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I am submitting herewith a dissertation written by Fujun Zhou entitled *"The Role of a Translation Initiation Factor Subunit, eIF3h, in Arabidopsis Shoot Apical Meristem Maintenance and Auxin Response."* I have examined the final electronic copy of this dissertation 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 Life Sciences.

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The Role of a Translation Initiation Factor Subunit, eIF3h, in Arabidopsis Shoot Apical Meristem Maintenance and Auxin Response

A Dissertation Presented for

The Doctor of Philosophy Degree

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Fujun Zhou

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Dedication

This dissertation is dedicated to my wife, Tingfen Yan, my son, Gene Zhou and my daughter, Maggie Zhou, for their supports on my study and research.

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Finaly I would like to thank my wife, Tingfen Yan for always supporting me on my study and research.

Abstract

The objective of this study was to dissect the function of the h subunit of translation initiation factor 3, eIF3h, in Arabidopsis shoot apical meristem (SAM) maintenance and auxin response. Translational regulation has been proposed to play important roles in plant development and environmental stress responses. eIF3, with 12-13 protein subunits the largest of the initiation factor complexes, is involved in many essential steps in translation initiation, but little is known about the structural and functional roles of most eIF3 subunits in any organism. Using various techniques, such as scanning electron microscopy and confocal microscopy, I revealed defects in the *eif3h* mutant that included an enlarged SAM and a pin-formed shoot and a variety of defects in leaf initiation and morphogenesis. Many groups of genes, such as those encoding auxin response factors (*ARFs*) and stem cell regulators (*CLAVATA1* and 3) were identified to have multiple upstream open reading frames (uORFs), which are generally inhibitory for the translation of downstream open reading frames and involved in translational control. Optimized translation assays indicated that *ARFs* and *CLAVATA* (*CLV1, 3*) genes were significantly less translated in the *eif3h* mutant, and removal of uORFs from *CLV1* and *CLV3* leaders significantly reduced their eIF3h dependence. Furthermore, the *eif3h* growth defects can be partially complemented by expressing the *CLAVATA3* gene in its native domain. These results indicated that, eIF3h is involved in efficient translation of *ARFs* and *CLV1, 3* genes by overcoming the translation repression by uORFs. The functional characterization of eIF3h provided the first evidence that translational control by eIF3h plays important roles in auxin signaling and shoot apical meristem maintenance. As part of this project I explored the utility of a viral

sequence element described as an internal ribosome entry site. Even though no robust IRES activity could be confirmed under the experimental conditions used here, the sequence had substantial promoter activity. Because the promoter activity was de factor eIF3h independent, the sequence could be utilized a component of a new generation of translational reporter genes.

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Chapter 1: Introduction

1.1 Translation initiation and translation initiation factors

The process of protein synthesis in living cells includes 3 major steps, translation initiation, elongation, and termination. Translation initiation is the rate-limiting step in protein synthesis (reviewed by Gingras et al., 1999; Gallie, 2002 and Sonenberg and Hinnebusch; 2009). The translation initiation for most eukaryotic mRNAs usually requires a cap structure in the 5' UTR, where cap-binding proteins bind and assist the loading of the translation pre-initiation complex. In contrast, many eukaryotic RNA viruses adopt a cap-independent translation initiation strategy, in which the small ribosomal subunit is loaded onto mRNAs through an internal ribosome entry sequence (IRES). IRESs are mRNA sequences that can attract a small ribosomal subunit, usually by virtue of their mRNA secondary structure, to initiate cap-independent translation initiation. While cap-dependent translation initiation requires multiple protein translation initiation factors, IRESs are less dependent on translation initiation factors than cap-dependent translation, in that eIF4E and sometimes other eIFs are dispensable for IRES activity (Pisarev et al., 2005).

1.1.1 Cap-dependent translation initiation and initiation factors

Many groups of proteins or protein complexes participate in eukaryotic translation initiation, playing structural, enzymatic, or regulatory roles **(Figure 1-1)** (reviewed by Pestova et al., 2007). eIF3, the largest and most complicated initiation factor complex, is involved in several steps in translation initiation. It binds to the 40S small ribosomal subunit, disassociating the small and large subunits and preventing their premature

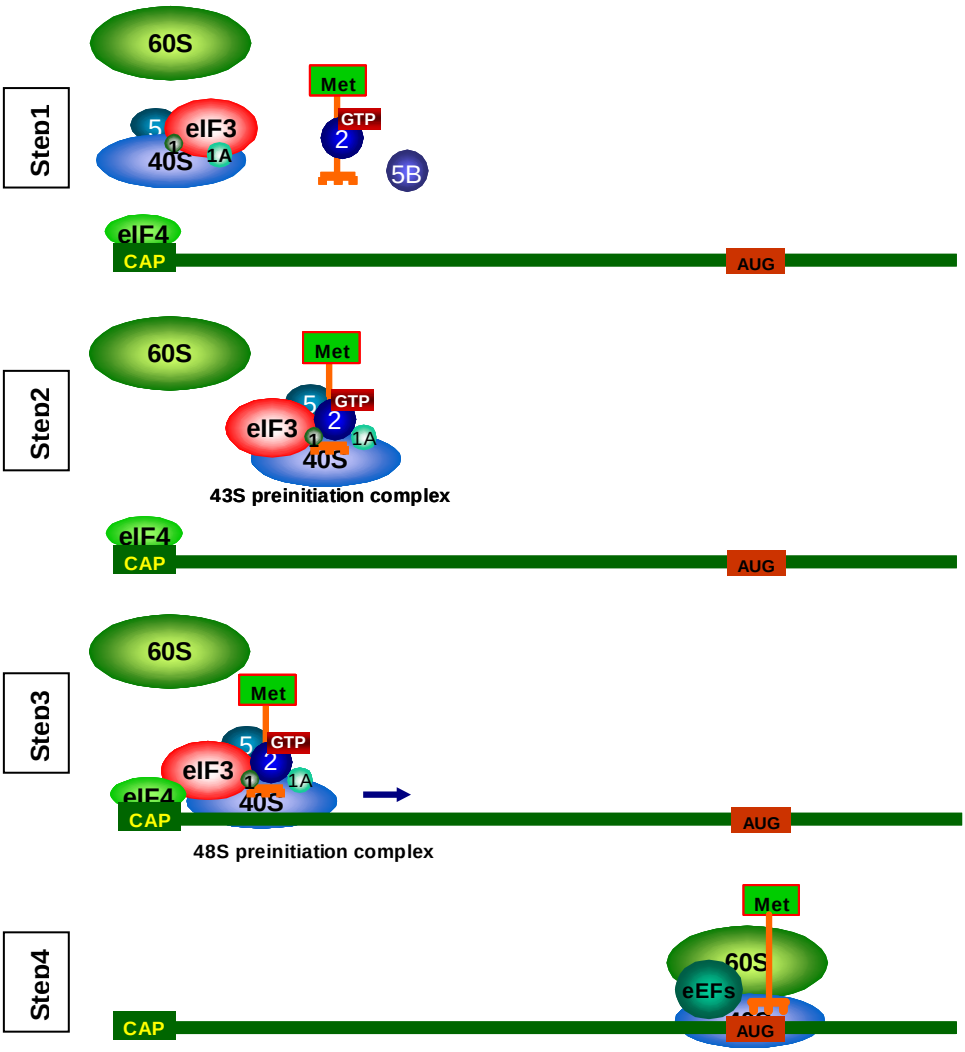

Figure1-1 The steps in cap-dependent translation initiation. Step1. The translation initiation factor eIF3 attaches to the small (40S) ribosomal subunit to block the interaction between the small and large subunits. eIF3 binds as part of a multifactor complex comprising eIF1, 1A and 5. Step2. The interaction between eIF3 and eIF2 helps the small ribosomal subunit to capture a methionine initiation tRNA and further form a 43S preinitiation complex. Step3. The 43S preinitiation complex attaches to the 5' cap of the mRNA based on the interaction between eIF3 and the cap-binding complex eIF4, and starts scanning downstream to find start codons. Step4. Once a start codon is recognized, the ribosomal large subunit and elongation factors join the complex to start translation elongation.

association; assisting the ternary complex (TC, formed by eIF2, the initiator methionine tRNA and GTP) in associating with the 40S subunit to form a 43S preinitiation complex; and promoting 48S initiation complex formation by loading the 43S complex onto the mRNA's 5' UTR (Untranslated Region) (Hershey et al., 2000; Kolupaeva et al., 2005; Hinnebusch, 2006). eIF3 also affects mRNA scanning and start codon recognition (Valásek et al., 2004). Most recently, eIF3 was proposed to have a principal role in dissociating the post-termination ribosomes into their 40S and 60S subunits and thus to facilitate ribosome recycling (Pisarev et al., 2007). Furthermore, eIF3 may act as a scaffold for the binding of mammalian target of rapamycin (mTOR), ribosomal S6 kinase (S6K) and eukaryotic initiation factor 4E, translational regulators that respond to nutrients, energy sufficiency and many other internal or external stimuli (Holz et al., 2005, Harris et al., 2006). The *A. thaliana* eIF3 protein complex consists of 12 or 13 subunits, all of which resemble mammalian eIF3 subunits (Burks et al., 2001; Unbehaun et al., 2004). Budding yeast has only 6 eIF3 subunits and 5 of them are conserved among plants and mammals. Those 5 conserved subunits are defined as "core" subunits. However, reconstitution of mammalian eIF3 from purified subunits indicates that 3 conserved subunits (eIF3a, b and c) and 3 non-conserved subunits (eIF3e, f and h) comprise the functional eIF3 core (Masutani et al., 2007).

The structural and functional roles of each individual eIF3 subunit are not well characterized (only one of the smallest subunits, eIF3k, has been crystallized, Wei et al., 2004), but Siridechadilok and coworker observed the entire eIF3 complex conformation using cryo–electron microscopy. eIF3 appears as a five-lobed particle with two arms, two legs and a head. They also proposed models for how eIF3 might interact with the complex of eIF4G-mRNA-40S ribosome subunit to direct cap-dependent translation

initiation, and with the complex of IRES-eIF4G-40S for cap-independent translation initiation **(Figure 1-2)** (Siridechadilok et al., 2005).

1.1.2 Cap-independent translation initiation

Other than cap-dependent translation initiation, there is an alternative cap-independent initiation pathway, mostly adopted by eukaryotic viruses. In the cap-independent initiation, instead of initiating translation at the mRNA 5' cap, an internal initiation can be achieved through an internal ribosome entry sequence (IRES). An IRES is a secondary structure of mRNA that can recruit the ribosome to start translation from a downstream start codon, bypassing 40S ribosomal scanning from the 5' end. The strategies adopted by different viral IRESs are diverse, especially in terms of factor requirements. Many IRESs, such as encephalomyocarditis virus (EMCV) and hepatitis A virus (HAV) IRESs require groups of initiation factors to achieve ribosomal protein binding (Lomakin et al., 2000; Borman et al., 1997), while others, such as cricket paralysis virus (CrPV) IRES and hepatitis C virus (HCV) can achieve the ribosomal protein loading without the help from initiation factors (Jan et al., 2002, Spahn et al., 2004, Lancaster et al., 2006).

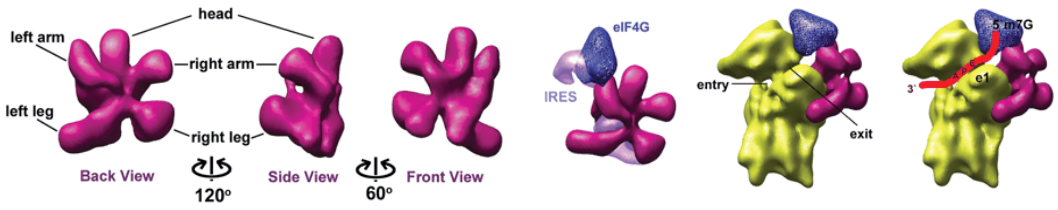

Figure 1-2 The five-lobed particle structure of the eIF3 complex observed using EM-microscopy (left) and models for its interaction with internal ribosome entry sequences (IRES) the 40S ribosome and eIF4G (right). (Siridechadilok et al., 2005)

Compared to animal viruses, very few plant virus IRESs have been identified and characterized. Among them are the tobacco etch virus (TEV) 5' leader sequence (TL) and an IRES identified in the crucifer infecting tobamovirus (crTMV). The 5’ leader of TEV has been observed to direct efficient translation from naturally uncapped viral mRNA in a manner dependent on eukaryotic initiation factor eIF4G (Gallie, 2001). The 148-nt sequence upstream of the 3′-proximal coat protein (CP) gene from crTMV contains an IRES described to promote efficient downstream translation through internal initiation (Ivanov et al., 1997).

1.1.3 eIF3h, a subunit of the eIF3 complex, is involved in efficient translation of mRNAs with multiple uORFs

As the largest complex among the initiation factors, eIF3 has drawn tremendous attention from researchers. The entire eIF3 complex conformation was observed using cryo–electron microscopy as a five-lobed particle with two arms, two legs and a head (Siridechadilok et al., 2005). However, the position of each subunit in the complex is mostly unknown. Due to genetic redundancy and complex complexity, the functions and structures for most individual subunits in any organism are still not characterized (only one of the smallest subunits, eIF3k, has been crystallized (Wei et al., 2004).

In our lab’s previous research, two *Arabidopsis* mutant lines harboring insertions in the eIF3h gene were confirmed. The mutant plants show pleiotropic growth defects, such as growth retardation and less developed roots. Translation profiling shows that the general translation in the *eif3h* mutant was not affected. Instead, specific mRNAs, such as *AtbZip11* (a leucine zipper (bZIP) type transcription factor) and *LHY* (*LATE ELONGATED HYPOCOTYL*, a component of the central oscillator of the circadian clock)

were less translated in the *eif3h* mutant. Further observations revealed that the mRNAs less translated in the *eif3h* mutant have a common feature, for they all harbor multiple upstream open reading frames (uORFs). Therefore eIF3h has been proposed to involve in efficient translation of mRNAs with multiple uORF (Kim et al., 2004), a notion that received further confirmation by a microarray comparison of polysome loading of mRNAs between the *eif3h* mutant and wild type. Compared to wild type, in the *eif3h* mutant, the mRNAs with multiple uORFs tend to be less abundant in the polysome fraction, indicating their low translation efficiencies (Kim et al., 2007).

eIF3h is absent in budding yeast, and was therefore not defined as a "conserved" core subunit. However, eIF3h forms part of the functional core of mammalian eIF3, based on in vitro reconstitution performed by Masutani and coworkers (2007), indicating that it might be a functional core subunit. In mammalian cells, eIF3h is overexpressed in many cancer cells, which enhances the protein synthesis rate and affects the growth characteristics of various cell types. Furthermore, the phosphorylation of eIF3h at Ser^{183} enhances its oncogenic activity (Zhang et al., 2008). *Arabidopsis* eIF3h shares about 40% sequence identity with human eIF3h, and shares even lower sequence identities with *Drosophila melanogaster* and *Caenorhabditis elegans* (about 36% and 26%, respectively) **(Table 1-1)**. An alignment for many eIF3h sequences (each from *Rattus norvegicus*, *Mus musculus*, human, *Arabidopsis thaliana*, *Medicago truncatula* and rice (*japonica cultivar-group))* indicates that 115 of a total of 337 residues in *Arabidopsis* eIF3h protein are identical in all 5 species (34% sequence identity) **(Figure 1-3)**.

The alignment showed higher sequence identity (about 45%) in the MPN (for Mpr1p and Pad1p Nterminal) domain of eIF3h sequences, which is located in the amino-terminal and conserved among eIF3h and eIF3f and many subunits in the COP9 signalosome (CSN) and 26S proteasome lid. The function of the MPN domain is not

Table1-1 The sequence identities between *A. thaliana* and other eIF3h proteins

Name	Sequence identity	Name	Sequence identity
O. sativa	0.80	*A. fumigatus*	0.39
R. norvegicus	0.40	*N. crassa*	0.36
M. musculus	0.39	*D. melanogaster*	0.36
H. sapiens	0.40	*C. elegans*	0.26
B. taurus	0.40	*T. annulata*	0.31
X. tropicalis	0.40	*C. albicans*	0.27
D. rerio	0.41	*C. parvum*	0.27

clear, but the two CSN subunits with MPN domain, CSN5 and CSN6 were proposed to have signalosome assembly activities and interact with Cullin3-Based E3 Ligases (Gusmaroli et al., 2007).

1.2 Translational control

In order to survive in different environments, gene expression must be tightly regulated in plants and other organisms alike. Plants face even more challenges than others, because they are immobile and thus unable to move themselves into favorable growth conditions. Eukaryotic cells regulate their gene expression at different levels, such as transcriptional regulation, mRNA degradation (siRNAs and miRNAs), translational control, and protein turnover. Among them, translational control is the least studied in almost all organisms, and only very limited information is available for the mechanisms of translational control in plant kingdom

Translational control is believed to be able to provide a more rapid response than transcriptional control, because it can bypass the processes of mRNA synthesis, processing and transport (Gingras et al., 1999). In eukaryotic cells, internal and external

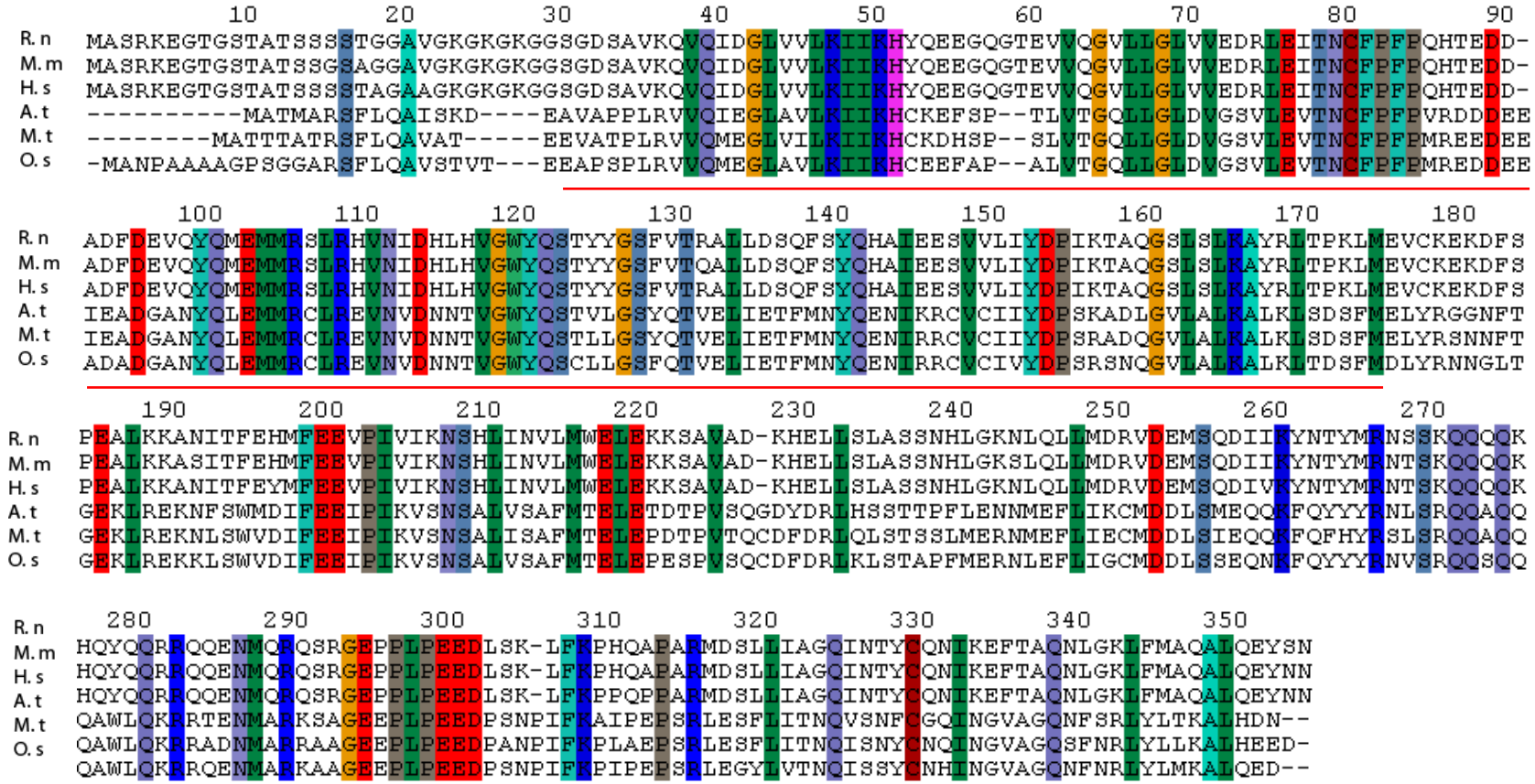

Figure 1-3, A protein sequence alignments for eIF3h protein sequences from following plants or mammals 1) *Rattus norvegicus* (R. n) 2) *Mus musculus* (M. m) 3) *Homo sapiens* (H. s), 4) *Arabidopsis thaliana* (A. t) 5), *Medicago truncatula* (M. t) 6) *Oryza sativa Japonica cultivar-group* (O. s), In aligned consensus columns' background are colored. The sequence of the MPN domain is underlined in red.

signals, such as nutrient availability and hormones, can regulate the translation efficiency of specific mRNAs. Translational control is generally achieved through the interaction of mRNA with specific proteins, although direct regulation of mRNA by a metabolite through a riboswitch has also been described (Wachter et al., 2007).

1.2.1 Cis-elements in translational control

During translation initiation according to the ribosome scanning model, the preinitiation complex formed by small ribosomal subunit, initiation tRNA and groups of initiation factors attach to the 5' cap, and scan downstream to initiate translation at the first recognizable start codon. After translation of the main open reading frame, the 80S ribosome separates and the peptide is released. A closed loop mRNA structure is formed based on the interaction between the poly-A binding protein and the cap binding protein **(Figure 1-4)**. This drives the two ends of the mRNA into close proximity, which not only significantly increases the posttranslational ribosomal recycling (Sonenberg and

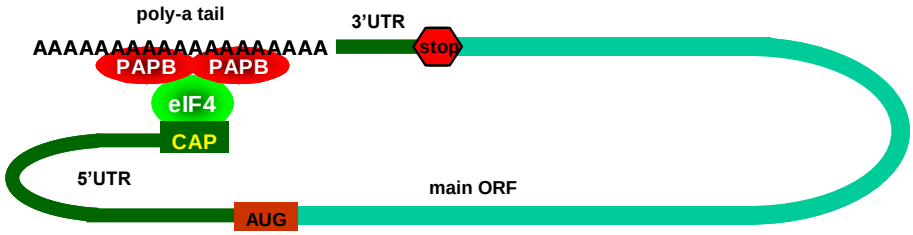

Figure 1-4 3' to 5' circularization of eukaryotic mRNA. The structure of a mature eukaryotic mRNA consists of a 5'UTR, main open reading frame, and a 3'UTR. The 5' UTR is protected by a cap structure, which is further attached by cap-binding proteins. The 3' UTR is extended by a poly-A tail, which is protected from ribonuclease cleavage by poly-A binding protein (PABP). The interaction between the cap binding protein and PAPB helps the mRNA to form a closed loop structure, which not only increases the mRNA stability but also facilitates post-translational ribosomal recycling.

Hinnebusch, 2009), but also makes the 3' UTR an important component in translational control. While structure and sequence elements in the 5' UTR contribute to translation initiation by controlling the preinitiation complex loading, scanning, and start codon recognition, the elements in the 3'UTR also control translation efficiency by translational repression or activation.

Many cis-elements have been observed to be involved in the translational control, such as MicroRNA (miRNA) biding site for translation repression (Chen, 2004; Gandikota et all. 2007), cytoplasmic polyadenytion element (CPE) in the 3' UTR for translation activation of specific mRNAs in spatial and temporal manner (Mendez et al., 2000; Pique et al., 2008) and iron regulatory protein (IRP) binding site (IRE, iron-responsive element) for regulating specific genes important for iron metabolism (Hentze et al., 1987; Casey et al., 1988). However, most characterized cis-elements are the sequence and structural elements in the 5' leader of mRNAs.

The upstream open reading frames in mRNA leaders

Various sequence and structural elements in the 5'UTR have been observed to affect translation efficiency. Upstream open reading frame (uORFs) are the most studied elements contributing to translation efficiency and translational control (Miller and Hinnebusch 1990; Watatani et al., 2008). An uORF in a mRNA leader is formed by the sequence between an upstream AUG (uAUG) and a following in-frame stop codon. The uORFs are either translated or bypassed by leaky scanning machineries. In the case of translation initiation by ribosomal scanning, if a uORF is translated, the downstream translation initiation will depend on whether the small ribosomal subunit resumes scanning after the upstream translation event (reviewed by Kozak 1999). Because resumption of scanning is not a given, uORFs generally repress downstream translation by reducing the available pool of scanning preinitiation complex on an mRNA.

A well-studied example for translational control by uORFs is the *GCN4* model system **(Figure 1-5)** (reviewed by Hinnebusch, 2005). The translation of *GCN4*, a transcription factor regulating more than 30 genes in amino acid synthesis is regulated by 4 uORFs in the 5' leader sequence. In response to amino acid availability and other regulators, the translation of *GCN4* mRNA can be turned on or off.

Base-paired secondary structures in the 5'UTR

It is believed that base-paired secondary structures near the cap are inhibitory for translation by blocking the ribosome entry (reviewed by Kozak, 2005; Pelletier and Sonenberg, 1987.). Although the helicase activity in the peinitiation complex can disrupt secondary structures, a long and structured 5' UTR can greatly reduce translation efficiency. In response to internal developmental or external environmental signals, cells may release translational repression by alternative splicing or activating downstream promoters (Kozak, 2005).

1.2.2 Trans-acting factors in translational control

As **figure 1-2** shows, during cap-dependent translation initiation, various proteins are involved in each step of initiation from preinitiation complex formation, mRNA-ribosomal association, scanning, to start codon recognition. eIF4, the cap-binding protein complex, eIF2, the protein component of the ternary complex, and eIF3, the largest complex among the initiation factors, were proposed to be important regulatory elements affecting initiation efficiency (Gingras et al., 1999; Qiu et al., 2000; Hinnebusch, 2005).

Subunits of the cap-binding protein eIF4 in translational control.

During cap dependent translation initiation, recognition of the 5' cap is a crucial step contributing to translation efficiency. The cap is initially recognized by eIF4E, a

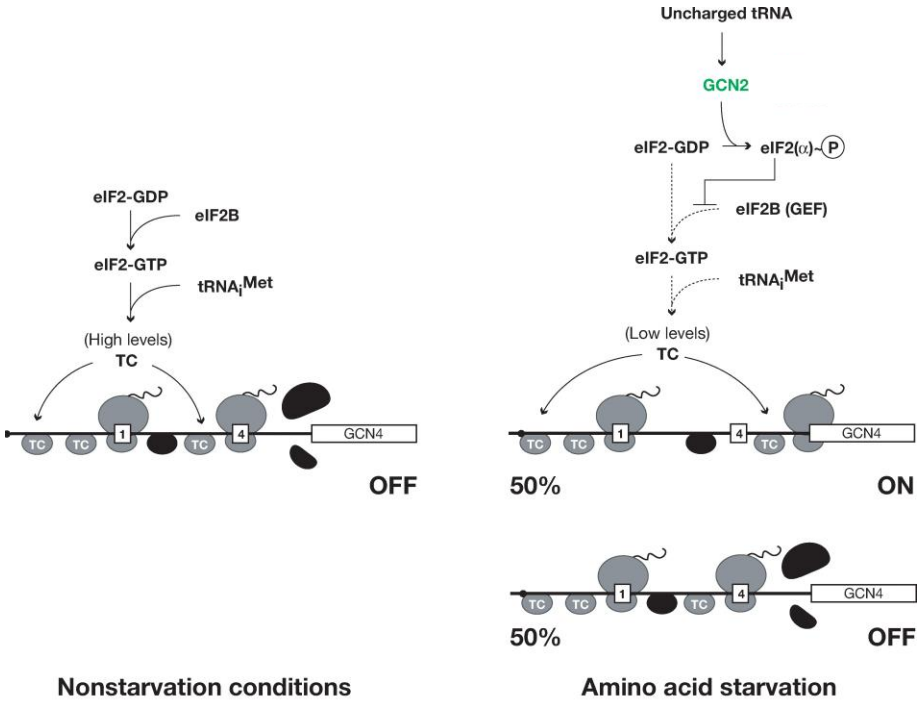

Figure 1-5 The Model for *GCN4* translational control described in Hinnebusch's review (2005). *GCN4* mRNA has 4 uORFs. Due to functional redundancy among uORF 2,3 and 4, only uORF1 and 4 are depicted in the model. After translation of uORF1, 50% of 40S ribosomal subunits remain attached to the mRNA for downstream reinitiation, initially without ternary complex (TC). The rate of TC acquisition depends on TC availability. Therefore, under nonstarved condition, TC can be rapidly acquired and reinitiation occurs at uORF4, which causes the bypassing of the translation of the main uORF. *GCN4* is repressed. However, when starvation conditions cause a drop in the level of free TC, 50% of the remaining 40S subunits fail to acquire a TC before they reach uORF4, and a subset of these will initiate at the main uORF (*GCN4*). A key regulator in this system is the starvation gene *GCN2.* GCN2 phosphorylates eIF2α to convert eIF2 from TC substrate to an eIF2B inhibitor. It in turn reduces the eIF2-GTP level in the cell.

component of the trimeric complex eIF4F, which also consists of eIF4G and eIF4A. eIF4G is a large scaffolding protein providing friendly binding surfaces to other initiation factors, such as eIF4A, eIF3 and polyA binding protein (PAPB). It is attached to the cap via eIF4E. eIF4A, a RNA helicase that associates with a cofactor, eIF4B, is responsible for unwinding the 5' proximal secondary structures, which might be inhibitory for preinitiation complex loading (Gingras et al., 1999). Phosphorylation of eIF4B by ribosomal protein S6 kinase (S6K) in response to insulin stimulation suggests its role in translational control (Shahbazian et al., 2006).

Compared to other initiation factors, the amount of eIF4E in the cell is relatively low. Therefore the availability of eIF4E is a key element in global translation efficiency. eIF4E-binding protein (4E-BP) can compete with eIF4G for binding to eIF4E and thus inhibit translation. Phosphorylation of 4E-BP triggers the dissociation of eIF4E and 4E-BP, thus permitting binding of eIF4G and efficient translation (reviewed by Sonenberg, 2008). However, in plants, the presence of eIF4E-BPs as well as the notion of global translational repression by controlling levels of eIF4E remains to be confirmed.

eIF3 subunits in translational control.

As the largest protein complex among the initiation factors, eIF3 consists of 13 subunits in human and at least 12 subunits in *Arabidopsis*. It is involved in almost all steps in translation initiation, from 43S preinitiation complex formation, association of the preinitiation complex with mRNA to form the 48S complex, to scanning and start codon recognition. Aberrant expression of specific eIF3 subunits, such as 3h, 3e and 3f has been observed in mammalian cancer cells. Moreover, manipulating the expression of many subunits also induces malignant cell transition (Zhang et al., 2007; 2008; Mack et al., 2007; Doldan et al., 2008), indicating the important roles of eIF3 subunits in controlling translation efficiencies and cell fate control. Individual eIF3 subunits, such as

eIF3a and eIF3h were observed to be essential for efficient translation of mRNAs with uORFs (Kim et al., 2004; Kim et al., 2007; Szamecz et al., 2008)

mTOR dependent translational control in mammalian cells.

Mammalian cells have adopted sophisticated pathways for translational control through a nutritional and energy sensor, the mammalian target of rapamycin (mTOR). In response to energy sufficiency, a high amino acid level, or hormone stimulation, mTOR and its cofactor, raptor regulate the phosphorylation of 4E-BP and other proteins, such as S6K to boost general translation. It is believed that eIF3 is the scaffolding protein complex for mTOR dependent translational control (Holz et al., 2005). The interaction between eIF3 and mTOR also enhances the association between eIF3 and eIF4G (Harris et al., 2006). Once mTOR is recruited onto eIF3, it phosphorylates 4E-BP and S6K1, which triggers the release of 4E-BP from eIF4E and S6K1 from the eIF3. Release of 4E-BP, frees the access for eIF4G to eIF4E, and increases the formation of translationally active eIF4F complex (Holz et al., 2005). The phosphorylation of S6K1 activates its kinase activity for the phosphorylation of ribosomal subunit S6 and eIF4B, the cofactor of eIF4A. Phosphorylated eIF4B has a higher affinity eIF3 complex, and associates with eIF4A for enhancing its RNA helicase activity **(Figure 1-6)**. To summarize, mTOR is an important checkpoint for general translational control in the cell. It regulates translation by targeting multiple components in the initiation pathway.

1.2.3 Translational control in plants

Compared to animals and yeast, plans have very different developmental characteristics, such as their autotrophic metabolism, enhanced cell rigidity, high plasticity of organ shape and size in response to environmental factors, and reduced

mobility. Although the factors involved in general translation are similar between plants and animals, plants may have significant differences in translational control machinery (Gallie, 2007).

Plant translation initiation factors

The components of plant and animal translation initiation factors are generally similar, but different at many specific factors. The plant not only has all subunits for the cap binding protein eIF4, but also has an additional isoform, which consists of eIFiso4G, and eIFiso4E. Plant eIF4 also has only a weak association with eIF4A. eIF4F and eIFiso4F may have functionally diverged in translation initiation, in that eIFiso4F prefers unstructured mRNA, while eIF4F also promotes translation from structured 5' leaders, uncapped mRNAs or mRNAs contain multiple cistrons (Metz and Browning, 1996; Gallie and Browning 2001; Gallie, 2007). The subunit composition of the eIF3 complex is similar between plant and animals, however the j subunit, which has been proposed to be important in the recruitment of eIF3 to the 40S ribosomal subunit, is absent from plant eIF3 (Burks, 2001, Fraser et al., 2004, 2007).

***Arabidopsis has a* rapamycin-insensitive Target of Rapamycin (AtTOR), but lacks 4E-BP**

In mammalian cells, the regulation of 4E-BP phosphorylation by mTOR plays important roles in translational control, but 4E-BP or even 4E-BP like activity has not been identified in plants, although the homologs for both mTOR and its cofactor raptor were identified in the plant genome. The Arabidopsis target of rapamycin (AtTOR) kinase is expressed in embryos, endosperm, and primary meristems, but not in differentiated plant cells. Disruption of AtTOR causes premature arrest of endosperm and embryo development, suggesting its role in early development (Menand et al.,

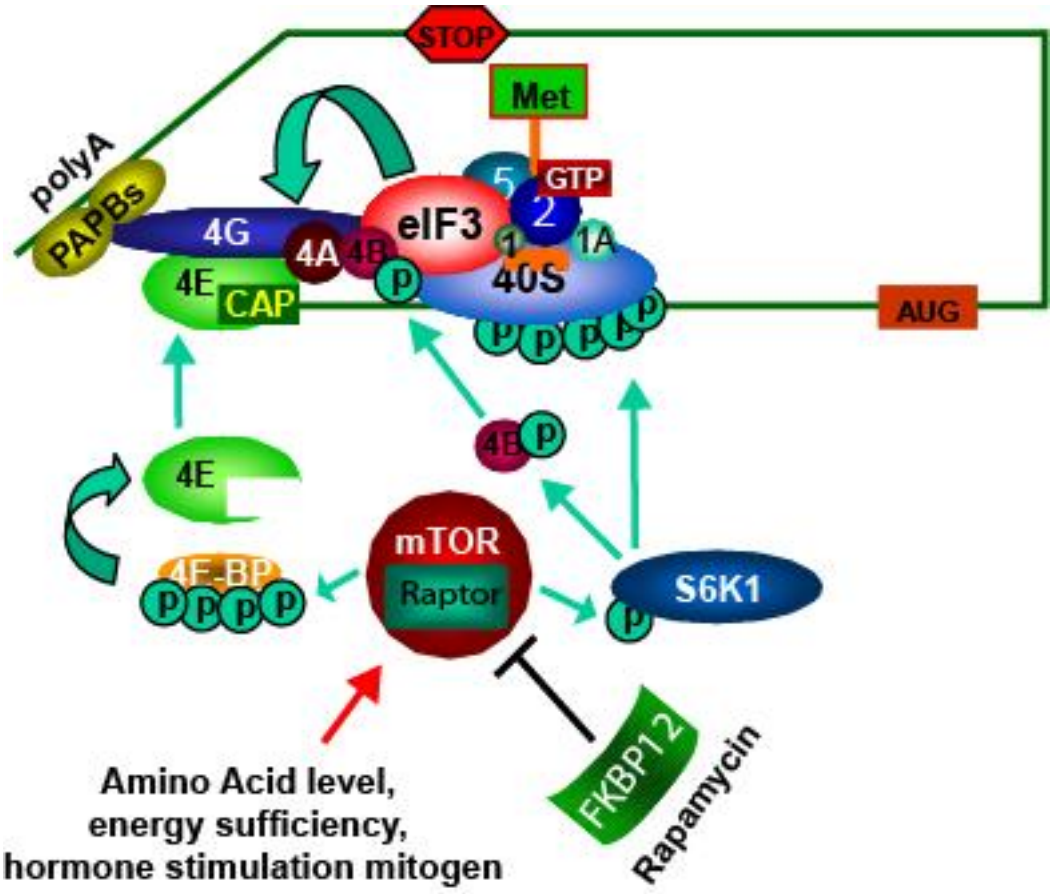

Figure 1-6 Mammalian target of rapamycin (mTOR) dependent translational control. As a nutritional and energy sensor, mTOR phosphorylates groups of proteins such as 4E-BP and S6K1 in response internal and external signals. The phosphorylation of 4E-BP control the eIF4E pull for forming translational active trimeric eIF4F complex. Phosphorylated S6K1 is the active form for eIF4B, and ribosomal subunit S6 phosphorylation. The mTOR is sensitive to the repression by FKBP12/rapamycin complex. (Holz et al. 2005; Harris et al. 2006; reviewed by Hinnebusch, 2006)

2002). AtTOR was also observed to participate in a S6K dependent stress response, and proposed to be involved in translational control (Mahfouz et al., 2006; Deprost et al.,2007). The Arabidopsis seedling is not sensitive to rapamycin, and the mechanism of TOR dependent translational control in plants is not characterized.

Plant proteins involved in efficient translation of mRNAs with multiple uORFs

UORFs are expected to repress translation of the main open reading frame downstream. In order to translate the main open reading frame efficiently, the translation machinery has to overcome the repression from the uORFs through leaky scanning or by enhancing translation reinitiation efficiency. Although the mechanism for the translation of poly cistronic mRNAs is not fully characterized, in plant many proteins are involved.

The h subunit of *Arabidopsis* eIF3, eIF3h, is involved in efficient translation of mRNAs with multiple uORFs, such as those of *AtbZip11* and *LHY*. The translation efficiency of the AtbZip11 leader is significantly reduced in the *eif3h* mutant, while removal of uORFs from its leader sequence by site directed mutagenesis reduced its eIF3h dependence (Kim et al., 2004; Kim et al., 2007). Interestingly, many proteins of the large ribosomal subunit have also been implicated in translational regulation of specific mRNAs (Nishimura et al., 2005; Yao et al., 2006; Pinon et al., 2006). Among them, RPL24/STV promotes translation of two uORF containing mRNAs, encoding auxin response factors ARF3/ETTIN and ARF5/MONOPTEROS (Nishimura et al., 2005). *Arabidopsis thaliana ACAULIS 5* (*ACL5*) a spermine synthase, was proposed to play roles in translation activation of another uORF containing mRNA, *SAC51*, which encodes a bHLH-type transcription factor, because the dwarf phenotype of the *acl5* mutant can be complemented by a mutation that disrupts a uORF in the *SAC51* untranslated leader (Imai et al., 2006).

In summary, plant has similar translation initiation factors to animals, but it may adopt different translational regulation machineries. Although mTOR dependent translational control plays essential roles in animal development and stress response, its role in plant cells might be different, since plant is not sensitive to rapamycin and does not have a homologue for 4E-BP or a protein with 4E-BP activity. In plant genome, about 1/3 mRNAs have uORFs, and some of them have multiple upstream open reading frames (kim et al., 2007). The machinery of the translational control of uORF harboring mRNAs is not fully characterized in plant, although many proteins have been implicated to play roles.

1.3 Stem cell maintenance in Arabidopsis

1.3.1 Shoot apical meristem (SAM) and root apical meristem (RAM)

For most animals, at least some of their organs were formed in the embryo stage, but most plant organs are developed after germination. Plant organs above ground develop from the stem cells in the shoot apical meristem (SAM), and roots develop from the stem cells in the root apical meristem (RAM). The rosette leaves are initiated during the vegetative stage, and the inflorescences and flowers develop during the reproductive stage.

The SAM of *Arabidopsis* includes a central zone (CZ) in the center of the shoot apex, which harbors the stem cell population. While some progeny of stem cell division retain the stem cell property and remain in the CZ, the others enter various differentiation pathways, to provide founder cells for leaf and flower primordia, and stems, eventually differentiating into a variety of cell and tissue types including

epidermis, cortex, mesophyll, and vasculature. (reviewed by Haecker and Laux, 2001). Within the SAM, the CZ is surrounded by a peripheral zone (PZ) for primordia initiation, and a rib zone lies underneath the CZ, providing cells for the stem. There is also a group of cells underlying the stem cells in the central zone, the organizing center (OC), which consistently provide signals for stem cell regulation and SAM maintenance **(Figure 1-7)**.

Similar to the SAM, the root apical meristem (RAM) also has an organizing center, termed the quiescent center, which provides signals for the maintenance of its surrounding stem cells **(Figure 1-7)**. The stem cells provide founder cells to central stele, ground tissue, cortex, epidermal cells, and root cap (reviewed by Laux, 2003).

1.3.2 Genes involved in SAM maintenance.

The stem cell number in the SAM is relatively constant from seed germination to plant death. The stem cell population in the SAM is tightly regulated by CLV/WUS pathway. CLAVATA3 (CLV3), a small peptide produced only in the outer two cell layers in the central zone, is the ligand for a heterodimeric receptor kinase encoded by *CLAVATA1* and *2* (*CLV1, 2*). In response to CLV3 perception, CLV1 represses the expression of *WUSCHEL* (*WUS*) in the organizing center, a homeodomain transcription factor responsible for the maintenance of stem cell identity. The expression of *CLV3* is also activated by WUS. Therefore a feedback regulation loop is formed to maintain a stable stem cell population in the SAM **(Figure 1-8)**(Fletcher et al., 1999; Brand et al., 2000; Lenhard et al., 2003; Schoof et al., 2000). Stem cell maintenance additionally requires the activity of SHOOTMERISTEMLESS (STM), which is a KNOTTED-like homeobox protein that represses the expression of organ formation genes (Long et al., 1996; Long et al., 1998; Scofield et al., 2007).

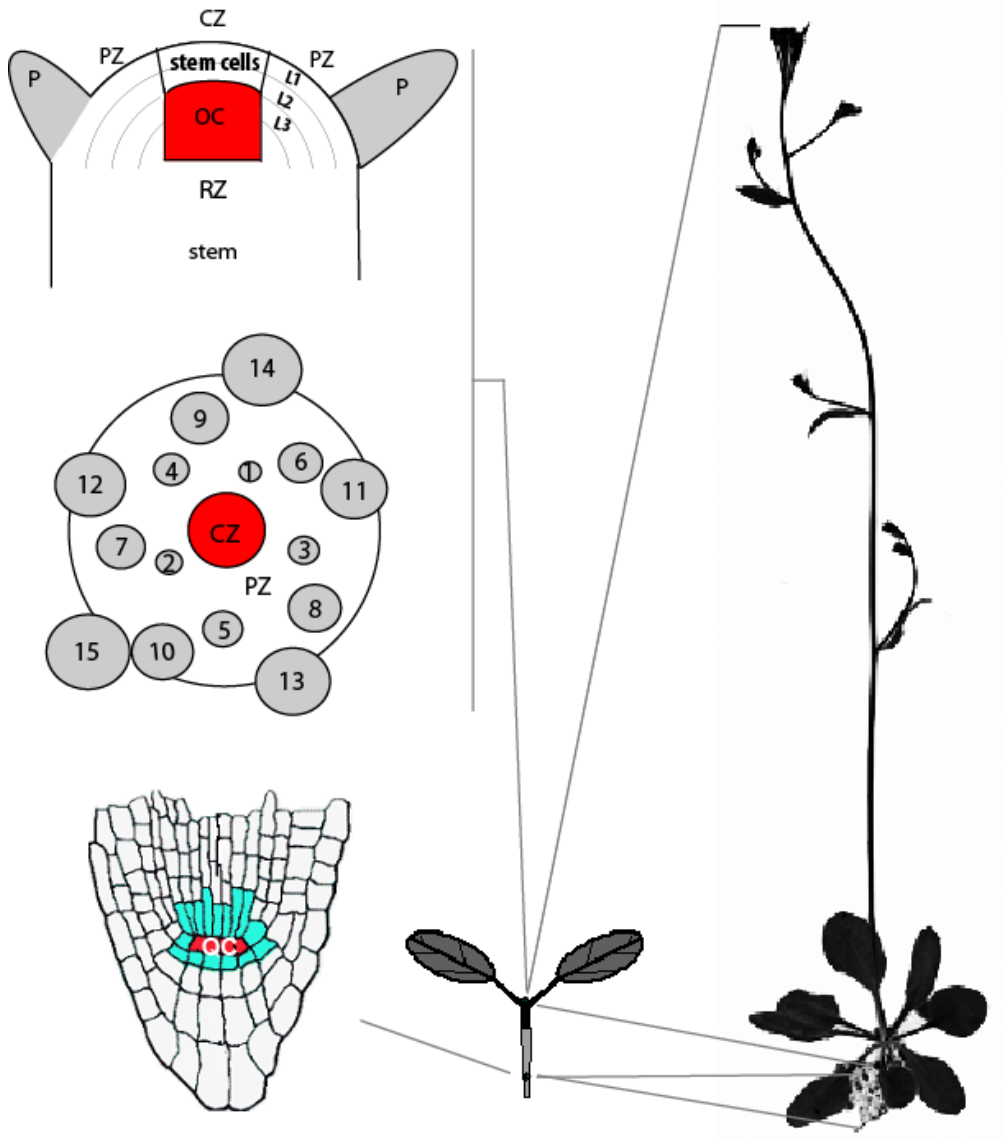

Figure 1-7. *Arabidopsis* SAM and RAM. Virtually all organs above and under ground develop from the stem cells in the SAM and RAM, respectively. The structure of the SAM includes a central zone (CZ), peripheral zone (PZ) and a rib zone (RZ). An organizing center (OC) in the L3 layer of the CZ provides signals for SAM maintenance. The primodia for lateral organs only initiate in the PZ. From the top view, the angle between two primodia is about 137.5^{o} in species such as Arabidopsis that follow a spiral phyllotaxis pattern The root Apical meristem also has an OC for RAM maintenance.

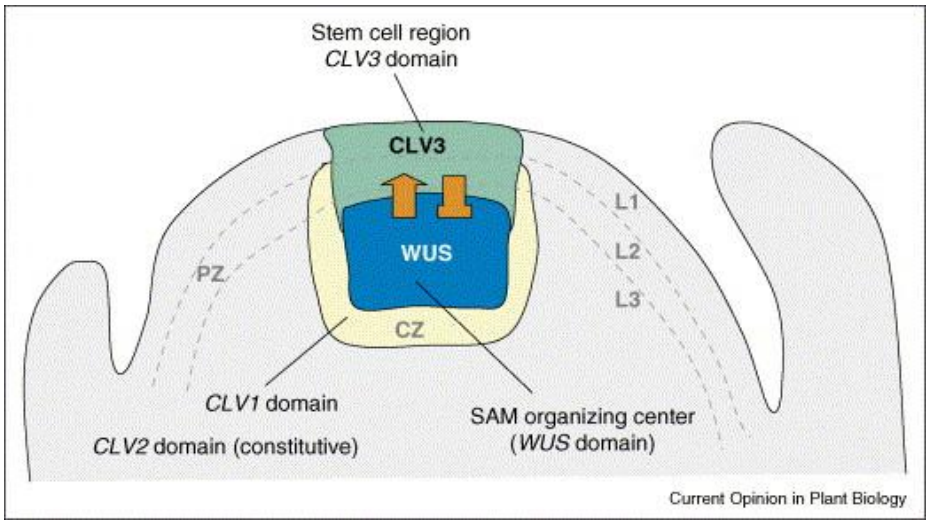

Figure 1-8 The expression domains of several crucial SAM-related genes. *CLV3* is mainly expressed in the first two cell layers in the central zone. *CLV1* and *CLV2* are expressed in the lower (L2 and L3) cell layers in the central zone. *WUS* expressed in the organization center, which is located in the *CLV1* expression domain. (Figure from Fiers et al., 2007)

CLV3 and its role in restricting WUS expression

CLV3 peptide is mainly produced in the L1 and L2 layers in the CZ of the SAM. It is responsible for restricting the number of stem cells in the central zone and involved in setting the CZ/PZ boundary. (Reddy and Meyerowitz, 2005; Fiers et al., 2007). CLV3 acts as a signal to repress *WUS* expression. WUS activates stem cell maintenance genes to promote stem cell identity and repress cell differentiation in the central zone. In the *clv3* mutant, *WUS* is overexpressed to induce an enlarged SAM. In a *wus* loss-of-function mutant the SAM terminates at an early stage **(Figure 1-9)**.

The endogenous, mature, CLV3 peptide contains 12 amino acids (RTVP^{h}SGP^{h}DPLHH; P^{h} = hydroxy-proline) was identified by in situ matrix-assisted laserdesorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) (Kondo et

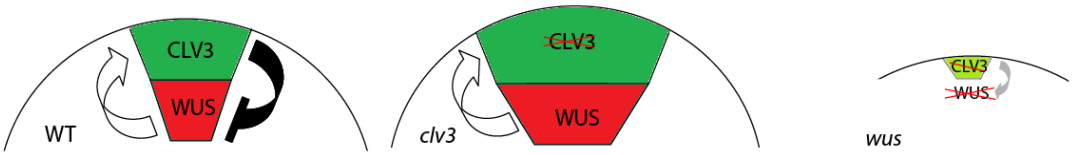

Figure 1-9 The feedback regulation loop in wild type, *clv3* and *wus* SAM. In the wild type (left), the *CLV3* expression, activated by WUS, provides a signal to restrict *WUS*. In the *clv3* mutant (middle), without the repression from *CLV3, WUS* becomes over expressed, causing an enlarged SAM. In the *wus* mutant (right), without both WUS and CLV3 activity, the SAM is terminated at an early stage.

al., 2006). It has been observed to bind to the extracellular domain of the receptor kinase CLV1 (Ogawa et al., 2008). However, the downstream pathway of *WUS* repression by the CLV3 signal is largely unknown, although two phosphatases, *POLTERGEIST* (*POL*) and POL-like1 have been proposed to be CLV1 signaling intermediates (Yu et al., 2003; Song et al., 2006).

Other CLE peptides in stem cell regulation.

The *CLE* gene family was named after the *CLV3* from *Arabidopsis* and the homologous *ESR* (*EMBRYO SURROUNDINGREGION*) from maize. The family consists of at least 31 members in Arabidopsis. They are expressed in diverse tissues and encode secreted proteins (Sharma et al., 2003). Other than the C terminal (or near C terminal) 14-amino acid CLE motif and the N terminal secretion signal (SS), the internal sequence in between is not very conserved among CLE proteins. The CLE motif of CLV3 is functionally independent from the nonconserved flanking sequence (Fiers et al., 2006), suggesting that the flanking sequence does not play roles in stem cell regulation. Most CLE genes have not been intensively characterized, but CLV3, CLE19 and CLE40 have been studied using both stable transgenic plants and synthetic peptides. The overexpression of these 3 genes in stable transgenic plants or treating plants with synthetic peptides leads to a terminal differentiation of the root meristem, indicating their

potential role in restricting root apical meristem (Casamitjana-Martinez et al., 2003; Hobe et al., 2003; Fiers et al., 2004; Fiers et al., 2005; Whitford et al., 2008). Ito and coworkers (2006) tested the effect of 26 synthetic dodecapeptides derived from all *Arabidopsis CLE* genes on xylem differentiation. The results indicate that some CLE peptides suppress xylem differentiation, whereas others promote cell differentiation. A complementation test of the *clv3-1* mutant with different *CLE* genes expressed in *CLV3* expression domain revealed that several, though not all, *CLE* genes rescued the *clv3* mutant defects to different degrees. For the *CLE* genes that rescued *clv3*, some required CLV1, but some did not, suggesting that another receptor may act redundantly with CLV1 in CLE peptide perception (Ni and Clark, 2006). In summary, the *CLE* genes in Arabidopsis may play different roles in regulating stem cell identity and cell differentiation. While some of them share functional redundancy with CLV3, others do not.

The role of STM in SAM maintenance

Another gene, *SHOOTMERISTEMLESS* (*STM*), encoding a member of the class I KNOX family homeodomain protein, also plays important role in SAM maintenance (Long et al., 1996). STM was suggested to maintain stem cell identity in the shoot apex through a WUS-independent pathway (Lenhard et al., 2002). It represses the expression of *ASSYMMETRIC LEAVES* genes (*AS1* and *AS2*) in the CZ. AS1 and AS2 promote leaf differentiation by repressing the expression of *KNAT1* and *KNAT2*, two homeobox genes responsible for promoting SAM identity **(Figure 1-10)**. Therefore, STM is required for maintaining SAM identity but not initiating the SAM (Byrne et al., 2000; Lin et al., 2003).

The roles of WUS and STM in promoting *CLV3* expression

Using inducible *WUS* and *STM* transgenic plants, Brand et al., (2002) observed the roles of WUS and STM in inducing *CLV3* expression. Although WUS expression is

required for the activation of *CLV3* gene in the embryo stage, and the induction of WUS expression in the seedling stage promotes *CLV3* expression in the shoot apex, but it is not sufficient to activate *CLV3* expression in non-meristematic tissues. Similarly, the induction of STM in Arabidopsis seedlings was also not sufficient to induce *CLV3* expression in non-meristematic tissues. However when both *WUS* and *STM* inducible genes were expressed in the seedling, *CLV3* expression can be observed throughout the leaves after induction, indicating both WUS and STM are required for activating *CLV3* expression in non-meristemic tissue.

The regulation WUS and STM expressions in the SAM

In Arabidopsis overexpression of *WUS* causes ectopic organ initiation (Xu et al., 2005), and induces somatic embryogenesis from various tissues (Zuo et al., 2002). Ectopic expression of *WUS* in the *CLV3* and *CLV1* domain induces an enlarged SAM (Brand et al., 2002; Lenhard et al., 2003). *AP3::WUS* and *AG::WUS* causes various defects on flower development, such as massive outgrowth of non-differentiated or differentiated organs and super enlarged gynoecium (Lenhard et al., 2001)

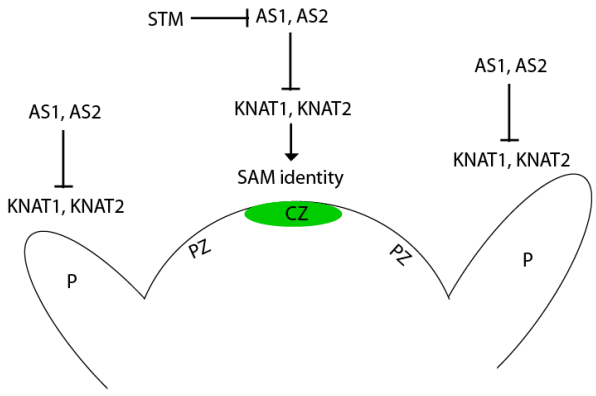

Figure 1-10 The Role of STM in maintaining SAM identity. In the SAM, STM represses the expression of *AS*1 and *AS2* to derepress the expression of *KNAT1* and *KNAT2*. In the primordium, since *AS1* and *AS2* are not repressed by STM, they are able to repress *KNAT1* and *KNAT2* expression to initiate leaf differentiation.

Figure 1-11 shows the regulation of *WUS* and *STM* in the SAM. The *WUS* expression in the OC is mostly regulated by the CLV3 signal from the stem cells through CLV1, CLV2, and possibly other receptor kinases, such as CORYNE (CRN) (Müller et al., 2008). *WUS* expression has been proposed to be regulated by other proteins, Such as transcriptional activation by SPLAYED dependent chromatin-remodeling (Kwon et al., 2005), positive regulation by a homeobox protein *STIMPY/WOX9* in the vegetative SAM (Wu et al., 2005), APETALA2 (AP2) (Würschum et al., 2005) and ZWILLE (an ARGONAUTE (AGO) family protein) (Tucker et al., 2008), and negative regulation by BARD1 (BRCA1 associated RING domain 1), and ULTRAPETALA1 (a SAND domain putative transcriptional regulator) (Han et al., 2008; Carles et al., 2005). It appears that various genes involved in the WUS expression regulation. The roles of these regulations are mostly inducing, stabilizing or restricting WUS expression in the OC, in which, WUS

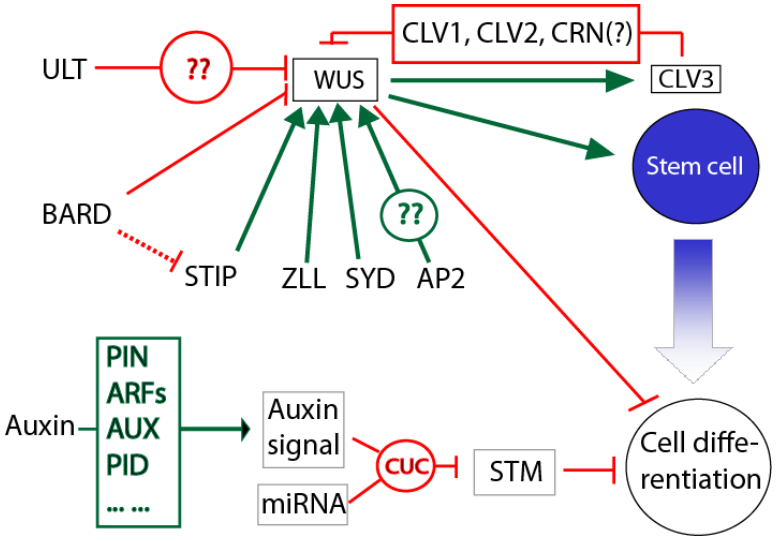

Figure 1-11. Genes regulating cell fate in the SAM. The maintenance of stem cell identity in the SAM is regulated by WUS and STM. *WUS* expression is regulated by CLV3 signal from stem cells and other factors. STM is regulated by auxin signaling and miRNA through *CUC* family genes.

expression can be further restricted by CLV signals. The expression of *STM* in the SAM is mostly regulated by auxin and microRNAs, through repression of *CUC (CUP-SHAPED COTYLEDON1*) family genes. (Aida et al.,1997, 1999, 2002; Laufs et al., 2004; Larue et al 2009).

1.3.3 The role of auxin in SAM maintenance and primordium initiation

Auxin is one of the most important plant hormones. It controls numerous aspects of plant growth and development, such as root development and organ specification. The auxin signal is perceived and processed through very complicated pathways **(Figure 1-12)**. An ubiquitin protein ligase, SCF^{TIR1}, responsible for triggering the degradation of the AUX/IAA proteins has been identified as auxin receptor (Dharmasiri et al., 2005; Kepinski et al., 2005). AUX/IAA proteins are transcriptional repressors regulating auxin response factors (ARFs), a group of transcription factors that regulate numerous auxin responsive genes. Auxin gradients across the plant are important for plant growth, primordium initiation and organ specification (Furutani et al. 2004; Schuetz et al., 2008; Rast and Simon, 2008; Reinhardt, 2005) **(Figure 1-13)**. The distribution of auxin is achieved by directional transport via auxin efflux carriers (PINs). PIN protein expression and their polar localization within the cell are regulated mostly by ARFs and PINOID protein kinases, respectively. The mutation of gene encoding an auxin efflux carrier, PIN1, causes various defects in organ generation, such as a pin-formed shoot and fused lateral organs. Similar phenotypes can also be observed upon mutation of specific ARFs (Teale et al., 2006).

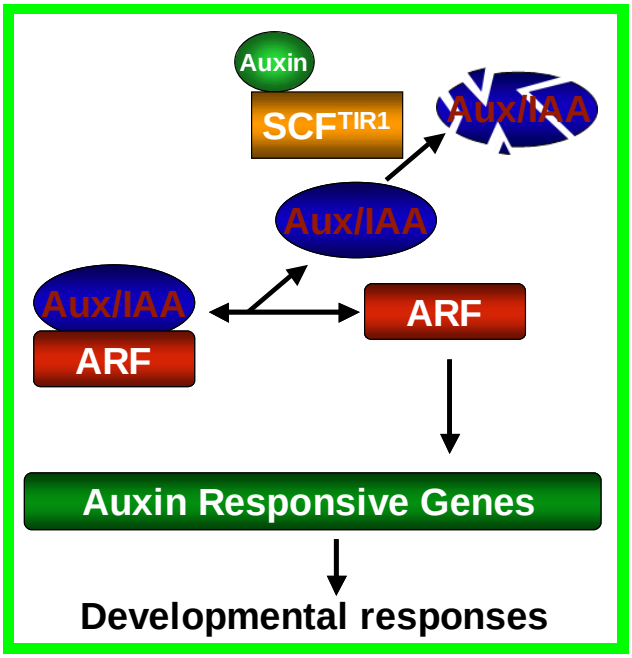

Figure 1-12. The auxin signaling in *Arabidopsis.* ARFs are transcription factors regulating a large group of auxin responsive genes. ARFs are regulated by Aux/IAAs, a group of transcription repressors. Aux/IAAs are regulated by ARFs, and a protein degradation pathway triggered by the SCF^{TIR1} E3 ubiquitin ligase, which functions as an auxin receptor (reviewed by De Smat and Jürgens, 2007; Lau et al., 208).

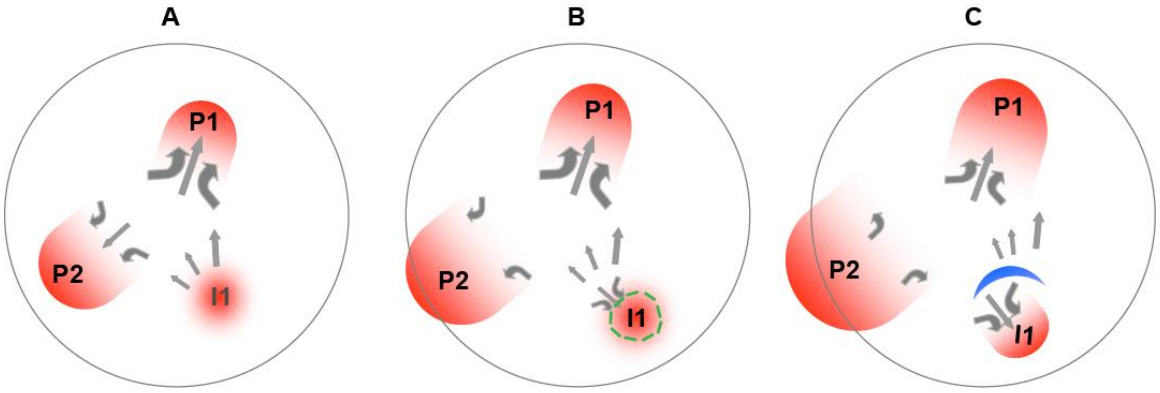

Figure 1-13 An auxin gradient mediates organ positioning and outgrowth and boundary definition. Auxin concentration is represented by red color and the direction of auxin transport indicated by gray arrows. Green bars in B represent *PIN1* activation and polar localization toward the center of I1. Blue shape in C indicates an auxin deprived zone for organ boundary determination.
(A) Newly initiated primordia (P1 and P2) act as auxin sinks to deprive auxin from the shoot apex. As a result, it establishes an auxin gradient, in which auxin is accumulated at a certain minimal distances from initiated premodia, defining a position for new premordium (I1) initiation. (B) At the incipient organ initiation site (I1) *PIN1* expression was induced and relocated to the cell side pointing the center of I1 to positively deliver auxin in to the center, which in turn triggers out growth of a primordium at the peak of the auxin gradient. (C) The sink activity of the newly initiated primordum (I1) and P1 (with reduced sink activity after I1 initiation) establishes an auxin deprived zone between them (Blue), in which *CUC* genes are expressed and *STM* is induced to define the organ boundary (reviewed by Reinhardt, 2005).

Chapter 2: Dual luciferase translation assay constructs supported by the intergenic element from the Crucifer-Infecting Tobamovirus.

This chapter is lightly revised version of a manuscript prepared by Fujun Zhou and Albrecht G von Arnim.

Dual luciferase translation assay constructs supported by the intergenic element from the Crucifer-Infecting Tobamovirus. (2009) *(Fujun Zhou[1)] and Albrecht G von Arnim[1)2)]*

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2.1 Abstract

Translational gene regulation plays important roles in plant development and in plant responses to metabolic signals and environmental stresses. However, compared to transcriptional regulation, there is a paucity of assay methods, especially assays that measure translational efficiency directly, *in planta,* and in multiple samples. In this study, we developed a dual-luciferase reporter gene expression assay to compare translation of a given coding sequence with that of a reference gene, both resident on the same expression plasmid. The reference gene was placed downstream of the experimental gene, separated by a sequence previously described as an internal ribosomal entry site from the crucifer strain of tobacco mosaic virus (crTMV IRES). With the intent of shielding the reference open reading frame from fortuitous cap-dependent translation, a stem-loop and stop codons were incorporated upstream of the IRES. The dual-reporter single-plasmid assay yielded results comparable to transformation of two independent plasmids or *in vitro* transcripts. In addition, I documented the expected reduction of translational efficiency on the 5' leader sequence of *Arabidopsis bZip11* in an *Arabidopsis* mutant of translation initiation factor *eIF3h*. Using this assay, it was further demonstrated that the 5' mRNA leaders of the stem cell regulatory genes, *CLAVATA1* (*CLV1*) and *CLV3* harbor uORFs that are inhibitory to translation. However, there is evidence that the crTMV element promotes primarily transcription initiation rather than internal ribosome entry. This finding must be taken into account when using the dual-reporter expression plasmids. I explored the utility of alternative internal ribosome entry sequences from cricket paralysis virus and tobacco etch virus. The CrPV element appeared to have robust IRES activity in wild type plants, but greatly reduced activity in

the Arabidopsis *eif3h* mutant, while the TEV element may yet turn out to be a viable IRES for constructing a dicistronic expression vector.

2.2 Introduction

Gene expression can be regulated at the DNA level, through chromatin structure and transcription, at the RNA level, through RNA processing and translation, and at the protein level, through posttranslational modification and protein turnover. By regulating translation, the cell pulls the reigns on the most energy-intensive step in gene expression, from a single mRNA molecule into multiple protein molecules. Translational repression coupled with efficient transcription can be a means to suppress the stochastic fluctuation inherent in gene expression (Kierzek et al., 2001; Blake et al. 2006). And translational control is a rapid and reversible means of gene regulation suitable for fine-tuning coarse patterns of expression imposed by hard-wired pathways of transcriptional regulation. To rapidly block expression of a gene, it is conceptually simpler to repress translation rather than stop transcription and induce mRNA degradation. Translational regulation of cytosolic mRNAs mediates responses to metabolites (Hanfrey and Michaels, 2005; Imai and Takahashi 2006; Rook and Smeekens 1998) and stress (Branco-Price et al., 2008; Kawaguchi and Bailey-Serres, 2005; Tang et al., 2003). However, the natural extent of translational control and its significance in gene regulation relative to transcriptional control are generally not well characterized, undoubtedly due, in part, to the paucity of reliable assay methods.

The 43S translation pre-initiation complex, which consists of the 40S ribosomal small subunit and a multifactor complex of initiation factors including a tRNA~Met, attaches to the eIF4 complex bound to the mRNA 5’ cap and scans along the mRNA 5’ untranslated region (5' leader) in search of a translation start codon (Pestova et al.,

2007). The contribution of the sequence and structure of the 5’ UTR to translational efficiency can be examined by fusing the 5’ UTR upstream of an easily quantifiable reporter gene. To measure translational efficiency *in vivo*, reporter gene expression is either quantified on the basis of the level of mRNA present, or alternatively compared with a co-transformed reference gene that should be insensitive to the experimental variable.

One inherent assumption of the reporter assay is that the mRNA levels of the query gene and that of the transformation control are the same or at least affected in the same way by the experimental treatment. Because translation state is coupled with mRNA stability (Shyu et al., 2008; Isken and Maquat, 2007; Kerenyi et al., 2008; Wu et al., 2007), mRNA levels may not be identical, however. This limitation has been addressed in metazoans by appending the reference ORF to the 3' end of the query mRNA, where it can be translated via an internal ribosome entry sequence (IRES), but no such system exists in plants.

IRESs have characteristic mRNA secondary structures or nucleotide composition, that have evolved to capture the small ribosomal subunit to initiate downstream translation at an associated start codon with or without assistance from translation initiation factors (reviewed in Doudna and Sarnow, 2007; Kieft, 2008). While metazoan IRESs of viral origin have been well characterized, plant IRESs took longer to discover (Ivanov et al., 1997, Skulachev et al., 1999, Niepel and Gallie, 1999; Gallie, 2001; Karetnikov and Lehto, 2007; Wong et al., 2008). The internal ribosome entry activity of a 148-nt sequence from the crucifer-strain of tobacco mosaic virus (crTMV) relies on polypurine stretches (Dorokhov et al., 2002), and has already been utilized for gene co-expression and gene trapping (Toth and Santa-Cruz, 2001; Yamamoto et al., 2003).

In this research, we constructed expression plasmids, in which the reporter construct and the transformation control were fused into a single plasmid via the crTMV IRES. Results using this expression plasmid were generally in good agreement with data from mRNA transformation and with previously published data from assays utilizing two separate transcription units (Kim et al., 2004). However, clear evidence that the reference ORF is expressed from the same, dicistronic, mRNA as the experimental ORF is still lacking. Instead, the results suggest that the crTMV element has fortuitous transcriptional activity. Nevertheless, with the new assay, plants defective in translation initiation factor eIF3h (*eif3h-1 and eif3h-2* carrying T-DNA insertion in the 10th exon and 11th intron, respectively) displayed reduced expression of the repressors of stem cell proliferation, *CLAVATA3* and *CLAVATA1*, an effect attributable to upstream open reading frames in their 5' leader sequences.

2.3 Material and Methods

2.3.1 Construction of dual-luciferase expression cassettes

Bona fide dicistronic dual-luciferase expression cassettes were generated in pBluescript II (Stratagene). pBluescript II was digested with *Xho*I and *Sal*I and religated to eliminate both sites. The 35S promoter, tobacco etch virus leader (TL) and Renilla luciferase coding region (RLUC) were released from pBS-35S::RLUC (Kim et al. 2004) using *Pst*I and inserted to form pBS-35S::TL-RLUC. A TA-cloning vector pKRX (Schutte et al., 1997) was digested with *Xba*I and *Spe*I and religated to eliminate the *XbaI* and *SpeI* sites. The TL and the firefly luciferase coding region were amplified from a previous

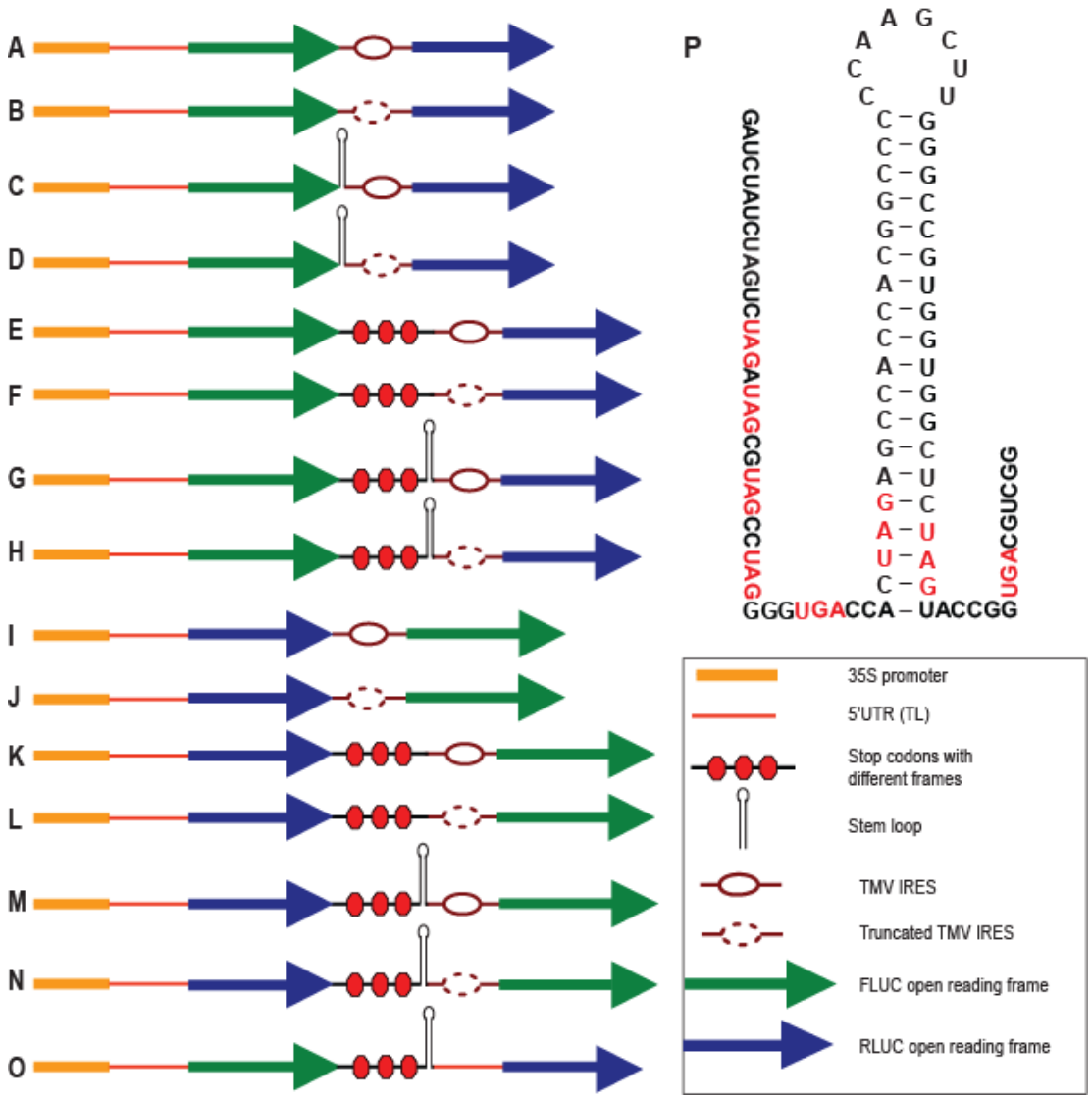

Figure 2-1. The crTMV element based DNA expression cassettes for the dual luciferase translation assay. A to H and O show constructs with FLUC upstream followed by RLUC downstream of the internal ribosome entry sequence from the crucifer infecting tobamovirus (crTMV IRES), and I-N show RLUC-FLUC constructs. See box for explanation of symbols. TL stands for the translational leader from tobacco etch virus. In O, the TMV IRES has been substituted by the TL sequence. Panel P shows the RNA sequence of the stem-loop and surrounding stop codons. The FLUC gene in constructs I-N is the codon-optimized LUC+ gene (Promega).

transient expression plasmid (pRTL2-35S::FLUC; Kim, et al. 2004) and inserted into pKRX by TA-cloning. The crTMV IRES sequence was amplified from plasmid pYY376 (Yamamoto et al., 2003) and, after passage through a fresh pKRX vector, was cloned into the *Spe*I/*Not*I sites downstream of FLUC to form pKRX-TL-FLUC-IRES. The TL-FLUC-IRES sequence was cloned into the *Xho*I/*Nco*I site of pBS-35S:TL-RLUC to form the plasmid pBS-35S:TL-FLUC-IRES-RLUC. A similar construct with a truncated IRES sequence (leaving only 7 bp on the left and 20 bp on the right end) was also generated as a negative control. A nos terminator from pBIN19 was inserted into the *Xma*I and *Bam*HI sites of both constructs to form constructs A and B in **Figure 2-1**.

To switch the FLUC and RLUC positions, both the full-length and truncated IRES sequences were inserted into the *Xho*I/*Nco*I sites upstream of the LUC+ cDNA in pGL3-basic (Promega, Madison, WI). The IRES-LUC+ sequence was excised with *Spe*I and *Xba*I and inserted into the *Xba*I site of pBS-35S:TL-RLUC to form pBS 35S:TL-RLUC-IRES-LUC+ and its truncated-IRES control (I and J in **Figure2-1**).

To introduce a series of stop codons between RLUC and the IRES, the construct I and J were further cut with *Bgl*II and *Asc*I and the gaps were filled in with two loop adaptor oligos (LOOPADP-for: 5' GATCTATC***TAG***TC***TAG***A***TAG***CG***TAG***CC***TAG***GGG***T GA***CC*AC**TAG**T*ACCGG***TGA***CGTCGG 3' and LOOPADP-rev 5' CGCGCCGACGTCA CCGGTACTAGTGGTCACCCCTAGGCTACGCTATCTAGACTAGATA 3' (K and L in **Figure 2-1***)*. A stem loop (□G= - 42.5 kcal/mol; determined by GeneBee service, Brodsky et al., 1995) was introduced into the *Spe*I site in the loop adaptor by annealing the two oligos LOOP-for: 5' CTAGAGCCACCACGGCCCCCAAGCTTGGGCCGTGGTG GC T 3' and LOOP-rev: 5' CTAGAGCCACCACGGCCCAAGCTTGGGGGCCGTGGT GGCT 3' (Kozak, 1986) to form plasmids M and N. The loop-adaptors with or without the stem loop were released from construct M and K, respectively, with *Xba*I and *Asc*I and

inserted into constructs A and B to form constructs G, H, E and F. The Loop-for and Loop-rev oligos were also annealed into the *Spe*I site in construct A and B to form constructs C and D respectively. Furthermore, the TMV IRES sequence in construct G was substituted with TL to form construct O. The constructs for the translation assays of specific mRNAs were generated by replacing the TL sequence in construct G and construct M with the mRNA 5' leader sequence to be tested. The expression cassettes A, B and E to H as well as those harboring the specific mRNA leaders were subcloned to the binary vector pFGC19 (Kim et al., 2007) *via* its *Hind*III and *Bam*HI sites for generating stable transgenic plants.

2.3.2 DNA based transient expression and stable gene transformation

Wild-type and *eif3h-1* mutant plants were grown on MS agar plates with 1% sucrose. Plasmids carrying dual-luciferase constructs were introduced into 10-day old wild-type and *eif3h-1* mutant seedlings by particle bombardment as previously described (Kim et al., 2004). Transfected seedlings were incubated at 22 ^{o}C in a lighted growth chamber for 8 hours, before assaying for luciferase activity. Luciferase activities were measured in a protein extract using the Dual-luciferase system (Promega, Madison, WI) in the TD-20/20 luminometer (Turnerdesigns, Sunnyvale, CA). The translation efficiencies of the 5' leader sequences were calculated as the ratio of FLUC to RLUC or RLUC to FLUC from 3 or 4 replicate experiments.

Plant gene transformations were performed with heterozygous *eif3h-1* mutant plants using floral dip with *Agrobacterium* strain GV3101. Wild type (ecotype WS) and

the *eif3h* mutant plants for translation assays were obtained from the segregation of second-generation transgenic plants.

2.3.3 Constructs and processes for *in vitro* transcription

The SP6 phage promoter was introduced into the pKRX-TL-FLUC construct by annealing two appropriate oligonucleotides (SP6 for 5’ TCGAAATTTAGGTGACACTATAGAAGTCGACA 3’ and SP6 rev 5’TCG ATGTCGACTTCTATAGTGTCACCTAAATT 3’) into the *Xho*I site. A 70 basepair poly-A tail was inserted downstream of FLUC between the *NotI* and *ScaII* sites by cloning a PCR product from two oligos (PolyA-for 5’ CCGCGGCCGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAGAGACCGAGCTCTT and PolyA-rev: AAGAGCTC GGTCTC*)* to form SP6:TL-FLUC (**Figure 2-6C**). The FLUC sequence was substituted with hRLUC (Subramanian et al., 2006) and LUC+ (Promega) to form SP6:TL-RLUC (**Figure 2-6B**) and SP6:TL-LUC+, respectively. The constructs for the translation assays with the 5’ leaders of *AtbZip11* were generated by replacing the TL with the *AtbZip11* leader (**Fig 2-9A, B**). The spacer-LUC+ and spacer-RLUC reference constructs (**Figure 2-9**) were generated by replacing TL with the multiple cloning site of the pGL3-basic vector (Promega). For observing the translational activity of the crTMV IRES in a 5’ proximal location, the PCR product of crTMV IRES from plasmid pYY376 TA was cloned to pKRX to form pKRX-IRES. crTMV IRES from pKRX-IRES was released with *Xho*I/*Bsp*HI and inserted into the *Xho*I/*Nco*I site of pGL3-basic to form pGL3-IRES-LUC+. A stem-loop was inserted by filling in the *Spe*I site with two oligos, Loop-for and Loop-rev. The Loop-IRES-LUC+ was cut out with *Xho*I/*Xba*I, and inserted into the *Xho*I/*Spe*I site of SP6:TL-FLUC to form SP6-Loop-IRES-LUC+. The IRES-RLUC

sequence was released from the dual-luciferase construct (**Figure 2-1A**) with *Asc*I/*Bam*HI, and inserted into the *Asc*I/*Bam*HI site of SP6-Loop-IRES-LUC+ to form SP6-Loop-IRES-RLUC (**Figure 2-6C**). A similar construct with truncated IRES was also generated for the negative control (**Figure 2-6C**). Plasmids for transcription of monocistronic or dicistronic mRNAs harboring the cricket paralysis virus IRES were kindly provided by Eric Jan (University of British Columbia; Jan et al., 2002).

For *in vitro* transcription, the plasmids harboring SP6 and T7 promoters were linearized by *Pvu*II and *Bam*HI digestion, respectively, and column purified (Qiagen). For cap-dependent translation initiation, mRNA was *in vitro* transcribed and capped using SP6 RNA polymerase and m^7G cap analog (New England Biolabs) following the manufacturer's protocols. Where indicated, cap analog was omitted. mRNA quality and quantity were estimated by agarose gel electrophoresis.

2.3.4 Protoplast preparation and PEG mediated mRNA transformation

Protoplasts were prepared from wild type or mutant 7 day old *Arabidopsis* seedlings (Yoo et al., 2007). 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 $CaCl_2$, 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 $CaCl_2$, 5mM KCl and 2 mM MES, *pH5.7*) and resuspended in 2ml W5 solution. The W5 solution was then substituted with MMG

solution (0.4 M mannitol, 15 mM $MgCl_2$ and 4mM MES, *pH5.7*) before mRNA transformation (Gallie, 1993). Eighty microgram of sheared and denatured salmon sperm DNA was added to 0.1 ml protoplasts in MMG solution in a 1.7 ml microcentrifuge tube. mRNA was added immediately before the addition of 0.11 ml PEG solution (40% PEG, 240mM mannitol and 100mM $CaCl_2$), which was subsequently mixed by gently inverting three to four times. The PEG transformation was terminated by addition of 0.43 ml 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.

2.4 Results

2.4.1 A stem loop is necessary for blocking IRES-independent expression of the downstream reading frame in a dual luciferase expression vector

Bona fide dicistronic expression cassettes were constructed for transcription in planta and placed between the cauliflower mosaic virus 35S promoter and a nopaline synthase terminator. A standard cassette contains an upstream firefly luciferase reading frame behind the generic 5' leader from tobacco etch virus (TL), and a downstream Renilla luciferase reading frame behind the IRES of tobacco mosaic virus coat protein (**Figure 2-1A**). The relative expression of each luciferase was determined initially in two transient assay situations, in Arabidopsis seedlings (**Figure 2-2A,B**) and in onion bulb epidermal cells (**Figure 2-2C**). In Arabidopsis, even the simplest IRES-containing

construct (**Figure 2-1A**) drove significantly higher expression of the downstream reporter protein (RLUC) than the construct harboring the truncated IRES (**Figure 2-1B**) both in absolute terms (**Figure 2-2B**) and also when expressed as the ratio of downstream:upstream ORF **(Figure 2-2A).**

2.4.2 An intergenic stem loop renders translation of the downstream ORF dependent on the crTMV IRES element.

A common concern for dicistronic expression constructs is that the downstream translation may not be entirely IRES dependent. Indeed, there was significant albeit low RLUC expression with the truncated IRES in both Arabidopsis (construct B in **Figure 2-2B and A**) and, more pronounced, in onion cells (**Figure 2-2C**). Translation of the downstream ORF could be due to a truncated transcript that lacks the upstream ORF, thus permitting ribosomes to reach the downstream ORF by cap-dependent scanning. To block ribosomes from reaching the downstream ORF by means other than internal ribosome entry, a stem-loop known to suppress scanning ribosomes (Kozak, 1988) was introduced upstream of the IRES (**Figure 2-1C and D**). In order to ensure that no translating ribosomes reach the stem-loop, multiple stop codons in all three reading frames were introduced between the upstream FLUC ORF and the stem-loop or the IRES (**Figure 2-1E-H**). The stem loop in particular suppressed the residual RLUC (downstream) expression from the truncated IRES construct, while the stop codons alone had no major effect, except in the onion cell assay (**Figure 2-2A-C**). From these data we conclude that the expression of the downstream ORF was largely caused by the crTMV IRES element.

Figure 2-2. Translation assays with FLUC-IRES-RLUC expression cassettes. Data from DNA plasmids harboring the constructs A to H in Figure 1. Error bars denote standard deviations. A-B. Transient assays using wild-type Arabidopsis seedlings, 10 days old. A. The RLUC-to-FLUC ratio indicates the expression of the downstream RLUC ORF relative to the upstream FLUC ORF. (n=3 replicates). B. Raw luminescence activities (in relative light units) for both FLUC (left scale) and RLUC (right scale) for data in (A). C. The RLUC-to-FLUC ratio for the transient assay using onion epidermal cells. D-E. Stable transgenic Arabidopsis plants. Luminescence activities from ten independent transgenic lines were measured in seedlings of the T2 generation. D. RLUC-to-FLUC ratios (n=3 replicates). E. Raw luminescence activities. Note that expression of the downstream RLUC ORF is entirely dependent on the intact crTMV element once it is preceded by the stem-loop sequence.

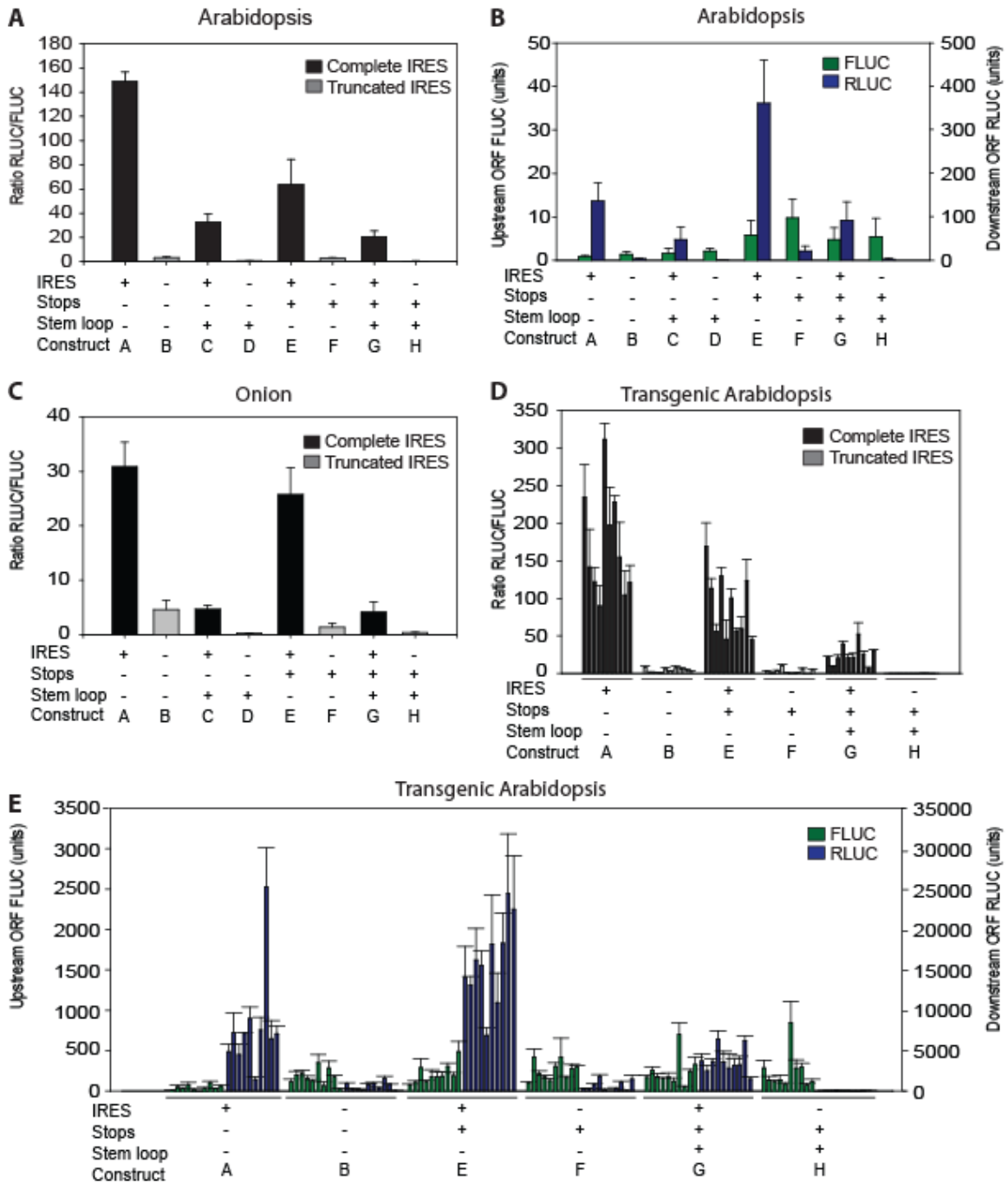
A
Arabidopsis
Ratio RLUC/FLUC
Complete IRES
Truncated IRES
IRES
Stops
Stem loop
Construct
B
Arabidopsis
Upstream ORF FLUC (units)
Downstream ORF RLUC (units)
FLUC
RLUC
C
Onion
D
Transgenic Arabidopsis
E
Transgenic Arabidopsis

Figure 2-2.

2.4.3 IRES activity in stably transgenic Arabidopsis

Since stable transgenic plants yield more reproducible and much higher luciferase activities than transient assays, we scored the crTMV IRES activity using the full length IRES and truncated IRES constructs in second-generation transgenic seedlings (T2 generation). Without stops and stem loop, the complete IRES in the dicistronic construct yielded much higher downstream RLUC activity than the truncated IRES (**Figure 2-2E**, construct A), again indicating that the crTMV element was active. However, the construct A lacking the stem-loop also showed the least upstream FLUC expression. Fortunately, with stop codons and stem loop introduced, the upstream FLUC activity showed no significant difference between full-length crTMV IRES and truncated IRES constructs (**Figure 2-2D and E**, see also **2-2B**), and downstream RLUC was overwhelmingly dependent on the crTMV sequence. In conclusion, a dual luciferase construct including the intergenic stop codon and stem-loop modules provides crTMV dependent expression of the downstream ORF (RLUC), and expression of the upstream ORF (FLUC) is robust and seems unaffected by the presence or absence of the crTMV element downstream.

2.4.4 The TMV element also functions in the context of firefly luciferase

The crTMV element showed significant activity, not only with the RLUC as the IRES-dependent reporter, but also with a codon-optimized firefly luciferase (LUC+ (Promega); **Figure 2-1I-N**; **Figure 2-3A-D**). Again, with the stop codons and the stem loop, the intact construct yielded about 20-fold higher FLUC activity than the truncated one when normalized to the expression of the upstream RLUC ORF.

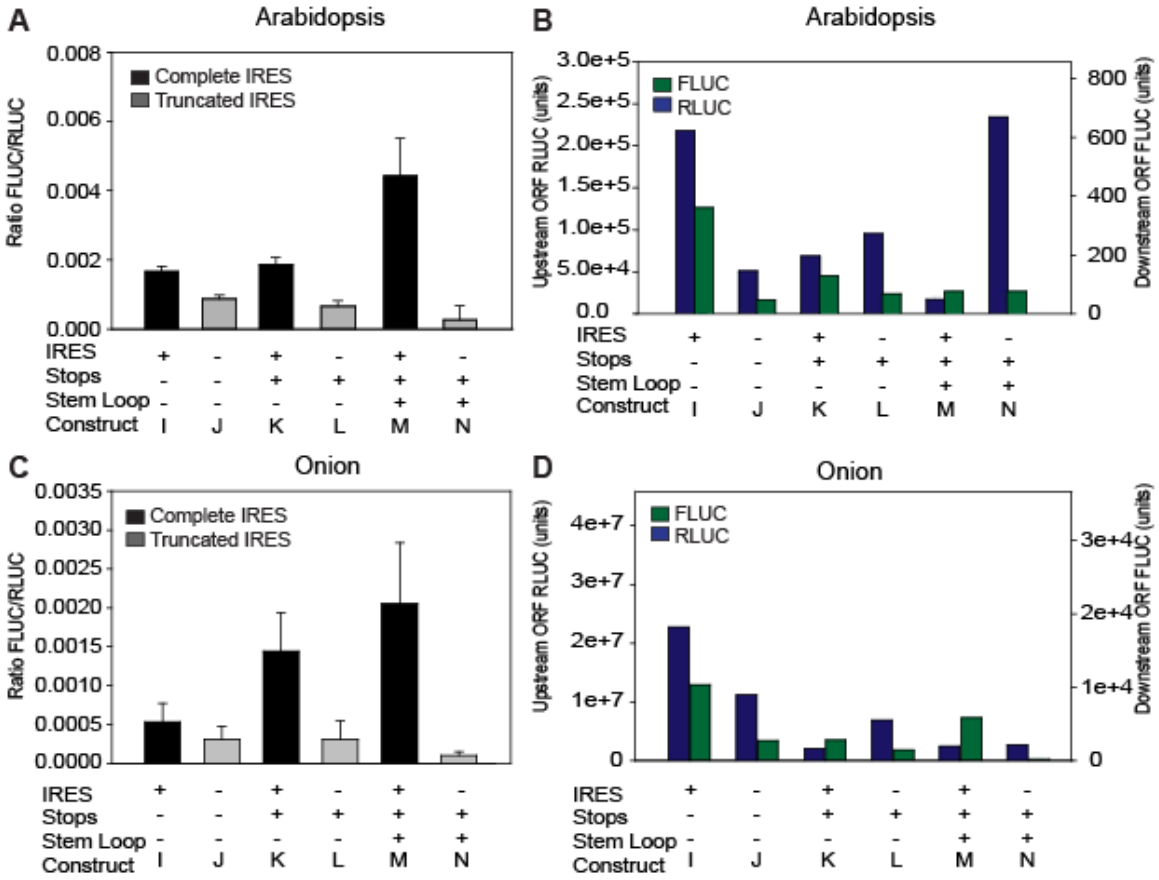

Figure 2-3. Translation assays with dicistronic RLUC-crTMV-FLUC expression cassettes. Data from plasmids harboring the constructs I to N in Figure 1. A-B. Transient assays with wild-type Arabidopsis seedlings (10 day old). A. The FLUC-to-RLUC ratio indicates the expression of the downstream FLUC ORF relative to the upstream RLUC ORF (n=3 experiments). Errors bars denote standard deviations. B. Raw luminescence values for the data shown in (A). Data are averages from 3 experiments. Variation exists primarily due to varying transformation efficiencies and is therefore omitted from the graph. Variation in IRES activity is reflected in panel A. C-D. Transient assays with onion epidermal cells. Data are presented as for panels A-B (n=3 experiments).

2.4.5 crTME element may have fortuitous promoter activity.

The 148nt CrTMV element has been characterized to have IRES activity (Ivanov et al. 1997), by virtue of driving cap-independent translation of a downstream ORF. However, in dicistronic constructs, the downstream luciferase gene activity may also be contributed from partial transcripts produced by fortuitous promoters (reviewed by Kozak 2003). In order to observe its fortuitous promoter activity, the DNA fragment carrying the crTMV element followed by RLUC in different sequence contexts **(Figure 2-4A)** (generated by restriction digestion and gel purification) was examined for expression in transient assays as described in the method and figure legend **(Figure 2-4)**. The crTMV element was also tested in tobacco (*Nicotiana tabacum*), the virus's original host **(Figure 2-4B)**. High RLUC activities were observed from tobacco and Arabidopsis wild type and *eif3h* mutant seedlings transformed with construct II and III but not construct V (with truncated crTMV element) **(Figure 2-4 B-D)**, indicating potential promoter activities upstream of RLUC gene. Interestingly, when the stem loop was removed, the downstream RLUC activities were dramatically increased (construct II and III, lower fragments compared to upper fragments), suggesting that the fortuitous transcription activity by the crTMV IRES is affected by the upstream DNA sequence, although the mechanism is not clear.

2.4.6 Evaluation of three different IRES sequences

We selected the crTMV IRES, because it had been successfully applied in gene co-expression and gene trapping constructs (Toth and Santa-Cruz, 2001; Yamamoto et al., 2003). However, to test whether other IRESs might be suitable as well, we decided to examine the leader from tobacco etch virus, which can drive cap-independent

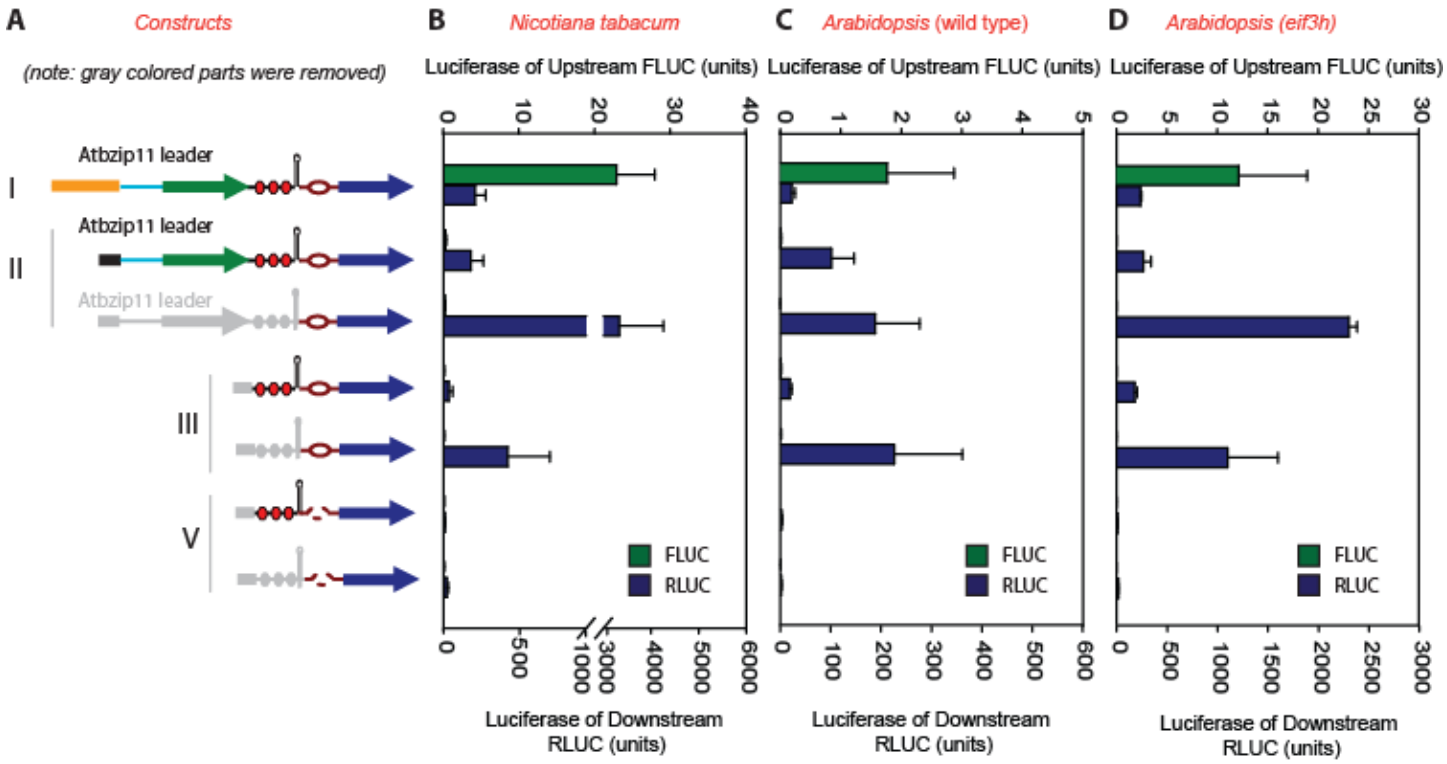

Figure 2-4. The crTMV IRES may have promoter activity. The SP6 promoter is represented by a black bar, and other symbols are the same as described in Figure 2-1. The construct I-V were digested with specific enzymes (I, *Kpn*I; II *Kpn*I (upper) and *Age*I (lower); III *Kpn*I+*Sal*I (upper) and *Age*I (lower); V *Kpn*I and *Sal*I (upper) and *Age*I (lower), DNA fragments were gel purified, bombarded into tobacco or *Arabidopsis* seedling and the luciferase activity was measured as described in Figure2. Note: The positions of the restriction site: *Kpn*I, before the promoters; *Sal*I, immediate behind the promoter; *Age*I, between the loop and the crTMV element.

translation initiation (TEV; Carrington and Freed, 1990; Gallie, 2001) and the IRES from cricket paralysis virus (CrPV; Jan and Sarnow, 2002). Compared to the TMV IRES, the TEV IRES (TL leader) had reduced activity (**Figure 2-1 construct O; Figure 2-5A**). Neither the TMV IRES nor the TEV IRES (TL) were affected by a mutation in the h subunit of Arabidopsis eIF3 (Kim et al., 2004), whether under transient assay conditions **(Figure 2-5A, C**) or in stably transgenic plants **(Figure 2-5B)**. TL is the default leader for many of our expression constructs, but it is usually present at the 5' end and therefore scanned in a cap-dependent manner. There is no evidence that this leader is eIF3h dependent in this setting.

The IRES from cricket paralysis virus (CrPV) is active in the wheat germ *in vitro* translation system (Jan and Sarnow, 2002; KS Browning, personal communication). To test the CrPV IRES in Arabidopsis, transformations of *in vitro* transcribed, uncapped mRNAs into Arabidopsis protoplasts were performed in order to eliminate confounding effects from transcription and RNA splicing. The CrPV IRES also displayed clear IRES activity in this assay **(Figure 2-6A)**, while a $CC_{6214\text{-}5}$ to GG substitution in pseudoknot I of the IRES was inactive, as expected (Jan and Sarnow, 2002). However, the CrPV IRES was severely compromised in the *eif3h* mutant plants. A similar result was obtained when the CrPV sequence was tested in a monocistronic setting **(Figure 2-6B)**. For comparison, the crTMV IRES was examined in a similar monocistronic setting with mRNA transformation **(Figure 2-6C)**, yet still did not reveal any eIF3h dependence. The strong stimulation of the CrPV IRES by eIF3h was unexpected, because this IRES is known to attract ribosomes without the help of initiation factors (Jan and Sarnow, 2002). In any event, despite its very strong activity in wild-type plants, the CrPV IRES was dropped from further consideration as a driver of reference gene translation.

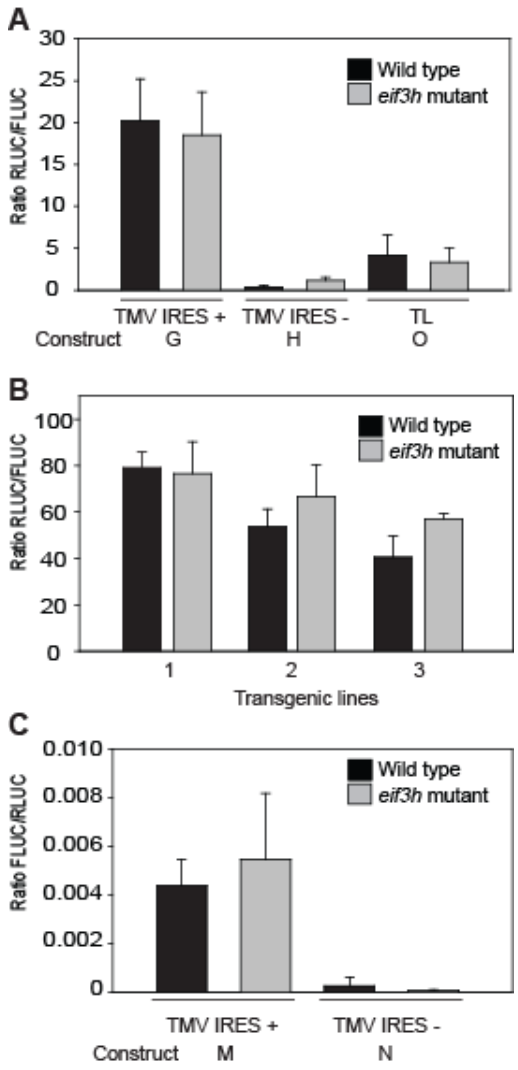

Figure 2-5. The crTMV element and the IRES of TL from tobacco etch virus are not dependent on eIF3h. A. RLUC-to-FLUC ratio for dicistronic constructs G, H, and O, measured in the transient assay using wild type and *eif3h* mutant seedlings, 10 days old. B. the RLUC-to-FLUC ratio for the complete dicistronic FLUC-IRES-RLUC construct (Figure 2-1G) transformed into stable transgenic Arabidopsis plants. Shown are data from wild type seedlings and *eif3h* mutants siblings segregating from the same transgenic parent. C. FLUC-to-RLUC ratio for dicistronic constructs M and N, measured in the transient assay.

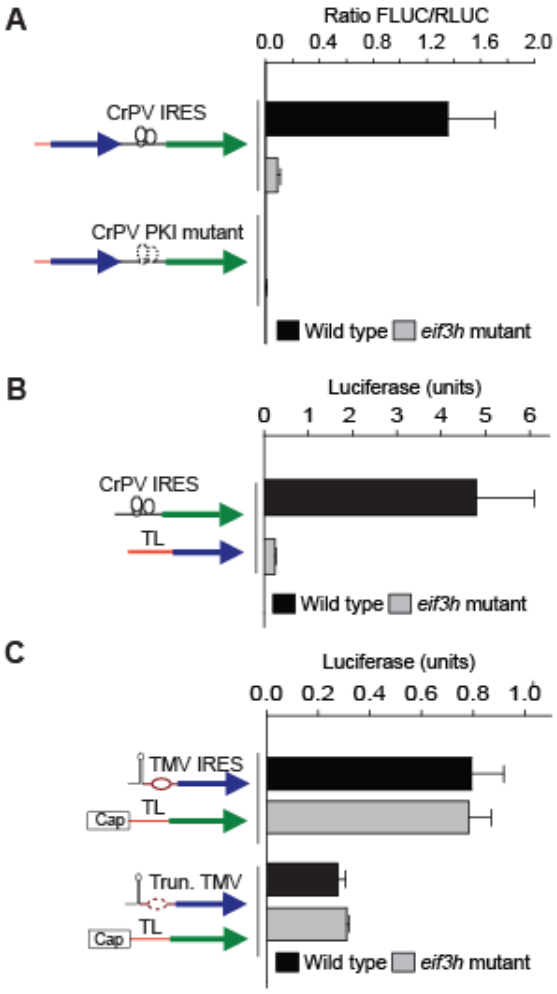

Figure 2-6. The cricket paralysis virus (CrPV) IRES. The cricket paralysis virus IRES was compared with the TMV IRES by mRNA transformation of Arabidopsis protoplasts prepared from wild-type or *eif3h* mutant plants. A. The FLUC-to-RLUC ratio for a dicistronic CrPV IRES construct and its control with a defective IRES (Pseudoknot I mutant, PKI). B. FLUC expression levels obtained with uncapped CrPV IRES-FLUC mRNA transformation normalized by co-transformed uncapped TL-RLUC mRNA and a separate transformation of TL-FLUC. C. RLUC expression levels obtained with an uncapped transcript harboring the complete or truncated IRES from TMV in a 5' proximal location. Data normalized by co-transformed capped TL-FLUC mRNA and a separate transformation of TL-RLUC. The CrPV expression plasmids were kindly provided by Eric Jan (Jan and Sarnow, 2002).

2.4.7 Comparison of translation efficiencies among different mRNA leaders using the dual-luciferase expression vector

The mRNA leaders of the Arabidopsis repressors of stem cell proliferation, *CLAVATA1* (AGI number At1g75820) and *CLAVATA3* (At2g27250) harbor multiple upstream open reading frames (**Figure 2-7A**), as does the 5' leader of *AtbZip11* (At4g34590; Kim et al., 2004). All three were cloned to the FLUC-IcrTMV-RLUC expression vector. All three showed significantly reduced translation efficiency compared with two representative leaders harboring no uORF (*PINFORMED1; At1g73590*) or only one uORF (LONG HYPOCOTYL5 (*HY5); At5g11260*; **Figure 2-7A, B**). Removal of the uORFs from the *CLV1* or *CLV3* leader dramatically increased expression of the FLUC reporter. This result indicates that the uORFs in *CLV1* and *CLV3* function as translational attenuators **(Figure 2-7B)**.

2.4.8 The dual-luciferase constructs can reveal translational defects in mutant plants

For further proof of concept, the dual-luciferase constructs were used to observe the translation efficiencies of specific mRNA leaders in a mutant defective in eIF3h, a subunit of the eIF3 complex. eIF3h boosts, in particular, the translation of mRNAs with multiple upstream open reading frames (uORFs), such as *AtbZip11* and *LHY* (Kim et al., 2004; Kim et al., 2007).

As expected, the *AtbZip11* leader gave less initiation in the *eif3h* mutant **(Figure 2-8A)**, while another bZip leader, *HY5*, was not significantly affected **(Figure 2-8B-C)**.

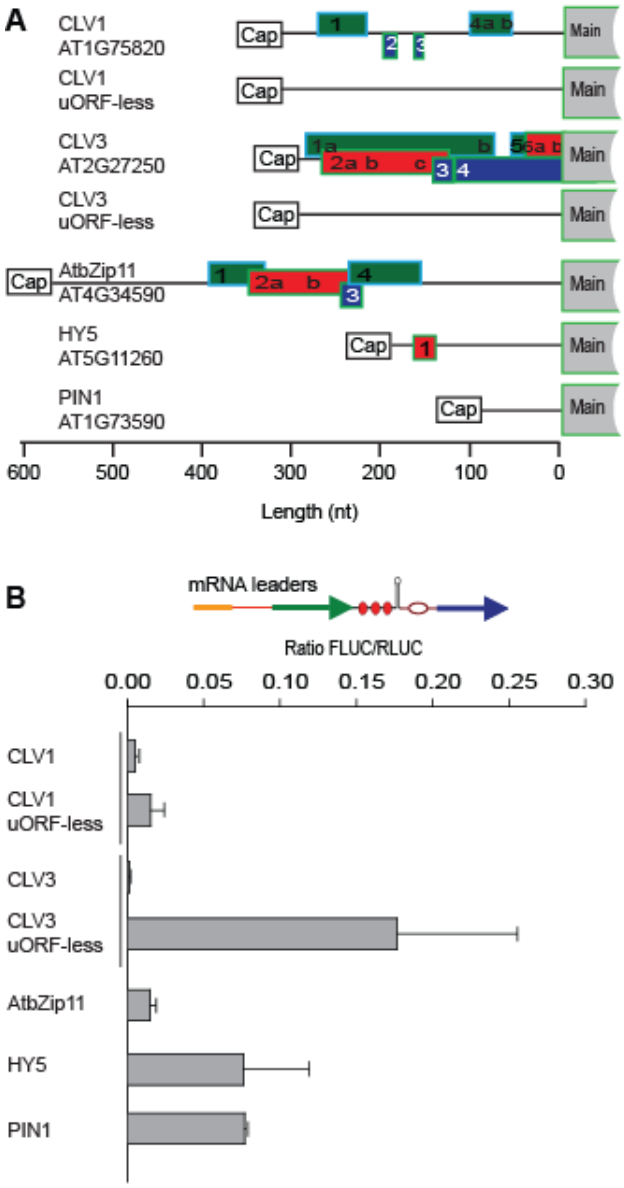

Figure 2-7. Measurement of translational efficiencies using the crTMV IRES construct. A. Schematic structures of the Arabidopsis mRNA leaders tested. Boxes indicate upstream open reading frames and multiple AUGs are labeled with letters. The uORFs in the *CLV1* and *CLV3* leaders were removed by mutating one nucleotide in each uAUG. B. Transient assay results for FLUC-IRES-RLUC constructs harboring the indicated mRNA leaders. The FLUC/RLUC ratio is a measure of the translation efficiency of the specific leader.

For additional validation, the *AtbZip11* 5' leader was also tested using *in vitro* transcribed luciferase mRNA, which was transformed into protoplasts together with a second mRNA encoding the alternative luciferase as a reference. Again, the *eif3h* mutant displayed a translational inhibition with the *AtbZip11* leader **(Figure 2-9A-B)**, but not with a generic control sequence **(Figure 2-9C)**.

2.5 Discussion

Evidence for translational regulation by plant developmental signals is sparse. A first step in addressing this question is to search for mRNA sequence elements that inhibit translation of key developmental regulator proteins, with the rationale that such elements might be the target of conditional translational derepression in specific developmental situations. Certain mutations in ribosomal proteins act as modifiers of developmental mutations (Pinon and Byrne, 2008; Yao et al., 2008), but the specific mRNAs whose translation is affected by the ribosomal protein mutation are generally unknown. As a rare exception, reduced translation of auxin response factors *ARF3/ETTIN* and *ARF5/MONOPTEROS*, which is caused by upstream open reading frames, may be responsible for the auxin related phenotypes of the mutation in ribosomal protein L24/*STV* (Nishimura et al., 2005). The mRNAs for the repressors of shoot stem cell division, *CLAVATA1* and *CLAVATA3*, harbor upstream open reading frames as well. Here I have presented evidence for the inhibitory role of the uORFs in *CLV1* and *CLV3* leaders (**Figure 2-7**).

Since the discovery of internal ribosome entry as a non-canonical mode of translation initiation in viruses (Jang et al., 1988; Pelletier and Sonenberg Nature 1988; Wilson et al., 2000) viral IRESs have become popular tools to express foreign mRNAs or

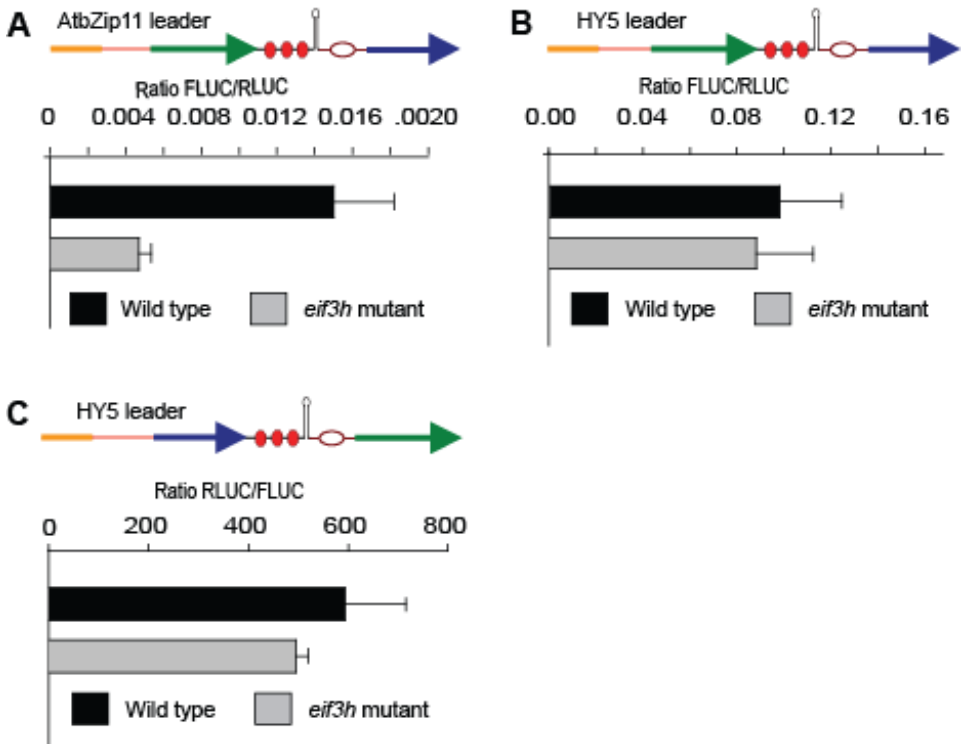

Figure 2-8. The crTMV IRES reveals differences in mRNA translation efficiencies between wild type and *eif3h* mutant plants. Dicistronic expression plasmids were transiently expressed via particle bombardment in seedlings of wild type or the *eif3h* mutant. The 5' leader in constructs G or M (Figure 1) was replaced with the new 5' leader to be tested. A. The 5' leader of Arabidopsis bZip11(At4g34590.1). B and C. The 5' leader of HY5 (At5g11260.1)

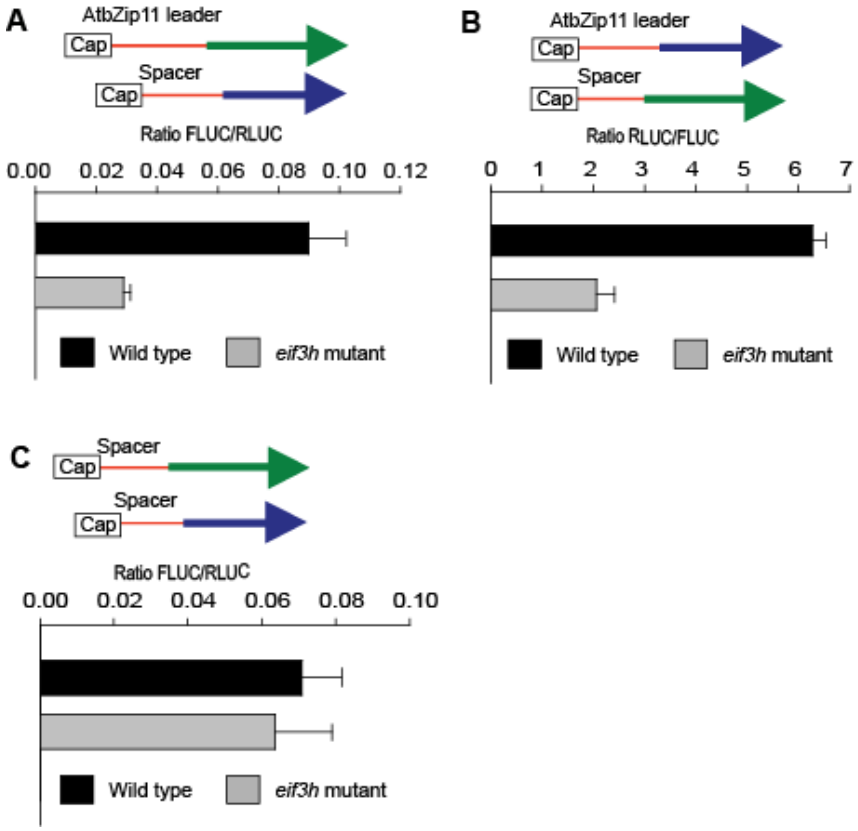

Figure 2-9. Results from the dicistronic assay using the TMV IRES (Figure 8) are compatible with those from transformation of *in vitro* transcribed mRNA. *In vitro* transcripts were transformed into Arabidopsis protoplasts. The reference mRNA has a generic leader derived from a plasmid polylinker sequence ('spacer'). A. The AtbZip11 leader was tested using FLUC as a reporter with RLUC serving as reference transcript. B. Same as (A) but with the reporter proteins swapped. C. Same as (A) but using the generic leader (spacer) rather than AtbZip11. n=3 replicates.

to construct dicistronic mRNAs (Elroy-Stein et al., 1989; Tahara et al., 1991; Kim et al., 1992). These strategies were confined to metazoans until IRESs of plant viral origin were characterized more recently (Carrington and Freed, 1990; Ivanov et al. 1997). Plant IRESs tend to be shorter and appear to rely more on biased base composition and simple secondary structures, rather than the elaborate structures that are common in metazoan IRESs (Niepel and Gallie, 1999; Dorokhov et al. 2002). On the whole, however, practical applications of plant IRESs as a component of dicistronic transcription units are few and far between (Toth and Santa-Cruz, 2001; Yamamoto et al., 2003) and their utility for measuring translation efficiency *in vivo* had not been established.

Cap-independent translation by internal ribosomal entry has been intensively studied for 20 years after the identification of an IRES in encephalomyocarditis virus (EMCV) (Jang et al., 1988). Numerous IRES elements have been identified and characterized from various viruses and even mammalian genes. Surprisingly the sequences and structure are not conserved among IRESs from different species. Therefore, very diverse mechanisms have been proposed for internal entry of ribosomes on different IRESs. Some of them require translation initiation factors, while others can associate with the 40S ribosomal subunit without the presence of any initiation factor (Jan and Sarnow, 2002). A factor-independent IRES, such as the CrPV IRES, would be a good choice for developing dicistronic constructs for translation assay purposes. However the CrPV IRES was significantly less translated in the *eif3h* mutant. Although the mechanism of CrPV IRES eIF3h dependence is not clear, we dropped the CrPV IRES from consideration for developing dual luciferase assay constructs for plant translation factor research.

The IRES dependent translation of a downstream gene in a dicistronic construct is usually very low, compared to its upstream cap-dependent translation. Therefore a lot

of IRESs have been questioned for their ribosomal entry activity, and their downstream translation has been criticized as being attributable to cryptic promoter activity or alternative splicing (Kozak, 2003). The transient assay using tobacco and Arabidopsis seedlings transformed with IRES-RLUC DNA fragments indicated that the crTMV element may have fortuitous promoter activity (Figure 2-4). However, since the promoter activity is affected by the upstream DNA sequence or structure, it is not clear how strong this fortuitous promoter could be in circularized plasmids or in the plant chromosome of a stable transgenic plant.

A direct way of measuring the efficiency of translation is to determine the rate of protein accumulation by pulse chase labeling using a radioisotope. This assay requires an affinity reagent to isolate the protein of interest from the bulk product and is therefore not conducive to high throughput. Another assay involves measuring the ribosome loading state of cellular mRNA using density gradient fractionation followed by detection of a specific mRNA by hybridization or polymerase chain reaction. This has the advantage of detecting the native mRNA rather than a reporter gene, yet assumes initiation to be the rate-limiting step. However, the number of ribosomes per mRNA is only an indirect measure of protein production. For example, ribosomes translating upstream open reading frames (uORFs) as well as delays in translation elongation or termination will increase the ribosome loading state while decreasing protein production. Sequestration of mRNAs into ribonucleoprotein particles such as P bodies is another confounding factor. Recently, Jonathan Weissman and coworkers suggested how deep sequencing can be applied to determine ribosome loading on all mRNAs (Ingolia et al., 2009). However, the general utility of this cutting-edge approach remains to be documented more broadly. In contrast, reporter assays directly monitor the production of the encoded protein and are easily tailored to test individual mRNAs or mRNA sequence

elements. In the reporter assay, selection of an appropriate reference gene is critical. By including a reference open reading frame under control of an IRES on the very mRNA that harbors the experimental reading frame, any variation in mRNA level will be corrected for and will not be a confounding factor. Initial results from our dual luciferase transcription units were encouraging in that, with the triple stop codons and the stem loop upstream of the IRES, translation of the downstream ORF was more than 99% independent of the upstream sequence (**Figure 2-2**). However, I then discovered that the crTMV element has strong transcriptional activation activity, an unexpected result, for crTMV is an RNA virus and has no need for transcription factor binding sites. The translation assay results indicated that the expression of the luciferase gene downstream of crTMV element is not affected by a mutation in the eIF3h gene (Figure 2-5). This allowed us to use the crTMV element based dual-luciferase construct to observe the translation efficiency of specific genes in the *eif3h* and other mutant.

Even though the original intent of driving translation of the reference using an IRES was not realized, the crTMV based dual-luciferase vector has certain benefits compared to the previous strategy of using separate constructs or a single construct with two 35S promoters. When two separate constructs are used, the reporter and the reference plasmids are premixed and bombarded into plant seedlings. Co-transformation efficiency is always a concern, which could introduce experimental variations. The crTMV based dual-luciferase construct has even more advantages in the translation assays using stable transgenic plant. A single construct is easy to transform and will usually yield the same copy numbers for the reporter and the reference gene. Most of all, since the reporter and reference gene are in a close sequence contact, they are likely to be affected equally by flanking sequence context and may undergo similar chromosome remodeling events. Similar dual-luciferase constructs can also be

constructed with two identical 35S promoters. However it is believed that multiple copies of identical sequences (35S promoters) can trigger or enhance homology-dependent transgene silencing (Mette et al., 1999; Dong and von Arnim, 2003). Thus having an alternative compact promoter that is not eIF3h-dependent can be advantageous.

Chapter 3:The translation initiation factor subunit, eIF3h, is involved in *Arabidopsis* shoot apical meristem maintenance and auxin response

This Chapter is a lightly revised version of a manuscript prepared by Fujun Zhou and Albrecht G von Arnim.

The translation initiation factor subunit, eIF3h, is involved in *Arabidopsis* shoot apical meristem maintenance and auxin response, *Fujun Zhou[1)] and Albrecht G von Arnim[1)2)]*

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3.1 Abstract:

Essentially all above-ground plant tissues develop from the stem cells in the primary shoot apical meristem. Proliferation of the stem cell population in the shoot apical meristem is tightly controlled by a feedback loop formed primarily by the homeodomain transcription factor WUSCHEL (WUS) and the CLAVATA (CLV) ligand-receptor system. In this study, we revealed how a translation initiation factor subunit, eIF3h, supports shoot apical meristem maintenance by ensuring the efficient translation of mRNAs for *CLV3* and *CLV1*, which harbor translational inhibitory elements in the form of a complex array of upstream open reading frames (uORFs). Consistent with the reduced translational efficiency of the *CLV3* and *CLV1* mRNAs, the *Arabidopsis eif3h* mutant possesses a strikingly enlarged shoot apical meristem and elevated WUS expression. Transformation with *CLV3* genomic DNA partially complemented the *eif3h* growth defects. The translational control by uORFs and eIF3h in the shoot apex is further supported by reduced translation of auxin response factors (*ARF*s) and concordant phenotypic observations. These results provide evidence that a translation initiation factor and translational control play important roles in *Arabidopsis* stem cell regulation.

3.2 Introduction

In eukaryotic cells, gene expression is highly regulated, often at multiple levels, such as transcription, mRNA structure and stability, translational control, and protein degradation. Translational regulation is arguably least well characterized and questions concerning the mechanism of translational control abound. In fungi such as budding yeast, translational control affords a rapid avenue to fine-tune metabolic regulation of

amino acid abundance and nutrient sufficiency (Hinnebusch, 2005; Sachs and Geballe, 2002). Similar (applications) for translational control exist in metazoans and plants (Hanson and Smeekens 2008; Hanfrey and Michael 2005; Deprost et al., 2007; Imai et al., 2006). In metazoans, translational control is closely connected to cellular responses to developmental and nutritional signals (reviewed by Mamane et al., 2006; Sonenberg and Hinnebusch, 2009) and plays a role in various diseases, such as obesity, diabetes, and tumor development (reviewed by Schneider and Sonenberg, 2007)). In contrast, how translational regulation contributes to the development of plants remains largely uncharted territory. Subtle mutations affecting specific proteins of the large ribosomal subunit (RPLs) that were discovered in genetic interaction screens suggest a role for translational control in leaf polarity (Yao et al. 2008; Pinon et al. 2008; Degenhardt et al., 2008). For example, mutations in RPL24/STV cause defects in organ initiation, vascular patterning, and gynoecium structure that could be attributed to undertranslation of mRNAs for transcription factors of the auxin response factor (ARF) class (Nishimura et al., 2004 and 2005). Auxin plays very important roles in apical-basal axis establishment and postembryonic organ initiation and specification (Aida et al., 2002 Friml et al, 2003; Muller and Sheen, 2008). The short-range directional auxin transport governs primordium initiation on the SAM, thereby affecting phyllotaxis (Heisler et al., 2005, Reinhardt et al., 2000 and 2003), while the long-range auxin transport from shoot to root is critical for root growth and patterning (Blilou et al. 2005; Dubrovsky et al., 2008).

Translation initiation is believed to contribute the most to translational efficiency (Gingras et al., 1999). Among the numerous eukaryotic translation initiation factors, eIF3, is by far the largest and most complex, consisting of 12-13 subunits in *Arabidopsis* and human. eIF3 is involved in almost all major steps during initiation, such as dissociating the small and large subunits and preventing their premature association;

assisting the ternary complex (TC, formed by eIF2, the initiator methonine tRNA and GTP) in associating with the 40S subunit to form a 43S preinitiation complex; and promoting 48S initiation complex formation by loading the 43S complex onto the mRNA's 5' UTR (Untranslated Region) (Hershey and Merrick, 2000; Kolupaeva et al., 2005; reviewed by Hinnebusch, 2006). eIF3 also affects mRNA scanning and start codon recognition (Valasek et al., 2004; Nielsen et al., 2004; Szamecz et al. 2008). Recently, eIF3 was proposed to have a principal role in dissociating post-termination ribosomes into their large and small subunits and thereby facilitating ribosome recycling (Pisarev et al., 2007). Studies in metazoans and yeast also attributed pivotal roles to eIF3 in the translational regulation in response to nutrients, energy sufficiency, and other internal or external stimuli (Holz et al., 2005), The *A. thaliana* eIF3 protein complex consists of 12 subunits, all of which resemble mammalian eIF3 subunits (Burks et al., 2001; Unbehaun et al., 2004).

The functions of the individual eIF3 subunits remain to be fully characterized. The h subunit of eIF3 is not conserved in budding yeast, but participates in forming the functional core of mammalian eIF3 based on in vitro reconstitution results (Masutani et al., 2007). Human eIF3h (P40) is over expressed in various cancer cells (Nupponen et al., 1999; Savinainen et al., 2004). Overexpression of eIF3h in human cells stimulate protein synthesis rate and promote oncogenesis (Zhang et al., 2008). Our previous research on the h subunit of *A. thaliana* eIF3 indicated that general translation is not dramatically compromised by a mutation in *eIF3h*; however, some specific mRNAs harboring multiple upstream open reading frames (uORF) in their 5' leader are less translated in the *eif3h* mutant (Kim et al. 2004; Kim et al., 2007). Therefore, we proposed that eIF3h is involved in the translational regulation of specific mRNAs, including but not limited to mRNAs with multiple uORFs. Although the *eif3h* mutant shows pleiotropic

developmental phenotypes, such as growth retardation, a less developed root and misexpression of auxin regulated genes (Kim et al., 2004), it has remained unclear how undertranslation of specific mRNAs causes these macroscopic phenotypes.

The plant tissues above and below ground ultimately develop from the stem cells in the shoot apical meristem (SAM) and root apical meristem (RAM), respectively. In *Arabidopsis,* the stem cell population in the SAM is tightly regulated by the CLV-WUS pathway, which includes an intercellular feedback loop. CLV3, a short extracellular peptide produced in the outer two cell layers in the central zone of the SAM, is the ligand for the receptor kinase CLV1 (Clark et al., 1997; Fletcher et al., 1999; Brand et al., 2000; Kondo et al. 2006; Ogawa et al., 2008). In response to the CLV3 signal, CLV1 and its functional partner, CLV2, restrict the spatial expression of a homeobox transcription factor, *WUSCHEL* (*WUS*), in the organizing center of the SAM. *WUS* plays a central role in stem cell population maintenance by transcriptionally regulating a large group of target genes. WUS also induces the expression of *CLV3*, whereby a negative regulatory feedback loop is formed to ensure the stability of the stem cell population (Laux et al. 1996; Clark et al. 1997; Mayer et al. 1998; Fletcher et al. 1999, Schoof et al., 2000). A recent study using a *CLV3* inducible expression system indicated that induction of *CLV3* mRNA at 70 fold (3 hours after induction (HAI)) or even >400 times (72 HAI) does not completely terminate *WUS* expression. Surprisingly the WUS expression was recovered after 72 HAI, indicating high level of *CLV3* transcript can be compensated over time (Muller et al. 2006). Such a robust mode of regulation suggests additional modes of regulation downstream of *CLV3* transcription.

Here we describe that a mutation in eIF3h causes variable defects in shoot apical meristem maintenance that range from subtle defects in phyllotaxis to a massively enlarged, yet largely quiescent, shoot apical meristem. Translation assays and

transgenic complementation experiments revealed that eIF3h supports the efficient translation of the mRNAs for *CLAVATA1* and *CLAVATA3* as well as multiple auxin response factors (*ARF*s), which can explain several of the phenotypic aberrations in *eif3h* shoot apical meristem maintenance and organ specification. Thus, the *eif3h* mutation amounts to a genetic perturbation that unveils a critical role for translational control in *Arabidopsis* shoot apical meristem function.

3.3 Results

3.3.1 The *eif3h* mutant plants have growth defects in the SAM

Unlike wild-type *Arabidopsis*, which always initiate a functional inflorescence from the shoot apex, a large proportion of *eif3h* mutant plants never initiated an inflorescence (**Figure 3-1A**). Instead the shoot apex started to enlarge (**Figure 3-1B**), often forming a dome-shaped structure (**Figure 3-1C**). Most plants that formed a dome-shaped apex died without initiating any additional leaves, but occasionally, some survived and initiated multiple inflorescences (**Figure 3-1D**). Scanning electron microscopy revealed that the *eif3h* mutant apex in a 3-week-old seedling (**Figure 3-1F**) is significantly larger than that of wild type (WS ecotype, **Figure 3-1E**). The enlargement was visible as early as 3 days after germination (**Figure 3-1H** compared to **Figure 3-1G**). It is common the see filamentous outgrowth or needle-like leaves on *eif3h* apex **(Figure 3-1I-L).** An enlarged meristem is characteristic of mutations in the regulators of stem cell development, *CLAVATA1* and *CLAVATA3* (Clark et al., 1997; Fletcher et al., 1999). Like *clv3* and *clv1* which often form fruits from more than the normal two carpels (3-5 in **Figure 3-2B**), the *eif3h* mutant occasionally produced extra carpels (**Figure 3-2A**). While the inflorescence

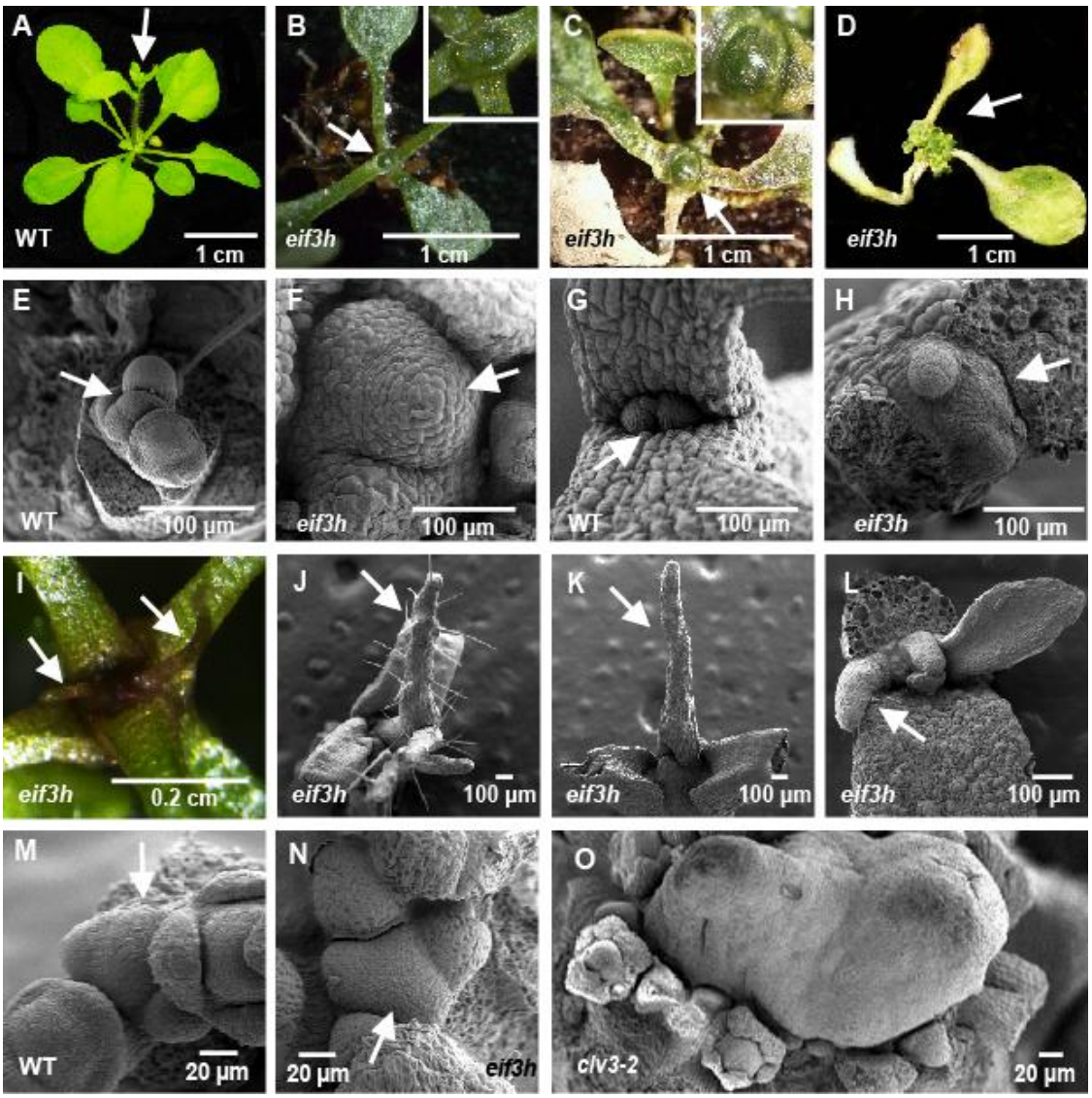

Figure 3-1. Defects of *eif3h* mutant plants in shoot apical meristem maintenance. (A-D) Enlarged shoot apex in *eif3h*. White arrows point to the inflorescence (in wild type) or the shoot apex (in *eif3h*). (A) Wild type (WS ecotype). (B) The enlarged quiescent *eif3h* SAM (inset shows a close-up of the apex). (C) Further enlarged dome-shaped *eif3h* SAM pior to senescence (inset shows a close-up). (D) Reactivated dome-shaped *eif3h* SAM with multiple inflorescences initiating. (E-K) Scanning electron micrographs for wild type, *eif3h* and clv3-2 SAMs. White arrows point to the apices. (E) 3 week old wild type. (F) 3 week old *eif3h.* (G) 3 days old wild type. (H) 3 days old *eif3h*. (I-L) Aberrant organ growth on *eif3h* SAM. White arrows point to the aberrant organs. (I) 3 Weeks old *eif3h* mutant with needle leaves. (J) Filamentous growth with trichomes on 3 weeks old eif3h apex. (K) Filamentous growth without trichome on 3 weeks old eif3h apex. (L) A needle-like leaf on week old eif3h apex. (M-O) Inflorescence apexes. (M) Apex of a wild type inflorescence. (N) Apex of an *eif3h* inflorescence SAM. (O) Apex of a 3 week old *clv3-2* inflorescence.

meristem of flowering *eif3h* mutants was not significantly enlarged, different to that of *clv3* (**Figure 3-1 M-O**), it was common to see *eif3h* plants with bifurcated shoots (**Fig 3-2C**) or fasciated stems (**Figure 3-2D**). Other abnormalities in *eif3h* included lateral inflorescences that were not subtended by a cauline leaf (**Figure 3-2E**) and reduced apical dominance upon spontaneous arrest of shoot apical meristem activity (**Figure 3-2F, arrow**).

3.3.2 Many *eif3h* mutant phenotypes are reminiscent of defects in auxin transport or response

Instead of a functional inflorescence with leaves and flowers, some *eif3h* mutant plants formed a naked shoot (**Figure 3-3A**). Pin-formed shoots are characteristic of defects in auxin transport or response, for example the *pin1* mutant (**Figure 3-3B**; Gälweiler et al., 1998) and mutations in the auxin response transcription factor, *MONOPTEROS/ARF5* (Przemeck et al., 1996). While pin-like shoots in the *eif3h* mutant still initiated rudimentary organs (**Figure 3-3A**), an *eif3h/ett-1* double mutant showed a pin-formed shoot without lateral organ primordia (**Figure 3-3C**). The enhancement of *eif3h* by *ettin*, which encodes *ARF3* (Sessions and Zambryski, 1995), to a classical pin-formed phenotype clearly suggests that eIF3h is specifically responsible for translation of mediators of auxin transport or auxin response in the shoot.

The siliques of the *eif3h* mutant are shorter than wild type, and often the carpels are initiated at an unusual distance from the node. Occasionally a carpel is missing from one side of the silique, which is reminiscent of the *ettin* mutant (**Figure 3-3D**; Sessions and Zambryski 1995; Nemhauser et al., 2000) and has also been seen in mutants of *STV*, which encodes ribosomal protein L24 and interacts with *ettin* (Nishimura et al.

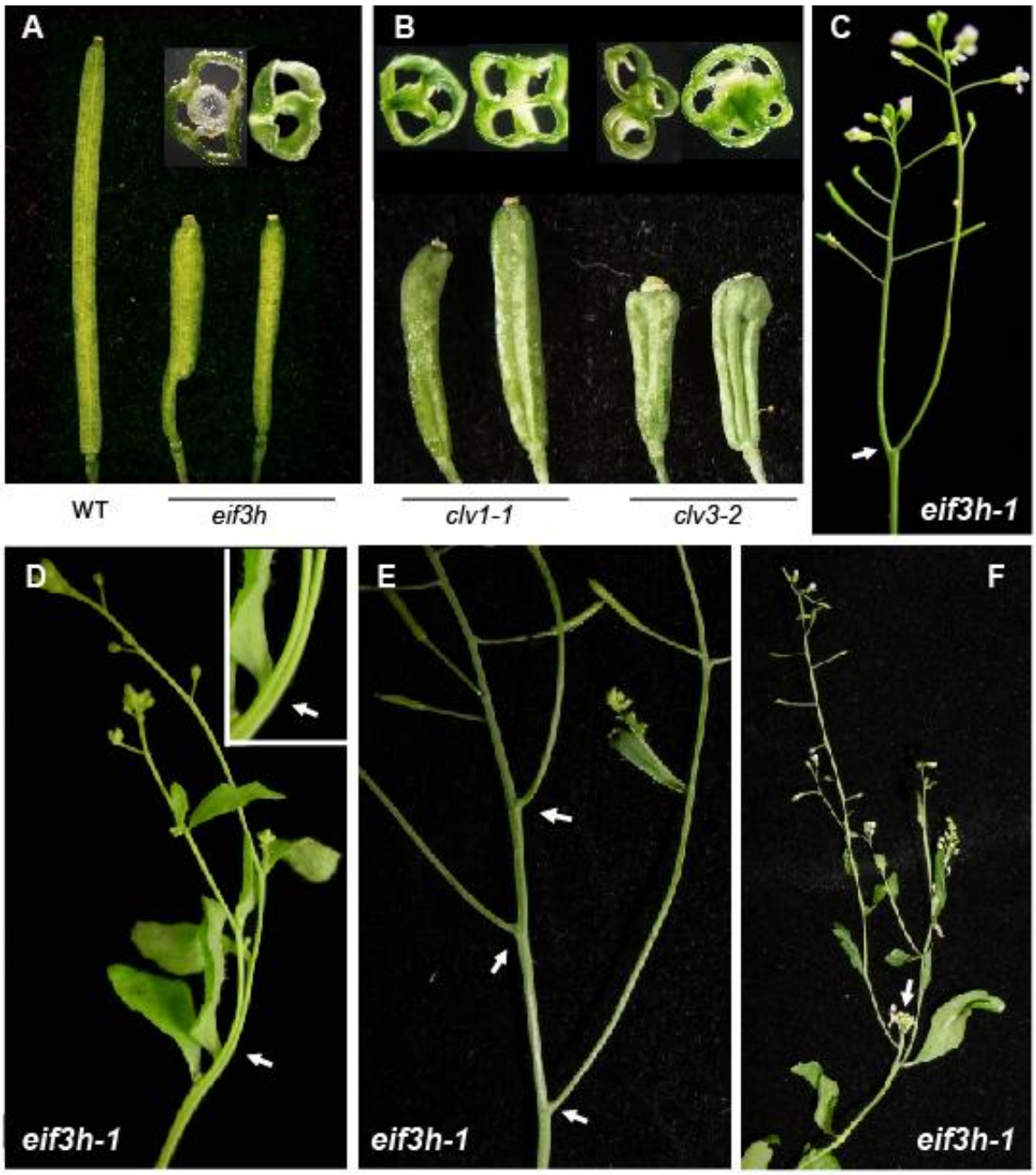

Figure 3-2. *eif3h* phenotypes possibly involved in stem cell regulation
(A) 3-carpel phenotype of *eif3h*. (B) *clv3-2* siliques showing 3 to 5 carpels. (C) *eif3h* inflorescence may be bifurcated. (D) *eif3h* inflorescence with fasciated shoots. (E) *eif3h* lateral branching initiated independent of cauline leaves. (F) *eif3h* inflorescence with aborted shoot apices (arrows) and defects on apical dominance.

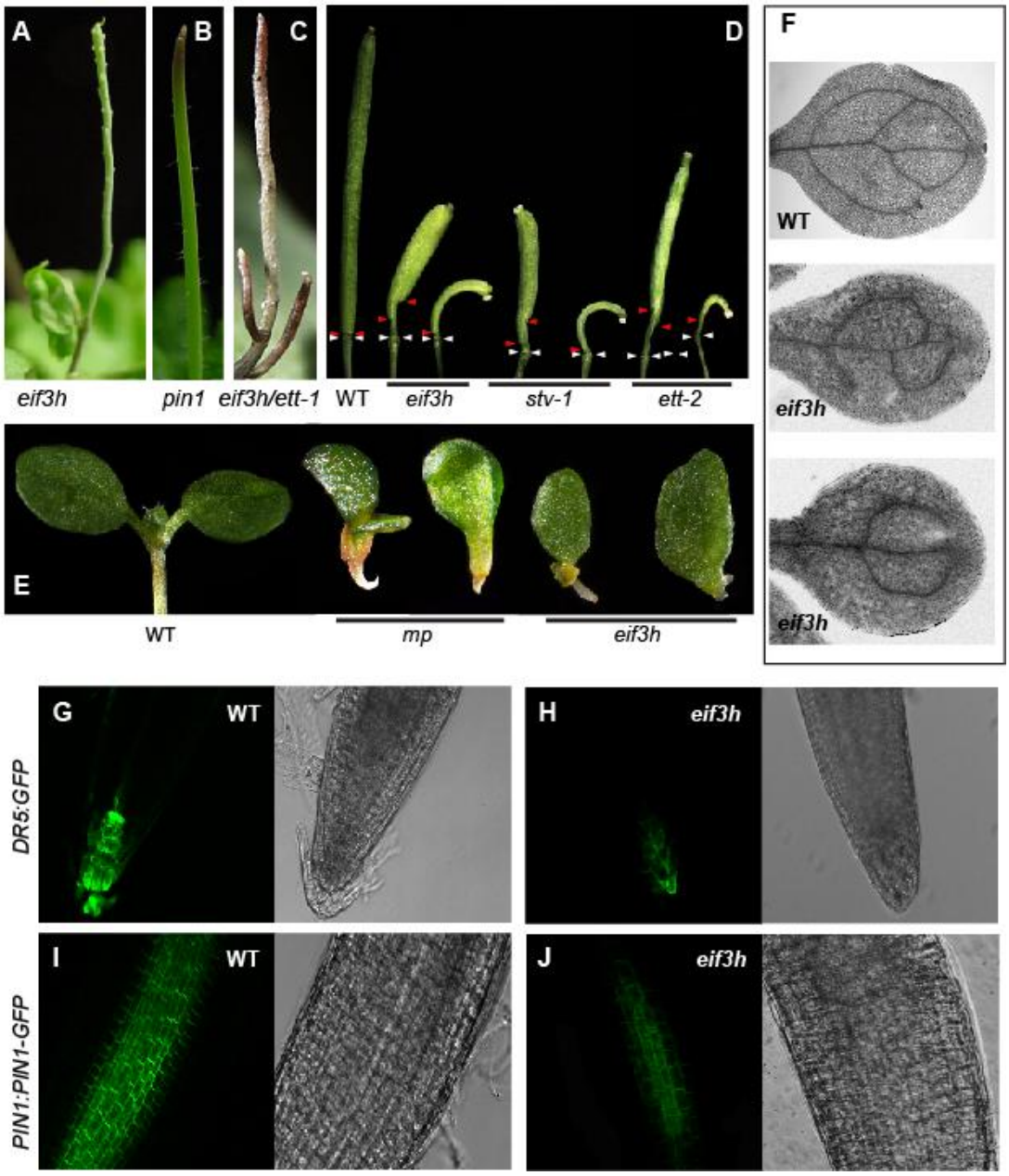

Figure 3-3. *eif3h* phenotypes reminiscent of defects in auxin transport or response. (A-F) *eif3h* phenotypes similar to auxin response or transport defects. (A) *eif3h* naked shoot. (B) *pin1* shoot. (C) *eif3h/ett-1* double mutant shoot. (D) *eif3h* fruits with defects in carpel development, similar to those of *stv-1* or *ett-1.* White arrowheads point to the last node carrying stamens and red arrowheads point to the lower end of the carpels. (E) Monocotyledon phenotype of *eif3h* seedlings, similar to that of the *mp* mutant. (F) *eif3h* defects on vascular development. (G-J) *DR5::GFP* and *PIN1::PIN1-GFP* expression in wild type and *eif3h* mutant roots. (G and H) *DR5::GFP* expression in wild type and *eif3h* root tips respectively. (I and J) *PIN1::PIN1-GFP* expression in wild type and *eif3h* root respectively.

2005). In further support of auxin response defects, some *eif3h* mutant seedlings have only a single cotyledon or two cotyledons with uneven growth, similar to the *mp (arf5)* mutant (**Figure 3-3E**, **Figure 3-4B**). Auxin defects often reveal themselves by cul-de-sac vascular elements in the cotyledons and by defects in phyllotaxis (Berleth and Jürgens, 1993; Hardtke and Berleth, 1998; Mattsson et al., 1999), and such defects are readily observed in the *eif3h* mutant (**Figure 3-3F**; **Figure 3-4A**). A *DR5::GFP* gene and a *PIN1::PIN1-GFP* have been introduced into the *eif3h* mutant by crossing the mutant to wild type *Arabidopsis* (Columbia) transformed with *DR5::GFP* and *PIN1::PIN1-GFP*, respectively. While *DR5::GFP* was highly expressed in the wild-type root tip, especially the quiescent center (QC) of the root apical meristem, it was significantly reduced in the *eif3h* mutant **(Figure 3-3H compared to G)**. Interestingly the *PIN1::PIN-GFP* expression was also significantly reduced in the *eif3h* mutant roots **(Figure 3-3J compared to I)**.

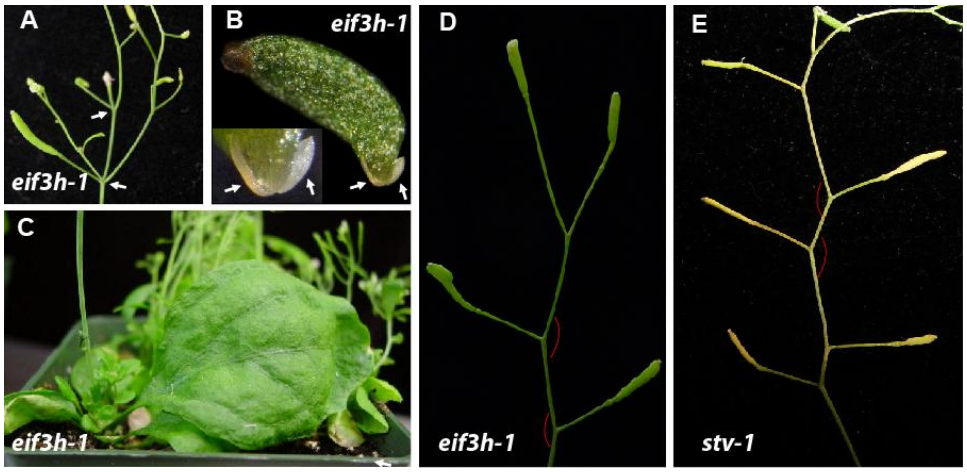

Figure 3-4. Other *eif3h* phenotypes suggesting defective auxin responses
(A) *eif3h* abnormal phyllotaxis. (B) *eif3h* seedling with one of the cotyledons and root development aborted. (C) *eif3h* with a dramatically enlarged rosette leaf. (D and E) Zigzag shaped *eif3h* and *stv-1* shoots respectively

3.3.3 The mRNAs of *CLV1*, *CLV3* and many *ARF*s harbor multiple uORFs

Our previous translation assays on specific mRNAs, such as *AtbZip11* and *LHY,* established that mRNAs harboring multiple uORFs were less translated in the *eif3h* mutant, suggesting that eIF3h may be involved in overcoming the translational repression by uORFs (Kim et al., 2004, Kim, et al. 2007). In order to identify candidate mRNAs whose translational control in shoot apical meristem maintenance and auxin response might be revealed by the phenotypes of the *eif3h* mutant, groups of genes involved in SAM maintenance and auxin signaling were examined for the presence of uORFs. Among the auxin-related genes, most *ARF*s harbor multiple uORFs. *ARF5*, *ARF6*, and *ARF11* have seven or more upstream AUGs (uAUGs) (**Figure 3-5**). In contrast, uORFs were much less abundant among *AUX/IAA* and *YUCCA* mRNAs, *TIR1* homologs, and *PIN* mRNAs (**Table 3-1**). Among genes involved in SAM maintenance, *CLV1* and *CLV3* harbor 5 and 10 uAUGs, respectively, in 5' leaders that are only about 300 nucleotides long. Other members of the *CLAVATA3* gene family (*CLE* genes), such as *CLE2* and *CLE25* also have multiple uAUGs in their 5' leaders (**Figure 3-5**). The abundance of uORFs in the *CLV* and *ARF* leaders indicates that they are potential clients of eIF3h.

3.3.4 *ARFs*, *CLV* and *CLE* mRNAs are less translated in the *eif3h* mutant

A translation assay system based on protoplast transformation of *in vitro* transcribed mRNA was adopted to observe the translation efficiency of specific mRNA

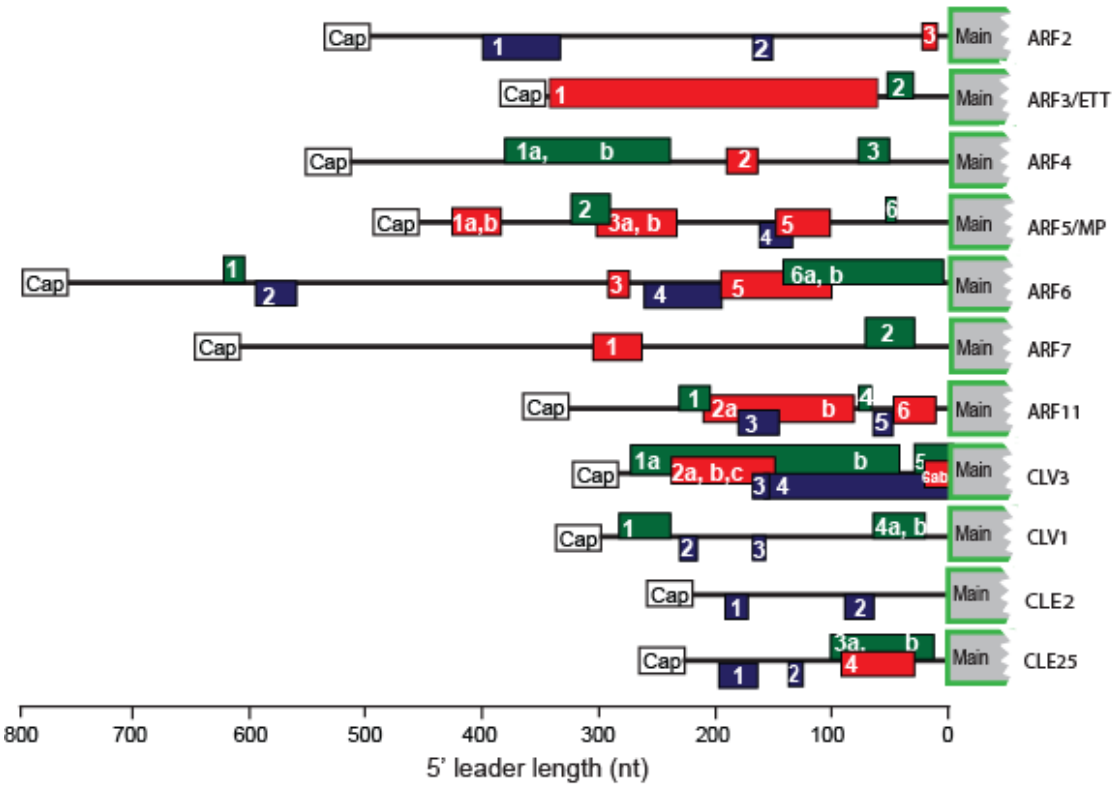

Figure 3-5. The leaders of many auxin response factors (*ARF*s), *CLAVATA*s and *CLE*s harbor multiple uORFs. Open gray boxes represent the main ORF; red boxes stand for uORFs in frame with the main ORF; green boxes for uORFs at –1 position to the main ORF; blue boxes for uORFs at +1 position to the main ORF. *Sources of cDNA sequence information are as follows: ARF2* (AT5G62000.1); *ARF3* (AT2G33860.1); *ARF4* (AT5G60450.1); *ARF5* (AT1G19850.1); *ARF6* (AT1G30330.1); *ARF7* (AT5G20730.1); *ARF11* (AT2G46530.1); *CLV3* (AT2G27250.1); *CLV1* (AT1G75820.1); *CLE3* (AT4G18510.1) *CLE25* (AT3G28455.1).

Table 3-1 uAUG abundance of many groups of auxin related genes

Gene family	leader sequences available	No. of uAUG/leader	No. of uAUG/100nt	% leaders with 2 or more uAUG	% leaders with 3 or more uAUG
ARF	14	2.71	0.75	64	36
IAA	26	0.42	0.27	12	0
AFB (Tir1)	5	0.80	0.31	20.	0
PIN	6	0.16	0.16	0	0
YUC	3	1.00	0.32	33	0

Note: Data based on mRNA 5'UTR sequences available in the *Arabidopsis* Information Resource (http://www.arabidopsis.org/).

leaders in the *eif3h* mutant. While translation with a *PIN1* leader, which lacks uORFs, was not significantly affected in the *eif3h* mutant, the *ARF* leaders with multiple uORFs were significantly less translated in the *eif3h* mutant. Likewise, the translational efficiency with a *CLV1* or *CLV3* leader was also dramatically reduced in the *eif3h* mutant. Two *CLE* leaders, *CLE2* and *CLE25,* also showed a significant translational reduction in the *eif3h* mutant **(Figure 3-6)**.

The translation efficiency of the *CLV* leaders was also measured using a dual-luciferase translation assay construct, in which the reporter construct (upstream gene) and the transformation control (downstream gene) were fused with a sequence from the crucifer strain of tobacco mosaic Virus (crTMV) reported to have activity as an internal ribosome entry site (IRES; Ivanov et al., 1997; Dorokhov et al. 2002, **Figure 3-7A**). Although the crTMV element was observed to have fortuitous promoter activity, since the expression activity of the crTMV element was not affected by the *eIF3h* mutation, the constructs were deemed adequate for determining the translation of specific mRNAs in the *eif3h* mutant (**see chapter 2 for details**). For validation of the dual-luciferase assay constructs, we measured translational efficiency with the leaders of *AtbZip11* and *HY5*.

Figure 3-6. The leaders of many *ARF*s, *CLVs and CLEs* are less translated in the *eif3h* mutant.

The reporter and control mRNAs were prepared by *in vitro* transcription with SP6 RNA polymerase. An equal volume of internal control (Spacer-LUC+) mRNA was added to reporter mRNA or the external control mRNA and vortexed to mix well prior to immediate transformation. The FLUC values from the LUC+ gene served as an internal control for evaluating transformation consistency. The ratio of RLUC values between wild-type and the *eif3h* mutant were used for normalizing the reporter RLUC value in the *eif3h* mutant against wild type. The bars with standard errors in Figure 6B and C represent the means of luciferase values from 3 replicate transformations.
(A) Schematic view of the mRNAs for protoplast transformation. Blue arrows represent RLUC gene, green arrows represent LUC+ gene. (B) Translation assay results for *CLV* and *CLE* genes (*CLV1, CLV3* and *CLE2, CLE25*). (C) Translation assay results for *ARF*s and *PIN1* genes (*ARF2-7, ARF11*, and *PIN1*).

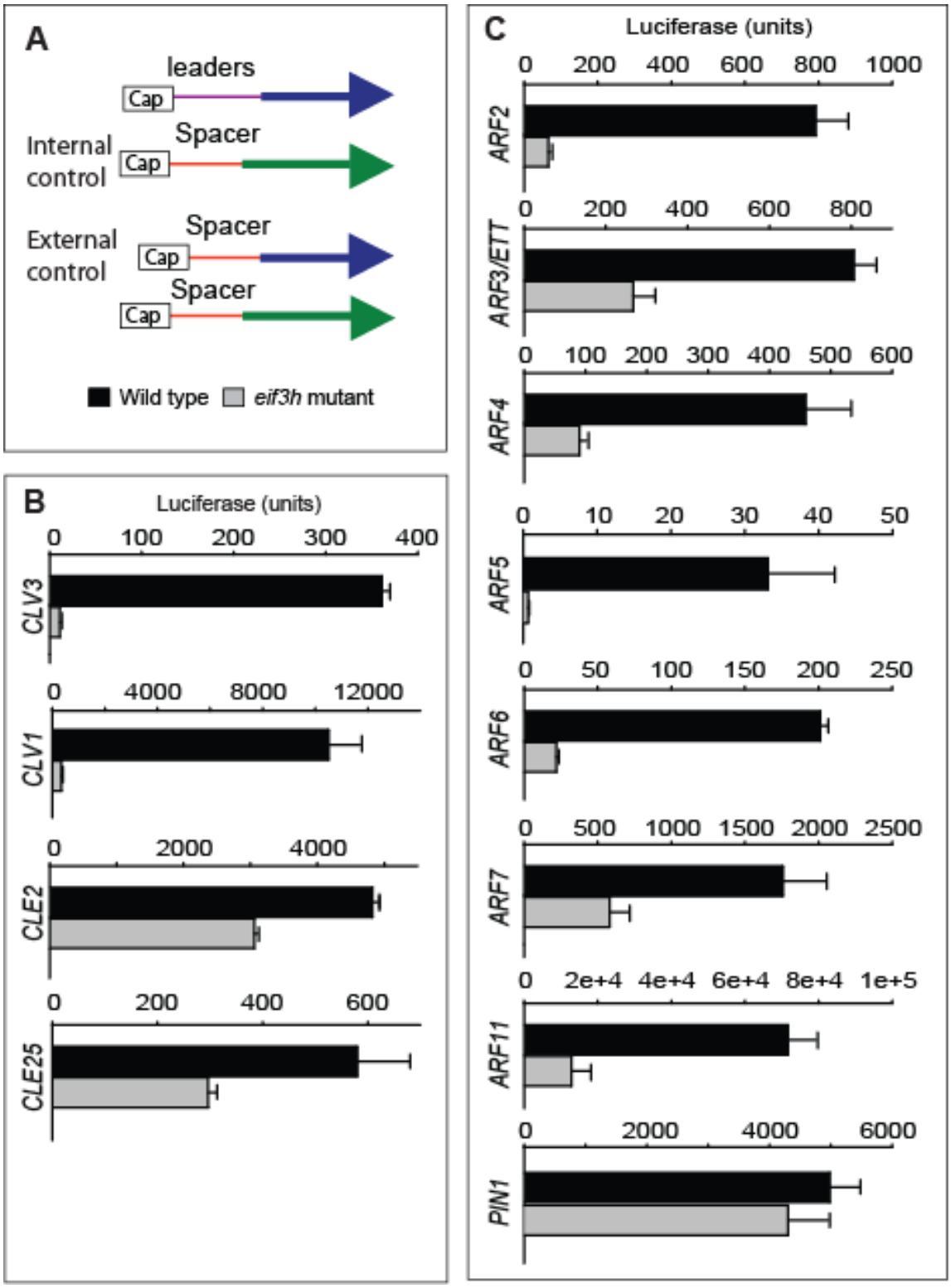
A
leaders
Cap
Internal control
Spacer
Cap
External control
Spacer
Cap
Spacer
Cap
Wild type
eif3h mutant
B
Luciferase (units)
CLV3
0 100 200 300 400
CLV1
0 4000 8000 12000
CLE2
0 2000 4000
CLE25
0 200 400 600
C
Luciferase (units)
ARF2
0 200 400 600 800 1000
ARF3/ETT
0 200 400 600 800
ARF4
0 100 200 300 400 500 600
ARF5
0 10 20 30 40 50
ARF6
0 50 100 150 200 250
ARF7
0 500 1000 1500 2000 2500
ARF11
0 2e+4 4e+4 6e+4 8e+4 1e+5
PIN1
0 2000 4000 6000

Figure 3-6

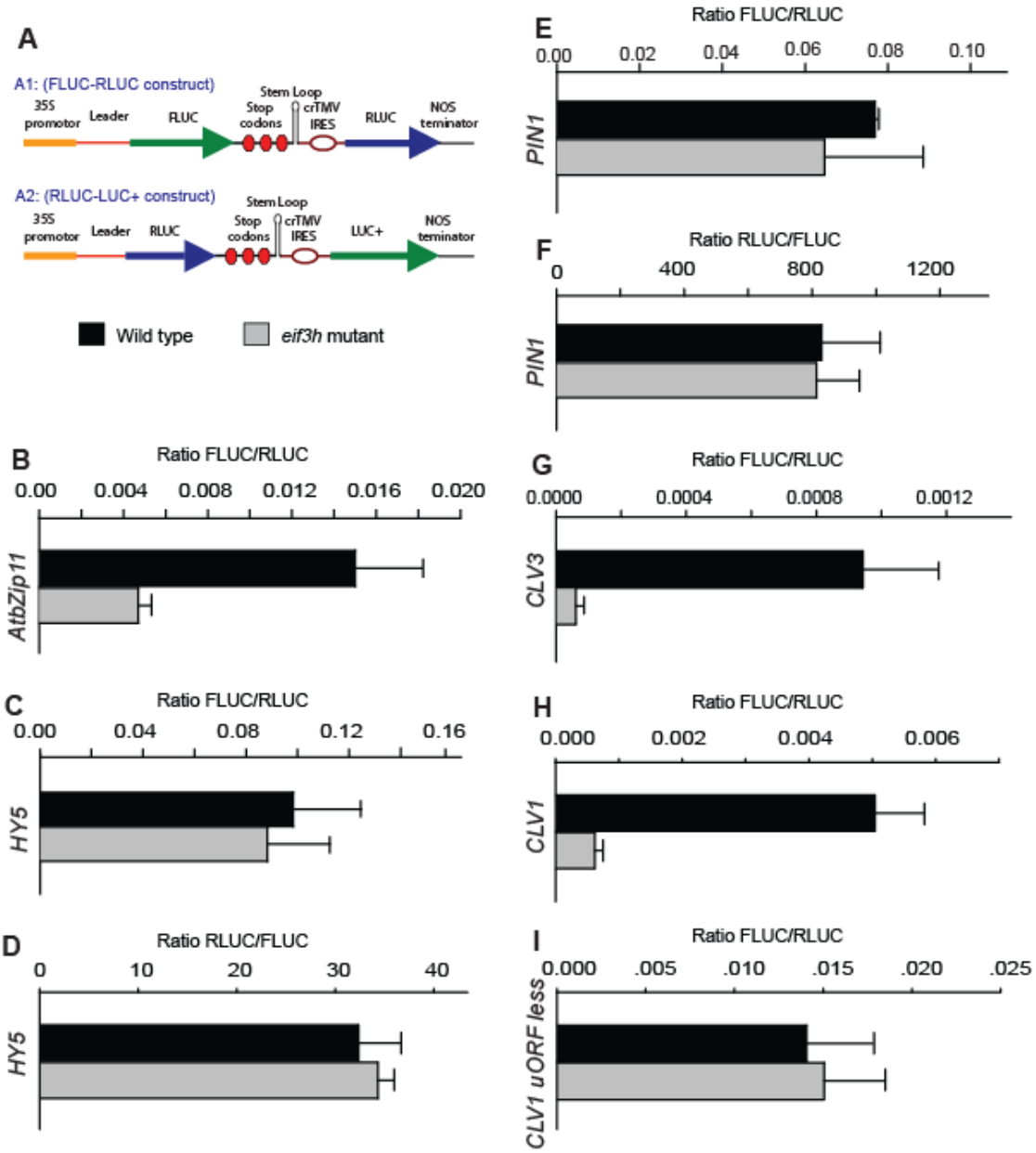

Figure 3-7. Translation assays using crTMV element based constructs. Plasmids carrying crTMV element based dual-luciferase constructs were bombarded into 10 days old wild type (WS ecotype) or *eif3h* seedling. The luciferase activity was measured after 8 hours incubation under florescent light at room temperature.

(A) FLUC-RLUC and RLUC-FLUC based constructs. (Described in chapter 2 (2.3.1)) (B-D) The translation assay results for *AtbZip11* and *HY5* leaders for evaluating the translation assay system. (B) *AtbZip11* leader in FLUC-RLUC construct; (C) *HY5* leader in FLUC-RLUC construct; (D) *HY5* leader in RLUC-LUC+ construct. (E-I) translation assays for *PIN1* and *CLV* genes. (E) *PIN1* leader in FLUC-RLUC construct; (F) PIN1 leader in RLUC-LUC+ construct; (G) CLV3 leader in FLUC-RLUC construct; (H) *CLV1* leader in FLUC-RLUC construct; (I) *CLV1* leader with all uAUGs mutated in FLUC-RLUC construct.

As expected (Kim et al., 2004), *AtbZip11* turned out to be *eif3h*-dependent in this single plasmid assay, while *HY5* was not (**Figure 3-7B-D**). The uORF-less *PIN1* leader was also not affected in the *eif3h* mutant, testing both FLUC-RLUC and RLUC-FLUC dual-luciferase constructs (**Figure 3-7E-F**). However, both *CLV3* and *CLV1* leaders showed significant eIF3h-dependence in the dual-luciferase construct (**Figure 3-7G-H**). Upon removal of all uAUGs by site directed mutagenesis, the uORF-less *CLV1* leader was translated equally in wild type and *eif3h* mutant (**Figure 3-7I**).

3.3.5 Dissection of the *CLV3* leader identifies inhibitory uORFs

The *CLV3* 5' leader was dissected to address which uORFs contribute to translational repression in wild type and *eif3h* mutant plants. The *CLV3* uORFs were clustered into 4 groups; uORF1a1b as Group 1; uORF 2a2b2c as Group 2; uORFs 3 and 4 as Group 3, because they are back to back in frame uORFs; and uORFs 5 and 6a6b, which lie closest to the main *CLV3* start codon, as Group 4. uORF4 in group 3 and uORF5 in group 4 overlap with the main ORF with 38 and 13 codons, respectively. The individual uAUGs were removed as described in section 3.5.2. The translation assays using in vitro transcribed mRNAs indicated that the eIF3h dependence was significantly reduced only if all four groups of uORFs were disrupted (**Figure 3-8A**, lower three constructs). Therefore the different groups are functionally redundant in terms of their eIF3h dependence.

The eIF3h dependence of the wild type and uORF-less *CLV3* leaders was further observed using stable transgenic plants. To this end, the *CLV3* wild-type leader and the uORF-less leader in the FLUC-RLUC dual-luciferase construct were transformed into *eif3h* heterozygous plants. Dual-luciferase assays were performed after Mendelian segregation of wild type and *eif3h* seedlings in the progeny. While the wild-type *CLV3*

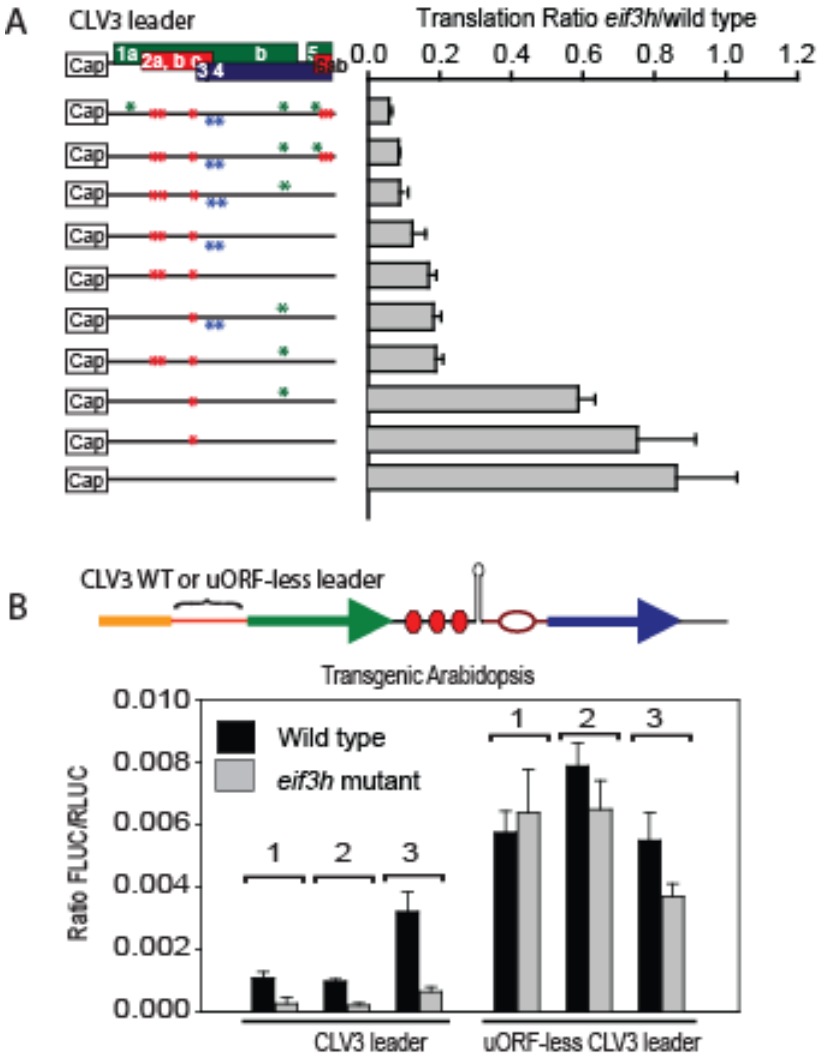

Figure 3-8. Removal of uORFs from *CLV3* and *CLV1* leaders reduces their eIF3h dependence. (A-B) The eIF3h dependence of wild type and mutated *CLV3* leaders. (A) mRNA transformation based assay for wild type and uAUG mutated *CLV3* leaders. (Color-coded * represent the presence of the uAUG in different reading frames. Green, red and blue represent uAUGs in –1 reading frame, in frame and +1 frame to the main AUG, respectively). Standard deviations are shown. (B) CrTMV-element based dual-luciferase construct for wild type and uAUG-less *CLV3* leaders in stable transgenic plant.

leader was less translated in the *eif3h* mutant for all three transgenic lines tested, the uORF-less *CLV3* leader was not significantly affected **(Figure 3-8B)**. The lower translation rate of the uORF-containing leader compared to the uORF-less version further underscores the translation repression roles of these uORFs.

3.3.6 *WUS* and *CLV3* expression in the *eif3h* mutant

Because *CLV3* suppresses expression of the stem cell regulator *WUSCHEL* (Schoof et al., 2000), the reduced translation of *CLV3* and *CLV1* anticipated in the *eif3h* mutant is expected to increase *WUS* expression. Indeed, RT-PCR results indicated that *WUS* mRNA was highly overexpressed in the *eif3h* mutant **(Figure 3-9A)**. Moreover, while expression of a *WUS:GUS* promoter:reporter transgene was restricted to a small domain in the shoot apex of wild-type seedlings, in the apex of *eif3h* mutant seedlings it was not only expressed more highly **(Figure 3-9C-D)**, but also ectopically around the base of fresh leaves **(Figure 3-9C)** and in the cotyledons **(Figure 3-9D)**. *WUS::GUS* expression was also elevated in the inflorescence tip in the *eif3h* mutant **(Figure 3-9I compared to Figure 3-9H)**, and could often be seen expressed ectopically in many organs of the flower and fruit, such as stamens and petals **(Figure 3-9K** compared to **3-9J)** and even seeds (data not shown). Elevated *WUS* expression might also alter *CLV3* transcription as part of a feedback loop. The *CLV3:GUS* expression in the *eif3h* mutant was variable. Some seedlings expressed a level similar to wild type **(Figure 3-9F** compared to **3-9E)**, while in others, *CLV3:GUS* was significantly reduced **(Figure 3-9G)**. *CLV3::GUS* was also variable in the *eif3h* inflorescence tip, but ectopic overexpression was seen besides normal expression **(Figure 3-9M-N** compared to **9L)**. These results are consistent with the notion that undertranslation of the uORF containing *CLV3* mRNA contributes to the meristem expansion observed in the *eif3h* mutant.

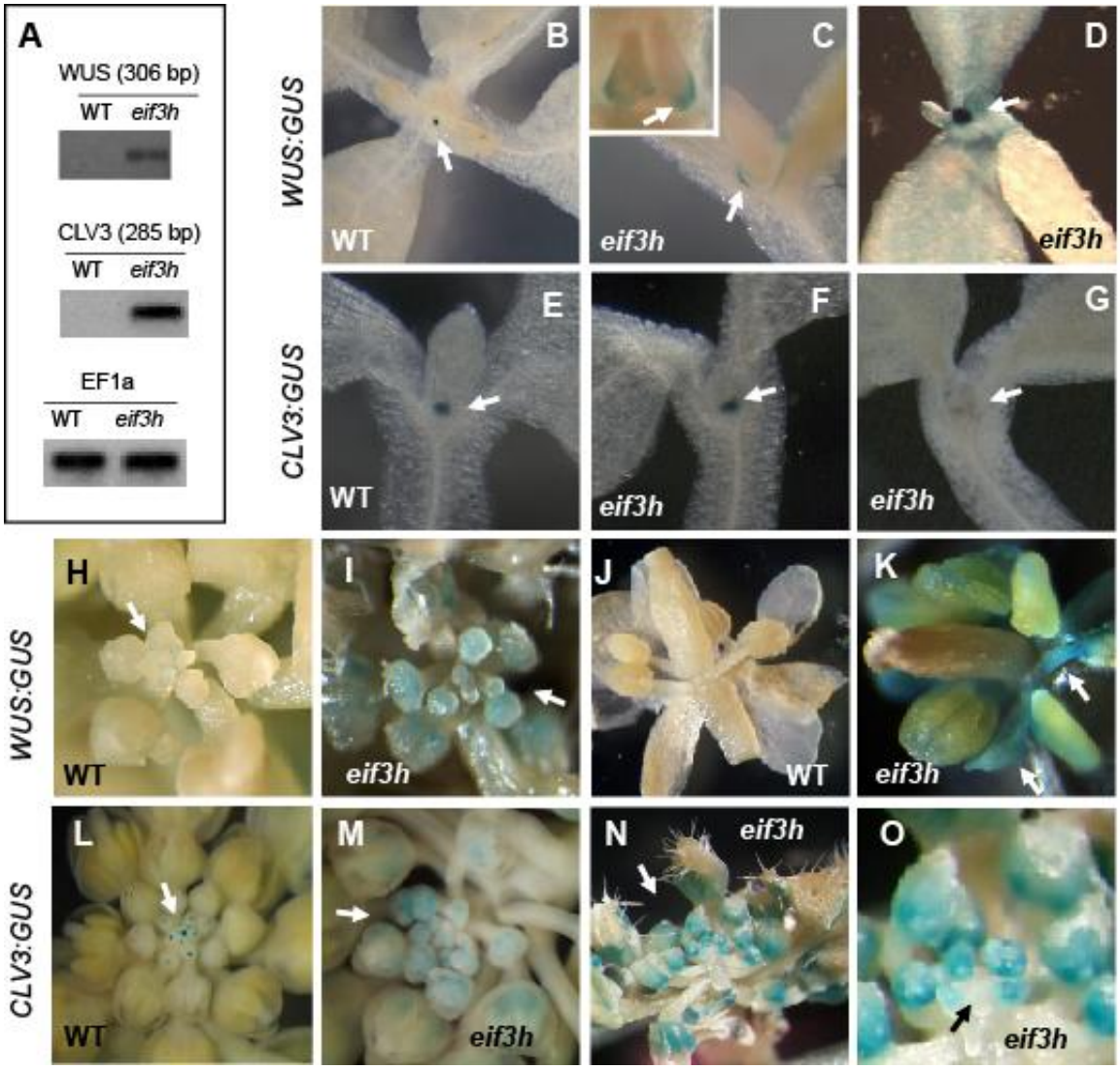

Figure 3-9. *WUS* and *CLV3* expression in the *eif3h* mutant.
(A) Reverse transcription (RT) PCR results for *WUS* and *CLV3* mRNAs. (B-D) *WUS:GUS* expression in wild type (B) and *eif3h* (C and D) seedling. (E-G) *CLV3:GUS* expression in wild type (E) and *eif3h* (F) seedling. (H and I) *WUS:GUS* expression in wild type (H) and *eif3h* (I) inflorescences. (J and K) *WUS:GUS* expression in wild type (J) and *eif3h* (K) flowers. (L-N) *CLV3:GUS* expression in the wild type (L) and *eif3h* (M-O) inflorescences. O is a close up view of N.

3.3.7 Expression of extra copies of *CLV3* genomic DNA partially complements the *eif3h* growth defect

The *CLV3* mRNA is less translated in the *eif3h* mutant, which in turn should trigger overexpression of the homeodomain transcription factor, *WUS*. In the associated feedback loop, *CLV3* itself is also regulated by *WUS* through transcriptional regulation. For some *eif3h* plants, the overexpression of *CLV3* at the mRNA level may partially compensate for the translational defect and thus remedy the defect of the *eif3h* mutation in SAM maintenance.

Our model also predicts that additional copies of the *CLV3* genomic DNA in the *eif3h* mutant will suppress the mutant phenotype by facilitating the feedback balancing. The *CLV3* genomic DNA with 1.76 kb sequence upstream of the start codon and 0.34 kb sequence after the stop codon was amplified and introduced into the *eif3h* mutant by Agrobacterium mediated transformation. The *eif3h* mutant transformed with *CLV3* genomic DNA showed significantly improved growth **(Figure 3-10A-C)**. However, many auxin related phenotypes, such as the *arf3*-like siliques and the increased fruit to stem angle were not significantly affected **(Figure 3-10D-G)**. These data underscore the model that undertranslation of *CLV3* contributes to the defects in meristem maintenance, but less so the defects in auxin responses, seen in the *eif3h* mutant plants.

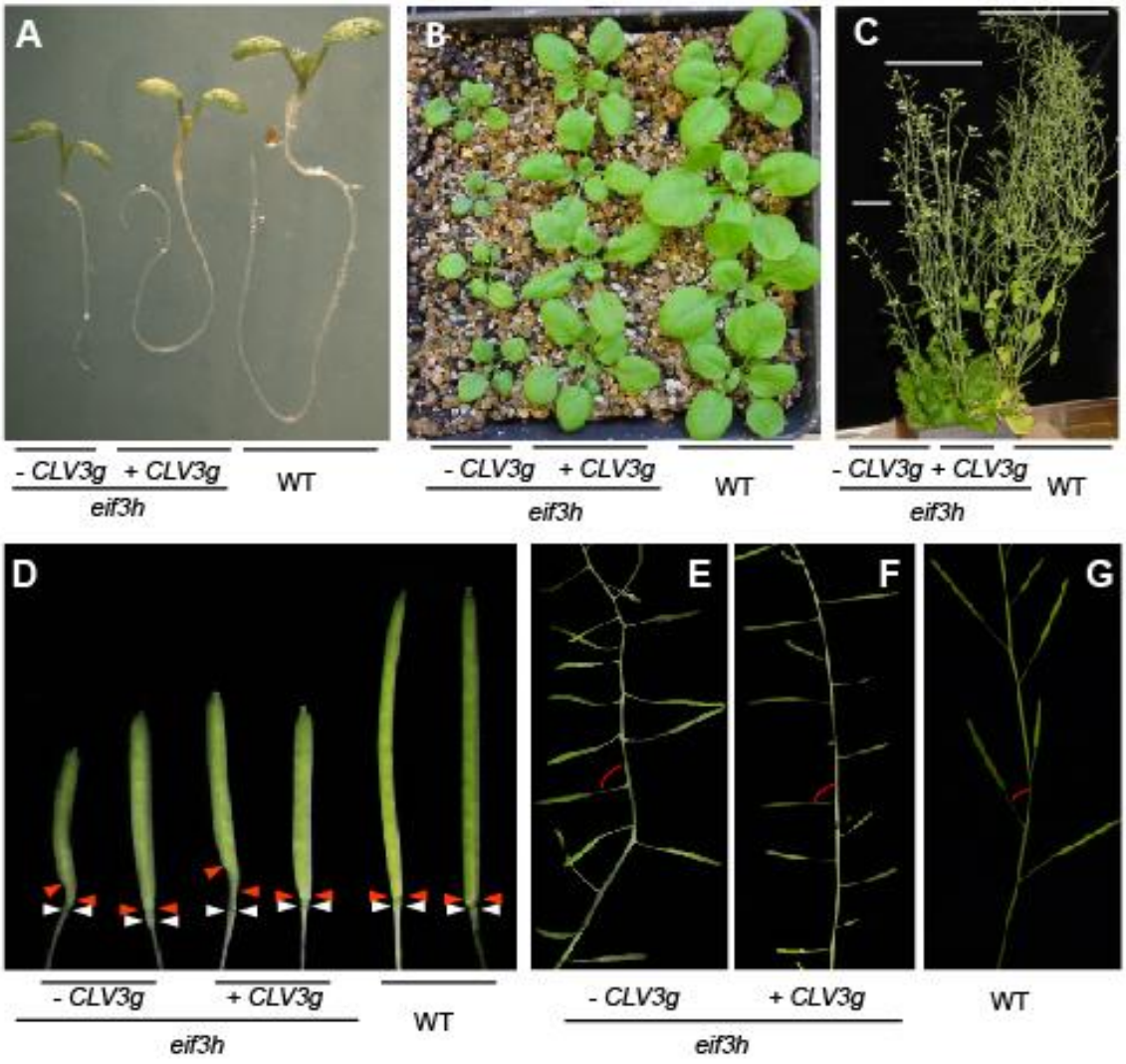

Figure 3-10. A transgene harboring the *CLV3* genomic DNA, including its native promoter partially complements *eif3h* growth defects. (A-C) The growth of *eif3h* mutant plants transformed with *CLV3* genomic DNA at different developmental stages compared to untransformed *eif3h* and wild type. (A) 5 day old seedlings. (B) 2 week old plants. (C) 8 week old plants. (D) The fruits of wild type, and *eif3h* with or without *CLV3* genomic DNA. Note, white arrows point to the abscission zone of the floral organs while red arrows point to the basal end of the carpel valves to indicate carpel initiation defects. (E-G) some *eif3h* mutants have enlarged silique to stem angles (E compared to that of wild type in G), which is also showing in the *eif3h* mutant transformed with *CLV3* genomic DNA (F).

3.4 Discussion

For *Arabidopsis* and other seed producing plants, initiating functional inflorescences with leaves and flowers is very crucial for the species to survive and proliferate. Therefore, the maintenance of the stem cell population in the SAM is especially important. The feedback regulatory loop by the *WUS* and *CLV* genes plays essential roles in SAM maintenance, and it has been intensively studied from different angles (Mayer et al., 1998, Fletcher et al., 1999; Schoof et al., 2000). Auxin, one of the most important plant hormones, is also involved in SAM maintenance and organ initiation (Aida et al., 2002; Reinhardt et al., 2003). Our study on the translation initiation factor subunit, eIF3h, revealed another previously unappreciated aspect of SAM maintenance. *Arabidopsis* eIF3h, whose human homolog is a functional core subunit of eIF3 (Masutani et al., 2007), plays important roles in *Arabidopsis* shoot apical meristem maintenance and organ initiation by promoting efficient translation of mRNAs containing multiple uORFs, specifically those encoding meristem regulators (CLV3 and CLV1) and auxin response factors **(**a model presented in **Figure 3-11)**.

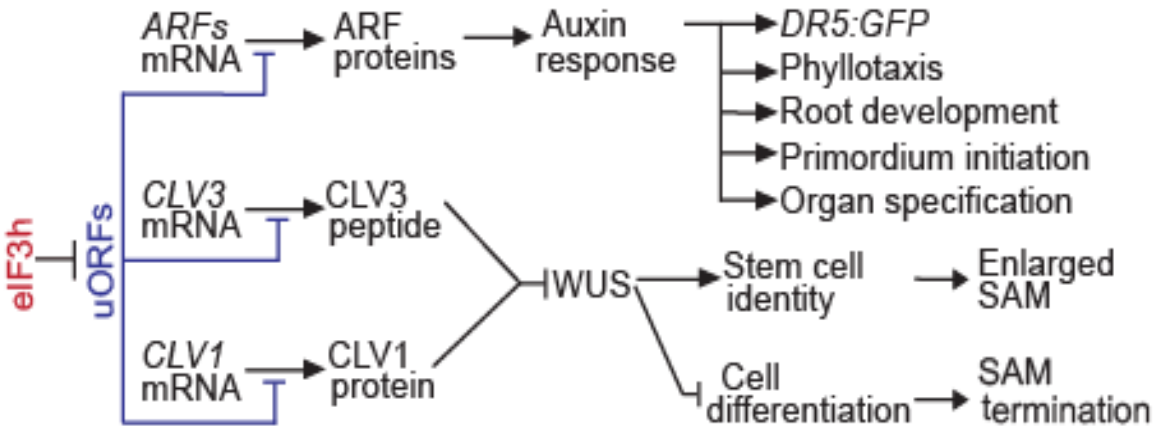

Figure 3-11. A model for the role of eIF3h in *Arabidopsis* shoot apical meristem maintenance and auxin response. By overcoming the translation repression of uORFs, eIF3h promotes the translation of *ARF*s, *CLV*1 and *CLV*3, and therefore plays an important role in SAM maintenance and organ initiation.

3.4.1 Reducing the translation of *CLV*s contributes to an enlarged SAM

Our previous research indicates that the general translation in the *eif3h* mutant was not affected, but specific mRNAs with multiple uORFs, including *AtbZip11* and *LHY* were significantly reduced, indicating the role of eIF3h in the efficient translation of uORF containing mRNAs (Kim et al., 2004). However, reduced translation of neither genes was likely responsible for *eif3h* phenotypes, especially the growth defect on SAM function **(Figure 3-1)**. Here, we identified two major groups of genes, *CLAVATA*s (*CLV3* and *CLV1*) and *ARF*s, whose translational defects in the *eif3h* mutant are responsible for *Arabidopsis* shoot apical meristem maintenance and organ initiation.

CLV3 and *CLV1* are uORF abundant mRNAs **(Figure 3-5)**. As we expected, the translation assay results indicated that both *CLV3* and *CLV1* mRNAs were dramatically less translated in the *eif3h* mutant **(Figure 3-6B and Figure 3-7G-H)**, which is consistent with overexpression of the homeodomain transcription factor WUS as observed by the *WUS::GUS* reporter assay **(Figure 3-9A, C-D)**. Since WUS is responsible for stem cell identity and represses cell differentiation, the overexpression of *WUS* is consistent with the enlargement of the stem cell population in the SAM (Fletcher et al., 1999, Reddy and Meyerowitz, 2005) starting from the early seedling stage, as **Figures 3-1B-C,F and H** show. The shoot apex was enlarged for almost all *eif3h* mutants observed in the vegetative growth stage. Some *eif3h* mutants died without initiating an inflorescence, but for those that survived and succeeded with the reproductive transition, the shoot apex on the inflorescence was similar in size to that of wild type. It suggests that, occasionally, the feed back regulation loop between *CLV3* and *WUS* can be rebalanced in the *eif3h* mutant, most likely by overtranscription of the *CLV3* gene as a consequence of *WUS*

overexpression. This interpretation is further supported by our promoter:reporter assays in **Figure 3-7**, in which both *WUS:GUS* and *CLV3::GUS* are highly expressed in the *eif3h* inflorescence.

3.4.2 Reduced translation of *ARF*s in the *eif3h* mutant affects auxin response and transport

Among many gene groups involved in auxin synthesis and signaling, the *ARF*s but not other gene families we observed have enriched uORFs **(Table 3-1)**, which are potential targets of eIF3h mediated translational control. Two *ARF* genes, *ARF3/ETT* and *ARF5/MP* were previously identified as targets for uORF-mediated translational control, in which a ribosomal subunit, RPL24 (STV) is involved (Nishimura et al., 2005). The translation assay results indicated that *ARFs*, but not *PIN1,* were less translated in the *eif3h* mutant **(Figure 3-6C)**. Because *ARF*s activate the *DR5* promoter (Wenzel et al., 2007), one expects underexpression of *DR5:GFP,* which was observed along with reduced *PIN1::PIN1-GFP* expression in the *eif3h* mutant **(Figure 3-3G-J)**, indicating auxin response and auxin transport were affected by the *eif3h* mutation. The underexpression of *PIN1* in the *eif3h* mutant can be attributed to the reduced translation of *ARF*s, especially *MP*, a positive regulator of *PIN1* and auxin transport (Wenzel et al., 2007; Schuetz et al., 2008). Many *eif3h* mutant phenotypes are reminiscent of defects in auxin transport or response. Among them are *pin*-like shoot with outgrowth of unspecified primordia, *arf3*-like fruit, *arf5*-like single cotyledon and abnormal phyllotaxis. Furthermore, introducing a homozygous *ett-1* allele in the *eif3h* mutant produced a smooth *pin1-like* shoot without primordial outgrowths. Taken together, it is likely that the

auxin related growth defect of the *eif3h* mutant are likely caused by reduced translation of *ARF*s.

3.4.3 The undertranslation of *ARF*s and extreme ectopic WUS expression affect organ initiation on the SAM.

Based on our model, the undertranslation of *CLV3* and *CLV1* may produce a *clv3* or *clv1* like phenotype, both of which have an enlarged SAM and an increased number of flowers initiated on the shoot apex (Clark et al., 1997; Fletcher et al. 1999). However, in *eif3h* mutants that fail to initiate an inflorescence, the shoot apices are continually enlarging (**Figure 3-1B-C**), without lateral organ initiation, or they only initiate a few filamentous leaves **(Figure 3-1 I-L)**. This can be explained by considering that *eif3h* mutant defects on SAM maintenance were caused by reduced translation of not only *CLV* genes, but also other genes, such as *ARF*s. Although direct evidence for the interaction between auxin signaling and *CLV/WUS* pathway is still lacking, auxin has been expected to be "the best-characterized long-range signal that influences stem cell niche specification" (Vernoux and Benfey 2005) and "might act as an upstream signal to maintain the SAM OC" (Fiers et al., 2007).

The establishment of an auxin gradient mediated by the auxin efflux carrier, PIN1, and the influx carrier, Aux1, on the shoot apex is very important for lateral organ initiation (Reinhardt et al., 2003; Benkova et al., 2003). The translation of the *PIN1* leader was not affected in the *eif3h* mutant, but the reporter *PIN1::PIN1-GFP* was significantly less expressed in the *eif3h* mutant, possibly caused by reduced translation of *ARF*s. Both *pin1* and *mp* form rosette leaves, however the *pin1/mp* double mutant or *mp* (but not *pin1*) mutant treated with auxin efflux inhibitor 1-*N*-naphthylphthalamic acid

(NPA) produce enlarged leafless meristem domes (Schuetz et al., 2008), indicating auxin response established by ARFs, especially by MP, on the SAM are responsible for leaf initiation. We believe that the translation reduction of *ARF*s, especially *MP* affects leaf initiation in the *eif3h* mutant, and as an important factor, it contributes to the formation of enlarged naked *eif3h* apical dome.

Another factor for producing naked *eif3h* apical dome is the ectopic expression of WUS. In the *clv* mutant, *WUS* is not only overexpressed, but also expressed ectopically in the shoot and flower meristems (Schoof et al., 2000). The *WUS* expression in the *eif3h* mutant was dramatically increased, and ectopic expression of *WUS* was observed in both shoot and floral meristems **(Figure 3-7)**. Some *eif3h* seedlings expressed *WUS* all over the shoot apex and even in cotyledons, and ectopic *WUS* expression was often seen in many organs of the flower, such as stamens and petals **(Figure 3-7K compared to 3-7J)** and even seeds (data not shown). The mechanism of extreme ectopic WUS expression is not clear, but we speculate that it might caused by the translational defects on not only *CLV3* and *CLV1*, but also other genes that regulate *WUS* (Kwon et al., 2005; Wu et al., 2005; Würschum et al., 2005 Müller et al., 2008; Tucker et al., 2008). Ectopic expression of *WUS* with the *CLV3* promoter induces an enlarged dome shaped SAM between two cotyledons with no lateral organ initiation. (Brand et al., 2002), indicating ectopic WUS expression on the SAM is sufficient to repress organ initiation, which might be also the case in the *eif3h* mutant.

3.4.4 uORFs in eIF3h dependent translational control.

uORFs are involved in translational control in all 3 major groups of eukaryotes (Miller and Hinnebusch 1990; Mehta et al., 2006; Nishimura et al., 2005; Watatani et al., 2008). In the *Arabidopsis* genome, only about 1/3 of mRNAs have uORFs and most of

them have only one uORF. The fraction of mRNAs that harbor a large number of uORFs is very low (Kim et al., 2007). Among mRNA sequences we observed, *ARFs* and *CLV* genes have obviously enriched uORF numbers **(Table 3-1 and Figure 3-5)**, especially *CLV3*, which harbors 10 uAUGs in a leader less than 300 nucleotides long. The high uORF content in *ARF*s and *CLV1,3* genes, suggests the potential regulatory roles of uORFs in SAM maintenance and auxin response. Translation assay have been performed to study the eIF3h dependence of a series of mutant *CLV3* leaders with specific uORF (or URFs) removed. As **Figure 3-8A** shows, the eIF3h dependence of the *CLV3* leader is significantly compromised as all groups of uORFs are removed. It was further proved by the translation assay using plants transformed with dual-luciferase constructs carrying wild type or uORF-less CLV3 leaders **(Figure 8B)**.

The role of eIF3h in the translation of uORF containing mRNAs is not fully understood. It is believed that, if a uORF is translated, the translation of downstream gene is largely dependent on the translation reinitiation. *eIF3h* may act as a regulator or adaptor in the translation reinitiation process (Kim et al., 2004).

3.4.5 Translation and the control of SAM stability.

As one of the most important aspects of plant development, the stem cell population in the SAM has to be tightly regulated for the production of lateral organs and a functional inflorescence. Stem cell fate in the central zone of the shoot apical meristem is tightly regulated by CLV/WUS feedback regulation loop mostly through transcription, as previously characterized (Mayer et al., 1998, Fletcher et al., 1999; Schoof et al., 2000 Lenhard et al., 2003). However, transcriptional regulation is believed to be noisy and stochastic in living cells (Ozbudak et al., 2002; Blake et al., 2003), which may not be sufficient to maintain stable *WUS* level in the feedback regulation loop. The stability of

the stem cell population and *WUS* expression indicates that an additional regulation system is present in the *CLV/WUS* pathway. Interestingly, the leader sequences for both *CLV3* and *CLV1* harbor multiple uORFs. Among the uORFs in the *CLV3* leader, uORF1 is a long uORF (80 codons) with a start codon in a good initiation context (AAA*ATG*G, based on Kozak, 1986), while uORF 4 and 5 overlap with the main ORF. The combinations of 10 uORFs would be detrimental to *CLV3* translation, unless a regulatory protein complex was present to help *CLV3* mRNA to overcome the inhibition by uORFs. The translation initiation subunit, eIF3h, may play this role as a regulator or adaptor in the regulation complex.

Since *WUS* ectopic expression was observed on both seedling apex and inflorescence tip of the *eif3h* mutant, we speculate that the uORF and eIF3h mediated translational control of *CLV3* mRNA may play important roles not only in stabilized WUS level, but also restrict the WUS expression domain in the OC.

3.4.6 Translational control in plant development.

Compared to transcriptional regulation, translational control is believed to be able to provide a more rapid response, because it can bypass the processes of mRNA synthesis, processing and transport (Gingras et al., 1999). Translational control is proposed to play important roles in plant development. However, the mechanisms and target genes for translational control in plant genomes are largely unknown. Two groups reported that many ribosomal large subunits such as RPL5A, RPL5B, RPL10A, RPL9 and RPL28A are involved in leaf polarity and patterning, suggesting their potential roles in translational control of gene expression (Yao et al., 2008; Pinon et al., 2008). Our results indicated that the translation initiation factor subunit, eIF3h is involved in efficient translation of mRNAs encoding *ARFs, CLV1* and *3*. eIF3h likely acts through translation

reinitiation, a similar role was also proposed by Nishimura et al. (2005) for the ribosomal subunits RPL24. The investigations on how eIF3h and other proteins (such as these ribosomal proteins) mediate the translation of uORF containing mRNAs will further enlighten our understanding on the role of translational control in plant development.

In summary, here I presented the first evidence that translational control, and eIF3h, play important roles in *Arabidopsis* shoot apical meristem maintenance and organ initiation by ensuring efficient translation of mRNAs encoding ARFs and CLAVATA proteins. The work also uncovered another important element in the stem cell maintenance feedback regulation loop.

3.5 Methods

3.5.1 Plant growth conditions for phenotypic characterization

Arabidopsis thaliana growth conditions for wild type (Wassiliweskija ecotype) and the *eif3h-1* allele have been described (Kim et al., 2004). 7 day old seedlings growing on agar plates with MS salts and 1% sucrose (*pH 5.7*) were transferred onto vermiculite covered soil in 4 inch X 4 inch square pots. Soil grown plants were under fluorescent light at 22^{o}C with 8 hour light and 16 hour dark for the first two weeks, and switched to 16 hour light and 8 hour dark thereafter. A regular supply of water is important for the survival of *eif3h* mutant plants.

3.5.2 Molecular cloning and plant transformation

The molecular cloning for in vitro transcription constructs and the dual-luciferase assay system were described in Chapter 2.

For phenotypic complementation of the *eif3h* mutant with *CLV3* genomic DNA: CLV3 genomic DNA (including 1.76 kb of promoter 5' of the start codon to 0.34 kb after the stop codon) was amplified from wild type *Arabidopsis* genomic DNA with primers (CLV3g-For: 5' CCCCCGGGATCCCGTGGACTTGGAGTTGATGCAG 3' and CLV3g-Rev: 5' ATAACTAGTCCCAACGGTACATTGCTTTGGAG 3'). The PCR product was cloned into the *EcoR*I and *Xba*I site of binary vector pFGC19, using an *EcoR*I site in the *CLV3* promoter.

Site directed mutagenesis for removing uAUGs from the CLV3 leader: Wild type *CLV3* leader was amplified with primers AT2G27250-FOR1 and REV1, and cloned between the SP6 promoter and RLUC in a pKRX vector as described in chapter 2 (2.3.3). The *CLV3* leader was re-amplified with primers AT2G27250-FOR2, REV2 and cloned between SP6 and RLUC as before, but now with uAUG1a, 5, 6ab removed. To further remove uAUG 2ab, two short PCR products made with M13-FOR, AT2G27250-REV3 and AT2G27250 FOR3, RLUC-REV were fused by a double template PCR (DT-PCR) with primer M13-FOR and RLUC-REV. Using the DT-PCR product as template, a similar approach was applied to further remove uORF 2c, 3 and 4 with primers AT27250-FOR4, REV4 and uORF1b with primers AT27250-FOR5, REV5. The final uORF-less *CLV3* leader and the cloning intermediates were cloned into SP6 based constructs for translation assays. Using the same strategy, uAUGs in *CLV1* the leader were also removed.

Primer list: (Underlined letters represent the substituted nucleotides)

AT2G27250-FOR1: GGGctcgagCACTCAGTCACTTTCTCTCTAAA

AT2G27250-REV1: GGGccatggCTACATGAACATAACACATGAA

AT2G27250-FOR2: GGGctcgagCACTCAGTCACTTTCTCTCTAAAA***C***T

AT2G27250-REV2: GGGccatggCTACA***A***GAACA***A***AACACA***G***GAA

AT2G27250 FOR3 TTCCTTCAT***CT***TGCTTCTGGT

AT2G27250 REV3 ACCAGAAGCAAGATGAAGGAA

AT2G27250 FOR4 AT***T***TGTCTATAACTCAT***C***TAA***T***TGG

AT2G27250 REV4 CCA ATT AGA TGA GTT ATA GAC aaAT

AT2G27250 FOR5 CTC AAG CTC A***A***G CTC ACG TTC

AT2G27250 REV5 GAA CGT GAG CTT GAG CTT GAG

3.5.3 Plant transformation

The *CLV3* genomic DNA in binary vector pFGC19 was transformed to the *eif3h* mutant using floral dip of *Agrobacterium.* The transgenic plants were selected by geminating T1 seeds on MS agar plates with 10 mg/l Basta and verified by PCR. The dual-luciferase expression constructs harboring the *CLV3* leader were transformed into heterozygous *eif3h* mutant plants using the same approach. Wild type (ecotype WS) and the *eif3h* mutant plants for translation assays were obtained from the segregation of second-generation transgenic plants.

3.5.4 DNA based expression assay after transient or stable transformation

Wild type and *eif3h* mutant plants were grown on MS agar plates with 1% sucrose. Plasmids carrying dual-luciferase constructs were introduced into 10-day old wild type and *eif3h* mutant seedlings by particle bombardment as previously described (Kim et al., 2004). Transfected seedlings were incubated at 22^{o}C in a lighted growth chamber for 8 hours before assaying for luciferase activity. Luciferase activities were measured in a protein extract using the Dual Luciferase system (Promega, Madison, WI)

in the TD-20/20 luminometer (Turnerdesigns, Sunnyvale, CA). The mean FLUC to RLUC ratio (for construct **A1** in **Figure 3-7A**) or the RLUC to FLUC ratio (for construct **A2** in **Figure 3-7A**) from 3 or 4 replicates were used to compare the translation efficiency between wild type and *eif3h* mutant.

3.5.5 GUS staining

Arabidopsis seedlings or inflorescences were prefixed in 90% acetone for 20 min, rinsed briefly in staining buffer without X-Gluc and infiltrated in staining buffer (0.05M phosphate buffer, *pH 7.2*; 0.2% Triton X-100; 2mM potassium ferrocyanide and 2mM potassium ferricyanide; 2mM X-Gluc) in vacuum for 30 min, followed by incubation at room temperature for 6 hours. After dehydration with an ethanol series up to 50% *EtOH* (20%, 35% and 50%), tissue was fixed in FAA (50% EtOH, 10% acetic acid and 5% formaldehyde) for 30 min at room temperature, then dehydrated completely with 70%, 85% and 100% EtOH.

3.5.6 Reverse transcription PCR

First strand cDNA were synthesized with M-MLV reverse transcriptase (Promega) and oligo (dT) primers using RNAs prepared form 7 day old *Arabidopsis* seedlings. PCR for *WUS* and *CLV3* was for 40 cycles and for *EF1 α* was 28 cycles. The gene specific primers for each gene are: *WUS* (forward: 5'-CCCAGCTTCAATAACGGGAAT-3', reverse: 5'-ACCGTGCATAGGGAAGAGAG-3'), *CLV3* (forward: 5'-ATGTCCGGTCCAGTTCAACA-3, reverse: 5'-TCAAGGGAGCTGAAA GTTGT-3') and *EF1*α (forward: 5'-GATGAGACTTTCGTTATGA TCGAC-3'; reverse: 5'-ATTGAAAACCATAATAAAAAGTCT CAGA-3').

The protoplast preparation and PEG mediated mRNA transformation were described in chapter 2 (2.3.4)

Chapter 4: Discussion and future directions

The human eIF3h is involved in forming the functional core of eIF3, based on in vitro reconstitution results (Masutani et al., 2007), indicating it may play an important role in translation initiation. The human eIF3h has been observed to affect protein translation efficiency, and is involved in oncogenesis (Zhang et al., 2008), indicating it may play important regulatory role in translational control. The fission yeast eIF3h does not affect general translation, but the eif3h mutant has a defect on spore formation and is hypersensitive to caffeine (Ray et al., 2008). eIF3h is not present in budding yeast. Therefore, while clearly conserved through evolution, eIF3h is not an essential component of the eukaryotic cell.

Although *Arabidopsis* mutants harboring insertions in the *eIF3h* gene are able to survive and proliferate, they show various severe growth defects, such as growth retardation, a less developed root and reduced fertility, indicating that eIF3h, indeed affects many aspects of plant development. This study on identifying candidate genes for eIF3h mediated translational control reveled an important role of eIF3h in *Arabidopsis* stem cell regulation, and therefore provided the first evidence for the presence of translational control in SAM maintenance.

4.1 Reduced translation of *CLV1, 3* and SAM enlargement

Our results indicated that reduced translation of genes encoding stem cell regulators (*CLV1* and *CLV3*) and auxin response factors (*ARF*s) contribute to the growth defects of the *eif3h* mutant during shoot apical meristem maintenance and organ initiation. As a stem cell marker gene, *CLV3* is expressed predominately in the outer two cell layers in the central zone of the SAM. CLV3 peptide restricts *WUS* expression in the

organizing center of the SAM through its receptor complex consisting of CLV1 and CLV2. WUS maintains stem cell identity in the CZ, and also promotes *CLV3* expression to form a feedback regulation loop (Fletcher et al., 1999; Brand et al., 2000). The *clv* loss-of-function (Clark et al., 1993, 1995) and *wus* gain-of-function (Schoof et al., 2000) mutants develop an enlarged SAM. The dynamic and quantitative effects of *CLV3* on SAM stability and WUS expression have been observed using both inducible *CLV3* silencing (Reddy and Meyerowitz, 2005) and *CLV3* overexpression systems (Müller et al., 2006). With the inducible RNAi silencing system, Reddy et al. revealed that a dramatic increase in SAM size occurs as early as 48 hours after induction (HAI) of *CLV3* silencing, and WUS activity (as indicated by *pCLV3:mGFP5-ER* expression) was induced as early as 24 HAI. The enlargement of the CZ was caused not merely by increased cell division but also by switching the fate of cells in the peripheral zone to revert to, or remain as, stem cells. These results indicate that the reduction of *CLV3* expression was able to rapidly promote *WUS* expression and trigger SAM enlargement. To observe the effect of *CLV3* overexpression on *WUS* activity, Müller et al. (2006) applied an inducible system to overexpress *CLV3* in its native domain. The results indicate that WUS expression responded to *CLV3* induction in a rapid, but transient manner. *WUS* expression was significantly reduced as early as 3 HAI of *CLV3*, but recovered after 72 HAI, although the *CLV3* mRNA was still very high (>400 times above uninduced level), indicating that the *CLV/WUS* feedback regulation loop is able to tolerate a broad range of *CLV3* mRNA fluctuation. In the *eif3h* mutant, both *CLV3* and *CLV1* mRNAs were significantly less translated. Reduced translation of *CLV* genes at early stage should be able to promote WUS expression and affect SAM size.

4.2 Elevated *CLV3* transcription was unable to compensate the defect on *eif3h* SAM size

We proposed the function of uORF-eIF3h-mediated translational control in SAM maintenance **(Figure 4-1)**. As Figure 4-1A shows, in the wild type, eIE3h might function as an adaptor for a regulator or a group of regulators, which are able to overcome the translation repression of uORFs in mRNAs. By regulating *CLV3* translation efficiency, a relatively stable CLV3 peptide level can be maintained despite a fluctuating *CLV3* transcript level, and the *WUS* expression and stem cell stability can be tightly controlled. The organ initiation on the SAM is triggered by auxin responses mediatedby ARFs, another group of genes whose efficient translation requires eIF3h.

In the *eif3h* mutant, *CLV1, 3* and *ARFs* were less translated as observed **(Figure 3-6 and 3-7).** Reduced *CLV* translation in vegetative seedlings enhances the expression of *WUS*, and therefore produces a larger shoot apex **(Figure 4-1B)**. The overexpression of *WUS* also boosts *CLV3* transcription, which may even compensate for the *CLV3* translational defect and recover a higher CLV3 peptide level. However, the translational reduction of *CLV1* will reduce the effectiveness of the CLV3 peptide on *WUS* repression. The *CLV/WUS* feedback regulation will be reestablished with elevated *WUS* and *CLV3* transcript levels, as observed. The early *eif3h* phenotype might not be caused only by underttranslation of *CLV1* and 3, but may also involve upregulation of *WUS* by other means that are not compensated by the transcriptional induction of CLV3 by elevated WUS expression. For example, I believe that undertranslation of *ARFs* and other mRNAs in the leaf primordia (ARFs, AS1, AS2 etc) leads to slow leaf blade outgrowth; evidence being that mutations in *arf5/pin1* can contribute to overgrowth of the SAM (Schuetz et al., 2008), similar to WUS overexpressors (Brand et al., 2002) and the

eif3h mutant **(Figure 3-1)**. It is possible that the slow leaf outgrowth is sensed by the SAM and interpreted as a sign of insufficient stem cell division activity. *WUS* then becomes overexpressed through an unknown pathway as a way for the SAM to remediate this problem.

Elevated *CLV3* transcription by overexpressed *WUS* was not always sufficient to overcome the defect on the SAM in *eif3h* mutants (Alarge portion of *eif3h* plants never initiate a functional inflorecence), but introducing an additional *CLV3* transgene into the *eif3h* genome significantly improved *eif3h* growth **(Figure 3-10)**. As explained in **Figure 4-1C**, addition of one or more *CLV3* genes changed the *CLV/WUS* feedback regulation loop so that multiple *CLV3* genes now feed forward onto a single *WUS* gene. *WUS*expression is repressed by CLV3 signals provided by multiple gene copies, and *WUS* activates transcription of multiple *CLV3* genes simultaneously. This will facilitate the reestablishment of feedback regulation (especially in early vegetative growth) with reduced *WUS* expression and also reduced *CLV3* transcripts from each *CLV3* gene. The SAM size will be reduced compared to *eif3h* mutants without the *CLV3* transgene (**Figure 4-1C** compared to **B**).

uORF-eIF3h-mediated translational control may be also involved in the compensation of *WUS* expression after *CLV3* induction (Müller et al., 2006). While the induction of *CLV3* silencing by RNAi causes an enlarged SAM and expanded *WUS* expression (Reddy and Meyerowitz, 2005) **(Figure 4-1D)**, the induction of *CLV3* expression had a rapid but transient effect on *WUS* repression (Müller et al., 2006). *WUS* transcripts were significantly reduced after 3 hours of *CLV3* induction, but bounced back within 72 hours of continuous *CLV3* induction (6 hours a day). The authors proposed the presence of a second feedback regulation loop in *WUS* expression regulation, for overcoming the effect from strong *CLV3* fluctuation and ensuring stem cell

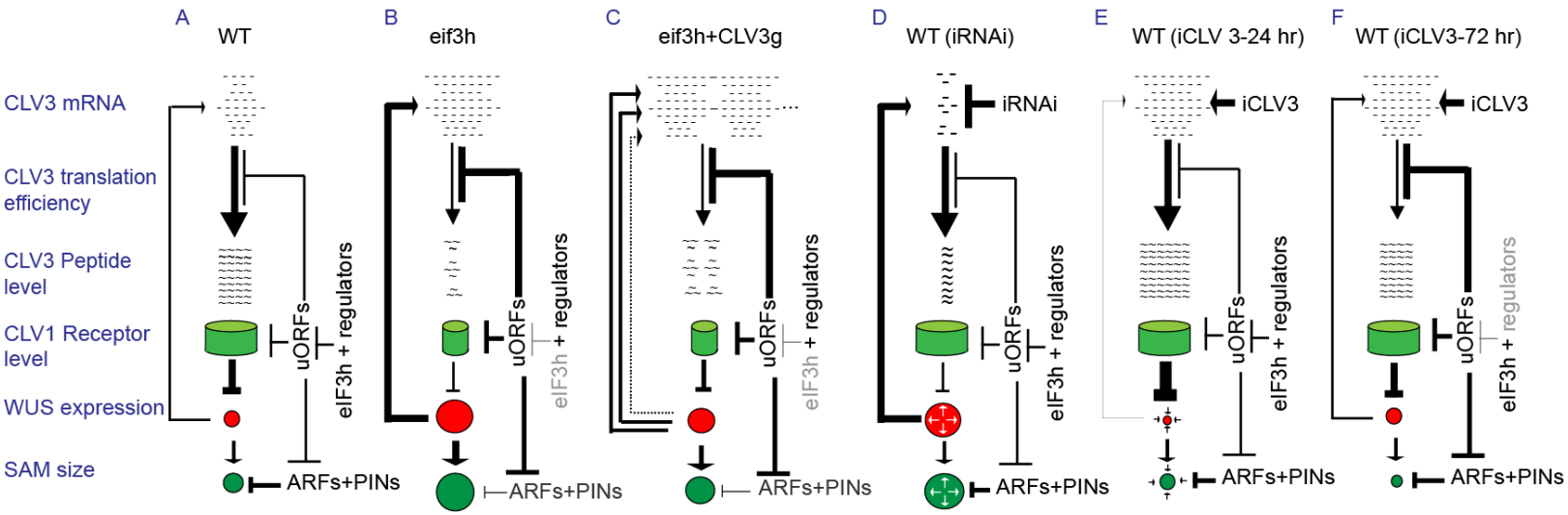

Figure 4-1 The potential role of uORF-eIF3h-mediated translational control in SAM maintenance. A. In the wild type, eIF3h might act as an adaptor for a regulator or regulators, by which *CLV1 and CLV3* translation can be regulated to ensure the stable *WUS* expression and constant stem cell population. The auxin signal established by ARFs and PINs also participates in SAM size control by promoting organ initiation to incoporate sells in the shoot apex to lateral organs. B, In the *eif3h* mutant, reduced *CLV3* and *CLV1* translation enhances *WUS* expression and induces SAM enlargement. Reduced auxin response by undertranslation of *ARFs* hinders organ initiation, therefore boost SAM enlargement. C. Introducing additional *CLV3* genes into the *eif3h* genome makes the feedback regulation loop changed to multiple *CLV3* genes and single *WUS* gene. As a result the feedback loop will established with reduced *WUS* level. D. In the inducible RNAi *CLV3* silencing by Reddy and Meyerowitz (2005) *CLV3* mRNA depletion cause SAM enlargement. E and F show the *CLV3* transcription induction by Müller and coworkers (2006). The reduction of *WUS* transcription (E) caused by *CLV3* induction (3-24 hours after induction) can be compensated over time, 72 hours after *CLV3* induction (F). The regulator may respond to high *CLV3* mRNA level and reduce *CLV3* translation.

stability. We speculate that the uORF- eIF3h-mediated translational control in the shoot apical meristem may be partly responsible **(Figure 4-1 E-F)**. In response to a high *CLV3* mRNA level, the regulator in **Figure 4-1E** reduced its activity as a translational activator. Therefore the translation of *CLV3* is repressed by uORFs and *WUS* level is recovered **(Figure 4-1F)**.

4.3 Ectopic expression of *WUS* affects organ initiation

Ectopic expression of *WUS:GUS* was observed in the *eif3h* mutant seedlings and flowers as shown in **figure 3-9C-D and K**, indicating that not only the expression level, but also the expression domain of *WUS* were affected in the *eif3h* mutant. It is consistent with the ectopic *WUS* expression observed in *clv* mutants (Schoof et al., 2000), suggesting that ectopic WUS expression in the *eif3h* mutant at least partially resulted from the undertranslation of *CLV1* and *CLV3.* However, we do not rule out the involvement of other undertranslated genes, since *WUS* expression is also affected by other genes in different pathways (Kwon et al., 2005; Wu et al., 2005; Würschum et al., 2005 Müller et al., 2008; Tucker et al., 2008).

Ectopic *WUS* expression is sufficient to induce *CLV3* expression and promote SAM enlargement in the vegetative seedlings (Schoof et al., 2000). The Arabidopsis seedling expressing *WUS* in the *CLV1* domain displays an enlarged and fasciated shoot meristem, and expression of *WUS* under the *AINTEGUMENTA* promoter produces a mass of meristem cells on the shoot apex without leaf formation (Schoof et al., 2000). Similarly, expression of *WUS* in the *CLV3* domain induces a large dome-shaped SAM without leaf formation (Brand et al., 2002). In the flowers, the *WUS* expression and stem cell identity are terminated by a MADS domain transcription factor AGAMOUS (AG),

which is also activated by WUS in the center of the floral meristem (Lenhard et al., 2001). The mutation of *AG* causes ectopic *WUS* expression and induces continuous interior flower formation inside the third whorl. Ectopic *WUS* expression by the *APETALA3* (*AP3)* promoter induces floral organ transformation, in which, the second whorl of the flower was transformed into numerous stamens like organ or even a gynoecium surrounded by large number of stamens (Lenhard et al., 2001).

The mechanism of ectopic *WUS* expression in the *eif3h* mutant seedling apex and flowers is still not clear. I propose, however, that the ectopic *WUS* expression is likely to induce SAM enlargement and affect organ initiation as described above (Schoof et al., 2000; Brand et al., 2000). Extremely high ectopic WUS expression observed in the second and third whorls of *eif3h* flowers **(Figure 3-9K)** is likely to induce organ transformation as described by Lenhard and coworkers (2001). Consistent with our prediction, the *eif3h* mutant does form a flower-within-flower phenotype, in which the third whorl organs (stamens) in the flower switched their identity to small flowers (with all 3 inner whorls intact) **(Figure appendix II-3)**.

4.4 Translational control of auxin response factors

Due to its complexity, the mechanism of auxin response in the plant is still not fully characterized. Especially the biological function of the individual auxin response factors is largely unknown. It is not clear to what degree the ARFs are functional redundant, but based on phenotypic observation of single and double mutants many ARFs were proposed to be functional sister pairs, such as ARF1 and 2, ARF3 and 4, ARF6 and 8, ARF7 and 19 (reviewed by Guifoyle and Hagen et al., 2007). Collectively, ARFs regulate a large group of genes by binding to the auxin response element

(TGTCTC). While many ARFs are transcriptional activators, others are transcriptional repressors (Ulmasov et al., 1999; Wilmoth et al., 2005).

ARFs are negatively regulated by a group of transcriptional repressors, the Aux/IAA (Auxin/indole acetic acid) proteins through physical interaction in an auxin dependent manner (**Figure 4-2**). Aux/IAAs are regulated in a feedback regulation loop by ARFs, and a protein degradation pathway triggered by SCF^{TIR1}, an E3 ubiquitin ligase later observed to be an auxin receptor (Dharmasiri et al., 2005; Kepinski et al., 2005). Many ARFs, such as ARF6, ARF8, ARF3 and ARF4, are regulated by miRNA or siRNAs (Wu et al., 2006; Hunter et al., 2006, Wang et al., 2005) or by uORF-mediated translational control based both on our results and those of Nishimura and coworkers (Nishimura et al. *2005*). ARFs also affect directional auxin transport by regulating the polar subcellular and cellular localization of the PIN auxin efflux carrier proteins (Sauer et al., 2006; Wenzel et al., 2007). The long-range and short-range directional auxin transport mediated by PIN proteins is responsible for the establishment of auxin gradients. The maximal auxin accumulation in an initiating primordium activates specific ARFs and promotes cell differentiation (Reinhardt et al., 2003; Friml et al., 2003; Benkova et al., 2003).

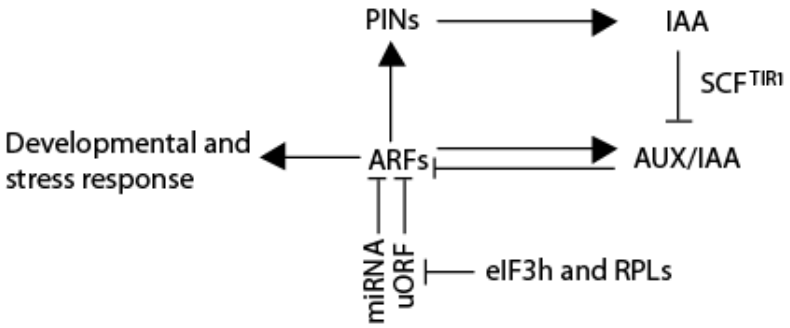

Figure 4-2. The regulation network of ARFs

In addition to *ARF3* and *ARF5*, which were also reported to be less translated in the *stv-1* mutant (Nishimura et al., 2005), we found other *ARF*s, including *ARF2, ARF4, ARF6, ARF7* and *ARF11,* that were also significantly less translated in the *eif3h* mutant. The following discussion will focus on the effect of *ARF5* undertranslation on *eif3h* development, since *ARF5* showed the most dramatic translation reduction in the *eif3h* mutant.

ARF5, one of the best-characterized ARFs, plays essential roles in embryogenesis and vascular development (Hardtke and Berleth, 1998). The interaction between *ARF5* and its regulator *IAA12* is the best established pair among the ARF-Aux/IAA feedback regulation loops (Hamann et al., 1999; Weijers et al., 2006). The *arf5* mutant also showed significantly reduced *PIN1* and *DR5::GFP* expression (Wenzel et al., 2007). The *arf5* and *pin1* mutants show similar defects on *Arabidopsis* apical patterning and perturbed *CUC* gene expression (Aida et al., 2002). Furthermore, the *arf5/pin1* double mutant or *arf5* treated with the auxin efflux inhibitor, NPA (1-*N*-naphthylphthalamic acid) forms an enlarged leafless shoot apical dome between the cotyledons (Schuetz et al., 2008). Those results indicate that ARF5 plays a very important role in shoot apex maintenance by promoting leaf initiation. Since ARF5 and many other *ARF*s were significantly less translated in the *eif3h* mutant **(Figure 3-6),** it is possible that reduced translation of *ARF*s in *eif3h* also affects PIN-mediated auxin canalization on the shoot apex (Sauer et al., 2006, Wenzel et al., 2007). One can hypothesize that, under certain circumstances, the *eif3h* mutant failed to accumulate auxin (or auxin response) to a sufficiently high level in the I1 primordium, and is therefore unable to promote cell differentiation for initiating a leaf (**Figure 1-13**).

In conclusion, two groups of uORF containing genes, encoding stem cell regulators (CLV1 and 3) and ARFs, were significantly less translated in the *eif3h* mutant. We propose that, uORF-eIF3h-mediated translational control is involved in *Arabidopsis* shoot apical meristem maintenance and organ initiation.

4.5 Future directions

The regulation network in uORF mediated translational control

In the budding yeast, the uORFs in the mRNA encoding the transcription factor GCN4 have been characterized as a sensor for the translational control. A model has been proposed for the coordinate regulation of uORF1 and 4 in the *GCN4* leader, based on our understanding of the translation reinitiation machinery **(Figure 1-5)** (reviewed by Hinnebusch, 2005). Although uORFs are involved in translational control of specific mRNAs in *Arabidopsis*, the mechanism of uORF-mediated translation is largely unknown.

eIF3h as a translation initiation factor involved in the efficient translation of uORF containing mRNA in *Arabidopsis* cells may provide us an important clue for characterizing the regulation network of uORF mediated translation. I have attempted to identify the interaction partners of eIF3h using yeast two hybrid (Y2H) and bi-molecule florescent complementation (BiFC) approaches. The yeast two-hybrid experiments were unsuccessful, because of the self-activation of the bait construct consisting of the eIF3h ORF and GAL4 DNA binding domain. Although BiFC experiments were able to document the interaction between eIF3h and other proteins, such as CSN1, the BIFC technique is not suitable for high throughput screening.

Recently many ribosomal proteins, such as RPL24 RPL5A, RPL5B, RPL10A, and RPL9 were proposed to play roles in translational control of genes involved in specifying leaf polarity and fruit development (Nishimura et al., 2005; Yao et al., 2008; Pinon et al., 2008). Among them, RPL24 was observed to affect the translation of uORF containing genes encoding auxin response factor 3 and 5 (ETT and MP, respectively) (Nishimura et al., 2005), which were also less translated in the *eif3h* mutant. Both eIF3h and RPL24 mediated translation were proposed to involve the translation reinitiation machinery, but it is not clear if they act in the same or separate pathways. Studying the protein interactions between eIF3h and many identified translational regulators such as RPL24 will provide us important information for understanding the mechanism of translational control in plants.

Other candidate genes contribute to the *eif3h* growth defect.

Our lab has identified the reduced translation of many genes in the *eif3h* mutant, including *AtbZip11, LHY,* many *ARFs* and *CLVs*. The microarray experiment for comparing the polysome loading of mRNAs between wild type and the *eif3h* mutant indicates that many functional groups of mRNAs tend to be in reduced translation state in the *eif3h* mutant, such as genes encoding transcription factors and proteins for protein modification. We believe that the translation of groups of other genes is also affected by the eIF3h mutation, and they are likely to contribute to various aspects of *eif3h* defect.

The *eif3h* mutant also shows growth defects on other aspects of plant development, such as root elongation, leaf development and reduced tropisms **(Appendix II)**. Interestingly, many *eif3h* phenotypes are surprisingly similar to the plants with mutations in genes encoding ribosomal proteins, such as RPL24. Among them are needle-like leaves and ectopic outgrowths (Yao et al., 2008; Pinon et al., 2008). The phenotypic similarity on leaf development between *eif3h* and the mutants with defects on

genes encoding ribosomal proteins suggests that those ribosomal proteins and eIF3h may be involved in the translational control of the same genes in leaf development, such as those genes encoding *ASYMMETRIC LEAVES* (*AS1* and *AS2*), which also harbor multiple uORFs.

Many *eif3h* defects shown in appendix II may provide us clues for studying other aspects plant development involving eIF3h, such as ectopic organ initiation on the leaf surface **(Figure appendix II-1)**, aberrant lateral organ specification **(Figure appendix II-2)** and flower-within-flower phenotypes **(Figure appendix II-3)**.

It is common to see abnormal outgrowth on abaxial midrib of the *eif3h* mutant rosette leaves. While some outgrowths look like adaxalized or abaxialized leaves or even a adventitious shoot with few small rosette leaves, some just look like the extra extension from the midrib **(Figure appendix II-1)**. It is not clear what causes those ectopic outgrowths, but I expect the ectopic *WUS* expression and reduced *ARF* translation may be involved, since ectopic expression of *WUS* causes ectopic organ initiation (Xu et al., 2005), and even induces somatic embryogenesis from various tissues (Zuo et al., 2002). We did not observe STM (another homeodomain transcription factor for SAM maitenance) expression, but reduced auxin response may affect STM expression domain, since auxin is a negative regulator of STM (Heisler et al., 2005). If that is the case, ectopic expression of *STM* and *WUS*, is likly to trigger aberrant organ initiation (Gallois et al., 2002).

Occationally *eif3h* forms needle-like rosette **(Figure 3-1 I-L)** or cauline leaves **(Appendix II-2 A)**, and sometime the lateral organs in the inflorescence tip (the position for flower initiation) failed to develop to flower, but form abaxialized-leaf-like structure **(Appendix II-2B-C)**. Reduced translation of *ARF*s, especially *ARF*3 and ARF4, may be involved in this phenotype, since the *arf3/arf4* double mutant shows defects on leaf

symmetry (Pekker et al., 2005). Other uORF contaning genes, such as *AS1* and *AS2*, may also less translated in the *eif3h* mutant, and affecte *eif3h* leaf polarity.

Rarelly, *eif3h* mutant plants also have flower within flower phenotype (FWF) **(Appendix II-3B1-B2)**, in which, one or a few stamens in the *eif3h* mutant flower switched their identities to small whole flowers. The small flower has almost all parts intact, except sepals. Interestingly the *eif3h* mutant carrying dual-luciferase constructs with *CLV3* leader or *CLV3* uORF-less leader (**Appendix II-3A1 and A2 respectively**) showed dramatically elevated chances of having FWF flowers (**Appendix II-3 C1-C4 and D1-D4 respectively**). The mechanism causing *eif3h* FWF phenotype is not clear, but, the ectopic *WUS::GUS* expression in **Figure 3-9K** may give us some clue, since *WUS* is highly expressed in the stames of *eif3h* flower. It is possible that ectopic expression of WUS, with other unknown factors contribute to FWF phenotype.

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Appendices

Appendix I mRNA transformation of *Arabidopsis* protoplasts prepared from seedlings

The Arabidopsis seedling protoplasts preparation protocol is based on Jen Sheen's protocol for preparing protoplast from rosette leaves. (http://genetics.mgh.harvard.edu/sheenlab/protocols_reg.html)

1) Plant Growth

The *Arabidopsis* seedlings were grown on MS plates (*pH 5.7*) with 1% sucrose. Wild type seed or the *eif3h-1* mutant *Arabidopsis* seed were sterilized with 30% bleach and washed with double distilled water (DD H_2O) for 3 times before plating. Plated seeds were incubated in 4 ^oC at least 1 day for WT, and 1-2 weeks for the *eif3h* mutant before transferred to fluorescent light growth chamber. The seed germination of the *eif3h* mutant was dramatically improved after long cold treatment.

2) Prepare Cellulose Solution

Digestion Medium	**for 10 ml**	**for 20 ml**
0.4 M mannitol	5 ml 0.8 M	10.0 ml
20 mM MES pH 5.7	1 ml 0.2 M pH 5.7	2 ml
1.5% R10 cellulase	150 mg	300 mg
0.3% R10 macerozyme	30 mg	60 mg
20 mM KCl	0.2 ml 1 M	0.4 ml
55oC for 10 min incubation, then cool down to RT and add,		
10 mM $CaCl_2$	0.1 ml 1 M	0.2 ml

0.1% BSA	10 mg	20 mg
5 mM mercaptoethanol	3.5 µl 14.2 M	7µl
H_2O	3.7 ml	7.2 ml

For sterilization filter the digestion medium through a 0.20 or 0.45 µm syringe-filter.

3) Seedling Cutting

Pour 10ml cellulase solution into a petri dish. Generally use 1-2 petri plates of 1 week old seedlings. Place 20-50 seedlings with roots removed on a piece of paper and slice them into thin strips (~0.5 mm) with a single edge razor blade. The cutting was not performed in the digestion solution. Therefore the sliced tissue has to be immerged in digestion solution immediately after cutting. Vacuum infiltrate it for 30 min with bench vacuum. Cover the box or wrap with foil, incubate at room temperature for 3 hours.

4) In vitro Transcription for mRNA Preparation.

The in vitro transcription follows the manufacturer's protocol from Promega (http://www.promega.com/tbs/9pip108/9pip108.pdf) For a 50 µl reaction: 1X RNAPol reaction buffer (5 µl); 1.25 µl RNAsin (50 units) (PROMEGA Cat. N251B); 10 µl rNTP cap mix (5mM rATP, 5mM rUTP, 5mM rCTP, 0.5mM rGTP, PROMEGA, Cat. # P1221) 2.5 µl 10 mM 7mG(ppp)G RNA Cap Structure Analog (NEB, Cat: S1404S, resuspended in 131 µl H_2O as the manufacture recommended); 2 µl SP6 RNA polymerase (40 units) (NEB Cat. # M0207S), and 4-10 µg *Pvu*II digested plasmid DNA (for transcripts that have a *Pvu*II site, *Pci*I will be used for digestion).

After 2 hours incubation at 40 ^{o}C 1 µl addtional SP6 polymerase was added for 1 hour incubation. The mRNA was observed by agarose gel electrophoresis for quantity and quality, and was used for transformation immediately after in vitro transcription.

5) Prepare W5, PEG and MMG solutions

W5 Solution (Stock at 4^{o}C, bring 50 ml Falcon tube to RT for exp)

	for 50 ml	***for 500 ml***
154 mM NaCl	7.70 ml of 1.0 M NaCl	4.50 g
125 mM $CaCl_2$	6.25 ml of 1.0 M $CaCl_2$	9.19 g
5 mM KCl	0.25 ml of 1.0 M KCl	2.5 ml
2 mM MES pH 5.7	0.5 ml of 0.2 M MES	5.0 ml
	35.3 ml of H_2O	H_2O to final volum

PEG Solution (Vortex 30 min to mix.)	**for 10 ml**
40% PEG 4000 (Fluka 81240)	4.0 g PEG
240 mM mannitol	3.0 ml of 0.8 M mannitol
100 mM $CaCl_2$ or $Ca(NO_3)_2$	1.0 ml of 1.0 M $CaCl_2$
	2.5 ml of H_2O

MMg Solution	**for 10 ml**
0.4 M mannitol	5.0 ml of 0.8 M mannitol
15 mM $MgCl_2$	0.15 ml of 1.0 M $MgCl_2$
4 mM MES pH 5.7	0.2 ml of 0.2 M MES
	4.65 ml of H_2O

6) Protoplast harvest

The protoplasts are harvested after 3-4 hours digestion, after the solution changed to green. Add 8-10 ml W5 solution into the digestion buffer, and release protoplasts by gently swirling, filter with Nylon filters (Mesh, 20-40 µm) (SPECTRUM LABORATORIES, INC. Lot. # 3203823) into a 30 ml plastic centrifuge tube on ice. Spin at 800 rpm (Speed 3) for 1 min (table top centrifuge with swinging bucket). Resuspend pellet in 10 ml W5 (ice chilled) and repeat centrifugation. Again, resuspend in 2 ml W5, and let it sit on ice for 30 min. At this stage the protoplast quality and concentration are observed under the microscope. Good quality protoplasts will settle down into the bottom of the tube over a period of 30 minutes. Replace W5 solution with room temperature MMG solution (1.8 ml, will be adjusted based on protoplast concentration, ~10^5/ml) for PEG mediated transformation.

7) Transformation of mRNA to protoplasts

Prepare denatured sheared salmon sperm DNA (SuperArray BIOSCIENCE CORPORATION Cat. # GA-007) by boiling it in water for 20 min, and immediately chilling on ice. 8 µl at 10mg/ml is used for each transformation.

To reduce damage to the protoplasts during pipeting, the yellow tips (for 200 µl pipette) were cut to remove the narrow ends. 100 µl protoplast (in MMG solution) was pipetted into a 1.5 ml tube with 8 µl denatured sheared salmon sperm DNA preloaded. Then add 10-20 µl mRNA (mixed with reporter and control mRNAs). Mix well by gently flipping 3 times, immediately add 110 µl PEG solution and mix well by inverting the tube 3 or 4 times. After 30 min incubation at room temperature, the transformation is terminated by adding 0.43 ml W5 (RT) solution. Gently invert the tube for 2 or 3 times

and let sit at room temperature for another 5 min (to help the protoplasts to settle down to the buttom of the tube.) Spin at 800 rpm for 1 min (table top with swinging bucket), pipette out the supernatant and resuspend the pellet into 1ml W5 solution in a 4X6 well flat bottom plate. After 3 hours incubation in the dark (RT) the protoplast are harvested for luciferase assays. For my experiments, 3 replicate transformations were performed for each sample.

8) Luciferase assays

For luciferase assays the protoplasts were collected by centrifugation. Transfer the incubation solution (W5) to a 1.5 ml tube on ice and let sit for 10-20 min, then spin down the protoplasts at 800 rpm for 1 min (table top with swinging bucket). Remove supernatant and add 25 µl 1 X lysis buffer (provided in the Dual-luciferase reporter assay system, PROMEGA, Cat. # 1980) for immediate homogenization. Cell lysates were centrifuged 10 min in a banch top centrifuge in the cold room at maximal speed (14X1000/min).

The luciferase assays were performed in a TD-20/20 luminometer (TURNER BIOSYSTEMS, Sunnyvale, CA). Reset the sensitivity, integration time, and delay time. For my experiments I used 50.1%, 10 seconds and 3 seconds respectively. 10 µl supernatant was pipetted into a 1.5 ml tube and applied to the luminometer for measuring background luciferase. 30 µl FLUC substrate was added, mixed well by brief vortexing, and applied to the luminometer for reading FLUC activity (collect two readings in quick succession). Immediately afterwards, 30 µl RLUC substrate in stop buffer was added, and vortexted briefly to collect two readings of RLUC activity.

Appendix II Pictures for other phenotypes of the *eif3h* mutant

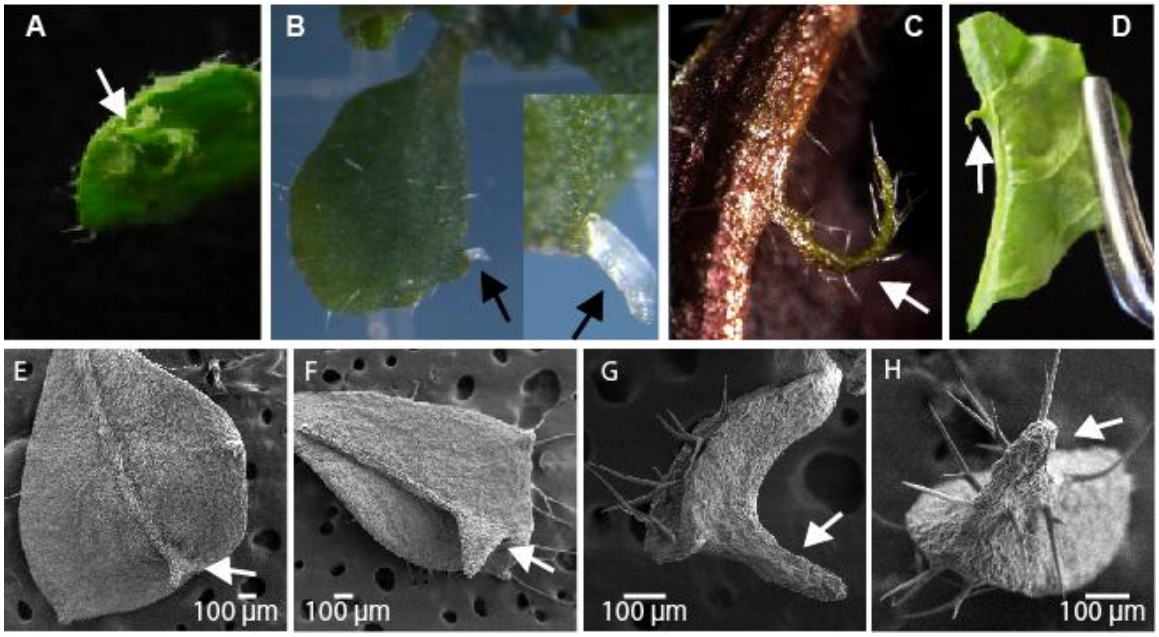

Figure Appendix II-1. The ectopic organ initiation on *eif3h* leaves. White or black arrows point to tissue outgrowth. **(A)** A shoot with a few leaves initiated at the margin of a rosette leaf. **(B)** A white unspecified tissue initiated at the margin of a rosette leaf. **(C)** An adaxialized leaf initiated on the abaxial side midrib of a rosette leaf. **(D)** A hook-like outgrowth on the abaxial side midrib of a rosette leaf. **(E-H)** Electron scanning microscopic images for rosette leaves with various outgrowths on the abaxial side of the midrib, either without **(E-G)** or with trichomes (H).

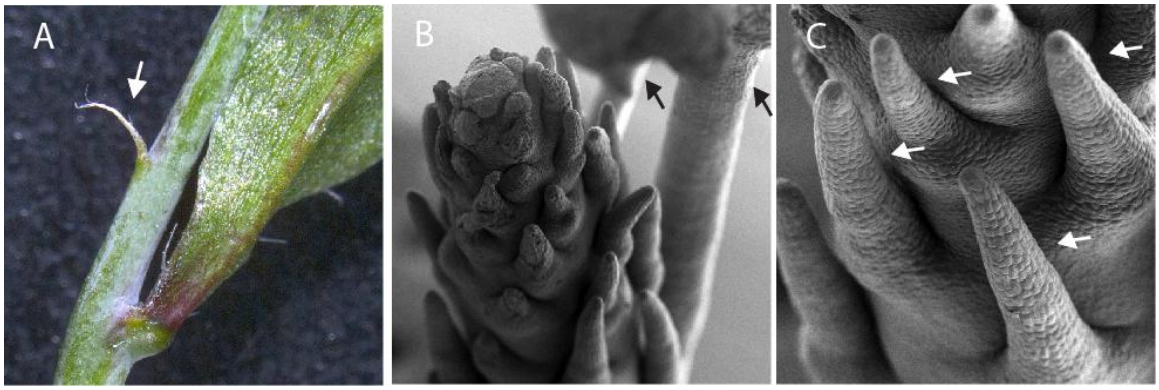

Figure Appendix II-2. *eif3h* defects on lateral organ specification. White arrows point to unspecified organs. **(A)** A needle-like *eif3h* cauline leaf. **(B-C)** A shoot apex with unspecified lateral organs and 2 flower buds (highlighted by black arrows). C is a close-up of B

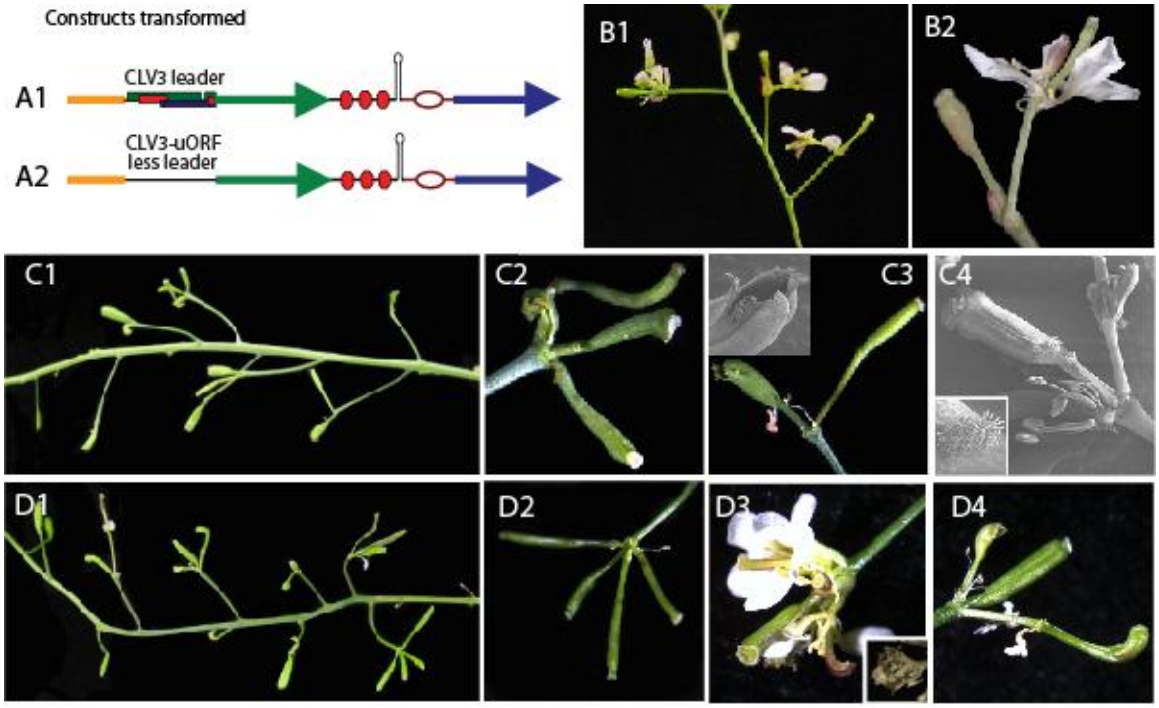

Figure Appendix II-3. Flower-within-flower (FWF) phenotype in *eif3h* and *eif3h* transformed with luciferase constructs carrying the *CLV3* leader. **(A1)** Dual-luciferase translation assay construct for the wild type (A1) and the uORF less (A2) *CLV3* leader. **(B1-B2)** FWF phenotype in the *eif3h* mutant. **(C1-C4)** FWF phenotype in *eif3h* transformed with the dual-luciferase construct for *CLV3* wild type leader (A1). **(D1-D4)** FWF phenotype in *eif3h* transformed with the dual-luciferase construct for *CLV3* uORF-less leader (A2).

VITA

Fujun Zhou was born in Nongan County, Jinlin Province, P.R.China on August 23, 1970. He went to Northeast Forestry University, Harbin, Heilongjiang Province in 1990, received his B.S. degree in Animal Science in 1994. After that he worked for Northeast Forestry University as a research assistant and lecturer. He started graduate school in the same university from 1996 and received his M.S. in Plant Molecular Ecology in 1999. He also worked for the University of Tokyo (Japan) from 2000 to 2001 as a visiting scholar, and for Oak Ridge National Laboratory as a research assistant from 2002-2003. He started his graduate study in the Graduate School of Genome Science and Technology in August 2003, graduated and received his PhD degree in May 2009.