Eukaryotic initiation factor 3 (eIF3) and 5' mRNA leader sequences as agents of translational regulation in Arabidopsis

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SUMMARY

This report summarizes results from the project period 2009-2014. Progress on prior project periods under the same award number between 1996 and 2009 were reported previously. For this report, emphasis is placed on yet unpublished results, while published data are summarized only briefly.

Protein synthesis, or translation, consumes a sizable fraction of the cell's energy budget, estimated at 5% and up to 50% in differentiated and growing cells, respectively. Plants also invest significant energy and biomass to construct and maintain the translation apparatus. Translation is regulated by a variety of external stimuli. Compared to transcriptional control, attributes of translational control include reduced sensitivity to stochastic fluctuation, a finer gauge of control, and more rapid responsiveness to environmental stimuli. Yet, our murky understanding of translational control allows few generalizations. Consequently, translational regulation is underutilized in the context of transgene regulation, although synthetic biologists are now beginning to appropriate RNA-level gene regulation into their regulatory circuits. We also know little about how translational control contributes to the diversity of plant form and function.

This project explored how an emerging regulatory mRNA sequence element, upstream open reading frames (uORFs), is integrated with the general translation initiation machinery to permit translational regulation on specific mRNAs. The regulatory potential of uORFs is realized by their length as well as their RNA sequence. Translational control by uORFs is mediated in part by eIF3h, one of 13 subunits of the largest and arguably least understood of the eukaryotic initiation factors. The h subunit of eIF3 is important for translation reinitiation, as determined by gene specific reporter assays and further confirmed by computational modeling (Roy et al., 2010). In Arabidopsis, eIF3h can be deemed responsible for a form of molecular memory that allows the ribosome to measure the length of uORFs in order to calibrate the translation reinitiation process. At the whole-plant level, eIF3h governs metabolic and developmental processes in stem cell and meristem maintenance, in auxin signaling, and in pollen development (Zhou et al., 2010; Roy et al., 2011). In several of these cases, the translational regulation by eIF3h is founded on uORFs (Zhou et al., 2010; Zhou et al., 2014). In addition to our work on eIF3h, we continued to investigate the genetic architecture of the eIF3 protein complex, in the process assembling the most comprehensive collection of eIF3 mutants outside of yeast (Roy and von Arnim, unpublished).

Independently of the eIF3h research, we built a framework for the comparative genomics of uORFs in dicotyledonous plants as a first step to understand how the rapid evolution of mRNA sequence elements has accompanied the diversification of plants. Specifically, the genomes of flowering plants harbor hundreds of conserved RNA sequence motifs, including uORFs, that are likely to serve as a nexus for RNA-level gene regulation (Vaughn et al., 2012). A comprehensive review article summarizes the contributions of uORFs to plant translational control (von Arnim et al., 2014), and a second review synthesizes the literature on translational control in Arabidopsis (Roy and von Arnim, 2013).

Meanwhile, we extended work from a prior funding period on regulation of gene expression by darkness. Interestingly, two different genes discovered in this process both function in RNA level regulation (see list of publications for details; Kim et al., 2009; Kim et al. 2012).

Finally, we added to the compendium of global genome-wide measurements of translation. Using polysome gradient fractionation of mRNAs and microarray hybridization, we discovered that the protein kinase GCN2, a sensor of uncharged tRNA, regulates translation in response to herbicide-induced amino acid starvation (Guan and von Arnim, unpublished). The project also contributed to a similar global analysis of translation in response to the diurnal light-dark cycle. We newly discovered that the circadian clock modulates the ribosome loading of mRNAs (Missra et al., submitted). In summary we have contributed critical and valuable data that deepen our understanding of how plants organize and regulate one of the most energy-intensive aspects of their life history. The translation machinery is highly conserved. Therefore, discoveries in Arabidopsis are likely to be relevant for biofuel crops. These data may inform and constrain future applications of plants in the bioenergy sector.

Note: References in blue font were supported by this grant.

BACKGROUND

Gene regulation at the level of translation initiation occurs in response to numerous environmental stimuli, including drought, hypoxia, heat, light, photosynthate, and viral infection (Kawaguchi and Bailey-Serres, 2002; Floris et al., 2009; Roy and von Arnim, 2013), yet little is known about the underlying cellular signaling pathways or the responsible mRNA sequence motifs. Protein synthesis is a major energy sink. Moreover, the translational machinery *per se* represents a sizable fraction of cellular protein, which must be regenerated for each growing season, maintained, and protected from denaturation during abiotic stress. The translation initiation factor eIF3 comprises 13 known subunits in plants. eIF3 binds to the small (40S) ribosomal subunit near the mRNA exit channel and coordinates the interaction between the 40S and the mRNA as well as the loading of other eIFs as part of a multifactor complex (Hinnebusch, 2006). Although eIF3 remains associated with the 40S as it scans along the mRNA 5` leader, its precise role and especially the functions of its individual subunits remain poorly understood.

In plants, about 30% of mRNAs possess one or more AUG triplets upstream of the main coding region. These uAUGs are particularly prominent in regulatory mRNAs (kinases, transcription factors; Kim et al., 2007). The associated upstream open reading frames (uORFs) typically repress translation and may impart translational regulation in response to an external stimulus. Once a uORF has been translated, `reinitiation` downstream of it poses a problem because, first, the 40S subunit must be coaxed into resuming the scanning process. And, second, the 40S subunit must be replenished with a new set of initiation factors (eIF1, 1A, 2, 3, 5, 5B) many or all of which were ejected from the ribosome during the original 40S-60S subunit joining step on the uAUG. Building on prior work in Arabidopsis (Kim et al., 2004; Kim et al., 2007; Yahalom et al., 2008), we investigated how eIF3 coordinates the regulation of translation by uORFs.

REPORT

CONTRIBUTIONS OF EIF3H AND OTHER TRANSLATION FACTORS TO TRANSLATION INITIATION

The *eif3h* mutation was one of the first mutants in an eIF3 gene in any multicellular organism. In our previous research we established that eIF3h supports translation re-initiation, a deviation from the standard scanning model of translation initiation, the mechanics of which are incompletely understood (Roy et al., 2010). In this project period, we modeled the role of eIF3h using a probabilistic approach that treats the initiation process as a linear series of decisions, made by the ribosome, representing events such as start codon recognition, loss of reinitiation competence during translation elongation, and regaining of reinitiation competence. After parameterizing the model with our existing experimental gene expression data, using a genetic algorithm, we concluded that the eIF3h translation factor helps to maintain competence for reinitiation while the ribosome is translating a long uORF (see Roy et al., 2010 for details).

We began to compare *eif3h* with other translation factors. To date, no other mutation in an eIF3 gene has revealed a similar phenotype to *eif3h*. This could indicate that our available alleles are too severe (embryo or gametophytic lethality, true for *eif3b1*, *c1*, *e*, *i1*) or too mild, due to genetic redundancy (*eif3b2*, *c2*, *d*, *g1*, *g2*, *m1*, *m2*) or due to a residual truncated, yet functional, gene product (*eif3g1*, *j*, *k*). More likely, however, eIF3h plays a unique role in the spectrum of eIF3's activities. Hence we do not yet know the genetic function of these subunits. For one eIF3 gene (eIF3a), no loss-of-function mutants whatsoever have been confirmed, despite numerous leads and attempts. These data have yet to be published.

In the interest of following the gametophytic defects among eIF3 genes in more detail, we developed a genetic labeling technique, utilizing a genome-wide collection of fluorescent protein transgenes expressed in pollen. For proof of concept, the technique was applied to track the precise gametophytic defect in *eif3h*-1, *eif3e*-1, and *eif3i1* (Roy et al., 2011).

Although no other *eif3* mutation resembled *eif3h*, a mutant in a ribosomal protein, RPL24B (also known as SHORT VALVE1), did. *Rpl24b/stv1* mutants have similar defects in leaf shape, inflorescence architecture, vascular development, and fruit morphogenesis. Gene expression assays showed that *rpl24b*

displays a strikingly similar translation reinitiation defect as *eif3h*, indicating that eIF3h and RPL24B cooperate closely. This is a satisfying result because it explains many aspects of the *eif3h* mutant phenotype. The reinitiation module that encompasses eIF3h and RPL24 plays a key role in developmental biology because many regulatory transcripts, for example those for auxin response transcription factors, possess uORFs and depend on RPL24 as well as eIF3h for proper gene expression (Zhou et al., 2010). Global analysis of the mRNA translation state in the *rpl24b* mutant revealed that *eif3h* and *rpl24b* have certain resemblances in their translation profile. For example, these two mutations together suggested that ribosomal protein mRNAs form a tightly defined regulon of translational control, a notion confirmed by other recent studies. However, *rpl24b* and *eif3h* also have distinct translational defects (Tiruneh et al., 2013); these defects differ from those seen in a mutant defective in poly(A) binding protein. In the future, such data will serve as a springboard to explore the global architecture of translational control in Arabidopsis.

The *eif3h* mutant also displays a striking shoot meristem overgrowth defect. While some of these phenotypes can be understood as defects in translation of auxin response factors (ARFs), which contain uORFs and are known targets of eIF3h, other phenotypes go beyond this. Numerous other meristem regulatory genes harbor uORFs, for example the CLAVATA1 receptor kinase (Zhou et al., 2014). Future work may leverage the *eif3h* mutant to crack open the translational dimension of growth regulation in the plant meristem.

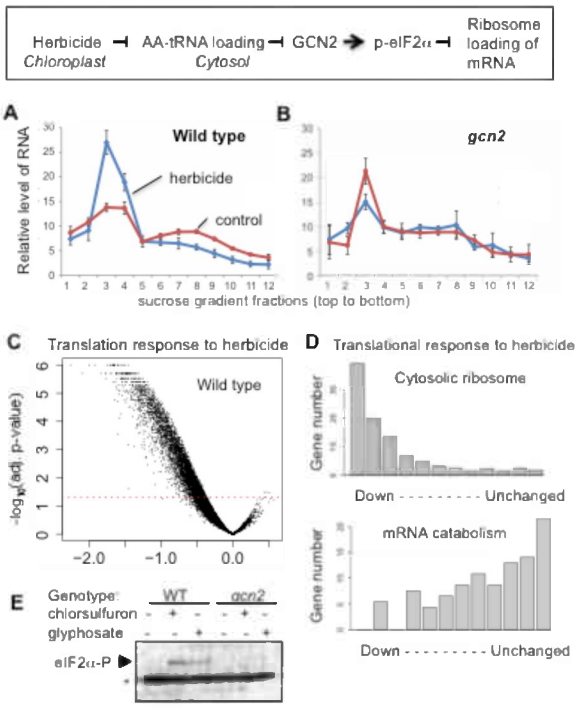

Fig. 2 *Global repression of ribosome loading upon herbicide treatment is GCN2 dependent. Overall RNA abundance across polysome gradient in* ***(A)*** *wild type and* ***(B)*** *gcn2. Red line: untreated. Blue line: Treated for 2hr with chlorsulfuron. Fraction 1: Non-polysomal RNA. F12: Highly polysomal RNA.* ***(C)*** *'Volcano plot' showing loss of ribosome occupancy of mRNAs in response to chlorsulfuron. x-axis value of 0.0 indicates that ribosome loading is unchanged. Dotted line: Significance threshold.* ***(D)*** *Gene set enrichment analysis shows functional bias among herbicide responsive mRNAs.* ***(E)*** *GCN2 phosphorylates eIF2α in response to herbicide.*

COMPARATIVE GENOMICS OF 5' LEADER SEQUENCES

The functional significance of uORFs and related RNA sequence elements for plant growth can only be fully appreciated if we capture and interpret their distribution and evolution across the emerging genomes of plants. The diversification of plants has been accompanied by the rapid evolution of mRNA sequence elements. Interestingly, in the rapidly evolving 5'UTRs of flowering plants, the AUG triplet is in fact the most highly conserved of all the 64 triplets, implying that uAUGs are subject to negative selection. In turn, this result implies that the majority of uORFs are functionally significant, even though their peptide sequences are generally not conserved. We performed a large-scale comparative genomics analysis of the 5' and 3' UTRs of six dicot plant genomes in search of evolutionarily conserved RNA sequence motifs. Several hundred motifs were identified. In the 5' UTR, where motifs are generally shorter, we found purine rich elements, as well as uAUGs and conserved-peptide uORFs. In the 3' UTR, more classical motifs such as potential Pumilio-type RNA binding motifs were identified, as well as a potential motif that mediates a rare case of subcellular targeting of a plant mRNA, namely for extensin cell wall proteins (Vaughn et al., 2012). This work suggests the interesting hypothesis that RNA motifs such as uORFs contribute to the diversity of phenotypic attributes among plant species.

TRANSLATOMIC ANALYSIS IN ARABIDOPSIS

Our analyses of conserved RNA sequence motifs and conserved mechanisms of translation reinitiation continuously raised questions about the extent of translational control in plant development and physiology. It is known that translation is regulated in a global fashion in response to various abiotic stresses, such as heat, darkness, and hypoxia (Roy and von Arnim, 2013). Much less well understood is how translation may be regulated by metabolic constraints including fixed carbon, energy status, diurnal light-dark changes, and fixed or inorganic nitrogen. We therefore initiated two global studies to specifically examine the extent of translational control by the diurnal light-dark cycle, which is governed by the circadian clock, and by the amino acid status of the cell, which is governed by the protein kinase, GCN2. The diurnal experiment was supported primarily by a separate award from the National Science Foundation.In response to amino acid starvation, uncharged tRNA activates the GCN2 kinase (GENERAL CONTROL NONDEREPRESSIBLE2). GCN2 in turn phosphorylates the critical eukaryotic initiation factor, eIF2. eIF2 binds GTP and delivers initiator-tRNA-Met to the 40S ribosomal subunit, which is needed for start codon recognition. In yeast and vertebrates phosphorylated-eIF2α tightly binds and inhibits another rate limiting initiation factor, eIF2B, which functions as the guanine nucleotide exchange factor for eIF2. Phosphorylation of eIF2α thus causes a global downregulation of translation. This regulatory mechanism appears to be conserved and operational in Arabidopsis as well. We treated Arabidopsis seedlings with chlorsulfuron herbicide, which inhibits synthesis of branched-chain amino acids, activating GCN2. We then examined the polysome loading of mRNAs in wild type plants and in the *gcn2* mutant. The global response to herbicide was scored gene by gene, and genes were characterized for their GCN2-dependent translational repression. We detected a global response, which however, affects certain mRNAs more than others. These data are still in preparation for publication (Guan and von Arnim, in preparation).

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** Kim, B.H., Malek, P., von Arnim, A.G. 2012. The BPG2 regulator of chloroplast ribosomal RNA processing binds specifically to chloroplast ribosomal RNA. Planta 236:677-690. *-> BPG2 was identified with prior DOE support as a phytochrome-regulated Arabidopsis gene that responds rapidly to a shift from light to darkness. We found that bpg2 mutants improperly process their chloroplast 25S rRNA and that BPG2 binds directly and specifically to ribosomal RNA.*
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-> FIERY1 and BPG2 (see below) are two genes that we identified in a screen for dark-responsive transcripts. Coincidentally, both of them code for elements of RNA level gene regulation (FIERY1, turnover of pNp, a ribonuclease inhibitor; BPG2, rRNA processing). Both are localized to the chloroplast.

TRAINING ACTIVITIES

Major portions of the work were carried out by one Masters student (Bayu Tiruneh) and three PhD students who have moved on to successful postdoctoral positions (Bijoyita Roy, UMassMedical; Fujun Zhou, NIH and Johns Hopkins University, and Justin Vaughn, University of Georgia). Several additional students have since contributed to the project but have not yet graduated (Ramya Enganti, Will Jordan, Qidong Jia, Benjamin Ernest). The early stages of the project were advanced by Dr. Byung-Hoon Kim, who now holds a faculty position at a primarily undergraduate institution. Two more recent postdocs (Anamika Missra, Ju Guan) performed the genome-wide analyses of translation between 2010 and 2013. We also hosted multiple graduate rotation students (Zhang, Utturkar, Nair, Zhu) and undergraduate students (not named in accordance with federal FERPA rules). A lab manager (Xiuhua Han) provided continuous technical support until 2013.

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