Metabolite Control of Translation by Conserved Peptide uORFs: The Ribosome as a Metabolite Multisensor[1][OPEN]

Sjors van der Horst,[a] Teodora Filipovska,[a] Johannes Hanson,[a,b] and Sjef Smeekens[a,2,3]

[a]Molecular Plant Physiology, Institute of Environmental Biology, Utrecht University, 3584CH Utrecht, The Netherlands

[b]Umeå Plant Science Center, Department of Plant Physiology, Umeå University, SE-901 87 Umea, Sweden

ORCID IDs: 0000-0002-1959-561X (S.v.d.H.); 0000-0002-5605-7984 (J.H.); 0000-0001-6631-9426 (S.S.).012020

The regulation of gene expression is intensely investigated in diverse biological systems. Gene expression involves RNA transcription, RNA splicing, RNA stability, translation, posttranslational modification, and protein stability. Particular attention has been given to mRNA levels due to advances in microarray analysis and RNA-sequencing techniques. However, transcript levels do not necessarily correlate with protein levels or functionality (Conrads et al., 2005; Gibon et al., 2006; Bianchini et al., 2008), and complex layers of posttranscriptional regulation have been uncovered, foremost mRNA translation.

Translation can be regulated both globally and in a transcript-specific manner. Examples of global mRNA translational regulation include availability of ribosomes and translation initiation, elongation, and termination factors. In transcript-specific translational regulation, individual mRNA species or mRNA groups are selectively translated. For example, mRNAs can be sequestered in stress granules, removing them from the translatable mRNA pool (Chantarachot and Bailey-Serres, 2018). mRNA sequence or structural features can affect translatability directly or indirectly, the latter via small RNAs or mRNA-binding proteins (for review, see Merchante et al., 2017). Upstream open reading frames (uORFs) have been shown to participate in both global and transcript-specific regulation (von Arnim et al., 2014). Here, recent advances in translation regulation by uORFs are discussed, focusing on uORFs encoding sequence-conserved peptides (CPuORFs).

THE PROCESS OF PROTEIN TRANSLATION

The general concept of protein translation has been reviewed extensively (Browning and Bailey-Serres, 2015; Merchante et al., 2017). Eukaryotic mRNAs consist of a 5′ leader (also referred to as the 5′ untranslated region) followed by the protein encoding the main open reading frame (mORF) and the 3′ trailer sequence (also referred to as the 3′ untranslated region). mRNA translation initiates with the 43S preinitiation complex (PIC) recognizing the 5′ cap structure present on most eukaryotic mRNAs. Additional eukaryotic initiation factors (eIFs) are then bound to form the 48S PIC (for review, see Browning and Bailey-Serres, 2015). The 48S PIC consists of the 40S ribosomal subunit, multiple eIFs, and the initiator Met-tRNA$_i^{met}$. Once formed, the 48S PIC starts scanning mRNA for a start codon. The initiation factors dissociate upon start codon recognition and the 60S ribosomal subunit joins the small subunit to form the 80S ribosome,

ADVANCES

- Many different mRNA species contain upstream open reading frames (uORFs) in their 5′ leader sequence that regulate translation of the main ORF.
- Bioinformatics analysis indicates that over 100 different plant genes encode conserved peptide uORFs (CPuORFs) in their mRNA 5′ leader sequences.
- Ribo-seq databases show that CPuORFs can be translated.
- CPuORFs can regulate gene expression at the mRNA level through a mechanism that involves ribosome stalling.
- For several CPuORFs, it was found that metabolites such as sucrose, polyamine and ascorbate induce ribosome stalling, thus enabling dynamic and immediate control of main ORF translation.
- The plant ribosome exit tunnel appears to present a regulatory environment that allosterically affects peptidyl transfer or peptide release in a CPuORF- and metabolite-dependent manner.

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[2]Author for contact: j.c.m.smeekens@uu.nl.

[3]Senior author.

S.v.d.H., T.F., J.H., and S.S. all contributed to the writing and artwork.

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which is followed by commencement of protein translation.

During translation elongation, an amino acid-charged tRNA will enter the aminoacyl (A) site of the ribosome. Next, the nascent peptide in the peptidyl site will be transferred to the charged tRNA in the A site in the peptidyl transferase reaction. The tRNA present in the peptidyl site will then transfer to the exit site and leave the ribosome, concomitant with the transfer of the peptidyl-tRNA to the peptidyl site, freeing up the A site for entry of the next charged tRNA (for review, see Browning and Bailey-Serres, 2015).

Elongation ends when a stop codon is perceived in the A site. The stop codon recruits eukaryotic release factors to the A site, resulting in termination of translation. Translation termination is usually followed by disassembly of the 80S ribosome and release from the mRNA. However, ribosomes can reinitiate translation at downstream start codons (von Arnim et al., 2014; Browning and Bailey-Serres, 2015).

Novel technologies enable detailed investigation of the genome-wide translational landscape. Ribosome protection (Ribo-seq) experiments (Liu et al., 2013; Juntawong et al., 2014; Merchante et al., 2015; Hsu et al., 2016; Bazin et al., 2017) and analysis of truncated RNA ends (Hou et al., 2016; Yu et al., 2016) can be used to map the positions of ribosomes on mRNAs with nucleotide precision. Such analyses show the remarkable breadth of translation regulation, which includes both regular mRNAs and other RNA species. Progression of the translating ribosome on RNA occurs in steps of three nucleotides. This triplex repeat is the hallmark of protein translation and can be readily studied using Ribo-seq.

uORFS

Twenty percent to 50% of eukaryotic mRNAs contain uORFs in the 5′ leader sequence preceding the main functional protein-encoding ORF (Kochetov, 2008). Generally, these uORFs repress translation of the mORF as the ribosomes terminate and are released from the mRNA after translation of the uORF. However, the inhibitory effect of uORFs on mORF translation can be bypassed by leaky scanning or translational reinitiation (Hellens et al., 2016; Merchante et al., 2017; Zhang et al., 2019; Box 1).

During leaky scanning, the 48S PIC fails to recognize the uORF start codon usually due to a suboptimal context and proceeds scanning to initiate translation at the mORF start codon. An optimal start codon context contains an AUG start codon, an adenine at the −3 position, and a guanine at the +4 position relative to the A_{+1}UG position (Browning and Bailey-Serres, 2015; Diaz de Arce et al., 2018). Non-AUG initiation codons also present a suboptimal initiation context.

Translation reinitiation involves translation of the uORF but, following termination, the 40S ribosomal subunit remains associated with the mRNA and acquires initiation factors needed for continued mRNA scanning and reinitiation of translation at a downstream start codon. Whether reinitiation occurs depends on multiple factors, including uORF length, whereby short uORF mRNAs are more likely to retain eIF3h to the ribosome that is needed for translation reinitiation (von Arnim et al., 2014). Reinitiation is enhanced by TOR kinase activity, which activates eIF3h via S6 kinase1 (S6K1). TOR kinase in turn can be regulated by multiple factors, including hormones and energy, and nutrient availability (Schepetilnikov et al., 2013; Schepetilnikov and Ryabova, 2017).

uORF-containing genes are prone to translation reinitiation, depending on conditions. For example, in *AUXIN RESPONSE FACTOR* (*ARF*) transcripts, uORFs regulate the translation of the mORF depending on auxin levels. Such reinitiation is stimulated by auxin activation of TOR activity (Schepetilnikov et al., 2013; Schepetilnikov and Ryabova, 2017).

CPuORFS

Bioinformatics analyses uncovered a significant number of genes with uORF-encoded peptides that are highly conserved among multiple plant species, named CPuORFs. In the plant kingdom, such observed CPuORF homology groups can be evolutionarily ancient, pointing to their functional significance. Different bioinformatics approaches (Box 2) have been used to identify homology groups (Hayden and Jorgensen, 2007; Tran et al., 2008; Takahashi et al., 2012, 2019; Vaughn et al., 2012; Lorenzo-Orts et al., 2019; van der Horst et al., 2019). Ribo-seq analysis shows that such CPuORFs can be translated (Hsu et al., 2016). Relatively few of the Arabidopsis (*Arabidopsis thaliana*) CPuORFs have a known biological function; those characterized CPuORFs are involved in translational regulation of the mORF, mostly in a metabolite-dependent manner (Table 1). In the following examples, metabolite levels control mRNA translation in a CPuORF-dependent manner.

Suc

The CPuORF homology group presented by mRNAs of the five Arabidopsis S1-group bZIP transcription factors (bZIP1, bZIP2, bZIP11, bZIP44, and bZIP53) is a well-studied example of metabolite-controlled translation by CPuORFs (Rook et al., 1998; Wiese et al., 2004; Hummel et al., 2009; Weltmeier et al., 2009). These bZIPs are regulators of metabolism and control amino acid and sugar metabolism as well as resource allocation (Hanson et al., 2008; Ma et al., 2011; Thalor et al., 2012; Dröge-Laser and Weiste, 2018). Translation of the mORF is regulated by the CPuORF in a Suc-dependent manner (Rook et al., 1998; Rahmani et al., 2009). Increasing Suc levels promote ribosome stalling on the CPuORF and, consequently, due to steric hindrance on the 5′ leader, ribosomes translating or scanning the uORF are unable to reach and translate the mORF (Fig. 1; Juntawong et al., 2014; Hou et al., 2016; Yamashita et al., 2017). Single amino acid mutations

BOX 1. The CPuORF start and stop codons

uORFs generally inhibit translation of downstream mORFs by allowing fewer ribosomes to reach the mORF. uORF start codon skipping by leaky scanning overcomes mORF translation repression by uORFs. The CPuORFs of S1 bZIPs have suboptimal start codon contexts (CACAUGUC) and substitution with an optimal start codon (AAAAUGGC) greatly reduces mORF translation, independent of sucrose levels (Rahmani et al., 2009). Similarly, exchanging the GGP CPuORF ACG start codon with AUG strongly inhibits mORF translation, independent of ascorbate levels (Laing et al., 2015). The ACG start codon has an optimal surrounding (ATCACGGC) and has an initiation competency comparable to AUG start codons having a suboptimal initiation context (De Arce et al., 2018). A recent study has shown that AUG-initiating CPuORFs generally have a poor codon context (van der Horst et al., 2019). In this study, 15 additional Arabidopsis CPuORFs possessing a non-AUG start codon were discovered, next to the two GGP paralogues, demonstrating the importance of non-AUG start codons in CPuORF regulation.

Sequence conservation at the CPuORF stop codons varies. In seven of the CPuORFs shown to be regulated by metabolites (Table 1), four have a conserved stop codon position (Hayden and Jorgensen, 2007; Rahmani et al., 2009; Alatorre-Cobos et al., 2012; Uchiyama-Kadokura et al., 2014). For these CPuORFs, ribosome footprint and mRNA degradome data revealed that the ribosome stalls directly in front of the stop codon (Juntawong et al., 2014; Hou et al., 2016).

Positioning the AtbZIP11 CPuORF stop codon 13 codons downstream of the normal stop position abolished sucrose-mediated translational regulation (Rahmani et al., 2009). Moreover, in vitro translation studies showed that in the stalled bZIP11 CPuORF, the codon before the stop codon contains the peptidyl tRNA, suggesting that in the presence of sucrose the ribosome stalls with this codon in the P-site and the stop codon in the A-site (Yamashita et al., 2017). Similarly, for the CPuORF of the AdoMetDC1 gene, in vitro translational and ribosome toeprinting experiments revealed that spermidine-induced ribosome stalling occurs with the final codon in the P-site of the ribosome and the stop codon in the A site (Uchiyama-Kadokura et al., 2014). The function of the stop codon in ribosome stalling remains unclear. L-Trp-induced tnaC ribosome stalling triggers a conformational change that prevents release factor binding. A similar mechanism might trigger ribosome stalling at the above-mentioned CPuORFs. Alternatively, the slow process of peptide release presents a time window for CPuORF peptide-co-factor interaction to occur.

in the CPuORF abolish Suc-dependent ribosome stalling (Rahmani et al., 2009; Yamashita et al., 2017). Thus, the CPuORF peptide and not the nucleotide sequence plays a role in ribosome stalling. Moreover, RNA sequencing and mRNA degradation experiments revealed the stalling site of the bZIP CPuORFs to be close to the stop codon (Juntawong et al., 2014; Hou et al., 2016). Recent in vitro translation studies using wheat germ extract revealed that the peptidyl-tRNA of the last amino acid of the CPuORF is present on the ribosome during Suc-induced stalling (Yamashita et al., 2017). Overexpression of S1 bZIPs lacking the CPuORF in plants results in generally altered metabolism, including the accumulation of Suc and several amino acids (Hanson et al., 2008; Ma et al., 2011; Thalor et al., 2012; Sagor et al., 2016).

Polyamines

Polyamines (PAs) provide essential functions in diverse biological processes, including the stability

BOX 2. Discovering plant CPuORFs

CPuORFs were found to function as mORF translational regulators; however, the extent to which genes are regulated by such CPuORFs remains poorly understood. In a first report on CPuORF genome-wide identification, Arabidopsis and rice orthologous CPuORFs and Arabidopsis paralogous uORFs, extracted from available cDNA sequences, were compared. This resulted in the identification of 58 CPuORF-containing genes, belonging to 26 distinct homology groups (HGs) (Hayden and Jorgensen, 2007). Further analysis using the same methodology with updated Arabidopsis and rice data identified four additional CPuORF HGs (Jorgensen and Dorantes-Acosta, 2012). In a different study that used sequence information from five cereal genomes, another four CPuORFs HGs were discovered in Arabidopsis (Tran et al., 2008).

A pipeline using expressed sequence tags (EST) derived from thousands of species, named BAIUCAS, (Takahashi et al., 2012) added 18 novel Arabidopsis CPuORFs. An upgraded version of the BAIUCAS pipeline, named ESUCA, delivered another 17 Arabidopsis CPuORF HGs (Takahashi et al., 2019).

The above protocols required the presence of an AUG start codon. However, CPuORFs can be translated using non-AUG start codons (Laing et al, 2015). Therefore, van der Horst et al (2019) searched for CPuORFs regardless of the start codon in combination with stringent filtering methods. This pipeline uses genomic information of 32 plant species and is based on comparison of stop codon to next in-frame stop codon regions. In addition, this protocol identifies CPuORFs with a stop codon in the mORF. In total 29 novel CPuORFs were discovered, of which 15 do not initiate with a canonical start codon (van der Horst et al., 2019). This brings the Arabidopsis CPuORF number to 119, present in 81 distinct HGs (Table 2). It should be noted that some of these CPuORFs were identified as part of the mORF in a different splice variant or in a overlapping mRNA. In addition, Jorgensen and Dorantes-Acosta (2012) predicted that two of their 30 identified homology groups are likely part of a bicistronic transcript, which was subsequently shown for the AT2G11890 HG, encoding CDC26 (Lorenzo-Orts et al., 2019). However, the majority of CPuORFs with proven functionality act as translational regulators, often inducing ribosome stalling in response to a specific metabolite. This means that predicted CPuORFs could respond to distinct metabolites and induce ribosome stalling. Thus, ribosomes appear to operate as metabolite multi-sensors with CPuORFs providing metabolite specificity.

of nucleic acids and membranes, and general protein translation (Poidevin et al., 2019). In plants, PAs are essential for growth and stress tolerance. Translational control is important for PA homeostasis through the involvement of CPuORFs in key metabolic steps. PA biosynthesis initiates with the conversion of Orn or Arg to putrescine. Aminopropyl units derived from decarboxylated *S*-adenosylmethionine (dcAdoMet) are added to putrescine to produce spermidine and the higher order PAs spermine and thermospermine. The enzyme *S*-adenosylmethionine decarboxylase (AdoMetDC) decarboxylates adenosylmethionine (AdoMet) to produce dcAdoMet. CPuORF-mediated translational control of AdoMetDC has been well documented in different systems (Ivanov et al., 2010; Uchiyama-Kadokura et al., 2014) and was recently reviewed in detail (Poidevin et al., 2019).

In the presence of spermidine or spermine, ribosomes stall on the CPuORF of the *AdoMetDC* mRNA. Similar to the S1-bZIP CPuORFs, it was shown that the peptide was necessary to induce the PA-dependent ribosome stalling at the stop codon of the CPuORF (Uchiyama-Kadokura et al., 2014). Interestingly, the *AdoMetDC* CPuORF is accompanied by an additional smaller uORF, also known as the tiny uORF (Hanfrey et al., 2002, 2005). In Arabidopsis, the tiny uORF is three amino acids long and overlaps with the start codon of the CPuORF in a different frame. When the PA level is low, the ribosome initiates translation on the tiny uORF, thereby skipping the CPuORF start codon,

Table 1. *List of CPuORF genes with known interacting metabolites*

Gene Containing CPuORF + Homology Group	Brief Description of mORF Function	Biological Role of mORF	CPuORF-Interacting Metabolite	References
S1-group bZIPs (HG1)	bZIP transcription factor	Controls amino acid and sugar metabolism	Suc	Rook et al., 1998; Wiese et al., 2004; Rahmani et al., 2009; Peviani et al., 2016; Yamashita et al., 2017
SAC51 (HG15)	bHLH transcription factor	Involved in xylem differentiation	Thermospermine	Imai et al., 2006, 2008; Cai et al., 2016; Ishitsuka et al., 2019
HsfB1/TBF1 (HG18)	HSF transcription factor	Involved in heat tolerance and growth-to-defense transition	Galactinol	Pajerowska-Mukhtar et al., 2012; Zhu et al., 2012, 2018; Xu et al., 2017
AdoMetDCs (HG3)	Polyamine biosynthesis	AdoMetDC decarboxylates AdoMet to produce dcAdoMet, a precursor of spermidine and spermine	Spermine and spermidine	Hanfrey et al., 2002, 2005; Uchiyama-Kadokura et al., 2014
PAO (HG6)	Polyamine degradation	Involved in degradation of PAs	Spermine	Guerrero-González et al., 2014, 2016
XIPOTL1/PEAMT (HG13)	Phosphocholine biosynthesis	Encodes a phosphoethanolamine *N*-methyltransferase involved in phosphocholine biosynthesis	Phosphocholine	Tabuchi et al., 2006; Alatorre-Cobos et al., 2012
GGP (HG131)	Ascorbate biosynthesis	Involved in the first dedicated step in ascorbate production	Ascorbate	Laing et al., 2015; Zhang et al., 2018

and then successfully reinitiates on the mORF due to the short length of the tiny uORF (Hanfrey et al., 2005). When PA levels rise, the tiny uORF start codon is skipped and the CPuORF is translated, inducing ribosome stalling that then prevents *AdoMetDC* mORF translation and dcAdoMet production. Exactly how translation initiation on the tiny and CPuORF AUGs is regulated by PAs is unresolved.

CPuORFs in conjunction with their specific metabolites usually promote ribosome stalling, often close to or at the stop codon of the CPuORF. However, metabolites can also promote the release of ribosomes stalled by CPuORF peptides, thereby promoting mORF translation. Polyamine degradation involves Polyamine Oxidase2 (PAO2) to PAO4 that catalyze the oxidative deamination of spermidine and spermine, contributing to PA homeostasis (Guerrero-González et al., 2014, 2016). Particularly, the CPuORF-regulated translation of *AtPAO2* mRNA has been well investigated. The *AtPAO2* CPuORF constitutively inhibits translation of the mORF, allowing PAs to accumulate. Accumulating PAs then release CPuORF-mediated repression of mORF translation, resulting in AtPAO2 production and PA degradation. Mutational analysis showed that the *AtPAO2* CPuORF must be translated in order to repress mORF translation (Guerrero-González et al., 2014, 2016). Again, the ribosome in combination with the CPuORF somehow presents a sensor function to fine-tune cellular PA levels. However, the molecular mechanism of how PAs promote *PAO2* translation remains unresolved.

Thermospermine

In Arabidopsis, thermospermine is produced by the thermospermine synthase ACAULIS5 (ACL5) that converts spermidine to thermospermine (Knott et al., 2007).

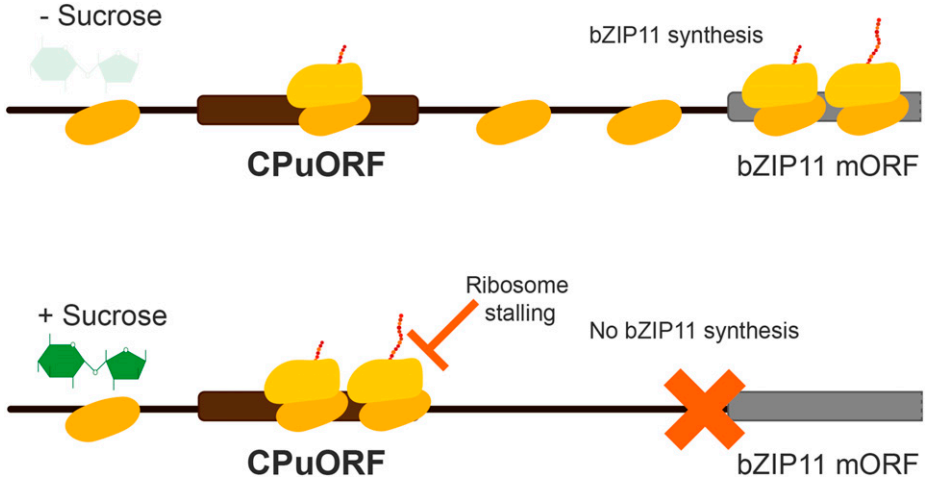

Figure 1. Schematic representation of Suc-induced repression of translation of the *bZIP11* mRNA. When Suc levels are low, ribosomes translating the CPuORF terminate at the stop codon. Translation of the mORF involves translational reinitiation or ribosome leaky scanning. Increasing Suc levels will induce ribosome stalling near the stop codon of the CPuORF, thereby blocking upstream translating or scanning ribosomes and inhibiting bZIP11 protein production. Lines and bars indicate RNA and ORFs, respectively.

ACL5 mutants display severe defects in stem elongation due to the overproliferation of xylem vessels. In an *acl5* suppressor mutagenesis screen, multiple dominant or semidominant *suppressor of acaulis* (*sac*) mutants were identified (Imai et al., 2006). One of these mutants, *sac51-d*, has a premature stop codon mutation in the *SAC51* CPuORF that fully restores the wild-type phenotype (Imai et al., 2006). Interestingly, in the *sac51-d* mutant, the *SAC51* mORF is translated, resulting in restoration of function. Thus, the presence of thermospermine promotes *SAC51* mORF translation, whereas low thermospermine levels result in poor *SAC51* translation. In the *sac51-d* mutant, a dysfunctional CPuORF peptide is produced that is incapable of inhibiting *SAC51* mORF translation (Imai et al., 2006; Ishitsuka et al., 2019). Similar to the AtPAO2 situation, the SAC51 CPuORF peptide inhibits mORF translation that is released by increasing thermospermine levels.

Phosphocholine

Phosphatidylcholine is an essential lipid that functions as a membrane structural lipid and as a signaling molecule. The Arabidopsis *XIPOTL1* gene (*AT3G18000*) encodes a phosphoethanolamine *N*-methyltransferase involved in phosphocholine biosynthesis. Phosphocholine is considered as a rate-limiting substrate for phosphatidylcholine biosynthesis. Translation of the *XIPOTL1* mRNA is regulated by a CPuORF that encodes a peptide of 26 amino acids. Physiological phosphocholine levels repress mORF translation in a concentration-dependent manner without significantly affecting mRNA levels. This translational repression depends on translation of the CPuORF peptide (Alatorre-Cobos et al., 2012).

Ascorbate

Enzyme-encoding mRNAs seem ideal targets for metabolite-regulated translation via CPuORFs, since feedback loops are thus readily established for maintaining metabolite homeostasis. The first dedicated step in ascorbate biosynthesis is catalyzed by GDP-L-Gal phosphorylase (GGP). Ascorbate levels control GGP expression independently of transcription or *GGP* mRNA levels (Laing et al., 2015). Phylogenetic analyses of the 5′ leader of the mRNA revealed a CPuORF that initiates with a highly conserved ACG codon. Increasing cellular ascorbate levels greatly reduce the translation of *GGP* mORFs in a CPuORF-dependent manner (Laing et al., 2015). Mutation of a conserved His codon in the CPuORF abolished the ascorbate-mediated regulation of translation. Moreover, ribosome footprinting revealed ribosome stalling on the CPuORF (Laing et al., 2015). Interestingly, in a recent study, CPuORFs of two lettuce (*Lactuca sativa*) *GGP* orthologs were mutated using CRISPR-Cas9, which caused cellular ascorbate levels to increase (Zhang et al., 2018). This study demonstrates the potential of CPuORF modification for crop improvement.

Galactinol

Plants induce the secretion of antimicrobial proteins when challenged with microbe-associated molecular patterns. The heat shock factor-like transcription factor HSFB1/TBF1 (*AT4G36990*) specifically binds to a promoter region (the TL1 element, GAAGAAGAA) present in the genes encoding such antimicrobial proteins (Pajerowska-Mukhtar et al., 2012). *HSFB1/TBF1* is itself transcriptionally induced by microbial pathogens or the elf18 microbe-associated molecular pattern. Importantly, HSFB1/TBF1 protein accumulation is highly dependent on the presence of two uORFs in its mRNA, the second of which is a CPuORF. Particularly, this uORF2 inhibits protein translation of the *HSFB1/TBF1* mORF (Zhu et al., 2012); however, this inhibition is released following pathogen infection or elf18 treatment (Xu et al., 2017). HSFB1/TBF1 protein promotes the all-important growth-to-defense transition. Recently, it was shown that the *HSFB1/TBF1* CPuORF is responsive to galactinol (Zhu et al., 2018), a Gal-inositol disaccharide that is the substrate for the biosynthesis of the raffinose series oligosaccharides involved in adaptation to stressful growth conditions. Galactinol in the micromolar range suppresses *HSFB1/TBF1* mORF translation, probably through CPuORF peptide-dependent ribosome stalling. Possibly, pathogens reduce galactinol levels, thereby releasing stalled ribosomes and allowing *HSFB1/TBF1* mORF translation and the induction of defense mechanisms. However, galactinol has been implicated previously as an inducer of plant pathogen defense (Kim et al., 2008). Recently, the *HSFB1/TBF1* mRNA leader sequence harboring the CPuORF (named the *TBF1* cassette) was used to induce pathogen resistance upon infection only by the immediate translation of downstream mORF resistance genes. This powerful translational control system circumvents deleterious effects of the constitutive expression of resistance genes (Bailey-Serres and Ma, 2017; Xu et al., 2017).

Boron

Boron (B) is an essential element for plants but is toxic at high concentrations. A remarkable uORF-dependent translation regulatory system was reported for the B diffusion facilitator *NODULIN26-LIKE INTRINSIC PROTEIN5;1* (*NIP5;1*), which is required for efficient B uptake in roots. In the *NIP5;1* mRNA leader, an AUG-stop uORF mediates ribosome stalling under high-B conditions, preventing mORF translation and thereby the production of NIP5;1 and B uptake (Tanaka et al., 2016). Here, ribosome stalling promotes mRNA degradation at a conserved nucleotide sequence immediately upstream of the stalled ribosome. Transfer of

the *NIP5;1* AUG-stop region (ATGTAA) to an unrelated mRNA imposed B-dependent ribosome stalling. Two additional B-responsive genes (*SKU5* and *ABS/NGAL1*) were identified that are regulated by ribosome stalling at the AUG-stop sequence. The B-dependent AUG-stop-mediated regulation system also functioned in cell-free and animal translation systems. When B is low, translation reinitiation appears to promote *NIP5;1* mORF translation. mRNA translation of another B transporter, BOR1, similarly depends on translation reinitiation (Aibara et al., 2018).

mORF RIBOSOME STALLING

AdoMet

Metabolites and their corresponding CPuORF peptides control mORF translation through a mechanism involving ribosome stalling in the mRNA leader region. Such metabolite-induced stalling can also occur in the mORF itself. The *CGS1* gene (*AT3G01120*) encodes the enzyme cystathionine γ-synthase, which catalyzes the first committed step in Met biosynthesis. In the N-terminal region of the Arabidopsis *CGS1* gene, a short sequence-conserved peptide (MTO1, ^{77}RRNCSNIGVAQIVA^{90}) is located that promotes ribosome stalling at the Ser-94 position, immediately downstream of the MTO1 region, in response to AdoMet, a direct metabolite of Met. Mutations in the MTO1 region of the *CGS1* gene no longer respond to AdoMet, resulting in unrestricted CGS1 enzyme production and Met overaccumulation (Chiba et al., 1999). The MTO1 region of the nascent peptide is present in the ribosomal exit tunnel when translation elongation is halted by AdoMet, following formation of the peptidyl tRNA^{Ser} located in the A site of the ribosome (Onoue et al., 2011). As a result of ribosome arrest, the *CGS1* mRNA is degraded specifically at the MTO1-encoding region (Chiba et al., 2003). AdoMet-induced stalling imposes a compact conformation of the nascent peptide in the exit channel (Onoue et al., 2011).

PF-06446846

An interesting example of mORF-associated, small molecule-induced ribosome stalling is presented by animal proprotein convertase subtilisin/kexin type 9 (PCSK9), a key enzyme involved in regulating levels of plasma low-density lipoprotein cholesterol (Lintner et al., 2017). Chemical library screening for cholesterol-lowering drugs affecting PCSK9 identified an inhibitory compound that was functionally optimized to the active molecule PF-06446846, operating in the low micromolar range. The mechanism of PCSK9 inhibition was investigated and, remarkably, it was observed that the compound inhibits *PCSK9* mRNA translation though ribosome stalling near amino acid position 34 of the mORF. Recently, details of the stalling mechanism as obtained by cryo-electron microscopy (cryo-EM) were reported (Li et al., 2019). Ribo-seq experiments showed that PF-06446846 is highly selective for translation inhibition of the *PCSK9* mRNA. This finding suggests that, in principle, translation of any stretch of amino acids can mediate ribosome stalling, provided that the matching chemical compound is provided. The challenge is to devise high-throughput chemical compound screening systems whereby translation stalling is readily displayed.

The above examples of small molecule-induced mORF stalling demonstrate the adaptability of the ribosome and suggest a dynamic and immediate way to control the expression of specific genes by ribosome stalling.

THE RIBOSOMAL EXIT TUNNEL PRESENTS A REGULATORY ENVIRONMENT

The ribosomal exit channel is part of the large ribosomal subunit and is structured by the 28S rRNA and several ribosomal proteins. The tunnel accommodates a stretch of approximately 40 amino acids. Peptides in the tunnel can already assume a partially folded state, particularly near the tunnel exit site. The peptidyl transferase center (PTC) at the entry site of the tunnel transfers amino acids to the nascent chain, as the ribosome progresses toward the 3′ end of the mRNA. Studies have shown that the passage of peptides through the tunnel can be highly regulated and involves interactions between the peptide and the tunnel wall. Important in this respect is a constriction at approximately one-third of the tunnel length counting from the PTC that involves r-proteins uL4 and uL10 (Ito and Chiba, 2013; Dao Duc et al., 2019). Interestingly, CPuORFs often are best conserved at the C-terminal 15 to 20 amino acids, spanning the PTC-to-constriction region. Translated peptides can have the intrinsic capacity to stall translation, whereas other peptides require an effector molecule such as a metabolite or drug to induce ribosome stalling (for review, see Ito and Chiba, 2013).

Technological advances in 3D single-particle cryo-EM allow for the near-atomic resolution of biological structures as complex as the eukaryotic ribosome (for review, see Nogales and Scheres, 2015). Particularly, the Beckmann laboratory in Munich has used cryo-EM to study the structural basis of nascent polypeptide-mediated ribosome stalling (for review, see Wilson et al., 2016), and the structural basis for cofactor-induced translational stalling was resolved for several bacterial systems (Arenz et al., 2016; Wilson et al., 2016). Of particular interest is the bacterial tryptophanase (*tnaCAB*) operon that controls bacterial Trp (L-Trp) levels by L-Trp-induced production of tryptophanase and Trp permease. *tnaCAB* regulation involves L-Trp-induced ribosome stalling of the tnaC nascent peptide. Such stalling allows for the production of L-Trp-removing enzymes. Cryo-EM structural resolution of the L-Trp-stalled tnaC ribosomal complex showed the presence of two L-Trp-binding pockets in the exit tunnel, created by the tnaC peptide and the

Table 2. *Arabidopsis CPuORF homology groups, classified according to Gene Ontology*

Homology Group	Gene ID	Gene Name
Transcription regulation		
HG1	*AT1G75390*	*bZIP44*
	AT2G18160	*bZIP2*
	AT3G62420	*bZIP53*
	AT4G34590	*bZIP11*
	AT5G49450	*bZIP1*
HG2	*AT1G06150*	*EMB1444*
	AT2G27230	*LHW*
	AT2G31280	
HG4	*AT4G25670*	
	AT4G25690	
	AT5G52550	
HG14	*AT3G01470*	*HB-1*
HG15	*AT1G29950*	
	AT5G09460	
	AT5G50010	
	AT5G64340	*SAC51*
HG18	*AT4G36990*	*HSF4*
HG21	*AT1G25470*	*CRF12*
	AT1G68550	*CRF10*
	AT3G25890	*CRF11*
HG34	*AT2G27350*	*OTLD1*
HG36	*AT3G15430*	
HG41	*AT5G09330*	*NAC082*
	AT5G64060	*NAC103*
HG43	*AT5G46590*	*NAC096*
HG44	*AT5G60450*	*ARF4*
HG50	*AT1G01060*	*LHY*
HG51	*AT4G15180*	*SDG2*
HG58	*AT2G24530*	
HG59	*AT2G35940*	*BLH1*
HG61	*AT5G55600*	
HG71	*AT3G61970*	*NGA2*
HG72	*AT4G34610*	*BLH6*
HG73	*AT5G53660*	*GRF7*
HG132	*AT1G07640*	*OBP2*
HG136	*AT1G68920*	
HG145	*AT4G17980*	*NAC071*
Metabolism		
HG3	*AT3G02470*	*SAMDC*
	AT3G25570	
	AT5G15950	
HG6	*AT2G43020*	*PAO2*
	AT3G59050	*PAO3*
HG11	*AT4G12430*	*TPPF*
	AT4G22590	*TPPG*
HG13	*AT1G48600*	*PMEAMT*
	AT1G73600	
	AT3G18000	*XPL1*
HG17	*AT3G53400*	
	AT5G01710	
	AT5G03190	
HG28	*AT1G67480*	
HG29	*AT2G22500*	*UCP5*
HG38	*AT4G10170*	
HG39	*AT4G12790*	
HG45	*AT5G63640*	
HG46	*AT1G14560*	
HG47	*AT1G68100*	*IAR1*

(*Table continues on following page.*)

Table 2. (*Continued from previous page.*)

Homology Group	Gene ID	Gene Name
HG48	*AT1G72820*	
HG54	*AT1G65320*	*CBSX6*
HG70	*AT3G52490*	
HG131	*AT4G26850*	*VTC2*
	AT5G55120	*VTC5*
HG133	*AT1G11820*	
HG134	*AT1G57680*	*Cand1*
HG135	*AT1G66540*	
	AT5G35715	*CYP71B8*
HG137	*AT2G23570*	*MES19*
HG142	*AT3G57170*	
HG143	*AT3G62040*	
HG146	*AT5G26140*	*LOG9*
Protein translation or degradation		
HG7	*AT1G36730*	
	AT1G77840	
HG22	*AT1G16860*	
	AT1G78880	
HG26	*AT3G10910*	
	AT5G05280	
HG42	*AT5G27920*	
HG141	*AT3G49430*	*SR34a*
HG147	*AT5G55100*	
HG148	*AT5G63190*	
Kinase or phosphatase		
HG10	*AT4G19110*	
	AT5G45430	
HG16	*AT3G51630*	*WNK5*
HG23	*AT1G64630*	*WNK10*
	AT5G41990	*WNK8*
HG25	*AT3G45240*	*SNAK2*
	AT5G60550	*SNAK1*
HG27	*AT4G30960*	*SIP3*
HG31	*AT5G01810*	*CIPK15*
HG32	*AT3G08730*	*S6K1*
HG33	*AT1G30270*	*CIPK23*
HG35	*AT2G42880*	*MPK20*
HG37	*AT3G55050*	
HG49	*AT5G14720*	
HG52	*AT3G08850*	*RAPTOR1*
HG53	*AT1G62400*	*HT1*
HG144	*AT4G03260*	
Other/unknown		
HG5	*AT5G07840*	*PIA1*
	AT5G61230	*ANK6*
HG8	*AT3G12010*	
HG9	*AT1G64140*	
	AT5G09670	
	AT5G64550	
HG12	*AT1G23150*	
	AT1G70780	
HG17	*AT1G58120*	
HG19	*AT5G53590*	
HG20	*AT2G37480*	
	AT3G53670	
HG24	*AT3G22970*	
	AT4G14620	
HG30	*AT2G11890*	
HG40	*AT5G02480*	
HG57	*AT1G54095*	
	AT1G72510	

(*Table continues on following page.*)

Table 2. (*Continued from previous page.*)

Homology Group	Gene ID	Gene Name
HG60	*AT3G12570*	*FYD*
HG138	*AT2G29290*	
HG139	*AT2G42490*	
HG140	*AT3G23010*	*RLP36*

tunnel wall. L-Trp binding causes a conformational change that blocks release factor entry (Bischoff et al., 2014; Wilson et al., 2016). Thus, the tnaC peptide together with the exit tunnel functions as an L-Trp receptor, regulating the expression of downstream enzymes. The presence of a particular peptide in the exit channel structural environment together with its cognate cofactor inhibits peptidyl transfer or release by inducing conformational changes in proximity to the PTC, thereby stalling the ribosome. Cryo-EM resolution of erythromycin-induced stalling of the ErmCL peptide in the exit channel presents another example of cofactor-induced ribosome stalling (Wilson et al., 2016).

METABOLITE SENSING BY THE RIBOSOME

The CPuORFs discussed in detail above either promote or release stalling, depending on the concentration of their cognate cofactors. How do these different CPuORF homology groups, which bear no apparent amino acid or structural similarity, promote ribosome stalling or release from stalling in the presence of their cognate cofactors? Similar to the tnaC bacterial ribosome stalling system, the eukaryotic ribosome also functions as a metabolite sensor, a prime example being the fungal Arg attenuator peptide (Bhushan et al., 2010).

Bioinformatics analysis using stringent parameters (van der Horst et al., 2019) shows that 81 highly conserved CPuORF homology groups, and likely many more, are present in plants (Table 2; Box 2). Conceivably, many of these groups respond to a cofactor. Such a cell-autonomous ribosome stalling-based translational regulatory mechanism is a most powerful one, as it operates as a dynamic and immediate metabolite sensor, comparable to allosteric regulation of enzymes (Fig. 2). Cryo-EM information of metabolite-induced stalling complexes in plants is currently lacking, but accumulated evidence clearly points to the ribosome as a metabolite sensor, operating in a similar way as discussed above for the tnaC peptide.

Translational stalling systems often can be reconstituted in in vitro translation systems (Onouchi et al., 2005; Ebina et al., 2015; Hayashi et al., 2017). Such cell-free system reconstitutions point to a sensor function for the ribosome. Moreover, genetic evidence for the involvement of the plant ribosome in metabolite sensing comes from the *SAC51* CPuORF peptide discussed above. This CPuORF promotes ribosome stalling that is then released by increasing thermospermine levels.

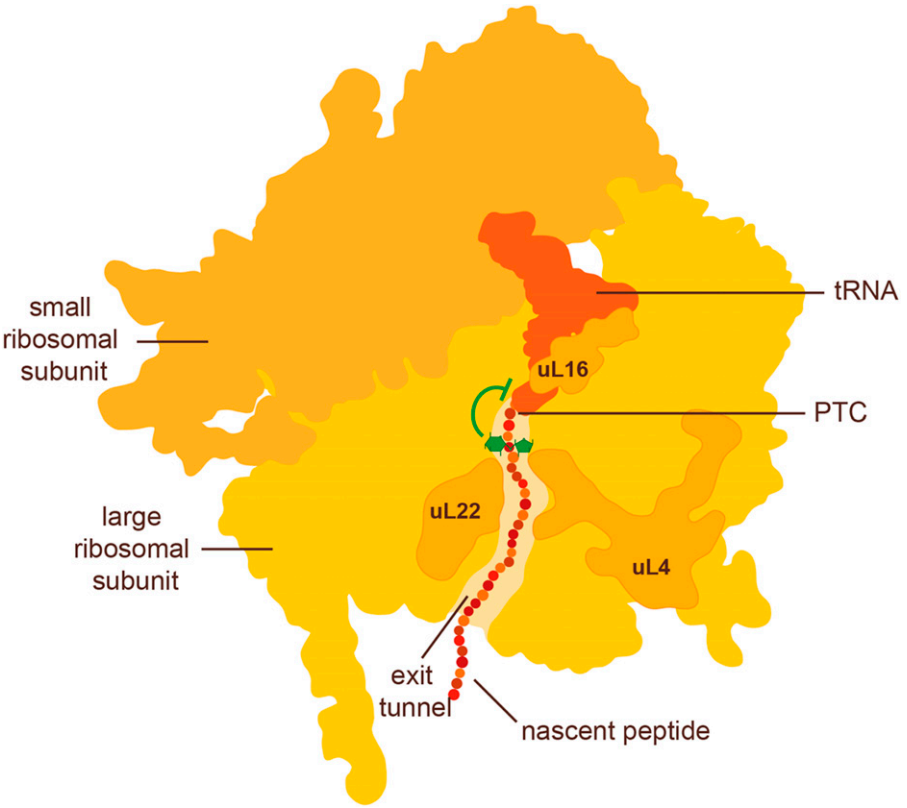

Figure 2. Hypothetical model of the stalled plant ribosome. The CPuORF peptide and the ribosomal exit tunnel wall create a specific metabolite-binding pocket. The presence of the metabolite (green color) in the pocket allosterically controls the peptidyl transferase reaction. uL4 and uL22 are ribosomal proteins present in the exit tunnel constriction. uL16 is adjacent to the PTC.

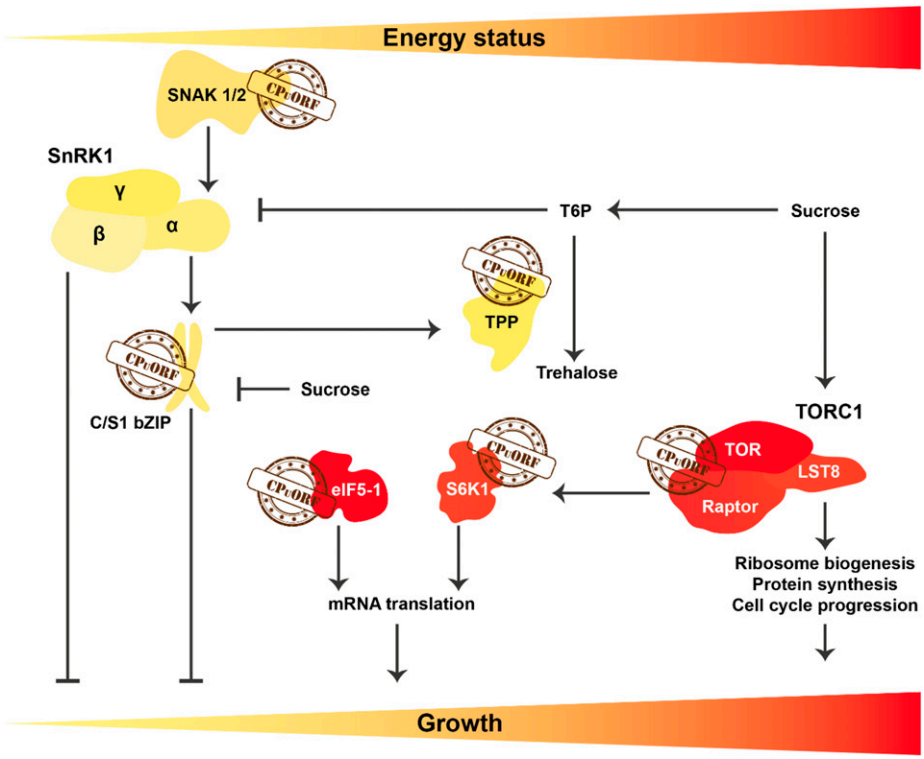

Figure 3. CPuORFs in the plant energy signaling network. Genes of the energy signaling network regulated by CPuORFs include the S1 group bZIP transcription factors, TPP, SnAK (SNAK 1/2), the RAPTOR subunit of the TOR kinase, S6K1, and eIF5-1. CPuORFs of the S1 group bZIPs are responsive to Suc. Presumed ligands of the other CPuORFs in the network are unknown.

In the SAC51 system, suppressor mutants were identified that promote SAC51 production in the absence of thermospermine. In addition to mutations in the *SAC51* CPuORF itself (Imai et al., 2006), mutations in the ribosomal tunnel wall-associated r-proteins uL16, uL4, and RACK1 were also retrieved, named *sac52-d*, *sac53-d*, and *sac56-d*, respectively (Imai et al., 2008; Kakehi et al., 2015). Each of these three semidominant mutants is able to restore the thermospermine-deficient *acl5-1* dwarf phenotype at least partly. In these *sac* ribosomal mutants, repression of the *SAC51* mORF translation was alleviated. These results suggest a role for both the ribosome and the CPuORF peptide in *SAC51* translational regulation by thermospermine.

In plants, r-proteins are encoded by up to six paralogs. In Arabidopsis, growth conditions can determine the paralog composition of ribosomes (Hummel et al., 2012, 2015). Such different ribosome isoforms might respond with different kinetics to a CPuORF and its cofactor. In Arabidopsis, many mutants affected in plant development turned out to be mutated in r-protein paralogs (Byrne, 2009; Horiguchi et al., 2011). Such mutants in plant development might be affected in receptor or signaling functions of the ribosome.

For optimal cellular function, ribosome stalling complexes should be dynamic (i.e. under certain cellular conditions, stalling must be released). For spermidine-stalled ribosomes translating the *AdoMetDC1* CPuORF in a wheat germ system, it was found that stalled complexes were spontaneously released and peptidyl-tRNA hydrolysis was started within a 30-min time frame of inhibition of translation initiation (Uchiyama-Kadokura et al., 2014).

CPuORF CONTROL OF THE PLANT ENERGY SIGNALING NETWORK

Genes involved in the plant energy signaling network seem particularly prone to expression regulation by CPuORFs (Jorgensen and Dorantes-Acosta, 2012; van der Horst et al., 2019). The S1 group of bZIP transcription factors adapt general metabolism to a low-energy state (Ma et al., 2011; Dröge-Laser and Weiste, 2018). As mentioned above, S1 bZIPs are Suc responsive through their CPuORFs, whereby elevated Suc levels promote ribosome stalling, allowing carbon utilization and growth. Other genes in the plant energy signaling network harboring CPuORFs include those encoding the activating kinases (SnAK1 and SnAK2) of the Suc nonfermenting-related protein kinase1 (SnRK1), trehalose 6-phosphate phosphatase (TPP), the regulatory-associated protein of TOR (RAPTOR), S6K1, and translation factor eIF5-1 (Fig. 3).

The SnRK1 and TOR protein complexes are conserved regulators of growth and development in eukaryotes and are responsive to carbon availability. SnRK1 can be activated by SnAK-mediated phosphorylation of the Thr-175 residue in the SnRK1 activation loop (Jossier et al., 2009; Shen et al., 2009; Glab et al., 2017). CPuORFs are present in both *SnAK1* and *SnAK2* (Jorgensen and Dorantes-Acosta, 2012). Activated SnRK1 regulates the expression of a multitude of genes and

OUTSTANDING QUESTIONS

- Do all CPuORFs bind their cognate metabolite in the exit channel molecular environment?
- What is the structural basis of metabolite-induced stalling of plant ribosomes? How is the activity of the PTC controlled?
- What is the identity of the compounds that interact with orphan CPuORFs?
- Can chemical entities be identified that specifically induce main ORF translation stalling of any gene?

metabolic enzymes during adaptation to a low-energy state (Crepin and Rolland, 2019). TOR responds to nutrient abundance by promoting ribosome biogenesis, cell cycle progression, and growth (Shi et al., 2018). The TOR complex consists of the TOR kinase, LST8, and RAPTOR. A CPuORF initiating with a CUG codon was found in the dominantly expressed *RAPTOR1* gene (*AT3G08850*; van der Horst et al., 2019). SnRK1 can phosphorylate RAPTOR, thereby inhibiting TOR activity (Nukarinen et al., 2016). Following uORF translation, activated TOR phosphorylates S6K1 that subsequently phosphorylates eIF3h to promote reinitiation at the mORF (Schepetilnikov et al., 2013). Cereal species have a CPuORF upstream of S6K (Tran et al., 2008); however, cognate start codons were not identified for CPuORFs in S6Ks of Brassicaceae species (van der Horst et al., 2019).

The signaling molecule trehalose-6-phosphate (T6P) inhibits SnRK1 activity (Crepin and Rolland, 2019). T6P is synthesized by T6P synthase from UDP-Glc and Glc-6-P and is metabolized by TPP to trehalose. CPuORFs were found in two of the 11 Arabidopsis *TPP* genes, *TPPF* and *TPPG* (Jorgensen and Dorantes-Acosta, 2012). The S1 group bZIP11 transcription factor induces TPP expression, thereby lowering T6P levels (Ma et al., 2011) and enabling SnRK1 activity.

The essential translation initiation factor eIF5-1 (*AT1G36730*) is involved in start codon selection. eIF5-1 is conserved in eukaryotes and harbors a CPuORF in plants (Jorgensen and Dorantes-Acosta, 2012). In human cells, eIF5 is autoregulated via negative feedback involving uORFs. Overexpressing eIF5 in mammalian cells increases translation initiation at upstream non-AUGs and weak AUG initiation contexts (Loughran et al., 2012).

From the above, it is evident that the energy signaling network is tightly interwoven and controlled at multiple points by CPuORFs. In this signaling network, the metabolites that control CPuORF-mediated stalling are known only for S1 bZIPs. Uncovering the controlling cofactors that regulate translation of the other CPuORF-containing genes is urgently needed to improve our understanding of this key signaling network.

CONCLUDING REMARKS

In plants, the large number of CPuORFs and their broad evolutionary conservation reflect their importance as a regulatory principle. Where known (Table 1), CPuORFs respond to metabolites within the context of the ribosomal exit tunnel to regulate mORF translation. The ribosome, the CPuORF, and the metabolite in a *ménage a trois* control mORF translation and in turn biological function.

It is likely that other CPuORFs in addition to those listed in Table 1 will similarly respond to metabolites, and it will be exciting to uncover such metabolites or, perhaps, cofactors such as oligosaccharides or small peptides, and the processes that are under CPuORF control (see Outstanding Questions). Usually, CPuORFs are embedded in complex uORF ensembles with non-conserved uORFs in front, overlapping and downstream of the CPuORF. How such uORF ensembles affect CPuORF function and mORF translation requires further investigation.

Metabolite-regulated CPuORFs present a direct regulatory mechanism for gene expression, dynamically operating within the physiological range of the controlling metabolite. Several of the experimentally confirmed metabolite-controlled CPuORFs concern mRNAs encoding metabolic enzymes involved in their biosynthesis. Such homeostatic feedback loops allow for direct fine-tuning of metabolite status and cellular function. Other CPuORFs appear to operate indirectly by regulating the expression of regulatory proteins such as transcription factors.

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