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"Régulation post-transcriptionnelles dans l'adaptation des plantes aux stress abiotique"

"Post-transcriptional regulation of the plants in adaptation to abiotic stresses"

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ABREVIATIONS

AGO	Argonaute
AML	Arabidopsis Mei2-like 1
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ARE	AU-rich elements
ATP	Adenosine triphosphate
BAC	Bacterial artificial chromosome
BLAST	Basic Local Alignment Search TOOL
Bp	Base pair
BSA	Bovine Serum Albumin
CaMV	Cauliflower mosaic virus
CDK	Cyclin dependent kinase
cDNA	complementary DNA
CDS	Coding sequence
CIP	calf intestinal phosphatase
DNA	deoxyribonucleic acid
dNTP	deoxy-nucleotide triposphate
DS	dehydration stress
EDTA	Ethylene diamine tetraacetate
eIFs	eukaryotic initiation factors
4E-BPs	eukaryotic initiation factor 4E-binding proteins
EST	expressed sequence tag
FAT	FRAP/ATM/TOR
FKBP	(FK506-Binding Protein): protein FK506
FRB domain	FKBP12-rapamycin binding domain of TOR
GCN2	general control non-derepressible-2
GDP	guanine diphosphate
GEF	guanine nucleotide exchange factor

GSP	gene specific primer
HEAT	Huntington-elongation factor 1A-protein phosphatase 2A (PP2A) subunit and TOR
IRES	internal ribosomal entry sites
Ka	kanamycin
kD	kilodalton
L	liter
LAT59	late anther tomato 59
LB	Luria Broth medium
LHCB	light–harvesting chlorophyll binding
LST8	Lethal with sec thirteen 8
M	molar
MES	2- (N-morpholilino) ethanesulfonic acid
mg	milligram
min	minute
MiP1	Mei2 interacting protein
ml	milliliter
mM	millimolar
mRNA	messenger RNA
Mya	millions of years ago
ng	nanogram
NMD	nonsense-mediated decay
OD_{λ}	Optical density (subscript = wavelength in nm)
ORF	open reading frame
P5R	pyrroline-5-carboxylate reductase
PCR	polymerase chain reaction
PI3K	Phosphatidyl inositol-3-kinase
Pol I	RNA polymerase I
Pmol	pico moles
PPT	phosphinotricin
rbcs	ribulose1, 5-bisphosphate carboxylase small subunit
rDNA	ribosomal DNA

RACE	rapid amplification of cDNA ends
Raptor	Regulatory associated protein of mTOR
RBR1	Retinoblastoma-related 1
RBPs	RNA-binding proteins
Rictor	Rapamycin-insensitive companion of mTOR
RISC	RNA induced silencing complex
RL	ribosome loading
RLM-RACE	RNA ligase-mediated RACE
RNA	ribonucleic acid
RNAi	RNA interfernce
rRNA	ribosomal RNA
RNP	ribonucleoprotein
RT PCR	reverse transcriptase PCR
SDS	sodium dodecyl sulfate
SE	standard error of the mean
SEL	size exclusion limit
SIRT	sucrose-induced repression of translation
S6K	S6 kinase
SnF1	sucrose non-Fermenting 1
SnRK1	SnF1-related kinase 1
Sut1	sucrose transporter 1
Tm	melting point
TAP42	PP2A Associated protein
TAP46	2A phosphatase associated protein of 46kDa
TAT2	tryptophan Permease
TEL	Terminal Ear-Like
TOP	terminal oligopyrimidine tract
TOR	Target of Rapamycin
TORC	TOR complex
TSC	Tuberous sclerosis complex
T-DNA	Transferred DNA
Tris	tris (hydroxymethyl)-aminomethane

uORF	upstream open reading frame
WS	Wassilewskija
%	Percent
Δ	change

RÉSUMÉ

Les plantes sont ancrées au sol pendant la majorité de leur cycle de vie et doivent donc constamment adapter leur croissance et leur métabolisme aux stress abiotiques. Ainsi, la subsistance des plantes dépend de leur capacité à réguler rapidement l'expression des gènes afin d'adapter leur physiologie à l'environnement. L'expression d'un gène peut être contrôlé à plusieurs niveaux; transcriptionnel, post-transcriptionnel, traductionnel et post-traductionnel.

De nombreux processus cellulaires vitaux tels que la réplication de l'ADN, la transcription, la synthèse protéique, et la dégradation des protéines, sont régulés par les signaux environnementaux. Des études chez la levure, la drosophile et les animaux ont montré que la protéine kinase TOR (Target Of Rapamycin) est impliquée dans le contrôle de la croissance cellulaire et de la prolifération en réponse à différents signaux tels que les nutriments, les acides aminés, les hormones et les facteurs de croissance. Chez *Arabidopsis thaliana*, TOR est nécessaire au développement de l'embryon et de l'endosperme. De plus, des modifications du niveau de protéine AtTOR affectent la croissance végétative et la reproduction.

Le principal objectif de cette thèse est de caractériser les mécanismes qui contrôlent l'expression de *AtTOR* en déterminant les éléments de régulation situés sur le la région 5' non traduite (5′UTR) de l'ARNm de *AtTOR,* puis de manipuler ces éléments de régulation afin d étudier leur rôle. Nous avons choisi de nous focaliser sur la région 5′UTR de AtTOR, et sur une microORF (uORF) située en amont de l'ORF principale de *AtTOR*. Il s'agit de la première tentative d'étude de la régulation de l'expression de TOR par ces éléments chez les eucaryotes.

Trois constructions chimériques ont été réalisées pour cette étude et transformée transitoirement est de manière stable dans des plantes. La première construction (contrôle positif) incluse le promoteur de *AtTOR*, la région 5′UTR, le premier intron et le début du premier exon fusionné au gène rapporteur *GUS*. La seconde construction (microORF mutée) est présente une mutation du codon start de la microORF (ATG changé en TTG). Enfin, la troisième construction (5′UTR délétée) contient la même séquence que le contrôle positif mais sans la région 5′UTR. Ces constructions ont également été placée sous le contrôle du promoteur 35S au lieu du promoteur de *AtTOR* afin d'étudier un lien éventuel entre la 5′UTR et la microRF et le promoteur de *AtTOR*

Nos résultats indiquent une régulation généralement négative exercée par la 5′UTR, et dans une moindre mesure par la microORF, sur l'expression de *AtTOR*. Cette régulation semble avoir lieu au niveau transcriptionnel ou au niveau de la stabilité de l'ARNm, mais pas au niveau de la traduction. En effet, les modifications du niveau de transcrit GUS sont suivie d'un changement équivalent de l'activité GUS. De plus, nous avons observé que l'auxine et le sucrose ont un effet positif sur l'expression de *AtTOR*. Dans le cas de l'auxine, cet effet semble lié à la présence de la région 5′UTR de *AtTOR*.

D'autres études de la fonction de la région 5'UTR et de la microORF de *AtTOR*, ainsi que de leur relation avec d'autres éléments régulateurs localisée dans le promoteur de *AtTOR,* permettront de mieux comprendre comment ces éléments régulateurs contrôlent finement l'expression de *AtTOR*.

ABSTRACT

Land plants are anchored in one place for most of their life cycle and therefore must constantly adapt their growth and metabolism to abiotic stresses. Thus, plants' subsistence depends on their ability to regulate rapidly gene expression in order to adapt their physiology to their environment. The expression of a gene can be controlled at many levels, including transcription, post-transcription, translation, and post-translation.

Many vital cellular processes like DNA replication, transcription, protein synthesis, and protein degradation are regulated by environmental signals. Studies in yeast, Drosophila, and mammals showed that the target of rapamycin (TOR) protein is involved in control of cell growth and cell proliferation in response to different types of environmental signals such as nutrients, amino acids, hormones, and growth factors. In *Arabidopsis thaliana*, TOR is necessary for both embryo and endosperm development in, and changes of TOR protein level affect both vegetative and reproductive growth.

The main purpose from this thesis is to highlight the mechanisms that control *AtTOR* expression at the post-transcriptional level through determination of the possible regulatory elements within the 5′ untranslated region (5′UTR) or the first intron of *AtTOR* mRNA itself, and through manipulation of these regulatory elements to study their precise role. We have chosen to focus on the small upstream open reading frame (uORF) as well as the 5′UTR region. This is the first attempt to study the regulation of TOR kinase expression in eukaryotes through these small uORF or the sequence of 5′ untranslated region (5′UTR).

To achieve this purpose, three chimeric constructs have been established and transformed in *Nicotiana benthamiana* leaves and *Arabidopsis thaliana* plants. The first construct (the positive control) contains the *AtTOR* promoter, the 5′UTR, the first intron, and the beginning of the second exon fused to the *GUS* reporter gene. The second construct (mutated uORF) have the same sequence as the positive control construct except the start codon of uORF was changed from ATG to TTG. The third construct (deleted 5′UTR) have the same sequence as the positive control construct without the 5′UTR. These constructs have also been placed under the activity of CaMV 35S promoter instead of *AtTOR* promoter to investigate whether there is a link between the 5′UTR/or uORF and the promoter.

Our work show an overall negative regulation exerted by the 5′UTR and, to a lesser extent, by the uORF on *AtTOR* gene regulation. This regulation is likely at the level of transcription or mRNA stability, since the changes in GUS transcript level was followed by the same changes in GUS activity. In addition we found that external inducers like auxin or sucrose exert a positive effect on AtTOR expression. This effect appears somehow linked to the presence of the 5′UTR of *AtTOR* mRNA.

Greater insight into the molecular mechanisms of *AtTOR* 5′UTR/or uORF function and its relationship with other regulatory elements located in *AtTOR* promoter will be required to understand how these regulatory elements work either individually or in combination to achieve the fine and accurate regulation of their gene expression.

1-INTRODUCTION

1.1. Plant growth and environmental signals

Plants start their life as simple organisms and develop to form new organs to reach elaborate complex and adaptive architectures during their life cycle. The growth of plant cell involves two successive events, the first is cell proliferation, and the second is cell expansion, in which cells reach a large size through expansion of their vacuole and their cell wall **(Menand *et al*., 2004; Robaglia *et al*., 2004)**.

Plant cells are produced mainly from tissues located at the tips of growth axes in shoots and roots. The meristematic cells represent the microenvironment that allows stem cells to grow optimally (**Figure 1**). Stem cells division generate new cells that are displaced from the stem cell niche, undergo several additional rounds of cell divisions and are then permitted to differentiate into specific cell types (**Sablowski, 2007)**. Meristematic cells are small (approximately 5µm), densely packed with cytoplasm, and in defined regions of the meristem, grow to specific size before dividing. For example, in the apical half of the *Arabidopsis thaliana* root meristem, cells divide and expand at approximately equal rates (**Figure 2**). Thus, cell size remains approximately constant with increasing distance from the tip. On the other hand, at the basal half of the meristems, cells expansion rates rapidly increase as a function of the distance from the root tip. This change in growth mode is accompanied by a decrease of the division potential. As a result, cell size starts to increase progressively. Vacuoles appear and partly drive growth in this region. In elongation zone, cells no longer divide, but rapidly expand **(Traas *et al*., 1998)**, up to 40 % per hour **(Beemster and *Baskin,* 1998)**, causing cell length to increase rapidly. In mature part of the root, that represent the largest part of the root system, cells no longer divide or grow except to initiate lateral roots **(Beemster *et al*., 2003).** The plant cell expansion can be viewed as an adaptation to increase in size economically because plants had to optimize their capacity to explore their environment to collect essential resources such as light, mineral nutrients, and water **(Cosgrove, 1997)**. Meristematic cells continuously divide and expand to produce new cells and new organs for plant development.

In general, the growth of a cell depends on the perception of the surrounding nutrients. Several conserved pathways are involved in the coordination between cell growth and division and

nutrient availability. The Target of rapamycin (TOR) pathway appears as one of these conserved pathways in most eukaryotes, including plants. TOR is necessary for both embryo and endosperm development in Arabidopsis (**Menand *et al.*, 2002**), and changes of TOR protein level affects both vegetative and reproductive organ growth in Arabidopsis plants (**Deprost *et al.*, 2007**).

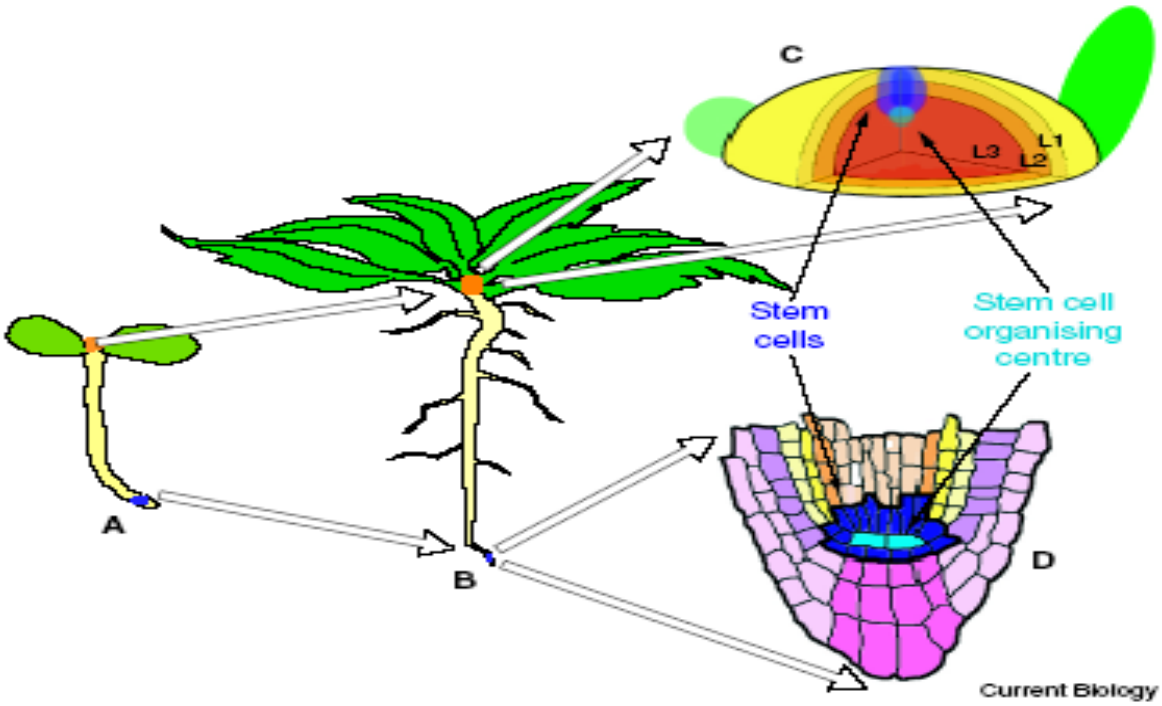


Figure 1: Functions of meristematic cells in development. (A) At germination stage, apical meristems differentiate into shoot meristem and root meristem. (B) Aerial tissues are produced from shoot meristem and the primary root generate from the root meristem. (C) Layers of shoot apical meristems – L1 (epidermal), L2 (subepidermal), and L3 (corpus) – are specified during embryogenesis and are maintained throughout development. Stem cells inhibit proliferation in the central zone, while organ primordial are initiated at the flanks. (D) Stem cells maintain indeterminate state of the root apical meristem, because they possessed the capacity for unlimited proliferation and self-maintenance. From Doerner, 2003.

Many vital cellular processes like DNA replication, transcription, protein synthesis, and protein degradation are regulated by environmental signals. Regulation of all these processes is essential for proper cell growth and development. Studies in yeast, Drosophila, and mammals showed that the target of rapamycin (TOR) protein is involved in control of cell growth and cell proliferation in response to different types of environmental signals such as nutrients, amino acids, hormones,

and growth factors (**Beretta *et al*., 1996; Thomas *et al*., 1997**). Also, TOR controls the expression of some genes responding to stress signals, like heat shock and hydrogen peroxide treatments, via down-regulation of their regulatory transcription factors in yeast (**Gasch *et al*., 2001**). Indeed, *Saccharomyces cerevisiae* cells that lack the *ScTOR1* gene appears to be more sensitive to high salts concentrations than wild type cells.

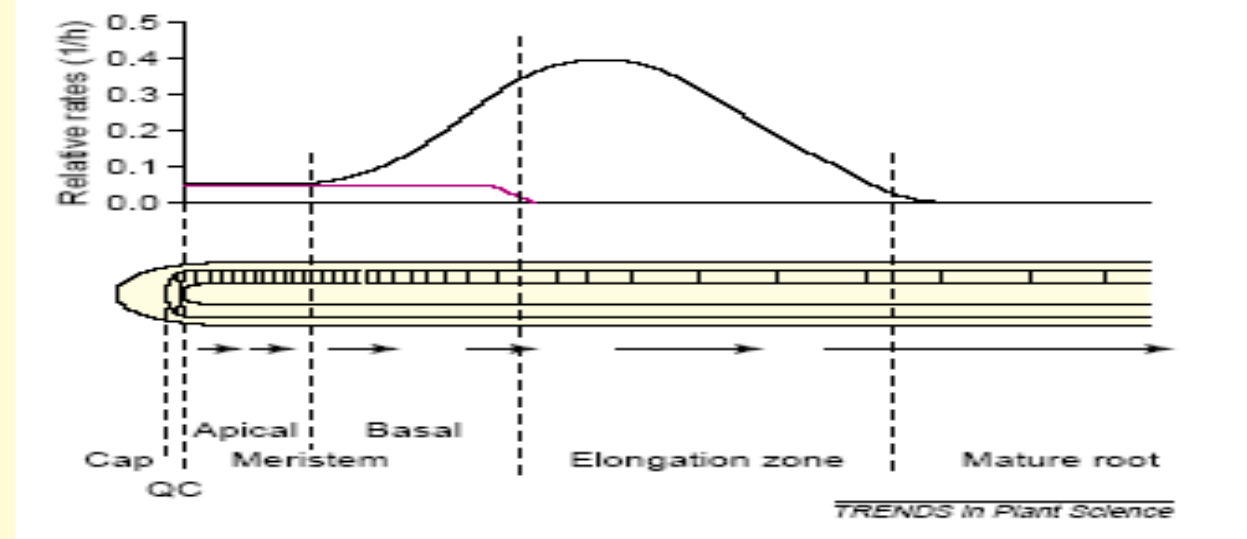


Figure 2: Cell files originate at the quiescent centre (QC) directly below the root cap. In each file, the following regions can be distinguished: apical half of the meristem, in which the cells divide (pink line) and expand (black line) at approximately equal rates (typically, 5% per hour). Basal half of the meristem, in which the cells divide at approximately the same rate as they do in the apical half of the meristem, but expansion rates rapidly increase as a function of distance from the root tip. Elongation zone, in which the cells no longer divide, but cell length rapidly increases. From Doerner, 2003.

Together, these data suggest that TOR might be one of the important regulators of suitable cellular response to stress in eukaryotes. Thus, we will discuss in details the structural features of the conserved TOR proteins as well as their functional roles for three major groups of eukaryotes; yeast, mammals, and plants.

1.2. TOR structure and functions

1.2.1. TOR and rapamycin

TOR is an acronym for Target of Rapamycin. TOR is a conserved Ser/Thr protein kinase belonging to the phosphatidylinositol kinase-related kinases (PIKKs) family **(Cardenas and**

Heitman, 1995; Sabers *et al.*, 1995). This family includes the mammalian ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia related), and DNA-dependendent protein kinase (**Abraham, 2001**). TOR was found in most eukaryotes including *Saccharomyces cerevisiae* (ScTOR1 and Sc TOR2; **Heitman *et al.*, 1991)** *Drosophila melanogaster* (dTOR; **Oldham *et al.*, 2000, Zhang *et al.*, 2000**), *Caenorhabditis elegans* (CeTOR; **Long *et al.*, 2002**), *Arabidopsis thaliana* (AtTOR; **Menand *et al.*, 2002**), mammals (mTOR; **Brown *et al.*, 1994** and **Sabatini *et al.*, 1994**), and *Cryptococcus neoformans* (CnTOR; **Cruz *et al.*, 1999**).

TOR was first identified in *Saccharomyces cervisiae* from a screen of rapamycin-resistant mutants (**Heitman *et al.*, 1991a).** Rapamycin is a lipophilic macrolide produced by a strain of *Streptomyces hygroscopicus* isolated from a soil sample collected on Easter Island, also known as Rapa Nui and hence the name rapamycin **(Abraham and Wiederrecht, 1996)**. Rapamycin blocks cell proliferation in response to nutrients or mitogens, as well as it blocks cell cycle and promotes autophagy **(Noda *et al.*, 1998; Kamada *et al.*, 2000; Abeliovich *et al.*, 2000)**.

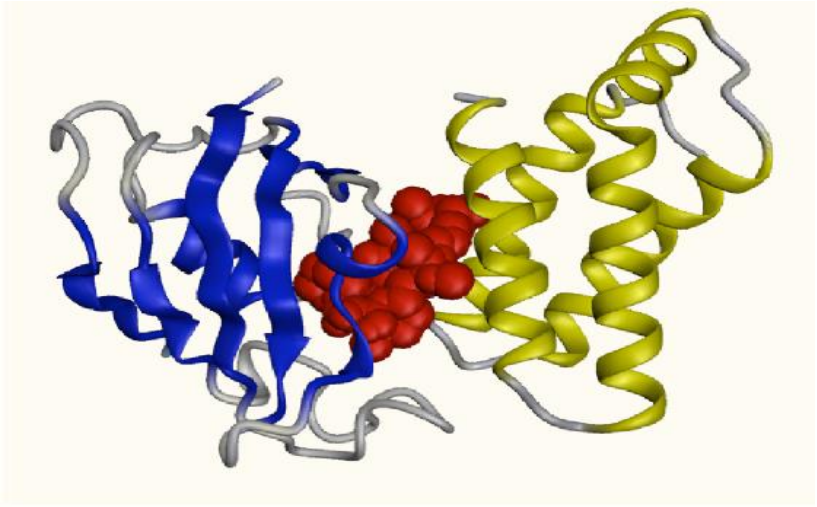


Figure 3: The structure of the ternary complex between mFKBP12-rapamycin and the mTOR (FRB domain). mFKBP12 – rapamycin - mTOR (rapamycin-binding domain). From Choi *et al.,* 1996.

Rapamycin goes through the plasma membranes to the cytoplasm to bind with high affinity to a 12 kDa protein named FK506-binding protein (FKBP) 12 encoded by the *FPR1* gene in *Saccharomyces cerevisiae* **(Wiederrech *et al.*, 1991)**.

The sensitivity of yeast cells to rapamycin is related to presence of FKBP12 protein, thus the yeast mutants lacking FKBP12 are viable and rapamycin resistant (**Heitman *et al.*, 1991b; Koltin *et al.*, 1991)**. Recent studies suggest that free rapamycin is capable of binding and

inhibiting mTOR, but the affinity of this interaction is increased approximately three times in the presence of FKBP12 **(Leone *et al.*, 2006; Shor *et al.*, 2008)**. The inhibition of TOR kinase by rapamycin results from the formation of a ternary complex between rapamycin, the FRB (FKBP12 Rapamycin Binding) domain of TOR and the mFKBP12 protein. (**Choi *et al.*, 1996**; **Figure 3)**.

1.2.2. Structure of TOR protein

TOR is a highly conserved protein of approximately 300 kDa composed of HEAT repeats, FAT (FRAP, ATM, and TRRAP), FRB (FKBP12–rapamycin binding), kinase, FIT (found in TOR), and FATC (FAT-C terminal) domains (**Figure 4**).

The N-terminal and central region of TOR contain multiple tandemly organized HEAT repeats named from Huntington, Elongation factor 3 [EF3], the A subunit of type 2A protein phosphatase [PP2A] and TOR. Each HEAT motif comprises approximately 40–50 amino acids, and contains several conserved hydrophobic residues. These HEAT repeats have been proposed to mediate protein–protein interactions (**Hemmings *et al.*, 1990; Andrade and Bork, 1995; Andrade *et al.*, 2001**). HEAT repeats are also required for association with raptor (regulator associated protein to Tor) (**Kim *et al.*, 2002**) and for the localization of mTOR at the endoplasmic reticulum and Golgi apparatus (**Liu and Zheng, 2007**).

Mammalian TORβ (m TORβ), a truncated version of mTOR produced by alternative splicing has been described recently (**Panasyuk *et al.*, 2009**). It consists of FRB, kinase, FIT, and FATC domains fused to the 23 N-terminal amino acids of full length mTOR.

The most highly conserved region of the TOR protein is the C-terminal region composed of the FAT, FRB, kinase, FIT, and FATC domains. The FAT domain (FRAP-ATM-TRRAP) consists of approximately 500 residues, located between HEAT repeats and the FRB domain and is proposed to contain additional HEAT motifs (**Alarcon *et al.*, 1999; Perry and Kleckner, 2003; Bosotti *et al.*, 2000**). TOR proteins contain an FKBP12-rapamycin binding domain (FRB), which consists of ~ 100 residues and is a hallmark of mammalian and yeast TORs.

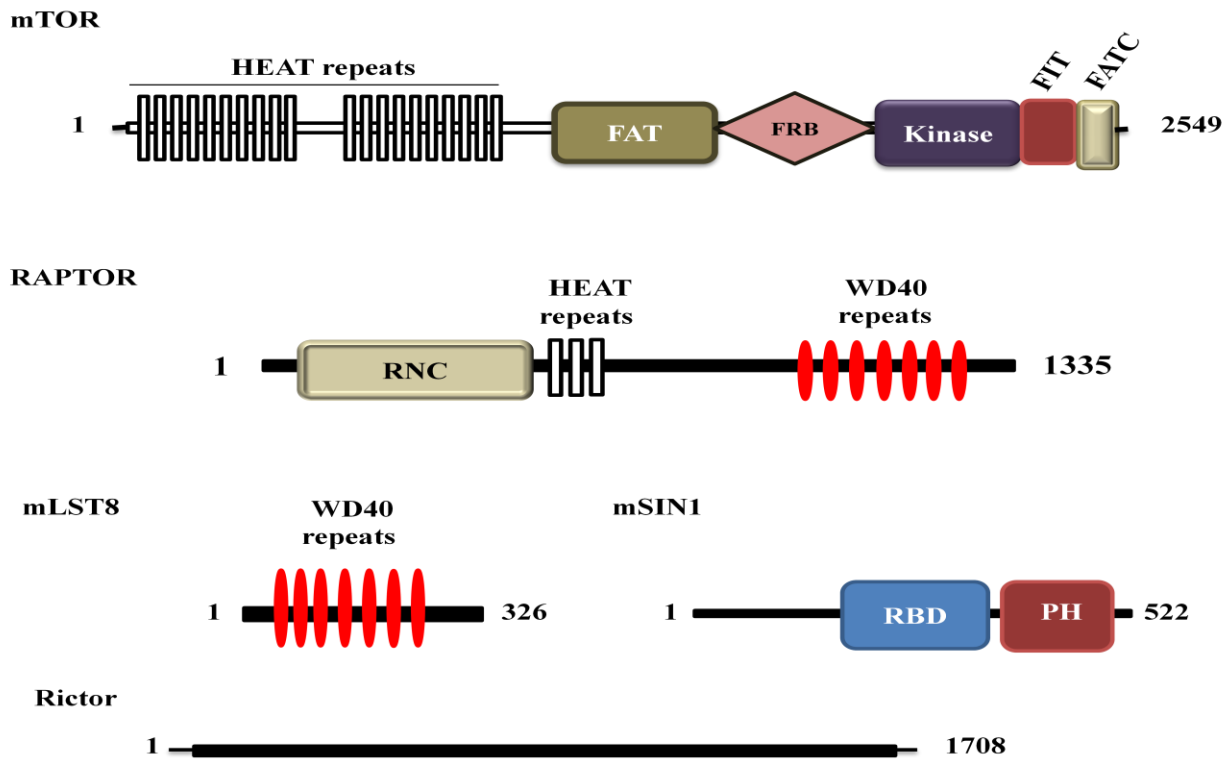


Figure 4: Schematic representation of the domain structures of TOR protein and members of mTOR complexes. Modified from Zinzalla *et al.*, 2010.

TOR kinase (catalytic) domain consists of ~ 300 residues. GTPase proteins bind to N-terminal part of the kinase domain in mTOR (**Long *et al.*, 2005**). FIT (Found In TOR) is a new domain located between the kinase and FATC domains and it is not highly conserved among different species (**Sturgill and Hall, 2009**).

1.2.3. TOR complexes in eukaryotes

Target of rapamycin (TOR) protein appears in two complexes termed TOR complex 1 (TORC1) and TOR complex 2 (TORC2) in most eukaryotic organisms **(Jacinto and Hall, 2003; Sabatini, 2006)**. These two complexes control a wide-range of cellular processes in response to different types of developmental and environmental signals.

The members of TOR complex1 (ScTORC1) in yeast are ScTOR (ScTOR1 or ScTOR2), ScKOG1, and ScLST8. In animals, mTOR, Raptor (regulatory associated protein in mTOR) and mLST8/GβL represent the member of mTORC1 (**Figure 5**).The members of TORC1 are

therefore conserved between yeast and mammals. TOR complex 1 is activated by growth factors, such as insulin, amino acids, and energy sufficiency, and is sensitive to rapamycin in mammals. The members of TOR complex2 (TORC2) are ScTOR2, AVO1, AVO3, and LST8 in yeast. While, mTOR, Rictor, mSIN1, and mLST8 represent the members of mTORC2 in mammals (**Figure 5**). It appears that these members are conserved between yeast and mammals. TORC2 show resistance to rapamycin treatment in budding yeast due to binding of AVO1 (adheres voraciously) to ScTOR2 protein in TORC2 complex that masks the FKBP12-rapamycin binding site (**Wullschleger *et al.*, 2005**). The mSIN1, ortholog to yeast AVO1, is an important member of mammalian TOR complex2 (mTORC2), which is required for its kinase activity (**Jacinto *et al.*, 2006; Yang *et al.*, 2006**). Also, mTORC2 is activated by growth factors and is insensitive to rapamycin.

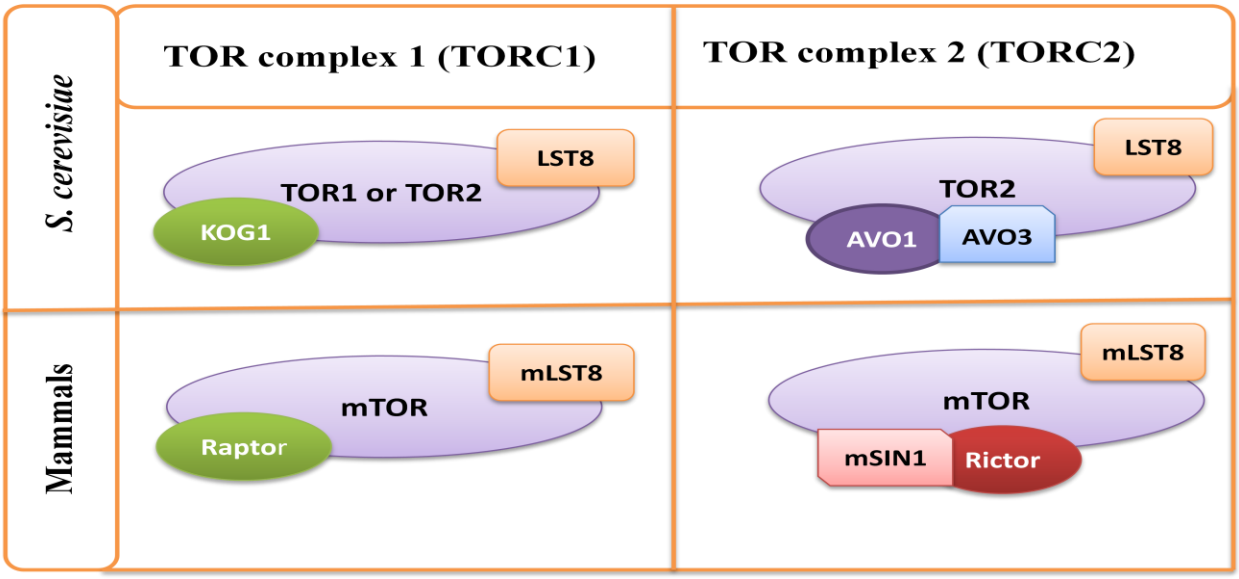


Figure 5: Composition of TOR complex 1 (TORC1) and TOR complex 2 (TORC2) of *S. cerevisiae* and mammals. Modified from Hall and Tamanoi, 2010.

1.2.4. TOR complexes in plants

Many vascular plants like *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays*, but also mosses like *Physcomitrella patens* and several algae possess a single copy of *TOR* gene as in mammals. Recent results suggested that plant TORC1 complex is composed of TOR, RAPTOR and LST8 (**Moreau *et al.*, 2010**). *Arabidopsis* TOR protein (*AtTOR*, At1g50030) has about 40 % of sequence identity with TOR sequence from other eukaryotic organisms. Two Raptor (regulatory

associated protein of TOR) proteins were characterized in *Arabidopsis.* The first is called AtRAPTOR1 (gene *AtRAPTOR3g*) and the latter is called AtRAPTOR2 (gene *AtRAPTOR5g*) **(Deprost *et al.*, 2005; Figure 6).** *AtRAPTOR3g* is ubiquitously expressed and at a much higher level than *AtRAPTOR5g*. It was demonstrated that AtRAPTOR1 can interacts with the HEAT repeats of TOR and modulate the activity of S6 kinase -1 (S6K 1) an homolog of a downstream target of mTORC1, in response to osmotic stress **(Mahfouz *et al.*, 2006)**.

Like Raptor, LST8 is encoded by two genes known as At3g18140 and At2g22040 for *AtLST8-1* and *AtLST8-2* genes respectively in *Arabidopsis*. *AtLST8-1* is expressed ubiquitously and at a much higher level than *AtLST8-2* (**Figure 6)**. Recently, it was observed that the AtLST8-1 protein has the ability to restore the complete growth of *lst8* mutant in *Arabidopsis* (**Moreau *et al.*, 2010).**

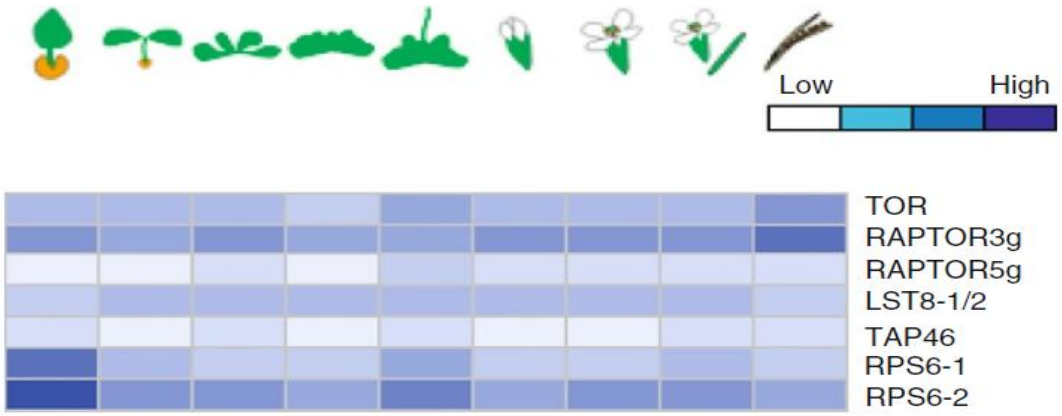


Figure 6: Expression pattern of Arabidopsis genes involved in the formation of the TORC1 complex or in the TOR signaling pathway in various plant organs or developmental stages. Microarray expression data are from Genevestigator (https://www.genevestigator.com, Hruz *et al*., 2008). From Moreau *et al.*, 2010.

Moreover, AtLST8-1 can complement the yeast lst8 mutant. By complementation experiments with *lst8* mutant from yeast, the function of LST8 from *Chlamydomonas* was also confirmed as similar to yeast LST8 protein **(Díaz-Troya *et al.*, 2008)**. Otherwise, there are no clear homologs in the plant genomes of the TOR complex 2 (TORC2) specific components like AVO1/hSIN1 or AVO3/RICTOR. This might be related to low degree of similarities that may have prevented their identification in plants and algae.

1.2.5. TOR localization

TOR proteins appear to be associated with the plasma membrane and membranous structures within yeast cells **(Kunz *et al*., 2000; Wedaman *et al*., 2003)**. **Lie *et al*., (2006)** demonstrated a nuclear localization pattern for ScTOR1. **Sturgill *et al*., (2008)** have detected that ScTOR1 is distributed in all the cytoplasm and it is more concentrated near vacuole membranes. Recently, **Berchtold and Walther (2009)** shown that ScTORC1 and ScTORC2 complexes are localized at different compartments of the cell. ScTORC1 is localized in vacuole while, ScTORC2 is localized at cytoplasmic membranes.

Localization of mammalian TOR (mTOR) was reported, and many discrepancies were found. Mammalian TOR (mTOR) was found located at the periphery of the endoplasmic reticulum (ER) and Golgi apparatus **(Drenan *et al*., 2004),** in nucleus (**Zhang *et al*., 2002**), and associated to external membrane of mitochondria (**Desai *et al*., 2002**). Mutation of these sequence cause delocalization of TOR protein and also affects signaling pathways mediated by TORC1 and TORC2 (**Liu and Zheng, 2007**).

It was observed that both LST8 and TOR proteins complexes are associated with internal membranes in high-molecular mass structures in *Chlamydomonas* **(Díaz-Troya *et al*., 2008)**.

In general, localization of TOR proteins is not clear and seemed to be variable between organisms and also between the different organelles inside each organism. Most studies have shown that TOR proteins localize on the external membranes either in yeast or in mammals, except for the new splicing TOR in mammals that is called mTORβ and localizes predominantly in the cytoplasm (**Panasyuk *et al*., 2009**). The localization of mTORβ protein in the cytoplasm is due to the absence of HEAT domains that are implicated in mediating mTORα membrane localization.

1.2.6. TOR functions in eukaryotes

Yeast (*Saccharomyces cerevisiae*) is one of the main eukaryote model organism used to study the TOR pathway. Several evidences indicate that ScTOR1 and ScTOR2 proteins are important for normal cell cycle and cell proliferation (**Heitmam *et al*., 1991a; Kunz *et al*., 1993; Helliwell *et al*., 1994**). Inactivation of TOR arrested larval development in *Drosophila* and yields particular cellular phenotypes resembling that produced by amino acid deprivation (**Oldham *et al*., 2000; Zhang *et al*., 2000**). *Caenorhabditis elegans* TOR (*CeTOR*) disruption shows some

traits in common with starvation, but is accompanied by a starvation-independent atrophy of the intestine (**Long et al., 2002**). *Schizosaccharomyces pombe* contain two *TOR* genes. *SpTOR2* is important for viability, while *SpTOR1* is necessary for viability under temperature, pH, osmotic, and oxidative stress conditions (**Weisman and choder 2001**). Both rapamycin treatment and *SpTOR1* disruption inhibit sexual differentiation under low nutrient conditions. TOR complex1 (TORC1) appears to be sensitive to nutrients or rapamycin and involved in regulation of cell growth. TOR complex2 (TORC2) is insensitive to nutrients or rapamycin and involved in regulation of cell growth as well as cytoskeleton organization **(Jacinto *et al.*, 2004; Wullschleger *et al*., 2006)**.

Increasing the rate of cell growth need highly active rate of protein synthesis. TOR plays an important role in coupling nutrient availability to the transcription of genes involved in the formation of ribosomes. Inhibition of the TOR pathway by rapamycin treatment or nutrient starvation rapidly and severely reduces transcription of genes associated with the three classes of RNA polymerases I, II, and III. In absence of rapamycin, ScTORC1 promote the expression level of these genes via phosphorylation of the yeast SCH9 protein (a kinase from the AGC family) (**Urban *et al.*, 2007; Huber *et al*., 2009**). Similarly mammalian TOR (mTOR) controls ribosomal biogenesis through transcriptional control of ribosomal RNA (rRNA) transcript with RNA polymerase I (Pol I) **(Tsang *et al*., 2003; Mayer and Grummt, 2006)**. Transcription of rDNA was found rapidly inhibited by nutrient starvation and rapamycin treatment in both yeast and mammalian cells **(Freckleton *et al*., 2009; Zhu *et al*., 2009)**.

Collectively, the effect of TORC1 on cell growth involves assembly of all the inputs which promote ribosome biogenesis, translation initiation and transcription.

In yeast, rapamycin treatment or inactivation of both *TOR* genes results in an early and severe decrease in translation initiation. As a consequence of this translation defect, the rapamycin treated cells are arrested in the early G1 phase of the cell cycle. Several observations suggest that the TOR pathway positively controls translation initiation through activation of translation factors eIF4E and eIF4G **(Berset *et al.*, 1998)**. A significant amount of evidence indicates that ribosomal protein S6 kinase (S6K) is a key downstream target of TOR. **(Avruch *et al*., 2001; Thomas *et al*., 2002)**. Many results indicate that S6K and 4E-BP1 are key TOR pathway elements, which mediate the regulation of cell size through protein translation **(Boyer *et al.*, 2008; Huang and Manning, 2008; Proud, 2010)**. Mammalian TOR (mTOR) activates S6K by

phosphorylation. The phosphorylation S6K of results in an elevated phosphorylation of 40S ribosomal S6 protein. The subsequent phosphorylation steps lead to the recruitment of the 40S ribosomal subunit into actively translating form. In the presence of amino acids, mTOR activates S6K, resulting in up-regulation of translation initiation. Eukaryotic initiation factor 4E-binding proteins (4E-BPs) include three members 4E-BP1, 4E-BP2 and 4E-BP3 that repress mammalian translation via inhibition of ribosome assembly. 4E-BPs binds to the surface of eIF4E to inhibit its interaction with eIF4G and block ribosome binding. Phosphorylation of 4E-BPs by mTORC1 results in the releasing of eIF4E, allowing it to bind eIF4G and its partners. Thus, mTORC1 activation promotes cell growth via increasing the process of protein synthesis through activation of S6K and inhibition of 4E-BP.

Several evidences indicated that different metabolic pathways are connected to TOR proteins. Amino acid permeases mediate the transport of nutrients across the plasma membrane, and play an important role in cell growth and viability. Studies in *S.cerevisiae* have revealed that the TOR pathway plays a remarkable role in regulation of amino acid permease activity. TOR proteins appeared to regulate inversely the high-specificity permeases such as tryptophan permease TAT2 and histidine permease HIP1, and the broad-specificity permease GAP1 **(Beck *et al*., 1999b)** in response to nutrient availability. Under good nitrogen source, TOR keeps NPR1 phosphorylated and in an inactive form, thereby protecting GAP1 from ubiquitination. In response to a poor nitrogen conditions, NPR1 becomes dephosphorylated and activated in a SIT4- and TIP41-dependent manner **(Jacinto *et al.*, 2001)**. Recently, permeases were identified as phosphoproteins that are rapamycin-sensitive (**Huber *et al*., 2009**).

Inhibition of TORC1 with rapamycin causes changes at the transcriptional levels for genes encoding enzymes involved in essential metabolic cycles. Inactivation of TORC1 by rapamycin resulted in transformation of metabolic pathways from fermentation (glycolytic cycle) to respiration (citric acid cycle) in yeast (**Bonawitz *et al*., 2007**).

TOR complex1 (TORC1) plays an important role in protecting cell from harmful stress signals. It regulates the transcription level of a number of stress-responsive genes in yeast (**De Virgilio and Loewith, 2006**). Most of these genes are transcription factors involved in the control of the expression of nitrogen and carbon assimilation genes. TOR negatively controls the transcription of stress-responsive genes by sequestering the general stress transcription factors MSN2 and MSN4 (zinc finger transcription factor) in the cytoplasm **(Beck *et al.*, 1999a; Swinnen *et al*.,**

2006). Mammalian TOR complex 1 (mTORC1) also exhibits a high sensitivity to environmental signals and different types of stresses. A lot of these external signals are integrated by the mTORC1 inhibitor TSC1/2 (tuberous sclerosis tumor suppressor proteins). In mammalian cells, mTOR signaling also controls transcription of stress-responsive genes. Biochemical analysis of mammalian cells has shown that mTOR regulates UR1 (unconventional prefolding RPB5 interactor) phosphorylation. This result suggested that TOR regulates nutrient-dependent transcription at least partially through UR1 phosphorylation and that this mechanism might be conserved from yeast to humans **(Gstaiger *et al*., 2003)**.

Environmental stress responses appear to be an important factor determining lifespan of yeast cells. Several studies indicated that partial inhibition of TORC1 increases chronological lifespan in a RIM15-and MSN2/4- dependent manner (**Wanke *et al*., 2008; Wei *et al*., 2008**). Lifespan increase was also observed in TOR repressed *C.elegans*, *Drosophila* and mouse (**Vellai *et al*., 2003; Kaeberlein *et al*., 2005; Kapahi *et al*., 2004**). Inactivation of TOR in all these organisms with rapamycin resulted in extended lifespan. Rapamycin effects appeared related in part on decreased or altered translation process. In yeast, the autophagy was induced even in rich nutrient conditions when TOR function was inactivated by rapamycin treatment. This finding indicates that TOR inhibits autophagy **(Noda *et al*., 1998)**. The autophagy is triggered by activation of the APG1-APG13 protein complex **(Kamada *et al*., 2000)**. The protein kinase APG1 associates with dephosphorylated APG13 and APG17 to form the APG1 protein complex **(Mastsuura *et al*., 1997)**. TOR inhibits autophagy by maintaining APG13 in a phosphorylated state with low affinity for APG1 and thereby inhibiting APG1 activity. In contrast, inactivation of TOR by rapamycin treatment or nutrient starvation enhances APG1 kinase activity **(Kamada *et al*., 2000)**. As rapamycin induces autophagy in cultured mammalian cells, even in nutrient-rich medium, mTOR was also supposed to play a role in the regulation of autophagy. The mechanism of mTOR inhibition of autophagy was hypothesized to involve the S6K signaling branch **(Blommaart *et al*., 1995; Shigemitsu *et al*., 1999)**. In yeast, it was reported that induction of autophagy via activation of TORC1 resulted in an extend aging, however, blocking autophagy through inhibition of TORC1 lead to reduced lifespan (**Stanfel *et al*., 2009**).

We can conclude that, in both yeast and animals, TOR pathway involves two complexes, TORC1 and TORC2, performing different functions. TORC1 exhibits sensitivity to rapamycin and nutrients, but TORC2 is insensitive to rapamycin and nutrients. TORC1 promotes cell

growth via increasing the process of protein synthesis through activation of S6 kinase (S6K) and inhibition of eukaryotic initiation factor 4E-binding protein (4E-BP). Even more, TORC1 complex participate in regulation of the transcription of genes encoding enzymes involved in essential metabolic cycles like glycolytic and citric acid cycles. Moreover, TORC1 complex also participates in protection yeast and mammalian cells from harmful stress signals via regulation of the transcriptional level of a number of stress-responsive transcription factors. It was reported that TORC1 control of lifespan in *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila*, and mouse cells. TORC2 complex is involved in regulation of cell growth as well as cytoskeleton organization.

1.2.7. TOR functions in Arabidopsis

Recent results suggested that Arabidopsis TORC1 complex is composed of TOR, RAPTOR and LST8 partners (**Moreau *et al.*, 2010**). The vegetative growth of Arabidopsis and other land plants such as the monocot *Oryza sativa*, the dicots *Nicotiana tabacum* and *Brassica napus* were found naturally resistant to rapamycin even at high concentrations. This resistance might be the consequence of the inability of plant FKBP12 to bind to rapamycin and form the active rapamycin-FKBP12 complex that potentially inhibits TOR activity (**Xu *et al*., 1998; Sormani *et al.*, 2007**). It was observed that the AtTOR FRB domain was able to interact with either yeast or human FKBP12 proteins in the presence of rapamycin but that it was unable to interact with AtFKBP12 and rapamycin in a yeast two hybrid essay (**Mahfouz *et al*., 2006; Sormani *et al*., 2007**). In contrast to *Arabidopsis*, the unicellular green alga *Chlamydomonas reinhardtii* appears highly sensitive to rapamycin. Furthermore, *Chlamydomonas* FKBP12 interacts with rapamycin in vivo to form a complex with the FKBP12-rapamycin binding (FRB) domain of TOR **(Crespo *et al.*, 2005)**. Rapamycin resistance makes the study of the TOR signaling pathways more complicated in plants comparing to yeast or mammals.

The first study on TOR pathway in plants showed that disruption of *TOR* gene in Arabidopsis via insertion of a T-DNA inside the coding sequence of AtTOR is lethal for embryo at early developmental stage**.** Heterozygous plants, however, display no detectable phenotype (**Menand *et al.*, 2002**). The development of *AtTOR* null embryos is arrested at the globular stage. The results suggested that the AtTOR inactivation was not harmful for the cell division machinery due to presence of some cells in divided state within these arrested embryos. Therefore, AtTOR

has been proposed probably required for premitotic cytoplasmic growth during cell proliferation **(Robaglia *et al*., 2004)**.

Two Arabidopsis homologs of *RAPTORs*, *AtRAPTOR1* and *AtRAPTOR2* (also termed *AtRAPTOR1B* and *AtRAPTOR1A*, respectively), were cloned and analyzed by two groups independently (**Deprost *et al.*, 2005; Anderson *et al.*, 2005a**). Both studies show that disruption of the *AtRaptor 2* (*AtRaptor 1A*) gene does not leads to visible phenotypes. Although both studies show that the *AtRAPTOR* genes are necessary for normal development, the phenotypes of knocked out *AtRaptor 1* (*AtRaptor 1B*) are not similar from these two groups. One report showed that the null AtRaptor 1 leads to seed abortion and to a complete arrest of embryo development at an early stage, (**Deprost *et al.*, 2005**). The other study, however, found that *AtRaptor 1B* disruption lines display slow leaf initiation, late flowering, increased branching and a normal growth of the embryo (**Anderson *et al.*, 2005a**). AtRaptor 1B was reported to bind the AML protein (Arabidopsis Mei2-like 1) in vitro (**Anderson *et al.*, 2005b**). Mei2-like RNA binding proteins were identified as putative TOR substrate and a meiosis signaling molecule in *Schizosaccharomyces pombe* **(Watanaba and Yamamoto, 1994)**. Mei2 interacting protein (Mip1) promotes TOR kinase activity under high nutrient conditions in *S.pombe* and is involved in meiotic development **(Shinozaki-Yabana *et al.*, 2000)**. These findings suggest that TOR protein affects indirectly the meiotic development of *S.pombe* via inactivation of the potential activity of mei2 proteins that inhibits the meiosis promoting activity **(Alvarez and Moreno, 2006)**. The interaction of AML1 (Arabidopsis Mei2-like) with Raptor1, suggests that it might be one of the downstream members of plant TOR pathway (**Anderson and Hanson, 2005; Figure 7)**. Mei2 –like proteins are found in plants, some fungi, but neither in mammals nor yeast. Computer analysis for transcripts and putative ORFs revealed presence of nine *mei2*-like genes in Arabidopsis and six in *Oryza sativa*. These predicted genes were divided into four major clades, based on sequence similarity One of these clades, AML1 (*Arabidopsis* mei2-like), is highly expressed in reproductive tissues and seems to be strongly implicated in both meiotic and gametophytic development (**Kaur *et al.*, 2006)**. Another of these clades, TERMINAL EAR-like (TEL) is particularly expressed in central zone of the shoot and root apical meristems, suggesting that the function of these genes might be important to maintain indeterminancy in these tissues (**Anderson *et al*., 2004)**.

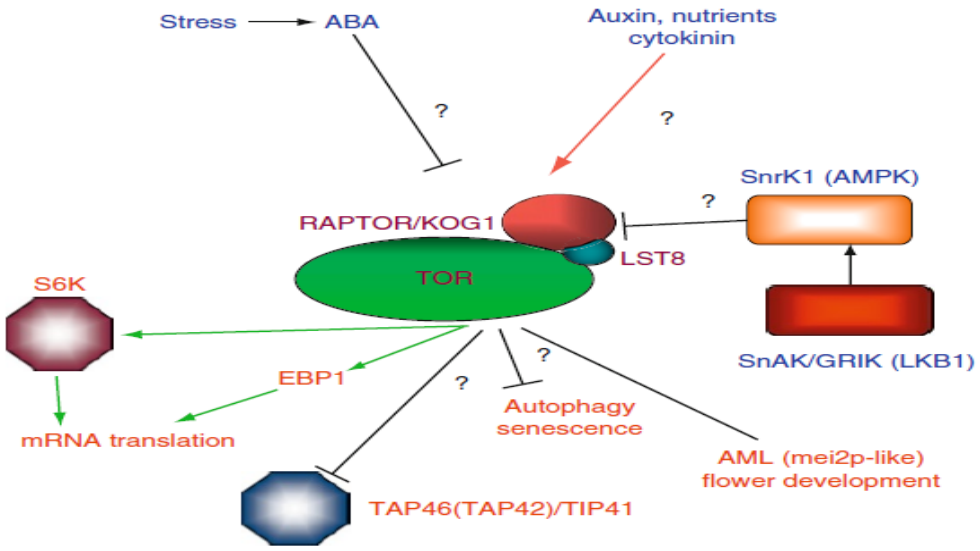


Figure 7: A tentative model of the Arabidopsis TOR signaling pathway. Homologous proteins in other organisms are indicated in parentheses. From Moreau et al., 2010.

Mammalian p70S6K is a downstream component of the mammalian TOR signaling pathway and phosphorylates RPS6 would activates protein translation initiation. In *Arabidopsis*, two S6K homologs with highly similar sequences (87 % identity), originally named AtPK6 and AtPK19 and later referred to as AtSK61 and AtS6K2, respectively were identified **(Zhang *et al.*, 1994; Mizoguchi *et al.*, 1995; Turck *et al.*, 1998)**. A GFP-fusion protein analysis showed subcellular localization of GFP-S6K2 was mainly confined to nucleus/nucleolus (**Mahfouz *et al.*, 2006**). However, p70S6K, which is mostly cytoplasmic, is involved in the phosphorylation of RPS6 in the cytoplasmic ribosomes. In contrast with AtS6K2, AtS6K1 exhibited the distribution toward cytoplasm. It has been proposed that the two Arabidopsis S6K homologs may play distinct functional roles in different subcellular locations (**Mahfouz *et al.*, 2006**). Furthermore, it was reported that AtRaptor 1 interacts with the HEAT repeats of TOR and regulates the activity of S6 kinase (S6K) in response to osmotic stress (**Mahfouz *et al.*, 2006).** Recent study suggested that the 40S ribosomal protein S6 kinase (S6K) represses cell proliferation in Arabidopsis plants through their associated with the Retinoblastoma-related 1 (RBR1)-E2FB complex (**Henriques *et al.*, 2010**). The same study revealed also that S6K is important for maintenance the chromosome stability. 5′TOP (5′ terminal oligopyrimidine tract) mRNAs contain a short (4-14 nucleotides) located adjacent to their 5′ end. Some components of the translational apparatus, like ribosomal proteins, other translational regulators, and elongation factors are encoded by

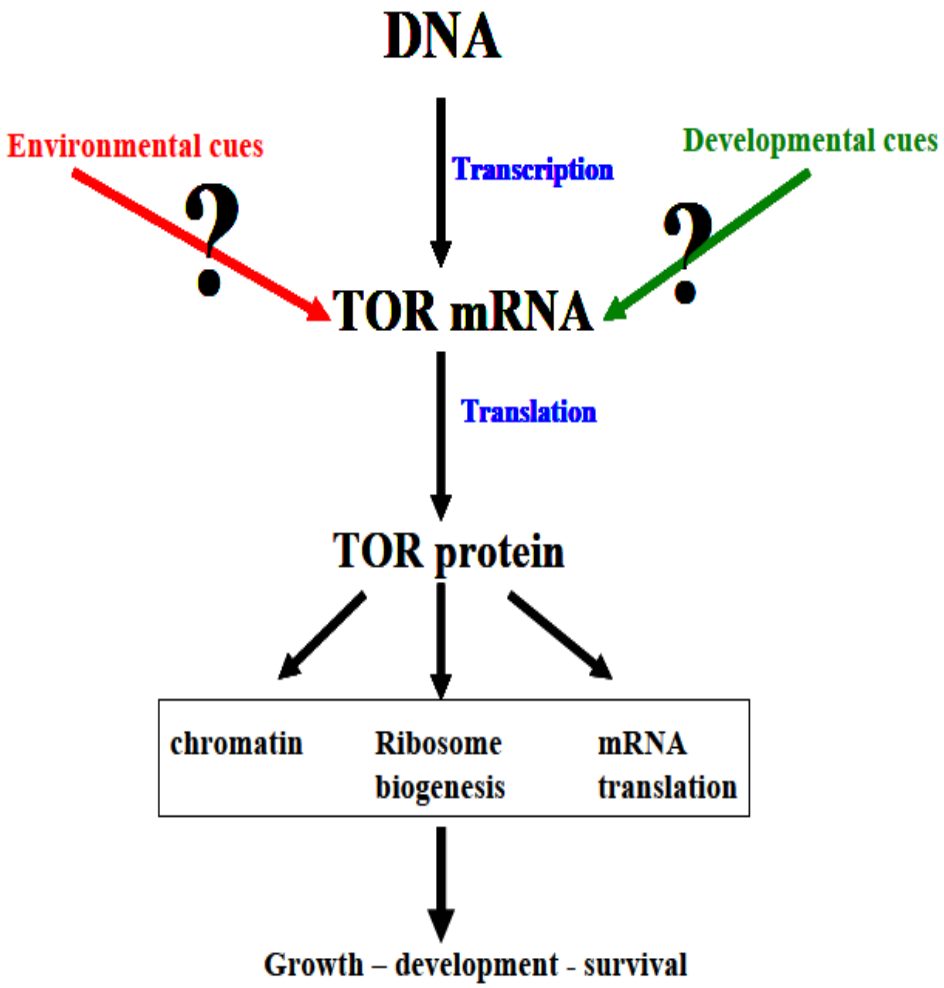
these TOP-containing mRNAs. (**Meyuhas and Hornstein, 2000**). A number of evidences indicate that activated S6K1 affects cell growth control through increased a family of 5′ TOP mRNAs translation. The results revealed that *S6K1* gene has a role in regulating cell division and the expansion of petals and stamens in transgenic Arabidopsis by translational regulation of the 5′TOP sequences in the 5′UTR of the corresponding mRNAs **(Tzeng *et al.*, 2009)**. As in animal cells, Raptor interacts *in vivo* with S6 kinase and regulates its activity towards the ribosomal S6 protein **(Mahfouz *et al.*, 2006; Otterhag *et al.*, 2006; Figure 7)**. In yeast, transduction of TOR kinase signals occurs mainly through two different types of substrates. The first is Sch9 kinase (a functional homolog of S6K) and the second is TAP42/TIP41 phosphatase interaction protein complex **(Huber *et al.*, 2009; Figure 7)**. Both TAP42 (named AtTAP46) and TIP41 were identified in *Arabidopsis* (**Harris *et al*., 1999**).

SnRK1 [Snf1 (Sucrose non-fermenting-1)-related protein kinases act as metabolic sensors to coordinate energy balance and nutrient supply in plants. SnRK1 and their homolog SNF1 and AMPK in budding yeast and animals, respectively are regulated in vitro by phosphorylation of threonin residues within the activation loop or the so called ‘’ T-loop’’. It has been observed that LKB1 kinase regulates TOR activity via phosphorylation of AMPK kinase in animals **(Wullschleger *et al*., 2006; Soulard *et al*., 2009)**. Two kinases were isolated from *Arabidopsis thaliana* called *AtSNAK1* and *AtSNAK2* which are homolog to LKB1 in animals. A recent study reported that both GRIK1 and GRIK2 proteins (also called SNAK2 and SNAK1, respectively) bind to SnRK1 catalytic subunit and phosphorylate the equivalent threonin residue in its activation loop in vitro (**Shen *et al.*, 2009)**. These result suggest that the plant SnRK1 kinase may participate in regulation of the plant TORC1 complex via direct phosphorylation of RAPTOR (**Gwinn *et al.*, 2008; Figure 7**), but it is not yet confirmed experimentally. It was also suggested that function of SnRK/GRIK kinases is similar to the function of LKB1 kinase that is known to regulate TOR activity in animal cells through regulation of AMPK (**Soulard *et al.*, 2009**).

We can summarize that Arabidopsis and other flowering plants seem naturally resistant to rapamycin. It was found that Arabidopsis TOR (AtTOR) kinase is probably required for premitiotic cytoplasmic growth during cell proliferation. Two Arabidopsis RAPTOR genes are important for normal growth and development. The ability of AtRAPTOR1B to bind with Arabidopsis Mei2-Like (AML) protein in vitro, suggested that AML protein might be one of the

downstream targets of TOR pathway in plants. Moreover, it was recorded that Arabidopsis S6 kinase1 (AtS6K1) that is distributed in cytoplasm is another downstream targets of TOR pathway. Even more, it was proposed that plant TORC1 kinase might be regulated by plant SnRK1 kinase like the regulation of animal TOR activity by AMPK kinase.

Figure 8: Diagram represents the regulation of growth and development in eukaryotes with TOR signaling pathway. This regulation by TOR activity is dependent on both inside factor represented by developmental cues and outside factot represented by environmental cues for fine control of vital process like ribosomal biogenesis, and transcript translation.



1.2.8. Expression pattern of AtTOR protein in Arabidopsis

The expression of AtTOR-GUS fusion protein revealed that the AtTOR protein is expressed in embryo, endosperm, primary meristems, and primordia but not in differentiated organs such as fully expanded leaf cells and root tissue (**Menand *et al.*, 2002; Figure 9**).

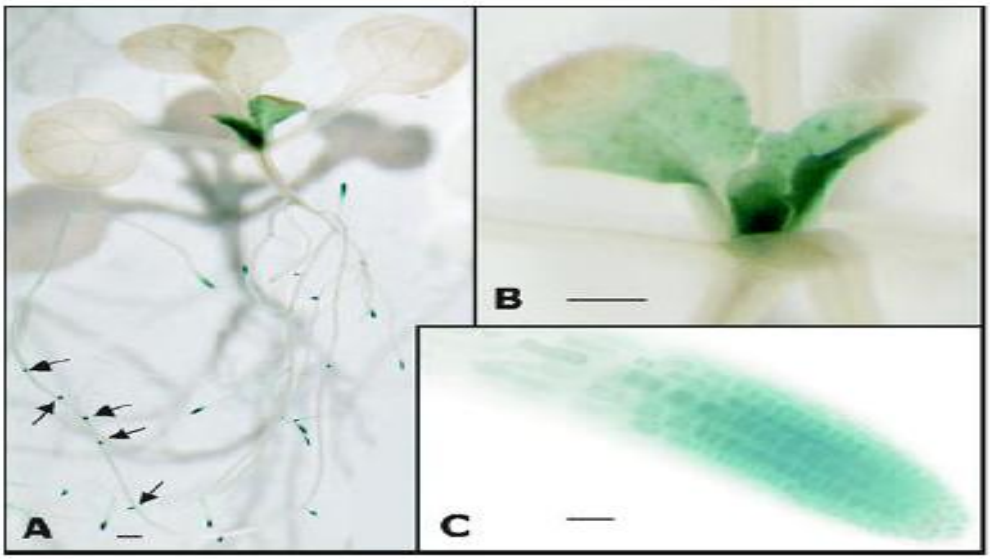


Figure 9: Expression pattern of AtTOR. GUS staining was performed on the heterozygous *TOR/tor-1* mutant (10 days old) to localize the AtTOR::GUS fusion protein encoded by the *tor-1* allele. (A) Whole plant, arrows show the emergence of secondary root meristems. (B) Zoom of leaves primordial. (C) Root meristem. Bar: 1 mm for (A and B); 20µm for (C). From Menand et al., 2004.

This contrast with TOR expression in mammalian cell where mTOR is expressed in all cells whatever their differentiated status (**Brown *et al.*, 1994; Chiu *et al.*, 1994**). Reverse transcriptase-PCR (RT-PCR) experiments designed to detect the *AtTOR* mRNA reveal that it is expressed at nearly equal levels in all plant tissues including differentiated cells at the tip of the leaves and fully expanded leaves where AtTOR-GUS protein is undetectable (**Robaglia *et al.*, 2004**). This leads to the proposal that *AtTOR* is regulated post-transcriptionally by translational repression in differentiated cells.

It has appeared from the previous discussion that the expression of *AtTOR* gene is subjected to different types of regulatory mechanism at different levels, among them the post-transcriptional level. Thus, we will discuss in some details the aspects of these mechanisms with a focus on translation processes.

1.3. Post-transcriptional regulation of gene expression

1.3.1. Post -transcriptional Regulatory System

For any organism, normal growth and development as well as the ability to adapt to environmental changes require the carefully regulated expression of many genes. Gene regulation includes transcriptional, posttranscriptional, translational, and post-translational control. Although more studies were pointed to transcriptional level, post-transcriptional mechanisms also play a fundamental role (**Bertone *et al.*, 2005; Sandelin et al., 2007; Bernstein *et al.*, 2007)**. Post-transcriptional regulation mechanism provides the cell with an extra and fine level of regulation not available through transcription alone. This fine regulation provided by post-transcriptional mechanisms may be particularly important for plants because of their sessile lifestyle.

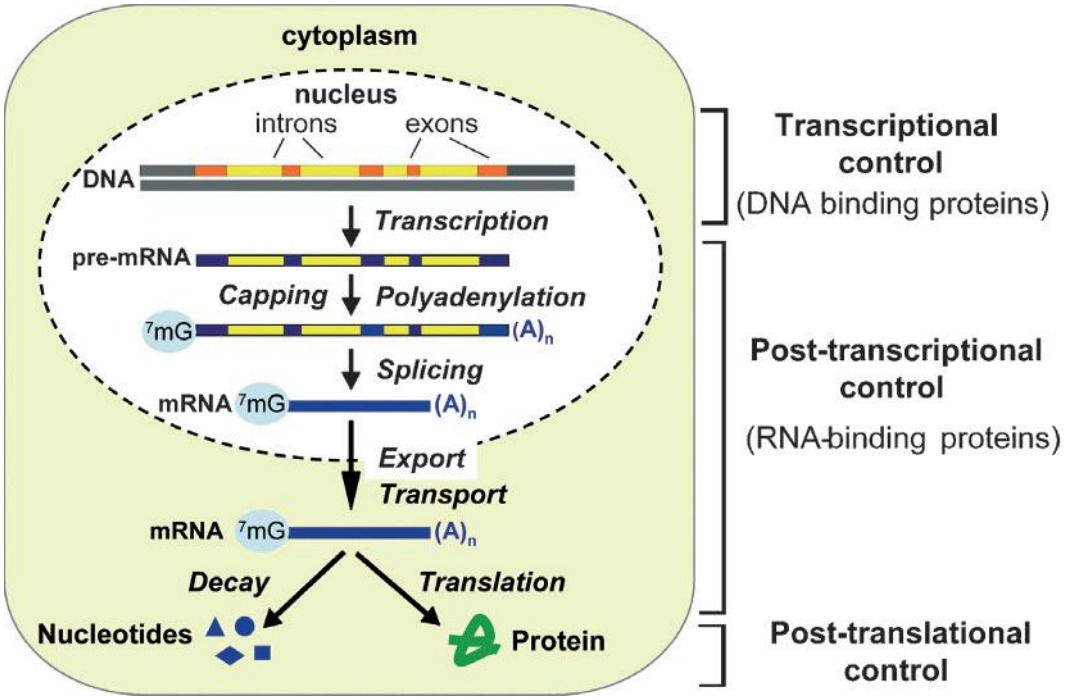


Figure 10: Schematic figure showing regulational levels of gene expression. From Halbeisen *et al.*, 2007.

Gene expression starts in the nucleus and finish in the cytoplasm **(Figure 10)**. New precursor mRNAs forms ribonucleoprotein (RNP) complexes and enter mRNA processing step. RNA processing includes 5′-capping, splicing, editing, and polyadenylation **(Moore, 2005)**. Mature mRNA, go to the cytoplasm across nuclear pores for localization, protein synthesis and

degradation **(Gebauer and Hentze, 2004; St Johnston, 2005)**. Each step of post-transcriptional processing might be regulated with different types of RNA binding proteins or small RNAs **(Mata *et al.*, 2005; Keene, 2007)**.

Three areas from the post-transcriptional regulatory mechanism including localization, degradation, and translation will be discussed in details.

1.3.1.1. Localization of mRNA

The RNA localization allows the presence of gene products in particular cellular compartment. This process is essential for control of gene expression. RNA localization pathway starts with formation of ribonucleoprotein (RNP) transport complex that move to anchoring site in the cell (**Wilhelm and Vale, 1993**). Most RNAs are distributed in active ways to spatially and temporally discrete sites within the cells.

Most information about RNA localization came from yeast, drosophila, or mouse. ASH1 mRNA, encoding for a transcription repressor protein which inhibits mating type switch, is the best examples illustrating the importance of mRNA localization in the determination of cell fate in yeast (**Figure 11 A**). During the production of a new daughter cell (bud) in yeast, *ASH1* mRNA is transferred from mother cell and translated into ASH1 protein in a new daughter cell before division of the cell. Localization of ASH1 protein blockes the distribution of endonuclease HO protein from mother cell to a new daughter cell, thus protecting the daughter cell from the same fate of the mother cell **(Bobola *et al.*, 1996; Long *et al.*, 1997; Cosma *et al.*, 2004)**.

RNA localization is important for maintaining cellular asymmetry and in some cases helps the assembly of protein complexes in polarized somatic cells. β-actin mRNA is targeted to leading edge of lamellipodia in different types of motile cells. These cytoplasmic outgrowths are rich in actin filaments that help in more extension during cell motility. β-actin mRNA is transported to the thin lamellipodia via zipcode binding protein 1 (ZBP1) and translated to β-actin protein monomers. The presence of high concentrations of β-actin monomers at the thin lamellipodia enhances formation of actin polymerization that maintains polarity and motility of motile cells (**Coneelis and Singer 2005, Figure 11 B**).

Localization of mRNAs around particular organelles like nucleus, ER, or mitochondria, plays a role in increasing the efficiency of organelles import of protein encoded by these mRNAs. For example, metallothionein-1 (*MT-1*) mRNA is localized at the nuclear periphery and associated

with perinuclear cytoskeleton in Chinese hamster ovary cells. MT-1 protein is imported to nucleus during G1/S transition phase of the cell cycle.

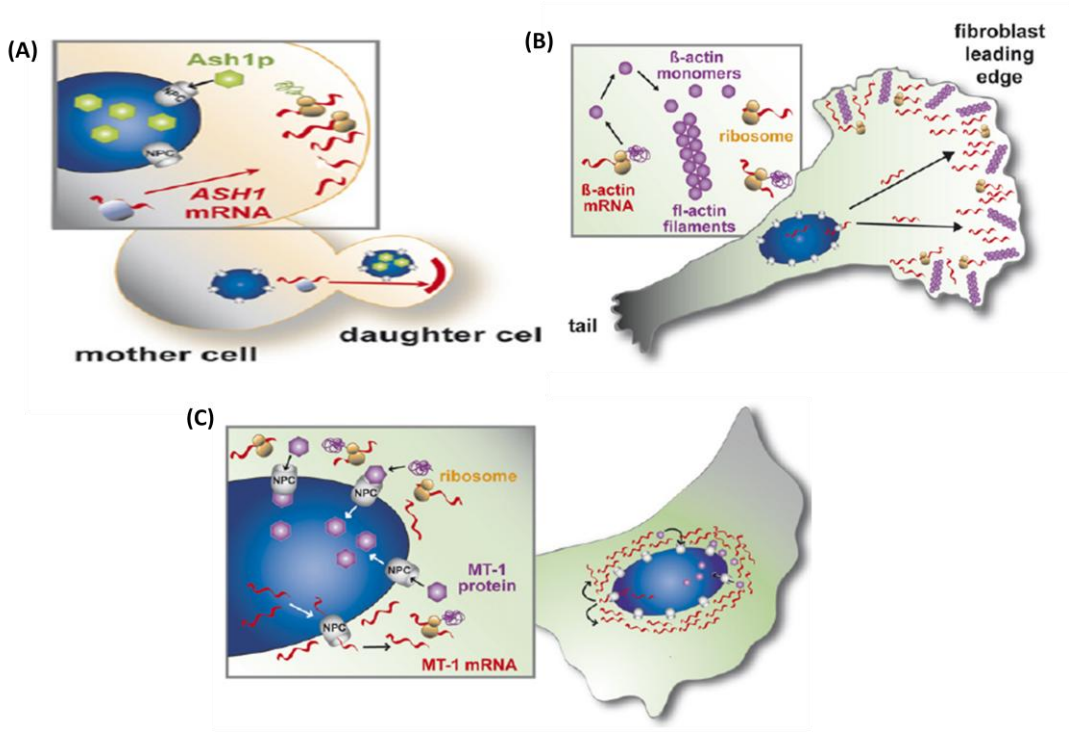


Figure 11: Localization of mRNA performs different functions. (A) Localization of yeast *ASH1* mRNA maintaining asymmetric distribution of cell fate determinants. (B) β-Actin mRNA localizes to the leading edge of chicken embryonic fibroblasts. Local expression of β-actin results in the assembly of this isoform into actin filaments. (C). Localization of Metallothionein-1 (MT-1) mRNA to the perinuclear cytoplasm facilitate import of MT-1 protein into the nucleus. From Tung-Gia *et al.*, 2007.

Replacement of 3′-UTR of *MT-1* mRNA prevents his localization around the nucleus during G1/S transition phase of the cell cycle. These results indicated that the functional role of the MT-1 protein is linked to localization of its mRNA around the nucleus (**Nahon *et al.*, 1997; Levadoux *et al.*, 2006, Figure 11 C**). In *Drosophila*, localization of *bicoid* and *mago nashi* mRNAs is essential for embryo development (**Bashirullah *et al.*, 1998; Palacios and Johnston, 2001**). Little is known about RNA localization in plants, but, some observations about long distance movement of RNA molecules in plants have emerged. The phloem elements (one component of plant vascular system) are responsible for the transport of many signaling molecules such as hormones, proteins and also RNA molecules. RNA silencing mechanism

depends on systematic distribution set of RNA molecules like small RNAs through phloem elements (**Baulcombe, 2004**). In some cases, movement and localization of mRNA is important for control of the developmental events that occurs in response to environmental changes.

Transport of mRNA from cell to cell in plants is mediated by movement protein (MP) **(Lazarowitz, 1999; Lazarowitz and Beachy, 1999)**. Movement proteins (MP) interact with plasmodesmata, microtubules, and ER to induce an increase of their size exclusion limit (SEL). Sucrose transporter 1 (*SUT1*) and Knotted-1 (*KN-1*) mRNAs are transcription factors, important for determination of the cell- fate in vegetative and floral meristems. They are promoting their self transport and also the transport of other RNA molecules through increasing SEL of plasmodesmata. *KN-1* is transported between shoot apical meristem cells as well as the cell layer within the leaf. *SUT1* mRNA has been found at either ends of the plasmodesmata between companion cell (CC) and sieve elements (SE). The transport of SUT1 RNA into the SE supports the idea of long-distance transport of RNA throughout the plant. (**Lazarowitz, 1999; Lazarowitz and Beachy, 1999; Kim *et al.*, 2002**).

Localization and distribution of mRNAs between subcellular structures or organelles have been investigated globally using DNA microarray analysis **(Diehn *et al.*, 2000)**. This analysis allowed to identify and classify about 300 new transcripts as membrane-bound in Arabidopsis (**De Jong *et al.*, 2006)** and more than 5000 membrane-bound and 6000 cytoplasmic/nuclear in human (**Diehn *et al.*, 2006).** The same approach has been applied in yeast to determine transcripts associated with free and mitochondrial-associated ribosomes **(Sylvestre *et al.*, 2003)**. Two groups of RNAs have been identified in different fractions (mitochondrial and cytoplasmic fractions). Transcripts enriched in mitochondrial fraction were preferentially longer and were homologous to bacterial genes, whereas mRNAs in free cytoplasmic polysomes were shorter and of of eukaryotic origin **(Czaplinski and Singer, 2006; Muller *et al.*, 2007)**.

In conclusion, localization of mRNA appeared as a mechanism to optimize gene expression at the post-transcriptional level. Besides their cellular functions such as formation of protein gradients, assembly of protein complexes and determination of cell fate and cell polarity, mRNA localization seems also to perform multiple functions in long-distance signaling transport and coordination with environmental and developmental events.

1.3.1.2. Stability of mRNA

Stability of mRNA appeared as a particular regulatory level from post-transcriptional mechanism. Plants can benefit from this mode of regulation to adapt their patterns of gene expression rapidly in response to different changes in their surrounding environment. Such level of regulation has been indicated through discrepancies between transcription rates and mRNA degradation rates (**Walling *et al.*, 1986**). The balance between mRNA synthesis rate and mRNA degradation rate (i.e. its chemical half-life) represent the accurate steady-state level of a transcript. A majority of transcripts in plants appeared inherently stable with half-lives extended to hours unless they are actively destabilized (**Brawerman, 1993; Sullivan and green, 1993)**. A direct way for measure mRNA decay rates is to measure the degradation kinetics (the half-life) of transcript *in vivo* after treatment with a transcription inhibitor. This is the approach taken by **seely *et al.*, (1992)** to investigate the stability of the phytochrome (*PhyA*) transcript in oat. The steady-state level of *PhyA* mRNA rapidly decreased when the etiolated seedlings were treated with red light. Suggesting that the *PhyA* transcript must be relatively unstable, either in a red-light dependent or constitutive manner. After treatment of oat coleoptiles with cordycepin, a general transcription inhibitor, and measuring the half-life of the *PhyA* mRNA in both the dark and the red light, the results indicated that the *PhyA* transcript is inherently unstable and this regulatory pathway is independent on treatment with red light.

Unstable mRNAs undergo a rapid disappearance from cells under certain conditions. For example, phytochrome mRNA rapidly disappears from coleoptiles of oat seedlings after shafting it from darkness to light (**Quail *et al.*, 1986; Seely *et al.*, 1992**). In tomato fruit, several mRNAs accumulate during ripening in response to the hormone ethylene. Whereas many of these mRNAs appear to be induced at the transcription level, one of them, *E17* shows little or no increase in transcription rate, despite a six fold increase of its mRNA accumulation **(Lincoln *et al.*, 1988)**. This indicated that stabilization of *E17* mRNA occurs in the presence of ethylene. Some studies also suggest that heat shock can cause the selective destabilization of the α-amylase mRNA in barley aleurone cell. It appears that light affects the stability of certain transcripts. For example, it was observed that the ribulose-1, 5-bisphosphate carboxylase small subunit (*rbcS*) transcript is less stable in the light than in the dark in soybean seedlings (**Shirley and Meagher, 1990)**. Further work proposed that this stability effect may also be developmentally regulated **(Thompson and Meagher, 1990)**, because measurements of the

steady-state of *rbcS* mRNA in mature soybeans revealed that the *rbcS* mRNA is more stable in light than in darkness. The light-regulated Fd (*Fed-1*) gene of pea is one of plant genes that are regulated post-transcriptionally at the level of mRNA stability in response to light (**Dickey *et al.*, 1992**). Light regulatory sequences located within the first 230 bp of transcribed region of the *Fed-1* mRNA confer light responsiveness when inserted to the 5'to *cat* or *luc* reporter genes. Run-on transcription assay performed with nuclei from light- and dark- adapted transgenic plants suggest that these sequences do not affect transcription initiation rates. The study concluded that *Fed-1* transcribed sequences cause mRNA degradation in the dark. The 5′-UTR can be involved in regulating mRNA stability. In pea seedlings, for example, it was observed that the 5'UTR of the *LHCB* (light–harvesting chlorophyll binding) transcripts induced mRNA destabilization (**Anderson *et al*., 1999; Gutiérrez *et al*., 1999).**

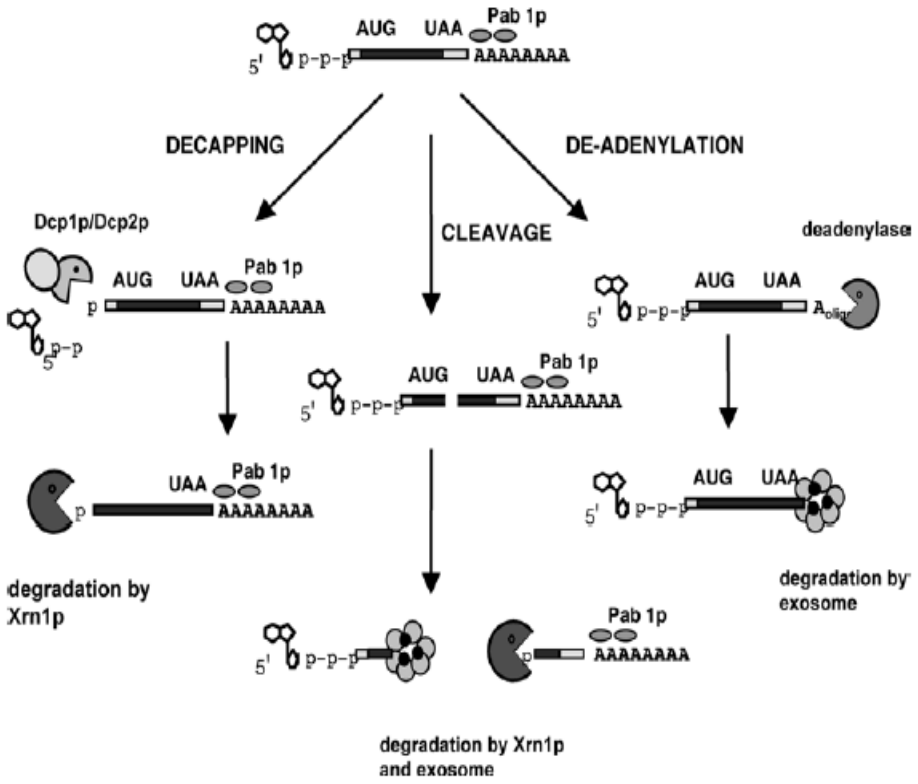


Figure 12: Pathways of mRNA degradation in eukaryotes. From Newbury**,** 2006.

There are several different mechanisms of eukaryotic mRNA decay. The more common mRNA decay pathway is initiated with shortening of the poly (A) tail by deadenylases followed by

decapping the 5' cap structure by the decapping enzymes, Dcp1p and Dcp2p which exist as a complex. The mechanism of action is not completely understood, but it seemed that Dcp2p cleaves the RNA and that this cleavage is stimulated by Dcp1p. m^7 GDP and a 5′-monophosphate mRNA are products of both shortening and decapping process. The decapped intermediates are then degraded either by the cytoplasmic exosome in the 3' to 5' direction or by an exonuclease Xrn1p, the critical exoribonuclease enzyme that is well characterized from yeast *Sacharomyces cerevisiae*, in the 5' to 3' direction **(Parker and Song, 2004; Figure 12)**. Degradation of mRNA can also be initiated by deadenylation-independent decapping via a variety of deadenylases proteins such as the adenylase complex containing the nucleases Ccr4p and Pop2p/Caf1p. Ccr4p and Pop2p/Caf1 are conserved among eukaryotes (**Meyer *et al.*, 2004; Tucker *et al.*, 2001**; **Figure 12**).

There is some eukaryotic mRNA that can be degraded via endonucleolytic cleavage before deadenylation. Two examples of endonuclease cleavage providing access to exoribonucleases are nonsense-mediated decay (NMD) and RNAi. For NMD in *Drosophila*, mRNA containing premature stop codons is cleaved in the vicinity of the stop codon by the ribosome itself or by a ribosome-associated endonuclease. Two fragments will be produced; the 5′ fragment is degraded from the 3′ end by the exosome, the 3′ fragment is degraded from the 5’ end by Xrn1p (**Gatfield and Lzzaurralde, 2004)**. In the case of RNAi, small RNAs (siRNA or miRNA), of 21 – 24 nt length, are produced from double-stranded RNAs by the double-stranded endonuclease Dicer. Then, the siRNAs are incorporated into a RISC (RNA-induced silencing complex) that cleaves mRNAs at a site complementary to the siRNAs. This results in the production of two fragments that are degraded by the exosome and by XRN1 (**Orban and Lzaurralde, 2005; Figure 12**).

Some works have shown that the combination of Xrn1p with the decapping proteins Dcp1 and Dcp2 forms a multicomponent complex known as Lsm proteins that form a heptameric ring around the RNA. Dcp1 and Dcp2 proteins are associated with two other proteins, Pat1p and Dhh1p, forming particular cytoplasmic structures known as P-bodies (**Sheth and Parker, 2003**; **Figure 13)**. When yeast cell grow under stress conditions, mRNA translation is repressed and the arrested mRNAs assemble with particular proteins in form of messenger ribonucleoproteins (mRNPs) and move into P-body for degradation or storage (**Brengues *et al.*, 2005**). Decapping of mRNA represents an irreversible step in mRNA degradation that occurs inside P-bodies. In both yeast and human, P-bodies act as sites where mRNAs are specifically decapped and

degraded. Three proteins named Decapping1 (DCP1), (DCP2), and Varicose (VCS) that represent decapping complex were found to be localize in P-bodies in yeast cells, with only DCP2 having the ability for removing the m^7GDP cap (**Iwasaki et al. 2007**).

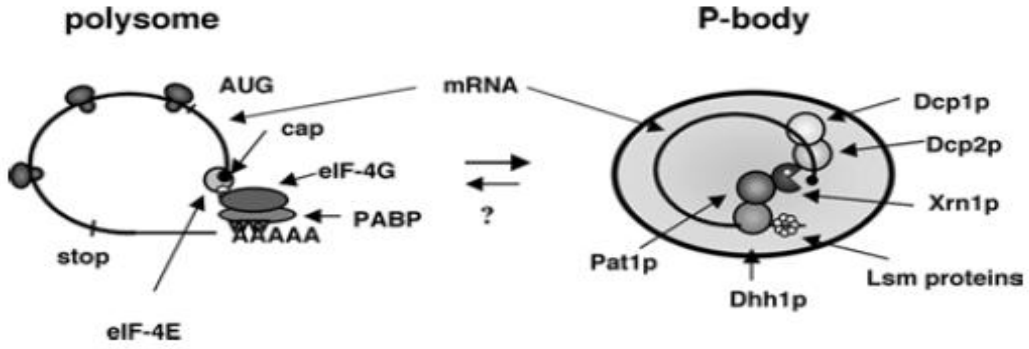


Figure 13: **Transport of mRNAs from polysomes to P-bodies**. Active mRNAs are bound to ribosomes, the initiation factors eIF4E, and eIF4G and to polyadenylated-binding protein (PABP). Once translation of the mRNA is repressed, the RNA moves to a P-body to be degraded or stored. The proteins that move mRNAs into or out of P-bodies are, as yet, unknown. From Newbury, 2006.

Many proteins suchs as SM4 (Lsm4P) and Lsm16/EDC3 are implicated in the formation of yeast P-bodies (**Decker *et al.*, 2007**). However, these two proteins are absent from plants. A recent study suggested that mechanism of P-body formation in plants is different from that in yeast and different protein domains are used for mRNP assembly (**June Xu and Chua, 2009**). The authors identified a new decapping protein in Arabidopsis called DCP5 and having the ability to interact with DCP1 and DCP2 through its C-terminus. Thus, P-body formations, mediated largely by DCP5, play an important role in mRNA decapping during Arabidopsis postembryonic development.

There are particular sequences that govern the rates of mRNA decay. Cis-acting elements located in the 5'-UTR, the coding sequence (CDS) and the 3'-UTR of mRNAs can function as binding sites for RNA-binding proteins that regulate decay **(Pierrat *et al.*, 1993; Raghavan and Bohjanen, 2004; Floris *et al.*, 2009)**.

The implications of specific RNA-binding proteins (RBPs) on RNA turnover have been investigated. **Grigull *et al.*, (2004)** examined the global mRNA turnover in mutant yeast cells from deadenylase components Ccr4p and Pan2p and putative RNA-binding proteins Pub1p and Puf4p after inhibition their transcription by chemicals and/or heat stress. The result of the

examination showed that Ccr4p participates in degradation of transcripts encoding both ribosomal proteins /rRNA synthesis. Pan2p and Puf4p also contribute in the degradation of these mRNAs, while Pub1p, a yeast RNA-binding protein, preferentially stabilized transcripts encoding ribosomal proteins. A second study focused on the role of the interaction of RNA-binding protein Pub1p with 3′ UTR AU-rich elements in destabilization of mRNA. The global decay profile in Pub1 mutants revealed a significant destabilization of proteins involved in ribosomal biogenesis and cellular metabolism, while the genes involved in transporter activity remain stable. Therefore, this result indicates that ARE-binding proteins may differentially determine the fate of mRNA depending on the cellular and environmental context (**Raghavan *et al.*, 2004; Duttagupta *et al.*, 2005**).

Thus, the control of gene expression at the level of mRNA stability occurs in all eukaryotes, including plants. The stability of transcripts is related to changes in exogenous or endogenous stimuli. Multiple factors affects the stability of a given mRNA; cis-acting factors such as specific nucleotide sequences located in different regions of a transcript and trans-acting factors such as RNA-binding proteins that bind many mRNAs or are mRNA-specific. The half-life of many mRNAs can fluctuate in response to developmental or environmental stimuli. Moreover, the global analysis of mRNA turnover revealed the importance of RNA decay in the control of mRNA levels and suggests the presence of specific RNA turnover programs.

1.3.1.3. Translation of mRNA

Translation of mRNA into protein is the last step in gene expression pathway. The process of translation involves three stages: initiation, elongation, and termination. The first stage (initiation) determines the efficiency of translation that includes mRNA interaction with translation factors and ribosomes. More than 25 proteins are needed to achieve proper translation initiation compared to few factors needed for elongation and termination. Thus, translation initiation is considered to be an important determinant for both global and mRNA-specific gene regulation. Protein synthesis is regulated globally via modification of eukaryotic initiation factors **(Gebauer and Hentze, 2004; Holcik and Sonenberg, 2005 Pestova *et al.*, 2007; Floris et al., 2009)**. Total protein synthesis is only slightly affected and the translational efficiency of most mRNAs is not changed when cells are treated cells with elongation inhibitors (e.g.

cycloheximide), supporting the idea that initiation is the rate-limiting step for translation (**Mathew's *et al.*, 2007**).

Most information about molecular mechanisms of translation initiation came from in vitro studies, in which components of translation were purified and used to reconstitute the translational events (**Hinnebusch *et al.*, 2007; Pestova *et al.*, 2007**). Most cellular mRNAs are translated through cap-dependent mechanism, although there is an alternative cap-independent initiation mechanism, responsible for translation of a small number of mRNAs.

1.3.1.3.1. Cap-dependent translational initiation

Translation initiation via cap-dependent mechanism requires at least nine eukaryotic initiation factors (eIFs) and includes two steps. The first step is the formation of 48S preinitiation complexes and the second step involves joining 48S complexes with 60S ribosomal subunits. The 48S preinitiation complexes are composed of ternary complex, 40S ribosomal subunit, eIF1, eIF1A, eIF3, and eIF5 initiation factors (**Jackson *et al.*, 2010; Figures 15**). The ternary complex consists of initiation factor 2 (eIF2), a hetero-trimer of α, β, and γ subunits; methionyl-initiator tRNA (Met-tRNAi Met), and GTP. The assembly of ternary complex components is regulated by the guanine nucleotide exchange factor (GEF) eIF2B. Hydrolysis of GTP to GDP produces inactive eIF2 that results in reduced Met-tRNAi Met affinity**.** Eukaryotic initiation factor 2 **(**eIF2) consists of three subunits- α, β, and γ phosphorylation of the α subunit at residue Ser51 blocks the GTP-exchange reaction by reducing the dissociation rate of eIF2 from eIF2B (**Figure 14**).

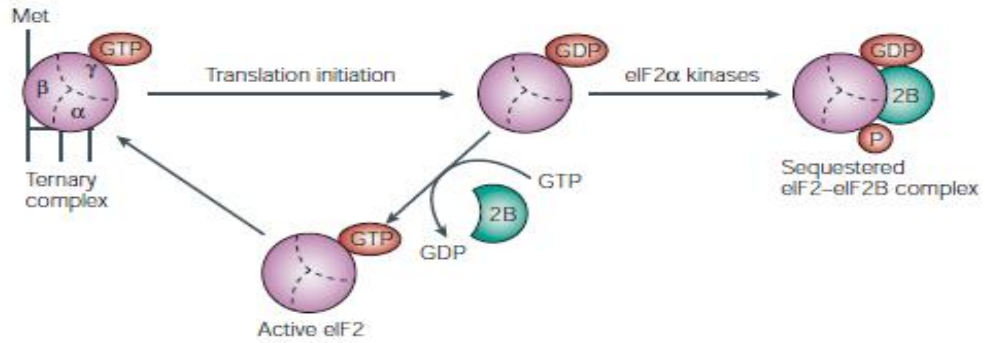


Figure 14: Schematic representation of the components of ternary complex and their activity. Translation initiation factor 2 (eIF2), composed of of α, β, and γ subunits, the initiator tRNA (Met-tRNAi Met) and GTP are components of the ternary complex. After recognition of the AUG start codon GTP is hydrolyzed to produce inactive eIF2 bound to GDP. EIF2B promotes the regeneration of active eIF2. From Gebauer and Hentze, 2004.

Under amino acid starvation, the activated GCN2 (general control non-derepressible-2) kinase promote the phosphorylation of translation initiation factor eIF2 resulting in a global decrease of translation.

Then, the 43S pre-initiation complex starts scanning the 5′UTR and recognizes AUG start codon. Recognition on cap structure at 5′- end of the mRNA is mediated by eIF4F, that consists of the three subunits eIF4E, eIF4G, and eIF4A **(Figure 15**). Translation initiation factor G (eIF4G) act as a scaffold protein for associated cap binding protein (eIF4E) that binds directly to the m7G cap structure and also eIF3 factor in mammalian cells. Translation initiation factor A (eIF4A) is an RNA helicase responsible for removing of secondary structures from the 5'- untranslated region. Removing of this secondary structure allows 43S preinitiation complex to scan along the mRNA more easily. PABP protein is associated with the poly (A) tail at the 3'UTR of the mRNA and also implicated in initiation process. It promotes formation of circular mRNA through binding to the eIF4G scaffold protein.

After binding of the 43S initiation complex to the 5′- end of the mRNA and formation of the 48S initiation complex, the scanning process starts after recognition of the the start codon **(Kozak , 1999)**. Recognition of the start codon occurs through formation of base pairs between the anticodon loop of the initiator tRNA and the AUG start codon (**Figure 15**). Selection of the correct start codon is dependent on eIF1.

Scanning of mRNA with unstructured 5′UTR does not require eIF4F, eIF4A, and ATP, However, structured 5′-UTR mRNA need eIF4F, eIF4B, ATP, and eIF1A. Translation initiation factors like eIF4A helicase and eIF4F promote unwinding of the secondary structure of the mRNA, while eIF1 and eIF1A promote a structural conformation of the 43S preinitiation complex for scanning in the 5′ – 3′ direction. Then, 60S ribosomal subunit joins to the 48S complex and forms the complete unit, i.e. 80S ribosome. Eukaryotic initiation factor 5 (eIF5) and eIF5B play an important role in joining the two ribosome subunits together via promotion of GTP hydrolysis and also dissociation of translation initiation factors (eIFs).

After the translation process is completed, ribosomes must dissociate from the mRNA to restart a new cycle from initiation of translation. Ribosome-recycling factor is responsible for dissociation of ribosome subunits in prokaryotes (**Kisselev and Buckingham, 2000**); however no homolog

for this protein is known in eukaryotes. Some studies suggested that dissociation of ribosome is directly linked to 43S preinitiation complex formation not by eIF3, eIF1, eIF1A, and eIF6 eukaryotic initiation factors (**Pestova *et al.*, 2007; Preiss and Hentze, 2003**).

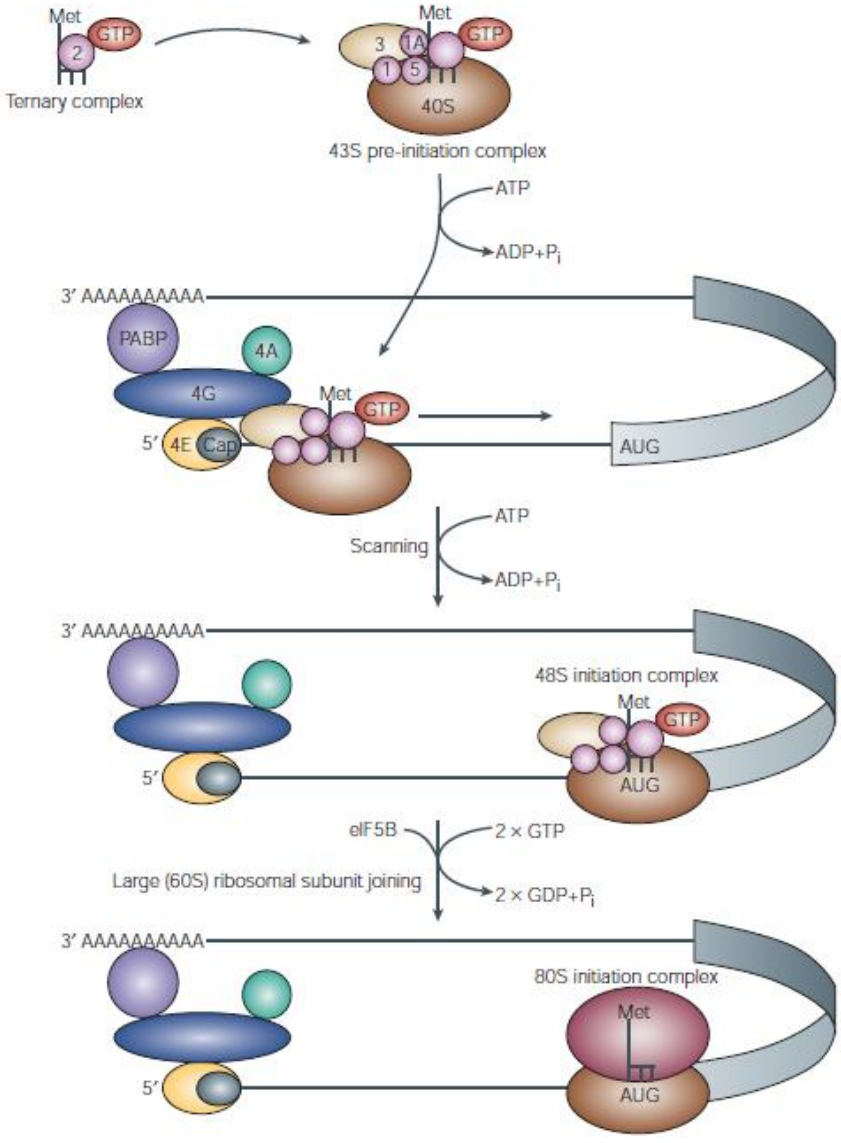


Figure 15: Major molecular events that lead to cap-dependent translation initiation. From Gebauer and Hentze, 2004.

1.3.1.3.2. Cap-independent translation initiation

Most cellular mRNAs are initiating translation by cap-dependent manner, while, some of mRNAs can initiate translation via sequence elements located in the 5′UTR known as internal

ribosomal entry sites [IRES] (**Baird *et al.*, 2006**). Initiation of translation by this mechanism that is called a cap-independent manner was discovered firstly in picornaviruses **(Pelletier *et al.*, 1988)**. IRES sequences were found in many cellular mRNAs encoding regulatory proteins and growth factors. Many developmental possess such as differentiation and apoptosis were shown to be regulated by certain mRNA containing IRES elements (**Doudna and Sarnow, 2007; Elroy-Stein and Merrick, 2007**). In-depth reviews on the topic of IRES are available (**Jackson, 2005; Fraser and Doudna, 2007).**

1.3.2. Untranslated regions (UTRs) and post-transcriptional regulation

1.3.2.1. Characteristic of 5′- and 3′-UTR structures

Untranslated regions (UTRs) play important roles in the post-transcriptional regulation of gene expression. Their roles include modulation of the transport of mRNAs out of the nucleus and modulation of translation efficiency, subcellular localization and stability (**Velden and Thomas, 1999; Jansen, 2001; Bashirullah *et al.*, 2001**). The regulation by UTRs involve several ways, such as regulation via interaction between motifs located in 5′UTRs and 3′UTRs with specific RNA-binding proteins or interaction between sequence elements located in the 5′UTRs and specific complementary non-coding RNAs (**Sweeney *et al.*, 1996**). Some conserved features of the structure of UTRs have been observed by comparison of the various completed genome sequences. The average length of 5′UTR ranges between 100 and 200 nucleotides, whereas the average length of 3′UTRs is much more variable, ranging from about 200 nucleotides in plants and fungi to 800 nucleotides in humans and other vertebrates. The length of both 5′ and 3′UTRs, also, varies a lot within a species, ranging from a dozen nucleotides to a few thousand (**Pesole *et al.*, 2001**). It has been observed using mammalian cells in vitro that only one nucleotide is a sufficient 5′UTR for the initiation of translation (**Hughes and Andrews, 1997**).

Figure 16 shows most of the structural features of both 5′ and 3′ UTRs. Some of these structures play a role in promotion of translation whereas other structures inhibit and prevent translation of mRNA. For example, the 5′-cap structure at the 5' end and the poly (A) tail at the 3'end of the mRNA strongly promote translation initiation, while, secondary structures, such as hairpins, inhibit translation. Internal ribosome entry sequences (IRESs) mediate cap-independent translation. Upstream open reading frames (uORFs) are implicated in reducing translation of the main ORF. The binding sites for proteins or RNA regulator sequences usually inhibit, but

occasionally promote, translation. Other elements are participating in activating poly (A)-tail lengthening of an mRNA such as CPE and AAUAAA signals.

We will focus here on 5′UTR features and their roles in translational efficiency. The 5′-untranslated region (5′-UTR) act as a potent sequence able to influence directly the gene expression in eukaryotes. It can affect the efficiency of translation by particular structures distributed along the 5′-UTR **(Figure 16)**. The efficiency and fidelity of translation from the optimal initiation codon depend, in most cases, on five aspects of mRNA structure. 1- the 5′-cap structure and the poly(A)tail, which are strong promoters of translation initiation; 2- internal ribosome entry sequences (IRESs), which mediate cap-independent translation initiation; 3- upstream open reading frames (uORFs), which normally reduce translation from the main ORF; 4- secondary or tertiary RNA structures, such as hairpins and pseudo- knots, which commonly block initiation, but can also be part of IRES elements and therefore promote cap-independent translation; and 5′- specific binding sites for regulatory complexes, which are crucial determinants of mRNA translation **(Gebauer and Hentze, 2004)**.

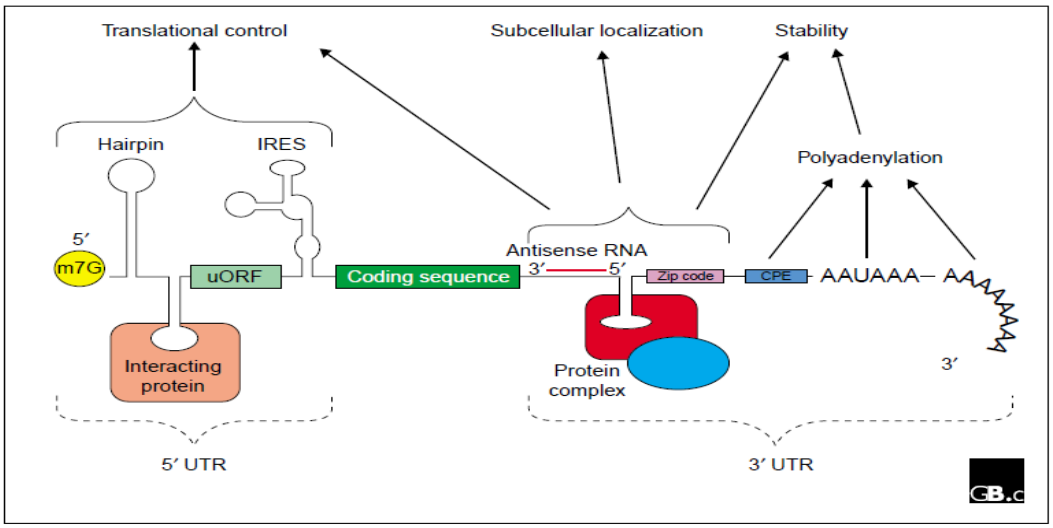


Figure 16: The generic structure of a eukaryotic mRNA, illustrating some elements that influence translation of mRNA. Abbreviations (from 5′ to 3′): UTR, untranslated region; m7G, 7-methyl-guanosine cap; hairpin, hairpin-like secondary structures; uORF, upstream open reading frame; IRES, internal ribosome entry site; CPE, cytoplasmic polyadenylation element; AAUAAA, polyadenylation signal. From Mignone *et al*., 2002.

1.3.2.2. Characterizations of the 5′- untranslated region (5′ UTR)

Kozak (1990) reported that shortening of the 5′-UTR of eukaryotic mRNA usually promotes leaky scanning (i.e. the ribosome bypass around the first AUG on mRNA and start translation at the following AUG) unless there is a well-positioned downstream secondary structure. Lengthening the 5′-UTR cause an increase in translational efficiency due to increased loading of the 40S subunits on the longer 5′-UTRs **(Kozak, 1991a)**. A computational analysis reported that the average length of complete leader sequences of mRNA from dicotyledonous and monocotyledonous plants was 98 and 113 nucleotides, respectively. The same analysis shown that the length of the majority of 5′-UTRs varies from 30 to 110 nucleotides in dicots and from 50 to 120 nucleotides in monocots **(Kochetov *et al.*, 2002)**.

The interaction of mRNAs with translation initiation complex occurs at the 5′ untranslated region sequence. A computational study of mRNA 5′-UTRs from monocots and dicots revealed that the G + C content of 5′-UTR differ between the two groups (**Montero *et al.*, 1990**). Another computational study has shown that leader regions are enriched with C and have a lower content of U in both monocot and dicot genes (**Kochetov *et al.*, 2002**). Also, the same authors reported that 5′-UTR is more riche with G and C residues than the 3′-UTR, introns, and promoters for a plant gene.

G/C and A/U di-nucleotide were characterized in the leader sequences and the computer analysis revealed a pronounced imbalance of their frequencies within the 5′leader (**Kochetov *et al.*, 2002**). The correlation between the 5′-UTR nucleotide composition (mono- and di-nucleotides) and the efficiency of ribosome loading (RL) on the 5′UTR was established for *Arabidopsis* leaves growing under normal and mild dehydration stress (DS) conditions (**Kawaguchi and Bailey – Serres, 2005)**. The results revealed that adenine A, AU, and AC contents positively affect RL, however, G and GU content possess a negative influence on RL under both conditions**.** Also, it was observed that GC has a negative effect on translation. The authers concluded that 5′UTR with high percentage of GC content (above the average 39%) impaired the translation of mRNAs under stress condition, while uracil (U) and cytosine (C) contents had a slightly effect at the same condition.

A considerable number of eukaryotic mRNAs contain 5′ upstream AUG. The presence of upstream AUG (uAUG) in the 5′-UTR, before the main translation start codon in mRNA causes decrease of mRNA translation **(Kozak, 2000 and 2001)**. A bioinformatics analysis have been

carried out on sequences consisting of the 5′UTRs and five nucleotides of the coding sequence from ten species including (*Homo* sp., *Mus* sp., *Rattus* sp., *Gallus* sp., *Xenopus* sp., *Drosophila* sp., *Arabidopsis* sp., *Zea* sp., *Aspergillus* sp., *Saccharomyces* sp.) (**Rogozin *et al*., 2001)**. The analysis shown that from 15 to 53 % of 5′-UTRs in the available cDNA sequences contains upstream AUGs. The sequence context surrounding the first AUG codon also plays an important role in regulation of the translation efficiency when uAUG is recognized as a translation initiation codon. In most cases, a 'weak' start codon context of the main AUG is correlated to the presence of a long 5′-UTR with upstream AUG; however, the transcripts with strong start codon context have short 5′- UTRs without upstream AUGs. These observations suggest that the presence of upstream AUGs might have a role in keeping the low basal level of translation of a gene. **Kawaguchi and Bailey – Serres, (2005**) also recorded that the presence of upstream AUGs (uAUGs) significantly impaired ribosome loading. Generally, mRNAs containing long 5′-UTRs with multiple upstream AUGs have a weak start context, and in contrast, mRNAs containing short 5′-UTRs without uAUGs have a strong start context.

The sequences surrounded AUG initiation codon is not random but fit a consensus sequence. Kozak established that an A or G residue at the -3 position and a G residue at the + 4 position (AUG codon is numbered +1 to +3) strongly contributes to the initiation step in vivo and in vitro based on a collection of 699 vertebrate mRNAs (**Kozak, 1987**). (GCC) GCC (A/G) CCAUGG is the consensus sequence for the context of functional AUG codon. When the purine base at the -3 and + 4 positions were replaced by pyrimidine base a dramatic decrease of over 20-fold in the translation rate of a vertebrate mRNA occurred (**Kozak, 1987**). Similar observation to the vertebrate AUG context sequence have been recorded for 5074 plant genes, where purines (A or G) are present at the -3 and +4 positions in about 80% of the sequences **(Joshi *et al*., 1997).** Detailed analysis between the two major groups of angiosperms recorded that the context of the AUG codon in dicot mRNAs is aaA(A/C) aAUGGCu but monocot mRNAs have c(a/c)(A/G)(A/C)c**AUG**GCG as a consensus that exhibits an overall similarity with the vertebrate consensus. In a recent study, it was shown that *Arabidopsis* exhibits AT-richness around the start codon whereas *Oryza* exhibits GC-richness **(Jaiswal and Rangan, 2007)**.

Secondary structures or base-paired structures in 5′- UTRs are also one of the structural features that influence translational efficiency. The presence of stable RNA secondary structures in the 5′-UTR impaire the ribosome scanning *in vitro* (**Kozak, 1994**). It is assumed that the ability of

RNA to form stable secondary structure is correlated with the G+C content **(Kozak, 1999)**. The most inhibitory effect for base-paired structures appears when their proximity to the 5′-end blocks ribosome entry (**Wang and Wessler, 2001**). Great reduction in translation efficiency occurred for transcripts harboring long 5′-UTR with substantial secondary structure but ribosome scanning was not prevented completely (**Van-der Velden *et al*., 2002)**. Some results recorded that the mRNAs with weak potential secondary structure in 5′-UTR (> -20 kcal/mole) had an advantage in ribosome recruitment, while mRNAs with a higher potential for secondary structure (< -55 kcal/mole) had a significant disadvantage in ribosome recruitment **(Kawaguchi and Bailey – Serres, (2005**). A recent study shown that the secondary structure produced from of *PtDrl02* exon1 sequence, that is relatively high (ΔG = -8.00 kcal mol^{-1}) results in 40.5% suppression on translation (**Zheng *et al*., 2009**).

1.3.3. Upstream open reading frames (uORFs) and translational efficiency

1.3.3.1. Upstream open reading frame (uORF) and ribosomes

Upstream open reading frame (uORF) are considering one of the most important translational control signal located in the 5′ leader of many eukaryotic mRNAs (**Lovett and Rogers, 1996**). Upstream open reading frames are small coding sequences composed of a start codon (uAUG), at least one additional sense codon, and a termination codon. They are found in approximately 20 % of plant genes (**Kochetov *et al*. 2002**). Upstream open reading frames are also common in mammalian and fungal genomes **(Iacono *et al*., 2005; Galagen *et al*. 2005; Zhang and Dietrich, 2005)**. Human genes analysis revealed that about 30% contain uORFs (**Suzuki *et al*., 2000**). **Kim *et al*. (2007)** reported that *Arabidopsis* transcription factors and protein kinases appeared to contain uORFs in high frequencies. In *S.cerevisiae*, it was estimated that 6 % of the expressed genes contain uORFs (**Nagalakshmi *et al*. 2008**). The comparative analysis of full length cDNA 5′UTR sequences from Arabidopsis and rice has revealed new homology groups of conserved peptide uORFs (**Hayden and Jorgensen, 2007**). The majority of them were discovering for the first time, indicating that the predicted conserved peptide-uORFs is widespread between plant genes **(Hayden and Jorgensen, 2007)**. In parallel, the comparison analysis of the human and mouse genomes suggested that the majority of uORFs are strongly conserved at the peptide level (**Crowe *et al*., 2006**).

Translation of uORF sequences occurs according to ribosome scanning model, in which the ribosome recognizes uORF start codon and start translation initiation. During the uORF scanning with ribosome, it will face different fates after uORF translation (**Morris and Geballe, 2000; Figure 18**). Ribosomes either remain associated with the mRNA to reach either a proximal or distal AUG codon (**Figure 18**, options 1 and 2), or stall during uORF translation preventing new ribosomes from scanning (**Figure 18,** option 3). In some cases, uORFs can also affect mRNA stability through the nonsense-mediated mRNA decay (NMD) pathway (**Figure 18,** option 4) as in yeast *CPA1* transcript (**Ruiz-Echevarria and Peltz, 2000**). Ribosome can dissociate after completing uORF translation (**Figure 18**, option 5).

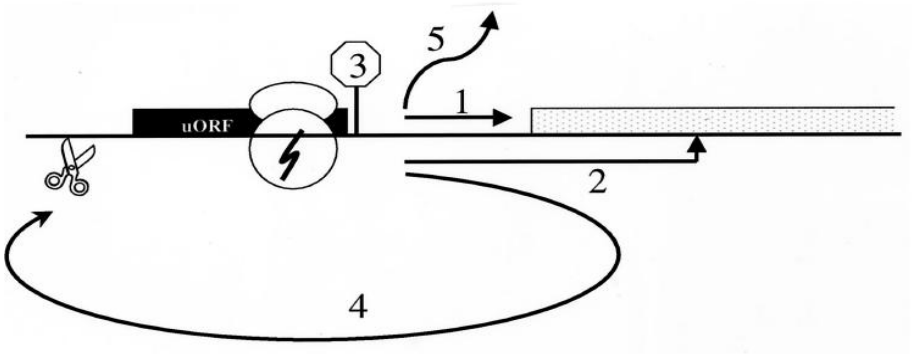


Figure 18: **Alternative fates available to a ribosome after translating a uORF**. From Morris and Geballe, 2000.

The starting of translation at downstream main ORF and control of translation rate are governed by presence of upstream functional uORF. In general, inhibition of efficient initiation at the start codon of the main ORF resulted from translated uORFs.

1.3.3.2. Control of translation by uORFs

Upstream open reading frames (uORFs) were recognized to play an important role in control of gene expression at level of translation. The upstream open reading frames have been grouped into two types according to their mode of action either *in-vitro* or *in-vivo*. The first is sequence-dependent uORF; in which uORF encodes bioactive peptides that directly affect translation via either ribosomal stalling during translation of the uORF or termination of translation via inhibiting formation of the peptide bond (**Gaba *et al*., 2001; Luo and Sachs, 1996**). The second

is sequence-independent uORF, where the peptide encoded by the uORF is not important for translational control. Other factors like uORF length, stop codon sequence context, uORF start codon context, as well as, both the length and nucleotide composition for the region between stop codon of uORF and start codon of the main ORF, can affect re-initiation efficiency at the main downstream ORF (**Vilela and McCarthy, 2003; Meijer and Thomas, 2002**). Also, translation can be affected indirectly by sequence-independent uORF by allowing ribosomes to bypass inhibitory stem-loop secondary structures or activate dormant internal ribosome entry sites (IRES) (**Hemmings-Mieszczak *et al.*, 2000; Yaman *et al.*, 2003**). The majority of studies revealed that the action of uORFs is an amino acid sequence-independent manner (**Kozak, 2000; Hayden and Jorgensen, 2007; Tran *et al.*, 2008**). The few examples of sequence-dependent uORFs involve the arginine-responsive mRNAs encoding the small subunit of arginine-specific carbamoyl phosphate synthetase (*CPA-1*) of S.cerevisiae (**Delbecq *et al.*, 1994**), *arg-2* of *Neurospora crassa* (**Fang *et al.*, 2000**), the cytomegalovirus gpUL4 mRNA (**Alderete *et al.*, 1999**), the mammalian AdoMetDC mRNA (**Raney *et al.*, 2000**), the *Medicago truncatula* HAP2-1 (MtHAP2-1) transcription factor (**Combier *et al.*, 2008**), and Arabidopsis transcription factor bZIP11 (**Rahmani *et al.*, 2009**).

Translational control by uORFs was identified in a number of plant genes. Opaque-2 (O2) mRNA is a transcription factor regulating the synthesis of zeins, the major endosperm proteins of maize. **Lohmer *et al.* (1993)** investigated the effect of the three uORFs present in O2 mRNA on its translation *in vivo* by a transient assay. They reported that these uORFs inhibit the activity of O2 and this inhibiting effect needs at least the presence of uORF1 and uORF2. *Leaf colour* (*Lc*) mRNA encodes a transcription factor required for activation of the anthocyanin biosynthetic pathway. It was found that the presence of 38 codon uORF at their 5′UTR resulted in repressed translation of the downstream *Lc* ORF (**Wang and Wessler, 1998 and 2001**).

Translational regulation with uORF can be important for normal growth and development. One of these important processes for plant growth and their development is polyamine biosynthesis that is regulated by S-Adenosylmethionine decarboxylase (AdoMetDC) enzyme. Two overlapping uORFs are located on the long 5′ UTR (about 500 bp) of plant AdoMetDC mRNA. The upstream tiny uORF (encoding for 3-4 amino acids) and downstream small uORF (encoding for 50-54 amino acids) overlap by one nucleotide (**Franceschetti *et al.*, 2001**). The disruption of small uORF by mutation resulted in increased translation of the downstream AdoMetDC ORF in

transgenic tobacco plants. Increasing the translation rate of AdoMetDC mRNA resulted in an increased the enzyme activity causing a high accumulation of decarboxylated AdoMet and disruption in polyamine concentrations. The presence of high concentrations from polyamine resulted in severe growth perturbations (**Hanfery *et al*., 2002**).

Some studies reported that the nutrients and metabolites also control translation through uORFs. For example, translation of the yeast GCN4 (general control nonderepressible 4) gene is induced by amino acid starvation (**Hinnebusch, 1997**). Also, higher concentrations of nutrients and metabolites control translation of transcription factor AtbZIP11/ATB2 in Arabidopsis. The 5′ UTR of the bZIP11 mRNA contains four uORFs. The most important one is the second uORF (uORF2), which is coding for 42 amino acids and is well conserved among bZIP11 homologous genes. It was observed that the translation of the bZIP11 mRNA is repressed through a general mechanism known as sucrose-induced repression of translation (SIRT) (**Wiese *et al*., 2004**). Recently, it was found that the uORF2 element encodes a sucrose control peptide (Sc-peptide) of 28 residues that is required and sufficient for SIR. This SC-peptide in combination with high concentration of sucrose leads to ribosome stalling and translational inhibition of the main ORF of bZIP11 mRNA (**Rahmani *et al*., 2009**).

MtHAP2-1 is a transcription factor from the model legume *Medicago truncatula*. The HAP protein binds to the CCAAT motif that is found in 30 % of eukaryotic promoters (**El Yahyaoui *et al*., 2004**). *HAP* genes play an important role in regulation of some developmental processes such as embryogenesis, flowering –time control, and abscisic acid responses (**Ben-Naim *et al*., 2006; Warpeha *et al*., 2007**). **Combier *et al* (2008)** recorded that alternative splicing occurs for the intron sequences in the NtHAP2-1 5′ leader sequence during the development of root nodules, causing the generation of a small peptide called uORF1p. The results revealed that uORF1p causes a reduction in MtHAP2-1 transcript accumulation and may influence local expression pattern of MtHAP2-1 within the nodule. Interestingly, they discovered that uORF1p is able to act in a trans-acting manner differently to all other known examples, in which the uORF acts only in cis-acting manner to down-regulate gene expression.

1.3.4. Implication of 5′UTR and uORF in control of transcription and mRNA stability

As we mentioned above, 5′UTR or uORF act as a regulatory elements controlling gene expression at the level of mRNA translation. However, few papers showed that these regulatory

elements impact gene expression via the stability or mRNA abundance before translation. Thus, some lines of evidence suggest that 5′-UTR can act in transcription regulation. For example, LAT59 (late anther tomato) mRNA is expressed in the pollen tissues of tomato plants. **Curie and McCormick, (1997**) shown that accumulation of LAT59 mRNA strongly decreased in the presence of their 5'UTR sequenc, while, both of their translation and stability were not affected. The authors proposed that the 5′UTR is responsible for transcription inhibition of LAT59 mRNA through participation of a potential stem-loop secondary structure located within their 5'UTR.

The pyrroline-5-caboxylate reeducates (P5R) is the last enzyme of the biosynthetic pathway of amino acid proline. This enzyme is regulated during developmental processes and also during salt stress (**Verbruggen *et al*., 1993**). Some results demonstrated that the stabilization and translational inhibition of *Arabidopsis P5R* (At-P5R) mRNA is related to the first 92 bp of AtP5R 5′UTR region during salt and heat stresses **(Hua *et al*., 2001)**. Also, they showed that this 5′ UTR region (the first 92 bp) is involved in transcription efficiency in a promoter-dependent manner. A recent study showed that the 5′-UTR sequence of *PtDrl02* gene, one of the 13 toll/interleukin-1 receptor domain – nucleotide binding site domain (TIR-NBS) genes in *Populus trichocarpa*, confer a negative effect on gene expression at both the transcription and translation level (**Zheng *et al*., 2009**).

Upstream open reading frame (uORF) can influence mRNA stability through two known pathways. The first one was found in yeast in which, the presence of uORF in the 5′UTR trigger the nonsense-mediated mRNA decay pathway and lead to decapping of the mRNA (**Ruiz-Echevarria and Peltz, 2000**). Alternatively, the decay pathway of mRNA depends on the termination codon of uORF. In this case, the 40S ribosome units dissociate from the mRNA due to stop codon flanking sequence (e.g., GC rich) or short intercistronic sequence containing a secondary structure. Dissociation of the 40S ribosomal units prevents reinitiation of translation downstream of the uORF and consequently subjects the mRNA to decay (**Vilela *et al*., 1999**).

In conclusion, uORFs are one of the most important translational control signals found in the 5′UTR of mRNAs in both eukaryotes and prokaryotes. MicroORFs are widespread in the eukaryotic genomes but their impact on translation has been studied in detail in relatively few cases. The mechanism of uORF action in translational repression depends on characteristics of uORF and their surrounding sequences not on amino acids-encoded by uORF for the most examples studied.

1.3.5. Post-transcriptional regulation in response to abiotic stress (Int. J. Mol. Sci., 2009)

The different modes of post-transcriptional regulation of gene expression described previously are particularly important for plant adaptation to abiotic stress. This aspect is discussed in the following review published in the International Journal of Molecular Sciences (2009).

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Review

Post-transcriptional Regulation of Gene Expression in Plants during Abiotic Stress

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Abstract: Land plants are anchored in one place for most of their life cycle and therefore must constantly adapt their growth and metabolism to abiotic stresses such as light intensity, temperature and the availability of water and essential minerals. Thus, plants' subsistence depends on their ability to regulate rapidly gene expression in order to adapt their physiology to their environment. Recent studies indicate that post-transcriptional regulations of gene expression play an important role in how plants respond to abiotic stresses. We will review the different mechanisms of post-transcriptional regulation of nuclear genes expression including messenger RNA (mRNA) processing, stability, localization and protein translation, and discuss their relative importance for plant adaptation to abiotic stress.

Keywords: plants; abiotic stress; gene expression; post-transcriptional regulations

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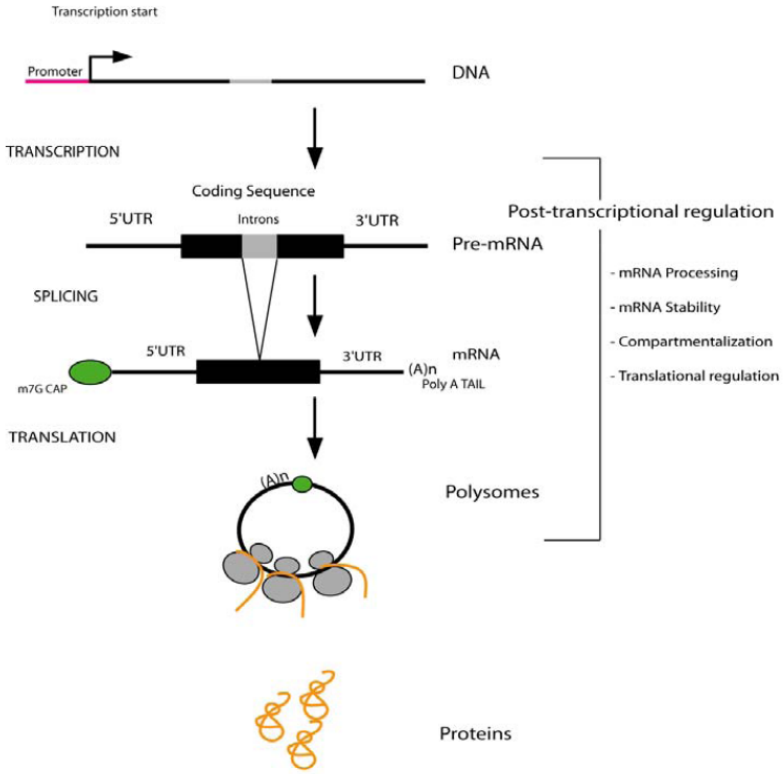
1. Introduction

Land plants are anchored in one place for most of their life cycle and therefore must adapt their physiology and development to environment variables such as light intensity, temperature and the availability of water and nutrients. For example, plants always have to deal with sunlight intensity, which varies along the day and also at the seasonal scale. On one hand, excess of light leads to cellular damage due to oxidative stress triggered by reactive oxygen species (ROS) accumulation. On the other hand, low light intensity results in a reduced growth of the plant due to diminished photosynthesis. Another example of the big impact of environment on plant growth is the availability of nutrients (such as phosphate and nitrate) and water, which are often limiting in the soil. Thus, subsistence of plants depends on the rapid regulation of gene expression in order to adapt their physiology to abiotic stresses. The expression of nuclear genes is highly regulated at both transcriptional and post-transcriptional level. Post-transcriptional regulations of gene expression occur at the levels of pre-messenger RNA (mRNA) processing (capping, splicing, and polyadenylation), mRNA stability, and mRNA translation (Figure 1). Here we will review the evidences of these mechanisms of post-transcriptional regulation during abiotic stress response in plants.

Figure 1. Post-transcriptional regulations of gene expression.



Gene expression should be controlled both at transcriptional and post-transcriptional level in order to fine-tune protein production. We define post-transcriptional regulations by all the regulated steps from mRNA to protein synthesis (mRNA processing, stability, compartmentalization translation).

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2. Regulation of mRNA Processing

Transcription of protein-encoding genes gives rise to precursor mRNAs that are capped, polyadenylated and spliced before being translated into proteins. Splicing results in the excision of introns sequences from the pre-mRNA and is mediated by the spliceosome. Alternative splicing allows production of more than one mRNA from a single gene. Bioinformatics and experimental data indicate that 30% of the *Arabidopsis thaliana* (thale cress) transcripts may be alternatively spliced [1,2].

SERINE/ARGININE-RICH (SR) proteins are a part of the spliceosome and act as splicing regulators in eukaryotes. In *A. thaliana*, cold and heat stresses regulate the alternative splicing of the pre-mRNAs for many SR encoding genes with different splicing functions under stress conditions [3]. Using a genetic screen, Lee *et al.* identified *STABILIZED1 (STA1)*, a gene coding for a nuclear pre-mRNA splicing factor that is important under cold stress conditions in *A. thaliana* [4]. The *Sta1-1* mutant shows a defect in the splicing of the *COLD-REGULATED 15A* (*COR15A*) pre-mRNA leading to hypersensitivity to chilling, to salt stress and to the stress responsive hormone abscissic acid (ABA). Expression of *STA1* itself is up-regulated under cold stress. These results show that STA1 is involved in regulating the splicing and turnover of transcripts and allows resistance to cold stress.

Several lines of evidence indicate that RNA-BINDING PROTEINS (RBPs) also have a crucial role in the regulation of mRNA splicing. RBPs are characterized by the presence of an RNA Binding Domain made of an RNA recognition motif. GLYCINE-RICH-RNA BINDING PROTEINS (GR-RBPs) have an N-terminal RNA recognition motif and a C-terminal tail of variable length enriched in glycine residues [5]. Plant GR-RBPs have been implicated in responses to changing environmental conditions, particularly cold stress [6,7]. Plants over-expressing *GR-RBP* show a better tolerance to cold stress. GR-RBPs might regulate the processing and/or the stability of mRNAs that are highly expressed during stress conditions. They can act as chaperones that modulate RNA-RNA interactions or shuttle the mRNA for efficient processing. Under stress conditions, GR-RBPs may stabilize mRNA either during the transfer from the nucleus to cytoplasm or directly in the cytoplasm, allowing efficient mRNA processing.

OLIGO URIDYLATE BINDING PROTEIN 1 (UBP1) is another protein implicated in mRNA stability and pre-mRNA splicing. UBP-ASSOCIATED (UBA) proteins are *A. thaliana* nuclear RBPs that interact with UBP1 [8]. Transient expression of *UBA1* in protoplasts leads to an increased accumulation of mature reporter mRNA, independently of splicing efficiency [9]. This data suggests that UBA proteins may stabilize mRNA in the cytoplasm. ABA-ACTIVATED PROTEIN KINASE (AAPK) - INTERACTING PROTEIN 1 (AKIP1) from *Vicia faba* (bean) is a homolog of *A. thaliana* UBA1. AKIP1 interacts with the guard cell protein AAPK that controls stomatal pores aperture and ions channels [10,11]. AAPK phosphorylates AKIP1 after treatment with ABA. Once phosphorylated, AKIP1 binds and stabilizes mRNA encoding the DEHYDRIN protein, which is implicated in cell protection. Together these data suggest that splicing is a step at which post-transcriptional regulation occurs during stress.

Another level of regulation through mRNA processing was revealed by a forward genetic approach. Zhang *et al.* identified *oxt6* (*oxidative stress tolerant 6*), an *A. thaliana* mutant that tolerates oxidative stress [12]. The *oxt6* mutation was caused by a T-DNA insertion in *At1g30460,* a gene encoding the *A. thaliana* ortholog of the 30-kD subunit of the CLEAVAGE AND POLYADENYLATION

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SPECIFICITY FACTOR CPSF30. Wild-type growth and stress susceptibility of *oxt6* could be restored by expression of *CPSF30*. The authors suggest that the CPSF30 protein is involved in the processing of pre-mRNA prior to polyadenylation [13]. Therefore, a deficit of CPSF30 might alter the site of polyadenylation for particular mRNAs. Zhang *et al*. showed that the poly(A) site choice is different between *oxt6* and the wild-type plants and is dependent on the presence of CPSF30 [12]. Together, these results indicate that a polyadenylation factor subunit can influence stress tolerance responses. All these data reveal the importance of mRNA processing regulation for stress tolerance in plants.

3. Regulation of mRNA Stability by RNA Silencing

RNA silencing is a mechanism involved in gene expression regulation during plant development, responses to virus infection and in response to abiotic stress [14]. While this mechanism has been first described in transgenic plants, it was further shown to be conserved among eukaryotes [15]. RNA silencing implicates short RNA molecules that inhibit gene expression in a sequence specific manner at the level of transcription, mRNA stability or translation. Endogenous small RNAs (sRNAs) from 20 to 25 nt are processed from non-coding double-stranded (ds) RNA precursors by RNAses of the DICER-LIKE (DCL) family [15]. One strand of the sRNAs duplex is then loaded into an ARGONAUTE (AGO) protein to form the so-called RISC (RNA Induced Silencing Complex) complex. This ribonucleoproteic complex recognizes mRNAs that present a partially or fully complementary sequence to the sRNA. Once sRNA and mRNA hybridize, the RISC complex silences expression of the target mRNA by triggering its cleavage and/or inhibition of its translation [16,17]. There are various classes of sRNAs, mainly differing by their biogenesis. Most known post-transcriptional regulations mediated by RNA silencing involve microRNAs (miRNAs). miRNAs are produced from *MIRNA* precursors, called pri-miR, that form an intramolecular double strand hairpin structure[18].

3.1. Implication of sRNAs in Abiotic Stress

The first indication that RNA silencing could be involved in abiotic stress responses was provided by the computational identification of plant sRNAs and their corresponding mRNA targets [19]. Several of these potential targets encode stress response proteins such as SUPEROXIDE DISMUTASES, LACCASES and ATP SULFURYLASES (APS). At the same time, sRNAs differentially regulated or specifically expressed under stress were cloned from *A. thaliana* seedlings grown under stress conditions [20]. More recently, a microarray-based large-scale analysis identified fourteen stress-regulated miRNAs that were induced by high salinity, drought or cold [21]. A computational approach in *A. thaliana*, confirmed by transcriptome experiments, has also shown that eight miRNAs are differentially expressed in response to low temperature [22]. Further analysis of the *MIR* loci encoding these miRNAs revealed known stress-related *cis*-regulatory elements in their promoter regions [22]. These data suggest that RNA silencing is an important component of the plant stress response pathway.

Few sRNAs have been characterized in detail for their involvement in abiotic stresses such as oxidative, nutrient and salt stress. Among these few examples, we can distinguish two mechanisms:

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(1) sRNAs that are induced under stress conditions and which repress negative regulators of stress tolerance, and (2) sRNAs whose expression is downregulated by stress to allow the accumulation of positive regulators of stress tolerance. Below we present three examples that illustrate the action of these two mechanisms in response to abiotic stress conditions.

3.2. sRNAs Up-Regulated by Stress

3.2.1. miR399 and Phosphate Starvation

Phosphorus (P) is an essential nutrient for plant growth and development. Therefore plants have developed a set of responses to enhance inorganic phosphate (Pi) uptake under phosphate starvation. These responses involve increasing Pi uptake activity, modification of root architecture, secretion of organic acids or phosphatase, remobilization of internal P and association with mycorrhizial fungi [23]. Among the large number of genes involved in the phosphate metabolism pathway, *UBC24* (*At2g33770*) encodes UBIQUITIN CONJUGATING ENZYME 24, which is involved in the targeted protein degradation pathway [24]. UBC24 regulates Pi transporters availability to prevent nutrient overloading. Bioinformatics analysis identified six loci in *A. thaliana* and eleven in *Oryza sativa* (rice) that might be targeted by a miRNA called miR399 [19]. This miRNA has five potential target sites in the 5'-untranslated region (5'UTR) of the *UBC24* transcript and two sites appear predominant for miR399-guided cleavage. Pi deficiency induces reduction of the *UBC24* transcript amount in parallel to an induced transcription of the *MIR399* precursor [25,26]. Accumulation of miR399 appears to be a specific response to Pi deficiency, as it does not occur under any other stresses tested [26] [27]. Transgenic plants overexpressing *MIR399* do not accumulate *UBC24* mRNA under high Pi, confirming that miR399 negatively regulates *UBC24* expression [26]. Furthermore miR399 overexpression results in the over-accumulation of Pi in the shoot and the appearance of the symptoms of Pi toxicity. These experiments suggest that the regulation of *UBC24* by miR399 plays an important role in the Pi signalling pathway and is part of the adaptive response to Pi starvation (Figure 2).

3.2.2. Natural Antisense sRNAs and Salt Stress

Another class of sRNAs, called natural antisense small interfering RNAs (nat-siRNAs), has been identified from plants exposed to salt stress [28]. Nat-siRNAs are produced from intermolecular dsRNA formed by complementary transcripts. In the example described by Borsani *et al.* the *P5CDH* (*Δ-PYRROLINE-5-CARBOXYLATE DESHYDROGENASE*) gene, that encodes a proline catabolism enzyme, is constitutively transcribed under normal conditions [28]. Another gene, *SRO5* (*SIMILAR TO RADICAL-INDUCED CELL DEATH ONE 5*), is transcribed from the same DNA locus as *P5CDH* but on the opposite direction. Whereas only *P5CDH* is transcribed under normal conditions, salt stress activates transcription of both genes, leading to the accumulation of two partially complementary mRNAs. The resulting dsRNA is cleaved by DCL in order to produce nat-siRNAs duplexes. These siRNAs are incorporated into the RISC complex and trigger silencing of *P5CDH* and *SRO5*. This partial inhibition of proline catabolism allows a better tolerance to salt stress. Bioinformatics analyses predict the existence of more than two thousand pairs of natural antisense transcripts in *A. thaliana*

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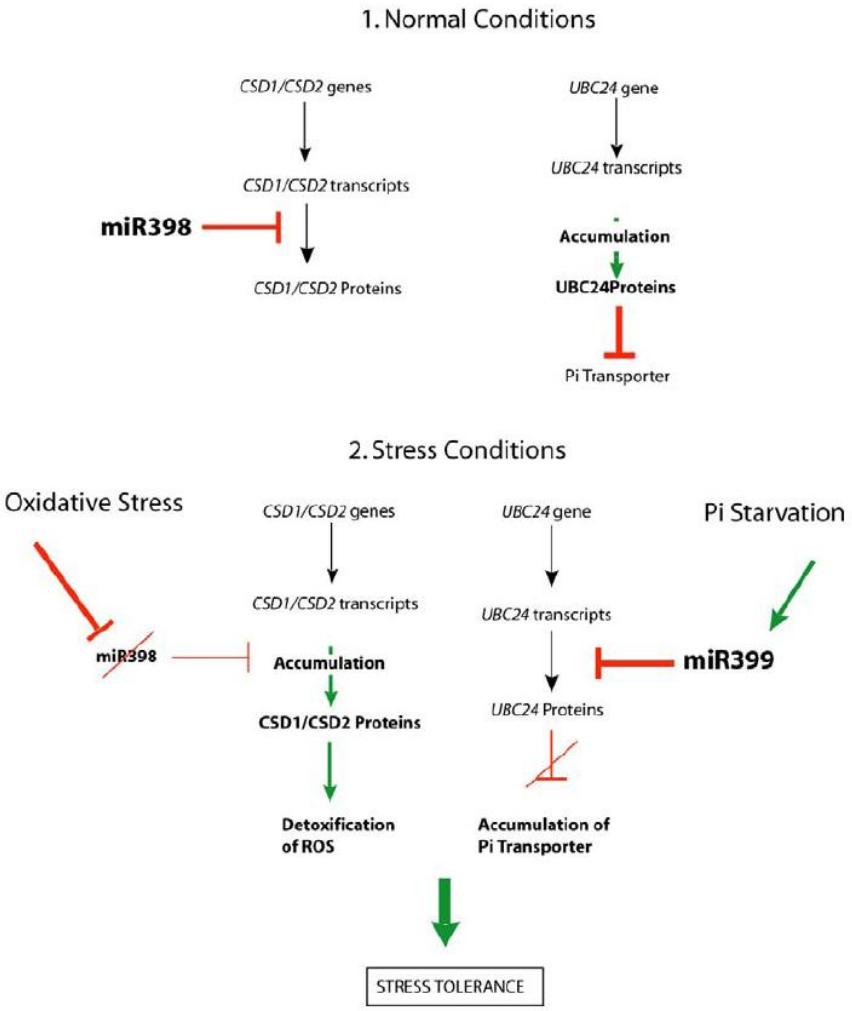
[29,30]. It can be expected that nat-siRNAs represent a common way for environmental stress response in plants and possibly in other eukaryotes.

Figure 2. Stress conditions can induce opposite regulations of miRNAs accumulation.



The two miRNAs miR398 and miR399 are implicated in post-transcriptional regulation of gene expression under stress conditions. They are oppositely regulated. miR398, which targets *COPPER SUPER OXIDE DISMUTASES* (*CSD1* and *CSD2*), is constitutively expressed in normal conditions and repressed under oxidative stress. [31]. By contrast, miR399 is produced only under phosphate starvation in order to repress the negative regulator of Pi import *UBC24* (*UBIQUITIN CONJUGATING ENZYME 24*) [25-27].

3.3. sRNAs Down Regulated by Stress (miR398)

Stress conditions such as drought, cold, salinity, high light or metal toxicity result in the accumulation of ROS in plant cells [32]. To prevent excess cellular damage, reactive radicals are scavenged at the site of their synthesis in the chloroplast. SUPEROXIDE DISMUTASES (SOD) are

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the firsts scavengers for the detoxification of superoxide radical O_2^- allowing conversion of O_2^- into $H_2O_2^-$ [32]. SODs are expressed under stress conditions to enable detoxification and are repressed under normal conditions. In *A. thaliana*, *CSD1* and *CSD2* mRNA, that encode Cu/Zn-SOD (CSD) proteins, accumulate in response to treatments inducing oxidative stress, such as high light or excess $Cu^{2+}$and Fe^{3+}. However, nuclear run-on assays indicate that *CSD1* and *CSD2* are equally transcribed under normal or oxidative conditions, indicating that their mRNAs are post-transcriptionally regulated [31]. Indeed, under normal growth conditions, the two *CSD* genes are transcribed but their mRNAs are silenced through the action of miR398. miR398 is present in *A. thaliana* and *O. sativa* and could be encoded from three loci [31]. In response to oxidative stress, the locus *MIR398* is transcriptionally down regulated, resulting in the absence of miR398 (Figure 2). Reduction of miR398 abundance allows the accumulation of *CSD1* and *CSD2* mRNAs, which can therefore be translated. Transgenic plants carrying a miR398-resistant mutation in the *CSD2* mRNA show better tolerance to oxidative stresses than wild type plants. This suggests that the fine regulation of *CSDs* transcript stability by miR398 is important to rapidly reduce ROS production in response to oxidative stress. Thus, in *A. thaliana*, miRNA mediated down-regulation of positive regulators of stress tolerance appears to be an important mechanism controlling oxidative stress responses.

4. General Evidences of Translational Regulations in Plants

In all organisms, regulation of mRNA translation allows fine modulation of the level of protein synthesized from its corresponding mRNA. Translation efficiency of individual mRNA can be estimated through the evaluation of the amount of mRNA associated with translating ribosomes [33]. mRNAs that recruit multiple ribosomes are actively translated and form ribonucleotidic complexes known as polysomes. Fractionation of polysomes on a sucrose gradient is an ancient method that has been adapted for plant material [34] (Figure 3). Differential mRNA translation occurs in response to numerous environmental stimuli such as heat stress [35], salt stress [36], water deficit [37], oxygen deprivation, pathogen infection [38], and sucrose starvation [39]. In one these studies, leaves of *A. thaliana* have been placed under normal or dehydration conditions [40]. The proportion of individual mRNA in polysomes has been measured for over two thousand genes. The authors reported that the majority of mRNAs show a significant decrease in polysomes association in response to dehydration stress. While some transcripts are upregulated under stress, their association with polysomes is maintained at the same level as in normal conditions. These results suggest that the effect of dehydration on translational level varied between mRNA species.

In a similar microarray experiment, the differential translation of 25,607 *A. thaliana* transcripts was analysed in response to sucrose starvation in cell culture [39]. This study identified 224 mRNAs that were regulated translationally and 268 mRNAs that were regulated transcriptionally. Most of the translationally regulated mRNAs are repressed by sucrose starvation, which is consistent with a general decrease of metabolic activity. The authors also observed that transcripts related to the protein synthesis machinery and to cell cycle control were particularly abundant among the translationally regulated transcripts. These results suggest that translational control may be important for gene regulation in response to sugar starvation.

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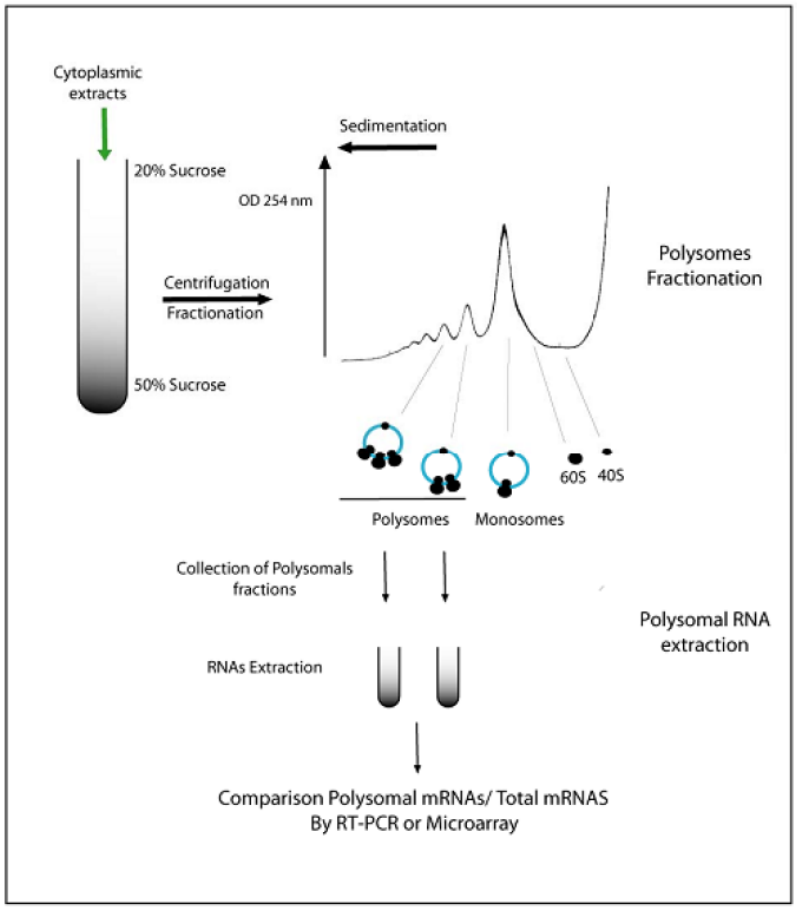
Figure 3. Polysomes fractionation on sucrose gradients allows the isolation of actively translated mRNA.



Cytoplasmic extracts are separated on a sucrose gradient. After centrifugation, fractions are collected from the bottom to the top of the gradient under continuous reading of optical density at 254 nm. The major peak corresponds to monosomes (entire 80S ribosome assembled on mRNA). Lower in the gradient are polysomes (translationally active mRNAs associated with more than one ribosome). Free RNAs and other low molecular weigh components of the cytoplasm sediment at the top of the gradient. RNAs can be extracted from the different fractions and quantified by RT-PCR or microarray to determine which mRNAs are translated. Comparing accumulation of mRNA in polysomal RNA *vs*. total RNA allows the identification of potential translational regulations.

A more recent study has shown that hypoxia stress followed by reoxygenation, which is linked with cellular ATP content, promoted adjustment in the level of polysomes in *A. thaliana* seedlings [41]. These data suggest that translational regulations contribute to the adaptation of plants to environmental perturbation by limiting consumption of ATP and directing the synthesis of specific proteins. Therefore differential translation of mRNAs appears to be a key component in the response to oxygen deprivation and reoxygenation.

Overall, we can conclude from the data mentioned above that many mRNAs undergo translational changes during environmental adaptation. Although the amount of polysomes bound mRNAs has been

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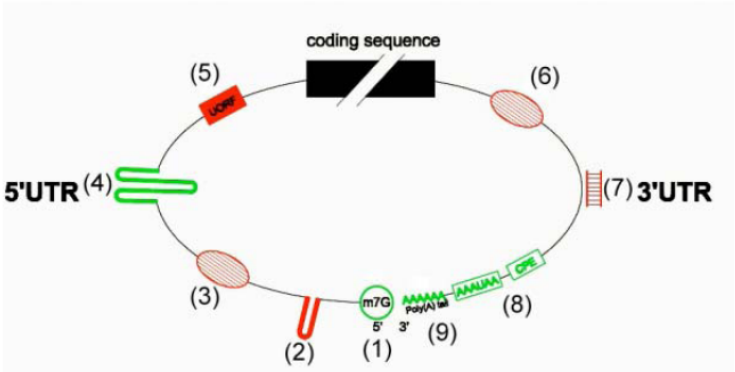
rarely correlated with the amount of the corresponding protein, polysomes analysis appears to give an acceptable estimation of translational efficiency.

5. Involvement of Untranslated Regions (UTRs) in Translational Regulations

mRNA features that contribute to translational regulation under abiotic stress have been studied using a comparison of the abundance of the total mRNAs versus polysomal mRNAs in leaves of *A. thaliana* [42]. It was shown that translation of an mRNA is mostly affected by control elements located within untranslated regions (5'UTR and 3'UTR). In addition, nucleotides surrounding the initiation codon also influence translational efficiency (figure 4). The interactions between 5' and 3' UTR might promote a synergistic enhancement of mRNA translation, ensuring that scanning proceeds from the correct end. Electroporation of mRNAs constructs into protoplasts was used to investigate involvement of maize *ADH1* (*ALCOHOL DEHYDROGENASE-1*) mRNA features in translation efficiency. The authors found that the 5'UTR, a portion of the coding sequence and the 3'UTR of *ADH1* mRNA are all required for its efficient expression in hypoxic protoplasts [43]. A more recent analysis of *ADH1* expression indicates that an internal ribosome entry site (IRES) in the 5'UTR is implicated in translational regulations [44]. In heat-shocked protoplasts, mRNA electroporation studies have also shown that the 5'-UTR of *HSP70* (*HEAT-SHOCK PROTEIN 70*) mRNA is sufficient for translational enhancement under stress [45] (Figure 5). Another study investigated the role of 5'UTR on translation of *FERREDOXIN-1* (*FED-1*) mRNA. Light can induce an increased association of *FED-1* mRNA with polysomes which is mediated by an internal light-regulatory element (iLRE) located in the 5'UTR and in the coding sequence [46]. In addition they proposed that *FED-1* mRNA stability is correlated with its association with polysomes [47]. Overall, all these data indicate that the features of the 5'UTR and the 3'UTR of mRNAs are involved in their translation in response to environmental stresses.

Figure 4. Generic structure of a eukaryotic mRNA, illustrating some of the post-transcriptional regulatory elements that affect gene expression.



Numbers on the 5'UTR refer to: (1) m7G, 7-methyl-guanosine cap; (2) hairpin-like secondary structures; (3) interacting repressor protein; (4) internal ribosome entry sites (IRES); (5) upstream open reading frame (uORF). Numbers on the 3'UTR refer to: (6) repressor protein complex; (7) sRNAs binding sites; (8) cytoplasmic polyadenylation elements (CPE) and hexanucleotide AAUAAA polyadenylation signal; (9) poly (A) tail. Red-coloured elements usually down-regulate translation of the main coding sequence, whereas green-coloured elements are usually favourable for translation of the main coding sequence.

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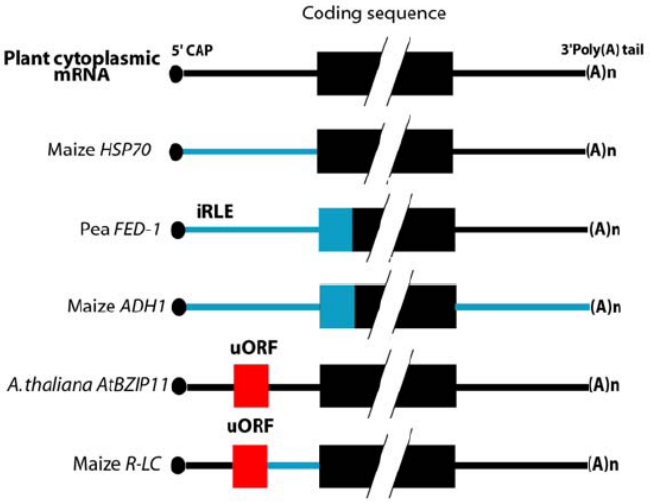
Figure 5. Cis-acting elements involved in translation of plant cytoplasmic mRNAs.



The 5' 7-methyl-guanosine cap structure is indicated by a filled circle. Regions of mRNAs with cis-acting sequences that regulate translation are indicated by blue boxes. Upstream open reading frames (uORFs) are indicated by red boxes. *HSP70*, *HEAT SHOCK PROTEIN 70* [45]; *FED-1*, *FERREDOXIN* -1; iLRE, internal light-regulatory element [47]; *ADH1, ALCOHOL DEHYDROGENASE-1* [43]; *AtBZIP11, A. thaliana BASIC LEUCINE ZIPPER 11* [48].

5.1. Upstream Open Reading Frames (uORFs) in Plants

Some of the most important translational control signals in eukaryotes are upstream open reading frames (uORFs) that are located in the 5'UTR [49]. The earliest example of uORF-guided translational regulation under stress was that of *GCN4* mRNA in yeast [50]. The presence of uORFs in 5'UTR modulates translation efficiency of the main ORF due to preferential recruitment of ribosomes. Factors such as uORF length, the nucleotide context of the start and stop codons, and the sequence between the uORF and the main ORF, all contribute to affect the efficiency of translation of the main ORF [51]. Depending of these characteristics of the uORF, ribosomes may reach the main ORF through reinitiation or leaky-scanning [52,53].

Five uORFs have been characterized in monocots, including two examples in rice that are uORFs of *S-ADENOSYLMETHIONINE DECARBOXYLASE* (*ADOMETDC*) gene [54] and *MYB7* (*MYB DOMAIN PROTEIN 7*) gene [55], and two examples in maize: *OPAQUE-2* gene [56], *R-Lc* gene [29]. Also, uORFs have been found in *A. thaliana* genes such as *AtBZIP11* (*A. thaliana BASIC LEUCINE ZIPPER 11*) [48], and *ARF* (*AUXIN RESPONSIVE FACTOR*) genes [57]. A recent study has used *O. sativa* and *A. thaliana* full-length cDNAs sequences to determine the prevalence of uORFs [58]. These bioinformatics analyses predict the presence of uORFs in the 5'UTR of a subset of genes conserved between both species. Many of these genes encode proteins that have a regulatory function including transcription factors, signal transduction factors, developmental signal proteins, a homolog of the translation initiation factor eIF5 (eukaryotic TRNASLATION INITIATION FACTOR 5), and a RING finger protein. Another recent study has identified several genes whose translation may be regulated by

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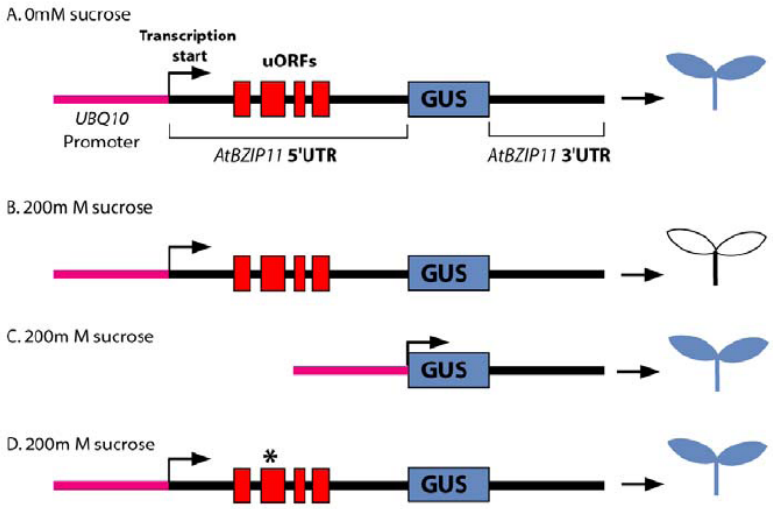
uORFs in monocotyledons [59]. The uORFs identified in these studies are strong candidates to trigger translational control.

The data mentioned above suggest that uORFs are important cis-acting regulatory elements present in the 5'UTR of many plants mRNAs. Several examples illustrate the role of uORFs in translational regulation during development, but only a few examples illustrate their role in response to abiotic stress. We will now discuss examples of uORFs that repress the translation of mRNAs under abiotic stress conditions in plants.

5.2. Examples of Translational Regulation Mediated by uORF

S-ADENOSYLMETHIONINE DECARBOXYLASE (SAMDC) is a key enzyme of the polyamine biosynthesis pathway [60]. Two overlapping small uORFs consisting of 3 and 52 codons respectively are present in the 5'UTR of *SAMDC* genes. The 52 codons uORF is responsible for the translational repression of *SAMDC* gene under normal growth conditions [61]. Another study demonstrated that both uORFs are involved in translation repression in response to stress conditions and negative feedback controlled by polyamines [62]. This is an example illustrating the metabolite-dependent translational control involving conserved uORFs in plants.

Figure 6. Effect of the *AtBZIP11* (*A. thaliana BASIC LEUCINE ZIPPER 11*) 5'UTR on sucrose-induced repression of translation in *A. thaliana* seedlings.



Schematic illustration of an *AtBZIP11* 5'UTR controlled -*GLUCURONIDASE* (*GUS*) chimera terminated by the *AtBZIP11* 3'UTR and fused to the *POLYUBIQUITIN10* (*UBQ10*) promoter (A and B). Histochemical staining of 5 days-old seedlings grown without sucrose (A) shows GUS expression in the root and shoot, while seedlings grown with 200 mM sucrose (B) show repression of GUS expression via a mechanism called sucrose-induced repression of translation. Deletion of the *AtBZIP11* 5'UTR destroys sucrose-induced repression of translation as indicated by presence of GUS expression in the root and shoot (C). A single mutation (*) in uORF2 is sufficient to destroy sucrose-induced repression of translation (D). Adapted from [48].

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Another study reported that the expression of the gene coding for the AtBZIP11 transcription factor is post-transcriptionally regulated by sucrose [63]. High sucrose concentrations result in translational repression of the *AtBZIP11* expression. The 5'UTR of *AtBZIP11* mRNA is necessary for the sucrose-induced repression of translation (SIRT) (figure 6). The long *AtBZIP11* 5'-UTR (547nt) contains four uORFs called uORF1, uORF2, uORF3 and uORF4 coding for polypeptides of respectively 18, 42, 5 and 18 amino acids. The upstream ORF2 is highly conserved in 5'UTRs of other *A. thaliana* BZIP genes as well as in other dicotyledons and monocotyledons plants [48]. The involvement of *AtBZIP11* uORFs in sucrose-induced repression of translation (SIRT) has been investigated via the introduction of point mutations in single uORFs in transgenic plants. These plants carry the *A. thaliana POLYUBIQUITIN10* (*UBQ10*) promoter and the *AtbZIP11* 5'UTR fused to the reporter gene coding for β-GLUCURONIDASE (GUS) (Figure 6) [48,64]. The exchange of the internal putative start codon of uORF2 to a stop codon leads to GUS activity when plants are grown in high sucrose concentration (Figure 6D). This indicates that SIRT activity is dependent on translation of the C-terminal part of uORF2. Thus, the authors have proposed the name "Sucrose Control uORF" (SC-uORF) for the uORF2 of *AtBZIP11* and any orthologous uORF from other plants or other *A. thaliana* genes. A recent study investigated in detail the mechanism of translational regulation of *AtBZIP11* by SIRT [65]. These authors show that the SC-peptide is required and sufficient for SIRT. They also suggest that the SC-peptide interacts with other molecules to repress translation of the main ORF. Overall, these results suggest that a sucrose-sensing pathway controls translation of several plants *BZIP* mRNA harbouring the conserved uORF in their 5'UTRs. This illustrates an example of a metabolite-dependent translational control system involving a conserved uORF in plants.

6. Compartmentalization of mRNAs in the Cytoplasm

During the recent years, a new aspect of post-transcriptional regulation of gene expression has been uncovered: the sequestration of mRNAs in the cytoplasm. Using tomato cell cultures, Nover *et al.* provided the first evidence of electron dense cytoplasmic foci, assembled under heat stress, called Heat Stress Granules (HSG) [66]. A recent study using tobacco and *A. thaliana* shows that HSG can be distinguished from stress granules (SG) that are not dependent on heat shock proteins (HSP) [67]. Following stress exposure, a subset of mRNAs aggregate with specific proteins, allowing physical separation of these mRNAs from the translational machinery and resulting in transient translational repression. Specific RNA binding proteins and the eukaryotic TRANSLATION INITIATION FACTOR (eiF4E) were identified as markers of stress granules in plants and in animals [68]. Understanding mechanisms of mRNA sorting by localization of mRNAs in distinct structures within the cytoplasm, and the resulting reversible regulation of translation, will be an important challenge for future studies on post-transcriptional regulation [68,69].

7. Conclusions

The work reviewed here indicates that, under abiotic stress, plant genes are regulated at all post-transcriptional stages, from mRNA processing to protein translation. Similar stresses can induce

different mechanisms of post-transcriptional regulation, indicating that there is no preference for one regulatory mechanism for a particular stress.

mRNA stability seems to be an important mode of regulation both during and after mRNA processing. However, the precise function of RNA binding proteins during mRNA processing is not yet very well understood and needs more investigation. Responses to abiotic stress highlight fascinating examples of the different modes of action by which sRNAs can regulate mRNA stability. On the one hand, a sRNA induced by stresses represses negative regulators of stress tolerance and, on the other hand, repression of the expression of a sRNA by stress allows the accumulation of positive regulators of stress tolerance. The role of sRNA might also have been overlooked given that they can regulate translation as well as RNA stability [16,17]. New examples of sRNAs-mediated regulation of translation in response to stress might be reported soon.

Most of the examples of translational regulation in response to stress involve the 5'UTR and particularly uORFs. However, there may be a bias because uORFs are particularly easy to identify and study compared to other elements in the 5' and 3'UTRs, such as secondary structure or sites for recognition by regulatory RNA binding proteins.

We anticipate that future studies will uncover that more plant genes are post-transcriptionally regulated in response to abiotic stress and that several mechanisms are operating together. For example, during the root nodule development induced by symbiotic bacteria in the legume plant *Medicago*, *truncatula* the spatial and temporal expression of transcription factor MtHAP2-1 (*M. truncatula* HEME ACTIVATOR PROTEIN homolog 2-1) is regulated by both a miRNA and a uORF [70,71]. This highlights the importance of investigating the combinatory, possibly synergistic, roles that multiple regulatory mechanisms may play in the regulation of plant gene expression during environmental variations.

Many papers mentioned in this review argue that post-transcriptional regulations of gene expression may be particularly important during stress responses because it allows more rapid adaptation in the proteome than transcriptional regulation can provide. Therefore, post-transcriptional regulations seem to be important for rapid adaptation of gene expression in response to environmental variations in plants.

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1.4 - Aim of the thesis

In plants, it was found that expression of *Arabidopsis TOR* (*AtTOR*) mRNA is restricted to particular tissues such as embryo, endosperm, primary meristems, and primordia and absent in differentiated organs, although, *AtTOR* mRNA was detected nearly in all tissues proliferative and differentiated (**Menand *et al.*, 2002**). This finding encouraged us to hypothesis that expression of *AtTOR* mRNA may be subjected to a regulation at the post-transcriptional level. Indeed, the analysis of 5'UTR of *AtTOR* mRNA revealed the presence of a small upstream open reading frame (uORF) encoding for 2 amino acids and recent studies recorded that more than 30 % of Arabidopsis transcripts contain uORF, suggesting presence of a regulatory role for these uORFs in regulation of gene expression. These uORF are widespread among transcripts having crucial role within the cell such as transcription factors, regulatory protein, and oncogenes. According to the essential regulatory function of AtTOR kinase in control of gene expression, protein synthesis, and cell growth, we consider it as one of the important regulatory proteins within a plant cell.

The main purpose from this thesis is to highlight the mechanisms that control *AtTOR* expression at the post-transcriptional level through determination of the possible regulatory elements within the 5' untranslated region (5′UTR) or the first intron of AtTOR mRNA itself, and through manipulation of these regulatory elements to study their precise role. We have chosen to focus on the small upstream open reading frame (uORF) as well as the 5′UTR region.

This is the first attempt to study the regulation of TOR kinase expression in eukaryotes through these small uORF or the sequence of 5′ untranslated region (5′UTR).

2 – RESULTS

2.1. Study of *AtTOR* expression at the post-transcriptional level

2.1.1. Presence of *AtTOR* mRNA not TOR protein in all tissues of Arabidopsis

RT-PCR was used to investigate the expression level of the Arabidopsis *TOR* (*AtTOR)* gene in mature tissues like old and young leaves, old and young stems and inflorescences as well as seedlings. The expression profile of *AtTOR* mRNA has been investigated from the mature tissues selected from heterozygous *TOR/tor-1* plants, in which the *tor-1* allele is fused with the *GUS* gene at amino acid 1555 of AtTOR (**Menand *et al.*, 2002**), and from the wild-type Arabidopsis ecotype Wassilewskija (WS). Total RNA was extracted from the specified mature tissues of the individual mature plants (one-month old) as well as a bulk composed of about 20 seedlings (10-days old) using the TriReagent solution (as described in material and method). PCR have been performed using *AtTOR* gene specific primers and actin2 gene primers as endogenous control. The *AtTOR* specific primers GSP2 and GSP3 were designed to overlap an intron-exon junctions to avoid amplification of contaminant genomic DNA and allow only to amplify the cDNA. RT-PCR results have revealed that expression of *AtTOR* mRNA was found nearly in equal intensity levels for all tested tissues selected from heterozygous *TOR/tor-1* plants either at 20 or 30 PCR cycles and also from the wild-type Arabidopsis ecotype wassilewskija (WS) at 30 PCR cycles. However, the expression of *AtTOR* mRNA for all tested tissues selected from wild-type Arabidopsis ecotype wassilewskija (WS) at 20 PCR cycles was not found in equal level (**Figure 19**). The variation in AtTOR mRNA expression between heterozygous *TOR/tor-1* and the wild-type Arabidopsis ecotype wassilewskija (WS) might result from inserted T-DNA and their transcription with the native genome of heterozygous *TOR/tor-1*. These results are consistent with the results that have been reported before by **Robaglia *et al.*, (2004)** who observed that *AtTOR* mRNA is expressed approximately at equal level for all tested mature tissues.

On the other hand, the expression of TOR fusion GUS protein from *TOR/tor-1* heterozygous plants was detected via histochemical GUS assay. The tissues of transgenic plants were immersed into the GUS staining solution (X-Gluc) and then it was incubated at 37 °C overnight.

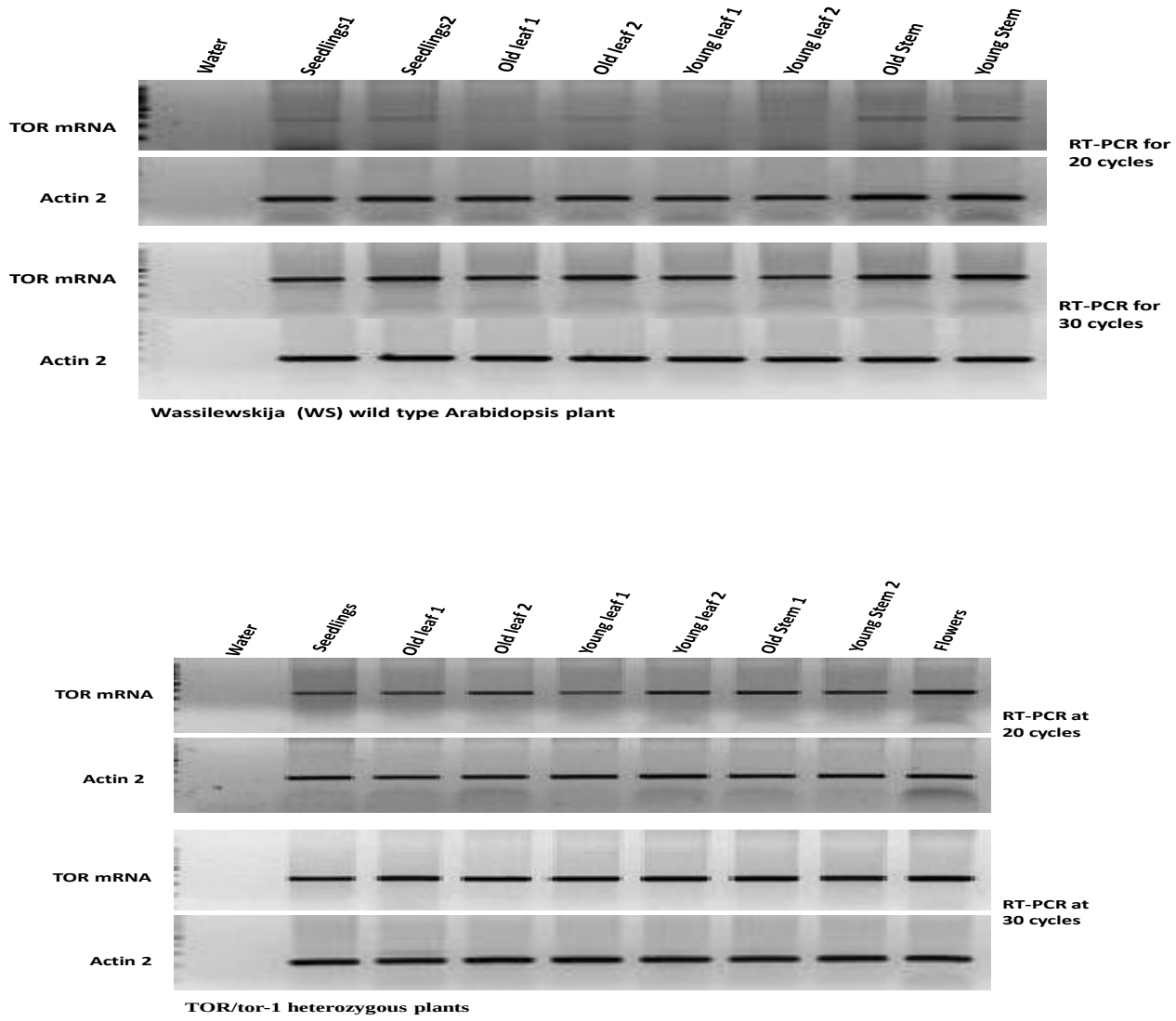


Figure 19: **Semi-quantitative RT-PCR analysis of the *AtTOR* gene in various tissues from the wild-type Arabidopsis wassilewskija (WS) ecotype [upper gel] and *TOR/tor-1* heterozygous [lower gel] Arabidopsis plant.** TOR primers used for analysis are designed as TORseq5′ forward primer and TORseq3′reverse primer located near to the 3′- end of *AtTOR* gene. The Arabidopsis *ACTIN2 (AtACTIN2)* gene was used as the endogenous control. 20 and 30 PCR cycles were performed.

Blue color appeared in the tissues and the staining became more obvious after extraction of the chlorophyll. It was observed that AtTOR protein fused with *GUS* is restricted only to undifferentiated tissues from *Arabidopsis thaliana* seedling such as meristematic root tissues,

primordia, and cotyledon leaves as well as the inflorescences and silics (Arabidopsis fruits) from the flowering and seed producing Arabidopsis plants (**Figure 20, Menand *et al.*, 2002**).

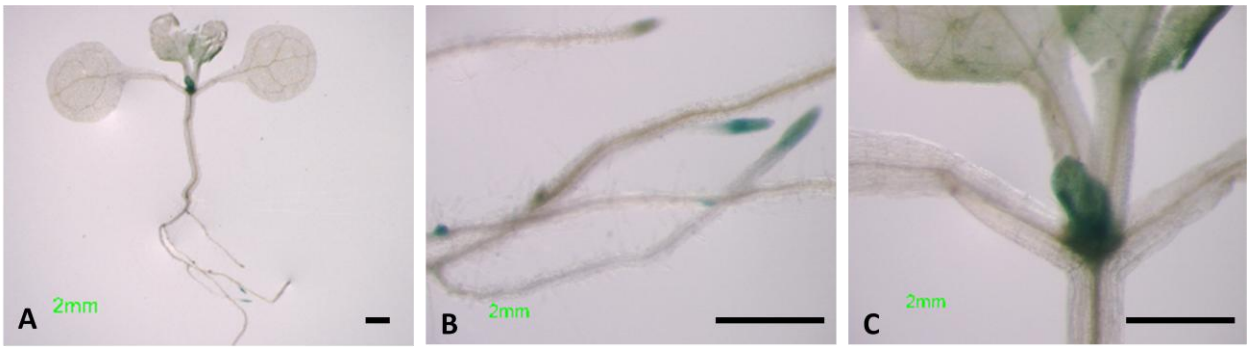


Figure 20: **Expression pattern of AtTOR::GUS fusion protein of the TOR /*tor-1* heterozygous Arabidopsis seedling. A -** Intact seedling showing the zones of expression in root meristems and in shoot meristem only, not in other tissues. **B -** Zoom on apical root meristem, TOR-GUS fusion protein expression is restricted to apical root meristem but absent in the rest of root tissue. **C -** Zoom on apical shoot meristem in which TOR-GUS fusion protein is expressed only in the primordial meristematic tissue. Scale bars: 2 mm.

Together, these results revealed that the *AtTOR* transcript is approximately expressed in all tissues either differentiated or undifferentiated tissues, while, TOR protein is expressed only in undifferentiated tissues. The pattern of expression of AtTOR is different from the pattern of expression of TOR in mammalian cells (**Brown *et al.*, 1994; Chiu *et al.*, 1994**) that occurs in all tissues. The absence of AtTOR protein expression in mature plant cells such as expanding root and leaves may be related to the fact that these cells undergo growth through expansion which is a different process to premitotic growth, which involves the synthesis of cytosolic components (**Schmelzle and Hall**, **2000**).

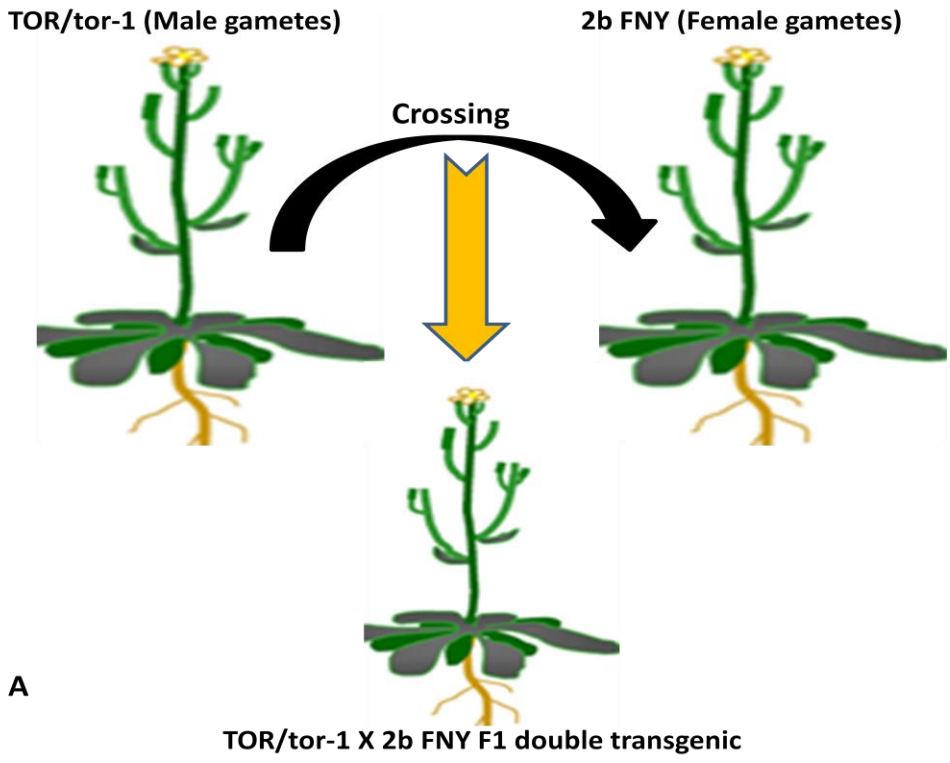
According to these results, we have suggested that *AtTOR* is regulated post-transcriptionally either at the level of mRNA translation or at the level of mRNA processing such as mRNA splicing or mRNA stability. Translational repression can occur by RNA silencing or through stabilization/destabilization of *AtTOR* mRNA. This hypothesis will be discussed in details in the following section.

2.1.2. Investigation of the impact of micro RNA (miRNA) on *AtTOR* expression

We first hypothesized that translation of *AtTOR* mRNA is subjected to repression via mechanism known as RNA silencing. RNA silencing is a mechanism involved in regulation of gene

expression through small RNA molecules in a sequence specific manner at the level of transcription, mRNA stability or translation (**Voinnet, 2002**). Most known post-transcriptional regulation mediated by RNA silencing involves microRNAs (miRNAs). Micro RNAs (miRNAs) are non-coding RNA sequences composed of 21 to 23 nucleotides long that act as a negative regulators of gene expression at the post-transcriptional level (**Bartel, 2004)**. Our hypothesis is based on bioinformatics information suggesting that *Arabidopsis* TOR (AtTOR) mRNA is target for one of the predicted miRNA called [miR34] composed of 20 nucleotides (**Bonnet *et al.*, 2004**). The miR34 and its complementary sequence were aligned with the *AtTOR* genomic sequence to detect the precise site for this miRNA within the TOR sequence. We found that this predicted miR34 sequence is completely complementary with the *AtTOR* cDNA sequence at exon number 21 and 22. In order to investigate if the potential predicted miR34 affect *AtTOR* expression, we created double-transgenic plants expressing TOR::GUS fusion protein in 35S-FNY2b background via crossing between *TOR/tor-1* (as a male plant) and transgenic *Arabidopsis* plants expressing cucumber mosaic virus (CMV) 2b (strain FNY) (as a female plant). We have chosen CMV 2b protein as it has been shown that the 2b protein purified from cucumber mosaic virus (CMV, severe strain FNY) is a strong suppressor of miRNA-mediated cellular mRNA turnover through its direct binding with ARGONAUTE protein (**Zhang *et al.*, 2006**; **Lewsey *et al*., 2007)**. ARGONAUTE (AGO) proteins are critical effectors protein of RNA silencing, they bind miRNA and form a RNA induced silencing complex (RISC) to cleave or repress translation of target mRNA (**Qi *et al.*, 2005**). It was observed that the CMV 2b (FNY strain) protein has the ability to bind directly with AGO1 protein (one of the well characterized member from AGO protein family) and interfere with RISC complex to block intrinsic cleavage activity of AGO1.

The purpose of this experiment is to test whether *AtTOR* mRNA is regulated by miR34 or not, through observation of any changes on the expression pattern of AtTOR::GUS fusion protein that could occurs in the new double transgenic plants expressing CMV 2b. A modification of TOR::GUS fusion protein expression will support this hypothesis and lead to the conclusion that AtTOR mRNA actually can be regulated by miR34. In contrast, the absence of clear and proper changes in TOR::GUS fusion protein expression would support the idea that AtTOR mRNA is not regulated by miR34 or any other miRNAs.



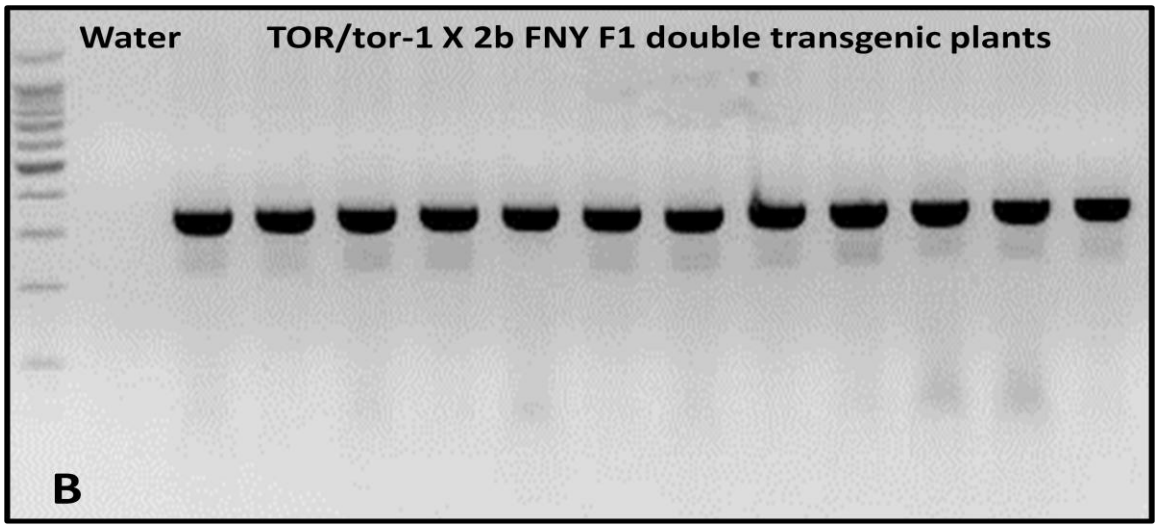


Figure 21: Production of double transgenic plants carrying *TOR/tor-1* allele and expressing CaMV 2b FNY protein. A - Schematic representation of the cross between heterozygous *TOR/tor-1* plant (male gametes) and transgenic plant expressing FNY 2b suppressor viral protein (female gametes). **B -** PCR to check the presence of 2b mRNA using 2b specific primers from flowers of the F1 generated plants. The number of PCR cycles was 30 and water has been used as a negative control.

Crossing between *TOR/tor-1* heterozygous plants as a male gametes [that is resistant to both kanamycin and phosphinotricin (PPT)] and the transgenic CMV 2b (Fny severe strain) plants as a female gametes has been achieved. The pollen grains from *tor-1* mutant plant were transferred to the ovules of Fny 2b transgenic plants (that is resistant to kanamycin only) and it was allowed to form the F_0 seeds. The produced seeds were screened by germination on MS medium supplemented with PPT (10µg/ml) to select the seedling containing the AtTOR::GUS fusion protein as well the phenotype characteristic of Fny 2b plants, since the expression of the phenotype is dominant and appears in the first generation (**Figure 21 A**). About 12 individual double transgenic (*TOR*/*tor-1*/2b) lines representing F1 generation have been isolated through segregation on Basta and also through observation of the 2b phenotype on resulted seedlings. The ratio of segregation was 2 kanamycin resistant : 1 kanamycin sensitive. As *tor-1* homozygous embryos are lethal, some seeds were aborted during embryo development, so we cannot obtain the double transgenic plants harbouring two copies of *tor-1* allele. PCR has been done to check the presence of 2b mRNA using 2b specific primers from flowers of the F1 generated plants (**Figure 21 B**). F2 and F3 generations have been produced from self-pollination of F1 and F2 generation, respectively. The produced seeds were segregated, also, on Basta to select the double transgenic lines having homozygous alleles from *2b* and heterozygous alleles of *TOR/tor-1*. Furthermore, the clear phenotype of 2b transgenic protein was used to select these transgenic plants (**Figure 22 A**). This F3 generation transgenic plants were used to investigate the effect of miRNA on *AtTOR* gene expression level via detection of the presence of *AtTOR* gene fusion with *GUS* reporter gene in the mature and old tissues through histochemical GUS expression assay.

The result revealed that the expression pattern of AtTOR:: GUS fusion protein does not change between the *TOR/tor-1* mutant plants (as a control) and the new double transgenic plants generated from crossing between *TOR/tor-1* mutant and transgenic plants expressing CMV 2b repressor protein (**Figure 22 B**).

These observations suggest that the *AtTOR* gene expression might not be targeted by the potential predicted miRNA (miR34) or that the effect of this potential miR34 appears having particular developmental or environmental conditions. Thus, this result suggests that *TOR* gene from Arabidopsis is not regulated by miRNA.

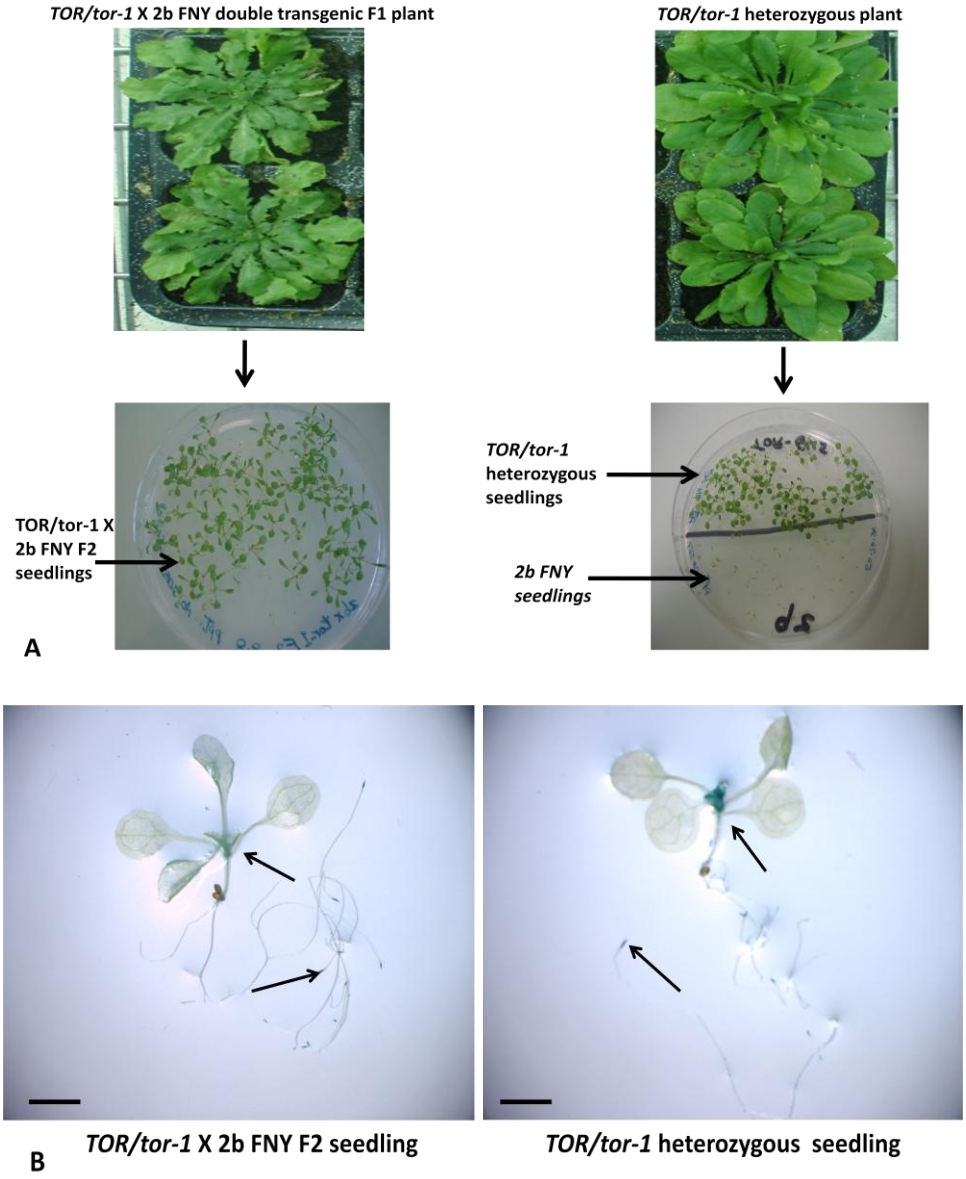


Figure 22: Expression of the 2b FNY protein does not change the expression pattern of AtTOR::GUS fusion protein in comparison with *TOR/tor-1* heterozygous plants. A- Pictures for mature 3 weeks old plants from double transgenic (left) with a clear FNY 2B phenotype in comparison to *TOR/tor-1* mutant plants (right). The lower panel, show two pictures for F2 seedlings from both plants (double transgenic and *TOR/tor-1* mutant) selected on antibiotic phosphinotricin [PPT] (10 µg/ml). 2b FNY plants are sensitive to PPT while *TOR/tor-1* plants are resistant. We selected the double transgenic plants expressing FNY 2b protein on the basis of their 2b phenotype and their resistance to PPT. **B-** Some seedling (10 days old) from these double transgenic plants wer placed in GUS solution to investigate the GUS expression pattern comparing to *TOR/tor-1* mutant plants. The same pattern of GUS expression was observed for both plants as indicated by arrows after incubation overnight. The arrows refer to the TOR::GUS protein expression either in root apical meristem or in primordial shoot meristem.

This conclusion is supported by the reproduction of the spatial expression pattern of TOR::GUS in transgenic lines expressing GUS under the control of the *AtTOR* promoter in the experiments described in chapter 2.3.

Having shown that the repression of TOR translation in mature leaves is probably not due to RNA silencing. I wanted to explore if it can be caused by translational regulation by the 5'UTR.

2.2. Determination of 5′UTR features of *TOR* gene from different plant species

2.2.1. Characteristic features of TOR 5′UTR from *Arabidopsis thaliana*

In order to study the role of the 5′UTR of *Arabidopsis* TOR (*AtTOR)* gene on its expression, we needed to identify the transcription start site of *AtTOR* gene. First, we have carried out a series of RT-PCR experiments to determine the length of *AtTOR* 5′UTR using different forward primers located at different distances from the ATG start codon, denoted as (+1), of *AtTOR* coding sequence. Different reverse primers have been designed to be specific for cDNA to avoid any contamination by genomic DNA. We have given a universal name to these primers such as gene specific primer (GSP) 1, 2, and 3 according to the position of GSP 1 at exon number 4. GSP1 sequence was designed from the fourth exon to use in generation of cDNA from *TOR* mRNA, GSP2 sequence was selected to overlap between the third and the second exon boundaries, and GSP3 sequence was selected to overlap between the second and the first exon boundaries (**Figure 23**).

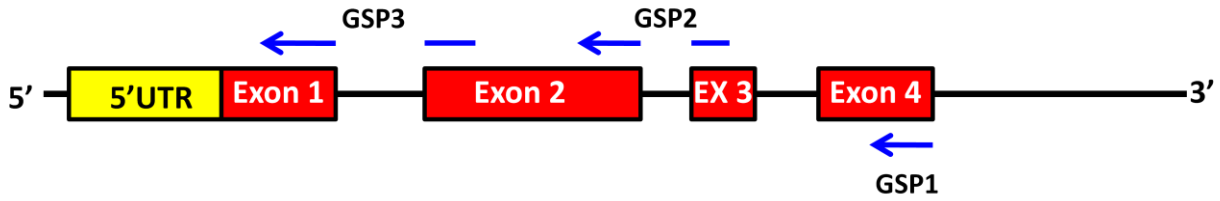


Figure 23: Diagram represents the beginning of Arabidopsis *TOR* (*AtTOR*) gene at the 5′end. It involve the 5′UTR (yellow box), the first four exons (red boxes), and the first three introns (black line). Blue arrows denote the positions of reverse primers GSP1, GSP2, and GSP3 that are used in both RT-PCR and 5′RACE to amplify and determine the 5′UTR length.

Amplification of *AtTOR* cDNA using 5′UTR-245-F3 forward primer and GSP3 reverse primer, including the first exon (166 bp) resulted in a clear PCR band at approximately 400 bp.

However, amplification of TOR cDNA using both forward primers 5′UTR-363-F4 and 5′UTR-405-F5 with reverse primer GSP3 did not give any PCR band, indicating that the length of *AtTOR* 5′UTR may be about 230 bp (**Figure 24**). This suggests that the length of *AtTOR* 5′UTR may expand to 230 bp only from the of ATG start codon and that it does not expand to 376 bp as it was documented in National Center of Biotechnology Information (NCBI; AF178967) and The Arabidopsis Information Resource (TAIR; AT1G50030).

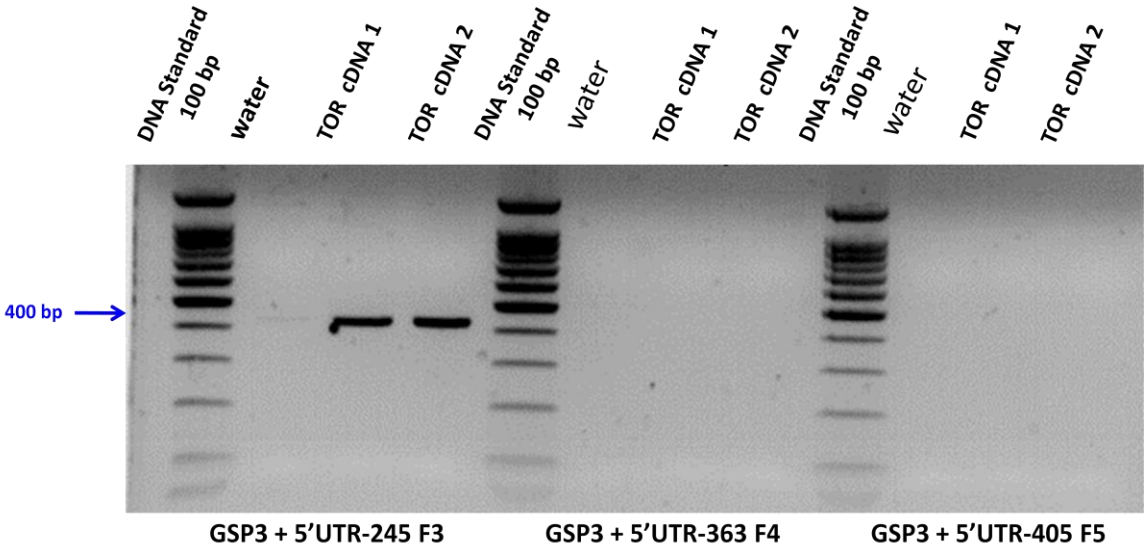


Figure 24: Semi-quantitative RT-PCR for amplification of the 5'end of *AtTOR*. Different forward primers named 5′UTR-245 F3, 5′UTR-363 F4, and 5′UTR-405 F5 have been used to amplify the 5′end of AtTOR cDNA in the presence of reverse gene specific primer 3 (GSP3). The number of each forward primer indicates the distance between each primer and the ATG start codon (+1). 5′UTR-245 F3 is the nearest to ATG and 5′UTR-405 F5 is the farthest. The size of PCR fragments equal nearly 405 bp corresponding to the summe of first exon (166 bp) and the minimal 5′UTR (230 bp).

The 7mGpppG cap of eukaryotic mRNA allows complete amplification of 5′UTR sequences with a modified 5′- RACE (**R**apid **A**mplification of 5′- **c**DNA **E**nd) technique called RNA ligase-mediated RACE (RLM-RACE). In RLM-RACE, an RNA sample was first treated with calf intestinal phosphatase (CIP) to remove the 5′-phosphate from all RNA species except those with a cap structure.

Tobacco acid pyrophosphatase (TAP) was then used to remove the cap structure from RNA, leaving a 5′-phosphate. Next, a synthetic RNA adaptor was ligated to the CIP/TAP treated RNA (**Figure 25 A**). Because the adaptor ligates only to RNA containing a 5′-phosphate, RLM-RACE ensures that cDNA was amplified from decapped RNA predominantly, starting at the ultimate 5′ terminus. Reverse transcription was made for total RNA using reverse primer GSP 1, located within exon number 4, in presence of AMV reverse transcriptase for 1 hour at 37 C to create a cDNA. Two PCR were carried out to obtain a proper and precise band that represents the true size of the AtTOR cDNA 5′end. For the first PCR, we have used the reverse gene specific primer 2 (GSP2) and the forward 5′GeneRace primer. The size of the produced PCR fragment was about 745 bp, corresponding to the size of the second exon (347 bp), the first exon (166 bp), and the estimated 5′UTR (230 bp). We have made an additional PCR using the first PCR product as a template, in which, 1 µl from the first diluted PCR (1:100) was added to PCR reaction containing reverse gene specific primer 3 (GSP3) and the forward GeneRacer nested primer. The PCR product from this PCR was 405 bp in length (**Figure 25 B)**. These result confirmed the result that has been obtained by RT-PCR in Figure 24.

The product of the second PCR was cloned into pGEM-T Easy vector (TA cloning) after addition of poly (A) nucleotides to this PCR product to facilitate the cloning process. The pGEM-T plasmid with the cloned AtTOR 5′UTR fragment was send for sequencing using 5′ UTR – 245-F3 forward primer and GSP 2 reverse primer to determine the exact length of the AtTOR 5′UTR.

The result of sequencing indicates that the length of 5′UTR is 230 bp, and the transcription start site for Arabidopsis TOR (*AtTOR*) cDNA starts at AAAGCAAAA nucleotides at position denoted by -230 upstream from ATG start codon (denoted by +1). These results are consistent with other results that have shown using another 5′RACE method that the length of 5′UTR of *AtTOR* mRNA is 230 bp (**Menand *et al.*, 2002**). Both results (our result and the Menand's result) indicate that the length of AtTOR 5′UTR is 230 nucleotides.

An EST sequence from NCBI under the name (AV565959) indicates that the length of AtTOR 5′UTR is 376 bp. This sequence was amplified from *Arabidopsis thaliana* green siliques and cloned into pBluescript II SK- between *EcoRI* and *XhoI* restriction sites.

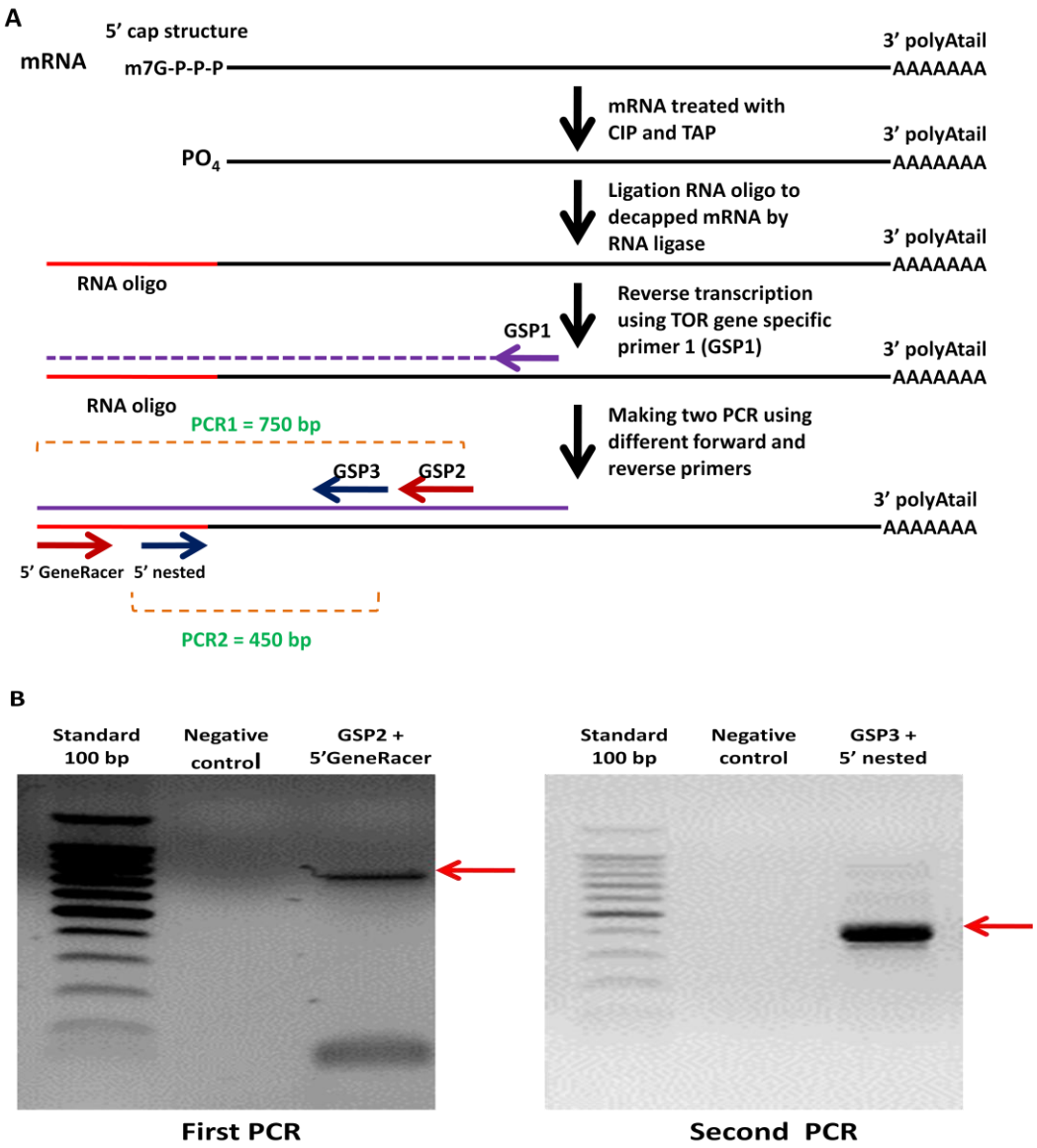


Figure 25: 5′RLM-RACE for 5'UTR of *AtTOR* gene. A- Schematic figure of RACE strategy showing primers and products. RNA oligo (red line) contains the sequence of 5'GeneRacer and 5'GeneRacer nested forward primers. Gene specific primer 1(GSP1; arrow in pink) was selected from the third exon *AtTOR* gene to make cDNA from treated mRNA. GSP2 and GSP3 reverse primers were chosen to include exone-intron-exon junction from *AtTOR* gene (intron 3, exone 2, and intron 1, respectively). **B-** Left panel: the product obtained in the first PCR using GSP2 (reverse primer) and 5'GeneRacer (Froward primer). Lane 1, 100 bp ladder; Lane 2: negative control without template RNA; Lane 3: RNA template treated with CIP and TAP. The length of PCR product from the first PCR equal approximately 725 bp. Right panel: the product obtained in nested PCR using GSP3 (reverse primer) and 5′GeneRacer nested (forward primer). Lane 1, 100 bp ladder; Lane 2: negative control without template RNA; Lane 3: RNA template treated with CIP and TAP. The length of PCR product from the second PCR equal approximately 410 bp. Red arrows refer to the amplified PCR band from each PCR reaction.

We have obtained these cDNA sequence (AV565959) from Kazusa DNA Research Institute in Japan. The plasmid was subjected to PCR amplification using M13 forward and reverses primers, and the PCR product has sequenced. The sequence observed was identical to the one in the database, corresponding to a fragment of – 376 to + 1. However, this fragment does not cover any intron and could therefore be artifact due to genomic DNA contamination. This hypothesis fit with our data.

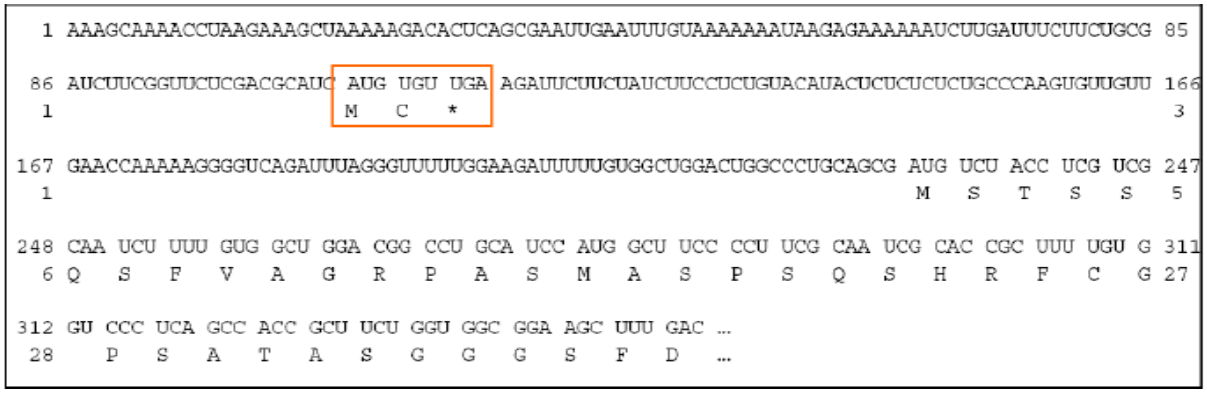


Figure 26: **Sequence of *AtTOR* 5'UTR and the beginning of the coding sequence.** The length of the 5'UTR is 0230 bp with a small uORF marked by the red box.

The sequence of the 5'RACE product revealed the presence of a short upstream open reading frame (uORF) encoding only 2 amino acids (ATG TGT TGA) located approximately in the middle of the 5'UTR (**Figure 26**).

This short uORF is conserved within Arabidopsis ecotypes (**Robaglia *et al.*, 2004**), suggesting that it may be conserved as well in another plant closely related to *Arabidopsis* such as *Brassica sp*, *Caryica sp*. *Cucumus sp*, etc. Thus, we will discuss in details in the following section the possibility of the conservation of TOR 5′UTR and/or uORF between different plant species according to a phylogenetic tree constructed with Phytozome database (**Phytozome v6.0, 2010**).

2.2.2. Identification and characterization of TOR 5′UTR from different plant species

In order to investigate the features of *TOR* gene, particularly the 5′untranslated region (5′UTR) region, we need to select the *TOR* gene sequence from different plant species. There are different databases used in comparative genomic studies, one of them is Phytozome database (Phytozome v.6., http://www.pytozome.net/ **Figure 27**).

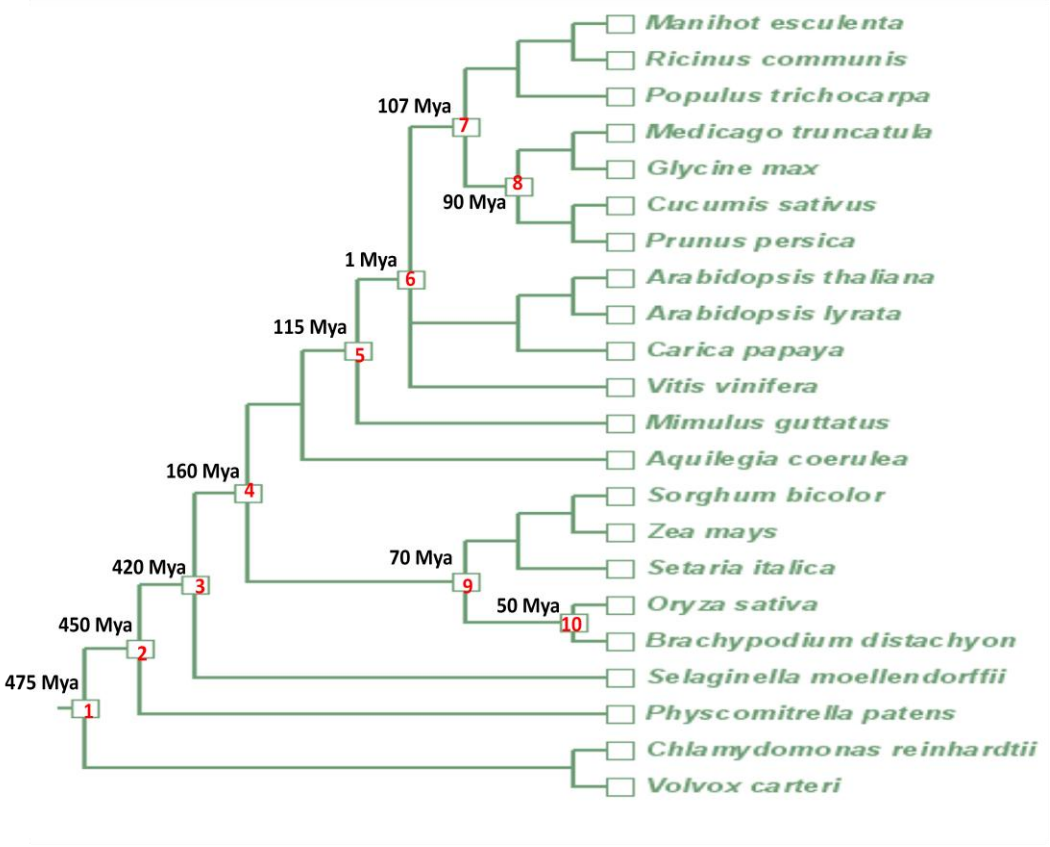


Figure 27: **Clustering overview representing sets of extant genes distributed in gene families.** Nodes were grouped to 10 internal nodes (the number in red color) and 22 crown nodes. The internal nodes represent the following events and the distance between each one by a million year (Mya) has been marked in a red colour.1: Viridiplantae (475 Mya); 2: Embryophyte (450 Mya); 3: Tracheophyte (420 Mya); 4: Angiosperm (160 Mya); 5: Core eudicot (115 Mya); 6: Rosid (1 Mya); 7: Fabid (107 Mya); 8: Nitrogen-fixing (90 Mya); 9: Grass (70 Mya); 10: BEP clade (one major lineage of grasses) (50 Mya). The crown nodes represented the different 22 plant species.

The Arabidopsis *TOR* (*AtTOR*) gene (At1G50030, **Menand *et al.*, 2002**) has been selected from TAIR (The Arabidopsis Information Resource) database and used as standards sequence to collect the predicted *TOR* gene sequences from other plant species from Phytozome v.6 database (http://www.pytozome.net/). According to the similarity of TOR amino acid residues between *Arabidopsis thaliana* (*At*) and other different plant species presented in the phylogenetic tree created by Phytozome, approximately eleven plant species distributed between dicots, monocots and mosses have been selected. These species are the dicots *Arabidopsis lyrata* (*Al*) [99.8 %], *Cucumis sativus* (*Cs*) [90 %], *Populus trichocarpa* (*Pt*) [79.9 %], *Vitis vinifera* (*Vv*) [86.1 %], *Glycine max* (*Gm*) [90.4 %], *Mimulus guttatus* (*Mg*) [88.3 %], the monocots, *Brachypodium*

distachyon (*Bd*) [81.7 %] and *the moss Physcometirella patens* (*Pp*) [75.4 %]. The numbers in brakets denotes the percentage of similarity of TOR amino acids from these plants species compared with the TOR amino acids from *Arabidopsis thaliana*. *Brassica rapa* TOR *(BrTOR)* was selected from *Brassica rapa* subsp. Pekinensis colne kBrB065N20 from the national center of biotechnology information (NCBI) sequence viewer v 2.0 under accession number of AC189427 after a blast with AtTOR amino acid sequence.

Alignment of TOR protein between the different plant species was achieved to confirm the corrected *TOR* gene sequence from these different species in comparison with *AtTOR* gene sequence. Alignment results revealed that the beginning of predicted TOR amino acid residues (about 200 aa) from *Arabidopsis lyrata* (*Al*); *Gm*: *Glycine max*; *Vv*: *Vitis vinifera*; *Mg*: *Mimulus guttatus*; *Pt*: *Populus trichocarpa*; *Cs*: *Cucumis sativus,* and *Bd: Brachypodium distachyon* have a high similarity to TOR amino acid residues from *Arabidopsis thaliana* (*At*), except that approximately the first 40 deduced amino acid residues from *Brachypodium distachyon* TOR protein (*BdTOR*) have low similarity with TOR protein from Arabidopsis and the rest of the other species **(Figure 28)**. This may be related to the important divergence between Arabidopsis and Brachypodium which are separated by nearly 160 million years. Moreover, TOR amino acid residues from another species such as *Ricinus communis* (*Rc*), *Medicago truncatula* (*Mt*), *Carica papaya* (*Cp*), and *Physcomitrella patens* (*Pp*) were subjected to alignment with TOR amino acid residues from *Arabidopsis thaliana* (*At*). The result showed a lower similarity between the beginning of the predicted TOR protein (about 200 amino acid residues) from these species and the beginning of TOR amino acid residues from *Arabidopsis thaliana*.

The low similarity between the beginning (about 200 amino acid residues) of TOR protein from *Ricinus communis* (*Rc*), *Medicago truncatula* (*Mt*), *Carica papaya* (*Cp*), and *Physcomitrella patens* (*Pp*) and Arabidopsis TOR protein may be related to the fact that TOR mRNA prediction at their translation starting codons was inaccurate. It may be due to some mistakes occurring during predicted of intron/exon junctions.

We will explore the features of 5′UTR of *TOR* RNA for nine plant species including *Arabidopsis thaliana, At*) having a high similarity in TOR protein sequence (as shown in figure 27), as well as *Brassica rapa* (from Brassicaceae) and *Carica papaya* (Caricaceae). We also choose *Physcomitrella patens* to represent one of the basal land plants to compare its TOR 5′UTR with

other plant species, because we obtained the PpTOR 5′UTR experimentally in our laboratory (Milena Mozzo).

```
At   MSTSSQSFVAGRPASMASPSQSHRFCG-PSATASGGGSFDTLNRVIADLCSRGNPKEGAP 59
Al   MSTSSQSFVAGRPASMASPSQSHRFCG-PSATASGGGSFDTLNRVIADLCSRGNPKEGAP 59
Gm   ---------------MATASQSHRYIG-PPSVAPGPG--DALNRILADLCTRGNPKEGAS 42
Vv   ---------------MASTAQSIRFGA-PAAG----SSLDALNRILADLCARGPPKDGAA 40
Mg   -------------MALATTSQSIRYIA-PAGAT-GASNLDALNRVLADLCIRGNPKDGAA 45
Pt   ---------------MASTSQSLRFLVGPATTAPGGGSFDALNRILADLCTRGNPKEGAT 45
Cs   ---------------MATSGQSLRSSS--AATS--GGNFDSLNRILSDLCTRGHPKEGAP 41
Bd   ----------------MKPSPHFPEIG--KKSKDLVAKDHSFN--IGAYISSG--ADAIS 38
                        ...                  .  .::*  :.     *   :. .
At   LAFRKHVEEAVRDLSGEASSRFMEQLYDRIANLIESTDVAENMGALRAIDELTEIGFGEN 119
Al   LAFRKHVEEAVRDLSGEASSRFMEQLYDRIANLIESTDVAENMGALRAIDELTEIGFGEN 119
Gm   LALKKHLEEEARDISGEAFSRFMDQLYDRISGLLDSSDVAENLGALRAIDELIDVALGEN 102
Vv   LALKIHLEEEARDLSGEAFSRFMDQLYDRISNLLDSNDVAENMGALRAIDELIDVALGES 100
Mg   SALRRHVEEQARDLSSEAFSRFVDHLYDRITNLLESNEVAENLGALRAIDELIDVGIGEN 105
Pt   LALRKHLEEEARDISGEAFPRFMDHLYERISSLLESNEVAENLGALRATDELIDVALGEN 105
Cs   SALKKHIEEAARDLNGEAFSRFMDQLYDRISTLLESNDVAENLGALRAIDELIDVALGEN 101
Bd   AALRKHVEEEARDLSGETFLRFMDQLYEQIYILLQSNDVSENLLALRAIDALIDMPFGEG 98
      *:: *:** .**:.:*:  **::::*::*  *::*.:*:**: **** * * :: :**.
At   ATKVSRFAGYMRTVFELKRDPEILVLASRVLGHLARAGGAMTSDEVEFQMKTAFDWLRVD 179
Al   ATKVSRFAGYMRTVFELKRDPEILVLASRVLGHLARAGGAMTSDEVEFQMKTAFDWLRVD 179
Gm   ASKVSRFSSYMRIVFDTKRDPEILVLASRVLGHLARAGGAMTADEVERQVKIALDWLRGN 162
Vv   ASKVSKFSGYVRTVFEAKRDRDVLILASTVLGHLARAGGAMTADEVECQVQNALEWLRGE 160
Mg   AVKVAKIANYMRTTFEAKRDPETLVTASKVLGHLARAGGAMTADEVERLVKVALEWLRGE 165
Pt   ASKVSKFAIYMRSVFEVKRDPEVLTHASRVLGHLARAGGAMTADEVEFQVKMALDWLHND 165
Cs   ASKVSKFSNYIRSVFELKRDPEILVLASRVLGHLARAGGAMTADEVEHQVKIALDWLRGE 161
Bd   ASKVSKFASFLRNVFEVKRDPEILVPASTVLGHLAKAGGAMTAEEVERQIKTALGWLGGD 158
     * **:::: ::* .*: *** : *  ** ******:******::***  :: *: **  :
At   RVEYRRFAAVLILKEMAENAS---------------------- 200
Al   RVEYRRFAAVLILKEMAENAS---------------------- 200
Gm   RVEYRRFAAVLILKEMAENASTVFNVHVPEFVDAIWVA---- 200
Vv   RIEYRRFAAVLILKEMAENASTVFNVHVPEFVDAIWVALR-- 200
Mg   RVEYRLFAAVLILKEMAENASTVFNVHVPEFVDAI------- 200
Pt   KAEFRLFAAVLILKEVAENASTVFNVHVPEFVDAIWVAL--- 204
Cs   RIEYRRFAAVLILKEMAENASTVFNVHVPEFVDAIWVAL--- 200
Bd   RVEYRRFAAVLILKEMAENASTLFNVHVTEFVDAIWVALRDP 200
     : *:* *********:*****
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Figure 28: Alignment between the first 200 amino acids residues of TOR amino acid residues from different plant species using ClustalW software. Alignment of the beginning of TOR amino acids for seven plant species has revealed a high similarity for TOR protein in respect to TOR amino acid residues deduced from *Arabidopsis thaliana* that was used as a reference. The first 40 amino acids residues from *Brachypodium distachyon* (*Bd*) protein appeared to have a low similarity with TOR protein sequence from other species including Arabidopsis. The name of TOR protein from the different plant species is as following: *At*: *Arabidopsis thaliana*; *Al*: *Arabidopsis lyrata; Gm*: *Glycin max*; *Vv*: *Vitis vinifera*; *Mg*: *Mimulus guttatus*; *Pt*: *Populus trichocarpa*; *Cs*: *Cucumis sativus,* and *Bd: Brachypodium distachyon*. All plant species belong to dicotyledon except *Brachypodium* from monocotyledon. The beginning of TOR protein is denoted by amino acid methionine (M). In case of *At* and *Al,* the first methionine is located at the same position, however, the TOR protein starts after approximately fifteen amino acids in other plant species comparing with *At* or *Al* .

About 500 bp upstream of the ATG from *Cucumis sativus* (*CsTOR*) and *Brachypodium distachyon* (*BdTOR*) mRNAs were selected. Alignment between the *Cucumis sativus* (*CsTOR)* genomic DNA and *CsTOR* mRNA and the same alignment for *Brachypodium distachyon* TOR (*BdTOR*) genomic DNA and *BdTOR* mRNA have allowed us to determine the positions of exons and introns.

Thus, we were able to design the forward and reverse primers needed for amplification of the TOR 5′UTR sequences from both species by RT-PCR to determine the transcription start sites of *TOR.*

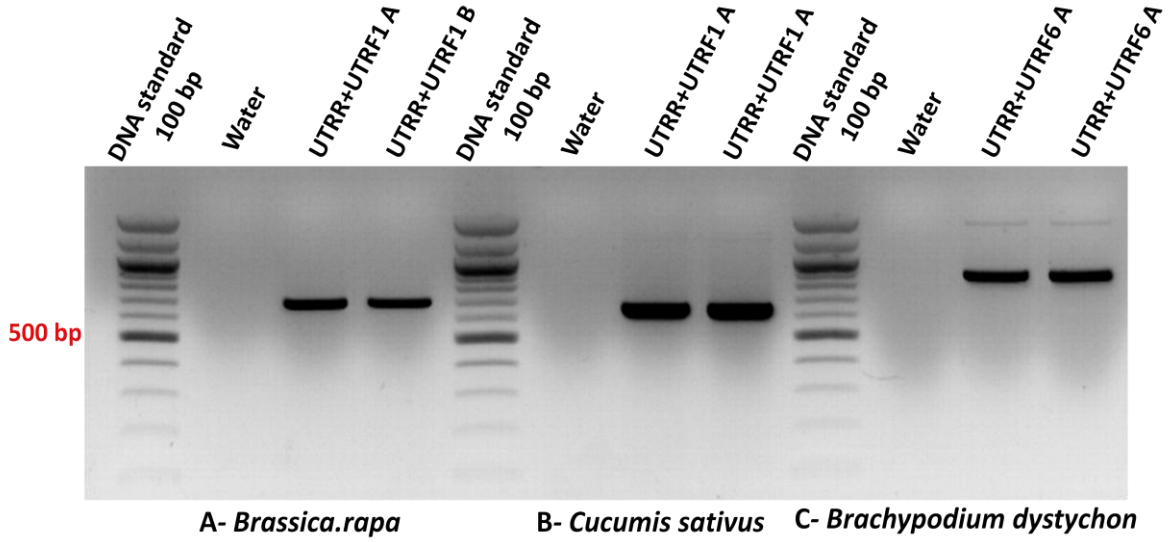

Figure 29: RT-PCR for detection of the length of 5′-UTR from *Brassica rapa* (A), *Cucumus sativus* (B), and *Brachypodium distachyon* (C) TOR cDNA. Two cDNA samples (A and B) from each species have been used for a PCR amplification using the same forward and reverse primers. PCR reaction with water instead of a cDNA template was used as a negative control for each one.

By determination of the position of exons and introns in *BrTOR* gene, we have designed the different forward and reverse primers needed for amplification of *BrTOR* 5′UTR sequence using RT-PCR. The names and sequences of each primer were described in material and methods Total RNA has been extracted from *Brassica rapa*, *Cucumis sativus* and *Brachypodium distachyon*. Reverse transcription has been carried out using gene specific primer located proximal to the 5′end from each species, in presence of AMV reverse transcriptase to amplify cDNA.

For *Brassica rapa*, RT-PCR amplification from BrTOR cDNA has been done to determine the length of TOR 5′UTR using Br + 528-R1 and Br -180-F1 primers. The resulted PCR fragment was 708 bp in length. By calculation the length of PCR sequence, we have found that minimal length of *BrTOR* 5′UTR approximately 180 bp, where, the length of the first and second exons were 162 and 366 bp, respectively (**Figure 29 A**). For *Cucumis sativus*, RT-PCR amplification for *CsTOR* cDNA using Cs +447-R1 and Cs -220-F1 revealed that the minimal length of their 5′UTR is about 220 bp in length. The PCR product was measured about 667 bp (**Figure 29 B**), in which the first and the second exons measure 108 and 339 bp, respectively. For *Brachypodium distachyon*, a clear RT-PCR fragment of 884 bp has been obtained from *BdTOR* cDNA using Bd +524-R1 and Bd -360-F6 primers. This correspond to 360 bp for the minimal length of the BdTOR 5'UTR only, while 46 bp for the first exon, 65 bp for the second exon, and 422 bp for the third exon (**Figure 29 C**).

From all these results we have concluded that the length of TOR 5′UTR varied between different plant species from about 180 to 230 bp for dicots plants that have been studied and it reach to nearly 360 bp for Brachypodium (monocots) and 400 bp for Physcometirella (mosses, data from Dr. Milena Mozzo).

```
At ------------------AAGCAAAACCTAAGAAAGCTAAAAAGACACTCAGCGAATTGA 42
Al -------------GAACAAAGCAAAACCTAGGAAAGCTAAAAAGACACTTAGCCAATTGA 47
Br ATTTACACTGCCTGAAAGAACCTGCACAAAAAAAACCTAAAAA-------AGCGAATTGA 53
                     ** *   **  *  *** *******       *** ******
At ATTTGTAAAAAAATAAGAGAAAAAATCTTGATTTCTTCTGCGATCTTCGGTTCTC----- 97
Al ATTTGTAAAAAAA-----AAAAAAATCTTGATTTCTTCTGCGATCTTCGCTTCTC----- 97
Br TTTTTGTAAAAAA------AAATAATCTTATTTTCTCCTGCGATCTCCGCTTCTCTCTCT 107
   ***   ******      *** ******  ***** ********* ** *****
At ------GACGCATCATGTGTTGAAGATTCTTCTATCT------TCCTCTGTACATACTCT 145
Al ------GACGCATCATGTGTTGAAGATTCCTCTCTCT------TCCTCTGTACATACTCT 145
Br CTCTTCGACGCACGATGTATTGAAGATACCTCTCTCTCTCTTCCTCTGTACATACCCT 167
       ******  **** ******** * *** ***      ************** **
At CTCTCTGCCCAAGTGTTGTTGAACCAAAAA--GGGGTCAGATTTAGGGTTTTTGGAAGAT 203
Al CTC--TACCCAAGTGTTGTTGAACCAAAAAAAGGGGTCAGATTTAGGGTTTTTGGAAGAT 203
Br CTCTAATACCCACTGTTGCTGAACCAAAAA--GGGGTTAGATTTAGGGTTTTTGCGAGAT 225
   ***     ** * ***** ***********  ***** ****************  ****
At TTTTGTGGCTGGACTGGCCCTGCAGCGATG 233
Al TTTTGCGGTTGGACCGGCCCTGCAGCGATG 233
Br TTCTGATG---------------------- 233
   ** **  *
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Figure 30: Alignment of the 5'UTR from *Arabidopsis thaliana* (*At*), *Arabidopsis lyrata* (*Al*), and *Brassica rapa* (*Br*) TOR mRNA using ClustalW program. The pink color box denotes the conserved uORF of 9 nucleotides between the different four species. The sequence downstream to the uORF appears to be more conserved than the sequence upstream of the uORF.

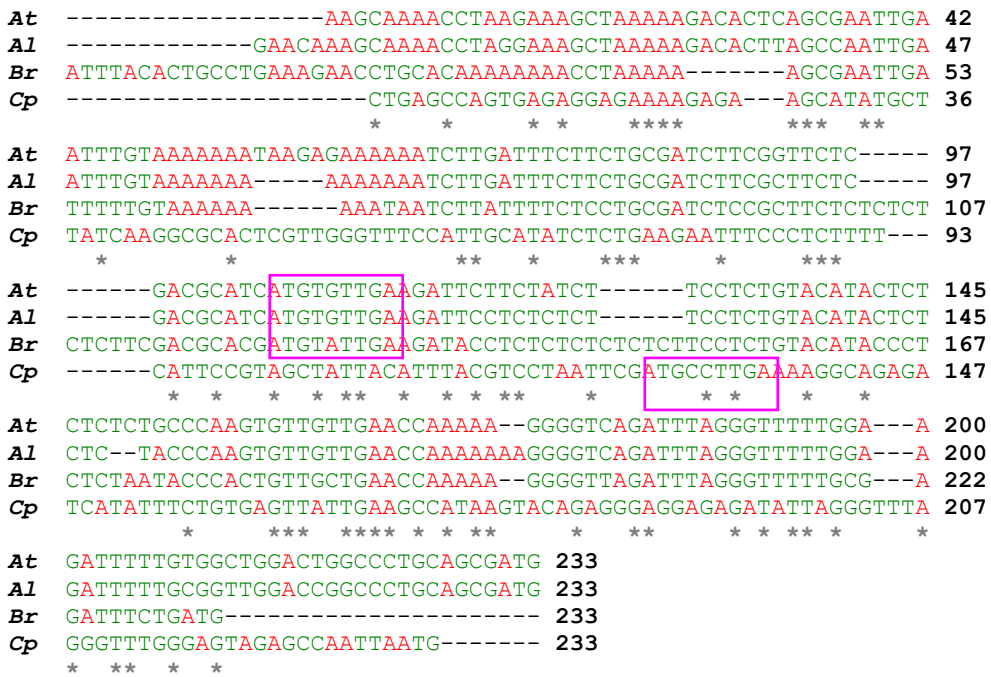


Figure 31: Alignment of the 5'UTR from *Arabidopsis thaliana* (*At*), *Arabidopsis lyrata* (*Al*), *Brassica rapa* (*Br*), and *Carica papaya* (*Cp*) TOR cDNA using ClustalW program. The pink color box denotes the uORF of 9 nucleotides which is not conserved between the different four species.

Our results were in line with other results recorded that leader length of mRNA for dicotyledonous plants is shorter than the leader length of mRNA for monocotyledonous plants (**Kochetov *et al.*, 2002**).

Many studies shown that structural features of the 5′UTR such as presence of an upstream AUG codons (uAUGs), upstream open reading frames (uORFs), and stable secondary structures have a major role in the control of mRNA translation (**Mignone *et al.*, 2002**).

According to our results related to the differences in 5′UTR lengths of TOR mRNA that we have obtained between different plant groups, we investigated the distribution frequency of upstream open reading frames (uORFs) along their 5′UTR and the presence of stable secondary structures.

We have made alignment between the 5′UTR nucleotide sequence between three different plant species *Arabidopsis thaliana*, *Arabidopsis lyrata*, and *Brassica rapa* because they belong to the same family that is Brassicaceae. We also added *Carica papaya* that belongs to the nearest family to Brassicaceae (**Figures 30 and 31**).

Alignment results have revealed that the 5′UTR nucleotide sequences from *Arabidopsis lyrata* (*Al*) and *Brassica rapa* (*Br*) share high similarity with the 5′UTR from *Arabidopsis thaliana* (*At*). Furthermore, the small uORF is conserved between the three species. The uORF is found at the same location and has the same number of deduced amino acids (2 amino acids with the start codon), suggesting that their function might be also conserved. The presence of uORF at the same position and with the same size (2 amino acids) is not surprising because the three plant species belongs to the same family.

For the plant species from other families, we have build a schematic structure to represent the 5′UTR lengths and their different uORFs that are distributed along their *TOR* mRNA from the plant species that have a high degree of similarity within their AtTOR amino acid residues. The arrangement of the different plant species within this schematic structure is the same as the distribution of plant species in Phytozome dataset in Figure 27 (**Figure 32**).

The effect of uORFs on gene expression has mainly been studied at the translational level. The action of uORF is related somehow with their position and their size within the 5′UTR. Thus, we examined both their positions and their size within their 5′-UTR. We have divided the studied plant species into four different groups on the basis of the position and the number of their uORFs. The first group includes *Arabidopsis thaliana*, *Arabidopsis lyrata*, *Brassica rapa*, and *Cucumis sativus*, in which TOR 5′UTR nucleotide sequences have a high similarity and have been detected by RT-PCR. Only one uORFs has been detected in this group. It is located at a nearly at equal distance from the start of their 5′UTR and the start of the TOR coding region (**Figure 32**). The predicted uORF from TOR mRNA is highly conserved in this subgroup.

The second group includes two sub-groups in their TOR 5′UTR. Sub-group 2a include the plant species in which the TOR 5′UTR contains just one uORF located at 193 and 174 nucleotides upstream of the ATG start (*Vitis vinifera* and *Mimulus guttatus*, respectively).

5′UTR Sub-group 2b contains plant species in which the TOR 5′UTR contains two uORFs distributed at different distances within their 5′UTR. For *populus trichocarpa* (group 3), it was observed that their TOR 5′UTR mRNA contains three uORFs in which the first one located at the middle of their 5′UTR and the second is located at 166 and 200 nucleotides upstream of the ATG start codon respectively. The fourth group involve both Brachypodium *distachyon* and *Physcomitrella patens*.

The analysis of the TOR 5′UTR from these two plant species has revealed presence of some differences in their nucleotides composition and their lengths.

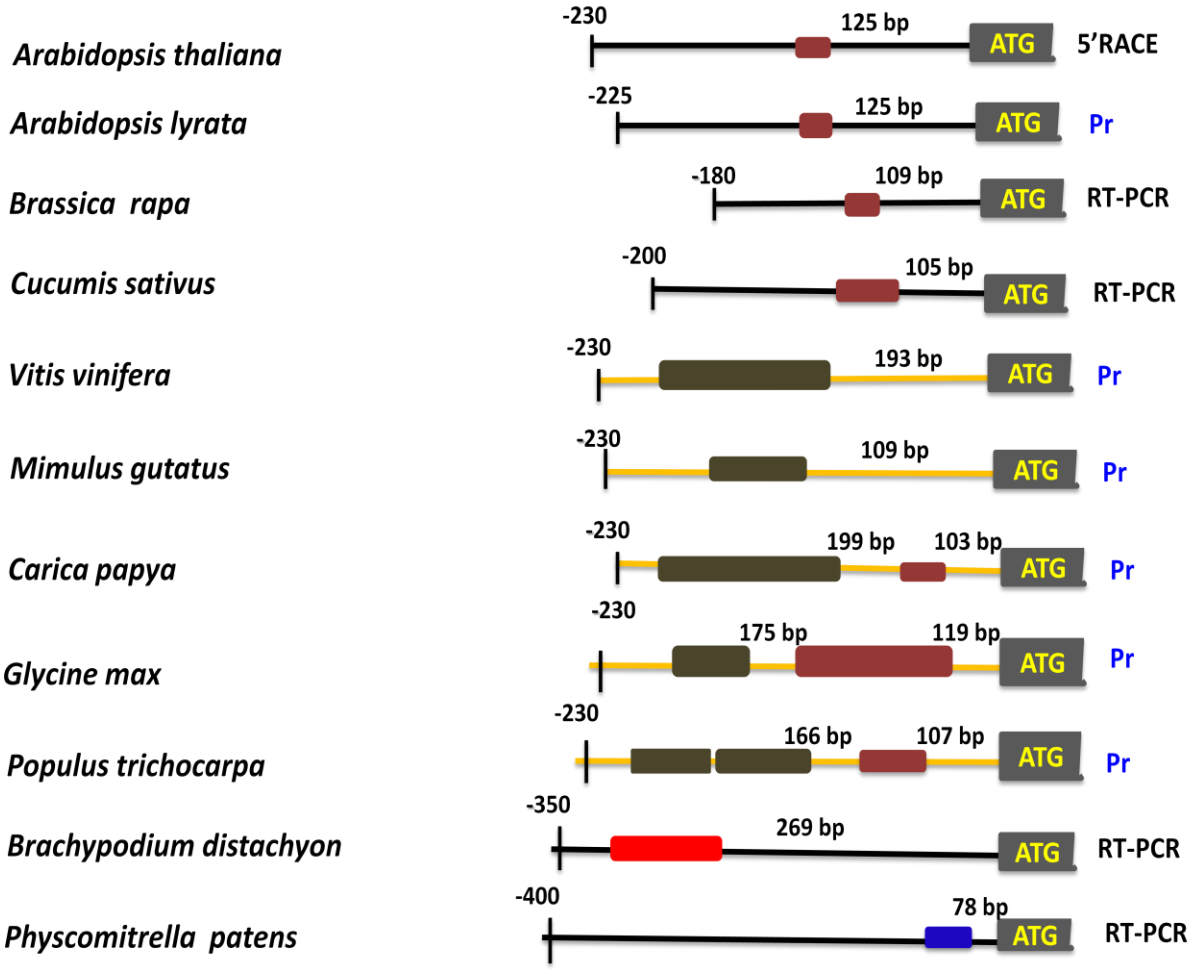


Figure 32: Diagram showing the occurrences of uORFs along the *TOR* 5′UTR from different plant species. *TOR* 5′UTR from *Arabidopsis thaliana* (*AtTOR*) has been determined by 5′RACE (as described in the previous section), while in *Brassica rapa, Cucumis sativus and Brachypodium* distychon it was determined by RT-PCR. For other species, TOR 5'UTR was selected from the Phytozome dataset with reference to TOR protein sequences from *Arabidopsis thaliana*. The length of predicted TOR 5′UTR from dicots species (yellow lines) is 230 bp upstream the start codon ATG in reference to TOR 5′UTR of *Arabidopsis thaliana*. Black lines refer to the length of 5′UTR that has been determined experimentally by RT-PCR; however, yellow lines refer to predicted 5′UTR only. The different colour boxes within the 5′UTRs denote the different position of upstream open reading frames (uORFs). The different uORFs were represented by larger and smaller size according to their length for it. The intercistronic distances values among the uORFs and ATG start codon were determined and placed.

We observed that sequences of TOR 5′UTR contained more G and C than A and T nucleotides in *Physcomitrella patens* and *Brachypodium distachyon* than in other species (**Figure 33**). This result is in accordance with results obtained from analysis of 5′UTRs and showning that the leader sequences of the dicot genes are enriched in A and T, and those of monocots in C.

As we mentiond above the G + C content correlates with the ability of RNA to form a stable secondary structure and many experiments have revealed that the presence of secondary structure on the leader sequence resulted in decrease of its translational efficiency (**Kozak, 1999; Niepel *et al.*, 1999**). The length of their 5′UTR is longer than for other plant species, measuring approximately 350 bp and 400 bp for *Brachypodium distachyon* and *Physcomitrella patens* respectively. These two species possess just one uORF. Brachypodium TOR 5′UTR uORF is located 269 nucleotides upstream the ATG start codon and encodes 25 amino acids. The *Physcomitrella* TOR 5′UTR uORF is located close to the ATG start codon just 78 nucleotides and encodes for 6 amino acids.

Upstream open reading frame (uORF) position and size are considered a fundamental point in mechanism through uORF-containing transcripts can be regulated at translational level. The ribosome scanning and reinitiation of translation are dependent on the uORF length and the uORF position with respect to the main start codon (mAUG). Some studies reported that plant uORFs can vary in length from 6 to 156 nucleotides (**Wang and Wessler, 2001; Franceschetti *et al.*, 2001; Hanfrey *et al.*, 2002**), we examined the length of the uORFs that are distributed along 5′UTRs from the *Arabidopsis thaliana TOR (AtTOR*), *Arabidopsis lyrata TOR* (*AlTOR*), *Brassica rapa* TOR *(BrTOR*), Cucumis sativus *TOR CsTOR,* Brachypodium distachyon *TOR BdTOR,* and *Physcomitrella patens TOR* (*PpTOR)* mRNAs.

The results indicated that 5′UTR from *Arabidopsis thaliana, Arabidopsis lyrata*, and *Brassica rap* have a tiny uORF with 9 nucleotides in length, while, the TOR mRNA from *Cucumis sativus* and *Physcomitrella patens* havs a uORF of 18 nucleotides, however, the 5′UTR from *Brachypodium distachyon* TOR mRNA was found to have a large uORF of 75 nucleotides. This result is in line with the results from computer analysis of 5′UTR mRNA from higher plants indicating that uORFs in mRNA 5′UTRs of dicots are short with the mean length of 36 nucleotides. Together, we can conclude that Arabidopsis TOR (*AtTOR*) mRNA and other species from Brassicaceae contain in their 5′UTR a small uORF encoding for a 2 amino acids only and located approximately within the middle of 5′UTR sequences. Other species from monocots and

dicots that have more than one uORF and different sizes from each one, that thought might have a potential effect on translational efficiency of the main coding sequences but these theoretical results need to be verified experimentally.

***Arabidopsis thaliana* (230 bp)**

AAAGCAAAACCTAAGAAAGCTAAAAAGACACTCAGCGAATTGAATTTGTAAAAAAATAAGAGAAAAAATCTTGATTTCTTCTGCG
ATCTTCGGTTCTCGACGCATCATGTGTTGAAGATTCTTCTATCTTCCTCTGTACATACTCTCTCTCTGCCCAAGTGTTGTTGAACCAAA
AAGGGGTCAGATTTAGGGTTTTTGGAAGATTTTTGTGGCTGGACTGGCCCTGCAGCG**ATG**

***Brassica rapa* (180 bp)**

GAATTGATTTTTGTAAAAAAAAAATAATCTTATTTTCTCCTGCGATCTCCGCTTCTCTCTCTCTCTTCGACGCACGATGTATTGAAGATA
CCTCTCTCTCTCTCTTCCTCTGTACATACCCTCTCTAATACCCACTGTTGCTGAACCAAAAAGGGGTTAGATTTAGGGTTTTTGCGAG
ATTTCTG**ATG**

***Cucumis sativus* (200 bp)**

CAGGACGCGGACAAATTAACAAAGCCTTTCTTCTCTTCATCTTCACGCTCAACAATACAGCTCTTCATCTCACTATGAAAATTATGCT
GATTTCAATGCTTCTGAACATTTAGGGCTCAAACACAAAATCCAAGGAATGAGTTAGAAAGCAGTTCTAATACAAGAAACCTTCTT
CTGGGGTTTAGGGTTTTTGTGGCCTG**ATG**

***Brachypodium distachyon* (350 bp)**

GACGCCGCATCCCAGCTCCGCGCCGGCTCATCCTTCTCCGACACGCCGCCGCCTCTCTCTCCTCCGCGACGTCGTCTTTACGAGCC
CATGCGCCCGGCGCACCCTGCACAAGGCGCTCCTTGGCATGCGGCAGCCGGCAGCCGGCAGGGTGTAGCGCCCTAGCTTCGCCT
TCCTGCTTTAGAGCCCCGTCCCTCGCTCCGTCTCGACGATTCGATCCCCACGCGCAGCAGCTCCCCCCACCCCGCCTTCCTGCGCT
GCCTCTCCAGCCGCGCCCCTCCTTCTCTCAGTTTTCCCCCACCCCACTATTTTGTTCTCTGCCTCTGGCCGCGGCGGCGGCTCTGGA
GGCGCCGTCGAG**ATG**

***Physcomitrella patens* (400 bp)**

CATTTCGGGTCGGGGTAACAGCAGGAATAATCACGATAACCAGGAACACGAAGCCATCATGTAGGAGGAGGAGGAGTTGGAAG
AGAAGGAGCACGGAAGACGACGACTGCGACCAAGACTAGACATCAAAGAGGAGGAGCAGGAGCAGGAGGACGAGGAGGAG
GAGGGAGGAGAGAGGAAGAAGAAGGGAGGAAAGAGGAAGAAGAAGGGAGGAGAGAGGAAGGAGAAGGGAGGAGGAGG
AGGAGAAGGTGGACTCGGAAGAGGAGGTGGAGCTGCTGCAGATGGTGGCTATGGCGACGACGGGGGAGTTTGCAATGGCGTG
TTTCATGTAGAGGGTAGAGGCGAGTCGACACACAGAGAGGCGAGGGAGCCGCGTGCTTCTTATTGCGCTTGCCTGCC**ATG**

Figure 33: TOR 5′UTR nucleotide sequences from 5 plant species determined by RT-PCR. ATG start codon was denoted by a red color and the length of the 5′UTR was placed under parentheses beside the name of each plant species. The different plant species are *Arabidopsis thaliana, Brassica rapa, Cucumis sativus, Brachypodium distachyon,* and *Physcomitrella patens.*

As we mentioned above that the secondary structures of the 5′UTR act as down-regulators of gene expression, where it have the ability to inhibit gene transcription process (**Curie and McCormick, 1997**), accelerating mRNA degradation rate (**Cannons and Cannon, 2002**), or reduce translation efficiency (**Bunimov *et al.*, 2007**). Using mfold program (Zuker et al., 1999), the predicted 5′UTR secondary structures from *Arabidopsis thaliana* TOR *(AtTOR,* **Figure 34***)*,

Brassica rapa TOR *(BrTOR,* **Figure 35***), Cucumis sativus* TOR *(CsTOR,* **Figure 36***), Brachypodium distachyon* TOR *(BdTOR,* **Figure 37***), and Physcomitrella patens* TOR *(PpTOR,* **Figure 38***)* mRNAs has been achieved. The analysis revealed that 5′UTRs from *AtTOR*, *BrTOR*, and *CsTOR* have the ability to form slight stable secondary structures as demonstrated by the value of a change in free energy (ΔG) for each one as shown in figure 33 A, B and C. The value of ΔG for 5'UTR secondary structure was - 46.99 kcal/mol, - 28.93 kcal/mol, and – 27.61 kcal/mol for *AtTOR*, *BrTOR*, and *CsTOR*, respectively. These values were correlated to the amount of A + T and G + C for nucleotide composition for each 5′UTR. It was found that the amount of A + T for all three 5′UTR sequences are quite similar and estimated at 60.5 %. This results support the idea that a stable secondary structure is correlated with presence of a high content of G + C nucleotides. For the predicted 5′UTR secondary structure from *BdTOR* and *PpTOR*, the results suggest that the 5′UTR from these species have the ability to form a more stable secondary structure than that predicted from dicots species due to presence of a high content of C + G. It was observed that change of free energy (ΔG) of the 5′UTR secondary structure produced from *BdTOR* mRNA is strong and it was estimated with – 97.08 kcal/mol due to presence of a high amount of G + C content in their 5′UTR (68.86 %). The value of change of free energy (ΔG) of the 5′UTR secondary structure produced from *PpTOR* mRNA was moderate and it was estimated at – 60.61 kcal/mol due to presence of a higher content of G + C.

Analysis of structural features of untranslated regions from diverse taxonomic classes suggested that the presence of moderately stable secondary structures (ΔG below – 30 kcal/mole), as in BrTOR 5'UTR and CsTOR 5'UTR, do not stall the migration of 40S ribosomal subunit and thus their effect on efficiency of translation is low. However, the formation of very stable secondary structure (ΔG below – 50 kcal/mol), as in AtTOR 5'UTR, BdTOR 5'UTR and PpTOR 5'UTR, have the ability to affect significantly and decrease the translational efficiency of a transcripts (**Mignone *et al.*, 2002**).

Our results suggest that secondary structure of TOR 5′UTR have a functional significance. This functional significance may be involving the maintenance of translational activity of TOR mRNA at low level and preventing from deleterious excessive production.

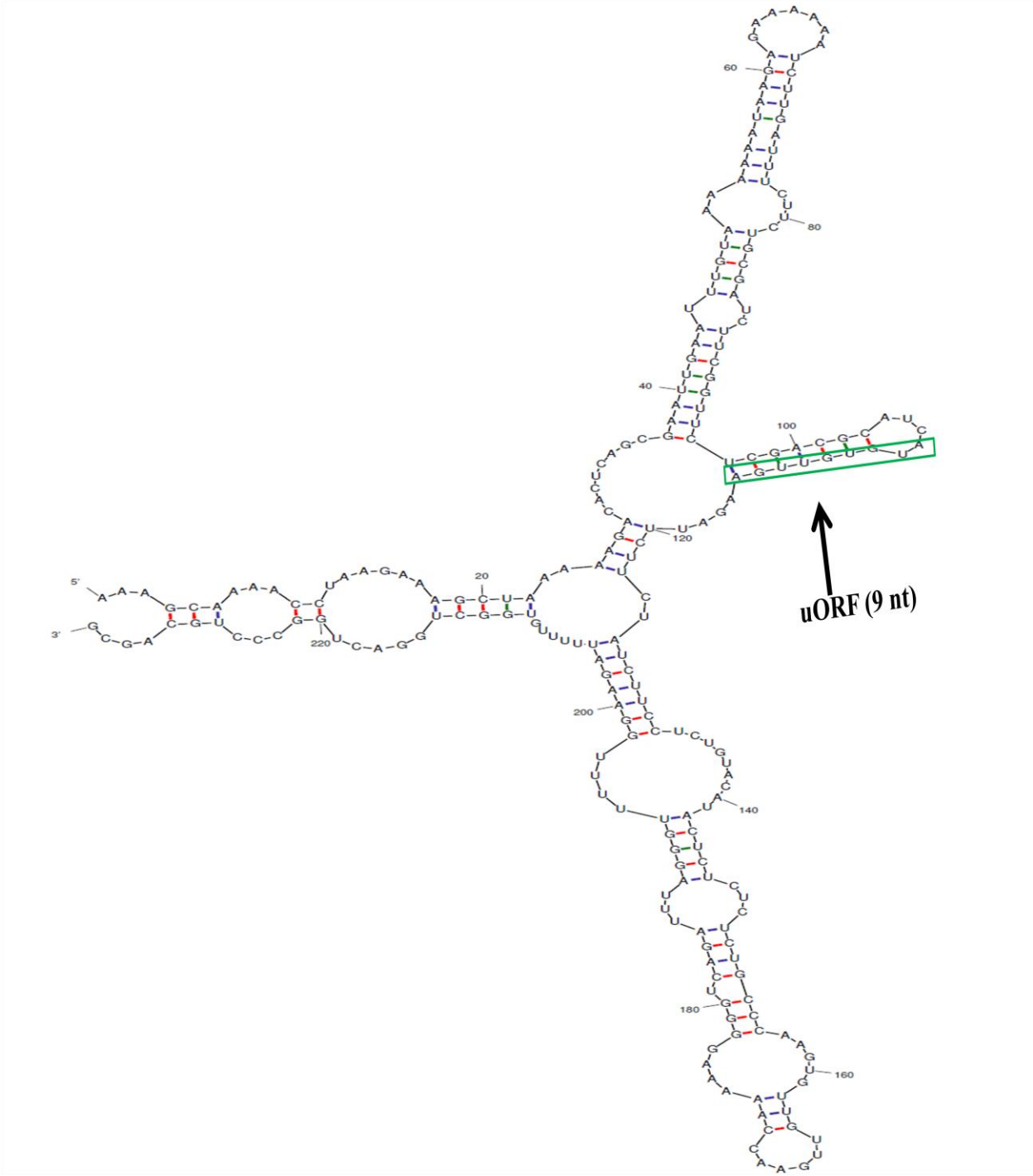


Figure 34: **Predicted local secondary structure from *Arabidopsis thaliana* TOR 5′UTR using mfold_util 4.4 programs.** Green boxes refer to the uORF position within the 5′UTR (230 bp, ΔG = - 46.99 kcal/mol).

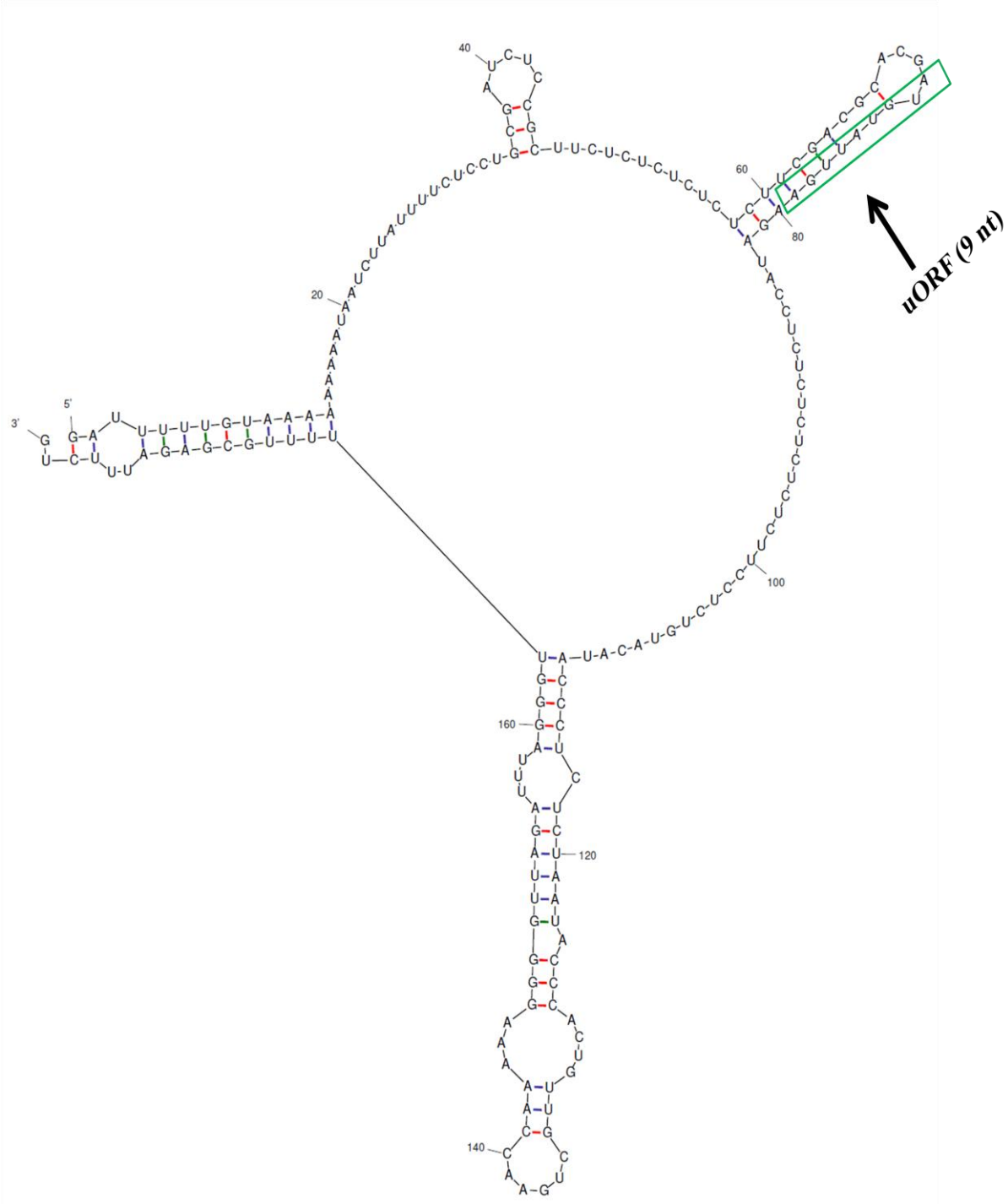


Figure 35: **Predicted local secondary structure from *Brassica rapa* TOR 5'UTR using mfold_util 4.4 programs.** Green boxes refer to the uORF position within the 5′UTR (180 bp, ΔG = - 28.93 kcal/mol).

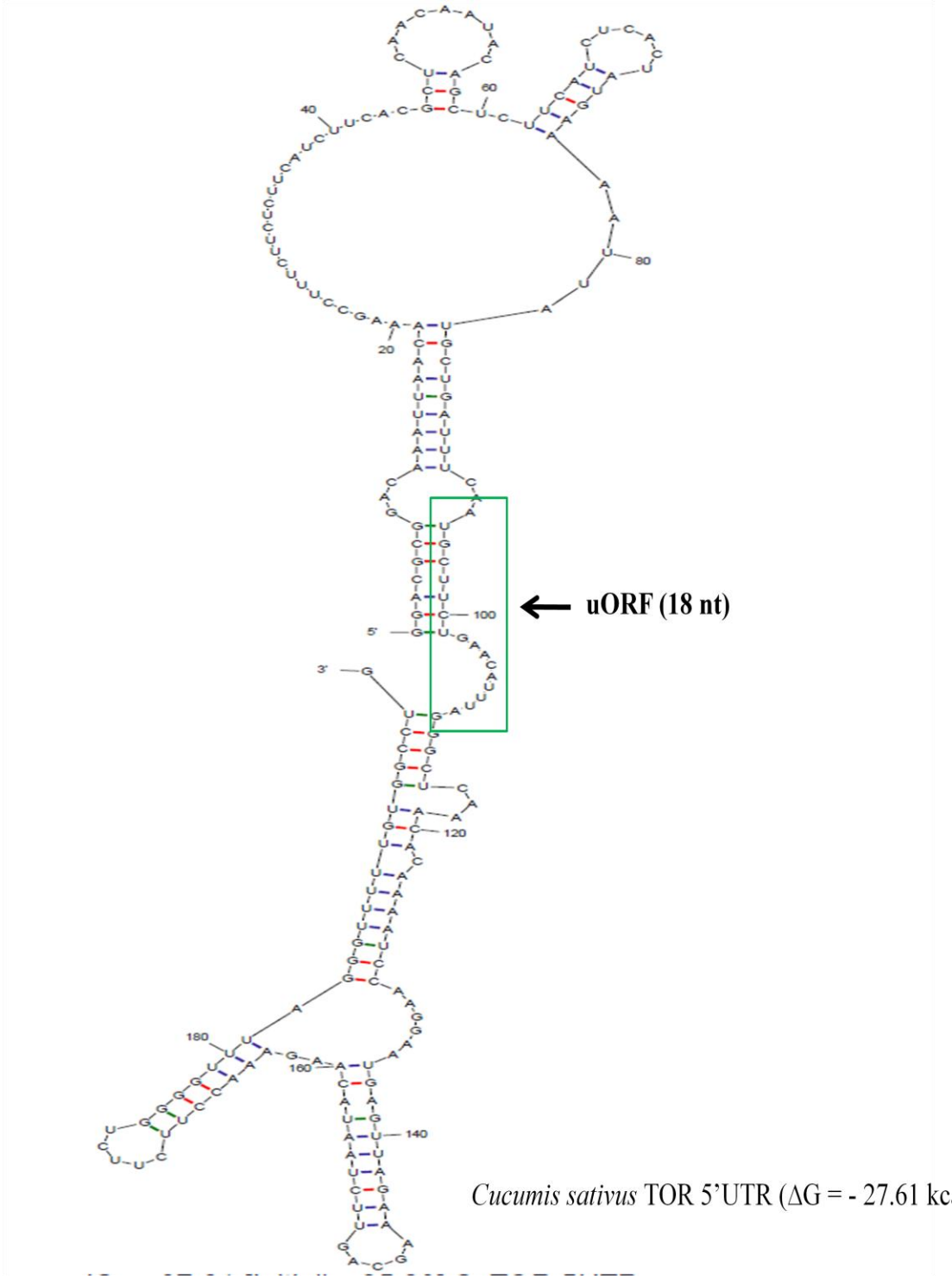


Figure 36: Predicted local secondary structure from *Cucumis sativus* TOR 5'UTR using mfold_util 4.4 programs. Green boxes refer to the uORF position within the 5′UTR (198 bp, ΔG = - 27.61 kcal/mol).

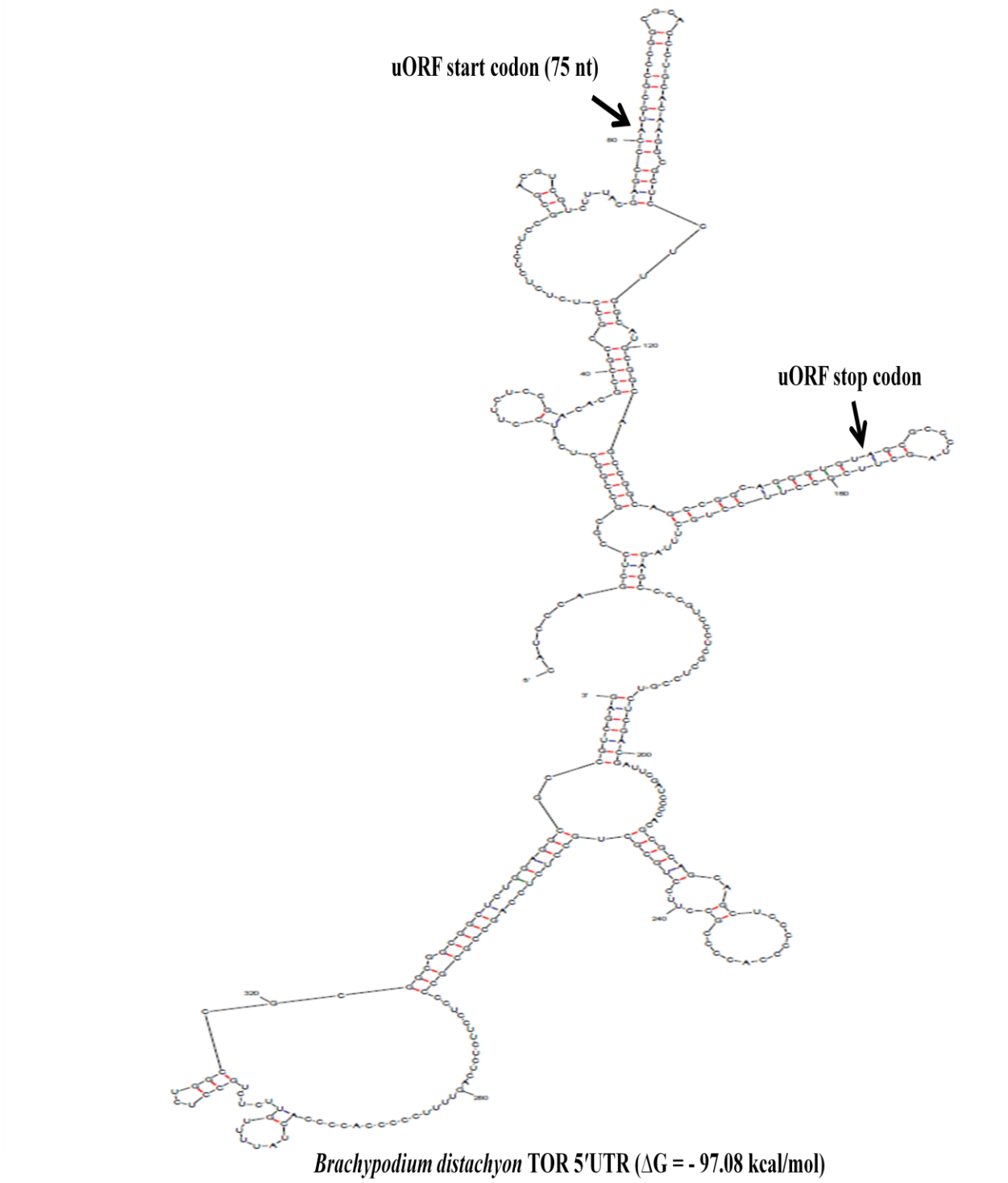


Figure 37: **Predicted local secondary structure from *Brachypodium distachyon* TOR 5'UTR using mfold_util 4.4 programs.** Black arrows refer to the start and the stop codons of the uORF position within the 5′UTR (360 bp, ΔG = - 97.08 kcal/mol).

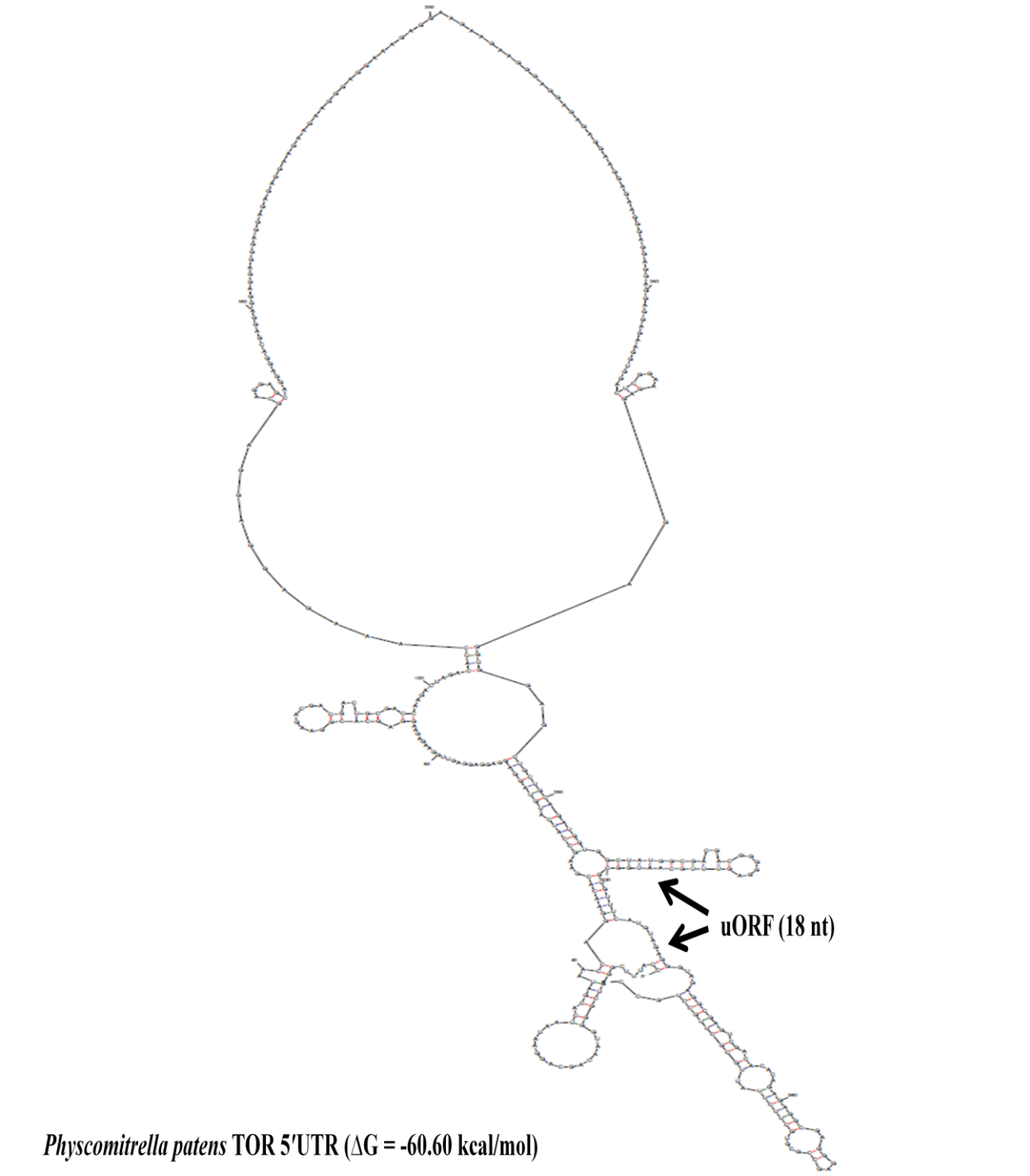


Figure 38: **Predicted local secondary structure from *Physcomitrella patens* TOR 5'UTR using mfold_util 4.4 programs.** Green boxes refer to the uORF position within the 5′UTR (400 bp, ΔG = - 60.60 kcal/mol).

2.3. Investigation of the role of 5′UTR and uORF in regulation of *AtTOR* expression

In order to investigate the role of 5′- untranslated region (5′-UTR) and the conserved small upstream open reading frame (uORF) in the regulation of *AtTOR* expression, three chimeric constructs have been established. The sequence used for production of these constructs was selected from the bacterial artificial chromosome (BAC) (gene F2J10.9; accession number AC015445) (**Figure 39 A**). Two groups of constructs were produced depending the type of promoter. One group was driven by the TOR promoter and based on the pBI101 binary vector; the second was driven by the CaMV 35S RNA promoter and based on the pBI121 binary vector **(Figure 39 b)**. The predicted promoter sequence of Arabidopsis TOR (*AtTOR,* At1G50030*)* gene was identified by determination the annotated coding sequence from TAIR (http://arabidopsis.org). Upstream nucleotides (approximately 2200 bp) located between the transcription start site of *AtTOR* gene and the perivious gene was detected as a TOR prmoter sequence.

2.3.1. Constructs with TOR promoter

The first construct (TORP-5′UTR; the positive control) was designed to contains the promoter of the Arabidopsis TOR (*AtTOR*) gene, the 5′UTR, the first exon, the first intron, and the beginning of second exon sequences fused to the GUS reporter gene ORF (2871 bp in length).

TOR sequences were amplified by PCR and cloned upstream of the β-glucuronidase (*GUS*) reporter gene in pBI101 binary vector (**Jefferson *et al.*, 1987**) at *Sal1* and *Sma1* restriction sites (**Figure 40**). Different colonies were checked by restriction enzyme digestion to select one that contains the 2872 bp fragment then confirmed by sequencing as described in methods. Plasmid DNA was transformed to *Agrobacterium tumeficens* for transformation to Arabidopsis plants.

The second construct (TORP-ΔuORF) was designed to study the effect of uORF on *AtTOR* gene expression, in which, the uORF start codon ATG has been point - mutated to become T instead of A (**A**TG changed to **T**TG; **Figure 40**).

To achieve this mutation, a 1860 bp fragment containing the 5′ UTR sequence was amplified by PCR using Hpa1A – 1200F and Sma1 + 540R1 forward and reverse primers and cloned into pGEM-T Easy vector. The pGEM-T vector sequence with mutated-uORF has been confirmed by sequencing.

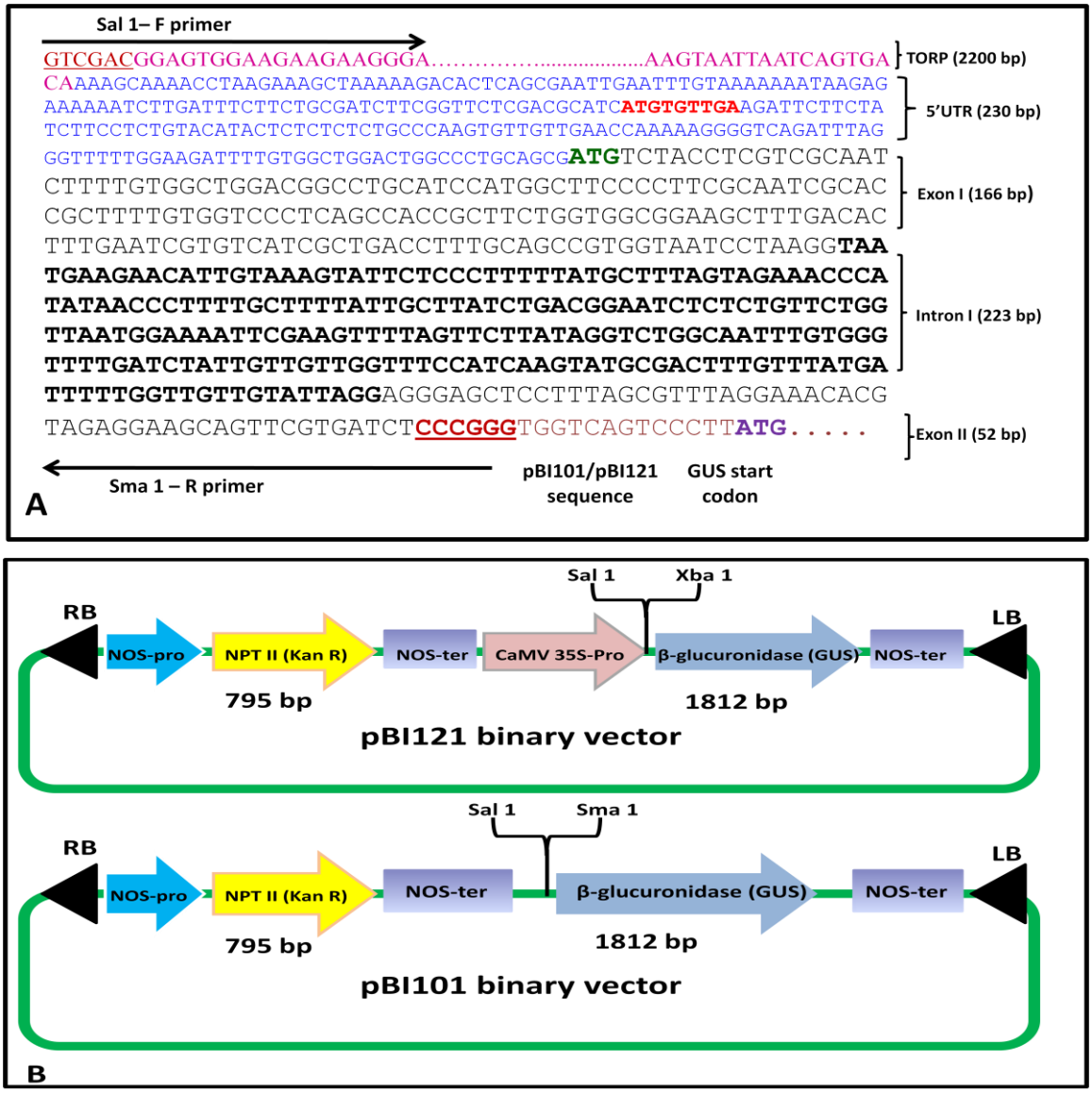


Figure 39: **Schematic representation of nucleotide sequence from *AtTOR* gene and the principal components of the T-DNA sequences within both pBI101 and pBI121 binary vectors.** (A) The *AtTOR* sequences involved the following: the TOR promoter (pink), the 5′UTR (blue), the first exon (normal black), the first intron (bold black), and beginning of the second exon (normal black color). The length of each region is placed enter parentheses beside their names. The small uORF located approximately at the center of 5′UTR expand for 9 nucleotides ATGTGTTGA, and it is marked by red color. The black arrows indicate the forward and reverse primers used in cloning. ATG start codon (+1) of TOR that is labeled in green. The start codon of GUS coding sequence is represented in violet and the rest sequences from pBI101 and pBI121 binary vectors are in brown. *SalI* and *SmaI* restriction sites are underlined and in bold brown. (B) The main features of pBI121 and pBI101 T-DNA. The NPT II kanamycin resistance gene is under control of nopaline synthetase (NOS) promoter and terminator. pBI121 contains a CaMV 35S promoter. The TOR sequences are cloned between the *Sma1* and *Sal1* restriction sites.

Then, the mutated uORF fragment was cloned to pBI101 binary vector at the *Hpa1* and *Sma1* restriction sites for transformation to Arabidopsis plants.

The third construct (TORP-Δ5′UTR) was designed to study the effect of the deletion of the whole 5′ UTR on *AtTOR* gene expression and was constructed using a similar strategy.

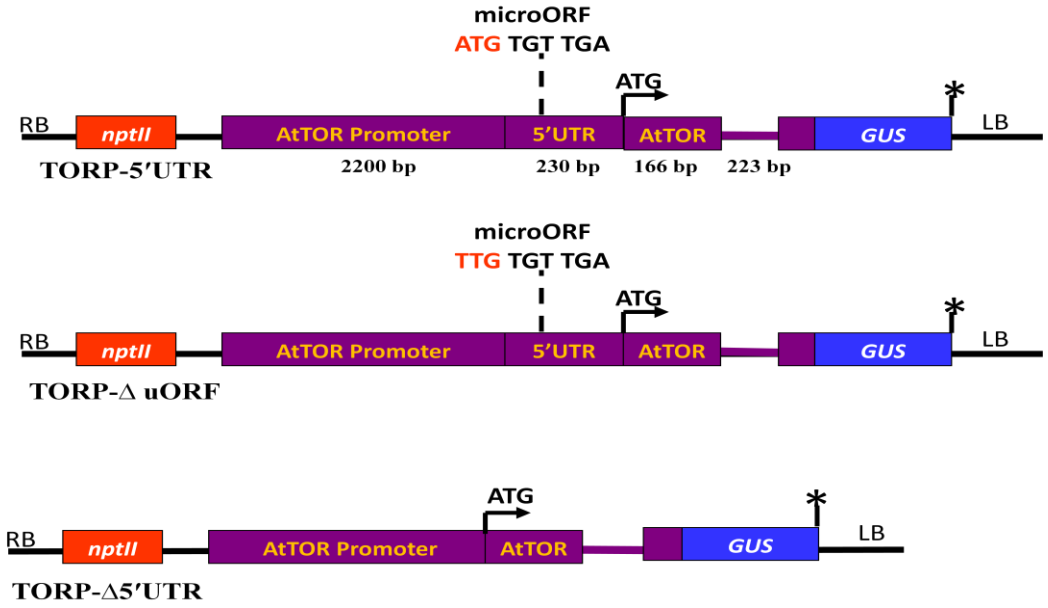


Figure 40: **Schematic structure showing the features of the three different constructs used in all experiments under expression of AtTOR promoter.** The upper construct, called full-length 5′UTR construct (TORP-5′UTR), is composed of the *AtTOR* promoter, the *AtTOR* 5′UTR, the first exon of AtTOR, the first intron of AtTOR and the beginning of the second exon of AtTOR fusion translationnally with GUS reporter gene. The length of each part as following: TOR promoter (2200 bp), the 5′UTR (230 bp), the first exon (166 bp), the first intron (223 bp) and the beginning of second AtTOR exon (52 bp). The middle construct is called uORF mutated construct (TORP-ΔuORF) that is involve the same components of the first construct except the uORF start codon ATG has been changed to TTG. The lower construct is called deleted 5′UTR construct (TORP-Δ 5′UTR) and involve the same component of the first construct except the 5′UTR (230 bp).

2.3.2. Constructs with 35S promoter

The same three constructs mentioned above have been placed under control of the CaMV 35S Promoter in pBI121 binary vector (**Figure 41**), in order to investigate whether the 5′UTR and/or uORF activity is dependent on the type of promoter or not. *XbaI* and *XmaI* restriction sites have been used to cloning the 5′UTR, the first exon, the first intron, and the beginning of the second

exon within pBI121 binary vector downstream of 35S promoter and upstream of the GUS coding sequence.

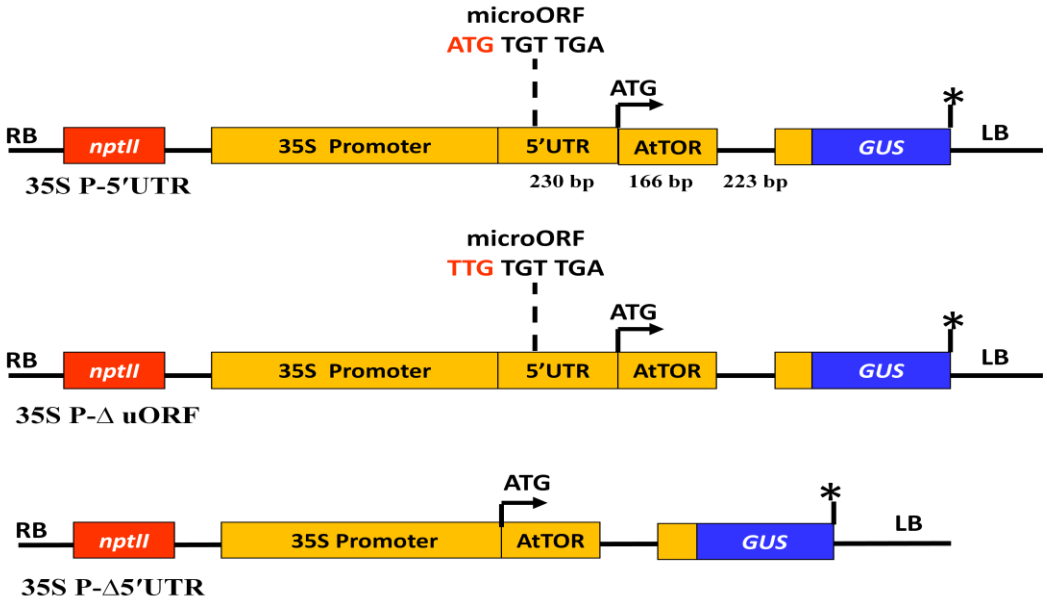


Figure 41: Schematic structure showing the components of the three different constructs under expression of CaMV 35S promoter. The same components of the TOR promoter constructs have been placed between 35S promoter and *GUS* reporter gene in pBI121 binary vector.

2.4. Transient expression with 35S and TOR promoter

2.4.1. Qualitative analysis of GUS expression

The functionality of these three constructs was used in transient expression assay in *Nicotiana benthamiana* leaves by Agroinfiltration method to investigate the impact role of *AtTOR* 5′UTR and uORF on *GUS* gene expression under control of CaMV 35S and AtTOR promoters. Transient expression system is a rapid method widely used in defining the mechanisms involved in eukaryotic gene expression (**Yang *et al.*, 2000**). Thus, we have investigated the impact of *AtTOR* 5′UTR and/or upstream open reading frame (uORF) on *GUS* mRNA accumulation and the amount of GUS protein using transient expression system. It provides informations more rapidly than analogous experiments in stable transgenic plants.

The functional expression of these six constructs was tested transiently using *Nicotiana benthamiana* leaves as described in methods. In all experiments, we have used *Agrobacterium* C58C1 carrying the 35S::p19 construct (**Voinnet *et al.*, 2003**) to repress RNA silencing.

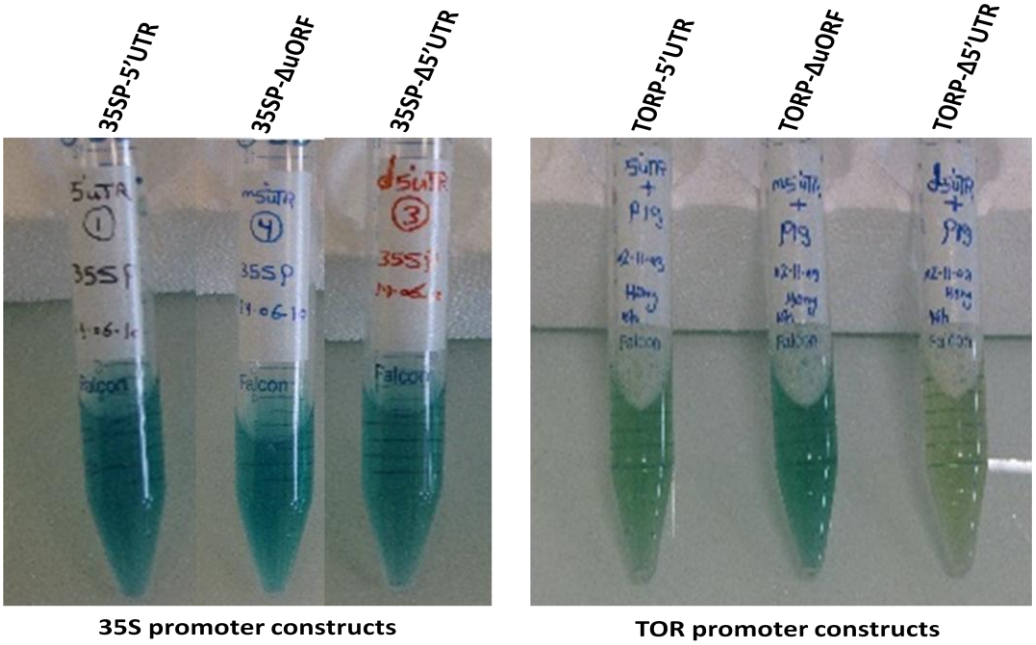


Figure 42: Transient qualitative analysis of GUS expression from three different constructs fewer than two different promoters. Left picture showing the degree of GUS activity (as indicated by intensity of the blue color) for three different constructs under expression of CaMV 35S promoter. The name of constructs is indicated at the top of the figure.

The results suggested that the deletion of 5′UTR from construct under the activity of AtTOR promoter (TORP- Δ5′UTR) caused a decreased in GUS activity, while the mutation in uORF caused an increased in GUS activity in comparison with the full-length 5′UTR construct (TORP-5′UTR) (**Figure 42)**. For the constructs under expression of CaMV 35S promoter, it was difficult to evaluate the differences possibly due to the strongest power of the constitutive 35S promoter.

2.4.2. Quantitative analysis of GUS activity and mRNA under control of 35S promoter

GUS expression from the three different constructs in *N. benthamiana* leaves was measured quantitatively using GUS fluorimetric assay.

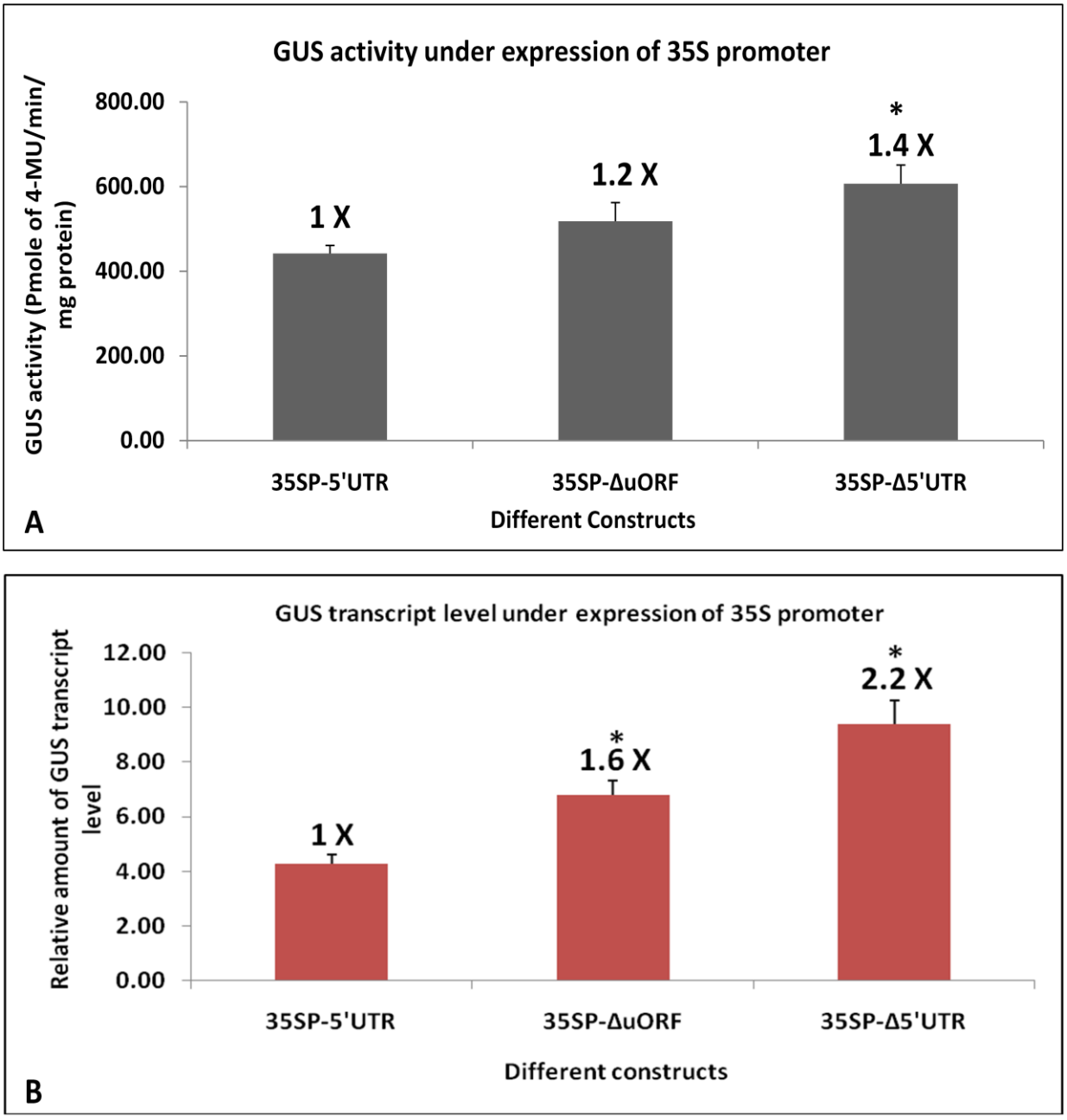


Figure 43: Effect of upstream open reading frame (uORF) and the 5′UTR of *AtTOR* mRNA on GUS expression in a transient expression assay in *Nicotiana benthamiana* leaves. The three different constructs containing the full-length 5′UTR, mutated uORF, and deleted 5′UTR under control of the CaMV 35S promoter were analysed. (A) GUS mRNA accumulation (B) GUS protein activity. Means and standard errors of the relative amount of *TOR::GUS* transcript levels and the amount of GUS activity have been calculated from six biological replicates. The value denoted with X on the top of each column represents the fold increase in respect to the full-length 5′UTR construct, which was arbitarily assigned as 1X. Asterisc refer to significant change in *GUS* mRNA level and in GUS activity in comparison to ful-length 5′UTR construct.

Six independent experiments from each construct have been carried out. *GUS* mRNA accumulation was analyzed using quantitative RT-PCR. In parallel, quantitative RT-PCR has been carried out for detection of *TOR::GUS* cDNA**.**

We have observed that the deletion of 5′UTR resulted in a significantly increased in GUS activity that was estimated by 1.4 folds (+ 40 %) compared to amount of detected GUS activity from the full-length 5′UTR (35SP-5′UTR). For the mutated uORF construct (35SP-ΔuORF), the amount of GUS activity increases slightly (1.2 fold; +20 %) compared to GUS activity from full-length construct (35SP-5′UTR) (**Figure 43 A**).

Our results shown that the deletion of AtTOR 5′UTR (35SP-Δ5′UTR) construct that is derived under expression of a constitutive 35S promoter caused a significant increase in the relative amount of *TOR::GUS* transcript level by 2.2 folds (+ 120 %) compared to the *TOR::GUS* transcript level from the full-length 5′UTR construct (35SP-5′UTR). For the mutated upstream open reading frame (35SP-ΔuORF) construct, we observed that the amount of *TOR::GUS* transcript level was increased by 1.6 fold (+ 60 %) compared to of the *TOR::GUS* transcript level from the full-length 5′UTR construct (35S-5′UTR), and this increase was significant (**Figure 43 B**). These results indicate that both 5′UTR and uORF of *AtTOR* possess negative effects on *TOR::GUS* mRNA accumulation level.

We can conclude that deletion of *AtTOR* 5′UTR resulted in an increase in both *GUS* transcript level and GUS protein activity and this increase was significant. Moreover, the mutation in uORF resulted in significant increase in *TOR::GUS* transcript level despite its modest effect on GUS activity. The increase in mRNA accumulation resulting from the deletion of TOR 5′UTR or mutation of uORF further suggests that these 5′UTR and/or uORF have a negative effect on transcription or RNA stability.

We will then investigate the impact of *AtTOR* 5′UTR and uORF on *TOR::GUS* mRNA level and GUS activity under control of the native *AtTOR* gene promoter to know whether the 5′UTR and/or uORF activity is independent or dependent on the type of promoter.

2.4.3. Quantitative analysis of GUS activity and mRNA under control of TOR promoter

The experiment with the three different constructs including the full-length 5′UTR (TORP-5′UTR), the mutated uORF (TORP-ΔuORF), and the deleted 5′UTR (TORP-Δ5′UTR) in *N. benthamiana* leaves was done as for 35S promoter experiments**.**

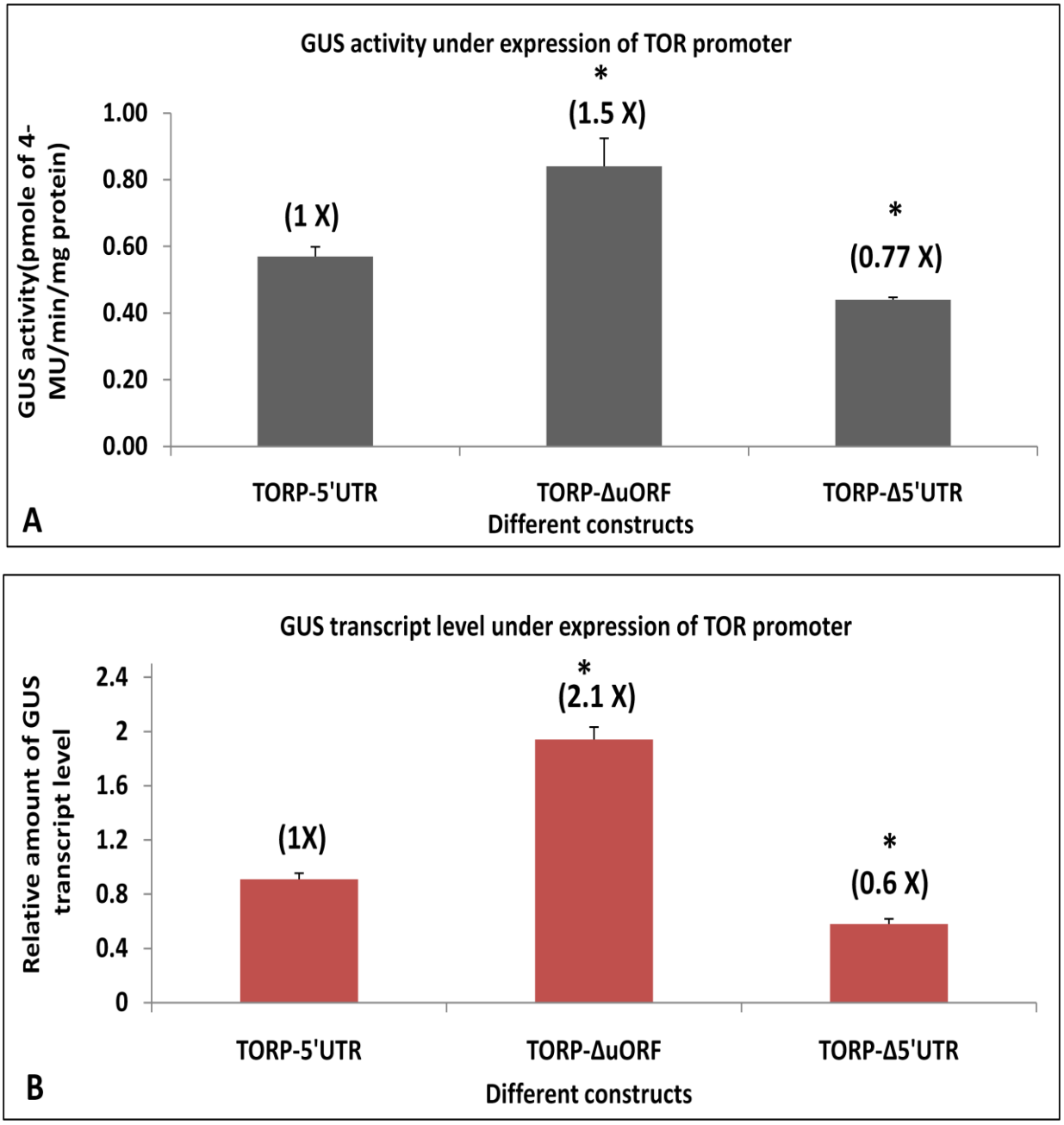


Figure 44: Effect of upstream open reading frame (uORF) and the 5′UTR of *AtTOR* mRNA on GUS expression in transient expression assay in *Nicotiana benthemiana* leaves. (A) *TOR:: GUS* transcript level ; (B) GUS activity. Means and standard errors of relative amount of *TOR::GUS* transcript accumulation and GUS activity have been calculated for six biological replicates from each construct. The fold change in reference to the full-length 5′UTR construct is indicated at the top of each bar. Asteriscs refer to significant changes.

GUS activity increases 1.3 fold (+ 30 %) in the mutated uORF construct (TORP-ΔuORF) compared to the full-length 5′UTR construct (TORP-5′UTR). For the deleted 5′UTR construct

(TORP-Δ5′UTR), GUS activity decreases by 0.8 fold (- 20 %) compared to the full-length 5′UTR (**Figure 44 A**).

The mutated uORF construct (TORP-ΔuORF), produces 2.1 fold (+ 120 %) more mRNA than the full-length 5′UTR construct (TORP-5′UTR). In contrast the deleted 5′UTR construct (TORP-Δ5′UTR) revealed 0.6 fold less (- 40 %) compared to *TOR::GUS* mRNA from full-length 5'UTR construct (**Figure 44 B**). This decrease in *TOR::GUS* mRNA level in the deleted 5′UTR construct is in opposition to what we observed using CaMV 35S promoter. This suggests a synergistic effect of the TOR promoter and the TOR 5'UTR in transcription.

These results suggest that the AtTOR 5′UTR uORF inhibits GUS expression when it is driven by the native TOR promoter. This effect may not occur at the level of translation since the amount of GUS mRNA also increases when the uORF was removed. However mRNA stability may increases as a results of increased translation. The AtTOR 5′UTR plays a positive role on *TOR::GUS* mRNA level and even more in GUS activity, suggesting that it act together with the AtTOR promoter to increase the transcription process of *TOR::GUS* mRNA or to increase the stability of *TOR::GUS* mRNA. Further experiments will be required to clarify these hypothesis.

When the expression levels of 35S and TOR promoters are compared, the GUS activity resulting from 35SP-5′UTR, 35SP-ΔuORF, and 35SP-Δ5′UTR constructs was approximately 1000-fold, 700-fold, and 1600 fold higher than the respective activities of equivalent TOR promoter constructs.

2.4.4. Translational efficiency in transient expression with 35S and *AtTOR* promoter

We have estimated the translational efficiency of GUS mRNA from the six independent experiments representing each construct either with 35S or TOR promoter to know the correlation between *TOR::GUS* mRNA levels and amount of GUS protein. Translational efficiency (TE) was defined as ratio of GUS protein activity to the amount of GUS mRNA level.

Our results show that both mutated uORF or deleted 5′UTR constructs under expression of CaMV 35S promoter exhibited a significant decrease in average of translational efficiency of the *TOR::GUS* mRNA (25 % and 35 %, respectively) compared to full-length 5′UTR construct (**Figure 45 A**). However, these constructs lead to an increased mRNA accumulation.

This supports our hypothesis that AtTOR 5′UTR or uORF appeared to play an important role in regulation of GUS expression either at RNA transcription or RNA stability level.

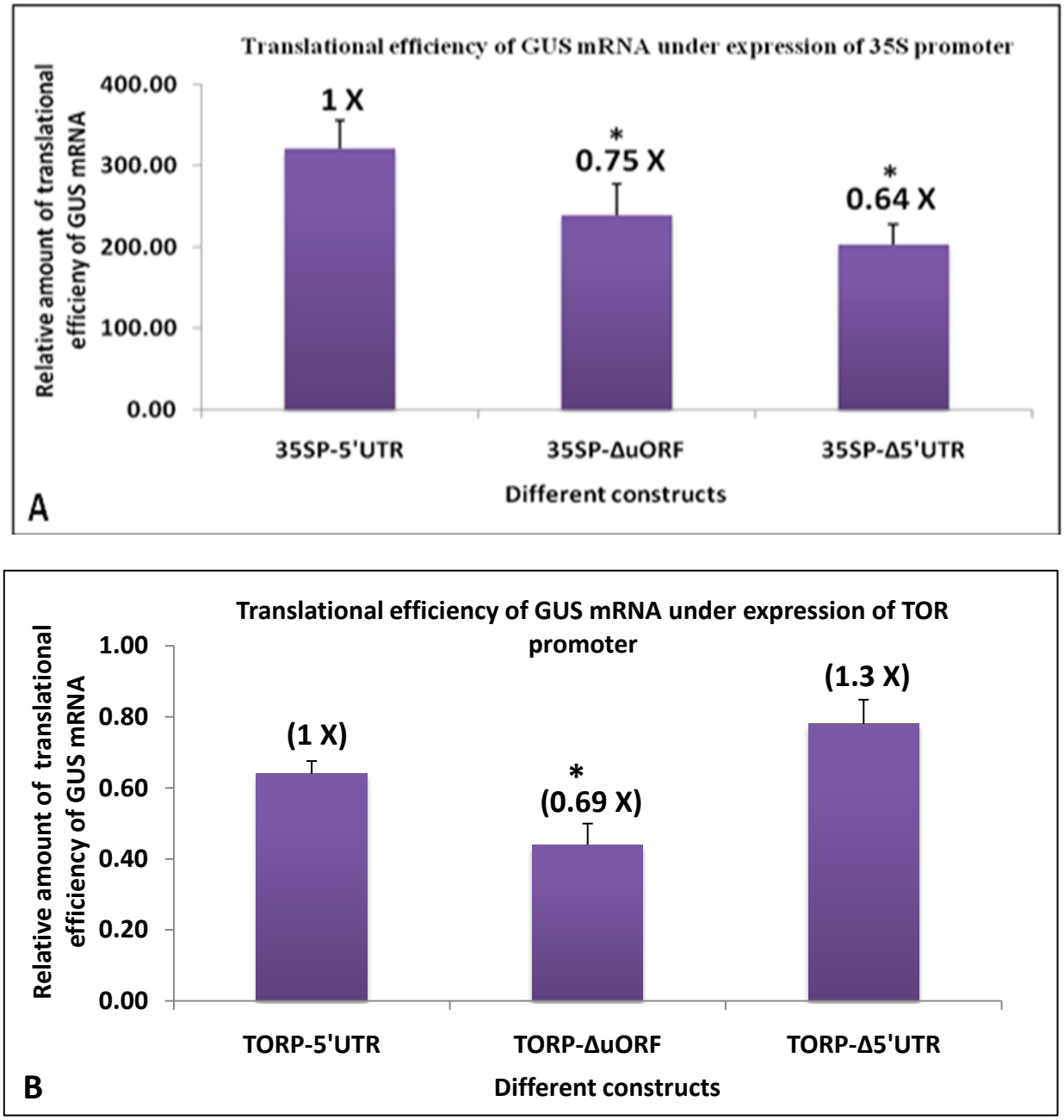


Figure 45: Average translational efficiency of GUS mRNA for each construct under expression of A) 35S promoter and B) TOR promoter. T-test analysis showed that the difference between mutated uORF and deleted 5′UTR constructs in relation to full-length 5′UTR construct was significant. The value of each change was put in the top of each column.

For constructs under expression of TOR promoter, it was observed that removal of uORF resulted in a significant decrease (30 %) in average translational efficiency of the *TOR::GUS*

mRNA. However, deletion of 5′UTR caused an increased by 30 % in translational efficiency of the *TOR::GUS* mRNA compared with full-length 5′UTR construct (**Figure 45 B**).

These results suggest that the AtTOR 5′UTR uORF promotes translational efficiency of GUS transcript when it is driven either by the CaMV 35S promoter or the native TOR promoter. We suggest that promotion of GUS mRNA translation by uORF depends on the stability of GUS transcript, since, we have observed alo increased in accumulation of GUS mRNA when the uORF is mutated. Our results also revealed that the effect of deletion of 5′UTR constructs depends of the promoter: a decrease in translational efficiency of GUS mRNA and increased in translational efficiency of GUS mRNA under expression of either CaMV 35S promoter or AtTOR promoter, respectively. These result suggest that AtTOR 5′UTR acts positively with TOR promoter for activation of GUS translation or GUS mRNA stability, while it is acts negatively with 35S promoter for repression of GUS translation or GUS mRNA stability.

2.5. Study the role of *AtTOR* 5′UTR and uORF on *GUS* transcript and activity in transgenic plants

2.5.1. Characterization and screening of stable homozygous transgenic plants

The three constructs containing the TOR promoter were used for *Arabidopsis thaliana* (Col-0 ecotype transformation by the floral dip method. The transgenic seeds resulted from these transformations (represented T1) were sterilized and sowed on MS/2 medium supplemented with sucrose and kanamycin to select the resistant plants that contain the desired T-DNA inserted. Approximately, 30, 40, and 35 individual transformed seedling with wild type-5′UTR, mutated-uORF, and deleted -5′UTR constructs, were obtained. Then, these seedlings were transferred to soil and allowed to flower. Genomic DNA has extracted to screen these transgenic individual plants by PCR. PCR products of ~ 490 bp fragments were obtained from the transformed plants with full-length 5′UTR and mutated -uORF constructs and 258 bp fragments were obtained from the transformed plants with deleted-5′UTR construct. We have performed additional PCR that were designed to amplify the 120 bp (80 bp from *GUS* gene and 40 bp from *TOR* gene) to confirm from the presence of the chimeric TOR::GUS fusion.

Full-Length 5′UTR lines	Actual value		Expected value		Total plants	X^2
	K^R	K^S	75 % K^R	25 % K^S		
FL-1	189	61	187.5	62.5	250	0.83
FL-2	174	54	171	57	228	0.65
FL-3	122	38	120	40	160	0.72
FL-4	140	45	138.75	46.25	185	0.83
FL-5	151	48	149.25	49.75	199	0.77
FL-6	225	72	222.75	74.25	297	0.76
FL-7	115	39	115.5	38.5	154	0.93
FL-8	170	55	168.75	56.25	225	0.85
FL-9	212	68	210	70	280	0.78
FL-10	92	33	93.75	31.25	125	0.72

Table 1: Kanamycin resistance segregation and chi-square (X^2) test of independence for transgenic lines TORP-5′UTR.

Mutated uORF lines (TOR-ΔuORF)	Actual value		Expected value		Total plants	X^2
	K^R	K^S	75 % K^R	25 % K^S		
Mut-1	98	31	96.75	32.25	129	0.80
Mut-2	112	39	113.25	37.75	151	0.81
Mut-3	123	38	120.75	40.25	161	0.68
Mut-4	130	46	132	44	176	0.73
Mut-5	106	38	108	36	144	0.70
Mut-6	135	45	135	45	180	1.00
Mut-7	123	39	121.5	40.5	162	0.79
Mut-8	120	38	118.5	39.5	158	0.78
Mut-9	125	39	123	41	164	0.72
Mut-10	130	44	130.5	43.5	174	0.93

Table 2: Kanamycin resistance segregation and chi-square (X^2) test of independence for mutated uORF transgenic lines (TORP-ΔuORF).

Deleted 5′UTR lines (TOR-Δ5′UTR)	Actual value		Expected value		Total plants	X^2
	K^R	K^S	75 % K^R	25 % K^S		
Del-1	181	64	183.75	61.25	245	0.68
Del-2	85	29	85.5	28.5	114	0.91
Del-3	105	33	103.5	34.5	138	0.77
Del-4	180	63	182.25	60.75	243	0.74
Del-5	90	28	88.5	29.5	118	0.75
Del-6	112	40	114	38	152	0.71
Del-7	109	34	107.25	35.75	143	0.74
Del-8	75	27	76.5	25.5	102	0.73
Del-9	105	37	106.5	35.5	142	0.77
Del-10	66	24	67.5	22.5	90	0.72

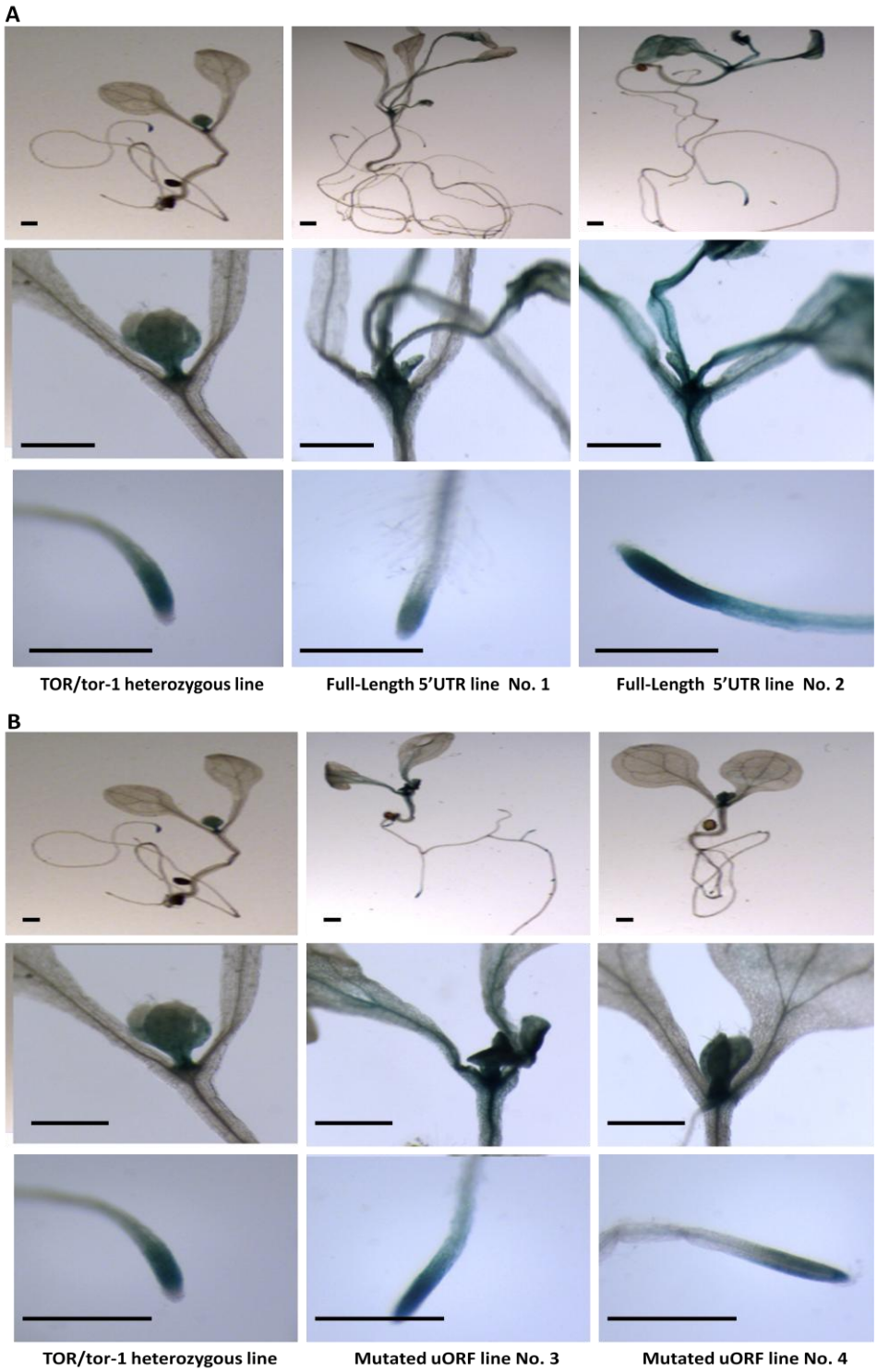
Table 3: Kanamycin resistance segregation and chi-square (X^2) test of independence for deleted 5′UTR transgenic lines (TORP-Δ5′UTR).

T2 positive transgenic seeds, derived from self-pollination of the T1 progenies, were harvested and allowed to germinate on kanamycin. A segregation analysis has been done at the T2 generation. T1 seeds derived from self-pollination of the primary transformants (T1) where it was germinating on kanamycin-containing MS medium. The transgenic lines have been analyzed to select those that have only one locus from inserted T-DNA using X^2 analysis (**Tables 1, 2, and 3**).

For each construct, a 3:1 ratio of segregation for kanamycin resistance (a marker carried by the T-DNA) was observed for at least 10 independent transgenic lines. X^2 analysis shows it is consistent with a Mendelian segregation of a single dominant gene at one locus inside the Arabidopsis genome.

2.5.2. Spatial pattern of GUS in transgenic plants

Individual homozygous transgenic lines were selected according to their resistance to kanamycin and theit pattern of GUS expression was compared to that of *TOR/tor-1* heterozygous plants (**Figure 46 A, B, and C**).



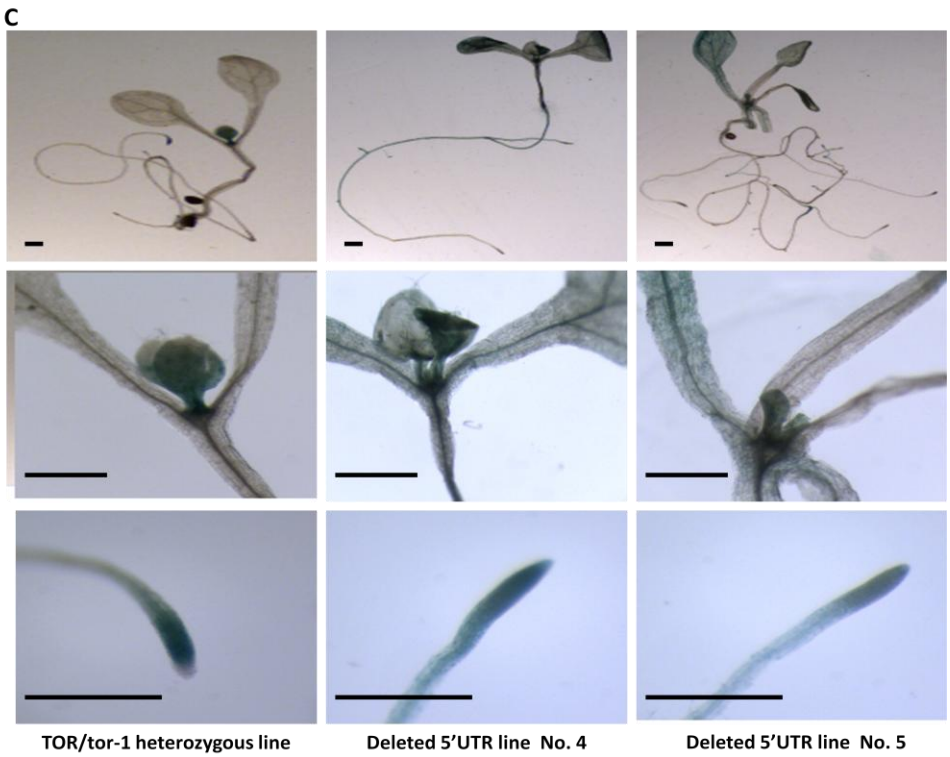


Figure 46: Histochemical GUS staining of selected transgenic lines from each construct at seedling stage in comparison to *TOR/tor-1* heterozygous seedling. (A) Full-length 5'UTR (FL-5′UTR) lines (B) mutated uORF (ΔuORF) lines (C) deleted 5'UTR (Δ5′UTR) lines. Scale bar is 4 mm.

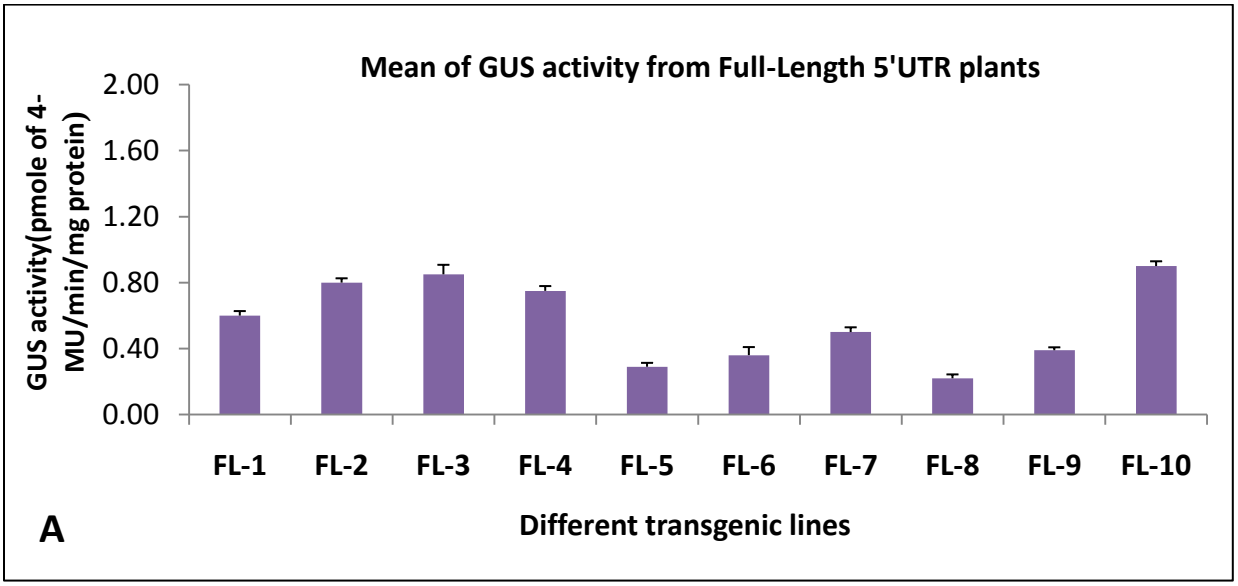
GUS expression was found in the root meristems, the vascular bundles of root and shoot system leaf primordia, and leaf cotyledons. There are variations in terms of the intensity of the GUS staining among individual transgenic plants (i.e. high and low expressers). The GUS expression pattern was essentially similar to that of the *TOR/tor-1* mutant line in which GUS was fused in frame with the TOR protein (**Menand *et al,* 2002**).

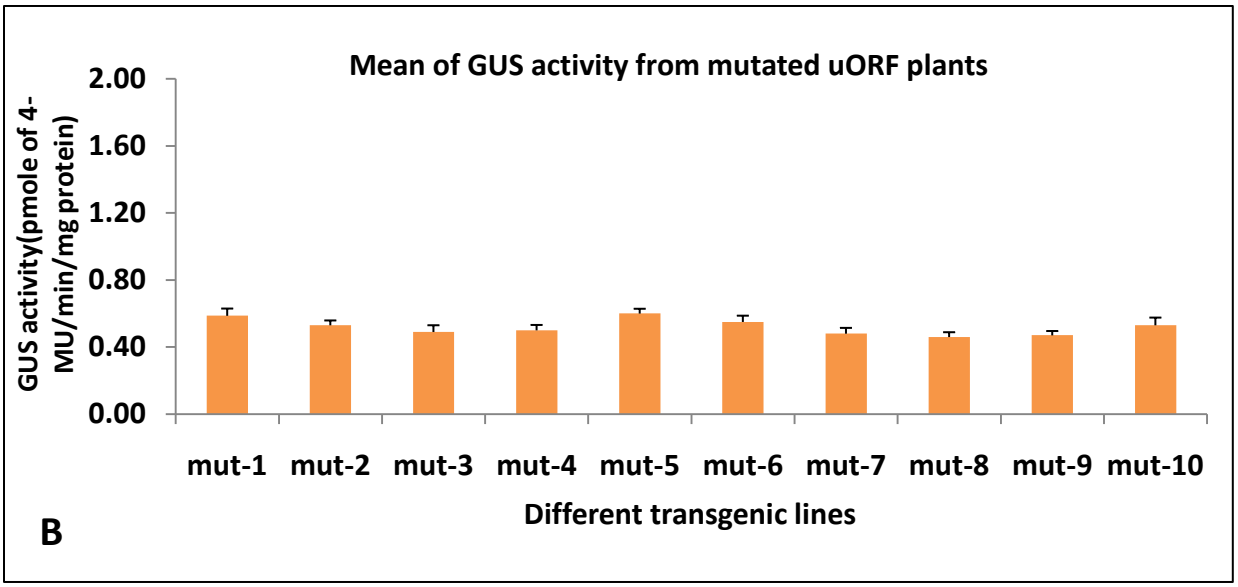
The presence of blue staining in vascular tissues and the basal of primary leaves may be due to diffusion via phloem or xylem vascular elements during prolonged incubation period for more than 8 hours at 37 ^{o} C. Altogether the use of 2.2 kb promoter of the *AtTOR* gene with the first intron drive the expression of *GUS* reporter gene in transgenic Arabidopsis seedling in a manner similar to the native *AtTOR* gene and this spatial pattern is not deeply affected by the 5'UTR.

A strongest GUS expression was generally observed for the majority of deleted 5′UTR transformed lines and will be discussed below.

2.5.3. Quantitative estimation of 5′UTR and upstream open reading frame (uORF) on GUS activity

In this paragraph, we will study the implication role of both 5′UTR and uORF from *AtTOR* mRNA on GUS expression quantitatively in transgenic plants. We will measure the amount of GUS activity, the relative amount of *TOR::GUS* mRNA level, and try to estimate the value of GUS translational efficiency for ten independent transformed lines from each construct at seedling stage.





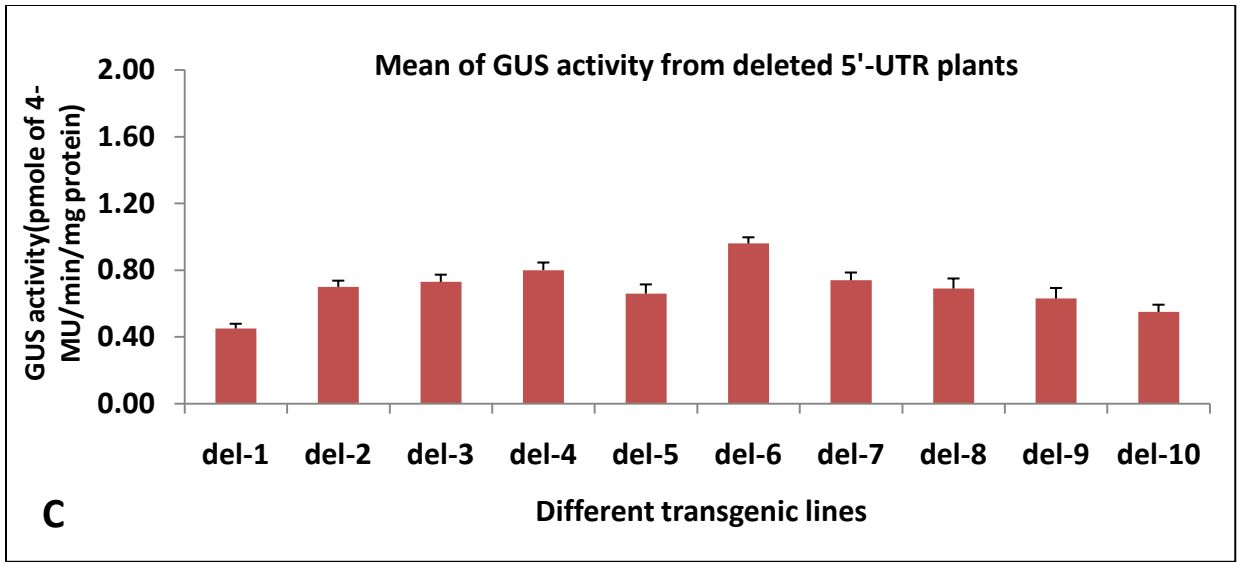


Figure 47: Quantitative measurements of the GUS enzyme activity from ten independent transgenic lines from each construct at 10 days old seedling stages. The three different constructs involve: (A) The full-length 5′UTR (FL-5′UTR), (B) mutated-uORF (ΔuORF) and (C) deleted-5′UTR (Δ5′UTR). Ten independent transgenic lines represented each construct have been selected for measurement of GUS activity. The standard errors (SE) for each individual line construct have been calculated in three biological replicates.

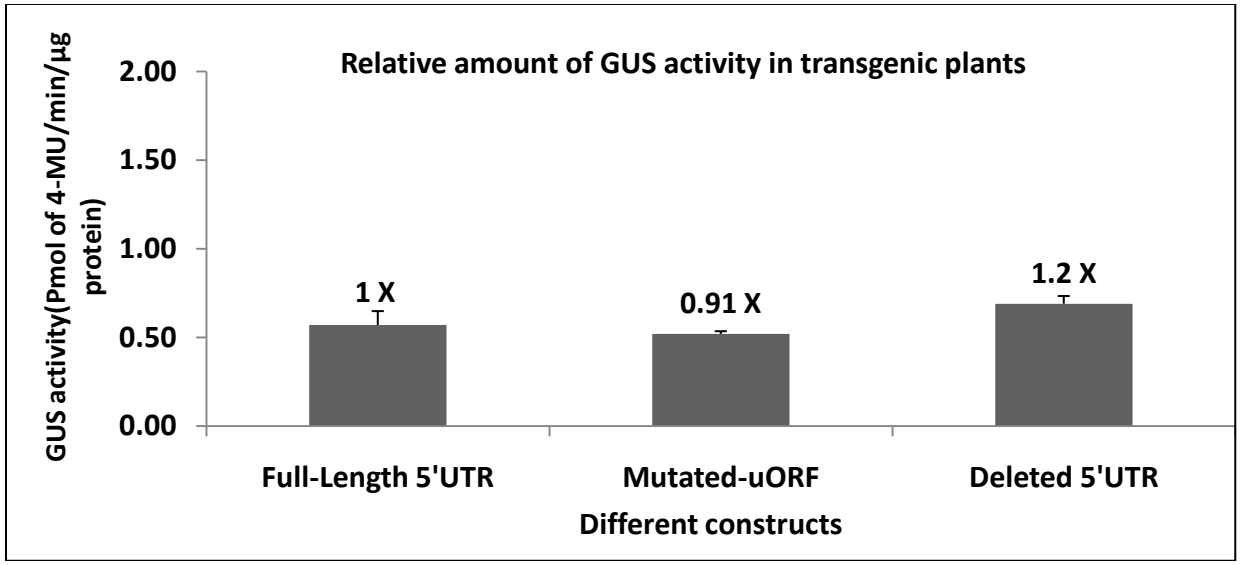
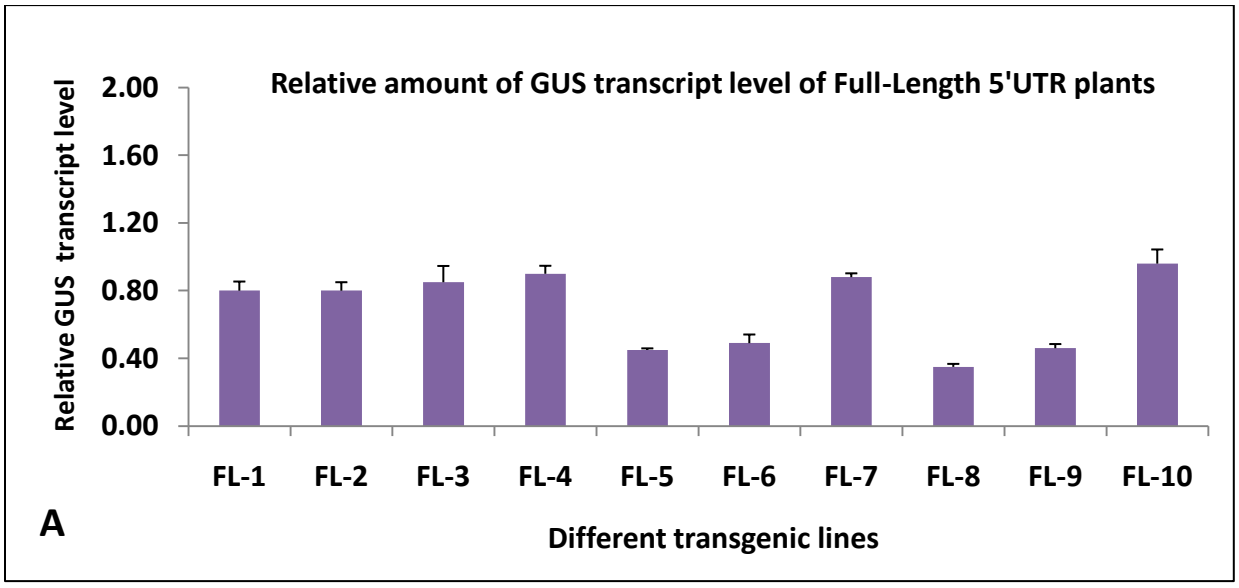


Figure 48: Average of relative amount of GUS activity for each construct, calculated from data of figure 47. The standard error between different 10 lines for each group was calculated. T-test analysis showed that the difference between mutated uORF and deleted 5′UTR constructs in relation to full-length 5′UTR construct was non-significant. The value of change was put in the top of each column.

The results revealed that full length (FL) 5′UTR transformed lines exhibited a variation in the amount of GUS activity ranged from 0.2 to 1.0. This variation may be attributed to the presence of more than one copy at the same location from the inserted T-DNA that contains GUS reporter gene. Two groups can be classified from these plants, the first one include lines 1, 2, 3, 4, 7, and 10, in which the amount of GUS activity is ranging from 0.5 to 1.0. The second group includes the lines 5, 6, 8, and 9, in which the amount of GUS activity is ranging from 0.2 to 0.5 (**Figure 47 A**). For the mutated uORF transformed lines, we have observed that there are no differences in the amount of GUS activity that was approximately 0.7 among all ten tested lines (**Figure 47 B**). For the deleted 5'UTR transformed lines, the estimated amount of GUS activity was ranging from 0.5 to 1.0 for all 10 tested lines (**Figure 47 C**). Average relative amount of GUS activity for each construct has been shown in **Figure 48.** Altogether GUS activity was found rather consistent in the three different constructs.

2.5.4. Quantitative estimation of 5′UTR and uORF on GUS mRNA

The relative amount of *TOR::GUS* mRNA level has been estimated for ten independent transformed lines with wild-type 5′UTR, mutated uORF, and deleted 5′UTR constructs, respectively by quantitative real-time PCR (qRT-PCR) method.



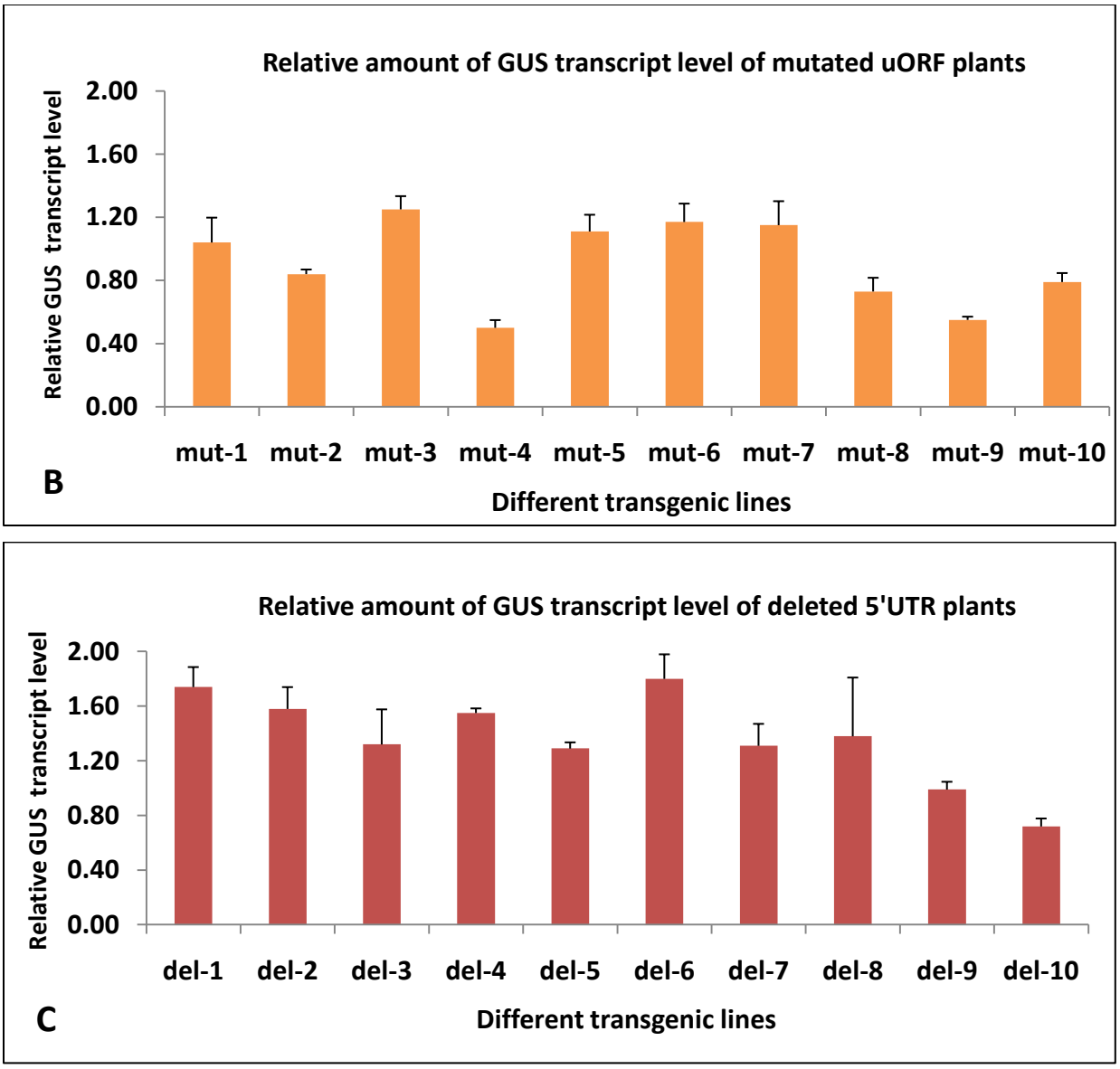


Figure 49: Quantitative measurements of the *TOR::GUS* mRNA level from ten independent transgenic lines from each construct at 10 days old seedling stages. (A) The Full-Length 5′UTR, (B) mutated-uORF and (C) deleted-5′UTR. The relative amount of GUS mRNA level was normalized to that *PDF2* mRNA as internal reference gene. The standard errors (SE) for each individual line from each construct have been calculated in three biological replicates.

To prevent contaminated signals from genomic DNA, the forward primer was designed to overlap the first exon and the second exon boundaries. *TOR::GUS* gene expression was normalized to PDF2 gene expression. Standard curves for each primer pair were derived from dilution series, and the relative amount of RNA in each sample was determined from the standard curves.

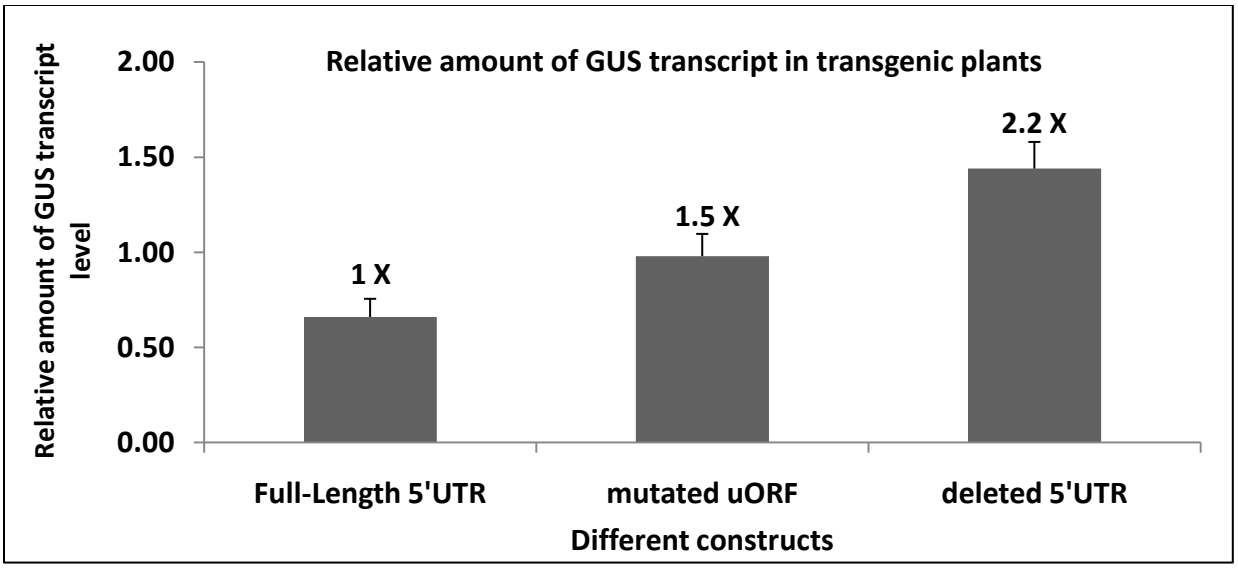


Figure 50: Average of GUS mRNA level for each construct, calculated from data of figure 49. The standard error between different 10 lines for each group was calculated. T-test analysis showed that the difference between mutated uORF and deleted 5′UTR constructs in relation to full-length 5′UTR construct was significant. The value of change was put in the top of each column.

The results revealed that the estimated relative value of *TOR::GUS* mRNA level from the wild-type 5′UTR transformed lines define two groups. A first group include lines 1, 2, 3, 4, 7, and 10; in which the relative amount of *TOR::GUS* mRNA level range from 0.8 to 0.9 and a second group (lines 5, 6, 8, and 9) in which the relative amount of *TOR::GUS* mRNA level is ranging from 0.4 to 0.5 (**Figure 49 A**). For the mutated uORF transformed lines, two groups can also be defined. The first one involve lines 1, 3, 5, 6, and 7; in which the relative amount of *TOR::GUS* mRNA level is ranging from 1.0 to 1.3, the second group (lines 4, 8, 9, and 10) in which the relative amount of *TOR::GUS* mRNA level is ranging from 0.5 to 0.8 (**Figure 49 B**). In case of the deleted 5′UTR transformed lines, one group includes lines 1, 2, 3, 4, 5, 6, 7, and 8; in which the mRNA level was between 1.3 to 1.9. While, the second group (lines 9 and 10) with a level of *TOR::GUS* transcript ranging from 0.8 to 1.0 (**Figure 49 C**). Average relative amount of GUS transcript for each construct is shown in **Figure (50).**

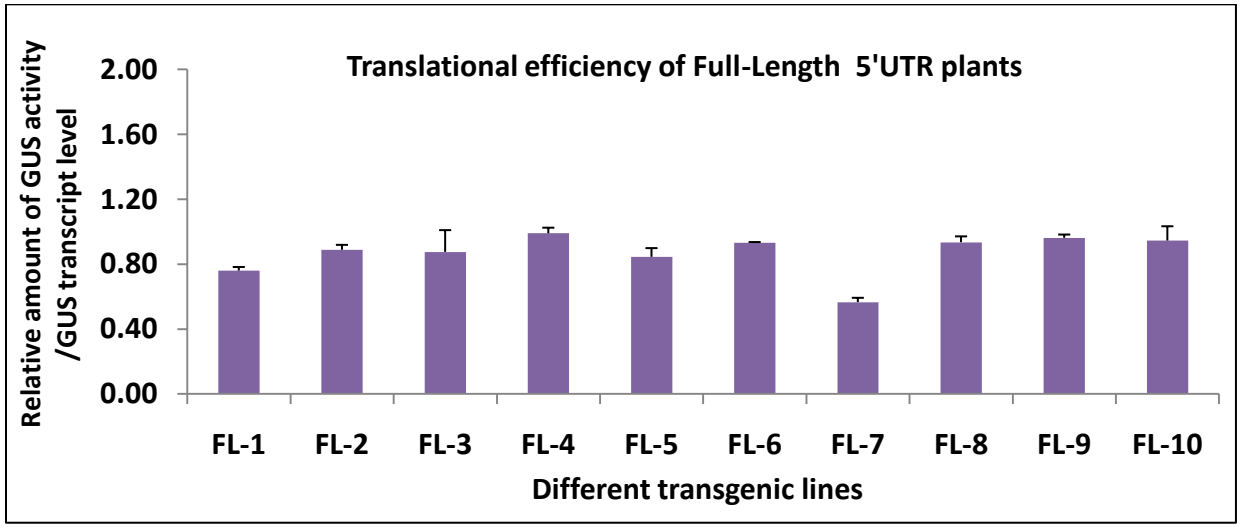
Our results show that both uORF and 5′UTR from the *AtTOR* mRNA possess a negative effect on the accumulation of *TOR::GUS* transcripts in comparison with the full-length 5′UTR transformed lines. This is consistent with with the results that we have obtained from the same constructs in transient expression assay using CaMV 35S promoter.

Our first hypothesis supposed the possibility that 5′UTR from the *AtTOR* mRNA may possess regulatory elements, particularly binding sites for repressor protein implicated in down-regulation of the main ORF. Another hypothesis is that TOR uORF and 5′UTR are acting as destabilizing elements of the *TOR::GUS* mRNA. Interestingly, in transient experiments, the increase in *TOR::GUS* mRNA accumulation is lost when the TOR promoter is used suggesting interactions between the promoter and the 5'UTR on accumulation of TOR mRNA.

2.5.5. Quantitative estimation of 5′UTR and uORF on translational efficiency of GUS mRNA

To know the correlation between *TOR::GUS* mRNA levels and amount of GUS protein, we have estimated the relative amount of GUS translational efficiency for ten transformed lines from each construct. Translational efficiency (TE) was defined as ratio of GUS protein activity to the amount of GUS mRNA levels for each line. **Figure 51** shows the TE for each transgenic lines and **figure 52** show the average TE for each construct.

This analysis shows that removal of the uORF or deletion of the 5′UTR both reduces the average translational efficiency (TE) of the *TOR::GUS* mRNA. However, these constructs allow for an increased mRNA accumulation compared to the full-length 5′UTR. This supports our hypothesis that AtTOR 5′UTR or uORF appeared to play an important role in this regulation.



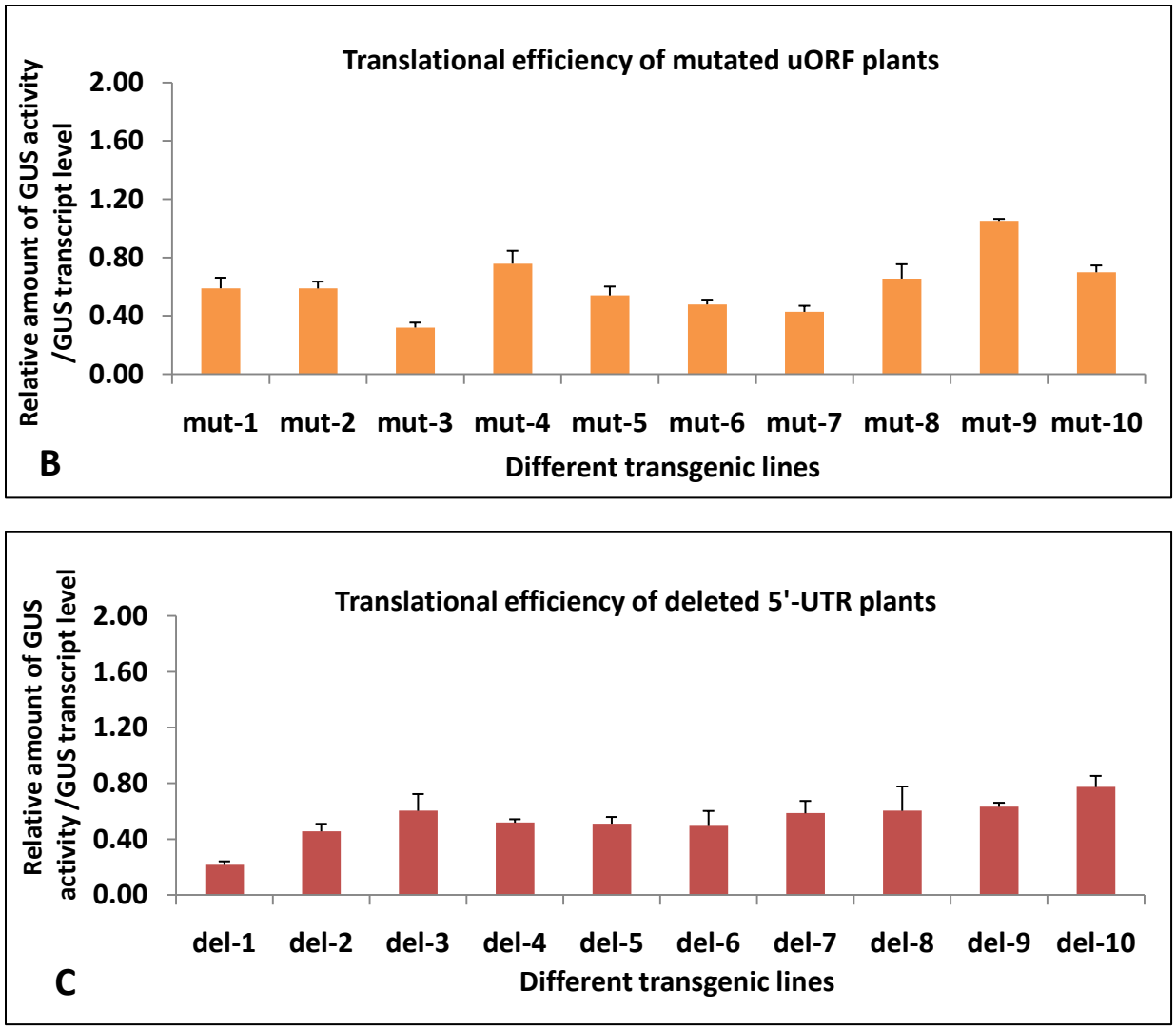


Figure 51: Estimation of relative value of GUS translational efficiency (TE) from ten independent transgenic lines from each construct at seedling stages. (A) Full-Length 5′UTR, (B) mutated-uORF and (C) deleted-5′UTR. TE was determined by dividing the amount of GUS activity by the relative amount of TOR::GUS transcript accumulation. The standard errors (SE) for each individual line from each construct have been calculated in three biological replicates.

The observed reduction in value of TE for these deleted 5′UTR transformed lines may be interpreted in the light that lower amount of TOR::GUS protein are produced from higher amount of TOR::GUS mRNA, suggesting that *TOR::GUS* mRNA may be subjected to either destabilization via endonuclease or exonuclease enzymes or reduction in mRNA synthesis. Since removal of the 5′UTR reduces translation efficiency, it seems that the 5′UTR has a positive role in translation but it has a negative role on transcription or stability of *TOR::GUS* mRNA.

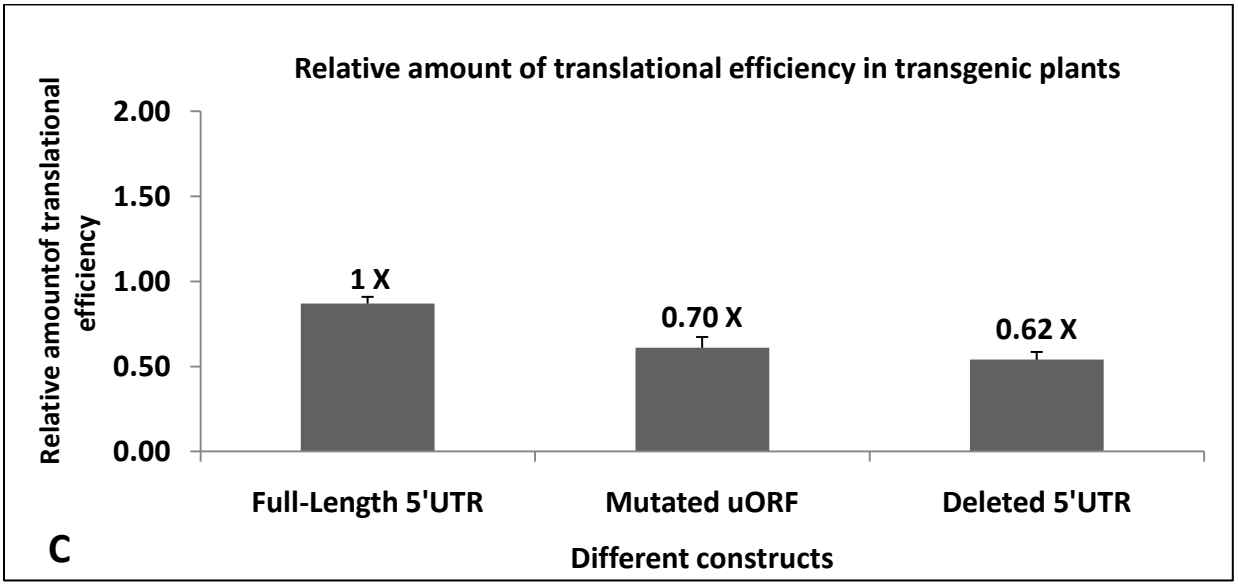


Figure 52: Average TE for each construct, calculated from data of figure 51. The standard error between different 10 lines for each group was calculated. T-test analysis showed that the difference between mutated uORF and deleted 5′UTR constructs in relation to full-length 5′UTR construct was significant. The value of change was put in the top of each column.

2.5.6. Comparison of translational efficiency between transient and stable transgenic plants

The results of translational efficiency obtained from stable transformed plants with mutated uORF and deleted 5′UTR constructs were completely consistent with the results of transient expression assay obtained from mutated uORF and deletion 5′UTR constructs under expression of CaMV 35S promoter. We observed that mutated uORF construct either under expression of Arabidopsis TOR promoters caused decrease in the relative amount in translational efficiency of *TOR::GUS* mRNA in both stable transgenic plants and transient expression assay. However, the deleted 5′UTR construct under expression of Arabidopsis TOR promoter exhibited increases in relative amount of translational efficiency of *TOR::GUS* mRNA in transient expression assay and caused decreased in translational efficiency of TOR::GUS mRNA in case of stable transgenic plants. Together, these data suggest that Arabidopsis TOR promoter with 5′UTR play an important role in control of GUS expression either at level of GUS transcription or at level of GUS mRNA stability.

2.6. Effect of 5′-UTR and uORF under particular growth conditions

2.6.1. Effect of exogenous sucrose

In plants, sugar is produced mainly through photosynthesis and then transported to different tissues to regulate many aspects of their growth and metabolism. Sucrose is the main transported form and it is implicated in regulation of different vital processes within plant cell. Sucrose acts not only as a nutrient but also as a signaling molecule alters expression of many genes (**Wiese *et al.*, 2004**). Indeed, starvation for sugar leads to a wide array of physiological responses and growth arrest (**Rolland *et al.*, 2002**).

Previous studies linked the AtTOR signaling pathway and sugar sensing. It was found that AtTOR RNAi plants display an altered susceptibility to exogenous sugars, suggesting that AtTOR positively regulate the plants response to sugar (**Yao thesis, 2006**).

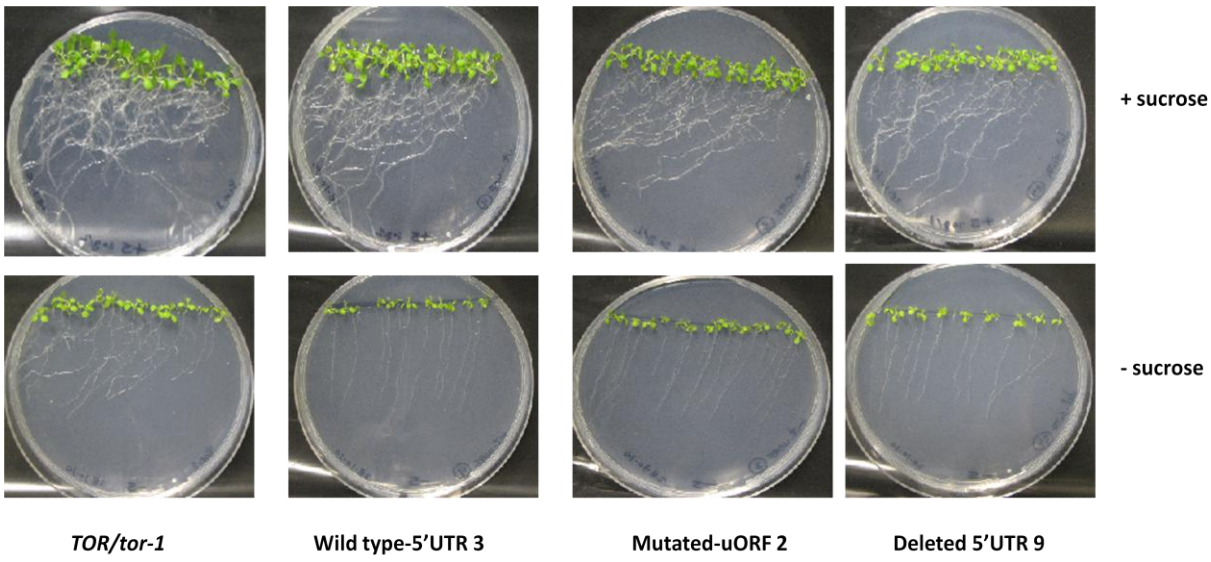


Figure 53: Transformed seedling from three different constructs grown on Hoagland/2 medium with and without sucrose. One independent transformed line was selected from three independent lines to represent each construct. These lines involve full-length - 5′UTR 3, mutated -uORF 2, and deleted -5′UTR 9. Sucrose (2 % g/L) was added to Hoagland /2 medium and the plates were placed in vertical position. Seedlings from TOR/tor-1 heterozygous were used as a reference for transformed lines.

Thus, we tried to test the effect of sucrose on pattern of *TOR::GUS* mRNA expression from transformed lines with the full-length 5′UTR, mutated uORF and deleted 5′UTR constructs.

Two independent transgenic lines representing each construct were selected for having the same level of the chimeric *TOR::GUS* mRNA. Seeds from transformed full-length 5′UTR, mutated uORF, and deleted 5′UTR lines have been sowed on Petri dishes contained Hoagland/2 medium supplemented with 2 % and 0 % sucrose, respectively. Then, the plates were placed in vertical position for allow roots to grow easily. After 7 days, the seedlings from each transformed line have been subjected to GUS histochemical analysis to examine the pattern of GUS expression qualitatively. The heterozygous *TOR/tor-1* seeds were grown in the same time as a positive control.

Transformed seedling from different constructs grown on Hoagland/2 medium supplemented with 2 % sucrose have a clear increase in growth of both shoot and root system, particularly secondary root in combination to seedling grow without sucrose (**Figure 53**).

This result can be explained since sucrose is the main transported sugars implicated in regulation of different vital processes within plant cell. Many papers recorded those high levels of sucrose leads to inhibition of photosynthesis, while the situation is reversed during sucrose starvation (**Koch, 1996**). Indeed, secondary metabolism as well as some developmental process like flowering and root development is affected by sucrose level (**Takahashi *et al.*, 2003; Solfanelli *et al.*, 2006**). We hypothesize that Arabidopsis TOR kinase signaling pathway may participate in sugar signaling in different plant tissues, particularly the proliferative tissues.

We tested the effect of sucrose on the spatial pattern of GUS activity from these transformed lines using GUS histochemical analysis assay. In the presence of sucrose, the main observation was that, with the exception of deleted 5′UTR line number 10, GUS activity was found in the root meristem. Full-length 5′UTR transformed line number 4 shows a higher GUS activity compared with other lines including the *TOR/tor-1* line. Besides this increase in the root meristem, the overall spatial pattern of GUS activity was not found to be affected by sucrose (**Figure 54 A and B**). This result suggests that TOR transcriptional activation occurs in proliferative cells in contact with sucrose of the medium.

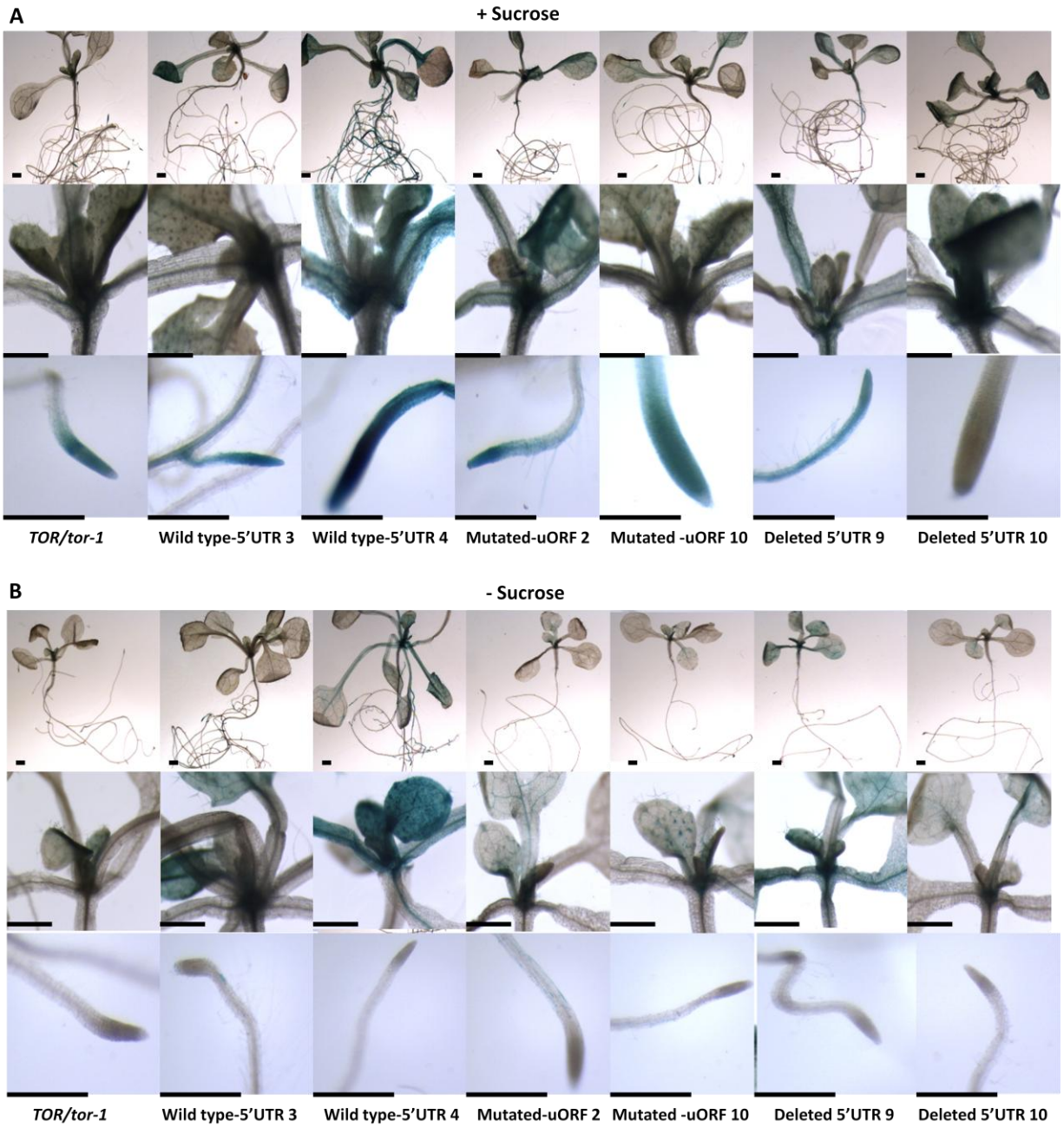


Figure 54: Histochemical analysis of TOR::GUS expression of two transformed lines from each construct growing on Hoagland/2 medium with sucrose (A) and without sucrose (B). Two independent transformed lines from each construct have been used and the number of each line was represented under each picture. Each line was represented by three views including the whole seedling, zoom of the shoot tip, and zoom of the root tips. Hoagland/2 medium-contained Plates supplemented with 2% sucrose were placed in vertical position to allow seedlings roots to grow on the surface of the medium. Seedlings have been transferred after 7 days to X-Gluc solution and incubated them at 37 ^{o} C for 8 hours. *TOR/tor-1* heterozygous plants were use as a positive control. Scale bare is 2 mm.

2.6.2. Effect of exogenous auxin

Auxin is involved in a wide array of developmental processes, such as initiation of meristems, secondary root emergence and the formation of foliar primordial at the whole plant level. It is known to be involved in cell division, cell expansion, and cell differentiation at the cellular level (**Teal et al., 2006**). It was reported that auxin activate expression of *AtTOR* gene (**Menand, PhD thesis, 2002**), as well as the S6 kinase signal transduction pathway in Arabidopsis (**Turck et al., 2004**). From these results, we hypothesized that 5′UTR and upstream open reading frame (uORF) of Arabidopsis TOR (*AtTOR*) mRNA are implicated in control of *TOR::GUS* mRNA expression through overlap between auxin and TOR signaling.

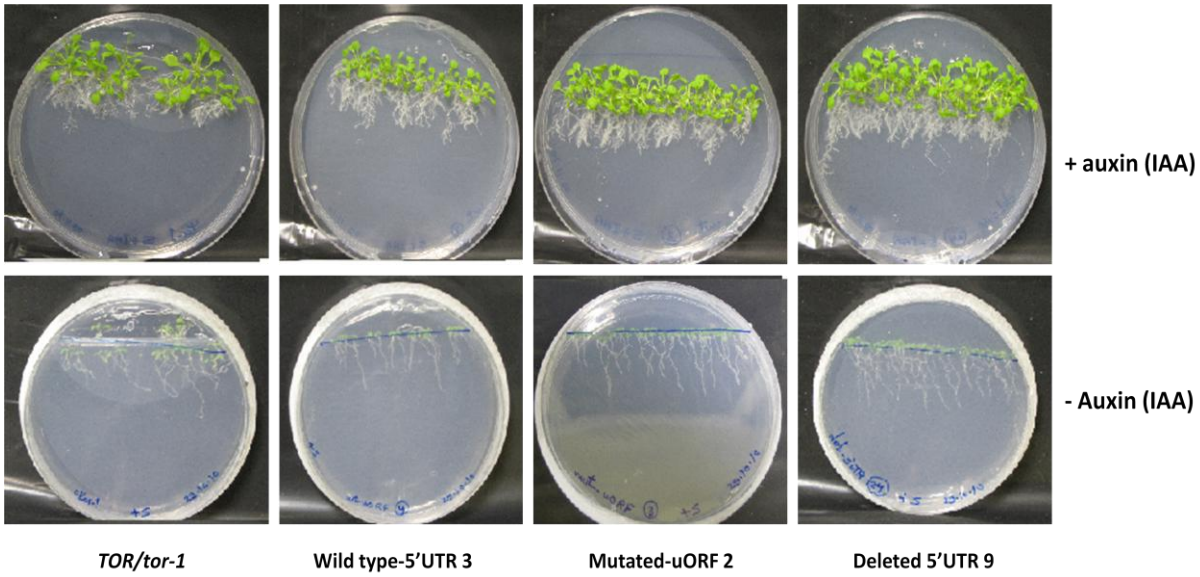


Figure 55: **Transformed seedlings from three different constructs grown on Hoagland/2 medium with and without 10^{-7} mM indole acetic acid (IAA).** One independent transformed line was selected from each construct that include full-length 5′UTR 3, mutated -uORF 2, and deleted -5′UTR 9. Seedlings were grown on cellophane bag placed on the surface of Hoagland/2 medium supplemented with 1 % sucrose. Seedlings from *TOR/tor-1* heterozygous were used as a reference for transformed lines.

The effect of both 5′UTR or uORF of *AtTOR* mRNA in presence of the phytohormone auxin on *TOR::GUS* mRNA expression from different independent transformed lines with full-length 5′UTR, mutated uORF, and deleted 5′UTR constructs has been examined. We have added indole acetic acid (IAA), that is consider the principle auxin in higher plants, to Hoagland/2 culture medium supplemented with 1gm/L sucrose in Petri dishes.

Treatment of transformed seedlings with 10^{-7} mM indole acetic acid was promotes the formation of numerous adventitious roots and activates the growth of the shoot system (**Figure 55**).

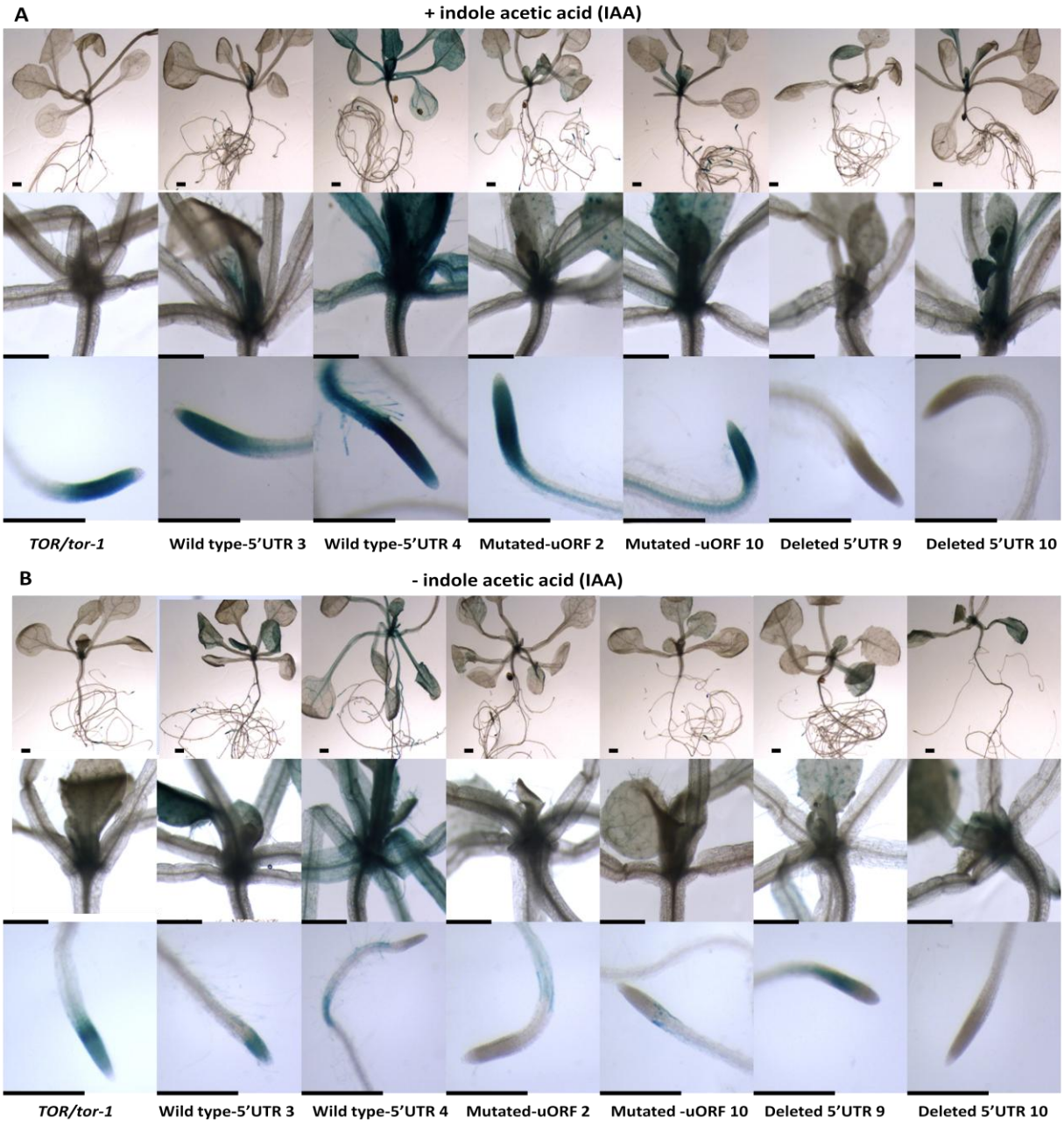


Figure 56: Histochemical analysis of TOR::GUS expression from full-length 5′UTR, mutated uORF and deleted 5′ UTR transformed lines with indole acetic acid (+ IAA; A) and without indole acetic acid (-IAA; B). Two independent transformed lines from each construct have used and the number of each line was represented under each picture. Each line was represented by three views include the whole seedling, zooming of the shoot tip, and zooming of the root tips *TOR/tor-1* heterozygous plants were use as a positive control. Seedlings were grown horizontally on cellophane bag placed at the surface of Hoagland/2 medium. Seedlings have been transferred after 5 days from IAA treatment to X-Gluc solution and incubated them at 37 ^{o} C for 8 hours. IAA was added in concentration of 10^{-7} mM to Hoagland/2 medium supplemented with 1% sucrose. Scale bare is 1 mm.

GUS histochemical analysis of auxin treated lines shows an increased overall TOR::GUS expression in meristematic cells of both the primary and lateral roots as well as shoot apical meristems from full-length 5′UTR and mutated-uORF transformed lines. However, it was observed that transformed lines with deleted 5′UTR construct did not have any indicator of TOR::GUS expression in root merestematic cells treated with auxin although they exhibited a clear pattern of *TOR::GUS* mRNA expression at the shoot apical meristems (**Figure 56 A**).

In the absence of auxin, the spatial pattern of TOR::GUS expression in root apical meristems appears stronger in the *TOR/tor-1* line. These suggest that *AtTOR* coding or intronic sequences may be implicated together with AtTOR promoter and the 5′UTR in regulation of *AtTOR* gene expression. Our results are consistent with the result of **Menand Ph.D. thesis, (2002)**, in *TOR/tor-1* heterozygous plants.

We hypothesized that the *AtTOR* 5′UTR acts to promote *TOR::GUS* mRNA transcription or protect the TOR::GUS mRNA from degradation and hence increase their stability in the presence of auxin. Positive induction of TOR::GUS mRNA by 5'UTR and auxin suppose presence of an auxin binding site sequence within the 5'UTR that will be recognized by auxin response factor and regulate the regulation of *AtTOR* mRNA transcription (**Figure 56 B**). To distinguish between the two levels of regulation, quantitative analysis of *TOR::GUS* mRNA and TOR::GUS activity are required from the root apical meristems of suitable numbers of selected transformed lines. Even more, the half-life of TOR::GUS mRNA needs to be investigated to determine the degree of stability of this transcript with and without auxin.

3 - DISCUSSION

The Target of rapamycin (TOR) pathway is conserved in most eukaryotes, including plants. Studies in yeast, Drosophila, and mammals have shown that the target of rapamycin (TOR) protein is involved in control of cell growth and cell proliferation in response to different types of environmental signals such as nutrients, amino acids, hormones, and growth factors (**Beretta *et al*., 1996; Thomas *et al*., 1997**). The change of *TOR* mRNA level affects both vegetative and reproductive organ growth in Arabidopsis plants (**Deprost *et al*., 2007**).

Our goal was to study the regulation of Arabidopsis TOR (*AtTOR*) gene expression at the post-transcriptional level. Expression of many genes is controlled by different regulatory elements distributed along the gene (i.e. cis acting regulatory elements) or depends on other regulatory genes (i.e. trans-acting regulatory elements). From the potential cis-acting regulatory elements of *AtTOR* gene, we have selected the 5′untranslated region (5′UTR) and their upstream open reading frame (uORF) to be the axis of our study which was the first attempt to study the mechanism of *TOR* gene regulation in eukaryotes.

3.1. Control of AtTOR gene expression at the post-transcriptional level

We have investigated the expression profile of *AtTOR* mRNA in heterozygous *TOR/tor-1* mature tissues, in which *tor-1* allele is fused with the GUS reporter gene at amino acid 1555. Our results revealed that *AtTOR* mRNA is expressed in all tissues, while, TOR protein (as estimated from GUS activity) is expressed only in undifferentiated tissues such as root and shoot meristems, primordia, and cotyledons. These results are consistent with the results of previous reverse transcription PCR which revealed that *AtTOR* mRNA is expressed in all plant tissues including differentiated cells where TOR-GUS protein is undetectable (**Robaglia *et al*., 2004**).

The absence of AtTOR protein expression in mature plant cells such as expanding root and leaves may be related to the fact that these cells undergo growth through expansion which is a different process to premitotic growth, which involves the synthesis of ribosomes and other cytosolic components (**Schmelzle and Hall, 2000**).

Our results suggests that *AtTOR* mRNA is regulated post-transcriptionally by translational repression in differentiated cells .Thus, we have investigated the role of micro RNA (miRNA) in

repression of *AtTOR* mRNA translation since one paper has reported that a predicted miRNA (miR34) may be implicated in regulation of *AtTOR* mRNA expression (**Bonnet *et al.*, 2004**). Cucumber mosaic virus (CaMV) 2b (strain FNY) is a strong suppressor of miRNA-mediated cellular mRNA turnover via its direct binding with ARGONAUTE 1(AGO1) protein (**Lewsey *et al.*, 2007**).We have made a cross between heterozygous *TOR/tor-1* plants and transgenic Arabidopsis plants expressing cucumber mosaic virus (CaMV) 2b (strain FNY). The expression pattern of TOR::GUS fusion protein from the new double transgenic plants expressed CaMV 2b was examined and compared with the expression pattern of TOR::GUS fusion protein from *TOR/tor-1* heterozygous plants as a control. The results revealed that there are no changes in the expression pattern of TOR::GUS fusion protein between the *TOR/tor-1* mutant plants and the double transgenic plants. These results suggest that *AtTOR* expression might not be targeted by the potential predicted miRNA (miR34) or that the effect of this potential miR34 is linked to particular developmental or environmental conditions. In addition the direct detection of miR34 by northern blotting was not conclusive (E. Delannoy, E. Lanet, unpublished).

Overall, these results show that translation of *AtTOR* mRNA is not subjected to regulation by micro RNAs (miRNAs) and thus, we can conclude that repression of TOR translation in mature leaves is probably not due to RNA silencing. We then explore if it can be caused by translational regulation by the 5′UTR.

3.2. Impact of TOR 5′UTR on expression of GUS reporter gene

3.2.1. Identification of regulatory sequences within 5'UTR of *TOR* gene

5′RACE analysis revealed that the precise length of Arabidopsis TOR (*AtTOR*) 5′UTR is 230 bp. Moreover, the sequencing analysis of 5′RACE product shows a small upstream open reading frame (uORF) encoding only 2 aa (ATG TGT TGA) beside the start codon located approximately in the middle of the 5′UTR. Our results are consistent with previous results reporting that the length of *AtTOR* 5′UTR is 230 bp and that it contains a small uORF encoded for 2 amino acids (**Menand *et. al.*, 2002**). Even more, **Robaglia *et al.*, (2004)** recorded that these small uORF is highly conserved among 12 different Arabidopsis ecotypes. Together, these results encouraged us to search conserved TOR 5′UTR and uORF between other plant species that are located within the family Brassicaceae or that are closely related to this family.

Our analysis has also revealed that the minimal length of TOR 5′UTR varied between different plant species, where it ranges from 180 bp for dicot *Brassica rapa* to 350 and 400 bp for monocot *Brachypodium distachyon* and the moss *Physcomitrella patens*, respectively. These results support our findings of TOR 5′UTR length for the studied dicots plants such as *Arabidopsis thaliana*, *Arabidopsis lyrata*, *Brassica rapa* and *Cucumis sativus*; in particular that *TOR* gene is considered one of the main important regulatory genes in most eukaryotes. RT-PCR experiments indicated that level of *TOR* mRNA is lower than internal reference genes such as *ACTIN2* or *PDF2*. Furthermore, expression profile of Arabidopsis TOR (*AtTOR*, At1G50030) signaling pathway in Arabidopsis using Genevestigator has indicated that expression of the *AtTOR* mRNA at different tissues is quite low as shown in figure 6.

Over than 30 % Arabidopsis mRNAs possess uORFs in their leader sequence and are enriched among mRNAs for transcriptional factors and protein modifying enzymes. An interesting finding was the high similarity in nucleotide sequences of *TOR* 5′UTR including the uORF with 9 nucleotides in length located approximately within the middle of 5′UTR between *Arabidopsis thaliana* (*At), Arabidopsis lyrata* (*Al*) and *Brassica rapa* (*Br*). Its phylogenetic conservation suggests a conserved function. The minimal length of the TOR 5′UTR from *Cucumis sativus* and *Physcomitrella patens* haves a uORF of 18 nucleotides, however, the TOR 5′UTR from *Brachypodium distachyon* TOR (*BdTOR*) mRNA was found to have a long uORF of 75 nucleotides. This result is consistent with the results obtained from computer analysis of 5′UTR mRNA from higher plants which revealed that uORFs from 5′UTRs mRNA of dicots are short with the mean length of 36 nucleotides and their uAUG codon is usually found in a nonoptimal context (**Kochetove *et al.*, 2002**).

Secondary structures are another feature of 5′UTR and can act as down-regulators of gene expression. They have the ability to inhibit gene transcription (**Curie and McCormick, 1997**), accelerate mRNA degradation rate (**Cannons and Cannon, 2002**), or reduce translation efficiency (**Bunimov *et al.*, 2007**). The predicted 5′UTR secondary structures from *Arabidopsis thaliana* TOR *(AtTOR), Brassica rapa* TOR *(BrTOR), Cucumis sativus* TOR *(CsTOR), Brachypodium distachyon* TOR *(BdTOR), and Physchomitrella patens* TOR *(PpTOR)* has been achieved. Our analysis revealed that 5′UTRs from *AtTOR*, *BrTOR*, and *CsTOR* have the ability to form slightly stable secondary structures. We recorded that the 5′UTR from *BdTOR* and *PpTOR* have the ability to form a more stable secondary structure than the predicted 5′UTR from

AtTOR, *BrTOR*, and *CsTOR* due to a high content of C + G. The presence of high content from G + C within the 5'UTR sequence is important factor in producing a stable secondary structure with high change in free energy. The value of ΔG for 5′UTR secondary structure was - 46.99 kcal/mol, - 28.93 kcal/mol, and – 27.61 kcal/mol for *AtTOR*, *BrTOR*, and *CsTOR*, respectively. The 5′UTR secondary structure of *BdTOR* mRNA is strong and it was – 97.08 kcal /mol and it was –60.61 kcal /mol for *PpTOR* 5′UTR. Analysis of structural features of untranslated regions from diverse taxonomic classes suggested that presence of stable secondary structure (ΔG below – 50 kcal/mol) has the ability to affect significantly and decrease the translational efficiency of transcripts (**Mignone *et al.*, 2002**). Our results may be interpreted on the light that secondary structure of *AtTOR* 5′UTR has a functional significance and may be involved in the maintenance of translational activity of *TOR* mRNA at low level, preventing the plant from deleterious excessive production.

3.2.2. AtTOR 5'UTR affects GUS mRNA stability or transcription

The *AtTOR* gene expression can be regulated by multiple elements at various levels, including post-transcriptional control leading to a low TOR activity as we have discussed above. We, therefore, hypothesized that native *AtTOR* gene expression might be tightly controlled by cis regulatory elements located in the 5′UTR sequence, such as the presence of uORF, the potential effect of stable secondary structure. The 5′UTR, the first exon, the first intron, and the beginning of the second exon of *AtTOR* mRNA has been fused with *GUS* reporter gene in pBI101 and pBI121 binary vectors under either *AtTOR* promoter or CaMV 35S promoter, respectively to create full-length 5'UTR chimeric GUS reporter as a reference construct. Mutation of the uORF and deletion of the 5′UTR have been done in order to study the impact of absence of AtTOR 5′UTR and uORF sequences on *AtTOR::GUS* mRNA expression qualitatively and quantitatively using both transient and stable expression assays.

Histochemical GUS analysis of stable transformed Arabidopsis seedlings lines containing reporter GUS constructs that are derived by the native *AtTOR* gene promoter revealed that the GUS expression pattern was essentially similar to that of the *TOR/tor-1* mutant line in which GUS was fused in frame with the AtTOR protein (**Menand *et al.*, 2002**). This result suggested that all regulatory elements implicated in the control of *AtTOR* gene expression are contained in TOR promoter, the 5′UTR and the first intron.

This result can be used to denote that *AtTOR* mRNA is not subjected to regulation by micro RNAs (miRNAs), as the construction do not contain the portion of *TOR* mRNA that is targeted by the putative miR34. We can add this argument to the first argument related to crossing of *TOR/tor-1* heterozygous plants with CaMV 2b FNY transgenic plants to support our hypothesis suggesting that *AtTOR* is not subjected to regulation by miRNA

When the gene constructs were driven by CaMV 35S promoter in transient expression, our results show that deletion of the *AtTOR* 5′UTR caused an increase in both *TOR::GUS* transcript level and TOR::GUS activity. These increases in *TOR::GUS* mRNA accumulation suggested that the 5′UTR has a negative effect either on GUS transcription level or *GUS* mRNA stability. In contrast, quantitative transient expression analysis of constructs driven by the *AtTOR* gene promoter revealed that deletion of *AtTOR* 5′UTR resulted in a decrease in both *TOR::GUS* transcript level and TOR::GUS activity. Since the only differences between these constructs are the promoter, these results suggest that *AtTOR* 5′UTR possess a positive effect either on transcription or RNA stability when it is linked to the *TOR* promoter. We may exclude the hypothesis that mRNA stability is differentially affected since the mRNA produced by the two kind of constructs are identical. However, we cannot exclude the possibility that mRNA transcribed by the *AtTOR* promoter in the presence of the 5′ UTR might be somehow marked and that it increases its stability.

In stable transgenic lines, our results show that the 5′UTR from the *AtTOR* mRNA possess a slight negative effect on *TOR::GUS* transcripts accumulation, which was consistent with the quantitative analysis of *TOR::GUS* mRNA expression from transient expression assay using CaMV 35S promoter. In transient expression, the amount of TOR::GUS activity take the same trends related to the changes in the relative amount of *TOR::GUS* transcript level, indicating that *TOR::GUS* mRNA is regulated either at RNA stability or RNA transcription level, but probably not at the translational level.

When comparing TOR::GUS translational efficiency for stable transformed lines, it appears that both 5′UTR and uORF of *AtTOR* mRNA possess a positive effect on translational effeciency of *TOR::GUS* mRNA. This positive effect for 5′UTR and/or uORF on translational efficiency can be mainly attributed to an increased *TOR::GUS* mRNA accumulation and not to changes in TOR::GUS activity which is maintained at quite similar level.

Together, these results strongly suggest that 5′UTR or uORF are implicated in regulation of *AtTOR* through stabilization/destabilization of *AtTOR* mRNA or through an increase in their transcription rates. In addition, our results suggest the possible integration between the 5′UTR sequence and the *AtTOR* promoter in regulation of GUS expression through control either of GUS transcription rate or *GUS* mRNA stability. The hypothesis that 5′UTR may affect *TOR::GUS* mRNA stability still exist, in particular in case of *AtTOR* promoter.

The transcription rate of *TOR::GUS* mRNA from both type of transformed plants having the 5′UTR of AtTOR fusioned with GUS reporter gene and either under activity of CaMV 35S or *AtTOR* promoters could be measured using a nuclear run-on transcription assay. Furthermore, we could distinguish between the transcription rate of *TOR::GUS* mRNA and the stability of *TOR::GUS* mRNA through treatment of transformed seedlings by chemical transcription inhibitor such as actinomycin D and measurement of the half-live of *TOR::GUS* mRNA at different period. The increased in mRNA half-life after treatment with transcriptional inhibitor indicate the increased stability of this transcript.

Indeed, the 5′ untranslated region (5′UTR) of plant mRNA where already found to affect either mRNA transcription or mRNA stability. For example, it was recorded that expression of *LAT59* transcript was inhibited by 5′UTR sequence at the transcriptional level by a putative stem-loop region that impede the transcription elongation process (**Curie and McCormick, 1997).** Another study shown that Arabidopsis pyrroline-5-carboxylate reductase (*At-P5R*) transcripts increased under salt and heat stress due to an enhanced mRNA stability (**Hua *et al.*, 2001**). The analysis revealed that the first 92 bp of the *At-P5R* 5′UTR mediate transcript stabilization and translation inhibition during salt and heat stresses. Moreover, the same region from *At-P5R* 5′UTR (92 bp) was also able to interact with a sequence in the *At-P5R* promoter to increase transcription. According to these data, it was proposed that the *At-P5R* 5′UTR might enhance transcription initiation rather than elongation because the activation element is located within the first 92 bp of the *At-P5R* 5′UTR close to the transcription initiation site. Analysis of the effect of 5′UTR on expression of TIR-NBS-encoding gene from triploid white poplar [(*Populus tomentosa* x *P.bolleana*) x *P. tomentosa*]**,** *PtDrl02***,** revealed that these 5′UTR sequence decreased the transcript level of the GUS reporter gene by 13.3-fold, assuming a regulatory role of 5′UTR in transcription and/or mRNA destabilization (**Zheng *et al.*, 2009**).

5′UTR are also known to mediate transcript degradation rates of some RNAs. For example, ferredoxin transcripts exhibited stabilization in light and destabilization in dark via the internal light-regulatory element located within the 5′UTR of the ferredoxin mRNA (**Bhat *et al.*, 2004**). Recently, it was reported that both 5′ and 3′ untranslated regions contribute in maintaining the transcript stability of *Solanum tuberosum* BEL-like transcription factor (StBEL5) (**Banerjee *et al.*, 2009**). StBEL5 transcription factor and its knotted1-like homeobox (Knox) protein partner regulate tuber formation by targeting genes that control growth.

In view of these data, we can speculate that *AtTOR* 5′UTR sequence may control the expression of *TOR::GUS* mRNA at level of transcription via their interaction with sequences within *AtTOR* promoter.

Stable and transient expression experiments have revealed that AtTOR promoter and CaMV 35S promoter do not appeared their activity in the same tissue. Expression is found in mature leaves of *N.benthamiana* for 35S promoter, while it was restricted to proliferative tissue in Arabidopsis seedlings for *AtTOR* promoter. Therefore, 5′ UTR regulation of *TOR::GUS* mRNA expression may only be active in proliferative tissue not in differentiated tissue. One may hypothesize that repression of the *AtTOR* promoter occurs in differentiated cells of *N.benthamiana* leaves. In addition, we can postulate the possible presence of binding sites for RNA-stabilizing proteins within the *AtTOR* 5′UTR sequence in Arabidopsis.

3.2.3. Upstream open reading frame affects both translation and stability of GUS

Upstream open reading frames (uORFs) can regulate post-transcriptional gene expression by translational inhibition of mRNA or degradation of mRNA via non-sense mediated decay, but no study describe their role in transcription regulation. Translational inhibition by upstream open reading frame (uORF) was studied in details in a number of plant genes (**Hanfery *et al.*, 2005; Hummel *et al.*, 2009**). In general, the mechanism of uORF inhibition depends on either the features of uORF or the peptide encoded by uORF and interaction with ribosomes during scanning process. The features of uORF involve their length, their number, their position within the 5′UTR, and the presence of a stable secondary structure. Both two mechanisms were investigated in eukaryotes included yeast, mammals and plants.

Our results revealed that the uORF has a negative effect on both *TOR::GUS* mRNA accumulation and TOR::GUS activity for the CaMV 35S or AtTOR promoter constructs in

transient expression. It was also observed that uORF has a negative effect on *TOR::GUS* mRNA level in transformed plants. This result encourage us to hypothesize that uORF is acting as destabilizing element of the *TOR::GUS* mRNA or it represent a binding site for interaction with transcription repressor factor that lead to reduction of the transcription rate of *TOR::GUS* mRNA.

Our results suggest that the *AtTOR* 5′UTR uORF promotes translational efficiency of *GUS* transcript when it is driven either by the CaMV 35S promoter or the native TOR promoter and in stable transformed plants with *TOR* promoter constructs. We suggest that promotion of *GUS* mRNA translation by uORF depends on the stability of *GUS* transcript, since we have observed an increased accumulation of *GUS* mRNA mutated at the uORF.

Upstream open reading frames (uORFs) may exert a negative role on *GUS* mRNA translation and *GUS* mRNA stability, since the two processes mutually influence each other. For example, in yeast, it was observed that introduction of an uORF encoding for 7 amino acids in the 5′UTR of the *cat* transcripts was responsible for both inhibition of translation and acceleration of degradation of the *cat* mRNA. It was concluded that destabilization of specific mRNA is linked to changes in translational initiation on the same transcript (**Oliveira *et al.*, 1995**).

Thus, in view of these data, we propose that presence of a small uORF located in middle of the 5′UTR and the presence of secondary structures within and downstream of uORF might exert a repressor effect on mRNA stability. Actually, we observed that the mutation introduced in the uORF does not cause a change in secondary structure.

Another mechanism related to regulation of mRNA stability by uORF is called nonsense-mediated mRNA decay (NMD). It is a quality mechanism that plays an important role in recognition and degradation of abnormal transcripts containing premature termination codons (PTC). PTCs are identified as premature if they are located more than 50-55 nucleotides upstream of the last exon-exon junction (**Isken and Maquat, 2007; Kerenyi *et al.*, 2008)**. Little is known about the impact of plant uORFs on transcript accumulation through the nonsense-mediated mRNA decay pathway. Some recent reports proposed that NMD regulates only a small fraction of uORF containing transcripts since only ~ 2% of annotated Arabidopsis genes contain more than 33 amino acids long uORF (**Nyikó *et al.*, 2009**).

It is conceivable that *TOR::GUS* mRNA accumulation might be subjected to regulation by NMD mechanism via uORF. Our results have shown that removing the uORF resulted in increasing the

amount of reporter GUS transcript level. However the length of uORF in the AtTOR 5′UTR is only 9 nucleotide and the distance between the uORF and the first intron is approximately 270 bp long. The introns either in 5′UTR or at the 3′UTR appeared to play a role in trigger NMD decay in plants (**Hori and Watanabe, 2007; Kerenyi *et al.*, 2008**). More studies are required to explore a possible role of *AtTOR* uORF in control of *AtTOR* stability by this mechanism.

Another possibility would be that 5′UTR uORF sequence of *AtTOR* mRNA acts as a binding site for unknown repressor transcription factors that are associated with *AtTOR* promoter leading to decreases in transcription rate of *AtTOR* mRNA and thus decreases in *AtTOR* mRNA stability. But actually, no evidence to date refers to this type of correlation between uORF and *TOR* promoter in regulation of AtTOR expression.

3.3. Impact of 5′UTR and uORF on expression pattern of GUS under particular growth conditions

3.3.1. Effect of exogenous sucrose

Sugar is both a nutrient and a signaling compound and is required for growth, either through photosynthesis or supplied externally. Sugar sensing modulates and coordinates internal regulators in response to environmental cues, and in turn governs plant growth and development (**Smeekens, 2000**). Plant cell proliferation can be induced by sucrose (**Van't Hof, 1966**).

The spatial pattern of *TOR::GUS* reporter gene of transformed plants with mutated uORF and deleted 5′UTR constructs and driven by *AtTOR* promoter was observed in the presence of exogenous sucrose. Our results show that sucrose generally increases TOR::GUS activity, more particularly in the root meristem (**Figure 54 A and B**).

Sugar may directly activate the *AtTOR* promoter, or indirectly activate it through the cell cycle. Indeed, some studies demonstrated that sugars play a main role in control the expression of D-type cyclin gene during the G1 phase of the cell cycle in Arabidopsis (**Gaudin *et al.*, 2000; Riou-Khamlichi *et al.*, 2000**). Also, when the Arabidopsis cyclin D2 is overexpressed in transgenic tobacco it causes an increased accumulation of biomass and enhanced root growth (**Cockcroft *et al.*, 2000**). **Riou-Khamlichi et al.** (**2000**) observed that the level of cyclin D2 and D3 mRNAs, and also the activity of the corresponding CDKs, strongly increase when sucrose is

added to starved Arabidopsis cells. Thus, the sugar availability could be sensed at the level of the cell cycle by a signaling pathway involving D cyclins.

In mammals, it was found that this activation is mediated by TOR/eIF4E signaling pathway. It was found that eIF4E is important for cyclin D expression by enhancing the transport of its mRNA in mammalian cells (**Rousseau *et al.*, 1996**). Therefore, the positive response of *AtTOR* expression to sugars may be mediated by the effect of sucrose on expression of D-type cyclin genes during the G1 phase of the cell cycle. The increase in expression activity of D-type cyclin genes might result in activation of transcription factors which control expression of some important genes including *AtTOR* gene via feedback mechanism (**Figure 57**).

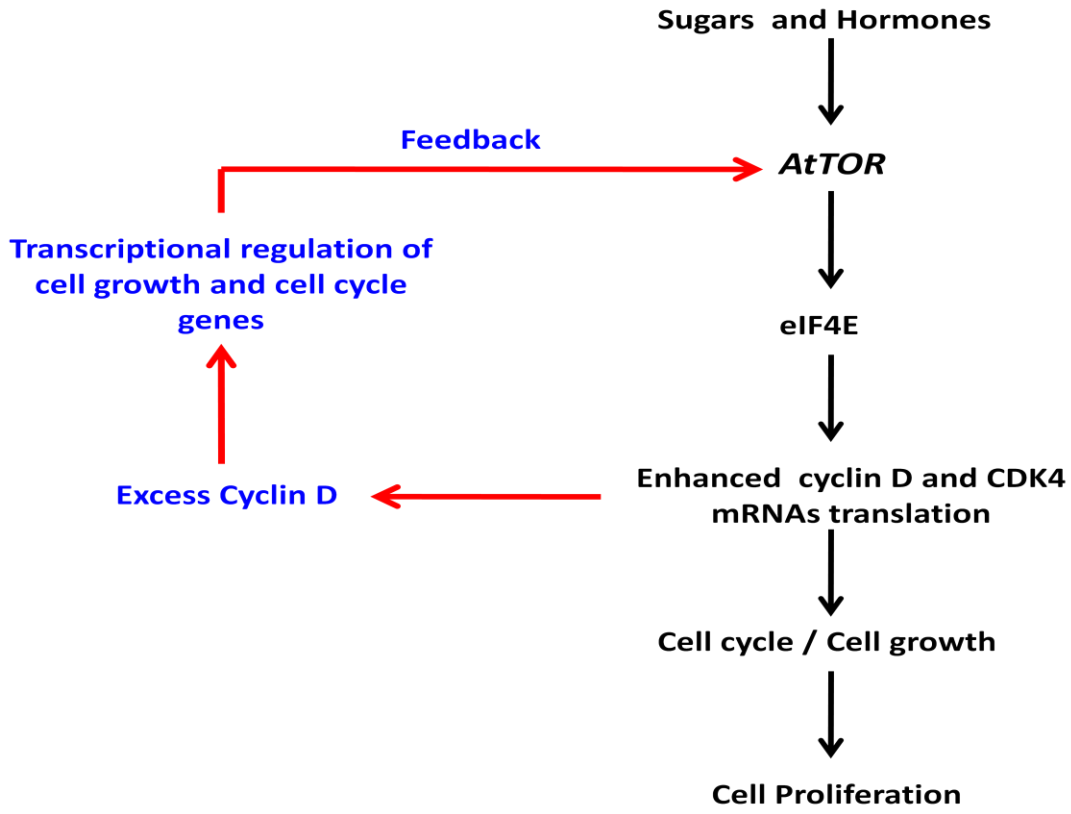


Figure 57: Proposal functions for cyclin D in feedback regulation of Arabidopsis TOR (*AtTOR*) expression in response to sugars and hormones. Black arrows denote the role of cyclin D and CDK4 in cell cycle and cell growth, while the red arrows denote the role of cyclin D in feedback mechanism that regulates transcription of *AtTOR* gene.

We can test the proposed role of D-type cyclin genes in regulation of AtTOR expression in presence of sucrose through determination the level of *AtTOR* mRNA in the meristematic root tissue from *cyclin D* mutant plants, particularly *cyclin D2* and *cyclin D3* compared to cyclin D 2

and 3 wild types. Even more, we can investigate whether there are a link between the cyclin D genes and both uORF and the 5′UTR of *AtTOR* mRNA in presence of sucrose through crossing between our full-length 5′UTR, mutated uORF, and deleted 5′UTR transgenic plants and the *cyclin d2* and *cyclin d3* mutant plants.

3.3.2. Effect of exogenous auxin

Auxins and cytokinins are linked to the capacity of plant cells to proliferate. Addition of auxin is sufficient to reactivate cell division of differentiated cells and tissues (**Kende and Zeevaart, 1997**).

We tested the influence of indole acetic acid (IAA) on the spatial pattern of *TOR::GUS* mRNA expression of the transformed plants with the full-length 5′UTR, mutated uORF, and deleted 5′UTR constructs under the expression activity of *AtTOR* promoter. In the root of seedlings grown on medium without auxin, it was observed that GUS is located in elongation zone of root tissue and its presence in meristematic tissues was weak. A different TOR::GUS pattern is observed with auxin, which induce a strong GUS activity in the root meristem. However, in the presence of auxin, it was observed that the transformed lines with the deleted 5′UTR do not have *AtTOR::GUS* expression in root meristematic cells, although the pattern of *AtTOR::GUS* expression was not affected in the shoot apical meristem. The pattern of GUS expression in uORF mutated lines was not different than that of control lines.

This may be attributed to presence of an auxin dependent sequence located in the *AtTOR* 5′UTR that mediate the *AtTOR::GUS* mRNA expression in response to auxin. Its removal resulted in non-response to exogenous auxin.

The spatial pattern of TOR::GUS protein was similar for *TOR/tor-1* heterozygous seedlings whether it was grew with or without auxin, suggested that TOR coding sequence is not implicated in regulation of *AtTOR* mRNA expression in presence of auxin and all regulatory elements are restricted either to the TOR promoter or to the 5′UTR.

These results show that addition of external IAA exert a positive role in regulation of the *AtTOR::GUS* mRNA expression via their positive activation of *AtTOR* transcript. These results supported our previous hypothesis that the 5′UTR acts together with the TOR promoter of *AtTOR* to increase the level of the *AtTOR::GUS* transcript either through increase the transcription rate of *AtTOR::GUS* mRNA or increased the *AtTOR::GUS* mRNA stability.

One study has shown that, in Arabidopsis suspension cultured cells, 1-naphthaleneacetic acid [1-NAA] and kinetin phytohormones participate in regulation of Arabidopsis S6 kinase (AtS6K) activity (**Turck *et al.*, 2004**). Furthermore, it was reported that AtRaptor 1 interacts with the HEAT repeats of TOR and regulates the activity of S6 kinase (S6K) in response to osmotic stress (**Mahfouz *et al.*, 2006).** Many transcription factors and signal transduction coding genes involved in auxin and gibberellins response have been identified in transcriptomic analysis of *AtTOR* RNA interference (RNAi) mutant plants (**Moreau, Ph.D., 2009**). For exemple, transcriptomic analysis revealed that the expression of both *ATAUX 2-11* and *AA16* transcription factors that encoded auxin responsive proteins are decreased. In addition to these two genes, expression of *HAT2* gene, which is stimulated by auxin, is also decreased in TOR RNAi lines. Moreover, Benoît Menand has shown during his thesis that addition of auxin to the growth medium caused an increased in the level of *AtTOR* gene expression. These data suggest a link between TOR signaling pathway and auxin signaling pathway.

We searched for putative DNA or RNA binding motifs within the promoter or the 5′UTR of *AtTOR* using the PLACE program (http://www.dna.affrc.go.jp/PLACE/signalscan.html).This shows the absence of auxin response elements from both of *TOR* promoter and the 5′UTR, indicating that AtTOR expression may not be regulated directly by auxin. On possibility would be that AtTOR is indirectly regulated by auxin dependent cell cycle (or cell growth) activation. A recent study suggested that auxin affects both activity and cellular distribution of CYCD2,1 via reducing the protein level of the interactor of CDK/kip-related protein 2 (ICK/KRP) (**Sanz *et al.*, 2011**). The presence of high levels of ICK2/KRP2 protein inhibits the G1/S phase transition of cell cycle.

From these result, we can propose that auxin enhanced the potential activity of cell division via increase the activity of CYCD2,1-CDK complex and this in turn activates TOR transcription through unknown activators. Investigation the level of *TOR* mRNA inside the cycd2,1 mutant will be reveal whatever *TOR* is regulated by CYCD2,1 or not.

In addition, we have identified a set of putative DNA binding motifs located in both TOR promoter and the 5'UTR of *AtTOR* gene (At1G50030, **Menand *et al.*, 2002**). From these motifs the AGATT DNA-binding sites may be involved in the regulation of AtTOR transcription through association with ARR1 and CCA1 transcription factors.

ARR1 has ability to bind DNA via their ARRM domain and activate transcription in plant cells. Furthermore, It was found that CCA1 protein regulating the circadian rhythm (**Wang *et al.*, 1997**), have an ARRM DNA binding- domain because DNA bound to CCA1 contains 5'-AGATT-'3 sequence that is identical to the target sequence of ARR1 (**Sakai *et al.*, 2000**).

Figure 55: Analysis of TOR promoter of *Arabidopsis thaliana* using PLACE program. Letters in black representing the sequence of TOR promoter, letters in thick blue representing the 5′UTR sequence. The beginning of coding sequence is indicated by ATG in red thick letter. Letters in orange representing the DNA-binding sequences for both ARR1 and CCA1 transcription factors. These motifs are distributed enter both TOR promoter and *TOR* mRNA 5′UTR.

An interesting study suggested that ARR1 control root meristem size in interaction with SHY2 and is a target of cytokinin (**Dello Ioio *et al.*, 2008**). SHY2 acts as a repressor of auxin via preventing activation of the auxin/indole-3-acetic acid inducible (Aux/IAA) responsive genes by forming heterodimers with the ARF (auxin response factor) transcription factors (**Guilfoyle and Hagen, 2007; Mockaitis and Estelle, 2008**).

From all these data, we can postulate that *AtTOR* gene may be regulated at the transcriptional level through ARR1 and SHY2 transcription factors at least in meristematic tissues. Further analysis are required to improve this hypothesis. We can investigate the level of *TOR* mRNA expression in meristematic and mature tissues of *arr1* and *shy2* mutant plants. Even more, we can make a cross between our transgenic plants having deleted 5′UTR construct with ARR1 and SHY2 wild type plants and *arr1* and *shy2* mutant plants to determine links between the 5′UTR of *AtTOR* and these transcription factors.

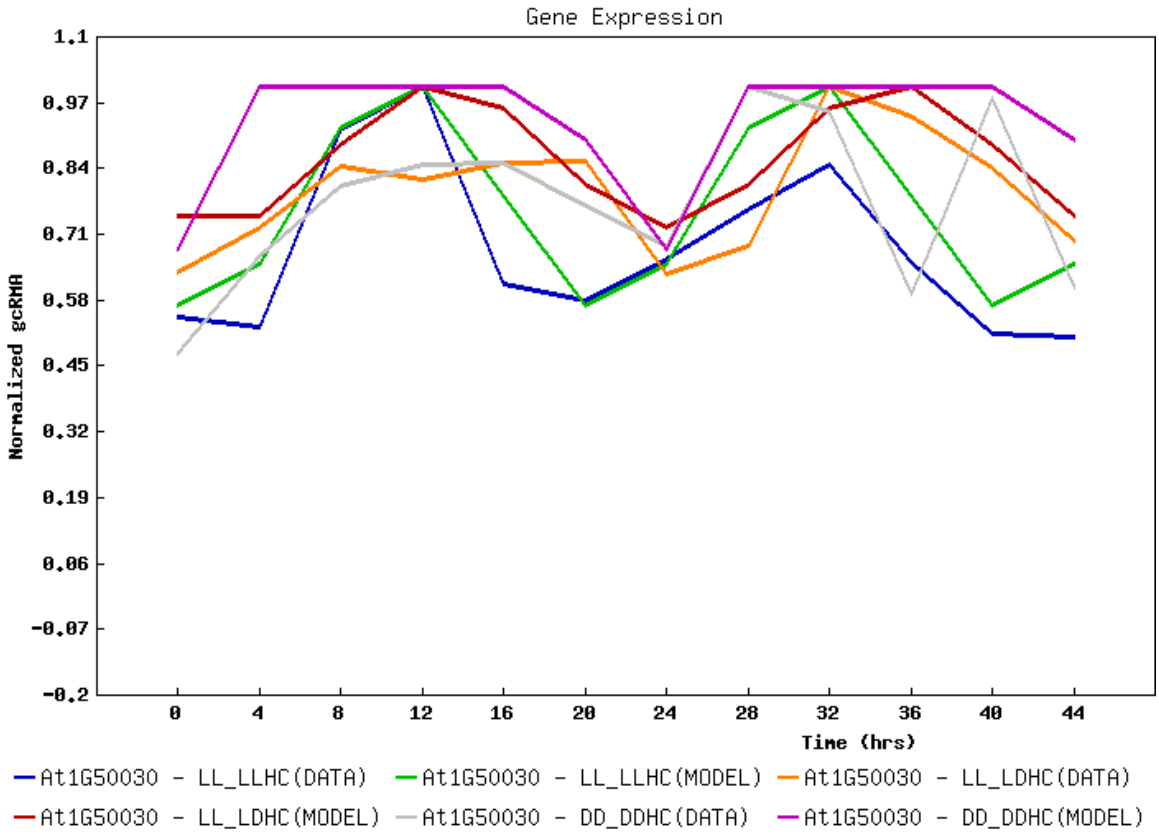


Figure 56: Expression profile of Arabidopsis TOR (*AtTOR*) gene at different photocycles and thermocycles entrainment. LLHC, is continuos light thermocycles; LDHC, is light/dark photocycles; and DDHC, is continous dark. Thermocycles for all photocycles include either 22 ^{o}C/18 ^{o}C or 22 ^{o}C/12 ^{o}C. LL: light/light; DD: dark/dark; LD: light/dark.

Circadian clock associated 1 (CCA1) is considered one of the main transcription factors located in the core of the circadian oscillator in *Arabidopsis* (**Wang and Tobin, 1998**). We identified two CCA1 binding sites in the 5′UTR and five in the *AtTOR* promoter. CCA1 binding sites in Arabidopsis *TOR* gene 5′UTR and promoter are composed of AGATTCTT or AGATTTTT nucleotide sequenc, but, the first 5 nucleotides only (AGATT) is similar to the ARR1 binding sites nucleotide sequence. We searched the circadian gene expression profile of *AtTOR* gene using the diurnal web tool (**Mockler *et al.*, 2007; Michael *et al.*, 2008**). We found that expression of AtTOR change during the hours of the day and night (**Figure 56**), suggesting that *AtTOR* mRNA expression can be regulated by CCA1 circadian transcription factor. CCA1 can activate the transcription of *TOR* mRNA and deletion of its 5′UTR would result in reduction in TOR transcription level. The transcriptional regulation of *TOR::GUS* mRNA either by ARR1 or CCA1 transcription factors may explain the possible role for the 5′UTR of *AtTOR* in this regulation mechanism.

Future experiments will address the regulation of *TOR* mRNA in *cca1* mutant plant. Another experiment involve a cross between our deleted 5′UTR transgenic plants with *cca1* mutant and CCA1 wild type plants to elucidate the role of 5′UTR of *AtTOR* on regulation of *TOR* gene expression.

4 – Conclusion

Our results suggest that expression of the Arabidopsis TOR (*AtTOR*) gene is not targeted by the predicted micro RNA (miRNA 34). Furthermore, the direct detection of miR34 by northern blotting was not conclusive (E. Delannoy and E. lanet, unpublished). Thus, repression of AtTOR mRNA translation in differentiated tissues is probably not due to RNA silencing.

The impact of AtTOR 5′UTR and uORF on expression of *TOR::GUS* mRNA and TOR::GUS activity was investigated in transient assay and in stably transformed Arabidopsis plants, as these regulatory sequences are expected to play an important role in regulation of *AtTOR* gene expression. Our work show an overall negative regulation exerted by the 5′UTR and, to a lesser extent, by the uORF on *AtTOR* gene regulation. This regulation is likely at the level of transcription or mRNA stability, because changes in GUS transcript level are followed by the same changes in GUS activity. It would be necessary to distinguish between a regulatory role of AtTOR 5′UTR on either RNA transcription or RNA stability. Nuclear run-on transcription assays can be done; either in transient or stable expression to determine if the 5′UTR affect the rate of transcription (**Kanazawa *et al.*, 2000; Meng and Lemaux, 2003**). In parallel, measurement of the amount of uncapped *TOR::GUS* mRNA versus capped mRNA will allow determining the mRNA stability. Increased level of uncapped *GUS* mRNA is an indicator of increased level their degradation and vice versa (**Jiao *et al.*, 2008**).

In addition, we found that external inducers like auxin or sucrose exert a positive effect on AtTOR expression. This effect appears linked to the presence of the 5′UTR of *AtTOR* mRNA in the case of auxin. To know more about the role of the 5′UTR on regulation of AtTOR expression the effect of others hormones and nutrients could be analyzed. Our results open up the possibility that the 5′UTR and the uORF sequence may acts as binding site for unknown activators of transcription (or mRNA stability factors). But, we think that the hypothesis that transcription factors binds to the 5′UTR is more probable than transcription factor binds to the uORF, particularly given the presence of CCA1 and ARR1 binding sites in the 5′UTR.

Greater insight into the molecular mechanisms of *AtTOR* 5′UTR/or uORF function and its relationship with other regulatory elements located in *AtTOR* promoter will be required to

understand how these regulatory elements work either individually or in combination to achieve the fine and accurate regulation of their gene expression.

5-Materials and Methods

5.1. Materials

5.1.1. Bacterial strains

The *Escherichia coli* strain used for all the constructions in this study is DH5α. The strain of *Agrobacterium* used for the transformation of *Arabidopsis* is C58C1 (pMP90) (**Koncz and Schell, 1986**) containing the pTiC58-based helper plasmid pMP90.

5.1.2. Plant material

Wild type *Arabidopsis thaliana* (L.) Heyn, ecotypes Columbia (Col-0) and Wassilewskija (WS) were used in this study. Wild type *Nicotiana benthemiana* plants were used for transient expression experiments. *Brassica rapa, Cucumis sativus, and Brachypodium distychon* plants were used for determination the 5′-untranslated region (5′UTR) sequence of *TOR* gene using RT-PCR.

5.1.3. Plant culture media

5.1.3.1. MS/2

MS/2 medium contains 10.35 mM NH_4NO3, 9.4 mM KNO_3, 0.75 mM $MgSO_4$, 1.5 mM $CaCl_2$, 0.6 mM KH_2PO_4, 100 µM $MnSO_4$, 100 µM H_3BO_3, 30 µM $ZnSO_4$, 5 µM KI, 1 µM Na_2MoO_4, 0.1 µM $CuSO_4$, 0.1 µM $CoCl_2$, 100 µM $FeSO_4$, 100 µM Na_2-EDTA, 0.3 µM Thiamine, 2.4 µM Pyridoxine, 4 µM Nicotinic acid, 550 µM Myo-Inositol (**Murashige and Skoog, 1962**).

5.1.3.2. Hoagland/2

Hoagland/2 medium contains 1 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 1.7 mM KNO_3, 0.5 mM $NH_4H_2PO_4$, 1.6 µM Fe, 46.2 µM H_3BO_3, 9.1 µM $MnCl_2$, 0.87 µM $ZnSO_4$, 0.32 µM $CuSO_4$, 1.03 µM $NaMoO_4$ (**Arnon and Hoagland, 1939**) and 2% sucrose.

5.1.4. Primers used for this study

A – Primers used to check *TOR* mRNA level in WS wildtype and *TOR/tor-1* mutant

TOR- intron-4583F1 5’ GCGCTGGTTTTGGAGAGCTA ‘3

TOR-intron-4229F2	5’ GCCACATTAGGACAAATGAGAT ‘3
GUS-80-R1	5’ ACAGTTTTCGCGATCCAGAC ‘3
TORseq5-cDNA-7070	5’ TTCCTGAAAAGGTTCCATTCCGCCT ‘3
TORseq3-cDNA-7421	5’ CCAAGCATATTTACAGCCTGAAGAA ‘3
Actin2-F	5’ GCACCCTGTTCTTCTTACCG ‘3
Actin2-R	5’ AACCCTCGTAGATTGGCACA ‘3

B – Primers used to check the presence of 2b gene in *TOR/tor-1* mutant

2b – F1	5’ ATGGAATTGAACGTAGGTGCAA ‘3
2b - R1	5’ TCAGAAAGCACCTTCCGCCCA ‘3
2b – F2	5’ GACCGAATGTCTCAGTCGTGTA ‘3
2b – R2	5’ CTGCTGGCGTAGATTCCTCTA ‘3

C – Primers used for identification of transcription start site of *AtTOR* 5′UTR

1 - Primers used to determine the length of 5′UTR of *AtTOR* using RT-PCR

5’UTR cDNA+621 -R0	5’ GGACATGAACGTTAAAGACAGTA’3
5’UTR cDNA+514-R1	5’ GCTGTTTTCATCTGAAACTCCA’3
5’ UTR cDNA+173-R2	5’ AGGAGCTCCCTCTTAGGATT ‘3
5’ UTR cDNA– 78-F1	5’ GCCCAAGTGTTGTTGAACCAA ‘3
5’ UTR cDNA– 148-F2	5’ GCGATCTTCGGTTCTCGACGCAT ‘3
5’ UTR cDNA – 245-F3	5’ GGTCTCTGAAAGAACAAAGCAA ‘3
5’ UTR cDNA– 412-F4	5’ GACCCTTTCAGGGTTTTAAGTA‘3
5’ UTR cDNA –519- F5	5’ GTCCAGTGAACAAATATGAGTTTT‘3

2 – Primers used for Amplification of the 5′end using 5′RACE kit (Invitrogen)

5’UTR- cDNA+ 621-R0	5’ GGACATGAACGTTAAAGACAGTA ’3
5’UTR cDNA+514-R1	5’ GCTGTTTTCATCTGAAACTCCA ‘3
5’UTR cDNA+173- R2	5’ AGGAGCTCCCTCTTAGGATT ’3

3 – Primer used to check the AV565959 cDNA sequence containing *AtTOR* 5′UTR from pBluscript II SK-

M13 vector – R	5’ GTCCTTTGTCGATACTG ‘3
5’ UTR cDNA – 245-F 3	5’ GGTCTCTGAAAGAACAAAGCAA ‘3
5’ UTR cDNA– 412-F 4	5’ GACCCTTTCAGGGTTTTAAGTA‘3

D - Primers used for the wild-type construct in pBI101 binary vector

1 - Primers for cloning TOR P + 5’-UTR+1stexon +1st intron+ begining of the 2nd exon of *AtTOR* gene (3165 bp) into pBI101 binary vector

SalI – F- primer	5’ CCGGTCGACGGAGTGGAAGAAGAAGGATAT’3

SmaI – R - primer 5’ CCGCCCGGGAGATCACGAACTGCTTCCTCTA’3

2 - Primers for sequencing of pBI101+TOR P+ 5’-UTR+ the first exon + the first intron of *AtTOR* into pBI101 binary vector using GENOME express company

TOR genome -2200-F1	5’ GCAGCGACGGTTGAGACGTTA’3
TOR genome +380-R1	5’ AGATCACGAACTGCTTCCTCTA’3
TOR genome -1690-F2	5’ TCGCCTGTCAATTTCCAGAT’3
TOR genome – 677-R2	5’ CGCAATCCCATAACGTGACAA ’3
TOR genome – 640-F3	5’ GGACCCTGTCTATTCACCAT ‘3
TOR genome – 210-R3	5’ CGCTGAGTGTCTTTTTAGCTT ‘3
GUS-80R1	5’ ACAGTTTTCGCGATCCAGAC ‘3
GUS-180R2	5’ AATATCTGCATCGGCGAACT ‘3

E - Primers for making mutated constructs in pBI101 binary vector

1 - Primers for PCR amplification of the fragment containing 1000 bp of *AtTOR* promotor + 5’UTR +1st exon +1st intron + begining of the 2nd exon of *AtTOR* and cloning in pGEM-T Easy vector using *Hpa*I and SmaI restriction enzymes (1750 bp)

HpaI 1 – 1200-F	5’ CCGACATTCTTGTCACGTTATG ‘3
SmaI + 540-R**1**	5’ TCGCGATCCAGACTGAATGC ‘3

2 – Primers used to make a point mutation in *AtTOR* uORF (ATG changed to TTG).

Mutation uORF–140-F 5’CTTCGGTTCTCGACGCATCTTGTGTTGAAGATTCTTCTA‘3
Mutation uORF–140-R 5’TAGAAGAATCTTCAACACAAGATGCGTCGAGAACCGAAG ‘3

3 - Primers for sequencing the mutated uORF cloned in pGEM-T Easy vector

HpaI 2 – 690-F	5’ CGATTACCATACAACTATTTTAC ‘3
UORF –246- F	5’ GGTCTCTGAAAGAACAAAGCA ‘3
UORF + 90-R	5’ CGGTGGCTGAGGGACCACAA ‘3

4 – Primers used for making the deletion of the 5’UTR

Del 5’UTR-3-F	5’ CCGAGATCTCGCGATGTCTACCTCGTCGCA ‘3
Del 5’UTR-231- R	5’ CCGAGATCTCCGTTCTTTCAGAGACCAATTATCT ‘3
Del uORF-R	5’ CCGAGATCTCCGATGCGTCGAGAACCGAAGA ‘3

F – Primers used for cloning the different constructs into pBI121 binary vector

1 - Primers for sequencing pBI121+ 35SP + GUS binary vector

PBI121-870- F2	5’ GTGAGTTAGCTCACTCATTAGG’3
PBI121-110-F1	5’ GACGTAAGGGATGACGCACA’3

GUS+80-R1 5’ ACAGTTTTCGCGATCCAGAC ‘3

2 - Primers for cloning three different constructs in pGEM-T Easy vector (800 bp)

pPI121-Xba 1-252- F 5’ CCGTCTAGAAGATAATTGGTCTCTGAAAGAAC ‘3
pBI121-Sma 1+440- R 5’ CCGCCCGGGAGATCACGAACTGCTTCCTCTA ‘3

G – Primers used for quantification of GUS mRNA by qRT-PCR

1- Primer used for making cDNA from mRNA

Poly-dT 5’ TTTTTTTTTTTTTTTTTTTTTTTT ‘3

2 - Primers for qRT-PCR to detect GUS mRNA level in stable transgenic plants

GUS +80-R 5’ ACAGTTTTCGCGATCCAGAC ‘3
TOR-cDNA+218-F1 5’ GTAATCCTAAGGAGGGAGCTC ‘3
PDF2-F 5’ TCACTCAATCCGTGAAGCTGCTG ‘3
PDF2-R 5’ GTTCTCCACAACCGCTTGGTCG ‘3

3 - Primers for qRT-PCR to detect GUS mRNA level in transient expression experiments

GUS +80-R 5’ ACAGTTTTCGCGATCCAGAC ‘3
TOR-cDNA+218-F1 5’ GTAATCCTAAGGAGGGAGCTC ‘3
NPt II – F 5’ GATGGATTGCACGCAGGTTCTC’3
NPt II – R 5’ CAGCCACGATAGCCGCGCTG ‘3
GUS – F60 5’ CAGTCTGGATCGCGAAAACTGTG ’3
GUS – R 200 5’ GACTTCGCGCTGATACCAGACG ‘3

H – Primers used for determine TOR 5’- UTR from different plant species by RT-PCR

1 - Primers used to amplify and sequence the BrTOR 5’UTR from *Brassica rapa*

Br cDNA- R0 5’ GACATGAACGTTAAAGACAGTAG ‘3
Br 5’UTR-R1 5’ GCAGTTTTCATCTGAAACTCCA ‘3
Br 5’UTR-F1 5’ CTCCTGCGATCTCCGCTTCT ‘3
Br 5’UTR-F2 5’ GTCAACAGTTATTTATTTACACTG ‘3

2 - Primers used to amplify and sequence CsTOR 5’UTR from *Cucumis sativus*

Cs cDNA –R0 5’ TCAATAACACGAAGGCAAGCACGC ‘3
Cs 5’UTR-R1 5’ AGCAATTTTTACCTGGTGTTCAAC ‘3
Cs 5’UTR-F1 5’ CGCGGACAAATTAACAAAGCCT ‘3
Cs 5’UTR-F2 5’ GAGGACGATTGTTGCAATTTTGA ‘3

3 - Primers used to amplify and sequence BdTOR 5'UTR from *Brachypodium distychon*

Bd cDNA –R0	5' ACTGTACACGCCACCGCGTC '3
Bd 5'UTR-R1	5' TCGGCCATCTCTTTGAGAATAAG '3
Bd 5'UTR-F1	5' GCCTTCCTGCGCTGCCTCTC '3
Bd 5'UTR-F2	5' CGCTCCGTCTCGACGATTCGA '3
Bd 5'UTR-F3	5' GCAGCCGGCAGGGTGTAGC '3
Bd 5'UTR-F4	5' CGCACCCTGCACAAGGCGCT '3
Bd 5'UTR-F5	5' CTCTCTCTCCTCCGCGACGT '3
Bd 5'UTR-F6	5' GCGACGCCGCATCCCAGCT '3

5.1.5. Vectors

A vector for constructing gene fusions was made preiviously by ligating the coding region of GUS (**Jefferson *et al.*, 1986**) 5' of the nopaline synthase polyadenylation site (**Bevan *et al.*, 1983a**) in the polylinker site of pBIN19 (**Bevan, 1984**). This vector, pBI101 (Figure 55), contains unique restriction sites for *HindI*, *Sall*, *XbaI*, *BamHI* and *SmaI* upstream of the AUG initiator-codon of GUS, to which promoter DNA fragments, can be conveniently ligated. The cauliflower mosaic virus (CaMV) 35S promoter (**Odell *et al.*, 1985**) described in the expression vector pROKi (**Baulcombe *et al.*, 1986**) was ligated into the Hindlll and BamHI sites to create pBI121.

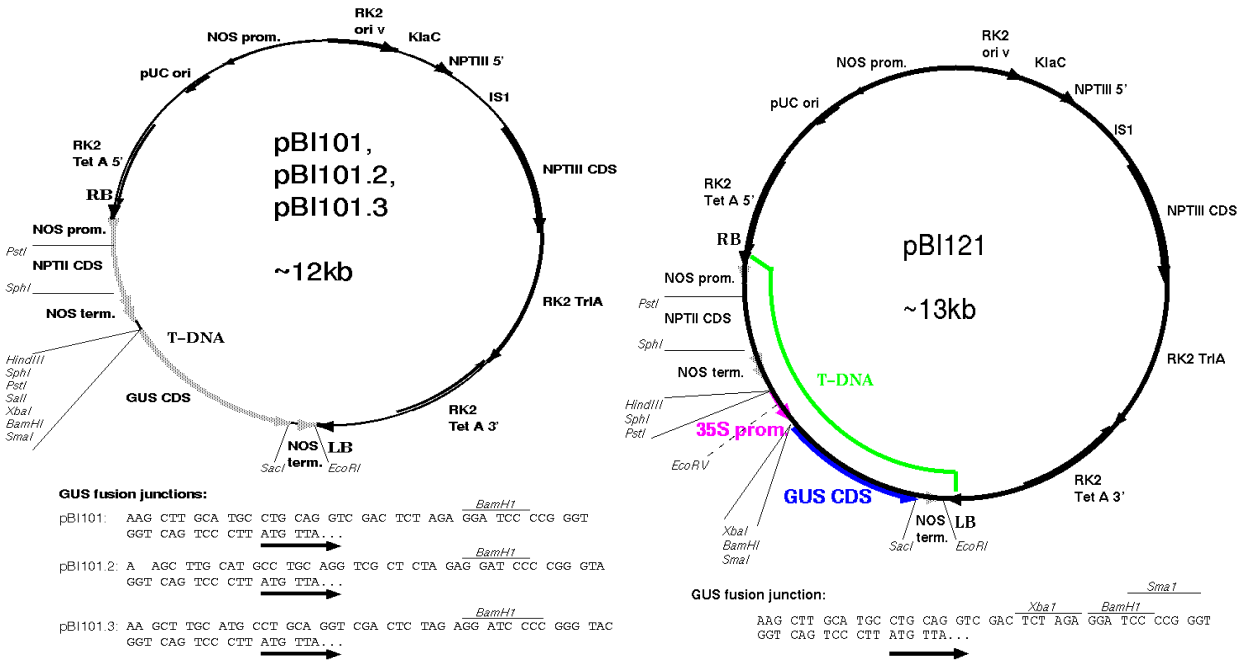


Figure 55: pBI101 and pBi121 binary vectors and GUS fusion site sequence showing the polylinker cloning sites

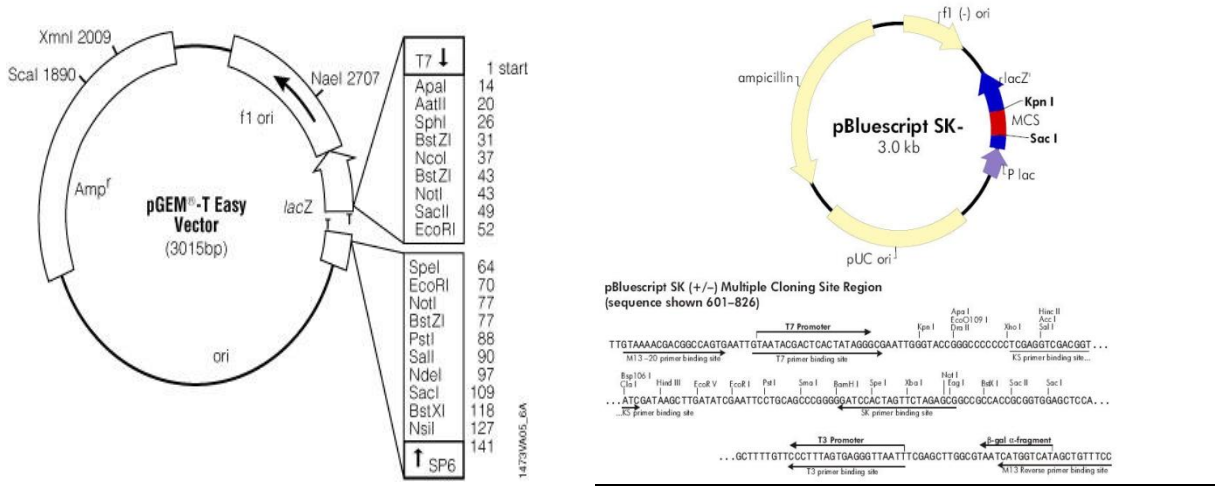


Figure 56: pGEM-TEasy vector and pBluescript SK- showing the polylinker cloning sites.

The pBI101 (Kan^R) binary vector was used for plant transformation. The pBI121 (Kan^R) binary vector under control of the CaMV 35S promoter was used in transient expression experiments in *Nicotiana benthemiana* leaves. The pGEM-T Easy vector (Amp^R) (Figure 56) was used for making different mutagenized constructs **[www.promega.com/citations].** pBluscript II SK- vector contains the Express sequence Tag (EST) sequence (AV565959) sequence.

5.2. Methods

5.2.1. Culture conditions

5.2.1.1. Bacterial culture

E.coli was grown in LB medium (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter water) supplemented with appropriate antibiotics at 37^{o}C. Agar was added to 1% (W/V) for solid medium. The concentrations of antibiotics used are: kanamycin 50 mg/ml, Ampicilline 100 mg/ml. *A. tumefaciens* was grown in LB medium supplemented with 25 mg/ml rifampicin, 25 mg/ml gentamycin, and 50 mg/ml kanamycin for 14 h at 28 ^{o}C.

5.2.1.2. Plant culture

5.2.1.2.1. *In vivo* growth conditions

All *in vivo* plant materials were grown in phytotron at 21 – 25 ^{o}C / 18 – 22 ^{o}C (day/night) with 14 h to 16 h light for long day and 8 hours for short day.

5.2.1.2.2. *In vitro* growth conditions

Seeds were surface sterilized using gas evaporation from hydrochloric acid (HCl) and Javel 12.5 for 6 – 10 hours. All in vitro plants were grown in circular Petri dishes with 25 ml Hoagland/2 medium supplemented with 1 % sucrose. For auxin signaling experiment, the plants were grown on base medium 7 days on a cellophane discs, and then transferred to the medium containing 10^{-7} M indole acetic acid IAA (a kind of Auxin). Seven days after transfer to auxin medium, the plants were stained with GUS solution and the images were taken with the microscope.

5.2.2. Generation and characterization of transgenic plants

5.2.2.1. Transformation of Arabidopsis plants using *Agrobacterium*

Floral dipping transformation was performed as described in **Clough and Bent (1998)**. *Arabidopsis* plants were grown to flowering stage in long day phytotron. To obtain more floral buds per plant, inflorescences were clipped after most plants had formed primary bolts, relieving apical dominance and encouraging synchronized emergence of multiple secondary bolts. Plants were dipped when most secondary inflorescences were about 1 – 10 cm tall (4 – 8 days after clipping).

Agrobacteria were grown to stationary phase in liquid culture at 28 ^{o}C, 200 rpm. Cultures were typically started from 1:100 dilutions of smaller overnight cultures and grown for roughly 18 – 24 hours. Cells were harvested by centrifugation for 10 min at 4 ^{o}C and 5500 g and then resuspended in infiltration medium to a final OD600 of approximately 0.8 prior to use. The floral dip infiltration medium contained 5.0 % sucrose and 0.05 % (i.e. 500 µl / L) Silwet L-77 (OSi Specialties, Inc., Danbury, CT, USA).

The plants were inverted into beaker containing infiltration medium, and all above-ground tissues were submerged in infiltration medium for 2 minutes. After 2 minutes, dipped plants were removed from the beaker, placed in a plastic bag to maintain humidity. Plants were left in plastic bag overnight and returned to the phytotron the next day. Plants were grown for a further 3-5

weeks until siliques were brown and dry, keeping the bolts from each pot together and separated from neighboring pots using tape. Seeds were harvested by gentle pulling of grouped inflorescences through fingers over a piece of clean paper. The majority of the stem and pod material was removed from the paper by gentle blowing and seeds were stored in microfuge tubes and kept at room temperature.

5.2.2.2. Selection of the transgenic plants

To select for kanamycin resistance transformed plants, sterilized seeds were placed on kanamycin selection plates at a density of approximately 2000 seeds par 12x12 cm petri dishes, and then grown for 7 – 10 days in a controlled environment at 24 ^{o}C with 16 hours light 50 – 100 mEinsteins m^{-2} s^{-1}. Selection plates contained MS/2 medium, 0.8% agar, 50 mg/ml kanamycin. Transformants were identified as kanamycin resistant seedlings that produced green leaves and well established roots within the selective medium. Some transformants were grown to maturity by transplanting, preferably after the development of 3 – 5 adult leaves, into heavily moistened potting soil.

5.2.3. Molecular biology

5.2.3.1. Identification of transcription start site of *AtTOR* gene by 5′- RACE

The 5′ RACE (**R**apid **A**mplification of 5′- **c**DNA **E**nd) was carried out with the GeneRacer kit (Invitrogen) following the manufacturer's instructions (Figure 57). Briefly, 2 µg of extracted total RNA was treated with calf intestinal phosphatase (CIP) in a total 20 µL reaction mixture containing 2 µL of 10× CIP buffer and 2 µL of CIP for 1 h at 50 ^{o}C. After extracting with phenol/ chloroform, RNA was resuspended in 6 µL of nuclease-free water. The 6 µL of CIP-treated RNA was treated with tobacco acid pyrophosphatase (TAP) in a 10 µL of reaction mixture containing 1 µL of 10× TAP buffer and 2 µL of TAP for 1 h at 37 ^{o}C. Again after extraction with phenol/chloroform, the CIP/TAP-treated RNA was resuspended in 6 µL of nuclease-free water and ligated to 250 ng of RNA adaptor by T4 RNA ligase in a 10 µL reaction mixture for 1 h at 37 ^{o}C. Then the CIP/TAP/ Ligated-RNA was extracted with phenol/chloroform and resuspended in 20 µL nuclease-free water and reserved at -70 ^{o}C. Another 6 µL non-treated RNA was ligated to 250 ng of RNA adaptor directly by T4 RNA ligase as a control.

Ten microliters of the ligated RNA or control RNA was used as a template to synthesize cDNA with AMV reverse transcriptase for 1 h at 42 °C. The outer reverse primer was used to prime the cDNA synthesis. The cDNA was then amplified by nested PCR with high fidelity system DNA polymerase. The PCR reaction mixture was incubated for 2 min at 94 °C followed by 30 amplification cycles, comprising denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. The reaction was extended for another 7 min at 72 °C to insure the full extension. PCR products were analyzed on 20 g/L agarose gel.

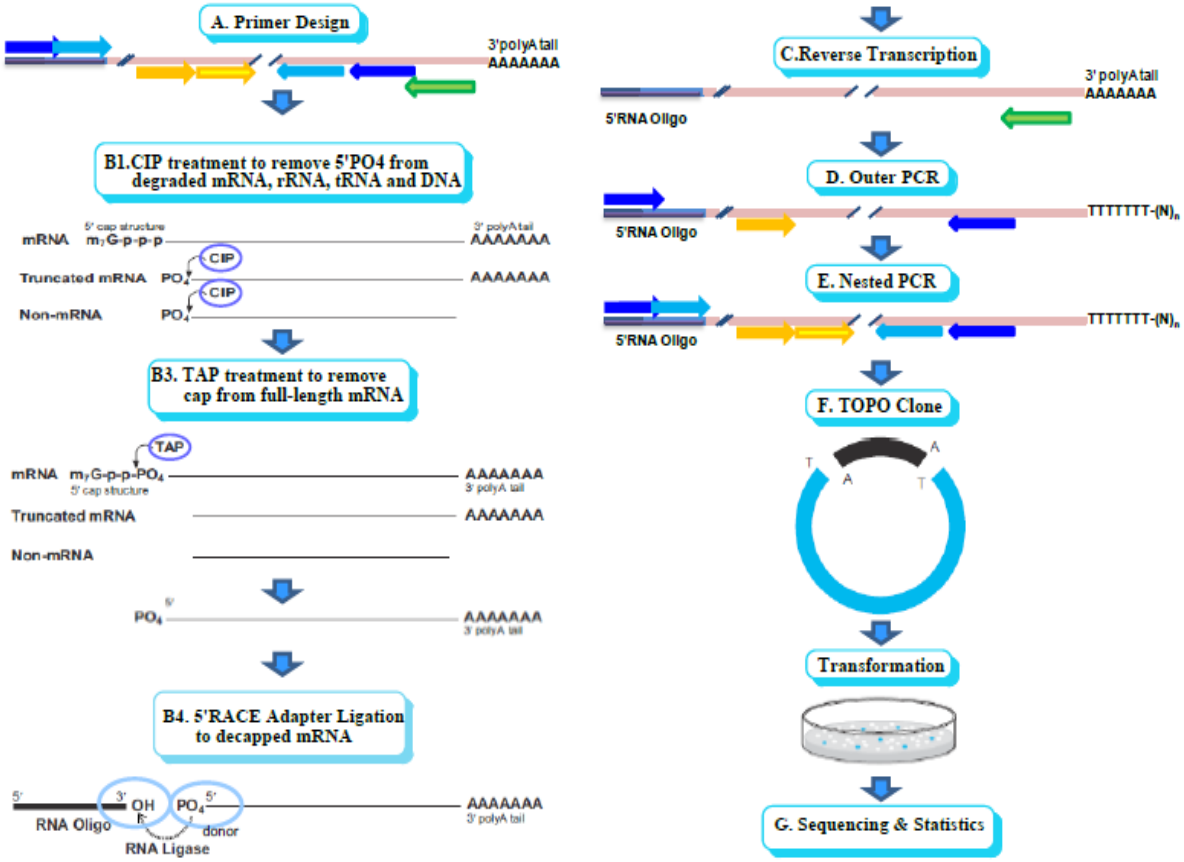


Figure 57: Steps diagram for 5'RACE protocol.

From http://www.natureprotocols.com/nucleic_acid_based_molecular_biology.

5.2.3.2. DNA cloning

5.2.3.2.1. DNA ligation and purification

Almost all DNA ligations were carried out with the classical methods. The reaction system of DNA ligation was set up as below:

Insert……..X µl

Vector…….....y µl

10x DNA ligation buffer……...1 µl

T4 DNA ligase (5u / µl)…… 1 µl

Distilled H_2O……...up to 10 µl

The molar ratio of insert / vector was adjusted to 3:1 or precisely ratio, then the ligation reaction was mixed and incubated at room temperature for 3 ~ 5 hours.

For *E.coli* transformation by $CaCl_2$ method, the ligation mixture can be applied directly without further purification. However, efficient transformation of *E.coli* by electroporation required purification of the ligation product. In this case, the ligated DNA was purified by Qiagen PCR purification kit and eluted in 20 µl of H_2O.

5.2.3.2.2. DNA transformation of bacteria (*E.coli*)

5.2.3.2.2.1. Preparation of competent cells of *E.coli*

Competent cells for transformation by electroporation (*E.coli* strain DH10B, from Invitrogen) were made by the following procedure.

We have started with 10 ml pre-culture in LB liquid medium. We made dilution for 1 volume of the pre-culture (2ml) to 100 volumes of fresh LB medium (200 ml) and then we leave the bacterial cells to grow at 37 ^oC on a shaker at 200 rpm. When cell growth reaches an O.D. at 600 nm of 0.5 – 1.0 (10^{10} cells / ml), it is chilled on ice immediately and it is centrifuged in four 50 ml tubes at 5000 rpm for 15 minutes at 4 ^oC and discard the supernatant. After that, the pellet is resuspended in 25 ml sterile cold H_2O in each tube and then, centrifugation is repeated at 5000 rpm for 15 minutes at 4 ^oC and the supernatant is discarded with two repeats. Finally, the pellet is resuspended in 400 µl sterile cold H_2O in each tube and all suspensions are collected together. Approximately, 10% of cold glycerol is added to each pellet and it is resuspended and mixed

gently but thoroughly. Aliquot 50 ~ 100 µl in each 1.5 ml sterile fresh Eppendorf tube is achieved, then it is freezed quickly in liquid nitrogen and keep at – 70 ^{o}C.

5.2.3.2.2.2. Transformation to *E.coli* by electroporation

An aliquot of competent cells are subjected to thaw on ice for 10 min. 1 to 5 µl of purified ligation reaction mixture are mixed with the competent cells by pipetting gently, and then it is loaded into a 2 mm pre-chilled electroporation cuvette, and kept on ice for 1 min. The pulse is applied to the cuvette on the electroporation unit 'Equibio Easyject' (Peqlab, Erlangen) with the pre-programmed setting: 2500 V (voltage), 25 µF (capacitance), 200 ohms (resistance), and 5msec (calculated pulse time). After that, the cuvette is transferred quickly to ice in less than 15 seconds, and then 1 ml of SOC medium is added to a 2 ml Eppendorf tube and incubated at 37 ^{o}C for 1 hour. Then, a proper volume of the culture is spreaded on LB plates with appropriate concentration of antibiotics (e.g. 50 µg/ml kanamycin). At the end, the plates are placed in a 37 ^{o}C incubator overnight (at least 18 hours).

5.2.3.3. Construction of plasmids for transformation of plants with GUS fusions.

Target of Rapamycin (*TOR*) promoter with the *AtTOR* 5'leader and the first intron fragment (3250 bp) was PCR amplified using the primer TORF1(5'-CCG**GTCGAC**GACTCTGATTCCGATT-CGGTT- '3; *SalI* site underlined) plus primer TORR1 (5'- CCG**CCCGGG**AACCTAGAGGAA-GCTTCAC-'3; *SmaI* site underlined). This amplified PCR fragment was fused upstream of the β-glucuronidase (GUS) reporter gene into *Sal1/Sma1* site of the binary vector pBI101 **(Jefferson *et al.*, 1987)** to produce TOR promoter + 5'UTR (Wt uORF) +1st intron :: GUS construct (WT construct). For making point mutation in the small upstream open reading frame (uORF) located in the 5'leader, and deletion of the all 5'leader, a PCR fragment (1860 bp) having the AtTOR 5' leader with the 1st intron as well as a part of the AtTOR promoter (possess *HpaI* and *SmaI* restriction sites) has been amplified using the *HpaI*-F primer (5'-CCGACATTCTTGTCA-CGTTATG-'3) and *SmaI*-R primer (5'-TCGCGATCCAGACTGAAT-GC -'3). The PCR product was purified using the Qiagen PCR purification kit (Qiagen, Hilden, Germany) and then 3'-A (dATP 10 µM) have been added to the PCR product to produce 3'-A overhangs in the presence of GoTaq DNA polymerase enzyme. The PCR product (TORP+5'UTR + 1st intron sequence) was cloned into pGEM-T Easy vector (Promega, Madison, WI). The PCR product was confirmed by DNA sequencing. Subcloning of

PCR fragment in pGEM – T Easy vector have been done in order to facilate the different manipulation at 5'leader and to make a new mutated constructs.

A site-directed mutagenesis for small uORF (**A*TGTGTTGA**) of the AtTOR 5'UTR fragment was produced by PCR. Mutagenic primers employed for small uORF were uORF-F1 (5'-CTTCGG-TTCTCGACGCATCT*TGTGTTGAAGATTCTTCTA-'3) and uORF-R1 (5'-TAGAAGAAT-CTTCAACACAA*GATGCGTCGAGAACCGAAG-'3); asterisks represent the mutated points). The produced mutated plasmid was confirmed by DNA sequencing. Then the 5'UTR with mutated uORF (**A**TG change to **T**TG) fragment was cut out from pGEM-T Easy vector with *HpaI* and *SmaI* restriction enzyme and ligated into the pBI101 binary vector, which was precut with the same enzymes and dephosphorylated. The resulting positive clones were double confirmed by PCR and DNA sequencing and named mut-uORF, representing the uORF start codon (ATG) has been changed to (TTG).

The third construct (pBI101-Δ5'UTR) was designed to study the effect of 5′ UTR on *AtTOR* gene expression. The 5′ UTR from the first construct (TORP-5′UTR) was deleted by PCR method (**Pérez-Pinera *et al.*, 2006**). The deleted sequence is replaced by the sequence of a restriction enzyme that is introduced as non-complementary, non-overlapping strands in the 5'end of each primer. Two primers were designed to amplify just all sequences within the pGEM-T Easy vector except the 5′ UTR (231 bp) part. The forward primer is called (Del 5′ UTR-3-F) 5′ CCG**AGATCT**CGCGATGTCTACCTCGTCGCA ′3 while, the reverse primer is called (Del 5′ UTR-231- R) 5′ CCG**AGATCT**CCGTTCTTTCAGAGACCAATTATCT ′3. *BglII* sequence (sequences at bold and underline) was introduced to pGEM-T vector within the 5'end of deletion primers for making a digestion profile to distinguish plasmids with deleted 5′UTR form other with full 5'UTR. Following PCR amplification, the plasmid is digested with *DpnI* to eliminate the template DNA. The PCR product is a linear DNA, thus the DNA fragment must be ligated using *T4 DNA* ligase to recirculirize the DNA fragment for use to transform *E.coli* cells. The new vector with deleted 5′UTR was confirmed by digestion with *BglII* restriction enzyme and the mutated PCR product was confirmed by DNA sequencing. Then, the sequence including the deleted 5′UTR region was cut out from pGEM T-Easy vector at *HpaI* and *SmaI* restriction sites and ligated into the pBI101 binary vector, which was precut with the same enzymes and dephosphorylated. The resulting positive clones were double confirmed by PCR and DNA sequencing and named TORP-Δ5′UTR construct, representing the 5′UTR deletion construct.

All the three above mentioned constructs (TORP-5′UTR, TORP-ΔuORF, and TORP-Δ5′UTR) were used for production of stable transgenic *Arabidopsis thaliana* plants, as well as to study the impact of 5'UTR and uORF of *AtTOR* gene on translational efficiency of GUS reporter gene in transient expression assay in tobacco (*Nicotiana benthemiana*) leaves. The same three constructs have been placed under the effect of the stronger CaMV 35S promoter and upstream of GUS reporter gene in pBI121 binary vector to study the role of 5'UTR and small uORF on translation of *GUS* reporter gene in transient expression assay in *Tobacco benthemiana* leaves under expression of stronger promoter. The names of 35S promoter constructs are 35SP-5′UTR, 35SP-ΔuORF, and 35SP-Δ5′UTR.

5.2.3.4. Agroinfiltration for transient expression in Nicotiana leaves

Transient protein expression in tobacco leaves was performed as described in the method of **Voinnet *et al.*, (2003)**. Individual colonies from *Agrobacterium tumefaciens* strain C58C1 carrying binary constructs were grown for 20 h in 5-ml culture at 29^{o} C in Luria-Bertani (LB) medium supplemented with 25 µg/ml rifampicin and 50 µg/ml kanamycin antibiotics. Bacterial cells were pelleted by centrifugation at 5000g for 10 min at room temperature and resuspended in infiltration medium 10 mM of 2-(N-morpholilino) ethanesulfonic acid (MES), pH 5.7/10 mM $MgCl_2$ /150 µM acetosyringone) to 0.5 OD at 600 nm, and incubated at room temperature for a minimum of 3 h (**Tai *et al.*, 1999**). The cell suspension was then used to infiltrate *Nicotiana* 3-week-old leaf tissue by pressing the syringe tip against the leaf underside and applying gentle pressure. The inoculated *Nicotiana* was then allowed to grow under containment glasshouse conditions. In all experiments, *Agrobacterium* C58C1 carrying the 35S::p19 construct (**Voinnet et al., 2003**) was cotransfected to achieve maximum level of protein expression.

5.2.3.5. DNA extraction

5.2.3.5.1. DNA plasmid extraction from *E.coli* and *Agrobacterium*

A single bacterial clone from *E.coli* growing on a solid LB plate is inoculated using a 10µl short pipette tip bacterial culture into 4ml LB liquid medium in a Falcon tube and then it is incubated on a shaker at 200 rpm, 37^{o}C overnight. After that, the bacteria are centrifuged at 14000 rpm for 2 minutes and then, the supernatant is discarded. Then, the plasmid DNA is extracted and purified as described in the manual of this Qiagen kit. Finally, the DNA from the QiaGene

column is eluted in 50 µl distilled H_2O and then, about 0.5 µl is analyzed by agarose gel electrophoresis. For plasmid in *Agrobacterium*, 6 ml of Agrobacteria culture were used for each sample.

5.2.3.5.2. Extraction of genomic DNA from *Arabidopsis* plants

Arabidopsis genome DNA was extracted from seedling (10 days old) growing in petri dishes. From 1 to 2 g plant tissue were freezed in liquid nitrogen (material not immediately used can be stored at – 80 ^{o}C). The plant tissue is grinded with mortar and pestle (in liquid nitrogen) to a fine powder. The fine powder is transfered to 50 ml centrifuge tubes containing 15 ml extraction buffer, (500 mM NaCl, 100 mM Tris-HCl pH 8.0, 50mM EDTA pH8.0, 70µl/100 ml mercaptoethanol). After that, about 1 ml SDS (20% W/V pH7.2) is added to extraction buffer, then, it is shaked and it is incubated at 65 ^{o}C for 10 minutes. About 5 ml 5M potassium acetate is added to the reaction and it is placed in ice for 20 – 30 minutes and centrifuged at 25,000 g for 20 minutes. The supernatant is poured through Miracloth (Calbiochem) filter into second tube containing 10 ml isopropanol, it is mixed together gently and then, it is placed in – 20 ^{o}C freezers for 20 – 30 minutes. It is centrifuged at 20,000 g for 20 minutes. The supernatant is removed and then the pellet is drained for few minutes. The pellet is resuspended in 0.7 ml 50 TE (50 mM Tris-HCl pH 8.0, 10 mM EDTA pH8.0), then it is transferred to eppendorf tube. About 20µl RNase (10mg/ml) is added to remove RNA and then it is incubated for 30 minutes at 37 ^{o}C. About 75 µl from sodium acetate (3 M) is added and it is centrifuged for 15 – 20 minutes. The supernatant is transferred carefully using a pipette into a clean eppendorf tube and 0.5 ml isopropanol is added. The eppendorf tube with isopropanol is leaved at room temperature for 5 minutes and centrifuged for 15 minutes. The supernatant is removed and the pellet is drained in air and resuspended in 200 µl TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH8.0).

5.2.3.5.3. DNA purification

5.2.3.5.3.1. Purification from agarose gels

DNA purification from agarose gels was done with the QiaGen gel extraction kit as described in the user protocol. In the last step, the DNA was typically eluted in 30 µl distilled water from the column, and 1µl was used for determination the DNA concentration.

5.2.3.5.3.2. Purification from other sources

DNA from other applications such as restriction enzyme digestion, DNA ligation, PCR amplification and rude preparation could be purified by the QiaGen PCR purification kit according to the user manual. About 0.5~1 µl was used for determination of the DNA concentration if necessary.

5.2.3.5.4. Determination of DNA concentration

A certain amount of DNA mixed with 6X DNA loading buffer was loaded in a 1% mini-agarose gel with 0.5 or 1 µl DNA standard (100 ng /µl 1kb DNA fragment, promega) as the reference. Then, it is run at moderate voltage for a proper time and it is seen under UV illumination.

5.2.3.6. RNA isolation

Total RNA was isolated from seedling (10 days of growth) *in vitro* using the TriReagen (Guanidine thiocyanate 0.8M, Ammonium thiocyanate 0.4 M, Sodium acetate pH 5 0.1 M, Glycerol 5%).

5.2.3.6.1. Homogenization and Phase separation

From 100 to 200 mg plant tissue is grounded in liquid nitrogen using morter with pestle to a fine powder, and then the fine powder is transfered to 1.5 ml eppendorf tube. About one ml TriReagent extraction buffer is added to powder and then, the homogenate is incubated for 5 minutes at 15 ~ 30 ^{o}C to permit the complete dissociation of nucleoprotein complexes. The homogenate is centrifuged at 12000 rpm for 10 minutes at 2~ 8^{o}C. The supernatant is transfered carefully into a sterilize 1.5 ml Eppendorf tube using a pipette and about 0.2 ml Chloroform per 1 ml TriReagent extraction buffer is added, and vortex strongly, then it is incubated at 15~ 30 ^{o}C for 10 minutes. The samples is centrifuged at 12000 rpm for 15 minutes at 2~ 8 ^{o}C. The mixture is separated into a lower red, phenol-chloroform phase, a turbid interface and a colorless upper aqueous phase after centrifugation. RNA remains exclusively in the aqueous phase.

5.2.3.6.2. RNA precipitation

The aqueous phase is transfered to a fresh 1.5 ml Eppendorf tube. To precipitate the RNA, the aqueous phase is mixed with 0.9 volumes of isopropyl alcohol thoroughly, then it is stored at 15~ 30 ^{o}C for 10 minutes and after that it is centrifuged at no more than 12000 rpm for 10 minutes at 2~ 8 ^{o}C.

5.2.3.6.3. RNA wash step

The supernatant is removed and then, the RNA pellet is washed once by adding 1 ml 75% (v/v) ethanol per 1 ml of TriReagent reagent used for the initial homogenization. Then, the RNA pellet is subjected to vortex and centrifuged at 7500 rpm for 5 minutes at 2~ 8 ^{o}C. **5.2.3.6.4. RNA suspention**

In the end of this procedure, the RNA pellet is subjected to dry in air for 5 ~ 10 minutes. Then, the RNA pellet is dissolved in 20 ~ 30 µl RNase-free H_2O and stored at -70 ^{o}C.

5.2.3.7. Quantitative Real - Time PCR (QRT-PCR)

For quantitative PCR, 4µg of total RNA treated with DNase (Promega) were used for reverse transcription with AMV (Avian Myeloblastosis Virus) reverse transcriptase according to the manifacturer's protocol. Quantitative real-time PCR (Q-RT–PCR) was carried out using the ABI PRISM 7000 and Bio-Rad iCycler and the SYBR Green I kit (ABI) according to the manufacturer's protocol. Specific primers for each gene selected were designed from cDNA sequence using primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). For each gene amplified, a standard curve was generated from duplicate series of 3 template dilutions to test PCR efficiencies. For quantification, the cDNA was amplified from the different pool of mRNA. Each PCR was realized three times in the presence of 1 ng cDNA, 0.4 µM of each primer, 0.4 µl of ROX, 10 µl Syber GREEN master mix, and distilled water to a final volume of 25 µl. The PCR conditions were 10 min at 95°C (to activate the hot-start recombination Taq DNA polymerase) followed by 45 cycles at 95°C for 10 sec, 64°C for 5 sec and 72°C for 34 sec. The results were standardized by comparing the data with the *PDF2* reference gene encoding protein phosphatase 2A [PP2A] (**Czechowski *et al.*, 2005**). The quantification of gene expression was performed using the comparative CT method.

5.2.4. Biochemical analysis

5.2.4.1. GUS assay

5.2.4.1.1. Histochemical assay (qualitative assay)

As described by **Jefferson et al. (1987)**, the colorless substrate X-Gluc (5-Brom-4-chlor-3-Indolyl β-D-glucuronide) can be converted into 5-Brom-4-chlor-3-Indolyl and glucuronide by the catalysis of the enzyme β-Glucuronidase (GUS). Dimers of 5-Brom-4-chlor-3-Indolyl are

subsequently formed as blue 5, 5'-Dibrom-4, 4'-Dichlor-Indolyl by oxidation (Figure 58). Tissues such as leaves of transgenic plants can be directly immersed into the GUS staining solution (X-Gluc) for whole mount staining reaction at 37°C.

Arabidopsis seedlings were submerged into 1.5 ml Eppendorf tube containing 0.5 ~ 0.8 ml X-gluc staining solution. Then, the Eppendorf tubes were incubated at 37°C for 6 ~ 10 hours. Then the tissue is removed by discarding the fixation buffer and incubating in 70% or 80% ethanol at RT overnight with alterations of the ethanol for 3 times.

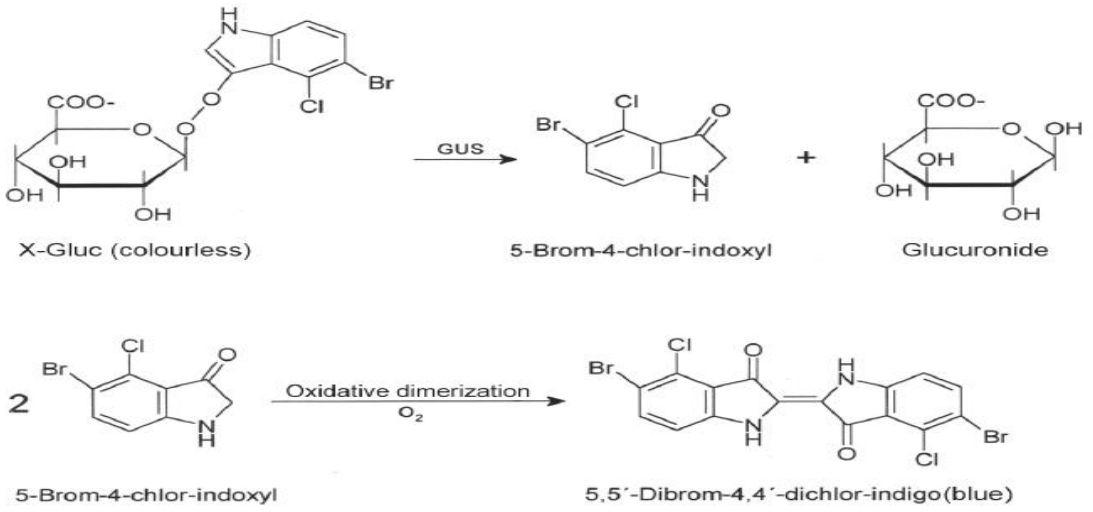


Figure 58: Chemical conservation of x-Gluc substrate (colourless) to 5,5′-Dibrom-4,4′-dichlor-indigo (blue) activated with GUS enzyme .

5.2.4.1.2. Fluorimetric assay (quantitative assay)

Like X-Gluc, the substrate 4-MUG (4-Methyl-umbelliferyl-β-D-glucuronide) can be converted into 4-MU (4-methyl-umbelliferon or 7-hydroxy-4-methyl-cumarin) and glucuronide by the catalysis of GUS. Fluorescence of 4-MU can be obtained at a wavelength of 365 nm for excitation, 445 nm for emission, and measured in a fluorimeter.

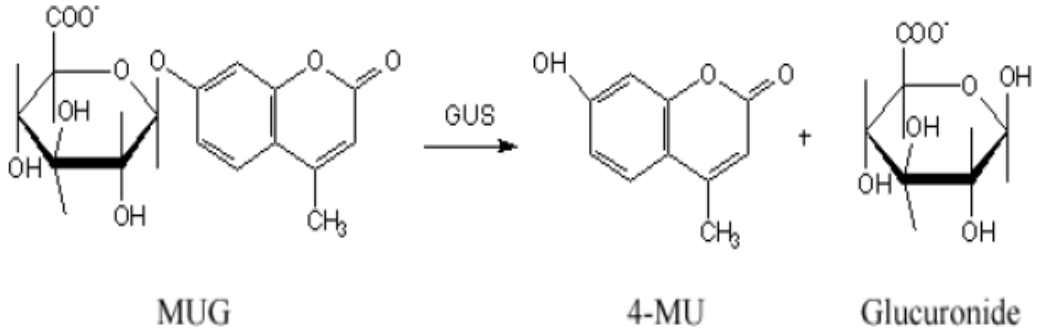


Figure 59: Reduction of MUG substrate of GUS enzyme to 4-MU and glucuronide.

5.2.4.1.2.1. Solutions

- GUS extraction buffer:

Phosphate buffer (Na_2HPO_4/NaH_2PO_4), pH 7.0……50 mM

Na_2 - ethylenediamineteteracetic acid (EDTA)……..10 mM

Triton X-100……...0.1% (v/v)

Sterilizes it and then it can store at 4°C.

Before directly use, B-Mercaptoethanol (70 µl/100 ml buffer) will add ...…..10 mM

- Fluorescence assay buffer:

4-MUG (4-methyl-umbelliferyl-β-D-glucuronide)……...1 mM

About 3.523 mg from 4-MUG will dissolve into 10 ml GUS extraction buffer and mix thoroughly to form a concentration of 1 mM, then, it will keep on ice.

- Stop buffer:

Na_2CO_3……0.2 M, autoclaved

5.2.4.1.2.2. Preparation of GUS extract from plant tissue

Two ml Eppendorf tube (pre-coled on ice) are filled with 200 µl GUS extraction buffer. About from 50 to 100 mg plant tissue is homogenized in extraction buffer using a Diller. Then, the homogenate is centrifuged at 15000 rpm for 3 minutes. The supernatant is transfered to a new Eppendorf tube and placed on ice. Additional 200 µl GUS extraction buffer is added to the sample and mixed vigorously for 2 minutes, and then it is centrifuged again. The two parts of supernatant for the same sample are

combined and is centrifuged at 15000 rpm for 5 minutes, and the centrifugation is transferred to a new tube. The sample is kept on ice for protein extraction. GUS extracts can be kept at -70°C for a long time or on ice for 4°C for several days.

5.2.4.1.2.3. Determination of protein concentrations by the Bradford method

The protein concentration of the GUS extract is measured by the Bradford method (Bradford 1976).

Bradford solution:

Coomassie Brilliant Blue G (Sigma)……100 mg

95-100% ethanol……50 ml

85% phosphate acid (H_3PO_4)……100 ml

Distilled water……to 1000 ml

Then, it is Kept at 4^oC in a brown glass container.

One ml of Bradford solution is added to X µl of GUS extracts into the cuvette and mix thoroughly. Then, the reaction is left to incubate in the dark for 15 minutes and then the absorption of the samples is measured at a wavelength of 595 nm. To calibrate the concentration of the samples, we have used a set of BSA dilutions at as 0, 1.0, 2.0, 4.0, 6.0, 10.0, and 20.0 µg to make the standard curve.

5.2.4.1.2.4. GUS measurement

X µl GUS extract is mixed with (450 – X) µl GUS assay buffer thoroughly and then, it is incubated at 37 ^oC, 100 µl is take rapidly into 900 µl stop buffer and mixed well. The fluorescence value is measured by the Fluorescence Spectrophotometer (CARY-VARIAN).

5.2.4.1.2.5. Evaluation of GUS activity

The GUS activity in pmole 4-MU per minute and µg protein is calculated by the following equation:

GUS activity (pmole 4-MU/min/µg protein) =

$$\frac{\text{Conc (4-MU in stopped sample) [nmol/L X dilution factor X volume (assay mix) [µl]}}{\text{Vol (extract in assay mix) [µl] X incubation time [min] X conc. (protein in extract) [µg/ml]}}$$

Where:

Conc (4-MU): the concentration of 4-MU of the stopped sample in cuvette;

Dilution factor: the ratio of the volume (stop buffer + assay mixture) to the volume of assay mixture;

Vol (assay mix): the total volume of assay mixture;

Vol (extract in assay mix): the volume of GUS extract used for assay;

Incubation time: the time durian of the assay;

Conc (Protein in extract): the concentration of total protein in the GUS extract.

5.2.4.1.2.6. Evaluation of GUS translational efficiency

Translational efficiency (protein/mRNA) is calculated by the following formula:

$$\text{Translational efficiency} = \frac{\text{the amount of GUS activity}}{\text{the amount of GUS mRNA}}$$

5.2.5. Staining and binocular optics

GUS staining was performed as described in chapter 4.1.1 with 8 hours incubation at 37 °C. Observation was performed with a ZEISS stereomicroscope (SteREO – Discovery. V12) for seedlings.

5.2.6. Data Retrieval and Data Handling

5.2.6.1 Statistical Analysis

Where applicable, significance of results was tested using student's t-test. Results were treated as significant if $p < 0.05$. In graphs error bars indicate ± one standard error (SE) from the mean. Standard error is calculated as the standard deviation divided by the root of the total number of measurements.

5.2.6.2 Sequence Analysis

1 – The software BLAST from NCBI (National Center for Biotechnology Information) was used for simple alignment between TOR amino acid sequence from *Arabidopsis thaliana* and TOR amino acid sequence from *Brassica rapa and other plant species*. It is available at the internet under the address: http://www.ncbi.nlm.nih.gov:80/blast/Blast.cgi (**Altschul *et al*., 1990**).

2 – For the multiple alignments, the software clustalw was used (hht://www.ebi.ac.uk/Tools/clustalw).

3 – The database TAIR (The Arabidopsis Information Resource, http://www.arabidopsis.org/) was used to search Arabidopsis genome sequences. For other plants, the database TIGR (The Institute for Genomic Research, http://tigrblast.tigr.org/tgi/) was used.

4– We used a data Pases of Plant Cis-acting Regulatory DNA Elements (PLACE) to identify the putative sequences within the Arabidopsis TOR (AtTOR, At1G50030)) promoter (http://www.dna.affrc.go.jp/PLACE/signalscan.html).

5 – DIURNAL web tool to search and visualize diurnal and circadian gene expression profiles for Arabidopsis TOR (AtTOR) http:://www.diurnal.cgrb.oregonstate.edu/.

6 - We used Phytozome v.6 to select the TOR genomic, TOR cDNA, and TOR protein sequences for 11 different plant species. For more details about the method used for construction of gene family and distribution of a given node, you can visit the Phytozome site (http://www.pytozome.net/).

7 – All the other manipulations of DNA sequences including alignment of the cDNA sequences with genomic DNA, making restriction maps to determine the sites of restriction enzymes for cloning, and many other functions was achieved using the Serial Cloner 1.3-11 software and DNA strider™ 1.3 developed by CEA Saclay.

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