Reverse genetic analyses of *TERMINAL EAR*-like RNA-binding protein genes in *Arabidopsis thaliana* (L.) Heynh.

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Suzanne Claire Lambie

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ABSTRACT

In maize, a loss-of-function mutation in a *MEI2*-like gene, *terminal ear1* (*te1*), leads to morphological defects able to be traced back to the shoot apical meristem. One *MEI2*-like gene has been identified in maize, while six have been identified in rice and nine in *Arabidopsis thaliana.* In this thesis, a programme of reverse genetic analysis has been designed to investigate if *Arabidopsis* genes most closely aligned in parsimony trees with *TE1*, *TERMINAL EAR-LIKE 1* (*TEL1*), *TERMINAL EAR-LIKE 2* (*TEL2*), perform the same function as *TE1*. The expression pattern of *TEL1* and *TEL2* genes is restricted to the Shoot Apical Meristem (SAM) and the Root Apical Meristem (RAM) suggesting these genes are important in meristem maintenance or function. Results of the molecular genetic analysis of *TEL* genes in *Arabidopsis* support models in which these genes help maintain cells in a pluripotent state. For the first part of the thesis, analysis of lines carrying single knockouts of *TEL1* and *TEL2* and double knockout lines reveals a slightly accelerated rate of organogenesis, consistent with these genes normally acting to inhibit terminal differentiation pathways. Plants grown on medium containing gibberellic acid and sucrose, at higher than normal concentrations, present a further accelerated rate of organogenesis.

As the second part of the thesis, *in situ* and promoter/reporter GUS fusion analyses indicate *TEL1* is preferentially expressed in both the root and shoot apical meristems. Deletion analysis using GFP reporter constructs show that 5' sequences are sufficient to drive quiescent centre (QC) expression in the root while additional sequences are required for central zone (CZ) expression in the SAM. Physiological studies indicate expression of *TEL1* in the root is sensitive to the hormones, auxin, gibberellic acid and zeatin, when added at physiological concentrations. To confirm the auxin effect, GFP expression is no longer visible after 12 hours of exposure to auxin transport inhibitors in plants containing GFP under the control of the *TEL1* promoter, suggesting, in common with other QC markers, that *TEL* expression is sensitive to auxin levels. Analysis of mutant plants with altered root patterning suggests QC specific expression of *TEL1* requires early acting genes, such as *PLETHORA 1* and *2*, but does not depend on later acting genes such as *SCARECROW* or *SHORTROOT*.

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1 INTRODUCTION

Plants are principally indeterminate and are characterised by having plasticity of form able to be modified in response to environmental conditions (Steeves and Sussex, 1989). The fate of cells in plants and the architecture, such as phyllotaxy and leaf polarity, of higher plants is determined to a large extent by the activities of apical meristems (Steeves and Sussex, 1989). Apical meristems are the source of the structures of the sporophyte (Steeves and Sussex, 1989; Howell, 1998; Clark, 2001; Veit, 2003a). Apical meristem cells are maintained in a largely undifferentiated state and direct processes controlling the determination of cell fate (Steeves and Sussex, 1989). Both the root and shoot apical meristem occupy a central position near the tip of the growing point (Lyndon, 1998). Cytohistological, fate-mapping and surgical studies (Schmidt, 1924; Foster, 1938; Satina et al., 1940; Buvat, 1952; Newman, 1965; Steeves and Sussex, 1989; Dolan et al., 1993; Fujie et al., 1993; van den Berg et al., 1995; van den Berg et al., 1997; Howell, 1998; Reinhardt et al., 2003b) describe how apical meristems are initiated and maintained and these studies are described in more detail below. For the purposes of this thesis, the term apical meristem refers to the slowly dividing and undifferentiated cells in both the root and shoot apex. Molecular genetic techniques have facilitated progress towards the identification of specific genes regulating aspects of plant development such as cell differentiation and, within the meristem, the coordination of the development of these cells is controlled by a number of genetic interactions (Nowak et al., 1997; Pruitt et al., 2003).

1.1 MERISTEM INITIATION AND MAINTENANCE

The fertilisation of an egg, by a sperm cell to form the zygote, signals the beginning of the formation of a new organism (Figure 1-1). Following the fusion of the egg and the sperm the fertilised cell divides into two giving rise to the two cell stage (Mayer et al., 1993; Jurgens and Mayer, 1994; Jurgens, 2001). Further divisions result in a four-cell stage an eight-cell stage and a 16-cell stage (Goldberg et al., 1994; Scheres et al., 1994). At the 16-cell stage, a division of each of the eight cells of the eight-cell stage produces inner and outer cell layer (Mayer et al., 1993; Jurgens and Mayer, 1994; Jurgens, 2001). The outer cell layer is termed the protoderm, and is the precursor of

the epidermis (Mayer et al 1991). In the early globular embryo the first transverse division of the terminal cell results in the embryo dividing into an upper layer and a lower layer (Figure 1-1 globular) (Scheres et al., 1994). The cells of the lower layer elongate in the apical basal direction and the embryo develops a morphologically recognizable axis (Jurgens, 2001). Transverse divisions will subdivide this lower layer into elongated procambium cells, one layer of protoderm and ground meristem cells (Berleth and Jurgens, 1993). The hypophysis (the lowermost cell of the zygote) divides asymmetrically at the globular stage to produce a lens-shaped cell and a larger basal cell (Berleth and Jurgens, 1993). It is the lens-shaped cell that will eventually form the quiescent centre, the origin of root apical meristem (displayed in brown in Figure 1-1). The larger basal cell forms the suspensor (Scheres et al., 1994; Vroemen et al., 1996). The upper layer of cells forms a small zone of cells extending into the abaxial cotyledon shoulder at later stages (Scheres et al., 1994). Heart stage embryos have a defined cellular pattern in the hypocotyl and root (Scheres et al., 1994). It is at this heart stage that the lower layer of cells is able to be identified as a root meristem as these cells undergo periclinal division that characterises the lateral root cap (Scheres et al., 1994). It is also at the heart stage that the presumptive Shoot Apical Meristem (SAM) consists of three layers of cells, and these are precursors of the clonal layers found in the shoot apical meristem (Long and Barton, 1998). By the walking stick stage, the three clonal layers in the SAM show the typical organisation associated with the true SAM and the Root Apical Meristem (RAM) includes the characteristic regular cell files (Long and Barton, 1998). That is, the SAM is divided into three layers, with division in the first clonal layer (L1) and the second clonal layer (L2) being strictly anticlinal, resulting in clonally separate populations of cells in each layer. The SAM L1, L2 and third clonal layer (L3) therefore contain separate groups of meristem cells. The RAM contains the organised arrangement of tissue types with a formed quiescent centre (QC) surrounded by the apical initials and defined columella cells (Jurgens, 2001; Paquette and Benfey, 2001). By the walking stick stage there is further expansion of the cotyledons and hypocotyl and the cotyledons are bent over (Jurgens and Mayer, 1994).

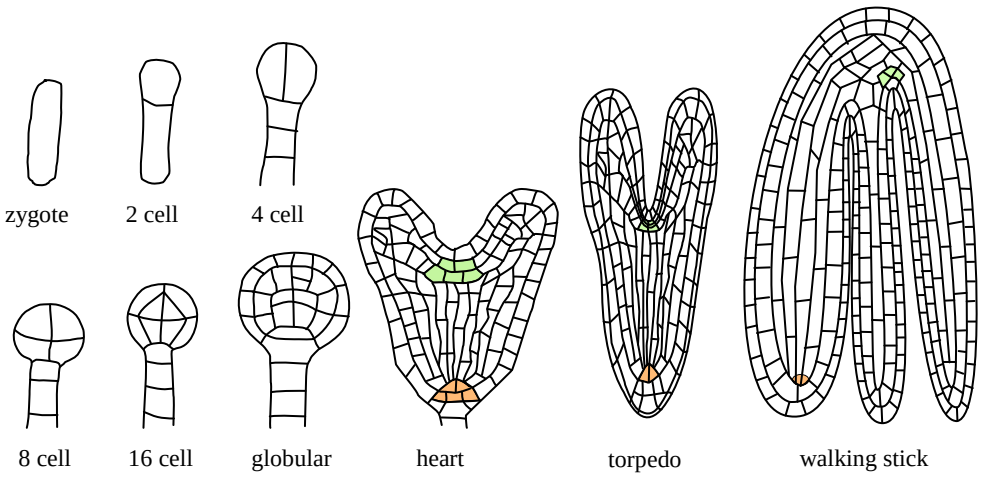


Figure 1-1: Stages of *Arabidopsis* embryogenesis (from (Jurgens and Mayer, 1994). The SAM is green and the RAM is brown.

The apical pattern of the globular embryo is divided into domains, with distinct developmental fates demarcated by specific gene expression patterns but it is not until the torpedo stage that the domains have become defined (Jurgens and Mayer, 1994). However the complex gene expression patterns of the SAM arise gradually during embryogenesis and at the heart stage most of the genes functioning to maintain the shoot and root apical meristems become active (Scheres et al., 1995; Kim et al., 2005a; Saiga et al., 2008). The development of the embryo is a progressive process, whereas the adult form is a series of reiterative processes resulting in repeated structures arising from meristems (Steeves and Sussex, 1989). Embryonic cells have meristematic characteristics at specific developmental stages, but what triggers a cell to become meristematic or to function as a meristem is still unresolved. A study of the morphology of the embryo cannot, in most cases, be related to adult plant morphology as post-embryonic development does not follow the same process that is observed during embryo formation.

1.2 THE SHOOT APICAL MERISTEM

The elegance and function of meristems has been noted in the literature as, for example, “the Shoot Apical Meristem (SAM) is the growing tip of the plant and comprises both the apical meristem and leaf primordia” Wardlaw and Cutter (1956), “the shoot apex, though tiny in stature, is a remarkable structure that gives rise to the whole shoot” (Lyndon, 1998) or “meristems make the plant” (Weigel and Jurgens, 2002). Thus an appreciation of how the shoot meristem functions is essential to understanding plant growth and development, and so the genes that are expressed specifically in the meristem and their function is discussed below.

1.2.1 SAM organisation

The pattern of cells in the SAM is laid down during embryogenesis and is maintained throughout the life of the plant and repeated as the plant grows (Steeves and Sussex, 1989). Schmidt (1924) looked at cytohistological divisions in the SAM and concluded that the SAM can be divided into layers characterised by the plane of division and the tissue types generated. The outermost region of the SAM, the tunica, divides so new cell walls form perpendicular to the surface and the inner region of the SAM, the corpus divides both perpendicular and parallel to the surface (Schmidt, 1924). Cells in the centre, the corpus, are undifferentiated but as they are displaced proximally, they undergo differentiation (Satina et al., 1940). Foster (1938) characterised the SAM into zones that include apical initial, transition, peripheral and rib zones. The rate of cell division in the peripheral zone is increased and cell structure has become more differentiated. The cells in the rib zone form the pith and vascular tissue. The apical initial zone is characterised by slowly dividing cells termed apical initials (Foster, 1938).

Satina et al., (1940) identified three distinct clonal layers in *Datura* meristems by mapping the fate of cells in the SAM. By inducing polyploidy in a particular cell (by the addition of colchicine) and then tracking the fate of each cell, Satina et al. observed that the polarity of cell division near the shoot tip was largely restricted to an anticlinal plane leading to the establishment of clonally distinct layers. These clonally distinct layers were then identified through analysis of marked cell lineages

and labelled as Layer 1 (L1), Layer 2 (L2), and Layer 3 (L3). The outermost layer, the L1, constitutes epidermal tissue. The adjacent layer, the L2**,** forms the sub-epidermal layer and the gametes (L1 and L2 together equal Schmidt's tunica layer). The innermost layer, the L3, has periclinal and anticlinal planes of division and forms the pith and the vascular structures (akin to Schmidt's corpus) (Figure 1-2). Daughter cells within each layer move from the centre to the periphery and are clonally distinct from other layers (Satina et al., 1940).

Attempts have also been made to classify shoot apical meristems according to how many apical initials are maintained in each cell layer (Newman, 1965). Newman (1965) looked at vascular cryptograms, and based on rates of cell division observed that the number of apical initials varied between plant classes. Gymnosperms, in general, had many initials clonally related to each other, suggesting the polarity of cell division is less constrained in gymnosperms than in angiosperms where there appears to be relatively stable clonally distinct layers (Newman, 1965). Grasses such as maize and rice tend to have two clonally distinct layers whereas *Arabidopsis* tends to have several layers and several initials in each layer (Newman, 1965).

Bowman and Eshed (2000) have reviewed the use of the terms demarcating the meristem into three zones and three layers, and agree the SAM can still usefully be defined by cytoplasmic differences and cell division rates, and that the behaviour of meristematic cells is position dependent. Plants all have a distally located initiating zone (protomeristem) and two derivative zones (the outer and the inner) in which histogenesis and organogenesis begin. They also agree, in *Arabidopsis* at least, the peripherial zone is the zone which gives rise to lateral organs. The central zone contains a reservoir of non-permanent meristematic cells whose behaviour is governed by position-dependent factors and these cells occupy all three layers. The rib zone is where the stem tissue forms and is comprised mainly of cells in the L3. Thus despite the ongoing debate surrounding the usefulness of demarcating the SAM into specific regions, the terms central zone, peripheral zone, and rib zone and L1, L2 and L3 are still in use today and are relevant when discussing the role of genetic interactions in the meristem. The meristem has been further dissected based on zones and layers. The central zone is important for maintaining and initiating meristems and the peripheral zones are important regions for the production of organs, but not for

meristem formation (Reinhardt et al., 2003a). For this thesis, the SAM is divided into three layers, the first clonal layer (L1), the second clonal layer (L2) and a third internal layer (L3). The L3 is divided further into peripheral and central zones. There are apical initials in each of the clonal layers. A schematic representation of zones, layers, tissue types and direction of growth is shown in Figure 1-2.

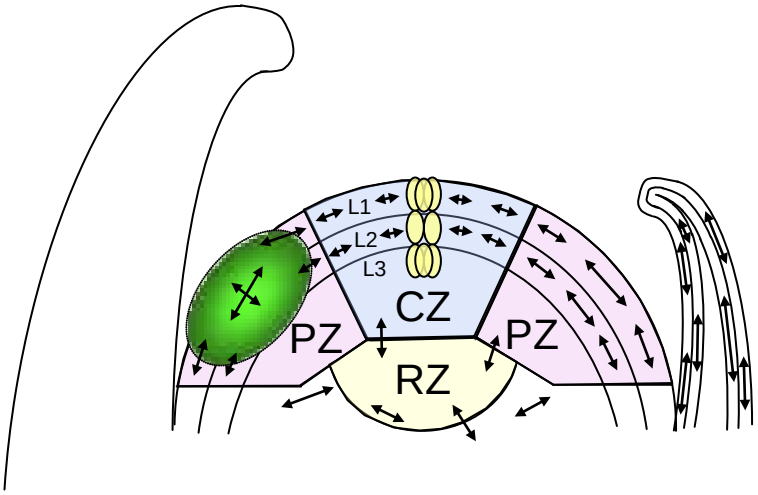


Figure 1-2: Longitudinal section through an *Arabidopsis* shoot apical meristem (adapted from (Veit, 2003b) and (Baurle and Laux, 2003)). L1 – layer 1, L2 – layer 2, L3 – layer 3, PZ – peripheral zone, CZ – central zone, RZ – rib zone. Arrows indicate direction of cellular division. The green region indicates a leaf primordium and the yellow regions the location of the apical initials.

Generally cell position determines cell fate in the meristem but plant cells do not migrate to a final position (van den Berg et al., 1995; van den Berg et al., 1997; van den Berg et al., 1998; Reinhardt and Kuhlemeier, 2002; Reinhardt et al., 2003a). Above-ground organs are formed by cells dividing and being pushed to the periphery of the SAM (Vernoux et al., 2000). As the role of the cell depends on the position in the plant, a constantly updated measurement of position relative to other cells is required (van den Berg et al., 1997; Reinhardt et al., 2003a). Once in position the cells expand and undergo further divisions determined by the polarity of the various parts of the organ (Lyndon, 1998). Laser ablation of L3 central zone cells of a tomato SAM did not affect organ formation, but laser ablation of L1 central zone cells resulted in terminal differentiation and no organ formation (Reinhardt et al., 2003a). Laser ablation of a few L1 peripheral zone cells changed cell division orientation and led to a replacement of the L1 layer cells (Reinhardt et al., 2003a). If a large number of L1 cells were ablated then organogenesis stopped and was not resumed (Reinhardt et al., 2003a). In addition, plant cells have a capacity to continuously respond to

positional information based on the cells relationship to developing organs (Lyndon, 1998). The SAM is the ultimate source of all above-ground post-embryonic organs. However, lateral organs are usually initiated by lateral meristems (Ozawa et al., 1998; Otsuga et al., 2001; Konishi and Sugiyama, 2003). As the development of the plant continues there is a change from determinate to indeterminate cells that result in the formation of meristems lateral to the primary meristem (Otsuga et al., 2001). The overall morphology and reproductive capacity of *Arabidopsis* is determined by the position, number and growth of these lateral meristems (Otsuga et al., 2001). Newly initiated lateral meristems do not retain meristem identity as they become separated from the apex, but instead meristem identity is reactivated shortly after initiation (Otsuga et al., 2001).

What can be concluded from the studies outlined above is the apical meristem plays an important role in balancing cell proliferation and cell differentiation, with positional information acting to specify the fate of new cells in meristems and that cell fate determinants are present in heart stage embryos. However surgical studies, such as those reviewed by Steeves and Sussex (1989) show meristematic cells can also be flexible. This flexibility is confirmed in studies where the capacity to respond to positional information is seen in leaves and stems of periclinal chimeras with the same form (Satina et al., 1940) and root cells change fate when the position they occupy changes due to laser ablation (removal) of adjacent cells (van den Berg et al., 1995; van den Berg et al., 1998).

1.3 THE ROOT APICAL MERISTEM

The Root Apical Meristem (RAM) is just as significant as the shoot apical meristem for determining the overall architecture of the plant. The RAM is the source of all below-ground post-embryonic organs. The RAM is radially organised and is located at the basal end of the plant. A group of cells in the central region of the growing tip of the RAM have a slow rate of division and maintain the apical meristem as an undifferentiated structure. Cell division and cell elongation occurs in zones apical to the RAM. The regions of the root where cell division and elongation occurs are described below.

1.3.1 RAM organisation

The mature primary root in *Arabidopsis* has a radial geometry and is comprised of concentric organised tissue layers (Dolan et al., 1993; Jurgens and Mayer, 1994; Berger et al., 1998; Paquette and Benfey, 2001; Reinhardt and Kuhlemeier, 2002). In the root the cells are arranged in regular columns arising from the RAM (Howell, 1998; Kaya et al., 2001; Casson and Lindsey, 2003). In the centre of the RAM a group of cells, in common with the apical initials in the SAM, are initiated early during embryogenesis (section 1.1) and are organised both radially and longitudinally (Berger et al., 1998). The apical initials in the RAM are located between the columella and the stele initials, slightly distal to the growing tip and can be considered analogous to the central zone cells in the SAM (Berger et al., 1998). In *Arabidopsis*, the RAM comprises a cluster of four cells arranged radially, which are surrounded by other initials. These four cells are termed the Quiescent Centre (QC) and have been shown to be mitotically inactive (Dolan et al., 1993; Fujie et al., 1993). Adjacent to the QC are the cortex/epidermal initials. Asymmetric division in these initials then gives rise to two columns of cells, the cortical cells and the epidermal cells (Steeves and Sussex, 1989; Dolan et al., 1993; Scheres et al., 1994; Scheres and Berleth, 1998). Proximal to the QC are stele initials that give rise to vascular tissue cells (the central core of tissue that consists of the xylem and phloem cells) and the pericycle; below the QC are the columella cells. The outer layer of the columella cells (the root cap) periodically sloughs off (Dolan et al., 1993). Periclinal divisions of the epidermal initial give rise to the lateral root cap layers that surround the epidermis (Dolan et al., 1993). Root growth occurs in transverse zones comprising a division, elongation and then

differentiation zone (Howell, 1998). The zone of division is proximal to the QC and the elongation zone is adjacent and proximal to the division zone (Howell, 1998). Differentiation occurs proximal to the elongation zone and is the zone in which root hairs form (Howell, 1998). Initials do not generate the root pattern (van den Berg et al., 1995), rather the root pattern is laid down during embryogenesis and initials perpetuate that pattern (Dolan et al., 1993).

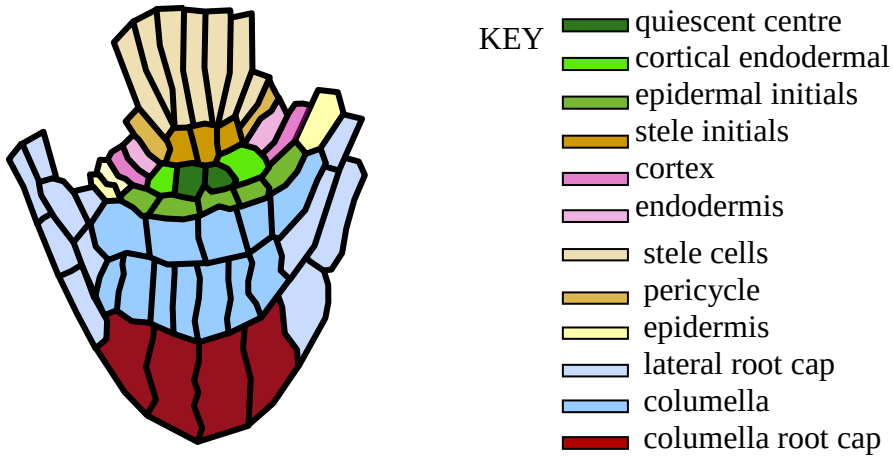


Figure 1-3: Longitudinal section through an *Arabidopsis* root. The lateral root cap (light blue) terminates at the beginning of the elongation zone and the rest of the files of cell continue. This picture is composed from a confocal image obtained during the course of this research.

Studies to determine the lineage of root cells have shown that a switch in cell type occurs when an invading cell crosses the clonal boundaries. Cells in the root, located below the QC, are determined to be columella cells, whereas cells above the QC are determined to be stele cells (van den Berg et al., 1995; van den Berg et al., 1997; van den Berg et al., 1998). Ablation studies in the RAM (van den Berg et al., 1995) show that when QC cells are killed they become compressed and pushed to the exterior of the root. An internal cell then assumes the dead cell's position and role. Ablation of specific cell types results in differing patterns of cellular compensation (van den Berg et al., 1995). If cortical initials are ablated then the pericycle cells invade and begin dividing anticlinally (van den Berg et al., 1995). Pericycle cell files are maintained and new cortical files are generated from the invading cell (van den Berg et al., 1995). Ablation of epidermal initials results in cortical cells dividing and occupying the epidermal cells position, to eventually give rise to lateral root cap cells (van den Berg et al., 1995). Individual cell layers generate new cells radially and towards the tip (van den Berg et al., 1995). A further study showed that ablation of one of the QC

initials prevents differentiation of the columella cells that are in direct contact with the ablated cell (van den Berg et al., 1997). Studies, observing whether cells adjacent to the QC or remote from the QC divide or not, determined that the QC does not directly regulate cell division in the columella cells, but that the QC prevents differentiation (van den Berg et al., 1997).

The RAM does not produce lateral root organs. Instead lateral roots arise from the pericycle in more mature regions of the root (Dolan et al., 1993). Auxin is not derived solely from the shoot but is synthesised *de novo* in the root to facilitate the emergence of lateral root primordia (Ljung et al., 2005). The initiation of replacement cells in the RAM differs from the SAM, but the genes required for apical and lateral meristem formation in the RAM may have similar relationships in terms of establishment and maintenance to those present in the SAM (Veit, 2003a). However, in recent years the use of molecular genetics has provided useful insights into genes controlling meristem establishment and maintenance.

1.4 GENES INVOLVED IN MERISTEM MAINTENANCE

An analysis of features shared by the RAM and SAM may indicate that similar functions can be performed by a common mechanism (Veit, 2003a). The ability of meristems to continuously produce new organs depends on the activity of the meristem cell population (Brand et al., 2002). This ability is determined by genes which are instrumental in meristem initiation or maintenance and is further influenced by genes involved in determining the switch from vegetative to floral meristems (Veit, 2003a). This common mechanism may have arisen independently in shoots and roots, but it is more likely the mechanism would have been co-opted from one of these regions for use in the other region (Veit, 2003a). An analysis of the possible interactions of these genes that are thought to act in concert, especially those expressed in both the RAM and SAM, aids in understanding how the population of meristematic cells in the apical portions of plants are initiated and maintained.

1.4.1 Shoot apical meristem identity genes

Populations of meristematic cells are maintained in an undifferentiated mitotically inactive state in the SAM through interactions between genes. *WUSCHEL* (*WUS*) is required to maintain the SAM through meristem cell proliferation and to control meristem cell numbers (Laux et al., 1996; Mayer et al., 1998; Brand et al., 2000; Schoof et al., 2000; Lenhard et al., 2002). *WUS* is first expressed in the apical sub-epidermal cells during embryogenesis at the 16-cell stage and expression shifts deeper into the SAM as the embryo matures, suggesting cell-to-cell interactions probably dictate the expression domain boundaries (Laux et al., 1996; Mayer et al., 1998). Three *CLAVATA* genes; *CLAVATA 1*(*CLV1*), *CLAVATA 2* (*CLV2*) and *CLAVATA 3* (*CLV3*) together promote the differentiation of cells into organs (Lenhard and Laux, 2003). The CLV1 protein is a receptor-like kinase, with extracellular leucine-rich repeats, a single transmembrane domain, and a functional intracellular serine protein kinase domain (Clark et al., 1997; Williams et al., 1997; Stone et al., 1998). *CLV2*, like *CLV1*, encodes a receptor-like protein with predicted extracellular leucine-rich repeats. However, CLV2 only has a very short cytoplasmic tail domain suggesting a different function, possibly to stabilise CLV1 (Clark et al., 1997; Williams et al., 1997; Stone et al., 1998). *CLV3* encodes a potential ligand in the form of a small,

secreted protein (Fletcher et al., 1999). Further CLV1/CLV3 signalling is still partially functional and able to repress WUS in outer cell layers in meristems of *clv2* mutants (Lenhard and Laux, 2003). The three genes in the *CLAVATA* complex (*CLV1*, *CLV2* and *CLV3*) interact with *WUS* to form a directional signalling system regulated by negative feedback to maintain meristem cells in the correct location (Schoof et al., 2000). This negative feedback system is primarily by transcriptional mediation of the signalling peptide *CLV3* (Schoof et al., 2000). *CLV3* represses *WUS* transcription through the *CLV1* receptor kinase signalling pathway (Schoof et al., 2000; Brand et al., 2002; Lenhard and Laux, 2003). *WUS*, in turn, represses the *CLV* complex and thereby represses the promotion of cells into organs, resulting in the maintenance of meristem cell numbers and position (Lenhard and Laux, 2003). As previously outlined the apical meristem plays an important role in balancing cell proliferation and cell differentiation, however positional information acts to specify the fate of new cells in meristems. The position of a new cell depends on where it is located within the meristem (van den Berg et al., 1998; Aida and Tasaka, 2006). The specification of boundaries within the meristem is another aspect of apical meristem maintenance.

An alteration of the gene expression patterns determining the identity of domains and boundaries can alter plant architecture. Organ separation is defined in part by *CUP SHAPED COTYLEDONS1* and *2* (*CUC1* and *CUC2*) (Aida and Tasaka, 2006). *CUC2* has overlapping domains with *SHOOT MERISTEMLESS* (*STM*) during embryogenesis (Bowman and Eshed, 2000). *STM* prevents the incorporation of central meristem cells into organ primordia (Endrizzi et al., 1996; Long et al., 1996). The embryo develops boundaries at the torpedo stage and *CUC1* is restricted to the boundary regions between the cotyledons and the SAM, but is dependent on *STM* activity (Aida et al., 1999). *CUC1* is required for expression of *STM* to form the SAM and to give proper spatial expression of *CUC2* to separate the cotyledons from each other (Aida et al., 1999). *CUC1*, *CUC2* and *STM* also interact with *WUS* and *CLV1*, *CLV2* and *CLV3* to form and maintain the meristem (Endrizzi et al., 1996; Laux et al., 1996; Mayer et al., 1998).

1.4.2 Root apical meristem identity genes

Like the *CUC* genes in the shoot, *SCARECROW* (*SCR*) and *SHORT ROOT* (*SHR*), define boundaries in the root (Benfey et al., 1993). Mutants of S*CR* and *SHR* have irregular cell layers where a distinct endodermis and the cortex would normally form (Scheres et al., 1995). Plants mutant for *SCR*, a transcription factor, lack a distinct endodermis and cortex (Nakajima and Benfey, 2002). The endodermis and cortex appear to be fused (Nakajima and Benfey, 2002), hence *SCR* is predicted to influence pattern formation through the regulation of morphogenesis (Scheres et al., 1995). A later study suggests *SCR* and *SHR* are required to regulate stem cell fate because *SCR* expression in the QC appears to regulate the identity of neighbouring meristem cells (Sabatini et al., 2003). SCR sequesters SHR by restricting the movement of SHR to the endodermis, which in turn halts induction of SCR in the cortex and prevents additional asymmetric divisions (Heidstra et al., 2004). Together with *SCR* and *SHR* *PLETHORA1* (*PLT1*) and *PLETHORA2* (*PLT2*) genes define the quiescent centre (QC) in the RAM and meristem cell positions (Heidstra et al., 2004). *PLT1* and *PLT2* genes encode AP2 class putative transcription factors and are essential for QC specification and stem cell activity (Aida et al., 2004).

In common with *SCR*, *SHR*, *PLT1* and *PLT2* having similar functions in the root to *CUC* genes in the shoot, genes comparable to *WUS* and *CLV3* exist in the RAM and control meristem cell proliferation and cell numbers (Kamiya et al., 2003). The *WUS* type homeobox gene *QUIESCENT-CENTRE-SPECIFIC HOMEOBOX* (*QHB*), isolated from rice, is expressed specifically in the QC (Kamiya et al., 2003). Over-expression of *QHB* results in the proliferation of new roots (Kamiya et al., 2003). However, no novel phenotypes were observed in *QHB* silenced lines suggesting there is genetic redundancy (Kamiya et al., 2003). After more recent research the gene name has been changed to *WUSCHEL-RELATED HOMEOBOX 5* (WOX5) (Gonzali et al., 2005; Sarkar et al., 2007), and WOX5 has been shown to maintain meristem cells in the root *via* interactions with other root specifying genes such as *SCR*, *SHR* and *PLT* (Sarkar et al., 2007). *CLE19* is a potentially secreted protein belonging to the *CLE* family of which *CLV3* is the founding member (Casamitjana-Martinez et al., 2003; Fiers et al., 2005). *CLE19* expression has been detected at low levels in roots of seedlings but not in the root apical meristem (Fiers et al., 2005). Localised over-

expression of *CLE19* restricts the size of the root apical meristem (Casamitjana-Martinez et al., 2003) in a similar way to *CLV3* in the SAM (Schoof et al., 2000; Brand et al., 2002; Lenhard and Laux, 2003).

These studies on *CLE19*, *CLV3*, *WOX5* and *WUS* suggest there are similar mechanisms regulating the apical meristem in the RAM and the SAM (Veit, 2003a). The *CLE* family is an example of genes with shared mechanisms in the root and the shoot and whose functions have been elucidated *via* a molecular genetic approach. *CLV3/ESR 19* (*CLE19*), one member of the *CLE* family, is expressed in the root and *CLAVATA3* (*CLV3*), another member of the *CLE* family, is expressed in the shoot and both appear to regulate the size of the meristem (Casamitjana-Martinez et al., 2003). The interactions such as those seen with the *CLE19*, *CLV1*, *CLV2*, and *CLV3* genes, where there is more than one member in the gene family and where each gene functions in a different way as part of a gene complex to synergistically regulate organ proliferation *via* interactions with other genes, suggests there may be more genes, as yet uncharacterised, that could form additional gene complexes that contribute to the maintenance of apical meristem regions.

1.4.3 Gene expression determines root and shoot meristem identity

FASCIATA genes are involved in the maintenance of chromatin and are expressed in both the RAM and SAM (Kaya et al., 2001). *FASCIATA1* (*FAS1*) and *FASCIATA2* (*FAS2*) mutants have normal body plans and organ morphology, but the cellular organisation of RAM is not maintained during growth after the formation of the RAM (Kaya et al., 2001). The regular arrangements of cell files in the RAM are lost and the QC and surrounding initials are hard to identify based on their position or characteristics (Kaya et al., 2001). As a result *fas1* and *fas2* plants have disorganised phyllotaxy, suggesting *FAS1* and *FAS2* are not required for embryogenesis, but to ensure meristem cell genes such as *SCR* and *WUS* are transcribed (Kaya et al., 2001). In *fas1* and *fas2* plants there is a reduction in the cell population in the elongation zone (Kaya et al., 2001). This suggests there is a defect in either the regulation of cell proliferation in the elongation zone or a defect in the activity of the initials supplying cells to the elongation zone. The irregular arrangement of the columella cells coupled

with the range of phenotypes suggests the meristem cell state is not strictly maintained (Kaya et al., 2001). Leakiness of meristem cell maintenance leads to morphologically different plants with the same genotype.

When *fas1* or *fas2* plants are crossed with *SCR*- and *WUS*-reporter gene constructs there is a range of expression patterns presented depending on the number and position of cells in the SAM that are affected (Kaya et al., 2001). The more cells that are affected the stronger the mutant phenotype. As *fas1* and *fas2* plants have distorted *SCR* and *WUS* expression, and since both *SCR* and *WUS* genes play a role in meristem maintenance, *FAS* genes are likely to facilitate stable maintenance (Kaya et al., 2001) *via* interactions with meristem maintenance genes. *FAS* genes appear to be required for the fundamental organisation of both the RAM and the SAM (Kaya et al., 2001). *FAS1* and *FAS2* expression is associated with the G1/S transition in the cell cycle, is important in nucleosome assembly or chromatin remodelling and is required to maintain meristem cells (Kaya et al., 2001; Endo et al., 2006). This is a different level of regulation from what is seen with transcriptional and translational regulation. *FAS1* and *FAS2* regulation occurs before transcription and prevents transcriptional regulators targeting DNA thereby preventing gene transcription and ensuring stable epigenetic states of chromosomes. This lends weight to the idea that mechanisms controlling the maintenance of meristem cell populations in the SAM are similar to those in the RAM (Kaya et al., 2001). This idea is supported by the studies done on the *CLE* family of genes and may be supported by studies looking at other genes present in both the root and shoot apical meristem and by considering other factors, such as hormones, that influence growth and development in the apical regions. Signalling molecules such as plant hormones, which contribute to overall plant development, are active in both the RAM and SAM.

1.4.4 Plant hormones specify root and shoot meristem identity

Several studies looking specifically at plant hormones have helped elucidate the role that these signals play in plant growth and development. Auxin was the first plant hormone to be identified, and since then several more have been described to reveal five classes of major plant hormones (auxin, cytokinin, gibberellin, abscisic acid, and ethylene).

Perturbations of hormone levels have a profound effect on plant growth and meristem identity and are implicated in many aspects of plant development (Salisbury and Ross, 1992).

Auxin (as indole-3-acetic acid, IAA; the major naturally occurring compound) in embryos is important for establishing the apical-basal gradient and the correct formation of the embryonic poles (Weijers et al., 2005). At the heart shaped stage, the point where the meristems become defined, auxin becomes localised to the meristems and the flow between the two meristems is initiated (Berleth et al., 2004). Auxin is transported directionally through the plant from the shoot apex to the root apex (Friml et al., 2002; Blilou et al., 2005). This movement is regulated by polar efflux regulators such as *PIN-FORMED1* (*PIN1*) (Friml et al., 2002; Blilou et al., 2005). Auxin flow can be altered by auxin transport inhibitors such as 2,3,5-triiodobenzoic acid (TIBA) and 1-naphthylphthalamic acid (NPA) (Reed et al., 1998). Auxin moves down the plant from the shoot *via* cell-to-cell movement into the root and accumulates in the columella initials just below the QC (Friml et al., 2002; Friml et al., 2003b; Blilou et al., 2005). This suggests the QC is defined by the location of the auxin maxima just distal to the QC. Genes active in the QC, such as those described by Sabatini and colleagues (Sabatini et al., 2003), could therefore interact with auxin to maintain meristem cell identity. Auxin and auxin transport inhibitors influence the expression patterns of *SCR*, *SHR*, *PLT1* and *PLT2* by enhancing their expression pattern or by altering the location of the expression in particular cells (Blilou et al., 2005). Auxin transport inhibitors prevent endogenous auxin from reaching the root tip by stopping transportation through the plant (Reed et al., 1998; Weijers et al., 2005). However auxin inhibitors do not prevent the *de novo* synthesis of auxin at the site of lateral root formation (Bhalerao et al., 2002). In lateral root primordia where auxin can be synthesised, the impact of auxin transport inhibitors is reduced due to transported auxin being less important for root primordia initiation (Bhalerao et al., 2002). Auxin levels can also be altered by the addition of synthetic auxins such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthylacetic acid (NAA) (Weijers et al., 2005). 2,4-D is a very powerful synthetic auxin and any change it initiates is rapid (Weijers et al., 2005). Response to NAA approximates the response to IAA, but NAA is more persistent and the effect is more pronounced as the response to the hormone occurs over a longer time period (Weijers et al., 2005).

In early globular stage embryos, PIN1 accumulates at the inner cell boundaries but becomes polarised mid-globular stage (Steinmann et al., 1999). This expression becomes narrower as the embryo matures (Steinmann et al., 1999). In heart stage embryos the vascular precursor cells accumulate PIN1 in the basal portion of the cells and the developing cotyledons and in the embryo axis PIN1 accumulates in the apical portion of the cells (Steinmann et al., 1999). Since the identification of *PIN1*, further research has identified and characterised *PIN2*, *PIN3*, *PIN4*, *PIN5*, *PIN6*, *PIN7* and *PIN8* (Friml et al., 2003a; Blilou et al., 2005). This research on the *PIN* genes, namely *PIN1* and *PIN7*, indicates that the interaction between auxin and the *PIN1* and *PIN7* genes control cell size, cell division zone size, cell expansion, and root enlargement through regulation of genes active in the meristem (Friml et al., 2003a; Blilou et al., 2005). Interestingly, the *PIN* family of genes, in particular *PIN1* and *PIN7*, appear to focus the auxin maximum just below the QC *via* an interaction with the *PLETHORA 1* (*PLT1*) and *PLETHORA 2* (*PLT2*) genes (Friml et al., 2003a; Blilou et al., 2005). The *PLT1* gene is required with *PIN* family gene transcription to stabilize the auxin maximum at the distal root tip (Blilou et al., 2005). Plants mutant for *pin1pin4pin7* have proximal displacement of starch granules that mark the columella cells and correspondingly *PLT1* mRNA expression is also shifted in the same direction (proximal to the usual location of the starch granules) (Blilou et al., 2005). In *pin3pin4pin7* mutants, the displacement of starch granules is a lateral shift and this is reflected in the *PLT1* mRNA expression patterns also moving laterally (Blilou et al., 2005).

Genes may be up-regulated or down-regulated by hormones, such as auxin, and may be either early-regulated (within 3 hours of exogenous auxin application) or late-regulated (after 3 hours of exogenous auxin application) inducible genes, or both early and late-inducible genes (Goda et al., 2004). Auxin response elements (AuxREs) have been shown to be sufficient to confer auxin responsiveness (Ulmasov et al., 1995). Synthetic AuxRE and DR5 (an auxin response synthetic promoter) responded to exogenously applied IAA (synthetic auxin) independent of the endogenous auxin level (Nakamura et al., 2003). The function of the *PIN1*, *PIN7*, *PLT1* and *PLT2* genes has been assessed by looking at interactions with markers specific to the root tip such as *QC25* (quiescent cell specific), *J0631* (mature epidermal cells adjacent to root meristem cells), *J0481* (lateral root cap and epidermal cell), and *DR5* (auxin accumulation in root cap, columella and QC cells) (van den Berg et al., 1995; van den Berg et al., 1998; Sabatini et al., 2003; Mo et

al., 2006). The use of the construct P_{DR5}::ER-GFP provides an indication of where auxin responses are occurring in the plant and its expression is considered to provide an approximation of auxin distribution. The use of P_{DR5}::ER-GFP therefore provides a useful means to compare auxin related phenomena in wild type versus mutant plants.

Auxin and cytokinin can be thought of as working complementarily towards organ initiation and growth and the proliferation of the overall architecture of the plant (Laplaze et al., 2005). Auxin moves from the shoot apex down through the cells to the roots whereas cytokinins move from the root apex up through the xylem to the shoots (Laplaze et al., 2005). Auxin and cytokinin change cell fate by altering cues relating to the position of cells in the meristem, thereby affecting cell types and the function of those cells within the meristem (Ljung et al., 2005). Cytokinins have been shown to have a role in nutrient sensing, in particular the presence of nitrogen and are positive regulators of cell division (Takei et al., 2001; Werner et al., 2003; Nishimura et al., 2004; Riefler et al., 2006). The current model for cytokinin signalling is a multi-step phosphorelay system that is comprised of sensor kinase receptors that have a kinase and a receiver domain, Histidine (His) phosphotransfer proteins and response regulators. The auto-phosphorylation activity of the sensor kinase is altered by ligand binding and results in the transferral of a phosphoryl group from the sensor kinase to the receiver domain *via* an auto-phosphorylated His residue (Ferreira and Kieber, 2005). The phosphoryl group is transferred, *via* a His residue in a His-phosphotransfer protein, from an Asp residue within a fused receiver domain to an Asp residue in a response regulator (Ferreira and Kieber, 2005).

The presence of nitrogen results in an increase in cytokinin accumulation which is then transported up the plant to the shoot (Takei et al., 2001; Takei et al., 2002). Once in the shoot, cytokinins stimulate cell division, shoot initiation and organ formation (Salisbury and Ross, 1992). Cytokinins are also implicated in lateral root growth (Werner et al., 2003; Mason et al., 2005). An increase in cytokinin levels reduces the number of lateral roots, presumably as the plant has sufficient nutrients to then put energy into the above ground portion of the plant. However studies looking at the endogenous application of cytokinins show plants have an active root meristem and a larger root system as a result (Nishimura et al., 2004). Ferreira and Kieber (Ferreira and Kieber, 2005) in their review of cytokinins propose these two pieces of

information are not mutually exclusive and suggest the effects of cytokinin can be thought of as being normally distributed about a point optimal for shoot growth and root growth. Cytokinin levels either side of this peak can cause either an increase in root production or a decrease depending on which side of the peak the concentration falls.

Gibberellins can regulate cell division through interactions with *KNOX* and *knotted1*-like genes such as *STM*, *KNAT* (meristem identity genes) (Kim et al., 2003; Kim et al., 2005b; Kessler et al., 2006). Kessler et al. (2006) showed that the mis-expression of *KNOX* genes induces cell division and meristem formation and this mis-expression can be reversed through the application of endogenous gibberellin. *LEAFY* interacts with gibberellin, *AGAMOUS* and phytochrome to regulate the transition to flowering (Okamuro et al., 1996) thereby specifying meristem identity. Plant hormones interact with genes such as *LEAFY* by increasing or decreasing transcription rates.

1.4.5 Developmental processes are regulated by controlling transcription

Analysis of spatial and temporal patterning in development can also be achieved through understanding how the transcriptional domains of regulatory genes are activated or deactivated (Watanabe and Okada, 2003; Baurle and Laux, 2005). DNA is transcribed within the nucleus into mRNA, which then moves from the nucleus to the cytoplasm (Ringo, 2004). This mRNA is then translated *via* tRNA and the resulting amino acid chain becomes folded into a functional protein (Ringo, 2004). Regulation of which genes are transcribed in a particular cell depends on the condensation state of the chromatin (euchromatin or heterochromatin) and whether the proteins associated with the gene have been methylated, phosphorylated or acetylated (Ringo, 2004). Methylation of the DNA may also influence the level of gene transcription, with actively transcribed genes typically being hypomethylated when compared to non-transcribed DNA (Schauer et al., 2002; Tran et al., 2005). Studies on *FAS1* and *FAS2*, chromatin-remodelling genes (Kaya et al., 2001), and a histone H3 gene family in *Arabidopsis*, where a mutation in a H3 gene was shown to cause decreased expression and ectopic RNA splicing (Okada et al., 2005), have

shown chromatin remodelling affects the regulation of a wide range of genes and that this level of regulation occurs prior to transcription.

Regulation occurring once the DNA has been transcribed into mRNA is termed post-transcriptional RNA processing and involves splicing out introns and/or microRNAs or the recruitment of multiple mRNAs into a complex (Ringo, 2004). Messenger RNA may not be translated or the incorporation by tRNA of specific amino acids may be regulated (Ringo, 2004). Regulation of proteins is termed post-translational modification and entails a change in the conformational folding of the protein, compartmentalisation, protein-protein interactions, or ligand binding (Ringo, 2004). An RNA binding protein may be used to regulate the splicing of a particular gene, to bring together mRNAs comprising a complex, to facilitate the translation of a particular mRNA or to hold mRNA in storage until it is required (Jeffery and Nakielny, 2004). An RNA binding protein, NAB1 is located in the cytosol in complexes where they bind and sequester a particular RNA (Mussgnug et al., 2005). This sequestering could exist so that should environmental conditions change the mRNA required for the organism to respond is already present and can be activated immediately. Messenger RNA binding proteins have also been shown to move RNA around the nucleus through dense patches of chromatin and out into the cytoplasm to facilitate translation (Vargas et al., 2005).

Regulation may also occur through interactions with microRNA (Schauer et al., 2002). Several studies have demonstrated microRNA can regulate cellular processes such as cell growth, cell division and proliferation (Achard et al., 2004; Kidner and Martienssen, 2004; Laufs et al., 2004; Guo et al., 2005; Wang et al., 2005b; Sieber et al., 2007). MicroRNA are small (approximately 22 nucleotides in length) forms of RNA generated from endogenous hairpin-shaped transcripts that prevent translation of the mRNA into a protein by attaching to the corresponding mRNA to inhibit translation or promote mRNA degradation. In the case of microRNA miR159, which is involved in the regulation of short-day photoperiod flowering time and anther development *via* GA regulation, RNA transcripts of *LEAFY* are targeted by the miRNA thereby regulating the transition to flowering (Achard et al., 2004). An analysis of the transcriptional domains of regulatory genes such as *WUS* and *AGAMOUS* (*AG*) indicates there are distinct, often short, regulatory regions that

control tissue specificity and levels of transcription (Sieburth et al., 1995; Baurle and Laux, 2005).

1.4.6 Molecular mechanisms of *MEI2*-like genes

Most of what is known about the molecular mechanisms of *MEI2*-like genes is based on work conducted in the yeast, *Schizosaccharomyces pombe* involving the elimination of gene function (MacNeill and Fantes, 1995; Hirayama et al., 1997; Watanabe et al., 1997; Yamashita et al., 1998; Shimada et al., 2003). A protein in *Schizosaccharomyces pombe*, MEI2, was shown to have dual function in regulating the initiation of meiosis and the progression of meiosis towards completion (Hirayama et al., 1997; Watanabe et al., 1997). MEI2 has three RNA recognition motifs (RRMs) and RNA binding activity essential for function (Hirayama et al., 1997; Watanabe et al., 1997) (see 1.4.8 for a more detailed description of RRMs). A deletion analysis of the *MEI2* gene showed the sequence surrounding RRM3 is essential for function of the MEI2 protein (Watanabe et al., 1997). RRM1 and 2 can be deleted and functionality is not seriously impaired. However, a mutation in RRM1 makes the protein heat sensitive and a mutation in RRM3 inactivated the protein and the cells did not enter meiosis (Watanabe et al., 1997). This work lead to postulation the MEI2 protein is required at least twice, once prior to pre-meiotic DNA synthesis and again prior to meiosis 1 (Watanabe and Yamamoto, 1994; Watanabe et al., 1997). How the MEI2 protein works to activate the switch from mitosis to the meiosis pathway is not clear. However, it is known transcription of *MEI2* is activated through starvation or by reception of a pheromone signal and its activity is regulated at transcription and post-translation (Watanabe et al., 1997). In addition, when *MEI2*, in *S. pombe*, is linked to the reporter gene *GREEN FLUORESCENT PROTEIN* (*GFP*), MEI2 moves to the nucleus adjacent to the microtubule organising centre and telomere cluster in cells proceeding to meiotic prophase (Watanabe et al., 1997). Specifically, MEI2 is localised to the *SME2* gene on chromosome one and occupies a fixed position in the horse-tail nucleus (Watanabe et al., 1997; Shimada et al., 2003). Subsequent research (Harigaya et al., 2006) has shown this fixed position in the nucleus is critical for sequestering RNA and maintaining the cell in a mitotic state. If the function of *MEI2* orthologs in multi-celled organisms is similar, then genes in this

family could be expected to influence differentiation to floral state through regulation of a particular gene or the organisation of the cellular machinery regulating transcription. Examples available in maize (TE1) (Veit et al., 1998; Kawakatsu et al., 2006) and rice (PLA2) (Kawakatsu et al., 2006) enable comparisons to be made with MEI2 in yeast.

1.4.7 Non functional MEI2-like genes affect organ initiation

TERMINAL EAR1 (*TE1*) located in the maize SAM and *PLASTOCHRON 2* (*PLA2*) located in the rice SAM are *MEI2*-like genes and are involved in meristem processes (Veit, 1998; Kawakatsu et al., 2006). Mutations in *TE1*, a *MEI2*-like gene isolated from maize, and in *PLA2*, a *MEI2*- like gene from rice, results in an alteration in phyllotaxy and growth pattern (Veit, 1998; Guo et al., 2006; Kawakatsu et al., 2006). The observed deviation from wild type phyllotaxy of *TE1* and *PLA2* mutants suggests that *MEI2*-like genes have a role in organ initiation or meristem maintenance (Veit, 1998; Kawakatsu et al., 2006). Knocking out *PLA2* in rice results in a reduced plastochron length (65 % reduction), corresponding to an increased rate of organogenesis and an increased rate of leaf maturation (Kawakatsu et al., 2006). In addition, in rice, the inflorescence is sterile (Kawakatsu et al., 2006). Knocking out *TE1* in maize results in a reduced plastochron length (35 % reduction), corresponding to an increased rate of organogenesis (Veit, 1998; Kawakatsu et al., 2006). However, there is no increase in the rate of leaf maturation and while fertility is reduced the inflorescence is not sterile (Kawakatsu et al., 2006). *PLA2* and *TE1* transcripts are tightly regulated to specific regions of the meristem (Veit et al., 1998; Anderson et al., 2004; Kawakatsu et al., 2006). *TE1* expression in maize is restricted to semicircular bands surrounding the incipient leaf (P0), whereas *PLA2* transcripts in rice are present in the leaf margins of the incipient leaf (P0) as well in central regions of the previous leaf (P1). The *PLA2* and *TE1* model proposed by Kawakatsu et al. (Figure 1-4) suggests that these genes prevent differentiation (Kawakatsu et al., 2006). *PLA2* acts as a repressor of leaf initiation, as once the *PLA2* signal is removed, leaf initiation occurs. In *pla2* mutant plants there is no repressor of leaf initiation and leaves form earlier than in *PLA2* plants (Kawakatsu et al., 2006).

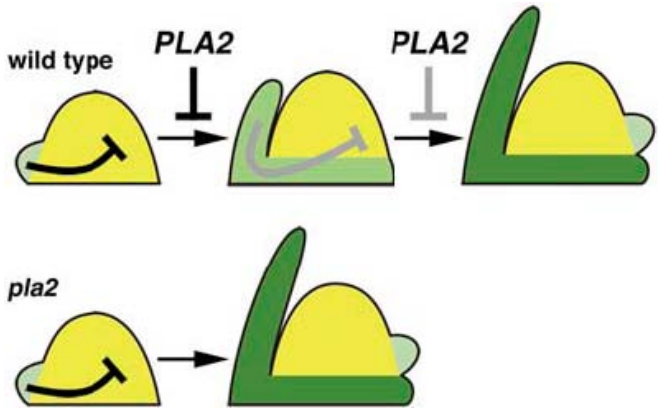


Figure 1-4 *PLA2* inhibits leaf development. Arrows indicate activation and -| indicates inhibition. Reproduced from (Kawakatsu et al., 2006)

TE and *PLA2*, and more generally the *MEI2*-like group of genes (genes with a predicted RNA binding function), could be argued to be involved, in plants, in the initiation and maintenance of the shoot meristem based on data presented by Kawakatsu, et al. (2006). An alteration in *te1*, a *MEI2*-like gene in maize, modified the phyllotaxy possibly through a perturbation of the meristem (Veit et al., 1998). In rice, *pla2*, shows a similar phenotype to *te1* (Guo et al., 2006; Harigaya et al., 2006; Kawakatsu et al., 2006). Studies on these *MEI2*-like genes, *te1* and *pla2*, lead to the conclusion the *MEI2* class of genes is probably important in aspects of meristem maintenance (Veit et al., 1998; Kawakatsu et al., 2006).

TERMINAL EAR (*TE1*) was characterised originally in maize. However maize has a large genome, a generation time of approximately 100 days, is hard to transform and is difficult to grow in a glasshouse due to its large stature. In contrast *Arabidopsis thaliana* is an easier plant to work with to assess the role of *MEI2*-like genes in meristem maintenance. *Arabidopsis* is a small, fast growing plant producing thousands of seeds (Glazebrook and Weigel, 2004). It has a fast generation time of approximately six - eight weeks which allows for several generations to be assessed in a year (Glazebrook and Weigel, 2004). The flowers are perfect, self fertile and generally do not open pollinate (Glazebrook and Weigel, 2004). The *Arabidopsis* genome has been fully sequenced which allows for a comprehensive analysis of genes and gene orthologs using a reverse genetic approach (The Arabidopsis Information Resource, http://www.arabidopsis.org/). In addition T-DNA insertion lines are available (The Arabidopsis Information Resource, http://www.arabidopsis.org/).

Arabidopsis thaliana (L.) Heynh. is widely recognised as being a good model for understanding plant processes (Howell, 1998).

1.4.8 MEI2 members in *Arabidopsis*

As described previously the *MEI2*-like class of genes are RNA-binding protein genes and are characterised by the presence of RNA Recognition Motifs (RRM's) (Jeffares et al., 2004). The best described and most commonly found of the RNA binding motifs is the RRM (Burd and Dreyfuss, 1994; Dreyfuss et al., 2002). Generally, the RRM comprises a RNA binding motif consensus sequence (RNP-CS) of about 90 – 100 amino acids and a consensus sequence RNA binding domain (CS-RBD) containing two short sequences, one 8 amino acids long and the other six amino acids long (Burd and Dreyfuss, 1994; Dreyfuss et al., 2002). In addition, there are hydrophobic conserved amino acids interspersed throughout the motif (Burd and Dreyfuss, 1994). The transportation of messenger RNA from its site of production in the nucleus to the protein synthesis machinery in the cytoplasm is regulated by proteins that bind RNA. What RNA the MEI2-like group of proteins binds is yet to be identified but speculatively, the MEI2-like group of proteins could bind a specific RNA to a precise location in apical initial nuclei for the purpose of directly controlling cell differentiation or protecting a pool of undifferentiated cells. Literature on Mei2p (Watanabe et al., 1997; Harigaya et al., 2006; Harigaya and Yamamoto, 2007) suggests MEI2-like proteins could a have role in regulating or in sequestering particular RNA or gene products. In yeast Mei2 prevents untimely and unstable expression of Mmi1 (Harigaya et al., 2006).

The *MEI2* gene family in *Arabidopsis* has nine identified members which were identified based on sequence similarity with the highly conserved RRM3 serving as a hallmark of the family (Jeffares, 2001; Anderson et al., 2004; Jeffares et al., 2004). Based on further patterns of sequence conservation, the family can be subdivided further into five *AML* (*ARABIDOPSIS MEI2 LIKE*) genes, two *TEL* (*TERMINAL EAR LIKE*) genes and two *MCT* (*MEI2 C-TERMINAL*) genes (Figure 1-5). Genes of the *MEI2* class are conserved across kingdoms and there appears to be redundancy in the functions of the genes (Jeffares, 2001; Alvarez, 2002).

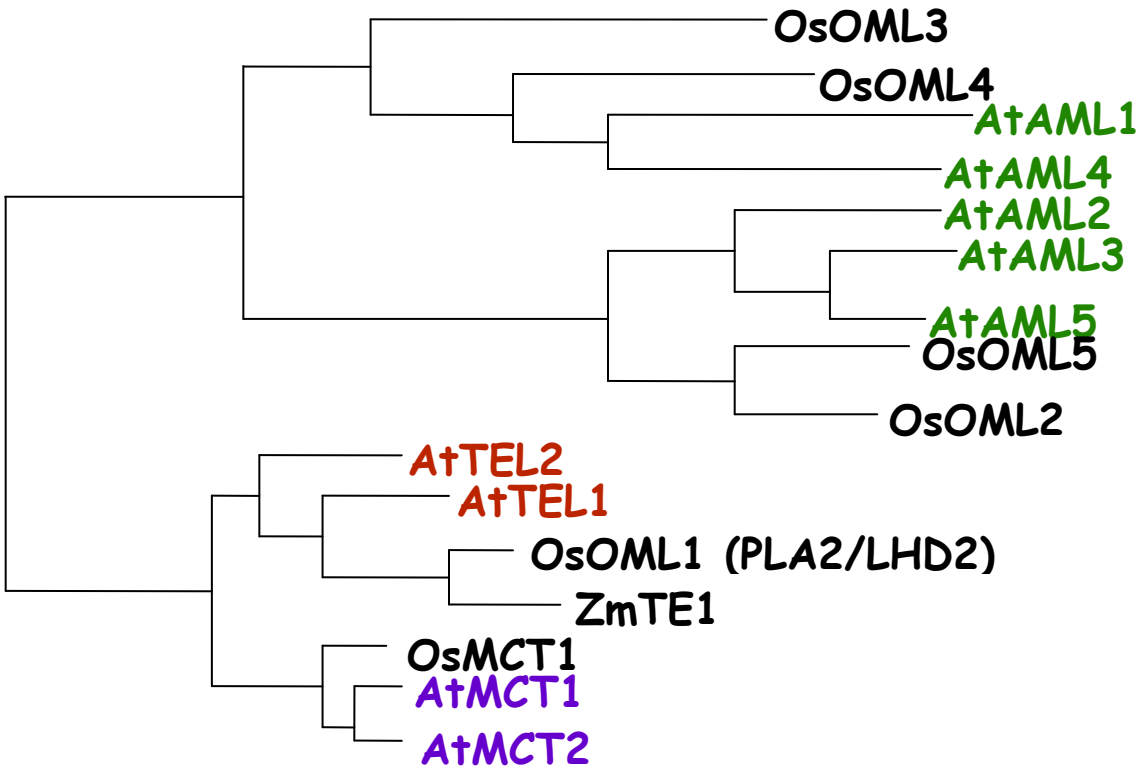


Figure 1-5: The phylogenetic relationship of *Mei2*-like genes from (Jeffares et al., 2004). Abbreviations: The first two letters in the name indicate plant species, Os = *Oryza sativa*, At = *Arabidopsis thaliana* and Zm = *Zea mays*. The second three letters refer to the gene class, OML = *Oryza* Mei2-like, AML = *Arabidopsis* Mei2-like, TEL = terminal ear-like, MCT = Mei2 C-terminal, and TE = terminal ear. The number after the letters refers to the gene number. There are 5 *OML* genes, 5 *AML* genes, 2 *TEL* genes, 2 *MCT* genes and 1 *TE* gene placed on this phylogenetic tree. OsOML1 is also known as *PLA2* (*Plastrochron 2*) or *LHD2* (*Leafy Head 2*).

MCT (*MEI2 C-TERMINAL*) genes contain the highly conserved C-terminal *MEI2*-like RRM3 but lack the rest of the gene. Conservation and redundancy are characteristics lending plausibility to the argument that *MEI2* genes play a critical role in some aspect of plant development. Based on the diagram above (Figure 1-5), an understanding of the *MEI2-like* gene function in *Arabidopsis* may only be achieved through knocking out the two most closely related genes to *TE1* and *PLA2*, *TEL1* and *TEL2*. Given RRM3 in *MEI2* was shown by deletion analysis to be essential for function, and *AML* genes appear to be functionally redundant (Watanabe et al., 1997), it follows both *TEL1* and *TEL2* genes should be active and have some degree of functional redundancy in *Arabidopsis*. In order to achieve a complete knockout of the *MEI2* clade of genes, both *TEL* members (*TEL1* and *TEL2*) of the *MEI2* gene family

may need to be inactivated. In addition, there may be functional redundancy spreading across the entire *MEI2*- like class of genes in *Arabidopsis*. Given there are nine identified *Arabidopsis* members in the *MEI2*-like gene family, the role of *TEL1* and *TEL2* may be as part of a gene complex, where the function of *TEL1* and *TEL2* genes may be to synergistically regulate organ proliferation *via* interactions with characterised meristem maintenance genes such as *WUS* and *CLV1*, *CLV2*, and *CLV3*.

The point at which the shoot meristem becomes histologically distinct during embryogenesis in dicotyledonous plants, specifically *Arabidopsis*, is at the transition from the globular to the heart shaped embryo (Barton and Poethig, 1993). *In situ* hybridisation results (Alvarez, 2002) (graphically represented below in Figure 1-6) showed that *TEL1* expression is localised to the quiescent centre in the root apical meristem and to the apical initials in the shoot apical meristem as early as the heart shaped embryo but is throughout the globular shaped embryo. *TEL1* expression is in the central and peripheral zones in all three clonal layers in the vegetative meristem and in the floral meristems in the developing floral buds. The expression pattern for *TEL2* is restricted to the central zone in all three clonal layers in both the vegetative and floral meristems.

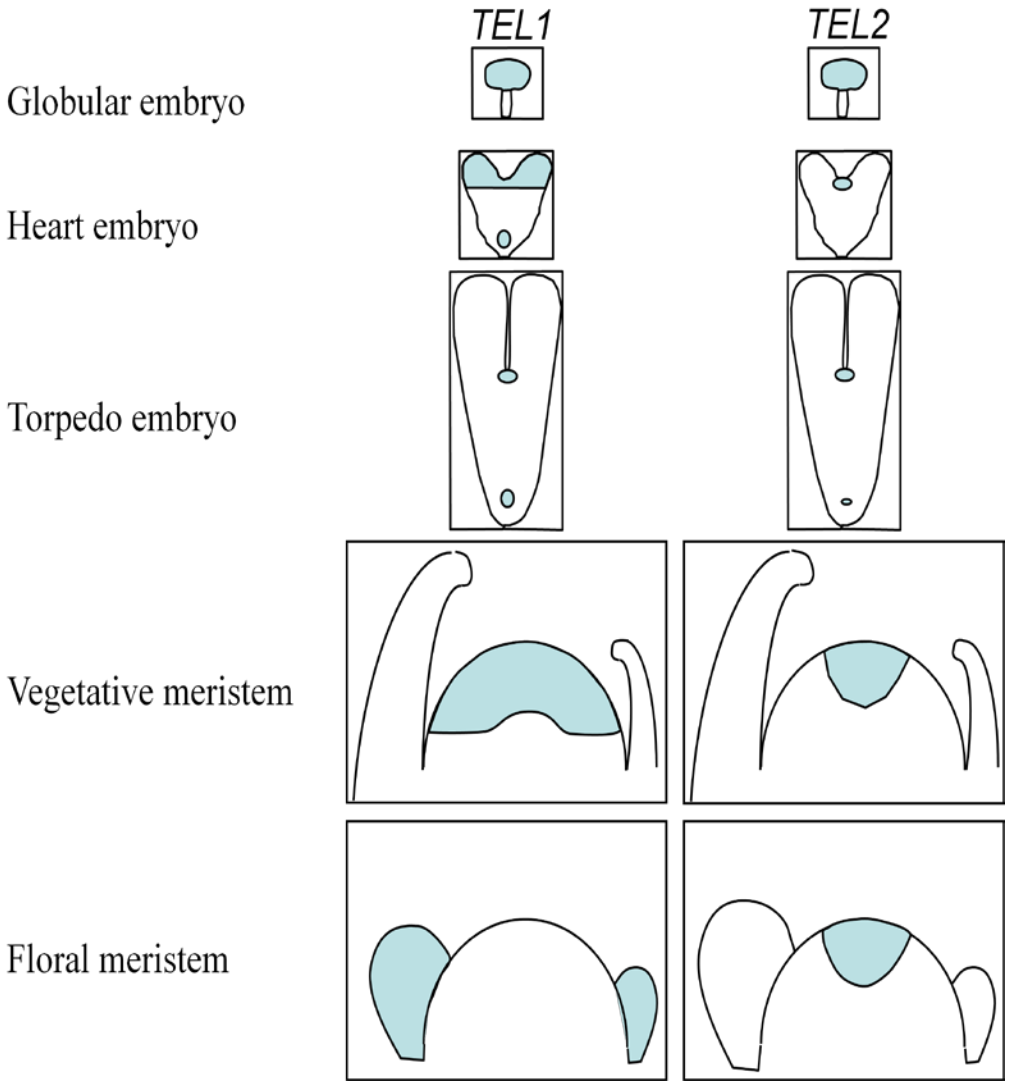


Figure 1-6: Localisation of *TEL1* and *TEL2* transcripts in embryos, vegetative and floral meristems. Schematic based on *in situ* hybridisation results (Alvarez, 2002; Anderson et al., 2004).

AML1 was identified during a study looking for sporulation recovery in a *Schizosaccharomyce pombe* (yeast) mutant (Hirayama et al., 1997). A population of *S. pombe,* with a disrupted pheromone receptor which failed to enter meiosis, was transformed with cDNA from *Arabidopsis*, and screened to see if there was any sporulation recovery (Hirayama et al., 1997). One of the clones, containing a DNA sequence similar to MEI2, showed a partial entry into the meiosis pathway under starvation conditions (Hirayama et al., 1997). The sequence was named *AML1* due to the presence of three RRM's similar to those found in *MEI2* (Hirayama et al., 1997). Sequence analysis revealed *AML1* has different phosphorylation sites to *MEI2,* and

phenotype analysis confirmed *AML1* regulation was different because *AML1* can partially restore yeast strains deficient in a pheromone receptor, but full complementation of MEI2 deficient strains is not achieved (Hirayama et al., 1997). Hirayama et al. (1997) predicted that *AML1* activity would be located specifically in the *Arabidopsis* reproductive organs because its role in *S. pombe* is to direct cells into the meiosis pathway. However, *AML1* is found in all organs in *Arabidopsis*, suggesting a role in the formation of all organs, not just the gametes (Anderson et al., 2004). In addition, Southern analysis showed more than one *AML* sequence hybridised to *MEI2*, further supporting the notion there is redundancy of *MEI2*-like genes in plants (Anderson et al., 2004).

In *Arabidopsis*, the single mutants of the most two closely related *TE1* orthologs, *TEL1* and *TEL2* (*TERMINAL EAR LIKE 1* and *2*) have no gross observable change in phenotype. Similarly single mutants of the next two most closely related *TE1* orthologs, *MCT1* and *MCT2* (*MEI2 C-TERMINAL*), and the more distantly related *AML1* and *AML4* have no observable altered phenotype either (Alvarez, 2002; Anderson et al., 2004). This lack of altered phenotype could be explained by genetic redundancy. This hypothesis, as it applies to the *AML*s, has received support from data showing combinations of these genes must be functionally deficient before a phenotype change can be observed. Kaur et al. (2006) observe an altered phenotype of the *aml1aml4* mutants. The *aml1aml4* double mutant showed evidence of meiotic failure and gamete defectiveness and, if seedlings did form, growth became arrested before flowering occurred. *AML* expression patterns are broader than *TEL* expression patterns. *AML* genes are expressed throughout the embryo and vegetative and floral meristems (Anderson et al., 2004). This broader expression pattern coupled with the data on *aml1aml4* mutants (Kaur et al., 2006) suggests there is functional redundancy in the *MEI2*-like gene family. A summary of data on each member of the MEI2-like transcripts in terms of expression in developing tissues is given in Table 1.

Table 1: location of MEI2 like transcripts in *A. thaliana* compiled from (Veit et al., 1998; Alvarez, 2002; Anderson et al., 2004; Kawakatsu et al., 2006).

tissue	location	TEL1	TEL2	AML1	AML2	AML3	AML4	AML5	TE1	PLA2
Globular embryo	basal	√	√	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	apical	√	√	N/A	N/A	N/A	N/A	N/A	N/A	N/A
	suspensor	x	x	x	x	x	x	x	N/A	N/A
Heart embryo	SAM	√	√	√	√	√	√	√	N/A	N/A
	RAM	√	√	√	√	√	√	√	N/A	N/A
	cotyledons	x	x	√	√	√	√	x	N/A	N/A
	venation	x	x	√	√	√	√	√	N/A	N/A
Torpedo embryo	SAM	√	√	√	√	√	√	√	N/A	N/A
	RAM	√	√	√	√	√	√	√/x	N/A	N/A
	cotyledons	x	x	x	x	√	√	x	N/A	N/A
	venation	x	x	√	√	√	√	√	N/A	N/A
Vegetative meristem	L1	√	√	√	√	√	√	√	√	√
	L2	√	√	√	√	√	√	√	√	√
	L3	√	√	√	√	√	√	√	√	√
	CZ	√	√	√	√	√	√	√	√	√
	PZ	√	x	√	√	√	√	√	√	√
	RZ	x	x	√	√	√	√	√	x	x
	P0	√	x	√	√	√	√	√	√	√
	P1	√	x	√	√	√	√	√	√	√
	P2	√	x	√	√	√	√	√	√	√
Floral meristem	L1	√	√	√	√	√	√	√	N/A	√
	L2	√	√	√	√	√	√	√	N/A	√
	L3	√	√	√	√	√	√	√	N/A	√
	CZ	√	√	√	√	√	√	√	N/A	√
	PZ	√	x	√	√	√	√	√	N/A	√
	RZ	x	x	√	√	√	√	√	N/A	x
	B0	√	x	√	√	√	√	√	N/A	√
	B1	√	x	√	√	√	√	√	N/A	√
	B2	√	x	√	√	√	√	√	N/A	√

√ = present, x = absent, N/A indicates tissue was not assessed. L1, L2, and L3 are three clonal layers in the meristem. CZ = central zone, PZ = peripheral zone, RZ = rib zone. P0 = presumptive leaf primordia, P1 = first leaf primordia, P2 = second leaf primordia. B0 = presumptive bract primordia, B1 = first bract primordia, B2 = second bract primordia.

Nevertheless, the MEI2 clade of genes could have a role in maintenance or initiation of the shoot and root apical meristem given *TEL1* and *TEL2* have a similar expression pattern to other identified and characterised meristem maintenance genes. Alternatively the role of these genes in plants could be similar to what is described for yeast (to prevent untimely meiosis) and *TEL1* and *TEL2* genes could be involved in the transition from vegetative to floral state. Given *TEL1* and *TEL2* have a similar

expression pattern in the shoot and root apical meristem to other identified and characterised meristem maintenance genes (Anderson et al., 2004). A question arising from looking at the interactions between these genes is whether, given the location of *TEL1* and *TEL2* gene expression in the meristem, *TEL1* and *TEL2* genes contribute to *CUC1*, *CUC2*, *STM*, *WUS*, *CLV1*, *CLV2* and *CLV3* interactions. However *TEL1* and *TEL2* genes are expressed in both the RAM and SAM so the function of *TEL1* and *TEL2* may be different to genes that are not expressed in both apical meristem regions.

SHR, *SCR*, *PLT1*, and *PLT2* have similar or overlapping domains of expression to *TEL1* and *TEL2* (Figure 1-7) raising the question of whether *TEL1* interacts with *SHR*, *SCR*, *PLT1* and *PLT2* to specify the identity of cells in the RAM. Based on *in situ* hybridisation results *TEL1* transcripts accumulate in the QC but not in surrounding cells (Alvarez, 2002; Anderson et al., 2004). As *TEL* genes are located in meristematic regions it is plausible to expect *TEL* genes interact with factors influencing the position of the QC such as auxin, cytokinin and gibberellin to regulate cellular differentiation in the meristem. In addition, given that *PLT1* and *PLT2* interact with auxin (Blilou 2005) and the location of *PLT1* and *PLT2* gene expression is similar to the location of *TEL1* and *TEL2* gene expression auxin could impact on *TEL1* and *TEL2* expression in a comparable manner to that seen with *PLT1* and *PLT2*.

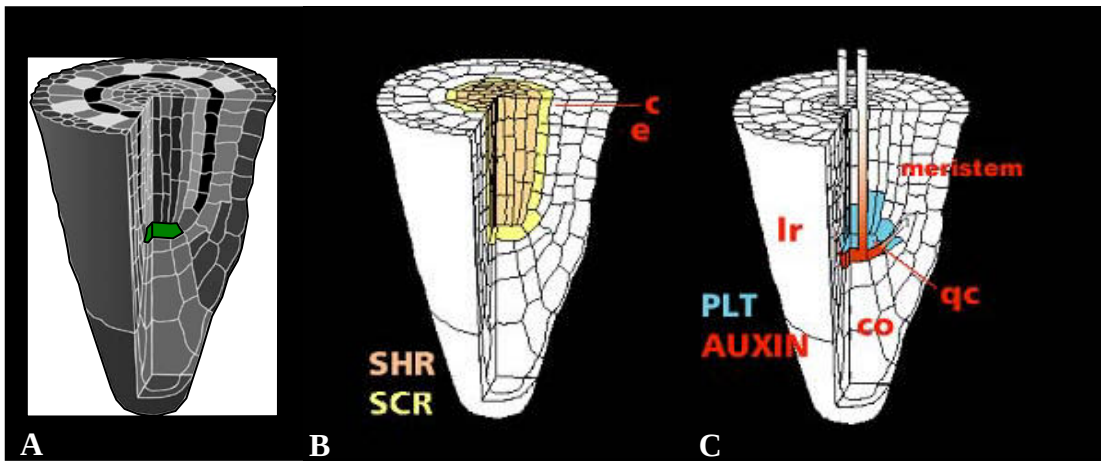


Figure 1-7: Location of *TEL1*, *SCR*, *SHR*, and the *PLT* genes. A: TEL1 signal is located specifically in the QC of the RAM. B: SCR expression is in the QC, cortical endodermal initials and in the endodermal cells (yellow) and SHR expression is in the QC, stele stem cells and in the stele cells (brown). C: PLT genes are expressed in the QC, in all initial cells and in the stele cells (blue) and auxin accumulates in the cells just distal to the QC (red). A is adapted from and B and C pictures are reproduced from (http://www.bio.uu.nl/mg/pd/index.html, 2003). C= cortex cells, e= endodermal cells, QC= quiescent centre cells, co= columella cells and lr= elongation zone

1.5 OBJECTIVES OF THIS RESEARCH

The purpose of the prior research in *Arabidopsis*, maize and rice (Veit et al., 1998; Alvarez, 2002; Anderson et al., 2004; Jeffares et al., 2004; Kawakatsu et al., 2006) was to investigate the role *MEI2*-like genes on overall plant growth and architecture. Research carried out previously indicated there are nine identified MEI2-like gene family members in *Arabidopsis* (Jeffares, 2001; Anderson et al., 2004; Jeffares et al., 2004) and that, based on in situ hybridization results (Alvarez, 2002), there are similarities in expression domains of the family members in *Arabidopsis* and in maize (Veit et al., 1998) and rice (Kawakatsu et al., 2006).

This research, in this thesis, builds on the knowledge obtained from this prior research on the MEI2-like genes but focuses on the role of two specific MEI2-like genes, *TEL1* and *TEL2*. Based on the phylogenetic tree and the observable altered phenotype in maize and rice, where disruption of a single gene appears sufficient to produce an obvious phenotype, it seems plausible that knocking out the two *TEL* genes, the most related *Arabidopsis* members would be sufficient to produce an observable change in phenotype. As well, investigating the transcriptional domains present in the *TEL1* gene and studying *TEL1* gene interactions with *SCR*, *SHR*, *PLT1* and *PLT2* and the effect of adding synthetic auxin, auxin mimics, and auxin transport inhibitors could also provide information about the role of *TEL1* in the maintenance of meristem cell populations.

This thesis, therefore, has a primary objective of characterising the role of *TEL1* and *TEL2* in *Arabidopsis*, as well as more generally seeking to understand the mechanisms involved in meristem maintenance in plants. To achieve this, the experimental programme is divided into the following aims.

Aim 1: To provide knowledge about biological function of *TEL* genes through characterisation of *TEL* mutant plants.

Aim 2: To provide knowledge about biological function of *TEL* genes through an analysis of cis-acting and trans-acting factors.

Aim 3: To determine whether shoot and root apical meristems use *TEL1* and *TEL2* genes to promote differentiation or to maintain a population of undifferentiated cells.

Aim 4: To investigate the transcriptional domains present in the *TEL1* gene.

Aim 5: To study *TEL1* gene interactions with *SCR*, *SHR*, *PLT1* and *PLT2*.

Aim 6: To assess the effect of adding synthetic auxin, auxin mimics, and auxin transport inhibitors on *TEL1* gene regulation.

2 METHODS AND MATERIALS

2.1 Plant material

2.1.1 Plant propagation

Seeds to be sown straight to soil were first stratified over 48 hours in water at 4 ^{o}C in a plastic microfuge tube before being shaken onto damp seed-raising mix in plastic pots. The pots and their contents were then thoroughly watered, placed under plastic to maintain high humidity, and transferred to a glasshouse. The plastic was removed once the seeds had germinated. The glasshouse operated a 16 hour day regime with a daytime temperature of 22 ^{o}C and a night time temperature of 17 ^{o}C.

Seeds planted to be grown in a growth room were first surface-sterilised (70 % (v/v) ethanol for one minute, 50 % (v/v) sodium hypochlorite for one minute, and three washes of sterilised water) before being transferred to plates containing Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium and antibiotic selection (if required) and stratified at 4 ^{o}C for 48 hours. Growth rooms operated a 16 hour day regime with a daytime temperature of 22 ^{o}C and a night time temperature of 17 ^{o}C with light levels at 100-150 µE / m^2/ s. Once the plants had been characterised as containing the T-DNA insert and had reached a suitable transplanting size (generally four true leaves) the plants were potted in the glasshouse. The plants were transferred from the tissue culture plate to a small hole in pre-wetted seed-raising potting mix, making sure the roots went into the hole. The potting mix was then squeezed together around the roots and the pots and the contents watered thoroughly. The plants were then placed under plastic for 48 – 72 hours in continuous light and ambient temperature. After 48 – 72 hours, the plastic was removed and the plants transferred to 16 hour days in the glasshouse under PC2 containment under ERMA development approval number #GMO 2001/AGPN007 and import approval number GMC99006; MAF #20022015068.

2.1.2 Generation of out-crossed progeny

Healthy plants were selected and the sepals and petals of unopened flowers were gently prised apart using size four ultra fine antistatic antimagnetic tweezers (Geneva Importers Ltd, Wanaka, New Zealand) to reveal anthers and style. The anthers were removed with tweezers taking care not to damage the style or stigma and the stigma was brushed with pollen from anthers of the pollen donor. The pollen donor was selected based on its genotype and pollen quality. Anthers with copious amounts of white fluffy pollen were considered ideal paternal parents.

2.1.3 Root phenotype analysis

Lines carrying T-DNA insertions were sown on large plastic plates (22 x 22cm). The plates were sterilised with 50 % (v/v) sodium hypochlorite overnight and rinsed three times with sterile water, once with 70 % (v/v) ethanol and finally 100 % ethanol. The plates were left to dry completely in a laminar flow cabinet before MS medium with 2 % (w/v) sucrose and 1 % (w/v) agar was poured into them and allowed to set. Seeds were surface sterilised (70 % (v/v) ethanol for one minute, 50 % (w/v) sodium hypochlorite for one minute, and three washes of sterilised water) and placed along one edge of the plate, 1.3 cm apart. The plates were placed at 4 ^{o}C for 48-72 hours before being transferred to the growth room and maintained under 16 hour day regime with a daytime temperature of 22 ^{o}C and a night time temperature of 17 ^{o}C with light levels at 100-150 µE / m^2/ s. The plates were initially placed horizontally to encourage the roots to grow into the medium. Once the roots had penetrated the medium the plates were inclined to the vertical (approximately two days after germination). Roots were scored nine days later. Plates were either scanned or photographed with a digital camera. The Image Processing and Analysis in Java (ImageJ, http://rsb.info.nih.gov/ij) programme was used to determine root length, alpha angle (Figure 2-1 A), beta angle (Figure 2-1 B) and the number of lateral roots.

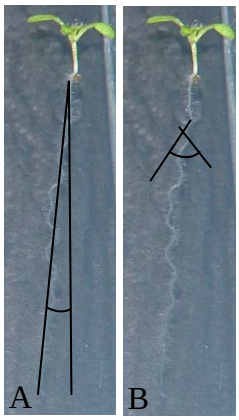


Figure 2-1: Root angle measurements. A: α angle, the angle roots deviate from the vertical, B: β angle, the angle between one root bend and another.

2.1.4 Pollen viability

Alexander's stain was used to test for pollen viability. Alexander's stain stock solution was stored in the dark at room temperature until use (Table 2).

Table 2: Formulation of Alexander's (1969) stain. The reagents were used to make a 50 x stock solution. (Johnson-Brousseau and McCormick, 2004)

Reagent	Amount
95 % (v/v) ethanol	10 ml
1 % (w/v) malachite green in 95 % (v/v) ethanol	5 ml
phenol	5 g
1 % (w/v) acid fuschin in water	5 ml
1 % (w/v) orangeG in water	0.5 ml
glacial acetic acid	2 ml
glycerol	25 ml
water	50 ml

A modified Alexander's stain (Alexander, 1969, 1980) stock solution without Chloral hydrate (50 x) was diluted (1:50) with water before use. Pollen collected from plants grown in the glasshouse was placed in a microfuge tube containing 1 ml of a working concentration Alexander's stain and allowed to sit for two minutes. Pollen was then removed from the microfuge tube and placed on a slide under a coverslip and assessed with a light microscope for colour. Pollen scored as red was deemed viable. Pollen scored as blue was deemed non viable.

2.1.5 Reporter gene visualisation

Apical root tips of plants grown on MS medium with 1 % (w/v) sucrose in a 16 hour day growth room with a daytime temperature of 22 ^{o}C and a night time temperature of 17 ^{o}C with light levels at 100-150 µE / m^{2}/ s were assessed for GFP expression 7-10 days after germination (DAG). The seedlings were pulled gently from the plate and the root tip and lateral roots were stained with 10 µg/ml propidium iodide for one minute at room temperature followed by three washes of sterile water. The shoot tip and inflorescence meristem were dissected using size four tweezers (Geneva Importers Ltd, Wanaka, New Zealand) and an ultra fine antistatic antimagnetic dissecting knife. Siliques containing embryos of all stages were selected from healthy plants. The siliques were slit open using an ultra fine dissecting knife and placed on a slide, covered with a coverslip and gently squashed until the embryos popped out of the seed coat.

All material was assessed with a Leica DMRBE confocal microscope with a mixed argon krypton laser. The excitation wavelength used was 488 nm in conjunction with a short pass beam splitter at 510 nm and a pinhole of 90. The detector used was a short pass 580 nm beam splitter and a BPFITC barrier filter. Any remaining light was then directed *via* a mirror to a 590 nm long pass barrier filter.

Cotyledons and true leaves of plants containing GUS constructs were grown for 5 -10 days on MS medium in a growth room under16 hour days with a daytime temperature of 22 ^{o}C and a night time temperature of 17 ^{o}C with light levels at 100-150 µE / m^{2}/ s. The seedlings were pulled gently from the plate and placed in wash solution (Table 3) for six hours at 37 ^{o}C. The solution was made (Table 3) as required and stored at -20 ^{o}C for no longer than two weeks. The concentration of potassium ferricyanide and potassium ferrocyanide was adjusted to restrict GUS to site of production. The stain was removed using a pipette and replaced with 70 % (v/v) ethanol and a drop of acetic acid (0.1 %) and left over night to remove the chlorophyll and discolour the tissue. To visualise the SAM the leaves and cotyledons were carefully removed using an ultra fine dissecting knife and tweezers. Whole plants and plant sections were visualised on an Olympus BX50 light microscope and photographed with Colour View 111 soft imaging system and analysisB software (from Olympus, Singapore).

Table 3: Formulation of GUS wash solution.

Reagents	**Final concentration**
500 mM sodium phosphate buffer (Na_2HPO_4 + NaH_2PO_4 pH7.0)	50 mM
100 mM potassium ferricyanide ($K_3Fe(CN)_6$)	4 mM – 8 mM
100 mM potassium ferrocyanide ($K_4Fe(CN)_6.3H_2O$	4 mM – 8 mM
10 % (v/v) triton X 100	0.1 % (v/v)
methanol	20 % (w/v)
100 mM X-Gluc in DMF	2 mM
water	to volume

2.1.5.1 Plant fixative; Formaldehyde:acetic acid (FAA)

Tissue was prefixed with 90 % (v/v) ice-cold acetone at room temp for 20 minutes then washed twice with cold water before the GUS wash solution (Table 3) was added. The tissue was then infiltrated on ice until everything sunk before being incubated at 37 ˚C overnight. The tissue was then taken through an ethanol series (30 min at room temperature of 20 % 9v/v) and 35 % (v/v) ethanol) before fixing with FAA (50 % (v/v) EtOH, 3.7 % (v/v) formaldehyde, 10 % (v/v) glacial acetic acid) for 30 minutes. The material was stored in 70 % (v/v) ethanol at room temperature.

2.2 Molecular techniques

2.2.1 Extraction of *Arabidopsis thaliana* DNA

Arabidopsis thaliana DNA was extracted using a quick DNA extraction protocol (Xin et al., 2003). This quick DNA extraction protocol was used throughout this project when DNA was extracted from a small number of plants.

2.2.2 Quick DNA extraction protocol

A single cotyledon was ground with a plastic RNase/DNase free pestle (Invitrogen, Carlsbad, USA) in a microfuge tube containing 50 µL of extraction buffer (Table 4) and spun at top speed for five minutes in an Eppendorf (Hamburg, Germany) 5417R bench top centrifuge. The supernatant (35 µl) was then transferred into a new microfuge tube containing 35 µL of 100 % isopropanol. The resulting solution was mixed by inversion to precipitate the DNA and then spun at top speed for 10 minutes in an Eppendorf 5417R bench top centrifuge. The supernatant was removed by inverting the microfuge tubes, leaving the DNA pellet collected at the bottom. The pellet was dried for 10 minutes at room temperature, re-suspended in 40 µL of filter sterilised MilliQ water and then stored at 4 ^{o}C.

Table 4 Quick DNA extraction buffer

Reagent [stock]	**Reagent [final]**
1 M Tris HCl (pH9.0)	0.2 M Tris HCl (pH 9.0)
2 M LiCl	0.4 M LiCl
0.5 M EDTA	25 mM EDTA
10% (w/v) SDS	1% (w/v)SDS
N/A	H_2O

2.2.3 High Throughput DNA preparation (Xin et al., 2003)

This high throughput DNA extraction method was used when DNA was extracted from a large number of plants, in particular for the genotyping of segregating double mutant population. A 6 mm diameter leaf disc was placed into a 96-well PCR plate sitting on ice. Fifty µL of extraction buffer A (Table 5) was added to each well and the 96-well PCR plate placed in a thermocycler machine set at 95 ^{o}C for 10 minutes before neutralisation with buffer B (Table 5). After gentle agitation, the plate was left to rest for an hour before being diluted five-fold with sterile MilliQ water. The samples and the dilutions were stored at 4 ^{o}C.

Table 5: High Throughput DNA buffers (Xin et al., 2003).

Buffer A (made fresh)	**Buffer B**
100 mM NaOH	100 mM Tris HCl (pH 2.0)
2% (v/v) tween20	2 mM EDTA

For Buffer B the 100 mM Tris HCl (pH 2.0) was made 10 x and diluted just prior to the addition of EDTA

2.2.4 DNA amplification from the BAC clone

The MJL14 BAC clone (obtained from TAIR, Carnegie Institution of Washington, Stanford, USA) was cultured in 10 ml of Lysogeny Broth (LB) (Bertani, 1951) overnight at 37 ^{o}C with shaking and the DNA extracted using a plasmid mini prep Qiagen kit (obtained from Biolab Ltd, Albany, Auckland) as per the manufacturer's instructions.

2.2.5 Polymerase Chain Reaction (PCR) amplification

Primers used for PCR were dissolved in filter sterilised MilliQ water to a final concentration of 1 mM and stored at -20 ^{o}C. As required, primers were diluted further with filter sterilised MilliQ water to a working concentration of 10 µM and stored at -20 ^{o}C. PCR reactions were set up in thin-walled 0.2 ml PCR tubes with a total reaction volume of 20 µL (Table 6). The thermal cycler (BioRad iCycler, Bio-Rad Laboratories Pty Ltd, Auckland, New Zealand) programmes are detailed below in

section 2.2.6. HiFidelity Taq (Invitrogen) was used in place of Platinum Taq (Invitrogen) when long range accuracy was required.

Table 6: PCR reaction constituents

Reagents	**Reagent [final concentration]**
DNA	≈ 0.5 ng
10 mM dNTPs mixture (Invitrogen™)	0.4 nM of each dNTP
50 mM $MgCl_2$	2.5 mM
Primers	0.2 µM of each primer
10 x PCR buffer (Invitrogen™)	1x
Platinum Taq / HiFi Taq (Invitrogen™)	0.25 units
MilliQ water	to 20 µl

2.2.6 Polymerase Chain Reaction thermal cycler programme

An initial denaturing step of 96 ^{o}C for 10 minutes was followed by 40 cycles of 96 ^{o}C for 20 seconds (denaturation), 57-60 ^{o}C for 20 seconds (annealing), and 72 ^{o}C for 30 – 60 seconds (extension). After the 40 cycles, there was a final extension time of four minutes at 72 ^{o}C before the reaction was held at 12 ^{o}C. The extension time varied depending on the expected product size (product sizes are displayed in (Table 10), but were calculated based on a 30 seconds requirement for every 500 base pairs. The annealing temperature varied depending on the primer combinations used and was calculated based on the GC content of the primer (annealing temperature was calculated at twice the number of G's and C's plus the number of A's and T's) (White, 1997).

2.2.7 Gel electrophoresis

PCR products less than 1.5 kb were electrophoresed, alongside a 1 Kb plus DNA ladder (Invitrogen), on a 1 % (w/v) agarose gel containing 1 x TAE and ethidium bromide (5 µg/ml). PCR products greater than 1.5 kb were electrophoresed, alongside a 1 Kb plus DNA ladder (Invitrogen), on a 0.8 % (w/v) agarose gel containing 1xTAE

and ethidium bromide (5 µg/ml). The gels were photographed using a Gel Doc trans-illuminator (Bio-Rad).

2.3 Vector construction

2.3.1 Restriction digests

The appropriate PCR product was excised from the 1 % (w/v) or 0.8 % (w/v) TAE agarose gel with a sharp scalpel blade and purified using a Qiagen© gel (obtained from Biolab Ltd) purification kit according to the manufacturer's instructions. This material was used to transform pTOPOv2.1® using a TOPO TA Cloning® Kit (Invitrogen) according to the manufacturer's instructions. Dilutions of the transformations were spread onto LB plates containing 50 mg / mL amplicillin and grown overnight at 37 ^{o}C. Colonies were picked off and assessed by colony digestion for *TEL1* promoter DNA.

The TOPO clones containing the desired fragment were cut using an appropriate restriction enzyme. A total of 2 µL of *TEL1* promoter DNA was digested with 20 units of *Bam*HI for two hours at 37 ^{o}C. A 500 ng aliquot of the digested DNA was electrophoresed on a 1 % (w/v) agarous gel to check digestion had proceeded as expected. A 10 µg aliquot of the *TEL1* promoter TOPO clone was digested with 20 units of *Bam*HI and *Hin*dIII restriction enzymes in a total volume of 20 µL of two hours at 37 ^{o}C to provide *TEL1* promoter DNA with *Bam*HI and *Hin*dIII sticky ends suitable for ligating into the vector of choice.

2.3.1.1 DNA / plasmid ligation

A 10 µg aliquot of pBINmGFP-ER (Figure 2-2) was digested with 20 units of *Bam*HI and *Hin*dIII restriction enzymes in a total volume of 20 µL of two hours at 37 ^{o}C. The products of digestion with *Bam*HI and *Hin*dIII left DNA ends that were complementary with the genomic DNA ends created by the primers on the ends of the *TEL1* promoter DNA. These ends were unable to self-ligate.

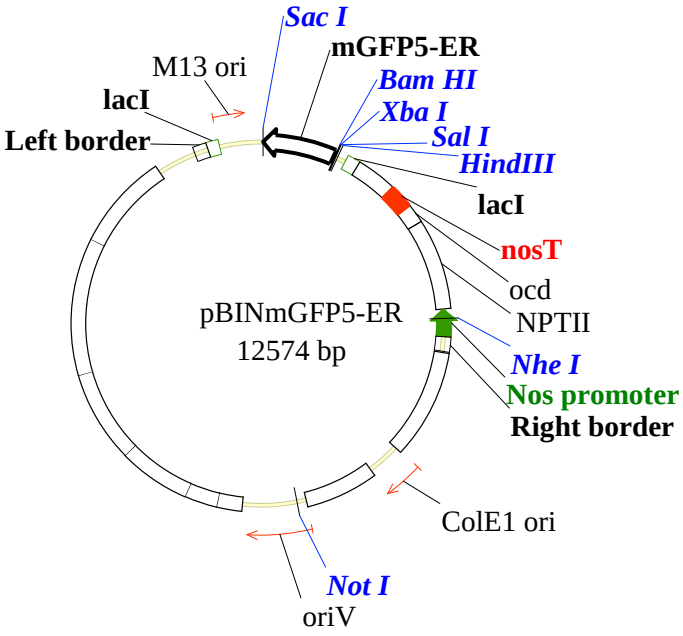


Figure 2-2: Schematic of pBINmGFP5-ER vector. This vector is a modified pBIN19 vector containing the mGFP5-ER version of GFP and kanamycin resistance gene (NPTII) being driven by a Nos promoter. The *Bam*HI and *Hin*dIII restriction sites preceding the mGFP5-ER sequence are the restriction sites of choice for the cloning strategy.

The gel purified *Bam*HI and *Hin*dIII digested *TEL1* promoter PCR product (150 ng) was ligated with 50 ng of the *Bam*HI and *Hin*dIII digested pBINmGFP-ER vector alongside the control reactions of vector only and *TEL1* promoter DNA only. Vector DNA (50 ng) was added to a tube containing 150 ng of *TEL1* promoter DNA, 1 µL of T4 DNA ligase enzyme, 4 µL of 5 x T4 ligase buffer and MilliQ water to a final volume of 20 µl. Control reactions were made to a total of 20 µl, that had either the vector DNA only or the *TEL1* promoter DNA only. The reactions were incubated at 4 ^{o}C overnight.

A total of 1 µg *TEL1* promoter DNA in pBINmGFP-ER was digested with *Bam*H1 in a total volume of 100 µL for two hours at 37 ^{o}C to confirm the insert could be removed. A total of 1 µg *TEL1* promoter DNA in pBINmGFP-ER was digested with *Not*1 and *Sal*1 in a double digest in a total volume of 100 µL for two hours at 37 ^{o}C as a diagnostic restriction mapping procedure. The products were electrophoresed, as outlined above on, a 1 % (w/v) TAE agarose gel to check the digestion had proceeded as expected.

A 10 µg aliquot of pCambia1391Xb vector was digested with 20 units of *Bam*HI and *Eco*R1 restriction enzymes in a total volume of 20 µL of two hours at 37^{o}C. The products of digestion with *Bam*HI and *Eco*R1 left DNA ends that were complementary with the genomic DNA ends created by the primers on the ends of the *TEL1* genomic DNA. These ends were unable to self-ligate.

The gel purified *Bam*HI and *Eco*R1 digested *TEL1* genomic DNA (150 ng) was ligated with 50 ng of the *Bam*HI and *Eco*R1 digested pCambia1391Xb vector alongside the control reactions of vector only and *TEL1* genomic DNA only. 50 ng of vector DNA was added to a tube containing 150 ng of *TEL1* promoter DNA, 1 unit of T4 DNA ligase enzyme, 4 µL of 5 x T4 ligase buffer and water to a final volume of 20 µl. Control reactions were made to a total of 20 µl, but had either the vector DNA only or the *TEL1* genomic DNA only. The reactions were incubated at 4 ^{o}C overnight.

A total of 1 µg *TEL1* genomic DNA in pCambia1391Xb was digested with *Bam*H1 and *Eco*R1 in a total volume of 100 µL for two hours at 37 ^{o}C to confirm the insert could be removed. A total of 1 µg *TEL1* genomic DNA in pCambia1391Xb was digested with *Not*1 and *Sal*1 in a double digest in a total volume of 100 µL for two hours at 37 ^{o}C as a diagnostic restriction mapping procedure. The products were electrophoresed on a 1 % (w/v) agarous gel to check the digestion had proceeded as predicted.

2.3.2 Plasmid DNA sequencing

Plasmid DNA from colonies identified as containing the correct DNA (by restriction digest analysis) was isolated using a Qiagen© mini DNA plasmid kit (Invitrogrn) according the manufacturer's instructions and the DNA used for sequencing to confirm the sequences were error free. DNA was prepared for sequencing with Big DyeV3.1 sequencing reagents. DNA (200 ng) was added to a tube containing 1 µL of Big DyeV3.1, 3.5 µL of Big DyeV3.1 dilution buffer, and a primer (final concentration of 3.3 mM). The primers used were a combination of forward and reverse primers as appropriate, listed in Table 7.

Table 7: List of primers and their sequences used for sequencing of *TEL1* DNA. Genomic bases are in capital letters, restriction sites in lower case.

Primer name	**Primer sequence**
TEL1Pf - forward	ggatccAATAATTTTGGTTCAGTGGTGG
TEL1KOL - forward	TAAGCCGCAGACAAACCCTAAGCTACATT
Pro1000 - forward	gacaagcttCTTGATCCCAAACTAAAG
Pro500 - forward	gacaagcttCTTCAAATTATGAGTGAG
TELKU - forward	ACTTTCCGTACACTCCTCCTCCTCCACAG
ATE1 - forward	AGCTCACCTTCTACTTCATC
TEL1GAPF - forward	ATTCCTCCTTTGAGGCGG
TEL1SL2 - reverse	CCAAACAAGTACAGGTTGGGCTT
TEL1Wt3 - reverse	CCGTAAACTTGGAAAATCTGTCTGAGAGT
TEL1SR3 - reverse	TCTCGCATATGTGATTTGGCA

The DNA was amplified using PCR conditions recommended by the manufacturer of the Big DyeV3.1 sequencing reagents (Applied Biosystems, Foster City, USA) of 25 cycles of 94 ^{o}C for 15 seconds, 55 ^{o}C for 15 seconds and 60 ^{o}C for four minutes. The resulting PCR product was cleaned up using the reagents and procedure as recommended by the manufacturer (Applied Biosystems). Added to 50 ng of PCR product was sodium acetate to a final concentration of 1.5 M, EDTA to a final concentration of 250 mM, 80 µL of EtOH 100% and water to 100 µL. The clean up reagents were added in the order they are listed to a microfuge tube containing the DNA, inverted to mix and incubated at room temperature for 15 minutes. The contents were then spun at 11700 xg for 15 minutes, the supernatant removed with a super fine hypodermic needle (0.4 grade) and then washed with 70 % (v/v) ethanol pre-chilled to -20 ^{o}C and spun for a further 15 minutes at 11700 xg. The wash with 70 % (v/v) ethanol step was repeated twice. The final wash was done with 100 % ethanol and the pellet air dried overnight at 4 ^{o}C. The pellet was re-suspended in BigDye V3.1^{tm} buffer and placed in the ABI3100 sequencing machine (Applied Biosystems) for sequencing.

2.3.3 *Escherichia coli* (*E. coli*) DH5α transformation

The *TEL1* DNA inserts and vector ligation products (from 2.3.1 and 2.3.2) were then transformed into *E. coli* strain DH5α. Chemically competent *E. coli* DH5α (Invitrogen) was thawed slowly on ice then incubated for 20 minutes on ice with 50-100 ng of the *TEL1* DNA and vector cloning mix from above. The mix was heated for 40 seconds at exactly 42 ^{o}C, before resting on ice for a further two minutes. The cells were added to 1 ml of LB medium and incubated for one hour at 37 ^{o}C with shaking. After the recovery period the cells were plated to LB medium containing 1 % (w/v) bactoagar and the appropriate antibiotic selection, allowed to dry and incubated at 37 ^{o}C for 8-16 hours. Colonies resistant to the antibiotics were screened for the presence of the required sequence by STET prep DNA isolation followed by PCR with M13 forward (5′GTAAAACGACGGCCAGT3′) and M13 reverse (5′GGAAACAGCTATGACCATG3′) primers. The M13 forward and M13 reverse primers were used to amplify the insert / vector junction to confirm insert presence in the plasmid.

2.3.4 Plasmid purification

The selected *E. coli* DH5α colonies were grown in 5 ml of LB medium, containing the appropriate antibiotic, selection for eight hours. An aliquot of the 1.5 ml of the cells were then poured into a 1.5 ml microfuge tube and spun at 14000 rpm in an Eppendorf 5417R bench top centrifuge for one minute to pellet the cells. The supernatant was poured off and the pellet re-suspended in the remaining 5 µL of LB until a homogenous cell paste was obtained. STET solution (100 µL comprising 8 % (w/v) sucrose, 5 % (v/v) Triton X-100, 50 mM Tris pH 8.0, and 50 mM EDTA pH 8.0) and 10 µL of lysosome (final concentration 0.5 mg/ml in STET buffer) was added and the tube mixed by vortexing briefly. The tubes were then placed in a boiling water bath for 30 seconds before being spun for 10 minutes at 14000 rpm in an Eppendorf 5417R bench top centrifuge. The pellet was removed with a sterile toothpick and discarded before the addition of 110 µL of 100% isopropanol. The mix was spun in an Eppendorf 5417R bench top centrifuge for 10 minutes at 14000 rpm to pellet the DNA. The supernatant was removed and the DNA pellet washed briefly with 0.4 ml of 70 % (v/v) ethanol, pre-chilled to 20 ^{o}C. The ethanol was aspirated

with a micropipette tip and the pellet left to dry at room temperature (22 ^{o}C). Once dry, the pellet was dissolved in 40 µL MilliQ water.

2.3.5 Transformation of *Agrobacterium tumefaciens*

The vectors detailed above (section 2.3.1 and 2.3.4) were used to transform competent *Agrobacterium tumefaciens* strain GV3101 (Invitrogen). *Agrobacterium tumefaciens* strain GV3101 was thawed slowly on ice before 500 ng of the construct DNA was added to the cells. This was incubated on ice for five minutes, then in liquid nitrogen for five minutes, and then at 37 ^{o}C for five minutes. One ml of LB was added to the tube, the resulting mix was incubated for two hours at room temperature with gentle shaking before plating on LB plates containing antibiotic selection and gentamycin (for selection of helper plasmid, pMP90RK). The plates were incubated at 28 ^{o}C for two days before assessing for presence of the construct as indicated by the presence of discrete colonies. Five of the discrete colonies were selected for amplification of the plasmid by PCR with specific primers (PCR conditions described in section 2.2.5 and primers described in Table 7).

2.3.6 Transformation of *Arabidopsis thaliana*

Columbia ecotype plants with five day old primary inflorescences were selected. An overnight culture of *Agrobacterium tumefaciens* strain GV3101 containing either pCambia1391Xb and 5' region, introns and exons of *TEL1* or pBIN-mGFP-er -1900, -1000, -840, -600 or -500 was re-suspended in a 4 % (w/v) sucrose / 0.1 % (v/v) Silwet solution and pipetted onto unopened buds. Any open flowers were plucked off and discarded. The plants were then placed under a plastic sheet for one day before being transferred to the glasshouse. The procedure was repeated at four day intervals for two weeks. Plants were grown in a glasshouse, with a 16 hour day with a daytime temperature of 22 ^{o}C and a night time temperature of 17 ^{o}C, until they flowered and set seed before being dried and the seeds harvested. The seeds were sown onto MS medium containing antibiotic selection (see section 2.3.1.1). Resistant plants were potted and maintained in a glasshouse (16 hour day with a daytime temperature of 22

^{o}C and a night time temperature of 17 ^{o}C) and the seed produced was screened for the presence of an insert and assessed for GFP or GUS expression.

2.3.7 Statistical analyses

The data was analysed using the Restricted Maximum Likelihood (REML) method. The method of residual maximum likelihood (REML) was introduced by Patterson & Thompson (Patterson and Thompson, 1971) and is used for estimating variances and covariance and deals with linear combinations of both fixed and random effects of the observed values whose expectations are zero. REML is considered to be equal to Analysis of Variance (ANOVA) when assessing balanced data but is a better model to use when using unbalanced or correlated data and large populations, such as the data presented here. REML estimates of variances and covariance are known to be unbiased and can be used when there is more than one source of variation or correlation in the data and the relative size of different sources of variability needs to be assessed. It allowed estimates of treatment effects and combined information from all the strata of a partially balanced design enabling an estimate of variance that makes use of the information from all the experiments, as well as the separate estimates from each individual experiment.

3 RESULTS

3.1 TEL1 AND TEL2 PHENOTYPIC ASSESSMENT

This section presents the results of a functional analysis of *TEL1* and *TEL2* genes in *Arabidopsis* by assessing knockout alleles. It includes the identification of knockout alleles and preliminary phenotype assessment, root experiments, pollen viability assessment, and telomere maintenance evaluation of both *tel1* and *tel2* single mutants and the double mutant. Results of putative multiple mutants of the *tel* and the *aml* genes are also presented. Previous *Arabidopsis in situ* hybridisation patterns have shown that *MEI2*-like gene expression is in the RAM and SAM, in floral buds, in pollen and in embryos (Anderson et al., 2002). Knocking out *TE1* in maize results in plants of short stature with irregular internodes and altered phyllotaxy (Veit et al., 1998). To determine whether knocking out *MEI2*-like genes in *Arabidopsis* has a similar effect on architecture or results in other phenotypic changes, plants were obtained containing T-DNA inserts within six *MEI2*-like genes (*tel1*, *tel2*, *aml1*, *aml4*, *mct1* and *mct2*). See section 3.1.6 and 3.1.7 for further discussion on the *AML* and *MCT* alleles used in this research.

3.1.1 Procurement of knockout alleles

A putative knockout allele of *tel1* (SM_3.41081, Columbia ecotype) and a putative knockout allele of *tel2* (SM_3.717, Columbia ecotype) were identified as being available from the Arabidopsis Biological Resource Centre (ABRC) from which seeds were obtained. A putative knockout allele of *tel1* (salk_089810, Columbia ecotype) was also identified as being available from the Nottingham Arabidopsis Stock Centre (NASC) and seeds were obtained. Another putative allele of *tel2* (Wisconsin Alpha, Wassilewskija ecotype) was identified as being available from the Wisconsin Stock centre from which seeds were obtained. The seeds obtained from these stock centres are now available from a central location, TAIR (The Arabidopsis Information Resource, http://www.arabidopsis.org/). The lines obtained (SM_3.41081, SM_3.717, salk_089810 and Wisconsin Alpha) were at least three generations or

more from the initial transformation (T3 or greater) (The Arabidopsis Information Resource, http://www.arabidopsis.org/).

The identification of the point of insertion into the genome was determined by alignment of the flanking sequence with the known sequence from the TAIR *Arabidopsis thaliana* database. The genomic sequences shown (Table 8) are the sequences occurring at the 3' end of the T-DNA insertion. The first base (indicated in bold) is the predicted point of insertion. If the insertion is present, the base in bold will be at the 5' end of the T-DNA insertion and the remaining bases will be at the 3' end of the T-DNA insertion.

Table 8: T-DNA insert sequence.

Knockout line	**Genomic DNA sequence**
SM_3.41081 (*TEL1*)	**G**TCTCTTCCATTACTCTCAG
Wisconsin Alpha (*TEL2*)	**C**CCCTCCTTAATTCCGACAA
Salk_089810 (*TEL1*)	**A**TTCTTCTTATGATTTCGTG
N56613 (*TEL2*)	**A**CGGCTCTGGTTGGTGTTAC
Salk_015088 (AML1)	**C**TTAATGAACGAAGACAAA
Salk_019467 (AML4)	**T**GATTCTTCTATATCCAATG
Salk_075516 (MCT1)	**A**CAAAGCTTATCTTCTCAAA
Salk_082209 (MCT2)	**G**AACTTGAGATTACTTATGC

The first base (in bold) is the last base of genomic DNA.

3.1.2 Identification of knockout alleles

The two putative knockout alleles of *tel1* (SM_3.41081 and salk_089810) and two of *tel2* (SM_3.717 and Wisconsin Alpha) were checked by PCR for the presence of the T-DNA insert. Those plants with a band corresponding to the T-DNA insert and no band corresponding to wild type were propagated to ensure the use of homozygous plants.

The DNA extracted using the quick DNA extraction protocol was used as a template for Polymerase Chain Reaction (PCR) to check for the presence of the T-DNA insert in the lines salk_089810, SM_3.41081, Wisconsin Alpha and SM_3.717. Three

primers were designed (with the help of the computer Vector NTI Suite 7) for each putative T-DNA insert line (Table 9). The forward and reverse primers were designed to give a band when there was no T-DNA present. The forward and T-DNA primers were designed to give a band when T-DNA was present (as outlined in Figure 3-1). To allow the wild type primers and the knockout primers to be used in the one PCR reaction, primers were designed based on the predicted point of insertion to give wild type and insertion products of different sizes (Table 10).

Table 9: Primer sequences used for the amplification of either the wild type genomic DNA sequence or the T-DNA insert sequence from the target gene, as indicated. Primers are written 5' to 3'.

Primer name	**Primer sequence**
Salk_089810 (*TEL1*) forward	TTCACATGGATTCGTGTGCTA
Salk_089810 (*TEL1*) reverse	CGATGCTCGATTACTTGAAATC
Salk_089810 (*TEL1*) T-DNA	GCGTGGACCGCTTGCTGCAACT
SM_3.41081 (*TEL1*) forward	GTCAAATACTCCGACGCGATCTCTCTCTT
SM_3.41081 (*TEL1*) reverse	CCGTAAACTTGGAAAATCTGTCTGAGAGT
SM_3.41081 (*TEL1*) T-DNA	TGGGAAAACCTGGCGTTACCCAACTTAAT
Wisconsin Alpha (*TEL2*) forward	GACGAAATAGTACGATCACTGA
Wisconsin Alpha (*TEL2*) reverse	GGAGGAAGAATCTTGTCGGAAT
Wisconsin Alpha (*TEL2*) T-DNA	CATTTTATAATAACGCTGCGGACATCTAC
SM_3.717 (*TEL2*) forward	GTCCATAGTCATTACCTGTCACCGGAAAA
SM_3.717 (*TEL2*) reverse	ACAGCATTGAGTTGAGGAAACACGAAATG
SM_3.717 (*TEL2*) T-DNA	CTTATTTCAGTAAGAGTGTGGGGTTTTGG

The two inserts in *TEL1* were spatially distinct from each other. Salk_089810 is in RRM3 and within exon 3, SM_3.41081 is in exon 1 (Figure 3-1). The two inserts in *TEL2* were in close proximity to each other in exon 1 (Figure 3-1). However, the second insert (Wisconsin Alpha) contained a complex rearrangement involving exon 1, exon 5 and the 3' downstream region (Figure 3-2). This complex rearrangement was identified by two previous lab members (Vernon Trainor and Rob Baker, personal communication) and confirmed and characterised during the course of this research by analysing in detail the sequence information provided by Vernon Trainor

(personal communication) and by PCR amplification of the T-DNA. *TEL2* DNA corresponding to RRM3 could not be amplified by PCR suggesting that the TEL2 gene was knocked out.

There is no evidence in the literature to suggest that T-DNA inserts within an exon can be cleanly removed from mRNA transcripts by the cellular machinery; alleles with T-DNA inserts located within an exon are likely to be non-functional. The generally accepted view of gene functionality is that introns and exons are transcribed into mRNA and then the introns are spliced out. A break in exon DNA by the addition of a large portion of extra DNA (a T-DNA insert) cannot be transcribed and thus the function of the gene can be considered to be impaired. In addition, as the sequence corresponding to RRM3 is considered to confer functionality (Watanabe and Yamamoto, 1994; Watanabe et al., 1997), a T-DNA insert located within this sequence is more than likely to be non-functional. As indicated, Figure 3-1 denotes the location of the T-DNA inserts, relative to the position of the RRM's and exons, while Figure 3-2 shows the nature of the complex rearrangement in the Wisconsin allele.

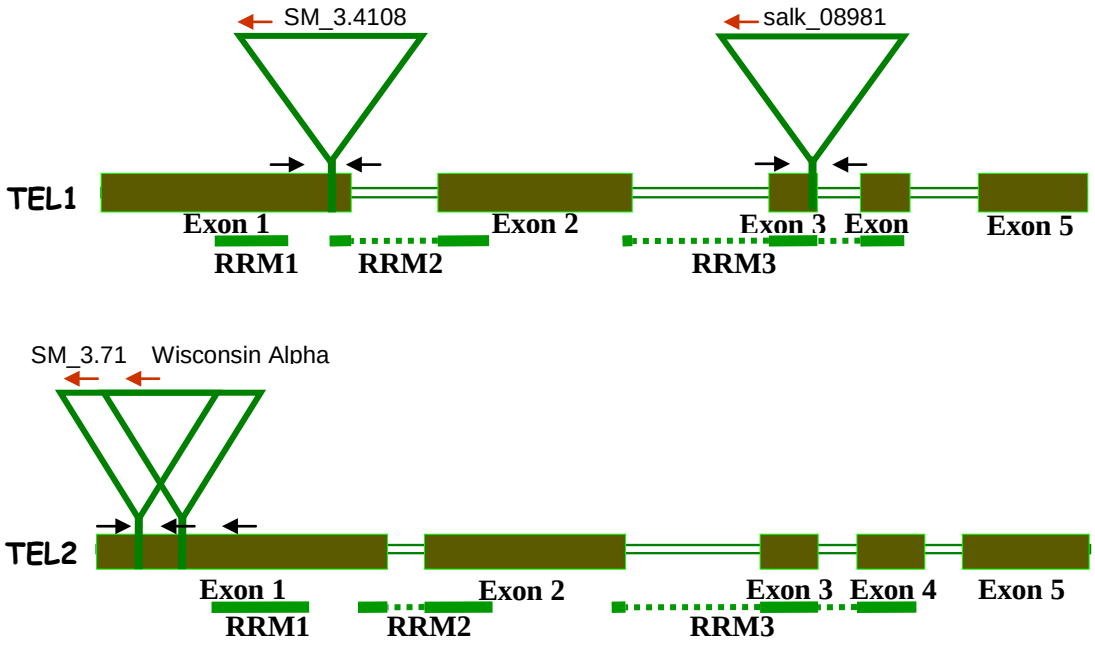


Figure 3-1: Location of the T-DNA insertion sites in *TEL1* and *TEL2*. Triangles represent the T-DNA insertion location, double lines represent introns, coloured boxes represent exons and green lines under the gene show the location of the RNA recognition motifs (RRM's). The dotted green lines within the RRM's correspond to introns spliced out during transcription. Black arrows indicate primers used to amplify genomic DNA and red arrows indicate primers located within the T-DNA. Arrows pointing left to right are forward primers and are used in conjunction with the T-DNA insert primers to amplify a section of the T-DNA insert. The forward primers in conjunction with reverse primers amplify wild type DNA. See Table 9 for primers.

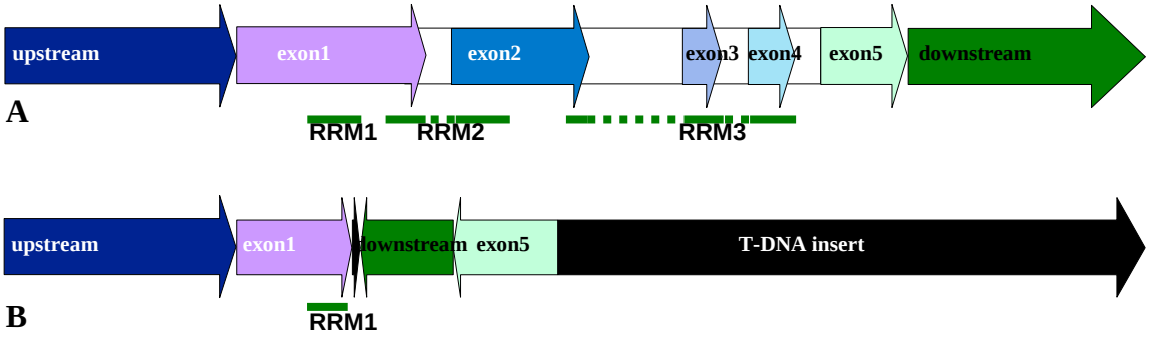


Figure 3-2: Schematic overview of *TEL2* and *tel2*. A: uninterrupted gene, B: interrupted gene. Arrows indicate the direction the DNA is read. The small black arrow between exon1 and the 3'downstream region in B is extra DNA that does not match any *TEL2* sequence.

Table 10: Expected product sizes for the amplified *TEL1* and *TEL2* alleles used in this research to create the *tel1tel2* double mutant.

primer combination	expected product size
Salk_089810 (*TEL1*) forward and T-DNA insert	582 base pairs
Salk_089810 (*TEL1*) forward and reverse	1000 base pairs
Wisconsin Alpha (*TEL2*) forward and T-DNA insert	355 base pairs
Wisconsin Alpha (*TEL2*) forward and reverse	1190 base pairs

To ensure the use of plants homozygous for the T-DNA inserts, those plants with a band corresponding to the T-DNA insert and no band corresponding to wild type were propagated and used to create the *tel1tel2* double mutant.

3.1.3 Preliminary phenotype assessment

A cross was made between homozygous *tel1* (SM_3.41081) and homozygous *tel2* (Wisconsin Alpha) mutant plants and the progeny visually screened for an altered phenotype. A population of 5233 *tel1* (SM_3.41081) *tel2* (Wisconsin Alpha) (*tel1tel2*) seeds was sown. Of the 5068 germinated seeds, 58 (1.14 %) were disorganised with distorted leaves and an irregular phyllotaxy. Based on Mendalian genetics, the population can be expected to contain homozygous *tel1* and *tel2* T-DNA inserts at ratio of 1/16 or six percent. As the percentage of the population exhibiting a disorganised appearance was lower than expected, a similar population of Columbia (Col) and Wassilewskija (Ws**)** wild type seeds were sown and assessed to rule out the possibility that naturally occurring mutations in the wild type population could account for the altered growth pattern at a frequency of one percent of plants. Of the 987 Col seeds and the 618 Ws seeds that germinated, 3.14 % and 6.15 % respectively had a similar disorganised appearance. Therefore from the genotype data and the population assessment it was concluded the altered growth pattern could not be attributed to the presence of T-DNA inserts.

During the assessment of a repeat *tel1* (SM_3.41081, Col ecotype) and *tel2* (Wisconsin Alpha, Ws ecotype) cross, an altered phenotype (Figure 3-3) appeared in the population at a frequency similar to the expected six percent (16 out of 300 plants) and could not be attributed to mutation events in a wild type population. These plants

had a phenotype similar to *gnom* mutants (Mayer et al., 1993) and population genotyping revealed the plants were not carrying the T-DNA inserts (data not shown). The parents were crossed with *gnom* plants and the resulting progeny assessed. The plants, based on their phenotypic characteristics and the segregating ratios of the offspring, were identified as *gnom* contaminants and were discarded.

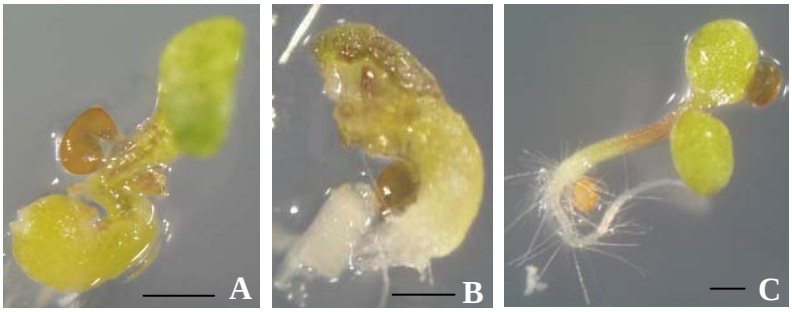


Figure 3-3: *gnom* mutants. Plants A and B are *gnom* mutants, plant C is wild type. Scale bar = 0.5mm

The progeny from the cross between *tel1* (SM_3.41081) and *tel2* (Wisconsin Alpha) were allowed to self and were genotyped to identify plants homozygous for both *tel1* and *tel2*. The resulting double homozygous plants had a wild type appearance in the vegetative portion of the plant, but the roots of the plant was not analysed. Given the expression pattern of *TEL1* and *TEL2* in the RAM and SAM, additional progeny were planted to determine if an altered phenotype was apparent in the basal portion of the plant instead of the apical portion.

3.1.4 Root experiments

TEL1 and *TEL2* genes are expressed in root, vegetative, and floral meristems and in the pollen. Thus a phenotype might be visualised in root structures and a series of root experiments were conducted to see if there was any difference in the root structure, length or phyllotaxy. Twenty six plants homozygous for *tel1* (salk_089810), *tel1* (SM_3.41081), *tel2* (Wisconsin Alpha) and *tel2* (SM_3.717), and 26 plants homozygous for both *tel1* (SM_3.41081) and *tel2* (Wisconsin Alpha) were sown, interspersed with appropriate wild type seeds, (i.e. *tel1* (salk_089810) was interspersed with Col and the other crosses with Ws). Knock out seedlings were alternated with wild type seedlings on the same MS medium plates as cousins to eliminate plate or replicate effects. Seedlings on plates were scored 10 days after

germination for differences in root length and number of lateral roots (Table 11). There was no difference between plants homozygous for *tel1tel2*, plants homozygous for one T-DNA insert or wild type plants. For each treatment, two sample t- tests were used to compare *TEL1TEL2* wild type plants with *tel1tel2* cousins for differences in root length and number of lateral roots (Table 11). The two sample t-tests indicate there was no difference between the two genotypes.

Table 11: Average root length and average number of lateral roots of *tel1* and *tel2* single mutant plants compared with their wild type cousins and *tel1tel2* double mutants compared with their wild type cousins.

plant genotype	root length (cm) (average)	t-test score	lateral roots #(average)	t-test score	number of plants assessed
tel2 SM_3.717 (backcross #3)	6.24	t= 0.16 p=0.872	11.37	t= 0.16 p=0.872	46
Ws wild type	7.43		13.53		19
tel1 SM_3.41081 (backcross #3)	4.62	t= 0.84 p=0.403	14.46	t= 0.99 p=0.838	53
Ws wild type	5.44		16.77		13
tel1tel2 (SM_3.41081 Wisconsin Alpha)	5.34	t= 0.22 p=0.825	5.60	t= 2.12 p=0.024	16
Col wild type	4.92		2.00		4
tel1tel2 (salk_089810 Wisconsin Alpha)	4.78	t= 0.55 p=0.589	5.23	t= 1.6 p=0.936	26
Ws wild type	4.34		9.29		14

Data is in pairs, with each pair comprising a knock out population and a wild type population. Knock out plants were interspersed on the same plates with wild type. Two sample t-test probabilities are presented alongside the average measurement column.

3.1.5 Pollen viability and telomere maintenance

The apparent lack of an altered phenotype similar to that seen with the maize *MEI2*-like gene equivalent suggested that either gene function may be different in *Arabidopsis*, and /or that the gene function may be similar to what was observed with *aml1aml4* double mutant plants (Kaur et al., 2006) and / or that an altered phenotype would be apparent in the gametes. Pollen viability could indicate that *TEL* gene function is important in the transmission of heritable material or during gametogenesis. Pollen was assessed for viability using the Alexander stain (Alexander, 1969, 1980) to determine if there was likely to be differences in

transmission of heritable material. The Alexander stain is a vital stain and indicates whether pollen is viable or not. Pollen collected from *tel1tel2* (SM_3.41081 Wisconsin Alpha), *tel1* (salk_089810), *tel2* (SM_3.717), *tel1* (SM_3.41081), and *tel2* (Wisconsin Alpha) showed no change in viability. Five flowers from each plant were selected and stained with Alexander stain. In each case 95-100 % of the pollen stained red indicating viability.

An alternative explanation for a lack of observable phenotypic change is if *TEL1* and *TEL2* are required to maintain chromosome stability during meiosis or maintain telomere length during growth. An altered phenotype may only become apparent over successive generations of allowing plants mutant for *tel1*, *tel2* or *tel1tel2* to self. On the basis of work on the Mre protein complex (comprising of Mre11, Rad50 and Nbs1) which showed that the Mre protein complex is important in repairing double stranded DNA breaks and aligning chromosome domains during meiosis (Puizina et al., 2004) the possibility exists that *tel1* and *tel2* plants could have a subtly altered phenotype attributable to unstable DNA or RNA. Alternatively telomere length and stability can also have a profound effect on plant morphology over selective generations; as the telomeres shorten and are not maintained the effects become more pronounced after increasing number of generations assessed (Tremousaygue et al., 1999). To ascertain whether *TEL* genes are important in maintaining chromosome or telomere stability, plants homozygous for either *tel1* (SM_3.41081) or *tel2* (Wisconsin Alpha) were allowed to self for seven generations. At each generation plants were observed for changes in inflorescence and leaf size. Seeds were collected just prior to silique dehiscence, allowed to dry before being sown in pots in the glasshouse. Seed collection and seed harvesting was conducted seven times. Seeds from the original parent plant and seeds from generation seven plants were sown in pots in the glasshouse and observed. No morphological changes in appearance from the parent plants could be observed suggesting that *TEL* genes are not important in telomere maintenance or chromosome maintenance (data not shown).

3.1.6 Putative multiple mutants

Given there are nine identified members of the *MEI2*-like gene family in *Arabidopsis* some redundancy in function might be expected. To determine if the function of the *MEI2*-like genes depends on having multiple members of the family rendered non-functional, the *tel1tel2* (SM_3.41081 Wisconsin Alpha) double mutant was also crossed to the *aml1aml3aml5* triple and the *aml1aml3aml4aml5* quadruple T-DNA insertion mutants (kindly supplied by Garret Anderson, Cornell University).

An examination of the location of the *MEI2*-like genes on the *Arabidopsis* chromosomes revealed an even distribution of the genes across the chromosomes (Figure 3-4). Only *AML4* and *MCT2* appear likely to segregate together in crosses because of their proximity to each other. *MCT1* and *AML5* may also segregate together. The approach of crossing homozygous *tel1tel2* mutants with the *aml1aml3aml5* triple and the *aml1aml3aml4aml5* quadruple T-DNA insertion mutants was therefore going to require a large population of F2 plants to be screened to provide an adequate likelihood of obtaining a plant that contained five (1 in 1024 plants) or six (1 in 4096) genes knocked out.

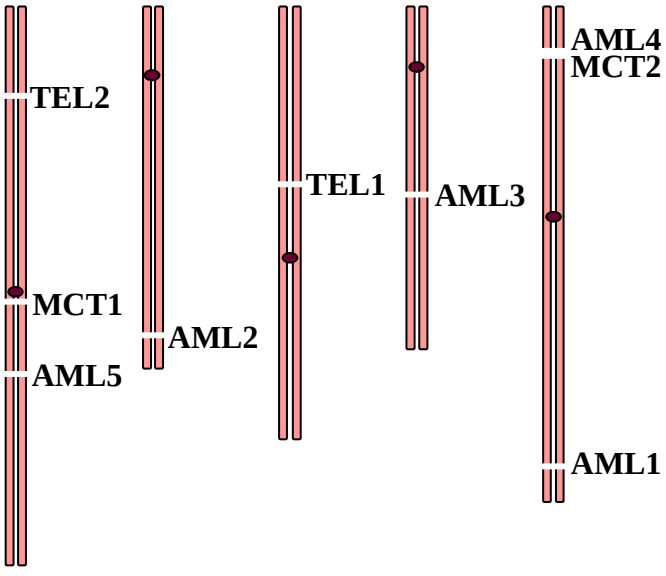


Figure 3-4: Schematic of the five *Arabidopsis* chromosomes. The locations of the MEI2-like genes are displayed.

To ensure the material used for the crosses was of the right genotype the *aml1aml3aml5* triple and the *aml1aml3aml4aml5* quadruple T-DNA insertion material (the F1 population) was assumed to be heterozygous for the inserts and the plants were allowed to self before any phenotyping or genotyping was carried out. The F2 material was then checked by PCR and plants identified as having T-DNA inserts in all the genes of interest were used as a parent plant for crossing to the *tel1tel2* double mutant plants. Given *aml1aml4* mutants may have unviable pollen (Kaur et al., 2006), *tel1tel2* plants were used as the paternal parent. Once the F2 material from these crosses (*aml1aml3aml5* x *tel1tel2* and *aml1aml3aml4aml5* x *tel1tel2*) was obtained the progeny were visually screened for abnormal plants on the chance a phenotype may be apparent in the heterozygous state. As expected, no plants appeared to differ from their wild type cousins and the plants were allowed to self, the seeds were harvested and sown to MS medium so that all seeds could be assessed. Of the 10 000 seeds sown to MS medium and visually assessed three seedlings looked different from the parents and these were genotyped. These three seedlings were shown to be at best heterozygous for some of the alleles. A representative proportion of the remainder of the population was screened. None of the plants screened were homozygous for the inserts (data not shown). The possibility remained therefore, that no plants in the population were double knockout due to pollen viability issues or that the alleles used were not true knockout alleles.

Given that *tel1* and *tel2* genes are predicted to have a significant phenotypic change (based on *te1* and *pla2* studies) (Veit et al., 1998; Kawakatsu et al., 2006) it is possible the alleles were not null. Watanabe et al. (1997) ascertained that in yeast, RRM3 was essential for the gene to function and an intact RRM3 was sufficient to confer functionality. With this knowledge, subsequent analyses shifted to a different *tel1* insertion allele (salk_089810), in which a T-DNA insert occurred in an exon encoding part of the RRM3 domain. However analyses continued to use the *tel2* (Wisconsin Alpha) allele, as its complex rearrangement effectively disrupts the exon encoding RRM3. This complex rearrangement resulted in the exon1 sequence containing the T-DNA insert sequence, and a reverse portion of exon5 and 3'UTR (3' untranslated region) sequence. To confirm that gene functionality was impaired, PCR was performed on *tel1* and *tel2* genomic DNA to amplify the region corresponding to

RRM3. The sequences could not be amplified confirming that gene functionality was likely to be impaired.

3.1.7 Identification of knockout alleles

The alleles used in this study are schematically represented in Figure 3-5 and can be considered null alleles due to the nature of the T-DNA insertions. Where T-DNA inserts are in RRM3, (the RRM considered to provide functionality), *tel1*, *mct2* and *aml1* genes are considered to be null on the basis of a disrupted RRM3. As described previously, *tel2* contains a complex rearrangement and is considered to be null on the basis that a large chunk of the DNA cannot be amplified and the orientation of the DNA that can be amplified is reversed. The *aml4* allele, with the T-DNA insert in RRM2, is the same allele used in studies looking at meiotic defects of *MEI2*-like genes (Kaur et al., 2006) and has been identified previously as being null. As only one allele is available from the *Arabidopsis* stock centre for *mct2*, there was no choice and this is the allele that was used.

An analysis *in silico* of all available TAIR stock centre putative *AML1, AML4, MCT1,* and *MCT2* knockout lines was used to determine which alleles would be more suitable for creating a multiple *MEI2*-like gene knockout plant on the basis of the location of T-DNA inserts within the gene (Figure 3-5). The presence *in silico* of T-DNA in *mct1, mct2, aml1, aml4* sequence that codes for RRM3 should correspond to a lack of function of that gene. Where T-DNA inserts were not available in the sequence coding for RRM3, the allele was selected based on T-DNA proximity to RRM3. Primers were designed (with the help of the Vector NTi suite 7 programme) to all T-DNA insert lines (Table 12) and confirmed by PCR. Those plants that had PCR products that were of the correct size (Table 13) were harvested for seed.

Table 12: Primer sequences used for the amplification of either the wild type genomic DNA sequence or the T-DNA insert sequence from the target gene, as indicated. Primers are written 5' to 3'.

Primer name	Primer sequence
Salk_015088 (*AML1*) forward	GGGAGGACAGGAGGACAACATTGA
Salk_015088 (*AML1*) reverse	CACCCCCCGATTTACAGAACTCTAGG
Salk_015088 (*AML1*)T-DNA	GCGTGGACCGCTTGCTGCAACT
Salk_019467 (*AML4*) forward	ACCTGCGTTTCTGGTTACAG
Salk_019467 (*AML4*) reverse	AACCGTGTGCACTATCTTACCCATGT
Salk_019467 (*AML4*)T-DNA	GCGTGGACCGCTTGCTGCAACT
Salk_075516 (*MCT1*) forward	AAGAAACACTACAAATGGTAGTCAGT
Salk_075516 (*MCT1*) reverse	GATACCATGATAAAGCCACGA
Salk_075516 (*MCT1*)T-DNA	GCGTGGACCGCTTGCTGCAACT
Salk_082209 (*MCT2*) forward	TCGCAATATACCGAACCGATAC
Salk_082209 (*MCT2*) reverse	CATTGCATTTACCGACCATTG
Salk_082209 (*MCT2*) T-DNA	GCGTGGACCGCTTGCTGCAACT

Table 13: Expected product sizes for *AML1, AML4, MCT1* and *MCT2* alleles from *Arabidopsis* genomic DNA.

primer combination	expected product size
Salk_015088 (*AML1*) forward and T-DNA insert	700 base pairs
Salk_015088 (*AML1*) forward and reverse	1150 base pairs
Salk_019467 (*AML4*) forward and T-DNA insert	400 base pairs
Salk_019467 (*AML4*) forward and reverse	920 base pairs
Salk_075516 (*MCT1*) forward and T-DNA insert	600 base pairs
Salk_075516 (*MCT1*) forward and reverse	800 base pairs
Salk_082209 (*MCT2*) forward and T-DNA insert	640 base pairs
Salk_082209 (*MCT2*) forward and reverse	900 base pairs

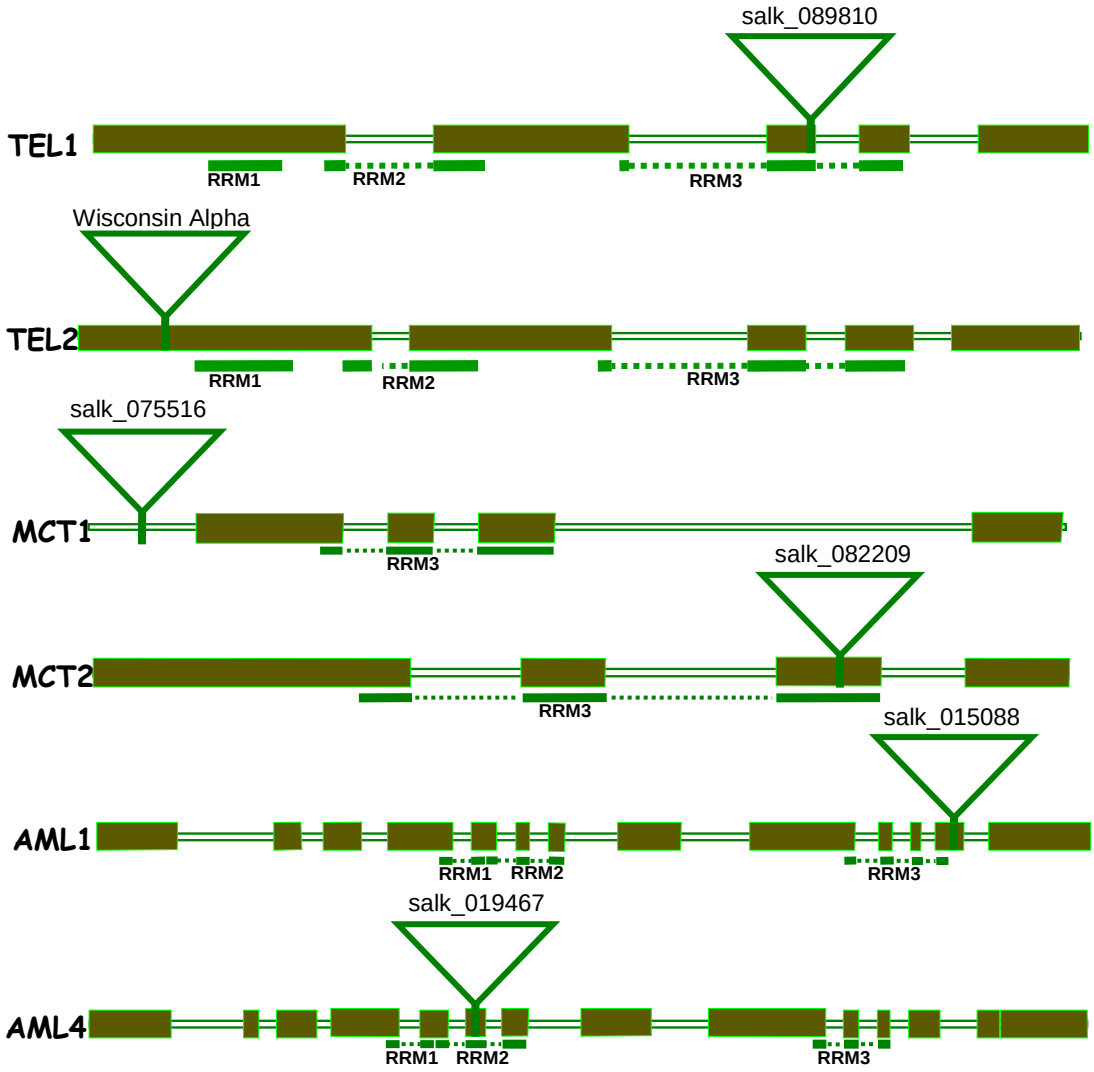


Figure 3-5: The location of the T-DNA insertion sites used in this research. Triangles represent the location of T-DNA inserts, solid blocks are exons and double lines are introns. The RRMs are indicated by solid green lines. *tel1*, *mct2* and *aml1* genes have T-DNA inserts in RRM3 (the RRM considered to provide functionality) and are therefore considered to be null. *tel2* contains the complex rearrangement (as described previously) and is considered to be null. The *aml4* allele, with the T-DNA insert in RRM2, is the same allele as used in studies looking at meiotic defects of *MEI2*-like genes (Kaur et al., 2006) and therefore can also be considered null.

For the remainder of this dissertation, the alleles used are referred to by their gene name eg *tel1* (salk_089810) will be referred to as *tel1*, and *tel2* (Wisconsin Alpha) will be referred to as *tel2*. The *tel2* allele was introgressed into Columbia five times to provide a uniform background which would be conducive to detecting small changes, should the altered phenotype be subtle. The *tel1* allele was backcrossed three times with Columbia to remove any unlinked T-DNA's. *mct1* and *mct2* lines and *aml1* and *aml4* lines were crossed to create the double mutants in preparation to cross to *tel1tel2* mutant lines in the event that functional redundancy within the gene family meant more than two members had to be knocked out before an effect was

observed. Plants homozygous for either a*ml1* or *aml4* were crossed with each other, allowed to self and the F2 progeny harvested as for the *aml1aml4* double mutant. Plants homozygous for either *mct1* or *mct2* were crossed with each other, allowed to self and the resulting F2 progeny harvested as for the *mct1mct2* double mutant. Progeny of these crosses were harvested but not assessed for double mutant plants. This material is not discussed further as time constraints dictated this material could not be assessed or used in subsequent crosses during the course of this project. However, this material is available for future work to determine if these genes are functionally redundant.

3.1.8 Phenotypic analysis of the *TEL1* single mutant

A population of 23 plants segregating 3:1 for homozygous *tel1* T-DNA allele were screened for altered phenotype. There was no apparent difference between siblings in leaf number, rosette number, inflorescence branching and root mass (Figure 3-6).

Figure 3-6: Phenotypic analysis of single mutants. Pairs of plants assessed for leaf number, rosette number, inflorescence branching and root mass. Plants on the left of each pair are *TEL1* wild type plants. Plants on the right of each pair are *tel1* knockout plants. The wild type *TEL1* plant in the middle pair had an additional rosette and this is displayed alongside the primary rosette, between the two plants.

3.1.9 Multiple mutants

Backcrossed homozygous *tel1* and introgressed homozygous *tel2* plants were crossed with each other to make the *tel1tel2* double mutants.

Primers were used to screen for the presence of T-DNA inserts (Table 14). A primer that should amplify all DNA samples was included as a template control (internal genomic primers).

Table 14: Primers used to check for the presence or absence of T-DNA inserts. Primer combinations are listed together and are written 5'to 3'.

DNA sequence	Primer name	Sequence 5' to 3'	Expected size
tel1 T-DNA insert	pROK2 (LBb1)	GCGTGGACCGCTTGCTGCAACT	292 base pairs
	SLa 36 tel1wtr	CGATGCTCGATTACTTGAAATC	
TEL1 wild type	SLa 39 tel1wtf	TTCACATGGATTCGTGTGCTA	380 base pairs
	SLa 36 tel1wtr	CGATGCTCGATTACTTGAAATC	
tel2 T-DNA insert	JL202	CATTTTATAATAACGCTGCGGACATCTAC	355 base pairs
	TEL2K5	CAGTCACCCAACAAACCTTAACCCAACAG	
TEL2 wild type	SLa 37 tel2wtr	GGAGGAAGAATCTTGTCGGAAT	203 base pairs
	SLa 38 tel2wtf	GACGAAATAGTACGATCACTGA	
Internal genomic	SLa 35 wtf	GCTACTGTTGGATATGTTGGAC	146 base pairs
	SLa 36 wtr	CGATGCTCGATTACTTGAAATC	

Parents were selected on the basis of the presence of a PCR band that was amplified using a T-DNA insertion primer and a primer located within the gene of interest. The F1 double heterozygous offspring were allowed to self. F2 plants were screened using PCR to identify plants homozygous for one T-DNA insertion. These plants were allowed to self, and were checked by PCR (Figure 3-7). A representative *tel1* knockout plant (Figure 3-7, lane D) and a representative *tel2* knockout plant (Figure 3-7, lane L) were selected as parent plants for crossing together to make the double *tel1tel2* mutant.

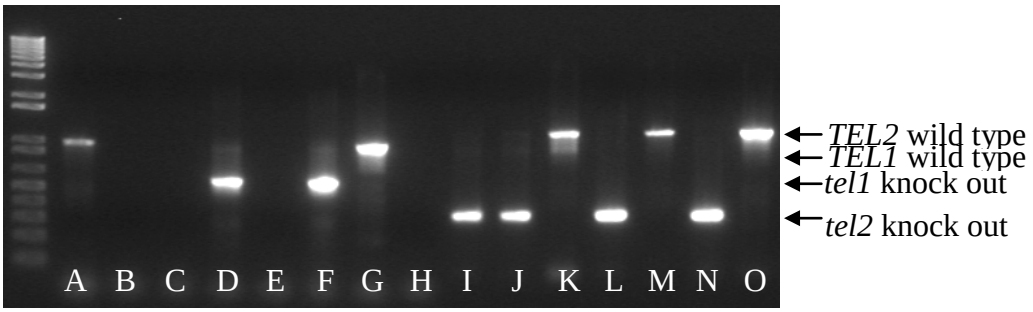


Figure 3-7: Parent plant PCR. Lanes A, B, C, D, and E contain DNA from plants expected to be *tel1* knock out plants. F contains DNA from a previously identified *tel1* knockout plant, G contains DNA from a Columbia wild type plant, H is the water control and contains no DNA. I, J, K, L and M contain DNA from plants expected to be *tel2* knock out plants, N contains DNA from a previously identified *tel2* knock out plant, O contains DNA from a Columbia wild type plant. Lanes A and G contain bands that correspond to wild type *TEL1* DNA. E and F contain bands corresponding to *tel1* knock out DNA. Lanes I, J, L and N contain bands corresponding to *tel2* knock out DNA. Lanes K and M contains bands corresponding to *TEL2* wild type DNA. Lane O contains a band corresponding to Columbia wild type DNA. Ladder is 1Kb Plus DNA.

These single mutant parent plants (D and L) were crossed both ways: i.e. both plants were used as paternal and maternal parents in case one plant was a better maternal or paternal parent than the other. A selection of the progeny was screened by PCR to confirm double knockout plants were obtained (Figure 3-8).

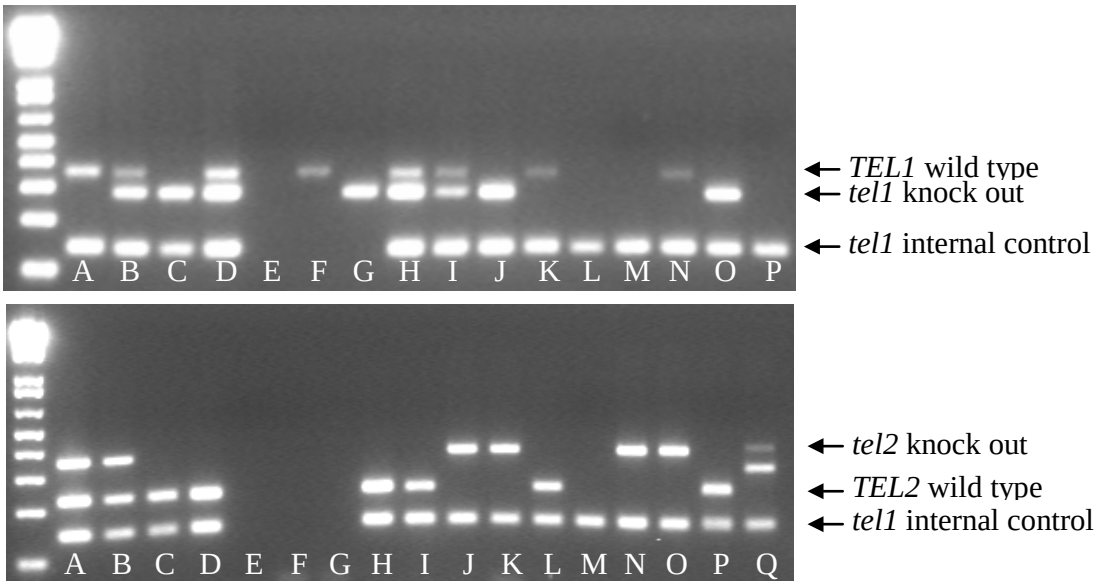


Figure 3-8: *tel1 tel2* progeny PCR. PCR on a subset of the progeny from *tel1* and *tel2* (D and L above) parent plants. Top gel shows amplification of *tel1* DNA and bottom gel shows amplification of *tel2* DNA. Lane E is water control. The extra band in lane Q in the bottom gel is *TEL1* wild type DNA loaded as a control. Lanes J and O represent homozygous *tel1tel2* knock out T-DNA insert plants. Lanes with incomplete compliment of band (missing either *tel1* and *TEL1* DNA or *tel2* and *TEL2* DNA) indicate that some DNA was unable to be amplified using these primer combinations. Ladder is 1Kb Plus DNA.

Representative plants (Figure 3-8, lanes J and O) were confirmed as being double knockout plants on the basis of the presence of PCR bands and were used as parent plants for subsequent analyses that required *tel1tel2* double knockout plants.

3.1.10 Phenotype of *tel1tel2* double mutant plants

From the *tel1tel2* F1 heterozygous double mutant population of 93 plants, *tel1tel2*, *tel1TEL2*, *TEL1tel2*, and their wild type *TEL1TEL2* siblings were genotyped by PCR. Plants in each genotypic class are therefore cousins of each other. The genotyping of these 93 cousins allowed observations on the transmission of heritable material to be made. The observed ratios of inheritance followed the expected Mendelian pattern where co-dominant segregation of alleles occurs (Table 15). Transmission of heritable traits was analysed using a Chi-squared test and shown to follow the predicted pattern for co-dominant alleles at the 5% level. The P value (0.125) indicates the predicted pattern is the correct pattern but that there is a suggestion of a deviation from this pattern (Table 15). The Aabb and AABb genotype classes are different than expected (four Aabb observed instead of the predicted 11.63 (2/16) and 18 AABb observed instead of the predicted 11.63) but still within range for the overall Chi-squared test (12.63).

Table 15: Chi-squared test of *tel1tel2* double knockout plants.

progeny class	expected	observed	Chi-Square statistic
AABB	5.81	8	0.82
aabb	5.81	3	1.36
AAbb	5.81	7	0.24
aaBB	5.81	5	0.11
aaBb	11.63	15	0.98
Aabb	11.63	4	5.00
AaBB	11.63	13	0.16
AABb	11.63	18	3.50
AaBb	23.25	20	0.45
Chi-Square statistic			12.63
P-value:			0.125

A close look at the population of 93 plants in this segregating population revealed no differences in dry weight or numbers of leaves between individuals that could be

attributed to the genotype class of that individual (data not shown). The aabb genotype class was assessed and compared with the AABB class as it was expected that a change in phenotype would only be observed in the double knockout class. However as the number of plants in each class was small (aabb = 3 and AABB = 8) meaningful comparisons could not be made and further experiments were required to determine if the lack of an observable change in phenotype was a consistent result.

Given the lack of an observable change in phenotype from the wild type progeny class to the double mutant progeny class, plants were grown under a range of environmental conditions in the anticipation that differences between the classes would be exaggerated and be able to be identified. Two plants were selected from the 93 plants genotyped (outlined above) on the basis of their genotype and the quantity of seed they produced. The seeds from these plants were sown for assessment. The plates for each treatment were divided in half and each half plated with either the *tel1tel2* genotype or the *TEL1TEL2* cousins to allow comparisons to be made between the two genotypes without having to account for variation between MS medium plates. Plants were assessed 10 days after sowing to MS plates for leaf number, root length, root number and plant height (hypocotyl base to apical point and included any inflorescences that formed). Since the data presented is not normally distributed but assumed to come from a binomial distribution, where direct comparison between measurements was not possible, the measurements are given on a transformed scale (logit transformation was used). The means back-transformed are italicised in brackets where relevant. Since the differences between *tel1tel2* and *TEL1TEL2* plants are expected to be subtle, they are described below in terms of whether they are statistically significant differences or not.

3.1.10.1 Comparison between progeny classes

Plants were grown on MS medium with different concentrations of mannitol and sucrose to place the plants under osmotic stress, and in the case of 0 % (w/v) mannitol/sucrose to remove the carbon source. The standard MS medium for growing *Arabidopsis* is 2 % (w/v) sucrose. Two progeny classes (60 seeds of each) were sown to MS medium containing 0 % (w/v) mannitol and sucrose, 2 % (w/v) mannitol, 5 %

(w/v) mannitol, 2 % (w/v) sucrose (standard medium used), or 5 % (w/v) sucrose. Ten plates were planted with six seeds of each progeny type, giving a total of 60 seeds per treatment. Plants were grown in long day conditions (16 hour day, eight hour night at 22 ^{o}C) and then assessed as described previously. When plants were grown on medium with 0 % (v/v) mannitol/sucrose *tel1tel2* had significantly more leaves than *TEL1TEL2* (3.08 ± standard error (S.E.) 0.23 compared with 2.45 ± S.E.0.23) and on the 2 % (w/v) sucrose (control) treatments, *tel1tel2* had significantly more leaves than *TEL1TEL2* (5.19 ± S.E.0.24 compared with 4.52 ± S.E.0.24) (Table 16). Significant differences were not observed for plants grown under 2 % (w/v) mannitol, 5 % (w/v) mannitol, and 5 % (w/v) sucrose treatments. There was some indication that 2 % (w/v) sucrose (control) (1.71 ± S.E.0.08 compared with 1.55 ± S.E.0.08) and 5 % (w/v) sucrose treatments (1.81 ± S.E.0.09 compared with 1.64± S.E.0.9) resulted in longer stems for *tel1tel2* plants compared with *TEL1TEL2* plants ($p< 0.1$). In addition, *tel1tel2* plants had longer roots than *TEL1TEL2* in the 0 % (w/v) mannitol/sucrose (1.83 ± S.E.0.21 compared with 0.76 ± S.E.0.21) and 2 % (w/v) mannitol treatments (0.71 ± S.E.0.23 compared with 0.17 ± S.E.0.23 ($p< 0.05$). *tel1tel2* plants also had more roots than *TEL1TEL2* plants (0.89 ± S.E.0.26 compared with 1.47 ± S.E.0.26) when grown on 2 % (w/v) sucrose (control) treatments ($P< 0.05$). Germination was significantly different between *tel1tel2* plants and *TEL1TEL2* plants on 0 % (w/v) mannitol/sucrose (4.09 ± S.E.0.98 compared with 2.03 ± S.E.0.98), 2 % (w/v) mannitol (2.44 ± S.E.0.5 compared with 0.87 ± S.E.0.5), 5 % (w/v) mannitol (-0.54 ± S.E.0.47 compared with -2.11 ± S.E.0.47), 2 % (w/v) sucrose (3.07 ± S.E.0.59 compared with 0.50 ± S.E.0.59) and 5 % (w/v) sucrose (2.06 ± S.E.0.44 compared with 0.63 ± S.E.0.44) treatments ($p< 0.05$) (Table 16).

Table 16: Comparisons between double-mutant and wild-type plants grown on sucrose and mannitol treatments.

Number of leaves	*tel1tel2*	*TEL1TEL2*	SED	Approx. LSD	difference between means	significance
0% mannitol/sucrose	3.08	2.45	0.23	0.46	0.63	P<0.05
2% mannitol	2.44	2.15	0.25	0.49	0.28	P>0.1
5% mannitol	2	2	0.52	1.03	0	P>0.1
2% sucrose	5.19	4.52	0.24	0.48	0.67	P<0.05
5% sucrose	3.77	3.94	0.25	0.5	-0.17	P>0.1
Stem length (mm)						
0% mannitol/sucrose	1.14	1.1	0.08	0.15	0.04	P>0.1
2% mannitol	1.07	1.03	0.08	0.17	0.04	P>0.1
5% mannitol	1.1	1.02	0.2	0.4	0.08	P>0.1
2% sucrose	1.71	1.55	0.08	0.17	0.16	P<0.1
5% sucrose	1.81	1.64	0.09	0.17	0.17	P<0.1
Primary root length (mm)						
0% mannitol/sucrose	1.83 *(3.35)*	0.76 *(0.58)*	0.21	0.42	1.07	P<0.05
2% mannitol	0.71 *(0.50)*	0.17 *(0.03)*	0.23	0.45	0.54	P<0.05
5% mannitol	0.46 *(0.21)*	0.17 *(0.03)*	0.51	1.02	0.29	P>0.1
2% sucrose	4.37 *(19.11)*	4.03 *(16.25)*	0.22	0.44	0.34	P>0.1
5% sucrose	4.15 *(17.20)*	4.16 *(17.32)*	0.23	0.46	-0.02	P>0.1
Number of secondary roots						
0% mannitol/sucrose	2	2	0.99	1.98	0	P>0.1
2% mannitol	2.43	2	1.39	2.77	0.43	P>0.1
5% mannitol	2	*	*	*	*	*
2% sucrose	0.89	1.47	0.26	0.52	-0.58	P<0.05
5% sucrose	0.55	0.59	0.27	0.55	-0.04	P>0.1
Germination rate						
0% mannitol/sucrose	4.09 *(0.98)*	2.03 *(0.88)*	0.98	1.97	2.06	P<0.05
2% mannitol	2.44 *(0.92)*	0.87 *(0.70)*	0.5	1	1.57	P<0.05
5% mannitol	-0.54*(0.37)*	-2.11*(0.11)*	0.47	0.94	1.57	P<0.05
2% sucrose	3.07 *(0.96)*	0.50 *(0.62)*	0.59	1.17	2.57	P<0.05
5% sucrose	2.06 *(0.89)*	0.63 *(0.62)*	0.44	0.88	1.43	P<0.05

***tel1tel2* plants grown on MS medium with different osmotic and carbon potentials (sucrose and mannitol levels) were compared with their *TEL1TEL2* cousins. * denotes no data obtained as plant did not grow or comparisons could not be made.**

SED = Standard error of the difference between (two) means. LSD = Least significant difference between (two) means. If the difference between means was larger than the approx LSD (=2 x SED) then P< 0.05 (i.e. strong evidence of a difference between means). If the difference between means was larger than the SED then P< 0.1 (i.e weak or little evidence the means differ). If the difference between means was larger than the SED but smaller than the approx LSD then P< 0.1 (i.e. weak evidence the means differ). If the difference in means was smaller or only just larger than the SED then P> 0.1 (i.e. there is no difference between the means).

Subjecting plants to temperatures that are higher or lower than their optimal growing temperature is another way of providing a stressful growing environment. *Arabidopsis* is generally grown at 25 – 30 ^{o}C. The treatments of 4 ^{o}C and 40 ^{o}C are therefore well out of the normal temperature range for these plants. Short days increase the growing time of *Arabidopsis* and subtle differences may become apparent over a longer time period. Therefore the plants grown in short days were assessed after 20 days instead of 10 days. Two progeny classes (60 seeds of each) were sown to MS medium containing 2% (w/v) sucrose. Ten plates were planted with six seeds of each progeny type, giving a total of 60 seeds per treatment. Plants were grown in long day conditions (described previously). To temperature shock plants, plates on day six were shifted to the fridge (4 ^{o}C) for three days before being shifted back to the long day growth chamber for two days to recover. To temperature shock plants at 40 ^{o}C, plates on day six were shifted to a 40 ^{o}C incubator for five hours before returning to the long day growth chamber for two days to recover. Plants were then assessed as described previously.

In the temperature shock treatments there is some evidence ($p< 0.1$) that stem length is longer for *tel1tel2* than *TEL1TEL2* (3.01 ± S.E.0.24 compared with 2.53 ± S.E.0.24) following treatment at 4 ^{o}C (Table 17). No significant difference was observed between *TEL1TEL2* and *tel1tel2* when exposed to a 40 ^{o}C shock. In addition there is some evidence to suggest *tel1tel2* has longer roots than *TEL1TEL2* (14.90 ± S.E.1.42 compared with 11.80 ± S.E.1.42) when treated with a 4 ^{o}C (5 % level). In short day conditions, evidence (5 % level) suggests *tel1tel2* plants have more leaves (3.82 ± S.E.0.19 compared with 3.40 ± S.E.0.19) and longer roots than *TEL1TEL2* plants (18.91 ± S.E.0.97 compared with 16.82 ± S.E.0.97) (Table 17).

Table 17 Comparisons between double-mutant and wild-type plants grown under different growth room conditions.

Number of leaves	*tel1tel2*	*TEL1TEL2*	SED	Approx. LSD	difference between means	significance
4 degrees	2.71	2.7	0.22	0.45	0.02	P>0.1
40 degrees	3.53	3.33	0.22	0.44	0.2	P>0.1
LongDay	4.26	4.35	0.23	0.47	-0.09	P>0.1
ShortDay	3.82	3.4	0.19	0.38	0.42	P<0.05
Stem length (mm)						
4 degrees	3.01	2.53	0.24	0.49	0.48	P<0.1
40 degrees	2.62	2.73	0.24	0.48	-0.11	P>0.1
LongDay	0.15 *(1.16)*	0.05 *(1.05)*	0.12	0.24	0.1	P>0.1
ShortDay	1.80 *(6.02)*	1.74 *(5.70)*	0.04	0.08	0.05	P>0.1
Primary root length (mm)						
4 degrees	14.9	11.8	1.42	2.84	3.1	P<0.05
40 degrees	17.55	15.61	1.41	2.82	1.94	P>0.1
LongDay	17.58	17.6	1.26	2.52	-0.02	P>0.1
ShortDay	18.91	16.82	0.97	1.94	2.09	P<0.05
Number of secondary roots						
4 degrees	2.6	*	*	*	*	*
40 degrees	3	2.67	*	*	*	*
LongDay	-0.24 *(0.79)*	-0.75 *(0.47)*	0.44	0.88	0.51	P>0.1
ShortDay	-2.11 *(0.12)*	-1.87 *(0.15)*	0.69	1.39	-0.24	P>0.1
Germination rate						
4 degrees	2.83 *(0.94)*	2.28 *(0.91)*	0.96	1.91	0.55	P>0.1
40 degrees	2.40 *(0.92)*	1.19 *(0.77)*	0.71	1.41	1.21	P>0.1
LongDay	2.67	1.01	0.34	0.67	1.66	P<0.05
ShortDay	9.54	4.86	3.58	7.16	4.68	P>0.1

*** denotes no data obtained as plant did not grow or comparisons could not be made.**

Phytohormones can increase growth rates (Salisbury and Ross, 1992). In particular, for *Arabidopsis* GA_3 is added to MS Medium to promote inflorescence bolting. It was expected that adding GA_3 would indicate whether *tel1tel2* were regulating growth in the same manner as *TEL1TEL2* plants. In addition, a synthetic auxin (NAA), auxin transport inhibitor (NPA) and a cytokinin (zeatin) were also assessed. Two progeny classes (60 seeds of each) were sown to MS medium containing 10 µM NAA, 10 µM NPA, 10 µM GA, or 10 µM Zeatin. Ten plates were planted with six seeds of each progeny type, giving a total of 60 seeds per treatment. Plants were grown in long day conditions (16 hour day, eight hour night at 22 ^{o}C) and then assessed as described previously. *tel1tel2* plants treated with GA_3 had more leaves (4.41 ± S.E.0.34 compared with 2.99 ± S.E.0.34), longer stems (3.56 ± S.E.0.33 compared with 2.41 ± S.E.0.33), and more roots (1.19 ± S.E.0.27 compared with 0.93 ± S.E.0.27) than *TEL1TEL2* plants treated with GA_3 ($p < 0.05$) (Table 18).. The *tel1tel2* plant in the

control treatment for this experiment (no treatment) also had significantly more roots than *TEL1TEL2* plants (0.93 ± S.E.0.24 compared with 0.31 ± S.E.0.24) ($p < 0.05$) (Table 18).

Table 18: Comparisons between double-mutant and wild-type plants grown on medium containing hormones.

Number of leaves	***tel1tel2***	***TEL1TEL2***	**SED**	**Approx. LSD**	**difference between means**	**significance**
no treatment	4.48	3.9	0.31	0.61	0.58	P<0.1
10 µM GA_3	4.41	2.99	0.34	0.69	1.41	P<0.05
10 µM NAA	0.9	1.43	0.61	1.23	-0.53	P>0.1
10 µM NPA	2.44	2.43	0.38	0.76	0.01	P>0.1
10 µM Zeatin	1.87	1.93	0.33	0.66	-0.05	P>0.1
Stem length (mm)						
no treatment	1.55	1.2	0.31	0.63	0.35	P>0.1
10 µM GA_3	3.56	2.41	0.33	0.66	1.15	P<0.05
10 µM NAA	0	0	0.48	0.96	0	P>0.1
10 µM NPA	1.3	0.93	0.36	0.73	0.37	P>0.1
10 µM Zeatin	1.54	1.21	0.32	0.65	0.32	P>0.1
Primary root length (mm)						
no treatment	2.77 *(16.15)*	2.48 *(12.09)*	0.17	0.35	0.29	P>0.1
10 µM GA_3	2.54 *(12.83)*	2.32 *(10.37)*	0.2	0.4	0.22	P>0.1
10 µM NAA	-0.28 *(0.96)*	-0.24 *(0.98)*	0.37	0.74	-0.04	P>0.1
10 µM NPA	-0.14 *(1.07)*	-0.33 *(0.92)*	0.22	0.43	0.18	P>0.1
10 µM Zeatin	0.45 *(1.77)*	0.28 *(1.52)*	0.19	0.38	0.18	P>0.1
Number of secondary roots						
no treatment	0.93	0.31	0.24	0.47	0.62	P<0.05
10 µM GA3	1.19	0.64	0.27	0.53	0.55	P<0.05
10 µM NAA	0	0	0.6	1.2	0	P>0.1
10 µM NPA	0	0.27	0.36	0.72	-0.27	P>0.1
10 µM Zeatin	0	0	0.26	0.52	0	P>0.1
Germination rate						
no treatment	91.7	43.3	9.6	19.2	48.4	P<0.05
10 µM GA3	51.7	41.7	9.6	19.2	10	P<0.1
10 µM NAA	15	11.7	9.6	19.2	3.3	P<0.05
10 µM NPA	56.7	28.3	9.6	19.2	28.4	P<0.05
10 µM Zeatin	56.7	43.3	9.6	19.2	13.4	P<0.1

There is strong evidence the germination rates differ between genotypes, where *tel1tel2* has a significantly higher germination rate than *TEL1TEL2* in the no hormone treatment (91.70 ± S.E.9.60 compared with 43.30 ± S.E.9.60), NPA treatment (56.70 ± S.E.9.60 compared with 28.30 ± S.E.9.60), long day conditions (2.67 ± S.E.0.34 compared with 1.01 ± S.E.0.34) (Table 17) and all of the osmotic stress treatments ($P< 0.05$) (Table 16). For other treatments, *tel1tel2* generally had higher germination rates but the differences were not significant at the 5 % level. While germination rates were not consistent with that normally seen in wild type populations (95 % germination), comparisons between the two populations can still be made because *TEL1TEL2* plants were grown at the same time and in the same conditions in the glasshouse as *tel1tel2* plants.

Based on the data presented above, a second analysis using four different progeny lines and MS medium plates with either 0 % (w/v) sucrose and long days, 2 % (w/v) sucrose and short days, or 2 % (w/v) sucrose with 10 µM GA_3 and long days was conducted (Table 19). A subsequent set of experiments were designed using additional plants from the wild type progeny class and the double knockout class. MS plates were divided into quarters; each quarter contained one progeny line and each plate contained two progeny lines from each of the classes. Plants grown in short day conditions (eight hour day, 16 hour night at 22 ^{o}C) were assessed 28 days post germination for leaf number, root length, root number and plant height.

tel1tel2 (plant number E8), *tel1tel2* (plant number H10) and *TEL1TEL2* (plant number C1) plants grown on the 0 % (w/v) sucrose media, have on average, a larger stature than *TEL1TEL2* (plant number G5) at $P<= 0.05$ (4.41 ± S.E.0.24, 4.44 ± S.E.0.24 and 4.31 ± S.E.0.24 compared with 3.92 ± S.E.0.24), whereas *tel1tel2* (plant number E8), (plant number H10) and *TEL1TEL2* (plant number C1) have very similar stature. After GA_3 treatment, *tel1tel2* (plant number E8) plants had significantly longer stems (21.15 ± S.E.2.07) than *TEL1TEL2* (plant number C1) (16.24 ± S.E.2.07) and *TEL1TEL2* (plant number G5) plants (15.15 ± S.E.2.07) ($P< 0.05$), but not *tel1tel2* (plant number H10) plants (18.76 ± S.E.2.07) and the means for *TEL1TEL2* (plant number C1) and (plant number G5) plants were not significantly different from *tel1tel2* (plant number H10) plants.

Root length of *TEL1TEL2* (plant number G5) plants were shorter than the other three genotypes ($P < 0.05$) when grown on 0 % (w/v) sucrose (29.46 ± S.E.4.30 compared with 35.79 ± S.E.4.30, 38.28 ± S.E.4.30 and 35.58 ± S.E.4.30) and short-day treatments (3.45 ± S.E.0.07 compared with 3.63 ± S.E.0.07, 3.61 ± S.E.0.07 and 3.61 ± S.E.0.07). Root length of *tel1tel2* (plant number E8) & (plant number H10) and *TEL1TEL2* (plant number C1) genotypes grown on 0 % (w/v) sucrose media showed no significant differences from each other at the 5 % significance level.

The number of roots in the *tel1tel2* (plant number E8) plants grown with GA_3 is significantly more than *TEL1TEL2* (plant number C1) and (plant number G5) (2.30 ± S.E.0.18 compared with 1.84 ± S.E.0.18 and 1.94 ± S.E.0.18) ($P <= 0.05$) but the differences were not significant between *TEL1TEL2* (plant number C1), (plant number G5) and *tel1tel2* (plant number H10). The other treatments showed no evidence of any differences in root number.

The number of leaves on the *TEL1TEL2* (plant number G5) plants (5.81 ± S.E.0.28) grown on 0 % (w/v) sucrose were significantly less than the *tel1tel2* (plant number E8) (6.43 ± S.E.0.28) and (plant number H10) plants (6.53 ± S.E.0.28) ($P <= 0.05$), but the difference between *TEL1TEL2* (plant number C1) plants (6.13 ± S.E.0.28) and *tel1tel2* (plant number E8) and (plant number H10) plants was not significantly different.

After GA_3 treatment, *TEL1TEL2* (plant number G5) plants have significantly fewer leaves than *tel1tel2* (plant number E8) and (plant number H10) plants (7.70 ± S.E.0.23 compared with 8.51 ± S.E.0.23 and 9 ± S.E.0.23) ($P < 0.05$) and there is an indication of a significant difference ($P \sim 0.05$) between *TEL1TEL2* (plant number C1) and *tel1tel2* (plant number E8) plants. *TEL1TEL2* plants tended to have fewer leaves than *tel1tel2* plants (8.04 ± S.E.0.23 compared with 8.51 ± S.E.0.23). In the short day treatment there was little evidence of differences in the number of leaves among the genotypes.

Table 19: Phenotypic analysis of an additional two sets of plants with the same genetic background.

stem length (mm)	***E8*** ***(tel1tel2)***	***H10*** ***(tel1tel2)***	***C1*** ***(TEL1TEL2)***	***G5*** ***(TEL1TEL2)***	**signficant differences at P<0.05**
10 µM GA_3	21.15	18.76	16.24	15.15	*tel1tel2*(E8) > *TEL1TEL2*(C1) and (G5), but not *tel1tel2*(H10)
0% sucrose	4.41	4.44	4.31	3.92	*tel1tel2*(E8) and (H10) > *TEL1TEL2*(G5)
ShortDay	9.06	8.64	9.44	8.82	none
root length (mm)					
10 µM GA_3	56.55	52.44	50.49	52.05	none
0% sucrose	35.79	38.28	35.58	29.46	*tel1tel2*(H10) > *TEL1TEL2*(G5)
ShortDay	3.63	3.61	3.61	3.45	*tel1tel2*(E8) and (H10) and *TEL1TEL2*(C1) > *TEL1TEL2*(G5)
number of roots					
10 µM GA_3	2.307	2.03	1.84	1.94	*tel1tel2*(E8) > *TEL1TEL2*(C1) and (G5), but not *tel1tel2*(H10)
0% sucrose	1.75	1.68	1.79	1.49	none
ShortDay	1.45	1.51	1.50	1.45	none
number of leaves					
10 µM GA_3	8.51	9	8.04	7.70	*tel1tel2*(E8) & (H10) > *TEL1TEL2*(G5)
0% sucrose	6.43	6.53	6.13	5.81	*tel1tel2*(E8) & (H10) > *TEL1TEL2*(G5)
ShortDay	8.67	8.64	8.60	8.19	none

This suggests that there is a general trend for plants with double insertions in *TEL* genes to have longer stems (inflorescence stems), an increased number of leaves and an increased number of roots. However these differences, while significant, are not large.

In addition, 60 seeds of each progeny class were sown to soil and placed in long days (16 hour day, eight hour night at 22 ^{o}C). Plants grown in soil in long days were assessed at floral bolt for leaf number and at seed dehiscence for the number of inflorescences and the number of branches on the inflorescences. Sixty seeds of each progeny class were sown to soil and placed in short days (eight hour day, 16 hour night at 22 ^{o}C) for 28 days before shifting to long days and then assessed at the time of seed dehiscence for the number of inflorescences (Table 20). *tel1tel2* genotyped plants (47) showed, on average, an increase of one leaf at floral bolt, one additional branch on the primary inflorescence, an additional branch on the secondary

inflorescence and two additional branches on the third inflorescence (Figure 3-9 schematic) when compared with a comparative population of 18 *TEL1TEL2* plants. The increase in leaves and branches is not statistically significant. There is a lot of variation in the population (Figure 3-9 photo) that is not able to be attributed to differences in genotype.

Table 20: Number of inflorescences and branches on *tel1tel2* plants compared with *TEL1TEL2* plants.

plant genotype	Primary Inflorescence (arising from the rosette) (mean #)	Primary branch (mean # of branches)	Secondary branch (mean # of branches)	Tertiary branch (mean # of branches)	Quaternary branch (mean # of branches)
tel1tel2	1	3	2	2	2
TEL1TEL2	1	2	2	2	
tel1tel2	2	2	1	1	1
TEL1TEL2	2	2	1	1	
tel1tel2	3	2	1	1	
TEL1TEL2	3	2			

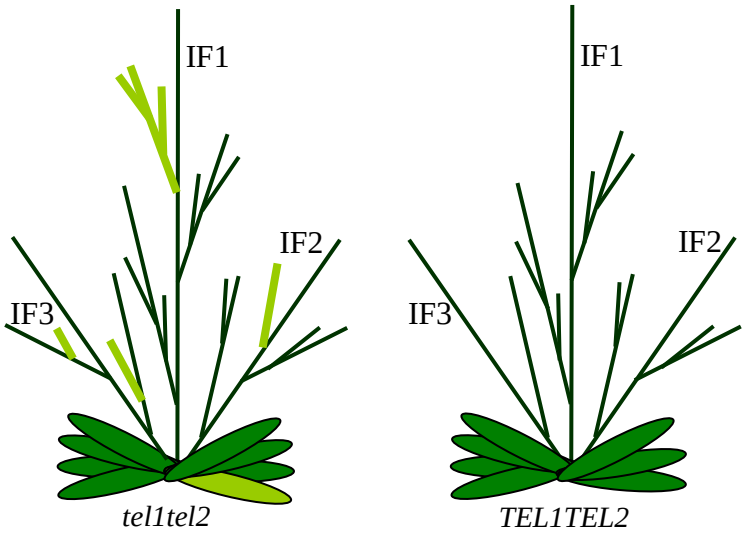


Figure 3-9: ***tel1tel2*** **plants grown on soil showed an increase in leaf number at floral bolt and in the number of inflorescence branches (IF). The top panel shows the range observed in the population;** ***tel1tel2*** **(left)** ***TEL1TEL2*** **(right). The bottom panel is a schematic of the differences (on average) between the** ***tel1tel2*** **and** ***TEL1TEL2*** **populations as indicated. Additional leaf and inflorescence branches are shown in pale green.**

3.2 TEL1 AND TEL2 GENE REGULATION

This section presents the results of the second part of this thesis which examines *TEL1* gene regulation sequence analysis to identify regulatory elements responsible for directing *TEL1* expression to the RAM and SAM. Results of *TEL1* promoter driven GFP expression under hormonal treatment and in a characterised mutant genetic background are also presented in this section, as are additional physiological studies, and the investigation *in silico* into the members of the *MEI2*-like gene family in *Arabidopsis*.

3.2.1 Gene regulation

In plants, transcriptional control plays an important role in determining the development of all plant parts. Insight into mechanisms defining spatial and temporal boundaries and organ recruitment can be obtained through understanding sequences regulating transcription and post transcriptional modifications by identifying upstream motifs and characterising the nature of these upstream signalling inputs.

3.2.2 *TEL1* gene regulation sequences

A broadly focused deletion analysis of the 5' upstream region of *TEL1* (At3G26120) was initiated to define regulatory elements responsible for directing *TEL1* to the RAM and SAM. For the purposes of this thesis, this region is defined as sequences extending from the predicted ATG start codon of *TEL1* (A=1) upstream of the *TEL1* gene to the 3' end of the adjacent gene (At3G26115.2). A series of 5' deletion *TEL1* promoter GFP reporter constructs was made to explore whether there are motifs required for normal *TEL1* gene function *in vivo*. The design of the constructs used to analyse the requirements of 5' upstream region for *TEL1* gene expression in the QC of mature roots and in the SAM (a total of five constructs were made, with no gene or 3' sequences included, but with increasingly truncated 5' DNA sequences) are shown in Figure 3-10 and Figure 3-12.

As mentioned, this series of deletion constructs were designed to determine which sequences of the *TEL1* upstream region were important for directing *TEL1* expression

to the root and shoot apical meristem. A further construct was also made to examine the influence of the coding and intron sequences of the *TEL1* gene (Figure 3.11). An analysis *in silico* of the *TEL1* gene (At3G26120) using The Arabidopsis Information Resource (TAIR) web page (http://www.arabidopsis.org/) identified a region upstream of the gene predicted to contain sequences necessary for the expression of the gene. Primers designed to correspond to the sequence downstream of the 3' end of At3G26115.2, the gene upstream of *TEL1*, and the TAIR predicted 3' terminal end of *TEL1* gene (At3G26120) were used to amplify the region from the Bacterial Artificial Chromosome (BAC) MJL14. A nested primer approach was taken to ensure the region amplified contained all of the sequences predicted by the TAIR web page. A forward primer (GCACTCTAGGGATGTTTGGTC) and an initial reverse primer (GGGTCTAAGTTTCCGACGAA) were used initially.

The nested forward primer (GACaagcttTAATTTTGGTTCAG) and the nested reverse primer (GACggatccTAATACCAGAG), corresponding to the 5' and 3' end of the TAIR specified region upstream of the *TEL1* gene, were designed to enable specific amplification of only the promoter region of *TEL1*. Restriction sites (shown in lower case) were added to facilitate cloning of the amplified product into a binary vector (pBIN19mGFP-ER) *via* the *Bam*H1 and *Hin*d111 restrictions sites (2.3.1.1).

The region upstream of the *TEL1* ATG start codon to the 3' end of the preceding gene is designated -2023. Deletions of this region are written with a minus sign followed by the number of base pairs present eg -1000 refers to 1000 bases upstream from the ATG of *TEL1*. However, there are also two constructs where the sequence does not terminate at the ATG. The first of these two constructs, a β-Glucuronidase (GUS) fusion containing the region upstream of the ATG (-2023), and the coding regions and the introns of the *TEL1* gene, is designated -2023 *TEL1*::GUS (Figure 3-11). The second construct, -1900 P_{TEL1}::ER-GFP, contains 1900 bases of the upstream region but is missing 112 base pairs at the 5' (adjacent to the preceding gene) and 11 base pairs immediately upstream of the start ATG of *TEL1* (i.e. -1900 to -11 P_{TEL1}::ER-GFP), a total of 123 base pairs.

A piecemeal approach was taken to reduce the number of constructs made. The promoter -1900 P_{TEL1}::ER-GFP, which gave GFP expression in the RAM (Figure 3-16

and Figure 3-18), was cut roughly in half to give the -1000 P_{TEL1}::ER-GFP construct (Figure 3-10 and Figure 3-12) and used to determine if the region between -2023 and -1000 of the *TEL1* promoter was required for RAM GFP expression. As GFP expression is still evident in the RAM (Figure 3-19) this region was reduced by half again to give a *TEL1* promoter region of -500 bases (-500 P_{TEL1}::ER-GFP, Figure 3-10 and Figure 3-12). The -500 base pair section of the *TEL1* promoter was deemed to be insufficient to direct GFP expression to the RAM (Figure 3-10 and Figure 3-12) so two additional constructs were made, between -1000 and -500, to further divide the *TEL1* promoter. These additional constructs did not have GFP expression in the RAM, suggesting elements between -1000 and -840 (the larger of the two additional *TEL1* promoter fragments) are essential for the correct RAM expression. More details of these expression studies are provided in section 3.2.4.

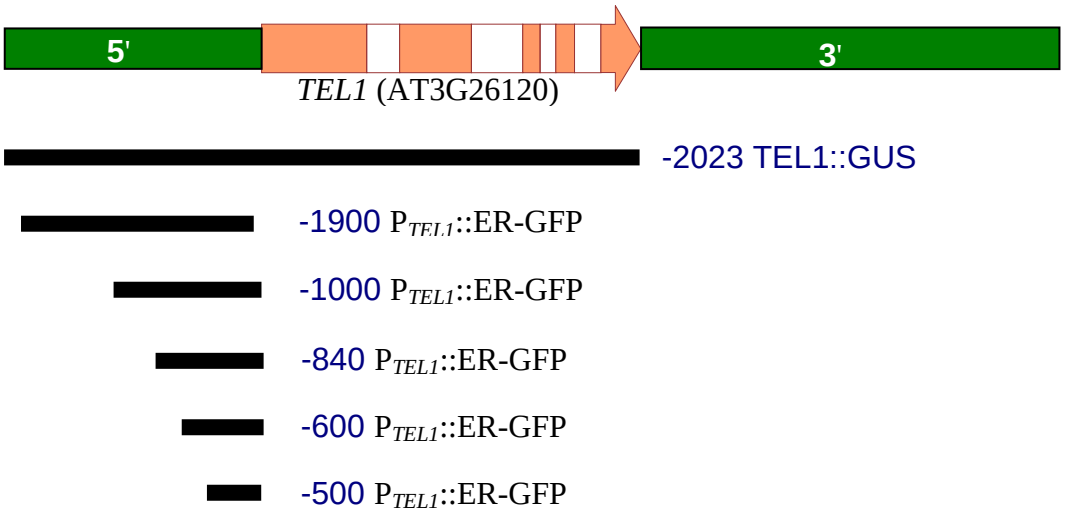


Figure 3-10: TEL1 gene structure and the regions used to assess expression. Green boxes represent 5' and 3' regions and are annotated as such. Orange boxes represent exons of the AT3G26120 gene and white spaces represent introns as predicted by the TAIR web site. -2023 TEL1::GUS includes the TEL1 coding region (AT3G26120) including introns and sequences extending 5' from the coding region to the next adjacent annotated coding region (AT3G26115). The other constructs are increasingly truncated promoter sequence (sequences 5'of the *TEL1* ATG) and do not include the coding region.

The pBIN vector containing -1900 of TEL1 promoter DNA confirmed as correct was used as the template for all subsequent promoter deletion constructs, and the truncated promoter constructs were amplified using the primers as shown in Table 21. Restriction sites (shown in lower case) were added to the ends to allow cloning into

the same pBIN19 vector as described above. The reverse primer used was the same for all additional PCR products (Table 21).

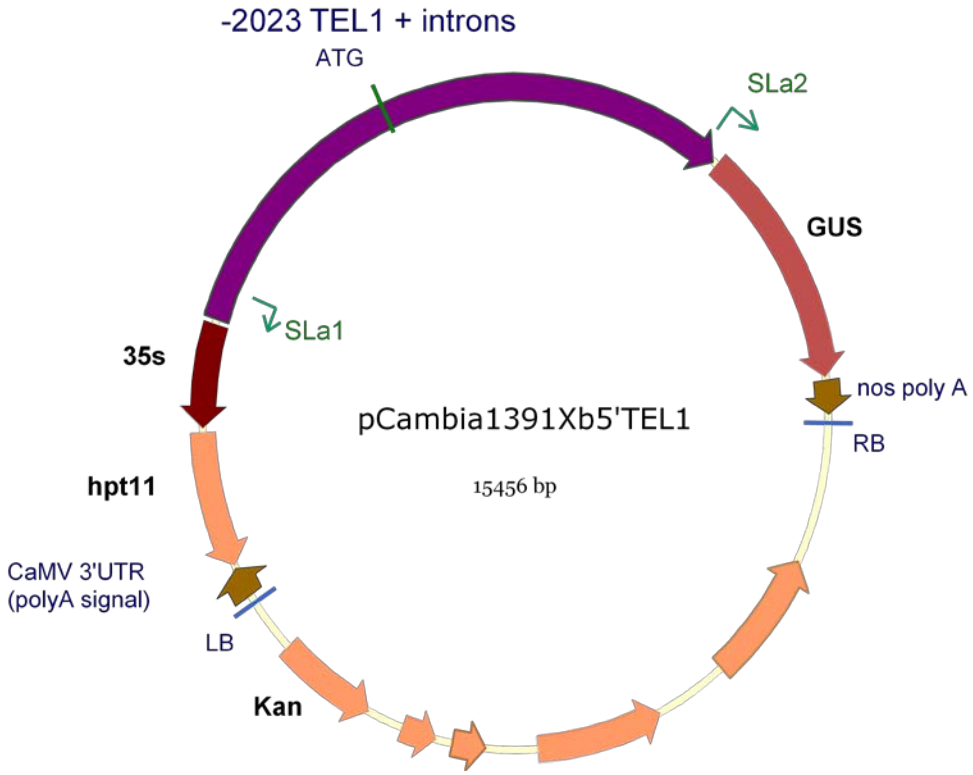


Figure 3-11: Plasmid for assessing expression of the *TEL1* upstream region and coding region including exons. The *TEL1* upstream region and coding region including exons are a translational fusion to GUS.

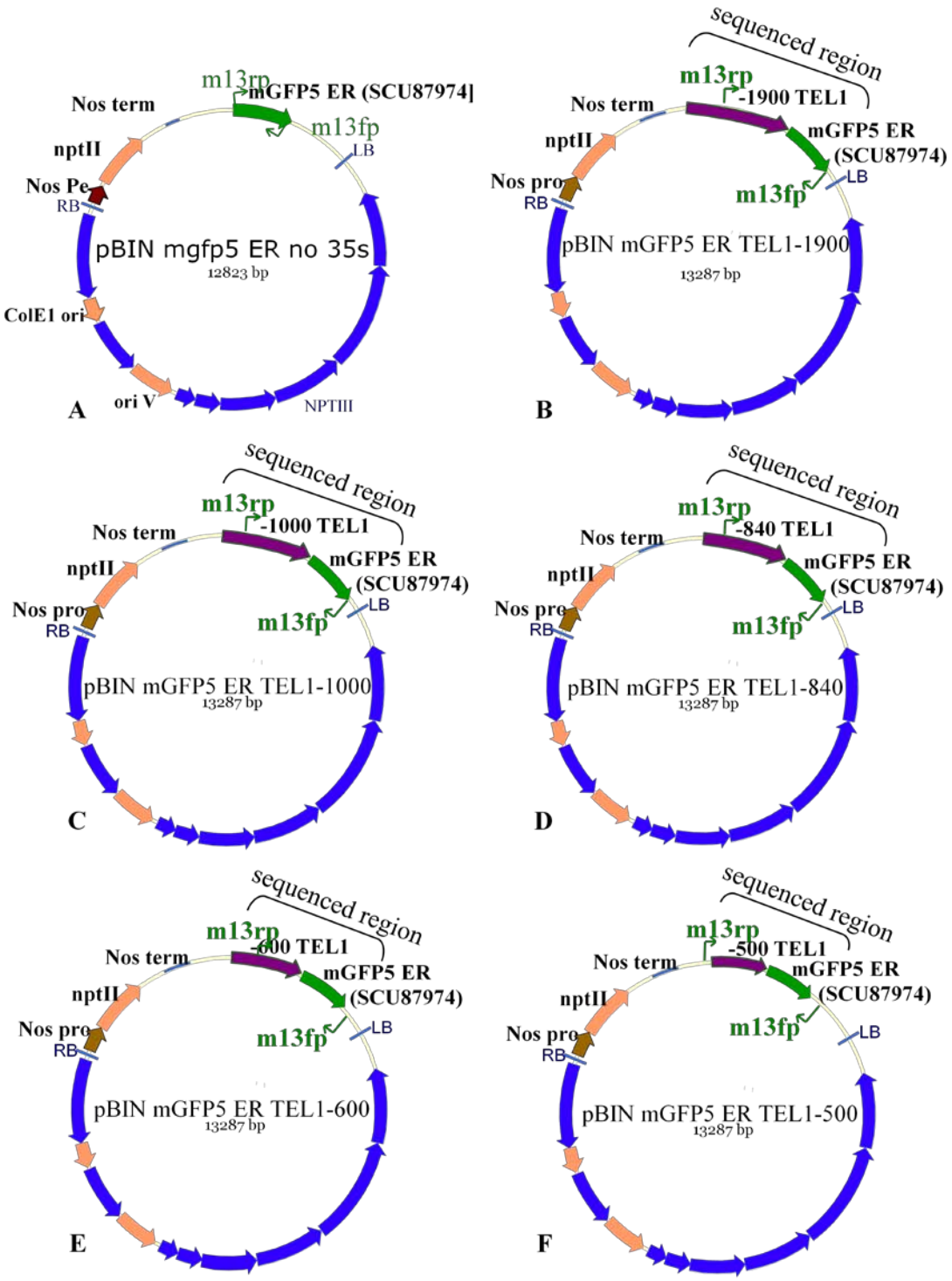


Figure 3-12: Plasmids for assessing *TEL1* expression. A: the parent pBIN mGFP5 ER has no 35s and contains an endoplasmic reticulum targeted GFP. B, C, D, E, F: increasingly truncated upstream regions in the pBIN mGFP5 ER vector.

Table 21: Primers used to amplify *TEL1* truncated promoter regions from the BAC MJL14 PCR product.

Primer name	Primer sequence
-500 P_{TEL1}::ER-GFP	ggatccTAATCCTCCGATATATTACC
-600 P_{TEL1}::ER-GFP	GCggatccTACATTGATACGATACGACGAT
-840 P_{TEL1}::ER-GFP	GCggatccCAATGCAGCATGTGGTGAA
-1000 P_{TEL1}::ER-GFP	ggatccTTTTCCTTCTATGATGTGAGATTCC
Reverse primer	GGGTCTAAGTTTCCGACGAA

The reverse primer was used in conjunction with each of the other primers. The name for each primer refers to the length of promoter DNA sequence amplified; -500 contains 500 base pairs upstream of the ATG, -600 contains 600 base pairs upstream of the ATG, -840 contains 840 base pairs upstream of the ATG, -1000 contains 1000 base pairs upstream of the ATG.

3.2.3 Construction of the *TEL1* gene including introns pCambia1391Xb plasmid

A fifth construct was generated using the *TEL1* gene including the promoter region and introns using the methods outlined above, and two new primers (Table 22) with restrictions sites allow the PCR product to be cloned into a pCambia fuse and use vector. The pCambia1391Xb binary vector allows a sequence to be fused in frame with the β-Glucuronidase (GUS) gene. PCR was used to amplify the region upstream of the *TEL1* genomic sequence including the 5' UTR (5' untranslated region) using the primers set out in Table 22.

Table 22: Primers used to amplify the *TEL1* gene including the 5' promoter region and introns from BAC MJL14.

Primer name	Primer sequence
5' upstream region	GACggatccTGGTTCAGTGGTGGTTTG
3' end of *TEL1* gene	gaattcgcGAAAGATGTTTCTCCTTCCACG

Lower case letters are restriction sites added to facilitate cloning into the pCambia 1391Xb plasmid.

The original primers (5' upstream region GACggatccTTTGGTTCAGTGGTGGTTTG and 3' end of TEL1 gene gaattcgcGAAAGATGTTTCTCCTTCCACG) were used for sequencing to confirm the beginning and end of the sequence.

3.2.4 *TEL1* expression

Eight of the 25, -2023 *TEL1*::GUS lines assessed gave GUS expression in the RAM and SAM of 10-day-old *Arabidopsis* plants (Figure 3-13). The remaining 17 lines did not display GUS staining. In the eight lines where GUS staining is observed in the RAM and SAM, staining is apparent in the early stages of embryo development and is absent from the suspensor (Figure 3-13 B). As the embryo develops, the GUS staining becomes more defined, and at the heart stage GUS staining is present in the presumptive SAM, RAM and vascular tissue (Figure 3-13 C and D). This staining pattern persists throughout all further stages of embryo development (Figure 3-13 A-E).

Plants (10 days old) containing the -2023 *TEL1*::GUS construct show a RAM and SAM staining pattern that matches the *TEL1 in situ* hybridisation pattern obtained by (Anderson et al., 2004), although additional GUS staining was obtained in the leaves and vascular tissue. By increasing the specificity of the GUS staining, *via* adjustment of the concentration of potassium ferricyanide and potassium ferrocyanide, expression in the vascular tissue was lost (Figure 3-14 F and G).

The -2023 *TEL1*::GUS gene fusion expression in the mature root is restricted to the initials and in particular to the QC (Figure 3-14 A). There are traces of GUS staining seen in the vascular tissue surrounding areas of high (GUS) expression such as the vascular tissue adjacent to lateral root primordia (Figure 3-14 C) that may be attributed to GUS persistence or "leakiness". Using *in situ* hybridisation in the vegetative meristem, *TEL1* expression has been shown to be in the central zone and rib zone but is absent from the peripheral zone (Anderson et al., 2004). In the inflorescence meristem, *TEL1* is only present in the peripheral zone where floral buds are becoming established (Anderson et al., 2004). This pattern is also observed in this thesis (Figure 3-15 C). In 10-day-old plants, GUS expression is limited to true leaves and is absent from the cotyledons (Figure 3-15). The unexpanded leaves, enclosed by the first true leaves, also show GUS expression (not shown due to difficulties dissecting out and photographing the material). No conclusions can be drawn about

the location in the apical meristem, as the material could not be assessed at this level of analysis using unfixed material. Resource constraints dictated that the material could not be fixed, dissected and visualised. In summary, the -2023 *TEL1*::GUS construct pattern shows *TEL1* is expressed in both the RAM and SAM in specific cell types.

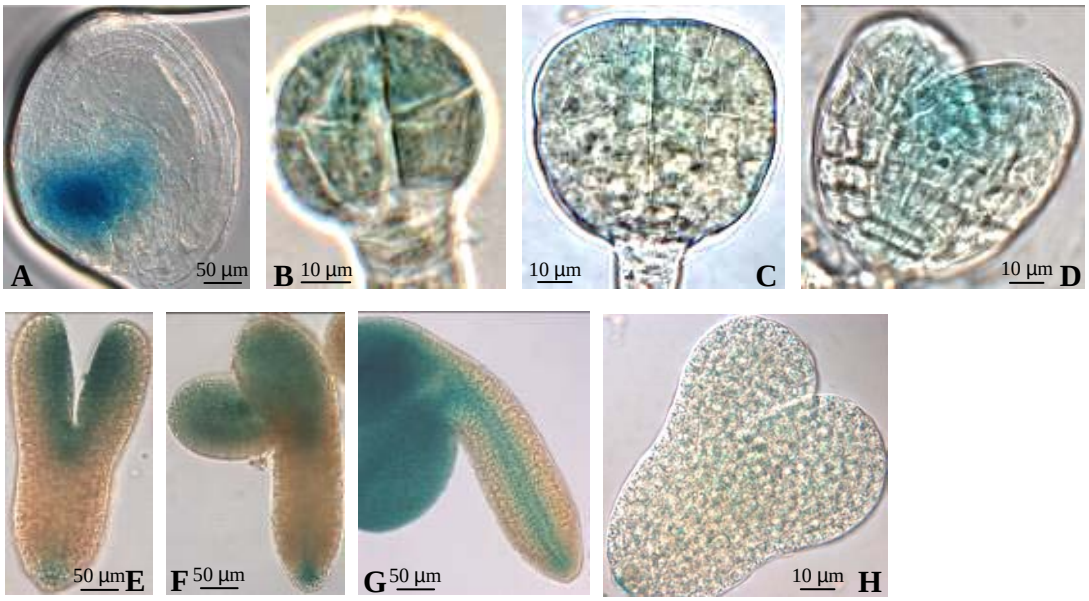


Figure 3-13: Embryos of transgenic plants transformed with the -2023 *TEL1*::GUS construct. A: globular embryo enclosed within the seed. B: eight cell embryo. C: early globular embryo. D: heart stage embryo. E: torpedo stage embryo. F/G: early walking stick stage embryo. Expression in the vascular tissue (G) disappears when increasing amounts of potassium ferrocyanide and potassium ferricyanide were added (F). H: empty vector.

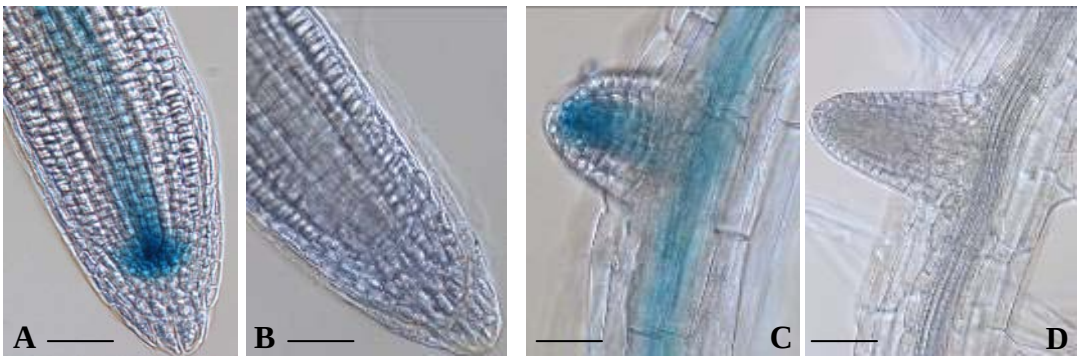


Figure 3-14: Mature and lateral roots of transgenic plants transformed with the -2023 *TEL1*::GUS construct. A: GUS staining in the mature root. C: GUS staining in the lateral root. B: mature root of an empty vector line. D: lateral roots of an empty vector line. Scale bar = 10 µm.

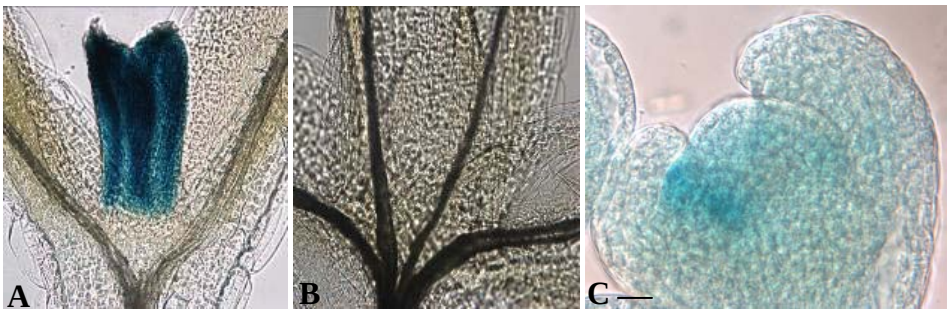
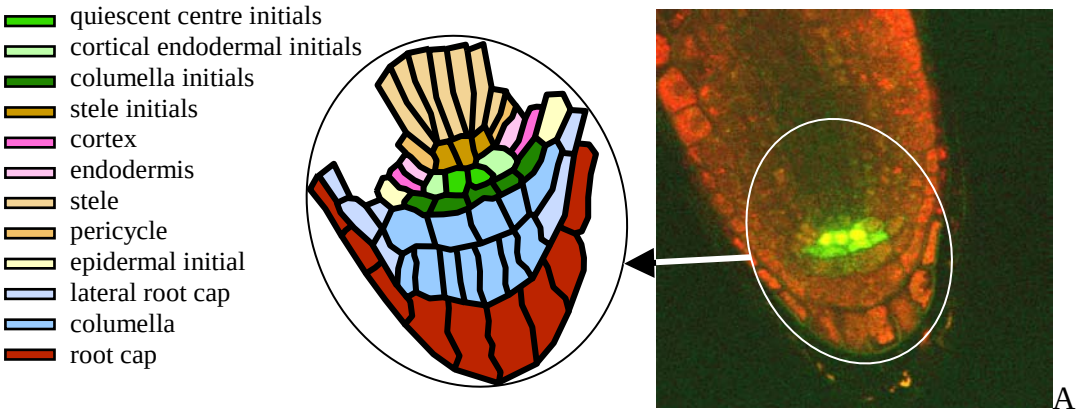


Figure 3-15: -2023 *TEL1*::GUS staining in the SAM of transgenic plants transformed with the -2023 *TEL1*::GUS construct. A: The first true leaves of a transgenic -2023 *TEL1*::GUS plant. B: the first true leaves of plant from an empty vector line. C: floral meristem of a transgenic -2023 *TEL1*::GUS plant. Scale bar in C = 10 µm.

Forty two lines obtained from the -1900 PTEL1::ER-GFP transformations were screened for Green Fluorescent Protein (GFP) expression. Eight lines gave consistent and bright expression in the RAM (Figure 3-16 and Appendix 1). GFP expression was not detected in the SAM (Figure 3-18 A-C and G-J). Two lines (lines 2 and 12) with expression in the RAM were selected and analysed in more detail. These lines were selected for further assessment because the GFP location was consistent with GFP expression observed in all expressing lines and with the *in situ* hybridisation results obtained by (Anderson et al., 2004). In these lines, GFP expression was limited to the initials with the majority of the expression in the quiescent centre initials (Figure 3-16). Expression in the surrounding initials may be a result of the GFP persisting in the cells as they divide rapidly rather than it being a true reflection of TEL1 expression. Expression came on very early in lateral root development (Figure 3-17). Root primordia three cells deep had localised GFP expression to the cells of the presumptive QC. Expression is not detected earlier in lateral root formation. GFP expression was detected in the early to late globular stage embryos in a non specific pattern (Figure 3-18 A). GFP expression was only detected in the RAM and specifically localised to the QC cells in heart to walking stick stage embryos (Figure 3-18 B and C). The *in situ* results (Figure 3-18 D, E, and F; reproduced with permission from Anderson et al. 2004) demonstrate the expected GFP expression pattern. *TEL1* mRNA is present throughout the late globular stage embryo, is in both the RAM and broadly in the SAM in heart stage embryos and limited to the SAM and RAM in torpedo stage embryos.



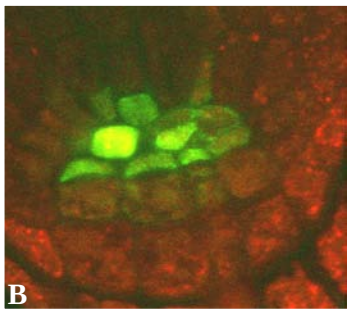


Figure 3-16: GFP expression in 10 day old -1900 P_{TEL1}::ER-GFP transgenic plants. A: 10-day-old root displaying GFP expression (right panel) with schematic insert (left panel). B: higher magnification of the RAM in A. The schematic was drawn by tracing over the cell lines of the confocal image.

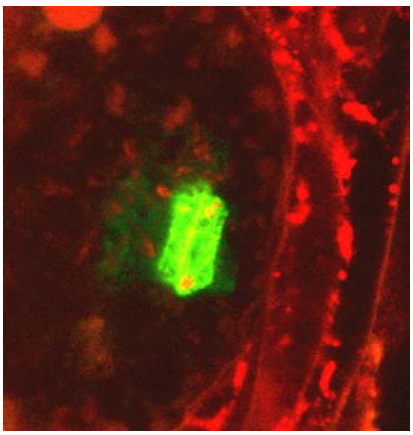


Figure 3-17: GFP expression in lateral root primordia. 10 day old plants transformed with the -1900 P_{TEL1}::ER-GFP construct display GFP expression in the early lateral roots but limited to the presumptive QC. The root primordium is approximately eight cells deep and GFP expression is limited to one or two cells.

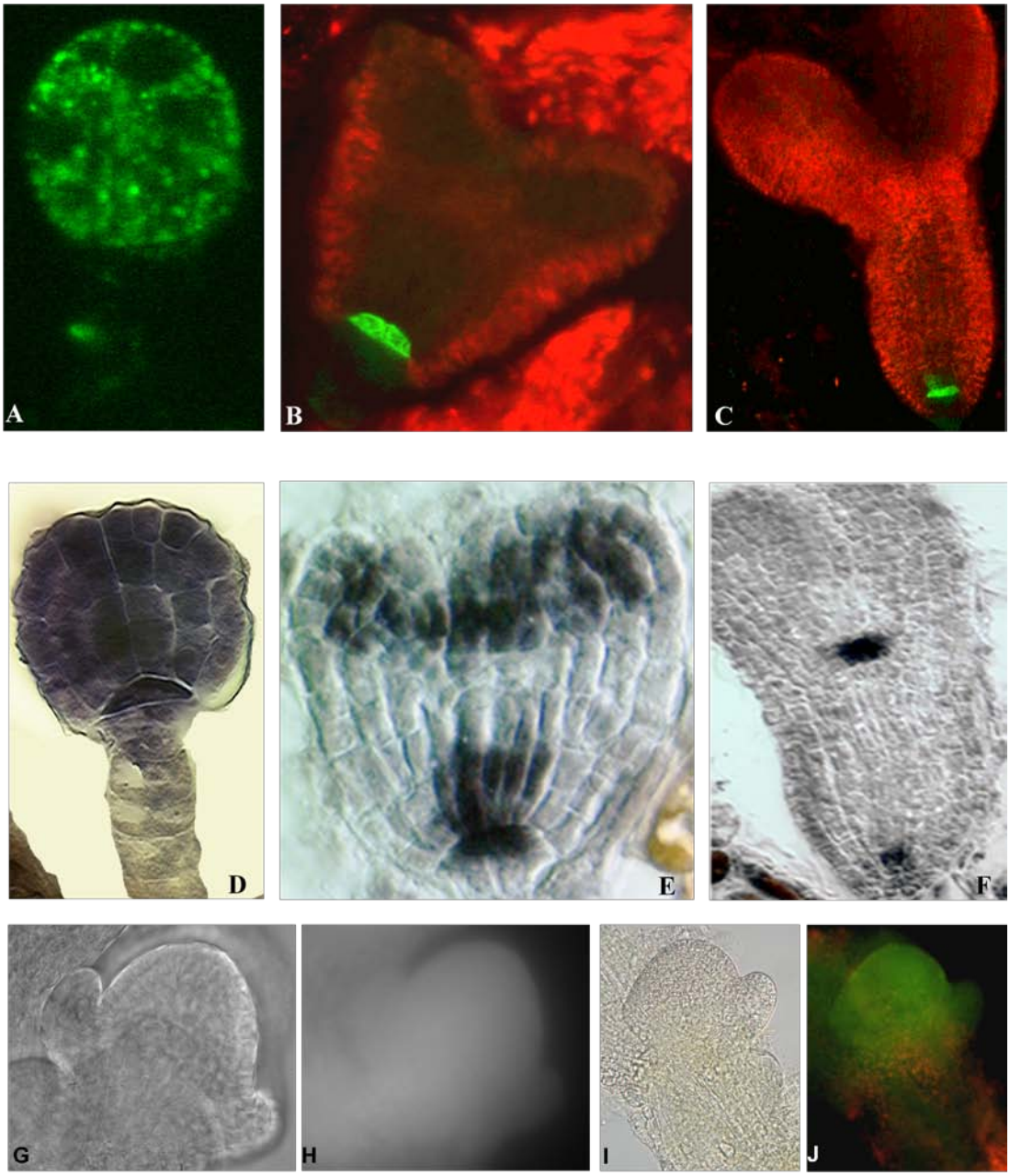


Figure 3-18: *TEL1* driven GFP expression in embryos. A: late globular stage embryo. B: heart stage embryo. C: early torpedo stage embryo. D, E, and F: the *in situ* results (reproduced with permission from Anderson et al 2004) demonstrate the expected SAM GFP expression. G: brightfield image of the SAM vegetative meristem and H: UV image of the SAM vegetative meristem. I: brightfield image of the floral meristem and J: confocal image of the floral meristem.

From the -1000 P_{TEL1}::ER-GFP transformations, 26 lines were screened for GFP expression. Of those, five lines gave expression in the root apical meristem in common with the -1900 P_{TEL1}::ER-GFP lines (see appendix 1). The remaining lines did not display any GFP expression. The expression observed with the -1000

P_{TEL1}::ER-GFP construct is similar to that seen with the -1900 P_{TEL1}::ER-GFP construct (Figure 3-19), with expression in the QC region.

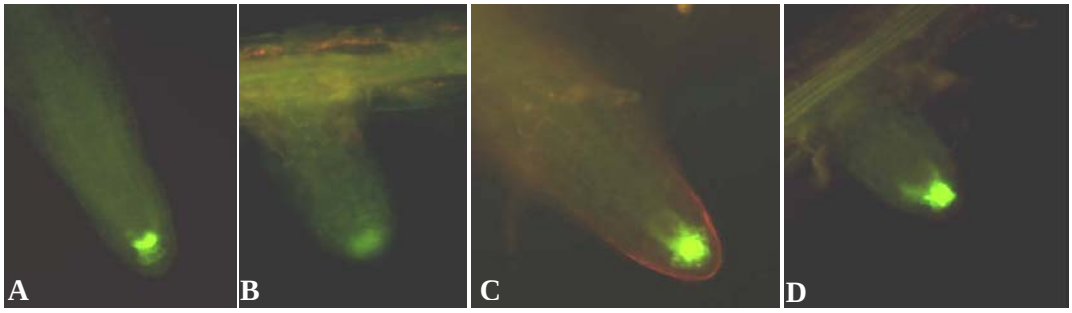


Figure 3-19: *TEL1* driven GFP expression in plants containing the -1000 construct. A: strong QC GFP expression in a plant containing the -1000 P_{TEL1}::ER-GFP construct. B: weak QC GFP expression in a plant containing the -1000 P_{TEL1}::ER-GFP construct. C: Strong and specific QC GFP expression in plants containing the -1900 P_{TEL1}::ER-GFP construct. D: Weak and specific QC GFP expression in plants containing the -1900 P_{TEL1}::ER-GFP construct. Images were viewed with an Olympus light microscope.

For the -840 P_{TEL1}::ER-GFP transformants, -600 P_{TEL1}::ER-GFP and -500 P_{TEL1}::ER-GFP transformants, 14, 16 lines and 25 lines respectively (see Appendix 1) were screened for GFP expression, but in none of those lines could expression be detected in the RAM (Appendix 1) or in any other cells in the plant. Two lines were randomly selected for each construct and screened by PCR for the presence of the construct using the M13R primer and M13F primer. The lines tested positive for the construct DNA (data not shown) and were then sequenced and confirmed as containing the construct (Appendix 2).

Plants containing the -1900 P_{TEL1}::ER-GFP construct gave the expected pattern of GFP expression in the root but not in the shoot. Given the SAM expression pattern was not seen in the -1900 P_{TEL1}::ER-GFP construct, the -1900 P_{TEL1} sequence was scrutinised for errors and the construct was shown to be missing 112 base pairs at the 5' end (adjacent to the upstream gene, At3G26115.2) and 11 base pairs at the 3' end of the sequence (immediately upstream of the ATG). The possibility the 11 base pairs missing from the 3' end of the upstream region results in the lack of observed SAM expression cannot be discounted. Alternatively, the 112 base pairs missing from the 5' end of the upstream region may have resulted in a lack of SAM expression. Subsequent deletions (-840 P_{TEL1}::ER-GFP, -600 P_{TEL1}::ER-GFP, -500 P_{TEL1}::ER-GFP constructs) which did contain the 11 base pairs at the 3' end of the upstream sequence did not restore the shoot pattern. Therefore, an analysis *in silico* of the *TEL1* gene and

5' upstream region was undertaken next to identify any sequences known to be important in gene regulation which would provide a theoretical framework for the deletion series and offer some background for assessing the promoter for motifs that may be important for correct *TEL1* expression in the SAM and RAM.

3.2.5 Analysis *in silico* of the promoter region

An assessment *in silico* of the missing 112 base pairs at the 5' end of the promoter region could bring to light motifs that may be required for SAM expression (data not shown). However, the list of motifs in *Arabidopsis* is not comprehensive and the programmes designed to identify motifs are only as good as the information contained within them. Therefore it was not surprising that the analysis *in silico* of the missing 112 base pairs did not reveal motifs predicted to specifically target the SAM. A further analysis *in silico* looking at some of the *MEI2*-like gene family (*TEL1*, *TEL2*, *AML1*, *AML4*, *MCT1* and *MCT2*) also revealed no common motifs other than the already established RNA Recognition Motifs (RRMs). As expected there was a match to the sequences coding for the RRMs, but there were no matches to any sequences predicted to code for microRNA. Given the similarity between the patterns of *TEL1* and *TEL2* mRNA accumulation (Anderson et al., 2004), a PLACE search of the entire region upstream of *TEL1* and *TEL2* was conducted. The PLACE search identified motifs common to both, but the analysis reported no statistical support for the common motifs (data not shown). In light of the lack of statistical support the results cannot be supported.

A Multiple Em for Motif Elicitation / Motif Alignment and Search Tool (MEME/MAST) search was also conducted on both *TEL1* and *TEL2* genes (exons and introns). Three motifs were identified in both *TEL1* and *TEL2*. The first motif has not been fully characterised. The second motif returned two potentially important motifs embedded within it. The first acts as a transcriptional repressor and is essential for expression of the β-phaseolin gene during embryogenesis, while the second regulates the transcription of CBF/DREB1 genes under cold conditions in *Arabidopsis*. The third predicted motif is thought to be involved in controlling expression of endosperm-specific genes *via* transcriptional regulation. Only the first and third motifs are located in the region deemed

to influence RAM expression (based on the deletion study results). Because motif one (β-phaseolin) is not characterised and the predicted function of motif three (endosperm specific gene) is not aligned with RAM function, the MEME/MAST identified motifs were not investigated further in this thesis.

The analysis *in silico* then shifted focus to the promoter region between -1000 and -840 that was identified as being important for correct RAM expression in the root. The PLACE search results could be used as a basis to investigate the likelihood that some of the predicted motifs could be the motifs required for *TEL1* or *TEL2* gene function. A PLACE motif search of the -1000 and -840 region indicated 12 potential motifs but of those, eight were also found in the region between -840 and the ATG and so were not considered further (data not shown). The remaining four motifs: the CATATGGMSAUR motif at -1296 and -867; the TELOBOXATEEF1AA1 motif at -964; the RAV1AAT motif at -1721 and -864; and the UP2ATMSD motif at -964 are of interest because their location (between -1000 and -840) suggest these motifs may influence the regulation of *TEL1* in the RAM. However, only the RAV1AAT motif is contained within other *MEI2* gene upstream sequences.

There is no empirical or statistical support for any of these identified motifs to be significant, and so no further assessment was conducted to determine how common these motifs are in the rest of the *Arabidopsis* genome, or if there are any similar motifs present in genes at nearby loci on the chromosome. These motifs were therefore only considered in context of the deletion construct results and as a possible place to start if further deletions were to be undertaken in the future. However time constraints dictated further deletion constructs could not be created. The identification of these motifs (CATATGGMSAUR, TELOBOXATEEF1AA1, RAV1AAT and UP2ATMSD) did provide a concept that could be tested experimentally, including whether multiple copies of a motif may be required for correct *TEL1* and *TEL2* expression. However, anecdotal reports suggested that the CATATGGMSAUR motif may be required for responses to auxin and the UP2ATMSD motif is present in genes that are up regulated after main stem decapitation (and so removal of the auxin source). Experiments looking at whether the *TEL1* mRNA or TEL1 protein is transported from the RAM to the SAM were not undertaken but could provide the basis for future research. On the basis of these

anecdotal accounts, a series of experiments looking at the effects of hormones on *TEL1* expression in the RAM were conducted. If *MEI2*-like genes are important in meristem maintenance or initiation then hormones are likely to interact with *MEI2*-like genes in a quantifiable manner through either direct interactions or through intermediaries to control meristem size and the rate of differentiation into organs.

3.2.6 Interaction with phytohormones

If *MEI2-like* genes are important in determining plant architecture through regulation of the meristematic regions, *TEL* genes may be sensitive to hormone levels and the activity of developmental genes. Phytohormones such as auxin and cytokinin are known to play an important role in defining the RAM stem cell population (Woodward et al., 2005; Lindsay et al., 2006; Riefler et al., 2006). The appropriate concentration of plant hormones to use in this study were determined experimentally following a literature search of hormone applications to tissue or plants of *Arabidopsis thaliana* (Kerk and Feldman, 1995; Sabatini et al., 1999; Kerk et al., 2000; Dharmasiri et al., 2003; Friml et al., 2003b; Jiang and Feldman, 2003; Reinhardt et al., 2003b; Bao et al., 2004; Geldner et al., 2004; Clay and Nelson, 2005; Guo et al., 2005). Generally the concentration of synthetic plant hormones used in these studies was between 1 -100 µM depending on the plant hormone and how it was applied. A pilot study using a concentration series of 1 - 100 µM of each of the hormones was undertaken to determine suitable hormone application and concentration. Plants grown over a period of six hours on MS medium containing 10 µM 2,3,5-triiodobenzoic acid (TIBA), 1-naphthylphthalamic acid (NPA), 1-naphthylacetic acid (NAA), gibberellic acid (GA_3), or zeatin gave a measurable response. In addition, a short (one minute) stain with 10 µg/ml propidium iodide facilitates cell wall visualisation by confocal microscopy.

Plants containing the -1900 P_{TEL1}::ER-GFP construct were thus treated with 10 µM 2,3,5-triiodobenzoic acid (TIBA), 1-naphthylphthalamic acid (NPA), 1-naphthylacetic acid (NAA), Gibberellic acid (GA), Zeatin or 10 nM 2, 4-dichlorophenoxyacetic acid (2,4-D) for 6 hours at room temperature before staining with 10 µg/ml propidium iodide for one minute at room temperature followed by two washes of sterile water. To determine if plants could recover from TIBA and NPA treatment, plants containing the -1900

P_{TEL1}::ER-GFP construct were treated with 10 µM TIBA or NPA for 6 hours before treatment with GA or Zeatin.

Normal -1900 P_{TEL1}::ER-GFP expression is in the QC (Figure 3-20 A). The images for expression analysis are medial sections. In some cases GFP expression in the apical initials hides the usual indicators of medial sections, (for example the cortex / endodermal division). However, the normal appearance of the columella, root cap and lateral root cells indicate these sections are medial. With the addition of synthetic auxin and auxin mimics such as NAA and 2,4-D and with added cytokinin (zeatin) there is an increase in expression of GFP in all root apex initials and in the columella cells (Figure 3-20 C and D). However, plants treated with gibberellic acid have GFP expression in the QC, cortex and endodermal cells (Figure 3-20 B). Plants treated with NPA or TIBA (auxin transport inhibitors) for 6 hours then treated with zeatin for two hours showed a similar pattern to the zeatin-only treated plants (Figure 3-20 O and P). In the -1900 P_{TEL1}::ER-GFP plants auxin transport inhibitors such as TIBA and NPA appear to restrict GFP expression to the QC, and with continued exposure to auxin transport inhibitors, GFP expression disappeared completely (Figure 3-20 I, J and K).

To provide an indication of auxin activity in the root, *Arabidopsis* transformed with P_{DR5}::ER-GFP construct were assessed (seeds kindly donated by Ben Scheres, Utrecht University, Netherlands). The P_{DR5}::ER-GFP plants displayed GFP expression in the QC and columella cells(Figure 3-20 E). The application of GA_3 does not change the location of DR5 driven GFP expression(Figure 3-20 F), as GFP expression is still located within the QC and columella cells and not in surrounding cells. With the addition of auxin, the DR5 promoter driven GFP expression becomes visible in the cortex and endodermal cells as well as the columella cells (Figure 3-20 G). With the addition of zeatin, the location of DR5 promoter driven GFP expression (Figure 3-20 H) is similar to that seen with the addition of auxin in that there is some GFP expression evident on the cortex and endodermal cells. With the addition of auxin the transport inhibitor, NPA, DR5 promoter driven GFP expression become less visible in the QC (Figure 3-20 L, M and N). This expression is no longer evident in the QC (Figure 3-20 N) after continued exposure to NPA.

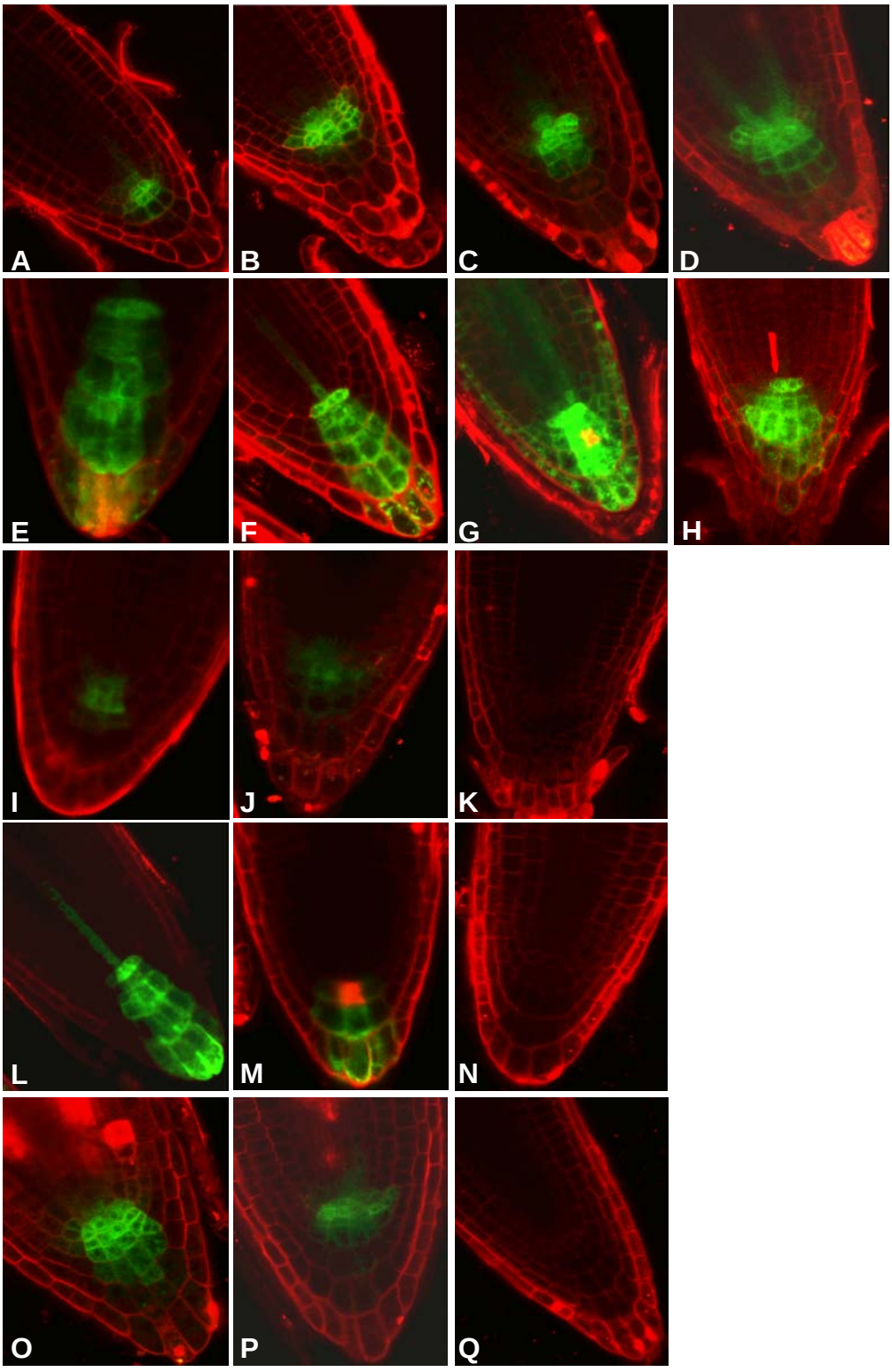


Figure 3-20: Plant hormone and auxin transporter inhibitor studies. *DR5* is a synthetic auxin response element used to drive GFP and reports on the location of auxin in the plant.

A: -1900 P_{TEL1}::ER-GFP transgenic plant prior to treatment.

B: gibberellic acid treated 10 day old -1900 P_{TEL1}::ER-GFP transgenic plant.

C: NAA (synthetic auxin) treated 10 day old -1900 P_{TEL1}::ER-GFP transgenic plant.

D: zeatin treated 10 day old -1900 P_{TEL1}::ER-GFP transgenic plant.

E: 10 day old P_{DR5}::ER-GFP transgenic plant prior to treatment.

F: gibberellic acid treated 10 day old P_{DR5}::ER-GFP transgenic plant.

G: NAA treated 10 day old P_{DR5}::ER-GFP transgenic plant.

H: zeatin treated 10 day old P_{DR5}::ER-GFP transgenic plant.

I: 10 day old -1900 P_{TEL1}::ER-GFP transgenic plant prior to NPA treatment

J: 10 day old -1900 P_{TEL1}::ER-GFP transgenic plant after six hours of NPA treatment

K: 10 day old -1900 P_{TEL1}::ER-GFP transgenic plant after 12 hours of continued exposure to NPA.

L: 10 day old P_{DR5}::ER-GFP transgenic plant prior to NPA treatment

M: 10 day old P_{DR5}::ER-GFP transgenic plant after six hours NPA treatment.

N: 10 day old P_{DR5}::ER-GFP transgenic plant after 12 hours NPA treatment.

O: 10 day old -1900 P_{TEL1}::ER-GFP transgenic plants treated with NPA for six hours then treated with zeatin for two hours.

P: 10 day old -1900 P_{TEL1}::ER-GFP transgenic plants treated with TIBA for six hours then treated with zeatin for two hours.

Q: 10 day old empty vector transgenic plant where GFP is not under control of the *TEL1* promoter.

Similar to the GFP expression pattern observed with the -1900 P_{TEL1}::ER-GFP lines is the expression pattern observed with the -1000 P_{TEL1}::ER-GFP lines (Figure 3-21). The -1000 P_{TEL1}::ER-GFP lines show GFP expression in the QC of the RAM. Images were observed using light microscopy as the confocal microscope was not available.

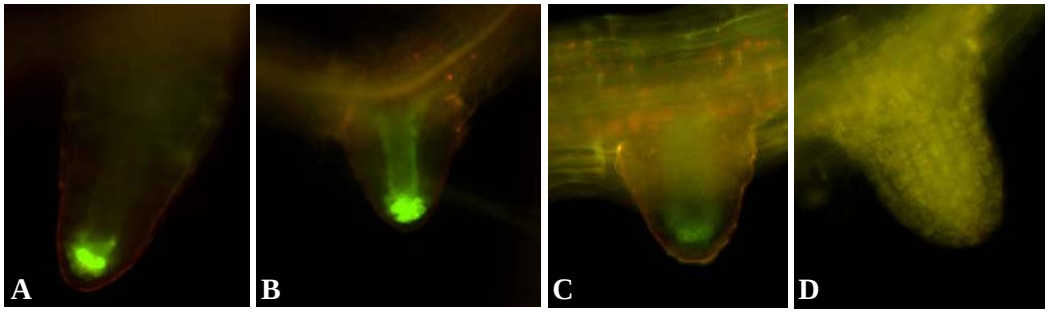


Figure 3-21: Plants containing -1000 P_{TEL1}::ER-GFP construct respond to auxin and auxin transport inhibitors. A: -1000 P_{TEL1}::ER-GFP transgenic plant treated with NAA. B: -1000 P_{TEL1}::ER-GFP transgenic plant treated with 2, 4-D. C: -1000 P_{TEL1}::ER-GFP transgenic plant treated with NPA. D: -1000 P_{TEL1}::ER-GFP transgenic plant treated with TIBA.

3.2.7 Assessment of -1900 P_{TEL1}::ER-GFP in a *short root*, *scarecrow*, or *plethora1* and *plethora2* mutant background

Specific genes such as *SCARECROW* (*SCR*), *SHORTROOT* (*SHR*), *PLETHORA1* (*PLT1*) and *PLETHORA2* (*PLT2*) specify the regular arrangement of cells found in *Arabidopsis* roots and have similar or overlapping zones of expression to *TEL1* and *TEL2* suggesting there may be an interaction between these genes to specify QC cells (Nakajima and Benfey, 2002; Aida et al., 2004; Heidstra et al., 2004). Seeds of *scr-3* (CS3997), *shr* (CS2972), *plt1* (Salk_007654) and *plt2* (Salk_128164) were sown to MS Medium plates and assessed at the two true leaf stage for an altered root morphology consistent with that previously described in the literature (Benfey et al., 1993; Sabatini et al., 2003; Aida et al., 2004; Heidstra et al., 2004; Sena et al., 2004). Plants exhibiting abnormal root morphology were transplanted to soil and grown in 16 hour days in the glasshouse. Once plants were flowering healthy plants were selected and the sepals and petals of unopened flowers were gently prised apart using size four ultra fine antistatic antimagnetic tweezers (Geneva Importers Ltd) to reveal anthers and style. The anthers were removed with tweezers taking care not to damage the style or stigma and the stigma was brushed with pollen from anthers of the pollen donor. The pollen donor plants, -1900 P_{TEL1}::ER-GFP containing plants, were sown

at the same time as the *scr-3*, *shr*, *plt1* and *plt2* seeds to ensure plants would be flowering at the same time. Pollen donor plants were selected based on pollen quality after visualisation GFP in one root tip. Anthers with copious amounts of white fluffy pollen were considered ideal paternal parents.

Seeds were harvested just prior to seed dehiscence to ensure all seed was captured. This seed was then sown to plates containing MS medium and the plants were assessed at the two seedling stage for GFP expression in the roots and root morphology consistent with there being a mutation in scr, *shr*, *plt1* or *plt2*. Plants identified as being -1900 P_{TEL1}::ER-GFP scr or -1900 P_{TEL1}::ER-GFP *shr* were planted to the glasshouse and grown in 16 hour days until seed dehiscence. The seeds were harvested and sown to MS Medium. The seedlings with the expected altered root morphology and GFP expression were assessed.

As *plt1* and *plt2* plants have a weak phenotype the progeny of the -1900 P_{TEL1}::ER-GFP *plt1* and -1900 P_{TEL1}::ER-GFP *plt2* were reciprocally crossed (plants were used as both maternal and paternal parents) as described above. The seeds were harvested just prior to dehiscence and sown to soil. The resulting F1 plants were allowed to self and the seed harvested. This F2 seed was sown to MS Medium and seedlings assessed at the two leaf stage for root morphology consistent with that previously described for *plt1plt2* and for GFP expression.

In -1900 P_{TEL1}::ER-GFP *scr-3* and *shr* plants, GFP expression is present and corresponds to the presumptive QC (Figure 3-22 A and B). As the cell files are altered in the scr-3 and shr mutant plants (Figure 3-22), the GFP expression pattern changes, but still reflects the position of the presumptive QC. In -1900 P_{TEL1}::ER-GFP *plt1plt2* homozygous plants, GFP expression is absent (Figure 3-22 C). In heterozygous siblings, a true QC is formed and GFP expression can be detected in these cells (data not presented). Empty vector lines indicate there is no background GFP expression (Figure 3-22 D).

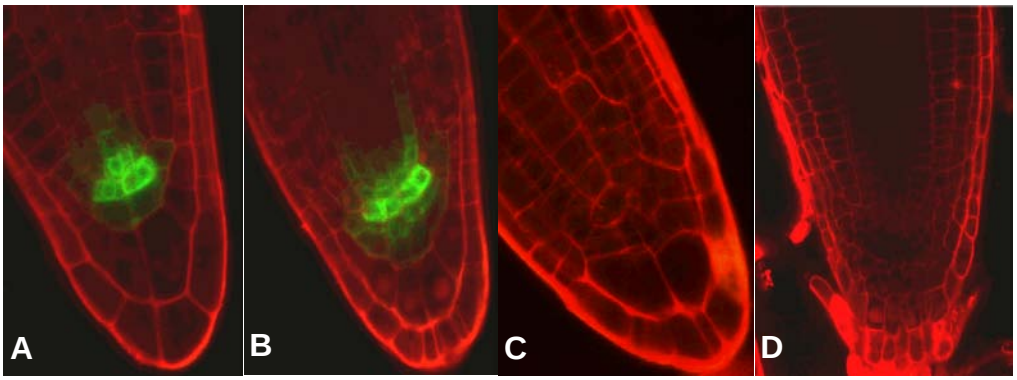


Figure 3-22: Roots from 10 day old -1900 P_{TEL1}::ER-GFP lines in characterised mutant backgrounds. A: 10 day old -1900 P_{TEL1}::ER-GFP in *shr* mutant background. B: 10 day old -1900 P_{TEL1}::ER-GFP in *scr-3* mutant background. C: 10 day old -1900 P_{TEL1}::ER-GFP in *plt1plt2* mutant background. D: empty vector.

Light microscopy photographs (brightfield) show abnormal root morphology of plants with mutant *shr* or *scr-3* genes. Florescent image of the same roots shows GFP expression in the presumptive QC cells (Figure 3-23). The cortex and endodermal tissues are ill defined and have characteristics of both cell types in plants carrying mutation in both the *shr* and *scr-3* genes.

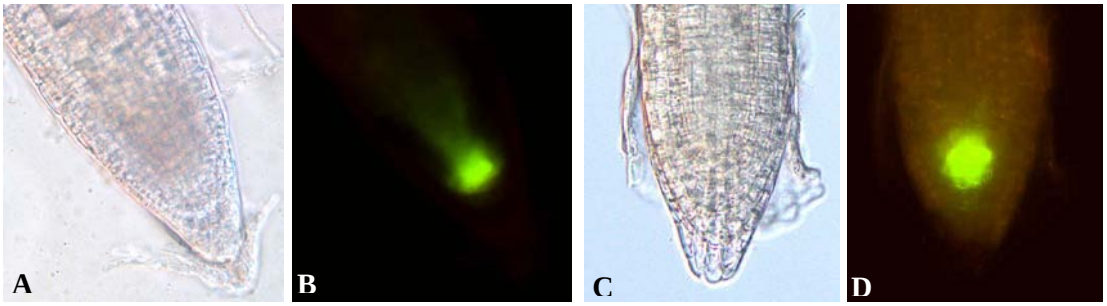


Figure 3-23: Abnormal roots in *shr* and *scr-3* mutant plants. A: Brightfield image of a -1900 P_{TEL1}::ER-GFP *shr* plant. B: Florescent image of the same -1900 P_{TEL1}::ER-GFP *shr* plant. C: Brightfield image of a -1900 P_{TEL1}::ER-GFP *scr-3* plant. D: Florescent image of the same -1900 P_{TEL1}::ER-GFP *scr-3* plant.

4 DISCUSSION

4.1 TEL1 AND TEL2 PHENOTYPIC ASSESSMENT

4.1.1 Mutant analysis

Comparison between wild type and mutant plants with a specific non-functional gene can reveal the role of that gene during the life of the plant. The use of foreign DNA (T-DNA, or transferred-DNA, insertion mutants), to interrupt a gene sequence, and subsequently its function in the plant, has enabled a targeted reverse genetic approach to assessing the function of a number of meristem maintenance genes (Sussman et al., 2000). Plants containing T-DNA insertions in the *TEL1* and *TEL2* genes were assessed, characterised and compared with what is described for the *MEI2*-like equivalents in rice (*PLA2*) and maize (*TE1*).

To address whether *TEL1* expression is linked to meristem activity and to provide clear evidence of the biological function of *TEL* genes comparison was made between plants wild type for *TEL1* and *TEL2* and plants with non-functional *tel1* and *tel2* genes. As single mutant plants and double mutant plants did not have an obvious phenotype, double mutant plants were grown in short-day conditions, exposed to higher or lower temperature than normal, grown on media with or without mannitol or sucrose, or grown on media containing NAA or GA_3 to elucidate the role of *TEL1* and *TEL2* genes during plant development. The correlation between *TEL1* and the QC suggests *TEL* genes are responding to factors influencing QC identity and leads to questions about whether physiological factors will also influence *TEL* expression. Treatments, such as increased temperature, perturbing the tight regulatory control normally existing in an organism may alter the amount of gene products present and may produce observable differences. The analysis performed here found the phenotypes associated with mutant plants was subtly different to wild type and observed to be sensitive to growth conditions.

4.1.1.1 *tel1tel2* mutant plants have more leaves and longer roots

Detailed phenotypic analysis of *tel1tel2* plants (Figure 3-9) revealed a subtle accelerated rate of leaf initiation, as there is a small increase in the number of leaves prior to flowering. This subtle accelerated rate of leaf initiation could be considered to be consistent with these genes normally acting to inhibit terminal differentiation pathways and is consistent with changes seen in loss of function mutants to equivalent genes in maize and rice (Veit, 1998; Kawakatsu et al., 2006). This data does not distinguish between whether there is a decrease in plastochron or a delay in flowering and as the differences are subtle there remain doubts as to whether this change in phenotypic characteristics can be attributed to knocking out the function of *TEL1* and *TEL2*.

Changing nutrient availability, such as sucrose and mannitol, and hormone levels can alter plant growth. *tel1tel2* plants grown on medium without mannitol or sucrose had significantly more leaves, more roots and longer roots than *TEL1TEL2* plants. Further, GA_3-treated *tel1tel2* plants generally had longer stems (this measurement included any inflorescences that formed during the growing period), longer roots, more roots and more leaves than their *TEL1TEL2* cousins (Table 16, Table 17 and Table 18). Plants of the same genotype under the same treatment conditions showed variation across all categories. However as *tel1tel2* plants were interspersed with *TEL1TEL2* plants on the same plate across all treatments the variability was able to be accounted for by the REML statistical test used to determine significance of results. This data suggests plants with non-functional *TEL* genes have slightly, but significantly, different patterns of growth than their wild type counterparts in tissue culture conditions and in the presence of growth promoters such as GA_3.

4.1.2 *Arabidopsis* genotypes used in this research

The genetic background of *tel1tel2* mutants may also account for the observed subtle increase in floral organogenesis. *Arabidopsis* is a facultative long-day plant, requiring long days in which to flower (Michaels et al., 2005). In short-day conditions (eight hours light and 16 hours darkness), the ecotype Columbia flowers after the production of approximately 50 leaves (Michaels et al., 2005). An increased number of leaves

correlates to an increased growing time so that subtle *Arabidopsis* phenotypes may become more apparent in short-days. However *Arabidopsis* will also flower in short-days if other cues, such as exposure to cold, are present or the flowering time genes are not functional (Moon et al., 2003). The genetic background of *tel1tel2* mutants may contain Wassilewskija (Ws) ecotype DNA, as the *tel2* T-DNA insertion allele was in a Ws background prior to introgression into a Columbia ecotype. The Ws ecotype used in this study may have a non-functional *PHYTOCHROME C*, *FRIGIDA*, or *FLOWERING LOCUS C* (Balasubramanian et al., 2006) which could lead to early flowering. However, as these flowering time genes are located on chromosomes 5, 4 and 5 respectively. As well, it is unlikely they are co-segregating with *tel1* and *tel2* genes, which are located on chromosomes 3 and 1 respectively, and the *tel2* plants used in this study were introgressed five times into *Arabidopsis* Columbia ecotype background to remove Ws ecotype DNA from the population. As *TEL1TEL2* cousins did not appear to have an early flowering phenotype (on the basis of leaf number) it is likely therefore that *tel1tel2* plants also contained functional flowering time genes.

The observed phenotype of a subtly increased rate of vegetative organogenesis (Table 16, Table 17 and Table 18) may also be attributable to the presence of other Ws ecotype DNA in the population. Plants of the Ws ecotype have been shown to have an increased rate of cell expansion when compared to other genotypes (Beemster et al., 2002). If Ws DNA, such as a-type cyclin-dependent kinase DNA specifying cell division activity (Verkest et al., 2005), is linked to the *tel2* knockout then the increased rate of leaf initiation may be explained by the difference in genetic contribution from each grandparent. This possibility was explored through genotyping a population of 96 cousins (Table 15). The observed Mendelian ratio follows the expected pattern of equal segregation by *tel1* and *tel2* alleles assuming they are co-dominant. Co-dominance is assumed as both genes have different but overlapping patterns of expression (Figure 1-6) and are predicted to be functional. Therefore transmission of heritable material occurs as predicted and the observed phenotype is attributable to *tel1tel2* genes.

4.1.3 *Arabidopsis TEL* genes have genetic redundancy

Finally, genetic redundancy, where a mutation in a gene may have little effect on an organism's phenotype if its activity is compensated for, or enhanced by, the function of a different gene or genetic pathway (Nowak et al., 1997; Kafri et al., 2006), may account for the absence of an observable phenotype in the single insertion *tel1* or *tel2* plants. The more similar the genes or predicted gene function is, the more likely these genes are to compensate for the loss of the partner (Kafri et al., 2006). Genes involved in maintaining precise regulation of plant organogenesis, such as those genes found in the SAM and RAM of plants (*TEL1* and *TEL2*), could reasonably be expected to have genes to compensate for their loss. On this premise genetic redundancy may also explain the subtle phenotype observed with *tel1tel2* plants (there are nine identified members in the *MEI2*-like gene family (section 1.4.8 and (Jeffares et al., 2004)) possibly compensating for the *tel1tel2* genes or contributing to masking a phenotype).

4.1.3.1 Members of the gene family compensate for the loss of other members

Evidence presented by Kaur et al., (2006) and Garrett Anderson (Cornell University, personal communication) support the concept of genetic redundancy and show that knocking out the *AML's* members will produce an increasingly evident phenotype. Garrett Anderson (Cornell University) identified an early flowering phenotype in *aml1aml4* mutants and Kaur et al. (2006) failed to obtain the *aml1aml4* mutants due to meiotic failure. However as functionally equivalent *TE1* and *PLA2* plants have profoundly affected architecture, it was expected that knocking out *TEL1* and *TEL2* (the *Arabidopsis* equivalents) would be sufficient to identify the role of this class of genes in *Arabidopsis* through mutant characterisation. The possibility exists that the alleles used in this study were not true knockouts but the nature of insertions suggests *tel1* and *tel2* genes are likely to be non-functional. RT-PCR failed to amplify transcripts corresponding to the sequences coding for the region of the gene conferring functionality (RRM3), suggesting *tel1* and *tel2* genes contain T-DNA inserts disrupting their function.

Each member of the *MEI2*-like gene family may have a different role to play during plant development. Jeffares, (2001), Anderson et al., (2004) and Jeffares et al., (2004) present a comparison of *AML* and *TEL* expression data highlighting that there are many similarities in the expression patterns of the *MEI2*-like genes suggesting these genes may have overlapping functions in the SAM and RAM. The presence of an additional seven members in the *MEI2*-like gene family in *Arabidopsis* mean there may be a degree of functional redundancy within the gene family in *Arabidopsis*. *MCT1* and *MCT2* then *AML1* and *AML4* are the next closely related to *TEL1* and *TEL2* (Figure 1-5).

Jeffares et al., (2004) show, with their comparison between *MEI2*-like proteins, there is low (RRM1 24 % and RRM2 28 %) to moderate (RRM3 57 %) conservation in amino acid residues between maize and rice members of the *MEI2*-like family. Gene products, closely related by sequences coding for similar proteins, can be expected to have a common function. Based on conservation at the protein level (Jeffares et al., 2004), the function of TEL, TE1 and PLA2 proteins are predicted to bind RNA. Both the *MCT*s and the *AML*s are thought to be functional in *Arabidopsis* because there appears to be conservation between *Arabidopsis* and sequences coding for equivalents proteins identified in rice (Jeffares et al., 2004). These conserved amino acids imply that genetic redundancy is a possibility.

4.1.3.2 *MEI2*-like transcript location is correlated to a phenotype

PLA2 and *TE1* transcripts are tightly regulated to specific regions of the meristem (Veit et al., 1998; Anderson et al., 2004; Kawakatsu et al., 2006). *TE1* expression in maize is restricted to semicircular bands surrounding the incipient leaf (P0), whereas *PLA2* transcripts in rice are present in the leaf margins of the incipient leaf (P0) as well in central regions of the previous leaf (P1). This SAM-focused expression of *PLA2* and *TE1* is consistent with the observed mutant phenotypes of reduced plastochron length and increased leaf maturation. Studies on *PLA2* in rice and *TE1* in maize show plants with non-functional *PLA2* and *TE1* have a reduced plastochron length, (65 % and 35 % respectively), which corresponds to an increased rate of organogenesis. Further, there is an increased rate of leaf maturation. Non-functional

pla2 genes in rice result in leaf maturation occurring over approximately 13 days in standard environmental conditions instead of the normal 30 days (Kawakatsu et al., 2006). No differences were documented in maize or rice roots, but as root phenotypes were not a focus, subtle phenotypes may have been missed.

Analysis of MEI2-like genes from Arabidopsis also reveals highly specific patterns of expression which are associated with the populations of cells comprising the SAM and RAM which are normally maintained in an undifferentiated state. TEL1 mRNA expression is broadly distributed in a non-uniform manner across the apical dome and has no obvious relationship to morphological landmarks. However expression can be characterised to occur in both the SAM and RAM; broadly in the apical dome in the incipient leaf (P0) and previous leaf (P1) of the vegetative meristem and in the central zone of the SAM and the QC of the RAM in embryos (Figure 1-6 and Alvarez 2002). *tel1* single T-DNA insertion mutants have no obvious phenotype (section 3.1.8) which could, based on previous in situ results, reasonably be attributed to its partner (TEL2) sharing functionality. TEL2 expression is in the apical initials in the central zone of the SAM and in the QC of the RAM (Figure 1-6 and Alvarez 2002). Closely related genes with similar expression patterns could be expected to have some overlap in function, and might at least partially compensate for each other. *TEL1* and *TEL2* could be expected to have overlapping functions and are more closely related by sequences and transcript location to *TE1* and *PLA2* than to other *Arabidopsis* members of the gene family (Figure 1-5).

4.2 TEL1 AND TEL2 GENE REGULATION

Previous analysis by *in situ* hybridisation shows *TEL* genes are expressed early in development and continue to be expressed throughout the life of *Arabidopsis* in populations of undifferentiated cells contained in the root apical meristem (RAM) and shoot apical meristem (SAM) (Alvarez, 2002; Anderson et al., 2004). During embryogenesis, *TEL1* expression is initially uniform in the globular embryo but becomes resolved to broad apical and basal domains in the early heart stage, and finally becomes restricted to the SAM and RAM in the late heart and torpedo stages.

Against this background, it seems expression could be at least partly be determined by factors influencing the establishment or function of apical meristems.

4.2.1 Sequences within the coding region of *TEL1* are required for expression in the SAM

The tissue specific accumulation of mRNA transcripts can be influenced by one or more processes, including mRNA production, mRNA degradation and mRNA movement between cells or between organs (Lucas et al., 1995; Kim et al., 2005a). Experimentally, it is possible to distinguish processes that influence the rate of mRNA production, or transcription, by fusing elements such as promoters, or enhancers to heterologous reporter genes. Recapitulation of the mRNA expression pattern by such a reporter can be taken as evidence for regulation at the level of transcription since in most cases, tissue specific patterns of reporter movement, or transcript stability can be discounted. To gain insight into whether regulated transcription might account for the patterned expression of the *TEL1* gene, a series of reporter fusions were analysed. Interestingly the -1900 P_{TEL1}::ER-GFP construct containing most of the *TEL1* promoter sequence did not give expression in the SAM suggesting elements essential for correct SAM expression were missing. However the RAM and SAM expression pattern was observed with the -2023 *TEL1*::GUS construct containing all sequences predicted by TAIR to be associated with *TEL1* (compare Figure 3-13 with Figure 3-18). The possibility remains that there were promoter elements present in the 11 base pairs that were missing adjacent to the ATG start codon or in the 112 bases missing in the 5' region of the promoter sequence. These bases are present in the GUS construct. Further the 11 bases adjacent to the ATG were present in subsequent promoter deletion constructs (-1000, -840, -600 and -500) and SAM expression was not restored suggesting these 11 bases were not instrumental for the SAM expression pattern. The 112 bases at the 5' end of the promoter could conceivably contain motifs directing expression to the SAM but it is more likely there are sequences in the introns of the gene that perform this role (Sieburth et al., 1995; Deyholos and Sieburth, 2000; Baurle and Laux, 2005). The results showing SAM expression in plants containing the GUS construct but absent in plants containing the -1900 construct indicate that elements required for the specific transcription of *TEL1* in the RAM versus the SAM can be separated. Furthermore this data suggests sequences

within the transcribed region of the gene are required for expression in the SAM. However these results do not distinguish between several alternative explanations for the SAM expression since the transcript contains *TEL1* sequences.

The recapitulation of the *TEL1* SAM pattern by the -2023 *TEL1*::GUS construct cannot distinguish between transcript up-regulation, stability or movement since the reporter transcript also contains *TEL* sequences that might influence its stability, or movement. The -2023 *TEL1*::GUS fusion protein might contribute to the pattern if *TEL1* protein usually moves or is stable in certain tissues. A second consideration relates to how the behaviour of the TEL1 protein might influence the pattern of reporter activity seen. In this case these sequences affect transcript behaviour but they also lead to the production of a reporter molecule, which may have novel characteristics not seen with the native reporter protein, including mobility and stability related properties.

Precedence for sequences contained within transcribed regions of the gene affecting transcript levels is provided by *AGAMOUS*. The data presented in this thesis show SAM GFP expression is not evident in plants containing the -1900 P_{TEL1}::ER-GFP reporter construct but is present in plants containing the -2023 *TEL1*::GUS reporter construct, suggesting *TEL1* DNA regulatory sequences may be present within intragenic regions of the gene. Research looking at deletions of the *AGAMOUS* gene determined enhancer elements (which increase the rate of transcription) were important for regulation and providing determinacy to the floral meristem (Sieburth et al., 1995; Deyholos and Sieburth, 2000). Furthermore normal *AGAMOUS* expression patterns are dependent on small regions, not necessarily adjacent to each other. Finally, transcript levels were still able to be enhanced when control sequences within the transcribed regions, that were acting as enhancer element, were placed upstream of the transcriptional start (Deyholos and Sieburth, 2000). An approach, similar to *AGAMOUS* studies where small regions of control sequences were placed upstream of the translation start, could be used to assess whether *TEL1* acts in a similar way.

4.2.2 *TEL1* promoter deletion analysis shows regulated transcription explains QC limited expression

Recapitulation of the QC pattern by plants containing the -1900 P_{TEL1}::ER-GFP construct (Figure 3-18) is a strong indication that *TEL1* promoter sequences account for *TEL1* QC expression pattern. Alternatively the GFP reporter might not accurately represent the location of TEL1 protein because GFP is responding to the signals from *TEL1* promoter DNA in a different manner from which TEL1 protein would respond. On the basis of the deletion studies the *TEL1* promoter region between -1000 and -840 was identified as containing elements necessary for RAM expression. Regions upstream of the coding regions of *TEL1* and *TEL2* (compared using the PLACE programme analysis, section 3.2.5) did not contain any common statistically significant motifs. Given the similarity between the promoters of *TE1* and *PLA2* (Jeffares et al., 2004), it is surprising there are only three regions of similarity shared between *TEL1* and *TEL2*, and indeed the rest of the gene family that encode the RRMs (RNA Recognition Motifs). While it is conceivable that sequences within these elements could function as enhancers, with the lack of other evidence, their conservation is most reasonably viewed as reflecting constraints on protein function. In view of not identifying anything of significance with the analysis *in silico* (presented by the PLACE programme, section 3.2.5) motifs were not investigated further due to time constraints but could be the basis of future studies.

4.2.2.1 Considerations on the use of reporters

Research in this thesis used two comparable reporter constructs for reporting on the location of the *TEL1* transcript. GUS (a 69 kDa protein) and GFP (a 27 kDa protein) reporters can be thought of as comparable as previous studies have reported only minor differences between them. GFP is the reporter of choice for detection of *TEL1* in the RAM as GFP permits imaging of living tissue. However GFP is not as useful as a reporter in the shoot because GFP expression becomes masked by chlorophyll autofluorescence and layers of leaf primordia effectively mask internal tissues which are difficult to dissect and resolve in an unfixed state. GUS is used as a reporter to monitor the regulation of *TEL1* in the SAM because GUS can be visualised in dissected and non-living material.

The addition of GUS and GFP to the C-terminal end of a gene in a gene -fusion construct is generally accepted to accurately represent the location of the protein of interest (Mantis and Tague, 2002). However the ER-GFP version of the GFP reporter, which is the version used in this study, is targeted to the endoplasmic reticulum and has been shown to be restricted to the original site of gene expression (Kim et al., 2005a). In addition, some mRNA or proteins may undergo intercellular movement in the early stages of embryo development (Kim et al., 2005a). However, the GFP signal seen here is localised to the RAM and reflects the *in situ* pattern (Anderson et al., 2004) so cellular movement within the embryo can be discounted.

There are constraints to be considered when interpreting information on the location of a transcript based on the vector used for the reporter constructs. The backbone used for the GUS reporter constructs is 1391Xb, a "use and fuse" vector from pCAMBIA. This backbone contains a 35S promoter driving a kanamycin antibiotic resistance marker (Figure 3-12 A). The 35S promoter has been shown to up-regulate the expression of the gene of interest and alter the location and intensity of the reporter gene product (Yoo et al., 2005). The 35S promoter driving an antibiotic resistance marker (such as kanamycin) has been demonstrated to result in GUS expression in vascular tissue. The *in situ* results (Anderson et al., 2004) indicate that *TEL1* expression is limited to the RAM and SAM and is not evident in the vascular tissue. The presence of GUS in the vascular tissue can, on the basis of previous *in situ* results and on the presence of the 35S promoter in the GUS construct, be considered to be 'leaky' GUS expression.

Another form of 'leaky' GUS expression is where diffusion of GUS occurs. The GUS expression in the vascular tissue seen in this research is 'leaky' in that the vascular tissue pattern disappeared with treatments (increasing concentrations of potassium ferricyanide and potassium ferrocyanide) limiting the diffusion of GUS to within the cells it is produced (Figure 3-13 F and G). The GUS expression in the adaxial side of the cotyledons is not consistent with what is seen by *in situ* hybridisation and can be ignored because it can be attributed to persistence of GUS (in heart embryos *TEL1* expression pattern is broadly across the apical dome including in the cotyledon region).

The GUS pattern in the RAM and SAM (Figure 3-13 F and G) is then similar to the expression seen with *in situ* hybridisation techniques (Alvarez, 2002; Anderson et al., 2004) and the GUS expression pattern in the root is similar to the GFP expression (the backbone used for GFP was a pBIN vector which does not contain a 35S promoter).

4.2.3 Plant hormones regulate *TEL1* expression

In addition to defining regulatory elements that are part of the *TEL1* gene that influence its expression, an analysis was conducted to examine the influence of hormones. Many researchers have looked at the role of plant hormones during plant growth and development and have concluded hormones regulate a wide variety of developmental processes ranging from patterning to regulating cell division and cell expansion in the RAM and SAM (Evans and Poethig, 1995; Gomez-Cadenas et al., 2001; Rashotte et al., 2003; Reinhardt et al., 2003b; Cheng et al., 2004; Wang et al., 2005a; Wang et al., 2005b; Weijers et al., 2005; Woodward et al., 2005; Lindsay et al., 2006; Riefler et al., 2006). Plant hormones are internally-secreted small signal molecules which can cause an alteration in cellular processes even when present at low concentrations (Salisbury and Ross, 1992). The results presented in section 3.2.6 in chapter 3 tests the effect *in vivo* of NAA, zeatin, gibberellic acid on the GFP reporter driven by the *TEL1* promoter. TIBA or NPA, auxin transport inhibitors, were assessed as an aid to understand the role of auxin on *TEL1* gene expression. *Arabidopsis* plants containing the P_{DR5}::ER-GFP construct were assessed to confirm whether treatments were altering endogenous levels of auxin during plant development. P_{DR5}::ER-GFP has been shown to report on the presence of natural auxin (IAA) in the plant root tips (Sabatini et al., 1999; Friml et al., 2003b; Weijers et al., 2005). The P_{DR5}::ER-GFP construct has nine tandemly-arranged auxin response elements (AuxREs) so higher cellular concentrations of auxin will be reflected as increased GFP expression which can then be detected through the use of confocal microscopy (Friml et al., 2003b). GFP expression, when driven by DR5, in the QC and columella cells of the RAM, is proposed to represent the site of pooled auxin (IAA) in plant root tips (Friml et al., 2002; Friml et al., 2003b).

4.2.3.1 Exogenously applied auxin increases *TEL1* promoter activity

Given one of the hallmarks of *TEL1* is its specific expression in the QC it was of interest to explore factors essential for establishment and maintenance of the QC tissue (Friml et al., 2002; Friml et al., 2003b). As previously discussed GFP expression, when driven by the *TEL1* promoter, is normally restricted to the QC of the RAM (Figure 3-16 A). On application of NAA, *TEL1* promoter driven GFP became evident in cells surrounding the QC, in the apical initials and in the columella initial cells (Figure 3-20 C) indicting this GFP expression in mature roots may normally be restricted to the QC by limiting concentrations of auxin. Wild type plants containing the auxin responsive element (DR5) driving the GFP reporter had GFP expression in the QC and columella cells (Figure 3-20 E), reiterating the GFP pattern of expression in the QC and columella cells seen in other research (Friml et al., 2003b). In the presence of exogenous NAA, GFP expression when driven by the DR5 promoter is evident in the columella cells and the stele cells as well as in the QC (Figure 3-20 G). This expansion of GFP expression suggests NAA treatments augment auxin levels in specific parts of the plant, and are consistent with previous reports that this expression reflects the location of auxin pools in the RAM.

4.2.3.2 Exogenously applied auxin transport inhibitors reduces *TEL1* promoter activity

To further explore the dependence of QC-limited *TEL1* promoter activity on auxin, the affect of auxin transport inhibitors TIBA and NPA were tested (Fukaki et al., 2005). To confirm TIBA and NPA prevented endogenous auxin from reaching the root tip, plants containing the P_{DR5}::ER-GFP construct were also assessed. Prior to exposure to NPA, GFP expression, when driven by the DR5 promoter, was restricted to the outer layer of columella cells and the columella root cap cells (Figure 3-20 L). With continued exposure to NPA (six hours of exposure), the GFP expression became less detectable in the roots (Figure 3-20 M) and undetectable in the root after 12 hours (Figure 3-20 N). Therefore, in the conditions used in these studies, NPA leads to the depletion of endogenous auxin from the root tip.

In plants treated with exogenous NPA, *TEL1* promoter driven GFP expression decreased over a period of 12 hours (compare Figure 3-20 I to K) suggesting *TEL1* expression in the QC depends on auxin or some auxin inducing factor(s). Together with results showing expansion of expression when exogenous auxin is added, these results suggest the *TEL1* promoter activity appears to be dependent and positively regulated by auxin. Indeed, by this model, the restricted activity of the *TEL1* promoter to the QC might be explained by the auxin maximum that is thought to be located over this region (Weijers et al., 2005) but also indicates other factors limit expression of *TEL1* beyond what is observed with auxin.

4.2.3.3 Exogenously applied zeatin increases *TEL1* promoter activity

Cytokinins are a group of compounds named for their ability to promote cell division and plant growth (Salisbury and Ross, 1992). Kinetin was the first cytokinin discovered to promote cell division but zeatin, isolated from maize (*Zea mays*) is the most common form of naturally occurring cytokinin. Cytokinins, such as zeatin, are thought to influence the identity of cells in the meristematic regions of the plant, the SAM and RAM, by regulating root growth and lateral root development, nutrient mobilisation and seed germination by promoting growth (Werner et al., 2003; Rashotte et al., 2003). Cytokinins control the exit of cells from the root meristem through interactions with cytokinin receptors such as *CRE1*, *AHK2* and *AHK3* and mediate the induction of cell division by increasing transcription of type-A *Arabidopsis* response regulators, which in turn degrades cytokinins (Rashotte et al., 2003; Bishopp et al., 2006). *CRE1* (allelic to *WOODEN LEG*) was also described as having an altered root pattern (Scheres et al., 1995). Thus the application of exogenous zeatin, to *Arabidopsis* roots expressing GFP under the *TEL1* promoter, was expected to alter GFP expression. The application of exogenous zeatin to mature roots of plants containing the P_{TEL1}::ER-GFP construct results in GFP expression expanding to include the QC, cortex and endodermal cells, not just the QC cells (Figure 3-20 D). The application of exogenous zeatin to mature roots of plants containing the P_{DR5}::ER-GFP construct gave a similar result to that observed by the addition of exogenous NAA – GFP expression is present in columella root cap cells and the stele cells as well as in the QC and columella cells (Figure 3-20 H).

Cytokinin and auxin are thought to work in concert toward organ initiation and growth (Laplaze et al., 2005). When treated with cytokinin the presence of GFP, when driven the DR5 promoter, in the QC and columella cells indicates auxin is still located in the QC and distal to the QC, but that the columella cells containing auxin are not as tightly defined (Figure 3-20 H). When treated with cytokinin the presence of GFP, when under the *TEL1* promoter, in the QC, cortex and endodermal cells indicates that cytokinin has an effect on *TEL1* that is different to auxin and may be mediated independently of auxin (Figure 3-20 D). Further research investigating *TEL1* in an auxin insensitive and in a cytokinin insensitive mutant background may reveal whether the response to cytokinin and auxin are coupled. In addition using a repressor of auxin response factors (such as INDOLE-3 ACETIC ACID 14) (Fukaki et al., 2005) or a cytokinin receptor (such as *Arabidopsis* HISTIDINE KINASE 4) (Inoue et al., 2001; Ueguchi et al., 2001) under the control of *TEL1* promoter could provide further information about the response of *TEL1* to auxin and cytokinin.

4.2.3.4 Exogenously applied GA_3 increases *TEL1* promoter activity

Gibberellins are derived from the ent-gibberellane skeleton and are diterpene acids synthesized from acetyl CoA *via* the mevalonic acid pathway (Salisbury and Ross, 1992). They consist of 19 or 20 carbon units grouped into either four or five ring systems. Gibberellins are named $GA_{1,}$ GA_2....GA_n in order of discovery. Gibberellic acid (GA_3) belongs to a complex family of diterpenoid compounds and have been shown to be endogenous regulators of plant growth (Salisbury and Ross, 1992). Gibberellic acid, which was the first gibberellin to be structurally characterised, is GA_3. There are currently 136 GAs identified from plants, fungi and bacteria. Gibberellins, such as gibberellic acid (GA_3) are thought to influence the identity of cells in meristematic regions of the plant (Evans and Poethig, 1995; Fu and Harberd, 2003; Achard et al., 2004; Cheng et al., 2004) through promoting cell expansion. Gibberellins also actively repress light-regulated genes in dark grown seedlings and during seed germination (Alabadi et al., 2004). KNOX proteins repress transcription genes encoding GA 20-oxidases which in turn represses GA biosynthesis at the shoot apex of *Arabidopsis* and promotes cell division and meristem function by activating

cytokinin (Jasinski et al., 2005). Negative feedback regulation of GA_5 expression, a GA 20-oxidase gene, operates at the level of transcription in elongating stems of *Arabidopsis* suggesting active GA's play an important role in maintaining the plant's height within a certain range and the deactivation of GA results in plants of smaller stature (Xu et al., 1999). The height of plants with non-functional *tel1tel2* genes could become altered when treated with GA_3. Furthermore severely GA-deficient *Arabidopsis* mutants, such as ga1-3, exhibit retarded growth of roots reflecting the removal of the moderating effects of DELLA growth-repressing protein on GA levels (Fu and Harberd, 2003). The data presented in this thesis shows the presence of exogenous GA_3 gives GFP expression in all apical initial cells and in the columella cells in mature roots in plants containing the -1900 P_{TEL1}::ER-GFP construct (Figure 3-20 B). GFP expression, when driven by the *TEL1* promoter, is no longer restricted to the QC in the RAM suggesting GA_3 also influences the expression of *TEL1*. However there is no difference in the location of auxin in the GA_3 treated P_{DR5}::ER-GFP containing plants (Figure 3-20 F). Once again the results presented here provide no direct evidence of the role of *TEL1* during plant development and this interaction needs to be studied further, perhaps using GA insensitive plants.

4.2.4 *TEL1* promoter activity reflects changes in hormone levels

The hormone interaction results outlined above show *TEL1* expression levels are affected by a cytokinin (zeatin), a gibberellin (GA_3), an auxin (NAA), and auxin transport inhibitors (TIBA and NPA). Extrapolation from this data suggests hormones causing a perturbation in the QC or promoting growth can be thought of as influencing *TEL1* gene transcription. Hormones may increase transcription, the rate of degradation of proteins, or alter the capacity of the protein to move throughout the plant. While this study was unable to determine the functional significance of these hormones on *TEL1*, the addition of NAA, GA, and zeatin results in *TEL1* promoter driven GFP expression being evident not only in the QC but also in the surrounding apical initials (and in some cases the cells surrounding those), suggesting *TEL1* responds to changes in hormone levels, perhaps as a reflection of the change in cellular identity. A change in the level of NAA, GA, and zeatin could be considered to cause an alteration of the identity of the cells surrounding and including the QC as

reflected by a change in gene expression corresponding to a particular cell type or the division, expansion, or other properties of those cells (Berleth et al., 2004; Woodward and Bartel, 2005). Because of the limited time frame in which these studies were conducted, it is difficult to distinguish whether the changes in *TEL1* promoter activity reflect changes in cellular identity, versus more direct responses to the hormone treatments themselves which are less dependent on cellular identity cues.

4.2.5 Genetic regulation of expression

The research outlined above considered whether *TEL1* gene expression is influenced by factors, such as auxin and cytokinins, which might provide positional information and thus specify cellular location. In addition to this type of physiological approach, a genetic approach was used to assess how the regulation of *TEL1* might be depend on the activity of other genes. By identifying such genes, and noting their function, further clues to *TEL1* function might be gained. Given the highly localised activity of the *TEL1* promoter in the QC, it was of interest to examine the influence of genes such as *SCARECROW* (*SCR*), *SHORTROOT* (*SHR*), and *PLETHORA* (*PLT*) which have roles in defining the QC and the regular cell files found in *Arabidopsis* roots. *SCR*, *SHR*, and the *PLT* genes together specify meristematic cells and have similar or overlapping domains of expression to the *TEL* genes (Figure 1-7). To determine where *TEL* genes fit in the regulatory cascade, *TEL1* expression was assessed in *scarecrow* (*scr*), *shortroot* (shr), and *plethora* (*plt*) mutant backgrounds by assessing GFP expression, when driven by the *TEL1* promoter, in plants containing the -1900 P_{TEL1}::ER-GFP construct and mutant for *scr*, *shr*, or *plt* genes.

4.2.5.1 Characterised root mutant analysis

PLT, an AP2 class putative transcription factor, is expressed in root apical initial cells (cortical endodermal stem cells, epidermal initials, and stele stem cells) but not in the QC. Plants mutant for *plt* also have irregular cell files in the root and terminate growth prematurely, but have many lateral roots that also terminate prematurely (Aida *et* al., 2004). *SCR* is expressed in the QC, cortical endodermal stem cells, and endodermal cells. Plants mutant for *scr*, a transcription factor, appear to have fused

endodermis and cortex cells (Nakajima and Benfey, 2002). *SHR* is expressed in stele stem cells and stele cells. Plants mutant for *shr* have irregular cell files in the root, usually terminate growth prematurely and produce more root hairs (Heidstra et al., 2004). Sabatini et al., (2003) show QC25 (QC marker gene) expression is absent in *shr* and *scr* mutant plants and suggests this indicates partial loss of QC identity in these mutants.

Plants containing the -1900 P_{TEL1}::ER-GFP construct in the *scr* mutant background, and plants containing the -1900 P_{TEL1}::ER-GFP in the *shr* mutant background had a *TEL1* promoter driven GFP expression range (Figure 3-22) corresponding to the location of the presumptive QC. The tightness of GFP expression appeared to be reduced in *shr* and *scr* mutant backgrounds (Figure 3-22). GFP expression in an increased number of cells corresponds to cells appearing to have QC properties and not to cells becoming differentiated, suggesting the transcription of *TEL1* in the QC does not require the activity of either *SHR* or *SCR*. However, because of the mis-specification of the cortex and endodermal cells in the corresponding mutants, the activities of these genes are capable of influencing *TEL1* expression indirectly.

In plants containing -1900 P_{TEL1}::ER-GFP in a *plt1plt2* mutant background, GFP expression is absent at all stages of growth (Figure 3-22 C). In *plt1plt2* where the QC fails to form, *TEL1* promoter driven GFP expression is no longer evident, suggesting factors required for QC specification and QC identity may be involved in promoting *TEL1* transcription. This dependence relationship can be contrasted with what is seen with *WUSCHEL LIKE HOMEOBOX5* (*WOX5*) (Sarkar et al., 2007). Like *TEL1*, *WOX5* is expressed in the QC of the RAM and is postulated to be required to maintain the QC. Sarkar et al., (2007) show *SHR / SCR* activity is required for *WOX5* expression. However in *plt1plt2* double mutants *WOX5* expression is occasionally slightly expanded suggesting *plt1plt2* has a minor role in confining *WOX5* expression to the quiescent centre (Sarkar et al., 2007). However, *TEL1* is influenced by auxin differently to *WOX5*. With the addition of auxin *TEL1* expression is in all apical initials (Figure 3-20 C) but *WOX5* expression is only in the QC and columella initials (Sarkar et al., 2007) suggesting *TEL1* has a different controls to *WOX5*. As *PLT* genes are involved in the initiation of the meristem and *SHR* and *SCR* are important in the maintenance of the regular cell files found in the roots it is not surprising *TEL*

gene expression (with the predicted function of maintaining the meristem) should be thus affected in a *plt1pl2* mutant background where there is no meristem.

4.3 BIOLOGICAL FUNCTION OF TEL GENES

This research set out to clarify if the biological function of *TEL1* and *TEL2*, members of the *MEI2*-like gene family, is to contribute to the growth and development of *Arabidopsis* by preventing cellular differentiation. An account of how this ascribed function fits with what is portrayed for two other members of the *MEI2*-like family, *TE1* and *PLA2* is presented.

TEL1 and *TEL2* genes may function by enabling plants to respond in a controlled manner to growth promoting factors, such as plant hormones, which under certain conditions may be more pronounced. Results obtained with *tel1tel2* mutant plants grown in altered environmental conditions, such as with the addition of exogenous NAA, zeatin, GA_3, or in short days, suggest *TEL* expression levels are affected by anything causing a perturbation in the apical meristem. Moreover *TEL1* expression in the QC and surrounding initials responds to changes in hormone levels and to perturbations in growing conditions (high temperatures and high sugar availability) suggesting *TEL1* could confer one aspect of QC identity. Furthermore in *shr* and *scr* plants where cortex or endodermal cells are misrepresented, *TEL1* expression is retained in the population of undifferentiated cells, the presumptive QC, but in *plt1plt2* plants where the QC fails to form, *TEL1* expression is missing.

Sequences included in the upstream region of *TEL1* gene are sufficient to drive *TEL1* gene expression in the RAM but not in the SAM (Figure 3-13 and Figure 3-18). Additional intragenic sequences are required for SAM expression. *TEL1* expression occurs in early embryos in all undifferentiated cells but is absent in differentiated suspensor tissue and only limited expression in the undifferentiated RAM and SAM in the heart stage embryo implies the role of *TEL1* and *TEL2* is likely to be crucial for plant development and suggests removing *TEL* gene function could result in embryo lethality at the young heart stage embryo. However, knockout data has shown functionally knocking out *TEL1* and *TEL2*, two members of the gene family, affects organogenesis only slightly.

The model proposed for the maize and rice gene equivalents suggests *TE1* and *PLA2* act to prevent differentiation *via* the negative regulation of leaf initiation (Veit, 1998; Paquet et al., 2005; Kaur et al., 2006). Conversely, research looking at *AML1* and *AML4* suggests *MEI2*-like genes may have a role to play during meiosis through an involvement in chromatin organisation as well as vegetative growth (Kaur et al., 2006). However as *AML1* and *AML4* are more distantly related to *TE1* and *PLA2* than *TEL1* and *TEL2*, the evidence presented by Kaur et al. (2006) may only reflect that there is genetic redundancy in the *MEI2*-like gene family. Furtherthe role of *TEL1* and *TEL2* in plant development is different to *AML1* and *AML4* and more closely aligned with preventing differentiation similar to *TE1* and *PLA2*.

4.3.1 Comparisons of gene activity between plant species

Angiosperms (flowering plants) split from Gymnosperms (cone bearing or naked seed plants) approximately 345 to 280 million years ago during the Carboniferous period (Bold et al., 1987; Schmidt and Schneider-Poetsch, 2002). The dicotyledonous (dicot) and monocotyledonous (monocot) clades of angiosperms separated approximately 150 million years ago in the late Jurassic–early Cretaceous period (Wikstrom and Kenrick, 2001; Chaw et al., 2004). While comparisons may be drawn between monocot plants, rice and maize and a dicot plant, *Arabidopsis* there are differences in plant developmental processes between monocot and dicot plants to be acknowledged (Sylvester et al., 1990; Aida et al., 1997; Aida et al., 1999). These are discussed below in relation to how members of the *MEI2*-like gene family are described as functioning in both this research and in previous studies conducted by Kawakatsu et al. (2006) and Veit et al. (1998).

4.3.1.1 *MEI2*-like genes prevent differentiation

Kawakatsu et al. (2006) propose a model where *TE1* and *PLA2* act non-cell-autonomously in the shoot apical meristem to inhibit organogenesis. During leaf initiation in maize (a monocot) *TE1* expression is restricted to semicircular bands surrounding the incipient leaf (P0) indicating *TE1* is involved in regulating the initiation of new leaves (Kawakatsu et al., 2006 and Veit et al., 1998). Furthermore,

Kawakatsu et al. (2006) suggest the formation of 65 % more leaves in rice compared with 35 % more leaves in maize is because *PLA2* is expressed in rice at a later developmental stage than *TE1* is expressed in maize. *PLA2* transcripts in rice are present in central regions of the previous leaf (P1) as well in the leaf margins of the incipient leaf (P0) (Kawakatsu et al., 2006). Kawakatsu et al., (2006) suggested this is because leaf maturation rates, as well as leaf initiation, may be affected if *PLA2* is not functional. In *pla2* mutants there is an increase in the number of leaves (65 % more leaves) and a concomitant decrease in leaf size (the length of blade and sheath and the width of leaf blade is decreased due to a reduction in cell number), suggesting the rate of leaf maturation is increased. In rice, with non functional *pla2* genes, leaves mature over approximately 13 days versus 30 days (Kawakatsu et al., 2006). There is also a shortening of internode length. However, as *PLA2* is not expressed in the stem, plants with shortened internode lengths are thought to be a reflection of the shortened leaf maturation time.

Following the model proposed by Kawakatsu et al. (2006), where the location of *TE1* results in an increased rate of leaf initiation and the location of *PLA2* results in an increased rate of leaf initiation and maturation, it could be expected that *Arabidopsis* plants with non-functional *TEL1* and *TEL2* genes will also have an increased rate of leaf initiation and maturation due to the absence of *TEL1* and *TEL2* gene expression in regions of the meristem analogous to rice *pla2* location. Increased leaf initiation is supported by the slight increase in number of leaves observed in *tel1tel2* mutants (Table 16, Table 17 and Table 18). However during leaf maturation developmental processes differ between monocots and dicots (Sylvester et al., 1990; Aida et al., 1997; Aida et al., 1999; Zimmermann and Werr, 2007). As measurements were not made on *tel1tel2* leaf size (width and length) direct comparisons cannot be drawn regarding rate of maturation and is an area for further research.

Root growth occurs in defined regions: the root cap where cells periodically get sloughed off as the root pushes through the soil; the root meristem where cells divide and form the regular cell files found in *Arabidopsis*; the elongation zone where those cells formed from the root meristem elongate and expand; and the differentiation zone where lateral root formation is initiated. Expression work in the root, carried out during this study, indicates *TEL1* is in the QC in the RAM once the meristem

becomes defined and the regular cell files are established (Figure 3-13, Figure 3-17 and Figure 3-18). *Arabidopsis tel1tel2* mutants had longer roots than their wild type counterparts suggesting there is unregulated root growth occurring in plants mutant for *tel1* and *tel2*, possibly as a result of QC cells constantly differentiating. However the differences were subtle, and as root length was not assessed in maize and rice, comparisons cannot be made between root growth in *Arabidopsis tel1tel2* mutants and *pla2* or *te1* mutants. Caution is required when interpreting these results and conclusions about unregulated growth should be reassessed after looking at *pla2* and *te1* roots. Live imaging of root development in *TEL1TEL2* and *tel1tel2* plants would provide interesting information about root growth and QC maintenance.

The comparison between rice, maize and *Arabidopsis* is complicated by rice and maize being monocots and *Arabidopsis* being a dicot where leaf developmental processes can differ. *TEL1* and *TEL2* genes in *Arabidopsis* may be acting in a similar manner to *PLA2* in rice and *TE1* in maize but the effects may be masked by the differences in growth and development of leaves in monocot and dicot plants. While there is a reduction in plastochron length in rice and maize plants with either non-functional *PLA2* (65 %) or *TE1* (35 %) genes, *TEL1* and *TEL2* may not be expected to reduce plastochron length to a corresponding level in *Arabidopsis.* The level of reduction in plastochron length in *Arabidopsis* may be subtle as the rate of leaf maturation could already be accelerated as indicated by the compact rosette growth habit of *Arabidopsis*.

4.3.1.2 *MEI2*-like genes regulate progression towards meiosis

Research on *MEI2* in yeast (Hirayama et al., 1997; Watanabe et al., 1997) suggests *TEL1* may be regulated at the gene sequence level rather than at the protein level. *MEI2* was first described as a master regulator of meiosis when it was shown MEI2 protein is required at least twice, once prior to pre meiotic DNA synthesis and again prior to meiosis 1 (Watanabe and Yamamoto, 1994; Watanabe et al., 1997). Specifically, *MEI2* has been shown to be localised to the *SME2* gene on chromosome one and occupies a fixed position in the horse-tail nucleus (Watanabe et al., 1997; Shimada et al., 2003). The mechanism enabling MEI2 protein in yeast to function as

a master regulator of meiosis has recently been described (Harigaya et al., 2006). Harigaya et al. (2006) show MEI2 protein interacts with *MMI1* (and other meiosis-specific genes such as *SSM4*, *REC8* and *SPO5*) in mitotic cells and sequesters them at a specific location in the nucleus. Once the cells switch from the mitotic to meiotic state, MEI2 protein was no longer sequestering *MMI1* and the cells underwent meiosis. This raises the question of whether *TEL* genes function to prevent untimely meiosis in *Arabidopsis* like *MEI2* does in yeast. Previous research suggests the function is likely to be different as *AML1* (one member of the *MEI2*-like gene family) was shown to be involved in meiosis similar to *MEI2*, but *MEI2* had a wider functional control (Hirayama et al. 1997).

The research present in this thesis coupled with the data on maize and rice (Veit et al., 1998; Kawakatsu et al., 2006) suggests *TEL* genes are important in regulating organ development. However MEI2 in yeast regulates the switch from mitotic to meiotic state. Perhaps instead of regulating organ development, *TEL* genes also regulate the transition from vegetative to floral state *via* the sequestering of inflorescence specific genes in a manner similar to what occurs in yeast. If *TEL* genes regulate organ development then the stature of *Arabidopsis* inflorescences and the number of organs could be expected to be altered in plants with non-functional *TEL* genes. This research provides no evidence to suggest non-functional *TEL* genes affect the height of the inflorescence but there is a subtly increased rate of organogenesis corresponding to subtly delayed flowering (more leaves form before the inflorescence forms and flowering time is calculated with respect to number of leaves (Michaels et al., 2005). This data supports the MEI2 model where *MEI2*-like genes could be expected to result in an earlier transition from mitosis (vegetative meristem) to meiosis (floral meristem and floral determinacy). The possibility remains that *TEL* genes may not be the only regulators involved in sequestering meiosis-specific genes in *Arabidopsis* and that other members in the *MEI2*-like gene family may be compensating for the *TEL* genes.

5 FUTURE WORK

5.1 MUTANT ANALYSIS

Looking at the single mutants under stressful conditions may tease out the single mutant phenotype. Experiments using NAA, GA_3, zeatin, mannitol, sucrose, long days and short days would provide a comparison with the results presented for *tel1tel2* double mutants. Leaf width and length measurements should be taken to allow comparison between *Arabidopsis* and the data obtained on maize and rice (Kawakatsu et al., 2006). This would then provide clues as to whether *TEL1* and *TEL2* are acting redundantly or whether they have different roles in plant development.

To provide further evidence *tel1* and *tel2* genes are responsible for the observed phenotype the *tel1tel2* double mutant should be recreated using different knockout alleles and the *tel1tel2* plants complemented through the addition of functional genes.

To complement root mutant studies, plants containing the -2023 *TEL1*::GUS construct should be assessed with respect to known shoot mutants such as *wus*, *clv*, and *stm*. For characterisation during early developmental stages, within embryos, GFP could be used as the reporter to provide a direct comparison with the studies already conducted in the root. This second analysis of the root material would provide confirmation of whether the root and shoot process are able to be separated.

Knocking out increasing numbers of *MEI2*-like genes would test the idea of genetic redundancy occurring within the gene family. Knockout plants with increasing numbers of *MEI2*-like genes could be made by crossing *tel1tel2* mutants with *mct1mct2* and *aml1aml4* mutants. This may result in the *tel1tel2* observed phenotype becoming more pronounced. However based on the phenotype described by Kaur et al. (2006) these plants may have disrupted meiotic divisions which could lead to lethality. To fully explore the possibility of meiotic failure with non-functional *MEI2*-like genes, studies similar to those conducted by Kaur et al. (2006) should be

undertaken. One way of creating plants with multiple genes knocked out is to use RNA-interference (RNAi). The function of over 4000 genes in Caenorhabditis elegans has been determined through a process known as RNAi (Fraser et al., 2000; Gonczy et al., 2000). RNAi, where double stranded RNA inserted into an organism triggers specific RNA degradation, facilitates targeted post-transcriptional gene silencing (Fire et al., 1998). Alternatively transgenes designed to express double-stranded or single-stranded self-complementary (hairpin) RNA have a similar and effective post-transcriptional silencing effect in plants (Waterhouse et al., 1998; Smith et al., 2000; Wang and Waterhouse, 2000; Wesley et al., 2001). Sequences required to knock out the *AML*, *MCT* and *TEL* genes would have to be stacked into one construct as there is insufficient similarity between the members of the *MEI2*-like family to make just one construct that would knock out all members of the *MEI2*-like gene family. If this approach was to be taken it would be advisable to place the RNAi or hairpin RNA within an estradiol or a dexamethasone inducible system in case silencing results in embryo death. Both these inducible systems allow genes to be over-expressed or silenced at specific stages in development after the addition of inducing substance (either estradiol or dexamethasone) which activates gene expression when desired (Craft et al., 2005; Brand et al., 2006).

The *tel1tel2* phenotypic assessment presented in this thesis shows a subtle increase in number of leaves and longer roots. The research detailed in this thesis did not determine whether *TEL1* expression is up-regulated prior to or post cell division. Looking at the point at which *TEL1* becomes regulated during the cell cycle could provide more information on how these genes are preventing differentiation by identifying the point of regulation. To determine if this subtle increase in rate of differentiation is through perturbation of a stage of the cell cycle, an analysis of cellular differentiation and the promotion of cells to divide should be undertaken. This could be done using a confocal microscope and other live imaging techniques (Lee et al., 2006; Reddy et al., 2007) in conjunction with isolated cells that divide synchronously.

Comparison between mutant and wild type plants through the use of a scanning electron microscope could clarify if the increased production of leaves is through a change in cell size in the apex. However, given that the predicted function of TEL1

protein is to bind RNA and possibly sequester products to a specific location, a better approach would be to determine what *TEL* genes interact with. Further work using *tel1tel2* mutants could be undertaken to clarify interactions with *PLT*. Characterisation of *PLT* expression and location in a *tel1tel2* mutant background will determine the nature of the *tel1tel2* interaction with *PLT*.

Given that the observed phenotype is subtle, an enhancer screen may provide additional information on interacting partners of the gene(s) of interest. This could be conducted through the screening of an ethane methyl sulfonate (ems) treated double mutant population. Plants showing an enhanced phenotype could be assessed through the identification and cloning of the interacting genes.

Arabidopsis is a eudicot with a compressed rosette. To determine if the differences between the phenotype observed in the monocots (maize and rice) and the eudicot (*Arabidopsis*) is because of the compressed growth habitat an additional eudicot could be assessed. These *MEI2*-like genes are likely to have homologues in tobacco, tomato and other model eudicot plants. These other eudicot plants have larger leaves and longer plastochron so that effects may be more noticeable. A search using sequences similar to *TE1* in tobacco and tomato may reveal *MEI2*-like homologues which could then be silenced using T-DNA inserts or RNAi techniques.

This study did not determine the nature of *TEL1* interaction with hormones. Looking at the impact of plant growth regulators on plant development in *tel1tel2* mutants with GFP expression marking the QC (such as QC25) would provide clarification on whether these genes are important in regulating growth *via* the prevention of differentiation. Techniques such as live imaging of apices after the addition of exogenous hormones in plants lacking *tel1tel2* gene expression and in plants over expressing *TEL1* and *TEL2* under an inducible system could provide information on how hormones impact on *TEL1* and *TEL2* gene expression.

In addition it remains to be determined if NAA is acting through auxin response elements. This could be investigated by doing an analysis of deletions involving auxin response elements. At -1296 and -867 in the *TEL1* promoter sequence there is a predicted auxin response element (section 3.2.5) in addition there is a ARF (auxin

response factor) binding site found at -1705. This ARF (TGTCTC) predicted auxin response element is found in the promoters of primary/early auxin response genes of *Arabidopsis thaliana* and is described as being enriched in the 5'-flanking region of genes up-regulated by both indole-3-acetic acid (IAA) and brassinolide (Goda et al., 2004). Deletions targeting specifically these regions containing predicted auxin response elements in the promoter sequence could provide information on whether *TEL1* responds directly to auxin and maybe add to information about how auxin response elements function in promoter regions of genes that are expressed in plant meristematic regions.

5.2 REGLATORY SEQUENCES

Analysis of *TEL1*::GUS and P_{TEL1}::ER-GFP expression patterns suggests the region directing expression to the SAM is contained within the intragenic sequences of the gene and the region directing expression to the RAM is between -1000 and -840. To identify specific motifs or sequences involved in the regulation of *TEL1* in both the root and shoot additional deletions should be completed. A closer examination of the intragenic regions of the *TEL1* gene to identify those regions contributing to SAM expression and an assessment of the region between -1000 and -840 to reveal motifs specific to *MEI2*-like gene sequences or QC specifying genes could then be used to predict regions in the other *MEI2*-like genes conferring site specific expression.

The closer examination of the intragenic regions of the *TEL1* gene should begin by looking at *TEL1* cDNA, because cDNA is missing introns this would confirm whether intragenic regions are required for correct SAM expression. If the SAM expression pattern is still missing then a series of deletion constructs sequentially eliminating introns would provide information on where the intragenic sequences were located. Alternatively the sequences important for SAM expression may by located in adjacent genes. This could be investigated if removing the introns does not eliminate the SAM pattern. Subsequent analysis using *TEL2* could be conducted to determine if there are intragenic sequences shared by family members.

While the identification of cis-acting regulatory sequences by promoter deletion analysis in this thesis did not provide conclusive evidence of motifs important for RAM and SAM expression, the identification of the RAV1AAT motif in *TEL1*, *TEL2*, *AML1*, *AML4*, *MCT1*, and *MCT2* suggests there may be motifs conserved among the *MEI2*-like class of genes that could be used to identify other genes functioning in a similar way. A deletion analysis, similar to that outlined in this thesis, should be conducted on other members of the *MEI2*-like gene family, starting with the closest relative of *TEL1*, *TEL2*. Once the regions important for SAM and RAM expression have been identified, an in-depth *in silico* analysis of those regions including the information from the *TEL1* deletion analysis may highlight novel motifs in all *MEI2*-like genes required for SAM and/or RAM expression.

The identification of the UP2 motif in the *in silico* analysis, coupled with the data on the non-cell autonomous nature of *TE1* and *PLA2*, suggests *TEL1* may be mobile. Whether the RNA or the protein is mobile and how important transportation is to the function could be determined through the use of grafting techniques (Turnbull et al., 2002) where a combination of single-hypocotyl grafts and two-shoot grafts could be used. The single-hypocotyl grafts, constructed with or without a supporting collar, would determine if there is root–shoot communication. The two-shoot grafts would identify any shoot–shoot communication.

TEL1 is predicted to function as an RNA binding protein. The *in silico* analysis coupled with the promoter deletion data identified motifs likely to be important in the regulation of *TEL1*, but provide no clue as to the RNA the protein is likely to bind or its function within the plant. A study of the TEL proteins and TEL's binding partners or protein complexes could elucidate TEL function in the cell and in the plant. To confirm *TEL1* binds RNA, a study similar to that described in Harigaya et al (2006) looking at protein binding partners should be conducted. Confirmation of the RNA binding function will provide more information on the specific function of *TEL1* and its role in the QC.

Chromatin immunoprecipitation (ChIP) could be used to confirm whether TEL proteins bind RNA, which RNA is bound and the localisation of the protein. The DNA ChIP assay combines cross-linking of whole cells (to freezes protein-protein

and protein-DNA interactions) and immunoprecipitation of protein-DNA complexes with specific antibodies to evaluate the association of proteins with specific DNA regions. A variation on this technique, RNA-ChIP has been successfully used in mammalian cells to examine the relationship of noncoding RNAs with histone proteins (Gilbert et al., 2000). In RNA-ChIP, RNA-protein interactions are fixed by reversible chemical cross-linking with formaldehyde followed by immunoprecipitation with antibodies against the candidate protein(s). RNAs that are associated with the protein are detected by reverse transcriptase-PCR (RT-PCR).

In addition a pull-down assay could be conducted to elucidate if there are any protein:protein interactions involving the TEL proteins. Pull-down assays are used to confirm previously suspected or to identify unknown protein:protein interactions (Einarson, 2001). Pull-down assays are a form of affinity purification, similar to immunoprecipitation, where a tagged bait protein is captured on an immobilized affinity ligand specific for the tag. The minimal requirement for a pull-down assay is the availability of a purified and tagged protein which is used to capture and 'pull-down' a protein-binding partner. The pull-down assay therefore requires purified TEL1 protein with a tag. Multiple tags are available including GST, His, Flag, HaloTag and Biotin. The tagged protein is then immobilized on a resin and pulls down the interacting protein. The challenge would then be to identify the protein or proteins that associate with TEL1.

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7 LIST OF ABBREVIATIONS

2,4-D	2, 4 dichlorophenoxyacetic acid, synthetic auxin
5' upstream region	The region upstream of the *TEL1* ATG start codon to the 3′ end of the preceding gene (includes the promoter and the 5' untranslated region)
cDNA	complementary DNA synthesized from a mature mRNA template
Col	Columbia ecotype
CZ	central zone
DAG	days after germination
dicot	dicotyledonous plant typically with two embryonic leaves (cotyledons)
DNA	Deoxyribonucleic acid
GA_3	gibberellic acid
L1	clonal layer 1 in the SAM
L2	clonal layer 2 in the SAM
L3	clonal layer 3 in the SAM
LB	Lysogeny Broth
MAST	Motif Alignment and Search Tool
MEME/MAST	Multiple Em for Motif Elicitation / Motif Alignment and Search Tool
microRNA	microRNA are small (approximately 22 nucleotides in length), forms of RNA generated from endogenous hairpin-shaped transcripts
monocot	monocotyledonous plant typically with one embryonic leaf (cotyledon)
MPSS	programme for Gene Analysis in Arabidopsis
mRNA	messenger RNA
MS medium	Murashige and Skoog medium, a complete medium for tissue culture of plants
NAA	1-naphthylacetic acid, synthetic auxin
NPA	1-naphthylphthalamic acid, auxin transport inhibitor
P0	incipient leaf
P1	youngest leaf
pBIN-mGFP-er	modified binary vector used for root studies
pCambia1391Xb	modified binary vector used for shoot studies
PCR	Polymerase Chain Reaction
PLACE	Plant Cis-acting Regulatory DNA Elements database
PZ	peripheral zone
QC	quiescent centre
RAM	root apical meristem
REML	Restricted Maximum Likelihood method, a statistical test similar to ANOVA used when analysing unbalanced or correlated data and large populations
RNA	Ribonucleic acid
RRM	RNA recognition motif
RRM1	RNA recognition motif 1
RRM2	RNA recognition motif 2
RRM3	RNA recognition motif 3
RZ	rib zone
SAM	shoot apical meristem

TAIR	The Arabidopsis Information Resource
T-DNA	transferred-DNA, DNA placed inside the plant genome resulting in an insertion mutant
TIBA	10 µM 2,3,5-triiodobenzoic acid, auxin transport inhibitor
Ws	Wassilewskija ecotype

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9 APPENDIX 1

10 APPENDIX 2