

ABSTRACT

TOR, target of rapamycin, is an atypical kinase, which controls the regulatory network in yeast and animal cells to regulate their growth in response to environmental cues. In response to nutrients, starvation, and hormones, TOR protein regulates ribosome biogenesis, and translation and transcription of a subset of genes involved in translational machinery (Cutler et al., 2001). It has been shown that TOR modulates translation in eukaryotes by phosphorylation of the downstream effectors and thus regulates the abundance of ribosomes and the translation factors (Dennis et al.1999). Integration of energy levels, nutrient sufficiency and stress information to regulate the translational machinery allows TOR to act as a “master regulator” that strikes a balance between nutrient availability, metabolic processes, protein synthesis, and cell growth (Abraham, 2002). Such a balance is crucial for the cell to ensure its survival. TOR is also at the heart of growth signaling pathways with its substrate AtS6K mediating translational regulation by phosphorylation of ribosomal protein S6 (RPS6;Turck et al., 2004). There is another regulatory role of TOR at the transcriptional level; TOR through its interaction with a phosphatase affects the phosphorylation status of some transcriptional factors and thereby changing their localization from cytoplasm to the nucleus (Cutler et al. 2001 and Beck et al. 1999). Under osmotic

stress or nutrient limitation, these transcription factors may shuttle into the nucleus to drive the expression of a set of stress related genes. However, the identification of such transcription factors is yet to be determined in plant cells. It is likely that based on the source of the signal AtS6K in a TOR dependent and TOR independent manner. For example, PDK1 may activate AtS6K under a certain set of signals that are different from that of TOR.

Despite the importance of this pathway in eukaryotes, little is known of the regulation of this central signaling mechanism in plants. This pathway may link various stress signals to the growth signaling pathway optimizing plant growth under a variety of environmental conditions. In this study, we cloned all plant TOR kinase genes from *Arabidopsis thaliana*. In order to study the regulation of TOR kinase pathway in plants, the interaction of FKBP12 and the FRB domain of pTOR in the presence of rapamycin was tested. pFKBP12 does not interact with the pTOR-FRB in a yeast two hybrid and *in vitro* translation experiments. However, the human FKBP12 (hFKBP12) was found to interact with pTOR-FRB domain in a rapamycin-dependent manner. The immunoprecipitated AtS6K was found to be active and phosphorylated the RPS6 in the kinase reactions. Among various treatments tested, osmotic stress was found to inhibit the kinase activity of AtS6K. AtS6K was found to interact with the regulatory TOR associated protein (RapTOR) *in vivo* when both constructs were expressed in tobacco leaves.

TABLE OF CONTENTS

	Page
Abstract.....	ii
Dedication.....	iv
Acknowledgments.....	v
Vita.....	vi
List of Figures.....	xii
List of Tables.....	xv
Abbreviations.....	xvi
Chapters:	
1.1 Identification of TOR proteins.....	1
1.2 Domain structure of TOR proteins.....	7
1.3 Inhibition of TOR through the formation of rapamycin/FKBP12 complex.....	10
1.4 TOR senses and integrates nutrient and growth factor signals.....	11
1.4.1 Nutrient sensing of amino acid and energy signals.....	12
1.4.2 Regulation of TOR kinase by growth factor.....	13
1.5 Translational effectors, S6K and 4E-BP1, coordinated control by TOR	

and PI3K.....	14
1.5.1 TOR signaling to ribosomal protein S6K regulation of ribosome biogenesis	17
1.6 Phosphatases and their TOR dependent regulation	23
1.7 Identification of TOR-interacting proteins.....	24
1.7.1 Regulatory associated protein of TOR RapTOR	27
1.7.2 GβL	29
1.8 PDK1.....	31
1.9 TOR-dependent regulation of cellular processes other than translation...	32
1.10 Control of cell growth and cell cycle progression by the TOR and PI3K	38
1.11 Conclusion and perspectives	43
2 Molecular cloning of the plant homologs of TOR kinase pathway genes	44
2.1 Introduction.....	44
2.2 Results and discussion.....	46
2.2.1 Cloning of TOR cDNA from Arabidopsis genome.....	49
2.2.2 Cloning of the Arabidopsis homolog of RapTOR.....	54
2.2.3 Cloning of the Arabidopsis homolog of ribosomal S6 kinase AtS6K.....	58
2.2.4 Cloning of the Arabidopsis cDNA homologs of ribosomal protein S6	62
2.2.5 Cloning of the Arabidopsis homolog of the PDK1	

	protein	65
2.2.6	Cloning of the plant homolog of human FKBP12.....	71
2.2.7	Transient expression in tobacco leaves.....	76
2.3	Materials and methods.....	80
2.3.1	Cloning of an Arabidopsis homologue of TOR kinase protein	80
2.3.2	Cloning and expression of Arabidopsis S6K1	81
2.3.3	Cloning and expression of Arabidopsis ribosomal protein S6.....	82
2.3.4	Cloning of Arabidopsis homologue of FKBP12.....	83
2.3.5	Transient expression in tobacco leaves	84
2.3.6	Plant materials, genomic DNA extraction and PCR screening	86
3	Plant TOR interacts with human FKBP12.....	89
3.1	Introduction.....	89
3.2	Results and discussion	93
3.2.1	Rapamycin inhibits Arabidopsis seed germination only at supraphysiological concentrations.....	93
3.2.2	Demonstration that plant FKBP12 does not interact with Rapamycin.....	93
3.3	Materials and methods.....	101

3.3.1	Cloning of plant homolog of human FKBP12	101
3.3.2	Subcloning of pTOR FRB domain into <i>in vitro</i> transcription and translation vector	101
3.3.3	Purification of recombinant FKBP12	101
3.3.4	Yeast strain and methods	102
3.3.5	Quantitative β -galactosidase assay	103
3.3.6	Transcription and translation of radiolabelled proteins ...	104
3.3.7	FKBP12-Rapamycin binding assays	104
4	AtS6K1 interacts with RapTOR and its activity is regulated by osmotic stress.....	106
4.1	Introduction.....	106
4.2	Results and discussion.....	108
4.3	Materials and methods.....	122
4.3.1	Growth and transformation of Arabidopsis plants	122
4.3.2	Growth and transformation of tobacco BY-2 cells.....	122
4.3.3	Binary vector construction for overexpression	123
4.3.4	Microscopy.....	124
4.3.5	Immunoprecipitation and kinase assay	125
5	TOR-HEAT repeats interact with RapTOR and regulate the AtS6K activity	127
5.1	Introduction.....	127

5.2	Results and discussion.....	130
5.2.1	RapTOR interacts with the HEAT repeats of pTOR.....	130
5.2.2	RapTOR reverses the effect of osmotic stress on AtS6K activity.....	133
5.2.3	Co-overexpression of pTOR-HEAT repeats reverses RapTOR effect on the activity of AtS6K.....	136
5.2.4	The regulation of AtS6K is mediated through TOR not through PDK1.....	139
5.3	Materials and methods.....	144
5.3.1	Agroinfiltration and overexpression.....	144
5.3.2	Immunoprecipitation and kinase assays.....	144
6	Discussion	147
	Bibliography	156

ABBREVIATIONS

aa	amino acids
AMP	Adenosine Mono Phosphate
ATP	Adenosine Tri Phosphate
bp	base pairs
cDNA	complementary DNA
CNBR	Cyanogen bromide
C	degree centigrade
DNA	Deoxyribo Nucleic Acid
DTT	Di Thio Threitol
EDTA	Ethylene Diamine Tetra Acetic Acid
FKBP12	FK506-Binding Protein 12
GST	Glutathione-S-Transferase
IPTG	Isopropyl Thio Galactoside
kb	kilo base
kDa	kilo Dalton
MALDI	Matrix assisted Laser Desorption Ionization
mM	milliMolar
mRNA	messenger RNA
m RNP	messenger Ribo Nucleo Protein complex
MS	Mass Spectrometry
μ M	microMolar

NLS	Nuclear Localization Signal
nM	nanoMolar
ORF	Open Reading Frame
PAGE	Poly Acrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PDK1	Phosphatidylinositol Dependent Kinase 1
PI3K	Phosphatidyl Inositol 3 phosphate Kinase
PKB	Protein Kinase B
PKC	Protein Kinase C
PP2A	Protein Phosphatase
PP4	Protein Phosphatase type 4
PP6	Protein Phosphatase type 6
PTEN	Phosphatase TENSIN homologue
PVP	Poly Vinyl Pyrrolidone
RNA	Ribo Nucleic Acid
rDNA	ribosomal DNA
rRNA	ribosomal RNA
SDS	Sodium Dodecyl Sulfate
Ser	Serine
Tap42	TOR associated Protein 42
TCA	Tri Carboxylic Acid
Thr	Threonine
TOF	Time Of Flight
TOR	Target of Rapamycin

TOS

TOR Signaling

tRNA

transfer RNA