Functional Analyses of the TERMINAL EAR 1-Like RNA Binding Proteins of *Arabidopsis thaliana*

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Abstract

In the Shoot Apical Meristem (SAM) the position at which leaf primordia arise on the periphery, and their subsequent differentiation, have been shown to be (at least in part) to be directed by genetic programs of development. A candidate gene associated with this regulation is *TERMINAL EAR 1* (*TE 1*) a maize gene identified by the irregular phyllotaxy of its mutant lines. Unlike most other genes associated with meristem function, *TE 1* is a novel RNA binding gene of the RRM type. It has been shown to have orthologues in a variety of plants including *Arabidopsis thaliana* as well as unicellular eukaryotes including MEI2, a gene whose product is associated with the regulation of meiosis in *Schizosaccharomyces pombe*.

In order to more fully understand *TE1*'s role, a functional characterisation of two of the so-called Mei2-like genes was undertaken in the model plant *A. thaliana*. These genes are called Terminal Ear-Like 1 and 2 (*TEL1* and *TEL2*). Constitutive overexpression of the cDNA of *TEL2* using the Cauliflower Mosaic Virus 35S promoter (CaMV35S) revealed a phenotype involving an apparently prolonged vegetative phase. However this was only observed in a limited number of lines of the total screened, and the next generation did not reiterate this phenotype. These difficulties were overcome using the LhGpOP construct system for ectopic misexpression in specific domains as well as inducible ubiquitous expression. Ectopic expression of either *TEL* cDNA is shown to lead to a pleiotrophic spectrum of phenotypes, which in general, were associated with reduced determinant development outside the apical meristems and as well as a delayed overall developmental progression. This provided some evidence that the normal function of *TEL* genes within the apical meristems is the repression of differentiation associated with the regulation of plant growth and architecture.

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Abbreviations

2-ME	2-mercaptoethanol
Amp	Ampicillin, followed by concentration in µg/ml
CLSM	Confocal Laser Scanning Microscope
CZ	Central Zone
DEPC	Diethylpyrocarbonate
DEX	Dexamethasone, followed by concentration in µM
DIG	Dioxygenin
DMPC	Dimethylpyrocarbonate
DMSO	Dimethylsulphoxide
dNTPs	deoxyribonucleotide triphosphates
DTT	dithiothreitol
GUS	β-Glucouronidase
Kan	Kanamycin, followed by concentration in µg/ml
Hyg	Hygromycin, followed by concentration in µg/ml
IPTG	Isopropyl-beta-D-thiogalactopyranoside
mg	milligram
µg	microgram
µM	micromolar
ng	nanogram
NaOAc	Sodium Acetate
PCR	Polymerase Chain Reaction
QC	Quiescent Centre
PZ	Peripheral zone
RAM	Root Apical meristem
rpm	Revolutions per minute
RRM	RNA Recognition Motif
RT-PCR	Reverse Transcriptase polymerase chain reaction
RZ	Rib zone
§	Section
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS Polyacrylamide gel electrophoresis
SAM	Shoot Apical Meristem

Spec	Spectinomycin, followed by concentration in μg/ml
SSC	sodium chloride and sodium citrate
TEL	Terminal Ear Like
Tm	Timentin, followed by concentration in μg/ml
Tris	Tris(hydroxymethyl)aminomethane
DAG	Days after germination
xg	multiples of gravitational force
X-Glc	5-Bromo-4-Chloro-3-Indolyl-B-D-Glucuronic Acid
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
ZD	Zone of Differentiation (Root)
ZE	Zone of Elongation (Root)

Chapter 1: Introduction

1.1 An Introduction to Differentiation

In plant embryos, the beginning of differentiation is marked by a prominent asymmetrical cell division of the fertilized zygote creating two unequally sized cells. This first division also marks the beginning of apical basal asymmetry, which eventually leads to the formation of distinct shoot and root domains first in the mature embryo. The process of embryogenesis in animals is also generally the same; asymmetrical cell divisions as well as changes in gene expression lead to the formation of a complex multicellular embryo from a relatively simple unicellular zygote.

However here the similarities end. In most cases the animal embryo is a miniature of the adult, having all the organs and tissues that it will have in maturity. The primary exception are invertebrates which undergo larval or instar stages before maturity. In general though upon hatching or birth animal embryos simply get bigger. The plant embryo, on the other hand, is not a preformed adult but a 'prototype'. The distinctive architecture characteristic of any plant species only becomes apparent after germination. This distinction is a direct consequence of one of the important differences between animal and plant development. Unlike animals, plants produce lateral organs continuously and repetitively throughout their lifespan. This is partly because, unlike animals, plants continue to increase in size throughout their lifespan and also must be able to replace any organs which may be periodically eaten or fall off (Foster and Veit, 2000). Therefore plants must maintain 'caches' of totipotent cells in structures called apical meristems, throughout their lifespan.

The cells within these domains known as the Shoot and Root Apical Meristems (SAM and RAM) are able to provide a continuous supply of cells, which differentiate into the organs and tissues that compose the growing plant both below and above ground, throughout the lifetime of the plant. In addition to these so-called 'primary' apical meristems there are the secondary or axillary meristems which form post-embryonically as required (Steeves and Sussex, 1989). It is now well established that the apical meristems have key roles in regulating the timing and position at which leaves and other organs

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arise, as well as their subsequent differentiation. This includes parameters such as the distance in space and time between leaves (i.e. the internodal distance), the position of axillary shoots and roots, and details as leaf shape. Therefore it is the meristems' role to enable the progressive elaboration of the characteristic architecture of each plant species post-embryonically.

However, while it is well accepted that this is the case, many details of the way in which these processes are unknown or unclear. For example at the cellular level the process by which cells emerging from either apical meristem become recruited, or 'fated', to form a leaf primordium in the mathematically precise fashion evident in the phyllotaxy of plants remains unclear. Within these incipient primordia individual cells within a leaf primordium will 'decide' to differentiate into the tissue types appropriate for their position whether it be a leaf or flower, and the details of this process is only just becoming clear.

In this chapter an outline of the current understanding in regard to these general questions is provided. Within this overview the genetic and physical factors involved in processes of cell identity and differentiation are discussed in relation to their roles in the regulation of the SAM. The overview concludes with a discussion of the possible role/s of the Mei2-like family of RNA binding proteins in the development of *Arabidopsis* sp.

1.1.1 Phyllotaxy

The mathematically precise position at which leaves arise is highly regular in all plants, and has captured the imaginations of poets and scientists alike for nearly two hundred years (and probably the Greeks, and the Egyptians before them (Adler et al., 1997)). The central fascination of phyllotaxy relates to the observation that the angle of divergence between leaves in many plants, the so-called genetic spiral, is related to the Fibonacci sequence.

Leonardo Fibonacci proposed the following equation in 1201 to account for the monthly growth of the local rabbit population (Adler et al., 1997). Using the number of the months in succession he generated the following sequence, 1,1,2,3,5,8... F_k, F_{k+1} where each term after the second is the sum of the two preceding terms, and F_k is the kth term of the sequence. However

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the Fibonacci sequence can equally used to describe the consecutive units of opposed spirals such as those in the flowers of daisies and sunflowers. For example the equation $360°\lim F_{k-1}/F_{k+1}$ predicts the divergence between consecutive leaves in the genetic spiral to be $137.5°$, which corresponds very closely to that observed.

Ultimately, it was this mysterious mathematical relationship present in phyllotaxis that led to the identification of the SAM and axillary meristems as the source of all plant organs above ground, and provided the impetus to understand their role in the regulation of leaf positioning.

For convenience three broad (but not necessarily distinct) historical approaches to understanding phyllotaxis, and the SAM's role therein, can be delineated (Douady and Couder, 1996):

(1) Geometrical: In the early nineteenth century, attempts to understand phyllotaxis involved analysis of the geometry of the shoot apical meristem from direct observation of leaf positions in mature stems.

(2) Cell division and growth: The cells immediately at the apex divide much more slowly than those immediately around them and their axis of division is perpendicular to the surface of the plant. Below the most apical cells, groups of cells at specific points on the circumference undergo atypical axes of division during mitosis. The axis of division in these cells changes to periclinal or parallel to the surface. Consequently these cells begin to form mounds or extrusions from which the leaf primordia are composed.

On this basis Hofmeister in 1868,(reviewed in (Adler et al., 1997)) proposed a 'dynamic' model to explain the observed patterns of leaf initiation. The formation of leaf primordia occurs in a 'periodic' fashion relating to the rate of mitotic division outside the apex and thus the 'velocity' at which the primordia descend from the apex. The 'plastochron' concept of Richards (Richards, 1948), similarly involves an invariable 'periodicity' between the emergent primordium of one leaf (P0) and the next leaf (termed P1) and so on (Pn+1).

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The dynamic model was further developed by the legendary Snows, and their classic surgical experiments to manipulate leaf initiation. Using the consistent divergence angle between successive leaves arise it is possible to predict the position of the'incipient' leaf primordium. When this incipient primordium (I_1) was ablated in a number of plant species, the position at which the next incipient primordium arose (i.e. I_2) was not affected but the position of I_3 was perturbed: It was found to arise much closer to the now (assumed) extinct I_1 (Snow M. and R., 1931). This seemed to indicate that the position at the primordia arose was affected negatively by the position at which older primordia had already arisen. In other words, periodicity wasn't an essential element of leaf initiation, but a consequence of the repressive influence of older leaves. The position of leaf primordia was and is therefore defined by the availability of space in the apex.

Subsequently Keuhmert (1967) found that when he amputated the third leaf primordia (P3) of *Osmundea* and cultured them in the presence of amputated P10 or P11 primordia they were twice as likely to form leaves, than shoots, than if they were not cultured in the presence of older leaves. Thus the older primordia also appeared to have influence over the organ identity and location of younger primordia. These results, and those outlined above, imply that phyllotaxy as well as organ differentiation probably involved physiological signals that emanate from older leaves.

Consequently this led to a third approach to understanding phyllotaxis, that of the physiology of the apical meristem and in particular the nature of chemical signals between leaf and SAM. While it is now generally accepted that space in the SAM determines the position of the next incipient primordia the question remains as to how or what might influence or regulate leaf initiation. Two particularly influential models are the Generalised Field theory (i.e.(Wardlaw, 1949)) and buckling instability due to differential cell growth on the apical surface (Green, 1976). The key difference in these models is a difference in opinion about the nature of the 'morphogen' or signal that recruits cell/s to undergo organogenesis. In the generalised field hypothesis, the signal was presumed to be a diffusible chemical such as Indole-3-acetic acid (IAA or auxin). Initially it was thought that the older leaf primordia were a source of IAA, which was supposed to inhibit morphogenesis where it was present at

greatest concentration such as the vicinity of more mature leaf primordia. Consequently those cells furthest away from the oldest leaf primordia (where the signal would be most dilute) were more likely to be recruited to form incipient primordia. This appeared to explain why leaf primordia apparently form at the furthest point from more mature leaf primordia.

The 'biophysical' hypothesis on the other hand, was based on the observation that although all mathematical models of phyllotaxy are fairly accurate, the best predictions come from models based on cell volume. This suggested that the physical pressures exerted on expanding cells by their 'cellulose corsets' act as the morphogen that 'informs' cells of their fate. Indeed the biophysical forces 'experienced' by the cell, precede biological signals such as hormones (i.e. IAA) and the movement of RNA and protein (Green, 1994). Increased frequency of division and expansion of cells 'moving' away from the shoot apex was proposed to lead to "wrinkles" or mounds of cells resulting not from changes in cell division but intense torsional stress. The cells at the position of highest stress react to this pressure by dividing parallel to the surface of the meristem creating cells whose progeny begin to project outward and so form a new leaf primordia.

Supporting evidence for both models will be discussed in the following sections. This preceeding section has looked at how the study of phyllotaxy, specifically leaf initiation, altered the perspective of investigations into plant development. The focus turned from plant architecture as a whole, to specific sets of cells in tiny domains at the apex and their physico-chemical interactions. The unique cytological characteristics of the SAM and RAM are the subject of the next section. Subsequent sections will briefly touch on how these characteristics are are thought to relate to the function of these meristems.

1.1.2 Cytohistology of the SAM

Despite the huge variety apparent in plant architecture, the general organisation of the SAM is highly conserved amongst plant species. Histologically and cytologically the SAM is highly distinctive from the regions which subtend it (Steeves and Sussex, 1989). In angiosperms the shoot apical meristem is usually composed of two or three distinct layers. The first or

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outermost layer/s are the 'tunica' while more internal, less stratified, cells are termed the 'corpus' (Schmidt, 1924). The tunica is composed of the epidermal layer (L1) and sometimes there is a sub-epidermal layer (L2). In both layers, division is almost entirely anticlinal or perpendicular to the surface. The innermost cells (L3) by contrast appear to divide more or less in random axes, and these form the corpus or body.

The significance of this layered arrangement in the SAM was not readily apparent though it did seem likely to be involved in the specification of cell fate. Careful study of graft hybrids and naturally variegated plant species indicated that the layers to a large extent, represented separate clonal lineages (Tilney-Basset, 1986). Could genetic variability between lineages actually be the source of information that predetermined the 'fate' of cells, the position of leaf primordia and in turn explain the peculiar cytological organisation of the shoot apical meristem? Cytochimeras were one approach taken to studying this phenomenon. Cytochimeric SAM's are composed of groups of cells, which are marked in some fashion by manipulations that in turn generate genetic mutations which visibly alter their morphology or other characteristics. An example of this are the so-called periclinal chimeras where one layer of the meristem is genetically distinct from the subtending layer/s leading to variegation.

The phenomenon of variegation is a natural example of cytochimeric plants. The Pelargoniums described by Baur for example were mosaic for green or white plastids in the L3 and L2 respectively (reviewed in (Steeves and Sussex, 1989)). However while plastid variegation was useful to study the contributions of the lower layers to the lateral organs, it isn't possible to determine the L1 contribution because this lineage does not contain differentiated plastids. Therefore in order to visualise the contribution of all the layers, Satina, Blakeslee and Avery (Satina et al., 1940) applied colchicine to *Datura* meristems. Individual layers were thus marked by polyploidy and contained correspondingly enlarged nuclei. The key observation in this work was that that all three layers contribute to the tissues of a lateral organ, corresponding to the contributions observed in variegated leaves. However despite this, the actual contribution of each layer to the various tissues of a leaf

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was not invariable. Infact they varied within members of a plant species and between leaves of an individual plant.

This in turn meant that cell lineage in the SAM (i.e. L1, 2 or 3) does not equal cell fate, and not dictate whether any individual cell was 'destined' to be incorporated into a leaf primordium (or any lateral organ). The question remained, therefore, if genetic differences did not determine relative contribution by any lineage or indeed any one cell within a lineage, then what does. The beginnings of an answer to this question came from the analysis of a large number of plant species in which individual cells within a plant embryo were mutated and thus marked. To achieve this, seeds heterozygous for albinism were irradiated. This irradiation caused random deletions of sections of the chromosomes contained in some cells of the plant embryos. Consequently, in a subset of such embryo's, the remaining functional *ALBINO* gene locus was eliminated from cells within the L2 or L3 within the shoot meristem. Thus these cells and their progeny could not produce differentiated plastids and would appear to be white in the mature plant. This led to plants which had albino periclinal sectors starting at the SAM and in some cases traversing many nodes of the plant, up into the flower.

The important observation was that where the albino cell arose near or at the apex, the albino region frequently extended from top to bottom of the plant. In contrast much shorter sectors were generated from albino cells induced further away from the apex. Moreover sectors near or at the apex were usually 1/3, or 2/3 the width of the plant, but could abruptly stop, narrow or widen, as the plant grew bigger. This indicated that the contribution of cells to the lateral organs within a lineage, were not equal in the apical meristems. Infact cells at the very apex of the meristem, the so-called apical initial cells, produced most of the plant through their progeny. Furthermore because of the characteristically fractional widths of these sectors there were probably only 3 or 4 such cells in the meristem in each lineage or layer. Because these sectors could widen or abruptly stop it was concluded that these apical initial cells could occasionally be 'dislodged', and be replaced by the progeny of cells in layers below or above it. Thus it seemed that while the angle of division appeared highly regulated, the layers could not be entirely clonal as proposed previously. Furthermore it seemed that the position of a cell rather than its

lineage determines its fate. This was also observed in early studies of rates of division where cells in the putative apex were marked with charcoal (i.e.(Ball, 1960)). Occasionally these cells were displaced away from the apex but the meristem itself didn't abruptly abort. Taken together it was concluded that the function of the meristem is therefore independent of the fate of any cell in the apex (Furner and Pumfrey, 1992; Poethig, 1989; Steeves and Sussex, 1989).

Consequently leaf or meristem 'identity' is not a function of lineage but of position. The purpose of the clearly distinct layers in the SAM is still unclear. One possibility is that it may help cells within the SAM determine their relative positions via intercellular communication, and this will be discussed further in § 1.1.2b.

(a) Domains of Fate

The importance of position, over lineage, implies that any cells fate is dictated to some extent by its distance from the apex. This was also apparent when cell-cycle parameters of cells at different positions in the SAM were examined. On the basis of apparent differences in cell division rate but also cytoplasmic density, Foster divided the SAM into three radial 'zones' or domains (Foster, 1938). These domains also roughly corresponded to the 'role' of each region in the development of the shoot. The cells in the region immediately surrounding the apical initials, as well as the apical cells themselves, were termed the Central Zone. This region is identifiable by its lack of cellular differentiation and considerably lower rates of division. Flanking the CZ is the so-called Peripheral zone (PZ) where the leaf primordia first emerge. The region, which underlies both of these areas, is the so-called Rib Zone or Rib Meristem. From the RM is derived the stem and its internal vasculature (Figure 1.1 C, overleaf).

New leaf primordia of angiosperms are first visible in the PZ as a mound on the flank of the shoot apical meristem (SAM). The cells which comprise this mound are said to have to been recruited to form a leaf and will undergo organogenesis. That is, they and their progeny will eventually form the highly determinate tissues and cell-types characteristic of a leaf. In contrast, cells of the CZ are 'indeterminate' being capable of forming any part of a plant. As the apical initials divide, their progeny are slowly pushed from

the apex, through the CZ, and out into the PZ. During this progession the cells are presumed (from changes in cytohistology) to progressively lose their indeterminate state (Bowman and Eshed, 2000). The basis of the zonation model was that cells undergo a progressive change in cellular morphology and cell cycle parameters, which is in proportion to their distance from the apex. These changes in cell morphology were accompanied by an apparent increase in the rate of division and a decrease in cytoplasmic density, inferring that there is a link between the onset of differentiation in cells and the rate and axis of cell division and expansion.
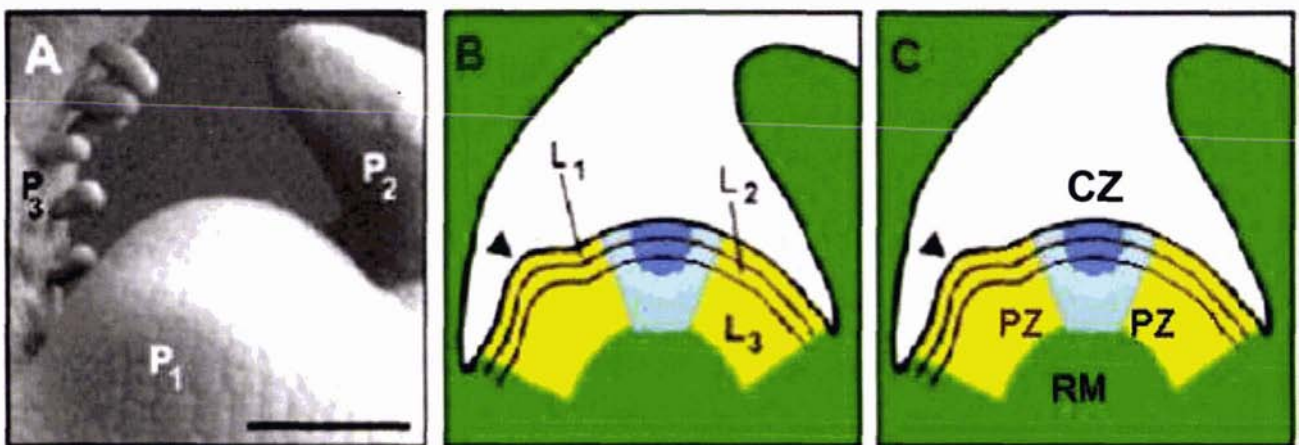
Figure 1.1 Three views of the SAM. (A) Exterior: SEM micrograph of a Tomato SAM. The dome is the SAM and the leaf primordia are labelled P1-3 according to age. **(B) Lineage:** Vertical section of SAM showing the cell layers of the SAM, labelled L1 – 3. **(C) Zonation:** Yellow domains indicate approximate position of the Peripheral Zone (PZ), light blue the area of the Central Zone (CZ) with the apical initial cells highlighted in darker blue. The underlying Rib Meristem or RM is shown in green. In both B and C the arrowhead indicates the position of the emergent leaf primordium or P1 shown in A (Modified from Reinhardt et al (2003)) .

One of the early markers of leaf fate in cells of the peripheral zone is the switch from anticlinal to periclinal divisions. Marked differences in the polarity of cell expansion within groups of cells are also coincident with the formation of primordial clusters (see (Reddy et al., 2004)). Studies of mutants of genes where the regulation of the axis of cell division is specifically altered or abolished (e.g. FASCIATA, KNOLLE and KEULE (Assaad et al., 2001; Kaya et al., 2001; Waizenegger et al., 2000)) indicate that this leads to altered subsequent development of the plant. Studies where cell expansion is specifically manipulated have supported its role both in leaf initiation and its subsequent organogenesis (i.e. (Fleming Andrew et al., 1999)). There is clearly a role for cell division, rate and axis, in regulating organ differentiation and

initiation and this will be further discussed in § 1.3.0. The subject of the next section briefly examines possible mechanisms which individual cells within the apical meristem could precisely co-ordinate their cellular activities both spatially and temporally.

(b) Communication

As mentioned earlier the layered topography of the SAM is now thought to assist cells to determine their position within the SAM by virtue of the movement of information within and between layers (Hobe et al., 2001). The first indication of this possibility were experiments by Szykowiak and Sussex (Szymkowiak and Sussex, 1992). Periclinal chimeras were assembled using different combinations of layers from tomato wild type plants and the tomato mutant *fasciated*. When the L3 was the mutant and the overlying layers were transplanted from wild type the plant developed as the mutant, indicating that the L3 was somehow communicating with the wild type layers, determining their fate.

More recent studies into the mechanism of this communication have indicated that this communication may actually involve the movement of protein and/or mRNA between cell layers. The transcripts of the homeobox gene KNOTTED1 (KN1) for example are normally found in the second inner layer (L2) or the innermost layer and cells (L3) of the SAM. In contrast the protein itself is found throughout the meristem including the outermost layer (L1). The protein of this gene, as opposed to the mRNA, therefore appears to be upwardly mobile within the SAM, able to migrate across cell walls (Lucas et al., 1995).

These observations were further refined by work demonstrating the role of plasmodesmata in the trafficking of different sorts of information between cells. Fluorescent dyes were injected into the apical cells of Birch seedlings and the movement (or lack of movement) of the dye indicated that symplasmic domains existed in the SAM that roughly corresponded to the CZ and PZ. Furthermore these domains appeared to be temporally and developmentally regulated. Cold treatment mimicking winter conditions initiated sealing of the links whereas the initiation of a leaf temporarily caused a brief connection between the CZ and PZ (Rinne and Van Der Schoot, 1998). The

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plasmodesmata are therefore cytoplasmic linkages which appear to function to connect or isolate subsets of cells within specific domains of the SAM.

The trafficking of information may not be restricted to different cell layers of the SAM, but also between different parts of a plant. An elegant experiment involving the grafting of pumpkin scions to cucumber parents, demonstrated the movement of both protein and RNA through the phloem (Ruiz-Medrano et al., 1999). The mRNA of the NACP gene of *Curbita* sp. was found in the phloem, and then at the SAM of the pumpkin graft. A number of different cucumber transcripts were found in the phloem, though most were excluded from the SAM. Thus there was also some specificity in the movement of transcripts to this location from the phloem (Ruiz-Medrano et al., 1999).

The distinctive cell layers of the SAM are therefore thought to assist communication by providing a structure, which allows cells to distinguish their position both radially and apical-basally and thus assist in cell fate. Within the SAM there would be short range signals between cell layers of the SAM. There also seem to be long distance signalling between, for example, between a leaf and the SAM, or between the apical meristems of the root and shoot. Such short and long range communication could function to regulate the recruitment of cells to form primordia and ultimately the organisation of apical basal pattern in the mature plant (Berleth and Sachs, 2001). Speculatively the balance of differentiation and cell division within the SAM (i.e. the length of the plastochron) might be responsive to signals from the RAM regarding growth conditions, and vice-versa.

1.1.3 Cytohistology of the RAM

The focus of this review is primarily leaf initiation, and the role of the SAM. However for the sake of completeness a brief description of the cytological organisation of the Root Apical Meristem (RAM), and the overall root morphology of angiosperms (reviewed in (Jiang and Feldman, 2005) and (Mylona and Dolan, 2002)) will be given in this section.

The area near the tip of the root can be separated into three general domains or zones which are common to angiosperms and many other plant species (Fig 1.2A, pg 12 (Mylona and Dolan, 2002)). These are (in ascending

order) the zone of Meristematic activity (M) which includes the RAM and the Root Cap (RC). Above this is the Zone of Elongation (E) where in which cells leaving the apex begin to expand in size. Finally there is the Zone of Differentiation (D) where the histologically distinct tissues characteristic of the interior and exterior of the root (such as the vasculature and trichoblasts) are evident. All angiosperm roots contain cells arrayed in cell files. These cell files originate from the region just above the RC and appear to be the result of generally highly regulated divisions within cells within the RAM.
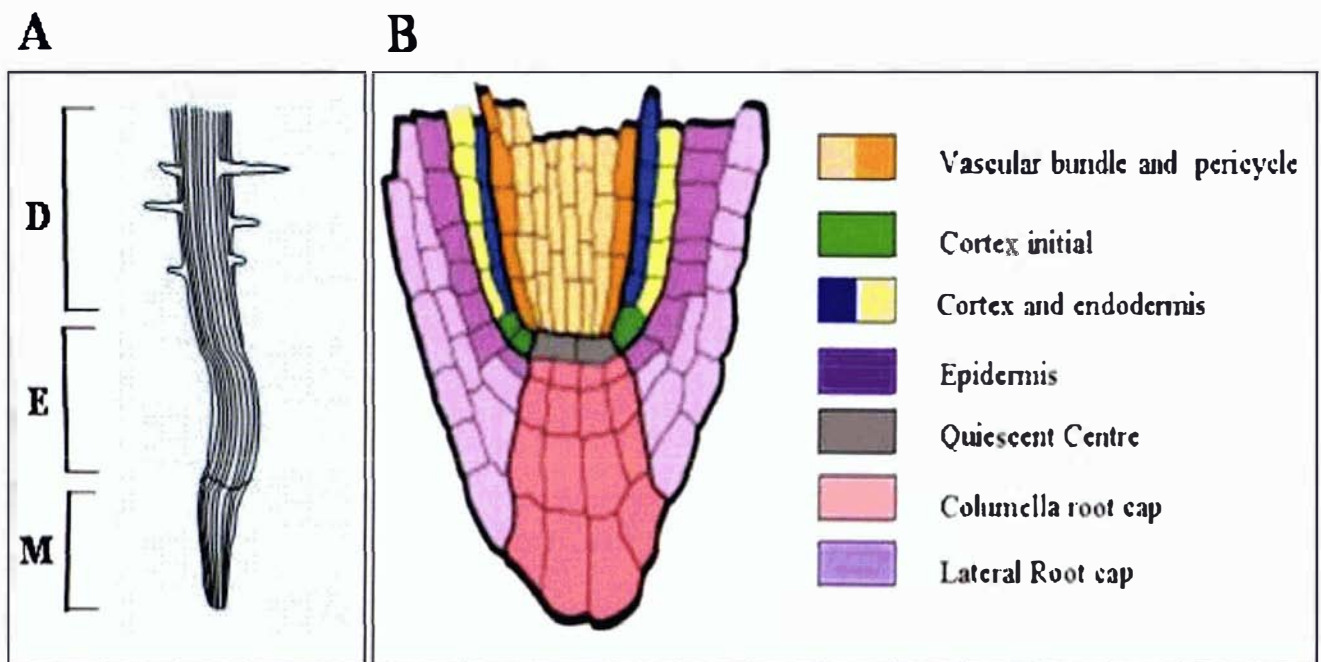
Figure 1.2: Zones and domains of the Root: Organisation of the *Arabidopsis* primary root and RAM. (A) Pictorial representation showing the different Zones of the root: Zones are D, Differentiation, E, Elongation and M, Meristematic (drawn by author). (B) Cytological arrangement of the RAM showing the location of the QC (grey), the Lateral and Columella RC, and the apical initials. The colour code indicates the different tissues of the root (Figure in B modified from illustration taken from **www.bio.uu.nl/mg/pd/research/anatomy.html**).

In *Arabidopsis* the centre of the RAM characteristically contains the four so called 'Quiescent Centre' cells or QC (Figure 1.2 b) which are largely if not entirely mitotically inactive throughout the development of the root (Clowes, 1956). However if manually excised, in most plant species the region of the QC is capable of complete regeneration of a new root (Feldman and Torrey, 1976).

Nevertheless since the QC is mostly mitotically inactive it cannot be the source of the cell files, which compose the root. To fulfil this role, a large

number of so-called 'apical initials' surrounding the QC were hypothesized to fulfil the generative role initially ascribed to the QC (Clowes, 1956). It is the highly regulated divisions with the apical initials that establishes and perpetuates the characteristic pattern of the root and the cell files. Radially, the cell files resolve into concentric rings or layers. The innermost region is the stele, which contains the vascular bundles (i.e. the xylem and phloem) and the pericycle layers, the latter being the starting point for root branches. Beyond that is the cortical layer and endodermis, and outside these layers is the epidermis. The outermost layer is the lateral root cap (Figure 1.2B), which at the apex lies adjacent to the QC and its initials. The apical initials are identifiable on the basis of highly regulated and asymmetric divisions. The result of these divisions is usually one larger daughter cell that will form or enter into a specific tissue layer/cell file, and a smaller sibling that remains an apical initial. For example the epidermis and lateral root cap are formed from periclinal divisions in a protodermal initial. The cortex and endodermis meanwhile, arise from the division of the cortical initials. The cortical initial divides anticlinally creating one larger and one smaller daughter cell. The smaller daughter cell remains a cortical initial while the larger daughter cell undergoes a further asymmetric periclinal division. This generates two cells, one of which will fo   the cortex (Fig. 1.2B previous page, smaller inner cell, blue) and endodermis (outer cell, yellow) (Dolan et al., 1993).

As outlined for the SAM, the identity of central and apical initial cells versus the tissue layers is believed to be based on position rather than lineage (reviewed in (Steeves and Sussex, 1989) and (Jiang and Feldman, 2005)). Recent work using laser ablation has elegantly supported this hypothesis. In these experiments a confocal laser was used to kill individual cells within the RAM in order to examine how this would affect the identity and subsequent division of remaining cells in this domain.

In one such experiment the cortical initials of a root tip were killed. The dead cells were then displaced towards the end of the root by advancing cells from the pericycle, undergoing division. These cells then underwent asymmetric periclinal division's, characteristic of the cortical daughter cell. Furthermore they lost the histological characteristics of their original position (Berger et al., 1998). It seemed therefore that these cells, which had replaced

the ablated cells, had assumed the identity and function of cortical daughter cells. Cell identity in the RAM was concluded to be positionally determined rather than lineage based.

In addition to this, the activities of individual cells depends on their proximity to the apex. When a central cell of the QC is ablated, cells of the Columella RC have been shown to prematurely differentiate based on molecular markers and physiological changes (van den Berg et al., 1997). Thus it appears that the central cells in the QC are able to repress differentiation of the adjacent columella initials by virtue of cell contact. Similarly when laser ablation was used to isolate a cortical initial from three adjacent daughter cells, the initial was still able to divide to produce a further daughter cell but it could not divide asymmetrically to create the cortex and endodermis. Thus it was inferred that contact with the more differentiated daughter cells was important in perpetuating differentiation in the progeny of an apical initial , and that this is based on short range communication requiring cell-to-cell contact (van den Berg et al., 1995).

Although the RAM has considerable cytohistological and morphological differences in comparison with the SAM, there are also profound similarities in their formation and function. The determination of cell identity through position and the role of short-range communication are indicative of this, as well as confirming their general importance in meristem function. The recent identification of specific genes (and gene families) which are expressed in both root and shoot (i.e. *TONSOKU* (Suzuki et al., 2004)) confirms the existence of underlying similarities at the level of the genetic program of development.

1.2 The cell cycle and the SAM

Some of the notable distinctions between cells in different domains of the shoot apical meristems are, polarity, the frequency of mitotic division, and the rate (and direction) of cell expansion. Changes in these parameters are most obvious in cells that are recruited to form leaf primordia. Such cells switch from predominantly anticlinal division to periclinal, and begin to expand vertically rather than horizontally. The new leaf primordium thus emerges from the flank of the SAM.

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Because plant cells are relatively fixed in place by rigid cell walls, and programmed cell death is uncommon as a mechanism of development, it seems obvious that cellular differentiation and organogenesis should be strongly influenced by the progression and parameters of the cell cycle. Indeed there is some evidence in the literature which supports this presumption, primarily in the description of mutants thought to be defective in one or several aspects of the cell cycle.

1.2.1 Axis and frequency of cell division

Arabidopsis seedlings which contain mutant alleles of both *KNOLLE* *(KN)* and *KEULE* *(KE)* (Assaad et al., 2001; Heese et al., 2001; Lauber et al., 1997; Waizenegger et al., 2000) cease to grow very early in development. Their organogenesis is either highly aberrant, or absent, and is associated with the presence of multinucleate overly large cells. *KN* and *KE* are therefore thought to be involved in the completion of cytokinesis, specifically the formation of the cell wall after cell division.

Maize lines containing mutant alleles of *LILIPUTIAN* (Dolfini et al., 1999) have an over large root, and crinkly leaves, all of which appear to be due to increased cell divisions, altered plane of cell division as well as expansion. Similarly two *Arabidopsis* mutants, *tonsoku* (*tsk*, (Suzuki et al., 2004)) and *fasciata* (*fas1/2*, (Kaya et al., 2001; Suzuki et al., 2004)) also have disordered planes of divisions in the SAM and RAM, disturbed morphogenesis, as well as overall architecture. *Tsk* plants are characterised by having shorter roots and fasciated stems which seems to be the result of increasingly random planes of cell division within both the shoot and root meristems. This causes a profoundly disorganised corpus and tunica in both meristems and eventually highly indeterminate architecture and growth of the seedling.

On the other hand mutants have also been described as having defects in the cell cycle, but the overall effect is not as disastrous as described above (Fleming, 2002; Reddy et al., 2004). The *Arabidopsis tonneau* mutant has defective cell division. Mitotic cells in the shoot and root apex of *ton* seedlings lack any kind of organised cytoskeletal structure and fail to form the pre-prophase band that defines the axis and position of cell division (Traas et al., 1995). Consequently the cell shape, and axis of division, in these cells is

highly irregular. Nevertheless the cellular histology, organ specification and phyllotaxy are not different to that of the wild type plants(Traas et al., 1995). A similar, if less extreme, example is that of the maize gene *TANGLED1*. The *tangled1* mutant alleles have normal transverse cell division, but atypical planes of division in the longitudinal plane(Smith et al., 1996). Nevertheless leaves are relatively morphologically normal if much smaller and rougher in appearance.

In order to examine the effect/s of modifying cell division more closely other investigators have sought to directly alter individual parameters of the cell cycle using gain-of-function studies. The overexpression of cell cycle regulators, such as the D-cyclins which are known to regulate the commitment of cells to division, are one example (Cockcroft et al., 2000). Unexpectedly this does not directly affect morphogenesis or phyllotaxy. Infact the primary effect observed was accelerated growth of the shoot and root systems, and flowering time. This was found associated with a significant increase in leaf initiation but there were no apparent changes in meristem size or structure. Therefore it appeared that the overexpression of Cyc-D2 lead to decreased plastochron length but no other changes within the meristem, or in the morphology of the plant overall. More localised increased cell divisions within the SAM itself lead to increased cell numbers, but also do not alter subsequent morphogenesis or meristem function (Fleming, 2002; Wyrzykowska et al., 2002).

On the other hand localised induction of increased cell division in a tobacco primordium on the flank of the SAM, led to reduced laminal expansion. Moreover the effect was more pronounced after, rather than before primordium emergence (Wyrzykowska et al., 2002). Consequently it seems that the effect of increased cell division is most significant only after the specification of leaf primordia, and does not significantly alter phyllotaxy or meristem function. Moreover in both cases the overall morphology and development of the plants is unaffected.

In contrast the constitutive expression of inhibitors of the CDK complex, were found to have more distinct affects on plant development. Overexpression of one such inhibitor, *Kip Related Protein 1* (*KRP1*), led to

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reduced stature and smaller leaves with increased serration along their edges. Furthermore it was reported that apart from reduced cell numbers, the leaves also had larger epidermal and internal cells (De Veylder et al., 2001; Wang et al., 2000). This led to three conclusions. Firstly that *KRP1* (De Veylder et al., 2001) is normally involved in the regulation of cell proliferation within leaves during organogenesis, thus defining the final shape of the leaves. Secondly it suggested that that leaf epidermal cells were able to compensate for their reduced number by increasing their size. Thirdly leaf morphogenesis is regulated both at the level of the individual cell, and also within the organ itself by the rate of mitotic division, (c.f localised increases in cell division).

Taken together these results imply that altered frequencies and axis of cell division and in particular increased cell division (as opposed to reduced cell division) does not in itself necessarily translate into a defective SAM or irregular leaf initiation, though localised effects were observed when cell division is inhibited or accelerated in leaves. A recent study involving a real time lineage analysis of *Arabidopsis* primary floral meristems,(Reddy et al., 2004) indicated that organogenesis does not (as had previously been thought) coincide with increases in mitotic activity. Infact no differences in rates of cell division within the primordia were observed in comparison to those outside the primordium. However it was noted that when cells of the three layers were recruited to form a primordium, these cells synchronised their divisions. Furthermore they underwent co-ordinated changes in the polarity of division and expansion according to their position within the primordium. This may explain why alterations in cell division only have minor effects on leaf initiations and no effect whatsoever on phyllotaxy. It is the co-ordination of the parameters of the cell cycle which is actually significant. So although cell divisions may be altered within a plant (whether local or throughout the plant), if cells are still able to co-ordinate their cell division, leaf intiation and organogenesis will be relatively normal.

1.2.2 Cell expansion and phyllotaxy

The third parameter of the cell cycle, which has been implicated in differentiation and leaf initiation, is cell expansion. The biophysical hypothesis of Green mentioned earlier (§ 1.1.1) was based on the assumption that cell expansion directly affects cell fate in the SAM (Green, 1976). Recently

biological evidence has emerged which supports this hypothesis. The evidence relates to the activities of a super family of wall-loosening agents termed expansins. In order for plant cells to grow their cellulose 'corset' has to be progressively loosened to accommodate cellular expansion. It has been proposed that expansins are the agents of this process since their *in vitro* activity involves the relaxation of the cellulose fibrils of the cell wall (Cosgrove, 2000). Indeed the presence of expansin transcripts and the protein itself appear to predict the recruitment and formation of leaf primordia (Fleming Andrew et al., 1999; Pien et al., 2001; Reinhardt et al., 1998).

If changes in torsional pressure experienced by cells in the meristem are the signal for the recruitment of cells to form leaf primordia, then localised induction of expansins at these locations should be able to generate leaf primordia. Several studies have indeed shown that both constitutive and localised expression of expansins affects both phyllotaxy and organogenesis. Induction of Tomato expansins in cells on one flank of tobacco leaf primordia increased laminal expansion of that leaf, leading to outgrowths reminiscent of a second leaf tip. Local induction of expansin expression within tobacco apices caused the emergence of a normal leaf which subsequently alters the phyllotactic pattern of the whole plant (Fleming Andrew et al., 1999; Pien et al., 2001). Similarly over- and under-expression of one Arabidopsis expansin, AtEXP10, increased and reduced petiole length and rosette leaf size respectively. In addition, from the fifth leaf onwards, the antisense lines had a twisted morphology. The differences in size of the leaves were accounted for by a increase or decrease in cell length particularly in the petiole (Cho and Cosgrove, 2000). These observations suggested that local induction of cell expansion can not only alter phyllotaxy and influence cell fate but also alter the formation of the leaf itself.

Further information regarding the effect of cell expansion comes from mutant alleles for two *Arabidopsis* genes *ANGUSTIFOLIA* (*AN*), and *ROTUNDIFOLA* (*ROT*) (Tsuge et al., 1996) . These mutant alleles were found to cause defective cell expansion, within leaves and other lateral organs, but in different axes of expansion. *An* leaves and floral organs are much narrower than WT. While the total number of cells is the same, cells in the mutant are smaller in the medio-lateral axis and larger in the abaxial/adaxial dimension

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than WT. In contrast *rot* leaves are shorter, smaller, and rounder than WT. Yet again, though the total number of cells is the same, the cells have reduced expansion in the proximal-distal axis (i.e. lengthwise). The double mutant (*an rot*) was found to be the sum of these two phenotypes (Tsuge et al., 1996). This suggested that the development of these two axes was regulated independently. Moreover because these mutants were both recessive it was proposed that these genes negatively regulated suppressors of cell expansion in the different axes.

Apart from defects in the axis of cell expansion additional phenotypes were observed for these mutants. The *an* seedlings have twisted stems, carpels, and two-pronged trichomes similar to that observed in another Arabidopsis trichome branching mutant *zwichel* (Schnittger and Hulskamp, 2002). *Rot* mutant seedlings were also found to have more rosette leaves, and slightly more cauline leaves than WT. Furthermore irregular leaf boundaries apparent in the single *rot* mutant were enhanced in the double an rot mutant(Tsuge et al., 1996).

Subsequently the gene product of *ROT* was identified as a novel cytochrome P450 suggestive of its involvement in hormone regulation of leaf morphogenesis and initiation (Kim et al., 1999). The latter appeared to corrobated by the observation that *rot* mutant lines showed increased leaf numbers, relative to wildtype. The gene product of *AN* encodes a homologue of human CtBPs/BARS, associated with transcriptional repression and Golgi dynamics, as well as containing motifs associated with cell-cycle regulation (Schnittger and Hulskamp, 2002). The aberrant cell expansion phenotype of *an* mutants was found to be the result of apolar arrangement of cortical microtubules, which was resolved by overexpression of *AN* . It therefore was concluded that its role in cell cycle is associated with the organisation and polar arrangement of microtubules within the cell (Folkers et al., 2002; Kim et al., 2002).

The defects present in *an rot* mutant lines, and the results of localised and constitutive expression of tomato EXPANSINS, provides the most direct evidence of the role of cell expansion in organogenesis, leaf initiation and phyllotaxy. Localised induction of cell expansion in the SAM, as well as

within a leaf primordia, has demonstrated that this can both alter leaf organogenesis as well as the signal recruiting cells to form the primordium, regardless of the previously established pattern.

1.2.3 Hormonal regulation of phyllotaxis

Early surgical and pharmacological manipulations implicated plant hormones, in the specification of phyllotaxy, as well as leaf morphology (reviewed in (Steeves and Sussex, 1989),(Lyndon, 1998)). Pre-existing primordia influenced the position of future primordia apparently through a diffusible agent. It is beyond the scope of this review to discuss the various hormones known to be involved in both phyllotaxis and organogenesis. However one of the key suspects implicated as a potential morphogen is auxin (IAA), and this will be briefly discussed.

Initially auxin was a candidate for the role of inhibitor of organogenesis as described in the Fields Hypothesis above. Until relatively recently, auxin was thought to be synthesized in the vicinity of the SAM, the older leaves acting as sinks to into the basal portion of the plant (for example (Sachs, 1993)). Consequently, because auxin would accumulate in the shoot meristem in the absence of developed vasculture, it was believed to suppress organogenesis of leaves in this domain. That is, with the exception of the peripheral zone, at the point farthest from the nearest and oldest leaf where the levels of auxin was presumed to be low. At this location cells could be recruited into new primordia and form the basis of a new leaf.

Recently new evidence has turned this model on its head. Several *Arabidopsis* mutants have been described which are defective in their response to this hormone. *MONOPTEROS*, *BODENLOS*, and *GNOM* (*GN*) mutant lines are all unable to form a proper apical-basal axis (Aida et al., 2002; Busch et al., 1996; Hamann et al., 1999). The first two were found to encode positive and negative regulators of auxin responsive genes (Hamann et al., 2002), while *GN* encodes a gene that regulates the asymmetric positioning of *PIN FORMED-1* proteins in cells (Geldner et al., 2003). *Pin1* mutants in turn are known to be deficient in auxin efflux (and thus deficient in the removal but not influx of auxin) and form leaves which were larger than normal (Okada et al., 1991). The correct translocation of auxin was therefore clearly important in

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the establishment of the apical basal asymmetry as had always been suspected. However the defects present in the *pin1* mutants and their ascribed cause suggest that auxin acts positively to recruit cells to form leaves rather than negatively. Consequently when auxin was directly applied to the predicted position of the first incipient primordium (I_1) of tomato apices, this elicited larger leaves with thicker petioles than usual, phenocopying the pin-1 mutants (Reinhardt et al., 2000).

Infact any kind of interference with the movement of auxin both within the meristem, and between apical meristems inteferes or abolishes both phyllotaxy and organogenesis. This is most aptly demonstrated in tomato seedlings transplanted to grow in tissue culture in the prescence of an inhibitor of Auxin transport (1-naphthylphthalamic acid or NPA). These seedlings produce no further leaves and resemble a pin. The shoot apical meristem of the so-called NPA 'pins' appears to be relatively normal apart from their size and lack of expansion. However the application of Auxin on the flank or at the tip of the shoot apex of the pins was found to induce leaf primordia at a position corresponding radially to the point of application but at an exact distance from the centre of the SAM. Clearly therefore, application of external auxin acts as a signal to recruit individual cells, while its local concentration determines the total number of cells recruited to a new leaf primordia. Furthermore, it is seems that cells in the apical region only become responsive to Auxin at a fixed distance from the apex, and that this region probably corresponds roughly to the peripheral zone described earlier in the Zonation model (Reinhardt et al., 2003; Stieger et al., 2002).

In summary Auxin has been shown to have several positive roles in the SAM. It promotes organogenesis, regulates differentiation by assisting the formation of the apical-basal axis, and has an instructional role in the determination of phyllotaxy (Reinhardt et al., 2003). Auxin in the meristem is the signal to recruit cells to form leaf primordia and also where these primordia should be located on the radial axis. The precise distance at which cells become competent to receive these instructions does appear to be independent of the location of the hormone provided. The fixed nature of this distance is believed to be directed by the genetic programme of development within the

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SAM. Investigations in the key players in this genetic programme are discussed in the next section.

1.3 Genetic programs of development

The acquisition of fate, as previously described, is first expressed in cells of the apical meristem/s by changes in the cell-cycle, particularly in the SAM. Of the three parameters of the cell cycle discussed, alterations in cell expansion were found to most profoundly affect leaf initiation and organogenesis. Moreover leaf initiation (and presumably cell expansion) appears to be determined by the responsiveness of cells to the presence of plant hormones such as auxin, which in turn appears to be genetically determined. Cell fate is considered therefore an integrated function of its position relative to the apex, physiological signals, and the genetic programme being expressed in the cell (Foster, 2000).

Many genes are now known to be involved both in the specification of the meristematic regions, and their subsequent elaboration of lateral organs and it is beyond the scope of this review to comprehensively review them. Key genes known to be involved in the establishment of the SAM, regulation of leaf initiation and the axial patterning of these primordia are discussed in order to introduce the current understanding of how development in these areas is maintained and regulated.

1.3.1 Meristem identity and maintenance

Many genes have been characterised from mutant lines whose SAM has altered SAM function and/or organogenesis. Despite this, details regarding the pathways within which these genes function, their immediate interactions (upstream and downstream) are not well understood (Lenhard and Laux, 1999; Schoof et al., 2000; Trotochaud et al., 2000). Nevertheless it is generally accepted that genetic programs of development within the SAM involve two fundamental processes. The first is the promotion of meristem formation and identity in a small number of apical cells within a specific domain, and the second is meristem maintenance, against the demands of organogenesis.

In relation to this first process, current models of apical meristem formation (Carles and Fletcher, 2003; Lenhard and Laux, 2003; Tooke and Battey, 2003) invoke a gradual process based on the maintenance and establishment of domains generated by differential gene expression. These domains appear to correspond to those described in the Zonation model. Some of the key genes implicated in meristem identity are discussed in the next sections.

1.3.2 The *KNOX* genes

SHOOTMERISTEMLESS (*STM*) is a member of an extended family of atypical homeobox genes known as the Knotted-like Homeobox or KNOX genes (Hake et al, 2004). As the name implies, mutants homozygous for the severe *stm-5* allele "reveal no evidence [of a] shoot apical meristem", while nonetheless still forming cotyledons that are fused at their petioles. Most plants of this allele die before producing any leaves at all. With weaker alleles such as *stm-2*, ~20% of the plants produce leaves at the position corresponding roughly to the location of the SAM in wild type (Endrizzi et al., 1996). The phyllotaxy is highly disorganised since those shoot meristems that do form, abruptly terminate and reinitiate several times in the region normally occupied by the primary SAM of a wild type plant. Less severe alleles of *STM* also produce axillary meristems but again these terminate after only 2-7 leaves being formed. The leaves produced, like the cotyledons, are fused at their base.

STM is, as mentioned earlier, an *Arabidopsis* orthologue of *KNOTTED1* (*Kn1*) a maize gene originally identified on the basis of the 'grotesque' knotty leaves elaborated by mutants (Freeling and Hake, 1985). *Kn1* is a founding member of this atypical KNOX gene family who are known to be essential to meristem identity and function(Barton and Poethig, 1993; Reiser et al., 2000, Hake et al, 2004). Ectopic overexpression of *Kn1* in maize SAMs led to abnormal lobate leaves and the formation of 'shoot meristem-like' regions on the adaxial surface of these lobes (Sinha et al., 1993). Similarly overexpression of another *Arabidopsis* KNOX gene *KNAT1* generated ectopic meristems and highly lobate leaves (Chuck et al., 1996; Lincoln et al., 1994).

The phenotypes described for the mutant alleles of *stm* imply that this is also the case with *STM;* Defective alleles lead to reduced meristem indeterminacy. As mentioned above partial loss of function alleles of *stm* do eventually manage to initiate a SAM but not at its usual location, and the emergence of leaves is considerably delayed. The fusing of the petioles of the cotyledons means that those leaves which are produced must literally force their way out through this tissue (Long et al., 1996). These leaves are mostly normal in morphology but highly abnormal in position with little or no organised phyllotaxy. The mutant alleles of *stm* therefore contributed to a highly disorganised or absent SAM, as well as a highly irregular phyllotaxy, where leaves were able to form.

Hybridisation *in situ* for the *STM* transcripts led to two key observations. Firstly, the transcripts of STM were initially observed in the periphery of the SAM and only later in the centre. Subsequently *STM* transcripts are produced through out the wild type embryonic and shoot apical meristem, except cells from which leaf or cotyledon primordia would subsequently arise. Thus it would seem that the function of *STM* involves defining the boundary region of the apical meristem (which will be discussed later), but is subsequently also required in the CZ as well. This latter function is inferred from the early termination of lines containing severe mutant alleles. This early termination is consistent with the identity of the apical initial cells of the SAM not being maintained as in wild type, and therefore not proliferating to the same extent. This in turn means that these cells are completely 'consumed' after the formation of only a few leaves.

The lack of expression of *STM* at the position of incipient leaf primordia is highly suggestive of a role in the regulation of leaf position. In the least severe *stm* alleles, the position and timing of leaf emergence is randomised, and much slower. This is consistent with a role for *STM* in regulating lateral organ emergence both spatially and temporally. This decline in expression of *STM* at the position of future leaf primordia is associated with the increased expression of *ASYMMETRIC LEAVES1* (*AS 1*). *AS1* is a Myb gene, and acts to inhibits the expression of KNOX genes such as *KNAT1*, assisting the specification of leaf founder identity in these cells (Byrne et al., 2000; Byrne et al., 2002). Ectopic expression of *STM*, as previously described

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for *KNOTTED1,* is sufficient to induce limited cell proliferation, along with ectopic expression of the KNAT genes (Byrne et al., 2002). Thus it is generally believed that STM plays a role in the specification and maintenance of meristem identity. To date it is not known how the expression of STM is downgraded in specific subsets of cells. The obvious candidate is *AS,* but its mutant lines show similar down regulation of STM at the position of incipient primordia so it cannot be responsible. However one possibility is that this requires a third as yet unknown Myb gene related in function perhaps to *PHAN* (Byrne et al., 2000).

Taken together *STM* is believed to restrict differentiation at the flank, while also promoting meristem maintenance against organogenesis in the central zone (Clark, 2001; Long and Barton, 1998). In regard to this latter activity, *STM* is known to interact with the genes, *WUSCHEL* and *CLAVATA1, 2* and *3* which will be described in depth in the next section. In general terms, *WUS* and *CLV* form a signalling pathway which is generally accepted to directly specify the subset of cells that form the apical initials of the shoot meristem. In this pathway the expression of *STM* is required to maintain normal levels of *CLAVATA3* expression. However the converse is not true: Ectopic localised expression of STM, although able to produce limited proliferation, is not sufficient to induce *CLV* expression outside the apical meristem. Infact co-expression of both *WUSCHEL* and *STM* is required for the induction of *CLV1* and *3* genes outside the apical meristem (Brand et al., 2002; Gallois et al., 2002; Lenhard et al., 2002). Thus while *STM* is required for the specification of the CZ in the apical meristem, it is not in itself sufficient to induce meristematic identity. Therefore *STM* and the KNOX family of genes are believed to be primarily meristem maintenance genes, functioning particularly in the repression of differentiation in favour of proliferation.

1.3.3 The *CLAVATA / WUSCHEL* Signalling Pathway

The genes of the *CLAVATA / WUSCHEL* group interact in a signalling pathway which determines 'apical initial' identity and thus meristematic function as opposed to maintenance. Genetic characterisation of *clv* mutants clearly showed that the genes of this class normally limit meristem growth. Single mutants of all the *CLV* genes and particularly *clv1,* and *3,* produce

phenotypes predominately associated with a grossly enlarged SAM to the extent that it is visible to the naked eye. Leaf initiation is delayed and phyllotaxy is erratic with frequent reversals. To some extent the enlarged meristem alters phyllotaxy because it is much larger than wild type, and there is more space for primordia to arise (Clark et al., 1996; Foster, 2000).

A further consequence of the enlarged SAM is a fascinated stem, and moreover, many more floral meristems (FM) than wild type. Within the FMs there are defects in lateral organ number rather than size. For example normally there are only 2 carpels in a wild type flower, but in a *clv 3* mutant there may be 4-7 carpels, and the silique is frequently distorted because of undifferentiated tissue proliferating within the gynoecium (Brand et al., 2000). These morphological defects in *clv* mutants are associated not just with an increase in meristem size but an increase in cell number in both vegetative and floral meristems (Laufs et al., 1998b). However this increase is thought to be the result of a defect in domain specification (i.e. a broader CZ leading to fewer cells transitioning to the PZ state), rather than simply increased cell division.

Genetic analysis has revealed that the *CLAVATA* genes all act on the same pathway, and that the products of *CLV1* and 3 probably physically interact to form a complex (Lenhard and Laux, 2003; Trotochaud et al., 1999; Trotochaud et al., 2000). However the expression domains of *CLV1* and *3* within the SAM don't appear to coincide. *CLV3* transcripts are found mainly in the L1 and L2 of the central zone, whereas *CLV1* is found in the L3. The expression of *CLV2* in contrast is less restricted, being found throughout the SAM, FM and other tissues. *CLV1* and *2* encode membrane associated Leucine-rich Receptors Like protein (LRR), and kinases, respectively which are associated or involved in many signal transduction systems. *CLV2* although otherwise identical to *CLV1* lacks the kinase activity of *CLV1*. In turn *CLV3* encodes a small secreted protein (Fletcher et al., 1999) which is assumed to be the ligand of the other two. The currently accepted model is that where the proteins of *CLV1* and *2* overlap, they assemble to form a heterodimer in the L3. The product of *CLV3*, the secreted ligand is then somehow able to migrate to join the heterodimer and thus complete the signalling cascade (Sharma et al., 2003).

The importance of this signalling complex and its components relates to its function in the regulation of another component, a gene called *WUSCHEL* (*WUS*). The protein product of *WUS* is a homeodomain transcription factor distantly related to *STM*. It was identified from the inability of its mutant to maintain a functional meristem terminating after a few leaves in a fashion similar to that seen in less severe alleles of *stm*. Unlike *STM* however, the transcripts of the intact *WUS* are normally found in 3-4 cells immediately beneath the putative apical initials in the three upper layers (Mayer et al., 1998). In lines containing the *clv* mutant alleles the expression of *WUS* expands both laterally and horizontally which in turn leads to a broader zone with apical initial identity and thus an oversupply of cells. This in turns creates the oversized SAM and the consequent morphological defects described for the *clv* mutants. In contrast ectopic expression of *CLV3*, alone, is sufficient to completely repress WUS and phenocopy the wus mutant phenotype. Consequently it seems that the intact *CLV* signalling complex is required to restrict expression of *WUS* to its central position below the L3 which in turn restricts apical initial identity to a small subset of cells at the apex of the SAM. It is still not clear how *WUS* is able to exert this effect in the three overlaying cell layers of the meristem. Nevertheless the result is that maintenance of the meristem is compromised, and the cells available for organogenesis are rapidly consumed as demand outstrips supply. This explains why organogenesis ceases very early in these plants and indicates the importance of the interaction of *WUS* with the *CLV* genes to promote meristem function and identity.

While ectopic expression of *CLV 3* is sufficient to completely repress *WUS* (and generate a *wus*-like phenotype) the normal levels of expression of *CLV 3* also require expression of *WUSCHEL* (and *STM*) in order to be maintained (Schoof et al., 2000). Thus all of these genes form a feedback loop, which balances 'stem-cell', or apical initial identity against proliferation and differentiation primarily within the CZ.

1.3.4 MGOUN and Other Genes that Specify the Boundary Domains

As described earlier, leaf initiation occurs in the boundary domain surrounding the CZ termed the Peripheral Zone (PZ) in the Zonation model.

In addition to those genes associated with CZ identity, there are also genes which have been associated with Boundary or PZ identity. The double mutants of *CUP-SHAPED COTYLEDON 1/2* (Aida et al., 1997) and *MGOUN1/2* (Laufs et al., 1998a) for example appear to derive their respective phenotypes from an inability to define correct boundary domains. Initial expression of the *CUC* family defines the divide between the cotyledons and also the future position of the apical meristem. *CUC* is also required for the expression of *STM*, whose expression also defines the boundary domain but is also required for meristem identity. In floral development CUC expression also appears to define the boundaries between lateral organs, including those of the flower.

MGOUN in turn also appears to determine where cells become competent to undergo differentiation by specifying the domain of the boundary or PZ within the meristem. The *mgo* mutants were both identified as having fewer leaves than wild type at 10 days. Subtle defects were also observed in leaf morphogenesis, floral organogenesis as well as stem fasciation. The *mgo* mutants were also found to have larger, more disorganised, shoot apical meristems. This was attributed in part to the accumulation of unspecified cells in the periphery of the meristem. *MGO* was therefore proposed to be involved in the recruitment of cells in the PZ to form leaf primordia by relieving the repression of differentiation exerted by KNOX class I genes (Laufs et al., 1998a).

A third member of this group, *MGOUN3,* has recently been characterised (Guyomarc'h et al., 2004). The *mgo3* mutant alleles demonstrate similar phenotypes to *mgo1* and *2* but also lead to disorganised root meristems, which eventually abort. Hybridisation in-situ analysis revealed multiple foci of *WUS* expression within the SAM, suggesting it was fragmented, rather than enlarged as observed in the *clv* mutants. This along with the histological disorganisation of the meristem was taken to imply that this gene is involved in the regulation of meristem identity. The presence of the LRR motif in the intact MGO 3 gene suggests that this protein interacts with other proteins and may therefore transmit or receive signals that regulate cell proliferation and differentiation.

In summary, a range of genetic and molecular analyses suggests overall that the shoot apical meristem is established progressively. The genes *CUC1* and *2* define the boundaries between the cotyledons and promote the expression of *STM*. Expression of *STM* and other representatives of the KNOX family repress organogenesis in favour of mitosis, and define the location of the apical meristem. Within the apical domain, the expression of *STM* as well as the more localised expression of *WUS,* is required for the specification of the apical initials. In turn the location of the expression of *WUS* within the central zone is regulated directly by that of the *CLV* complex, and indirectly via *STM* and its positive regulation of *CLV3*. Outside the CZ the expression of the *MGOUN* genes defines the domain of the PZ, and thus where cells can become competent to respond to signals such as auxin, as well as appearing to regulate meristem identity in both the SAM and RAM. In the cells of the boundary domain, *STM* expression declines as *AS1/2* are up-regulated and this begins to define the boundaries of the putative organ primordia.

The changes in cell histology seen in the SAM are therefore associated with gradients in gene expression within the cells in the vicinity of the meristems. These gradients in gene expression appear to inform cells of their position within the shoot apex in the apical-basal dimension and consequently their fate. The meristem specific functions of apical initial identity and maintenance are generated by the expression of these genes in specific domains which are themselves specified progressively.

However the functionality of shoot apical meristems also depends on normal organogenesis. This has been observed in the analysis of mutants with defects in the acquisition of polarity in lateral organs, which will be discussed in the next section.

1.4 Axial patterning of the Leaf

In this section the relationship between the acquisition of polarity in lateral organs (i.e. axial patterning), and its significance in the formation and maintenance of apical and non-apical meristems will be briefly examined. Some mutant lines with organ polarity defects specifically in the abaxial-

adaxial dimension will be discussed in this context and their function reviewed. This will lead onto the recently identified contribution of so-called microRNA species to the activity of these genes, and a discussion of their significance in leaf polarity.

The transition of a leaf primordium to mature leaf is associated with the progressive elaboration of asymmetry. Mature leaves have two surfaces, the abaxial (the underside, facing away from the SAM) and the adaxial sides (the upper side, facing the SAM). Externally and internally these 'surfaces' can be identified by the arrangement of tissues. The following sections deal with specific genes identified as having roles in the formation of distinct domains which specify the abaxial and adaxial fates respectively, and how these domains affect meristem function differently.

1.4.1 *PHANTASTICA(PHAN)*

PHANTASTICA encodes a putative Myb domain transcription factor which is required for the elaboration of polarity in lateral organs of Snapdragon (*Antirrhinum majus*). Severe mutant alleles of *PHAN* (Waites et al., 1998) cause radially symmetrical leaves and petals which have been interpreted as 'abaxialised'. The organs possess predominantly abaxial cells types and internal arrangement (Bowman and Eshed, 2000). They also appear to have proximo-distal defects because there is no distinction between petiole and leaf blade. Weaker alleles are characterised by discrete sectors of abaxial tissue occurring on the adaxial surface, bordered by ectopic tissue reminiscent of leaf margins. Strikingly the *phan* alleles are temperature sensitive, and when grown at the non-permissive temperature, both apical and axillary meristems cease dividing suggesting PHAN has roles in both leaf formation and meristem function.

The expression of *PHAN* is throughout the lateral organ primordia, but not the SAM, in a pattern complementary to *AmSTM*. That is, *PHAN* transcripts begin to be detected as *AmSTM* expression declines in the incipient leaf primordia, exactly as described for *AS1* in *Arabidopsis*. Therefore it seems likely that PHAN is involved both in the specification of leaf fate, as well as its subsequent organogenesis. Moreover this appears to involve recruiting cells to

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the adaxial domain and also proximo-distal fate. The specification of these domains in the leaf seems to be required cell non-autonomously for the activity of both the shoot meristem itself and the axillary meristems (Bowman and Eshed, 2000; Christensen and Weigel, 1998; Waites et al., 1998).

1.4.2 *PHABULOSA (PHB),PHAVOLUTA(PHV)* and *REVOLUTA (REV)*

PHABULOSA, PHAVOLUTA and *REVOLUTA* are all *Arabidopsis* genes that encode products which contain motifs characteristic of Class III HD-ZIP genes. Members of this family are characterised by an amino terminal HD-ZIP motif (associated with transcription activation) followed by a domain with similarity to a mammalian sterol/lipid binding domain (Baima et al., 1995; Carabelli et al., 1993; Koizumi et al., 2000; Otsuga et al., 2001; Soderman et al., 1999). In terms of mutant analysis, defective alleles of *PHB* and *PHV* (but not *REV*) are similar to *PHAN* in that they have defects associated with leaf polarity. Both *phb-1d* and *phv* mutants have radially symmetrical leaves, but unlike *phan* these organs are largely adaxialised. With the *phb-1d* allele, the SAM itself is enlarged and the radialised leaves are encircled by a profusion of axillary meristems. Waites and Hudson (Waites et al., 1998) therefore proposed that fully abaxialised leaves as seen in *phan* mutants are insufficient for meristem maintenance in *Antirrhinum*. Conversely, completely adaxialised leaves such as in *phab-1d* mutants, promotes the formation and maintenance of meristems (McConnell and Barton, 1998). Hence the correct organogenesis of leaves requires the acquisition of polarity by the leaf primordium as represented by the emergence of abaxial and adaxial domains. These domains in turn which in turn appear to have opposing roles in relation to the creation of axillary meristems and the maintenance of the SAM itself.

Oddly, missense mutation within the coding regions of the *PHB* gene did not reduce expression. Infact initially it appeared that the mutations in the *phb-1d* mutant alleles had rendered them constitutively active, because these mutations were dominant and all clustered in a particular region of the sterol-binding domain. This was substantiated by the overexpression phenotype of the *PHB* cDNA which, when modified with a similar lesion as detected in *phb-1d*, created a similar dominant phenotype(McConnell et al., 2001).

Recent work has identified that the defect characteristic of this mutant allele prevents the post-transcriptional silencing of the mRNA of *PHB/PHAV*. The site of the lesion in the coding region of *PHB/PHV* appears to be the target of a microRNA whose normal role is to direct the elimination of these transcripts in the abaxial domains of the leaves. Because this region is deleted in the mutant alleles, their expression domains are not limited to the adaxial side of the leaf. Consequently the phenotype observed is the result of this expanded domain of expression leading to the radially symmetrical largely adaxialised leaves (Mallory et al., 2004). This phenomenon will be discussed further in the following sections relating to the functions of RNA binding genes in plant development.

The phenotypic affects of *revoluta* mutant alleles, also known as *interfasiscular fibreless* (*rev/ifl1*), arise during or after bolting since the development of the plant up to this point is indistinguishable from wild type (Talbert et al., 1995; Zhong and Ye, 2001). However after bolting, new rosette leaves are 'revolute' (i.e. downwardly curved at their edges and on their long axis), as well as very long and narrow. The cauline leaves repeat this pattern being revolute and unusually large. The axils of these unusual leaves may have buds but this is increasingly less likely acropetally. Instead they either possess a filamentous or leaf-like structure or are empty. Interestingly, where the axillary bud did form, the leaf, which subtended it, was up to 1/5 smaller than normal. This indicated an antagonistic relationship between leaf size and the formation of the axillary bud. The absence of axillary buds in other places was in part attributed to reduced adaxial/ abaxial identity in the leaves. The stems of the mutants are unusually thickened and a broad range of effects are observed in the structure of the flowers which varies between individual plants (Talbert et al., 1995). In the case of the severe *rev-1* allele, the flower organs range from oversized, (but fertile), to dwarf and incomplete, i.e. floral organs are replaced by tapered filaments, to absent. In some cases the floral meristem abruptly aborts within a couple of internodes of bolting, terminating as a cluster of primordial like knobs. The aberrant leaves produced in *rev* mutants have nearly twice the number of cells of the wild type as well as additional vascular tissue and larger intercellular spaces. The stem was also found to have additional layers of cortical tissue, and the distance between the leaves (i.e. the internode length) was drastically reduced. Therefore in general

REV/IFL-1 was proposed to have reciprocal effects in the later leaves compared to the associated meristem/s. It regulates or represses cell growth and differentiation in the leaf while promoting or maintaining division in nearby meristematic tissue. In the stem, the absence of this activity leads to orderly but increased cellular proliferation, as indicated by the presence of additional cortical layers and defects in vascular patterning.

Subsequently it has been shown that *REV/IFL* null mutants have reduced or ineffectual Auxin transport in their lateral organs and reduced expression of Auxin efflux genes. Furthermore it is possible to generate a phenocopy of the *rev* mutant phenotype in wild type plants using Auxin transport inhibitors (Zhong and Ye, 2001). Thus the function of these genes is associated with the transport of auxin within the leaves, and stem.

Overall the observed defects in the mutant seedlings of *rev/ifn*, *phb*, *phav* and *phan* indicate that there is a reciprocal relationship between leaf organogenesis (and its tissues) with the subsequent formation of meristematic tissue at their base. Furthermore the polarity of their expression appears to determine the location and identity of adaxial and abaxial domains during organogenesis. In turn, adaxial identity is required for the formation and maintenance of both primary and axillary meristems. Finally, organ polarity, and meristem activity, are associated with the correct translocation of auxin within the leaf, and the plant as a whole.

1.4.3 *ARGONAUTE(AGO)* and *PINHEAD / ZWILLE (PNH /ZWL)*

Initial characterisation of *ARGONAUTE* mutant lines indicated some similarities with the phenotypes observed in the *phb* mutants described above. Instead of cauline leaves, *AGO* plants have filamentous structures which might correspond to radialised leaves (Bohmert et al., 1998). The rosette leaves had less defined abaxial and adaxial identity, were longer, narrower, more pointed and thicker than wild type. Unlike *rev*, however, the leaves weren't grotesquely large but did lack axillary meristems.

In contrast ectopic overexpression of the intact *AGO-1* cDNA led, in some plants, to goblet like leaves and trumpet like petals. The latter being a

deformity strikingly reminiscent of the leaves produced by the Snow's in their attempts to isolate the leaf from the SAM (reviewed in (Steeves and Sussex, 1989)). In addition some lines produced ectopic shoot-like structures possibly indicating ectopic meristematic activity. This lead Bohmert et al to conclude that *AGO* was a factor required for the proper morphogenesis of leaves, which doesn't depend on the presence of a functional meristem (Bohmert et al., 1998). However because of the occasional ectopic meristem, it was also concluded that *AGO* was involved in generating positional information for meristematic identity. Finally because *ago* plants lack axillary meristems and produced filamentous structures instead of cauline leaves, it was inferred that *AGO* has a role in the generation of the adaxial domain in leaves and the determination of leaf polarity.

Genetic analysis has shown that *AGO* activity is partially redundant with a second gene, *PINHEAD/ZWILLE* (*PNH/ZLL*). *Pnh/zwille* loss-of-function alleles cause 'little or no loss of [internal] polarity' in the leaves, but the epidermal cells have little or no recognisable abaxial or adaxial differentiation. The meristem frequently terminates in a pin-like structure, and in general *pnh* mutants lack an persistent apical meristem (Lynn et al., 1999; Moussian et al., 2003; Moussian et al., 1998). The double mutant of *ago/pnh* is substantially more severe. It fails early in embryogenesis to develop bilateral symmetry. Instead cells in the suspensor divide inappropriately creating extra layers of tissue. Germination is considerably delayed (up to 14 days) and the resulting shoot produces 'slender filamentous organs' in a highly disorganised array. These filamentous structures do have stomata on their surfaces, as well as vasculature within, but the latter is disjointed and its individual elements misshapen. Finally, the plant doesn't appear to form a SAM or axillary meristems, nor is *STM* expression detectable during embryogenesis. Consequently *PNH* is also thought to be involved in the activation of *STM*, and the establishment of the shoot meristem. That the "leaves" produced are distinctly abnormal both in phyllotaxy and polarity may in part be due to the absence of *STM* and the absence of an organised apical meristem. However the lack of polarity in these organs in turn also means in turn that the axillary and apical meristems cannot be maintained.

Both *pnh/zwille* and *ago* mutants phenocopy mutants of a third gene called *DICER LIKE1* (*dcl1*) (S. E. Schauer, 2002). *DCL1* is a paralogue of the gene *DICER* which is believed to generate the short interfering RNAs required for post-transcriptional silencing in animal models. Thus *PNH* and *AGO* probably have complementary functions in the metabolism of specific RNA's in particular domains of the plant. Furthermore it has been inferred that *PNH*, like *AGO*, is also required for the domain specific expression of for example *PHB*. In the absence of either gene, polar expression becomes less consistent generating the pleiotrophic phenotypes observed in this mutant (i.e. (Bartel and Bartel, 2003) and see below).

Homologues of *AGO* have been identified in a broad range of eukaryotes and prokaryotes. The motifs, which define members of the Argonaute family, are the carboxyl terminal PAZ and PIWI domains. Some evidence exists that the PAZ domain is important for the binding of RNA (J. - J. Song et al., 2004) but otherwise the biochemical role of either motif is not well defined. Nevertheless it is now generally accepted that the functions of this extended family of genes, (which includes AGO, REV and PHB1D/PHV) involve either the regulation of microRNA metabolism or targeting (Bartel and Bartel, 2003; Liu et al., 2004). This will be discussed in more depth in § 1.4.5.

1.4.4 MicroRNA Mediated Gene Regulation

In the previous section, microRNA metabolism has been invoked as a key regulator of gene expression associated with organogenesis, and specifically acquisition of abaxial and adaxial cell identity. The elucidation of this mechanism relates to the characterisation of another phenomenon, that of sequence specific silencing of expressed genes in RNA interference (RNAi).

This pathway is thought to be an ancient mechanism for resisting viral invasion associated with the accumulation of dsRNA in the cytoplasm (Hannon, 2002). The triggers of silencing are short interfering dsRNAs of ~21-25nt cleaved from the captured dsRNA, by DICER (Ketting et al., 2001). These stRNAs then become the template by which the RNA-Induced Silencing Complex (RISC) identifies the transcript targeted for disposal (Hamilton and Baulcombe, 1999; Hammond et al., 2001; Ketting et al., 2001). The RIS Complex is able to cleave the mRNA while leaving the template intact,

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allowing continuing cycles of degradation (reviewed in (Sontheimer and Carthew, 2004)). The human paralogue Argonaute2 was the first identified component of the RISC (Hammond et al., 2001). Recently *AGO2* has been implicated to be the theoretical 'Slicer' endonuclease, executing the initial cleavage of transcripts within RISC via the PIWI domain (Liu et al., 2004). Other *Argonaute* genes may also undertake a similar function based on their absolute conservation in their characteristic domains but this remains to be determined.

There is evidence accumulating that this RNAi system has been adapted for the domain specific regulation of endogenous gene expression within an organism. First described in *C. elegans* this regulation was shown to occur from the expression of two short non-coding RNA's (i.e. lin-4 and let7) which shut down expression of specific genes during larval development (Grishok et al., 2000). In contrast to RNAi these short RNA's were endogenously expressed, and processed from a longer RNA transcript. However the same components identified as part of the RNAi pathway were also required for this pathway implying a common mechanism. The tiny non-coding RNA's termed microRNAs (or miRNAs) are now known to be part of a much larger class of short non-coding RNA's which are thought to have broad regulatory functions outside those defined for *lin-4* and *let-7,* including translational regulation, ((Lai, 2002) and reviewed in (B. J. Reinhardt, 2002)).

In plant systems, the elements of the RNAi pathway involved in the metabolism of miRNA are conserved, and include homologues of *Argonaute* and *Dicer*. Furthermore some miRNAs identified in plants have striking sequence similarities to existing animal miRNAs (B. J. Reinhardt, 2002). However certain aspects of their function may be specific to plants, such as RNA-directed DNA methylation involved in sustained transgene silencing of plants. In particular there is evidence accumulating that the polar or domain specific expression of genes such as *REV*, *PHAB / PHAV* as well as *CUC1/2* are regulated by the RNA degradation activities of *AGO* as directed by the polar accumulation of specific miRNA elements (Achard et al., 2004; Laufs et al., 2004; Mallory et al., 2004).

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This hypothesis emerged from the identification of two microRNA elements, miRNA165/166, which had direct sequence complementarity to essential domains in *PHAB* and *PHAV* respectively. Overexpression of *PHAB*, modified to remove the miRNA165 target site, was found to recapitulate the mutant phenotype. In addition the position of the point mutation determined the extent of the perturbation suggesting that the complementarily of the miRNA determines the efficiency of transcript degradation (Mallory et al., 2004). Examination of the lesions in the mutant alleles of *PHAB* (as mentioned previously) revealed that they were all deletions near or at the location of the miRNA target site within the Sterol Binding domain of *PHAB*. Consequently the apparent polar expression of *PHAV* and *PHAB* is created by transcript degradation in the abaxial domain and the shoot apical meristem directed by these miRNAs. Furthermore the similarity of the mutant phenotypes of *ago*, *pnh/zll* and *rev* is thought to reflect their inability to restrict the expression of genes such as *PHAB*, *REV* and *PHAV* to their normal domains at different stages of the pathway (Bartel and Bartel, 2003; Kidner and Martienssen, 2004; Reinhardt et al., 2002) as discussed earlier.

The potentially broad importance of miRNAs in the differential regulation of gene expression and its consequential effects on meristem activity and organogenesis is becoming clearer. As will be discussed in the next section there is considerable information on the regulatory activities of RNA binding proteins and their RNA transcripts in animal systems but very little information on this category of proteins in plants. The possible regulatory roles for non-coding RNA's such as miRNA, especially in plants, implies that RNA binding proteins may have important roles in both organogenesis and meristem function.

The remainder of this review will therefore consider the possible roles of RNA binding proteins in general as well as their known roles in plant development. Examples from the literature associated with animal, insect and plant development will be used to introduce the known roles of these genes in other systems. Finally members of a unique family of RNA binding genes, the Mei2-like genes of Arabidopsis will be discussed along with the roles in the regulation of morphogenesis.

1.5 RNA binding proteins and cell fate

RNA binding proteins associated with development have been most thoroughly characterised in animal and insect model organisms. In the literature they have been shown to have many roles in the regulation of the expression of RNA. They assist and supervise the RNA transcripts of particular genes, in all their forms, from synthesis to degradation. Specific RNA binding proteins are known to be required for the transcription, translation, localisation, degradation, as well as stable storage before translation of specific transcripts. RNA binding proteins are therefore involved in all the key processes which affect the level, and location of gene expression within cells, and within population of cells (reviewed in (Federoff, 2002; Moore, 2005)).

1.5.1 The RNA Recognition Motif (RRM)

In terms of protein structure, the motif that 'defines' the RNA binding proteins as a class is the presence of the RNA Binding Domain or RBD. However because RNA binding proteins can have many other domains, the presence of an RBD is not a reliable indicator of function (Federoff, 2002). RNA binding proteins are therefore broadly segregated into catalytic (those that modify RNA directly) and non-catalytic, those which only interact with RNA. Within this latter category there are three sub-categories. These are the RNA-recognition Motif, the dsRBD (double stranded RNA Binding Domain), and the K-homology domain. Of these three the RRM (also known as the RNP-CS domain) is by far the most common in eukaryotic genomes, and in particular the model plant *Arabidopsis* sp. (Bandziulis et al., 1989; Burd and Dreyfuss, 1994; Federoff, 2002; Lorkovic and Barta, 2002; Varani and Nagai, 1998).

Typically both the sequence and the spatial structure of the RRMs are highly conserved between proteins from highly divergent species. All RRM domains consist of a 4 stranded antiparallel β-sheet with 2 α-helices that lie on one face of the sheet (Burd and Dreyfuss, 1994; Lorkovic and Barta, 2002). Comparison of the structure of seven RRM domains from RNA binding proteins whose structure has been solved, indicate that both the helices and sheet are very highly conserved (Jeffares, 2001). Instead variability generally

occurs in the regions that connect these structures so presumably the function of these RRM regions is closely linked to the structure, and this function is highly conserved amongst eukaryotes. The structure determined for one of the only RNA binding proteins so far crystallised interacting with its cognate RNA species (The 2 RRM *SEX LETHAL* from *Drosophila* sp.-see § 1.5.2), indicates that its RRMs interact directly with single stranded RNA and possibly mediate specificity (Handa et al., 1999).

One of the most abundant RNA binding protein complexes in animal cells are the hnRNPs or heterogeneous nuclear RiboNuclear Particles (Moore, 2005; Varani and Nagai, 1998). HnRNP complexes mediate the post-transcriptional modification of most pre-mRNAs through RNA interactions with their RRM domains. A characteristic feature of the RRM domains in hnRNP proteins is that they have different specificities (*in vitro*) individually, as opposed to the full length protein (Shamoo et al., 1995). Thus regions in-between the RRM domains are thought to act as flexible linkers between allowing them to be functionally independent units. In the case of the SEX LETHAL protein, the binding of RNA changes the conformation of the RRM (reviewed in (Penalva and Sanchez, 2003)). Thus it has been proposed that as in dsRBD proteins (i.e. STAUFEN see § 1.5.2), the individual RRMs are functionally independent, but also 'function' differently depending on the particular transcript they have bound (Federoff, 2002).

1.5.2 *Drosophila* RNA binding Proteins

One of the best characterized examples of an RNA binding protein is *SEX-LETHAL* (*SXL*) from *Drosophila* sp. This gene is one of the key players in sex determination and dosage compensation in *Drosophila* (reviewed in (Penalva and Sanchez, 2003)). Apart from promoting female morphological development, it is also involved in dosage compensation. Because female embryos have twice as many X chromosomes as the male, the latter must undergo hyperactivation of gene expression in order to match the female level of expression. The activities of *SXL* in sex determination and dosage compensation are described below along with those of *Staufen,* another RNA binding protein from Drosophila. This will therefore served to introduce examples of three key functions known to be undertaken by RNA binding

proteins within the cells of animal systems. These are alternative splicing, repression of translation, and compartmental localisation of transcripts.

Sxl contains 2 RNA Recognition Motifs (RRM) and a Glycine rich domain implicated in protein:protein interactions. It regulates sex-determination primarily through the creation of sex-specific splice variants of its own transcript and that of two other genes, *TRANSFORMER* 1 and 2 (*tra1* and *tra2*). This requires specific interactions with members of the RNA spliceosomal complex, which alters the processing of the transcripts in either sex. The overall result is that males produce transcripts of these genes that encode truncated and non-functional proteins, whereas in females the transcripts are full length and produce functional proteins. Thus the primary role of these genes is differential splicing of the transcripts of *tra* and *tra2* in order to alter expression of this gene leading to the two different sexes and associated morphological development.

In the case of *Sxl* own expression, the male specific transcript includes the third or 'male' exon 3 that contains a premature STOP signal. The presence of functional *SXL* (as well as a number of other spliceosomal associated genes) excludes this exon in the mature transcript. Therefore functional *Sxl* proteins self-regulate their own synthesis, and that of the *tra* genes, by direct interaction with their transcripts during processing.

The second process that *Sxl* is known to regulate is dosage compensation. One of the genes involved in this is the gene *MALE-SPECIFIC LETHALITY2* (*MSL2*). In this case *Sxl* binds elements in the 3' and 5'UTR of the *Msl-2* transcript and thereby suppresses translation of this gene as well as destabilising the transcript in the female (Penalva and Sanchez, 2003).

Finally the third function identified for some RNA binding proteins is intra cellular compartmental localisation of mRNA. In *Drosophila* embryos the asymmetrical expression of, for example the transcripts of the genes *Bicoid (Bcd)* and *Oskar* (*Osk*), is required to determine anterior and posterior identity respectively. It is the role of *STAUFEN* to chaperone transcripts of these genes to the correct pole of the embryo. In terms of its structure *Stau* is a double stranded (ds) RNA binding gene with 5 dsRNA Binding domains (dsRBDs). Its localisation function in the fertilized egg includes the attachment of the

translationally repressed transcripts of *osk* and *bcd* to different poles of the cell. Specific RBDs are required to bind specific transcripts, but also their subsequent translational de-repression upon arrival at the appropriate location in the cell. Thus *Stau* not only creates the appropriate polar distribution of *bcd* and *osk*, but also is required to overcome their translation repression. Studies of *bcd* localisation lead to a further important observation. Localised expression of *Bcd* requires a 625bp sequence in the 3'UTR of the *Bcd* transcript. However it is the shape of the three hairpin structures formed by this region, rather than its actual sequence, which drives specificity by *Stau*. Thus the affinity for specific transcripts is not necessarily sequence specific, but conformation sensitive and this seems to be a characteristic common to RNA binding genes (Federoff, 2002; Moore, 2005).

Although these examples come from an insect model organism, they serve to illustrate important themes that characterise RNA binding proteins in all eukaryotic systems. Firstly, as stated at the beginning of this section, they participate in the regulation and localisation of gene transcripts both within cells and also populations of cells. Individual RNA binding proteins often (as in the case of STAU) regulate both translation and localisation of multiple transcripts. Because they regulate the expression and localisation of more than one protein they can integrate the activities of multiple pathways. STAU for example regulates the axial differentiation of the embryo, but subsequently participates in neural crest formation through the correct localisation of transcripts of another gene *Prospero*. Biochemically, RNA binding proteins are characterised by high substrate specificity *in vivo*, in that they interact with a limited pool of RNA's. Furthermore individual RBDs within the protein can be dedicated to the binding of specific transcripts alone, or in association with other RBDs. In *SXL* both RRM domains are required for the binding of the *TRA* and *MSL2* transcripts. Finally in many cases the substrate specificity of RNA binding proteins is related to specific elements in their target transcript. However this specificity is dependent less on their sequence and more on the topography of the duplexed RNA in the element.

1.5.3 *Arabidopsis* RRM Proteins

Based on analysis of genomic sequence from *Arabidopsis thaliana* it has been estimated that this model plant has nearly two hundred RNA binding genes containing the RRM motif, about twice as many as any other eukaryotes surveyed so far (Federoff, 2002). Moreover only two of these, *FCA* (Macknight et al., 1997) and *FPA* (Schomburg et al., 2001) have been functionally characterised on the basis of mutant phenotypes. In both cases the mutants of these genes lead to delays in flowering largely through disturbance of the autonomous flowering pathway as indicated by the rescue of this phenotype by vernalization.

Overexpression of *FPA* generated a dominant phenotype of delayed floral transition which closely resembled the mutant alleles of *fpa* (Schomburg et al., 2001). In contrast overexpression of the *FCA* cDNA (as opposed to the genomic region) accelerated flowering in comparison to WT (Macknight, 2002). Both *FCA* and *FPA* are thought to reduce levels of the floral repressor *FLOWERING LOCUS C* (*FLC*) to exert their effect. How these genes regulate the levels of FLC and other unknown targets is unknown but post-transcriptional silencing has been invoked as a formal possibility (Macknight, 2002; Schomburg et al., 2001). In addition to the floral phenotypes described, reduced root length and root branch number in *FCA* mutants imply that *FCA* may also have functional roles outside the floral domain. Other than *FCA* and *FPA* there is very little specific information about the possible functions of RRM RNA binding genes in *Arabidopsis* despite their profound over-representation in the genome of this plant. In particular, to date, there is nothing in the literature regarding their potential roles in meristem function and phyllotaxy.

1.5.4 *TERMINAL EAR 1*: A Novel Maize RRM RNA Binding Gene Involved in the Regulation of Phyllotaxy

An RRM RNA binding gene was identified in a monocotyledonous plant species which has been shown to be involved in both leaf initiation and phyllotaxy. The gene *TERMINAL EAR 1* (*TE1*) was identified by transposon mutagenesis in Maize (Veit et al., 1998). Mutants of *TE1* have a characteristically stunted phenotype with a partial feminisation of the tassel. This phenotype arises because the tassel is enclosed in leaves and resembles an ear. More specifically this 'feminisation' appears to be the result of an irregular internodal length associated with reduced restrictions on leaf initiation. The internodal distance varies enormously but is usually shorter than in wild type lines. Moreover the leaves often appear to initiate at random around the axis of the shoot. Consequently the normally distichous phyllotaxy (leaves present on opposite sides of the stem) usually observed in maize, becomes spiral in these plants. Occasionally two leaves will initiate close enough together to be fused (Veit et al., 1998). Thus TE1 would appear to regulate the length of stem between leaves or length of plastochron, as well as the position and number of leaf primordia.

In situ analysis demonstrated that expression of *TE1* was throughout the SAM. As the sections descended from the meristem this expression resolved into discrete "horse-shoe" bands encircling the stem which persisted until P3 (Veit et al., 1998). Notably expression of *TE1* was absent where the incipient primordia were expected to arise, as well as the positions at which older primordia did arise. The phenotype of the mutant and the localisation of the transcripts suggest that both inside, and outside the apical meristem, *TE1* regulates or represses leaf initiation. Furthermore, it also seems to regulate the position at which leaf primordia arise in both the radial and apical-basal dimensions, meaning it has a role in regulating phyllotaxy in space and time.

When the intact *TE1* gene was cloned it was found to encode a novel RNA binding protein containing three RNA Recognition Motifs (RRM) characteristic of the RRM/RNP class of RNA binding proteins. At that time it was the only RNA binding gene known to be associated with the regulation of

plant phyllotaxy. Moreover the only other characterised RRM protein with significant sequence similarity to *TE1* was Mei2p from *Schizosaccharomyces pombe*. This gene was known for its pivotal role in the switch from mitosis to meiosis, ((Watanabe and Yamamoto, 1994) and see below) a function different to that suggested for *TE1*. Finally the protein sequences, derived from these two genes, were found to have high similarity particularly in the third RRM. Moreover the third RRM of each gene had considerably less similarity than the other two RRMs had with each other, suggesting a distinct and conserved function for this domain.

The significance of these observations will be further discussed in the following section. Mei2p is well characterised both in terms of its function and its biochemical mechanism of action. These mechanisms will be outlined in the following section and provide a background for the possible developmental functions of *TE1* and the family of Mei2-like genes identified in *Arabidopsis* sp.

1.5.5 Mei2p and meiRNA

Mei2p is one of several factors known to be involved in the regulation of meiosis of *Sc. pombe*. However, despite being shown to have a 'critical role' for the entry into meiosis, as well for premeiotic DNA synthesis (reviewed in (Ohno and Mattaj, 1999)) it is still unclear if these are its only functions. *MEI2* was originally identified from fission yeast mutant strains incapable of entering meiosis and thus also unable to sporulate. Subsequently a high copy number suppressor of this allele was identified, and this was found to encode a polyadenylated non-coding RNA, the so-called meiRNA. Though Mei2p naturally has general affinity for RNA's, it binds meiRNA with high specificity both *in vivo* and *in vitro*. MeiRNA was later shown to be required (by association with Mei2p) for meiosis, though only for meiosis I, and not premeiotic DNA synthesis.

Fusion of Mei2p to GFP showed that a fraction of cytoplasmic Mei2p and meiRNA migrated to a specific point in the nucleus early in meiosis (Watanabe and Yamamoto, 1994). This "nuclear dot" was not observed in mutant strains in which the locus encoding meiRNA was deleted, or those

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containing Mei2p modified to be defective in RNA binding. However where Mei2p was still intact, but meiRNA was deleted, Mei2p was still able to execute its role in promoting pre-meiotic DNA synthesis despite seemingly being restricted to the cytoplasm. Addition of a nuclear localisation signal overcame the need for meiRNA but only intact versions of Mei2p were able to form the nuclear 'dot' and undergo meiosis I (Yamashita et al., 1998). Surprisingly deletion of the first 429 amino acids (leaving only RRM3 intact) from Mei2p did not impair its function. In contrast deletion of residues 711 to 750 inactivated the protein, as did any mutation within the RRM3. This lead to the conclusion that the RRM3 is essential for the promotion of Meiosis I though exactly why is unknown.

The role of meiRNA is still speculative but seems to be one of targeting the Mei2p to a specific location in the nucleus via its affinity to RRM3. When Mei2p::GFP and meiRNA were expressed in a heterologous system (i.e. primate COS-7 cells) they also co-localised to a nuclear 'dot' that corresponded to the nucleolus. While Mei2p/meiRNA doesn't migrate to the nucleolus in *Sc. pombe* it still points to a conserved mechanism present in widely divergent eukaryotic lines. It may be that in animal cells Mei2p is indeed involved in pre-mRNA processing (a speculation suggested by its accumulation in the nucleolus of COS-7 cells) or it may simply accumulate in the regions of highest RNA content in the host (Yamashita et al., 1998).

More recently the point of localisation of the Mei2p::GFP in Sc. pombe has been identified in an elegant experiment using artificial chromosomal markers (Shimada et al., 2003). The fusion protein was found to localise to the chromosomal locus that encodes meiRNA. Moreover when this locus was deleted localisation still occurred but to a different position in the chromosome suggesting that meiRNA is not absolutely required.

Regarding the question of how Mei2p executes either of its attributed roles in meiosis, putative protein associates have been identified (Shinozaki-Yabana et al., 2000) which appear to assist in these functions. A novel protein, Mip1p, was isolated as a suppressor of ectopic meiosis induced by high expression of Mei2p. This protein appears to interact physically with Mei2p, and a mutant version, Mip1-15p, appeared to bind more strongly than Mip1p

and thus was able to inactivate Mei2p in-vivo. For this reason it was postulated that its normal role is repression of Mei2p when not required. In turn homologues of Mip1p are found throughout animal and plant systems. Little is known about this gene's function in plants. However in animal systems Mip1 orthologues such as RAPTOR interact with a protein called mTOR whose ascribed function include the regulation of the translational complex and thus the regulation of cellular growth.

The *Arabidopsis* homologue, *AtTOR*, has been investigated. In contrast to the mammalian gene, expression of *AtTOR* was found in apical shoot and root meristems, regions where cell proliferation is linked to cell expansion, but not in differentiated tissues (Menand et al., 2002). Thus there exists the possibility that the pathway by which Mei2p and others appear to participate, is a fundamental pathway in specific eukaryotic cell systems associated with the regulation of both cell growth and cell division.

On this basis and assuming there is some conservation of function, one interpretation of the Mei2-like *te1* maize phenotype and normal expression patterns of *TE1* is as follows: In Maize this fundamental pathway has been adapted to regulate temporal and spatial parameters of cell differentiation and in particular leaf identity of cells within the SAM. This in turn regulates the elaboration of the architecture of the whole plant and its developmental progression. In order to confirm this, a functional analysis of the gene was required, and consequently a model plant system more amendable (than maixe) to molecular genetic techniques was required. The model plant chosen was *Arabidopsis thaliana.*

1.5.6 The Mei2-like Genes of *Arabidopsis sp.*

Searches of genomic sequence databases of *Arabidopsis thaliana* yielded seven MEI2-Like genes (Anderson et al., 2004a; Jeffares, 2001; Jeffares et al., 2004). These were named the Mei2-like family because of sequence similarities to Mei2p especially in the third RRM of the encoded protein (Anderson et al 2004, Jeffares et al 2004). However Mei2-Like genes hwere also identified in other dicots such as *Petunia*, monocots other than

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maize (such as Rice and Wheat) as well as a range of unicellular eukaryotes such as *Plasmodium sp* and *Chaemocystis sp*.

One Mei2-like gene had earlier been identified through its ability to complement a meiosis deficient pheromone receptor mutant strain of *Sc. pombe*. The cDNA of 'Arabidopsis Mei2 Like 1' or *AML1*, when expressed in this mutant strain allowed this strain to bypass a mating type defect (Hirayama et al., 1997). The particular deletion present in the *Sc. pombe* mutant was found to act 'upstream' of Mei2 in this particular meiosis pathway so *AML1* was apparently complementing unknown factors which were required for its function. Apart from casting an intriguing angle on the function of genes in this family it further reinforced the highly conserved nature, and function, of a hypothetical pathway being retained in highly divergent eukaryotic species.

Sequence comparisons of the *Arabidopsis* Mei2-like genes found that they formed two distinct clades. The so-called TE-Like or *TEL* genes were most similar to *TE 1* while the *AML* genes were most similar to Mei2p. In addition to the seven orthologues, truncated versions consisting primarily of the RRM3 have also been identified and named *MCT1* and *2*. Currently it is not known whether or where these truncated forms are expressed in *Arabidopsis* (Anderson et al., 2004a). Additional mei2-like genes have also been identified in rice (*OML1-6*), wheat, tomato, soybean, petunia and maize. Of these, only *OML1* sits with the *TELs* while the rest cluster within the *AML* clade (Anderson et al., 2004a; Jeffares et al., 2004).

As part of the initial characterisation of the *Arabidopsis* Mei2-likes, *in situ* hybridisation analysis was undertaken to determine the domains of expression of members of both clades (Anderson et al., 2004a). The results showed primarily that these Mei2-likes genes are expressed in the shoot apical meristem from a very early stage of embryogenesis. Of the two clades in this family, only the *TELs* showed expression that was restricted to the apical meristems. The *AMLs* are expressed throughout the globular embryo and its subsequent stages. In comparison the expression of the *TEL* transcripts remains generally in the vicinity of the apical or axillary meristem/s throughout embryogenesis and subsequent stages.

TEL1 transcripts were first detected in the late globular to heart stages, in the regions corresponding to the incipient cotyledons and future SAM as well as the putative QC of the future RAM. Subsequently these transcripts were found in a relatively broad domain between the putative cotyledons within the domain of the future shoot apical meristem. In comparison *TEL2* was found in a much more restricted domain within the apex possibly corresponding to the vicinity of presumptive apical initials, and this was maintained after germination. After the vegetative phase both *TEL1* and 2 are expressed in the floral meristem but not generally to specific domains within these domains (Alvarez et al., 2002; Anderson et al., 2004a).

The locations in which the Mei2-likes transcripts are found in *Arabidopsis* embryos and seedlings clearly do not exactly reiterate those observed for *TE1*. However the highly limited expression domains observed for the *TELs* in the shoot apical meristem does provide at least circumstantial evidence that of the Mei2-likes, that the *TELs* may have functions that relate to the meristem and its activities. For this reason, and their sequence similarity to *TE1*, the *TEL* genes were selected for functional characterisation.

1.6   Aims of this Research

At the onset of the current work there was very little known about the *TEL* genes, either biochemically or functionally. The primary aim therefore was to learn more about the genes both in terms of their biochemical properties and in the context of their effect on the development progress of the whole plant.

One strand of the current work was therefore create plant lines in which the *TEL* genes are ectopically over-expressed beyond their normal domains. Changes in the morphology and histology of seedlings from these lines could then be investigated in order to provide information about the function of these genes in their normal domain of expression. Initially this expression would involve constitutive ectopic expression under a viral promoter, but subsequently different expression constructs were used which allowed ectopic expression in localised domains as well as inducible ubiquitous expression.

The second strand of this work was the biochemical characterisation of the *TEL* genes. To this end constructs were created to examine the localisation

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of *TEL2* fused to GFP within the plant cell. Furthermore expression of the cDNA clones of these genes was undertaken both recombinantly as well as *in planta,* to obtain the purified protein products of these cDNAs.

Underlying these approaches, is the hypothesis that the *TEL*s (and by association *TE1*) have role/s in the suppression of cellular differentiation within the meristem, consistent with their previously determined expression domains. In attempting to test this hypothesis it was expected that these general approaches should lead to a functional characterisation of these novel RRM RNA binding genes.

Chapter 2: Localisation of *TEL* within plant cells

Introduction

Initial analysis of the MEI2 gene in *S. pombe* showed that this gene carried out functions in both the cytoplasm and the nucleus (Watanabe and Yamamoto, 1994). However the switch from vegetative to meiotic division required the localisation of Mei2p within the nucleus. This was elegantly shown by the expression of a Mei2::GFP fusion, resulting in the so-called 'nuclear dot', in these cells. The ability of Mei2p to form this dot, and induce meiosis, requires only the region including the third RRM, and not the preceding 2 RRMs or N terminal region. Therefore nuclear localisation, and a functional RRM3, were considered to be tightly correlated with the induction of meiosis by Mei2p (Watanabe and Yamamoto, 1994). It has already been noted that there is a high degree of sequence similarity between the RRMs of the Mei2-likes and Mei2p. Moreover because this similarity is highest in the third RRM (Jeffares et al., 2004), it is suggestive of conserved function within this gene family.

An important aspect of the functional characterisation of the TEL genes is to determine where, and to what degree their protein products were localised both intracellularly and throughout the whole Arabidopsis plant. The determination of localisation to a specific intracellular compartment such as the nucleus would provide evidence for conservation of function. Beyond this, localisation of the *TEL* proteins within individual cells, or within specific domains of the plant, was also expected to be informative about their function. For example if they are detected beyond the known expression domains of their mRNA, then this might imply that the proteins are able to migrate between cells and thus able to exert their effect on a much broader area.

Two approaches to *TEL* protein localisation were undertaken. These were the use of a reporter gene fusion, to examine localisation in a living plant, and the use of antibodies for immunolocalisation. In the first approach the *TEL* cDNAs were to be translationally fused with the reporter gene GFP and

placed in front of the CaMV35S constitutive promoter. In addition to allowing localisation of the GFP fused versions of the *TEL* proteins within the living plant, it was expected that any phenotypes from overexpression would be evident. In the second approach, in order to generate antibodies the *TEL* cDNAs were to be expressed recombinantly and purified using affinity tags. When sufficient protein was extracted this would be used to generate antibodies which would allow *in situ* immunolocalisation within plant tissues. This protein would also allow characterisation of the properties of the *TEL* proteins *in vitro*.

While the affinities of Mei2p are well known, the biochemical specificities of the *TEL* proteins are largely a matter of speculation. It is assumed that the *TEL* proteins would be able bind RNA based primarily on their possession of the RRM motif and their sequence similarity with Mei2p. However there is no biological evidence for this assumption. Using recombinant as well as plant expressed *TEL* proteins it would be possible to gather data regarding the RNA affinities of these proteins.

General Methods

2.1.1 General Culture of *Escherichia coli*

Liquid cultures of *E. coli* (DH5α and BL21 CodonPlus) were grown in Luria-Bertani medium (1% w/v bacto-tryptone, 0.5% yeast extract, 1% NaCl, pH 7.2) (LB) at 37°C shaking at 200 rpm. Solid LB media was poured into Petri dishes and contained 1.5% Agar. Antibiotics were included as selective agents as required. Antibiotic and substrate (i.e. X-Gal and IPTG) stocks were made up at 1000 times the concentration used in the media and were filter sterilized (using syringe fitted 0.22 μm Millex GS filter units (MILLIPORE)) where necessary. Glycerol stocks of recombinant *E.coli* lines were prepared by mixing 0.2 ml of sterile glycerol to 0.8 ml of usually stationary phase liquid culture in a screw-top cryovial, and freezing in liquid nitrogen.

2.1.2 Preparation of Heat shock competent *E. coli* strains

10 Colonies were taken from a fresh overnight plate, and inoculated into 250 ml of SOB medium (2% Bacto-tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCL, 10 mM $MgCl_2$, 20 mM $MgSO_4.7H_20$, 20 mM Glucose) in a 2L flask. The culture was grown with shaking at 250 rpm at ambient temperature (~25°C) until the optical density (OD600) reached 0.5. The flask was incubated on ice for 10 minutes then the cells were harvested at 2500 xg at 4°C. The pellet was then washed and resuspended in ice-cold TB solution (10 mM PIPES, 33 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, pH 6.7, filter sterilised). DSMO was added to 7%, and the cells dispensed to individual microcentrifuge tubes and flash-frozen in liquid nitrogen.

2.1.3 Transformation of E. coli by heat shock

Aliquots of competent cells were thawed on ice, and dispensed into pre-chilled 1.8 ml microcentrifuge tubes containing the DNA to be transformed. The cells were gently mixed with the DNA then left on ice for 10 minutes, then transferred to a 42°C-heated block for 30 seconds. After the heat-shock, the cells were then incubated with 1 ml of LB with shaking for 1 hour at 37°C.

After incubation 250μl and 600 μl of the cells were plated onto LB Agar plates containing the appropriate antibiotic/s and/or substrates. These plates then incubated overnight at 37°C and examined for single colonies the next day.

Agrobacterium Methods

2.2.1 Growth of *Agrobacterium sp.*

Liquid culture of *Agrobacterium* sp. were grown in YEB broth (0.1% (w/v) Yeast extract, 0.5% Peptone, 0.5% Beef extract, 0.5% Sucrose, 1 mM $MgSO_4.7H_20$, pH 7.3) at 28°C and 200rpm shaking for 42-48 hours. Solid YEB media contained 1% (w/v) agar. Antibiotics and glycerol stocks for Agrobacterium lines were made up as described in § 3.1.

2.2.2 Preparation of electrocompetent *Agrobacterium* sp.

A single colony of *Agrobacterium* sp from a fresh plate was inoculated into 100 ml of LBS broth (1% (w/v) bacto-tryptone, 0.5 % yeast extract, 0.5% NaCl, 0.1% glucose) and incubated at 30°C overnight with shaking of 250 rpm. The cells were pelleted (~4000 x g for 5 minutes) and washed three times with 50 ml of ice-cold sterile 10% glycerol. Finally the washed cells were resuspended in a final volume of 1 ml and divided into aliquots of 40μl then frozen in liquid Nitrogen and stored at -80°C.

2.2.3 Electroporation of competent *Agrobacterium* sp.

The competent cells were allowed to thaw on ice and then gently mixed with the DNA to be transformed. This mixture was transferred to an ice-chilled electroporation cuvette with a 2 mm gap (EquiBio Ltd) and incubated on ice for 10 minutes. The chilled cuvettes were then pulsed in a Biorad GenePulser with the preset parameters recommended for *Agrobacterium* by the manufacturer (2.5 kV potential, 25 μF capacitance, 400 Ω resistance). 1 ml of YEB broth was then rapidly added, mixed, and transferred to a microcentrifuge tube and incubated at room temperature (with shaking) for 2-4 hours. Aliquots of this mixture were then plated onto selective media and examined for putative transformed colonies. Candidates were streaked onto a second selective plate and incubated as described earlier.

2.2.4 Preparation of Freeze/Thaw "competent" *Agrobacterium* cells

This method, and § 2.2.5 below, were based on that described in (Holsters et al., 1978). 1 ml of an overnight culture of *Agrobacterium* sp. is inoculated into 200 ml of LB broth in a 1L flask. The cells were then incubated at 30° with vigorous shaking until the OD_{550} is ~0.5-0.8. The pellet was harvested at 4000 x g for 10 minutes, washed in sterile TE (10 mM Tris, 1mM EDTA, pH 7) and resuspended in 1/10th the original volume of LB. The cells were then split into aliquots, flash-frozen in liquid nitrogen, and stored at -80°C.

2.2.5 Freeze/thaw transformation of *Agrobacterium* cells

The competent cells were allowed to thaw on ice and aliquots of 250 µl placed into microcentrifuge tubes and mixed briefly with the DNA to be transformed. The cells were placed on/into ice, liquid nitrogen and a 37°C water bath, for 5 minutes each. 1 ml of LB was added to each tube and they are incubated with general mixing at ambient temperatures (~20°C) for 2-4 hours.

The mixture was subsequently plated onto two solid YEB plates containing the appropriate selective antibiotics, and then incubated for 48 hours at 28°C. Putative transformant colonies were restreaked onto selective solid media as well as liquid culture. The latter was then used for a minipreparation of plasmid DNA to verify the presence of the construct and its sequence.

General DNA/RNA methods

2.3.1 Plasmid DNA extraction from bacterial lines

Plasmid DNA was either obtained by modified versions of Alkaline Lysis (based on (Sambrook et al., 1989) and see below) or using the QIAPrep Spin minprep kit (cat no. 27104, QIAGEN) as per the instructions of the manufacturer.

2.3.2 Alkaline lysis for plasmid DNA extraction

A 5ml LB culture was grown overnight with the appropriate antibiotics was harvested by centrifugation at 2000xg for 5 minutes at 4°C. The pellet was washed with 5 ml of ice-cold STE (10 mM Tris, 1 mM EDTA, 0.1M NaCl, pH 8.0) then recovered by centrifugation. The cells were then resuspended in 200 µl of Solution I (25 mM Tris, 10mM EDTA, 50mM Glucose pH 8.0) and placed on ice. To this 400µl of solution II is added (0.2 N NaOH, 1% (w/v) SDS) and gently mix. Finally 300µl of solution III is added, (3M sodium acetate, 11.5% (v/v) glacial acetic acid) and the mixture was incubated on ice for 5 minutes.

The cell debris was pelleted by centrifugation at 26 800 x g for 5 minutes at 4°C, and the supernatant transferred to a fresh microcentrifuge tube. This was mixed with 900µl of isopropanol at room temperature, and

immediately centrifuged at 20 800 x g for 5 minutes to collect the precipitated DNA. The supernatant was discarded and the pellet rinsed with 1 ml of 70% ethanol, finally centrifuging the sample to remove the ethanol by aspiration. The DNA was allowed to dry at ambient temperatures for approximately 10 minutes. The pellet was dissolved in an appropriate volume (depending on apparent size of the pellet) of 10mM Tris pH 8.0.

2.3.3 Genomic plant DNA Extraction

Whole plants, or tissue samples from whole plants, were harvested and frozen in liquid nitrogen. Tissue was finely ground in approximately twice the volume of liquid nitrogen using a chilled mortar and pestle. The DNA was extracted from the powdered tissue using the DNeasy Plant Mini Kit (cat no. 69104, QIAGEN) as described in the manufacturer's instructions.

2.3.4 Total plant RNA extraction and general precautions

Plant tissue was acquired and treated as described for Genomic DNA extraction. Total RNA was extracted from ~0.1g of finely ground tissue using the RNAEasy Plant Mini kit (cat. no. 74904, Qiagen) according to the manufacturer's instructions using the supplied guanidium isothiocyanate lysis buffer ("RCL" buffer). In addition specific precautions were practised to avoid contamination of the samples with RNase. These precautions were applied whenever RNA was being used or manipulated

Wherever possible, items to be used for RNA work were dedicated to that purpose, meaning that they were never handled without at least one set of gloves and were kept separate from all other pieces of equipment. Aerosol resistant tipped disposable RNase-free pipettes (Molecular BioProducts) were used whenever RNA or RNase free solutions were aspirated. Metal implements (i.e. tweezers and scalpels) and glass containers were baked at 180°C for a minimum of 8 hours. Microcentrifuge tubes were obtained from unopened packages and placed into baked containers. 'RNase free' MQ water was obtained directly from the filtration unit and placed into baked Schott bottles. Both the microcentrifuge tubes and MQ water were then autoclaved at 121°C for 45 minutes.

Plasticware such as the lids and sealing ring from the Schott bottles and the electrophoresis units, were soaked in 0.3% H_2O_2 (MERCK) for a minimum of 8 hours. In the case of the lids they were then wrapped in foil and

autoclaved for 45 minutes. Solutions used for RNA work were made using dedicated chemical stocks and water treated as described above and put into baked glassware.

2.3.5 Quantification of RNA and DNA

Nucleic acids were initially quantified by electrophoresis, and by spectrophotometric methods. In the latter case, DNA and RNA were diluted in quartz cuvettes and the absorbance readings at 260nm and 280nm recorded. These were used to calculate the quantity and quality of nucleic acids. Quantity was calculated according to the formula $C=OD_{260}$ x F x dilution ratio where F=50 for dsDNA or 40 for RNA. Purity was determined by the ratio of 260nm/280nm, and a value >= 1.8 was considered reasonably pure for DNA. In addition a scan of the wavelengths from 220 to 300nm was routinely obtained to confirm purity and quality.

Subsequently spectrophotometric measurements have been performed on the Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies). Instead of dilution in a quartz cuvette, 1μl of the undiluted sample is applied to the light source, and the absorbance determined at 260 and 280nm wavelengths. The same formula and method described above was then used to determining concentration and purity.

2.3.6 Electrophoresis of DNA and RNA

While the quantity of RNA was assessed spectrophotometrically, it was usually also necessary to assess the degree of degradation of RNA samples before proceeding with any manipulations. This was assessed by gel-electrophoresis, electrophorescing 5μl of each sample as described below. The appearance of the ribosomal bands in the minigel, (i.e. fuzzy versus distinct) relative to the marker, were taken to indicate the degree of degradation.

Electrophoresis of nucleic acids was generally preformed in a Biorad mini-sub cell. DNA and RNA were electrophoresed in 0.8-1.5% agarose gels made using 1x TAE (40mM Tris-acetate, 1mM EDTA, pH 8.0). The RNA or DNA was generally compared to 250ng of the 1Kb Plus DNA ladder (Invitrogen Life Technologies) and/or 211ng of a λ HindIII digest as molecular weight markers. Gels were stained in 0.5-5 μg /ml ethidium bromide and

visualised using the Biorad Gel Doc 2000 UV trans-illuminator and Quantity One software version 4.2.1. The preparation of samples for electrophoresis of RNA samples to be used subsequently in a Northern hybridisation differs slightly and will be outlined in the following section.

(a) Electrophoresis of glyoxylated RNA

A 0.1M sodium phosphate buffer (NaPB) stock was prepared by mixing 1 M NaH_2PO_4 (42 ml) and 1 M Na_2HPO_4 (58ml) to make a pH 7.0 buffer then diluting 1:10 with RNase-free water. Approximately 10 μg of RNA for each sample to be electrophoresed was glyoxylated in a glyoxylation mix containing 1 M deionised glyoxal (Sigma), 50% DSMO (Fluka Molecular Biology grade), 10 mM NaPB pH 7 for 1 hour at 50°C. Glyoxal was deionised as described in (Sambrook et al., 1989).

The gel apparatus and silicon tubing for a peristaltic pump (Cole Parmer Instrument Co.) was treated with 0.3% H_2O_2 (Merck) overnight to inactivate RNases. Molten 1.0% agarose was prepared in baked glassware in 10 mM NaPB buffer. To inactivate RNases in the molten agarose, solid sodium iodoacetate (Merck-Schuchardt) was added to make a concentration of 10mM, once the agarose had cooled to ~ 70°C. Once the agarose had set, 10mM NaPB buffer was circulated over the gel for an hour prior to electrophoresis.

Glyoxylated RNA was mixed with 1μl of RNA loading buffer (50% glycerol, 10mM NaPB, 0.25% bromophenol blue, 0.25% xylene cyanol) per 15 μl of sample and loaded onto the agarose gel. RNA was electrophoresed at 45mA for 30 minutes without buffer circulation, and for 4 hours at 45mA with vigorous circulation using the peristaltic pump(approximately 150 ml/minute, total buffer volume was 1.2 L). 3 μl of Gibco BRL 0.24-9.5kb RNA molecular weight ladder was run in the first lane of the gel.

The portion of the gel that was to be stained for visualisation (including the weight markers) was sectioned off with a sterile blade, while those lanes to be blotted were not stained. Staining was carried out in trays protected from light with aluminium foil, gels were soaked for 10 minutes in 50mM NaOH, stained for 30 minutes in 10μg/ml ethidium bromide in 0.1 ammonium acetate(NH_4OAc), and destained for 30 minutes in 0.1M NH_4OAc. Images were acquired as described at beginning of this section, but with a ruler to allow molecular weight calibration.

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(b) Transfer of RNA to membranes

RNA was transferred to positively charged nylon membranes (Nylon+, Roche) with a downward transfer blotting stack. This consisted of, (from the bottom up) a stack of 10-15 cm of paper towels, four sheets of Whatman 3MM Chromatography paper the same size as the paper towels, the receiving nylon membrane, the gel itself, 4 sheets of Whatman 3MM paper all the same size as the gel being be blotted. Overlaying the stack there was a wick of two sheets of Whatman paper, which draws from two buffer reservoirs on either side of the stack containing 1L of 20x SSC. A weight of approximately 200g is placed to sit evenly on top of this stack. All layers apart from the paper towels were pre-wetted with 20xSSC (3M NaCl, 0.3 M sodium citrate pH 7.0).

Stacks were usually left overnight to transfer. After blotting, the RNA was fixed to the membrane by crosslinking with 120,000 $\mu J/cm^2$ ultraviolet light in a UV Stratalinker 2400 (Stratagene). Membranes were then carefully dried at ambient temperatures, sealed in a plastic bag, and stored at 4°C until hybridisation.

(c) Synthesis of Dioxygenin Labelled DNA Probes

Dioxygenin (DIG) labelled ROC 1 DNA probes were amplified from ~100 ng of template (the vector containing the *ROC 1* cDNA (Lippuner et al., 1994)) using a DIG labelling mix and the standard PCR protocol (§ 2.3.7a).

Labelled products were compared to unlabelled products using gel-electrophoresis, with the expectation that the DIG labelled probe would be delayed relative to its unlabelled counterpart.

(d) Hybridisation of RNA Membranes

The hybridisation, and other treatments, were carried out in H_2O_2 treated hybridisation tubes or plastic boxes. Immediately prior to pre-hybridisation, RNA was de-glyoxylated by treating the membrane with 20mM Tris-HCl pH 8.0 at 65oC for 5 minutes. Membranes were then pre-hybridised for an hour in at least 20 ml of DIG Easy Hyb (Roche) at 65°C. Probes were boiled for 10 minutes in 1 ml of sterile MQ, chilled on ice for 5 minutes, and then added to 10ml of heated 65°C Church-Gilbert hybridisation buffer (Church and Gilbert, 1984). Membranes were hybridised overnight at 65°C. The membranes were then washed twice in 2 x SSC, 0.5% SDS in the

hybridisation tubes at 65°C, then twice in 2 x SSC, 0.5%SDS at ambient temperature to remove excess probe. Stringency washes were preformed in 0.1 x SSC, 0.1% SDS at 65°C for 15 minutes.

(e) Detection of DIG Labelled Probes

The membrane was washed briefly in washing buffer (100mM maleic acid, 0.15mM NaCl, 0.3 % Tween 20 pH 7.5) then covered in blocking buffer (1% blocking reagent in maleic acid buffer). Anti-DIG-AP was added at 1:20 000 to the membrane in the blocking solution, and incubated at room temperature for 30 minutes. The membrane was then washed twice with wash buffer, to remove unbound antibody. Detection of DIG labelled probes was performed as recommended by Roche. The CDP-Star^{TM} substrate was used. Membranes were wrapped in plastic-wrap and exposed to Kodak BioMax film for 30 minutes to 2 hours.

2.3.7 Standard PCR protocol

The PCR protocols used in the experiments described in this thesis generally had very similar conditions, varying primarily in the primers used, annealing temperature, extension time and cycle number, the template, and the type of polymerase used.

a) *Taq* Polymerase reactions

Standard reactions for *Taq* polymerase were performed in 50 μl final volume, containing a final concentration of 1x PCR buffer (10 mM Tris-HCl pH 8.3, 50mM KCl, 1.5 mM MgCl$_2$)(Roche), 1x PCR Enhancer solution (Gibco BRL) or 0.5M Betaine, 400 pM of each primer, 250 μM of each deoxynucleotide triphosphate (Invitrogen Life technologies) and 2.5 U *Taq* Polymerase. 'Hot start' reactions were initially used, and thus the *Taq* polymerase was added just prior to the extension step.

b) High fidelity DNA Polymerase reactions

The reactions used with High Fidelity Polymerases were largely identical to those used for *Taq* except that MgSO$_4$ was included in the reaction buffer and the extension temperature was 68° instead of 72°C. In general either

an MJ Research PTC-200 DNA engine or a Biorad iCycler™ was used to execute the PCR programs.

Obtaining cDNAs

2.4.1 Selection of tissue for RNA extraction

Plant tissue was harvested from 2 week old Arabidopsis plants grown under long day conditions. Vegetative SAMs, floral apices, and young siliques (<2 mm long) were primarily harvested. The harvested tissue samples were flash frozen in liquid N_2 and stored in Falcon tubes at -80oC until extraction. At extraction, tissue was then be ground using a baked mortar and pestle in the presence of liquid nitrogen. Total RNA was then extracted from this powder using the RNAEasy mini kit (Qiagen) according to the recommendations of the manufacturer as described earlier. Previous work (Jeffares, 2001) suggested that the transcripts of *TEL1* and *TEL2* might be rare. Therefore to improve isolation of full-length transcripts, enrichment for mRNA was undertaken.

2.4.2 mRNA Enrichment and Extraction

A modified version of the PolyATract mRNA isolation system III (Promega) method was used to enrich for mRNA. Samples of ~100mg of tissue were placed in RNase free plastic bags, dipped into liquid nitrogen and then thoroughly crushed using a plastic roller. 1ml of Lysis/Binding buffer (Tris (pH 8)100 mM, EDTA 10 mM, LiCl 0.5 M, SDS 1% (w/v), DTT 5 mM, DEPC treated water) was then added to the contents of the bag. This suspension was then transferred to a microcentrifuge tube containing streptavidin-paramagnetic beads annealed to biotinylated oligo dT primers. From this point the method specified by the manufacturer was generally followed, with the exception that glycogen was not included as a carrier. The quantity and quality of the resulting RNA was determined by spectrophotometric analysis and Northern hybridisation respectively, using the methods described previously in § 2.3.6 a-e.

2.4.3 Reverse Transcriptase PCR

Gene-specific primers (see Appendix 1) were designed based on predictions of the start and termination codons in the sequence of the genes of

interest based on gene predictions derived from the genomic sequence of *A. thaliana* guided by sequence homology to *TERMINAL EAR 1* (Jeffares, 2001).

The primers for *TEL 2* also included EcoR1 and BamH1 sites (Appendix 1) to facilitate future cloning into the pRoEX-1 expression vector (Life Technologies GIBCO BRL).

RT-PCR was carried out using the AmpliTaq Gold RNA PCR Core system (Applied Biosystems). 1μg of mRNA enriched RNA was reverse-transcribed using oligo-dT. An aliquot of this reaction was used in a PCR reaction with gene specific primers in order to amplify full length cDNAs in the mixture.

2.4.4 Cloning and confirming the cDNA

The putative cDNA and subsequent versions generated by PCR were generally cloned by ligation into the pGEM T-Easy vector (Promega) according to the method of the manufacturers. Where necessary (i.e. where a proofreading DNA polymerase or Taq polymerase had been used) the products were A-tailed then ligated into the pGEM vector.

The clones were then transformed into *E. coli* DH5α using heat shock (§ 2.1.3). Subsequently colonies harbouring the pGEM plasmid including the cDNA were identified by blue-white selection as per the manufacturer's instructions. Plasmids from white colonies were obtained by alkaline lysis and initially checked by restriction analysis to see if the fragments generated by restriction were of the expected sizes. Subsequently clones found to yield the size of insert were then sequenced and compared to the predicted cDNA sequences (obtained from Daniel Jeffares). Those found to be correct were given an alphanumerical designation and stored in *E.coli* lines in cryovials at -80°C.

2.4.5 Sequencing Protocol

To verify products of PCR as well as of RT-PCR, sequencing reactions were prepared to a 10μl volume and contained 150-300 ng of template, 200 pM of a gene-specific primer, and 1 μl of ABI Prism BigDye Terminator Ready Reaction mix. Thermocycling of the dye-termination products used the following program: 25 cycles of (i) 95°C for 20s, (ii) 50°C for 15s, 60°C for 1

minute, then 4°C indefinitely). The reaction/s were then purified according to the following procedure. 2 μl of NaOAc/EDTA and 80 μl of 95% Ethanol were added to each sample, vortexed briefly then microcentrifuged at 20,600 x g for 25 minutes. The supernatant was discarded and the pellet washed in 70% ethanol and centrifuged for 10 minutes at 20 600 x g. The ethanol was removed and the pellet dried at room temperature. 10 μl of Formamide was added, mixed briefly, and this was either stored at 20°C wrapped in foil, or immediately sequenced in an ABI Prism 3100 Genetic Analyser (Applied Biosystems Hitachi).

Cloning of cDNA into expression vectors

2.5.1 General Vector construction outline

It was necessary to create a variety of cDNAs with different restriction sites to allow them to be cloned into the different vectors used in this work. These were generated by PCR, using oligonucleotide primers containing the required restriction sites. The different versions of the cDNA were then re-sequenced to ensure that they had not acquired any unwanted mutations.

The cloning vector and the insert DNA (to be cloned) were digested with the appropriate restriction enzyme/s. To avoid religation of the sticky ends of the digested vector, the reaction was phosphatased using a heat labile alkaline phosphatase (TsAP, Gibco BRL) followed by deactivation as recommended by the manufacturer. Prior to ligation both the vector and insert were gel-electrophoresed to get an approximation of their quantity prior to ligation.

Where a blunted end was required, the restriction enzyme/s would be heat-killed (if possible) and 2U of T4 Large fragment of DNA Polymerase and dNTPs added to the mixture. It was then incubated briefly during which time the sticky ends would be filled in. In most cases the insert would then be gel-purified after gel-electrophoresis(as described earlier) using either Amicon gel-purification or the Qiagen gel-purification kit according to the manufacturers instructions.

Ligation reactions were set up based on the principle that the ration of theconcentrations of vector:insert is optimally at least 1:3 .(Sambrook et al., 1989). Contrary to the manufacturers instructions (Invitrogen Life

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technologies), ligations were usually incubated either at ambient temperatures or at 16°C overnight. An aliquot of a ligation reaction/s were introduced into *E. coli* DH5α using heat shock transformation. Well separated colonies were restreaked onto fresh plates and checked for the successful insertion of the target DNA in the most appropriate fashion. Initially this usually involved a Quick check (described in next section) but also restriction analysis, and finally sequencing of the completed construct.

2.5.2 Quick Check by Phenol 'cracking'

If it was not possible to check successful ligation of the insert using blue-white selection, vector size shift would be examined through phenol cracking. Single colonies identified on transformation plates were inoculated into 5 ml LB broths containing the appropriate antibiotic, and incubated overnight at 37°C with shaking. An aliquot (0.5 ml) was taken from these cultures and the cells pelleted at 20,817 x g in a bench top microcentrifuge. 0.45 ml of the supernatant was removed and 50 μl of PCI (Phenol:Chloroform:Isoamyl alcohol, 25:24:1) added. The mixture is briefly vortexed and 10 μl of the aqueous phase analysed by electrophoresis.

If the DNA fragment to be cloned would be >10% the size of the accepting vector a successful ligation caused a shift in the apparent size of the non-linear forms of the plasmid DNA relative to the intact vector without insert after gel-electrophoresis. In this way lines containing a successful ligation were quickly identified.

2.5.3 Colony PCR

For screening of *E. coli* transformants containing ligated plasmids in which the orientation of the insert was equally likely to be inverted as correct, colony PCR would be used as verification. A sterile toothpick was touched to the colony and streaked onto a LB plate with the appropriate antibiotic. The remaining adherent cells were added by agitation to a PCR reaction mix containing all components except Taq polymerase (see § 2.3.7a, Hotstart reactions). Alternatively the E.coli colonies were grown overnight in a 5ml LB broth with selection, and 2 μl of the culture added to a standard PCR reaction mix immediately before putting the samples into the thermocycler.

In both cases the Taq polymerase is added after the initial denaturation (95°C) step. The PCR reaction was then conducted according to the standard protocol. The absence of any product after amplification was taken to mean that the ligated insert in that clone was inverted and therefore that line could be discarded.

Recombinant expression

2.6.1 General Protein Methods

(a) The Bradford Assay Estimation of Protein Concentration

Estimations of total protein concentrations were performed in microtitre plates in a total volume of 200 µl. 40 µl of Bradford reagent was mixed with a dilution of the protein extract in a total volume of 160 µl. The reagent was mixed by aspiration and allowed to incubate for 5 minutes at room temperature. The absorbance of the samples was then measured at 595 nm on an Anthos HTll microtitre plate reader (Anthos). Values were compared to a Bovine serum albumin (BSA) standard curve generated with known quantities, and the concentration of the extract estimated.

(b) SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE were carried out as described by Sambrook et al (1989) in a mini-gel format. A 10% resolving gel was generally used (10% acrylamide mix, 375 mM Tris-HCl pH 8.8, 0.1% SDS, 0.1% ammonium persulphate, 4×10^{-4}% TEMED) with a 4% stacking gel (4% acrylamide mix, 125 mM Tris-HCl pH 6.8, 0.1%SDS, 0.1% SDS, 0.1% ammonium persulphate, 4×10^{-4}% TEMED). The acrylamide mix consists of 37.4% acrylamide and 2.6% *N,N'*-methylenebisacrylamide (Biorad). The mini-gels were poured into a vertical electrophoresis apparatus (Biorad Mini-Protean® III Cell).

Protein samples were boiled for 5 minutes in SDS loading buffer(50 mM Tris 6.8, 2% SDS, 0.2 % bromophenol blue, 20% (v/v) glycerol, 70 mM 2-Mercaptoethanol) then centrifuged for 5 minutes at maximum speed at room temperature in a bench-top microfuge. Equal volumes were loaded onto the gel, typically between 10 – 20 µl. Molecular weight markers, Biorad Broad

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range Molecular Weight markers or Invitrogen BenchMark™ Prestained Protein Ladder were always included on the first and left-most lane of the gel. Proteins were electrophoresed in Tris-glycine electrophoresis buffer (25mM Tris-HCl, 250mM glycine pH 8.3, 0.1% SDS) at 75V until the loading dye front was within the resolving gel and then they were run at 150V until the dye reached the bottom of the apparatus.

Gels were either stained for 1 hour in Coomassie stain (5g Coomassie Brilliant blue, 90 ml ethanol:water (1:1), 10 ml glacial acetic acid) or transferred to PVDF for western blotting (see following section). Coomassie stained gels were destained in 'Destain solution' (90ml ethanol:water, 10 ml glacial acetic acid) usually overnight with one or two changes. Gels were then placed between Mylar sheets and photographed.

2.6.2 Western blotting and Chemiluminescent Detection

(a) Semi-dry western blotting

The acrylamide gels were soaked in 'Bottom Transfer Buffer' (BTB: 25mM Tris, 14.4% Glycine, 20% (v/v) Methanol) for 30 minutes. Meanwhile the PVDF membrane (Immobilon-P, Millipore) was pre-wet in Methanol for approximately 2 minutes then placed into BTB for 20 minutes.

Transfers were undertaken using a Trans Blot SD semi-dry transfer cell (Biorad) and associated power cell. The blot itself was assembled as follows: Three pieces of Whatmann paper cut to be smaller than the gel itself were briefly soaked in BTB and placed on the transfer plate. The membrane and then the gel, were placed on top of this stack. Finally three pieces of Whatmann paper approximately the size of the gel, and briefly soaked in 'Top Transfer Buffer' (TTB: 25mM Tris, 14.4% Glycine, 1% SDS), were placed on top. The lid was gently placed onto this stack and the transfer commenced at 15V for 24 minutes.

(b) Chemiluminescent Detection

The blotted membranes were placed into Blocking solution (PBST: [1 x Phosphate Buffered saline, 0.2% Tween 20], 5% milk powder) for a minimum of 1 hour at room temperature, or overnight at 4°C. The primary antibody was usually added to the blot soaking in Blocking solution at a dilution of 1:10000 depending on the sensitivity of the antibody. This would

be incubated for up to 2 hours at room temperature, then washed in PBST twice for 5 minutes at room temperature.

The membrane was then exposed to the secondary antibody (in this case anti mouse IgG20a conjugated to Horse radish peroxidase) at a dilution of 1:2000 in blocking solution, for 1 hour at room temperature. Finally the membrane was washed 4 times in PBST for a minimum of 10 minutes each, with shaking. Chemiluminescent reagents 1 and 2 were mixed in equal volumes (Western Lightning™, Perkin Elmer) and the membrane was immersed in this solution for 1 minute with shaking. The membrane was then briefly blotted and transferred to a plastic sheath for timed exposures to BioMax Scientific Kodak film.

2.6.3 Recombinant Expression Constructs

Two different sets of expression constructs were made for expression in *E. coli*. The first was made using the construct pRoEX-1 which contains the poly Histidine affinity tag (Life Technologies). This construct allows the expression of a recombinant protein fused to a 6 x Histidine tag at the C-terminal end. The complete cDNA of *TEL2* was cloned into pRoEX-1 as a EcoR1/BamH1 fragment. The cDNA of *TEL1* was not cloned into pRoEX.

The second expression construct utilised was pGEX 6P-3 (Amersham Biosciences) allowing expression of recombinant proteins fused to the Gluthione S-transferase tag at the C-terminus. The *TEL1* cDNA was directly cloned into this vector as a Bgl II/ EcoR1 fragment. However the *TEL2* cDNA could not be directly cloned into pGEX 6P-3 because of incompatible sites. To facilitate cloning, an adapter was designed to reverse the order of the BamH1/ EcoR1 sites (and alter the frame of the EcoRI site) in the multi-cloning region. The adapter was ligated into pGEX 6P-3 at the XhoI site, creating pVTEX-P-1. The *TEL2* cDNA was then cloned into this modified construct as an EcoR1/ BamH1 fragment.

Both sets of constructs were transformed into heat-shock competent Epicurian™ *E. coli* BL21 CodonPlus RIL cells (Stratagene). Fourteen individual transformed lines were assessed for expression of the construct. 10ml cultures of each line containing the constructs and grown at 37°C overnight, were diluted 1/100 and grown at 37°C till their absorbance reached 0.5-1.0. At this point 1.0 mM of IPTG was added and the cultures grown at 28°C or 37°C. 1ml Samples were taken at 4 hours and overnight, their OD

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determined, the pellet flash frozen in liquid N2 and stored at -80°C until required for SDS-PAGE electrophoresis.

Subsequently the recombinant plasmids were re-isolated from each line and sequenced to check for correct frame and any sequence modifications, which might affect expression.

2.6.4 Purification of His Tagged Protein

50ml cultures of pRoEX containing the gene of interest were grown as described above and harvested at 10000 x g after growth for 5 hours at 30°C post induction. The pellet was chilled, weighed and resuspended 1g to 4 volumes of Lysis buffer (50mM Tris pH 8.5, 10 mM 2-Mercaptoethanol). It was then sonicated for 3 repeats of 1 minute duration on ice and this lysate was centrifuged twice at 11 000 x g to clear the sample of cell debris. The supernatant was then transferred to a column containing the Ni-NTA resin. Use of the Ni-NTA column and subsequent purification steps were executed as per the manufacturer's instructions.

Much younger seedlings and plant embryos were not vacuum infiltrated. The embryos were harvested in the following way. Siliques were harvested from mature plants and opened using fine needles. The seeds were removed and opened with fine needles. Gentle pressure was applied to the seed to extract the embryo from the seed-coat. These were then placed into a microcentrifuge tube containing stain solution without infiltration and left at 37°C overnight. To examine the domains of GUS expression, pictures of T3 seedlings were obtained using a Leica Wild M3Z binocular microscope.

General plant transformation protocols

2.8.1 Terminology

Transformation of *Arabidopsis* Col 0 plants, as described here, involves the assisted infiltration of *Agrobacterium* into the developing flowers of mature plants (Clough and Bent,1998) causing transformation at an expected rate of ~0.1% of the ovules. This transformation leads to hemizygous/heterozygous transgenic seeds which carry a resistance marker,

(e.g. the antibiotic Kanamycin). This allows identification of these transgenic lines by virtue of their ability to grow in the presence of the lethal compound.

The infiltrated plants i.e. those that are dipped into the *Agrobacterium* culture are termed the T0 generation. Seeds from these plants are screened (e.g. via growth on media containing Kanamycin), allowing the identification of the T1 hemizygote seedlings, the primary transformants. When the T1 seed is germinated, and the T2 plants are self-fertilised, they generate the T2 seed lines. Because the primary transformant T1 plants are hemizygous with single or double inserts of the construct, seedlings germinated from the T2 seeds (i.e. the T3 generation) are expected to segregate 1:3 in the case of a single insertion or 1:15 where there are two independently inherited (i.e. non-linked) T-DNA inserts. This latter case is relevant in manipulations involving the LhG-pOP system of vectors.

2.8.2 Transformation of *Agrobacterium*

All plant transformations were carried out using the *A tumiefasciens* strain GV3101 containing the helper plasmid pMP90. This strain was maintained on YEB plates (1.5 % (w/v) beef extract, 0.3 % yeast extract, 0.3 % peptone, 1.5 % sucrose, and 1.5 % agar) containing 20δ gentamycin as selection for the binary-helper plasmid pMP90.

Competent *Agrobacterium* cells were prepared and electroporated as described in § 2.2.3. Cells were transformed with the appropriate construct and then screened by selection on YEB plates containing the plasmids selective marker (i.e. Kanamycin 50δ) and Gent 20δ as selection for the helper plasmid. In the case of the HTOP-G based constructs (§ 3.2.1), a different helper, pSOUP was required which was maintained in strain GV3101 with Tetracycline 10δ. To confirm that no rearrangements had occurred within the constructs after electroporation, plasmid DNA was obtained from the *Agrobacterium* lines and analysed by restriction.

2.8.3 Transformation of *Arabidopsis* plants

Two types of transformation were used to generate transgenic *Arabidopsis* lines in the course of these experiments. These were the simplified Floral dipping procedure of Clough and Bent (Clough and Bent,

1998), and the 'hairy root' transformation proceedure which is described in §
2.8.3b.

The plants used for floral dipping were grown in the following way.
Seeds of *A. thaliana* Col 0 were imbibed in MQ for a minimum of 48 hours at
4°C then sown onto moistened Seedling Raising mix (Oderings, Palmerton
North) and covered with plastic until germination was apparent. The plants
were then transferred to glasshouse conditions with supplementary lighting
providing a 16 hr day. Approximately three weeks after germination or when
axillary floral inflorescences were apparent at the base of the newly emergent
bolts, the plants were ready to be 'dipped'.

The *Agrobacterium* lines containing the construct to be transformed
were grown as described previously, and diluted 1:1000 in 2 x 500ml YEB
containing the appropriate antibiotics in 1L flasks. These cultures were grown
at 28°C for 24 hours with selection, or until mid-log phase (i.e. OD_{600} ~0.8)
then the cells harvested by centrifugation at 8000 x g for 10 minutes at 4°C.
The pellet was then resuspended in roughly one-third its original volume of 0.5
x MS with 5 % (w/v) sucrose and 1δ 6-Benzylaminopurine (BAP).

The resuspended cultures were poured into baked glass bowels and
0.005% Silwet L77 (Lehle seeds) added. The plants were immersed in this
mixture for periods up to 5 minutes, and then placed on their sides in a plastic
tray and covered with plastic overnight. The next day the plants were removed
from the plastic tray and placed right way up in a new plastic tray. Usually
watering was not necessary for a few days. When the oldest siliques from these
plants began to yellow the seed is harvested. The bolts and siliques are cut off
the plant, bagged then dried at 42°C for a week and it is from this material that
the T0 seed is harvested.

(a) Screening for transgenic plant lines

The T0 seed is surface sterilised (1min in 80% ethanol, 5 minutes in
2% Bleach with 0.05% Tween 20 and three washes with sterile MQ water) and
plated onto MS agar containing the appropriate antibiotics. Upon germination
successful (i.e. viable) T1 candidates are transferred to soil pots. Depending on
the construct being transformed these plants will then undergo further
screening to determine the levels of expression. Reporter constructs, which
should be expressing GUS or GFP, are assayed as appropriate. In addition

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rosette leaf tissue may be sampled and prepared for RT-PCR analysis or Western analysis.

(b) Ri-mediated transformation protocol

Ri mediated transformation was achieved using the *Agrobacterium rhizogenes* strain A4T (Grant et al., 1991; Henzi et al., 2000). This was used to transform the roots of transgenic seedlings containing the *TEL1*::TAP construct.

Seedlings were germinated and grown on selective media until the first 3 leaves were visible. The roots of these seedlings were then dissected off at the base of the hypocotyl with a scalpel dipped into an A4T culture. The seedling was then inserted into MS plates (without antibiotics) and incubated, until a new root was visible. The seedling was then transferred to media containing the broad spectrum antibiotic Timentin 150δ to remove any remaining Agrobacterium contamination.

To test the transformation rate of the procedure a A4T strain containing the binary construct P35S:GFP was used for transformation. In subsequent experiments the 'hairy-root' phenotype resulting from Ri integration was sufficient to identify successful transformants. Upon establishing a putative hairy root phenotype, the upper parts of the putative transformant were removed. The roots were then cultured independently and assayed for the presence of the tagged *TEL1* protein.

2.8.4 Plant protein extraction

A range of tissues was assayed, including young siliques, floral inflorescences and root-tips. The plant material was dissected from the plant, flash-frozen in liquid N2 and ground using frozen micropestles. When wet tissue, such as root tips was flash-frozen, grinding was very difficult. Therefore, upon harvesting, the fresh root tissue was placed directly into extraction buffer (EB: 50mM Tris (pH7.5), 5mM EDTA, 5mM DTT, 0.1% (w/v) SDS, 0.15 (v/v) 2-Mercaptoethanol) and ground, using sterile sand and micropestles (Eppendorf). Once processed, the extracts of the roots were electrophoresed and blotted as described in § 2.6.2. The TAP tag includes the Prot A domain, which has high affinity for IgA. Therefore the Western immunoblots of these extracts were probed with IgA linked to Alkaline

phosphatase to determine the abundance of the TAP protein fusion in the transgenic lines.

Localisation of *TEL2* using a GFP fusion construct:

2.8.5 Construction of *TEL2::GFP* expression vector

High fidelity PCR and specific primers were used to modify the cDNA, removing the stop codon as well as adding *Nco* I sites at both ends (Primers in Appendix I). This allowed the fusion of the *TEL2* cDNA to the N-terminus of *gfp*-5 (Haseloff et al., 1997) in the sub-cloning vector pAVA393 (von Arnim et al., 1998).This vector has several elements of note. Firstly the promoter is a dual Cauliflower Mosaic Virus 35S promoter (CaMV35S). Secondly a translational enhancer (TE) fromTobacco Etch virus follows after the dual viral promoter. Both of these elements ensure high level expression of the construct. The final element is a CaMV terminator sequence (see Fig 2.2).

The fusion of *TEL2* to the coding sequence of *gfp* was achieved by cloning the cDNA into the Nco I site at the N-terminus of the *gfp* sequence. Clones were checked for correct orientation using colony PCR and those found to be correct were further verified using restriction and sequence analysis.

For transformation into plants the 35S:GFP and 35S:*TEL2*::GFP cassettes were subcloned from pAVA393 (as *BamH*I-*Hind*III fragments) into pBIN19(Bevan et al 1983). This generated the constructs p35S:*TEL2*::GFP & p35S:GFP shown in Fig 2.2. Subsequently the T-DNA of these constructs was transformed into *A. thaliana* using floral dipping as described in § 2.7.3.
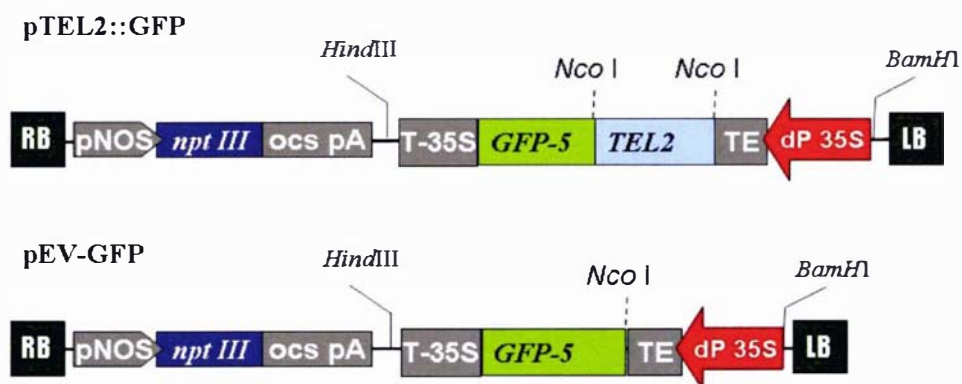
Figure 2.2: GFP and *TEL*2::GFP fusion constructs: The T-DNAs of the constructs are shown in diagrammatic form. The upper T-DNA is p*TEL2*::GFP. This was constructed as an N-terminal fusion of *TEL2* to gfp-5 cloned into pBIN-19. The lower T-DNA represents pEV-GFP which is the empty vector. **Abbreviations: TE:** Tobacco-etch virus Translational Enhancer; **dP 35S**: dual 35S CaMV promoter.

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RB/LB: Left and Right Border sequence respectively of the T-DNA. **pNOS- nptIII**-nos-pA: Kanamycin resistance marker.

2.8.6 Visualisation of GFP

Kanamycin resistant T1 and T2 lines were examined using a Leica UV stereomicroscope to determine the level of GFP fusion expression. This was compared to that observed in empty vector lines as well as WT plants. Subsequently a selection of lines was examined using the Leica TCS 4d Confocal Laser Scanning Microscope (Excitation 480nm, Emission 515nm). Part, or whole, seedlings were placed onto slides, mounted in 50% glycerol and examined under coverslips.

2.9.0 Results

2.9.1 cDNAs of the *TERMINAL EAR*-like genes

a) *TEL2* cDNA

A 1500bp partial *TEL2* cDNA was isolated from floral mRNA after increasing the $MgCl_2$ in the PCR to 2mM. This initial cDNA turned out after sequencing to be lacking a total of 84bp of sequence at two sites. The second attempt isolated three cDNA s of the expected length (1584 bp) from floral mRNA, one of which was found to have no errors.

b) *TEL1* cDNA

An 1858bp *TEL1* cDNA was isolated from floral mRNA when the Mg^{2+} concentration was increased to 3mM. This sequence was found to have a error at base-pair 424 (RRM1) converting Leucine142 to Phenylalanine (i.e. CTT became TTT).

As a replacement cDNA could not be generated from the original RT-PCR or RNA sample, the cDNA was repaired in the following ways. In the case of the construct pVT622, the error was patched using a PCR product amplified from genomic DNA(see Appendix 1). This 'patch' was the region within the genomic *TEL1* from the ATG to the Xho I site at position 1068, and included the first intron. The product was cloned into pVT622 as a Bgl II / Xho I fragment.

However the recombinant expression of *TEL1* in *E. coli* still required a correct and intron-less cDNA. Therefore a second method of repair used site

mutagenesis to correct the single bp error. Primers in which the known error was corrected, were used to produce a repaired version of the *TEL1* cDNA (Appendix 1). Further amplification of the repaired fragments with primers directed at the 5' and 3' ends of the cDNA allowed the generation of a complete cDNA for *TEL1* (Horton et al., 1990; Yon and Fried, 1989) which was subsequently verified as correct. This cDNA was therefore subsequently used for the attempt to express *TEL1* as a GST fusion in *E. coli* (see §2.6.3, pg 65) and the OP-*TEL1* construct described in §3.2.2 (pg 92).

2.9.2 Recombinant expression

It was not possible to see the expression of His tagged *TEL2* expression in the lPTG induced, versus uninduced, crude cell lysate (Figure 2.3 lane 2). These lysed cells were processed and passed through a column containing Ni-NTA resin. When the purified eluate was electrophoresed it contained a single band assumed to be 6xHis::*TEL2* because it was approximately the expected size of ~66.8kDa (Fig. 2.3 lanes 3-5). However the mean yield of recombinant *TEL2*p as determined by the Bradford protein assay (§ 2.6.1a) was low, ~100 μg harvested from 50ml of culture and was entirely used in the gel depicted in Fig 2.3. Subsequent attempts to upscale this yield (i.e. extraction from 0.5 l) yielded no detectable protein. Attempts to determine whether the protein being expressed was insoluble, through electrophoresis of the lysate pellet, revealed no difference between the uninduced and induced lines.

The construct was re-isolated from the *E.coli* strains used, and sequenced. No errors were found either in the *TEL2* cDNA or surrounding elements which could account for the lack of expression observed under induction.

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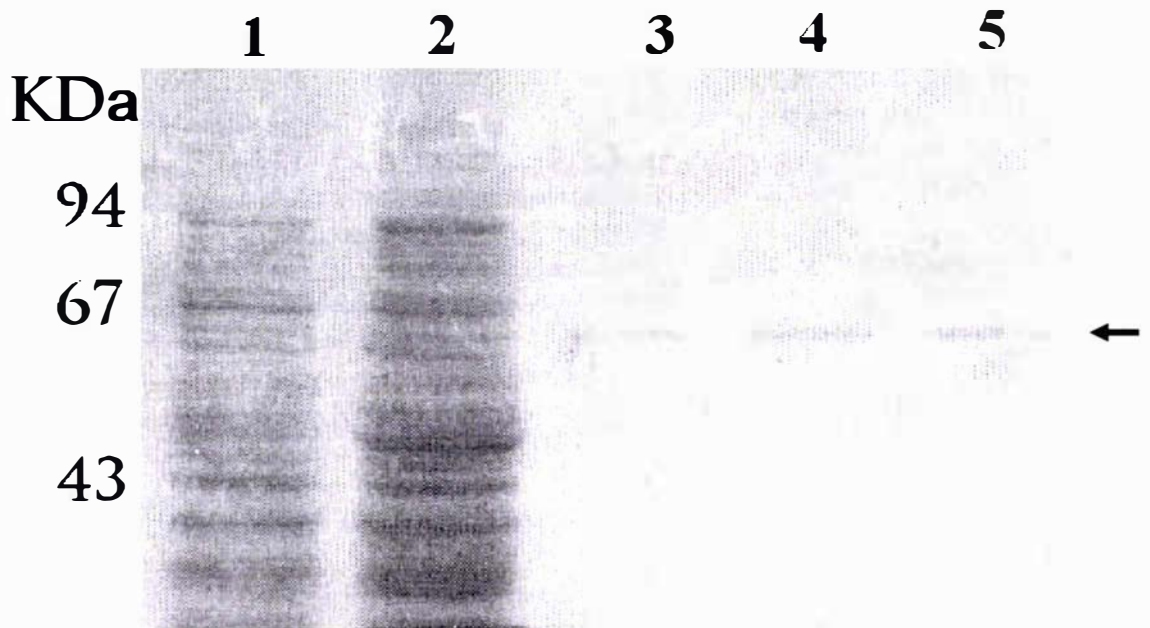
Figure 2.3. Recombinant Expression of His-tagged *TEL*2: 10% SDS PAGE Gel. Lanes: (1) Sample of crude lysate of cells prior to induction, (2) 5hrs post-induction, (3-5) sequential samples eluted from Histidine column. Arrow points to band of ~65kDa.

The *TEL1* and *TEL2* cDNAs were subsequently cloned into a different vector pGEX-6P-3 which fuses the GST tag to the protein. Expression levels of *TEL1* and *TEL2* in the GST constructs were not significantly better. Attempts to improve yields of either set of constructs using lower temperatures, lower and higher IPTG concentrations, shorter incubation times, protease inhibitors or different lysis methods (i.e. French Press) did not improve yields.

2.9.3 Unable to locate expression of *TEL2*::GFP in any line examined

56 T1 Kanamycin resistant lines containing P35S:*TEL2*::GFP were generated and examined for their level of expression relative to the Empty vector (i.e. P35S:GFP) line 14 and WT. Of these, 38 putative GFP expressing T1 lines survived to self. The T2 seedlings were then examined for GFP expression using a confocal scanning microscope as described. None of the lines demonstrated fluorescence greater than present in WT, or equivalent to that observed in P35S:GFP-line 14.

To determine if *TEL2*::GFP was being expressed but at levels below the sensitivity of the confocal microscope, whole plant protein extracts were

obtained from leaves of eight T2 lines (based on apparent fluorescence) and Western-blotted. The blot was then probed with Anti-GFP Antibody (Roche, cat no. 1 814 460) and detected using Horse radish peroxidase (Amersham). Although GFP protein was detected using this method in the EV line, GFP fusions were not detected in the putative GFP fusion lines tested (data not shown).

2.9.4 Is The *TEL2*::GFP Construct Translationally Functional?

One possible explanation of the lack of detectable GFP in the stably transformed lines was that the vector backbone of the construct contained errors (outside the *TEL2* coding sequence), or was not a translational fusion leading to a truncated and thus non-functional protein product. To determine if this was the case for the P35S:*TEL2*::GFP construct, a transient assay was undertaken for GFP activity. The construct was biolistically bombarded into onion epidermal cells for transient expression studies by Susanna Leung as part of her MSc studies.

GFP activity was observed in these cells which can be assumed to indicate that expression can occur from this construct (Fig. 2.4, overleaf). Because the GFP coding sequence was fused to the N-terminal end of the *TEL2* cDNA, visible GFP would not have been observed if errors in this construct prevented correct translation. Thus, the observation of GFP in these cells suggests the construct is translationally functional.
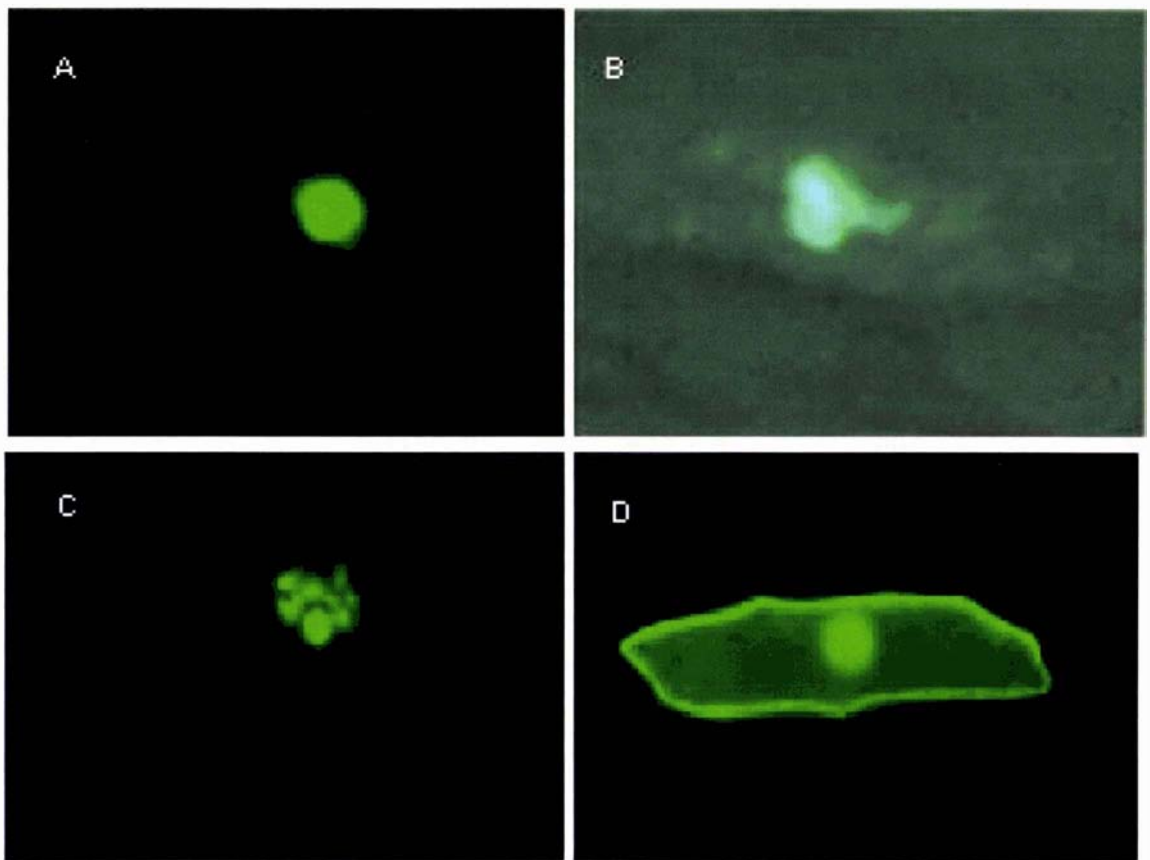
Figure 2.4 The P35S:*TEL2*::GFP construct is translationally functional:
Transient expression of *TEL2*::GFP was observed in onion epidermal cells. Images
depict cells expressing 35S:*TEL2*::GFP **(A,C)**, **(B)** DAPI stained nucleus of an onion
cell and **(D)** a cell expressing 35S:GFP (Pictures courtesy of Susanna Leung).

2.10 Discussion

The aim underlying the work outlined in this chapter was to determine
localisation and biochemical properties of the *TEL* proteins. The first step
towards this was successfully achieved, being the identification of cDNAs
containing the coding regions of both *TEL* genes. Subsequently localisation
was to be initially observed in the live cells using transgenic lines containing a
translational fusion with the Green Fluorescent Protein. The expression of the
construct was driven by the constitutive viral promoter Cauliflower Mosaic
Virus 35S as described in §2.8.5. Therefore, apart from localisation, these
transgenic lines were also expected to allow insights into the nature of the
overexpression phenotype. Subsequently the position of the GFP fusions in
cells and the plant overall would be confirmed by immunolocalisation using
antibodies directed at the native *TEL* proteins *in planta*. The antibodies would

be generated using purified protein derived from recombinant expression constructs. In turn the recombinantly expressed *TEL* proteins would also be used for *in vitro* investigations of their RNA binding properties.

Initial attempts to isolate intact cDNAs isolated examples with significant errors. In the case of *TEL2* this was resolved by isolating a new cDNA without deletions. This was not possible for the *TEL1* cDNA which was repaired using PCR in two different ways. One version was repaired using a patch derived from the genomic sequence of this gene, and this was used in a plant expression construct. The second version was repaired using primers designed to correct the single bp error directly, and this cDNA was subsequently used in both recombinant expression and transgenic constructs.

Of the two cDNAs, *TEL2* was the first to be isolated which was verfiably correct, so this was duly placed into front of the GFP gene, and into recombinant expression vectors. However attempts to visualise the GFP tagged *TEL2* proteins in T1 lines identified by screening, as having the antibiotic marker, were not successful. The fluorescence observed in 38 T2 lines were found to be considerably lower than the empty vector line. Furthermore *TEL2*::GFP protein was not detected in Western blots using antiGFP antibodies against a selection of 8 T2 lines. Consequently it appeared that the construct was not functional in these stable lines. As GFP was neither detectable either under epifluorescence or immunoblots it was concluded that the construct was not being expressed, although direct investigation of construct expression was not undertaken

No errors in the sequence of the construct itself were found and transient expression of the 35S:*TEL2*::GFP construct was observed in onion epidermal cells biolistically transformed by Susanna Leung as part of her Masters research. This indicates that the construct is a functional translational fusion at least in transient expression. The difficulties with this construct were concluded to be associated with the overexpression of the *TEL2* protein itself. When attempts have been made to overexpress members of other Mei2-likes have been made, similar difficulties have been encountered. A recent paper described attempts to isolate lines constitutively expressing *AML1*::GFP fusions. These workers also failed to identify lines with visible fluorescence,

nor could the transcripts of the fusion construct be detected in any tissues (Anderson and Hanson, 2005). The authors of this study concluded that the difficulty in obtaining constitutive overexpression of *AML1*::GFP was probably due to toxicity of constitutive expression of *AML1*. While a similar conclusion cannot be directly made in this case (because levels of expression were not determined) the results outlined in this chapter are consistant with their findings.

In this vein it is also notable that the phenotypes of some T1 *TEL2*::GFP lines (which did not survive to produce seed) had similarities to those described in the next chapter in the OP-*TEL1* and *2* expression lines. The most consistent aspect of these lines was that they were highly variable in appearance and did not survive to produce seed. In many lines the plants had a very unhealthy appearence and it was unclear whether their fluorescence was due to callose deposition, (due to cell death) or expression of the construct. In hindsight one possibility is that those lines which had the highest expression of the fusion proteins were those with the most variable appearance and were eliminated primarily by their low viability and inability to produce seed. Consequently screening was biased towards the isolation of T1 and T2 lines with low, or zero expression of the tagged *TEL2* protein which were able to produce seed.

The second set of experiments outlined in this chapter were those involving the expression and purification of the *TEL* proteins from *E.coli*. As mentioned previously the *TEL* cDNAs were put into a a severalrecombinant and plant protein expression constructs. These constructs contained protein tags to allow purification of the protein products after extraction. *TEL2* cDNA was the first to be cloned into the pRoEX-1 vector which adds a hexamer of Histidine at the 5' end of the protein. Initial results from expression of this construct, after purification, revealed a band of the expected size, albeit at low overall yields. Subsequent attempts at expression and purification of the soluble fraction were not however successful. It is possible that the protein was being retained in the insoluble fraction. However when samples of the lysate pellet for induced and uninduced bacterial lines were compared there was no evidence of overexpression of the *TEL2* protein in either case. This was despite attempts to alter growth and induction conditions including for

example growth at low temperatures. A similar result was found when the *TEL1* and *2* cDNAs were cloned into the pGEX construct. The possibility that the lack of expression may be due to construction or sequence errors was investiagated. No sequence errors were found in either construct. In consequence, regardless of solubility, there was no evidence that the protein was being expressed, or if it was, that it was being expressed at low levels. It was decided that this point that attempting to improve expression or solubility might be a prolonged process, and that the priority was evaluation of the plant expression constructs.

The aim was to express the whole protein. However expression of a, or several peptides, may in retrospect have been sufficient for the purposes of this work. Sections of the protein sequence which exclude the regions containing the rare codons and in particular the prolines could have been cloned for recombinant expression. That is to say, peptides, encoding for example the third RRM of the *TEL* proteins, could conceivably have been more readily expressed. However it was also possible that such peptides would not fold in a native fashion. Consequently they might have aberrant or reduced RNA affinities compared to the full protein and they might not the same epitopes in solution, increasing the possibility that antibodies raised against these peptides might have cross reactivity or not be able to detect the native protein at all. Given the investment required for experiments investigating RNA binding and the generation of antibodies from purified peptides attempts involving recombinant expression were not further pursued.

One possibility as to why expression in bacterial hosts was so difficult to detect might be codon bias. The strain of *E.coli* used in this work to express the constructs contained a plasmid encoding additional tRNAs for rare codons encoding Arginine, Isoleucine and Leucine. However in addition to these rare codons, the *TEL2* protein has also a relatively high number of CCA (Prolines) near the 5' end of the gene, which is again uncommon in bacterial proteins. Recent work by Karen Wilson, in the BV laboratory, has shown improvements in the yield of recombinant *TEL* expression through the use of the Rosetta™ strain of *E.coli* (Novagen). These strains contain the pRARE plasmid which encodes the tRNA genes for these uncommon codons rarely used in high level expression of *E.coli* proteins (e.g. (Kurland and Gallant, 1996)) . These rare

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codons include the tRNA for Proline, which was not present in the Epicurean RIL strains used in this work. Therefore the lack of success may in part to the inability of the bacterial host to cope with the codon usage present in the sequence of the *TEL* cDNAs.

Overall then the aims of this chapter were not met. The localisation of the *TEL* proteins, within the plant, as well within the cells of the plants remains to be determined. In hindsight some alternative experimental approaches are evident now which could form the basis of future work. One example is of course the expression of peptides containing the domains of interest from the *TEL* proteins which was mentioned earlier. Functional characterisations of *FCA* (for example) have used this approach to generate effective antibodies (Richard Macknight *pers com*) though it is unclear whether these same peptides have been used to investigate their biochemical properties.

In addition, the use of the hairy root transformation procedure, along with a 35S::*TEL*1::GFP construct might be more effective tool for localisation at least in the root. This could be further refined by the use of an inducible expression system (such as described in the next chapter) to avoid difficulties associated with overexpression of the *TEL* proteins themselves. The success, or otherwise, of these approaches remains to be determined.

Chapter 3: Constititutive and ectopic over-expression of *TEL* cDNAs

3.0 Introduction

A standard approach in the characterisation of a genes' function, in terms of cellular development, is to analyse the consequences for development where that gene is no longer functional. Primarily this involves the identification of a mutant allele or alleles of the gene in question or else expression of antisense constructs that decrease or eliminate its expression post-transcriptionally. In plant development there are, for example, collections of *Arabidopsis* T-DNA insertional mutants where the locations of insertions have been identified (e.g. (José M. Alonso, 2003)). These collections have greatly facilitated the systematic characterisation of gene function in this model dicot (José M. Alonso, 2003; Rios et al., 2002).

The interpretation of phenotypes from such insertional mutants may however be complicated. For example the number of insertions of the T-DNA in the genome of such mutant lines may not necessarily be known at the onset. Furthermore the insertion may generate complex rearrangements in its vicinity, potentially inactivating a broader range of genes. These problems are dealt with by repeated backcrossing with the desired WT background to eliminate any unlinked mutations, but this in turn considerably delays the characterisation of the allele. A particularly relevant problem in the present work relates to determining whether an insertional mutant allele would be truly be a 'knockout' or null allele. This is especially problematic in this case because these genes are known to be expressed in very restricted domains, and at very low levels, that detection of the expression of the intact gene in WT is challenging. This difficulty is compounded where the gene of interest belongs to a multi-member family whose sequences have high levels of similarity, and may be at least partially redundant (such as in the case in the Mei2-likes). This would mean that even if it is possible to confirm a null allele, there might be no obvious phenotype because even partial complementation might be sufficient to allow normal development.

Assuming a clear null allele is identified, and an associated phenotype/s is identified for a gene of interest, it is still necessary to confirm that it is the

interruption of that gene which has created the phenotype. This is traditionally done using gain-of-function or complementation studies. In essence, the gene is reintroduced into a mutant allele background, either as a genomic fragment, or as a cDNA version under the control of the Cauliflower Mosaic Virus 35S promoter (CaMV35S). Thus, if the null allele is responsible for the phenotype, then the ectopic overexpression of a functional copy of the gene could be expected to 'rescue' development, and thus complement the defect. If it does, then this is considered evidence towards attributing the nature of the phenotype to the function of the gene. Any additional phenotypes observed under constitutive expression of the gene of interest can be considered informative about the function of the gene product outside its normal domains.

As noted previously functional characterisation of the *TEL* genes by loss of function analysis was considered unlikely to be informative. The Mei2-like family consists of seven members, all of whom have high sequence similarity and overlapping domains of expression. Given their sequence similarity it was considered likely there would be some degree of functional complementarity between members of this family. Furthermore the difficulties in obtaining lines with single insertions in their genomes that are also indisputably knockout alleles of both *TEL* genes (given their limited expression domains, and the difficulties in detecting the transcripts even in wildtype plants) were considered prohibitive.

Instead an analysis of gain-of-function lines was undertaken, as this seemed most likely to be informative regarding the activities of the *TEL* genes both within their normal domains and without. Given the difficulties described (Chapter 2) when using the 35S:*TEL2*::GFP construct, a number of different approaches to ectopic expression was undertaken. This chapter presents the results of ectopic expression using a range of constructs. This in turn allowed the hypothesis, outlined at the beginning of this work that the *TEL* genes are associated with the repression of organogenesis in their normal domain/s, to be directly tested.

3.1 Methods

3.1.1 Ectopic expression of the *TEL* genes.

Two different sets of vectors were used to create the constructs used in the experiments described in this chapter. One set were constructed in pHTOP-

G, from the LhG:pOP system which is described in §3.1.2 & 3.1.3 (Fig 3.1 below). The other was made using the pART7 and 27 expression vectors and is the subject of this section.

The pART27 vector was designed to drive constitutive overexpression

I. (a) **p35S::TEL2**

II. (a) **pHTOP-G (EV)**

(b) **pOP-*TEL1***
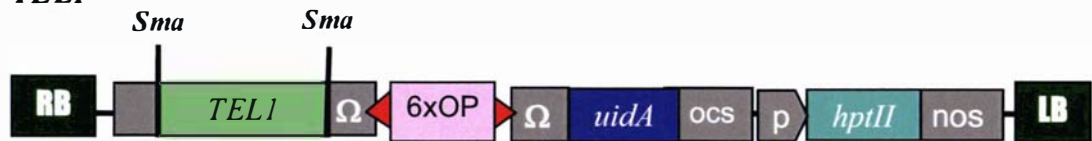
(c) **pOP-*TEL2***
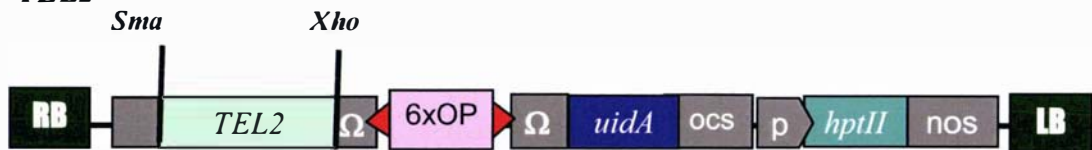
Figure 3.1:Constructs used in plant ectopic expression experiments: Constructs are shown diagrammatically as T-DNAs.**(I)** p35S:*TEL2* in pART27 for constitutive ectopic expression under the control of CaMV 35S promoter. **(IIa) pHTOP-G** is the Empty vector of the LhGpOP system. **(IIb) pOP-*TEL1*** and **(IIc) pOP-*TEL2*** are pHTOP-G vectors containing the cDNAs of *TEL1* and *TEL2* respectively. **Abbreviations: RB/LB**: Right and Left border respectively; **6XOP**: 6 repeats of operator sequence; **P35S in large red arrow**: CaMV 35S promoter**; Small red arrow:** minimal 35S-TATA; **Ω: TMV** translational enhancer; ***uidA*** β-Glucuronidase or GUS; **ocs-PA**: Octopine polyA terminator and adenylation sequence; **pN/ nos:** Nopaline promoter / terminator and adenylation sequence; **NPTII:** neomycin phospho-transferase II (Kanamycin resistance marker);**HPTII:** hygromycin phospho-transferase II.

of plant genes under the control of the CaMV35S promoter (Gleave, 1992). Unlike the GFP fusion construct there is only a single CaMV35S promoter driving expression and no enhancer elements. The *TEL2* cDNA was cloned into pART7 as an Xba I/EcoR1 fragment, and the correct orientation of the *TEL2* insert confirmed using colony PCR as described previously. Once

confirmed a NotI fragment containing the 35S:*TEL2*:ocs region was then subcloned into pART27 using standard methods described in § 2.5.1. The completed T-DNA insert, termed p35S:*TEL2*, is shown in Figure 3.1(1a) on the previous page.

The original *TEL1* cDNA was also cloned into pART27 but it was subsequently found to have a non-silent missense error in the vicinity of the RRM1. Consequently this construct was not used to transform *Arabidopsis* seedlings.

3.1.2 Characterisation of P35S:*TEL2* lines

Wildtype *Arabidopsis* plants were transformed with the P35S:*TEL2* binary construct via floral dipping. The T1 seed derived from these plants was then screened for resistance to Kanamycin. Seedlings found to be resistant were given a numerical designation (i.e. Line 51) transferred to soil and assessed for phenotypes compared with those transformed with the empty vector (pART27).

Lines that were identified as having a phenotype (distinct from EV lines) were then assessed for expression of the transgene via RT-PCR. Total RNA was extracted from samples of mature rosette leaves (as described in §2.3.4, pg 52) as these tissues are normally devoid of native *TEL2* transcripts. Alongside these reactions two negative controls were included. These were an EV line and a putative P35S:*TEL2* line with Kanamycin resistance but no distinctive phenotype. The primers used in the RT-PCR were designed to bridge two intron boundaries in the genomic sequence of *TEL2* to exclude false positives from genomic DNA contamination. If the cDNA was being expressed the size of product expected (from these primers) was 280bp.

Expression lines (i.e. found to have both a phenotype and expression of the construct), and examples of the EV lines, were allowed to self and the T2 seed of all these lines collected. In order to determine if these lines represented single or multiple insertions of the T- DNA a segregation analysis was undertaken. The T2 seed of the lines was germinated on selective media and the segregation of the resistance marker in these seedlings was then compared to the Mendelian ratio of 3:1 expected of a single unlinked insertion.

Seedlings of the 35S: *TEL2* lines were also transferred to growth in soil and growth under glasshouse conditions. Thirty six resistant T2 seedlings from selected 35S:*TEL2* lines were planted out into potting mix (Oderings, Palmerton North) from selective tissue culture at approximately 5 days after germination. During subsequent growth the seedlings were examined for the presence of the phenotype observed in the previous generation. As part of this experiment quantitative measurements were also made at ~6 weeks, regarding the length of floral bolts, the length, and number of siliques.

3.1.3 Developmental Progression

As will be described in the results section of this chapter all the expression lines displayed considerable variability in the degree and nature of the phenotypes seen. In an attempt to discern if there was a consistent developmental effect underlying these phenotypes a quantitative assay of developmental progression was used.

The general hypothesis is that the primary function of the *TEL* genes relates to meristem function such as organogenesis. Consequently one result of ectopic *TEL* expression might be delays in leaf initiation and overall developmental progression. To address this possibility, the growth of populations of T2 seedlings was examined for the timing of developmental landmarks. For the P35S:*TEL2* lines these were the completion of germination (G) and emergence of the first true leaves (Leaf set 1). Extended versions of this experiment were also conducted with LhG4>>OP-*TEL* and HTOP-G lines, which included the emergence of the second set of true leaves (Leaf set 2), Leaf 5 (Ls3) and the floral bolt.

Germination was defined as the appearance of the root and cotyledons of the seedling. Leaf set 1 was defined as the separation of the cotyledons and the visible emergence of the first true leaves. Subsequent leaf sets were scored in a similar way (Fig 3.2, next page). The floral inflorescence was identified by the emergence of the floral bracts and the subsequent appearance of floral buds as visualised by a binocular micro-scope (not shown). These experiments were
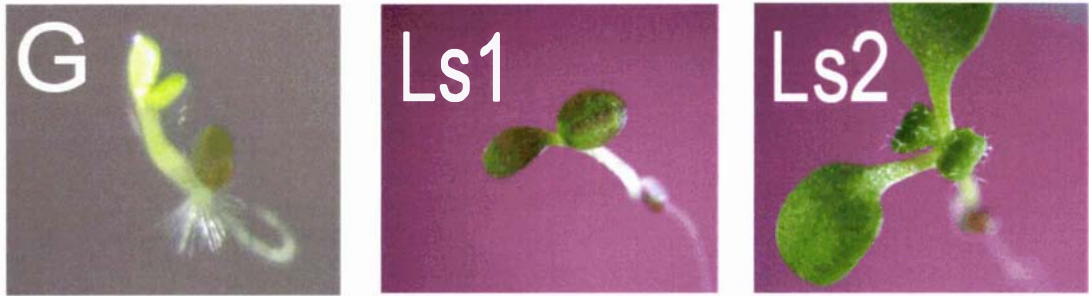
Figure 3.2: Examples of Developmental "landmarks": (G) Germination, (Ls 1) Leaf set 1, (Ls 2) Leaf set 2.

undertaken in tissue-culture and grown under long days. Seed of each line was plated onto selective media at the axes of a grid composed of 1 cm^2 squares to ensure even spacing of seedlings. Each plate contained between 24-26 seeds. After cold treatment, the plates were placed in the growth chamber. Thereafter the seeds (and later seedlings) were examined at 24 hour intervals for up to 14 days, at 40x magnification of a Leica Wild binocular microscope. Where necessary (i.e. leaves sticking together) seedlings were manipulated in order to determine whether the next leaf set was visible. Counts were therefore made from these examinations recording the progression of T2 seedlings through the stages of germination, Ls1 and subsequent leaves. These counts were noted as a percentage of the total population on a given day, and expressed as a cumulative frequency, since the percentage of plants at Ls1 depends on the percentage of plants which have germinated.

The statistical significance of the differences observed in these assays was only examined in the domain specific crosses and this is described in §3.3.1e (pg 99).

3.2.1 LhG:pOP, a two component expression system

The second set of constructs used in these experiments were derived from vectors of the LhG-pOp system (Moore et al., 1998, Samalova et al 2005, Craft et al 2005). This is a plant transgene expression system with two components, the Activator construct and the Reporter construct. A schematic of how the Reporter construct, pHTOP-G, and the two different Activator constructs, pLhG4 or pLhGR-N, interact is shown in Fig 3.3 (pg 92). The two activator constructs are designed to promote domain specific misexpression

(i.e. pLhG4) or inducible over-expression (i.e. pLhGR-N) respectively, of the reporter gene and the gene of interest in HTOP-G. The pLhG4 construct encodes a chimeric transcriptional activator (LhG4, Fig 3.3, pg 92) composed of a fusion of the transcription-activation domain-II from Gal4 (*Gal 4AD*) from *Saccharomyces cerevisiae*, and a mutant *lac*-repressor that binds its Operator (OP) with increased affinity (*mLac 1*).

In turn HTOP-G contains a sequence of 6 six palindromic repeats of the Operator sequence (6XOP) between the Reporter gene, and a multi-cloning site where the *TEL* cDNAs were inserted. The LhG4 protein is able bind to these sequences and promotes expression in either direction via its Gal 4 domain activity. In principle the expression of the gene of interest is co-ordinately linked to that of the reporter gene, which, in this construct is β-Glucuronidase or GUS (*uidA*). This point will be further discussed in reference to the inducible constructs (Craft et al 2005, Samalova et al 2005).

Expression of the LhG4 protein is controlled by native *Arabidopsis* promoters whose expression is restricted to specific domains of the developing plant (as listed in Table 3.2, pg 94). This allows the targeted misexpression of the gene of interest in specific domains of the plant and/or stages of development that the gene of interest may not normally be expressed in. Moreover if ubiquitous expression of this gene throughout development is suspected to be detrimental or even lethal, domain specific expression could circumvent this problem.

Another common problem with ectopic expression is the wildly variable levels of expression up to and including complete suppression via post-transcriptional silencing. The *Arabidopsis*-derived promoters of the LhG4 system avoid this problem as these are less likely to undergo silencing or co-suppression as might be seen with a viral promoter such as CaMV35S. Furthermore the histochemical activity of GUS in pHTOP-G provides a quick indication of the levels and spatial location of expression within any line. If expression of LhG4 is suppressed then this will be indicated by the absence of histochemical staining in that plant, or region of the plant.

The final advantage of this system that it also allows inducible ubiquitous expression via a driver construct pLhGR-N (Craft et al 2005, Samalova et al 2005, Figure 3.3). In this construct the LhG4 protein has been

fused to the Rat Glucocorticord receptor (GR) at its N terminus and hence the name pLhGR-N. The LhGR-N protein requires the synthetic steroid Dexamethasone (DEX) in order to promote expression from the pHTOP-G construct. In turn expression of the pLhGR-N transcriptional factor is under the control of the CaMV35S promoter, and thus in the presence of DEX activates ubiquitous expression throughout the plant of both the reporter and the gene of interest. This means that ubiquitous expression can be switched on after early stages of development which might be sensitive to an overabundance of the gene of interest. This means that potentially high-level expressing plant lines are not eliminated before they can produce seed which improves the chances of identifying a phenotype associated with expression especially with the assistance of the GUS reporter gene.
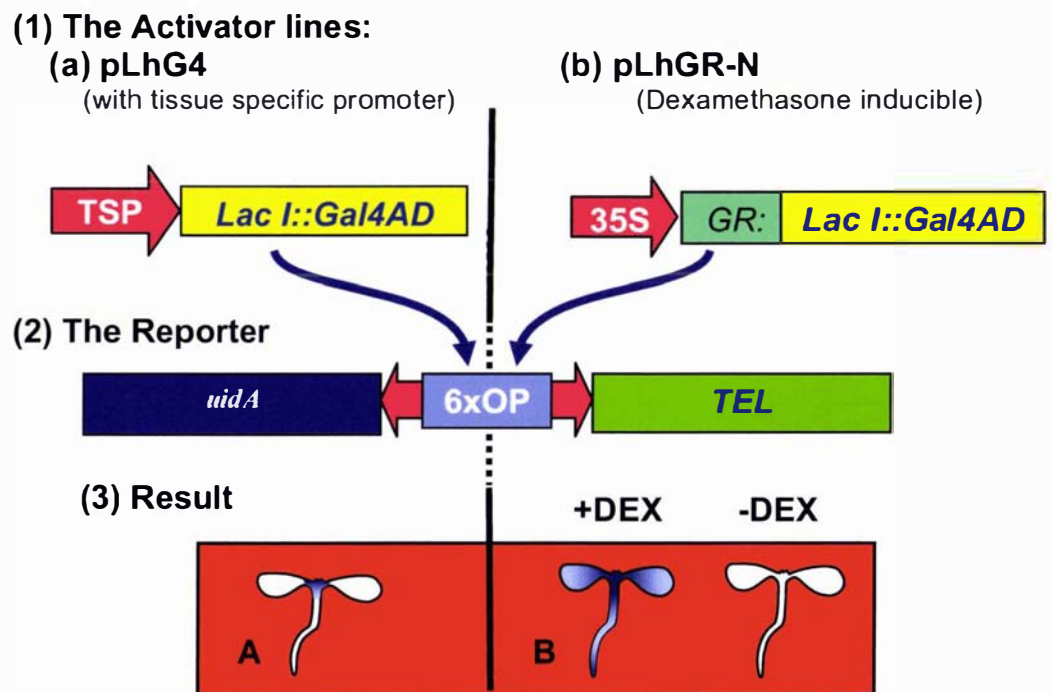
Figure 3.3: The two component LhG-pOP system: The two components are (1) the Activator lines which expresses a synthetic transcription factor (LhG or LhGR-N) which 'drives' expression of the second component, (2) the Reporter construct, through the binding of LhG/LhGR-N at 6 repeats of the lac Operator sequences (6XOP). The result (3) is either ectopic domain specific expression (3A) or (3B)inducible ectopic expression throughout the plant.

3.2.2 Construction and nomenclature of OP-*TEL* lines

The constructs described in this section including pHTOP-G were kindly provided by Dr Ian Moore of Oxford University. To generate the OP-*TEL1* construct, the *TEL1* cDNA was cloned into the pHTOP-G vector as a

blunted *EcoR*1 fragment. The OP-*TEL2* construct was generated by cloning the *TEL2* cDNA as a *Xho* I *Xba* I (blunted) fragment into the *Xho-Sma* I sites of pHTOP-G. The schematic layout of these constructs is shown in Fig 3.1(II)a-c (pg 87). As mentioned in the previous section two different types of activator line were available to use with these OP-*TEL* constructs. The S7 Activator lines contain the pLhGR-N construct as a homozygous stable insertion. Seeds of this line were kindly provided by Dr Ian Moore of Oxford University. The OP-*TEL* constructs and the empty vector construct pHTOP-G were super-transformed into the S7 background and subsequently selected for their resistance to Hygromicin, the marker carried by the HTOP-G construct and Kanamycin, the marker carried by pLhGR-N. Lines containing both markers were used in inducible expression experiments.

The second set of activators were provided as *Arabidopsis* sp. seed lines that contained different pLhG4 constructs. The primary difference between these lines was that the expression of pLhG4 was under the control of different plant specific gene promoters rather than the viral CaMV35S promoter (as described above). Pollen from seedlings identified by selection as containing the pOP-*TEL* constructs were then manually crossed to plant lines containing different plant promoters (see §3.2.4).

Inducible lines were supertransformed into the S7 background, and therefore are denoted by '-S7' as part of their designation. In general the inducible lines were used primarily to examine the effect of constitutive *TEL* expression after germination. However where inducible lines were found to have good GUS activity and consistent phenotypes, these lines were also included in manual cross pollination experiments to the domain specific driver lines and these are also denoted by the S7 suffix.

All of the promoters used with LhG4 in this work were identified based on the characterisation of their associated genes. The domains of expression attributed to these genes are based entirely on *in situ* hybridisation analyses published as part of their original functional characterisation. The names of these mutants and their promoters are given in Table 3.1 (pg 94), as well as their expected temporal and spatial activities. Descriptions of the expected expression domains are also outlined in the following sections.

The nomenclature used in this work to describe crosses using different combinations of activator and reporter lines follows the convention of Eshed et al (2001). The Promoter construct and Expression construct combination are denoted as Promoter name>>OP-*Gene*-line number. For example, when the plant line containing the promoter of AINTEGUMENTA driving LhG4, (i.e. ANT:LhG4), is crossed to the pOP-*TEL2* WT Line 3, the F1 seedling 'progeny' would be written as ANT>>OP-*TEL2*-3. In turn, the seedlings obtained from crosses of lines containing the empty vector construct (i.e. HTOP-G line 3) with the ANT:LhG4 line, will be referred to as ANT>>HTOP-G-3.

3.2.3 Domain specific expression

(a) *AINTEGUMENTA*

The promoter of AINTEGUMENTA (ANT:LhG4) was included to examine the effect of ectopic expression in a range of tissues and organs, from embryogenesis through to the formation of the floral organs and the siliques. The mutant alleles of *AINTEGUMENTA* (*ANT*) were initially characterised for

Table 3.1: Activator lines selected for analysis of tissue specific ectopic expression.

Activator (promoter)	Expected primary expression pattern	Construction details (Source)
AINTEGUMENTA (ANT:LhG4)	Organ anlagen and primordia	6.5kb::LhG4 (Shoof et al (2000))
APETALA1 (AP1:LhG4)	Margins and axils of cauline leaves and young flower buds. Later restricted to sepals and petals	1.7kb (M. Yanofsky, unpublished)
APETALA3 (AP3:LhG4)	Petal and stamen whorls only	0.42kb (T. Jack, unpublished)
AGAMOUS (AG:LhG4)	Stamens and carpels	3kb HindIII fragment of AG second intron (D. Wiegel, unpublished)

their defect in ovule formation and floral organs (Elliott et al., 1996; Klucher et al., 1996). An examination of the expression domains of this gene, by *in situ* hybridisation, revealed high expression both in the early floral meristem and the organs of the developing flower. However, expression was also observed, albeit at much lower levels, in early development of the embryo. Discreet domains appeared at the positions of the incipient cotyledons in globular embryos. Subsequently, in the walking stick embryo, these resolved into internal domains within the cotyledons, apparently in the vasculature. In turn expression of the *ANT* transcript was not seen in either shoot or root apical meristem, the hypocotyl or mature rosette leaves,(Elliott et al., 1996), although expression was detected in the root vasculature (Klucher et al., 1996). Consequently apart from its role in ovule morphogenesis, *ANT* was ascribed a general role in the initiation and growth of lateral organs. Therefore expression of the *TEL* cDNAs under the control of ANT:LhG4 was expected in all lateral organs, including those of the floral domain but not in the shoot apical meristem. The crosses of OP-*TEL* to this promoter were expected to reveal what if any effect these genes might have in organogenesis (i.e. outside the SAM) and later in the floral lateral organs.

(b) *APETALA1*, *APETALA3* and *AGAMOUS*

The mutant alleles of these genes all display defects in the specification of floral meristem identity, determination and organ identity (Bowman et al., 1989; Irish and Sussex, 1990; Ng and Yanofsky, 2001). Expression of these genes outside the floral meristem has not been described in the literature.

APETALA1 (*AP1*) is associated with the specification of floral meristem identity and is expressed throughout the floral meristem from early on in its formation but not in the inflorescence meristem. Subsequently *AP1* is also involved in organogenesis, but its expression is restricted to the first and second whorls (sepals and petals). *AGAMOUS* (*AG*) in turn is also expressed very early at the apex of the floral meristem, and is eventually associated with the third and fourth whorls (stamens and carpels) during organogenesis. The third gene in this set, *APETALA3* (*AP3*), is activated by *AP1* and is therefore expressed only during floral organogenesis specifically in the second and third whorl. *AP3* is associated with the specification of petals and stamens (i.e. (Ng and Yanofsky, 2001)).

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These three genes therefore represent both early and late stages of floral development, as well as overlapping domains within the whorls of the flower. Promoters from these three genes were expected to allow ectopic expression targeted at specific whorls of the floral domains. If the *TEL* genes do have function/s in one or all of these floral domains then it was expected that domain specific expression would provide insight into what this might be, and what aspect/s of floral development are affected.

3.2.4 Manual cross-pollination

Plants for crossing were either germinated in pots in the glasshouse (as in the case of Basta selectable driver lines such containing ANT:LhG4) or on selective tissue culture, then transferred to the glasshouse (as was the case for the OP-*TEL* lines and both APETALA:LhG4 driver lines). At 2-3 weeks under glasshouse conditions there were sufficient unopened floral buds for crossing. Reciprocal crosses were carried out between a 'male' donor line and 'female' accepter line. Occasionally non-reciprocal crosses were made where necessary. Unopened floral buds of the female line were emasculated under a dissection microscope using tweezers. The tips of the sepals and petals were removed to allow access to the six stamens, which were then removed. Stamens were obtained from the intended male line and applied to the stigmatic tissue of the gynoecium. The reverse cross was then also usually carried out. Siliques that survived this process were harvested as they began to yellow.

3.3.0 Characterisation of LhG:pOP lines

3.3.1 Domain Specific Expression

(a) Terminology

The seed resulting from the transfer of pollen from a F0 donor plant to the female parts of an F0 accepting plant is termed the F1 seed. The seedlings germinated from this seed are the F1 seedlings of the cross. Each F1 seedling which is allowed to self, then produces the F2 generation seeds, and when these are germinated the resulting seedlings are the F2 generation.

(b) Screening of the F1 seed

The F1 seed obtained from manual crosses was screened according to the expected marker of the male donor. Individual F1 seedlings which were found to have the appropriate antibiotic or herbicide resistance for the promoter construct, were then checked for histochemical activity of the reporter gene, and planted directly to soil to collect the F2 seed.

The extent to which expression of each PROMOTER:LhG4 construct conformed to that expected was determined by examination of crosses of the promoter with the empty vector lines (HTOP-G). The extent of domain specific expression were determined from histochemical staining by the reporter gene in individual F1 or F2 seedlings, for each cross. These were determined at three general stages, 5DAG, 12 DAG and within the Floral domain.

(c) Segregation of the Reporter construct

There were three possibilities expected for the segregation of the reporter and OP-*TEL* constructs in the F2 generation (see Table 3.4, pg 116). Firstly the two lines being crossed are heterozygous for one of the two constructs and thus contain a single unlinked copy of this construct. Secondly the one of the two lines is homozygous for the construct, and thirdly, one or both lines contains multiple copies of each construct which are either linked or unlinked.

In general the lines containing the activator and driver constructs were found to be segregating their respective antibiotic resistances at frequencies indicating a single heterozygous insertion so the assumption of heterozygosity seemed warranted. However it is still possible that any individual seedling from these lines, used in a cross, could have been homozygous for that construct.

In order to determine if this was infact the case the segregation of GUS activity was determined in populations of F2 seedling. If the F1 progeny of any cross were infact heterozygous for both constructs (i.e. Aa Bb where A are the OP-*TEL* constructs and B is the LhG4 construct), then upon selfing, their F2 progeny would be expected to segregate positive GUS staining at a frequency of 10/16 or 62.5%. In contrast if one of the lines used in the initial cross is

homozygous, then the frequency of GUS staining would be expected to be ¾ or 75%. Similarly if there were two unlinked versions of one of the constructs in one partner of the cross then the expected frequency of GUS staining would be 19/25 or 76%. In this way the segregation of GUS staining in populations of F1 seedlings was used as an indication of whether there were multiple insertions of the OP-*TEL* or HTOP-G construct.

A minimum of 72 F2 seedlings were therefore examined for GUS activity for each OP-*TEL* /HTOP-G line crossed to the promoter. In addition within this population of plants the frequency of specific phenotypic categories observed was determined as described below. In some cases only the F1 generation seedlings from a cross were able to characterised in this fashion.

(d) Phenotypic categories

F2 seed of the crossed lines were sterilised and plated into a grid on tissue culture plates without selection (in triplicate), at a densities of 24-26 seeds per plate. Phenotypes which were consistently associated with GUS histochemical activity, in independent OP-*TEL* lines, were used to define phenotypic 'categories'. Four such categories of phenotype were created based on consistent elements of these phenotypes. These were Callus-like tissue (CLT), Extremely delayed (ED), Abnormal Cotyledons (Ab. Cotyledon) and Tendril like leaves.

The CLT category referred to the observation that many of the crosses produced highly dedifferentiated tissue similar in appearance (at the cellular level) to callus cells. In turn ED seedlings characteristically were much smaller, slow to elaborate leaves and had vestigial, or did not produce recognizable cotyledons. Seedlings of the Ab. Cotyledon category elaborated aspects of both the preceding categories, but were otherwise often normal in development. They were thus characterised by the localisation of phenotypes to the cotyledons. The fourth category, the tendril-like leaves referred to plants with very narrow or strap-like leaves, giving the seedling a spider-like appearance. This also included a subset of seedlings which produced leaves with tendril like extensions from the underside of the tip of a some leaves.

To some extent the delineation of these categories were artificial since their occurrence (in particular CLT) were associated to varying degrees with one or more of the other categories. Despite this the categorisation was useful

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in to defining the frequency at which the specific elements of these phenotypes arose within any population of any cross. Cytological characterisation of the most frequent phenotypes was then undertaken using Scanning Electron Microscopy and Confocal Laser Scanning Microscopy (CLSM) as described in §3.2.8.

(e) Quantitative characterisation

Finally the crossed lines were assessed quantitatively for the emergence of developmental landmarks in the F2 generation as described in §3.1.3 (pg 89). The developmental landmarks used in these assays were extended to include Leaf set 2, and Leaf 5 in PAp1 crosses. Only PAp3 crosses were assessed for the emergence of the Floral bolt. Cumulative frequencies for the number of seedlings at a particular landmark were obtained from populations grown without selection. For each Promoter >>OP-*TEL* or HTOP-G combination, the number of seedlings examined was approximately equivalent. Finally after the final time point, all the seedlings were harvested and examined for GUS activity. The progression of populations of GUS positive OP-*TEL* lines crossed to the individual promoters was then be compared to that observed in GUS positive OP-GUS lines, and the statistical significance of these differences was determined. Because of the discontinuous nature of the data it was analysed using Friends Exact Test of significance. This is a Chi-square test which compares the difference in percentage in a control versus a variable population. For example, on any given day the percentage of promoter>> OP-*TEL* seedlings which have germinated (i.e. x over the total number of seed) is compared with the percentage observed with promoter>>HTOP-G. From this comparison a probability is given that this difference could have occurred by chance (i.e. $P<0.001$).

3.3.2 Cytohistological examination

(a) Epidermal Cell counts from the cotyledon

Cell counts were made from the adaxial surface of cotyledons (both GUS positive and negative) from F2 seedlings of each of the ANT>>OP-*TEL2* crosses. Samples taken for examination under a bench top microscope were generally prepared in the following way. The sample was transferred to a modified Hoyers solution (chloral hydrate: glycerol:MQ, at the ratio of 8:4:1)

for clearing, usually for 2-3 days. After clearing the samples were mounted in Hoyers solution, onto slides and examined using the 20x objective on a Olympus BX50 light microscope. The cotyledon was generally mounted adaxial side up. A transect was measured across the middle of the cotyledon, which was considered to be the widest part of the cotyledon and was determined by sequential measurements. The number of pavement cells, stomata and the length of the transect were recorded for 5 cotyledons of each line examined. Length was determined using a eyepiece micrometer calibrated for the 20x objective or with calibrated software.

(b) Preparation of samples for SEM

Plant tissue was either dissected, or whole seedlings were fixed by placing into aliquots of 2.5% Glutaldehyde in 50mM NaPB (pH 7.0) on ice. These were briefly infiltrated (~30s) to remove air-bubbles, then put at 4°C overnight. Subsequently the samples were dehydrated, stained, mounted, sputter-coated, and viewed under a Cambridge 250 Mark 3 scanning electron microscope at the HortResearch SEM Unit.

(c) Epifluorescence using the Scanning Confocal Laser Microscope (CLSM)

Cotyledons and roots were dissected from plants at a given time-point, and then vacuum infiltrated with FAA (Formaldehyde 3.8%, Ethanol 50% and Glacial acetic acid 5%) for 15 minutes. The samples were then either left in fixative for 4 hours or overnight. This was followed by an ethanol dehydration series (60, 70, 80, 90, 96, 100, 100%). To minimise tissue damage cach increase was 10%, till 96%, and tissue was left in the 'step' for a minimum of 2 hours. The samples were left at room temperature overnight in 100% ethanol.

Clearing was undertaken using a Roticlear or Histoclear series in 25% increments and a minimum of two hours per step. Samples were then re-hydrated back for staining in two repeat stainings with Fast Green (3 hrs), destained in 50% ethanol for a period of between 2-5 days. The tissue was then temporally mounted in 50% glycerol for optical sectioning with the Leica TCS 4d Scanning Confocal Laser Microscope. Where necessary digital images of these sections were processed for quantitative cell measurements obtained using ImageJ 1.32j image processing software.

3.3.3 Inducible Expression

(a) Inducible lines

Inducible expression were initially identified by screening for Hygromicin resistance, the marker carried on the OP-*TEL* constructs. Characterisation of these lines was two-fold: Firstly the optimal method of application of the inducer (for the most consistent phenotype and GUS activity) needed to be determined and this is described in this section. Secondly, there was the question as to whether induction was coincident with ectopic expression of the *TEL* cDNAs and the method used in this case is described in the next section.

Experiments to determine the optimal method of induction initially used Dexamethasone (DEX), dissolved in 100% ethanol. Subsequently working stocks were prepared using Dimethylsulfoxide (DSMO) as the solvent and this was found to much less phytotoxic than ethanol at the concentrations used. A standard control were mock inductions, using the DSMO solvent at a final concentration equivalent to that used in the DEX inductions (i.e. 0.01-0.05% depending on the concentration of DEX used).

Two distinct methods of induction were used. Inducible lines (both OP-*TEL* and pHTOP-G (EV)) were either exposed to DEX or a mock inducer while growing under glasshouse conditions (see §3.3.3c) or under tissue culture conditions. In the latter case T2 seeds were germinated on tissue culture plates without antibiotic selection but containing 5 μM DEX, or 0.01% DSMO / Ethanol. Levels of apparent GUS expression in seedlings of these lines both with, and without, induction were qualitatively assessed. Subsequently OP-*TEL* seedlings showing GUS staining were examined for phenotype variation relative to the empty vector and the mock induced seedlings. Those lines with the most apparent *β-Glucuronidase* activity in the presence of DEX, (as well as the least phenotype and GUS activity under mock induction) were selected as candidates for further trials. Six independent lines of each construct underwent further characterisation.

The concentration of DEX and the timing of DEX exposure required to generate phenotypes were determined empirically on the basis of phenotype and GUS staining of lines in the tissue culture experiments and also glasshouse

trials. For tissue culture experiments the concentration of DEX which gave the most consistent phenotype and GUS activity was found to be in the range between 10μM and 15μM. Glass-house trials on the other hand were found to require much higher concentrations (30 μM) and frequent spraying in order to elicit the same degree of staining in part because a wetting agent could not be used.

In both glasshouse and tissue culture experiments, it was noted that exposure from germination appeared to generate obvious phenotypes. In contrast transferral of seedlings onto DEX-containing media post germination, or spraying of established seedlings led to evident reporter gene activity but no phenotypes compared to that observed with exposure from germination. Consequently in glasshouse trials, spraying commenced as soon as germination was evident. This in turn meant it was not possible to use a wetting agent in the spray since this was highly detrimental to the survival of the young seedlings.

(b) Expression levels of OP-*TEL* inducible lines

To determine the level of induction achieved by individual inducible lines exposed to DEX (as well as background levels of expression) a tissue culture trial was undertaken. Inducible lines were germinated on tissue culture plates containing Hygromycin 20δ and transferred to growth media without selection. The seedlings were grown without induction to the 6 leaf stage. Prior to induction, (the zero time-point) rosette leaves were harvested from 10 plants of each of the T2 *TEL* lines and an EV line prior to induction. Plants were then either induced (10μM DEX) or mock-induced (0.01% DMSO) by direct application of 2μl of the relevant solution, (by autopipette) to all the fully expanded leaves. Treated leaves were then harvested at 4 hours and 24 hours post-induction. All tissue harvested was immediately flash-frozen, and prepared for RT-PCR as described in earlier sections.

(c) Glasshouse analysis

Inducible lines grown under glasshouse conditions were sowed onto potted soil, suspended in 0.1% agar at a rate of ~10 seeds per pot in trays of twelve. These trays were placed randomly within two different treatment

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groups (+ DEX / - DEX) to reduce variance based on position. Upon visible germination of individual seedlings, the pots were thinned to one plant per pot. The seedlings were then either sprayed with 30 μM DEX or 0.01% DSMO twice a day for 20 days. In initial spraying experiments, the wetting agent Silwet L100 was used at a rate of 0.01% but this was not used in subsequent trials as it was found to be detrimental to the survival of young seedlings. Instead a higher concentration of DEX was used with more frequent spraying and this was found to be adequate for induction.

During the 20 day period of the trial, total leaf counts were obtained for each seedling at 48hr intervals until day 10. In addition to leaf counts the emergence and growth of the floral bolt was determined at 24 hour intervals from day 5 to 14 then at days 17 and 20. Because of the large number of seedlings and lines being inspected, the quantification of floral bud emergence and bolt growth for each line was achieved using a coding system. This was based firstly on the presence or absence of the floral bud, and subsequently on the approximate length of the bolt in millimetres (Table 3.2 below). The approximation of length was made from the base of the last rosette leaf to the tip of the inflorescence. After the completion of the trial the presence or absence of GUS activity was determined for each plant. The mean floral index and leaf count, over the period of the experiment, was then determined for a minimum of 30 GUS positive plants from each inducible line.

Table 3.2 Categories used in classifying stage of floral development: The code used to categorise seedlings on the stage of floral development is shown here.

Category	description	Category	description
0	No Floral bolt visible	4	1.0-1.5 cm
1	Floral bolt <=0.2cm	5	1.5-2 cm
2	<=0.5cm	6	2.5-3.0 cm
3	0.5-1.0cm	7	3.0-3.5 cm

(d) Root elongation analysis

A quantitative analysis of the effect of inducible OP-*TEL* transgene expression on root length was undertaken. This involved measurements of root elongation over time and consequently root growth kinetics. Surface sterilised seeds of the inducible lines were placed aseptically along one edge of square plates (Nunc, 225mm x 225mm) containing MS media with/without DEX-15μM and 1.5% agar. This level of agar was expected to encourage growth along the surface without reducing the amount of moisture available. The seed was arrayed as a single line of 21 seeds spaced 1 cm apart and 3 cm from the top of the plate. The opposite edge of the plates were sealed with waterproof tape (Sleek, Smith+ Nephew) and the entire edge of the plate was then wrapped with sealing film (Whatman) to reduce contamination, leakage, and dehydration of the media. During incubation at 25°C the plates were positioned vertically under lights programmed for long days (16/8hr light dark cycle).

During the course of the experiment the seeds were examined at 24 hour intervals in the first 3 days to determine the date of germination. In addition the length of visible root was measured at 48 hour intervals from the beginning of incubation, using a 20x objective of the Olympus SZX12 binocular microscope and a calibrated eye-piece micrometer. At day 10, plates were photographed using a digital camera and the roots measured using the Java graphical analysis program ImageJ (http://rsb.info.nih.gov/ij).

Whole plants demonstrating phenotypes not present in the control plates were examined for histochemical staining then photographed using a Leica Wild microscope camera. At the end of the experimental period all the roots were excised and examined for GUS activity to determine activity of the reporter constructs. Individual roots were stained for epifluorescence microscopy as described previously in §3.3.2c (pg 100).

3.4 Results

3.4.1 Constitutive ectopic overexpression of *TEL2* causes developmental delays

Approximately 250,000 T0 seeds were plated on MS media containing Kanamycin 50δ. Screening of this seed identified 128 putative 35S:*TEL2* T1 lines (0.051%) and these were transferred to soil. Of those transferred, 89 survived to produce seed, compared with 40/48 empty vector lines transferred. Some examples of individual T1 lines which did show some abnormalities but did not survive to set seed are presented in Figure 3.4 (below). The abnormalities presented in this figure, although striking, were weakly penetrant. Tendril extensions from the underside of the leaf (Fig. 3.4 C) were only observed in a single leaf of three individual seedlings, but not in subsequent leaves. A more extreme phenotype is shown in 3.4 B, where rosette leaves acquired a tendril-like morphology but again this was observed in only four lines. Seedlings showing these phenotypes grew slowly and eventually died without setting seed. A third phenotype, (Fig 3.4 A, E/F) termed disorganised leaves, was observed in another eight lines. In contrast, only 3 of
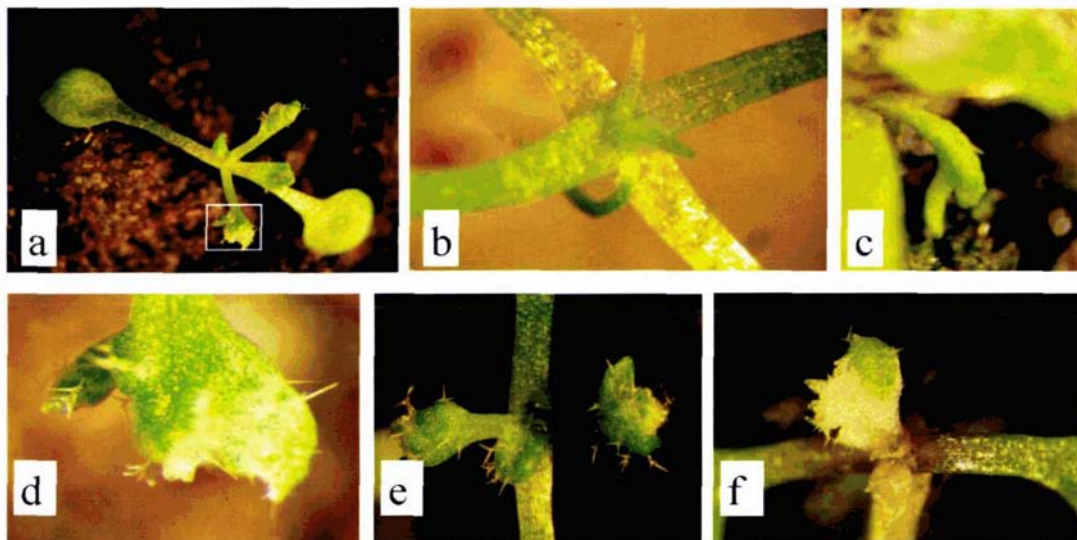
Figure 3.4: Abnormalities observed in some pTART2 T0 lines: These pictures represent two frequently observed (but inconsistent) features in these lines. The first was the presence of malformed leaves (a. d (closeup of inset)and e) This was often but not always associated with callus-like tissue (ie e and f). The second feature was the presence of ten-dril-like extensions, either from the abaxial surface of leaves as in c, or in place of leaves (as in b). An empty vector plant of the same age is shown in (g) as a comparison.
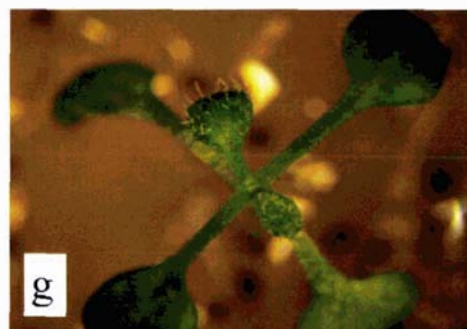
the lines died before setting seed, the remaining 5 lines eventually both elaborated normal leaves and produced seed normally. However T2 seedlings germinated from these seeds showed no obvious morphological defects, nor did they reiterate the observed phenotype in the T1 ancestor.

Of the remaining 84 seed lines, 5 T1 seedlings were identified with a fourth phenotype, whose most notable feature was an apparently prolonged delay before transition to the floral stage (see Figure 3.5 overleaf). At 54 days after germination (DAG), Line 161, had very small rosette leaves and no evident bolt compared to the EV lines and other putative *TEL2* expression lines. The other 4 T1 Lines (69, 113, 115,and 165) had larger more normal leaves, but also did not have floral inflorescences or floral buds (Figure 3.5 A/B). Twenty days later the floral transition had occurred as short bolts were now visible. Of the five T1 seedlings, Line 69 had the longest bolt (i.e. Fig 3.5D) but did not have open flowers at this point.

In comparison, the empty vector lines and the 79 other putative 35S:*TEL2* lines had bolted within 12 days of transfer to soil, and by day 54 all had mature siliques (i.e. yellowing), as well as open flowers (c.f. Line 10 with the front row in 3.5D). Therefore the normal period before the emergence of floral bolts was considerably lengthened in these five T1 lines. This delay was associated with a variable reduction in plant size in these five lines.
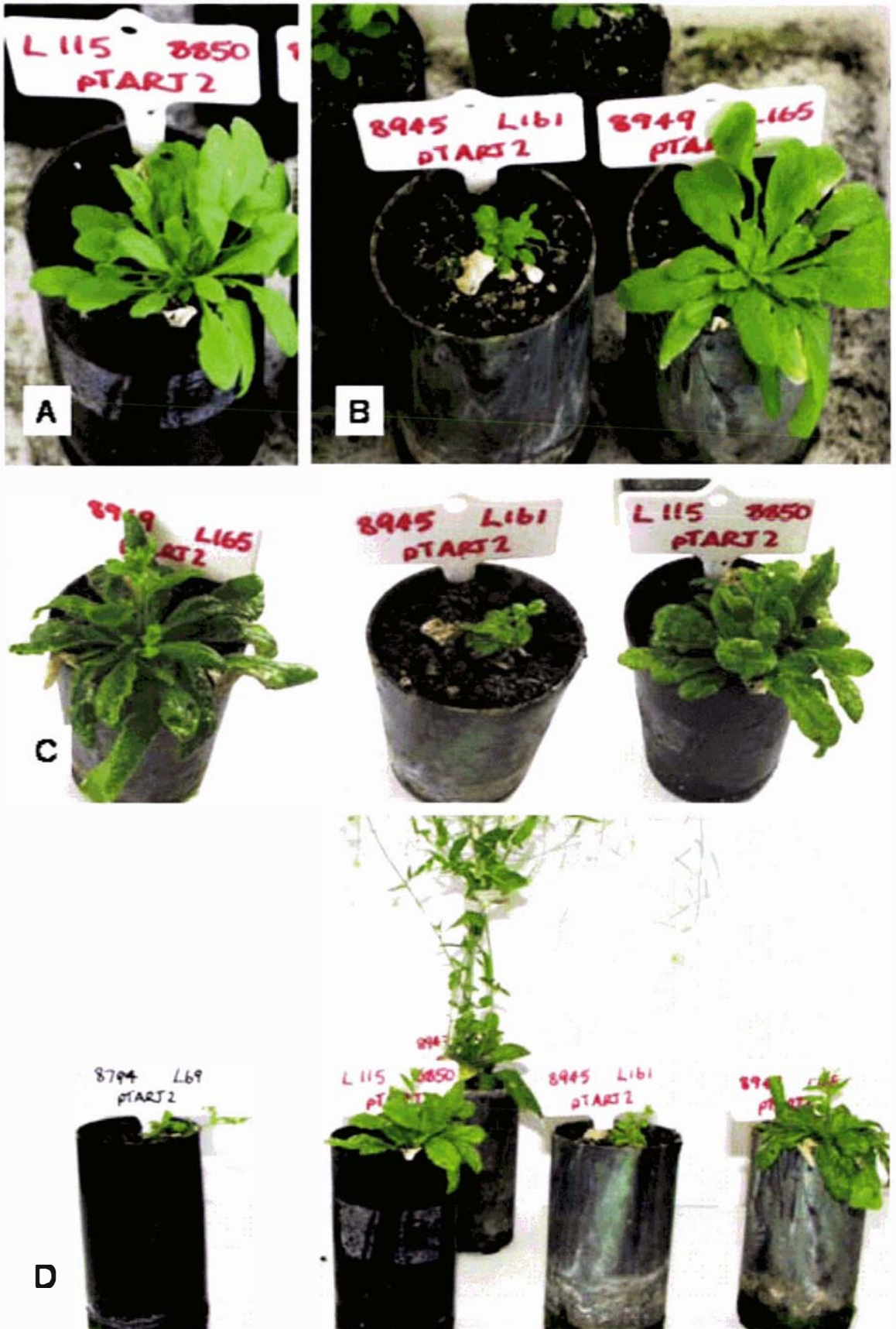
Figure 3.5: Delayed development of 35S:*TEL2* lines: 35S:*TEL2* T1 lines at day 54 (A) Line 115 (B) Line 161 and Line 165. (C)Close up of T1 lines at day 74: (D) Front row from left to right: Line 69, 115, 161 and 165. Behind is an empty vector line (L10) with elongated bolts and mature siliques.

3.4.2 Delayed phenotype coincides with constitutive *TEL2* expression

It was necessary to determine whether the delayed phenotype observed in these five lines was attributable to expression from the P35S:*TEL2* construct in these T1 lines. Total RNA was extracted from samples of rosette leaves as previously described in §3.1.2 (pg 88). This was then assayed for the presence of the *TEL2* transcript using RT-PCR. Two negative controls were included in this expression analysis. These were rosette leaves from a putative P35S:*TEL2* transgenic line (163) identical in appearance to EV, and the Empty Vector line 10.

RT-PCR of the yielded a 280bp product from all the lines, except 163, and EV (Line 10) (Fig 3.6, below). This indicated that the phenotypes observed in the various lines were at least coincidental with the expression of P35S:*TEL2*. Although the level of expression in the expressing lines was not determined quantitatively a positive control for the PCR, the amplification of 10pg of a synthetic *TEL2* transcript allowed some qualitative comparison.
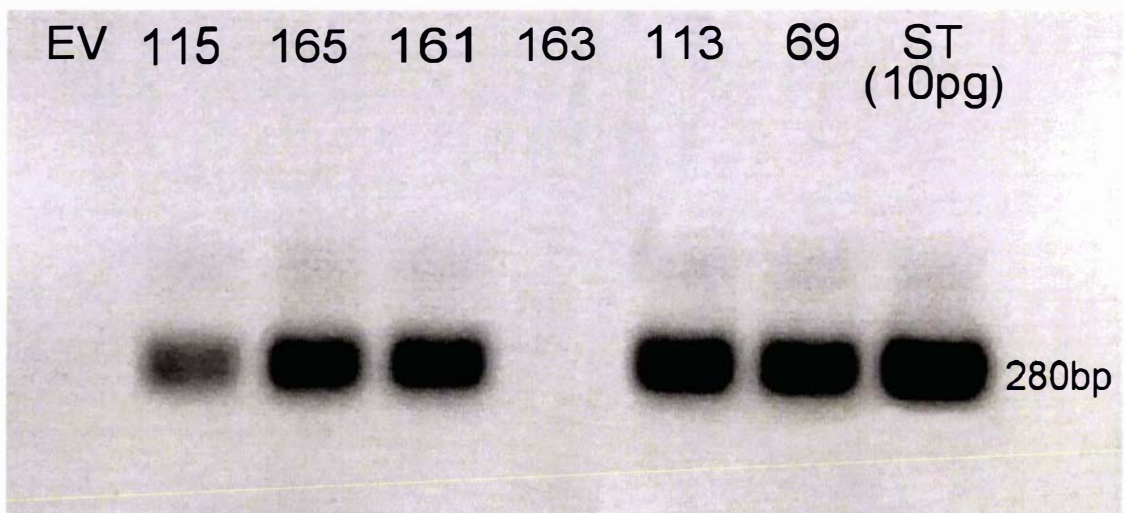
Figure 3.6: RT-PCR analysis of *TEL2* transgene expression. RT-PCR amplification derived from 1 μg of RNA of 35S:*TEL2*/EV T0 lines. The presence of a 280bp product (as indicated in the figure) confirmed the presence of the *TEL2* transcript. 10pg of a synthetic transcript (ST) of *TEL2* was included as a positive control for the RT-PCR reaction.

3.4.3 The delayed phenotype is not reiterated in the T2 generation

The T2 seedlings of six lines (113, 115, 69, 161, 165 and 163) were examined for segregation of the marker and the phenotype/s described above. This included the five lines showing the delayed phenotype, and line 163 which does not have either expression of the *TEL2* construct or phenotype.

Two of these lines (113 and 163) were found to have segregation ratios for the resistance marker that suggested the presence of multiple unlinked insertions of the construct. The remaining four lines (69, 115, 161, and 163) demonstrated a ratio of approximately 3:1 as expected for a single dominant insertion (Table 3.3 below). Both 115 and 113 were excluded from further analysis while 163 was retained as a non-expressing control.

The morphology of 36 T2 Kan^R seedlings from the five lines (including 163) was investigated. These were compared to an equal number of seedlings from two empty vector lines, 8 and 10.

Construct	Germinated	Total	%
P35S:*TEL2*			
L113	15	15	100
L115	104	127	81.9
L161	46	65	70.8
L165	54	73	74
L163	46	49	93.9
L69	54	76	71.1
pART27(EV)			
L8	66	87	75.9
L10	64	80	80
L12	28	41	68.3

Table 3.3: Segregation of the marker in 35S:*TEL2* T2 lines: Segregation of the marker gene (Kanamycin Resistant) was determined in T2 lines containing the 35S:*TEL2* construct.
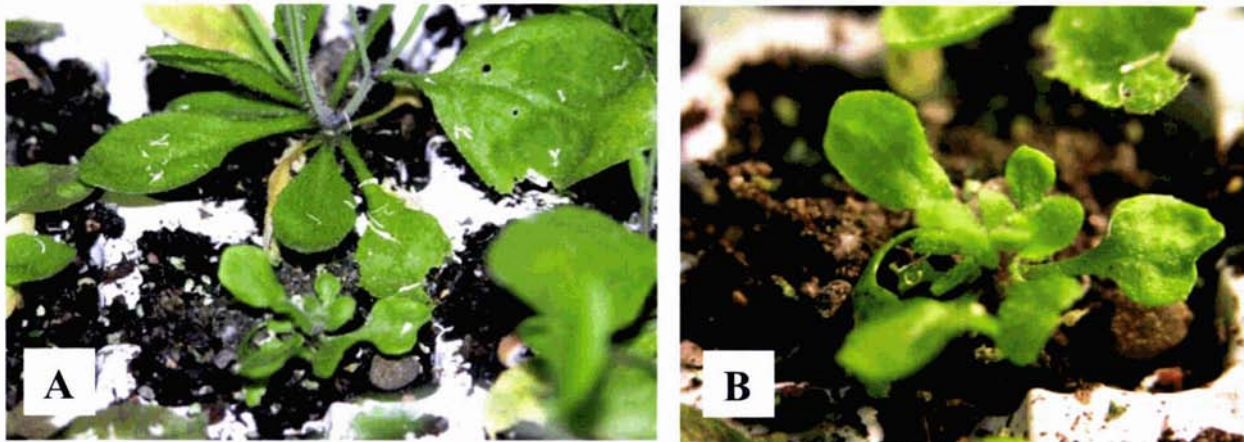
Figure 3.7: Occasional phenotype in T2 seedlings of one 35S:*TEL2* line. **(A)** Comparison of small seedling of Line 165 (foreground) with normal seedling to rear, at three weeks. **(B)** Close-up on seedling showing irregular leaf morphology and reduced size in this seedling.

Only T2 seedlings of Line 165 demonstrated a phenotype similar to that described in §3.4.2 but not at the frequency of antibiotic resistance in these seedlings. Three of the 36 seedlings planted (8.6%) showed irregularly shaped leaves and were overall much smaller (Fig 3.7A/B). As all the plants planted were Kan^R, approximately 12 of these plants could be expected to be homozygous for the construct. Therefore the observed frequency also did not match the expected frequency if the phenotype was associated with plants having two copies of the transgene.

It was possible that the remainder of the lines (as well the 33 other seedlings of 165) did have more subtle phenotypes. In order to exclude this possibility, measurements were made of the floral bolt length, as well as length and number of siliques in the remaining 4 lines. None of the expression lines measured demonstrated any statiscally significant differences between each other, with 163, or with the empty vector lines (data not shown).

Thus the phenotypes identified in the T0 *TEL2* expression lines did not reappear in the T1 seedlings of 3 of the 4 lines. In the only line in which this phenotype did reappear in the T2 seedlings, Line 165, its occurrence (<10%) was not the same as the frequency of the resistance marker (74%).

3.4.4 Developmental progression is mildly delayed

Although the delayed phenotype was not observed in T2 seedlings of 4 of the *35S:TEL2* lines, and measurements had revealed no differences, the

question remained as to whether there were other subtle phenotypes in these lines.

Given that the original T1 lines appeared to have delayed developmental progression it was possible that this was also the case in the T2 generation but it was more subtle. In order to measure this, an assay of developmental progression (see § 3.1.3, pg 89) was used. Two developmental landmarks, germination and the first set of true leaves (Leaf set 1 or Ls1) were evaluated in T2 seedlings of three expression lines (69, 163, 165) and the empty vector, line 10. 80.8% of EV seedlings were found to have completed germination by day 2, and the remainder by day 4 (Fig. 3.8 below). The percentages of Line 163 and Line 69 were not different from EV in this regard (i.e. ~80-90% germinated by Day 2).

Only Line 165 demonstrated a modest delay in germination, but was identical to the other lines by day 2. Subsequently both 165 and 69 showed a reduced frequency of the onset of Leaf-set 1 compared to the EV line and 163. At day 4, 85.7 and 88% of germinants of 163 (n=74) and EV (n=68) respectively had visible true leaves, compared to 41.7% and 50% of L165 (n=74) and 69 (n=69) respectively. By day 6 this had increased to 88 – 90% respectively compared to 100% in the two control lines.

Therefore T2 seedlings of two of the *TEL2* overexpression lines did appear to take longer to produce visible leaves than the controls. While it was clearly not of the same scale as that observed in the previous generation it was consistent with generally slower development in the expression seedlings compared to the empty vector lines. Overall this was taken to imply that while the lines did not have obvious morphological phenotypes, there was a slight developmental delay in some expression lines relative to the empty vector.
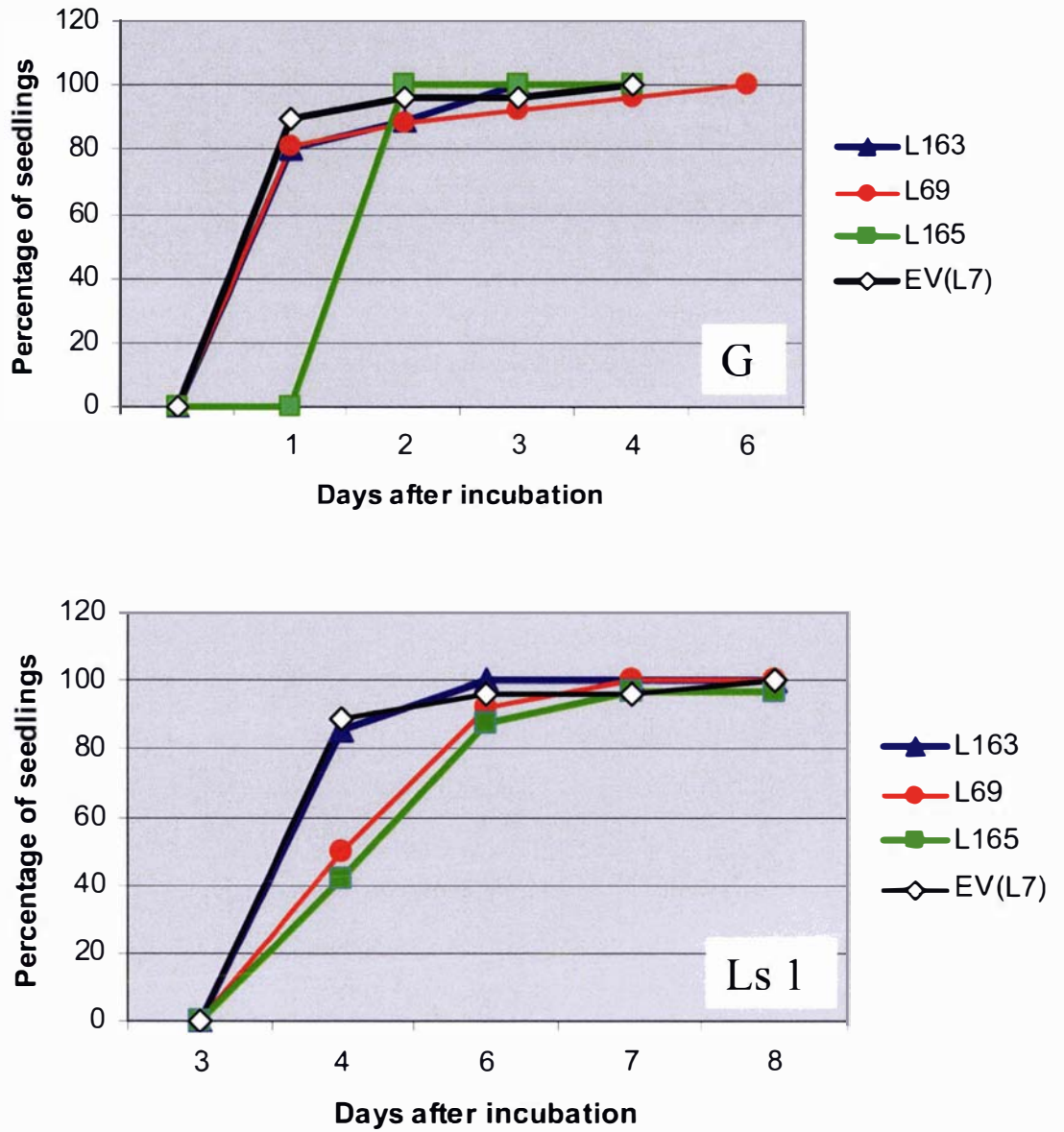
Figure 3.8: Developmental Progression of 35S::*TEL2* lines. The progression of 35S:*TEL2* lines from Germination (G) to Leaf set-1(Ls 1) is shown as the percentage of T2 seedlings reaching a particular developmental landmark on a given day after incubation (n=64).

3.4.5 Domain-specific expression

(a) Confirmation of correct expression domains

The sites of activity of the promoters used in these experiments were determined in the F2 progeny of HTOP-G crosses, with the exception of AGAMOUS. At the time of writing, the *AGAMOUS* promoter had only been successfully crossed with an OP-*TEL2* line. Therefore GUS staining resulting from this combination are represented by an F1 seedling of the AG>>OP-*TEL2*-3 cross. Figures 3.9 (overleaf) and 3.10, (pg 115) present examples of the observed domains of GUS activity in crosses of each of the promoters AP1, AP3, ANT and AG.

Apart from their expected activity in the floral domains, both *APETALATA* and *AGAMOUS* promoters were observed to drive GUS activity in the cotyledons of young plants, the earliest true leaves and to varying extents the roots. Consequently their actual domains of expression were broader than was expected. This was especially true for the APETALA promoters whose domains of expression were comparable that observed for the ANT>>HTOP-G crosses, at least in young seedlings (Fig 3.9 c.f. AP1 with ANT at 5 DAG).
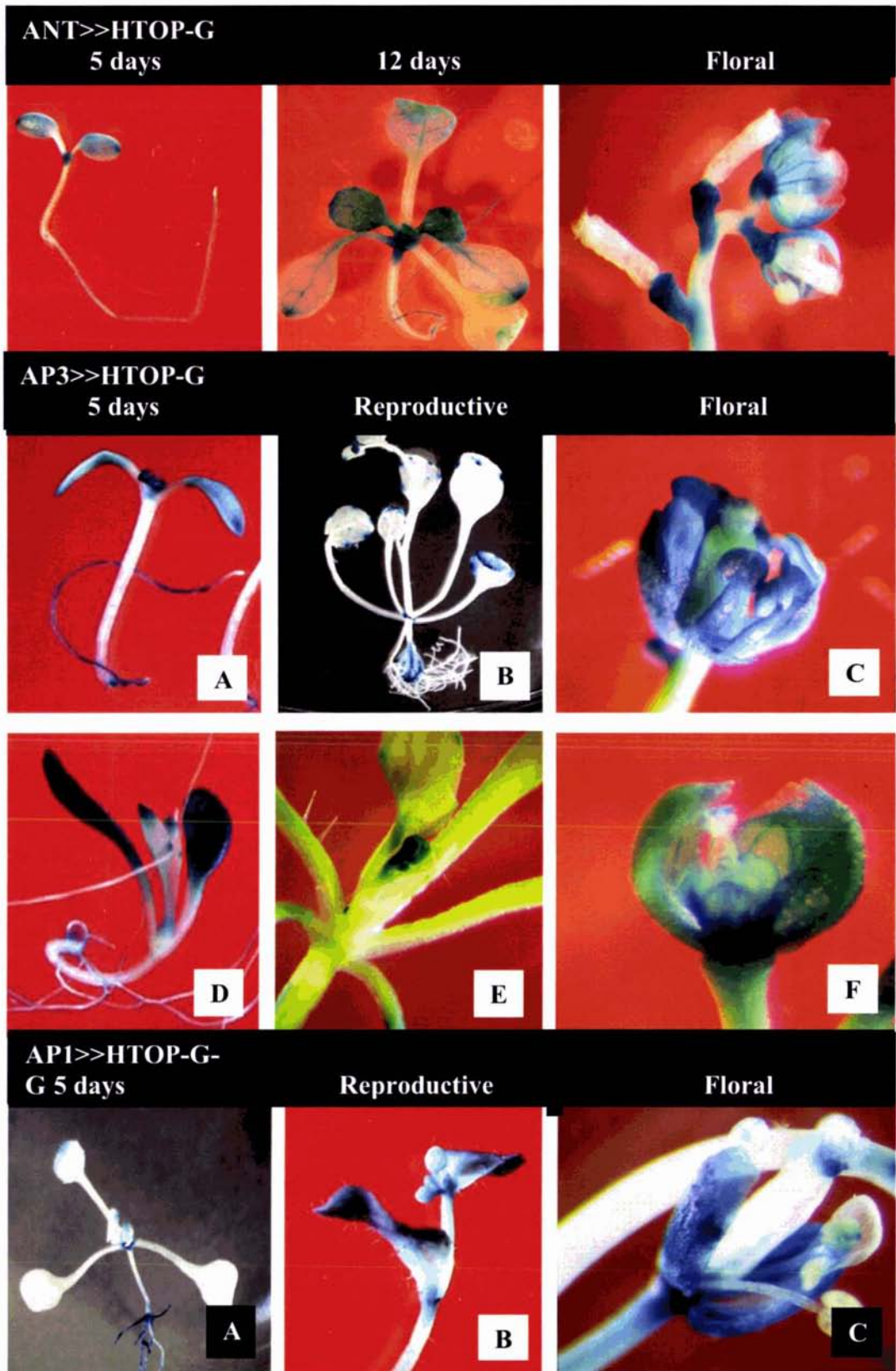
Figure 3.9: Domains of GUS staining in HTOP-G crosses: ANT>>HTOP-G
(A-C): Seedlings are shown at 5 DAG, 12 DAG and in the floral domain. **AP3>>HTOP_G**
(A-F): Staining in representative seedlings shown at 5 DAG (A and D) indicating variable
GUS activity in cotyledons and early leaves; 'Reproductive' stages showing floral bud (E) and
rosette leaf expression in defined domains; Floral: Two representative flowers showing intense
staining in the sepals and stamens. **AP1>>HTOP-G (A-C):** Representative seedlings at 5 days
(A), and 'Reproductive'(B) showing staining in cauline leaf and its axil, as well as the floral
buds. Floral domains (C) showing expression in sepals and petals.

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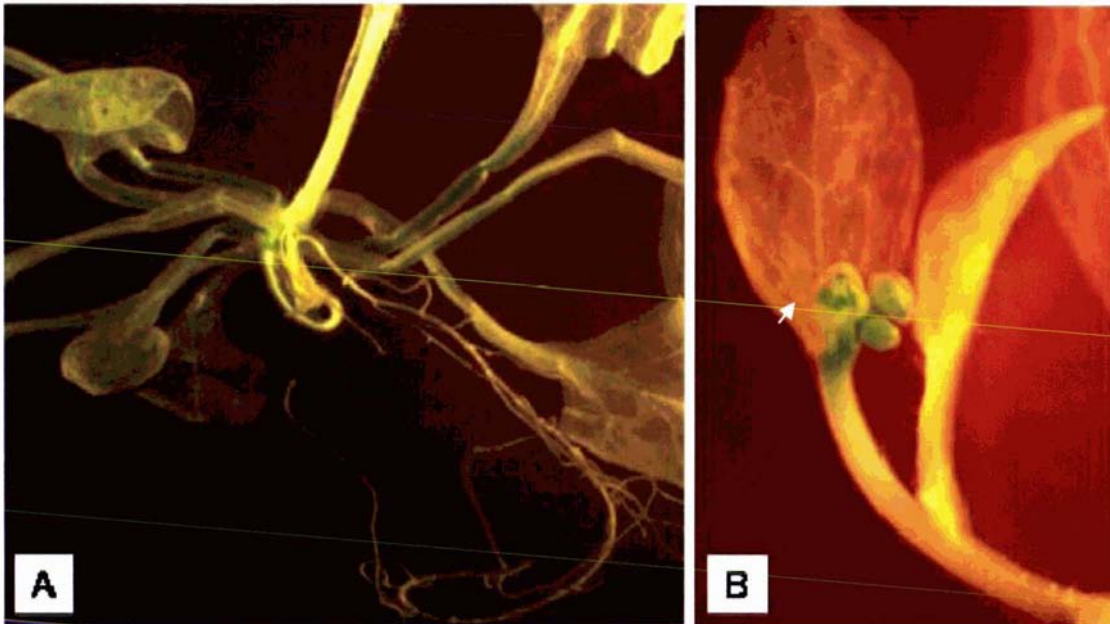
Figure 3.10: Domains of GUS activity in AG>>OP-*TEL2*-3 cross: GUS staining domains of AG>>OP*TEL2* F1 seedlings for which no empty vector crosses were available. F1 Seedlings were stained at 14 days post-germination. **(A)** Faint staining was observed in the petioles and vasculature of the cotyledons, and rosette leaves, but not the roots. **(B)** GUS staining was also observed in the stem of the floral inflorescence, but not in the subtending cauline leaves. Within the early floral buds, staining was evident in the inner whorls, particularly the stamens (white arrow).

However the levels of apparent GUS staining in the cotyledons, and to a lesser extent the later leaves, of the *APETALA* crosses, (compared with ANT) was highly variable within individual F2 seedlings. This is illustrated in Figure 3.9, AP3>>HTOP-G, A and D and also AP1>>HTOP-G, (A). This staining pattern was found to be independent of the HTOP-G line used for the cross meaning this is not likely to be a positional effect of the reporter construct. In contrast although GUS staining was observed in the cotyledons and rosette leaves of the AG>>OP*TEL2* seedlings it was comparatively very faint (Fig 3.10A) and primarily associated with the vasculature.

Furthermore expression of the two AP promoters was distinctive in the true leaves, when compared with ANT and AG, but also between the two AP promoters themselves. The AP1 crosses typically displayed GUS staining at the distal tips of both rosette and cauline leaves. In addition expression was evident throughout the cauline leaves as well as their axils which was not evident in AP3 crosses. Moreover in the AP3>>HTOP-G seedlings, expression in the later leaves rapidly resolved into discrete domains around the perimeter of the leaves corresponding to the position of gnathodes (i.e. AP3>>HTOP-G (B) in Fig 3.9 pg 114). In comparison ANT>>HTOP-G

expression persisted throughout the youngest leaves, and was notably reduced in mature leafs.

In the floral domains, the observed expression of GUS was generally as expected. The highest expression of *AP3>>OP*-GUS lines was observed (as predicted) first in the floral buds and then whorls 2 and 3 of the open flower. Similarly *AP1* produced GUS staining in the early floral buds and then was restricted primarily in the sepals and petals. Whether the AP1 and AP3 drivers produced contrasting GUS expression patterns in the early inflorescence and floral meristems as has been described (for example (Guan et al., 2002)) was not examined in these lines. *ANT* drove expression in the sepals and pedicel as expected but was not evident in organs of the inner whorls at 15 days post germination. *AGAMOUS* was expected to be restricted to the floral meristem and subsequently whorls 3 and 4 of the mature flower, which was what was observed; GUS staining was observed in the pedicels of the primary floral inflorescence as well as within axillary floral inflorescences, indicative of its early expression in the floral meristem. In the mature flowers, GUS activity was most obvious in the stamens and only weakly in the carpels. Apart from faint GUS staining in the rosette leaves, AG did not drive staining in either the cauline leaves, or roots as was observed in the AP and ANT promoter.

As described for the expression noted in the cotyledons, expression in the roots of AP crosses was highly variable (compare AP1 and AP3 in Fig 3.9) both within plants and between crosses. In the ANT crosses this expression was primarily with the vasculature of the primary and root branches and but was excluded from the root apical meristems which was as expected.

In conclusion, the observed domains of GUS staining under these drivers were generally as expected. The primary exceptions mentioned were the activity of both *APETALA* promoters early in vegetative development, and within sectors of the roots. In contrast the expression of ANT>>HTOP-G was largely as expected apart from the absence of strong expression in the carpels. However this probably represents an artifact of poor infiltration by the substrate of GUS into these organs.

(b) Segregation of the Reporter construct

The segregation of GUS staining, and thus the Reporter construct, was investigated in the domain specific crosses. These results are presented in Table 3.4 below. As outlined in §3.3.1c, if the lines which were used in a cross, were both heterozygous, (i.e both contained single unlinked insertions of the constructs) then the F2 seedlings should segregate the reporter construct at a frequency of 9/16 or 56.25%. If not, (i.e.one or other of the constructs is present as multiple unlinked copies) then the frequencies of GUS staining would be expected to be 70%.

Promoter>>	GUS staining				
OP-Gene cross	Line	Generation	Observed	Expected	Chi Square (P)
ANT>>OP-_TEL1_	2	F1	75 (12/16)	8/16 (50)	0.046
ANT>>OP-_TEL2_	2	F2	75.6 (65/86)	48/86(56.25)	_P<0.0001_
	3	F2	67.9 (55/81)	46/81	0.044
	4	F2	69.2 (68/100)	56/100	0.016
	7	F2	51.3 (41/80)	45/80	**0.367**
	57	F2	72.6 (61/84)	47/84	0.002
ANT>>HTOP-G	1	F2	52.4 (44/84)	47/84	**0.51**
	3	F2	75 (66/80)	47/84	_P<0.0001_
AP1>>OP-_TEL1_	1	F2	43.7(52/119)	67/119	0.006
	2	F1	75 (12/16)	8/16 (50)	0.046
AP1>>OP-_TEL2_	2	F2	73.8(59/80)	45/80 (56.5)	0.002
	3	F2	73.1 (51/70)	39/70	0.004
	5	F2	64.4(47/73)	41/73	**0.157**
	17	F1	57.1 (16/28)	14/28(50)	**0.45**
AP1>>HTOP-G	1	F2	57.4 (31/54)	30/54(56.25)	**0.784**
	3	F2	74.1 (83/112)	63/112	_P<0.0001_
AP3>>OP-_TEL1_	1	F2	49.1(55/112)	63/112	0.078
	68	F2	52.0 (39/75)	42/75	**0.488**
AP3>>OP-_TEL2_	2	F2	73.6 (53/72)	41/72	0.004
	3	F2	76.2(48/63)	35/63	0.001
	14	F1	not tested	50	n/a
AP3>>HTOP-G	1	F2	52.3(22/42)	24/42	**0.533**
	3	F2	75.0 (27/36	20/36	0.019

Table 3.4: Frequencies of GUS histochemical activity in F1/F2 seedlings of crosses: The number of seedlings expected and observed to stain positive for GUS are shown. Numbers in parentheses show the expected, and observed percentage, respectively. The P result from Chi Square tests of the expected distribution is shown in the final column. Highly significant results are in italics while low significant results are bolded.

In the ANT crosses, OP-TEL1-1, OP-*TEL2*-2, 3, 4, and the control line ANT>>HTOP-G 1 showed an observed frequency of GUS staining close to (or exceeding) 70% which the Chi-Square result was found to be signicant (P<0.0001 to <0.05). In contrast HTOP-G-1, and ANT>>OP-*TEL2*-7, were found to have frequencies not significantly different from that expected indicating these were the only crosses not to contain unlinked copies of the OP-TEL T-DNA.

In the AP1 crosses, only OP-*TEL2*-5 and 17 were close to the expected frequencies. In contrast HTOP-G-3 and three of the OP-*TEL* crosses, (OP-*TEL1*-2, OP-*TEL2*-3, & 4), segregated the reporter at frequencies exceeding 70%, while OP-*TEL1*-1 and HTOP-G-1 were not significantly different from the expected frequency. This contrasted with the AP3 crosses where OP-*TEL1*-1 (and HTOP-G-3) were found to have lower than expected frequencies (OP-*TEL1*-1 at 49%) while OP-TEL1-68 was not different from expected. However of the first two, neither of these frequencies, were found to be highly significant. In particular the observed difference in AP3>>OP-*TEL1*-1 was only found to be mildly significantly (P<0.1). In line with observations with other crosses, both AP3>>OP-*TEL2* crosses had frequencies exceeding 70% and these were confirmed to not be the result of chance.

In summary most of the OP-*TEL* lines examined were found to have frequencies indicating they contained multiple unlinked copies of the OP construct. Crosses involving the lines, OP-*TEL1*-2, OP-*TEL2*-2, 3, 4, 2-57 and HTOP-G-3 lines consistantly yielded GUS activities at frequencies which were close to 70% and significantly different from that expected for a single insertion of the reporter T-DNA. In contrast the crosses involving the lines OP-*TEL1*-68, OP-*TEL2*-5, 17 and HTOP-G-1 showed frequencies which were not different from the expected frequency. However while AP1 crosses with OP-TEL1-1 indicated that this line contained multiple copies of the reporter construct (P=0.006) in the AP3 cross the observed difference was considerably less significant (P<0.1). This probably represents unknown factor/s relating to the activity of these crosses in the two different crosses.

3.4.6 AP1>>OP-*TEL*

(a) Phenotypic characterisation

The AP1:LhG4 driver was crossed with three OP-*TEL1* lines (OP-*TEL1*-1, 2 and 1-68-S7) and four OP-*TEL2* lines (OP-*TEL2*-2, 3, 5, and 17-S7). Of these crosses only OP-*TEL1*-2 was characterised in the F1 generation, the rest being characterised in the F2 generation. Specific phenotypic catergories, and their frequency of occurrence, are presented in Figure 3.11 and Table 3.5 (pg 119 & 120 respectively).

The APETALA>>OP-*TEL1* crosses described in this section and §3.4.7 were generally distinct from those made with OP-*TEL2* lines, because only the OP-*TEL1* crosses produced the Tendril like phenotype. This was especially true in the AP1 crosses, where seedlings of this category elaborated rosette leaves with very narrow laminae, although tendril like cauline leaves were also observed (Fig 3.11 cf. C/D with A/B pg 118). The primary distinction between tendril like versus strap like leaves was that laminae of the leaf was evident, but highly reduced, in the latter. In contrast the tendril like leaves lacked evident laminae and appeared to lack normal abaxial/adaxial polarity although this was not investigated at a histological level. It is not clear for example whether these leaves were composed mostly or entirely of midvein tissue and simply lacked a lamina. In the AP1>>*OP-TEL1*-1 F2 seedlings, and OP-*TEL1*-2 F1 seedlings, the phenotype was seen at
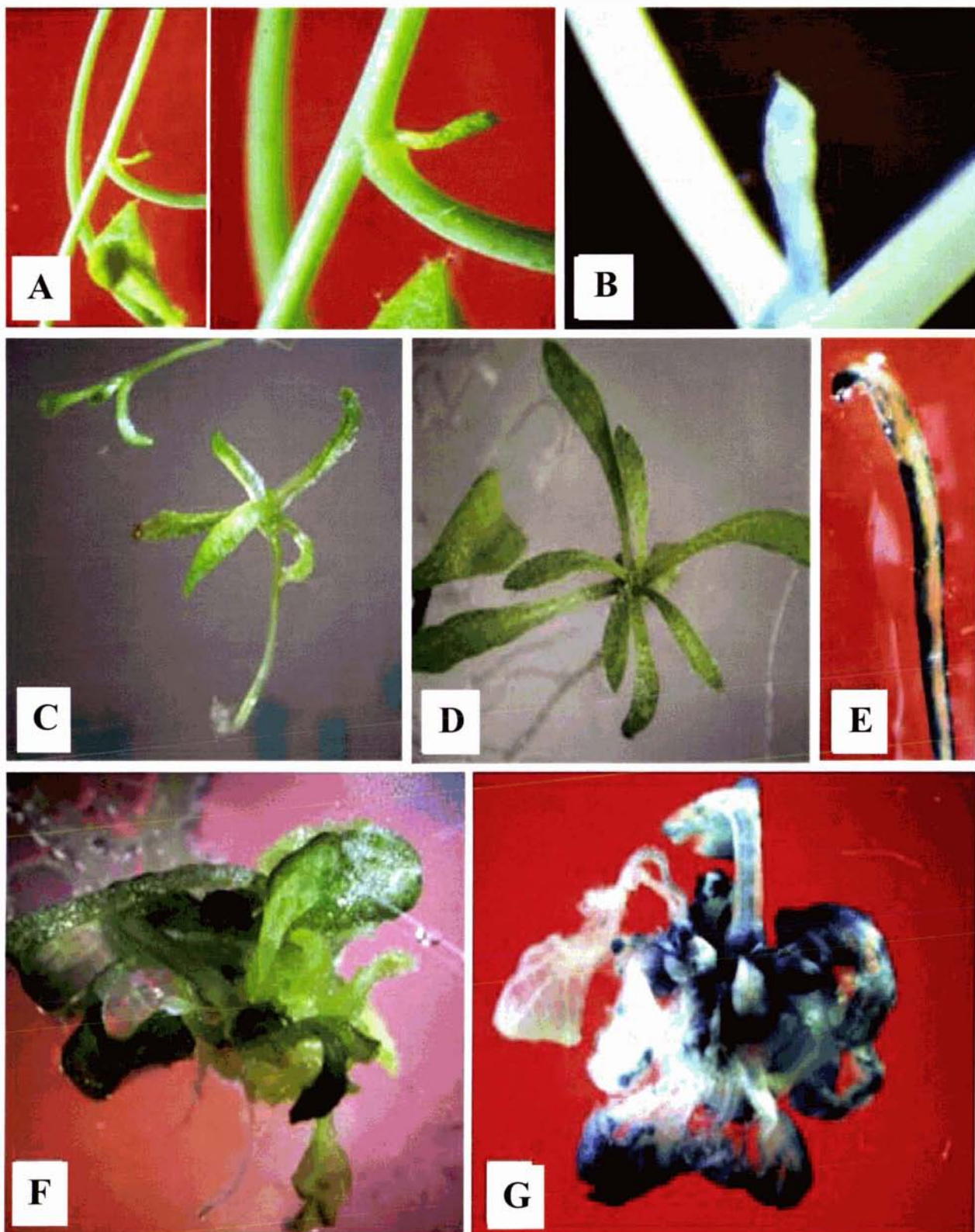
Figure 3.11: Phenotypes observed in AP1>>OP-*TEL* lines. (A) Occasional tendril-like cauline leaves observed in 2 F1 lines of AP1>>OP-*TEL1*-2 shown with a normal cauline leaf of the same plant. **(B)** Histochemical GUS staining of the tendril. **(C)** 10 day old seedlings showing narrow leaved phenotype of AP1>>OP-*TEL1* line 1. **(D)** Older seedlings at 28 days after germination. **(E)** GUS staining characteristic of narrow leaves. **(F)** Second phenotype observed in both AP1>>OP-*TEL1* and 2 lines; disorganised plants with callus-like tissue and fused leaves. **(G)** GUS staining characteristic of disorganised plants.

Cross	Total seedlings		Frequency of Phenotypes (%) in GUS + seedlings			
	n	**GUS+**	Ab.cotyledon (n)	Ex.delayed Seedling	CLT	Tendril-like leafs
ANT>>OP-*TEL1*-2	16	12	8.333% (1/12)	8.33% (1/12)	0%	*16.6%(2/12)
ANT>>OP*TEL2*-2	86	65	53 (34/65)	25% (16/65)	0	0
-3	81	55	49.1 (27/55)	21.0 (13/60)	18.2 (10/55)	0
-4	100	68	63.2 (43/68)	23.5 (16/68)	5.9 (4/68)	0
-7	80	41	0	0	0	0
-57	84	61	35.7 (22/61)	26.23(16/61)	8.2 (5/61)	0
ANT>>HTOP-G-1	**84**	**44**	0	0	0	0
ANT>>HTOP-G-3	**88**	**66**	0	0	0	0
AP1>>OP-*TEL1*-1	119	52	17.3 (9/52)	0	15.4 (8/52)	26.9 (14/52)
-2	16	12	25 (3/12)	0	8.33 (1/12)	16.6 (2/12)
AP1>>OP*TEL2*-2	80	59	0	0	5.1 (3/59)	0
-3	70	51	0	0	15.8 (9/51)	0
-5	73	47	0	0	0	0
-17	28	16	0	0	0	0
AP1>> HTOP-G-1	**54**	**31**	0	0	0	0
AP1>> HTOP-G-3	**112**	**83**	0	0	0	0
AP3>>OP-*TEL1*-1	112	55	14.5(8/55)	0	10.9(6/55)	18.2(10/55)
-68	75	39	7.7(3/39)	0	0	0
AP3>>OP*TEL2*-2	72	53	0	0	3.8 (2/53)	0
-3	63	48	0	0	16.7 (8/48)	10.41 (5/48)
-14	Not recorded		0	0	0	0
AP3>> HTOP-G-1	**42**	**22**	0	0	0	0
AP3>> HTOP-G-3	**36**	**27**	0	0	0	0

Table 3.5: Frequency of Phenotypes in F1 and F2 crosses: Percentages indicate the frequency of the four defined phenotypic categories observed in GUS positive seedlings. The numbers in parentheses indicate number of seedlings with the phenotype over the total number of seedlings examined.

a frequency of 26.9% (14/52 plants) and 16.6 % respectively (2/12 plants,

cauline leaves only). In contrast this phenotypic category was not observed in

any of the AP1>>OP-*TEL2* crosses.

In terms of the other phenotypic categories both AP1>>OP-*TEL1*

crosses, and 2 of the 5 OP-*TEL2* crosses, showed varying degrees of Callus

-like Tissue at relatively low frequencies. In contrast only the OP-*TEL1*

crosses showed abnormal cotyledons in both F2 and F1 seedlings. In this case

the Abnormal cotyledons phenotype category referred to the observation that

AP1>>OP-*TEL1* seedlings produced callus like tissue both in their cotyledons

and on all subsequent leaves, whereas this was not observed in the correspond-

ing OP-*TEL2* crosses (i.e the cotyledons were normal).

Where callus like tissue was produced on subsequent true leaves this

lead to seedlings which were small compact plants with a highly disorganised

appearance and were most prevalent in OP-*TEL2*-3 and OP-*TEL1*-1 lines (~15%). Examples of the latter are presented in Fig 3.11 F/G).

The final observation made for these crosses was that no obvious morphological phenotypes were observed in the floral domains of either F0 or F2 seedlings containing either OP-*TEL* construct in combination with the AP1 driver. It should also be noted at this point however that the callus like seedlings displayed in Figure 3.11 (F/G) did not produce recognisable floral bolts or flowers despite prolonged cultivation.

(b) Developmental progression

Assays of developmental progression were undertaken for three AP1>>OP-*TEL2* crosses (2, 5, 7), two AP1>>OP-*TEL1* crosses (1, 1-68-S7) and one AP1>>HTOP-G cross (1). The results of this analysis are depicted in Figure 3.12 on the next page (pg. 121/122).

Initial rates of germination were low. The AP1>>OP-*TEL1*-1 seeds showed a very low frequency of germination, which was significantly different up to day 5 (Fishers Exact Test, $P<0.00001$). In contrast, the OP-*TEL1*-68 and both OP-*TEL2* lines were significantly different from the empty vector line only on days 1 and 3 ($P<0.005$-0.01). Overall the OP-*TEL1* crosses showed a slower increase in the frequency of germination than those including the OP-*TEL2* lines and the EV.

Subsequent the emergence of the true leaves was also found to be slower than the empty vector. By day 2, 80% of the AP1>>HTOP-G germinants had their first true leaves (Ls1) and by day 3 this was 100%. In contrast, on Day 3 between 20-60 % of the OP-*TEL2* crosses, and 0-10% of the OP-*TEL1* crosses had achieved Ls 1. By Day 4, ~80% of OP-*TEL2* germinants had evident leaves, compared with 50-60% of the OP-*TEL1* lines. These latter crosses only breached >= 80 % on day 5. Consequently the seedlings of the OP-*TEL* crosses took twice as long (2 days versus 4 and 5 days respectively) as the empty vector lines to produce their first true leaves.

By day 6, ~75% of the EV seedlings were found to have their second leaf set (Ls 2), which compared to ~20-40% of the OP-*TEL2*, and only 10-20% of the OP-*TEL1* crosses. However this frequency rapidly increased in both OP-*TEL2* crosses, so that by day 7-8 they were both equivalent to EV. In contrast both the AP>>OP-*TEL1* crosses were found to be significantly different from the OP-*TEL2* lines and empty vector during this period. At day 7, ~60% of the

118
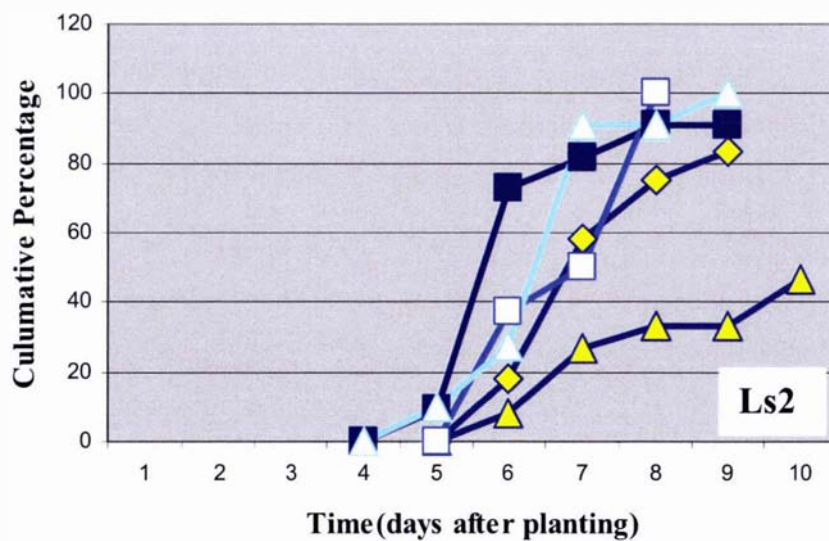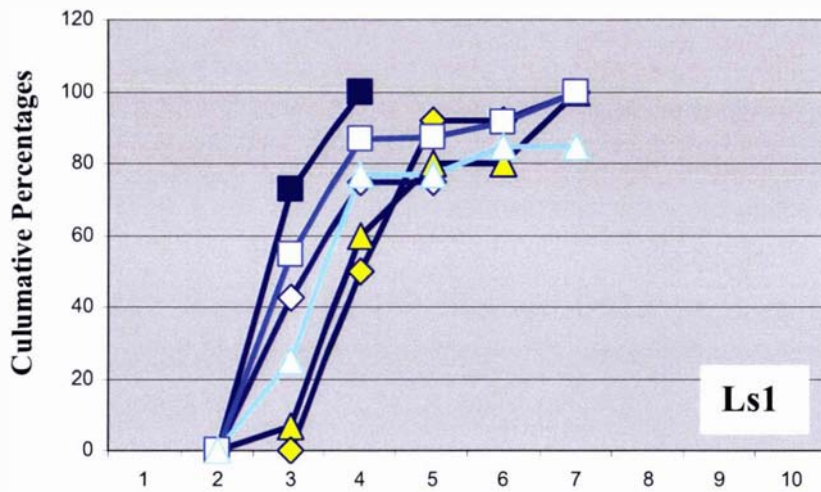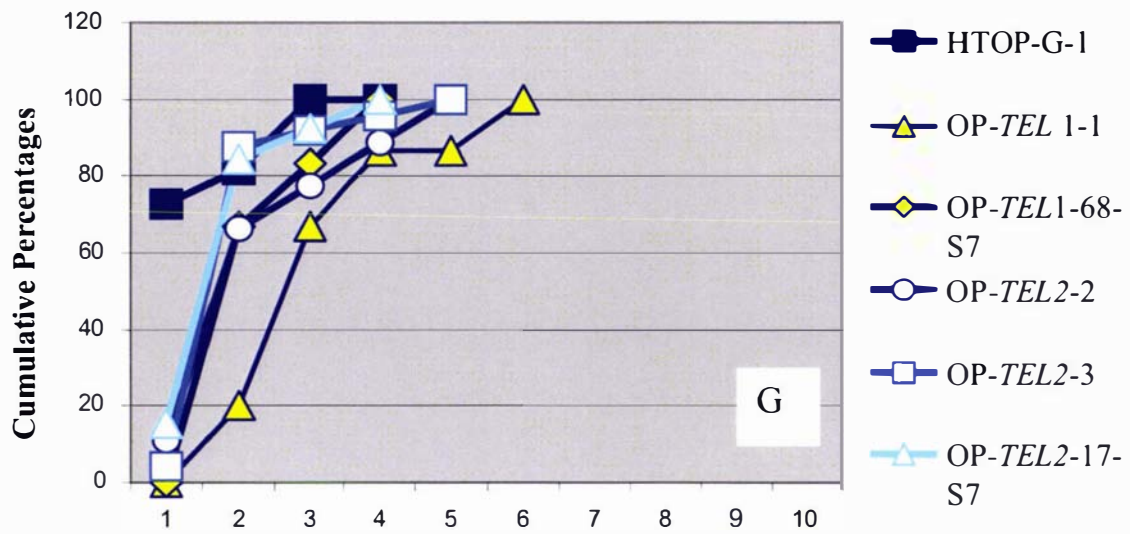
Fig 3.12: See Figure description overleaf for explanation.

Figure 3.12 (continued from previous page): Developmental progression of *AP1>>OP-TEL* **crosses:** Four developmental landmarks were examined for five OP-*TEL* crosses and one OP-GUS line. These were Germination (**G**), Leaf set 1, 2 (**Ls1, 2**). The frequency of Leaf 5 (**L-5**) was not followed in AP1>>OP-*TEL2*-2. The graphs depict the frequency (%) within a population of GUS positive F2 plants, which passed a particular development landmark versus time after planting (i.e. placement of tissue culture plates into incubation conditions). For comparison purposes the numbers of GUS positive seedlings, of different lines, used to generate these frequencies were generally similar but were not identical; HTOP-G 1 n=78, OP-*TEL1*-1, n=84, OP-*TEL1* 68-S7 n=82; OP-*TEL2*-2 n=75; OP-*TEL2*-3, n=68, OP-*TEL2*-17-S7, n=62.

OP-*TEL1*-68 seedlings possessed Ls2 compared to 25% of the OP-*TEL1*-1 versus 90% of the Empty vector (Fisher's Exact Test, day 7, P=0.014; day 8 P=0.0015). Only the OP-*TEL1-1* crosses remained below 50% showing their second leaf set up to day 9.

In the final developmental landmark (L5) there was no definable pattern discerned in terms of the progression in relation to the empty vector. For example the fifth leaf of ~18% of the OP-*TEL2*-3 seedlings was evident a day earlier than the empty vector, reaching 70% by day 10, while only 10% of AP1>>OP-*TEL2*-17-S7 seedlings had their fifth leaf by day 10. 55 % of the OP-*TEL1*-68 cross had their fifth leaf by day 9 compared to 30% the OP-*TEL1*-1 cross on day 10. In comparison the visible emergence of the fifth leaf is largely linear in the seedlings of the empty vector during the period of observation. The first seedlings with their fifth leaf in this cross were seen on day 8 (34%) and this increases to 75% by day 10. Consequently two of the

OP-*TEL2* crosses rapidly converge with the empty vector while OP-*TEL2*-17 and OP-*TEL1*-1 remained below 50% until the final day.

3.4.7 AP3>>OP-*TEL*

(a) Phenotypic characterisation

Two lines of OP-*TEL1*(1, 1-68-S7) and three OP*TEL2* lines (4, 17-S7) respectively were crossed with the AP3:LhG4 promoter line. The results of the phenotypic observations are presented in Table 3.5 (pg 119) and Fig 3.13 (pg 124).

F2 seedlings of the OP-*TEL1*-1 cross, yielded further examples of the Narrow or Tendril like-leaf phenotypic category (as described previously in §3.4.6a), and these are shown in Fig 3.13 (C-E,G pg 124). This phenotype was observed in 18.2 % of GUS positive plants of this cross, but not at all in the OP-*TEL1*-68-S7 cross. Similarly the presence of callus-like tissue in the petiole of leaves occurred in 10.9% of AP3>>OP-*TEL1*-1 F2 seedlings but was not observed in seedlings of the AP3>>OP-*TEL1*-68 cross. The only phenotypic category observed in this latter cross were Abnormal Cotyledons (i.e. abnormal shape, presence of callus like tissue but only on the cotyledons) and this only in a small number of plants (8/55).

Of the three OP-*TEL2* lines crossed with AP3, only AP3>> OP-*TEL2*-3 showed narrow or tendril like leaves (9.6%). The other two lines were not observed to have this phenotype. The predominant phenotype in the OP-*TEL2* crosses was callus like tissue as per the examples in Fig 3.13F and G. Abnormal cotyledons were also not noted in the OP-*TEL2* crosses. Moreover the seedlings of the cross AP3>>OP-*TEL2*-14-S7 although GUS positive, did not shown any features of the four phenotypic categories. They also did not show any phenotypes in the floral domains.

(b) Developmental progression

The developmental progression of two AP3>>OP-*TEL1* cross (1, 1-68-S7), AP1>>OP-*TEL2*-4 and AP1>>HTOP-G-3 were examined. The results of this analysis are depicted in Figure 3.14, (pg 125 -126).

On day 1 80% of the control cross had germinated. By day 2 this had increased to over 90%, and of these plants, >90% had produced their first true
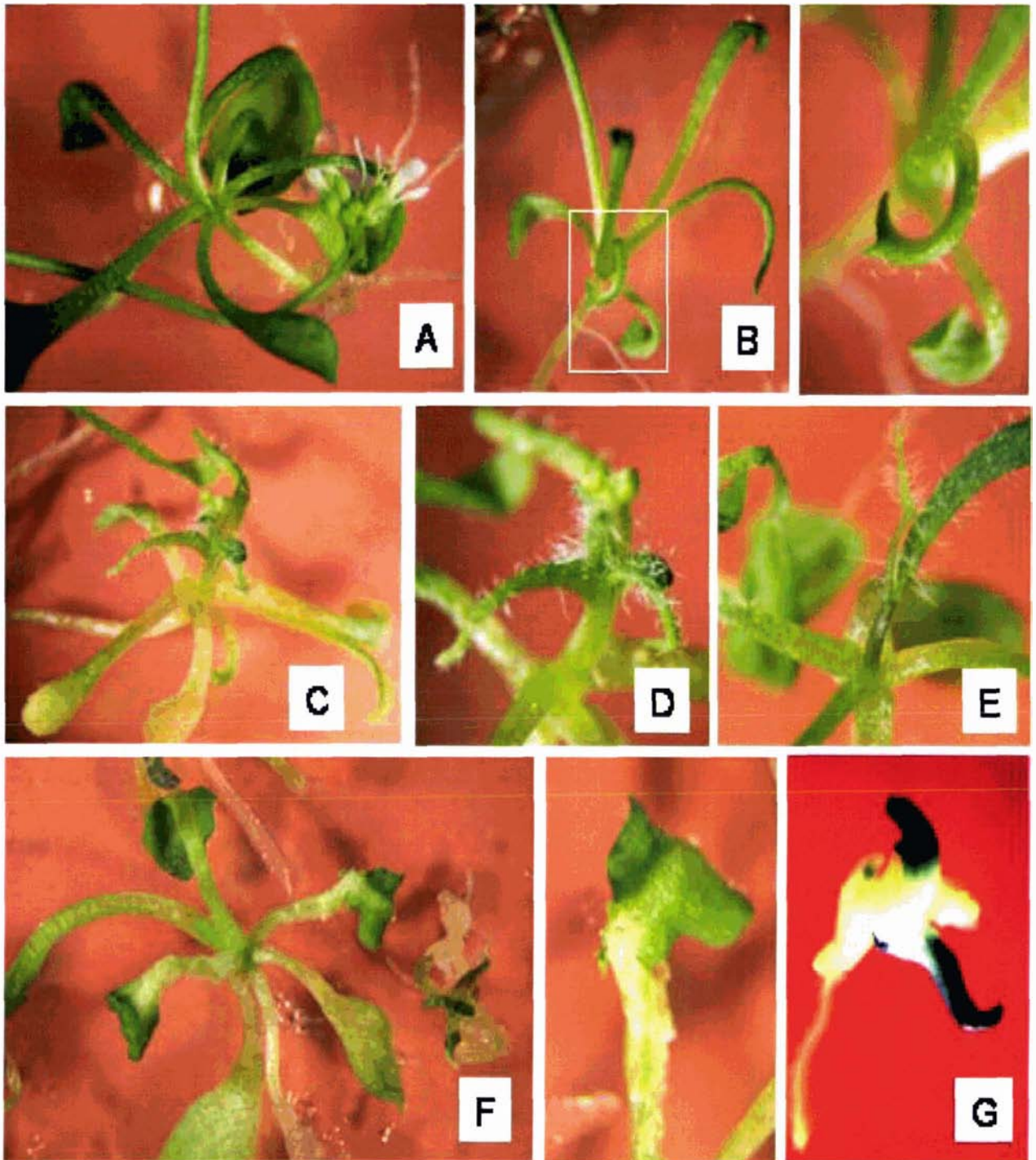
Figure 3.13: Morphological phenotypes in *AP3>>OP-TEL* **crosses: (A)** *AP3>>HTOP-G-*3 F2
seedling at day 15, showing rosette leaves and bolt with inflorescence and open flowers. **(B)**
'Tendril like leaves' phenotype in 15 day old seedling of **AP3>>OP-*TEL1-1*.** Inset box is
magnified in following frame and shows morphology of the highly curved narrow "leaf". **(C/D)**
AP3>>OP-*TEL1-1* seedlings with narrow leaf phenotype with tendril cauline leaf, and tendril
extension from second cauline leaf, and floral buds at 15 days. **(E) AP3>>OP-*TEL1-1*** seedling
with tendril-like putative cauline leaf and absence of subtending floral bud. **(F)** Two
AP3>>OPTEL2-3 seedlings with callus-like tissue showing the range of phenotype. Both
seedlings were photographed 15 days after germination. Closer view of callus like tissue in petiole
of one rosette leaf from larger seedling in **(F). (G)** Smaller seedling to left in (F), shown here
stained for GUS activity, Note tendril like 'leaves' similar to those displayed in (B).

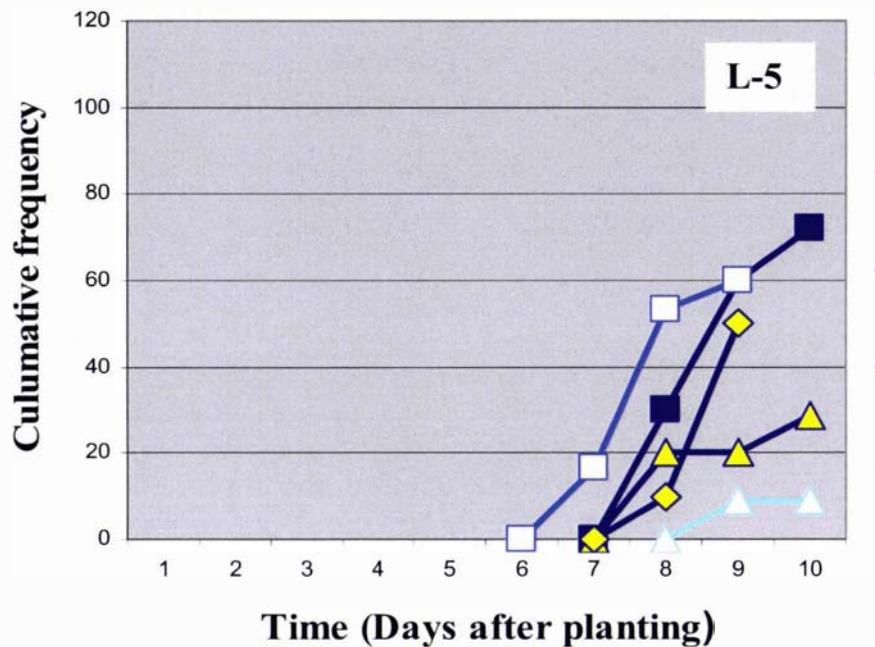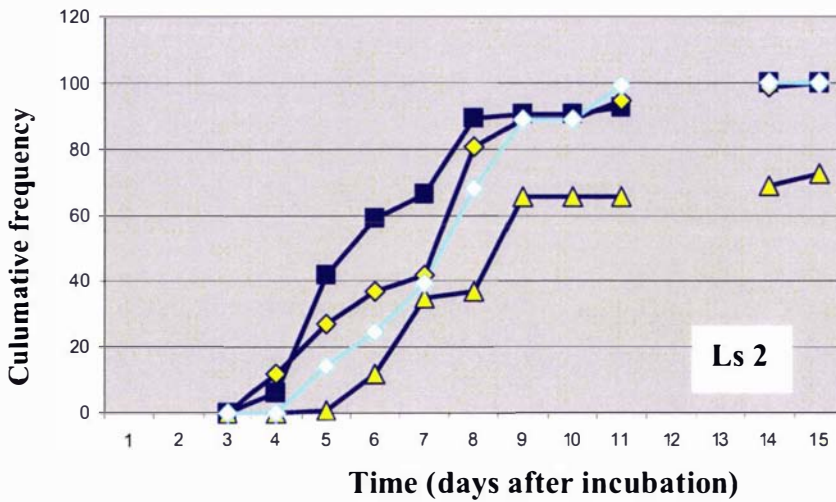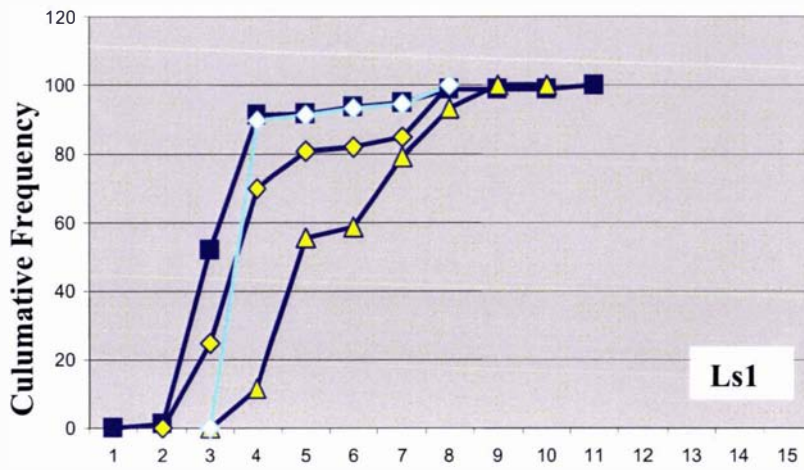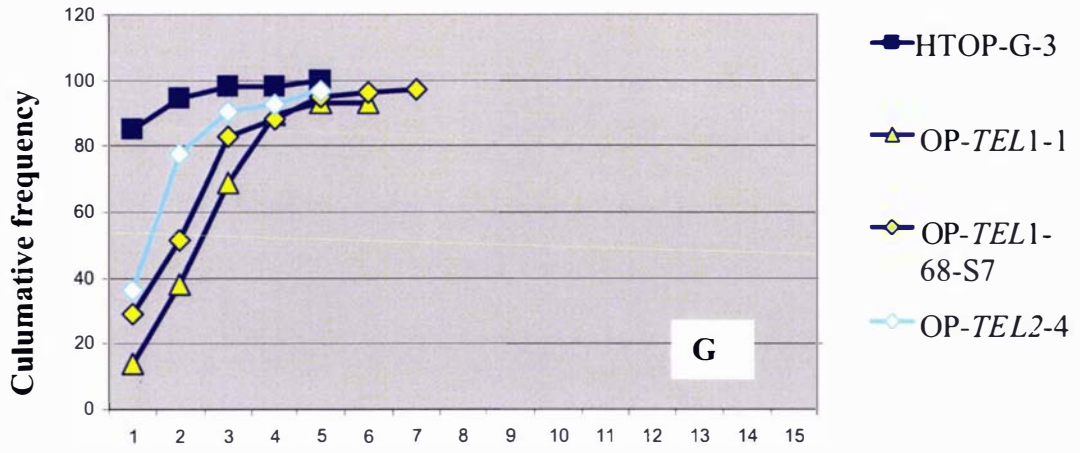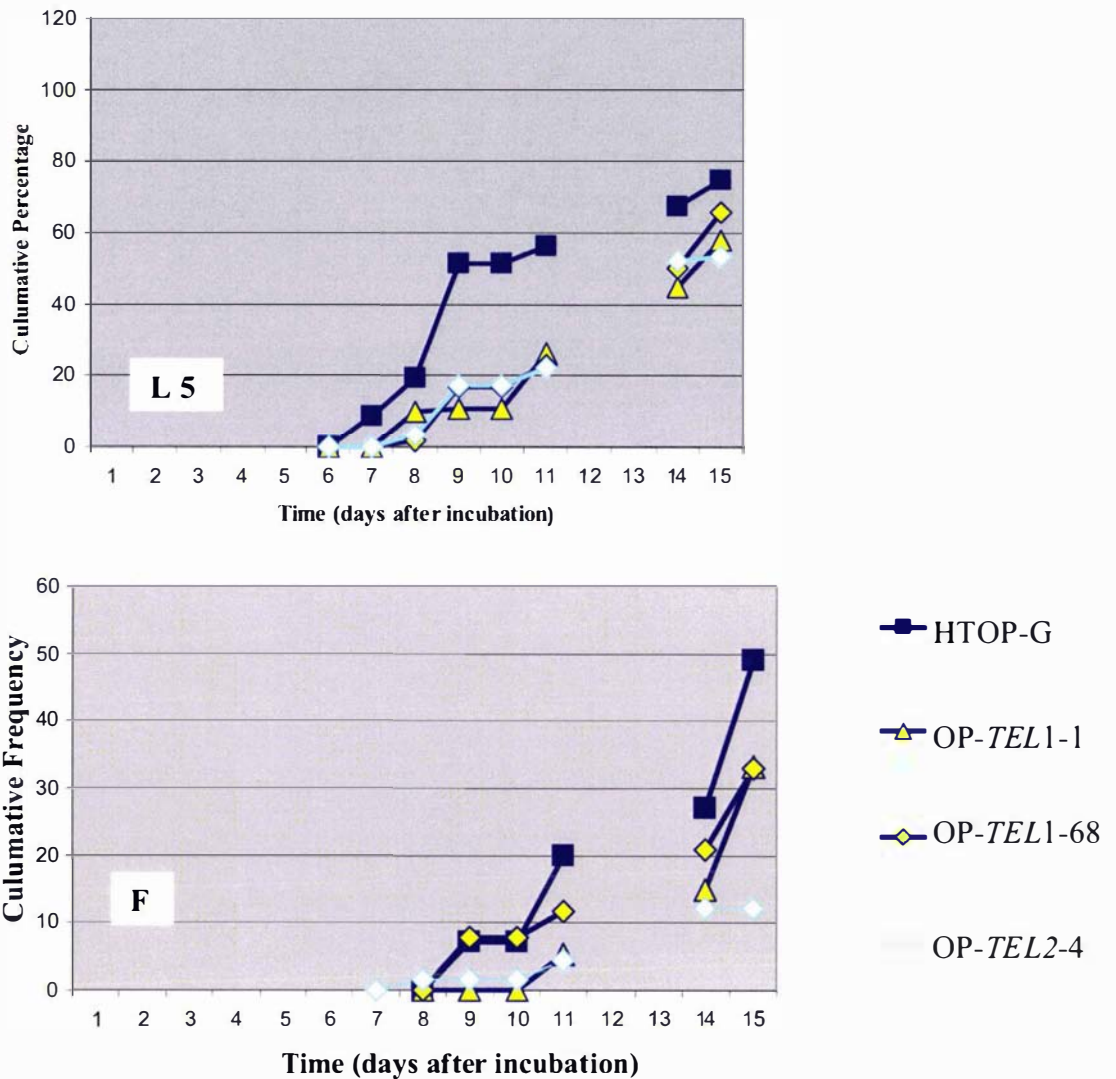**Figure 3.14 (and previous page): Developmental progression of
AP3>>OP-*TEL* crosses:** Five developmental landmarks were examined.
These were Germination (**G**), Leaf sets 1 and 2 (**Ls1- 2**, previous page), Leaf 5
(**L 5**) and Floral (**F**, this page). The graphs depict the frequency of each
developmental landmark against Time (from the beginning of incubation) for a
population of GUS positive F2 seedlings of each cross. Population numbers for
each line: OP-GUS-3, n=72; OP-*TEL1*-1, n=56; OP-*TEL1*-68, n =62; OP-
TEL2-4, n=71.

leaves (Ls-1) by day 4. In comparison, 53 and 78% of the OP-*TEL1* crosses

(1, 1-68-S7) had germinated by Day 2. By day four the percentage was ~90-

95% and were not significantly different from the control lines. In addition, on

day 4, 70% of the OP-*TEL1*-68 and 15% of OP-*TEL1*-1 plants were found to

have their first leaf. In comparison, while the germination frequency of the

AP3>> OP*TEL2*-4 cross was somewhat slower compared to the control line,

by day 2 it was not significantly different. Similarly although Ls1 was not

observed in these seedlings until day 4, there was no significant differences compared to EV on this day, or subsequent days.

In comparison more pronounced differences in frequency were observed in the subsequent developmental landmarks. At day 6, 59% of the EV seedlings had produced their second leaf set, compared to 15, 23 and 39% of OP-*TEL1*-1, 1-68-S7 and 2-4 crosses respectively. By day 7 all three were at ~40% (Ls2) at which point the EV cross was at 68%. The following day, over 90% of AP3>>HTOP-G-3 seedlings had their second leaf set. At the same time point, (day 8) the AP3>> OP-*TEL* crosses were at 38, 68 and 80% (1, 2-4, 1-68-S7) respectively. Thereafter, only seedlings of the OP-*TEL1*-1 seedlings did not appear to have evident Ls2, the frequency only reaching 75% by day 15 when all other crosses had reached 100%.

Ls5 was evident in the control line cross on day 7 and by day 9, this had increased to >50% reaching just under 75% by the end of the experiment. In comparison Ls5 was evident in the OP-*TEL* crosses on day 8 and had only reached ~20% in all three crosses by day 11. However on day 14, this frequency was 55-65% in the OP-*TEL* lines, and thus not significantly different.

The AP3 crosses were the only lines were evaluated for the emergence of the floral buds. Floral buds were evident in the empty vector and OP-*TEL1*-68-S7 seedlings at day 8 whereas in the other crosses they were first noted on day 9 (1-68-S7) and 10 (1-1) respectively. By day 15 approximately 50% of the EV seedlings had produced a visible floral inflorescence. In comparison only 30% of the OP-*TEL1* lines and approximately 12% of the OP-*TEL2*-4 seedlings had evident floral buds. These differences in the frequency of floral seedlings were highly significant (P<0.001) on days' 14 and 15.

3.4.8 AG>>OP-*TEL*2-3

Only one cross was successfully made with the AGAMOUS promoter and this was with OP-*TEL2*-3. The domains of staining were examined in 'older' seedlings i.e. 14 DAG. Phenotypically, GUS positive plants appeared more compact in comparison to GUS negative Basta^R plants. In addition there appeared to be slight differences in the size and shape of mature siliques (i.e. the 10th silique from the tip of the primary bolt in this case) of 8 GUS positive
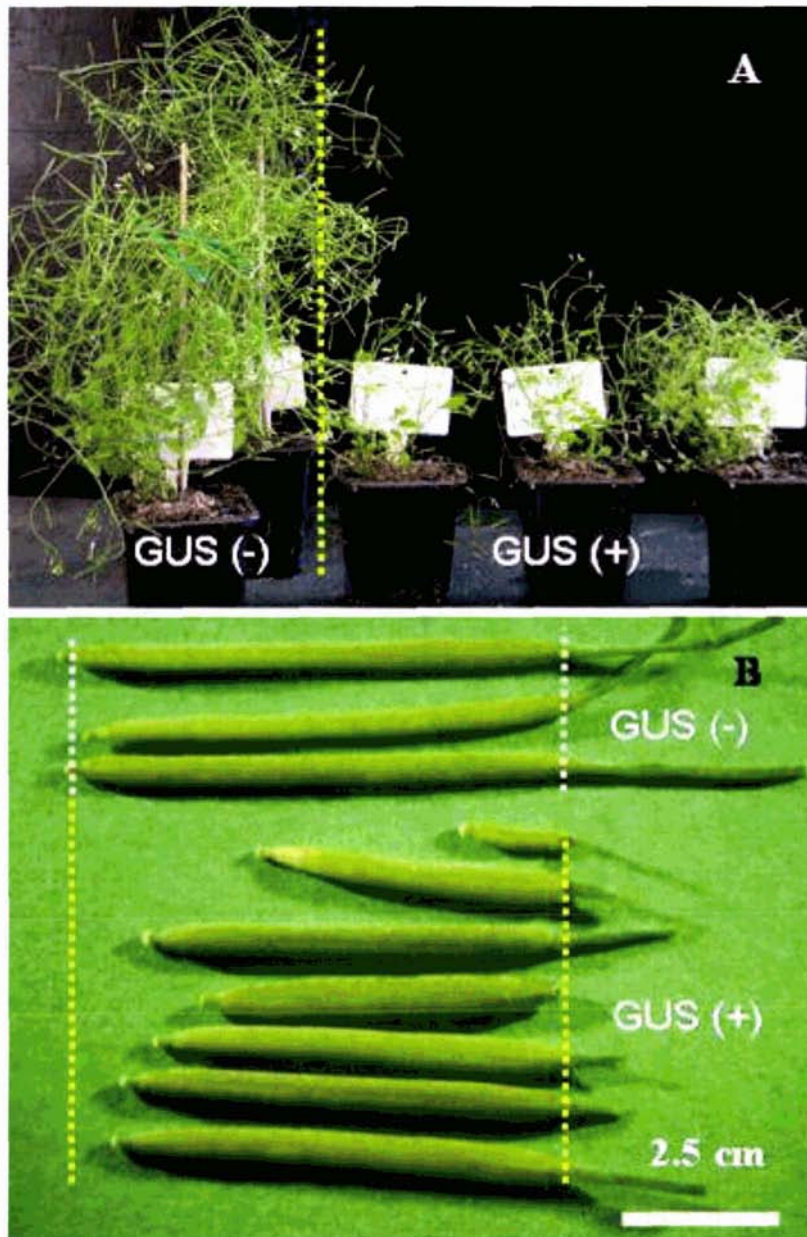
Figure 3.15: Morphology of F1 AG>>OP-*TEL2*-3 seedlings: (A) 26 day old
F1 seedlings of this cross are shown here alongside GUS minus Basta resistant
seedlings. **(B)** Mature siliques (i.e. all from position 10 from first flower of bolt) taken
from 26 day old F1 seedlings of this cross, both GUS negative and GUS positive.

seedlings of this cross, compared to GUS negative plants. In general the

siliques obtained from seedlings containing the OP-*TEL2* cross appeared

shorter and wider (Figure 3.15 above). Further investigation of these

observations was not undertaken.

3.4.9 ANT>>OP-*TEL*

(a) Phenotypic characterisation

Five OP-*TEL2* lines (2, 3, 4, 7, 2-57-S7) and a single OP-*TEL1* line, OP-*TEL1*-2, were successfully crossed with the ANT:LhG4 promoter line and characterised in the F2 generation.

Characterisation of the ANT>>OP-*TEL1*-1 cross was undertaken only in the F0 generation. Sixteen Basta resistant plants were generated from this cross and twelve stained positively for the activity of GUS. One of the 12 seedlings was found to have a comparatively abnormal morphology (see

Figure 3.16: Phenotypes observed in ANT>>OP-*TEL1*-2 seedlings. (A) Seedling at 28 DAG. Dotted line indicates probable region of hypocotyl, recognisable cotyledons and root are not obvious (bar is 3mm). **(B)** Seedling at 52 days old. It now has a number of axillary shoots (white arrows) arising in the vicinity of the putative apex. **(C)** At 72 days the seedling has a bolt with a single open flower and one floral bud (bar is 1cm). **(D)** Cauline leaf of an otherwise normal ANT>>*TEL1*-2 F1 seedling. The leaf is narrow, and has a tendril like extension from the underside of its distal tip.

Figure 3.16A-C pg 129). This 'seedling' had a short hypocotyl, did not produce cotyledons or a histologically normal root. The apical-basal polarity of the seedling could only be confirmed after the appearance of a shoot at five weeks in the vicinity of the apex (white arrows in Fig 3.16B). The leaves of the largest shoot were normal in appearance but much smaller than normal. Ten days later the plant produced a short bolt comprising two flowers, the terminal one of which eventually produced a short silique. In four of the remaining 11 sibling plants with a more normal appearance, a secondary observation was tendril-like extensions were observed in some cauline leaves (as shown in Fig 3.16 D). Apart from these observations no further investigations were undertaken.

The remainder of this section deals with the characterisation of F1 and F2 seedlings of the ANT>>OP-*TEL2* crosses. Two of the F0 seedlings from the ANT>>OP-*TEL2*-4 cross had strikingly irregular cotyledons and some of the leaves had a glossy 'wet' appearance. An example is shown in Fig 3.17A (pg 131). Scanning electron micrographs of the surface (Fig 3.17 B-E) revealed discreet sectors within the petiole, and the leaves, of a distinct appearance compared to other epidermal cells. Cells within these sectors were small, irregular, and globular in appearance. It was also noted that the size of these sectors was highly variable between leaves within a seedling. For example, in the seedling shown in Fig 3.17A, one of the leaves possessed discrete sectors while another smaller leaf (3.17D) has irregular cells over the entire adaxial surface. Because these globular cells had a striking resemblance to callus tissue in terms of their disorganised arrangement and lack of recognisable histological identity, this phenotype was dubbed Callus-Like Tissue, or CLT.

In the F2 generation of the ANT>>OP-*TEL2* crosses, three general categories of morphological defect were identified. Of these CLT was the least frequently observed, being found in only three of the crosses. The most frequently observed phenotype was associated with variability in the morphology of cotyledons (Abnormal cotyledons) and to a lesser extent the earliest leaves. In four of the five OP-*TEL2* crosses examined, abnormalities in the cotyledons were the most commonly observed phenotypic category (i.e. AbCot, Table 3.5 pg 119). These abnormalities ranged in severity from
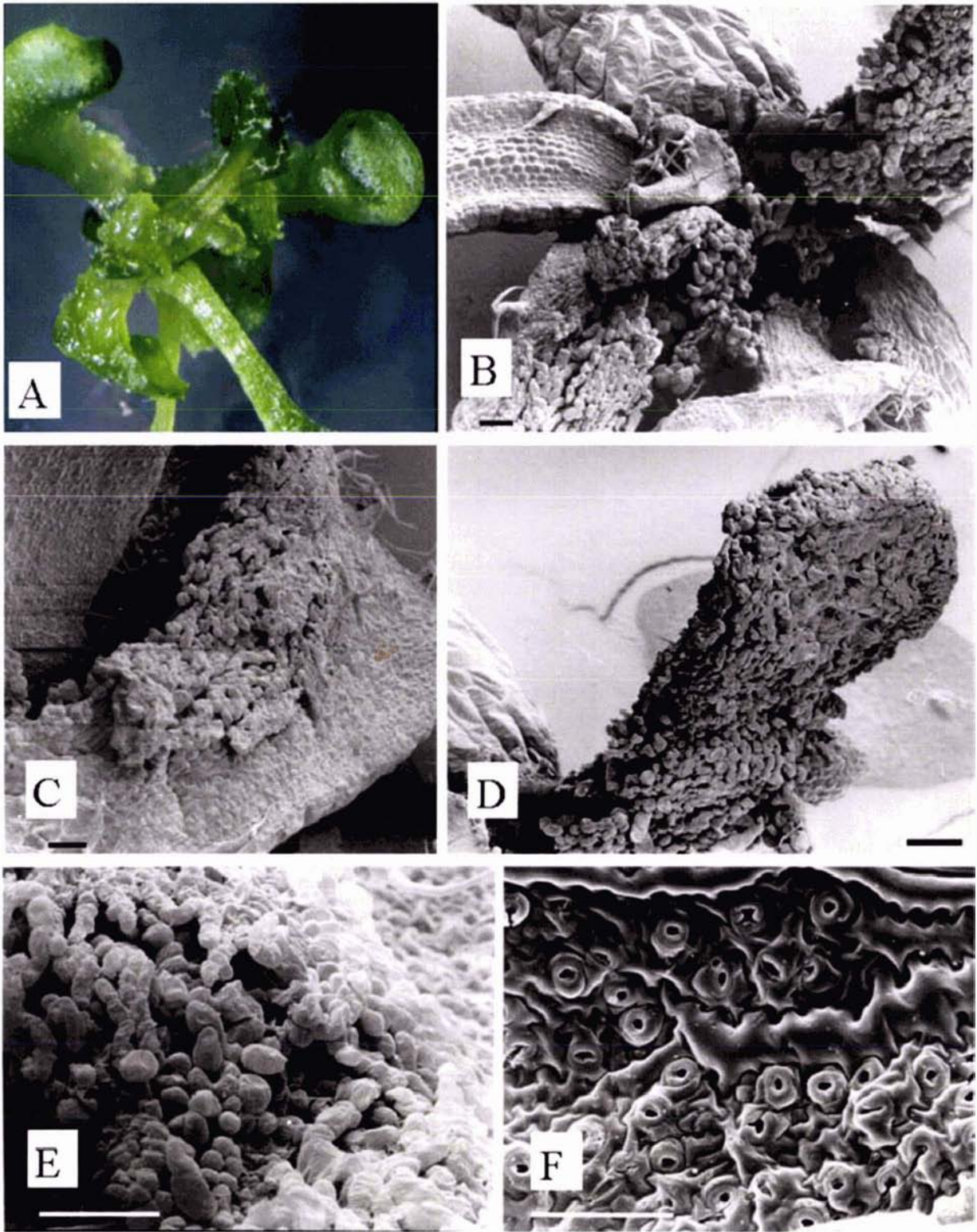
Figure 3.17: Callus-like tissue in an F1 ANT>>*TEL2*-3 seedling: Micrographs of cellular morphology of an F1 seedling from the cross ANT>>OP-*TEL2*-3. **(A)** The living plant prior to processing. **(B)**View from above looking down on the disorganised shoot apical region. **(C)** Globular tissue forming a domain within **(C)** or covering a whole leaf, **(D)** with irregular cells lacking normal epidermal histology. **(E)** A closer view of some domain of irregular cells (from C) shows that they resemble callus like tissue.**(F)** Comparatively more normal epidermal cells from the same leaf but displaying an overabundance of stomata (scale bar in all pictures = 200μm).
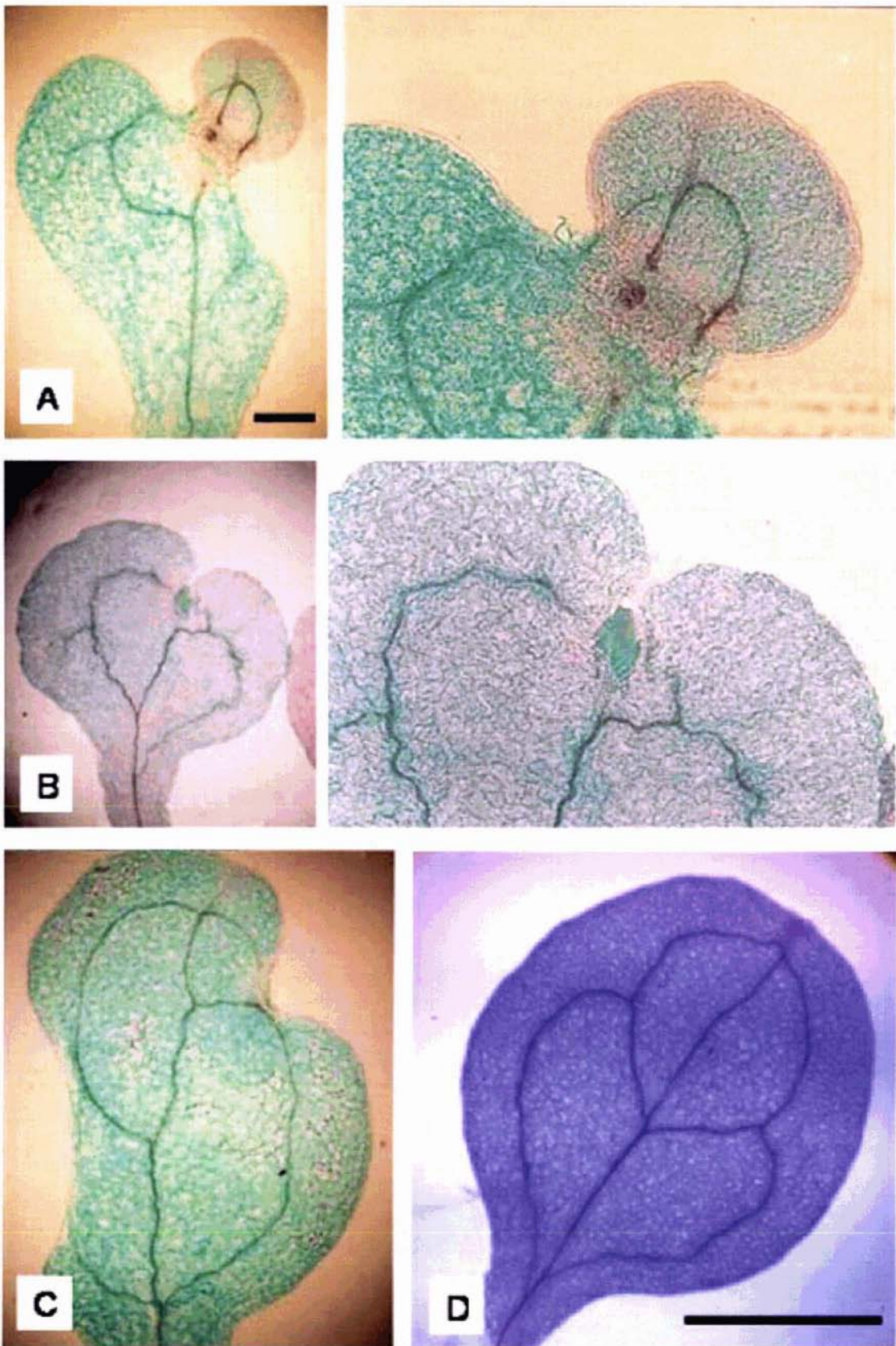
Figure 3.18: Irregular morphology of ANT>>OP-*TEL2* cotyledons: (A) Leaflet like structure emerging to one side of the distal tip of the cotyledon (scale bar is 0.3 mm). **(B)** Cotyledon with dense 'beak' of cells at the distal tip (scale same as A). **(C)** Cotyledon with reduced laminal expansion in two locations. **(D)** ANT>>HTOP-G cotyledon showing a more regular shape and vasculature characteristic of empty vector lines.
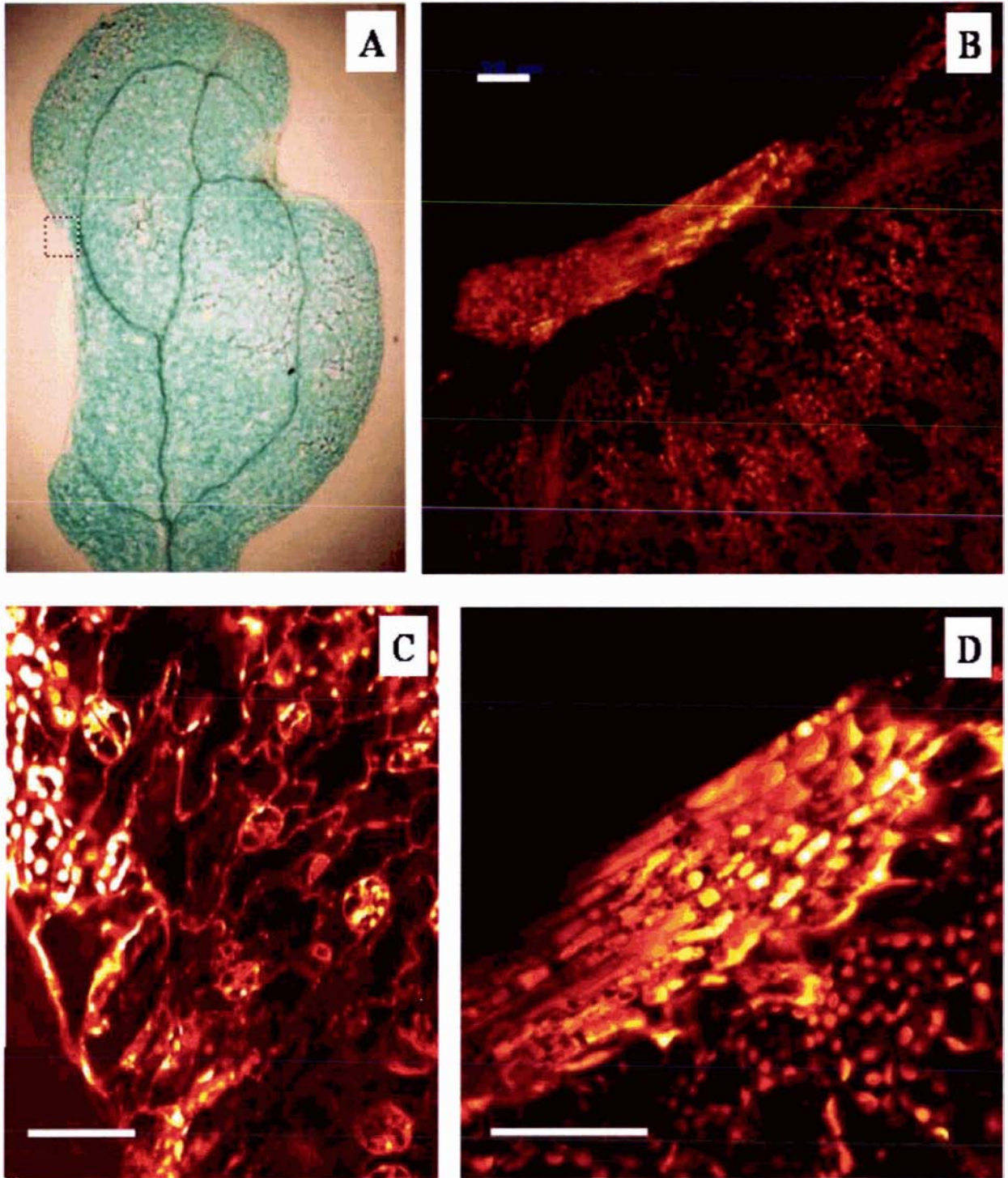
Figure 3.19: Morphology of ANT>>OP-*TEL2* cotyledon epidermal "sectors": (A) Gross morphology of a ANT>>OP-*TEL2*-3 cotyledon showing abnormal overall shape (cf. with Fig. 3.17 overleaf). Box inset indicates position of image in (B). In **(B)** a domain of highly fluorescent cells is obvious at the edge of the cotyledon, surrounded by normal tissue beyond. **(C)** Closer view of relatively irregular epidermal cells at the boundary. **(D)** Section through cells at the edge domain. These consist of small irregular cells lack typical differentiation of the epidermis or inner tissues. All scale bars represent 50μm. Images obtained using Scanning Confocal Laser microscopy and fast-green staining.

irregularities in shape (i.e. Figure 3.18, pg 132) to the cotyledon being largely
vestigial or absent (i.e. see Fig 3.22 (pg 137) & 3.23 (pg 138)).

The specific defect/s observed in the abnormal cotyledons of these F2
seedlings varied considerably. In general however, the cotyledons were
asymmetrical, smaller, and possessed irregular vasculature. Moreover this was
associated with distinct sectors being present near or at the tip of the
cotyledon. These sectors were either beak-like protrusions, or recessed sections
(c.f. B and C in Fig 3.18 pg 132) and their position was highly variable. They
could be to one side of the distal tip of one cotyledon (Fig 3.20, pg 135),
recessed domains on either side of the distal tip, (Fig 3.19, pg 133) or a
combination of both (Fig 3.21, pg 136). Another alternative to the beak like
domains, which was occasionally observed, was an emergent leaflet structure
with independent vasculature was observed at the putative site of the beak (as
shown in Fig 3.18A pg 132).

Examination of the beak-like or recessed sectors using epifluorescence
microscopy and CLS Microscopy, indicated that they were composed of small
cells distinct from nearby epidermal cells by their irregular shape and smaller
size. The lack of histological differentiation was also noted which was similar
to that observed in SEM of the callus like tissue previously described. Figure
3.20 and 3.21 show examples of CLSM images of beak like sectors. Fig 3.20
shows two optical sections of an emergent beak like structure. In these the
beak appears to be composed of small oblate cells. In Fig 3.21, the beak like
structure is also shown to be composed of small irregular cells that lack
histological identity to the surrounding tissue.

Finally in Fig 3.19C (pg 133) is shown an example of the recessed edge
domains. From these images it is apparent that the region is composed of
narrow, elongated cells, at the edges of the cotyledon. As observed for the
beak structures, these cells are distinct in appearance from the surrounding
epidermal cells and appear not to have undergone the same histodifferentiation
evident in nearby cells.

In the examples provided so far the cotyledons although abnormal
were recognizable. In more extreme cases, this was not the case. Vestigial
cotyledons arose as beak or tooth-like structures in place of one or both

132
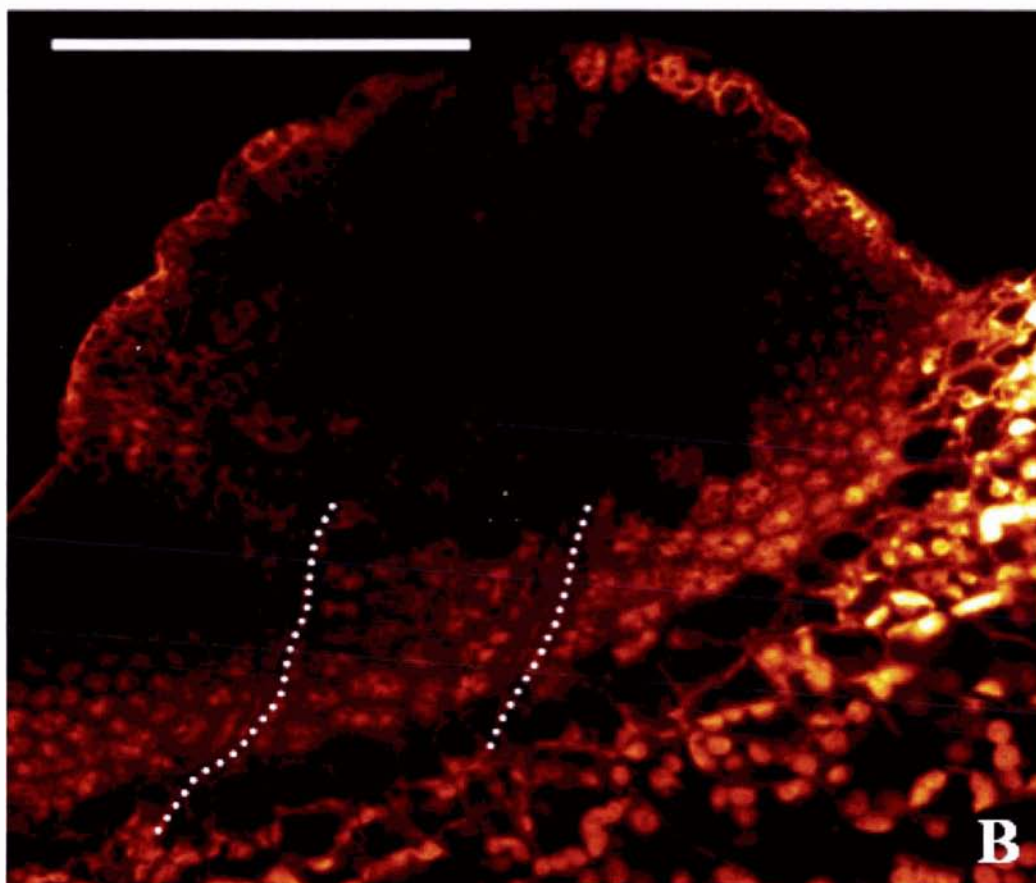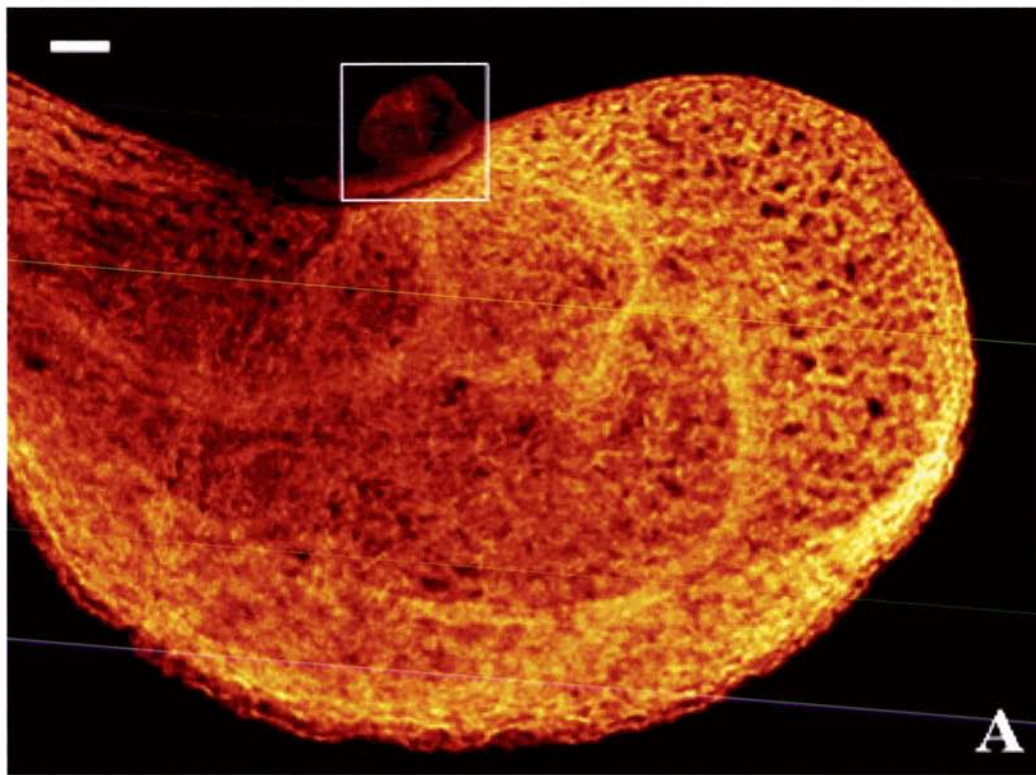
Figure 3.20 Morphology of ANT>>OP-*TEL2* cotyledon with distal "beak":
Cotyledon from an F2 seedling of the ANT>>OP-*TEL2*-2 cross with 'beak'. **(A)**
Image depicts a beak like structure located to one side of the distal tip of this
cotyledon. **(B)** Optical section of A at a depth of 5µm within the beak structure;
shows the small irregular dense cells of this structure and at the border. Dotted lines
indicate location of vasculature entering the structure from the cotyledon.
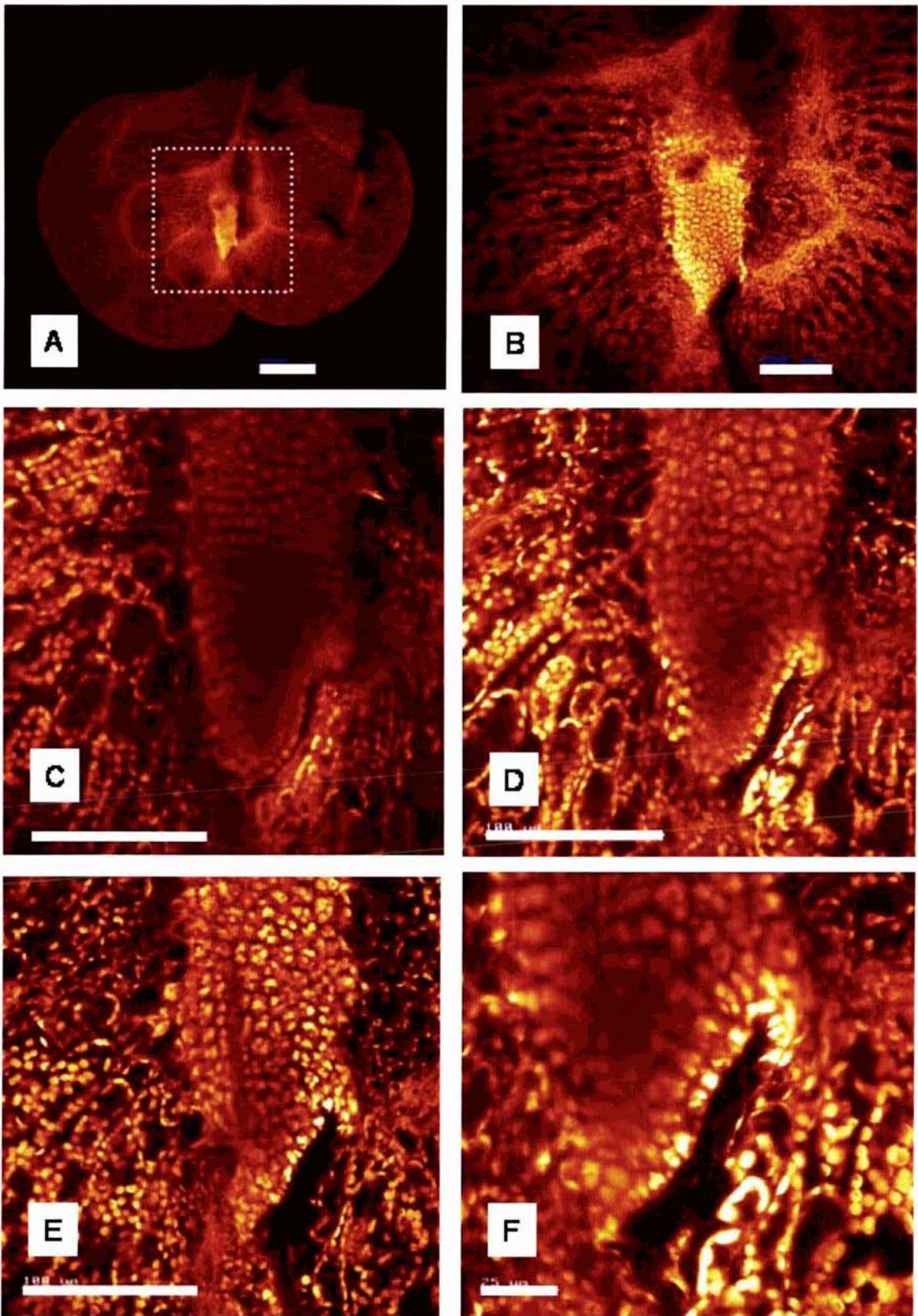
Figure 3.21 Morphology of ANT>>OP-*TEL2* cotyledon with recessed "beak":(A) One cotyledon of an F2 ANT>> OP-*TEL* 2-4 seedling showing a similar morphology to that shown in Fig. 3.16 b (pg 120): **(B)** Higher magnification view of inset in **A**, shows the small irregular dense cells in this beak. **(C-E)** Consecutive 10 μm optical sections starting at a depth of 20μm, from top (C) to bottom (E). In (E) the vasculature is apparent within the densely packed cells. **(F)** Closer view of 'beak' structure (in D) showing a regular layered arrangement of small cells.
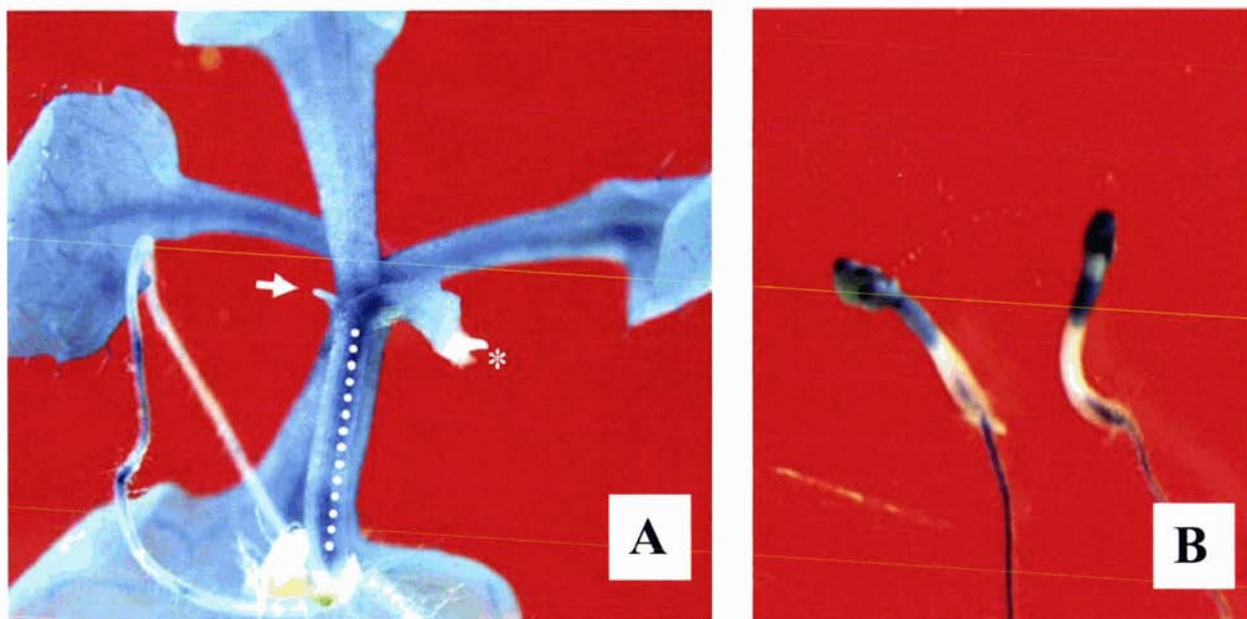
Figure 3.22: The vestigial cotyledons of ANT>>OP-*TEL2* seedlings (A) Callus cotyledon and beak cotyledon of a ANT>>OP-*TEL2*-4 16 day old seedling. Arrow indicates beak structure in the position of the cotyledon, asterisk indicates vestigial cotyledon along with callus like tissue at tip. **(B)** ANT>>OP-*TEL2*-2 seedlings at 10 DAG whose cotyledons have failed to expand. Note vivid GUS expression at tips of cotyledons and roots which is largely absent from the hypocotyl.

cotyledons. Examples of the second phenotype, the vestigial cotyledon phenotypes are presented in Fig 3.22-3.23 (pg 137 & 138). Fig. 3.22A shows a representative example of a 16 day old OP-*TEL2*-3 seedling with a characteristically vestigial beak-like cotyledon (white arrow) on one side, and a malformed cotyledon with callus like tissue at its tip (white asterisk), on the other.

The scanning electron micrographs in Figure 3.23, (pg 138) shows views of some examples of vestigial cotyledons and associated callus-like tissue. In Fig 3.23C, the top picture shows the apical region of a seedling. The foremost cotyledon of this seedling has formed as a beak-like structure, and the rear cotyledon is thickened and composed of large oblate cells lacking the normal cellular morphology of the epidermal cells. A leaf emerging from a shoot to one side of the presumptive apex, (white box) shows callus-like cells 'emerging' from its otherwise normal abaxial surface. In Fig 3.23B a seedling is shown with cotyledons where the laminal surface is absent, replaced by callus-like tissue along the edge and tips. A closer view of these shows that they are large oblate cells of varying sizes similar to those observed in the F0 seedling described earlier.

135
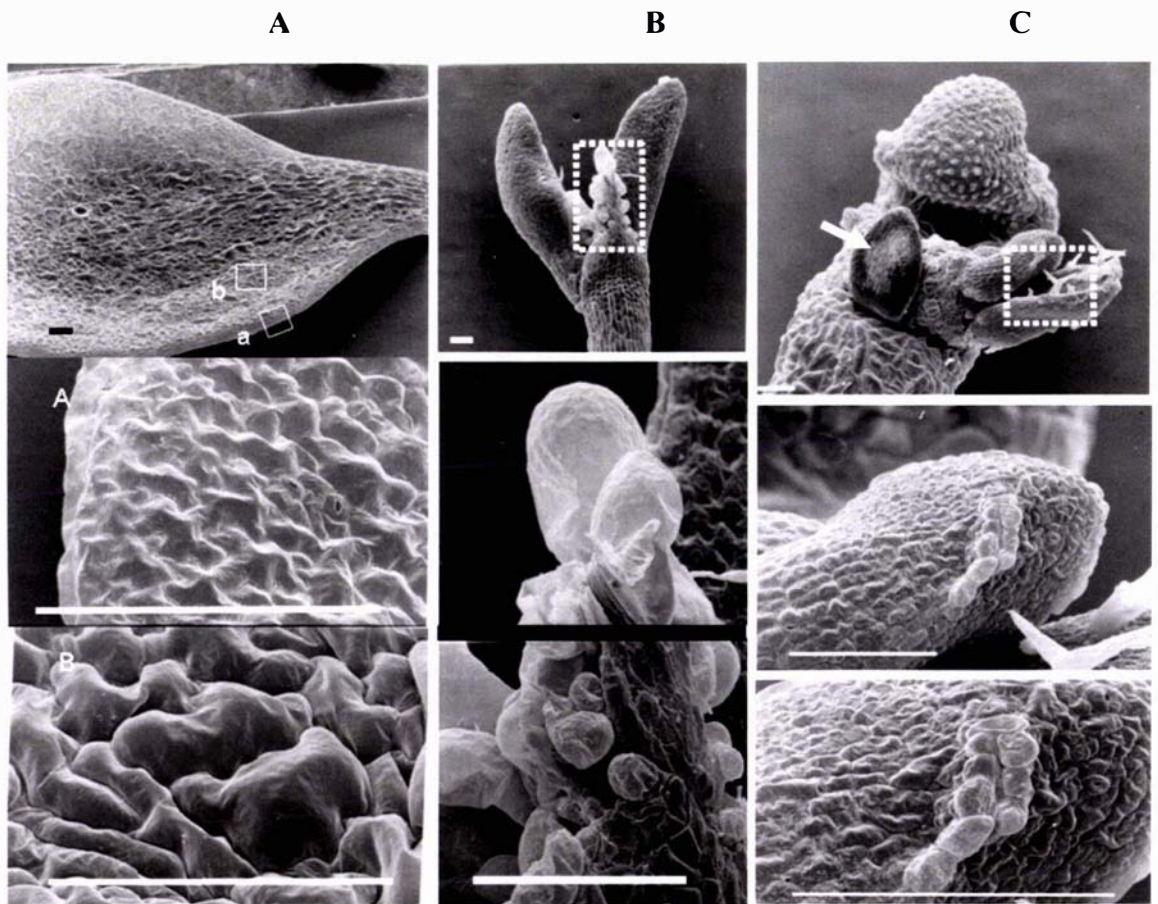
Figure 3.23: Cellular morphology of callus like tissue in cotyledons of ANT>> OP-seedlings: (A): Far view of ANT>>HTOP-G cotyledon (Top), close view of epidermal cells at edge (middle) and (bottom) towards middle, showing characteristic 'pavement' cell morphology. **(B)** Cotyledons of an ANT>> OP-*TEL2* seedling (Top) is composed of irregular globular cells. Inset is shown enlarged below. **(C)** Top: Second ANT>>OP-*TEL2* seedling displaying cotyledons which are small and callus-like in appearance. Arrow indicates beak-like cotyledon in foreground. Inset indicates leaves arising to one side of the putative apex and shown enlarged in middle and lower frames. (Lower) Callus-like cells are apparent on the abaxial surface of one leaflet. (scale bars are 100µm).

Therefore a key characteristic of the AbCot phenotypic category was its variability of extent and affect. The common theme was a reduction in the histodifferentiation of part or the whole of one or both cotyledons.

The third phenotypic category identified was termed the Extremely Delayed seedlings (Table 3.5, Figure 3.24, pg 139). The EDS were observed in four of the five ANT>>OP-*TEL2* crosses, and also the ANT>>OP-*TEL1*-2 cross as previously described. Seedlings of this type were initially identified on the basis of a characteristically prolonged delay before visible shoot formation, up to 50 days after germination. These seedlings were also noted to have comparatively normal root systems (see Fig 3.24A). Cotyledons and leaves were vestigial, or absent, and associated with varying degree of callus-like tissue in the vicinity of the shoot apex. Examples
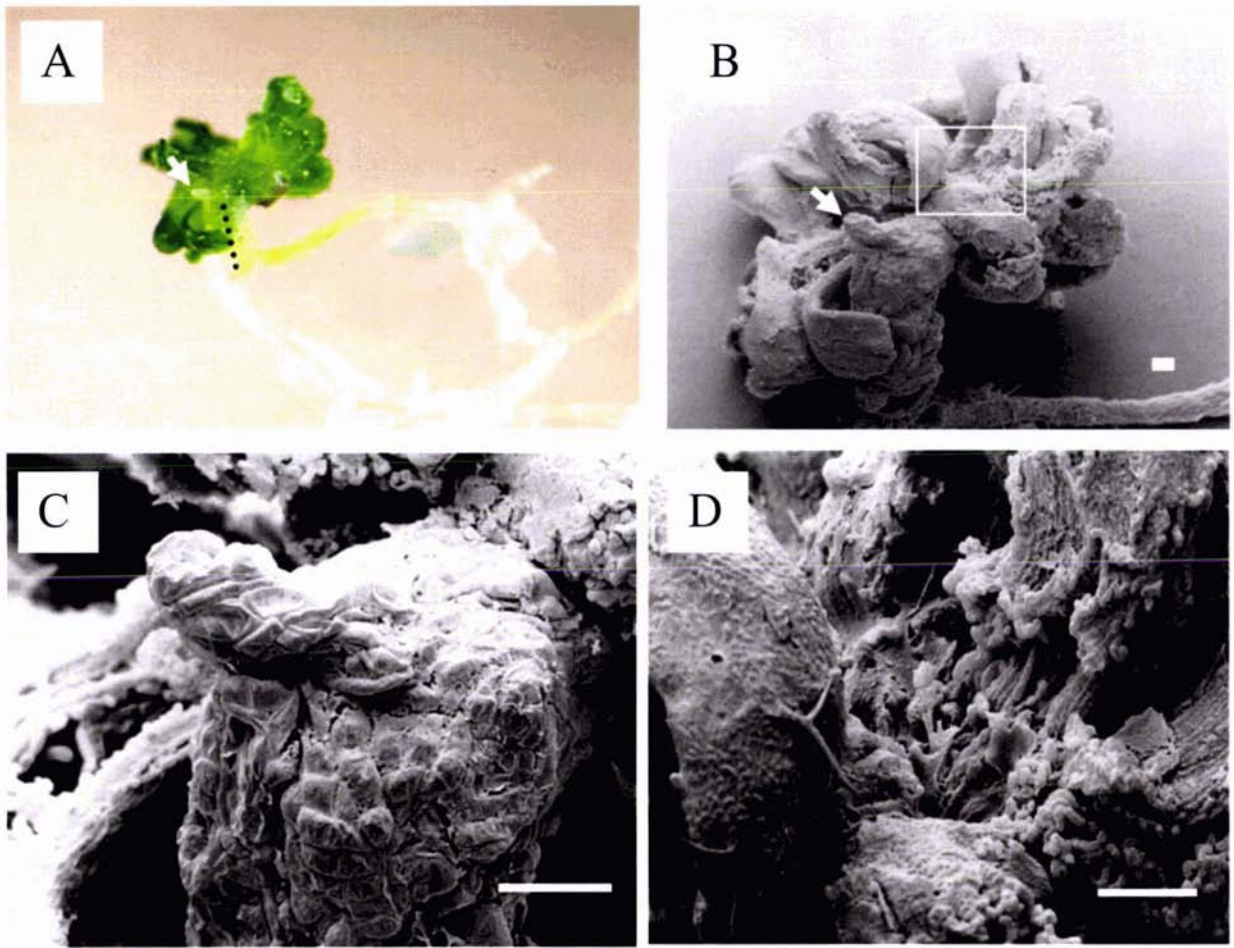
Figure 3.24: Irregular morphology of ANT>>*TEL2* Extremely Delayed Seedlings:
Clockwise from top (A): white arrow indicates presumptive position of cotyledon. Black
dotted line indicates hypocotyl. (B, C) The presumed cotyledon is a nub of rectangular cells.
(D) The box in B is shown in D emphasises the disorganised nature of the cells (bar =100μm).

showing the range of morphology in F2 seedlings of this category are shown in

Fig 3.24 & 25 (pg 139 & 140). Figure 3.24 above shows an F2 ANT>>OP-

TEL2-3 seedling at 55 DAG. In 3.24A the black dotted line indicates the

position of the hypocotyl and the roots below. The white arrow in A indicates

the presumed position of a beak-like cotyledon. In the SEM micrographs (B-

D) this beak appears to a nub of cells which is not distinct from those cells

surrounding it. To one side of the presumed 'apex', an axillary shoot appears

to have arisen. The leaves of this shoot are lumpy and have callus-like tissue

evident on their adaxial epidermal surface (Fig 3.24 D). Figure 3.25 shows

another example of an F2 ANT>>OP-*TEL2*-4 seedlings of this category. In

this figure the seedling is shown at 38 DAG. Recognisable cotyledons are not

evident, and prolific callus-like tissue is present in the vicinity of the apex. The
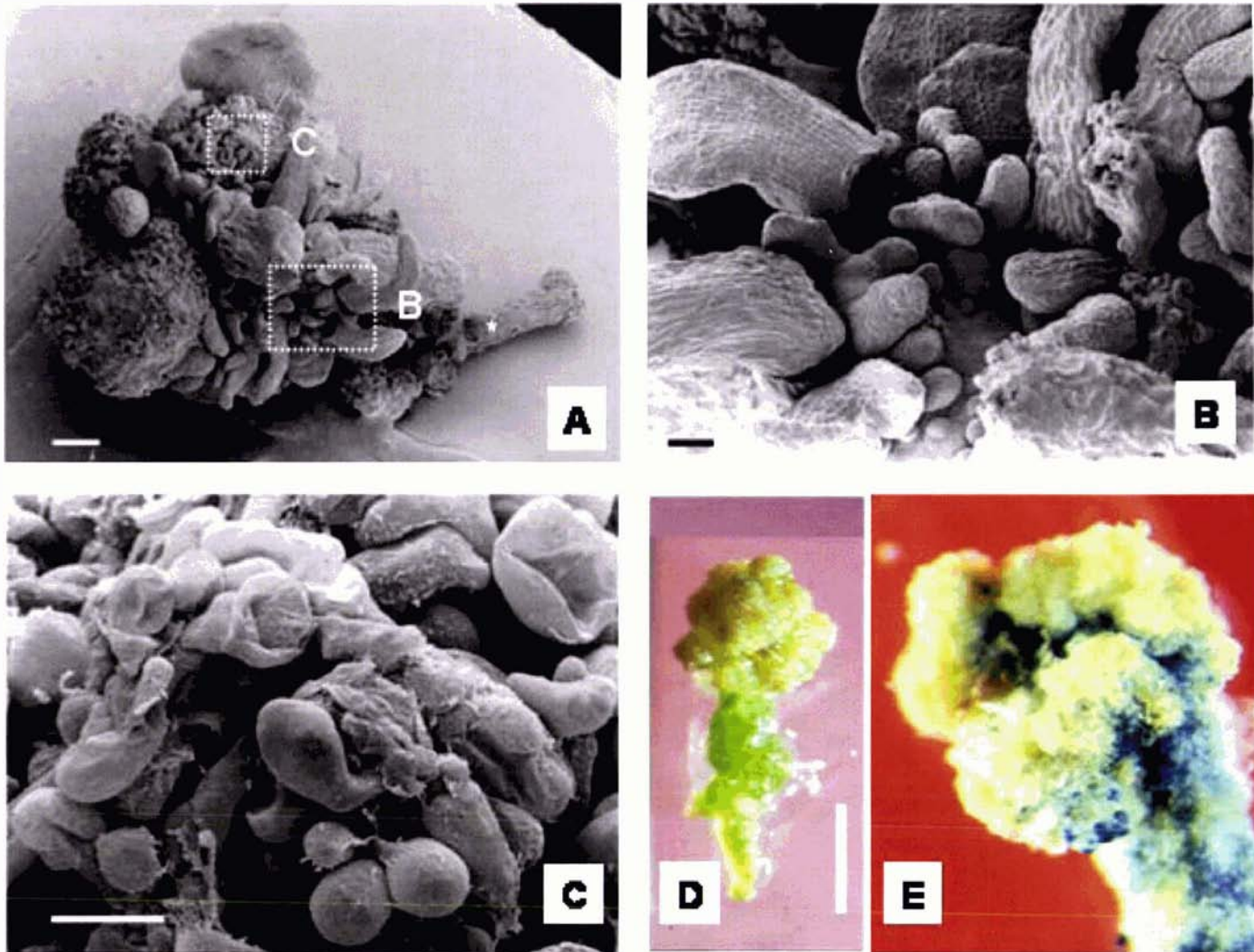
Figure 3.25: Further examples of extremely delayed seedlings: Two examples of the extremely delayed seedling phenotype seen in ANT>>OP-*TEL2* lines. (A) SEM micrograph of a 43-day-old OP-*TEL2*-3 seedling. Shoot architecture is evident but disorganised (Inset B). Asterisk indicates position of root (tip was broken at transfer). Bar = 400µm. (B) Magnification of inset B. Leaf like structures with some callus like tissue in a shoot-like formation near apex. (C) Magnification of inset C (bar =100µm). Cells lack normal epidermal identity. (D) Living example (at 28 days) showing callus like tissue over entire apex of seedling (bar = 3mm). (E) Typical domains of GUS staining in representative of this phenotype.

seedling has callus-like tissue proliferating across the apical region amongst which a number of axillary shoots are evident. In 3.25 D a third example is shown at 25 days post germination, with green callus-like tissue present at the apex of the seedling. The hypocotyl is thickened and the root very much shorter. Histochemical staining of this seedling is shown in E and is characteristic for this phenotypic category.

Figure 3.26 (pg 141) shows two optical sections from an extremely delayed ANT>>OP-*TEL2*-3 seedling harvested at 14 days after germination. The putative cotyledons or 'beaks' in this case are indicated by white asterisks and the apical meristem was presumed to be located between these two organs.
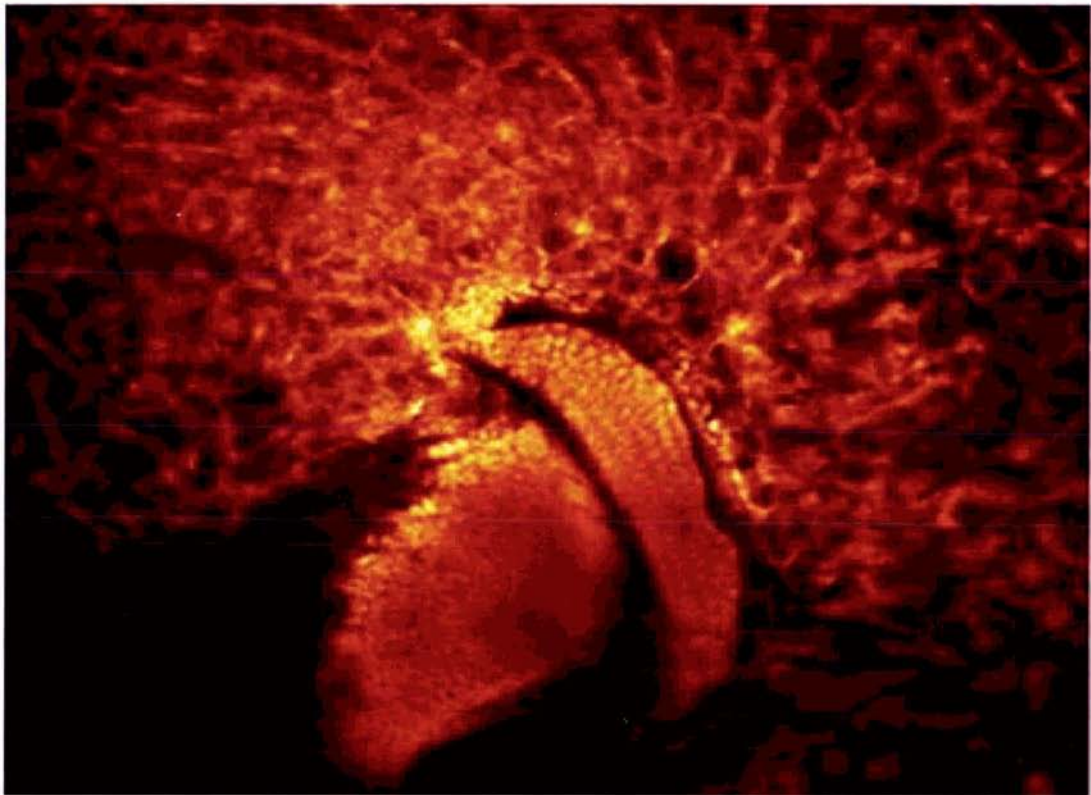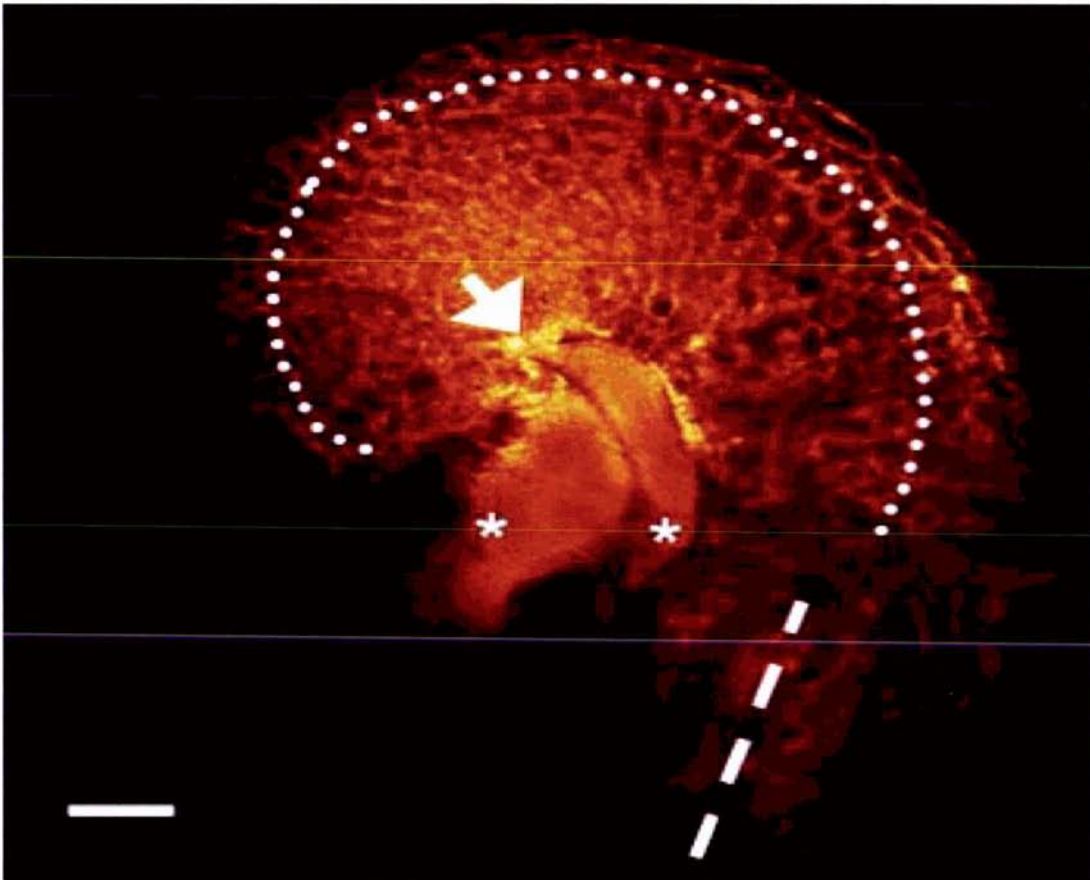
Figure 3.26: Cellular morphology of Extremely Delayed phenotype: Upper frame shows a transverse optical section of an irregular seedling at a depth of 30μm. Dashed-line indicates the region of the root, and dotted line indicates the hypocotyl. The arrow indicates the presumptive shoot apical meristem, and the asterisks, the beak-like cotyledons. The lower frame is a section taken at a depth of 40μm(scale bar is 100μm).

However it was not possible to identify either the SAM or the cell layers characteristic of a SAM. The cells of the 'hypocotyl' are large and irregular compared to normal cells at that location. The cells of the interior of the cotyledons show none of the tissue specific differentiation characteristic of cotyledons.

(b) Developmental progression

Assays of developmental progression were undertaken for four of the five ANT>>OP-*TEL2* crosses (2, 3, 4, 7, 2-57), and one ANT>>HTOP-G cross (3). Most of these crosses (OP-*TEL2*-2, 3, 7 and 57-S7) and the empty vector cross, were followed up to Leaf set 2. Seedlings of one cross, ANT>>OP-*TEL2*-4 , were assayed only up until the emergence of Leaf set 1. The results of this analysis are depicted in Figure 3.27 on the next page (pg. 143) the legend on page 144.

Germination took longer to occur in the ANT>>OP-*TEL2* lines compared to the empty vector. At 24 hours approximately 76% of the OP-HTOP-G-1 seedlings had germinated. Forty eight hours after transfer to growth conditions, ~97% of these seeds had germinated. In comparison, none of the seeds of the ANT>>OP-*TEL2* crosses had germinated at day 1. By day 2 two groups were evident by their markedly different rates of increase in germination. Group I crosses (OP-*TEL2*-2, -7 and 57 (S7)) had reached 61-85% germination by Day 2 (i.e. similar frequency to the empty vector on day 1). In contrast only 16% of the Group II crosses (OP-*TEL2*-3 and 4) had germinated by day 2. By day 4, both the empty vector and Group I crosses were > 90% germination. In turn, under a third of the seeds of the Group II crosses had germinated (OP-*TEL2*-3, 21.2%;OP-*TEL2*-4, 31.8%) by day 4, and did exceed 50% for the remainder of the experiment.

This distinction between Group 1, 2 and the empty vector was also evident in Leaf set 1. By day 4, ~96% of the EV cross had visible leaves (Ls 1) compared with ~46-64% of Group I, and 11.8-15 % of Group II crosses. The frequency of Ls 1 in all Group I crosses did not exceed 90% until day 6. In the group II crosses, this frequency was ~70% at this time. One plate of ANT>>OP-*TEL2*-4 seedlings was removed from the assay because of contamination which led to the decrease in frequency (Ls1) evident in Fig 3.26 from day 7 onwards. Subsequently the empty vector cross and all of the Group I crosses reached 100% on day 7 and days 8-9 respectively. In contrast the

140
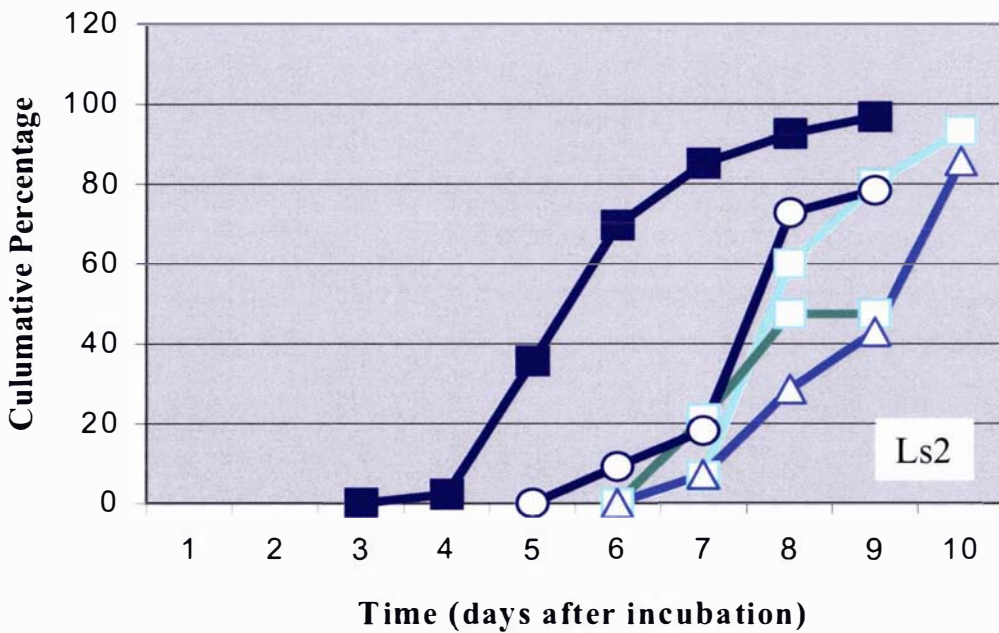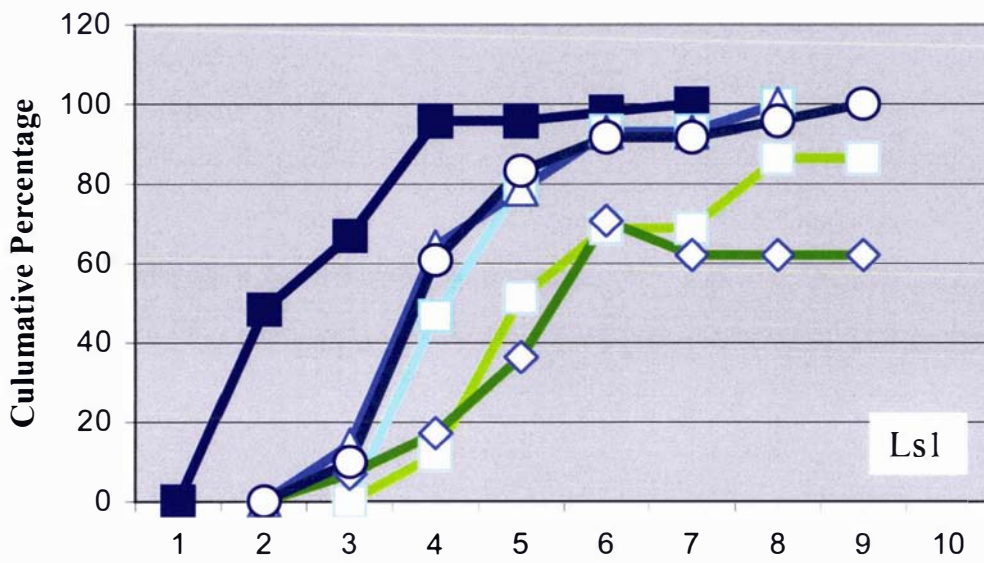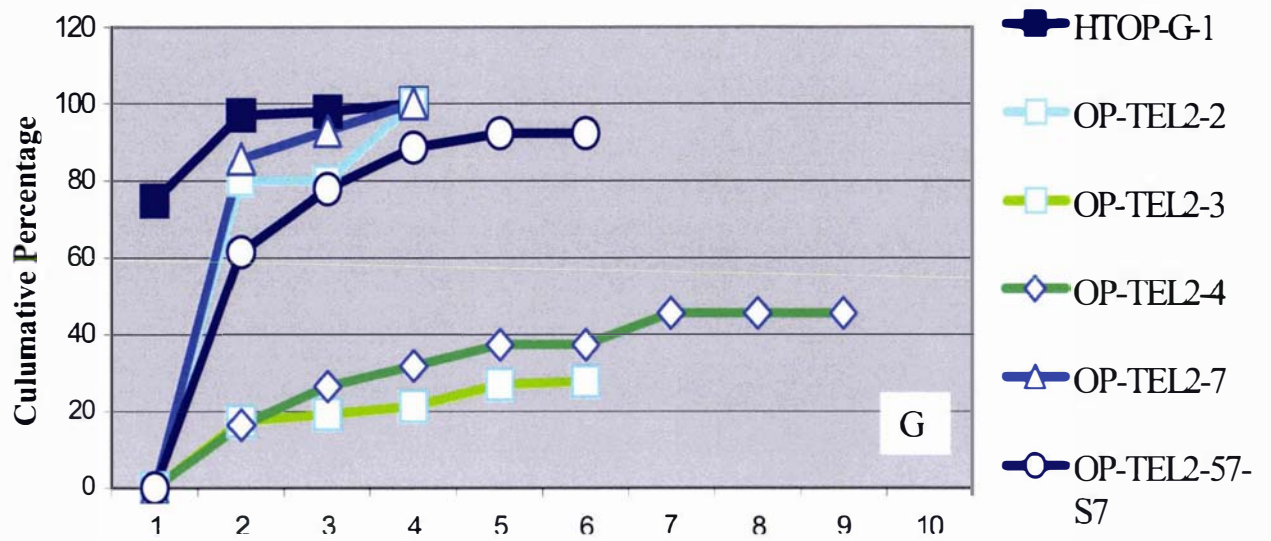
Figure 3.27 (previous page): Developmental progression of ANT>>OP-*TEL2* **crosses:** Three developmental "landmarks" were examined for five of six OP-*TEL2* lines, and one OP-GUS line. These were Germination (**G**), Leaf set 1 and 2 (**Ls 1**, and **Ls 2**). Only G and Ls 1 were examined for the sixth line, OP-*TEL2*-4. Numbers for each line: OP-GUS-1, n=86; OP-*TEL2*-2, n=96, OP-*TEL2*-3, n=72; OP-*TEL2*-4, n=62, OP-*TEL2*-7, n=86; OP-*TEL2*-57-S7, n=92.

frequency of Ls1 in the Group II crosses reached 62 (OP-*TEL2*-4) and 86.4 % (OP-*TEL2*-3) by day 9. Consequently there were Group II germinants which did not produce any leaves during the nine days of this experiment.

The second leaf set (Ls2) became evident in the empty vector F2 seedlings on day 4. By day 5, over a third of the EV seedlings (35.6%) had their second set of leaves. By day 9, this frequency had increased to 96.8%. In comparison the appearance of Ls2 was not noted in 3 of the 4 remaining OP-*TEL2* crosses until day 7. Only ANT>>OP-*TEL2*-57-S7 was found to have Ls2 on day 6 (9.1%). From day 8 onwards the increase in frequency of Ls2 was once more distinct between Group I and II crosses. The exception in the Group I crosses was ANT>>OP-*TEL2*-7 which was more similar to the Group I cross (ANT>>OP-*TEL2*-3) in days 7-9. By day 10 however the *TEL2*-7 cross was not significantly different from the empty vector or other members of Group I. In comparison the seedlings of the ANT>>OP-*TEL2*-3 (Group II) cross were stable at slightly under half of seedlings with their second leaf set (47.4 %) on days 8 and 9.

The differences in the developmental progression of the ANT>> OP-*TEL2* crosses compared to empty vector outlined in this section and Fig 3.26 (previous page) were analysed for statistical significance. In general the development progression described for these crosses were found to be highly significantly different ($P > 0.0005$, Friend's Exact Test) versus empty vector, at the beginning of each developmental landmark. The statistical significance of this difference decreased with time in the Group I, but not in the Group II crosses. For example the frequency of germination of Group I lines ceased to be statistically different from empty vector on day 4, but this was not the case in Group II. Similarly the frequency of Ls 1, and Ls 2, in the Group I lines were found to be not significantly different from EV by days 5, and 6 respectively. In comparison the frequency of these two developmental landmarks remained significantly different throughout the course.

3.4.10 Cotyledons have a reduced size but increased cell number at the distal tip

A common feature of the ANT>>OP-*TEL2* crosses were smaller cotyledons (example in top frame of Fig 3.28 next page). In order to investigate this observation counts of the epidermal cell number, and measurements of width were made across the mid point of the adaxial surface of cotyledons as described in §3.3.2a. The results of these measurements are summarised in Table 3.6. The mean width of OP-*TEL2* cotyledons was found to range from equivalent to approximately half (1 - 0.6 x) the mean width of cotyledons from the GUS negative and empty vector crosses. However the mean epidermal cell count / mm showed that all of the crosses had cotyledons with between 1.5 -2.2 times as many pavement cells compared to the controls.

This raised the question as to whether this is reflected in the histology of the cells of the adaxial surface. The CLSM images presented in Fig 3.28 A-D show examples of epidermal cells at the distal most tip and midpoint of an OP-*TEL2*-4 GUS-positive cotyledon with a control for verification.

ANT>>	HTOP-G		OP-*TEL2*			
	-1	-3	-2	-3	-4	-57
GUS negative seedlings Mean cell count +/- 1SD	29.4	28.2	26.2	25.4	26.8	25.5
	+/-1.1	+/- 0.8	+/-1.4	+/-2.1	+/-1.5	+/-1.9
Mean width of transect (mm)	2.7	2.85	3.1	3.4	2.8	2.7
Mean cells/mm	10.9	9.9	8.45	7.47	9.57	9.44
GUS positive seedlings: Mean cell count +/- 1 SD	28.6	27.4	42.5	64.3	62.8	48
	+/-1.0	+/-1.4	+/-2.9	+/- 7.1	+/-5.7	+/-5.2
Mean size of transect(mm)	2.6	2.8	2.6	1.6	1.8	2.3
Mean cells/mm	11	9.8	16.35	40.2	34.9	20.87

Table 3.6: Mean width and epidermal cell count data for the midpoint of cotyledons from ANT>>OP-*TEL2* crosses: means are derived from measurements of five cotyledons(n=5)+/-1 Standard Deviation.
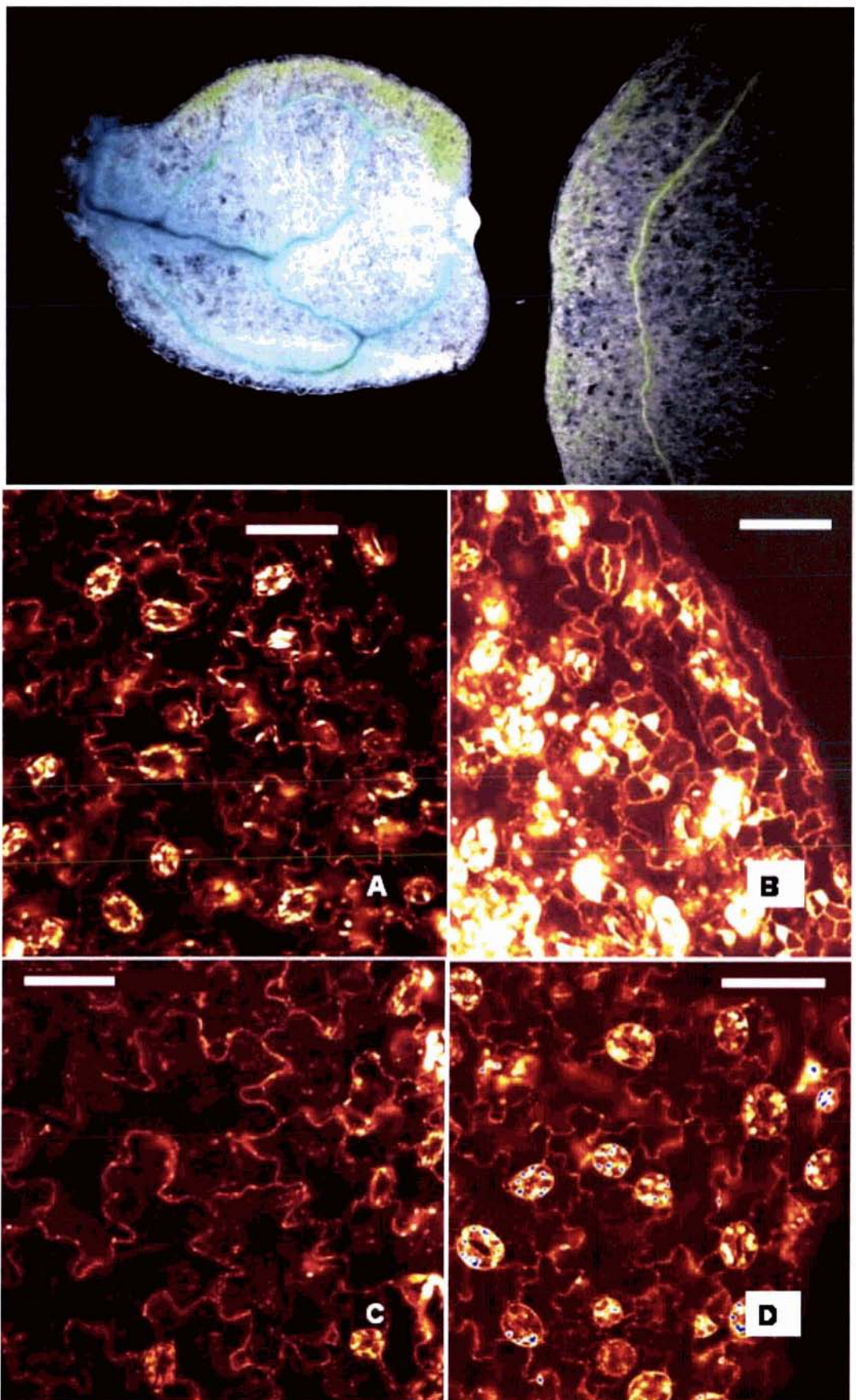
.**Figure 3.28 Epidermal morphology of ANT>>OP-*TEL2* seedlings.** Top frame shows a ANT>>OP-*TEL2-4* cotyledon (left) alongside an EV (right side). Below this are confocal optical sections of the abaxial surface of cotyledons of an ANT>>OP-*TEL2-4* F2 seedling (**A, B**) compared to empty vector(**C, D**). Pictures, were obtained from two locations, the midpoint of the cotyledon (A, C) and the distal edge of the cotyledon (B,D). Scale bars = 50 μm.

The primary observation was that the cells across the midpoint are smaller but not different in shape, whereas at the distal tip they have a comparatively more irregular shape at the cell tip (relative to the EV). No direct measurements of cell volume were made. Nevertheless these results confirm that there is a reduction in cell determinancy in these cotyledons at the site of prolonged ectopic *TEL2* expression, associated with increased cell numbers (nearer the midpoint), reduced cell size, and differentiation.

3.4.11 Characterisation of OP-*TEL(*S7) lines

(a) Levels of inducible expression

The OP-*TEL*1 & 2 and HTOP-G constructs were successfully transformed into individual seedlings of the S7 activator line. A minimum of 40 T1 lines was generated for each construct. Five lines were selected from each combination based on apparent levels of GUS expression under DEX induction. These lines underwent further characterisation in the T1 generation.

Initial experiments indicated that the timing of DEX application was important. If the inducing chemical was applied after emergence of the cotyledons, then no morphological effects on the architecture of the plant were observed. However if exposure to DEX occurred from (or prior to) germination, then phenotypes were observed with similarities to those observed in the ectopic misexpression crosses. Therefore germination on tissue culture media, with, and without DEX was used in preference to glasshouse spraying experiments. The concentration of DEX used in such experiments, was also determined empirically based on the consistency of GUS expression observed in plants after induction. Apparent suppression of GUS activity in sectors of leaves and roots, as well as variability of observed phenotypes, was observed at the highest DEX concentrations used (20-30μM). In turn, plants treated with DEX at concentrations <=5μM while staining evenly, had no apparent phenotype relative to EV. Intermediate concentrations between 10-15 μM were found to yield the most consistent results.

Once the optimal induction parameters had been determined the inducibility of the *TEL* transcript was determined for representative lines of OP-*TEL* -S7 by RT-PCR as described in §3.3.3b. OP-*TEL1-43-S7* and *2-21-S7* were selected as
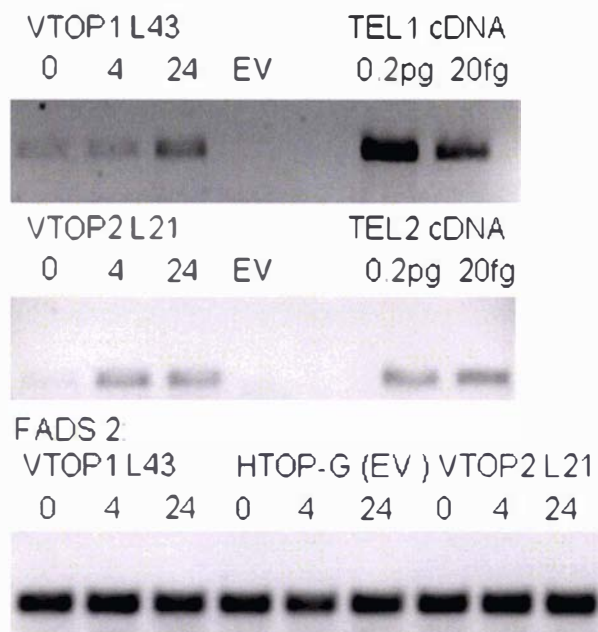
Figure 3.29: Inducibility of OP-*TEL*-S7 lines: Representative OP-*TEL* inducible lines 0, 4, and 24 hours after application of 10μM DEX compared to an induced EV line (24hr sample). RT-PCR amplification of a FADS2 product was used as an indicator of equivalence of RNA samples used.

representatives because of their vigorous GUS expression and consistent phenotypes under induction. The results are presented in Figure 3.29. OP-*TEL1–43-S7* was found to have faint background expression prior to DEX induction. Nevertheless expression does appear to increase at 4 and is clearly apparent at 24 hours post induction suggesting that this line is still inducible. Similarly in OP-*TEL2*-21-S7 the *TEL2* transcript is clearly present under induction and not in the empty vector line.

(b) Evaluation of OP-*TEL*(S7) lines in glasshouse spraying experiments

The effects of DEX induced *TEL* expression on plant development were examined under glasshouse conditions. Three OP-*TEL1*-S7 lines (24, 43 and 66) and three OP-*TEL2* lines (10, 21 and 32) were compared to three EV lines. No morphological differences were found which was characteristic of spray induction experiments with these lines. Consequently a quantitative approach was undertaken. Leaf counts and floral stage were determined over a 17 day period and these results are presented in Figure 3.30 (pg 149).
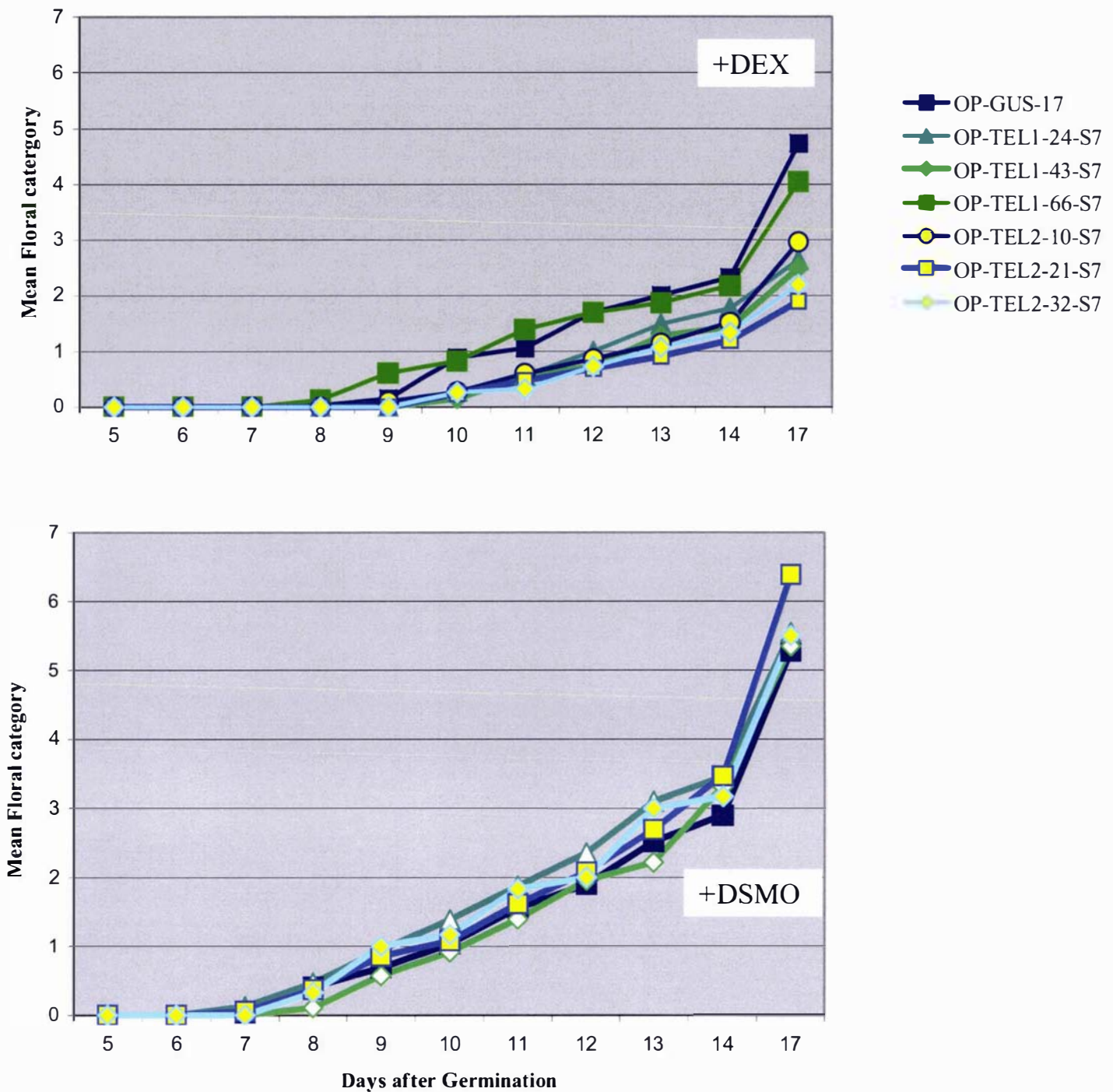
Figure 3.30: Delays in floral development in inducible *TEL* lines. Treatments: **(+DEX)** 30uM dexamethasone sprayed twice daily for 17 days;(+**DSMO**) 0.01% DMSO sprayed twice day for 17 days. Mean floral index derived from a minimum of 25 plants for each line.

In general the total leaf count of OP-*TEL*-S7 seedlings with or without DEX did not differ significantly from HTOP-G-S7 during the experiment (data not shown). However the frequency of seedlings with evident floral buds and their subsequent growth did appear to be delayed. The mean floral class for 5 of the 6 DEX treated OP-*TEL* lines, (versus the empty vector lines) was consistently lower than the two EV lines (Fig 3.30) from day 9 as indicated by the slow appearance of the floral buds within each group. The majority of

plants in the DEX induced OP-*TEL-S7* lines had evident floral buds (i.e. a mean index value of 1 means 100% of the plants were floral) by days 12 to 13. This compares with an index value of 1 by day 11 for the control line (and OP-*TEL1-66*).

After flowering, the index value corresponds to the approximate length of the bolt. The slow increase in the mean floral category of the OP-*TEL* lines corresponds to a relatively slow increase in bolt elongation versus the controls. In general OP-*TEL1*-66 was the one line which was not obviously different from the empty vector lines, with or without induction, at any stage during the experiment. In general the mean floral index of mock treated OP-*TEL* lines were found not to be different from each other or the EV control lines.
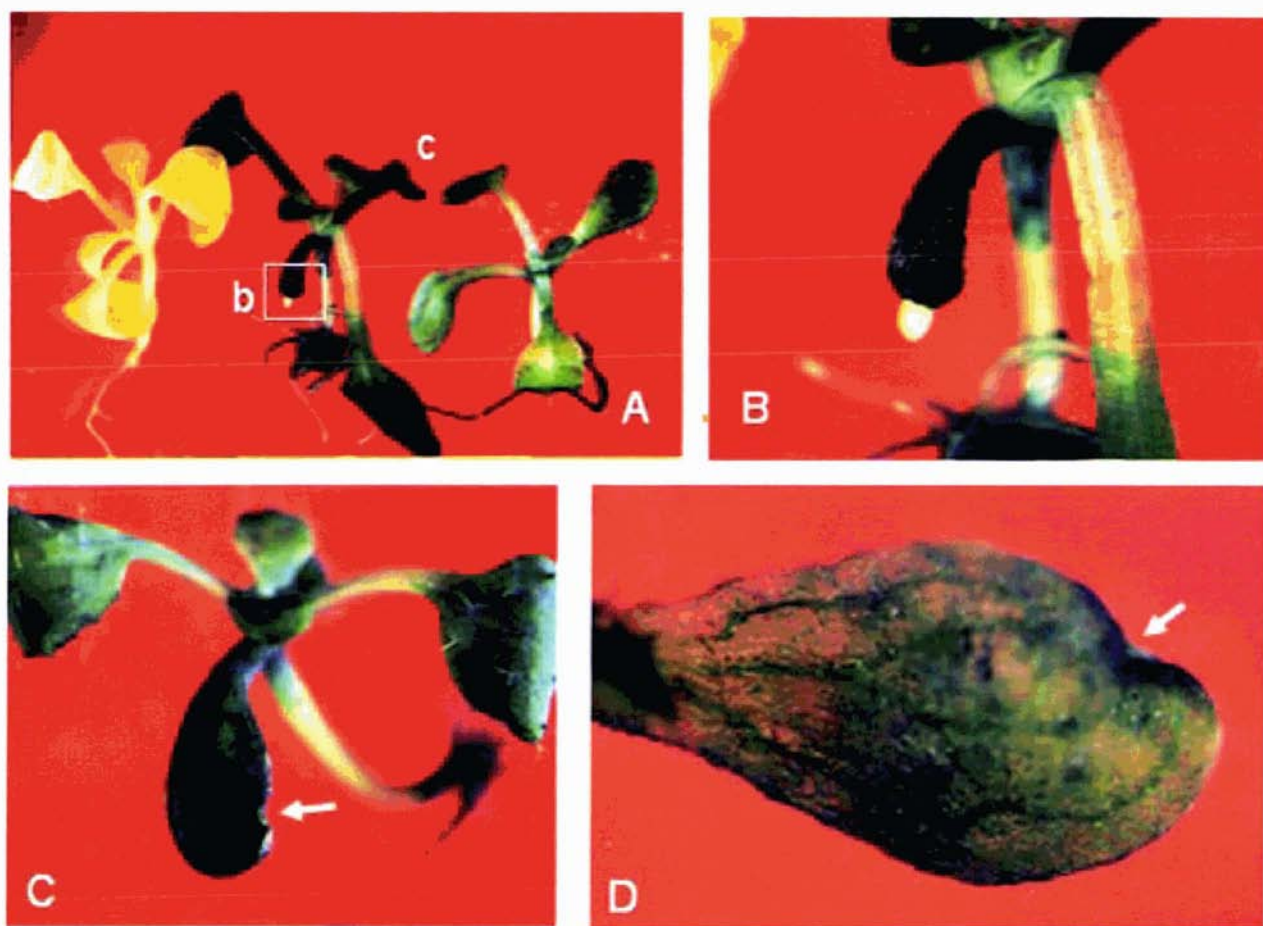
Fig 3.31: Representative phenotypes of OP-*TEL2*-S7 lines: Seedlings displayed are 10 days old and were grown on media containing 15µM DEX. **(A)** GUS negative seedling alongside two GUS positive OP-*TEL2*-21-S7 seedlings, displaying cotyledon abnormalities. **(B)** Close-up of white beak structure on unexpanded cotyledon of central seedling. **(C)** Adaxial view of OP-*TEL2*-32 cotyledon. Arrow indicates small beak forming at tip of cotyledon. **(D)** shows the abaxial view of the cotyledon in (c).
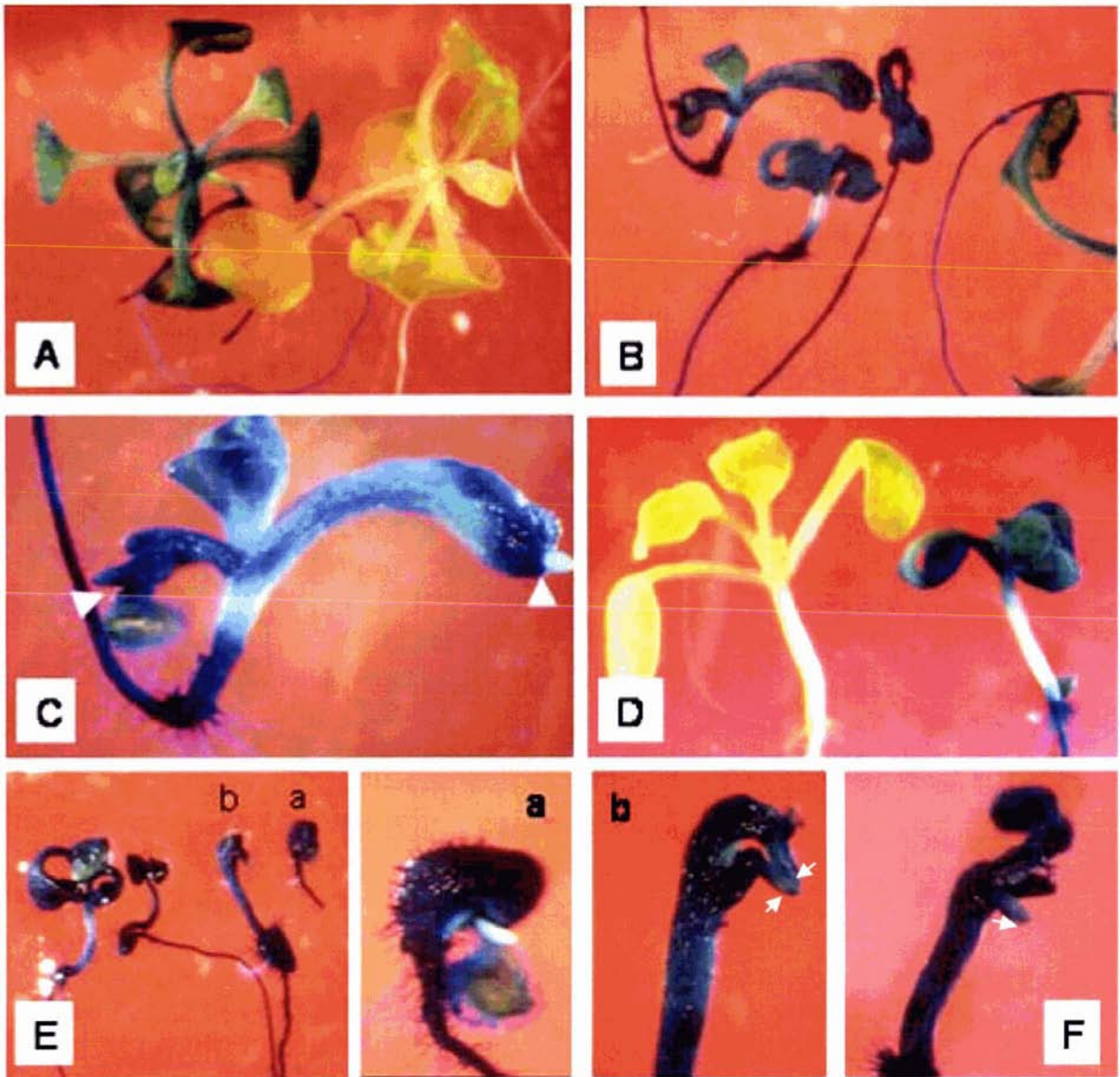
Fig 3.32: Representative phenotypes of OP-*TEL1*-S7 lines. Seedlings displayed are shown 10 days post-germination and were grown on tissue culture media containing 15μM DEX. **(A)** HTOP-G-8: GUS positive and negative seedlings at day 10. **(B)** Line-up of OP-*TEL1*-24 plants. **(C)** Close view of first seedling in line-up. Arrows indicate beak structures on characteristically abnormal cotyledons. **(D)** OP-*TEL1*-24 plant alongside a GUS negative plant from the same plate. **(E)** Line-up of OP-*TEL1*-43 plants. **(a/b)** Close-up of seedlings from (E). **(F)** Example from OP-*TEL1*-24, arrow highlights putative cotyledon.

(c) Tissue culture induction of OP-*TEL*(S7) lines

Abnormalities in the morphology of T2 seedlings of the OP-*TEL*-S7 inducible lines were most apparent when they were germinated on tissue culture media in the presence of DEX, as opposed to later application. Moreover these abnormalities were most evident in the cotyledons and the length of the root.

In the case of the former, one or both cotyledons were narrower, with shorter petioles (Fig's 3.31, overleaf and 3.32, this page). Furthermore

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individual seedlings of both OP-*TEL* lines were found to have beak-like structures (or sectors) at the tip or to one side of the distal end of the cotyledon. The size of the beak, its location, as well as the size of the cotyledon was highly variable. The beak structure did not always stain positively for GUS activity but was always associated with GUS activity in the nearby tissue (See Fig 3.31B). Finally some seedlings were observed in which both cotyledons were vestigial or beak-like, and produced no true leaves during culture. Consequently these phenotypes were considered to be reiterating aspects of the phenotypic categories described for the domain specific crosses i.e the ANT>>OP-*TEL2* extremely delayed seedling phenotypic category.

The third observation was a distinct reduced root length in OP-*TEL*-S7 seedlings grown with the inducer (i.e. Fig. 3.32E, seedling 'a' at far right). A reduction in root length was first noticed in seedlings showing the more extreme phenotypes (i.e abnormal cotyledons, absence of leaves, small overall size). Given that expression of the OP-TEL constructs in the inducible lines would be expected to be ubquitous throughout the roots of the seedlings (Fig 3.32C) including the RAM, this suggested that ectopic expression of these genes might be affecting the function of the RAM. The extent of the reduction in root length was the subject of further investigation.

(d) Root growth rate of OP-*TEL*(S7) lines under induction

The kinetics of root growth was examined through sequential measurements at two-day intervals as described in §3.3.3d. The results described in this section are presented in Table 3.7 (pg 153) and Fig. 3.33 (pg 154). No significant differences were found between mock treated inducible OP-*TEL*-S7 lines and the empty vector lines. From day 2, the mean root length of the OP-*TEL*-S7 lines was approximately half (range 0.57 – 0.44 x EV) that of the empty vector line on day 2 and this continued through to day 10 (range 0.62 – 0.44 x EV).

Comparisions of root growth rate (Δcm per two-day interval, Table 3.7 below) gave a slightly different perspective. On day 4 the growth rates were comparable between all lines and infact higher in 3 of the four OP-*TEL* lines compared to EV. Subsequently on day 6, while the mean growth rate of EV more than doubled, the growth rate of the OP-*TEL* lines had remained static or

	Mean Length (cm) / Mean Growth rate									
Construct	Day 2		Day 4		Day 6		Day 8		Day 10	
HTOP-G-8	0.57	-	1.50	0.75	2.73	1.67	3.92	1.40	4.75	1.66
OP-*TEL1*-43	0.33	-	1.02	0.67	1.67	0.67	2.52	0.85	2.93	0.39
OP-*TEL1*-24	0.25	-	1.15	0.91	1.57	0.65	2.13	0.55	2.12	0
OP-*TEL2*-21	0.26	-	1.08	0.82	2.016	1.08	2.93	0.92	2.78	0
OP-*TEL2*-32	0.32	-	1.13	0.81	1.70	0.57	2.27	0.57	2.55	0.28

Table 3.7: Summary of growth rate of roots: The change in growth rate under induction of the empty vector line is compared to 4 OP-*TEL* expression lines under induction. Mean Length and Growth rate are shown in centimetres per 2 day period.

declined. The exception was OP-TEL2-21 whose growth rate peaked on day 6. Over the next 4 days, the growth rate of OP-*TEL* seedlings generally declined and this is evident in the slow increase in the mean length of the roots of these lines. It should be noted that although the mean length of the roots of OP-*TEL2-21* OP-*TEL2-21* roots appears to decline at day 10, this is because the total population of seedlings was reduced from 63 to 42, due to fungal contamination of the growth medium and seedlings of one plate.
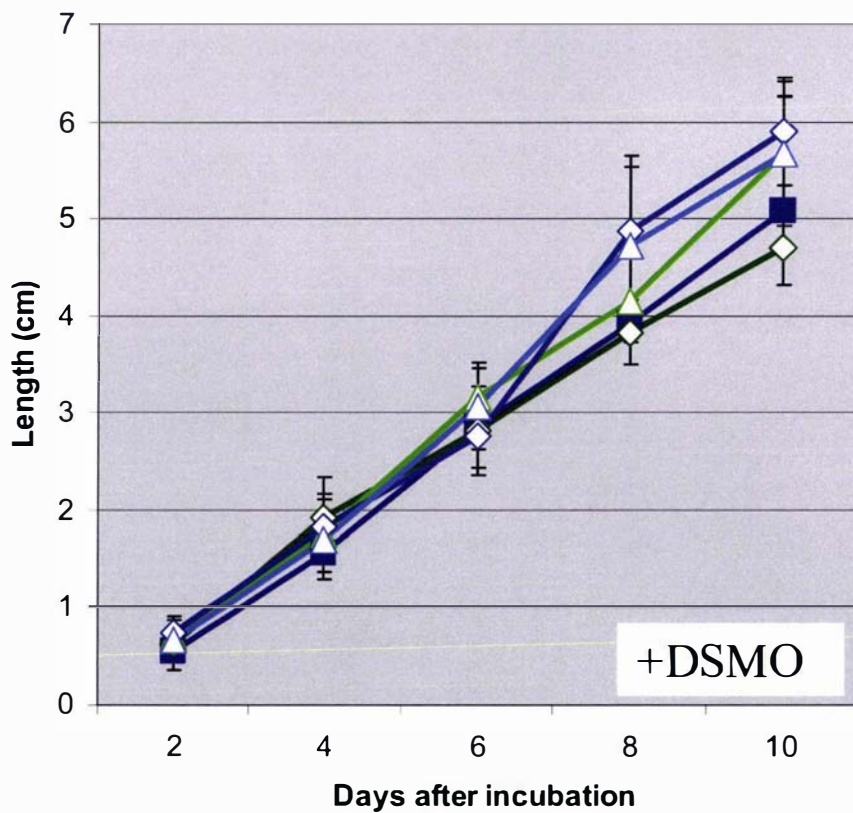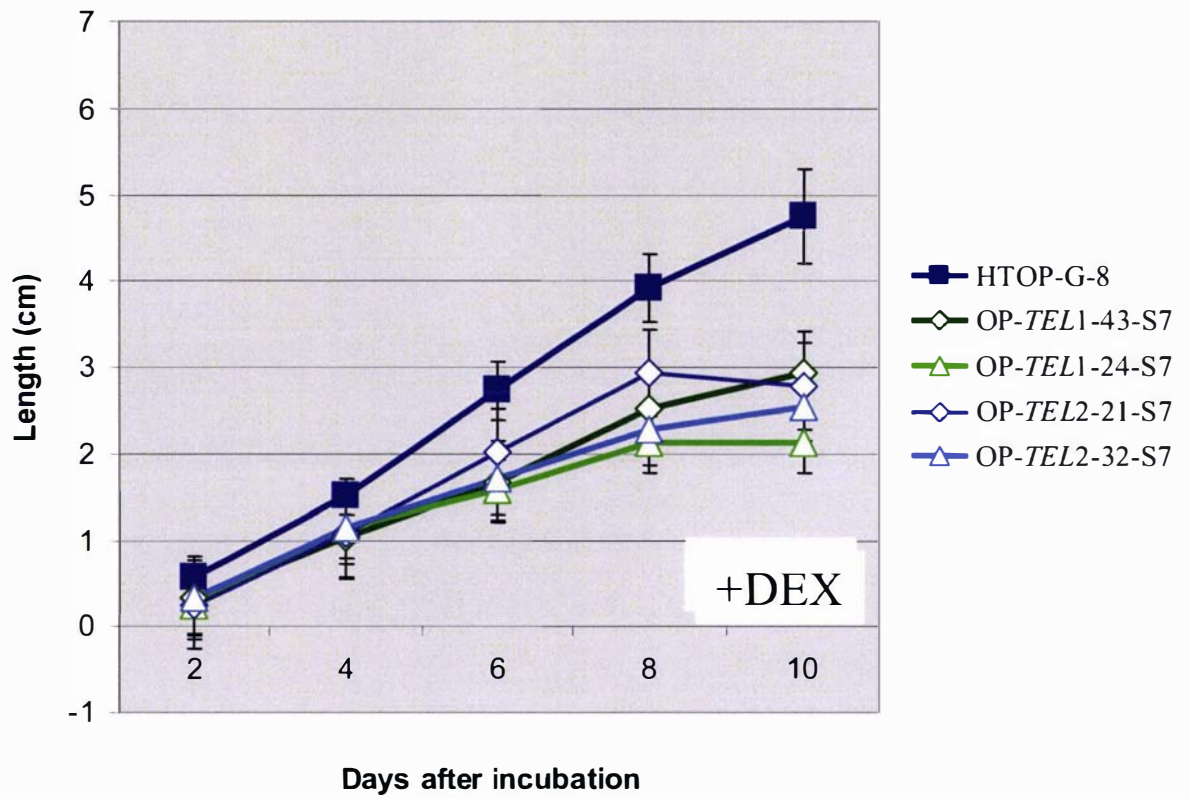
Fig 3.33 Mean root length of OP-*TEL*-S7 lines versus EV: The mean root length (at two day intervals) of Dex treated (**+DEX**) inducible and control lines, is compared with the same lines treated with DSMO (**+DSMO**). Error bars for both figures are +/- 1 standard deviation of the mean.

(e) Cytohistology of OP-*TEL*-S7 roots grown with induction

The observations presented in the preceding section, i.e. reduced root growth rate of induced OP-*TEL*-S7 seedlings, raised the question as to whether this was related to changes in the root cellular structure. This was investigated in root samples fixed and prepared as described in §3.3.2c and viewed using the CLS Microscope. Optical transverse sections were made of primarily the root apex, and the Zone of Differentiation (ZD) to identify any differences in cell morphology in these roots. Representative images are shown in Fig 3.34 (pg 156).

The expectation was that it would be possible to determine from these images whether the organisation of the RAM is affected by inducible expression of either OP-*TEL* construct. However in the sections taken in the vicinity of the RAM the cell walls are indistinct. In particular the central cells of the QC were not identified in many sections of either control or expression lines. This suggests that these images are not true longitudinal sections and therefore it is not possible to firmly state whether or not the structure of the RAM is abnormal, or furthermore measure cell volumes accurately.

Nevertheless, within these limitations, no clear differences in the cellular layout of the root apical meristem were observed in the OP-*TEL* lines compared to empty vector (Figure 3.34A, C, E, pg 157). However, above the RAM, more obvious differences, between the EV and expression lines were apparent in the cell files. As mentioned earlier in the introduction, outside the RAM cells are organised into distinct cell files, which upon exiting the vicinity of the apical meristem, eventually begin to differentiate in size and shape to form distinct tissue layers. An example of this is clearly evident in the HTOP-G-S7 roots shown in Fig 3.34B (pg 157). In contrast cells in the same region of the roots of the OP-*TEL* lines, appear to expand longitudinally much closer to the root cap. Furthermore the histological differentiation between the inner three layers of the ZD is less pronounced, the distinction between the epidermis and cortex not as obvious as in the empty vector lines (Fig 3.34 C, E). As it was not possible to directly measure cell size it was not possible to estimate volume and therefore determine if cells were smaller. Nevertheless measurements of root width, (as an estimate of cell width overall in these

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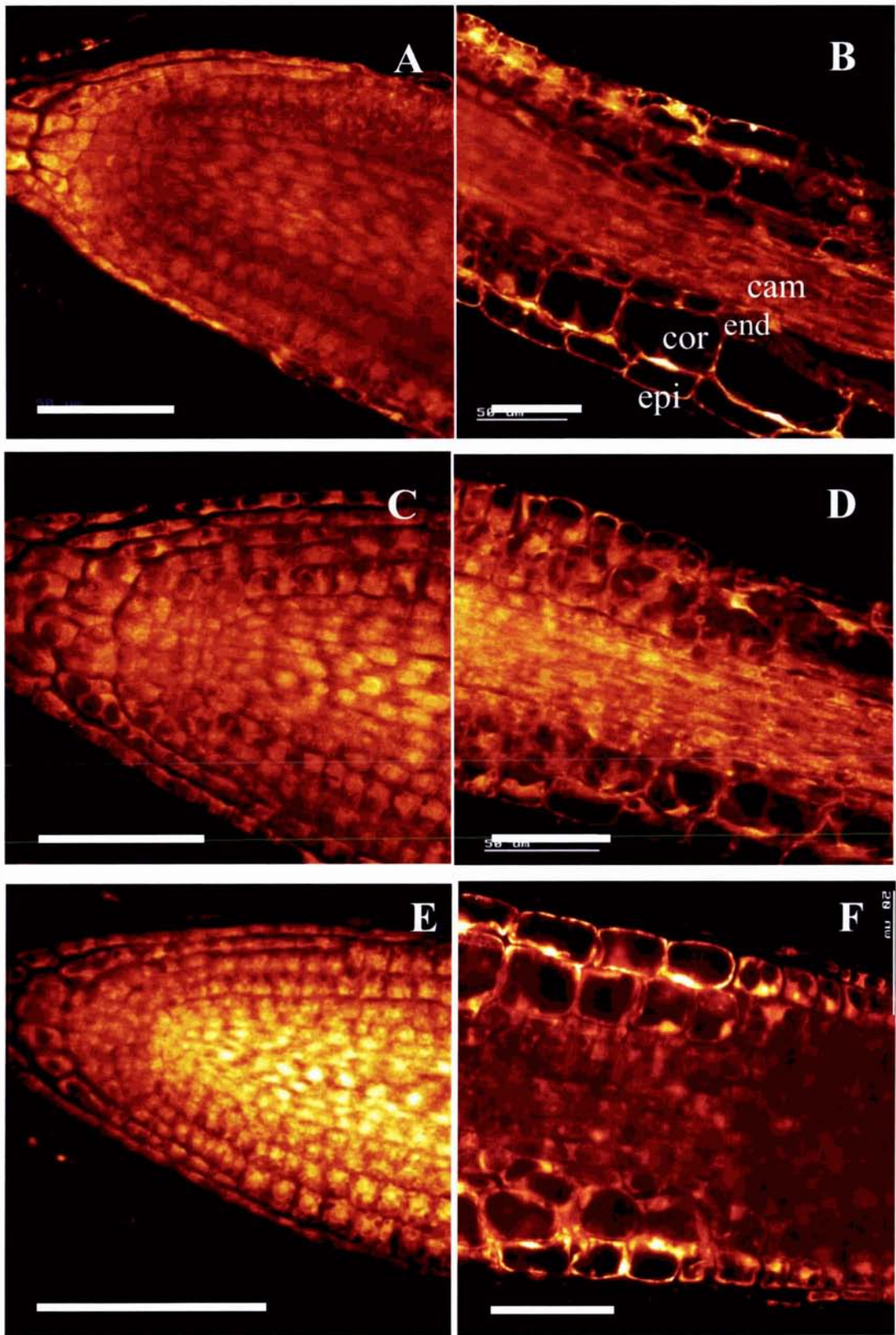
Figure 3.34: Cellular morphology of roots from representative OP-*TEL*-S7 and HTOP-G-S7 seedlings: (A, B) Root apex (A) and 'zone of cell differentiation'(ZD) (B) of HTOP-G root. In (B) tissue layers are labelled for comparison **(C, D)** Root Apex (C) and Zone of Differentiation (D) of induced OP-*TEL1*-24. **(E, F)** Root Apex (E) and ZD(F) of OP-*TEL2*-32. All scale bars are 50μM, except (E) which is 100μm. Tissue layers in (B): **epi**: epidermis; **cor**: cortex; **end**: endodermis, **cam**: cambium.

	Root apex (μm)		ZE* (μm)	
	mean	+/- SD	mean	+/- SD
HTOP-G-8	57.87	6.96	156.11	5.01
*TEL*1-24	53.54	5.9	132.86	13.46
*TEL*1-43	61.43	3.4	118.54	8.7
*TEL*2-21	53.07	5.4	133.61	7.65
*TEL*2-32	56.21	10.05	133.75	11.21

Table 3.8: **Mean width of roots from the inducible expression lines:** Mean width (+/- standard deviation) of the RAM and Zone of Elongation (ZE), from roots of inducible OP-*TEL* lines is compared to empty vector lines.

regions) were taken from three roots of each line, and these results are presented in Table 3.8. These measurements showed that the mean width of the apical region (excluding the root cap) of the OP- *TEL* inducible lines were not different from that observed in the HTOP-G line. However the mean width of the Zone of Differentiation (ZD) in the *OP-TEL* lines was found to be narrower versus empty vector. This coincided with the observation that the zone of elongation, (which normally sits between the meristematic region and the zone of differentiation) was considerably less obvious in roots of the OP-*TEL* lines (c.f. Fig 3.34 B, C and E, OP-*TEL1*-24 page 157).

In general terms ectopic expression of *TEL* does not appear to cause disorganisation of the RAM as judged by cellular layout. Instead the morphology of the cells outside the meristematic zone was suggestive of a lack of comparable differentiation in OP-*TEL*-S7 lines as opposed to the EV lines. Measurements in these areas were indicative of a reduced width overall which is circumstantial evidence of reduced cellular expansion. Thus there appeared to be some evidence that the shorter length, and decreased width of the OP-*TEL*-S7 relative to EV roots is the result of a reduction in cell differentiation that in turn may be associated with reduced cell expansion or growth along the length of the root.

3.5 Discussion

3.5.1 The weakly penetrant phenotypes of P35S:*TEL2* in transgenic lines

The characterisation of 35S:*TEL2* T2 lines lead to two general observations. The first was that while screening of 35S:*TEL2* T0 seed produced a reasonable number of KanR T1 lines only a small proportion of these had morphological phenotypes of any kind. In all cases these phenotypes were associated with the organisation of their leaves, but they were highly variable in nature (Figure 3.4 pg 105) and the majority did not survive under glasshouse or tissue culture conditions. Of the remaining T1 lines that did continue to grow, 5 exhibited a 'slow-midget' phenotype distinct from the empty vector. These plants showed an overall reduced size and a delayed transition to the floral state of up to 60 days. That the expression of the transgene was found in only these 5 lines, confirmed by RT-PCR, was evidence that the phenotype was the result of ectopic expression of *TEL2*.

The second general observation was that not only was the 'slow-midget' phenotype rare in the T1 generation, it was even rarer in the next. . Of the four T2 lines characterised, only seedlings of Line 165 demonstrated a characteristic 'slow-midget' phenotype in this generation. The observed frequency of this phenotype was much lower (~8%) than that observed for the segregation of the resistance marker in this line (74%) as well as the expected frequency of homozygous plants in this KanR population (12/36). Infact the majority of plants were not discernibly different in growth or morphology from the empty vector lines.

Given the difficulties noted in Chapter 1 regarding expression of the *TEL2*::GFP construct it was concluded that both of these observations, and in particular the weak penetrance of the midget phenotype, might be due to cosuppression or post-transcriptional gene silencing of this construct. In retrospect this hypothesis should have been investigated directly using RT-PCR, but at the time it was considered more important to obtain positive results, rather than confirm negative ones. Nevertheless it is a failing of this section of the work that it cannot be unequivocally stated whether the lack of

phenotype was associated with a reduction or absence of *TEL2* transgene expression in these lines.

One possibility that was investigated was that although the lines did not present the extreme appearance of the T1 plants, there might be quantitative differences in developmental progression, and thus the phenotype might be more subtle in the T2 generation. Based on the hypothesis that overexpression of *TEL2* might delay germination, and the emergence of leaves, an assay was developed to quantify the developmental progression of the overexpression lines as previously described. Consequently 2 *TEL2* expression lines (165, and 69) were found to have a mild delay in the emergence of Leaf set 1. In addition 165 also found to have a slight delay in germination. This suggested that the pronounced delays in growth observed in the previous generation were present in the T1 seedlings of these lines, but the degree of effect was considerably reduced and this was assumed to be associated with a reduction in ectopic expression of the construct. Given that 165 was also the only line to produce slow-midget seedlings, it was perhaps not surprising that it also showed developmental delays in both germination and the emergence of the first leaf.

However this also meant that of the five original lines only one produced elements of the postulated slow-midget phenotype presumed to be the result of constitutive *TEL2* expression and thus presumably retained some expression of the construct. The validity of pursuing investigations the function of *TEL2* based on a single expression line, especially when the frequency of the phenotype of interest was so low, was dubious. Consequently, in order to proceed, a different approach to transgene expression was required. On the basis of the previous experiments with 35S:*TEL2* and the GFP fusion it was hypothesized that ubiquitous high levels of *TEL2* expression was detrimental to continued development in seedlings. The presence of a construct with high levels of expression could act negatively selection during screening, both in terms of the seedlings survival, or selection for subsequent cultivation, e.g. T0 high expressors do not look healthy on the plate or do not produce seeds. A construct system was required which could bypass these difficulties and allow investigation of ectopic expression of the *TEL* genes.

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3.5.2 The LhG>>OP-*TEL* crosses

In order to avoid these problems, the LhG-pOP construct system was used. These constructs enabled a comparison of domain specific expression, with inducible ubiquitous expression.

The domain specific expression driver constructs contain *Arabidopsis* promoters whose expression domains have been previously characterised by *in situ* hybridisation analysis of the intact mRNA of the genes. The initial step was to examine whether the observed expression of the reporter construct was as expected under these promoters and in broad terms this appeared to be the case. However the *APETALA* and *AGAMOUS* promoters were also found to have leaf, root, and occasionally cotyledon expression domains which are not described in the literature. Despite the lack of published data regarding *APETALATA* expression outside the floral domains, there is unpublished microarray data on these promoters which supports ectopic expression (albeit at a much lower level) in the vegetative domains (M. Yanofsky, D. Weigel pers.com). Moreover, inclusion of the first two exons and introns of the coding region in the *AP1* promoter has been shown to more completely restrict GUS activity to the expected domains (M. Yanofsky pers.com). In addition it is also known that transcriptional regulation of *AGAMOUS* requires elements which span its second intron (Table 3.2 pg 103 (Sieburth and Meyerowitz, 1997)). That there is low-level expression by particularly the *APETALATA* floral promoters outside the floral domains is particularly noteworthy in the light of subsequent results and this will be discussed in the next section regarding the phenotypes observed in OP-*TEL* crosses involving these promoters.

While the *AINTEGUMENTA* promoter produced reporter activity largely as expected, it was also frequently observed that there was poor or absent GUS staining within the carpels of the silique. The recent observation that broken whole siliques do stain positively for GUS activity suggested this was probably the result of poor penetration by the histochemical substrate of the reporter into this organ.

The observed frequency of reporter gene activity in the HTOP-G and OP-*TEL* crosses was determined. This was necessary as the frequency obtained would be indicative as to whether these lines contained multiple insertions of their respective constructs. This in turn might affect what interpretations could be placed on the resulting phenotypes (or lack thereof) since multiple unlinked

insertions might lead to rapid silencing or else phenotypes unrelated to the ectopic expression. In general the majority of the crosses yielded frequencies greater than or equivalent to 62.5%. In those crosses where the GUS positive seedling were in the 60-68% range, taking into account the numbers involved it is unlikely that these are significantly different from the expected frequency and therefore are likely to be both heterozygous in the F0 generation. In those crosses (both HTOP-G and OP-*TEL* crosses) where the frequency exceeded, or was equal to 75%, it seems likely that the seedling used in the cross may have been homozygous for the activator construct as comparisons between crosses showed that this was dependent on the activator line used.

3.5.3 Phenotypes in the Domain specific expression lines

(a) The Floral promoters

The most surprising observation derived from the F2 seedlings for the APETALA crosses was the apparent lack of a phenotype in the floral domains. Of the floral promoters used, only the F1 seedlings derived from the AG>>OP-*TEL2*-3 cross appeared to have a mild phenotype in the floral domains, specifically in the size of the siliques, but it was not furthered investigated due to time restrictions. The other aspect noted, e.g. the compact nature of the AG>>OP-*TEL*2-3 seedlings, relative to GUS negative vector lines cannot be easily explained based on the observed and expected expression domains of this promoter. However it is conceivable that there is also early ectopic expression as observed in the F2 seedlings of the APETALA crosses.

The phenotypes that were observed in both AP1 and 3 crosses, were associated with the early vegetative development of the seedlings, and thus related to the low level ectopic expression mentioned previously. The phenotypes, though variable in extent, were found to fit into three general categories. These were 'Tendril-like leaves or leaf extensions', 'Callus-like tissue'(CLT) and 'Abnormal Cotyledons'. In general the first category was mostly observed in crosses involving AP1 or AP3>>OP-*TEL1* lines, and to a lesser extent in AP3>>OP-*TEL2* lines. In the F1 generation, there was some variation in the presentation of the tendril-like feature. In some crosses the tendril like feature was observed extending from the tip of leaves, in others the tendril replaced individual rosette or cauline leaves (e.g. AP1>>OP-*TEL1*-2). It has been noted that tendril-like extensions/leaves were observed albeit

infrequently in 35S:*TEL2* T0 lines (See Fig 3.4 B and C, pg 105) . In F2 plants of the *APETALA* crosses this phenotype presented predominantly as narrow strap-like leaves, which appeared to have reduced laminal expansion in the medio-lateral (midrib to leaf edge) dimension. Individual plants which possessed strap-like or tendril like leaves did not produce flowers, and consequently no seed. The appearance of the strap-like and tendril leaves was strongly remiscent of the appearence of leaves in certain polarity mutants. However it is also evident that the phenotype was comparatively rare in those crosses in which it was observed. The exception was AP1>>OP-*TEL1*-1, but as mentioned previously this line also displayed lower reporter gene activity implying a degree of silencing. It is worth noting the observation regarding the expression of the reporter in the empty vector crosses versus expression in the strap-like leaves of the AP1>>OP-*TEL1*-1 cross. In the rosette leaves of AP1>>HTOP-G crosses the expression of the reporter gene was noted in specific individual domains around the edges of the leaf. In contrast this expression was continuous along the edges of the strap-like leaves, (Fig 3.11E pg 118) which were highly suggestive that ectopic expression of the OP-*TEL1* construct was the cause of the narrow leaves.

Despite this, and the observations of tendril like leaves in the F1 AP1>>OP-*TEL1*-2 seedlings, the possibility that this phenotype is infact the result of (for example) cosuppression of other genes cannot be ruled out. Therefore, although this phenotype was striking, it was not further investigated and the significance of this phenotype remains to be determined.

The remaining categories, Callus-like Tissue (CLT), and Abnormal Cotyledon (Ab.Cot.) formed the bulk of phenotypes observed in the floral crosses with OP-*TEL1* and 2. The occurence of callus-like tissue in some or all rosette leaves of a seedling denoted membership of the CLT category. Where only the cotyledons had an abnormal appearance but the seedling itself was normal in appearance this led to catergorisation as Ab.Cot. Consequently these phenotypic categories represented two extremes of a pleiotrophic spectrum of phenotypes which were not always mutually exclusive or consistent. At one end of this spectrum, the F2 seedlings of the OP-*TEL* crosses consisted primarily of callus like tissue and did not flower. At the other end there were plants with cotyledons composed of callus like tissue but subsequently might

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be otherwise normal. In general it seems that the majority of phenotypes in these floral lines are associated with the early ectopic expression as opposed to the later floral expression. The absence of any discernible phenotype in the floral domains confirms this conclusion.

(b) ANT>>OP-*TEL* seedlings

The majority of the results and observations in the domain specific expression experiments are derived from the examination of seedlings from the ANT>>OP-*TEL2* crosses. Only one cross was successfully made with OP-*TEL1*-2, and this was not followed past the F1 generation.

A subset of the F1 seedlings of the ANT>>OP-*TEL2*-4 cross were distinctively smaller and disorganised in appearance. Examination by SEM revealed that the epidermal cells of these seedlings were irregular in appearance. The surface of these lateral organs appeared to be composed of variably sized sectors comprised of oblate irregular cells. These cells were highly indeterminate and looked similar to callus tissue.

In the F2 generation, there was a spectrum of morphological defects associated with the early development of the seedlings. The primary phenotypic category was that of Aberrant cotyledon which had the highest frequency in all but one of the ANT>>OP-*TEL2* crosses. This category covered a range of defects associated with the size, shape and structure of the cotyledons. Direct measurements of the cotyledons from the ANT>>OP-*TEL2* crosses indicated that they ranged in size from equivalent to approximately half the size of EV lines depending on the OP-*TEL2* line used in the cross. In contrast the mean epidermal cell number per millimetre across the centre of the cotyledon was approximately 1.5 (OP-*TEL2*-2) –2.2 times (OP-*TEL2*-4) that of the empty vector. This implied that there is a much higher surface cell density in the expression lines and this is associated with a reduced cell size. However given that the expression of the promoter was expected to be restricted to the internal regions of the cotyledon it is unclear why ANT>>OP-*TEL2* expression might cause this effect.

Examination of epidermal cell morphology in these cotyledons, confirmed that the cells across the middle are smaller, but also that the cells near the tip of the cotyledon are more irregular in appearence. This irregularity was suggestive of a decreased histodifferentiation in these cells compared to the empty vector lines.

These counts and observations were made using cotyledons of a relatively normal appearence in the Ab.cot category. There were however more extreme presentations worthy of note, usually associated with the distal tip, or replacing the cotyledons themselves. These included vestigal cotyledons which lacked a recognizable lamina or petiole but usually had callus-like tissue and/or beak like structures. An infrequent observation, in cotyledons with callus like tissue at their tip, were leaflet like structures. These were found to have an independent vasculature suggesting they had arisen *de-novo* rather than being an extension of the cotyledon itself. This implied that the callus-like tissue at these points while indeterminate in appearance was able to elaborate determinate structures.

These extreme presentations though striking were not however the main focus of subsequent investigation. The main focus was the emergent (beak-like) or recessed domain/s near or at the distal tip of the cotyledon. Although perplexing in their variability, the sectors at the tips of the cotyledons shared a common feature. Whether recessed or emergent, these sectors were found to consist of smaller less differentiated cells, apart from an innervating vasculature arising from within the main cotyledon.

The observed expression of the reporter gene in the HTOP-G crosses revealed expression throughout the cotyledon but also a persistant concentration at the distal tip (Fig 3.11A-C pg 118). Expression of the ANT promoter is known to predict the position of the incipient cotyledons in the globular embryo, so consequently begins very early in development. However expression of the reporter is clearly visible at the distal tips of the cotyledons of plants at 14 DAG, so expression at this point persists long after germination. This persistant expression at the distal tip of the cotyledon therefore could explain the comparative consistancy of phenotypes observed in this organ in the ANT>>OP-*TEL2* crosses. It also suggests that there is a common theme to all the Ab.Cot sub-categories, in that they vary primarily in the extent to which subsets of cells in the cotyledon are able to differentiate.

The next phenotypic category of note in the ANT>>OP-*TEL* crosses are the Extremely Delayed seedlings. These were identified on the basis that they fail to make cotyledons and leaves, but often produce proliferating CLT at their apex. They are termed Extremely Delayed Seedlings (EDS) because they do eventually produce axillary shoots and leaves but only after a prolonged

period of incubation of up to, or more than 4 weeks. Of those displayed in Fig 3.24/ 3.25, (pg 139, 140) most formed one or several axillary apical meristems at ~ 30 days post germination. Where leaves did form, they were found to have varying degrees of callus like tissue on their surface. This phenotype was observed at very similar frequencies (~20-25% of the GUS positive seedlings) in all but one of the ANT>>OP-*TEL2* crosses. It was also noted in the progeny of the ANT>>OP-*TEL1*-2 cross. Thus the ectopic expression of OP-*TEL* in these seedlings appears to not only prevent the formation of the cotyledons but also prevented the concerted formation of the SAM. However it is unclear how ectopic expression within the incipient cotyledons could disrupt the formation of the SAM itself unless the activities of *TEL* proteins in these cells is somehow sufficient to interfere with the expression of genes associated with the determination of the domains required for the shoot apical meristem.

It should be noted at this point that these phenotypes provide a possible explanation in the difficulties in identifying *TEL2* expression lines. It seems likely that if such seedlings had been observed during screening of the 35S:*TEL2* / *TEL2*::GFP T0 seed, they would not have been selected for further screening because they do not look healthy. This point will be further discussed in subsequent sections.

The third and least frequent phenotypic category were seedlings which demonstrated an extremely disordered morphology equivalent to that described in the F1 generation of the ANT>>OP-*TEL2*-4 cross. Unlike the situation in the floral crosses they formed a minority of the aberrant phenotypes observed. Seedlings of this category were observed in 3 out of 5 OP-*TEL2* lines and generally at ~10% (the exception being OP-*TEL2*-3 with 20%). Unlike the EDS, these plants are able to form a shoot apex but subsequently produce disordered leaves. The appearance of these plants was similar to that observed in the F1 generation. It remains unclear what their significance is as detailed examinations of their histology were not undertaken. It is however possible that they represent an intermediate stage in a continuum ranging from the EDS to the Ab.Cot plants.

The general conclusions derived from these crosses, (and also the floral crosses, are as follows. The phenotypes observed (with the possible exception of the strap-like and tendril leaves) share two general themes. The most

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extreme and highest frequency phenotypes are all associated with early ectopic expression of the OP-*TEL* constructs (e.g in the incipient cotyledon and early leaves) and they are all associated with variable degrees of indeterminancy in these organs both within a cross, and between different lines in a particular cross. The beak-like structures for example were shown to be accumulations of small irregular cells lacking the differentiation characteristic of their location. In one example present the beak achieved an internal organisation strikingly reminiscent of the tunica-corpus of the shoot apical meristem. Similarly SEM micrographs of different Ab.cot seedlings showed that this phenotype was the result of varying degrees of indeterminacy in either cotyledon. In the Extremely delayed seedlings, the shoot apex itself appears unable to form in an organised fashion suggesting that the normal developmental progression of embryogenesis has been derailed by the early ectopic expression of OP-*TEL2*.

3.5.4 Developmental assays

In order to examine more subtle effects of expression, an assay of developmental progression in populations were applied. Because of the observations made regarding the growth of the slow-midget phenotype this assay measured the emergence of specific developmental landmarks. The hypothesis underlying this assay was that in addition to generating histological variability, the ectopic expression of *TEL* might also generally delay overall development in the young seedlings. As noted previously the T2 generation of the 35S:*TEL2* expression lacked the striking small midget phenotype. Simlarly a subset of seedlings of the OP-*TEL* crosses had minimal or no phenotypes despite vivid staining of the reporter protein. The prime example of this was the Ab.Cot phenotypic category. In many crosses the seedlings elaborated abnormal cotyledons but subsequently were comparatively normal in their development. The use of the developmental assay was expected to address the question of whether ectopic *TEL* expression had more subtle developmental consequences for seedlings of the various expression constructs.

In general, this does seem to be the case. In almost all the crosses that were assayed, delays in the emergence of developmental landmarks were observed versus the HTOP-G line. The notable exception was API>>OP-*TEL2*-3 at Ls 5, where the fifth leaf was noted a day earlier than the control in a percentage of seedlings. Otherwise the timing of Germination, the emergence

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of the first and second leaves was shown to be significantly different from the empty vector.

The other notable result related to a delay in flowering in the AP3 crosses. This was considered significant because no histological phenotypes had been noted specifically associated with ectopic expression of *TEL* in the floral domain. It remains unclear whether this is associated with the expression of the construct during flowering, or the early low-level expression in the cotyledons and first leaves. Nevertheless given that the most extreme histological phenotypes were associated with absent or delayed flowering, this provides further evidence that ectopic expression of *TEL* is able to interfere with the transition to flowering. Whether this interference is direct, or indirect, remains to be determined.

Delays in developmental progression were particularly evident in the ANT>>OP-*TEL2* crosses. These separated into two classes on the basis of the extent of these differences. Class I was OP-*TEL2*-2, 5 and -57-S7 and Class II included OP-*TEL2*-3 and *4*. An example of this is Germination. In the class II crosses the frequency of germination did not exceed 50% in these two crosses during the duration of the experiment. In contrast class I crosses achieved this by day 2, and 98% of seedlings of the empty vector had completed germination at this time point. The clear separation of class I and II and EV remained throughout the rest of the experiment. In one sense the result is unsurprising given the expected expression domains throughout the lateral organs. It is in line with the general observation that ectopic expression of *TEL* delays developmental progression. What is not clear in these assays, is whether this delay represents an increased plastochron (e.g. increased time between leaf initiations) or simply slower overall growth and differentiation. If this was determined this would go some way to answering whether ectopic *TEL* expression directly interferes with the floral transition, or whether it is simply the result of delayed leaf differentiation which delays the overall progression of the seedling to maturity. These points will be discussed further in the next chapter.

3.5.6 Inducible OP-*TEL* expression

The second line of enquiry used in this analysis of ectopic expression was ubiquitous inducible expression responsive to dexamethasone (DEX). In these experiments it became obvious that timing and the concentration of DEX application were important to the overall observed result of induction. Specifically if seedlings were induced significantly after germination (e.g. sprayed when cotyledons are visible, or transferred after germination) they typically showed comparable phenotypes with the empty vector and non-induced lines. Thus experiments involving either glasshouse cultivation and spray induction, or transfer of seedlings to DEX containing media failed to produce contrasting phenotypes in the OP-*TEL*-S7 lines versus HTOP-G regardless of concentration of inducer. However when such lines were germinated on media containing the inducer then a variety of phenotypes were noted and these required a concentration of inducer of between 10-15 μM.

These phenotypes bore some similarities with those observed in the domain specific crosses. Particularly noteworthy was the reiteration of the beak like structures in the cotyledons, and the EDS category. An additional feature present in the induced OP-*TEL* lines, and not noted in the crosses, was a decreased root length (see §3.4.11d). Investigations of this phenotype are discussed in the following section. Interestingly prominent callus-like tissue as described in the crosses was not evident in the inducible seedlings either in tissue culture or spray experiments. It was therefore concluded then that early expression of the OP-*TEL*–S7 construct is a required aspect of these highly indeterminate phenotypes (especially in the cotyledons), and also that ubiquitous expression does not seem to be able to generate callus like tissue at least to the same extent. This may mean this is a feature of ectopic expression in specific domains. Alternatively it may represent difficulties in providing sufficient inducer to provide the required level of expression in order to generate this characteristic. This is hinted at in the degree of induction obtained (see δ3.4.11a) for the OP-TEL lines. This was lower than was expected given the clearly visible activity of the reporter gene in these lines. It seems likely that the level of expression of the gene of interest does not correlate directly with the level of GUS activity (Samalova et al, 2005; Craft et al, 2005). Consequently it is possible that the variability of phenotypes

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observed relates directly to the lower expression of these constructs under induction relative to the activities of LhG4 (Craft et al, 2005)

The inducing chemical Dexamethasone is light sensitive so this represents another difficulty in spray induction experiments in the glasshouse, especially because of the higher concentrations required to generate phenotypes in plant culture media. However although few phenotypic variances were observed in the glasshouse experiments, (amongst the inducible OP-*TEL*-S7 plant lines versus the controls) the OP-*TEL* did show a degree of delay in the emergence of the floral buds. This was in line with the general observations of AP3>>OP-*TEL* domain specific expression crosses and was matched with a slower increase in bolt length as indicated by the mean floral index. For two of the three OP-*TEL1* lines the mean floral bolt category was significantly lower from day 10 (P<0.001). For the OP-*TEL2* lines the difference was significant from day 12. While not of the same order of magnitude observed in the initial 35S:*TEL2* lines, this delayed flowering and growth of the bolt provides evidence that induced lines also have slower emergence of the floral bud and subsequent elongation of the floral bolt.

3.5.7 Root growth kinetics

The apparent reduction in root length of OP-*TEL* inducible lines was assessed quantitatively. Initially the differences in mean root length between OP-*TEL*-S7 and the EV were not significant. By day 8 the growth of the OP-*TEL*-S7 had levelled out and by day 10 they were half or two thirds the length of the control lines in the presence of DEX.

When the rate of root growth (Δlength per two days) was determined this indicated that root growth was reduced from the beginning (Table 3.7, pg 154). All inducible OP-*TEL* lines had comparable rate of elongation relative to empty vector line at day 2. By day 4 however while the growth rate had doubled in the empty vector line, it was constant or declining in the inducible lines. Inducible expression of the *TEL* constructs therefore seems to be strongly inhibitory to the elongation and growth of the root in a fashion similar to that observed for the upper half of the plant. Furthermore there is no distinction in the degree of this effect between OP-*TEL1* and 2 which might be

expected given their contrasting domains of normal expression. This may therefore be evidence for their functional equivalence in the root.

In hindsight the association of reduced root length and growth could adversely affect the survival of, for example, strongly expressing lines of 35S:*TEL2* when they were transferred to soil. This would therefore be a further contributory factor to the difficulties observed in identifying high expressing *TEL*2 lines.

Regarding root growth kinetics the question then raised was how ectopic expression of OP-*TEL* might be causing this strong inhibitory effect. In *Arabidopsis* mutants which show reduced root length (e.g. *fas* (Kaya et al., 2001)) defective divisions have been identified in the RAM which are presumed to lead to defects in the arrangement of cells within the root meristem interfering with its normal patterning and growth. To identify if such defects exist in the RAM of inducible expression lines sample roots of induced seedlings were examined using CLSM. However difficulties in identifying the QC in many of these samples made identification of the longitudinal axis impossible. Therefore because it was unclear whether the optical sections were truly longitudinal it was not possible to make definitive statements about whether there were defective divisions within or near the RAM, or accurately measure cell volumes (specifically in the length dimension).

Despite these limitations, the cytohistology of the roots of the OP-*TEL* lines does appear to show the effects of ectopic *TEL* expression in the both the apex and beyond. This is particularly obvious in the region immediately above the meristematic zone. The developing histological distinction between tissue layers such as the endodermis, cortex and epidermis in this region were not as evident in OP-*TEL* lines. In general the outer two layers appeared less distinct in size and shape than in the control lines.

Finally although the length of cells could not measured, measurements of root width, both at the putative root apex, and subsequently in the putative ZE provided an estimate of overall size. The results of these measurements showed that, while the root apex of the OP-*TEL* lines were not different to EV, beyond this area the roots of the expression lines were narrower. This implies that cell expansion is reduced. Taken together these results were suggestive, in general, of reduced cellular determinancy and possibly cell growth. This reduced determinism in the roots may interfere with the subsequent expansion

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of cells both radially and in the apical basal dimension. It is generally held that overall root length is based on root cell expansion in the zone of elongation the region directly above the region of the meristem. Consequently decreased cell expansion could lead to decreased overall root length. Another potential contributor would be a reduction in overall cell divisions both in the RAM but particularly as the cells leave the meristematic region, which could be expected to be a consequence of reduced determinancy evident in these cells. This remains to be determined.

Chapter 4.0: Discussion

4.1 The Mei2-likes of Arabidopsis

One of the primary themes in the introduction was the study of leaf position, or phyllotaxy, and some of the genes believed to regulate this in plants. In this context *TERMINAL EAR 1* was introduced as an RNA binding gene from Maize, believed to regulate leaf position both radially and apically-basally. Furthermore it was noted that *TE1* has sequence similarity to *MEI2* from the fission yeast *Schizosaccharomyces pombe*. MEI2 in turn is known to be required for the switch from mitosis to meiosis in this same yeast (i.e. (Kitamura et al., 2001; Watanabe, 1997; Watanabe and Yamamoto, 1994)). The inference made was that the function of TE1 is also in some way associated with the cell cycle, but specifically with the commitment to differentiation in leaf primordia as opposed to cell division. Furthermore because tMei2p::GFP was shown to migrate to the nucleus of animal cells, just as was observed in the fission yeast itself, it is possible that there may be conserved function between animal, fungal and plant cells.

Following on from this, further plant homologues of MEI2 were introduced and in particular a family of seven related genes, the Mei2-Like's of *Arabidopsis thaliana,* were described. These were identified on the basis of sequence similarity (particularly in the RRM3) to *TE1* (the TE-Like genes 1, and 2) and MEI2 (the Arabidopsis Mei2-likes or AMLs) respectively (Anderson et al., 2004a; Jeffares et al., 2004). In addition two truncated versions consisting of only the third RRM (Anderson et al., 2004a; Jeffares et al., 2004) have also been described recently. Apart from *Arabidopsis* a number of other plant species (including wheat, tomato, petunia and rice) and unicellular organisms have been found to have homologues encoded in their genomes. That so many homologues exist in such widely disparate species and kingdoms, has been taken as further evidence supporting the inference of a conserved function/s in a phylogenetically broad range of organisms and therefore may serve a fundamental role in the activities of these cells.

Some features of the function of the MEI2 gene were highlighted in the context of the possible function of these plant homologues. Firstly the activities of Mei2p are known to require a non-coding RNA called meiRNA,

which assists in the nuclear localisation of Mei2p. Moreover it has been been reported that Mei2p associated with meiRNA, goes to the *SME2* locus which encodes meiRNA (Shimada et al., 2003). As yet it is not clear what Mei2p might do, once at this location, but it is clear that it is in some way associated with promoting meiosis.

This leads onto the underlying question of this work; what is the function of the *TEL* genes in the overall development of *Arabidopsis sp.* Considering the functional characterisation of the mutant alleles of *tel* (Veit et al., 1998), and the *in situ* hybridisation data obtained from the *TEL* cDNAs (Anderson et al., 2004a), it seemed likely that these Mei2-like genes are involved in aspects of the regulation of leaf position. Furthermore it was postulated that this regulation would involve repression of the onset for example leaf founder cell identity and subsequent differentiation. This was based in part on the observations in Maize. In particular the description of random plastochron lengths lead to the hypothesis that the function of these genes may not simply involve 'where' a leaf primordia may arise, but more specifically 'when' it can arise.

In order to investigate this hypothesis ectopic over-expression was chosen primarily because analysis of mutant alleles was considered unlikely to be informative. This was based on the high sequence similarity present within the Mei2-likes, which suggested that there would be at least partial complementation in lines containing mutant alleles of both *TEL* genes. Consequently the phenotype of mutant lines was, at the time, considered likely to be less informative than the phenotypes of ectopic expression of the intact gene. The latter was expected to generate data relevant to the function of these genes relating to its role in the architecture of the whole plant as well its role within individual cells.

This data was to be supported by investigations of the localisation of the tagged protein, as well as the native protein. Localisation to the nucleus by GFP tagged *TEL* proteins would for example confirm that they may migrate to a specific site/s in the chromosomes just as was observed for Mei2p. Recombinantly expressed *TEL* proteins were to be used to determine the identity of their cognate RNA partner/s if possible. In plants the association of

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MEI2 with meiRNA and its role in its function is particularly notable because of the recently identified regulatory prominence of short non-coding RNAs, in a variety of plant developmental processes (e.g.(Achard et al., 2004; Bartel and Bartel, 2003; Federoff, 2002; Lorkovic and Barta, 2002)). Consequently, if there was conservation of function between Mei2p, *TE1* and the Mei2-likes, then this might involve the movement or metabolism of short non-coding RNAs within specific domains. This was to be investigated using the recombinantly expressed *TEL* proteins but ultimately to be examined *in vivo* using the TAP tagged construct.

In summary an investigation into the function of TE1 was undertaken using two *A. thaliana* homologues, TEL1 and 2. On the basis of previous work undertaken to characterise MEI2 and TE1 (as well as expression domains of the AtMei2-likes) it was proposed that the function of these genes might involve regulation of leaf position both spatially and temporally. To investigate this, two strands of inquiry were undertaken. The first, ectopic expression of the TEL's in *Arabidopsis* thaliana Col0, would address whether the *TEL* genes are able to exert positive effects on cells outside their normal domain, and thus perturb the normal development of a plant and its organs. Furthermore any kind of organ or stage specific effects could provide evidence that the function of *TEL* does require specific cognate factors whereas more general phenotypes present throughout development (or throughout the plant) might imply the opposite. The second strand was biochemical characterisation which would yield more detailed information about the function protein within a cell and its cycle. Consequently this would allow a deeper understanding of the mechanism of these genes and ultimately which pathway/s they interact with.

4.2 Expression of the *TEL* protein products from the cDNAs

Attempts to extract the entire protein product, encoded by the *TEL* cDNAs, from recombinant yielded very low or undetectable quantities of protein. In the case of recombinant expression in *E. coli*, two different tags were used and a variety of expression and extraction parameters examined. However expression did not improve to any measurable extent. Difficulties in

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expressing the entire *TEL* proteins are not restricted to the current work. Expression of the complete protein has remained recalcitrant despite ongoing efforts in the BV laboratory.

The possibility of expressing segments of the coding region, e.g. short peptides, such as the region of the RRM3 or areas specific to either *TEL* gene, were considered as an alternative. However it was unclear whether (a) the peptide would fold correctly if expressed and therefore (b) whether this peptide would retain its native function and consequently(c) whether antibodies derived from such peptides would detect epitopic regions available in the native protein. Consequently this approach was not undertaken. It should be noted that such an approach has been used for FCA (Macknight *pers.com*) and found to be acceptable. In hindsight this approach was the logical next step and should have been investigated further.

Lacking any protein to progress further investigations of the *TEL* proteins biochemical properties (i.e. RNA affinity) their RNA affinities and biochemical properties remain unknown.

4.3 Localisation of *TEL2*::GFP

One of the first plant expression constructs used experimentally was a translational fusion created between the *TEL2* cDNA and the reporter gene m*GFP-5*. Expression of this fusion protein in stably transformed plant lines was to be the first approach made towards determining the localisation of these proteins in living cells, and if possible, in the respective meristems of these plants. There were however difficulties in isolating lines with visible *TEL2*::GFP fusion. Fifty-six T1 lines were identified by antibiotic resistance to contain the construct. However none were found to have discernible levels of GFP fluorescence when compared to empty vector lines. Nor could the fusion protein itself be detected by immunoblot analysis in a limited selection of these T2 seedlings of these lines. Thus this approach failed to provide any information regarding localisation of the *TEL2* protein.

One explanation for this was that expression of the fusion construct itself was defective. Sequence from the construct showed no errors in either cloned gene or the construct itself. Nevertheless this possibility was further investigated using transient expression assays. This was undertaken in onion epidermal cells by Susanna Leung as part of her thesis work. From these experiments it was found that the GFP tag was visible confirming that the

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construct was indeed translationally functional in this context. Despite the appearance of Fig.2.4, (pg 77) nuclear localisation of the *TEL2*::GFP fusion protein could not be confirmed in all cells examined and thus no conclusions could be made in this regard. However this does represent a potentially fruitful avenue for future experiements and this will be discussed in a latter section.

A likely explanation, regarding the difficulties in isolating stably transformed lines, was that overexpression of *TEL2* itself is highly detrimental to the survival of any seedling which contains this construct. In initial screens a number of T1 *TEL2*::GFP seedlings were eliminated from consideration on the basis of adverse morphologies and reduced viability. Similar phenotypes were later found to be associated with ectopic misexpression of OP-*TEL* in the LhG:pOP constructs. Prime examples are the phenotypes observed under inducible ubiquitous expression and in particular the disorganised leaves/seedlings and EDS like phenotype. Consequently GFP fusion overexpression lines with the highest expression levels were likely to have been overlooked on the basis of their apparent low viability, and strong resemblance to kanamycin sensitive plants. This would have inevitably biased the screening procedure towards selection of lines with the least expression of the fusion construct. The inability to detect the GFP protein fusion in extracts from a subset of T2 seedlings implies that infact these lines were unlikely to have even very low expression of the construct which is at least consistant with this possibility. It should be noted at this point that other investigators, working with a different clade of the Mei2-like family, have reported similar problems. A recent paper describes attempts to isolate lines constitutively expressing *AML1*::GFP fusions, which also failed to identify lines with visible fluorescence. They were unable to detect the transcripts of the fusion construct in any tissues (Anderson and Hanson, 2005). The authors of this study concluded that the difficulty in obtaining constitutive overexpression of *AML1*::GFP was probably due to toxicity of constitutive expression of *AML1*.

Therefore there is at least some corollary evidence that other members of the Mei2-likes are readily silenced when expressed in this manner. It is unfortunate that expression levels of the *TEL2*::GFP transcript were not directly tested since this would have confirmed that this was the case in the current work. Nevertheless it is possible to conclude that intracellular localisation of the *TEL2*::GFP fusion protein is not easily determined in

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ubiquitous expression lines. Consequently the next best option would be using inducible expression lines. This possibility will be further discussed in a later section.

4.4 The 35S:*TEL2* expression lines and the slow midget phenotype

The disappearance of the slow midget phenotype from the majority of T2 seedlings of these lines, and the difficulties with the *TEL2*::GFP fusion lines, were discussed the previous section. Based on the observations made with the LhG:pOP lines these may reflect the same underlying problem. The constitutive overexpression of *TEL2* is deleterious to seedling survival and/or viability which creates a bias during screening. It is possible for example that it would be difficult to discern the difference between the antibiotic sensitive seedlings and those with high levels of ectopic *TEL* expression. Furthermore even if high expressing line/s were correctly identified its likely they would not flower or produce seed. Therefore the lower viability of high expressing lines would reduce the likelihood of such lines being identified on a tissue plate, or surviving once transferred to soil. Furthermore, it also seems that even if lines showing ubiquitous expression are identified and do survive, that the phenotype is eliminated or markedly reduced in the following generations. This is in line with the observations and explains the low numbers of putative expression lines observed to have altered development in the initial screen, as well as the lack of phenotype in subsequent generations.

Unfortunately there is no direct evidence that this is the result of markedly reduced expression between generations, because the levels of transgene expression was not directly verified by RT-PCR in the T1 generation of these lines. Without this of course it is not possible to state categorically whether it is infact silencing of the construct or whether for example it is silencing of other crucial genes or even if there are other unknown factors that contributed to these observations. The only evidence that this is not the case is a single T2 line which continued to demonstrate phenotypes equivalent to that previously observed although at a frequency lower than the inheritance of the resistance marker. Further investigation of the phenotypes described in this

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single transgenic line was of course of limited utility. Therefore investigation of the nature of the effect of ectopic expression of *TEL2* required a different approach to ectopic expression and this is discussed in the following section.

4.5 Ectopic expression delays determinative development

In order to further investigate the effects of ectopic *TEL* expression, domain specific and ubiquitous inducible expression constructs based on the LhG-pOP vector system were constructed (Moore et al., 1998, Samalova et al 2005, Craft et al 2005). The bulk of the results presented in this section come from the analysis of phenotypes in ANT>>OP-*TEL2* seedlings and therefore associated with early expression driven by the *AINTEGUMENTA* promoter (ANT). Very similar but more variable phenotypic effects were seen in the leaves and cotyledons, with ectopic expression utilizing the floral promoters. However because of their variability and because relatively few crosses were completed, further analysis of the phenotypes was confined to the ANT>>OP-*TEL2* crosses.

As described in Chapter 3, (§3.3.2a) *ANT* transcripts are first observed in the globular embryo at the positions of the incipient cotyledons (Elliott et al., 1996). This contrasts with the described native expression of both *TEL1* and *TEL2,* which have been shown to occupy a more central position in the globular embryo in the presumed central zone of the SAM (Anderson et al., 2004b). Subsequently the ANT transcripts are found in the internal regions of the cotyledons, during the walking stick phase. After germination the activity of the reporter gene product (in the control lines) was found to be throughout the cotyledon but most pronounced in a region at the distal tip of the cotyledon. Expression of the ANT>>OP-*TEL2* constructs in these organs can be presumed to start upon their specification and to persist throughout their subsequent development.

The F1 progeny of the ANT>>OP-*TEL2*-4 cross included some seedlings that were small and disorganised in appearance relative to their more normal siblings. SEM of the cotyledons and early leaves epidermal surface of the cotyledons and early leaves, revealed sectors as well as whole leaves completely lacking histodifferentiation on their surfaces. The cells in these

areas were callus-like in appearance, by virtue of their irregular shape and size, and were thus dubbed callus-like tissue. The sectored occurrence of the callus-like and normal tissue was a consistent aspect of this phenotype. Its variability resembles that of the well known phenomenon 'position effect variegation' first observed in *Drosophila* and Maize (reviewed in (Lippman and Martienssen, 2004)) whose significance will be further discussed in a later section.

In the F2 generation the effect of *TEL2* expression localised to the incipent cotyledons produced a spectrum of effects in the elaboration of the cotyledons. These effects ranged from vestigial (or absent) cotyledons, and the proliferation of callus-like tissue at the apex, to the presence of beak like structures at the distal tip or in place of the cotyledon. Similar phenotypes to the F1 generation (i.e. more general callus like tissue) were less frequently observed in subsequent vegetative development and not observed at all during the floral phase. No phenotypes were observed in the floral stage in seedlings containing either *ANT* or *APETALA*>>OP-*TEL* combinations. This latter observation will be discussed in the following section.

The phenotypes observed in the F2 cotyledons of ANT>>OP-*TEL2* appears to be linked by a common theme, specifically that ectopic expression in the cotyledons (and less frequently in the rosette leaves) was associated with discrete accumulations of indeterminate cells in the form of beak like structures, or wet irregular tissue. In the cotyledons of the ANT>>OP-*TEL* lines, this often manifested as beak-like structures composed of apparently undifferentiated cells of variable size but usually smaller than neighboring, (and recognizably histodifferentiated) cells. These domains of reduced determinancy in both the cotyledons and following leaves were also observed in the AP>>OP-*TEL* crosses but at a lower frequency. In the case of the *ANT* crosses, the predictability of these phenotypes in the cotyledons and particularly at the tip of the cotyledons, (versus for example callus-like tissue in any part of the plant) is suggestive that cells in these organs are most receptive to the activities of *TEL*. Alternatively because expression of *TEL* under the *ANT* promoter occurs at a very early stage in embryo development it may be that ectopic expression of *TEL* at this stage embryogenesis, is more able to alter or interfere with the differentiation of the cells in this area.

While the beak like structures and callus like tissue were the most obvious effects more subtle phenotypic effects were noted. In the least affected ANT>>OP-*TEL2* seedlings, cell counts across the abaxial surface of the middle but not across the tip also showed an increased cell number approximately 1.5 -2.4 times that of the controls. In turn these cotyledons ranged from equalto 0.6 times the width of the empty vector cotyledons. Furthermore CLSM images showed that epidermal cells of this region were reduced in overall size but also had relatively normal histology suggesting they were able to differentiate relatively normally which was in contrast to the less differentiated cells observed at the distal tip. This in turn that while not all the cotyledons of the different lines possessed the beak-like structure they did show greater cell densities at least beyond the distal tip associated with ectopic expression. One element which is unclear is that the expression domain expected for the ANT promoter late in development of the cotyledon is within the internal layers of this organ. Consequently it is unclear whether the increased cell numbers and size are the direct result of expression of the OP-*TEL2* construct or an indirect or downstream consequence. Speculatively it would likely that it is a consequence of the early expression of the ANT>>OP-TEL2 construct in the cells of the incipient cotyledon, but this remains to be confirmed.

Nevertheless these observations are consistant with the literature regarding the effects of both misexpression and mutant alleles of certain genes thought to be associated with different facets of the cell cycle. The *an rot* single and double mutants (Tsuge et al., 1996) were reported as showing compensatory increases in cell number when cell expansion (and thus overall size) is reduced. Contrasting effects were reported for *Arabidopsis* plants containing mutant alleles of the Heterotrimeric G-Protein. The lines containing the putative null *gpa* mutant alleles were reported to have fewer larger cells in their hypocotyls and leaves, and this increase in cell size suggested to be compensating for the reduction in overall cell numbers(Ullah et al 2001). In contrast inducible misexpression of the G-Protein resulted in reduced growth correlating with both reduced leaf and cell size (Ullah et al 2001) creating plants similar to appearence to the EDS phenotype described in this work. Thus the increased cell densities, reduced size, and proliferation of undifferentiated cells at the tips of the cotyledons of the ANT>>OP-*TEL2*

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crosses appear consistant with TEL2 having a role in promoting active division versus cell differentiation in these organs.

Previously the role of KNOX genes in plant development, (i.e. STM and the KNAT genes) have been mentioned in the context of promoting meristem function, specifically the proliferation of indeterminate cells in the central zone (Hake et al, 2004). Aspects of the ectopic phenotypes described here resemble that described, for example, of overexpression of the Class I KNOX <u>N</u>icotiana <u>t</u>abacum <u>h</u>omeobox (NTH) genes in Tobacco (Nishimura et al, 2000). Given the general similarities of the described expression domains of KNOX and *TEL* genes, one possibility is *TEL* and the KNOX family may be different parts of the same pathway/s. The observations of the proliferation of callus like tissue in various locations, even the delayed elaboration of leaves under *TEL* ectopic expression, is consistant with what would be expected in plants where cell proliferation is favoured over differentiation.

4.6 The Floral promoters

Three floral promoters were selected for crosses with the OP-*TEL* constructs, being p*APETALA1*, *AP3* and *AGAMOUS*. Unexpectedly it was noted that crosses with the promoters of *AP1* and *AP3* had additional ectopic expression of GUS in the cotyledons, the first true leaves and also the cauline leaves of the floral bolts. Subsequently it was found that this represented unreported domains of expression for these promoters, based on unpublished microarray data (M. Yanofsky, D. Weigel *pers.com* and see §3.5.2).

The most unexpected observation was that despite vivid staining in the floral domains no direct histological effects were observed in the floral organs (i.e. petals, stamens and gynoecium) of either the F1 or F2 generations of these crosses. Instead the phenotypes observed were associated with the alternative early expression under the *APETALA* promoters in the cotyledons and rosette and cauline leaves. The only exception to this were the siliques or gynoeciums of F1 seedlings from the AG>>OP-*TEL2*-4 cross. The plants were smaller than their GUS negative siblings, and their siliques appeared marginally shorter and more compact, although direct measurements were not made. In principle this seems inconsistent with the expected expression domains by this promoter (Coen and Meyerowitz, 1991; Mizukami and Ma, 1997). It is unclear why

ectopic expression of OP-*TEL2* in this area should apparently affect gynoecium formation (creating shorter siliques) rather than that of the carpels and petals.

Based on the lack of phenotype observed in the floral organs of seedlings under all the floral promoters (and the inducible ubiquitous expression lines in the next section) at this stage it seems reasonable to conclude that *TEL* function are not associated with either floral meristem or organ identity, although there may be downstream effects in the siliques themselves. The possibility of more subtle cellular effects in the floral domains is discussed later in this section.

In contrast a variety of phenotypes were observed in the cotyledons and true leaves, which seemed comparatively responsive to the presence of ectopic *TEL* expression. The phenotypic category most frequently observed in the cotyledons/leaves of the AP>>OP-*TEL* crosses was Ab.Cot and CLT respectively. However, as few lines were successfully crossed to the floral promoters, and the nature of these phenotypes was similar in nature to that seen in the ANT>>OP-*TEL* 2 crosses, no further investigations were undertaken. There was however a fourth phenotypic category which is worthy of note being the Tendril-like leaf phenotype, which was observed primarily in F1 and F2 seedlings of AP1/AP3 crosses with the OP-*TEL1*-1 line, and subsequently in the F1 seedlings of both AP1>>OP-*TEL1*-2 and ANT>>OP-*TEL1*-2 . In general, the precise appearance and penetrance of the phenotype varied according to the promoter and generation. For example, tendril-like cauline leaves were noted in two F1 seedlings of the AP1>>OP-*TEL1*-2 cross, which lacked a subtending floral inflorescence. In two F1 seedlings of the ANT>>OP-*TEL*1-2 cross, narrowed cauline leaves with tendril like extensions from their abaxial surface at the distal tip were observed. The F2 seedlings of the AP1>>OP-*TEL1-1* cross produced narrow strap-like leaves and did not flower whereas the F2 seedlings of the AP3>>OP-*TEL*1-1 cross produced tendril like leaves which appeared to have a midrib but little or no lamina. Similar phenotypes to this category (i.e. narrow leaf/tendril leaf phenotype) had previously observed amongst putative *TEL2*::GFP lines, and promptly excluded from further analysis on the basis of their unusual morphology and low viability. Taken together this implies that ectopic expression of primarily *TEL1* under either *APETALA* promoter interferes with the normal expansion of

the lamina in some or all leaves. Strap-like or tendril like leaves were not observed in any of the *ANT>>OP-TEL2* crosses, and this is despite the previous observation of 35S:*TEL2* lines with this appearance (although they themselves were rare). Nor were strap-like leaves observed in any of the inducible OP-*TEL1* or *2* lines. This could be interpreted to mean that the tendril-like leaves are a function of domain specific expression specific to that seen under either *APETALA* promoter. Alternatively it could be argued based on its low frequency in the large number of 35S:*TEL2* T0 lines identified, and absence from inducible lines or domain specific OP-*TEL2* crosses, that it is in reality a position effect, an artifact of the location at which the OP-*TEL1* construct has inserted.

The only counter to this latter possibility is the observation of the tendril cauline leaves in the *ANT >>OP-TEL1-2* and *AP1>>OP-TEL1-1* and *1-2* F1 seedlings. Under the assumption that any construct is inserted randomly into the plant genome during transformation it is unlikely that two lines could insert randomly to create the same position effect. It is still however possible that the tendril leaf phenotype is the result of interactions not related to the normal function of *TEL*, created only by its presence in a domain that it does not normally occupy. Therefore while the phenotype is itself intriguing, more OP-*TEL*1 and 2 crosses are required in order that it can be confirmed that this phenotypic category is not the result of unrelated interactions or a position effect.

It is difficult to conclude much about the function of *TEL* in the floral domains given the comparatively few OP-*TEL* lines crossed to the AP and AG promoters. Nevertheless two main conclusions can be inferred from these experiments. Firstly there is a common theme, especially if the observations made in the ANT>>OP-*TEL2* crosses are included. Early ectopic vegetative expression, rather than floral expression, leads to obvious phenotypes, which are generally associated with reduced histodifferentiation in the leaves and cotyledons as well as slowed growth. Finally there is no evidence that ectopic *TEL* expression in specific areas within the flower or stages in floral development can alter the observed arrangement of the whorls or their organogenesis regardless of the stage at which the floral promoter is active. The only evidence that there may be some effect were the observations

outlined for the AG>>OP-*TEL2* T0 crosses, in particularly the siliques. The latter naturally require further investigation but in generally no obvious phenotypes were seen in the floral organs themselves.

From this it would therefore seem that the developemental sequence of the floral buds is not in itself affected by ectopic *TEL* activity. This may mean that the *TEL*'s have little or no role in the developmental processes in the flowers. Alternatively the effects of ectopic *TEL* expression on the organs of the flower are vastly more subtle than that seen elsewhere. For example Kaur and colleagues (Kaur et al., 2006) recently reported that *aml* insertional mutant alleles and antisense constructs allele cause no obvious external effects but more in-depth investigation revealed sterility and other defects in gametogenesis. So while ectopic expression of OP-*TEL* might not appear to be obviously involved in either floral organogenesis or floral meristem activity more in-depth characterisation could reveal more subtle cellular effects within these domains.

4.7 The inducible expression constructs

The inducible expression driver pLhG4 (Craft et al, 2005) was used to examine ubiquitous expression of *TEL* in *Arabidopsis*. The expectation was that because ectopic expression driven by LhG4 occurs in the presence of the GR ligand, dexamethasone, that this would mean that T1 lines could be identified without the rigors of ubiquitous *TEL* expression, avoiding the problems encountered with the CaMV35S construct.

Subsequently the degree of histochemical staining by the reporter gene was used to identify potentially high level OP-*TEL*-S7 expression lines. RT-PCR confirmed that induction of the reporter gene was associated with expression of the *TEL1 / 2* transcript from the OP-*TEL*-S7 lines (but not the HTOP-G construct) in selected lines. Despite this, it was found that if seedlings were transferred to DEX containing media, or sprayed immediately after germination in a glasshouse situation, no obvious morphological phenotypes comparable to those described in the domain specific crosses or indeed the empty vector and uninduced controls were seen. Furthermore no morphological defects were seen in the structure of the flowers of seedlings of the inducible lines despite persistant spraying.

Instead it was found in the glasshouse experiments that a more subtle phenotype was present in the developmental progression of these lines which

was apparently in line with that seen in the 35S:*TEL2* lines. When spray induced plants were compared to empty vector and uninduced lines there was no discernible differences in leaf count, but the emergence of the floral buds was found to be delayed and similarly the elongation of the floral bolt was slower, in expression lines. For example in the case of the former the majority of the induced OP-*TEL* lines had evident floral buds (index value of 1) by days 12 (OP-*TEL*1-24) or 13 (the remainder) compared to day 10/11 in the empty vector and days 9/10 in the uninduced lines. In terms of floral bolt elongation, on day 17 the majority of bolts in the induced expression lines were in the range 0.5 cm – 1 cm (i.e. an index value of 2-3) on day 17 while the majority of EV seedlings were in the range of 1.5-2.0mm (index value of 6). Because these values are derived from rough measurements and general observations it is not possible to put too much weight on these results. It is reasonable however to infer that the length of time before flowering is prolonged in these expression lines under induction, as described previously in other lines.

However because there were no morphological effects on seedlings at any stage and the degree of developmental delay was not as extreme, (as in the 35S:*TEL2* lines) it is reasonable to ask whether this observations are infact significant and not the result of random factors. This may infact still be the case but it is also consistant with the conclusions made with the ANT>>OP-TEL2 lines i.e. that the extent of phenotypes produced relates to the timing of induction and thus the developmental stage when ubiquitous *TEL* expression commences. Where OP-*TEL*-S7 seeds were germinated on media containing DEX, this evoked a range of morphological effects comparable in general to the ANT>>OP-*TEL2* crosses. This included the presence of the beak-like structures like that described for the Ab.cotyledons, and Extremely delayed seedlings. In contrast callus-like tissue (as seen in the EDS catergory), or the tendril-like / narrow leaf phenotypic categories were notable by their absence in inducible lines of either OP-*TEL* construct.

This infers that induction of ubiquitous expression of *TEL* must occur before a certain stage of development is reached in order to evoke indeterminate organ development in seedlings. Induction at any point after this stage, such as post-germination, acts only to retard growth and leads to considerably less striking effects. Moreover it may be inferred that the absence of callus like tissue and tendril like leaves described in previous

sections are features primarily associated with the domain specific expression of *TEL* as opposed to ubiquitous expression throughout the seedling as alluded to in § 4.5. It is also pertinent to note that this point that while ANT>>OP-TEL2 seedlings bear some resemblance to inducible GPA-1 lines, inducible OP-TEL2 lines do not. Consequently it would seem that there are distinct differences in the apparent effect of TEL depending on its spatial and temporal dimensions.

Ubiquitous expression in the inducible lines was also associated with a feature not seen in the domain specific crosses. This was the observation of shorter roots in expression lines under induction. Measurements of root growth rate showed that the OP-*TEL*-S7 seedlings roots under induction grew at approximately half the rate observed in the control lines from day 4. By day 10 the growth rate ranged from zero to 1/4 that of the empty vector line. Consequently by 10 DAG the mean length of OP-*TEL-S7* roots growing in DEX containing media, were between a third to just over half (55%) the length of the OP-GUS lines (Table 3.7 pg 153).

This had not been observed in any of the domain specific expression crosses although histochemical staining had been noted in sectors of the roots in both floral and ANT>>OP-GUS control lines. Crucially staining was never observed in the RAM and so it appeared that the shorter roots were associated with the ectopic expression of *TEL* in the RAM itself.

Arabidopsis mutants such as *fasciata*, *tonsoku* and *hydra/fackel* (Kaya et al., 2001; Schrick et al., 2002; Souter et al., 2002; Suzuki et al., 2004) are examples of lines with characteristically short roots. Furthermore the morphology of the roots of these mutants does resemble (to variable extents) that observed for the more extremely affected seedlings of the inducible OP-*TEL* lines. In these and other such mutants, the shorter root phenotype has been found to be associated with defects in the radial organisation (and tissue identity) within the RAM and subsequent domains. The mutant seedlings of *fackel*, for example, have additional cell files in the root itself, as well as atypical cell divisions, and disorganised apolar cells within the RAM. Thus, the shorter length of the root might be the result of malfunctions within the RAM that lead to disorganised radial patterning and defective cell identity.

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When the roots of OP-*TEL*-S7 lines were examined for similar defects they were not found. Confocal optical sections did not show any evidence of additional cell layers or atypical cell divisions in either the RAM or in subsequent domains of these roots. However because sections with clearly identifiable central cells in either control or expression lines were were also not found defects in cells of QC or RAMmay have been present but not recognized as such.

Beyond the root meristem, more obvious differences were noted in the morphology of cells in the roots of OP-*TEL* lines (compared to control lines) compared to that observed in the empty vector lines. In the region of the root known as the zone of differentiation, the cell files established in the meristematic region and zone of cell division begin to differentiate to form distinct tissue types, such as the endoderm, cortex and epidermal layers (i.e. (Howell, 1998)). In the HTOP-G lines these layers were histologically distinct and clearly present, while in the OP-*TEL* lines, the distinction between these cell layers was considerably less obvious.

This effect would appear to be similar in nature to that described for the beak like structures associated with the cotyledons, i.e. the accumulation of cells showing reduced histodifferentiation in the cotyledons of the ANT>>OP-*TEL*2 crosses. As noted previously the cells of the beak structures also appeared to be smaller, so it was possible this was the case in the roots as well. However because of the difficulties previously mentioned regarding the angle of section, it was not possible to accurately measure their length or determine differences in volume. Nevertheless, measurements of width across the region of reduced differentiation in a subset of root samples did show they were narrower in the expression lines compared with the empty vector lines. This contrasted with the observation that the width of the region of the RAM was not different to that of empty vector.

It is generally accepted that root length is largely determined by the degree of expansion of cells as they leave the meristematic region (Howell, 1998). Consequently, apart from reduced differentiation, the reduced length of these roots is most likely to relate to reduced cell expansion outside the RAM a possibility supported by the reduced width of these roots. It is not possible at this point to say whether for example rates of cell division are affected either in the RAM or outside it in the presence of OP-*TEL* expression. Evidence of

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this sort of effect i.e. total number and volume of cells in the different zones which are obvious indicators of both changes in cell division and growth, remain to be quantified. Nevertheless it does seem reasonable to conclude that as observed in the domain specific crosses, that inducible ectopic expression of either *TEL* gene in either the RAM or SAM is capable of slowing overall growth as well ascellular differentiation, and moreover that this effect is most pronounced when the ectopic expression includes the meristematic region itself. Foreshortened roots along the same lines were not described in any of the domain specific crosses which implies that the effect is related to the ectopic expression of *TEL* both within and beyond its normal boundaries in the root..

4.8 Developmental progression of ectopic expression constructs

The first set of domain specific expression lines characterised showed a strongly delayed developmental progression, as well as a variety of phenotypes. Aspects of this delay were manifested in the extreme by the ED seedlings which were very slow to produce a floral bolt. However there were many seedlings of the F1 generation which did not show characteristics of the phenotypic categories, while others after producing abnormal cotyledons did not subsequently vary qualitatively from the empty vector in their subsequent development.

Given the previous observations in the T1 35S:*TEL2* lines and the inducible lines it seemed possible that there was a more subtle underlying effect uniting all the F1 domain-specific expression seedlings. In order to observe this effect a quantitative assay of developmental progression was used. In this context progression was defined as the percentage of seedlings, within a group, which reach a defined developmental landmark. The general result from these experiments was that the percentage of OP-*TEL* seedlings at a particular developmental landmark, at any given time point, was significantly lower relative to the empty vector. This was most evident in the *ANT*>>OP-*TEL2* lines but was also seen in the floral promoters.

Because seedlings of the narrow leaf phenotype never flowered, this prompted the question as to what extent flowering time was delayed overall. This was assayed only in the *AP3* crosses and in this case the developmental

landmark was the visible emergence of the floral buds. Significant delays were apparent in both the formation of leaves and the presentation of the floral bolts in all the *AP3>>OP-TEL* crosses which confirms the association of ectopic *TEL2* expression with a delayed onset of flowering. However this delay did not lead to the production of more leaves. The same number of leaves was produced but over a longer period (data not shown).

While the assay of developmental progression used was indirect and amounts in essence to an estimate of developmental progression, there does appear to be a relationship between growth of the plant, presence of ectopic expression, and the delays observed in general developmental progression. At the very least this is evidence which correlates with the observations made in the 35S:*TEL2* lines, that ectopic expression of TEL2 significantly slows overall growth and emergence of leaves.

There is also precedent in the reported phenotype for members of the Arabidopsis Mei2-like or AMLs. Characterisation of quintuple *aml* mutant lines has shown that they have a reduced time to bolting (Anderson and Hanson, 2005) consistant with an acceleration of developmental progression. This is the corrollary of the observations in this work of both delayed flowering and delayed leaf specification described for ectopic expression of *TEL*, in that it appears this retards both the differentiation of individual cells and the development of the whole plant. Overall the delayed elaboration of leaves in the inducible lines, is consistent with the conclusions from the observations in the domain specific expression lines, specifically that they are the result of increased overall cell division, versus differentiation.

4.9   A role for *TEL* in repressing cell growth and differentiation?

In the introduction the maize gene *TERMINAL EAR1* was introduced as being thought to be associated with the regulation of both the position and timing of leaf formation (Veit et al., 1998). It was hypothesized that the *TELs* might have some degree of conservation of function with *TE1* and therefore repress or regulate the differentiation of cells within the SAM, RAM and the Floral meristems.

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The main method by which this hypothesis was tested was investigation of the phenotypes associated with ectopic overexpression and misexpression. This is because attempts to express the intact protein recombinantly were not successful, so direct investigations of the protein function were not possible.

Initially the CaMV35S promoter was used for *TEL2* overexpression and this generated a variety of extreme phenotypes, as well as the identification of the slow midget phenotype in the first generation. The significance of this phenotype could not be investigated because it all but disappeared from the succeeding generation. This problem was overcome by the use of the LhG:pOP constructs for ectopic expression.

Once more a spectrum of phenotypes was observed but this time it was possible to identify specific categories of phenotype within populations of F2 seedlings. Initially a subset of the F1 seedlings from the ANT>>OP-TEL2 crosses were identified with sectors of tissue composed of undifferentiated cells on their leaves. In the next generation a number of phenotypic categories were observed associated with these undifferentiated cells in the cotyledons, less often with their true leaves and not at all in the floral inflorescences. Ectopic expression under the *ANT* promoter in the cotyledons and early leaves was associated with sectors of in indeterminant cells development as well sloweroverall growth within these organs. The most extreme manifestation of this effect was the Extremely delayed (ED) seedlings which were characterised by the proliferation of callus-like tissue (usually at the apex) and the absence of cotyledons. Even in the least affected seedlings, the cotyledons possessed (or were replaced by) accumulations of cells lacking evident histodifferentiation. Furthermore these seedlings characteristically do not produce true leaves for up to at least 28 days after germination.

Thus it seems that prolonged expression of *TEL1* and *2* in the incipient cotyledons from a very early stage of development interfered with the subsequent differentiation of these cells to varying extents.
The precise effect observed in this later case depended on whether the expression of the relevant construct was domain specific or throughout the seedling. In both inducible and domain specific lines cotyledons often produced beak like structures composed of a small number of undifferentiated cells at the distal tip of the cotyledon (or cotyledon like structure), or located at the position where the cotyledon ought to be. The early expression of *TEL* at

the location of the incipient cotyledon (from the globular stage in embryogenesis)therefore appears able to impede the differentiation (to various extents) all or some of the cells initially recruited to the incipient cotyledon. For example vasculature elements are visible extending into the beak like structure in Figure 3.20 (pg 138) so it appears some differentiation has occurred. The nature of this impediment and what cells are affected is unclear.

However the sectors of undifferentiated cells in the beak like structures, as well as the the accumulation of callus like tissue shown in the examples of the EDS is suggestive that *TEL* acts to promote cell proliferation against cell differentiation. This speculation is given some foundation by the observations, regarding increased cell counts associated with overall reduced cotyledon width which is consistant with the observations mentioned earlier regarding overexpression of GPA-1 (Ullah et al, 2001).

In this work the authors found that ectopic expression of the G protein increased cell numbers and decreased cell size in leaves. Furthermore seedlings induced to express GPA-1 constitutively were dwarf plants with slow growth and initiated multiple meristems at their apex. These plants therefore bore similarities to that observed in the equivalent experiments in this work with the notable exception of multiple ectopic meristems at the apex. There is some evidence that overall the domain specific lines were have much slower elaboration of leaves, and in the AP3>>OP-*TEL* constructs, delayed emergence of the floral buds. This result was also consistant with initial observations in the 35S:*TEL2* T1 lines and replicated in the spray induction trials described earlier. Finally further corrobative evidence comes from the inducible OP-*TEL* seedlings which were germinated on Dexamethasone. Apart from some similarities in phenotype the prime observation was a much reduced root length which in turn was found to be associated with reduced tissue differentiation in these roots.

When Ullah et al (2001) expressed *GPA-1* in synchronised cell cultures they found that its primary effect was to shorten the duration of the time between the end of one cell division and the beginning of the next (G-S1). Thus they concluded that the observed phenotypes of the ectopic expression of GPA-1 were the result of generalised and increased active cell division within

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the plant as a whole causing both the reduced statue, slow growth and increased cell numbers.

In the case of *TEL* there does seem to be some evidence that the ectopic expression is associated with increased cell proliferation, versus differentiation comparable to that observed with *GPA-1*. The similarities inferred with G protein and the overexpression phenotypes of KNOX genes suggest that the function of *TEL* may involve promoting or regulating indeterminate cellular growth versus organogenesis just as was described for *TE1*. The recent analysis of the rice homologue of *TE1*, *PLASTOCHRON2* (*PLA2*) (Kawakatsu et al., 2006), provides further evidence in this regard.. Overall, the *pla2* mutant was found to have accelerated cell and tissue differentiation that is associated with a considerably reduced plastochron length. This suggested to the authors that the normal function of this gene includes the timing of organ initiation and subsequent formation. This is similar in nature to the observations made by Anderson et al regarding the quintuple *aml* mutant line (Anderson and Hanson, 2005) and thus represents a corollary of the findings outlined in this work. However whether ectopic expression of the OP-*TEL* constructs does infact affect plastochron length remains to be determined.

The mechanism by which the TEL genes might exert this effect might be speculatively inferred from reports of other mutant lines which phenocopy aspects of those observed in the ectopic expression lines. One of the best examples is the double mutant of the *SERRATE* (*SE*) (Prigge and Wagner, 2001) and *FASCIATA* (*FAS*) loci. Both *FAS* and *SE* are thought to involved in the aspects of the regulation of chromatin remodelling (Kaya et al., 2001). Seedlings of this combination have a range of phenotypes, (depending on the allele), but predominantly they have narrow leaves, floral defects and pronounced proliferating callus like tissue in the apical regions (Figure 4.1 next page). The authors stated that the 'synergistic interactions' of the *se/fas* double mutant most likely represents the convergence of two different pathways associated with the regulation of chromatin structure (Prigge and Wagner, 2001) associated with organogenesis. Apart from the floral defects there do appear to be many similarities to the effects observed particularly in the domain specific expression lines.
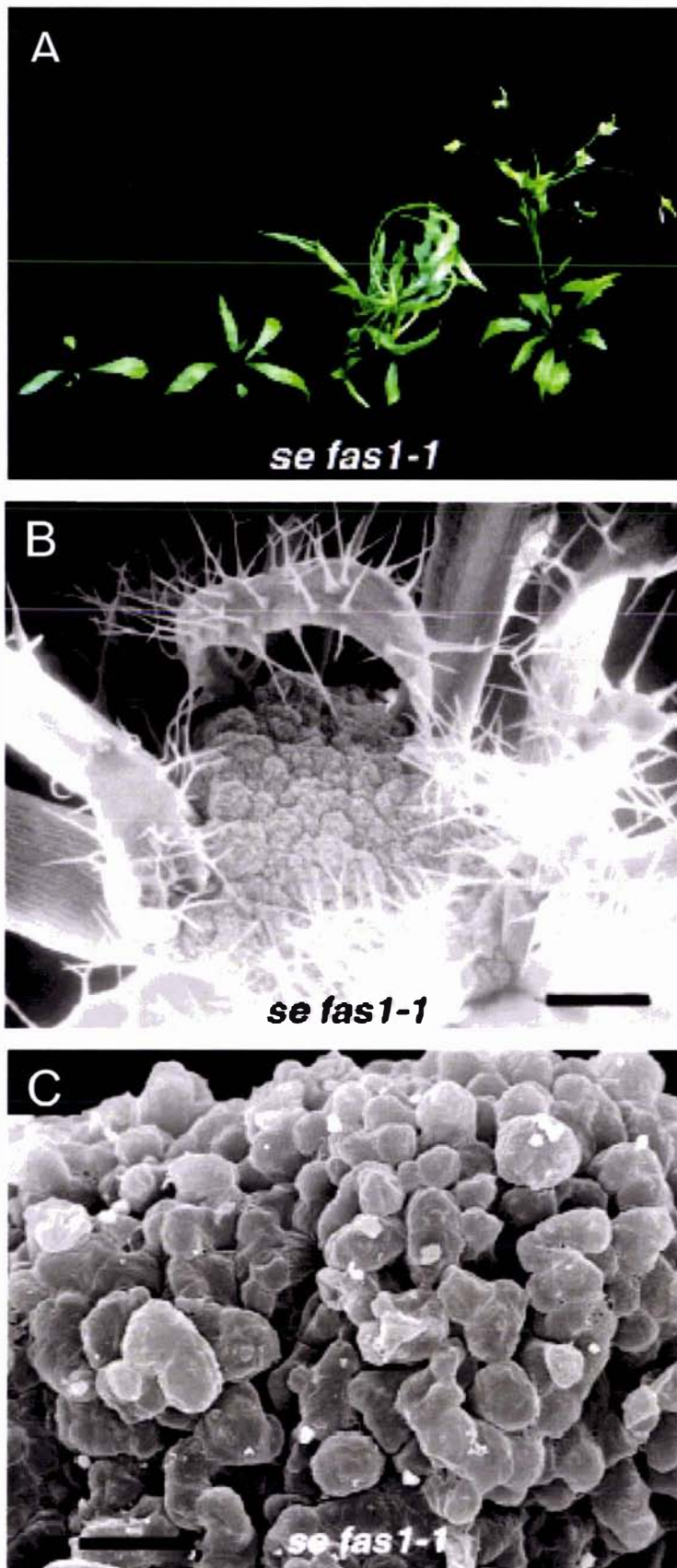
Fig 4.1 Phenotypes of *se fas 1-1* double mutants. (reproduced from (Prigge and Wagner, 2001)) with modifications. **(A)** Range of phenotypes exhibited by ~28 day old se fas 1-1 double mutants. Note the narrow leaves and that these plants exhibit arrested growth at different developmental times. **(B)** and **(C)** Scanning Electron micrograph of arrested apex of ~30 day old seedling, composed of callus like tissue (cf. Fig 3.15 pg 110). Bar in **(B)**=~500µm; bar in **(C)** =20µm.

Other examples of mutants which resemble the effects of ectopic *TEL* expression include hormonal mutants such as *detiolated-2*(Fujioka et al., 1997), *diminuto* (*dwarf-1*)(Klahre et al., 1998), the *pasticcino* family (Faure et al., 1998) and *shrink-1D* (Takahashi et al., 2005). The specific similarities include reduced stature of the seedlings, the shortened root, slow growth, as well as patterning defects in the cotyledons. Of these four mutants, those of the *PASTICCINO* family of genes most strongly resemble the ED seedling phenotype, with 'vitreous' leaves and finger like and beak like projections.

Assumin that there is a link between these genes and the activities of ectopic *TEL* expression then the implication is their function may involve, or be upstream of the activities of a gene or genes that are involved in the integration, or regulation, of many different pathways associated with cell differentiation, including the response of cells to hormones. Furthermore it would imply that this regulation might be exerted through active chromatin organisation via interactions with RNA, such as RNA directed silencing(Lippman and Martienssen, 2004) and in particular shutting down genetic programmes associated with differentiation. There is precedent for this possibility based on recent findings regarding the function of Mei2p. It has previously been mentioned that migration to a set location in the chromosome is a feature of the function of Mei2p (Shimada et al., 2003). Furthermore the specific destination in the chromosome has been identified as the *sme2* locus that encodes meiRNA a short non-coding RNA which acts as a signal to recruit the MEI2 protein into the nucleus. Although it is currently unknown what Mei2 does at this site, this nuclear migration is required for the switch to meiosis and one possibility is that it is required to promote chromatin remodelling to allow Meiosis I to begin (Hirota et al., 2004).

Another feature of the phenotypes of ectopic *TEL* expression which points to an association with chromatin structure which has been alluded to earlier in this chapter, is the pronounced sectorisation / variability of phenotype described for domain specific *TEL* ectopic expression i.e. the sectorised callus-like tissue and abnormal cotyledons. This is similar in nature to a mechanism identified in other systems. This is the phenomenon known as 'Position Effect Variegation' or PEV. It was first described in *Drosophila* and latter in maize (reviewed in (Lippman and Martienssen, 2004)). In *Drosophila*

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PEV is apparent as the juxtaposition of albino against pigmented sectors in the eye, giving them a mottled appearance. This was found to be the result of differential downregulation of pigmentation during eye formation of *Drosophila*. Subsequently a similar effect was identified in maize via transposon mediated variegation in maize. In essence PEV is believed to be the result of differential regulation of the formation of heterochromatin within the cells of these sectors The role of heterochromatin is controversial but is now becoming more generally accepted as being important in the process of RNAi and in particular the RNA directed silencing of specific genetic programs during development of animals, plants and fungi (Lippman and Martienssen, 2004). The processes of chromatin remodelling, methylation and histone modification are also believed to be part of what is known as RNA dependent heterochromatic silencing, since it requires the presence of siRNA to reach its targets on the chromosomes. The inference is therefore that the sectorisation is the result of RNA silencing within specific cells of a leaf primordium promoted by the presence of either *TEL* and in particular that this silencing leads to or directly prevents cells from undergoing differentiation relevant to their position in the organ and/or plant overall.

There is also evidence in the literature for the activity of RRM proteins in chromatin remodelling. Other RRM proteins have been reported as executing aspects of their function through chromatin remodelling. The conserved C-terminal region of FCA has been shown to be able to interact with At SWI3B (Sarnowski et al., 2002). This is one of four *Arabidopsis* homologues (AtSWI3A-D) of the Swi/Snf family, core proteins of the chromatin remodelling complex of *Schizosaccharomyces pombe*. It has therefore been suggested that the regulatory activities of FCA in the autonomous pathway of flowering involve RNA directed silencing through chromatin modification (Sarnowski et al., 2002).

In conclusion there appears to be some evidence that the hypothesis postulated regarding the function of the *TEL* genes is associated with the repression of differentiation. They do appear able when expressed at an early enough stage in the embryo's development to repress cell specification (admittedly to variable extents) in favour of cell proliferation except it seems in the floral domains. The nature of the observed phenotypes i.e. the

sectorisation, and the characterisation of mutant lines which are similar in appearance or are related, provide evidence that the normal function of the TEL's is to repress differentiation and that this might be associated with chromatin organisation.

Any mechanistic model based on these results is however doomed to be pure speculation at present. This is because the basis of the fore-going conclusion is the ectopic expression data which predominantly describes the effects observed from expression of the gene outside its normal domain of activity. A valid question raised by this would be whether these phenotypes represent a toxic or abnormal effect of the overabundance of an RNA binding protein in an inappropriate location especially since it is unclear whether the appropriate cognate RNA would actually be present outside the normal expression domains of the *TEL* genes. In essence a major hurdle in accepting the foregoing conclusions is whether these morphologies are actually the result of the normal function of *TEL*.

As has been mentioned previously it is possible that *TEL* genes are involved in miRNA metabolism. It has been reported recently that outside the SAM the regulation of the domains of expression of certain genes associated with the processes of organogenesis and morphogenesis of leaves (for example CUC 1 and 2 (Kidner and Martienssen, 2003; Laufs et al., 2004)) are the result of miRNA directed degradation of their transcripts. It is possible that the early ectopic expression of *TEL* in cotyledons or leaf primordia might interfere with this normal regulation in a fashion that is not related to its normal role in the apical meristems, but nonetheless drastically alters the subsequent differentiation and development of its ectopic domain. For example the tendril like leaves described for the *APETALA*>>OP-*TEL* crosses may represent interference with the metabolism of the target RNA of CUC1/2.

The literature was cited reguarding the possibility of RRM proteins such as encoded by *TEL1/2* being involved in chromatin reorganisation. Equally however there is very little in the literature associated with plant development which provides any precedent regarding the effect of ectopic expression of RRM proteins outside their normal domain. Infact very few RRM type plant RNA binding genes have been functionally characterised using overexpression analysis, and there is little specific information as to whether such non-specific toxicity in overexpression of RRM binding proteins does infact occur in

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plants.In the literature there are only two reports regarding RRM RNA binding genes which have been functionally characterised in *Arabidopsis* using overexpression and these are FCA and FPA (Macknight et al., 1997; Schomburg et al., 2001). While neither gene was reported to generate any morphological phenotypes resembling for example the CLT or leaf tendrils resulting from overexpression, examples were noted by Richard Macknight of small plants with crinkley leaves (Macknight *pers.com*). The primary effect of overexpression described was related to delays in flowering.

There are, unfortunately, specific examples relating to interference in normal RNA metabolism do exist in both yeast and plant systems. Overexpression of the RBD of *Srp1p* in its yeast host interfered with the intronic processing of pre-mRNA transcripts. This led to the accumulation of preRNA which caused cessation of growth (Gross et al., 1998). Similarly the overexpression of a transgene in the chloroplasts of tobacco, (deliberatively constructed to be defective in its pre RNA processing) caused severe developmental defects in these seedlings including reduced size (Hegeman et al., 2005). It is therefore possible that overexpression of the *TEL* genes might cause non-specific defects in RNA metabolism relating to specific genes and stages of development and this might be an alternative explanation for the generalised reduction in cell expansion and growth as well as the indeterminate seedling morphologies.

Determining the normal function of these genes in their normal domains remains a considerable obstacle to any conclusions regarding this data. Nevertheless it is clear that the effects described are significant and limited by unknown factors to specific parts of the seedling and its organs. This in itself argues that these phenotypes are not impertinent or artifactural, and therefore deserve further investigation. This will be discussed in the next section.

4.10 Future directions

There are a number of observations from the current work which could form the basis of immediate experiments. It has been noted that only the domain specific expression crosses produced the abundant callus like tissue, or

highly disorganised plants (i.e. Figure 3.11/13) so it seems this latter feature required restricted expression in order to be generated. However given that the expected expression of the construct was localised to the incipient cotyledons, and throughout the subsequent organs, it might be asked as to why it was the shoot apex that produced callus like tissue, and why also did leaves sometimes have sectors of callus like tissue and sometimes not. Detailed investigations of the callus like tissue in these different locations both histologically and by *in situ* hybridisation might begin to address this question as it would be possible to look at whether these cells are infact complete undifferentiated histologically and what identity they are expressing.

Another element which might contribute to more answers regarding the spectrum of phenotypes observed is examining the level of expression achieved in particular crosses. As noted in the previous chapter the frequencies of GUS staining in specific crosses indicated that most of the OP-*TEL* lines contained at least two unlinked reported constructs. It is likely that this also contributed to the variability observed in the phenotypes of these crosses. However this might also mean that the potential level of expression of the OP-*TEL* constructs in these lines could be higher than was achieved in the inducible lines and therefore able to promote dramatic effects. The levels of expression of the OP-TEL cross relative to the inducible lines need to be determined particularly in the cotyledons in order to make sense of the contrasting phenotypes observed.

As stated earlier one challenge of future work in this regard will be establishing that the normal function of the *TEL* genes within its normal domain is related to that already observed under ectopic expression outside it. The traditional approach to answering this sort of criticism is overexpression of the intact cDNA, within a background containing a null allele of the gene of interest. If the overexpression of the transgene compensates for the null allele then the transgene is assumed to be executing its 'normal' function. Direct complementation of a double *TEL* mutant would be challenging given that (to date) mutant alleles have no obvious phenotype. However the descriptions of mutant alleles of PLA2 and AML1-5 provide some indication that the ectopic phenotypes are as might be expected.. Furthermore an alternative approach,(pending an in-depth characteristion of the double *TEL* mutant lines)

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would be to complement the multiple AML mutations by crossing the quintuple mutant line with both OP-*TEL*-S7 inducible expression lines (as well as domain specific expression crosses) to see if complementation occurs. This may provide an answer to the question of whether the phenotypes are infact related to the normal function of this gene.

An alternative possible approach would be microarray analysis associated with *in situ* analysis. The activity of the transcriptome could be investigated in inducible *TEL* expression lines and compared with non-induced and wildtype lines. This would be used firstly if there are genes whose expression is up or down-regulated in the presence of ectopic *TEL* expression. Depending on the nature of the genes identified by this analysis, this would very informative about firstly the function of these genes. For example it should be possible to assay whether the presence or absence of ectopic *TEL* expression leads to the upgrading or repression of genes such as STM or WUS versus wildtype genes. More specifically the expression those genes whose mutant lines were mentioned as being similar in appearance could be directly investigated. The identification of such effects would likely assist in determining whether *TEL*'s function is infact associated with genes in known pathways associated with meristem function or not. It might even give an indication as to whether there is an association with chromatin modifications or silencing.

The possibility that *TEL* may interact with multiple pathways has previously been alluded to. Biochemical characterisation of the *TEL* proteins is therefore crucial to understanding the function of these genes. The identity of cognate RNA/s for example must be addressed in order to understand the mechanism by these proteins act. Furthermore, given the possibility of its role in chromatin remodelling and/or non-coding RNA metabolism, the nature of any cognate RNA partner/s will be crucial in identifying the pathways in which these proteins may act as well important to corrobate results from for example microarray analysis. This work continues in the BV lab.

In terms of the phenotypes obtained from over-or ectopic expression of the *TEL* genes there is much that must be followed up. One consistent feature of the ectopic expression phenotypes was an overall delay in developmental progression which may relate to lengthening of the plastochron. Direct

measurement of the plastochron in inducible lines would confirm whether the timing of leaf initiation overall is affected, and confirm whether the results described for PLA2 and the multiple AML mutant lines are infact direct corollaries.

The results from the inducible lines are also a promising area for continuing studies of the effect of *TEL* expression particularly in the RAM but also the SAM. It should be possible to determine whether infact there defects present in the RAM, and whether there are reduced cell numbers and cell expansion in these roots compared to the empty vector. Moreover using resources available for the study of Arabidopsis development it would be possible conduct more elegant experiments to examine this. One example would be to examine whether ectopic expression of TEL changes the identity of specific cells within the cell files of the root. This could involve *Arabidopis* lines in which GFP is produced in specific domains of the root according to the specification of identity i.e. pSCARECROW:GFP in the root. These lines could be transformed with the inducible construct and then examined for visible changes in the expression of GFP within these roots. The effects of inducible *TEL* expression on cellular identity in these domains could then be examined.

Another variant of this experiment would be to create an inducible lines containing either *TEL* gene tagged with GFP.This construct could then be transformed into roots and when appropriate induced to visualise the localisation of the tag within cells of the RAM and elsewhere in the root.

In a different vein the inducible lines could be used to investigate the role of the *TEL*s in differentiation of primordia at the apex. In this case seedlings of the inducible expression lines would be cultured on the auxin inhbitor NPA (as described in §1.2.3 pg 21). This would create pins onto which DEX and auxin could then be administered together or separately to examine the effect on subsequent organogenesis. Ideally this system might reveal whether the *TEL* genes do have a role in the responsiveness of cells in the SAM to hormones and their subsequent differentiation.

Regarding the domain specific crosses there is much that could be done on existing crosses, and many other promoter>>OP-*TEL* crosses which could be created and which would be highly informative. For example continuing

investigation of the tendril leaf phenotype, using more OP-*TEL1* lines would be merited. Further crosses involving the promoter of AGAMOUS may be fruitful regarding the role of the *TEL*s in the siliques. Moreover investigations of the effects of *TEL* expression in the stamens would also be useful especially given the effects on gametogenesis recently described for the AML insertional mutants (Kaur et al., 2006).

There are a number of plant promoters which were not used in the course of the ectopic expression characterisation and which would be useful. Given the results observed with ANT, other promoters suggest themselves in future studies to enhance the conclusions already discussed. In this regard, the prime candidates are CLV (the promoter of CLAVATA), and promoters which direct expression in specific polar domains of the leaf such as pPHAB. The first would be expected to direct expression within a very limited domain within the central zone of the SAM, and thus provide an indication of the effect of overexpression of these genes within their normal domain. In contrast polar expression of *TEL* within the adaxial or abaxial domains would be a useful comparison to the results observed under expression driven by ANT. Furthermore these promoters would provide finer detail regarding the whether the effects already described for *TEL* expression are restricted to early stages of development, and whether highly localised expression might have a different effect from that in progressively less specific domains.

In conclusion although there appears to be evidence that *TEL* is involved in or regulates the balance between differentiation and cell division in plant cells the specifics of its role remain unclear. Further work should break into the unresolved complexities of the function of the *TEL* genes in the development of *Arabidopsis thaliana* and thus reveal insights into one of the most fundamental aspects of plant development.

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Appendix I:
Primers used in this work:

Primers used to amplify *TEL* cDNAs for insertion in expression constructs and expression analysis by RT-PCR. Red letters in primer sequence indicate the addition of an restriction site as indicated in final column. Where the restriction site modification overlaps with the beginning or end of the cDNA these codons are indicated by blue letters. Brown letters indicate the inclusion of putative 3'/5 UTR in the sequence of the primer.

Gene		Purpose	Direction	Name	Length	Tm	Sequence (5'->3')	modifications/comments		Description (**chapter**:page)
TEL2		cDNA	forward	*TEL2*-5'	31	76.19	AGGAATTCTCATGTCTGTCACCGGACCATTC	EcoR1 site		**2**: 13
		cDNA	reverse	ATE2-3'	29	80.3	CGGATCCTAAACGCCGCCGTTTTCGATTC	BamH1site; removes UAG		
		GFP	forward	N2-5	27	78.2	TGCCATGGGTGTCACCGGACCATTCAG	Nco I site		**2**: 27
		GFP	reverse	N1-3	25	78.4	TACCATGGCGCCGCCGTTTTCGAT	Nco I site		
		RT	forward	*TEL2*-RTF	20	43.3	CAAGTACACACACAGAAGCT	NA		**3**: 5
		RT	reverse	*TEL2*-RTR	16	46	AAGACCCTGGATACGA	NA		
TEL1		cDNA	forward	ATE1L	27	59.5	ATATCAACTCTGGTATTAATGGAAGAC	NA		**2**: 13
		cDNA	reverse	ATE1R	27	57.5	ACATTACAGATAACACCTAGAAAGATG			
		repair	forward	*TEL1*-SOE(F)	21	59.8	GAATCTCTGAAGGAATCGTG	Repairs 1bp mutation.		**2**: 28
		repair	reverse	*TEL1*-SOE(R)	20	53.8	GATTCCTTCAGAGATTCTCT			
		repair	reverse	*TEL1*Xho	20	56	TTCCTCGAGTCTTATTATCG	Patch repair		**2**:28
		TAP	forward	*TEL1*-FB	30	67.9	ggaagatctTTAATGGAAGACTCCAGACTT	BamHI site		**2**: 22
		TAP	reverse	*TEL1*-RX	28	64.2	gctctagACCTAGAAAGATGTTTCTCCT	Xba I site		
		promoter	forward	prm*TEL1*-5'	30	74.7	tacccgcggggAATAAGAGATTGAATTACG	Sac II site		**2**: 20
		promoter	reverse	pr*TEL1*Bgl-3*	27	67.7	gagatctccTGATATTTTCCGACGAAT	Bgl I site		
		RT	forward	*TEL1*-TAP-RTF	19	56.9	TGGATTTCAACAACAAGTG	NA		**2**: 33; **3**: 5
		RT	reverse	*TEL1*-RTR	20	45.4	CTAGCTTTTGACTGTACTTG	NA		