Title: Translation regulation in plants: an interesting past, an exciting present, and a promising future.

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Summary

Changes in gene expression are at the core of most biological processes, from cell differentiation to organ development, including the adaptation of the whole organism to the ever-changing environment. Although the central role of transcriptional regulation is solidly established and the general mechanisms involved in this type of regulation are relatively well understood, it is clear that the regulation at a translational level also plays essential roles in modulating gene expression. Despite the large number of examples illustrating the critical role played by translational regulation in determining the expression levels of a gene, our understanding of the molecular mechanisms behind such types of regulation is slow to emerge. With the recent development of high-throughput approaches to map and quantify different critical parameters affecting translation such as RNA structure, protein-RNA interactions and ribosome occupancy at the genome level, a renewed enthusiasm toward studying translation regulation is warranted. The use of these new powerful technologies in well-established and uncharacterized translation-dependent processes holds the promise to decipher the likely complex and diverse, but also fascinating mechanisms behind translation regulation.

Significance Statement

In this review, we provide an extensive list of examples of translation regulation studies in plants and what is known about the molecular mechanisms involved. We hope this review will serve as a reference point for those researchers interested in implementing new technological advances to the study of classical and novel translation-dependent processes in plants.

We start by providing some background information about the structural components of the translational machinery and the general mechanisms of translation. To facilitate the comprehension of the different types of translational regulation, we have grouped them into two broad categories, global or whole-genome translation regulation and gene-specific translation control, although as we will see in some cases the separation between these two classes is not completely clear. Thus, we will describe some of the examples in the general regulation of translation section where most of the transcripts in the cell are similarly regulated, but some specific mRNAs escape such regulation indicating certain level of gene-specific regulation. Finally, we have further classified the types of translational regulation based on the structural features involved, highlighting existence of some common, although only partially understood, general mechanisms.

We provide a comprehensive literature overview of plant studies on translational regulation, with special focus made on those situations were active regulation of translation is needed to explain the differences between the transcript and protein abundance. We have only considered translation of endogenous nuclear genes, whereas translation in chloroplasts and mitochondria or cellular translation of viral RNAs were not included.

A) THE TRANSLATION MACHINERY: THE RIBOSOME AND tRNAs

The ribosome is an enormously complex “molecular machine” that has the universal role of catalyzing protein synthesis remarkably fast (one amino acid is added to the polypeptide every 60 ms) and in a highly accurate fashion (Zaher and Green, 2009).

The plant ribosome, as in other eukaryotes, is composed of two subunits, four ribosomal RNA (rRNA) species and approx. 70-80 different ribosomal proteins (RPs) (Chang *et al.*, 2005; Giavalisco *et al.*, 2005; Perry, 2007; Carroll *et al.*, 2008; Hummel *et al.*, 2012). In all organisms the two subunits of the ribosome play two distinct roles during the translation process: the 40S subunit decodes the mRNA, whereas the 60S catalyzes the peptidyl transferase reaction (reviewed in Browning and Bailey-Serres, 2015). The large 60S subunit contains three rRNA species, 25-26S, 5.8S and 5S, and over 40 RPs, while the small 40S subunit consists of an 18S rRNA and over 30 RP. A proper balance between rRNA and RP biosynthesis is therefore needed to ensure an efficient production of this complex cellular machinery. There are over 80 RPs identified in the Arabidopsis genome and each RP is encoded by two to seven paralogs, resulting in a total of more than 230 proteins identified as RPs in the Arabidopsis genome (Barakat *et al.*, 2001) (a complete list of all Arabidopsis RP genes is listed in Browning and Bailey-Serres, 2015). Interestingly, with few exceptions, only one member of each RP gene family is present in a ribosome (Perry, 2007; Yusupova and Yusupov, 2014). In both monocots and dicots, some RP are phosphorylated and this phosphorylation is regulated by developmental and environmental cues (Beltrán-Peña *et al.*, 2002; Williams *et al.*, 2003; Turck *et al.*, 2004; Chang *et al.*, 2005; Khandal *et al.*, 2009; Turkina *et al.*, 2011; Boex-Fontvieille *et al.*, 2013). Although the biological significance for this post-translational regulation is not yet understood, the phosphorylation status of RPS6 via the TOR signaling pathway is involved in the selective translation of a subset of

mRNAs (reviewed in Muench et al., 2012). Generally, Arabidopsis mutants in RPs share some developmental phenotypes such as reduced shoot and root growth and cell proliferation, increased ploidy in leaf cells, and a characteristic leaf defect called *pointed first leaf* (*pfl*, also called *pointed leaves* or *denticulata*) (reviewed in Byrne, 2009; Horiguchi *et al.*, 2012). However, the facts that the effect of mutations in RP range from embryo lethality to no-phenotype, that some of the RP mutant phenotypes are unique, and that many RP paralogs exhibit different spatial and/or developmental expression patterns (reviewed in Byrne, 2009; Horiguchi *et al.*, 2012; Xue and Barna, 2012) raise the possibility that the specific spatiotemporal patterns of expression or even the "paralog composition" of the ribosomes exert some translational control. Although this paralog specificity question remains open, the deployment of techniques such as TRAP-seq and Ribo-seq holds the promise to clarify this interesting aspect of translation regulation.

In preparation for protein synthesis, cells need to charge transfer RNAs (tRNAs) with their respective amino acids in a process catalyzed by aminoacyl-tRNA synthetases. There are hundreds of tRNA genes in the genomes of plants (Michaud *et al.*, 2011). For example, in Arabidopsis out of 637 tRNA-like genes present in the nuclear genome, 599 are considered as potentially functional. In contrast, there are only 45 aminoacyl-tRNA synthetase genes in the Arabidopsis genome (Duchêne *et al.*, 2005; Berg *et al.*, 2005), implying that multiple tRNAs can be charged by a single synthetase. Remarkably, several of these nuclear-encoded tRNA-charging enzymes in Arabidopsis appear to function in multiple compartments, as they display dual or even triple subcellular targeting specificities for mitochondria, plastids and/or the cytosol (Duchêne *et al.*, 2005) and multiple mechanisms exist for the import of tRNA to the organelles (Salinas *et al*., 2006; Murcha *et al*., 2016). Many of the aminoacyl-tRNA synthetases are non-redundant in function, as loss-of-function mutants lead to gametophyte or embryo lethality (Sun *et al.*, 1998; Berg *et al.*, 2005).

B) STRUCTURAL mRNA FEATURES THAT INFLUENCE TRANSLATION

As mentioned above, the ribosomes are the decoding machinery translating information contained in mRNAs into amino acid sequences in the proteins. Not all RNAs are substrate for the ribosome and therefore translated. In fact, transcripts that are going to be translated present a number of intrinsic features that determine how well they would be translated (reviewed in Gebauer and Hentze, 2004; Arnim *et al*., 2014) (Figure 1). These features include the 5´ leader or 5´UnTranslated Region (5´UTR) at the 5´end of the mRNA upstream of the translation start codon, and the 3´ UTR downstream of the stop codon. Other important features are the 7-methyl guanosine (m^7GpppN) cap at the 5´ end of the mRNA, and the poly(A) tail at the 3'; both are strong promoters of translation. The secondary structure adopted by an mRNA can also have a strong influence on translation. Hairpins and pseudoknots block translation if the translating ribosome cannot resolve the structure. Other features of the mRNA that regulate its translation are the presence of recognition sites for various RNA binding proteins (RBPs) and for small RNAs. Upstream Open Reading Frames (uORFs), which are short protein coding regions located upstream of the main genic ORF (mORF) in the 5´UTR are among the most ubiquitous gene-specific elements affecting an mRNA's protein production level, typically by negatively influencing translation of the downstream mORF. The sequence context in which the start codon is located within the mRNA also influences greatly how often the ribosome will be able to recognize it as a site for translation initiation and therefore affects the protein production rates.

The structural features do not only determine how well an mRNA is translated, but also whether specific proteins and other RNA can interact with it. Thus, although capped and polyadenylated mRNAs exported to the cytoplasm are in principle ready for translation, they can be sequestered in stress granules and processing bodies (P-bodies) (Roy and Arnim, 2013) by interacting with specific proteins or small RNAs. Stress granules form transiently during a particular stress, such as hypoxia (Sorenson and Bailey-Serres, 2014; Lordakshi *et al*., 2016) or heat stress (Nguyen *et al*., 2016), sequestering mRNAs that should not be translated, and then disassemble after the stress is over, making mRNAs available for translation. P-bodies, on the other hand, are the sites of accumulation of decapping enzymes, ribonucleases, and occasionally the miRNA-dependent endonuclease AGO1 (Maldonado-Bonilla, 2014) and are responsible for the inactivation and degradation of mRNAs. However, in some cases, P-bodies can also serve as sites of transient reversible storage of intact mRNAs (Merchante *et al.*, 2015). In addition, transcripts are also subject to mRNA surveillance mechanisms such as the no-stop, no-go, and nonsense-mediated decay, to ensure that only error-free mRNAs are translated, while structurally aberrant transcripts are eliminated without producing potentially toxic truncated proteins (Lykke-Andersen and Bennett, 2014).

C) THE GENERAL PROCESS OF TRANSLATION

The process of eukaryotic translation has been reviewed recently in detail (Jackson *et al.*, 2010; Dever and Green, 2012; Andreev *et al*., 2017) and plant-specific aspects of translation have been extensively discussed

(Muench *et al*., 2012; Roy and Arnim, 2013; Echevarría-Zomeño *et al.*, 2013; Gallie, 2014; Browning and Bailey-Serres, 2015; Moore *et al.*, 2016). Translation is divided in three phases: initiation, elongation, and termination. Initiation is the most studied and divergent step between eukaryotes and is considered the main stage of regulation of translation. Thus, herein we provide a short description of the key steps of eukaryotic translation relevant to this review summary with a focus on initiation as the main step for translation regulation (Figure 2). Translation in eukaryotes relies on a complex machinery of RNA-protein and protein-protein interactions. A comprehensive list of all Arabidopsis translation factors and their subunit composition is listed in Browning and Bailey-Serres (2015).

The process of translation starts when the cap-binding complex recognizes the cap structure and the poly(A) binding protein, PABP, binds to the mRNA poly(A) tail. In plants, the cap-binding complex, or eIF4F, is composed of the cap-binding protein eIF4E and the scaffolding protein eIF4G (Browning and Bailey-Serres, 2015). eIF4G brings the helicase eIF4A, eIF4E, PAPB and eIF3 together circularizing the mRNA. Circularization of the mRNA serves as quality control to check for mRNA integrity, stimulates translation initiation and promotes 40S recycling (Gallie *et al*. 2004; Muench *et al*. 2012). eIF4A is a helicase that, in association with its cofactor eIF4B, unwinds secondary structures of the mRNA allowing for the recruitment of the 43S preinitiation complex (43S PIC). The 43S PIC consists on the 40S small ribosome subunit associated with the multi-subunit initiation factors eIF3, eIF1, eIF1A and eIF5, and with the ternary complex (TC). The TC is composed of the GTP-binding protein eIF2, GTP, and the Met-tRNA^{Met} initiator (Met-tRNA$^{Met}{}_{i}$), which is specific for initiation and can be distinguished from the Met-tRNA^{Met} used in elongation. eIF3, composed of twelve subunits, is the most complex and largest initiation factor. eIF3 participates in nearly all major steps of initiation, such as the charging of 40S with the TC, and is responsible for bringing the 43S PIC to the cap structure through its interaction with eIF4G. eIF3 also participates in the scanning and start codon recognition processes, inhibits the premature association of the 40S subunit with the 60S until an initiation codon is reached, and it is involved in reinitation (reviewed in Browning and Bailey-Serres, 2015). The mRNA molecule with all these factors assembled at the mRNA cap is called the 48S PIC (pre-scan) (Figure 2).

With the assistance of eIF4A, eIF4B, eIF4G, eIF1 and eIF1A, the ribosome starts scanning the 5´UTR of the transcript in the 5´-to-3´ direction until the AUG initiation codon is identified (Figure 2). eIF1 plays a key role in the fidelity of start codon recognition (Jackson *et al.*, 2010). After recognition, eIF1 is displaced, leading to the hydrolysis of the GTP of eIF2 promoted by eIF5. The eIF5B GTPase is now recruited and mediates the joining of the 60S subunit to the 40S and the dissociation of eIF1 and eIF2-GDP, yielding the elongation-competent 80S ribosome. eIF6 is also involved in subunit binding: it prevents premature binding of 60S to the 48S PIC complex, but once phosphorylated, loses its affinity and dissociates from 60S, thus enabling the formation of the 80S ribosome (Browning and Bailey-Serres, 2015). After subunits assemble, eIF5B and eIF1A dissociate (Jackson *et al.*, 2010). In fact, at this point all of the eIFs have dissociated from the ribosome.

At this stage, the methionyl-tRNA$_i$ resides in the Peptidyl (P) site of the assembled 80S ribosome and the Aminoacyl (A) site of the ribosome is free to recruit the second charged tRNA. eEF1A forms a ternary complex for elongation, delivering GTP and the aminocyl-tRNA to the A-site. The codon-anticodon recognition between the mRNA in the A-site and the aminoacyl-tRNA results in the hydrolysis of GTP, and eEF1A-GDP gets released and eventually regenerated by eEF1B. Peptide bond formation between the initiator Met (or, in the future rounds of elongation, the peptide) in the P-site and the second (or, in later rounds, third, forth, etc.) amino acid in the A-site is catalyzed by the 25-26S rRNA of the large ribosomal subunit (the energy for this transesterification reaction comes from the aminoacyl-tRNA ester linkage) and transfers the growing peptide to the A-site. Subsequent ribosomal translocation by one codon in the 5' to 3' direction carried by eEF2-GTP (at the expense of its GTP which gets hydrolyzed) leaves the A-site empty, with the peptidyl-tRNA residing in the P-site and the deacylated tRNA ejected from the exit (E) site. The A-site is now ready to accept another aminoacyl-tRNA to match the third mRNA codon.

Elongation continues until a stop codon (UAG, UGA, or UAA) enters in the A site of the ribosome. Termination is mediated by the release factors eRF1 and eRF3 that together with a GTP molecule form a ternary complex that enters the A-site of the ribosome. The polypeptide chain and tRNA are released from the ribosome, the 80S ribosome dissociates into its subunits, and can now be recycled for new rounds of translation. Interestingly, not all three stop codons work equally well in promoting the termination of protein synthesis (Yu *et al.*, 2016). This difference might result either from the different affinities of specific termination complexes to the ribosomes encountering different stop codons, or from the different termination kinetics imposed by each particular termination codon.

All higher plants possess three different forms of cap-binding proteins, eIF4E, nCBP, and the plant-specific eIFiso4E (reviewed in Browning and Bailey-Serres, 2015). In addition, *Brassicaceae* contains two more paralogs of eIF4E, eIF4E1b and eIF4E1c (Patrick and Browning, 2014). These cap-binding proteins have different patterns of expression, with the canonical eIF4E, eIF4E1b and eIF4E1c binding the cap stronger than eIFiso4E or nCBP

(Patrick and Browning, 2014; Kropiwnicka *et al*., 2015). Finally, plants also have an alternative version of eIF4G, eIFiso4G, so in addition to the universal eIF4F, plants make a second, plant-specific, cap-binding complex called eIFiso4F (Patrick and Browning, 2012). Research suggests that the two complexes have different affinities for certain mRNAs, providing a potential mechanism to regulate the translation of specific mRNA populations (Mayberry *et al*., 2009; Martínez-Silva *et al*., 2012; Gallie, 2016).

As mentioned previously, the predominant mode of translation initiation is via mRNA scanning by the ribosome recruited to the cap. While this seems to be the case for a majority of transcripts, translation does not always start at the first AUG encountered by the ribosome. Other mechanisms of initiation, leaky scanning, reinitiation, initiation at non-AUG codons, internal ribosomal entry, and shunting, have also been described in plants.

***Leaky scanning*.** The sequence context around AUG plays an important role in start codon recognition by the ribosome and some AUGs in weak translation context can be skipped (Kozak, 2002). Studies that looked for the most favorable context for translation initiation in eukaryotes established that purines (A or G) at the -3 and a G at the +4 position were the most important nucleotides that determined AUG recognition (Kozak, 1987). In a study of over 5074 sequences from plants, the context of the translation initiator codon was found to be AC-rich, with the consensus of caA(A/C)aAUGGCg. It was also found that the sequence contexts for the initiation in dicots (aaA(A/C)aAUGGCu) and monocots (c(a/c)(A/G)(A/C)cAUG) are slightly different (Joshi *et al*., 1997). Very recently, with the whole-genome data from many plant species, a similar survey was done using 7 monocots and 7 dicots. The most favorable context for translation initiation determined by comparisons with protein abundance was GCNAUGGC in monocots, AANAUGGC in dicots and GCNAUGGC in plants in general (Gupta *et al*., 2016).

Reinitiation. In some cases, after ribosome dissociation, the 40S subunit can remain bound to the mRNA and translate another ORF present in the mRNA. This happens in polycistronic RNAs or in uORFs-containing transcripts (Roy and von Arnim, 2013). For reinitiation to happen after translation termination, the 40S subunit has to reacquire some lost initiation factors, including eIF3 and eIF4 (Schepetilnikov *et al*., 2013). After 43S PIC reassembly, the scanning resumes and translation starts at a downstream ORF (Roy *et al*., 2010; Arnim *et al*., 2014). Reinitiation is more likely to occur after translation of a small ORF and when the intercistronic regions are relatively long (Kozak, 2002). This process of termination and re-initiation can take place five or more times in a single transcript under optimal conditions (Wang and Rothnagel, 2004).

Initiation at non-AUG codons. Translation initiation events at non-AUG codons have been described for some Arabidopsis mRNAs, including *AGAMOUS* (Riechmann *et al*., 1999), *FCA* (Simpson *et al*., 2010), *GDP-L-GALACTOSE PHOSPHORYLASE* (Laing et al., 2015), and have been recently confirmed by Ribo-seq (Hsu *et al*., 2016).

IRESs. Internal ribosomal entry sites (IRESs) are structured elements within an mRNA that can recruit 40S ribosomal subunits to an internal site in the 5´UTR, bypassing the need of scanning and cap-dependent translation, and initiate translation very efficiently with only partial dependence on canonical initiation factors. The vast majority of IRES-mediated translation events have been described in metazoans, and, in plants, cap-independent elements are found in viral transcripts that often lack the cap structure (Miller *et al*., 2007). Although the evidence of classic IRESs in plant genomes is poor, there are some examples of endogenous plant cytoplasmic RNAs being translated through IRES-like mechanisms (Dinkova *et al*., 2005; Cui *et al*., 2015).

Ribosome shunting. This is a translation initiation mechanism that combines 5´end scanning and internal initiation, that allows the 40S subunit to bypass a large section of highly-structured 5´UTR by apparently shunting. This mechanism was first discovered in plant viruses and allows the assembly of the ribosome at an initiation codon downstream of stem-loop structures that would otherwise have not been accessible for the translational machinery (Ryabova and Hohn, 2000; Babinger *et al*., 2006; Poogin *et al*., 2012).

D) GLOBAL CHANGES IN TRANSLATION

Under certain conditions, such as severe stress, the energetically demanding translation machinery is turned down. In this section, we will review several examples where the levels of translation of large numbers of transcripts are coordinately regulated in response to a particular stimulus. In most of these cases, however, gene-specific translation regulation is also likely to play a key role in the plant survival, allowing for the translation of specific sets of transcripts.

There are several mechanisms governing translation regulation at a global level. Several stimuli trigger phosphorylation of PABP and specific eIFs, and this can have drastic effects on translation, either by repressing or upregulating translation globally (reviewed in Muench *et al*., 2012 and in Browning and Bailey-Serres, 2015). Specific kinases phosphorylate different eIFs. For example, CK2 phosphorylates eIF2α, eIF2ß, eIF3c, eIF4B, and eIF5, and this enhances the interaction between these proteins (Dennis and Browning, 2009; Dennis *et al*., 2009), and TOR phosphorylates eIF3 (Schepetilnikov *et al*., 2013, see below). One of the best characterized

phosphorylation-mediated translation regulation events in yeast and mammalian cells affects the eIF2α subunit of eIF2, which is only active in delivering Met-tRNA$^{Met}{}_{i}$ and GTP to the ribosome when eIF2α is unphosphorylated (Figure 3A). Upon GTP hydrolysis and eIF2-GDP release from the ribosome, eIF2 needs to be recharged with GTP by its guanine exchange factor, eIF2B. If eIF2α is phosphorylated, eIF2 binds too tightly to eIF2B and cannot be regenerated and, thus, recycled in the future rounds of translation initiation (Jackson *et al.*, 2010). The only kinase known to phosphorylate eIF2α in plants is GENERAL CONTROL NONDEREPRESSIBLE2 (GCN2) (Lageix *et al.*, 2008; Zhang *et al.*, 2008) which reduces global protein synthesis and plays a crucial role in plant growth and development (Lageix et al., 2008; Liu *et al.*, 2015), however, no direct evidence for GCN2 regulating the formation of the ternary complex exists in plants (Browning and Bailey-Serres, 2015). AtGCN1 interacts with GCN2 to mediate the phosphorylation of eIF2α. The repressive function of GCN1 is witnessed by the increase in polysomal loading in the the *gcn1* mutant and the demonstration of this gene's role in Arabidopsis cold tolerance (Wang *et al.*, 2017). Phosphorylation of eIF2α has been implicated in the down-regulation of translation after amino acid and purine starvation, UV radiation, cold stress, wounding, cadmium treatment and in the response to various phytohormones (jasmonate, ethylene, and salicylic acid), but not in the plant responses to heat, osmotic or oxidative stresses (Lageix *et al.*, 2008; Zhang *et al.*, 2008; Sormani *et al.*, 2011; Wang et al., 2017). The helicase eIF4A was also known to be phosphorylated in response to heat and hypoxia (Webster *et al.*, 1991; Le *et al.*, 1998). It has recently been found that the phosphorylation of eIF4A in proliferating cells is mediated by CYCLIN-DEPENDENT PROTEIN KINASE A (CDKA), and the results from this research suggest that this phosphorylation down-regulates the activity of eIF4A, and therefore, affect translation negatively (Bush *et al.*, 2016).

In addition to the phosphorylation of general eIFs or RPs, global changes in translation can also be achieved by altering the protein composition of the translational machinery itself. It is becoming clear that certain eIF and RP combinations can affect the translation of specific groups of transcripts. Thus, differential translation regulation may be achieved through the presence of non-core subunits of the eIFs in the translation initiation complex or by altering the ribosome composition through utilizing different RP family members. For example, some eIFs are necessary for the efficient translation of uORF-containing genes, as is the case of eIF3 (Kim *et al*, 2004; Kim *et al.*, 2007; Roy *et al.*, 2010). As mentioned, the presence of uORFs limits translation of the main ORF due to inefficient reinitation. For translation reinitiation to occur, the ribosome needs to terminate translation, re-acquire lost eIFs, and resume scanning. The eIF3h subunit of eIF3 is able to overcome the inhibitory effect of uORFs by promoting reinitiation competence (Kim *et al.*, 2004; Kim *et al.*, 2007; Roy et al., 2010; Zhou *et al.*, 2014). The Arabidopsis *eif3h* hypomorphic mutants show growth arrest, enlarged shoot apical meristem, defects in organ polarity, and a series of auxin-related defects, i.e. pin-formed inflorescences, mis-expression of auxin-related genes and poor translation of *AUXIN RESPONSE FACTOR* (*ARF*) mRNAs (Kim *et al.*, 2004; Zhou *et al.*, 2014). Thus, translation of *bZIP11*, *LHY*, *CLV1*, and *AS1,* all genes containing multiple uORFs, is compromised in the *eif3h* mutant (Kim *et al.*, 2004; Kim *et al.*, 2007; Zhou *et al*. 2014). The function of eIF3h is necessary for the proper translation of several additional genes containing multiple uORFs, as eIF3h can overcome the repressive effect of uORFs by supporting efficient translation reinitiation after uORF translation (Kim *et al.*, 2007) (Figure 3B). The capacity of eIF3h to reinitiate after translation termination at uORFs depends on its specific phosphorylation by AtS6K1, which is mediated by TOR in the presence of auxin (Schepetilnikov *et al.*, 2013). TOR is a conserved master coordinator of nutrient, energy, and stress signaling in the cell (reviewed in Xiong and Sheen, 2014).

Regarding translational regulation through ribosome composition, as previously mentioned, the eukaryotic ribosome consists of four rRNA species and around 80 RPs. Interestingly, the catalytic role of the ribosome in peptide-bond formation is thought to only require ribosomal RNAs (Noller *et al.*, 1992), so this large set of RPs may have evolve to provide not only structural support, but also possibly contribute to specific regulatory functions. Translation regulation affected by specific ribosomal proteins can be illustrated by the *short valve1* (*stv1*) mutant that harbors a mutation in the *RPL24B* gene. In addition to the aforementioned "general" phenotypes of Arabidopsis ribosomal mutants, *stv1-1* shows defects in gynoecium patterning that resemble those of auxin signaling mutants. In fact, in the *stv1* mutant translation of the auxin signaling genes *MP/ARF5* and *ETT/ARF3*, that contain uORFs in their 5´UTR, is impaired (Nishimura *et al.*, 2005). RPL4A, RPL4D and RPL5A also play a role in the translation of *ARF3*, *ARF5* and *ARF7* through their uORFs (Rosado *et al.*, 2012). In contrast, translation of *ARFs* that do not possess uORFs is not affected by these mutations. Another informative example, this time involving the biosynthetic route of polyamines, is the effect that mutations in the ribosomal proteins RPL4, RPL10 and RACK1 have on the translation of the uORFs of *ACAULIS5 (ACL5)* (Imai *et al.*, 2008; Kakehi *et al.*, 2015).

eif3h displays similar phenotypes to those of *stv1/rpl24* (Zhou *et al.*, 2010). Global analysis of the translatome of *rpl24* and *eif3h* showed a major overlap in the affected mRNAs, although the targets of the two were not identical (Tiruneh *et al.*, 2013). Thus, although some of the phenotypes observed in *eif3h*, *stv1* and other RP mutants can be explained by defects in the translation of genes involved in specific processes, such as auxin response or polyamine

biosynthesis, the transcript specificity of the mutant defects seems to be determined by rather common structural features, such as the presence of uORFs in these mRNAs and, thus, considered here as examples of global translational regulation.

The aforementioned examples not only highlight the role of specific RPs and eIFs in the control of translation, but also the capacity of common structural features such as the uORFs to provide this type of global regulation. In addition to uORFs, the degree of secondary structure of an mRNA can also have an impact on its translation rates. RNA structures of individual transcripts had been studied *in vitro*. Now, the development of new techniques allows the study of the RNA structure *in vivo* at a global level. Two recent genome-wide studies have made a significant advance in the field. Li *et al.* (2012) combined high-throughput sequencing-based structure mapping with RNA-seq (see Glossary of techniques), small RNA-seq, and polysomal mRNA-seq to study the secondary structure of all RNAs (except rRNA) in Arabidopsis flower buds. Ding *et al.* (2014) developed Structure-seq (Glossary of techniques) to study the RNA secondary structure in Arabidopsis seedlings *in vivo*. Both groups found that, in general, the UTRs in an mRNA were less structured than the coding regions, with the most significant decrease in structure appearing at the places of the start and stop codons of the transcript. This trend is also conserved in yeast, Drosophila and *C. elegans*, and may represent a conserved feature of eukaryotic protein-coding transcripts that affects translation, as ribosome accessibility could be increased in the regions where protein translation starts and ends. In fact, in highly translated mRNAs the region situated approx. 5 nt upstream of the translation initiation codon was significantly less structured, whereas this feature was not that evident in mRNAs with low translation efficiency (Ding *et al.*, 2014), clearly linking mRNA structure with translation efficiency. Interestingly, both studies found that the structural features detected by their corresponding assays display a 3-nt periodicity within the coding regions which represents the codon periodicity and is, therefore, absent in the UTRs. This periodicity was significantly higher in transcripts with high translation efficiency, while was absent in the poorly translated transcripts (Ding *et al.*, 2014). Although this periodicity could reflect the effects of ribosome occupancy on the output of the assays employed to measure secondary structure, it has been suggested it may also represent an intrinsic triplet periodicity in the structure of the CDS.

When analyzing specific structures within Gene Ontology (GO) categories, it was found that mRNAs coding for proteins involved in pathogen-response were among the most structured ones, while mRNAs involved in RNA regulation were the least structured (Li *et al.*, 2012). Stress-related RNAs had little correlation between the *in vivo* structure and that predicted *in vitro*. This could reflect that the stress-related mRNAs are more flexible and capable of adopting different conformations in response to the changing cellular conditions (Ding *et al.*, 2014), which would in turn determine their translation rates. It would be very interesting to know if, indeed, different conformations allow for different translation efficiencies in response to environmental changes.

Together, these examples illustrate different mechanisms used to alter translation at a global scale, but remarkably, still affecting different subsets of transcripts to different degrees. In the following sections, we will provide several examples where such types of global translation regulation seem to be at work, although in many cases the exact mechanisms behind the observed translational changes are not yet known.

Translation regulation in response to light

Light is a key environmental signal that has profound effects on plant development and physiology, so it is not surprising that plants respond to this factor by also regulating translation. In fact, the overall translation activity in plants is higher in light than in the dark (Berry *et al.*, 1986; Petracek *et al.*, 1997; Hansen *et al.*, 2001; Tang *et al.*, 2003; Bailey-Serres and Juntawong, 2012; Liu *et al.*, 2012; Liu *et al.*, 2013; Pal *et al.*, 2013; Floris *et al.*, 2013) and this is correlated with a higher energetic status of the plant cells under light conditions (Pal et al., 2013). Early studies already found that translation of the large and small subunits of RuBisCO was activated in response to light and rapidly repressed under dark conditions without changes in the mRNA levels, stability, secondary structure, or protein turnover (Berry *et al.*, 1985; Berry *et al.*, 1986). In addition, whole-genome studies have shown that plants transferred from dark to light growth conditions experience a massive increase in translation (Liu *et al.*, 2012; Liu *et al.*, 2013) that preceded the changes at the transcriptional level (Floris *et al.*, 2013). And *vice versa*, darkness rapidly decreased translation, even outside of the regular light-dark cycle. So an unanticipated 1h of darkness at midday during a regular light-dark cycle caused a sudden 15% decrease in global translation consistent with inhibition of translation at the level of initiation (Juntawong and Bailey-Serres, 2012). Out of many transcript species that showed reduced polysomal levels in response to darkness, only 10% also had a decrease in their mRNA levels, which means that translational inhibition mechanisms were activated in this response. This rapid decrease in translation was reversible, and only after 10 mins after re-illumination, polysomal loading increased back to the initial levels, while the monosomal levels decreased accordingly.

Light intensity also affected translation levels, with high light increasing polysomal levels transiently until plants become acclimatized and low-light conditions reducing polysomal loading (Floris *et al.*, 2013).

Translation regulation and the circadian clock

By having control of the circadian clock, organisms can foresee environmental cues that affect translation. By following translation over the course of the daily cycle, it was found that translation was higher during the day than at night (Piques *et al.*, 2009; Pal *et al.*, 2013; Missra *et al.*, 2015). Translation begins at its lowest level at the end of the night, rapidly increases in the morning, remaining elevated until midday and returning to low levels again by the next dawn, and it correlates with the sucrose content in the cell and its expected effects on translation (Pal *et al.*, 2013). The strongest group of genes translated at night was that encoding ribosomal proteins. Since ribosome biosynthesis occupies a large part of the transcriptional and translational machinery of the cell, it seems logical to produce them when there are fewer processes going on despite the lower energetic state of the plant cells at night (Missra *et al.*, 2015).

In plants overexpressing *CCA1* the circadian clock is disrupted (Wang and Tobin, 1998). The comparison of the translation in wild-type Arabidopsis plants in a light-dark cycle and in constant light, as well as to the *CCA1* overexpression plants, revealed that translational cycling was not only light-dependent but also clock-dependent (Missra *et al.*, 2015). The clock-associated gene *LHY* constitutes an interesting example of circadian regulation of translation at the level of initiation. The Arabidopsis *lhy-1* mutant harbors a gain-of-function mutation caused by the insertion of a Dissociator element (Ds) from the Ac/Ds transposons in the 5´UTR of *LHY* (Schaffer *et al.*, 1998). In the *lhy-1* Arabidopsis mutant, *LHY* transcript is constitutively expressed, however, its protein levels were found to be light dependent, with the protein accumulating during the day and disappearing at night (Kim *et al.*, 2003). Since light does not change the rate of protein turnover, *LHY* translation is thought to be regulated by light.

Interestingly, posttranslational modifications of RPs may also play a role in the modulation of diurnal translational changes as it was found that RPs are differentially phosphorylated between day and night (Turkina *et al.*, 2011).

Translation in response to sucrose

Given that translation is a highly energy-demanding process (Buttgereit and Brand, 1995), it needs to be very finely coupled with the energetic status of the cell, and in this the availability of sucrose plays an important role. Indeed, a general decrease in translation is observed in plants under sucrose-starvation conditions, which is concomitant with a general drop in the metabolic activities (Nicolaï *et al.*, 2006; Gamm *et al.*, 2014).

AtHD1 histone deacetylase is among the most translationally repressed mRNAs in the absence of sugar. AtHD1 induces deacetylation of histones H3 and H4, which is linked to transcriptional repression. In fact, under sucrose starvation, the amount of acetylated histone H4 that associates with DNA increases, establishing therefore a direct link between translational control and chromatin activity (Nicolaï *et al.*, 2006). Another translationally repressed mRNA in response to sugar is *bZIP11* that is regulated via a uORF-dependent mechanism (see below). Importantly, genes involved in protein synthesis, cell cycle, and growth increase their transcript presence in polysomes in sugar-supplemented media, again suggesting a complex regulation that combines both global and gene-specific translational control.

Development and cell-type specific translation regulation

The global changes of translation during plant development and growth have been also studied, finding stage- and cell-type specific differences. In general, age causes a decrease in translation (Kawaguchi *et al.*, 2003; Yamasaki *et al.*, 2015) and polysome content is higher in actively dividing tissues (Mason *et al.*, 1988; Bensen *et al.*, 1988).

Using TRAP (Translating Ribosome Affinity Purification (Zanetti *et al.*, 2005), see Glossary of techniques) with tissue-specific promoters, the translatomes of specific cell populations, flower developmental domains, and germinating pollen tubes were studied in Arabidopsis, uncovering cell-type specific differences in the populations of mRNAs being translated (Mustroph *et al.*, 2009; Jiao and Meyerowitz, 2010; Lin *et al.*, 2014).

The effects on translation during seed germination were investigated by comparing dormant versus non-dormant seeds in sunflower and Arabidopsis. Polysomal loading increases after imbibition and is higher in non-dormant seeds, although the amount of total RNA does not differ between the two seed populations, showing that germination is mainly regulated at the translational level (Layat *et al.*, 2014; Basbouss-Serhal *et al.*, 2015). Very recently, Bai *et al.* (2016) studied the dynamics of total and polysomal RNA populations, as well as polysome profiles, of five consecutive stages of germination, from 0 to 72 hours after imbibition (HAI). This study found extensive translation regulation during two specific temporal phases of the germination process, from 0 to 6 HAI

and from 48 to 72 HAI, which the authors referred to as hydration and germination translation shifts, respectively (HTS and GTS). These two phases correspond to key stages of the seed-to-seedling transition. Interestingly, the two shifts are regulated by distinct mechanisms, as is shown by the different trends in the ratios of transcription and translation, and by different sequence features of the mRNAs identified as translationally regulated (Bai *et al*., 2016). Interestingly, ABA, a hormone that promotes seed dormancy, has been shown to cause global decreases in translation (Bensen *et al.*, 1988; Guo *et al.*, 2011). ABA treatment down-regulated the expression of *eIF6A* and of all three *RACK1* members involved in 60S biogenesis and 80S assembly. This decrease in the expression occurred very shortly after ABA application, and provides a possible explanation of the mechanisms ABA employs to decrease translation initiation (Guo *et al*., 2009; Guo *et al.*, 2011).

Translation and abiotic stress

Various types of stress, including high salt (Matsuura *et al.*, 2010; Matsuura *et al.*, 2013), water deficit (Hsiao, 1970; Dhindsa and Cleland, 1975; Rhodes and Matsuda, 1976; Mason *et al.*, 1988; Bensen *et al.*, 1988; Kawaguchi *et al.*, 2003; Kawaguchi *et al.*, 2004; Kawaguchi and Bailey-Serres, 2005; Lei *et al.*, 2015), heat-shock (Gallie *et al.*, 1995; Matsuura *et al.*, 2010; Yángüez *et al.*, 2013; Merret *et al.*, 2015), cold (Wang *et al*., 2017), hypoxia (Branco-Price *et al.*, 2005; Branco-Price *et al.*, 2008; Juntawong *et al.*, 2014; Sorenson and Bailey-Serres, 2014) reactive oxygen species (Benina *et al.*, 2015), gravistimulation (Heilmann *et al.*, 2001), and heavy metals (Sormani *et al.*, 2011), resulted in a very significant decrease in global translation rates. Despite the genome-wide inhibition of translation, the mRNAs coding for proteins that were necessary for the survival under the stress situation were found in polysomes and, thus, likely actively translated. And most of these well-translated genes were also the ones upregulated at the transcriptional level under the stress, suggesting a well-coordinated adaptive response (Lukoszek *et al.*, 2016).

Over the years, the effects of dehydration on global translation have been studied in multiple species, including maize (Hsiao, 1970; Lei *et al.*, 2015), oats (Dhindsa and Cleland, 1975), pumpkin, pea, barley, wheat, and safflower (Rhodes and Matsuda, 1976), soybean (Mason *et al.*, 1988; Bensen *et al.*, 1988), tobacco (Kawaguchi *et al.*, 2003) and Arabidopsis (Kawaguchi *et al.*, 2004; Kawaguchi and Bailey-Serres, 2005), using different techniques that range from monodimensional Western blots to ribosome profiling. In these studies, a decrease in polysomal loading and protein synthesis was observed upon water stress, which could be reversed after rehydration. The extent of the polysomal decrease and recovery time depended on the severity of the stress, the species, and tissue employed. Despite the global decrease in translation, most of the stress-induced mRNAs were still properly translated. Remarkably, while there was no regulation of RP genes at the transcriptional level, ribosome loading for some RP transcripts was severely affected by the stress.

Heat stress produces a massive global decrease in translation of approx. 50% (Gallie *et al.*, 1995; Matsuura *et al.*, 2010; Yángüez *et al.*, 2013; Merret *et al.*, 2015), that can be observed as a decrease in polysomal fractions and an increase in the monosomal 80S peak. The severity of the change in the polysome profile in response to the stress is directly proportional to the stress duration (Gallie *et al.*, 1995). However, transcripts related to the stress response are actively translated upon heat treatment (Yángüez *et al.*, 2013). The global decrease in translation after heat stress is thought to be the consequence of ribosome pausing. This pausing is likely produced because of the down-regulation of HSP70 in the ribosomes after heat stress, which helps with the folding of the nascent peptides. Abnormal folding of the nascent protein under heat stress causes the ribosomes to pause, leading to the mRNA decay, as in the loss-of-function *hsp70* mutant such arrest in translation and induction of mRNA decay can be observed even at 20ºC (Merret *et al.*, 2015).. The co-translational regulation of mRNA degradation observed under heat requires the cytosolic exoribonuclease XRN4 (Merret *et al*., 2015).

The plant response to cold stress includes a decrease in the global levels of translation, which is, in part, achieved by the previously mentioned phosphorylation of eIF2α by AtGCN1 and GCN2 (Lageix *et al*., 2008; Zhang *et al*., 2008; Wang *et al*., 2017). In addition to this route, another mechanism exists that is mediated by the inactivation of TOR activity at the early stages of cold stress. As explained before, TOR phosphorylates AtS6K1, which then activates translation of uORF-containing genes (Schepetilnikov *et al.*, 2013). The inducible *tor* knockdown mutant does better under cold stress than at room temperature, a phenomenon likely related to the decreased levels of protein synthesis in *tor* (Wang *et al*., 2017). In fact, cold treatment transiently reduces the kinase activity of TOR in plants that are wild-type for this gene and, thus, phosphorylation of AtS6K1, decreasing translation rates concomitantly (Wang *et al*., 2017). However, TOR activity recovers and increases after a prolonged cold treatment.

Finally, oxygen deprivation also causes enormous changes in global translation. 2h of hypoxia result in up to a 50% decrease in global translation (Branco-Price *et al.*, 2005; Branco-Price *et al.*, 2008; Mustroph *et al.*, 2009; Juntawong *et al.*, 2014). This is thought to be an energy conservation response that is rapidly reversed to almost

normal levels within 1h of reoxygenation (Branco-Price *et al.*, 2008; Juntawong *et al.*, 2014). Not all the plant tissues respond equally to hypoxia. Roots are more affected than shoots, consistent with a more severe decline in the cellular ATP levels in roots. Similarly, roots experience a more drastic translatome remodeling with changes affecting genes such as *ADH1*, promoting fermentation to allow for ATP production under anaerobic conditions (Mustroph *et al.*, 2009). It was recently found that OLIGOURIDYLATE-BINDING PROTEIN1 (UBP1) plays a key role during hypoxic stress. Under hypoxia, UBP1 sequesters mRNA into stress granules, decreasing their translation potential. Importantly, once the stress conditions have subsided, these mRNA are released and re-start translation (Sorenson and Bailey-Serres, 2014).

Translational regulation of ribosomal proteins

In many of the stresses described above, the transcript levels of RPs were not affected, however their translational efficiency was drastically changed. This down-regulation of translation of mRNAs encoding RPs upon certain stimuli seems to be a logical strategy to prepare the cell for the general effect of those stimuli on translation. Under sucrose starvation, for example, mRNAs for RPs are drastically removed from polysomes, and the opposite happens when sucrose concentration increases (Hummel *et al.*, 2012; Pal *et al.*, 2013; Gamm *et al.*, 2014). Interestingly, not all members of a certain RP gene family behaved similarly: some members do not change, some members change both in mRNA and protein levels, and some other members only respond in total mRNA or polysomal levels (Gamm *et al.*, 2014). Hypoxia has also been shown to greatly alter the accumulation of RP mRNAs in polysomes (Branco-Price *et al.*, 2005; Juntawong *et al.*, 2014).

Translation regulation also takes place during normal plant development. In maize, in the first 24h of germination seedlings experience a dramatic global upregulation of transcription and protein synthesis, while the mRNA levels for most RPs remain constant. This observation suggests that the concomitant increase in the synthesis of new RPs has to depend on pre-existing RP mRNAs and, therefore, on changes in their translation efficiency (Jiménez-López *et al.*, 2011). Interestingly, these mRNAs had in common the presence of 5´TOP (track of pyrimidines) suggesting a possible mechanism for the translational regulation of these genes (Jiménez-López *et al.*, 2011).

Finally, it is noteworthy that the *rpl4d* and *rpl5a* mutants in RP genes show very mild phenotypes in the Arabidopsis Columbia background but very severe defects in Landsberg *erecta* (L*er*), suggesting that the genetic background has a strong influence on the ribosomal regulatory mechanisms that control developmental programs (Rosado *et al.*, 2012).

E) GENE-SPECIFIC TRANSLATION REGULATION

Gene-specific translation regulation occurs when the translation rate of a particular mRNA species does not correlate with the concentration of that mRNA in a cell, implying that the transcript is regulated in a particular way independent of the overall translational status of the cell. The activation of this type of regulation, although not fully understood in most cases, is thought to involve specific trans-factors such as RNA binding proteins, specific translation factors, ribosomal protein isoforms, small RNAs, certain metabolites, as well as sequence- and/or structure-based *cis*-regulatory elements in the target mRNAs.

Regulation of translation through uORFs

As stated above, uORFs are short translated open reading frames present in the 5´ leaders of mRNAs that usually repress translation of the main downstream ORF. The effectiveness of a uORF in repressing translation depends on several characteristics such as the sequence context around its initiation codon, the length, the distance between its stop codon and the next ORF in the transcript, and/or the overlap of the uORF with the mORF. Thus, when the AUG codon of a uORF resides in a very weak translation initiation sequence context, most ribosomes would skip it and continue scanning (Kozak, 2002). Similarly, although the length of the uORF is also important, the correlation between the length of the uORF and the inhibition of translation of the main uORF is moderate and only for uORFs longer than 16 amino acid such correlation can be expected (Arnim *et al.*, 2014). In addition, the uORFs that overlap with the mORF are the ones with the strongest inhibitory effect (Yun *et al.*, 2012). Liu et al. (2013) found that translated uORFs tend to be longer, located more proximately to the 5´ end of the transcript and have stronger AUG contexts than the untranslated uORFs. In general, the higher the number of uORFs present in a transcript, the stronger the inhibition of the mORF (Liu *et al.*, 2013).

There are two general types of uORFs: sequence-dependent uORFs, also known as conserved peptide uORFs (CPuORFs), and sequence-independent uORFs (SIuORF). In CPuORFs the sequence is conserved among different taxa or gene families and the peptide encoded by the uORF is thought to be important for the inhibitory function of the uORF. While in SIuORFs the sequence of the uORF is not conserved across related species and is not important

for the repressive function, the presence and position of the SIuORF may still be conserved across related species (Vaughn *et al*., 2012, and examples below). SIuORFs are more frequent and the inhibition of translation by SIuORFs of the downstream mORF is generally due to inefficient translation reinitiation. In contrast, CPuORFs are more rare and generally inhibit translation of the mORF by causing ribosome stalling during translational elongation or premature translation termination (Gaba *et al.*, 2001; Arnim *et al.*, 2014; Hou *et al*., 2016). Global analyses of 5´-truncated mRNA ends by GMUCT (Genome-wide Mapping of Uncapped and Cleaved Transcripts) and PARE (Parallel Analysis of RNA Ends) (see Glossary of Techniques) revealed that CPuORFs predominantly accumulate reads at two sites upstream of the CPuORF stop codon, -16 and -45 to -47, implying the stalling of ribosomes in tandem and co-translational decay (Hou *et al*., 2016, Yu *et al*., 2016). Tandem occupancy was found frequently at uORFs, but is almost absent in mORFs (Yu *et al*., 2016).

Although the exact number of genes with functional uORFs is difficult to determine, it has been estimated that 20-35% of all Arabidopsis genes contain one or more uORFs, therefore implying the presence of uORFs in a range of 5000-9000 genes (Pesole *et al.*, 2000; Rogozin *et al.*, 2001; Kim *et al.*, 2007; Arnim *et al.*, 2014), with only 60-100 of those genes possessing CPuORFs (Hayden and Jorgensen, 2007). These numbers are probably underestimations, as many active uORF can start with non-canonical start codons that would have escaped the previous calculations (Simpson *et al.*, 2010; Hsu *et al.*, 2016). The experimental validation even for the CPuORFs has proven challenging due to the technical difficulties to detect low abundant small peptides by standard mass spectrometry approaches (Arnim *et al.*, 2014). Nowadays, with the development of the Ribo-seq technology, empirical identification of uORFs has been facilitated tremendously. Searches for actively translated uORFs by Ribo-seq in 3- and 4-day-old Arabidopsis seedlings detected approx. 2000 genes that contained 5360 and 3177 uORFs, respectively (Liu *et al.*, 2013). Interestingly, a total of 3615 translated genes, containing 6927 non-translated uORFs have been also reported. These non-translated uORFs could represent non-active uORFs or, more likely, uORFs that have a regulatory role but that were inactive under the experimental conditions used. A recent Arabidopsis Ribo-seq study identified, with high confidence, 187 translated uORFs among the 8,745 translated genes (Hsu *et al.*, 2016) examined. Finally, although the translational effects of only a handful of uORFs have been studied, studies in Arabidopsis have found an enrichment of uORF-containing genes among GO categories related to regulatory activities, such as catalytic and transferase activities, transcription regulation, or kinases, among others (Kim *et al*., 2007; Hu *et al.*, 2016).

In the next sections, we will highlight some of the most significant examples where the mode of action or biological significance of these uORFs have been determined.

uORFs in the polyamine metabolism: Polyamines, with their most abundant forms spermine, spermidine and putrescine, are small organic polycations involved in a variety of cellular processes that affect growth and development. Polyamines have been implicated in cell proliferation, gene expression, post-translational regulation (including the hypusine modification of eIF5A/eEF5 (Balabanov *et al.*, 2007)), nucleic acid and membrane structure and functionality, control of the activity of ion-channels, and cell stress, with defective regulation of the polyamine metabolism leading to cell malfunction and disease (reviewed in Takahashi and Kakehi, 2010; Pegg and Casero, 2011; Miller-Fleming *et al.*, 2015). Accordingly, cellular polyamine content is tightly regulated by biosynthesis, transport, conjugation, and degradation (Guerrero-González *et al.*, 2016). One of the components of this complex mode of control is the uORF-dependent translational regulation of polyamine metabolism genes.

S-Adenosyl methionine decarboxylase (AdoMetDC) is a key enzyme in the polyamine biosynthesis pathway. It decarboxylates S-AdoMet to dcAdoMet, which is then utilized for the synthesis of spermidine from putrescine. The *AdoMetDC* mRNA from plants possesses two highly conserved uORFs in their 5´UTRs, the 5´tiny and the 3´small, that overlap by 1 nt (Franceschetti *et al.*, 2001) (Figure 4A). The removal of these uORFs in transgenic tobacco causes dramatic phenotypes, highlighting the importance of these elements in the regulation in the polyamine metabolism (Hanfrey *et al.*, 2002). The 48-54 amino-acid-long small uORF is a CPuORF, CPuORF9, and is involved in the constitutive repression of the *AdoMetDC* mORF independently of the polyamine concentrations. In the presence of normal polyamine conditions, translation starts at the 3-4 amino-acid-long tiny uORF, the ribosome gets through the AUG of the second small uORF without translating, because it is in a different frame, and gets dissociated at the stop codon of the tiny uORF. Due to the very short length of tiny uORF, reinitiation at mORF is very efficient and the 40S subunit scans until the AUG of the *AdoMetDC* ORF initiating its translation. In the presence of high levels of polyamines, the ribosome does not initiate translation at the tiny uORF's AUG, which is in a weak translation context, and starts directly at the small uORF. Translation of the small uORF does not permit reinitiation at a downstream mORF, causing ribosomes to stall at the stop codon of the small uORF, and thus preventing the translation of *AdoMetDC* (Hanfrey *et al.*, 2005; Uchiyama-Kadokura *et al.*, 2014). The effect of the tiny uORF is sequence independent, while the polyamine-dependent translation repression of the

small uORF is sequence dependent. RNA degradation after ribosome stalling was observed using reporter fusions of the *AdoMetDC1* small uORF. This degradation was reduced in *upf* mutants, linking uORF-mediated ribosome stalling with mRNA degradation through the nonsense-mediated mRNA decay (NMD) pathway (Uchiyama-Kadokura *et al.*, 2014) (Figure 4A). Indeed, when analyzing mRNAs degradome, protected fragments appeared at position -16 from the CPuORF stop codon, consistent with a ribosome-protected fragment from the ribosomes stalled at this uORF stop codon (Hou *et al*., 2016).

Another case of translation regulation within the polyamine biosynthetic pathway was discovered in a mutant screen looking for suppressors of the *acl5* mutant in Arabidopsis, which exhibits severe dwarf phenotypes (Imai *et al.*, 2004). *ACAULIS5* (*ACL5*) encodes a thermospermine synthase and *suppressor of acaulis51* (*sac51-d*) restores the defects caused by the *acl5* mutation in the absence of thermospermine. *sac51-d* is dominant and harbors a mutation that causes a premature stop codon in the 4th uORF of *SAC51*, a bHLH transcription factor (Figure 4B). The 5´UTR of *sac51-d* was found to enhance translation compared with the WT 5´UTR (Imai *et al.*, 2006). It is likely that in WT plants thermospermine releases the inhibition caused by the uORFs in the 5´UTR of *SAC51,* whereas the enhancement of translation in *sac51-d acl5* double mutant is due to the premature stop codon in the 4th exon in *sac51-d* that facilitates ribosome reinitiation and thus the efficient translation of the *SAC51* mORF even in the absence of thermospermine (Imai *et al.*, 2006) (Figure 4B). Other suppressors of *acl5*, such as *sac52-d*, *sac53-d* and *sac56-d,* correspond to dominant or semidominant mutant alleles of the ribosome components *RPL10*, *RACK1* and *RPL4*, supporting the role of these proteins in the translation of uORF-containing transcripts under thermospermine-dependent translation control (Imai *et al.*, 2008; Kakehi *et al.*, 2015).

Recently, translation regulation has also been found for the polyamine oxidases (PAO), enzymes involved in the catabolism of polyamines. There are five PAO genes in Arabidopsis, and three of them, *PAO2*, *PAO3* and *PAO4*, possess uORFs that are conserved in a wide array of plant species, ranging from mosses to monocots and dicots (Vaughn *et al*., 2012; Guerrero-González *et al.*, 2014). *PAO2* was studied in more detail, and the peptide encoded by its uORF was found to exert control by repressing translation of the mORF in the presence of exogenously added polyamines (Guerrero-González *et al.*, 2014; Guerrero-González *et al.*, 2016).

uORFs and metabolite-dependent translation regulation: ATB2/bZIP11 is a small transcription factor that belongs to the group S of basic region leucine zipper (bZIP) genes and directly controls the expression of *ASN1* and *ProDH2* genes that code for enzymes involved in amino acid metabolism (Baena-González *et al.*, 2007; Hanson *et al.*, 2008). The *bZIP11* mRNA contains an unusually long 5´UTR that harbors four small uORFs. The expression of *bZIP11* was strongly repressed at the translational level in the presence of physiological levels of sucrose, although its mRNA expression was not affected (Rook *et al.*, 1998). Glucose or fructose had no effect on *bZIP11* translation. The second uORF (uORF2) was responsible for this repression and was able to confer sucrose-dependent translation regulation to a reporter gene. So, uORF2 was called Sucrose Control uORF (SC-uORF) and the mechanism referred to as Sucrose-Induced Repression of Translation (SIRT) (Wiese *et al.*, 2004; Rahmani *et al.*, 2009) (Figure 5). uORF2 possesses an internal AUG codon that allows the synthesis of two peptides, a longer (42 amino acid) or a shorter (28 amino acid) one, with the second shorter peptide being sufficient for SIRT (Wiese *et al.*, 2004). Interestingly, this uORF2 starts at an AUG in a weak translation initiation context, which presumably allows leaky scanning and translation of mORF to occur (Rahmani *et al.*, 2009). The SC-uORF is conserved in four other members of group S of *bZIP*s, and their 5´UTRs also mediate translational repression in response to sucrose (Jakoby *et al.*, 2002). And this regulatory mechanism is also conserved in other dicot and monocot plant species (Wiese *et al.*, 2004; Hummel *et al.*, 2009; Thalor *et al.*, 2012). Likely, the SC-peptide works by stalling the ribosomes on the uORFs in high sucrose concentrations preventing translation of the mORF (Hummel *et al.*, 2009). This was recently confirmed when analyzing the mRNA degradome, when PARE reads appeared in *bZIP2*, *bZIP11* and *bZIP53* at positions -16, -46 and -17 of the stop codon of the SC-uORF, consistent with one, two or three adjacent ribosomes stalled at the CPuORF stop codon (Hou *et al*., 2016). However, the mode of action and the proteins involved in the recognition are still unknown.

The next case is a beautiful example of metabolite-mediated translation regulation that depends on a uORF that starts with a non-canonical start codon. GDP-L-galactose phosphorylase (GGP) encoded by the gene *GGP,* also known as *VTC2*, works as a central control point in vitamin C biosynthesis (Bulley *et al.*, 2009). Vitamin C concentrations are regulated according to the demands of the cell, and *GGP* transcripts show strong circadian rhythms, with maximal expression pre-dawn, so that the transcript is available when photosynthesis begins (Müller-Moulé, 2008). *GGP* contains a long 5´UTR that is able to regulate translation of the transcript in an ascorbate-dependent manner, limiting GGP protein production and, thus, ascorbate biosynthesis under high vitamin C conditions (Laing *et al.*, 2015). This 5´UTR encodes a highly conserved yet newly discovered 60 to 65-amino acid peptide that initiates at a non-canonical ACG initiation codon in all plants, from mosses to angiosperms. Disruption

of this CPuORF stops the ascorbate feedback on *GGP* translation regulation, and moreover, removal of the 5´UTR or inactivation of the *GGP* CPuORF increased ascorbate levels in leaves. Although the peptide is translated, as indicated by the presence of ribosome footprints at a density even higher than that found in the *GGP* CDS (Liu *et al.*, 2013), Laing and co-authors (Laing *et al.*, 2015) were not able to detect the peptide *in vivo* nor show interaction between the peptide and ascorbate. Thus, the exact mechanism by which ascorbate represses the translation of *GGP* is still unknown. Interestingly, this conserved peptide escaped from the searches of conserved peptides in CPuORFs (Hayden and Jorgensen, 2007), most likely because the search was done for canonical origins of translation. So, this raises the possibility that there are many more examples of uORFs that initiate at non-canonical start codons that are yet to be identified. By Ribo-seq it has been very recently found that this CPuORF starts 14 amino acids upstream of the ACG start codon described herein, at another non-AUG codon (Hsu *et al.*, 2016).

Translation repression control mediated by a metabolite and a uORF has also been found in the methylation pathway of phosphatidylcholine biosynthesis. *XIPOTL1* encodes a phosphoethanolamine N-methyltransferase (PEAMT) that synthesizes phosphocholine, an intermediate of phosphatidylcholine biosynthesis that affects root meristem size, cell division, and cell elongation. *XIPOTL1* contains a CPuORF that represses translation of the mORF in response to phosphocholine (Tabuchi *et al.*, 2006; Alatorre-Cobos *et al.*, 2012) and this regulation is critical for maintaining proper levels of this metabolite during plant development, yet once again, the molecular mechanisms of this regulation are not fully understood.

Although boron is not a metabolite, translation regulation in a uORF and boron-dependent manner has been recently uncovered. *NIP55;1* encodes a boric acid channel that is required for efficient boron uptake by the roots and essential for growth under low boron conditions (Takano *et al.*, 2006). *NIP5;1* carries two minimal uORF (AUG-Stop: AUGUAA) in its 5´UTR that are responsible for the translational regulation of the mORF in a boron-dependent manner (Tanaka *et al.*, 2016). Under high-boron conditions, ribosomes stall at the AUG-Stop sites inhibiting translation, with each of the two minimal uORFs being sufficient for this inhibition. Ribosomes stalled at these sites caused boron-dependent degradation of the transcript in an NMD-independent mechanism that depended on a 5´UTR sequence upstream of the stalled ribosome. Importantly, just the minimal uORFs were able to confer boron responsiveness to a non-boron responsive transcript. Additionally, two other boron-responsive genes presented this type of regulation, that is also conserved in animals. Interestingly, around 23% of Arabidopsis genes possess minimum uORFs. It would be of much interest to determine whether the same AUGUAA sequence could mediate translation regulation under different environmental and developmental conditions.

uORFs in biotic and abiotic stress responses: When facing a severe stress, plants need to decide whether to focus on the response to the stress or combine the response with plant growth. HsfB1/TBF1 (*At4g36990*) acts as a molecular switch that inhibits plant growth and development upon pathogen attack or strong environmental stress (Pajerowska-Mukhtar *et al.,* 2012). *HsfB1/TBF1* is an Arabidopsis heat shock factor that increases its translation in response to heat stress and that contains two uORF in its 5´UTR. The second uORF is conserved (*At4g36988*, *CPuORF49*). Both uORFs repress translation of a downstream ORF, although the repression exerted by the second uORF is stronger and epistatic over the first one. Interestingly, this repression is alleviated upon pathogen attack or heat shock, allowing the transcript to be translated (Pajerowska-Mukhtar *et al*., 2012; Zhu *et al*., 2012). Both uORFs of *HsfB1/TBF1* are rich in phenylalanine (Phe) and CPuORF49 was shown to exert a stronger repression of translation in the presence of high levels of Phe (Pajerowska-Mukhtar *et al*., 2012). Pathogen atack, on the other side, released the inhibition. Interestingly, pathogen attack was found to alter the Phe metabolism and to elevate the levels of phosphorylated eIF2α. This lead to the hypothesis that upon pathogen attack, the phosphorylation of eIF2α would difficult ribosome reassembly after translation of the first uORF, and as happens in yeast with the translation of *GCN4*, the 40S would continue scanning through the second uORF and reinitiate translation at the AUG of the mORF (Pajerowska-Mukhtar *et al*., 2012). In addtion, this CPuORF49 was proven to be sufficient to confer heat-mediated translation regulation to a non-heat-regulated gene (Zhu *et al*., 2012).

Translation regulation in response to metabolites: *CGS1* codes for a cystathionine gamma-synthase that catalyzes the first committed step of methionine biosynthesis (Thompson *et al.*, 1982). The reversible translational regulation of this gene in response to S-Adenosylmethionine (AdoMet) is mediated by a CPuORF and represents an unusual case of ribosomal arrest at the step of elongation (Onouchi *et al.*, 2005). First, it was observed that the *CGS1* transcripts are degraded in response to Met. A highly conserved amino acid stretch called MTO1 located approx. 80 residues from the N-terminus of CGS1 is necessary for this feedback regulation and acts in *cis* controlling the expression of *CGS1* (Chiba *et al.*, 1999). It was found later that the actual molecule that causes the regulation is AdoMet, a metabolite of Met, and that a translational arrest occurs prior to mRNA degradation (Chiba *et al.*, 2003).

Further characterization showed that the ribosome arrest in response to AdoMet happens during translation at the Ser94 codon positioned immediately downstream of the MTO1 region. The distance from the initial Met to Ser94 was shown to be important (Onouchi *et al.*, 2005). When the ribosome stops at Ser94, the MTO1 peptide is in the ribosome exit tunnel. In the presence of AdoMet, the MTO1 peptide inside of the tunnel adopts a more compacted conformation causing the reversible arrest of the translating ribosome (Onoue *et al.*, 2011). Although an exciting possibility of AdoMet interacting directly with the nascent MTO1 peptide in the ribosome tunnel has been proposed, experimental support for that is still lacking. After the AdoMet-dependent translational arrest at position Ser94, the accumulation of ribosomes at this and preceding codons is thought to trigger mRNA degradation, and, in fact, ribosomal pausing at a second (Val85) and third (Ala76) positions has also been observed (Yamashita *et al.*, 2014).

Translation regulation mediated by structural features of the mRNA

As mentioned above, evidence of IRES-mediated translation in plants is poor, and most of the cases have been found in plant viruses. However, there are some indications suggesting IRES-mediated translation occurring during plant development. WUSCHEL (WUS) is an homeodomain protein that controls the proliferation and cell identity of the cells in the shoot apical meristem (Mayer *et al.*, 1998). The La protein is a nuclear-localized RNA-binding factor with roles in RNA metabolism and processing in all eukaryotes. It was described that La can bind to 5´UTRs of target transcripts and mediate IRES-dependent translation (Kim *et al.*, 2001; Wolin and Cedervall, 2003). Interestingly, in Arabidopsis, the lack of *AtLa1* resembles the *wus* mutant phenotype. Furthermore, *WUS* contains an IRES in its 5´UTR. Thus, it was not a total surprise when it was found that under environmental stress a fraction of AtLa1 protein moves to the cytoplasm, where it binds to the 5´UTR of *WUS* and increases its IRES-dependent translation (Cui *et al.*, 2015). This mechanism may have evolved to assure the proper translation of essential genes, such as *WUS,* in the meristem maintenance under stress-conditions when cap-dependent translation is down-regulated.

Other plant cellular mRNAs are also thought to be translated through IRES-like mechanisms. For example, during heat stress, canonical cap-dependent translation decreases, however, transcripts for certain proteins, heat-shock proteins (HSPs) among them, resist this down regulation, most likely through a feature in their 5´UTRs that renders cap-independent translation. Dinkova *et al.* (2005) found that the increase of maize HSP101 during heat stress reveals translation regulation. The translation of *HSP101* during heat stress depended on its 5´UTR and could be carried out in wheat germ extracts from which the eIF4E and eIFiso4E had been depleted, simulating cap-independent conditions. This mechanism seems to be an efficient way to ensure the translation of *HSP101* upon heat stress conditions when cap-dependent translation is inhibited.

Hairpin structures in the 5' end of transcripts have been known for a long time to affect translation in bacterial and animal systems (Kozak, 1999), and evidence for this type of translational regulation is much more scarce in plants. There are, nevertheless, some examples of translationally regulated mRNAs in Arabidopsis where the regulation appears to be conferred by the formation of a hairpin structure. Thus, the *PHYTOENE SYNTHASE* (*PSY*) gene in Arabidopsis constitutes a beautiful example of sophisticated transcriptional and translational regulation resulting in functional diversification in just one gene. *PSY* catalyzes the first committed and rate-limiting step in the synthesis of carotenes and, therefore, highly regulated (Hirschberg, 2001; Álvarez *et al.*, 2016). Recently, two alternative splice variants (ASV) of the Arabidopsis *PSY* that differ in their length and the presence of an intron in their 5´UTR have been identified. The 5´UTR is longer in ASV1 than in ASV2 and lacks an intron (Álvarez *et al.*, 2016). In addition, ASV1 and 2 differ in their expression patterns and biosynthetic roles. Thus, while ASV2 is responsible for the immediate phytoene biosynthesis in response to environmental stresses, ASV1 is developmentally regulated and responsible for carotenogenesis in processes such as de-etiolation. There is, however, an additional second layer of regulation. The long 5´UTR of ASV1 inhibits translation of *PSY,* presumably due to the formation of a hairpin loop in the 5'UTR. ASV2 lacks this hairpin and is translation-permissive. Interestingly, in most plant taxa, *PSY* constitutes a small three-member gene family, while in *Brassicaceae* there is only one *PSY* gene (Walter *et al.*, 2015). This sophisticated mechanism of gene function duplication is likely a way that *Brassicaceae* have evolved to perform multiple activities [that in other species require several genes] with only one gene copy. How the translation machinery can overcome the hairpin loop and translate the main ASV1 ORF of *PSY* is still unknown.

The formation of secondary structures within the 5´UTR of the pollen gene *npt303* also contributes to its specific translation during pollen germination. *npt303* was shown to be translationally regulated, as its mRNA accumulates to high levels during pollen development, but the protein is only detectable later during pollen germination. The 5´UTR of *npt303* is responsible for the specific repression of translation during the early stages of

pollen development. This 5´UTR forms two stem-loop structures, I and II. Both have been found necessary for the proper translational regulation of *npt303*, with the removal of structure II affecting both transcript translation and stability, and structure I reducing translation with no effect on transcript levels (Hulzink *et al.*, 2002). Additional work would be however required to understand the molecular mechanism behind this 5'UTR-mediated regulation and the *trans*-factors involved.

Although, as exemplified in the previous section, strong hairpin structures in the 5'UTR of an mRNA typically have a negative impact on its translation, there are some cases in which the formation of such secondary structures may have a positive impact on protein synthesis. This positive effect on translation can be achieved by ribosomal shunting. The best studied cases of shunting are described in plant viruses (Ryabova and Hohn, 2000; Ryabova *et al*., 2006; Pooggin *et al*., 2012) and it is a rare phenomenon outside of viral transcripts. One such example of ribosome shunting on cellular mRNAs can be found is the multicellular green alga *Volvox carteri* during the translational regulation of the *regA* mRNA. *regA*s a master regulator gene that represses the transcription of genes involved in growth and reproduction in somatic cells (Kirk *et al.*, 1999). It was found that *regA* mRNA and protein levels do not correlate, and that *regA* possesses 8 potential uORFs in its 5´UTR. When tested, the only uORFs with capacity to repress translation of the mORF were uORF1 and 2. These two uORFs are within a region of very strong secondary structure that resembles the viral structures known to promote ribosome shunting. Deletion of the putative ribosome landing site completely abolished the *regA* regulation, and transformation with a construct harboring an artificial hairpin strong enough to repress regular ribosome scanning and inserted upstream of the ribosome landing site was able to rescue the *rega* mutant defects, indicating that the transgene was translated and, therefore, providing support for the possible translation of *regA* through a shunting mechanism (Babinger *et al.*, 2006).

Translation regulation mediated by small RNAs

Small RNAs, including microRNAs (miRNAs) and small interfering RNAs (siRNAs), are important regulatory components of many developmental and physiological processes in both plants and animals. They typically repress gene expression by guiding the RNA-induced silencing complex (RISC) to their target mRNAs and induce transcript cleavage or translational inhibition.

The biogenesis of miRNA in plants depends on Dicer-like proteins (DCL). DCL1 is responsible for the generation of miRNAs, that are then loaded onto AGO1 to form the miRNA-RISC complex that recognizes its target mRNA by base-pairing. In animals, perfect mRNA-miRNA base-pairing is associated with target mRNA cleavage, while lower complementary is associated with translation inhibition. Plant miRNAs in general show high degree of complementarity to their targets and were assumed to act exclusively by cleavage. However, it is becoming increasingly evident that translation inhibition is also a mode of action for some plant miRNAs (Brodersen *et al.*, 2008; Eckardt, 2009; Dalmay, 2013; Bologna and Voinnet, 2014). It has been shown that the broad majority of plant miRNA targets are located within the coding sequence of the mRNA, although they can inhibit translation regardless of the position (5´UTR, CDS, or 3´UTR), and the process of translation inhibition can be genetically uncoupled from miRNA-directed slicing.

An early report of a plant miRNA with high sequence complementarity to their target sequence to also act by regulating translation came from the studies in which *miR172* was shown to inhibit translation of *AP2* and a small subfamily of *AP2*-like transcription factors (Aukerman and Sakai, 2003; Chen, 2004). *AP2*-like genes act as floral repressors. *miR172* keeps the expression of *AP2* genes low during early wild-type plant development, resulting in the promotion of flowering. Overexpression of *miR172* in rice phenocopies the knockout mutant of its target, *SUPERNUMERARY BRACT (SNB),* without altering the mRNA levels, which is consistent with *miR172* regulating *SNB* in rice via translational repression (Zhu *et al.*, 2009).

A similar case was found with *miR15*6, *miR157*, and *miR398*. *miR156* and *miR157* that target 11 of the 17 members of the *SBP* box gene family. Gandikota *et al.* (2007) showed that, in addition to the described role of these miRNAs in mRNA cleavage, *miR156* and *157* also inhibit the translation of the *SBP* box gene *SPL3* accounting for the proper regulation of this gene during floral transition. Similarly, *miR398* was shown to repress the translation of the Cu/Zn superoxide dismutases *CSD1* and *CSD2* under low copper conditions (Dugas and Bartel, 2008).

Interestingly, *AP2, SPL3* and *CDS1*, targeted by *miR172, miR156,* and *miR398*, respectively, have shown to be transcripts with very high efficiency of miRNA-mediated cleavage, implying that miRNAs can efficiently silence the expression of genes by both mechanisms, cleavage and translational repression (Yu *et al.*, 2016). *PLANTACYANIN (ARPN),* that encodes a blue copper protein, and the miRNA metabolism genes *AGO1* and *SERRATE (SE)* are also subjects of this type of regulation, being translationally repressed by *miR408, miR168,* and *miR863*, respectively (Reis *et al*., 2015).

How exactly miRNAs repress translation is still not fully understood, but some of the pieces of the puzzle are starting to be unveiled. For example, *SUO* was found as a necessary component of the *miR156/157*-mediated repression of *SPL3* and *miR398*-mediated repression of *CSD2*. *SUO* encodes a protein with two conserved GW domains that co-localizes with DCP1 in P-bodies (Yang *et al.*, 2012). Other pieces of the puzzle were uncovered during the study of two DOUBLE-STRANDED RNA-BINDING proteins, DRB1 and DRB2, which are partners of DCL1 in miRNA biogenesis. It has recently been shown that *DRB1* is required for miRNA-dependent mRNA cleavage and that *DRB2* is required for translational inhibition. This is important as it suggests each miRNA could be predetermined for a specific role from the moment of its biogenesis (Reis *et al.*, 2015). In addition, the fact that *DRB2* is evolutionary conserved makes it possible to speculate that translational repression may have been a primitive form of miRNA-directed gene regulation in ancient plants.

Other necessary proteins for this type of translation regulation are AGO1, AGO2, AGO10, and the decapping enzyme VARICOSE (VCS) (Brodersen *et al.*, 2008; Fátyol *et al.*, 2015). AGO1 and miRNAs can associate with actively translating polysomes. The AGO1-RISC complex is capable of repressing translation of the target mRNA without promoting deadenylation or mRNA decay by blocking the 48S complex formation. In addition, when bound to the mRNA, the complex can sterically block the recruitment or movement of ribosomes (Lanet *et al.*, 2009; Iwakama and Tomari, 2013). However, no specific patterns of PARE reads that could reveal ribosome stalling appeared at a region of approx. 55 nt upstream of the putative miRNA-guided cleavage site, which suggests that the mechanism by which miRNAs repress translation may not be by acting as physical barriers to impair ribosome movement (Hou et al., 2016). However, the binding of AGO7 to a non-cleavable target of *miR390* seems to arrest ribosomes at the step of elongation (Hou et al., 2016). AGO2, in addition to slicing, has also been implicated in miRNA-dependent translation repression, with the activity of AGO2 being independent of the degree of sequence complementarity between miRNA and its target (Fátyol *et al.*, 2015).

In a genetic screen looking for necessary components for miRNA-mediated translation inhibition, *miRNA action deficient mutants5 and 6* (*mad5/6*) were found (Brodersen *et al.*, 2008). *MAD5* encodes KATANIN, a microtubule-severing enzyme, which suggests that proper microtubule dynamics are also needed for this regulation. Another protein that has been found necessary for miRNA-mediated regulation of translation is ALTERED MERISTEM PROGRAM1 (AMP1) (Li *et al.*, 2013). The *amp1* mutant is affected in translation inhibition, but not in cleavage. AMP1 is needed to send the miRNA target mRNAs to the ER-bound polysomes, where it co-localizes with AGO1. This suggests that the ER is the place where miRNA-mediated translation repression takes place. The fact that target genes of multiple miRNAs are de-repressed at the translational level in the *mad5*, *mad6*, and *amp1* mutants indicates that translational repression by miRNA is more widespread in plants than originally thought (Ma *et al.*, 2013). Evidences for miRNA-mediated translational regulation were also found in Ribo-seq experiments by Liu and co-workers (Liu *et al.*, 2013). In that study, miRNA targets had significantly lower translation efficiencies than the untargeted transcripts, whereas the steady-state levels of transcripts of miRNA-target and non-target genes were similar. The fact that the density of footprints across the CDS was uniformly lower for miRNA targets than for non-targets suggests that miRNA may repress translation at the initiation or elongation steps.

Recently, a putative endogenous siRNA involved in translational regulation was also described. Maize *small1* is a 24-nt small RNA, processed by DCL3 and RDR2, that was shown to interfere with the translation of *Outer Cell Layer1 (OCL1)* when transiently expressed in *Nicotiana benthamiana* without altering *OCL1* mRNA levels (Klein-Cosson *et al.*, 2015).

<u>Translation regulation mediated by RNA binding proteins</u>

Two independent groups uncovered an RNA-binding-protein-based translation regulation module within the ethylene-signaling pathway that regulates translation of the signal repressors *EBF1* and *EBF2* in the presence of the hormone ethylene (Merchante *et al.*, 2015; Li *et al.*, 2015). mRNA levels of *EBF2* were known to increase upon ethylene treatment, but no concomitant increase in protein levels was detected (Guo and Ecker, 2003; Potuschak *et al.*, 2003; Konishi and Yanagisawa, 2008). By studying the genome-wide effects of ethylene on translation by means of Ribo-seq, it was found that translation of *EBF1* and *EBF2* was down-regulated in response to the hormone (Merchante *et al.*, 2015). This ethylene-mediated effect on the translation of *EBF1/2* transcripts was found to be due to their very long 3´UTRs that are both necessary and sufficient to confer ethylene-dependent translation regulation to a non-ethylene responsive gene (Merchante *et al.*, 2015; Li *et al.*, 2015). In the presence of ethylene, the C-terminal end of the ER-localized protein EIN2 is released from the membrane (Wen *et al.*, 2012; Ju *et al.*, 2012; Qiao *et al.*, 2012) and travels to the nucleus where it potentiates a transcriptional cascade that leads to the up-regulation of *EBF2* transcription (Figure 6). In the cytoplasm, directly or indirectly, EIN2 C-terminus binds to the 3´UTR of *EBF1* and *EBF2* and, by collaborating with the UPF proteins of the NMD machinery, sequesters the *EBF* transcripts into P-bodies, impeding their translation and explaining the lack of EBF protein accumulation upon

ethylene treatment despite high *EBF2* transcript levels (Figure 6B). Translation of the stored *EBF* transcripts can resume once ethylene is withdrawn (Merchante *et al.*, 2015; Li *et al.*, 2015). The recent finding of EIN2 as an RNA-binding protein (Reichel *et al.*, 2016) suggests that the interaction of EIN2 with the 3´UTR of *EBF*s is indeed direct.

Another example of translation regulation through protein binding, in this case to the 5´UTR of its target, comes from the study of the *PENTA1 (PNT1)* gene and its regulation within the phytochrome signaling pathway. Phytochromes, the red and far-red light receptors, can interconvert between the Pr to Pfr form and vice versa, in response to red and far-red light, respectively (Inoue *et al.*, 2017). The Pfr form of the phytochromes moves to the nucleus to promote gene expression changes leading to photomorphogenesis and so, the nucleus was considered the focal site of phytochrome signaling (Lorrain *et al.*, 2006; Toledo-Ortiz *et al.*, 2010). However, a translation repression role for the Pfr form of PhyA and PhyB in the cytosol has been also uncovered. Pfr forms bind the cytosolic protein PNT1. PNT1 can repress translation of the protochlorophyllide reductase *PORA* gene by binding to its 5´UTR. This repression does not occur in the *phyA phyB* Arabidopsis double mutant, but is present in the *fhy1/fhl* mutant that is impaired in the transport of PhyA to the nucleus. This double regulation of the Pfr form of the phytochromes inhibiting *PORA* transcription in the nucleus and translation in the cytosol is an effective and rapid way to reduce the levels of PORA upon the dark to light transition (Paik *et al.*, 2012). What structure in the 5´UTR of *PORA* is recognized by PNT1 and how the translation inhibition occurs remain unknown.

In *Toxoplasma gondii*, Alba proteins have shown to regulate their own translation. In the absence of TgAlba1 protein, *TgAlba2* is properly transcribed, although the protein cannot be detected, while under normal TgAlba1 levels, the TgAlba2 protein accumulates. It was found that the proper translation of *TgAlba2* depends on its 3´UTR and on the presence of active TgAlba1 (Gissot *et al.*, 2013), and most likely is mediated by TgAlba1 directly or indirectly binding to the 3´UTR of *TgAlba2* and promoting its translation. Although nothing is known about Alba proteins in plants, the recent discovery that they directly bind RNA in Arabidopsis (Reichel *et al.*, 2016) opens the door for a translation regulation role of Alba proteins in plants.

Translation regulation mediated by unknown mechanisms

There are also many cases in which a certain event of translation regulation has been found to be determined by, for example, the 5´UTR of a specific mRNA, however, the exact mechanisms remain unknown and no uORF or IRES seem to be involved. One of such unresolved cases happens with the enzyme delta 1-pyrroline-5-carboxylate reductase (P5R) that catalyzes the final step in the biosynthesis of proline from glutamate and ornithine (Smith and Greenberg, 1956). Transcript levels of Arabidopsis *P5R (AtP5R)* are up-regulated upon heat and salt stresses due to increased half-life of the *AtP5R* mRNA in these conditions. However, protein accumulation does not occur, nor does the Pro accumulation in the cell correlate with the mRNA levels under these stresses (Hua *et al.*, 2001). In fact, during these stresses, and opposite to the mRNA increase, the polysome loading of *AtP5R* is reduced consistent with a partial decrease in translation initiation. The 5´UTR of *AtP5R* was found to be responsible for both the increase in mRNA stability and the down-regulation of translation under these conditions, however, the biological significance of this regulation remains unknown (Hua *et al.*, 2001). The 5´UTR of *AtP5R* is longer than average, with a GC rich, highly structured sequence, and with no canonical uORFs (Hua *et al.*, 2001).

Translational regulation of *Ferredoxin-1* (*Fed-1*), a nuclear encoded component of photosystem I in response to light, also falls in this category of 5'UTR-dependent but otherwise unknown mechanisms. *Fed-1* mRNA abundance and polysome loading decrease in the dark and are restored upon re-illumination. High-polysome loading in the light correlates with an increase in mRNA stability and depends on active photosynthesis (Petracek *et al.*, 1997). An internal light regulatory element (iLRE) within the 5´UTR and first third of the *Fed-1* CDS is necessary for the light-dependent post-transcriptional regulation of *Fed-1*, mediating a decrease in the translation efficiency and ribosome dissociation from the *Fed-1* mRNA in dark conditions (Hansen *et al.*, 2001). This effect can be reversed upon light treatment. The *Fed-1* iLRE that regulates translation and mRNA abundance of *Fed-1* can confer light-responsiveness to normally non-light-responsive transcripts (Dickey *et al.*, 1992). Like *Fed-1*, *Lchb*, a component of the light-harvesting complex b, is also translationally regulated by photosynthesis by an unknown mechanism (Petracek *et al.*, 1997).

F) CONCLUDING REMARKS

Translation is the single most energy demanding process in the cell (Buttgereit and Brand, 1995). As such, fine regulation of this process is essential not only for providing a fast mechanism to control protein production from an mRNA and determining where in the cell and when this mRNA should be translated, but also to precisely adjust the levels of protein synthesis to the actual demand. We have described multiple examples indicating that indeed regulation at the translational level represents an important point in the control of gene expression in plants.

Although for some of the examples we have discussed there is enough information to pinpoint, at least in general terms, the type of regulation responsible for the translational changes observed, in many cases the detailed molecular mechanisms involved remain unknown. This lack of mechanistic understanding of many of the processes described in this review provides both a challenge and an opportunity to put to the test new technologies, such as Ribo-Seq or Structure-Seq, and to fill the knowledge gap in our comprehension of how plants regulate translation under different conditions and the consequences of perturbing such mechanisms. These latest methods and, hopefully, newly developed state-of-the-art approaches will shed exciting new light on the role and molecular mechanisms of translational control in plants and beyond.

GLOSSARY OF TECHNIQUES TO STUDY PROTEIN SYNTHESIS AND TRANSLATION REGULATION

Several different techniques have been employed to study translation regulation, from the old (but still used) pulse-chase labeling to the more modern techniques that take advantage of the whole-genome sequencing methods. Here several of the techniques are explained and some protocols cited.

Pulse labeling is a technique employed for the identification of the rate of protein synthesis per unit of time. The method is based on the incorporation by the plant of radioactively labeled amino acids, usually [35^{S}]methionine/cysteine, over a series of time points. Proteins are then extracted and the newly synthesized proteins that have incorporated the labeled amino acid are detected by SDS-PAGE followed autoradiography and densitometry or by scintillation counting. Protocols that avoid the use of radioactive labeling and employ unnatural amino acids instead have also been developed (Schmitd *et al*., 2009; Wang et al., 2017), but have not yet been implemented in plants.

Cell-free translation systems are useful to study the molecular mechanisms of translation *in vitro*. The most widely utilized cell-free system employed in the studies of plant translation is wheat germ extract (Olliver et al., 1996), but a cell-free Arabidopsis system has also become available (Murota et al., 2011).

Polysome fractionation is a classical technique that allows for the study of the translational state of a whole plant or a specific tissue, i.e. to infer the translational efficiency at the global level or of a particular mRNA of interest. This method is still widely practiced and involves the use of sucrose gradients and ultracentrifugation to separate the polysomes based on the number of ribosomes that they contain. The centrifuged sample resolved in the gradient is then split into fractions (with the lower denser fractions containing heavier polysomes and upper lighter fractions harboring individual ribosomal subunits) and the RNAs are isolated from the fractions to proceed to expression studies, such as Northern blot, RT-PCR, microarray or next-generation sequencing (Mustroph *et al.*, 2009).

Translating Ribosome Affinity Purification (TRAP) is an affinity-chromatography-based ribosome purification method in which a specific ribosomal protein is tagged and then immunoprecipitated to isolate the mRNAs bound by the tagged ribosomes. Next-generation sequencing of the ribosome-bound mRNAs provides information about the translational state of the cell (Zanetti et al., 2005; Mustroph et. al., 2009). This technique, like polysome fractionation, relies on polysomal RNA as a proxy for translation, but cannot distinguish between translating ribosomes and those stalled on mRNAs non-productively.

Ribo-seq (aka ribosome footprinting) is a higher-resolution technology that reveals the exact position of the ribosomes on an mRNA and allows to discriminate between ribosomes stalled in 5´UTRs and those positioned in coding regions. Polysomal RNAs in this case are isolated either using ultracentrifugation through a sucrose cushion or using column-based gel filtration methods and then treated with an RNase. As each translating ribosome shields a short stretch on its respective mRNA, with the exposed parts of all transcripts getting degraded by the RNase treatment, sequencing of the surviving footprints using next-generation approaches provides codon-level information on where the ribosomes were on each and every transcript. Initially developed for yeast (Ingolia et al., 2009), efficient Ribo-seq protocols have been also implemented in plants (Merchante *et al.*, 2016; Hsu *et al.*, 2016).

Title?? Sequencing based, structure mapping approach to study RNA secondary structure with double-strand and single-strand-specific nucleases. This technique allows a genome-wide analysis of RNA secondary structure by mapping double stranded (ds) and single stranded (ss) RNA by the use of ds and ss-specific nucleases. Out of an RNA extraction, ssRNAs are degraded by the use of ssRNases and dsRNA is purified and converted into

sequencing libraries. By the use of dsRNases, ssRNAs are isolated and converted into sequencing libraries. The analysis of the reads allows the generation of an unbiased and comprehensive collection of RNA secondary structure models (Li *et al*., 2012b)

Structure-seq is a state-of-the-art technique that combines dimethyl sulfate (DMS) methylation with next generation sequencing to quantitatively measure RNA secondary structure at genome-wide level and single nucleotide resolution, both *in vivo* and *in vitro*. DMS methylates unpaired As and Cs (all paired nucleotides are protected) and this results in the termination of reverse transcription products. Comparison of the sequencing results between DMS-treated and untreated samples pinpoints unpaired As and Cs. Thus, high DMS reactivity indicates less structured regions which account for loops, bulges, mismatches or joining regions (Ding *et al.*, 2014)

In addition, new techniques that study mRNA degradation intermediates at a genome-wide level, such as **Parallel Analysis of RNA Ends (PARE)** (Zhai *et al*., 2014) or **Genome-wide Mapping of Uncapped and Cleaved Transcripts (GMUCT)** (Gregory et al., 2008), have been developed and aid in the study of ribosome positioning, translation, and co-translational degradation. These techniques involve the capture of the 5´monophosphorylated ends of cleaved 3´ end mRNAs products and make sequencing libraries by ligating RNA adapters.

FIGURE LEGENDS

Figure 1. Structural mRNA features that influence translation. Translation of a particular mRNA can be influenced by structural features contained within the same molecule. The 5´Cap (m7GpppN) and the poly(A) tail strongly enhance translation. IRESs promote cap-independent translation; hairpins and uORFs generally reduce translation of the mORF. Yellow ovals represent RNA-binding proteins that recognize specific sequences in the transcript, marked as blue bands, and can either inhibit or enhance translation. Red bands represent small-RNA binding sites, that can be present in the mORF and 5´ and 3´UTRs. The grey boxes represent ORFs and this is maintained in all figures of this review.

Figure 2. The process of translation initiation. Only the eIFs that are referred to in the text are represented. Elements are not drawn to scale. To be translatable, mRNAs need to be capped and polyadenylated. Multiple subunits of the poly(A) binding protein (PAPB, light blue) bind to the poly(A) tail. The cap-binding complex, eIF4F (orange), composed of the cap-binding protein eIF4E and the scaffolding protein eIF4G, is recruited to the mRNA cap. The DEAD-box helicase eIF4A (red), assisted by eIF4B (light green), unwinds the secondary structures of the mRNA to allow binding of the 43S pre-initiation complex (43S PIC). The 43S PIC is composed of the 40S ribosome subunit (40S, dark green), eIF3 (pink), eIF1 (turquoise), eIF1A (dark blue), the ternary complex (TC), and possibly eIF5 (purple). The TC is formed by the GTP-binding protein eIF2 (yellow), GTP (red solid star) and the Met-$tRNA^{Met}_i$. The complex formed by the mRNA, eIF4F, eIF4B and the 43S PIC is called 48S. With the assistance of eIF4A, eIF4B, eIF1, eIF1A, the 40S scans the 5´UTR in search of the initiation codon. Upon AUG recognition, eIF5 promotes the hydrolysis of the eIF2-bound GTP to GDP (faded red star) catalyzed by eIF2. The eIF2-GDP is replaced by eIF5B, that along with eIF1A, assists in the 60S ribosome subunit recruitment, as other eIFs dissociate. The GTP of eIF5B is hydrolyzed, eIF5B-GDP and eIF1A leave the complex, yielding a translation-competent 80S ribosome (adapted from Jackson *et al.*, 2010; for additional plant-specific information, see Browning and Bailey-Serres (2015)).

Figure 3. Regulation of translation through phosphorylation of eIFs. A) General mechanism of global down-regulation of translation through phosphorylation of eIF2α. After a regular round of translation initiation, the GTP (red star) of eIF2γ subunit gets hydrolyzed into GDP (faded red star) upon AUG recognition. The guanine exchange factor eIF2B (green oval) replaces the GTP on eIF2, making eIF2 active again for the next round of initiation. After certain stresses or developmental signals, GCN2 (red rectangle) in association with AtGCN1 (blue hexagon) phosphorylates eIF2α. Phosphorylated eIF2 has high affinity for eIF2B, that cannot dissociate from the complex and restore eIF2-GTP, thus reducing translation globally (adapted from Gebauer and Hentze, 2004). **B**) Phosphorylation of eIF3h is involved in reinitiation after uORF translation. The structure of a generic transcript is shown first to facilitate the interpretation of the figure. In response to auxin TOR is phosphorylated and recruited to polysomes, which triggers the phosphorylation of eIF3h through S6K1. After the ribosome subunits disassemble at the uORF stop, the phosphorylation on eIF3h allows 40S to recruit new 60S at the AUG of the mORF, reinitiating translation (adapted from Schepetilnikov *et al.*, 2013).

Figure 4. Translation regulation in the polyamine metabolism. A. Translation regulation of *AdoMetDC*. The *AdoMetDC* mRNA contains two overlapping uORFs. The AUG of the first "tiny" uORF is in a weak context, whereas the AUG of the second "small" uORF is strong. Under low levels of polyamines, translation starts from the tiny uORF, which does not repress translation of the main ORF; the small uORF does not get translated as it is in a different frame. Reinitiation at the main ORF is efficient and *AdoMetDC* is translated. Under high levels of polyamines, the ribosome skips the first uORF and starts from the second small uORF, which is a repressive CPuORF. Ribosomes stall at the small uORF, triggering mRNA degradation mediated by the UPF proteins (blue), which are the key components of the nonsense-mediated decay machinery. Adapted from Hanfrey *et al.* (2005) and Uchiyama-Kadokura *et al.* (2014). **B**. Translation regulation of *SAC51*. *SAC51* mRNA contains five uORFs that repress translation. In the presence of polyamines, the ribosomes overcome the repression and translate the mORF. In the *acl5* mutant, the amount of polyamines decreases and *SAC51* cannot be translated due to the ribosomes getting stuck in the uORFs. The *sac51-d* mutant, found in a screen for suppressors of *acl5*, harbors a premature stop codon in the 4th uORF that releases the translational inhibition, so that the *SAC51* mRNA can be translated in the absence of polyamines. This explains why *sac51-d* suppresses the *acl5* mutation. Adapted from Imai *et al*. (2008).

Figure 5. Sucrose-Induced Repression of Translation (SIRT). *bZIP11* contains four uORFs. For simplicity, only the regulatory uORF2 is represented that is responsible for SIRT and is referred to as SC-uORF for Sucrose Control. The SC-uORF contains two in-frame AUGs that produce two peptide species, a 42-aa-long peptide from uAUG1 and a 28-aa-long peptide from uAUG2, with the latter one responsible for SIRT. uAUG1 is in a weak context that allows for leaky scanning by the ribosome under low levels of sucrose, leading to translation of the mORF and bZIP11 protein production. In high levels of sucrose, translation starts from uORF2, SC-peptide is produced and ribosomes stall, thus, impeding translation of the *bZIP11* mORF (Wiese *et al*., 2004; Rahmani *et al*., 2009).

Figure 6. EIN2-mediated translation regulation of *EBF2*. A. In the absence of ethylene, EIN2 (in bright pink) is phosphorylated, which keeps it in an inactive state. In this situation, there is an active translation of the *EBF2* mRNA (3´and 5´UTRs are represented as a black line, the CDS as a black box, and the stop codon marked with a red hexagon) in the cytoplasm. EBF2 protein (in black) is produced and moves to the nucleus (in light yellow), where it targets the transcriptional activators EIN3/EIL1 (in orange) for their degradation by the proteasome (in grey), preventing the transcriptional activation of ethylene responsive genes. **B.** In the presence of ethylene, the phosphorylation of EIN2 does not occur. EIN2 is re-activated, its C-terminal end (C-end) is cleaved off and moves to the nucleus, where it directly or indirectly promotes the activity of the transcriptional master-regulators EIN3/EIL1. EIN3/EIL1 are now able to bind to the promoters and activate transcription of ethylene-responsive genes, *EBF2* among them. The transcription of *EBF2* is up-regulated, and the newly synthesized *EBF2* mRNA moves to the cytoplasm. The released C-end of EIN2 is able to recognize and bind to the 3´UTR of the *EBF2* transcripts, and this binding stimulates the recruitment of the UPF proteins (blue diamonds) to the long 3´UTR of *EBF2*. The UPF proteins, the key components of the nonsense-mediated decay machinery, inhibit the translation of *EBF2* by sending its EIN2-marked transcripts to the P-bodies (in light orange) for temporary storage. Thus, in the presence of ethylene the synthesis of the EBF2 protein is inhibited, EIN3/EIL1 are stabilized, and the transcriptional cascade is active. The *EBF2* translation is not resumed and EIN3/EIL1 are not degraded until the ethylene signal is withdrawn. Molecules marked with grey stripes (EIN3/EIL1 in "no ethylene", or EBF2 in "ethylene") correspond to unstable proteins targeted to proteasome-mediated degradation. Adapted from Merchante *et al*. (2015).

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