

To the Graduate Council:

I am submitting herewith a dissertation written by **Bijoyita Roy** entitled "**Translational Regulation in *Arabidopsis thaliana*: Genetic and Functional Characterization of Eukaryotic Initiation Factor 3**". I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Botany.

Albrecht G. von Arnim, Major Professor

---

We have read this dissertation  
and recommend its acceptance:

Barry D. Bruce

---

Bruce D. McKee

---

David A. Brian

---

Accepted for the Council:

Carolyn R. Hodges

---

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records)

**Translational Regulation in *Arabidopsis thaliana*:**  
**Genetic and Functional Characterization of**  
**Eukaryotic Initiation Factor 3**

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Bijoyita Roy

August 2010

Copyright © 2010 by Bijoyita Roy  
All rights reserved.

## Acknowledgements

This thesis owes itself to a number of people and I would like to begin by acknowledging my advisor, Dr. Albrecht von Arnim. Foremost, I thank him for giving me the opportunity to work in his lab in a very interesting project and for being extremely patient and supportive through these years. I am indebted to him for pampering me with absolute freedom on my project that I realize not many graduate students enjoy. I would like to thank him for training me to be an independent researcher. From him, I have not just learnt how to do experiments, but also lessons of critical analysis, balanced approach to science, attention to details and organization. Finally, I will always be grateful to him for introducing me to this field. For my new endeavors, I take with me the experiences that came with working in his lab that have enriched me in many ways.

I would like to thank the amazing faculty members who were part of my advisory committee and were never short of encouragement, Drs. David Brian, Barry Bruce and Bruce McKee. Their patience, interest, and generosity with time and advice are gratefully acknowledged. Thanks are due to Dr. Andreas Nebenführ for his suggestions, advice on work and life in general, and for being a great critic. The efforts and the concerns are greatly appreciated. I have been fortunate to have worked with a bunch of wonderful colleagues, past and present. I would like to thank all of them for sharing knowledge and good company. Thanks are due to Byung-Hoon Kim, Chitra Subramanian and JongChan Woo for their help during the initial days, Fujun Zhou for his friendship and humor, Lindsay Staron for checking back even after leaving the lab, and, Xiuhua Han for her warmth. I would also like to acknowledge the contributions Justin Vaughn made on the eIF3h project that we collaborated on.

On the personal front, the list of people whom I must thank is considerable. I am thankful to my high school biology teacher, Dr. Tanuja Samal, for being a captivating teacher. She

introduced me to the subject that fascinates me even now as much as it did all those years. Most importantly, for showing how science is a way of life and not just about experiments. This journey would have been dull and uneventful if it were not for my friends, both old and new, friends back home in India for their unflagging support and also their concerns. Debasri, Banishree, Pragya, thank you for being such wonderful people. I made a bunch of friends here at UT, Eunsook, Sukanya, Non, Fujun were a great support; Gai, for his constant supply of great food, sometimes at wee hours. Looking back, it was so much fun at times; the midnight walks to the riverside and the pep talks lighted up many days and nights.

Words seem so inadequate to express my feelings for my family; I am particularly thankful to my grandmother, for being an exceptional role model in so many ways. She amazes me more and more everyday and I would like to dedicate this dissertation to her. I am indebted to my parents for having given me the freedom to do whatever I wanted and their unflagging support and confidence in whatever I have chosen to do. I am most fortunate that I have a sister who has also been my closest friend and confidante ever since I can remember. My heart-felt appreciation goes to K-man who has never been short of encouragement and more than anything, his unfaltering confidence in me.

Finally, I am grateful for the many other things that have enriched my life during the course of this degree and the time spent in Knoxville. My volatile hobbies have ensured that the journey has been as interesting and valuable as the end.

## Abstract

Molecular functions of eukaryotic initiation factor 3 (eIF3) in translation are manifold, encompassing events from initiation complex assembly to translation termination. The contribution of the individual subunits of eIF3 to its multiple activities is quite unclear. It has been hypothesized that several of its 13 subunits contribute to mRNA specific regulation. Prior research had established that the h subunit of eIF3 in *Arabidopsis* was required for translation of specific mRNAs as well as for organ formation and meristem development. This study aims towards understanding the functions of individual subunits of eIF3 in the context of plant development and to further define the role of eIF3h at the molecular level.

This dissertation describes an effort to identify mutations affecting each of the 13 eIF3 subunits. Using a panel of pollen-specific fluorescent marker genes, eIF3 subunits e, h and i1 were demonstrated to be essential for normal male gametophyte development. Furthermore, subunits b and c proved to be essential for embryo development. In contrast, a mutation in eIF3k revealed no phenotypic abnormalities. This work represents a systematic effort to attribute functions to many of the eIF3 subunits in growth and development in a multicellular eukaryote.

The h subunit of eIF3 is necessary for the efficient translation of specific mRNAs in *Arabidopsis*. In particular, eIF3h fosters the translation of those mRNAs that harbor multiple upstream open reading frames (uORFs) in their 5' leader. The specific molecular activity of eIF3h was investigated by structure-function analysis of the 5' leader of the *Arabidopsis* AtbZip11 mRNA, which harbors a set of four uORFs that is evolutionarily conserved. By pairing extensive mutagenesis of the AtbZip11 5' leader with gene expression analysis in *Arabidopsis* seedlings, it was revealed that eIF3h helps the ribosome to retain its reinitiation competence during uORF translation. These data establish a function for the h subunit of eIF3 in a special case of translation initiation, reinitiation.

Finally, the molecular events during translation reinitiation were investigated further for a functional cooperation between eIF3h and the large subunit of the ribosome, given that the large ribosomal subunit had been implicated in reinitiation in other biological contexts. Reinitiation profiling using the AtbZip11 leader demonstrated that a protein of the large ribosomal subunit, RPL24B, bolsters reinitiation similar to eIF3h. Taken together, there exists a functional cooperation between the large ribosomal subunit and eIF3 that helps ribosomes to reinitiate after translation of uORFs.

# Table of Contents

|                                                                                                             |           |
|-------------------------------------------------------------------------------------------------------------|-----------|
| <b>Chapter 1</b>                                                                                            | <b>1</b>  |
| <b>Introduction</b>                                                                                         | <b>1</b>  |
| I. Translational regulation of gene expression                                                              | 2         |
| I.1 Targets and mechanisms of translational regulation                                                      | 3         |
| II.1 Cap mediated canonical scanning mode of translation initiation                                         | 6         |
| II.2 Cap independent translation initiation                                                                 | 11        |
| II.3 Special cases of translation initiation – reinitiation and shunting                                    | 11        |
| II.4 Controls during translation initiation                                                                 | 14        |
| III. eIF3 (eukaryotic initiation factor 3)                                                                  | 16        |
| III.1 Structure and composition of eIF3                                                                     | 16        |
| III.2 Functions of eIF3 in translation initiation                                                           | 19        |
| III.3 eIF3 in later stages of translation                                                                   | 21        |
| III.3 eIF3, functions beyond the translation apparatus                                                      | 24        |
| IV. Upstream open reading frames (uORFs)                                                                    | 26        |
| IV.1 Effect of uORF on translation – uORFs as translation barriers                                          | 30        |
| IV.2 Sequence dependent uORFs                                                                               | 33        |
| IV.3 Effect of uORFs on mRNA stability and structure                                                        | 34        |
| IV.4 uORF function in response to stress and metabolite signaling                                           | 35        |
| IV.5 Translational control of development by uORFs                                                          | 38        |
| V. Aims of the dissertation                                                                                 | 42        |
| <b>Chapter 2</b>                                                                                            | <b>44</b> |
| <b>eIF3 in Arabidopsis – Reverse genetic approach to characterize functions of individual eIF3 subunits</b> | <b>44</b> |

|                                                                                                                                           |           |
|-------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| II.1 Summary.....                                                                                                                         | 45        |
| II.2 Introduction .....                                                                                                                   | 46        |
| II.2.1 eIF3 in Arabidopsis.....                                                                                                           | 46        |
| II.3 Results .....                                                                                                                        | 50        |
| II.3.1 Generation of eIF3 mutant strains by T-DNA insertion screening .....                                                               | 50        |
| II.3.2 RNAi for eIF3 .....                                                                                                                | 71        |
| II.4 Discussion .....                                                                                                                     | 74        |
| II.5 Materials and methods.....                                                                                                           | 78        |
| II.5.1 Assembly of T-DNA insertion mutants .....                                                                                          | 78        |
| II.5.2 PCR genotyping .....                                                                                                               | 78        |
| II.5.3 RNAi lines .....                                                                                                                   | 78        |
| II.5.4 RNA analysis .....                                                                                                                 | 79        |
| II.5.5 Polysome analysis .....                                                                                                            | 79        |
| <b>Chapter 3.....</b>                                                                                                                     | <b>86</b> |
| <b>The h subunit of eIF3 promotes reinitiation competence during translation of<br/>mRNAs harboring upstream open reading frames.....</b> | <b>86</b> |
| III.1 Abstract .....                                                                                                                      | 87        |
| III.2 Introduction .....                                                                                                                  | 88        |
| III.3 Results .....                                                                                                                       | 90        |
| III.3.1 Mutant eIF3h protein does not associate tightly with 43S complexes.....                                                           | 90        |
| III.3.2 eIF3h supports translation of the uORF-containing AtbZip11 mRNA .....                                                             | 92        |
| III.3.3 Identification of inhibitory uORFs in the AtbZip11 leader .....                                                                   | 96        |
| III.3.4 Function of eIF3h in uAUG recognition .....                                                                                       | 96        |
| III.3.5 eIF3h and the AtbZip11 attenuator peptide .....                                                                                   | 104       |
| III.3.6 eIF3h controls the efficiency of reinitiation events .....                                                                        | 104       |

|                                                                                                                              |            |
|------------------------------------------------------------------------------------------------------------------------------|------------|
| III.3.7 eIF3h supports the resumption of scanning, not tRNA acquisition .....                                                | 109        |
| III.4 Discussion .....                                                                                                       | 113        |
| III.4.1 The AtbZip11 leader as a model system for assigning roles to translation factors .....                               | 114        |
| III.4.2 Transit routes of ribosomes on the AtbZip11 leader .....                                                             | 114        |
| III.4.3 The H subunit of eIF3 is a reinitiation factor .....                                                                 | 115        |
| III.5 Materials and methods .....                                                                                            | 118        |
| III.5.1 Molecular cloning and generation of AtbZip11 5' leader mutants.....                                                  | 118        |
| III.5.2 Transient expression and translation assay.....                                                                      | 118        |
| III.5.3 RNA expression analysis .....                                                                                        | 120        |
| III.5.4 Polysomal protein isolation and eIF3 association .....                                                               | 120        |
| <b>Chapter 4.....</b>                                                                                                        | <b>123</b> |
| <b>Efficient reinitiation after uORF translation requires cooperation between eIF3 and the large ribosomal subunit .....</b> | <b>123</b> |
| IV.1 Abstract.....                                                                                                           | 124        |
| IV.2 Introduction .....                                                                                                      | 125        |
| IV.3 Results .....                                                                                                           | 127        |
| IV.3.1 Requirement of eIF3h for translation of AtbZip11 mRNA is specific.....                                                | 127        |
| IV.3.2 RPL24B is required for efficient translation of AtbZip11 leader .....                                                 | 130        |
| IV.3.3 RPL24B is required for efficient reinitiation after uORF translation .....                                            | 133        |
| IV.3.4 Molecular function of RPL24B in reinitiation.....                                                                     | 133        |
| IV.4 Discussion .....                                                                                                        | 137        |
| IV.4.2 Cognate partners of eIF3 and RPL24 .....                                                                              | 142        |
| IV.5 Materials and methods.....                                                                                              | 143        |
| IV.5.1 Transient expression and translation assay .....                                                                      | 143        |

|                                                                                                                                                    |            |
|----------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| IV.5.2 RNA expression analysis.....                                                                                                                | 143        |
| <b>Chapter 5.....</b>                                                                                                                              | <b>145</b> |
| <b>Use of fluorescent-tagged lines to study male gametophyte developmental defects in Arabidopsis translation factor mutants.....</b>              | <b>145</b> |
| V.1 Abstract .....                                                                                                                                 | 146        |
| V.2 Introduction .....                                                                                                                             | 147        |
| V.2.2 Gene expression in male gametophyte and molecular events controlling male gametophyte development.....                                       | 149        |
| V.3 Results .....                                                                                                                                  | 151        |
| V.3.1 Mutation in eIF3e results in reduced transmission through the female gametophyte and loss of transmission through the male gametophyte ..... | 151        |
| V.3.2 Phenotypic characterization of male gametophytic developmental defects in eIF3 mutants .....                                                 | 154        |
| V.3.3 <i>eif3e</i> and <i>eif3h</i> pollen grains develop normally .....                                                                           | 156        |
| V.3.4 eIF3e and eIF3h mutations affect pollen germination .....                                                                                    | 156        |
| V.3.5 Pollen tube elongation is reduced in <i>eif3h</i> but not in <i>eif3e</i> .....                                                              | 158        |
| V.4 Discussion .....                                                                                                                               | 165        |
| V.4.1 Translational regulation of pollen germination and pollen tube elongation.....                                                               | 165        |
| V.4.2 eIF3 in pollen development.....                                                                                                              | 166        |
| V.5 Materials and Methods .....                                                                                                                    | 168        |
| V.5.1 Mutant isolation and growth conditions.....                                                                                                  | 168        |
| V.5.2 Genetic analysis and generation of <i>eif3x</i> +/- FTL +/-.....                                                                             | 168        |
| V.5.3 Alexander's viability staining .....                                                                                                         | 169        |
| V.5.4 DAPI staining.....                                                                                                                           | 169        |
| V.5.5 Fluorescein diacetate staining .....                                                                                                         | 170        |

|                                                                           |            |
|---------------------------------------------------------------------------|------------|
| V.5.6 <i>In vitro</i> pollen germination assay.....                       | 170        |
| <b>Chapter 6.....</b>                                                     | <b>174</b> |
| <b>Perspectives and future directions.....</b>                            | <b>174</b> |
| VI.1 Molecular functions of eIF3 in translation.....                      | 175        |
| VI.1.1 How does eIF3h foster reinitiation?.....                           | 176        |
| VI.1.2 What other factors promote reinitiation together with eIF3h? ..... | 180        |
| VI.2 Significance of eIF3 in Arabidopsis development .....                | 183        |
| <b>References.....</b>                                                    | <b>185</b> |
| <b>Vita.....</b>                                                          | <b>213</b> |

## List of Tables

| Table              |                                                                                      | Page |
|--------------------|--------------------------------------------------------------------------------------|------|
| <b>Table I.1</b>   | Interacting partners of eIF3                                                         | 26   |
| <b>Table I.2</b>   | Experimentally confirmed uORFs in <i>Arabidopsis</i>                                 | 39   |
| <b>Table II.1</b>  | Comparison of eIF3 subunit composition across different species                      | 47   |
| <b>Table II.2</b>  | Gametophytic transmission of <i>eif3b1</i>                                           | 54   |
| <b>Table II.3</b>  | Embryo lethality in <i>eif3b1</i> and <i>eif3c1</i>                                  | 55   |
| <b>Table II.4</b>  | Gametophytic transmission of <i>eif3c1</i>                                           | 60   |
| <b>Table II.5</b>  | Genetic analysis of <i>eif3i-1</i>                                                   | 67   |
| <b>Table II.6A</b> | Delivery clones for RNAi from AGRIKOLA                                               | 73   |
| <b>Table II.6B</b> | Oligonucleotides to confirm the gene specific tag in RNAi destination<br>vectors     | 73   |
| <b>Table II.7</b>  | Available confirmed T-DNA insertion lines for eIF3                                   | 75   |
| <b>Table V.1</b>   | Gametophytic transmission of <i>eif3e-1</i>                                          | 153  |
| <b>Table V.2</b>   | Germination efficiency of <i>eif3e</i> pollen grains in the <i>qrt+/-</i> background | 163  |
| <b>Table V.3</b>   | Germination efficiency of <i>eif3h</i> pollen grains in the <i>qrt+/-</i> background | 164  |

## List of Figures

| Figure              |                                                                                      | Page |
|---------------------|--------------------------------------------------------------------------------------|------|
| <b>Figure I.1</b>   | <i>Cis</i> -regulatory elements in the mRNA                                          | 4    |
| <b>Figure I.2</b>   | Eukaryotic cap-mediated translation initiation                                       | 8    |
| <b>Figure I.3</b>   | Possible events leading to gaining of reinitiation competence after uORF termination | 13   |
| <b>Figure I.4</b>   | eIF3 structure                                                                       | 18   |
| <b>Figure I.5</b>   | Upstream open reading frame in 5' leader of mRNAs                                    | 29   |
| <b>Figure I.6</b>   | uORF as translation barrier                                                          | 32   |
| <b>Figure I.7</b>   | Translational regulation of the GCN4 mRNA in yeast                                   | 36   |
| <b>Figure I.8</b>   | Translational regulation of the ATF4 mRNA                                            | 37   |
| <b>Figure II.1</b>  | eIF3 subunits in <i>Arabidopsis</i>                                                  | 48   |
| <b>Figure II.2</b>  | PCR genotyping strategy for identification of T-DNA insertion alleles                | 51   |
| <b>Figure II.3</b>  | T-DNA insertion screening for eIF3b                                                  | 52   |
| <b>Figure II.4</b>  | Embryo lethality in <i>eif3b1</i>                                                    | 56   |
| <b>Figure II.5</b>  | T-DNA insertion screening for eIF3c                                                  | 58   |
| <b>Figure II.6</b>  | Embryo lethality in <i>eif3c1</i>                                                    | 59   |
| <b>Figure II.7</b>  | T-DNA insertion screening for eIF3d                                                  | 61   |
| <b>Figure II.8</b>  | T-DNA insertion screening for eIF3f                                                  | 63   |
| <b>Figure II.9</b>  | T-DNA insertion screening for eIF3g                                                  | 65   |
| <b>Figure II.10</b> | T-DNA insertion screening for eIF3i                                                  | 66   |
| <b>Figure II.11</b> | T-DNA insertion screening for eIF3j                                                  | 69   |
| <b>Figure II.12</b> | T-DNA insertion screening for eIF3k                                                  | 70   |

|                     |                                                                                                                     |     |
|---------------------|---------------------------------------------------------------------------------------------------------------------|-----|
| <b>Figure II.13</b> | T-DNA insertion screening for eIF3m                                                                                 | 72  |
| <b>Figure III.1</b> | In <i>elf3h</i> mutant plants, 43S complexes lack eIF3h                                                             | 91  |
| <b>Figure III.2</b> | eIF3h dependent translational regulation of the AtbZip11 5' leader                                                  | 93  |
| <b>Figure III.3</b> | Contribution of individual uAUGs to translational repression and eIF3h-dependent translation of AtbZip11-Fluc       | 97  |
| <b>Figure III.4</b> | uAUG recognition                                                                                                    | 101 |
| <b>Figure III.5</b> | Stimulation of translation across uORFs 2 and 3 by eIF3h is not dependent on the uORF peptide sequence              | 105 |
| <b>Figure III.6</b> | Requirement of eIF3h for efficient reinitiation                                                                     | 107 |
| <b>Figure III.7</b> | Translation initiation on the 5' leader from yeast GCN4 in <i>Arabidopsis</i>                                       | 108 |
| <b>Figure IV.1</b>  | Translation efficiency of the AtbZip11 leader in <i>elf3x</i> mutants                                               | 128 |
| <b>Figure IV.2</b>  | Translation efficiency of the AtbZip11 leader in <i>rpl</i> mutants                                                 | 131 |
| <b>Figure IV.3</b>  | RPL24B is not required for AUG recognition and is independent of the peptide sequence of the uORF                   | 134 |
| <b>Figure IV.4</b>  | RPL24B along with eIF3h is required for efficient reinitiation after uORF translation                               | 136 |
| <b>Figure IV.5</b>  | <i>rpl24b</i> and <i>elf3h</i> have a similar reinitiation defect on the GCN4 leader                                | 138 |
| <b>Figure IV.6</b>  | ATF4-like leader to elucidate function of RPL24B in reinitiation                                                    | 139 |
| <b>Figure V.1</b>   | Schematic representation of the distinct morphological stages of male gametophyte development in <i>Arabidopsis</i> | 148 |
| <b>Figure V.2</b>   | eIF3e and eIF3h T-DNA insertion mutants                                                                             | 152 |
| <b>Figure V.3</b>   | Schematic representation of the FTL- <i>elf3x</i> genetic analysis                                                  | 155 |
| <b>Figure V.4</b>   | Viability of <i>elf3x</i> pollen                                                                                    | 157 |
| <b>Figure V.5</b>   | Phenotype analysis of male gametophyte development for <i>elf3e</i>                                                 | 159 |
| <b>Figure V.6</b>   | eIF3h affects pollen tube growth                                                                                    | 161 |

|                    |                                                                                                                                                                                         |     |
|--------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| <b>Figure VI.1</b> | Schematic illustration of the hypothesized functions of eIF3h in reinitiation                                                                                                           | 177 |
| <b>Figure VI.2</b> | Schematic illustration of proposed ribosome density mapping for the precise molecular function of eIF3h in reinitiation                                                                 | 179 |
| <b>Figure VI.3</b> | Schematic representation of proposed experimental approach using micrococcal nuclease digestion for understanding the molecular function of eIF3h in translation of the AtbZip11 leader | 181 |

## List of Appendices

| Appendix              |                                                                                                  | Page |
|-----------------------|--------------------------------------------------------------------------------------------------|------|
| <b>Appendix II.1</b>  | Tissue specific expression profile of eIF3                                                       | 80   |
| <b>Appendix II.2</b>  | Oligonucleotides used for confirmation of T-DNA<br>insertion                                     | 82   |
| <b>Appendix II.2</b>  | Oligonucleotides used for RT-PCR                                                                 | 84   |
| <b>Appendix III.1</b> | AUG2a is not recognized by ribosome back-scanning                                                | 121  |
| <b>Appendix III.2</b> | Reinitiation on the maize R-Lc leader                                                            | 122  |
| <b>Appendix V.1</b>   | eIF3 expression pattern in floral organs based on<br>publicly available gene expression analysis | 171  |
| <b>Appendix V.2</b>   | Transmission of <i>eif3h-1</i> allele through the male and<br>female gametophyte                 | 172  |
| <b>Appendix V.3</b>   | Oligonucleotides used for confirmation of the<br>fluorescent transgene in FTL                    | 173  |

## **List of Abbreviations**

ATF4: Activating Transcription Factor 4  
CaMV: Cauliflower Mosaic Virus  
CrPV: Cricket Paralysis Virus  
CSN: COP9 signalosome  
eIF: Eukaryotic Initiation Factor  
eIF4E-BP: eIF4E Binding Protein  
EM: Electron Microscopy  
ERAD: Endoplasmic Reticulum Associated Degradation  
GCN4: General Control Nondepressible  
GTP: Guanosine 5'-Triphosphate  
HCV: Hepatitis C Virus  
IRES: Internal Ribosome Entry Site/Sequence  
ITAF: IRES Trans-acting Factor  
MAPK: Mitogen Activated Protein Kinase  
MFC: Multi-Factor Complex  
MPN: Mpr1p Pad1p N-terminal  
mRNA: Messenger RNA  
mRNP: messenger Ribonucleoprotein Particle  
NLS: Nuclear Localization Signal  
NMD: Nonsense Mediated Decay  
NT: Nucleotides  
PABP: Poly (A) Binding Protein  
PIC: Pre-Initiation Complex  
RNA: Ribo-Nucleic Acid

RRF: Ribosome Release Factor

SIRT: Sucrose Induced Repression of Translation

TAV: Transactivator

TC: Ternary Complex

tRNA: transfer RNA

uORF: upstream Open Reading Frame

UV: Ultra-Violet

# **Chapter 1**

## **Introduction**

## **I. Translational regulation of gene expression**

The organization and functioning of a cell requires the coordination of a battery of different processes, all equally important and necessary for life. But by dint of sheer numbers, protein synthesis usurps the maximum resources in a cell. Protein synthesis (translation) is a sophisticated process involving extensive biological machinery. Taking into account the resources invested in translation, it would be surprising if this cellular process was not closely regulated and monitored. Regulation of translation has been recognized as an important concept since investigations began to resolve long-standing problems in areas as diverse as learning and memory, embryonic development, and human diseases such as cancer, diabetes and obesity as well as viral infections (Sonenberg and Hinnebusch, 2009). These studies have highlighted that our understanding of the basic molecular machinery of translation is rather incomplete. Regulating gene expression during synthesis of the peptide products offers the advantage of responding to external stimuli without invoking preceding cellular processes that extend from messenger RNA (mRNA) synthesis and processing, to mRNA transport. Also, the immediacy of the modifications during translation, their reversibility, and the spatial control offers an edge over transcriptional regulation.

Protein synthesis is a multistep, multi-factorial pathway and regulation is exerted at many levels (Jackson et al., 2010; Kapp and Lorsch, 2004), although regulation of translation is most common at the initiation stage. Translation regulation encompasses global regulation affecting most of the repertoire of cellular mRNA, by modulating general components of the protein synthesis machinery in unison (Mathews, 2007). Specific regulation on the other hand affects only a subset of mRNAs by exploiting specific components of the machinery or by targeting specific sequence elements in the mRNA. Conceptually, regulation can be achieved by either activating or repressing translation. But the paucity of translational activation mechanisms suggests that under regular conditions mRNAs are translated efficiently, and, only under certain cellular circumstances, expression is downregulated. However, that does

not imply that all mRNAs are continually associated with ribosomes and are translationally active. Cytosolic mRNAs are distributed between ribosome-associated (actively translated) and a ribosome-free (non translated) pool (Balagopal and Parker, 2009; Gebauer and Hentze, 2004).

### **I.1 Targets and mechanisms of translational regulation**

The process of protein synthesis in eukaryotes can be divided into distinct phases that involve initiation, elongation, termination, and ribosome recycling. Translation initiation is modulated through the *cis*-acting structural features of the mRNA, the translation apparatus *per se*, *trans*-acting molecules – usually proteins, small RNAs, or, occasionally, small molecules and RNA factors for regulation (Gebauer and Hentze, 2004; Jackson et al., 2010; Kapp and Lorsch, 2004; Meijer and Thomas, 2002; Mignone et al., 2002; Sonenberg and Hinnebusch, 2007, 2009). The likening of the messenger ribonucleoprotein particles (mRNPs) to ‘posttranscriptional operons’ indicates the extent to which *cis*-acting elements and their *trans*-acting factors affect the fate of the mRNA (Keene and Tenenbaum, 2002).

#### ***Cis*-acting features of mRNA in translational regulation**

Structural features and regulatory sequences inherent to a mRNA have a critical role in determining the translation efficiency. These include canonical modifications (poly A tail and cap structure) that are strong enhancers of translation initiation; the internal ribosome entry sequences (IRESs) that mediate cap independent translation initiation; upstream open reading frames (uORFs) acting as translational barriers by reducing translation from the main ORF; RNA secondary structures, generally blocking efficient initiation; and finally, sequence elements for binding of specific proteins (**Figure I.1**). These inherent regulatory elements can act either through the general translation apparatus or require specific *trans*-acting factors.

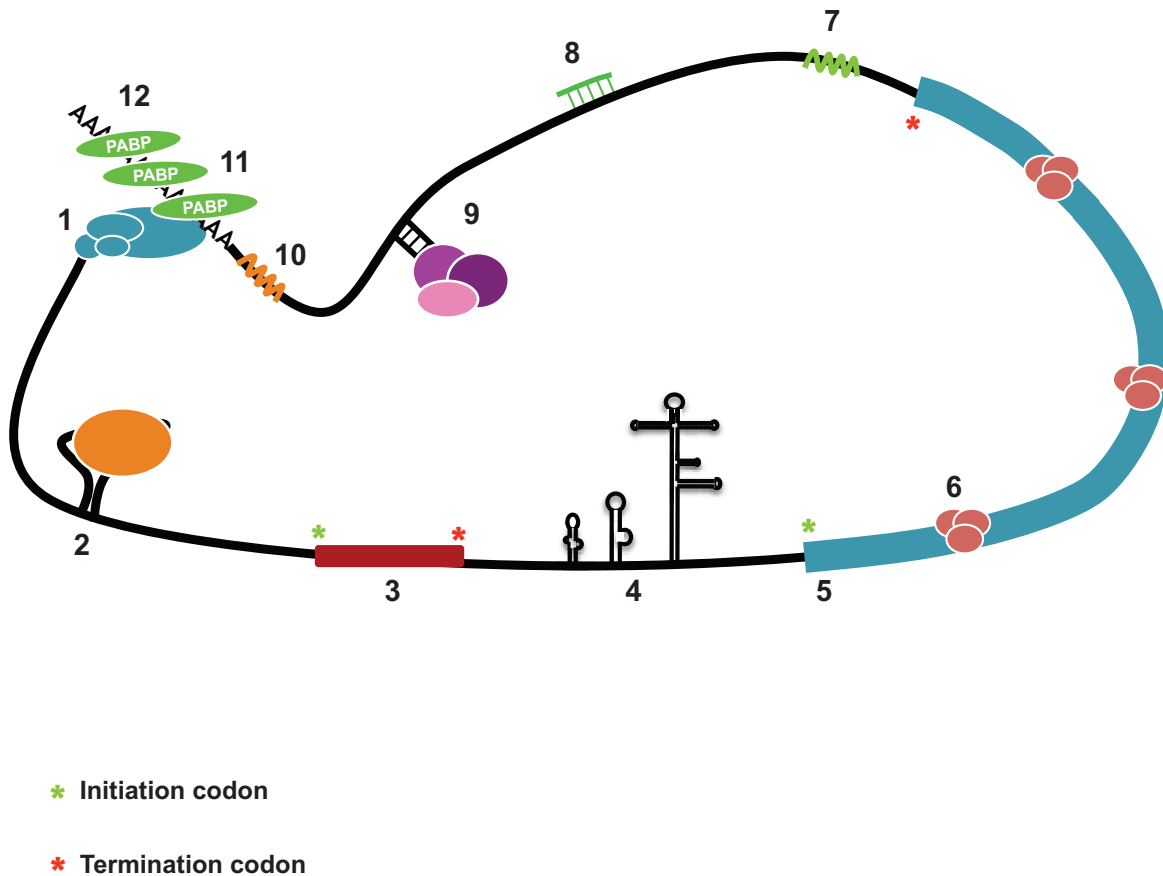


**Figure I.1. *Cis*-regulatory elements in the mRNA.** Representation of a hypothetical, eukaryotic, closed-loop, cytosolic mRNA illustrating the variety of sequence elements and *trans*-acting factors that may be present and affect gene expression. The 5' end of the mRNA consists of the 7-methyl-guanosine cap bound by the eIF4 complex (**1**). The 5' leader with an RNA stem loop and stem loop binding protein (**2**), an upstream open reading frame (uORF)(**3**), an internal ribosome entry sequence (IRES)(**4**) is represented. The main open reading frame (**5**) with exon junction complex (**6**) is followed by the 3' untranslated region (3'UTR). The 3'UTR bears a zipcode element (**7**) for mRNA localization, binding sequence for complementary-small RNA (**8**), a structural element bound by RNA binding protein (**9**), a cytoplasmic polyadenylation element (**10**). The poly A tail (**12**) is bound with poly-A binding protein (PABP)(**11**).

The cooperation between the regulatory elements not just affects translation, but is also closely coupled with mRNA stability (Mathews, 2007).

### **Control via translation factors**

The *trans*-acting eukaryotic initiation factors (eIFs) provide a focal point for the translational regulation of gene expression in response to developmental regulatory elements as well as environmental cues (Hanson et al., 2008; Harding et al., 2000; Hinnebusch, 2005; Kawaguchi et al., 2004; Meijer and Thomas, 2002; Petracek et al., 1997). Regulation is imparted by initiation factors mainly via phosphorylation and is known to act in conjunction with *cis*-acting elements of the message to be translated (Kapp and Lorsch, 2004; Mazumder et al., 2003; Meijer and Thomas, 2002; Morris and Geballe, 2000; Sachs and Geballe, 2006). Eukaryotic initiation factors eIF4G, eIF4B, eIF2, eIF2B, eIF3, eIF5 and Poly A binding protein (PABP) are some of the factors that are targeted by specific kinases to regulate translation efficiency (Mathews, 2007). As an example, eIF2 phosphorylation in response to stress provides a means to modulate the translational status of the cell by downregulating global translation, and, it initiates a reconfiguration of the gene expression profile that can effectively manage the altered cellular conditions (Sonenberg and Hinnebusch, 2009). Apart from phosphorylation, translation factors are also subject to diverse post-translational modifications, eIF5A modification by hypusine and eEF2 modification by diphthamide. Modification of ribosomal proteins by ubiquitination, methylation and phosphorylation also impart accessory regulation on the translation apparatus (Mathews, 2007).

## **II. Translation initiation in eukaryotes**

Translation initiation being the key target of regulation, understanding the molecular structure and the biochemical functions of the initiation machinery holds the clue for uncovering regulatory strategies in the cell. The main objective of the process of translation initiation is

the assembly of the 80S ribosome on an mRNA in which the initiation codon is base paired to the 5'-CAU anticodon of the aminoacylated initiator methionyl-transfer RNA (Met-tRNA<sub>i</sub><sup>Met</sup>) in the ribosomal peptidyl (P) site (Hinnebusch, 2006; Jackson et al., 2010; Kapp and Lorsch, 2004). Initiation can be categorized into two distinct phases, (1) the formation of the translation competent 48S initiation complex, and, (2) the joining of the 60S ribosomal subunit. Translation initiation in eukaryotes is orchestrated by the interaction of initiation factors with tRNA<sub>i</sub><sup>Met</sup>, ribosomal subunits, and messenger RNA in order to stimulate mRNA recognition and commencement of protein synthesis. With the rare exception of internal ribosome entry sites (IRESs), initiation is predominantly accomplished via recognition of the 5' cap followed by unidirectional movement of the ribosomes along the 5' leader of the mRNA in a 5'-to-3' direction that forms the basis of the 'scanning' mechanism of translation initiation in higher eukaryotes (Kapp and Lorsch, 2004; Kozak, 2002; Spirin, 2009). However, both these processes involve the coordinated interaction between the ribosomal subunits as well as the eIFs.

### **II.1 Cap mediated canonical scanning mode of translation initiation**

The structural feature of the mRNA is a key determining factor for regulating initiation efficiency as well as for deciding the course of translation initiation. The nucleotide sequences in the mRNA determine the efficiency as well as the mode of ribosome recruitment, scanning efficiency, and initiation codon recognition. All mRNAs from the eukaryotic nucleus with the exception of metazoan histone mRNAs (Dominski and Marzluff, 2007) have two key elements, a (m<sup>7</sup>G[5']ppp[5']N cap at the 5' end and a 50-300 nucleotides long poly A tail that is not coded from the DNA but added post-transcriptionally in the nucleus. These elements ensure that the mRNA is not susceptible to exonucleases that are abundant in the cell. Also, the physical interaction between the 5' cap and the poly (A) tail boosts translation via a synergistic interplay between the two ends (Gallie, 1991).

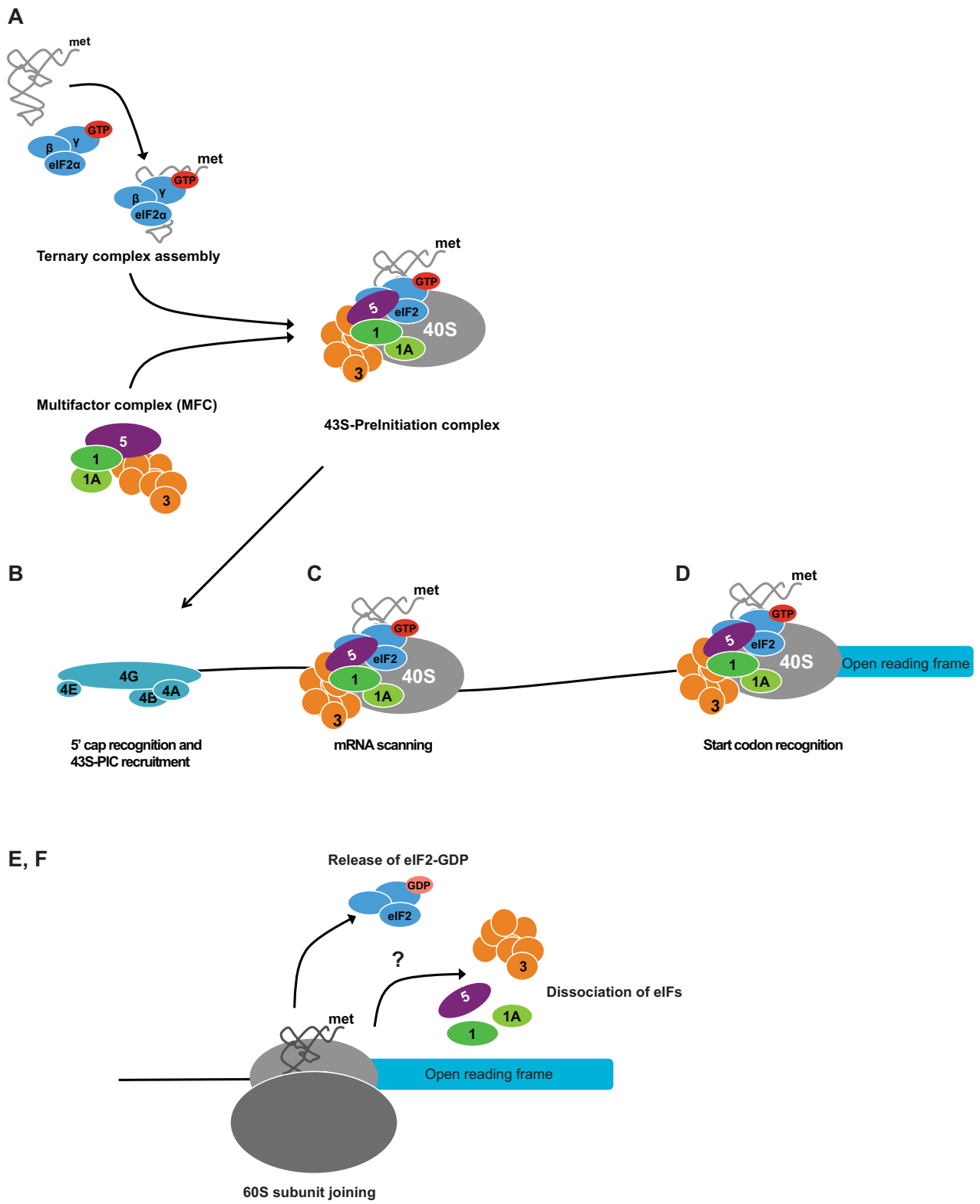
### **Pre-assembly of the initiation competent 43S complex**

Translation initiation requires free separated cytosolic ribosomal subunits and a plethora of initiation factors (eIFs) (Jackson et al., 2010). The translation initiation pathway commences with the binding of the free 40S cytosolic ribosomal subunits with the ternary complex (TC) comprised of eIF2, GTP, Met-tRNA<sub>i</sub><sup>Met</sup> (**Figure I.2A**). Crucial for initiation is the assembly of the 43S pre-initiation complex (43S-PIC) by the cooperation between eIF1, eIF1A, eIF3, eIF5, TC and the 40S ribosomal subunit. The assembly of the 43S-PIC is stimulated by binding of the TC to the 40S ribosome, which in turn is an additive effect of eIFs 1, 1A, 3 and 5 (**Figure I.2A**). In *Saccharomyces cerevisiae*, the 43S-PIC is helped both in assembly and function by a higher order complex consisting of these initiation factors dubbed as the multifactor complex (MFC)(Asano et al., 2000). Whether a pre-formed MFC binds the 40S or whether the interactions between the components of the MFC form after binding to 40S to stabilize the PIC is not clear. Similar complexes have been reconstituted *in vitro* with plant factors meaning that MFCs might be a feature of all evolved eukaryotes (Dennis et al., 2009).

### **43S complex attachment and recruitment to mRNA**

Recognition as well as attachment of the 43S-PIC to the 5' cap of the mRNA is a fundamental feature of the scanning mode of translation initiation. The eIF4F cap-binding complex of the cytosolic, translation-competent, mRNPs opens up secondary structures near the 5' cap via the eIF4A Dead-box RNA helicase (Jackson et al., 2010; Kapp and Lorsch, 2004). Thus, the 5' cap becomes more accessible for ribosome attachment. The attachment of the 43S-PIC in mammals is mediated via a chain of interactions consisting of cap-eIF4E-eIF4G-eIF3-40S (**Figure I.2B**).

**Figure I.2. Eukaryotic cap dependent translation initiation.** Translation initiation in eukaryotes involves formation of the 43S-PIC (**A**); followed by 5' cap recognition (**B**), mRNA scanning (**C**) and start codon recognition (**D**). The codon-anti codon base pairing is followed by GTP hydrolysis and eIF2-GDP release (**E**), and release of eIF1, 1A, and 5 and subsequent 60S subunit joining (**F**). The release of eIF3 during subunit association is debated and therefore has been denoted by a question mark (?). For simplicity, eIF5B, required for subunit joining, has been omitted from the illustration.



### **Ribosome scanning, start codon recognition and formation of the 48S complex**

The 43S PIC, after its attachment to the 5' cap scans the mRNA downstream to the initiation codon (**Figure I.2C**). Scanning of the mRNA is attained by the combined function of mRNA secondary structure unwinding together with the movement of the ribosome using molecular Brownian ratchet machines (Spirin, 2009). A requirement for ATP and the helicase activity of eIF4A is evident in 5' leaders with weak secondary structures. Scanning is reduced in the absence of eIF1A in a reconstituted mammalian system, and in the absence of eIF1 it is completely abolished. Thus, eIF1 and eIF1A may be critical for conferring a scanning permissive conformation on the PIC (Nanda et al., 2009).

As the nucleotide sequence of the mRNA is scanned by the PIC, it discriminates between canonical initiation codons and non-authentic ones. The fidelity of start codon recognition depends on the base pairing of triplets in the 5' leader with the Met-tRNA<sub>i</sub><sup>Met</sup> anticodon. AUG codons in optimum context, with a purine in -3 and +4 positions (relative to the AUG codon) are recognized efficiently by the scanning PICs as initiation codons (Kozak, 1984, 1986). eIF1 is a central mediator of start codon recognition (Mathews, 2007). Upon establishment of a codon-anti codon base pairing, eIF1 is displaced from the ribosome peptidyl site. Mutations that weaken binding of eIF1 to the PIC decrease the fidelity of start codon recognition by releasing eIF1 at non-AUG codon (Cheung et al., 2007). In addition to eIF1, eIF2, eIF3 and eIF5 have also been implicated in fidelity of start codon selection (Jackson et al., 2010). Recognition of the start codon is then followed by release of phosphate from the G-protein factor eIF2, after the GTPase-activating protein eIF5 has triggered GTP hydrolysis. And this event presumably triggers the release of the other eIFs as well. As a final step, 60S subunit joining is promoted by eIF5B in a GTP dependent fashion. The joining of the 60S ribosomal subunit opens the door to translation elongation.

## II.2 Cap independent translation initiation

The conventional scanning mechanism of initiation is bypassed by some exceptional mRNAs, particularly in viruses. Here the 40S ribosome is directly recruited to the mRNA via a highly structured element present in the 5' leader - an internal ribosome entry site/sequence (IRES). The mechanism of initiation via IRESs varies with respect to factor requirement. Some IRESs rely on *trans*-activating factors (ITAFs) that stabilize the IRES conformation. However, the requirement for canonical eIFs varies. eIF participation is divergent, with hepatitis C virus (HCV) IRES dispensing with eIF4F and requiring only eIF3 and eIF2/eIF5, and the extreme case of cricket paralysis virus (CrPV) IRES, which dispenses with all eIFs and even Met-tRNA<sub>i</sub><sup>Met</sup> (Lomakin et al., 2000). Cellular mRNAs active during conditions (apoptosis and mitosis) when the cap dependent translation machinery is shut off use IRESs to bypass the cap dependent initiation mechanism for efficient expression. Compared to animal viruses, only few plant viruses have been reported to implement IRESs. The 5' leader (TL) of tobacco etch virus (TEV) has been observed to direct efficient translation from naturally uncapped viral mRNA dependent on eukaryotic initiation factor eIF4G (Gallie, 2001). IRES mediated translation initiation has also been reported in the crucifer infecting tobamovirus (Ivanov et al., 1997).

## II.3 Special cases of translation initiation – reinitiation and shunting

While the scanning model of translation initiation adequately explains initiation on the majority of cellular mRNAs, it cannot explain the efficient translation of a substantial number of plant, animal and human mRNAs that harbor uORFs (Calvo et al., 2009; Kim et al., 2007; Lawless et al., 2009; Resch et al., 2009). Specifically, it turns out that the 5' leader sequences of a large fraction of cellular mRNAs in higher eukaryotes possess one or more AUGs upstream of the main AUG. According to the scanning model, translation of a uORF will impede, or in extreme circumstances, even abolish translation of the main ORF (Meijer and Thomas, 2002; Morris

and Geballe, 2000; Sachs and Geballe, 2006). For, after translating the uORF, the 40S ribosome would no longer be competent to initiate at the main ORF because (1) no Met-tRNA<sup>Met</sup> is bound to the 40S, and, (2) the initiation factors responsible for loading the TC have been dispersed from the ribosome. This shortfall of the classic scanning model could be reconciled by postulating that the translating ribosomes retain some of the factors that may stimulate reloading of a TC. Evidence from different systems has substantiated that after translation of an upstream ORF, ribosomes resume scanning and *reinitiate* downstream (Kapp and Lorsch, 2004; Meijer and Thomas, 2002; Morris and Geballe, 2000; Poyry et al., 2007; Poyry et al., 2004; Rajkowitsch et al., 2004; Sachs and Geballe, 2006; Szamecz et al., 2008). What determines efficient reinitiation after the translation of these uORFs is still not fully understood. However, based on the current understanding of translation initiation, reinitiation can be accomplished only if, (1) the 40S ribosomal subunit remains attached to the mRNA after uORF termination and resumes scanning, (2) fresh eIF2-GTP-methionyl-tRNA<sup>Met</sup> and eIF5 are reacquired *de novo*, (3) eIF1 and eIF1A are reacquired to ensure AUG specificity to the reinitiating complex, (4) the secondary structures in the 5' leader can be melted, and (5) scanning is directional (**Figure I.3**). A complete list of factors pivotal for reacquiring reinitiation competence is still lacking, although subunits of eIF3 are crucial (discussed in detail in Chapters 3 and 4).

In contrast to reinitiation involving linear scanning by 40S ribosomes, translation in viral mRNAs (and some rare eukaryotic mRNA) occurs by discontinuous scanning of the ribosomes (Babinger et al., 2006; Chappell et al., 2006; Pooggin et al., 2008; Pooggin et al., 2001; Racine et al., 2007). What it implies is that ribosomes can be shunted from an upstream site to a downstream element in the mRNA. The structural features in the mRNA that facilitate

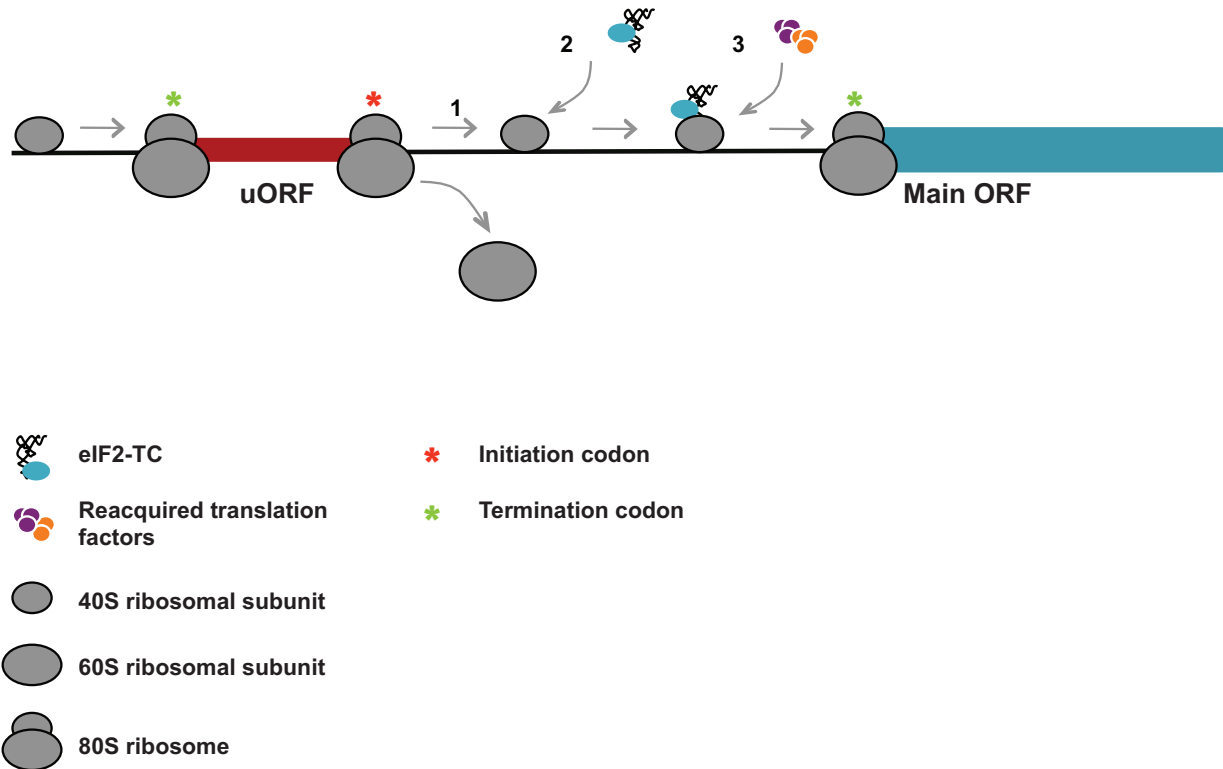


**Figure I.3. Possible events leading to gaining of reinitiation competence after uORF termination.** For efficient reinitiation to take place, the 40S subunit should remain bound to the mRNA and resume scanning (resumption of scanning) (**1**); reacquire eIF2-GTP-methionyl-tRNA<sup>Met</sup> (TC) (**2**); and reacquire other factors such as eIF1, 1A, 3 and eIF5 (**3**) so as to recognize the downstream AUG of the main open reading frame (ORF) efficiently and with high fidelity.

ribosome shunting are, presence of a large stem-loop and a uORF preceding the stem loop. It is still puzzling how ribosomes acquire the TC for efficient translation downstream.

## **II.4 Controls during translation initiation**

### **Global control**

The best characterized mechanism of translation regulation in eukaryotic cells involves the phosphorylation of eIF2. eIF2, a trimeric complex, binds to the initiator methionyl tRNA in a GTP dependent manner and cycles between its GTP-bound state and GDP-bound state that is triggered by eIF5 (a GTPase activating protein) and eIF2B (a guanine nucleotide exchange factor). The phosphorylation of the  $\alpha$  subunit of eIF2 at Serine-51 regulates its exchange reaction. Phosphorylation at Ser<sup>51</sup> does not affect the ability to form a functional initiation competent eIF2-TC. But after its release following eIF5 mediated GTP hydrolysis, phosphorylated eIF2 binds tightly to eIF2B and abrogates eIF2B function. The sequestration of eIF2B by phosphorylated eIF2 to an inactive eIF2-GDP-eIF2B form inhibits protein synthesis because of diminution of eIF2 charged with GTP. Surprisingly, gene specific consequences of eIF2 $\alpha$  phosphorylation have been reported as well. The mRNA encoding the yeast bZIP transcription factor, general control nonderepressible 4 (GCN4) and the mammalian activating transcription factors 4 and 5 (ATF4 and ATF5) are subject to paradoxical translational up-regulation when levels of active eIF2-TC decline (Hinnebusch, 2005; Wek et al., 2006). The stimulation of translation on these mRNAs is explained by a particular inhibitory uORF configuration that represses the translation under normal condition (discussed in later sections).

The significance of this pathway is further apparent from the diversity of the kinases that are involved. In *S. cerevisiae* only one eIF2 $\alpha$  kinase has been reported, GCN2. GCN2 is activated by nutrient limiting conditions that include low levels of amino acid, purine, glucose

deprivation, as well as by high sodium concentration and the drug rapamycin (Hinnebusch, 2005). However, four eIF2 $\alpha$  kinases exist in mammals; typically, each kinase has a predominant role in response to a specific cellular stress condition (Wek et al., 2006). On the other hand, plant homologs of eIF2 $\alpha$  kinases were identified only recently (Zhang et al., 2003). The *Arabidopsis* GCN2-like kinase responds to amino acid deprivation conditions as well as purine starvation, UV, cold shock and wounding (Lageix et al., 2008; Zhang et al., 2008b). Thus, the phosphorylation of eIF2 $\alpha$  and signaling downstream represent a well-conserved adaptation to cell stress.

### **mRNA specific regulation during initiation**

Initiation on specific mRNAs can also be restrained by binding of a specific RNA binding protein to the 3' UTR of the mRNA. This is generally inhibitory, with the exception of poly (A) binding protein (PABP) which stimulates translation of cytosolic mRNAs. The translational advantage of polyadenylated transcripts over non-polyadenylated ones suggests that the binding of PABP to the poly (A) tail provides stimulation to translation via formation of a 'closed-loop' (Amrani et al., 2008; Gallie, 1991; Kahvejian et al., 2001). Binding of the *trans*-acting factors to the 3'UTR disrupts the closed loop configuration. For example, binding of Maskin and Pumilio to the cyclin B1 mRNA in *Xenopus*; Bicoid to the *caudal* mRNA in *Drosophila*; Bruno to the *oskar* mRNA in *Drosophila*, all represent cases where the cap binding complex is targeted by 3'UTR binding proteins for translation inhibition (Gebauer and Hentze, 2004; Jackson et al., 2010). Regulation is also imparted during the ribosome subunit joining step – either during 40S recruitment or during 60S recruitment as evidenced from the regulation of Msl2 translation of the sex lethal mRNA in *Drosophila* (Duncan et al., 2006; Gebauer and Hentze, 2004).

### **III. eIF3 (eukaryotic initiation factor 3)**

With up to 13 subunits, eIF3 is by far the largest eIF. The association of a large mass of non-ribosomal proteins with free 40S ribosomal subunits in rabbit reticulocyte lysates led to the identification of a complex of polypeptides of approximately 500-600KDa that could further be removed by high salt washes (Freienstein and Blobel, 1975; Sundkvist et al., 1974; Sundkvist and Staehelin, 1975). The removed fraction retained its activity of accelerating initiation in a cell-free globin synthesizing system and thus led to its characterization as an initiation factor. Furthermore, based on *in vitro* studies of its biochemical activities, it was implicated in Met-tRNAi stabilization, mRNA binding of the ribosome, and promoting ribosome dissociation (Benne and Hershey, 1978; Peterson et al., 1979; Sundkvist et al., 1974; Sundkvist and Staehelin, 1975; Thompson et al., 1977; Trachsel et al., 1977).

The association of this mass of protein (eIF3) with the cytosolic 40S ribosomal subunit gained further credence from electron micrograph studies where the physical association between the two was revealed (Emanuilov et al., 1978). eIF3 association with the 40S *in vitro* and *in vivo* was further evident from biochemical assays including sucrose density gradients and chemical crosslinking of eIF3 with 18S rRNA. The identification of eIF3 in the early 1970s in rabbit reticulocyte lysates was then followed by identification of a similar high molecular weight complex from Krebs ascites (Trachsel et al., 1979), HeLa cells (Brown-Luedi et al., 1982), rat liver (Benne and Hershey, 1976), wheat germ lysates (Checkley et al., 1981) and a lower molecular weight complex from yeast (*S. cerevisiae*) (Naranda et al., 1994), suggesting a universal function of this protein complex in translation.

#### **III.1 Structure and composition of eIF3**

eIF3 is a multi-subunit protein complex consisting of up to 13 non-identical subunits and is indispensable for the canonical scanning mode of translation initiation (Hinnebusch, 2006). The mammalian eIF3 complex is composed of 13 different subunits (eIF3a-m) and has a

molecular mass of ~600 KDa (Damoc et al., 2007; Hinnebusch, 2006; Phan et al., 1998; Zhou et al., 2008). A similar protein complex has been identified in plants (*Arabidopsis* and wheat), and the plant eIF3 complex has at least 12 subunits similar to that in mammals (Asano et al., 1997; Burks et al., 2001; von Arnim and Schwechheimer, 2006). Furthermore, mass-spectrometric analyses of eIF3 complexes purified from HeLa cells has identified 13 subunits in stoichiometric amounts present in three structural modules within the complex (Damoc et al., 2007; Zhou et al., 2008) (**Figure I.4**). A preliminary inter-subunit interaction map has been inferred from the mass spectrometric analysis.

In contrast, budding yeast (*S. cerevisiae*) has 5 subunits for basal translation, which are also present in mammalian and plant eIF3 complexes (Hinnebusch, 2006), and one non-essential subunit - eIF3j. The five subunits (a, b, c, g and i), tightly conserved among a wide range of organisms, were considered to be the 'core' subunits and were perceived to mediate the basal functions of eIF3. The apparent absence or the viability of knockout mutations in genes of the other 8 'non-core' eIF3 subunits in budding yeast and fission yeast respectively, insinuated that the five-subunit core complex might be sufficient to carry out the basic functions of eIF3. The additional, less well-conserved 'non core' subunits were anticipated to possess accessory or regulatory roles in higher eukaryotes. However, reconstitution of mammalian eIF3 complex as reported by Masutani *et al.* has provided new insights into the functional relevance of these subunits (Masutani et al., 2007). The formation of the functional eIF3 "core" required subunits a, b, c (conserved core subunits), and also the non-core subunits e, f and h as visualized by the formation of a 48S initiation complex (Masutani et al., 2007). Possibly, the non-core subunits perform vital functions in higher eukaryotes. However, the essentiality of these subunits *in vivo* needs to be substantiated.

Very little information is known about the molecular structure of eIF3 and the stoichiometry of individual eIF3 subunits. Apart from the crystal structure of the smallest human subunit (eIF3k) (Wei et al., 2004) and the solution structure of the eIF3b RNA binding domain

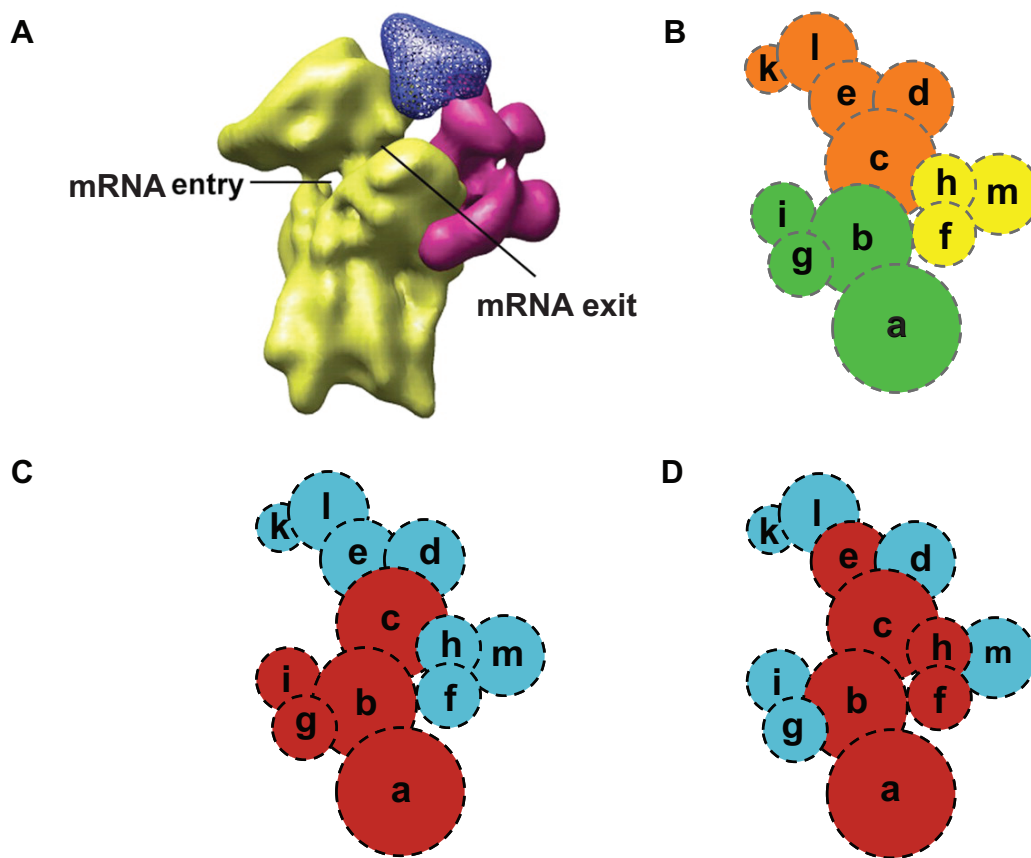


**Figure I.4. eIF3 structure.** **A.** Model of a 40S subunit (in yellow, viewed from the 60S-40S subunit interface) with eIF3 (magenta) on its solvent exposed side based on cryoelectron microscopy analysis. eIF4G (purple) is bound to eIF3 near the E-site (Siridechadilok et al., 2005). **B.** Potential intersubunit map based on the mass spectrometric analysis of eIF3 (Damoc et al., 2007; Zhou et al., 2008). The three structural modules defined by physical association within the complex are color coded. eIF3 subunit map is color coded based on subunit conservation across species including budding yeast (**C**) and *in vitro* reconstitution (Masutani et al., 2007) (**D**). Red denotes conserved subunits and blue non-conserved subunits (in *S. cerevisiae*) in panel C. In panel D, red denotes the essential subunits for reconstitution of a functional 48S-PIC whereas blue denotes the accessory subunits.

(ElAntak et al., 2007), not much structural detail has come forth. However, the cryoelectron microscopy reconstruction of eIF3 on the small ribosomal subunit has yielded considerable insight into the structure. eIF3 has been revealed as a five lobed particle that binds to the 40S subunit of the ribosome on the solvent side, close to the mRNA exit channel (Siridechadilok et al., 2005).

### **III.2 Functions of eIF3 in translation initiation**

As described in previous sections, critical biochemical steps in eukaryotic translation initiation involve formation of a translation competent 43S-PIC and its binding to the mRNA, scanning of the mRNA, and finally, recognition of the initiation codon. Much of our knowledge about the functions of eIF3 subunits in translation is based mainly on the analysis in budding yeast and mammals (Asano et al., 2000; Asano et al., 1998; Hinnebusch, 2006; Nielsen et al., 2004; Phan et al., 1998; Sonenberg and Hinnebusch, 2009; Valasek et al., 2001; Valasek et al., 2002; Valasek et al., 2004). The following sections discuss the function of eIF3 in each of the biochemical steps in initiation.

#### **eIF3 binding to 40S ribosomal subunit**

Crucial for the assembly of the 43S PIC is the availability of free cytosolic 40S ribosomal subunits. eIF3 binding to the 40S ribosomal subunit is stimulated by the loosely associated subunit, eIF3j *in vitro* (Fraser et al., 2004). Mammalian eIF3d can crosslink to 18S rRNA in 40S complexes and hence can provide additional 40S binding domains (Nygard and Westermann, 1982). Furthermore, deletion of the N and C termini of eIF3c and N-terminal domain of eIF3a in budding yeast impaired the binding of eIF3 complexes to 40S subunits even though the integrity of the eIF3 complex was not affected (Valasek et al., 2003). Mutations in the RNA recognition motif in the eIF3b N-terminal domain (NTD) also abrogated eIF3 binding of 40S subunits in yeast (Nielsen et al., 2006). These observations are

suggestive of multiple contacts between eIF3 and the 40S subunit. However, the exact position of individual eIF3 subunits on the 40S ribosomal subunit has not been mapped yet. Binding of eIF3 to the 40S is critical as it prevents the premature association of the large ribosomal subunit; the anti association activity being aided by cooperative action of TC, eIF1 and eIF1A.

### **eIF3 in 43S-PIC assembly**

The formation of the 43S-PIC is facilitated by the connections among the factors in the MFC, eIF3c-NTD and eIF3a C-terminal domain (CTD) being important among others (Singh et al., 2004; Valasek et al., 2002; Valasek et al., 2004). eIF3 promotes the binding of TC to the 40S in reconstituted mammalian systems and mutations affecting eIF3b-eIF5 interactions reduce TC binding in yeast (Valasek et al., 2002). Disruption of eIF2-eIF3 contacts by mutations in the C-terminal domains of eIF5 and eIF3a results in a slow growth phenotype that is rescued by overexpression of TC (Asano et al., 2000; Valasek et al., 2002). Therefore, it is evident that for optimal PIC formation, the MFC and interactions between its components are required *in vivo* and there are multiple interactions between eIF3 subunits and the MFC.

### **eIF3 in 43S complex recruitment to mRNA**

Several lines of evidences are consistent with a function of eIF3 in mRNA loading. eIF3 stimulates the binding of the 43S-PIC to the mRNA by, (1) fostering TC binding to the 40S and therefore stimulating mRNA binding indirectly (Jivotovskaya et al., 2006), (2) the high affinity interaction between eIF3e and eIF4G in mammals (LeFebvre et al., 2006), and, (3) RNA binding activity of individual eIF3 subunits (Asano et al., 1997). The credibility of the eIF3-eIF4G interaction is questioned in lower eukaryotes by the lack of a consensus eIF3 binding domain in eIF4G. Nevertheless, recruitment of the 43S-PIC to the mRNA under these circumstances is possibly facilitated by RNA-binding activities of the eIF3 subunits.

## **Functions of eIF3 in ribosome scanning, start codon recognition and 48S complex formation**

After the recruitment of the 43S-PIC to the mRNA, genetic studies in yeast have revealed that mutations in eIF3 can also affect scanning and start codon recognition (Nielsen et al., 2004). Mutations in eIF3b in yeast also cause a slow scanning phenotype. Functions of eIF3 in start codon recognition are apparent from the *suppressor of initiation (sui-)* phenotype on mutating the CTD of eIF3c, suggesting that eIF3 might have an indirect role in fidelity of start codon recognition via eIF1 or eIF2. How eIF3 promotes scanning of the 43S-PIC is not understood, but interaction with eIF1, which is the main regulator of scanning, might be critical. Also, other studies have yielded evidence in favor of a role for eIF3 subunits in these later stages of translation initiation. For example, Unbehaun and coworkers demonstrated that individual eIFs remain associated with the scanning ribosome *in vitro* at least until the 60S subunit joins in (Unbehaun et al., 2004).

The joining of the 60S subunit displaces among others, eIF1, eIF1A and eIF2. However, whether or not eIF3 is dissociated from the initiation complex is debated. The anti-association property of eIF3 argues for the loss of eIF3 as a prerequisite for subunit joining. However, eIF3 position on the 40S ribosome argues against the requirement for eIF3 dissociation.

### **III.3 eIF3 in later stages of translation**

Unlike what would be expected for a translation initiation factor, surprisingly, eIF3 has been implicated in post-termination ribosome disassembly. It has been reported that eIF3 is required for the release of the 60S ribosomal subunit after translation of a short ORF (Pisarev et al., 2007). The sequence of events that take place during termination starts with eIF3 promoting the release of the 60S subunit. The 40S ribosome is still bound to the mRNA with a deacylated tRNA in the P-site. The eIF3/40S complex is then acted upon by eIF1. eIF1 helps dislodge the deacylated tRNA and also weakens eIF3/40S association with the mRNA. The

complete release of the 40S subunit however requires eIF3j. With this seminal work, Pisarev et al. have not just provided titillating insights into the functions of eIF3, but have also provided a fundamental understanding of the mystery surrounding the disassembly of post-termination 80S ribosomes. It also helped explain the presence of eIF3j in the mRNA binding channel as observed by Fraser et al. and affirmed a role for this loosely bound eIF3 subunit in the dissociation function (Fraser et al., 2007).

Furthermore, this could help in clarifying the long debated question of how reinitiation takes place after the translation of the uORFs in the mRNAs. It is plausible that the termination of a short uORF does not proceed beyond the deacylated tRNA ejection step where the 40S ribosome is still attached to the mRNA. It implies that eIF3 might physically remain associated with the 40S subunit after termination and the mRNA may still be attached to the ribosome. This would resemble the 48S-PIC, the difference being the absence of TC, eIF1A, eIF5. eIF3 thus might substitute for the ribosome release factors (RRF) in prokaryotes as the efforts to find cytoplasmic eukaryotic RRF homologs have failed extensively. Thus, eIF3 may hold a key function in determining the fate of the ribosome after termination. With the involvement of eIF3 as a termination promoting factor on short uORFs, a scenario is emerging where a greater complex consisting of canonical and ancillary factors are involved during termination that could potentially affect the behavior of the ribosome towards reinitiation or shunting (Jackson, 2007; Pisarev et al., 2007; Pisarev et al., 2010).

### **eIF3 as a reinitiation factor**

In line with the alleged functions of eIF3 in termination, specialized functions have been designated to eIF3 during reinitiation in yeast, metazoans and plants. Reinitiation on the GCN4 leader in yeast is dependent on the general functions of eIF3 in scanning (Hinnebusch, 2005). Furthermore, functional interaction between yeast eIF3a and GCN4 mRNA sequences have been observed to enhance reinitiation (Szamecz et al., 2008). eIF3h, a non-core subunit

in *Arabidopsis*, is also indispensable for the efficient translation of specific uORF containing mRNAs (Kim et al., 2004). A follow up study involving microarray analysis verified the polysome loading of mRNAs with multiple uORFs to be reduced in *eif3h* (Kim et al., 2007). Furthermore, eIF3g interacts with the transactivator protein (TAV) of plant cauliflower mosaic virus that stimulates reinitiation on its polycistronic mRNA (Park et al., 2004a; Park et al., 2001; Ryabova et al., 2004; Thiebauld et al., 2009). Finally, eIF3 was also found to bind a *cis*-acting sequence element that fosters reinitiation after long uORFs (Poyry et al., 2004). Taken together, functions of eIF3 in special translation initiation processes involving specific mRNAs are emerging. All these pieces of evidence, however, do not easily reconcile with the preexisting notion of eIF3 dissociation from the ribosome during initiation at AUG. Instead, they provide evidence for the sustained association of eIF3 with the elongating ribosome for a few rounds of elongation, though the interaction might be labile. Alternatively, eIF3 may be recruited *de novo* during termination after short uORF termination. Because uORFs often have regulatory significance (discussed in the next section), these data give evidence for the notion that eIF3 subunits might have a regulatory role in the translation process.

### **eIF3 as a translational regulator of specific mRNAs**

Apart from participating in global translation regulation as evidenced from the lethality in eIF3 depleted cells, there is strong evidence implicating eIF3 subunits in translation initiation of specific mRNA subpopulations. Zhou *et al.* ascertained the existence of two eIF3 complexes in *S. pombe* that, in addition to the core subunits, differ in non-core subunit composition (Zhou et al., 2005). Interestingly, these eIF3 complexes were found to be associated with different transcript sets and hence suggested that the non-core subunits confer a specific function to the eIF3 complex and modulate the translation status of different mRNA species. Although this is the strongest evidence for the presence of an mRNA specific regulation by eIF3, it has also been reported that eIF3a, a core subunit, plays a similar role in regulating the translation of

specific mRNA in mammals (Dong and Zhang, 2006). Finally, a similar regulation has been documented for eIF3h, a non-core subunit of eIF3 in *Arabidopsis*. eIF3h may not be vital for global protein synthesis, but the translation efficiency of mRNA characterized by the presence of uORFs in their 5' UTR were compromised in the absence of functional eIF3h (Kim et al., 2007; Kim et al., 2004).

The functional role of the eIF3 multisubunit complex is not well defined. For example, it is unclear whether all of eIF3's biochemical activities are indeed relevant for its function *in vivo*. Conversely, genetic data implicate eIF3 in late stages of initiation, i.e. downstream from PIC assembly, during scanning and start codon recognition, functions which have not been characterized *in vitro*. The involvement of eIF3 in dissociation of post-termination ribosomes and its function in promoting reinitiation, lend credence to the hypothesis that eIF3 remains associated with the ribosome, at least for a few rounds of translation, and therefore might not be displaced from the translating ribosomes.

The functions of individual subunits of eIF3 also remain poorly understood and the molecular mechanism by which eIF3 exerts its functions has been elusive (discussed in later sections).

### **III.3 eIF3, functions beyond the translation apparatus**

Functions of eIF3 have been reported that are apparently independent of its function in initiation. In concert with upregulated protein synthesis during the G1 phase of cell cycle, several eIF3s have been implicated in regulating cell cycle progression (Dong and Zhang, 2006). Given that eIF3 has been implicated in cell division and cell differentiation, misexpression of eIF3 subunits has often been detected during tumorigenesis. Specifically, increased mRNA and protein levels for eIF3a, b, c, h and i have been detected in a wide variety of human tumors (Zhang et al., 2007; Zhang et al., 2008a). In contrast, reduction in expression of eIF3e and f accompanies several cancer types (Shi et al., 2006).

It will be of considerable interest to understand the underlying functions of eIF3 in tumorigenesis and to investigate its potential use as a therapeutic target. The phosphorylation of eIF3h and eIF3f seems to be important for its function in tumor development (Shi et al., 2006; Zhang et al., 2008a); but whether or not the functions of the other eIF3 subunits are just limited to aberrantly activated translation or might require a more complicated signaling pathway remains to be unveiled.

### **eIF3 and PCI complexes**

Another interesting aspect of eIF3 is its evolutionary relatedness to two other multisubunit protein complexes, the regulatory lid of the 26S Proteasome and the COP9 signalosome (CSN), which is also involved in protein turnover (Pick et al., 2009). These three complexes share two structural domains – the PCI domain (19S **P**roteasome lid, **C**SN, and eukaryotic translation **I**nitiation factor 3) and the MPN domain (**M**pr1p, **P**ad1p **N**-terminal domain) (Kim et al., 2001). Six of the eIF3 subunits (eIF3 a, c, e, k, l, m) have a PCI homology domain and two subunits (eIF3 h, f) an MPN domain; thus bringing eIF3 in line with the postulated 6+2 configuration of the other PCI complexes (Pick et al., 2009; von Arnim and Schwechheimer, 2006). The significance of these domains is not known but it may be possible that it mediates a biochemical cross talk between the otherwise distantly related protein complexes. The protein-protein interactions between eIF3 and the CSN, and between eIF3 subunits and the proteasome lid (Karniol et al., 1998; Kim et al., 2001) may tell a tale of biochemical coordination between translation initiation and protein turnover processes.

### **Versatility of eIF3, interaction with other cellular components**

Interactions of eIF3 with components of the PCI complex as well as the regulatory functions of eIF3 in cell growth and differentiation exhibit the multifaceted functions of eIF3 outside of its function in protein synthesis. Since eIF3 is critical for these processes, targeting eIF3 is a way

to exert and fine-tune the control over these processes. Over the years, a number of interactions of other cellular as well as non-cellular proteins with eIF3 have been observed. A summary of these interactions of eIF3 with factors beyond the translation apparatus and the PCI complexes is summarized in **Table I.1**. Some of these interactions might relate to its function in translation (hijacking of the host translation machinery by viral protein by targeting eIF3 subunits) and some might be relevant to the mRNP life cycle - mRNA transport, localized translation, mRNA decay. Even though the exact mechanism by which eIF3 affects all these processes is far from being clear, but there is definitely evidence for multiple functionality of eIF3 and its subunits.

#### **IV. Upstream open reading frames (uORFs)**

As discussed in the previous sections, several elements in the mRNA control the efficiency of protein synthesis after the mRNA has been activated for translation via addition of covalent modifications. Among these are initiation codons (AUG codons) within the leader of the transcript (upstream AUG, uAUG) and in many cases the associated open reading frame (upstream open reading frame - uORF). uORFs are defined as structural elements in the 5' leader of the mRNA formed with a start codon and an in-frame stop codon (Morris and Geballe, 2000; Sachs and Geballe, 2006). In extreme cases, the termination codon of the uORF lies downstream of the start codon of the main open reading frame and thus overlaps with the main ORF (**Figure I.5**). uORFs generally have coding sequences of fewer than 50 codons and are present either singly or in clusters. The prevalence and abundance of these uAUGs were studied as early as 1987 when Marilyn Kozak reported 10% of vertebrate mRNAs (out of a total of 699 mRNAs that were studied) to harbor upstream initiation codons in a favorable context that would mean efficient translation from these AUGs (Kozak, 1987). Moreover, the majority (~70%) of these uAUGs were in proto-oncogenes and other transcripts critical for controlling of cellular growth and differentiation. However, misrepresentation of the

**Table I.1. Interacting partners of eIF3.**

| <b>eIF3 subunit</b> | <b>System</b>        | <b>Interacting partner</b> | <b>Function of interacting partner</b> | <b>Reference</b>                         |
|---------------------|----------------------|----------------------------|----------------------------------------|------------------------------------------|
| eIF3a/TIF32/RPG1    | <i>S. cerevisiae</i> | Microtubule                | Localization, transport                | (Hasek et al., 2000)                     |
| eIF3a/TIF32         | Mammals              | Microtubule                | Localization, transport                | (Shanina et al., 2001)                   |
| eIF3a/TIF32         | <i>S. cerevisiae</i> | Mkp1                       | Protein kinase                         | (Asp et al., 2008)                       |
| eIF3a/TIF32         | <i>S. cerevisiae</i> | Sty1                       | Stress activated MAPK <sup>1</sup>     | (Asp et al., 2008)                       |
| eIF3a               | <i>Mus musculus</i>  | MILI                       | PIWI protein                           | (Unhavaithaya et al., 2009)              |
| eIF3b/PRT1          | <i>S. cerevisiae</i> | Gle1                       | mRNA transport                         | (Bolger et al., 2008)                    |
| eIF3c               | Mammals              | P56                        | Viral stress response                  | (Hui et al., 2005; Terenzi et al., 2005) |
| eIF3c               | Mammals              | Merlin/<br>Schwannomin     | Cell proliferation                     | (Scoles et al., 2006)                    |
| eIF3d/Moe1          | <i>S. pombe</i>      | Cdc48                      | ERAD <sup>2</sup> , cell division      | (Otero et al., 2010)                     |
| eIF3e               | <i>Mus musculus</i>  | P56                        | Viral stress response                  | (Guo and Sen, 2000)                      |
| eIF3e               | Mammals              | MCM7                       | Stabilization during DNA replication   | (Buchsbaum et al., 2007)                 |
| eIF3e               | Mammals              | UPF1                       | Nonsense mediated decay                | (Morris et al., 2007)                    |

---

|               |                               |                             |                                                  |                              |
|---------------|-------------------------------|-----------------------------|--------------------------------------------------|------------------------------|
| eIF3e         | <i>S. pombe</i>               | Cdc48                       | ERAD, cell division                              | (Otero et al., 2010)         |
| eIF3f         | HEK293 cell line              | CDK11                       | Inhibition of protein synthesis during apoptosis | (Shi et al., 2009)           |
| eIF3f         | HeLa cell line                | Corona virus spike protein  | Inhibition of host anti-viral reaction           | (Xiao et al., 2008)          |
| eIF3f         | <i>Drosophila</i> and mammals | TRC8                        | Ubiquitination                                   | (Lee et al., 2010)           |
| eIF3g         | HeLa cell line                | AIF                         | Inhibition of protein synthesis during apoptosis | (Kim et al., 2006)           |
| eIF3g         | CaMV host                     | CaMV TAV                    | Translation reinitiation                         | (Park et al., 2001)          |
| eIF3h         | Yeast two hybrid assay        | Rabies virus matrix protein | Inhibition of host cap dependent translation     | (Komarova et al., 2007)      |
| eIF3h         | <i>Drosophila</i> and mammals | TRC8                        | Ubiquitination                                   | (Lee et al., 2010)           |
| eIF3i/TRIP1   | <i>Arabidopsis</i>            | TGF beta receptor           | Cell differentiation and growth                  | (Jiang and Clouse, 2001)     |
| eIF3k/PLAC24  | HeLa cell line                | 5-HT <sub>7</sub>           | Serotonin receptor                               | (De Martelaere et al., 2007) |
| eIF3m/Tango 7 | <i>Drosophila</i>             | Caspase 9                   | Caspase mediated programmed cell death           | (Chew et al., 2009)          |

---

<sup>1</sup> Mitogen activated protein kinase; <sup>2</sup> Endoplasmic Reticulum associated degradation



**Figure I.5. Upstream open reading frames in 5' leader of mRNAs.** Schematic representation of an mRNA without any uORF in the 5' leader (upper panel) and a uORF containing mRNA (lower panel). The green and the red asterisk denote the initiation and termination codons respectively. The uORFs might be present singly, or in clusters. In extreme situations, the uORFs can overlap the main ORF as well.

5' ends on the mRNA could have precluded authentic uAUGs from being identified. With the wealth of increasing sequence data, together with more information about the alternative transcription start sites and alternative promoter usage, a more accurate estimate from independent studies is that 49% of human and 44% of mouse transcripts have at least one uORF in the 5' leader (Calvo et al., 2009; Kim et al., 2007; Lawless et al., 2009; Resch et al., 2009). Among other eukaryotes, 30% of *Arabidopsis* transcripts have uAUGs in their 5' leader (Kim et al., 2007). In contrast, uORFs are relatively rare in yeast with only 13% of the transcripts representing this class (Lawless et al., 2009). Even though there is a disparity in the abundance of uORF containing transcripts among different organisms, there is a significant enrichment of specific functional classes of genes. Stress responsive genes, transcription factors, protein modifying enzymes, proto-oncogenes and cell growth regulators are overrepresented (Calvo et al., 2009; Kim et al., 2007; Lawless et al., 2009). Hence, it is apparent that uAUGs are ubiquitous elements in genes with critical cellular roles and are probably important for regulation of gene expression.

#### **IV.1 Effect of uORF on translation – uORFs as translation barriers**

uORFs have both quantitative as well as qualitative effects on downstream translation and impart their regulation by governing the fate of the ribosome in translation initiation downstream. uORFs are generally inhibitory to translation as the scanning ribosome first encounters the uAUGs that are proximal to the 5' end of the mRNA in comparison to the main AUG. The question is how will the main ORF get translated if there is a uAUG in the 5'UTR and the ribosome encounters the uAUG. One thing to be borne in mind is that not all proteins are translated efficiently in a cell. Some have a regulated expression, and probably the ones that are under the control of uAUG are minimally expressed under normal physiological conditions. The translation of this category of inefficiently translated proteins can be explained by the leaky scanning model and the rarer case of reinitiation (**Figure I.6**). According to the

scanning mechanism of translation initiation, ribosomes translating the uORFs might be affected in the following ways. First, the uORF could preemptively initiate translation (uORF recognition; **Figure I.6**). Due to scanning from the 5' end, when ribosomes have the option of initiating at a uAUG, the probability of initiating downstream of a start codon is correspondingly reduced. It is likely that many uORFs affect translation by reducing the ribosome flow to the downstream ORF. The efficiency with which a uAUG captures the ribosome will further depend on the sequence context of the AUG. In absence of a favorable context around the uAUG, the initiation complexes may reach the downstream start codon by leaky scanning (leaky scanning, **Figure I.6**).

Secondly, the uORF can also govern the ribosome reinitiation efficiency, i.e. determine whether or not the ribosomes, after translating the uORF, will resume scanning and initiate at a downstream initiation codon. Both the length of the uORF and the elongation rate are critical parameters that determine the reinitiation efficiency in *S. cerevisiae* and this has been recapitulated in mammals as well (Hinnebusch, 2005; Kozak, 1987; Rajkowitsch et al., 2004). Also, the initiation factors that transiently associate with the ribosome during uORF translation may be crucial for determining reinitiation competence. Taking into considerations these parameters, translation of a long uORF is more inhibitory than a shorter one, because of prolonged transit time of the ribosome on the uORF that results in loss of reinitiation potential. Finally, a longer intercistronic spacer promotes reinitiation compared to a shorter one via *de novo* reacquisition of factors (TC, and probably eIF1, eIF1A) (Kozak, 2001).

Additionally, two other mechanisms important for the regulation of downstream translation have been reported and both involve uORF mediated ribosome stalling at the termination codon of the uORF - an indirect effect of the nascent peptide specified by the uORF (**Figure I.6**).

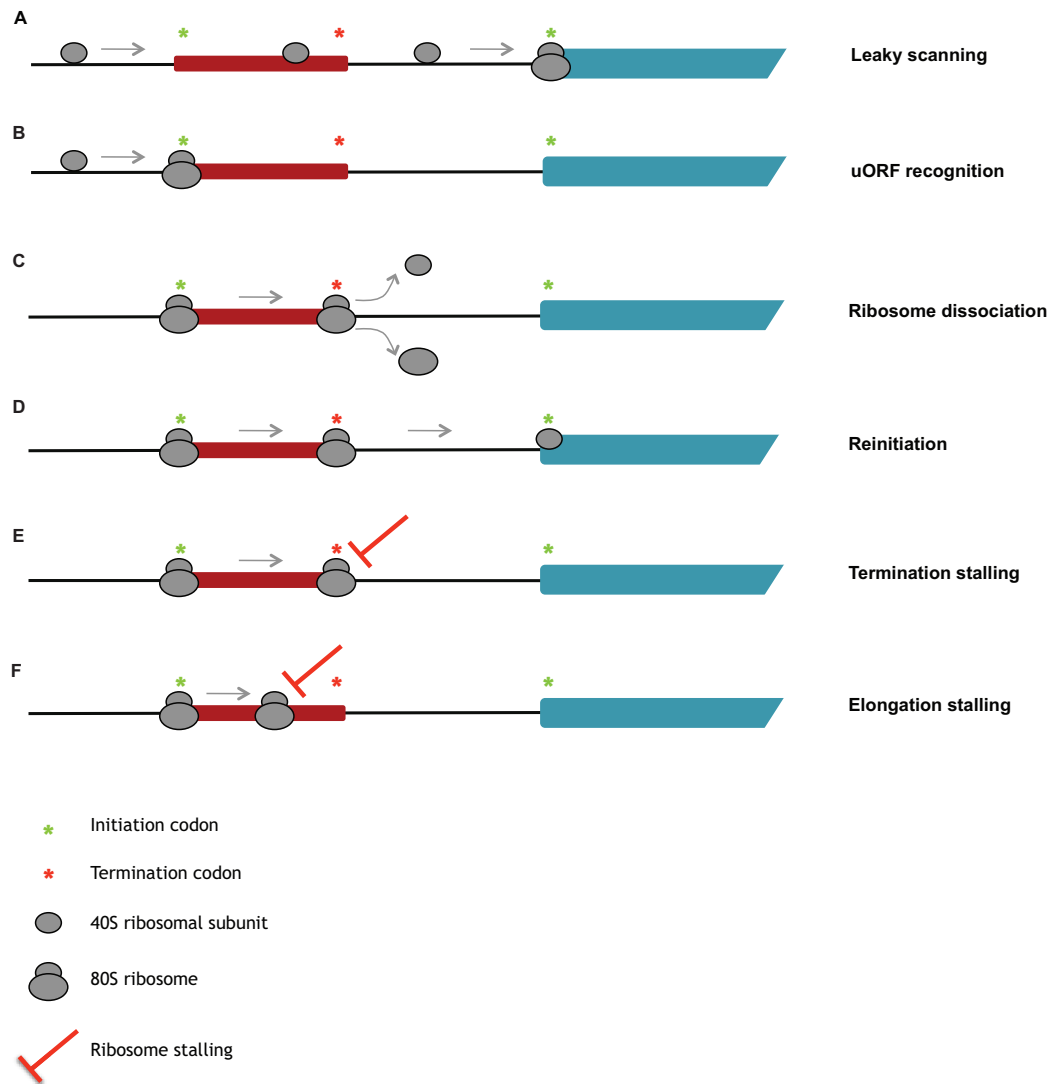


**Figure I.6. uORFs as translation barrier.** Possible outcomes on a leader harboring uORF. uORFs are represented in red boxes and blue boxes denote the main ORF. In presence of an uAUG in weak context, the 40S ribosome can leaky-scan the uORF and initiate at the main open reading frame (**A**). However, if the uAUG is in a favorable context, the 40S initiates at the uORF rather than the main ORF (**B**). After translation of the uORF the ribosome can either dissociate from the mRNA (**C**), or reinitiate downstream (**D**). Ribosomes stalling during termination (**E**) or elongation (**F**) in response to attenuator peptides can block the ribosome transit pathway as well.

Such a ribosomal pause at a eukaryotic stop codon acts as a barrier to the scanning ribosome from traversing to the downstream start codon. Finally, a ribosome translating a uORF can stall within the coding region mainly as a function of codon usage or because of the nascent peptide that has been synthesized. This, like the previous situation, will block the transition of the ribosomes.

#### **IV.2 Sequence dependent uORFs**

Vertebrates have a greater propensity of bearing a favorable context around the main AUG as compared to the sequence context around the upstream AUGs (Meijer and Thomas, 2002). Conceptually, this implies that not 100% of the ribosomes recognize these uAUGs. However, even if the recognition of the uORF is not efficient and if the initiation complex can leaky-scan past the uAUG, the uORF can have a substantial effect on the downstream translation in a sequence dependent manner via ribosome stalling. Ribosome stalling on uORFs is either a consequence of the rare codons in the uORF coding region or a result of the nascent peptide that is synthesized from these uORFs. In contrast to bacterial antibiotic resistance genes that are affected by the nascent peptide product of a short uORF (Lovett, 1994; Lovett and Rogers, 1996), very few transcripts have been found in eukaryotes where translation is repressed by uORFs in a sequence dependent manner. uORFs in the fungal *Arginine-2*, plant *AtbZip11* and plant and animal S-adenosylmethionine decarboxylase mRNAs are some of the examples of sequence specific uORFs in the eukaryotic kingdom (discussed in later sections). Some of these peptides are believed to interfere with translation termination. For the fungal *arg-2* uORF, it has been observed that the arginine attenuator peptide stalls the ribosome either during elongation or termination in response to high arginine concentration (Fang et al., 2000). Exactly which biochemical event during termination is affected is not clear. Moreover, the fact that uORF peptide sequences are generally not evolutionarily conserved also complicates the assessment of how these peptides might be affecting the ribosome function

(Hayden and Jorgensen, 2007).

### **IV.3 Effect of uORFs on mRNA stability and structure**

Manipulation of the ribosome transit pathway by the uORF affects not just the downstream translation, but also has a profound effect on the fate of the transcript. Because of a prolonged pause of terminating ribosomes in the CPA-1 uORF, the stability of the transcript is altered and becomes an active substrate for the nonsense mediated decay (NMD) pathway (Gaba et al., 2005). Similarly, a uORF in the *Arabidopsis* AtMHX 5' leader renders the transcript unstable via NMD (Saul et al., 2009). In accordance, a large-scale analysis of *S. cerevisiae* transcripts has revealed that uORF containing transcripts represent a major class of NMD substrate. However, not all transcripts with uORFs are subject to NMD, the well studied GCN4 leader in *S. cerevisiae* being one example. Efficient reinitiation events after uORF translation have the capacity to abrogate the effect of NMD on uORF containing leaders (Zhang and Maquat, 1997). Also, stabilizing elements that might bind to specific proteins were found to be important for reducing instability caused by uORFs (Ruiz-Echevarria and Peltz, 2000).

Prevalence of uORFs not just make the mRNA more prone to decay by NMD, but parallel to the situation in prokaryotes, it may also affect the structure of the mRNA by unmasking elements in the mRNA that activate gene expression. Translation of the uORF in the Cat-1 (cationic amino acid transporter-1) mRNA unmasks an IRES element and thus activates the protein synthesis from the main ORF (Fernandez et al., 2005; Yaman et al., 2003). The ribosome transit pathway is altered after uORF translation in other ways as well. It has been observed in the cauliflower mosaic virus (CaMV) that translation of a uORF enables the ribosomes to be shunted to a downstream initiation codon (Pooggin et al., 2000). The mammalian  $\beta$ -secretase mRNA also adopts a similar fate (Chappell et al., 2006). Thus, the ribosome behavior during uORF translation can trigger alternative initiation mechanisms.

#### IV.4 uORF function in response to stress and metabolite signaling

The functions of uORFs are not just relevant during normal growth conditions, but under cellular stress, they impact (in most of the cases stimulate) the translation of specific transcripts. Also during evolution, organisms developed different translational control mechanisms so as to adapt to changing nutrient conditions. Even though stress and nutrient deprivation conditions generally inhibit translation, stimulation of specific mRNAs is also evident.

Metabolite mediated translational control has been best studied for yeast GCN4 (general control non-derepressible 4) that responds to amino acid starvation (**Figure I.7**). GCN4 is a transcription factor of a large group of genes important for amino acid biosynthesis. Under normal conditions, the GCN4 translation is repressed as ribosomes fail to reach the GCN4 coding region. However, under starvation conditions when the TC levels are low, GCN4 translation is increased. The translation derepression of GCN4 under starvation conditions turns on the amino acid biosynthetic genes thus modulating the cellular gene expression pattern (Hinnebusch, 2005).

The mammalian activating transcription factor 4 and 5 (ATF4 and ATF5) mRNA are subjected to paradoxical translational up-regulation in response to cellular stress. The stimulation of translation on these mRNAs is explained by a particular inhibitory uORF configuration that represses the translation under normal condition. Declining ternary complex during stress conditions derepresses translation on these mRNAs by a mechanism involving regulated reinitiation (**Figure I.8**) (Wek et al., 2006).

Elevated concentrations of nutrients and metabolites also affect uORF mediated translation. In response to excess arginine, the translation of the small subunit of the arginine-specific carbamoyl phosphate synthetase (CPA-1 and *arg-2*) is repressed in *S. cerevisiae* and *Neurospora*, respectively (Wang et al., 1998; Wang and Sachs, 1997). Mechanistically, these

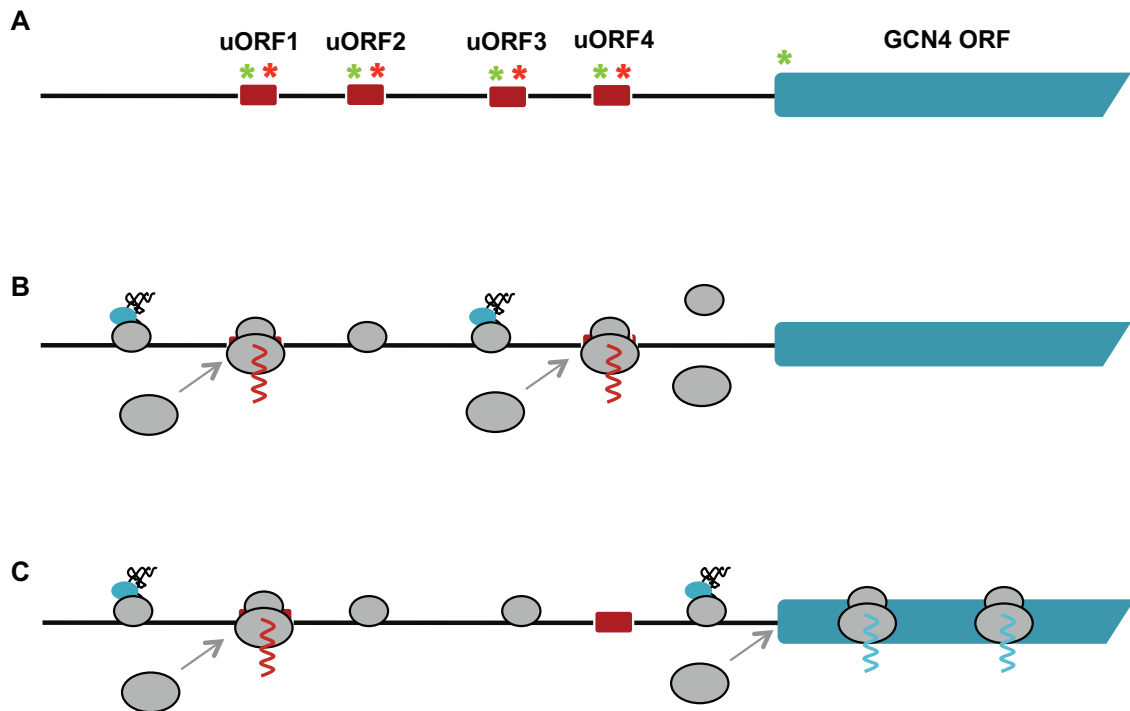


**Figure I.7. Translational regulation of the GCN4 mRNA in yeast.**

**A.** Schematic representation of the GCN4 mRNA with the 5' leader harboring four uORFs (red boxes). The initiation and the termination codons are represented by green and red asterisk and the blue box denotes the GCN4 ORF.

**B.** Translation pattern during normal (non starvation) conditions. For simplicity, only the most repressive uORFs 1 and 4 are represented. Small ribosomal subunits are represented by gray circles and blue circles denote ternary complex (TC). Zigzag lines denote the nascent peptide synthesized and are color coded with the (u)ORF that is translated. After translation of uORF1, small ribosomal subunits (small gray circles) remain attached to the mRNA and resume scanning. Scanning 40S ribosomes bind active eIF2-GTP-methionyl-tRNA<sup>Met</sup> (TC) that are abundant in the cell and initiate translation at uORF4. The ribosomal subunits dissociate from the mRNA after translation of uORF 4 and GCN4 ORF remains untranslated.

**C.** Under amino acid starvation conditions, when eIF2-GTP-methionyl-tRNA<sup>Met</sup> levels are low, many 40S ribosomes skip uORF4 after translation of the upstream ORF1. The scanning ribosomes reinitiate at GCN4 ORF and thus translation is derepressed.

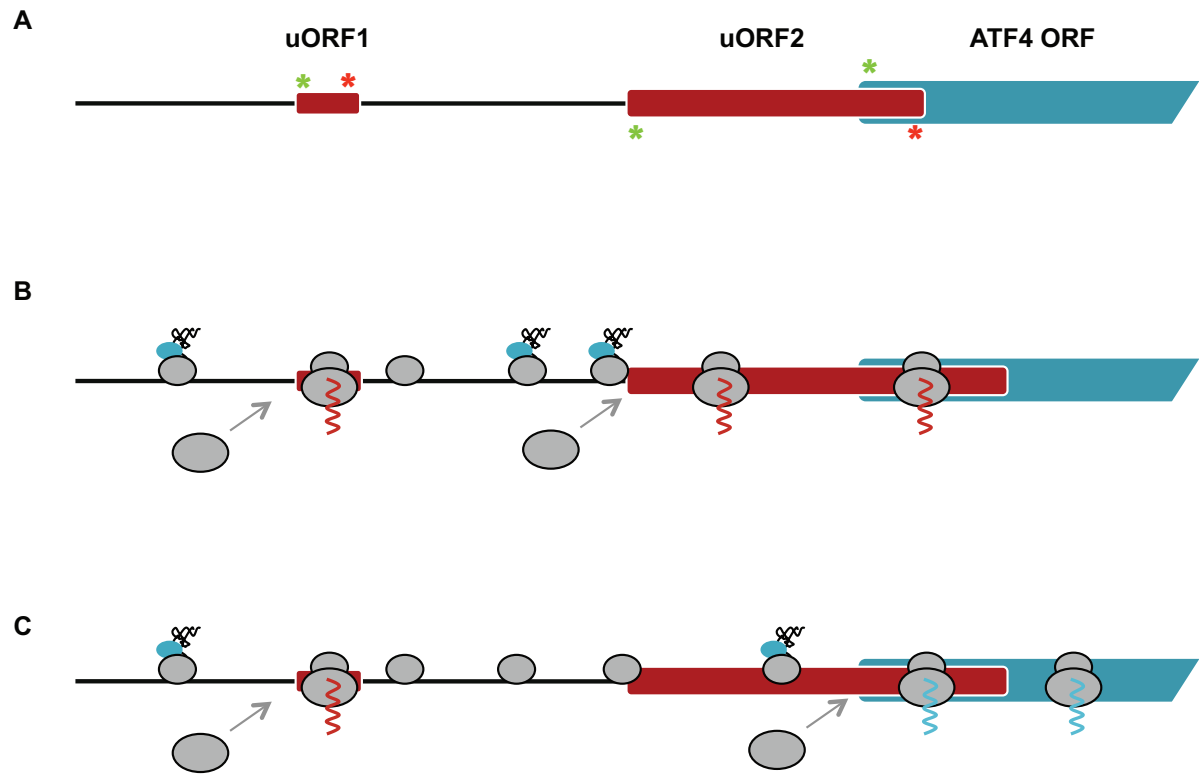


**Figure I.8. Translational regulation of the ATF4 mRNA.**

**A.** Schematic representation of the ATF4 mRNA with the 5' leader harboring two uORFs (red boxes). The initiation and the termination codons are represented by green and red asterisk and the blue box denotes the ATF4 ORF.

**B.** The pattern of translation under normal (unstressed) conditions, when eIF2-GTP-methionyl-tRNA<sup>Met</sup> (TC) is abundant. Small ribosomal subunits are represented by small grey circles and TC with blue circles. Nascent peptides are represented by zigzag line color coded with the (u)ORF that is translated. When TC is abundant in the cell, most ribosomes that resume scanning after uORF1 initiate at uORF2. The expression of ATF4 thus remains low under these conditions.

**C.** Translation pattern under stressed conditions. As a result of low TC levels, uORF2 is bypassed by scanning 40S ribosomes and translation is initiated at ATF4 ORF.

uORFs affect translation differently from GCN4. In both these cases, the sequence of the uORF is crucial as the uORF peptide attenuates and stalls the ribosome in presence of high arginine concentration. Translational regulation of the mammalian S-adenosylmethionine decarboxylase (AdoMetDC) is also due to an uORF encoded hexapeptide sequence, MAGDIS (Hanfrey et al., 2005; Hanfrey et al., 2003; Hanfrey et al., 2002). Ribosomes translating the uORFs stall during termination and prevent the access to the downstream AdoMetDC initiation codon.

Another illustrative example of a uORF-mediated response to metabolites is the *Arabidopsis* transcription factor bZip11 (Rook et al., 1998a; Wiese et al., 2004). AtbZip11 is translationally repressed in response to sucrose, and the downstream consequence is a change in amino acid metabolism, reminiscent of yeast GCN4 (Hanson et al., 2008). The 5' leader of the bZip11 mRNA harbors four upstream open reading frames (uORFs), of which the second uORF is well conserved among bZip11 homologous genes. The uORF2 element encodes a sucrose control peptide (SC-peptide) of 28 residues that is sufficient for imposing sucrose induced repression of translation (SIRT). The peptide acts as an attenuator peptide presumably by stalling translating ribosomes (Hanfrey et al., 2005; Hanfrey et al., 2003; Hanfrey et al., 2002; Rahmani et al., 2009).

Hence, the uORFs are not just mere structural features of the mRNA, but regulatory functions of uORFs extend to coordinating gene expression in response to external cues as well.

#### **IV.5 Translational control of development by uORFs**

As described in the previous sections, uORFs are prevalent in mRNAs for transcription factors and protein modifying enzymes, such as kinases (Kim et al., 2007). They may play an important role in regulating growth and development by (1) inhibition by the uORF encoded leader peptide, and, (2) acting as a translational barrier for the main ORF. In doing so, the uORF may render translation responsive to cellular signals (sucrose, polyamine) to modulate

**Table I.2. Experimentally confirmed uORFs in *Arabidopsis***

| <b>Gene name</b> | <b>Gene function</b>                               | <b>Mechanism of regulation</b>                            | <b>Regulatory signal</b> | <b>Reference</b>                                                     |
|------------------|----------------------------------------------------|-----------------------------------------------------------|--------------------------|----------------------------------------------------------------------|
| AtbZip11         | bZip transcription factor; amino acid biosynthesis | Inhibition of downstream translation by ribosome stalling | Sucrose                  | (Rahmani et al., 2009; Rook et al., 1998a; Wiese et al., 2004)       |
| AtMHX            | Tonoplast transporter                              | Reinitiation, NMD                                         |                          | (David-Assael O et al., 2005<br>Saul et al., 2009)                   |
| AdoMetDC         | Polyamine biosynthesis                             | Ribosome stalling, programmed -1 frameshifting            | Polyamine                | (Hanfrey et al., 2005)                                               |
| PEAMT            | Choline biosynthesis                               | Not determined                                            | Choline                  | (Tabuchi et al., 2006)                                               |
| ACL-5            | Spermine synthase                                  | <i>Trans</i> -acting peptide, mRNA stability              |                          | (Imai et al., 2006, 2008)                                            |
| CGS1             | Cystathionine gamma-synthase                       | Elongation arrest, mRNA degradation                       |                          | (Haraguchi et al., 2008; Onouchi et al., 2005; Onouchi et al., 2008) |
| ARF3/Ettin       | Auxin response factor                              | Inhibition of downstream translation                      |                          | (Nishimura et al., 2005)                                             |
| ARF5/Monopteros  | Auxin response factor                              | Inhibition of downstream translation                      |                          | (Nishimura et al., 2005)                                             |
| ARF7             | Auxin response factor                              | Inhibition of downstream translation                      |                          | (Zhou and von Arnim; unpublished)                                    |
| ARF11            | Auxin response factor                              | Inhibition of downstream translation                      |                          | (Zhou and von Arnim; unpublished)                                    |
| AS1              | Leaf differentiation                               | Inhibition of downstream translation                      |                          | (Zhou and von Arnim; unpublished)                                    |
| AS2              | Leaf differentiation                               | Inhibition of downstream translation                      |                          | (Zhou and von Arnim; unpublished)                                    |
| CLV1             | Stem cell maintenance                              | Inhibition of downstream translation                      |                          | (Zhou and von Arnim; unpublished)                                    |

gene expression (**Table I.2**).

Interestingly, there are few reports where the peptide encoded from the uORF acts in *trans* to affect translation. *Medicago truncatula* nodule development requires the controlled expression of a HAP-2 type transcription factor of the CCAAT-box –binding family in the meristematic zone (Combier et al., 2006). Binding of the uORF encoded peptide to the 5' leader leads to transcript degradation in this rare example (Combier et al., 2008). In contrast, most plant uORFs either attenuate the translation of the main ORF (discussed in next section) or destabilize the mRNA independent of the uORF peptide (Nyiko et al., 2009; Saul et al., 2009).

### **uORFs in organ initiation and auxin perception**

uORFs are over-represented in the Auxin Response Factor (ARF) family of genes. To a large extent, the post-embryonic organ specification and initiation is under the regulation of auxin. Auxin also plays an important role in the establishment of the apical-basal axis (Aida et al., 2002; Friml et al., 2003). Auxin response in plants is mediated by short-range directional transport mediated by the PIN proteins and conversion of the local auxin gradient into a gene expression profile by transcription regulators, ARFs (Teale et al., 2006). Prominent among the ARFs are ARF3/ETTIN, which defines dorsoventrality of the leaves and gynoecium development, and ARF5/MONOPTEROS mediating apical-basal axis establishment. Among the auxin regulatory genes, uORFs are over-represented among the ARFs but rare among the AUX1/IAA mRNA (F. Zhou, personal communication). In contrast to ARFs, neither the PIN mRNAs nor the auxin receptor, TIR1, has any uORFs in the 5' leader. The uORFs in the ARF mRNA are inhibitory to translation of the main protein product (Nishimura et al., 2005). Hence, it can be envisioned that auxin response factors form a module whose expression is translationally regulated by uORFs for organ specification and auxin perception.

## **uORFs in integrating metabolite signaling**

Apart from their plausible function as regulatory modules for specific developmental processes, uORFs also function in integrating growth signals in plants - like polyamines and sucrose.

Polyamine homeostasis is important for vascular development, embryo development, and is also involved in physiological processes such as tolerance to stress. A corollary, downregulation of polyamine biosynthesis causes dwarfism in plants and also enlargement of the vasculature (Gill and Tuteja, 2010; Kusano et al., 2008; Vera-Sirera et al., 2010). S-adenosylmethionine decarboxylase (AdoMetDC) catalyzes a rate limiting step in the polyamine biosynthesis pathway. Similar to mammals, plant AdoMetDC mRNA is also translationally regulated in response to polyamines (Hanfrey et al., 2005; Hanfrey et al., 2003; Hanfrey et al., 2002) via two conserved overlapping uORFs in the mRNA 5' leader (Hanfrey et al., 2003). Another aspect of uORF mediated regulation of the polyamine biosynthesis pathway was revealed from the mutational screening of the ACAULIS 5 (ACL5) gene encoding the thermospermine synthase (Imai et al., 2006). Disruption of ACL5 results in severe dwarfism because of a defect in stem elongation (Imai et al., 2006). The intricacy in the translational regulation of ACL5 is further evident from a possible destabilization of the 80S complex translating the inhibitory uORF of ACL5 in a mutation disrupting the large ribosomal protein RPL10A (Imai et al., 2006; Imai et al., 2008).

uORF mediated sucrose sensing has come forth as a second post-transcriptional regulatory mechanism for mediating growth and development in plants (Hummel et al., 2009). AtbZip11 is a member of the S1 class of bZip transcription factors and harbors highly conserved uORFs in its 5' leader (Rook et al., 1998a; Rook et al., 1998b). Of the four uORFs, the uORF2 peptide sequence is conserved and encodes a sucrose control (SC) peptide (Hummel et al., 2009; Rahmani et al., 2009). The translation of *Arabidopsis* AtbZip11 mRNA is repressed in response to sucrose, and repression is mediated during translation of the uORF containing leader.

AtbZip11 further modulates the amino acid biosynthetic pathway. The expression of asparagine synthase1 (AS1) and proline dehydrogenase2 (ProDH2) is induced under sucrose repressive conditions (Hanson et al., 2008). Thus, modulation of amino acid biosynthesis pathway in response to sucrose deprivation offers a way of integrating and coordinating the carbon and nitrogen metabolic pathways.

Thus, it is evident that certain developmental aspects in the life cycle of plants are under the control of similar modules - uORF mediated auxin perception and uORF mediated polyamine homeostasis. Probably this way, the cell ensures that similar pathways respond to external cues using the same regulatory elements.

## **V. Aims of the dissertation**

Our current understanding of the process of translation has highlighted that it regulates gene expression in much more details rather than just fine-tuning it. Translational control encompasses events that regulate the availability of the message from the nucleus to the protein synthesis machinery. As highlighted in the preceding sections, translational regulation is mediated by the co-action of *cis*-acting elements and *trans*-acting factors that, via their regulation of the general translation, affect the development as well as physiology of the cell. This study attempts to understand the functional significance of initiation factor, eukaryotic initiation factor 3. Functions of eIF3 are diverse but the consequences are largely unknown. What are the molecular activities of eIF3 and its individual subunits? What are the consequences of these activities at the cellular level? What are the contributions of eIF3's activities during development? Answers to these questions are few. Furthermore, the mRNA specific translational regulation by eIF3h (Kim et al., 2004) led to the hypothesis that eIF3 might have regulatory functions in translation and the regulatory functions might be critical for certain developmental and physiological events in *Arabidopsis*. In this study, this hypothesis and its associated questions have been addressed.

**Chapter 2 (*eIF3 in Arabidopsis – reverse genetic approach to characterize functions of individual eIF3 subunits*)** describes the screening, identification, and initial characterization of mutants affecting individual eIF3 subunits in *Arabidopsis*.

**Chapter 3 (*The H subunit of eIF3 promotes reinitiation competence during translation of mRNAs harboring upstream open reading frames*)** aims at elucidating the molecular function of the H subunit of eIF3 in translation of mRNAs with upstream open reading frames in their 5' leader.

**Chapter 4 (*Efficient re-initiation after uORF translation requires cooperation between eIF3 and the large ribosomal subunit*)** identifies factors of the translation machinery that act in conjunction with eIF3h to bolster ribosome rescanning after translation of upstream open reading frames.

**Chapter 5 (*Use of fluorescent-tagged lines to study male gametophyte developmental defects in Arabidopsis translation factors*)** aims at identifying the earliest developmental events that are under the regulation of eIF3 by using a novel genetic tool.

## Chapter 2

**eIF3 in *Arabidopsis* - Reverse genetic approach to characterize functions of individual eIF3 subunits**

## II.1 Summary

Eukaryotic Initiation Factor 3 (eIF3) is the largest of initiation factors whose role in translation is well established. However, apart from yeast, the function of individual subunits in a biological context has been elusive. Furthermore, the functionally cryptic role of eIF3 in translation initiation has put a spotlight on the potential regulatory roles of the eIF3 subunits. In an effort to better understand the functions of eIF3 in a multicellular eukaryote, this study focuses on a reverse genetic approach to functionally characterize eIF3 subunits in *Arabidopsis*. In this study, a collection of *eif3* mutant strains using the conventional method of insertion screening was assembled. Mutational screening of eIF3 subunit genes led to identification of 9 lines with homozygous *eif3x* mutations and several other lines that failed to yield homozygotes. Several rather extreme situations were encountered during the analysis; either substantial functional redundancy between duplicated family members (*eif3d*, *g*, *m*) or gametophytic lethality (*eif3e*, *i-1* and *h*) or embryo lethality (*eif3b1* and *c1*) was observed. The other extreme was the least severe effect of disruption of the k subunit. This assembled collection can further be exploited to understand the molecular functions of eIF3 in cellular processes.

## II.2 Introduction

### II.2.1 eIF3 in *Arabidopsis*

Eukaryotic initiation factor 3 is the largest of eIFs, and is arguably the least understood of the initiation factors. The functional studies in yeast have highlighted the significance of individual subunits in translation (Hinnebusch, 2006). A previous study in our lab demonstrated the regulatory functions of one subunit of eIF3 that otherwise is absent in budding yeast (Kim et al., 2004). What is apparent is that higher eukaryotes implement different levels of translational regulation as compared to lower eukaryotes, and a complete understanding of the functions of eIF3 is yet to emerge. Furthermore, even though the molecular functions of eIF3 in yeast are understood to some extent, the fact that *Arabidopsis* eIF3 has 7 additional eIF3 subunits (**Table II.1, Figure II.1**) leads to the speculation that these accessory, non-core subunits execute additional levels of regulation to the various cellular processes.

*Arabidopsis* eIF3 was first purified from cell suspension cultures and was observed to have 11 subunits, 10 of which were common with mammalian eIF3 and a novel subunit was reported (Burks et al., 2001). However, identification of homologs of eIF3m and the loosely bound eIF3j brings eIF3 in line with the mammalian complex (Damoc et al., 2007; von Arnim and Schwechheimer, 2006) and therefore points towards a unified eIF3 structure in higher eukaryotes. This further strengthens the use of *Arabidopsis* eIF3 as a model system for functional analysis of eIF3.

The *in vitro* analysis of reconstituted eIF3 complex in mammals has reflected the basal functions of some of the eIF3 subunits in translation (Masutani et al., 2007). However, in absence of an established reconstituted *in vitro* system in *Arabidopsis*, I undertook a genetic approach to assign *in vivo* functions to eIF3 and to address the question of what the exact functions of eIF3 are, in a biological context. It is worth mentioning that only one mutant model for eIF3 has been reported in any other multicellular eukaryote (Koyanagi-Katsuta et al., 2002) (apart from the *eif3h*

**Table II.1. Comparison of eIF3 subunit composition across different organisms.**

Conserved, essential subunits in yeast are highlighted in red (Mathews, 2007; von Arnim and Schwechheimer, 2006). Non-essential subunits in budding yeast are represented in blue.

| <i>Saccharomyces cerevisiae</i> | <i>Arabidopsis</i> | Mammals     |
|---------------------------------|--------------------|-------------|
| <b>TIF32</b>                    | <b>eIF3a</b>       | <b>p170</b> |
| <b>PRT1</b>                     | <b>eIF3b</b>       | <b>p116</b> |
| <b>NIP1</b>                     | <b>eIF3c</b>       | <b>p110</b> |
|                                 | eIF3d              | p66         |
|                                 | eIF3e              | p48         |
|                                 | eIF3f              | p47         |
| <b>TIF35</b>                    | <b>eIF3g</b>       | <b>p44</b>  |
|                                 | eIF3h              | p40         |
| <b>TIF34</b>                    | <b>eIF3i</b>       | <b>p36</b>  |
| <b>HCR1</b>                     | eIF3j              | p35         |
|                                 | eIF3k              | p25         |
|                                 | eIF3l              | HSPC021     |
|                                 | eIF3m              | GA17        |

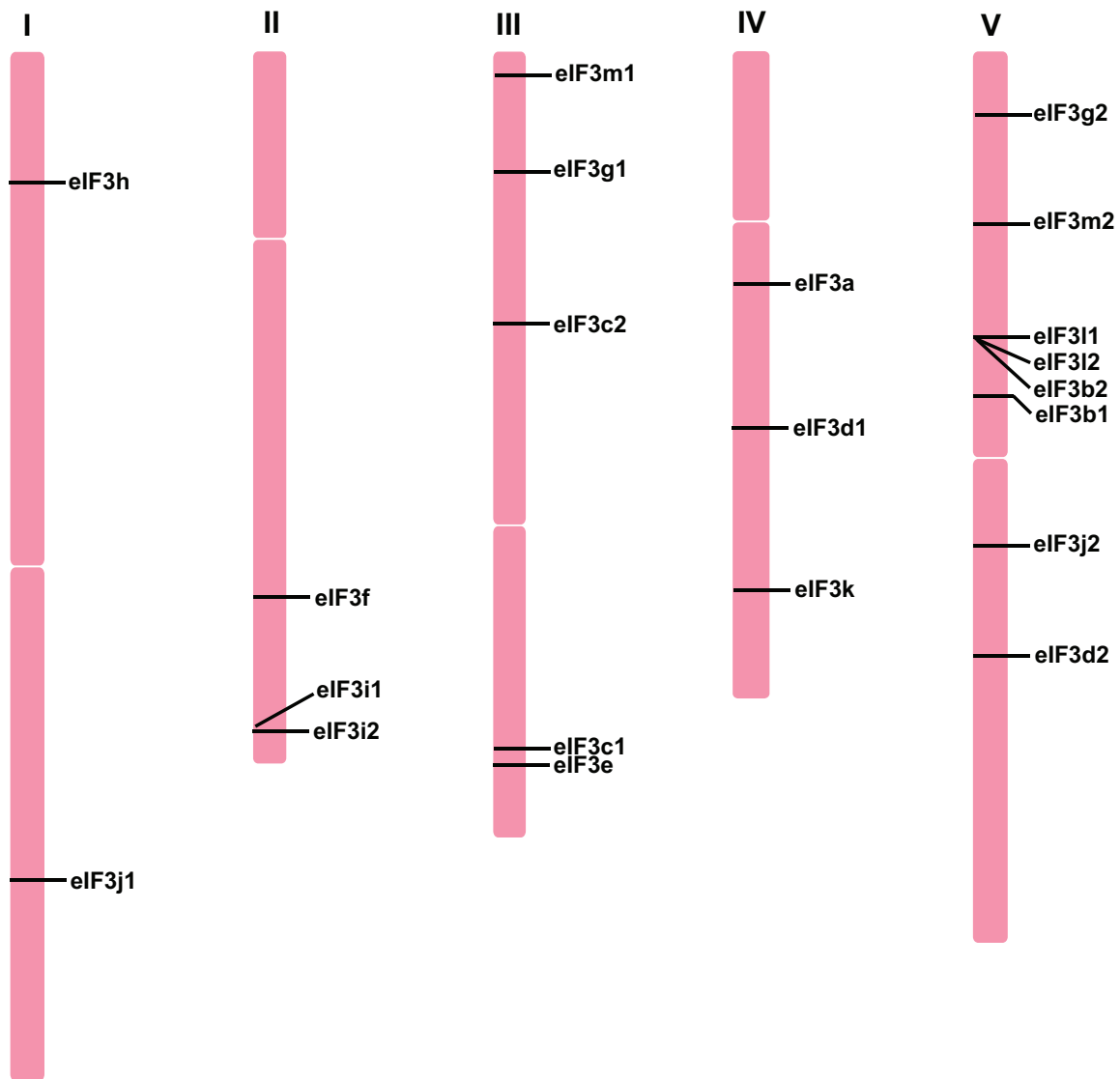


**Figure II.1. eIF3 subunit genes in *Arabidopsis*.** Schematic representation of the positions of eIF3 genes in *Arabidopsis* chromosome.

hypomorphic mutant reported from our lab). This further prods the establishment of mutant models for other eIF3 subunits.

For the functions of eIF3 subunits, especially the regulatory subunits, one possibility is that they contribute to a single biological function. In the extreme case, the absence of any one subunit will result in the same biochemical defect and the same mutant phenotype as the absence of the other. A second logical hypothesis is that there are different classes of eIF3 complexes that in turn have different combinations of accessory subunits. Each sub-complex might regulate a specific set of mRNAs, as reported for fission yeast where there are two distinct eIF3 complexes sharing the same core subunits but distinguished by the PCI proteins eIF3e and eIF3m (Sha et al., 2009; Zhou et al., 2005). Thirdly, it is also plausible that each subunit, or at least several of the subunits, exercise a distinct control mechanism by virtue of the presence of specific domains. For example, it might be possible that the two gene copies of the *Arabidopsis* non-core subunit, eIF3d, which have an mRNA binding domain (Nygard and Westermann, 1982), may have regulatory roles on distinct sets of client mRNAs.

This is an ongoing project whose long-term goal is to elaborate the current understanding that eIF3 is composed of core subunits as well as accessory subunits that may perform specialized functions in translation. In this study, mutants have been identified for 19 of the 21 eIF3 genes. The requirement of specific eIF3 subunits in translation of uORF containing mRNAs is discussed in Chapter 4 and therefore has been omitted from discussion in this chapter. Here, the reverse genetic screen conducted for isolation of the eIF3 mutant strains has been emphasized to highlight the current status of the available mutant collection that has been generated during the course of this study. As will be discussed in later sections, the genetic characterization of eIF3 subunit genes have highlighted the absolute requirement of some of the conserved subunits in *Arabidopsis* development and the non-essential functions of some of the other subunits.

## **II.3 Results**

### **II.3.1 Generation of eIF3 mutant strains by T-DNA insertion screening**

As a first step to study the biological functions of individual eIF3 subunits, collections of T-DNA insertion mutants were screened for insertions in the genes of interest (Alonso et al., 2003; Rosso et al., 2003). The insertion alleles were selected in such a way that both weak (3' insertions) and severe mutants (5' insertions) could be obtained wherever possible. The putative insertion lines were then examined by PCR to confirm the presence of the T-DNA insertion, and were subsequently genotyped to distinguish heterozygotes from homozygotes (**Figure II.2**). The characterization of each individual eIF3 gene is described separately.

#### **eIF3a**

The largest of the eIF3 subunits, AteIF3a, is encoded by a single gene in *Arabidopsis* (At4g11420). Insertion lines were screened from SALK as well as GABI-Kat collections for eIF3a. Unfortunately, the insertion could never be confirmed for any of the lines. The lack of recovery of any T-DNA insertion in the eIF3a locus led to the adoption of another reverse genetic approach that will be discussed in a later section.

#### **eIF3b**

The B subunit of eIF3 is a 82kDa protein (Burks et al., 2001). eIF3b in *Arabidopsis* has two paralogs, AteIF3b1 (At5g27640) and AteIF3b2 (At5g25780). The two paralogs share 91.6 % amino acid sequence identity and are highly similar at the nucleic acid level (73% identity) and differ mainly in the 3<sup>rd</sup> exon sequence (**Figure II.3A**). The Spalign tool from NCBI to identify putative alternatively spliced forms did not suggest an alternative splicing event giving rise to the two paralogs.

Insertion lines were confirmed for both AteIF3b1 and AteIF3b2. Insertion in the 8<sup>th</sup> exon (out of 11 exons) and insertion in the 10<sup>th</sup> exon (out of 11) were identified for AteIF3b1 and AteIF3b2

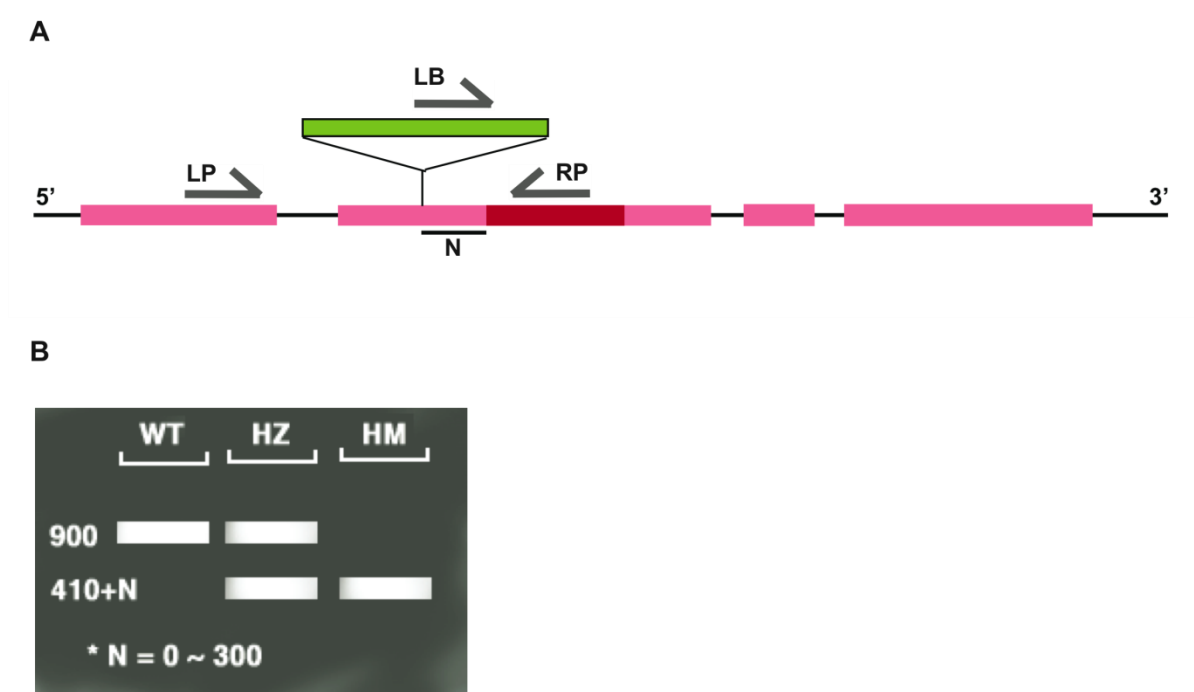


**Figure II.2. PCR genotyping strategy for identification of T-DNA insertion allele.** T-DNA (green box) is inserted in the second exon (pink box) of a hypothetical gene.

**A.** Primers were designed using the iSect tool in SIGnAL

(<http://signal.salk.edu/tdnaprimers.2.html>). Briefly, T-DNA allele is amplified using T-DNA left border primer (LB) and a gene specific primer (RP, right primer) in the flanking region of the T-DNA (red region in the exon). The wild-type allele is amplified with two gene specific primers, LP (left primer) and RP (right primer).

**B.** Representation of expected results from PCR genotyping. The wild-type allele is amplified as ~900 base pair product. The length of the PCR product from the T-DNA allele depends on the distance between the actual site of insertion and the flanking sequence (N), usually 0-300 base pairs.

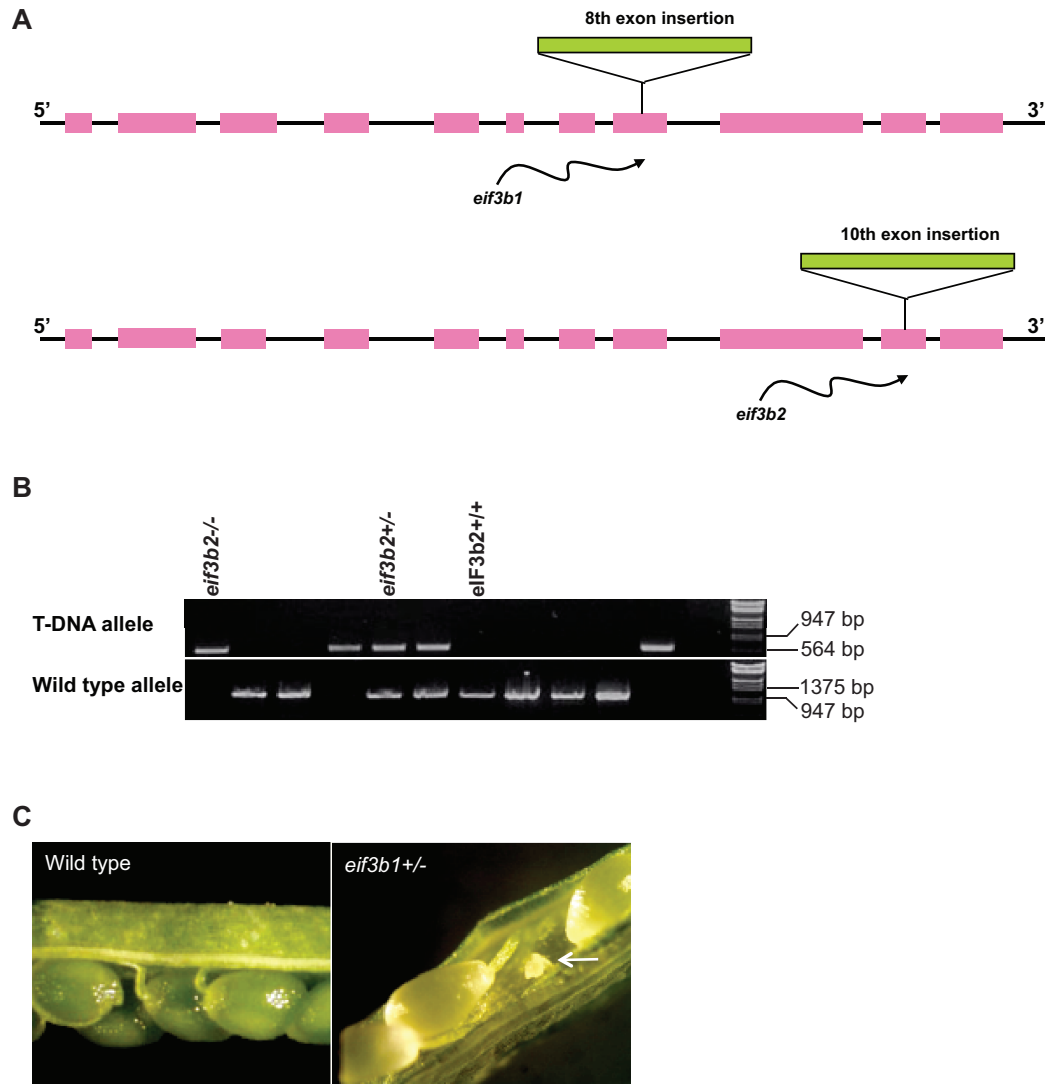


**Figure II.3. T-DNA insertion screening for AteIF3b.**

**A.** Schematic representation of AteIF3b1 and AteIF3b2 loci with confirmed T-DNA insertions. Pink boxes represent exons and green boxes T-DNA.

**B.** A representative ethidium bromide stained agarose gel showing confirmation of *eif3b2* in homozygous condition. The upper panel represents the presence of the T-DNA allele and the lower panel represents the wild-type allele.

**C.** Abnormal seeds in *eif3b1* heterozygous plants are highlighted with an arrow.

respectively (**Figure II.3A**). *elf3b1* was identified only in the heterozygous condition even after maintaining the plants for 3 consecutive generations. Hence, the presence of the *elf3b1* allele preferentially in the heterozygous state implies that the homozygous knockout of eIF3b1 is lethal in *Arabidopsis*. Also, it is absolutely critical to have at least one functional copy of the gene. This can be attributed to, (1) defect in the transmission of the mutant allele through the gametophytes, (2) embryo lethality, or, (3) defect in growth and development during later stages where requirement of EIF3b1 becomes critical. In order to identify the developmental event that requires at least one functional copy of eIF3b1, transmission of the T-DNA was analyzed after reciprocal crosses with wild-type plants (**Table II.2**). Outcrossing experiments revealed that *elf3b1* did not have a reduced transmission through the male or the female gametophyte (**Table II.2**). This also suggests that events after fertilization might be affected by mutations in AteIF3b1.

In order to check if loss of AteIF3b1 leads to a developmental abnormality in the embryos, the developing seeds from *elf3b1* and wild-type plants were analyzed. *Arabidopsis* seed development undergoes characteristic morphological changes starting with change in the outer appearance. Immature seeds are white in color, which then transit to green, and finally a brown appearance as the seeds mature. Siliques of different lengths were removed from the plants and were sliced open to check abnormal seed development. Analysis of developing seeds showed that in comparison to wild type, that had 2% average frequency of abnormal seeds (represented by small ovules or empty positions), *elf3b1* had an average of 13%, lower than the expected 25% abortion if homozygous mutation is lethal (**Table II.3**)(**Figure II.3C, II.4**). To further investigate the seed developmental abnormality associated with AteIF3b1 mutation, developing seeds were observed under light microscope using DIC optics. In comparison to normal seeds, the abnormal seeds do not seem to be having any developing embryo within (data not shown), thus suggesting that the embryos are aborted early on.

**Table II.2. Gametophytic transmission of *elf3b1*.** The transmission of the *elf3b1* allele through the male and the female gamete was tested using reciprocal crosses with wild type. The transmission of the *elf3b-1* allele is normal when *elf3b1* heterozygous plants are used as either male or female parent. Transmission of the T-DNA allele was tested by PCR genotyping of F1 progeny.

| Parental Genotype |                  | Allele transmission to F1 progeny |            |   |
|-------------------|------------------|-----------------------------------|------------|---|
| Female            | Male             | <i>elf3b1</i> /Total              | Percentage | n |
| <i>elf3b1</i> /+  | +/+              | 21/46                             | 46         | 6 |
| +/+               | <i>elf3b1</i> /+ | 23/41                             | 56         | 6 |
| <i>elf3b1</i> /+  | <i>elf3b1</i> /+ | 32/45                             | 71         | 2 |

**Table II.3. Embryo lethality in *elf3b1* and *elf3c1*.** Plants were genotyped using PCR to confirm heterozygosity. Wild-type and mutant siliques were opened to reveal seed content. Normal seeds as well as aborted seeds were scored. *elf3b1* and *elf3c1* heterozygous plants showed higher seed abortion as compared to wild type. The siliques were grouped according to the developmental stage of the embryos. Immature siliques consisted of embryos in globular stage, intermediate siliques had early and late heart stage embryos and mature siliques had torpedo stage embryos.

|                      | <b>Group I<br/>(immature)</b><br>Silique length<br>0.5cm-0.9cm<br>n=31 (siliques) |                               | <b>Group II<br/>(intermediate)</b><br>Silique length<br>1.0cm-1.4cm<br>n=50 (siliques) |                               | <b>Group III<br/>(mature)</b><br>Silique length<br>> 1.5 cm<br>n=42 (siliques) |                               |
|----------------------|-----------------------------------------------------------------------------------|-------------------------------|----------------------------------------------------------------------------------------|-------------------------------|--------------------------------------------------------------------------------|-------------------------------|
|                      | Normal<br>seeds<br>(average)                                                      | Aborted<br>seeds<br>(average) | Normal<br>seeds<br>(average)                                                           | Aborted<br>seeds<br>(average) | Normal<br>seeds<br>(average)                                                   | Aborted<br>seeds<br>(average) |
| <b>Wild type</b>     | 39                                                                                | 0                             | 43                                                                                     | 1                             | 49                                                                             | 1                             |
| <b><i>elf3b1</i></b> | 35                                                                                | 3                             | 37                                                                                     | 3                             | 45                                                                             | 4                             |
| <b><i>elf3c1</i></b> | 32                                                                                | 2                             | 36                                                                                     | 9                             | 41                                                                             | 7                             |

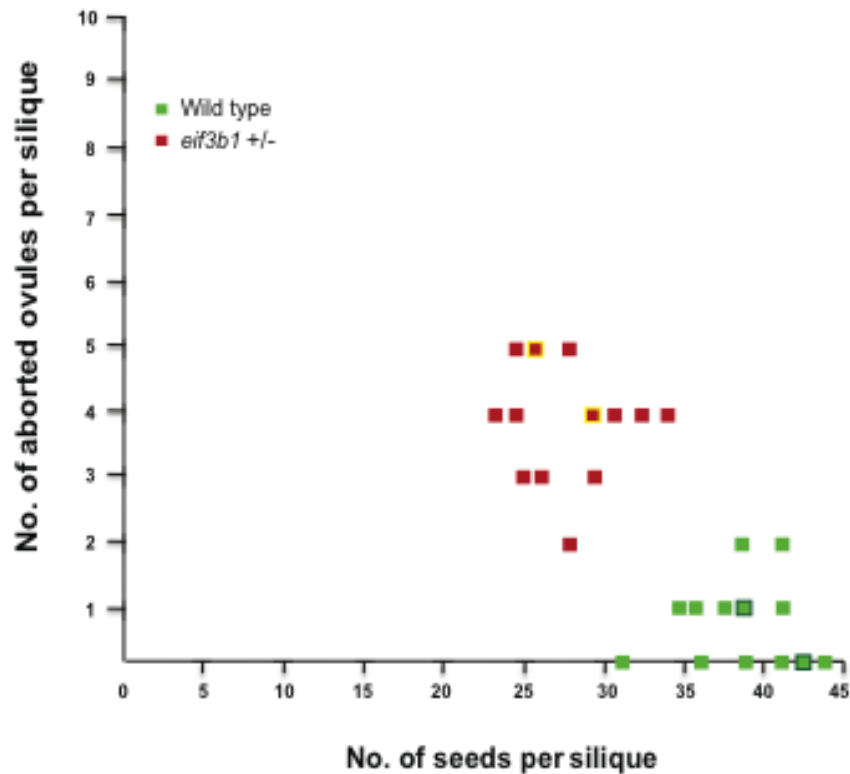


**Figure II.4. Embryo lethality in *eif3b1*.** Reduced seed set in mature siliques of *eif3b1*+/- plants were scored by clearing the silique walls with chloral hydrate and counting normal vs abnormal seeds (often represented as empty position). Treatment with chloral hydrate without slicing open the siliques ensured that seeds are not lost during sample preparation. Also, random abortion events (usually represented by loss of seeds from the base of the silique) could be differentiated from abortion due to loss of eIF3b1 (represented by unbiased distribution of the aborted seeds in the silique). Comparison with wild type (average number of seeds = 40; n=15) showed a reduced number of seeds in *eif3b1* (average number of seeds = 29; n=15). Furthermore, there is a higher representation of aborted seeds in *eif3b1*. Multiple data points are represented by solid lines.

In contrast to *eif3b1*, *eif3b2* mutant allele was recovered in homozygous condition (**Figure II.3B**). The characterization of the homozygous T-DNA insertion line was further extended by investigating the mRNA expression levels. The full-length eIF3b2 transcript was however detected in the homozygous progeny.

### eIF3c

Similar to eIF3b, eIF3c in *Arabidopsis* is encoded by two paralogs AteIF3c1 (At3g56150) and AteIF3c2 (At3g22860) sharing 74.9% amino acid sequence identity. Insertion lines were identified for both the genes, eIF3c1 with an insertion in the 5' UTR and two independent insertion lines for eIF3c2 (*eif3c2-1* and *eif3c2-2*), both with insertion in exon 1 (out of 2) (**Figure II.5A**).

Outcrossing *eif3c1*, isolated only in heterozygous condition, revealed that eIF3c1 was dispensable for the male and female gametophyte development (**Table II.4**). However, the loss of AteIF3c1 affects the development of embryos and results in early embryo abortion similar to AteIF3b1, though the extent is more severe (**Figure II.5C; II.6, Table II.3**). In contrast to wild type where seed abortion frequency was around 2%, *eif3c1* had 21% seed abortion frequency. Furthermore, analysis of the developing embryos suggested an early abortion (data not shown). As for eIF3c2, both the independent lines for AteIF3c2 were recovered in the homozygous condition. However, further characterization of *eif3c2* was complicated by the fact that the full-length transcript could never be detected with RT-PCR in either wild type or mutant. Available information from a gene expression database shows that eIF3c2 is expressed at very low levels in *Arabidopsis* with the exception of its expression in endosperm (Genevestigator (Hruz et al., 2008); **Appendix II.1**).

### eIF3d

AteIF3d1 (At4g20980) and AteIF3d2 (At5g44320) encode eIF3d in *Arabidopsis*. The two paralogs share 87.9% identity at the amino acid level. Insertion alleles from the SALK T-DNA collection

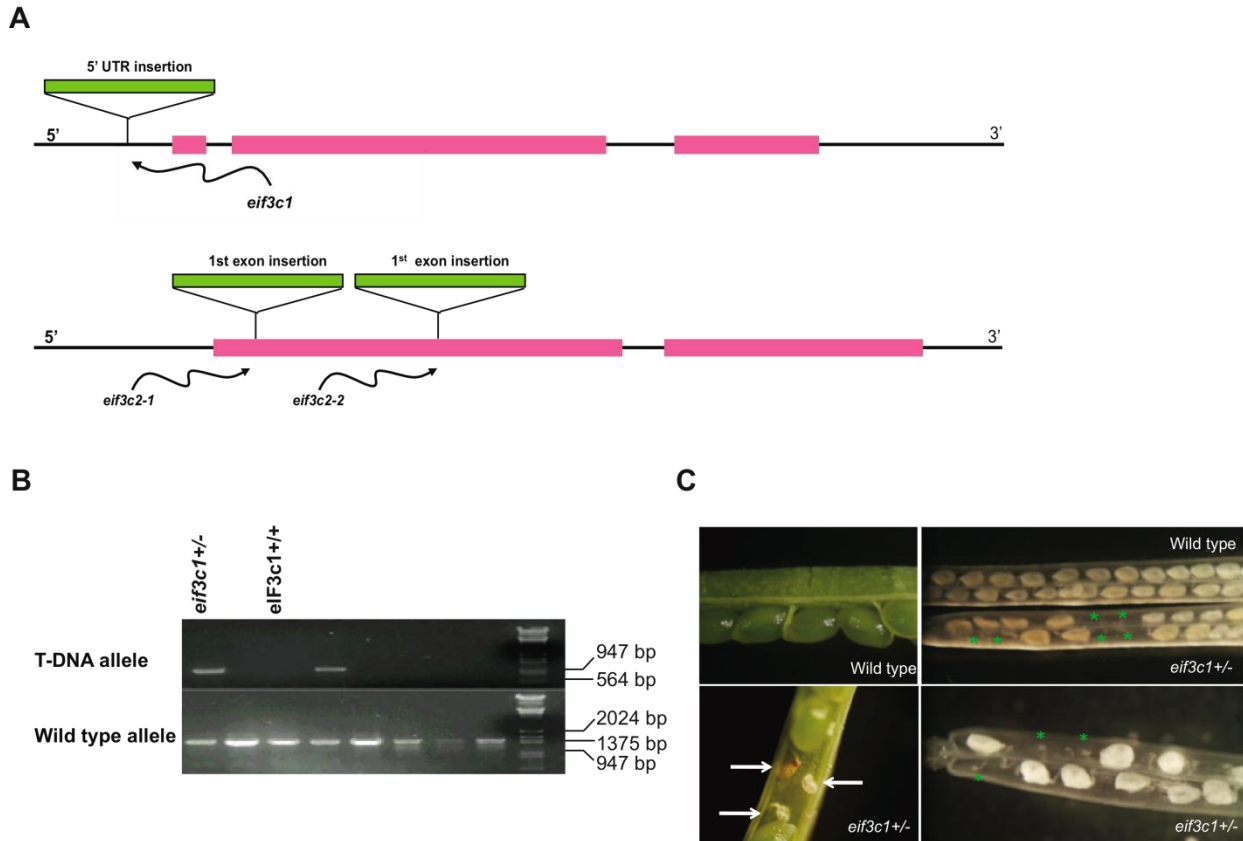


**Figure II.5. T-DNA insertion screening for AteIF3c.**

**A.** Schematic representation of AteIF3c1 and AteIF3c2 loci with confirmed T-DNA insertions. Pink boxes represent exons and green boxes T-DNA.

**B.** A representative ethidium bromide stained agarose gel showing confirmation of *eif3c1* allele and identification of *eif3c1*<sup>+/-</sup> plants. The upper panel represents the presence of the T-DNA allele and the lower panel represents the wild-type allele.

**C.** Abnormal seeds in *eif3c1* heterozygous plants are highlighted with an arrow. Cleared siliques showing empty positions in the mature siliques of *eif3c1*<sup>+/-</sup> plants in the right panel with asterisk representing empty seed spots.

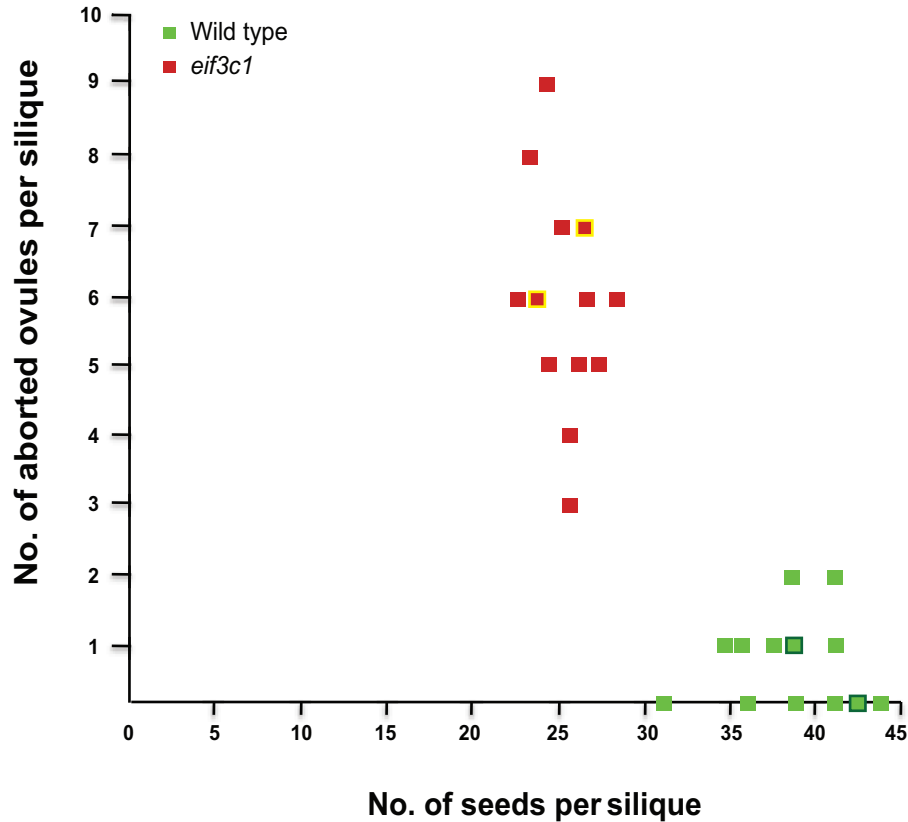


**Figure II.6. Embryo lethality in *eif3c1*.** Reduced seed set in mature siliques of *eif3c1*+/- plants were scored by clearing the silique walls with chloral hydrate and counting normal vs abnormal seeds (often represented as empty positions). Treatment with chloral hydrate without slicing open the siliques ensured that seeds are not lost during sample preparation. Also, random abortion events (usually represented by loss of seeds from base of the silique) could be differentiated from abortion due to loss of eIF3c1 (represented by unbiased distribution of the aborted seeds in the silique). Comparison with wild type (average number of seeds = 40; n=15) showed a reduced number of seeds in *eif3c1* (average number of seeds = 26; n=20). There is a higher representation of aborted seeds in *eif3c1*. Multiple data points are represented by solid lines.

**Table II.4. Gametophytic transmission of *elf3c1*.** The transmission of the *elf3c1* allele through the male and the female gamete was tested using reciprocal crosses with wild type. The transmission of the *elf3c1* allele is normal when *elf3c1* heterozygous plants are used as either male or female parents. Transmission of the T-DNA allele was tested with PCR genotyping of F1 progeny.

| Parental Genotype |                  | Allele transmission to F1 progeny |            |   |
|-------------------|------------------|-----------------------------------|------------|---|
| Female            | Male             | <i>elf3c1</i> /Total              | Percentage | n |
| <i>elf3c1</i> /+  | +/+              | 26/49                             | 54         | 4 |
| +/+               | <i>elf3c1</i> /+ | 19/43                             | 44         | 5 |
| <i>elf3c1</i> /+  | <i>elf3c1</i> /+ | 25/37                             | 67         | 3 |

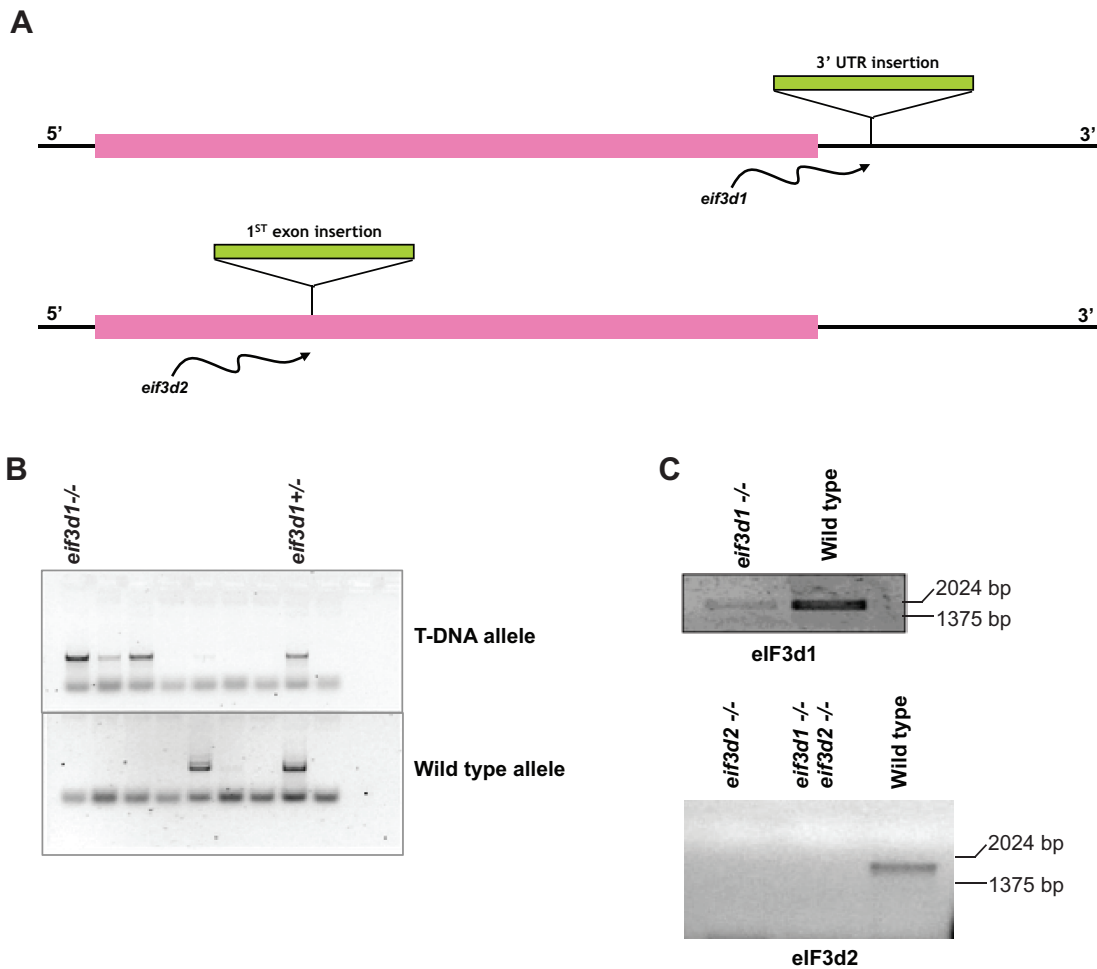


**Figure II.7. T-DNA insertion screening for AteIF3d.**

**A.** Schematic representation of AteIF3d1 and AteIF3d2 loci with confirmed T-DNA insertions. Pink boxes represent exons and green boxes T-DNA.

**B.** A representative ethidium bromide stained agarose gel showing confirmation of *eif3d1* allele and identification of *eif3d1*<sup>-/-</sup> plants. The upper panel represents the presence of the T-DNA allele and the lower panel represents the wild-type allele.

**C.** RT-PCR analysis for testing the mRNA expression level in the *eif3d*<sup>-/-</sup> plants. Upper panel shows the presence of the eIF3d1 transcript in the *eif3d1*<sup>-/-</sup> plants. Bottom panel represents the RNA analysis for eIF3d2 transcript. eIF3d2 transcript is absent in *eif3d2*<sup>-/-</sup> plants as well as in the putative *eif3d* double mutant (*eif3d1*<sup>-/-</sup> *eif3d2*<sup>-/-</sup>).

were obtained for both eIF3d1 and eIF3d2 and mutant alleles were confirmed to be present in homozygous condition for both the genes (**Figure II.7B**). Insertion in the 3' UTR in *eif3d1* (**Figure II.7A**), however, did not affect the gene expression drastically as a full-length eIF3d1 transcript could still be detected albeit at a lower level when gene specific primers were used to amplify the full-length transcript using RT-PCR (**Figure II.7C**). In contrast, the full-length eIF3d2 transcript was not amplified when primers across the insertion were used (**Figure II.7C**) and truncated transcript for eIF3d2 could not be detected as well.

Gross phenotype analysis of the single homozygous plants did not reveal any difference as compared to wild type. The lack of any significant phenotype in the single mutants might be because of redundancy in function shared between the two genes of the same family. To rule out such a redundancy, double mutants were generated for eIF3d and is under investigation.

### **eIF3e**

eIF3e in *Arabidopsis* is encoded from a single gene (At3g57290). Two insertion lines were used in this study, one with an insertion in exon 3 (out of 8) and another with an insertion in the 3' UTR. *eif3e* *-/-* progenies were not identified for both the alleles and were isolated in heterozygous condition in three consecutive generations. Further analysis revealed that eIF3e is important for male gametophyte development (discussed in Chapter 5).

### **eIF3f**

eIF3f is another single gene (At2g39990) encoded protein in *Arabidopsis* that shares the conserved MPN domain (**M**pr1p, **P**ad1p **N**-terminal domain) with eIF3h. Unfortunately, screening of SALK as well as GABI Kat insertion collections was unsuccessful in identifying confirmed insertion lines. However, an insertion line from INRA Versailles with an insertion in the 3<sup>rd</sup> exon was obtained and the insertion was confirmed with PCR (**Figure II.8A**). The lines have been screened for one generation and only heterozygous insertions were identified.

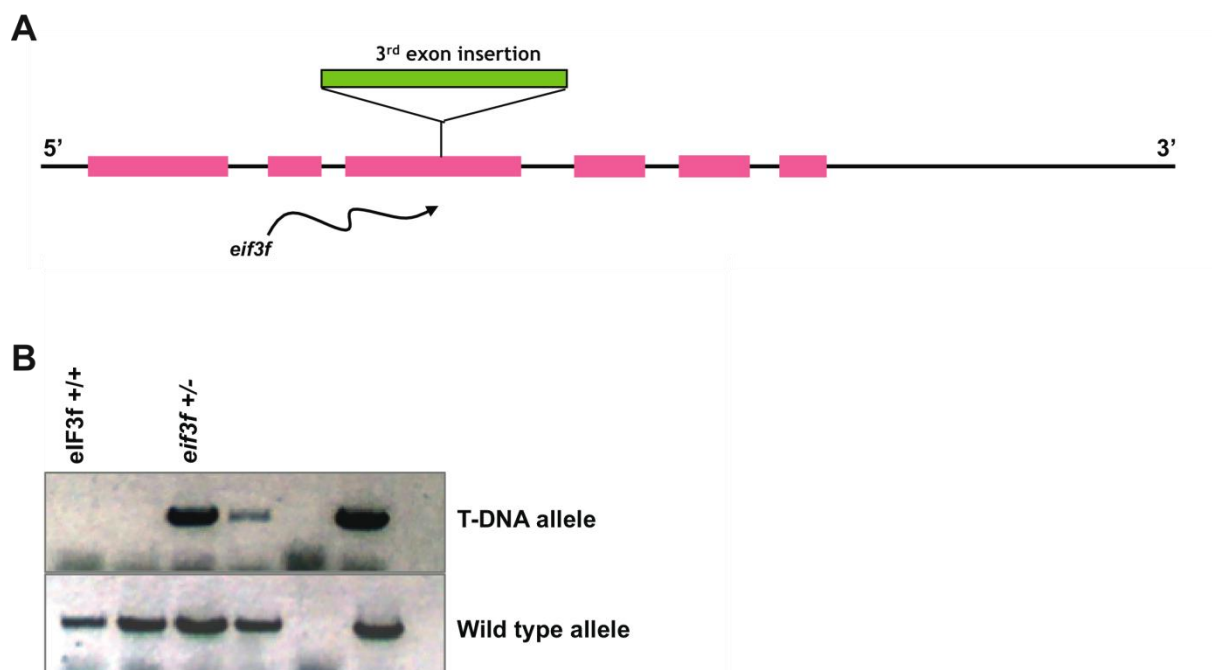


**Figure II.8. T-DNA insertion screening for AteIF3f.**

**A.** Schematic representation of *eIF3f* locus with confirmed T-DNA insertion in the 3<sup>rd</sup> exon. Pink boxes represent exons and green box T-DNA.

**B.** A representative ethidium bromide stained agarose gel showing confirmation of *eif3f*+/- allele and identification of *eif3f*+/- plants. The upper panel represents the presence of the T-DNA allele and the lower panel represents the wild-type allele.

### eIF3g

*Arabidopsis* has two paralogs for eIF3g, AteIF3g1 (At3g11400) and AteIF3g2 (At5g06000) sharing 60% amino acid sequence identity. Two independent T-DNA insertion alleles for AteIF3g1 were confirmed, *elf3g1-1* with insertion in exon 2 (out of 2) and *elf3g1-2* with insertion in the 5' UTR (**Figure II.9A**). Homozygous *elf3g1-1* and *elf3g1-2* progenies were identified (**Figure II.9B**) and RNA analysis revealed that the insertion in exon 2 disrupts expression of the transcript (**Figure II.9C**).

Insertion in intron 1 (out of 1) was confirmed for AteIF3g2 and homozygous progenies were identified. Similar to *elf3g1*, the full-length transcript was absent in *elf3g2* (**Figure III.9C**). The single eIF3g mutants resembled the wild type under standard growth conditions and therefore double mutants were generated to rule out possible functional redundancy and is currently under investigation.

### eIF3i

Another family of duplicate genes, eIF3i, is encoded by AteIF3i-1 (At2g46280) and AteIF3i-2 (At2g46290). Two insertion lines were identified for eIF3i-1; insertion in exon 1 (out of 5) (*elf3i1-1*) and exon 2 (out of 5) (*elf3i1-2*) (**Figure II.10A**). Both these insertion alleles were preferentially present in the heterozygous condition and outcrossing experiments with wild-type plants demonstrated a defect in the transmission of the *elf3i-1* allele through the male gametophyte (**Table II.5**). However, technical difficulties in confirming the insertion restricted the population size that was screened.

No insertion alleles were identified for eIF3i-2. Moreover, the tight linkage between the two genes would further complicate the generation of double mutants that might be necessary to rule out functional redundancy. However, an RNAi line for eIF3i has been reported before (Jiang and Clouse, 2001), that results in severe morphological defects (**Figure II.10C**).

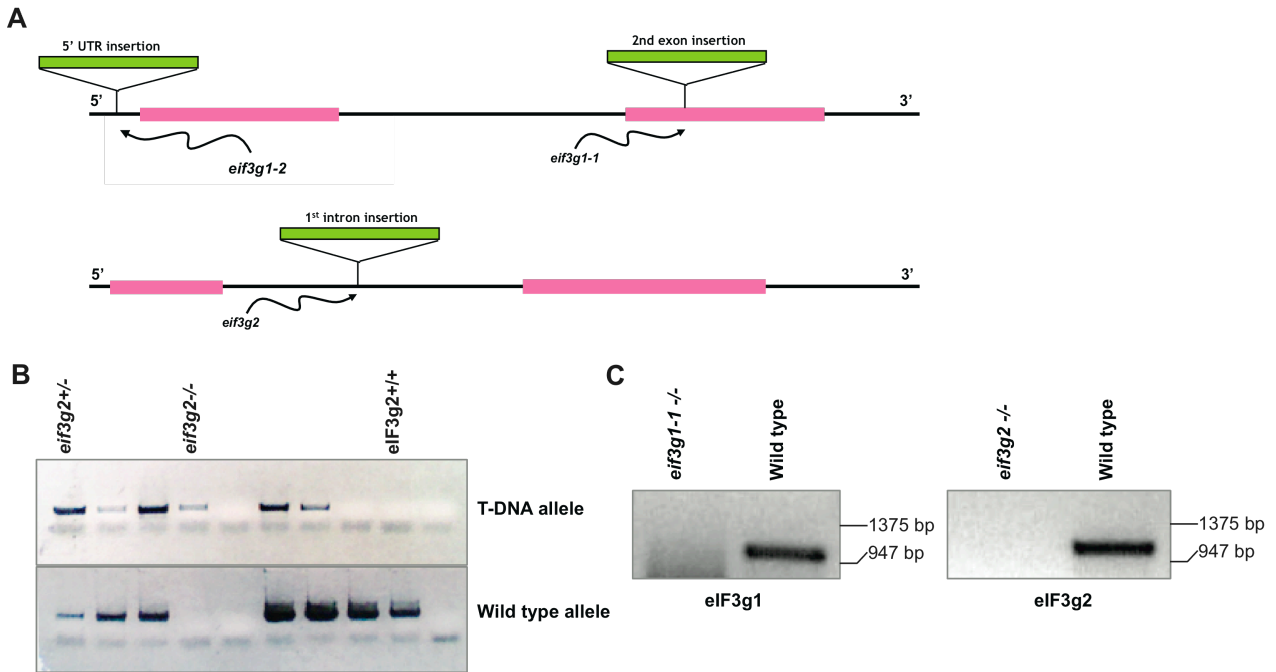


**Figure II.9. Insertion screening for AteIF3g.**

**A.** Schematic representation of AteIF3g1 and AteIF3g2 loci with confirmed T-DNA insertions. Pink boxes represent exons and green boxes T-DNA.

**B.** A representative ethidium bromide stained agarose gel showing confirmation of *eif3g2* allele and identification of *eif3g2*<sup>-/-</sup> plants. The upper panel represents the presence of the T-DNA allele and the lower panel represents the wild-type allele.

**C.** RT-PCR analysis for testing the mRNA expression level in the *eif3g*<sup>-/-</sup> plants. Left panel shows that the eIF3g1 transcript was not amplified in the *eif3g1-1*<sup>-/-</sup> plants that were identified using PCR. Right panel represents the RNA analysis from *eif3g2*<sup>-/-</sup> and wild-type plants showing an absence of the eIF3g2 transcript in homozygous plants.

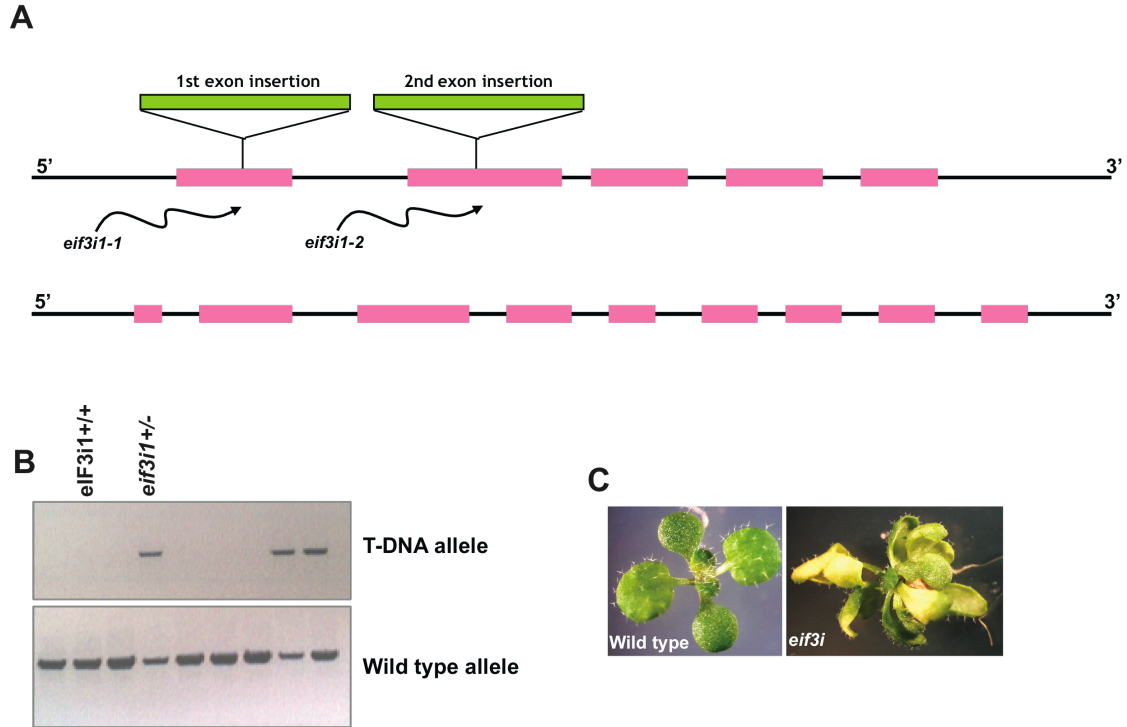


**Figure II.10. Insertion screening for AteIF3i.**

**A.** Schematic representation of AteIF3i1 and AteIF3i2 loci with confirmed T-DNA insertion in the AteIF3i-1 locus. Pink boxes represent exons and green boxes T-DNA.

**B.** A representative ethidium bromide stained agarose gel showing confirmation of *eif3i1-1* allele and identification of *eif3i1-1*+/− plants. The upper panel represents the presence of the T-DNA allele and the lower panel represents the wild-type allele. Note that there is a reduced representation of the T-DNA among the progenies.

**C.** *eif3i* (RNAi line) shows morphological defects as compared to wild type.

**Table II.5. Genetic analysis of *elf3i-1* plants – gametophytic transmission of *elf3i-1* allele.** The transmission of the *elf3i-1* allele is reduced when *elf3i-1* heterozygous plants were used as male parents. Transmission of the T-DNA allele was tested by PCR genotyping of the F1 progenies.

| Parental Genotype |                   | Allele transmission to F1 progeny |            |   |
|-------------------|-------------------|-----------------------------------|------------|---|
| Female            | Male              | <i>elf3i-1</i> /Total             | Percentage | n |
| <i>elf3i-1</i> /+ | +/+               | 6/15                              | 40         | 2 |
| +/+               | <i>elf3i-1</i> /+ | 4/17                              | 23         | 2 |

### eIF3j

At1g66070 and At5g37475 encode AteIF3j1 and AteIF3j2 in *Arabidopsis*. Insertion alleles have been identified for both the genes. For eIF3j1, insertion in exon 4/6 in heterozygous condition has been identified (**Figure II.11A**). The confirmation of the genotype for this line was complicated by the fact that the detection of the insertion was difficult. Initially, putative homozygous lines were identified but they segregated into wild type, and heterozygous in the next generation. Furthermore, the newly released status of Salk T-DNA lines (after PCR confirmation) also lists this insertion to be present in homozygous condition. The inconsistency with this selected line led to the screening of other available T-DNA lines for eIF3j1. Another insertion line with putative insertion in intron 1/5 was obtained but unfortunately, the insertion could not be confirmed. Multiple insertions were confirmed for eIF3j2, two with insertion in exon 2 (out of 6) (*eif3j2-1* and *eif3j2-2*) and another with insertion in exon 4 (out of 6) (*eif3j2-3*). *eif3j2-1* and *eif3j2-2* were both present in the heterozygous condition whereas *eif3j2-3* was found in homozygous condition (**Figure II.11B**).

### eIF3k

*Arabidopsis* eIF3k (At4g33250) is a 25kDa protein. Insertion in intron 6 (out of 6) (**Figure II.12A**) of eIF3k was identified from the Gabi-Kat collection and the insertion was recovered in the homozygous condition. Further analysis of the transcript level showed that even though the full-length transcript was absent in *eif3k* mutants, a partial transcript was still being made (**Figure II.12B**). The homozygous eIF3k mutants looked similar to wild type under regular growth condition (**Figure II.12A**) and overall translation state compared by polysome analysis resembled the wild type as well (**Figure II.12C**).

### eIF3m

Sharing about 76% sequence identity, AteIF3m1 (At3g02200) and AteIF3m2 (At5g15610) encode the m subunit in *Arabidopsis*. For AteIF3m1, insertion in exon 6 (out of 9) (*eif3m1-1*) and 3' UTR

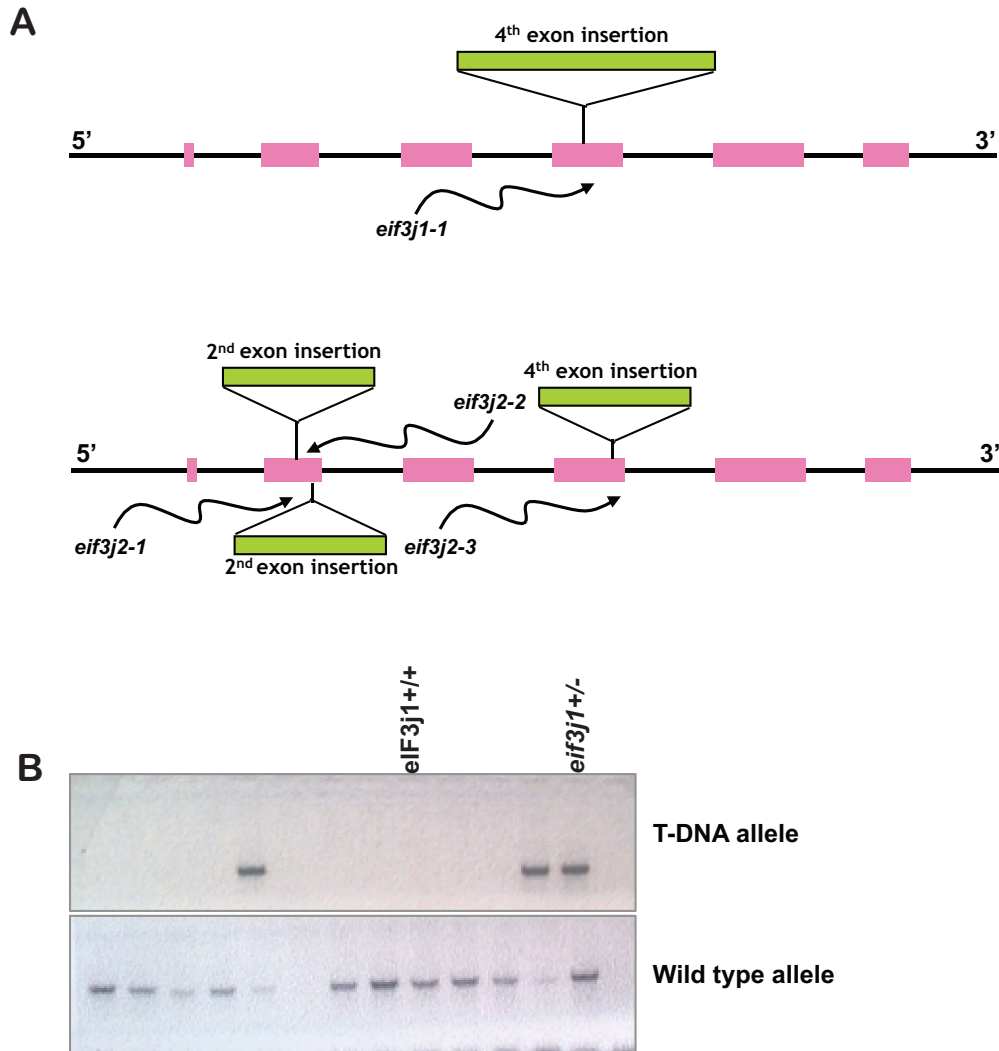
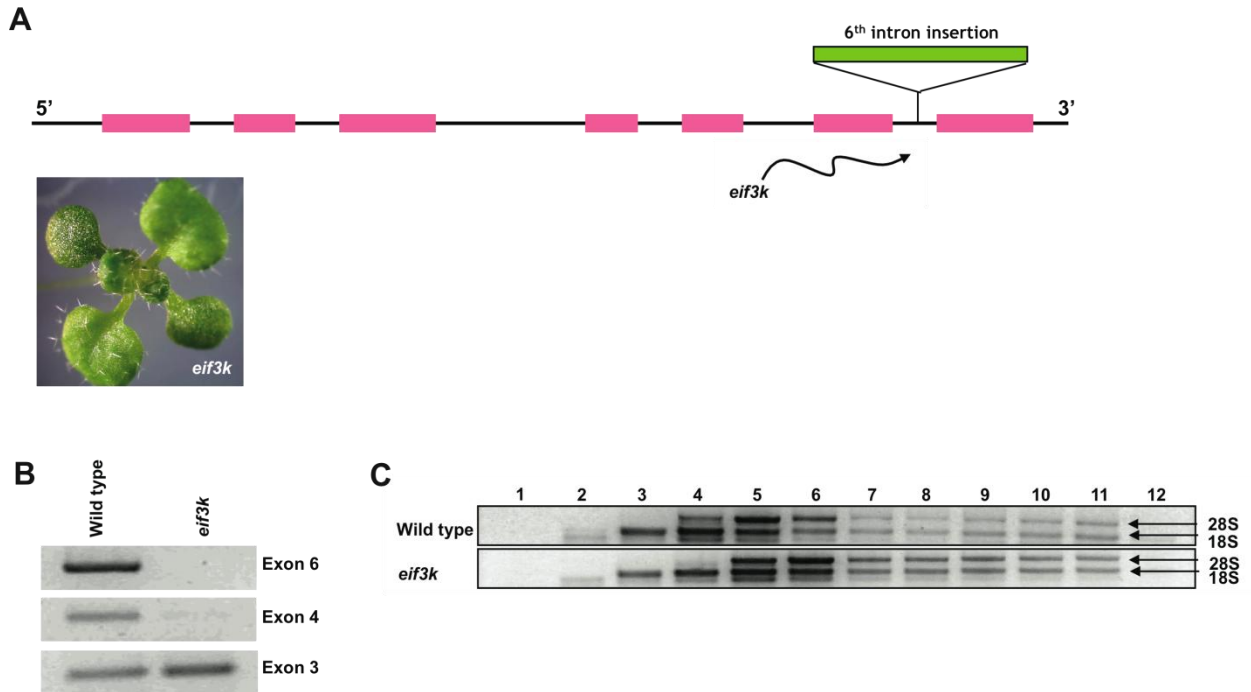


**Figure II.11. Insertion screening for AteIF3j.**

**A.** Schematic representation of AteIF3j1 and AteIF3j2 loci with confirmed T-DNA insertions.

Pink boxes represent exons and green boxes T-DNA.

**B.** A representative ethidium bromide stained agarose gel showing confirmation of *eif3j1* allele. The upper panel represents the presence of the T-DNA allele and the lower panel represents the wild-type allele.



### Figure II.12. Insertion screening for AteIF3k.

**A.** Schematic representation of AteIF3k locus with confirmed T-DNA insertion. Pink boxes represent exons and green box T-DNA.

**B.** RNA analysis for confirmation of the transcript level. Presence of full-length transcript was examined using primers in the 1<sup>st</sup> and 8<sup>th</sup> exon. A full-length transcript is absent in *eif3k* but a truncated transcript was detected. Presence of partial transcripts were tested using primer in the 3<sup>rd</sup> exon and 4<sup>th</sup> exon.

**C.** The translation state of *eif3k* is similar to wild type. Distribution of 18S and 28S rRNA after sucrose density gradient fractionation revealed similar polysome profiles in wild type and *eif3k*.

(*eif3m1-2*) were confirmed in heterozygous and homozygous conditions respectively (**Figure II.13A**). *eif3m1-2* allele however does not affect the expression of eIF3m1 as evident from the transcript analysis (**Figure II.13B**). For AteIF3m2, insertion in exon 8 (out of 9) was identified in the homozygous condition and the full-length transcript was not detected (**Figure II.13C**) meaning that the insertion disrupts eIF3m2 expression.

eIF3m single mutants did not show any phenotypic difference from the wild type and double mutants were generated. The transcript analysis of the double mutants confirmed the RNA analysis from the single mutants; full-length transcript was detected for eIF3m1 but not for eIF3m2. However, it needs to be tested if a partial transcript is still present in the *eif3m2* mutants. The double mutants are currently being analyzed for phenotypic defects.

### **II.3.2 RNAi for eIF3**

The lack of any T-DNA insertion mutant for some of the eIF3 subunits led to the adoption of another reverse genetic approach to address the function of eIF3 subunits. A large collection of RNAi silencing constructs available via the AGRIKOLA (Arabidopsis Genomic RNAi Knock-out Line) project was taken advantage of for obtaining silencing lines for the eIF3 genes (Hilson et al., 2003). However, delivery clones with gene specific tag (GST) cloned into a hairpin construct were available only for eIF3a, eIF3d1, eIF3d2, eIF3f, eIF3k at that time (**Table II.6**) (as of July, 2007). The available delivery clones were requested and the presence of the GST was confirmed using PCR. The RNAi T-DNA was transformed into *Arabidopsis* to obtain stable transgenics. First generation transgenics were screened for Basta resistance. 10-15 transgenic plants were obtained for eIF3a, eIF3f and eIF3k. The transformation efficiency was low for eIF3d1 and eIF3d2 and consequently only 2-3 transgenic lines were obtained. The knockdown of the eIF3 gene of interest in the silencing lines was then examined by checking the corresponding eIF3 transcript level by RT-PCR. Unfortunately, none of the lines recovered showed a knockdown of the transcript and therefore was not pursued further.

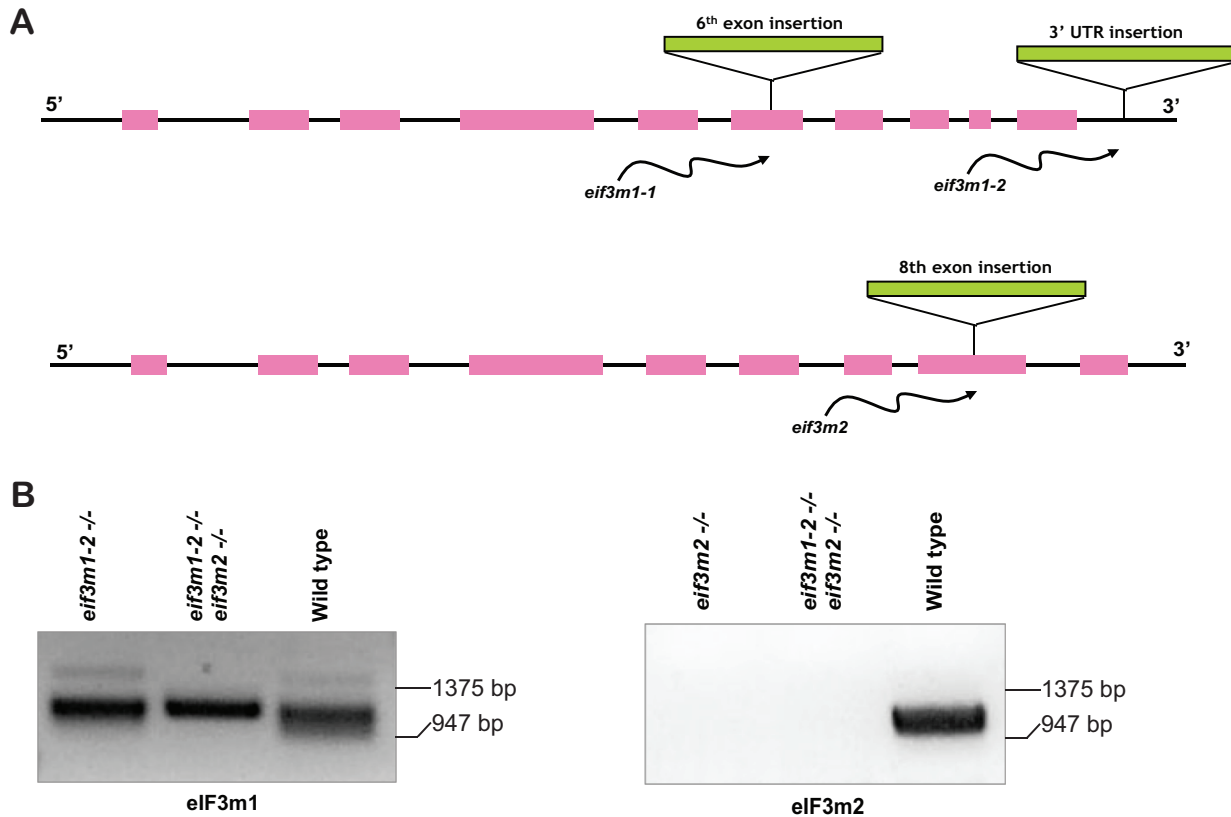


**Figure II.13. Insertion screening for AteIF3m.**

**A.** Schematic representation of AteIF3m1 and AteIF3m2 loci with confirmed T-DNA insertions. Pink boxes represent exons and green boxes T-DNA.

**B.** RNA analysis for mRNA expression level in *eif3m1* and *eif3m2*. Left panel shows detection of the full-length eIF3m transcript in *eif3m1-2 -/-* plants as well as *eif3m* double mutants by RT-PCR. Panel on right shows the amplification of eIF3m2 transcript from wild-type plants but not from *eif3m1-/-* and *eif3m* double mutants.

**Table II.6A. Delivery clones for RNAi from AGRIKOLA.**

| <b>eIF3 gene</b> | <b>Stock No</b> | <b>Catalog No</b> |
|------------------|-----------------|-------------------|
| eIF3a            | N227836         | Catma4a1155       |
| eIF3d1           | N226617         | Catma4a2252       |
| eIF3d2           | N248605         | Catma5a4008       |
| eIF3f            | N243340         | Catma2a3821       |
| eIF3k            | N228095         | Catma4a3500       |

**Table II.6B. Oligonucleotides to confirm the gene specific tag in destination vectors**

|                | <b>Oligonucleotide sequence</b>  |
|----------------|----------------------------------|
| <b>Agri 51</b> | 5' CAA CCA CGT CTT CAA AGC AA 3' |
| <b>Agri 56</b> | 5' CTG GGG TAC CGA ATT CCT C 3'  |
| <b>Agri 64</b> | 5' CTT GCG CTG CAG TTA TCA TC 3' |
| <b>Agri 69</b> | 5' AGG CGT CTC GCA TAT CTC AT 3' |

## II.4 Discussion

Using the available genetic resources in *Arabidopsis*, the main objective of this ongoing study is to characterize the eIF3 subunits functionally. Currently, out of the 21 eIF3 subunit genes, mutants have been screened for 19 of them and at least one mutant allele has been confirmed for 17 eIF3 genes (**Table II.7**).

Surprisingly, the genetic analyses revealed that many of the viable *eif3x* mutants are not morphologically compromised, in contrast to what has been observed for *eif3h* (Kim et al., 2004) and *eif3i* (Jiang and Clouse, 2001). For some of the single mutants (*eif3b2*, *c2*, *d1*, *d2*, *g1*, *g2*, *m2*), the functional redundancy shared with duplicate genes of the same family can explain the reduced severity. However, double mutants for eIF3d and eIF3m did not show any striking phenotype as well. It needs to be considered that, for both eIF3d1 and eIF3m1, the insertion in the 3' UTR does not affect the transcript status. In absence of any other available T-DNA insertion in these two loci, it is worth checking the developmental defects of the double mutants under varying physiological conditions when the requirement for one isoform might be critical as compared to the other.

In contrast to subunits encoded by duplicate genes, *eIF3k* in *Arabidopsis* is a single gene and homozygous insertion mutants were identified for *eIF3k*. Surprisingly, though a mild transcriptional defect has been observed in *eif3k*, it does not affect the morphology or the overall translation state. Therefore, the function of this non-conserved subunit is still not clear and might not affect any general housekeeping processes. Furthermore, a null *eif3k* allele in *Caenorhabditis elegans* is viable (Lin et al., 2008). The regulatory functions of eIF3k, if any, might only be apparent under specific conditions and need to be investigated further.

In contrast to the homozygous mutants, several other insertion mutants have only been recovered as heterozygotes (*eif3b1*, *c1*, *e*, *f*, *i-1*), suggesting that these genes are essential for development and absence of a functional copy is detrimental. These eIF3 subunit genes were observed to belong to two distinct groups, (1) important for gametophyte development (eIF3e, h,

**Table II.7. Available confirmed T-DNA insertion lines for eIF3.** The table below summarizes the status of the T-DNA insertion screening. \* AGI (Arabidopsis Genome Initiative Number), <sup>&</sup>Insertion in exon 8 out of 11 exons, <sup>§</sup>SALK insertion collection, <sup>¶</sup>GABI KAT insertion collection, <sup>#</sup> FLAG insertion collection.

| eIF3 Subunit<br>Gene |           | Insertion allele         |                                            |                  |              |
|----------------------|-----------|--------------------------|--------------------------------------------|------------------|--------------|
| No                   | AGI       | Line No.                 | Annotated site of insertion                | Insertion status | Genotype     |
| a                    | AT4G11420 |                          |                                            |                  |              |
| b1                   | AT5G27640 | SALK_047620 <sup>§</sup> | Exon 8/11 <sup>&amp;</sup>                 | Confirmed        | Heterozygous |
| b2                   | AT5G25780 | SALK_126794              | Exon 10/11                                 | Confirmed        | Homozygous   |
| c1                   | AT3G56150 | SALK_120072              | 5'UTR<br>(321 bp upstream of start codon)  | Confirmed        | Heterozygous |
| c2                   | AT3G22860 | SALK_101653              | Exon 1/2                                   | Confirmed        | Homozygous   |
|                      |           | SALK_143551              | Exon 1/2                                   | Confirmed        | Homozygous   |
| d1                   | AT4G20980 | SALK_150238              | 3' UTR<br>(238bp downstream of stop codon) | Confirmed        | Homozygous   |
| d2                   | AT5G44320 | SALK_085397              | Exon 1/1                                   | Confirmed        | Homozygous   |
| e                    | AT3G57290 | SALK_121004              | Exon 3/8                                   | Confirmed        | Heterozygous |
|                      |           | SALK_003909              | 5'UTR                                      | No Insert        |              |
|                      |           | SALK_113234              | (399bp upstream of start codon)            |                  |              |
|                      |           |                          | 3'UTR<br>(352bp downstream of stop codon)  | Confirmed        | Heterozygous |
| f                    | AT2G39990 | FLAG_41307               | Exon 3/6                                   | Confirmed        | Heterozygous |
| g1                   | AT3G11400 | SALK_029432              | Exon 2/2                                   | Confirmed        | Homozygous   |
| g2                   | AT5G06000 | SALK_070257              | Intron 1/1                                 | Confirmed        | Homozygous   |

|    |           |                       |                                          |                 |              |
|----|-----------|-----------------------|------------------------------------------|-----------------|--------------|
| h  | AT1G10840 | Wisconsin             | Exon 10/12                               | Kim et al. 2004 | Homozygous   |
|    |           | SALK_012027           | Intron 11/11                             | Kim et al. 2004 | Homozygous   |
|    |           |                       |                                          |                 | Heterozygous |
| i1 | AT2G46280 | SALK_028797           | Exon 2/5                                 | Confirmed       | Heterozygous |
|    |           | SALK_015367           | Exon1                                    | Confirmed       |              |
| i2 | AT2G46290 | SALK_122372           | 5'UTR<br>(230bp upstream of start codon) | No insert       |              |
| j1 | AT1G66070 | SALK_052620           | Intron 1/5                               | No insert       |              |
|    |           | SALK_076067C          | Exon 4/6                                 | Confirmed       | Heterozygous |
| j2 | AT5G37475 | SALK_031883           | Exon 4/6                                 | Confirmed       | Homozygous   |
|    |           | SALK_116617           | Exon 2/6                                 | Confirmed       | Heterozygous |
|    |           | SALK_124700C          | Exon 2/6                                 | Confirmed       | Heterozygous |
| k  | AT4G33250 | AL943500 <sup>¶</sup> | Intron 6/6                               | Confirmed       | Homozygous   |
| l1 | AT5G25754 |                       |                                          |                 |              |
| l2 | AT5G25757 |                       |                                          |                 |              |
| m1 | AT3G02200 | SALK_016789           | Exon 6/8                                 | Confirmed       | Heterozygous |
|    |           | SALK_065494           | 3'UTR                                    |                 | Homozygous   |
| m2 | AT5G15610 | SALK_003895           | Exon 8/9                                 | Confirmed       | Homozygous   |
|    |           |                       | Exon 7/8                                 |                 |              |

i-1) (discussed in Chapter 5), and, (2) important for embryo development (eIF3b1, c1). The requirement of eIF3 genes in early stages reflects the relevance of translation in growth and development. Defect in embryogenesis upon disruption of a single copy of eIF3b (eIF3b1) is a little surprising in presence of the other functional copy of the gene (eIF3b2). Comparison of the co-expression pattern of these two paralogs revealed high correlation including in the gametophytes and embryo (*Arabidopsis* coexpression data mining; Expression Angler (Toufighi et al., 2005)). On the other hand, lethality of eIF3c1 alone is less surprising as eIF3c2 (having 65.8% sequence identity with eIF3c1) is expressed at very low levels, and the transcript for eIF3c2 was never detected with RT-PCR in the wild type. Also, eIF3c1 is expressed highly in carpels (Genevestigator (Hruz et al., 2008; Zimmermann et al., 2004); *Arabidopsis* eFP browser (Winter et al., 2007); **Appendix II.1**). Given that these lines were backcrossed, it is unlikely that another unlinked mutation contributes to the embryo lethal phenotype.

In summary, several different situations were encountered regarding the function of eIF3 in *Arabidopsis*. Firstly, some of the eIF3 subunits have been identified as essential in *Arabidopsis* as they affected the early development – during gametogenesis and early embryo development. Functional redundancy has been observed for eIF3d, g and m. In comparison to eIF3h, which is indispensable for efficient translation of specific mRNAs and which exhibits severe developmental defects, requirement for some of the other eIF3 subunits varied (eIF3k for instance). It will be of considerable interest to investigate these mutant strains for their specific expression patterns as their expression might be critical in a cell or tissue type, or in a temporal dimension. Furthermore, the requirement for a specific subunit might be critical under specific regulatory condition, stress response or response to external cues, where control imparted during protein synthesis is critical. With the available eIF3 mutant strains, potential translational regulation by eIF3 subunits can be unveiled by studying the specific developmental and physiological phenotypes. Also, the translation reporters designed to study the various stages of translation in eIF3h (Chapter 3) can be used to narrow down the function of these individual subunits in translation.

## II.5 Materials and methods

### II.5.1 Assembly of T-DNA insertion mutants

The Salk, Gabi KAT, and FLAG T-DNA collections were considered for selecting insertion alleles for eIF3 subunit genes. The insertion alleles were selected in such a way that both weak (3' insertions) and severe mutants (5' insertions) can be obtained wherever possible. Seeds were ordered from the Arabidopsis Biological Resource Center (<http://www.arabidopsis.org/>) for Salk lines; INRA Versailles ([http://dbsgap.versailles.inra.fr/agrobactplus/English/Accueil\\_eng.jsp](http://dbsgap.versailles.inra.fr/agrobactplus/English/Accueil_eng.jsp)) for the FLAG line and GABI-KAT (<http://www.gabi-kat.de/>) for their collection.

### II.5.2 PCR genotyping

Genomic DNA was isolated from inflorescence of lines to be screened. Flowers were ground in homogenization buffer with the following composition: 100mM Tris-Cl (pH 7.0); 10mM EDTA; 100mM NaCl; 1% SDS. DNA was extracted with phenol-chloroform-isoamyl alcohol and precipitated with 100% ethanol.

Putative insertion lines were genotyped following the guidelines from SIGnAL (Salk Institute Genomic Analysis Lab). Primers were designed using the Primer design tool (<http://signal.salk.edu/tdnaprimers.2.html>). Primers used for confirming insertion lines are listed in **Appendix II.2**.

### II.5.3 RNAi lines

RNAi silencing constructs with gene specific tag (GST) cloned into a hairpin construct were ordered via the AGRİKOLA (Arabidopsis Genomic RNAi Knock-out Line) project. Delivery clones were available only for eIF3a, eIF3d1, eIF3d2, eIF3f, eIF3k. These were requested and the presence of the GST was confirmed by PCR using pAgri primers (**Table II.6B**), and the RNAi T-DNA was transformed into *Arabidopsis* to obtain stable transgenics.

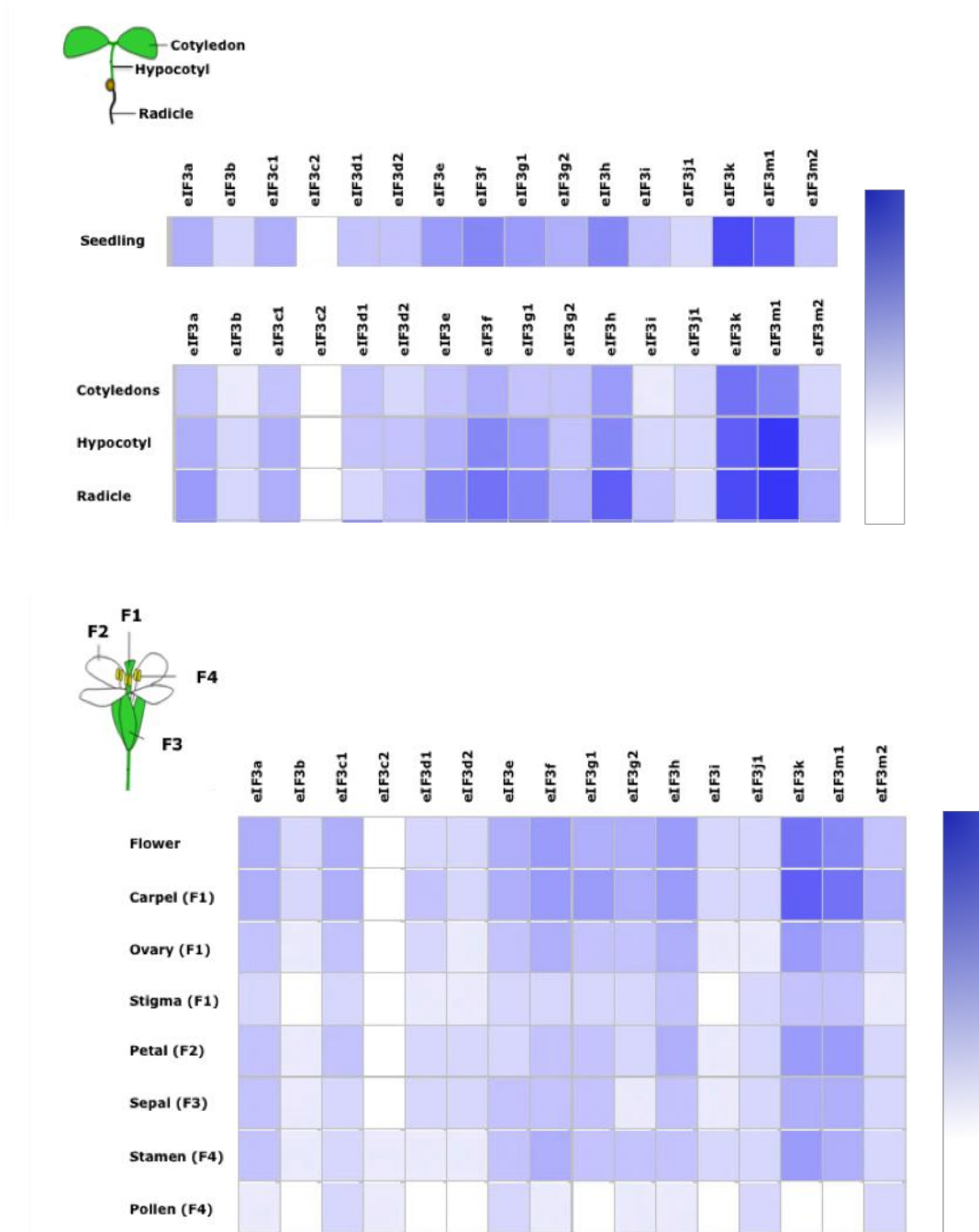
#### **II.5.4 RNA analysis**

Total RNA was isolated from either seedlings or inflorescence by harvesting tissues in liquid nitrogen and extraction with Trizol (Sigma). For RT-PCR analysis, 1 µg of total RNA was treated with RNase-free DNase I (Promega), and cDNA was synthesized with a 15-mer oligo(dT) primer using MMLV reverse transcriptase (Promega). Amplification was performed using gene specific primers (**Appendix II.3**).

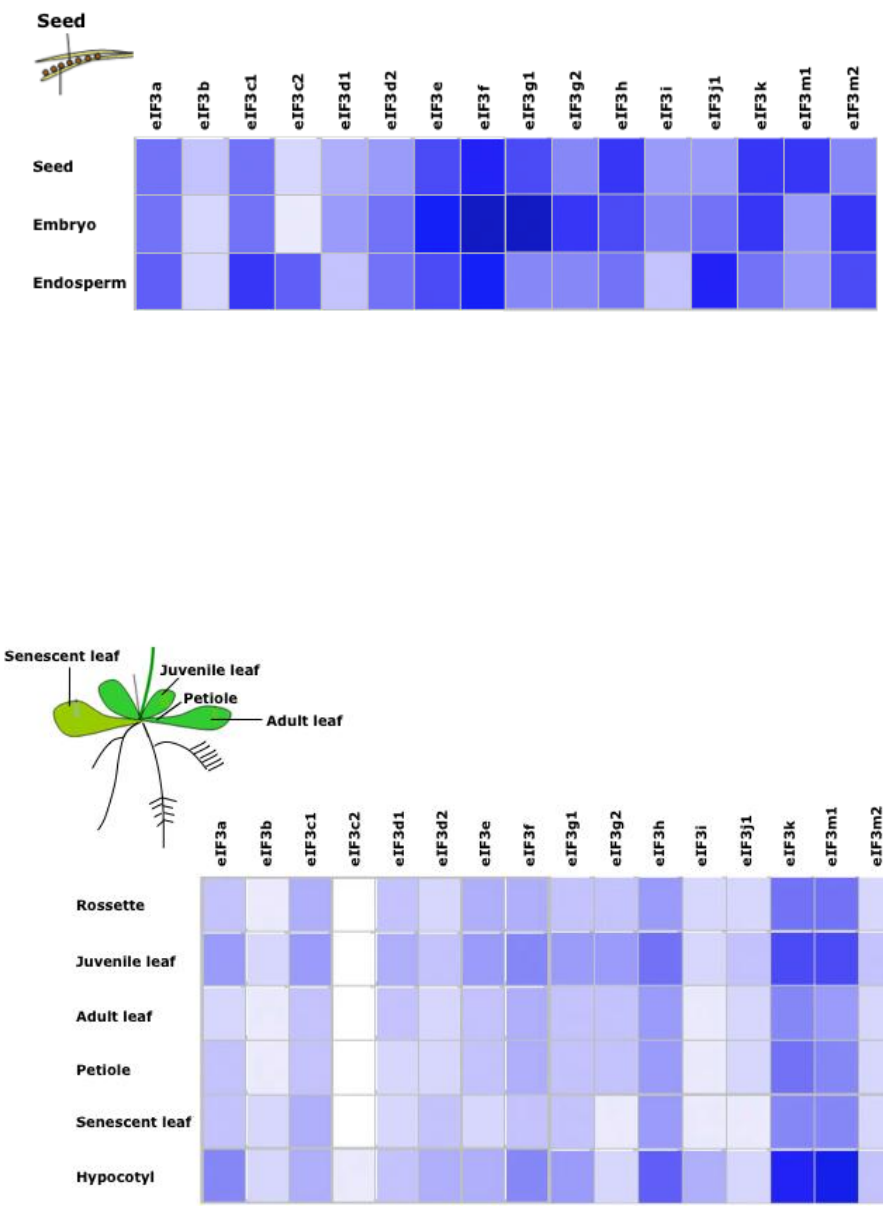
#### **II.5.5 Polysome analysis**

*Arabidopsis* polysomes were fractionated over sucrose gradients as described previously (Kim et al., 2004). Approximately, 1 g of 10-day old seedlings were ground in liquid nitrogen with 1 mL of extraction buffer (0.2 M Tris-HCl, pH 8.0, 50 mM KCl, 25 mM MgCl<sub>2</sub>, 1% Triton X-100, 400 units/mL of RNasin, and 50 mg/mL of cycloheximide). After spinning for 5 min, 900µL of supernatant was loaded onto a 10-mL 15 to 50% sucrose gradient. After centrifugation at 4°C in a Beckman SW41Ti rotor for 3.5 hours at 35,000 rpm, 11 fractions were collected manually. RNA was extracted from the collected fractions with phenol/chloroform/isoamyl alcohol, and precipitated with isopropanol.

**Appendix II.1. Tissue specific expression profile for eIF3.** Results are given as heat maps in white/blue coding that reflect mean signal values with darker representing strong expression. Modified from Genevestigator (Hruz et al., 2008; Zimmermann et al., 2004) and Arabidopsis eFP browser (Winter et al., 2007).



Appendix II.1 (continued)



## Appendix II.2. Oligonucleotides used to confirm T-DNA insertion

| Oligonucleotide  | AGI       | eIF3   | Oligonucleotide sequence (5'-3')                    |
|------------------|-----------|--------|-----------------------------------------------------|
| Salk LB          |           |        | TGGTTCACGTAGTGGGCCATCG                              |
| Gabi Kat LB      |           |        | GCAATGAGTATGATGGTCAATATG                            |
| FLAG LB          |           |        | TCCGATTCAGTACAATCGATT                               |
| SALK_047620(RP)  | At5g27640 | eIF3b1 | ACCTGGTGGTGTGCTGG                                   |
| SALK_047620(LP)  | At5g27640 | eIF3b1 | GCTGTGGCCATTGTCTCG                                  |
| SALK_126794 (RP) | At5g25780 | eIF3b2 | AAAGGTTTCAATGGCCAGC                                 |
| SALK_126794 (LP) | At5g25780 | eIF3b2 | TGTGGAATGAGTTTTGATCGC                               |
| SALK_120072 (RP) | At3g56150 | eIF3c1 | TTTAGCTGGAAGCGTAAGCAAC                              |
| SALK_120072 (LP) | At3g56150 | eIF3c1 | AGCAATGAAACCAATGCTGTG                               |
| SALK_101653 (RP) | At3g22860 | eIF3c2 | AGACTACTTGAGCGGACAGG                                |
| SALK_101653 (LP) | At3g22860 | eIF3c2 | GAAGTTTCCTTTGATCAGGGC                               |
| SALK_143551 (RP) | At3g22860 | eIF3c2 | TGCGGAGATTGTTCTCG                                   |
| SALK_143551 (LP) | At3g22860 | eIF3c2 | TCCTTGTTGATCATCATCTTGC                              |
| SALK_150238 (RP) | At4g20980 | eIF3d1 | TTATGAGGTTCCACCTGATGC                               |
| SALK_150238 (LP) | At4g20980 | eIF3d1 | TATGGTTTTTGCCGTTGCTAC                               |
| SALK_085397 (RP) | At5g44320 | eIF3d2 | TGAGACTCAGAGAGGTGCGG                                |
| SALK_085397 (LP) | At5g44320 | eIF3d2 | GGAGCCGAAGTTAACCAGC                                 |
| SALK_121004 (RP) | At3g57290 | eIF3e  | CCTCTAGATTATCATGAAAGCAGTT<br>GCCAAGTAGC             |
| SALK_121004 (LP) | At3g57290 | eIF3e  | GAAGATCTATGGAGGAAAGCAAACAGAA<br>CTATGACCTGACGCCACTA |
| SALK_113234 (RP) | At3g57290 | eIF3e  | TTGGAACACACACAGGCG                                  |
| SALK_113234 (LP) | At3g57290 | eIF3e  | ATGCTGAAATTTAAAAGCTAAATCATA                         |
| FLAG_413H07 (RP) | At2g39990 | eIF3f  | TACTGCATCTGCAATGAATCG                               |
| FLAG_413H07 (LP) | At2g39990 | eIF3f  | CTTCGTGAGACGTCCTGACTC                               |
| SALK_029432 (RP) | At3g11400 | eIF3g1 | ACCAACCGATGTCTTCATTGAC                              |
| SALK_029432 (LP) | At3g11400 | eIF3g1 | TGATATATATGCAGTTAAAAATAAATGG                        |

|                   |           |        |                               |
|-------------------|-----------|--------|-------------------------------|
| SALK_007584 (RP)  | At3g11400 | eIF3g1 | GCACCGTTTTGATTCCTTCAG         |
| SALK_007584 (LP)  | At3g11400 | eIF3g1 | CATGTGGTTTGTTATGGGTCC         |
| SALK_070257 (RP)  | At5g06000 | eIF3g2 | TGGCTACTGTTTGTGTGAATGG        |
| SALK_070257 (LP)  | At5g06000 | eIF3g2 | AATCTGGTCCACGGGTATCTTC        |
| SALK_028797 (RP)  | At2g46280 | eIF3i1 | TAAAAGGTTGACGTTCAAGTAGTCGGAAT |
| SALK_028797 (LP)  | At2g46280 | eIF3i1 | TCGCATCAAACCCTAAGAACTGAAGATG  |
| SALK_015367 (RP)  | At2g46280 | eIF3i1 | TAAAAGGTTGACGTTCAAGTAGTCGGAAT |
| SALK_015367 (LP)  | At2g46280 | eIF3i1 | TCGCATCAAACCCTAAGAACTGAAGATG  |
| SALK_122372 (RP)  | At2g46290 | eIF3i2 | GTGGAATGATGAAAAAATGGAAAGACAT  |
|                   |           |        | G                             |
| SALK_122372 (LP)  | At2g46290 | eIF3i2 | AATTCGGTTCCCAAAGCAACAAGTCTTG  |
| SALK_052620 (RP)  | At1g66070 | eIF3j1 | TAGCTGTTTTTCCTGGGTGTG         |
| SALK_052620 (LP)  | At1g66070 | eIF3j1 | AGACCTTCACAGCCTTCTTCC         |
| SALK_076067C (RP) | At1g66070 | eIF3j1 | GGTCATGAAAAACGGATTCAG         |
| SALK_076067C (LP) | At1g66070 | eIF3j1 | TTTCTGATTCCAGAGGACGAG         |
| SALK_031883 (RP)  | At5g37475 | eIF3j2 | TTGAGATTGCGGCTAACAATC         |
| SALK_031883 (LP)  | At5g37475 | eIF3j2 | AGGACCCGAAACAAGATCATC         |
| SALK_116617 (RP)  | At5g37475 | eIF3j2 | CCAGTGAGAGCCTTTGTTCTG         |
| SALK_116617 (LP)  | At5g37475 | eIF3j2 | CCTCAAATGGTACAAGTCTCTGAG      |
| SALK_124700C (RP) | At5g37475 | eIF3j2 | TGCAGGTTTTTCAGTTGGATTC        |
| SALK_124700C (LP) | At5g37475 | eIF3j2 | CCTCAAATGGTACAAGTCTCTGAG      |
| AL943500 (RP)     | At4g33250 | eIF3k  | ATGGGAGTAGAGATCCAATCGCCG      |
| AL943500 (LP)     | At4g33250 | eIF3k  | GGGAAGATGCGGGCAATGTGTTCCAAGG  |
| SALK_016789 (RP)  | At3g02200 | eIF3m1 | AATACTCCAGCTTGGTGAATGA        |
| SALK_016789 (LP)  | At3g02200 | eIF3m1 | GAGCGGCTGTAAGATATGAATC        |
| SALK_065494 (RT)  | At3g02200 | eIF3m1 | GAAAGGTTGGGCGTTTCCAAG         |
| SALK_065494 (LF)  | At3g02200 | eIF3m1 | TGAACCAAGTTTTAATTGTGTCAGGCA   |
| SALK_003895 (RT)  | At5g15610 | eIF3m2 | AAAGAAACGAACACGACTGGGG        |
| SALK_003895 (LT)  | At5g15610 | eIF3m2 | CGCCTCAGATGATTCTGGCAA         |

### Appendix II.3. Oligonucleotides used for RT-PCR

| AGI       | eIF3   | Oligonucleotide sequence (5'-3')   |
|-----------|--------|------------------------------------|
| At4g11400 | eIF3a  | ATGGCGAATTTTGCCAAACCA              |
| At4g11400 | eIF3a  | ATCTTAGACACCTGTTGAAGT              |
| At5g27640 | eIF3b1 | ATGGAGGTCGTTGATATTGATGCCCGAG       |
| At5g27640 | eIF3b1 | TTACGACTCTTGAACGATTTCCTC           |
| At5g25780 | eIF3b2 | ATGGCGGCGGTTGTTGATATTGATG          |
| At5g25780 | eIF3b2 | TTACATCAACTCTTGAACAATTTTC          |
| At3g56150 | eIF3c1 | ATGACGTCTCGTTTTTCACTCAGGTTGGAAGT   |
| At3g56150 | eIF3c1 | ATTAGTACGAACACCTCGGTTAAGACT        |
| At3g22860 | eIF3c2 | ATGTTTCGTTTACAGAGTGGAAGT           |
| At3g22860 | eIF3c2 | TTACGCAGAAACGCCTCGGGTAAG           |
| At4g20980 | eIF3d1 | ATGGTAACCGAAGCTTTTCAAT             |
| At4g20980 | eIF3d1 | TCAAGCTTGAGCATCATCAGCT             |
| At5g44320 | eIF3d2 | ATGGTGTTTGAAGCATTGGAAG             |
| At5g44320 | eIF3d2 | TCAGGCTTCAACTTCGGATTTC             |
| At3g57290 | eIF3e  | ATGGAGGAAAGCAAACAGAACTATGACCTGACGC |
| At3g57290 | eIF3e  | GTGTGTTCCAAGAGCTGATTCACTAACT       |
| At2g39990 | eIF3f  | ACGAGCGAGCACACCATCTTGCAATTT        |
| At2g39990 | eIF3f  | TTATAGCATTTGAGCAGCTGTGTTT          |
| At3g11400 | eIF3g1 | ATGACGATCGATTGCGAGCAAA             |
| At3g11400 | eIF3g1 | CTAGGTTGGTCTTGAGTTGCC              |
| At5g06000 | eIF3g2 | ATGATTAGTCCGACCAGAAC               |
| At5g06000 | eIF3g2 | TCATGAATGACATATATTCAACAACCTTACAAAG |
| At1g10840 | eIF3h  | ATGGCAACCATGGCTAGGTCGTTTCTG        |
| At1g10840 | eIF3h  | TCAGGTAAAGCCTGCTGAAGTTCT           |
| At2g46280 | eIF3i1 | ATGAGGCCGATCCTGATGAAGGGAC          |

---

|           |        |                                          |
|-----------|--------|------------------------------------------|
| At2g46280 | eIF3i1 | TAAATCTTGATGTTGTAGTCGGAGTC               |
| At2g46290 | eIF3i2 | ATGACAATTGAGTGCGTTAATCCCGAAGGTC          |
| At2g46290 | eIF3i2 | CTAAATCTTGATGTTGAAGTAATTGGAGTC           |
| At4g33250 | eIF3k  | ATGGGAGTAGAGATCCAATCGC                   |
| At4g33250 | eIF3k  | TCAACCAAGGATGGGGAAGATG                   |
| At5g15610 | eIF3m1 | ATGACGACGATTGTTCCACCTCCG                 |
| At5g15610 | eIF3m1 | TGAGCTGCTGATGATGTCTGAGTAC                |
| At4g33250 | eIF3k  | CCATAAGAGCCTTGACCAAGATTTCGAGCCACGATATGAG |
| At4g33250 | eIF3k  | TGTGATAGAACAATTAGTGACTTGAAGTGC           |
| At4g20980 | eIF3d1 | ATTCTGATTAATATAAGCAGCCTCCA               |
| At5g44320 | eIF3d2 | TGGGAGAGGCGGAGGAGGAA                     |
| At5g44320 | eIF3d2 | ATGGTAACCGAAGCTTTCGAATTCGT               |
| At5g25780 | eIF3b2 | ATGGCGGCGGTTGTTGATATTGATGTC              |
| At5g25780 | eIF3b2 | CATCAACTCTTGAACAATTCCTCAGTAACATCAA       |
| At1g66070 | eIF3j1 | ATGGATGATTGGGAGGCCGAG                    |
| At1g66070 | eIF3j2 | TCACATGAAATCATCATCGTC                    |

---

## **Chapter 3**

**The h subunit of eIF3 promotes reinitiation competence during translation of mRNAs harboring upstream open reading frames**

Results presented in this chapter have been published as part of the following paper:

Roy B., Vaughn J.N., Kim B.H., Zhou F., Gilchrist M.A., von Arnim A.G., 2010. The h subunit of eIF3 promotes reinitiation competence during translation of mRNAs harboring upstream open reading frames. *RNA* 16: 748-761

### **III.1 Abstract**

Upstream open reading frames (uORFs) are structural elements in the 5' leader of messenger RNA that impede downstream translation. The prevalence of uORFs suggests the existence of a mechanism during initiation of protein synthesis to overcome the inhibitory effects. In a previous study, eukaryotic initiation factor 3 (eIF3) subunit h was found to be indispensable for the translation of mRNAs harboring multiple uORFs in their 5' leader. In addition, microarray comparisons of mRNA polysome loading between wild-type and *eif3h* mutant plants confirmed uORFs as determinants of eIF3h dependent translation.

In this study, the specific requirement for eIF3h to translate mRNAs with uORFs was dissected by studying the *Arabidopsis* AtbZip11 (At4g34590) and yeast GCN4 5' leader harboring multiple uORFs. Of the four phylogenetically conserved uORFs in AtbZip11, three are inhibitory to translation, while one is anti-inhibitory. The results from transient gene expression assays furnish evidence for the involvement of eIF3h during the later stages of translation. The requirement for eIF3h was independent of uORF start codon recognition as well as the *cis*-inhibitory effects of the uORF encoded peptide. Instead, efficient reinitiation after uORF translation was compromised in the absence of functional eIF3h. eIF3h ensures that a fraction of uORF-translating ribosomes retain their competence to resume scanning. Experiments using the yeast GCN4 leader provided no evidence that eIF3h fosters tRNA reacquisition. Together, these results provide compelling evidence for the function of eIF3 downstream of 48S complex formation and establish eIF3 as a reinitiation factor.

### III.2 Introduction

Upstream open reading frames (uORFs) are short protein coding regions located in the 5' leader of many eukaryotic mRNAs. Upstream ORFs are found in about one third of experimentally supported 5' leaders in *Arabidopsis* and occur at even higher frequencies among transcription factors and protein kinases (Kim et al., 2007). According to the scanning model of translation initiation, uORFs are expected to generally suppress efficient initiation at the start codon of the main ORF (Kozak, 2002). However, by compensating for the translational repression in response to specific signals, translation can become regulated (Hanfrey et al., 2005). For example, in *Arabidopsis*, a uORF-encoded peptide in the mRNA for the basic leucine zipper transcription factor, AtbZip11, mediates translational inhibition by sucrose (Rahmani et al., 2009; Rook et al., 1998a; Wiese et al., 2004). In yeast, the uORFs in the 5' leader of the bZip transcription factor GCN4 cause a translational derepression in response to amino acid starvation (Hinnebusch, 2005). A mechanistically similar regulatory module exists in mammalian cells, where phosphorylation of eIF2 $\alpha$  by one of a number of different eIF2 kinases depresses general translation, all the while boosting translation of mRNAs such as ATF4 with suitably balanced uORF patterns (Harding et al., 2000). Upstream ORFs also function in complex regulatory modules dedicated to internal ribosome entry (Yaman et al., 2003) as well as translation reinitiation or shunting (Park et al., 2004a; Park et al., 2001; Ryabova et al., 2004).

The great majority of uORFs are not conserved at the peptide level (Hayden and Jorgensen, 2007). However, occasionally, the peptide sequence encoded by the uORF determines the fate of the mRNA (Hanfrey et al., 2005; Rahmani et al., 2009), in particular if, (1) rare codons are present, (2) the peptide blocks elongation while in the ribosome exit channel (Wang et al., 1998), or, (3) if translation termination is stalled (Gaba et al., 2005; Gaba et al., 2001).

According to the scanning model of translation initiation, the 40S ribosomal subunit in association with eIF3 and the ternary complex consisting of eIF2-GTP-methionyl-tRNA<sup>Met</sup> scans

along the mRNA and is poised to recognize the first AUG start codon it encounters (Kozak, 2002). The start codon is recognized primarily through codon-anticodon pairing with tRNA<sup>Met</sup> although the accuracy of AUG recognition is enhanced by eIF1 and eIF1A (Fekete et al., 2007; Maag and Lorsch, 2003; Pestova et al., 1998) and eIF3 (Nielsen et al., 2004). At this point, the 60S large ribosomal subunit joins in, followed by translation elongation. A uORF poses a barrier to the scanning 40S ribosome because, upon recognition of the uORF start codon, the uORF peptide must be translated and terminated. A ribosome whose 40S subunit disassociates from the mRNA after termination can be regarded as having suffered a permanent loss of reinitiation competence. Conversely, a 40S ribosome that resumes scanning downstream of the uORF displays a conditional loss of reinitiation competence because it lacks a ternary complex. The regaining of full reinitiation competence requires, at the least, that the 40S reacquire a fresh ternary complex, in order to successfully recognize the start codon of the main ORF further downstream (Kozak, 1987, 2001). The molecular factors responsible for resumption of scanning and acquisition of a fresh ternary complex are largely unknown. The efficiency with which scanning resumes is affected by a number of factors, including the length of the uORF, the time it took to translate it (Kozak, 2001; Rajkowitsch et al., 2004), as well as the translation initiation factors involved in the initial initiation event (Poyry et al., 2004). These data point to a form of molecular memory, possibly in the form of initiation factors clinging to the 80S ribosome for a limited period of time during translation elongation on a uORF. Because eIF2 loses its tRNA and GTP cofactor in the process of start codon recognition, and because eIF1 and 1A bind at the 40S-60S interface and must surely be displaced before subunit joining can take place (Lomakin et al., 2003; Maag and Lorsch, 2003), the most likely known eIF to maintain a physical or functional association with the ribosome during the initial phase of elongation is eIF3. This notion gained additional credibility when cryoelectron microscopy of the 40S-eIF3 complex located eIF3 on the back side of the 40S ribosome, near the mRNA exit channel (Siridechadilok et al., 2005). Reinitiation requires, first, that the post-

termination 40S ribosome has not permanently lost its competence to resume the scanning mode, and second that the 40S overcome its conditional loss of reinitiation competence by regaining a fresh ternary complex. Studies in yeast have implicated specific eIF3 subunits in this process (Nielsen et al., 2004; Szamecz et al., 2008; Valasek et al., 2002), extending earlier biochemical data demonstrating that eIF3 bolsters ternary complex loading onto the 40S (Benne and Hershey, 1978).

The eIF3 complex is the largest initiation factor. While functions have been assigned in yeast to the five subunits shared between yeast and other eukaryotes, the individual contributions of the remaining seven eIF3 subunits, which are not recognizably conserved in budding yeast, are largely unknown (Hinnebusch, 2006). The h subunit of eIF3 is a 38kDa MPN (Mpr1-Pad1-N-terminus)-domain protein conserved between fission yeast, plants, and metazoans. It was previously detailed in our lab that plants mutated in *eif3h* show defects in translation of mRNAs harboring uORFs (Kim et al., 2007; Kim et al., 2004). The specific translational regulation of mRNAs with uORFs in *eif3h* leads to the hypothesis that eIF3h may be involved in (1) the fidelity of start codon recognition of uAUGs, or, (2) overcoming the repression exerted by the translation product of an upstream ORF which may be inhibitory, (3) it may act by favoring the reinitiation after uORF translation by aiding in resumption of ribosome scanning or acquisition of new protein factors *viz.* TC and eIFs. To identify the role of eIF3 more precisely, a detailed mutational dissection of the *Arabidopsis* AtbZip11 leader which harbors a cluster of phylogenetically conserved uORFs was performed and is discussed here.

### **III.3 Results**

#### **III.3.1 Mutant eIF3h protein does not associate tightly with 43S complexes**

Plants harboring T-DNA insertions in *eIF3h* continue to express carboxyl-terminally truncated eIF3h protein (**Figure III.1A**) (Kim et al., 2004). It is important to examine whether the truncated eIF3h-1 protein is still associated with multifactor complexes harboring eIF3, such

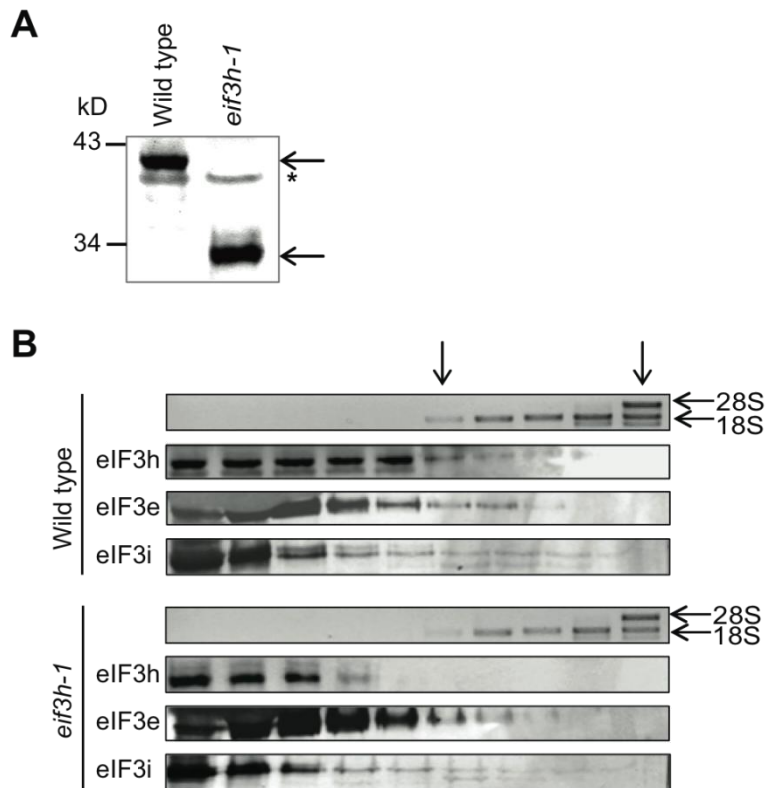


**Figure III.1. In *eif3h* mutant plants, 43S complexes lack eIF3h.**

**A.** Wild-type eIF3h and truncated eIF3h expressed in *eif3h-1* mutant plants were visualized by immunoblotting with anti-eIF3h antibody. The asterisk denotes a crossreacting protein.

**B.** Sucrose gradients from wild type and *eif3h* mutant plants were examined for 18S ribosomal RNAs to identify 40S subunits using electrophoresis and ethidium bromide staining. Corresponding protein extracts were examined for eIF3 subunits by SDS-PAGE and immunoblotting. The topmost fractions containing 40S and 60S subunits are highlighted with arrows.

as the 43S complex (**Figure III.1B**). In wild-type plants, eIF3e, eIF3i, and wild-type eIF3h were detected in sucrose gradient fractions containing fast sedimenting complexes (fractions 5-9), including 40S ribosomes marked by the 18S rRNA. However, in *eif3h-1* mutant plants, eIF3e and eIF3i were found in similar fractions, while the truncated eIF3h-1 protein was found primarily at the top of the gradient. This led to the conclusion that the eIF3h-1 mutant protein has a reduced tendency to associate with other eIF3 subunits into multifactor complexes, and association with the 43S and 48S preinitiation complexes is weaker as compared to that of the full length protein.

### **III.3.2 eIF3h supports translation of the uORF-containing AtbZip11 mRNA**

To elucidate the molecular mechanism of the h subunit of eIF3 in a living biological context, the 5' leader of the AtbZip11 leader, which is dependent on the wild-type eIF3h for optimal expression (Kim et al., 2004), was used as a model system.

In transient expression assays using firefly luciferase (FLUC) as a reporter, the cluster of four uORFs in the AtbZip11 leader repressed expression by about two-fold in the wild type but by about 10-fold in *eif3h* mutant seedlings (**Figure III.2A**). The dependence on eIF3h can be attributed primarily to direct translational inhibition, rather than reduced abundance or decreased stability of the AtbZip11-FLUC mRNA as observed from measurements of the transcript level as well as a transcription inhibition assay (**Figure III.2A, B**). In order to test the involvement of the uORFs in impeding translation of the AtbZip11 leader in an eIF3h dependent fashion, the uAUGs in the leader were removed by site directed mutagenesis. Notably, translation of the AtbZip11 leader in the *eif3h* mutant was no longer compromised once all five upstream AUG start codons (uAUGs) were removed (**Figure III.2A**). Introducing a stable hairpin ( $\Delta G = -42.8$  kcal/mol; (Kozak, 1986) near the 5' end dramatically reduced expression, thus ruling out that translation occurs by internal ribosome entry or from a truncated transcript (**Figure III.2A**). Thus, the ribosomes initiate in the AtbZip11 leader in a

**Figure III.2. eIF3h dependent translational regulation of the AtbZip11 5' leader.**

**A. (Top)** Schematic of the FLUC reporter coding region fused to original and mutated 5' leaders of AtbZip11 (590nt long). Only the four uORFs are drawn to scale (white boxes; 18, 42, 5, and 19 amino acids, respectively). **(Middle)** Transient expression data from 10-day-old wild-type and *elf3h* mutant seedlings. The efficiency of translation was calculated as the activity ratio of FLUC versus *Renilla* luciferase (RLUC) as a co-transformed reference gene. Bars denote standard error. \*,  $P < 0.002$  for wild type (WT) versus *elf3h* in a two-sided Student's t-test. **(Bottom)** Respective transcript levels of FLUC reporter and translation elongation factor 1 $\alpha$  (EF1 $\alpha$ ) as a control were compared by RT-PCR.

**B.** Turnover of AtbZip11-FLUC mRNA expressed in stable transgenic plants (Kim et al., 2007) was measured by RT-PCR after blocking transcription with 0.1mg/ml cordycepin. EF1 $\alpha$  served as a control.

**C.** Translation of the AtbZip11-FLUC reporter construct with the default 3' UTR from cauliflower mosaic virus was similar to that with the native AtbZip11 3'UTR. \*,  $P < 0.002$  (n=5).

**D.** Undertranslation of the AtbZip11 and  $\Delta$  AtbZip11 5' leader in the *elf3h* mutant in *Arabidopsis* protoplasts transformed with *in vitro* transcripts.

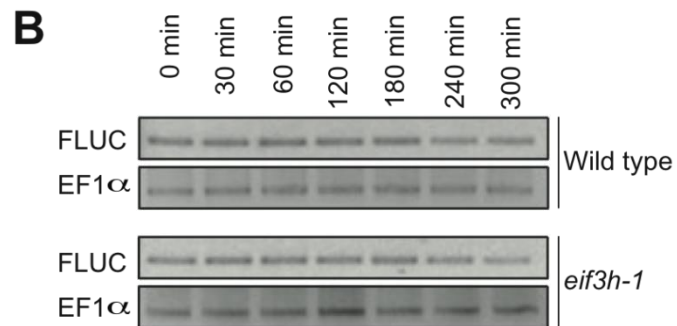
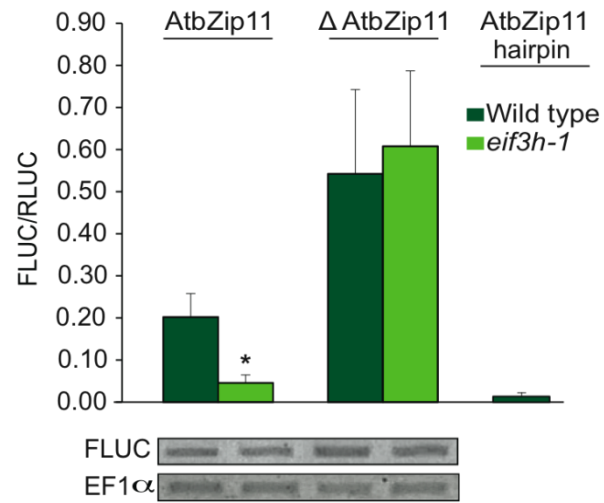
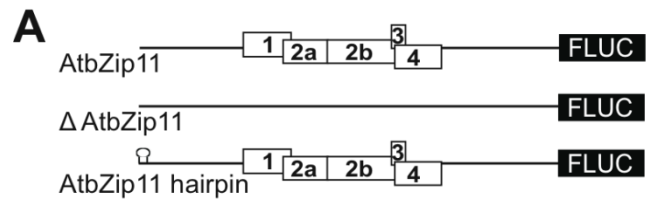
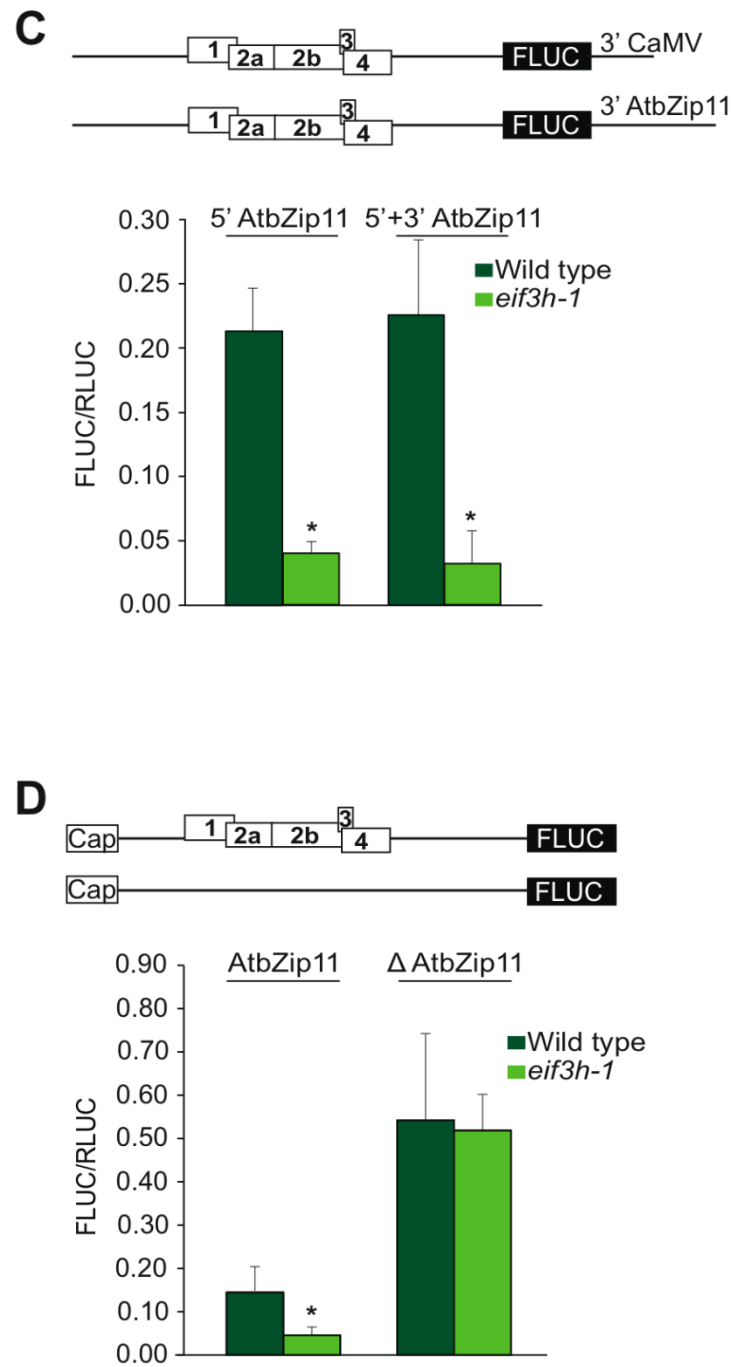


**Figure III.2 (continued)**



cap dependent manner. Repression by uORFs is sometimes controlled by elements in the 3' UTR (Mehta et al., 2006). However, the translational inhibition was not rescued by including the native 3' untranslated region (UTR) from AtbZip11 instead of the heterologous 3' UTR from cauliflower mosaic virus (**Figure III.2C**). Similar results were obtained using transformation of *Arabidopsis* seedlings with expression plasmids and transformation of *Arabidopsis* protoplasts with capped *in vitro* transcripts (**Figure III.2D**), which rules out that wild-type expression is high because of splicing. In summary, the data indicate that initiation on the AtbZip11 leader occurs by ribosome scanning from the 5' cap. Moreover, the wild-type ribosome must traverse the hurdle imposed by the uORF cluster, either by leaky scanning across the uAUGs or by uORF translation followed by a new initiation event at the main AtbZip11 AUG.

### **III.3.3 Identification of inhibitory uORFs in the AtbZip11 leader**

To determine whether the translational defect in the *eif3h* mutant could be attributed to one specific uORF, each uAUG in AtbZip11 was mutated systematically (**Figure III.3A**). The only uORF that was clearly essential for eIF3h-dependent translation was uORF2a. However, individual removal of uORFs 2b or 3 or 4 also caused derepression, especially in *eif3h*. The presence of uORF1, which should impede recognition of uORF2 by virtue of overlapping with it, had an anti-inhibitory effect (**Figure III.3A, C**). The simplest uORF arrangement with the most clearcut eIF3h dependence consisted of uORFs 2 and 3 (**Figure III.3B**). These results suggest that all uORFs affect expression and contribute to the requirement for eIF3h, either directly or indirectly, with uORFs 2 and 3 playing a predominant role.

### **III.3.4 Function of eIF3h in uAUG recognition**

One possible role of eIF3h that would be consistent with the observations is that eIF3h helps wild-type plants to disregard certain uAUG start codons, in particular uAUGs in a weak

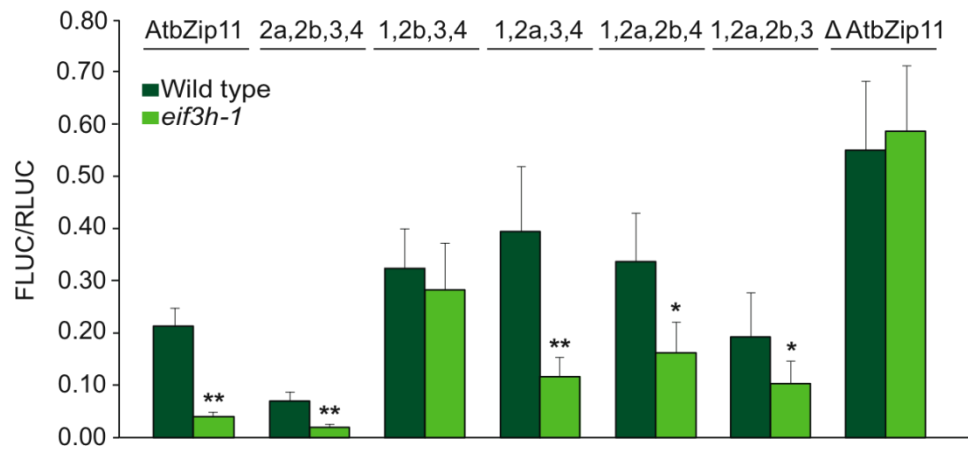
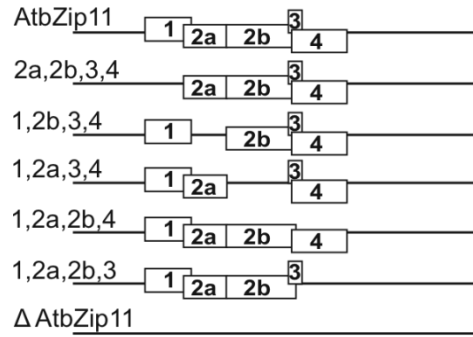
**Figure III.3. Contribution of individual uAUGs to translational repression and eIF3h-dependent translation of AtbZip11-FLUC.**

**A.** Single uAUGs were eliminated by site directed mutagenesis. Note the anti-inhibitory effect of uORF 1 (also see **C**). Error bars represent standard error (n = 8-11). Data on the original and uORF-less leaders are included from **Figure III.2A** for comparison. \* Statistical significance at  $P < 0.01$ , \*\*  $P < 0.002$ .

**B.** The combination of uORFs 2 and 3 caused a robust dependence on eIF3h, in contrast to uORFs 1 and 4 together (n = 8-12). eIF3h dependence of uORF2 alone was also significant but that of uORF3 alone was not. \* Statistical significance at  $P < 0.01$ . For the right panel, AtbZip11-FLUC and endogenous EF1 $\alpha$  mRNA levels were estimated by RT-PCR.

**C.** A pairwise comparison demonstrating the anti-inhibitory effect of uORF1 in the AtbZip11 leader. Note that leaders harboring uORF1 are still eIF3h-dependent. Bars represent standard error (n = 9-22). \* Statistical significance at  $P < 0.04$ , \*\*  $P < 0.01$ .

**A**



**B**

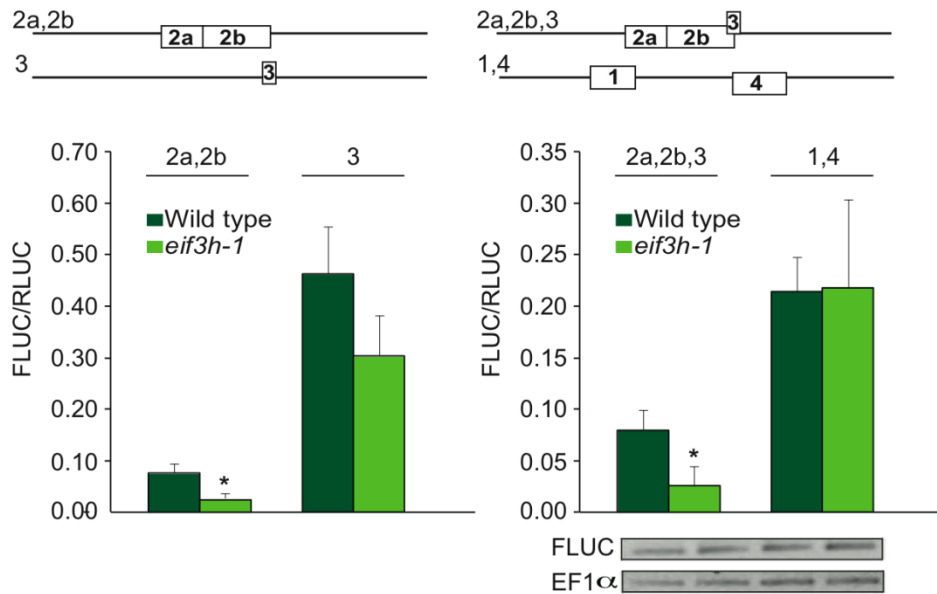
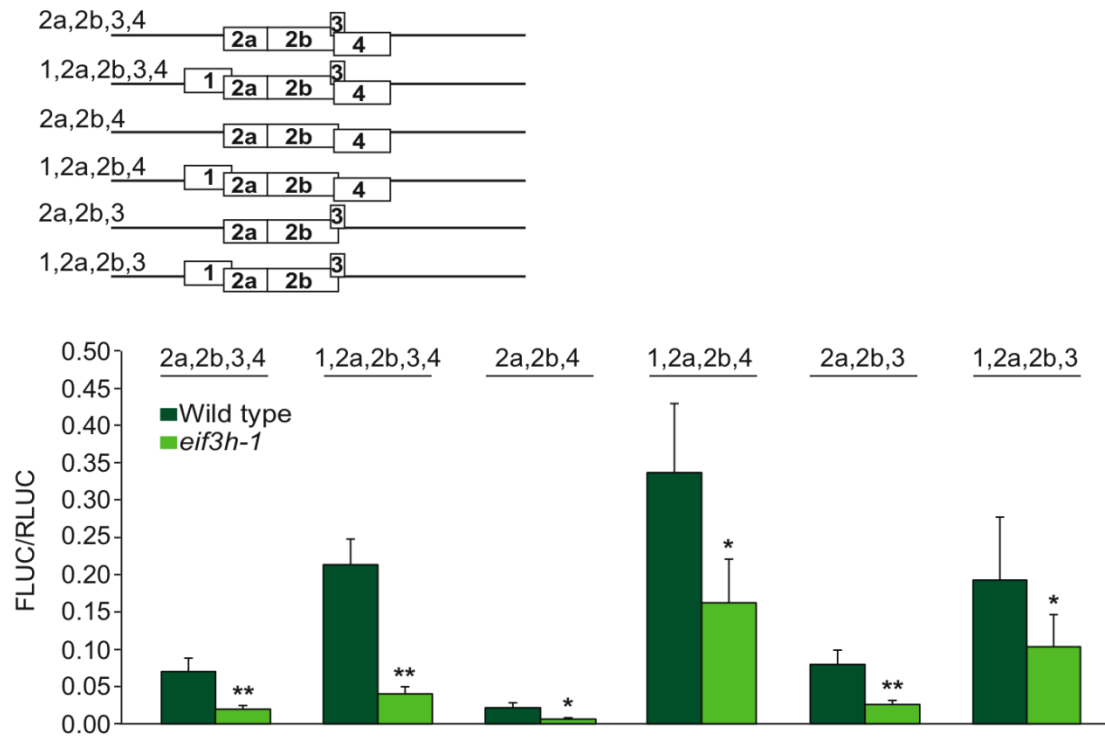


**Figure III.3 (continued)**

**C**



sequence context (Kozak, 1986). To examine this, each uAUG start codon in the AtbZip11 leader was coupled directly to the FLUC coding sequence. Because FLUC expression was indistinguishable between wild-type and mutant seedlings it can be concluded that eIF3h does not affect uAUG start codon recognition in AUG contexts considered weak (uAUG1, 2a, 2b, 4), medium (uAUG3) or strong (modified uAUG4; **Figure III.4A**). Even though the AUGs are recognized, the weak or medium contexts around the AUGs suggest that not all ribosomes transiting the AtbZip11 leader will recognize the uAUGs and there might be a substantial amount of leaky scanning around the AUGs. To examine leaky scanning around uAUGs 2a, 2b and 3, uORFs 2 and 3 were extended so as to overlap the FLUC start codon by 44 and 45 codons, respectively (2a,2b,3 ovlp; **Figure III.4B**), an overlap sufficient to suppress any hypothetical backward scanning (Jackson et al., 2007). In the case of rampant leaky scanning, the 2a,2b,3 ovlp transcript should be expressed at the same level as 2a,2b,3. If the uORF cluster was recognized every time, FLUC expression would be zero. Because expression was reduced dramatically, the majority of the ribosomes recognize the uORFs, and translation of FLUC downstream of the AtbZip11 leader must require a reinitiation event. Moreover, since the ribosomes got trapped by the overlapped uORFs, it can also be concluded that ribosomes do not simply bypass the entire uORF cluster by shunting (discontinuous scanning). Leaky scanning from individual uAUG and reinitiation after each uORF were further examined using the uORF arrangements shown in **Figure III.4C**. Compared to the number of successful initiation events at uAUG1, gradually fewer ribosomes initiated at a uAUG downstream of uAUG1. Also, construct AUG2b, which reports on initiation at either 2a or 2b, did not show eIF3h dependence, and neither did AUG3. Initiation at uAUGs 3 and 4 was much lower than at the main ORF, which argues for poor reinitiation immediately downstream of uORF1 in these constructs. The apparent eIF3h dependence of initiation at uAUG2a was unexpected given the prior data. eIF3h-dependent backward scanning from the Stop of uORF1 toward uAUG2a was also considered. However, an additional construct in which the stop codon of uORF1 was

**Figure III.4 uAUG recognition.**

**A.** The uAUGs of the AtbZip11 uORFs were fused directly to FLUC while retaining the start codon context up to the +4 position as well as their distance from the transcription start site as drawn in the schematic. Note that all uAUGs appear to be recognized in an eIF3h-independent fashion (n = 7-14). Experimental conditions are as in **Figure III.2**.

**B.** To delineate leaky scanning across uORFs 2 and 3, all in-frame stop codons were eliminated by point mutations, thus causing them to overlap the main FLUC ORF by 44 and 45 amino acids respectively (2a,2b,3 ovlp). Residual expression of FLUC is attributed to ribosomes that have leaky-scanned uORFs 2a, 2b and 3.

**C.** uAUG-FLUC fusion constructs to examine the (re-)initiation potential at individual uAUGs downstream of uORF1. Standard error is represented by bars (n = 5-6). \* Statistical significance at  $P < 0.02$ .

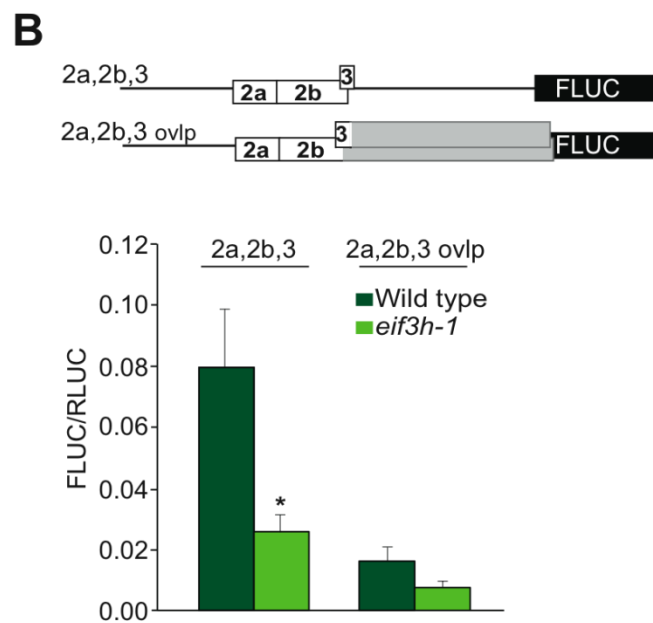
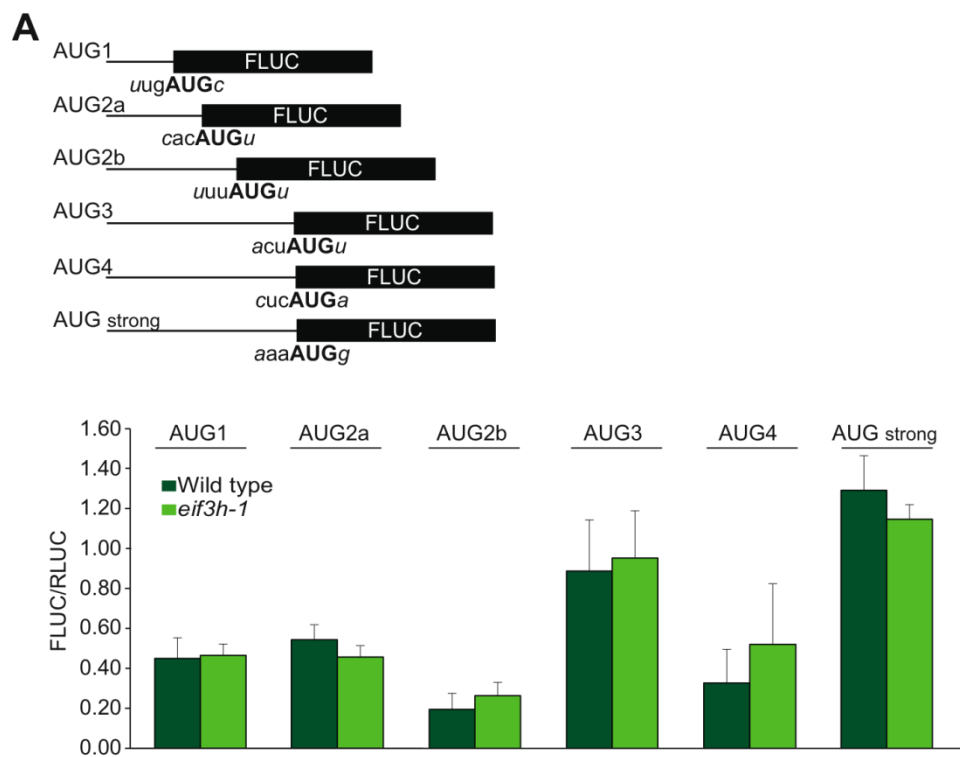
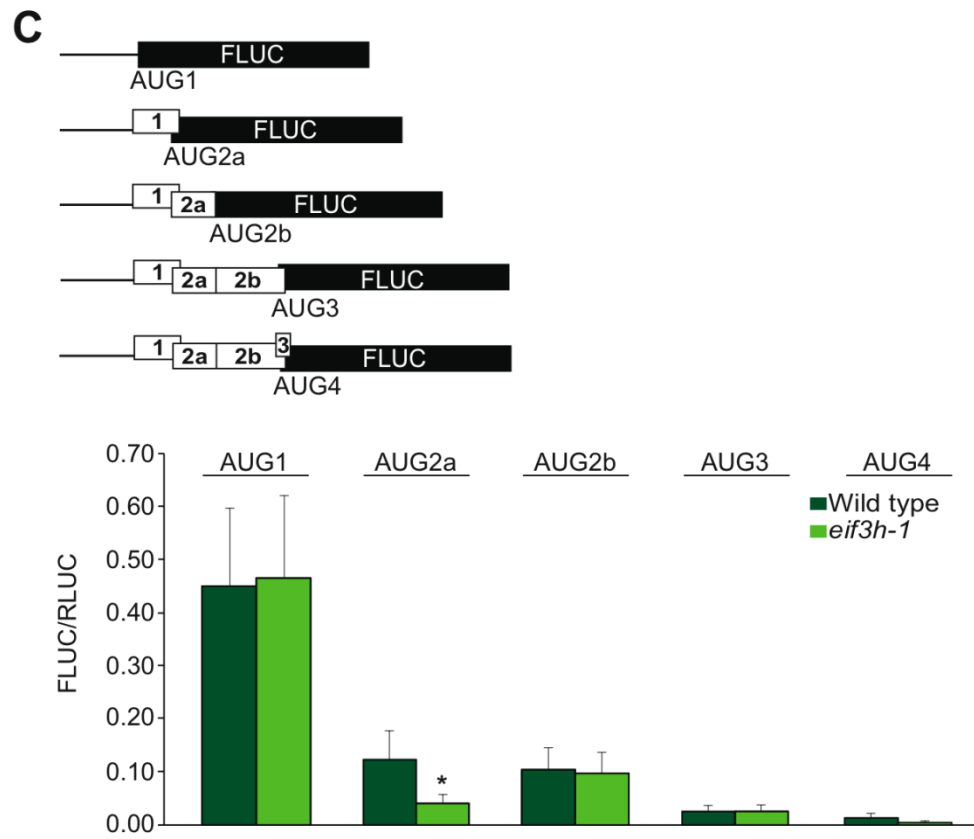


**Figure III.4 (continued)**



moved 5' of uAUG2a, which should eliminate backward scanning, continued to express the reporter gene, but in an eIF3h-independent fashion (**Appendix III.1**). This result favors leaky forward scanning over backward scanning as the primary route for initiation at uAUG2a.

### **III.3.5 eIF3h and the AtbZip11 attenuator peptide**

The 2b portion of uORF2 is an inhibitory attenuator peptide that steps into action when AtbZip11 translation is repressed by sucrose (Rahmani et al., 2009). Although a comparatively low sucrose concentration of 1% was used in these assays, changing the peptide sequences of uORFs 2 and 3 via compensatory frameshift mutations resulted in translational derepression, the extent of which was more pronounced in *eif3h* than in wild type. The AtbZip11 uORF2b harbors three consecutive arginine residues encoded by rare codons in *Arabidopsis* and derepression after introduction of frameshift mutation might be a result of the difference in codon usage from the new frame generated. Despite alteration of the uORF's peptide sequences, translation remained dependent on eIF3h albeit at a diminished level (**Figure III.5A**). Several versions of the frameshifted uORF with slightly different coding sequences gave similar results (**Figure III.5B**). In conclusion, the role of eIF3h in overcoming the AtbZip11 uORF is not restricted to the uORF2 peptide sequence.

### **III.3.6 eIF3h controls the efficiency of reinitiation events**

To directly test whether eIF3h is responsible for reinitiation after uORF translation, the cluster of uORFs 2a, 2b, and 3 was first altered to commence with start codons in a strong Kozak context (\* in **Figure III.6A**) in an effort to minimize leaky scanning. Reinitiation is generally poor if the spacer between the uORF stop codon and the main ORF's start codon is too short, an effect attributed to rate-limiting Met-tRNA<sup>Met</sup> acquisition (Abastado et al., 1991a; Abastado et al., 1991b; Kozak, 1987). Varying the spacer length between 25 and 213 nucleotides (nt) clearly demonstrated such a relationship. Reinitiation in the wild type reached a plateau with a

**Figure III.5. Stimulation of translation across uORFs 2 and 3 by eIF3h is not dependent on the uORF peptide sequences.**

**A.** Two independent frameshift mutations were introduced into uORF2a/2b to change the sequence of the encoded peptide while retaining its native length and also the flanking sequences. For uORF3, point mutations were introduced to change three out of its five amino acids. The frameshift data (wild type versus *eif3h*) were statistically significant by paired t-test ( $P < 0.002$ ).

**B.** Another version of the frameshift mutation with the internal AUG removed resulting in a long extended uORF2.

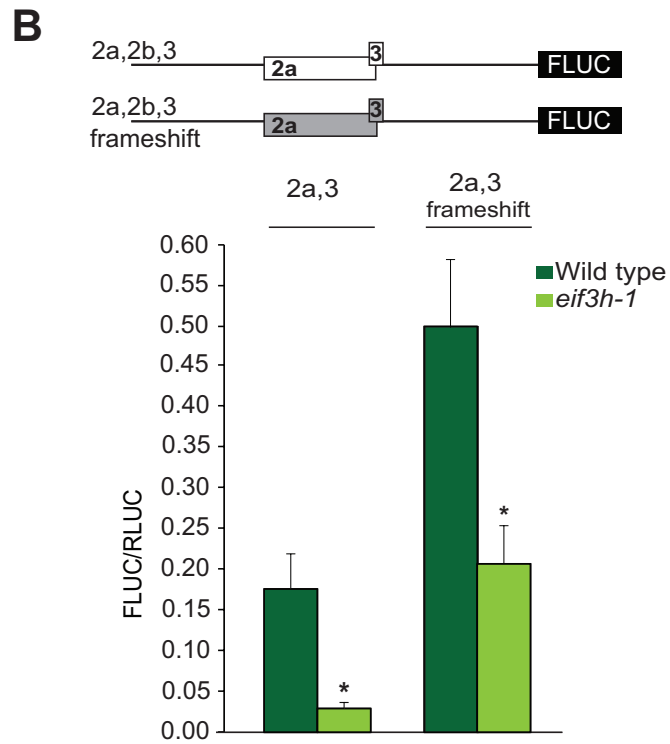
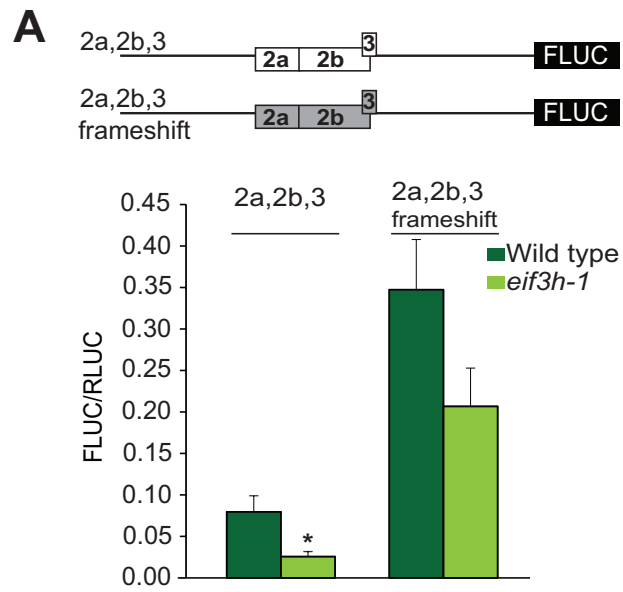
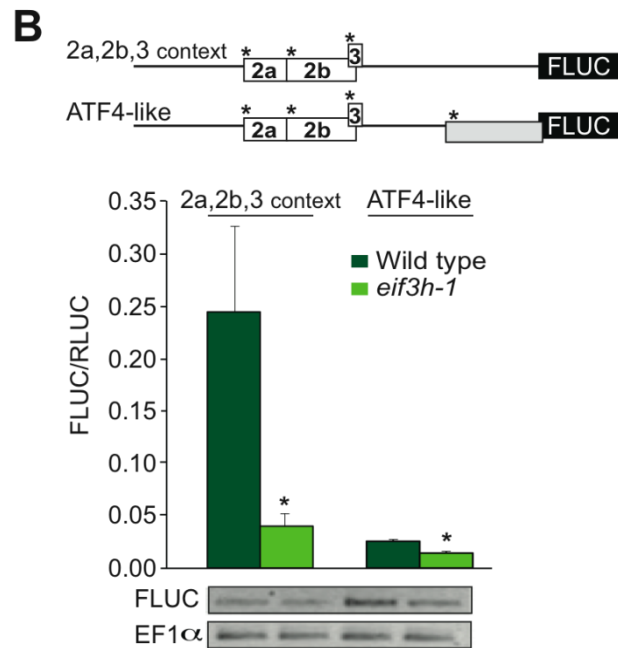
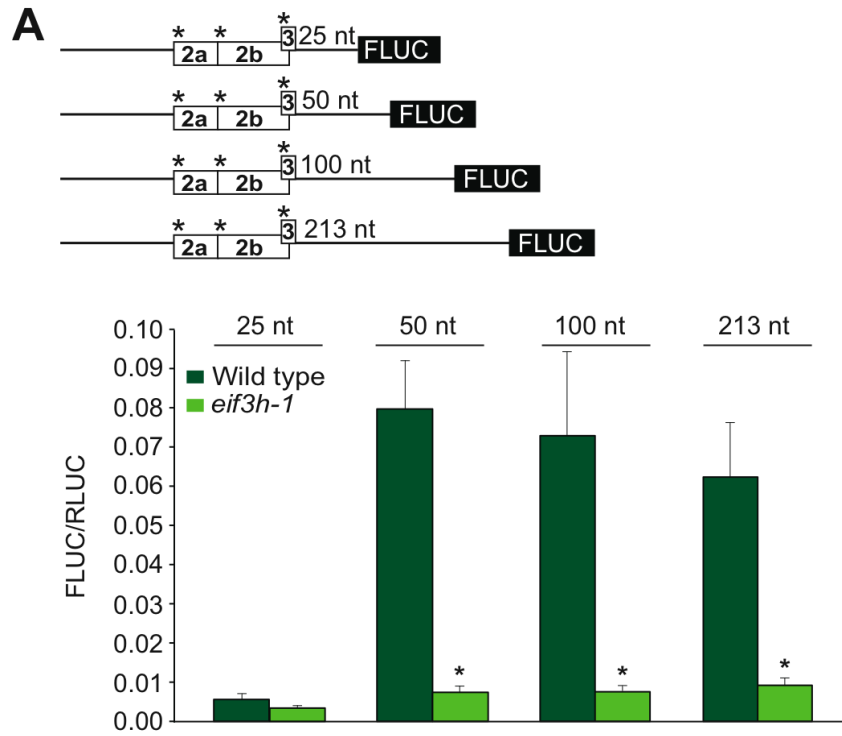


**Figure III.6. Requirement of eIF3h for efficient reinitiation.**

**A.** The effect of eIF3h on reinitiation downstream of uORF 2a 2b 3 was tested by shortening the intercistronic spacer length in the AtbZip11 leader by deletion from the 3' end of the spacer. uORFs 2a, 2b and 3 were placed in a strong Kozak context ( $_{-3}aaaAUGg_{+4}$ ) to minimize the effect of leaky scanning. Error bars denote standard error (n = 5-8). \* Statistical significance at  $P < 0.002$ .

**B.** Inhibition of translation by an ATF4-like (Harding et al., 2000) overlap-uORF downstream from uORF2 demonstrates consistent reinitiation in wild type but marginal reinitiation in *eif3h*. A uORF (gray box) in a strong Kozak context ( $A_{-3}AAA\underline{AUG}G_{+4}$ ) was created starting 60nt upstream of the FLUC ORF to overlap the main ORF. Asterisks mark AUGs in a strong context. Bars represent standard error (n = 8-12). AtbZip11-FLUC and endogenous EF1 $\alpha$  mRNA levels were estimated by RT-PCR.



spacer length of 50 nt. For comparison, the *eif3h* mutant showed only marginal expression, regardless of the spacer length (**Figure III.6A**). This result indicates that eIF3h is specifically required for reinitiation downstream from uORF2 or 3. Placing a uORF that overlaps the main ORF downstream of the uORF2, 3 cluster greatly diminished the residual expression, especially in the wild type. Neither the *eif3h* mutation nor the addition of the overlap uORF affected mRNA accumulation (**Figure III.6B**). This result confirms that ribosomes resume scanning after uORFs 2, 3 and do acquire a fresh ternary complex over the time needed to scan across the ~150 nt spacer between the uORFs.

### **III.3.7 eIF3h supports the resumption of scanning, not tRNA acquisition**

The function of eIF3h in reinitiation can pertain either to its function in ternary complex reacquisition or resumption of scanning after uORF termination. As described in Chapter I, the yeast GCN4 leader reports on ternary complex reacquisition by ribosomes that have resumed scanning after translating uORF1. A delay in ternary complex reacquisition, as occurs in yeast *general control derepressed* (*gcd*) mutants, results in skipping of the inhibitory uORF4, which in turn permits some translation initiation at the main ORF (Hinnebusch, 2005). Hence, the GCN4 leader was tested to check whether *eif3h* shows a *gcd*-like delay in tRNA acquisition on the GCN4 leader (**Figure III.7A**). uORFs 1 and 4 were both inhibitory when compared to the uORF-less leader (**Figure III.7B, C**). uORF4 was not as strongly inhibitory as it is in yeast, however, and neither uORF caused significant eIF3h dependence in *Arabidopsis*, as expected because these uORFs are short. Defects in resumption of scanning and tRNA reacquisition make opposite predictions about the expression from mRNAs containing both uORF1 and uORF4 (**Figure III.7A**). Experimentally, FLUC expression downstream of uORF 1 plus 4 was reduced at least as strongly in *eif3h* as in wild type, a result more consistent with a defect in resumption of scanning than a delay in tRNA acquisition (**Figure III.7C**). Much of the residual expression from 1,4 in the *eif3h* mutant can be ascribed to leaky scanning past uAUG4, as

**Figure III.7. Translation initiation on the 5' leader from yeast GCN4 in *Arabidopsis***

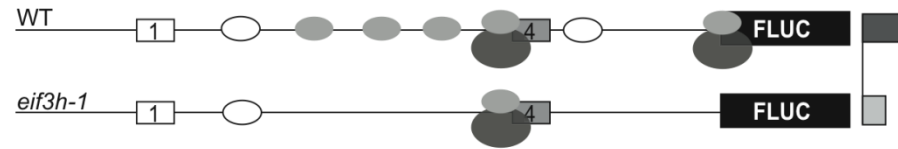
**A.** The schematic illustrates the predictions of two contrasting hypotheses concerning the molecular function of eIF3h (after Hinnebusch, 2005). uORF4 is shaded in gray for the sake of emphasis. If eIF3h supported resumption of scanning, few ribosomes would scan the intercistronic spacer in the *eif3h* mutant, but those that do would easily acquire a fresh ternary complex (TC) and initiate at the inhibitory uORF4. Hence, adding uORF4 to uORF1 will reduce FLUC translation to similar degrees in wild type and *eif3h* (bars on the right symbolize predicted FLUC expression levels). In contrast, if the *eif3h* mutation delayed TC acquisition, some mutant ribosomes would leaky-scan past uORF4 and thus reach the main FLUC ORF. Meanwhile, wild-type ribosomes, having acquired their TC early, will be preferentially intercepted by uORF4, resulting in a reversal of the eIF3h dependence (bars on right).

**B.** Schematic drawings of the GCN4 5' leader sequences tested. The version with four uORFs is the original.

**C.** Expression data for the constructs shown in panel B. Error bars denote standard error ( $n > 4$ ). \* Statistical significance at  $P < 0.05$ . Where shown, GCN4-FLUC and endogenous EF1a mRNA levels were estimated by RT-PCR.

**A**

Hypothesis 1: eIF3h stimulates resumption of scanning after uORF translation



Hypothesis 2: eIF3h stimulates reacquisition of ternary complex after uORF translation

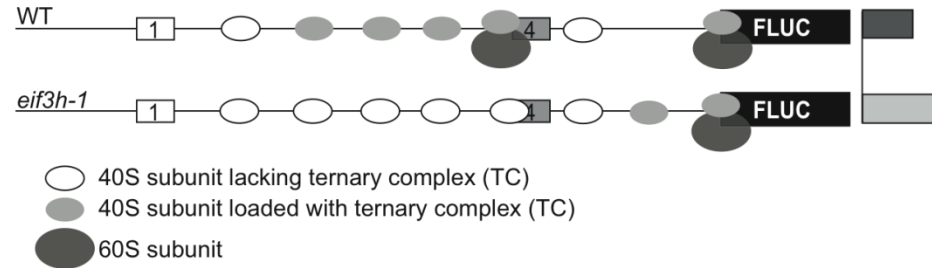
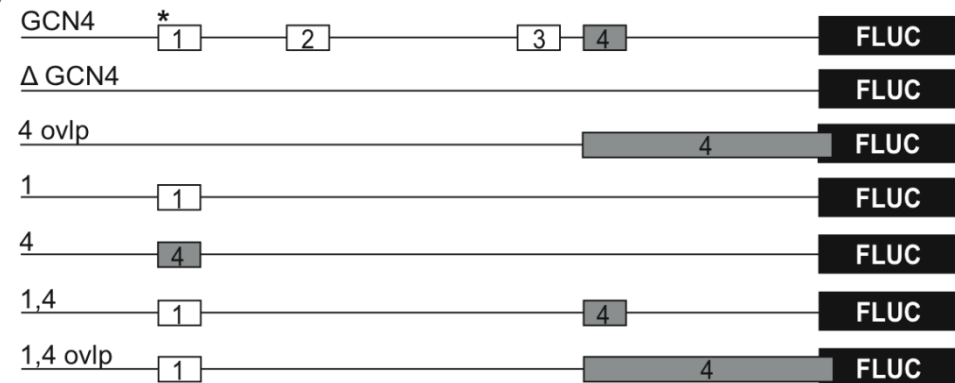
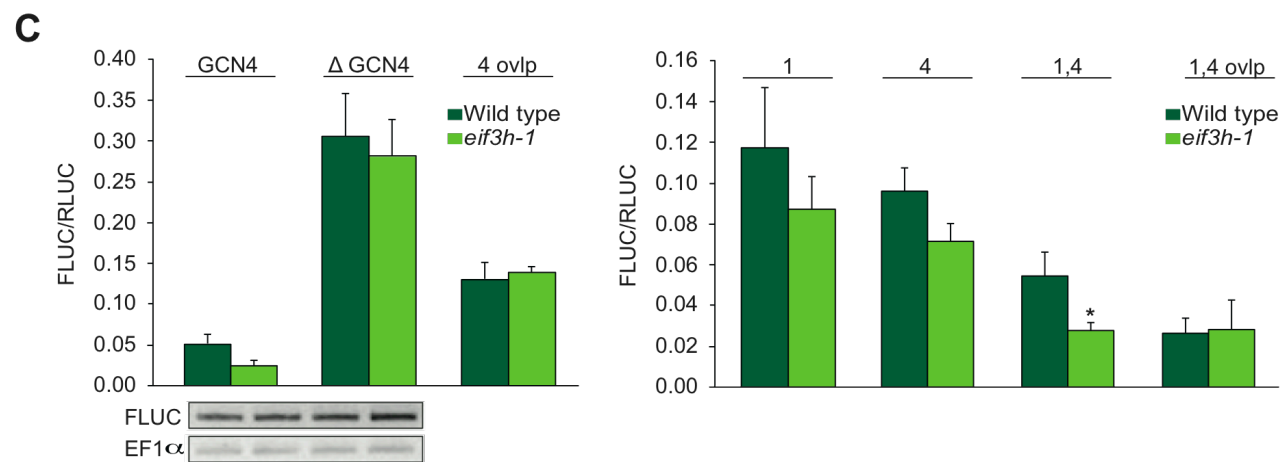
**B**

**Figure III.7 (Continued)**



judged from the 1,4 ovlp construct. The near-background expression with 1,4 ovlp also demonstrates that ribosomes reinitiating after uORF1 reacquire a fresh tRNA, soon enough to be efficiently trapped by uAUG4 (**Figure III.7C**). These data are consistent with the scenario that the wild-type initiation machinery can traverse the GCN4 leader with two reinitiation events, whereas in the *eif3h* mutant, resumption of scanning downstream of the uORFs is reduced.

These data favor the notion that resumption of scanning rather than tRNA acquisition is defective in *eif3h*.

### **III.4 Discussion**

AtbZip11 encodes a basic leucine zipper transcription factor capable of heterodimerizing with other bZips and binding to G-box promoter motifs. Some of the direct targets of AtbZip11 include genes in the amino acid biosynthesis pathway like proline dehydrogenase and asparagine synthetase (Hanson et al., 2008). The uORF cluster in AtbZip11 is highly conserved among homologs from other angiosperms (Hayden and Jorgensen, 2007; Wiese et al., 2004). Among these uORFs, uORF2b is responsible for translational repression by sucrose (Rahmani et al., 2009; Rook et al., 1998a; Wiese et al., 2004), subsequently affecting the expression of its target genes (Hanson et al., 2008). The role of AtbZip11 in amino acid biosynthesis and its translational regulation by sucrose level is similar to other bZips, such as GCN4 in yeast and ATF4 in mammalian cells. Thus, understanding the molecular mechanism of translation in this leader together with the function of eIF3h in regulating its expression has physiological relevance.

#### **III.4.1 The AtbZip11 leader as a model system for assigning roles to translation factors**

The structure-function analysis of the AtbZip11 leader revealed that translation is cap dependent and does not involve any internal ribosome entry or ribosome shunting. Furthermore, the uORFs in the 5' leader are recognized by the scanning ribosomes and are translated. The translation of the main ORF is impeded by the uORF translation as evident from the derepression of translation efficiency in the wild type after the uORFs are removed (**Figure III. 2A**). The amount of initiation observed at the main AUG is higher than expression from the ribosomes leaky scanning past the uAUGs (wild type expression in **Figure III.3B** and in **Figure III.4B**); this provides evidence that translation of the uORFs is followed by a reinitiation event. Together, AtbZip11 is translated according to the scanning model, involving at least one reinitiation event.

Of the four uORFs, uORF1 masks the start codon of uORF2a. uORF1 is anti-inhibitory because it prevents the ribosomes from initiating at AUG2a, which represses translation more than any other uORF in the AtbZip11 leader. The overlapping nature of the uORFs suggests that not all uORFs will be recognized efficiently and some uORFs will be more inhibitory than the others. For reinitiation dependent recognition of the main AUG, uORFs 2 and 3 conferred more inhibition than uORFs 1 and 4.

#### **III.4.2 Transit routes of ribosomes on the AtbZip11 leader**

Structure-function analysis of the AtbZip11 leader leads to the following scenario for ribosome transit routes on the wild type AtbZip11 leader. The majority of ribosomes translate uORF1, notwithstanding its weak AUG context. Reinitiation after uORF1 is moderately eIF3h dependent. Of the ribosomes that skip uAUG1, most initiate at uAUG2a or 2b. Translation of uORF2 makes initiation at uAUGs 3 or 4 unlikely, because their start codons lie upstream of the uORF2 stop codon. Reinitiation downstream of uORF2 at the main FLUC start codon is

overall inefficient (compare wt expression in **Figure III.3A** 'AUG 2a' with **Figure III.6A** '213nt'). It is also eIF3h dependent, first, because uORF2 is long enough to make reinitiation highly inefficient in the mutant, and second because uORF2 encodes an attenuator peptide (Rahmani et al., 2009) that appears to be more inhibitory in the mutant (**Figure III.5**). However, in the AtbZip11 leader, there are reinitiation events that seem to be independent of eIF3h – leaders containing uORF1 but not uORF2a (**Figure III.3A** and **III.3B**). This observation leads to the unconventional idea that reinitiation after uORF1 is inherently not eIF3h-dependent. But that, even in the presence of uORF1, ribosomes often translate uORF2a, resulting in the typical eIF3h dependent reinitiation seen with the uORF2 containing leader sequences. Among the possible mechanisms for this, backscan-reinitiation event from the stop of uORF1 into uAUG2a, was ruled as unlikely. The other possibility is that ribosome occupancy on uORF2 affects the route of upstream ribosomes that are poised to reinitiate after uORF1. The block on uORF2 by the attenuator peptide would block 40S ribosomes that are scanning downstream from uORF1 and this might cause them to dissociate from the mRNA, possibly in an eIF3h-dependent way. Another plausible mechanism is that stacking of initiation-competent 40S ribosomes may foster AUG recognition at uAUG2a or 2b. Because one predicts that very few *eif3h* ribosomes traverse the spacer downstream of uORF2, it is difficult to discern whether these ribosomes can be trapped by an overlap uORF. For this reason, the estimate of tRNA acquisition in the *eif3h* mutant remained tenuous (**Figure III.6B**). However, complementary experiments with the GCN4 leader, which has short uORFs that should allow resumption of scanning in the mutant, suggested that the *eif3h* mutant is not defective in tRNA acquisition.

### **III.4.3 The H subunit of eIF3 is a reinitiation factor**

Reinitiation after translation of a short ORF is not uncommon, but the molecular events needed for successful reinitiation are still not well understood. As discussed in Chapter 1,

reinitiation after translation of long ORFs is exceedingly rare but involves eIF3 as well (Park et al., 2001; Poyry et al., 2007). Budding yeast has a poor reinitiation capacity on all but short uORFs, and lacks eIF3h. However, several yeast eIF3 mutants have defects in reinitiation, for instance a decrease in scanning during reinitiation observed on the GCN4 leader in a point mutant of eIF3b (Nielsen et al., 2004). An interaction between eIF3a and the small ribosomal subunit protein S0 is necessary to interpret the specific sequence context at uORF1 of GCN4. Presumably eIF3 recognizes the specific mRNA motif as it emerges from the mRNA exit channel once the ribosome terminates translation of uORF1 (Szamecz et al., 2008). Again, very few yeast *eif3* mutants display the general control derepressed (*gcd*) phenotype that is suggestive of defects in tRNA acquisition (Nielsen et al., 2004). In yeast, the reinitiation efficiency is poor by default, but can be enhanced by sequence context. With the conclusions drawn from the reinitiation on the AtbZip11 leader, it is apparent that factor requirements for reinitiation also vary depending on the uORF that is being translated and the 'challenge' provided by the uORF. The eIF3a mediated reinitiation after GCN4 uORF1 is a case of reinitiation after a short uORF and it seems to be a specific phenomenon, as the same sequence element could not be identified in the mammalian ATF4 leader (Szamecz et al., 2008). On the other hand, reinitiation on the AtbZip11 leader is a case of reinitiation after a long extended uORF. Reinitiation after long uORFs might differ in factor requirement and well as might involve specialized RNA sequences that have been envisioned to capture the wiggling ribosome efficiently after termination (Park et al., 2004a; Park et al., 2001; Poyry et al., 2007; Poyry et al., 2004).

It is unclear whether all reinitiation events are enhanced by eIF3h. Several AtbZip11 uORFs showed little eIF3h dependence when tested individually. A similar lack of eIF3h dependence was seen with a number of single-uORF leaders such as HY5 and ABI5 (Kim et al., 2004). The 5' leader of maize basic helix-loop-helix transcription factor R-Lc possesses a single uORF of 38 codons, which demands reinitiation for effective expression of maize R-Lc (Wang and

Wessler, 1998), yet the maize R-Lc leader was also not eIF3h dependent in our *Arabidopsis* assay system (**Appendix III.2**). These results could be explained by invoking that (i) eIF3h is most important on leaders with multiple uORFs, as suggested by polysome microarray data (Kim et al., 2007), while single uORFs cause eIF3h dependence only in exceptional cases, as with uORF2 of AtbZip11.

For *Arabidopsis* eIF3h, there are two canonical biochemical activities of eIF3 that may underlie its role in reinitiation; effective recruitment of a fresh ternary complex to the 40S subunit, which is not supported by the data described here; and separation of the 40S and 60S subunits (Benne and Hershey, 1978; Pisarev et al., 2007). Studies on the AtbZip11 leader indicate that eIF3h assists in efficient resumption of scanning. This function of eIF3h might be related to eIF3's role in ribosomal subunit separation by virtue of which it helps the 40S ribosome to remain attached to the mRNA.

Which ribosomal subunits or proteins might partner with eIF3h? One might expect that the cognate partner of eIF3h in the ribosome display a similar mutant phenotype as *EIF3H*.

*Arabidopsis* STV1 encodes ribosomal protein RPL24B, mutation of which interferes with translation of some uORF containing mRNAs. The AtbZip11 mRNA requires both eIF3h and RPL24b (discussed in Chapter 4). One other plant eIF3 subunit, eIF3g, has been linked to reinitiation because it interacts with the cauliflower mosaic virus reinitiation factor, TAV (Park et al., 2004a; Park et al., 2001). Furthermore, eIF3 and TAV also interact with RPL24 and with a recently discovered cellular protein that is auxiliary to reinitiation (Thiebauld et al., 2009). Taken together, eIF3h appears to be part of a larger functional module that is responsible for efficient reinitiation after uORF translation.

### **III.5 Materials and methods**

#### **III.5.1 Molecular cloning and generation of AtbZip11 5' leader mutants**

The AtbZip11-leader/FLUC reporter plasmid for testing translation efficiency in transient expression assays (Kim et al., 2004) served as the template to generate AtbZip11 5' leader mutants by site directed mutagenesis, which were confirmed by sequencing. Primer sequences are available upon request. To introduce multiple uAUG mutations, single mutants were used as PCR template. For the set of constructs to test initiation at uAUGs, the AtbZip11 5' leader till +4 position with respect to the uAUG was amplified with *XhoI*/*NcoI* sites and fused to FLUC. Frameshift mutations in uORF2 were produced by introducing +1 and -1 mutations in uORFs 2a and 2b separately while maintaining the internal AUG in frame. Spacer lengths were varied by truncating its 3' end. To study the extent of leaky scanning, uORFs 2 and 3 were extended past the start of the main ORF by mutating all in-frame stop codons. The *AtbZip11* 3'UTR was amplified as a *BglII*/*XbaI* fragment from *Arabidopsis* genomic DNA and subcloned so as to replace the cauliflower mosaic virus 35S terminator in the AtbZip11-FLUC construct. The 5' leaders from the GCN4-LacZ constructs described previously as B180, B976, B235, B227, pM114, B1002, B1010, and B1014 (Abastado et al., 1991a; Abastado et al., 1991b) were amplified with *XhoI* and *NcoI* sites and sub-cloned to the 35S:FLUC plasmid and confirmed by DNA sequencing.

#### **III.5.2 Transient expression and translation assay**

*Arabidopsis* seedlings were grown in MS medium for up to 20 days and were transformed via particle bombardment (PDS-1000/He; Bio-Rad) with plasmid DNAs harboring either AtbZip11 or GCN4 5' leaders and firefly luciferase (FLUC) coding sequence. The promoter (35S) and 3' UTR are from cauliflower mosaic virus. A 35S-driven *Renilla* luciferase construct containing the translational leader of tobacco etch virus served as a reference (Kim et al., 2004). Luciferase activities were measured in protein extracts prepared 15-17 hours after bombardment using

the Dual Luciferase System (Promega, Madison, WI) and a TD 20/20 luminometer (Turner designs, Sunnyvale, CA). The ratio of FLUC to RLUC activity indicates the translation efficiency. P-values for two-sided t-tests were routinely calculated after log-transformation of the FLUC/RLUC ratios.

For *in vitro* transcription, expression plasmids harboring a SP6 promoter and a 70 nucleotide poly(A) tail were linearized by *Pvu*II digestion and column purified (Qiagen). For cap-dependent translation initiation, mRNA was *in vitro* transcribed and capped using SP6 RNA polymerase and m7G cap analog (New England Biolabs) following the manufacturer's protocols. mRNA quality and quantity were estimated by agarose gel electrophoresis. Protoplasts were prepared from wild type or mutant 7 day old *Arabidopsis* seedlings. Seedlings with roots removed were cut into 0.5 mm slices and digested with digestion buffer (1.5% cellulase R10, 0.3% macerozyme R10, 0.4M mannitol, 20mM KCl, 20mM MES pH 5.7, 10mM CaCl<sub>2</sub>, 5mM mercaptoethanol, 1% BSA) for 3 hours after 30 min vacuum infiltration. Protoplasts were released by gently swirling, and filtered through a 40µm mesh into a plastic centrifuge tube on ice. After centrifugation in a tabletop centrifuge, the protoplast pellets were washed with 10 ml W5 solution (154 mM NaCl, 125 mM CaCl<sub>2</sub>, 5mM KCl and 2 mM MES, pH 5.7) and resuspended in 2ml W5 solution. The W5 solution was then substituted with MMG solution (0.4 M mannitol, 15 mM MgCl<sub>2</sub> and 4mM MES, pH5.7) before mRNA transformation. Eighty microgram of sheared and denatured salmon sperm DNA was added to 0.1 ml protoplasts in MMG solution in a 1.7ml microcentrifuge tube. Two hundred nanogram of mRNA was added immediately before the addition of 0.11 ml PEG solution (40% PEG, 240mM mannitol and 100mM CaCl<sub>2</sub>), which was subsequently mixed by gently inverting three to four times. The PEG transformation was terminated by addition of 0.43ml W5 and centrifugation. The protoplasts were resuspended in 1 ml W5 solution and incubated in a 24 well plate for 3 hours in the dark at room temperature, then harvested by centrifugation for luciferase assays.

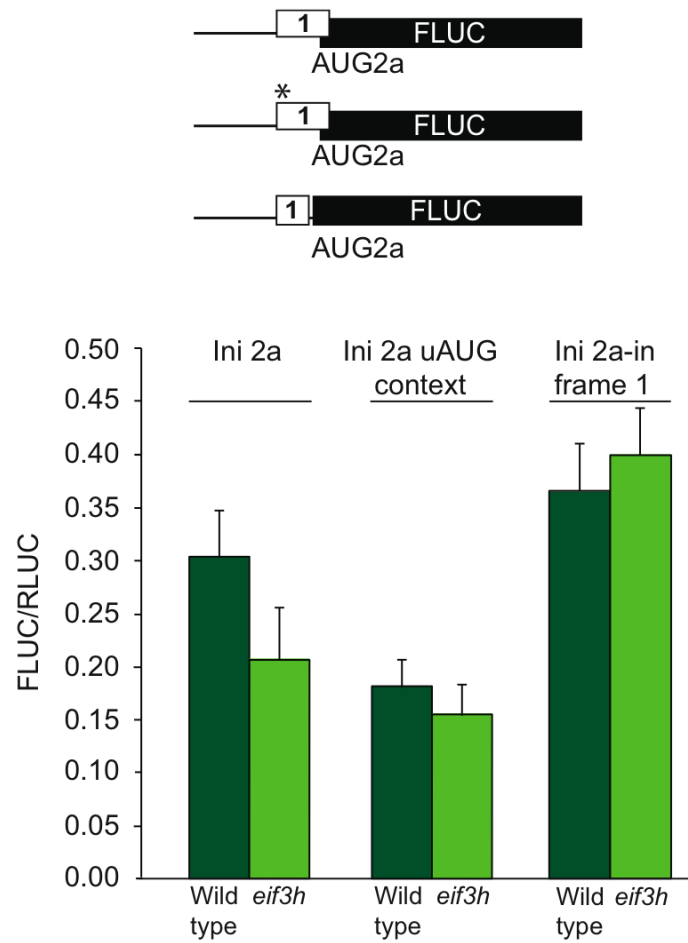
### **III.5.3 RNA expression analysis**

Total RNA was isolated from seedlings transiently expressing AtbZip11 5' leader constructs by harvesting tissues in liquid nitrogen after 15-17 hours and extraction with Trizol (Sigma). For RT-PCR analysis, 1 µg of total RNA was treated with RNase-free DNase I (Promega), and cDNA was synthesized with a 15-mer oligo(dT) primer using MMLV reverse transcriptase (Promega). Amplification was performed as described before (Kim et al. 2004). Half-life of mRNAs was determined after blocking transcription with cordycepin. Transgenic *Arabidopsis* seedlings harboring the AtbZip11:FLUC transgene (Kim et al., 2007) were grown for two weeks in germination medium and were transferred to incubation buffer (1mM Pipes, pH 6.25, 1mM sodium citrate, 1mM KCl, 15mM sucrose) for 30 minutes, followed by addition of cordycepin (Sigma, St. Louis) to a final concentration of 100µg/mL. RNA samples were harvested at specific time points, treated with DNase, and analyzed by RT-PCR for the levels of FLUC and eukaryotic elongation factor 1a. Control experiments demonstrated that the amplifications had not reached saturation and that no DNA contamination was present.

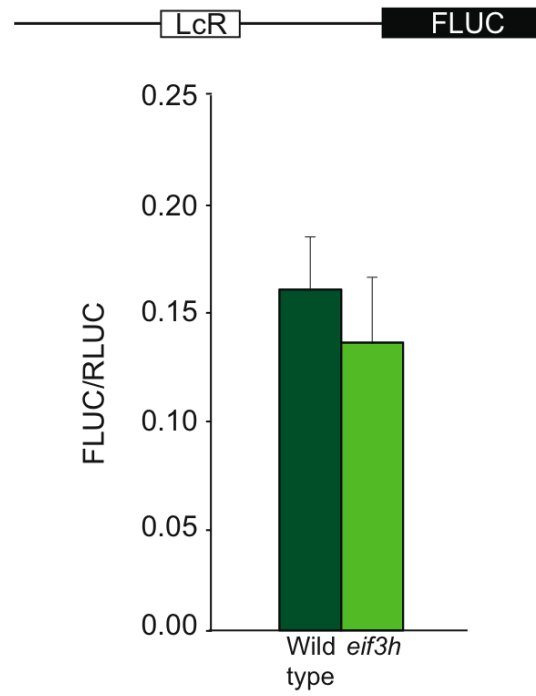
### **III.5.4 Polysomal protein isolation and eIF3 association**

Seedling extract was prepared in 1mL extraction buffer containing 200mM Tris-Cl (pH 8.4), 50mM KCl, 25mM MgCl<sub>2</sub>, 50µg/ml cycloheximide, and 1X protease inhibitor cocktail (Sigma) (Park et al., 2001). The extract was layered on a 10mL 7.5-30 % sucrose gradient. After centrifugation at 4°C in a Beckman SW41Ti rotor for 5 hours at 39,000 rpm, 11 fractions were collected manually (Nielsen et al., 2004). Fractions were concentrated using an Amicon centrifugal filter device (Millipore) at 6500 rpm for 10 minutes. Concentrated fractions were resolved on 10% acrylamide gels and immunoblots performed with anti eIF3h (Kim et al., 2004) and anti eIF3 (Lauer et al., 1985) antibody.

**Appendix III.1. AUG2a is not recognized by ribosomes back-scanning from uORF1 stop codon.**



**Appendix III.2. Reinitiation on the maize R-Lc leader is not eIF3h dependent.**



## **Chapter 4**

### **Efficient reinitiation after uORF translation requires cooperation between eIF3 and the large ribosomal subunit**

Some of the results presented in this chapter have been submitted for publication as part of the following paper:

*The translation initiation factor eIF3h cooperates with the large ribosomal subunit in mediating translation reinitiation on mRNAs harboring upstream open reading frames.*

*Fujun Zhou, Bijoyita Roy and Albrecht G. von Arnim*

#### **IV.1 Abstract**

Constraints are imposed on the translation machinery when an upstream open reading frame (uORF) precedes the main protein coding region. During special initiation events, ribosomes encountering these roadblocks translate the uORF and then reinitiate downstream to synthesize the functional peptide. A complete understanding of the molecular events and cellular factors that direct the reinitiation efficiency on elongating ribosomes is still elusive. The h subunit of the eukaryotic initiation factor 3 (eIF3) is required for maintaining reinitiation competence during translation of multiple upstream open reading frames for the bZip11 transcription factor AtbZip11. Using the *Arabidopsis* AtbZip11 leader as a model system, the requirement for other components of the translation machinery in retaining reinitiation competence was tested in this study. Mutations in genes for the d, i, k, and m subunits of eIF3 did not impede translation across uORFs. Large ribosomal protein, RPL24B, was previously shown to foster translation of certain uORF containing mRNAs. Here I provide evidence that like *eif3h* mutants, *rpl24b* mutants undertranslate the AtbZip11 mRNA. The spectrum of deficiencies across a panel of mRNA constructs is indistinguishable between *eif3h* and *rpl24b* suggesting that RPL24B, like eIF3h, bolsters reinitiation competence of the uORF-translating ribosomes. Overall, this suggests that eIF3h cooperates functionally with the 60S ribosomal subunit for reinitiation after uORF translation.

## IV.2 Introduction

Translation initiation in eukaryotes is based on the notion that ribosomes generally cannot translate an open reading frame that resides downstream of a previously translated ORF (Jackson et al., 2010; Kapp and Lorsch, 2004). That said, however, there are exceptions to this general rule that fall into the following categories, (1) skipping of the upstream ORF, (2) translation of the uORF followed by reinitiation, and, (3) occasional trapping of ribosomes in the intercistronic spacer by an internal ribosome entry site/sequence (IRES) (Jackson et al., 2010). Non-canonical translation initiation mechanisms via IRESs are widely employed by viruses, and are occasionally found during mRNA specific translational control (Sonenberg and Hinnebusch, 2009). This however, relies on the presence of specific regulatory *cis*-acting structural elements in the messenger RNA. About 45-50% of mammalian genes and 30% of genes in *Arabidopsis* (compared to only 13% of yeast genes) encode mRNAs that have at least one open reading frame upstream of the principal protein coding region (Calvo et al., 2009; Kim et al., 2007; Lawless et al., 2009; Resch et al., 2009). As described in Chapters 1 and 3, uORFs are generally inhibitory as they block the path of the scanning ribosome; however, a few ribosomes that have translated the uORF resume scanning and reinitiate downstream. According to the scanning model of translation initiation, the initiation competent 43S pre-initiation complex recognizes the 5' cap and starts scanning till it encounters the first initiator codon, an uAUG in case of uORF harboring mRNAs, and starts translating it (Kozak, 2001, 2002). It can be assumed that the termination event at the uORF proceeds conventionally with the release of the 60S ribosomal subunit and the deacylated t-RNA; whereas the 40S ribosome remains bound to the mRNA and scans further downstream. The exact mechanism of reinitiation is far from being deciphered. Reinitiation and rescanning efficiency are diminished as the length of the uORF increases or the ribosome pauses during elongation (Kozak, 2001; Morris and Geballe, 2000; Rajkowitsch et al., 2004; Sachs and Geballe, 2006). Hence, the time taken to translate the uORF is pivotal in determining the

reinitiation potential. Also crucial is the participation of eIF4F during initiation; probably because of its interaction with eIF3 (Poyry et al., 2004). A critical question concerns which molecular factors are associated with the elongating ribosome that determine a preferential rescanning of 40S terminating ribosomes over dissociation from the mRNA. The most plausible model to explain this is that translating ribosomes have a molecular memory in the form of eIFs that remain loosely associated for a couple of cycles of elongation, but are eventually lost/ejected from the 80S, eIF3 being one of the critical components. Evidence in favor of this model comes from both genetics and biochemistry (discussed in Chapter 3). Furthermore, because eIF3 is bound to the solvent face of the 40S subunit rather than the 40S-60S subunit interface, 60S subunit joining might not break the transient eIF3-40S interaction, and eIF4G-eIF3-40S interaction may persist during translation elongation (Siridechadilok et al., 2005). Second, translation of polycistronic RNA of cauliflower mosaic virus (CaMV) and other pararetroviruses using transactivator viroplasm (TAV) engages eIF3 in reinitiation via an interaction between eIF3g and a large ribosomal subunit protein, RPL24 (Park et al., 2004a; Park et al., 2001; Ryabova et al., 2004; Thiebauld et al., 2009). These data suggest that interaction between eIF3 and 60S ribosomal proteins is critical for reinitiation after long uORFs. Till date, the function of eIF3 in reinitiation has been attributed to its involvement with the 40S subunit. However, the interaction between eIF3-TAV-RPL24 corroborates the critical role of 60S-eIF3 cooperation in deciding the fate of the terminating ribosomes.

Here, I am going to investigate how eIF3 cooperates with the 60S subunit in the context of cellular reinitiation. The rationale for this study is that, (1) uORF containing mRNAs (AUXIN RESPONSE FACTOR3 and AUXIN RESPONSE FACTOR5) are undertranslated in an *rpl24b* mutant (Nishimura et al., 2005), and, (2) *eif3h* mutants show phenotypic defects similar to *arf3* and *arf5* mutants (Zhou and von Arnim, unpublished observations). If eIF3 (via h subunit) and RPL24B function together, then a mutation in RPL24B should essentially have the same defect in morphology and gene expression as *eif3h*. Hence, the function of RPL24B in

reinitiation after uORF translation was tested using reagents as described in Chapter 3. For comparison, several other *eif3* mutants were examined for a reinitiation defect on the AtbZip11 mRNA.

## **IV.3 Results**

### **IV.3.1 Requirement of eIF3h for translation of AtbZip11 mRNA is specific**

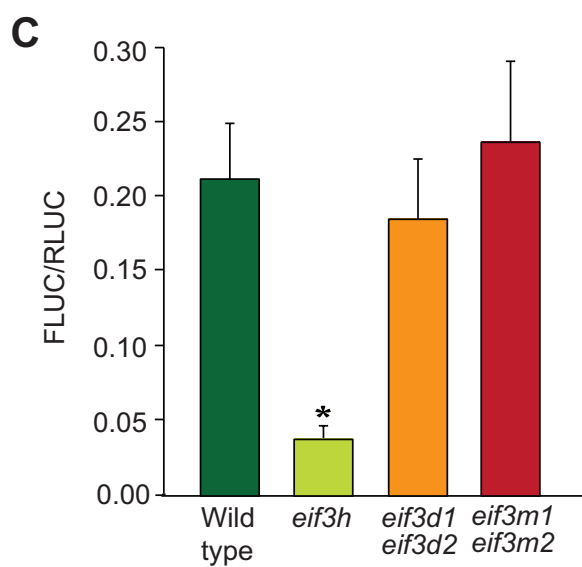
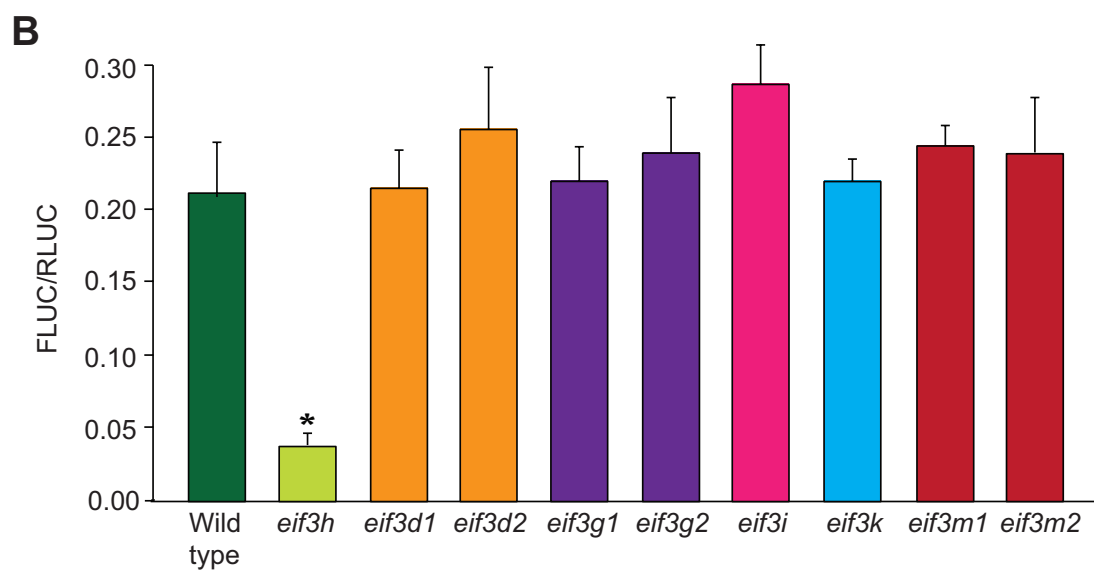
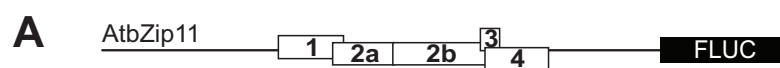
In order to investigate whether mutations in other eIF3 subunits display gene expression defects similar to *eif3h*, translation reinitiation efficiency on the AtbZip11 mRNA was compared (**Figure IV.1A**). The rationale being, a similar spectrum of defects would give evidence of closely related function and *vice versa*.

The requirement of the non-conserved subunits d, k, and m were tested for their involvement in translation across uORF containing AtbZip11 mRNA. As described previously (Chapter 2), a T-DNA insertion in the 6<sup>th</sup> intron of the k subunit was identified; homozygous lines resembled the wild type under normal growth conditions and had subtle differences in the transcript level (Chapter 2). The d subunit of eIF3 in *Arabidopsis* has 2 paralogs – AteIF3d1 (At4g22980) and AteIF3d2 (At5g44320). Individual homozygous lines with insertion in the 3' UTR for *eif3d1* and insertion in the single exon for *eif3d2* were identified (characterization of mutant lines is discussed in Chapter 2). Similar to the d subunit, mutations in AteIF3m1 (At3g02200) and AteIF3m2 (At5g15610) were identified as well (Chapter 2 for characterization of mutants). Using the transient reporter gene expression assay, translation efficiency of the AtbZip11 leader was tested in the different *eif3* mutant backgrounds. Notably, translation efficiency in all the single mutants was found to be similar to wild type. Therefore, no specific requirement for any of the eIF3 subunits was observed (**Figure IV.1B**). In order to rule out functional redundancy between duplicate members of the family, translation efficiency of the AtbZip11 leader was also tested in *eif3m1 eif3m2* and *eIF3d1 eIF3d2* double mutant background.

**Figure IV.1. Translation efficiency of the AtbZip11 leader in *eif3x* mutants.**

**A.** Schematic representation of the firefly reporter coding region fused to the original leader of *Arabidopsis* AtbZip11. Boxes represent uORFs. Only the uORFs are drawn to scale.

**B.** Transient gene expression assay in ten days old seedlings. The respective 5' leader is fused to firefly luciferase and is expressed in the presence of a reference gene expressing *Renilla* luciferase as an internal control for transformation efficiency. Both genes are transcribed from a CaMV 35S promoter. Bars denote standard error. \* Statistical significance at  $P < 0.002$  in a two sided Student's t-test. (n=5-7).



Translation efficiency was not altered even when both the copies of eIF3m and eIF3d were disrupted (**Figure IV.1C**). To test for the involvement of eIF3g in translation reinitiation after uORF translation, translation efficiency was tested in the single mutants of AteIF3g1 (At3g11400) and AteIF3g2 (At5g06000). Translation efficiency of the AtbZip11 leader was not compromised in either (**Figure IV.1B**). However, a possible functional redundancy between AteIF3g1 and AteIF3g2 may exist. Apart from this, another conserved subunit of eIF3 was tested for its function in translation reinitiation. The i subunit of eIF3 is highly conserved from *Saccharomyces cerevisiae* to *Arabidopsis*. RNAi lines disrupting eIF3i expression (Jiang and Clouse, 2001) were used to test the requirement of eIF3i for translation reinitiation. Loss of eIF3i activity did not affect translation efficiency downstream of the uORF cluster in the AtbZip11 leader (**Figure IV.1B**). Taken together, among all the eIF3 subunits tested, the h subunit has a specific role in overcoming the inhibitory effects imposed by upstream open reading frames.

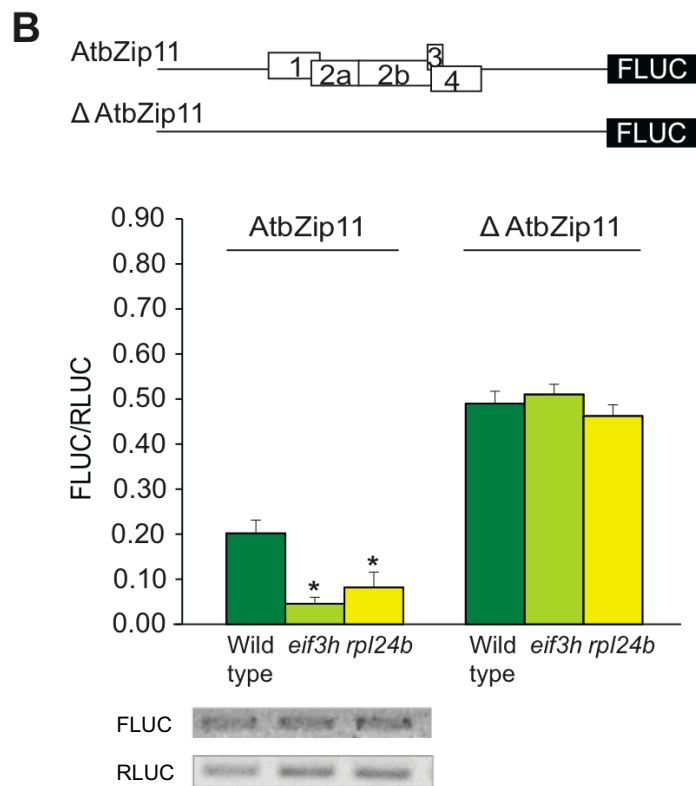
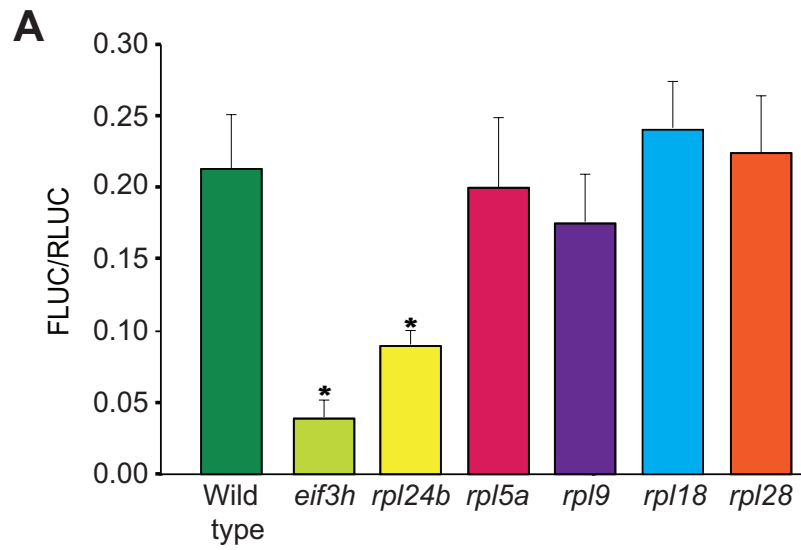
#### **IV.3.2 RPL24B is required for efficient translation of AtbZip11 leader**

Developmental defects reported for *rp124b* (Nishimura et al., 2005), *rp15*, *rp19*, *rp118* and *rp128* (Byrne, 2009) led to the speculation that these proteins might cooperate in the translation of the same client of mRNAs as eIF3h. This hypothesis was examined by testing the translation efficiency of the AtbZip11 mRNA that shows absolute eIF3h dependence. Indeed, the translation efficiency of the AtbZip11 leader was reduced by about 2 fold in the *rp124B* mutant (**Figure IV.2A**). Notably, translation of the AtbZip11 leader was no longer dependent on the presence of functional RPL24B once all the five uAUGs were removed by site directed mutagenesis (**Figure IV.2B**). Requirement of RPL24B for efficient translation of the uORF containing AtbZip11 leader was very specific and was not observed when other ribosomal proteins (RPL10, RPL5A, RPL28, RPL18) were disrupted (**Figure IV.2A**). This indicates that RPL24B is specifically required for the efficient translation of the uORF containing AtbZip11 leader.

**Figure IV.2. Translation efficiency of the AtbZip11 leader in *rpl* mutants.**

**A.** Transient expression data from 10-day old seedlings. The efficiency of translation was calculated as the activity ratio of firefly luciferase versus *Renilla* luciferase (RLUC) as a co-transformed reference gene. Bars denote standard error. \*,  $P < 0.002$  in a two sided Student's t-test. (n=3-15).

**B.** eIF3h and RPL24B are required for efficient translation of the uORF-containing AtbZip11 mRNA. Translation efficiency in the uORF-less AtbZip11 leader; uAUGs are removed as described in Chapter 3. Bars denote standard error. \*,  $P < 0.002$  in a two sided Student's t-test. (n=3-10). Respective transcript levels of FLUC and RLUC reporter (Bottom).



### IV.3.3 RPL24B is required for efficient reinitiation after uORF translation

To ascertain the function of RPL24B in translation of uORF containing leaders, a series of different expression cassettes described in Chapter 3 were employed. Mutation in RPL24B did not affect recognition of the uAUGs, similar to *eif3h* (**Figure IV.3A**) nor was it sensitive to the attenuator peptide encoded from uORF2 (**Figure IV.3B**). To address the question whether RPL24 and eIF3h function in a similar or distinct way, reinitiation efficiency after uORF translation was measured. In order to measure reinitiation, the uORFs in the AtbZip11 leader were altered to commence with start codons in a strong context in an effort to minimize leaky scanning (**Figure IV.4**) (Kozak, 1986). One important parameter guiding reinitiation is the length of the spacer between the uORF stop codon and start of the main ORF, attributed to the efficiency with which Met-tRNA<sup>Met</sup> is acquired (Abastado et al., 1991a; Abastado et al., 1991b; Kozak, 1987). Varying the spacer between 25 and 213 nucleotides (nt) showed increase in reinitiation potential in the wild type after traversing 50 nt after which it reached a plateau (**Figure IV.4**). Similar to *eif3h*, *rpl24b* showed only a marginal increase in translation efficiency even after traversing 213 nt (**Figure IV.4**). This indicates that reinitiation downstream of the uORF cluster in the AtbZip11 leader requires both eIF3h and RPL24.

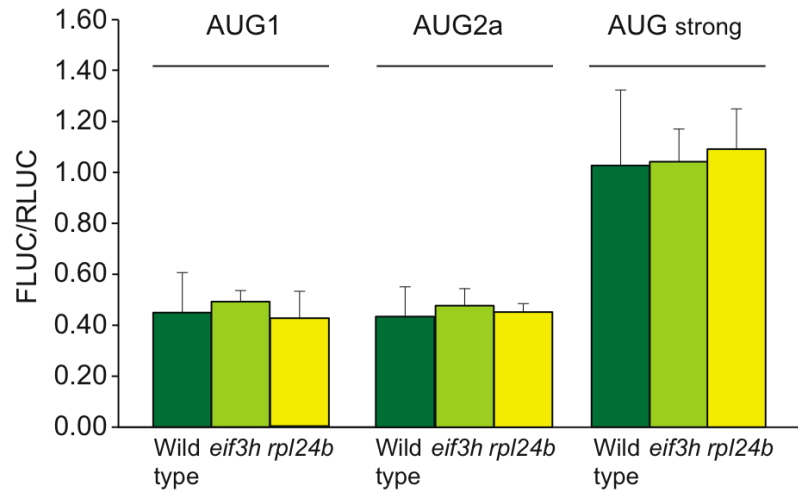
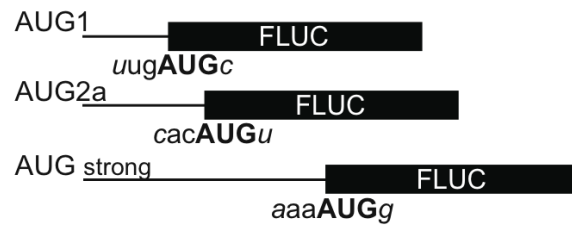
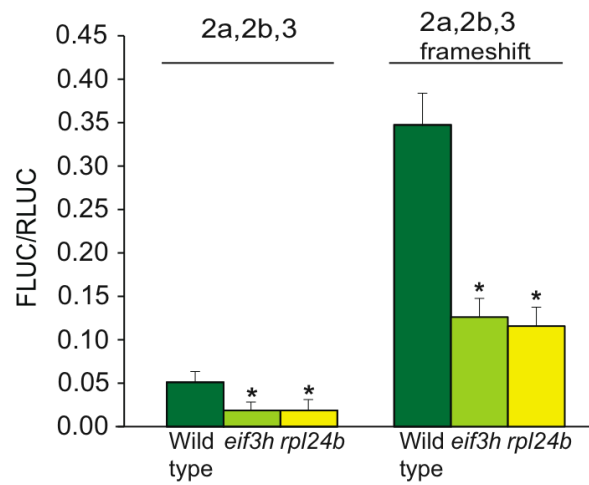
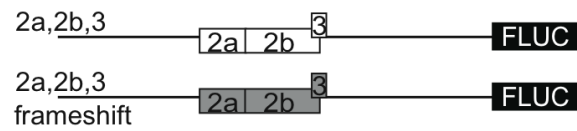
### IV.3.4 Molecular function of RPL24B in reinitiation

In order to gain insight into the function of RPL24B in reinitiation, translation of the yeast GCN4 leader that requires reinitiation for expression was examined. The four uORFs in the GCN4 leader, in contrast to AtbZip11, are short and therefore translation defects are less pronounced in *eif3h* (Chapter 3). In yeast, the GCN4 leader reports on ternary complex reacquisition by ribosomes that have resumed scanning after uORF1. A delay in reacquisition of ternary complex, as occurs in *gcd* mutants, results in skipping of uORF4, and hence an elevated expression compared to wild type is observed (Hinnebusch, 2005). Translation of the

**Figure IV.3. RPL24B is not required for AUG recognition and is independent of the peptide sequence.**

**A.** uAUG1, 2a of the AtbZip11 uORFs were fused directly to FLUC while retaining the start codon context up to the +4 position as well as their distance from the transcription start site as drawn in the schematic. A synthetic leader for initiation at a favorable context was tested as well. Note that all uAUGs appear to be recognized in an RPL24B-independent fashion (n = 3-5).

**B.** The dependence of translation on the uORF peptide sequence was tested by introducing a frameshift mutation as described in Chapter 3. Stimulation of translation across uORFs 2 and 3 by RPL24B is not dependent on the uORF peptide sequences similar to eIF3h. Both *rpl24b* and *eil3h* are inhibited to a similar degree by uORFs encoding the original uORF2, 3 peptide sequences and frame-shifted peptide sequences. (n=6-11)

**A****B**

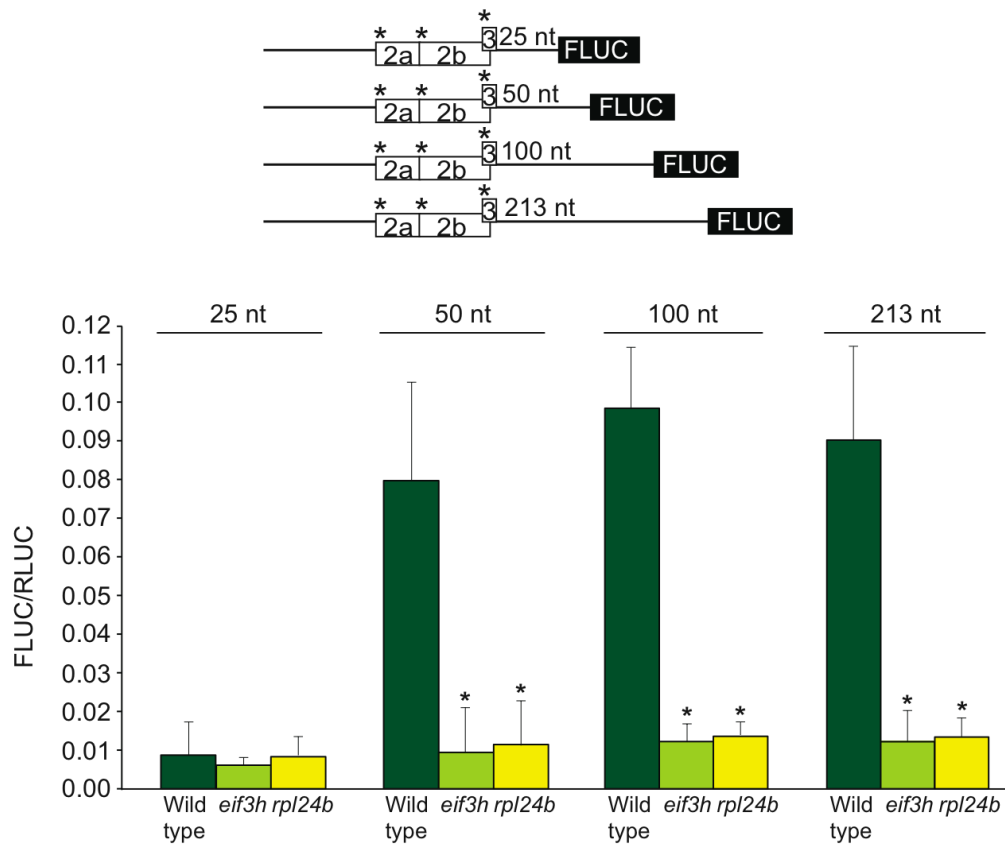


**Figure IV.4. RPL24B along with eIF3h is required for efficient reinitiation after uORF translation.**

The molecular function of RPL24B in reinitiation was tested by shortening the intercistronic spacer region in the *AtbZip11* leader. Translational efficiency on varying spacer length constructs. *rpl24b* exhibits a similar reinitiation defect as *eil3h*. Bars denote standard error; n = 5-10; \* P<0.002 by Student's t-test. Error bars denote standard error (n=5-10); P<0.002.

GCN4 leader in *rpl24b* showed no derepression and resembled *elf3h*, consistent with a closely related molecular function (**Figure IV.5**).

Introducing an inhibitory uORF in the AtbZip11 leader that overlaps with the main ORF affects the translation of the main ORF negatively (**Figure IV.6**), because the wild-type ribosomes can reinitiate at the overlapping uORF after translating the uORF cluster. However, the addition of the overlap-uORF did not further repress expression in *rpl24b*, as in *elf3h*, suggesting that reinitiation after uORF translation is poor. This observation further bolstered the hypothesis that, like eIF3h, RPL24B facilitates resumption of scanning of the post-termination ribosomes (**Figure IV.6**). Hence, RPL24B acts together with eIF3h in maintaining reinitiation competence during translation of uORFs. Because a role for RPL24B (and eIF3h) in tRNA<sup>Met</sup> acquisition would have led to a relatively mild translation defect on the GCN4 leader, the data suggest that the function of RPL24B in reinitiation is not related to *de novo* reacquisition of the ternary complex. Instead, RPL24B, similar to eIF3h, helps retain the reinitiation competence.

#### **IV.4 Discussion**

Reinitiation after short upstream open reading frames might not be a common phenomenon, and, the molecular events determining reinitiation competence are not well understood. eIF3 has emerged as an important component of the translation machinery pivotal for retention of reinitiation competence of uORF translating ribosomes (Park et al., 2004a; Park et al., 2001; Ryabova et al., 2004; Szamecz et al., 2008; Thiebauld et al., 2009) (Chapter 3). Our current understanding of translation reinitiation suggests that reinitiation after short uORFs is inhibited less than reinitiation after long extended uORFs. The factor requirements for reinitiation as well as the efficiency can vary drastically depending on the uORF that the ribosome translates.

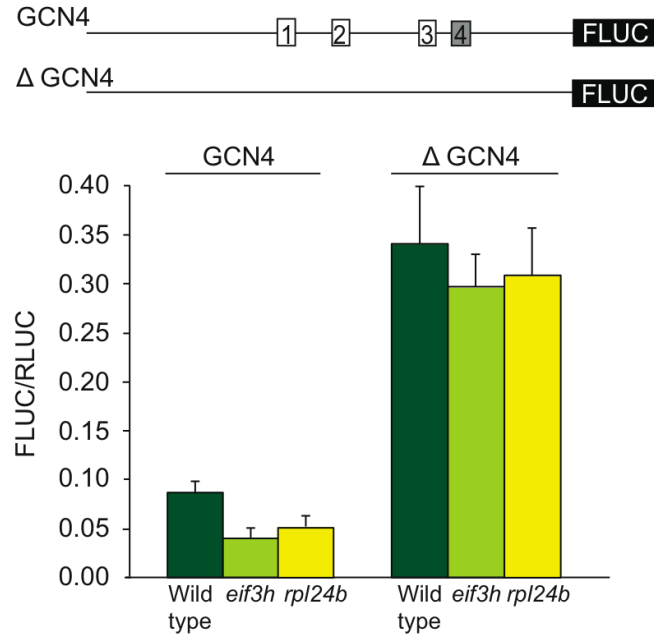


**Figure IV.5. *rpl24b* and *eif3h* have similar reinitiation defect on the GCN4 leader.**

**A.** Schematic representation of the GCN4 5' leader sequences tested. Boxes represent uORFs. Only the uORFs are drawn to scale.

**B.** Expression data for wild-type GCN4 leader and mutated GCN4 leader. Translational activity is similar in *rpl24b* and *eif3h*. Error bars denote standard error (n=5).

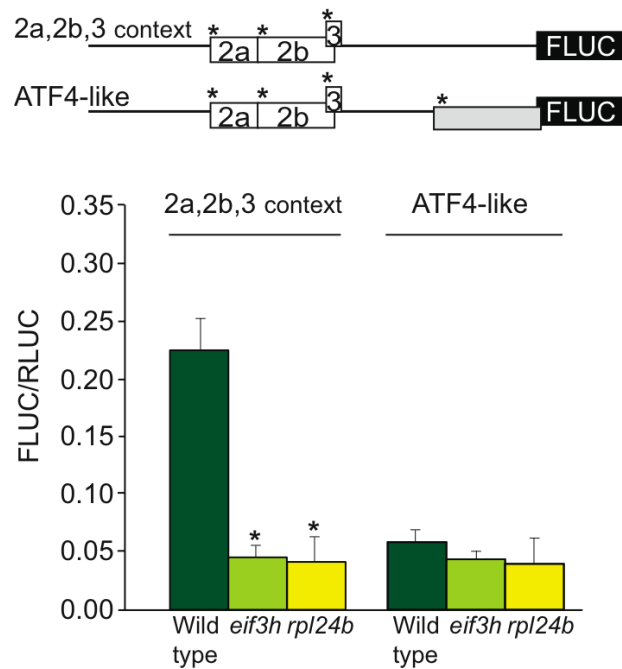


**Figure IV.6. ATF4-like leader to elucidate function of RPL24B in reinitiation.**

Inhibition by an ATF4-like overlap-uORF downstream from a uORF cluster demonstrates efficient reinitiation in wild type but marginal reinitiation in both *eif3h* and *rpl24b*. A uORF (white box) in a strong Kozak context (A<sub>-3</sub>AAAUGG<sub>+4</sub>) located 60nt upstream of the FLUC ORF and 150 nt downstream of the uORF cluster is introduced as described in Chapter 3. Asterisks mark AUGs in a strong context. Bars represent standard error (n = 8-12).

Also, it is becoming increasingly apparent that certain uORFs possess inherent properties that in turn dictate the reinitiation process. Surprisingly, eIF3 is necessary for maximal reinitiation on a panel of different mRNAs and its requirement is generic. The exceedingly rare event of reinitiation after a long uORF has been reported to be eIF3 dependent as well (Poyry et al., 2007; Poyry et al., 2004). As mentioned in Chapter 3, the h subunit of eIF3 helps ribosomes retain reinitiation competence during translation of moderately long uORFs in *Arabidopsis*. On the other hand, eIF3a has been reported to be important for making crucial contacts with the yeast GCN4 mRNA and thus facilitates resumption of scanning after translation of the short uORF1 (Szamecz et al., 2008). Hence, the functions of eIF3 in promoting reinitiation are multifaceted and it might assist reinitiation by (1) binding to RNA sequence elements (Poyry et al., 2007; Szamecz et al., 2008); (2) interacting with cellular and non-cellular reinitiation factors (Park et al., 2004a; Park et al., 2001; Ryabova et al., 2004; Thiebauld et al., 2009); and, (3) possibly via the canonical biochemical functions in reacquisition of a ternary complex after uORF translation. However, the longstanding question is whether eIF3 functions by itself or whether there are other factors that might cooperate with eIF3 in promoting reinitiation. Another interesting aspect of reinitiation that has surfaced is the possible cooperation of the large ribosomal subunit proteins. Specifically, studies on the reinitiation mechanism on CaMV RNA have suggested that RPL24 (ribosomal protein L24) interacts with TAV, and overexpression of RPL24 stimulates TAV-dependent reinitiation of translation in plant protoplasts (Park et al., 2001; Ryabova et al., 2004). However, the loss of reinitiation due to a mutation in RPL24 has not been shown for the CaMV RNA. This observation has further been complemented by the translation defects observed in uORF containing ARF3 and ARF5 mRNAs when RPL24B was disrupted (Nishimura et al., 2005). Thus, these parallel pieces of evidence lend support to the notion that reinitiation involves proteins of the large ribosomal subunit. The mistranslation of uORF containing transcripts in *Arabidopsis* due to disruption of eIF3h is associated with pleiotropic developmental defects (Kim et al., 2004) (F. Zhou and A. G. von

Arnim, unpublished observations). Pronounced defects are observed in meristem maintenance and auxin perception that can be attributed to under-translation of meristem maintenance genes and auxin response factors, mRNAs that are enriched in uORFs (F. Zhou and A. G. von Arnim, unpublished observations). Applying Occam's razor, factors that might act synergistically with eIF3h in retaining reinitiation competence on these mRNAs will display similar phenotypic defects when disrupted. Mutations of several ribosomal proteins in *Arabidopsis* cause defects in leaf patterning (Degenhardt and Bonham-Smith, 2008; Fujikura et al., 2009; Nishimura et al., 2005; Pinon et al., 2008; Van Lijsebettens et al., 1994; Weijers et al., 2001; Yao et al., 2008). A subtle defect in leaf development leading to a change in the rosette leaf shape has been observed for *eif3h* as well (F. Zhou and A. G. von Arnim, unpublished observations). However, translation efficiency of the uORF containing AtbZip11 transcript was altered only when RPL24B was disrupted, but not in any other *rpl* mutant. Hence, RPL24B might be important for efficient reinitiation, a function similar to eIF3h. Though disruption of other ribosomal proteins showed similar phenotypic defects, it is not clear if the developmental defects overlapping with the other ribosomal proteins are a direct consequence of translational defects. It should be noted that the function of many of these ribosomal proteins in translation are not known and may go beyond reinitiation (Byrne, 2009). One of the ribosomal proteins closely associated with RPL24 is RPL23 (Ban et al., 2000; Spahn et al., 2001). Disruption of RPL23A in *Arabidopsis* shows disruption of auxin homeostasis and defect in meristem maintenance (Degenhardt and Bonham-Smith, 2008; McIntosh and Bonham-Smith, 2005). It is tempting to hypothesize that the close proximity of RPL23 with RPL24 and similar phenotypic defects might be a result of their similar molecular function.

#### **IV.4.1 Molecular mechanism of RPL24B – eIF3h cooperation in promoting efficient reinitiation**

The functional overlap between eIF3h and RPL24B in translation reinitiation on the AtbZip11 leader (**Figures IV.4, IV.5, IV.6**) suggests a co-operativity between eIF3 and the large ribosomal subunit for efficient re-initiation after translation of long extended uORFs. Not much is known about how these two factors might function together. However, the RPL24-TAV-eIF3 interaction on the ribosomes translating CaMV polycistronic messages provides some intriguing insights.

Possibly, TAV acts as a scaffold and prevents the dissociation of eIF3 from the translating 80S ribosome during a prolonged elongation event by sequestering eIF3 to the translating ribosome. Recruitment of Reinitiation Supporting Protein (RISP) (Thiebeauld et al., 2009) and its interaction with RPL24 and TAV might strengthen the interaction between the large ribosomal subunit and eIF3. Whether a strong interaction between the two ribosomal subunits is crucial for maintaining the processivity of the elongating ribosome or whether it might have significance post-termination, is not clear.

Identification of RISP as a cognate partner in TAV mediated translation reinitiation raises the question of whether there are indeed ancillary proteins that are recruited onto the translating ribosome and aid translation of specific cellular mRNAs as well. It has to be borne in mind that these interactions were observed under viral reinitiation mechanism; what still needs to be elucidated is whether all re-initiation events involve RISP like proteins or whether RISP functions exclusively for viral translation machinery.

#### **IV.4.2 Cognate partners of eIF3 and RPL24**

The involvement of RISP like proteins in cellular reinitiation events is not known. It is tempting to speculate that the cellular machinery might have a similar mechanism to ensure reinitiation downstream of uORFs. Contrary to CaMV mediated viral reinitiation, cellular reinitiation depends on the length of the uORF. The interaction between the same components of the large ribosomal subunit (RPL24) and eIF3 might be weak in the context of cellular translation

event as a result of which this does not persist for a long time and the reinitiation competence is lost during translation of long uORFs. Moreover, the interaction of RISP with the  $\alpha$  and  $\gamma$  subunits of eIF3 raises the possibility that other eIF3 subunits might be important in forming the 'reinitiation bridge' between the 60S and 40S. Bimolecular fluorescence complementation (BiFC) showed interaction between eIF3h and RPL24 (F. Zhou and A. G. von Arnim, unpublished observations). However, it is not clear if the interaction is direct or other components of the translation machinery might be involved. Collectively, future work will elucidate the biochemical basis of this cooperation to deduce if other eIF3 subunits act in concert with eIF3h and RPL24 to promote reinitiation.

## **IV.5 Materials and methods**

### **IV.5.1 Transient expression and translation assay**

*Arabidopsis* seedlings were grown in MS medium for up to 20 days and were transformed via particle bombardment (PDS-1000/He; Bio-Rad) with plasmid DNAs harboring either AtbZip11 or GCN4 5' leaders and firefly luciferase (FLUC) coding sequence. The promoter (35S) and 3' UTR are from cauliflower mosaic virus. A 35S-driven *Renilla* luciferase construct containing the translational leader of tobacco etch virus served as a reference (Kim et al., 2004). Luciferase activities were measured in protein extracts prepared 15-17 hours after bombardment using the Dual Luciferase System (Promega, Madison, WI) and a TD 20/20 luminometer (Turner designs, Sunnyvale, CA). The ratio of FLUC to RLUC activity indicates the translation efficiency. P-values for two-sided t-tests were routinely calculated after log-transformation of the FLUC/RLUC ratios.

### **IV.5.2 RNA expression analysis**

Total RNA was isolated from seedlings transiently expressing AtbZip11 5' leader constructs by harvesting tissues in liquid nitrogen after 15-17 hours and extraction with Trizol (Sigma). For

RT-PCR analysis, 1 µg of total RNA was treated with RNase-free DNase I (Promega), and cDNA was synthesized with a 15-mer oligo(dT) primer using MMLV reverse transcriptase (Promega). Amplification was performed as described before (Kim et al. 2004). RNA samples were harvested at specific time points, treated with DNase, and analyzed by RT-PCR for the levels of FLUC and RLUC.

## **Chapter 5**

**Use of fluorescent-tagged lines to study male gametophyte developmental defects in *Arabidopsis* translation factor mutants**

Some of the results in this chapter have been published as part of the following paper:

Yahalom A., Kim T.H., Roy B., Singer R., von Arnim A.G., and Chamovitz D.A., 2008.

*Arabidopsis eIF3e is regulated by the COP9 signalosome and has an impact on development and protein translation. Plant J. 2008 Jan;53(2):300-11*

## **V.1 Abstract**

Translational control plays an important role in regulation of growth and development in multicellular eukaryotes. Eukaryotic initiation factor 3 (eIF3) is a multisubunit complex whose role in translation is well established across a wide array of organisms. Mutational analyses of eIF3 subunit genes have revealed the relevance of eIF3 in *Arabidopsis* development. A previously detailed carboxy-terminally truncated T-DNA insertion allele of the h subunit of eIF3 (At1g10840) develops a strikingly enlarged shoot apical meristem, and has defects in organ initiation and auxin responsive gene expression. Here I report that eIF3 is required early during the life cycle and is important for male gametophyte development. Mutations in the e and h subunits of eIF3 were transmitted poorly through the male gametophyte.

Transmission through the female gametophyte was also aberrant in mutations affecting eIF3e. A transgenic marker linked to the eIF3 gene, expressing a pollen specific fluorescent protein, was used in the *quartet* mutant background to study the defect in gametophyte development. Using these lines it was observed that the atypical transmission of *eif3e* and *eif3h* is in part a consequence of reduced pollen germination and, for *eif3h* specifically, reduced pollen tube growth. Hence, the translation factors, eIF3e and eIF3h are critical for male gametophyte development in *Arabidopsis*.

## **V.2 Introduction**

The complex life cycle of higher plants involves alternation between a diploid sporophytic phase and a haploid gametophytic phase. The formation of the two distinct phases in higher plants comprises two sequential processes, sporogenesis and gametogenesis. Gametophytes arise from the proliferation of haploid spores after meiotic divisions. Functionally, the gametophyte is responsible for the formation of haploid gametes; fusion of the haploid gametes results in the diploid zygote. The zygote then proliferates and differentiates into a complex multicellular sporophyte. Genetic studies in *Arabidopsis* have revealed mutations that affect the gametophyte development and therefore have started to unveil a complex genetic framework that maintains the short-lived gametophytic phase.

### **V.2.1 Male gametophyte development in higher plants**

In flowering plants, the male gametophyte plays a vital role in plant fertility. Male gametophyte development is often divided into two major phases, an early phase consisting of the microspore and bicellular pollen and a late phase consisting of the mature pollen (**Figure V.1**). Meiotic division in the diploid pollen mother cell during microsporogenesis results in the formation of haploid microspores in tetrads that further undergo an asymmetric cell division, Pollen Mitosis I (PMI), to form the vegetative cell and the generative cell. Mutational analyses in *Arabidopsis* have revealed the importance of this asymmetric cell division as it determines the fate of daughter cells evident from differences in cell cycle progression as well as cell specific gene expression (highly transcriptionally active vegetative cell and transcriptionally repressed generative cell) (Honys and Twell, 2003, 2004; Pina et al., 2005). For example, upon disruption of GEMINI1, encoding a microtubule associated protein, both daughter cells adopt the vegetative cell fate (Park et al., 1998; Park et al., 2004b). Several other mutants have also been isolated that authenticate the importance of the

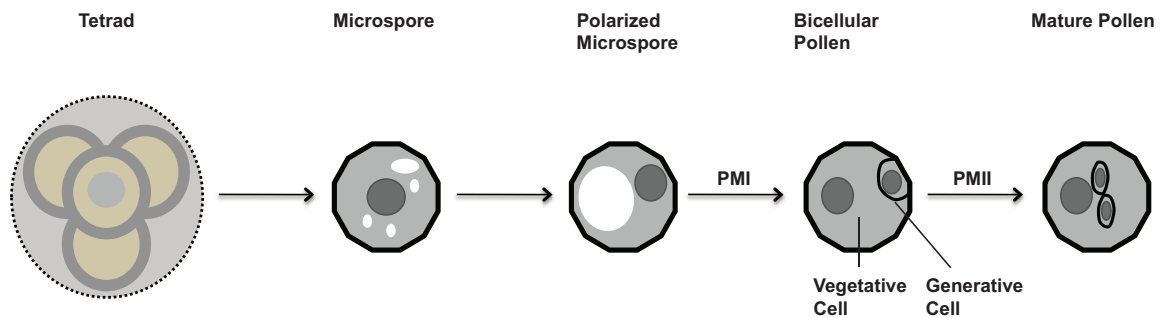


**Figure V.1. Schematic representation of the distinct morphological stages of male gametophyte development in *Arabidopsis*.** Microsporocytes undergo a meiotic division to produce a tetrad of four haploid microspores during microsporogenesis. During microgametogenesis, the released microspores undergo asymmetric division, called Pollen Mitosis I (PMI), to produce a bicellular pollen grain with a small germ cell within the cytoplasm of a large vegetative cell. The germ cell undergoes a further mitotic division at Pollen Mitosis II (PMII) to produce twin sperm cells.

asymmetric division (*sidecar pollen 1; tio; kinesin-12A/kinesin-12B*) (Chen and McCormick, 1996; Oh et al., 2005). The vegetative cell then develops to form the pollen tube after pollination takes place. On the other hand, the generative cell undergoes a mitotic division (Pollen Mitosis II-PMII) to produce twin sperm cells required for the double fertilization of the egg and the central cell nuclei of the female gametophyte. The germ cell division is highly orchestrated and a number of factors have been identified that affect PMII and consequently the development of the male gametophyte. Mutations in Cyclin Dependent Kinase A (*cdkA-1*) as well as the F-box-like 17 (*fb17*) gene result in a failure of germ cell division (Gusti et al., 2009; Iwakawa et al., 2006; Nowack et al., 2006). The *fb17* mutant is not male sterile. Other mutations have been identified where aberration in the germ cell division results in loss of egg cell fertilization. Furthermore, mutations in cell wall patterning and pollen guidance have been identified that have furthered our understanding of the dynamic cellular as well as extra-cellular events required for pollen function (Johnson et al., 2004; Nishikawa et al., 2005; Palanivelu and Preuss, 2006; von Besser et al., 2006).

### **V.2.2 Gene expression in male gametophyte and molecular events controlling male gametophyte development**

Comparative transcriptome analysis between sporophytic datasets and the male gametophyte transcriptome has revealed pollen specific gene expression patterns in higher plants (Becker et al., 2003; Honys and Twell, 2004; Pina et al., 2005). The large number of pollen specific transcripts discovered, suggest functional specialization of the male gametophyte. Transition from PMI to germ line specification is also accompanied by a distinct expression profile – higher transcriptional activity is evident during the early stages, whereas transcriptional activity is simplified at the bicellular stage. Genes involved in cellular processes such as cell signaling, cytoskeletal functions and cell division are highly expressed (Borg et al., 2009). Also

active are a group of genes functioning in general metabolism, cellular organization, DNA synthesis and protein degradation.

Transcriptome analysis of the male gametophyte has provided insight into the cellular processes that are critical for development. Compared to transcriptome profiling very little proteomic analysis has been performed for the male gametophyte development, thus limiting our understanding of the critical molecular events. However, increase in protein synthesis independent of control at the transcript level has been observed in *Nicotiana* during pollen germination (Capkova et al., 1997). Studies of ribonucleoprotein particles in tobacco pollens have furthered the notion that ribosomal subunits, translation factors and cytoskeletal proteins are present together with translationally silenced mRNAs in pollen grains. It is thought that mRNAs are stored in the form of mRNPs and their translation is activated in response to an appropriate trigger, hydration for instance (Honys et al., 2009). Specific translational control during pollen development also gains credence from the identification of pollen specific translation factors (Belostotsky and Meagher, 1996; op den Camp and Kuhlemeier, 1998). Furthermore, comparison of the publicly available microarray dataset for *Arabidopsis* genes shows that certain translation factors are preferentially expressed in the male gametophyte (**Appendix V.I**).

Here, I describe how mutations in two translation factors, eIF3e and eIF3h affect male gametophyte development. Using a novel genetic tool to distinguish wild-type eIF3 pollen from *eif3* mutant pollen grains with the aid of a tightly linked fluorescent tag, it is demonstrated that eIF3 subunits e and h affect pollen germination and eIF3h is also important for pollen tube elongation. This is the first report of gametophytic developmental defects due to mutations in eIF3 subunits.

## V.3 Results

### V.3.1 Mutation in eIF3e results in reduced transmission through the female gametophyte and loss of transmission through the male gametophyte

In an effort to understand the molecular functions of the individual eIF3 subunits in the context of plant development, a reverse genetic screen was undertaken to isolate mutants from the publicly available T-DNA collections (described in details in Chapter 2). Two insertion lines were identified for AteIF3e (At3g57290) with insertion in the 3<sup>rd</sup> exon (SALK\_121004) (Yahalom et al., 2008) and 3'UTR (SALK\_113234) (**Figure V.2**). After maintaining these insertion lines for 3 consecutive generations, genotyping revealed that none of these mutants were present in the homozygous condition; suggesting eIF3e is an essential gene in *Arabidopsis*. That said, heterozygous plants were morphologically normal during sporophyte development under standard growth conditions. Segregation analysis of the integral kanamycin resistance gene present in the T-DNA revealed approximately 25% (2/8; **Table V.1**) transmission of the *eif3e-1* and *eif3e-2* allele when heterozygous plants were selfed instead of the expected 75%; consistent with a defect in gametophytic transmission (**Table V.1**). Moreover, open siliques from self-pollinated *eif3e* heterozygous plants did not reveal embryo abortion or any significant reduction in seed set, suggesting a defect prior to fertilization. To identify the exact developmental event that is affected by eIF3e during gametogenesis, the T-DNA transmission was analyzed after reciprocal crosses with wild-type plants. PCR based genotyping for the identification of the T-DNA allele revealed a reduced transmission of the T-DNA allele when *eif3e-1* heterozygous plants were used as the maternal parent and a complete absence of transmission of the mutant form of the allele when used as the paternal parent (**Table V.1**). Hence, eIF3e is important for both male and female gametogenesis in *Arabidopsis* and disruption leads to a severe loss of transmission through the male gametophyte whereas transmission through the female is reduced by about 50% (**Table V.1**).

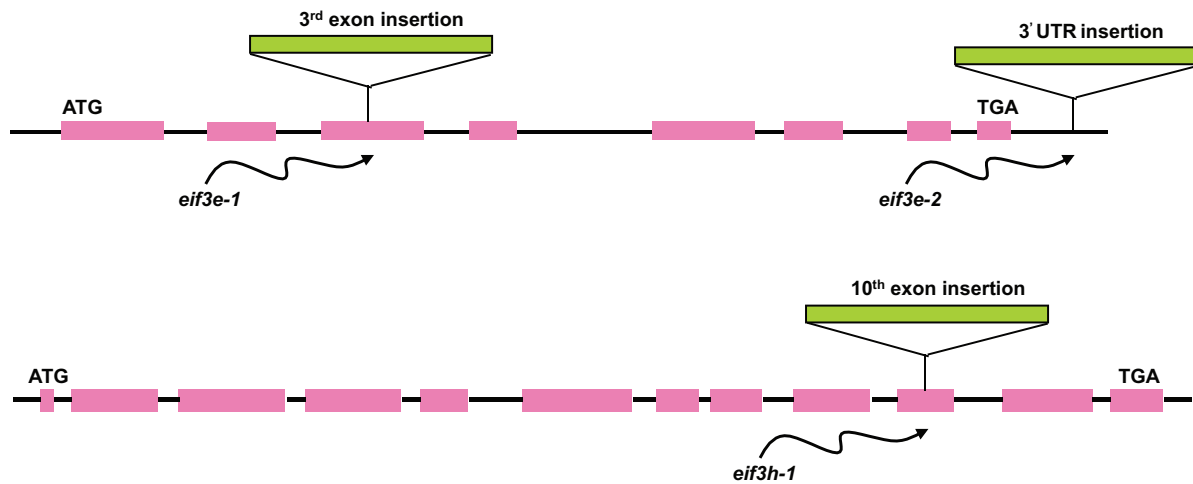


**Figure V.2. eIF3e and eIF3h T-DNA insertion mutants.** Schematic representation of eIF3e and eIF3h loci. Pink boxes represent exons and green boxes represent the T-DNA insertions.

**Table V.1. Gametophytic transmission of *elf3e* allele.** The transmission of the *elf3e* allele is lost when *elf3e* heterozygous plants were used as male parents and reduced to about 24% when *elf3e* plants were used as female parents. Transmission of the T-DNA allele was tested by PCR genotyping of the F1 progenies.

| Parental genotype |                 | Allele transmission to F1 progeny |            |   |
|-------------------|-----------------|-----------------------------------|------------|---|
| Female            | Male            | <i>elf3e</i> /Total               | Percentage | n |
| <i>elf3e</i> /+   | +/+             | 13/54                             | 24         | 8 |
| +/+               | <i>elf3e</i> /+ | 0/65                              | 0          | 6 |
| <i>elf3e</i> /+   | <i>elf3e</i> /+ | 2/8                               | 25         | 1 |

### V.3.2 Phenotypic characterization of male gametophytic developmental defects in eIF3 mutants

A previous study on the developmental characterization of another eIF3 gene showed that disruption of the h subunit results in reduced transmission through the male gametophyte (Kim et al., 2004) (**Appendix V.II**). In contrast to *eif3e* mutants where transmission was completely abolished, *eif3h-1* did not abrogate the transmission completely. In order to test whether mutations in eIF3e and eIF3h affect the same developmental process, the male gametophyte development was studied in detail. Failure to transmit the mutant alleles through the male gametophyte can be caused by early post-meiotic defects in the development of the pollen grain or during the germination of the pollen. Defects can also occur during fertilization when the pollen tube reaches out to the ovules. To facilitate the phenotypic characterization, the wild-type eIF3e and eIF3h alleles were marked by tightly linked fluorescent genes as visible markers. The fluorescent tagged eIF3 lines were selected from a collection of T-DNA insertion lines where LAT52:YFP/dsRED transgene under the control of the pollen specific LAT52 promoter had been randomly inserted into the genome (Francis et al., 2007). *eif3e* and *eif3h* heterozygous insertion lines were crossed with the fluorescent tagged lines as depicted in **Figure V.3** (FTL1413 for eIF3e and FTL567 for eIF3h). Because of the tightly linked fluorescent protein gene (FTL567 ~1 centimorgan units; FTL1413 ~7 centimorgan units), wild-type eIF3 pollens will be fluorescent but mutant pollen grains will not be (**Figure V.3**). The FTLs are further present in the *quartet-1* background that allowed comparison of the wild-type and *eif3* mutant pollens at the same developmental stage (**Figure V.3**). Because of a mutation in *QUARTET-1*, encoding a pectin methyl esterase, the pollen mother cell wall is not degraded and the four meiotic products remain together in a tetrad (Francis et al., 2006). F2 progenies from the crosses were screened for the segregation of the quartet mutation and the mutant eIF3 allele and plants in the *qrt-/-* and *eif3x+/-* genetic background were considered for phenotypic characterization.

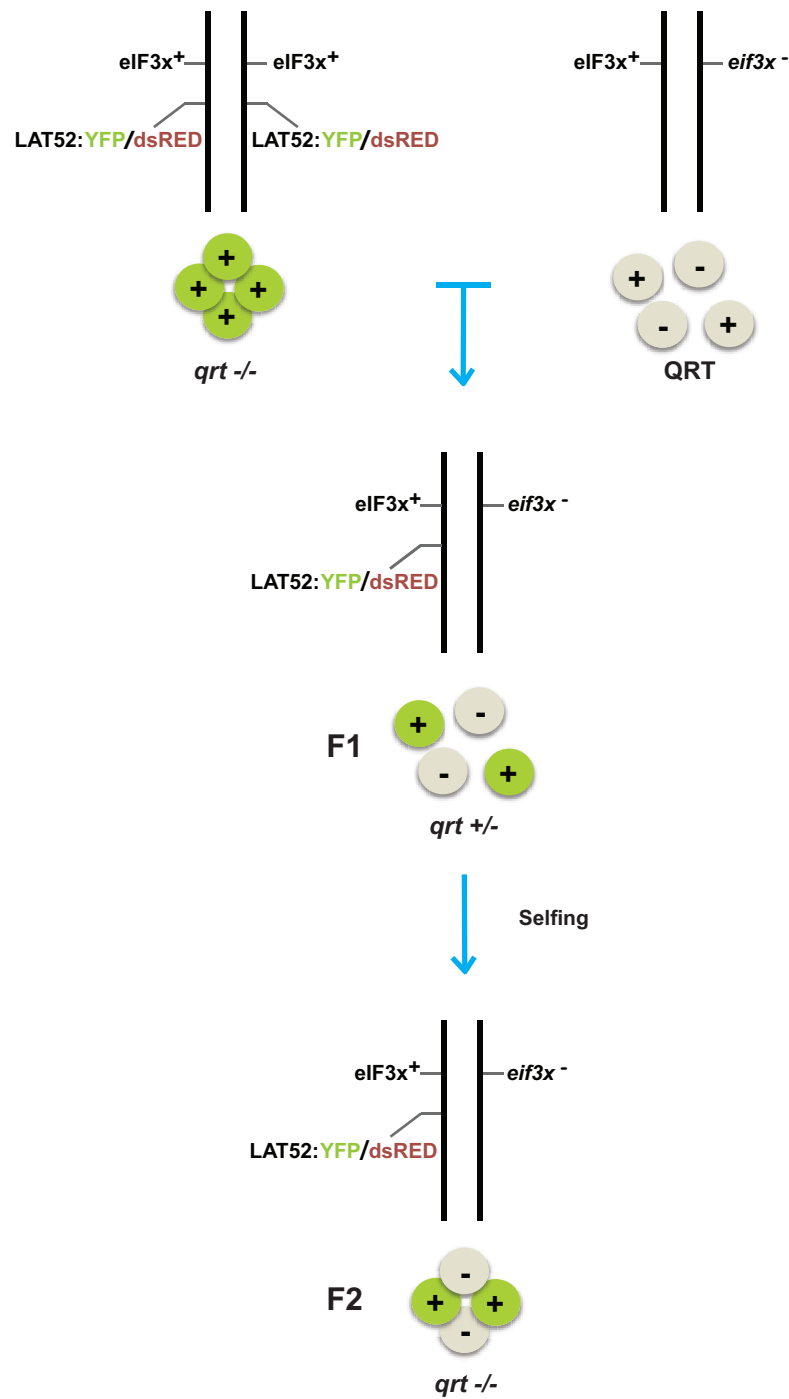


**Figure V.3. Schematic representation of the *FTL-eif3x* genetic analysis.** *eIF3x*<sup>+</sup> refers to the wild-type *eIF3* allele and *eif3x*<sup>-</sup> refers to the T-DNA allele of the given *eIF3* gene.

### **V.3.3 *elf3e* and *elf3h* pollen grains develop normally**

Viability of mature pollen grains with disruption in eIF3e and eIF3h was tested with Alexander's stain (Alexander, 1969, 1980). Morphological comparisons were performed in the *quartet-1* background. Similar to the wild-type pollen grains, *elf3e* and *elf3h* pollen were round, of normal size and took purple stain indicative of viability. They did not reveal any obvious cytoplasmic anomaly or defect in the pollen wall ornamentation (**Figure V.4**). Vital staining with fluorescein diacetate (FDA) also showed high pollen viability for both the mutants similar to wild type (not shown). 4',6-diamidino-2-phenylindole (DAPI) staining further revealed no difference in the nuclear content as well as any aberration in the mitotic divisions in the developing pollen grain. Furthermore, aniline blue staining of the pollen grains revealed no further striking defect in the callose deposition as has been observed with some other gametophytic mutants such as *gemin1-1*. Hence, disruption of eIF3e or eIF3h does not have any obvious effect on mature pollen morphology and viability prior to germination. Hence, the reduced transmission of the mutant allele through the male gametophyte is not because of aberrant pollen grains, but must be because of developmental defects later during the growth and development of the pollen tube and subsequent fertilization.

### **V.3.4 eIF3e and eIF3h mutations affect pollen germination**

To define the defect in male gametophyte development in *elf3e* and *elf3h* further, pollen grains from both these mutants were compared to wild-type pollen grains with regard to their germination efficiency. The germination efficiencies were compared using an *in vitro* germination assay. Efficiency was scored in the *quartet-1* *-/-* background as well as in the *quartet-1* *+/-* background. Germination efficiency was slightly reduced in the *qrt* *-/-*

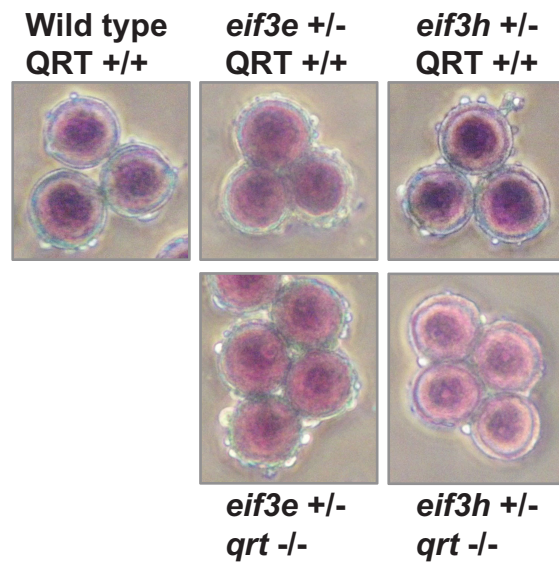


**Figure V.4. Viability analysis of *elf3x* pollen grains.** Staining with Alexander's stain for pollen viability.

background (Boavida and McCormick, 2007). Fluorescent pollen grains representing wild type were scored against non-fluorescent pollen grains representing *elf3e* and *elf3h*. Compared to wild type that had an average of 67% germination (**Table V.2**), *elf3e* pollen grains germinated less efficiently with an average of 25% (**Table V.2**). The reduced germination of *elf3e* was reflected in tetrads as well (**Figure V.5**). Parallel assays with *elf3h* demonstrated reduced germination (17%-23%) as compared to wild type (**Table V.3**). Therefore it was concluded that poor male gametophyte transmission of *elf3e* and *elf3h* is in part a consequence of reduced pollen germination.

#### **V.3.5 Pollen tube elongation is reduced in *elf3h* but not in *elf3e***

To investigate whether reduced germination is also accompanied by a defect in pollen tube growth and elongation for those that manage to germinate, pollen tube lengths were measured. Pollen tube lengths were measured after 3 hours, 5 hours and after 10 hours of dabbing pollen grains onto the germination media. Average pollen tube length for wild-type pollens was 400 $\mu$ M after 10 hours incubation in germination media. Pollen tube length was comparable between wild type and *elf3e* with average pollen tube length of 370 $\mu$ M for *elf3e*. Contrary to observations made for *elf3e*, pollen tube elongation was reduced in *elf3h*. Average pollen tube length for *elf3h* was observed to be around 150 $\mu$ M. When the frequency distribution of the tube length was plotted, wild-type pollen tubes were highly represented in the 300-500 $\mu$ M classes (**Figure V.6A**), whereas *elf3h* pollen tubes measured from 100-200 $\mu$ M (**Figure V.6B**). However, no significant aberration in pollen tube morphology (bifurcated pollen tubes, bulbous tip) was observed for either *elf3e* or *elf3h*.

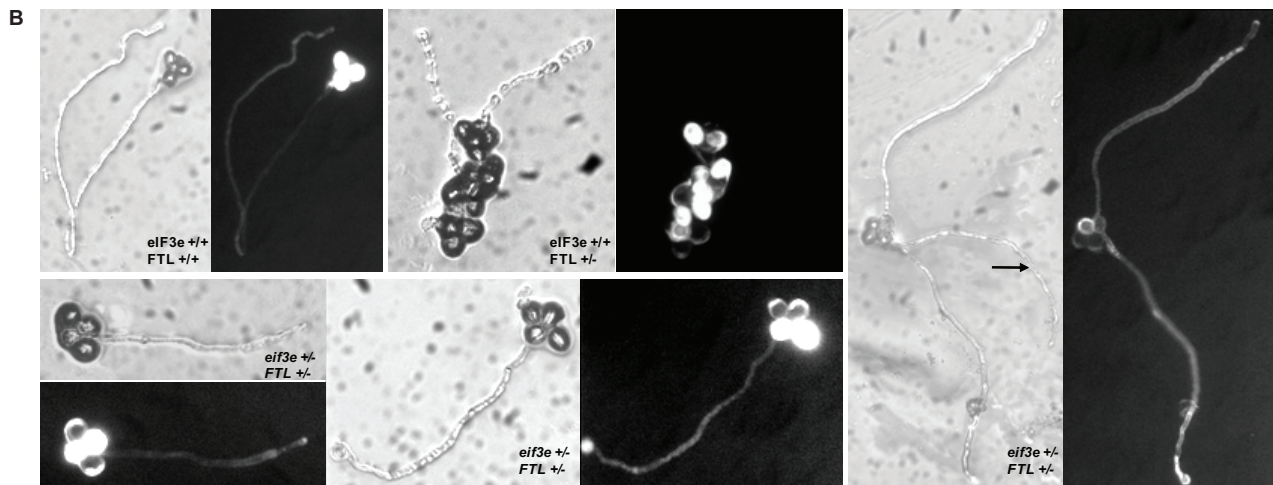
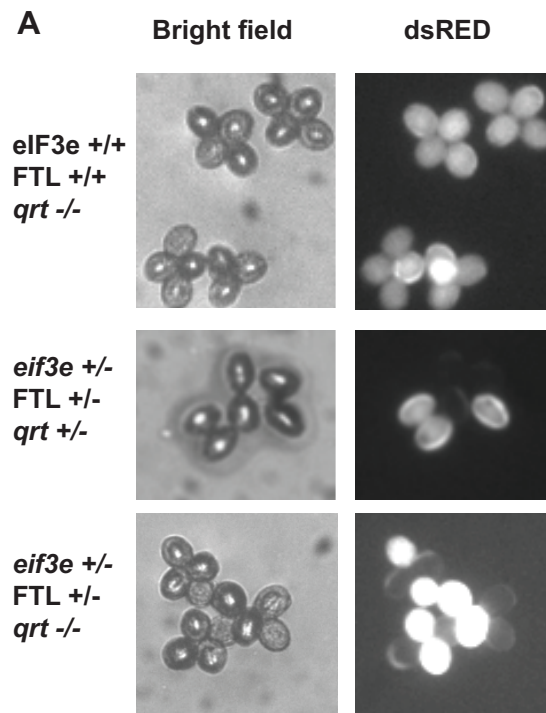
**Figure V.5. Phenotype analysis of male gametophyte development for *eif3e*.**

**A. (Upper)** Wild type fluorescent eIF3e pollen grains in tetrad (*quartet*<sup>-/-</sup> with FTL: dsRED).

**(Middle)** Segregation of *eif3e*<sup>-</sup> mutant allele and eIF3<sup>+</sup> wild-type allele linked to FTL:dsRED in the progeny of a non quartet plant (*qrt-1* +/-).

**(Lower)** Segregation of *eif3e*<sup>-</sup> mutant allele and eIF3<sup>+</sup> wild-type allele linked to FTL:dsRED in the progeny of a *quartet-1* plant (*qrt-1* <sup>-/-</sup>) and subsequent generation of tetrad with wild-type and *eif3e* pollens differentiated by expression of the fluorescent protein.

**B.** In vitro pollen germination showing underrepresentation of the *eif3e*<sup>-</sup> pollen tubes. Wild-type pollen tubes are fluorescent and arrow highlights the non-fluorescent *eif3e* pollen tubes.



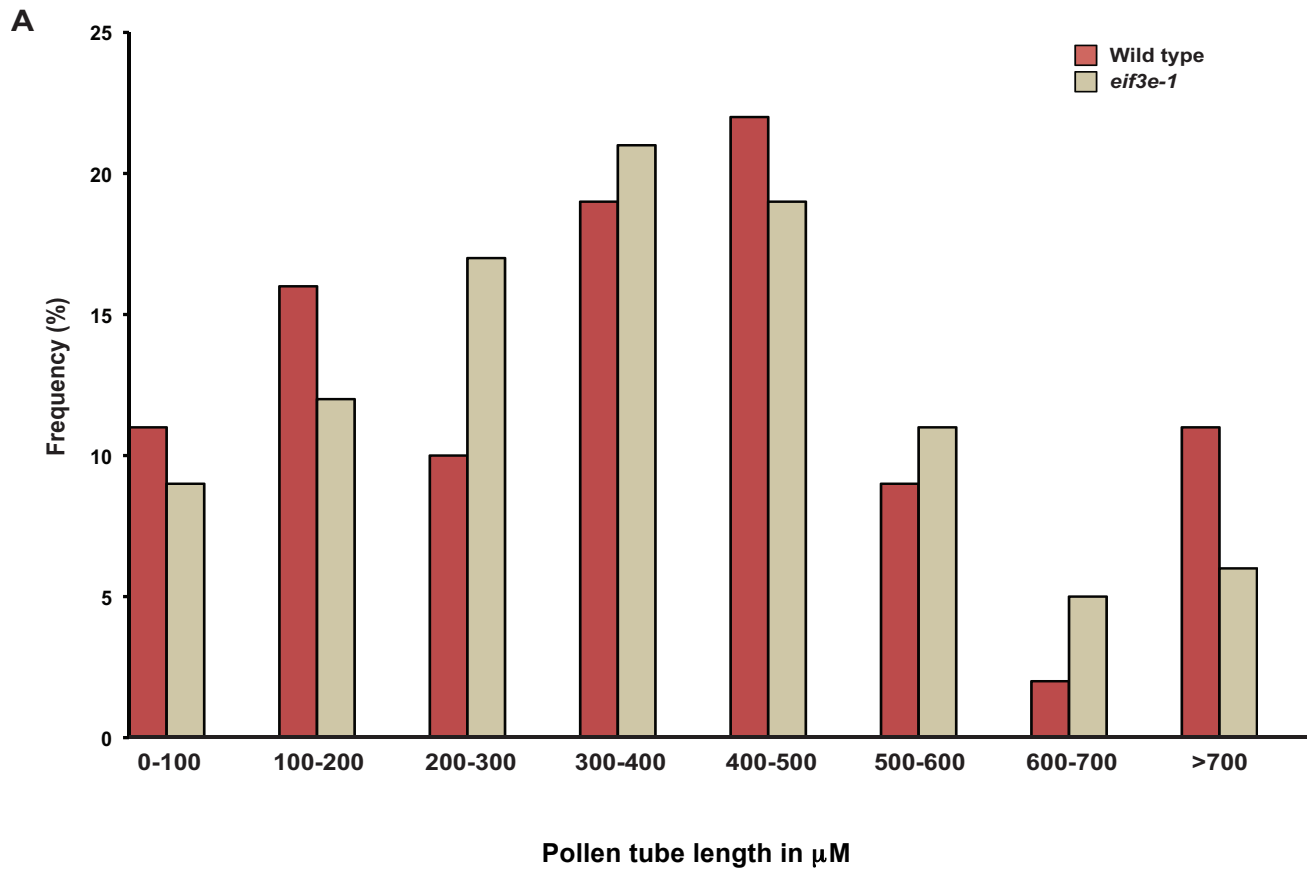


**Figure V.6. *eif3h* affects pollen tube growth.** Comparison of pollen tube lengths between wild type and *eif3e* (**A**) and *eif3h* (**B**) after 6 hours of germination.

**Figure V.6 (continued)**

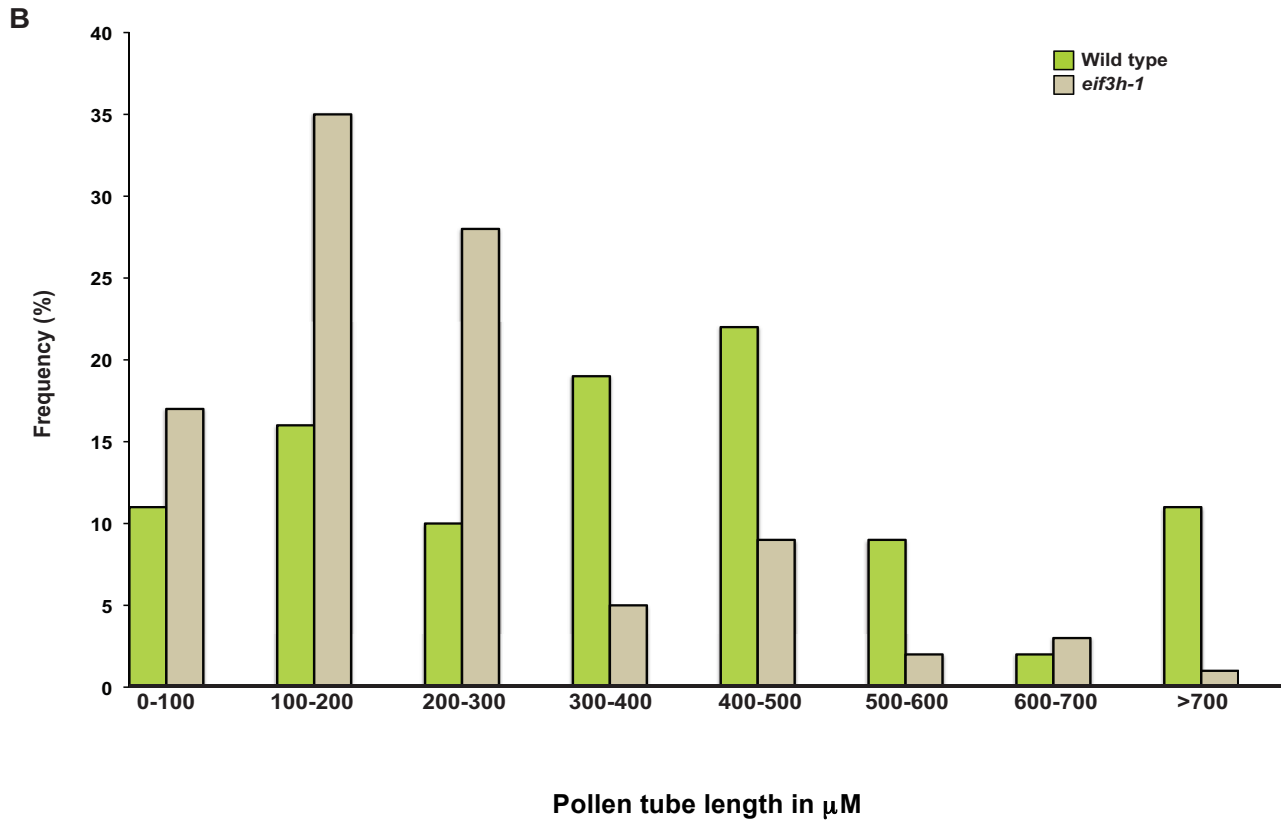


**Table V.2. Germination efficiency of *eif3e* pollen grains in the *qrt-1* +/- background. Data are from 5 replicates.**

| No of pollen grains scored |                                         | Pollen grains germinating  |                                         | Percentage germinating     |                                         |
|----------------------------|-----------------------------------------|----------------------------|-----------------------------------------|----------------------------|-----------------------------------------|
| Fluorescent<br>(wild type) | Non-<br>fluorescent<br>( <i>eif3e</i> ) | Fluorescent<br>(wild type) | Non-<br>fluorescent<br>( <i>eif3e</i> ) | Fluorescent<br>(wild type) | Non-<br>fluorescent<br>( <i>eif3e</i> ) |
| 92                         | 86                                      | 57                         | 21                                      | 62                         | 25                                      |
| 34                         | 48                                      | 24                         | 11                                      | 71                         | 23                                      |
| 67                         | 61                                      | 43                         | 17                                      | 65                         | 28                                      |
| 59                         | 67                                      | 37                         | 16                                      | 63                         | 24                                      |
| 41                         | 39                                      | 25                         | 9                                       | 61                         | 22                                      |
|                            |                                         |                            |                                         | <b>Average: 64</b>         | <b>25</b>                               |

**Table V.3. Germination efficiency of *eif3h* pollen grains in the *qrt-1* +/- background. Data are from 5 replicates.**

| No of pollen grains scored |                                         | Pollen grains germinating  |                                         | Percentage                 |                                         |
|----------------------------|-----------------------------------------|----------------------------|-----------------------------------------|----------------------------|-----------------------------------------|
| Fluorescent<br>(wild type) | Non-<br>fluorescent<br>( <i>eif3h</i> ) | Fluorescent<br>(wild type) | Non-<br>fluorescent<br>( <i>eif3h</i> ) | Fluorescent<br>(wild type) | Non-<br>fluorescent<br>( <i>eif3h</i> ) |
| 122                        | 109                                     | 84                         | 17                                      | 69                         | 16                                      |
| 26                         | 39                                      | 15                         | 6                                       | 61                         | 17                                      |
| 59                         | 61                                      | 43                         | 13                                      | 73                         | 22                                      |
| 22                         | 36                                      | 13                         | 7                                       | 63                         | 19                                      |
| 31                         | 27                                      | 20                         | 5                                       | 67                         | 15                                      |
|                            |                                         | <b>Average: 67</b>         |                                         | <b>18</b>                  |                                         |

## **V.4 Discussion**

Development of the male gametophyte is an orchestrated process involving a number of factors that determine its fate. Most of our current understanding about the developmental regulation comes from genetic and mutational analyses that have helped identify the important components. But the underlying molecular mechanisms that might be regulatory in the developmental process are not well established. Two studies of the *Arabidopsis* transcriptome (Becker et al., 2003; Honys and Twell, 2003, 2004) demonstrated 10% of the genes in *Arabidopsis* to be pollen specific. What remains to be investigated is whether all these mRNAs are translated into proteins during pollen development. It is plausible that developmental stage specific transcriptional regulation of gene expression is coupled with translational fine-tuning.

### **V.4.1 Translational regulation of pollen germination and pollen tube elongation**

Cycloheximide dependent blockage of pollen tube elongation was one of the earliest observations to implicate active translation in pollen germination and growth (Hao et al., 2005; Picton and Steer, 1983). The requirement for protein synthesis during pollen tube growth can be justified by the constant demand of cell wall modifying proteins and cytoskeletal proteins at the growing tip of the pollen tube. Also, proteomic comparison between mature and germinating pollen in *Brassica napus* has demonstrated translational upregulation of translation initiation and elongation factors in germinating pollen (Sheoran et al., 2009).

A recent study deciphering the components of the ribonucleoprotein particles (RNPs) in tobacco pollen has provided evidence for translational control in pollen development. Together with eIF3, several other translation factors including eIF4A, EF1 and PABP were found to be associated with the silenced RNPs (Honys et al., 2009), suggesting that a core translation machinery might be present. The identification of NTP303 (translationally regulated mRNA in

pollen (Hulzink et al., 2002; Weterings et al., 1995)) with the RNPs furthers this idea that specific mRNAs are present in storage granules and upon appropriate trigger and in the presence of right factors are translated and affect the male gametophyte development. The mechanics of translational regulation are not well understood. Possible elements of such regulation include *cis*-acting regulatory sequences in the 5'UTR that are important for pollen germination and growth (Hulzink et al., 2002; Hulzink et al., 2003); pollen specific miRNAs (Grant-Downton et al., 2009); polypyrimidine tract binding proteins (PTBs)(Sawicka et al., 2008; Wang and Okamoto, 2009); pollen-specific PABPs (Belostotsky and Meagher, 1996; op den Camp and Kuhlemeier, 1998); and, phosphorylation of eIF4A (Belostotsky and Meagher, 1996; op den Camp and Kuhlemeier, 1998). All these pieces of evidence point towards a fine tuned translational control that is implemented during pollen germination via *trans*-acting factors, some of which are inherent components of the translation machinery.

#### **V.4.2 eIF3 in pollen development**

Circumstantial evidences, as described in the previous section, has suggested the importance of translational regulation in pollen development. However, no direct genetic or biochemical evidence has demonstrated a defect in the male gametophyte development due to disruption of components of the translation machinery.

The results discussed in this chapter demonstrate that mutations disrupting functions of two subunits of eukaryotic initiation factor 3 (eIF3e and eIF3h) resulted in reduced transmission of the mutant allele through the male gametophyte. A possible function of eIF3 in cell cycle regulation (Dong and Zhang, 2006; Otero et al., 2010) and involvement of eIF3e and eIF3h in meiotic division in *S. pombe* (Bandyopadhyay et al., 2000; Ray et al., 2008) leading to abnormality in microspore fate was investigated using cytological examination of pollen grains, but any gross morphological defect was ruled out. *In vitro* pollen germination assay

revealed pollen germination to be eIF3 dependent. Pollen germination was reduced for both *eif3e* and *eif3h* as compared to wild type and pollen elongation was affected in *eif3h*.

Disruption of eIF3h did not abolish the transmission of the mutant allele completely (**Appendix V.II**). This can be explained by the reduced, but not complete loss of germination efficiency that was observed. A few of the pollen grains that managed to germinate, yet have a reduced pollen tube length, might still be able to fertilize the ovules. Transmission of the *eif3e-1* allele through the male gametophyte however was completely abrogated. Surprisingly, the developmental events that were examined do not reflect the same magnitude of severity. A considerable percentage of *eif3e-1* pollens do germinate and germinating *eif3e-1* pollen grains do not have any tube growth defect. Therefore, the complete loss of *eif3e-1* allele transmission cannot be explained exclusively by a reduced germination efficiency. The *in vitro* assay used to study the male gametophyte development might not be sufficient to dissect all possible defects and responses of the male gametophyte. The pollen guidance in the pistil is a tightly regulated developmental pattern that is dictated by a number of external cues, the female gametophyte being one crucial determinant (McCormick, 2007; Palanivelu and Preuss, 2000, 2006). Signal molecules from the ovules have been demonstrated to be critical for efficient pollen tube guidance and fertilization. It is understandable that the *in vitro* system limits these responses. Possibly, *eif3e* pollen tubes germinating on the stigma might be (1) defective in tube growth in the pistil where the conditions and pollen tube growth is different from *in vitro* conditions and more complex and challenging, (2) unable to fertilize the ovule, and, (3) outcompeted by the wild type on the stigmatic surface. *In vivo* examination of pollen growth and development might hold a clue, for gaining a comprehensive understanding of the defect imparted by mutation in eIF3e.

Mature pollen grains have an enormous store of proteins and carbohydrates. However, the dynamic change in the gene expression profile during pollen germination suggests that

synthesis of new proteins is also crucial. Thus, one possible function of eIF3 in germination might relate to its function in general protein synthesis. Changes in gene expression pattern are not restricted to pollen germination alone. Specific expression pattern and transcriptional upregulation of specific classes of genes is observed during pollen tube elongation and growth. It can be speculated that translational regulation might become eminent during pollen tube growth. Interestingly, mutation in eIF3h affected pollen tube elongation thus suggesting that the requirement for eIF3 continues well after pollen germination. How and why eIF3h affects pollen tube elongation while eIF3e does not, is not clear and needs further investigation. But in accordance to the molecular functions of eIF3h in translation of specific mRNAs harboring upstream open reading frames (Chapter 3), suggest that eIF3h might be required for a specific set of mRNA that is crucial for pollen tube elongation. Some of the possible targets of eIF3h might be regulated by *cis*-elements in the 5' leader, probably uORFs, that are highly regulated during pollen tube growth and development. It will be important to test if the functions of eIF3e and eIF3h in pollen germination pertain to their regulatory functions in translation or reflect a function in general translation. Hence, a proteomic approach will clarify and complement the transcriptome analysis in understanding regulation during pollen development. Furthermore, it will be of considerable interest to check the translation efficiency of pollen specific transcripts harboring uORFs in *eif3h* mutants.

## **V.5 Materials and Methods**

### **V.5.1 Mutant isolation and growth conditions**

The general growth conditions for *Arabidopsis* were as described in Chapter 2. T-DNA screening and PCR genotyping was performed as described in Chapter 2.

### **V.5.2 Genetic analysis and generation of *eif3x* +/- *FTL* +/-**

*eif3e* heterozygous plants were selected after PCR genotyping. For segregation analysis, selected plants were allowed to self fertilize and F1 progenies were screened for the

transmission of the T-DNA allele by scoring kanamycin resistance as well as confirmation of the T-DNA by PCR. For gametophytic transmission analysis, heterozygous plants were used for reciprocal crosses with wild-type plants. Stage 12 flowers from wild type and insertion mutants were emasculated and cross-pollinated after 24 hours. F1 progenies were genotyped by PCR as described in Chapter 2.

The transgene in the FTL was confirmed using oligonucleotides listed in **Appendix V**.

Confirmation of the FTL was followed by crossing with heterozygous *elf3x* mutant plants. The FTL were used as the paternal parent and *elf3x* as the maternal parent. F1 progenies were screened for the segregation of the fluorescent protein marker and the *quartet* mutation. Some of the F1 plants were allowed to self fertilize and some were crossed with the parental FTL. The F2 progenies were screened for *qrt*-/- and *ftl* +/- phenotype and identified plants were then PCR genotyped for confirmation of the *elf3x* +/- genotype.

### **V.5.3 Alexander's viability staining**

Stock solution was prepared with: 10mL 95% ethanol; 1% malachite green in 95% ethanol; 5g of phenol; 5mL 1% acid fuchsin in H<sub>2</sub>O; 0.5mL 1% orange G in H<sub>2</sub>O; 2mL glacial acetic acid; 25mL glycerol and 50mL H<sub>2</sub>O (*note*: chloral hydrate was omitted due to restriction in use). Working solution was diluted 1:50 in H<sub>2</sub>O. Pollen grains were stained on a glass slide and observed under microscope after incubating in staining solution for 15 minutes.

### **V.5.4 DAPI staining**

Anthers from fresh flowers were dissected in coloration buffer (1mg/mL DAPI solution in Nonidet P40 (1%); DMSO (10%); PIPES (50mM, pH 6.9); EGTA (5mM, pH 7.5)) and observed after 30 minutes incubation.

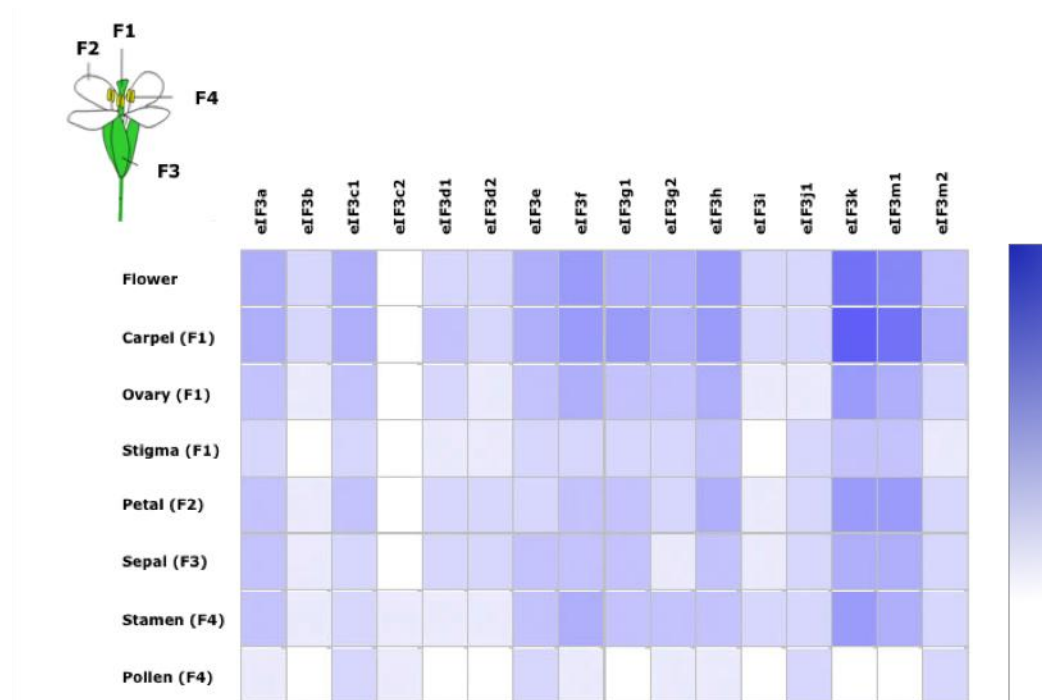
#### **V.5.5 Fluorescein diacetate staining**

Pollens were incubated in a humid chamber for approximately 30 minutes. Staining solution was prepared by adding drops of FDA (2mg/mL acetone) to pollen growth media until the media got turbid. Pollen grains were then incubated in the FDA containing growth media for 10 minutes before observing under an epifluorescence microscope using the FITC filter.

#### **V.5.6 *In vitro* pollen germination assay**

Pollen germination media was prepared with: 18% Sucrose; 0.01% Boric acid; 1mM  $\text{CaCl}_2$ ; 1mM  $\text{Ca}(\text{NO}_3)_2$ ; 1mM  $\text{MgSO}_4$  (pH adjusted to 7.0). For germination in liquid media, pollens were gently dabbed on the media in a chamber slide and incubated for 6hours to overnight. Solid germination media was prepared by adding 0.5% low melting agarose. The molten media was dropped onto a glass slide and after solidification, pollens were dabbed on the surface gently and incubated in a humid chamber for 6hours to overnight.

**Appendix V.I. eIF3 expression pattern in floral organs based on publicly available gene expression analysis.** Results are given as heat maps in white/blue coding that reflect mean signal values with darker representing strong expression. Modified from Genevestigator (Hruz et al., 2008; Zimmermann et al., 2004) and *Arabidopsis* eFP browser (Winter et al., 2007).



**Appendix V.II. Transmission of *elf3h-1* allele through the male and the female gametophyte** (Kim et al., 2004).

| Parental genotype |                 | Allele transmission to F1 progeny |            |   |
|-------------------|-----------------|-----------------------------------|------------|---|
| Female            | Male            | <i>elf3h</i> /Total               | Percentage | n |
| <i>elf3h</i> /+   | +/+             | 73/155                            | 47.1       | 3 |
| +/+               | <i>elf3h</i> /+ | 3/116                             | 2.6        | 3 |
| <i>elf3h</i> /+   | <i>elf3h</i> /+ | 381/683                           | 55.8       | 3 |

**Appendix V.III: Oligonucleotides used for confirmation of the fluorescent transgene in the fluorescent tagged lines.**

| <b>FTL</b>           | <b>Oligonucleotide sequence (5' – 3')</b> |
|----------------------|-------------------------------------------|
| FTL 1413 For (eIF3e) | GAC CAT TGC TGA CAT TGA CAT GG            |
| FTL 1413 Rev (eIF3e) | GGC AAC TCT GTC GAG GCA CAT               |
| FTL 567 For (eIF3h)  | TGG TCG GCC CTA AAT GTT TG                |
| FTL 567 Rev (eIF3h)  | ACC GAC ACA AGA ATC TGT GGA ACC           |
| T-DNA LB             | CAA TTC GGC GTT AAT TCA GTA C             |

## **Chapter 6**

### **Perspectives and future directions**

This current study has been prodded by our limited understanding of the regulatory roles of the multisubunit complex eIF3. Most of our knowledge about the functions of eIF3 comes from budding yeast where the subunit composition is different from higher eukaryotes. The main objectives of this study were to (1) assemble a collection of mutant eIF3 strains in a multicellular eukaryote, (2) assign molecular functions to the eIF3 subunits in translation, and, (3) understand how these molecular functions relate to development in *Arabidopsis*.

### **VI.1 Molecular functions of eIF3 in translation**

During the initial stages of this project, even though the biochemical properties of eIF3 in general translation were somewhat understood, a clear understanding of the other regulatory functions of eIF3 was elusive. Genetic analysis in yeast and biochemical interactions of eIF3 with other components of the translation machinery reflected the functions of eIF3 in stabilization of the pre-initiation complex; recognition of the mRNA via interaction with the eIF4F cap binding protein complex, and eIF3 was speculated to be involved in the 40S-60S dissociation (Hinnebusch, 2005, 2006). eIF3 was also implicated in scanning of the 5' leader of mRNAs as well as start codon recognition (Nielsen et al., 2004). Therefore, *functions of eIF3 in early translation initiation events were well established.*

However, over the course of this study, a broader spectrum of eIF3 functions has emerged, restricted not only to translation but also to other quality control mechanisms as well as signal transduction pathways of the cell (Hinnebusch, 2006; Sonenberg and Hinnebusch, 2009). Overall, it is becoming increasingly apparent that the functions of eIF3 are more deeply rooted than initially perceived.

Functions of eIF3 in translation have been extended to translation termination (Pisarev et al., 2007). Also, the cryoelectron microscopy reconstruction of mammalian eIF3 and a model for its interaction with the 40S subunit (Siridechadilok et al., 2005) helped gain insight into eIF3 position on the 40S ribosome. Genetic and biochemical evidences have suggested functions of

eIF3 in reinitiation (Poyry et al., 2007; Poyry et al., 2004; Szamecz et al., 2008). Also, as described in Chapters 3 and 4, our understanding of the molecular functions of the h subunit of eIF3 in *Arabidopsis* revealed that eIF3 is required for retaining the reinitiation competence on uORF translating ribosomes possibly involving a functional cooperation with the large ribosomal subunit protein, RPL24B. All these pieces of evidence suggest that the *functions of eIF3 are not restricted till 48S initiation complex formation, but can extend to later stages of translation, during termination and reinitiation*. Possibly, eIF3 can either remain attached to the translating ribosomes or, alternatively, eIF3 might be acquired *de novo* during termination (similar to a ribosome release factor).

#### **VI.1.1 How does eIF3h foster reinitiation?**

eIF3h functions to retain the reinitiation competence on uORF containing mRNAs, for instance in AtbZip11. However, how exactly it does so, or which specific biochemical step or molecular event is affected by eIF3h still needs to be investigated. Future work needs to explore the hypotheses of whether eIF3h is, (1) involved in translation termination, or, (ii) required after termination, when the 40S ribosomal subunit resumes scanning. Defect in termination will lead to ribosome stalling near the uORF termination codon and subsequent ribosome stacking in the uORF-coding region (**Figure VI.1A**). Alternatively, termination may proceed as expected, but eIF3h might be required for the 40S ribosomes to remain bound to the mRNA after 60S dissociation so that it can resume scanning (**Figure VI.1B**). Mapping the ribosome occupancy on the AtbZip11 leader can address this crucial question and multiple approaches can be taken to accomplish this.

**1.** Toeprint analysis (Kozak, 1998; Sachs et al., 2002) can reveal preferential stalling of ribosomes at a single position *in vitro*. Briefly, cycloheximide treatment is used to freeze ribosomes on the mRNA and the 3' boundary of the stalled ribosome can be detected by a primer extension assay using a downstream oligonucleotide. The predictions about eIF3h

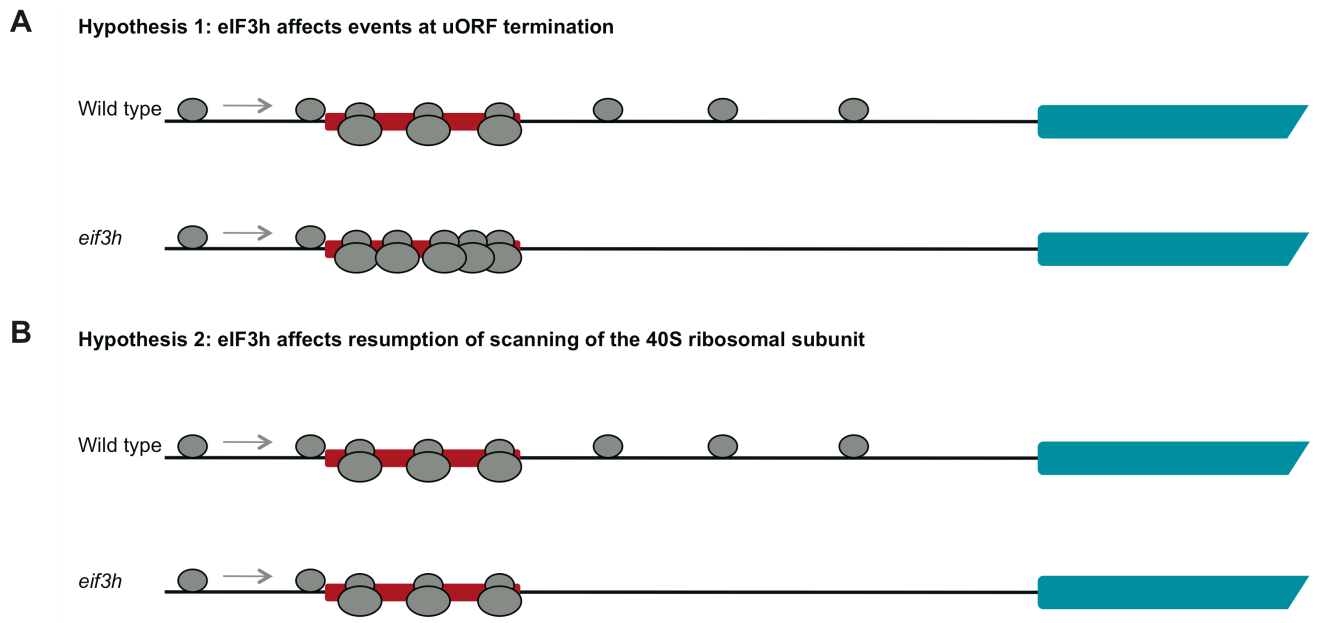


**Figure VI.1. Schematic illustration of the hypothesized function of eIF3h in reinitiation.** uORF2 cluster of the AtbZip11 leader is represented by red box and the main open reading frame by blue box. Small gray circles denote 40S ribosomal subunits.

**A.** Function of eIF3h in termination will stall ribosomes in the uORF termination codon and will result in subsequent ribosome stacking. Note that the ribosome occupancy on the uORF is higher in *eif3h* than the wild type.

**B.** Function of eIF3h in resumption of the 40S ribosomal subunit after uORF termination predicts low ribosome occupancy in the intercistronic spacer.

function from this assay would be, if termination defects cause ribosome stalling in *elf3h*, there will be an overrepresentation of the toeprint product in *elf3h* as compared to wild type. Preliminary work that I did on toeprinting AtbZip11 in *Arabidopsis* extracts (data not shown) and also the fact that there is just one report of *in vivo* toeprinting (Cao and Geballe, 1996), illustrates the technical challenge. Using *in vitro* transcripts in wheat germ translation extracts can help to optimize and standardize the conditions required for efficient toeprinting on the AtbZip11 leader (data not shown).

**2.** An alternative technique for mapping ribosome density (Arava et al., 2005; Eldad and Arava, 2007, 2008) on mRNAs can also be used to reveal a snapshot of ribosome occupancy on the Atbip11 uORF. This assay depends on RNase H mediated site-specific cleavage of polysomal RNA followed by separation on a sucrose density gradient (**Figure VI.2**). Depending on the number of ribosomes bound, the cleaved fragment will have a differential sedimentation pattern. The expected results would be, if eIF3h is required for efficient translation termination, then the ribosome density on the uORF will be higher than wild type because of a stalling event (**Figure VI.2**). Therefore, the uORF fragment will occupy heavier fractions as compared to wild type when subjected to a sucrose density gradient centrifugation. If eIF3h helps the 40S subunit to scan downstream, then the number of 80S ribosomes bound to the uORF will be same but the number of 40S ribosomes on the intercistronic spacer will be reduced as compared to wild type. Ribosome density mapping on the AtbZip11 leader was also attempted. Site specific cleavage of *in vitro* transcribed AtbZip11 leader was successfully accomplished, but it could not be recapitulated in *Arabidopsis* extracts. One possibility might be the presence of some other RNA binding protein or the highly structured AtbZip11 leader that prevents efficient oligonucleotide annealing and thus affecting the efficiency of cleavage. This needs to be optimized further by annealing the oligonucleotides to different regions in the AtbZip11 leader. Another important consideration

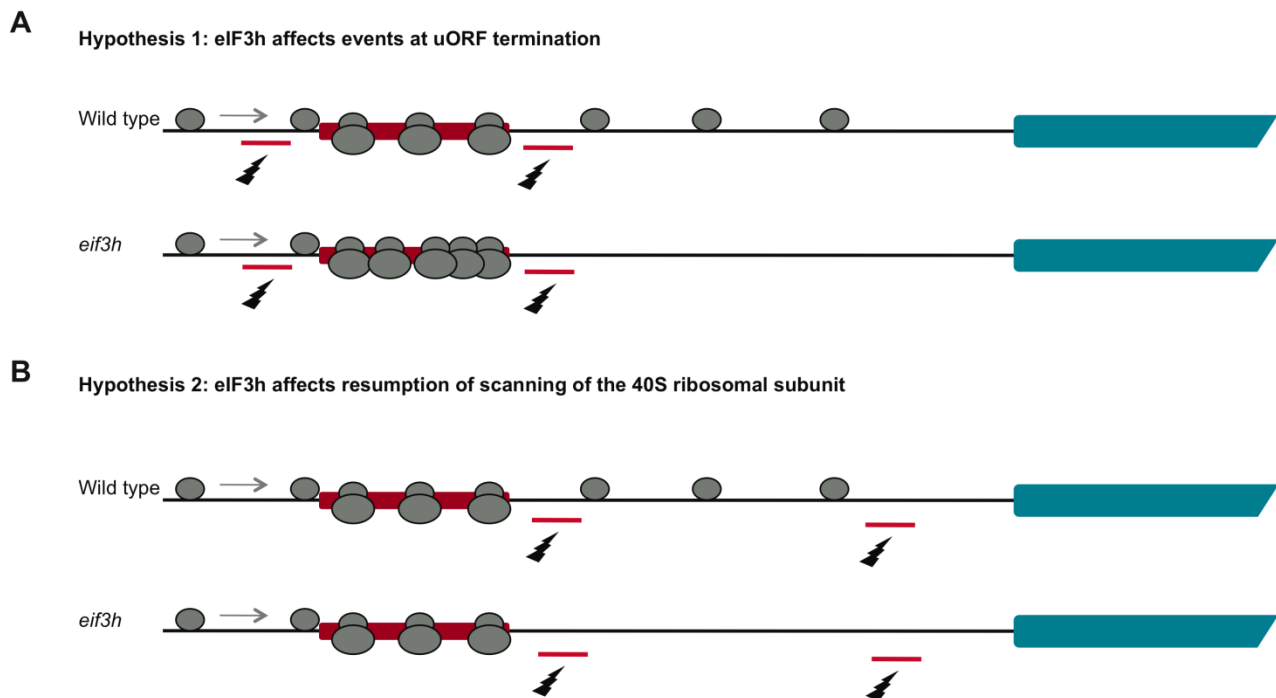


**Figure VI.2. Schematic illustration of proposed ribosome density mapping for understanding the precise molecular function of eIF3h in reinitiation.**

**A.** Hypothesis 1 predicts higher ribosome occupancy on the uORF (represented by red box) in *eif3h*. Site specific cleavage on the AtbZip11 leader is represented by red line representing oligonucleotide annealing and solid arrow denoting RNase H cleavage. Cleaved fragment will have differential sedimentation profile on the sucrose gradient because of difference in ribosome occupancy.

**B.** According to hypothesis 2, function of eIF3h in 40S ribosomal subunit scanning after uORF termination predicts low ribosome occupancy in the intercistronic spacer and lower ribosome density in *eif3h*.

is that cycloheximide treatment is used to freeze ribosomes on the mRNA. But, how efficiently 40S ribosomes are acted upon by cycloheximide is not clear.

**3.** The *AtbZip11* mRNA, for its ribosome occupancy, can also be tested using a ribosome protection assay using micrococcal nuclease followed by primer extension (Wolin and Walter, 1988). The predictions will be similar to that discussed for the toeprint assay.

**4.** Both toeprinting and ribosome density mapping provide the ribosome occupancy for a single mRNA at one time. However, deep sequencing of ribosome protected fragments on the other hand can provide a high resolution nucleotide level information on the ribosome occupancy for majority of cellular mRNAs (Ingolia et al., 2009). If the function of eIF3h pertains to translation termination, uORF termination codon sequences will be overrepresented in *eif3h* and the intercistronic sequences can be predicted to be underrepresented (**Figure VI.3**).

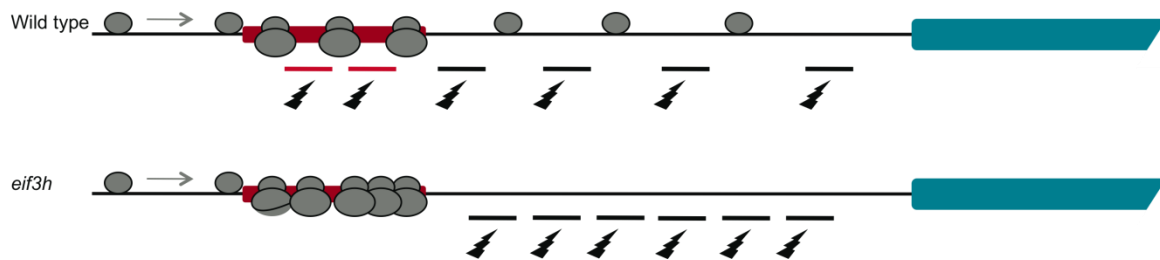
**5.** With the advent of real space polysome imaging of translation over time using atomic force microscopy (Mikamo-Satoh et al., 2009), visualization of translation on the uORF containing transcripts is another way of getting a precise, high-resolution translation profile. Though this will not address the question of a termination defect in *eif3h*, the overall ribosome occupancy/translation profile of a specific mRNAs can be compared. However, the requirement for a cell-free translation system from wild type and mutants is a limiting factor at this point of time.

### **VI.1.2 What other factors promote reinitiation together with eIF3h?**

The requirement of RPL24B for reinitiation on the *AtbZip11* leader suggests a functional cooperativity between eIF3h and RPL24B.

Recently, yeast eIF3a has also been reported to be important for rescanning of ribosomes after GCN4 uORF1 translation (Szamecz et al., 2008). Interaction of yeast eIF3a NTD with RPS0A and eIF3c with RPS10A (Valasek et al., 2003), places these two subunits on the solvent exposed side of the 40S ribosome. Furthermore, interaction of eIF3a with helices 16

**A** Hypothesis 1: eIF3h affects events at uORF termination



**B** Hypothesis 2: eIF3h affects resumption of scanning of the 40S ribosomal subunit

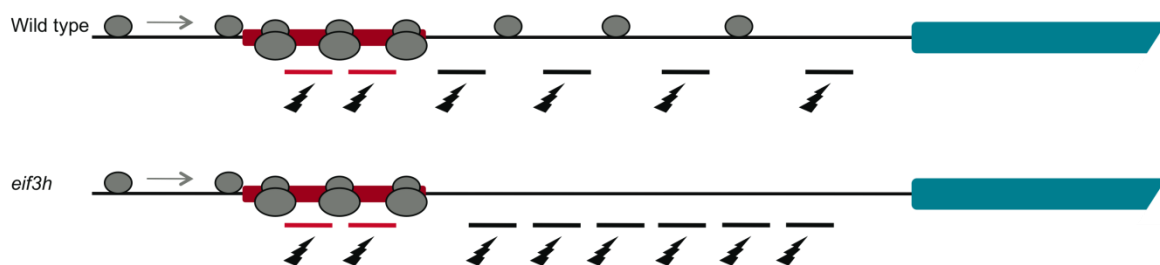


**Figure VI.3 Schematic illustration of proposed experimental approach using micrococcal nuclease digestion for understanding the precise molecular function of eIF3h in translation of AtbZip11 leader.**

**A.** According to hypothesis 1, ribosome stalling will protect the uORF in *eif3h* from micrococcal nuclease digestion and therefore predicts higher representation of the uORF sequence in the ribosome protected fragment.

**B.** Prediction from hypothesis 2 with equal representation of uORF sequence in the ribosome protected fragments, but overrepresentation of intercistronic sequence in wild type.

and 18 of the 18S rRNA substantiates the position of eIF3a on the solvent side (Szamecz et al., 2008). Even though functions of eIF3a in reinitiation have been proposed, this seems unlikely to involve RPL24. Independently, the g subunit of eIF3 (because of its interaction with TAV) has been speculated to be important for efficient reinitiation on the polycistronic RNA of CaMV (Park et al., 2004a; Park et al., 2001). Taken together, these findings suggest reinitiation function for other eIF3 subunits.

Based on the available structure information, eIF3 binding to the solvent side of the 40S ribosome has been suggested to disrupt two intersubunit bridges, B4 (between RPS13 of the 40S and helix 34 of the 28S rRNA in the 60S) and B2a (between helix 44 of 40S and helix 69 of 60S) (Siridechadilok et al., 2005). Interestingly, RPL24 also forms part of an intersubunit bridge, B7, with helix 44 of the 40S subunit (Spahn et al., 2001; Spahn et al., 2004). It can be speculated that eIF3 subunits that are in physical proximity of helix 44 of the small ribosomal subunit will be favorable in interacting with RPL24.

The question is whether eIF3h is in close physical proximity of RPL24. In absence of a detailed map of eIF3 subunit interaction with the 40S, it is challenging to predict a possible molecular map for the 'reinitiation bridge' between eIF3h and RPL24. The eIF3h-RL24B interaction can be used as a bait to fish out other cellular components that might be involved.

Another interesting option is that both eIF3h and RPL24B function to foster the 40S-mRNA interaction independently. The involvement of RPL24B in forming a peripheral protein-RNA intersubunit bridge that is connected to the P-site suggests that mutations affecting RPL24B might trigger premature release of mRNA during 40S-60S dissociation. However, very little is known about the molecular events and biochemical steps that lead to termination and subsequent reinitiation. Therefore, it is hard to rule out any of the possibilities discussed.

## **VI.2 Significance of eIF3 in *Arabidopsis* development**

The study of the h subunit of eIF3 exemplified the significance of specific regulatory controls imparted by individual eIF3 subunits. When this study was initiated, not much was known about the functions of the 13 subunits of eIF3 in *Arabidopsis* (with the exception of the h and i subunit), or rather in any other higher eukaryote. As described in Chapter 2, a reverse genetic approach was initiated to gain insight into the functions of individual eIF3 subunits. Genetic analyses revealed the critical requirement for eIF3 subunits in early development (eIF3b, c, e, h, i). Furthermore, a considerable amount of functional redundancy can be suspected between several of the eIF3 genes. The requirement of eIF3 in development is apparent from the fact that disruption of the h subunit exhibits a spectrum of growth defects (Kim et al., 2004)(Zhou and von Arnim, unpublished data). The gametophytic transmission defects observed for mutations in eIF3e, h, and i-1 together with embryo lethality for eIF3b1 and c1 might show the basal requirement of duplicated eIF3 in translation initiation (Chapters 2 and 5). The fluorescently tagged eIF3 alleles can be exploited further to better understand how individual subunits affect translation during pollen development. Fluorescence activated cell sorting has been used for differentiating fluorescent pollen grains and their subsequent transcriptome analysis (Becker et al., 2003). A similar approach involving differentiating the wild-type pollens from the *eif3* pollen grains followed by protein profiling can highlight whether specific eIF3 subunits have specific clients of mRNA or whether they target all transcripts equally. The identification of downstream targets of these genes will provide insight into whether general translation is affected by these factors or whether specific regulations are imparted. Also, for understanding the functions of individual eIF3 subunits in *Arabidopsis*, it has to be borne in mind that functions of eIF3 may not be confined to translation, but may impinge on related processes. Even though the eIF3 subunits have been reported to be present stoichiometrically in the complex, there is evidence that eIF3 subunits might also act independently of the complex. The i subunit of eIF3 has been reported to be involved in

brassinosteroid signaling (Jiang and Clouse, 2001). Therefore, a comprehensive understanding will require monitoring growth and response under specific conditions (brassinosteroid for *eif3i*, as an example).

Finally, eIF3 is emerging as a versatile protein factor associated with the messenger RNA whose function is limited not just to translation (**Table I.1**). The challenge that lies ahead is to unravel the exact interactions between different complexes, and understanding the mechanistic details of how these processes are connected and coordinated.

## References

Abastado, J.P., Miller, P.F., and Hinnebusch, A.G. (1991a). A quantitative model for translational control of the GCN4 gene of *Saccharomyces cerevisiae*. *New Biol* 3, 511-524.

Abastado, J.P., Miller, P.F., Jackson, B.M., and Hinnebusch, A.G. (1991b). Suppression of ribosomal reinitiation at upstream open reading frames in amino acid-starved cells forms the basis for GCN4 translational control. *Mol Cell Biol* 11, 486-496.

Aida, M., Vernoux, T., Furutani, M., Traas, J., and Tasaka, M. (2002). Roles of PIN-FORMED1 and MONOPTEROS in pattern formation of the apical region of the Arabidopsis embryo. *Development* 129, 3965-3974.

Alexander, M. (1969). Differential staining of aborted and non-aborted pollen. *Stain Technol* 44, 117-122.

Alexander, M. (1980). A versatile stain for pollen, fungi, yeast, and bacteria. *Stain Technol* 55, 13-18.

Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., *et al.* (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* 301, 653-657.

Amrani, N., Ghosh, S., Mangus, D.A., and Jacobson, A. (2008). Translation factors promote the formation of two states of the closed-loop mRNP. *Nature* 453, 1276-1280.

Arava, Y., Boas, F.E., Brown, P.O., and Herschlag, D. (2005). Dissecting eukaryotic translation and its control by ribosome density mapping. *Nucleic Acids Res* 33, 2421-2432.

Asano, K., Clayton, J., Shalev, A., and Hinnebusch, A.G. (2000). A multifactor complex of eukaryotic initiation factors, eIF1, eIF2, eIF3, eIF5, and initiator tRNA(Met) is an important translation initiation intermediate in vivo. *Genes Dev* 14, 2534-2546.

Asano, K., Phan, L., Anderson, J., and Hinnebusch, A.G. (1998). Complex formation by all five homologues of mammalian translation initiation factor 3 subunits from yeast *Saccharomyces cerevisiae*. *J Biol Chem* 273, 18573-18585.

- Asano, K., Vornlocher, H.P., Richter-Cook, N.J., Merrick, W.C., Hinnebusch, A.G., and Hershey, J.W. (1997). Structure of cDNAs encoding human eukaryotic initiation factor 3 subunits. Possible roles in RNA binding and macromolecular assembly. *J Biol Chem* 272, 27042-27052.
- Asp, E., Nilsson, D., and Sunnerhagen, P. (2008). Fission yeast mitogen-activated protein kinase Sty1 interacts with translation factors. *Eukaryot Cell* 7, 328-338.
- Babinger, K., Hallmann, A., and Schmitt, R. (2006). Translational control of *regA*, a key gene controlling cell differentiation in *Volvox carteri*. *Development* 133, 4045-4051.
- Balogopal, V., and Parker, R. (2009). Polysomes, P bodies and stress granules: states and fates of eukaryotic mRNAs. *Curr Opin Cell Biol* 21, 403-408.
- Ban, N., Nissen, P., Hansen, J., Moore, P.B., and Steitz, T.A. (2000). The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution. *Science* 289, 905-920.
- Bandyopadhyay, A., Matsumoto, T., and Maitra, U. (2000). Fission yeast Int6 is not essential for global translation initiation, but deletion of *int6(+)* causes hypersensitivity to caffeine and affects spore formation. *Mol Biol Cell* 11, 4005-4018.
- Becker, J.D., Boavida, L.C., Carneiro, J., Haury, M., and Feijo, J.A. (2003). Transcriptional profiling of *Arabidopsis* tissues reveals the unique characteristics of the pollen transcriptome. *Plant Physiol* 133, 713-725.
- Belostotsky, D.A., and Meagher, R.B. (1996). A pollen-, ovule-, and early embryo-specific poly(A) binding protein from *Arabidopsis* complements essential functions in yeast. *Plant Cell* 8, 1261-1275.
- Benne, R., and Hershey, J.W. (1976). Purification and characterization of initiation factor IF-E3 from rabbit reticulocytes. *Proc Natl Acad Sci U S A* 73, 3005-3009.
- Benne, R., and Hershey, J.W. (1978). The mechanism of action of protein synthesis initiation factors from rabbit reticulocytes. *J Biol Chem* 253, 3078-3087.

- Boavida, L.C., and McCormick, S. (2007). Temperature as a determinant factor for increased and reproducible in vitro pollen germination in *Arabidopsis thaliana*. *Plant J* 52, 570-582.
- Bolger, T.A., Folkmann, A.W., Tran, E.J., and Went, S.R. (2008). The mRNA export factor Gle1 and inositol hexakisphosphate regulate distinct stages of translation. *Cell* 134, 624-633.
- Borg, M., Brownfield, L., and Twell, D. (2009). Male gametophyte development: a molecular perspective. *J Exp Bot* 60, 1465-1478.
- Brown-Luedi, M.L., Meyer, L.J., Milburn, S.C., Yau, P.M., Corbett, S., and Hershey, J.W. (1982). Protein synthesis initiation factors from human HeLa cells and rabbit reticulocytes are similar: comparison of protein structure, activities, and immunochemical properties. *Biochemistry* 21, 4202-4206.
- Buchsbaum, S., Morris, C., Bochard, V., and Jalinot, P. (2007). Human INT6 interacts with MCM7 and regulates its stability during S phase of the cell cycle. *Oncogene* 26, 5132-5144.
- Burks, E.A., Bezerra, P.P., Le, H., Gallie, D.R., and Browning, K.S. (2001). Plant initiation factor 3 subunit composition resembles mammalian initiation factor 3 and has a novel subunit. *J Biol Chem* 276, 2122-2131.
- Byrne, M.E. (2009). A role for the ribosome in development. *Trends Plant Sci* 14, 512-519.
- Calvo, S.E., Pagliarini, D.J., and Mootha, V.K. (2009). Upstream open reading frames cause widespread reduction of protein expression and are polymorphic among humans. *Proc Natl Acad Sci U S A* 106, 7507-7512.
- Cao, J., and Geballe, A.P. (1996). Coding sequence-dependent ribosomal arrest at termination of translation. *Mol Cell Biol* 16, 603-608.

Capkova, V., Fidlerova, A., van Amstel, T., Croes, A.F., Mata, C., Schrauwen, J.A., Wullems, G.J., and Tupy, J. (1997). Role of N-glycosylation of 66 and 69 kDa glycoproteins in wall formation during pollen tube growth in vitro. *Eur J Cell Biol* 72, 282-285.

Chappell, S.A., Dresios, J., Edelman, G.M., and Mauro, V.P. (2006). Ribosomal shunting mediated by a translational enhancer element that base pairs to 18S rRNA. *Proc Natl Acad Sci U S A* 103, 9488-9493.

Checkley, J.W., Cooley, L., and Ravel, J.M. (1981). Characterization of initiation factor eIF-3 from wheat germ. *J Biol Chem* 256, 1582-1586.

Chen, Y.C., and McCormick, S. (1996). sidecar pollen, an *Arabidopsis thaliana* male gametophytic mutant with aberrant cell divisions during pollen development. *Development* 122, 3243-3253.

Cheung, Y.N., Maag, D., Mitchell, S.F., Fekete, C.A., Algire, M.A., Takacs, J.E., Shirokikh, N., Pestova, T., Lorsch, J.R., and Hinnebusch, A.G. (2007). Dissociation of eIF1 from the 40S ribosomal subunit is a key step in start codon selection in vivo. *Genes Dev* 21, 1217-1230.

Chew, S.K., Chen, P., Link, N., Galindo, K.A., Pogue, K., and Abrams, J.M. (2009). Genome-wide silencing in *Drosophila* captures conserved apoptotic effectors. *Nature* 460, 123-127.

Combier, J.P., de Billy, F., Gamas, P., Niebel, A., and Rivas, S. (2008). Trans-regulation of the expression of the transcription factor MtHAP2-1 by a uORF controls root nodule development. *Genes Dev* 22, 1549-1559.

Combier, J.P., Frugier, F., de Billy, F., Boualem, A., El-Yahyaoui, F., Moreau, S., Vernie, T., Ott, T., Gamas, P., Crespi, M., *et al.* (2006). MtHAP2-1 is a key transcriptional regulator of symbiotic nodule development regulated by microRNA169 in *Medicago truncatula*. *Genes Dev* 20, 3084-3088.

Damoc, E., Fraser, C.S., Zhou, M., Videler, H., Mayeur, G.L., Hershey, J.W., Doudna, J.A., Robinson, C.V., and Leary, J.A. (2007). Structural characterization of the human eukaryotic initiation factor 3 protein complex by mass spectrometry. *Mol Cell Proteomics* 6, 1135-1146.

De Martelaere, K., Lintermans, B., Haegeman, G., and Vanhoenacker, P. (2007). Novel interaction between the human 5-HT7 receptor isoforms and PLAC-24/eIF3k. *Cell Signal* 19, 278-288.

Degenhardt, R.F., and Bonham-Smith, P.C. (2008). Arabidopsis ribosomal proteins RPL23aA and RPL23aB are differentially targeted to the nucleolus and are desperately required for normal development. *Plant Physiol* 147, 128-142.

Dennis, M.D., Person, M.D., and Browning, K.S. (2009). Phosphorylation of plant translation initiation factors by CK2 enhances the in vitro interaction of multifactor complex components. *J Biol Chem* 284, 20615-20628.

Dominski, Z., and Marzluff, W.F. (2007). Formation of the 3' end of histone mRNA: getting closer to the end. *Gene* 396, 373-390.

Dong, Z., and Zhang, J.T. (2006). Initiation factor eIF3 and regulation of mRNA translation, cell growth, and cancer. *Crit Rev Oncol Hematol* 59, 169-180.

Duncan, K., Grskovic, M., Strein, C., Beckmann, K., Niggeweg, R., Abaza, I., Gebauer, F., Wilm, M., and Hentze, M.W. (2006). Sex-lethal imparts a sex-specific function to UNR by recruiting it to the msl-2 mRNA 3' UTR: translational repression for dosage compensation. *Genes Dev* 20, 368-379.

ElAntak, L., Tzakos, A.G., Locker, N., and Lukavsky, P.J. (2007). Structure of eIF3b RNA recognition motif and its interaction with eIF3j: structural insights into the recruitment of eIF3b to the 40 S ribosomal subunit. *J Biol Chem* 282, 8165-8174.

Eldad, N., and Arava, Y. (2007). Detecting ribosomal association with the 5' leader of mRNAs by Ribosome Density Mapping (RDM). *Methods Enzymol* 431, 163-175.

Eldad, N., and Arava, Y. (2008). A ribosomal density-mapping procedure to explore ribosome positions along translating mRNAs. *Methods Mol Biol* 419, 231-242.

Emanuilov, I., Sabatini, D.D., Lake, J.A., and Freienstein, C. (1978). Localization of eukaryotic initiation factor 3 on native small ribosomal subunits. *Proc Natl Acad Sci U S A* 75, 1389-1393.

Fang, P., Wang, Z., and Sachs, M.S. (2000). Evolutionarily conserved features of the arginine attenuator peptide provide the necessary requirements for its function in translational regulation. *J Biol Chem* 275, 26710-26719.

Fekete, C.A., Mitchell, S.F., Cherkasova, V.A., Applefield, D., Algire, M.A., Maag, D., Saini, A.K., Lorsch, J.R., and Hinnebusch, A.G. (2007). N- and C-terminal residues of eIF1A have opposing effects on the fidelity of start codon selection. *EMBO J* 26, 1602-1614.

Fernandez, J., Yaman, I., Huang, C., Liu, H., Lopez, A.B., Komar, A.A., Caprara, M.G., Merrick, W.C., Snider, M.D., Kaufman, R.J., *et al.* (2005). Ribosome stalling regulates IRES-mediated translation in eukaryotes, a parallel to prokaryotic attenuation. *Mol Cell* 17, 405-416.

Francis, K.E., Lam, S.Y., and Copenhaver, G.P. (2006). Separation of Arabidopsis pollen tetrads is regulated by QUARTET1, a pectin methylesterase gene. *Plant Physiol* 142, 1004-1013.

Francis, K.E., Lam, S.Y., Harrison, B.D., Bey, A.L., Berchowitz, L.E., and Copenhaver, G.P. (2007). Pollen tetrad-based visual assay for meiotic recombination in Arabidopsis. *Proc Natl Acad Sci U S A* 104, 3913-3918.

Fraser, C.S., Berry, K.E., Hershey, J.W., and Doudna, J.A. (2007). eIF3j is located in the decoding center of the human 40S ribosomal subunit. *Mol Cell* 26, 811-819.

Fraser, C.S., Lee, J.Y., Mayeur, G.L., Bushell, M., Doudna, J.A., and Hershey, J.W. (2004). The j-subunit of human translation initiation factor eIF3 is required for the stable binding

of eIF3 and its subcomplexes to 40 S ribosomal subunits in vitro. *J Biol Chem* 279, 8946-8956.

Freienstein, C., and Blobel, G. (1975). Nonribosomal proteins associated with eukaryotic native small ribosomal subunits. *Proc Natl Acad Sci U S A* 72, 3392-3396.

Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa, R., and Jurgens, G. (2003). Efflux-dependent auxin gradients establish the apical-basal axis of *Arabidopsis*. *Nature* 426, 147-153.

Fujikura, U., Horiguchi, G., Ponce, M.R., Micol, J.L., and Tsukaya, H. (2009). Coordination of cell proliferation and cell expansion mediated by ribosome-related processes in the leaves of *Arabidopsis thaliana*. *Plant J* 59, 499-508.

Gaba, A., Jacobson, A., and Sachs, M.S. (2005). Ribosome occupancy of the yeast CPA1 upstream open reading frame termination codon modulates nonsense-mediated mRNA decay. *Mol Cell* 20, 449-460.

Gaba, A., Wang, Z., Krishnamoorthy, T., Hinnebusch, A.G., and Sachs, M.S. (2001). Physical evidence for distinct mechanisms of translational control by upstream open reading frames. *EMBO J* 20, 6453-6463.

Gallie, D.R. (1991). The cap and poly(A) tail function synergistically to regulate mRNA translational efficiency. *Genes Dev* 5, 2108-2116.

Gebauer, F., and Hentze, M.W. (2004). Molecular mechanisms of translational control. *Nat Rev Mol Cell Biol* 5, 827-835.

Gill, S.S., and Tuteja, N. (2010). Polyamines and abiotic stress tolerance in plants. *Plant Signal Behav* 5.

Grant-Downton, R., Le Trionnaire, G., Schmid, R., Rodriguez-Enriquez, J., Hafidh, S., Mehdi, S., Twell, D., and Dickinson, H. (2009). MicroRNA and tasiRNA diversity in mature pollen of *Arabidopsis thaliana*. *BMC Genomics* 10, 643.

Guo, J., and Sen, G.C. (2000). Characterization of the interaction between the interferon-induced protein P56 and the Int6 protein encoded by a locus of insertion of the mouse mammary tumor virus. *J Virol* 74, 1892-1899.

Gusti, A., Baumberger, N., Nowack, M., Pusch, S., Eisler, H., Potuschak, T., De Veylder, L., Schnittger, A., and Genschik, P. (2009). The *Arabidopsis thaliana* F-box protein FBL17 is essential for progression through the second mitosis during pollen development. *PLoS One* 4, e4780.

Hanfrey, C., Elliott, K.A., Franceschetti, M., Mayer, M.J., Illingworth, C., and Michael, A.J. (2005). A dual upstream open reading frame-based autoregulatory circuit controlling polyamine-responsive translation. *J Biol Chem* 280, 39229-39237.

Hanfrey, C., Franceschetti, M., Mayer, M.J., Illingworth, C., Elliott, K., Collier, M., Thompson, B., Perry, B., and Michael, A.J. (2003). Translational regulation of the plant S-adenosylmethionine decarboxylase. *Biochem Soc Trans* 31, 424-427.

Hanfrey, C., Franceschetti, M., Mayer, M.J., Illingworth, C., and Michael, A.J. (2002). Abrogation of upstream open reading frame-mediated translational control of a plant S-adenosylmethionine decarboxylase results in polyamine disruption and growth perturbations. *J Biol Chem* 277, 44131-44139.

Hanson, J., Hanssen, M., Wiese, A., Hendriks, M.M., and Smeekens, S. (2008). The sucrose regulated transcription factor bZIP11 affects amino acid metabolism by regulating the expression of ASPARAGINE SYNTHETASE1 and PROLINE DEHYDROGENASE2. *Plant J* 53, 935-949.

Hao, H., Li, Y., Hu, Y., and Lin, J. (2005). Inhibition of RNA and protein synthesis in pollen tube development of *Pinus bungeana* by actinomycin D and cycloheximide. *New Phytol* 165, 721-729.

Harding, H.P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., and Ron, D. (2000). Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol Cell* 6, 1099-1108.

Hasek, J., Kovarik, P., Valasek, L., Malinska, K., Schneider, J., Kohlwein, S.D., and Ruis, H. (2000). Rpg1p, the subunit of the *Saccharomyces cerevisiae* eIF3 core complex, is a microtubule-interacting protein. *Cell Motil Cytoskeleton* 45, 235-246.

Hayden, C.A., and Jorgensen, R.A. (2007). Identification of novel conserved peptide uORF homology groups in *Arabidopsis* and rice reveals ancient eukaryotic origin of select groups and preferential association with transcription factor-encoding genes. *BMC Biol* 5, 32.

Hinnebusch, A.G. (2005). Translational regulation of GCN4 and the general amino acid control of yeast. *Annu Rev Microbiol* 59, 407-450.

Hinnebusch, A.G. (2006). eIF3: a versatile scaffold for translation initiation complexes. *Trends Biochem Sci* 31, 553-562.

Honys, D., Renak, D., Fecikova, J., Jedelsky, P.L., Nebesarova, J., Dobrev, P., and Capkova, V. (2009). Cytoskeleton-associated large RNP complexes in tobacco male gametophyte (EPPs) are associated with ribosomes and are involved in protein synthesis, processing, and localization. *J Proteome Res* 8, 2015-2031.

Honys, D., and Twell, D. (2003). Comparative analysis of the *Arabidopsis* pollen transcriptome. *Plant Physiol* 132, 640-652.

Honys, D., and Twell, D. (2004). Transcriptome analysis of haploid male gametophyte development in *Arabidopsis*. *Genome Biol* 5, R85.

Hruz, T., Laule, O., Szabo, G., Wessendorp, F., Bleuler, S., Oertle, L., Widmayer, P., Gruissem, W., and Zimmermann, P. (2008). Genevestigator v3: a reference expression database for the meta-analysis of transcriptomes. *Adv Bioinformatics* 2008, 420747.

Hui, D.J., Terenzi, F., Merrick, W.C., and Sen, G.C. (2005). Mouse p56 blocks a distinct function of eukaryotic initiation factor 3 in translation initiation. *J Biol Chem* 280, 3433-3440.

Hulzink, R.J., de Groot, P.F., Croes, A.F., Quaedvlieg, W., Twell, D., Wullems, G.J., and Van Herpen, M.M. (2002). The 5'-untranslated region of the ntp303 gene strongly enhances translation during pollen tube growth, but not during pollen maturation. *Plant Physiol* 129, 342-353.

Hulzink, R.J., Weerdesteyn, H., Croes, A.F., Gerats, T., van Herpen, M.M., and van Helden, J. (2003). In silico identification of putative regulatory sequence elements in the 5'-untranslated region of genes that are expressed during male gametogenesis. *Plant Physiol* 132, 75-83.

Hummel, M., Rahmani, F., Smeekens, S., and Hanson, J. (2009). Sucrose-mediated translational control. *Ann Bot* 104, 1-7.

Imai, A., Hanzawa, Y., Komura, M., Yamamoto, K.T., Komeda, Y., and Takahashi, T. (2006). The dwarf phenotype of the *Arabidopsis* *acl5* mutant is suppressed by a mutation in an upstream ORF of a bHLH gene. *Development* 133, 3575-3585.

Imai, A., Komura, M., Kawano, E., Kuwashiro, Y., and Takahashi, T. (2008). A semi-dominant mutation in the ribosomal protein L10 gene suppresses the dwarf phenotype of the *acl5* mutant in *Arabidopsis thaliana*. *Plant J* 56, 881-890.

Ingolia, N.T., Ghaemmaghami, S., Newman, J.R., and Weissman, J.S. (2009). Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling. *Science* 324, 218-223.

Iwakawa, H., Shinmyo, A., and Sekine, M. (2006). *Arabidopsis* CDKA;1, a *cdc2* homologue, controls proliferation of generative cells in male gametogenesis. *Plant J* 45, 819-831.

Jackson, R.J. (2007). The missing link in the eukaryotic ribosome cycle. *Mol Cell* 28, 356-358.

Jackson, R.J., Hellen, C.U., and Pestova, T.V. (2010). The mechanism of eukaryotic translation initiation and principles of its regulation. *Nat Rev Mol Cell Biol* 11, 113-127.

Jiang, J., and Clouse, S.D. (2001). Expression of a plant gene with sequence similarity to animal TGF-beta receptor interacting protein is regulated by brassinosteroids and required for normal plant development. *Plant J* 26, 35-45.

Jivotovskaya, A.V., Valasek, L., Hinnebusch, A.G., and Nielsen, K.H. (2006). Eukaryotic translation initiation factor 3 (eIF3) and eIF2 can promote mRNA binding to 40S subunits independently of eIF4G in yeast. *Mol Cell Biol* 26, 1355-1372.

Johnson, M.A., von Besser, K., Zhou, Q., Smith, E., Aux, G., Patton, D., Levin, J.Z., and Preuss, D. (2004). Arabidopsis hapless mutations define essential gametophytic functions. *Genetics* 168, 971-982.

Kahvejian, A., Roy, G., and Sonenberg, N. (2001). The mRNA closed-loop model: the function of PABP and PABP-interacting proteins in mRNA translation. *Cold Spring Harb Symp Quant Biol* 66, 293-300.

Kapp, L.D., and Lorsch, J.R. (2004). The molecular mechanics of eukaryotic translation. *Annu Rev Biochem* 73, 657-704.

Karniol, B., Yahalom, A., Kwok, S., Tsuge, T., Matsui, M., Deng, X.W., and Chamovitz, D.A. (1998). The Arabidopsis homologue of an eIF3 complex subunit associates with the COP9 complex. *FEBS Lett* 439, 173-179.

Kawaguchi, R., Girke, T., Bray, E.A., and Bailey-Serres, J. (2004). Differential mRNA translation contributes to gene regulation under non-stress and dehydration stress conditions in *Arabidopsis thaliana*. *Plant J* 38, 823-839.

Keene, J.D., and Tenenbaum, S.A. (2002). Eukaryotic mRNPs may represent posttranscriptional operons. *Mol Cell* 9, 1161-1167.

Kim, B.H., Cai, X., Vaughn, J.N., and von Arnim, A.G. (2007). On the functions of the h subunit of eukaryotic initiation factor 3 in late stages of translation initiation. *Genome Biol* 8, R60.

Kim, J.T., Kim, K.D., Song, E.Y., Lee, H.G., Kim, J.W., Chae, S.K., Kim, E., Lee, M.S., Yang, Y., and Lim, J.S. (2006). Apoptosis-inducing factor (AIF) inhibits protein synthesis by interacting with the eukaryotic translation initiation factor 3 subunit p44 (eIF3g). *FEBS Lett* 580, 6375-6383.

Kim, T., Hofmann, K., von Arnim, A.G., and Chamovitz, D.A. (2001). PCI complexes: pretty complex interactions in diverse signaling pathways. *Trends Plant Sci* 6, 379-386.

Kim, T.H., Kim, B.H., Yahalom, A., Chamovitz, D.A., and von Arnim, A.G. (2004). Translational regulation via 5' mRNA leader sequences revealed by mutational analysis of the Arabidopsis translation initiation factor subunit eIF3h. *Plant Cell* 16, 3341-3356.

Komarova, A.V., Real, E., Borman, A.M., Brocard, M., England, P., Tordo, N., Hershey, J.W., Kean, K.M., and Jacob, Y. (2007). Rabies virus matrix protein interplay with eIF3, new insights into rabies virus pathogenesis. *Nucleic Acids Res* 35, 1522-1532.

Koyanagi-Katsuta, R., Akimitsu, N., Hamamoto, H., Arimitsu, N., Hatano, T., and Sekimizu, K. (2002). Embryonic lethality of mutant mice deficient in the p116 gene. *J Biochem* 131, 833-837.

Kozak, M. (1984). Point mutations close to the AUG initiator codon affect the efficiency of translation of rat preproinsulin in vivo. *Nature* 308, 241-246.

Kozak, M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* 44, 283-292.

- Kozak, M. (1987). Effects of intercistronic length on the efficiency of reinitiation by eucaryotic ribosomes. *Mol Cell Biol* 7, 3438-3445.
- Kozak, M. (1998). Primer extension analysis of eukaryotic ribosome-mRNA complexes. *Nucleic Acids Res* 26, 4853-4859.
- Kozak, M. (2001). Constraints on reinitiation of translation in mammals. *Nucleic Acids Res* 29, 5226-5232.
- Kozak, M. (2002). Pushing the limits of the scanning mechanism for initiation of translation. *Gene* 299, 1-34.
- Kusano, T., Berberich, T., Tateda, C., and Takahashi, Y. (2008). Polyamines: essential factors for growth and survival. *Planta* 228, 367-381.
- Lageix, S., Lanet, E., Pouch-Pelissier, M.N., Espagnol, M.C., Robaglia, C., Deragon, J.M., and Pelissier, T. (2008). Arabidopsis eIF2alpha kinase GCN2 is essential for growth in stress conditions and is activated by wounding. *BMC Plant Biol* 8, 134.
- Lauer, S.J., Browning, K.S., and Ravel, J.M. (1985). Characterization of initiation factor 3 from wheat germ. 2. Effects of polyclonal and monoclonal antibodies on activity. *Biochemistry* 24, 2928-2931.
- Lawless, C., Pearson, R.D., Selley, J.N., Smirnova, J.B., Grant, C.M., Ashe, M.P., Pavitt, G.D., and Hubbard, S.J. (2009). Upstream sequence elements direct post-transcriptional regulation of gene expression under stress conditions in yeast. *BMC Genomics* 10, 7.
- Lee, J.P., Brauweiler, A., Rudolph, M., Hooper, J.E., Drabkin, H.A., and Gemmill, R.M. (2010). The TRC8 ubiquitin ligase is sterol regulated and interacts with lipid and protein biosynthetic pathways. *Mol Cancer Res* 8, 93-106.
- LeFebvre, A.K., Korneeva, N.L., Trutschl, M., Cvek, U., Duzan, R.D., Bradley, C.A., Hershey, J.W., and Rhoads, R.E. (2006). Translation initiation factor eIF4G-1 binds to eIF3 through the eIF3e subunit. *J Biol Chem* 281, 22917-22932.

Lin, Y.M., Chen, Y.R., Lin, J.R., Wang, W.J., Inoko, A., Inagaki, M., Wu, Y.C., and Chen, R.H. (2008). eIF3k regulates apoptosis in epithelial cells by releasing caspase 3 from keratin-containing inclusions. *J Cell Sci* *121*, 2382-2393.

Lomakin, I.B., Kolupaeva, V.G., Marintchev, A., Wagner, G., and Pestova, T.V. (2003). Position of eukaryotic initiation factor eIF1 on the 40S ribosomal subunit determined by directed hydroxyl radical probing. *Genes Dev* *17*, 2786-2797.

Lovett, P.S. (1994). Nascent peptide regulation of translation. *J Bacteriol* *176*, 6415-6417.  
Lovett, P.S., and Rogers, E.J. (1996). Ribosome regulation by the nascent peptide. *Microbiol Rev* *60*, 366-385.

Maag, D., and Lorsch, J.R. (2003). Communication between eukaryotic translation initiation factors 1 and 1A on the yeast small ribosomal subunit. *J Mol Biol* *330*, 917-924.

Masutani, M., Sonenberg, N., Yokoyama, S., and Imataka, H. (2007). Reconstitution reveals the functional core of mammalian eIF3. *EMBO J* *26*, 3373-3383.

Mathews, M.B., Sonenberg, N. and Hershey, J.W.B. (2007) *Origins and Principles of Translational Control*, In M. B. Mathews, N. Sonenberg and J. W. B. Hershey, ed, *Translational Control in Biology and Medicine*, CSHL Press, Cold Spring Harbor.

Mazumder, B., Seshadri, V., and Fox, P.L. (2003). Translational control by the 3'-UTR: the ends specify the means. *Trends Biochem Sci* *28*, 91-98.

McCormick, S. (2007). Plant science. Reproductive dialog. *Science* *317*, 606-607.

McIntosh, K.B., and Bonham-Smith, P.C. (2005). The two ribosomal protein L23A genes are differentially transcribed in *Arabidopsis thaliana*. *Genome* *48*, 443-454.

Mehta, A., Trotta, C.R., and Peltz, S.W. (2006). Derepression of the Her-2 uORF is mediated by a novel post-transcriptional control mechanism in cancer cells. *Genes Dev* *20*, 939-953.

Meijer, H.A., and Thomas, A.A. (2002). Control of eukaryotic protein synthesis by upstream open reading frames in the 5'-untranslated region of an mRNA. *Biochem J* 367, 1-11.

Mignone, F., Gissi, C., Liuni, S., and Pesole, G. (2002). Untranslated regions of mRNAs. *Genome Biol* 3, REVIEWS0004.

Mikamo-Satoh, E., Takagi, A., Tanaka, H., Matsumoto, T., Nishihara, T., and Kawai, T. (2009). Profiling of gene-dependent translational progress in cell-free protein synthesis by real-space imaging. *Anal Biochem* 394, 275-280.

Morris, C., Wittmann, J., Jack, H.M., and Jalinot, P. (2007). Human INT6/eIF3e is required for nonsense-mediated mRNA decay. *EMBO Rep* 8, 596-602.

Morris, D.R., and Geballe, A.P. (2000). Upstream open reading frames as regulators of mRNA translation. *Mol Cell Biol* 20, 8635-8642.

Nanda, J.S., Cheung, Y.N., Takacs, J.E., Martin-Marcos, P., Saini, A.K., Hinnebusch, A.G., and Lorsch, J.R. (2009). eIF1 controls multiple steps in start codon recognition during eukaryotic translation initiation. *J Mol Biol* 394, 268-285.

Naranda, T., MacMillan, S.E., and Hershey, J.W. (1994). Purified yeast translational initiation factor eIF-3 is an RNA-binding protein complex that contains the PRT1 protein. *J Biol Chem* 269, 32286-32292.

Nielsen, K.H., Szamecz, B., Valasek, L., Jivotovskaya, A., Shin, B.S., and Hinnebusch, A.G. (2004). Functions of eIF3 downstream of 48S assembly impact AUG recognition and GCN4 translational control. *EMBO J* 23, 1166-1177.

Nielsen, K.H., Valasek, L., Sykes, C., Jivotovskaya, A., and Hinnebusch, A.G. (2006). Interaction of the RNP1 motif in PRT1 with HCR1 promotes 40S binding of eukaryotic initiation factor 3 in yeast. *Mol Cell Biol* 26, 2984-2998.

Nishikawa, S., Zinkl, G.M., Swanson, R.J., Maruyama, D., and Preuss, D. (2005). Callose (beta-1,3 glucan) is essential for Arabidopsis pollen wall patterning, but not tube growth. *BMC Plant Biol* 5, 22.

Nishimura, T., Wada, T., Yamamoto, K.T., and Okada, K. (2005). The Arabidopsis STV1 protein, responsible for translation reinitiation, is required for auxin-mediated gynoecium patterning. *Plant Cell* 17, 2940-2953.

Nowack, M.K., Grini, P.E., Jakoby, M.J., Lafos, M., Koncz, C., and Schnittger, A. (2006). A positive signal from the fertilization of the egg cell sets off endosperm proliferation in angiosperm embryogenesis. *Nat Genet* 38, 63-67.

Nygard, O., and Westermann, P. (1982). Specific interaction of one subunit of eukaryotic initiation factor eIF-3 with 18S ribosomal RNA within the binary complex, eIF-3 small ribosomal subunit, as shown by cross-linking experiments. *Nucleic Acids Res* 10, 1327-1334.

Nyiko, T., Sonkoly, B., Merai, Z., Benkovics, A.H., and Silhavy, D. (2009). Plant upstream ORFs can trigger nonsense-mediated mRNA decay in a size-dependent manner. *Plant Mol Biol* 71, 367-378.

Oh, S.A., Johnson, A., Smertenko, A., Rahman, D., Park, S.K., Hussey, P.J., and Twell, D. (2005). A divergent cellular role for the FUSED kinase family in the plant-specific cytokinetic phragmoplast. *Curr Biol* 15, 2107-2111.

op den Camp, R.G., and Kuhlemeier, C. (1998). Phosphorylation of tobacco eukaryotic translation initiation factor 4A upon pollen tube germination. *Nucleic Acids Res* 26, 2058-2062.

Otero, J.H., Suo, J., Gordon, C., and Chang, E.C. (2010). Int6 and Moe1 interact with Cdc48 to regulate ERAD and proper chromosome segregation. *Cell Cycle* 9, 147-161.

Palanivelu, R., and Preuss, D. (2000). Pollen tube targeting and axon guidance: parallels in tip growth mechanisms. *Trends Cell Biol* 10, 517-524.

Palanivelu, R., and Preuss, D. (2006). Distinct short-range ovule signals attract or repel *Arabidopsis thaliana* pollen tubes in vitro. *BMC Plant Biol* 6, 7.

Park, H.S., Browning, K.S., Hohn, T., and Ryabova, L.A. (2004a). Eucaryotic initiation factor 4B controls eIF3-mediated ribosomal entry of viral reinitiation factor. *EMBO J* 23, 1381-1391.

Park, H.S., Himmelbach, A., Browning, K.S., Hohn, T., and Ryabova, L.A. (2001). A plant viral "reinitiation" factor interacts with the host translational machinery. *Cell* 106, 723-733.

Park, S.K., Howden, R., and Twell, D. (1998). The *Arabidopsis thaliana* gametophytic mutation *geminipollen1* disrupts microspore polarity, division asymmetry and pollen cell fate. *Development* 125, 3789-3799.

Park, S.K., Rahman, D., Oh, S.A., and Twell, D. (2004b). *geminipollen 2*, a male and female gametophytic cytokinesis defective mutation. *Sex Plant Reprod* 17, 63-70.

Pestova, T.V., Borukhov, S.I., and Hellen, C.U. (1998). Eukaryotic ribosomes require initiation factors 1 and 1A to locate initiation codons. *Nature* 394, 854-859.

Peterson, D.T., Merrick, W.C., and Safer, B. (1979). Binding and release of radiolabeled eukaryotic initiation factors 2 and 3 during 80 S initiation complex formation. *J Biol Chem* 254, 2509-2516.

Petracek, M.E., Dickey, L.F., Huber, S.C., and Thompson, W.F. (1997). Light-regulated changes in abundance and polyribosome association of ferredoxin mRNA are dependent on photosynthesis. *Plant Cell* 9, 2291-2300.

Phan, L., Zhang, X., Asano, K., Anderson, J., Vornlocher, H.P., Greenberg, J.R., Qin, J., and Hinnebusch, A.G. (1998). Identification of a translation initiation factor 3 (eIF3) core complex, conserved in yeast and mammals, that interacts with eIF5. *Mol Cell Biol* 18, 4935-4946.

- Pick, E., Hofmann, K., and Glickman, M.H. (2009). PCI complexes: Beyond the proteasome, CSN, and eIF3 Troika. *Mol Cell* 35, 260-264.
- Picton, J.M., and Steer, M.W. (1983). The effect of cycloheximide on dictyosome activity in *Tradescantia* pollen tubes determined using cytochalasin D. *Eur J Cell Biol* 29, 133-138.
- Pina, C., Pinto, F., Feijo, J.A., and Becker, J.D. (2005). Gene family analysis of the *Arabidopsis* pollen transcriptome reveals biological implications for cell growth, division control, and gene expression regulation. *Plant Physiol* 138, 744-756.
- Pinon, V., Etchells, J.P., Rossignol, P., Collier, S.A., Arroyo, J.M., Martienssen, R.A., and Byrne, M.E. (2008). Three PIGGYBACK genes that specifically influence leaf patterning encode ribosomal proteins. *Development* 135, 1315-1324.
- Pisarev, A.V., Hellen, C.U., and Pestova, T.V. (2007). Recycling of eukaryotic posttermination ribosomal complexes. *Cell* 131, 286-299.
- Pisarev, A.V., Skabkin, M.A., Pisareva, V.P., Skabkina, O.V., Rakotondrafara, A.M., Hentze, M.W., Hellen, C.U., and Pestova, T.V. (2010). The role of ABCE1 in eukaryotic posttermination ribosomal recycling. *Mol Cell* 37, 196-210.
- Pooggin, M.M., Futterer, J., and Hohn, T. (2008). Cross-species functionality of pararetroviral elements driving ribosome shunting. *PLoS One* 3, e1650.
- Pooggin, M.M., Futterer, J., Skryabin, K.G., and Hohn, T. (2001). Ribosome shunt is essential for infectivity of cauliflower mosaic virus. *Proc Natl Acad Sci U S A* 98, 886-891.
- Pooggin, M.M., Hohn, T., and Futterer, J. (2000). Role of a short open reading frame in ribosome shunt on the cauliflower mosaic virus RNA leader. *J Biol Chem* 275, 17288-17296.
- Poyry, T.A., Kaminski, A., Connell, E.J., Fraser, C.S., and Jackson, R.J. (2007). The mechanism of an exceptional case of reinitiation after translation of a long ORF reveals

why such events do not generally occur in mammalian mRNA translation. *Genes Dev* 21, 3149-3162.

Poyry, T.A., Kaminski, A., and Jackson, R.J. (2004). What determines whether mammalian ribosomes resume scanning after translation of a short upstream open reading frame? *Genes Dev* 18, 62-75.

Racine, T., Barry, C., Roy, K., Dawe, S.J., Shmulevitz, M., and Duncan, R. (2007). Leaky scanning and scanning-independent ribosome migration on the tricistronic S1 mRNA of avian reovirus. *J Biol Chem* 282, 25613-25622.

Rahmani, F., Hummel, M., Schuurmans, J., Wiese-Klinkenberg, A., Smeekeens, S., and Hanson, J. (2009). Sucrose control of translation mediated by an upstream open reading frame-encoded peptide. *Plant Physiol* 150, 1356-1367.

Rajkowitsch, L., Vilela, C., Berthelot, K., Ramirez, C.V., and McCarthy, J.E. (2004). Reinitiation and recycling are distinct processes occurring downstream of translation termination in yeast. *J Mol Biol* 335, 71-85.

Ray, A., Bandyopadhyay, A., Matsumoto, T., Deng, H., and Maitra, U. (2008). Fission yeast translation initiation factor 3 subunit eIF3h is not essential for global translation initiation, but deletion of *eif3h+* affects spore formation. *Yeast* 25, 809-823.

Resch, A.M., Ogurtsov, A.Y., Rogozin, I.B., Shabalina, S.A., and Koonin, E.V. (2009). Evolution of alternative and constitutive regions of mammalian 5'UTRs. *BMC Genomics* 10, 162.

Rook, F., Gerrits, N., Kortstee, A., van Kampen, M., Borrias, M., Weisbeek, P., and Smeekeens, S. (1998a). Sucrose-specific signalling represses translation of the Arabidopsis ATB2 bZIP transcription factor gene. *Plant J* 15, 253-263.

Rook, F., Weisbeek, P., and Smeekeens, S. (1998b). The light-regulated Arabidopsis bZIP transcription factor gene ATB2 encodes a protein with an unusually long leucine zipper domain. *Plant Mol Biol* 37, 171-178.

Rosso, M.G., Li, Y., Strizhov, N., Reiss, B., Dekker, K., and Weisshaar, B. (2003). An *Arabidopsis thaliana* T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based reverse genetics. *Plant Mol Biol* 53, 247-259.

Ruiz-Echevarria, M.J., and Peltz, S.W. (2000). The RNA binding protein Pub1 modulates the stability of transcripts containing upstream open reading frames. *Cell* 101, 741-751.

Ryabova, L., Park, H.S., and Hohn, T. (2004). Control of translation reinitiation on the cauliflower mosaic virus (CaMV) polycistronic RNA. *Biochem Soc Trans* 32, 592-596.

Sachs, M.S., and Geballe, A.P. (2006). Downstream control of upstream open reading frames. *Genes Dev* 20, 915-921.

Sachs, M.S., Wang, Z., Gaba, A., Fang, P., Belk, J., Ganesan, R., Amrani, N., and Jacobson, A. (2002). Toeprint analysis of the positioning of translation apparatus components at initiation and termination codons of fungal mRNAs. *Methods* 26, 105-114.

Saul, H., Elharrar, E., Gaash, R., Eliaz, D., Valenci, M., Akua, T., Avramov, M., Frankel, N., Berezin, I., Gottlieb, D., *et al.* (2009). The upstream open reading frame of the *Arabidopsis* AtMHX gene has a strong impact on transcript accumulation through the nonsense-mediated mRNA decay pathway. *Plant J* 60, 1031-1042.

Sawicka, K., Bushell, M., Spriggs, K.A., and Willis, A.E. (2008). Polypyrimidine-tract-binding protein: a multifunctional RNA-binding protein. *Biochem Soc Trans* 36, 641-647.

Scoles, D.R., Yong, W.H., Qin, Y., Wawrowsky, K., and Pulst, S.M. (2006). Schwannomin inhibits tumorigenesis through direct interaction with the eukaryotic initiation factor subunit c (eIF3c). *Hum Mol Genet* 15, 1059-1070.

Sha, Z., Brill, L.M., Cabrera, R., Kleinfeld, O., Scheliga, J.S., Glickman, M.H., Chang, E.C., and Wolf, D.A. (2009). The eIF3 interactome reveals the translasome, a supercomplex linking protein synthesis and degradation machineries. *Mol Cell* 36, 141-152.

Shanina, N.A., Ivanov, P.A., Chudinova, E.M., Severin, F.F., and Nadezhkina, E.S. (2001). [Translation initiation factor eIF3 is able to bind with microtubules in mammalian cells]. *Mol Biol (Mosk)* 35, 638-646.

Sheoran, I.S., Pedersen, E.J., Ross, A.R., and Sawhney, V.K. (2009). Dynamics of protein expression during pollen germination in canola (*Brassica napus*). *Planta* 230, 779-793.

Shi, J., Hershey, J.W., and Nelson, M.A. (2009). Phosphorylation of the eukaryotic initiation factor 3f by cyclin-dependent kinase 11 during apoptosis. *FEBS Lett* 583, 971-977.

Shi, J., Kahle, A., Hershey, J.W., Honchak, B.M., Warneke, J.A., Leong, S.P., and Nelson, M.A. (2006). Decreased expression of eukaryotic initiation factor 3f deregulates translation and apoptosis in tumor cells. *Oncogene* 25, 4923-4936.

Singh, C.R., He, H., Ii, M., Yamamoto, Y., and Asano, K. (2004). Efficient incorporation of eukaryotic initiation factor 1 into the multifactor complex is critical for formation of functional ribosomal preinitiation complexes in vivo. *J Biol Chem* 279, 31910-31920.

Siridechadilok, B., Fraser, C.S., Hall, R.J., Doudna, J.A., and Nogales, E. (2005). Structural roles for human translation factor eIF3 in initiation of protein synthesis. *Science* 310, 1513-1515.

Sonenberg, N., and Hinnebusch, A.G. (2007). New modes of translational control in development, behavior, and disease. *Mol Cell* 28, 721-729.

Sonenberg, N., and Hinnebusch, A.G. (2009). Regulation of translation initiation in eukaryotes: mechanisms and biological targets. *Cell* 136, 731-745.

Spahn, C.M., Beckmann, R., Eswar, N., Penczek, P.A., Sali, A., Blobel, G., and Frank, J. (2001). Structure of the 80S ribosome from *Saccharomyces cerevisiae*--tRNA-ribosome and subunit-subunit interactions. *Cell* 107, 373-386.

Spahn, C.M., Gomez-Lorenzo, M.G., Grassucci, R.A., Jorgensen, R., Andersen, G.R., Beckmann, R., Penczek, P.A., Ballesta, J.P., and Frank, J. (2004). Domain movements of elongation factor eEF2 and the eukaryotic 80S ribosome facilitate tRNA translocation. *EMBO J* 23, 1008-1019.

Spirin, A.S. (2009). How does a scanning ribosomal particle move along the 5'-untranslated region of eukaryotic mRNA? Brownian Ratchet model. *Biochemistry* 48, 10688-10692.

Sundkvist, I.C., McKeehan, W.L., Schreier, M.H., and Staehelin, T. (1974). Initiation factor activity associated with free 40 S subunits from rat liver and rabbit reticulocytes. *J Biol Chem* 249, 6512-6516.

Sundkvist, I.C., and Staehelin, T. (1975). Structure and function of free 40 S ribosome subunits: Characterization of initiation factors. *J Mol Biol* 99, 401-418.

Szamecz, B., Rutkai, E., Cuchalova, L., Munzarova, V., Herrmannova, A., Nielsen, K.H., Burela, L., Hinnebusch, A.G., and Valasek, L. (2008). eIF3a cooperates with sequences 5' of uORF1 to promote resumption of scanning by post-termination ribosomes for reinitiation on GCN4 mRNA. *Genes Dev* 22, 2414-2425.

Teale, W.D., Paponov, I.A., and Palme, K. (2006). Auxin in action: signalling, transport and the control of plant growth and development. *Nat Rev Mol Cell Biol* 7, 847-859.

Terenzi, F., Pal, S., and Sen, G.C. (2005). Induction and mode of action of the viral stress-inducible murine proteins, P56 and P54. *Virology* 340, 116-124.

Thiebauld, O., Schepetilnikov, M., Park, H.S., Geldreich, A., Kobayashi, K., Keller, M., Hohn, T., and Ryabova, L.A. (2009). A new plant protein interacts with eIF3 and 60S to enhance virus-activated translation re-initiation. *EMBO J* 28, 3171-3184.

Thompson, H.A., Sadnik, I., Scheinbuks, J., and Moldave, K. (1977). Studies on native ribosomal subunits from rat liver. Purification and characterization of a ribosome dissociation factor. *Biochemistry* 16, 2221-2230.

Toufighi, K., Brady, S.M., Austin, R., Ly, E., and Provart, N.J. (2005). The Botany Array Resource: e-Northerns, Expression Angling, and promoter analyses. *Plant J* 43, 153-163.

Trachsel, H., Erni, B., Schreier, M.H., Braun, L., and Staehelin, T. (1979). Purification of seven protein synthesis initiation factors from Krebs II ascites cells. *Biochim Biophys Acta* 561, 484-490.

Trachsel, H., Erni, B., Schreier, M.H., and Staehelin, T. (1977). Initiation of mammalian protein synthesis. II. The assembly of the initiation complex with purified initiation factors. *J Mol Biol* 116, 755-767.

Unbehaun, A., Borukhov, S.I., Hellen, C.U., and Pestova, T.V. (2004). Release of initiation factors from 48S complexes during ribosomal subunit joining and the link between establishment of codon-anticodon base-pairing and hydrolysis of eIF2-bound GTP. *Genes Dev* 18, 3078-3093.

Unhavaithaya, Y., Hao, Y., Beyret, E., Yin, H., Kuramochi-Miyagawa, S., Nakano, T., and Lin, H. (2009). MILI, a PIWI-interacting RNA-binding protein, is required for germ line stem cell self-renewal and appears to positively regulate translation. *J Biol Chem* 284, 6507-6519.

Valasek, L., Hasek, J., Nielsen, K.H., and Hinnebusch, A.G. (2001). Dual function of eIF3j/Hcr1p in processing 20 S pre-rRNA and translation initiation. *J Biol Chem* 276, 43351-43360.

Valasek, L., Mathew, A.A., Shin, B.S., Nielsen, K.H., Szamecz, B., and Hinnebusch, A.G. (2003). The yeast eIF3 subunits TIF32/a, NIP1/c, and eIF5 make critical connections with the 40S ribosome in vivo. *Genes Dev* 17, 786-799.

Valasek, L., Nielsen, K.H., and Hinnebusch, A.G. (2002). Direct eIF2-eIF3 contact in the multifactor complex is important for translation initiation in vivo. *EMBO J* 21, 5886-5898.

Valasek, L., Nielsen, K.H., Zhang, F., Fekete, C.A., and Hinnebusch, A.G. (2004).

Interactions of eukaryotic translation initiation factor 3 (eIF3) subunit NIP1/c with eIF1 and eIF5 promote preinitiation complex assembly and regulate start codon selection. *Mol Cell Biol* 24, 9437-9455.

Van Lijsebettens, M., Vanderhaeghen, R., De Block, M., Bauw, G., Villarroel, R., and Van Montagu, M. (1994). An S18 ribosomal protein gene copy at the Arabidopsis PFL locus affects plant development by its specific expression in meristems. *EMBO J* 13, 3378-3388.

Vera-Sirera, F., Minguet, E.G., Singh, S.K., Ljung, K., Tuominen, H., Blazquez, M.A., and Carbonell, J. (2010). Role of polyamines in plant vascular development. *Plant Physiol Biochem*.

von Arnim, A.G., and Schwechheimer, C. (2006). Life is degrading--thanks to some zomes. *Mol Cell* 23, 621-629.

von Besser, K., Frank, A.C., Johnson, M.A., and Preuss, D. (2006). Arabidopsis HAP2 (GCS1) is a sperm-specific gene required for pollen tube guidance and fertilization. *Development* 133, 4761-4769.

Wang, L., and Wessler, S.R. (1998). Inefficient reinitiation is responsible for upstream open reading frame-mediated translational repression of the maize R gene. *Plant Cell* 10, 1733-1746.

Wang, S., and Okamoto, T. (2009). Involvement of polypyrimidine tract-binding protein (PTB)-related proteins in pollen germination in Arabidopsis. *Plant Cell Physiol* 50, 179-190.

Wang, Z., Fang, P., and Sachs, M.S. (1998). The evolutionarily conserved eukaryotic arginine attenuator peptide regulates the movement of ribosomes that have translated it. *Mol Cell Biol* 18, 7528-7536.

Wang, Z., and Sachs, M.S. (1997). Ribosome stalling is responsible for arginine-specific translational attenuation in *Neurospora crassa*. *Mol Cell Biol* 17, 4904-4913.

Wei, Z., Zhang, P., Zhou, Z., Cheng, Z., Wan, M., and Gong, W. (2004). Crystal structure of human eIF3k, the first structure of eIF3 subunits. *J Biol Chem* 279, 34983-34990.

Weijers, D., Franke-van Dijk, M., Vencken, R.J., Quint, A., Hooykaas, P., and Offringa, R. (2001). An Arabidopsis Minute-like phenotype caused by a semi-dominant mutation in a RIBOSOMAL PROTEIN S5 gene. *Development* 128, 4289-4299.

Wek, R.C., Jiang, H.Y., and Anthony, T.G. (2006). Coping with stress: eIF2 kinases and translational control. *Biochem Soc Trans* 34, 7-11.

Weterings, K., Schrauwen, J., Wullems, G., and Twell, D. (1995). Functional dissection of the promoter of the pollen-specific gene NTP303 reveals a novel pollen-specific, and conserved cis-regulatory element. *Plant J* 8, 55-63.

Wiese, A., Elzinga, N., Wobbes, B., and Smeekeens, S. (2004). A conserved upstream open reading frame mediates sucrose-induced repression of translation. *Plant Cell* 16, 1717-1729.

Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G.V., and Provart, N.J. (2007). An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. *PLoS One* 2, e718.

Wolin, S.L., and Walter, P. (1988). Ribosome pausing and stacking during translation of a eukaryotic mRNA. *EMBO J* 7, 3559-3569.

Xiao, H., Xu, L.H., Yamada, Y., and Liu, D.X. (2008). Coronavirus spike protein inhibits host cell translation by interaction with eIF3f. *PLoS One* 3, e1494.

Yahalom, A., Kim, T.H., Roy, B., Singer, R., von Arnim, A.G., and Chamovitz, D.A. (2008). Arabidopsis eIF3e is regulated by the COP9 signalosome and has an impact on development and protein translation. *Plant J* 53, 300-311.

Yaman, I., Fernandez, J., Liu, H., Caprara, M., Komar, A.A., Koromilas, A.E., Zhou, L., Snider, M.D., Scheuner, D., Kaufman, R.J., *et al.* (2003). The zipper model of translational control: a small upstream ORF is the switch that controls structural remodeling of an mRNA leader. *Cell* 113, 519-531.

Yao, Y., Ling, Q., Wang, H., and Huang, H. (2008). Ribosomal proteins promote leaf adaxial identity. *Development* 135, 1325-1334.

Zhang, J., and Maquat, L.E. (1997). Evidence that translation reinitiation abrogates nonsense-mediated mRNA decay in mammalian cells. *EMBO J* 16, 826-833.

Zhang, L., Pan, X., and Hershey, J.W. (2007). Individual overexpression of five subunits of human translation initiation factor eIF3 promotes malignant transformation of immortal fibroblast cells. *J Biol Chem* 282, 5790-5800.

Zhang, L., Smit-McBride, Z., Pan, X., Rheinhardt, J., and Hershey, J.W. (2008a). An oncogenic role for the phosphorylated h-subunit of human translation initiation factor eIF3. *J Biol Chem* 283, 24047-24060.

Zhang, Y., Dickinson, J.R., Paul, M.J., and Halford, N.G. (2003). Molecular cloning of an arabidopsis homologue of GCN2, a protein kinase involved in co-ordinated response to amino acid starvation. *Planta* 217, 668-675.

Zhang, Y., Wang, Y., Kanyuka, K., Parry, M.A., Powers, S.J., and Halford, N.G. (2008b). GCN2-dependent phosphorylation of eukaryotic translation initiation factor-2alpha in Arabidopsis. *J Exp Bot* 59, 3131-3141.

Zhou, C., Arslan, F., Wee, S., Krishnan, S., Ivanov, A.R., Oliva, A., Leatherwood, J., and Wolf, D.A. (2005). PCI proteins eIF3e and eIF3m define distinct translation initiation factor 3 complexes. *BMC Biol* 3, 14.

Zhou, M., Sandercock, A.M., Fraser, C.S., Ridlova, G., Stephens, E., Schenauer, M.R., Yokoi-Fong, T., Barsky, D., Leary, J.A., Hershey, J.W., *et al.* (2008). Mass spectrometry reveals modularity and a complete subunit interaction map of the eukaryotic translation factor eIF3. *Proc Natl Acad Sci U S A* 105, 18139-18144.

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L., and Gruissem, W. (2004).  
GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. *Plant Physiol*  
*136*, 2621-2632.

## **Vita**

Bijoyita Roy was born in Calcutta, India. After graduating from the University of Calcutta with a Masters degree, she began graduate school at the University of Tennessee in 2004. She was employed as a GTA (graduate teaching assistant) at the University of Tennessee from August 2004 to May 2010. She received her Ph.D. in 2010.