CHAPTER 5

Summary and Conclusions
RNA binding proteins have been known to regulate various aspects of plant development. These genes are involved in regulation of gene expression at the post-transcriptional level, which involves processing (splicing, polyadenylation), mRNA transport, translation and stability/degradation of RNA. Regulation is mainly achieved either directly by RNA binding proteins or indirectly where the RNA binding proteins modulate the function of other regulatory proteins (Lorkovic and Barta, 2002). *Arabidopsis* genome analysis for identifying RNA binding proteins has shown that there are more than 200 proteins, which contain the RRM (RNA recognition motif) and KH (K Homology) RNA binding motifs (Lorkovic and Barta, 2002). There is a growing body of evidence that the RNA binding proteins are involved in plant development, hormonal signalling, and circadian rhythms (Reviewed in Fedoroff, 2002).

5 *AML* genes in *Arabidopsis* constitute a family of RNA binding proteins, which show homology to the Mei2 protein of *S. pombe*. Mei2p is indispensable for the initiation of premeiotic DNA replication and entry of cell into meiosis I. Homologues of Mei2 gene have been identified in many other plant species like *Hordeum*, *Gossypium*, *Glycine max*, *Cynadon*, and petunia, suggesting that these genes are conserved through evolution. The objective of this study was to determine the role of the *AML* gene family in plant development using *Arabidopsis* as a model system. Further it also aimed at investigating if the conservation of homology between *AML* and Mei2 at the sequence level can be extended to the functional level.

As a first step towards elucidating the function of the *AML* genes during plant development spatial (for *AML2* and *AML3*) and temporal (for *AML1-4*) expression of these genes was characterized employing *in situ* hybridization, *AML3* promoter-GUS fusion, and semi-quantitative RT-PCR analysis. The expression pattern of this uncharacterized gene family gave clues towards the possible roles of these genes in various events during plant development. In addition, reverse genetic approaches were also used to understand the role of this gene family in plant development.

Results from this study demonstrate the involvement of the *AML* gene family in multiple stages during vegetative and reproductive phases of plant development. Two members of the *AML* gene family, *AML1* and *AML5*, were shown to be required during vegetative development of the plant. Plants homozygous for *aml1 aml5* showed
aberrant phyllotaxy indicating that the wild type products of AML1 and AML5 were required for determining the position of leaf primordia initiation. These genes are also required for leaf patterning as suggested by the distorted morphology and radialization of the leaves. The position of the new primordia on the flanks of the SAM is determined by the pre-existing leaf primordia. In Arabidopsis the maximum angle of divergence between successive leaves is ~140° (Furner and Pumfrey, 1992). In the double mutant plants the disruption in the phyllotaxy from spiral to alternate in some plants indicates an increase in the angle of divergence form 140° to 180°. Such defects in phyllotaxy can result from reduction in the size of the SAM, which would result in extending the limits of the inhibitory zone defined by the existing leaf primordia. Moreover, Wyrzykowska et al., (2002) have shown that rate of cell proliferation, in particular at the flanks of young leaf primordia is critical for proper development of lamina and leaf shape. This indicates a possibility that defects in leaf morphology observed in am/1 am/5 could be a result of defective signalling and cell proliferation.

The am/1 am/5 double mutant shares these phenotypes with the terminal ear1 (te1) mutant of maize. TE1 also encodes an RNA binding protein and shows homology to the Mei2 gene in the RNA recognition motif (RRM) domains. TE1 in maize is required for regulation of leaf initiation (Veit et al., 1998). Mutation of te1 results in abnormally shortened internodes and defects in leaf initiation and development. Some of the leaves in the te1 mutant also show large-scale pattern defects (Veit et al., 1998).

A clue regarding the possible function of two other members (AML2 and AML3) comes from the expression pattern analysis of these genes using in situ hybridisations and pAML3--GUS transgenic lines. The expression of these genes in the regions of active cell division: shoot apical meristem, leaf primordia, young leaves, and root tips (primary and secondary) suggested that they might have a role similar to AML1 and AML5. It would be therefore worthwhile to examine the am/2 am/3 double mutants for developmental defects. The lack of phenotype in am/1 am/3, am/1 am/4, am/3 am/4, am/3 am/5 could be due to genetic redundancy and analysing triple mutant combinations can test this possibility.

Similar intense expression in the actively dividing meristematic cells have also been reported for other RNA binding proteins (AtRBP1, RBP37, Poly A RNA Binding...
protein, PAB2) of yet unknown function (Hecht et al., 1997; Suzuki et al., 2000; Palanivelu et al., 2000)

A relatively uniform expression of AML2 and AML3 was detected in the embryo at all the stages of embryogenesis. The expression was associated with the growing and actively dividing embryo and was not observed in the suspensor cell. At the later stage of vegetative development a low basal expression was observed in all the plant tissues with intense expression detected in the actively dividing shoot and root apical meristem. Taken together this expression pattern indicates an association between AML2 and AML3 expression and actively dividing cells. This correlation leads us to hypothesize that these genes might have a general role (direct or indirect?) in cell proliferation. Moreover this hypothesis, to a certain extent, can explain the defective leaf morphology and altered phyllotaxy observed in the aml1 aml5 double mutant plants.

Besides their role in the vegetative development, multiple lines of evidence suggest the involvement of this gene family in reproductive development. Single and double mutant combinations examined, including aml1 aml5, did not show any defects in reproductive development. However, arrest in male and female gametophyte development, in multiple anti-sense and RNAi transformants, revealed a potential role of these genes during gametogenesis. Severe defects in male and female gametophyte development in the plants harbouring AML2 anti-sense or RNAi construct points to the possibility of the wild type AML2 gene products being involved in male and female gametophyte development. These developmental defects observed in the transgenic lines are heritable and dominant as expected for anti-sense and RNAi approaches.

The aml1 aml5 double mutant plants were recovered at a lower frequency in a F2 and F3 segregating population. The AML1\(^+\)/AML5\(^+\) and AML1\(^+\)/AML5\(^-\) mutant showed normal ovule development, seed set and germination which rules out the possibility of embryo lethal effects of aml1 aml5 double mutants. These observations indicate a possibility of defective transmission of aml1 aml5 combination through the male gametes. The pollens carrying aml1 AML5 have an advantage over the aml1 aml5 pollen during pollination.
Expression pattern for two representative members AML2 and AML3 provide additional support to our hypothesis of a possible role for AML genes during reproductive development. Intense expression of AML2 and AML3 in actively dividing cells of the inflorescence meristem (primary and secondary), floral meristem and floral organ primordia suggest a general role for these genes during plant reproductive development. These expression patterns are broadly similar to those observed for HUA2 (a RNA helicase), and HUA1 (nuclear RNA binding protein with CCCH type Zinc finger motifs) — proteins that participate in the complex regulation of flower development (Li et al., 2001; Western et al., 2002).

AML2 and AML3 mRNA accumulated in the early stages of anther and ovule development. In the anther, the sporogenous cells maintained expression of these genes as they form the pollen mother cells and proceed through meiosis to form tetrads of microspores. Though the expression pattern of AML2 and AML3 appear to be overlapping during sporogenesis, there were differences observed during gametogenesis. The AML2 expression reduced in the free microspores whereas AML3 expression was maintained through later stages of pollen maturation, suggesting distinct roles for these two genes.

During ovule development AML2 and AML3 transcripts accumulated in the cells on the adaxial faces of the two carpels. Intense expression of these genes was detected in the developing ovule including the megaspore mother cell, the emerging integuments, and the chalazal region. Later during ovule development the expression was observed in the embryo sac. The pattern of AML2 and AML3 expression during sporogenesis and gametogenesis is consistent with the gametophytic defects observed in the antisense and RNAi transgenic lines.

Collectively all these observations strongly suggest that AML genes have a broad role in vegetative and reproductive development. The expression pattern, double mutant analysis and anti-sense results indicate the requirement of these genes during cell proliferation (mitotic as well as meiotic division). Mei2p in S. pombe is required for pre-meiotic DNA replication and initiation of meiosis though how it actually brings about replication is not known. Moreover, Mei2p-meiRNA complex has been shown to
be involved in the meiosis-specific splicing of mes1 pre-mRNA. Mes1p function is required for the second meiotic division (reviewed in MacNeill and Fantas, 1995). Mei2p is known to be indispensable for meiosis but the molecular mechanism underlying this function is not known. Theoretically, meiosis is believed to be a modified version of mitosis. The genes defining the DNA replication machinery are conserved between both the S phases. Mutations that block enzymes of general cell cycle progression can block meiosis S phase and subsequent divisions. As AML proteins show homology to the Mei2 protein, it could be speculated that these genes might play a similar role in DNA replication or might be required for post-transcriptional regulation of an important component in cell cycle progression. AML genes might have a general role in cell proliferation common to both mitosis and meiosis cycles. A schematic integrating the findings from expression and functional analysis indicating different stages of plant development where the AML genes might have a probable function is shown in Fig. 5.1.

Possible functions of the AML proteins

There is a growing body of evidence that RNA binding proteins are involved in various developmental processes in plants. Recent reports provide evidence for the involvement of RNA binding proteins HUA1 and HEN4 and a RNA helicase HUA2, in flower mophogenesis. These genes are proposed to act by specifically facilitating the processing of AGAMOUS pre mRNA. Mutations in these genes lead to reduction in the level of AG transcripts (Cheng et al., 2003). The AG pre mRNA has a polyadenylation signal in the second intron, which when recognized results in premature polyadenylation and synthesis of a truncated mRNA. HEN1/HUA4 either bind to the pre mRNA and mask the recognition of this polyadenylation signal or promote correct splicing of the second intron.

Similarly FCA another nuclear RNA binding protein is involved in promoting flowering in Arabidopsis (Macknight et al., 1997). A recent study on the mechanism by which FCA regulates floral transition reveals its function in alternate splicing of its own transcript. FCA protein forms a complex with another protein FY and negatively regulates its own expression by promoting alternate splicing and polyadenylation within the 3rd intron of the nascent FCA pre mRNA (Simpson et al., 2003; Quesada et al.,
A schematic summarising evidence for involvement of AML genes during vegetative and reproductive development. Functional information obtained from reverse genetic approaches (T-DNA mutant analysis and anti-sense) is marked in red * and that inferred from expression analysis is shown in blue * 

Figure 5.1
The FCA/FY complex is also required for the down regulation of the floral repressor FLC. It is speculated that the FCA/FY complex might regulate the levels of FCA by prematurely polyadenylating the FCA transcripts.

*HUA1, HUA2, HEN4, and FCA/FY* regulate plant development by post-transcriptional processing of their own transcript and/or specific components of the development pathway. *AML* genes also encode RNA binding proteins and are potentially involved in multiple events during plant development. *AML1* and *AML5* required for determining the position of leaf primordia initiation and leaf development may probably regulate the signal emanating from the SAM by post-transcriptional processing of the target transcripts.

An alternate hypothesis for explaining the mode of action of *AML* genes in development is through its involvement in RNA localization. In recent years RNA localization has emerged as an important process in cellular and developmental biology. There is evidence supporting the role of RNA as a non-autonomous messenger in controlling plant growth and development (reviewed in Lucas et al., 2001). Several reports show that short range (through plasmodesmata) and long range (through phloem) trafficking of specific mRNA and protein signals is an important regulatory mechanism in plant development (Kim et al., 2001; Ruiz-Medrano et al., 1999). Knotted-1 (KN-1) a homeodomain transcriptional regulator is involved in cell fate determination in the vegetative and floral meristem. The KN-1 protein and mRNA undergo intracellular trafficking through plasmodesmata. For transport through plasmodesmata or phloem the RNAs associate with RNA binding proteins to form ribonucleoprotein complexes to facilitate their transport.

The development of the leaf and flowers is regulated by signals emanating from the SAM or received through the phloem (Reviewed in Foster et al., 2002). *AML1* and *AML5* proteins might be involved in non-cell autonomous signalling from the SAM to determine the position of leaf primordia initiation on its flanks and also for leaf development. *AML1* and *AML5* are also the two most similar members of the *AML* gene family. Hence, disruption of AML1 and AML5 proteins might result in a block in localization of the signalling molecule from the SAM to the primordia initiation site. This defective signalling can thus result in altered phyllotaxy and abnormal leaf morphology.
Figure 5.2

A schematic explaining the hypothesis that if the signaling between the SAM and the initiating the leaf primordia is disrupted in *aml1 aml5* mutant, it would lead to defective leaf positioning and leaf growth.

*Flowering time program induces AML gene expression in the mature rosette leaves*

From the results (Chapter 2) it is evident that the *AML* genes are targets of the flowering time program in the leaves and their expression is induced in the rosette leaves at the time of flowering. Most of the flowering time genes like *CO*, *LD*, *AGL20*, and *AGL24*, are expressed in the leaves and the SAM but their expression is induce prior to the conversion of the SAM into inflorescence meristem. In the case of AML the expression in the leaves is induced in response to initiation of the flowering process. All the four members examined showed a similar pattern of induction in expression in response to flowering. These results show (to my knowledge for the first time) the existence of targets of flowering time in the leaves. Moreover, the excised leaf experiments show that increase in *AML3* transcripts in the leaves is autonomous and does not need signals from the SAM. The increase in *AML3* expression in the mature leaves is independent from its requirement in leaf primordia initiation and development. Hence the induction of *AML* genes in the rosette leaves in response to flowering suggest yet another role of this gene family in the later stages of plant development.
To summarize, the AML gene family has 5 members and all the members are probably active as they encode for expressed mRNAs. Although the broad spatial and temporal expression pattern for these genes is similar suggesting a common function in plant development, yet these genes seem to have distinct functions too.

Of the ones tested, only aml1 aml5 double mutant combination resulted in defects in the vegetative growth. Other double mutant combinations showed a normal growth. AML1 and AML5 are the most similar members showing 75% identity and 84% similarity at the protein level.

There were slight differences observed in the temporal expression of AML2 and AML3 during male gametogenesis, suggesting that besides overlapping functions AML3 might have some additional functions too. Results from semi-quantitative RT-PCR analysis show that all the members of the AML family respond to similar stimuli (environmental and endogenous).

Detailed characterization of the defects observed in the aml1 aml5 double mutants and analysis of triple mutants will give further insight about the distinct and overlapping functions of the AML gene family. I have analyzed the RNA expression profile; additional information regarding the localization of the proteins would also be informative towards understanding the function of this gene family.