TOR SIGNALING AND ARABIDOPSIS DEVELOPMENT

A Dissertation
Presented to the Faculty of the Graduate School
of Cornell University
in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy

by
Garrett H. Anderson
January 2005
TOR (target of rapamycin) proteins are central regulators of cell growth in eukaryotes. Acting as protein kinases, they integrate nutrient, amino acid and hormonal signals to regulate ribosomal activity, mRNA recruitment to the ribosome and other processes via the phosphorylation of downstream activators. Their ability to phosphorylate target substrates depends on the activity of a second conserved eukaryotic protein, Raptor (regulatory associated protein of TOR), which as a TOR binding partner presents substrates to TOR for phosphorylation.

Mei2 is a putative TOR substrate in the fission yeast Schizosaccharomyces pombe. It acts in concert with a noncoding, mRNA-like molecule meiRNA to trigger meiosis in conjugated diploids under low nutrient conditions. Mei2 is present as a single-copy locus in a variety of eukaryotes and as a small conserved gene family in land plants.

This thesis describes efforts to characterize the Arabidopsis Raptor and Mei2 homologues as a means of understanding TOR signaling in land plants. Using a reverse-genetics approach, I have isolated Arabidopsis lines harboring disruptions of the Raptor homologues AtRaptor1A and AtRaptor1B, and crossed these lines to generate AtRaptor double mutants. AtRaptor1B mutants show subtle defects to root and shoot development; AtRaptor double-mutants show normal embryonic development but arrest growth as seedlings with minimal post-embryonic meristem-based growth.
AtRaptor1B interacts with the Arabidopsis Mei2 homologue AML1 (Arabidopsis Mei2-like 1) in a yeast two-hybrid assay, in an interaction mediated by the AML1 N-terminus. This implicates AML1 as a substrate for AtTOR in Arabidopsis TOR signaling. Using reverse-genetics complemented by a phylogenetic analysis of the AML gene family in Arabidopsis and elsewhere in the angiosperms, I show that there are two conserved clades of AML-like gene products in the angiosperms. There is considerable redundancy among and between members of these clades. Single and higher-order mutants harboring disruptions of the AML loci yielded subtle defects in the timing of the transition to flowering. A line homozygous for disruptions in all five AML loci did not differ dramatically from lower-order insertion allele homozygotes; this may be due to accumulation of transcript fragments expressed from the disrupted AML5 locus.
BIOGRAPHICAL SKETCH

Born in Pasadena, California on October 11, 1972, Garrett Anderson was raised in Washington, Pennsylvania. He graduated from Trinity High School at the head of his class in 1991. He attended Princeton University from 1991-1995, graduating with an A.B. degree, *cum laude*, in Ecology and Evolutionary Biology and earning certificates in both East Asian History and Chinese Language and Literature. From 1995-1996 he taught English at the Hubei Education Academy in Wuhan, P.R. China; in 1997 he worked with the Nature Conservancy and the Government of Yunnan province to establish the Great Rivers National Park in Northwest Yunnan province, China. In the summer of 1997 he began graduate work at Cornell University, first in the Field of Plant Biology and later in the Field of Genetics, and was a member of Dr. Maureen R. Hanson’s lab from spring 1998 to the completion of his degree.
This work is dedicated to my wife and my parents.
ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Maureen Hanson, for giving me the freedom and the resources to pursue my own interests and for giving me the guidance to bring these interests together into a single thesis. The intellectual free reign did much to maintain my enthusiasm in my research.

Thanks also go to my committee members Dr. David Stern and Dr. Jian Hua for their timely advice and considerable technical assistance, and to former member Dr. Jeff Doyle for his advice and training. Thanks also to Dr. Eric Alani for his frank discussions both academic and otherwise.

Many thanks go also to Dr. Bruce Veit of AgResearch New Zealand for a long and productive collaboration, and for his enthusiasm in Mei2-like proteins. Thanks also go to Nena Alvarez, his former student and collaborator. Special acknowledgment must also be made to Masayuki Yamamoto and Yoshinuri Watanabe, whose work on Mei2 signaling in fission yeast guided much of my early thinking on this project.

Much of my work was facilitated by the DNA Sequencing Facility and IDT DNA for providing timely sequencing results and primers. My plants were well-taken care of by Paul Cooper, who was both an excellent worker and a refreshing source of conversation. Mutant lines were made available by Joseph Ecker and the Salk Institute as well as Michael Sussman and the University of Wisconsin Arabidopsis Knockout Facility.

Support for my work was provided by the a fellowship for the NSF/DOE/USDA Training Group in Molecular Mechanisms of Plant Processes and USDA Hatch Program and DOE Energy Biosciences (DE-F602-89ER14030) grants to Dr. Hanson.
All members of the Hanson lab have at some point or another contributed to my work; I would like to single out Ernie Kwok for sharing 303B Biotech with me for six years and Yan Xu for leaving me with what became a very interesting project.

Outside of lab I am indebted to Ali Alan, Jason Palter and Paul Mason for organizing the Smashed Tomatoes soccer team. It provided me with so many fond memories and so much well-needed exercise.

Finally I would like to thank my wife Qinhong Anderson for her patience and her support of my pursuit of science.
TABLE OF CONTENTS

CHAPTER 1: TOR SIGNALING – A REVIEW  1

CHAPTER 2: THE ARABIDOPSIS AtRaptor GENES ARE ESSENTIAL FOR POST-EMBRYONIC PLANT GROWTH  15

CHAPTER 3: DIVERSICATION OF GENES ENCODING Mei2-LIKE RNA BINDING PROTEINS IN PLANTS  43

CHAPTER 4: THE Arabidopsis Mei2 HOMOLOGUE AML1 BINDS AtRaptor1B, A MAJOR REGULATOR OF PLANT GROWTH  72

BIBLIOGRAPHY:  90
LIST OF TABLES

Table 3.1  Mei2-like gene products in Arabidopsis, Rice and Chlamydomonas  51
Table 3.2  Other Full and Near Full length Mei2-like Gene Products in the Viridiplantae  54
Table 3.3  Alternatively Spliced and Genomic Variant AML Transcripts  61
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>TOR complexes in mammals</td>
</tr>
<tr>
<td>2.1</td>
<td>Raptor proteins in eukaryotes are highly conserved</td>
</tr>
<tr>
<td>2.2</td>
<td>AtRaptor loci and insertion allele characterization</td>
</tr>
<tr>
<td>2.3</td>
<td>Seedling root phenotype of <em>AtRaptor1B-/-</em> mutants</td>
</tr>
<tr>
<td>2.4</td>
<td><em>AtRaptor1B-/-</em> plants grow slowly</td>
</tr>
<tr>
<td>2.5</td>
<td><em>AtRaptor1B-/-</em> plants show altered shoot architecture</td>
</tr>
<tr>
<td>2.6</td>
<td><em>AtRaptor</em> accumulation pattern</td>
</tr>
<tr>
<td>2.7</td>
<td><em>AtRaptor1A-/- 1B-/-</em> double mutants</td>
</tr>
<tr>
<td>2.8</td>
<td>TOR functions in two complexes in eukaryotes</td>
</tr>
<tr>
<td>3.1</td>
<td>Mei2-like C-terminal RNA Recognition Motif</td>
</tr>
<tr>
<td>3.2</td>
<td>Phylogeny of the Mei2-like proteins in the Viridiplantae</td>
</tr>
<tr>
<td>3.3</td>
<td>Conserved motifs and local alignments for Arabidopsis, Oryza and Chlamydomonas AML protein sequences</td>
</tr>
<tr>
<td>3.4</td>
<td>Conserved motifs and local alignments for TEL protein sequences</td>
</tr>
<tr>
<td>3.5</td>
<td><em>In situ</em> hybridization of TEL and AML probes to Arabidopsis developing embryos and apical meristems</td>
</tr>
<tr>
<td>3.6</td>
<td>Quantification of <em>AML</em> transcript levels by quantitative PCR</td>
</tr>
<tr>
<td>4.1</td>
<td>AML1 interacts with AtRaptor1B in a yeast two-hybrid assay</td>
</tr>
<tr>
<td>4.2</td>
<td>The AML1 N-terminus harbors transcriptional activation domain activity</td>
</tr>
<tr>
<td>4.3</td>
<td><em>AML</em> loci and insertion alleles</td>
</tr>
<tr>
<td>4.4</td>
<td><em>AML</em> mutant allele characterization</td>
</tr>
<tr>
<td>4.5</td>
<td><em>AML</em> insertion mutants bolt early</td>
</tr>
</tbody>
</table>
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4EBP</td>
<td>eukaryotic initiation factor 4E binding protein</td>
</tr>
<tr>
<td>AD</td>
<td>transcription activation domain</td>
</tr>
<tr>
<td>Ade</td>
<td>Adenine</td>
</tr>
<tr>
<td>AML</td>
<td>Arabidopsis Mei2-like</td>
</tr>
<tr>
<td>AtRaptor</td>
<td>AtRaptor; <em>Arabidopsis thaliana</em> Raptor homologue</td>
</tr>
<tr>
<td>AtTOR</td>
<td>Arabidopsis thaliana Target of rapamycin</td>
</tr>
<tr>
<td>AVO3</td>
<td>Adheres voraciously to TOR; yeast Rictor homologue</td>
</tr>
<tr>
<td>BD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>Bp</td>
<td>base pair</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>Col-0</td>
<td>Arabidopsis thaliana ecotype Columbia</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>eIF4E</td>
<td>eukaryotic initiation factor 4E</td>
</tr>
<tr>
<td>EST</td>
<td>Expressed sequence tag</td>
</tr>
<tr>
<td>Et</td>
<td>Etiolated</td>
</tr>
<tr>
<td>FKBP</td>
<td>antibiotic FK56 binding protein</td>
</tr>
<tr>
<td>GbetaL</td>
<td>G-protein beta subunit like</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HEAT</td>
<td>Huntington, elongation factor3, A subunit of protein phosphatase 2A and TOR1 repeat motif</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>Kb</td>
<td>kilobase pair</td>
</tr>
<tr>
<td>KOG1</td>
<td>Kontroller of growth; yeast Raptor homologue</td>
</tr>
<tr>
<td>Leu</td>
<td>Leucine</td>
</tr>
<tr>
<td>LST8</td>
<td>Lethal with sec13; yeast GbetaL homologue</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Mei2</td>
<td>Meiosis inhibited</td>
</tr>
<tr>
<td>Mip1</td>
<td>Mei2 interacting protein; fission yeast Raptor homologue</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian Target of rapamycin</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAUP*</td>
<td>phylogenetic analysis using parsimony and other techniques</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositol-3 kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>polII</td>
<td>RNA polymerase II</td>
</tr>
<tr>
<td>Q-line</td>
<td>Quintuple insertion allele homozygotes</td>
</tr>
<tr>
<td>RAM</td>
<td>root apical meristem</td>
</tr>
<tr>
<td>Raptor</td>
<td>Regulatory associated protein of TOR</td>
</tr>
<tr>
<td>Rictor</td>
<td>Rapamycin-insensitive companion of TOR</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNC/C</td>
<td>Raptor N-terminal conserved / caspase</td>
</tr>
<tr>
<td>RT</td>
<td>reverse-transcription</td>
</tr>
<tr>
<td>SAM</td>
<td>shoot apical meristem</td>
</tr>
<tr>
<td>S6K</td>
<td>ribosomal protein S6 kinase</td>
</tr>
<tr>
<td>Sme2</td>
<td>suppressor of mei2, encoding meiRNA</td>
</tr>
<tr>
<td>Ste7</td>
<td>sterile; fission yeast GbetaL homologue</td>
</tr>
<tr>
<td>Suc</td>
<td>sucrose</td>
</tr>
<tr>
<td>TE1</td>
<td>Terminal ear 1</td>
</tr>
<tr>
<td>TEL</td>
<td>Terminal ear-like</td>
</tr>
<tr>
<td>TOR</td>
<td>Target of rapamycin</td>
</tr>
<tr>
<td>TORC1</td>
<td>TOR complex 1</td>
</tr>
<tr>
<td>TORC2</td>
<td>TOR complex 2</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>Trp</td>
<td>tryptophan</td>
</tr>
<tr>
<td>WD40</td>
<td>tryptophan/aspartic acid repeat motif</td>
</tr>
<tr>
<td>Ws</td>
<td><em>Arabidopsis thaliana</em> ecotype Wassilewskija</td>
</tr>
<tr>
<td>YEPDA</td>
<td>Yeast extract with peptone, dextrose and adenine</td>
</tr>
</tbody>
</table>
CHAPTER ONE
TOR SIGNALING – A REVIEW

Cell fate determination in metazoans and plants – dramatically different strategies for multicellularity

Cell fates are determined very early in metazoan development. In mammals and most other metazoans, embryogenesis essentially produces a miniature version of the adult organism, and all (or nearly all) post-embryonic growth results from the expansion in size and number of cell types which have already been established. Some post-embryonic differentiation occurs, but rarely if ever is there a complete reversion to totipotency among differentiated metazoan cells. The situation is only slightly more complicated in metazoans like Drosophila melanogaster, which undergo considerable rearrangement of their body plan in the transition from larvae to adult, because this rearrangement results not from the reversion to totipotency but from the delayed development of previously differentiated cell types.

In dramatic contrast, cell fate determination in angiosperms is integrally related to post-embryonic cell growth. The development of the angiosperm embryo initially resembles that of the metazoan: an apical-basal axis is established, and cells differentiate into organs which superficially resemble those of the adult. However, whereas the metazoan grows post-embryonically through the expansion in size of these embryonically established, terminally differentiated cell types, angiosperms grow post-embryonically through the formation of novel organs from preserved totipotent cell lines called meristems.

As a result, angiosperm development is remarkably plastic in comparison to that of metazoans. Because totipotent cells are preserved through all stages of development, the size and even the number of organs produced by a given individual
can vary dramatically among genetically identical populations in response to environmental cues such as day length, temperature, and availability of light.

Also as a result of the maintenance of totipotent cells throughout plant development, angiosperm cell differentiation is linked to overall plant growth. Thus, conserved eukaryotic signaling mechanisms which in metazoans regulate cell growth can be seen in the angiosperms as candidates for the regulation of post-embryonic cell differentiation.

**TOR is a regulator of growth in yeast and metazoans**

One such candidate for the regulation of post-embryonic growth and differentiation is the TOR signaling pathway. In both yeast and mammalian systems, TOR (target of rapamycin) proteins act in a signaling pathway to regulate cell growth in response to nutrients and hormones. TOR proteins are large (about 2,400 residues) protein kinases comprised of a series of helix-turn-helix HEAT repeats followed by a more highly conserved phosphoinositol-3 kinase (PI3K)-like kinase domain. Though they belong to the PI3K family of kinases, they are thought to act exclusively as protein kinases (Thomas et al., 2004).

TOR proteins perform two distinct functions in yeast and mammals. In a complex with other proteins, TOR regulates cell growth in response to nutrient and hormone signals (Helliwell et al., 1998; Hara et al., 2002; Kim et al., 2002; Loewith et al., 2002; Kim et al., 2003; Kim and Sabatini, 2004) as mentioned above. In a second complex, TOR regulates cytoskeletal organization (Helliwell et al., 1998; Loewith et al., 2002; Sarbassov et al., 2004). TOR kinase activity is essential for its participation in nutrient signaling (Schmelzle and Hall, 2000; Thomas et al., 2004) and may be for its participation in cytoskeletal organization as well.

TOR proteins are encoded by one or occasionally two loci in all eukaryotes for which sufficient sequence information is available. They were originally identified in
Saccharomyces cerevisiae (baker’s or budding yeast), and have been extensively characterized in both yeast and mammalian cells. Significant work has also been done in Schizosaccharomyces pombe, Caenorhabditis elegans, Drosophila and Arabidopsis thaliana. TOR proteins have been extensively reviewed (Schmelzle and Hall, 2000; Raught et al., 2001; Gingras et al., 2004; Hay and Sonenberg, 2004; Thomas et al., 2004); the following will provide a brief summary of the information on TOR signaling to date.

TOR proteins were identified as the targets of the antiproliferative toxin rapamycin. Rapamycin was isolated from a strain of Streptomyces hygroscopicus originally found on Easter Island (Rapa Nui in the language of the island’s ancestral inhabitants) but no longer known in the wild (Thomas et al., 2004). There is not yet consensus on the mechanism by which rapamycin perturbs TOR function (Hara et al., 2002; Kim et al., 2002; Kim and Sabatini, 2004; Oshiro et al., 2004), but it is known that TOR is only sensitive to rapamycin complexed with the immunophilin FK506-Binding Protein 12 (FKBP12) (Bierer et al., 1990; Heitman et al., 1991). Sensitivity to rapamycin varies among eukaryotes: it triggers cell cycle arrest due to a defect in cell growth in budding yeast (Heitman et al., 1991) and mammalian T cells (Thomas et al., 2004), inhibits meiosis but does not interfere with vegetative growth in S. pombe (Weisman et al., 1997), and has no effect on Arabidopsis (Menand et al., 2002). Rapamycin-FKBP12 complexes bind TOR at a C-terminal region of the protein which is highly conserved across eukaryotes (Choi et al., 1996); variability in sensitivity to rapamycin is thought to result in part from variability in the ability of various species’ FKBP12 proteins to bind rapamycin, rather than variations in the TOR proteins themselves.

**TOR signaling in yeast**

The two S. cerevisiae TOR loci, TOR1 and TOR2, were identified through gain
of function mutants which conveyed resistance to rapamycin (Heitman et al., 1991; Kunz et al., 1993). tor1 null mutants show a mild hypersensitivity to rapamycin. tor2 null mutants show cell cycle arrest within four generations, accompanied by cytoskeletal depolarization. Double mutants, like rapamycin-treated cells, show a G1 cell-cycle arrest due to an inability to sustain cell growth (Heitman et al., 1991; Kunz et al., 1993; Barbet et al., 1996). These phenotypes indicated that TOR1 and TOR2 share a rapamycin-sensitive role in cell growth, and that TOR2 possesses a second role in the regulation of cytoskeletal polarity.

The role of the TOR proteins in yeast cell growth is far-reaching. They have been implicated in protein translation initiation, upregulation of the processivity of the ribosome, upregulation in ribosomal RNA translation, downregulation of protein degradation and downregulation of RNA degradation (Chan et al., 2000; Schmelzle and Hall, 2000). TOR2’s role in cytoskeletal reorganization is not well understood, though work has implicated a RHO1 GTPase (Schmeltze et al 2002) and the kinase PKC1 (Schmidt et al., 1997).

Major insight into the biochemistry of TOR signaling came from the finding that there are two distinct TOR complexes in yeast (Loewith et al., 2002). The first of these, TORC1, involves TOR1 or TOR2, LST8 and KOG1, the yeast Raptor homologue. This complex binds FKBP12-rapamycin, and selective depletion of this complex via the repression of KOG1 transcription phenocopies rapamycin treatment.

The second TOR complex, TORC2, involves TOR2, LST8 and AVO1-3. This complex does not bind rapamycin, and selective repression of AVO1 transcription mimics the tor2 mutant phenotype. Repression of LST8 transcription mimics the phenotype of tor1 tor2 double mutants.

**TOR in mammals**

The single mammalian TOR protein mTOR (also known as FRAP – FKBP12-
rapamycin associated protein, RAFT – rapamycin and FKBP12 target and RAPT – rapamycin target) was first identified as a binding partner of a rapamycin-FKBP12 complex (Brown et al., 1994). mTOR disruption in mice is lethal; neither pleuripotent stem cells nor placental cell precursors are able to grow from mTOR disruption homozygotes. Rapamycin treatment phenocopies only part of the mTOR disruption phenotype: rapamycin-treated embryos share the defect in placental cell growth but are able to begin pleuripotent stem cell growth (Murakami et al., 2004). mTOR plays a well-characterized role in translation (Schmelzle and Hall, 2000) and a recently uncovered role in cytoskeletal regulation (Sarbassov et al., 2004).

Like TOR2, mTOR is involved in two distinct protein complexes. The first of these involves Raptor (Hara et al., 2002; Kim et al., 2002) and GβL (Kim et al., 2003) (the mammalian KOG1 and LST8 homologues). This complex is nutrient-sensitive and is destabilized by raptor-FKBP12 (Kim et al., 2003). The second of these complexes involves GβL, Rictor (rapamycin-insensitive companion of mTOR), a weak homologue of yeast AVO3, and may contain other proteins as well (Sarbassov et al., 2004). This complex does not interact with rapamycin-FKBP12. RNAi-mediated knockdown of Rictor expression causes defects in cytoskeleton and a decrease in the phosphorylation of PKCa, the mammalian homologue of yeast PKC1 implicated in yeast TOR2 cytoskeletal regulation.

mTOR is known to regulate translation in response to nutrients via the upregulation of ribosomal processivity and the upregulation of mRNA recruitment to the ribosome (Schmelzle and Hall, 2000). Increased ribosomal processivity is accomplished via the phosphorylation of S6K, a kinase which directly phosphorylates ribosomal protein S6; phosphorylated S6 is more active. Upregulation of mRNA recruitment is accomplished via the phosphorylation of the eukaryotic initiation factor 4E (eIF4E) binding protein (4EBP). Unphosphorylated 4EBP binds eIF4E, preventing
it from recruiting mRNA to the ribosome. mTOR phosphorylates 4EBP, decreasing its affinity for eIF4E. Phosphorylation of both of these substrates is rapamycin-sensitive \textit{in vivo}. mTOR phosphorylates both of these substrates weakly or not at all \textit{in vitro}; addition of Raptor increases the phosphorylation of these substrates significantly (Hara et al., 2002).

**TOR in other metazoans**

In both Drosophila and \textit{C. elegans}, disruption of the single \textit{TOR} locus phenocopies nutrient starvation (Oldham et al., 2000; Zhang et al., 2000; Long et al., 2002). The Drosophila \textit{dTOR} disruption phenotype mimics rapamycin treatment and can be restored by overexpression of \textit{dS6K}, the Drosophila ribosomal protein S6 kinase homologue (Oldham et al., 2000; Zhang et al., 2000). \textit{C. elegans} is rapamycin insensitive. \textit{CeTOR} disruption shows some traits in common with starvation, but is accompanied by a starvation-independent atrophy of the intestine (Long et al., 2002).

Homologues of Raptor, Rictor and GβL are present in both Drosophila and \textit{C. elegans}, but the formation of TOR complexes in these organisms has yet to be shown. Selective reduction of \textit{dRictor} and \textit{dTOR} but not of \textit{dRaptor} causes a reduction in the accumulation of \textit{dPKCa}, the Drosophila homologue of the kinase thought to mediate TOR action on the cytoskeleton (Sarbassov et al., 2004). Deletion (Jia et al., 2004) and selective depletion of \textit{CeRaptor} phenocopy \textit{CeTOR} depletion and \textit{Ce4E-BP} depletion (Hara et al., 2002).

**TOR signaling in \textit{S. pombe}**

\textit{S. pombe} contains two \textit{TOR} loci. \textit{Tor2} is essential for viability (Weisman and Choder, 2001). \textit{Tor1} is not essential for growth, but is apparently critical for deciding when to stop growing: \textit{tor1} mutants grow normally under high nutrients but rapidly lose viability upon reaching saturation (Kawai et al., 2001; Weisman and Choder, 2001). \textit{Tor1} is also essential for viability under temperature, pH, osmotic and
oxidative stress conditions (Kawai et al., 2001), and tor1 mutants are slightly more sensitive to rapamycin during vegetative growth than are wild-type cells (Weisman and Choder, 2001). Both rapamycin treatment and tor1 disruption inhibit sexual differentiation under low nutrient conditions (Weisman et al., 1997; Kawai et al., 2001; Weisman and Choder, 2001; Weisman et al., 2001).

Raptor, Rictor and GβL have clear homologues in S. pombe. The S. pombe Rictor homologue was independently identified as Ste20 (Hilti et al., 1999). Ste20 null mutants arrest their cell cycles in G₂ upon transfer to a nitrogen-free medium, in contrast to wild-type cells which arrest in G₁. Ste20 null mutants are sterile, and this results from the inability to induce mei2, a transcript whose protein product is a potent meiosis signaling molecule.

The S. pombe Raptor homologue was also independently identified as Mip1 (Mei2 interacting protein)(Shinozaki-Yabana et al., 2000); indeed, both Rictor and Raptor were first described in S. pombe. An allele encoding an N-terminally truncated Mip1 protein was identified through a screen for suppressors of a super-active allele of mei2, the potent meiosis signaling molecule whose expression was abolished in the Rictor mutant Ste20 discussed above. Full-length Mip1, as well as the truncated allele, interact with full length Mei2 protein. mip1 null mutants, like tor2 mutants, are inviable (Shinozaki-Yabana et al., 2000), suggesting that Tor1 and Tor2 both form a complex with Mip1; if Mip1 formed a TOR-Raptor complex with Tor1 only, one would expect mip1 null mutants to phenocopy tor1 mutants.

Summary – TOR signaling in yeast and mammalian systems

The consistent story recently emerging from work on TOR signaling in a wide range of eukaryotes (mostly metazoans) is that there are two distinct TOR activities: cell growth regulation in response to nutrient signals, and cytoskeletal regulation. The two activities correspond to two TOR complexes in both yeast and mammalian cells.
The first complex includes homologues of GβL and Raptor, and is sensitive to rapamycin in some species. It acts consistently through S6K, 4EBP, or both, to regulate translation. The second involves homologues GβL and the less strongly conserved Rictor, and perhaps other proteins, and acts through a PCK1 homologue (Figure 1.1).

Work from *S. pombe* supports this general story but also points to novel avenues of research. Tor1 and Tor2 both appear to form a TOR-Raptor complex to sense nutrients, as is the case with TOR in budding yeast; however, whereas TOR elsewhere appears to promote growth in the presence of nutrients, *S. pombe* TOR activity is critical for the cessation of growth, and the promotion of low-nutrient developmental pathways, in the absence of nutrients. The lethality of *tor2* disruption indicates that it also has a second, rapamycin-independent activity which does not overlap with Tor1.

**TOR in plants**

The single Arabidopsis TOR homologue, AtTOR, is critical for plant development (Menand et al., 2002). *AtTOR* mutants undergo cell division but fail to gain cell volume or to undergo any cell differentiation beyond the formation of a suspensor and globular embryo. The *AtTOR* transcript accumulates in all tissues assayed (Robaglia et al., 2004), but an *AtTOR:GUS* fusion is only translated in dividing and growing cells (Menand et al., 2002). This apparent post-transcriptional regulation of *AtTOR* expression may be regulated by a TOR-specific microRNA conserved between Arabidopsis and rice (Bonnet et al., 2004).

There are readily identifiable homologues of the Raptor and GβL, components of the eukaryotic nutrient-sensitive growth regulation complex, in the completed genome sequences of both Arabidopsis (AGI, 2000) and rice (Goff et al., 2002; Yu et al., 2002). Rictor homologues, components of the cytoskeletal regulation complex,
**Figure 1.1.** TOR complexes in mammals. The complex at left regulates translation and cell growth in response to nutrient signals. It is comprised of TOR (depicted as a zig-zag of HEAT repeats and a square kinase domain), Raptor and GβL. Raptor functions in this complex to recruit substrates for phosphorylation by TOR. The homologous yeast complex involves TOR1 or TOR2, KOG1 and LST8, homologues of Raptor and GβL. The complex at right regulates cytoskeletal organization in response to unknown signals. It is comprised of TOR, GβL and Rictor; it may involve other proteins as well. The homologous yeast complex involves TOR2, LST8, AVO1, AVO2, and AVO3. AVO3 is the yeast Rictor homologue.
are not found in either plant genome; however, this may be due to the low degree of sequence conservation among Rictor homologues (mammalian and S. pombe Rictor homologues, for example, share only 24 percent similarity, compared to 38 percent for the corresponding Raptor homologues) rather than the absence of all Rictor homologues from these organisms.

Much insight into TOR function in yeast and mammals has come from the fact that TOR nutrient-sensing activity, but not its cytoskeletal regulation activity, is sensitive to rapamycin complexed to the immunophilin FKBP12. Arabidopsis is insensitive to rapamycin (Menand et al., 2002); this is apparently due to the inability of Arabidopsis FKBP12 to form a complex with rapamycin (Robaglia et al., 2004) rather than due to a change in AtTOR compared to other eukaryotic TOR proteins. Thus, though TOR function is critical early in plant embryonic development, is it not clear if plant TOR forms two distinct complexes with distinct signaling functions as it does in yeast and metazoans.

**TOR effectors as a means of identifying TOR function**

As a kinase, TOR acts in the regulation of cell growth through the phosphorylation of substrates which effect TOR signaling. Identification of TOR substrates in plants, then, may serve as a means toward understanding the role of TOR in angiosperms. All known TOR substrates in the growth regulation pathway act to regulate the translation machinery directly (Schmelzle and Hall, 2000), either by increasing the processivity of the ribosome itself or by decreasing the inhibition of mRNA recruitment to the ribosome - however, it must be mentioned that genetic evidence implicates TOR in a broad range of processes (Chan et al., 2000), and the list of TOR substrates will undoubtedly expand considerably in the coming years.

One intriguing putative TOR substrate is the Mei2 protein of *S. pombe*. Mei2
is a potent signaling molecule in the *S. pombe* meiosis signaling pathway, and homologues of this protein are present in all land plants (see below). Mei2 has not been shown to be a TOR substrate; however, multiple lines of evidence point strongly to Mei2 as a TOR substrate.

First, Mei2 is regulated by phosphorylated at three sites (Watanabe et al., 1997). The kinase governing two of these sites has been identified. The function of the third of the three Mei2 phosphorylation site, and its governing kinase, are unknown (Watanabe et al., 1997). However, *S. pombe* Tor1 kinase activity is essential for the generation of the meiosis-inducing nutrient signal upstream of Mei2 (Kawai et al., 2001; Weisman et al., 2001), indicating that it regulates Mei2. Secondly the *S. pombe* Raptor homologue Mip1 interacts with Mei2 (Shinozaki-Yabana et al., 2000). In all systems where Raptor binding partners have been characterized, Raptor binds two classes of proteins: TOR complex partners and TOR substrates (Hara et al., 2002; Kim et al., 2002; Loewith et al., 2002). Thus, an interaction with a Raptor homologue implicates a protein as an effector of TOR signaling.

**Mei2 in *S. pombe***

Mei2 is the terminal signaling molecule in the fission yeast *S. pombe* meiosis signaling pathway (Watanabe et al., 1988; Watanabe and Yamamoto, 1994). The Mei2 protein is comprised of three RNA Recognition Motifs (RRMs), the third of which is critical for protein activity (Watanabe et al., 1997). Mei2 accumulates in response to low nutrient conditions (Watanabe and Yamamoto, 1994). Detection of these nutrient conditions is rapamycin sensitive and requires Tor1 (Kawai et al., 2001; Weisman and Choder, 2001) and, surprisingly, requires the *S. pombe* Rictor homologue Ste20 (Hilti et al., 1999). In haploid cells, Mei2 is inactivated by phosphorylation at two sites by Pat1 kinase (McLeod and Beach, 1988; Watanabe et al., 1997). The function of, and kinase responsible for a third phosphorylation site is
unknown (Watanabe et al., 1997). Pat1-phosphorylated Mei2 is sequestered in the cytoplasm (Yamashita et al., 1998; Sato et al., 2002), ubiquitinated at an undetermined site in its N-terminal half and degraded (Kitamura et al., 2001).

Following conjugation between compatible mating types and karyogamy (nuclear fusion), Pat1 is inactivated (McLeod and Beach, 1988; Li and McLeod, 1996) allowing Pat1-unphosphorylated Mei2 to accumulate. Mei2 unphosphorylated by Pat1 is active at very low levels of accumulation; though transcriptionally upregulated under low nutrient conditions, Mei2 driven by a minimal promoter is able to complement mei2 locus lesions (Peng et al., 2003). Pat1-unphosphorylated Mei2 accumulates at a specific focus in the nucleus called a ‘Mei2 dot,’ where it binds the noncoding, polyadenylated, mRNA-like molecule meiRNA (Yamashita et al., 1998). This interaction occurs concurrently with meiRNA transcription resulting in the accumulation of Mei2 proteins tethered to the meiRNA locus (Shimada et al., 2003). Accumulation of Mei2 at a single focus within the nucleus triggers chromatin remodeling (Mizuno et al., 2001) and immediately precedes meiosis I.

**Phylogenetic distribution of Mei2-like proteins**

Like other TOR downstream effectors but unlike TOR complex members, Mei2 homologues are not universally present in eukaryotic genomes. They are absent altogether from the metazoans. There is a single mei2-like locus in a broad range of fungal genomes, such as *Aspergillus nidulans* and *Neurospora crassa*, but not in budding yeast. One or at most two mei2-like loci are found in the alveolates *Paramecium tetraurelia*, *Plasmodium falciparum*, *Toxoplasma gondii*, and *Neospora hughesii*, but not in *Cryptococcus neoformans*. Single mei2 loci are found in the diatom *Thalassiosira pseudonana*, a cryptomonad with a secondarily acquired red algal chloroplast, but not in the primary red alga *Cyanidioschyzon merolae*. There is a single mei2 locus in the available nuclear genome of *Chlamydomonas reinhardtii*. 
A small conserved gene family of mei2-like loci has been found in all vascular plants for which sufficient sequence information is available. This stands in sharp contrast to all other organisms known to contain mei2-like loci, and suggests that they have proliferated in response to some developmental need unique to land plants.

**Summary of Following Chapters**

In this thesis the following aspects of TOR signaling are addressed in an effort to understand both TOR signaling and its role in angiosperm cell differentiation. In Chapter 2, consequences of partial and total disruption of AtRaptor on plant growth are shown. As Raptor is a component of the TOR nutrient-sensitive protein complex but not of the cytoskeletal-regulatory complex in yeast and mammals, disruption of Raptor activity in Arabidopsis can be compared to determine, first, if the loss of Raptor activity mimics the loss of TOR activity. If it does not, then the AtRaptor and AtTOR mutant phenotypes can be compared to determine what aspects of TOR signaling in plants is Raptor-independent and presumably due to TOR activity in a Raptor-independent complex, and what role the TOR-Raptor nutrient-sensing complex plays in plants.

In Chapter 3 the published report of a survey of mei2-like genes in plants is presented. Unlike *S. pombe* and all other organisms whose genomes encode a single Mei2-like protein, plant genomes contain a small gene family of loci encoding Mei2-like proteins. Mei2-like proteins from a broad range of plants, predicted from their genomic loci or from their transcripts, are analyzed to determine their phylogenetic distribution. In Arabidopsis, the expression patterns of genes encoding Mei2-like proteins are assayed through quantitative RT-PCR and through *in situ* RNA hybridization.

In Chapter 4 it is shown that AML1 (Arabidopsis Mei2-like 1) interacts with AtRaptor1B. As Raptor proteins recruit substrates to TOR for nutrient-regulated
phosphorylation, this result implicates the AML proteins in TOR signaling. Also in Chapter 4 the phenotype of plants homozygous for perturbations of all five AML loci are discussed, and reasons why these plants may not have lost all AML activity are presented.
CHAPTER 2
THE ARABIDOPSIS AtRaptor GENES ARE ESSENTIAL FOR POST-EMBRYONIC PLANT GROWTH

ABSTRACT

Flowering plant development is wholly reliant on the maintenance of totipotent cell lines called meristems from which all post-embryonic organs arise. By synthesizing organs de novo throughout their lifespan, plants are uniquely able to alter their development in response to external signals. To identify genes that regulate meristem-based growth in plants, we considered homologues of genes that are known to regulate growth in other eukaryotes. Raptor proteins in yeast and metazoans regulate cell growth in response to nutrients as part of a signaling complex with the kinase TOR. We identify AtRaptor1A and AtRaptor1B, two loci predicted to encode plant Raptor proteins. Disruption of AtRaptor1B yields plants with a wide range of developmental defects: roots grow slowly, leaf initiation and bolting are delayed, and the shoot shows reduced apical dominance. AtRaptor1A AtRaptor1B double mutants show normal embryonic development but are unable to maintain meristem-driven development. AtRaptor transcripts accumulate in dividing and expanding cells and tissues. The data implicate the TOR signaling pathway, a major regulator of cell growth in yeast and metazoans, in the maintenance of growth from the shoot apical meristem in plants. These results provide insights into the ways in which TOR/Raptor signaling has been adapted to regulate plant growth and development, and indicate that in plants, as in other eukaryotes, there is some Raptor-independent TOR activity.

* Anderson, G. H., B. Veit, and M. R. Hanson, manuscript under review. B. Veit contributed the in situ RNA hybridization image in Figure 2.6A, B.
INTRODUCTION

Plant development is remarkably plastic. Totipotent cell lines called meristems are maintained throughout the life of the plant, and give rise to all post-embryonic organs from roots and leaves to petals and fruit. This allows plants, unlike metazoans, to change their final body plans dramatically in response to environmental, hormonal and nutritional cues. While much has been learned about the determination of cell fates in the embryo (Willemsen and Sheres, 2004) and the apical meristems (Poethig, 2003; Hake et al., 2004; Zhao et al., 2004) less is known about the nuclear genes that control the maintenance of plant meristem activity. We report here our finding that a two-member Arabidopsis gene family, comprised of AtRaptor1A and AtRaptor1B, is essential for post-embryonic plant development.

TOR proteins TOR1 and TOR2 were originally identified in budding yeast as the targets of rapamycin, a potent antibiotic that disrupts cell growth (Thomas et al., 2004). In both yeast and metazoans, TOR proteins mediate translation in response to nutrients (Schmelzle and Hall, 2000). Yeast TOR2 and mammalian mTOR also regulate cytoskeletal organization (Heitman et al., 1991; Kunz et al., 1993; Barbet et al., 1996; Helliwell et al., 1998; Jacinto et al., 2004; Murakami et al., 2004).

In both yeast and mammalian cells, TOR proteins are involved in two distinct protein complexes. In the first of these, TOR forms a complex, TORC1, with GβL (Kim et al., 2003) and Raptor (regulatory associated protein of TOR) (Hara et al., 2002; Kim et al., 2002; Loewith et al., 2002). Both GβL and Raptor consist of protein-binding domains; the Raptor N-terminus additionally shows similarity to a caspase domain (Ginalska et al., 2004), though catalytic activity has yet to be shown. The strength of the TOR-Raptor interaction has been reported to be regulated by nutrients, though there is not yet consensus on this point (Hara et al., 2002; Kim et al., 2002; Loewith et al., 2002). Raptor functions in this complex to recruit substrates for
phosphorylation by TOR; Raptor binds TOR substrates S6kinase and eIF4E-BP, and is necessary for full TOR phosphorylation of these substrates in vitro (Hara et al., 2002; Nojima et al., 2003). The TOR-Raptor complex is thought to mediate the nutrient-sensitive regulation of cell growth; mutants in yeast lacking a functional complex cease cell growth in a manner that mimics rapamycin treatment (Loewith et al., 2002). A second TOR complex, TORC2, involves GβL, Rictor (rapamycin-insensitive companion to TOR) and perhaps other proteins (Loewith et al., 2002; Jacinto et al., 2004; Sarbassov et al., 2004). This complex is unaffected by rapamycin, and is thought to mediate TOR cytoskeletal regulation.

In Arabidopsis, the single TOR homologue, AtTOR, is critical for plant development (Menand et al., 2002; Robaglia et al., 2004). AtTOR insertion homozygote embryos undergo cell division but are unable to gain cell volume or undergo apical-basal differentiation. AtTOR transcripts accumulate in all tissues assayed (Robaglia et al., 2004), but an AtTOR:Gus fusion transcript is only translated in meristematic tissue and the immediately adjacent expanding cells (Menand et al., 2002). A TOR-specific microRNA conserved between Arabidopsis and rice (Bonnet et al., 2004) implicates post-transcriptional regulation in AtTOR expression.

Rapamycin perturbs the activity of the nutrient-sensitive TOR complex in yeast and mammals. Contrasting the phenotype of rapamycin treatment with the phenotype of TOR disruption has provided insights into rapamycin-sensitive and insensitive TOR signaling functions and protein complexes in these organisms. Unlike budding yeast and mammals, Arabidopsis is insensitive to rapamycin. AtTOR forms a complex with rapamycin in a yeast three-hybrid assay (Menand et al., 2002); however, in order to perturb TOR activity, rapamycin must be bound to a second protein, FKBP12 (Schmelzle and Hall, 2000). None of the Arabidopsis FKBP12 homologues bind rapamycin (Robaglia et al., 2004). Rapamycin is therefore not
useful for the characterization of TOR signaling in plants, and the degree of similarity between TOR signaling in plants and other eukaryotes has been difficult to ascertain. Here we report the characterization of \textit{AtRaptor1A} and \textit{AtRaptor1B}, encoding the two Arabidopsis Raptor homologues. We describe subtle defects in both root and shoot growth in \textit{AtRaptor1B} disruption lines, resulting in slow leaf initiation, late flowering and increased branching. We report that \textit{AtRaptor1A AtRaptor1B} double mutant homozygotes undergo normal seedling development, but exhibit only minimal post-embryonic meristem activity. We contrast these results with those found earlier for the \textit{AtTOR} mutant, and discuss the implications of this work with regard to TOR signaling in plants compared to other eukaryotes.

**MATERIALS AND METHODS**

**Insertion lines**

\textit{Agrobacterium}-mediated T-DNA insertion mutagenized lines SALK_042920 and SALK_078159, tagging the \textit{AtRaptor1A} and \textit{AtRaptor1B} loci in the Columbia (Col) genetic background were generated by Joseph R. Ecker and the Salk Institute Genomic Analysis Laboratory and distributed by the ABRC (Alonso et al., 2003). Lines were genotyped using the following primers: 1Asm5 5’aaaaagtctttgtagtttcagatg3’ and 1Asm3 5’attcagaatatacaatccaagcattagt3’ to identify the \textit{AtRaptor1A} wild-type locus; 1Bsm5 5’ctgaccataacattctcttgtaggtaagg3’ and 1Bsm3 5’aggcctgaactctaatgaacaaactctcc3’ to identify the \textit{AtRaptor1B} wild-type locus, and B5 or A5 and pROK-737 (5’gggaattcactggccgtttttataaa3’) to identify the respective \textit{AtRaptor} allele. Mutant lines were crossed to Col wild-type plants; the \textit{AtRaptor1B} mutant phenotype cosegregated with the 1B insertion allele in the F2 population.
**Growth Conditions**

Plants were grown in a greenhouse with supplemental light to 16 hrs, with temperatures held at 22°C days and 17°C nights. Seeds for plate-grown seedlings were surface sterilized with 20% Chlorox, washed in H2O stratified for four days at 4°C and grown on 1/2x Murashige and Skoog (MS) salts, 0.3% Phytagel under 12 hr. daylight cycles.

**RT-PCR**

Buds from Col-0 and insertion line homozygotes were snap-frozen in liquid N2. Total RNA was extracted from 0.3g of tissue using TRIzol Reagent (Invitrogen) according to manufacturer’s instructions. Resuspended RNA was thrice-treated with DNA-free™ DNase treatment (Ambion). RNA was quantified spectrophotometrically. cDNA was generated from 2µg of total RNA using Omniscript Reverse Transcriptase (Qiagen) and a poly-dT18 primer. PCR was performed using ExTaq polymerase (Takara) in a PTC-100 thermocycler. Primer sequences were as follows: 1A+1828 5’gctgcgtttattttggctgttattgtc3’, 1A-2800 5’ctaggccagccagaggagtgtgagatg 3’; 1B+1379 5’aggccggcaaaacgatcgtaagacatt 3’, 1B-2774 5’catcagcccagaggagccaagagg3’. PCR cycling parameters were 95°C, 5 min followed by 35 cycles of 94°C, 30 sec, 62°C, 30 sec, and 72°C, 1 min.

**AtRaptor1B cDNA clone**

EST clone RZL03b06 corresponding to the 5’ end of AtRaptor1B was ordered from Kazusa DNA Research Institute and sequenced (Accession number AY769948). RNA ligase-mediated RACE was performed on total RNA extracted from bulk shoot tissues using a GeneRacer™ Kit (Invitrogen) according to manufacturer's instructions to confirm that RZL03b06 represented a full length clone.

**Assembly of the AtRaptor1B complementation vector**

Primers were designed to amplify the region from the end of the transcript
adjacent to the \textit{AtRaptor1B} locus to a site within the ORF, spanning a region from 1145 bases upstream of the transcript initiation site through to the sixth exon of the \textit{AtRaptor1B} transcript. Primers used, and restriction sites added, are as follows: 1B-8189	extbf{Bgl}II 5’ AGA TCT GAG GAA CCA GAA GAA CCC 3’; 1B+5104	extbf{Hind}III 5’ AAG CTT CGG CGG AGT AGG AAA AC 3’. PCR was performed using ExTa polymerase (Takara) in a PTC-100 thermocycler on Col-0 genomic DNA with the following parameters: 96°C, 5 min, then 35 cycles of 94°C 30 sec, 60°C 30 sec, 72°C 2 min; 72°C for 10 minutes. \textbf{Hind}III \textbf{Bgl}II digested fragments were ligated into pCambia1301.

To assemble the \textit{AtRaptor1B} complementation vector, the 1301B fragment was digested with \textit{PmlI} and \textit{PmeI}. A clone containing the \textit{AtRaptor1B} ORF was digested with \textit{PmeI} and \textit{SmaI}, and the fragment was ligated into digested 1301B to create the complementation vector 1301B:Raptor. 1301B:Raptor was transformed into \textit{Agrobacterium} line GV3101, and into Arabidopsis Col-0 or 1B/- via floral dip (Clough and Bent, 1998).

\textbf{Microscopy}

SEM fixation was performed using standard methods (Jackson et al., 1994). Bright field microscopy was performed on a Zeiss microscope and images were collected on a BioRad Confocal System.

\textbf{In situ hybridization}

Digoxigenin-labelled probes corresponding to the antisense strand cloned \textit{mei2}-like genes of interest were hybridized to paraffin-sectioned material using previously described methods (Weigel and Glazebrook, 2002).

\textbf{In silico analysis}

Locus identifiers were submitted to the Genevestigator \textit{Arabidopsis thaliana} microarray database and analysis toolbox (Zimmermann et al., 2004) at
https://www.genevestigator.ethz.ch, where they were assayed against 1434 developmental and tissue-specific Arabidopsis microarray experiment results (Edgar et al., 2002; Brazma et al., 2003; Rocca-Serra et al., 2003; Craigon et al., 2004).

RESULTS

Characterization of the Arabidopsis Raptor homologues

To search for plant homologues of mammalian Raptor, we assayed the completed Arabidopsis genome and found two loci that might encode proteins highly similar to Raptor at the north ends of chromosomes three and five. The AtRaptor1A locus (At5g01770) is not represented by any EST sequence. However, we used 5’ RACE to establish that the locus is transcribed to an mRNA with a 5’ end consistent with the predicted ORF. The transcript predicts a protein of 1,346 residues. An expressed sequence tag (EST) (accession no. AV554844) for the putative transcript of one of these, At3g08850, encoding AtRaptor1B, was ordered and sequenced. 5’ RACE was used to confirm that the tagged clone represented the full ORF. The AtRaptor1B locus is transcribed to a 4.8kb mRNA of 23 exons. The cloned cDNA differed from the previously predicted ORF at the 3’ end of exon 8. The transcript predicts a protein of 1,344 residues. Predicted AtRaptor1A and AtRaptor1B proteins show 80% identity over their entirety.

To gain a clearer understanding of the time of divergence between the two AtRaptor loci, we searched for Raptor homologues in available plant genome data using AtRaptor1A and AtRaptor1B as query sequences. Results were similar for the two queries. Full length Raptor loci were discovered in the available rice (Oryza sativa ssp. japonica) and alfalfa (Medicago truncatula) genome sequences. Using AtRaptor1B and available partial cDNA sequence as a guide, we determined putative protein sequences from these loci and aligned these sequences with AtRaptor1A,
AtRaptor1B, mammalian Raptor, the budding yeast Raptor homologue KOG1 and the fission yeast Raptor homologue Mip1p. There is a striking degree of conservation among all Raptor homologues (Fig. 2.1A). AtRaptor1B and the S. cerevisiae Raptor homologue KOG1, the most divergent member included in the analysis, show 28% identity throughout their length. All plant Raptor homologues encode the Raptor N-terminal Conserved / Caspase (RNC/C) motif, HEAT repeats and WD40 motifs first identified in fission yeast Mip1 and characteristic of all Raptor proteins (Shinozaki-Yabana et al., 2000; Yonezawa et al., 2004) (Fig. 2.1B). From this alignment we generated a phylogeny of the Raptor homologues (Fig. 2.1C). The predicted AtRaptor proteins resolve to a single clade with a high degree of confidence, indicating that the duplication of the AtRaptor loci post-dates the divergence of Arabidopsis from Medicago, and that the loci are likely to encode functionally redundant proteins.

Identification of knockout mutants in AtRaptor1A and AtRaptor1B

To gain insight into the function of the AtRaptor proteins, we searched for insertion alleles of each locus among the publicly available sequenced T-DNA insertion lines. Insertion mutant lines SALK_042920 and SALK_078159, representing disruptions to the AtRaptor1A and AtRaptor1B loci were obtained from the ABRC. Lines homozygous for each insertion (referred to as 1A-/- and 1B-/-, respectively) were identified via PCR, and RNA from floral buds of insertion homozygotes was used for RT-PCR to assay for accumulation of wild-type transcripts from the disrupted locus. AtRaptor1A transcripts could not be detected in 1A-/- buds; AtRaptor1B transcripts were not detected in 1B-/- buds. AtRaptor1A was detected in 1B-/-, and AtRaptor1B was detected in 1A-/-. Both transcripts were detected in wild-type Columbia (Col) buds (Fig. 2.2).
Figure 2.1. Raptor proteins in eukaryotes are highly conserved. (A) Similarity plot of Raptor homologues from the vascular plants Arabidopsis, Medicago truncatula and Oryza sativa, the fungus S. pombe (Mip1p), and mammals. The X-axis represents residue number; the Y-axis represents percent identity at that residue. (B) Schematic diagram showing the position of the Raptor N-terminal Conserved / putative Caspase domain (RNC/C) region, HEAT repeats (H), and WD-40 repeats (WDx7) common to all Raptor proteins. (C) Phylogeny of plant, animal and fungal Raptor proteins. Bootstrap values, calculated using both parsimony (left) and maximum likelihood (right) are shown to the left of the clades they describe. The two Arabidopsis Raptor proteins, AtRaptor1A and AtRaptor1B, resolve as a single clade with 100% confidence. The alignment was generated using Megalign (DNASTar); bootstrap values were calculated using PAUP*.
**AtRaptor1B -/- seedling roots grow slowly**

As a first step toward characterizing these insertion allele homozygote lines (mutant lines), Col, 1A-/- and 1B-/- seedlings were germinated on culture medium. Col and 1A-/- seedlings were phenotypically indistinguishable. *AtRaptor1B*-/- seedlings showed distinct root growth defects. Roots were thick, coiled, densely covered with hairs, and had difficulty penetrating the medium (Fig. 2.3 A). Plate-grown 1B-/- seedlings were repeatedly shorter than Col or 1A-/-, and this defect was largely due to reduced root growth; dark grown 1B-/- seedlings were again smaller than Col or 1A-/-, but the difference was much reduced (Fig. 2.3 B). Dark-grown etiolated seedlings grow primarily through the expansion and division of embryonic hypocotyl cells. Light-grown seedlings grow primarily through the *de novo* production of cells from the root apical meristem. Thus the difference between Col 1A-/- and 1B-/- seedlings results primarily from a reduction in root apical meristem growth rather than a general defect in cell expansion or metabolism.

1B-/- seedlings grown on 90° inclined plates were morphologically wild-type (the roots were not thick, coiled, or more hairy than Col) but roots were shorter than Col roots. When these plates were rotated to lay flat horizontally, new growth from the root apex of 1B-/- seedlings showed thick, coiled growth (though not the profusion of root hairs); previously produced root tissue remained morphologically wild-type (Fig. 2.3 C). Thus the root growth disturbance was a defect in growth into agar rather than in gravitropism, though in the absence of resistance roots still grew more slowly than wild-type. Also, the root hair growth (Fig. 2.3A) was a secondary effect of not being in contact with the growth medium, rather than being a direct result of the *AtRaptor1B* lesion.
Figure 2.2. *AtRaptor* loci and insertion allele characterization. (A) *AtRaptor1A* and *AtRaptor1B* loci. Genomic sequence is depicted as a thin central line. Thick blocks indicate exons. Coding exons span the central line; exons encoding untranslated regions are fully below the central line. The positions of the T-DNA insertions are depicted with inverted triangles. (B) Reverse-Transcribed RNA-template Polymerase Chain Reactions (RT-PCR) on plants homozygous for both wild-type *AtRaptor* alleles (Col), the *AtRaptor1A* insertion allele (A-) or the *AtRaptor1B* insertion allele (B-), using primers spanning the *AtRaptor1A* insertion site, the *AtRaptor1B* insertion site, or control primers. Both AtRaptor insertion alleles abolish accumulation of the wild-type transcript from their locus.
Figure 2.3. Seedling root phenotype of *AtRaptor1B/-* mutants. (A), (B). Col and B-seedlings on growth medium, four days after germination. The B- root has not penetrated the medium and is thick, hairy and coiled. (C) Col, A- and B- seedlings at 8 days after germination in light. Scale bar is in mm. (D) Same genotypes and age as C, grown in the dark. (E) Measurements of populations grown as in C, D. Root length is indicated in tan; shoot length is dark green. (F) B- and Col seedlings grown on vertical plates for 12 days, and then returned to horizontal growth for three days. B- roots are thin, straight and hairless on vertical plates (compare to 3B), and revert to coiled growth only in tissue generated after being placed horizontally. (G) Quantification of results in (F). B- seedlings grown on vertical plates are intermediate in length between flat-grown B- seedlings and Col seedlings. (H), (I) Col and B- root tips, viewed under bright field microscopy. Scale bar=100µm. B- root tips contain all the cell types seen in Col root tips, but the overall morphology is blunt and rounded compared to Col.
Figure 2.3 (Continued)
Col and 1B-/- root apical meristems (RAM) were investigated via bright-field microscopy (Fig. 2.3 D). 1B-/- seedling RAMs contained all cell types present in Col RAMs but their root morphology was much more blunt than Col; this difference appeared to be localized to the 1B-/- zone of elongation. 1B-/- root tips showed a tendency to shed their root caps; shedding was sometimes observed in Col root tips as well.

**AtRaptor1B-/- line shoots show developmental and morphological defects**

To measure developmental defects resulting from *AtRaptor* insertions, we grew Col, 1A-/- and 1B-/- lines on soil, and scored their rates of leaf initiation, time to floral bud initiation, rate of cauline leaf initiation, time to flowering and number of floral shoot apices. 1B-/- plants appeared smaller, had a slower rate of leaf initiation, and bolted and flowered later than did Col or 1A-/- (Fig. 2.4).

Mature 1B-/- plants were conspicuously bushier than Col or 1A-/- homozygotes (Fig. 2.5). The primary shoot apex was shorter than that of Col, ending prematurely in a terminal inflorescence of infertile flowers. The growth of the plant was then taken over by axillary shoots and by secondary shoots from the basal rosette.

To quantify this phenotype, Col and 1B-/- lines were grown to maturity in 16-hr days, and shoot architecture for an individual plant was scored at the shattering of the first silique. 1B-/- mutant plants showed reduced shoot height, reduced primary stem length, increased axillary branch number, and an increased number of secondary shoots compared to Col and 1A-/- (Fig. 2.5 C, D). Axillary branch length did not differ significantly from Col values (Fig. 2.5 E). This phenotype became more pronounced later in plant development and was more conspicuous under short days.
**Figure 2.4.** *AtRaptor1B-/-* plants grow slowly. (A), (B) Col and B- plants at 15 days after germination on soil. (C) B- plants bolt later than Col or A-. Shown are shoots from plants 1 month after germination. (D) Growth curve of Col, A- and B- plants. The X-axis represents time after production of the first leaf. The Y-axis represents the number of rosette leaves up to 11; presence of a floral bud is 12; number of cauline leaves plus 12 is 13-16, and values above 16 are the number of shoot apices harboring flowers plus 15. B- plants show slower leaf initiation, later bolting (though at a similar rosette leaf number as Col and A-) and later flowering.
Figure 2.5. *AtRaptor1B/-* plants show altered shoot architecture. (A) B- plant at flowering. The primary shoot apex, center, has ceased growth and is surpassed by axillary branches. Compare to Col, A- in 3C. (B) Mature B- plant, showing a bushy phenotype due to decreased primary shoot growth and increased branching. (C) Col, A- and B- primary shoot length. (D) Col, A- and B- cauline and rosette branch number. (E) Col, A- and B- cauline and rosette branch length. B- primary shoots are smaller than Col or A-, and secondary shoots initiate more frequently than Col or A- but are not significantly longer than Col or A-.
Complementation of the *AtRaptor1B*-/ phenotype

To confirm that the collection of phenotypes observed in the *AtRaptor1B*-/ homozygotes was due to the mutant 1B allele, 1B/- mutants were transformed with a construct containing the *AtRaptor1B* ORF driven by the *AtRaptor1B* promoter and 5’ UTR. Among these transformants we recovered plants which showed wild-type transition to bolting, shoot branching and root growth (data not shown). We conclude that the *AtRaprot1B*/- phenotypes described above result from homozygosity for the lesion at the *AtRaptor1B* locus.

*AtRaptor* transcripts accumulate in dividing and expanding cell tissues

A combination of *in situ* RNA hybridization and *in silico* expression analysis was used to determine the RNA accumulation pattern of *AtRaptor* transcripts in Arabidopsis. The *AtRaptor1B* cDNA sequence was used to generate an *AtRaptor* probe to assay transcript accumulation in the shoot tips of wild-type plants. *AtRaptor* transcripts were detected throughout the shoot tip, in all organs of the differentiating floral bud, and deep into the differentiated inflorescence stem (Fig. 2.6A). Signal intensity faded with the distance from the shoot apex. This accumulation pattern differed from that of actin (Fig. 2.6B), which was more prominent in dividing cells of the apex. Notably, *AtRaptor* accumulation is not restricted to the primary shoot apex.

*AtRaptor1A* and *AtRaptor1B* show 80% identity through the length of their transcripts. To obtain an estimate of the relative levels of *AtRaptor1A* and *AtRaptor1B* in the signal seen in Fig. 2.6A, *AtRaptor1A* and *AtRaptor1B* locus IDs were used to query the Genevestigator *Arabidopsis thaliana* Microarray Database and Analysis Toolbox (Zimmermann et al., 2004). *AtRaptor1A* and *AtRaptor1B* accumulate in all developmental stages (Fig. 2.6C). *AtRaptor1B* accumulation levels are consistently fourfold higher than those of *AtRaptor1A*. A second analysis of accumulation by tissue rather than developmental stage produced similar results. We conclude that
AtRaptor1A and AtRaptor1B show similar expression patterns.

**AtRaptor1A-/- 1B-/- double homozygote mutant seedlings show minimal meristem growth**

The high degree of similarity between AtRaptor1A and AtRaptor1B led us to suspect that they may be at least partially functionally redundant. To eliminate all AtRaptor activity in a single line, 1A-/- and 1B-/- lines were crossed. No 1A-/- 1B-/- plants were identified among the F2 of this cross on soil. Phenotypically wild-type 1A-/- 1B +/- plants were isolated and their progeny were examined on agar plates. 165 of 205 seedlings (80%) appeared wild-type. 40 of 205 seedlings germinated but showed minimal postembryonic shoot meristem growth (Fig. 2.7A). Genotyping of wild-type seedlings and seedlings showing minimal post-embryonic growth confirmed that the seedling arrest mutant phenotype cosegregated with homozygosity for the AtRaptor1B T-DNA mutant allele in the AtRaptor1A mutant background. We concluded that the seedling phenotype corresponded to the AtRaptor1A-/- 1B-/- double mutant.

We examined 1A-/- 1B-/- double mutant seedling roots via bright field microscopy to ascertain the extent of their post-embryonic growth defect. 1A-/- 1B-/- roots were conspicuously narrower than wild-type. The columella, quiescent center, vasculature, pericycle, endodermis and cortex are present though all smaller than wild-type; the epidermis and root cap were not evident. Further up the root, root hairs were clearly visible, indicating that the RAM had produced some mature root cell types (Fig. 2.7D, E).

To gain a better understanding of the double mutant shoot apical meristem (SAM) defect, Col and 1A-/- 1B-/- 7-day seedlings were fixed, coated and their shoot apexes were observed with a scanning electron microscope. In Col seedlings, leaves one and two, with trichomes, were clearly visible; primordia for leaves three, four and
**Figure 2.6.** *AtRaptor* accumulation pattern. (A) *AtRaptor* transcripts accumulate throughout the floral shoot apex, stem and differentiating floral buds. Accumulation is not confined to dividing or meristematic cells, but fades in intensity away from the apex. (B) Adjacent tissue slice, probed with actin. *AtRaptor* and actin transcript accumulation patterns differ. (C) *In silico* analysis of *AtRaptor1A* (left) and *AtRaptor1B* (right) accumulation from 1434 developmental gene chip experiments. Results are given by developmental stage (X-axis) and in terms of gene chip-normalized expression levels (Y-axis). Expression levels are shown to scale. Developmental stages are as follows: 1, 1.0-5.9 days; 2, 6.0-13.9 days; 3, 14.0-17.9 days; 4, 18.0-20.9 days; 5, 21.0-24.9 days; 6, 25.0-28.9 days; 7, 29.0-35.9 days; 8, 36.0-44.9 days; 9, 45.0-50.0 days. Analyses performed *via* the genevestigator website (https://www.genevestigator.ethz.ch).
five were visible under higher magnification. 1A-/- 1B-/- shoot apexes showed minimal SAM activity: leaves one and two were produced, but were smaller than wild-type and did not separate to reveal any further leaf primordia (Fig. 2.7 F,G,H,I).

To visualize the shoot apex directly, we generated a vertical section of a 1A-/- 1B-/- seedling. Leaf primordium 1 is prominent; beneath it, primordia for leaves 3 and 4 are visible (leaf 2 was cut away in the sectioning process). The meristem is flat rather than dome shaped, indicating an exhaustion of totipotent cells.

To assay for a plant hormone or signaling molecule able to restore post-embryonic growth to these arrested seedlings, progeny of 1A-/- 1B+-/- individuals were germinated on plates containing plant hormones and signaling molecules and scored for their total seedling lengths at 10 days post germination. None of these treatments restored SAM growth to the 1/4 of the progeny showing seedling arrest. 1A-/- 1B+-/- seedlings did show a significant but nonmeristematic response to sucrose (Fig. 2.7 B,C). Addition of 1% sucrose, which is a growth signaling factor as well as a carbon source (Rolland et al., 2002), to the growth medium promoted growth in Col, 1A-/-, 1B-/- and 1A-/- 1B-/--. In sibling seedlings, the addition of sucrose elicited a twofold increase in seedling length; in the double mutants the increase was five-fold. Germination on 6% sucrose, which signals the arrest of growth in Col plants compared to 1% sucrose (Rolland et al., 2002), yielded sibling plants 1-1.5x the size of seedlings grown on 1/2 MS salts with no sucrose added. 1A-/- 1B-/- seedlings on 6% sucrose were 2.3x the length of those grown on 1/2 MS salts. A similar result was observed among 1A-/- 1B+-/- progeny germinated in the dark on 1/2 MS salts plates. Length of 1A-/- 1B-/- seedlings was increased more than 3x. The absolute length of these double mutant seedlings was in all cases still substantially smaller that that of sibling seedlings for a given treatment, and the increase in length was exclusively due to hypocotyl elongation or root growth. Only minimal SAM activity was observed.
Figure 2.7. *AtRaptor1A-/- 1B-/-* double mutants. (A) Col, A-, B- and A-B- seedlings at seven days on growth medium with no sucrose. (B) 1A-/- 1B+/- progeny germinated on growth medium supplemented with 0%, 1% or 6% sucrose. Shown for each treatment is an A-B- seedling and an A-/ B+ sibling. A-B- seedlings on 1% sucrose show significant root growth and minimal leaf buds. Scale bar for A, B = 5 mm. (C) Quantification of results in (B). (D) A-B- root tips grown on 1% sucrose lack an epidermal cell layer. (E) A-B- roots form root hairs on 1% sucrose. Scale bar=100µm. Compare to 3H, I. (F, G, H, I) Scanning electron microscopy on Col, A-B- shoot apices from growth medium plates. As in (B), A-B- seedlings show minimal (SAM) activity. Primordia for leaves 1 and 2 form but do not expand significantly. Scale bar=50 µm (F, G) or 15 µm (H, I).
Figure 2.7 (continued)
DISCUSSION

Axillary branch growth in Arabidopsis and other vascular plants is governed indirectly by auxin produced at the primary shoot apex, which acts through an undetermined secondary messenger to repress axillary meristem growth (Ward and Leyser, 2004). AtRaptor1B-/− plants’ shoot morphology indicates a defect in primary SAM maintenance. The primary shoot is shorter than wild-type and ends in a whorl of sterile flowers. The number and length of axillary shoots, both cauline and emerging from the basal rosette, is significantly increased. Notably, axillary meristems are quite viable, producing branches as long as or longer than wild-type. The viability of axillary shoot apices points to a failure to maintain primary SAM activity specifically, and a subsequent failure to repress axillary meristem activity. This defect is not shared by the axillary SAMs, which match or surpass the activity of their wild-type counterparts. A similar phenomenon of a shoot apex defect becoming more pronounced in the primary apex than in axillary SAMs is seen in the erecta mutants (Shpak et al., 2004) and may be quite common. The increased branching in AtRaptor1B mutant plants may result directly from reduced auxin production at the shoot apex. However, AtRaptor1B-/− mutants did not show defects in their ability to sense exogenous auxin (data not shown). AtRaptor1B-/− mutants evidently represent a partial disruption of AtRaptor activity. The AtRaptor1A gene product, under the regulation of its own promoter, cannot fully complement the disruption of AtRaptor1B despite the fact that their predicted proteins are 80% identical, share all conserved Raptor motifs and strongly resolve to a single clade in phylogenetic analysis. In silico analysis suggests that the failure of AtRaptor1A to mask AtRaptor1B lesions may be due to lower levels of AtRaptor1A expression globally rather than limited tissue-specific accumulation of AtRaptor1A. In contrast, AtRaptor1B can fully complement the loss of AtRaptor1A expression; indeed, a single
wild-type *AtRaptor1B* allele in 1A/- - 1B+/+ plants is sufficient for wild-type growth.

Disruption of both *AtRaptor* loci resulted in growth-arrested seedlings, which undergo normal embryonic organogenesis but are unable to maintain post-embryonic growth from their SAMs. *AtRaptor1A/- AtRaptor1B/-* double mutants’ SAMs cease activity after the production of a few leaf primordia. We conclude that AtRaptor activity is essential for the maintenance of SAM activity but not for SAM assembly or the initiation of SAM activity.

AtRaptor activity is not essential for embryonic organogenesis. *AtTOR/-* mutants, in contrast, arrest development early in embryogenesis (Menand et al., 2002). The disparity between the *AtRaptor1A/- 1B/-* and the *AtTOR/-*-phenotypes indicates that AtTOR activity in embryonic development does not require AtRaptor. Thus if AtTOR is acting in a complex in embryonic development, this complex does not require AtRaptor.

As previously discussed, in both yeast and mammalian cells TOR functions in two distinct complexes (Fig. 2.8A). The first of these, TORC1, involves Raptor and regulates cell growth and translation in response to nutrients (Hara et al., 2002; Kim et al., 2002; Loewith et al., 2002; Kim et al., 2003). The second of these complexes, TORC2, regulates cytoskeletal organization and does not involve Raptor (Loewith et al., 2002; Jacinto et al., 2004; Sarbassov et al., 2004).

AtRaptor-independent AtTOR activity in the plant embryo is consistent with the existence of two AtTOR complexes in Arabidopsis: 1) a Raptor-independent complex critical for early embryogenesis (and perhaps all stages of plant development), and 2) a Raptor-dependent complex that is dispensable for embryonic development but which is necessary for post-embryonic, meristem-driven plant growth (Fig. 2.8B).

By identifying the phenotypes resulting from partial and total disruption of AtRaptor activity, we have generated valuable tools for the study of plant TOR
Figure 2.8. TOR functions in two complexes in eukaryotes. (A) TOR participates in two complexes in yeast and mammals. The first of these, TORC1, regulates cell growth in response to nutrient and hormonal signals. Raptor is integral for TORC1 activity. The second of these, TORC2, regulates cytoskeletal organization. Its activity is nutrient-independent, and Raptor is not a component of TORC2. (B) Model of TOR function in plants. Embryonic development is indicated by the single horizontal arrow from zygote to seedling; meristem-driven post-embryonic development is indicated by the arrows emanating from the seedling root and shoot apices. TOR, acting independent of Raptor in a putative complex homologous to yeast and mammalian TORC2, is essential for embryonic development. TOR via TORC2 may play a role in post-embryonic development as well; the embryonic lethal *AtTOR* knockout phenotype precludes a definitive answer on this point. Raptor activity, in a putative plant homologue of TORC1, is dispensable for embryonic development but is essential for meristem-driven post-embryonic growth.
signaling. When viewed in the context of previous work on AtTOR (Menand et al., 2002; Robaglia et al., 2004), our work provides evidence for Raptor-independent TOR activity in land plant embryonic development – development which, notably, occurs in an environment made nutritionally and environmentally stable via maternal input to growth. We propose that in plants as in other eukaryotes there are two (or more) TOR complexes, only one of which involves Raptor. We further propose that the plant homologue of TORC1, involving TOR and Raptor, has been co-opted in plants from its ancestral role in nutrient sensing and cell growth to regulate the highly plastic post-embryonic growth driven by the plant shoot apical meristem.
CHAPTER 3
DIVERSICATION OF GENES ENCODING Mei2-LIKE RNA BINDING PROTEINS IN PLANTS

ABSTRACT

A predominantly plant-based family of genes encoding RNA binding proteins is defined by the presence of a highly conserved RNA binding motif first described in the mei2 gene of the fission yeast Schizosaccharomyces pombe. In silico analyses reveal nine mei2-like genes in Arabidopsis thaliana and six in Oryza sativa. These predicted genes group into four distinct clades, based on overall sequence similarity and subfamily-specific sequence elements. In situ analysis show that Arabidopsis genes from one of these clades, TEL1 and TEL2, are specifically expressed in central zone of the shoot apical meristem and the quiescent center of the root apical meristem, suggesting that they may somehow function to maintain indeterminancy in these tissues. By contrast, members of two sister clades, AML1 through AML5, are expressed more broadly, a trend that was confirmed by Q-PCR analysis. mei2-like transcripts with similar sequences showed similar expression patterns, suggesting functional redundancy within the four clades. Phenotypic analyses of lines that contain T-DNA insertions to individual mei2-like genes reveal no obvious phenotypes, further suggesting redundant activities for these gene products.

* Anderson GH, Alvarez ND, Gilman C, Jeffares DC, Trainor VC, Hanson MR, Veit B (2004) Plant Mol Biol 54: 653-670, by permission of Kluver Academic Publishers. Veit and Alvarez contributed Figure 3.5; Gilman and Trainor cloned TEL1 and TEL2; Jeffares performed sequence analysis in parallel with the analysis presented here.
INTRODUCTION

Eukaryotes feature a variety of mechanisms which regulate the effective concentration of mRNA, including those that act at the level of synthesis, processing, localization and turnover (Burd and Dreyfuss, 1994; Guhaniyogi and Brewer, 2001; Mallory and Vaucheret, 2004). Recently, novel modes of RNA-focused regulation have emerged in which non-coding RNAs affect the stability or translation of specific mRNAs, and which may also act to direct chromatin remodeling (Volpe et al., 2002; Hunter and Poethig, 2003). Over 200 *Arabidopsis thaliana* protein-encoding genes are currently annotated as having an RNA recognition motif (RRM) (Lorković and Barta, 2002) and with the inclusion of other putative RNA binding motifs - pentatricopeptide repeats, or PPRs, for example (Small and Peeters, 2000) - the number could easily approach 500 or higher. Although molecular studies have clarified the function for some of these proteins, for most little is known.

One intriguing class of plant RNA binding protein genes can be identified by their similarity to the *mei2* gene of the unicellular fungus *S. pombe* (Watanabe et al., 1988; Watanabe and Yamamoto, 1994; Lorković and Barta, 2002). The protein encoded by *mei2*, Mei2, is characterized by three RNA Recognition Motifs (RRMs) (Birney et al., 1993). Genetic and biochemical analyses have indicated that the most C-terminal of these, RRM3, is essential for the normal meiosis-promoting activity of the protein. Under meiosis-inducing conditions, haploid *S. pombe mei2* mutant lines undergo conjugation and karyogamy (nuclear fusion), but fail to initiate pre-meiotic DNA synthesis or meiosis I (Watanabe et al., 1988; Watanabe and Yamamoto, 1994). In wt haploid cells, the *mei2* transcript accumulates in meiosis-inducing conditions, but its product, Mei2, is maintained in an inactive state by phosphorylation at two sites between RRM2 and the C-terminal RRM (Watanabe et al., 1997). Following conjugation, karyogamy and the formation of a compatible mating type complex,
unphosphorylated Mei2 accumulates and somehow triggers pre-meiotic DNA synthesis (McLeod and Beach, 1988; Watanabe et al., 1988; Watanabe and Yamamoto, 1994; Li and McLeod, 1996; Watanabe et al., 1997) and chromatin remodeling (Mizuno et al., 2001). Unphosphorylated Mei2 localizes to the nucleus (Watanabe et al., 1997; Yamashita et al., 1998), where it binds the non-coding, polyadenylated mRNA-like molecule meiRNA (Watanabe and Yamamoto, 1994; Yamashita et al., 1998). This binding of Mei2 to meiRNA is mediated by the Mei2 C-terminal RRM (Watanabe et al., 1997) and occurs as the meiRNA is being transcribed (Shimada et al., 2003), resulting in the localization of Mei2 at a 'Mei2 dot' at the locus that encodes meiRNA, sme2. Formation of this dot coincides with the onset of meiosis I (Watanabe and Yamamoto, 1994; Watanabe et al., 1997; Yamashita et al., 1998).

In plants, mei2-like genes were first reported with the characterization of AML1 (Arabidopsis Mei2-like). This gene was first identified in a heterologous screen for Arabidopsis cDNAs that could enable meiosis in an S. pombe line defective in mating and consequently blocked for meiosis (Hirayama et al., 1997). Like mei2, AML1 encodes 3 RRMs, with the third showing the highest degree of conservation. The third RRM alone was sufficient for complementation of the mating defect. However, AML1 cannot rescue disruptions of mei2 itself, suggesting that the two genes are not equivalent. The ability of AML1 to overcome the mating type block, but not mutations to mei2 itself could reflect AML1 interfering with elements that negatively regulate Mei2 activity. In Arabidopsis, the function of AML1 has not been previously addressed beyond expression analyses, which show accumulation of its transcripts in roots, leaves, siliques and flowers (Hirayama et al., 1997).

To date, the terminal ear 1 (te1) mutant of Zea mays (Veit et al., 1998) offers the only functional analysis of a plant mei2-like gene. Like mei2 and AML1, te1 encodes two N-terminal RRMs and a single, more highly conserved C-terminal RRM.
In loss of function mutants, the terminal and normally free tassel becomes enclosed in a series of husk-like leaves, a change associated with altered patterns of leaf initiation in the shoot apical meristem (SAM). Leaves are initiated more frequently, often with abnormal phyllotaxy, and the normally long vegetative internodes of the main shoot that precede the tassel are abnormally short. Molecular analysis of te1 in wild-type plants show an accumulation of transcripts in a series of semi-circular rings in the SAM that bracket sites of leaf initiation (Veit et al., 1998). This negative correlation between te1 expression and sites of leaf initiation, the more frequent and irregular initiation of leaves and the truncated internode development in the mutant are consistent with a model in which te1 acts to prevent precocious differentiation events.

Regarding possible RNA targets of Mei2-like proteins, no clear sme2 homologues in plants have been detected by sequence similarity. There are however at least 18 polyadenylated, mRNA-like transcripts which have either no conserved ORF or only a very small ORF which may not be translated (MacIntosh et al., 2001). Characterized examples include the CR20/GUT15 (Taylor and Green, 1995; Teramoto et al., 1995, 1996; van Hoof et al., 1997), and ENOD40 (Crespi et al., 1994).

To gain additional insight into mei2-like genes in plants, we have undertaken a survey of additional family members. Using the highly conserved C-terminal RRM as a family hallmark, we identify nine Arabidopsis and six Oryza sativa mei2-like loci. We describe how the plant based family of mei2-like genes groups into four subfamilies by sequence and expression criteria. We observe that the Terminal Ear-like (TEL) subfamily expression pattern is highly specific to groups of pluripotent cells (so-called stem cells) in both the root and shoot apical meristems, while the AML subfamily is more broadly expressed. We conclude by discussing how these genes may influence processes that regulate tissue differentiation in plants.
METHODS

RNA extraction

Tissues were collected, snap-frozen in liquid N2 and stored at -80°C. Total RNA was extracted according to manufacturer’s instructions from 0.3g of tissue using TRIzol Reagent (Invitrogen). Resuspended RNA was thrice-treated with DNA-free™ DNase treatment (Ambion). RNA was quantified spectrophotometrically and 2µg were run on a 1xTAE agarose gel to confirm the quantification. cDNA was generated from 2 µg of total RNA using Omniscript Reverse Transcriptase (Qiagen). Reverse-transcription reactions were performed in triplicate for each tissue; three mock RT reactions lacking Reverse Transcriptase were also performed for each tissue. Reactions were RNase treated, diluted tenfold and stored at -20°C until use.

Rapid Amplification of 5' cDNA Ends

RNA ligase-mediated RACE was performed on total RNA extracted from bulk shoot tissues using a GeneRacer™ Kit (Invitrogen) according to manufacturer's instructions.

Alignment, Phylogenetic Analysis and Genome Analysis

Sequences were aligned using ClustalW (DNASTar) with default parameters. Cladograms were constructed and bootstrap values calculated using PAUP*4.0b10 (Sinauer Associates, Inc.) with parsimony as the set criterion and sequences added randomly to a heuristic search. Analyses were repeated with maximum likelihood as the set criterion; the results did not differ qualitatively from those shown. Similarity plots were generated using Vector NTI Suite 8. Alignment images were generated in GeneDoc (www.psc.edu/biomed/genedoc) (Nicholas et al., 1997). Fig. 3.2 was generated using TreeView (Page, 1996). The MIPS Interactive Redundancy Viewer (mips.gsf.de/proj/thal/db/gv/rv/viewer.html; Martin Ruopp & Dirk Haase) was used to map loci to the Arabidopsis nuclear genome, and to identify loci that may have been
recently duplicated.

**Primer Design**

Primers were designed using PrimerExpress (ABI Prism). Primer annealing regions were selected to minimize the likelihood of paralog amplification and to generate amplicon melting temperatures of 75-80°C. Primer specificity was confirmed in a PTC-200 thermocycler using a Quantitect SYBR-Green PCR Kit (Qiagen) on target template and nearest paralog clone templates. Primer sequences and annealing temperatures were as follows: *AML1* upper primer 5’ gaccacaaaccttttacctcaggtt 3’, lower primer 5’ tagccagcaaagaagatgagtgta 3’, 63°C; *AML2* upper primer 5’ gatgggcaagagccgaatgatc 3’, lower primer 5’ gcagcattctggagttaaccgt 3’, 68°C; *AML3* upper primer 5’ ccaagaagggaggactacgctgagacgaca 3’, lower primer 5’ aagactcgggtggctgagac 3’, 64°C; *AML4* upper primer 5’ gtgccgaatctaatcggagtta 3’, lower primer 5’ ttctgcttcctctgacggaca 3’, 68°C; *AML5* upper primer 5’ ggcagagagaagctgacacag 3’, lower primer 5’ gcctttcaccaatatgtacggttaa 3’, lower primer 5’ tcttgcttcctctgacggaca 3’, 68°C; *TEL1* upper primer 5’ gatgggcatcatgttgagtaa 3’, lower primer 5’ atgtttctctctctgacggttaa 3’, 67°C; *TEL2* upper primer 5’ accattatatcagttac 3’, lower primer 5’ ctctgcttcctctgacggaca 3’, 68°C; *FAD2* upper primer 5’ ccaggtagtggtctctgtgatcttgaaggtcctacggttaa 3’, lower primer 5’ cagttgctctctgacggttaa 3’, 65°C.

**In situ RNA hybridization**

The distribution of transcripts for specific *mei2*-like genes was determined by *in situ* RNA hybridization experiments. Digoxigenin-labelled probes corresponding to the antisense strand cloned *mei2*-like genes of interest were hybridized to paraffin-sectioned material. Tissue fixation, hybridization, washes and detection of hybridization were performed using previously described methods (Jackson et al., 1994). To confirm the specificity of the probes used, control hybridization
experiments were performed in which individual probes were hybridized to membranes onto which a dilution series of synthetic sense transcripts for target transcripts had been fixed. In all cases, probes showed at least a tenfold higher signal with target transcripts compared to signal obtained for transcripts of other mei2-like genes.

**Q-PCR**

PCR reactions were performed in an ABI Prism 7900 Sequence Detection system using a Quantitect SYBR-Green PCR Kit (Qiagen). For each tissue, three RT+ and three RT- template reactions were run. Controls and standards, all performed in triplicate, include water as a template, a 10-9-fold dilution series of the target template, and a 10-3-fold dilution series of the target's nearest paralog. Thermocycling included 5 minutes at 95°C; 94°C for 15 seconds, annealing for 30 seconds, and 72°C for one minute, 35 cycles; and a final melting curve step of 50°C to 95°C.

Values for each reaction were obtained basically according to manufacturer's instructions (ABI Prism Sequence Detection System User Bulletin #2, updated 10/2001). Briefly, a standard curve is constructed using the log of the template standard (in moles of single-stranded template) plotted against the Threshold Cycle (CT) for a given reaction. A slope of ~3.3 on this curve corresponds to a doubling of template every cycle; results of runs whose standard curve slopes were greater that 3.4 or less than 3.0 were discarded as artifactual.

To control for variability in the quality of template, values were normalized against those of the internal standard *FATTY ACID DESATURASE 2 (FAD2)*. *FAD2* was used because its encoded protein performs an essential function in all cell types, it is highly expressed in a broad range of tissues, and it is a single copy gene in Arabidopsis.
RESULTS

Mei2-like proteins are well-represented in the Viridiplantae

To identify Mei2-like proteins in vascular plants, the essentially complete Arabidopsis and rice genomes (AGI, 2000; Goff et al., 2002; Yu et al., 2002) were searched with tBlastn (Altschul et al., 1997) for sequences encoding the highly conserved C-terminal RRM common to S. pombe Mei2, Arabidopsis AML1 and maize tel1. This search yielded nine Arabidopsis and six rice loci. 12 of these loci (seven Arabidopsis and five rice) encode proteins with two N-terminal RRMs preceding a C-terminal Mei2-like RRM. cDNA from Arabidopsis loci were cloned to confirm or correct gene predictions and RACE was used to confirm 5' ends. cDNAs for AML3 and AML5 differed from their predicted ORFs: an AML5 3’ exon was mispredicted, and a coding AML3 exon was skipped due to a single base error in the genomic sequence. Oryza gene predictions were confirmed by comparison to available rice (Kikuchi et al., 2003) or other monocot cDNA and EST sequences. A further two Arabidopsis and one rice loci encode proteins which contain the C-terminal Mei2-like RRM but no other Mei2-like features. A search of the nearly complete nuclear genome of Chlamydomonas reinhardtii, a photosynthetic alga, revealed one additional mei2-like locus. Table 3.1 lists Mei2-like proteins in Arabidopsis, rice and Chlamydomonas. An alignment of the C-terminal RRMs of these sequences is shown in Fig. 3.1.

Comparisons of these clones emphasize the high degree of conservation of the third RRM, which was then used in a second round of searches against ESTs and unfinished genomic sequence from other vascular plants. cDNAs corresponding to ESTs that appear to tag full-length or near full-length mei2-like ORFs were ordered and sequenced. Genomic loci from Hordeum vulgare and Medicago truncatula were compared to available ESTs and cDNAs to assess the validity of predicted ORFs.
Table 3.1. Mei2-like gene products in Arabidopsis, Rice and Chlamydomonas

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Gene Structure</th>
<th>Number of Exons</th>
<th>Protein length - residues</th>
<th>Sequence source</th>
<th>BAC / Contig</th>
<th>Chr</th>
<th>locus or cDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML1</td>
<td>14</td>
<td>915</td>
<td>cDNA</td>
<td>K22G18</td>
<td>V</td>
<td>AT5G61960</td>
<td></td>
</tr>
<tr>
<td>AML2</td>
<td>13</td>
<td>830</td>
<td>cDNA</td>
<td>F14N22 / F7D19</td>
<td>II</td>
<td>AT2G42890</td>
<td></td>
</tr>
<tr>
<td>AML3</td>
<td>11</td>
<td>760</td>
<td>cDNA</td>
<td>F15J5</td>
<td>IV</td>
<td>AT4G18120</td>
<td></td>
</tr>
<tr>
<td>AML4</td>
<td>14</td>
<td>907</td>
<td>cDNA</td>
<td>T28J14 / T2I1</td>
<td>V</td>
<td>AT5G07290</td>
<td></td>
</tr>
<tr>
<td>AML5</td>
<td>12</td>
<td>800</td>
<td>cDNA</td>
<td>F15D2</td>
<td>I</td>
<td>AT1G29400</td>
<td></td>
</tr>
<tr>
<td>TEL1</td>
<td>5</td>
<td>615</td>
<td>cDNA</td>
<td>MJL14</td>
<td>III</td>
<td>AT3G26120</td>
<td></td>
</tr>
<tr>
<td>TEL2</td>
<td>5</td>
<td>527</td>
<td>cDNA</td>
<td>F12A21</td>
<td>I</td>
<td>AT1G67770</td>
<td></td>
</tr>
<tr>
<td>MCT1</td>
<td>3&lt;sup&gt;p&lt;/sup&gt;</td>
<td>233</td>
<td>genomic</td>
<td>F28L22</td>
<td>I</td>
<td>AT1G37140</td>
<td></td>
</tr>
<tr>
<td>MCT2</td>
<td>4&lt;sup&gt;p&lt;/sup&gt;</td>
<td>282</td>
<td>genomic</td>
<td>MXM12 / F13G24</td>
<td>V</td>
<td>AT5G07930</td>
<td></td>
</tr>
<tr>
<td>OsAML1</td>
<td>12&lt;sup&gt;p&lt;/sup&gt;</td>
<td>933</td>
<td>genomic</td>
<td>AAA01004807</td>
<td>II</td>
<td>AK062177&lt;sup&gt;α&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>OsAML2</td>
<td>15</td>
<td>955</td>
<td>cDNA</td>
<td>AAA01005965</td>
<td>V</td>
<td>AK074004</td>
<td></td>
</tr>
<tr>
<td>OsAML3</td>
<td>15</td>
<td>811</td>
<td>cDNA</td>
<td>AAA01000531</td>
<td>II</td>
<td>AK068695</td>
<td></td>
</tr>
<tr>
<td>OsAML4</td>
<td>13&lt;sup&gt;p&lt;/sup&gt;</td>
<td>848</td>
<td>genomic</td>
<td>AAA01003321</td>
<td>II</td>
<td>no rice EST</td>
<td></td>
</tr>
<tr>
<td>OsTEL1</td>
<td>6&lt;sup&gt;p&lt;/sup&gt;</td>
<td>672</td>
<td>genomic</td>
<td>AAA01003386</td>
<td>I</td>
<td>no rice EST</td>
<td></td>
</tr>
<tr>
<td>OsMCT1</td>
<td>4</td>
<td>242</td>
<td>cDNA</td>
<td>AAA01000466</td>
<td>IX</td>
<td>AK064107</td>
<td></td>
</tr>
<tr>
<td>ChlMei2</td>
<td>6&lt;sup&gt;p&lt;/sup&gt;</td>
<td>790</td>
<td>genomic</td>
<td>scaffold 377</td>
<td>unk</td>
<td>no EST</td>
<td></td>
</tr>
</tbody>
</table>

Explanations: Chr - Chromosome, p - predicted, unk - unknown, n - not full length. For gene structures, the midline represents the genomic locus, solid blocks below the midline indicate untranslated regions (where known), while solid blocks spanning the midline indicate coding exons.
Figure 3.1. Mei2-like C-terminal RNA Recognition Motif. Shown are the C-terminal RNA Recognition Motifs (RRMs) of *S. pombe* Mei2, *Zea mays* TE1, the 9 Arabidopsis and 6 Oryza Mei2-like RRMs and the single Chlamydomonas Mei2-like RRM. RNA Binding Domains (RBDs) are underlined. Sequences containing the variable length insert between RBDs 1 and 2 are listed immediately after ZmTE-1. At bottom is RRM2 of Arabidopsis Poly-A Binding Protein2 (PABP2), included to illustrate that the high degree of sequence similarity among the Mei2-like proteins is not a characteristic of RRRMs generally.
This analysis yielded a further 16 full or near-full length Mei2-like predicted proteins, which are listed in Table 3.2.

**Phylogenetic Analysis divides the plant mei2-like gene family into four clades**

A phylogenetic analysis of the transcripts and putative ORFs from Arabidopsis, rice and Chlamydomonas is shown in Fig. 3.2. The vascular plant sequences fall into four distinct clades: two Arabidopsis Mei2-like clades, named AML14 and AML235, a third Terminal Ear 1-like (TEL) clade and a fourth Mei2 C-Terminal RRM only (MCT) clade. All four clades have representatives from both Arabidopsis and rice. The Chlamydomonas ORF resolves as a sister to the two AML clades, but not as a sister to all vascular plant Mei2-like proteins. The two AML clades together form a larger clade with a bootstrap value of 97. The TELs and MCTs form a larger clade with a bootstrap value of 100. The MCTs do not form a clade with a bootstrap value above 55, and do not always resolve as distinct from the TELs. Their depiction as a distinct clade is supported by the motif analysis below.

A second phylogenetic analysis (not shown) of all sequences listed in Table 3.2 found the following: i) all vascular plant mei2-like transcripts (including a single partial transcript from the gymnosperm *Pinus taeda*) resolve into one of the four clades of Fig. 3.2; and, ii) the two AML clades were well-represented from a variety of species, but the TELs and MCTs were not. Only a single TEL transcript was reported (inferred from a contig of 5 Glycine max ESTs out of 357,720 nucleotide sequences available). Similarly, the MCT clade is represented by only a single Triticum aestivum EST (found among 504,102 nucleotide sequences available). This poor representation suggests that both TEL and MCT gene classes are expressed at very low levels, and/or have a very limited tissue distributions.

**Sequence Analysis Reveals Common and Clade-specific Motifs**

All of the Mei2-like proteins in Table 3.2 share a highly conserved C-terminal
<table>
<thead>
<tr>
<th>Species</th>
<th>Order</th>
<th>Transcript</th>
<th>Protein length</th>
<th>clade</th>
<th>source</th>
<th>sequence ID</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pinus taeda</em></td>
<td>Coniferales</td>
<td>PtAML1^p</td>
<td>632</td>
<td>AML235</td>
<td>cDNA</td>
<td>NXCI_108_B01</td>
</tr>
<tr>
<td><em>Beta vulgaris</em></td>
<td>Caryophyllales</td>
<td>BvAML1</td>
<td>617^i</td>
<td>AML14</td>
<td>cDNA</td>
<td>23-E9135-006-006-M06-T3</td>
</tr>
<tr>
<td><em>Glycine max</em></td>
<td>Fabales</td>
<td>GmTEL1</td>
<td>529</td>
<td>TEL</td>
<td>EST contig</td>
<td>Gm-c1036-7660, Gm-c1052-5123, Gm-c1036-11113, Gm-c1036-12445</td>
</tr>
<tr>
<td><em>Medicago truncatula</em></td>
<td>Fabales</td>
<td>MIAML1</td>
<td>856</td>
<td>AML235</td>
<td>cDNA</td>
<td>NF012c12PH</td>
</tr>
<tr>
<td><em>Medicago truncatula</em></td>
<td>Fabales</td>
<td>MIAML5</td>
<td>865</td>
<td>AML235</td>
<td>cDNA</td>
<td>NF028G07PH</td>
</tr>
<tr>
<td><em>Medicago truncatula</em></td>
<td>Fabales</td>
<td>MIAML2</td>
<td>961</td>
<td>AML14</td>
<td>genomic</td>
<td>mtgsp002h08</td>
</tr>
<tr>
<td><em>Aegyptes speltoides</em></td>
<td>Poales</td>
<td>AsAML1</td>
<td>869</td>
<td>AML235</td>
<td>cDNA</td>
<td>WHE2239F10L19</td>
</tr>
<tr>
<td><em>Hordeum vulgare</em></td>
<td>Poales</td>
<td>HvAML6</td>
<td>919^i</td>
<td>AML14</td>
<td>cDNA</td>
<td>HVSMEi0006M19f</td>
</tr>
<tr>
<td><em>Hordeum vulgare</em></td>
<td>Poales</td>
<td>HvAML3</td>
<td>921</td>
<td>AML14</td>
<td>genomic</td>
<td>BAC011009</td>
</tr>
<tr>
<td><em>Sorghum bicolor</em></td>
<td>Poales</td>
<td>SbAML1</td>
<td>818^i</td>
<td>AML14</td>
<td>cDNA</td>
<td>PI191F03.b1A002</td>
</tr>
<tr>
<td><em>Triticum aestivum</em></td>
<td>Poales</td>
<td>TaMCT1</td>
<td>223</td>
<td>MCT</td>
<td>EST</td>
<td>WHE3506C01E02</td>
</tr>
<tr>
<td><em>Triticum aestivum</em></td>
<td>Poales</td>
<td>TaAML15</td>
<td>870</td>
<td>AML235</td>
<td>cDNA</td>
<td>TaLr1151E12R</td>
</tr>
<tr>
<td><em>Zea mays</em></td>
<td>Poales</td>
<td>TE-1</td>
<td>656</td>
<td>TEL</td>
<td>cDNA</td>
<td>AF047852</td>
</tr>
<tr>
<td><em>Cirtus unshiu</em></td>
<td>Sapindales</td>
<td>CuAML1</td>
<td>858</td>
<td>AML235</td>
<td>cDNA</td>
<td>pcMAIM1105-48</td>
</tr>
<tr>
<td><em>Lycopersicon esculentum</em></td>
<td>Solanales</td>
<td>LeAML1</td>
<td>971</td>
<td>AML14</td>
<td>cDNA</td>
<td>cTOD20J23</td>
</tr>
<tr>
<td><em>Solanum tuberosum</em></td>
<td>Solanales</td>
<td>SIAML1</td>
<td>843</td>
<td>AML235</td>
<td>cDNA</td>
<td>cSTS5O17</td>
</tr>
</tbody>
</table>

^p - transcript is not full length; ^i - predicted protein lacks one or more adjacent internal motifs
Figure 3.2. Phylogeny of the Mei2-like proteins in the Viridiplantae. Shown is a parsimony tree of Mei2-like proteins from Arabidopsis, Rice and Chlamydomonas; *S. pombe* Mei2p is the outgroup. Bootstrap values of 55 or greater are shown to the right of the clade they describe. Branch lengths reflect the number of substitutions between sequences.
Mei2-like RRM. At many sites in the C-terminal RRM, conservation is absolute across all genes compared. The domain is further distinguished by a conserved extension at its C-terminus of about 50 amino acids which is not associated with other RRMs (Birney et al., 1993; Burd and Dreyfuss, 1994). Like Mei2 itself, the TELs and AMLs (with exceptions to be discussed below) also possess two N-terminal RRMs. By contrast, the MCTs show no recognizable motifs beyond their single RRMs.

mei2-like genes belonging to the TEL and MCT clades can be distinguished from AML members by the distance between two elements contained in RRM3, ribonucleoprotein consensus sequence 2 (RNP 2) and RNP 1 (Birney et al., 1993; Burd and Dreyfuss, 1994). In some characterized RRMs, the region between RNP2 and RNP1 plays a role in sequence recognition. In the AMLs, this distance is absolutely fixed at 32 highly conserved residues. In the TELs and MCTs, an additional 6-20 residue insert separates the RNPs.

Members of both AML clades share the following motifs: a small 'Tri-Serine' element; a region rich in acidic residues containing an IGNLLPD consensus (absent from Chlamydomonas); a second region rich in acidic residues containing a GGMEELD consensus; two N-terminal RRMs, a variable region of 200-400 residues containing a double tryptophan element and an HIGSAP element (not recognized in Chlamydomonas); and the C-terminal Mei2-like RRM, with the RNP 2 - RNP 1 distance fixed at 32 residues (Fig. 3.1, 3.3).

The location of the double tryptophan and the HIGSAP elements within the AML variable region is similar to that of two critical sites in S. pombe Mei2 whose phosphorylation state indirectly governs RNA binding, sub-cellular localization and activity of Mei2 (Watanabe et al., 1997).

Members of the TEL clade share a small N-terminal GNL motif followed by a hydrophobic proline-rich stretch (26 of 53 residues are proline in TEL1; 11 of 14 in
**Figure 3.3.** Conserved motifs and local alignments for Arabidopsis, Oryza and Chlamydomonas AML protein sequences. For the similarity plot, the X-axis represents residue number; the Y-axis is percent identity. Labeled regions shown below the plot are as follows: a) TriSer; b) IGN; c) GGME LD; d1, d2) RRM tandem repeat; e) VGSP; f) LHPH; g) WW-motif; h) HIGSAP; i) Mei2p-like RRM (see Figure 3.1). The IGN motif, b, is absent from the Chlamydomonas protein.
GmTEL, 21 of 32 in TEL2, 11 of 22 in ZmTE1, and 17 of 27 in OsTE1), two RRMs, a variable region with no clear motifs, and a C-terminal RRM with a variable RNP2-RNP1 distance (Fig. 3.1, 3.4).

**Alternatively spliced AMLs and genomically encoded motif deletions are common in the vascular plants**

Alternative splicing is commonly observed in plants (Simpson and Filipowicz, 1996). However, until recently there were relatively few examples with clear functional significance (Macknight et al., 1997; Lopato et al., 1999; Mano et al., 1999; Reddy, 2001; Jasinski et al., 2002; Macknight et al., 2002; de la Fuente van Bentem et al., 2003; Kazan, 2003; Quesada et al., 2003; Savaldi-Goldstein et al., 2003; Staiger et al., 2003; Zhang and Gassmann, 2003). In the Arabidopsis Mei2-like family, alternative splicing was observed in transcripts of two of the five AML loci and one of the two TEL loci. Splice variant AML3 and AML4 transcripts were cloned from bulk shoot tissue. Only the AML3 variants alter the coding potential of the transcript. One AML3 variant lacks a Tri-Serine motif due to alternate intron 5’ end selection; a second variant retains an intron, interrupting the open reading frame with a stop codon. Retention of intron three in TEL1, containing a stop codon and polyadenylation signal, yields a transcript encoding a truncated open reading frame.

A bioinformatics-based approach was used to detect splice variants elsewhere in the available vascular plant EST database. cDNA sequences from Table 3.2 were used to query vascular plant ESTs at NCBI (www.ncbi.nlm.gov). Splice variants were identified when a given pair of near-identical sequences differed only by a gap or abrupt border containing one or more consensus intron border sequences. A full list of splice variants detected is given in Table 3.3.

In several cases, AML class cDNAs that lack one or more motifs can be attributed to genomically encoded variants. The *Hordeum vulgare* locus encoding
Figure 3.4. Conserved motifs and local alignments for TEL protein sequences. The similarity plot is as in Figure 3.3. Labeled regions shown below the plot are as follows: a) GNL-motif; b) proline-rich region; c1, c2) RRM tandem repeat; i) Mei2p-like RRM (see Figure 3.1). Glycine TEL and Zea tel were included to increase the resolution of the TEL similarity plot.
Table 3.3. Alternatively Spliced and Genomic Variant AML Transcripts

<table>
<thead>
<tr>
<th>Sequence Pair</th>
<th>Organism</th>
<th>Event</th>
<th>EXON/intron...</th>
<th>How often?</th>
<th>Effect on ORF</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML3, variant</td>
<td>Arabidopsis</td>
<td>Intron 5' end</td>
<td>ACA274/guau vs CUA325/guga</td>
<td>6/10</td>
<td>Tri-Serine deleted</td>
<td>Tri-Serine</td>
</tr>
<tr>
<td>AML3-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HvAML6, Hordeum vulgare</td>
<td>standard splicing event</td>
<td>ACC217/guga... cag/CGA</td>
<td>All</td>
<td>Tri-Serine absent</td>
<td>Tri-Serine</td>
<td></td>
</tr>
<tr>
<td>SbAML1, Sorghum bicolor</td>
<td>standard splicing event</td>
<td>GAG114/guau... uag/AUC</td>
<td>All</td>
<td>RRM1, RRM2 not genomically encoded</td>
<td>RRM1, RRM2</td>
<td></td>
</tr>
<tr>
<td>BvAML1, Beta vulgaris</td>
<td>standard splicing event</td>
<td>GCC30/guau... cag/UUU</td>
<td>All</td>
<td>RRM1, RRM2 not genomically encoded</td>
<td>RRM1, RRM2</td>
<td></td>
</tr>
<tr>
<td>AML3, variant</td>
<td>Arabidopsis</td>
<td>Intron retained</td>
<td>GAG109/guau... cag/AUA</td>
<td>3/10</td>
<td>Premature STOP</td>
<td>after RRM2</td>
</tr>
<tr>
<td>AML3-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MtAML1, BF645880</td>
<td>Intron retained</td>
<td>AGC34/guau... cag/UAA</td>
<td>1/6</td>
<td>Premature STOP</td>
<td>After Tri-Serine</td>
<td></td>
</tr>
<tr>
<td>MtAML1, AW690292</td>
<td>Intron retained</td>
<td>...unknown/G341 UG vs. ...cag/G350GC</td>
<td>1/6</td>
<td>Residues AGG deleted</td>
<td>N-terminus</td>
<td></td>
</tr>
<tr>
<td>BQ970227, BQ971840</td>
<td>Intron retained</td>
<td>...cag/G114/UA vs. ...unknown/UGU</td>
<td>1/8</td>
<td>Frameshift</td>
<td>RRM3</td>
<td></td>
</tr>
<tr>
<td>BQ968715, BU028561</td>
<td>Intron retained</td>
<td>AAG/guau ...cag/AAC</td>
<td>1/7</td>
<td>Premature STOP</td>
<td>RRM3</td>
<td></td>
</tr>
<tr>
<td>HvAML6, CB880137</td>
<td>Intron excised</td>
<td>CAA239/guac ...gcu(^{\text{3'UTR}})GUA</td>
<td>1/17</td>
<td>Premature STOP</td>
<td>RRM3</td>
<td></td>
</tr>
<tr>
<td>AML4, AML4-2</td>
<td>Arabidopsis</td>
<td>Intron retained</td>
<td>AAG229/guau ...cag/AGG</td>
<td>2/3</td>
<td>None</td>
<td>3' UTR</td>
</tr>
<tr>
<td>LeAML1, BE354132</td>
<td>Intron excised</td>
<td>GCC291/guau ...cag/AAA</td>
<td>1/9</td>
<td>None</td>
<td>5' UTR</td>
<td></td>
</tr>
<tr>
<td>MtAML5, BG453336</td>
<td>Intron retained</td>
<td>...cag/AA vs. ...unknown/U(149-8)GC</td>
<td>1/2</td>
<td>None</td>
<td>5' UTR</td>
<td></td>
</tr>
<tr>
<td>CF051251, BU037082</td>
<td>Intron excised</td>
<td>CAA/guua ...aag/ACT</td>
<td>3/30</td>
<td>None</td>
<td>3' UTR</td>
<td></td>
</tr>
</tbody>
</table>

n - non-canonical intron 3' end. A subscript indicates the base of the corresponding sequenced clone, if known, or the effect of the alternative intron end selection on one EST sequence with respect to another.
HvAML6 does not encode a Tri-Serine motif. The *Sorghum bicolor* *SbAML1* and the *Beta vulgaris* *BvAML1* loci have independently lost their two N-terminal RRM, but retain all other clade-specific motifs.

**One pair of Arabidopsis mei2-like genes is found in a large genomic duplication**

The nine Arabidopsis *mei2*-like genes are distributed throughout the genome. *AML1* and *AML4* appear to be products of a recent gene duplication of the region spanning At5G60990 (Replication protein A1-like) to At5G62230 (a putative protein) at the north end of chromosome V and At5G07170 (a putative protein) to At5G08020 (Replication factor A-like) at the south end of chromosome V. 14.5% of the annotated genes in the northern block (18 of 124 genes in 300kb) show significant similarity to 21.2% of the genes in the southern block (18 of 85 genes in 240kb). The paralogous loci are collinear and span but are not confined to two small duplicated clusters identified by the MIPs Interactive Redundancy Viewer. No other *mei2*-like genes could be linked to identified duplications within the Arabidopsis genome.

**Putative Mei2-like proteins are not plant specific, but are absent from Metazoans and *S. cerevisiae***

tBlastn searches (Altschul et al., 1997) using the *S. pombe* Mei2 sequence and keyword searches of annotated genomic sequence reveals a number of Mei2-like proteins within several eukaryotic groups, but none within the Archaea or Eubacteria. No examples of *mei2*-like loci could be found in metazoans, nor were any detected in *S. cerevisiae*, though examples were evident in other fungi.

Among unicellular eukaryotes, Mei2-like C-terminal RRMs were detected in the genomic sequences of the Apicomplexan Alveolates *Plasmodium falciparum*, *P. yoelii*, and *Toxoplasma gondii*; and as ESTs in *T. gondii* (two paralogous transcripts) and *Neospora hughesi*; and in the Ciliophoran Alveolate *Paramecium tetraurelia* genomic survey sequence. At least one Mei2-like C-terminal RRM is present in the
available genome sequence of the Mycetozoan *Dictyostelium discoideum*. As previously mentioned, there is a single mei2-like locus in the *Chlamydomonas reinhardtii* nuclear genome sequence.

Few of the ORFs encoding Mei2-like RRM in these unicellular organisms are well resolved beyond the motif itself, precluding any analysis of the overall structure of their encoded proteins. Within the RRM the RNP 2- RNP 1 linear distance is absolutely conserved at 32 residues. This suggests that the variable-length sequence separating the RNPs in the TEL clade of Mei2-like proteins is unique to the vascular plants.

With respect to the expression of these unicellular genes, two examples suggest a meiotic function. The *Plasmodium falciparum* mei2-like orthologue MAL6P1.195 accumulates to significantly higher levels in the gametocyte compared to other developmental stages (Bozdech et al., 2003; Le Roch et al., 2003). Similarly, the Chlamydomonas mei2-like transcript is absent from existing EST collections, which are largely focused on vegetatively growing cells under a variety of environmental conditions and for which no meiosis-specific EST collections have been reported ([www.biology.duke.edu/chlamy_genome](http://www.biology.duke.edu/chlamy_genome)). This stands in sharp contrast to the vascular plant AML transcripts, which are highly represented in EST collections from vegetative tissues.

**Expression analysis shows clade specific patterns of transcript accumulation**

To gain insight into the possible function of mei2-like genes in Arabidopsis, we analyzed the tissue localization of mei2-like transcripts in embryos and SAMs by *in situ* hybridization. Earlier studies of the maize *te1* gene had shown a close correlation between its patterned expression in the SAM and defects to this structure in *te1* loss of function mutants (Veit et al., 1998). *TEL1* and *TEL2* transcripts were localized predominantly to apical meristems. Beginning with relatively uniform
expression throughout the globular embryo, transcripts later resolved to the apical and basal regions of the heart stage embryo (Fig. 3.5). In the apical region, expression includes precursors of the cotyledons and SAM (shoot apical meristem), while the basal region shows expression in the quiescent center and adjacent founder cells of the vascular system. Later, in the torpedo stage embryos, $TEL1$ signal becomes limited to the SAM and RAM (root apical meristem). $TEL2$ transcripts exhibit a similar distribution, but are detected at lower levels than their $TEL1$ counterparts and appear more focused over the SAM of heart and torpedo staged embryos. In contrast to $TEL1$, $TEL2$ accumulation is much more intense in the shoot than in the root of torpedo stage embryos. Later in development, $TEL2$ expression becomes confined to central regions of the apical dome in both vegetative and floral shoot apices. By contrast, $TEL1$ transcripts show a broader and more patchy distribution within the meristem.

$AML$ transcripts exhibit a variety of distributions, which in general are less distinct than their $TEL$ counterparts. $AML1$ transcripts are relatively uniformly distributed in the heart stage embryo, but become more focused in vascular precursor tissue along the apical/basal axis in later stages. $AML1$ is expressed weakly throughout the vegetative shoot apex, but is later focused in organogenic regions of floral apices. $AML2$ shows a widely distributed but patchy accumulation in the heart stage embryo that later resolves to an $AML1$-like pattern in the torpedo stage embryo. Higher levels of transcripts are observed throughout the vegetative embryo, while no significant accumulation is observed in floral apices. $AML3$ transcripts show a relatively uniform distribution in the heart and torpedo stage embryos. Unlike other $AML$ and $TEL$ transcripts, $AML3$ accumulation appears restricted primarily to nuclei in these early stages, and was not detected in the SAM during vegetative or floral development. Notably, $AML3$ is also the transcript with the highest frequency of alternative splicing. $AML4$ transcripts appear to be relatively uniformly distributed
**Figure 3.5.** *In situ* hybridization of TEL and AML probes to Arabidopsis developing embryos and apical meristems. Shown are heart/globular embryos, torpedo stage embryos, vegetative shoot apical meristems (SAMs) and floral shoot apical meristems.
throughout the meristem during embryonic and vegetative development; like *AML1*, *AML4* transcripts accumulate in floral organogenic regions. *AML5* transcripts exhibited a similar broad distribution, but showed higher levels along the apical/basal axis of the embryo, especially in the SAM region of the torpedo staged embryo.

**Q-PCR reveals *AML* expression across a broad range of tissues**

Using *FAD2*, a gene which is highly expressed across a broad range of tissues, as an internal standard, *AML* and *TEL* transcript accumulation was observed among a panel of distinct tissues. *TEL1* and *TEL2* were found only in buds, flowers, and siliques, and never more than 10-fold higher than background. No SAM- or RAM-enriched tissues were used. This indicates that *TEL1* and *TEL2* are expressed at very low levels, in a very limited set of cells, or both.

*AML1* through *AML5* were expressed ubiquitously at levels of 1000x or more than *TEL1* or *TEL2*. Expression was generally high in leaves and lowest in seedlings, and was higher in actively growing leaf tissue (cauline leaves and leaves of rosettes which had not yet bolted) than in more mature basal leaves of plants which had bolted (Fig. 3.6).

A correlation test was performed on above-ground tissue *AML* transcript levels to assay for relationships among *AML* accumulation patterns. Data sets which rise and fall in unison have a correlation value of up to 1, unrelated data sets have a correlation of 0, and data sets which are inversely related have a correlation value of minus 1. Results of this analysis (Table 4) show that transcripts encoding similar proteins show similarly varying patterns of accumulation. Control reactions confirmed that this correlation is not due to non-specific amplification of nearest paralog transcripts.

**DISCUSSION**

This survey of *mei2*-like genes in Arabidopsis, rice and other eukaryotes provides useful insights into the origin and possible functions of this unusual family.
Figure 3.6. Quantification of *AML* transcript levels by quantitative PCR. *AML1*, *AML2*, and *AML5* transcript values are described by the Y-axis. *AML3* values are 0.2x the values on the Y-axis; *AML4* values are 0.1x the values on the Y-axis. *AML2* accounts for over 50% of total transcript accumulation; the *AML235* clade accounts for 85% of total transcripts. All transcripts values were normalized using accumulation of the internal standard FAD2, a transcript highly expressed in all tissues.
As defined by the presence of an unusual RRM initially described in the *Schizosaccharomyces pombe mei2* gene, this family has undergone a dramatic expansion in the vascular plant lineage. Their ubiquity among all highly sequenced vascular plant species, as well as in the green algae Chlamydomonas, suggests *mei2*-like genes are present in all green plants.

The essentially complete genome sequences of rice and Arabidopsis afford a consistent view of the *mei2*-like family in angiosperms. Four distinct clades are revealed, which can be grouped by absolute sequence similarity as well as by clade specific sequence motifs. By definition, all share a motif similar to the C-terminal RRM of Mei2. Comparisons between these clades and Mei2 emphasize the highly conserved nature of this motif. By contrast, RRM1 and RRM2, though similarly positioned to those of Mei2, appear less well conserved and are entirely absent in the MCT clade and some AML clade members. The presence of Arabidopsis and rice representatives in all four clades suggests that these clades were an early feature in the evolution of the Angiosperms. Looking back further, the closer affinity of ChlMEI2 with the AML clades suggests the existence of distinct TEL and AML clades prior to the evolution of vascular plants, or that the TEL and AML clades diverged later, can also be supported, but requires that the TEL clade in some way evolved more rapidly.

With respect to the biological functions mediated by *mei2*-like genes, data reported here support roles other than the meiosis promoting activity of Mei2. Arabidopsis *mei2*-like genes show a diverse range of tissue specific expression patterns throughout the life of the sporophyte, suggesting these genes perform specialized functions during mitotic growth. Attempts to define these functions by analysis of loss of function phenotypes reveal no obvious phenotypes, possibly due to genetic redundancy as suggested by phylogenetic and expression analyses of the gene family. However, the highly focused and apically limited expression of *TEL1* and
TEL2 would be consistent with these genes acting as the maize te1 gene does, to somehow limit differentiation processes in the SAM. By contrast, the broader expression patterns seen for members of the AML clades suggest more diverse functions. The distinct character of the AML and TEL clades is further reinforced by the presence of clade specific motifs which presumably mediate different sets of protein interactions.

The simultaneous and specific expression of TEL1 and TEL2 genes in regions in both the shoot and root deserves special comment given the uncommon nature of this pattern. Previous examples of transcripts with a similar expression pattern include the tomato defective embryo and meristems (dem) mutant (Keddie et al., 1998), a gene critical to both root and shoot apical meristem development, and the FASCIATA 1 and 2 (FAS1 and FAS2), genes thought to encode chromatin assembly factors, which are also essential for the normal maintenance of the root and shoot (Kaya et al., 2001). In view of recent work that suggests the indeterminate growth of shoot and roots may rely on similar mechanisms (Casamitjana-Martinez et al., 2003; Hobe et al., 2003; Kamiya et al., 2003; Veit, 2004), the expression of TEL1 and TEL2 in both locations would be consistent with a role for these genes in maintaining cells in an undetermined state.

At present, there is no direct evidence for how mei2-like genes might function at a molecular level in plants. In fission yeast, however, several lines of evidence suggest that Mei2 may act, at least in part, through an RNA dependent chromatin remodeling activity (Mizuno et al., 2001; Shimada et al., 2003) that critically depends on the RRM3, the most highly conserved element of the gene family. Such regulation would add to a list of precedents where chromatin remodeling involves an RNA component, including X chromosome dosage compensation in both humans and Drosophila, as well as gene silencing in S. pombe.
To reconcile the clearly defined meiotic activity of Mei2 in yeast with what appear to me more diverse roles of related genes implants, two general perspectives can be offered. The first would suppose that the molecular mechanism by which mei2-like genes act is well suited to certain processes. For example, a Mei2 mediated chromatin remodeling activity would have clear relevance to meiosis, in which chromatids must replicate and pair. Similarly, the proposed role for TEL genes in maintaining cells in an undetermined state may also reflect a chromatin related activity. The essential role of the chromatin assembly factors FAS1 and FAS2 in roots and shoots suggests regulation at this level may be an integral feature of stem cell maintenance.

A second and not mutually exclusive perspective focuses on how cell division and growth might be linked to nutritional status. As a single cell, the normal response of heterothallic S. pombe diploid cells to nutrient stress is to undergo meiosis, a process in which Mei2 plays a pivotal role. In multicellular organisms, however, such a direct coupling of cellular behavior and nutrient might preclude more complex patterns of development. Perhaps the role of mei2-like genes reflects their ancestral role in coupling nutrient status and cell division which has been adapted to permit different cellular responses in a multicellular context. Thus, the specific expression of TEL genes in both the shoot and root might somehow condition the characteristically slow division of apical initials in these structures.

CONCLUSION

This analysis suggests mei2-like genes have found several functional niches in the plant kingdom that extend beyond the meiotic signaling function of the fission yeast mei2 gene, and which have no parallel in metazoans. Phylogenetic analyses of this family in plants suggest they had diversified in ancestor of the angiosperms, with four distinct subfamilies found in both monocots and dicots. Expression analyses
support roles for these genes throughout vegetative development. The expression of 
TEL genes in domains of both the shoot and root which are thought to contain 
pluripotent stem cells is especially intriguing, and offers support for models in which 
the uncommitted state of these cells in both apical meristem types is maintained by 
similar mechanisms.

Acknowledgement of materials
Clones from EST collections were kindly made available from the following sources: 
PtAML1 - Dr. Arthur Johnson, North Carolina State University; BvAML1 - Dr. Bernd 
Weisshaus, Max-Planck-Institute for Plant Breeding Research; MtAML1, MtAML5 - 
Dr. Joe Clouse, Noble Foundation; AsAML1 - Dr. Olin Anderson, US Department of 
Agriculture, Agriculture Research Service, US Department of Agriculture, Albany, 
CA; HvAML6 - Dr. RA Wing, Clemson University Genomics Institute; SbAML1 - 
Dr.s L. H. Pratt and M. M. Cordonnier-Pratt, University of Georgia; TaAML15 - Dr. 
Sylvie Cloutier, Cereal Research Centre, Agriculture and Agri-food Canada; CuAML1 
- Dr. Mitsuo Omura, National Institute of Fruit Tree Science, Okitsu, Japan; LeAML1 
- Dr. Steven Tanksley, Cornell University via Clemson University Genomics Institute; 
StAML1 - Dr. Steven Tanksley, Cornell University and Dr. Robin Buell, TIGR, via 
Clemson University Genomics Institute.  *Hordeum vulgare* cultivar Tadmore genomic 
DNA was kindly provided by Dr. Mark Sorrells, Cornell University;  *Sorghum bicolor* 
genomic DNA was kindly provided by Caroline Kellogg, Cornell University;  *Beta 
vulgaris* genomic DNA was extracted by Jason Gillman, Cornell University. 
Unfinished Medicago genomic sequence was made available by the *Medicago 
truncatula* Genome Sequencing Project at the Advanced Center for Genome 
Technology of the University of Oklahoma, Norman, Oklahoma and the Noble 
Foundation, Ardmore, Oklahoma.
CHAPTER 4

THE Arabidopsis Mei2 HOMOLOGUE AML1 BINDS AtRaptor1B, A MAJOR REGULATOR OF PLANT GROWTH

ABSTRACT

TOR is a potent cell growth regulator in all eukaryotes. It acts through the phosphorylation of downstream effectors that are recruited to it by the binding partner Raptor. In Arabidopsis, Raptor activity is essential for postembryonic growth. Though comparative studies suggest potential downstream effectors, no Raptor binding partners have been described in plants.

AtRaptor1B, a plant Raptor homologue, binds the AML1 protein in a yeast two-hybrid assay. This interaction is mediated by the N-terminal 219 residues of AML1, and marks AML1 as a candidate AtTOR kinase substrate in plants. The AML1 N-terminus additionally carries transcriptional activation domain activity. Plants homozygous for insertion alleles at the AML1 locus, as well as plants homozygous for insertion alleles at all five loci in the AML gene family, bolt earlier than wild-type plants.

AML1 interacts with AtRaptor1B, homologue of a protein that recruits substrates for phosphorylation by the major cell-growth regulator TOR. Identification of AML1 as a putative downstream effector of TOR gives valuable insights into the plant-specific mode of action of this critical growth regulator.

INTRODUCTION

TOR, the target of the antibiotic rapamycin in both yeast and mammalian cells, is a major regulator of cell growth and translation (Schmelzle and Hall, 2000). TOR is
a large (over 2,400 residues) protein kinase (Thomas et al., 2004) present in all eukaryotes analyzed. It acts in a nutrient-sensitive complex with Raptor (regulatory associated protein of TOR) and another protein to regulate cell growth in response to nutrients (Hara et al., 2002; Kim et al., 2002; Loewith et al., 2002; Kim et al., 2003; Kim and Sabatini, 2004). Raptor, a protein with HEAT and WD-40 protein interaction domains, recruits substrates for phosphorylation by TOR in yeast and mammals (Hara et al., 2002; Loewith et al., 2002). TOR also acts in a second, nutrient-insensitive complex without Raptor to regulate the cytoskeleton (Loewith et al., 2002; Sarbassov et al., 2004).

Disruption of the Arabidopsis TOR homologue AtTOR is lethal early in plant embryonic development (Menand et al., 2002). Disruption of AtRaptor (encoded by two paralogous loci in Arabidopsis) causes seedling developmental arrest but allows normal embryonic development (Chapter 2), suggesting that AtTOR embryonic activity is independent of AtRaptor and that the TOR-Raptor complex has been adapted in the ancestor of the angiosperms to regulate growth from the shoot apical meristem (SAM). Thus, identifying downstream activators of TOR signaling may provide insights into the activity of the SAM.

Mei2 is a putative TOR substrate and potent signaling molecule identified in the fission yeast Schizosaccharomyces pombe (Watanabe et al., 1988; Watanabe and Yamamoto, 1994). Mei2 is bound by the Raptor homologue Mip1 (Shinozaki-Yabana et al., 2000), and is an inactive phosphoprotein under high nutrient conditions (Li and McLeod, 1996; Watanabe et al., 1997) - conditions which promote TOR kinase activity. The kinase governing two of the three Mei2 phosphorylation sites is known (Watanabe et al., 1997); TOR is a strong candidate for the kinase governing the third. In mated diploids under low nutrient conditions, unphosphorylated Mei2 accumulates and localizes to the nucleus, where it binds to a noncoding, mRNA-like RNA
molecule meiRNA in an interaction mediated by the third of its three RNA Recognition Motifs (RRMs)(Watanabe and Yamamoto, 1994; Watanabe et al., 1997; Yamashita et al., 1998). The Mei2-meiRNA interaction occurs as meiRNA is being transcribed, tethering Mei2 to the meiRNA locus (Shimada et al., 2003). Accumulation of Mei2 at this focused point immediately precedes meiosis.

Mei2-like proteins are predicted in a wide range of organisms (Anderson et al., 2004; Jeffares et al., 2004) including some fungi, alveolates, a diatom, and all land plants, but they are absent from metazoans and budding yeast. In land plants, predicted Mei2-like proteins form a small conserved gene family, many of whose members’ transcripts accumulate in the shoot apical meristem (Anderson et al., 2004).

Arabidopsis Mei2-like 1 (AML1), the first member of this family to be described, was isolated in a screen for plant cDNAs whose expression could complement defects in the fission yeast meiosis signaling pathway (Hirayama et al., 1997). Like Mei2, AML1 has three RRs. Expression of a protein fragment containing only the third AML1 RRM was sufficient for restoration of meiosis signaling in fission yeast lines with defects upstream of Mei2, but not in lesions of the mei2 locus itself. Terminal Ear 1 (te1), a more divergent member of the mei2-like family of genes, regulates leaf initiation in maize; the tassel of mutant plants is encased a whorl of leaves superficially resembling a maize ear (Veit et al., 1998).

Given the potential role of downstream AtTOR effectors in SAM activity, the intriguing signaling activity of Mei2 in fission yeast, and the known interaction between Mei2 and the fission yeast Raptor protein, we asked if the Mei2-Raptor interaction was conserved between its Arabidopsis orthologues AML1 and AtRaptor1B.
METHODS

Generating the Two-hybrid Constructs

AML1 was cloned via RT-PCR. The cDNA template was reverse-transcribed using Omniscript (Qiagen) from RNA extracted from bulk shoot tissue using Trizol Reagent (Invitrogen). Restriction sites NcoI and XmaI/SmaI were added to the 5’ and 3’ ends of the ORF and of all smaller AML1 fragments via PCR using ExTaq high-fidelity polymerase (Takara). An EST clone (RZL03b06) tagging AtRaptor1B was obtained from Kazusa DNA institute and sequenced. Restriction sites NcoI and EcoRI were added at the 5’ and 3’ ends of the ORF via PCR. pGBK7 and pGADT7 are distributed by BDBiosciences.

Yeast two-hybrid assay

AH109 cells (leu2-trp1-ade2-his3-) were grown in YEPDA liquid plates or on YEPDA plates with 17 g/L Agar-Y (Bio101 Systems). Cells were transformed using the Yeastmaker Yeast Transformation System2 (BD Biosciences) and plated on medium lacking the appropriate macronutrients (Bio101 Systems). Colonies were observed 3-7 days after transformation.

Genotyping of Insertion Alleles

DNA from lines harboring insertion alleles was extracted using the alkaline boiling method (Klimyuk et al., 1993). Provisional homozygotes were confirmed via a second extraction using the C-TAB DNA extraction protocol. PCR to assay for wild-type and insertion alleles was performed in 20uL volumes using ExTaq polymerase and buffers and the following cycling parameters: 94°C, 15 seconds; 61°C, 30 seconds; 72°C, 2 minutes; 35 cycles. Genotyping primers were as follows: AML1-5sm 5’atagaagaaacaaaaaggaagggaa3'; AML1-3sm 5'tagcatatactcctgtacgccgactg3'; AML2-5sm 5’atgctctgtctctgtatgatgattttgtc3';
AML2-3sm 5’gcagcaatatcataagcctcgggttca3’; AML3-5sm 5’cttgtaaccttctctgtgtgat3’; AML3-3sm 5’ctgccaagcctggaaacaaacataaa3’; AML4-5sm 5’ttgcaagcctgtgatcatataacctc3’; AML4-3sm 5’atgctaccgggagaacctaagtgaaatc3’; AML5-5sm 5’tctttagccacatcaacattcctctcc3’; AML5-3sm 5’atcagcgtcaagtctccctcctctccac3’; JL-202 5’cattttataataacgctgcggacatctac3’; JL-270 5’tttctccatattgaccatctcattg3’; pROC-737 5’gggaattcactggccgtcgttttacaa3’. The wild-type loci were assayed with the above pairs. The insert was assayed using the following pairs: AML1-5sm or AML1-3sm with JL-270 or JL-202, AML2-5sm with pROC-737, AML3-5sm with JL-270 or JL-202, AML4-3sm with JL-270 or JL-202, AML5-5sm with JL-202.

Insertions in the $AML_1$ and $AML_5$ loci were obtained from the University of Wisconsin alpha collection using their described protocol and are in the Wassilewskija (Ws) ecotype background. The $AML_1$ insertion was found in pool CSJ8-46-H35. The $AML_5$ insertion was found in pool CSJ1091-H45. Insertions in the $AML_3$ and $AML_4$ loci were obtained from the University of Wisconsin Basta collection and are in the Ws background. The $AML_3$ insertion was found in pool 67-6-F. The $AML_4$ insertion was found in pool 18-2-H. The insertion in $AML_2$ was obtained from the line 029713 from the Salk collection and is in the Columbia (Col-0) ecotype background. Aside from regions genetically linked to any of the insertion loci, the $AML$ quintuple insertion allele homozygote lines are in the genomic background of a Col-0 x WS F3 individual once backcrossed to the WS background.

**RNA extraction, RT-PCR**

RNA extraction was performed using TRIzol™ Reagent (Invitrogen) essentially according to manufacturer’s instructions. Total RNA was treated with DNA-free™ DNase (Ambion) and reverse transcribed using Omniscript reverse transcriptase (Qiagen) with an oligo-dT primer. Primer pairs spanning the insertion
site for RT-PCR on all five lines are as follows: AML1 117-138
5’gtgatgatgagtggattgata3’, AML1 556-534rc 5’atgggctggtagctagt3’; AML2 86-109
5’ttggctctctctctctctct3’, AML2 456-435rc 5’agcatcgggtctctctctctc3’; AML3 548-568
5’gtagcggaggagctgttga3’, AML3 1060-1039rc 5’tctctctgatcctgctata3’
AML4 1932-1955 5’aagcggtagttccctctc3’, AML4 2944-2927rc
5’tccctctgaatccctatctc3’; AML5 104-124 5’cctgCatagctcctgctctctc3’, AML5 1047-
1024rc 5’ctgcaagcttctctctctc3’. Reactions were performed using Takara ExTaq
and 35 cycles of 94°C for 30 sec, 58°C annealing (AML1,2,4) or 60°C annealing
(AML3,5) for 30 sec, and 72°C for one minute.

Primer pairs amplifying a region 3’ of the insertion site for RT-PCR on AML1,
AML2, AML3 and AML5 insertion homozygotes are as follows: AML1 +1635
5’aggctctgcggcctata3’, AML1 -2466 5’ctggctatgctctgctat3’; AML2 +1779
5’acccggttctagtcctatcta3’, AML2 -2107 5’ctgctctgctgtgctaata3’; AML3 +1756
5’tctgctctgctgctgctatgta3’, AML3 -2326 5’cctgctgctgctgctgctgctgta3’; AML5 +1268
5’gacccgctgtgctgctgctgta3’, AML5 -1869 5’acccgctgctgctgctgctgta3’. All reactions
were performed with a 59°C annealing temperature.

RESULTS

AML1 and AtRaptor1B interact in a yeast two-hybrid assay

To test for an interaction between the Arabidopsis proteins AML1 and
AtRaptor1B, the open reading frames (ORFs) of each of the transcripts predicted to
encode these proteins were amplified via PCR with primers carrying suitable
restriction sites for cloning into the yeast two hybrid vectors pGADT7 and pGBK7.
pGADT7 encodes a transcriptional activation domain (AD) which can recruit the yeast
transcription machinery. pGBK7 encodes a DNA binding-domain (BD) which binds
to the promoters of ADE2 and HIS3, genes essential for the synthesis of the purine
adenine and the amino acid histidine. pGADT7 and pGBK7 additionally carry the genes LEU2 and TRP1, essential for the synthesis of leucine and tryptophan, respectively, which can be used to select for successful transformation. When transformed into the yeast line AH109, which is leu2-trp1-ade2-his3-, co-transformed cells will grow on yeast medium lacking leucine and tryptophan. Cells co-transformed with two-hybrid constructs encoding proteins that interact will grow on media additionally lacking histidine and adenine (selective media).

AH109 yeast cells co-transformed with clones encoding AML1 and AtRaptor1B in complementary two-hybrid vectors regained the ability to grow on selective media. Co-transformants of control pGBK7 and pGADT7 empty vectors, or either control vector co-transformed with its complement harboring the AML1 or AtRaptor1B ORF, could not grow on selective media.

Evidence from other systems indicates that Raptor protein fragments lose the ability to bind substrates (Kim et al., 2002). Therefore, only full length AtRaptor1B was tested in this assay. Mei2, the fission yeast AML1 homologue, is highly modular. It is divided into distinct N-terminal and C-terminal domains. The N-terminal half of the protein appears to play a regulatory role (Kitamura et al., 2001). The C-terminal half of Mei2 is sufficient to complement lesions in the mei2 locus (Watanabe et al., 1997). Additionally, the AML1 C-terminal half, expressed in fission yeast meiosis signaling mutants, is able to complement meiosis signaling defects upstream of Mei2 (Hirayama et al., 1997). Therefore we generated clones encoding fragments of AML1 and assayed them for interactions with full length AtRaptor1B.

AML1 fragments N412 and N219, comprised of the first 412 or the first 219 residues of the 915 residue AML1 protein, restored growth on media lacking leucine, tryptophan, histidine and adenine when cloned into pGADT7 and co-transformed with pGBK-Raptor (Fig. 4.1). Neither N163, nor 155-219, (the fragments which together
Figure 4.1. AML1 interacts with AtRaptor1B in a yeast two-hybrid assay. A. Schematic diagram of AML1 fragments cloned into pGADT7. The AML1 RNA Recognition Motifs (RRMs) are indicated. B. Yeast two-hybrid results. Numbers on each plate refer to the pGAD construct in A. For each plate, the set of six cultures on the top half were co-transformed with pGBK:Raptor; the set of six cultures on the bottom half were co-transformed with pGBKTK7 vector. The plate at left lacks leucine, tryptophan, histidine and adenine and selects for a protein-protein interaction. The control plate at right lacks leucine and tryptophan, and indicates co-transformation of the yeast cells with both a pGAD and a pGBK plasmid.
comprise N219), nor 155-412 (which with N163 comprises N412), could restore growth when cloned into pGADT7 and co-transformed with pGBK-Raptor. AML1 fragment 402C, comprised of residues 402 to the C-terminus of the protein, and all C-terminal fragments tested (695C, 402-704) failed to restore growth on selective medium.

AML1 fragments N412 and N163 harbor activation domain activity
AML1 N412, cloned into pGBKT7, restored growth on selective medium to cells co-transformed with either pGAD:Raptor or pGADT7. To investigate this result further, we tested all AML1 fragments for native transcriptional activation domain activity in a yeast one-hybrid assay. AML1 fragments were cloned into pGBKT7, singly transformed into AH109 yeast cells, and assayed for growth on media lacking tryptophan (to confirm transformation) and media additionally lacking adenine. The DNA binding-domain of the pGBKT7 tethers any C-terminally fused fragments to the ADE2 promoter. In the absence of a binding partner, BD-fusion chimeric proteins trigger transcription of ADE2 only if the protein fused to the BD contains native transcriptional activation activity.

AML1 N412 and AML1 N163, but not full length AML1, AML1 N219 or any C-terminal AML1 fragments, were able to restore growth on media lacking tryptophan and adenine (Fig. 4.2). AML1 N122 was similarly unable to restore growth. AML1 fragments 155-219 and 155-412, in pGADT7, could not be stably transformed into yeast.

Plants homozygous for insertion alleles of AML1 and of all five AML family members show early flowering

An interaction with AtRaptor1B points to AML1 as a downstream effector of TOR signaling in plants. Additionally, the dramatic phenotype from mei2 disruption and the intriguing mode of Mei2 action led us to ask what the consequences are of
**Figure 4.2.** The AML1 N-terminus harbors transcriptional activation domain activity. One-hybrid assay on AML1 fragments cloned into pGBK. The plate at left lacks tryptophan and adenine, and growth indicates transcriptional activation domain activity in the tested fragments. The control plate at right lacks adenine, and indicates transformation of the cells with the desired pGBK construct.
disruption of the AML1 locus and of all five AML gene family members. To obtain insertion alleles in AML1 and other AML gene family members, we screened the insertion allele populations at the University of Wisconsin Arabidopsis Knockout facility (Sussman et al., 2000), obtaining alleles harboring insertions in AML1, AML3, AML4 and AML5 (Fig. 4.3). An AML2 insertion allele was obtained from the SIGnAL collection at the Salk Institute (Alonso et al., 2003).

By RT-PCR using primers which anneal to the cDNA at sites spanning the insertion site of each insertion allele, we established that no wild-type transcripts accumulate in homozygous mutants (Fig. 4.4). By a series of crosses, we then generated higher-order insertion homozygotes, culminating in the quintuple insertion homozygotes Q6 and Q17. All lines were viable and fertile.

AML insertion homozygotes bolted earlier than wild-type lines (Fig. 4.5). This effect was independent of the number of insertion alleles carried by the mutants; AML ‘quint’ lines Q6 and Q17 were not qualitatively different than lower order insertion allele homozygotes.

Additionally, AML insertion homozygote seedlings were assayed for a differential response to a range of signaling molecules. Seedlings were germinated on culture medium supplemented with the gibberellic acid GA3, paclobutrazol, the auxin 2,4-D, 1-amino-cyclopropane-1-carboxylic acid, 1% sucrose, 6% sucrose and kinetin, and in the dark. Quintuple insertion mutants responded slightly more than wild-type seedlings to GA3 as measured by the change in seedling length in the presence vs. in the absence of the hormone. This effect was repeatable but weak, and no other differential hormonal response was observed (data not shown).

**Sequence downstream of the mutant allele insertion sites is transcribed**

Given the mild phenotype of the AML insertion homozygotes, we further investigated the extent of the effect of the insertions at each locus. RT-PCR, as
Figure 4.3. *AML* loci and insertion alleles. The position of the insertion in each locus is indicated by a half-triangle below the locus picture. The wild-type loci were described in Chapter 3. The thin central line indicates genomic DNA. Solid blue blocks spanning the central line indicate coding exons. Solid blue blocks below the line indicate untranslated regions. T-DNA insertion left borders are indicated with a half-triangle below the locus. Binding sites for primers used to genotype the wild-type allele of each locus are indicated with arrows. Binding sites for primers used to ascertain the effect of homozygosity at the mutant allele are indicated with arrows having a single hash line if the PCR product spans the insertion site, or a doublet hash line if the PCR product is from a region downstream of the insertion site.
Figure 4.4. *AML* mutant allele characterization. i) PCR product to identify the wild-type allele of each *AML* locus. ii) PCR product to identify the mutant allele of each *AML* locus. iii) PCR product to assay for accumulation of cDNA from transcripts transcribed across the insertion site. iv) PCR product to assay for accumulation of cDNA from transcripts transcribed from the AML locus downstream of the insertion site. AML4 was excluded from this assay because the mutant allele is disrupted in the center of its coding region.
Figure 4.5. *AML* insertion mutants bolt early. A. Wassilewskija (Ws) and AML1 insertion homozygotes (*AML1-/-*) grown under long days. B. Bolting time and number of rosette leaves at time of bolting for AML single insertion and higher order insertion mutants. Plants were grown under 16 hour days.
previously stated, showed that no wild-type transcripts accumulate in lines Q6 and Q17. In fission yeast, however, the C-terminal half of the protein is sufficient to complement lesions of the mei2 locus (Watanabe et al., 1997). The insertion alleles of all but AML4 are disrupted at or near the 5’ ends of their predicted coding regions. We therefore designed primers which anneal to the region downstream of the insertion site in each allele and performed PCR reactions to assay for accumulation of fragments capable of encoding the C-terminal half of any of the AML proteins (Fig. 4.4). To determine whether the amplified fragments corresponded to AML cDNAs, we performed restriction digests on the PCR products, which confirmed that the cDNAs originated from AML gene transcripts. Weak amplification of cDNA representing transcripts originating downstream of the insertion site was observed for AML1, AML2 and AML3; amplification of the AML5 3’ region was indistinguishable from the amplification seen from of wild-type cDNA template.

**Transgenic lines overexpressing AML1:GFP or GFP:AML1 fusion proteins could not be recovered**

The AML1 ORF was separately cloned into the pCambia1302 35S::GFP plant transformation vector both 5’ and 3’ of the GFP ORF, and the construct was transformed into Arabidopsis via Agrobacterium-mediated floral dip (Clough and Bent, 1998). Transformants, identified by resistance to the antibiotic hygromycin and confirmed through PCR, were recovered at a very low rate of less than .01%. No fluorescence was observed in any tissues of any transformants assayed, and AML1:GFP transcripts could not be detected via RT-PCR performed on cDNA transcribed from RNA extracted from bulk shoot tissue (data not shown).
DISCUSSION

Raptor proteins in yeast and mammals function by recruiting substrates for TOR, a central regulator of cell growth in response to nutrients (Hara et al., 2002; Kim et al., 2002; Yonezawa et al., 2004). An interaction with Raptor therefore strongly suggests that a given protein is a TOR substrate and downstream effector of TOR signaling. Plants homozygous for lesions at both \textit{AtRaptor} loci show normal embryonic development but are unable to maintain shoot meristem activity (Chapter 2). TOR substrates, then, may play a role in regulating meristem-driven post-embryonic growth. The interaction between AML1 and AtRaptor1B implicates the AML family of proteins in TOR signaling. It points specifically to a role for the AML proteins in regulation of shoot meristem activity.

An interaction between Mei2 and the fission yeast Raptor homologue Mip1 has been reported previously; indeed, Mip1 (Mei2 interacting protein 1) was the first Raptor homologue characterized in any eukaryote (Shinozaki-Yabana et al., 2000). The conservation of this interaction from fission yeast to plants suggests that the well-characterized Mei2 signaling pathway may provide insight into the function of the AMLs.

Mei2 is a potent signaling molecule which triggers pre-meiotic cell differentiation and meiosis in response to nutrient stress (Watanabe et al., 1988; Watanabe and Yamamoto, 1994). Meiosis signaling in fission yeast is a model for cell differentiation in response to external nutrient cues. Thus the AMLs may also play a role in cellular differentiation or in meiosis signaling.

Aside from the effect of Mei2 in development, there is the intriguing issue of its mode of action. Mei2 sub-cellular localization is mediated by an interaction with a noncoding, mRNA-like molecule (Watanabe and Yamamoto, 1994; Shimada et al., 2003). There is a fairly large population of mRNA-like transcripts conserved among
land plants despite lacking large conserved open reading frames (MacIntosh et al., 2001). Of these, the conserved alfalfa transcript ENOD40 has been shown to mediate the sub-cellular localization of an RNA-binding protein (Campalans et al., 2004) and to mediate phytohormone responses (Dey et al., 2004). AML1 may be a binding partner of one or more of these mRNA-like noncoding molecules in plants.

The transcriptional activation activity of the AML1 N-terminus has not been ascribed to Mei2 and may represent a novel activity of plant Mei2-like proteins. This activation activity localizes to the N-terminal 163 residues, but is strongly influenced by the adjacent residues. Activity is lost in N219, regained on N412 and lost again in full length AML1. This suggests that the AML1 N-terminal half has multiple configurations, and that the accessibility of the activation domain varies among configurations.

AtRaptor1B binding to AML1 is also localized to the N-terminus, and appears to be mediated by multiple sites in this region. This suggests that the N-terminus may contain a TOR phosphorylation site, and that this site may influence the configuration of the N-terminus.

The repeated failure to recover transgenic lines expressing AML1 suggests that its unregulated overexpression is lethal. Future efforts to characterize the AML proteins in transgenic plants may benefit from the use of inducible promoters driving transgene expression to circumvent the putative lethality of unregulated AML expression.

Disruption of any of the AML loci causes early bolting in plants grown under long days. However, lines homozygous for insertions in all five AML loci did not differ dramatically from lower-order insertion homozygotes, despite the fact that RT-PCR reactions performed with primers spanning the insertion sites show that the wild-type transcript does not accumulate.
Transcripts originating downstream of the insertion sites but still capable of encoding the C-terminal half of the wild-type protein accumulate from all loci but AML4. This raises the possibility that the AML quintuple insertion homozygote lines do not represent total disruption of AML activity. Four of the five AML open reading frames in the insertion mutant are apparently truncated and all are divorced from their native promoters, but some promoter activity (perhaps from the 35S viral promoters harbored within the inserted DNA) remains and may be sufficient to cause transcription of AML coding region DNA downstream of the insertion site.

Viewed in this light, the early flowering phenotype of AML insertion homozygotes may arise not from the total disruption of AML activity but from the accumulation of AML proteins which, due to the truncations in their N-termini caused by the insertions, are no longer bound by AtRaptor1B, no longer phosphorylated by AtTOR, or no longer able to activate transcription of floral repressors.

Finally, these results provide a cautionary tale. RT-PCR performed using primers which span an insertion site may not be sufficient to conclude that all activity of a protein of interest is abolished.

TOR is a major regulator of cell growth in eukaryotes, but little is known about its downstream effectors in plants. This work shows that AML1 binds AtRaptor1B, and suggests that the AML protein family may be phosphorylated by AtTOR in an AtRaptor1B-mediated interaction. The interaction with AtRaptor1B implicates AML1 as a downstream effector of AtTOR kinase signaling, and provides insight into the mode of action of this critical growth regulator.


Choi J, Chen J, Schreiber SL, Clardi J (1996) Structure of the FKBP12-rapamycin

complex interacting with the binding domain of human FRAP. Science 273: 239-242


Hirayama T, Ishida C, Kuromori T, Obata S, Shimoda C, Yamamoto M, Shinozaki K,


rapamycin sensitive, have distinct roles in cell growth control. Mol Cell 10: 457-468


Lorković ZJ, Barta A (2002) Genome analysis: RNA recognition motif (RRM) and K homology (KH) domain RNA-binding proteins from the flowering plant Arabidopsis thaliana. Nucleic Acids Research 30: 623-635


McLeod M, Beach D (1988) A specific inhibitor of the ran1+ protein kinase regulates
entry into meiosis in *Schizosaccharomyces pombe*. Nature 332: 509-514


van Hoof A, Kastenmeyer JP, Tatlor CB, Green PJ (1997) GUT15 cDNAs from Tobacco (Accession No. U84972) and Arabidopsis (Accession No. U84973) Correspond to Transcripts with Unusual Metabolism and a Short Conserved Open Reading Frame. Plant Physiology 113: 1004


Yonezawa K, Tokunaga C, Oshiro N, Yoshino K (2004) Raptor, a binding partner of
target of rapamycin. Biochem Biophys Res Commun 313: 437-441


