes the *vch1* phenotype is therefore discounted.

7.14 The defect in *vch1* might be in the determination of the axial direction of cell expansion

The following question was posed in Section 7.5: is the primary defect in *vch1* one of cells not receiving a signal required for axial elongation, perhaps in the form of a polar gradient of a hormone (discussed in Section 7.5) or other signalling molecule; or, is the defect a failure to mechanically respond to such a signal, for example by cytoskeletal organization or assembly/yielding of the cell wall (discussed in Section 7.11-7.12).

One might also ask whether radial expansion is a default state of expanding cells lacking the putative "longitudinal expansion-determining signal" or, whether inappropriate radial expansion somehow inhibits longitudinal expansion Benfey *et al.* (1993) suggest that radial expansion is due to a lack of control over the interplay between the cytoskeleton and cell wall material deposition. This could be the case in *vch1* also. Several other broad explanations have also been proposed. The putative "longitudinal expansion-determining signal" could fail to be transmitted or perceived.

Overexpression of phytochrome A in transgenic tobacco resulted in dwarfing by reducing cell elongation in hypocotyls and stems (Jordan *et al.*, 1995). Overexpression of phytochrome C in *Arabidopsis* resulted in reduced cell lengths in both hypocotyl and leaves (Qin *et al.*, 1997). Phtochrome A and B have been shown to be involved in the hypocotyl-specific downregulation of the *TUB1* (tubulin) gene in light-grown seedlings (Leu *et al.*, 1995). A recently identified suppressor of phytochrome signalling, *SPA1*, has been shown to be involved in downregulation of the phytochrome A response (Hoeker *et al.*, 1998). *spa1* mutants exhibit a dwarfed phenotype in the light. A model is proposed whereby the *VCH1* gene could play a similar role in downregulation of

phytochrome signalling. An excessive response to phytochrome-mediated signals as a result of mutation in such a gene might result in the observed phenotype of reduced cell elongation. The role, and effects of, phytochrome D and E, for example, are not well characterized and might be found to be involved in control of cell elongation in all organs.

Alternatively, the defect might be at the level of perception or response to the "longitudinal-expansion determining signal", again, maybe in the the assembly of the cytoskeleton or cell wall; interplay between the cytoskeleton and cell wall; or in wall yielding. One possible hypothesis is that reduction of lateral tightening by peroxidase (Goldberg *et al.*, 1987) might result in a tendency towards isodiametric expansion. It has been suggested that "hot spots" of expansin activity may exist in cell walls (Cosgrove, 1997) and an increase in the number or position of such "hot spots" could result in the tendency towards isodiametric expansion in mutants such as *hydra1* (Topping *et al.*, 1997). This is also possible in *vch1*.

7.15 Summary of the *vch1* mutant

In summary, the *vch1* mutant exhibits reduced cellular elongation throughout the plant, resulting in a dwarf with very short roots. Despite its overall morphology being defective, the relative spatial positioning of cells and organs is normal: the abnormality is only in the shape of those cells and organs. This is interpreted to suggest that morphogenesis can be regulated independently of correct cellular pattern formation. There was also no abnormality in the expression pattern of *gus*-fusion genes regulated by positional signalling. *vch1* follows a normal life history by all parameters examined, including time of bolting, flowering and onset of senescence, demonstrating that the timing of these events can be uncoupled from correct morphogenesis. The phenotype of *vch1* cannot be rescued by the exogenous supply of a range of hormones, signalling inhibitors, or by alteration in growth conditions, including growth in the dark. However, it can respond to each hormone tested in a proportionately similar manner to

wild type seedlings. No defect in cell wall architecture nor in cytoskeletal organization in *vch1* were detected during this study.

The question regarding the molecular nature of the defect was not resolved during the course of this study, however, speculative models for the role of the *VCH1* gene have been proposed. Two broad alternative explanations of the *vch1* phenotype have been described. The first is that the defect lies in the control of elongation growth at the level of the mechanics of cellular expansion, possibly in cytoskeletal organization or in wall extensibilty. The second is that the defect may be at a supracellular level of control, in the perception or signalling of the normal axial direction of elongation growth.

7.16 The *asf1* mutant is embryonic-lethal

How the growth of the suspensor is regulated and what causes it to degenerate at a defined stage of development is not well understood. Several pieces of evidence from studies in which the embryo proper was induced to degenerate suggest that the normal development of the embryo proper restricts further growth of the suspensor, since this caused failure of the suspensor to degenerate, or it even to proliferate (Haccius, 1963). More recently, many embryo-defective mutants in both maize (Clark & Sheridan, 1991) and *Arabidopsis* (Yeung & Meinke, 1993; Marsden & Meinke, 1985; Schwartz *et al.*, 1994; Yadegari *et al.*, 1994) have been identified, in which embryogenesis is disrupted at an early stage. A significant number of these exhibit an abnormally large suspensor late in development. In the case of each of a number of such mutants now identified, including the *sus* mutants (Schwartz *et al.*, 1994) and the *raspberry* mutants (Yadegari *et al.*, 1994), the abnormality is always first manifest as the arrest of the embryo proper in a globular state, followed by inappropriate cell divisions within the embryo proper. This is later followed by ectopic cell divisions within the suspensor.

The *asf1* mutant was fortuitously identified whilst examining the embryos of the *vch1* line. It was initially thought that *asf1* represented the embryonic phenotype of the *vch1* mutant. However, in the next generation following selfing it was found that the embryo

mutant. However, in the next generation following selfing it was found that the embryo (*asf1*) and the seedling (*vch1*) mutant phenotypes segregated independently of one another. Individual lines found to be heterozygous for *vch1* and for *asf1*, respectively, were selected for further experiments.

The *asf1* mutant described here is an embryo-lethal mutant which arrests as an enlarged globular-shaped embryo proper with an inappropriately enlarged suspensor. This character places it in the general class of mutants described above (Marsden & Meinke, 1985; Schwartz *et al.*, 1994; Yadegari *et al.*, 1994). Complementation analysis with the *sus1* mutant (Schwartz *et al.*, 1994), to which *asf1* is most phenotypically similar, showed *asf1* not to be allelic and it is proposed that *asf1* represents a mutation at a novel genetic locus.

7.17 The *ASF1* gene is not T-DNA tagged

asf1 segregated out at a ratio of 1:3 mutant:wild type in subsequent generations of selfed heterozygotes (Section 3.2), suggesting that it represented a homozygous recessive loss-of-function mutation at a single locus. Although T-DNA was present in the genome of the first generation of *asf1* examined, it was later proven that the mutation was not linked to the presence of T-DNA (Section 6.4).

When embryos of *asf1* were subjected to histochemical analysis for GUS no activity was detected (Section 3.2) suggesting no promoter-GUS fusion had occurred in either case (discussed in Section 6.4).

The possibility that the mutation represented a partial T-DNA integration was discussed in relation to *vch1* in Section 7.3. The same argument applies here - that fragments of the T-DNA not containing *gusA* gene sequences, and so not detected when this was used as a probe in Southern analysis, was causative of the mutation.

Again, because the gene was not T-DNA tagged it was necessary to adopt an alternative stategy towards cloning the *vch1* gene. Progress towards mapping of the

chromosomal location of the *vch1* gene has been made (Section 6.5) as a first step towards cloning the gene (Section 1.8.1).

7.18 The *ASF1* gene is required for embryonic morphogenesis and maintenance of suspensor cell fate

The developmental profile of *asf1* presented in Section 5.2; Fig 5.1 establishes that the mutant phenotype first becomes apparent at the transition from the globular- to heart-stage of embryogenesis. Prior to this no deviation from the normal cell patterning of the early embryo was observed, with correct organization of the embryo proper into an upper and lower tier of cells, and a suspensor of a single file of approximately 8 cells. This is consistent with the other mutants of this class: all the *sus* and *raspberry* mutants become abnormal at the transition stage. Like these, *asf1* fails to make the transition to the heart-stage with no evidence of the establishment of bilateral symmetry. Instead it begins to undergo apparently random cell divisions in all orientations relative to the axes of the embryo. The definition of the O' boundary between the upper and lower tiers disappears and there is apparently no bilateral, radial nor apical-basal patterning. In both longitudinal and transverse section there is little evidence of heterogeneity in cell size in an organised pattern.

One experiment which was initiated, but for which results are not yet available, was the introduction of the *AtLTP2-gus* gene into the *asf1* mutant background (with kind permission of Prof. C. Somerville (Stanford University, USA). *AtLTP* encodes an *Arabidopsis* homologue of the carrot *EP2* lipid transfer protein (Sterk *et al.*, 1991; Thoma *et al.*, 1994), which is strongly expressed in the protoderm/epidermis of embryos and seedlings. The rationale here was to look for its expression in the embryo proper and suspensor of *asf1* embryos as a biochemical marker of protoderm/epidermis cell differentiation (Vroemen *et al.*, 1996). *AtLTP* is not expressed in the wild type suspensor (Yadegari *et al.*, 1994). However, it was expressed in the peripheral cells of the *raspberry* embryo proper and suspensor (Yadegari *et al.*, 1994) It was also expressed in the correct spatial context in *knolle* and *keule*, in their grossly abnormal

outer cell layer. Its expression, therefore, is not dependent on correct protodermal cell morphology (Vroeman *et al.*, 1996).

In the *sus2* mutants, for example, there is some apparent cytodifferentiation in the middle of the embryo to form enlarged, vacuolate cells which appear to have some vascular cell character (Schwartz *et al.*, 1994). Cells of the *asf1* embryo appear quite homogeneous in size and shape, being small, rounded and cytoplasmically dense, with no zones of increased vacuolation evident. Cell patterning is apparently random but the overall shape of the embryo proper always remains approximately spherical. At approximately the time at which wild type embryos within the same silique reach the torpedo stage, the first evidence of inappropriate cell divisions within the *asf1* suspensor is seen. The cell divisions within the suspensor start at the hypophysis and progress along the length of the suspensor towards its basal (micropylar) end. When the suspensor begins to divide, the embryo proper elongates in the apical-basal axis to become slightly ovoid. It does, then, have rudimentary apical-basal polarity (Section 5.2).

In the *sus* and *raspberry* mutants, the point at which the first inappropriate divisions of the suspensor occurred was at different, but quite characteristic, points along its length and subsequent divisions effectively spread out from the point of this first division. The cell in which ectopic divisions started was not as predictable as in *asf1*. Why the "wave" of cell divisions proceeds in this manner is intriguing. *raspberry, sus2* and *sus3*, for example, generally begin with suspensor cell divisions at the basal end of the suspensor, and *sus-1* generally with the cells towards the middle. This will be discussed further in Section 7.26.

7.19 Ectopically divided suspensor cells of *asf1* exhibit embryo proper-like characteristics

There is some evidence that the structure produced from ectopic suspensor cell division in *asf1* takes on some embryo proper-like character. This phenomenon has previously been noted in other mutants of this class.

In *asf1* it has been observed that the ectopically divided suspensor cells are cytoplasmically dense, like embryo proper cells and unlike the highly vacuolate cells of the normal suspensor. They also accumulated large protein bodies and lipid bodies (Section 5.4.1; Fig. 5.3), had smaller vacuoles and smaller cell sizes, compared to those of the wild type suspensor. Also, these cells accumulate mature chloroplasts (Section 5.4.2). Development of mature chloroplasts from immature plastids requires the expression of a large number of genes in both the nuclear and plastid genome (Gruissem, 1989), again suggesting activation of a whole set of genes associated with this aspect of cytodifferentiation. All of these features are suggestive of their having acquired character more akin to that of the normal embryo proper than to the suspensor cells from which they derive.

Ultrastructural analysis of the *sus* (Schwartz *et al.*, 1994) and *raspberry* (Yadegari *et al.*, 1994) mutants by TEM revealed that, whilst the embryos arrested at and remained in a globular state, they acquired some ultrastructual features characteristic of wild type maturation stage embryos. These features included the accumulation of starch grains, protein bodies and lipid bodies. During normal embryogenesis there is no evidence of accumulation of these until after the heart-stage. Starch grains begin to appear by the torpedo-stage, becoming abundant by the cotyledonary-stage. Neither protein bodies nor lipid bodies normally appear until the cotyledonary embryo enters its maturation phase. Prominent vacuoles containing electron-dense material are characteristic of the stage just prior to storage protein deposition. These characteristics normally associated with late embryonic events were observed, in various combinations, in *sus* and *raspberry* embryos as in *asf1*. For example, the *raspberry* embryos accumulated prominent vacuoles containing electron-dense material in both their embryo proper and suspensor regions. They did not, however, accumulate detectable lipid bodies or protein bodies; neither did they accumulate mature chloroplasts, another feature of normal embryos from the heart-stage to late cotyledonary stage (Rodkiewicz, 1994). In the case of the *sus* embryos, accumulation of storage protein bodies, lipid bodies and starch grains was observed in both their embryo proper and suspensor regions. These

were present at varying densities in the mutants representing different *sus* alleles, but always at higher density than that observed in either a normal globular embryo proper, or a normal suspensor of any stage (Schwartz *et al.*, 1994). In both the *sus2* and *sus3* mutants it was observed that the starch grains and protein bodies had become restricted to the outer cell layers of the mutant. This corresponds to their distribution in wild-type embryos where these storage products are much more abundant in the cells of the epidermis and storage parenchyma, and much less so in provascular cells.

7.20 The altered genetic identity of *asf1* suspensor cells is confirmed by the expression of spatially restricted *gus*-fusion marker genes

Further compelling evidence for the acquisition of embryo proper-like genetic character in the suspensor-derived cells is suggested in this section discussing the expression patterns of *gus*-fusion marker genes introduced into the *asf1* background. The use of marker genes with spatially and/or temporally restricted expression patterns to study the organization of mutants was introduced in Section 1.1.2 and the results presented in Section 5.5.

It has been suggested from a study in this laboratory (Topping & Lindsey, 1997) in which the *POLARIS* and *EXORDIUM* genes were introduced into the *hydra1* and *emb30* mutants that these two *gus*-fusion genes mark different aspects of axial development. *EXORDIUM* was highly expressed in the correct position at root and shoot apices and at lower levels, as in the wild type, in the cotyledons in the *hydra1* seedling, which does have structurally organized root and shoot apices. In the *emb30* mutant, however, *EXORDIUM* was expressed at low level in the cotyledonary regions, but not at the expected position of root and shoot apices. This is consistent with the observation that, unlike *hydra1*, *emb30* lacks structurally differentiated root and shoot meristems, and supports the hypothesis that *EXORDIUM* marks cells with such meristematic identity. *POLARIS*, however, was expressed not only in those *hydra1* seedlings with a root, but also in the basal part of those more extreme sibling variant *hydra1* seedlings which lack an anatomically distinct root, and similarly in the embryos

of *hydra1*, all of which lack any structural evidence of embryonic root organogenesis. It is also similarly expressed in the basal part of *emb30* seedlings and embryos, both of which totally lack root organization. It was suggested from this that *POLARIS* differentiates positional aspects of polar development from structural aspects, in that its expression is independent of the structural differentiation of root cells, but is still positionally correct with respect to apical-basal polarity. *POLARIS* expression may reflect biochemical differentiation of cells in a position-related manner, suggesting activation by a signalling pathway that regulates position-dependent gene expression in the embryonic and seedling root. Expression of *POLARIS* has been shown to be upregulated by exogenous auxin and downregulated by cytokinin (Topping & Lindsey, 1997).

The expression patterns of both *POLARIS* and *EXORDIUM* in the *asf1* embryo not only supported these hypotheses, but yielded further information regarding both the organization of the *asf1* mutant and the regulation of the expression of these two genes.

In phenotypically wild type embryos *EXORDIUM* is expressed throughout the whole embryo proper. In seedlings it is restricted to the root meristem, in the shoot meristem and the cotyledons and young leaves. Section 5.5.2 described how *EXORDIUM* is expressed not only throughout the enlarged, globular embryo proper of *asf1*, but also in those cells derived from ectopic division of suspensor cells. *EXORDIUM* is never expressed in any cells of the suspensor of normal embryos. This provides a further line of evidence that the ectopically divided cells of the suspensor have taken on aspects of embryo proper genetic character.

Switching off of the expression of *276-1* was observed. During wild type embryogenesis, *276-1* is restricted to a single cell type, the suspensor cells. When the identity of these cells change following their ectopic division in *asf1*, the gene is switched off (Section 5.5.3). This repression appears to be cell identity dependent since, if it were position dependent, it would still be expressed in cells at least at the basal end of the ectopically divided cells. When the maintenance of suspensor

identity breaks down and the cells enter a default embryo proper-like genetic programme, this must involve activation of alternative cell differentiation pathways, including not only switching on of embryo proper specific genes but also switching off of suspensor specific genes.

Similarly, the expression of *POLARIS* in the correct spatial context within the the unit comprising the embryo proper and enlarged suspensor as a whole (at the basal end; Section 5.5.1) supports this idea of gene expression pattern change in suspensor-derived cells. This, in addition, strongly supports the idea that *POLARIS* is switched on in response to positional information specified in a dynamic manner during embryogenesis. Its expression is not lineage-dependent because it can be switched off in cells in which it was previously expressed (those at the basal part of the embryo proper) and switched on in (suspensor-derived) cells in which it was previously not. These undergo a gene expression pattern change, presumably in response to the signals which regulate *POLARIS* expression. Since *EXORDIUM* expression in *asf1* remains in all cells of the embryo proper, as it is in wild type embryos, its expression in the ectopically divided suspensor cells must again represent response to a cell fate-determining signal which can now be perceived by these cells. Again this signal must be transmitted in a dynamic manner during embryogenesis rather than just at a temporally restricted stage.

7.21 Other suspensor-proliferation mutants also exhibit altered patterns of gene expression in the suspensor

In situ localization was performed on *raspberry* embryos to detect the transcripts of genes expressed in a temporally and spatially restricted manner during embryogenesis. One of these was the lipid transfer protein gene, *AtLTP2* (Thoma *et al.*, 1994) This is discussed in Section 7.18. Several *LEA* storage protein mRNAs were also detected. These, which include *AT12S* and *At2S* mRNAs, accumulate specifically in parenchyma cells in wild type embryos from the torpedo-stage onwards (Yadegari *et al.*, 1994). All of these transcripts were also detected in *raspberry* embryos at the time at which wild type embryos within the same silique were in late maturation. They were in the correct

spatial context, *i.e.* in the outer and some inner cell layers, and not in the central region of the embryos (Yadegari *et al.*, 1994). It was suggested that switching on of their expression is effectively regulated by an "internal developmental clock" which does not require inductive cues regarding the stage of morphogenetic development achieved. This will be discussed further in Section 7.31. It has been suggested, therefore, that the *sus* mutants are able to interpret positional information during storage tissue differentiation even in the absence of correct morphogenesis (Schwartz *et al.*, 1994).

In *sus1,* cells at the centre of the embryo proper degenerate late in development. Programmed cell death does occur in the normal degeneration of the suspensor and also during vascular tissue formation in adult plants (Section 7.29), but is not normally a feature of the embryo proper. *sus2* occasionally showed precocious xylem differentiation in the middle of the embryo proper, again suggesting that positional cues are being interpreted even in the absence of correct morphogenesis. The nature of such cues is of considerable interest. Whilst hormonal and other types of intercellular signalling between different parts of the plant are becoming increasingly well characterised at many stages of development (Kende & Zeevaart, 1997), very little is yet known about such intercellular interactions within the early embryo (Schwartz *et al.*, 1994). The disrupted interactions between embryo proper and suspensor, and the establishment of cellular diversity despite the failure of morphogenesis in mutants such as these may yield valuable insights into normal control mechanisms.

Another feature taken to suggest late-embryogenic character in the *raspberry* mutants was the presence of "raspberry-like protruberances" on cells on the outer surfaces of both the embryo proper and suspensor. Such convex protruberances of the outer wall of epidermal cells are normally characteristic of late maturation stage embryos and are not seen on globular stage embyos. This feature was not seen on the *sus* embryos nor on the *asf1* mutant described here.

7.22 Some *asf1* ovules can undergo maturation and germination

A significant proportion of *asf1* mutant ovules (12%) was able to complete maturation and desiccation (Sections 1.3.2; 5.3), producing a seed, the only difference from wild type seeds being a slightly paler brown colouration. This contrasts with *sus* and *raspberry* which all abort prior to desiccation of the wild type seeds within a heterozygous silique. It seems in some respects that the *asf1* phenotype is less severe than these other mutants. It would appear to exhibit more embryo proper like characteristics, both in its overall morphology, with the mutant suspensor apparently forming a morphological continuum with the embryo proper region, and in its expression of *POLARIS* in the correct spatial context within this unit as a whole. The *POLARIS* expression pattern implies a continuous gradient in signalling pathways which regulate it. This, though, could also prove to be the case in other mutants of this class. Other evidence is the accumulation of chloroplasts and the ability to withstand desiccation.

7.23 Some *asf1* seeds germinate but undergo very abnormal post-germinative development

asf1 homozygous seed was occasionally able to germinate, albeit at low frequency (< 1%). The phenotype of the 'seedlings' was defective (Section 5.3). Of those individuals which germinated with the suspensor-derived structure intact (Fig. 5.2), this structure became brown and died before the 'seedling proper'. This was reminiscent of apoptosis, and was interpreted to suggest some residual suspensor identity in those cells which had apparently acquired several aspects of embryonic character. Whether this represents a signal from the 'seedling' proper, inducing apoptosis, or the switching on in a 'suspensor'-autonomous manner of the apoptotic gene expression pattern is not obvious. This could, were sufficient material available to make it feasible, be investigated by dissecting the suspensor-derived structure away from the rest of the 'seedling' and determining whether it still underwent apoptosis.

The rest of the 'seedling' grew and developed villus-like projections on its surface, surviving, with periodic transfer to fresh medium, for 9-11 weeks. An explanation of this morphology remains obscure, although similar meristem-like nodules sometimes arise spontaneously in cultured callus (Huang & Yeoman, 1984). It would be interesting to determine whether it is possible to generate sustainable callus from these germinated individuals and whether it would then be possible to induce hormonally the formation of shoot and roots. If it were possible, this would indicate that the *ASF1* gene is expressed in an embryo-specific manner and is not necessary for post-germinative development (Franzmann *et al.*, 1989).

Schwartz *et al.* (1994) attempted to rescue the *sus* embryos by dissecting them out from immature seeds and transferring them to nutrient medium. Both *sus2* and *sus3* were able to undergo a limited amount of organogenesis, however, neither were able to regenerate fertile plants. This suggests that the gene may be required for vegetative development, although it could not be discounted that the culture conditions were inappropriate for complete rescue. Such experiments can only be conclusive where a fertile, normal mature plant is produced.

7.24 Polyembryony

Even more dramatic evidence that cells of the suspensor can alter their fate and take on embryo proper-like characteristics is the identification of the *twin* mutants (Vernon & Meinke, 1994; Zhang & Somerville, 1997) in which second or even third embryos are able to develop from a cell of the suspensor of the first. A similar phenotype of secondary embryos is sometimes seen in the *häuptling* mutant, in which they are derived from supernumerary basal tiers of the primary embryo (Ploense, 1997). There are some abnormalities in cell division patterns within these and the first embryos, but they are nevertheless able to complete embryogenesis, germinate and form viable, fertile adult plants.

7.25 The primary defect in most suspensor mutants is in the embryo proper

It has been suggested that *twn1* may represent mutation directly in a component of a signalling pathway from the embryo proper to the suspensor, which normally represses suspensor division and its embryonic potential and/or ultimately results in its programmed cell death (Schwartz *et al.*, 1997). Another possible model, in addition to that postulating disrupted signalling *per se*, is that the genetic defect in all, or some, of this class of inappropriately proliferated suspensor mutants may be in genes involved in controlling either embryonic morphogenesis, or in basic biochemical processes essential to the normal development of the embryo proper (so-called "housekeeping" genes). For example, a biotin auxotrophic mutant of *Arabidopsis*, *bio1*, results in embryonic lethality (Schneider *et al.*, 1989). *häuptling* is allelic with *amp1*, a cytokinin overproducing mutant (Chaudhury *et al.*, 1993), and so fits the model of "housekeeping" mutations in the embryo proper. It might be that, in the suspensor proliferation mutants, whatever the genetic lesion that causes the embryo proper to arrest, or at least develop abnormally, has a knock-on effect on production or transduction of the putative signal which normally maintains the suspensor in its non-dividing state.

It is suggested that this is a likely explanation for the phenotypes of mutants including *sus* and *raspberry* as this model predicts a large number of target genes and so accounts for the relative abundance of such abnormal suspensor mutants observed. The first mentioned model, of disruption to genes involved in signalling *per se*, might still be applied to the *twin* mutants since the lack of a major defect in the embryo proper might suggest that the defect is largely suspensor autonomous, or at least in response to a specific signal to the suspensor. The small number of mutants of this type identified (two) is consistent with the view that such a specific signalling pathway may involve only a relatively small number of genes (Schwartz *et al.*, 1997). There are, however, occasional cellular patterning defects in the embryo proper at the globular stage in *twin1* and this can either be interpreted to suggest that the primary defect is in fact in

the embryo proper, or that the *TWN1* gene product not only signals to the suspensor but also plays some role in the embryo proper (Schwartz *et al.*, 1997).

The only enlarged suspensor mutant for which the disrupted gene has been cloned is *sus2*. Cloning was facilitated by virtue of the gene being T-DNA tagged. Sequence analysis of the cloned gene revealed close homology with the yeast *PRP8* gene. *PRP8* encodes a protein component of the yeast spliceosome complex and is though to play a role in removal of introns from pre-mRNAs (Brown & Beggs, 1992). The identity of this gene is consistent with the model whereby mutants of this type are due to mutations in general "housekeeping" genes, in this case in a basic component of the gene expression apparatus.

One of the *twin* genes, *twn2,* has recently been cloned (Zhang & Somerville, 1997) and found to encode a putative valyl tRNA synthetase gene. They suggest that, as this gene presumably performs an indispensable function in transcription, it may be differentially expressed from the first zygotic division in the apical and basal cells and their derivatives: the apical cell derivatives arrest after one or two divisions and partially or completely duplicated embryos arise from the basal cell derivatives. This again represents an embryonic "housekeeping" type gene, mutation of which indirectly affects embryo proper-suspensor signalling.

asf1 hydra1 double mutants (Section 5.6), the resultant double mutant embryos (Fig. 5.7) arrested at an enlarged globular stage, as *asf1*, but had "raspberry-like protruberances" on their surface, indicating uncontrolled expansion of the protodermal layer: this might be predicted from the affect of the *hydra1* mutation on direction of cell expansion. The effect of the mutations is additive in that aspects of both phenotypes were evident in the double mutant. They differed from *asf1* alone in that the suspensor of the double mutants was consistently observed to enter inappropriate divisions later than the suspensors of *asf1* embryos within the same silique. This lends support to the hypothesis that the primary defect leading to suspensor cell proliferation in *asf1* is embryonic, since *hydra1* itself specifies a normal suspensor: any differences

between the suspensor of double mutants and that of *asf1* embryos alone must, therefore, be indirectly due to the *hydra1* embryo-proper phenotype.

Both of the models described above, namely 1) that the primary defect is in the embryo proper; and 2) that the defect is in signalling between embryo proper and suspensor, seem much more likely than the suggestion that the genetic defect is suspensor-autonomous, since no suspensor mutants have been identified in which the suspensor takes on this type of phenotype in the absence of preceding embryonic arrest or abnormality.

7.26 The different phenotypes of the enlarged suspensor mutants might suggest different signalling pathways between embryo proper and suspensor

If suspensor cell division does begin as a result of the failure of production, transduction or perception of a signal from the embryo proper to the suspensor, the position at which suspensor cells begin to divide might, therefore, define a point at which the level of the signal falls below a critical threshold, releasing them from its inhibitory effect. This proposed signalling pathway is likely to involve a number of components in a cascade. It is possible that the different *sus* and *raspberry* alleles and *asf1* could simply represent mutations in genes affecting, either directly or indirectly, different components of this pathway.

What this model does not explain is why, in mutants such as *sus1* in which the suspensor begins to divide in the middle, or *asf1*, in which it begins to divide at the top, cells further down the suspensor do not begin to divide first. If starting to divide represents a point at which the level of the inhibitory signal from the embryo proper drops below a threshold, then why do the cells which are further away from the embryo proper, *i.e.* at the basal end of the suspensor, not always start to divide first? Explanation of this has to postulate the existence of an opposing gradient, initiated at the micropylar end of the suspensor.

This could be either a gradient of intrinsic "dividability potential" of the cells of the suspensor, favouring those nearer to the embryo proper end: cells could be differentially sensitive to the putative inhibitor. Such a gradient could be set up during development of the suspensor, possibly as a result of biochemical gradients established within the single cell zygote and maintained through cell partitioning. Alternatively, it could be that normal inhibition of suspensor cell division is not only in response to a signal from the embryo proper end moving down the suspensor, but also to a signal from the maternal tissue, or simply from the basal cell of the suspensor itself, moving up the suspensor towards the embryo proper. The point at which the suspensor divides would depend on the summation of these two signals in a given cell, determining the total level of inhibition of division.

According to this model, it could be postulated that mutants such as *sus2, sus3, raspberry1* and *raspberry2,* in which division starts at the bottom of the suspensor, simply represent loss of this maternal inhibitory signal. However, this possibility is discounted since the genetic lesion segregates with embryonic and not maternal genotype and because the primary defect is always in the embryo proper and only subsequently in the suspensor. A signal from the basal cell cannot be discounted. Alternatively, it could be that there are common components in a signalling pathway from maternal tissue to both the embryo proper and suspensor, and that there is a receptor to this maternally derived signal in both types of cells, the effect of mutation of which is first evident in the embryo proper and then later in the suspensor, perhaps due to the temporally regulated expression of other genes.

This model need not necessarily imply receptor defects: it could be response to the perceived signal or transmission of the signal are defective in *asf1*. There are examples of transcription factors which move from one cell type to another, including the maize *KNOTTED* protein which moves from inner to outer epidermal cells where it has a determinative role in meristem differentiation (Kerstetter & Hake, 1997). Similarly with some floral organ identity proteins (Perbal *et al.*, 1996). This movement involves movement through plasmodesmata. Alternatively, the signal may be a hormone or, for

example, a component of a protein kinase cascade. The differential response of different cell types may be due to a signal affecting the expression of different genes which have common promoter elements which bind the signalling molecule. For example, many genes have been identified which contain a common promoter motif which defines auxin-responsivity (*e.g.* Liu *et al.*, 1994).

The failure of an inhibitory signal model accounts well for all of the *sus* and *raspberry* mutants. It could also explain the *asf1* phenotype, suggesting that the threshold of "suspensor division inhibitor" is not met in the uppermost cell of the suspensor. Unlike for the *sus* and *raspberry* mutants, however, there is another alternative model which could explain the *asf1* phenotype. This is that the onset of division of the suspensor cells is due not to release of their latent division potential by failure of an inhibitory signal from the embryo proper, but to positive stimulation of suspensor cell division by the over-production of a cell division promoting signal by the embryo proper, again probably as an indirect result of the disrupted embryo proper phenotype.

Why does division proceed along the length of the suspensor once initiated, at whatever point this occurs? Subsequent divisions may represent either successive cells of the suspensor being released from division inhibition exactly as the first cell to divide, or in *asf1*, the putative division-promoting molecule accumulating above a threshhold level. Alternatively it may be that there is some form of positive feedback on division activity, with rapidly dividing cells somehow promoting the division of adjacent cells, through intercellular signalling.

Several gene products are involved in regulating rates of cell division. These include the mitotic cyclins and proteins with which they interact (Hemerly *et al.*, 1993; Doonan, 1996; Doerner *et al.*, 1996; Fuerst *et al.*, 1996). Also, the hormone cytokinin can have a stimulatory effect on rates of cell division. A class of mutants named *titan* have recently been identified (Liu *et al.*, 1997) which produce embryos comprising sometimes as few as 1-2 giant cells, in seeds with highly polyploid but undivided endosperm cells. These are a dramatic example of the importance of the control of cell

division during embryogenesis. It is postulated that they may represent mutation in a gene involved in cytoskeletal function, since they exhibit a failure of nuclear migration.

It is possible that the *ASF1* gene could somehow be involved in down-regulatory control of cell cycling in the suspensor and that when cell cycling is allowed to proceed unchecked in the absence of its product, then this might somehow override morphogenetic signals and disrupt not only morphogenesis of the embryo proper, but also be transmitted to the cells of the suspensor.

7.27 Polar auxin transport may be disrupted in *asf1*

Auxin is important in the establishment of polarity, particularly around the transition stage of embryogenesis. Mutants, including *pin-1* (Okada *et al.*, 1991) have defects in polar auxin transport, resulting in the formation of embryos with a single fused cotyledon and no bilateral symmetry. This is consistent with experiments in which inhibitors of auxin transport resulted in a fused cotyledon phenotype in cultured *Brassica juncea* zygotic embryos (Liu *et al.*, 1993). A model was proposed whereby blocking of basipetal auxin transport leads to it accumulating and diffusing around the apical region of the embryo, promoting formation of a ring of cotyledonary tissue around the top of the embryo. Polar auxin transport inhibitors in the culture medium of wheat embryos *in vitro* resulted in the formation of secondary embryos (Fischer *et al.*, 1997).

That auxin plays an important role in embryonic polarity is well established. Obviously, not all embryonic arrests represent failure of polar auxin transport. However, there are many genes involved in the synthesis, transport and degradation of auxin, and so mutation in such might account for a significant proportion of globular arrests. One mutant which directly links an ectopically divided suspensor phenotype to auxin defects is the auxin-resistent *axr6* mutant (Hobbie *et al.*,1997): this, as well as having a seedling lethal dwarf phenotype, sometimes forms a suspensor of a double file of cells.

That the *asf1* proliferated suspensor is not tumorous, in that it is often of approximately constant diameter along its length (Section 5.2), does suggest some control over radial division and expansion along an apical-basal axis. The globular embryo-proper often elongates to become slightly ovoid, again along this axis. This may or may not relate to polar auxin transport.

7.28 *asf1* was not phenocopied by the exogenous application of hormones and inhibitors to developing ovules of *Brassica napus*

Injection of hormones and inhibitors into the locule of *Brassica napus* siliques had no obvious effect on the embryos developing therein (Section 5.7). It is quite likely that the *asf1* mutation has some effect, directly or indirectly, on intercellular signalling between embryo proper and suspensor. It is likely that disruption of such a signalling pathway by the application of signalling inhibitors ought to phenocopy *asf1*. Why this did not occur is unknown. It might be informative to perform such an experiment on *in vitro* cultured embryos, where the practical problems associated with these injection experiments (Section 5.7) would be avoided. Another approach might be to use antisense technology or, for example, overexpression of hormone precursors/signalling components/inhibitors, under the control of tissue-/stage-specific promoters. It might be possible to use the *EXORDIUM* promoter (Sections 1.1.2; 4.8; 5.7), for example, to specifically drive the expression of such genes in the embryo proper. Cloning of the *EXORDIUM* promoter is currently underway (M. Evans & K. Lindsey, in this laboratory). The *276-1* promoter, similarly, could be used to drive such genes in a suspensor-specific manner (Schwartz *et al.*, 1997). Such promoters could also be used to ablate specific cells, by fusing with a cytotoxic gene such as diptheria toxin A chain (Worrall *et al.*, 1996). It has been suggested that mutants such as *twn2*, in which the embryo proper arrests after 1-2 divisions, might represent a genetic solution to performing embryo proper ablation experiments (Zhang & Somerville, 1997). The gene under the normal control of the promoter tagged in the *276-1* suspensor-specific *gus*-fusion line has been cloned (P. Gallois, pers. comm.) and this promoter might facilitate specific suspensor ablation experiments.

7.29 Programmed cell death, and control of cell proliferation in the suspensor may be regulated independently

The degeneration of the suspensor-derived structure in germinated *asf1* 'seedlings' (Section 5.3) supports a model that control of suspensor cell proliferation and suspensor cell degeneration are regulated independently.

Some mutants have been identified which do exhibit embryonic disruption and suspensor persistence, but not suspensor cell proliferation (Vernon & Meinke, 1995). Their existence suggests that maintenance of suspensor cell identity may be regulated separately from the initiation of apoptosis (Schwartz *et al.*, 1997). It might also be concluded that it is not simply failure of morphogenesis *per se* which releases the latent embryonic developmental potential of the suspensor, but perhaps failure of specific aspects of either morphogenesis or of biochemical processes in the embryo proper. There may even be parallel signalling pathways between embryo proper and suspensor involved in either apoptosis and/or repression of embryonic potential of the suspensor, which could account for the different mutant phenotypes observed.

Programmed cell death (*apoptosis*) has been assumed to be the mechanism by which suspensor cells normally degenerate. Apoptosis is common in animal development, during which it serves to remove unwanted cells during the sculpting of organs, and to remove inappropriately dividing cells which might otherwise form tumours. It is a metabolically active process involving the upregulation of specific genes and associated ultrastructural changes in cells, including membrane "blebbing", cytoplasmic compaction, chromatin condensation and DNA fragmentation (Schwartz *et al.*, 1997). Recent studies have provided positive evidence of apoptosis in the suspensor, including the increase in activity of certain hydrolytic enzymes (reviewed in Schwartz *et al.*, 1997). Genes probably involved in the regulation of apoptosis in plants have been identified. These include the *defender against apoptotic death* (*DAD1*) gene recently identified in *Arabidopsis* (Gallois *et al.*, 1997) which is highly homologous to an animal gene. *DAD1* has been shown to be a negative regulator of cell death in animal cells *in vitro*. Proteins which interact with this gene have also been recently

described (Wiig, 1997). Genes which may be important in signal transduction leading to apoptosis are being studied, particularly in relation to plant responses to pathogen attack, which can cause localised cell death resulting in lesion formation on infected leaves (Dietrich *et al.*, 1994). These signalling pathways may be in common with those initiating apoptosis during plant development - those in the suspensor, in vascular tissue formation and in anther development (Dietrich *et al.*, 1994).

7.30 Suspensor cell identity may be influenced by interactions with both embryo proper and maternal tissue

Normal differentiation of suspensor cells must involve not only the activation of suspensor-specific genetic programmes, but also the repression of embryo-specific programmes. Where this repression fails, as in the case of all of the *sus* and *raspberry* alleles, other mutants of this class described by Marsden & Meinke (1985), the *twin* and *häuptling* mutants and the *asf1* mutant described here, the suspensor cells are able, to varying degrees, to enter a genetic pathway normally restricted to the embryo proper. It is perhaps interesting to note that no mutants have been described which totally fail to specify the identity of the suspensor initially: it might be predicted that some such mutants may be identified. Even *gnom/emb30* , which exhibits extremely aberrant embryonic development from the failure of the asymmetry of the first zygotic division onwards (Section 1.3.1), does specify a normal suspensor, as does *hydra1* (Topping & Lindsey, 1997). It could be, if maternally-derived factors are indeed necessary for suspensor development, that maternal-effect mutations are lethal.

No mutants have been identified in which the suspensor alone exhibits a mutant phenotype. Only one suspensor-expressed gene has been identified by insertional mutagenesis (P. Gallois, pers. comm.). As mentioned above, no maternal effect mutations affecting the suspensor have been identified. These pieces of evidence may be interpreted to suggest that the expression of relatively few genes is necessary for suspensor development and function.

There are only two known mutants which initially specify an abnormal suspensor. The first is *fass*, the suspensor of which has irregularly oriented cell walls and fewer cells, however, they are still recognisable as differentiated suspensor cells on the basis of staining patterns (Torres-Ruiz & Jürgens, 1994). The second is *knolle*, which typically forms a suspensor of only one or two cells (Lukowitz *et al.*, 1996). This is consistent with the role of the *KNOLLE* gene as a syntaxin-related protein involved in cytokinesis (Lauber *et al.*, 1997): again, these are structurally recognisable as suspensor cells.

Mutants which successfully undergo the globular- to heart-stage transition but later arrest generally do not exhibit suspensor proliferation (Vernon & Meinke, 1995). Interactions with the embryo proper are apparently necessary from the globular stage, as suggested by the embryonic defects in *twin,* which exhibits abnormal cell divisions from this stage. *sus*, *raspberry, asf1* and others become defective at the transition to heart-stage, all subsequently resulting in suspensor cell proliferation (Schwartz *et al.*, 1997), suggesting that such interactions are not necessary beyond this transition stage. It could be that symplastic (plasmodesmatal) connections between embryo proper and suspensor become discontinued at this point, although there may still be later apoptosis-inducing signals. During early embryogenesis the whole embryo proper and suspensor cells form a single symplast (Schwartz *et al*, 1997). Symplastic uncoupling of root epidermal cells during development, for example, has been demonstrated (Duckett *et al.*, 1994).There must normally be a boundary to the signals which induce *POLARIS* and *EXORDIUM* expression in the embryo proper and not the suspensor. This boundary may be either a physical one preventing movement of signalling molecules, or a genetic mechanism whereby switching on of these genes in response to the putative signal is repressed in the suspensor as a result of the expression of other tissue-specific genes.

The scarcity of dominant embryo-defective mutations, which would imply maternal effect, suggests that fertilised eggs do not contribute much in terms of stored maternal transcripts essential to early embryogenesis (Meinke, 1991). This is in contrast to the situation in animal embryogenesis: in *Drosophila*, a number of stored maternally

encoded transcripts are essential for establishing embryonic body plan (reviewed in Frankel, 1992). This suggestion of autonomy of the early embryo is supported by the fact that somatic embryos do develop very similarly to zygotic embryos (Zimmerman, 1993), in the absence of maternal influence. It is interesting to note that somatic embryos rarely produce a structurally differentiated suspensor, perhaps indicating the requirement of a maternal factor for suspensor development. That embryonic factor(s) are required is evidenced by the embryo-lethal mutants described, including *asf1*. Support for this need for maternal factor(s) comes from the observation that one cell derived from a symmetrical division in carrot suspension culture strongly expresses the epitope recognised by JIM8 (JIM8+ cells), as do suspensor cells. Only JIM8- cells go on to give rise to "embryonic cell masses" (McCabe *et al.*, 1997) and no structurally organized suspensor development is seen. This cell may show some suspensor-like gene expression character, as evidenced by the expression of JIM8, but it is not structurally recognisable as a suspensor.

7.31 The *asf1* phenotype may be interpreted as an example of heterochrony

Heterochrony is an alteration in the timing of developmental events. This may result in the concurrent appearance of morphological characteristics which are normally temporally and/or spatially separated (Keith *et al.*, 1994). There are mutants which exemply this. *leafy cotyledon1* (*lec1*) is a mutant in which embryonic cotyledons acquire some leaf-like character, including trichome formation. Also, there is expression in the embryo of some genes characteristically expressed in post-germination seedlings, and switching off of some embryo-specific genes (West *et al.*, 1994). This suggests that activation of some embryo-specific developmental pathways must be coupled to the repression of the potential of cotyledons to become leaf-like.

The *det/cop/fusca* mutants also exhibit heterochrony: they continue to exhibit light-growth characteristics (photomorphogenesis) when grown in the dark, in which wild type seedlings exhibit a dark-growth phenotype (skotomorphogenesis) of hypocotyl etiolation, apical hook formation and pale colouration (Deng *et al.*, 1994; Chory *et al.*,

1992; Keith *et al.*, 1994). The dominance of such mutations suggests that skotomorphogenesis involves the negative regulation of photomorphogenesis potential.

Other examples include *embryonic flower* (Sung *et al.*, 1992), in which mutant SAMs enlarge precociously and produce inflorescence meristems on germination. This suggests that the dominant *EMF* allele is required for maintenance of the vegetative state of the SAM. A final example is a mutant designated *pickle*, in which the primary root meristem retains some characteristics of embryonic tissue (Ogas *et al.*, 1997). Excised roots produced callus-like growth which generated somatic embryos. Roots examined *in situ* retained some embryonic character, including accumulation of triacylglycerols, starch grains and transcripts of seed-specific genes. This could be suppressed by exogenous gibberellin, suggesting that *PICKLE* encodes a component of GA signalling of post-embryonic root cell fate.

It could be hypothesised that *asf1* represents an example of heterochrony. It is early embryo-like in its globular shape and persistence of the suspensor. Suggestions that it has also acquired some late embryo-like character is that it accumulates chlorophyll, which then disappears, as in wild type maturation stage embryos; it acquires desiccation tolerence and exhibits a late embryo-like spatially restricted pattern of *POLARIS* expression. These characteristics may be taken to suggest the concurrent expression of early and late embryo developmental pathways. The ectopically-divided suspensor cells exhibit several embryo proper characteristics, as discussed, but also undergo apoptosis in those individuals in which this structure germinates along with the 'seedling proper' (Section 5.3). This suggests the concurrent expression of genes characteristic of the wild type suspensor with wild type embryo proper-specific genes in these cells. It would be very interesting to look at the expression of other genes which are expressed in a temporally/spatially restricted manner throughout embryogenesis.

7.32 It would be interesting to study the expression of early embryonically expressed genes in *asf1*

It would be interesting to investigate the expression of genes which are expressed prior to the stage at which the *asf1* phenotype becomes apparent. It might be hypothesized that the expression pattern of such genes might be altered prior to the stage at which cellular patterning defect become apparent. One such gene, which is expressed in a spatially restricted manner in the embryo from the globular stage, is *STM* (Barton & Poethig, 1993).

SHOOT MERISTEMLESS (*STM*) is a gene of which expression is required for SAM initiation, both embryonically and post-embryonically (Barton & Poethig, 1993). It encodes a protein of the *KNOTTED* class of homeodomain proteins (Long *et al.*, 1996), which are known to be involved in SAM formation (Lincoln *et al.*, 1994; Barton & Poethig, 1993; Section 1.5.1). *In situ* localization revealed that *STM* is expressed from the early to mid-globular stage of embryogenesis (32-64 cell stage), first restricted to one or two cells and by late globular stage in a stripe across the upper half of the embryo. These cells are not clonally related (Mansfield & Briarty, 1991), suggesting that *STM* is switched on by intercellular signalling. It is restricted to the notch between the developing cotyledons from the heart-stage onwards. There is a high degree of correlation between the expression pattern of *STM* and the cells predicted, from histological analysis (Barton & Poethig, 1993) and fate mapping (Furner & Pumfrey, 1992), to give rise to the SAM.

Since *asf1* appears phenotypically normal until the transition stage, it might be predicted that *STM* would be expressed at least until this stage in the correct spatial context. It is suggested that *STM* specifies biochemical differentiation of the SAM in advance of morphological differentiation (Barton & Poethig, 1993). It was considered interesting to investigate whether, indeed, the *STM* gene is correctly expressed up to this stage in *asf1* and also what happens to the expression pattern once the *asf1* embryo becomes phenotypically abnormal. An *STM* cDNA clone was kindly supplied by Dr.

K. Barton (University of Wisconsin, Madison, USA) for use in this experiment. This experiment was unsuccessful and could not be pursued due to time constraints.

7.33 Summary of the *asf1* mutant

asf1 represents a new example of the expanding group of mutants described which arrest at a globular stage and exhibit suspensor cell proliferation. The phenotypes of mutants in this group vary in nature and severity and the extent to which the suspensor is affected. The relatively high frequency of mutants blocked at the transition from globular- to heart-stage (Meinke, 1991) is consistent with the expression of a large number of genes at this critical stage of development. During this stage bilateral symmetry is normally established and the differentiation of primary tissues along the longitudinal and radial axes begins (Mansfield & Briarty, 1991), laying down the basic body plan of the plant ready for further elaboration.

7.34 Final Summary and Future Work

The objective of this thesis was to identify and characterize T-DNA tagged *Arabidopsis* mutants defective in morphogenetic aspects of development. Two such mutants were identified and a phenotypic and genetic characterization has been presented.

Unfortunately, neither gene proved to be caused by a simple T-DNA insertion. Genetic mapping has therefore been initiated as a first step towards cloning the disrupted genes and data should be available soon. This should facilitate cloning by, either targeted transposon tagging (Coupland (1992; Section 1.8.3), or by chromosome walking from known markers close to the map position (Leyser & Chang, 1997).

The value of using marker gene fusion lines to study cellular organization of mutants has been demonstrated.

Particularly in the case of *asf1*, the value of a thorough phenotypic characterization even in the absence of a cloned gene is obvious. It is predicted that many suspensor

proliferation mutants will represent mutation in "housekeeping" genes: this may or may not be the case for *asf1*. Investigation of the phenotype alone has nevertheless yielded valuable information regarding not only gene expression patterns of cells comprising the mutant, but also regarding the regulation of the expression of the introduced marker genes. It can be argued that the phenotype may well be more interesting than the identity of the disrupted gene *per se*.

That *vch1* represents a single gene null mutation was strongly suggested by the identification of a second allelelic mutant, *vch1-2*. Interpretation of the *asf1* phenotype must be "with all the caveats associated with the use of single alleles and [....] insertion mutations" (Zhang & Somerville, 1997), until further alleles should be identified.

A fruitless attempt was made to perform *in situ* localization of the transcript of the *SHOOT MERISTEMLESS* (*STM*) gene in *asf1* during the course of this study. Success of this experiment might yield important information regarding positional signalling within the early *asf1* embryos, prior to the stage at which the mutant phenotype becomes apparent. It would also be interesting to perform such experiments with other genes expressed in a temporally and/or spatially restricted manner during embryogenesis. The use of GFP-fusion genes (Section 1.11) may facilitate the observation of changes in gene expression patterns in real time. It would be interesting to perform the *gus*-fusion gene introduction and analysis on the other suspensor proliferation mutants: also, creation of double mutants of *asf1* and other such mutants might reveal information regarding the disrupted control mechanisms.

Two monoclonal antibodies, JIM 8 and JIM 13, have been identified which recognise epitopes of a plasma-membrane arabinogalactan protein on various specific plant cell types (Pennell *et al.*, 1991, 1992; Southworth & Kwiatkowski, 1996; Toonen *et al.*, 1996) including the suspensor cells. In *Brassica napus* (Pennell *et al.*, 1991) and in *Arabidopsis* from the globular to the heart-stage of embryogenesis both bind specifically to cells of the suspensor and not the embryo proper. It would be interesting to investigate whether the epitopes were recognised on the suspensor of *asf1* in early

embryos, since at this stage the phenotype appears normal in terms of cellular organisation; also at later stages beyond the point at which the mutant phenotype becomes apparent. This experiment was attempted during this study but was not successful. The monoclonal antibodies were obtained from Prof K. Roberts (John Innes Centre, Norwich, UK). It would be desirable to repeat this experiment.

In vitro culture both of excised *asf1* mutant embryos and their potential regeneration, and of wild type embryos in the presence of various hormone and signalling inhibitor compounds may furnish us with further insights.

In the case of *vch1*, it would be desirable to investigate further the organization of the cytoskeleton. It might also be informative to look at the expression levels of known structurally important genes by Northern analysis. Care must be be taken in interpreting the results of such analysis though, since alteration in expression levels of such genes may be an indirect effect of the mutation (Section 7.11).

The possibility that *VCH1* might encode a phytochrome signalling component could be investigated through light-response experiments, such as investigating dose-response relationships to light of different wavelengths. That *VCH1* might be a negative regulator of the expression of a phytochrome which promotes elongation could be investigated by crossing with reduced phytochrome long-hypocotyl mutants (Whitelam *et al.*, 1993) which might potentially compensate for the *vch1* defect. Previously described phytochrome mutants/overexpression studies have not shown such a global reduction in cell elongation, the main effect being on hypocotyl elongation. There are other phytochromes, as yet uncharacterised in their physiological role which could prove to have a more global role in controlling cell elongation.

Were the genes cloned, this might open up new avenues, allowing investigation of the normal cellular expression patterns by *in situ* localization, the effects of ectopic expression of the gene and of the proteins which regulate their expression (Lindsey *et al.*, 1996). It would be especially interesting to clone the *VCH1* gene, since it obviously

plays a pivotal role in cell elongation and we have not really yet gained a significant insight into its role through phenotypic and physiological analysis of the mutant alone.

One unifying conclusion regarding these two mutants is that both may lend support to the concept that the control of body plan, at the level of cellular organization and cytodifferentiation and of spatially restricted gene expression, is supracellular. This is exemplified by the spatially correct expression, in *asf1*, of the *gus*-fusion marker genes, *POLARIS* and *EXORDIUM*, from which it is concluded that the expression of these genes is regulated by intercellular positional signalling. The *vch1* phenotype may also lend support to this concept: the independent regulation of correct cellular patterning and overall morphogenesis in *vch1* may prove to be due to disruption of a gradient in a longitudinal expansion-determining factor at the whole plant (supracellular) level.

The amazing plasticity of plant development has been well documented. One example is the ability of plants to regenerate cells of the appropriate type in the correct position following experimental cell ablations (*e.g.* Scheres *et al*, 1994), and *in vivo* in response to the mechanical damage or removal of whole organs. Another example is the ability of plants to alter their morphology in response to growth in the dark, which must involve the coordinate activation and repression of a number of condition-specific genes. Mutants such as *asf1*, in which inappropriate developmental pathways are activated by mutation will yield valuable insights into the genetic mechanisms whereby alternative developmental pathways are normally repressed in a stage- or tissue-specific manner.

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Appendix 1

Plant growth media

Murashige and Skoog basal medium (Murashige and Skoog, 1962)

Component	mg/litre
ammonium nitrate	1650.0
boric acid	6.2
calcium chloride (anhydrous)	332.2
cobalt chloride.6H2O	0.025
cupric sulphate.5H2O	0.025
EDTA (disodium)	37.26
ferrous sulphate.7H2O	27.8
magnesium sulphate	180.7
manganese sulphate.H2O	16.9
molybdic acid (sodium salt).2H2O	0.25
potassium iodide	0.83
potassium nitrate	1900.0
potassium phosphate monobasic	170.0
zinc sulphate.7H2O	8.6
glycine (free base)	2.0
myo-inositol	100.0
nicotinic acid (free acid)	0.5
pyridoxine-HCl	0.5
thiamine-HCl	0.1

1/2MS10
The seed germination medium 1/2MS10 was prepared by dissolving MS basal medium (half concentration) and 1% w/v sucrose in distilled water with the pH altered to 5.8 using 5 N KOH. The medium was solidified with 0.8 % w/v agar and sterilised by autoclaving.

MS20 medium
MS20 was prepared by dissolving MS basal medium and 2% w/v sucrose in distilled water and altering the pH to 5.8 with 5 N KOH. The medium was solidified with 0.8 % w/v agar and sterilised by autoclaving.

Root culture medium
Root culture medium was prepared by dissolving MS salts with minimal organics (Sigma), 3% w/v sucrose, 100mg/l *myo*-inositol, 1mg/l thiamine hydrochloride, 18µg/l w/v KH_2PO_4 in sterile water and autoclaving.

Callus Induction Medium
Callus Induction Medium was prepared by dissolving Gamborg's B5 medium (at suppliers recommended concentration; Sigma), 0.5g/l MES and 20g/l glucose in sterile water. pH was adjusted to 5.8 with 5 N KOH. The medium was solidified with 8g/l Difco Bacto Agar (Detroit, USA) and sterilized by autoclaving. After autoclaving and cooling to approx. 60°C, 5mg/l silver thiosulphate, 0.5mg/l 2,4-D and 0.05mg/l kinetin were added.

Appendix 2

Bacterial growth media

Luria-Bertani (LB) broth

Component	g/l
bacto-tryptone	10
bacto-yeast extract	5
sodium chloride	10

The pH was set to 7.4 with 1 N NaOH and the broth was autoclaved prior to use.

Appendix 3

gus-fusion marker lines used

The *pΔgusBin19* promoter trap *gus*-fusion lines, *POLARIS* and *EXORDIUM*, were first described in Topping *et al.* (1994), as *At EM101* and *AtEM201*, respectively. Their expression patterns are described in some detail in Sections 4.9 and 5.7.

VS-1 is described in Muskett (1998).

Genetic Mapping Lines

Genetic linkage analysis was performed by crossing plants heterozygous for each mutation with the DP23, DP24 and DP28 multiple tester lines (Franzmann *et al.*, 1995) obtained from the Nottingham Arabidopsis Seed Centre. Each of these lines exhibits 4/5 specific phenotypic traits associated with mutation at genes of known genetic map location. These three lines were chosen as the marker genes represent loci on each of the arms of the five *Arabidopsis* chromosomes.

Line (Stock no.)	Genotype	Phenotype	Background Ecotype
DP23 (N3078)	*chl1-1* (*chlorina*)	Yellow-green plant	Ler
	er-1 (*erecta*)	Reduced petiole length, plant of short stature, short, blunt siliques, thick inflorescence stem, clustered flower buds.	
	cer2-1 (*eceriferum*)	Bright green stem (due to reduced wax layer)	
	tt3-1 (*transparent testa*)	Yellow seeds	
DP24 (N3079)	*er-1* (*erecta*)	Reduced petiole length, plant of short stature, short, blunt siliques, thick inflorescence stem, clustered flower buds.	Ler
	yi-1 (*yellow inflorescence*)	Yellow flower buds, yellow-greyish sharper leaves.	
	bp-1 (*brevi-pedicellus*)	Short pedicel, causing droopy siliques.	
	ttg-1 (*transparent testa-glabra*)	Yellow seeds, no trichomes	
DP28 (N3080)	*dis1-1* (*distorted trichomes*)	Siliques are short, bent and club-like.	Ler
	er-1 (*erecta*)	Reduced petiole length, plant of short stature, short, blunt siliques, thick inflorescence stem, clustered flower buds.	
	clv2-1 (*clavata*)	Club-like siliques.	
	tt5-1 (*transparent testa*)	Yellow seeds	

Linkage analysis on this data is performed using MapMaker/Exp. 3.0 (Lander *et al.*, 1997; Lincoln *et al.*, 1992).