Chapter 2
The Arabidopsis-mei2-Like (AML) Genes Play a Role in Meiosis and Vegetative Growth in Arabidopsis

2.1 Abstract

Arabidopsis has five putative homologues of mei2 known as Arabidopsis Mei2 Like or AMLs. mei2 is considered to be a master regulator of meiosis in S.pombe. Each of the AML genes encode a putative RNA binding protein similar to mei2. In the current study, I have employed a combination of forward and reverse genetic approaches to functionally characterize the AMLs to examine their role in diverse aspects of plant development, and to assess their functional implications. Examination of AML4 expression at the cellular level indicated that it is expressed in both vegetative and reproductive tissues. I have also detected higher levels of AML4 transcripts both in the male and female reproductive tissues, especially in the meiocytes. Functional analysis of the AML gene family using an RNA interference (RNAi) approach and T-DNA insertion mutants suggested a likely role for AML genes during meiosis. Consistently, we observed sterility and a range of defects in meiotic chromosome behaviour in multiple independent AML5-RNAi lines and aml triple mutant combinations. Abnormalities in seedling growth and development were also observed at a low penetrance. In summary, our findings suggest a role for AML genes in meiosis and vegetative growth and development in Arabidopsis. This suggests a probable conservation in the basic molecular mechanism that controls/regulates meiosis in yeast and plants.
2.2 Introduction

The plant life cycle alternates between a long diploid sporophytic phase and a short haploid gametophytic phase. Meiosis acts as a switch in the plant life cycle, triggering the transition from the sporophytic to gametophytic generation. The understanding of meiosis and gamete formation in plants has advanced considerably in the last few decades. This is largely due to extensive studies in Arabidopsis, maize, and more recently in rice (reviewed in Yang and Sundaresan, 2000; Bhatt et al., 2001; Caryl et al., 2003). Many genes encoding basic structural components of the meiotic machinery such as chromosome organization, synapsis, chromosome separation and segregation are highly conserved across species (Hamant et al., 2006). However some meiotic genes appear to be unique to plants and they do not have any obvious counterparts in other species. This suggests that, even though meiosis as a whole is conserved across species certain aspects may be unique to plants.

The transition from mitosis to meiosis has been well characterized in yeast (Schizosaccharomyces pombe and Saccharomyces cerevisiae). It is currently believed that this transition, from the vegetative to reproductive phase involves a combination of nutritional and developmental controls (reviewed in Yamamoto, 1996; Honigsberg and Purnapatre, 2003). Several studies have shown that the inactivation of pat1 in S. pombe induces a mitotic to meiotic transition (Beach et al., 1985; Nielsen and Egel, 1990). Subsequent studies demonstrated that, Mei2p is the master regulator of meiosis and is critical for vegetative to reproductive transition in yeast (Watanabe et al., 1988). Pat1 kinase is involved in the whole process by determining the stability of Mei2 protein (Watanabe et al., 1988; Peng et al., 2003). During the vegetative phase of the life cycle, Pat1 kinase phosphorylates Mei2 protein and triggers its inactivation and thereby ensures the continuance of the mitotic cycle (Peng et al., 2003). When haploid yeast cells are placed in sporulation medium, they accumulate mei2 transcript but Mei2p remains inactive due to phosphorylation at two sites, S438 and T527 between RRM2 and RRM3 (RNA Recognition Motif) by the Pat1 kinase. This subsequently leads to its degradation in an ubiquitin mediated proteolysis.
pathway (Peng et al., 2003). Such a mechanism helps in keeping the endogenous Mei2p level below a threshold and in effect blocks the transition from mitosis to meiosis (reviewed in Yamamoto, 1996; Harigaya and Yamamoto, 2007). However, the onset of meiosis, which is usually triggered by nutrient mediated signaling, induces a cascade of events leading to the activation of another protein called Mei3 (McLeod et al., 1987). The Mei3p is a potent inhibitor of Pat1 kinase and it ensures stability of Mei2 protein leading to its accumulation in the cell (Kitamura et al., 2001; Peng et al., 2003). Once the cell accumulates sufficient levels of Mei2p, it switches from the mitotic to the meiotic cycle (reviewed in Yamamoto, 1996). It was well described in fission yeast that, a stable pool of non-phosphorylated Mei2 protein can facilitate the normal progression of meiosis (Watanabe et al., 1997). In other words after fusion between two haploid cells of opposite mating type, Mei2p accumulates in the unphosphorylated form which promotes meiosis. Further studies revealed that mei2 expression is directly regulated by ste11 (Shimoda et al., 1987). The ste11 gene under conditions of nutrient deprivation and low levels of intracellular cAMP was found to activate the transcription of mei2 (Watanabe et al., 1988).

2.2.1 Mei2 protein functions at two stages in meiosis

Analysis of two different mei2 mutant alleles, a deletion mutant and a temperature sensitive mutant (mei2-33), indicated that mei2 is required at two different stages during meiosis (Peng et al., 2003). The deletion allele of mei2 caused an arrest before premeiotic DNA synthesis, whereas the temperature sensitive mei2-33 led to arrest just before the onset of meiosis I, two distinct stages in the meiotic cell cycle (Watanabe and Yamamoto, 1994). From these findings Watanabe and Yamamoto proposed that, a stable form of Mei2 protein is essential for both premeiotic DNA synthesis as well as for the entry of cells into meiosis I (Watanabe and Yamamoto, 1994). The mei2 gene encodes an RNA binding protein with three RRMs. Each RRM in the Mei2p is believed to be involved in recognition and binding of a particular RNA molecule (Yamashita et al., 1998). At the beginning of meiosis I, Mei2p is localized to the nucleus, where
it is observed in the form of a dot (Yamashita et al., 1998; Sato et al., 2001). Subsequent studies described that nuclear localization of Mei2p in the form of a dot is promoted by a unique nuclear RNA called meiRNA. meiRNA is a small non-coding RNA molecule and by a mechanism, which is not fully understood, meiRNA entraps the Mei2p within the nucleus (Shimada et al., 2003). Afterwards it was demonstrated that this nuclear dot, which appears near to the nucleolus, corresponds to the location of the sme2 gene that encodes the meiRNA (Shimada et al., 2003).

2.2.2 Mei2 protein and nutrition mediated signaling

In another study, Mei2p was found to interact with Mip1p (Mei2-interacting protein 1). Mip1p contains WD-40 and HEAT repeat domains, believed to be involved in protein-protein interactions (Shinozaki-Yabana et al., 2000). In addition to being required for sexual development and meiosis, Mip1p is also essential for spore viability and cell growth in yeast (Shinozaki-Yabana et al., 2000). Mip1p belongs to the Raptor (regulatory associated protein of Tor) family of proteins that associates with the TOR kinase. TOR kinase is a major regulator of translation and cell growth found in all known eukaryotes (reviewed in Kim and Sabatini, 2004). Tor and Raptor proteins are believed to act as part of a complex known as TORC1 that regulates multiple aspects of cell growth and physiology in response to nutrient status (nutrient-activated signal transduction). Several studies suggested that Raptor might be acting as a scaffold for the recruitment of substrates that are then phosphorylated by Tor1p (Shinozaki-Yabana et al., 2000; Matsuo et al., 2007).

Tor1 protein kinase belongs to the family of phosphatidylinositol kinases (Keith et al., 1995). Detailed studies in yeast suggested that Tor protein and its kinase activities are necessary for normal meiotic progression (Zheng and Schereiber, 1997). Tor protein function was revealed to be critical during two distinct stages of meiosis such as, the transition from mitosis to meiosis and during the packaging of spores into asci (Zheng and Schereiber, 1997). Based on these findings, it was proposed that Mei2 protein could be one of the Tor
protein targets in yeast. Mei2 function may thus be connected to nutrient dependent signaling.

### 2.2.3 mei2 related genes in plants

The existence of mei2-like genes in plants was first revealed by the identification and characterization of the *AML1* (*Arabidopsis mei2-like1*) gene in *Arabidopsis* (Hirayama et al., 1997). *AML1* was isolated in a screen for *Arabidopsis* cDNAs that could allow meiosis to proceed in a *mam2 map3* mutant of *S. pombe*. The *mam2 map3* mutant is deficient in the mating receptors and hence defective in meiosis (Hirayama et al., 1997). Although *AML1* was found to rescue the mating receptor signaling requirement for meiosis, it could not complement a *mei2* deficient mutant of fission yeast. In other words *AML1* cDNA failed to perform a full-level trans-complementation but only effected a partial rescue of sterility. This suggested that rescue of the meiotic defect by *AML1* is not by replacement of endogenous *mei2* function but by some other mechanism (Hirayama et al., 1997). The molecular nature of this *AML* mediated rescue pathway is not understood.

*AML1* was also shown to be expressed in a number of different plant tissues such as leaves, roots, flowers, and siliques (Hirayama et al., 1997). The *TERMINAL EAR 1* (*TE1*) gene of maize was the first plant *mei2*-like gene to be functionally characterized (Veit et al., 1998). The *te1* mutant plants were defective in the regulation of leaf initiation (mutant plants appeared like a maize ear). Based on the mutant phenotype characteristics, it was proposed that *TE1* might be functioning in the control of phyllotaxy and leaf initiation in the meristem (Veit et al., 1998). *TE1* is expressed in both shoot and root meristems. However interestingly there was no expression of *TE1* in the meiocytes. Consistently the *te1* mutant phenotype does not include any meiosis associated defects (Veit et al., 1998; Jeffares et al., 2004). A subsequent bioinformatic study has shown that *mei2*-like genes are widespread in the plant kingdom. They constitute a diversified group of genes comprising of many distinct clades (Jeffares et al., 2004; Anderson et al., 2004). In *Arabidopsis*, there are nine *mei2*-related genes
distributed over three clades (Anderson et al., 2004). Of these nine genes, five genes (AML1-AML5), which are most similar to mei2, cluster together. These genes have a broad expression pattern during plant development, including vegetative and reproductive tissues (Anderson et al., 2004). In a subsequent study, the AML1 protein has been shown to bind AtRaptor1B in a yeast two-hybrid assay (Anderson and Hanson, 2005). This interesting finding implicates the AML family of proteins in TOR-dependent signaling in Arabidopsis.

2.3 Objectives of the current study

Despite this information the role of AMLs in meiosis and related events still remains obscure. Hence in this study, I have examined AML1-AML5 genes with respect to their likely involvement in plant meiosis. The first question was the expression pattern of these genes in Arabidopsis particularly during meiosis. Expression data was already available in the lab for four members of the AML gene family and hence I carried out analysis for AML4. To investigate the role of AMLs during plant development, an RNAi based gene silencing approach was employed. AML5-RNAi results were further validated using different aml T-DNA insertion mutants. Findings from this study and their implications are described in this chapter.

2.4 Results

2.4.1 AMLs show significant identity with Mei2 protein

Sequence analysis of AML1-AML5 and Mei2p using ClustalW revealed a significant identity between AMLs and Mei2p. The AMLs showed greatest similarity to Mei2p at the C-terminal portion of the protein. This includes a region that encompasses the RNA Recognition Motif 3 or RRM3 (Figure 2.1). I have carried out a phylogenetic analysis of AML1-AML5, AtTEL1-2, OML1-OML5, and ZmTE1 from Arabidopsis, maize, and rice respectively (using both experimental and predicted sequences), along with Mei2p to examine the phylogenetic relationship between different mei2-related genes in plants (Figure 2.2).
Figure 2.1 (A) Line diagram representing AML protein showing the approximate locations of RRM s. Bar indicates the region of highest identity (45%) between AMLs and Mei2p. (B) ClustalW alignment of AML1-AML5 and Mei2 proteins for RRM 3.
Figure 2.2 Phylogenetic tree of *mei2*-related genes from Arabidopsis, rice, maize and *S. pombe* generated using Phylip. Bootstrap values greater than 50 are indicated.
In the phylogeny analysis, AML2, AML3, and AML5 proteins were grouped together in one clade. This clade also included proteins such as OML2 and OML5 from rice. The AML1 and AML4 which are the most similar genes in the AML gene family clustered together with OML3 and OML4 from rice. AtTEL1 and AtTEL2 cluster together with OML1 and ZmTE1 (from rice and maize respectively) and formed another distinct clade.

2.4.2 AML4 shows expression in the meristems and meiocytes

To investigate the expression pattern of AML genes during plant development, I carried out RNA in-situ hybridization analysis for AML4. I have examined AML4 expression in both vegetative and reproductive tissues (RNA in situ data was available for rest of the AML genes). A basal level of expression was detected in most tissues. However, higher level of expression was seen in the shoot apical meristem and in the inflorescence meristem (Figure 2.3A and B). In flowers expression was observed in the developing anther and pistil primordia (Figure 2.3C). A relatively weak expression was also observed in the sepals (Figure 2.3C). High expression levels were detected in the male and female meiocytes (Figure 2.4). Higher levels of AML4 transcripts were also detected in the developing pollen and young ovules (Figure 2.4B and C) and in the embryo sac (Figure 2.4D). Altogether the AML4 expression pattern was similar to the other AML genes (data not shown).

A comparison of our AML expression data with the Genevestigator microarray database (https://www.genevestigator.ethz.ch; Zimmermann et al., 2004) revealed consistent results. The strong meiotic expression of AMLs was particularly interesting and this prompted to test for a function of AML genes during meiosis in Arabidopsis, similar to what is known for the related gene, mei2 of S. pombe.
Figure 2.3 Expression of *AML4* in apical meristems and reproductive organs

(A) Vegetative SAM. (B) Inflorescence SAM. (C) Floral stage 6 buds show increased signal in stamens and pistils. (D) Sense. Scale Bar: 10 μm.
Figure 2.4 Expression of *AML4* in meiocytes and gametophytes

(A) Transverse sections of anther showing strong expression in pollen mother cells. (B) Expression in developing pollen. (C) Expression in young ovules including the megaspore mother cell. (D) Mature ovules showing strong expression in the embryo sac. Scale Bar: 25 μm.
2.4.3 RNAi mediated knock down of AMLs cause sterility

I have attempted an RNAi based silencing approach to dissect out the possible role of the AML gene family in Arabidopsis. This decision was largely based on the assumption that the close similarity in expression pattern between the five AML genes might also indicate a redundancy in their function. Therefore I adopted a common-domain based RNAi approach using the region, which is most conserved among all the five AML genes to simultaneously knock down the different members of the AML family. After detailed sequence analysis, I selected a 572 bp portion of the AML5 cDNA (GenBank entry NM_179396: coordinates 2112-2683) encoding the C-terminal part of the protein for RNA interference. This portion contains the region, most conserved across all the AML genes and also includes RRM3, which appears to be critical in Mei2p function (Watanabe and Yamamoto, 1994). To further validate the selection for best RNAi results, we subsequently estimated similarity and specificity of the chosen 572 bp region in the Arabidopsis genome. This analysis was performed by means of nucleotide BLAST (using the short nearly exact matches option). BLAST analysis revealed that close similarity was only to each of the other four AML genes at E values between $1 \times 10^{-44}$ and $1 \times 10^{-11}$. The next level of similarity was at $E = 0.034$, which is well beyond any significant identity. Hence the RNAi strategy was designed to specifically target the five AML genes. A schematic of the AML5-RNAi construct is given in Figure 2.5.

Transgenic lines carrying the AML5-RNAi construct (RNAi expressed under the control of the CamV35S promoter) were subsequently generated (described in detail in the Material and Methods section) and examined for phenotypes. The main phenotype observed among the T1 RNAi transgenic plants was sterility (Figure 2.6). Out of 70 T1 transgenic plants analyzed, 11 of them showed >90% sterility (~ 5 seeds per silique). In comparison we did not observe sterility greater than 50% in any of the control transformant plants (0/80) carrying the empty vector (plants transformed with RNAi vector minus the 572 bp AML5 cDNA fragment).
Figure 2.5 Diagrammatic representation of the *AML5*-RNAi construct

The CaMV35S promoter symbolizes the Cauliflower Mosaic Virus promoter housed in the pKANNIBAL vector. AML and LMA stand for the sense and antisense *AML5* cDNA fragments respectively. Spacer represents the pyruvate orthophosphate dikinase (PDK) intron and OCS stands for the octopine synthase (OCS) terminator.
Figure 2.6 *AML5-RNAi* lines show sterility

(A) Wild type plant with elongated siliques. (B) *AML5-RNAi* line showing strong sterility. The siliques failed to elongate.
2.4.4 Reduced levels of AML transcripts in the AML5-RNAi lines

To test whether the sterility correlates with corresponding reduction in the mRNA in all or some of the AML genes, we (work done in collaboration with Jagreet Kaur) quantitated RNA levels for each of the five AML genes using semi-quantitative RT-PCR. Analysis revealed that those RNAi lines, which showed strong sterility correspondingly exhibited a 2-fold or greater reduction in mRNA level for at least 3 of the AML genes (Figure 2.7) whereas line R20 that did not show sterility failed to show any such reduction in the AML mRNA levels. R25 which showed moderate sterility exhibited less reduction in mRNA levels when compared to the sterile lines. Multiple independent control vector transformants also failed to show any reduction in the AML transcript levels (data not shown). Taken together these findings clearly suggested a correlation between AML down regulation and the sterility phenotype observed among the RNAi plants. Results from this study also indicated that AML1 and AML4 are the more important genes in the AML gene family as their expression levels were significantly reduced in all the five sterile RNAi lines. In fact AML1 and AML4 genes are the most closely related genes among all AML family members (75% amino acid identity). This also indicated a greater degree of redundancy in function between AML1 and AML4 genes. Surprisingly the levels of AML3 were found to be up-regulated (more than wild type) in multiple RNAi lines (Figure 2.7). The reason behind this observation is not clearly understood.

2.4.5 Male and female gametogenesis was impaired in the AML5-RNAi lines

To investigate the basis of sterility observed in the RNAi lines, we examined ovule (work done in collaboration with Jagreet Kaur) and pollen development in multiple T1 and T2 transformant lines. Our analysis revealed abnormalities in both male and female gametogenesis. Defects in pollen development were identified by the presence of shriveled and shrunken pollen (Figure 2.8A and B). Pollen from multiple RNAi lines were also greatly compromised in viability. Compared to wild type anthers which consist of mostly
Figure 2.7 Quantitation of AML1-AML5 transcript levels in multiple AML5-RNAi lines

The levels of each transcript are represented as percentages normalized against the levels of AML1-AML5 expression observed in control plants. In each line at least three members of the AML family show a >2 fold reduction in the transcript level. R20 is an RNAi line that did not show sterility. Percentage sterility indicates the observed degree of sterility in the corresponding AML5-RNAi lines.
viable (purple) pollen grains (Figure 2.8C) a large majority of pollen grains in the AML5-RNAi anthers were dead (green) (Figure 2.8D). This suggested that pollen development was severely compromised in the AML5-RNAi lines.

In the mutant ovules, the phenotype ranged from arrest of embryo sac development at the 1n or 2n stages of ovule development, to degeneration of the embryo sac (Figure 2.9). At a low frequency (approximately 5% of ovules) we also observed two enlarged cells along with degenerating material likely from the nonfunctional megaspores in few of the RNAi ovules (Figure 2.9F). These two cells could represent the products of an abnormal division of the functional megaspore, or they might possibly correspond to two surviving megaspores. Although the frequency of these two-celled structures was quite low, they were observed in multiple independent AML5-RNAi plants as well as in the antisense transformant plants (generated earlier in the lab by Jagreet Kaur). Similar observations were also made in aml triple mutant lines as well (see below). Moreover, we did not observe a comparable phenotype in wild type or in control transformants. Hence, we hypothesize that this observation in the mutant ovules is likely to be significant, but further experiments are needed to elucidate the implications of this finding.

2.4.6 AML5-RNAi causes seedling defects

Abnormalities were also observed in a number of T2 and T3 AML5-RNAi transgenic plants during seedling development. These defects were comparable to earlier observations for the aml1 aml4 double mutants (work done by Jagreet Kaur) and also in the aml1 aml2 aml4 triple mutants (see below). To investigate the cause of vegetative defects in the RNAi plants in detail, I undertook a scanning electron microscopy (SEM) approach (Figure 2.10). SEM analysis of arrested RNAi seedlings revealed that, the shoot apical meristem did initiate leaf primordia in the mutant seedlings. However these were much slower in growth and expansion when compared to wild type. This finding suggested a likely defect in meristem activity in the RNAi mutant seedlings. The AML5-RNAi mutant seedlings displayed retarded growth often leading to growth arrest and failure to
Figure 2.8 AML5-RNAi lines show defective male gametogenesis

Optical sections of cleared pollen from wild type (A) and AML5-RNAi line (B). Majority of the pollen in the RNAi anther were shriveled. (C and D) Alexander staining of anthers showing viable pollen (purple) in the wild type anther (C) as compared to a majority of non-viable pollen (green) in the AML5-RNAi anther. (D). Scale Bar = 25 μm.
Figure 2.9 *AML5*-RNAi lines show defective female gametogenesis.

Stages of female gametophyte development. (A to D) Wild type. (E to H) *AML5*-RNAi. (A) Ovule stage 2-1 wherein the megaspore mother cell (MMC) has differentiated. (B) Two-nuclear (ovule stage 3-2) and (C) and four-nuclear (ovule stage 3-4) embryo sac. (D) Ovule stage 3-6 showing a mature embryo sac. (E) Stage 3-6 ovule showing an embryo sac arrested at the uninucleate stage. (F) Two enlarged cells (arrows) separated by a cell wall (arrowhead) in place of a mature embryo sac. (G) Two nuclear embryo sac undergoing degeneration. (H) Degenerating embryo sac. Asterisk marks the apical epidermis below which MMC differentiates. Bar = 25 μm.
develop new leaves after cotyledon expansion (Figure 2.10D and G). Some of the RNAi mutant seedlings showed abnormalities in root development and few even completely lacked the primary root (Figure 2.10B).

2.4.7 Generation of aml triple mutants

To further validate the AML5-RNAi results, T-DNA insertions in different aml genes were obtained. These mutant aml lines were initially characterized in the lab by Jagreet Kaur. Subsequently, she used these different mutant lines to create various combinations of aml triple mutants such as aml1 aml2/+ aml3/+ and aml1/+ aml2 aml3/+, by crossing homozygous double mutants that had one mutant aml allele in common (aml1 aml2 X aml1 aml3). F2 seeds from these crosses were screened for homozygous triple mutant plants. For aml1 aml2 aml4 triple mutants, I screened around 160 F2 plants in a PCR based screening strategy. In brief, I isolated genomic DNA from all the 160 plants and subjected them to PCR analysis to confirm the presence of T-DNA insertion in each of the three AML genes. This was done using a left border outwardly directed primer (LB1) in combination with a gene-specific primer flanking the site of insertion. The gene-specific primers used were K22R1, F15D2F1, F7F1, F15F1 and T28F1 for aml1, aml2, aml3, aml4 and aml5 genes respectively. To genotype each locus, I used a pair of gene specific primers for each AML gene. The gene specific primer combinations K22F1 and K22R1, F15D2F1 and F15D2R1, F7F1 and F7R1, F15F1 and F15R1, T28F1 and T28R1 were used to test for the presence of the wild type allele of AML1 to AML5 respectively in these plants. Out of 160 plants screened, I identified five aml1 aml2 aml4 triple homozygous mutant plants. All of them exhibited reduced fertility (Table 2.1). The degree of sterility was approximately 30-60%. We also observed sterility in the following genotypes of aml mutant plants: aml1 aml2/+ aml4, aml1 aml2/+ aml4/+ and aml1 aml2 aml4/+ (Table 2.1).
Figure 2.10 Seedling arrest in *aml* triple mutants and RNAi lines.

(A) *aml*1 *aml*4 double mutant showing seedling arrest: normal seedling (black arrowhead) and arrested seedlings (white arrowhead). (B) Defective root development. (C) to (G) Scanning electron microscopy of 10 day old seedlings. (C and E) Wild type. (D and G) AML5-RNAi. (F) *aml1 aml2 aml4* triple mutant. (C) Low magnification; cotyledons have been removed. Leaves have expanded. (D) Low magnification; whole seedling leaves fail to expand. (E) High magnification; cotyledons and leaves up to 4 have been removed. Primordia of leaves 5 and 6 are visible. (F) Primordia of leaves 3 and 4 are indicated. (G) Cotyledons have been removed. Primordia of leaves 1 and 2 are indicated. Scale bar: (C and D) 100 μm, (E) to (G) 30 μm.
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<tr>
<td><code>aml1 aml2 aml4/+</code></td>
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Table 2.1 Reduced fertility in various `aml` triple mutants

The sterility was scored as the percentage of seeds in approximately 5 siliques assuming a wild type seed set of 55 seeds per silique.
In subsequent analysis I detected abnormalities in both male and female gametogenesis in the \textit{aml1 aml2 aml4} triple homozygous mutant lines (Figure 2.11). The female phenotypes comprising arrest at the 1n and 2n stages or degenerating embryo sacs were similar to what was observed for the RNAi lines. A quantification of mutant ovule phenotypes during female gametogenesis in both RNAi and \textit{aml} triple mutant lines is given in Table 2.2. Male gametogenesis was also defective as indicated by the presence of about 30-40\% shrunken pollen. In addition \textit{aml1 aml2 aml4} seedlings displayed abnormalities during seed germination, similar to what was observed in the RNAi mutant seedlings. However, the vegetative phenotype appeared to be less penetrant among the \textit{aml} triple mutant seedlings (data not shown). For isolating the \textit{aml1 aml2 aml3} triple homozygous plants, I screened around 135 \textit{F2} plants and obtained 6 triple homozygous mutant plants. But none of them displayed a visible phenotype. Similarly triple homozygous mutant plants for other \textit{aml} mutant combinations also failed to display a visible phenotype (data not shown). In brief, data from multiple insertion mutant analysis confirm our earlier findings with \textit{AML5-RNAi}, that the \textit{AML} gene family is required for male and female gametogenesis and vegetative growth in Arabidopsis. Moreover these findings further reinforce that \textit{AML1} and \textit{AML4} are important for gametogenesis.

2.4.8 \textbf{AML} genes play a role in meiosis in Arabidopsis

Defects in pollen development and arrest at an early stage of female gametogenesis (in both RNAi and \textit{aml} triple mutants) together with higher levels of \textit{AML} transcripts in the meiocytes (RNA in situ hybridization) prompted an examination of meiotic progression in the mutant plants. I therefore analyzed meiotic progression in multiple \textit{T2} and \textit{T3} generation RNAi lines and \textit{aml} triple mutant (\textit{F2} and \textit{F3} generation) plants using chromosome spreads. About 115/457 (25\%) of the male meiocytes from RNAi lines, covering diplotene to metaphase stages showed abnormalities. I did not observe any comparable defects in the
Figure 2.11 Defective male and female gametogenesis in *aml1 aml2 aml4* triple mutants

(A) Alexander staining of anther showing viable (purple) and non-viable (green) pollens. (B) Stage 3-6 ovule with embryo sac arrested at functional megaspore stage. (C) Two cells (arrows) present along with degenerating megaspores in place of a mature embryo sac. Arrowhead marks cell wall. (D) Degenerating embryo sac. Bar = 20 μm
### Table 2.2 Defective female gametogenesis in RNAi and triple mutants

1n, arrest at uninuclear stage; 2n, at two nuclear stage respectively; mes, mature embryo sac; deg, degenerating. Ovule stages are according to Smyth et al., (1990).

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<tr>
<td></td>
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wild type meiocytes at similar stages (0/156). The abnormalities included, pairing defects ranging from partial desynapsis to the formation of univalents (Figure 2.12K), fragmentation and appearance of acentric fragments, and clumping of chromosomes. Chromosome bridges were also observed resulting from exchange between what appeared to be nonhomologous chromosomes (Figure 2.12L). At prometaphase to metaphase there was clumping of chromosomes at the metaphase plate (Figure 2.12M). An interesting observation in many mutant meiocytes was the appearance of an acentric fragment prior to anaphase I, in otherwise normal looking mutant meiocyte (Figure 2.12N). The nature/implication of this finding is not clear.

Defects were also observed during meiotic progression in the triple mutant combinations of aml1 aml2 aml4, aml1 aml2/+ aml4/+ and aml1 aml2/+ aml4 plants. 181/738 meiocytes covering diplotene to metaphase stages in aml1 aml2 aml4 plants were defective. Abnormalities included desynapsis (data not shown) and clumping of homologous chromosomes (Figure 2.12P). In many mutant meiocytes, I observed the appearance of chromosome bridges (usually one or two) (Figure 2.12Q and T). Chromosomes were found to clump at the metaphase plate in few instances (Figure 2.12R and V). In several cases, I found laggard chromosomes that are remained at the metaphase plate in the mutant meiocytes (Figure 2.12U). Similar defects were also observed in mutant female meiocytes (Figure 2.12W-Z). A statistical data of meiotic defects in RNAi and aml triple mutants is provided in Table 2.3. In general the range of meiotic abnormalities observed in RNAi and aml triple mutants were comparable. In summary, the finding that disruption of AMLs causes meiotic defects provides strong evidence for a likely role of AML genes during the progression of meiosis in Arabidopsis.
Figure 2.12 Defective meiotic chromosome organization in AML5-RNAi and aml triple mutant plants

(A) to (J) Wild type. (K) to (O) AML5-RNAi. (P) to (Z) aml1 aml2 aml4 meiocytes. (A) Leptotene. (B) Zygotene. (C) Pachytene. (D) Late diplotene. (E) Diakinesis. (F) Metaphase I. (G) Anaphase I. (H) Metaphase II showing organelle band. (I) Anaphase II. (J) Telophase II. (K) Partial desynapsis resulting in a mix of univalents and bivalents at diplotene. (L) Chromosome bridge (arrowhead) between non-homologous chromosomes at diakinesis. (M) Clumping of chromosomes. (N) Acentric chromosome in addition to five condensed bivalents at metaphase I. (O) Chromosome bridge and laggard chromosome at telophase II. (P) Disorganized diplotene stage. (Q) Chromosome bridge at diakinesis. (R) Clumped chromosomes at metaphase I. (S) Two univalents and acentric chromosome at diakinesis. (T) Anaphase I bridges. (U) Laggard chromosomes at telophase II. (V) Clumped chromosomes at prometaphase I. (W) Mutant female meiocyte with ten univalent chromosomes. (X) Mutant female meiocyte showing chromosome bridge. (Y and Z) Chromosome bridges at diakinesis in mutant female meiocytes. Scale Bar: 10 μm.
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Table 2.3 Meiotic defects in RNAi and aml triple mutants
2.5 Discussion

The switch from mitosis to meiosis is a critical step in the plant life cycle however little is known about the molecular mechanisms which govern this decision in plants. In this study, through a candidate gene approach, I have tried to investigate the control of meiosis in plants. Several studies have shown that mei2 acts as a key positive regulator of meiosis in yeast (Watanabe and Yamamoto, 1994). Here I attempted to address the question of whether AML genes, the Arabidopsis homologues of mei2 also play a similar role in the control of meiosis in plants.

2.5.1 AML genes share identity with S. pombe mei2

Sequence analysis revealed significant similarity between each of the AMLs and mei2p. The similarity was strongest in the RRMs but also extends outside the RRMs at the C-terminal portion. Such a similarity at the sequence level is consistent with the hypothesis that AMLs are the true Arabidopsis homologues of mei2. For mei2, it is proposed that it binds to two different RNA molecules for performing its two critical functions in meiosis, S-phase DNA synthesis and entry of cells into meiosis I (Watanabe and Yamamoto, 1994). Mutations in the RRMs that disrupt RNA recognition and binding are found to severely compromise mei2 functions in yeast (Watanabe and Yamamoto, 1994). However, unfortunately the molecular identity and nature of these RNA molecules is not well understood.

2.5.2 AMLs have a distinct expression pattern different from mei2

Expression analysis of AML genes substantiated the likely role of these genes during plant meiosis. mei2 is more or less a meiotic specific gene in yeast, as its levels are actively downregulated during vegetative growth by ubiquitin mediated proteolysis pathway (Peng et al., 2003). However based on results from our and other studies, it appears that, it is not the case with AMLs as they exhibited strong expression in the vegetative tissues, especially in the actively dividing shoot and root meristems (Anderson et al., 2004). Such a strong AML
expression in both shoot and root meristems raises the argument that AMLs have a critical role in mitosis or in any other meristem related activities. This suggests that AMLs may have possibly acquired additional functions in plants, distinct from mei2. Strong expression was also observed in both male and female meiocytes. The expression patterns for the five AML genes were closely related and showed substantial overlap, suggesting a likely functional redundancy between the different members of the gene family.

2.5.3 RNAi mediated AML down regulation lead to reduced fertility

To study the role of AMLs during meiosis in plants, I used an RNAi approach. RNAi was directed to a conserved region common to all AMLs that could specifically target all members of the AML gene family for alteration of expression. This strategy also helped us to circumvent the issue of functional redundancy. In brief, RNAi analysis has shown that down regulation of at least three or more AML genes leads to visible abnormalities. Abnormalities include reduced fertility and defects in both male and female gametogenesis. These findings clearly support a role for AML gene family during reproductive development in Arabidopsis. However it is not clear, whether the gametophytic defects were due to a carry over from the abnormal meiosis or the gene family has additional roles during gametogenesis in plants as well.

2.5.4 Meiosis was compromised in the RNAi and aml triple mutant plants

I carried out chromosome spreads to investigate the cause of defective gametogenesis in the AML5-RNAi and aml triple mutant plants. These analyses revealed a range of meiotic defects in the mutant meiocytes from both RNAi and triple mutants. The mutant phenotypes included abnormalities in chromosome organization during meiotic prophase I and in later stages like desynapsis, formation of interchromosomal bridges, chromosome fragmentation. I have also observed defects in chromosome segregation in mutant meiocytes. Apparently similar abnormalities were detected in the mutant female meiocytes as well. The meiotic phenotypes were similar between the RNAi lines and aml triple mutants.
Therefore, these findings altogether provide functional evidence for the role of AML genes during meiosis in plants. Interestingly these phenotypes are much less severe than mei2 disruption in S. pombe, which results in a failure of cells to undergo premeiotic DNA synthesis and a complete block to entry into meiosis. Hence the contribution of AMLs in the control of meiosis in plants appears to be more limited than that of Mei2p in S. pombe.

There are two ways in which Mei2p has been shown to be involved in chromatin organization in yeast. The first is directly, through the formation of a nuclear dot in association with the sme2 locus which encodes meiRNA (Shimada et al., 2003). The second is reflected in the requirement of mei2-dependent signalling for chromatin remodeling at the ade2-M26 recombination hotspot (Mizuno et al., 2001; Wahls and Smith, 1994). The defects in meiotic chromosome organization in aml mutants and AML5-RNAi lines may similarly reflect a requirement for AML-dependent signalling in chromatin organization during meiosis. However further detailed studies are essential to work out the exact role of AMLs and their signaling pathways in aspects of meiotic chromatin organization in plants.

2.5.5 A potential role for nutritional signaling in plant meiosis

The finding that AML1 protein can bind to AtRaptor1B has important implications for the AML function, as Mip1p is an interacting partner of the Tor protein. Tor protein is a master regulator of cell growth and division in yeast and other organisms, via nutrition mediated signaling (Kawai et al., 2001; Weisman and Choder, 2001). Tor signaling connects the energy status of the cell to the protein synthesis apparatus and to cell growth. Mip1p was also shown to bind to Mei2p in vivo and is essential for the normal function of Mei2p to induce meiosis (Shinozaki-Yabana et al., 2000). In yeast, it was speculated that TOR kinase might be involved in the phosphorylation of Mei2p and the finding that Mip1p can bind to Mei2p supports the hypothesis that Mei2p could be a putative TOR substrate. Mip1p might be playing its role by recruiting Mei2p to the TOR complex. Several studies have provided information on the action of the TOR
signalling pathway in Arabidopsis (Menand et al., 2002; Deprost et al., 2005; Anderson et al., 2005). The Arabidopsis TOR homologue, AtTOR gene is essential for embryogenesis and endosperm development and is expressed in the embryo and meristematic regions (Menand et al., 2002). AtRaptor1B has also been proposed to play a role in early embryogenesis as the mutant shows defects in early embryo development leading to embryo lethality (Deprost et al., 2005). Unfortunately the likely role of these genes in plant meiosis has not been described. Mutant phenotypes in the Atraptor1B and Atraptor1A double mutants, such as seedling arrest, defective root growth and reduced fertility (Anderson et al., 2005) are similar to those found in aml triple mutants and RNAi lines. These findings are consistent with the possibility that AMLs and AtRaptor genes act in the same pathway. Further analysis of these genes will help to dissect out the role of TOR mediated signaling in plant growth and development. In summary, the findings that AML1 can bind to AtRaptor1S and the likely involvement of AMLs in meiosis, support a probable involvement of nutrition mediated signaling in meiosis in Arabidopsis.

2.5.6 Vegetative defects in RNAi and aml triple mutant lines

Apart from sterility I also observed vegetative phenotypes in RNAi and aml triple mutant plants at low penetrance. The vegetative phenotypes comprised seedling arrest and defects in root growth. It therefore appears that AML genes function in both vegetative and reproductive development. In the case of Mei2p, no function has been ascribed during vegetative development and the small amount of Mei2p present during vegetative growth is considered to be inactive (Watanabe et al., 1997). I therefore speculate that the ancestral function of the AML genes was in meiosis and that the vegetative function evolved subsequently. However the exact vegetative role of AMLs is not understood.

2.5.7 Likely nature of AMLs in plants

RNA binding proteins are known to play diverse roles in eukaryotes especially during posttranscriptional regulation of gene expression (Burd and
The posttranscriptional regulation of gene expression involves processes such as capping, pre-mRNA splicing and polyadenylation. Each of these processes has a major impact on the stability of the mRNA transcript (Dreyfuss et al., 1988; Wilusz et al., 2001). More recent reports described the role of RNA binding proteins in aspects of RNA synthesis, transport, translation and degradation (reviewed by Fedoroff, 2002). Role of RNA binding proteins in meiosis and gametogenesis as translational regulators of specific mRNAs is known in many organisms such as the DAZ family of proteins in animals (Yen, 2004).

The Arabidopsis genome contains approximately 196 putative RNA binding proteins with RRM domains (Lorković and Barta, 2002). Comparatively few RNA binding proteins have been characterized in plants. Based on the available information, RNA binding proteins act as multifunctional cellular regulatory proteins controlling diverse pathways during plant development such as hormone responses, plant morphogenesis and stability of chloroplast RNAs (Fedoroff, 2002; Rochaix, 2001). Findings from this study demonstrated a likely role of RNA binding proteins in aspects of meiosis in plants. However the exact function of AMLs during plant meiosis is still obscure. The possible role of AMLs in meiosis include, regulation of certain meiosis specific transcripts, mediation of meiotic specific splicing, acting as splicing enhancers for certain meiotic genes, or promoting the nuclear export of certain meiotic RNAs. More extensive studies are required to fully elucidate the targets of AMLs in Arabidopsis.

The exact role of mei2 during meiosis is still not fully understood. However mei2 was shown to be required for splicing and transcription of certain meiosis specific genes such as mes1 and mei4 (Kishida et al., 1994; Horie et al., 1998). On the basis of aml phenotypes the meiotic role of AMLs appears to be indirect in nature. In other words the observed defects during meiosis in AML5-RNAi and aml triple mutant plants could represent the improper regulation or splicing or transport of certain meiotic specific mRNAs, whose function is essential for successful progression of meiosis in Arabidopsis. However currently we have no evidence to prove the existence of such defect. But I believe that future studies
will shed light into the molecular identity of these probable downstream AMLs targets/effectors and also on the reasons for the difference in the severity of mutant phenotypes between yeast (causes meiotic arrest) and plants.

2.6 Conclusion

In conclusion, I have provided evidence in favour of the involvement of the AML genes in the control of meiosis in plants. Findings from aml triple mutants and RNAi lines suggested that there is a likely involvement of nutrition-dependent signaling pathways in the regulation of meiosis in plants as has been shown to be the case for lower eukaryotes like yeast. From these results, it appears that AMLs also have a role during the vegetative development of the plant. This is unlike mei2 which was shown to be meiotic specific in yeast; hence I hypothesize that the vegetative role of AMLs might represent an additional one, which they probably acquired in plants.
2.7 Materials and Methods

2.7.1 Plant Material and growth Conditions

The Col ecotype of Arabidopsis was used for generating transgenic lines expressing the AML5 RNAi construct. Lines carrying a T-DNA insertion in each of the AML1-AML5 genes (SALK_015088, SALK_029713, SALK_006041 SALK_019467, SALK_061664) were obtained from Arabidopsis Biological Resource Centre, USA (Alonso et al., 2003). Seeds were surface sterilized and germinated on Murashige-Skoog (MS) medium supplemented with 2% sucrose and containing 0.7% agar. Seedlings were transferred to potting mix comprising peat, vermiculite and perlite (1:1:1) and grown in a Conviron TC30 growth chamber at 20 °C under an illumination of 180 μmol m⁻² s⁻¹ and 16/8 h light/dark cycle. Plants were irrigated once with MS solution while sowing or transplanting and subsequently with water.

2.7.2 Bacterial strains used

E. coli (DH5α): supE44lacU169 (φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 (Sambrook and Maniatis, 1989).

E. coli (HB101): supE44hsdS20 (rBmB-) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 (Sambrook and Maniatis, 1989).

Agrobacterium tumefaciens (AGL1): It carries a hypervirulent, attenuated tumor-inducing plasmid pTiBo542 from which the T-region DNA sequences have been precisely deleted, allowing optimal DNA transformation of many dicotyledonous plants. It also carries an insertion in its recA recombination gene, which stabilizes the recombinant plasmids (Lazo et al., 1991).

2.7.3 Generation of AML5- RNAi Lines

A 572 bp fragment corresponding to the carboxy terminal portion of AML5 was amplified using primer pair AML2F1 and AML2R1, which engineered Xho1/Xba1 and EcoR1/BamH1 enzyme sites at the 5' and 3' ends respectively.
The 572 bp fragment was cloned in the sense orientation as a Xho1-EcoR1 fragment and in the anti-sense orientation as a BamH1-Xba1 fragment in the pKANNIBAL vector (Wesley et al., 2001). This AML5-RNAi construct was further sub-cloned into the binary vector pART27 and transformed into Agrobacterium strain AGL1 for in planta transformations. In planta transformation was carried out as described by (Bechtold and Pelletier, 1998) and is explained below. The AML5-RNAi transformants were selected on MS plates containing 2% sucrose and Kanamycin at 50 μg/ml and transferred to soil. The sterility was scored as the percentage of seeds in approximately 5 siliques assuming a wild type seed set of 50 per silique. Plants scored as >90% sterile gave less than 5 seeds per silique.

2.7.4 Triparental mating and plant transformations

Triparental mating was carried out to mobilize the binary vector harboring the construct of interest from E.coli (DH5α) to Agrobacterium (AGL1) using another E.coli strain (HB101) carrying a helper plasmid pRK2013. For this transfer, we co-cultured the recipient Agrobacterium strain, the E.coli donor strain carrying the construct of interest and the helper E.coli strain HB101 together on a non antibiotic TYA plate. The recipient, helper, and the donor strains were mixed in 3:1:1 ratio before co-cultivation and spotted on a non-antibiotic plate and incubated at 28 °C for 24 hrs. After incubation the cells were harvested from the plate and resuspended in 1ml of sterile water or TYM broth. Subsequently serial dilutions of the cells were prepared and plated on to a TYMA plate containing carbenicillin and donor specific antibiotic selection marker and incubated at 28 °C for 2 days. The positive colonies were identified by PCR. The positive clones were then stored as glycerol stocks (500 μl of Agrobacterium culture at log phase + 500 μl of 40% filter sterilized glycerol) at -80 °C.

For plant transformations, we followed the protocol described by Bechtold et al., (1993) with minor modifications. Agrobacterium cell cultures harboring the construct of interest were grown in TYM broth with the appropriate antibiotics at 28 °C in a rotary shaker for approx. 40-45 hrs. The cells were then gently spun at
8000 rpm for 10 min and pellet harvested, which was then resuspended in the infiltration media. The pot carrying the plants were then inverted and placed in a 250 ml beaker containing the Agrobacterium cells suspended in the infiltration medium. The entire setup was then transferred to a closed cabinet connected to a vacuum unit and subjected to 500-600 mm Hg vaccum for 15-25 min. After vacuum infiltration, pots were kept horizontally in a plastic tray and covered with saran wrap to maintain humidity. After recovery, the plants were placed upright and watered at regular intervals and grown till maturity.

2.7.5 Genomic DNA isolation (Dellaporta method)

Genomic DNA was isolated according to the method described by Dellaporta et al., (1983) with minor modifications. Approximately 100-200 mg of plant tissue (leaf tissue or inflorescence) was collected in a 1.5 ml eppendorf tube. The tissue was then snap frozen in liquid nitrogen and ground to a fine powder using a micro pestles. Subsequently around 200μl of freshly prepared DNA extraction buffer was added to the powderized tissue followed by an equal volume of 2X CTAB buffer. The mixture was then incubated at 65 °C for 10 minutes in a shaking water bath. After incubation, the sample was allowed to cool and an equal volume of 24:1 chloroform:isoamyl alcohol was added. After thorough mixing the mixture was centrifuged for 15 min at 13,000 rpm at 4 °C. Afterwards the aqueous phase containing the DNA was transferred to fresh eppendorf tube and 2/3 volumes of ice cold isopropanol was added to precipitate the DNA. The DNA was pelleted down by centrifugation at 13,000 rpm for 30 min at 4 °C. The harvested DNA pellet was subsequently washed with 70% ethanol (in 1 ml 70% ethanol for 10 min at 4 °C) and air dried for 30 minutes at room temperature. The dried DNA pellet was suspended in required amounts of sterile water or Tris EDTA buffer and stored at -20 °C until use.

2.7.6 Expression Analysis by Semi-quantitative RT-PCR

Plant tissue was frozen in liquid nitrogen and stored at−80 °C. Total RNA was isolated from the inflorescence using Trizol (Invitrogen) according to the
manufacturers protocol and treated with RNase free DNase1 (RQ1, Promega) before proceeding for reverse transcription. Equal amounts of RNA (approximately 1 µg) from different samples were reverse transcribed using MMLV-RT (Gibco) according to manufacturer’s instructions. cDNA product was diluted 10-50 times and 2 µl of these dilutions was used to determine the linear range for AML1-AML5. GAPC expression was measured using GAPC1 and GAPC2 primers after determining the linear amplification range and used for normalization. The PCR was carried out after equalizing the RT products for each sample with respect to GAPC. Primer combinations, AML1up and AML1sac were used for detecting expression of AML1, 140jUp and 140jD for AML2, Chr4F and Chr4R for AML3, AML5F and AML5R2 for AML4 and 337c and 40BD for AML5. Sequence information for all the primers used in this study is given in Table 2.4. RT-PCR products were separated on a 1% agarose gel, blotted onto Hybond N+ and hybridized with the respective α32P labeled probes for AML1-AML5 and GAPC. Hybridization signals were detected using a Fuji FLA-3000 Phosphorimager and quantified using Image Gauge software.

2.7.7 Microscopic analysis

Developmental analysis of whole mount anthers and ovules were performed after fixing and clearing the inflorescence in methyl benzoate as described previously (Siddiqi et al., 2000). Briefly, inflorescences were fixed in FAA overnight at 4 ºC or for a minimum of 2 hours at room temperature. The fixed inflorescence were then rinsed in 50% acetone and dehydrated in an acetone series (60%, 70%, 80%, 90%, 95%, 100%) for 45 minutes each. After dehydration, the tissues were cleared in methyl benzoate for 45 minutes followed by overnight clearing with methyl benzoate. Afterwards the ovules/anthers were dissected on a glass slide under a stereo dissecting microscope. The dissected tissues were mounted on the same medium used for overnight clearing.
### Table 2.4 List of primers used in this study

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The slides were observed on a Zeiss Axioplan 2 Imaging microscope under DIC optics using 40x oil immersion objective.

Pollen viability was examined using the method of Alexander staining (Alexander, 1969). Anthers from both wild type and mutant plants were dissected under a stereo microscope and mounted in required amounts of the staining solution. The staining solution was prepared as follows, 95% alcohol- 10 ml, Malachite green- 10 mg (1 ml of 1% solution in 95% alcohol), Distilled water- 50 ml, Glycerol- 25 ml, Phenol- 5 g, Chloralhydrate- 5 g, Acid Fuchsin- 50 mg (5 ml of 1% solution in water), Orange G- 5 mg (0.5 ml of 1% solution in water), Acetic acid- 2 ml. The pH of the final staining solution was adjusted to 2.3. Images were captured on an Axioplan CCD camera using Axiovision (version 3.1) and edited with Adobe Photoshop 6.0.

Meiotic chromosome spreads were prepared and analyzed according to the protocol of Ross et al., (1996) with minor modifications as described in Agashe et al., (2002). The enzyme digestion mixture contained 0.3% cellulase and 0.3% pectinase/pectolyase. 3% stock solutions were prepared in 10mM citrate buffer pH 4.5/ with 45% glycerol and stored at -20 °C. Chromosomes were stained with DAPI (1 µg/ml) and observed on a Zeiss Axioplan 2 Imaging microscope using a 365 nm excitation and 420 nm long pass emission filter and a 100x oil objective. The images were captured on an Axioplan CCD camera using Axiovision (version 3.1) and edited with Adobe Photoshop 6.0.

For SEM analysis, the plant material was fixed over night in FAA containing 1% triton X-100 at 4 °C, dehydrated through an acetone series, followed by critical point drying in liquid CO₂. The dried samples were mounted on stubs, and sputter coated prior to examination using a Leica 240 scanning electron microscope.

2.7.8 RNA in situ hybridizations

In situ hybridizations were done according to the protocol of Jackson (1991). The regions used for strand specific cDNA probes were AML1 (NM_125589): 1583-2120 and AML4 (NM_120811): 1590-2623. Respective
regions from AML1 and AML4 for probe synthesis were PCR amplified using primer combinations AML1F2 & AML1R2 and A4RTF3 and A4R2 respectively and cloned into the vector pGEM-T (Promega). Strand specific RNA probes (sense or antisense) for the AML genes were prepared by in vitro transcription using the DIG RNA labeling kit (Roche) following linearization of the plasmid harboring the cDNA fragment. We used both SP6 and T7 RNA polymerases for the in vitro probe synthesis (for both sense and antisense probe). We followed the same protocol for hybridization, washing, and blocking steps as described by Jackson (1991). After these steps the DIG detection was carried out using the DIG nucleic acid detection kit (Roche) according to the manufacturer's instructions. The slides were mounted in 50% glycerol with cover slips. Observation was done using a Zeiss Axioplan 2 microscope equipped with Differential Interference Contrast (DIC) optics. Images were captured using an AxioCam camera, then edited and annotated using Adobe Photoshop 6.0. Specificity of the probe was validated in cross-hybridization experiments using radioactively labeled probe under conditions of stringency that were the same as those used for in situ. For each gene the probe used showed greater than 12-fold specificity over hybridization to the related genes.

2.7.9 Reagent compositions

**Tryptone Yeast Mannitol (TYM) medium:** For 1 litre— Bacto tryptone (5 g), Yeast extract (0.5 g), Mannitol (10 g), CaCl$_2$ (1 mM/litre), Agar Agar 1.5%.

**Murashige and Skoog (MS) medium 1X:** CaCl$_2$ (4 mM), MgSO$_4$ (1.5 mM), KNO$_3$ (18.8 mM), NH$_4$NO$_3$ (20.6 mM), KH$_2$PO$_4$ (1.25 mM) pH 5.6, Fe-EDTA (20 mM), Minor Salts (1X) (see below for the recipe). pH adjusted to around 5.6-5.7.

**Fe-EDTA solution:** 2.5 g of FeSO$_4$.7H$_2$O was dissolved in 400 ml of water followed by 3.36 g of NaEDTA and heated in a water bath till boiling. The volume
was made up to 450 ml. 2.5 ml of this stock solution was added to a liter of 1X MS solution.

**Minor nutrients (1000X Stock):** \( \text{H}_3\text{BO}_3 \) (70 mM), \( \text{MnCl}_2 \) (14 mM), \( \text{CuSO}_4 \) (0.5 mM), \( \text{ZnSO}_4 \) (1 mM), \( \text{NaMoO}_4 \) (0.2 mM), \( \text{NaCl} \) (10 mM), \( \text{CoCl}_2 \) (0.01 mM). Added 1 ml of this mix to 1 litre of the MS solution to make 1X minor nutrients.

**Infiltration medium:** ½ strength MS, 2% sucrose, 0.05 \( \mu \)m Benzyl amino purine (BAP), 200 \( \mu \)l/litre Silwet (surfactant).

**DNA extraction buffer:** 100 mM Tris (pH-8.0), 50 mM EDTA (pH-8.0), 500 mM NaCl, 10 mM 2-mercaptoethanol and 1.4% SDS.

**CTAB buffer:** 2% CTAB, 100 mM Tris (pH-8.0), 20 mM EDTA (pH-8.0) and 1.4 M NaCl.

**Carnoy’s solution:** 6:3:1 (Ethanol: Chloroform: Acetic acid).

**8% PFA:** Adjusted pH of 100 ml PBS to 11 with NaOH. Heated to 60 °C in a water bath. In a fume hood, added 8 g of paraformaldehyde and stirred till it dissolved. Cooled on ice and readjusted the pH to 7 with \( \text{H}_2\text{SO}_4 \).

**FAA:** 4% freshly prepared paraformaldehyde (PFA), 5% acetic acid and 50% ethanol.

Composition and preparation of all the other reagents/chemicals, which are used in this study, are adopted from Sambrook and Maniatis manual (Sambrook and Maniatis, 1989). Protocols explained in Sambrook and Maniatis were used for plasmid DNA isolation, PCR, cloning and bacterial transformation.
2.7.10 Programs used for DNA and protein sequence analysis

Protein sequences were aligned using ClustalW (www.ebi.ac.uk/clustalw) with default parameters (Higgins et al., 1994). The RRMs were identified using the NCBI conserved domain search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi; Marchler-Bauer and Bryant, 2004) Phylogenetic analysis was carried out using the bootstrap neighbour-joining (N-J) method of the Phylip 3.6a3 package (Felsenstein, 1989; http://bioweb.pasteur.fr). The tree was bootstrapped 100 times. Mei2 protein S. pombe was used as the out-group for constructing the tree.

BlastN and Blast 2: Used for sequence comparison and alignment of two DNA sequences for comparative analysis (Altschul et al., 1990).

Amplify 2.5β: Used to design the primers used in this study (William Engels, Genetics department, University of Wisconsin).

Gene Tool: Used for editing and assembling chromatograms after DNA sequencing. Also used for in silico restriction analysis (Wishart et al., 2000).

DNA Strider: Used for in silico restriction analysis of sequences and in silico translation of coding regions (Mark, 1988).

2.7.11 GenBank accession numbers

The GenBank accession numbers of sequences used in this study are: X07180 (Mei2p), At5g61960 (AML1), At2g42890 (AML2), At4g18120 (AML3), At5g07290 (AML4), At1g29400 (AML5), At3g26120 (AtTEL1), At1g67770 (AtTEL2), BAB92568 (OML1), BAD12869 (OML2), AAW56930 (OML3), BAD46727 (OML4), BAD28947 (OML5), and AF047852 (ZmTE1).