Molecular genetic analysis of plant *Mei2*-like genes

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Thesis Abstract

Molecular and genetic methods were used to analyse how a novel class of genes, plant *Mei2*-like genes may be involved in the regulation of morphogenesis in plants. The study specifically aimed to 1) further characterise maize *te1* (the first plant *Mei2*-like gene to be genetically analysed) and understand the morphological basis of the *te1* mutant phenotype and 2) analyse the function of *Arabidopsis Terminal Ear Like* (*TEL*) genes using expression analyses and reverse genetics strategy.

te1 maize mutants are initially characterised by abnormal phytomer formation and development. A more detailed morphological analysis shows that mutant plants 1) have smaller vegetative shoot apices than the wild type, 2) initiate leaves at a higher, more distal position on the apical dome and 3) have higher plastochron ratio. Molecular analyses of *kn1* expression pattern, a marker of leaf founder identity, show that dowregulation of *kn1* transcripts occur higher up the dome. Clonal analyses show that fewer number of leaf founder cells are recruited to form the leaf.

TEL1 and *TEL2* are expressed in distinct overlapping domains in the undifferentiated region of the shoot apical meristems during the embryo, vegetative and reproductive stages of *Arabidopsis* development suggesting involvement of these genes in regulating meristem development and subsequent maintenance. The distinct expression of *TEL1* in both the embryonic SAM and RAM raises the possibility of a unifying regulatory mechanism in the formation of the root and the shoot. The absence of *TEL* single knockout phenotypes supports the idea of functional redundancy. When the *TEL* genes were both knocked out, double mutant phenotypes show apical-basal pattern defects, ectopic production of numerous secondary shoots, production of numerous leaves and basic embryonic pattern defects such as deletions of apical and/or basal region of the seedling.

Results of this study support the hypothesis that plant *Mei2*-like genes are important in regulating morphogenesis in plants and that they are required in the early patterning of the basic plant body.

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Gene nomenclature

Genes and gene products are named as follows:

Arabidopsis

GENES are capitalised and italicised

GENE TRANSCRIPTS are capitalised and italicised

PROTEINS are capitalised

mutant plants are named after the gene, in lower case, italicised

Maize

genes are written in lower case, italicised

PROTEINS ARE capitalised, italicised

Mutants are named after the gene, in lower case, italicised

Yeast (*S. pombe*)

genes are written in lower case, italicised ie. *mei2*

Proteins are written with the first letter capitalised, not italicised as in Mei2p

Groups of genes

Mei2-like

TEL Terminal Ear-Like

Abbreviations

Amp	ampicillin
BCIP	x-phosphate/5- bromo-chloro-indoyl-phosphate
DMPC	dimethylpyrocarbonate
DEPC	diethylpyrocarbonate
DIG	Digoxigenin
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EDTA	disodium ethylene diamine tetra acetate
Kan	kanamycin
LB	Luria Bertanni medium
NBT	4 Nitroblue tetrazolium chloride
ng	nanogram
NaOAc	sodium acetate
NTP	nucleotide triphosphates
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RAM	root apical meristem
rpm	revolutions per minute
RRM	RNA recognition motif
SAM	shoot apical meristem
SDS	sodium dodecyl sulphate
μ	micron
°C	degrees Celsius

Chapter 1. An overview of pattern formation and morphogenesis in plants

Plants exhibit a diverse array of characteristic forms and architectures. How these are specified and elaborated remains one of the most basic questions in plant development. Morphogenesis starts during embryogenesis and continues throughout the entire life of the plant through the activity of specialised groups of cells, called meristems. Thus, to a large extent, insight into the basis for plant development depends on understanding of how meristems function, including how a small number of undifferentiated, dividing cells that serve as the ultimate source of all plant cells are maintained in an apical position and how derivatives of these cells become recruited to form specific organs and tissues.

To gain insight into the genetic mechanisms that control meristem function, we have begun to analyse a new class of genes, the plant *Mei2*-like genes. A role for these genes in regulating meristem function was first suggested by an analysis of the *terminal ear 1* (*te1)* gene of maize, defined by a mutant in which the timing and position of leaf initiation becomes irregular (Veit *et al.*, 1998). This chapter summarises the principles and molecular mechanisms underlying morphogenesis in plants with particular emphasis on the organization and function of the apical meristem and reviews earlier studies on the *terminal ear 1* gene. With this review as a foundation, this chapter concludes with consideration of the questions and aims of this thesis.

1.1. Pattern formation in plants

Early studies of pattern formation in plants involved surgical manipulations, anatomical observations and in *vitro culture* procedures (reviewed by Steeves and Sussex, 1989). Later, genetic approaches using marked cell clones have been used to study morphogenesis in a number of species, including the model plants maize

and *Arabidopsis* (Christiansen *et al.*, 1986; Poethig *et al.*, 1986; Bossinger and Smyth, 1996; Irish and Sussex, 1992). More recently, the extensive use of molecular and genetic analyses have enhanced our understanding of the molecular mechanisms involved in patterning events in plants (reviewed by Lyndon, 1998 and by Aida and Tasaka, 2002).

Unlike in animals where the body plan is largely established during embryonic development, the body plan in higher plants is set up at two stages: 1) during embryogenesis, when the basic plant architecture is established with the formation of the apical, basal and radial axes and the formation of the shoot and root meristems; 2) during postembryonic development, when the majority of the plant body is formed from the activity of specialised groups of cells called the apical meristems. Thus, the problem of pattern formation and morphogenesis in plants can in many respects be addressed by focusing on how the shoot apical meristem functions.

1.1.1. The basic body plan is set up during embryogenesis

The seedling embodies the primary organisation of the plant generated by embryogenesis. It consists of two growing regions situated on opposite terminal regions of the apical-basal axis. The apical region consists of the shoot apical meristem (SAM) that subsequently gives rise to the above ground parts of the plant, while the root apical meristem (RAM) is found at the basal pole, which is responsible for the production of root structures. Along this apical-basal axis are other seedling parts: epicotyl (shoot meristem + cotyledon), the hypocotyl and radicle (root + RAM).

The establishment of the apical and basal axis is believed to be a key early event in embryogenesis. Some apical-basal polarity is already present in the eggs of most seed plants even before fertilisation, where the basal end is located against the embryo sac wall, near the micropyle, while the apical end projects into the fluid filled embryo sac, which contains high concentrations of growth substances and metabolites (Lyndon, 1990). In *Arabidopsis*, this apical-basal polarity is consequently inherited when the egg is fertilised, wherein a large vacuole is located at the basal end of the zygote while a nucleus is located toward the apical end

(Mansfield and Briarty, 1991). The apical-basal axis in *Arabidopsis* embryo is manifested in the first division of the zygote, which divides asymmetrically into small apical cell and large basal cell. The apical cell gives rise to the majority of the embryo proper while the basal cell gives rise to the suspensor (Figure 1.1).

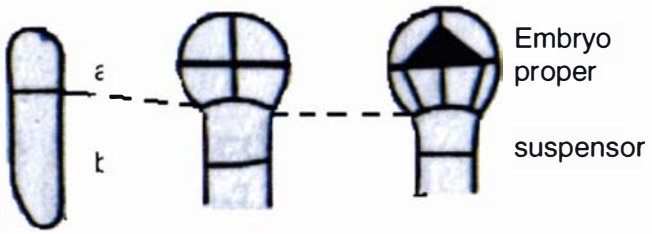


Figure 1.1. Cell fate specification in the one-celled embryo. Apical cell (a) gives rise to the embryo proper while the larger basal cell (b) gives rise mainly to the suspensor. (Source: Willemsen *et al.*, 1998)

In some plants, such as *Arabidopsis*, cell division of the zygote is highly regular (West and Harada, 1993) but much less so in others, such as maize and cotton (Poethig *et al.*, 1986; Christianson, 1986). Yet, all angiosperms progress through a similar series of morphological stages, arguing for the importance of positional rather than lineage based information in establishing plant form.

Some clues to how the basic body plan is established during embryogenesis have been offered by the characterisation of mutants that are altered in different aspects of seedling organization (Jurgens *et al.*, 1991; Mayer *et al.*, 1991). Mutations that lead to deletion of specific apical-basal pattern elements were traced back to defects in early embryos. Analyses of a variety of embryonic pattern mutants and clonal analyses have established that groups of cells in the early embryo make predictable but not invariant contributions to the initial plant form

suggesting early patterning mechanisms (Jurgens *et al.*, 1991; Irish and Sussex, 1992).

One of the early patterning genes identified in *Arabidopsis* is the *GNOM/EMB30m (GN)* gene, required for establishment of the normal apical-basal axis. Phenotypic analysis of loss of function mutants suggests that *GNOM/EMB30 (GN)* promotes asymmetric cell division of the zygote, one of the earliest stages in apical-basal formation (Mayer *et al.*, 1993). Mutants fail to form the apical and basal end regions of the shoot and root and some plants lack any obvious morphological features of apical-basal polarity. In the *gnom* plants, the division of the zygote into daughter cells of equal sizes instead of asymmetrically leads to the production of an abnormally large apical cell and smaller basal cells. Subsequent cell divisions are irregular resulting in abnormal number of cells in each region. Although the smaller basal daughter cell gives rise to a shortened suspensor, the apical cell still forms the embryo proper suggesting that unequal first cell division of the zygote is not necessary for the fate establishment of the two daughter cells.

That the gene is required to stabilise the apical-basal polarity was supported by the altered expression patterns *gnom* mutants of an apical-specific marker gene, *Arabidopsis thaliana Lipid Transfer Protein 1 (AtLTP)* (Vroemen *et al.*, 1996) and a protein marker for cell polarity, *PIN-FORMED 1 (PIN1)* (Hadfi *et al.*, 1998). *AtLTP* expression is normally restricted to the apical region of the later stage embryo. In *gnom* mutants, the expression of *AtLTP* expression is variable along the apical-basal axis suggesting that although a shortened suspensor and an embryo proper was formed in the mutant plant, the polarity was not sufficient to establish the apical-basal axis during embryogenesis. *PIN-FORMED 1 (PIN1)*, a member of the putative auxin efflux carrier is localised in a polar fashion accumulating in the basal region of the wild-type globular embryos. In the *gnom* mutants, *PIN1* distribution is altered suggesting that the distribution of *PIN1* promotes polar auxin transport and that polar auxin transport is necessary for the establishment of the apical-basal polarity (Steinmann *et al.*, 1999; Hadfi *et al.*, 1998).

Molecular analysis of *GN/EMB30* shows that it encodes a protein most similar to yeast guanine nucleotide exchange factors, which are members of the ADP-ribosylation factor (ARF) family (Busch *et al.,* 1996), indicating that the *GN/EMB30* gene may be involved in vesicle transport. This was later on supported by biochemical studies which indeed show *GN/EMB30* involvement in the localization of PIN1 (Steinman *et al.*, 1999).

The *Arabidopsis* gene *MONOPTEROS (MP)* also appears to be involved in the establishment of the basal structures such as hypocotyl, radicle and root meristem (Mayer *et al.*, 1991). *mp* seedlings lack roots and hypocotyl. Interestingly, the pattern defect results not from a mutant embryo having fewer cells than wild type but from an early stage embryo having more cells than normal. The octant stage embryo has four tiers of cells instead of two tiers (Berleth and Jurgens, 1993). In a normal embryo, the basal region normally derives from the lower tier of cells (Jurgens and Mayer, 1994; Mansfield and Briarty, 1991). Because of the increased number of cell tiers, the cells divide abnormally and the cells that would normally form the basal structures seem to lack instructions for the formation of the elongated cell files, which normally characterise the hypocotyl and the root. Because *MP* encodes AUXIN RESPONSE FACTOR 5 (ARF), a DNA-binding transcription factor (Hardtke and Berleth, 1998), the *MP* gene was associated with auxin mediated cell axialization in plants.

1.1.2. Pattern forming processes in plants

1.1.2a. Cell division patterns

The patterns of cell division have long been thought to be a key determinant in plant morphogenesis (reviewed by Meyerowitz, 1996). Plant cells, unlike animal cells, are immobilised in place by cell walls and hence do not migrate during embryonic development as they do in animals. This implies that morphogenesis in plants can be equated with cell activities particularly the frequency and planes of cell

division. Since morphogenesis in plants is highly regulated, it would appear that cell division patterns would be highly regulated as well.

Studies have shown that changes in the frequency of cell division do not necessarily alter the pattern of formation in plants. Over expression of a dominant, negative *Arabidopsis* CDC2a gene in tobacco (Hemerly *et al.*, 1998), for example, leads to plants in which the vegetative and shoot apical meristem have a reduced cell number. This, however, has little affect on overall plant structure with all cell types present in proper positions, indicating that plant development is not necessarily sensitive to a general reduction in cell number.

The plane of cell division is also a key aspect of growth. Anticlinal divisions (divisions in which the cell plates are perpendicular to the surface) result in an expansion of the surface while periclinal divisions (divisions in which the cell plates are parallel to the surface) are associated with the formation of new growth axes. Epidermal cell layers divide almost exclusively anticlinal hence the epidermis expands as a single layer to cover the surface of a plant organ. On the other hand, initiation of organ primordia or outgrowth of a lateral root start with the periclinal divisions of inner cells which results in the formation of a bulge and subsequent development of secondary growth. Mosaic analyses show that a generally fixed pattern of cell division polarity preserves the clonal layers in the meristem (Furner and Pumphrey, 1992; Irish and Sussex, 1992; Bossinger and Smyth 1996). Genetic mosaics also show that although occasional cell infiltration of one layer by another may occur, this does not significantly alter plant form (Tilney-Basset, 1986). In maize, a mutant disrupted in the *tangled1* gene that shows irregular divisions along the longitudinal plane, but normal divisions in the transverse plane, still maintains a typical overall leaf shape (Smith *et al.*, 1996). Similarly, the *tonneau* (*ton1*) (Traas *et al.*, 1995) and *fass* (Torres-Ruiz and Jurgens, 1994) mutants of Arabidopsis, which affect cell division planes, result in plants that are short and misshapen, but are otherwise, able to form all organs in a normal pattern. In both mutants, the abnormal cell form is associated with the irregularity in cell division patterns. The *ton* and *fs* phenotype suggest that oriented cell divisions are not associated with pattern formation.

1.1.2b. Cell fate specification and positional information

Since majority of the adult plant body is derived postembryonically from the activity of the shoot apical meristem, there is this issue whether cell fate specification in plants is similar to lineage dependent mechanisms in animals, where the identity of the cell depends upon the identity of its parent. That cell fate in plants is determined by positional information and not by cell lineage has been established from several lines of investigations (Tilney- Basset, 1986; Poethig *et al.*, 1986; Scheres *et al.*, 1994; Berger *et al.*, 1998).

Clonal analyses show that the fate of a meristem cell largely depends on its position rather than its lineage (Stewart, 1978; Poethig *et al.*, 1986; Ruth *et al.*, 1985; Irish and Sussex, 1992). A cell at the summit of the meristem, for example, plays its role as an apical initial and serves as a source of other cells only by virtue of its position; the cell ceases to be an initial cell once displaced from its position (Ruth *et al.*, 1985). Studies of periclinal chimeras show that although the meristem is stratified into clonally distinct layers, each layer being a separate cell lineage (Satina, *et al.*, 1940), occasional invasion of one layer by cells from another layer does not affect the organization of the plant. The invading cells continue to differentiate according to their new position (Tilney-Basset, 1986). That cell fate depends upon the position of the meristem cell is also shown in sector analyses (Irish and Sussex, 1992), wherein cells in different regions of the embryonic meristem differ in their probable contribution to the plant body. It appears that cells in the basal and the outermost region of the meristem display a more restricted fate compared with the cells in the meristem summit; these cells being more committed and determined compared with the central cells.

Cell ablation experiments in *Arabidopsis* roots show that gaps formed where ablated cells die (Berger *et al.*, 1998; van den Berg *et al.*, 1995). These gaps are then filled out by neighbouring cells that subsequently divide and differentiate according to their new position. Ablation of trichoblast in the root meristem, for example, results in the invasion of trichoblast position by atrichoblast cell (Berger *et al.*, 1998). These atrichoblast cells assume a new trichoblast identity. In a similar

experiment (van den Berg *et al.*, 1995), laser ablation of cortical cell initial resulted in the invasion of cortical position by pericycle cells. These invading cells soon differentiated and behaved as a cortical cell.

Genetic analyses also provide evidence that cell fate specification is position dependent. In the embryo, a very basic differentiation of embryonic organ types that depends on positional information rather than cell lineage can be seen along the apical/basal axis, where apical cells give rise to shoot organs while cells in the basal regions become the root. In some circumstances, however, it is clear that the fate of these cells is not yet fully determined by their position. For example, analysis of the *Arabidopsis twin* mutants (*twn1* and *twn2*) (Zhang and Sommerville, 1997) in which mutants develop secondary embryos from the basal cells, suggest that the basal cell is still capable of changing its fate into becoming embryo proper. This observation is also true with another *Arabidopsis* mutant, *raspberry* where the embryo proper arrests and embryo-like structures develop from the basal cells (Schwartz *et al.*, 1994).

Results from the above studies suggest that the ultimate development of tissues and organs depend upon interactions between cells and that mechanisms are present within the plant that enables a cell to assess its position relative to the other cells.

1.1.2c. Intercellular communication in plants

How is positional information transmitted from cell to cell? One model that was postulated regarding position-dependent cell fate specification is that cells gain "knowledge" of their positions via gradients of morphogens and interpret these gradients as "positional information" (Wolpert, 1969). This concept of diffusible morphogens has led to studies implicating diffusible chemical signals, such as auxin gradients, to relay positional information. The effect of auxin gradients has been demonstrated through the use of mutants and auxin inhibitors. The *pin1* mutants of Arabidopsis are defective in polar auxin transport (Okada *et al.*, 1991) resulting in the alteration of the bilateral symmetry in the embryo. Results of recent experiments

show that exogenous application of auxin is sufficient to induce organogenesis in vegetative tomato meristem and in the *Arabidopsis* inflorescence meristem (Reinhardt *et al.*, 2000) and inhibition of polar auxin transport blocks leaf formation resulting in pin-like structures. From these experiments, it has been proposed that a gradient of auxin provides information for the establishment of radial symmetry (Kuhlemeier and Reinhardt, 2001).

Another model that has emerged is that signals are transmitted between cell layers. Mosaic analyses show that some genes act non-autonomously (Dudley and Poethig, 1993; Foster *et al.*, 1999), suggesting that cell signalling mechanisms occur between cell layers. The observation that large macromolecules can be transported through plasmodesmata (Lucas *et al.*, 1995) has explained symplastic movements of some gene products. In maize, *kn1* mutants form knots that involve the epidermal layer even though *kn1* transcripts are restricted to the subepidermal layer (Sinha and Hake, 1990). The KN1 protein was found to be present in both the tunica (L1) and corpus (L2) layers of maize shoot apical meristems (SAMs) while *kn1* transcripts was limited only to the L2 layer (Jackson *et al.*, 1994). It was further observed that the production of knots is dependent on the genetic make-up of the subepidermal layer and not the epidermal layer expressing the phenotype, indicating a non-cell autonomous activity. Subsequent studies show that KN1 protein could shuttle through the plasmodesmata from one cell layer to another (Lucas *et al.*, 1995).

In *Arabidopsis*, the floral transcription factor LEAFY was found to move to adjacent cells, where it directly activated homeotic target genes (Sessions *et al.*, 2000). The observation that LEAFY protein moves within and between layers of the floral meristem although the patterns of LFY RNA and protein in the wild type was the same was taken as evidence for a redundant mechanism of specifying meristem fate to ensure complete conversion of the meristem into a flower.

In the roots, evidence has been presented that shows symplastic transfer of patterning information within cell files via plasmodesmata (Schiefelbein *et al.*, 1997). The *SHORT-ROOT* gene in Arabidopsis controls radial patterning of roots through radial signalling mechanism (Helariuta *et al.*, 2000). The SHORT ROOT protein has been shown to move between cells to regulate the transcription of *SCARECROW* gene in the target cells.

The above studies show that an intercellular network allows movement of certain molecules across the symplast and provides a mechanism of signalling positional information in plants.

1.2. Shoot apical meristem

The shoot apical meristem (SAM) in a typical flowering plant is responsible for the formation of all the aboveground portion of the plant. Unlike in lower plants, where there seems to be a distinctive single apical cell that serves as a source of all cells that form the shoot, the source of cells in higher plants is a group of specialised cells at the tip of the shoot apex called the shoot apical meristem. The SAM has the ability to divide and replenish itself, as well as produce daughter cells that differentiate into organs that soon define plant form (Steeves and Sussex, 1989). The wide diversity and variability seen in plant forms reflects the diversity, variability and complexity of activities in the SAM itself. Leaves may be initiated in different phyllotactic patterns, growth in the SAM may vary from determinate to indeterminate, branching may be enhanced or reduced, and growth modifications may be present or absent as the SAM respond to various internal and external cues. However, even with recent progress in the study of meristem function and maintenance, much is still unknown about how SAMs initiate organs, how daughter cells in the SAM acquire specific identities and how the SAM maintains itself.

The development of molecular genetic techniques such as RNA *in situ* hybridisation, immunolocalisation, transgenic expression of reporter genes, provided tools to study the structure of the SAM and how it functions. Analyses of a number

of meristem-specific gene expression patterns and mutant analyses indicate that the organization of the meristem into zones reflect differential expression of genes which correlate with functional domains within the meristem such as that of maize *knotted (kn)* (Smith *et al.*, 1992; Jackson *et al.*, 1994) and *Arabidopsis STM* (Long *et al.*, 1996).

1.2.1. SAM structure

Shoot apical meristems differ in size and morphology. The *Arabidopsis* meristem is about 100 microns while that of maize is about 250 microns in diameter. Some plants, as in some species of cycads, may have meristems as large as 3500 microns, while others have meristems as small as 50 microns (Gifford, 1954). The Arabidopsis SAM is mound-shaped (Medford *et al.*, 1992) while that of maize is finger-shaped (Steffensen *et al.*, 1968). Regardless of the meristem size and SAM morphology, however, the SAMs of higher plants exhibit the same basic structural organization.

The organization of the shoot apical meristem has often been described in two ways: 1) according to cytological and functional zonations (Steeves and Sussex, 1989); and 2) based on clonally distinct layers of cells (Satina *et al.*, 1940). The first classification subdivide the SAM into three regions: the central zone (CZ or the zone of stem cells), where the cells are large and exhibit prominent vacuoles; the peripheral zone (PZ or the organ forming region), where cells are smaller, densely staining and possess smaller vacuoles and the rib zone (RZ or the pith forming region), where cells also possess small vacoules. The cytological appearances have been associated with mitotic activity of the cells within the respective zones where cell division in the central zone is slow while in the peripheral and rib zone, cell divisions are rapid. These three zones may also represent a functional subdivision of the SAM (also discussed in Schoof *et al.*, 2000, Bowman and Eshed, 2000). The central zone acts as a source of stem cells, which replenish both the peripheral zone and the rib zone, the PZ is the direct source of cells recruited to form the lateral organs and the rib zone is the direct source of cells recruited to form the pith tissue.

Superimposed on the first classification is the "tunica-corpus" classification (Schmidt, 1924) that is based on the layered appearance of the meristem. In this second classification, the SAM is subdivided into distinct layers of cells in the meristem; 1) the tunica, where cell division is largely anticlinal and 2) enclosed by the tunica is the corpus, where cell division occurs in varied orientations. Analysis of periclinal chimaeras has extended the tunica-corpus concept by showing the clonally distinct nature of these tissues. Thus, in a dicot plant such as *Arabidopsis*, the tunica consists of 2 clonally distinct layers, L1 and L2. The corpus or the L3 layer lies beneath the tunica (Figure 1.2). Thus, the *Arabidopsis* SAM consists of three layers, L1, L2 and L3 (Irish and Sussex, 1992).

The predominantly anticlinal pattern of cell division in L1 and L2 layers results in the maintenance of separate lineage of cells in each layer. In maize, however, there are two layers in the SAM, the tunica (L1) and the corpus (L2) (Steffensen, 1968). This means that the central zone and peripheral zone contain cells from the clonally distinct layers.

Although the SAM exhibits a highly regular cell division pattern within its layered structure, chimeric studies show that the position of a cell rather than its lineage determines its fate (Satina *et al.*, 1940). Studying the fate of marked cell clones in *Datura* plants, it was shown that meristem layers give rise to specific tissues of the plant body. The L1 layer gives rise to the epidermis while the L2 and L3 give rise to internal tissues. However, the fate of the cells is quite flexible. For example, cells from one layer occasionally invade the adjacent layer, then subsequently divide and differentiate according to the new location emphasizing the position dependent specification of cell fate (Satina and Blakeslee, 1941).

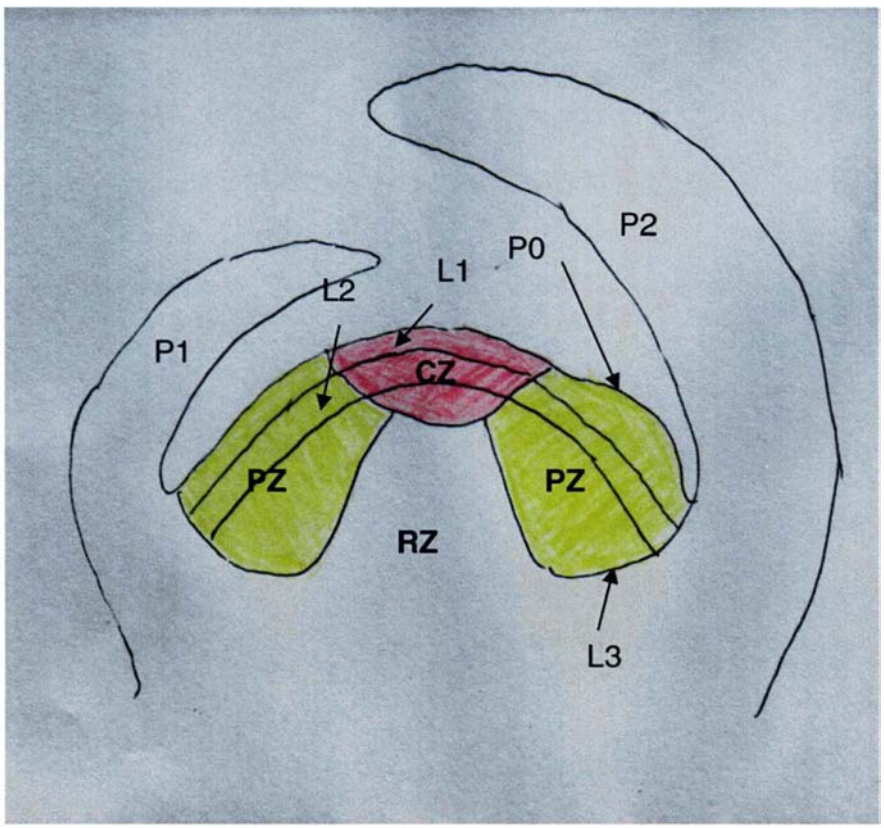


Figure 1.2. Schematic representation of a shoot apex showing the organisation of the meristem. The shoot apex is organised into clonal layers (L1, L2 and L3) and superimposed in this is an organisation according to cytological zonations; central zone (CZ), peripheral zone (PZ) and the rib zone (RZ). P0 is an emerging leaf primordium. P1 and P2 are older leaves. (Source: Laufs *et al.*, 1998b).

It is interesting to note that the functional domains in the SAM also show distinct patterns of gene expression, which in some cases have been shown to have functional significance. The indeterminate state of the cells in the central zone, for example, seems to be promoted by *kn1*-like homeobox genes (*knox)*(Jackson *et al.*, 1994; Chuck *et al.*, 1996). *On* the other hand, the expression of the *Arabidopsis AINTEGUMENTA (ANT)* gene is restricted to the peripheral region where organogenesis occurs (Elliot *et al.*, 1996). The involvement of these genes in SAM function will be discussed in more detail in the next topics.

1.2.2. Maintenance of self-renewing initial cells in the SAM

Clonal analyses have indicated that a pool of undifferentiated cells that resides in the summit of the meristem serves as the initial cells and ultimately the source of all plant parts (Steeves and Sussex, 1989). Surgical experiments also provide evidence that the central cells are particularly significant in maintaining the integrity of the meristem. These cells have been found to have a high degree of pluripotency, or the ability to regenerate many different tissues.

The molecular genetic approach in the analysis of meristem structure and function has provided further understanding of the fundamental processes that maintain populations of initial cells. Several genes expressed in the shoot meristem seem to specifically regulate the function of the central zone (Clark *et al.*, 1995; Laux *et al.*, 1996; Barton and Poethig, 1993; Jackson *et al.*, 1994). The maize *kn1*, the founding member of the *knox* family, is expressed in subepidermal cells of vegetative meristem but is not detected in differentiated tissues, such as leaves (Jackson *et al.*, 1994). In a similar manner, the *Arabidopsis SHOOTMERISTEMLESS (STM)* is expressed throughout the shoot apical meristem but is down regulated in organ primordia (Chuck *et al.*, 1996). The expression patterns of these genes and the mutant phenotypes suggest involvement of the *knox* genes in maintaining stem cell identity in the meristems. Maize plants expressing the dominat mutation of *kn1* show ectopic meristematic outgrowths or "knots" on leaves, a phenotype which is associated with ectopic expression of *KN1*

mRNA. In *Arabidopsis*, *stm* mutant phenotype is characterised by premature termination of growth due to the absence of embryonic shoot apical meristem.

A molecular genetic analysis of the *WUSCHEL (WUS)* gene, expressed early on during *Arabidopsis* embryogenesis plays a central role in the maintenance of stem cells within the center of the meristem (Laux, *et al.*, 1996). It is first expressed in the subepidermal layers in the L3 during the 16-cell stage of embryogenesis. As the embryo matures, the expression of *WUS* is limited to a small group of cells underneath the three most superficial layers. *wus* mutant embryos form a flat structure at the base of the cotyledon instead of the dome-shaped SAM (Laux *et al.*, 1996) in which a population of vacuolated and partially differentiated cells replaces the normal stem cells. This mutant structure does not form any more leaves, apparently because uncommitted cells from which these organs might form have been depleted. Taken together, the analysis of the mutant phenotype and the expression pattern of the gene suggests that *WUS* is required for specification of stem cells within the SAM (Mayer *et al.*, 1998).

In *Arabidopsis*, mutations in the three genes *CLAVATA*, *CLV1, CLV2* and *CLV3* result in an enlarged or fasciated meristem that is due to the meristem accumulating undifferentiated cells in the central zone (Clark *et al.*, 1993, 1995, 1997; Kayes and Clark, 1998). This accumulation appears to be the result of a failure to promote cells on the flanks of the meristem toward differentiation. Expression analyses show that the *CLAVATA* genes are expressed in the central zone of the meristem and genetic and biochemical analyses indicate that the three *CLV* genes encode components of a signalling pathway (Clark *et al.*, 1997; Fletcher *et al.*, 1999).

Another class of genes expressed early on during embryo development that are also required to maintain undifferentiated nature of the stem cells in the vegetative meristem are the *ZWILLE/PINHEAD (ZLL/PNH)* and *ARGONAUTE (AGO1)* (Jurgens *et al.*, 1994; Endrizzi *et al.*, 1996; Lynn *et al.*, 1999). *Arabidopsis* plants lacking *ZLL/PNH* activity terminate early due to defective shoot meristems.

1.2.3. Organ formation

1.2.3a. Specification of organ position

One of the major functions of the SAM is to make leaves. There are two important aspects of organ formation: 1) the specification of organ position or phyllotaxy and 2) the specification of group of cells to become leaf primordia. The arrangement of leaves along the shoot, phyllotaxy, reflects the regular pattern in which the leaf primordia are positioned in the meristem. Clonal and surgical analyses indicate that organs become determined on the flank of the meristem in the peripheral zone (Poethig and Symkoviak, 1995 and Steeves and Sussex, 1989). Surgical and pharmacological experiments provided some early insights into the mechanisms involved in leaf initiation. Snow and Snow (1931) performed surgical studies on *Lupinus* that provided evidence that the positioning of leaf primordia results from an interaction between the existing leaf primordium and the shoot apex such that the positioning of a new leaf primordium is inhibited by the presence of leaf primordia nearby. Hence, if an incision is made to physically isolate an existing primordium, the next leaf to be initiated will be closer to the site of the existing primordium than it would normally have been. A model was proposed whereby the positioning of leaf primordia depends upon "available space" in the meristem. This model states that a leaf arises in the first space that becomes available in the shoot apex farthest from the summit and from the existing primordia.

Similar surgical techniques applied in the study of *Dryopteris* (Wardlaw, 1949) gave similar results but were interpreted differently. A hypothesis was put forward that inhibitory substances are responsible for determining the position of the new leaf primordium at a given interval relative to an existing primordia. According to this hypothesis, each new leaf primordium initiated is surrounded by a physiological field within which initiation of new leaf primordium is inhibited.

An alternative model forwarded to explain the pattern of organ initiation is that biophysical factors generate pattern of leaf arrangement (Green *et*

al., 1996a). The theory supposes that physical processes, such as the reorientation of the cell wall cellulose microfibrils leads to a change in the polarity of cell division resulting in the growth of leaf primordium (Green *et al.*, 1996b). It further supposes that the regular positioning of primordia results from the regular patterns of tension imposed by the corpus cell turgor upon the tunica. This model has been supported by recent studies wherein application of expansin, a protein that promotes cell wall expansion, to the shoot apex of tomato causes localised outgrowths and changes in phyllotaxy (Fleming *et al.*, 1997), and by the observation that *expansin* gene expression is normally upregulated in the sites of incipient leaf primordia (Reinhardt *et al.*, 1998) before any other changes are apparent.

The "field of inhibition theory" has put forward the biochemical model for the regulation of phyllotaxy. The model proposes that the existing primordia and the summit of the meristem produce gradients of inhibitory substances or diffusible morphogens. Various interpretations have been offered about the identity and activity of this morphogen, but this has continued to be a subject of studies (Scwabbe, 1984; Meichenheimer , 1981). Recent experiments suggest that the plant hormone auxin has activities that suggest a role as a diffusible morphogen in the context of leaf initiation (Reinhardt *et al.*, 2000; Vernoux *et al.*, 2000). Application of auxin inhibitors to tomato apices cultivated *in vitro* completely suppressed leaf production, resulting in "pin-like" structures. Application of auxin restored leaf formation (Reinhardt *et al.*, 2000). This observation agrees well with the molecular genetic analysis of an *Arabidopsis* auxin mutant *pin1* (Vernoux *et al.*, 2000). *pin1* mutant has reduced polar auxin transport and the plant makes bare floral stem. Local application of auxin to *pin1*, showed that auxin induced formation of flower primordia, suggesting that auxin plays an essential role in the initiation of primordia.

Genetic studies show that the pattern by which leaves are formed is affected by meristem size. The *abphyll* mutant of maize, for example, which exhibits altered phyllotaxy by producing leaves in decussate pattern rather than the normal distichous phyllotaxy, has a larger SAM than the wild type (Greyson *et al.*, 1978). In *Arabidopsis*, the *clv* mutants, which are all characterised by enlarged meristems, produce numerous organs, which have abnormal spacing patterns (Clark *et al.*,

1993, 1995; Leyser and Furner, 1992). These studies offer further support for the notion that the positioning of organ primordia are sensitive to geometric parameters, which in turn reflect the activity of a diffusible morphogen.

The changes in the expression patterns of plant homeobox genes, like the maize *kn1* and *Arabidopsis STM* have often been used to predict the position whereby organs will form. The expressions of these genes are down regulated in leaf primordia sites. On the other hand, the expression of the *Arabidopsis AINTEGUMENTA (ANT)* gene is restricted to the peripheral region where organogenesis occurs and the accumulation of the *ANT* transcripts has often been used as a marker for organ formation (Elliot *et al.*, 1996).

A recent study suggests the involvement of a different class of genes, first described with the *terminal ear 1* of maize, in specifying organ position (Veit *et al.*, 1998). Loss of function mutation to the *te1* gene results in increased rate of leaf initiation and aberrant phyllotaxy. *te1* transcripts are expressed in a series of semi-circular rings along the shoot apex, with leaves initiated in regions where *te1* mRNA is lowest. A more detailed morphological characterisation of *te1* mutant and a discussion about the functional role of the gene is presented in Chapter 3.

1.2.3b. Specification of leaf founder cells

Anatomical studies show that a leaf begins its development as a localised protrusion on the flanks of the shoot meristem that gradually develops into a leaf primordium (Steeves and Sussex, 1989, Lyndon, 1994) through growth along preferred axes. Several questions arise that relate to primordia initiation: 1) how are cells recruited and determined to become a leaf? 2) what are the cues that promote the outgrowths? and 3) what regulates the developmental processes leading to organ formation?

Clonal analyses indicate that about 100-200 cells from the flank of the meristem are recruited to form each leaf primordium (Poethig and Symkoviak, 1995). Surgical experiments indicate that a group of cells are determined to become a leaf even before a distinct morphological change is observed (Wardlaw, 1949). A "bump" that grows out of the apical surface of the meristem has been associated with an increased rate of cell division at the site of primordia initiation (Lyndon, 1983). Anatomical studies indicate that changes in the planes of cell division accompany primordium initiation, but do not necessarily initiate the event (Cunningham and Lyndon, 1986; Foard, 1971; Lyndon, 1970). Surface extensibility is, however, considered an important factor in primordium initiation, especially with the localised expression of expansins, proteins that cause wall loosening in incipient leaf primordia (Reindhardt *et al.*, 1998).

Molecular markers have also been identified associated with identification of leaf founder cell population. The expression domain of the homoeobox gene, maize *knotted (kn1)* has often been used as marker for leaf identity in the shoot apical meristem (Smith *et al.*, 1992; Jackson *et al.*, 1994, Long and Barton, 1998). *kn1* transcripts accumulate to high levels throughout the shoot apical meristem, but are downregulated in leaves and leaf primordia. *kn1* transcripts are also absent in a ring of cells on whose position on the flank of the SAM in relation to older leaves suggests a leaf founder cell identity (Smith *et al.*, 1992, 1995; Jackson *et al.*, 1994). The number of cells in these regions clear of *kn1* transcripts was also found to be consistent with the number of founder cells predicted from clonal analyses. This down regulation of *kn1*-like genes occurs early on during leaf initiation even before any morphological indications of leaf initiation, which suggests that the negative regulation of these genes may be a prerequisite for these cells to assume a leaf founder cell identity. Similar expression patterns are also observed for *Arabidopsis STM*, a gene that encodes a KN1- like protein, which is necessary for meristem maintenance (discussed in more detail in Chap 3).

1.3. The maize terminal ear1 (te1) and the Mei2-like gene family of RNA binding proteins.

1.3.1. The maize terminal ear 1

The *te1* mutant plant was initially described in terms of its short stature and tassel feminisation, which gives the plant the appearance of having a "terminal ear" (Matthews *et al.*, 1974). More recent characterisation has shown that *te1* mutant initiates leaves more frequently than the wild type, often in abnormal positions on the shoot, with many leaves showing morphological defects. The short stature of the plant can be mostly attributed to abnormally short internodes, especially in the apical portion of the plant. The altered pattern of leaf initiation is sometimes accompanied by a change in phyllotaxy from distichous to essentially random leaf arrangement.

In situ hybridisation experiments show that the *te1* transcripts accumulate in a series of semi-circular rings that bracket sites of leaf initiation. The expression pattern and the mutant phenotype suggest a role for *te1* in specifying or delimiting time and position of leaf initiation (Veit *et al.*, 1998).

The gene was cloned and the deduced amino acid sequence of the *terminal ear 1* gene *(te1)* in maize (Veit *et al.*, 1998) shows that it belongs to a gene family of RNA binding proteins. Sequence analysis shows that the most similar gene at the time is *Mei2* in yeast, *Saccharomyces pombe* (Watanabe *et al.*, 1988). Mei2p is an RNA binding protein that acts late in the commitment pathway to meiosis and is required to complete Anaphase I. Both TE1 and Mei2p contain three RNA recognition motifs (RRM), of which the third one is highly distinctive and most conserved region. The presence of conserved RRMs suggested that *te1* may function in RNA binding activity, presumably in the post transcriptional regulation of leaf initiation.

1.3.2. The plant Mei2-like genes as RNA binding proteins

Initial database searches at the time this study was started revealed 7 plant genes showing sequence similarities to *mei2,* and hence were referred to collectively as plant *Mei2*-like genes. This new class of genes included the maize *te1* and 6 *Arabidopsis* orthologues. These plant *Mei2*-like genes are characterised by the presence of 3 RNA Recognition Motifs (RRM's) with the third one being highly distinctive (Jeffares, 2001)(Figure 1.3). Later, phylogenetic analysis (Jeffares, 2001) of the *Mei2*-like genes based on sequence homologies and intron positions revealed additional *Mei2*-like genes that could be grouped into the following 3 classes: 1) *terminal ear1*-like (*TEL* group; 2) *Arabidopsis Mei2-Like* (*AML* group); and 3) *mei2* of *Schizosaccharomyces pombe* (Figure 1.4).

A large number of proteins have been identified that contain one or more copies of the so-called RNA recognition motif (RRM) (Birney *et al.*, 1993). Proteins belonging to this class are involved in wide variety of functions in plants, including RNA splicing, polyadenylation, stabilisation, transport, and translation as well as the regulated alternatives such as alternative splicing and translational regulation (Alba and Pages, 1998; Burd and Dreyfus, 1994). In animals, the most studied is the Drosophila SEX-LETHAL protein (McKeown *et al.,* 1992) while in plants, this group of proteins include the flowering gene of *Arabidopsis FCA* which contains 2 RRMs, and control flowering time (Macknight *et al.*, 1997).

In the case of *S. pombe* Mei2p, the third RRM appears to be important in RNA binding and function with high sequence specificity (Watanabe *et al.*, 1997). That RRM3 is important for the nuclear localisation of Mei2p is indicated by the result of GFP fusion studies with the Mei2p carrying a mutation in RRM3 (Watanabe and Yamamoto, 1994). The mutation abolished not only RNA binding activity of the RRM3 but also localisation to the Mei2 dot, indicating the importance of the third RRM in the nuclear transport of the meiRNA-MEI2 complex. Mei2p is localized mainly in the cytoplasm of proliferating cells but in the nucleus, the mei2p-meiRNA complex is seen as a single spot close to the microtubule organizing centre in

Figure 1.3. The third RRM characterizes Mei2-like genes (source: Jeffares, 2001)**.** The C-terminal RRM of the Mei2-like proteins that have been identified when the study started. The region encompassed by the canonical RRM domain is shaded. Within peptide sequence, predicted b-sheets are shown in blue, a-helices in red. Gene names are preceded by source; Zm, *Zea mays*, At *Arabidopsis thaliana*, Sp *Schizosaccharomyces pombe*. Gene names *TEL1, TEL2, AML2, AML3 and AML4* are our conventions for predicted/cloned cDNAs.

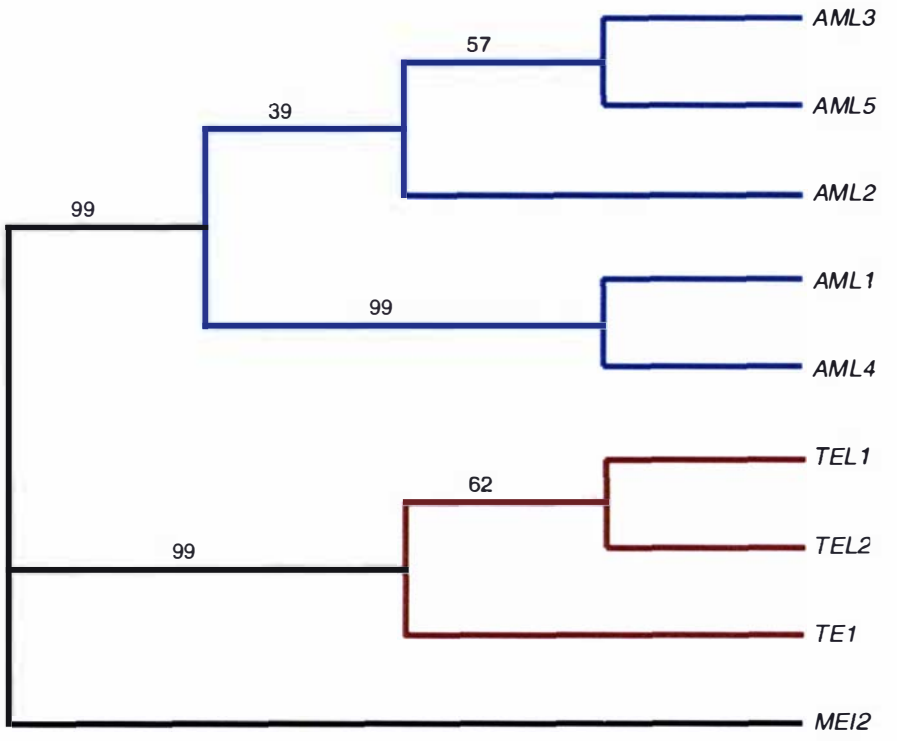


Figure 1.4. Three groups of *Mei2*-like genes have strong support in maximum parsimony trees (source: Jeffares, 2001). A maximum parsimony tree showing the relationships of *Mei2*-like genes. The TEL group (*TE1, TEL1, TEL2*), and the AML group are from plants, *MEI2* is from *Schizosaccharomyces pombe*. *AML1* and *AML4* are very similar and always group together.

prophase nuclei during meiosis emphasizing the role of Mei2p in the execution of meiosis (Watanabe *et al.*, 1997).

The RNA binding ability was found to be needed for Mei2p to get transported and perform its function in the nucleus. A specific non-coding RNA species, the meiRNA was shown to assist the movement of Mei2p in the nucleus. As a Mei2p-meiRNA complex, they form a characteristic dot in the nucleus before meiosis. This dot formation is necessary for the switching on of the cell cycle from mitosis to meiosis and that the meiRNA was found to be essential for this dot formation (Watanabe *et al.*, 1994, Yamashita *et al.*, 1998). Recent studies of Mei2p-meiRNA interaction showed that Mei2p undergoes nucleocytoplasmic shuttling (Sato *et al.*, 2001) and meiRNA facilitates the assembly of Mei2p into a dot structure to trap the Mei2p in the nucleus instead of just being an escort of Mei2p. It was suggested that Mei2p may shuttle by itself, and whether it binds with the meiRNA on the way to the nucleus or after both have reached the nucleus is not clear, but it seems that binding with meiRNA is important for the dot formation in the nucleus and this dot formation is important for the completion of Anaphase I.

In summary, the presence of three RRM's characterises the plant *Mei2*-like genes. The positional similarity of these regions with Mei2p indicates that this family of genes function to bind RNA. The third RRM which was found to be important for binding and subsequent nuclear localisation of Mei2p is also the most highly conserved region and this position is identical in all plant *Mei2*-like genes identified and referred to in this thesis. There are no evidences yet, at present, whether meiRNA-like RNAs are also in plants. Since meiRNA is important in nuclear localisation and stabilisation of Mei2p dot in the nucleus, it seems likely that these molecules are also present in plants.

1.4. Aims of the thesis

The main aim of the thesis is to gain further understanding of how plant *Mei2*- like genes function. The molecular genetic analysis of these genes will help us understand the molecular mechanisms that control morphogenesis in plants. Specifically, the thesis aimed to do the following:

1. to assess the degree of correspondence between *te1* expression and leaf initiation, expression analyses were done in the following contexts: *a) te1* expression during maize embryogenesis; b) *te1* expression in *abphyll*, a maize mutant, with altered pattern of leaf initiation and *c)* expression of a leaf marker gene, *kn1*, in *te1* mutants;

2. to test the hypothesis that changes in the pattern of leaf initiation and development caused by mutation in the *te1* gene result from characteristic changes in the shoot apex, comparisons of the maize shoot apices of *te1* mutant and their normal siblings were done. Clonal analysis was done to compare the number of leaf founder cells in *te1* mutants versus normal siblings;

3. to further understand how *te1* may play a role in organogenesis, a detailed morphological characterisation of *te1* mutant was done;

4. with the identification of additional *Mei2*-like genes in Arabidopsis, expression and genetic analysis were done to further understand how members of this RNA binding protein family may function in the regulation of morphogenesis in plants. Expression analysis was done to determine if members of this gene family particularly *Terminal Ear*- Like (*TEL*) genes in *Arabidopsis* are expressed in the same pattern as the maize *te;*

5. to provide evidence for the biological significance of *Mei2*-like genes in *Arabidopsis*, gene knockouts were identified and characterised.

Chapter 2. General experimental procedures

Abstract

This chapter presents the general methods used in the experiments. These include protocols used to manipulate DNA, RNA, plasmids and bacteria, and the method used for *in situ* hybridisation experiments. Because most of the plasmid, DNA and RNA protocols used in the study were standard protocols in the Veit's laboratory, these are presented in this study as described in Jefarres, 2001.

2.1. General plasmid and E.coli methods

The general plasmid and *E.coli* methods were done primarily to prepare plasmid DNAs for cloning experiments and DNA amplifications. All glassware materials used with plasmid manipulations and bacteria cultures were sterilised by baking.

2.1.1. Growth of bacterial culture

Plasmids are purified from bacteria (*E.coli*) grown either in liquid culture or solid medium containing the appropriate antibiotic. Liquid *E.coli* cultures were grown in LB Medium (1% bacto-tryptone, 0.5% yeast extract, and 1% NaCl pH7.0) at 37°C with vigorous shaking at 200rpm. Solid LB media were prepared by adding 1.5% agar into the liquid LB recipe. Appropriate antibiotics were added after autoclaving the media. Glycerol stocks were prepared by adding 0.2 ml of sterile glycerol to 0.8 ml of stationary phase liquid culture, vortexing, and freezing in liquid nitrogen and stored at - 80°C.

2.1.2. Harvesting and lysis of bacteria

Initially, plasmid extraction and purification was done using a modified alkaline lysis method, Sambrook (I989). When problems were encountered with the

transcription reactions which were done to generate RNA probes for in *situ* hybridisation experiments, plasmid preparation was done using available kits (Qiagen or Bresatek), the protocol based on the manufacturers recommendations.

2.1.2a. Alkaline lysis method based from Sambrook (l989)

In the alkaline lysis method (Sambrook, l989), a 2 ml LB culture grown overnight with appropriate antibiotics was harvested by centrifugation at 12 000 x g for 1 minute at 4°C in a microfuge. The pellet was resuspended in 100 µl of Solution I (50 mM glucose, 25 mM Tris-HCl pH8.0, 10 mM EDTA). Two µl of freshly prepared 10 mg/ml lysozyme in 10 mM Tris-HCl pH 8.0 was added and the mixture was mixed by rapid vortexing. After mixing, 200 µl of freshly prepared Solution II (0.2 N NaOH, 1% SDS) was added and mixed by gentle inversion, and the tube was stored on ice for 5 minutes. A 150 µl of ice-cold Solution III (5 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid, 28.5 ml of Milli-Q water) was added and the tube was mixed by gentle shaking. The tube was stored on ice for 10 minutes, and then centrifuged at 12 000 x g for 5 minutes at 4°C. The supernatant was removed, poured through cheesecloth wet with isopropanol into a new tube (taking care to avoid including any white precipitate with the supernatant). The DNA was precipitated with 0.6 volumes of isopropanol and the nucleic acids were recovered by centrifugation at 12 000 x g for 5 minutes at room temperature. The pellet was washed with 80% ethanol, and resuspended in 50 µl of TE10/1 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA samples were purified by further extractions with Tris-HCl (pH 7.5) saturated phenol:SEVAG (1:1), and then with chloroform. SEVAG contains isoamyl alcohol:chloroform (1:24). DNA samples were then precipitated with 1/10th volumes of 3 M NaOAc and 2.5 volumes of ethanol, rinsed with 80% ethanol, and resuspended in 50 µl TE10/1 containing 1 µl of 10mg/ml RNase A.

2.1.2b. Qiaprep Spin Miniprep Kit protocol (Qiagen)

The protocol is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt. The DNA is then washed with ethanol solution and eluted with the given Qiagen elution buffer or sterile glass-distilled water. This was designed for purification of up to 20 µg plasmid DNA from 1-5 ml overnight liquid culture of *E.coli* grown in LB medium. The bacteria were lysed under alkaline condition by addition of a given buffer solution, buffer P1. The lysate was subsequently neutralised and adjusted to high salt binding condition by the addition of to other buffer solutions, buffer P2 and buffer N3. The bacterial lysate was then cleaned by a quick centrifugation and the clear supernatant was passed through a membrane column to bind the DNA. The bound DNA was washed twice with ethanol solution, and eluted with warm sterile water. Purified DNAs were stored at – 20°C freezer.

2.1.3. Transformation of E. coli by heat shock

Aliquots of frozen *E. coli* cells were thawed on ice and dispensed into sterile 1.8 ml microcentrifuge tubes. The plasmid DNA was added and gently mixed with the cells, and they were incubated on ice for 10 minutes. Cell-DNA mixture was heat shocked at 55°C for 45 seconds then 0.5 ml of LB was immediately added. Cells were incubated for 1 hour at 37°C, and 250 µl was plated onto LB plates containing the appropriate antibiotic, then grown overnight at 37°C.

2.2. General DNA methods

2.2.1. Quantification of DNA

DNAs were quantified either by spectrophotometry, or by agarose gel electrophoresis and staining with ethidium bromide. For spectrophotometry quantification, DNA was diluted and dispensed into quartz cuvettes. Absorbance readings at 260 and 280 nm wavelength light were recorded. The quantity of DNA was calculated according the formula $C = OD_{260} \times 50 \times D$ for double stranded DNA,

D is dilution factor. 260/280 ratios were used as an indication of purity, a reading of 1.8 and above indicated relatively pure DNA samples.

To quantify DNA by agarose gel electrophoresis, DNA were run on 0.8-2.0% agarose gels along with known concentration of specific molecular weight markers. Gels were stained with ethidium bromide and visually compared to the known masses of particular fragments in molecular weight markers according to their molar ratios, either 211 ng of λ *Hind*III digest or 250 ng of 100 bp ladder (Molecular Weight Marker XIV, Roche) for DNA. For example, a 211 ng loading of λ *Hind*III contains 100 ng of the 23 kb fragment, and 25 ng of the 9.4 kb fragment.

2.2.2. Electrophoresis of DNA

DNA separation by electrophoresis was done by loading a given/approximate amount of DNA in 0.8 to 2.0% agarose gels in 1xTAE (40 mM Tris-acetate, 1 mM EDTA (pH8.0)) or 1xTBE (90mM Tris-borate, 1mMEDTA). Appropriate DNA molecular weight markers were used depending upon the sizing of the DNA fragments. Either 100bp ladder (Roche) (with 250 ng) and/or 211 ng of a λ *Hind*III digest were used as molecular weight markers. Ethidium bromide staining was done either by incorporating Ethidium bromide in the gels or by staining the gel in Ethidium bromide solution and visualised with either an IS1000 Digital Imaging System (Alpha Innotech Corp.), or an Ultralum Integrating CCD Camera (Ultralum Inc.).

2.2.3. Amplification of DNA by PCR

The PCR reactions used in this thesis were performed in 50 µl final volume containing a final concentration of 1x PCR buffer (10 mM Tris-HCl pH8.3, 50 mM KCl, 1.5 mM $MgCl_2$) (Roche), 1x PCR Enhancer Solution (Gibco BRL), 400 pM of each primer, 250 µM of each deoxynucleotide triphosphate (Roche) (250 µM dNTPs), and 2.5 units of Taq polymerase (Roche). 'Hot start' reactions were standard; Taq polymerase was excluded from the initial reaction mix of 40 µl

volume, a 10 µl volume enzyme mix containing 1x PCR buffer, 1x enhancer and 2.5 units of Taq polymerase was added to each tube during the first 95°C denaturation step. Most of the PCR reactions used in the experiments made use of the standard PCR recipe except that the templates and the set of primers used for each reaction varied. The PCR programs used very similar conditions except that when the primer set is varied, certain aspect of the PCR program is also varied. The annealing temperature, for example, depends on the melting temperature of the primers and the elongation time relates with the size of the amplification product. PCR reactions were done in an MJ Research PTC-200 DNA Engine.

2.2.4. DNA extraction protocol

The methods of DNA preparations and extractions used in this thesis varied depending upon the plant material used and the purpose for which the DNA was to be used. Maize genomic DNA was prepared using urea leaf DNA prep, while *Arabidopsis* DNA was prepared by using any one of the following: 1) urea leaf DNA prep, 2) the longer version of Shorty prep (from the Wisconsin Arabidopsis center protocol used for identifying knockouts) and 3) Qiagen plant DNeasy prep. For PCR genotyping purposes, *Arabidopsis* genomic DNA was extracted using a shorter version of the Shorty prep.

2.2.4a. Urea leaf DNA prep

For maize, approximately 1 g of leaf tissue was ground to a fine powder in liquid nitrogen, and scooped into a 15 ml falcon tube containing 5 ml of urea extraction buffer (6.9 M urea, 350 mM NaCl, 50 mM Tris-HCl pH8.0, 0. 20 mM EDTA, 1% N-lauroylsarcosine) and mixed. Five ml of Tris-HCl pH 7.5 saturated phenol:SEVAG (1:1) was then added and mixed by inversion then allowed to stand at room temperature for 15 minutes. The amount of the extraction reagents was reduced proportionately with less amount of plant tissues. For *Arabidopsis,* approximately 200-500 mg of plant tissue was used. Phases were separated by centrifugation at 4 000 rpm for 20 minutes at room temperature. The aqueous phase was pipetted to a new tube, and the nucleic acids were precipitated by adding

1/10th volume of 4.4 M NH_4OAc, and an equal volume of isopropanol, mixing gently. The strands of genomic DNA were spooled with a Pasteur pipette with a hook formed under a Bunsen burner flame, rinsed on the hook with 80% ethanol, and transferred to a 1.8 ml tube. The DNA pellet was air dried and resuspended in 100 µl of TE50/5 (50 mM Tris-HCl pH7.5, 5 mM EDTA).

2.2.4b. Qiagen plant DNeasy prep (Qiagen Cat No. 69104)

Using this procedure, a maximum of approximately 200 mg fresh weight starting material was used. The procedure involved 4 main steps. First, the plant material was disrupted by grinding in liquid nitrogen (in eppendorf) with a pestle made from pasteur pipettes.The DNA was then extracted by adding 400 µl of the extraction buffer, mixed by vortexing, incubated at 65 °C and centrifuged for 5 minutes at 14000 x g. The cell debris and salt precipitate were removed by passing the clear lysate through the Qiashredder spin column. The DNA was precipitated by adding 0.5 volume of Buffer AP3 and 1 volume of ethanol to the clear lysate, and mixed. The DNA was isolated and purified by filtration through a DNeasy minispin column. The bound DNA was washed twice with ethanol, centrifuged at 6000 x g and eluted with 50 µl warm sterile glass-distilled water.

2.2.4c. Shorty DNA Quick prep

Shorty DNA prep (from the Wisconsin Arabidopsis center). A cotyledon was nipped off the plant and placed into an eppendorf tube where leaf sample was ground in liquid nitrogen. To the ground sample, 500 µl of extraction buffer (0.2 M Tris-HCl pH 9.0, 0.4 LiCl, 25 mM EDTA, 1% SDS) was added, mixed thoroughly and placed on ice until all of the samples have been finished. After 5 minute centrifugation at 4°C, 350 µl of clear supernatant was transferred into a new tube containing 350 µl of isopropanol. The content of the tube was mixed by inversion, centrifuged, and then the pellet is dried by inverting the tube on a piece of clean paper towel. The DNA was resuspended in 40 µl sterile warm glass-distilled water. One µl of this was used for PCR reaction.

Longer version of Shorty DNA prep. Tissue samples in eppendorf tube were ground in liquid nitrogen. To the ground sample, 500 µl of extraction buffer (shorty buffer) and 500 µl of phenol (Tris-HCl)/Chloroform/ Iso-amyl alcohol (25:24:1) was added, vortexed and placed on ice until all of the samples have been finished. The mixture was centrifuged for 5 minutes at 4°C, the supernatant transferred into a new tube containing 500 µl of phenol mixture, mixed thoroughly by vortexing then transferred to ice until all samples have been finished. The mixture was centrifuged for 5 minutes and the supernatant was transferred to a new tube containing 500 µl of isopropanol, mixed by inversion, allowed to stand a minute at room temperature, then centrifuged for another 10 minutes. The pellet was dried by inverting the tube on a piece of paper towel, and resuspended in 500 µl of TNE buffer (0.01 M Tris-HCl, 0.1 M NaCl, 1 mM EDTA, pH 8.0) and 2 µl of Rnase (10 mg/ml stock). The solution was incubated for 10 min at 37 °C. Five hundred µl of phenol mix was added to the tube, mixed slowly by inversion, centrifuged at 4°C and the pellet rinsed with 80 % ethanol. The pellet was dried by inverting the tube on a paper towel and resuspended in sterile warm glass-distilled water.

2.2.5 Southern blotting with the DIG system

For maize, 1-2 µg of maize DNA was found to be optimal for detection of digoxygenin labelled probes with the DIG System (Roche), 100 ng of *Arabidopsis* DNA was sufficient. DNA was typically digested with 5 units of restriction enzyme, and 0.5 µl of 10 mg/ml RNase A at the recommended temperature for the restriction enzyme for at least three hours. Digested DNA was electrophoresed in 0.8% TAE gels at 70V overnight in an 20 cm x 25 cm gel tray, in a buffer circulating gel apparatus (Owl A5 Buffer Puffer™) alongside 4 µl of DIG-labelled λ *Hind*III digest (Roche DNA Molecular Weight Marker 2, DIG –labelled). The entire gel was then stained with Ethidium bromide, destained in water and an image captured as described in Section 2.2.2.

2.2.5a. DNA transfer to nylon membrane

The gels were first denatured and neutralised before transferring the DNA onto nylon membranes. Gels were soaked with gentle agitation twice for 15 minutes in 500 ml of denaturing solution (0.5 M NaOH, 1.5 M NaCl) and then twice for 15 minutes in 500 ml of neutralisation solution (1 M Tris-HCl pH7.2, 1.5 M NaCl). The gel was then transferred onto positively charged nylon membranes (Nylon+, Roche) by capillary transfer using a downward blotting stack using 10x SSC (3 M NaCl, 0.3M sodium citrate pH 7.0) as the transfer buffer. Downward transfer blotting stacks consisted of (from the bottom up); a 10-15 cm stack of paper towels, three sheets Whatman 3MM Chromatography paper the same size as the paper towels, nylon membrane, gels, two sheets of Whatman 3M paper the same size as the gel, a wick of two sheets of Whatman 3M paper that overlays the stack and feeds into two buffer reservoirs (containing 1 L of 10x SSC) on either side of the stack, the gel tray and a weight. All layers apart from the paper towels were pre-wet with 10x SSC. Blotting stacks were left to transfer overnight, and then the membranes were crosslinked with 120 000 μJ/cm^2 ultraviolet light in a UV Stratalinker 2400 (Stratagene). Membranes were then sealed in a plastic bag, and stored at 4°C until further use.

2.2.5b. DNA labeling

Digoxigenin probes were synthesised by PCR according to the standard PCR protocol (Section 2.2.3) except that a DIG labelling mix was used in place of dNTPs. Final concentrations of nucleotides were 250 μM dATP, 250 μM dCTP, 250 μM dGTP, 166 mM dTTP, 40 μM DIG-dUTP. Conditions for particular primer sets were optimised with standard (non labelled) PCR reactions, and 50 to 100 pg of the PCR product from the optimised reaction was used as templates for the labelling PCR reaction. Labelled PCR products were examined by gel electrophoresis for quality; expectations were of a single band of higher molecular weight than the unlabelled PCR product, and quantity by comparing to a known mass of a molecular weight marker.

2.2.5c. DNA-DNA hybridisation

Membranes were pre-hybridised for an hour in 10 ml of DIG Easy Hyb (Roche) at 65°C for maize and 55°C for *Arabidopsis.* Approximately 50 ng of DIG labelled probe was diluted in 0.5 ml of sterile glass-distilled water, boiled for 5 minutes, then immediately chilled on ice for 5 minutes. The probe was added to 10 ml of fresh DIG Easy Hyb, and hybridised overnight at 65/55°C. Membranes were washed twice in 2x SSC, 0.5% SDS in hybridisation tubes at 65/55°C, then twice in 2x SSC, 0.5% SDS in plastic containers at room temperature to remove excess probe. Stringency washes were performed in 0.1 x SSC, 0.1% SDS at 65/55°C for 15 minutes. Detection of DIG labelled probes was performed as recommended by Roche using the CDP-Star™ substrate. Anti-Digoxigenin-AP antibody was used at 1:20 000 dilution. Membranes were exposed to film for 30 min to 2 hours.

2.2.6. Southern blotting with radiolabeled probes

DNA was prepared, digested, electrophoresed, stained and transferred to nylon membranes as in Section 2.2.5. The only difference is the method used to synthesize probes.

2.2.6a. Synthesis of radiolabeled probes

Random-primed α^{32}P dCTP labelled probes were synthesised from templates of either PCR products or the entire *te1* cDNA cut from the vector with appropriate restriction enzymes and gel purified. The DNA insert was cut from the gel with a sterile scalpel blade, using a long wavelength UV lamp to visualise the band. The DNA was extracted from the gel slice with a Concert Gel Extractions Kit (Gibco BRL), and eluted in 30 µl of sterile glass-distilled water. Radiolabelled probes were synthesised as follows; probe templates were quantified by ethidium stained 100 ng of template DNA was diluted to 7 µl volume in sterile Milli-Q water, boiled for 5 minutes in a 1.8 ml micorcentrifuge tube, then immediately chilled on ice for 5 minutes. Templates were mixed on ice with a priming reaction mix to a final

concentration of 75 µM random hexamer primer, 375 µM of dATP, dTTP and dGTP, 2 units of Klenow enzyme (Roche) to a volume of 15 µl. Finally, 5 µl (50 µCi) of α^{32}P dCTP (NEN Research Products) was added and mixed. The reaction was incubated at 37°C for 1-2 hours. Probes were then diluted to 50 µl volume with 30 µl of sterile glass-distilled water, and the unincorporated nucleotides were removed with Sephadex G-50 spin columns (ProbeQuant™ G-50 Micro Columns, Pharmacia). Probes were used immediately or stored at -20°C.

2.2.6b. DNA-DNA hybridisation

Membranes were pre-hybridised for an hour in at least 10 ml of Church/Gilbert hybridisation buffer (0.5 M $NaHPO_4$ pH 7.2 [2 M $NaHPO_4$ stock is composed of 35.6g of Na_2HPO_4 and H_3PO_4 to pH 7.2], 7% SDS, 1 mM EDTA) (Church 1984) at 65°C for maize and 55°C for Arabidopsis. Probes were boiled for 5 minutes in 500 µl of sterile Mill-Q water, chilled on ice for 5 minutes, and added to 10 ml of 65/55°C Church/Gilbert hybridisation buffer. Membranes were hybridised overnight at 65/55°C. Membranes were washed twice in 2 x SSC, 0.5% SDS in hybridisation tubes at 65/55°C, then twice in 2 x SSC, 0.5% SDS in boxes at room temperature to remove excess probe. Stringency washes were performed in 0.1 x SSC, 0.1% SDS at 65°C for 15 minutes. Membranes were sealed in plastic bags before being exposed to film.

2.3. General RNA methodology

2.3.1. Precautions to avoid RNase contamination

Glasswares used for RNA work were rinsed with sterile water, baked overnight at 180°C and set aside primarily for RNA work. Plastic wares were either new (plastic pipettes) or washed thoroughly and rinsed with sterile water, then treated overnight or longer in 0.3% H_2O_2 (Andrew Industrial Ltd.). Solutions used for RNA work were prepared using baked glasswares, were not used for any other purpose, and were always handled with gloves. All solutions, except those

containing primary amines (such as Tris), were treated with diethylpyrocarbonate (DEPC) or dimethylpyrocarbonate (DMPC) (Sigma) prior to use. was added to solutions in baked bottles to a final concentration of 0.1% by volume, shaken for a minimum of two hours at 37°C, then autoclaved twice. The pH of critical solutions was checked, and readjusted after treatment with DEPC/DMPC. The electrode used with the pH meter was treated with 50 mN NaOH for 10 minutes, then rinsed with sterile glass-distilled water prior to use with RNase-free solutions. Solutions that could not be treated with DMPC/DEPC (such as Tris) or could not be autoclaved (such as SDS) were prepared from new containers set aside for RNA work using sterile glass-distilled water and baked glassware. For all *in situ* work, autoclaved glass distilled water was used.

2.3.2. RNA labeling

DNA templates were either prepared from a plasmid DNA linearised at restriction sites downstream of the cloned insert or synthesised by PCR using the general forward and backward primers M13. RNA are labelled using digoxigenin using either SP6, T7, or T3 polymerase according to Boehringer Mannheims protocol. The transcription reaction was done in microfuge consisting of the following: template DNA, 1x transcription buffer (40mM Tris-HCl, pH 8.0, 6mM MgCl, 10mM dithioerythritol (DTE), 2mMspermidine,10mM NaCl, 1 unit of RNase inhibitor), NTP labeling mixture(with a final concentration of 1mM ATP, 1mM CTP, 1mMGTP, 0.65mMUTP, 0.35 mM DIG-UTP; in Tris-HCl, pH 7.5), DMPC-treated water and RNA polymerase (final conc 2 units/ul). The reaction mixture was incubated at 37°C for a minimum of 3 hours. At the end of the reaction, a 2 ul of the reaction mixture was loaded onto a non-denaturing gel to determine whether there was a product. Two microliter of DNase was added to the transcription mixture then incubated further for 15 minutes to digest untranscribed DNA. With or without DNase treatment, the reaction was stopped with a mixture consisting of EDTA:LiCl. RNA was precipitated with ethanol, incubated at -20°C overnight. The RNA was washed with chilled 70% ethanol twice, then after a final wash with 100 %ethanol, dried and resuspended in 50 % formamide.

2.3.3. Quantification of DIG-labeled RNA

DIG-labeled RNAs were quantified by a spot test with DIG-labeled control or by electrophoresis of glyoxylated RNA and subsequent colorimetric determination colorimetric determination.

2.3.3a. Spot test method (Boehringer Mannheim protocol)

Dilution series of both the RNA sample and the control are prepared and spotted on a piece of nylon membrane (Boehringer Mannheim, DIG Users Guide). The RNA were fixed to the membrane by cross-linking in UV light and colorimetrically detected according to the DIG Users guide. The membrane was washed briefly with washing buffer (100 mM maleic acid, 0.15mM NaCL pH7.5, 0.3% Tween 20) before blocking (1% blocking reagent in maleic acid buffer). Anti-DIG-alkaline phosphatase at 1:5000 was added and the membrane incubated in the antibody for another 30 minutes at room temperature. The antibody was rinsed off by washing the membrane twice with washing buffer. After incubation in detection buffer for 5 minutes, a color substrate solution consisting of 45 µl NBT solution(75 mg/ml nitroblue tetrazolium salt in dimethylformamide) and 35 µl BCIP (50 mg/ml 5-bromo-4-chloro-3-indoyl phosphate toluidinium salt in dimethyformamide) in 10 ml of detection buffer. The amount of color substrate was approximated to have enough solution covering the whole surface of the membrane. After incubation in the dark, yield was estimated by comparing the color intensities of sample with the control.

2.3.3b. Electrophoresis and colorimetric detection of glyoxylated RNA

This was done not only to quantify the RNA but to confirm if the RNA probes are of the correct size. Spot test may reveal the yield of RNA but for efficient *in situ* hybridisation, the RNA have to be of the correct size, less than or equal to 150 base pairs. A 0.1 M sodium phosphate buffer (NaPB) stock was prepared by mixing 1M NaH_2PO_4 (approximately 42 ml), and 1 M Na_2HPO_4 (approximately 58 ml) until the pH is 7.0, diluting 1:10 with water to 0.1 M, and DMPC/DEPC treatment. RNA was

glyoxylated in a glyoxylation mix containing 1 M deionised glyoxal (Sigma), 50% DMSO (Fluka Molecular Biology grade), 10 mM NaPB pH7.0 for 1 hour at 50°C. Glyoxal was deionised as described in Sambrook (1989). The gel apparatus and silicon tubing for a peristaltic pump (Cole Parmer Instrument Co.) was treated with approximately 0.3% H_2O_2 (Andrew Industrial Ltd.) overnight to inactivate RNases. Molten 1.0% agarose was prepared in baked glassware in 10mM NaPB buffer. To inactivate RNases in the molten agarose, sodium iodoacetate (Merk-Schuchardt) to 10 mM concentration was mixed into the molten agarose once it had cooled to approximately 70°C. Once the agarose had set, 10 mM NaPB buffer was circulated over the gel for an hour prior to electrophoresis. Glyoxylated RNA was mixed with 1 µl of RNA loading buffer (50% glycerol, 10 mM NaPB, 0.25 % bromophenol blue, 0.25% xylene cyanol, DMPC treated) per 15 µl of sample and loaded onto the agarose gel. RNA was electrophoresed at 45 mA for 30 minutes without buffer circulation, and for approximately 4 hours at 45 mA with vigorous circulation with the peristaltic pump (approximately 150 ml/minute, total buffer volume was 1.2 L). Three µl of Gibco BRL 0.24-9.5 kb RNA molecular weight ladder was run in the first lane of the gel.

The portion of the gel that was to be stained with ethidium bromide (including the molecular weight marker) was cut from the remainder of the gel with a sterile scalpel blade; those lanes that were to be transferred to a membrane were not stained with ethidium bromide. Staining was carried out in trays protected from light with aluminium foil, gels were soaked for 10 minutes in 50 mM NaOH, stained for 30 minutes in 10 µg/ml ethidium bromide in 0.1 M ammonium acetate (NH_4OAc), and destained for 30 minutes in 0.1 M NH_4OAc. Images of ethidium stained gels were captured under ultraviolet light with an IS1000 Digital Imaging System (Alpha Innotech Corp.), with a ruler included for molecular weight calibration.

RNA was transferred to positively charged nylon membranes (Nylon+, Roche) with a downward transfer blotting stack wet with DMPC-treated 10 x SSC (3 M NaCl, 0.3M sodium citrate pH 7.0) without further treatment of the gel. Stacks were usually left to transfer overnight. RNA was fixed by crosslinking with 120 000 µJ/cm^2 ultraviolet light in a UV Stratalinker 2400 (Stratagene). Membranes were either

processed for colorimetric determination as described in Section 2.3.3a or sealed in a plastic bag, and stored at 4°C.

2.4. Hybridisation of DNA membranes with RNA probes

All experimental conditions referred to in here apply only for *Arabidopsis*. This was done to detect cross-hybridisations among the 3 *Mei2*-like RNAs (instead of using synthetic RNAs, however, DNAs were spotted onto a nylon membrane. Serial dilutions of DNA (PCR products) were first prepared in microtitre plates, the one µl each the different dilutions was spotted onto a nylon membrane. The RNA were fixed to the membrane by cross-linking in UV light.

All hybridisation procedures used H_2O_2 treated hybridisation tubes, or H_2O_2 treated plastic boxes. Immediately prior to pre-hybridisation, RNA probes were prepared as described in section 2.3.2. Prehybridisation and hybridisation procedures were performed as in Southern blotting using DIG-labeled probes (Section 2.2.5c) except for some modifications. Hybridisation in this experiment was between a DNA membrane probed with DIG-labeled RNA. Membranes were pre-hybridised for an hour in at least 10 ml of DIG easy hybridisation buffer at 55°C. Probes were heated for 5 minutes at 80°C in 500 µl of sterile glass-distilled water, chilled on ice for 5 minutes, and added to 10 ml of 55°C DIG easy hybridisation buffer. Membranes were hybridised overnight at 55°C. Membranes were washed twice in 2 x SSC, 0.5% SDS in hybridisation tubes at 55°C, then twice in 2 x SSC, 0.5% SDS in boxes at room temperature to remove excess probe. Stringency washes were performed in 0.1 x SSC, 0.1% SDS at 55°C for 15 minutes. Colorimetric detection of DIG labelled hybrid was done using NBT and BCIP solutions as described in Section 2.4.3a.

2.5. Microscopy methods

Tissues for light microscopic analysis were fixed with FAA fixative (ethanol, acetic acid, formaldehyde, water 50:5:3.7:41.3) for a minimum of five hours, rinsed

with 50% ethanol and dehydrated through ethanol series; 70, 85, 96, 100 % ethanol. After 2 hour incubation 2 x with 100 % ethanol, these were cleared with a xylene substitute, Histoclear (National Diagnostics), infiltrated and subsequently embedded in wax. Wax blocks were stored in the cold room (4 ^{o}C) until further processing. Serial sections through the apex were cut at 8 µm with Leica microtome using disposable blades, and stained with safranin-green or toluidine blue. Microscope slides were mounted with a xylene based mountant DPX after dehydration through ethanol and Histoclear (Shandon). Microscope slides were examined under bright field on a microscope and images were recorded using Kodak Ektachrome or digital camera attached to an Imaging system.

Scanning electron microscopic analysis of vegetative shoot apices was done using a modified replica technique (Sylvester *et al.,* 1990), details of which, is presented in Section 3.2.7a.

2.6. In situ hybridisation protocol

The general precautions to avoid RNAse contamination (Section 2.3.1) are observed throughout the conduct of the experiment. Gloves were changed often when gloved hands accidentally touched a " RNAse contaminated" working surfaces.

2.6.1. Fixation of plant tissue materials

Plant tissues were either fixed in 4 % paraformaldehyde (paraformaldehyde in 1x PBS, 130mM Na2HPO4, 3mM NaH2PO4) (Jackson 1991) or FAA (ethanol, acetic acid, formaldehyde, water, 50:5:3.7:41.3) (Drews and Okamura,1996).

2.6.1a. FAA fixation

Fresh fixative (prepared in the same day) was used in all tissue preparations. Plant tissue materials to be fixed were immersed in fixative as soon as

unwanted plant parts were removed. The materials were then vacuum-infiltrated for 15 minutes. The vacuum is pulled slowly to prevent damage of the tissues. The solution was never allowed to boil. The materials were incubated in the fixative for 3 hours at room temperature. After 3 hours, the fixative was removed and the materials were rinsed with 50 % ethanol and passed through subsequent dehydration steps.

2.6.1b. Paraformaldehyde fixation

Fresh paraformaldehyde fixative was prepared in the same day that the tissues were to be fixed. Paraformaldehyde (4%) is dissolved in 1x PBS (100 mM phophate buffered saline solution). To facilitate dissolution of paraformaldehyde, 1x PBS is first heated at 60-70 ^{o}C and the pH increased to 11.0 before paraformaldehyde is added. After paraformaldehyde has dissolved, the pH is reduced to 7.0 with H_2SO_4. The fixative was kept on ice all throughout the fixation process. Plant tissues to be fixed were immersed in the fixative after unwanted plant parts have been removed. The materials were then vacuum-infiltrated for 15 minutes. The vacuum is pulled slowly to prevent damage to the tissues. The materials were incubated in the fixative overnight (less than 16 hours) at 4 ^{o}C. After incubation, the fixative was removed and the tissues were rinsed with cold 0.85% NaCl. The tissues then passed through subsequent dehydration steps.

2.6.2. Dehydration

Dehydration was done with a series of ethanol solutions. When the tissues were fixed with FAA, dehydration starts with 50% ethanol. Incubations in ethanol solutions were done for a minimum of 1 hr. Ethanol solutions was prepared using sterile glass distilled water. When paraformaldehyde was used as the fixative, dehydration starts with 0.85% NaCl and subsequent ethanol solutions with the following concentrations: 15, 30 and 50 %in 0.85% NaCl. From 50 % ethanol solution, the tissues (fixed in either way) were transferred to a 50 % TBA (tertiary butyl alcohol) solution and left at 4 ^{o}C overnight. In the morning, the tissues were transferred and incubated for a minimum of one hour in a series of TBA solutions,

70, 85, 90, 100%, and 3 times 2 hour incubation in pure TBA (in any one of the pure TBA, the tissues were left in the solution overnight).

2.6.3. Infiltration of wax

Before wax infiltration, an equal volume of paraffin oil was added into the sample-TBA to prevent damage of the tissues when exposed to high temperature. The tissues were kept overnight in this solution. The next day, the tissues inTBA/paraffin mixture were transferred into a sterile container with molten wax and placed in 60 °C oven. Wax was changed 6 more times, (the change was done 2x a day) before wax embedding. A histochemical embedder was used to prepare wax blocks. These blocks were stored in 4 °C cold room when not immediately used. The blocks were kept in the cold room for a period of 6 months without any apparent loss in the signal.

2.6.4. Sectioning

Tissues were sectioned at 8-10 μm except for siliques of *Arabidopsis* which were sectioned at 12 μm. Sections were cut using disposable microtome blades (Feather Blades S 35) in a Leica microtome, floated on 42°C water and captured with precoated microscope slides (Fischer, Scientific, US and/or Esco superfrost plus coated microscope slides, Biolab Scientific). Microscope slides were baked at 42 °C in a warmer overnight and kept in a dessicator afterwards for a maximum of three days when not processed immediately.

2.6.5. Probe synthesis

Digoxigenin-labeled RNA probes were synthesised with in vitro transcription using T3 or T7 RNA polymerase according to manufacturers instruction (Boehringer Mannheim) as described in Section 2.4.2. Templates for transcription of probes were derived from PCR-amplified cDNAs using general primers M13F and M13R. Control experiments (negative control) were done using sense probes of each and no signal above background was detected. The RNA probes were hydrolysed to reduce probe size and allow better penetration of probes into the tissue. These were

chemically degraded to a mean length of 100 - 150 bp with sodium carbonate buffer, pH 10.2 using the given formula to calculate the incubation time for each probe.

Incubation time (in minutes) = (Lo-Lf) / (K) (Lo) (Lf)

Where t – hydrolysis time in minutes

Lo -- starting length in kb

Lf – final length in kb= 0.1kb

K – rate constant for hydrolysis = 0.11 kb^{-1} min^{-1}

The hydrolysis reaction was stopped with 10 µl of 1 M sodium acetate (pH 4.7) and the probes were precipitated with 1 ul glycogen, 10 µl 4 M LiCl and 300 ul chilled ethanol. This was incubated at -20°C overnight. The probe was then washed twice with cold 70% ethanol, dried and resuspended in 300 µl 50% formamide (Molecular Biology Grade) solution.

Probe concentrations were estimated according to Boehringer Mannheim procedure of probe quantification. About 200 ng/ml/kb of probe complexity was used in hybridisations.

2.6.6. Pre-treatments and RNA In situ hybridisation

2.6.6a. Pre-treatments

Section pre-treatments were done according to the method of Jackson et al. (1991). The microscope slides passed through the following solutions for section pre-treatments: First, the slides were dewaxed by passing through 2 separate solutions of histoclear (National Diagnostics), 10 minutes per change, then through sequential rehydration steps, a minute in each of 2 x 100 % ethanol, 96 % ethanol, 85%, 70% , 50%, 30%,15%, 0 ethanol solutions. All of the ethanol solutions except 96% were diluted with sterile 1 x PBS (phosphate buffered saline solution). The next step was pronase treatment (0.125 mg/ml pronase, Sigma type XIV) in 50 mM Tris-

HCl, pH 7.5, 5mM EDTA). This step degrades proteinaceous material and allows cell permeability for probe penetration. The optimal duration of the pronase treatment was determined by varying duration and concentration of pronase. The tissues were washed with 1xPBS before post fixation with 4% paraformaldehyde. Post fixation was followed by acetylation reaction (3 ml acetic anhydride in 0.1 M triethanolamine) which was supposed to reduce background through acetylation of exposed charges. After washes in 1x PBS the tissues were dehydrated back throught the ethanol solution with fresh ethanol asolution at the end. This allows easier probe penetration into the dehydrated cells. The slides were vacuum dried in a glass dessicator for an hour before probe application.

2.6.6b. Hybridisation

RNA probes were diluted at the required hybridisation concentration with hybridisation buffer (50% formamide, 300 mM NaCl, 10 mM Tris(7.5), 1 mM EDTA 5% dextran sulfate, 1% blocking reagent, 150 μg/ml tRNA) and Rnase inhibitor. Approximately 100 μl was used per slide using the sandwich method where the same RNA probe was applied to a set of two microscope slides and these were incubated facing each other with the probe between them like a sandwich. The slides were placed in a covered plastic container where the slides were laid down on an elevated level and the bottom of the container was lined with wet Whatman paper to maintain a humid atmosphere inside the box. The paper was wet with 2 X SSC in 50% formamide. Hybridisations were done at 42°C. After an overnight incubation, low and high stringency ashes were done according to Drews and Okamura (I996) protocol. The microscope slides were washed once with low stringent saline solution (2 x SSPE) at room temperature and twice with high stringency washes (0.2 x SSPE) at 55 °C for an hour per wash. RNase treatment was done in some cases but in most of the experiments, no RNase treatment was done. A final rinse was done using 1x TBS (Tris buffered saline solution) at room temperature for 10 minutes.

2.6.7. Immunological detection

Immunological detection was done according to Drews Lab and Okamuros Lab (l996) using Boehringer Mannheim DIG Nucleic Acid Detection Kit (# 1175041) with some modifications. Blocking solution was prepared using maleic acid buffer (100mM Maleic acid, 0.15mMNaCL) but blocking was done with 1 x blocking solution in 1x TBS. Antibody reaction was done using 1:1000 dilution of DIG-AP conjugate in BSA wash solution (1% Bovine Serum Albumin (Boehringer Mannheim), 0.3% Triton X-100, 100mM Tris-HCl, pH 7.5), 150 mM NaCl). Antibody was washed off the slides 3 x with BSA wash solution followed by incubation in detection buffer, TNM 50 buffer (100 mM Tris (9.5), 100 mM NaCl, 50 mM $MgCl_2$. Color reaction was done with color substrate solution (4.5 µl NBT and 3.5 ul BCIP in 1 ml of detection buffer). One hundred µl of the color substrate was applied onto each slide then covered with Esco coverglass. The slides were kept in a humidified plastic container and incubated in the dark. The progress of color reaction was monitored beginning at about 24 hours. In some cases, additional substrate was added to enhance color reaction. I normally stop the reaction after 3 days with T10E1. Microscope slides were either mounted with the use of a water based mountant (Immu-mount, Shandon). Sections were viewed with a Nomarski optics of a Zeiss Axiophot compound microscope. Digital images and photographic images were taken using an attached camera or a video digital camera. Images were captured with SGI imaging system.

Chapter 3. Morphological characterisation of the te1 mutant and localisation of gene transcripts

Abstract

This chapter describes molecular and genetic analyses that were done to further characterise the maize *terminal ear 1* gene. Loss of function mutation in *te1* results in increased rates of leaf initiation, which is associated with altered meristem size. Morphological characterisation and histological analyses show that the increased rate of leaf initiation and irregular phytomer development in the mutant plants was correlated with smaller meristems. Clonal analysis, likewise, shows a reduced number of leaf founder cells in the mutant plants. The downregulation of *kn1* transcripts, a molecular marker for leaf identity, occurs higher in the *te1* shoot apex than in the wild type. *In situ* hybridisation experiments showed a tight correspondence between *te1* expression and leaf initiation. The possible role of *te1* in the regulation of shoot development is discussed.

3.1. Introduction

Organogenesis is a major function of the shoot apical meristem. The pattern in which organs are positioned in the shoot axis is a key factor in specifying a plant's architecture (as discussed in Chapter 1). Leaves are initiated at the flank of the shoot apical meristem (Steeves and Sussex, 1989) from a predictable number of cells, which as indicated by clonal analyses is from about 100-200 cells from the peripheral zone spanning all three tiers of cells in the SAM (Poethig, 1987, Poethig and Symkowiak, 1995). Surgical experiments provided initial evidence that the pattern by which leaves are arranged in the meristem depends on position rather than lineage (Snow and Snow, 1931, Steeves and Sussex, 1989). In these experiments, incisions that isolate leaf primordia from organogenic domains within the SAM altered the position at which a new leaf emerged, supporting the hypothesis that positional information specifies the positions where leaves will

emerge. This view is further supported by genetic analysis that shows highly variable patterns of cell division can nevertheless produce leaves with a consistent form (Smith *et al.,* 1996).

Recent molecular genetic analyses have also increased understanding of the mechanisms involved in organ formation. The downregulation of *kn1*-like homeobox genes in regions corresponding to the position where a leaf is initiated is considered to be an early marker for leaf founder cell identity (Jackson *et al.*, 1994; Chuck *et al.*, 1996). Both *kn1* transcripts and proteins accumulate to high levels throughout the shoot apical meristem but is downregulated in leaves and leaf primordia. This downregulation of *kn1* occurs early during the leaf initiation process before any morphological signs that a leaf has formed. *kn1* is absent in ring of cells whose position on the flank of the SAM implies a leaf founder cell identity (Smith *et al.*, 1992, 1995; Jackson *et al.*, 1994). This interpretation is supported by the correspondence in the number of cells in which this down regulation occurs with the number of leaf founder cells estimated from clonal analyses (Poethig and Symkoviak, 1995). In the same manner, the *Arabidopsis SHOOTMERISTEMLESS (STM),* a member of *kn1*-like genes, is also expressed throughout the shoot apical meristem, where it maintains meristem cells in undifferentiated state but is downregulated in positions where leaves are initiated (Long *et al.*, 1996).

By contrast, the expression of some genes is restricted to these organ forming regions, suggesting they may serve some role in the patterning or differentiation process. In *Arabidopsis,* for example, *AINTEGUMENTA (ANT)* gene is specifically expressed in the peripheral regions where organs are formed (Elliot *et al.*, 1996). The loss of function mutations in *ANT* cause a reduction in lateral organ number and size associated with a decrease in cell number, while, overexpression of *ANT* results in enlarged organ size which was correlated with an increase in cell number. *ANT* appears to be involved directly in organogenesis by sustaining cell proliferation and cell growth (Mizukami and Fischer, 2000).

Although a number of experimental approaches have now provided insights into the process of leaf initiation, less is understood of mechanisms that determine

earlier events in the leaf initiation process, the timing and position of initiation. A number of mutations have been identified that disrupt the pattern of leaf initiation and leaf development in plants. The *abphyll* mutation alters the distichous phyllotactic pattern in maize. In the *abphyll* mutant plants, leaves are initiated in decussate pattern, wherein two leaves are formed per node and offset by 90° instead of the normal distichous phyllotaxy, where the leaves are initiated singly and separated by 180° (Greyson et al., 1978). The altered phyllotactic pattern was associated with increased meristem size (Jackson and Hake, 1999). The decussate shoot meristems were found to be larger than the normal meristems, though the general structure of the meristem is not altered. Another maize mutant, *narrow sheath*, produces narrow leaves, which was associated with less founder cell allotment for leaf formation (Scanlon *et al.*, 1996).

In an attempt to address the issue in more detail, we have analysed the *terminal ear 1* mutant of maize, which appears perturbed in both the timing and position of leaf initiation. The mutant plant was originally identified in 1957 and initially described in terms of its short stature and tassel feminisation, which results in the terminal ear appearance (Matthews *et al.*, 1974). In a later study, phenotypic characterisation showed that *te1* plants produce leaves more frequently than the wild-type, often times accompanied by abnormal leaf development and irregular phyllotaxy (Veit *et al.*, 1998). A role for maize *te1* in the regulation of leaf initiation has been postulated based on the characterisation of mutant phenotype and the patterned expression of the normal gene. *In situ* hybridisation experiment showed that the *te1* mRNA is expressed predominantly in the SAMs as series of semi-circular rings that bracket the sites of leaf initiation. Given that the *te1* mutant is recessive, it is assumed that the mutant phenotype reflects loss of function, and that the functional gene is thought to constrain positioning of leaf primordia by precluding leaf initiation at sites where the *te1* gene product is present (Figure 3.1).

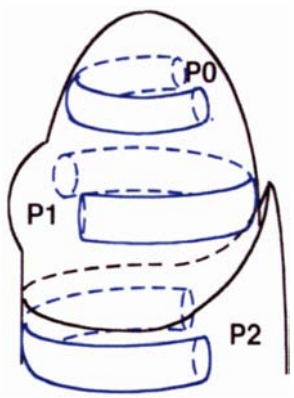


Figure 3.1. A model of *te1* function based on the expression of *te1*. (Veit *et al.*, 1998). The gene product is expressed as semi- circular rings at the base of each phytomer. It was proposed that leaf initiation is repressed in regions where the *te1* gene product is expressed.

Loss of *te1* function affects not only the rate of leaf initiation, but also phytomer development. Mutant plants are significantly shorter than their wild-type siblings, with abnormally shortened internodes concentrated in the apical region of the mature shoot. The irregularly shortened internodes show a pronounced length asymmetry with the short side of the internode located consistently directly below the midrib of the leaf above. Given that in the maize phytomer, a leaf is clonally related to the internode below it (Poethig, 1986; McDaniel *et al.*, 1988), the aberrant growth of the internodes might be attributed to the precocious initiation of leaves. A hypothesis was developed that linked abnormal internode development to precocious leaf initiation, supposing that as a consequence of a leaf being initiated higher on the apical dome than normal, there would be fewer uncommitted cells in this region to partition between the leaf and adjacent internode. The truncated and asymmetric internode that results would be attributed to its initiation from a reduced number of cells.

This study was done primarily to test elements of the above hypothesis, and to determine more precisely, the nature of morphological changes in maize shoot development that result from the loss of *te1*. Given the close linkage between the geometry of shoot apices and the process of leaf formation, microscopic analyses were done to compare the shoot apices of *te1* from their normal siblings. Clonal analysis was done to estimate the apparent cell number involved in leaf formation in

te1 mutant compared with their normal siblings. Expression analyses were done to determine the spatial distribution of the *te1* gene product during the early vegetative development of the plant, particularly during the initiation of the first few embryonic leaves. The relationship between *te1* expression and the leaf initiation was further explored by localising its mRNA in the *abphyll* mutant of maize, in which leaves are formed at abnormal positions. The expression pattern of a leaf marker gene, *kn1* was also analysed in *te1* mutant to assess how early, molecularly defined events in the leaf initiation process might be influenced by *te1* activity.

3.2. Materials and Methods

To make morphological comparisons between the mutant and the wild-type without the complications of genotypic variability, the reference allele, *te1-1,* was introgressed to B73 and A188 maize inbreds for three generations. Comparisons were made between mutant and the normal siblings from the progeny of a segregating third generation heterozygous plants. However, only the analyses of the plants introgressed to B73 are reported in detail in this thesis. The following 3 experiments were done under this phase of the study:

3.2.1. Experiment 1. Histological analysis of leaf initiation and meristem size

Seeds from a segregating population of *te1-1* were sown in seedboxes and grown under Glasshouse 2 of Massey University for the following purposes: 1) microscopic dissection to determine the frequency of leaf initiation; 2) tissue fixation for expression analyses; 3) light microscopic and SEM analyses of vegetative shoot apices to determine meristem morphology. Approximately 200 seeds were planted per unit time for each of the purposes enumerated above.

Field experiments were also done at the same time primarily to generate seeds. From one of these field experiments, (October 1998-March 2000 planting), controlled pollination was done to generate embryos for morphological and expression analyses. Plants heterozygous for *te1-1* were used in the analyses.

3.2.2. Experiment 2. Morphological characterisation of the te1 mutant

Seeds from a segregating population of *te1-1* were sown in plastic pots (size 18), thinned to 1 plant per pot, and grown in a glasshouse (Plant Growth Unit, Massey University, August-December 1997) for morphological analyses. The plants were grown under controlled temperature of 21°C/24°C. A total of eighty plants were maintained and grown to maturity under the glasshouse, 30 of which are *te1* mutants. Data were gathered on the following: 1) leaf sizes; 2) shoot juvenile traits; 3) internode lengths and; 4) flowering traits.

3.2.3. Experiment 3. Clonal analysis

Field experiments were done in 2 seasons (October 1998 – March 1999 and November 1999 – March 2000) for clonal analysis. A corn ear produced from a cross between a homozygous *te1-1* and heterozygous *wd (white deficiency,* an albino recessive marker*)* was used as a starting material. During the first growing summer season, these double heterozygous seeds were sown in the field and these were selfed to generate F2, a segregating seed population from which *te1* mutants heterozygous for the albino marker (*te1/te1, +/wd)* and normal plants carrying the heterozygous albino mutation (+/+, +/*wd* or +/te1, +/wd) were generated. During the second growing season, the seeds were irradiated for the clonal analysis (Table 3.1).

F2 seeds were first imbibed in water for 24 hours at room temperature (18-20 °C), allowed to germinate at radicle emergence in wet Whatman paper in 48 hours (kept under humidified condition) and were irradiated with 2000 rads x-rays at the Palmerston North General Hospital, New Zealand. Approximately 10,000 seeds were used in the experiment and grown in the field (AG Hort Nursery field area, Massey University). From Table 3.1, 1/8 of the population were expected to be *te1* mutants heterozygous for the albino marker and 3/8 of the population would be normal plants carrying *wd/+.* Plants were planted in the field immediately after irradiation, with a spacing of 0.25 m between hills and 0.5 m between rows. When the plants have grown and about 5 -7 leaves have been formed, the population was

scoured initially for sectored plants. Only plants that have single sectors were considered for the analyses. The leaves of these plants were tagged for identification. Sector measurements were done when the first leaves have fully expanded and while the plants were still standing in the field. When about 50 % of the plants were at anthesis (staminate flowers open), the population was scoured for the second and final time for sectored plants. The plants were properly tagged and the leaves of each plant numbered from base to the apex, where leaf number 1 is the most basal leaf.

3.2.4. Plant measurements

From a total of eighty plants grown and maintained under glasshouse conditions, ten mutant plants and 10 normal siblings were used in the plant measurements. Plant height was measured from the lowest node to the tip of the tassel. The leaves were tagged for identification as they emerged from the shoot. Leaves were numbered according to their position relative to the basal portion of the shoot. Leaf number 1 is the most basal leaf and the 1st leaf that came out of the shoot. Leaf size measurements of the 1st five leaves were done before they have senesced by carefully dissecting out each leaf from around the stem. Leaf dimension measurements were made of all leaves of the sample plants. Leaf blade length was measured along the midrib of the leaf from the ligule to the tip. The width was measured across three different points, ¼, ½, and ¾ of each leaf length. Unless specified, leaf width was taken as the width measurement along the midpoint (which also corresponds to the largest leaf width) of the leaf length. Sheath length was measured from the point of insertion to the ligule and sheath width was measured across the midpoint of the sheath length. The data were averaged and statistically analysed using completely randomised design (CRD). The plants were scored visually for the presence of wax on the leaf blade after the leaf had expanded fully (Moose and Sisco, 1996). The plants were harvested during

Table 3.1. Genotypes, phenotypes and phenotypic ratios of the maize plants used in the clonal analysis.

Seed generation	Genotypes	Phenotype	Phenotypic ratio
F1	a. *te1*/+,*wd*/+;	normal	
	b. *te1*/+, +/+	normal	
F2	1. +/+, +/+	normal	1/16
	2. +/*te1*,+/+	normal	2/16
	3. +/+,+/*wd*	normal	2/16
	4. +/*te1*,+/wd	normal	4/16
	5. +/+,wd/wd	Normal, albino leaves	1/16
	6. +/*te1*,wd/wd	Normal, albino leaves	2/16
	7. *te1*/*te1*,+/+	*te1* mutant	1/16
	8. *te1*/*te1*,+/wd	*te1* mutant	2/16
	9. *te1*/*te1*,wd,wd	*te1* mutant, albino leaves	1/16

Note: F1 seeds were planted during the October 1998 – March 1999 season. Individual plants were selfed to produce F2 seeds. F2 seeds produced from selfed "a" line were morphologically selected for clonal analysis. Maize ears from these selfed plants were morphologically distinct because of the presence of a mixture of white and yellow kernels. F2 seeds were planted and grown during November 1999 – March 2000 summer season. From Table 3.1, plants of the genotypes 5, 6 and 9 died during the seedling stage. Only plants having genotypes 3, 4 and 8 produce white sectors upon irradiation.

anthesis, and internode lengths were measured after the leaves were all removed from the plant. With the internodes showing length asymmetry, internode lengths were measured in both short and longer side of the internode and the average was determined. Data on the following were also determined: number of days to anthesis and relative position of the ear on the shoot (primary ear position).

3.2.5. Frequency of leaf initiation

Beginning at five days after seedling emergence (12 days after planting), plants from both mutant and normal plants were harvested and the total number of leaves including the youngest visible leaf primordium (morphologically visible 'bulge' along the flank of the meristem) was determined by dissecting the plants under a Olympus stereoscope. Sampling was done at 6-day intervals until the plants started to become floral as indicated by a morphological change in the shoot apex from round shaped dome into elongated one.

3.2.6. Expression analyses

The expression pattern of *te1* gene product during maize embryo development was determined in the maize inbred, B73. At defined intervals after self-pollination, ears were harvested, the caryopses were opened under a stereoscope, and the embryos fixed according to the protocol described in Section 2.6. For the vegetative shoot apices, fourteen-day-old seedlings grown under glasshouse conditions were used in the study. Tissue samples were prepared and sectioned according to the protocol described in Section 2.6. The roots and the plant parts just above the shoot meristem (this was determined empirically) were cut off from the plant. Vegetative shoot apical sections, about 2 cm long and 0.5 cm thick were immediately fixed in 4 % paraformaldehyde. Clearing and paraffin embedding were done as previously described in Section 2.6. Sense and antisense probes for the full-length *te1* cDNA (pBV432, 2.5kb) were prepared using either T3 or T7 polymerase. The expression of *kn1* in 19-day-old mutant and normal shoot apices was also determined using a 1.8 kb *kn1* antisense probe (pBV 432).

The same *te1* probe was used to determine the expression pattern of *te1* in a maize mutant with altered phyllotaxy, *abphyll*. Plant tissues used for this experiment were from 14 day old seedlings that were germinated from a segregating population for *abphyll* mutant and grown under the same glasshouse conditions. Because a high percentage of the *abphyll* mutants reverted back from the decussate pattern to the distichous phyllotaxy only a very limited number of *abphyll* mutant samples were actually studied.

3.2.7. Histological analyses

3.2.7a. SEM analysis

Shoot apices of mutant and normal plants were viewed under Scanning Electron Microscopy (SEM) using a modified replica technique (Sylvester *et al.*, l990). Two-week-old seedlings were dissected under an Olympus stereoscope and the leaves were carefully removed until the shoot meristem was visible. Impression casts were made using dental impression media (Exaflex). Once set, these were then filled with epoxy resin (Revell, Contacta Professional) that was allowed to polymerise overnight to a couple of days. The polymerised resin were mounted on SEM stubs, sputter coated and viewed under the scanning electron microscope (Cambridge 250 Mark 3, Hortresearch SEM facility).

3.2.7b. Light microscopy

For shoot meristem studies and measurements, nineteen-day-old seedlings of both mutant and their normal siblings were harvested, and tissues corresponding to the shoot apical region were fixed in FAA (Section 2.5). These were dehydrated in ethanol series, cleared and paraffin embedded. Eight µm serial longitudinal sections were prepared, stained with Saffranin-Green and mounted in polylysine coated slides. Using the median longitudinal section, the width and length of the shoot meristem were measured. The width of the meristem was taken as the width just above the most recently initiated leaf primordium, P0. Meristem lengths were

measured as the vertical distance from the summit to P0, and the vertical distance between the summit and P1 (Figure 3.2).

Epidermal replicas of both the leaves and the internodes were made using clear nail polish (California colours) and viewed under a Zeiss Axiophot compound microscope. Nomarski differential interference images were captured using a Leica 35mM camera attached to the microscope. Microscopic analyses of embryo shoot apices were done in two ways. In the first, forty mature maize embryos from a corn ear segregating for *te1* were harvested 45 days after pollination, fixed, stained and viewed under a compound microscope. The total number of leaves was counted including the youngest visible primordium. Embryos from a maize inbred, B73 were used as a control. In the second experiment, ears from a single *te1* mutant plant were pollinated separately. One was selfed while the other pollinated with an inbred, B73. This was done to generate *te1* mutant and normal embryos from similar genotypic background. The ears were harvested 45 days after pollination, fixed, stained and viewed under a Zeiss axiophot compound microscope. The total number of leaves was counted including the youngest visible leaf primordium.

3.2.8. Sector analysis

The sectored plants were dissected and the position, size and extent of the white sector were recorded. The width of leaf sectors was measured as the width measurement along the midpoint of the sector length.

The apparent cell number (ACN) of the internode was calculated by dividing the circumference of the internode by the width of sectors confined to the internode. The ACN of the leaf was calculated by dividing the width of the leaf sheath by the width of sectors in the leaf sheath. The calculated ACN was multiplied by a correction factor of 0.67 in consideration of the possibility that half of the sectors originated as single cells (G2 breaks) and half from 2 cells (G1 breaks) (McDaniel and Poethig, 1988).

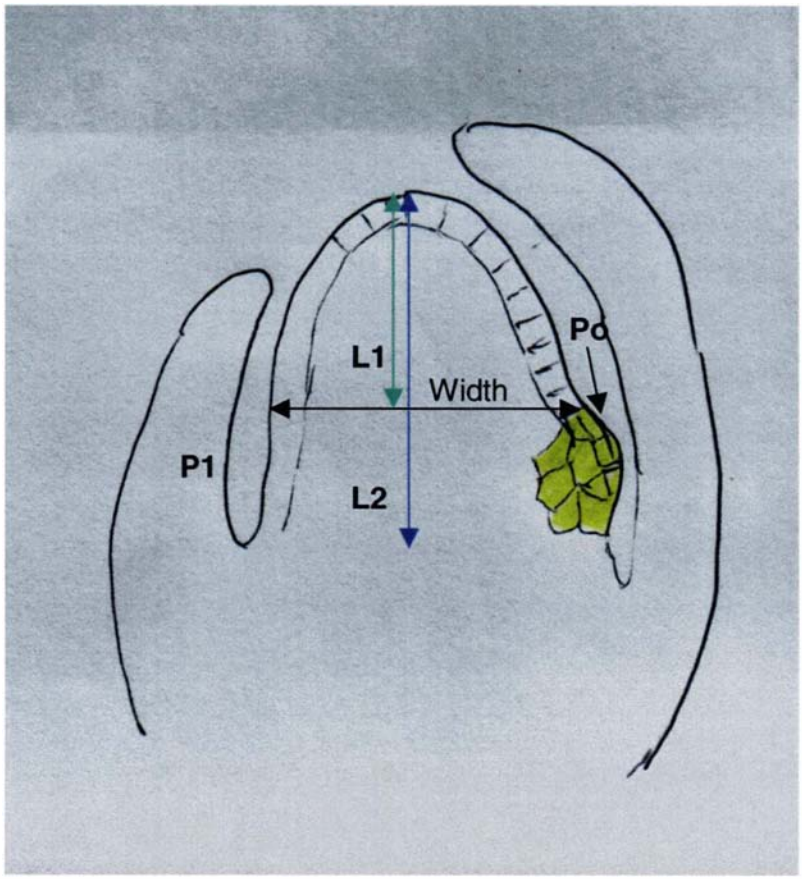


Figure 3.2. Schematic representation of a median longitudinal section of a maize vegetative shoot apex. P0 is used to indicate most recently initiated leaf primordium. P1 is the next older leaf. Meristem width is the width just above P0. L1 is the length from summit to P0( ). L2 is the distance from summit to P1( )

A sample of imbibed seeds at the time of irradiation was fixed and the number of leaves initiated at the time of irradiation was determined by light microscopic analyses.

3.3. RESULTS

3.3.1. Leaf initiation

The maize mutant *te1* was introgressed to the maize inbreds B73 and A188 for three generations before morphological comparison was made. Only the plants introgressed to B73 were analysed and the results presented in this study. The *terminal ear1* (*te1)* mutants are first distinguished from their normal siblings at about 7-10 days after sowing by the increased number of leaves initiated and the distinctively small size of the leaves. Throughout the vegetative growth of the plants, the mutants produced more leaves than their normal siblings (Figure 3.3, Figure 3.4A).

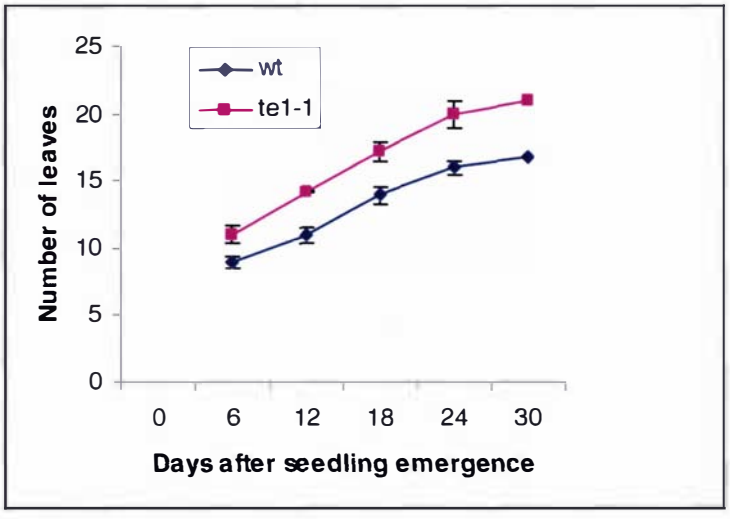


Figure 3.3. Total number of leaves initiated in normal plants and *te1* mutants during the vegetative development.
Vertical bars indicate s.e.m (ave. of 10 plants).

An initial experiment to determine the number of embryonic leaves in seeds of a *te1* segregating population showed all forty mature embryos sampled as having about 4-6 leaves. The control embryos, B73 also showed an equal range of values. From a different set of seed population, where *te1* mutant was both selfed and cross-pollinated with B73 (*te1-1* x B73) to generate mutant and normal embryos from the same mutant background, all mature embryos showed 4-6 leaves.

In addition to being shorter and narrower, mutant leaves sometimes exhibit distinctive pattern defects. Some leaves have double midribs, splitting of leaf margins and asymmetry across the blade of the leaf with respect to the central midrib (Figure 3.4B). The regular distichous arrangement of the leaves is altered to a spiral pattern.

The duration of the vegetative phase from germination to the reproductive phase (as indicated by a change in the meristem from round vegetative apex to elongated floral meristem) was shortened by about 2 days in *te1* mutants (personal observation). Under glasshouse growing conditions, at 23-25°C and long day condition (Experiment 1), some plants have turned floral at approximately 30-36 days after planting. At 30 days after planting, for example, approximately 75 % of the *te1* mutants sampled for measurements have gone floral compared with only 50% in their normal siblings.

3.3.2. Internode elongation

te1 mutants are significantly smaller than the wild type due to shortened and irregular growth of the internodes (Table 3.2). These shortened internodes are most frequently found at the apical portion of the shoot (Figure 3.4C and Figure 3.5). A characteristic aspect of the shortened internodes of the mutant is that the reduction in internode lengths can be sporadic in some plants, wherein very long internodes are interspersed in between shortened ones. In some mutant alleles, the internodes were extremely compressed, even in the basal portion, resulting in dwarf, stocky plants (N. Alvarez, unpublished data).

Table 3.2. Shoot traits of wild type and *te1* mutant siblings in B73 background (Average of 10 plants).

Trait	Wild-type	*te1-1*
Number of vegetative phytomers	17. 2 ± 0.9	21.6 ± 1.1
Plant height, cm	210.7 ± 9.9	89.4 ± 4.9
Internode length, cm (average of 4 in the apical portion of shoot)	15.3 ± 2.3	3.5 ± 1.0

Most of the shortened internodes, and especially uppers ones, initiated late in vegetative development, show an asymmetry with the shorter side of the internode located just below the site of leaf initiation. Light microscopic analyses show larger, irregularly shaped epidermal cells on the shorter side of these internodes, compared with relatively uniform, evenly spaced cells in the longer side of mutant internodes (Figure 3.6).

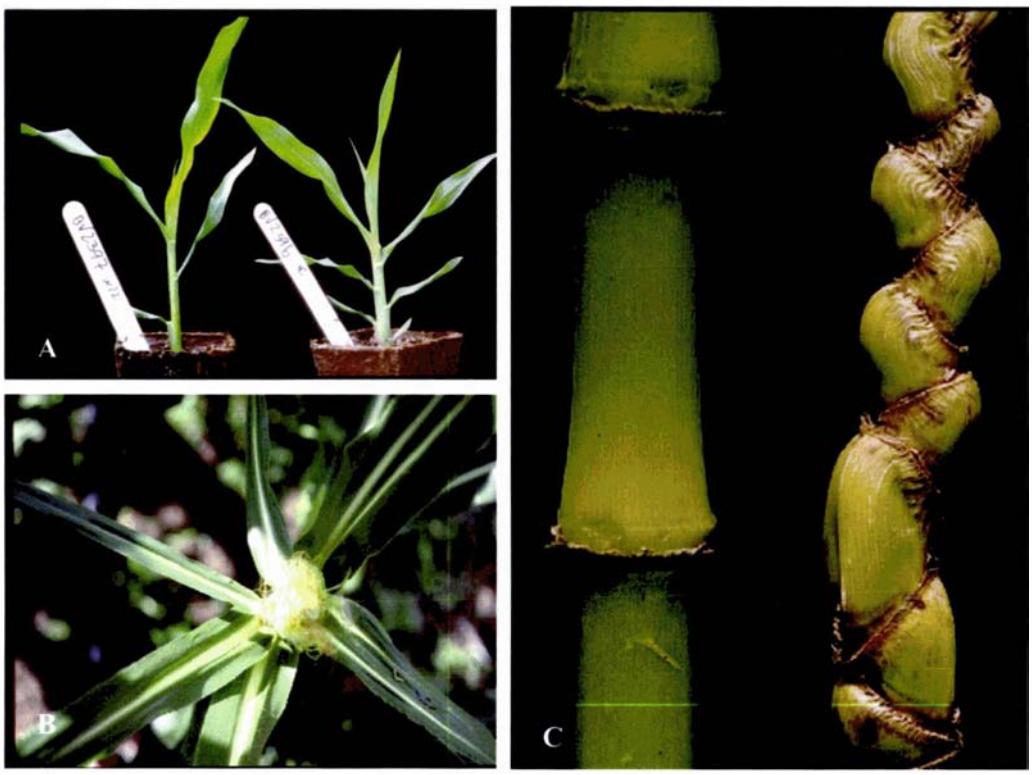


Figure 3.4. *te1* mutant phenotype. (A) Seedling phenotype showing increased number of leaves in *te1* plant. (B) *te1* plant showing altered phyllotaxy and double midribs. (C) Maize internodes showing irregular, shortened *te1* internode. (source: B. Veit)

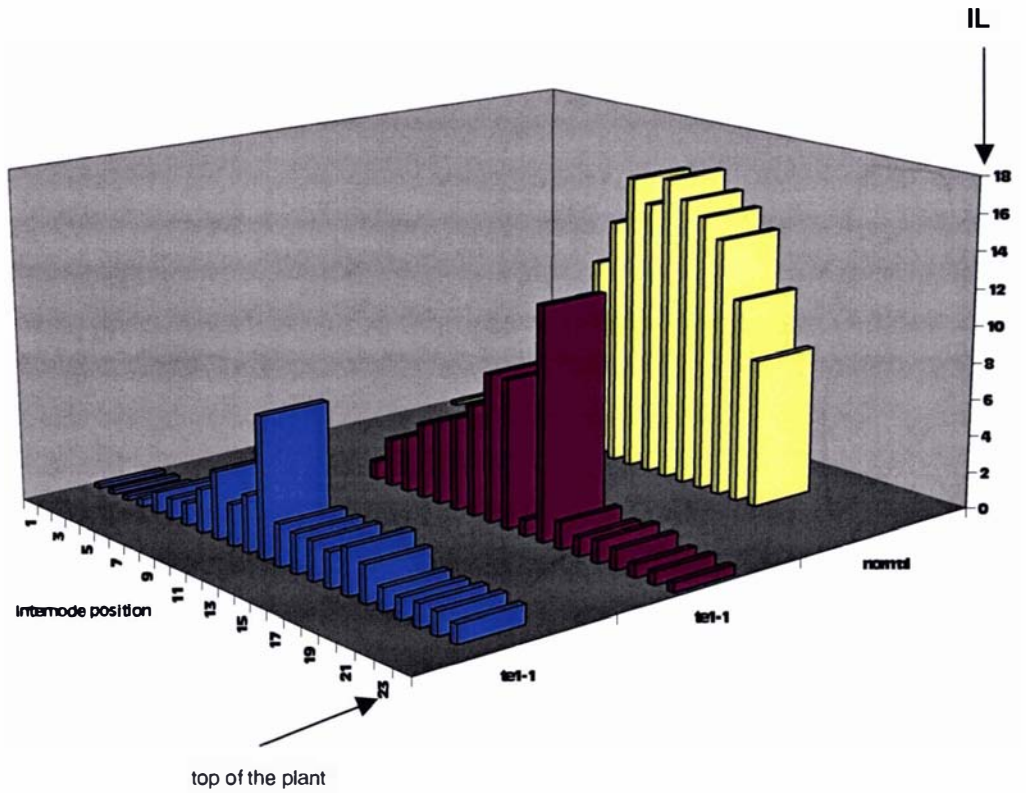


Figure 3.5. Internode lengths of normal and *te1* mutants at different internode positions from the base of the plant. X-axis, internode position from the base of the plant. Internode position 1 means that it is the first internode above the roots. Vertical axis, internode length (**IL**) in cm (profile of single plants).

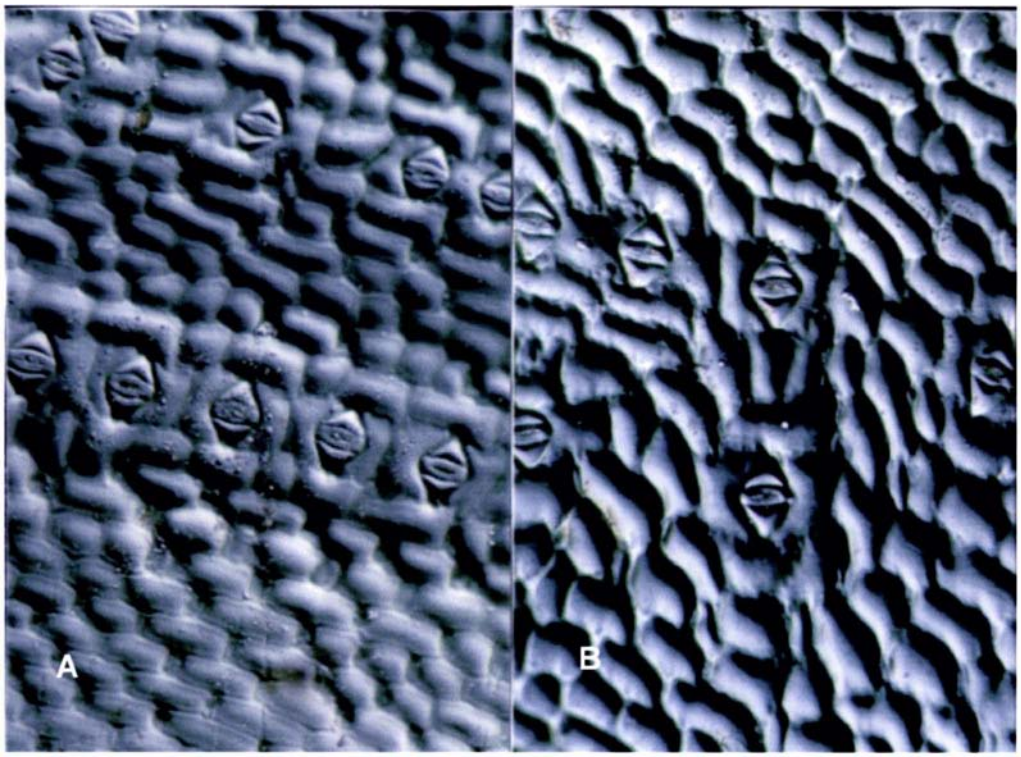


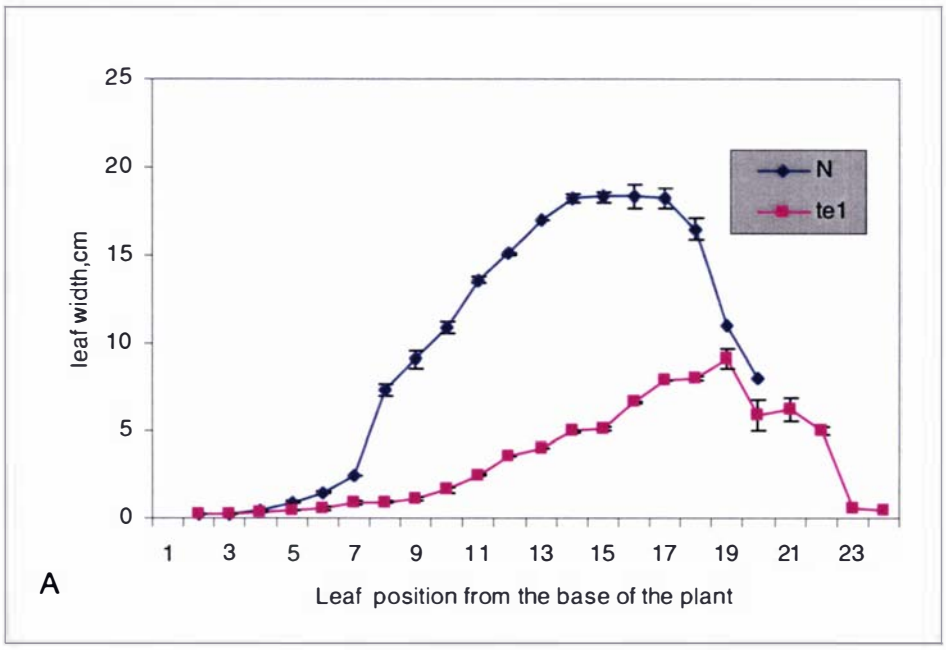
Figure 3.6. Light microscopy images of internode epidermal surface. Nail polish impression of internode epidermal surface from (A) normal side showing regular arrangement of epidermal cells and (B) irregular side of *te1* internode showing irregular distribution of bigger epidermal cells.

3.3.3 Leaf size during vegetative development

The leaves of *te1* mutants are generally smaller than those of normal plants (Figure 3.7) with both widths (Figure 3.7A) and lengths (Figure 3.7B) markedly reduced. However, the overall shape of the leaves and the pattern of change of leaf size as a function of position on the plant was the same in both the normal and the mutant plants (Figure 3.8). Both the *te1* mutant and the wild type show the same distribution pattern of leaf shapes along the main shoot, in which leaves are relatively narrow up to the 7th leaf from the base and become broader until the last leaf. Maize leaves are known to exhibit differences in shape during development. In previous studies (Moose and Sisco, 1996), leaf narrowing was associated with juvenility. Leaf shapes were also illustrated as normalised leaf areas (Figure 3.9), with a significant reduction in leaf areas of *te1* plants. To determine whether the *te1* gene may be involved in altering the phases of vegetative development, some additional morphological features that are indicative of juvenility were also noted (Table 3.3).

Table 3.3. Juvenile shoot traits of *te1* mutants and their normal siblings.

Traits	**Normal**	***te1***
Last node with prop roots	7.5 ± 0.4	7.9 ± 0.8
Last leaf blade with wax	5.3 ± 0.3	7.3 ± 0.5



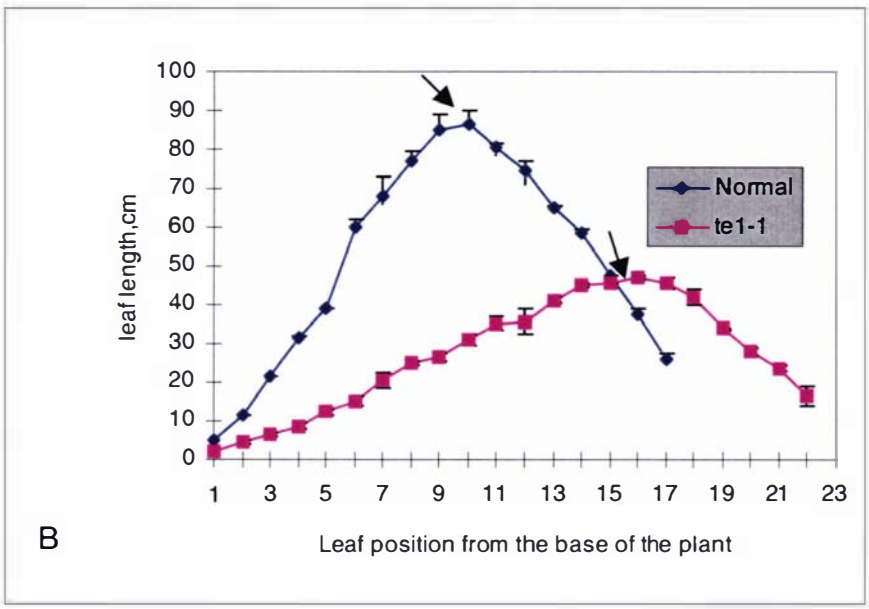


Figure 3.7. Leaf sizes of *te1* mutants and their normal siblings in B73 background. Leaf position is numbered from the base of the plant. A) Leaf width is measured across the widest region of the leaf blade. B) Leaf length is measured from the ligule to the tip of the leaf blade. Arrowheads indicate ear positions. Vertical bars indicate s.e.m.

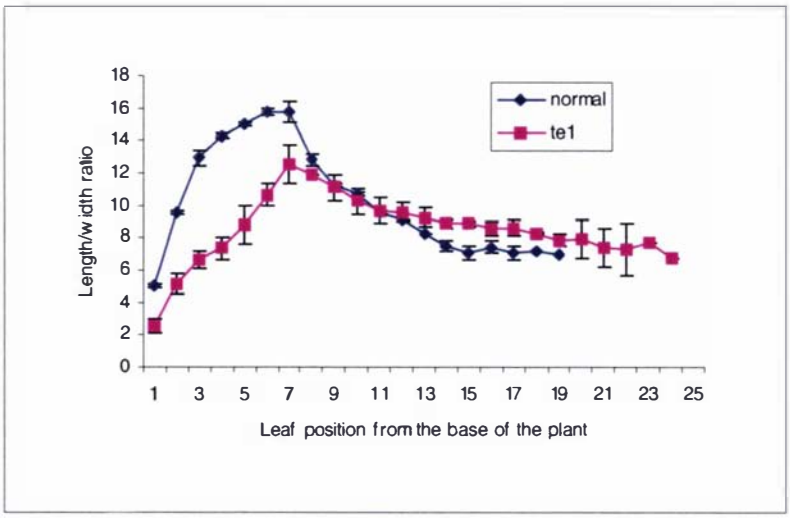


Figure 3.8. Leaf shapes of *te1* mutants and their normal siblings, illustrated as leaf length/width ratio. Vertical bars represent s.e.m (ave. 10 plants)

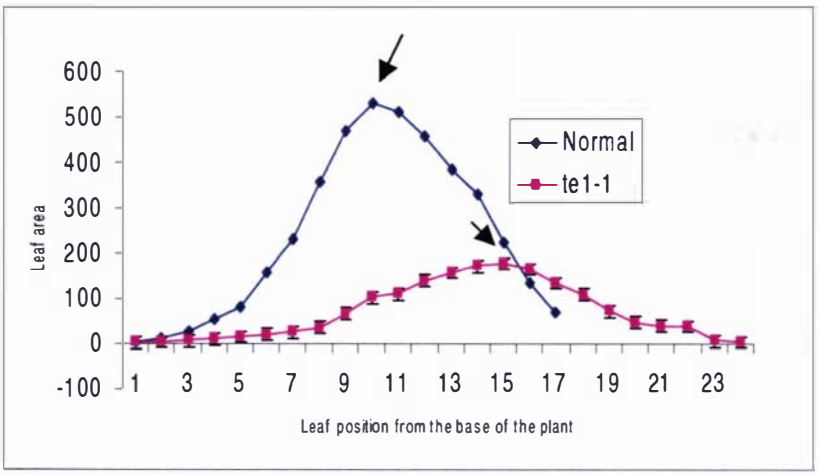


Figure 3.9. Leaf shapes of *te1* mutants and their normal siblings, illustrated as a normalised leaf area curve. Arrows indicate the positions of the primary ears (average of 10 plants).

3.3.4. Phenotype during reproductive development

One of the distinguishing features of *te1* plant is the high frequency of feminisation of the tassel. A normal maize plant is monoecious; the male and the female inflorescences are located in a single plant, but in different positions. The male inflorescence, called the tassel is located at the terminal portion of the shoot while the female inflorescence, referred to as the ear is initiated as an axillary shoot along the stem axis. In some *te1* plants, an ear (a female inflorescence consisting of unbranched kernel-bearing cob covered with husk leaves) is produced in the terminal inflorescence instead of the male branched tassel, and thus the tassel is said to be feminised. In those individuals that are not feminised, the tassels were observed to be either unbranched or not as branched as the wild-type (Figure 3.10).

The female inflorescences normally develop as axillary organs along the shoot axis. An individual plant may produce 2-3 fully developed ears depending upon the genotype and growth conditions, with the topmost axillary shoot (primary ear) completing development first. In this experiment, the primary ear was found at the 10^{th} position in the normal plant and 16^{th} in the mutant, with both these positions coinciding with the position of the longest leaf and largest leaf area (Figure 3.7B and 3.9).

The growth duration from seedling emergence to anthesis (pollen shedding) was also noted in this experiment. Since anthesis does not occur at the same time for all plants, days to anthesis was taken as the time when 50% of the plants had shown evidence of pollen shedding. Anthesis occurred earlier in *te1* mutants than in the wild type by about 2-3 days (personal observation).

3.3.5. Vegetative shoot apices

At approximately 19-21 days after germination, the vegetative shoot apices of *te1* mutants appear much smaller than those of their normal siblings (Figure 3.11) with the youngest leaf primordium in mutants located higher up the apical dome than in normal siblings. Scanning electron microscopy also showed a much smaller

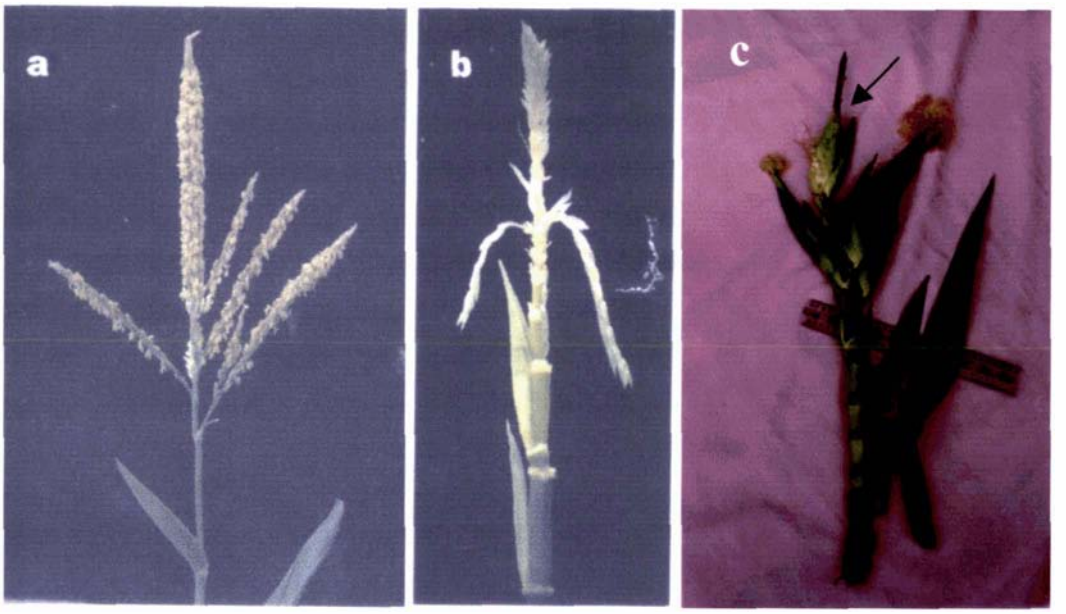


Figure 3.10. Phenotype of flowering maize plants. (a) In normal plant, the tassel (terminal male inflorescence) consists of several long tassel branches. In *te1* plant, the tassel is either (b) less branched or (c) feminised (arrowhead indicates feminised tassel. All plants are from B73 background (November 1997- March 1998, Massey University experimental area). (a and b from Veit, *et al.*, 1998)

mutant shoot apex compared with the larger, more rounded finger shaped shoot apex of the normal maize plant (Figure 3.12).

The morphology of normal and *te1* vegetative shoot apices was compared by measuring meristem size as summarised in Table 3.4. The mutant plants have smaller shoot apices and a higher plastochron ratio than the normal plants.

Table 3.4. Meristem size of maize vegetative shoot apices (μm). (see Fig.3.2 for reference points of the measurements).

	Normal	**te1**
Meristem width (just above P0)	70.4 ± 3.8	58.0 ± 6.2
Meristem height (MH):		
a. from summit to P0	50.5 ± 6.8	28.1 ± 8.1
b. from summit to P1	126.2 ± 16.8	55.9 ± 5.4
Plastochron ratio (a/b)	0.4	0.50
Height (a)/ Width	0.72	0.48

3.3.6. Expression analyses

In situ hybridization experiments were done at 20, 30, and 45 days after pollination to determine the expression pattern of *te1* mRNA during embryogenesis, when embryonic leaves are being initiated. The gene product was detected in the 21 and 30 day-old embryo, but not in the 45 day-old embryo. (data not shown)

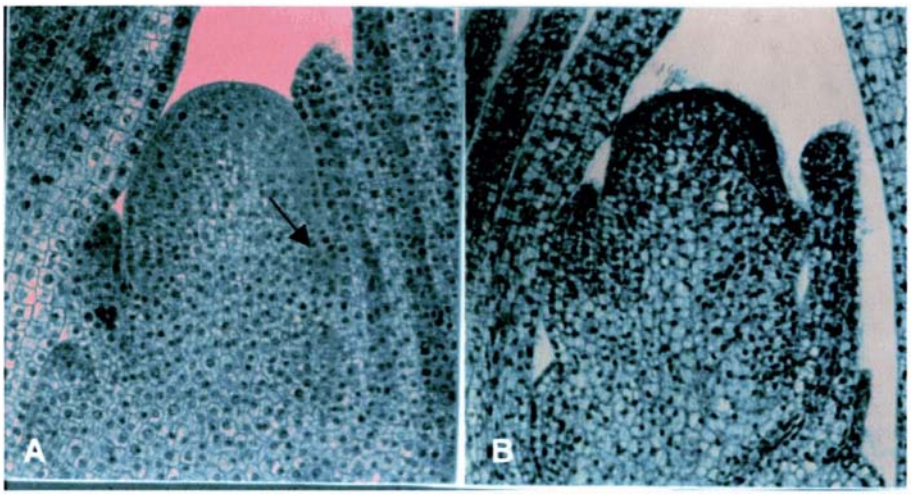


Figure 3.11. Longitudinal section of maize vegetative shoot apices . Median longitudinal section of (A) normal maize vegetative shoot apex and (B) *te1* mutant showing initiation of leaf primordium higher up the apical dome (marked by arrowhead higher up the apex) . (Both images were viewed under the same magnification, 160x. Print images are approx. 400x actual sizes).

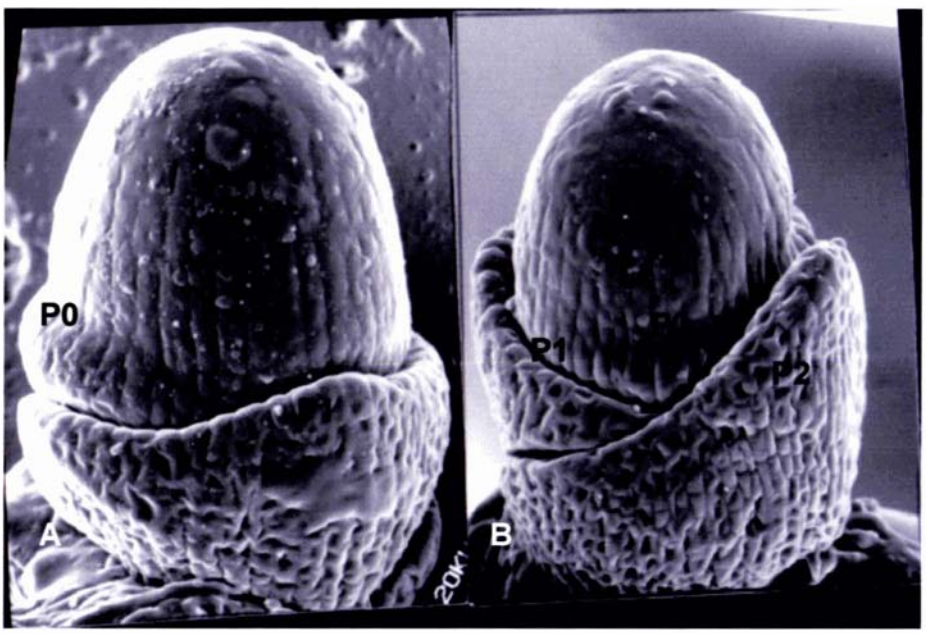


Figure 3.12. Scanning electron microscopy images of maize vegetative shoot apices . (A) Vegetative shoot apex of a normal maize showing P0 and P1 leaf primordia. (B) *te1* mutant vegetative shoot apex showing 3 young leaf primordia. P0-leaf anlagen, P1-youngest leaf primordium, P2 is an older leaf primordium. (Both SEM images were viewed under the same magnification, 250x. Print images are approx. 700x actual sizes).

Molecular aspects of the leaf initiation process in the *te1* mutant were investigated by examining the expression of a marker of leaf founder cell identity. The expression of *kn1* in 19 day-old normal and *te1* seedlings was similar except that *kn1* expression was downregulated higher up the dome, suggesting that leaf initiation occurs higher up the apical dome compared with the normal plant (Figure 3.13).

The pattern of *te1* expression was also determined in *abphyll* mutant, a maize mutant that initiates leaves in decussate pattern (an opposite pair of leaves are initiated simultaneously at one position, with a 90° divergence angle between successive leaf pairs, instead of the normal distichous leaf arrangement. Because of incomplete penetrance associated with the *abphyll* mutant, only a few *abphyll* plants were available in this study. Of the few *abphyll* vegetative tissues analysed, *te1* transcripts were expressed in shaded regions opposite each other in the SAM in the same positions, indicative of the decussate pattern of leaf initiation (Figure 3.14). It is quite difficult to analyse the staining pattern of *abphyll* because of some sectioning artefacts but *te1* expressions appear to be in patches opposite each other at the base of each leaf primordium.

3.3.7. Clonal analysis

Clonal analysis was done primarily to estimate the number of leaf founder cells in *te1* mutant compared with the wild type. This involved irradiating about 10,000 seeds from a double (*te1/wd*) mutant segregating population. Approximately 1/3 of this population was lost in the first week to Australian Magpies. From the remaining plants, a total of 187 and 90 single sectors were observed in normal and *te1*, respectively. In this experiment, although the dissected seeds show about 6-8 leaves, sectors induced in leaf 5 to 15 were considered for analyses because the seeds show non-uniform rate of germination. In this experiment, it is assumed that the white sectors, observed in the leaf and the internode are due to events involving the internal L2 layer. The possibility of the sectors originating from the L1 layer was

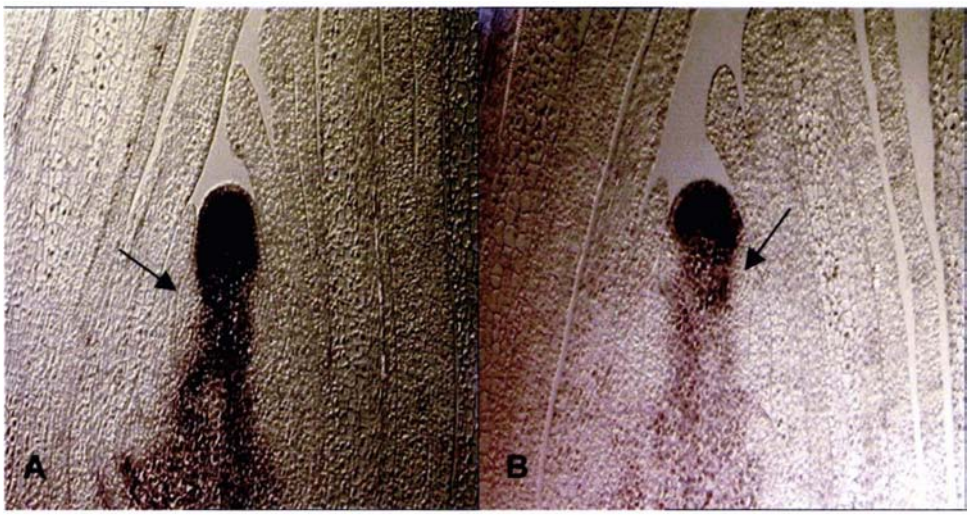


Figure 3.13. Expression of *kn1* in maize vegetative shoot apices. The patch of cells not expressing *kn1* (marked by arrowheads) corresponds to leaf initiation sites in (A) wild type and (B) *te1* mutant.

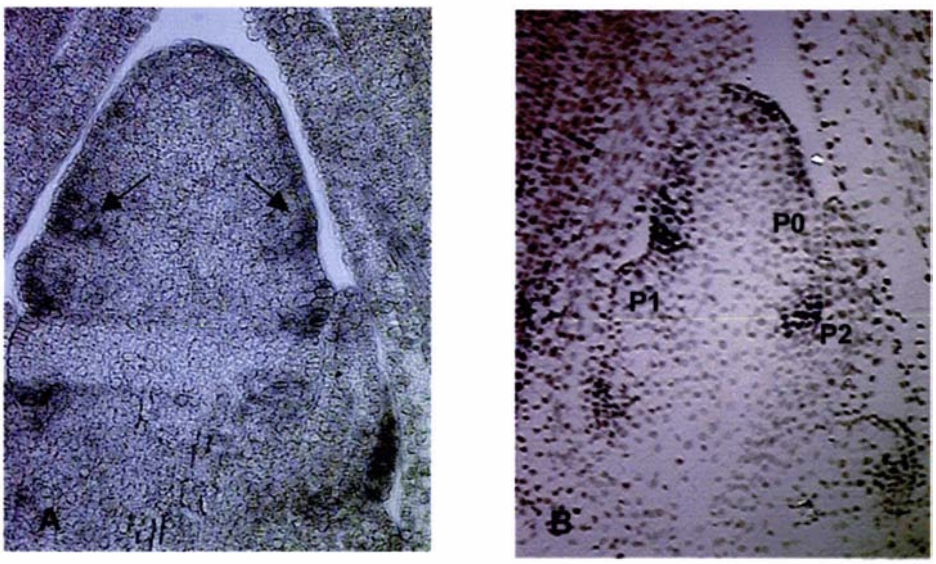


Figure 3.14. Expression of *te1* in maize vegetative shoot apices. (A) *te1* is expressed as dark patches below leaf primordia of *abphyll* shoot apex. Leaf primordia appear in decussate pattern (opposite sides on the flank of the meristem). (B) In wild type plant, *te1* is expressed as dark patch in alternate positions on the flank of the shoot apex. Arrowheads indicate *te1* expression in decussate pattern in abphyll. P0 is the leaf anlagen P1 is the youngest morphologically visible leaf primordium, P2 is an older leaf primordium.

not considered. Previous studies (Sharman, 1942; Poethig *et al.*, 1986) have indicated that sectors at the margin of the leaf often result from the introduction of L1 cells into the internal layer of the leaf.

Sectors were classified into 4 types based on their position in the phytomer: 1) sectors extending from the base of the internode into the leaf sheath and the leaf blade (I+L sectors); 2) sectors confined to a leaf, encompassing both the blade and the sheath (S+B); 3) sectors present only in the sheath (S); 4) sectors confined to the blade (B). Table 3.5 shows the frequency of these types of sectors in wild type and *te1* plants. In cases where sectors traverse multiple phytomers, the most basal phytomer showing the sector was considered.

The number of sectors is most frequent in leaf 6 and 7 in the normal plant, and leaf 8, 9, and 10 in *te1* mutant (Fig. 3.15). Sectors that encompass both the internode and the leaf are found in leaf number 7 and 8 of the normal plant and leaf 8-11 of *te1* plant. The wide range suggests that the seeds are not uniform in their germination and some plants may be more advanced in their growth at the time of irradiation.

The apparent cell number was calculated by using the formula:

Internode

$$ACN = \frac{\text{total circumference of the internode}}{\text{Sector width}}$$

Leaf

$$ACN = \frac{\text{Width of leaf sheath}}{\text{Sector width}}$$

Table 3.5. Frequency and position of sectors in leaves 5-15 of irradiated wild type and *te1* maize plants.

Leaf position	Internode + leaf		Sheath +Blade		Leaf sheath		leaf Blade	
	N	*te1*	N	*te1*	N	*te1*	N	*te1*
5	1	0	12		4	1	8	2
6	18	0	17	2	1		8	5
7	26	3	14	2		2	5	
8	23	11	5	1		1	2	1
9	21	12	1	2	3			
10	10	9	3	6		1		1
11	5	10				1		
12		8		1				
13		5						
14		3						1
15				1				

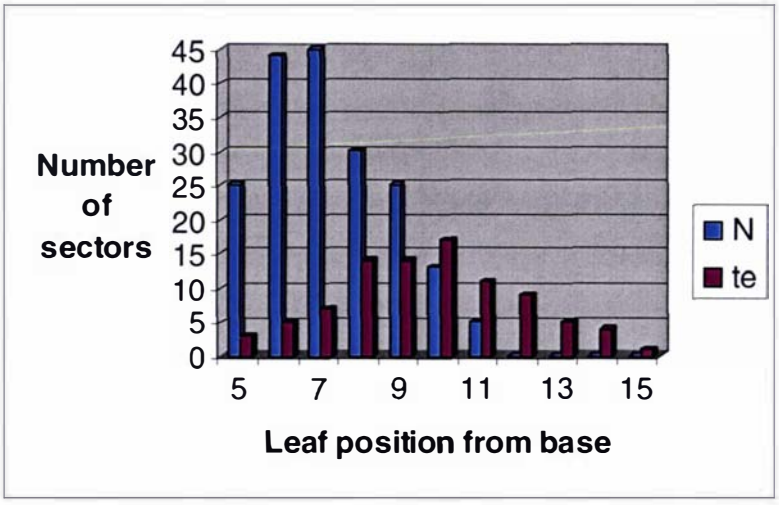


Figure 3.15. Total number and positions of sectors in the normal maize plant and *te1* maize.

The ACN indicates an estimate of the number of 'founder' cells at the time of irradiation. In both the internode and the leaf, a lower value of ACN for *te1* mutants was observed compared with the normal plants (Table 3.6). The lower values of ACN in *te1* indicates that there are fewer founder cells for that particular organ and this is reflected in a larger sector in the mutant plant compared with the normal plants (Figure 3.16).

Table 3.6. Apparent cell number of the leaf and the internode. Numbers in parentheses are corrected values by multiplying calculated ACN by 0.67.

Leaf position	**Internode**		**Leaf**	
	Normal	*te1*	Normal	*te1*
6	75 (50)		36 (24)	19 (13)
7	78 (52)	51 (34)	36 (24)	26 (17)
8	74 (50)	30 (20)	37 (25)	25 (17)
9	69(46)	29 (19)	46 (32)	32 (21)
10	35 (23)	38 (25)	60 (40)	36 (24)
11	32 (21)	23 (15)	60 (40)	24 (16)

Figure 3.16. Sector sizes in maize phytomers. In maize seedlings, white sectors are narrower in the leaves of (A) normal plant compared with that of (B) *te1* mutant. (C) Sector lengths extend through 3-4 internodes in both the normal and mutant plant. White sector in mutant internode is wider compared with that of its normal sibling. D) Sector size in leaf sheath is wider in *te1* mutant than in the normal plant.

3.4. Discussion

3.4.1. te1 regulates phytomer organization and development

In general, the vegetative development of plants consists of the regulated addition of metameric units called phytomers. In maize, the addition of these developmental units occurs during both embryogenesis and postembryonic vegetative development (Kiesselbach, 1949; Hanway and Richie, 1983). The first five or six leaves of a maize plant are formed during embryogenesis followed by another 11 to 17 leaves during vegetative development (depending on genetic background and growing conditions). Mutations in *te1* perturb several aspects of phytomer development including an increased number of leaves, shortened plastochron, reduced stature, and a reduction in leaf growth that is frequently associated with morphological abnormalities. In the following discussion, these changes are considered in detail in an attempt to understand the process in which *te1* participates.

One of the earliest phenotypes observed in *te1* mutants is an elevated frequency of leaf initiation. To determine whether this increase might also occur during embryo development, microscopic analyses of mature embryos were done. In terms of the total number of embryonic leaves, there was no significant difference between mutant and wild type embryos, with an analysis of 40 mature *te1* and wt embryos both showing between 4-6 leaves. A second approach to estimating the total number of embryonic leaves involved calculating the y–intercepts from the best fit-regression lines in Figure 3.3. The values of 4.07 and 5.58 for N and *te1,* respectively suggest the possibility that the *te1* mutation leads to a slightly higher frequency of leaf initiation during embryo development. These values, however, are just estimates and may not necessarily reflect the actual total number of leaves initiated during embryogenesis. It is possible that both the wild type and the mutant plants have initiated the same number of embryonic leaves, but that mutant plants begin producing postembryonic leaves sooner than the wild type. Another possibility is that the embryonic phase during which leaves are produced is longer in *te1* mutants than in *wt.* It is worth noting, however, that a greater number of leaves

showing juvenile features, such as prop roots and leaf wax, are present in *te1* mutants than in the wild type (Table 3.3), consistent with the possibility that a higher number of leaves are produced in *te1* mutants during embryo development. In a similar experiment in rice, in the characterisation of *pla1 (plastochron1)* mutants (Itoh et al., 1998), the mutant embryo could also not be distinguished from the wild type embryo; both seem to have produced the same number of embryonic leaves. *pla1* mutants start to initiate leaves more frequently only after germination. In this study, the result seems consistent with the possibility that embryonic leaves are also initiated more frequently in *te1* plants.

Another measure of the frequency of leaf initiation is provided by the term *plastochron*. A plastochron is a unit corresponding to the time interval between the initiations of successive leaf primordia (Esau, 1965). In this experiment, the plastochron was estimated from the slope of the best-fit regression lines in Figure 3.2. The values calculated were 0.51 for the normal plants and 0.62 for the mutant. From these values, plastochron indices were estimated for the wild type (1.92 days/leaf) and the mutant plants (1.60 days/leaf). It is important to note that while the rate of leaf production in the mutants is higher than that of the normal maize plant, it appears to be relatively constant from Figure 3.2, and thus may reflect a regular pattern of leaf initiation. Conversely, the greater standard deviation observed for these averages may reflect a greater variability in plastochron indices for single plants. In agreement with other studies (Itoh *et al.*, 1998; Poethig, 1989), these data show that there is not necessarily a strict correlation between the timing of phase change and the number of phytomers produced.

The duration of vegetative development of both *te1* mutant and wild type is similar, though it was noted that *te1* mutant showed earlier anthesis (about 2-3 days) compared with the wild type. As noted previously, the transition of the shoot apex from the vegetative to reproductive stage limits the total number of leaves produced (Poethig, 1990). The fact that the duration of vegetative phase in *te1* mutant is almost the same as that of the wild type indicates that the vegetative morphogenetic program of the plant is not strongly affected by the *te1* mutation. It can be inferred from these results that the increase in the number of leaves did not

have a strong effect on the duration of the developmental phases. Considering that the *te1* mutant shows extensive feminisation and extremely altered vegetative and inflorescence architecture, the slightly earlier flowering time may be an indirect consequence of these changes in growth.

The growth of the leaf appears to be affected by the *te1* mutation as indicated by the reduced sizes of the leaf lamina and leaf sheath. Previous studies have correlated changes in leaf shapes with changes in meristem patterning processes as in the *narrow sheath* mutant of maize (Scanlon *et al.*, 1996). Mutation in maize *narrow sheath*, whose activity is required for the establishment of lateral domains of the leaf, results in the deletions of the leaf lateral domains. A model for the *NS* function was postulated, in which *NS* products initialize meristematic cells to assume leaf founder cell identity, especially in marginal domains. If leaf development is dependent on the acquisition of regional identities by discrete domains within the organogenic region of the shoot apical meristem (Freeling, 1992, Scanlon and Freelingl, 1998) then the altered leaf sizes in *te1* mutant might be interpreted as a consequence of disruption of this patterning process.

In this study, the leaf, node, internode and axillary earshoot are terms used to describe clonally related portions of a maize phytomer (Poethig and Symkoviak, 1995). The reduction in leaf size in *te1* mutants is accompanied by a reduction in internode length. One thing unique about this mutant, however, is that the degree of shortening of the internodes is not the same for all internodes, but is highly variable, wherein very short and curved internodes are interspersed with relatively normal long ones. Previous studies on maize internode elongation pattern have indicated that the relative positions and the functional roles of the internodes in the stem development influences elongation pattern and development (Morrison *et al.*, l994). They have observed in their studies, for example, that the internodes that supported the developing ears have the longest duration for internode elongation, but shorter final lengths. This observation is consistent with the observed internode elongation patterns in both normal and *te1* mutants. In *te1,* some of the short internodes are highly curved, with larger, longer and disorganized cells along the shorter and curved side of the internode. These curved internodes in the *te1* mutant reflect

altered cell division pattern and this would indicate that phytomer organization and development is disrupted in *te1* mutants. Although the shortened internodes of *te1* mutants might be compared to those resulting from defects in the GA pathway, they differ in terms of their sporadic occurrence along the length of the shoot. The shortened internodes are further distinguished by a pronounced asymmetry in which the shorter side of the internode occurs just below the midrib of the leaf above. The irregular shortened internodes and the accompanying irregularity in leaf development such as the reduction in leaf size and presence of double midribs seem to reflect irregularity in phytomer development.

3.4.2. te1 gene product is expressed in "active" SAM

Results from the embryonic studies indicate that the *te1* gene product is expressed only in an "active" shoot apical meristem (i.e. organogenesis is actually in progress). It would seem that *te1* gene function is required only during the time when leaves are being initiated, as indicated by the continued presence of the gene product during the early embryonic development when the embryonic leaves are being initiated, in contrast to the absence of the same *te1* transcripts during the later stage of embryogenesis when embryo is fully mature. This is consistent with earlier studies, which also pointed to reduced meristematic activity towards the end of embryogenesis after the plants have initiated 4-5 leaves (Clowes, 1978); this stage of mitotic inactivity is reached even before the embryo has acquired its full size at seed maturity. This would have explained the lack of *te1* expression pattern during the latter part of embryogenesis, at about 45 days after pollination.

Expression of the *te1* gene transcripts throughout the vegetative stage in the same pattern that was previously reported (Veit *et al.*, 1998) is consistent with the earlier observation that the gene product is expressed only when organ formation is in progress. The expression of the gene transcripts in decussate pattern in *abphyll* mutant is consistent with the idea that *te1* constrains the position of leaf initiation as earlier postulated; however, it should be emphasised that this consistent relationship with initiation sites does not in itself imply that *te1* limits this process. Arguments in favor of a role for *te1* in controlling leaf initiation are based primarily on the

decreased plastochron index and altered positions of leaf initiation in the loss of function mutant.

That the total number of leaves initiated during embryo development may be constant and that differences in the frequency of leaf initiation due to *te1* mutation during the embryonic stage may not be significantly different from the *wt* suggests that the program controlling leaf initiation during embryogenesis is more constrained than that which operates during the vegetative stage. This could be attributed to the relatively short time frame in which embryogenesis occurs, in that small decreases in the plastochron might not be sufficient to produce extra leaves. However, this needs to be clearly established with more detailed histological studies coupled with molecular experiments. It would be helpful, for example, to determine the cell division patterns and cell behaviour during early embryonic leaf initiation.

3.4.3. te1 mutant has a smaller vegetative shoot apex and fewer leaf founder cells

Light microscopic analysis shows that *te1* plants have smaller shoot apices than the *wt* plants and that leaf initiation occurs higher up the apical dome. This is supported by molecular studies of the initiation process that show that down-regulation of *kn1*, a marker of leaf founder cell identity, occurs higher up the apex compared with the wild type. These results support a model in which loss of *te1* activity leads to leaf initiation higher up on the apical dome, where a smaller number of cells are available for recruitment to form the leaf and the associated phytomer elements. The decreased cell number and the abnormal partitioning of the cells among the phytomer elements are envisaged to lead to smaller leaves and irregularly shortened internodes. Recent studies show that the size or shape of the shoot apical meristem affects the shoot architecture. In maize, the *abphyll* mutant, which shows altered phyllotaxy, has enlarged meristem compared with the wild type (Greyson *et al.*, 1978). In rice, the *pla1* mutation results in enlarged meristem that is associated with an increased number and altered arrangement of leaves. In *Arabidopsis*, several mutations affecting the size of the shoot apical meristem also affect morphogenesis (Medford *et al.*, 1992; Barton and Poethig, 1993; Clark *et al.*,

1995; Clark *et al.*, 1996; Laux *et al.*, 1996). Mutations in *CLAVATA* genes result in enlarged meristems, which are associated with increased organ number and altered phyllotaxy. Conversely, mutations in *WUSCHEL* and *SHOOTMERISTEMLESS* result in smaller or nonexistent meristems, and consequently reduced organ number. These mutations illustrate a close relationship between the geometry of the shoot apex and patterns of leaf initiation.

Classical surgical experiments have established that the initiation of leaves by the SAM does not depend on the presence of existing primordia, but that existing leaf primordia inhibit the formation of new leaf primordia near them such that new leaves develop at the position in the SAM where inhibition is least (Ball, 1960). The model also postulates that inhibition occurs in the center of the SAM so that no leaves are formed in the central zone. This model is consistent with other models postulated wherein a morphogenetic field exists within the shoot apex that favors the initiation of leaves at points most distant from existing leaf primordia (Thornley, 1975; Mitchison, 1977; Steeves and Sussex, 1989). Consistent with this model, the possible role of plant hormones has been previously studied to explain the inhibition theory. Careful analyses of hormone or antagonist treatments on the shoot apex have led to the conclusion that the amount of space on the apical dome is not as critical for the phyllotactic switch as the plastochron index (measure of the frequency of leaf initiation and describes how far apart are the organ primordia formed on the flank of the meristem) (Schwabe, 1971; Meicenheimer,1981; Marc and Hacket, 1991). This means that a change in phyllotaxy results when these inhibitory factors are absent. The nature of this inhibitory substance, however, is still unknown (Wilkinson and Haughn, 1995). In *te1* mutants, as a leaf primordium is initiated higher up the dome, the plastochron index is also reduced (there is a shorter distance/ time between two successive leaf primordia), suggesting that somehow the constraints imposed on the timing and position of leaf initiation by the shoot apex and the existing primordia have been relaxed. While the regulatory mechanism by which *te1* functions is still unclear, the foregoing discussion suggests that the gene may specify a domain within the SAM in which leaf initiation is inhibited.

The model, in which *te1* normally inhibits leaf initiation, predicts that the loss of activity would be expected to lead to precocious leaf initiation higher on the apical dome where fewer cells are available for organogenesis. This situation could account for the irregular, asymmetric shortened internodes, especially if normal internode development requires some minimum number of founder cells. Given the progressive nature of the leaf determination process (Scanlon *et al.*, 1996), the consequences of precocious initiation might be expected to be most pronounced on the side of the internode directly below the initiation site of the leaf above (to which the internode is clonally related). By the time the marginal regions of the leaf are recruited, continued cell division in the SAM would lead to more cells being available for partitioning between the leaf and adjacent internode. The larger and are more disorganized appearance of cells on the short side of the internode would reflect the plant's attempt to compensate for the asymmetric partitioning of cells. This would appear to be consistent with the persistence of the *te1* transcripts in the margins of older leaf primordia within the shoot apex.

The results of the clonal analyses support the model that leaves in the *te1* mutant are initiated from a smaller number of founder cells compared to the wild type. The larger sectors observed in the mutant plants indicated a lower apparent cell number where the apparent cell number is the estimated number of cells required to give rise to the observed phytomer element. It also appears that the mutant plants have already initiated 1-2 more leaves than the wild type at the time of irradiation.

3.5. Conclusions

The results of this study shows that the smaller size of *te1* shoot apical meristem is associated with leaf initiation occurring higher up the apical dome and that the interval of time between the initiation of successive leaf primordia is less than in the wild type. The use of the leaf marker *kn1* supports the model presented in that the downregulation of *kn1* transcripts occur higher in the *te1* shoot apex than in the wild type. Clonal analyses further strengthen the hypothesis that leaf initiation occurs higher up the apical dome, showing that fewer cells are recruited to form the

leaf as indicated by large sectors in *te1* mutant compared with the wild type. Though the study established that in *te1*, the alteration in leaf initiation is correlated with shoot apical size and fewer founder cells, it would be helpful in the future work to analyse how the gene may affect the cell division patterns. The identification of the putative gene targets of the *te1* transcripts will also clarify the mechanisms by which the gene functions.

Chapter 4. Expression patterns of Mei2-like genes in Arabidopsis

Abstract

The *in situ* mRNA localisation patterns for 3 of the seven *Mei2*-like genes of *Arabidopsis* were determined to assess whether the genes might function in a manner similar to the *TE1* gene of maize. Two members of this gene family, named *TEL1* and *TEL2* (*Terminal Ear-* Like) are expressed in the SAM early during embryogenesis from the globular stage onwards. Significantly, *TEL1* is also expressed in the RAM. For both *TEL1* and *TEL2*, expression becomes limited to those regions of the apical meristem that contain pluripotent stem cells. The expression of these genes persisted in the SAM throughout the vegetative and reproductive development of the plant. *AML1* (*Arabidopsis Mei2*–like), a more *mei2*-like member of the gene family, is also expressed in the early embryo, but in a diffuse pattern. Later, during the torpedo stage, its expression in the SAM becomes more distinct. *AML1* is expressed throughout the vegetative meristem, but becomes more restricted in the apical meristems during the reproductive development. Given the similarity in the DNA sequences and expression patterns of these genes, a model is suggested in which *Mei2*-like genes act redundantly in specifying the undifferentiated nature of the meristem.

4.1. Introduction

Plants have two types of apical meristems, the shoot and root apical meristems (SAM and RAM), which are located at the tip of the shoot and root, respectively. These apical meristems consist of undifferentiated, indeterminate and actively dividing groups of cells. The primary SAM and RAM are formed during the early stages of embryogenesis, with the SAM derived from a subset of cells in the apical region of the globular embryo, while the RAM is derived from the central and basal cells of the globular embryo (Barton and Poethig, 1993; Jurgens *et al.*, 1995).

Further insights into the formation of the apical meristems have come from the isolation and analysis of mutants that affect embryonic organs and meristems (Bertleth and Jurgens, 1993; Long *et al.*, 1999; Willemsen *et al.*, 1998). Analyses of these embryonic mutants have provided means of reconstructing embryonic development from a functional perspective.

Much progress has also been made toward understanding the mechanisms controlling meristem formation and development through the use of molecular genetic methods (discussed in Chapter 1). Genes that were identified as important for the proper formation and function of the meristems were found to be expressed and persist in distinct regions within the meristems throughout the lifetime of the plant. Figure 4.1 illustrates the expression of early patterning genes during embryogenesis. The expression of *WUSCHEL (WUS)* (Laux *et al.* 1996), in a small group of cells in the central zone of the SAM, appears essential for maintaining a reservoir of undifferentiated cells. The expression of *SHOOTMERISTEMLESS (STM), CUP-SHAPED COTYLEDON 1* and *2 (CUC1* and *CUC2)* and *AINTEGUMENTA (ANT),* in the later stages of globular embryo development, mark specific domains in the apical region of the embryo (Long and Barton, 1998, Aida *et al.*, 1999, Takada *et al.*, 2001). *STM,* which is expressed in the central region, is required for SAM formation; *ANT,* which is expressed in the peripheral region, specifies the position of the cotyledons; *CUC1* and *CUC2,* which are restricted to the boundary regions between the cotyledons and the SAM, act redundantly in suppressing growth at cotyledon boundaries (Aida *et al.*, 1997; Aida *et al.*, 1999). As the embryo matures, *STM* becomes downregulated at the periphery and becomes restricted to the central SAM, while *CUC1* and *CUC2* are downregulated at the centre and become restricted to the region surrounding the centre. At this same early heart stage, *CLAVATA1 (CLV1)* gene becomes activated in the L2 and L3 layer and with *CLV2* and *CLV3* (Clark et al., 1993,1995,1997) acts to regulate *WUS* in a negative feedback loop to maintain the integrity and overall size of the meristem (Schoof *et al.,* 2000). These expression analyses indicate that differential expression of genes correlate with the functional domains within the meristem.

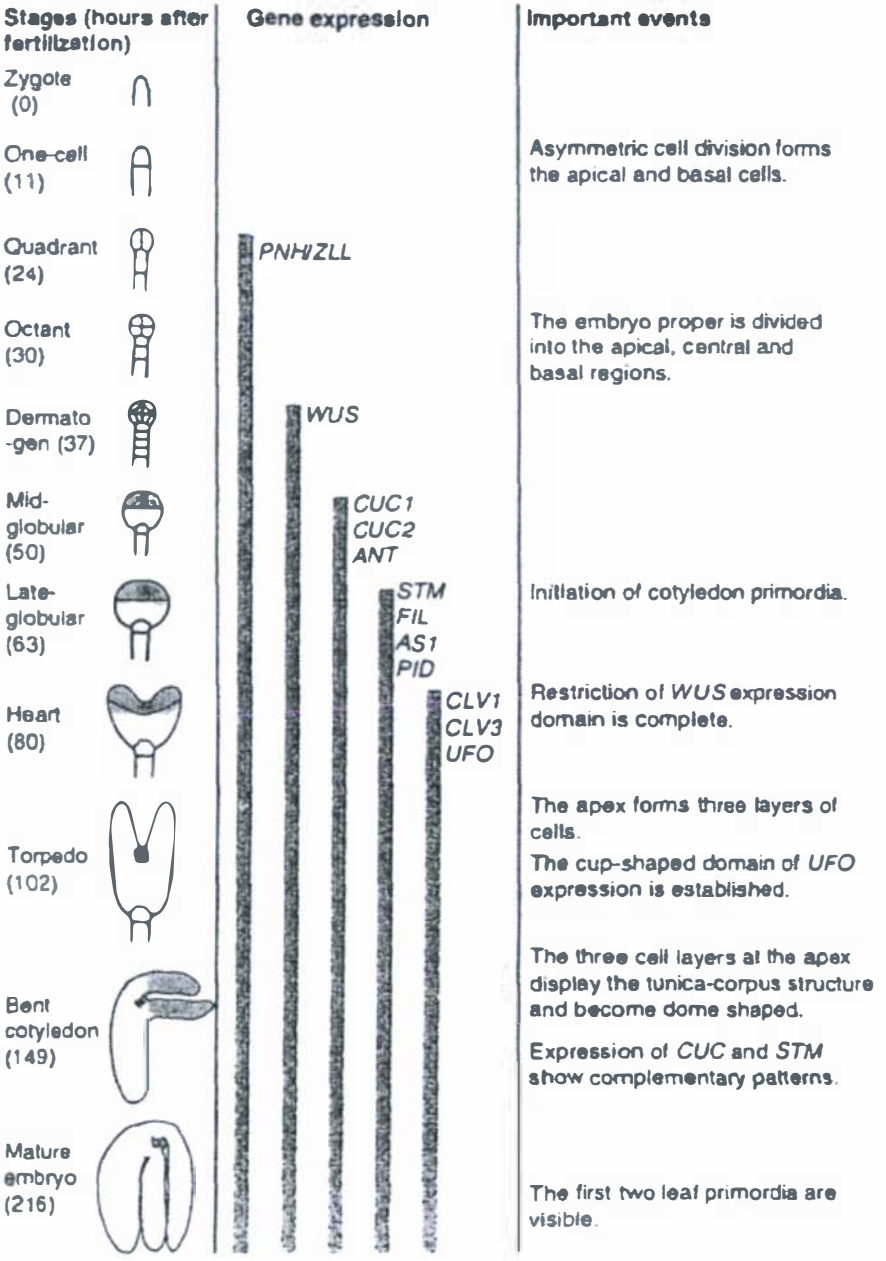


Figure 4.1. Shoot apical meristem (SAM) formation and expression of related genes (Source:Aida and Tasaka, 2002).

A new class of RNA binding proteins has recently emerged in plants, collectively referred to as plant *Mei2*-like genes, which we believe regulates meristem function, and in particular, acts to maintain cells in a undifferentiated, pluripotent state. The study of plant *Mei2*-like genes started with the initial work toward molecular and genetic characterisation of the *terminal ear* 1 *(te1)* mutant in maize (Veit *et al.*, 1998). Sequence analysis of *te1* showed similarity to the RNA binding protein-encoding *mei2* gene of the yeast, *Schizosaccharomyces pombe* (Watanabe *et al.*, 1988). The protein gene product of *mei2,* Mei2p, binds RNA and in some way switches the cell cycle from mitosis to meiosis.

Phylogenetic analyses (Jeffares, 2001) revealed that the 7 *Arabidopsis Mei2*-like genes, identified from the initial database searches, can be separated by DNA sequence similarity into 2 subfamilies. The first group, consisting of the two genes *TEL1* and *TEL2* (*Terminal Ear1*-Like), are more closely related to the *TE1* gene of maize, while the remaining 5 genes, *AML1-5,* more closely resemble *mei2* of *Schizosaccharomyces pombe* (Jeffares, 2001). (Fig. 4)

In general, very little is known about the function of *Mei2*-like genes. That they encode RNA binding proteins suggests that they may be involved in post-transcriptional regulation of gene expression, RNA transport or RNA processing events. Genes that encode the RRM (RNA recognition motif) type of RNA binding motif serve diverse cellular functions, including regulation of splicing, stabilisation and localisation of mRNAs (Burd and Dreyfus, 1994). A similar diversity is seen in the biological functions mediated by these genes, including control of flowering time by *Arabidopsis FCA* (Macknight *et al.,*1997), regulation of sex specific alternative splicing by the Drosophila SEX-LETHAL protein (McKeown, 1992) and regulation of meiotic cell division by *S.pombe mei2* (Watanabe *et al.*, 1988).

Among the *Mei2*-like genes, the product of *S. pombe mei2* has been the most intensively studied (Watanabe *et al.,* 1988; Watanabe and Yamamoto, 1994; Watanabe *et al.*, 1997; Yamashita *et al.*, 1998; Shinozaki-Yabana *et al.,* 2000; Sato *et al.,* 2001). Molecular genetic analyses have also been done with the maize *te1* (Veit *et al.*, 1998; Jeffares, 2001), but very little is known about the other plant *Mei2*-

like genes. *mei2* is required at two stages of meiosis, during premeiotic DNA synthesis and for the completion of Anaphase I (Watanabe *et al.*, 1988). The function of the Mei2 protein was found to be dependent on the presence of a non-coding RNA species, *meiRNA,* which triggers the localisation of Mei2p to the nucleus where it is presumed to function to promote meiosis (Yamashita *et al.*, 1998). It is not yet clear, however, what specific biochemical function Mei2p performs in the nucleus.

The *te1* gene has not yet been biochemically characterised, but the expression pattern and the mutant phenotype suggest a role for *te1* in specifying or delimiting time and position of leaf initiation. *te1* transcripts have been detected by RT-PCR in early tassel and ear tissues, but no evidence suggests that *te1* is required for meiosis, as is Mei2p. Whatever function *te1* may be doing in these tissues was not discussed, but it was speculated that the gene is involved in other developmental processes in addition to regulation of leaf initiation *per se* (Jeffares, 2001).

The function of the AML group genes is also still unclear. The analysis of *AML1* (Hirayama *et al.*,1997) indicated that the transcript was present at approximately equal levels in siliques, roots, leaves shoots and flowers, but until this current study, no mutant phenotypes had been described, nor had a gene function been suggested.

Because of the significant sequence similarity of *Terminal Ear like (TEL)* genes with maize *te1*, a focused effort was directed toward characterising these genes in *Arabidopsis.* How do the expression patterns of these *TEL* genes compare with those of maize *te1* ? Previous expression studies show that the *te1* transcripts are present in the SAM in a series of alternating, semi-circular rings that bracket sites of leaf initiation (Veit *et al.,* 1998). Using the same experimental approach, *in situ* hybridisation experiments were done in the present study to determine the expression patterns of the *TEL* genes (*TEL1* and *TEL2*) and as well as *AML1,* as a representative of the more *Mei2*-like *AML* family, during embryogenesis, vegetative development and inflorescence development. An analysis of how this family of

genes may interact with other meristem genes, such as *CLAVATA* genes, was also initiated, of which preliminary results are presented.

4.2. Materials and Methods

4.2.1. Plant growth

Seeds of *Arabidopsis thaliana* (ecotype Col 1-0) were sown in plastic pots containing commercially pre-mixed potting soil. The pots were kept in the cold room at 4°C for 48 hours. After this stratification treatment, the plants were maintained in a growth cabinet, maintained at 23°C under short day condition, 8 hours light and 16 hours dark. After 19-21 days, the plants were harvested for tissue processing. The remaining plants were moved out of the growth cabinet and maintained at the same temperature condition but under long days (continuous light). Plants were harvested when the plants began to initiate floral shoots, with some showing visible signs of bolting after about 25-29 days. The tissues were fixed immediately in either 4% paraformaldehyde or FAA (Chapter 2.6) after the outer leaves and the roots were removed from each plant sample.

A different set of plants were planted and grown up to maturity under long day condition (continuous light). These plants were allowed to produce flowers, self-pollinate and develop siliques (the fruits of *Arabidopsis*). When several siliques had formed in one inflorescence stalk, the siliques were harvested for tissue fixation and processing (an inflorescence branch bears siliques in a linear array that represent different stages of development). Stages of embryogenesis were identified using the criteria defined by Barton and Poethig (1993). The early heart stage is characterised by the initial growth of the cotyledons from the surface of the apex to form a heart shaped structure supported by the suspensor. The torpedo stage is defined as the time when the cotyledons account for about 1/3 of the length of the embryo. The bent stage embryo corresponds to the stage when the cotyledons start to bend over the apex. In this study, the mature embryos were those in which the cotyledons had completely folded over the apex and extended all the way to the root tip.

4.2.2. In situ hybridisation

In situ hybridisation experiments were done as previously described in Section 2.6. Plant and silique samples were fixed briefly in FAA (freshly made) under vacuum for 2 x 15 minutes and left in the fixative for 3-4 hours at room temperature. After fixation, the tissue samples were washed, dehydrated, cleared and embedded in paraffin wax (Paraplast X-TRA Product No. 88809-50302). Paraffin sections (8-10 μm thick for vegetative and inflorescence materials, 12 μm thick for siliques) were cut in a microtome and attached to pre-coated glass slides (Esco Superplus precoated slides). Anti-sense probes were synthesized using digoxigenin (DIG-UTP, Boehringer Mannheim) following the manufacturer's instruction. Immunodetection of the DIG-labelled probes was performed using an anti-DIG antibody coupled to alkaline phosphatase as described earlier. Sections were viewed with Nomarski optics on a Zeiss Axiophot compound microscope. Images were captured either with a photographic or a digital camera.

The following probes were used: *SHOOTMERISTEMLESS* (*STM*) antisense probe was used as a positive control in all of the experiments. *STM* antisense probe was synthesized from a cDNA (provided by H. Schoof from Thomas Laux lab) extending from *STM* codons 81-382 + 3' UTR. For *TEL1*, sense and antisense probes were synthesized from a full length *TEL1* cDNA (1.8kb). Sense and antisense probes of *TEL2* were synthesized also from a full length cDNA (1.5kb). Sense and antisense probes of *AML1* were synthesized from a 3.6 bp cDNA fragment.

4.3. Results

4.3.1. Expression patterns of Mei2-like genes during embryo development

TEL1 mRNA is expressed at high levels in discrete patterns in the shoot and root apical regions of late globular, transition, heart, torpedo and bent stage embryos (Figure 4.2A- F). During the early stages of embryogenesis, late globular to heart stages (Figure 4.2A- C), *TEL1* mRNA was detected in the sites encompassing both the presumptive embryonic shoot apical meristem and the cotyledon primordia.

In the root apex, *TEL1* mRNA was strongly expressed in the region corresponding to quiescent centre of the root apical meristem (RAM). A less intense staining, however, was detected in the neighbouring cell files above the quiescent centre. During the subsequent development of the embryo, torpedo stage (Figure 4.2D, E), when the cotyledons have increased in size and extend above the SAM region, *TEL1* expression had completely diminished from the cotyledons, but persisted strongly in the presumptive embryonic SAM. This pattern of *TEL1* expression remained confined to the presumptive embryonic SAM until the bent stage of the embryo. During these later embryonic stages, *TEL1* expression as indicated by the dark staining has increased in width, corresponding to the growth in size of the embryonic SAM (Figure 4.2E, F). While the pattern of *TEL1* expression slowly diminished from the cotyledons as these grew in size and became developed and fully differentiated, the transcripts persisted a bit longer in the adaxial sides of both the cotyledons. During both the torpedo and mature stages of the embryo, *TE1* transcripts continued to be expressed in the embryonic SAM and the root apical meristem.

TEL2 mRNA was also detected in the presumptive SAM of wild type *Arabidopsis* during the globular, torpedo and bent stages of the embryo (Figure 4.3A, C). Unlike *TEL1* expression, however, *TEL2* transcript was not detected in the cotyledon primordia and transcripts were not present in the root apical meristem. *TEL2* mRNA expression was confined to the shoot apical meristem region with a similar intensity and cell width as that seen for *TEL1*.

AML1, a member of a different subgroup of the *Mei2*-like gene family, has an expression pattern that is diffused throughout the embryo during the globular and heart stages (Figure.4.4A, B) with the transcript at high levels and uniformly distributed all over the tissues. During the torpedo stage, however, there is a more distinct and intense staining in the embryonic SAM (Figure.4.4C). *AML1* expression pattern was very similar to that of *TEL2* in that *AML1* mRNA expression was significantly more intense in the embryonic SAM of the embryo, with no distinct strong staining detected in the root apex.

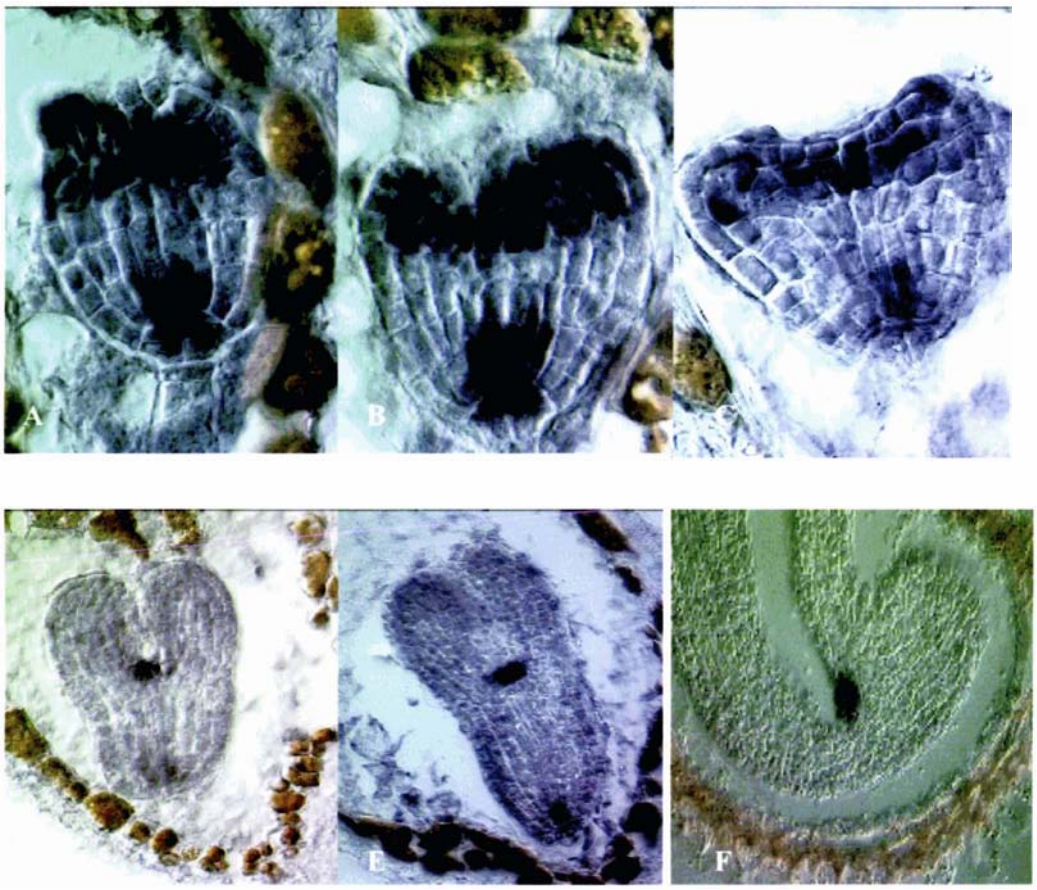


Figure 4.2. *TEL1* mRNA expression patterns during embryo development of wildtype *Arabidopsis.* In longitudinal sections (LS) of wildtype *Arabidopsis* embryos, *TEL1* mRNA is expressed in the presumptive shoot apical and root apical meristems of (A) late globular, (B) transition, (C) early heart, and (D, E) torpedo stage embryos. (F) *TEL1* expression in SAM persists in mature embryo. Sections are 12 *u* thick. The plants from which the siliques were taken had been grown at 23 C under long days for 24 days.

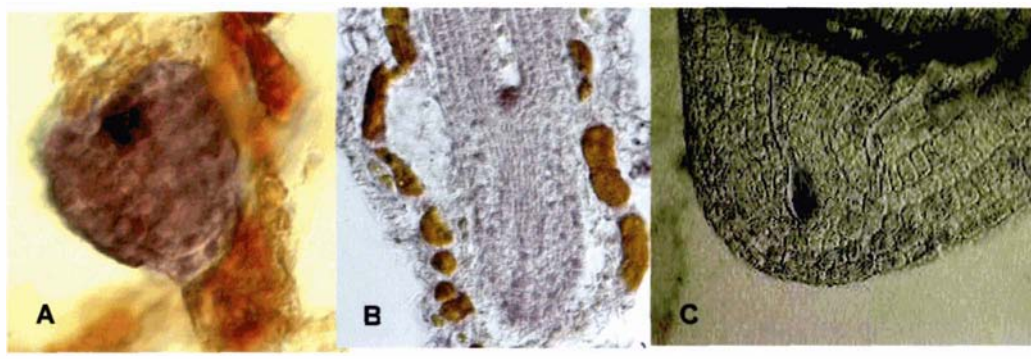


Figure 4.3 *TEL2* mRNA expression patterns during embryo development of wildtype *Arabidopsis.* The gene transcripts are distinctly expressed in the embryonic SAM during the (A) globular (B) torpedo and (C) bent stage embryos. Sections are 12 *u*m thick from siliques harvested from long day grown plants

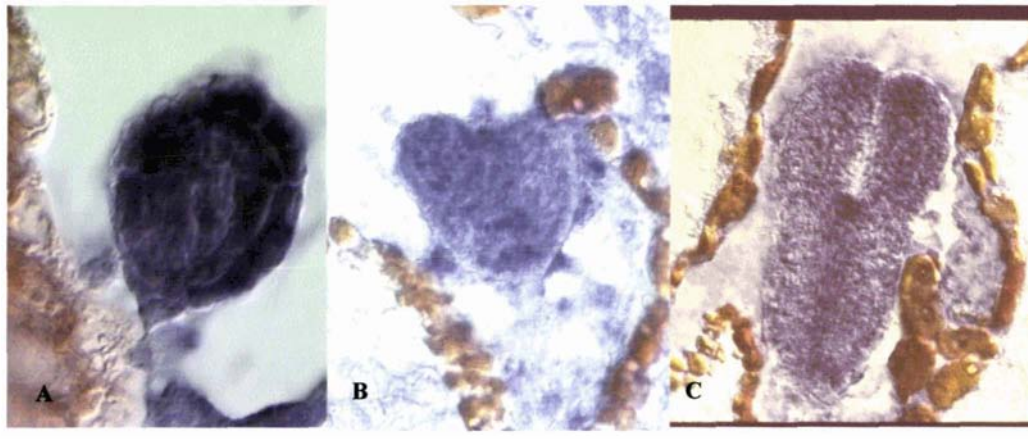


Figure 4.4. *AML1* mRNA expression patterns during embryo development of wildtype *Arabidopsis.* The gene transcripts are expressed throughout the embryo during (A) globular and (B) heart stage embryos. (C) During the torpedo stage, there is distinct higher expression in the presumptive SAM. Sections are 12 *u*m thick from siliques harvested from longday grown plants.

In summary, *TEL1* and *TEL2* both showed discrete and distinct patterns of expression during the early stages of the *Arabidopsis* embryogenesis. Interestingly, *TEL1* expression pattern is unusual in being expressed in both the shoot and root apex during an early patterning process. While *TEL1* was expressed in both the embryonic SAM and RAM, *TEL2* expression was confined to the embryonic SAM. The expression of *AML1* was more diffuse with a distinct higher expression in the embryonic SAM during the torpedo stage of embryo development.

4.3.2. TEL expression during vegetative development

Vegetative development of *Arabidopsis* is characterised by the formation of a series of rosette leaves in a spiral phyllotaxy. Molecular genetic studies have identified a number of genes that are expressed in vegetative SAMs that are involved in maintaining the SAM's self-renewing and organ forming activities (Smith *et al.,* 1992; Jackson *et al.,* 1994; Long *et al.,* 1996; Mayer *et al.,* 1998). Interestingly, *TEL* gene expression in the SAM persisted during the vegetative development, suggesting that in addition to playing a role in establishment of the SAM, *TEL1* may also have a maintenance function. In a median longitudinal and transverse sections of 19-23 day-old *Arabidopsis* vegetative shoot apices, *TEL1* is expressed in the vegetative shoot meristem and young leaf margins (Figure 4.5). *TEL1* mRNA expression appeared as diffuse staining throughout the whole meristem with some strong and light patches of expression in certain regions within the shoot apex (Figure 4.5A). It was also expressed in the margins of very young leaves (Figure 4.5B).

TEL2 mRNA expression was more localised. In a longitudinal section of the vegetative shoot apex, hybridisation with the *TEL2* antisense probe resulted in a distinct wedge-like staining of a group of cells within the central region of the shoot apical meristem (Figure 4.6A, B). This staining pattern appears to exclude peripheral cells as shown in the transverse sections (Figure 4.6C, D).

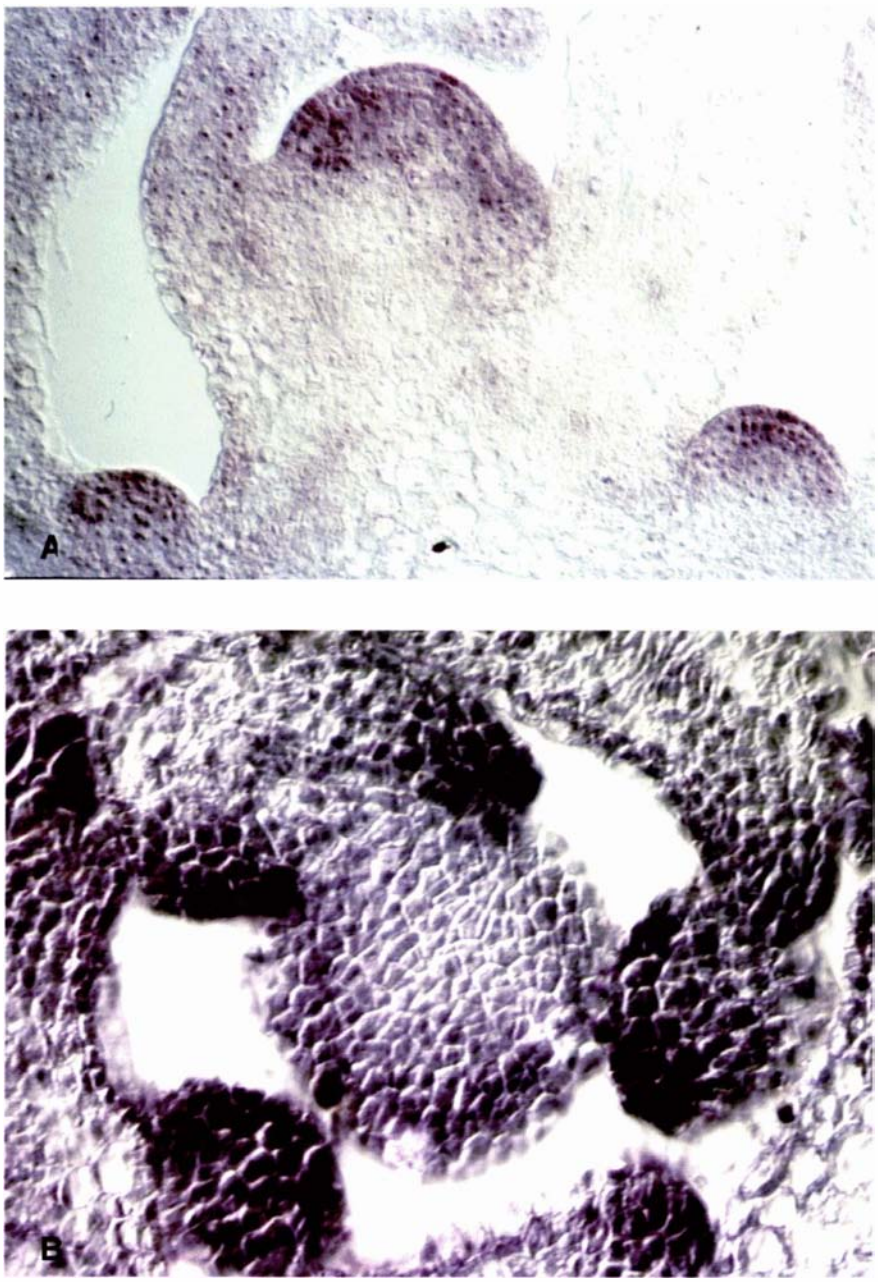


Figure 4.5. *TEL1* expression pattern in the vegetative meristem of wildtype *Arabidopsis*. (A) Longitudinal section of *Arabidopsis* vegetative shoot apex (VM) showing *TEL1* expression distributed throughout the shoot apical and axillary meristems. (B.) Transverse section showing *TEL1* mRNA expression in margins (marginal meristems) of young leaves. . Sections are 8 *u* thick .

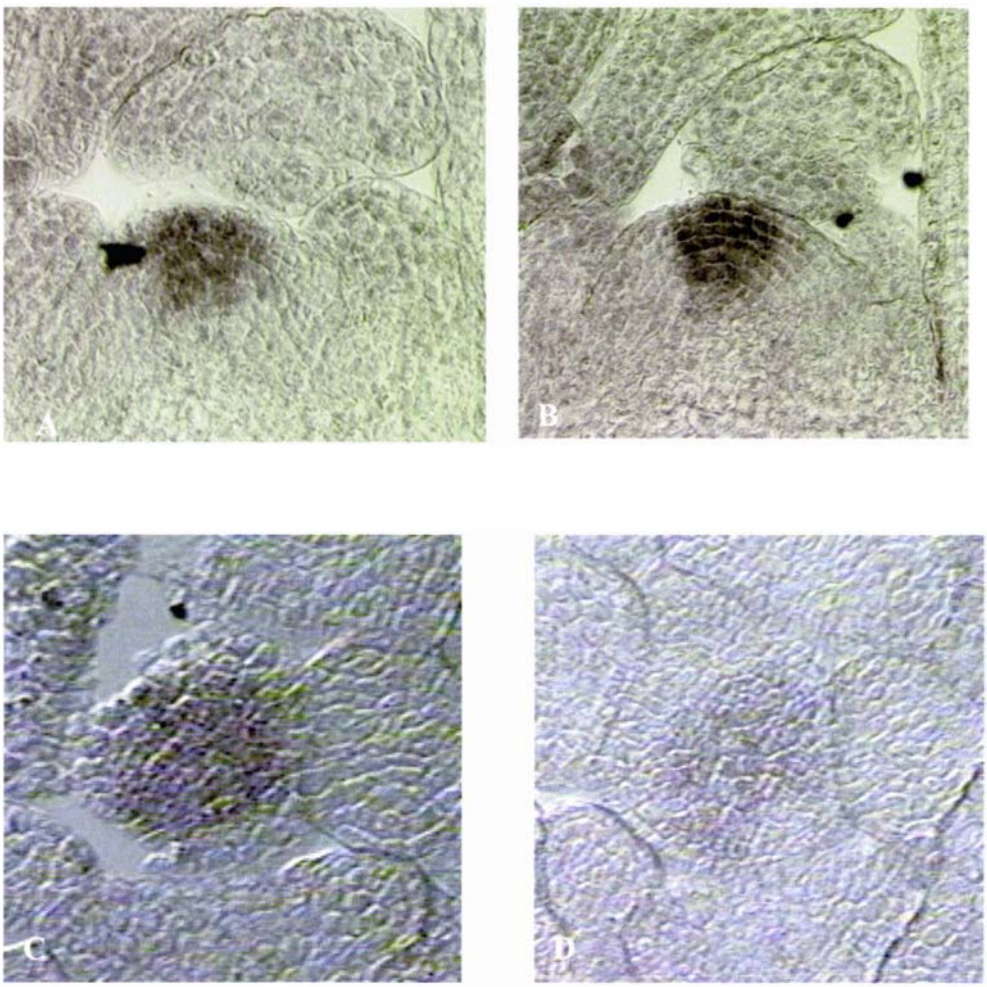


Figure 4.6 *TEL2* mRNA expression patterns in vegetative meristem of wildtype *Arabidopsis*. (A, B) Adjacent longitudinal sections of a vegetative shoot apex showing *TEL2* mRNA expression in a wedge pattern in the central region of the shoot apical meristem (SAM). (C, D) Adjacent transverse sections showing *TEL2* mRNA expression in the SAM. *TEL2* is expressed more intensely in the (C) uppermost apical SAM region and (D) diminishes in the lower adjacent section. Sections are 8 *u*m thick.

4.3.3. Expression patterns of Mei2-like genes during inflorescence and floral development

Shortly after transferring three-week old plants from short-day to long-day growth conditions, plant internodes start to elongate, and secondary inflorescence branches and floral primordia initiate at the periphery of the inflorescence meristem in a spiral pattern very much like the pattern whereby leaves of *Arabidopsis* are initiated. Floral meristems first emerge as small bulges along the flank of the inflorescence meristem but soon develop into domes of undifferentiated cells from where sepals, petals, stamens and gynoecium are initiated. The stages of flower development described in this study were identified following the same morphological identification used in *Arabidopsis*, Atlas of Morphology (Bowman, 1994).

TEL2 mRNA was expressed in the central region of the meristem and in the floral anlagen within the longitudinal section of the inflorescence meristem (Figure 4.7A). In a stage 5 flower, when the sepals have formed and the stamen are initiated (Figure 4.7D), *TEL2* is expressed very strongly in the stamen primordia. In an inflorescence meristem, *TEL1* mRNA was expressed more strongly in the developing flower primordia (Figure 4.7B). In a stage 5 flower, *TEL1* was also distinctly and highly expressed in the stamen and the central region where the carpels will form (Figure 4.7E). The expression of *AML1* in the inflorescence was more diffuse than either of the *TEL* genes (Figure 4.7C). It is expressed in the whole inflorescence meristem with some distinct patches of higher expression in some groups of cells. In a stage 6 flower, *AML1* is expressed in the stamen primordia (Figure 4.7F).

TEL2 expression in the inflorescence meristem is better visualised in a series of transverse sections where the dark stains corresponding to *TEL2* expression are located in specific regions in the inflorescence meristem, forming a distinct spiral pattern which correlates with the spiral arrangement of the flowers that form on the flanks of the SAM (Figure 4.8A-F). In the uppermost section of the inflorescence meristem (Figure 4.8A), the unstained group of cells appear to

correspond to regions where floral primordia will be initiated. In the lower adjacent section (B), the dark patch of stain would correspond to newly initiated floral primordia. In Figure 4.8D, *TEL2* is highly expressed in an axillary SAM. *TEL 2* is absent in the lowermost section of the inflorescence meristem (Figure 4.8F).

It is interesting to note that the pattern of *TEL2* expression is variable depending upon the stage of flower development (Figure 4.9A-C). In stage 2 flowers (Figure 4.9A), when the flower primordia have grown as distinct outgrowths from the inflorescence meristem, *TEL2* transcripts were present in the central regions of floral apices. When the flower had developed as in a stage 4 flower (Figure 4.9B), *TEL2* transcripts are expressed in the central region of the floral meristem. In a more mature flower (Figure 4.9C), the transcripts were expressed in both the stamen and gynoecium. *AML1* is expressed throughout the whole floral apices of stage 3 flowers (Figure 4.9D). In a stage 6 flower, it is expressed in the stamen and the gynoecial primordia (Figure 4.9F). *TEL1* was also expressed in floral organs. In a stage 8 flower, *TEL1* is expressed in stamens and gynoecium that have grown to be about of the same height at this stage (Figure 4.9G). In a stage 11 flower, *TEL1* mRNA is expressed distinctly in the pollen (Figure 4.9G) and the tip of the growing ovules (Figure 4.9I). One thing that is common in the expression patterns of the genes, though, is that the transcripts seem to be expressed in newly initiated primordia, be it the flower itself or a flower organ.

At the start of inflorescence development, secondary shoot meristems develop from the axils of leaves. Axillary meristems normally develop at the axils of both the cauline and rosette leaves with these meristems becoming visible first in the cauline leaves and spreading basipetally to the axils of the rosette leaves (Hempel and Feldman, l994). *TEL1* and *TEL2* mRNA are both strongly expressed in these axillary meristems (Figure 4.10). Just as in the vegetative meristems, *TEL2* mRNA is expressed most strongly at the central region of the axillary meristem (Figure 4.10A). In transverse sections (Figure 4.10B, C), *TEL2* is expressed in axillary meristems initiated at the adaxial region of the leaf (Figure 4.10B, C). *TEL2* is also strongly expressed in a secondary inflorescence meristem (Figure 4.10D). *TEL1* is expressed more diffusely in axillary meristem (Figure 4.10E).

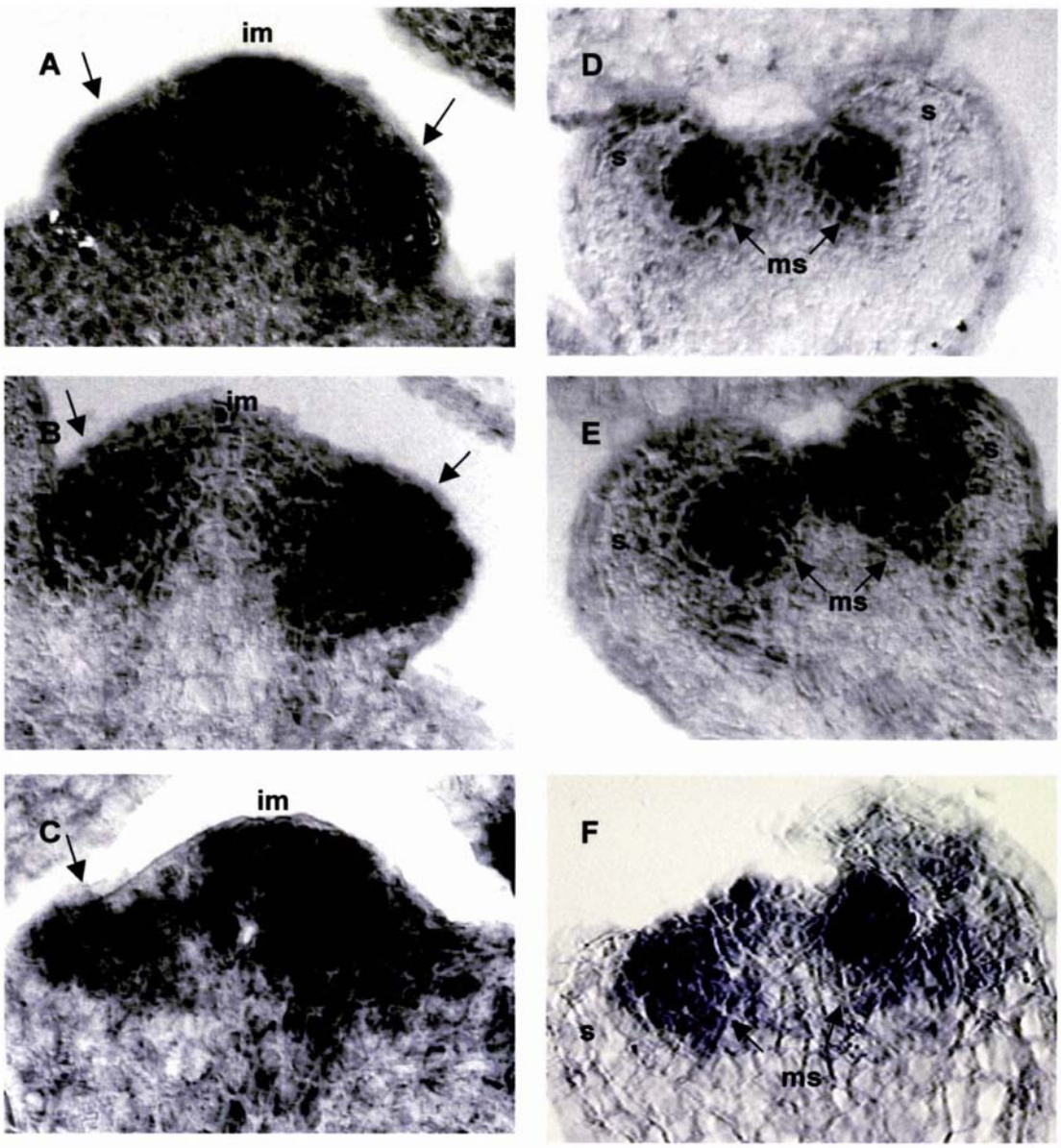


Figure 4.7. Expression patterns of *Arabidopsis Mei2*-like genes in inflorescence and floral meristems of wildtype *Arabidopsis.* (A) *TEL2* is expressed in the central region of inflorescence meristem and floral anlagens (indicated by arrowheads). (D) In a stage 5 flower, *TEL2* is expressed in developing stamen primordia (ms, medial stamen). (B) *TEL1* is expressed very strongly in the developing floral primordia. (E) In a stage 5 flower, *TEL1* is expressed in stamen primordia and the floral meristem. (C) *AML1* is expressed throughout the inflorescence meristem, while (F) in a stage 6 flower, *AML1* is expressed in developing stamen primordia. Arrowheads indicate floral primordia. (im-inflorescence meristem, s-sepal, ms-medial stamen).

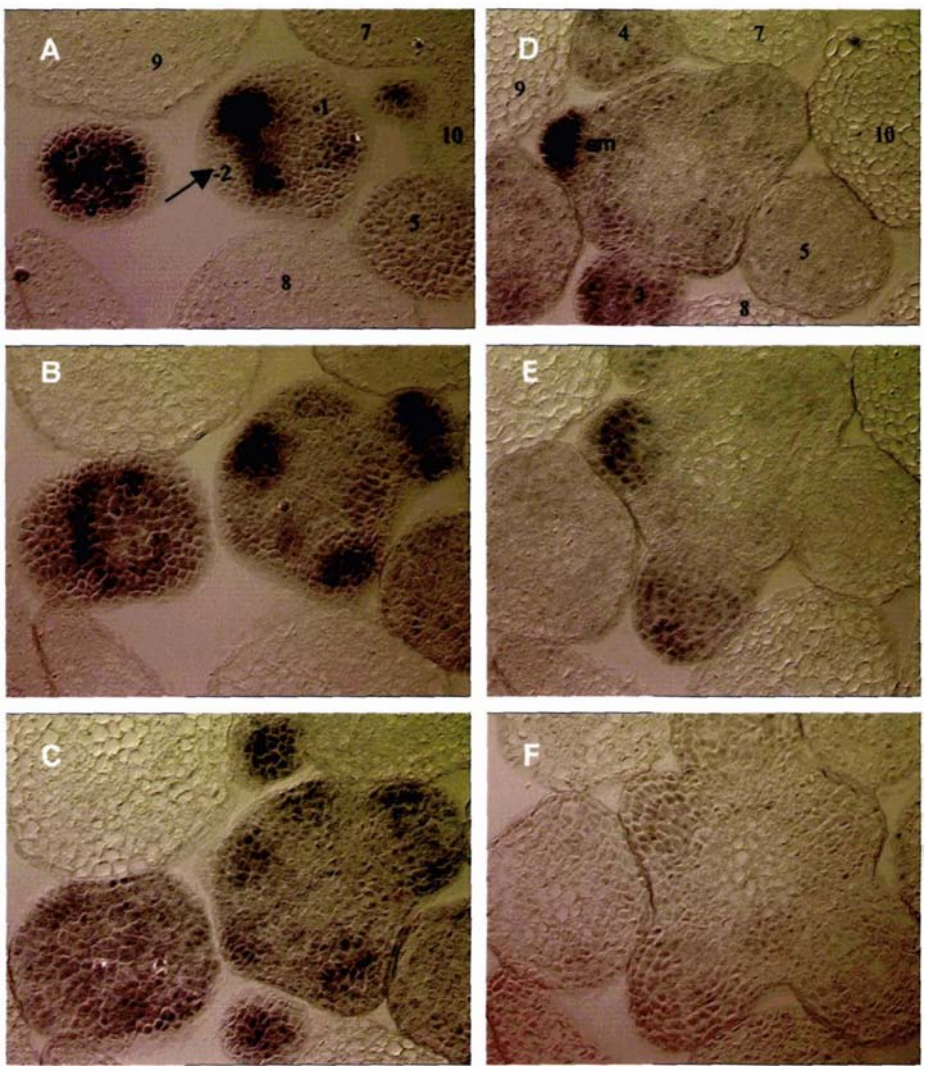


Figure 4.8. Serial, transverse sections of an inflorescence meristem (IM) of wild type *Arabidopsis* showing *TEL2* mRNA expression patterns in the inflorescence and floral meristems. Sections are 8 *u*m thick and are ordered from the most apical (A) to the basal section (F). *TEL2* is expressed in young and developing floral primordia and axillary meristem (am). Floral primordia are labeled 0, 1, 2, etc. in order of increasing age. The plant from which this inflorescence was taken had been grown at 23 °C under long days for 24 days. Arrowheads indicate the position of next primordia (-1 and -2) to be initiated.

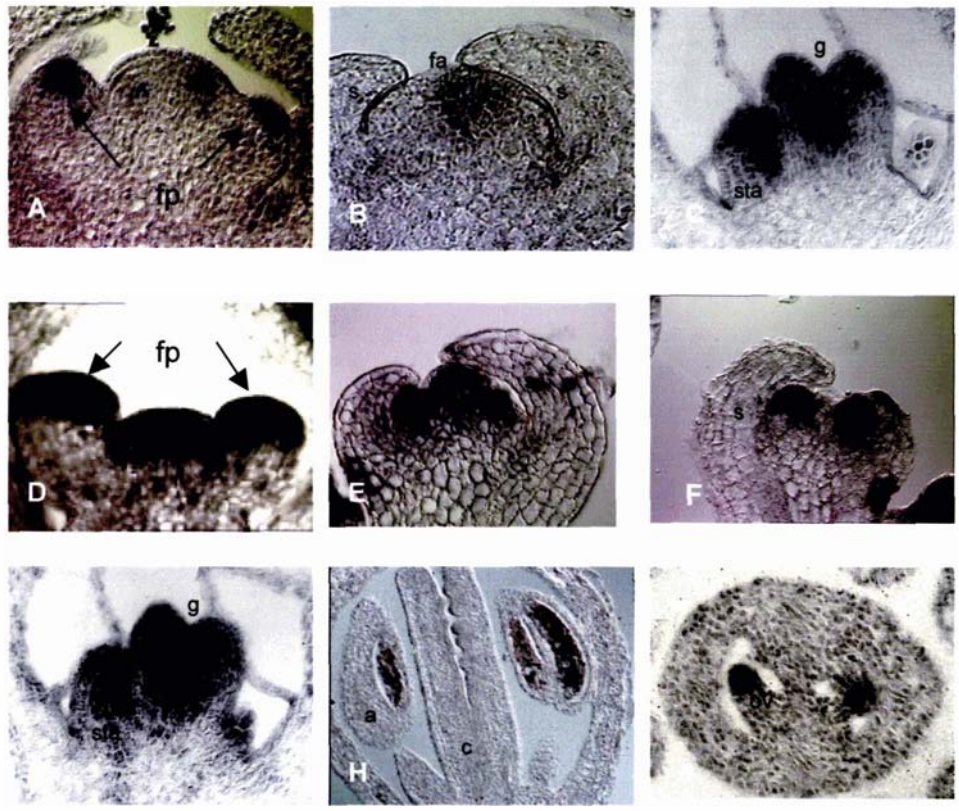


Figure 4.9. Expression patterns of *TEL2*, *AML1* and *TEL1* during flower development. *TEL2* is expressed in (A) stage 2 flowers (marked with arrowheads), (B) central region of floral apex in stage 4 flower, and (C) throughout developing stamen and gynoecium in a stage 8 flower. *AML1* is expressed (D) throughout floral primordia in stage 3 flowers, and (E, F) throughout floral organ primordia in stage 6 flower. (G) In a stage 8 flower, *TEL1* transcripts are detected in both the gynoecium and stamen. (H) In stage 11 flower, *TEL1* is expressed in pollens and (I) ovule tip. (fp-flower primordia, fa-floral apex, s-sepal, g-gynoecium, c-carpel, ov-ovule). Sections are 8 *u*m thick.

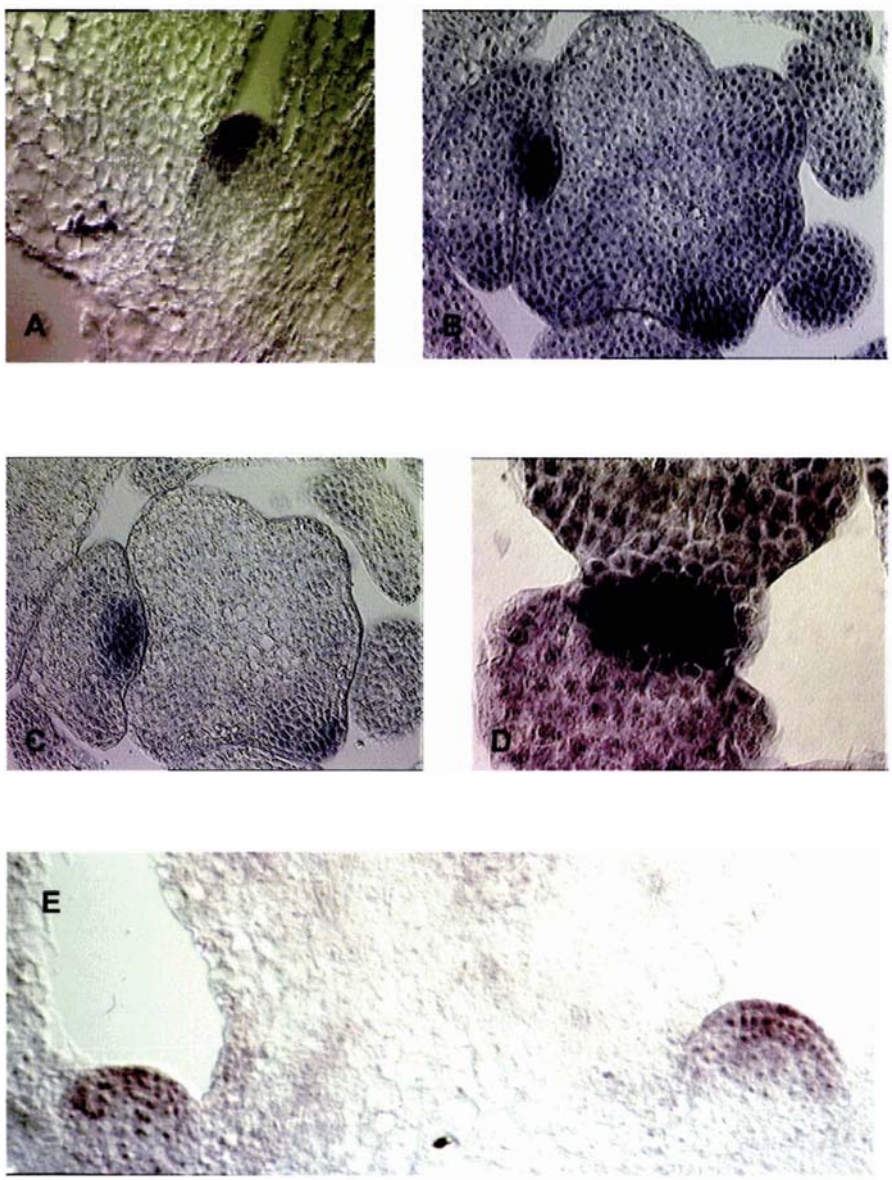


Figure 4.10. *TEL1* and *TEL2* mRNAs are expressed in axillary meristems. (A) Longitudinal and (B, C) adjacent transverse sections of a shoot apex showing expression of *TEL2* mRNA in the axil of a leaf. (D) Transverse section of a secondary shoot apical meristem showing *TEL2* expression in a secondary SAM. E*) TEL1* is expressed throughout the axillary meristems. .

4.3.4. Expression of TEL genes in clavata mutant

***c**lavata* mutants, *clv1, clv2* and *clv3* have very similar phenotypes, which are thought to reflect the interdependent way in which the normal gene products function. CLV1 and CLV2 proteins are members of the receptor complex and CLV3 is a ligand that activates the complex. *CLV1* encodes a protein containing extracellular leucine repeats (LRR's), a transmembrane domain and an intracellular serine/threonine kinase domain (Clark *et al.*, 1997). The CLV2 protein consists of extracellular LRR's, a transmembrane domain and a short cytoplasmic tail (Jeong *et al.*, 1999). CLV3 protein has been shown biochemically to bind specifically with the CLV1/CLV2 complex that indicates it to be a ligand (Trotochaud *et al.*, 2000).
All *clavata* mutants have enlarged meristems and initiate more organ primordia than the wild type (Clark *et al.*, 1993,1995; Jeong *et al.*, 1999). In a fasciated meristem of *clv3* mutant, the large size of the meristem is attributed to an accumulation of stem cells in the central zone. Given that these genes are normally involved in limiting the size of the meristem by promoting the progression of stem cells to organ formation, we wanted to analyse *TEL* expression in *clv* meristem. If the *TEL* genes specify undifferentiated region of the central zone domain, then we would expect to see corresponding enlargement of the *TEL* gene expression domain in the mutant meristems.

Figure 4.11 shows the expression patterns of *TEL* genes in the *clv3* mutant. *TEL1* and *TEL2* are both expressed in the bent stage of the *clv3* mutant embryo (Figure 4.11B and D, respectively) in a wider region than in the wild-type (Figure 4.11A and C). In a wild type inflorescence shoot apex, *TEL2* is expressed in a region corresponding to the undifferentiated region of the meristem (Figure 4.11E) while in a fasciated shoot apex of *clv3* mutant (Figure 4.11F), *TEL2* is expressed in a wider region which includes the epidermis and the cell layer below it corresponding to the central zone domain of the enlarged meristem but was not detected in the regions where leaf primordia are initiated. *TEL2* was also expressed in the inflorescence and floral meristems of *clv3* mutant in a distinct 'patchy' pattern (Figure 4.11G-I).

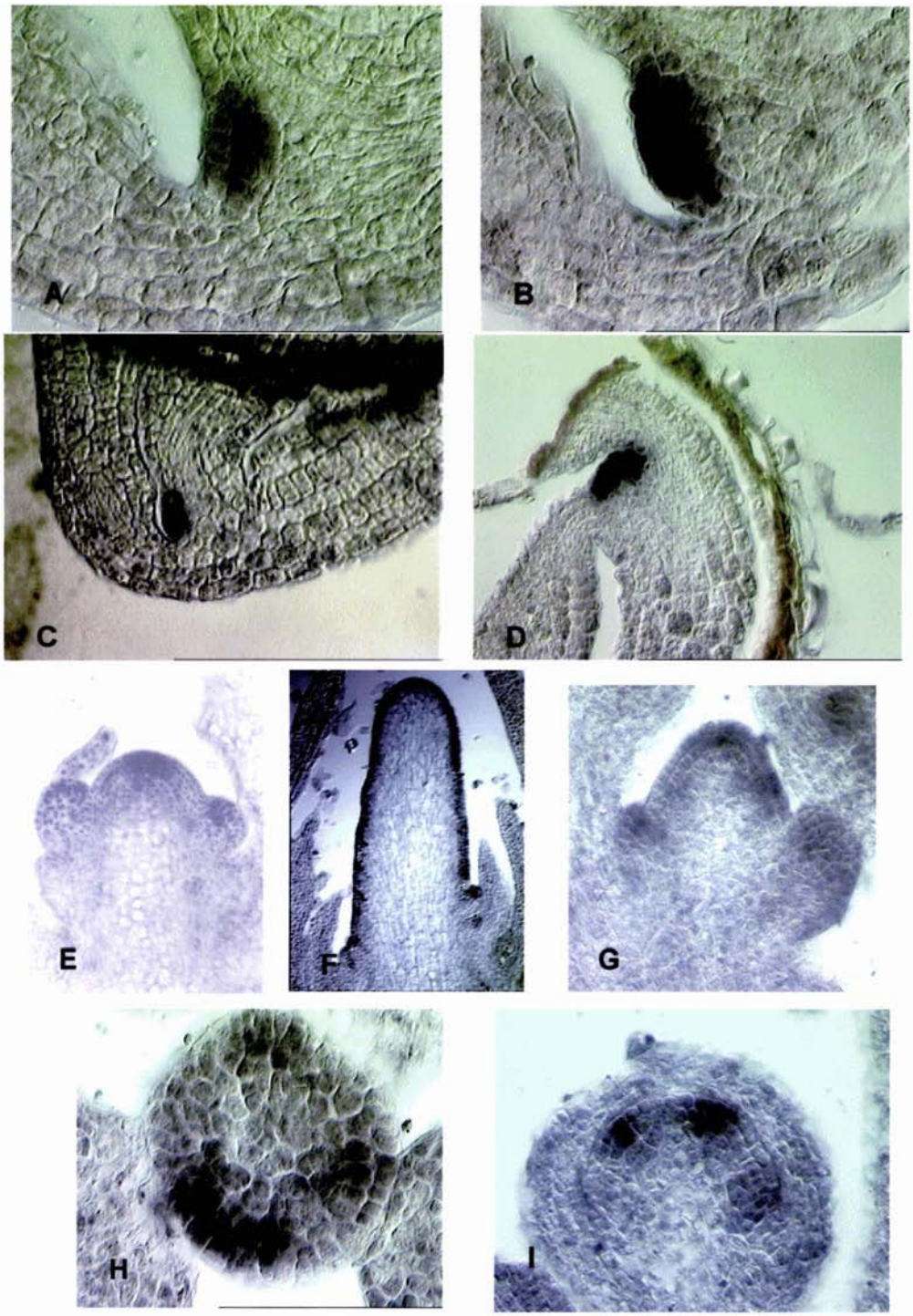


Figure 4.11. Expression patterns of *TEL* mRNAs in *clv* mutant. A) *TEL1* is expressed in the embryonic SAM of wild-type embryo. (B) *TEL1* is expressed in the enlarged SAM of *clv* mutant. C) *TEL2* is expressed in wild-type embryonic SAM. (D) *TEL2* expression domain is enlarged in *clv* mutant. (E) *TEL 2* is expressed in the central region of wild- type inflorescence SAM. (F) In a fasciated *clv* meristem, *TEL2* is expressed throughout the enlarged stem cell region. (G) In longitudinal and (H) transverse sections of *clv* inflorescence meristem, and (I) floral meristem, *TEL2* is expressed in distinct patches.

4.4. DISCUSSION

4.4.1. Function of plant Mei2-like genes

The expression domains of the plant *Mei2*-like genes suggest that they may be involved in the regulation of meristem development and function. *In situ* RNA-hybridisation experiments showed expression during the development of the embryo, seedling and inflorescence in distinct overlapping SAM domains which are thought to comprise the undifferentiated regions or stem cells. These results suggest that the Arabidopsis *Mei2*-like genes may act redundantly to specify the undifferentiated nature of the meristem.

4.4.1a. The roles of plant Mei2-like genes in embryo development

Results show that *TEL1* was highly expressed in both the SAM and the RAM throughout embryo development starting from the late globular stage to the mature embryo stage. During the early embryo stages, globular and heart stage, *TEL1* was detected in the whole upper apical region encompassing the cotyledon primordia and the presumptive shoot apical meristem. By late heart stage, the dark staining was limited to the embryonic SAM and the embryonic RAM. *TEL 2* was detected in the SAM as distinct dark staining in the apical sub-epidermal cells in the early globular stage of the embryo. By the transition stage, the expression became more distinct and limited to the SAM. *AML1* expression was more diffuse throughout the embryo proper during the early stages but was present more strongly and distinctly at the SAM during the torpedo stage.

The expression of these plant *Mei2*-like genes in the presumptive shoot apical meristems as early as when the embryonic SAM and/or RAM are being initiated and the persistence of the transcripts in the meristems throughout embryogenesis indicate that these genes may be involved in basic processes regulating the formation of the meristems and their subsequent activities. One possible interpretation is that these genes act to specify a region in which organogenesis does not occur by preventing or inhibiting cellular differentiation.

Previous molecular genetic studies indicate that the genes like *WUS, STM*, *PNH/ZLL*, and *CLV* (Laux, *et al.,* 1996; Endrizzi *et al.,* 1996; Moussian *et al.*, 1998; Clark *et al.*, 1996) expressed early on during embryogenesis were required in the formation and/or subsequent maintenance of the SAM. In a similar manner, the expressions of *TEL1, TEL2* and *AML1* as early as the globular stage suggest involvement of these genes in regulating SAM formation and/or development. The genes that are known to be expressed earliest during embryogenesis, for example, are *ZWILLE/PINHEAD* and *WUSCHEL* (Mayer, *et al.,* 1998). *WUS* is first expressed during the 16-cell stage in a small group of subepidermal cells. Mutational analysis suggests *WUS* is directly involved in conferring stem cell identity. The *ZWILLE* class of genes were also found to be required to specify stem cell identity (Moussian *et al.*, 1998). During late globular stage of the embryo, the genes *SHOOT MERISTEMLESS (STM), CUP-SHAPED COTYLEDON (CUC)* and *AINTEGUMENTA* are first expressed (Aida *et al.*, 1999, Long and Barton, 1998). These genes are all expressed in distinct patterns in the apical region of the globular embryo. *STM* and *CUC* have overlapping domains. *STM,* which was found to be involved in specification of stem cell identity, was detected in the central region of the SAM. *CUC*, which was proposed to have a role in the separation of organs from the meristem and from each other, was also expressed in the central region, while *ANT,* which specifies organ initiation, is expressed in the peripheral region of the SAM. The expression patterns of these genes were found to correspond to functional domains of the SAM. During the heart stage, the expression of *CLV* genes and their interaction with *WUSCHEL* was found to be important in maintaining the meristem where the *CLV* signalling complex negatively regulates *WUS* (Schoof *et al.,* 2000).

There are three observations which suggest that *TEL1* may be involved in specifying the "meristematic" nature of cells in the cotyledon precursors, the presumptive SAM and the root meristem. First, *TEL1* is expressed in the entire upper apical region during the globular stage of the embryo, a stage when the SAM and cotyledons become organised. Second, *TEL1* expression in the cotyledons slowly fades during the heart stage and then completely diminishes from the cotyledons at the onset of torpedo stage, when the cotyledons have supposedly

undergone differentiation. Third, *TEL1* is highly expressed in the quiescent centre/RAM, a region which is also a meristematic region.

The expression of *TEL1* in the root apical meristem (RAM) as early as the globular stage (the earliest stage examined in this study) and its persistence up to the mature stage of the embryo suggests the involvement of *TEL1* in establishing and maintaining the RAM. The root meristem contains a group of undifferentiated cells surrounding a group of mitotically inactive cells, the quiescent centre. *TEL1* transcripts persisted in these regions throughout embryo development. Previous studies (van den Berg *et al.,* 1997) suggest that the cells of the quiescent centre inhibit differentiation of adjacent cells.

The persistence of *TEL1* transcripts in the shoot and root apical meristem of the plant suggests that *TEL1* may be involved in specifying the identity of the undifferentiated regions of the meristems or that it may inhibit these groups of cells from being recruited into organ primordia. Although the shoot and root meristems have been shown to use different sets of regulatory genes, the expression data for *TEL1* suggests regulation of a process common to both meristem types. The restriction of *TEL2* expression in the SAM region at the same late globular stage, and its absence from the root apical region throughout embryo development, suggest that *TEL1* and *TEL2* genes may be acting together to regulate the SAM.

There have been controversies regarding the origin and development of the shoot meristem, and specifically, the origin of the cotyledons. One of these arguments maintains that the cotyledons arise independently of the embryonic shoot apical meristem and that the shoot apex is partitioned into two distinct regions when the embryonic SAM is first differentiated, the cotyledon primordia and the "apical cells" in the centre (Laux and Jurgens, 1997). A contrasting argument presents the cotyledons as the first products of the shoot meristem, just as the leaf is initiated from the vegetative SAM (Kaplan and Cooke, 1997). The second argument supports the concept of a pre-existing SAM; a view that is validated by the expression of *STM* mRNA in the meristem summit of the early to mid-globular stage embryo (Long *et al.,* 1996). However, the observation that *stm* mutants produce normal cotyledons

suggests that the mutant has produced a normal SAM, which it clearly has not. The question about SAM origin is not resolved by the present expression analysis. However, the expression of *TEL1* in the presumptive cotyledon primordia and the observation that *tel1 tel 2* double mutant do not initiate cotyledons (as discussed in more detail in Chapter 5) is interesting because it suggests involvement of the *TEL* genes in SAM formation. It will be interesting to study how the *TEL* genes may interact with *WUSCHEL* which is expressed early on during embryo development even before a shoot apical meristem is morphologically evident (Mayer *et al.,* 1998) and also to analyse the expression pattern of *TEL1* and *TEL 2* in *stm* embryos.

Sequence analysis show that *TEL1* and *TEL2* are in the same subgroup within the *Mei2* gene family and these genes are the most similar to the maize *TE1* gene (Jeffares, 2001). While both genes are detected in the shoot meristems, it is interesting to observe that only *TEL1* is expressed in the RAM. This is an unusual expression pattern, for a gene to be detected in both the apical meristems early on in the embryo development. In previous studies, different sets of regulatory genes appear to mediate development of the shoot and the root apical meristems. That *TEL1* was expressed in both meristems suggests that there may be a common regulatory mechanism governing early patterning events in the shoot and the root and that *TEL1* may also be involved in establishing and maintaining the stem cells of both. That there are differences in the expression domains between the two genes suggesting that the *TEL* genes may perform slightly different functions, but the function may overlap to various degrees at different stages of development. That the genes are redundant is supported by genetic analysis that shows the absence of any obvious phenotype in single gene knockouts (described in more detail in Chapter 5).

4.4.1b. The roles of plant Mei2-like genes in postembryonic development

To understand how the plant *Mei2*-like genes may be involved in postembryonic development, it will be helpful to briefly review meristem structure and function here, which was discussed in more detail in Chapter 1. Once the shoot meristem has been formed, it becomes a source of all the above ground parts of the plants, including the leaves, internodes, axillary shoots and flowers. The shoot meristem is divided into functionally distinct cell groups: (1) central zone, (2) peripheral zone and the (3) rib zone. The central zone consists of the stem cells, organs are formed in the peripheral zone while the pith tissues are derived from the rib zone. Since all the above ground parts are continually initiated from the peripheral region of the meristem, there has to be a continual replenishment of cells from the central region.

TEL2 expression pattern persisted in the vegetative meristem. In a pattern similar to that seen in the embryo, *TEL 2* continued to be expressed in the vegetative SAM in a wedge shaped pattern covering the central region. The expression of *TEL2* during the vegetative development appears similar to that of the maize *te1* gene, in the sense that both genes are expressed most strongly in regions of the SAM that are not recruited into leaf primordia. The expression of the two gene differs in the sense that while *te1* is expressed in semi-circular rings bracketing leaf initiation sites along the length of the maize shoot apex, *TEL2* expression domain corresponds primarily to the central zone of the shoot apical meristem.

TEL1 was expressed throughout the length of the vegetative SAMs, but there are some regions where staining was mottled, with patches of both lightly and darkly stained tissue. It is conceivable that if *TEL1* is involved in the regulation of basic developmental process like cell division, then the differences in the expression pattern observed may be attributed to the differential division rates of the expressing cells. It appears that just like in the embryonic stage, the region where *TEL1* is expressed is wider than *TEL2* but that the expression domains of the *TEL* genes continue to overlap.

In the inflorescence meristems, *TEL2* transcripts were detected in regions which bracket sites of floral initiation and the pattern of expression is reminiscent of the *te1* expression in maize. This observation supports the hypotheses that these genes may be involved in specifying stem cells or undifferentiated regions in the meristem. Regions where these genes are not expressed would correspond to determinate organ-forming regions. In the inflorescence meristems, when the floral primordia have been developed into discrete floral meristems and formed their own group of undifferentiated cells, the genes become expressed in these regions. Other genes such as the *CLV* genes, *CLV1, CLV2, CLV3, WUSCHEL* and *STM* were reported to be expressed only in the stem cells of the SAM and were down regulated in the organ-forming sites (Clark *et al.*, 1993, Clark *et al.*, 1995, Endrizzi *et al.*, 1996, Mayer *et al.*, 1998). One of the earliest markers for leaf initiation is *STM* where the regions of leaf initiation are marked by the downregulation of STM in these regions (Long and Barton, 1998). It would be interesting to know how the plant *Mei2*-like genes may interact with these patterning genes.

While the expression patterns of the 3 *Mei2*-like genes indicate overlapping expression domains during specific stages of the plants development, there are also differences in expressions at certain developmental stages. One common feature about the expression patterns of the 3 *Mei2*-like genes, for example, is that they are all expressed in shoot apical meristems, flower primordia, and floral organ primordia, which suggests involvement of these genes in promoting the "undifferentiated" nature of these structures. Whether they act in the form of a complex or redundantly is not addressed in this study but results of Chapter 5 gives a preliminary evidence of redundant function for *TEL1* and *TEL2*. That *TEL1* is expressed in the anthers and the ovule does not conclusively indicate that *TEL1* may be involved in meiosis in plants just as *S. pombe mei2* is required in meiosis, but *TEL1* could very well be involved in specifying the yet undifferentiated nature of these tissues. Results also show consistent expression of *TEL1* and *TEL2* in axillary meristems. Given that axillary meristems are also secondary shoot meristems which are initiated at the axils of leaves, the expression of the *TEL* genes in these

structures strengthens our hypothesis that these genes are involved in meristem function, particularly in specifying the undifferentiated nature of these meristems.

4.4.2. TEL expression in clv mutants

To test the hypothesis that the *TEL* genes expression domains mark undifferentiated cells, we analysed the expression of *TEL* genes in *clv* mutant. Molecular, genetic and biochemical data indicate that the *CLV* genes are involved in meristem maintenance by negatively regulating the expression of *WUSCHEL*, a meristem gene which confers stem cell identity (Schoof *et al.*, 2000). In the absence of *CLAVATA* genes, the domain of expression of *WUSCHEL* becomes enlarged and the region of stem cell population increase correspondingly. Since the *clv* mutants have an enlarged stem cell domain due to the massive accumulation of stem cells (Clark *et al.*, 1993, 1995 and 1997), we expected the *TEL* genes expression to be enlarged as well. The expression data in this study does not fully address the mechanism of interaction between the *TEL* genes and *CLV* nor conclusively establishes the function of the *TEL* genes, but the enlarged domain of *TEL* expression in *clv* mutants supports the model that the expression of *TEL* genes promotes or maintains cells in an undifferentiated state. It is specifically interesting to see that both *TEL1* and *2* transcripts were expressed in broader regions of the SAMs of *clv* embryos, most specifically in a broader stem cell region in a fasciated *clv* meristem. It will be interesting to pursue this aspect of the project to determine how the *TEL* genes may interact with meristem maintenance genes, including *CLV, STM* and *WUS*.

4.5. Conclusions

The expression of the plant *mei2* like genes, particularly the *TEL* genes, early during embryogenesis indicates that these genes may be involved in the early patterning events during embryogenesis in *Arabidopsis* and possibly in the formation of the SAM. The presence and persistence of the expression patterns in specific domains in the SAM during the vegetative and the inflorescence development suggest that they may be involved in maintenance of the meristem. Data presented here and the following chapter support the idea that these genes

may act redundantly to specify the undifferentiated region of the meristem and may act as a switch for these cells to assume new cell behaviour pattern or identities.

The expression data suggest that *TEL* and *AML* genes may be involved in basic developmental processes in the plant which involve meristem function. The expression of *TEL*1 in other plant parts especially the root meristem and both the pollen and the ovule suggest that the genes function appear to be more complex than was thought. These expression patterns suggest *TEL1* involvement in other developmental processes as well. The function of the genes is still less clear but mutant analysis and functional analysis may reveal more about how these genes function.

Chapter 5. Genetic analysis of Arabidopsis Mei2-like genes

Abstract

This chapter describes a reverse genetics strategy to identify and characterise knockouts of *Mei2*-like genes in *Arabidopsis.* Single homozygous knockouts *of tel1, tel2* and *aml1,* which were identified from a large pool of T-DNA insertional mutants using PCR methods (Arabidopsis Knockout Facility at the University of Wisconsin) showed no obvious phenotypes. When crosses were made between the single knockout lines to generate *tel1 tel2* and *tel1 aml1* double mutants, abnormal seedling development was observed. Initial analyses of *tel1 tel2* double mutants are presented and discussed in relation to possible roles of the *Mei2*-like genes in plant morphogenesis. The putative double mutants show variable phenotypes that all reflecting embryonic pattern defects. *tel1 tel2* double mutant phenotypes show apical-basal defects, arrested embryo development, proliferation of numerous secondary shoots and production of numerous leaves. Extreme *tel1 tel2* double mutant phenotypes show abnormal seedlings which lack both SAM and RAM and which also lack cotyledons. Together these data suggest that *Mei2*-like genes are involved in early embryonic patterning processes and more specifically in the formation of the shoot and root apical meristems.

5.1. Introduction

In *Arabidopsis*, the SAM is established during embryogenesis and together with other seedling parts (cotyledons, hypocotyl, root meristem and the root) makes up the basic body plan (Jürgens *et al.,* 1991; Endrizzi *et al.,* 1996; Laux *et al.,* 1996; Long and Barton, 1998). During postembryonic development, the SAM is responsible for the continued formation of organs. Thus, the shoot apical meristem serves two basic purposes: meristem self-renewal and organ formation (Steeves and Sussex, 1989). For normal development of a plant, the SAM has to function by

maintaining a group of pluripotent stem cells in the summit to replenish those that enter differentiation for organ initiation.

The relationship between the organisation of the SAM and behaviours of resident cells is especially well documented. Aside from its tunica-corpus organisation, the SAM is also subdivided into functional zones or domains; consisting of the central, peripheral and the rib zones (Steeves and Sussex, 1989). Cells in the central zone are often classified as stem cells and are characterised by their ability to renew themselves, multiply, and differentiate for a variety of specialized functions. Cells in the peripheral zone, on the other hand, by virtue of their position, are considered to have a determined fate related to programmes controlling organogenesis while those in the rib zone give rise to cortical and vascular tissues. The group of slowly dividing pluripotent cells maintained in the central zone serves as the ultimate source of all cells produced in the shoot. Their immediate daughter cells become displaced to the peripheral regions where they enter a more rapid division patterns, differentiate and assume new identities. As organs are initiated in the peripheral zone of the SAM, cells recruited to form the organs must be replenished through the production and displacement of uncommitted cells from the central zone.

The question of how the shoot apical meristem is stably maintained given the continued removal of cells for organogenesis has been addressed by different methods of developmental analyses. More recently, molecular genetic analyses have provided additional insights into the study of meristem function. In *Arabidopsis*, a number of mutants show basic pattern defects that have been interpreted as defects in the formation and function of the meristem. *stm* mutants, for example, are characterised by the failure of the SAM to form and the slight fusion of cotyledons at their bases, suggesting that *STM* gene is required for SAM formation (Long and Barton, 1998). Similarly, *cuc1 cuc2* (cup shaped cotyledons) double mutants not only fail to produce an embryonic SAM, but produce abnormal cotyledons which are fused at their bases, suggesting that *CUC1* and *CUC2* are required for SAM formation and suppression of growth at cotyledon boundaries (Aida *et al.*, 1997, 1999). Mutations in *WUS* results in premature termination of shoot and floral

meristems suggesting a role for the gene in specifying stem cell identity (Laux *et al.,* 1996*).* The *clavata* (*clv)* mutants are characterised by enlarged meristems, which is attributed to the unchecked accumulation of stem cells, indicating that the *CLV* genes are required in the maintenance of the SAM (Clark *et al.,* 1997). The analyses of these mutant plants have provided excellent means of dissecting the biological function of a particular gene and relating complex developmental processes. In this study, we have addressed the biological functions of *TEL1, TEL2* and *AML1* by characterising mutant phenotypes resulting from knockouts to these genes.

In conventional forward genetics, the study usually starts with the isolation of an individual with a mutant phenotype, progresses with phenotypic analysis and description of the gene as a heritable trait, and culminates with a functional analysis of the gene. Sometimes, the whole exercise ends with cloning of the gene. The opposite is done in reverse genetics, as the study starts with a given gene sequence, progresses with perturbation of that gene's activity, and culminates with a description of phenotypes that result from that altered gene activity (Krysan *et al.*,1999). In both forward and reverse genetics, however, the basic question remains the same, "what is the biological function of a particular gene?"

The popularity of reverse genetics can be traced to two recent developments. First, through large scale genomic and EST sequencing efforts, a large number of gene sequences has become accessible, though the biological function of many, if not most, remains unclear. Second, a variety of methods have made it possible to identify and isolate individuals in which the activity of a particular gene, defined at the DNA level, has been altered. One common approach involves the use of transgenic plants in which the expression of a gene is either suppressed or augmented by antisense or overexpression constructs. A somewhat different approach, detailed in this chapter, involves PCR identification of insertional mutants from large pools of individuals that have been mutagenised by transformation with T-DNAs. A variant of this method uses endogenous transposable elements as an insertional mutagen (Chandler and Hardeman, 1992).

In the case of *Arabidopsis,* reverse genetics has been greatly facilitated by the completion of its genome sequence and the availability of large populations of T-DNA transformed lines (Krysan *et al.,* 1996). Polymerase chain reaction (PCR) methods have been developed that allow one to identify individual plants that have T-DNA insertion in a particular gene of interest. By using PCR method, one is able to screen thousands of independently transformed lines by sample pooling (McKinney *et al.,* 1995; Winkler and Feldman, 1998; Krysan *et al.,* 1996). Two such populations, each consisting of approximately 60,000 independent T-DNA transformed lines, can be accessed through the Arabidopsis Knockout Facility at the University of Wisconsin (http://www.biotech.wisc.edu/arabidopsis). For a modest fee, the facility allows users to screen the population of lines for the presence of T-DNA inserts in the particular gene of interest.

The expression patterns of the plant *Mei2*-like genes *TEL1, TEL2* and *AML1* as early as the globular stage (as described in Chap 4 of this thesis) suggested involvement of these genes in regulating SAM formation and/or development. These genes were also expressed in the vegetative and inflorescence SAMs, axillary meristems and early floral primordia. The expression of these genes in distinct domains in the meristems suggested the possible involvement of the genes in specifying and maintaining the undifferentiated stem cells in the meristems and somehow restricting this population from entering an organ related differentiation pathway.

While these expression studies were suggestive, it is clear that the functional roles of these plant *Mei2*-like genes in the plant developmental process can only be conclusively demonstrated through analyses of mutants. If the genes were involved in early patterning events and or early shoot formation and maintenance, mutant phenotypes should be uncovered that reflect defects in embryogenesis and subsequent seedling development.

In situ RNA-hybridisation experiments showed that during the embryo, seedling and inflorescence development, *TEL1, TEL2* and *AML1* were expressed in specific regions of the shoot meristems where populations of undetermined cells are

maintained. The expression data also suggested that the genes may have different functions and may be required at different stages of development. The overlapping expression domains of these structurally similar genes also suggested the possibility that they may act in a functionally redundant manner and that double or triple mutants for these genes might be required before any visible phenotype is observed.

Sequence analysis (Jeffares, 2001) showed that *TEL1* and *TEL2* were most similar to maize *te1*. In terms of expression pattern (Chapter 4), *TEL2* appeared most similar to *te1* in that it is expressed in regions of the SAM that are not recruited into leaf primordia. In the inflorescence meristem, *TEL2* was expressed in regions that are not recruited for floral primordia in a pattern reminiscent of the phyllotactic pattern of *te1* expression in maize. *TEL1*, however, was expressed throughout vegetative SAMs and was also expressed in the transition stage embryo SAMs, cotyledon primordia and root meristem. *AML1* was expressed throughout the embryo, but at higher levels in the embryonic SAM during the later stages of embryonic development. During the vegetative stage, *AML1* expression was also diffuse, but became more distinct during inflorescence development when it was expressed in the floral organ primordia.

Because of the overlap in expression profiles, the lack of visible phenotypes in the single gene knockouts and structural similarities that suggested the possibility of overlap in function, double mutants were generated and scored for segregating mutant phenotypes. This chapter focuses on the genetic analysis and characterisation of double mutants.

5.2. Materials and Methods

5.2.1. The Arabidopsis Knockout Facility

Figure 5.1 outlines the organization and screening strategy of 60,480 T-DNA transformed lines (Krysan *et al.*, 1999). The strategy works on the assumption that once integrated into the plants chromosomes, the T-DNA with a known DNA sequence serves as an insertional mutagen for which PCR primers can be

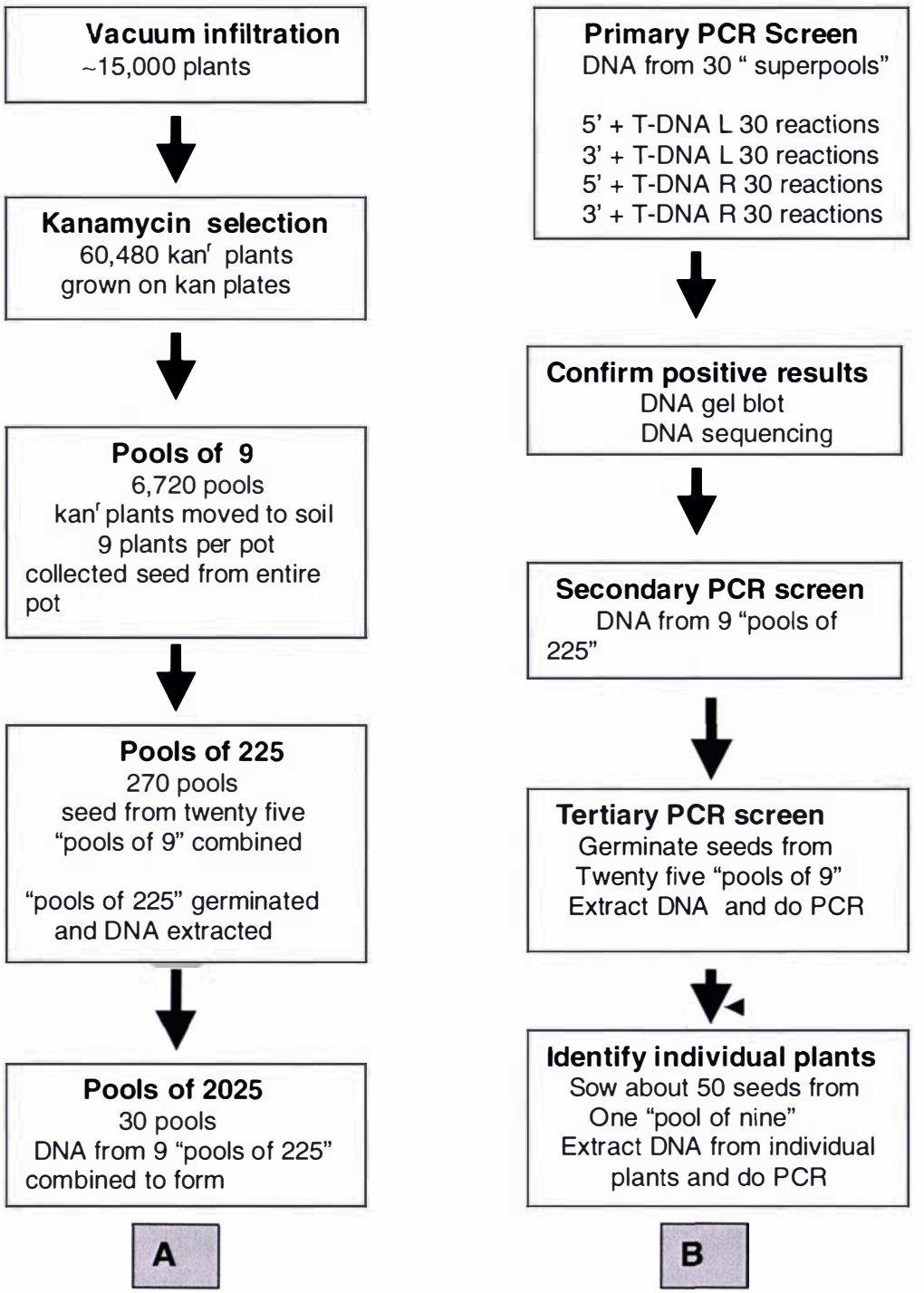


Figure 5.1. Organisation and screening of T-DNA transformed Arabidopsis lines.
(A) Pooling strategy.(B) T-DNA insertion screening strategy.

(Source: http://www.biotech.wisc.edu/arabidopsis/default.htm.

designed. By designing a specific primer targeted to a specific gene in combination with a T-DNA specific primer, lines containing a T-DNA insertion in a specific gene of interest can be identified in pooled populations by performing PCR reactions. The complete screening for *tel1* and *aml1* and initial screening for *tel2* single knockouts were done by Carmel Gilman (Veit lab) making use of the Wisconsin Arabidopsis Knockout Facility (http://www.biotech.wisc.edu/arabidopsis).

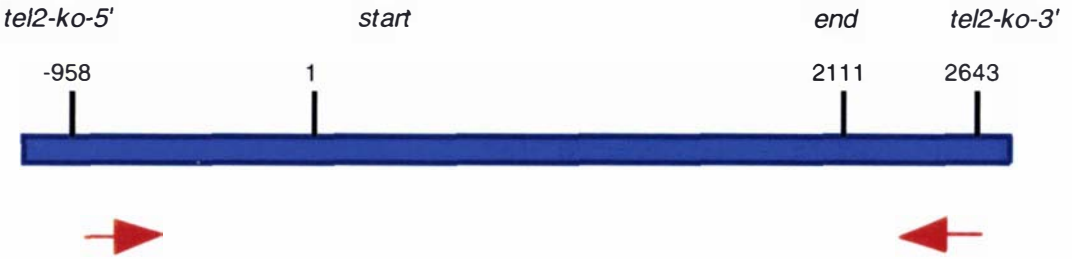
Single *tel1 and aml1* knockouts were screened from a population of T-DNA transformed *Arabidopsis* with pD991 vector. *tel2* was screened from a population of *Arabidopsis* transformed with pSK1019.

5.2.1a. Knockout primer design

The primary PCR screen was done at the Knockout Facility. Carmel Gilman of Massey University designed primers specific for the gene of interest and tested them for compatibility with the T-DNA specific primers under PCR conditions specified by the facility. The primers were tested first for compatibility with primers to the T-DNA left and right borders before these were sent to the facility where the primary PCR screen was done.

The following control primers were also synthesised: Con-1A, Con-1B, JL 202 and XR2. Con-1A and Con-1B amplify a 5 kb fragment of the Arabidopsis genome and served as a standard against which to compare the performance of the gene specific primers. JL 202 and XR2 are primers used by the UWBC facility for the T-DNA left and right borders, respectively. Two primers at the extreme 5' and 3' ends of the gene were designed based on the following recommended primer specifications: 29 base pairs in length with a GC content between 34 and 50% and zero to 1 G or C allowed at positions 28 and 29. A web-based primer selection program was used in primer design (http://www-genome.wi.mit.edu/cgi-bin/primer). The primers locations with respect to the ORF of the different genes are presented in Figure 5.2.

A. TEL 1 genomic DNA



B. TEL 2 genomic DNA

C. AML1 genomic DNA

Figure 5.2. Graphical illustration showing knockout primer locations in A) *TEL1*, B) *TEL2* and C) *AML1* genomic DNA. The locations of the primers are marked in relation to the transcription start site which was used as a reference point and was designated as 1.

5.2.1b. Hot start PCR condition

A 50 *ul* PCR reaction mixture was used in the experiment using Ex taq DNA polymerase (Source:Pan vera) (1x ex-taq buffer, 12pmol of each primer, 250 μM of each deoxynucleotide triphosphate, 0.4 ng Arabidopsis DNA, 2.5 U x-taq polymerase) with the following PCR conditions: 5 minutes initial denaturation at 96°C during which time the TAQ mixture was added, and 36 cycles of denaturation at 94° C for 15", annealing at 65°C for 30", and 2' extension at 72°C. A final extension time was done for 4' at 72° followed by holding at 4°. The following PCR reactions were initially done: (1) Con-1A + Con-1B, a gene specific reaction that tests PCR in the lab and provides a standard with which to judge the gene specific primers, (2) Con-1A + Con-1B + JL-202, a reaction which tests the compatibility of Con-1A and Con-1B with the border primer, (3) Gene-specific primers and (4) Gene specific primers + JL202, a reaction which tests the compatibility of the gene specific primers with the border primers. Five *μl* of the PCR product was loaded in agarose gels and compatibility assessed by comparing lanes 1 and 3 and lane 2 with lane 4. The PCR product in lane 1 should be similar with lane 3 and PCR product in lane 2 should be similar with lane 4. The primers were mailed to the KO facility once they had passed the compatibility test and used for the primary screening.

5.2.1c. DNA sequencing of PCR products

The PCR products were mailed back from the facility, run on a gel, photographed and southern blotted with a gene specific probe to identify "real knockouts". For those PCR reactions that a product hybridised to a gene specific probe for the target gene, DNA sequencing was performed to verify authentic DNA insertions within the different genes, *TEL1, TEL2* and *AML1*. This was done by performing sequencing reactions using the T-DNA border primer JL 202 or XR2 as the sequencing primer. A 50 sequencing reaction contains 4 *μl* Big Dye enzyme (Source: Applied Biosystems), 1x reaction buffer, 12 pmol primer and 50-100 ng DNA. The product was cleaned, precipitating first with 75% ethanol at RT for 15' and

washing with 85% ethanol. This was sent to Massey Sequencing Facility as a dry sample.

5.2.1d. Finding the knockout plant

The Southern blot and DNA sequencing results were used to identify superpool from which a secondary PCR screening was done in the facility to identify the pool of 225 plants. The PCR products were run on agarose gels, Southern blotted and the result submitted to KO facility to identify pool of 9 plants. A seed population from this 'pool of 9' was sent to us, germinated, DNA extracted and individual knockout plant identified by PCR. DNA was extracted using Shorty DNA quick-prep (Section 2.2.4c).

5.2.2. Genetic crosses

Plants used for generating double mutants were first tested by PCR for homozygosity and/or heterozygosity for the T-DNA insert. When this experiment was done, we only had recently identified a T-DNA insertion in *TEL2*, hence heterozygous plants for the *tel2* mutation were used in the crosses. The crosses that were made are summarised in Table 5.1. Only the F2 progenies of cross 2 were initially characterised, results of which are presented in this chapter.

Flowers of plants to be used as female parents were emasculated by using forceps to remove anthers. These flowers were pollinated on the same day by touching the stigma with anthers from the male parent. Generally, only 2-3 successive flowers of the same female plant were used in the same cross.

5.2.3. Genetic analyses of double mutants

F1 seeds were germinated in kan^r plates and putative double heterozygotes were confirmed by kan^r screening and PCR analysis. The plants were transferred in soil, grown and allowed to self pollinate to produce large number of F2 plants. F2 seeds from individual plants were harvested, germinated in both kan MS and

Table 5.1. A summary of the crosses made to generate double mutants.

	Female plant		Male plant
1.	*tel1/tel1*	x	*tel2/+*
2.	*tel2/+*	x	*tel1/tel1*
3.	*tel1/tel1*	x	*aml1/aml1*
4.	*aml1/aml1*	x	*tel1/tel1*
5.	*tel2/+*	x	*aml1/aml1*
6.	*aml1/aml1*	x	*tel2/+*

ordinary MS plates and observed for mutant phenotypes. When mutants were observed, segregation ratio was noted for that particular F1 line population. Ten individual F2 plants were genotyped for double T-DNA insertions and heterozygous plants (*tel1/+ tel2/+*, *tel1/+ aml1/+*) were allowed to grow to maturity to generate more seeds. Putative double mutants were submitted for SEM analysis and characterised. F3 progenies of the heterozygotes were planted and scored to determine if the double mutants would segregate across a further generation.

5.2.4. Analysis of embryo and seedling phenotypes

F2 seeds were surface sterilised with 80 % ethanol for 5 minutes, briefly rinsed with sterile water, washed in 50% commercial bleach for 5 minutes and rinsed three times with sterile water. The sterilised seeds were plated on 0.5 MS plates and kan MS plates. The plates were incubated at 4°C for 2 days and then transferred to a growth room maintained at 25 °C under continuous white light. Observation was done with an Olympus stereoscope at daily intervals over a 10 day period beginning from when the cotyledons first emerged. The appearance of abnormal seedlings was noted and segregation ratio based on the observed seedling abnormality was scored. Photographs were taken with a camera attached to the stereoscope. For embryos, siliques from genotyped heterozygous plants were

harvested and fixed in fresh FAA fixative, cleared with chloral hydrate/water, opened using an acupuncture needle under a stereoscope and observed for aborted or abnormal embryos. Ovules were fixed overnight, dehydrated and embedded in wax, according to the protocol described in Section 2. Serial sections, 12 um thick, were cut with a Leica microtome, transferred to polylysine coated slides, stained with 0.05% toluidine blue and coated with xylene based mountant (DPX). The slides were analysed with a Zeiss Axiophot microscope using bright-field illumination. Seedlings showing abnormal and normal siblings were submitted to Hortresearch SEM lab for processing where these were fixed, dehydrated in graded ethanol series, critical point dried, coated and viewed under Scanning Electron Microscope (Cambridge 250 Mark 3).

5.3. Results

5.3.1. Single knockouts

Initial screening of T-DNA transformed *Arabidopsis* lines using the T-DNA right border primer, XR2 and the 5' gene specific primer showed a 2.1 kb product from pool #25 (Screening for *tel1* and *aml1* single knockouts were done by CG Gilman, Veit's lab). This was chosen for the next round of screening because it predicted a T-DNA insert within the RRM1 region of the gene. Southern blot analysis showed the same 2 kb band hybridising with a *TEL1* specific probe. Subsequent sequence analysis shows that the T-DNA is inserted within the RRM1 region, about 670 bp downstream of the transcription start site (Figure 5.3). After the second round of PCR screening, a seed population from a pool of nine lines was sent to us by the Knockout Facility from which an individual plant, homozygous for the insert was identified.

Using similar approach, a 1.6 kb PCR product that hybridised with a *TEL2* specific probe was sequenced to locate a T-DNA insert within the RRM1 region of *TEL2* (Figure 5.4). Sequence analysis, however, revealed a rearrangement within the *TEL2* gene as indicated in the same illustration where there seemed to be an inversion event. In the screening for *aml1* knockout, a 6 kb product which hybridised with a *AML1* specific probe positions the T-DNA insert in the promoter region of

AML1 (Figure 5.5). Subsequent sequence analyses confirmed the authenticity of the T-DNA insertions.

PCR analysis showed the presence of T-DNA inserts in putative homozygous and heterozygous plants and the absence of the same insert in putative wild type plants. The wild type and heterozygous plants showed an amplified gene fragment while plants homozygous for the inserts gave no product when genotyped using gene specific primers which spanned through the T-DNA insertion sites. Phenotypic observation, however, showed no obvious phenotypes for single knockouts *tel1, aml1* and *tel2*.

Expression analyses were done to confirm that no transcripts were produced from the knockouts. Because only *tel1* knockout plants (flowering stage) were available at the time, *in situ* hybridisation experiments were done to determine the expression pattern of *TEL1* and *TEL2* in embryos of *tel1* knockouts. In plants that were homozygous for the *tel1* knockout, the normal expression of transcript in the SAM of torpedo stage embryos was absent. Hybridisations done with *TEL2* on the same material showed expression in the embryonic SAM, confirming the significance of the absence of *TEL1* in the knockout plants (Figure 5.6).

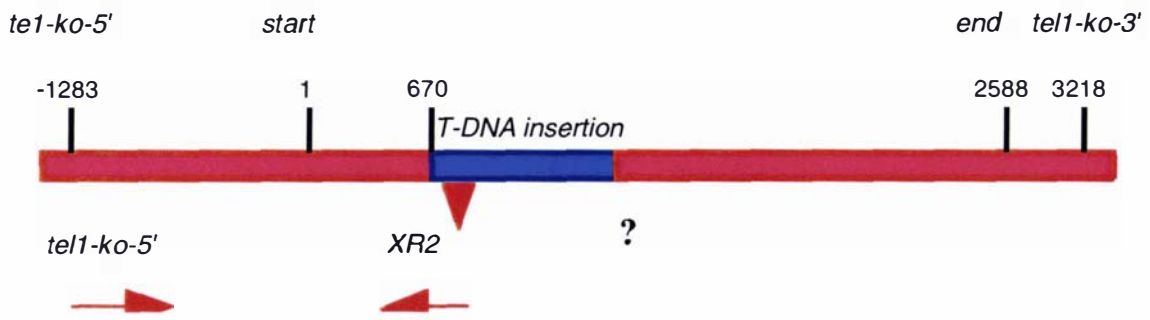


Figure5.3. Graphical illustration showing the T-DNA insertion and primers location in *TEL1* genomic DNA. Sequence analysis shows that the T-DNA right border primer, XR2 is nearest the T-DNA-*TEL1* gene junction, about 670 bp downstream of the start site. tel1-ko-5' and tel1-ko-3' were the gene specific primers submitted to the knockout facility to identify and isolate the knockout. The question mark (?) means that the junction region as depicted in the illustration was not sequenced.

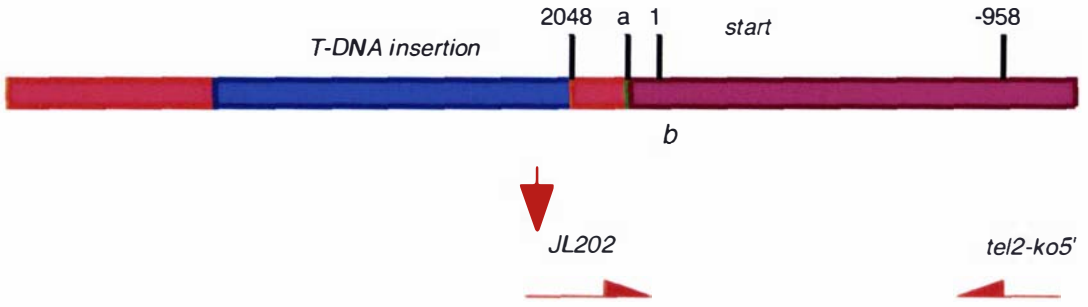


Figure 5.4. Graphical illustration showing T- DNA insertion and primer locations in *TEL2* genomic DNA. Sequence analysis shows that the T-DNA left border primer, JL 202 is nearest the T-DNA/*TEL2* gene junction, 2048 downstream of the transcription start site. However, sequence analysis also shows a rearrangement (inversion event) within the gene as indicated in the illustration (a) 2196 downstream of the start site and b) 69 bp from the start site.

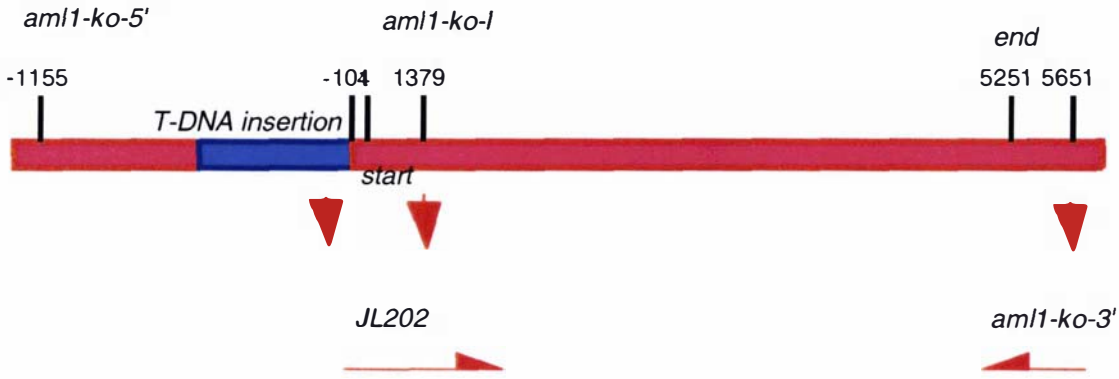


Figure 5.5. Graphical illustration showing T-DNA insertion and primer locations in *AML1* genomic DNA. Sequence analysis shows that the T-DNA left border primer, JL202 is nearest the T-DNA-*AML1* junction which is about 104 bp upstream of the start site. This places the T-DNA somewhere within the promoter region of the gene. The primers aml1-ko-5' and aml1-ko-3' were the original gene specific primers submitted to the knockout facility to identify and isolate the knockout. AML1-kol was subsequently used for routine PCR amplification of the gene-insert fragment.

5.3.2. Double knockouts

The lack of obvious phenotypes in the single mutants confirmed an earlier hypothesis that the plant *Mei2*-like genes might have redundant functions. This is supported by the similarity of sequence of these genes and the overlap in their domains of expression. Plants that carried *tel1*, *tel2* and *aml1* singly were crossed to make double mutants. Reciprocal crosses were made wherein an individual plant identified to be a putative homo/heterozygote for the mutation was used as both female and male parent. While F1 seeds from the different crosses were generated, only the phenotypic analyses for *tel1* combined with *tel2* is presented here.

The *tel1 tel2* double mutant is quite variable (Figure 5.7). All of the abnormal seedlings show disruption in the apical basal pattern. The following four groups of phenotypes were observed from the segregating population: 1) the root and callus group, where a mass of root or white callus emerges from the seed, appearing very extreme because there is no apparent resemblance to a normal seedling (Figure 5.7 A, B); 2) the heart-stage embryo-like seedlings, where the abnormal seedling is green and it resembles a heart stage embryo with both of the cotyledon primordia just protruding from the apical part of the seedling. This phenotype is variable, where some abnormal seedlings have grown past the heart-stage-like to torpedo-stage-like stage. This could be taken as progression from the first class of phenotypes because the abnormal seedling seems to show an apical-basal axis but then aborts further organised growth (Figure 5.7 C, D); 3) the cone-shaped seedling where the abnormal seedling seems to have formed a "normal" expanded cotyledons but which are sometimes either fused and have no visible SAM region or that have a multiple SAM structure that emerges out of the central region (Figure 5.7 E, F) and 4) shoot phenotype, where a single expanded cotyledon emerges from the seed with multiple shoots growing out of the "petiole" or the basal portion of the cotyledon (Fig.5.7 G-I). Out of 640 seeds germinated in kan^r or ordinary MS plates, about 28 abnormal seedlings were observed, with phenotypes distributed as follows: 4 mutants show root phenotype, 6 seedlings show the embryo-shaped phenotype, 8 of the abnormal seedlings are cone-shaped and 10 show variable shoot phenotype.

A SEM image of the mutant phenotype and the root tip is presented in Figure 5.8. Although the tissues show some artifactual shrinkage, the figure still illustrates an abberrant morphology of cells in the root end wherein instead of the normal cell files, small irregular disorganised cells are formed. In one of the mutants, root hairs appear at the basal end of the hypocotyl (Figure 5.8). In both of the mutants shown in Figure 5.8, no root growth was observed. The mutants have not yet been analysed further at the cellular level and the phenotypic analyses is being continued including growing the seedlings at varied environmental conditions in an effort to detect the basis of the variable phenotypes.

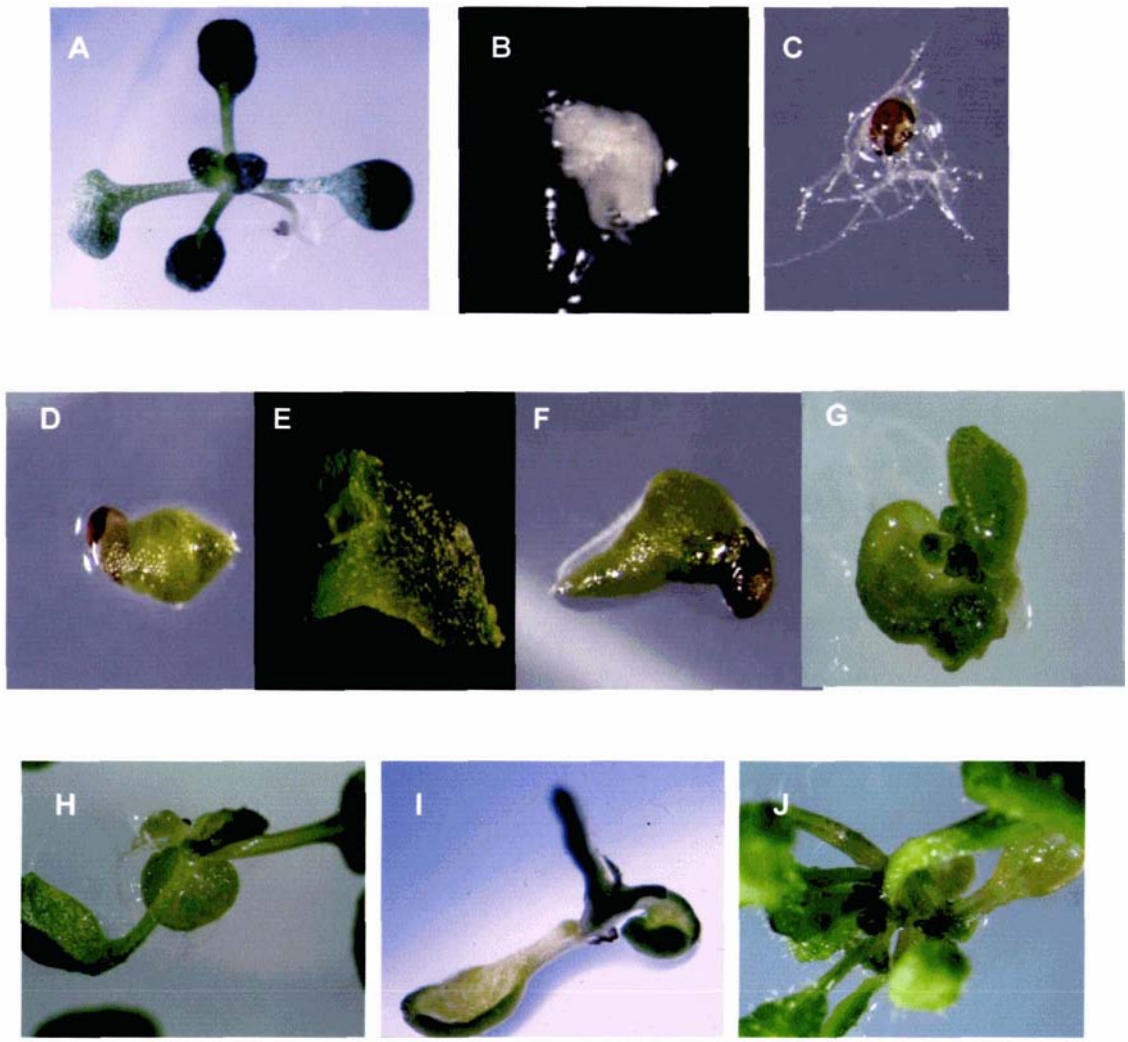


Figure 5.7. *tel1/tel2* double mutant phenotypes. (A) Wildtype seedling. (B-J) Variable *tel1 tel2* double mutant phenotypes. (B,C) Root/callus phenotype. A mass of root or callus emerges from the seed and the growth stops. (D,E) embryonic structure phenotype. The mutant seedling resembles either a heart stage or a torpedo stage embryo. The seedling growth is then arrested at this stage. (F,G) cone-like, where the seedling assumes variable cone-shaped structure. The abnormal seedlings form expanded cotyledons but there are no root growth and either the cotyledon is fused or there is a multiple growth in the SAM region. (H-J) 'shoot' phenotype. The seedlings form multiple secondary shoots from a single cotyledon and in (I) and (J) there are no roots formed.

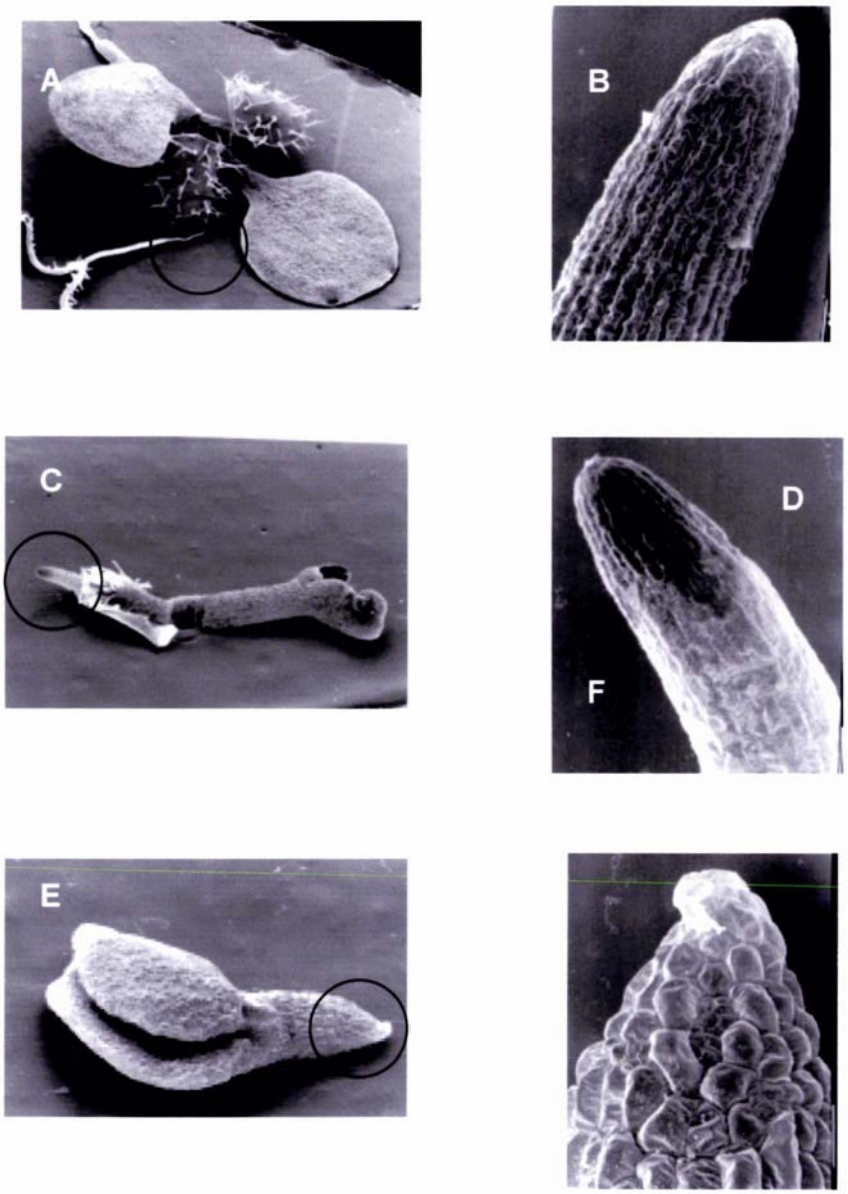


Figure 5.8. Scanning electron microscopy images of putative double mutant and the wildtype. (A) A normal seedling and its (B) root end. (C) One of the *tel1-tel2* double mutant phenotype forms cotyledon but the growth is very slow and while the growth stops at the cotyledonary stage, the normal plant has progressed to 6 leaf stage to about 500 x larger than the mutant. (D) root tip. (E) Cone-like mutant phenotype and (F) abnormal root tip that appears to have no RAM. The cone shaped abnormal 'seedling' is about the size of the Arabidopsis seed, the cotyledons are fused, the root does not grow in length and the growth of the seedling stops there. The mutants are magnified 500 x normal plant.

5.4. Discussion

The genetic analysis of a mutant phenotype can often provides an excellent means of dissecting the function of a gene in a complex developmental process. In order to do this, however, there must be a clear cut relationship between the mutation and the resulting phenotype. It has to be conclusively shown that the phenotype observed is due to a specific gene mutation. In this study, the function of the *Mei2*-like genes is explored by using the reverse genetics strategy. Results indicate no visible phenotype of each of the single *tel1, tel2* and *aml1* knockouts; by contrast a preliminary analyses of a *tel1 tel2* double mutant segregating population shows variable phenotypes which were characterised by apical-basal pattern defects, ectopic expression of numerous secondary shoots and random production of many leaves. The results suggest a disruption of the early embryonic patterning mechanism. Since the experiments done in this study are preliminary and largely exploratory, the discussion of results will be mainly speculative, drawing upon supporting data from the expression analyses (Chapter 4).

5.4.1. What are the functions of plant Mei2–like genes?

The expressions of *TEL1* and *TEL2* and *AML1* in embryonic SAM during the early stages of embryo development suggested that these genes may be involved in early patterning events. In *Arabidopsis*, the primary structure of the seedling is established during the early stages of embryogenesis. The apical-basal axis is established as early as the first asymmetric division of the zygote. Division of the zygote into two equal sized daughter cells results in irregular division in the apical cell which consequently result in *Arabidopsis* mutants with deletions in the apical-basal structures as exemplified by the *gnom* mutant in *Arabidopsis* (Mayer et al., l993).

The lack of visible phenotypes in the single mutants but variable phenotypes in the double mutant segregating population confirm the earlier hypothesis that the genes in the M*ei2*-like family may be performing redundant functions. The phenotype of the *tel1 tel2* double mutant is variable. One group consists of

seedlings that develop a mass of roots or a white callus with no distinct cotyledons and no apparent apical-basal axis (Figure 5.7A, B). The second and third group of the double mutants show apical-basal defects. There is a visible apical-basal axis, where the apical region consisting of the cotyledon is quite distinct from the root end region (Figure 5.7C, D), however, the mutants show only partially developed cotyledons and do not progress beyond the torpedo embryo stage of development.

It would appear that while the apical and basal regions have been established in the mutants, the ability of the meristems to renew themselves has been lost. The cotyledon primordia have started to protrude from the apical region but it seems that their growth stops prematurely. A similar outcome is seen in the root region with the primary root whose end is visible, but apparently arrested in development. The observation that the mutants failed to form the cotyledons raises the possibility that no SAM has been organised in these mutants. If the cotyledons were homologous to leaves and a functional SAM precedes cotyledon formation as postulated in one model of SAM initiation, then the failure of the cotyledons to form in the mutants raises the possibility that no SAM has been initiated in these mutants. An alternative explanation supposes that the cotyledons are not necessarily a product of the SAM, but their formation has a requirement for *TEL* gene function that is also seen in formation of the SAM. One *Arabidopsis* mutant which shows similar defects in the formation of the SAM and cotyledons and also shows an extreme phenotype where the mutants form all roots is the *topless* mutant (Evans and Barton, 1997; Long et al., 1999). It was suggested that the gene is required both to specify the fate of the apical region of the embryo and to suppress root fate.

The root and shoot apical meristems are formed during embryo development. Once the shoot meristem has been formed, it becomes a source of all the above ground parts of the plants, the leaves, internodes, axillary shoots and flowers. The root meristem, on the other hand is responsible for continued root growth and subsequent development of lateral roots. The shoot apical meristem is a highly regulated system where cells recruited to form the organ primordia are continually replenished for the SAM to maintain itself. Failure of the SAM to either replenish the organ forming cells or to supply the organ forming regions with new

cells leads to mutant plants with either large meristems, because of cell accumulation, or arrested growth because the plants can no longer sustain organ formation. Results of the genetic analysis suggest failure of SAM development, as indicated by the third group of mutant phenotypes in which cotyledons have been formed, but are fused, or in one case, numerous secondary "meristem-like" structures emerged from between the cotyledons in the central region (Figure 5.7E, F). In all of the cases observed in this class, there was no root growth. The last group of double mutant phenotypes observed were characterised by the emergence of a single flat structure which resembles one cotyledon with a basal "petiole" (Figure 5.7I). Numerous secondary shoots then grow ectopically at the basal region of the cotyledon (Figure 5.7G-I). In some cases, this single flat structure eventually loses its green colour, becomes white and dies. In most of the cases, no roots are formed and the plants just continue to form leaf primordia and numerous secondary shoots. This would seem to indicate that the specification of cell fate is grossly altered and that all of the cells seem to have gained a pluripotent ability. This phenotype is very similar to the 'stop and go' growth reminiscent of the *wus* mutants phenotype (Laux *et al.*, 1996), suggesting that like *WUS*, *TEL1* and *TEL2* may be involved in maintaining a population of uncommitted stem cells. In older double mutants, numerous secondary shoots arise from the region just below the base of the cotyledon without any visible root system. This observation raises the possibility that in the absence of the *TEL* genes, meristematic cells are randomly distributed throughout the embryo leading to basic pattern defects. Under favourable conditions and requirements for differentiation, these cells leave their undifferentiated state and organs are formed.

Results of genetic analyses support the working hypothesis that the *TEL* genes are involved in regulating SAM formation and function during both embryonic and postembryonic development. The wildtype *TEL* gene products would promote a meristematic identity for cells, and thus limit programmes leading to organogenesis. We also propose that the genes may act in tandem or in group to regulate morphogenesis in *Arabidopsis.* The mechanisms by which this group of genes regulate morphogenesis will be more clearly understood with continued molecular, genetic and biochemical analyses.

5.5. Conclusions

Genetic analyses of plant *Mei2*-like genes show that the genes within this gene family may be performing partially overlapping functions. *tel1 tel2* double mutants are characterised by apical-basal pattern defects and ectopic expression of numerous secondary shoots. These mutants often prematurely cease growth and exhibit an apparent random proliferation of numerous secondary shoots and leaves. It is speculated that the genes within this family particularly *TEL1* and *TEL2* may be involved in specifying the "pluripotent" nature of the meristems and thus prevent these groups of cells from being recruited. It is suggested that further genetic dissection coupled with molecular and biochemical analyses be done to further understand the biological functions of these genes. The project is continuing at present with the goal of characterising F2 and F3 progenies in more detail, characterising the other double mutant combinations, producing more double and triple mutants and/or generate other mutant alleles.

Chapter 6. Summary and Conclusions

6.1. Summary of aims and results of the study

This thesis describes how molecular and genetic methods were used to explore how a novel class of genes, collectively referred to as plant *Mei2*-like genes, regulates morphogenesis in plants. Named for their similarity to the *mei2* gene of *Schizosaccharomyces pombe*, these genes share 3 RNA recognition motifs (RRMs), with the unusual third motif providing the defining hallmark of the class. Since the initial description of *mei2* as a gene required for meiosis (Watanabe *et al.*, 1988), a combination of genetic and molecular approaches have confirmed that its gene product, Mei2p, is an RNA binding protein (Watanabe and Yamamoto, *et al.*, 1994). The same studies also revealed that its activity depends on an unusual pattern of nuclear localisation, which is triggered by the protein binding of a specific non-coding, polyadenylated RNA termed *mei*RNA. While it is unknown whether this form of interaction is common to other *Mei2*-like genes, the unusual third RRM, which mediates the binding, is the most highly conserved region of the protein. Thus, it seems possible that the molecular mechanism by which this class of genes acts is broadly conserved. While the nature of this mechanism presents an important question, the main aim of the thesis was to gain further understanding of how plant *Mei2*-like genes function, and in particular, to understand how the genes may be involved in the regulation of morphogenesis in plants. There were two main aspects of the thesis: 1) morphological characterisation of maize *te1,* the first plant *Mei2*-like gene to be genetically characterised; and 2) the molecular genetic characterisation of *TEL1* and *TEL2* genes, 2 *mei2*-like genes from *Arabidopsis* whose sequence, expression patterns and mutant phenotypes suggest an equivalence to the *te1* of maize.

The first phase of the thesis was specifically aimed at looking into the morphological basis of *te1* mutant phenotype. Results of morphological analyses show that plants that lack *te1* activity 1) have smaller vegetative shoot apices than the wild type; 2) initiate leaves at a higher, more distal position on the apical dome; and 3) have a higher plastochron ratio. Similarly, molecular analyses of *kn1*

expression, a marker for leaf founder cell identity, show that downregulation of *kn1* transcripts occur higher up the dome. Results of the clonal analyses support the notion that leaf determination is occurring higher on the apical dome by showing that a fewer number of leaf founder cells are recruited to form the maize phytomer. The initiation of leaf primordium higher up the apical dome and the higher plastochron ratio in mutant plants suggest that initiation of new leaf primordium is less constricted by the proximity of both the shoot tip and the position of older leaf primordia. The smaller population of leaf founder cells might also explain the narrow and smaller size of leaves and the shortened internodes that characterise the *te1* mutant. While the study did not address specifically the cellular basis of the mutant phenotype, initial characterisation of the shortened internodes revealed an alteration in cell division and expansion patterns.

In the second phase of the thesis, the expression patterns of *Terminal Ear Like* (*TEL*) genes, *TEL1* and *TEL2* were assessed by *in situ* hybridisation and genetic analyses were done to characterise the mutant phenotypes of the genes. Because of the significant sequence homologies between maize *te1* and the *TEL* genes, it was hypothesised that one or both of these might correspond to the *te1* orthologue (similar genes related by descent that have the same function). *In situ* RNA-hybridisation experiments showed that during the embryo, vegetative and reproductive stages of *Arabidopsis* development, the *TEL* genes and 4 other members of AMLs (Alvarez, unpublished data) were expressed in distinct and in some cases, overlapping domains in the shoot apical meristem, which are thought to comprise the undifferentiated regions or stem cells. These results are consistent with the possibility that the Arabidopsis *Mei2*-like genes act redundantly to maintain cells in an undifferentiated state. The expression of these plant *Mei2*-like genes in the presumptive shoot apical meristems and at early stages, during the initiation of the embryonic SAM and/or RAM, and the persistence of these transcripts in the meristems throughout embryogenesis indicate that these genes may be involved in basic processes regulating the formation of meristems, as well as their subsequent maintenance. The finding that *TEL1* is expressed in both the embryonic SAM and RAM, and the restriction of *TEL2* expression to specific domains within the same SAM implicates a broader and more complex role for these *Mei2*-like genes than

previously thought. The distinct and high expression of *TEL1* in both the SAM and RAM throughout embryogenesis suggests a unifying regulatory mechanism in the formation of the root and the shoot, which to a large extent appear to be regulated by different set of genes.

Genetic analyses show that there were no visible phenotypes in each of the *tel1*, *tel2* and *aml1* single knockouts. Preliminary phenotypic analyses of a population segregating *tel1 tel2* double mutants, however, show variable mutant phenotypes. The variable development of putative double mutants suggests a disruption of an early embryonic patterning mechanism. *tel1 tel2* double mutants were characterised by apical-basal pattern defects, ectopic expression of numerous secondary shoots and random production of many leaves. The lack of mutant phenotype for the single knockouts and the overlapping expression domains are consistent with the hypothesis that the genes within this family have partially overlapping functions. It was further hypothesised that genes within this family, particularly *TEL1* and *TEL2*, may be involved in specifying the "pluripotent" nature of the meristem in restricted domains.

To recapitulate, the expression pattern and the mutant phenotype of maize *te1* suggest a role for the gene in specifying or delimiting time and position of leaf initiation (Veit *et al.*, 1998). Expression analysis showed *te1* transcripts were present in the meristem as semi-circular rings that bracket sites of leaf initiation. In so far as leaves were seen to initiate at the open end of the ring, it was suggested that TE1 may function by preventing leaf development within its expression domain. How do the molecular and genetic analyses of other plant *Mei2*-like genes in this thesis impinge on this model? There are many aspects in the results of the study that need to be clarified and considered before a detailed model for *TEL* or plant *Mei2*-like gene function can be formulated. First of all, the distinct but overlapping expression patterns of the *TEL* genes and AMLs (unpublished data) suggest functional redundancy. This overlapping function of the genes was supported by the observation that mutant phenotypes were observed only when double knockouts were generated. Second, the expression of *TEL1, TEL2*, *AML1* and other AMLs (unpublished data) in the SAM early on during embryo development suggest

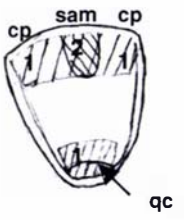
involvement of these genes in SAM formation and development. Third, *TEL1* is expressed not only in the embryonic SAM but also in the RAM throughout embryo development. Later, it is also expressed in the ovule and pollen. Fourth, while all of the other *Arabidopsis Mei2*-like genes are expressed throughout the vegetative, inflorescence and floral meristems, *TEL 2* is expressed in very discrete wedgelike patterns in the central region of the vegetative SAM and stage 2 flower, and as distinct patches arrayed in a spiral pattern in the inflorescence SAM. Fifth, *TEL* genes were highly expressed in axillary meristems. The expression data are consistent with an organogenesis repression function for *TEL2*, but suggest a different and broader function for the other *Mei2*-like genes. The expression of *TEL1* in the ovule and pollen does not indicate whether the gene performs a meiosis related function analogous to Mei2p in *S. pombe*, but its presence in these structures does suggest some function in reproductive processes.

6.2. Model for TEL function

A model for *TEL* function is proposed here which expands on the model proposed for *te1*. The expression patterns and the double mutant phenotypes suggest that the *TEL* genes act redundantly to specify the domain of meristematic cells. Their expression within distinct domains in meristems also restricts organogenesis to positions outside domains of expression. When the genes are knocked out, mutant phenotypes show unformed cotyledons (cotyledons appear to be just emerging out of the opposite sides of a 'heart stage embryo") resulting in a slowly growing malformed seedling very much the picture of a late heart stage embryo. In older double mutants that had somehow progressed beyond this stage numerous secondary shoots arise from the region just below the base of the cotyledon without any visible root system. This observation raises the possibility that in the absence of the *TEL* genes, meristematic cells are randomly distributed throughout the embryo, thereby disrupting the cellular organisation and intercellular communication and leading to the disruption of basic patterning mechanisms. It also raises the possibility that in *tel1 tel2* mutants, meristematic cells are randomly distributed throughout the seedling and that there are now no restrictive factors for organ formation, which implies that organogenesis does not require a highly

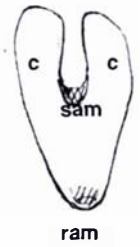
organised SAM structure. Figure 6.1 illustrates the model for TEL function in *Arabidopsis*.

Figure 6.1. Model for *TEL* function in *Arabidopsis*. In wild type development of *Arabidopsis*, the *TEL* genes are required to specify undifferentiated cells in meristems. The absence of both *TEL1* and *TEL2* activities leads to basic patterning defects and ectopic production of numerous secondary shoots, which suggest 1) failure of SAM and RAM to develop and 2) random and unrestricted proliferation of meristematic cells.



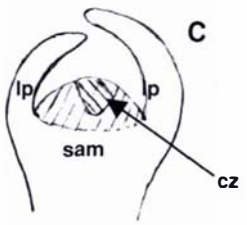
A

A. In a transition stage embryo, *TEL1(1)* and *TEL2* (2) are required to specify the undifferentiated nature of the SAM in the meristem center. *TEL1(1)* is also required to specify the undifferentiated nature of the quiescent center.



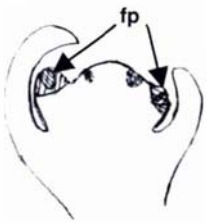
B

B. In an older embryo (torpedo stage), *TEL1* and *TEL2* expression are restricted to the SAM. *TEL1* continue to be expressed in the RAM. *TEL* genes are required to maintain SAM and RAM.



C

C. During postembryonic development, *TEL1* and *TEL2* specify the stem cell region in the central zone (cz) and the expression of both *TEL* genes in the overlapping domain restricts organogenesis outside these regions.



D

D. During the reproductive stage, *TEL1* and *TEL2* specify the undifferentiated nature of cells in the floral primordia (fp).

6.3. Limitations of the study and future experiment directions

This study was done with the ultimate aim of understanding how plant specifies form. Although results of the molecular genetic analyses suggest involvement of plant *Mei2*-like genes in the regulation of morphogenesis in plants, it is difficult to assess the biological role of these genes because of the following limitations: 1) very little is yet known about the biochemistry of TEL proteins, 2) cellular bases of the mutant phenotypes have not been studied in detail, 3) embryo and seedling lethality of double mutants makes phenotypic characterisation limited, and 4) in *Arabidopsis*, the overlapping expression patterns of the genes in the shoot meristem makes the analyses a bit more complicated because of redundancy.

Experiments are currently in progress to address the biological function of the genes: 1) Overexpression of *TEL* genes in *Arabidopsis* is being done to more clearly understand how the genes function; 2) Cellular localisation of TEL proteins and identification of RNA targets are currently being investigated to understand TEL protein biochemical activity; 3) Because of embryo and seedling lethality of double mutants, two component expression system experiments have been designed to allow phenotypic analysis of mutants at later stages of the plant's development.

The result of this thesis raises more questions regarding the biological function of the plant *Mei2*-like genes: 1) The expressions of *TEL1* in the root quiescent centre as well as in the SAM suggest processes common to both meristem types. Do *Mei2*-like genes regulate similar processes in both the SAM and RAM? 2) Is *TEL1* or any of the plant Mei2-like genes involved in the regulation of meiosis in plants? 3) Since organogenesis is very much coupled with changes in cell division patterns and spatially regulated patterns of differentiation, it would be helpful to address how *Mei2*-like genes influence these processes; 4) What are the biochemical processes in which MEI2-like proteins function, it would be helpful to address this issue as well as the biochemistry of the *te1* gene, in the future.

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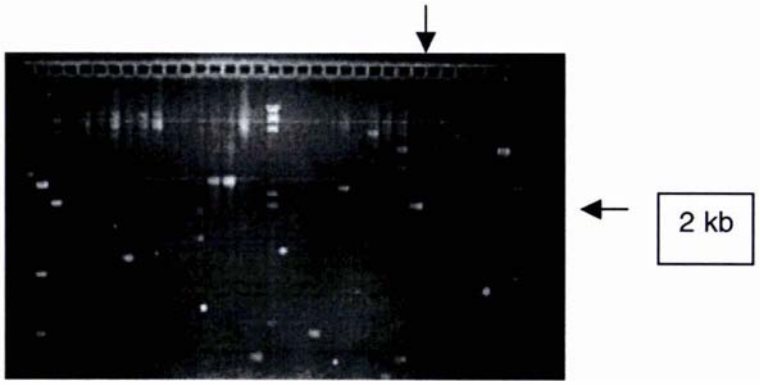
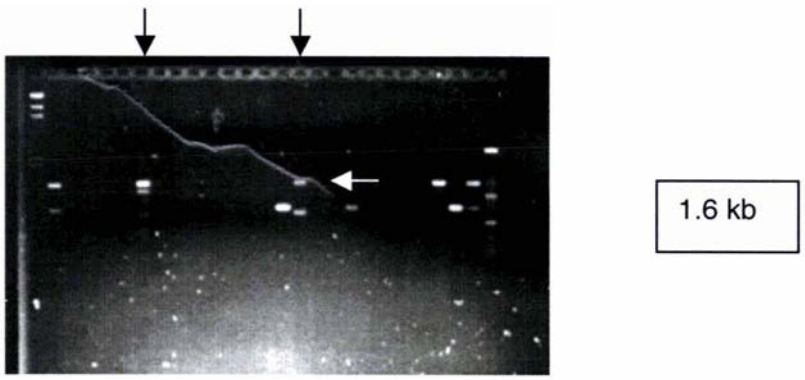
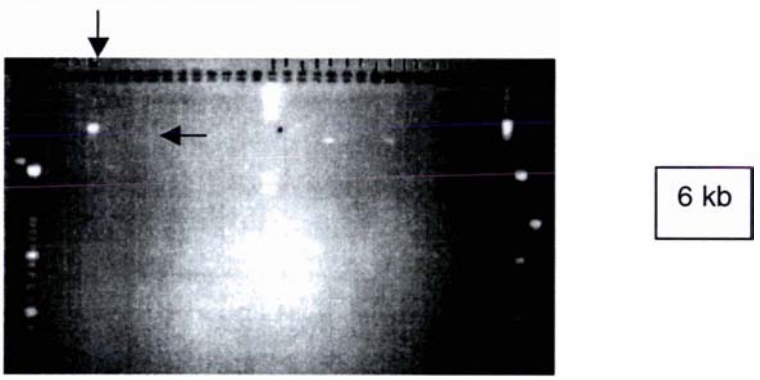


A. *TEL1* gel illustration

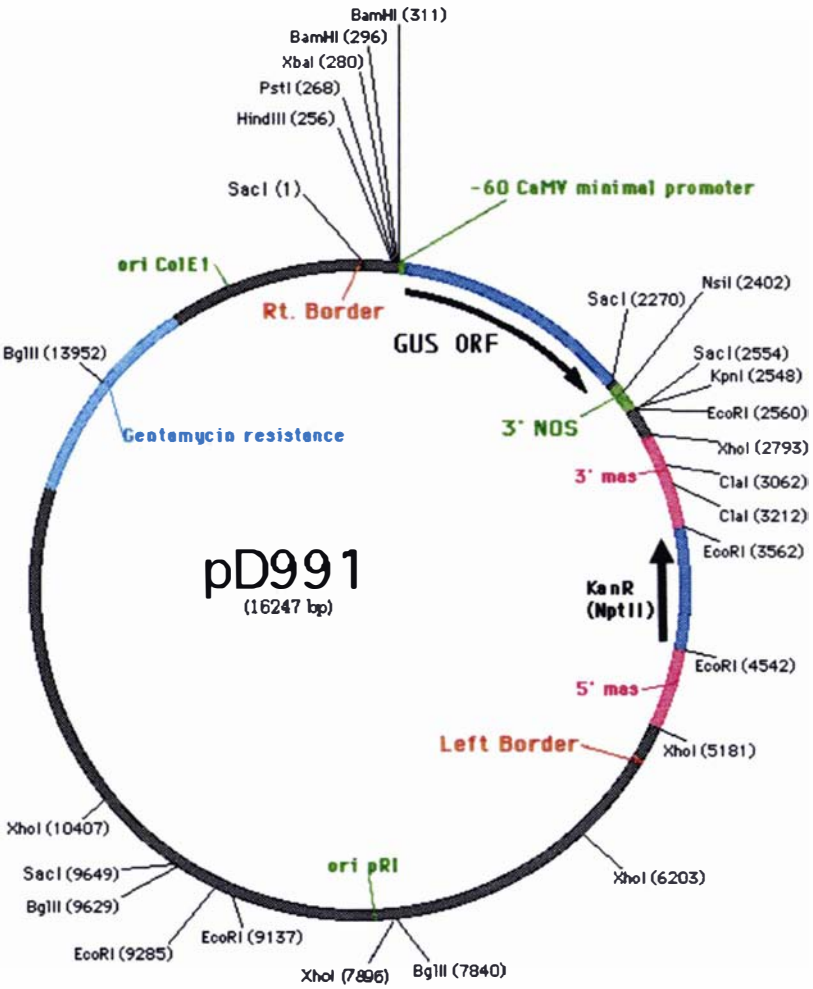


B. *TEL2* gel illustration

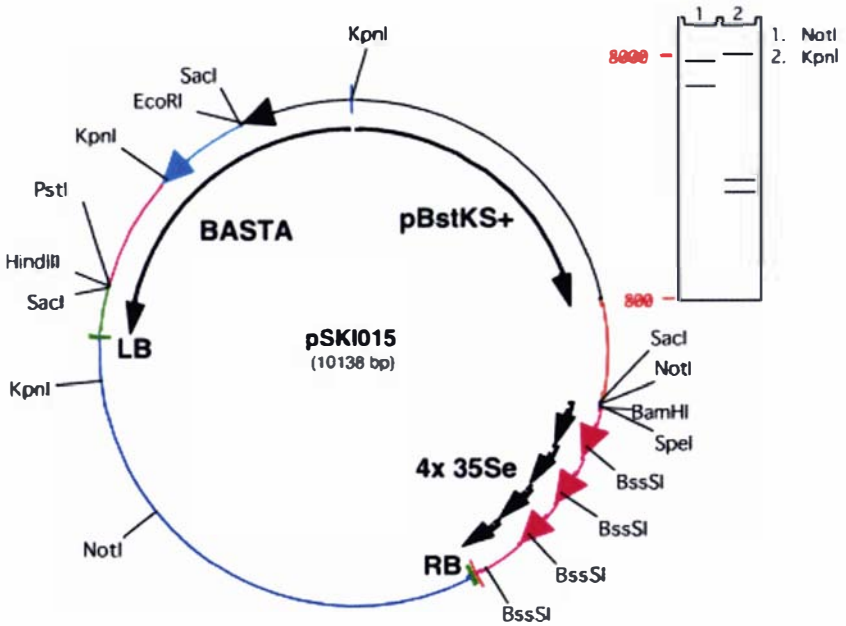


C. *AML1* gel

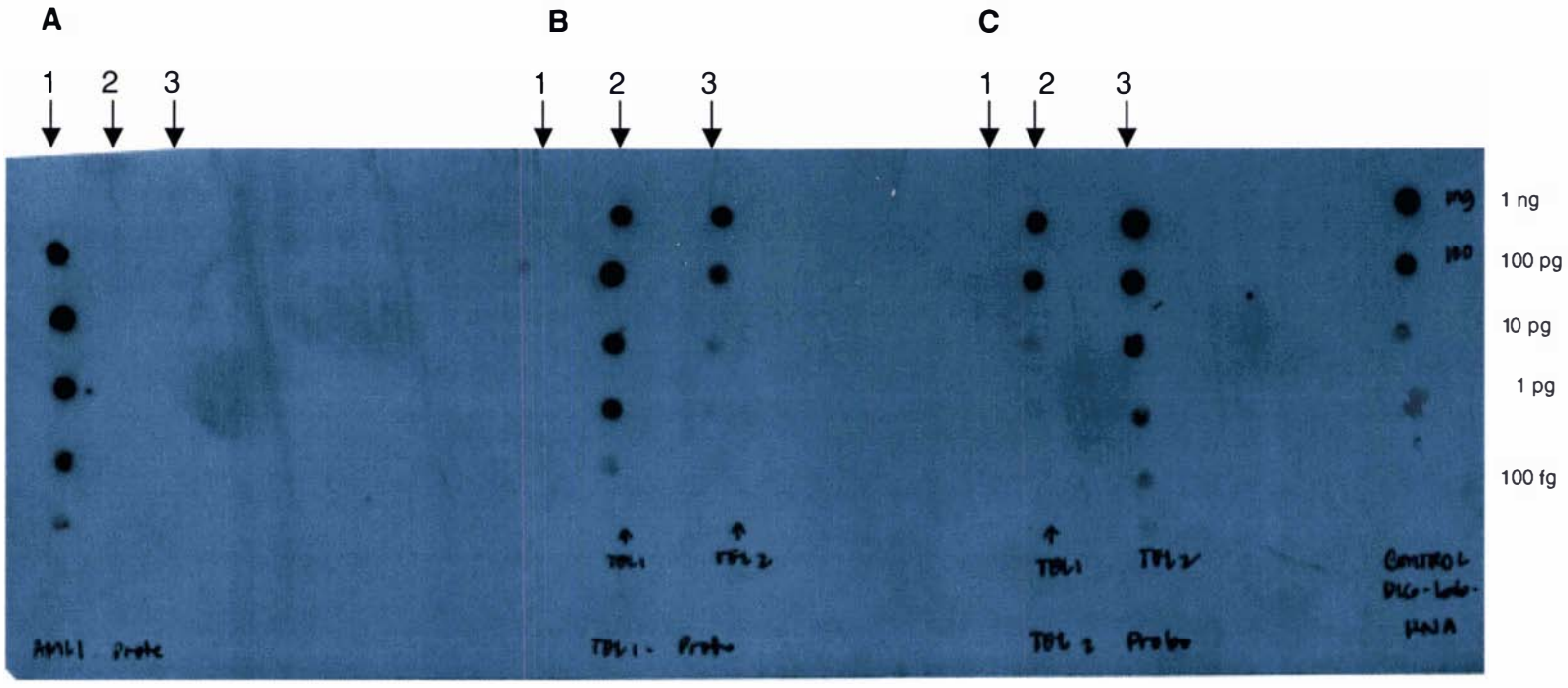
Appendix 1. Primary screening of T-DNA transformed Arabidopsis lines . A) *tel1* B) *tel2* and C) *aml1* knockouts using PCR. (CG Gilman). Arrowheads indicate the products for the knockout candidates.



Appendix 2. Map of vector pD991.
(Source: http://www.biotech.wisc.edu/arabidopsis/default.htm)



Appendix 3. pSKI015 map (Weigel et al., 2000)



Appendix 4. Southern blot to determine cross-hybridisations between plant Mei2 mRNAs. In this experiment, however, serial dilutions of DNA PCR products were spotted on the membrane instead of synthetic RNAs. 1) *AML1*, 2) *TEL1*, and 3) *TEL2* DNA. A) *AML1*, 2) *TEL1*, and 2) *TEL2* RNA probe. Approximately 100 ng probe diluted in 10 ml DIG-easy hyb was used for each tube. No cross-hybridisation was observed between AML1 and the TEL probes.