12-2013

Investigation of the Role of TOR and Nutrient Restriction on Lifespan of Arabidopsis thaliana

Debashree Banerjee
Dominican University of California

https://doi.org/10.33015/dominican.edu/2013.bio.08

Investigation of the role of TOR and nutrient restriction on lifespan of *Arabidopsis thaliana*

By

Debashree Banerjee

A thesis presented to the Dominican University of California
in fulfillment of the
thesis requirement for the degree of
Master of Science
In
Biology

San Rafael, California, USA

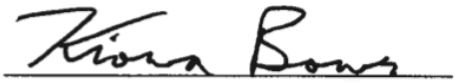
November 2013

CERTIFICATION OF APPROVAL

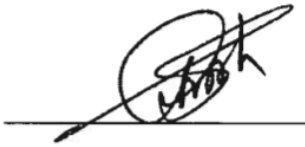
I certify that I have read **Role of Target of Rapamycin (TOR) in lifespan extension under low nutrient conditions in *Arabidopsis thaliana*** by Debashree Banerjee, and I approved this thesis to be submitted in partial fulfillment of the requirements for the degree: Master of Sciences in Biology at Dominican University of California.

Dr. Sibdas Ghosh
Graduate Research Advisor

Dr. LaKisha Barrett
Second Reader



Dr. Kiowa Bower
MS Biology Thesis Coordinator



Dr. Sibdas Ghosh
Graduate Program Director

ABSTRACT

The Target of Rapamycin (TOR) kinase is evolutionarily conserved and is a key regulator, controlling many cellular functions to promote survival and growth in all eukaryotes. Several model organisms have indicated a relationship between TOR signaling and life expectancy, such as yeast, fruit flies and mice. Abundant nutrient availability promotes rapid growth and development, whereas depletion of nutrients reduces the activity of the pathways that are involved in growth and nutrient processing. This reduction in activity increases life expectancy, which is supported by evolutionary theories. The confirmed presence of the TOR homolog (a major regulator of growth and cell proliferation) in *Arabidopsis thaliana* makes this plant an ideal candidate to study for the effects of reduced nutrients in the lifespan extension of plants. To study the role of TOR pathway in lifespan extension in *Arabidopsis thaliana*, mutant plants with reduced and overexpressed TOR activity were grown in hydroponic culture systems, to monitor the effects of varying nutrient conditions and several senescence associated phenotypes (SAP). During this study we found plants with reduced TOR activity displayed delayed flowering, shattering of siliques, and yellowing of leaves as compared to the wild type plants, whereas plants overexpressing TOR activity displayed early SAP. Further preliminary molecular and physiological analysis shed light on the link between the TOR signaling pathway and

nutrient restriction on *Arabidopsis* lifespan. We hypothesize that modulating TOR may slow the aging process in *Arabidopsis,* through downstream processes including mRNA translation, protein synthesis, autophagy, and stress responses. However challenges presented by plants to the general theories of biological aging as it differs in plants in several fundamental ways from the processes in animals. Therefore identifying the mechanisms through which *Arabidopsis* TOR (AtTOR) functions in plants may aid in developing stress resistant strains that could provide tools for improving crop yield and creating long lasting plants such as fruits and vegetables, as well as benefit the floral industry by providing fresh and long lasting flowers.

ACKNOWLEDGEMENTS

It is with immense gratitude that I acknowledge the help of my advisors, Dr. Sibdas Ghosh and Dr. LaKisha Barrett. Without their guidance, assistance, and encouragement throughout the course of this study, this thesis would have been a dream. I thank Dr. Maggie Louie, for her invaluable guidance during the course of my research. I deeply appreciate the efforts of Dr.Chris Endicott for his help, in various ways, during this study.

This thesis is dedicated to my family. It would not be possible without the efforts of my mother Anjana Banerjee, and my mother-in-law Tamasi Bhattacharjee who have always unremittingly supported my academic pursuits. Thank you Shloke, for being the best baby in the whole world. The final, most special thanks goes to my partner and best friend, my husband Sanjit, for giving me unconditional support throughout the duration of this endeavor.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

DR	Dietary restriction
TOR	Target of Rapamycin
AtTOR	*Arabidopsis* TOR
TORC1	TOR complex 1
4E-BP	Eukaryotic initiation factor elf4E binding protein
TORC2	TOR complex 2
mRNA	Messenger ribonucleic acid
tRNA	Transfer RNA
RAPTOR	Regulatory associated protein of TOR
PIKK	Phosphatidylinositol kinase-related kinase
S6K	S6 Kinase
SGK	Serum and glucocorticoid induced kinase
mLST8	Mammalian lethal with sec13 8
PRAS40	Proline rich AKT1 substrate
FKBP12	12-KDa FK506- binding protein
FRB	FKBP rapamycin binding protein
KOG1	Kontroller of growth 1
GβL	Protein G β subunit like
Rictor	RAPTOR independent companion of mTOR1
AVO1	Adheres voraciously to TORC2
AGC	Acylglycerol Kinase
PDK1	3-phosphoinositide dependent protein kinases 1
FATC	FRAP, ATM, TRRAP C terminal
FRAP	FKBP-12-rapamycin associated protein

ATM	Ataxia telangiectasia mutated
PI3K	Phosphatidylinositol 3- Kinase
SnRK1	SNF related kinase 1
PDK1	Phospholipid- dependent Kinase -1
eIF-4E BPs	Eukaryotic initiation factor – 4E binding protein
RPS6	Ribosomal protein S6
AMPK	AMP- activated protein kinase
Snf1	Sucrose non-fermenting 1
AMP	Adenosine mono phosphate
ATP	Adenosine triphosphate
Mbps	Mega base pairs
TORo/e	TOR overexpression
s6k	Reduced S6K expression
tor	Reduced TOR expression
WT	Wild type
Col-0	Columbia ecotype
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
BCA	Bicinchoninic acid
PVDF	Polyvinylidene fluoride
BSA	Bovine serum albumin
TRIS	tris (hydroxymethyl)- aminomethane
HCl	Hydrochloric acid
rpm	Revolutions per minute
d	Day
DAG	Days after germination
PCR	Polymerase chain reaction
kDa	Kilodaltons
EDTA	Ethylenediaminetetraacetic acid

NaCl	Sodium chloride
RT	Room temperature
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
ABA	Abscisic acid
MS	Murashige and skoog
SAM	Senescence associated marker
Sen	Senescence associated genes
DTT	Dithiothreitol
KPO_4	Potassium phosphate
qRT-PCR	Quantitative Real-time PCR
1X	Full strength Hoagland’s solution
0.5X	Half strength Hoagland’s solution
PK	Protein Kinase

1 INTRODUCTION

Growth and aging are mutually exclusive. Growth is a process that uses energy for the synthesis of macromolecules from nutrients and a decrease of entropy, whereas aging is the decay and damage of macromolecules and a rise of entropy. Therefore factors like dietary restriction that delay growth, can also delay the aging process. Dietary restriction can delay growth and increase lifespan in several model organisms ranging from yeast to mice. Dietary restriction can be defined as the reduction of nutrient intake without starvation. This could be applied as an overall reduction of caloric intake, restricting intake of a particular class of nutrient, variations of food intake or a combination of all of these.

1.1 ROLE OF DR IN PROMOTING LONGEVITY

Early studies on the effect of dietary restriction (DR) on lifespan extension were reported in 1935, where it was observed that DR causes stunted body size in rats (*JMcCay CM, et.al 1935*). Since then, several studies have supported the fact that reduced nutrient intake without causing malnutrition promotes lifespan extension and effects cell growth in many organisms (*Guarente,L.et.al. 2005 and Partridge,L et.al. 2002*). This process of lifespan extension by DR is an evolutionarily conserved

process among diverse organisms ranging from yeast to mammals. Studies have also shown that lifespan extension by DR can have beneficial effects on various age related pathologies, such as slowing down the progression of some cancers (*Hart RW and Turturro A, 1997*), neurodegeneration (*Mattson MP, et.al 2001*) and cardiovascular diseases (*Sung MM, et.al 2012*).

One of the underlying mechanisms of DR mediated lifespan extension is the shifting of resources from growth and reproduction towards somatic maintenance, thereby allowing organisms to survive under harsh environmental conditions until suitable reproductive conditions are available (*Holliday,R.1989 & Kirkwood,TL.et.al.2000*). In invertebrates, it has been a subject of detailed studies in the model organisms *Saccharomyces cerevisiae* (*Anderson et al., 2003; Jiang et al., 2000, 2002; Kaeberlein et al., 2004; Lin et al., 2000, 2004; Sinclair, 2005; Guarente, 2005*), in *Caenorhabditis elegans (Houthoofd et al., 2003; Johnson et al., 1990; Klass, 1977; Lakowski and Hekimi, 1998; Walker et al., 2005)* and in *Drosophila melanogaster (Chapman and Partridge, 1996; Chippindale et al., 1993; Partridge et al., 1987)*. Since the biochemical and molecular mechanisms of DR mediated lifespan extension are not fully understood, it has yet to be determined whether lifespan extension by DR in various model organisms occur by similar mechanisms. However lifespan extension due restricted nutrient intake, in all these model organisms, does suggest an evolutionary conservation of some fundamental mechanisms (**fig 1).**

Data gathered from a plethora of studies, have led to the identification of one of the conserved factors involved in DR mediated lifespan extension, the Target of Rapamycin (TOR). TOR is a highly conserved nutrient-sensing pathway among organisms ranging from fungi to humans and has been implicated in the regulation of life span. Several studies in *S.cerevisiae* (*Bonawitz et.al. 2007;Powers et.al., 2006*) *, D.melanogaster (Kapahi et.al., 2004) and C.elegens* (*Chen et.al., 2009; Hansen et.al.,2008*) have further provided evidences of the TOR signaling pathway being linked to DR . Therefore, albeit the underlying principles of DR mediated lifespan extension have not been fully explained, it remains that reduced TOR signaling may be a primary mechanism by which DR extends lifespan.

TOR is a protein kinase that is stimulated by certain nutrients such as Nitrogen, Phosphorus, etc, amino acids, and hormonal signals. TOR coordinates these signals to regulate ribosomal activity, recruitment of mRNA to the ribosome, and other processes via phosphorylating a number of downstream activators (*Schmelzle, T.et.al., 2000*). TOR's ability to phosphorylate its target substrates is mediated by the activity of the conserved eukaryotic protein, Regulatory Associated Protein of TOR (RAPTOR). RAPTOR binds to TOR allowing presentation of the target substrates to TOR for phosphorylation.

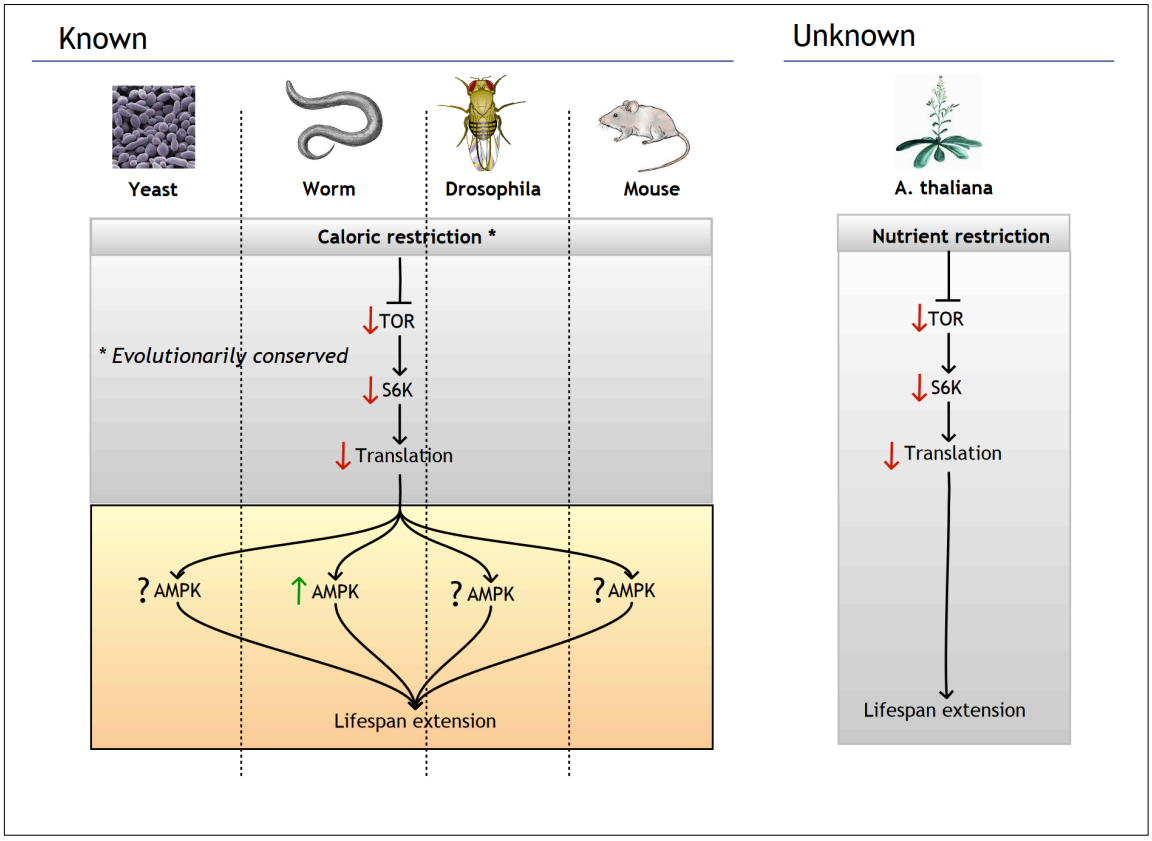


Figure 1: Model of DR mediated lifespan extension in various organisms-

a) Depiction of known and hypothesized lifespan extension mechanisms in Yeast, worms, flies and mice

b) Proposed DR mediated lifespan extension in *A.thaliana*

1.2 TARGET OF RAPAMYCIN (TOR)

TOR belongs to the conserved group of serine/threonine protein kinases from the phosphatidylinositol kinase-related kinase (PIKK) family. The TOR signaling pathway integrates nutrient, amino acids, and hormonal signals to regulate ribosomal activity, recruitment of mRNA to the ribosome and other processes. This regulates cell

growth and metabolism in response to nutrients and growth factors in all eukaryotic cells (*Wullschleger et.al, 2006*). TOR was first identified in Saccharomyces cerevisiae, as the target of the antifungal drug Rapamycin. This kinase is a naturally occurring antifungal, which also acts as an immunosuppressant. Rapamycin was first discovered as a byproduct of the soil bacterium *Streptococcus hygroscopicus* acting to inhibit growth of fungal competitors, from Easter island (Rapa Nui) (*Heitman et.al,.1991*). TOR proteins are known to have two distinct complexes with different functions.

(1) TOR complex 1 (TORC1) is nutrient and Rapamycin sensitive and acts as the central element of the TOR signaling network. TORC1 controls various aspects of cell size, proliferation and lifespan by a number of downstream pathways such as through S6 kinase 1 (S6K1) and the initiation factor 4E- binding protein 1 (4E-BP1) (*Nojima,H.et.al.,2003*). TORC1 consists of the serine/ threonine TOR kinase, RAPTOR (regulatory associated protein of TOR), mLst8 and PRAS40 (*Guertin and Sabatini, 2005, 2009; Wullschleger et.al., 2002*).

(2) The other complex, TOR complex 2 (TORC2) is nutrient and Rapamycin insensitive and is responsible for intracellular regulation such as activities of serum and glucocorticoid induced kinases (SGK) (*Alessi et.al., 2009*).

The inactivation of the TORC1 protein by Rapamycin is due to the formation of a ternary complex where Rapamycin binds with peptidyl-prolyl isomerase 12-kDa

FK506- binding protein (FKBP12) and the FKBP-rapamycin binding domain (FRB) of TOR proteins to form a toxic complex that mimics stress signals (*Choi.J.et.al.1996*), thereby inhibiting TOR activity. However some data also suggests that Rapamycin inhibits the TOR kinase function by dissociating the TOR regulatory protein, RAPTOR from TOR (*Oshiro et.al., 2004)*. Interaction with RAPTOR, is necessary for presentation of the target substrates via formation of a complex with TOR (*Hara et al., 2002; Kim et al., 2002; Nojima et al., 2003)*. In *Saccharomyces cerevisiae*, TOR is activated under favorable nutrient conditions to promote cell growth by maintaining a robust rate of ribosome biogenesis, translation initiation and nutrient import (*Wullschleger,S et.al, 2006*). In mammals, the TOR signaling pathway operates as the central part of the signal transduction network that controls metabolism, growth and differentiation by the several signals induced by different nutrients, energy, stress and hormones (*Zoncu, R.et.al.,2011;Inoki, K.et.al.,2003*). Spcifically, TOR modulates the key component of the translational machinery, including the ribosomal protein S6 Kinase (S6K) and the translation initiation factor 4E-binding protein (4E-BP) (*Ma and Blenis, 2009; Zid et.al., 2009*). S6K and 4E-BP act as positive and a negative regulator of translation respectively and inhibition of S6K extends lifespan in both worms and flies (*Kapahi,P.et.al.2004, Hansen,M.et.al.2007*) whereas inhibition of 4E-BP shortens lifespan in flies (*Tettweiler,G.et.al.2005*). The presence of a TOR homolog in *Arabidopsis* makes it an interesting model organism, to study and compare the functions of TOR. The various aspects of AtTOR are discussed below.

1.3 TOR SIGNALING PATHWAY IN PLANTS

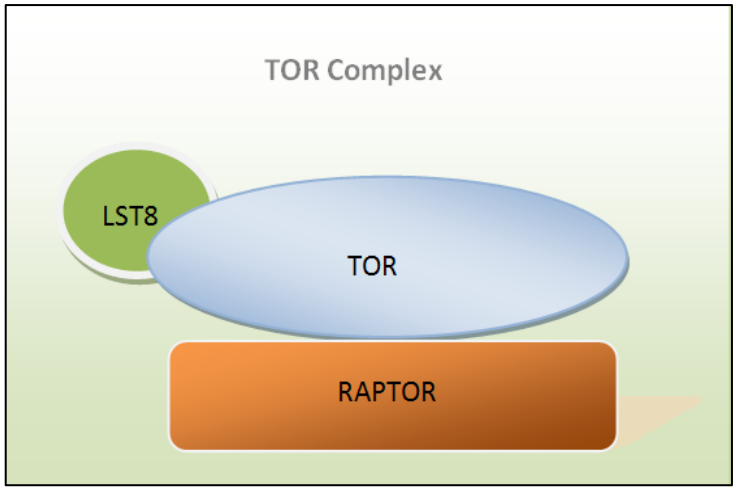
The conserved function of the TOR pathway in the regulation of cell growth in both yeast and mammals suggests that it may function similarly in all eukaryotes, including plants. Growth in plants can be defined as cell proliferation which is coupled with cell division and cell expansion in specialized meristematic zones to produce new tissues and organs. The meristematic activity and the embryonic development depend mostly on the availability of nutrients that is provided by the other parts of the plants, such as the roots and leaves. However, the regulatory mechanisms of the plant TOR pathway have yet to be fully understood. Discovering the molecular and regulatory mechanisms of the TOR signaling pathway in plants including the downstream and upstream regulators has been difficult due to the difficulty of molecular studies on endogenous TOR activity and the embryo lethality in null *Arabidopsis* tor mutants (*Meyer,C et.al., 2011*). Modulation of TOR expression levels suggests a correlation with the cell and organ size, seed yields and stress resistance in *Arabidopsis (Deprost,D.et.al.,2007; Ren, M.et.al.,2012)*. The expression levels of TOR are correlated with the growth of *Arabidopsis*, a reduced or increased expression of TOR gene, results in a decreased or increased cell and organ size, seed production, respectively (*Meyer, C. et.al., 2007*). Therefore it is hypothesized that the TOR pathway may have an important role in the generation of new tissues and organs in plants during embryonic development by sensing nutrients. Moreover, most of the mammalian targets of TOR have been identified in

the *Arabidopsis* genome (*Menand et al., 2002; Deprost et.al., 2005)*, suggesting a possibility of AtTOR's involvement in lifespan in *Arabidopsis thaliana*.

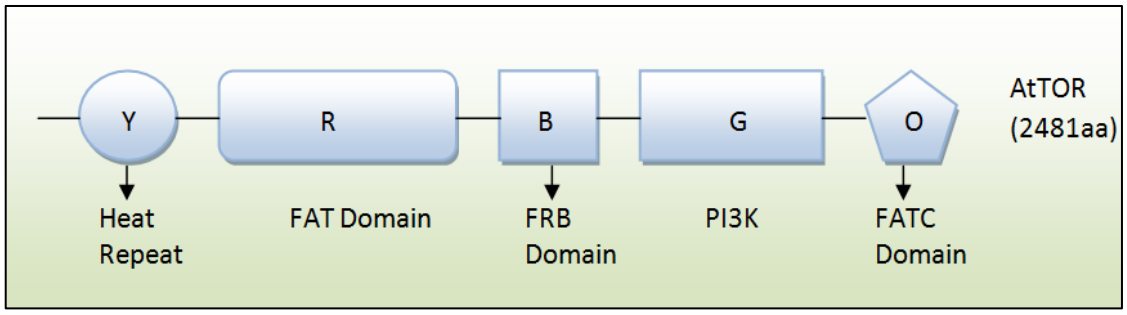
1.4 *Arabidopsis thaliana* TOR

The *Arabidopsis* genome contains a single TOR homolog, AtTOR, present on chromosome 1, It shares features with other eukaryotic TOR genes (*Robaglia, C.et.al.,2002*). The AtTOR, serine/threonine kinase contains 2,481 amino acid residues having a predicted molecular mass of 280 kDa and is a member of the phosphatidylinositol kinase-related kinase family. In *Arabidopsis*, experimental evidence revealed that AtTOR is constitutively expressed in actively dividing tissues such as endosperm, embryo, roots, and in primary meristems (*Robaglia,C. et.al., 2002*). This sheds light on the strategies plants may use to grow. Several components of the TOR signaling pathway have been found in *Arabidopsis*. Most of the conserved components of the TORC1 complex are found in plants as well such as (**Fig.2**) RAPTOR (*Anderson et.al.,2005; Deprost et.al.,2005*) and mLST8/GβL homologues (*Menand,B. et.al.2002*). Recent studies of the *Arabidopsis* TOR interaction partner RAPTOR and one of its downstream effector TAP46 suggests that they play vital roles in growth and development, stress resistance and autophagy (*Meyer,C.et.al.,2005; Hanson,M.R.et.al., 2005; Ahn,C.S.et.al., 2011*) . The TORC1 specific protein Raptor/KOG1 (Regulatory-associated protein of mTOR/ Kontroller of growth 1) is encoded in two copies on chromosome 3 and 5:

AtRaptor1A and AtRaptor1B, known as Raptor2 and Raptor1 respectively (*Verma, D.P.et.al., 2006*). Two binding proteins of the TOR complex, Raptor and GβL, have orthologues in plants (*Templeton & Moorhead, 2004*). Arabidopsis also encodes two LST8/Gβ1 (Lethal with Sec13 8/Protein G β subunit like) homologues, where the LST8 proteins are comprised of seven WD-40 repeats that mediate the protein–protein interactions and provide stability to the TOR complex (*Andrade,M.A.et.al.,1995*). This might function as a scaffold linking TOR to substrates such as S6K, whereas Rictor/AVO1–3 seems not to be present in plants (*Loewith, R.et.al., 2002)*. Furthermore there is also evidence that the TOR substrates and regulators such as S6K or other AGC kinases and 3-Phosphoinositide dependent protein kinases1 (PDK1), are also conserved in plants (*Deak,M. et.al., 1999*). However the TORC2- specific proteins have not been identified in any plant sequences. Despite having TORC1 which is rapamycin sensitive in other eukaryotes, plants are believed to be resistant to rapamycin. This is because plants are deficient in the FK506-binding domain12 (FKP12) and thus it fails to form a TOR inhibitory complex with rapamycin (*Xu,Q. et.al. 1998*), thus remaining insensitive to Rapamycin. Transgenic *Arabidopsis* lines, expressing FKBP12 from S.cerevisiae, have been created to overcome this problem. These bind with Rapamycin, making Arabidopsis sensitive to Rapamycin (*Robaglia,C. et.al., 2007*). Since, most of the components of the TOR signaling pathway are present in plants, the dynamics of the AtTOR signaling pathway and its link to longevity can be better understood now.



A



B

Figure 2: Components of the plant TORC1 complex (*Adapted from Robaglia, C.et.al., 2012*)

Animals	Yeast	Arabidopsis
mTOR	TOR1/TOR2	AtTOR
RAPTOR	KOG1	RAPTOR3g
		RAPTOR5g
mLST8/GβL	LST8	LST8-1
		LST8-2
TAP42/α4	TAP42	TAP46
TIP41	TIP41	AtTIP41
S6K	SCH9	S6K2/ATPK19
		S6K1/ATPK6
AMPKα subunit	SNF1	SnRK1α1/KIN10
		SnRK1α2/KIN11

Table 1: Comparison of genes among animals, yeast and Arabidopsis, involved in the TOR pathway (Adapted from Current Opinion in Plant Biology 2012, Sensing nutrient and energy status by SnRK1 and TOR kinases)

1.5 *ARABIDOPSIS* TOR SIGNALING PATHWAY

Experimental reports suggest the presence of a functional TOR signaling pathway in *Arabidopsis* and all the necessary components of the TOR pathway have homologues in *Arabidopsis* (*Menand,B.et.al.,2002*). The most well explained

downstream targets of TOR are the regulators of protein biosynthesis, S6K1 (protein S6 kinase 1) and 4E-BP1 (eukaryotic initiation factor 4E (eIF-4E) binding protein 1). With the depletion of the TORC1 downstream effectors S6K and eIF4E, lifespan extension has been observed (*Kapahi,P. et.al 2004; Hansen,M. et.al 2007*). When TOR is activated, it leads to the activation of S6K1 and 4E-BP1 by phosphorylation, leading to a higher rate of protein synthesis (*Wullschleger, S.et.al.,2006)*. Although plants lack orthologues of eIF4E-BPs, two plant homologues of the S6Ks, AtS6K1 and AtS6K2 which are highly homologous to the mammalian S6K with a sequence homology of 87%, have been identified (*Mizoguchi,T et.al., 1995*). The two *Arabidopsis* S6K homologs play distinct functions in different subcellular locations; S6K1 is located in the cytoplasm, whereas S6K2 is located in the nucleus, suggesting that S6K2 is the functional equivalent of p85S6K, and S6K1 is the functional equivalent of p70S6K (*Verma,D.P et.al.,2006*), and is able to phosphorylate the ribosomal S6 protein (RPS6) (*Zhang,S.H.et.al.,1994; Turck,F.et.al.,1998*), which is the key component of the 40S ribosomal subunit, integrating t-RNA recruitment to mRNA and translation initiation factors, and thus regulating translation of mRNAs *(Ruvinsky, I.et.al.,2006)*. An in vivo assay showed that RAPTOR1B interacts with S6K1 to present it to TOR as a substrate. The *Arabidopsis* genes AtS6K1 and AtS6K2 encode proteins that share about 87% amino acid sequence similarity with the human S6K1 and S6K2 (*Zhang, S.H.et.al 1994*). Both plant homologues of the S6Ks, AtS6K1 and AtS6K2 are proposed to act as PDK1 targets (*Deak,M.et.al.,1999*) and the PDK1 homolog, that has been identified in *Arabidopsis*, is AtPDK1. Very limited information is available about the

PDK1 signaling pathway in *Arabidopsis* (*Deak,M.et.al.,1999)*. In mammalian cells, the phosphorylation and activation of the S6K by PDK1 promotes the phosphorylation of the downstream target 40S ribosomal protein S6, which in response controls cell growth and proliferation via mRNA translation (*Otterhag, L.et.al.,2006*). The key mammalian phosphorylation sites for the activation of S6K are located at Threonine-229, Serine-371 and Threonine-389, are conserved in AtS6K1 and AtS6K2 (*Turck, F.et.al.,1998*). This confirms that phospho-specific anti-S6K1-T389-P antibodies are able to recognize *Arabidopsis* phosphorylated S6K1 in a Rapamycin- sensitive manner. This strongly provides evidences for the presence of a functional TOR kinase-signaling pathway in plants.

The essence of life is cell growth and proliferation, which is mediated by the availability of nutrients and energy from growth factors. This process requires metabolic checkpoints either halting or permitting cell growth when nutrients and energy are in limiting or are in abundance, respectively. In yeast and animal cells, the conserved TOR (Target of Rapamycin) and AMPK/Snf1 (AMP-regulated kinase/Sucrose non fermenting 1) signaling pathways have emerged as a central and crucial components regulating the perception and the responses to nutrients (sugars and amino-acids) and energy levels (*Ashrafi et al., 2000; Baena- Gonzalez, E.et.al., 2007; Hong and Carlson, 2007; Thelender, M. et.al.,2004*) . This regulatory role of AMPK in growth and metabolic regulations is widely conserved across eukaryotes. AMPK orthologs in plants KIN10/KIN11 plays a key role in response to nutrient deprivation *Baena- Gonzalez, E.et.al., 2007)*. The AMPK phosphorylation

sites are found in the *Arabidopsis* ortholog of RAPTOR. Arabidopsis KIN10/11 are collectively designated as SnRK1s and are the orthologs of the yeast SNF1 (Sucrose non-fermenting 1) and mammalian AMPK (AMP-activated PK), all members of a highly conserved PK family. The SnRK1 protein shares approximately 47% amino acid sequence identity with both SNF1 and AMPKα. SnRK1 plays a central role in coordinating energy balance and nutrient metabolism in plants Furthermore, new studies have now cleared that AMPK and TOR closely interact and primarily act in opposite ways, mainly by phosphorylation and through the activation of negative regulators, by several growth factors, in the regulation of nutrient-driven processes like autophagy (*Inoki, K.et.al.,2003*). AMPK/Snf1/SnRK1 kinases are activated by a decrease in energy level, sensed by an increase in the AMP/ATP ratio for AMPK, or by starvation (*Hardie, D.G.et.al.,1994)*. Conversely the TOR kinase is activated by favorable and nutrient-replete conditions. Activated Snf1 and AMPK have been linked to extended life span and dietary restriction (*Greer, EL.et.al.2007*).

1.6 POSSIBLE LINK BETWEEN NUTRIENT RESTRICTION AND LONGEVITY IN *Arabidopsis thaliana*

Plants are anatomically very distinct from most other multicellular eukaryotes. They are also different in their metabolic processes. Plants have totipotent stem cells that have the ability to regenerate new organs throughout lifetime. They are sessile in

nature and have an autotrophic lifestyle. They also possess very extensive biosynthetic capabilities and have the ability to adapt to various stress conditions. Hence the determinants for growth and lifespan are anticipated to be different in plants than other multicellular organisms (*Thomas, H., 2002*). Therefore the common functional regulator, the TOR pathway, serves as the ideal candidate for determining the possible role on lifespan extension studies in *Arabidopsis thaliana*. Some recent evidences have shown that TOR is a major regulator of growth and size in *Arabidopsis* and that several components of the TOR signaling pathway in *Arabidopsis* serves as ideal candidates for operating the link between nutritional sensing and the regulation of growth (*Menand, B.et.al., 2002*). Also the importance of TOR in embryo development makes it an ideal candidate for drawing parallels between the metabolic regulation of plant senescence and extension of life span by dietary restriction in heterotrophic organisms. It has been observed that the *Arabidopsis* mutants, lacking the TOR pathway components such as S6K, LST8, TAP46 and RPS6, were embryo lethal at an early developmental stage (*Creff,A.et.al.,2010; Henriques, R.et.al.,2010; Ahn,C.S.et.al., 2011; Moreau, M.et.al., 2012)*. A major part of the available nutrients and energy in proliferating cells is taken over by protein synthesis and ribosome biogenesis, that effects growth and longevity (*Byrne, M.E.et.al., 2009*; *Lempiainen, H.et.al., 2009)*. The TOR signaling pathway plays a vital role in coupling the available energy and nutrients for protein and ribosome biogenesis (*Wullschleger, S.et.al., 2006)*. During nutrient restricted conditions, the TOR signaling pathway is inhibited and it leads to rapid down-regulation of protein synthesis and ribosome biogenesis in diverse eukaryotes (*Li,*

H.et.al.,2006), and also negatively regulates autophagy in yeast and mammals (*Noda,T.et.al.,1998; Pattingre, S.et.al., 2008*) . Recent experimental evidences suggest that TOR signaling pathway also negatively regulates autophagy in *Arabidopsis* (*Bassham, D.C.et.al., 2010*). The downstream target of the mammalian TOR is S6K, that regulates growth and lifespan in mice (*Pende, M.et.al.,2004; Holz, M.K.et.al.,2005; Selmen, C.et.al.,2009*) and RPS6 in Yeast regulates cell size, growth and lifespan (*Fabrizio, P.et.al.,2001; Chiocchetti, A.et.al., 2007)* , which is suggestive of a strong link to the TOR signaling pathway. However, a direct link between TOR and S6K in plants, has not been clearly established, but the fact that *Arabidopsis* mutants lacking S6K, are embryo lethal at an early stage (*Creff,A.et.al.,2010; Henriques, R.et.al.,2010; Ahn,C.S.et.al., 2011; Moreau, M.et.al., 2012),* is suggestive of a link between the TOR pathway and S6K. In a proposed mechanism described in **Figure 3**, TOR activity is reduced by restricted nutrient conditions, which thereby induces a reduced AtS6K1 activity by reduced phosphorylation. A reduced AtS6K1 phosphorylation results in low levels of ribosomal protein S6 (RPS6), which thereby results in increased activation of SnRK1/AMPK, which eventually results in longevity. A reduction of TOR activity may increase autophagy, which in turn may aid in lifespan extension in *Arabidopsis.* Since downregulation of mTOR has been shown to promote lifespan extension in many model organisms (*Kapahi, p.et.al.,2010*), it is likely that same mechanisms may also act in *Arabidopsis*. Ribosomal protein S6 activation probably requires a complex array of separate concurrent phosphorylation of multiple sites catalyzed by several protein kinases, including PDK1 and TOR. These upstream kinases are

themselves activated by an array of growth factors including hormones and nutrients. The information related to the regulation of the TOR signaling pathway by AMPK/snf1/SnRK1 is at an early stage and therefore this may help in providing a likely mechanism through which SnRK1 could influence *Arabidopsis* lifespan under restricted nutrient conditions.

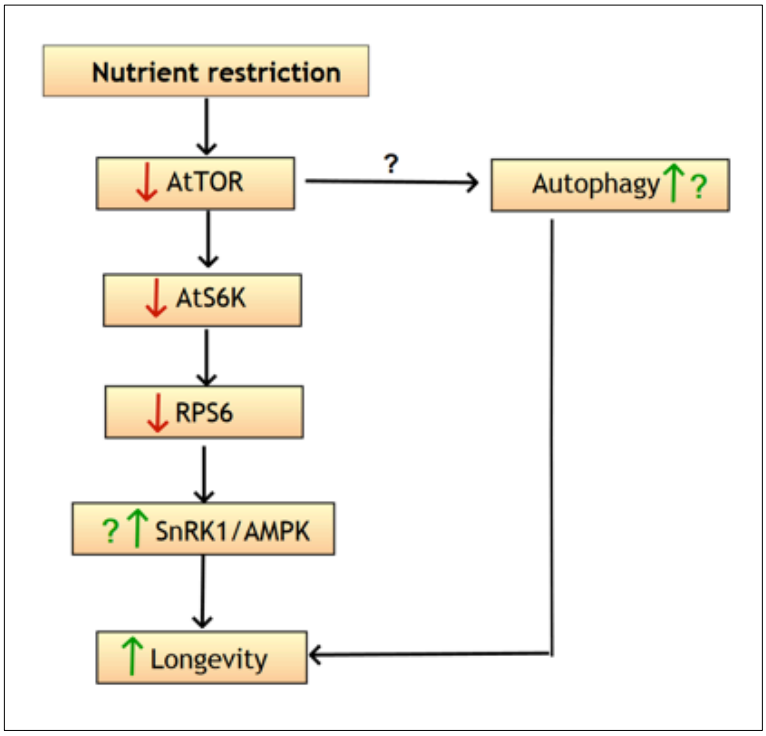


Figure 3: A possible link between nutrient restriction and Lifespan extension in *A.thaliana*

1.7 IMPLICATION OF AMPK ON LIFESPAN

Studies are still in progress to deduce the implication of AMPK activity in DR mediated lifespan extension. Apparently the AMPK- activated protein kinase has emerged as a key nutrient and energy sensor. AMPK is activated upon an increase in the AMP-to-ATP ratio, which reflects the energy status of the cell (*Canto, C.et.al.,2010*). The role of AMPK is to sense the energy stresses and act as a master regulator of mitochondrial biogenesis and metabolism (*Canto, C.et.al., 2011*). There are ongoing studies on the role of AMPK in the beneficial effects of DR. There is evidence that reduced nutrient intake reduces TOR signaling and increases AMPK activity, both of which increase the lifespan of model organisms (*Greer, EL.et.al.2007)*. There have been reports of increased AMPK activity upon reduced TOR mediated by DR in worms (*C. elegens*) (*Apfeld, J.et.al.,2004*), and similar studies in other organisms are still in progress.

Yeast: Snf1 is the mammalian AMPK homologue in yeast. In yeast the function of snf1 is very complex as both disruption and overexpression of Snf1 gene has been linked to reduced lifespan. (*Ashrafi, K.et.al.,2000; Lin, S.S.et.al., 2003; Lorenz, D.R.et.al., 2009).*

Drosophila: The early stages of the experimental data provide evidence, that AMPK is linked to the control of lifespan in some way but the mechanisms are still being tested (*Lee, J.H.et.al., 2007; Tohyama, D.et.al.,2010; Johnson, E.C.et.al.,2010).*

Mammals: Implications of AMPK on lifespan are still being studied.

1.8 *Arabidopsis thaliana* AS A MODEL ORGANISM AND THE IMPLICATIONS OF UNDERSTANDING THE LONGEVITY MECHANISMS IN PLANTS

Arabidopsis thaliana is a widely used model organism for studies involving plant biology. It is a small flowering plant in the mustard family. It has a short lifespan where the entire life cycle, from seed germination, development of rosette plant, bolting of the stem, flowering and maturation of seeds is all completed in 6 weeks. Its entire genome has been sequenced, and it has a genome size of 157Mbps and consists of only 5 chromosomes. Because of it's small size, it is very easily maintained and grown in smaller spaces, making it an excellent model to use to understand the relationship between nutrient restriction and longevity in plants. *Arabidopsis* plants can be grown in petri dishes and then maintained on either pots or in hydroponic culture systems under fluorescent light systems in the laboratory.

The specific nutritional capacities of plants, such as carbon autotrophy, make them attractive models for the comparative molecular genetics of nutrient regulations. A number of studies on how DR extends lifespan have been done on animal models and in invertebrates, and it has been assumed that extension of lifespan with nutrient restriction is a conserved phenomenon across many species. But it is still

unknown if restricting nutrients would also mediate similar lifespan extension in plants. Dissecting the control mechanisms of growth and longevity in plants has implications of considerable biological interest. Identifying the molecular mechanisms through which *Arabidopsis* TOR (AtTOR) works in plants may have several important applications, including the ones listed below

(1) Developing stress resistant plants that remain fresh for longer periods of time

(2) Providing a framework for addressing key components and regulators of lifespan in plants

(3) Opportunities to create long lasting horticultural plants

(4) Provide avenues to induce early maturation of plants during short seasons

(5) Overall improvement of crop yield

(6) Cost effective

2 OBJECTIVES AND HYPOTHESIS

Plant growth is intricately linked to nutrient availability. Nutrients and stress have an impact on growth rate, organ production, and developmental transitions. The conserved nutrient sensing TOR pathway is a prime study candidate to better understand the relationship between nutrient signaling and on lifespan in plants. Thus based on previous studies my hypothesis is

“*The reduction of nutrients increases lifespan in Arabidopsis thaliana by partially down regulating AtTOR.*”

The aims of the following study are:

i) Determine if restricting nutrients promotes longevity in *Arabidopsis thaliana*

ii) Determine how low nutrients effect the developmental growth stages of the tor and s6k mutant lines

iii) Determine how nutrient reduction effects TOR, S6K and Snf1 expression levels

3 MATERIALS AND METHODS

3.1 GROWTH OF PLANTS AND ACQUIRING MUTANTS

Arabidopsis thaliana plants were grown in a growth chamber at 21°C to 22°C for 16h day light and 8h dark, at a light intensity of 125umol photons $m^{-1}s^{-1}$ using fluorescent bulbs and maintaining 70% relative humidity in hydroponic culture systems.

3.1.1 PLANTS USED IN THIS STUDY

1) Wild type plants are of the Columbia (Col-0) ecotype, acquired from Lehle seeds.

2) T-DNA insertion mutant lines in Columbia background. TOR- low expression mutant (tor, SALK_007654), TOR- overexpression mutant (TORo/e, CS332607) and S6K-low expression (s6k, CS836868) obtained from the Arabidopsis resource center (ABRC).

3) RNAi-TOR null mutant was kindly donated by Dr. Diane C. Bassham.

3.1.2 HYDROPONIC CULTURE OF ARABIDOPSIS PLANTS

In order to observe the influence of nutrient restriction on plant development and lifespan, a hydroponic growth system was chosen. Transparent containers 2L, (24x14x5 cm), with lids were wrapped in aluminum foil.12 holes were drilled on the lids for seed holders. The seed holders were prepared using 1.5ml eppendorf tubes with the tips cut off from the bottom, sealed with parafilm at the top and filled with molten sterile 1% bactoagar medium.

Procedure:

The 1.5ml eppendorf tubes with molten agar were left to solidify for 30 minutes. An *Arabidopsis* seed, which was previously surface sterilized was then placed on the surface of the solidified agar medium in each seed holder. All the seed holders containing the *Arabidopsis* seeds were placed on an eppendorf tube rack and wrapped with saran to maintain moisture. The seed holders were held at 4°C for four days for vernalization, to break dormancy and improve synchronous seed germination.

Hoagland's solution (*Hoagland, DR.,1950)* was used for growing plants (described in Tocquin, P.et.al.2003, see **Table 2**). Concentrations were as follows: 2X, 1X, 0.5X and 0.25X. Following this the growth chambers were covered with lids. Cold -treated seed holders were inserted into the holes on the lid and then to the containers filled with liquid.

The containers containing the seed holders were then transferred to the growth chambers maintaining a relative temperature of 21^{o}C to 22^{o}C, 16h daylight and 8h dark, at a light intensity of 125umol photons $m^{-1}s^{-1}$, using fluorescent bulbs and maintaining 70% relative humidity. Most seeds germinated and grew into small seedlings with 2 cotyledons and 2 small true leaves within one week. The old Hoagland's solution was replaced once every two weeks, for the initial four weeks and then replaced on a weekly basis. The level of the nutrient solution was continuously checked to make sure the seed holder maintains contact with the liquid.

Component	Stock solution	mL of Stock solution/L
Macronutrients		
1M Ca(NO3)2.4H2O	236.1g/L	7
1M KNO3	101.1g/L	5
1M KH2PO4	136.1g/L	2
1M MgSO4.7H2O	246.5g/L	2
Trace elements (make up to 1L)		
H3BO3	2.8g	
MnCl2.4H2O	1.8g	
ZnSO4.7H2O	0.2g	1
CuSO4.5H2O	0.1g	
NaMoO4	0.025g	
FeEDTA (Make 1L)		
EDTA 2Na	10.4g	
FeSO4.7H2O	7.8g	1
KOH	56.1g	

Table 2: Chemical composition for Hoagland's nutrient solution

3.1.3 SURFACE STERILIZATION OF SEEDS

Seeds of *Arabidopsis thaliana* were surface sterilized in a sterile hood. Approximately 40 seeds were surface sterilized at a time in a 1.5ml eppendorf tube, serving as a reaction vial, by adding 1ml of sterilization solution (20% bleach + 30% TritonX-100 {dilution 1:1000}) and 10-minute incubation, with occasional rocking. The sterilization solution was removed and seeds were washed five times with 1ml of sterile distilled water, all steps occurred under sterile conditions.

3.2 PROTEIN ISOLATION AND DETERMINATION OF PROTEIN CONTENT

3.2.1 PROTEIN ISOLATION FROM *ARABIDOPSIS THALIANA* FOR SDS-PAGE AND WESTERN BLOT ANALYSIS

~1g or more of 28 d old bud tissues were used and all the extraction procedures were performed on ice. Approximately 2ml of extraction buffer [100mM 2M KPO4 (pH 7.8), 1mM 0.5M EDTA, 1% Triton X-100, 10% of 80% Glycerol, 1mM 1.0M DTT], per ~1g tissue, was used to grind the tissues until no large pieces of plant tissue

are visible, using a mortar and pestle. ~1ml of the liquid was removed into a microfuge tube and placed on ice. Samples were then spun at 12000 rpm in a microfuge at 4°C for 15 minutes. The liquid supernatant was stored at -80°C after being transferred to 1.5 ml eppendorf tubes for each sample.

3.2.2 PROTEIN MEASUREMENTS (BCA ASSAY)

Total protein content of plant tissues was measured using Bicinchoninic acid (BCA) assay (*Smith, P.K, et.al., 1985*). The supernatant from protein isolation step is diluted with the same extraction buffer and three replicates were measured for protein concentration. 10 µl of 1:20 diluted supernatant was mixed with 200 µl of the BCA reagent, incubated at 37°C for 30 min and measured photometrically at 562 nm on a plate reader (Bio-rad). Correspondingly a BSA series (2.0-0.125 mg/ml), diluted in the same extraction buffer, was measured at the same time and a standard curve was generated. Absorption values were calculated against the calibration line and absolute protein levels per fresh weight were determined

3.3 WESTERN BLOT ANALYSIS

3.3.1 SDS-PAGE protein analysis:

24µl of samples were taken and added to 6µl 5x SDS gel loading buffer (Laemmli buffer) to denature proteins (*Laemmli, 1970*). The samples were boiled for 5 min at 100°C. A total of 30µg protein was used. 10% Polyacrylamide gels were used with the Mini-PROTEAN 3 cell system (BioRad) according to the manufacturer's instructions with a running buffer consisting of 25 mM Tris, 192 mM glycine and 0.1% SDS (w/v). Electrophoretical separation was performed at 120V. Proteins in polyacrylamide gel were detected by Coomassie Blue staining.

3.3.2 PROTEIN TRASFER

Prior to their detection, proteins were transferred from the polyacrylamide matrix to a membrane based on Towbin et al. (1979). Protein transfer occurred in a wet blot chamber (Trans Blot Cell, BioRad) onto a PVDF membrane (Immun-Blot, Bio-rad) according to the manufacturer's instructions using 4°C chilled transfer buffer (20% methanol [v/v], 10 mM Tris, 77 mM glycine, 0.1% SDS [w/v]). Proteins were transferred at 90V for 100 min at 4°C.

Following transfer the membranes were washed 3 times with 1x TBST (150 mM NaCl, 10 mM Tris; pH 8.0, supplemented with 0.05% Tween-20 [v/v]) each for 5 min, followed by blocking the membranes with 5% BSA in 1x TBST for 1 hr at RT. Primary antibody (monoclonal mouse anti-HA 1:1000 and monoclonal mouse anti-RGSH6 1:1000 [Santa Cruz Biotechnology, Santa Cruz, CA, USA]) in 5% blocking solution (BSA) over night at 4°C. After washing the membranes with 1x TBST, incubated with secondary antibody (goat anti-mouse 1:5000 [Santa Cruz Biotechnology, Santa Cruz, CA, USA]) in 5% blocking solution (BSA) for 1 h at RT.. Visualization of detected proteins were done via chemiluminescence with ECL Plus Reagent (Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer's instructions. Phospho-p70 S6K (p-Thr389) polyclonal antibody (Cell signaling, cat # 9205) was used to detect the TOR PK phosphorylation of p-T449 in S6K1. Polyclonal antibody (Cell signaling Cat # 9202) was used for the detection of endogeneous levels of total S6K protein. Phospho- AMPKα (Threonine 172) rabbit monoclonal antibody (Cell signaling, Cat # 2535) was used for the detection of phosphorylated AMPK and polyclonal antibody (Cell signaling Cat # 2532) was used to detect total AMPK.

3.4 QUANTITATIVE PCR ANALYSIS

3.4.1 SAMPLING STRATEGY

For most experiments, *Arabidopsis* plants were grown in hydroponics and several plant tissues harvested on Day 24. Tissues from plants were combined and considered as one biological sample. The tissues were flash frozen placed in liquid nitrogen. At least 3 biological replicates were used for each data point.

3.4.2 RNA ISOLATION AND cDNA SYNTHESIS

Total RNA was isolated using the RNeasy plant mini kit protocol (Qiagen), and traces of DNA were removed with DNA-free kit (Ambion). RNA concentration was then determined by absorbance at 260 nm, using Nanodrop 2000 (Thermo Scientific) and all the samples were used at 1 µg/µl. 1 µg of RNA template was used to make cDNA with the Super Script first-strand cDNA synthesis kit (Invitrogen).

3.4.3 REAL-TIME PCR

Reactions were set up in a 96-well plate on ice as follows:

cDNA	1 µl
Primer F/R (100 µM)	0.4 µl
SYBR Green (2x)	0.4 µl
H2O	3.6 µl
	10 µl

Plate sealed with film (Biorad). The gene levels were detected using the ICycler (Biorad). For detecting a specific gene level, that particular corresponding primer was used, **Table 3**.

Gene (accession number)	Primer name	Primer sequence
AtTOR (At1g50030)	AtFKBP12 F	TCT CAG TTG CTG GAA GGT TAT C
	AtFKBP12 R	CTC TTC CCT GCA CTT GTT ATC T
AtS6K1 (At3g08730)	AtS6K1 F	AGC GGA GCA GAG GAG ATA AA
	AtS6K1 R	ATG CAT TGC CTT CCC GAT AC
Housekeeping gene (At5g15710)	HG F	GCT AAG AAT GGA GAC GAA GAG AG
	HG R	CGA CCC AAC TCG ACC TAA AG

Table 3: Primers used for Real time Quantitative PCR

4 RESULTS

In this study, our aim was to investigate how modulation of AtTOR activity via nutrient restriction would affect lifespan in *Arabidopsis thaliana.* To measure lifespan in *A.thaliana*, we used senescence markers such a decline in chlorophyll content, flowering time, the appearance of yellow leaves and silique shattering.

Growth stage definitions for crop plants and weeds were developed by BASF, Bayer, Ciba-Geigy, Hoechst (BBCH), providing a generic nomenclature for the assignment of various growth stages in plants. This BBCH scale was later used by Douglas C. Boyes et.al 2001, as a basis to generate a series of growth stages for the plate-based and the soil-based, phenotypic analysis in *Arabidopsis thaliana* (**fig.4**) **(table 4**). This study used a hydroponic growth system previously established and it has been demonstrated that both soil and hydroponic based conditions provide the same growth rates throughout the vegetative growth cycle (3-7 weeks) on (*Gilliham, M. et.al., 2013)*. Thus my further studies were based on these growth stages.

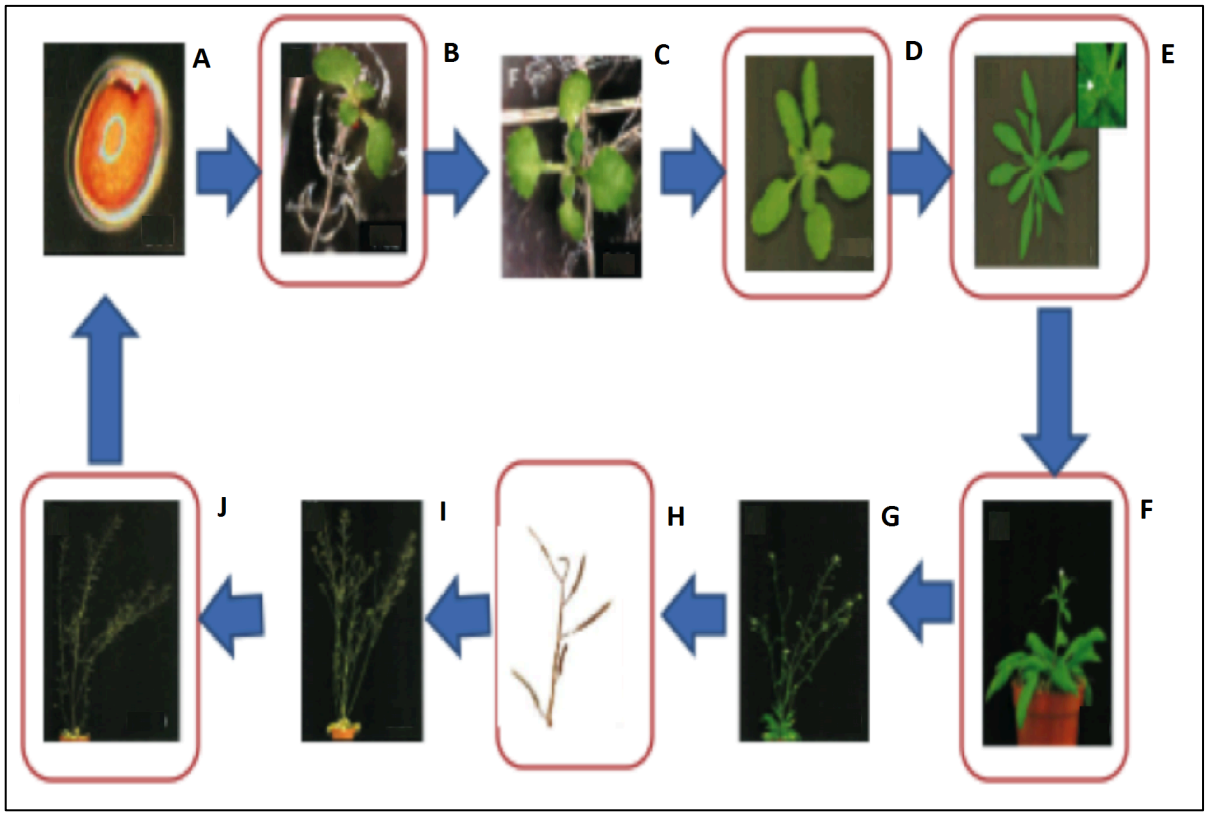


Figure 4: Various growth stages of *Arabidopsis thaliana* (*Adapted from Boyes DC.e.al.,2001).* (A) 0.1, imbibition (B) 1.02, Two rosette leaves (C) 1.04, Four rosette leaves (D) 1.10, Ten rosette leaves (E) 5.10, First flower buds visible (F) 6, First flower open (G) 6.50, Midflowering (H) Silique shattering (I) 6.90, Flowering complete (J) 9.70, Senescence and ready to harvest seeds.

Stage	Approximate number of days	Description
0		Seed germination
0.1	3 (on agar)	Seed imbibition
0.5	4.3 (on agar)	Radicle emergence
0.7	5.5 (on agar)	Hypocotyle and cotyledon emergence
1	6 (on agar)	Rosette growth
1.02	10.3	2 rosette leaves
1.03	14.4	3 rosette leaves
1.04	16.5	4 rosette leaves
1.05	17.7	5 rosette leaves
1.06	18.4	6 rosette leaves
1.07	19.4	7 rosette leaves
1.08	20	8 rosette leaves
1.09	21.1	9 rosette leaves
1.1	21.6	10 rosette leaves
1.11	22.2	11 rosette leaves
1.12	23.3	12 rosette leaves
1.13	24.8	13 rosette leaves
1.14	25.5	14 rosette leaves
3		Rosette growth
3.2	18.9	Rosette 20% of final size
3.5	24	Rosette 50% of final size
3.7	27.4	Rosette 70% of final size
3.9	29.3	Rosette growth is complete
5		Inflorescence emergence
5.1	26	1st flower buds visible
6	31.8	Flower production
6.1	35.9	10% flowers open
6.3	40.1	30% flowers open
6.5	43.5	50% flowers open
6.9	49.4	Flowering complete
8	48	Silique ripening
9	ND[a]	Whole plant senescence begins
9.7	ND[a]	Senescence complete

Table 4: *Arabidopsis thaliana* growth stages (*adapted from Boyes,DC.et.al.,2001*)

a ND- not determined

4.1 RESTRICTING NUTRIENTS PROMOTE LONGEVITY IN PLANTS

In a preliminary screening, the effects of nutrient restriction only in WT Col 0 ecotype were tested. Plants were grown in 2X, 1X, 0.5X and 0.25X concentrations of Hoagland's solution. The first signs of senescence were observed in plants growing in 0.25X specifically, rapid flowering and leaves turning reddish-brown after 35 days, possibly due to the production of Anthocyanin. Anthocyanin production is a characteristic response of flowering plants to unfavorable environmental conditions (*Chalker-Scott, 1999*). This might be possible due to the stress of persistent low nutrients of the 0.25X concentration. Plants growing in 0.5X characteristically had delayed flowering time, first silique shattering time and the appearance of yellow leaves as compared to the plants growing in 1X nutrient solution. Plants growing in 2X nutrient solution also showed symptoms of early senescence. By 65 d, all the plants growing in 1X nutrient solution had senesced, whereas the rosettes were still green in plants growing in 0.5X nutrient solution, indicating longer life span (**fig 5).** Therefore for my further analysis, only Plants growing in 1X (as control) and 0.5X (as nutrient limiting condition) were considered.

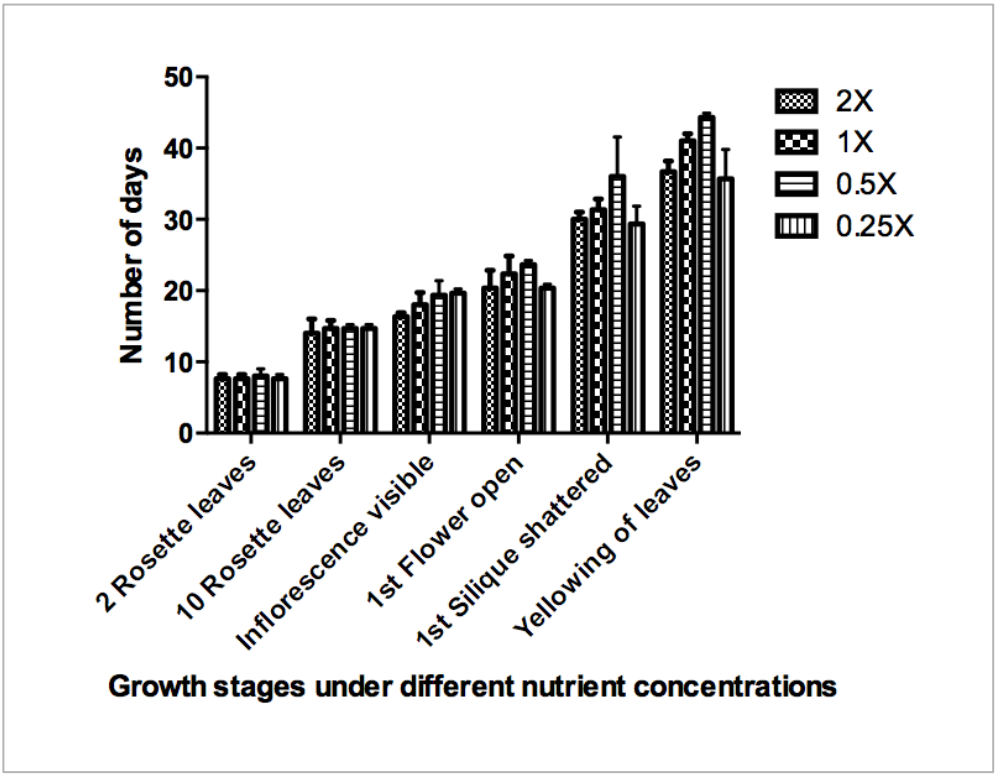
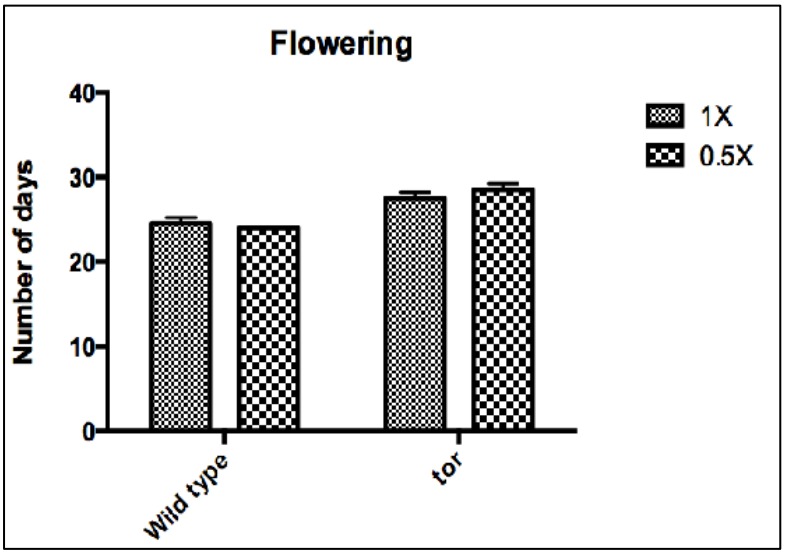


Figure 5: Growth stages of WT plants under various concentrations of Hoagland's nutrient solution. Each bar represents the mean of 3 replicates ±SD.

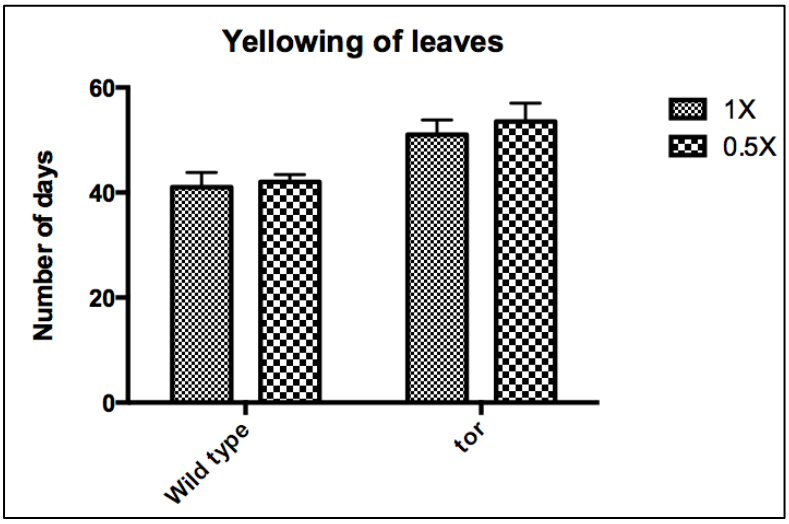
4.1.1 EFFECTS OF NUTRIENT RESTRICTION ON WT, S6K AND TOR LINES

To observe the effects of nutrient restriction on growth of *Arabidopsis*, various growth phase changes were examined in WT, s6k and tor (Reduced S6K and TOR activity, low expression constructs) plants. Growth phases such as flowering time, yellowing of leaves and shattering of siliques were observed. Plants with reduced TOR activity (tor) had delayed flowering time by 4 days as compared to the WT growing in 0.5X concentration. Whereas the tor mutants growing in 0.5X concentration flowered approximately 2 days later than the mutants growing in 1X concentration (**fig.6A)**. Reduced TOR mutants showed a significant delay in the appearance of yellow leaves growing in 0.5X concentration, where it delayed the response by approximately 14 days as compared to the WT growing in 0.5X and by 4 days as compared to the tor mutants growing in 1X (**fig.6B)**. Though not much of a difference in flowering time and yellowing of leaves has been observed in the WT and tor plants growing in 1X and 0.5X concentrations individually, however a delayed response has been shown in silique shattering in the WT growing in 0.5X concentration by approximately 4 days as compared to the plants growing in 1X, however not much difference has been observed in the tor plants (**fig. 6C).** Plants with reduced S6K activity (s6k) characteristically showed delayed flowering time as compared to the WT growing in 0.5X concentration delaying by approximately 3 days , also a slight delay by approximately 1 day has been observed in the flowering

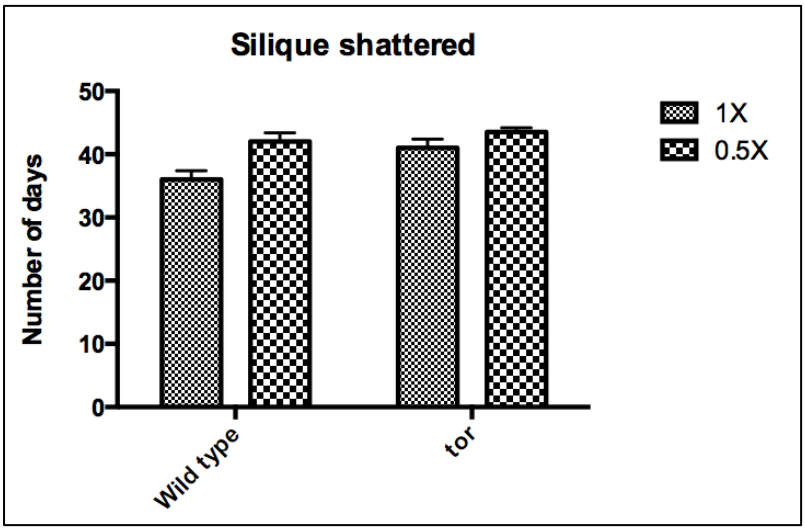
times of plants growing in 0.5X as compared to the ones growing in 1X, for both WT and s6k mutants (**fig.6D**). However not much difference has been observed for the time taken for the appearance of yellow leaves and for silique shattering in WT and s6k mutants growing in both 1X and 0.5X concentrations, the reasons for this behavior is apparently unknown (**fig. 6E & 6F).** By 70 d, the wild type plants had senesced, whereas the s6k and tor rosettes were still green, suggesting sustained growth and longer lifespan.



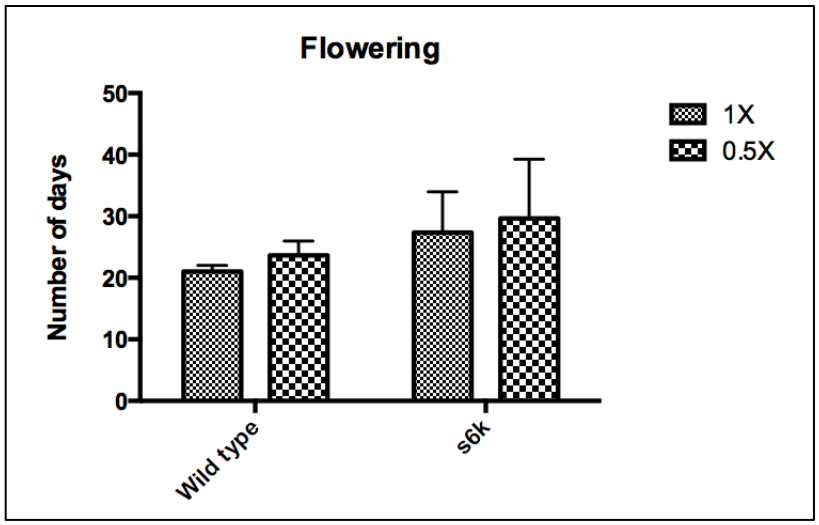
6A



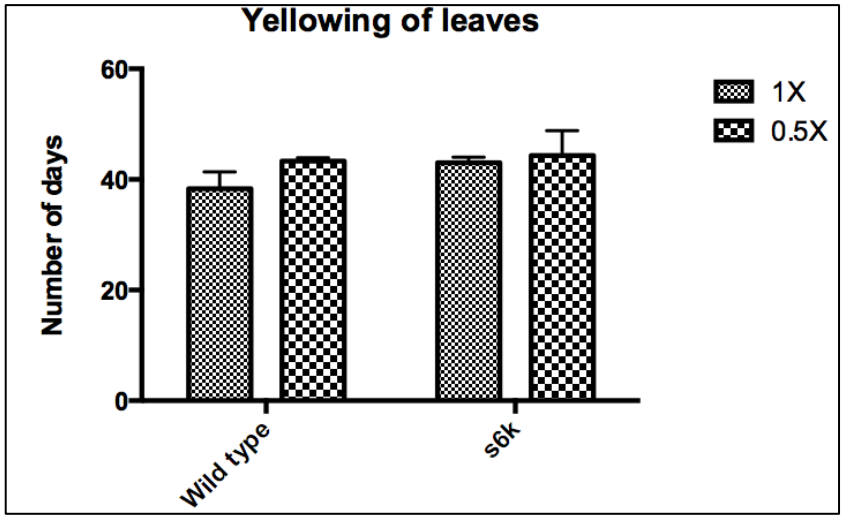
6B


Silique shattered
Number of days
50
40
30
20
10
0
Wild type
tor
1X
0.5X

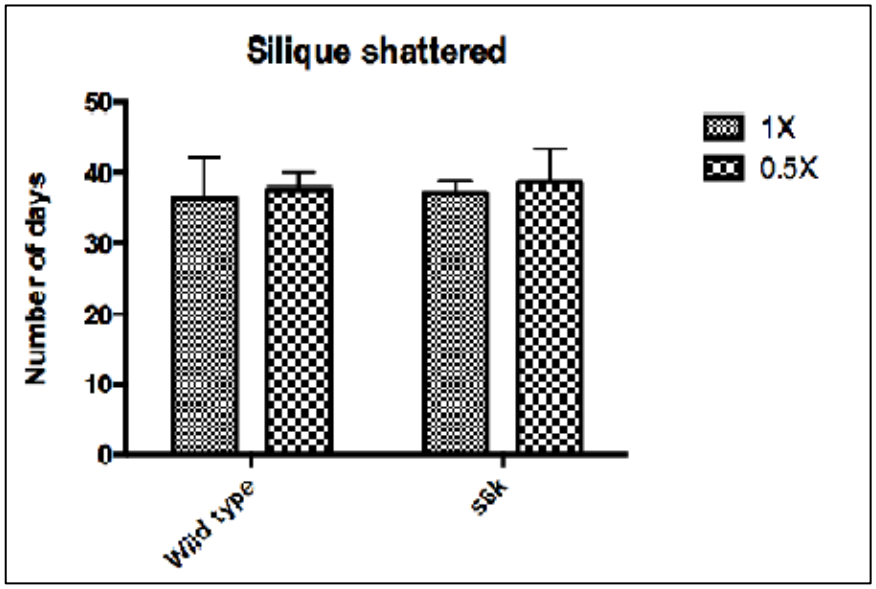
6C


Flowering
Number of days
50
40
30
20
10
0
Wild type
s6k
1X
0.5X

6D


Yellowing of leaves
Number of days
60
40
20
0
Wild type
s6k
1X
0.5X

6E



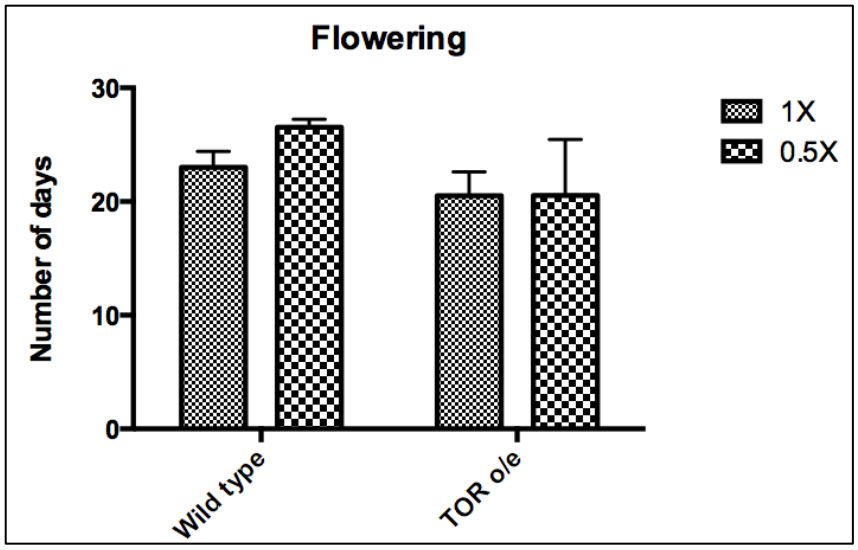
6F

Figure 6: Reduced TOR and S6K expression delays growth and physiological changes. The partial reduction of TOR activity (tor) delays flowering time in 0.5X concentration (A) Delays the appearance of yellow leaves in 0.5X concentration (B) and delays shattering of siliques in 0.5X (C), as compared to WT. The partial reduction of S6K activity (s6k), delays flowering time in 0.5X (D) however not much difference has been observed for yellowing of leaves and for silique shattering (E & F), when compared to WT. Each bar represents the mean of 3 replicates ±SD.

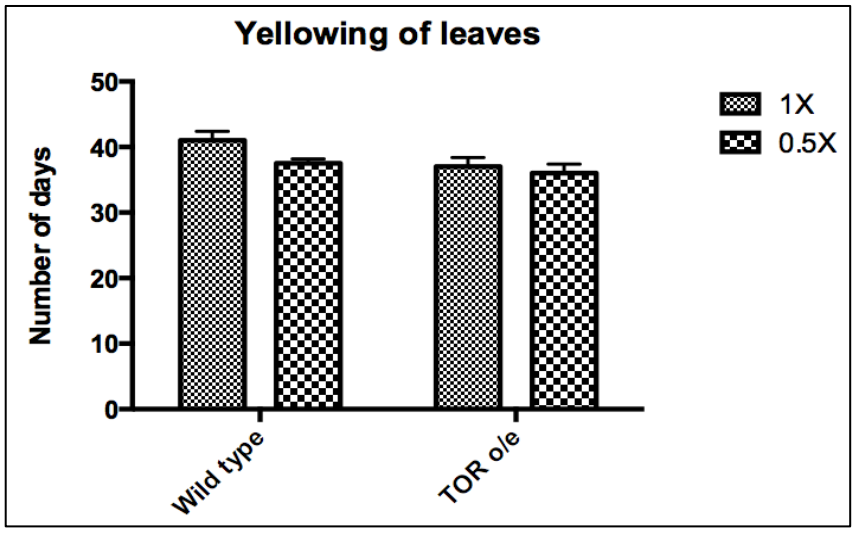
4.1.2 OVEREXPRESSION OF TOR LEADS TO ACCELERATED GROWTH PHASE CHANGE AND EARLY SENESCENCE

Since s6k lines (reduced expression) displayed delayed growth phase transitions in lower nutrient concentrations, we used transgenic lines overexpressing TOR (TORo/e), to analyze the involvement of TOR in response to restricted nutrient conditions on various growth phases. These TOR o/e lines should have increased activity of TOR's downstream targets such as S6K. We hypothesize that increased TOR activity will compensate for the growth delay in nutrient limiting conditions. Upon growing WT and TORo/e lines in 1X and 0.5X nutrient solution, TORo/e lines growing in 1X and 0.5X concentrations, clearly exhibited early flowering approximately by 3 and 4 days as compared to WT growing in 1X and 0.5X respectively (**fig.7A**). TORo/e lines growing in 1X clearly displayed early onset of senescence by developing yellow leaves as compared to WT growing in 1X by approximately 2 days. However TORo/e lines growing in 0.5X barely exhibited early yellowing of leaves as compared to WT growing in 0.5X (**fig. 7B**). Silique shattering was clearly observed early in TORo/e lines growing in 1X and 0.5X by approximately 6 and 2 days as compared to WT growing in 1X and 0.5X respectively. But the time taken to shatter siliques was not much different within the WT and TORo/e plants growing in 1X and 0.5X respectively (**fig. 7C)**. Therefore, comparing the approximate number of days for the SAP for the mutants s6k, tor and TORo/e lines

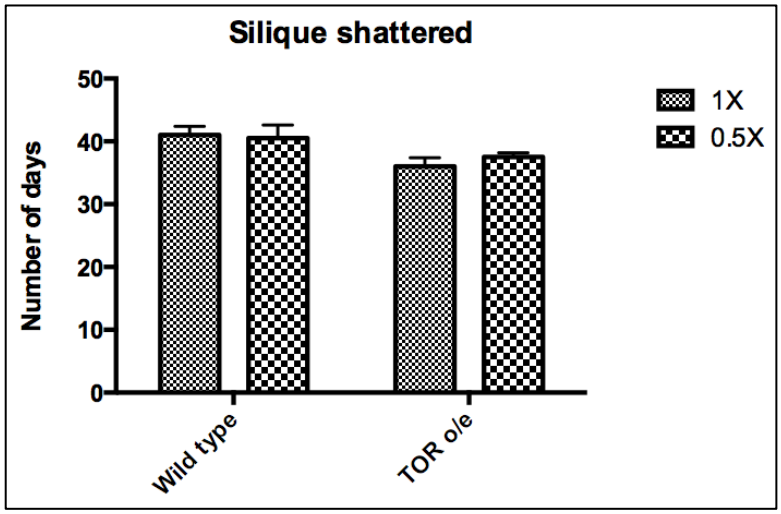
with WT (**),** it could be clearly seen that TORo/e lines displays an early SAP as compared to s6k and tor, which display a delayed SAP when compared to WT.



7A



7B



7C

Figure 7: Overexpression of TOR activity accelerates growth and physiological changes. TORo/e in 1X accelerates flowering time as compared to WT in 1X (A). TORo/e lines displays early appearance of yellow leaves in 1X concentration as compared to WT (B). Shattering of siliques happen early in TORo/e lines in 1X and 0.5X when compared to WT in 1X and 0.5X respectively (C). Each bar represents the mean of 3 replicates ± SD.

	Approximate number of days to					
	Flowering time		Yellowing of leaves		1st silique shattered	
	1X	0.5X	1X	0.5X	1X	0.5X
WT	24	25	39	41	37	40
s6k	28	30	44	46	37	39
tor	27	28	50	54	40	45
TORo/e	21	21	38	37	37	39

Table 5: Summary of the approximate number of days for the appearance of the senescence associated phenotypes (SAP) such as flowering, yellowing of leaves and for shattering of the first siliques for WT, s6k, tor and TORo/e mutant lines. Mean of 3 replicates.

4.2 GENE EXPRESSION UNDER RESTRICTED NUTRIENT CONDITIONS

To understand the molecular mechanisms of nutrient restriction on growth we measured the relative expression of TOR and S6K genes, assessed by qRT-PCR in Wild type, low expression s6k and TORo/e lines. The TORo/e, s6k and WT plants growing in 0.5X nutrient concentration of Hoagland's solution we see an unexpected increase in gene expression as compared to the 1X nutrient solution. This increased gene expression in the lower concentration of nutrient solution can be explained, as the lower concentration of nutrients may induce some stress in the plants growing in it and under stressed conditions, changes in gene expression take place. Changes in gene expression may be regulated directly by the stress conditions or may result from secondary stresses and/or injury responses via a complex series of signal transduction events (*Hanson and Hiltz, 1982*). This pattern has been observed in **figures 8 and 9** respectively.

4.2.1 TOR EXPRESSION LEVELS

Since AtTOR is primarily expressed in the meristematic tissues such as flower buds, root tips and apical meristems. Therefore to measure AtTOR expression, RNA was isolated from the bud tissues from 26 day old plants (approximately mid life) growing in 1X and 0.5X nutrient solutions. Wild type, TORo/e and low S6K expression plants, growing in 0.5X nutrient solution had significantly higher levels of TOR expression as compared to the plants growing in control 1X nutrient solution (**fig.8**). A low TOR expression was observed in tor (reduced TOR0 mutants growing in 0.5X concentration (data not shown). A higher TOR expression in the TORo/e mutants can be clearly observed in the control 1X nutrient solution as compared to the WT and s6k (reduced S6K). The significant increase in TOR gene expression in the lower nutrient concentration in 0.5X can be explained as expression and activity are not always synonymous, and that increased expression doesn't necessarily mean that TOR is actively phosphorylating S6K as our preliminary Western blot results suggest that phosphorylation could be downregulated and thus decreased activation of s6k may lead to delay of senescence. This phenomenon can also be explained as the lower concentration of nutrients may induce some stress in the plants growing in it and under stressed conditions, changes in gene expression take place. Changes in gene expression may be regulated directly by the stress conditions or may result from secondary stresses and/or injury responses via a complex series of signal

transduction events (*Hanson and Hiltz, 1982*). One of the signals involved is abscisic acid (ABA) which is a stress hormone and is regulated during stress (*Bray, EA.,1997*), which has been involved in changes of gene expression during stress conditions. Together this data and the early SAP in TORo/e lines growing in 0.5X, suggest that TOR is definitely involved in responding to low nutrients.

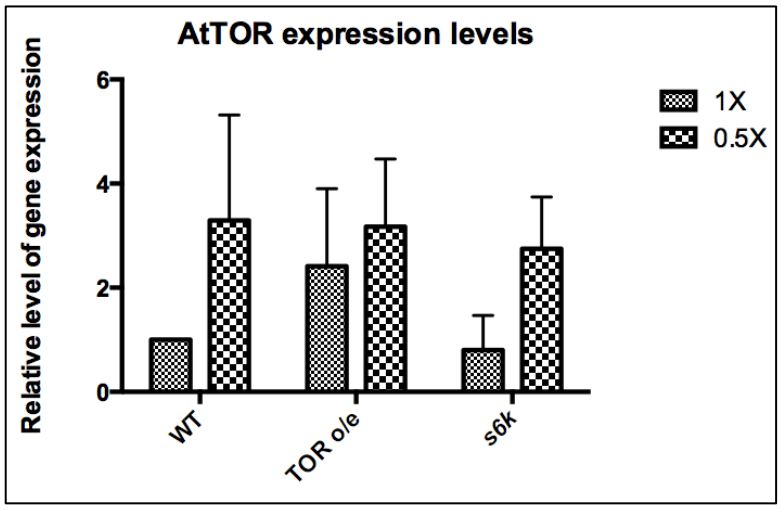


Figure 8: AtTOR expression in *Arabidopsis thaliana* growing in control (1X) and in reduced nutrient concentration (0.5X). Buds expressing AtTOR were harvested at 26 DAG. Each bar represents the mean of 4 replicates ±SD.

4.2.2 S6K EXPRESSION LEVELS

We next measured S6K expression levels. S6K being the direct downstream target of TOR, therefore when TOR is reduced via nutrient restriction S6K activity is reduced via lower phosphorylation. Therefore to assess this, S6K expression levels

were measured in WT, TORo/e and s6k mutant plants. From **figure 9** we can see that AtS6K1 expression levels were significantly higher in TORo/e lines as compared to WT growing in 1X and 0.5X nutrient concentrations. Where as we see that in s6k mutants the expression of S6K is significantly lower than TORo/e and WT growing in 1X and 0.5X nutrient concentrations. The reduced expression of S6K in the s6k mutant compared to the WT and TORo/e can be explained as a low expression mutant so we do not expect no expression as in a null mutant However expression of S6K in 0.5X is higher in WT, TORo/e and s6k mutants as compared to the 1X concentration, in s6k mutants. A higher expression of S6K was also observed in tor mutants growing in 0.5X concentration (data not shown). The higher expression of S6K in s6k mutants growing in 0.5X concentration can be explained, as there is an increased activity of TOR. This confirms the result above that not only is TOR activity increased but also it is truly affecting its downstream targets. Thus TOR effects (on ribosomal activity, cell growth etc.) are responsible for the growth phenotypes. Therefore these s6k results confirm that not only is TOR expression increased (**fig. 8**) but we have increased TOR activity as well (**fig. 9**). A S6K null mutant could not be acquired due to technical difficulties.

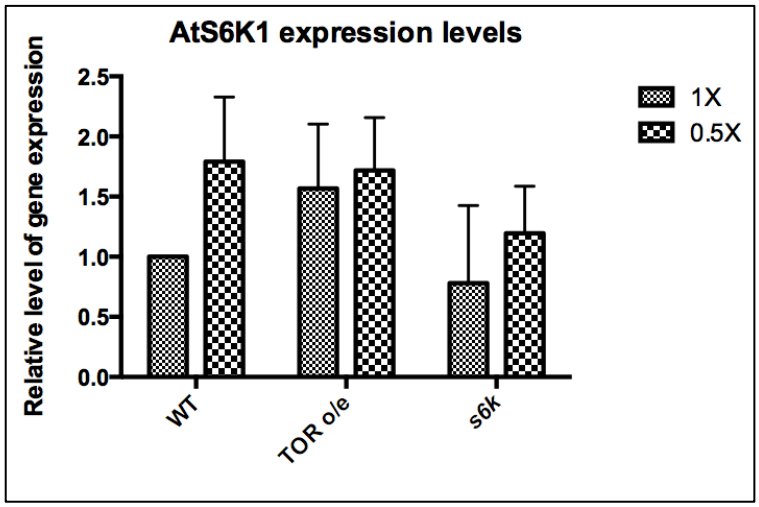


Figure 9: AtS6K expression in *Arabidopsis thaliana* growing in control (1X) and in reduced nutrient concentration (0.5X). Buds expressing AtTOR were harvested at 26 DAG. Each bar represents the mean of 4 replicates ±SD.

4.3 REDUCED NUTRIENTS EFFECTS THE TOR SIGNALING PATHWAY

4.3.1 S6K PHOSPHORYLATION

The gene expression levels led us to believe that TOR activity is upregulated under nutrient limiting conditions, however to confirm kinase activity we wanted to assess phosphorylation of TOR target substrates. A major function of TOR kinase involves the phosphorylation of its downstream target proteins, such as S6K. This target protein S6K is highly conserved in *Arabidopsis* and humans. Interestingly the TOR phosphorylation motif in human S6K1 and *Arabidopsis* S6K homologs is highly conserved (*Turck et al., 1998*). Therefore by using a specific antibody against the phospho T-389-peptide in human S6K1 phosphorylated by TOR, it is possible to monitor the endogenous AtTOR activity based on the phosphorylation status of the conserved S6K, T449 and T455 in *Arabidopsis* S6K1 and S6K2 respectively. Preliminary results show that S6K phosphorylation is higher in the TORo/e line as expected. Due to the low endogenous S6K expression levels we were unable to get a clear picture of what the phosphorylation state is in the other mutants. We can also see that the S6K phosphorylation is the faintest in s6k mutant since it is a low expression construct and there are naturally low levels of S6K in these tissues at this stage (**fig. 10- upper panel**). We can also see that S6K phosphorylation is slightly

reduced in the plants growing in reduced nutrient concentration 0.5X, in the wild type, s6k, tor and the TORo/e lines as compared to the control plants **(fig. 10- upper panel).**

Due to the unavailability of proper antibodies, it was difficult to assess the phosphorylation status of these mutant plants. Creating antibodies for *Arabidopsis thaliana* can circumvent this difficulty.

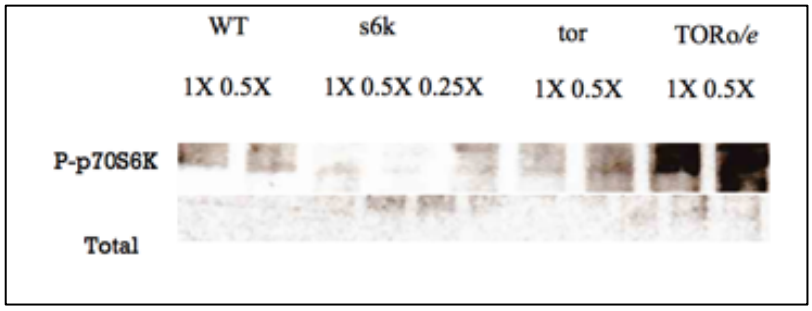


Figure 10: AtTOR activity based on T449 phosphorylation of S6K1. Upper panel represents the Phosphorylated p70S6K where TORo/e shows higher phosphorylation than WT, tor and s6k lines and the lower panel represents the Total S6K.

4.3.2 AMPK PHOSPHORYLATION DURING REDUCED NUTRIENT CONDITIONS

SnRK1/Snf1/AMPK kinases play crucial roles in nutrient and energy sensing. In plants recently it has been demonstrated that TOR and AMPK have opposite roles in the initiation of autophagy in response to limited nutrients (*Kim, J.et.al.,2011).* AMPK is activated upon an increase in the AMP to ATP ratio, which reflects the energy status of the cell. Though sufficient biochemical data is lacking, AMPK has been shown to be a downstream effector of S6K and may mediate a S6K dependent effect on growth and lifespan. We hypothesize that if S6K is reduced under low nutrient conditions via TOR, then AMPK will be activated with this reduced S6K activity.

The ability to sense nutrient stress and act as the master regulator of mitochondrial biogenesis and metabolism, makes AMPK a vital regulator to be studied for its beneficial effects of DR mediated lifespan extension. Therefore to assess AMPK activity, a western blot was performed for Total AMPK and Phospho AMPK, and the preliminary data suggests that AMPK phosphorylation is the faintest in the TORo/e lines (**fig. 11- upper panel),** which supports the findings that AMPK activity increases with a decrease in TOR activity (. Further from **figure 11**, we can see that tor and s6k mutant plants show darker bands as compared to the TORo/e mutants,

suggesting higher phosphorylation of AMPK. We also see that AMPK phosphorylation in WT is reduced when compared to the tor and s6k mutants. Though these findings are in their preliminary stage, these data may suggest a possible link between the TOR and AMPK signaling pathways on lifespan extension processes of *A.thaliana*. Though not very clear, the difference in total AMPK explains the phosphorylation levels seen.

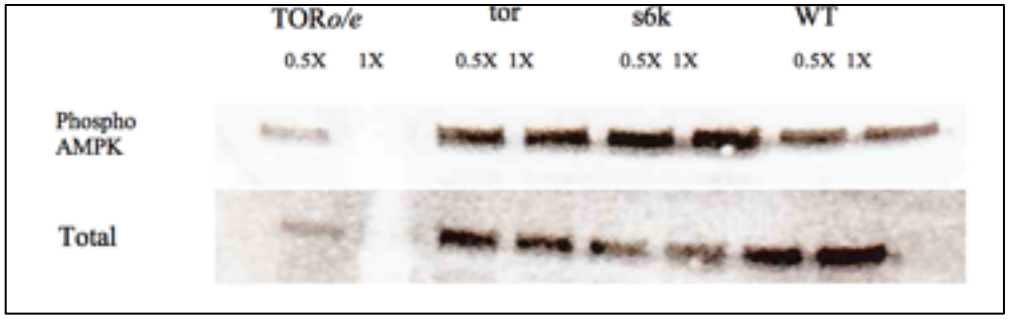


Figure 11: Upper panel represents AMPK/snf1/SnRK1 phosphorylation which shows that AMPK phosphorylation is higher in tor and s6k mutants as compared to the TORo/e and WT plants. Lower panel represents the Total AMPK, the difference in total AMPK explains the phosphorylation levels seen in the upper panel

5 DISCUSSION

Recognition of the TOR signaling pathway as a central integrator of nutrient sensing in all eukaryotes and the link between dietary restriction and the TOR pathway in lifespan extension has stimulated a flurry of research over the past few years. Studies have focused on defining the physiological, biochemical and molecular mechanisms underlying these regulatory functions of TOR kinase in both animals and plants. In plants the TOR signaling pathway is involved in various growth related processes, the mechanisms though, are mostly unknown (*Deprost, D.et.al.,2007; Caldana, C.et.al.,2013)*. As autotrophic organisms, plants are fundamentally different from their heterotrophic counterparts in many different ways such as in terms of growth, development and differentiation. Involvement of TOR in these processes has begun to be unraveled with the studies of TOR signaling in yeast and some animal model systems via developing molecular and genetic tools, and pharmacological experiments using Rapamycin dependent systems to repress TOR activity. Unfortunately such tools are limited in understanding the TOR pathway in plants.

The role of TOR in lifespan extension under limiting nutrient conditions has been studied in *S. cereviceae*, *D.melanogester* and mammals and represents potential for the investigation of the relationship between nutrient limitation and TOR signaling in plants. A recent scientific study suggests the importance of the TOR signaling

pathway in plant growth and longevity, particularly in the development of roots, root hairs, leaf and shoot. The results from this study suggest that growth rate, the various growth related factors and lifespan are regulated by the modulation of the TOR signaling pathways in *Arabidopsis* and also a link between nutrition and the TOR pathway have been suggested. Experimental data collected from this investigation, also suggested that modulation of the TOR levels alters the growth, flowering time and senescence to produce longer or shorter lifespan in *Arabidopsis* (*Ren, M.et.al.,2012).* These findings have provided new insights in this important field of plant TOR signaling pathway and this has helped in understanding the critical TOR pathway.

Preliminary results obtained from the present study with *Arabidopsis thaliana*, suggests that the model organism *A.thaliana*, growing in reduced concentration of Hoagland's solution (*Hoagland, DR.et.al.,1933*) induced early senescence, seen in the senescence associated phenotypic markers such as delayed flowering time, silique shattering and the appearance of yellow leaves. As decreased TOR activity causes a delay in senescence, the transgenic lines with reduced TOR and S6K activity induced a delayed response for the senescence associated phenotype (SAP) as compared to the wild type plants, where as the plant lines overexpressing TOR activity shows an early response for the SAP as compared to the wild type plants. Our preliminary Western blot results suggest that phosphorylation of S6K could be downregulated and thus decreased activation of S6K may lead to delay of senescence. These preliminary results are consistent with the previous observations

where Rapamycin sensitive transgenic lines that are grown in 0.5X MS media supplemented with Rapamycin, displayed delayed flowering time as compared to the wild type plants. In this study due to the lack of Rapamycin sensitive transgenic lines of *A.thaliana*, the reduced nutrient concentrations of the Hoagland's solution may have mimicked the effect of Rapamycin, which downregulates TOR and S6K activities.

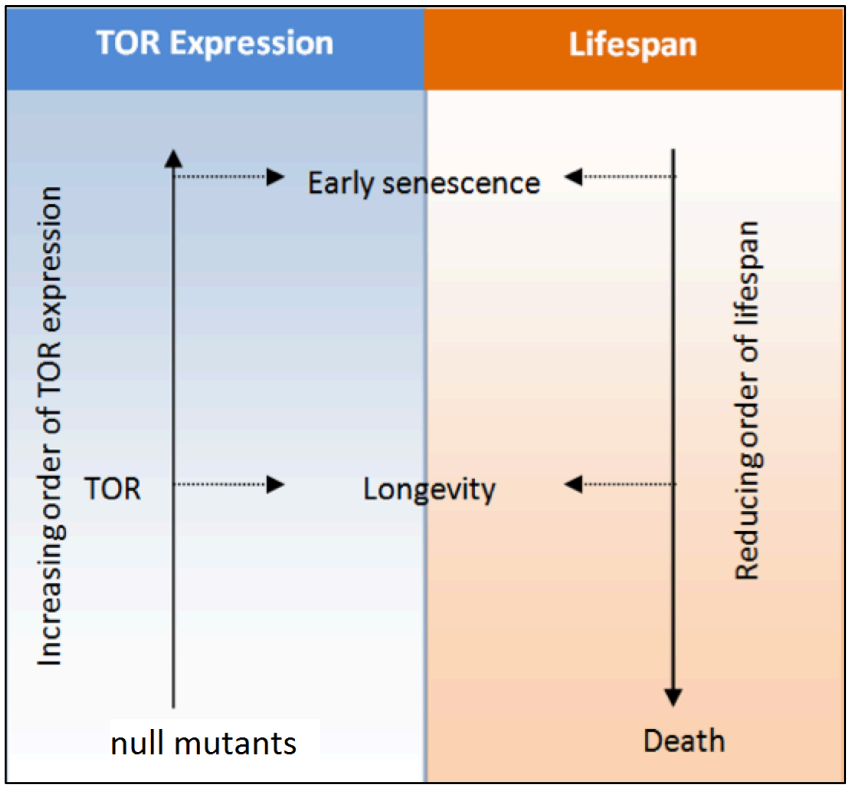


Figure 12: A model to explain the potential link between the TOR signaling pathway and lifespan in *Arabidopsis thaliana*. An increasing order of TOR expression leads to a decreasing order of lifespan, with overexpression of TOR gene leads to early senescence and mutants lacking the TOR gene leads to death. However a reduction of TOR gene, may lead to lifespan extension.

In addition to the effects of reduced nutrients on the senescence-associated markers in various mutant lines of *A.thaliana*, the gene expression levels were analyzed In contrast, both TOR and S6K, which is a direct effector of the TOR signaling pathway, were expressed at higher levels in the reduced nutrient conditions as compared to the control plants. Therefore this theory may provide a rational mechanism by which gene levels are up regulated. In this preliminary data the increased level of gene expression may be explained as an effect derived by the stress induced to the plants due to lower nutrients, resulting in higher expression of TOR genes, however may not be exclusively linked with increased activation of TOR kinase activity.

In this study we attempted to demonstrate the regulation of TOR activity under restricted nutrient conditions, by measuring the phosphorylation status of S6K, which is a direct TORC1 target (*Mahfouz, MM.et.al 2006*) and gene expression levels as well as measuring lifespan. Preliminary results suggest that TOR activity is down regulated since the levels of phosphorylated S6K were similarly down regulated in the bud tissues of *A.thaliana*, which supposedly has the higher levels of TOR gene expression due to its meristematic properties. Reduced nutrient concentration displays a slight reduction of S6K phosphorylation, indicative of reduced TOR activity. We cannot discount that increased expression of TOR might suggest that its activity would likewise be upregulated, however TOR could be stimulating its other targets during nutrient limiting conditions. Future research on the TOR signaling

pathway components, such as studying the phosphorylation status of RPS6 which is a direct downstream target of S6K (*Chung, J.et.al., 1992; Price, DJ.et.al., 1992; Turck, F.et.al , 1998; Dufner, A.et.al., 1999)*, this can be more clearly explained along with the phosphorylation of S6K by TOR kinase. Therefore, to summarize the results based on this study shows that with upregulating expression of TOR gene, lifespan decreases in *Arabidopsis*. We found that plants overexpressing TOR gene display early senescence associated phenotypes and previous studies have shown that null TOR mutants have embryo lethality (*Meyer, C.et.al.,2011),* These findings suggest that plants need reduced TOR activity to show a delay in the senescence associated phenotypes. We have found that nutrient restriction, without starvation decreases TOR activity and increases lifespan. We present a working model of TOR's role based on the data available from this study and published data (**fig.12**).

Based on our preliminary Western blots we cannot conclusively state that AMPK activation is reduced in the s6K mutant. However it is still highly plausible that AtSnRK1/AMPK has a role in DR mediated lifespan extension in *Arabidopsis*. Current studies in other eukaryotes support this theory. In this study the preliminary data suggest a link between the DR mediated lifespan extension, delayed senescence and down regulation of TOR activity. A better understanding of the role of TOR could potentially lead to research resulting in the engineering of crops to increase yield and better responses to nutrient stress conditions.

FUTURE RESEARCH

The goal of this study was to analyze the role of TOR kinase activity on longevity under reduced nutrient concentrations. We used Western blotting to determine phosphorylation levels of S6K, a direct downstream regulator of TOR. However better techniques are required to visualize these transient phosphorylation events. It would also be advantageous to measure expression levels of *A.thaliana* senescence associated genes (sen1), to have an additional marker for senescence (*Oh SA,et.al., 1996)*. Testing the effects of low nutrient conditions on sen1 would provide a strong correlation between senescence nutrient availability and TOR kinase activity. Another important question to address is the role of autophagy. As of date, autophagy is thought to be negatively regulated by TOR kinase and reduction/inhibition of TOR will likely result in increased autophagy enabling recycling of cellular nutrients (*Bassham,DC.et.al., 2012*). Therefore determining the expression levels of the autophagy associated genes under reduced nutrient conditions may shed light on the relationship between TOR and DR mediated lifespan effects. These future experiments will add to the broad picture of the role of TOR kinase in lifespan extension in the model plant *A. thaliana*.

6 REFERENCES

Andrade MA, Bork, P (1995) HEAT repeats in the Huntington's disease protein

Ashrafi K, Lin SS, Manchester JK, Gordon JI (2000) Sip2p and its partner snf1p kinase affect aging in S. cerevisiae

Alessi DR, Kozlowski MT, Weng QP, Avruch J (**1998)** 3-phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates and activates the p70 S6 kinase *in vivo* and *in vitro*

Ahn CS, Han JA, Lee HS, Lee S, Pai HS (2011) The PP2A regulatory subunit Tap46, a component of the TOR signaling pathway, modulates growth and metabolism in plants

Apfeld J, O'Connor G, McDonagh T, DiStefano PS, Curtis R (2004) The AMP-activated protein kinase AAK-2 links energy levels and insulin like signals to lifespan in C. elegans

Baena-Gonzalez E, Rolland F, Thevelein JM, Sheen J (2007) A central integrator of transcription networks in plant stress and energy signaling

Buchanan-Wollaston V, Page T, Harrison E, Breeze E, Lim PO, Nam HG, Lin JF, Wu SH, Swidzinski J, Ishizaki K, Leaver CJ (2005) Comparative transcriptome analysis reveals significant differences in gene expression and signaling pathways between developmental and dark/starvation-induced senescence in *Arabidopsis*

Bray EA (1997) Plant responses to water deficit

Bjedov I, Toivonen JM, Kerr F, Slack C, Jacobson J, Foley A, Partridge L (2010) Mechanisms of life span extension by rapamycin in the fruit fly *Drosophila melanogaster*

Boyes DC, Zayed AM, Ascenzi R, McCaskill AJ, Hoffman NE, Davis KR, Gorlach J (2001) Growth stage –based phenotypic analysis of *Arabidopsis*

Bonawitz ND, Chatenay-Lapointe M, Pan Y, Shadel GS (2007) Reduced TOR signaling extends chronological life span via increased respiration and upregulation of mitochondrial gene expression.

Corton JM, Gillespie JG, Hardie DG (1994) Role of the AMP-activated protein kinase in the cellular stress response

Creff A, Sormani R, Desnos T (2010) The two *Arabidopsis* RPS6 genes, encoding for cytoplasmic ribosomal proteins S6, are functionally equivalent

Choi G, Yi H, Lee J, Kwon YK, Soh MS, Shin B, Luka Z, Hahn TR, Song PS (1999) Phytochrome signalling is mediated through nucleoside diphosphate kinase 2

Chen EJ, Kaiser CA (2003) LST8 negatively regulates amino acid biosynthesis as a component of the TOR pathway

Chalker-Scott L (2008) Environmental significance of Anthocyanins in plant responses

Chung J, Kuo CJ, Crabtree GR, Blenis J (1992) Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases

Cheng M-C, Liao P-M, Kuo W-W, Lin T-P (2013) The *Arabidopsis* Ethylene response factor 1 regulates abiotic stress-responsive gene expression by binding to

different cis-acting elements in response to different stress signals

Chiocchetti A, Zhou J, Zhu H, Karl T, Haubenreisser O, Rinnerthaler M, Heeren G, Oender K, Bauer J, Hintner H, Breitenbach M, Breitenbach-Koller L (2007) Ribosomal protein Rpl10 and Rps6 are potent regulators of yeast replicative lifespan

Chippindale AK, Leroi AM, Kim SB, Rose MR (1993) Phenotypic plasticity and selection in Drosophila life-history evolution. I. Nutrition and the cost of reproduction.

Caldana C,Li Y, Leisse A, Zhang Y, Bartholomaeus L, Fernie AR, Willmitzer L, Giavalisco P (2013) Systemic analysis of inducible target of rapamycin mutants reveal a general metabolic switch controlling growth in *Arabidopsis thaliana*

Carles Cantó, Johan Auwerx (2011) Calorie restriction: is AMPK as a key sensor and effector?

Canto C, Auwerx J (2010) AMP-activated protein kinase and its downstream transcriptional pathways

Deprost D, Yao L, Sormani R, Moreau M, Leterreux G, Nicolai M, Bedu M, Robaglia C, Meyer C (2007) The *Arabidopsis* TOR kinase links plant growth, yield,stress resistance and mRNA translation

Deprost D, Truong HN, Robaglia C, Meyer C (2005) An *Arabidopsis* homolog of RAPTOR/KOG1 is essential for early embryo development

Deak M, Casamayor A, Currie RA, Downes CP, Alessi DR (1999) Characterisation of a plant 3-phosphoinositide-dependent protein kinase-1 homologue which contains a pleckstrin homology domain

Diaz-Troya S, Perez-Perez ME, Florencio FJ, Crespo JL (2008) The role of TOR in autophagy regulation from yeast to plants and mammals

Dufner A, Thomas G (1999) Review Ribosomal S6 kinase signaling and the control of translation

Dobrenel T, Marchive C, Sormani R, Moreau M, Mozzo M, Montané MH, Menand B, Robaglia C, Meyer C (2011) Regulation of plant growth and metabolism by TOR kinase

Elena Baena-González, Mol. Plant (2010) Energy Signaling in the Regulation of Gene Expression during Stress

Evans DS, Kapahi P, Hsueh WC, Kockel L (2011) TOR signaling never gets old: Aging, longevity and TORC1 activity

Fuyuhiko Tamanoi, Michael N Hall- The enzymes: Structure, function and regulation of TOR complexes from Yeasts to Mammals, Part 2 (Google e-book)

Fabrizio P, Pozza F, Pletcher SD, Gendron CM, Longo VD (2001) Regulation of longevity and stress resistance by Sch9 in yeast

Funakoshi M, Tsuda M, Muramatsu K, Hatsuda H, Morishita S, Aigaki T (2011) A gain-of-function screen identifies wdb and ikb1 as lifespan-extending genes in *Drosophila*

Garrett H. Anderson (2005) TOR signaling and *Arabidopsis* development

Greer EL , Dowlatshahi D, Banko MR, Villen J, Hoang K, Blanchard D, Gygi SP, Brunet A (2007) An AMPK–FOXO pathway mediates longevity induced by a novel method of dietary restriction in *C. elegans*

Guertin DA, Sabatini DM (2007) Defining the role of mTOR in cancer

Guarente L, Kenyon C (2000) Genetic pathways that regulate ageing in model organisms.

Gilliham M, et.al (2013) Protocol: optimizing hydroponic growth systems for nutritional and physiological analysis of *Arabidopsis thaliana* and other plants

Greer EL , Oskoui PR, Banko MR, Maniar JM, Gygi MP, Gygi SP, Brunet A (2007) The energy sensor AMP-activated protein kinase directly regulates the mammalian FOXO3 transcription factor

Hansen M, Taubert S, Crawford D, Libina N, Lee SJ, et al. (2007) Lifespan extension by conditions that inhibit translation in *Caenorhabditis elegans*

Hart RW, Turturro A (1997) Dietary restriction and cancer

Hahn-Windgassen A, Nogueira V, Chen CC, Skeen JE, Sonenberg N, Hay N (2005) Akt activates the mammalian target of rapamycin by regulating cellular ATP level and AMPK activity.

Hanson AD, Hitz WD (1982) Metabolic responses of mesophytes to plant water deficits

Hansen M, Chandra A, Mitic LL, Onken B, Driscoll M, Kenyon C (2008) A role for autophagy in the extension of lifespan by dietary restriction in *C. elegans*

Hong SP, Carlson M (2007) Regulation of snf1 protein kinase in response to environmental stress

Houthoofd K, Braeckman BP, Johnson TE, Vanfleteran JR (2003) Life extension via dietary restriction is independent of the Ins/IGF-1 signaling pathway in *Caenorhabditis elegens*

Holliday R (1989) Food, reproduction and longevity: is the extended lifespan of calorie-restricted animals an evolutionary adaptation?

Hoagland DR, Arnon DI (1950) The water culture method for growing plants without soil

Hardie DG (2007) AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy

Hara T, Takamura A, Mizushima N (2008) FIP200, a ULK-interacting protein, is required for autophagosome formation in mammalian cells

Hara T, Mizushima N (2009) Role of ULK-FIP200 complex in mammalian autophagy: FIP200, a counterpart of yeast Atg17?

Henriques R, Magyar Z, Monardes A, Khan S, Zalejski C, Orellana J, Szabados L, de la Torre C. Koncz C. Bögre L (2010) *Arabidopsis* S6 kinase mutants display chromosome instability and altered RBR1-E2F pathway activity.

Heitman J, Movva NR, Hall MN (1991) Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast.

Harrison D.E. et al., (2009). Rapamycin fed late in life extends lifespan in genetically heterogeneous mice

Hall MN (2008) mTOR-what does it do?

Holz MK, Ballif BA, Gygi SP, Blenis J (2005) mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events

Inoki K, Zhu T, Guan KL. (2003) TSC2 mediates cellular energy response to control cell growth and survival

Johnson EC, Kazgan N, Bretz CA, Forsberg LJ, Hector CE, Worthen RJ,

Onyenwoke R, Brenman JE (2010) Altered metabolism and persistent starvation behaviours caused by reduced AMPK function in *Drosophila*

Oh SA, Lee SY, Chung IK, Lee CH, Nam HG (1996) A senescence- associated gene of *Arabidopsis thaliana* is distinctively regulated during natural and artificially induced leaf senescence

Kapahi P, Zid B (2004) TOR Pathway: Linking Nutrient Sensing to Life Span

Kapahi,P,et.al, (2009) Regulation of Lifespan in Drosophila by Modulation of Genes in the TOR Signaling Pathway

Katewa SD,Kapahi P (2009) Dietary restriction and aging

Katewa SD, Kapahi P (2011) Role of TOR signaling in aging and related biological processes in *Drosophila melanogaster*

Kaeberlein M, Kapahi P (2009) The hypoxic response and aging

Kaeberlein M, Powers RW, 3rd, Steffen KK, Westman EA, Hu D, Dang N, Kerr EO,

Kirkland KT, Fields S, Kennedy BK (2005) Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients

Kim E, Goraksha-Hicks P, Li L, Neufeld TP, Guan KL (2008) Regulation of TORC1 by Rag GTPases in nutrient response

Kim J, Kundu M, Viollet B, Guan KL (2011) AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1

Kim DH, Sarbassov DD, Ali SM, Latek RR, Guntuk KV, et.al. (2003) GbetaL, a positive regulator of the rapamycin sensitive pathway required for the nutrient sensitive interaction between raptor and mTOR

Lakowski B, Hekimi S (1998) The genetics of caloric restriction in *Caenorhabditis elegans*

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4

Loewith R, Jacinto E, Wullschleger S, Lorberg A, Crespo JI, et.al (2002) Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control

Loewith R, Hall MN (2011) Target of rapamycin (TOR) in nutrient signaling and growth control.

Liu Y, Bassham DC (2012) Autophagy: Pathways for self-eating in plant cells

Leiber RM, John F, Verhertbruggen Y, Diet A, Knox JP, Ringli C (2010) The TOR pathway modulates the structure of cell walls in *Arabidopsis*

Lin SS, Manchester JK, Gordon JI (2003) Sip2, an N-myristoylated beta subunit of snf1 kinase, regulates aging in *Saccharomyces cerevisiae* by affecting cellular histone kinase activity, recombination at rDNA loci, and silencing

Lorenz DR, Cantor CR, Collins JJ (2009) A network biology approach to aging in yeast

Lee JH, Koh H, Kim M, Kim Y, Lee SY, Karess RE, Lee SH, Shong M, Kim JM, Kim J, Chung J (2007) Energy dependent regulation of cell structure by AMP-activated protein kinase

Masoro EJ (2005) Overview of caloric restriction and ageing

Ma XM, Blenis J (2009) Molecular mechanisms of mTOR-mediated translational control.

Mattson MP, Duan W, Lee J, Guo Z (2001) Suppression of brain aging and neurodegenerative disorders by dietary restriction and environmental enrichment: molecular mechanisms

Meyer C.et.al., (2012) Mutations in the *Arabidopsis* homolog of LST8/Gbeta L, a partner of the target of Rapamycin kinase, impair plant growth, flowering and metabolic adaptation to long days

Mahfouz MM, Kim S, Delauney AJ,DPS (2006) *Arabidopsis* TARGET OF RAPAMYCIN interacts with RAPTOR, which regulates the activity of S6 kinase in response to osmotic stress signals

Noda T, Ohsumi Y (1998) TOR, a phosphatidylinositol kinase homologue, controls autophagy in yeast

Nojima H, Tokunaga C, Eguchi S, Oshiro N, Hidayat S, Yoshino K, Hara K, Tanaka N, Avruch J, Yonezawa K (2003) The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif

Otterhag L, Gustavsson N, Alsterfjord M , Pical C, Lehrach H, Gobomb J, Sommarin M (2005) *Arabidopsis* PDK1: identification of sites important for activity and downstream phosphorylation of S6 kinase

Partridge L, et.al (2005) Dietary restriction in Drosophila

Pattingre S, Espert L, Biard-Piechaczyk M, Codogno P (2008) Regulation of macroautophagy by mTOR and Beclin 1 complexes

Price DJ, Grove JR, Calvo V, Avruch J, Bierer BE (1992) Rapamycin-induced inhibition of the 70-kilodalton S6 protein kinase.

Pende M, Um SH, Mieulet V, Sticker M, Goss VL, Mesten J, Meuller M, Fumagalli S, Kozma SC, Thomas G (2004) S6K(-/-)/S6K2(-/-) mice exhibit perinatal lethality and rapamycin sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen activated protein kinase dependent S6K pathway

Powers RW, 3rd, Kaeberlein M, Caldwell SD, Kennedy BK, Fields S (2006) Extension of chronological life span in yeast by decreased TOR pathway signaling

Raught B, Gingras AC, Sonenberg N (2001) The target of rapamycin (TOR) proteins

Robaglia C, Thomas M, Meyer C (2012) Sensing nutrient and energy status by SnRK1 and TOR kinases

Ruvinsky I, Meyuhas O (2006) Ribosomal protein S6 phosphorylation: From protein synthesis to cell size

Ren M, Venglat P, Qui S, Feng L, Cao Y, Wang E, Xiang D, Wang J, Alexander D, Chalivendra S, Logan D, Mattoo A, Selvaraj G, Datla R (2012) Target of rapamycin signaling regulates metabolism, growth and lifespan in *Arabidopsis*

Stanfel MN, Shamieh LS, Kaeberlein M, Kennedy BK (2009) The TOR pathway comes of age

Schmelzle T, Hall MN (2000) TOR, a central controller of cell growth

Sung MM, Dyck JR (2012) Age-related cardiovascular disease and the beneficial effects of calorie restriction

Sabatini DM (2006) mTOR and cancer: insights into a complex relationship

Sakamoto K, McCarthy A, Smith D, Green KA, Grahame Hardie D, Ashworth A, Alessi DR (2005) Deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction

Schulz TJ, Zarse K, Voigt A, Urban N, Birringer M, Ristow M (2007) Glucose Restriction Extends Caenorhabditis elegans Life Span by Inducing Mitochondrial Respiration and Increasing Oxidative Stress

Sormani R, Yao L, Menand B, Ennar N, Lecampion C, Meyer C, Robaglia C (2007) *Saccharomyces cerevisiae*FKBP12 binds *Arabidopsis thaliana* TOR and its expression in plants leads to rapamycin susceptibility

Stan R, McLaughlin MM, Cafferkey R, Johnson RK, Rosenberg M, Livi GP (1994) Interaction between FKBP12-rapamycin and TOR involves a conserved serine residue.

Selman C, et.al (2009) Ribosomal protein S6 kinase 1 signaling regulates mammalian lifespan

Smith PK, *et al.* (1985) Measurement of protein using bicinchoninic acid

Tettweiler G, Miron M, Jenkins M, Sonenberg N, Lasko PF (2005) Starvation and oxidative stress resistance in Drosophila are mediated through the EIF4E-binding protein, d4E-BP

Tocquin,P.et.al.(2003) A novel high efficiency, low maintenance, hydroponic system for synchronous growth and flowering of *Arabidopsis thaliana*

Turk BE, Hutti JE, Cantley LC (2006) Determining protein kinase substrate specificity by parallel solution-phase assay of large numbers of peptide substrates

Thelander M, Olsson T, Ronne H (2004) Snf1-related protein kinase 1 is needed for growth in a normal day-night light cycle

Thomas H. (2002) Ageing in plants

Turck F, Kozma SC, Thomas G, Nagy F (1998) A heat-sensitive *Arabidopsis thaliana* kinase substitutes for human p70s6k function *in vivo*

Turck F, Zilbermann F, Kozma SC, Thomas G, Nagy F (2004) Phytohormones participate in an S6 kinase signal transduction pathway in *Arabidopsis*

Thelander M, Olsson T, Ronne H (2004) Snf1-related protein kinase 1 is needed for growth in a normal day-night light cycle

Tohyama D, Yamaguchi A (2010) A critical role of SNF1A/dAMPK alpha (*Drosophila* AMP- activated protein kinase alpha) in muscle on longevity and stress resistance in *Drosophila melanogaster*

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications

Vander Haar E, Lee SI, Bandhakavi S, Griffin TJ, Kim DH (2007) Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40

Vellai T, Takacs-Vellai K, Zhang Y, Kovacs AL, Orosz L, Muller F (2003) Genetics: influence of TOR kinase on lifespan in C. elegans

Walker,G., et.al, (2005) Dietary restriction in *C. elegans*: From rate-of-living effects to nutrient sensing pathways.

Wingler A, Masclaux-Daubresse C, Fischer AM (2009) Sugars, senescence, and ageing in plants and heterotrophic organisms

Wullschleger S, Loewith R, Hall MN (2006) TOR signaling in growth and metabolism

Weaver L.M, Amasino RM (2001) Senescence is induced in individually darkened *Arabidopsis* leaves, but inhibited in whole darkened plants

Xiong Y, Sheen J (2012) Rapamycin and glucose-target of rapamycin (TOR) protein signaling in plants

Yang W, Hong YH, Shen XQ, Frankowski C, Camp HS, Leff T (2001) Regulation of transcription by AMP-activated protein kinase: phosphorylation of p300 blocks its interaction with nuclear receptors

Zang M, Zuccollo A, Hou X, Nagata D, Walsh K, Herscovitz H, Brecher P, Ruderman NB, Cohen RA (2004) AMP-activated protein kinase is required for the lipid-lowering effect of metformin in insulin-resistant human HepG2 cells

Zhang P, Hu X, Xu X, Fassett J, Zhu G, Viollet B, Xu W, Wiczer B, Bernlohr DA, Bache RJ, Chen Y (2008) AMP activated protein kinase-alpha2 deficiency exacerbates pressure-overload-induced left ventricular hypertrophy and dysfunction in mice

Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ, Moller DE (2001) Role of AMP-activated protein kinase in mechanism of metformin action

Zhang H, Zha X, Tan Y, Hornbeck PV, Mastrangelo AJ, Alessi DR, Polakievicz RD, Comb MJ (2002) Phosphoprotein analysis using antibodies broadly reactive against phosphorylated motifs

Zheng XF, Florentino D, Chen J, Crabtree GR, Schreiber SL (1995) TOR kinase domains are required for two distinct functions, only one of which is inhibited by rapamycin

Zid BM, Rogers AN, Katewa SD, Vargas MA, Kolipinski MC, Lu TA, Benzer S, Kapahi P (2009) 4E-BP extends lifespan upon dietary restriction by enhancing mitochondrial activity in Drosophila

Zoncu R, Efeyan A, Sabatini DM (2011) mTOR: from growth signal integration to cancer, diabetes, and ageing