

RELEVANCE OF TRANSLATIONAL REGULATION ON PLANT GROWTH AND ENVIRONMENTAL RESPONSES

EDITED BY : Alejandro Ferrando, M. Mar Castellano, Purificación Lisón,
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RELEVANCE OF TRANSLATIONAL REGULATION ON PLANT GROWTH AND ENVIRONMENTAL RESPONSES

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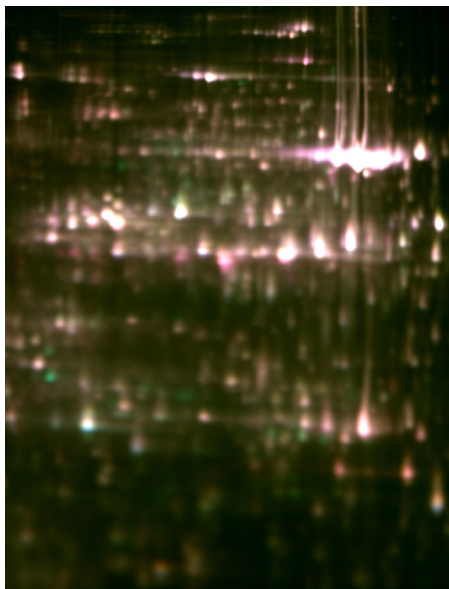
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Differential proteomic analysis of tomato leaves. Equal amounts of protein control sample (Cy5-labeled, red), hormone-treated sample (Cy3-labeled, green) and internal standard (Cy2-labeled, blue) were loaded in the same gel. The fluorescent-labeled proteins were separated by two-dimensional electrophoresis and scanned. The picture shows an overlay of the three fluorescence images. Proteins induced by treatment are green, red dots represent repressed proteins, and unaffected proteins are shown in white.

Image courtesy of Dr. Purificación Lisón and Dr. Susana Tárraga.

Plants, as sessile organisms, are exposed to a large array of challenging external and internal alterations that may restrict plant growth. These limiting growth conditions activate plant signalling responses which eventually target the protein synthesis machinery to rapidly reprogram plant metabolism to adapt to the new situation. Thus, the control of mRNA translation is one key regulatory step of gene expression and it is an essential molecular mechanism used by plants to bring about impressive growth plasticity. Compared to the vast number of studies aimed to identify plant transcriptional changes upon hormonal or environmental cues, the subsequent steps of mRNA transport, stability, storage, and eventually translational regulation, have been less studied in plants. This lack of knowledge concerns not only the fate of protein-coding transcripts in plants, but also the biogenesis and maturation of rRNAs, tRNAs and the plant translation factors involved.

In this eBook we have focused on how internal cues and external signals of either biotic or abiotic origin impact translation to adjust plant growth and development. We have collected altogether ten scientific contributions to extend the knowledge on plant post-transcriptional and translational events that regulate the production of proteins that execute the required cellular functions. We hope that this compilation of original research articles and reviews will provide the readers with a detailed update on the state of knowledge in this field, and also with additional motivation to improve plant growth adaptation to future environmental challenges.

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Editorial: Relevance of Translational Regulation on Plant Growth and Environmental Responses

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Keywords: mRNA translation, translation factors, post-transcriptional regulation, translome, organellar gene expression

Editorial on the Research Topic

Relevance of Translational Regulation on Plant Growth and Environmental Responses

One of the great challenges in the near future will be the sustainable production of sufficient amounts of safe food worldwide. A combination of adverse demographic factors and climatological perturbations is expected to impact on food systems globally (Vermeulen et al., 2012). To ensure food security in the coming years, multidisciplinary approaches are needed, and useful leads are likely to emerge from advances in plant biotechnology. However, improving plants' performance under restrictive growth conditions will require a deep understanding of the molecular processes that underlie their extraordinary physiological plasticity.

Much research in plant biology in recent decades has focused on phenomenological descriptions of changes in gene expression at the mRNA level. However, in eukaryotes, shifts in transcript levels do not always correlate with equivalent changes in protein levels, since a variety of post-transcriptional events may uncouple transcription from translation (Vogel and Marcotte, 2012). This is particularly relevant in plants, given the intrinsically complex properties of their translational apparatus, the presence of additional genetic systems in mitochondria and chloroplasts, and the occurrence of environment-dependent variation of cytosolic ribosome composition (Hummel et al., 2012). Recent advances in *next-generation-sequencing*, combined with breakthrough technologies like *ribosome footprint profiling*, may help to close the gap between transcriptional and translational studies and further elucidate plant responses to environmental factors.

In this Research Topic, we present reviews and original research articles that extend our knowledge of post-transcriptional and translational mechanisms that regulate the production of plant proteins which ultimately execute the cellular functions required for adaptation to environmental challenges.

Many of the contributions highlight the importance of translational regulation. Among the panoply of translational regulators, the TOR kinase emerges as a key regulatory element. Dobrenel et al. have used transgenic plants deficient in TOR activity to reveal its role in regulating levels of chloroplast ribosomal proteins, possibly by recognizing a sequence motif present in their mRNAs. This work also demonstrated that the TOR pathway is involved in phosphorylation of the ribosomal

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protein S6. Two reviews summarize our growing knowledge of the roles of the TOR signaling pathway in plants. Schepetilnikov and Ryabova review recent findings that link auxin signaling to TOR kinase activity via the small GTPase ROP2, and explain how this pathway contributes to translational regulation of mRNAs that harbor upstream ORFs within their 5'-leader region that inhibit translation of the main ORF. Sesma et al. describe recent advances in the regulation of TOR signaling, eIF4E activity and eIF2 α phosphorylation in plants. These authors highlight the paucity of our knowledge of the regulation of these important players in plants and the need for further studies to clarify the relevance of controls on translational initiation.

Another key regulator of translational initiation is the large protein complex eIF3. Wang et al. have investigated the physiological effects of reduced expression of the eIF3e subunit in transgenic rice plants. These transgenics grew more slowly and remained smaller in size than controls, showed impaired pollen maturation and were more sensitive to osmotic stress. The results indicate that eIF3e plays surprisingly specific roles in plant growth and development.

One of the key post-transcriptional steps in gene expression is the regulation of mRNA levels by microRNAs (miRNAs). Several studies have demonstrated that abiotic stress conditions induce aberrant expression of miRNAs that reduce steady-state levels of their target mRNAs. To shed more light on this subtopic Li et al. have used high-throughput sequencing and computational approaches to identify a large number of stress-related miRNAs involved in melatonin-mediated cold tolerance in watermelon. In a similar way Aravind et al. have studied inbred lines of subtropical maize to identify miRNAs involved in drought stress.

Two papers included in the Topic deal with aspects of organellar gene expression. Leister et al. review the link between the expression of chloroplast genes and whole-cell acclimation to environmental changes. Although only a small fraction of the genes present in the original cyanobacterial endosymbiont remains in the modern organelle, perturbation of their expression plays a major role in triggering acclimation and tolerance responses via signaling from the chloroplast to the nucleus. Zoschke et al. have investigated the effect of a maize chlorophyll-deficient mutant, *chl1H/gun5*, on the translation of plastidic transcripts coding for chlorophyll-binding apoproteins (CBPs). By comparing the positions and numbers of ribosomes on the

plastidic transcripts of wild-type and mutant plants, the authors concluded that chlorophyll availability modulates the stability rather than the synthesis of CBPs in plastids. Furthermore, the *chl1H* mutation had no effect on the partitioning of CBP footprints, suggesting that co-translational targeting of the nascent peptides into the thylakoid membrane is independent of chlorophyll binding by the CBPs.

Finally, the Topic includes a review of translational regulation during development and under stressful conditions, and an overview of the translation of viral RNAs. Sablok et al. summarize recent global analyses of mRNA populations associated with ribosomes (now referred to as the “translatome”), highlighting the importance of alternative splicing and the application of these technologies to polyploid plant species. Miras et al. focus on translation in plants infected with RNA viruses. Plant viruses have evolved subtle mechanisms, such as mRNA *cis*-translational enhancers, to recruit the host's translational machinery and initiate translation by non-canonical mechanisms. The authors highlight the diversity of these translational elements and focus on current knowledge of their structure and interactions with the host's translational initiation apparatus.

We believe that this compilation of original research articles and reviews will bring the reader up to date on the current state of the art in the field of post-transcriptional and translational regulation in plants. We are also convinced that advances in this area will be of the utmost importance for the development of biotechnological tools for yield enhancement.

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All authors listed have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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The Arabidopsis TOR Kinase Specifically Regulates the Expression of Nuclear Genes Coding for Plastidic Ribosomal Proteins and the Phosphorylation of the Cytosolic Ribosomal Protein S6

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Protein translation is an energy consuming process that has to be fine-tuned at both the cell and organism levels to match the availability of resources. The target of rapamycin kinase (TOR) is a key regulator of a large range of biological processes in response to environmental cues. In this study, we have investigated the effects of TOR inactivation on the expression and regulation of Arabidopsis ribosomal proteins at different levels of analysis, namely from transcriptomic to phosphoproteomic. TOR inactivation resulted in a coordinated down-regulation of the transcription and translation of nuclear-encoded mRNAs coding for plastidic ribosomal proteins, which could explain the chlorotic phenotype of the TOR silenced plants. We have identified in the 5' untranslated regions (UTRs) of this set of genes a conserved sequence related to the 5' terminal oligopyrimidine motif, which is known to confer translational regulation by the TOR kinase in other eukaryotes. Furthermore, the phosphoproteomic analysis of the ribosomal fraction following TOR inactivation revealed a lower phosphorylation of the conserved Ser240 residue in the C-terminal region of the 40S ribosomal protein S6 (RPS6). These results were confirmed by Western blot analysis using an antibody that specifically recognizes phosphorylated Ser240 in RPS6. Finally, this antibody was used to follow TOR activity in plants. Our results thus uncover a multi-level regulation of plant ribosomal genes and proteins by the TOR kinase.

Keywords: phosphorylation, plastid, proteomic, ribosome, RPS6, TOR kinase, transcriptomic, translational

INTRODUCTION

During their life, living organisms have to adapt their growth and development to exogenous factors such as stresses and nutrient availability. Therefore, they have evolved different regulatory pathways to increase the perception of environmental cues and to fasten the required metabolic modifications. These pathways employ conserved key players that link energy depletion, which is often the result of stresses and nutrient limitation, to anabolic and catabolic cellular activities. One of the most important pathway that is found in all eukaryotes is the one related to the target of rapamycin (TOR) protein kinase. TOR is a large kinase, which operates in at least two multi-protein complexes (TORC1 and TORC2; for reviews, see: Wullschleger et al., 2006; Laplante and Sabatini, 2012; Albert and Hall, 2015) and controls a wealth of biological outputs. In animals and yeast, it is well known that TOR positively regulates protein synthesis and anabolic activities when the growth conditions are favorable, while repressing the mechanisms implicated in recycling and catabolism (Laplante and Sabatini, 2012; Shimobayashi and Hall, 2014). Indeed, the production of proteins is particularly energy consuming since it requires ribosome biogenesis as well as mRNA translation (Warner, 1999).

In plants, there is so far only evidence for the presence of the TORC1 complex which comprises the conserved Regulatory-associated protein of TOR (RAPTOR) and the Lethal with Sec 13 (LST8) proteins, (for reviews, see: Robaglia et al., 2012; Henriques et al., 2014; Xiong and Sheen, 2014; Rexin et al., 2015; Dobrenel et al., 2016). TOR has already been shown to control a vast array of biological processes in plants (Deprost et al., 2007; Caldana et al., 2013; Xiong and Sheen, 2014; Dong et al., 2015) and a link between the TOR complex and protein translation has been evidenced. We have indeed shown earlier that TOR inactivation, either after silencing (Deprost et al., 2007) or by using a TOR inhibitor (Sormani et al., 2007) leads to a decrease in polysome abundance. It has also been shown that the translation reinitiation after a long upstream open reading frame by the plant viral reinitiation factor transactivator-viroplasm is mediated by its physical association with the TOR protein (Schepetilnikov et al., 2011). Furthermore, TOR activity appears essential for translation reinitiation of cellular mRNA containing short-ORFs in the 5' UTR (Schepetilnikov et al., 2013).

The biochemical analysis of the plant cytoplasmic ribosome showed that it contains 81 different proteins, 33 for the small 40S subunit and 48 for the large 80S subunit (Giavalisco et al., 2005; Carroll et al., 2008; Carroll, 2013; Browning and Bailey-Serres, 2015; Hummel et al., 2015). In animal cells, TOR regulates cap-dependent translation by phosphorylating and stimulating the activity of the ribosomal protein S6 kinase (S6K), a conserved target of TOR which phosphorylates the 40S ribosomal protein S6 (RPS6, Barbet et al., 1996; Holz et al., 2005), and by repressing the inhibitory effect of eIF4E-binding protein (Ma and Blenis, 2009; Albert and Hall, 2015). Consistently, in yeast, TOR inhibition by rapamycin leads to an 80% reduction in overall translation (Barbet et al., 1996). It has been shown in mammals that S6K is activated in a TOR-dependent manner

by phosphorylation of Thr389 and Thr229 (Ma and Blenis, 2009), resulting subsequently in the phosphorylation of serine residues in the C-terminal extremity of the ribosomal protein RPS6. Early on RPS6, which is located at the right foot of the 40S subunit, was identified as the only phosphorylated protein in the ribosome small subunit (Gressner and Wool, 1976).

It has been postulated that TOR regulates the translation of a particular sub-set of mRNAs containing a 5' terminal tract oligopyrimidine (TOP) motif (for a review, see Meyuhas and Kahan, 2015). Canonical TOP mRNAs harbor a C residue on position 1 followed by a stretch of 4–15 pyrimidines. The first evidence suggested that TOR activates TOP mRNA translation through phosphorylation of S6K and RPS6 but this hypothesis was later questioned since TOP mRNA are normally translated in S6K-deficient mice (Meyuhas and Kahan, 2015). The Arabidopsis genome contains two tandem-repeated S6K genes and the proteins encoded by these genes are directly phosphorylated by TOR (Mahfouz et al., 2006; Schepetilnikov et al., 2011, 2013; Xiong et al., 2013). The phosphorylation level of S6K proteins is positively correlated to their capacity to phosphorylate RPS6 (Turck et al., 1998, 2004; Mahfouz et al., 2006; Browning and Bailey-Serres, 2015). In yeast, untargeted phosphoproteomic analyses pointed RPS6 to be the main phosphorylation target of TOR in the ribosome (Huber et al., 2009). In plants, RPS6 was found to be the major phosphorylated ribosomal protein in tomato cells and this phosphorylation was found to be reduced after heat stress (Scharf and Nover, 1982) or in oxygen deprived maize roots (Bailey-Serres and Freeling, 1990). Later a survey of post-translational modifications of the Arabidopsis ribosomal proteins only identified Ser240 in RPS6 and Ser137 in RPL13, together with acidic proteins, as being phosphorylated (Carroll et al., 2008). Multiple phosphorylation sites were detected in the RPS6 C-terminal region including Ser238 and Ser241 for maize (Williams et al., 2003) and Ser237 and Ser240 for Arabidopsis (Chang et al., 2005). Moreover Ser240 phosphorylation was found to be induced by light and high CO₂ conditions (Turkina et al., 2011; Boex-Fontvieille et al., 2013). More recently, Nukarinen et al. (2016) have observed a strong induction of RPS6 Ser240 phosphorylation when the activity of the Sucrose non-fermenting 1-Related Kinase 1 (SnRK1) is decreased. However, despite the accumulation of data showing variations in plant RPS6 phosphorylation in response to several stresses, the precise role of this C-terminal phosphorylation in the regulation of translation remains largely elusive.

Since TOR was found to affect translation in plants, we undertook a global phosphoproteomic, transcriptomic and translational analysis of the ribosomal fraction after TOR inactivation. Interestingly, we observed a strong effect of TOR inactivation on the expression of nuclear-encoded plastidic ribosomal proteins (pRPs) and the main phosphorylation site controlled by TOR activity was found to be Ser240 in the cytoplasmic ribosomal protein (cRP) RPS6. Finally, we made use of this specific phosphorylation site to design a robust Western-based method for quantifying TOR activity in plant extracts.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Seeds of two independent ethanol-inducible TOR RNAi lines (5.2 and 6.3, described in Deprost et al., 2007) as well as an ethanol-inducible GUS overexpressing line (as a control) (Deprost et al., 2007) were grown *in vitro* under long day conditions (16 h light/8 h night) for 7 days on solid 1/5 Murashige and Skoog medium supplemented with sucrose 0.3% (w/v) at a constant temperature of 25°C and a light intensity of 75 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The plants were subsequently treated with ethanol vapor for either 3 or 10 days. Whole plantlets from two independent biological replicates of each condition were then harvested in the middle of the light period and directly snap frozen in liquid nitrogen, grinded and subjected immediately to the ribosome enrichment protocol.

Ribosome Enrichment

Ribosomal subunits (40S and 60S), monoribosomes (80S) and polyribosomes were isolated from the plantlet powder according to Bailey-Serres and Freeling (1990) with minor modifications. Freshly harvested and grinded plantlets were homogenized at a final concentration of 10% (w/v) in the ice-cold extraction buffer (0.2 M Tris-HCl [pH 9], 0.4 M KCl, 0.025 M EGTA, 0.035 M MgCl_2 , 0.2 M sucrose) supplemented with 2% (v/v) Triton X-100, 2% (v/v) Tween 20, 2% (v/v) NP-40 and 1% (w/v) sodium deoxycholate. The extracts were incubated on ice for 10 min to solubilize membrane-bound ribosomes and centrifuged at $2880 \times g$ for 15 min at 4°C. The supernatants were layered over a sucrose cushion (0.04 M Tris-HCl [pH 9], 0.2 M KCl, 0.005 M EGTA, 0.03 M MgCl_2 , 1.75 M sucrose) and ultracentrifuged at $225\,000 \times g$ for 14 h. The ribosome enriched pellet was resuspended in 300 μl of Laemmli buffer (Laemmli, 1970) and denatured at 100°C for 10 min.

LC-MS/MS Analysis

For the proteomic characterization, ribosome enriched fractions were first submitted to a short migration through the stacking gel of a SDS-PAGE, in order to remove the rRNA and the possible chemical contaminant, including detergents. After a Coomassie staining, the unique band of proteins, for each sample, was cut and divided into five pieces that were submitted, in gel, to the tryptic digestion, reduction and alkylation. Peptide containing fractions were then analyzed by nano LC-MS/MS as previously described (Boex-Fontvieille et al., 2013). Briefly on-line liquid chromatography was performed on a NanoLC-Ultra system (Eksigent). Eluted peptides were analyzed with a Q-Exactive mass spectrometer (Thermo Electron) using a nano-electrospray interface (non-coated capillary probe, 10 $\mu\text{i.d.}$; New Objective). Peptides and the corresponding proteins were identified and grouped with X!TandemPipeline using the X!Tandem Piledriver (2015.04.01) release (Craig and Beavis, 2004) and the TAIR10 protein library with the phosphorylation of serine, threonine and tyrosine as a potential peptide modification. Precursor mass tolerance was 10 ppm and fragment mass tolerance was

0.02 Th. Identified proteins were filtered and grouped using the X!TandemPipeline v3.3.4¹. Data filtering was achieved according to a peptide *E*-value lower than 0.01. The false discovery rate (FDR) was estimated to 0.92%. Relative quantification was performed using the MassChroQ software (Valot et al., 2011) by peak area integration on extracted ion chromatograms (XICs) within a 10 ppm window, after LC-MS/MS chromatogram alignment and spike filtering.

Phosphopeptide Enrichment

Arabidopsis seedlings grown on MS agar plates in standard 16/8 h and 21/17°C day/night conditions were transferred to liquid MS media supplemented with 10 μM NAA (Sigma-Aldrich). Total protein extracts were precipitated with 0.1 M ammonium acetate in 100% methanol, reduced, alkylated and digested overnight with trypsin (Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate. Resulting peptides were vacuum-dried and re-suspended in 250 mM acetic acid with 30% acetonitrile for phosphopeptide enrichment with Phos-Select Iron Affinity Gel (Sigma-Aldrich) according to the protocol from Thingholm et al. (2008). Eluted phosphopeptides were desalted and analyzed by nano LC-MS/MS on a TripleTOF 5600 (Sciex, Canada) coupled a NanoLC-2DPlus system with nanoFlex ChiP module (Eksigent, Sciex).

Transcriptome and Translatome Analysis

Transcriptomic and translational analyses were performed on two biological replicates using 7-day-old plantlets from the two independent TOR RNAi and GUS control lines grown *in vitro* and treated with ethanol for 24 h. Transcriptome analyses using CATMA arrays were performed on total RNA preparations as previously described (Moreau et al., 2012). For translational analyses total RNA was extracted and polysomal fractions were purified on sucrose gradients after ultracentrifugation as previously described (Deprost et al., 2007; Sormani et al., 2011). Polysome-bound RNAs were extracted using guanidinium hydroxylchloride and precipitated by isopropanol and linear acrylamide as a carrier. Subsequently, RNAs were reverse transcribed and hybridized on CATMA arrays as described above for the determination of differentially translated mRNAs (Sormani et al., 2011). Statistical analysis of each comparison was based on two dye swaps and followed by the analysis described by Gagnot et al. (2008) and Moreau et al. (2012). Briefly, an array-by-array normalization was performed to remove systematic biases. To determine differentially expressed genes, we performed a paired *t*-test on the log ratios averaged on the dye swap. The raw *p*-values were adjusted by the Bonferroni method, which controls the family-wise error rate to keep a strong control of the false positives in a multiple-comparison context. We considered as being differentially expressed the probes with a Bonferroni *P*-value ≤ 0.05 , as described by Gagnot et al. (2008). The results are available online in the CatDB database².

¹<http://pappso.inra.fr/bioinfo/xtandempipeline/>

²http://urgv.evry.inra.fr/cgi-bin/projects/CATdb/consult_expce.pl?experiment_id=302

Antibody Production

Antibodies directed against phosphorylated RPS6A were obtained by conjugating to keyhole limpet hemocyanin the SRLpSSAAAKPSVTA (phosphoSer240) peptide (produced by Proteogenix, Schiltigheim, France) and injecting two New Zealand White female rabbits (performed by Proteogenix). Seven injections were performed over a period of 56 days. Then a preliminary ELISA test was performed at day 63 to evaluate the titer of the antibodies and the rabbits were bled at day 70. The obtained antisera were first depleted against immobilized non-phosphorylated peptide then specific antibodies were purified using the phosphorylated SRLpSSAAAKPSVTA peptide. The specificity of the purified antibodies was evaluated using an ELISA test with the phosphorylated and non-phosphorylated peptides (produced by Proteogenix) (Supplementary Figure S3).

SDS-PAGE and Western Blotting

Primary antibodies used in this study are directed against mammalian RPS6 (Cell Signaling Technology #2317S), rapeseed RPL13 (Sáez-Vásquez et al., 2000) and Arabidopsis RPS14 (Agrisera AS09 477).

For detection of phosphorylated RPS6, total proteins were extracted from either wild-type (Col-0), TOR RNAi or control Arabidopsis lines using the Laemmli buffer and blotted with our RPS6 phospho-specific antibody. Bradford assay (Bio-Rad) was performed to quantify total protein concentrations. Ten micrograms of proteins were separated by SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (PVDF, Bio-Rad) by electroblotting. Membranes were probed with either antiphospho-RPS6 (P-RPS6) rabbit polyclonal antiserum (dilution 1:5000) or with anti-RPS6 mouse monoclonal IgG (dilution 1:1000). Goat anti-rabbit IgG-HRP (horseradish peroxidase 1:2000, Santa Cruz Biotechnologies) and Goat anti-mouse IgG-HRP (1:2000, Santa Cruz Biotechnologies) were used as secondary antibodies. Immunodetection was performed by using enhanced chemiluminescent (ECL) substrates for HRP as recommended by the manufacturer (Clarity Western ECL blotting substrate Bio-Rad). Transferred proteins on PVDF membranes were visualized by Ponceau S staining.

Motif Analysis

The 5' UTR sequences of the ribosomal proteins mRNAs were obtained from the TAIR10 database³. The identification of the motifs was performed with the online MEME software⁴ (Bailey et al., 2009) with a motif recognition size comprised between 6 and 50 nt. Only the representative isoforms of the genes were used.

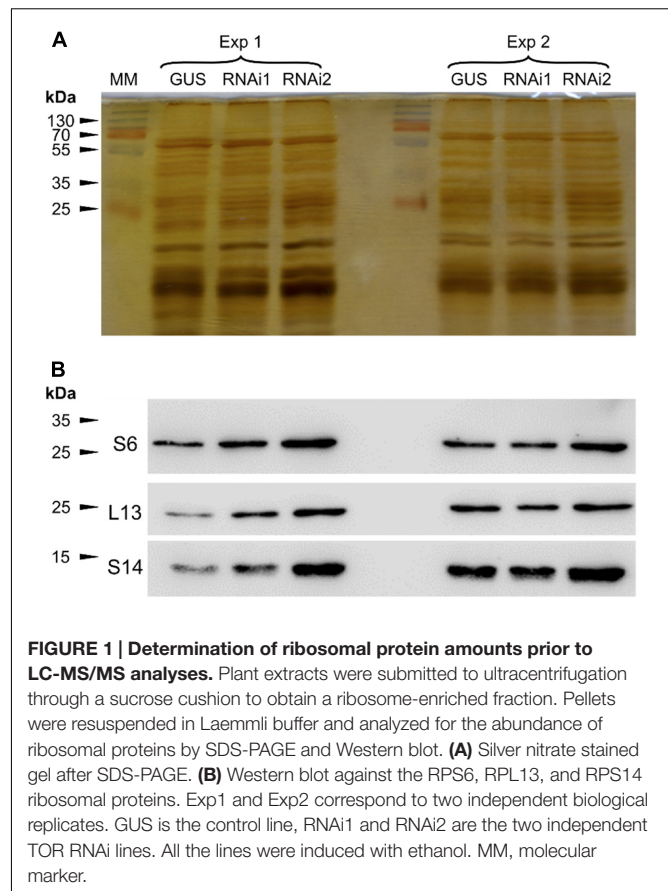
Accession Numbers

Arabidopsis RPS6A: At4g31700 and RPS6B: At5g10360. Proteomic raw data are available in the Protic database under the following accession name: tor_inactivation⁵.

³<http://www.arabidopsis.org>

⁴<http://meme-suite.org>

⁵moulon.inra.fr/protic/tor_inactivation



RESULTS

Ribosome Enrichment by Density Ultracentrifugation

The aim of this study was to identify, by an untargeted proteomic analysis, modifications in the Arabidopsis ribosome fraction in response to TOR inactivation. First, we evaluated the suitability of our ribosome extraction method for LC-MS/MS analysis. To do so, 7-day-old seedlings of Arabidopsis (Col-0) were harvested and, to prevent protease, phosphatase, or kinase activities, immediately submitted to the ribosome purification protocol (see, Materials and Methods). Finally, high molecular weight particles, including polysomes, were pelleted by ultracentrifugation through a sucrose cushion.

We then submitted the ultracentrifuged fraction to SDS-PAGE and resolved proteins were stained using silver nitrate (Figure 1A). The obtained protein profile is typical of purified plant ribosomal fractions (Carroll et al., 2008). The presence of ribosomal proteins in this fraction was confirmed by Western blot analysis which allowed to normalize the protein fractions by diluting the samples according to Western blot quantifications (Figure 1B).

To inactivate TOR, we used two independent ethanol-inducible TOR RNAi lines (based on the *Alcr/AlcA* operon) that we previously obtained and characterized (Deprost et al.,

2007) and compared them to a control line expressing an ethanol-inducible GUS gene (GUS control, Deprost et al., 2007; Dobrenel et al., 2011).

Identification of Ribosomal Proteins by LC-MS/MS Analysis

Seedlings of two independent RNAi lines and of the GUS control were grown for 7 days *in vitro* and then treated with ethanol to induce TOR inactivation. We repeated the same experiment twice and then analyzed the six samples together. In order to remove eventual contaminating rRNA as well as the chemicals, the samples were first submitted to a short migration through a SDS-PAGE stacking gel. Proteomic as well as phosphoproteomic analyses by LC-MS/MS identified a total of 5936 spectra, corresponding to 1508 unique peptide sequences (raw data are available in the Protic database). Peptides were matched to protein sequences from TAIR10 and grouped according to sequence homology using the X!TandemPipeline with the phosphorylation of serine, threonine and tyrosine as a potential peptide modification. By this method, 361 different proteins were potentially identified by at least two peptide sequences, belonging to 217 groups (corresponding presumably to protein families, based on sequence homologies). Among these 361 proteins, 210 were identified by the presence of at least two proteotypic (i.e., specific of a given protein) peptides. Based on the previous annotations of the ribosomal proteins (Sormani et al., 2011; Tiller and Bock, 2014; Hummel et al., 2015), we found that more than half of the 361 proteins were ribosomal proteins (147 correspond to cytosolic ribosomes and 46 to organelle ribosomes) (**Figure 2A**). Using an *in silico* analysis, Sormani et al. (2011) refined the annotation of ribosomal proteins (including plastidic and mitochondrial ones). We used this list to identify the proteins (and therefore the peptides) corresponding to the mitochondrial and plastidic ribosomes. All identified organellar peptides corresponded to plastidic ribosomes.

Target of Rapamycin inactivation in the RNAi lines did not largely affect the number of detected peptides originating from the cytosolic ribosomes and thus most peptides were found both in the RNAi and in the control lines (between 419 and 449 peptides for RNAi2 and GUS lines, respectively; **Figure 2B**). Conversely the pool of peptides coming from pRPs was specifically depleted in the TOR RNAi lines with only 116 and 111 peptides detected for the TOR RNAi1 and RNAi2 lines, respectively, compared to 201 peptides in the control GUS line (**Figure 2C**). One third of the pRPs peptides were only present in the control GUS line (74 out of 218) whereas a much smaller number of peptides (17) were specifically found in the RNAi lines.

Quantitative Analysis of the Expression of Ribosomal Protein Encoding Genes

In order to exclude biases that could be caused by potential technical issues, like the mass spectrometer being occupied by some abundant peptides eluting near the peptides of interest, we quantified the traces of the peaks corresponding to the identified peptides of the plastidic and cytoplasmic RPs. These peak areas were then compared between the RNAi and GUS

control lines for each peptide (**Figure 2D**). Such a quantitative approach confirmed that most of peptides resulting from the fragmentation of the pRPs are indeed less abundant in the TOR RNAi samples. This also confirmed that TOR inactivation did not have any strong effect on the accumulation of the cRPs peptides (**Figure 2D**). Thus these results suggest a global decrease in the abundance of pRPs resulting in a lower number and amount of peptides detected in the LC-MS/MS analysis.

We then used the number of spectra (or peptide hits) per protein to estimate the protein abundance for the pRPs and cRPs. Indeed it has been shown that there is a proportional relationship between these two values (Allet et al., 2004; Liu et al., 2004). We exclusively used the spectra corresponding to proteotypic peptides in order to avoid a bias that would be caused by the very high sequence homology within ribosomal protein families. By this method, we showed a coordinated down-regulation of the pRPs while the cRPs have a much less coordinated profile and are globally only slightly affected by the TOR inactivation (**Figure 3**). To better understand the role of TOR in regulating plant gene expression, we performed transcriptomic and translomic analyses after TOR inactivation, in which we monitored the mRNA levels on total and polysome-bound RNA samples. The variations in the abundance of polysome-bound mRNA were determined after purification of polysomes on a sucrose gradient, extraction of RNA and microarray hybridization. To better identify the primary TOR-regulated mRNA targets, we decided to shorten the time of ethanol-mediated RNAi induction. Two biological repetitions were performed using each time the two independent RNAi lines. The resulting four transcriptomic and translomic experiments were submitted to a statistical analysis to identify common differentially expressed genes when compared to the GUS control lines (see Materials and Methods). When focusing on the ribosomal proteins, we found a large difference in the expression profiles of the corresponding nuclear genes depending on whether the gene product is part of the cytoplasmic or the plastidic ribosome (**Figure 3**; Supplementary Table S1). First, almost all detected nuclear-encoded mRNA coding for pRPs, whether located in the small or in the large subunit, showed either no change in abundance or were down-regulated after TOR inactivation when compared to the GUS control line treated with ethanol (**Figure 3A**). On the opposite mRNAs coding for cRPs were mostly up-regulated (**Figures 3B,C**) except for RPL18a-1 which was the only transcript showing a reproducible decrease in abundance. The translomic analysis mostly mirrored the transcriptomic variations but for some genes coding for cytosolic ribosomes, like RPL40 and RPL41, the total mRNA abundance did not vary significantly whereas they were more engaged in polysomes compared to the GUS control, suggesting a higher translation of these genes (**Figure 3C**). Taken together, these data suggest that there is a coordinated down regulation of the nuclear genes coding for the pRPs at the transcriptional, translational (polysomal loading), and protein levels in response to TOR inactivation (**Figure 3A**).

Next we examined whether this co-regulation involves the recognition of a conserved motif in the 5' UTR sequences of the nuclear-encoded mRNA coding for pRPs. These sequences

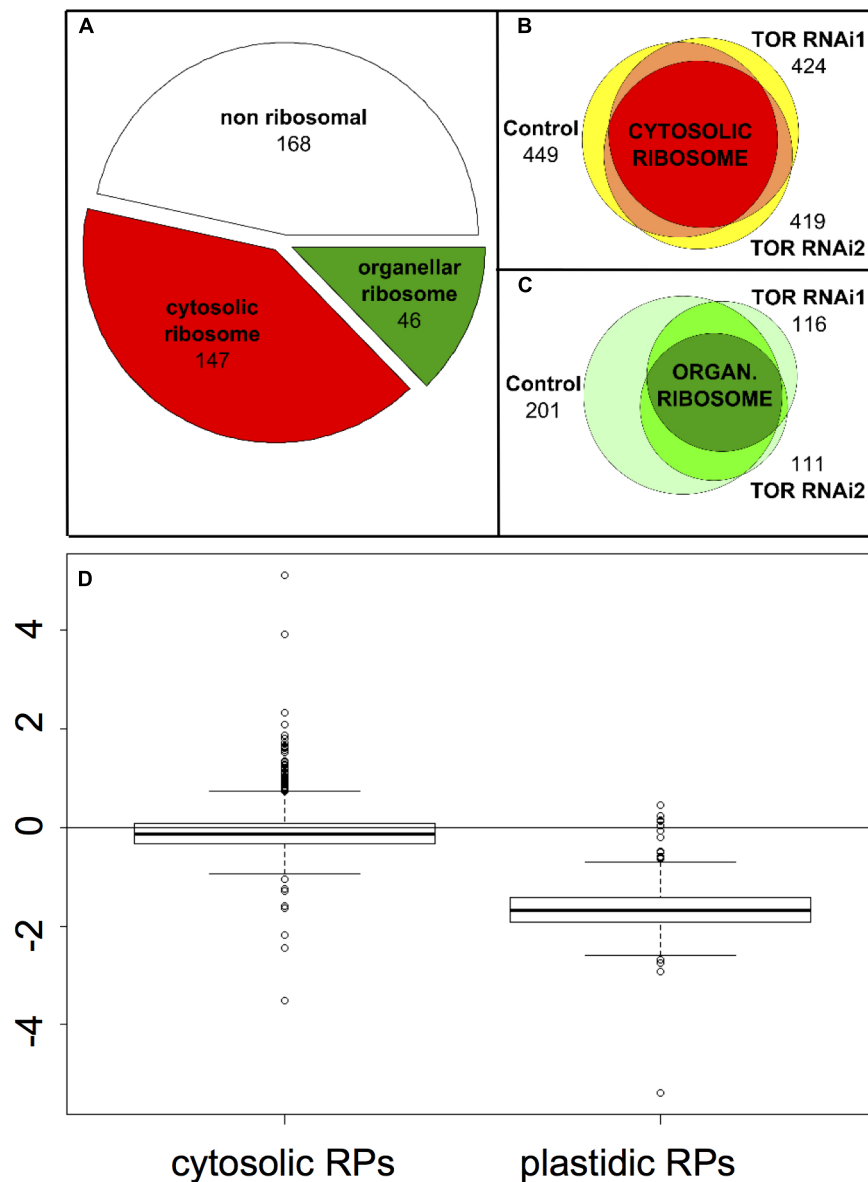


FIGURE 2 | Effect of TOR inactivation on the cytosol and organelle ribosomal proteins. (A) Distribution of the proteins identified in at least one of the samples depending on whether they are part of the cytosolic ribosome, the organelle ribosome or non-ribosomal. **(B)** Venn diagram showing the number of peptides corresponding to the cytosolic ribosomal proteins identified in the GUS control and in the TOR RNAi lines. The intersections of two peptide sets are shown in orange and the intersection of the three sets in red. The total numbers of peptides are shown and the areas are representative of the number of common or specific peptides. **(C)**, same as **(B)** for the organelle ribosome except that intersections are shown in medium and dark green for two or three peptides sets, respectively. **(D)** Boxplot representing the relative abundance (\log_2 fold change) of the peptides derived from ribosomal proteins in the TOR RNAi compared to the GUS control lines depending of their localization in the cytosolic or plastidic ribosomes. For each peptide a mean abundance was obtained from the four repetitions (two independent experiments using the two RNAi lines) and the abundance ratios were compared.

were analyzed for enriched motifs using the MEME software (Bailey and Elkan, 1994). A strongly significant motif composed of a stretch of pyrimidines was identified in this set of 5' UTRs (Figure 4A). This sequence is reminiscent of the animal TOP motif found within the 5'UTR of mammalian ribosomal protein encoding mRNAs, which confers translational regulation by TOR (Meyuhas and Kahan, 2015). The MEME analysis

also revealed the presence of a second A/G-enriched motif (Figure 4A). On the contrary, the 5' UTRs of the mRNAs coding for cRPs were significantly enriched for a TTTAGGGTTT motif (Figure 4B), which is similar to the telo-box consensus sequence already identified in the promoters of these genes (Figure 4B) (McIntosh et al., 2011; Wang et al., 2011). Next we analyzed the 5' UTRs of the nuclear genes coding for pRPs,

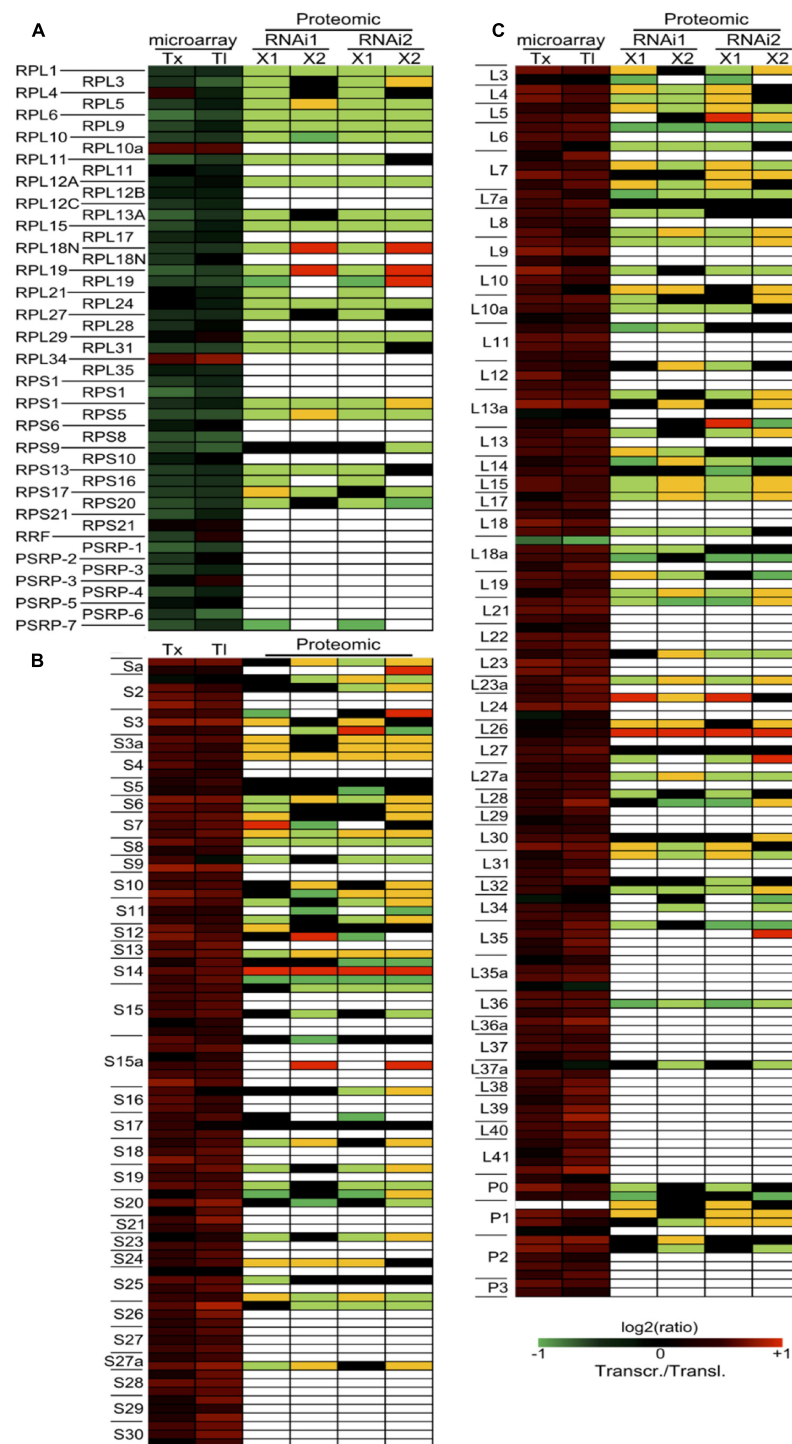


FIGURE 3 | Effect of TOR inactivation on the transcription, translation, and abundance of ribosomal proteins. (A) Expression of the nuclear genes coding for chloroplastic ribosomal proteins at the total mRNA level (transcriptomic analysis: Tx), at the polysome-bound mRNA level (translatomic analysis: TI) and at the protein level (proteomic analysis). **(B,C)** Expression level of the genes coding for the cytosolic ribosomal proteins and localized in the small or large ribosome subunit, respectively. For the transcriptome and translational experiments, mRNA abundances in the TOR RNAi lines were compared to the GUS control line treated with the same 24 h ethanol induction time. The results represent the mean of the two TOR RNAi lines in the two independent biological replicates. Intensity ratios are shown in log₂ scale according to the color scale shown in the figure on the bottom right for transcriptomic and translational experiments (Transcr./Transl.). For the proteomic experiment, two independent biological replicates (X1 and X2) using the two TOR RNAi lines are presented. Orange and pale green represent a quantitative up- and down-regulation, respectively, and red and dark green represent a qualitative regulation, respectively (peptides either present or absent). Only the proteotypic spectra were used. Black boxes indicate no change and white boxes represent missing data.

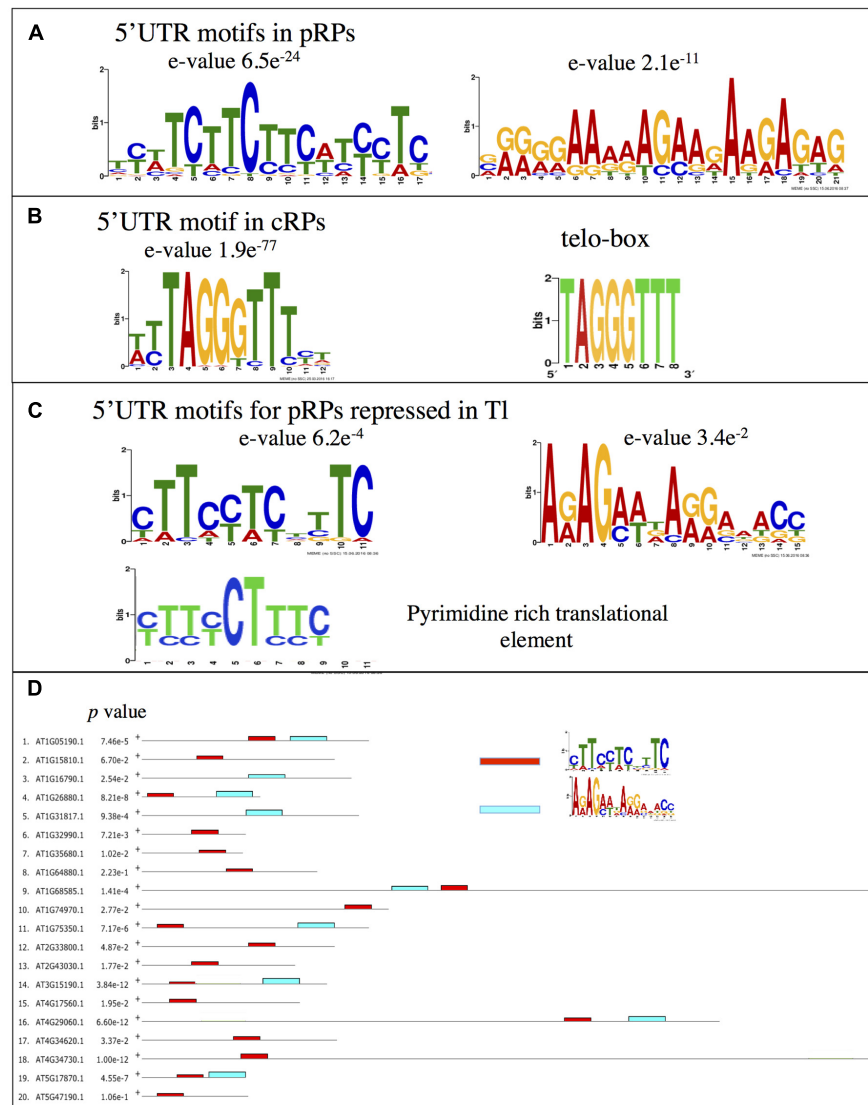


FIGURE 4 | Motifs identified in the 5' UTRs of genes coding for ribosomal proteins. (A) Motifs identified as being significantly enriched in the 5' UTRs of the nuclear genes coding for the plastidic RPs after analysis by the MEME software. **(B)** Same for genes coding for the cytosolic RPs. The telo-box motif found in cRPs promoters is shown. **(C)** Same as for **(A)** but only the 5' UTRs of the genes showing a down-regulation in the translome experiments following TOR inactivation were kept. **(D)** Positions of the motifs identified in **(C)** in the 5' UTRs of the analyzed sequences. See Supplementary Figure S1 for details.

which were less engaged in polysomes after TOR inactivation (Figure 3A). MEME analysis identified a shorter pyrimidine-rich motif that is more similar to canonical TOP motifs, but even more to the pyrimidine-rich translational element (PRTE), a motif identified in animal genes which translation is controlled by TOR (Figure 4C) (Hsieh et al., 2012). This motif is found in the majority of animal TOR targets and, unlike conventional TOP motifs, does not reside at the start of the mRNA sequence (Hsieh et al., 2012). Consistently, the motif we identified is also rarely present at the start of the analyzed mRNAs (Figure 4D; Supplementary Figure S1) but is significantly enriched in pRP transcripts translation of which is affected by TOR inactivation. A purine-rich motif was also

identified in this subset of genes and was often found 3' to the PRTE-like motif (Figure 4D).

We then mined the public Genevestigator transcriptome database (Hruz et al., 2008) for information about the expression profile of plastidic ribosomal genes encoded by the nuclear genome. Interestingly these genes were found to be down-regulated in estradiol-inducible TOR RNAi lines (Xiong et al., 2013; Supplementary Figure S2) which suggests that TOR inactivation reproducibly reduces their expression. Nuclear genes coding for pRPs are also strongly induced during germination or after light treatment, which is consistent with their role in the formation of the photosynthetic machinery. Conversely they were repressed in response to various stresses such as

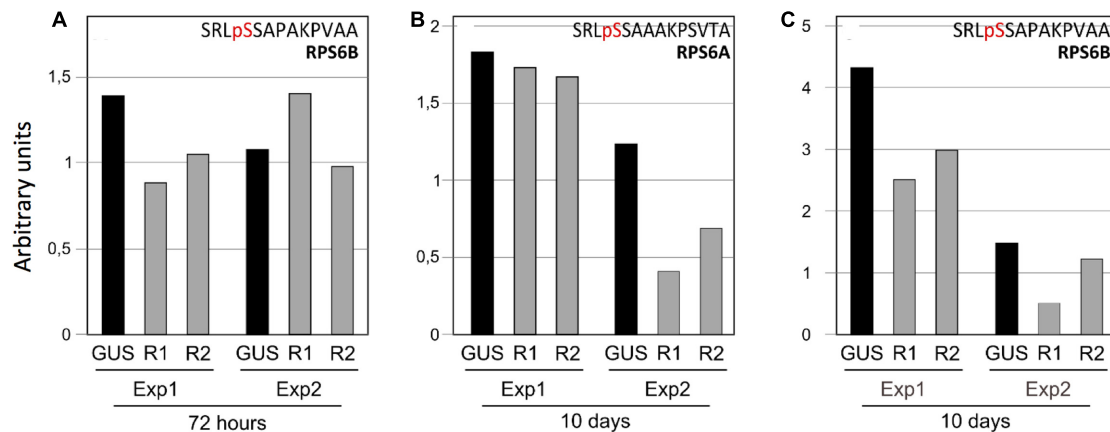


FIGURE 5 | Downregulation of RPS6A and RPS6B phosphorylation in response to TOR inactivation. (A) Phosphorylation level of the RPS6B C-terminal peptide after 72 h of ethanol treatment. (B) Same for the RPS6A and (C) for the RPS6B proteins after 10 days of ethanol treatment. Each bar corresponds to the peak intensity of the phosphorylated peptide normalized by a proteotypic peptide from the same protein (GENDLPLGLTDEKPR for RPS6A and GVSDLPGLTDEKPR for RPS6B). For each experiment, the results of the GUS control (GUS), the TOR RNAi1 (R1) and the TOR RNAi2 (R2) lines are presented. Exp1 and Exp2 correspond to two independent biological replicates with 72 h or 10 days of TOR inactivation.

pathogen infection, drought, hypoxia, or increased temperature as well as in response to extended night. Finally these genes were slightly induced in one experiment of sucrose feeding but globally repressed in several experiments of nitrogen starvation (Supplementary Figure S2).

TOR Dependent Phosphorylation of RPS6 on Ser240

Even if most of the cRPs were not affected by TOR inactivation (Figure 2D) we found 30 peptides which were significantly down regulated (t -test p -value < 0.05; Supplementary Table S2). In most of the case, these peptides are not proteotypic, thus making the conclusions more complicated. The two cytosolic RPS6 paralogs were identified with three different proteotypic peptides that are significantly down-regulated in a quantitative manner following TOR inactivation (Figure 3B). In total, 15 peptides were detected for this protein family. Among these 15 peptides, we identified a phosphorylated peptide corresponding to the RPS6B C-terminal extremity. The X!Tandem analysis predicted a phosphorylation site on Ser240 (SRLpSSAPAKPVAA: Figure 5; Supplementary Figure S3). This serine seems to be conserved in the eukaryotic RPS6 proteins, including plants, and was previously identified as being phosphorylated following TOR activation in yeast and animals (Pende, 2006; Meyuhas, 2008; Yerlikaya et al., 2016) (Figure 6). In order to clarify the actual position of the phosphorylated residue in this peptide, the phosphoproteomic results were submitted to a Phoscalc statistical analysis (MacLean et al., 2008). This analysis failed to clearly identify the phosphorylated site between Ser237, 240 or 241 (Supplementary Figures S3C,D). Nevertheless it showed that this RPS6B C-terminal peptide carries only one phosphate group. An independent phosphoproteomic analysis of Arabidopsis seedlings treated with auxin was also performed (see Materials and Methods). Indeed previous

studies suggested that auxin mediates TOR, and thus S6K1, activation in Arabidopsis as shown by phosphorylation of TOR at Ser2424 and S6K1 at Thr449 (Schepetilnikov et al., 2013). Since S6K1 is directly involved in the phosphorylation of RPS6, we asked whether this ribosomal protein is phosphorylated at Ser240 in Arabidopsis extracts treated by auxin. After purification of the phosphopeptides by immobilized metal affinity chromatography (IMAC), Ser240 was again identified as a potential phosphorylation site both in RPS6A and B (Supplementary Table S3; Supplementary Figures S3E,F). In some cases RPS6A Ser240 was identified together with Ser237 (pSRLpSSAPAKPVAA) but again the discrimination between phosphorylated Ser240 and Ser241 was not always possible (Supplementary Table S3).

Even if the precise localization of the phosphorylated serine residue in the RPS6B protein C-terminus could not be determined without ambiguities, we compared the abundance of the C-terminal monophospho-SRLSSAPAKPVAA peptide between the TOR RNAi lines and the GUS controls. After 72 h of exposure to ethanol we observed a modest decrease in the RPS6B C-terminal phosphorylated peptide (Figure 5A). This decrease in phosphorylation was only observed in one of two experiments. Since the abundance of the phosphorylated peptide was already low in the control line for the second experiment, it could be that the decrease in phosphorylation level was less obvious in this case. The corresponding RPS6A peptide was not found in this analysis. Thus to confirm this TOR-dependent decrease in RPS6 C-terminus phosphorylation, we performed a longer silencing induction by ethanol for 10 days. As for the 72 h treatment, biological duplicates have been used. The abundance of the monophospho C-terminal peptides was compared for both RPS6A and RPS6B proteins between the RNAi and the control GUS lines (Figures 5B,C). The amount of phosphorylated C-terminal peptide was lower (with a mean of 40% decrease) for both the RPS6A and the RPS6B

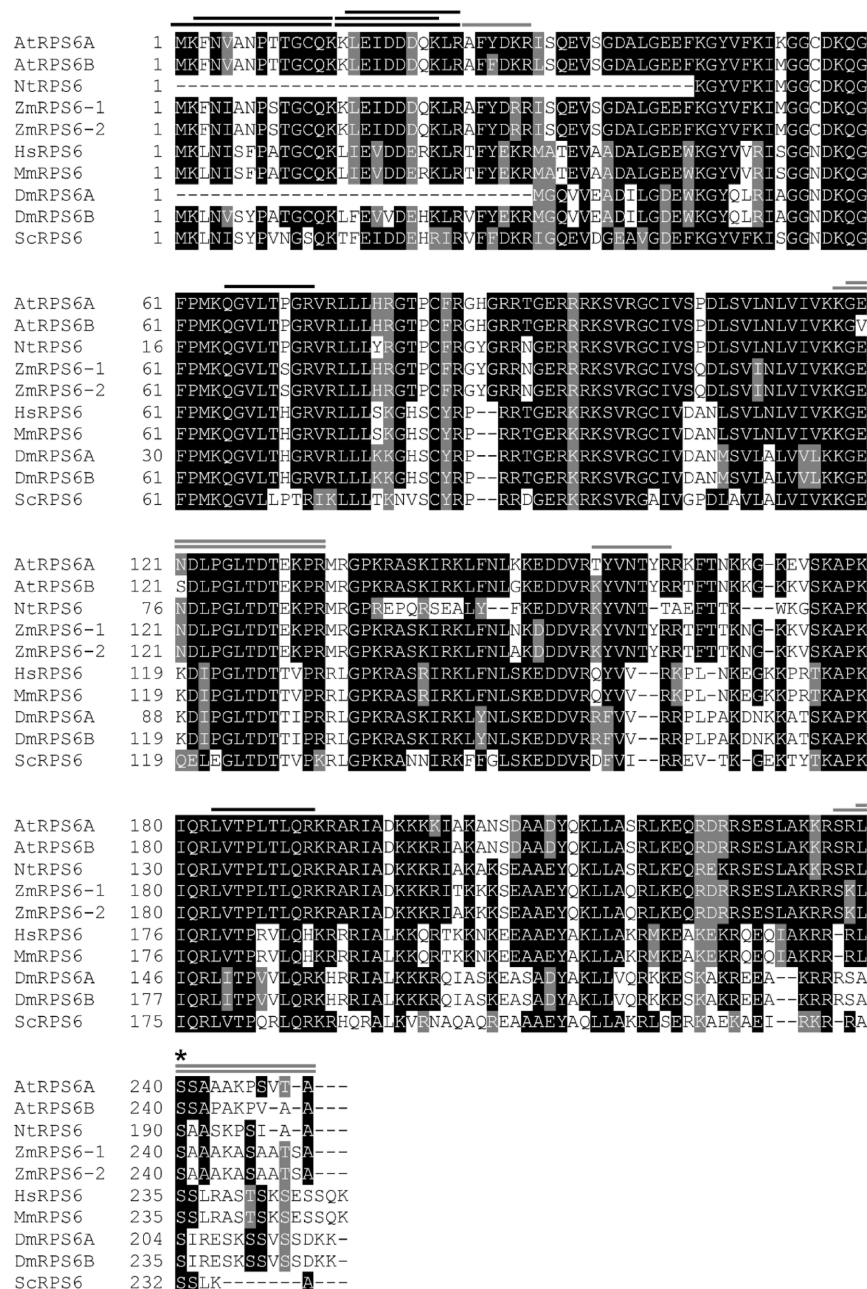


FIGURE 6 | Comparison of RPS6 amino acid sequences. Arabidopsis RPS6s (AtRPS6A, At4g31700.1, and AtRPS6B, At5g10360.1), *Nicotiana tabacum* RPS6 (NtRPS6, P29345.2), *Zea mays* RPS6s (ZmRPS6-1, NP_001105544.1 and ZmRPS6-2, NP_001105634.1), *Homo sapiens* RPS6 (HsRPS6, NP_001001.2), *Mus musculus* RPS6 (MmRPS6, NP_033122.1), *Drosophila melanogaster* RPS6s (DmRPS6A, NP_727213.1 and DmRPS6B, NP_511073.1) and *Saccharomyces cerevisiae* RPS6 (ScRPS6, NP_015235.1) protein sequences were aligned by T-Coffee (<http://tcoffe.org.cat>). The level of conservation is represented by a color code in which the most conserved residues are in black, those with intermediate conservation are gray and the least conserved are in white. RPS6 peptides identified in Arabidopsis by the proteomic analysis are represented by lines over the corresponding amino acid sequences (black for the peptides common to the two isoforms and gray for peptides specific to one isoform). An asterisk shows the conserved phosphorylated Ser240 residue.

proteins in the TOR RNAi lines when treated with ethanol for 10 days.

To confirm this phosphoproteomic analysis, a phospho-specific antibody was raised against a synthetic SRLpSSAAAKPSVTA peptide in which only Ser240 was phosphorylated.

The obtained polyclonal antibody was purified against this peptide and the eluted antibody fraction detected a single band corresponding in size to RPS6 proteins (Supplementary Figure S4A; **Figure 7**). Furthermore this antibody was found to be highly specific for the phosphorylated SRLpSSAAAKPSVTA peptide,

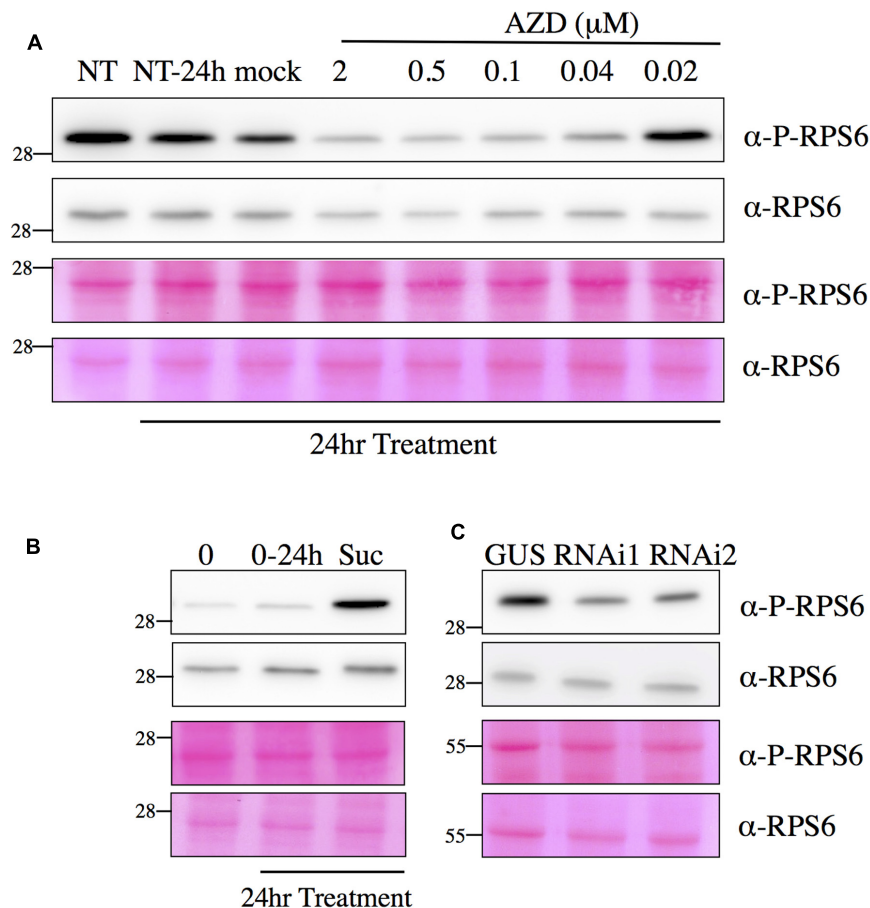


FIGURE 7 | Detection of RPS6 phosphorylation by Western blot assay. Total protein extracts obtained from seedlings were separated by SDS-PAGE and blotted onto a membrane. After incubation with the phospho-specific antibody against RPS6 Ser240 (P-RPS6) or a monoclonal antibody against total mammalian RPS6, blots were revealed by a secondary antibody linked to HRP activity and imaged with a CCD camera (see Materials and Methods for details and Supplementary Figure S4). **(A)** AZD treatments inhibit Ser240 RPS6 phosphorylation. Six day-old seedlings were either mock or AZD treated for 24 h. NT, non-treated plants at time 0 and 24 h. **(B)** Sucrose treatment induces RPS6 phosphorylation. Six day-old seedlings were transferred to sugar-free medium for 24 h and then either mock (0–24 h) or sucrose (0.5%) treated for 24 h. 0: plants before sucrose induction at time 0. **(C)** Silencing of TOR decreases RPS6 phosphorylation. The control (GUS) or TOR RNAi lines were grown for 7 days and induced with 5% (v/v) ethanol (EtOH). Bottom panels show Ponceau Red staining of the membranes.

showing no reaction with the control non-phosphorylated peptide in an ELISA test (Supplementary Figure S4B).

This antibody was used in an optimized Western blot assay and the obtained signal was very strong even when the antibody was diluted 1/5000. This band co-migrated with the band decorated by a specific monoclonal antibody against mammalian RPS6 (Figure 7) which was subsequently used to quantify the amount of total RPS6 in protein extracts. Both treating *Arabidopsis* seedlings with AZD-8055, a strong and specific second generation TOR inhibitor (Montané and Menand, 2013), or silencing the expression of TOR by a 7-day ethanol treatment, resulted in a significant and dose-dependent decrease in the signal obtained with the RPS6 phospho-specific antibody, whereas the total amount of RPS6 protein only decreased slightly (Figures 7A,C). Since this antibody is highly specific for the phosphorylated RPS6 protein, this confirms that there is a reproducible decrease in Ser240 phosphorylation

level following TOR inactivation. Accordingly it was previously shown in animals that AZD inhibits TOR, and as a consequence S6K activity and RPS6 phosphorylation (Chresta et al., 2010). TOR and S6K were previously shown to be activated by sugars like sucrose and glucose (Xiong et al., 2013). Consistently RPS6 phosphorylation was augmented by the addition of sucrose when supplied to sugar-starved *Arabidopsis* seedlings (Figure 7B). Next we examined the kinetic of changes in RPS6 phosphorylation after either AZD-8055 or sucrose treatments (Figure 8). As soon as 1 h after AZD addition, a significant decrease in Ser240 phosphorylation was observed (Figure 8A). For sucrose, an increase in phosphorylation was detected 2 h after supply. However it is difficult at this stage to discriminate between a direct signaling effect of sucrose and an indirect consequence of sugar metabolism (Figure 8B). Altogether these data suggest a strong positive correlation between the RPS6 phosphorylation level, as detected by this specific polyclonal

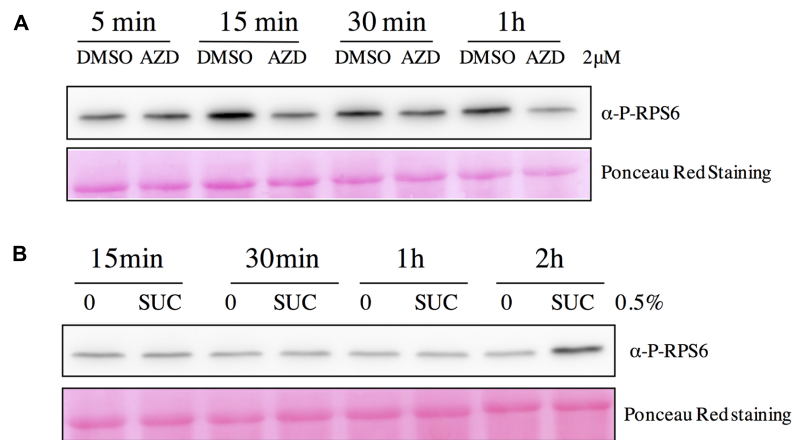


FIGURE 8 | Kinetics of variations in RPS6 phosphorylation following AZD-8055 or sucrose addition. Total protein extracts obtained from seedlings were separated by SDS-PAGE and blotted onto a membrane. After incubation with the phospho-specific antibody against RPS6 Ser240 (P-RPS6) blots were revealed by a secondary antibody linked to HRP activity and imaged with a CCD camera. **(A)** Kinetics of the inhibition of RPS6 Ser240 phosphorylation by AZD 8055. Seven day-old seedlings were either mock (DMSO) or AZD-8055 (2 μ M) treated and then harvested at the indicated time. **(B)** Kinetics of the induction of RPS6 Ser240 phosphorylation by sucrose. Seven day-old seedlings were transferred in sugar-free medium for 24 h and then either mock (0) or sucrose (0.5%) treated. Seedlings were harvested at the indicated time. Bottom panels show Ponceau Red staining of the membranes.

antibody, and TOR activity. Therefore, it seems that in plants, like in animals and yeast, RPS6 phosphorylation can be used as a robust and sensitive readout for TOR activity.

DISCUSSION

Several studies have investigated the impact of TOR inhibition in *Arabidopsis* on transcript or metabolite levels (Deprost et al., 2007; Moreau et al., 2012; Caldana et al., 2013; Xiong et al., 2013; Dong et al., 2015) but hitherto the global proteome has not been examined. In this paper, we investigated the expression of ribosomal proteins and genes using transcriptome, translome, proteome and phosphoproteome analyses following silencing of the TOR gene. Concerning the cytoplasmic ribosome, we identified in our proteomic experiments 65 families of ribosomal proteins, corresponding to 69 ribosomal protein isoforms identified with at least two proteotypic peptides. Hummel et al. (2015) identified 165 cRPs isoforms by LC-MS/MS after a tryptic digestion. We were able to find more than one third of these protein paralogs and only 16 families were not found in this analysis. Seven of these 16 families cannot be identified by LC-MS after a tryptic digestion mainly because of the small size of the resulting peptides due to their high content in lysine and arginine. Thus, only nine out of the 81 ribosomal protein families were missing in our analysis (RPL22, RPL35a, RPL38, RPP3, RPS27, RPS28, RPS29, RPS30, and RACK1). Moreover, this work has been focusing solely on young seedlings while Hummel et al. (2015) were also using rosettes in their analysis and some specific paralogs may be expressed only in specific tissues or at some specific developmental stages (Weijers et al., 2001; Sormani et al., 2011). Since we performed our proteomic analyses using plants silenced for TOR expression by a long ethanol treatment (3 and 10 days), our analysis of changes in protein abundance may reveal

steady-state long-term, and sometimes indirect, adaptation to a decrease in TOR activity.

In plastids translation occurs on 70S bacterial-type ribosomes. About half of the plastid small ribosome subunit (30S) proteins and most of the large subunit (50S) proteins are encoded by the nuclear genome (Yamaguchi and Subramanian, 2000). TOR silencing (Deprost et al., 2007; Xiong and Sheen, 2012; Caldana et al., 2013), inhibition by rapamycin (Sormani et al., 2007; Ren et al., 2011) or by AZD-8055 (Montané and Menand, 2013; Li et al., 2015) as well as mutations affecting the TORC1 complex (Moreau et al., 2012; Kravchenko et al., 2015) consistently result in leaf chlorosis and yellowing. We show here that TOR inhibition results in a coordinated decrease in pRP expression, at the level of protein abundance but also at the total and translated mRNA levels, which could explain these chlorotic phenotypes (Figure 3). Whether this is the result of a decreased synthesis or an increased degradation of the chloroplast components by autophagy, which is induced after TOR inactivation (Liu and Bassham, 2010), remains to be determined. Interestingly the expression of nuclear genes coding for cytosolic proteins was found to be mostly induced whereas the level of proteins often decreased. The same trends were observed in N-limited *Chlamydomonas* where the levels of pRPs as well as their corresponding mRNAs decreased in response to N starvation whereas only protein levels decreased for cRPs (Schmollinger et al., 2014). The same effect of TOR inactivation on the expression profile of the pRPs was confirmed by the comparison with transcriptomic data obtained using estradiol-inducible TOR RNAi line (Supplementary Figure S2) (Xiong et al., 2013). Interestingly, nuclear genes coding for pRPs were down-regulated in response to several abiotic or biotic stresses suggesting that they may play an important role in the adaptation to stresses (Supplementary Figure S2). These genes were also repressed by ABA but strongly induced by the application of

brassinolide. This is consistent with the inhibitory effects of ABA on the growth-promoting hormones like brassinosteroid and with the role of TOR in brassinosteroid (Zhang et al., 2009, 2016) and ABA signaling (Kravchenko et al., 2015; Li et al., 2015), which could result in TOR inhibition. As reported earlier by Pal et al. (2013) we found diverse and uncoordinated responses to variations in sugar supply for nuclear genes coding for pRPS while their expression was repressed by nitrogen starvation as observed in *Chlamydomonas* (Schmollinger et al., 2014). Altogether, these data suggest that nuclear genes coding for the pRPs are controlled by TOR at multiple levels to integrate environmental cues for the regulation of chloroplastic translation.

In animals TOR is known to regulate the translation of TOP-containing mRNAs (Hsieh et al., 2012; Thoreen et al., 2012; Meyuhas and Kahan, 2015). This motif is particularly present in the 5' UTR of genes coding for ribosomal proteins or components of the translation machinery (Meyuhas and Kahan, 2015). Using a MEME analysis we did not detect any specific enrichment for TOP motif in cRPs. The most abundant motif was related to the telo-box (**Figure 4A**). This DNA motif is found in the 5' regions, often close to the start codon, of nuclear genes coding for both mitochondrial and cytosolic RPs, but not in the genes encoding pRPs (Wang et al., 2011). Conversely, we found a highly significant occurrence of a pyrimidine-rich motif in the 5' UTR of nuclear genes coding for pRPs. This motif is reminiscent of TOP motifs (**Figure 4A**). A TOP-like motif was also previously identified in mRNA coding for ribosomal proteins in maize embryonic axes (Jiménez-López et al., 2011) but these motifs are so far poorly described in plants. Canonical TOP motifs are located at the start of the mRNA, which is not the case in our analysis. Nevertheless, a previous study has demonstrated the presence of several transcription start sites in genes coding for pRPs (Lagrange et al., 1993). Interestingly for the plastidic RPL21 gene, the start site specifically used in leaves produced a mRNA starting with a canonical TOP motif. The 5' UTRs of cRP genes which are less translated after TOR inactivation were enriched in a motif that is strikingly similar to the PRTE motif found within the 5' UTRs of animal genes controlled by TOR at the level of translation (**Figures 4C,D**) (Hsieh et al., 2012). It is thus tempting to hypothesize that TOR has been recruited in plants to regulate specifically in leaves the translation of nuclear mRNAs coding for chloroplastic ribosomal proteins. It was previously shown that translation of animal TOP-containing mRNAs can be differentially regulated *in vitro* in a wheat germ extract (Shama and Meyuhas, 1996) and that auxin stimulates S6 ribosomal protein phosphorylation on maize ribosomes and the recruitment of TOP-like mRNAs for translation (Beltrán-Peña et al., 2002). Since TOR is also activated by auxin (Schepetilnikov et al., 2013) these data suggest that plant mRNAs containing TOP-related motifs could also be regulated in a TOR- and phosphorylated S6-dependent manner.

Only phosphorylation of the RPS6 protein could be identified in a reproducible manner in previous unbiased phosphoproteomic analyses of the ribosomes performed in eukaryotes (Huber et al., 2009; Hsu et al., 2011; Yu et al., 2011). Nevertheless, despite a wealth of studies and several hypotheses,

the precise biological role of these conserved phosphorylation events remains disputed and unclear. For example, expression of human RPS6 containing alanine at all phosphorylated serine residues did not modify the overall translation rate, even for TOP mRNAs (Ruvinsky et al., 2005). Instead cell growth and size as well as ribosome biogenesis were affected (Ruvinsky et al., 2005; Chauvin et al., 2014). The same result was observed in yeast expressing non-phosphorylatable RPS6 (Yerlikaya et al., 2016). However, phosphorylation of the C-terminal Ser residues of RPS6 has been used as a robust and recognized readout for TOR activity in animals and yeast (Meyuhas, 2008, 2015; Yerlikaya et al., 2016). In this study, a decrease in RPS6 phosphorylation in response to TOR inactivation was observed (**Figures 5 and 7**). The phosphoproteomic analysis identified a C-terminal phosphorylation site in both the RPS6A and RPS6B proteins that is TOR activity-dependent without unambiguously determining which of the C-terminal serine residues is modified. In *Arabidopsis* Ser237 was previously identified by MALDI-TOF as being phosphorylated but the absence of fragmentation in the C-terminal region hindered the precise localization of the other modification sites by MS/MS analysis (Chang et al., 2005). Several phosphoproteomic studies of the plant ribosome have already shown the presence of a phosphorylation site in the C-terminal peptide of the RPS6 and have suggested that the Ser240 could be one of the modified residues together with Ser229, 231, or 237 (Carroll et al., 2008; Turkina et al., 2011; Boex-Fontvieille et al., 2013). A global phosphoproteome analysis of *Arabidopsis* identified Ser237 and 240 as being phosphorylated together with Ser247 and Thr249 (Reiland et al., 2009). Ser240 is conserved in all plant RPS6 sequences whereas Ser241 is missing in the maize and tobacco sequences (**Figure 6**). Conversely Ser237 is found in all plant sequences but only Ser240 can be aligned with one of the known phosphorylated serine in the yeast (Ser232) or human (Ser235) RPS6 sequence (**Figure 6**; Meyuhas, 2015). It is well known in yeast and animals that TOR activity controls RPS6 phosphorylation through activation of S6K (Wullschleger et al., 2006; Biever et al., 2015; Meyuhas, 2015) and Mahfouz et al. (2006) have established that TOR interacts with S6K through RAPTOR to activate RPS6 phosphorylation. Nevertheless it should be noted that S6K is also activated by the 3-phosphoinositide-dependent protein kinase 1 (PDK1) which operated after S6K phosphorylation by TOR (Mahfouz et al., 2006; Otterhag et al., 2006). Interestingly the SnRK1 kinase, which probably acts antagonistically to TOR (Dobrenel et al., 2016), was recently shown to interact with and phosphorylate RAPTOR in *Arabidopsis* (Nukarinen et al., 2016). Moreover, a strong increase in RPS6 Ser240 phosphorylation was also observed after SnRK1 inactivation. This is coherent with the hypothesis that SnRK1 inhibits TOR activity, and hence RPS6 phosphorylation, presumably through RAPTOR phosphorylation (Nukarinen et al., 2016).

Western blot assays using the RPS6 Ser240 phospho-specific antibody demonstrated that phosphorylation of this residue decreased following TOR inactivation either by silencing or by using a specific inhibitor (**Figure 7**). Therefore this assay could be used as a TOR readout in plants. Previous assays for TOR activity in plants were based on the detection of Thr449

phosphorylation in S6K by commercial antibodies directed against phosphorylated Thr389 in animal S6K. However, these antibodies produce many non-specific bands in Western blot assays (Schepetilnikov et al., 2011; Xiong and Sheen, 2012). Moreover the abundance of plant S6K is low in plants whereas RPS6 is present in large amounts.

Taken together these data show that the TOR-dependent C-terminal RPS6 phosphorylation is conserved in plants like in other eukaryotes. We have taken advantage of this conserved phosphorylation to design a sensitive and specific assay to monitor TOR activity in plants. The question that remains open is the biological role of RPS6 phosphorylation. Structural studies have shown that RPS6 is accessible to the solvent, and hence to kinases, but the disordered C-terminal region is unfortunately absent from the resolved ribosome structure (Khatter et al., 2015). Nevertheless the charge modifications produced by phosphorylation of RPS6 probably have important biological roles either within the ribosome or for extra-ribosomal functions of RPS6. Indeed it was recently reported that RPS6 affects ribosomal RNA production and interacts with the HD2B histone deacetylase (Kim et al., 2014). More work is therefore needed to elucidate the role of TOR in regulating translation or development through the conserved RPS6 phosphorylation.

AUTHOR CONTRIBUTIONS

TD, MS, MZ, CR, LR, JH, and CM conceived the research plan and supervised the experiments, TD, EM-M, CF, MA, MD, MM,

JC, and OL performed the experiments and analyzed the data, TD, CF, JH, CR, and CM wrote the article with contributions of all the authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.01611/full#supplementary-material>

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Auxin Signaling in Regulation of Plant Translation Reinitiation

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The mRNA translation machinery directs protein production, and thus cell growth, according to prevailing cellular and environmental conditions. The target of rapamycin (TOR) signaling pathway—a major growth-related pathway—plays a pivotal role in optimizing protein synthesis in mammals, while its deregulation triggers uncontrolled cell proliferation and the development of severe diseases. In plants, several signaling pathways sensitive to environmental changes, hormones, and pathogens have been implicated in post-transcriptional control, and thus far phytohormones have attracted most attention as TOR upstream regulators in plants. Recent data have suggested that the coordinated actions of the phytohormone auxin, Rho-like small GTPases (ROPs) from plants, and TOR signaling contribute to translation regulation of mRNAs that harbor upstream open reading frames (uORFs) within their 5'-untranslated regions (5'-UTRs). This review will summarize recent advances in translational regulation of a specific set of uORF-containing mRNAs that encode regulatory proteins—transcription factors, protein kinases and other cellular controllers—and how their control can impact plant growth and development.

Keywords: target of rapamycin TOR, small GTPases ROPs, S6K1, endosomes, signal transduction, translation-reinitiation

INTRODUCTION

Plant hormones (phytohormones) trigger complex growth and developmental processes. One of the most important plant growth regulators is auxin (from the Greek “auxein” meaning to enlarge/grow)—a small signaling molecule with great ability to induce growth responses throughout the plant life cycle. The auxin signaling pathway modulates diverse aspects of plant growth and development, such as responses to light and gravity, organ patterning, general root and shoot architecture and vascular development. Auxin elicits responses—cell division and expansion—depending on the cellular and developmental context in which it is perceived. The core components of auxin signaling differ in their expression patterns due to transcriptional and post-transcriptional regulation. Here, we review recent data describing auxin signaling in the cytoplasm of plant cells, and how auxin perception leads to activation of target of rapamycin (TOR), which promotes a protein synthesis pathway. In eukaryotes, TOR signaling is a key signaling pathway connecting environmental signal perception to growth decisions. Thus, TOR is a sensor that up-regulates cell growth and proliferation but also limits life span in yeast, mammals and plants. A hypothetical scheme linking auxin and TOR signaling with the G-protein (guanine nucleotide-binding proteins) family is described. This observation makes TOR an important part of the auxin signaling pathway that up-regulates translation, and, thus, plant growth and development.

It is clear that many environmental cues, such as nutrient and energy availability, instruct phytohormones to control plant growth, making it a very plastic process. We decided to travel along the recently discovered pathway from auxin to TOR via a small GTPase, ROP2, which ends by up-regulating production of critical effector proteins using a post-transcriptional mechanism via targeting of a specific translation initiation pathway: reinitiation.

AUXIN PERCEPTION AND SIGNALING

Auxin distribution is highly regulated in plants. Local auxin maxima and concentration gradients drive cell differentiation and embryogenesis. Auxin patterns form dynamically in response to environmental inputs (e.g., light and gravity). Thus auxin signal is converted into context-dependent developmental responses. Auxin perception is believed to be mediated by receptors that physically bind auxin, allowing it to travel from outside the cell into the cell cytoplasm, where it then initiates signal transduction cascades that trigger specific physiological auxin responses.

The best-characterized auxin pathway targets the nucleus (Mockaitis and Estelle, 2008), whereas the cytoplasmic role of auxin remains unexplored despite the existence of cytoplasmic auxin networks in the ER and plasma membranes (PMs) (Friml and Jones, 2010). The classical nuclear auxin signaling pathway relies on a molecular mechanism of action via auxin-dependent degradation of the transcriptional repressors Aux/IAA, which leads to gene activation outputs depending on the cellular spatio-temporal context. This degradation is dependent on the ubiquitin ligase Skp1-Cullin-F-box (SCF)^{TIR1} protein complex, where the associated F-box protein TIR1 confers target specificity (Lavy and Estelle, 2016). In the presence of auxin, the F-box protein TIR1 binds to Aux/IAA, resulting in the ubiquitination and degradation of the latter (Gray et al., 2001). By filling in a hydrophobic cavity at the protein interface, auxin enhances TIR1–substrate interactions by acting as a “molecular glue” (Tan et al., 2007). In this context, F-box protein TIR1 is a true auxin receptor, mediating transcriptional responses to auxin in plants (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). Each TIR receptor targets specific Aux/IAA proteins for degradation (Parry et al., 2009), thus switching on transcription of a multitude of genes, including auxin response factors (ARFs). The ARF transcription factors (23 members in *Arabidopsis*) contain DNA binding domains and interact specifically with tandem repeats, known as *Auxin-Responsive Elements* (AuxREs; Ulmasov et al., 1995, 1997a,b) that serve as either activators or repressors of transcription. ARFs regulate a multitude of critical steps in plant development by converting local auxin maxima into gene expression responses. Several ARF genes confer developmental phenotypes, and some possess interaction complexity and functional overlap. One example is ETTIN/ARF3, which is involved in establishment of organ polarity (Sessions et al., 1997; Garcia et al., 2006; Marin et al., 2010; Kelley et al., 2012). Recent data suggest the existence of a non-canonical direct

auxin effect on ETTIN without the ubiquitination and Aux/IAA-mediated degradation steps (Simonini et al., 2016), thus raising the question of whether alternative auxin pathways can exist. Another well-studied example of a transcription-activating ARF is MONOPTEROS/ARF5 (MP). Defects in MP result in aberrant seedling morphology, often with a single cotyledon and a loss of basal structures (Hardtke and Berleth, 1998). Current data indicate that ARF protein levels are regulated post-transcriptionally (Nishimura et al., 2005; Leyser, 2006; Zhou et al., 2010).

Auxin Binding Protein 1 (ABP1), which displays high affinity to chlorinated auxins (Reinard et al., 1998; Napier et al., 2002), was characterized as an auxin receptor and implicated in many aspects of growth and development, particularly mediating the fast, non-genomic effects of auxin (for a review, see Chen and Yang, 2014). Specifically, ABP1 was implicated in rapid cell surface-located auxin signaling as a sensor of cytosolic pH and K⁺ flux (Thiel et al., 1993; Gehring et al., 1998). Although ABP1 is a soluble auxin receptor, its partnering with membrane associated-receptor-like kinase TMK (transmembrane kinase) was proposed for perception of auxin and its travel to the cytoplasm (Xu et al., 2014). In 2015, however, several publications raised significant concerns about the role of ABP1 in both auxin signaling and *Arabidopsis* development (Gao et al., 2015; Strader and Zhao, 2016).

Since auxin efflux carriers bind auxin and promote its polar active transport (PAT) from cell to cell, it was suggested that the PIN-FORMED (PIN) family of auxin efflux carriers could be considered as auxin receptors (Hertel, 1995). PINs orchestrate polar cell-to-cell auxin transport via asymmetric subcellular concentrations. Moreover, PINs were implicated in the formation of auxin perception complexes when partnered with PID (PINOID) protein kinases (for a review, see Strader and Zhao, 2016). However, since PINs are not able to generate secondary messengers or the intermediate reactions required for signal transduction, this idea seemed to be non-productive. Interestingly, several PINs, including PIN5 and PIN8, are involved in cytoplasmic auxin trafficking, where PIN5 likely mediates auxin transport from the cytosol into the lumen of the ER (Mravec et al., 2009), and PIN8 from the ER to the cytosol (Ding et al., 2012). PIN8 is highly expressed during pollen development, and resides in the ER of pollen grains and germinated tubes (Bosco et al., 2012; Ding et al., 2012). Although PIN8 specific expression resulted in shorter root hairs likely due to auxin efflux activities that decrease accumulation of auxin, overexpression of PIN5 promotes root hair growth by increasing levels of internal auxin in the root hair cells (Ganguly et al., 2010). Therefore, both PIN5 and PIN8 can mediate auxin trafficking within the cytosol and the ER, but their output effects require further studies.

Auxin can alter plant development rapidly in response to different environmental stimuli acting at many diverse downstream target systems. In the cytoplasm, auxin is able to activate PM-associated ROPs (Rho-like GTPases from plants), which are involved in the regulation of endocytosis of auxin transport proteins and organization of the cytoskeleton (Tao et al., 2002). Although ROPs, as powerful signaling

molecules, coordinate many diverse signal transduction pathways, accumulating data suggest clearly defined crosstalk between auxin and ROP signaling (Tao et al., 2002; Xu et al., 2010; Schepetilnikov et al., 2017). ROP GTPases function as mediators of auxin-regulated gene expression, rapid PM auxin signaling, and directional auxin transport to link local auxin gradients with ROP regulation of cell polarity (for a review, see Wu et al., 2011). In *Arabidopsis*, ROPs are encoded by 11 genes that comprise a closely related, multigenic family that represents a subgroup of the Ras superfamily of small GTPases, and includes Rho, Rac, and Cdc42 subfamilies (Winge et al., 2000). Like other G-proteins, ROPs interact with their target proteins through conformation-specific states: a GTP-bound active state, a short-lived nucleotide free state, and a GDP-bound inactive state (Berken and Wittinghofer, 2008). ROPs efficiently bind GTP, but their hydrolysis activity depends on Rho GTPase-activating proteins RopGAPs (Berken et al., 2005; Gu et al., 2006; Berken and Wittinghofer, 2008). Plants contain a family of RhoGAPs that carries a conserved GAP-related domain and an N-terminal CRIB (Cdc42/Rac-interactive binding) motif that is involved in ROP binding (Wu et al., 2000), and REN1 (ROP1 ENHANCER1) protein, which, in addition to a GAP-related domain, carries an N-terminal pleckstrin homology (PH) domain (Hwang et al., 2008). Both RopGAPs have been shown to regulate ROP signaling (Wu et al., 2000; Klahre and Kost, 2006; Hwang et al., 2008). In contrast, guanine nucleotide exchange factors (RopGEFs) activate ROPs by promoting GDP-GTP recycling. The *Arabidopsis* genome contains a single ortholog of the mammalian DOC180 family protein, SPIKE1 (SPK1; Qiu et al., 2002), and 14 plant-specific RopGEF family members with the PRONE (plant-specific Rop nucleotide exchanger) domain required for GTP-GDP exchange (Berken et al., 2005; Gu et al., 2006). Several ROP downstream effectors—a family of CRIB-domain-containing proteins (RICs) that specifically interact with active GTP-bound ROPs—have been described in plants (Nagawa et al., 2010; Fehér and Lajkó, 2015). Although RICs are highly variable, their CRIB motifs are highly conserved (Nagawa et al., 2010), and the CRIB motif is used widely to estimate active ROP levels using a pull-down assay with a ROP-interactive CRIB motif-containing protein 1 (Ric1) that specifically targets activated forms of RAC/ROPs (Tao et al., 2002).

Although the role of the auxin signal transduction pathway in ROP signaling activation has been well documented, mechanisms and intermediate signaling components are not well known. Since RopGEFs are defined as molecules that activate ROPs, while RopGAPs prevents uncontrolled signaling of ROPs, both could be potential components of the auxin-ROP signaling axis. The PM localized receptor-like protein kinases (RLKs) play a critical role in transmission of extracellular signals to intracellular ROP signaling pathways, and function in regulation of fertilization and cell expansion mechanisms such as cell elongation, tip and hair growth (for a recent review, see Galindo-Trigo et al., 2016). We draw the reader's attention to the *Catharanthus roseus* RLK-1-like (CrRLK1L) protein kinase subfamily, which contains FERONIA (FER; Hématy and Höfte, 2008). FER specifically up-regulates ROP2 signaling activity through RopGEFs in *Arabidopsis* (Duan

et al., 2010); the FER and RopGEF-containing complex recruits an inactive form of ROP2 and converts it to an active form in a guanine nucleotide-responsive manner, while *fer* mutants accumulate the inactive (GDP) form of ROP2 (Duan et al., 2010). Moreover, it was suggested that a network of different RLKs, RhoGEFs, and ROPs can respond to diverse signals in various tissue and cell types (Schiller, 2006). Importantly, FER protein kinase interferes with several phytohormone pathways, including auxin signaling (Duan et al., 2010). Although auxin signaling stimulates root hair elongation (Pitts et al., 1998; Rahman et al., 2002), root hairs of *fer* mutants are not responsive to exogenous application of auxin (Duan et al., 2010). Taking into account that loss-of-function *fer* mutants are pleiotropic and display severe growth defects, FER is indispensable for plant growth and development (Duan et al., 2010). Future research will determine whether FER and RopGEFs function in ROP signaling control in an auxin-sensitive manner. Generally, RLKs can have broad functions in regulating cytoskeletal organization, vesicle trafficking and reactive oxygen species (ROS) production during plant growth (Wolf and Höfte, 2014).

TOR SIGNALING COMPLEXES AND THEIR UPSTREAM REGULATION

Cell growth requires protein synthesis—a process that consumes a huge amount of energy and therefore needs to be tightly regulated to keep a balance between cell demands and resources. Plants and animals share a common signaling pathway—the TOR pathway—connecting growth with environmental signal perception, where TOR accomplishes fine-tuning of the translational machinery, thus reprogramming translation rates in accordance with cellular needs. TOR operates as a hub in the signal transduction network that coordinates many critical molecular processes in eukaryotes, such as translation, proliferation, transcription, survival, aging, differentiation and autophagy, and is responsive to diverse signals, including nutrient and oxygen availability, energy sufficiency, stress, hormones, and growth factors. For two recent excellent reviews on the TOR signaling pathway in plants (see Barrada et al., 2015; Dobrenel et al., 2016).

Target of rapamycin belongs to the family of phosphatidylinositol kinase-related kinases (PIKKs), and is clearly related to PIK. However, TOR is atypical of PIK in that it appears not to phosphorylate lipid substrates, instead possessing a serine-threonine protein kinase activity. TOR was first described in yeast over 20 years ago as a target protein of the anti-fungal and immunosuppressant agent rapamycin (Heitman et al., 1991; Kunz et al., 1993). Rapamycin is a naturally occurring macrolide that acts as an allosteric inhibitor of TOR. Rapamycin forms a drug-receptor complex with the cellular peptidyl-prolyl *cis-trans* isomerase FKBP12, which, upon binding to TOR, inhibits its kinase activity (Sabatini et al., 1994; Choi et al., 1996). In contrast, most plants are insensitive to rapamycin-mediated inhibition of growth due to FKBP12, which is not efficient in rapamycin binding (Xu et al., 1998; Mahfouz et al., 2006; Sormani et al., 2007; Deng et al., 2016).

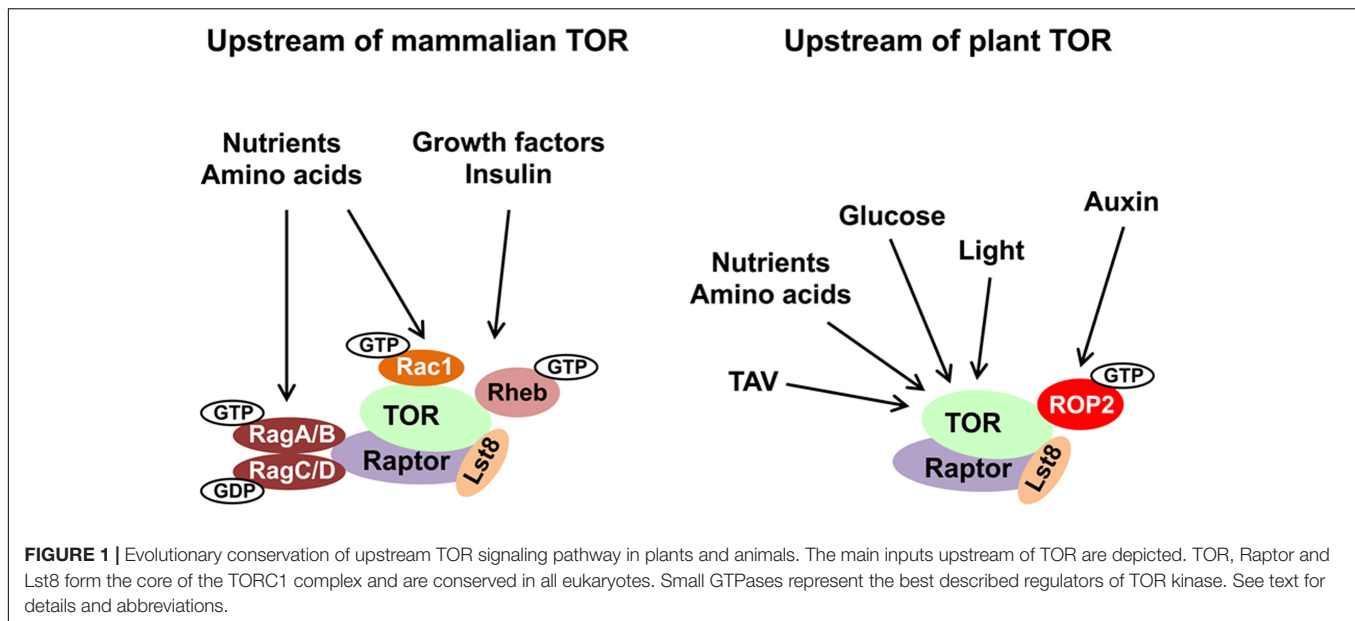
Mammalian TOR (mTOR) exists in two multiprotein complexes, mTORC1 and mTORC2, which differ in their composition, function, downstream substrates, and mode of action (direct or indirect) in many cellular processes. mTORC1 contains the TOR catalytic subunit, scaffold protein Raptor (regulatory associated protein of TOR), adaptor mLst8 (lethal with SEC13 protein 8) and regulatory protein DEPTOR (DEP domain-containing TOR-interacting). The mTORC2 complex—larger in size, with a molecular weight of about 1.4 MDa—contains TOR, scaffold protein Rictor (rapamycin-insensitive companion of TOR), mLst8, hSin1 (stress-activated protein kinase-interacting protein 1), PROTOR (protein observed with Rictor) and DEPTOR. mTORC1 is typically defined by a specific component, Raptor, and stimulates anabolic processes, including protein synthesis (Ma and Blenis, 2009), whereas mTORC2 contains Rictor and regulates cytoskeletal organization and survival (Hara et al., 2002; Kim et al., 2002; Loewith et al., 2002). mTORC2 is activated by the ribosome, where TORC2-ribosome interaction is a likely conserved mechanism that is physiologically relevant in both normal and cancer cells (Zinzalla et al., 2011). In addition, under most conditions, mTORC1 is sensitive to rapamycin, but mTORC2 is not (Loewith et al., 2002).

Plants depend greatly on signal perception by TOR (the *Arabidopsis* genome contains a single essential TOR gene; Menand et al., 2002; Deprout et al., 2007), which is required to adapt growth and development rapidly to changes in environmental inputs (Figure 1). The TOR pathway is a major growth regulator in plants. Previous research with transgenic *Arabidopsis* plants characterized by increased or decreased TOR cellular levels (Deprout et al., 2007) revealed a correlation between both root and shoot growth and TOR expression levels, thus confirming a role of TOR in growth regulation. Mutations in the TOR gene is lethal, and cause an early block in embryo development (Menand et al., 2002; Deprout et al., 2005). The *Arabidopsis* genome encodes two copies each of *Raptor* and *Lst8* genes. The *Arabidopsis* ortholog of Raptor contains HEAT repeats and WD40 domains responsible for protein–protein interactions, and serves as a binding partner of TOR in complex assembly (Anderson et al., 2005; Deprout et al., 2005). Lst8 consists of seven WD40 repeats, which form a propeller-like structure. Disruption of *Lst8* results in growth retardation phenotypes and extreme sensitivity to shifts in light conditions (Moreau et al., 2012). Recent data suggest that TOR signaling also affects cell wall biogenesis (Leiber et al., 2010) and negatively regulates autophagy in plants (Liu and Bassham, 2010; Zvereva et al., 2016)—a protein degradation process by which cells recycle cytoplasmic content under stress conditions or during senescence. The great enigma of plant TOR biology is the existence of a TORC2 complex, since no homologs of Rictor and Sin1 have been found in the genomes mono- or dicotyledonous plants to date. A search for TOR complex subunit paralogs revealed broad conservation, with a surprising lack of TORC2 in plants and some parasites (Dam et al., 2011; Dobrenel et al., 2016). Unlike TORC2, TORC1 shows a high degree of functional conservation in both multicellular plants and unicellular algae, as manifested by TOR protein–protein interaction experiments (Mahfouz et al., 2006; Díaz-Troya et al., 2008; Moreau et al., 2012).

In mammals, hormones and growth factors can directly activate mTOR signaling via phosphorylation of membrane-bound receptor kinases. Binding of insulin—a major energy control hormone—to receptor tyrosine kinase (RTK) triggers recruitment and phosphorylation of insulin receptor substrate (IRS) adaptors. IRSs activate phosphatidylinositol-3-kinase (PI3K) to generate phosphoinositol (3,4,5)-triphosphate (PIP₃) (Burke and Williams, 2015). PIP₃ binds pleckstrin homology (PH) domain and mediates the phosphoinositide-dependent kinase 1 (PDK1) and AKT kinase recruitment to the PIP₃-containing compartments in the PM (Pearce et al., 2010). PDK1-activated AKT phosphorylates TSC2 to inhibit the TSC complex by inducing its release from the lysosome (Alessi et al., 1997). The TSC complex functions as a GAP for Ras homolog enriched in brain (Rheb) small GTPase (Inoki et al., 2003). Rheb is located within the lysosomal compartment, where GTP-loaded Rheb activates mTORC1 via direct interaction with the catalytic domain of mTOR (Long et al., 2005). Availability of nutrients, in particularly amino acids, promotes mTORC1 activity via the conserved Rag family of small GTPases (González and Hall, 2017).

Due to their autotrophic lifestyle, plants lack several key upstream effectors of the TOR complex (e.g., TSC, AKT, and several classes of PI3K). In plants, the most critical environmental input comes from light energy, and suppression of TOR activity negatively affects light-energy-dependent growth (Ren et al., 2012). Upon nutrient deprivation conditions, TOR activity in plants is modulated via potential antagonistic crosstalk with SnRK1 kinase (sensor of cellular energy homeostasis) (Nukarinen et al., 2016); light and sugar signaling through TOR maintain the balance between hormone-promoted growth and carbon availability (Xiong et al., 2013; Dong et al., 2015). Active TOR promotes accumulation of the brassinosteroid-signaling transcription factor BZR1 in response to environmental signals and hormones (Zhang et al., 2016). Thus, TOR kinase represents an evolutionary conserved regulator of metabolism. In plants, disruption of the TOR signaling pathway affects sugar metabolism (Dobrenel et al., 2013). TOR senses and transduces photosynthesis-derived signals to specifically control root meristem proliferation. Glucose promotes primary root and root hair growth via the TOR pathway (Xiong and Sheen, 2012). Glucose-TOR signaling was implicated in transcriptional control of the cell cycle (Xiong et al., 2013).

Another integral part of the mammalian machinery that stimulates mTOR is phospholipase D (PLD) (Wiczner and Thomas, 2012). PLD enzymes harbor a phospholipid-binding Pox domain (PX) and catalyze the hydrolysis of phosphatidylcholine to phosphatidic acid (PA). PA is a metabolite and secondary lipid messenger, which regulates response to growth factors, stress and nutrients. In response to nutrients, PI3K generates PI3P species, which interact with the PX domain of PLD and promote production of PA. PA binds the FRB (FKBP12-rapamycin binding) regulatory domain of mTOR and displaces the DEPTOR subunit from mTOR to rapidly activate mTORC1 (Fang et al., 2001; Yoon et al., 2015). In plants, PLD mediates stress responses and signal transduction.



Plant TOR can be a key potential target of PA messengers produced by PLD. Changes in lipid composition and membrane integrity upon various abiotic stresses provoke PLD activity (Bargmann and Munnik, 2006). PA-mediated stomatal closure, root growth, tolerance to salinity and water deficits are the subjects of intensive research in plant science. In plants, PLD is induced by the stress hormone abscisic acid (Jacob et al., 1999). The involvement of PLD in ABA responses raises intriguing questions as to the potential role of abiotic stress and abscisic acid in TOR activation. Moreover, the TOR signaling pathway is involved in the regulation of ABA levels in *Arabidopsis* (Kravchenko et al., 2015). Strikingly, PLD and PA are required for auxin responses, providing hints of crosstalk between auxin and phosphatidylinositol signaling pathways (Li and Xue, 2007). There is now a growing body of evidence demonstrating that TOR acts as an essential factor for auxin signal transduction in *Arabidopsis* (Schepetilnikov et al., 2013; Dong et al., 2015; Deng et al., 2016). Auxin has been also identified as the cellular candidate for a role as an upstream TOR effector (Schepetilnikov et al., 2013). In response to auxin signaling, the TOR pathway is activated as manifested by phosphorylation of the 40S ribosomal S6 kinase 1 (S6K1; a direct downstream target of TOR) at TOR-responsive Thr449, and association of active TOR with polyribosomes. Recently, glucose and light signals as well as exogenously applied auxin were shown to activate S6K1, in shoot meristems (Li et al., 2017).

Phosphorylation is a common post-translational modification that indicates an active status of mTOR kinase. Only three phospho-sites have been reported to date in mTOR (Ser 1261, 2448, and 2481; Acosta-Jaquez et al., 2009). A Rheb-driven phosphorylation event at mTOR Ser1261 within the HEAT repeat domain promotes autokinase activity at Ser2481, resulting in mTOR activation, while the C-terminal Ser2448 is likely phosphorylated by S6K1 via a feedback loop. Mapping of orthologous phosphorylation sites in *Arabidopsis* reveals the high

conservation of mammalian Ser2448 and plant Ser2424 epitopes (Schepetilnikov et al., 2013). To date, Ser2424 is the only the TOR specific phospho-site with a confirmed biological function in auxin and ROP2 signaling (Schepetilnikov et al., 2013, 2017).

Many animal viruses have developed multiple mechanisms to activate mTOR signaling in favor of viral replication cycles. One such strategy results in stimulation of the PI3K-AKT pathway upstream of TOR kinase (for a review, see Walsh et al., 2013). The plant pararetrovirus, *Cauliflower mosaic virus* (CaMV), appears to be the first among plant and mammalian viruses known to trigger TOR activation (Schepetilnikov et al., 2011). Indeed, viral transactivator/viroplasm (TAV) protein binds TOR directly, triggering its activation and recruitment to polysomes. TAV represents a unique example of a pathogenicity effector that specifically targets a basal defense system of plants and suppresses innate immune responses to non-viral pathogens in a TOR-dependent manner (Zvereva et al., 2016).

SMALL GTPases CONTROL THE FUNCTION AND LOCALIZATION OF TOR COMPLEXES

The molecular mechanism of TOR activation is complex and diverse. Small GTPases emerge as the most significant direct upstream regulators of TOR complexes, and function as molecular switches, which, upon activation, interact with downstream effectors and stimulate multiple signaling pathways (Table 1). It is well established that yeast and mTOR are regulated by a plethora of small GTPases, including Rho, Rheb, Rag, Rac, Ral, Arf, and Rab, each responsible for perception of a unique type of stimulus. In mammals, small GTPases from the Rheb and Rag families are the two main direct upstream regulators of TOR complexes. Mammalian TORC1 is controlled primarily by Rheb GTPase. However, activation of mTORC1 in

TABLE 1 | Small GTPase regulators of TOR complexes.

GTPase	Function
Ras family (signal transduction)	
Rheb	Ras-homolog enriched in brain (Rheb) small GTPase is essential for mTORC1 activation. Rheb binds TOR directly. Rheb is not involved in TOR recruitment—this function is provided by Rags, which presents TOR to Rheb for proper activation. Rheb must be in a GTP-bound state to activate TOR (Long et al., 2005).
RalB	In response to nutrients, RalB GTPase activates mTORC1. RalB functions downstream of Rheb, suggesting cross-talk between amino acid-sensing signaling pathways (Maehama et al., 2008; Martin et al., 2014).
Rap1	In amoeba, Rap1 GTPase binds directly the regulatory subunit of Sin1 and activates TORC2 (Khanna et al., 2016).
RasC	In yeast and slime molds, RasC GTPase activates the TORC2 complex (Cai et al., 2010; Charest et al., 2010).
Rit1	In response to oxidative stress, Rit1 GTPase binds and activates mTORC2 (Cai and Andres, 2014).
Ras related family (signal transduction)	
RagA/B/C/D	Rag GTPases form a heterodimer RagA/B ^{GTP} and RagC/D ^{GDP} . Rags function as a central regulator of mTORC1 activation in response to amino acids via TOR complex recruitment to lysosomal membranes. Rags interact directly with the Raptor subunit of the mTORC1 complex. Proper GTP/GDP charging is crucial for their functioning (Kim et al., 2008; Sancak et al., 2008).
Rho family (signal transduction)	
Rac1	Rac1 activates both mTORC1 and mTORC2 complexes. Rac1 binds directly to TOR via its C-terminal positively charged lysine-rich motif. Rac1 recruits TOR complexes to the plasma membrane in a GTP-independent manner (Saci et al., 2011).
Rho1	In response to stress, Rho1 GTPase binds directly to the N-terminal domain of the Raptor subunit, resulting in inhibition of TORC1 activity. In yeast, Rho1 is a negative regulator of TOR activity. Rho1 disrupts membrane association of TORC1 (Yan et al., 2012; Yang et al., 2012).
ROP/RACs	In response to auxin, ROP2 GTPase binds directly to plant TOR via its C-terminal positively charged lysine-rich motif, which is similar to mammalian Rac1. GTP-bound active ROP2 recruits TOR complex on the membranes of early endosomes, which is similar to RAGs. ROP2-TOR interaction is indispensable of GTP/GDP charging, GTP is required for ROP2-mediated activation of TOR, which is similar to Rheb (Schepetilnikov et al., 2017).
Arf family (intracellular trafficking)	
Arf1	Arf1 activates mTORC1 on lysosomes, specifically in response to glutamine (Gln). GTP binding and hydrolysis by Arf1 is required for mTORC1 activation. Arf1 signaling to mTOR is specific to Gln and independent of ER-Golgi intracellular trafficking (Jewell et al., 2015).
Rab family (intracellular trafficking)	
Rab6	In yeast, Rhy1 GTPase, ortholog of mammalian Rab6, activates TORC2. In response to glucose, the GTP-bound active form of Rhy1 interacts physically with the TORC2 complex via its effector domain (Tatebe et al., 2010; Hatano et al., 2015).

response to amino acids requires GTPases of the Rag family (Sancak et al., 2008). Two heterodimeric Rag complexes (RagA/C and RagB/D) bind lysosomal membranes via a lysosomal adaptor RAGULATOR—a scaffold complex with GEF activity toward Rag GTPases (Bar-Peled et al., 2012). Amino acids promote the reciprocal charging of RagA/C and RagB/D with GTP and GDP, respectively, and their binding to mTORC1 via Raptor to relocate mTORC1 to lysosomes for mTORC1 presentation to GTP-bound Rheb (Kim et al., 2008; Sancak et al., 2008). Interestingly, Rac1, a member of the Rho family of small GTPases, affects signaling through both mTORC1 and mTORC2 complexes. Rac1 regulates TOR intracellular localization: upon serum stimulation, Rac1 binds mTOR directly via its C-terminal, lysine-rich motif in a GTP-independent manner and governs its movement from the perinuclear region to the PM (Saci et al., 2011). Thus, Rac1-mediated mTOR activation is independent of the PI3K-AKT-TSC axis. This is opposite to Rheb GTPase, which must be in the GTP-bound state to activate mTOR.

Several amino acids can stimulate mTORC1 in a Rag GTPase independent manner. Glutamine-mediated mTORC1 recruitment to lysosomes requires an alternative pathway via the Arf family GTPase Arf1, which is normally involved in intracellular vesicular trafficking, and vacuolar ATPase (v-ATPase) (Jewell et al., 2015). Several other small GTPases have been identified as indirect upstream actors in the TOR signaling pathway. In yeast, glucose activates TORC2 via the Rab family GTPase Rhy1 (Tatebe et al., 2010; Hatano et al., 2015). Moreover, a member of the Rho GTPase family, Rit, was suggested to bind directly to the hSin1 subunit and activate the mTORC2 complex in response to oxidative stress (Cai and Andres, 2014). Recent data suggest a cross-talk between GTPases RalB and Rheb in nutrient perception and mTORC1 control (Maehama et al., 2008; Martin et al., 2014). Among many small GTPases in yeast, Rho1 GTPase of the Rho family can negatively regulate mTORC1 under stress conditions. Rho1 GTPase is the master regulator of the yeast cell wall integrity (CWI) pathway that controls actin

polarization, cell morphogenesis, and cell wall expansion (Levin, 2005). In response to environmental or intracellular stresses, Rho1 binds directly to the Raptor subunit and inhibits mTORC1 activity (Yan et al., 2012; Yang et al., 2012).

In eukaryotes, small regulatory G domain proteins of Ras superfamily are divided into five main families based on their structure, sequence and function: Ran GTPases function in nuclear trafficking; Rab and Arf/Sar—in intracellular vesicular trafficking; Ras and Rho family members regulate signal transduction. ROPs of plants are structurally distinct from the proteins in the Rho, Rac, and Cdc42 subfamilies of Rho GTPases of other eukaryotes (Brembu et al., 2006), but were originally defined as RACs based on sequence similarity to animal Rac GTPases. Since plants lack orthologs of Rheb and Rag GTPases, the ROP/RACs are the only candidates for plant-specific TORC1 upstream regulators. Strikingly, ROP2 interacts directly with TOR both *in vivo* and *in vitro* in a manner independent of its GTP-bound state, but it activates TOR, when bound to GTP. Accordingly, *Arabidopsis* plants with high endogenous auxin levels, or *Arabidopsis* seedlings treated by auxin, or expressing high GTP-bound ROP2 levels, are characterized by increased TOR phosphorylation (Schepetilnikov et al., 2013, 2017). As expected, TOR phosphorylation in response to auxin is abolished in *rop2 rop6 ROP4 RNAi* plants (Schepetilnikov et al., 2017). As expected, *Arabidopsis* plants expressing constitutively active GTP-bound ROP2 (CA-ROP2 line) are more resistant to TOR inhibitors, and display a significant delay in AZD-8055-sensitive suppression of primary root growth and root hair elongation (Schepetilnikov et al., 2017), as normally occurs in WT seedlings in response to this TOR inhibitor (Montané and Menand, 2013). Similarly, active GTP-bound ROP2 triggered root hair elongation, but, in addition, ROP2 up-regulation promotes initiation of additional misplaced hairs (Jones et al., 2002).

In plants, as with most small GTPases, membrane association of ROPs is mediated by post-translational modifications, including prenylation and S-acylation (Hancock et al., 1989; Li et al., 1999; Fu et al., 2005, 2009; Sorek et al., 2011), similar to that shown for members of the Ras superfamily of small G-proteins (Michaelson et al., 2001). Recent research has revealed that ROPs 1–6 and mammalian Rac1 share a common sequence motif comprising several basic lysine residues that direct interaction with TOR (Saci et al., 2011; Schepetilnikov et al., 2017). The next important issue to be resolved is the intracellular compartmentalization of TOR upon auxin treatment. The PM operates as a platform for diverse receptor signaling and vesicle trafficking events. Active GTP-bound ROPs associate closely with the PM, which allows recruitment of ROPs from the cytoplasm (Sorek et al., 2011). ROP GTPases are not known to be localized in intracellular vesicles, suggesting rather a transient association with intracellular compartments or a unique redistribution in the PM. Since ROP2 interacts physically and functionally with TOR, it may participate in TOR relocation to the PM. Interestingly, ROP2 association with PM is indispensable for subsequent TOR activation—ROP2 GTPase lacking a prenylation domain is still capable of interacting with, but not activating, TOR (Schepetilnikov et al., 2017). Phosphorylated TOR accumulates in microsomal fractions of CA-ROP2 plants, and colocalizes

with endosomes in the cytoplasm in a ROP2-dependent manner. Note that the Lst8 subunit has been also found colocalized with endosomes (Moreau et al., 2012). TOR binding to endosomes is not sensitive to disruption of ER-to-Golgi intracellular vesicular trafficking, but may rely on the endocytic pathway. Primarily, ROP GTPases are considered to control cytoskeleton reorganization, thus interfering with vesicular trafficking. In response to auxin, GTPases of the ROP family coordinate the recycling of PINFORMED (PIN) transporters between the PM and endomembrane compartments (Chen and Friml, 2014). Accordingly, TOR may move to specific intracellular locations via interaction with appropriate subsets of small regulatory GTPases.

Many questions remain unanswered: what are the effects of ROP2 that increase intrinsic phosphorylation activity of TOR, and do other ROPs contribute to TOR activation? TOR complexes have been found at several subcellular locations, including the cytoplasm and the nucleus. Nevertheless, how TOR can mediate activation on lysosomes and be translocated to 40S preinitiation complexes (40S PIC) to regulate the cell translation machinery is still an open question. In addition, TOR is known to be localized in mitochondria, the PM and stress granules in response to different inputs (Betz and Hall, 2013). In the unicellular green alga *Chlamydomonas*, TOR activity is restricted to ER membranes (Díaz-Troya et al., 2008, 2011). Further work is obviously required to examine the intracellular location and trafficking of TOR, in both active and inactive states, and whether TOR activation takes place before or after its loading on endosomes.

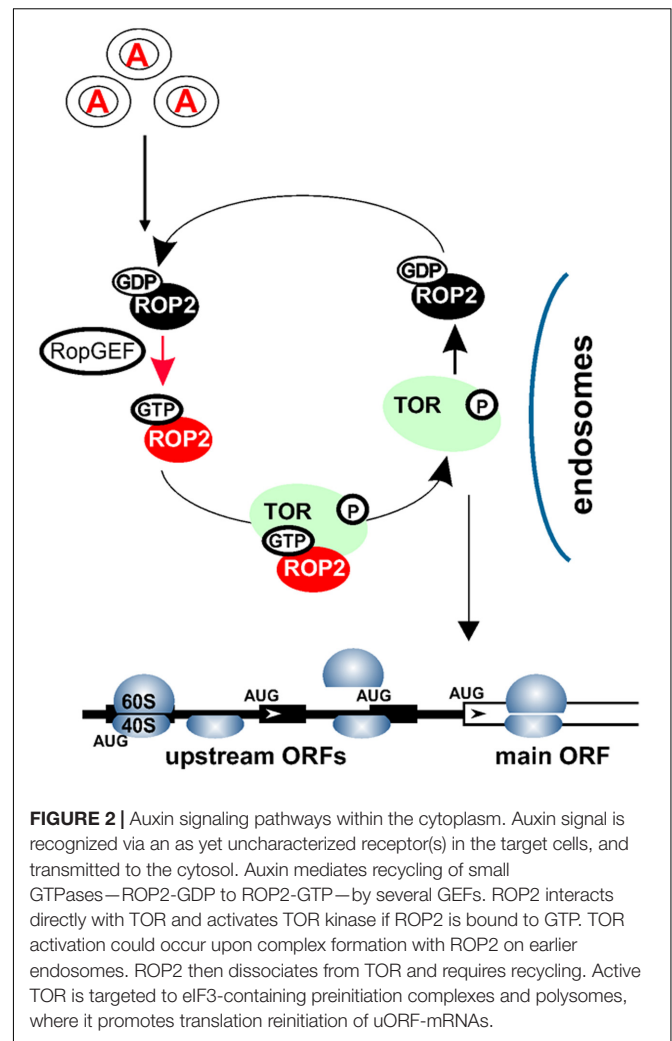
TOR PROMOTES TRANSLATION REINITIATION IN PLANTS

Plants are sessile organisms that continuously monitor and transduce environmental inputs into regulation of protein synthesis pathways. Indeed, much effort has been directed to demonstrate that translation of many mRNAs is affected by a multitude of environmental signals, for example, cold (Juntawong et al., 2013), heat (Matsuura et al., 2013), dehydration (Kawaguchi et al., 2004; Kawaguchi and Bailey-Serres, 2005; Park et al., 2012), salinity (Park et al., 2012), hypoxia (Branco-Price et al., 2005, 2008), and light (Khandal et al., 2009; Juntawong and Bailey-Serres, 2012; Floris et al., 2013). However, the underlying molecular mechanisms that affect protein synthesis efficiency are largely unknown and in need of further research. A recent study revealed that heat stress can rapidly induce an mRNA degradation process where involving LARPs (La and related Proteins) (Deragon and Bousquet-Antonelli, 2015). Strikingly, mammalian LARP1 was implicated in translation regulation of TOP (5'-terminal oligopyrimidine tract)-containing mRNAs under the control of TOR (Tcherkezian et al., 2014); however, whether translation of many plant TOP-containing mRNAs (Dobrenel et al., 2016) depends on TOR remains to be identified. Moreover, the contribution of TOR to the overall control of cap-dependent translation initiation via phosphorylation of eIF4E-binding proteins (4E-BPs)—the best

studied mechanism of translation control in response to stress in other eukaryotes (Siddiqui and Sonenberg, 2015)—has been questioned in plants due to the lack of data on plant 4E-BPs. A discussion of cap-dependent translation control in plants, including a key mechanism of down-regulation of translation by phosphorylation of eIF2 α , is beyond the scope of this review, and it is well described recently (Browning and Bailey-Serres, 2015).

Conversely, *Arabidopsis* plants silenced for TOR expression display significantly reduced polysomal abundance (Deprost et al., 2007), suggesting a role for TOR in plant translation. Additionally, it was reported that auxin signaling can affect translation, as manifested by phosphorylation of ribosomal protein S6 (RPS6) and up-regulation of polysomal levels in *Arabidopsis* suspension cultures (Beltrán-Peña et al., 2002; Turck et al., 2004). Accordingly, application of new generation TOR inhibitors, as well as existing TOR-deficient plants, has uncovered TOR function in the translation reinitiation of a specific pool of cellular mRNAs that harbor upstream open reading frames (uORFs) within their leader regions (uORF-mRNAs; Schepetilnikov et al., 2013). The current model suggests that TOR can receive signals from auxin via a small GTPase ROP2 to boost production of important regulatory proteins in a post-transcriptional manner by targeting a specific translation mechanism: reinitiation (Figure 2).

Upstream open reading frames are defined as 5'-UTR *cis*-elements of mRNAs defined by a start codon that is out-of-frame with the main ORF. Mounting data suggest the critical importance of post-transcriptional control via translation reinitiation (Roy et al., 2010; Schepetilnikov et al., 2013; von Arnim et al., 2014). Nowadays, uORFs are considered as prevalent translation repressors in eukaryotes (Johnstone et al., 2016). This is not surprising since more than 30% of eukaryotic mRNAs harbor relatively long leaders that contain multiple uORFs (Calvo et al., 2009). Among these are ARF family of transcription factors (von Arnim et al., 2014) and human tyrosine kinases (Wethmar et al., 2016); uORFs play a role of molecular switches in pathophysiology (Wethmar et al., 2010) and in stem cell regulation and organogenesis in plants (Zhou et al., 2014). To understand how translation reinitiation is controlled by upstream signals and contributes to overall protein synthesis, we first review briefly how uORFs can alter expression of the main ORF located downstream of the leader. The scanning model of eukaryotic translation initiation states that the 40S ribosomal subunit prebound by a multisubunit complex (eIF3, eIF1 and eIF1A, eIF5) and a ternary complex (TC, eIF2-GTP-Met-tRNA^{Met}) loads at the capped 5'-end of mRNA via eIF4F-bound to cap, scans in a 3'-direction until it recognizes an initiation codon in a suitable initiation context, where 60S joins and translation elongation begins (Browning and Bailey-Serres, 2015; Hinnebusch et al., 2016). The preceding translation event would negatively interfere with translation reinitiation at a downstream ORF, mainly due to loss of eIFs that have been recruited during the cap-dependent initiation event. It is generally accepted that reinitiation at the downstream AUG codon can occur, if (1) the initiation context of the 5' AUG codon is not optimal and is recognized only inefficiently by scanning ribosomes (leaky scanning mechanism—Kozak, 1986), and there



is no downstream secondary structure that would improve its recognition (Kozak, 1990); or (2) the initiation context is optimal, but is located in close proximity to the 5'-end of mRNA (Kozak, 1991); and (3) the preceding translation event was short (short uORF of 2 to ~30 codons) (Kozak, 1999). In the latter case, reinitiation is less efficient, but can be improved slightly by having a sufficiently long intercistronic distance between the uORF and the “main” ORF (Kozak, 1987; Luukkonen et al., 1995; Hinnebusch, 1997). The reinitiation potential of ribosomes depends on specific features of uORFs, as well as their amount and combination (von Arnim et al., 2014), and can be regulated by specific *trans*-acting factors (Rahmani et al., 2009; Medenbach et al., 2011).

Beside these features of uORFs, stalled translation of sequence-specific short uORFs can block translation reinitiation of a leader downstream ORF (Sachs and Geballe, 2006). Sequence-specific uORFs are common in genes involved in a variety of control mechanisms, and encode attenuator peptides that act in a sequence-dependent manner to inhibit its own translation termination, often through a delay of peptidyl-tRNA hydrolysis in response to saturating levels of a

regulatory signal, usually a metabolite. For example, a 48–55 codon uORF is responsible for the translational repression of the *SAMDC* (*S-ADENOSYLMETHIONINE DECARBOXYLASE*) gene in response to stress conditions and high polyamine levels (Hanfrey et al., 2005). Sequence-specific uORFs control the synthesis of AtbZIP transcription factors and several of their paralogs, as well as trehalose-6-phosphate phosphatase, in a manner sensitive to carbohydrates (Wiese et al., 2004; Hayden and Jorgensen, 2007). It seems certain that more short sequence-specific uORFs that would irreversibly abolish reinitiation of the main ORF translation in response to various regulatory signals or under certain conditions will be identified.

How does the 40S terminating subunit solve the problem of rapid loading of factors necessary for the reinitiation event, i.e., Met-tRNA^{Met} and 60S? The most likely explanation is that initiation factors that have been recruited during the cap-dependent initiation event dissociate from 40S gradually, and might remain associated with the translating ribosome for a few elongation cycles (Kozak, 1987). These reinitiation promoting factors (RPFs) could assist 40S ribosomal subunits to resume scanning, rapidly acquire TC and 60S *de novo* and thus stay reinitiation-competent. A new study using *in vivo* RNA-protein Ni²⁺-pull down assay directly demonstrated that eukaryotic initiation factor 3 (eIF3) physically associates with early elongating ribosomes on the GCN4 mRNA (Mohammad et al., 2017). eIF3 is composed of 13 distinct subunits in humans and plants, and facilitates rapid recruitment of TC to 40S and assembly of the 43S preinitiation complex on mRNA (Burks et al., 2001; Hinnebusch, 2006). Mammalian RPFs include, in addition to eIF3 (Cuchalová et al., 2010; Munzarová et al., 2011), the cap-binding complex eIF4F (Pöry et al., 2004).

In plants, eIF3 non-core subunit h (eIF3h) greatly elevates the reinitiation competence of mRNAs coding for the *Arabidopsis* basic zipper transcription factors (bZIPs) and several ARFs (Roy et al., 2010; Zhou et al., 2010); while the 60S ribosomal protein L24B (RPL24B), which is encoded by *SHORT VALVE1*, lifts translation of uORF-containing mRNAs that encode ARF3 (ETTIN) and ARF5 (MONOPTEROS; Nishimura et al., 2005; Zhou et al., 2010). Thanks to a mutant allele of *eif3h-1* carrying a C-terminally truncated eIF3h and a short valve1 (*stv1*) mutant lacking the RPL24-encoding gene, it was demonstrated that both mutants display similar defects in auxin-mediated organogenesis and undertranslate uORF-containing *bZip11* and several ARF mRNAs (Zhou et al., 2010). Although eIF3h can be dispensable for cap-dependent translation initiation (Kim et al., 2004; Roy et al., 2010), a global analysis of ribosomal loading confirmed that many mRNAs containing uORFs are less abundant in polysomes in the *eif3h-1* mutant (Tiruneh et al., 2013), thus confirming that translation of the majority of uORF-containing mRNAs depends on eIF3h. Future studies will clarify the mechanism of eIF3h function in reinitiation of translation.

Taken together, translation of mRNAs with several short uORFs is still possible, albeit with lowered efficiency, while reinitiation after long ORF translation is largely prohibited in eukaryotes. However, viruses often break basic cellular rules.

Indeed, there are a few abnormal cases of reinitiation after long ORF translation, best studied in mammalian caliciviruses (Royall and Locker, 2016) and plant caulimoviruses (Ryabova et al., 2006). The subgenomic mRNA of caliciviruses is bicistronic, with two long ORFs that encode structural proteins VP1 and VP2 overlapping by four nucleotides, and its translation relies on a termination-dependent reinitiation strategy, where expression of the downstream cistron is dependent on the ribosome binding site (TURBS) within the upstream VP2 ORF located close to VP1 ORF stop codon. The motif was shown to bind 40S ribosomal subunits and eIF3 (Luttermann and Meyers, 2007; Pöry et al., 2007). Thus, in caliciviruses, the ribosome might be held at the stop/restart region by base pairing of TURBS with the 18S rRNA (Luttermann and Meyers, 2009; Zinoviev et al., 2015), and can be further stabilized by binding of eIF3 to promote reinitiation by post-terminating 80S ribosomes (Pöry et al., 2007).

The second unique example of reinitiation after long ORF translation comes from CaMV, where reinitiation critically depends on a single viral protein TAV (De Tapia et al., 1993). To promote reinitiation, TAV interacts with the host translation machinery via eIF3 (Park et al., 2001), reinitiation supporting protein (RISP; Thiébeauld et al., 2009), and TOR, where TAV activates TOR via an as yet unknown mechanism (Schepetilnikov et al., 2011). According to the current model, TAV is responsible for retention of RPFs on translating ribosomes during the long elongation event, thus increasing the reinitiation competence of ribosomes. Indeed, sucrose gradient analysis of extracts isolated from *Arabidopsis* plants transgenic for TAV revealed greatly increased accumulation of eIF3, RISP, and TOR in addition to TAV in polysomes as compared with WT plants (Thiébeauld et al., 2009). Moreover, TAV function in reinitiation is strongly dependent on active TOR (Schepetilnikov et al., 2011). RISP appears to be a specific target of TOR/S6K1 signaling, and its phosphorylation promotes both its binding to TAV and TAV function in translation reinitiation. Indeed, TOR and RISP binding to polyribosomes correlates with RISP phosphorylation, while phosphorylation of RISP is abolished in polysomes isolated from plants transgenic for a TAV deletion mutant that failed to associate and thus activate TOR (Schepetilnikov et al., 2011). In conclusion, it was proposed that TOR function in polysomes would be to maintain the high phosphorylation status of RISP, and possibly other RPFs, to promote viral pathogenesis.

In mammals, eIF3 was identified as a platform for phosphorylation of S6K1 by TOR, where active mTOR or inactive mS6K1 enter the cell translation machinery via interaction with the eIF3-containing preinitiation complex in a dynamic order of events (Holz et al., 2005). Although the eIF3 complex is prebound by inactive mS6K1 in mTOR inactivation conditions, binding of mTOR, when it is activated, results in phosphorylation and dissociation of mS6K1. In yeast, TORC2 was detected in polysomes, where it maintains co-translational phosphorylation of Akt kinase (Oh et al., 2010). In *Arabidopsis*, TOR, when active, associates with polysomes also prebound by inactive S6K1, phosphorylates S6K1, triggering its dissociation (Schepetilnikov et al., 2011, 2013). Further phosphorylation of S6K1 may involve PDK1 (Deak et al., 1999).

In plants, active TOR accumulates mainly within 40S preinitiation complexes, and at significantly lowered levels in polysomes, which can explain the low reinitiation capacity of *Arabidopsis* plants. Although partial depletion of TOR revealed defects in polysomal loading of uORF-containing mRNAs that require reinitiation; TOR, when up-regulated in response to either auxin or by GTP-ROP2, promotes polysomal loading and translation of *ARF3*, *ARF5*, *bZIP11*, and other uORF-containing mRNAs (Schepetilnikov et al., 2013, 2017). Active TOR can up-regulate translation reinitiation via phosphorylation of the plant reinitiation factor eIF3h in polysomes to maintain the high phosphorylation status of eIF3h-promoting reinitiation events (Schepetilnikov et al., 2013). A new study has identified mTORC1 as the key factor contributing to translation of uORF-mRNA that encodes ATF4, a member of the CREB/ATF family of bZIP transcription factors, where TOR may regulate ATF4 mRNA translation through a uORF-dependent mechanism and 4E-BPs (Park et al., 2017).

Translation/reinitiation events within *bZIP11*, *ARF3*, and *ARF5* 5'-UTRs impede or block ribosomal movement toward the main ORF, causing inefficient translation of uORF-mRNAs under WT conditions (Zhou et al., 2010). Indeed, it was shown that uORFs downregulate main ORF translation for *ARF5* by 15-fold, *ARF3* by 2-fold (Zhou et al., 2010), and *bZIP11* by 4-fold, if only uORFs 1, 2, and 4 are removed (Kim et al., 2004). Accordingly, polysomes isolated from WT *Arabidopsis* are deficient in loading of active TOR and *bZIP11*, *ARF3* and *ARF5* uORF-mRNAs, and their levels are not much higher than in plants grown on medium containing the TOR inhibitor AZD-8055 (Schepetilnikov et al., 2013, 2017). Here, the classic means of determining whether up-regulation of gene expression at the translational level has occurred on mRNAs via a shift of these mRNAs into the polysomal fraction is not easily applied to mRNAs that carry multiple uORFs within their long leader regions that would require reinitiation events. Indeed, the increased abundance of initiating/reinitiating 40S, and likely uORF-translating 80S, within their long leaders shifts these mRNAs toward 80S or even light polysomal fractions, even when translation of the main ORF is strongly inhibited, depending on the number and arrangement of uORFs (Schepetilnikov et al., 2017). Upon introduction of TOR-activated conditions, TOR phosphorylation, and, consequently, uORF-mRNA loading into polysomes is increased (Schepetilnikov et al., 2017). Strikingly, studies of mRNA abundance across sucrose gradients in WT versus *CA-ROP2* plants (Schepetilnikov et al., 2017) revealed a high proportion of uORF-mRNA (about 64–80%) sedimenting to the top fraction of the gradient in WT conditions, while only 20–25% of uORF-mRNA remained in the top gradient fraction in *CA-ROP2* conditions, regardless of the fact that total transcript levels did not differ significantly between WT and *CA-ROP2* extracts. These data correlate with the high translation efficiencies of uORF-mRNAs in plant mesophyll

protoplasts prepared from plants expressing high active TOR levels.

TOR up-regulation of reinitiation events could be as harmful in plants as in mammals, where up-regulation of the protein synthesizing machinery contributes to the development of cancer (Ruggero and Pandolfi, 2003). In the opposite situation of reinitiation defects, the developmental abnormalities identified in *rpl24b* and *eif3h-1* mutants are largely similar to auxin-related developmental defects (Zhou et al., 2010). Further investigation is needed to understand the roles of ROP2 in TOR activation, as well as to identify other upstream TOR effectors in plants and their roles in translation.

CONCLUSION AND PERSPECTIVES

The last 10 years have witnessed striking advances and rapidly emerging data on the composition of the TOR complex, the TOR pathway, and its function and control in plants, in part due to the appearance of a new generation of TOR inhibitors that bind to the TOR kinase domain within the ATP-binding pocket and inactivate TOR (Chresta et al., 2010; Montané and Menand, 2013). Many critical questions remain unanswered. Recent work has revealed the role of TOR in sensing environmental conditions, including various stresses and phytohormones, but the molecular mechanisms underlying these signaling events remain unknown. It is not yet known whether, and how, TOR controls general translation by sensing amino acid levels. Finally, a key issue is the existence of functional ortholog of TORC2 in plants. Recent data have revealed that the molecular composition of the TOR complex varies in different cell types. Identification of a novel binding partner of TOR—GIT1 (G-protein-coupled receptor kinase-interacting protein 1)—suggested a unique mTOR complex lacking both Raptor and Rictor (Smithson and Gutmann, 2016). Therefore plants can contain more than one functional TOR complex. A challenge for future studies in plants will be to elucidate further TOR signaling pathways in plant translation, and to reveal how TOR can control mRNA translation at the initiation step.

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All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Regulation of Translation by TOR, eIF4E and eIF2 α in Plants: Current Knowledge, Challenges and Future Perspectives

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An important step in eukaryotic gene expression is the synthesis of proteins from mRNA, a process classically divided into three stages, initiation, elongation, and termination. Translation is a precisely regulated and conserved process in eukaryotes. The presence of plant-specific translation initiation factors and the lack of well-known translational regulatory pathways in this kingdom nonetheless indicate how a globally conserved process can diversify among organisms. The control of protein translation is a central aspect of plant development and adaptation to environmental stress, but the mechanisms are still poorly understood. Here we discuss current knowledge of the principal mechanisms that regulate translation initiation in plants, with special attention to the singularities of this eukaryotic kingdom. In addition, we highlight the major recent breakthroughs in the field and the main challenges to address in the coming years.

Keywords: translation initiation, stress, plant development, TOR, eIF2 α , eIF4E

INTRODUCTION

In eukaryotes, canonical cap-dependent translation begins with eIF4E recognition of the cap structure (7-methyl guanosine) at the 5'-end of the mRNA and formation of the eIF4F complex. Within this complex, eIF4G interacts with several factors, allowing mRNA recircularization and recruitment of the preinitiation complex 43S (PIC) to the mRNA. Once loaded, this complex, which consists of the small ribosomal subunit 40S, the ternary complex eIF2/GTP/tRNA_i^{met} and the factors eIF3, eIF1, and eIF1A, scans the mRNA in the 5'-3' direction until an initiation codon is found. At that point, the ribosomal subunit 60S is loaded and the elongation phase begins (Jackson et al., 2010; Hinnebusch et al., 2016).

Regulation of protein synthesis is a widespread, dynamic mechanism that controls gene expression in eukaryotes. This regulation takes place mainly, but not exclusively, during the translation initiation phase and involves the regulation of the activity of the master kinase target of rapamycin (TOR) and two important translation initiation factors, eIF4E and eIF2 α (Chu et al., 2013; Hinnebusch et al., 2016). Although regulation of these three main players has been studied profusely in other eukaryotes, the information available as to how these proteins regulate translation in plants is very limited. This review focuses on specific aspects of their involvement in translation initiation in plants, introducing what is known in other organisms, what we know about their regulation in plants, and how this regulation impinges on specific aspects of plant development and response to environmental cues.

THE TOR SIGNALING CASCADE

The TOR protein kinase is a central regulator of growth in response to nutrients in eukaryotic cells. The importance of this signaling pathway is shown by the large number of papers published annually (see Dobrenel et al., 2016a; Eltschinger and Loewith, 2016; Gonzalez and Hall, 2017 for recent reviews), and the celebration of the 25-year anniversary of its discovery (Hall, 2016). The two yeast TOR kinase genes were first identified during a screen designed to seek the targets of the antiproliferative drug rapamycin (Heitman et al., 1991). Soon after, it was observed that translation initiation was altered in yeast TOR mutants (Barbet et al., 1996). It is now well established that TOR integrates the signals that perceive the nutritional status of the cell and regulates downstream processes essential for proliferation and growth. These include the ability to modulate translation initiation (Ma and Blenis, 2009; Thoreen et al., 2012; Nandagopal and Roux, 2015), maintenance of lysosome identity (Yu et al., 2010; Munson et al., 2015), autophagy (Noda and Ohsumi, 1998), and synthesis of ribosomes and tRNAs (Ma and Blenis, 2009). Whereas yeast has two TOR proteins, some filamentous fungi, animals, and plants have only one (Franceschetti et al., 2011; Dobrenel et al., 2016a; Eltschinger and Loewith, 2016). Yeast and mammalian TOR proteins form two widely conserved multiprotein complexes that differ structurally and functionally, TORC1 and TORC2; only TORC1 is rapamycin-sensitive (Eltchinger and Loewith, 2016). The ability of these TORC complexes to interact with specific protein partners controls the diverse downstream outputs of the TOR cascade.

In plants, analysis of the TOR pathway has been a challenge because of the embryo lethality of knockout TOR mutants (Menand et al., 2002), and the less reliable rapamycin sensitivity of these eukaryotes (Rexin et al., 2015; Dobrenel et al., 2016a). The latter is due to the differences in amino acid residues in the 12 kDa FK506-binding proteins (FKBP12) of plants. The generation of TOR inducible mutants and silenced lines (Deprost et al., 2007; Caldana et al., 2013; Dobrenel et al., 2016b), the introduction of the yeast FKBP12 in Arabidopsis, which increases rapamycin sensitivity (Sormani et al., 2007), together with newly developed drugs that target TOR such as Torin and AZD-8055, have provided tools to dissect the role of TOR in plants. Although all TORC1 components are present, no clear orthologs of the TORC2 subunits AVO1 and AVO3 have yet been found in plants (Robaglia et al., 2012; Maegawa et al., 2015).

TORC1 AND THE CONTROL OF TRANSLATION INITIATION

In yeast and animals, TORC1 activation by nutrient signals coordinately controls various components of the translation initiation machinery by direct or indirect phosphorylation of a subset of proteins (Ma and Blenis, 2009). These include the translation initiation factors eIF4G, eIF4B, 4E-BPs, and the 40S ribosomal S6 kinases (S6K1 and S6K2). Furthermore, TORC1 can control general protein synthesis and the selective

translation of specific mRNAs, including those with 5' terminal oligopyrimidine (TOP) tracts (Thoreen et al., 2012). These TOP mRNAs encode ribosomal and other proteins that control translation. Although the mechanism by which these mTOR-dependent mRNAs are selected is not yet clear, several features of their 5' UTR has allowed their classification in two functional subsets of transcripts whose translation initiation is regulated differently (Gandin et al., 2016). Not only nutrient starvation, but also other stresses can modulate the TOR cascade and canonical translation initiation, as shown during hypoxia, when mTOR inactivation reduces the translation of several TOP mRNAs and overall protein biosynthesis (Spriggs et al., 2010).

Plant lines in which TOR is silenced have reduced polysomal content (Deprost et al., 2007), an observation that supports TOR involvement in translation regulation. TOR also reduces the transcription and translation rates of nuclear genes that encode plastidic ribosomal proteins, suggesting protein synthesis defects in chloroplasts. This correlates with the chlorotic phenotype observed in TOR-silenced plants (Dobrenel et al., 2016b).

Arabidopsis S6K conserves the main regulatory phosphorylation sites found in human S6K (Dobrenel et al., 2016a), and phosphorylation of S6K1 has been used to monitor TORC1 activity in plants (Xiong and Sheen, 2011). This is an important TOR effect, since the S6K pathway not only stimulates overall protein synthesis but also eIF3h-mediated translation reinitiation after an upstream open reading frame (uORF) (Schepetilnikov et al., 2013), a frequent feature found in plant mRNAs (von Arnim et al., 2014). Auxin treatment activates TOR in Arabidopsis seedlings, and stimulates TOR association with polysomes, where S6K1 is phosphorylated (Bogre et al., 2013; Schepetilnikov et al., 2013). The loading of the translation initiation factor eIF3h into polysomes in response to auxin is impaired in TOR-deficient mutants with an inactive S6K form, suggesting that eIF3h is possibly phosphorylated by the TOR/S6K1 pathway (Schepetilnikov et al., 2013). With respect to environmental pressures, TOR activity can modulate the plant response to osmotic stress through the S6K1 kinase pathway (Mahfouz et al., 2006; Deprost et al., 2007). Other evidence pointing to TOR participation in plant adaptation derives from the induction of the two S6K gene homologs in Arabidopsis by cold and salinity (Mizoguchi et al., 1995). Although the S6K pathway is conserved in plants, clear orthologs of the other main target of TOR, the eIF4E-binding proteins (4E-BPs), have not been identified in this kingdom (see below for details).

REGULATION OF eIF4E ACTIVITY IN ANIMALS BY ITS ASSOCIATION TO DIFFERENT PROTEINS

In animals, eIF4E translational activity is tightly regulated by a myriad of proteins that regulate eIF4E function by phosphorylation (Waskiewicz et al., 1997) or by binding directly to eIF4E. These latter proteins, which are one of the focus of this review, modulate general and specific translation.

Probably the best-known eIF4E translational regulators are the mammalian 4E-BPs (Lin et al., 1994; Pause et al., 1994; Poulin et al., 1998). These proteins interact with eIF4E through multiple contacts to the lateral and dorsal surface of eIF4E (Paku et al., 2012; Lukhele et al., 2013; Peter et al., 2015). The dorsal interaction comprises the so-called 4E-binding motif (4E-BM), a canonical sequence YXXXXLØ (where Ø denotes a hydrophobic amino acid) present in 4E-BPs. Since the same motif is used by eIF4G for eIF4E binding (Mader et al., 1995; Marcotrigiano et al., 1999), the output of 4E-BPs/eIF4E interaction is the displacement of eIF4G from the eIF4E-eIF4G complex, which leads to general inhibition of mRNA translation (Haghighat et al., 1995). 4E-BPs interaction with eIF4E is intimately coupled to their phosphorylation status, which is controlled and adapted to physiological conditions through the master kinase TOR (Nandagopal and Roux, 2015). After TOR phosphorylation, mammalian 4E-BPs dissociate from eIF4E, whereas in their hypophosphorylated state, 4E-BPs form a tight complex with eIF4E (Pause et al., 1994).

Along with the 4E-BPs, other proteins known as 4E-interacting partners associate to eIF4E through canonical 4E-BM or similar structures (Napoli et al., 2008). In general terms, these proteins support multiple protein-protein interactions that create bridges between the 5' and 3' UTR of specific mRNAs, rendering them inactive for translation (Wells, 2006; Rhoads, 2009). Although these eIF4E interactors control specific animal developmental programs, such a mechanism has not been found yet in plants.

In addition to these translational regulators, several eIF4E interactors were recently implicated in eIF4E-dependent mRNA export and degradation (Nishimura et al., 2015; Osborne and Borden, 2015). Some of these proteins, such as LRPPRC, PRH, or 4E-T, interact with eIF4E through the canonical 4E-BM (Dostie et al., 2000; Topisirovic et al., 2003, 2009), which highlights the importance of this domain in eIF4E binding and regulation.

REGULATION of eIF4E ACTIVITY BY ITS ASSOCIATION TO DIFFERENT PROTEINS IN PLANTS

As described above, the most common and powerful tool for regulation of eIF4E activity in animals is protein association to the dorsal surface of eIF4E. For this reason, it is surprising that despite the conservation of the amino acids involved in the eIF4E/eIF4G interaction and the precise regulation of translation in different developmental and environmental conditions, no clear homologs of these eIF4E regulators have yet been found in plants. This is especially surprising for 4E-BPs, which appear to have been conserved throughout the evolution of many eukaryotic species, but specifically lost in plants (Hernandez et al., 2010).

Besides the lack of plant orthologs for the 4E-BPs and eIF4E-interacting partners, the existence of proteins that regulate eIF4E activity through eIF4E association remains an open question. Different studies reported the identification of proteins

bearing the consensus 4E-BM that bind eIF4E and eIFiso4E (Freire et al., 2000; Freire, 2005; Lázaro-Mixteco and Dinkova, 2012), although their role in translation has yet to be elucidated. Apart from these proteins, an Arabidopsis database search retrieves more than 6900 proteins that contain one or more canonical eIF4E-binding domains (YXXXXLØ) (Toribio et al., 2016), that therefore might bind eIF4E and regulate its function. The number of possible plant eIF4E interactors could be larger if we consider that the canonical domain can have variations at the 3' end and that some structures like the reversed L-shaped motif can also promote eIF4E binding (Napoli et al., 2008). Other evidence that supports the existence of these eIF4E regulators are the presence of conserved RNA-binding proteins in plants as the case of Brn, which mediates eIF4E translational inhibition of targeted mRNAs in animals (Kim et al., 2013).

It is worth to mention that wheat eIF4E and eIFiso4E show different isoelectric isoforms that are compatible with changes in their phosphorylation state (Gallie et al., 1997). Although the kinases involved have not been identified, the existence of different isoelectric states opens the possibility that these modifications could regulate translation initiation during plant development and/or in response to environmental cues.

TRANSLATION REGULATION BY THE INITIATION FACTOR eIF2 α

Inhibition of canonical translation by eIF2 α phosphorylation has been analyzed exhaustively (Hinnebusch et al., 2016). Studies in yeast and other eukaryotes showed the eIF2 function in formation of the ternary complex Met-tRNA_i^{Met}-eIF2-GTP, needed to couple the initiating Met-tRNA_i^{Met} at the first AUG in the 5' leader of mRNAs. The resulting eIF2-GDP complex is recycled by eIF2B to eIF2-GTP, which binds a new molecule of Met-tRNA_i^{Met} and forms a new ternary complex to initiate translation. eIF2, one of the best-characterized translation initiation factors, is composed of three subunits, eIF2 α , eIF2 β , and eIF2 γ . Phosphorylation of the conserved Ser51 residue in the eIF2 α subunit inhibits eIF2B dissociation from the eIF2-GDP complex and thus, formation of a new ternary complex, whose depletion arrests initiation of protein synthesis. Phosphorylation of eIF2 α is a key mechanism that controls mRNA translation in eukaryotes in response to stress. In yeast, the general control non-derepressible 2 (GCN2) kinase phosphorylates eIF2 α during nutrient starvation. GCN2 is part of a complex also comprised of GCN1 and GCN20 proteins, necessary to trigger eIF2 α phosphorylation (Hinnebusch, 2005; Castilho et al., 2014). In mammals, protein kinases in addition to GCN2 phosphorylate eIF2 α in various stress conditions including nutrient starvation, protein misfolding, or immune responses (Harding and Ron, 2002; Baker et al., 2012; Donnelly et al., 2013).

In Arabidopsis, GCN2 mediates eIF2 α phosphorylation after stress treatments such as UV light, amino acid starvation, cadmium, oxidative stress, and wounding, and it is so far the

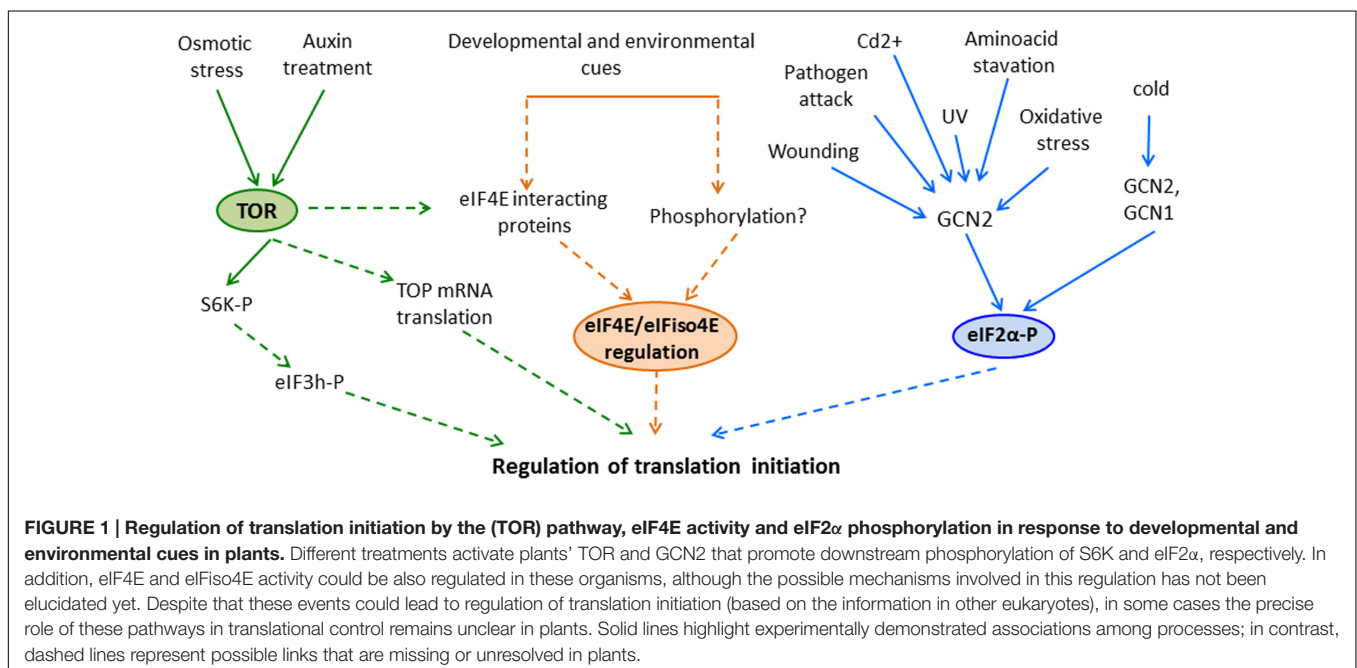
only eIF2 α kinase identified in plants (Lageix et al., 2008; Zhang et al., 2008; Sormani et al., 2011; Wang et al., 2016). In addition, ILITHYIA (ILA), the Arabidopsis homolog of yeast GCN1, is needed to promote eIF2 α phosphorylation in response to cold (Wang et al., 2016). Despite this evidence, the functional relevance of this regulatory pathway in plant adaptation to stress is not yet completely understood (see Echevarria-Zomeño et al., 2013; Browning and Bailey-Serres, 2015 for recent reviews). In plants, GCN2-dependent eIF2 α phosphorylation is reported to regulate protein synthesis, although this mechanism as a general inhibitor of translation is limited to the responses to the purine synthesis inhibitor 8-azaadenine and the amino acid synthesis inhibitor chlorsulfuron (Lageix et al., 2008). Moreover, data are contradictory regarding the role of GCN2 in plant adaptation to amino acid deprivation (Zhang et al., 2008; Faus et al., 2015).

In addition to abiotic and nutritional stresses, recent evidence suggests a function for GCN2 and eIF2 α phosphorylation in plant immunity, although their role remains elusive. eIF2 α phosphorylation is reported in response to *Pseudomonas syringae* pv. *maculicola* ES4326/avrRpt2 infection (Pajerowska-Mukhtar et al., 2012), but its influence on bacterial growth has yet to be determined. Adult *gcn2* plants show enhanced resistance to the necrotroph *Pectobacterium carotovorum* subsp. *carotovorum* and the biotrophic fungus *Golovinomyces cichoracearum*, a response that contrasts with the enhanced susceptibility of young *gcn2* plants to *G. cichoracearum* or *Hyaloperonospora arabidopsidis* inoculation (Liu et al., 2015). Other studies reported activation of eIF2 α phosphorylation in response to treatment with the defense-related hormones salicylic acid, jasmonic acid, the ethylene precursor ACC, and the priming agent β -aminobutyric acid (Lageix et al., 2008; Luna et al., 2014; Wang et al., 2016).

CHALLENGES AND FUTURE PERSPECTIVES

The recent development of techniques for obtaining ribosome footprints in plants, by direct isolation of monosomes (Ribo-seq) (Merchante et al., 2015, 2016; Hsu et al., 2016) or by TRAP-SEQ (translating ribosome affinity purification-RNA sequencing) (Wang and Jiao, 2014; Juntawong et al., 2015; Reynoso et al., 2015), have revolutionized translation studies; they allow determination of exact ribosome positions on a genome-wide scale at single-codon resolution. These techniques have already been used to identify global features in translating mRNAs (Hu et al., 2016; Zhao et al., 2016), translating mRNAs in chloroplasts (Zoschke et al., 2013; Chotewutmontri and Barkan, 2016) and mRNAs regulated at the translational level during developmental processes such as seed germination and in response to stress conditions or plant hormones (Mustroph et al., 2009; Juntawong et al., 2014; Merchante et al., 2015; Bai et al., 2016). In addition, the incorporation of a non-canonical amino acid, azidohomoalanine (AHA), has recently been used to monitor newly synthesized proteins in plants. The use of AHA was firstly reported by Echevarria-Zomeño et al. (2015), where AHA was described to mark *de novo* synthesized HSP90 and HSP70 proteins under heat stress conditions in Arabidopsis. This method, coupled to tandem liquid chromatography-mass spectrometry (LC-MS), has now been implemented to allow non-radioactive analysis of protein synthesis in plants (Glenn et al., 2017). All these techniques will be extremely helpful for identifying and characterizing the mechanisms that regulate translation in response to nutritional and environmental cues.

The recent development of chemical genetic tools and cellular assays for analysis of TOR pathway in plants will help to identify new targets of this pathway and to understand its involvement



in translation regulation. It will also be relevant to clarify the regulatory activity of TOR on TOP mRNAs, as well as its role in regulating plant adaptation through selective translation of ribosomal proteins. In addition to translation, glucose-mediated TOR signaling has been found to play an important role at transcriptional level in *Arabidopsis* (Xiong et al., 2013).

Since the function of putative plant eIF4E interactors has not been studied in detail, considerable effort is needed to determine the role of these proteins in mRNA translation, export or decay, and how such regulation could affect plant development or responses to environmental stimuli.

An exclusive feature of plant translational machinery is the presence along with eIF4E of eIFiso4E isoforms, which mediate the translation of specific mRNA populations as part of the eIFiso4F complexes (Mayberry et al., 2009; Martinez-Silva et al., 2012; Chen et al., 2014). Therefore, it would be of interest to analyze the possible specialization of the eIF4E putative regulators in the selective regulation of eIF4E and eIFiso4E proteins. It will also be important to study the nature of eIF4E and eIFiso4E post-translational modifications and their function in the control of translation initiation in plants.

The effort to define the role of phosphorylated eIF2 α during plant adaptation to environmental changes highlights current interest in this area. Reports with contrasting results nonetheless emphasize the need for additional studies to clarify the participation in plant immunity of eIF2 α phosphorylation and of the TOR pathway. As inhibition of translation mediated by eIF2 α phosphorylation is less severe in plants than in mammals, it is necessary to clarify its role in plant adaptation to stress; identification of plant mRNAs targeted by this regulatory mechanism would constitute a major breakthrough.

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In this review, we have focused on the regulation of the TOR pathway and eIF4E and eIF2 α translation initiation factors by developmental and environmental cues (Figure 1). Nevertheless, when analyzing translation regulation during plant response to environmental changes, other mechanisms including those that affect translation elongation and termination, or formation of cytoplasmic ribonucleoprotein foci must also be considered. Many important questions remain to be answered; indeed, we are just beginning to understand translational regulation in plants and can thus anticipate major findings in this field in coming years.

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All authors listed made substantial intellectual contribution to the work, wrote the manuscript together and approved it for publication.

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The Rice Eukaryotic Translation Initiation Factor 3 Subunit e (OseIF3e) Influences Organ Size and Pollen Maturation

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Eukaryotic translation initiation factor 3 (eIF3) is a large protein complex that participates in most translation initiation processes. While eIF3 has been well characterized, less is known about the roles of individual eIF3 subunits, particularly in plants. Here, we identified and characterized *OseIF3e* in rice (*Oryza sativa* L.). *OseIF3e* was constitutively expressed in various tissues, but most strongly in vigorously growing organs. Transgenic *OseIF3e*-silenced rice plants showed inhibited growth in seedling and vegetative stages. Repression of *OseIF3e* led to defects in pollen maturation but did not affect pollen mitosis. In rice, eIF3e interacted with eIF3 subunits b, d, e, f, h, and k, and with eIF6, forming homo- and heterodimers to initiate translation. Furthermore, *OseIF3e* was shown by yeast two-hybrid assay to specifically bind to inhibitors of cyclin-dependent kinases 1, 5, and 6. This interaction was mediated by the sequence of amino acid residues at positions 118–138, which included a conserved motif (IGPEQIETLYQFAKF). These results suggested although *OseIF3e* is not a “functional core” subunit of eIF3, it still plays crucial roles in rice growth and development, in combination with other factors. We proposed a pathway by which *OseIF3e* influence organ size and pollen maturation in rice, providing an opportunity to optimize plant architecture for crop breeding.

Keywords: *OseIF3e*, translation initiation, *OsICKs*, *Oryza sativa* L., reproductive development, pollen maturation

INTRODUCTION

In the process of translation initiation, eukaryotic initiation factors (eIFs) participate in the recruitment of initiator tRNA (Met-tRNA^{Met}) and mRNA to the 40S ribosomal subunit, as well in scanning for the AUG start codon (Browning et al., 2001; Kapp and Lorsch, 2004; Hinnebusch, 2006). Of the 12 known eIFs, eukaryotic translation initiation factor 3 (eIF3) is the largest and most complex. It is involved in assembling the eIF2-GTP-Met-tRNA^{Met} ternary complex and recruiting it to the 40S subunit, recruiting mRNA to the 43S pre-initiation complex, and scanning for and recognizing AUG start codons (Burks et al., 2001; Kawaguchi and Bailey-Serres, 2002; Siridechadilok et al., 2005; Hinnebusch, 2006).

Abbreviations: ABA, abscisic acid; ABREs, ABA response elements; BTF3, basal transcription factor 3; CDS, coding sequence; eIF3e, eukaryotic translation initiation factor 3 subunit E; eIF3h, eukaryotic translation initiation factor 3 subunit H; GA, gibberellin; ICK, inhibitors of cyclin-dependent kinases; *Os*, *Oryza sativa*; qRT-PCR, quantitative reverse transcription-PCR; RNAi, RNA interference.

Mammalian eIF3 contains 13 non-identical subunits designated eIF3a–m (Asano et al., 1997; Browning et al., 2001). In contrast, eIF in *Saccharomyces cerevisiae* comprises only six subunits (eIF3a, eIF3b, eIF3c, eIF3g, eIF3i, and eIF3j). Five of these (eIF3a, eIF3b, eIF3c, eIF3g, and eIF3i) are conserved in all eukaryotes (Phan et al., 1998; Browning et al., 2001). The non-conserved nature of subunit e indicates that it may not be essential for translation initiation (Asano et al., 1998; Burks et al., 2001; Zhou et al., 2005; Xia et al., 2010).

The eIF3e subunit, also known as *Int6*, is a common integration site for the mouse mammary tumor virus (MMTV) genome (Marchetti et al., 1995), which plays multiple roles in translation, as indicated by its association with the COP9 signalosome (CSN). The CSN is known to be involved in the regulation of proteolysis (Yahalom et al., 2001), control of 26S proteasome activity (Yen et al., 2003), and spindle organization (Yen and Chang, 2000; Morris and Jalinot, 2005). These findings suggest its potential as a regulatory subunit for gene translation (von Arnim and Chamovitz, 2003).

Few studies have examined the functions of the various eIF3 subunits in plants, some of those have been conducted in *Arabidopsis* (Kim et al., 2004; Yahalom et al., 2008; Xia et al., 2010). Two *Arabidopsis thaliana* eIF3e mutants are known (Yahalom et al., 2008). *AteIF3e-Tp*, which carries an insertion (T) 150 bp upstream of the first exon, leads to reduced fertility and reproductive defects (Yahalom et al., 2008). The mutant *eIF3e-Tnull*, containing an insertion (T) in the middle of the third exon, results in lethality of the male gametophyte. These results suggest that *AteIF3e* is necessary for male gametogenesis. Mutations in subunits *eIF3f* and *eIF3h* have also been characterized in *Arabidopsis*. A *Ds* (transposon element) insertion mutation in *AteIF3f* has been found to disrupt pollen germination and embryonic development (Xia et al., 2010). Plants homozygous for *AteIF3h* mutation exhibit pleiotropic growth defects throughout development, including low fertility, reduced stamen number, partial seed abortion, and inhibition of root hair formation (Kim et al., 2004, 2007). Subsequently, Zhou et al. (2014) described a mutation in *AteIF3h* led to expansion of shoot apical meristem (SAM) size accompanied by a failure to initiate new organs. Recently, the biological function of OseIF3f has been studied by Li et al. (2016). The OseIF3f-RNAi plants showed a higher percentage of arrested unicellular pollen at bicellular stage and aborted pollen at the tricellular stage, it is suggest that OseIF3f plays a vital role in microgametogenesis. Overall, even eIF3 subunits are not part of the functional core, it's also play important roles in the growth and development of *Arabidopsis* and rice (Li et al., 2016).

Organ size is controlled by two fundamental processes: cell proliferation and cell expansion, which are strictly regulated by cyclin-dependent kinases (CDKs) together with their specific cyclin partners (Mizukami and Fischer, 2000; Sugimoto-Shirasu and Roberts, 2003). Other factors act as inhibitors of CDK (ICK) during plant development and in response to environmental changes (Sherr and Roberts, 1999). Studies in plants, particularly *Arabidopsis* and rice, have shown that overexpression of various *ICK* genes results in phenotypic effects similar to those produced by mutations of eIF3 subunits, including small organ sizes,

reduced cell numbers, pollen sterility, and low seed setting (Wang et al., 2000; De Veylder et al., 2001; Zhou et al., 2002; Barroco et al., 2006; Bemis and Torii, 2007; Kang et al., 2007). For example, overexpression of either *AtICK1* or *AtICK2* in *Arabidopsis* induces cells to initiate endoreduplication earlier than normal, resulting in a higher ploidy numbers (Verkest et al., 2005; Weinl et al., 2005). Similarly, overexpression of rice *OsiICK6* results in multiple phenotypic effects on plant growth, pollen viability, and seed setting (Yang et al., 2011).

A previous study revealed that inhibition of *Osj10gBTF3* (*Oryza sativa* BTF3) results in dramatic plant miniaturization. Furthermore, pollen is completely sterile, an effect correlated with the altered expression of two Rf (fertility restorer)-like genes encoding pentatricopeptide repeat-containing proteins (OsPPRs); two translation initiation factors, OseIF3e and OseIF3h; and the heat shock protein OsHSP82 (Wang et al., 2012). The present study sought to confirm the functions of *OseIF3e* in plant growth and development. Specifically, protein–protein interactions demonstrated that OseIF3e plays important roles in rice growth and pollen development and interacts with eIF3 subunits OseIF3b, OseIF3d, OseIF3e, OseIF3f, OseIF3h, OseIF3k, as well as eIF6 and ICKs. Taken together, these results help to unravel a possible pathway for *OseIF3e* involvement in organ growth and pollen development in rice.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Stress Treatments

Rice (*O. sativa* L ssp *japonica* cv Nipponbare) was used in this study for various experiments. All plants were grown on the experimental field of Zhejiang University in Hangzhou (30°16'N, temperate climate, China) or Sanya (19°2'N, tropical climate, China) during the natural growing season. Rice seedling plant were grown at 28°C with 16 h light/8 h dark cycle, 75% relative humidity in greenhouse. For expression studies of *OseIF3e* in response to various treatments, 2-week-old seedlings were transferred to Yoshida solution (Yoshida et al., 1976) supplemented with 200 mM NaCl, 10 μM ABA, 100 μM GA. Seedlings grown in the same liquid medium without any supplementary component were used as controls. For cold stress, 4-week-old seed-derived seedlings were transferred from semi-solid 1/2MS medium (Murashige and Skoog, 1962) to Yoshida solution, were exposed in 4°C for 24 h.

Vector Construction and Rice Transformation

To generate *OseIF3e* knock-down transgenic lines, the *OseIF3e* cDNA fragments of 325 bp (from 174 to 499 bp of *OseIF3e*, Supplementary Figure S3) was inserted into pTCK303 vector (Wang et al., 2004) to produce RNAi repression vectors. The resultant vector was introduced into *Agrobacterium tumefaciens* strain EHA105, which was used to infect rice embryogenic calli from Nipponbare. Transgenic plants were screened by PCR

amplification with hygromycin B phosphotransferase gene (*Hpt*). All primers used in this study are listed in Supplementary Table S2.

Phenotypic Analysis of Transgenic Plants

The evaluation of phenotypic traits of three independent transformants *OseIF3e*^{Ri}-2, *OseIF3e*^{Ri}-4, *OseIF3e*^{Ri}-7 were performed in the T1–T3 generation. Seeds of *OseIF3e*^{Ri} and wild-type (WT) plants were collected and germinated by soaking in water for 2 days at 37°C. Germinating seeds were sown in experimental field as described above during the natural growing season at five-leaf and maturity stage, the phenotypic characteristics were measured and photographed, including plant height, tiller number, the internode length, panicle length, the spikelet number, the grain length and width. The data were analyzed by ANOVA, and mean values were separated by least significant difference at the 5 and 1% probability level using Statistical software (Sigmaplot10.0.).

RNA Extraction, cDNA Synthesis, and Quantitative Real-Time RT-PCR

Total RNAs were extracted from different tissues of the WT and *OseIF3e*^{Ri} plant using TRIzol reagent (Invitrogen). Reverse transcription (RT) was performed using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitative real-time RT-PCR (qRT-PCR) analysis was conducted with the Lightcycler 480 machine using SYBR Green I (TAKARA). *UBIQUITIN* (*Os03g0234200*) mRNA was used as an internal control. The specific primers for qRT-PCR are listed in Supplementary Table S2.

Yeast Two-Hybrid Assay

The yeast two-hybrid assay was performed using the Matchmaker Two-Hybrid System (Clontech¹). The full-length CDS and different truncations of *OseIF3e*, *OsICKs*, and other subunits of *OseIF3*, *OseIF1*, *OseIF2*, *OseIF4*, *OseIF5*, and *OseIF6* were amplified by PCR using the primers listed in Supplementary Table S2. The fragments were cloned into the pGBKT7 or pGADT7 vector. Then co-transformed into yeast strain AH109 first selected on SD/-Leu/-Trp (DDO) plates at 30°C for 3 days, signal colony from yeast transformants including different pair of constructs were diluted in 0.9% NaCl, and a 1/10th dilution was spotted on SD/-Ade/-His/-Leu/-Trp (QDO) plates and incubate at 30°C for 3 days. Yeast cells co-transformed with pGBKT7-53 and pGADT7-T were used as the positive control, pGBKT7-Lam and pGADT7T were used as the negative control.

I₂-KI and DAPI Staining

To analyze pollen viability, mature anthers were incubated with 1% (w/v) I₂-KI staining, with three biological repetitions. The stained pollen grains were observed and recorded using a Leica DMIRB fluorescence microscope. For 4',6-diamidino-2-phenylindole (DAPI) staining, pollen grains were fixed in DAPI staining solution (0.1 M sodium phosphate, pH 7.0, 1 mM EDTA,

0.1% Triton X-100 and 0.25 mg/ml DAPI) for 1 h at room temperature. Photography was performed using Leica DMIRB fluorescence microscope under UV light.

Bioinformatics Analysis

To investigate gene's structure, the exon/intron boundary were predicted with RGAP², and protein domains were predicted by PROSITE³, PLACE⁴ was used for analysis *cis*-elements of *OseIF3e* promoter region. The primers used in this study were designed by primer primer5.0 and the BLAST⁵ was used for sequence alignment. Alignment was performed using CLUSTALX1.8 (Thompson et al., 1994) with default settings. All amino acid sequences were obtained from the NCBI database⁶. Phylogenetic analysis was conducted using MEGA5 via the neighbor-joining method (Kolaczowski and Thornton, 2004). Motif 1,2 in *eIF3e* from different species using were identified by the MEME/MAST program⁷ (Bailey and Elkan, 1994; Bailey and Gribskov, 1998).

RESULTS

Characteristics and Expression Patterns of *OseIF3e*

In rice, *OseIF3e* was originally identified via its interaction with the basal transcription factor *Osj10gBTF3*, inhibition of which results in plant miniaturization and pollen abortion (Wang et al., 2012). Previous studies have characterized a multitude of *eIF3e* homologs from other species. We constructed a phylogenetic tree of *OseIF3e* according to sequence homology. This revealed that *OseIF3e* is most closely related to *ZmeIF3e*, while homologs in animals and fungi form separate clades (Figure 1a).

To investigate the expression profile of *OseIF3e*, we searched the CREP (Collection of Rice Expression Profiles) database⁸, which collects genome-wide expression data over the life cycles of two rice varieties (Wang et al., 2010). This revealed *OseIF3e* to be constitutively expressed in all tissues and organs, with particularly high expression levels in young and developing tissues (Supplementary Table S1 and Figure S1). We then performed qRT-PCR to confirm *OseIF3e* expression patterns in the following tissues: callus (Ca), shoot (Sh), root (Ro), stem (St), leaf (Le), sheath (Ls), lemma (Lm), palea (Pa), anther (An), pistil (Pi), and internode (In). The results were consistent with the CREP data, with higher *OseIF3e* expression occurring in vigorously growing tissues (Figure 1c). These results implicate *OseIF3e* in both vegetative growth and reproductive development in rice.

²<http://rice.plantbiology.msu.edu>

³<http://prosite.expasy.org/>

⁴<http://www.dna.affrc.go.jp/htdocs/PLACE/>

⁵<http://blast.ncbi.nlm.nih.gov/Blast.cgi>

⁶<http://www.ncbi.nlm.nih.gov/>

⁷<http://meme-suite.org/tools/tomtom>

⁸<http://crep.ncpgr.cn>

¹<http://www.clontech.com/>

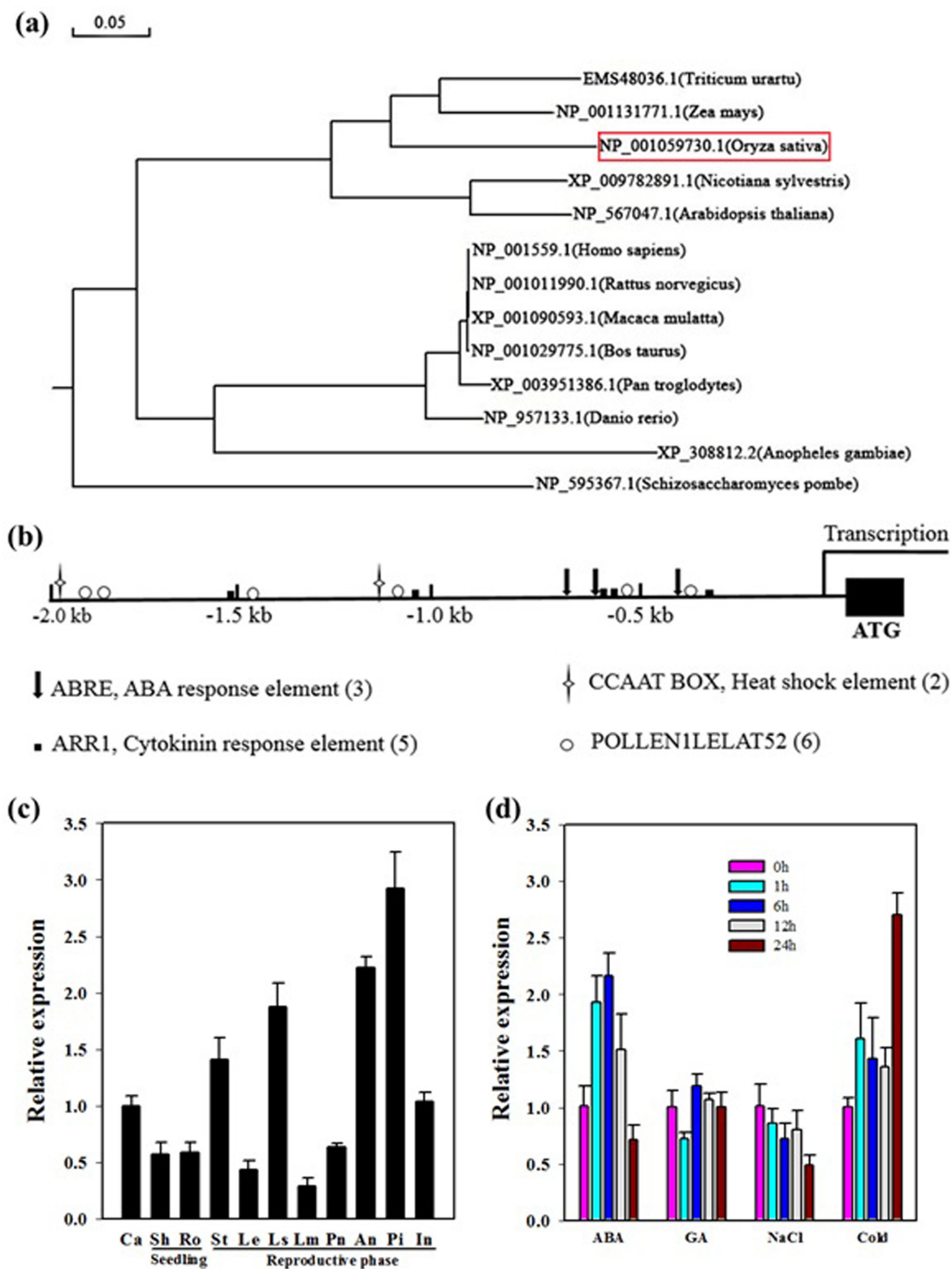


FIGURE 1 | Characteristics and expression profile of *OseIF3e*. (a) Phylogenetic tree constructed by MEGA5 software using the neighbor-joining method. (b) *Cis*-element analysis of the *OseIF3e* promoter region. (c) Expression of *OseIF3e* in various organs. Ca, callus; Sh, shoot; Ro, root; St, stem; Le, leaf; Ls, sheath; Lm, lemma; Pa, palea; An, anther; Pi, pistil; In, internode. (d) Expression of *OseIF3e* under hormone and stress treatments, including ABA, GA, salt, and cold (0, 1, 6, 12, 24 h) treatments in seedlings.

Next, we analyzed the 2.0-kb promoter region of *OseIF3e* and found several types of *cis*-acting elements, including several hormone response elements. These included three ABREs, five ARR1s (cytokinin response elements), and two heat shock elements (Figure 1b). Accordingly, we performed qRT-PCR to determine *OseIF3e* expression levels under different hormonal and abiotic stress treatments in seedlings (Figure 1d). The results showed *OseIF3e* to be induced by cold, but repressed by salt treatment. For the ABA treatment, *OseIF3e* transcripts increased within the first 6 h, but then decreased. GA treatment only slightly affected *OseIF3e* expression.

Transgenic *OseIF3e*-Silenced Rice Plants Show Inhibited Growth in Seedling and Vegetative Stages

To determine the function of *OseIF3e* in rice, we obtained nine *OseIF3e^{Ri}* knockdown lines, in which RNAi reduced the expression of *OseIF3e* (Figures 2a,c). Eight transgenic plants had significant decreases in *OseIF3e* compared with WT plants. Then three independent transformants *OseIF3e^{Ri}*-2, *OseIF3e^{Ri}*-4, *OseIF3e^{Ri}*-7 were used for further experiments. Within the first 10 days after germination, these lines did not differ significantly from the WT in terms of seed germination and phenotypic expression (Figure 2c). *OseIF3e^{Ri}* lines gradually became slower than that of the WT (Figure 2e), leading to shorter shoots (Figure 2b) and slightly shorter roots (Figure 2d) in *OseIF3e* plants. When the plants entered the vegetative period, other organs in the *OseIF3e^{Ri}* plants were reduced, e.g., the length and width of the first flag leaf were shorter than in the WT (Supplementary Figure S2). No differences were observed in tiller number. Overall, before maturity, *OseIF3e^{Ri}* transgenic plants differed most markedly from the WT in seedling and flag leaf phenotypes.

These phenotypic differences between WT and *OseIF3e^{Ri}* plants remained stable in generations T₀–T₃, confirming that they were indeed due to the suppression of *OseIF3e* (Figure 2c). The observation that *OseIF3e* suppression led to stunted rice suggests that *OseIF3e* is critical to the growth of seedling and vegetative-stage plants.

Aberrant Panicle Phenotype and Low Plant Biomass in *OseIF3e^{Ri}* Lines

OseIF3e^{Ri} plants remained notably shorter than WT plants due to reduced internode lengths (Figures 3a,b,m). At the mature stage, the panicle axis of *OseIF3e^{Ri}* was notably shorter than in WT (Figure 3d). We measured the lengths of panicle and primary branches and numbers of primary branches and spikelets. The *OseIF3e^{Ri}* plants displayed shorter panicles and reduced spikelet numbers (Figures 3e–i). Moreover, the grains of *OseIF3e^{Ri}* lines appeared thinner and shorter than WT grains, resulting in lower 100-grain weights (Figures 3c,j–l). These results demonstrated that *OseIF3e* influences not only panicle size and shape, but also overall plant biomass.

Repression of *OseIF3e* Affects Pollen Maturation

OseIF3e^{Ri} plants exhibited a high rate of sterility in generations T₀–T₃, which were grown in different locations (Figures 4a,l). Seed setting rate of *OseIF3e^{Ri}* plants ranged from 20.2 to 42.8%, compared to from 88.9 to 94.6% in WT plants (Figure 4l). In addition, *OseIF3e^{Ri}* plants exhibited abnormal anthers (Figures 4c,h). We examined the pollen viability of WT and *OseIF3e^{Ri}* plants with I₂-KI staining. Stained WT pollen presented full and black, while *OseIF3e^{Ri}* pollen appeared light brown (Figures 4b,d,e,g,i,j). To visualize possible mitotic defects, pollen grains were stained with DAPI. DAPI staining revealed two brightly stained sperm nuclei and a large, diffusely stained vegetative cell nucleus in both *OseIF3e^{Ri}* and WT pollen grains (Figures 4f,k, arrowhead). Therefore, while repression of *OseIF3e* led to defects in pollen maturation, it did not appear to affect pollen mitosis.

OseIF3e^{Ri} Seedlings Exhibited a Sugar-Sensitive Phenotype

In *Arabidopsis*, mutation of either of two eIF3 components, eIF3f and eIF3h, produced a biphasic response to exogenous sugars (Kim et al., 2004; Xia et al., 2010). The present study examined the role of the *OseIF3e* subunit in response to sugar, using the *OseIF3e^{Ri}* knockdown line. WT and *OseIF3e^{Ri}* seeds were germinated on 1/2MS agar plates containing either no sugar (control) or one of the following: 2% (w/v) sucrose, 2% (w/v) mannitol, 2% (w/v) maltose, 1% (w/v) glucose. The results showed nearly no differences in responses of WT seedlings to sugar treatments. However, *OseIF3e^{Ri}* seedlings exhibited stunted growth in 2% (w/v) mannitol, compared with the other sugar treatments (Figures 5a,b). Subsequently, WT and *OseIF3e^{Ri}* seedlings were grown on 1/2MS agar plates containing 0, 1, 2, 3, or 5% mannitol (w/v). As mannitol concentration increased, the growth of *OseIF3e^{Ri}* seedlings appeared more notably stunted, compared to WT (Figures 5c,d). In summary, repression of *OseIF3e* caused rice seedlings to become sensitive to exogenous mannitol, resulting in stunted growth of the transgenic plants.

Yeast Two-Hybrid Assays Reveal that eIF3e Interacts with itself, Other Subunits of eIF3, and eIF6

The components of eIF3 have been identified in many species. Previous studies show that the different subunits of eIF3 form complexes, which allows them to participate in gene regulation (Kim et al., 2004; Xia et al., 2010). In *Arabidopsis*, eIF3h interacts directly with the eIF3a, eIF3b, eIF3c, and eIF3e subunits (Kim et al., 2004). In addition, the eIF3f subunit has been confirmed to interact with eIF3e and eIF3h (Xia et al., 2010). We performed yeast two-hybrid assays, demonstrating that in rice, eIF3e is able to interact with itself, with other subunits of eIF3 (b, d, f, h, and k), and with eIF6, but does not interact with eIF1, eIF2;1, eIF4, or eIF5 (Figure 6). These protein–protein interactions suggest that the subunits of eIF3 and eIF6 form homo- and heterodimers, in different combinations, to initiate translation and regulate target gene expression in rice.

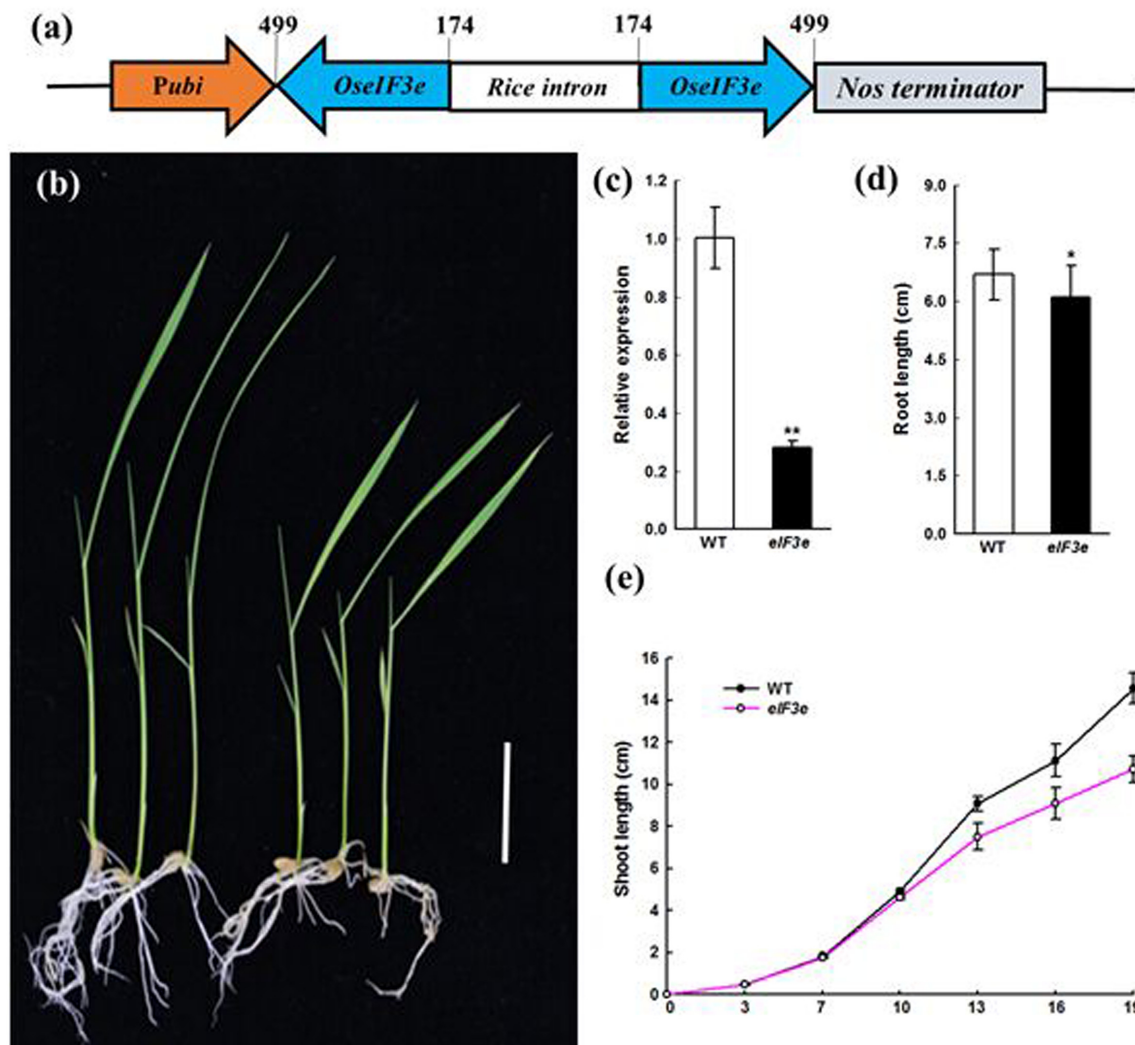


FIGURE 2 | Phenotype of the *eIF3e* knockdown seedling. (a) Schematic representation of the *OsIF3e* RNAi vector construction. The numbers indicate the nucleotide position in the *OsIF3e* CDS sequence. *Pubi*, maize ubiquitin promoter. (b) Wild-type (WT, left) and *OsIF3e^{Ri}* plants (right) at 15 days after sowing. Scale bars: 3 cm. (c) Relative expression level of *OsIF3e* in WT and *OsIF3e^{Ri}* plants. Two-week-old seedlings were used for the analysis. Rice ubiquitin (*OsUBQ*) was used as the reference. (d) Two-week-old seedlings root length of WT and *OsIF3e^{Ri}* plants. Values are expressed as means \pm SD. * $P < 0.05$; ** $P < 0.01$ compared with the WT plant using Student's *t*-test. (e) Height of WT and *OsIF3e^{Ri}* plants after sowing.

Targeting of the OsICK Family by the eIF3 Complex is Mediated by Amino Acids 118–138 of eIF3e

To determine whether the OsICK gene family is regulated by the eIF3 complex, the interaction between OsIF3e and OsICKs was investigated by yeast two-hybrid assay. Moreover, considering that the OsIF3e protein possesses relevant domains in its N- and C-terminal regions, we used fragments of OsIF3e encoding the eIF3_N domain (*OsIF3e Δ PCI*), the PCI domain (*OsIF3e Δ eIF3_N*), and the full-length cDNA as baits. The assay revealed that OsICK1, OsICK5, and OsICK6 interacted with OsIF3e and *OsIF3e Δ PCI*, both of which included the eIF3_N domain, while no interaction between *OsIF3e Δ eIF3_N* and any OsICK was observed (Figure 7a). These results suggest that

the OsICK family is a direct target of the eIF3 complex and that this interaction is mediated by the eIF3_N domain of OsIF3e.

We then identified conserved sequence motifs in OsICK1, OsICK5, and OsICK6. In these three OsICKs, two consensus sequence motifs were identified by the MEME/MAST program (Bailey and Elkan, 1994; Bailey and Gribskov, 1998; Torres Acosta et al., 2011; Figure 7d). Examination of *OsICK1*, *OsICK5*, and *OsICK6* gene expression in WT and *OsIF3e^{Ri}* lines showed that all three genes experienced various degrees of reduction in *OsIF3e^{Ri}* plants, compared with WT plants (Figure 7c).

In order to further characterize the OsIF3e N-terminal motif responsible for its interaction with OsICKs, we cloned fragments encoding different truncations of the OsIF3e N

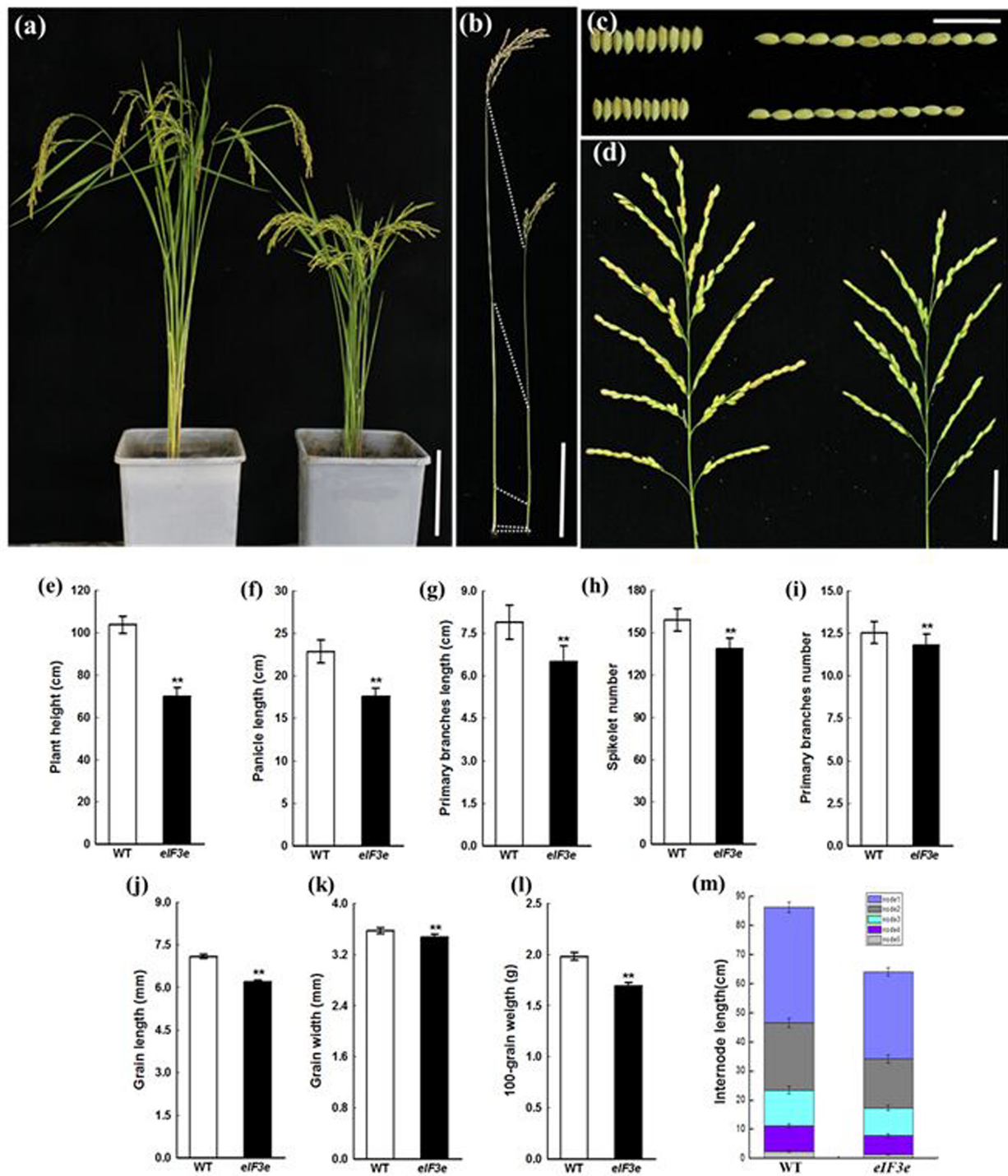


FIGURE 3 | Phenotype and statistical analysis of panicle and seed of the *OseIF3e^{Ri}* plant at the maturity stage. (a) Five-month-old wild-type (WT, left) and *OseIF3e^{Ri}* (right) plants. Scale bars: 20 cm. **(b)** Comparison of the internode in WT (left) and *OseIF3e^{Ri}* (right) plants. Scale bars: 4 cm. **(c)** Seed width and seed length in WT (upper) and *OseIF3e^{Ri}* (lower) plants. Scale bars: 2 cm. **(d)** Panicle branching in WT (left) and *OseIF3e^{Ri}* (right) plants. Scale bars: 5 cm. **(e)** Statistical analysis of plant height in WT and *OseIF3e^{Ri}* plants. **(f–i)** Statistical analysis of panicle types in WT and *OseIF3e^{Ri}* plants. **(j–l)** Statistical analysis of seed size in WT and *OseIF3e^{Ri}* plants. **(m)** Comparison of internode length of the main culm in WT and *OseIF3e^{Ri}* plants. Values are expressed as means \pm SD. ** $P < 0.01$ compared with the WT using Student's *t*-test.

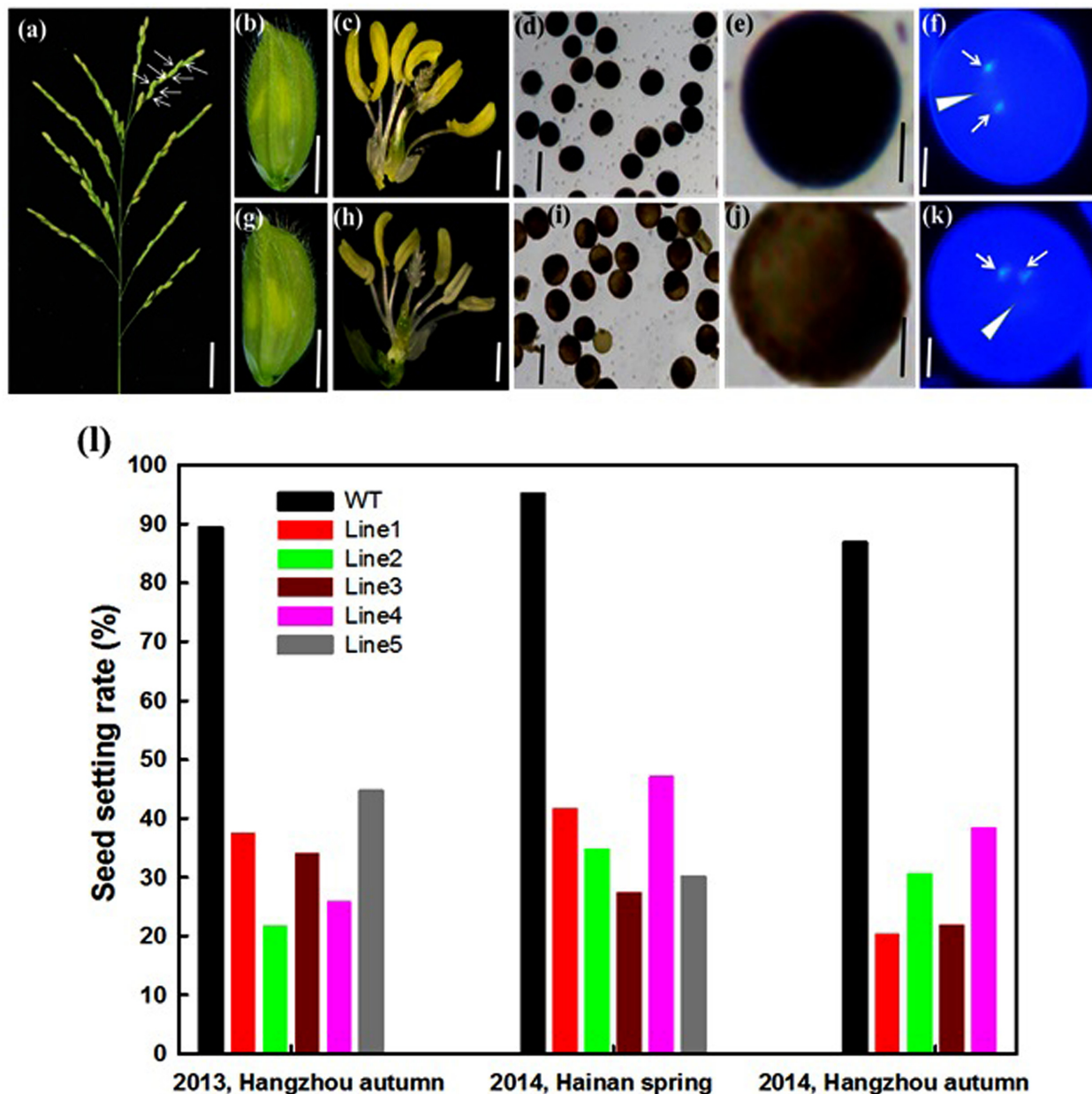


FIGURE 4 | Analysis of the sterile phenotype in the *OsIF3e* RNAi transgenic plants. (a) Panicle of *OsIF3e*^{RNAi} plants. The white arrow represents empty seeds. Scale bars: 3 cm. **(b,c,g,h)** Wild-type spikelet **(b,c)** and *OsIF3e*^{RNAi} spikelet **(g,h)**. The lemma and palea are removed in **(c,h)**. **(d,i)** I₂-KI staining showing pollen viability in the control plant **(d)** and *OsIF3e*^{RNAi} plant **(i)**. **(e,j)** The higher magnification of I₂-KI staining showing pollen viability in the control plant **(e)** and *OsIF3e*^{RNAi} plant **(j)**. **(f,k)** DAPI staining showing three nuclei of pollen grain in the control plant **(f)** and *OsIF3e*^{RNAi} plant **(k)**. Arrowheads indicate the vegetative nucleus, and arrows indicate sperm-cell nuclei in the pollen of the control plant **(f)** and abnormal pollen of *OsIF3e*^{RNAi} plant **(k)**. **(l)** Seed setting analysis of *OsIF3e*^{RNAi} transgenic plants generated in 2013 autumn, and 2014 spring and autumn, respectively. Scale bars: 2 mm in **(b,g)**, 1 mm in **(c,h)**, 100 μ m in **(d,i)**, 20 μ m in **(e,j)**, and 10 μ m in **(f,k)**.

terminus as baits and determined their interaction with OsICK5. As shown in Figure 7b, no interaction was detected if the cloned fragment lacked amino acids 118–138 (N4), suggesting that these 20 amino acids which included a conserved motif (IGPEQIETLYQFAKF, Figure 7e) are necessary for the interaction to occur.

DISCUSSION

OsIF3e Is Involved in the Regulation of Organ Size and Pollen Maturation

Plant organ size is controlled by two successive, overlapping types of cell growth: cell proliferation and cell expansion (Mizukami

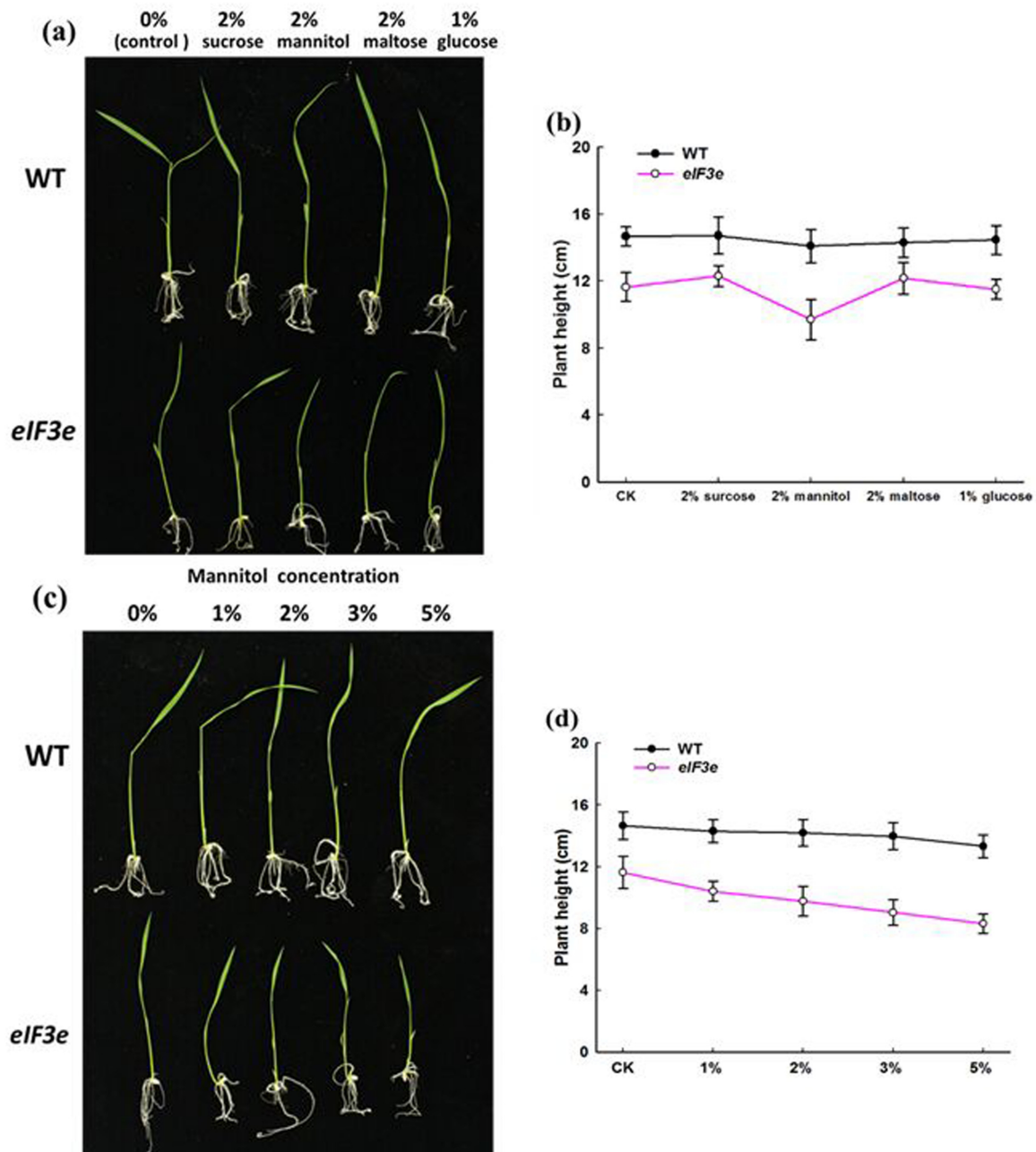
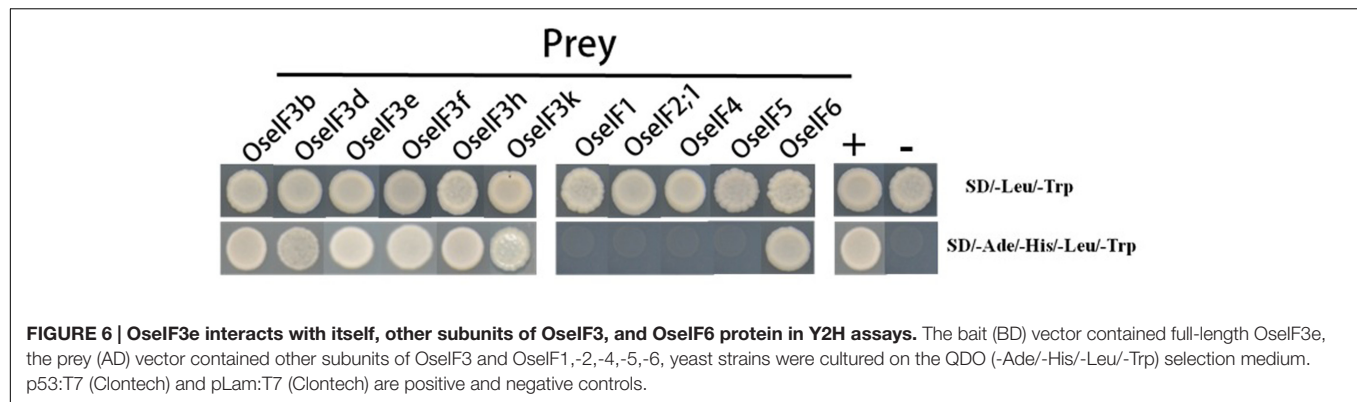


FIGURE 5 | *OseIF3e^{Ri}* seedlings are sensitive to exogenous sugars. **(a)** Wild-type (WT) and *OseIF3e^{Ri}* seedlings cultured on 1/2 MS medium containing various sugars. **(b)** Comparison of plant height in WT and *OseIF3e^{Ri}* seedlings cultured on 1/2 MS medium containing various sugars. **(c)** WT and *OseIF3e^{Ri}* seedlings cultured on 1/2 MS medium containing various concentrations of mannitol. **(d)** Comparison of plant height in WT and *OseIF3e^{Ri}* seedlings cultured on 1/2 MS medium containing various concentrations of mannitol.

and Fischer, 2000; Busov et al., 2008). To date, several positive and negative factors affecting organ size have been identified in *Arabidopsis* and rice. Positive factors include *AINTEGUMENTA* (ANT; Krizek, 1999; Mizukami and Fischer, 2000), ARGOS (Hu et al., 2003), *KLUH/CYP78A5* (Anastasiou et al., 2007), *ORGAN SIZE RELATED1* (Feng et al., 2011), and *XIAO* (Jiang et al., 2012). Negative regulators include *BIG BROTHER* (Disch et al., 2006),

PEAPOD1/2 (White, 2006), *DA1* (Li et al., 2008), and *MED25* (Xu and Li, 2011). However, the pathways involved in organ size regulation are not yet well understood.

The present study identified a translation initiation factor in rice, *OseIF3e*, which we found to influence organ size and pollen maturation. During both the vegetative and reproductive stages, all organs of *OseIF3e^{Ri}* plants exhibited significant reductions



in size, compared with WT plants. In addition, repression of *OseIF3e* led to defects in pollen maturation but did not affect pollen mitosis. These results implicate *eIF3e* as an essential gene in rice growth and development.

The *eIF3e* gene was first described as *Int-6*, a common integration site for the MMTV genome (Marchetti et al., 1995). In plants, *eIF3e* was originally identified as co-purifying with the CSN (Karniol et al., 1998), and its function was verified in *Arabidopsis*. Targeted expression of *AteIF3e* results in pleiotropic effects on development, including defects in seedling, vegetative, and floral development (Yahalom et al., 2008). In this respect, our results are consistent with those reported for *Arabidopsis*. *AteIF3f* and *AteIF3h* mutants also exhibit severe defects in plant growth and development (Kim et al., 2004; Yahalom et al., 2008; Xia et al., 2010). These phenotypes are similar to those of the *OseIF3h^{Ri}* plants examined in our study. Besides, repression of *OseIF3e* led to rice seedlings to become sensitive to mannitol, resulting in stunted growth of *OseIF3e^{Ri}* knockdown lines. These results imply that subunits of *eIF3*, even though not part of the functional core, are crucial for not only normal plant growth and development, but also abiotic stress response.

The Activity of the *OseIF3* Complex may be Regulated by *OseIF3e* in Combination with *OseIF3* Subunits b, d, e, f, h, and k, as well as *eIF6*

Research in plants has shown that different subunits of *eIF3* initiate translation and regulate gene expression through the formation of homo- and heterodimers (Karniol et al., 1998; Yahalom et al., 2001; Kim et al., 2004; Huang et al., 2005). For example, in *Arabidopsis*, the *eIF3h* subunit interacts directly with subunits a, b, c, and e (Kim et al., 2004). Similarly, *AteIF3f* interacts with both the e and h subunits (Xia et al., 2010).

The present study revealed *in vivo* protein-protein interactions between the *OseIF3e* subunit and subunits b, d, e, f, h, and k, as well as *eIF6*. Although it is not part of the highly conserved functional of *eIF3*, *OseIF3e* also plays a role in translation processes in combination with other subunits or *eIFs*. Karniol et al. (1998) first associated subunit *eIF3e* with the CSN in plants. Further examination by Kim et al. (2004) revealed that *AteIF3e* interacts directly with *AteIF3h*. Yahalom et al. (2008)

and Xia et al. (2010) subsequently demonstrated that *AteIF3e* exhibits subcellular co-localization with CSN and is negatively regulated by it. Binding between multiple subunits of *eIF3* in *Arabidopsis* suggests the possibility that its activity is regulated by these interactions. However, interactions between *eIF3e* and other proteins in plants are still largely unknown.

eIF6 was initially identified as a wheat protein capable of interaction with the 60S ribosome (Russell and Spremulli, 1980). In yeast, disruption of *eIF6* results in the abnormal processing of ribosomal RNA precursors and a reduction in abundance of the 60S subunit (Wood et al., 1999; Basu et al., 2001). In *Arabidopsis*, loss of the *AteIF6;1* gene results in embryonic lethality (Kato et al., 2010), suggesting that *eIF6* is an essential component of ribosome biogenesis (Si and Maitra, 1999).

Targeting of *OsICKs* by the *OseIF3* Complex, Mediated by Amino Acids 118–138, Is Responsible for Plant Growth and Development in Rice

We used the proteins *OseIF3e*, *OseIF3e_{ΔPCI}* (which included the *eIF3_N* domain), and *OseIF3e_{ΔeIF3_N}* (which included the *PCI* domain) as baits in yeast two-hybrid assays. We thereby determined that three members of the *OsICK* family (*OsICK1*, *OsICK5*, and *OsICK6*) interacted with *OseIF3e* and *OseIF3e_{ΔPCI}*, but not *OseIF3e_{ΔeIF3_N}*. This demonstrated that the interactions were mediated by the *eIF3_N* domain, as the deletion of this region resulted in the lack of interaction. Interestingly, the interaction between *eIF3* and CDK was confirmed during apoptosis (Shi et al., 2003), while *ICK* as inhibitor of CDK which also interact with *eIF3*, suggesting that *eIF3* play a vital role in processes which CDK and *ICK* participate in, such as cell cycle and cell proliferation.

The *ICK* family of CDK inhibitors have been identified as key genes in plant growth and development. Seven *ICK* genes, along with a pseudogene, have been reported in rice and several studies have reported notable effects on plant growth and development due to their over-expression (Wang et al., 2000; Barroco et al., 2006; Yang et al., 2011). For example, over-expression of *OsICK6* results in multiple phenotypic effects on plant growth, morphology, pollen viability, and seed setting (Yang et al., 2011). Transgenic overexpression of *OsICK1*

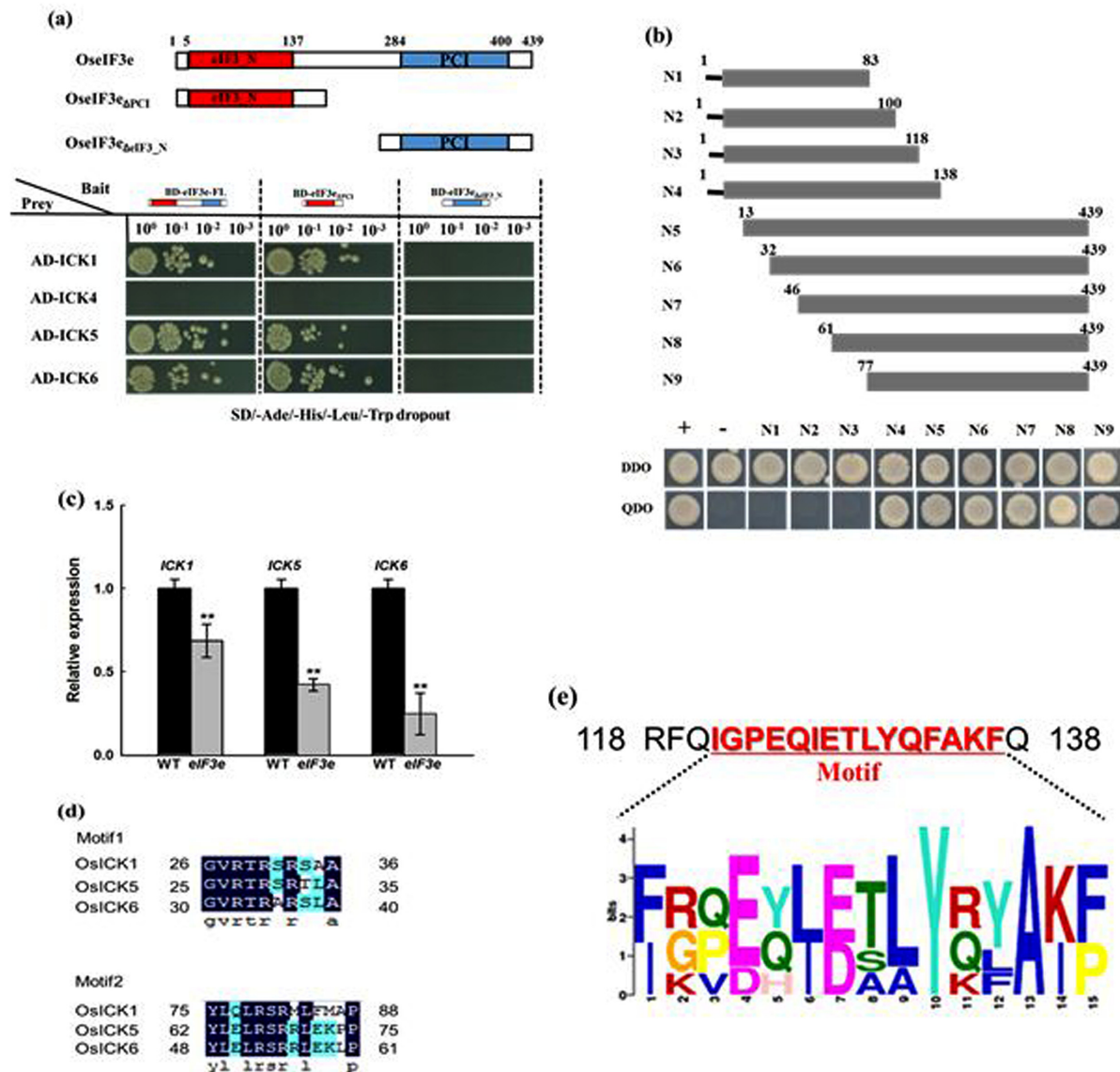
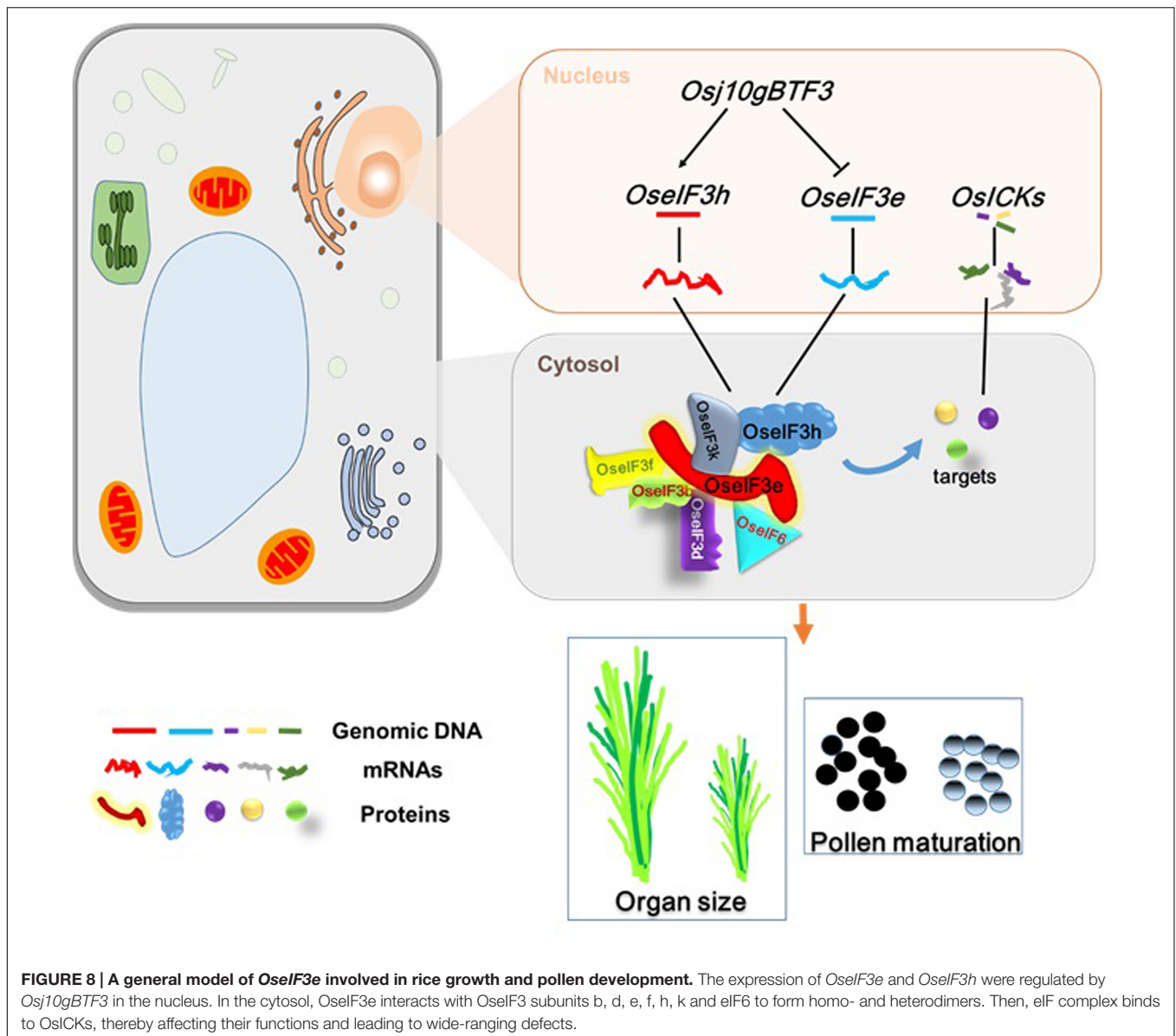


FIGURE 7 | Interaction of OseIF3e with OsICKs and identification of the binding region responsible for the interaction. (a) Interaction of OseIF3e with OsICKs in a yeast two-hybrid assay. A schematic diagram of OseIF3e and the truncations. The bait (BD) vector contained full-length OseIF3e, OseIF3e_{ΔPCI}, or OseIF3e_{ΔeIF3_N}, the prey (AD) vector contained ICKs (ICK1, -4, -5, -6), yeast strains were cultured on the QDO (-Ade/-His/-Leu/-Trp) selection medium. p53:T7 (Clontech) and pLam:T7 (Clontech) are positive and negative controls. **(b)** The interaction between OsICK5 and a series of truncated version of eIF3e_N domain (N1–N9) in yeast. The bottom panels show the growth of the transformed yeast cells on DDO (-Trp/-Leu) or QDO (-Ade/-His/-Leu/-Trp) selection medium. **(c)** Quantitative RT-PCR analysis of gene expression of *OsICK1*, *OsICK5*, and *OsICK6*. **(d)** Sequences of conserved motifs in *OsICK1*, *OsICK5*, and *OsICK6* proteins. **(e)** Consensus sequence of the conserved motif (amino acids 120–137 in rice) in eIF3e from different species. ** indicates *P* values generated by student's *t*-test < 0.01.

affects endosperm development and greatly reduces seed filling (Barroco et al., 2006). In the present study, we analyzed the phenotypes of *OseIF3e* RNAi-mediated knockdown transgenic rice plants, which revealed pleiotropic growth inhibition throughout development. The phenotypes of the *OseIF3e*^{Ri} plants were similar to those reported in transgenic plants that overexpress *OsICK1* and *OsICK6*.

Consistent with this finding, in the present study, knockdown of *OseIF3e* dramatically reduced the expression of *OsICK1*,

OsICK5, and *OsICK6*, suggesting that *OseIF3e* may influence the cell division cycle via interaction with ICKs. In addition, we found that the OseIF3e amino acids 118–138 are necessary for its interaction with OsICKs. This interaction may be mediated by either motif1 or motif2 in OsICKs and by amino acids residues 118–138 in OseIF3e (Figure 7d). The eIF3e subunit may interact with various proteins that possess different binding specificities to initiate translation and regulate the expression genes involved in the development of plants.



In summary, this study points to *OseIF3e* as a crucial regulator of rice seedling development and reproductive processes, including pollen maturation. Based on previous results and the present findings, we propose a possible regulatory model (Figure 8) for the role of *OseIF3e* in these processes: (i) *Osj10gBTF3* regulates transcription of *OseIF3e* and *OseIF3h* in the nucleus (Wang et al., 2012); (ii) in the cytosol, *OseIF3e* interacts with *OseIF3* subunits b, d, e, f, h, and k, and with *eIF6*, to form homo- and heterodimers; (iii) the *eIF* complex interacts with *OsICKs* to regulate cell division, affecting plant growth and development. These findings may lead to a better understanding of the factors influencing plant growth and pollen development. Moreover, *OseIF3e*-mediated regulation of *OsICKs* genes pleiotropically modulates several plant characteristics (e.g., plant height, spikelet number, and seed size), providing an opportunity to optimize crop architecture for crop breeding.

AUTHOR CONTRIBUTIONS

WW and JT conceived and designed the project, analyzed the data, and wrote the manuscript. MX and XL helped with data analysis. The manuscript was approved by all other authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.01399>

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High-Throughput MicroRNA and mRNA Sequencing Reveals That MicroRNAs May Be Involved in Melatonin-Mediated Cold Tolerance in *Citrullus lanatus* L.

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Transcriptional regulation of cold-responsive genes is crucial for exogenous melatonin-mediated cold tolerance in plants. Nonetheless, how melatonin regulates cold-responsive genes is largely unknown. In this study, we found that exogenous melatonin improved cold tolerance in watermelon by regulating expression of microRNAs (miRNAs). We identified a set of miRNAs that were regulated by melatonin under unstressed or cold conditions. Importantly, mRNA-seq analysis revealed that melatonin-induced downregulation of some miRNAs, such as *miR159-5p*, *miR858*, *miR8029-3p*, and *novel-m0048-3p* correlated with the upregulation of target genes involved in signal transduction (CDPK, BHLH, WRKY, MYB, and DREB) and protection/detoxification (LEA and MDAR) under cold stress. These results suggest that miRNAs may be involved in melatonin-mediated cold tolerance in watermelon by negatively regulating the expression of target mRNAs.

Keywords: cold stress, high-throughput sequencing, melatonin, microRNA, watermelon

INTRODUCTION

Plants have to endure various abiotic stresses due to their sessile life-style. In particular, cold stress is one of the destructive environmental stresses that considerably reduce both yield and quality of fruits and vegetables in tropics and subtropics (Rivero et al., 2001, 2002). Transitory as well as constant cold stress causes damage to cell membranes, which disrupts the balance between water uptake and transpiration, leading to dehydration in shoots. Cold stress decreases the photosynthesis rate by affecting stomatal movement (Foyer et al., 2002), and induces accumulation of reactive oxygen species (ROS) by disrupting electron transport system in both mitochondria and chloroplasts (Suzuki and Mittler, 2006). ROS at high concentration can damage membranes through lipid peroxidation, break DNA strand, and inactivate various vital enzymes (Cheng and Song, 2006).

To survive cold stress, plants have evolved a variety of stress response mechanisms that minimize damage, ensure proper cellular homeostasis, and enable plants to function under stressful condition. Molecular sensors distributed in different cellular compartments can sense any decrease in growth temperatures, and thereby generate secondary signals, such as Ca^{2+} , ROS, and inositol 1,4,5-trisphosphate (InsP), and activate different transcriptional regulators, such as basic helix-loop-helix (BHLH), INDUCER OF CBF EXPRESSION (ICE) 1, C-repeat-binding factor (CBF), WRKY, and MYB, via the activation of phosphoprotein kinases,

such as calcium-dependent protein kinases (CDPKs) and multiple mitogen-activated protein kinases (Xiong et al., 2002; Mahajan and Tuteja, 2005). Eventually, late embryogenesis abundant (LEA) proteins, chaperones, detoxification enzymes, pathogenesis-related proteins, mRNA/protein-binding proteins, proteinase inhibitors, transporters, lipid-transfer proteins, and enzymes required for osmoprotectant biosynthesis are induced to maintain normal physiological processes including photosynthesis (Janská et al., 2010).

Melatonin (*N*-acetyl-5-methoxytryptamine), a low molecular-weight molecule with an indole ring in its structure, is ubiquitous in living organisms (Hardeland et al., 2011; Tan et al., 2012). In vascular plants, melatonin was first identified in 1995 (Dubbels et al., 1995; Hattori et al., 1995) and numerous subsequent studies have established its important roles in plant growth, development, and defense against various abiotic and biotic stresses, such as salinity, drought, cold, excess copper, and pathogens (Zhang et al., 2014; Arnao and Hernández-Ruiz, 2015). Previously, the primary role of melatonin in stress mitigation was considered as a broad spectrum antioxidant that directly scavenges ROS and/or modulates cellular antioxidant system (Wang P. et al., 2012; Zhang and Zhang, 2014; Manchester et al., 2015). Recent studies have revealed that melatonin could also activate defense systems by regulating the expression of stress-responsive genes involved in signal transduction. For example, exogenous melatonin upregulates the expression of cold-responsive genes such as *C₂H₂-type zinc finger transcription factor (ZAT) 10*, *ZAT12*, *CBFs*, *cold-responsive gene (COR) 15*, and *calmodulin-binding transcription activator (CAMTA) 1* under cold stress (Bajwa et al., 2014). Furthermore, Shi and Chan (2014) reported that the AtZAT6-activated CBF pathway might be essential for melatonin-mediated response to freezing stress in *Arabidopsis*. However, the mechanism by which melatonin regulates the expression of stress-responsive genes to activate defense networks is unclear.

MicroRNAs (miRNAs) with a length of 19–25 nucleotides (nt), are highly conserved, endogenous, single-stranded non-coding RNA molecules. An increasing number of studies have demonstrated that miRNAs are important regulators in plant development and stress responses. Generally, miRNAs regulate the response to biotic and abiotic stresses by binding to reverse complementary sequences, resulting in the cleavage or translational inhibition of target mRNAs (Khraiwesh et al., 2012; Sunkar et al., 2012). For instance, *miR398* is downregulated to release its suppression of *CSD1* and *CSD2* in plant responses to oxidative stress (Sunkar et al., 2006). The increased sensitivity of *TamiR159*-overexpressing rice lines to heat stress suggests that the downregulation of *TamiR159*, which targets *TaGAMYB1* and *TaGAMYB2* in wheat, is involved in a heat stress-related signaling pathway, and therefore contributes to heat stress tolerance (Wang Y. et al., 2012). Transcriptional control of the expression of cold-responsive genes is well known, but miRNAs have recently been added to the suite of cold-responsive gene regulatory networks. Various miRNAs that target stress-related genes are significantly up- or downregulated during cold stress in a range of plant species (Khraiwesh et al., 2012; Sunkar et al., 2012).

Watermelon (*Citrullus lanatus* L.), one of the most economically important crops in the world, is highly sensitive to low temperatures (Rivero et al., 2002). In this study, by assessing the watermelon plants in terms of leaf phenotype, the maximum quantum yield of PSII (F_v/F_m), and relative electrolyte leakage (REL) under cold stress, we found that exogenous melatonin could enhance watermelon tolerance to cold stress. To understand the molecular mechanisms of melatonin-mediated cold tolerance in watermelon, we carried out a high-throughput miRNA and mRNA sequencing. The results showed that melatonin up- or downregulated a set of miRNAs under normal temperature or cold stress conditions. Importantly, under the cold stress, the downregulation of some miRNAs, such as *miR159-5p*, *miR858*, *miR8029-3p*, and *novel-m0048-3p*, by melatonin may cause the upregulation of putative target genes involved in signal transduction (CDPK, BHLH, WRKY, MYB, and DREB) and protection/detoxification (LEA and MDAR), suggesting that some miRNAs may be involved in melatonin-mediated cold tolerance in watermelon.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Treatments

Watermelon (*C. lanatus* L., cv. Y134) seeds were surface sterilized with 5% sodium hypochlorite (NaOCl) solution for 5 min and then rinsed with running water and distilled water. The sterilized seeds were soaked in distilled water for 8 h and sown directly in pots filled with a mixture of peat/vermiculite (3/1, v/v). Plants were grown in growth chambers with the following environmental conditions: a constant relative humidity of 60–70%, a 12-h photoperiod, 25/18°C (day/night), and a photosynthetic photon flux density of 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The plants were watered daily and fertilized with Hoagland's nutrition solution at 1-day interval.

Seedlings at the four-leaf stage were sprayed with 150 μM melatonin solution for 3 days, with distilled water used as the control. The melatonin (Sigma-Aldrich, St. Louis, MO, USA) solutions were prepared by dissolving the solute in ethanol followed by dilution with Milli-Q water [ethanol/water (v/v) = 1/10,000]. Each plant was sprayed with 20 mL of solution. Twelve hours after the third spray of melatonin, the plants were exposed to cold stress (i.e., 4°C) for 36 h with a 12-h photoperiod and photosynthetic photon flux density of 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Leaf samples were harvested at different time-points after imposition of cold stress, such as at 0, 3, 6, 12, 24, and 36 h to analyze *Clao20078 (CBF1)* and *Clao20702 (MYB)* transcript levels, at 6 h to sequence miRNAs and mRNAs, and at 36 h to analyze cold tolerance.

Analysis of Chlorophyll Fluorescence and REL

The maximum photochemical efficiency of PSII (F_v/F_m) was measured with Portable Chlorophyll Fluorometer (PAM2500; Heinz Walz, Effeltrich, Germany), after the whole plants were

dark adapted for 30 min. Minimal fluorescence (F_o) was measured during the weak measuring pulses and maximal fluorescence (F_m) was measured by a 0.8-s pulse light at $4,000 \mu\text{mol m}^{-2} \text{s}^{-1}$. F_v/F_m was determined using the third leaf of watermelon plants counting from the bottom to up. The calculation of F_v/F_m was done by the following formula as described by van Kooten and Snel (1990). $F_v/F_m = (F_m - F_o)/F_m$.

REL in the leaves was determined as previously described elsewhere (Zhou and Leul, 1998).

Library Construction and Sequencing of Small RNA

Eight independent small RNA libraries from plant leaves for four treatments (Control, CK; Melatonin, MT; Cold; Melatonin+Cold, MT-C), with two biological replicates for each treatment, were sequenced. The sequences have been deposited into the NCBI Sequence Read Archive database (SRP078211, SRA438995). Library construction and sequencing of small RNA were performed using a service provider Gene Denovo Co. (Guangzhou, China). Total RNA was extracted using miRNeasy Mini Kit (Cat#217004, QIAGEN GmbH, Germany) according to the manufacturer's protocol. Total RNA integrity was measured on an Agilent 2100 Bioanalyzer system (Agilent) for quality control. A total of 16–35 nt RNA fragments were excised, purified from a PAGE gel, and ligated with 5' and 3' adaptors using T4 RNA ligase. Reverse transcription followed by PCR was used to create cDNA constructs based on the small RNA ligated with 3' and 5' adaptors. Subsequently, the amplified cDNA constructs were purified from agarose gel, in preparation for sequencing analysis using the Illumina Genome Analyzer (Illumina, CA, USA) according to the manufacturer's instructions.

Identification and Differential Expression Analysis of Known and Novel miRNAs

The raw sequences were firstly processed by Illumina's Genome Analyzer Pipeline software to filter out the adapter sequences, low quality as well as low-copy sequences. Then, the extracted small RNA sequences with 15–26 nt in length were subjected to cucurbit species mRNAs, Rfam¹ and Repbase² to discard mRNA, rRNA, tRNA, snRNA, snoRNA and repeat sequences. Finally, the remaining unique sequences were analyzed by BLAST against miRBase (Release 21)³. Solexa sequences with identical or related (one mismatch) sequences from mature miRNAs were identified as known miRNAs (Meyers et al., 2008).

To identify potential novel miRNAs in watermelon, rest of the unmapped small RNA sequences were searched by BLAST against watermelon genome downloaded from cucumber genome database⁴. The mappable sequences were then folded into a secondary structure using UNAFold software⁵. Only the non-coding sequences which could form a perfect stem-loop structure and meet the criteria for miRNAs prediction (Meyers

et al., 2008) were then considered to be a potential novel miRNA candidate.

We chose the miRNAs increased or decreased by more than twofold and P -value < 0.01 in two treatments as the criterion for a melatonin or cold response. Then, the heat map of differentially expressed miRNAs expression profile was drawn with MultiExperiment Viewer version 4.0 and clustering analysis was performed using a hierarchical clustering method (Eisen et al., 1998).

Expression Analysis of Predicted Target Genes Based on High-Throughput Sequencing

Eight independent mRNA libraries from plant leaves for four treatments (Control, CK; Melatonin, MT; Cold; Melatonin+Cold, MT-C), with two biological replicates for each treatment, were sequenced. The sequences have been deposited into the NCBI Sequence Read Archive database (SRP078211, SRA438977). Library construction and sequencing of mRNA were performed using a service provider Gene Denovo Co. (Guangzhou, China). After removal of low quality sequences, the clean reads were mapped to the watermelon reference genome, allowing up to one mismatch. The differentially expressed genes were identified using the R package edgeR (Robinson et al., 2010). The expression level of each unigene was calculated and normalized to generate FPKM (fragments per kilobase of exon per million mapped fragments). The false discovery rate (FDR) was used to determine the threshold of the P -value in multiple tests. In this study, the FDR < 0.05 and fold change ≥ 1.5 were used as significance cut-offs of the gene expression differences.

Prediction of Putative Target Genes of Melatonin- or Cold-Responsive miRNAs

A plant miRNA target prediction server⁶, was used to predict putative miRNA target genes with default settings based on the library of Watermelon genome database, version 1. It reports all potential sequences complementary to an inquiring miRNA sequence with mismatches no more than a specified value for each mismatch type. The minimal weighed score ≤ 3.0 was applied in the prediction according to scoring schema of miRU by Zhang (2005). The other default settings as follows: length for complementarity scoring (hspsize), 20 bp; target accessibility-allowed maximum energy to unpair the target site (UPE), 25.0; flanking length around target site for target accessibility analysis, 17 bp in upstream/13 bp in downstream; range of central mismatch leading to translational inhibition, 9–11 nt (Brodersen et al., 2008).

RNA Extraction and Quantitative Real-time PCR Analysis

Two cold-sensitive genes *Cla020078* (*CBF1*) and *Cla020702* (*MYB*) were chose to perform quantitative real-time PCR (qRT-PCR). Total RNA was extracted using a RNA extraction kit (Tiangen, Beijing, China) according to the supplier's instructions.

¹<http://rfam.xfam.org/>

²<http://www.girinst.org/>

³<http://www.mirbase.org/>

⁴<http://cucumber.genomics.org.cn/>

⁵<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>

⁶<http://plantgrn.noble.org/psRNATarget/>

DNA contamination was removed using a purifying column. One microgram of total RNA was reverse-transcribed using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) following the supplier's instructions. The gene-specific primers for qRT-PCR were designed based on their cDNA sequences, as follows: *Cla020078* (F, AGCAGAGCCCTAACACAGGT; R, AATGGTCTTGAGTTGGG), *Cla020702* (F, GATCCATTGACGGCACTAAC; R, TCGCTACAACGTCCTTCATC), and watermelon β -actin gene (F, CCATGTATGTTGCCATCCAG; R, GGATAGCATGGGGTAGAGCA) was used as an internal control (Kong et al., 2014). The qRT-PCR assays were performed using an iCycler Iq Multicolor PCR Detection System (Bio-Rad, Hercules, CA, USA). PCRs were performed using the SYBR Premix ExTaq II (2 \times) Kit (Takara, Tokyo, Japan). The PCR conditions consisted of denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. The quantification of mRNA levels was based on the method of Livak and Schmittgen (2001).

Statistical Analysis

The experiment was a completely randomized design with three replicates. Each replicate contained at least 10 plants. Analysis of variance was used to test for significance, and significant differences ($P < 0.05$) between treatments were determined using Tukey's test.

RESULTS

The Effects of Melatonin on Cold Stress Tolerance and Cold-Responsive Gene Expressions in Watermelon

Melatonin plays important regulatory roles in plant defense against various biotic and abiotic stresses (Zhang et al., 2014). In this study, we first analyzed the effects of exogenous melatonin on watermelon tolerance to cold stress. As shown in **Figure 1**, melatonin had a minimal effect on the leaf phenotypes, the maximum quantum yield of PSII (F_v/F_m), and REL at optimal growth temperatures. After 4°C treatment for 36 h, the blade edges of watermelons were wilted, and the F_v/F_m and REL were significantly reduced and increased, respectively. However, pretreatment with melatonin obviously alleviated cold-induced wilting of blade edges and the reduction and increase in leaf F_v/F_m and REL, respectively. For example, the F_v/F_m and REL decreased and increased by 15.45 and 19.82%, respectively in melatonin-pretreated plants after cold treatment, far less than the changes of 31.06 and 50.51% in control plants (**Figure 1**). Taken together, exogenous melatonin significantly enhanced watermelon tolerance to cold stress.

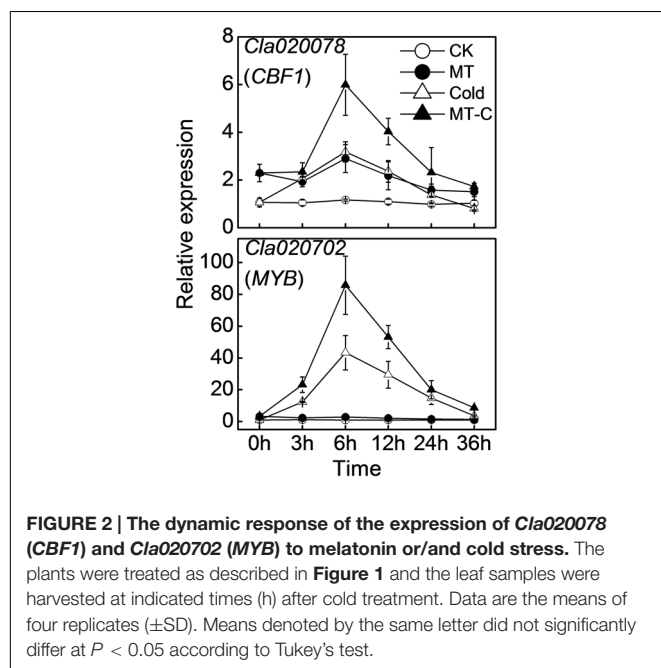
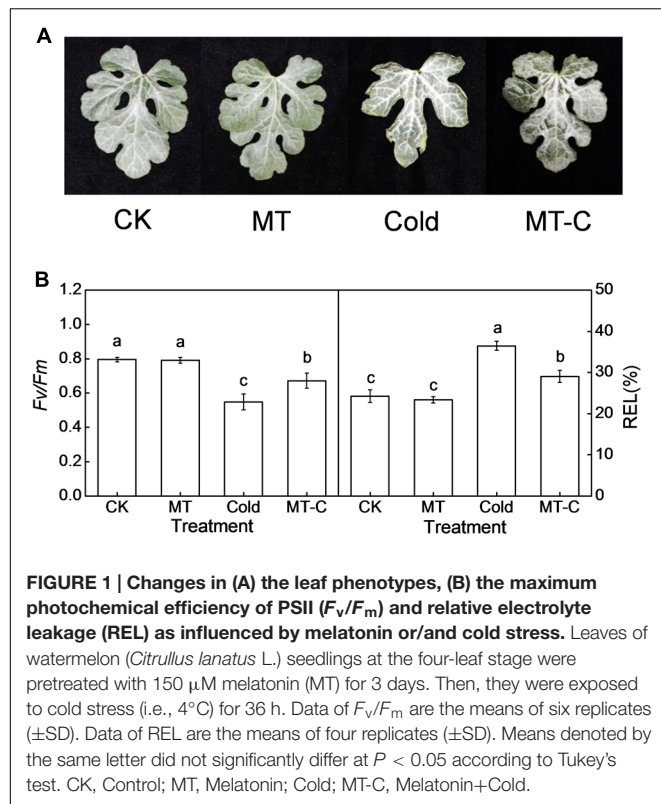
To analyze the effects of melatonin on the expression of cold-responsive genes and to choose the appropriate time point for miRNA expression profiling, we examined the dynamic responses of *Cla020078* (*CBF1*) and *Cla020702* (*MYB*) to melatonin or/and cold treatment. Transcript levels of *CBF1* and *MYB* were slightly

induced by melatonin at optimal growth temperatures (**Figure 2**). After cold treatment (4°C), they were rapidly upregulated, reaching peak levels at 6 h, and subsequently declining to original levels at 36 h. Furthermore, pretreatment with melatonin improved the induction of *CBF1* and *MYB* expression by cold stress. For instance, at 6 h after cold treatment, the expression levels of *CBF1* and *MYB* in melatonin-pretreated plants were upregulated by 5.99- and 85.69-fold, respectively, far more than the increases of 3.18- and 43.38-fold observed in control plants.

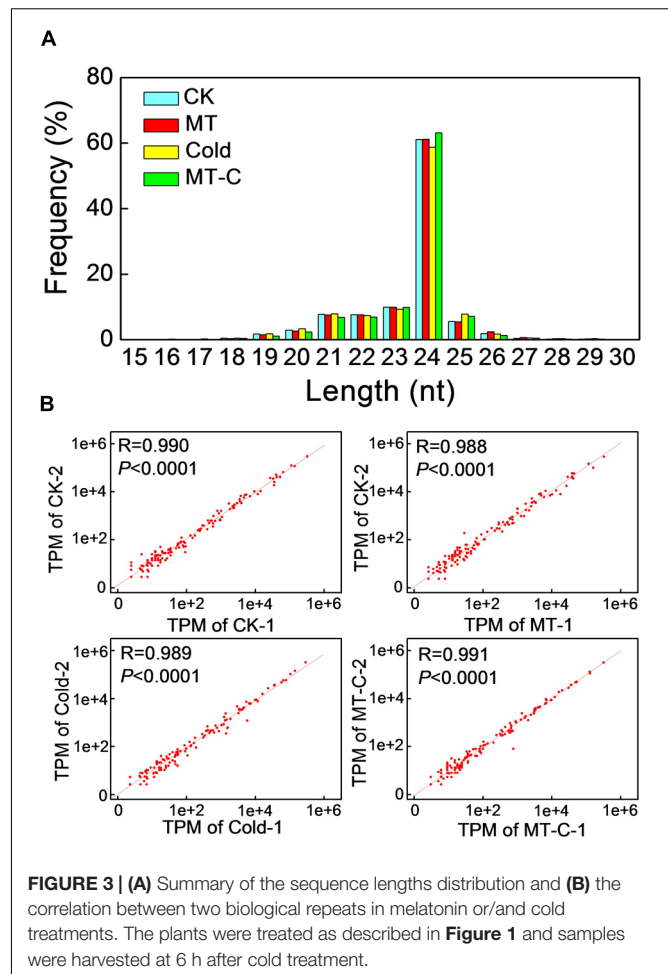
Analysis of MicroRNA Response to Melatonin or/and Cold Based on High-Throughput Sequencing

To examine whether miRNAs are involved in melatonin-mediated cold response in watermelon, we performed high-throughput sequencing analysis of miRNA in watermelon leaves treated with and without the melatonin and cold stress. A total of 9,704,684/10,831,539 (Control, CK-1/2), 10,124,858/12,383,237 (Melatonin, MT-1/2), 12,812,180/9,718,613 (Cold-1/2), and 9,806,063/11,489,173 (Melatonin+Cold, MT-C-1/2) raw reads were obtained (**Supplementary Table S1**). After the removal of rRNAs, tRNAs, snRNAs, and snoRNAs, a total of 5,327,079/5,914,071, 5,635,109/6,581,846, 6,219,825/5,085,935, and 5,409,138/6,272,887 sRNA sequences for CK-1/2, MT-1/2, Cold-1/2, and MT-C-1/2 were obtained, respectively. The majority of redundant reads were in the range of 19–26 nt, among which the most abundant sequences were 24 nt long in all libraries (**Figure 3A**). To identify miRNAs in watermelon, all sRNA sequences were compared to known plant miRNAs in miRBase Release 21. A total of 440 known unique miRNAs with high sequence similarity to known plant miRNAs were identified (**Supplementary Table S2**). By mapping all unique sRNA sequences to the watermelon genome and predicting the hairpin structures for their flanking sequences, 106 novel miRNAs candidates were identified. The expression level of each miRNA was normalized to generate TPM (tags per million) and the correlation of detected miRNA expression between two biological repeats for each treatment was analyzed. As shown in **Figure 3B**, there was a strong correlation ($R = 0.99$, $P < 0.0001$) between the two biological replicates for all four treatments, suggesting that the sequencing results are highly reliable.

A total of 50 miRNAs were significantly up- or downregulated by melatonin under unstressed or cold conditions (**Figure 4**). Among them, four miRNAs such as *miR170-5p*, *novel-m0074-3p*, *novel-m0044-3p*, and *novel-m0040-3p* were upregulated, while one miRNA (*miR399-5p*) were up- and downregulated by melatonin pretreatment, under both unstressed and cold conditions. Ten and nine miRNAs were specifically up- and downregulated by melatonin at optimal growth temperatures, respectively. Twelve and 20 miRNAs were specifically up- and downregulated by melatonin after cold treatment for 6 h, respectively. A total of 54 miRNAs were significantly up- or downregulated by cold stress in control or melatonin-pretreated plants, respectively. Among them,



four miRNAs (*miR399-5p*, *miR170-3p*, *miR482-5p*, and *novel-m0031-5p*) and three miRNAs (*miR159-5p*, *novel-m0033-5p*, and *novel-m0038-5p*) were up- and downregulated by cold stress, respectively, in both control and melatonin-pretreated plants. In total, 18 and 12 miRNAs were specifically up- and downregulated by cold in control plants,

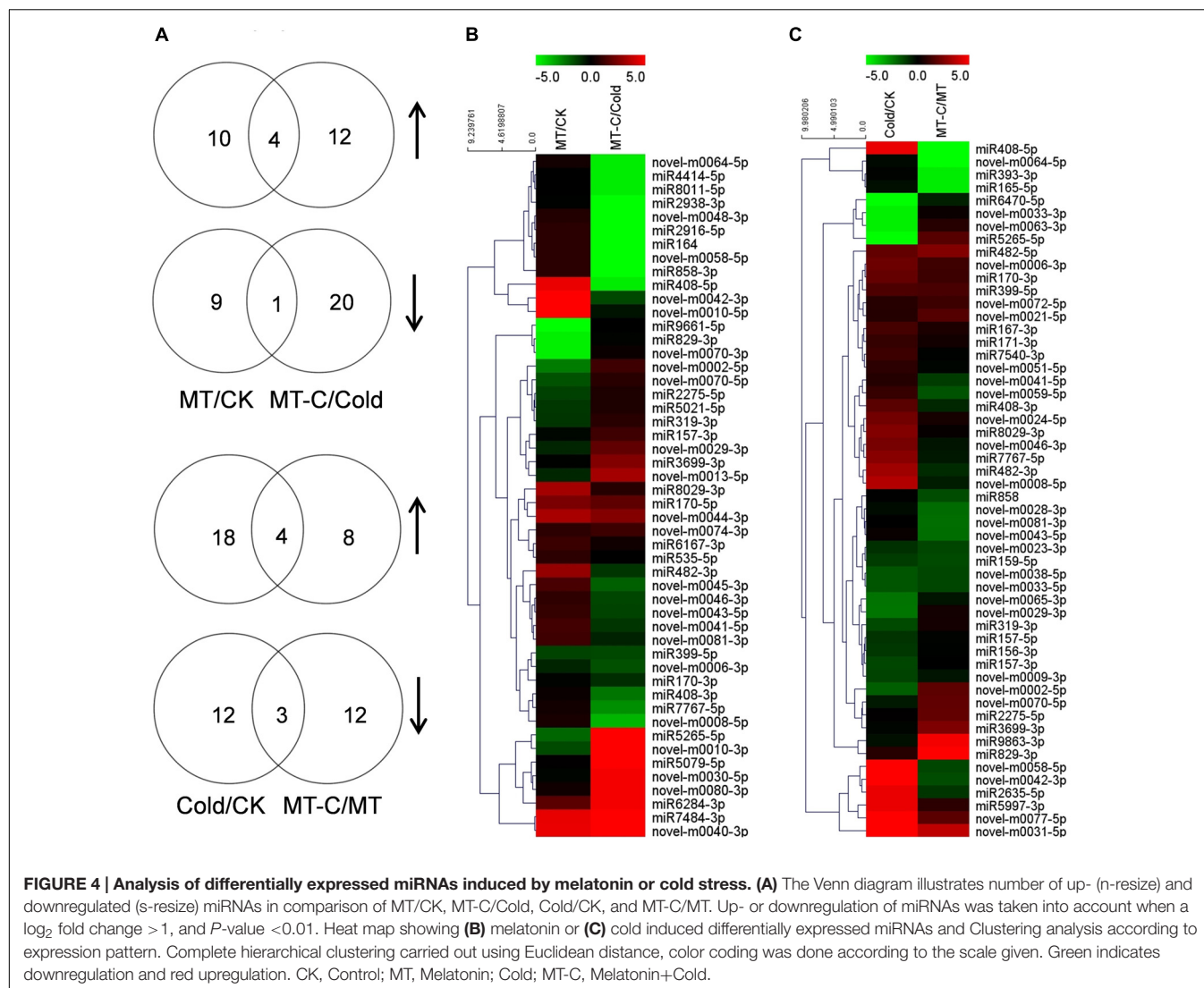


respectively. While 8 and 12 miRNAs were specifically up- and downregulated by cold in melatonin-pretreated plants, respectively.

Biological Function Analysis of Differentially Expressed miRNAs via Prediction of Target mRNAs

To examine the biological functions of differentially expressed miRNAs, the miRNA sequences were searched against watermelon genomic sequences using the plant miRNA potential target finder⁷ to predict target mRNAs. miRNAs negatively regulate mRNAs by inducing their cleavage or repressing their translation (Bartel, 2004). In this study, a total of 505 mRNAs were predicted to be cleaved by miRNAs. Then, we performed RNA-seq analysis in watermelon leaves treated with and without the melatonin and cold stress using the high-throughput Illumina Solexa system. Reads from each sample were aligned to the *C. lanatus* reference genome. The expressions of predicted mRNAs in comparison of MT/CK, MT-C/Cold, Cold/CK, and MT-C/MT were analyzed. The expression patterns of only 49 detected mRNAs were opposite

⁷<http://plantgrn.noble.org/psRNATarget/>



with respect to those of 21 corresponding miRNAs (Table 1). The negative correlation coefficients between miRNAs and their target mRNAs regulated by melatonin and cold were -0.74 ($P < 0.001$) and -0.48 ($P < 0.001$), respectively, suggesting that these 49 target mRNAs were likely cleaved by the corresponding miRNAs (Figure 5). Additionally, 34 miRNAs were searched against a total of 87 potential target mRNAs via translational inhibition (Supplementary Table S3). The functions of these mRNAs were annotated in the Cucurbit Genomics Database⁸.

Some of the 27 potential target mRNAs cleaved by melatonin-responsive miRNAs were induced or repressed by melatonin at optimal growth temperatures, but not under cold stress (Figure 6A; Supplementary Table S4). Interestingly, most miRNAs including some cold-responsive genes, such as *Cla011198* (*BHLH*), *Cla017355* (*WRKY*), and *Cla022458* (*DREB*, *dehydration-responsive element-binding gene*), were upregulated

by melatonin during cold stress, but were not affected by melatonin under unstressed conditions. Most of 25 potential target mRNAs cleaved by cold-responsive miRNAs were repressed or unaffected by cold stress, except for *Cla017745* (*LEA*) and *Cla017373* (*R2R3-MYB*), those were upregulated (Figure 6B). However, melatonin pretreatment alleviated cold-induced repression of target mRNAs or improved cold-induced increases in target mRNAs. For instance, *LEA* and *R2R3-MYB* in melatonin-pretreated plants were upregulated by 5.53- and 14.91-fold under cold stress, far more than the changes of 2.82- and 2.44-fold in control plants, respectively. *MYB*, *MDAR* (*monodehydroascorbate reductase*), and *PPRP* (*pentatricopeptide repeat protein*) were significantly repressed by cold stress in control plants, but were minimally affected in melatonin-pretreated plants. Accordingly, a set of potential target mRNAs cleaved by corresponding miRNAs might be involved in the response of melatonin-pretreated plants to cold stress.

We also subjected the predicted target mRNAs to Gene Ontology classification based on their involvement in process

⁸<http://www.icugi.org>

TABLE 1 | Potential target mRNAs cleaved by differentially expressed miRNAs.

miRNA	Target	Log ₂ fold change of miRNAs/targets				Description
		MT/CK	MT-C/Cold	Cold/CK	MT-C/MT	
miR159-5p	Cla001826	−0.14/0.21	−0.63/0.34	−1.19*/0.53	−1.45*/0.66*	Calcium-dependent protein kinase (CDPK)
miR170-3p	Cla006665	−0.08/−0.04	−1.09*/0.31	2.02*/−0.63*	1.20*/−0.28	GRAS family transcription factor
miR171-3p	Cla006665	0.20/−0.04	−0.31/0.31	1.04*/−0.63*	0.39/−0.28	GRAS family transcription factor
miR399-5p	Cla008388	−1.31*/0.65*	−1.43*/0.93*	1.55*/−3.83*	1.43*/−3.55*	Pentatricopeptide repeat protein 77 (PPRP77)
	Cla005858	/0.23	/−0.18	/−0.88*	/−1.29*	NEFA-interacting nuclear protein NIP30
miR482-5p	Cla014776	−0.05/0.10	0.54/−0.85	2.01*/−0.67	2.60*/−1.61*	Os10g0422600
	Cla013089	/0.43	/−0.97	/−3.27*	/−4.67*	Leucine-rich repeat family protein (LRR)
	Cla009419	/0.12	/−5.36	/2.59*	/−8.06*	Unknown
	Cla016187	/0.86	/−1.31	/−0.56	/−2.57*	C ₂ H ₂ zinc-finger transcription factor (C ₂ H ₂ -ZFTF)
miR858	Cla017745	−0.08/−2.84	−0.11/−0.23	−0.04/1.29	−1.02*/3.90*	Late embryogenesis abundant (LEA) protein like
	Cla017373	/−0.67	/0.30	/1.50*	/2.47*	R2R3 MYB transcription factor
miR2635-5p	Cla021237	7.03/−0.19	−7.96/0.14	4.64*/−0.70*	−1.04/−0.37	Cryptic precocious CRP/Med12
miR2916-5p	Cla001518	0.86/0.39	−5.18*/0.60*	8.51/−1.31*	0.00/−1.09*	DNA primase large subunit
miR5021-5p	Cla010003	−1.08*/0.64*	0.59/−0.02	−0.21/1.13*	1.47/0.46	3-ketoacyl-CoA reductase 2 (KCR2)
	Cla015503	/1.53*	/−0.69	/0.91	/−1.32	RING-H2 zinc finger protein (RING-H2 ZFP)
	Cla001461	/1.33*	/0.25	/0.02	/−1.06	Unknown
	Cla018974	/1.09*	/0.36	/−0.65	/−1.38	J023065D24
miR5997-3p	Cla009517	0.00/−0.12	−0.88/0.33	4.64*/−1.12*	0.88/−0.67	Vacuolar protein sorting-associated protein (VPS) 13
	Cla021487	/−0.09	/0.30	/−0.73*	/−0.35	Cysteine-rich repeat secretory protein (CRRS) 12
miR7540-3p	Cla015655	1.41/−0.15	0.17/1.01	1.19*/−1.33*	−0.05/−0.18	Fasciclin-like arabinogalactan protein (FLA) 2
miR8029-3p	Cla018379	3.29*/−0.17	0.80/0.70	2.60*/−0.68*	0.15/0.18	Monodehydroascorbate reductase (MDAR)
	Cla008020	/0.01	/0.49	/−0.68*	/−0.21	Cyclase/dehydrase
	Cla002580	/−0.10	/0.33	/−0.80*	/−0.36	Lysine-specific demethylase (LSD) 5A
	Cla012681	/−0.29	/0.36	/−0.92*	/−0.27	Pentatricopeptide repeat-containing protein (PPRP)
	Cla004188	/−0.42	/−0.04	/−0.62*	/−0.24	MYB transcription factor
	Cla004079	/−1.03*	/−0.50	/−3.11*	/−2.58	Unknown
	Cla011614	/−0.62*	/−0.18	/−0.27	/0.17	Kinesin like protein
	Cla014313	/−0.77*	/−0.23	/−0.40	/0.13	Peroxidase (POD)
novel-m0006-3p	Cla012226	−0.70/−0.11	−1.59*/0.59*	2.14*/−0.25	1.26/0.46	YABBY
hboxnovel-m0030-5p	Cla014681	−0.11/0.88	4.84*/−1.50*	−8.14/−2.35*	0.13/−4.73*	K ⁺ transporter
novel-m0045-3p	Cla003322	1.44/−0.47	−1.89*/0.71*	0.97/−1.11*	−2.40/0.08	Altered inheritance of mitochondria protein (AIMP) 32
novel-m0048-3p	Cla014357	0.71/0.11	−5.18*/0.60*	1.36/−2.46*	−7.90/−1.97*	Glycyl-tRNA synthetase (GARS)
	Cla015456	/−0.46	/0.65*	/−1.87*	/−0.76	Nitrate transporter (NRT)
	Cla022458	/0.48	/1.55*	/−1.03	/0.05	Dehydration-responsive element-binding protein (DREB)
	Cla017676	/−0.24	/1.22*	/−1.47	/−0.01	Kinesin-5 transcription factor
	Cla011198	/0.00	/0.74*	/0.89	/1.63	BHLH transcription factor
	Cla009841	/0.18	/0.73*	/−0.98	/−0.43	Ethylene-regulated nuclear protein ERT2-like
	Cla010176	/−0.08	/0.65*	/0.44	/−0.29	TCP transcription factor
	Cla006034	/0.66	/1.31*	/−0.15	/0.50	Ankyrin repeat-containing protein (Akr1p)
	Cla018866	/−0.14	/0.63*	/−0.49	/0.27	Nucleolar and coiled-body phosphoprotein (NOLC) 1
	Cla016714	/0.10	/1.82*	/−2.37	/−0.66	Os02g0202300
	Cla017355	/0.05	/0.71*	/−1.90*	/−1.23*	WRKY transcription factor
	Cla006039	/−0.31	/1.07*	/−2.55*	/−1.17	Protein forked1
novel-m0058-5p	Cla015802	0.86/0.10	−5.89*/1.19*	5.89*/−3.96*	−1.36/−2.87*	Pentatricopeptide repeat-containing protein (PPRP)
	Cla005115	/0.15	/0.80*	/−1.39*	/−0.74	Chromatin remodeling complex subunit (SWI/SNF)
novel-m0059-5p	Cla019857	1.98/−1.02	−0.82 / 1.01	1.10/0.35	−1.63*/2.38*	TIR-NBS-LRR disease resistance protein
novel-m0070-5p	Cla005664	−1.60*/0.24	0.83/−0.35	−0.50/0.00	1.93*/−0.59*	Nitrate transporter (NRT)
	Cla016054	/0.96*	/0.22	/−0.61	/−1.35	UDP-glucosyltransferase (GTase) 1
novel-m0077-5p	Cla004726	0.00/−0.13	−0.24/0.46	5.58*/−0.87*	1.82/−0.28	IQ-domain 10
novel-m0081-3p	Cla004682	1.25*/−0.46	−0.69/0.17	0.06/0.14	−2.17*/0.77*	Serine/threonine-protein phosphatase (Ser/Thr-PPs)

*Indicates significant difference.

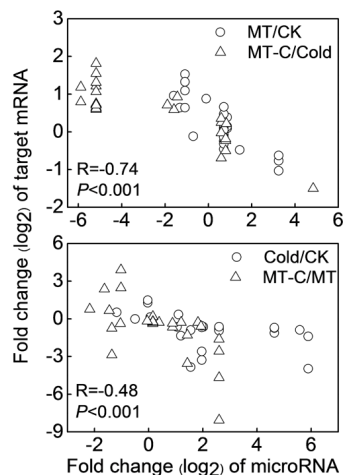


FIGURE 5 | Correlation analysis between the expression of miRNAs and predicted targets by cleavage in comparison of MT/CK, MT-C/Cold, Cold/CK, and MT-C/MT.

in Cucurbit Genomics Database⁹ with watermelon 97103 v1 (Figure 7; Supplementary Figure S1). The target genes inhibited by miRNAs via cleavage and translational repression were involved in various biological processes, with 19 and 38 mRNAs involved in cellular process regulation, 18 and 31 mRNAs involved in responses to biotic or abiotic stress, and 5 and 10 mRNAs involved in signal transduction, respectively. Thus,

⁹<http://www.icugi.org>

a number of potential target mRNAs might be involved in watermelon responses to cold stress by direct or indirect ways.

DISCUSSION

Melatonin Induces Cold Stress Tolerance and Cold-Responsive Gene Expressions in Watermelon

In recent years, melatonin has emerged as a focus of research in plant science. It functions in many aspects of plant growth and development, and regulates plant tolerance to abiotic stresses, such as salinity, drought, radiation, excess copper, and chemical stresses (Zhang et al., 2014). Recently, several studies have shown that exogenous melatonin with optimum concentrations enhances cold tolerance in *Arabidopsis* and *Triticum aestivum* L. (Bajwa et al., 2014; Shi and Chan, 2014; Turk et al., 2014), since the initial observation that exogenous melatonin attenuates cold-induced apoptosis in carrot suspension cells (Lei et al., 2004). In the current study, cold stress caused wilted blade edges, decreased maximum photochemical efficiency of PSII (F_v/F_m), and increased REL, which indicates membrane damage (Figure 1). Notably, in agreement with previous studies, the adverse effects of cold were significantly alleviated by the application of melatonin. Moreover, melatonin treatment up-regulated two cold-responsive genes *Cla020078* (*CBF1*) and *Cla020702* (*MYB*) in watermelon, which is in line with previous studies in *Arabidopsis* (Bajwa et al., 2014; Shi and Chan, 2014), suggesting that melatonin activates signaling pathways during cold stress via regulation of cold-responsive genes (Figure 2).

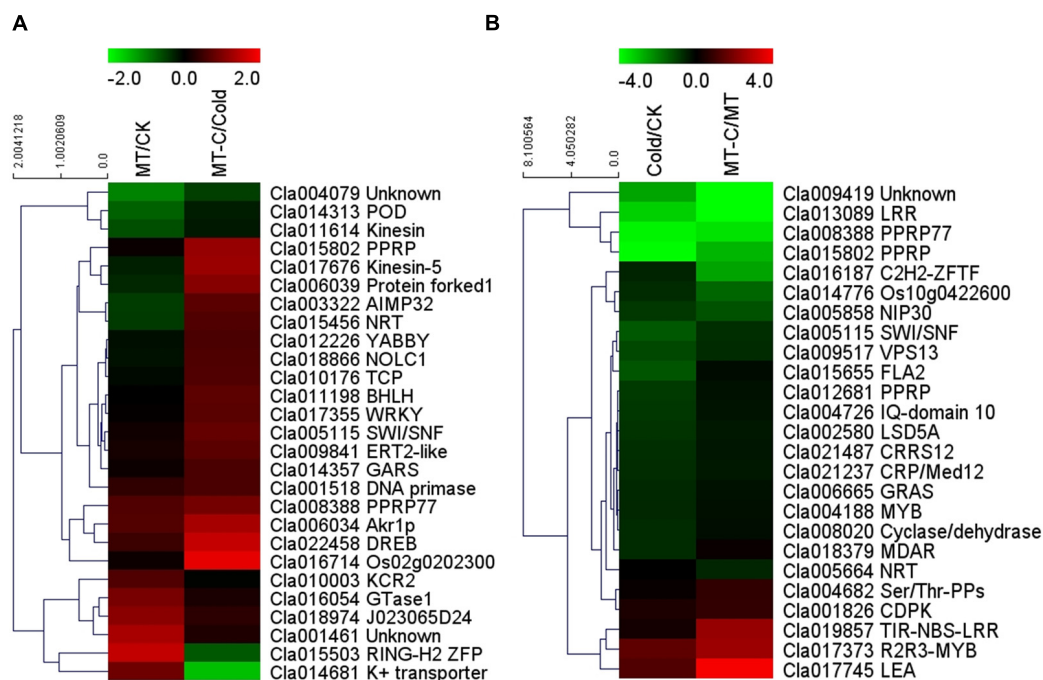


FIGURE 6 | Heat map showing (A) melatonin or (B) cold induced differential expression of the potential target mRNAs.

Melatonin Affects the Expression of a Number of MicroRNAs under Optimal Growth Conditions or Cold Stress

Transcriptional control of the expression of cold-responsive genes is well known (Chinnusamy et al., 2010), but miRNAs, which could regulate stress responses by controlling the expression of cognate target genes, have recently been added to the suite of cold-responsive gene regulatory networks. Comparative profiles of miRNA expression among various plant species (*Arabidopsis*, *Brachypodium*, and *Populus*) during cold stress revealed similarities and differences in miRNA regulation (Liu et al., 2008; Lu et al., 2008; Zhang et al., 2009). In this study, 440 known miRNAs and 106 novel miRNAs were identified using Solexa high-throughput sequencing (Supplementary Table S2). Based on an analysis of differentially expressed miRNAs, we identified a set of miRNAs including 16 known miRNAs and 21 novel miRNAs that were significantly up- or downregulated by cold stress, which exhibited similarities and differences with other species (Figure 4; Khraiweh et al., 2012; Sunkar et al., 2012). The discrepancies in cold-responsive miRNA expression might be attributed to the differential responses of unrelated plant species and variation in experimental conditions and methods. Importantly, pretreatment with melatonin affected the expression of a set of miRNAs under unstressed or cold conditions, and changed the expression of cold-responsive miRNAs. These results suggest that a number of miRNAs are involved in melatonin-mediated enhancement in cold tolerance in watermelon. To the best of our knowledge, this is the first report on the interaction between melatonin and miRNAs in regulation of plant tolerance to stress, although a few studies have reported such interactions in animal cell lines (Lee et al., 2011; Sohn et al., 2015).

MicroRNAs May Be Involved in Melatonin-Mediated Cold Tolerance by Negatively Regulating Target mRNAs

To examine the functions of melatonin- or cold-responsive miRNAs, we predicted their target mRNAs. We detected 49 mRNAs with opposite expression patterns with respect to their corresponding miRNAs, and inferred that these mRNAs are likely cleaved by the corresponding miRNAs (Table 1). Among the 49 potential target mRNAs, several mRNAs were closely associated with plant defense against cold stress by activating signaling transduction or encoding functional proteins. These mRNAs include *Cla001826*, *Cla011198*, *Cla022458*, *Cla004188/Cla017373*, *Cla017355*, *Cla017745*, and *Cla018379* which encode CDPK, BHLH transcription factor, DREB protein, MYB transcription factor, WRKY transcription factor, LEA protein, and MDAR, respectively. As Ca^{2+} sensors, CDPKs those can directly bind Ca^{2+} , are major players in coupling cold stress signals to specific protein phosphorylation cascades, leading to the activation of downstream cold-responsive genes, such as *ICE1*, a MYC-type BHLH transcription factor (Janská et al., 2010). *ICE1* is activated by low temperatures and triggers *CBF/DREB* expression, which promotes the accumulation of cold-responsive gene products

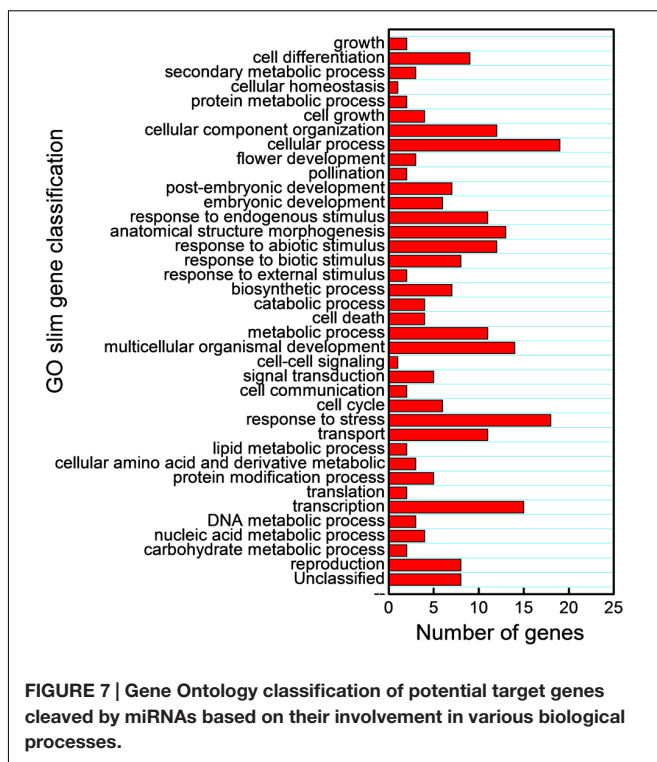


FIGURE 7 | Gene Ontology classification of potential target genes cleaved by miRNAs based on their involvement in various biological processes.

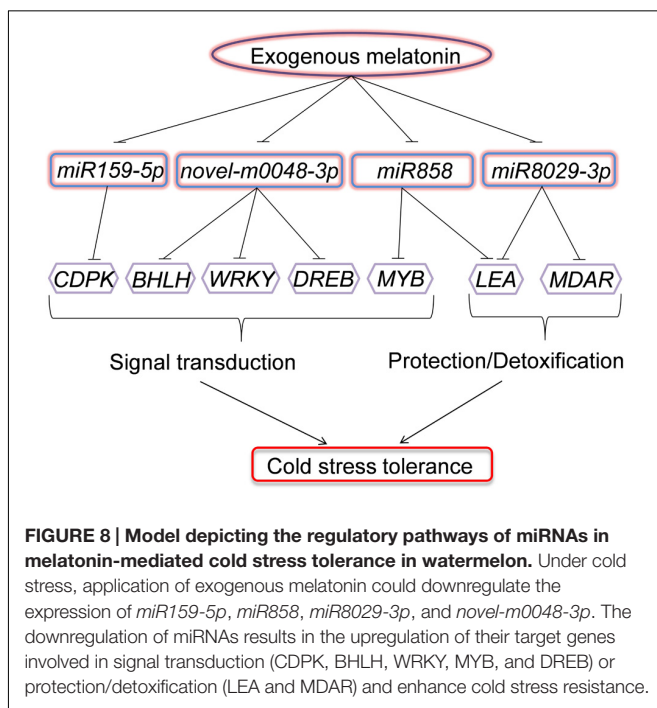


FIGURE 8 | Model depicting the regulatory pathways of miRNAs in melatonin-mediated cold stress tolerance in watermelon. Under cold stress, application of exogenous melatonin could downregulate the expression of *miR159-5p*, *miR858*, *miR8029-3p*, and *novel-m0048-3p*. The downregulation of miRNAs results in the upregulation of their target genes involved in signal transduction (CDPK, BHLH, WRKY, MYB, and DREB) or protection/detoxification (LEA and MDAR) and enhance cold stress resistance.

such as LEA, antioxidant enzymes, and molecular chaperones (Janská et al., 2010). MYB and WRKY transcription factor gene families have been suggested to play important roles in the regulation of transcriptional reprogramming associated with plant responses to cold stress (Shinozaki et al., 2003;

Zhu et al., 2005; Chen et al., 2012). Overexpression of some *MYB* or *WRKY* genes enhances plant tolerance to cold stress and activates the expression of a set of cold-responsive genes such as *LEA*, *DREB2A*, and genes encoding antioxidant enzymes (Dai et al., 2007; Niu et al., 2012; Yang et al., 2012). *LEA* is important for membrane stabilization and the protection of proteins from denaturation when the cytoplasm becomes dehydrated (Janská et al., 2010). *MDAR* is an important antioxidant enzyme that is critical to alleviate cold-induced oxidative damage (Chen and Paul, 2002). Importantly, these defense-related genes were significantly induced by melatonin, exhibiting contrasting expression patterns with respect to the corresponding miRNAs, including *miR159-5p*, *miR858*, *miR8029-3p*, and *novel-m0048-3p* (Figures 5 and 6). Accordingly, it could be inferred that some cold resistance pathways in melatonin-pretreated plants were promoted by a number of downregulated miRNAs under cold stress.

Some other potential target genes might also be involved in watermelon responses to cold stress by direct or indirect ways. For example, low temperatures can inhibit the uptake and transport of nitrate (Warren, 2009; Leffler et al., 2013; Dechatiwongse et al., 2014). The significantly upregulated expression of *Clat015456* (*NRT*) by melatonin during cold stress suggests that the active transport of nitrate could be helpful in the acclimation of plants under adverse environmental conditions (Zhang et al., 2005). Additionally, the differentially expressed miRNAs were predicted to target a set of mRNAs via translational repression (Supplementary Table S3). Some of these mRNAs, such as *MYB*, *MYC*, and *CYP450*, are important in transcriptional regulation and protection in cold responses, respectively. However, whether these predicted target mRNAs are regulated by corresponding miRNAs via translational inhibition requires further studies.

CONCLUSION

We demonstrated that exogenous melatonin induced watermelon tolerance to cold stress, and this induction was associated with the upregulation of cold-responsive genes. Moreover, we provided evidence for the alteration of the expression profile of a set of miRNAs regulated by melatonin with putative cold-responsive gene targets. In particular, the

downregulation of *miR159-5p*, *miR858*, *miR8029-3p*, and *novel-m0048-3p* by melatonin was coincident with the upregulation of putative target genes involved in signal transduction (CDPK, BHLH, WRKY, MYB, and DREB) and protection/detoxification (*LEA* and *MDAR*) under low-temperature conditions (Figure 8). To our knowledge, these results provide the first evidence for a potential regulatory role of miRNAs in melatonin-mediated cold tolerance in plants.

AUTHOR CONTRIBUTIONS

HL and XZ designed research; HL, YD, and JC performed research; HL, JH, HC, QL, CW, JM, YZ, JY, and XZ analyzed data; HL, YD, and XZ wrote and revised the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.01231>

FIGURE S1 | Gene Ontology classification of potential target genes of miRNAs with translational repression based on their involvement in process.

TABLE S1 | Reads abundance of various classification of small RNAs in control plants (CK) and plants treated by melatonin (MT), Cold, and melatonin+cold (MT-C).

TABLE S2 | Identified known and novel miRNAs in watermelon.

TABLE S3 | Predicted target mRNAs of differentially expressed miRNAs with translational inhibition.

TABLE S4 | The FPKM value of melatonin- or cold-responsive potential target mRNAs in each treatment.

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Identification, Characterization, and Functional Validation of Drought-responsive MicroRNAs in Subtropical Maize Inbreds

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MicroRNA-mediated gene regulation plays a crucial role in controlling drought tolerance. In the present investigation, 13 drought-associated miRNA families consisting of 65 members and regulating 42 unique target mRNAs were identified from drought-associated microarray expression data in maize and were subjected to structural and functional characterization. The largest number of members (14) was found in the zma-miR166 and zma-miR395 families, with several targets. However, zma-miR160, zma-miR390, zma-miR393, and zma-miR2275 each showed a single target. Twenty-three major drought-responsive cis-regulatory elements were found in the upstream regions of miRNAs. Many drought-related transcription factors, such as GAMYB, HD-Zip III, and NAC, were associated with the target mRNAs. Furthermore, two contrasting subtropical maize genotypes (tolerant: HKI-1532 and sensitive: V-372) were used to understand the miRNA-assisted regulation of target mRNA under drought stress. Approximately 35 and 31% of miRNAs were up-regulated in HKI-1532 and V-372, respectively. The up-regulation of target mRNAs was as high as 14.2% in HKI-1532 but was only 2.38% in V-372. The expression patterns of miRNA-target mRNA pairs were classified into four different types: Type I- up-regulation, Type II- down-regulation, Type III- neutral regulation, and Type IV- opposite regulation. HKI-1532 displayed 46 Type I, 13 Type II, and 23 Type III patterns, whereas V-372 had mostly Type IV interactions (151). A low level of negative regulations of miRNA associated with a higher level of mRNA activity in the tolerant genotype helped to maintain crucial biological functions such as ABA signaling, the auxin response pathway, the light-responsive pathway and endosperm expression under stress conditions, thereby leading to drought tolerance. Our study identified candidate miRNAs and mRNAs operating in important pathways under drought stress conditions, and these candidates will be useful in the development of drought-tolerant maize hybrids.

Keywords: drought, gene expression, maize, miRNA, mRNA, post-transcriptional changes

INTRODUCTION

Drought is one of the prevailing abiotic stresses that affect plant growth, development and grain yield (Ceccarelli and Grando, 1996). In particular, regions with insufficient water are more prone to drought due to uneven changes in weather conditions. Furthermore, shortages of water resources resulting from increasing human needs and growing climatic adversities exaggerate the effects of drought several fold (Rosegrant and Cline, 2003). Development of climate-resilient and drought-tolerant cultivars could help in sustaining food grain production in the present era of climate change. However, drought tolerance is a morphologically, physiologically, and genetically complex trait. Therefore, understanding the underlying molecular basis and regulation of drought tolerance could help in accelerating drought-tolerance breeding programs. Many reports have highlighted and proposed various genetic and molecular mechanisms of drought tolerance in different crops, including model plant systems (Ha et al., 2012; Shikha et al., 2017). Additionally, a functional understanding of stress responsive gene(s) and their regulation patterns can aid in devising new genetic tools (Langridge and Reynolds, 2015).

Post-transcriptional modification of RNAs is one of the major forms of regulation of gene expression and is primarily performed by microRNA (miRNA) molecules (Ding et al., 2009; Zhou et al., 2010). miRNAs belong to a non-coding family of RNAs and are known to play major roles in modulating gene expression to adjust plant metabolism to withstand stresses. The regulatory mechanism of miRNAs involves a change in self-concentration, targeting the mRNA quantity and modifying the mRNA expression *via* miRNA-protein complexes. These, in turn, change the ultimate expression of proteins upon exposure to stress (Ding et al., 2009; Wang B. et al., 2014). In plants, miRNA genes code for long pri-miRNA (primary miRNA) transcripts with imperfect stem-loop secondary structures, which are transcribed by RNA polymerase II (Lee et al., 2004; Xie et al., 2005). These transcripts are processed into ~70-nt pre-miRNAs and subsequently released as miRNA/miRNA duplexes by DCL-1 (dicer-like enzyme 1) in association with a dsRNA binding protein, HYL-1. Such duplexes are methylated by a dsRNA methylase, HEN1, and loaded into AGO1 (Kurihara and Watanabe, 2004; Vazquez et al., 2004; Yu et al., 2005). They are subsequently transported to the cytoplasm with the help of an exportin homolog, the HASTY protein (Bartel, 2004; Park et al., 2005), and cleaved into ~22-nt mature miRNAs. Mature miRNA strands are incorporated into a multiprotein complex, the RNA-induced silencing complex (RISC), where they guide the cleavage of complementary target mRNAs by AGO1, which possesses a PAZ domain and a catalytic PIWI domain (Vaucheret et al., 2004; Baumberger and Baulcombe, 2005).

The involvement of miRNAs in different abiotic stresses has been demonstrated in *Arabidopsis*. Overexpression of miR168, miR171 and miR396 under hypersalinity, mannitol, and cold stress was reported in *Arabidopsis* (Liu et al., 2008). Nevertheless, both the involvement of total miRNAs under drought stress and the systemic expression analysis of their

drought-related mechanism are still in progress and necessitate further exploration.

Maize is an important crop in the world, contributing significantly to food and nutritional security. However, the production of maize is most vulnerable to various abiotic stresses, especially drought. To date, functional genomics approaches have revealed large amounts of information on target mRNA control through miRNAs for various traits in maize. The expression of a class III homeodomain-leucine zipper (*HD-Zip III*) protein that functions in asymmetrical leaf development and that of a floral meristem transcription factor, *APETALA2*, responsible for meristem identity were found to be targeted by miR166 and miR172, respectively (Juarez et al., 2004). Similarly, miR156 is reported to target the expression of *tga1* (*Teosinte* glume architecture 1) (Chuck et al., 2007). Studies on differential expression of miRNAs have shed light on the regulatory roles of miRNAs in plant development (Kang et al., 2012; Liu et al., 2014) and stress responses in maize (Zhang et al., 2008; Ding et al., 2009; Wei et al., 2009; Zhai et al., 2013; Wu et al., 2014). Such investigations deciphering the regulatory control between miRNAs and target mRNAs will pave the way for a better understanding of the molecular mechanisms underlying drought stress responses. Differentially expressed genes (DEGs) have been identified in drought or low-moisture stress microarray studies (Yu, 2003; Yue et al., 2008; Hayano-Kanashiro et al., 2009; Li et al., 2009; Marino et al., 2009; Luo et al., 2010; Zheng et al., 2010; Lu et al., 2011; Hansen et al., 2013; Regulski et al., 2013). Such DEGs were found to be associated with drought-related miRNA targets.

Maize germplasm shows great genetic variability involving temperate, tropical, and subtropical groups, as well as dent, flint, semi flint and waxy types within germplasm groups. Efforts have been taken to characterize the drought-responsive miRNAs and target mRNAs in temperate maize germplasm (Li et al., 2013; Wang Y. G. et al., 2014). There have been no reports available on comprehensive characterization of drought-responsive miRNAs and target-mRNAs using subtropical maize germplasm. Therefore, in the present study, we identified putative regulatory miRNAs targeting drought-related mRNAs based on gene expression data from 12 drought/low-moisture stress experiments. The expression patterns of miRNA-target mRNA pairs were validated in the root and shoot tissues of two contrasting subtropical maize inbreds under drought stress. The functional annotation and the role of drought-related miRNA-target mRNA pairs were analyzed. Our study identified the differential interactions of miRNA-target mRNA pairs during drought stress in the tolerant genotype and explained their functional roles in drought tolerance.

MATERIALS AND METHODS

Plant Growth and Experimentation

Seeds of two contrasting subtropical maize inbreds, HKI-1532 (drought-tolerant) and V-372 (drought-susceptible), were grown in the National Phytotron Facility, Indian Agricultural Research Institute, New Delhi. Potting was done in triplicate

with sandy loam soil. The plants were maintained under controlled greenhouse conditions of 28/22°C (day/night) at a light intensity of 600 $\mu\text{mol m}^{-2}\text{s}^{-1}$ (16 h day/8 h night) with 50–55% relative humidity. Regular irrigation was provided for 15 days to the first set of plants (stress) after sowing and suspended for the next 5 days to induce severe drought stress (Kakumanu et al., 2012; Min et al., 2016; Nepolean et al., 2017). The second set of plants (control) was watered throughout the experiment. On the 21st day after sowing, leaf samples were collected for expression assays (Figure 1).

Prediction of miRNA-target mRNA Interactions

The entire set of maize mature miRNA sequences available (321) was downloaded from miRbase v19 (Griffiths-Jones et al., 2008; Kozomara and Griffiths-Jones, 2011). Four popular miRNA target prediction tools—RNAHybrid (Rehmsmeier et al., 2004), TargetFinder (Allen et al., 2005; <http://carringtonlab.org/resources/targetfinder>), TAPIR (FASTA search engine) (Bonnet et al., 2010), and psRNATarget (Dai and Zhao, 2011)—were used to identify targets for the miRNAs from among the 76,617 *Zea mays* B73 RegGen v2 mRNAs downloaded from NCBI (National Center for Biotechnology Information) Genome database identified by the GNOMON v2 gene prediction tool (Souvorov et al., 2010; ftp://ftp.ncbi.nlm.nih.gov/genomes/PLANTS/Zea_mays/Gnomon_v2/). Consensus predictions of miRNA-target mRNA interactions detected among the four tools were used for further analysis.

Identification of Putative Drought-Related miRNAs

DEGs identified in 12 microarray-based gene expression studies in maize under drought/low moisture stress and/or recovery irrigation conditions (Table S1) were selected for comparison with the target mRNAs identified in the consensus predictions of miRNA-target mRNA interactions. For the experiments where DEG lists were not available, the raw data from NCBI GEO (*Gene Expression Omnibus*) (Barrett et al., 2005) were reanalyzed to generate a list. The regulatory miRNAs of the DEG sequences that were also predicted to be miRNA targets were identified as putative drought-related miRNAs. For both the mature miRNA sequences and the target mRNA sequences with the respective target sites masked, multiple sequence alignment was done using the Clustal Omega program (Sievers et al., 2011). A distance matrix was constructed based on pairwise distances using an identity matrix with the “dist.alignment” function of the R package seqinR (Charif et al., 2008) and used to performing hierarchical clustering by complete linkage. The clusterings of miRNAs and their respective targets were compared visually by plotting a tanglegram using “cophyloplot” function of the R package ape (Paradis et al., 2004). Furthermore, the functional annotation of the corresponding target mRNAs was carried out by Blast2GO (Conesa et al., 2005).

Analysis of *cis*-Regulatory Elements (CREs) in miRNA Promoters in Maize

DNA sequences upstream of the starts of the precursor miRNA structures were identified and extracted using NCBI tools (<https://www.ncbi.nlm.nih.gov>). The transcription start sites (TSS) were predicted from the upstream regions of pri-miRNA sequences using softberry TSSP (Shahmuradov et al., 2005; <http://www.softberry.com/berry.phtml?topic=tss>). The promoter sequence, i.e., the 2-kb region upstream of the TSS, was retrieved using Perl Script. For families having >1 promoter, the sequence between the two promoters was considered for the identification of CREs using the PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) database.

RNA Isolation and qRT-PCR Assay

Total RNA was isolated from seedling leaf tissue using the Ultra Clean Plant RNA isolation kit (MO BIO Laboratories, USA). Primers specific to the target mRNA sequences were designed using BatchPrimer3 (You et al., 2008) and optimized to avoid primary and amplicon secondary structures using Beacon DesignerTM Free Edition (<http://www.premierbiosoft.com/qpcr/>) and IDT OligoAnalyzer (<http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/>), respectively (Table S2). Sequence specificity of the primers was assured by comparing their sequences with maize gene sequences using NCBI nucleotide BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). qRT-PCR for mature miRNAs was performed using a modified version of stem-loop qRT-PCR (Chen et al., 2005). The stem-loop primers, miRNA forward primers and universal reverse primers were designed according to Kramer (2011) (Table S3). The primer for the reference transcript U6snRNA was designed as previously described (Turner et al., 2013).

For target mRNAs, the first-strand cDNA synthesis was carried out with the RNA ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs, Ipswich, Massachusetts) using 50 ng total RNA and 1 μM Oligo d(T)23 VN primer. For miRNAs, reverse transcription reactions were carried out with 50 ng total RNA and 1 μM stem-loop RT primer. The reactions were incubated in a thermocycler for 30 min at 16°C, 30 min at 42°C and 5 min at 85°C and then held at 4°C.

For both mature miRNAs and their mRNA targets, real-time quantitative PCR with SYBR Green I was performed on an Agilent Technologies Mx3005P QPCR (Agilent Technologies, Santa Clara, California, United States) instrument. Briefly, each 25- μl PCR mixture contained approximately 100 ng cDNA, 9 ml Hotstart IT SYBR Green qPCR Master Mix (Affymetrix), ROX (Affymetrix) and 250 nM of each primer. The reactions were mixed gently and incubated at 94°C for 2 min (pre-heating), followed by 40 cycles of 94°C for 15 s (denaturation), 60°C for 30 s (primer annealing), and 72°C for 30 s (primer extension). Analysis was conducted on three biological replicates and two technical replicates for each treatment and genotype combination. U6 snRNA and 18S rRNA were used as internal controls for miRNA and mRNA, respectively. The $\Delta\Delta\text{Ct}$ method was used to determine the differences in expression levels among samples (Livak and Schmittgen, 2001).

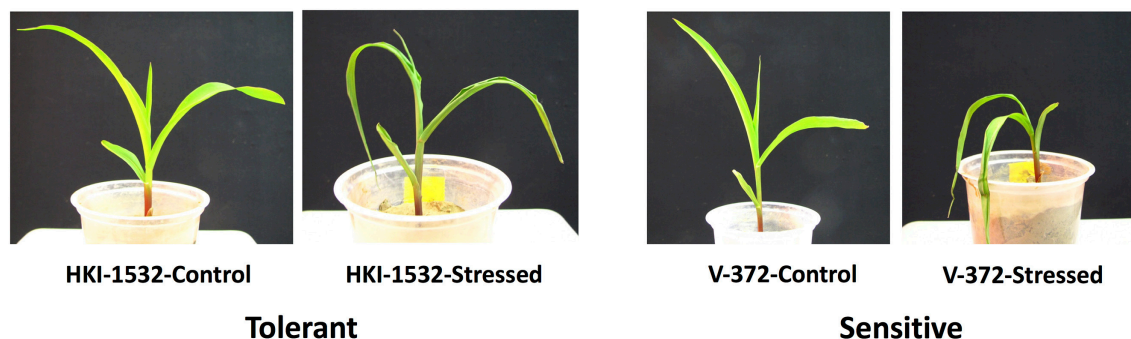


FIGURE 1 | Phenotypic response of tolerant (HKI-1532) and sensitive (V-372) genotypes under drought stress.

RESULTS

Identification of Putative Drought-Related miRNAs and Target mRNAs

Among the DEGs identified in 12 microarray-based gene expression studies under drought/low moisture stress and/or recovery irrigation conditions, 42 differentially expressed mRNA sequences were predicted to be putatively regulated by 65 miRNAs belonging to 13 families in 183 miRNA-target mRNA interactions (**Table 1**). These were considered for further expression analysis. The predicted interactions involved the same miRNAs targeting multiple mRNAs, as well as the same mRNA being targeted by multiple miRNAs. For most of the interactions, miRNAs with similar sequences targeted mRNA sequences that clustered together (**Figure 2**). The miRNA families with the largest numbers of members included zma-miR166, zma-miR395 and zma-miR156, with 14, 14, and 12 members, respectively. On the other hand, some miRNA families, such as zma-miR160, zma-miR399, zma-miR529, and zma-miR2275, had only one member each (**Figure 3**). The target distributions of miRNA families themselves were investigated, which showed that zma-miR166, zma-miR396, zma-miR529, zma-miR164, and zma-miR169 had the most targets, with 6, 6, 5, and 5 target mRNAs, respectively, while zma-miR160, zma-miR390, zma-miR393, and zma-miR2275 had the fewest target mRNAs, with a single target each (**Figure 3**).

Prediction of miRNA-Target mRNA Interactions

The four miRNA target prediction tools detected different but overlapping sets of miRNA-target mRNA pairs from among the 321 miRNAs and 76617 mRNAs of maize. Among these dissimilar sets, 594 consensus predictions of miRNA-target mRNA interactions could be identified. Compared to single prediction tool-based identification of miRNA-target mRNA interactions, we employed four prediction tools to choose the robust miRNA-target mRNA interactions. The total numbers of interactions supported by RNAHybrid, TargetFinder, TAPIR FASTA and psRNATarget were 31,262, 4,522, 3,406, and 3,072, respectively, including both unique and commonly identified interactions from each tool. The numbers of unique interactions

identified by RNAHybrid, TargetFinder, TAPIR FASTA and psRNATarget were 29869, 1264, 222, and 1293, respectively (**Figure 4**). The consensus predictions of miRNA-target mRNA interactions involved 156 miRNAs belonging to 25 families, and 150 unique target mRNAs.

GO Enrichment Analysis of miRNA Target Genes

To gain insights into the functional roles of miRNAs, annotation of their predicted target mRNAs was carried out. GO terms were identified under three different functional categories: biological (57.14%), cellular (23.80%) and molecular (30.95%). In the biological process category, major functions included anatomical structure morphogenesis, multicellular organismal development and post-embryonic development. In the molecular process category, major functions included DNA binding, hydrolase activity and nucleotide binding (**Figure 5**). Furthermore, most of the target genes were confined to the nucleus.

Analysis of *cis*-Regulatory Elements

The promoter regions of 65 drought-related miRNAs were examined to detect the occurrence of conserved *cis*-regulatory elements. A total of 61 conserved CRE motifs were identified from the miRNA promoters. Among these, 41 were major *cis*-regulatory elements that were observed in >10 members, including the drought-related *cis*-elements ABRE (ABA-responsive element) and MBS (MYB-binding site) (**Table 2**). In miR169n of rice, the ABRE *cis*-acting element resided in the promoter region, implying an ABA-mediated response to stress (Zhao et al., 2007). An ABRE *cis*-element was also found in sorghum ABA-responsive genes (Buchanan et al., 2005). Similarly, in *Arabidopsis*, MBS was found in the upstream regions of all the miRNA genes in the rice shoot apices. Of the 65 miRNA members, miR164f showed the highest number of CREs (41), whereas miR156d had the lowest number (8).

Expression Patterns of miRNAs and Their Target mRNA(s)

To analyze the responses of contrasting genotypes to drought, transcriptional profiling of the identified putative drought-responsive miRNAs and their target-mRNAs was done at the

TABLE 1 | List of 13 drought-related miRNA families and their respective targets with annotations.

Family specific target mRNAs			
miRNA family	miRNA	Target mRNAs	Annotations
miR156	zma-miR156-a,c,d,e,f,g,h,i,j,k,l	gnl GNOMON 13750094.m	tpa: squamosa promoter-binding (sbp domain) transcription factor family protein
	zma-miR156-a,c,d,e,f,g,h,i,j,k,l	gnl GNOMON 66064033.m	Squamosa promoter-binding-like protein 13-like
miR159	zma-miR159e-3p	gnl GNOMON 11944063.m	Transcription factor gamyb
	zma-miR159e-5p	gnl GNOMON 22442014.m	Transcription factor gamyb
	zma-miR159-c,d,g,h,i	gnl GNOMON 57248043.m	Transcription factor
miR160	zma-miR160-f	gnl GNOMON 46030063.m	gdsI esterase lipase at5g45910-like
miR164	zma-miR164-f	gnl GNOMON 10380054.m	Hypothetical protein
	zma-miR164-f	gnl GNOMON 10384054.m	Hypothetical protein
	zma-miR164-f	gnl GNOMON 18192014.m	Hypothetical protein
	zma-miR164-b	gnl GNOMON 4218083.m	psbp domain-containing protein Chloroplastic-like
	zma-miR164-h	gnl GNOMON 46106013.m	Wound responsive protein
miR166	zma-miR166-a,b,c,d,e,f,g,h,i,j,k,l	gnl GNOMON 1168013.m	Rolled expressed
	zma-miR166-a,b,c,d,e,f,g,h,i,j,k,l	gnl GNOMON 15104054.m	Partial
	zma-miR166-a,b,c,d,e,f,g,h,i,j,k,m,n,l	gnl GNOMON 35860043.m	Tpa: homeobox lipid-binding domain family protein
	zma-miR166-a,b,c,d,e,f,g,h,i,j,k,m,n,l	gnl GNOMON 52446103.m	Rolled leaf1
	zma-miR166-a,b,c,d,e,f,g,h,i,j,k,m,n,l	gnl GNOMON 54238013.m	Tpa: homeobox lipid-binding domain family protein
	zma-miR166-a,b,c,d,e,f,g,h,i,j,k,m,n,l	gnl GNOMON 8472093.m	Homeobox-leucine zipper protein athb-15-like
miR169	zma-miR169-l,j,k,l	gnl GNOMON 74364063.m	Nuclear transcription factor y subunit a-3
	zma-miR169-l,j,k,l	gnl GNOMON 74366063.m	Nuclear transcription factor y subunit a-3
miR390	zma-miR390-a,b	gnl GNOMON 30954063.m	Activator of 90 kda heat shock protein atpase
miR393	zma-miR393-a,c	gnl GNOMON 39086093.m	Protein transport inhibitor response 1-like
	zma-miR393-a,c	gnl GNOMON 5722063.m	Atpsulfurylase
	zma-miR393-a,c	gnl GNOMON 92168013.m	Atpsulfurylase
miR395	zma-miR395-b,d,e,f,g,h,i,j,m,n,p,l	gnl GNOMON 5722063.m	Atpsulfurylase
		gnl GNOMON 92168013.m	Atpsulfurylase
miR396	zma-miR396-c,d	gnl GNOMON 3258103.m	Growth-regulating factor 1
	zma-miR396-c,d	gnl GNOMON 41140073.m	Growth-regulating factor 1
	zma-miR396-c,d	gnl GNOMON 54764053.m	Growth-regulating factor 1-like
	zma-miR396-c,d	gnl GNOMON 74420063.m	Growth-regulating factor 9
	zma-miR396-c,d	gnl GNOMON 8836063.m	Growth-regulating factor 8
	zma-miR396-c,d	gnl GNOMON 9726103.m	Growth-regulating factor
miR399	zma-miR399-e	gnl GNOMON 61030013.m	60s ribosomal protein l7a-like
	zma-miR399-e	gnl GNOMON 92930013.m	Heavy metal-associated domain containing expressed
miR529	zma-miR529	gnl GNOMON 24048063.m	Squamosa promoter-binding (sbp domain) transcription factor family protein isoform 1
	zma-miR529	gnl GNOMON 24052063.m	Squamosa promoter-binding (sbp domain) transcription factor family protein isoform 1
	zma-miR529	gnl GNOMON 24056063.m	Squamosa promoter-binding (sbp domain) transcription factor family protein isoform 1
	zma-miR529	gnl GNOMON 86696013.m	p8mtcp1
	zma-miR529	gnl GNOMON 86698013.m	p8mtcp1
	zma-miR529	gnl GNOMON 86706013.m	p8mtcp1
miR2275	zma-miR2275-a	gnl GNOMON 55702013.m	Mitochondrial protein

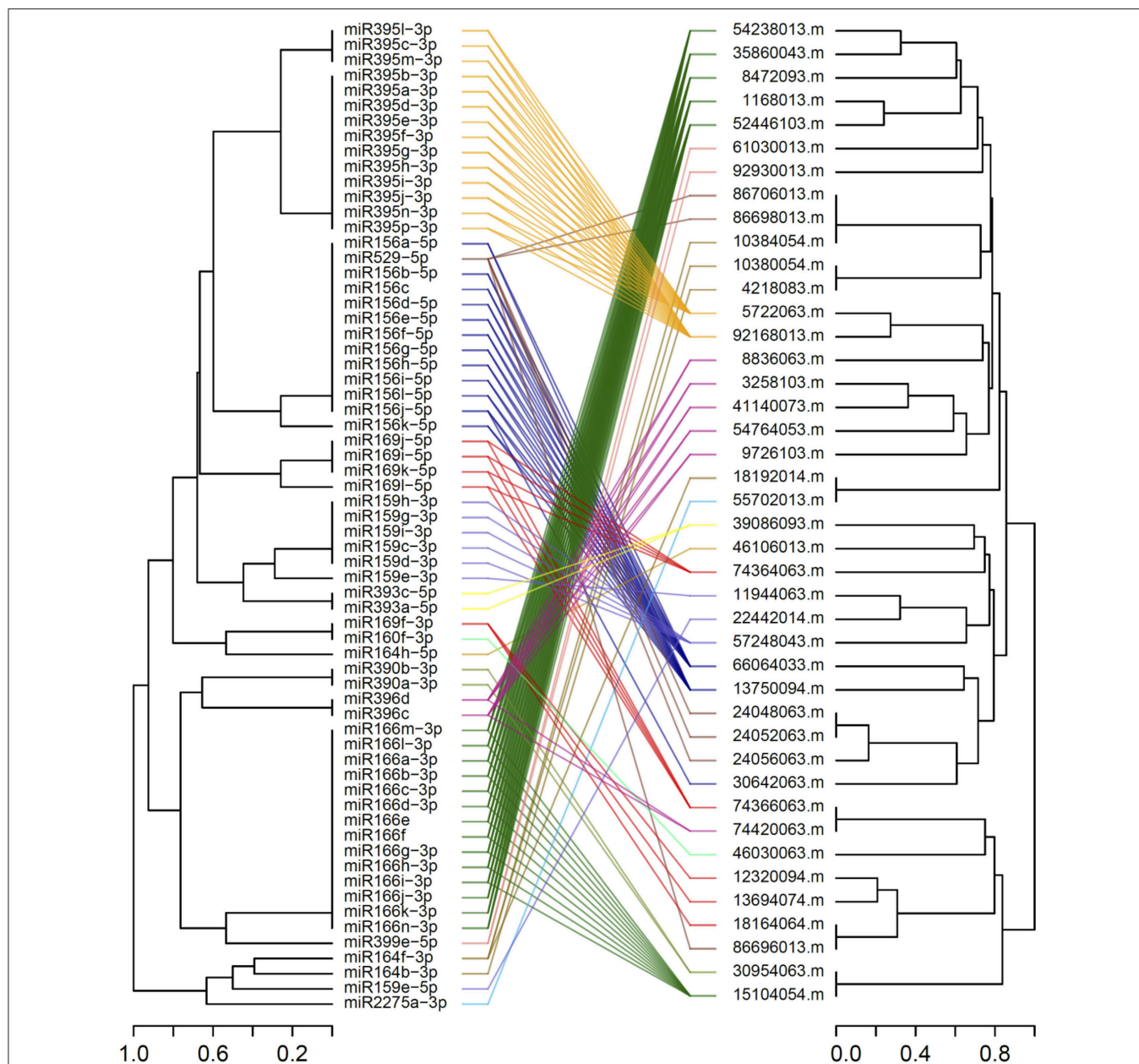


FIGURE 2 | Tanglegram of predicted mature miRNA and target mRNA sequences (with the target site masked) related to drought/low-moisture stress. Lines colored according to the miRNA family, connect the miRNA sequences to their respective target mRNAs.

seedling stage, revealing their interactions with each other. The significantly up-regulated miRNAs common to both genotypes (HKI-1532 and V-372) included zma-miR164h, zma-miR169l, zma-miR396c, zma-miR396d, and zma-miR399e. In tolerant genotype HKI-1532, 16 miRNAs belonging to the zma-miR159, zma-miR160, zma-miR164, zma-miR166, zma-miR169, zma-miR390, zma-miR395, zma-miR396, and zma-miR399 families were significantly up-regulated. However, zma-miR156 and zma-miR159 were significantly down-regulated. Conversely, in V-372, members of 7 families—zma-miR164, zma-miR169, zma-miR393, zma-miR396, zma-miR399, zma-miR529, and zma-miR2275—were significantly up-regulated; and zma-miR156,

zma-miR159, zma-miR166 and zma-miR395 families were significantly down-regulated. These results indicate disparate patterns of regulation in the tolerant and susceptible genotypes (Figure 6).

Furthermore, expression analysis of target mRNAs revealed six up-regulated, 18 down-regulated and 18 neutrally expressed mRNAs in HKI-1532. V-372, however, had one up-regulated, 10 down-regulated and 26 neutrally expressed mRNAs. The miRNA-target mRNA interactions were classified into four different groups: Type I, with miRNA-mRNA up-regulations; Type II, with miRNA-mRNA down-regulations; Type III, with neutral miRNA-mRNA expressions; and Type IV, with opposite

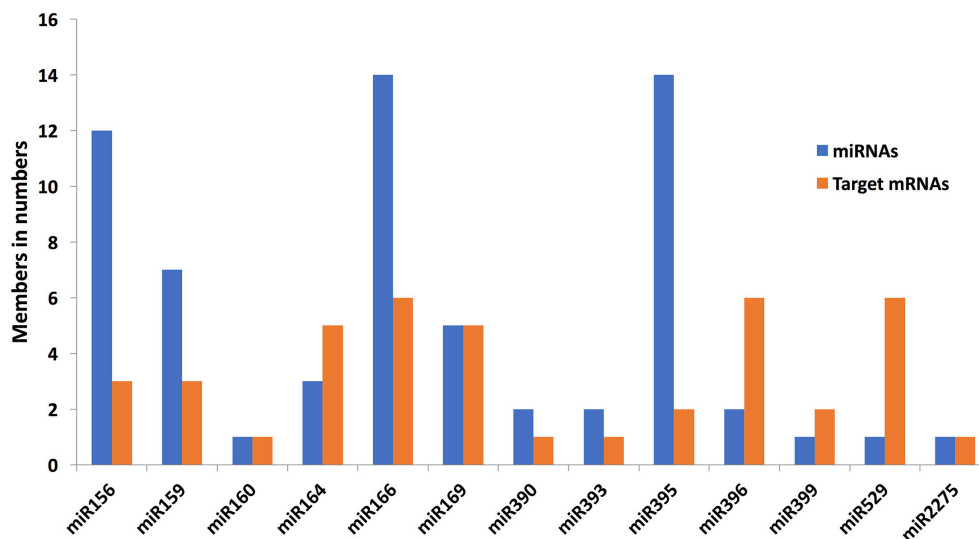


FIGURE 3 | Distribution of 13-drought related miRNA families members and respective target mRNAs. Blue bar represents the members count in each miRNA family and orange bar represents the total number of target mRNAs in each miRNA family.

interactions. The tolerant genotype, HKI-1532, had 46 Type I, 13 Type II, 23 Type III, and 101 Type IV combinations. The susceptible genotype, V-372, had eight Type I, four Type II, 20 Type III, and 151 Type IV combinations (**Figure 6**).

DISCUSSION

Several investigations have revealed the role of miRNAs in modulating gene expression under abiotic stresses—cold (Lv et al., 2010), salt (Ding et al., 2009), aluminum tolerance (Kong et al., 2014), waterlogging (Zhai et al., 2013), and drought (Li et al., 2013; Wang Y. G. et al., 2014). Among the several abiotic stresses, drought is the most prominent stress in the sub-tropical maize production systems. A number of plant parameters, such as growth, yield, membrane integrity, pigment composition, osmotic relations and photosynthesis, are commonly affected by drought stress (Praba et al., 2009). The change in miRNA expression pattern under drought is an indication of stress responses in plants. Such findings provide an insight into drought tolerance mechanisms and can potentially aid in designing drought-tolerant cultivars (Zhao et al., 2007; Chen et al., 2012). The role of miRNAs in drought tolerance has been previously explored in maize using temperate germplasm. Wang Y. G. et al. (2014) identified 301 drought-responsive miRNAs in temperate maize germplasm and Li et al. (2013) detected differentially expressed 68 miRNAs falling under 29 miRNA families in a maize inbred R09. In the present investigation, 42 differentially expressed mRNA sequences were predicted to be putatively regulated by 65 miRNAs belonging to 13 families. Furthermore, a combination of 183 miRNA-target mRNA interactions were identified and validated in a contrasting pair of subtropical maize germplasm. Four prediction tools were employed to reveal high-confidence predictions of miRNA-target mRNA interactions due

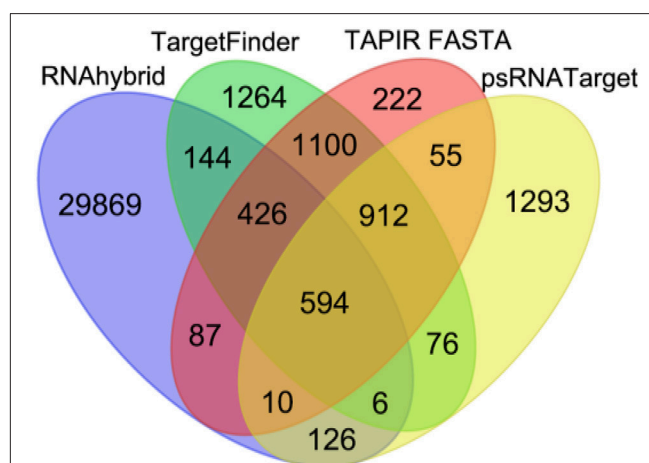


FIGURE 4 | Venn diagram illustrating the number of common and differently identified miRNA-target mRNA interactions through four different source tools (RNAhybrid, TargetFinder, TAPIR FASTA, and psRNATarget). The color of each leaf represents the total and overlapped number of miRNA-target mRNA interactions obtained from each source tool.

to enhanced accuracy and site recognition efficacy as compared to previous single tool-based prediction (Ding et al., 2012; Li et al., 2013; Wang Y. G. et al., 2014). All 13 of the miRNA families were found to be conserved across taxonomic groups related to drought, ABA or oxidative stress response (Kim et al., 2003; Ding et al., 2013). These results suggested that specific microarray expression profiling data could be effectively used for identifying regulatory miRNAs.

miRNA genes originated from the preexisting genes through duplication events (Nozawa et al., 2012). miRNAs that are evolutionarily conserved are usually encoded by miRNA gene

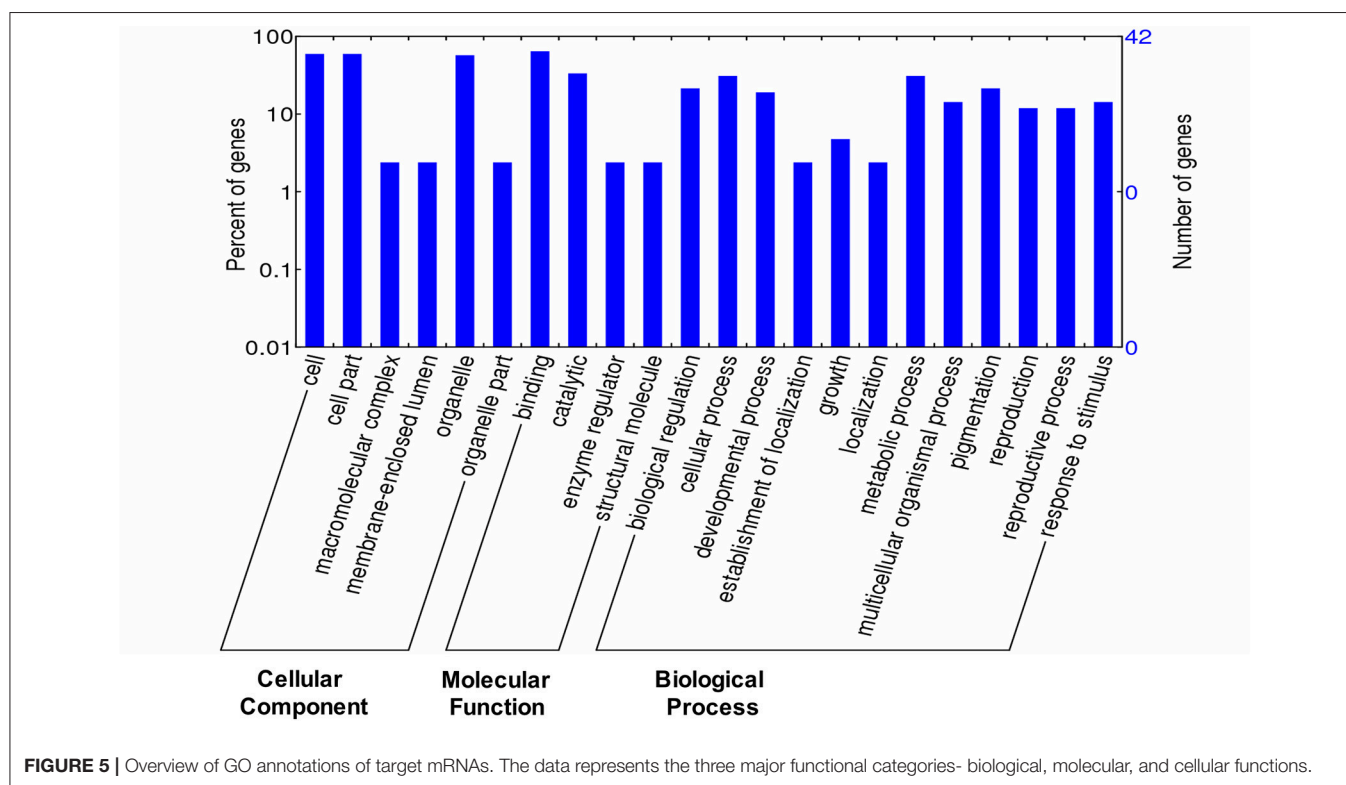


FIGURE 5 | Overview of GO annotations of target mRNAs. The data represents the three major functional categories- biological, molecular, and cellular functions.

families. This, coupled with the strong similarity requirements of plant miRNA - target site interactions, leads to overlapping functions of miRNAs belonging to the same families, buffering against loss of individual members (Jones-Rhoades et al., 2006). It was found that the families such as miR2118, miR395, and miR159 were highly conserved in maize and rice (Xu et al., 2017). In a few interactions, similar miRNA sequences belonging to the same families were found to target dissimilar target mRNA sequences, such as mature miRNAs from the 3' arm of miRNA family 166 and those from the 5' arm of miRNA family 169. In *Arabidopsis*, miR395 has been found to regulate an ATP sulfurylase and an unrelated sulfate transporter (Allen et al., 2005), and miR159 has been reported to target both *MYB101* and *MYB120* and two unrelated genes, *OPT1* and *ACS8* (Schwab et al., 2005). In maize, miR395 showed differential expression in response to salt stress, and was found to regulate *ATP sulfurylase*, *L-Isoaspartyl methyltransferase*, and *Beta-D-xylosidase* genes (Ding et al., 2009).

The promoter analysis of differentially expressed miRNAs indicated that they were predominantly involved in ABA signaling, the auxin response pathway, light-responsive pathways and endosperm expression. Among the 13 drought-related miRNA families, some families had common targets, i.e., zma-miR160, zma-miR390, and zma-miR393 were mostly related to the ARF (auxin response factor) transcription factor, which plays an active role in ABA and auxin mediated signaling under drought conditions (Ding et al., 2013). Similarly, miR164 targets the NAC transcription factor and *CUC* (cup shaped cotyledon) genes in *Arabidopsis* (Rhoades et al., 2002); these genes are

responsible for root and shoot development. Furthermore, zma-miR164 showed higher expression in crown roots but not in seminal roots of maize, suggesting that it could play a crucial role in development of crown roots (Kong et al., 2014).

Members of the miR166 family generally participate in regulation of their target, *HD-Zip III* (homeodomain-leucine zipper III), which is engaged in lateral root development, initiation of axillary leaf meristems and leaf polarity (Boualem et al., 2008) (Figure 7). In maize, it was reported that the leaf polarity was controlled by a subset of miR166 family members (Nogueira et al., 2009). The regulatory mechanism of miR390 is somewhat different, as it prompts the production of tasiRNA (TAS-3 derived small interfering RNA), which targets the ARFs (*ARF2*, *ARF3*, and *ARF4*) that function in lateral root emergence and organ polarity (Nogueira et al., 2009; Meng et al., 2010). miR399 is involved in regulation of a phosphate transporter that regulates the uptake of phosphate and its translocation (Pant et al., 2008). Our *in-silico* analysis identified various target mRNAs, including *SPL* (SQUAMOSA promoter-binding-like proteins), *GAMYB* (a gibberellin- and abscisic acid-related MYB), *ARF* (auxin response factor), *AST* (a sulfate transporter), and *GRFs* (growth regulating factors), among others. The majority of the identified targets are also conserved across other plant species, including model systems such as *Arabidopsis* (Adai et al., 2005) and rice (Wang et al., 2004; Luo et al., 2006).

The interactions of each miRNA with a specific target are of prime importance, as these interactions can help to discern the variation in drought-induced gene expression. For example, the miR156 family regulates the expression of *SPL*, leading

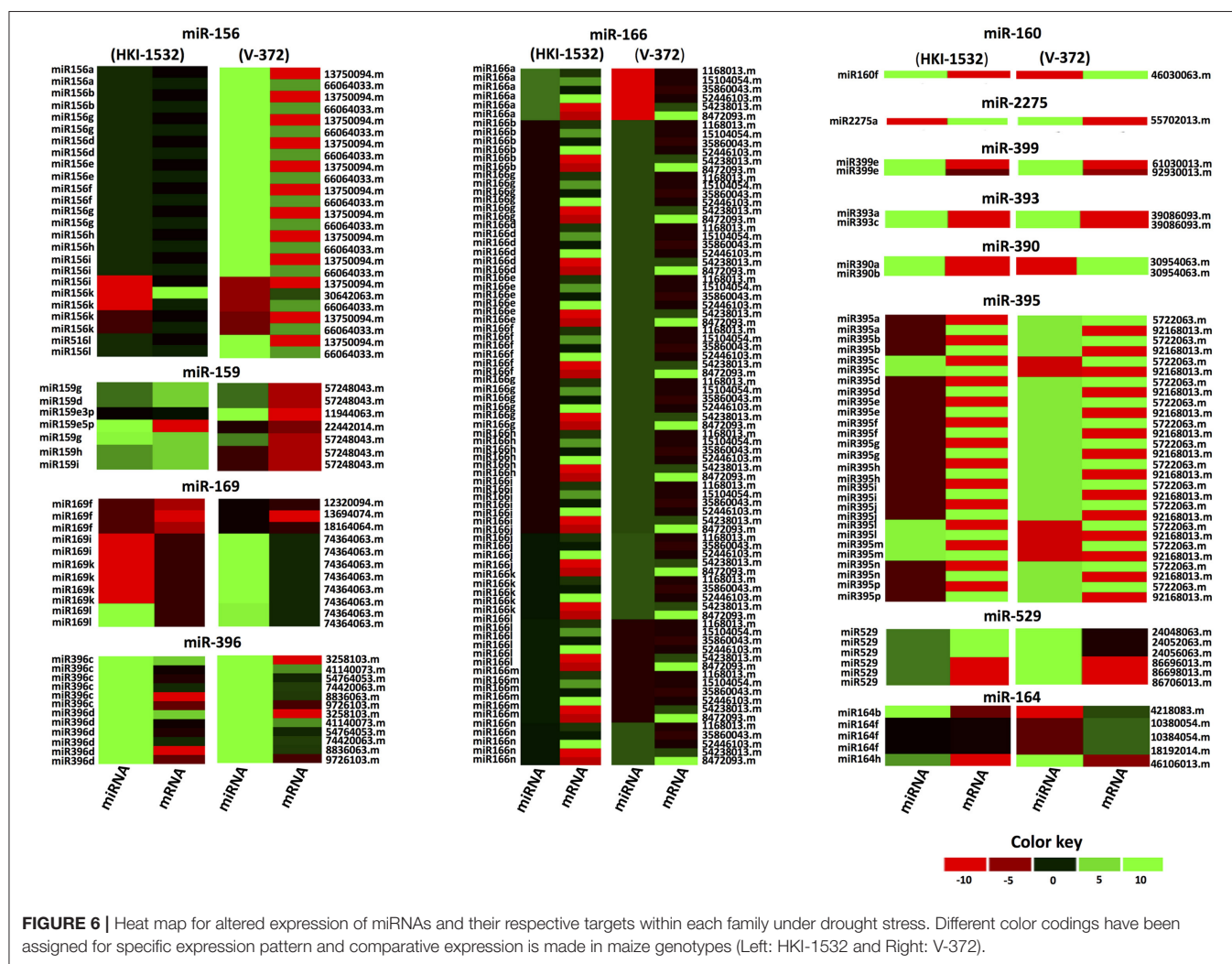
TABLE 2 | Putative *cis*-regulatory elements identified in the upstream region of drought responsive miRNA in maize.

<i>cis</i> -elements	Frequency	Sequence	Function
TATCCAT/C-motif	10	TATCCAT	<i>cis</i> -acting regulatory element; associated with G-box like motif; involved in sugar repression responsiveness
GA-motif	11	AAGGAAGA	Part of a light responsive element
LTR	12	CCGAAA	<i>cis</i> -acting element involved in low-temperature responsiveness
CCGTCC-box	12	CCGTCC	<i>cis</i> -acting regulatory element related to meristem specific activation
MNF1	12	GTGCCCTT	Light responsive element
ATCT-motif	12	AATCTAATCC	part of a conserved DNA module involved in light Responsiveness
GC-motif	14	CCCCCG	Enhancer-like element involved in anoxic specific inducibility
AE-box	14	AGAAACAT	Expression and repressed
GARE-motif	14	AAACAGA	Gibberellin-responsive element
TCT-motif	14	TCTTAC	Part of a light responsive element
RY-element	15	CATGCATG	<i>cis</i> -acting regulatory element involved in seed-specific regulation
5UTR Py-rich stretch	16	TTTCTTCTCT	<i>cis</i> -acting element conferring high transcription levels
TCA-element	16	CAGAAAAGGA	<i>cis</i> -acting element involved in salicylic acid responsiveness
ACE	17	GACACGTATG	<i>cis</i> -acting element involved in light responsiveness
Box I	17	TTTCAAA	Light responsive element
HSE	18	AAAAAATTC	<i>cis</i> -acting element involved in heat stress responsiveness
TGA-element	20	AACGAC	Auxin-responsive element
Box-W1	20	TTGACC	Fungal elicitor responsive element
W box	20	TTGACC	Elicitation; wounding and pathogen responsiveness. Binds WRKY type transcription factors
CCAAT-box	21	CAACGG	MYBHv1 binding site
CATT-motif	21	GCATTC	Part of a light responsive element
O2-site	22	GATGACATGG	<i>cis</i> -acting regulatory element involved in zein metabolism regulation
GCN4_motif	22	TGAGTCA	<i>cis</i> -regulatory element involved in endosperm expression
Box 4	22	ATTAAT	Part of a conserved DNA module involved in light responsiveness
CAT-box	24	GCCACT	<i>cis</i> -acting regulatory element related to meristem expression
GT1-motif	24	GGTTAA	Light responsive element
I-box	24	GATATGG	Part of a light responsive element
AAGAA-motif	26	GAAAGAA	–
TC-rich repeats	28	ATTTTCTTCA	<i>cis</i> -acting element involved in defense and stress responsiveness
GAG-motif	30	GAGAGAT	Part of a light responsive element
ABRE	31	GCAACGTGTC	<i>cis</i> -acting element involved in the abscisic acid responsiveness
circadian	31	CAANNNNATC	<i>cis</i> -acting regulatory element involved in circadian control
ARE	35	TGGTTT	<i>cis</i> -acting regulatory element essential for the anaerobic induction
CGTCA-motif	36	CGTCA	<i>cis</i> -acting regulatory element involved in the MeJA-responsiveness
TGACG-motif	36	TGACG	<i>cis</i> -acting regulatory element involved in the MeJA-responsiveness
Sp1	37	CC(G/A)CCC	Light responsive element
MBS	37	CAACTG	MYB binding site involved in drought-inducibility
G-Box	42	CACGTT	<i>cis</i> -acting regulatory element involved in light responsiveness
Skn-1_motif	45	GTCAAT	<i>cis</i> -acting regulatory element required for endosperm expression
TATA-box	52	ATATAAT	Core promoter element around -30 of transcription start
CAAT-box	53	CCAAT	Common <i>cis</i> -acting element in promoter and enhancer regions

to plant developmental phase transitions (Wang et al., 2009; Chen et al., 2010) (**Figure 6**). Altered expression of this family during drought is evidence of its functional novelty in drought stress. Li et al. (2013) reported the up-regulation of miR156 at the early stage of drought stress in the maize seedlings. The regulation of SPL TFs through miR156 was also reported during somatic embryogenesis of maize (Chávez-Hernández et al., 2015). Additionally, Liu et al. (2014) reported that miR156 controlled several SPL genes during the juvenile-to-adult phase

transition in maize. Over-expression of miR156 encoding the maize *Cg1* gene showed to prevent the flowering, and improved the digestibility and starch content in switchgrass (Chuck et al., 2011). Similarly, the expression of *GRF*, which plays important role in leaf growth by transforming cell proliferation, is regulated by miR396c (Kim et al., 2003).

It was interesting to note that the expression patterns of some miRNAs were genotype-specific under drought stress. In this experiment, the Type IV (opposite) pattern

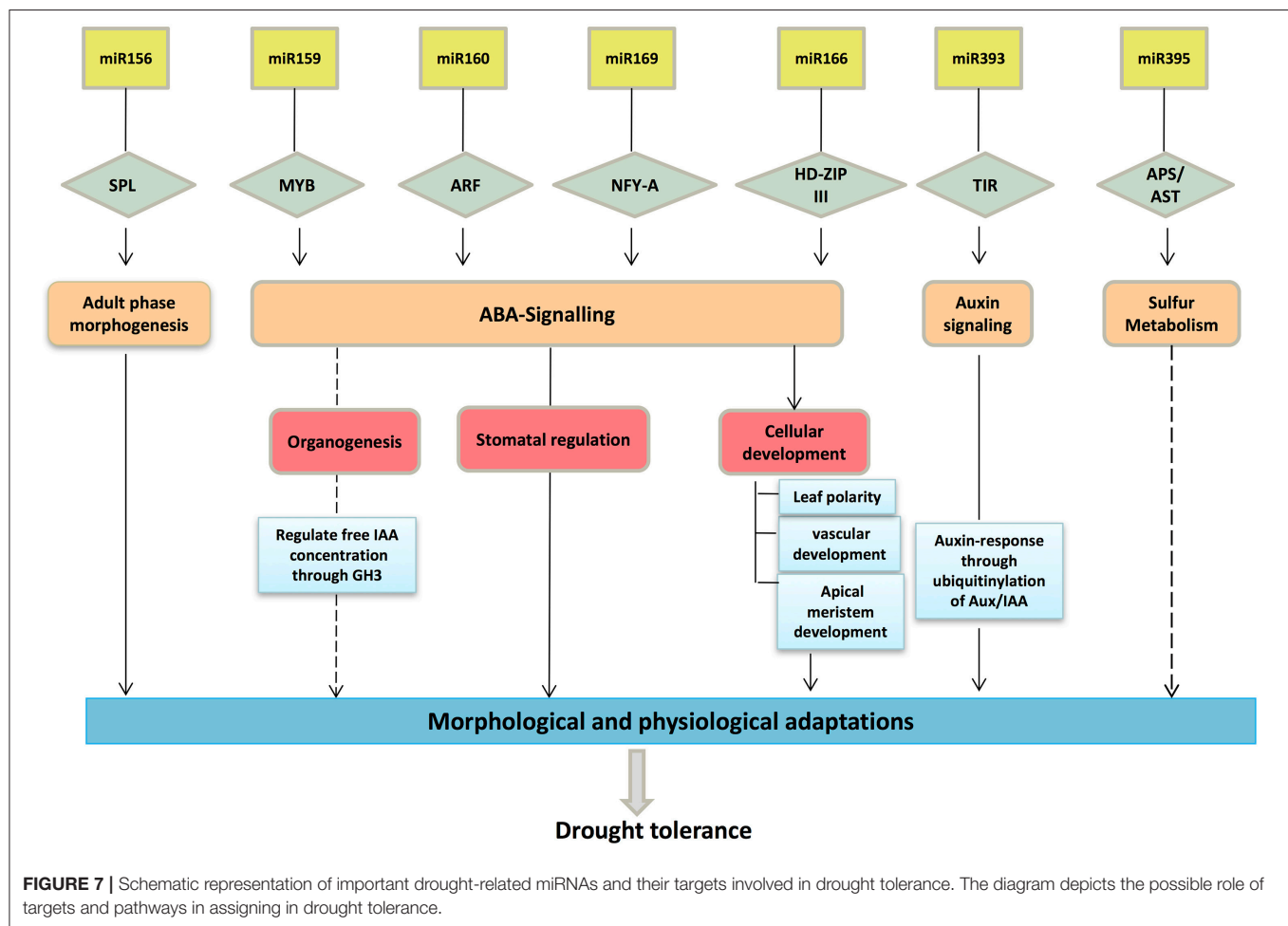


identified between miRNA and target mRNAs suggested negative regulation under drought stress. The up-regulation of miR396 in HKI-1532 suppressed the expression of its target, *GRF1*, whereas the up-regulation of miR396 in V-372 led to neutral expression of its target. This suggests that suppressed *GRF* expression under drought can provide tolerance by precluding leaf growth and function under stress (Figure 7). Our hypothesis is supported by the fact that *GRF8* reduces stomatal density in *Arabidopsis* (Liu et al., 2009). However, expression of miR396c and miR396d, targeting *GRFs*, was the same in both genotypes. It may be deduced that the small up-regulation of miRNAs in HKI-1532 contributes to drought tolerance. Similar results were obtained for miR396 in *Arabidopsis* (Liu et al., 2008) and tobacco (Frazier et al., 2011).

In the same way, Type I (up-regulated) miRNA-mRNA interaction was observed for miR169l, which targets transcription factor *NF-YA3* (nuclear transcription factor subunit A-3) in HKI1532. However, the same miRNA exhibited a Type IV interaction in V-372. Our hypothesis is supported by findings

in *Arabidopsis*, where the role of transcription factor *NF-YA* in drought tolerance is well documented (Liu et al., 2008). Similarly, Type IV interaction was also observed for the miR159 family, targeting the transcription factor *GAMYB* that is involved in flowering development and flowering time. Furthermore, overexpression of the miR159 family during stress aids in germination under stress conditions. In *Arabidopsis*, overexpression of *MYB* transcription factors (*MYB33* and *MYB101*) provides tolerance to drought by adapting ABA hypersensitivity (Reyes and Chua, 2007). Regulation of *MYB* transcription factors by the member of miR159 was also reported in temperate maize (Li et al., 2013) and was found to play a crucial role in stress tolerance (Wang Y. G. et al., 2014).

The miR393 family that targets the *TIR1* (transport inhibitor response 1) enzyme was up-regulated in V-372. This enzyme directly participates in the ubiquitinylation of inhibitors of the auxin response pathway (Dharmasiri and Estelle, 2002). miR160, representing a Type IV interaction in HKI-1532, plays a role in ABA-auxin interaction during drought (Liu et al., 2007).



Similarly, the Type IV interaction between up-regulated miR395 up-regulated and its down-regulated targets *ATP sulfurylase* and *HD-Zip* transcription factor (Buchner et al., 2004) were involved in seedling growth and seed germination in HKI-1532. This negative link indicates the role of miR395 in conferring drought tolerance. Our results are supported by the findings of studies conducted in *Arabidopsis* under drought stress, where overexpression of miR395 decreased seed growth and germination upon drought exposure (Kim et al., 2010). Similar to observations for miR156 family, Type II (down-regulated) interactions were found for *SPL* genes in both genotypes. *SPL* genes have been found to be involved in deferred blooming and the adult phase transition in *Arabidopsis* (Park et al., 2010). This can be correlated with the similar studies conducted in rice and maize (Wei et al., 2009; Zhou et al., 2010).

CONCLUSIONS

Drought-specific miRNAs and their target mRNAs were identified from existing drought-associated microarray expression profiling data, and their expression was assayed in two contrasting subtropical maize genotypes, HKI-1532 and V-372. The promoters of all drought-related miRNAs were

analyzed and confirmed the presence of important drought-responsive CREs. Eleven miRNAs belonging to nine families in HKI-1532 and seven miRNAs from three families in V-372 were differentially regulated in both genotypes. Among different miRNA-mRNA interactions, suppression of biologically important genes by miRNAs in V-372 suggests their weak performance under drought stress. It was noticed that the fact that some crucial genes being unaffected by regulatory miRNAs in tolerant genotype HKI1532 could have led to drought tolerance. Our experiment provided a clear understanding of miRNA regulation in drought response. To our knowledge, this is the first report on the role of miRNAs in regulation of drought tolerance in subtropical maize inbreds. Many of the identified candidate miRNAs and mRNAs from the present investigation could be used as potential candidates for development of drought-tolerant maize hybrids for the subtropical production system.

AUTHOR CONTRIBUTIONS

JA and TN: conceived and designed the experiments; JA, SK, AK, BP, and MGM: performed the experiments; SR, MS, and ARR: analyzed the data; All authors contributed to manuscript

preparation. All authors have read and approved the final manuscript.

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Organellar Gene Expression and Acclimation of Plants to Environmental Stress

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Organelles produce ATP and a variety of vital metabolites, and are indispensable for plant development. While most of their original gene complements have been transferred to the nucleus in the course of evolution, they retain their own genomes and gene-expression machineries. Hence, organellar function requires tight coordination between organellar gene expression (OGE) and nuclear gene expression (NGE). OGE requires various nucleus-encoded proteins that regulate transcription, splicing, trimming, editing, and translation of organellar RNAs, which necessitates nucleus-to-organelle (anterograde) communication. Conversely, changes in OGE trigger retrograde signaling that modulates NGE in accordance with the current status of the organelle. Changes in OGE occur naturally in response to developmental and environmental changes, and can be artificially induced by inhibitors such as lincomycin or mutations that perturb OGE. Focusing on the model plant *Arabidopsis thaliana* and its plastids, we review here recent findings which suggest that perturbations of OGE homeostasis regularly result in the activation of acclimation and tolerance responses, presumably via retrograde signaling.

Keywords: organellar gene expression, plastid, retrograde signaling, *Arabidopsis*, acclimation

INTRODUCTION: THE PLASTID GENE-EXPRESSION MACHINERY IS OF MIXED GENETIC ORIGIN

Like mitochondria, plastids – as descendants of cyanobacterium-like progenitors – are of endosymbiotic origin (Raven and Allen, 2003). During evolution, plastids have lost most of their genes to the nucleus, and the plastid genomes of embryophytes contain only 90 to 100 genes (Wicke et al., 2011). However, plastids contain 3000–4000 proteins which function in photosynthesis, the biosynthesis of fatty acids, amino acids, hormones, vitamins, nucleotides, and secondary metabolites, and intracellular signaling (Leister and Kleine, 2008). Thus, plastids encode only a small fraction of the proteins needed to sustain the processes they host. The relatively few genes remaining in the organelles code for proteins involved in plastid gene expression (PGE) or energy production. But while its gene complement is small, PGE is a very complex process. This is because plastids have retained a prokaryotic gene-expression apparatus which is combined with eukaryotic inventions, and its polycistronic transcripts must undergo numerous post-transcriptional maturation steps (**Figure 1**). In higher plants, plastid transcription is performed by three different RNA polymerases: two monomeric, nucleus-encoded (NEP) RNA polymerases and a plastid-encoded (PEP) *E. coli*-like enzyme (Lerbs-Mache, 2011; Börner et al., 2015). Moreover, the multisubunit enzyme requires a set of polymerase-associated proteins (PAPs) and sigma factors

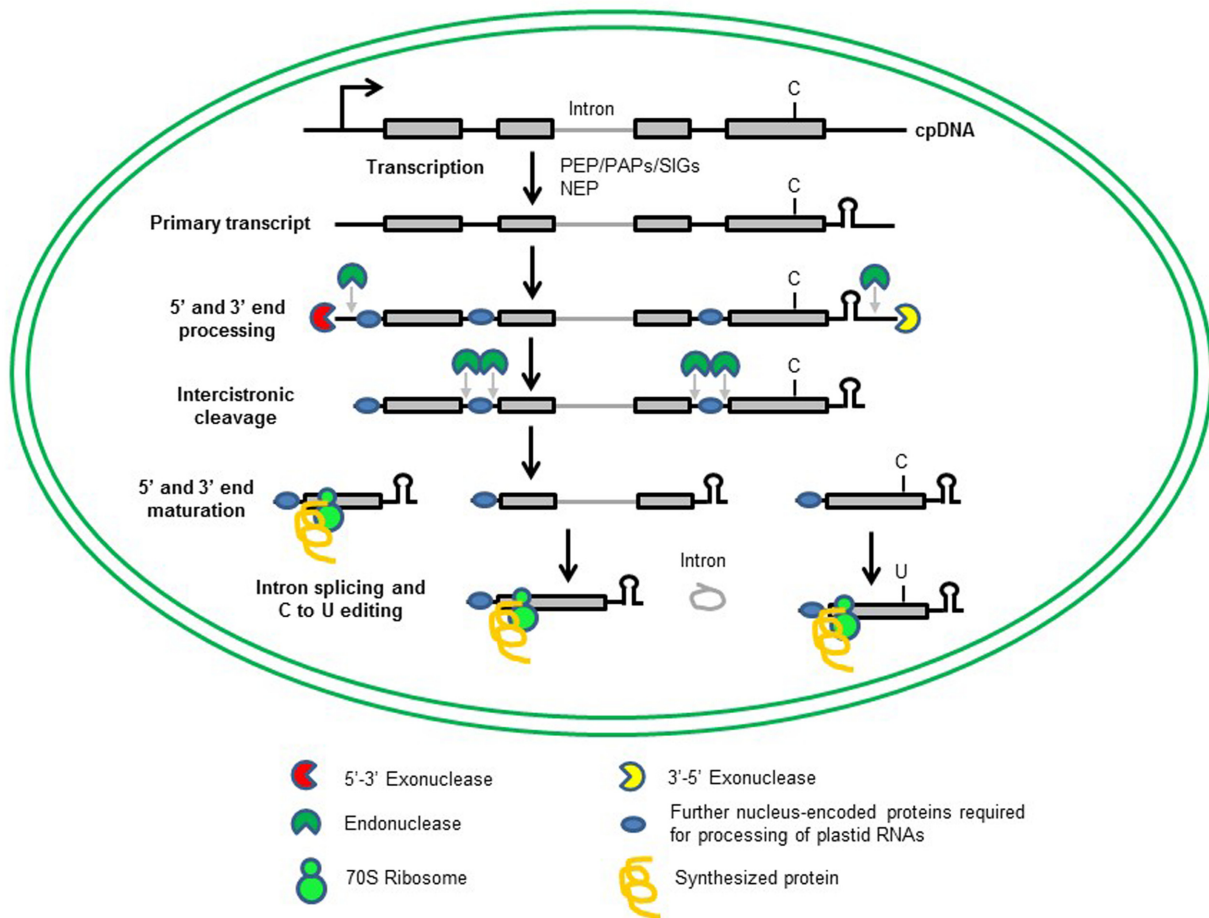


FIGURE 1 | Transcription of chloroplast genes and maturation of chloroplast RNAs. Most of the chloroplast genes are organized in operons, and are transcribed as polycistronic RNAs from single promoters (bent arrow). Transcription of chloroplast mRNA depends on two types of RNA polymerases, a plastid-encoded PEP and one or two nucleus-encoded NEPs. The primary transcript undergoes several steps of maturation that include 5' and 3' end processing, intercistronic cleavage, 5' and 3' end maturation, intron splicing and RNA editing to produce functional RNAs. For these events to take place, a whole series of nucleus-encoded proteins are needed (blue oval or segmented circles). Mature plastid RNAs are translated by bacterial-type 70S ribosomes using the set of tRNAs encoded by the plastid genome.

(SIGs) for function, which are themselves encoded in the nucleus (Lerbs-Mache, 2011; Börner et al., 2015; Chi et al., 2015).

The polycistronic RNAs synthesized by plastid polymerases require extensive processing, including 5' and 3' trimming, intercistronic cleavage, splicing and editing, for which a plethora of nucleus-encoded proteins are needed (Figure 1; del Campo, 2009; Stern et al., 2010; Hammani et al., 2014; Kleine and Leister, 2015; Schmitz-Linneweber et al., 2015). Plastid proteins are synthesized by bacterial-type 70S ribosomes using a set of tRNAs that is entirely encoded in the plastid genome (Tiller and Bock, 2014; Sun and Zerges, 2015). The plastid ribosome itself consists of the large (50S) and small (30S) multi-component ribosomal subunits, each comprising one or more plastid-encoded ribosomal RNA species (rRNAs), and furthermore, plastid- and nuclear-encoded proteins (Yamaguchi and Subramanian, 2000; Yamaguchi et al., 2000).

IMPACT OF ENVIRONMENTAL CHANGES ON THE PGE MACHINERY

Plastid gene expression is crucial for plant development and photosynthesis, and must therefore respond appropriately to developmental and environmental changes. It does so, in part, by modifying transcription levels. Thus, the hormone abscisic acid (ABA) represses the transcription of plastid genes (Yamburenko et al., 2013), and also the circadian clock (Noordally et al., 2013), light, temperature and plastid development differentially modulate transcription in the plastid (reviewed in: Börner et al., 2015). Recently, it was proposed that light-related plastid transcriptional responses are integrated by especially SIG5 (Belbin et al., 2017). In detail, the transcriptional response to light intensity, as well as the response to the relative proportions of red and far red light through phytochrome and photosynthetic signals, and the circadian regulation of

plastid transcription (which is predominantly dependent on blue light and cryptochrome), are regulated by SIG5 (Belbin et al., 2017). In bacteria, responses to stress rely mainly on phosphorylation-dependent signal transduction systems, which act upon transcriptional regulons either by activating DNA-binding two-component response regulators or sigma factors (Marles-Wright and Lewis, 2007). The example of the involvement of SIG5 in several light-dependent pathways (Belbin et al., 2017) and that in general, SIG5 and SIG6 are involved in multiple signaling pathways, suggest that this type of regulation may also be important in plants (reviewed in: Chi et al., 2015). Transcription rates of plastid genes have been shown to be modulated by electron-transfer inhibitors and whether incident light preferentially excites photosystem I or photosystem II (Pfannschmidt et al., 1999). Another PEP-associated protein is the plastid transcription kinase PTK, which responds to changes in the thiol/disulfide redox state mediated by glutathione (Baginsky et al., 1999), and has been shown to target SIG6 (Schweer et al., 2010). *In organello* run-on transcription and phosphorylation assays indeed suggest that the regulation of plastid transcription under different light intensities depends on both glutathione and phosphorylation status (Baena-Gonzalez et al., 2001).

Cluster analyses of plastid transcriptomes from mutants with severe photosynthetic defects or from plants exposed to stresses suggest that the accumulation of specific plastid RNAs is regulated in response to the physiological state of the organelle (Cho et al., 2009). Because organellar multiprotein complexes – including many components of PGE and the photosynthetic machinery – typically contain both plastid- and nucleus-encoded subunits, tight coordination of the activity of the two compartments is necessary. A part of this takes place at the transcript level, as revealed by an analysis of co-regulation based on 1300 transcription profiles obtained under different environmental conditions and in different genetic backgrounds (Leister et al., 2011). The tightest co-regulation was generally observed for genes located in the same compartment. Strikingly however, under stress conditions, nucleus-plastid coregulation could predominate over intracompartamental networks, i.e., specific sets of nuclear and organellar photosynthesis genes were co-expressed. Moreover, when genes were ranked according to the number of situations in which their expression levels were altered by at least twofold (Leister et al., 2011), *NDHF* (the plastid gene for a subunit of NADH dehydrogenase) was classified as “very highly responsive,” as it reacted in 104 of 413 tested states. Several other plastid genes were highly responsive, showing that coordinated transcriptional regulation occurs on a broader scale. The relevance of transcriptional control in the plastid is underlined by changes in the expression of nucleus-encoded sigma factors (which mediate transcription initiation by PEP): *SIG1* and *SIG5* mRNA levels are regulated in 110 and 65 conditions, respectively (Leister et al., 2011) and other studies confirm that sigma factors respond to environmental conditions and are involved in acclimation processes (see above; summarized in: Börner et al., 2015; Chi et al., 2015). Indeed, SIG5 is considered as a multiple stress-responsive sigma factor (Nagashima et al., 2004; Chi et al., 2015), because *SIG5* is

induced by exposure to high light, low temperature, high salt and high osmotic pressures (Nagashima et al., 2004), blue light (Tsunoyama et al., 2002), and ABA (Yamburenko et al., 2015).

Steady-state mRNA levels at any given time reflect the relationship between transcription rate and mRNA degradation rate. In bacteria, the latter plays an important role in controlling gene expression (Hui et al., 2014). Since sessile plant species cannot escape from unfavorable environmental conditions, it is conceivable that they have had to develop more flexible response mechanisms. Indeed, it is generally accepted that the control of PGE has shifted to post-transcriptional events over the course of evolution (Barkan and Goldschmidt-Clermont, 2000; Stern et al., 2010), especially in mature chloroplasts (Sun and Zerges, 2015). Thus, unlike redox regulation of transcription in mustard (Pfannschmidt et al., 1999) and ABA-mediated repression of transcriptional activity of chloroplast genes in barley (Yamburenko et al., 2013), levels of individual plastid mRNAs in spinach (Klauff and Gruijssem, 1991) and barley (Kim et al., 1993) during plant development are mainly determined by alterations in stability, with half-lives of many hours or even days – much more stable than bacterial mRNAs with typical lifetimes of seconds to hours (Radhakrishnan and Green, 2016). This suggests that the differential accumulation of chloroplast mRNAs – at least under these conditions – is primarily regulated at the post-transcriptional level. Consequently, RNA stability is probably the dominant factor governing mRNA levels in plastids. Interestingly, a genome-wide study of mRNA decay rates in *A. thaliana* cell cultures showed that nuclear transcripts encoding mitochondrial, chloroplast and peroxisomal proteins tend to have a high proportion of transcripts with long half-lives (Narsai et al., 2007). This may be largely due to the fact that many of the proteins known to be located in these organelles are associated with intermediate metabolism and energy. Interestingly, transcripts encoding pentatricopeptide repeat (PPR) proteins, which have short half-lives, are exceptions to this generalization (Narsai et al., 2007). The latter finding is corroborated by an analysis of mRNA half-life changes in response to cold stress in *Arabidopsis* (Chiba et al., 2013). When mRNA levels vary depending on developmental stage, environmental factors or intracellular signals, earlier processing events can be the main determining factor (Monde et al., 2000). PPR proteins are important here also, for they are mainly targeted to chloroplasts and/or mitochondria and, as RNA-binding proteins, they participate in RNA editing, splicing, stability, and translation (Barkan and Small, 2014).

ORGANELLE GENE EXPRESSION AND ACCLIMATION TO ABIOTIC STRESS CONDITIONS

Many of the genes on which plastid and mitochondrial gene expression (organellar gene expression; OGE) depends reside in the nuclear genome, which provides for direct control of OGE by nuclear factors (via “anterograde signaling”). Conversely, organelles transmit information relating to their developmental and metabolic states to the nucleus (“retrograde signaling”),

enabling nuclear gene expression to be modulated in accordance with their physiological needs (reviewed in: Kleine et al., 2009; Chi et al., 2013; Bobik and Burch-Smith, 2015; Kleine and Leister, 2016). Retrograde signals are presumed to originate from OGE itself, the tetrapyrrole pathway, the redox state of the organelles, levels of reactive oxygen species (ROS) in the organelles (such as singlet oxygen, hydrogen peroxide, superoxide anion radicals, and hydroxyl radicals) and metabolites [such as cyclocitral, 3'-phosphoadenosine 5'-phosphate (PAP) and methylerythritol cyclodiphosphate (MecPP)] (reviewed in: Kleine et al., 2009; Terry and Smith, 2013; Bobik and Burch-Smith, 2015; Colombo et al., 2016; Dietz et al., 2016). Recent research extends the previous view of retrograde signaling to mainly affect transcriptional reprogramming to also include posttranslational control, which involves the ubiquitin-proteasome system (reviewed in: Woodson, 2016). ROS signatures and metabolite signals control acclimation processes involving the alteration of gene expression and translation which is reviewed elsewhere (Dietz et al., 2016; Kleine and Leister, 2016). Furthermore, a picture emerges in which considerable cross-talk between established signaling pathways takes place. As examples, retrograde signaling pathways converge with photoreceptor pathways (Martin et al., 2016), regulation of flowering time (Feng et al., 2016) and/or hormonal signaling cascades (reviewed in: Bobik and Burch-Smith, 2015; Gollan et al., 2015). In this review, we focus on the relationship between OGE and acclimation responses to abiotic stresses.

The *gun* Mutants, ABA, and Abiotic Stresses

Treatment with inhibitors of OGE, such as chloramphenicol or lincomycin, or the carotenoid biosynthesis inhibitor norflurazon results in reduced expression of nuclear genes encoding plastid proteins (Oelmüller and Mohr, 1986). In the best-known screen for retrograde signaling mutants, *genomes uncoupled* (*gun*) seedlings were mutagenized and mutants that continued to express a nucleus-encoded plastid protein in the presence of norflurazon were selected (Susek et al., 1993). Recent research confirms that functioning chloroplasts are essential for plant acclimation to adverse environmental conditions (for an overview, see Table 1). Inactivation of the H-subunit of the plastid Mg-chelatase (GUN5) results in cold (4°C) sensitivity, and it was suggested that perturbation of plastid function in *gun5* mutants could result in inhibition of protein synthesis and impair plant performance at low temperatures (Kindgren et al., 2015). In this context, it is postulated that enhanced tetrapyrrole biosynthesis might confer drought tolerance via ROS detoxification (reviewed in: Nagahatenna et al., 2015).

A recurring feature of *oge* mutants is their atypical response to ABA. The tetrapyrrole biosynthesis proteins GUN4 and GUN5 (Voigt et al., 2010) and the plastid-targeted PPR protein GUN1 (Cottage et al., 2010) enhance seedling development in the presence of ABA. On the other hand, loss of the mitochondrial PPR protein PENTATRICOPEPTIDE REPEAT PROTEIN FOR GERMINATION ON NaCl (PGN) results in hypersensitivity to ABA, glucose, and salinity (Laluk et al., 2011).

It was suggested that *pgn* plants accumulate large amounts of ABA, and transcripts of ABA-related genes, as well as mitochondrial transcripts, are up-regulated. Levels of *ABI4* and *ALTERNATIVE OXIDASE1a* mRNAs, whose products are known for their roles in mitochondrial retrograde signaling, are particularly affected (Laluk et al., 2011). Thus, PGN is assumed to help neutralize ROS in mitochondria during abiotic and biotic stress responses, probably via retrograde signaling. Another mutant with perturbed RNA metabolism, *srrp1* (*S1 RNA-binding ribosomal protein 1*), in which intron splicing of plastid *trnL* and processing of 5S rRNA were altered, does not display any visible phenotype under normal growth conditions, but seedling development is impaired in the presence of ABA (Gu et al., 2015). Furthermore, mutants lacking WHIRLY1 were shown to be less sensitive to salicylic acid and ABA during germination (Isemer et al., 2012a). The DNA-binding protein WHIRLY1 can translocate from plastids to the nucleus, making it one of the most promising candidate mediators of signaling between organelles and the nucleus (Isemer et al., 2012b). WHIRLY1 was recently proposed to serve as a redox sensor in plastid-to-nucleus retrograde signaling and to mediate cross tolerance, including acclimation responses (Foyer et al., 2014). Finally, application of ABA can partially restore mRNA expression of the nucleus-encoded plastid protein Lhcb1.2 in NF-treated wild-type plants, supporting the view that OGE and ABA signaling are interconnected (Voigt et al., 2010). Indeed, the transcription factor ABSCISIC ACID INSENSITIVE4 (*ABI4*) which has emerged as a central player in many signaling processes during plant development (reviewed in: Léon et al., 2012), has been directly associated with retrograde signaling (Koussevitzky et al., 2007; Giraud et al., 2009). Interestingly, it was shown more than a quarter of a century ago that, while *abi* mutations had no apparent effect on freezing tolerance, cold-acclimated ABA biosynthesis (*aba*) mutants were markedly impaired in freezing tolerance (Gilmour and Thomashow, 1991), indicating that ABA levels can affect freezing tolerance. In addition, temporal and spatial interactions of ABA with ROS signals were shown to play a key role in the regulation of systemic acquired acclimation of plants to heat stress (Suzuki et al., 2013) and ABA is required for plant acclimation to a combination of salt and heat stress (Suzuki et al., 2016). The manifold links between ABA and acclimation responses, and the ABA phenotypes of *oge* mutants, imply that a functional OGE system is essential for proper acclimation responses.

The *hon*, *soldat*, and *mterf* Mutants in the Context of Abiotic Stresses

Treatments with synthetic inhibitors expose plants to highly artificial conditions and are only effective if applied at an early stage of seedling development (Oelmüller and Mohr, 1986). To approximate physiological conditions more closely, a screen was designed that used a reduced concentration of norflurazon and low light levels. This resulted in the identification of *happy on norflurazon* (*hon*) mutants, which remain green in the presence of a low dose of norflurazon (Saini et al., 2011). Because some *hon* mutations were mapped to genes coding for a subunit of

TABLE 1 | Phenotypes of *oge* mutants.

ATG number	Mutant name	Description	Localization	Mutant identification/ availability	Type of mutation	Mutant phenotype	Reference
AT2G31400	<i>gun1-1</i>	GENOMES UNCOUPLED 1, GUN1	Chloroplast	Screen of an EMS-mutagenized M2 population for mutants that still accumulate <i>LHCB1</i> mRNA when grown on norflurazon	Ala259Val	Defective in plastid-to-nucleus signaling, ABA tolerant	Koussevitzky et al., 2007; Cottage et al., 2010
AT3G59400	<i>gun4-1</i>	GENOMES UNCOUPLED 4, GUN4	Chloroplast	Screen of an EMS-mutagenized M2 population for mutants that still accumulate <i>LHCB1</i> mRNA when grown on norflurazon	Leu88Phe	Pale green and defective in plastid-to-nucleus signaling, ABA tolerant	Larkin et al., 2003; Voigt et al., 2010
AT5G13630	<i>gun5-1</i>	ABA-BINDING PROTEIN, ABAR, CCH1, CHLH, GENOMES UNCOUPLED 5, GUN5	Chloroplast	Screen of an EMS-mutagenized M2 population for mutants that still accumulate <i>LHCB1</i> mRNA when grown on norflurazon	Ala990Val	Defective in retrograde plastid-to-nucleus signaling, sensitive to low temperatures, ABA tolerant	Mochizuki et al., 2001; Voigt et al., 2010; Kindgren et al., 2015
AT1G56570	<i>pgn</i>	PENTATRICOPEPTIDE REPEAT PROTEIN FOR GERMINATION ON NaCl, PGN	Mitochondrion	ABRC	SALK_141937, truncated protein	Hypersensitive to ABA, glucose, and salinity	Laluk et al., 2011
AT3G23700	<i>srp1</i>	S1 RNA-BINDING RIBOSOMAL PROTEIN 1, SRP1	Chloroplast	ABRC	SAIL 299 A11, T-DNA is inserted approximately 100 base pairs upstream of the start codon of SRP1	Poorer seedling growth and less cotyledon greening on MS medium supplemented with ABA	Gu et al., 2015
AT4G17040	<i>hon5</i>	CLP PROTEASE R SUBUNIT 4, CLP4, HAPPY ON NORFLURAZON 5, HON5	Chloroplast	Screen of an EMS-mutagenized M2 population for mutants that were able to green when grown on a low dose of norflurazon under dim light	G to A exchange at the 3' end of first intron	Mutants are green in the presence of norflurazon and plastid protein homeostasis is disturbed	Saini et al., 2011
AT5G13650	<i>hon23</i>	SUPPRESSOR OF VARIATION 3, SVR3, HAPPY ON NORFLURAZON 23, HON23	Chloroplast	Screen of an EMS-mutagenized M2 population for mutants that were able to green when grown on a low dose of norflurazon under dim light	Arg438His	Mutants are green in the presence of norflurazon and plastid protein homeostasis is disturbed	Saini et al., 2011
AT2G36990	<i>soldat8</i>	RNAPOLYMERASE SIGMA-SUBUNIT F, SIG6, SIGF, SIGMA FACTOR 6, SOLDAT8	Chloroplast	Screen for second-site mutations of <i>fluorescent (flu)</i> that attenuate the <i>flu</i> phenotype	Gln354Stop	Accumulates reduced amounts of chlorophyll and delays chloroplast development, high light tolerant	Coll et al., 2009
At2g03050	<i>soldat10</i>	mTERF1, EMB93, EMBRYO DEFECTIVE 93, SINGLET OXYGEN-LINKED DEATH ACTIVATOR 10, SOLDAT10	Chloroplast	Screen for second-site mutations of <i>fluorescent (flu)</i> that attenuate the <i>flu</i> phenotype	Pro54Leu	Suppresses ¹ O ₂ -induced cell death, high light tolerant	Meskauskienė et al., 2009

(Continued)

TABLE 1 | Continued

ATG number	Mutant name	Description	Localization	Mutant identification/availability	Type of mutation	Mutant phenotype	Reference
AT4G14605	<i>mda1</i>	mTERF5	Chloroplast	Sequence similarity searches in Arabidopsis genome databases using Arabidopsis RUG2 protein as a query	<i>mda1-1</i> : SALK_097243, truncated protein lacking 2 mTERF motifs; <i>mda1-2</i> : SAIL_425_E03, truncated protein lacking 5 mTERF motifs	Altered chloroplast morphology and plant growth, reduced pigmentation of cotyledons, leaves, stems and sepals, salt and osmotic stress tolerant.	Robles et al., 2012
AT5G55580	<i>mterf9</i>	mTERF9	Chloroplast	Reverse genetics	<i>mterf9</i> : N857510, truncated protein lacking 2 mTERF motifs. <i>twr-1</i> : Q467Stop	Pale, stunted growth, and reduced mesophyll cell numbers, altered responses to sugars, ABA, salt and osmotic stresses	Robles et al., 2015
AT4G02990	<i>rug2-1</i>	mTERF4	Chloroplast, mitochondrion	Screen of an EMS-mutagenized M2 population for mutants affecting leaf morphology	Pro420Leu	Reduced growth, leaves with green and white sectors, altered chloroplast and mitochondrion development, hypersensitive to temperature stress	Quesada et al., 2011
At3g60400	<i>shot1</i>	mTERF18	Mitochondrion	Suppressor of <i>hot1-4</i> (a dominant-negative allele of <i>HSP101</i>)	<i>shot1-1</i> : Gly ₁₀₅ /Asp; <i>shot1-2</i> : premature stop codon, truncated protein.	Short hypocotyl in the dark, growth reduction, less oxidative damage, suppresses other heat-sensitive mutants, heat tolerant	Kim et al., 2012
AT5G30510	<i>rps1</i>	RIBOSOMAL PROTEIN S1, RPS1	Chloroplast	ABRC	<i>rps1</i> : CS874869, T-DNA is inserted 6-bp upstream of the 5'-untranslated region of the <i>RPS1</i> gene	Perturbation of HSF-mediated heat stress response, loss of heat tolerance	Yu et al., 2012
AT2G333800	<i>rps5</i>	RIBOSOMAL PROTEIN S5, RPS5	Chloroplast	Screen of an EMS-mutagenized M2 population for mutants with abnormal leaf color	Gly180Glu	Pale yellow inner leaves, reduced growth, reduced abundance of chloroplast 16S rRNA, hypersensitive to cold stress	Zhang et al., 2016
AT3G20930	<i>orm1</i>	ORGANELLE RRM PROTEIN 1, ORRM1	Chloroplast	ABRC	SALK_072648, T-DNA is inserted in first exon	Developmental delay and pale green inner leaves when grown at 4°C	Sun et al., 2013; Wang et al., 2016
At3g53460	<i>cp29a</i>	CHLOROPLAST RNA-BINDING PROTEIN 29, CP29	Chloroplast	Obtained from ABRC and GABI-Kat	<i>cp29a-1</i> : SALK_003066, T-DNA is inserted in third exon; <i>cp29a-6</i> : 001G06, T-DNA is inserted in second intron	Developmental delay and pale green leaves when grown at 8°C	Kupsch et al., 2012
AT4G24770	<i>cp37a</i>	31-KDA RNA BINDING PROTEIN, CP31, RBP31	Chloroplast	ABRC	<i>cp37a-1</i> : SALK_109613, T-DNA is inserted in third exon; <i>cp37a-3</i> : SAIL_258H02, T-DNA is inserted in first exon	Developmental delay and pale green leaves when grown at 8°C	Tillich et al., 2009

(Continued)

TABLE 1 | Continued

ATG number	Mutant name	Description	Localization	Mutant identification/availability	Type of mutation	Mutant phenotype	Reference
AT1G70200	<i>rbd1</i>	HIGH PHOTOSYNTHETIC EFFICIENCY 1, HPE1, RBD1	Chloroplast	ABRC	<i>rbd1-1</i> : SALK_041100, T-DNA is inserted in third exon; <i>rbd1-2</i> : SALK_012657, T-DNA is inserted in first exon	Pale green inner leaves when grown at 4°C	Wang et al., 2016
AT4G39040	<i>cfm4</i>	CRM FAMILY MEMBER SUBFAMILY 4, CFM4	Chloroplast	ABRC	<i>cfm4-1</i> : SALK_076439, T-DNA is inserted in third exon; SALK_126978, T-DNA is inserted in third exon	Retarded seed germination and growth under stress conditions	Lee et al., 2014
AT5G26742	<i>rh3</i>	ATRH3, EMB1138, EMBRYO DEFECTIVE 1138, RH3	Chloroplast	ABRC	<i>rh3-4</i> : SALK_005920, T-DNA is inserted in ninth intron	Retarded growth phenotype and defects in chloroplast biogenesis and photosynthetic activity	Gu et al., 2014

the plastid-localized Clp protease complex (*ClpR4 = HON5*) and a putative plastid translation elongation factor (*HON23*), *hon* mutations can clearly interfere with PGE (Saini et al., 2011). Interestingly, *hon* seedlings were more resistant than WT to simultaneous exposure to low temperature and high light (Saini et al., 2011). The *soldat8* and *soldat10* (singlet oxygen-linked death activator) seedlings identified in an earlier screen for second-site mutations that suppress the singlet oxygen ($^1\text{O}_2$)-mediated stress response of *fluorescent (flu)* seedlings (Coll et al., 2009; Meskauskiene et al., 2009) behave similarly. The two *soldat* lines are also mutated in genes for proteins related to PGE: *soldat8* is mutant for *SIG6* (see above; Coll et al., 2009) and *soldat10* is defective in the gene encoding mitochondrial Transcription Termination Factor1 (mTERF1) (Meskauskiene et al., 2009), thus linking PGE to the $^1\text{O}_2$ -mediated cell-death responses.

Most members of the mTERF family, which are found in metazoans (four each in human and mouse) and plants (35 in *A. thaliana*), are located in mitochondria and/or plastids, where they regulate OGE at different steps of transcription or translation (Kleine and Leister, 2015). Moreover, several *mterf* mutants have been linked to stress responses. Thus *mda1* (*mterf5*) and *mterf9* seedlings are less susceptible to salt and osmotic stresses, perhaps owing to reduced sensitivity to ABA (Robles et al., 2012, 2015). The *rug2-1* (*mterf4*) mutant is sensitive to temperature stress. When grown at 26°C, *rug2-1* growth is arrested, whereas at 16°C its mutant phenotype is fully suppressed (Quesada et al., 2011). The concept of ROS as retrograde signals in heat stress responses has been reviewed elsewhere (Sun and Guo, 2016). An example for a perturbation in OGE homeostasis contributing to enhanced thermotolerance is the *mterf18/shot1* mutant (Kim et al., 2012). The mitochondrial mTERF18/SHOT1 protein was identified as a suppressor of *hot1-4* (a dominant-negative allele of HSP101) (Kim et al., 2012). The increase in thermotolerance (after heat acclimation at 38°C for 90 min, followed by 2 h at 22°C, then heat-shocked at 45°C for several hours) in the *shot 1* mutant is associated with the accumulation of lower amounts of ROS, and thus a higher tolerance of oxidative stress. Moreover, the plastid ribosomal protein S1 (RPS1) is induced after 2 h of heat treatment (38°C) in the dark, and down-regulation of RPS1 has been shown to severely impair the heat stress-activated expression of *HsfA2* and its target genes, resulting in a loss of heat tolerance (Yu et al., 2012). Interestingly, the level of RPS1 is controlled by GUN1 (Tadini et al., 2016).

PGE and Chilling Tolerance

Plastid gene expression is also important for chilling (low but not freezing temperatures; 4–12°C) tolerance, as exemplified by the *rps5* mutant identified in a screen for genes required for plastid development. The missense mutation in plastid ribosomal protein S5 reduces growth rate and inner leaves remain pale yellow. Furthermore, a variety of photosystem I and II proteins, as well as plastid ribosomal proteins are underrepresented. Levels of proteins associated with stress responses to cold stress are decreased in *rps5*, and overexpression of plastid *RPS5* improves tolerance to cold (Zhang et al., 2016). In a systematic screen of 11,000 T-DNA *A. thaliana* insertion mutants for genes involved

in chilling tolerance, 54 lines defective in 49 genes had a chilling-sensitive phenotype. Of these genes, 16 encode proteins with plastid localization, of which four are plastid ribonucleoproteins (RNPs) (Wang et al., 2016). Three of the 16 – ORRM1, CP29A and CP31A – were previously characterized. CP31A and CP29A (for 31-kD and 29-kD chloroplast protein, respectively) are required for the stability of various mRNAs at low temperatures, and under these conditions they promote specific processing steps (Kupsch et al., 2012). The organelle RNA recognition motif (RRM) protein 1 (ORRM1) is an essential plastid editing factor in *A. thaliana* and maize (Sun et al., 2013). The newly identified RRM/RBD/RNP protein RBD1 binds directly to 23S rRNA, and more strongly under chilling conditions than at normal growth temperatures. Accordingly, the *rbd1* defect in chloroplast protein synthesis is particularly severe at low temperatures (Wang et al., 2016). Furthermore, the CRM (chloroplast RNA splicing and ribosome maturation) family member subfamily4 CFM4 (Lee et al., 2014) and the DEAD-box RNA helicase RH3 (Gu et al., 2014) play a positive role in seed germination and seedling growth under salt or cold stress conditions, because seed germination and seedling growth of the respective mutants are retarded under those conditions.

CONCLUSION

The importance of OGE in stress acclimation responses has become increasingly apparent in recent years. Perturbations in OGE homeostasis trigger abiotic acclimation and tolerance

responses, presumably via retrograde signaling. Thus, further studies on the molecular and physiological functions of OGE proteins should elucidate their roles in such responses. Moreover, as changes in proteins responsive to stress do not always reflect changes at the transcript level, posttranscriptional and translational mechanisms must be given more attention. Cell-fractionation experiments, together with metabolomics studies and the application of next-generation sequencing technologies like mRNA-Seq, global run-on (GRO)-Seq and global ribosomal profiling (Ribo-Seq), should ultimately allow us to assemble an integrated picture of how environmental changes regulate sub-cellular states and reveal the extent and nature of retrograde signal transduction.

AUTHOR CONTRIBUTIONS

TK drafted the manuscript, LW prepared the figure and the table, DL and TK finalized the manuscript.

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Translation and Co-translational Membrane Engagement of Plastid-encoded Chlorophyll-binding Proteins Are Not Influenced by Chlorophyll Availability in Maize

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Chlorophyll is an indispensable constituent of the photosynthetic machinery in green organisms. Bound by apoproteins of photosystems I and II, chlorophyll performs light-harvesting and charge separation. Due to the phototoxic nature of free chlorophyll and its precursors, chlorophyll synthesis is regulated to comply with the availability of nascent chlorophyll-binding apoproteins. Conversely, the synthesis and co-translational insertion of such proteins into the thylakoid membrane have been suggested to be influenced by chlorophyll availability. In this study, we addressed these hypotheses by using ribosome profiling to examine the synthesis and membrane targeting of chlorophyll-binding apoproteins in chlorophyll-deficient *chlH* maize mutants (*Zm-chlH*). *ChlH* encodes the H subunit of the magnesium chelatase (also known as GUN5), which catalyzes the first committed step in chlorophyll synthesis. Our results show that the number and distribution of ribosomes on plastid mRNAs encoding chlorophyll-binding apoproteins are not substantially altered in *Zm-chlH* mutants, suggesting that chlorophyll has no impact on ribosome dynamics. Additionally, a *Zm-chlH* mutation does not change the amino acid position at which nascent chlorophyll-binding apoproteins engage the thylakoid membrane, nor the efficiency with which membrane-engagement occurs. Together, these results provide evidence that chlorophyll availability does not selectively activate the translation of plastid mRNAs encoding chlorophyll apoproteins. Our results imply that co- or post-translational proteolysis of apoproteins is the primary mechanism that adjusts apoprotein abundance to chlorophyll availability in plants.

Keywords: translation, chloroplast, chlorophyll, ChlH, GUN5, ribosome profiling, maize

INTRODUCTION

Chlorophylls are crucial for the light reactions of photosynthesis. They harvest light energy in the antenna complexes of photosystems I and II (PSI and PSII), they transmit the energy to the reaction centers of both photosystems and they are the primary site of light-induced charge separation. Chlorophylls are tetrapyrroles whose synthesis starts with the reduction of an activated glutamate delivered by the glutamyl-tRNA inside chloroplasts (Masuda and Fujita, 2008; Tanaka et al., 2011).

Subsequent steps produce protoporphyrin IX, which is the substrate for the first committed step in chlorophyll synthesis: the insertion of a magnesium ion (Mg^{2+}) by the enzyme protoporphyrin IX magnesium chelatase (referred to as magnesium chelatase). Additional reactions generate chlorophyll *a* and its descendant chlorophyll *b*. The majority of chlorophylls are bound by plastid-encoded proteins located in the cores of PSI (PsaA/B) and PSII (PsbA/B/C/D, also known as D1, CP47, CP43, and D2, respectively) and by nuclear-encoded proteins that make up the light harvesting complexes (LHC) (Umena et al., 2011; Croce, 2012; Mazor et al., 2015).

Chlorophylls are highly photoreactive and their accumulation outside the context of a photosynthetic complex produces deleterious reactive oxygen species (Apel and Hirt, 2004). Hence, the synthesis of chlorophylls is coordinated with the availability of chlorophyll-binding apoproteins (Wang and Grimm, 2015). Chlorophyll synthesis is regulated at different steps and activated by light (Brzezowski et al., 2015; Gabruk and Mysliwa-Kurdziel, 2015). In turn, the expression of the nuclear-encoded LHC apoproteins is adjusted by retrograde chloroplast-to-nucleus signaling, which has been suggested to emanate, among other origins, from intermediates in chlorophyll synthesis (Kleine and Leister, 2016; Larkin, 2016). Two of the genes identified in a genetic screen for mutants with disrupted retrograde signaling, *genomes uncoupled* (*gun*) *gun4* and *gun5*, were found to encode components of the chlorophyll synthesis pathway (Susek et al., 1993; Mochizuki et al., 2001). GUN5 constitutes the catalytic H subunit of the magnesium chelatase (ChlH, Mochizuki et al., 2001) and its interaction partner GUN4 enhances the chelatase activity (Adhikari et al., 2011).

The accumulation of plastid-encoded chlorophyll-binding proteins strictly requires chlorophyll (e.g., Klein et al., 1988a; Herrin et al., 1992; Eichacker et al., 1996). Various lines of evidence implicate chlorophyll both as essential for the stability of chlorophyll-binding proteins and as an activator of their synthesis. For example, the results of *in vivo* and *in organello* pulse-labeling assays suggested that the rate of synthesis of chlorophyll-binding apoproteins increases upon a shift from dark to light, coinciding with the onset of chlorophyll synthesis (Fromm et al., 1985; Klein et al., 1988a,b; Malnoë et al., 1988; Mühlbauer and Eichacker, 1998). Furthermore, pulse-labeling experiments with chlorophyll-deficient *Chlamydomonas* and *Synechocystis* cells showed strongly diminished PsbA labeling, suggesting that chlorophyll activates *psbA* translation (Herrin et al., 1992; He and Vermaas, 1998). By contrast, other experiments provided evidence that chlorophyll-binding stabilizes nascent chlorophyll-binding proteins and does not influence their synthesis (Mullet et al., 1990; Herrin et al., 1992; Kim et al., 1994a; Eichacker et al., 1996). Specific ribosome pausing sites were identified on the *psbA* mRNA and were suggested to enable chlorophyll-binding (Kim et al., 1991). However, ribosome pausing was not detectably altered between dark-grown plants and plants illuminated for short periods, arguing against a chlorophyll-mediated pausing mechanism (Kim et al., 1994b). Taken together, the available data provide strong evidence that chlorophyll-binding apoproteins are highly

unstable in the absence of chlorophyll, and that several of the apoproteins are synthesized at normal rates in the absence of chlorophyll in barley or *Chlamydomonas* (Mullet et al., 1990; Herrin et al., 1992). Although reduced levels of radiolabeled PsbA in pulse-labeling assays in the absence of chlorophyll suggest that chlorophyll may, in fact, activate translation (Klein et al., 1988a; Herrin et al., 1992; He and Vermaas, 1998), the technical challenge of discriminating lack of protein synthesis from rapid protein turnover in pulse-labeling assays precludes firm conclusions.

The binding of chlorophyll to nascent chlorophyll-binding proteins has also been suggested to be coordinated with their insertion into the thylakoid membrane (Sobotka, 2014). Recently, we have shown that membrane engagement of nascent plastid-encoded chlorophyll apoproteins occurs shortly after the first transmembrane segment emerges from the ribosome (Zoschke and Barkan, 2015). Interestingly, an interaction between a chlorophyll synthesis enzyme and the ALB3 protein translocase in the thylakoid membrane has been demonstrated in cyanobacteria (Chidgey et al., 2014) and this provides a potential mechanism for linking chlorophyll attachment with membrane integration.

Altogether, there is a paucity of firm data that address the interconnection of chlorophyll availability with the synthesis and targeting of plastid-encoded chlorophyll apoproteins. To clarify this issue, we used ribosome profiling to comprehensively analyze (i) ribosome distributions on plastid mRNAs, and (ii) the co-translational membrane-engagement of plastid-encoded proteins in chlorophyll-deficient *chlH* mutants in maize. Our results show that chlorophyll deficiency has little if any effect on the abundance or positions of ribosomes on chloroplast mRNAs, nor on the co-translational membrane engagement of plastid-encoded chlorophyll apoproteins. Together, this implies that plastid apoprotein synthesis and membrane engagement are not regulated by chlorophyll-binding and that changes in protein stability account for adjustments of apoprotein accumulation to chlorophyll levels in plants.

MATERIALS AND METHODS

Plant Material

The *Zm-chlH* mutants were recovered from our large collection of mutants with defects in chloroplast development, the Photosynthetic Mutant Library (Belcher et al., 2015). An Illumina sequencing approach (Williams-Carrier et al., 2010) detected the insertions in the *GUN5/ChlH* ortholog GRMZM2G323024 (B73 genome v.3) in individual yellow seedlings. Gene-specific PCR confirmed that the insertions co-segregated with the phenotype [primers used for genotyping the mutants: et175GRM3230245 5'-gacgaggacacggacaaccta-3', et1082GRM3230243 5'-ggcgaagttgctggagttg-3' (*Zm-chlH-1* and *Zm-chlH-2*); et966GRM3230245 5'-CAATTGCTCGGGTGTTC-3', et1847GRM3230243 5'-AACGAATTGGGGTTGGTGTC-3' (*Zm-chlH-3*)]. The alleles are recessive and confer a seedling lethal phenotype. Plants were grown in soil in cycles of 16 h light

($\sim 300 \mu\text{mol m}^{-2} \text{s}^{-1}$)/28°C and 8 h dark/26°C. On the eighth day after sowing, leaf tissue was harvested and snap-frozen in liquid nitrogen one hour after the start of the light cycle. Plant tissue was stored at -80°C until use. The second and third leaves to emerge were used for ribosome profiling and chlorophyll measurements whereas the apical half of the second leaf was used for protein extraction and immunoblotting.

Protein Analysis and Chlorophyll Measurements

SDS-PAGE and immunoblotting used the methods and antibodies described previously (Barkan, 1998; Roy and Barkan, 1998). Chlorophyll content was examined in 80 % acetone by the method described by Porra et al. (1989) and normalized to fresh weight.

Ribosome Profiling

Microarray-based ribosome and transcriptome profiling experiments were carried out as in Zoschke et al. (2013). Spatially resolved analysis of stromal and thylakoid membrane-tethered ribosomes was performed as in Zoschke and Barkan (2015). For the latter approach, a micrococcal nuclease pre-treatment was performed to remove mRNA-tethered ribosomes from thylakoid membranes before pelleting the membranes (Zoschke and Barkan, 2015). The microarray figures for *Zm-chlH-1/-2* are based on one biological replicate including three technical replicates (Figures 2, 5, 6). The wild-type data in Figures 5, 6 come from two biological replicates including three technical replicates each, and were taken from Zoschke and Barkan (2015) according to the journal guidelines. The values used to generate the plots are available in Supplementary Datasets S1, S3. Due to the known difficulties of a reliable quantification of highly abundant RNAs (problem of saturation effects), signals for tRNAs and rRNAs were excluded from the plotting of total RNA (Figures 2C,F). To verify the microarray-based ribosome profiling results, ribosome profiling by deep-sequencing was performed with one biological replicate as described by Chotewutmontri and Barkan (2016) with minimal adjustments: ribosomes were pelleted through sucrose cushions by layering 0.82 ml lysate on a 0.33 ml sucrose cushion (1 M sucrose, 0.1 M KCl, 40 mM Tris acetate, pH 8.0, 15 mM MgCl_2 , 10 mM 2-Mercaptoethanol, 100 $\mu\text{g/ml}$ chloramphenicol, and 100 $\mu\text{g/ml}$ cycloheximide) in a 11 mm \times 34 mm tube and centrifugation in a Beckman TLA-100.2 rotor for 1.5 h at 55,000 rpm. Reads were aligned to the maize chloroplast genome using Genbank accession X86563.2 and the quality of the footprints was evaluated (Supplementary Figure S1). The data are normalized to ORF length (kilobase) per million reads mapping to nuclear genome coding sequences (rpkm). The data used for the plots are provided in Supplementary Dataset S2. RNA was extracted from an aliquot of the same tissue homogenate used for ribosome profiling, and used for transcriptome analysis by either microarray or RNA-sequencing as described previously (Zoschke and Barkan, 2015; Chotewutmontri and Barkan, 2016). Each of the abovementioned ribosome profiling experiments used plant tissue from independent mutant plants.

RESULTS

Identification of Transposon-induced *chlH* Mutant Alleles in Maize

The maize gene encoding the ortholog of *ChlH/GUN5* is designated GRMZM2G323024 in the B73 v.3 genome annotation¹. We identified three *Mu* transposon insertions in this gene during the systematic sequencing of *Mu* insertions in our large collection of non-photosynthetic maize mutants, the Photosynthetic Mutant Library (Belcher et al., 2015) (Figure 1A). *Zm-chlH-1* and *Zm-chlH-2* have insertions in the 5'-UTR, and represent hypomorphic alleles as shown by the reduction of chlorophylls in *Zm-chlH-1/-2* mutants to less than 10% of wild-type levels (Table 1). The insertion in *Zm-chlH-3* maps in the last exon and is flanked by a deletion of 11 base pairs. *Zm-chlH-3* is a null allele, based on the facts that chlorophylls are undetectable (Table 1) and that the insertion/deletion prevent translation of a highly conserved protein-coding sequence (Supplementary Figure S2). All three alleles condition a yellow seedling phenotype (Figure 1B). Experiments below used

¹<http://cas-pogs.uoregon.edu/#/pog/17223>

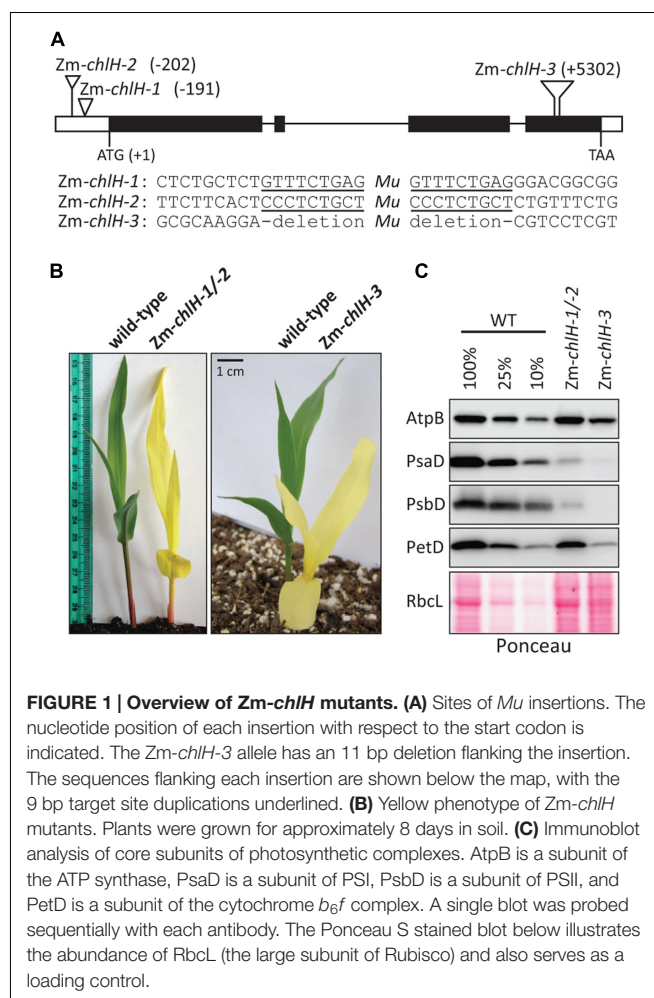


FIGURE 1 | Overview of *Zm-chlH* mutants. (A) Sites of *Mu* insertions. The nucleotide position of each insertion with respect to the start codon is indicated. The *Zm-chlH-3* allele has an 11 bp deletion flanking the insertion. The sequences flanking each insertion are shown below the map, with the 9 bp target site duplications underlined. **(B)** Yellow phenotype of *Zm-chlH* mutants. Plants were grown for approximately 8 days in soil. **(C)** Immunoblot analysis of core subunits of photosynthetic complexes. AtpB is a subunit of the ATP synthase, PsaD is a subunit of PSI, PsbD is a subunit of PSII, and PetD is a subunit of the cytochrome *b₆f* complex. A single blot was probed sequentially with each antibody. The Ponceau S stained blot below illustrates the abundance of RbcL (the large subunit of Rubisco) and also serves as a loading control.

TABLE 1 | Chlorophyll content in *Zm-chlH* mutants.

	Chlorophyll per 1 g fresh weight [μg]		
	Chlorophyll <i>a</i>	Chlorophyll <i>b</i>	Chlorophyll <i>a + b</i>
wild-type	771.8 \pm 80.3	163.4 \pm 2.8	935.2 \pm 82.9
<i>Zm-chlH-1/-2</i>	82.1 \pm 12.3	1.7 \pm 1.8	83.8 \pm 14.1
% wild-type	10.6	1.0	9.0
wild-type	937.6 \pm 180.0	193.4 \pm 39.4	1131.0 \pm 219.4
<i>Zm-chlH-3</i>	0.1 \pm 0.1	0.1 \pm 0.1	0.2 \pm 0.2
% wild-type	0.0	0.1	0.0

Chlorophyll *a* and *b* levels were measured for *Zm-chlH-1/-2* and *Zm-chlH-3* mutants and wild-type siblings as described in Section "Materials and Methods". Mean values and standard deviations are shown for three biological replicates.

the *Zm-chlH-3* null allele and the heteroallelic progeny of a complementation test cross between *Zm-chlH-1* and *Zm-chlH-2*. Mutants with any of these allele combinations die between the three and four-leaf stage (~ 2 weeks after germination), as is typical of non-photosynthetic mutants in maize.

We examined the abundance of the thylakoid membrane complexes PSII, cytochrome *b₆f*, PSI, and ATP synthase in the *Zm-chlH* mutants by immunoblot analysis of one core subunit of each complex (Figure 1C). The PsaD and PsbD subunits of PSI and PSII, respectively, were reduced more than ten-fold in the hypomorphic mutant and were undetectable in the *Zm-chlH-3* mutant. This is expected based on prior evidence that chlorophyll-binding proteins and the proteins with which they closely associate fail to accumulate in the absence of chlorophyll (e.g., Klein et al., 1988a; Herrin et al., 1992; Eichacker et al., 1996). Interestingly, the PetD subunit of the cytochrome *b₆f* complex was substantially reduced in the *Zm-chlH-3* null mutant ($\sim 10\%$ of wild-type levels). Reduced levels of the cytochrome *b₆f* complex were also observed in an *Arabidopsis chlM* mutant (Pontier et al., 2007), and may result from instability of the complex when its single chlorophyll is unavailable (Croce, 2012). The AtpB subunit of the ATP synthase and the large subunit of Rubisco (RbcL) accumulated to normal levels in the hypomorphic mutant but were reduced approximately four-fold in the *Zm-chlH-3* null mutant; the reduction of these proteins is less severe than that of subunits of PSI, PSII, and the cytochrome *b₆f* complex, consistent with the fact that the ATP synthase and Rubisco lack chlorophyll. It is interesting, however, that AtpB and RbcL were reduced at all, and possible explanations are discussed below.

Ribosome Placement on Plastid mRNAs Encoding Chlorophyll-binding Apoproteins Is Not Substantially Altered in *Zm-chlH* Mutants

To address whether chlorophyll alters ribosome behavior on apoprotein-coding mRNAs, we used ribosome profiling to compare the distribution of ribosomes among and within plastid ORFs in wild-type and *Zm-chlH* mutant leaf tissue. The original ribosome profiling method uses deep-sequencing to map and quantify ribosome footprints – small mRNA segments that are

protected by ribosomes from nuclease attack (Ingolia et al., 2009). Our initial experiments used a modified method that substitutes high-resolution microarrays for deep-sequencing to profile ribosome footprints (Zoschke et al., 2013; Figure 2). We hybridized microarrays to ribosome footprints (Figures 2B,E) and total RNA (Figures 2C,F) from wild-type and *Zm-chlH-1/-2* samples; translational efficiencies were then calculated as the ratios of ribosome footprints to RNA abundances (Figure 2D). Genotype-dependent differences in the abundance of ribosome footprints from several genes are apparent, the largest of which mapped to the *psbA* and *atpF* coding regions (Figures 2B,E,G; ratios > 3). However, these result from a difference in mRNA abundance (Figures 2C,F,H). A several-fold decrease in *psbA* mRNA had previously been observed in other maize mutants with diverse chloroplast biogenesis defects and is, therefore, likely to result from pleiotropic effects of the photosynthesis defect (Zoschke et al., 2013; Williams-Carrier et al., 2014). The calculated translational efficiencies for all ORFs encoding chlorophyll-binding apoproteins (Figure 2I), and in fact for all other ORFs, were very similar in the wild-type and the *Zm-chlH-1/-2* mutant (Figure 2D). These results strongly suggest that there are no substantive differences between the wild-type and the *Zm-chlH* mutant in the number of ribosomes bound per mRNA for the plastid-encoded chlorophyll apoproteins or any other chloroplast ORF.

To validate and expand on these findings, we repeated the experiment by using deep-sequencing to profile ribosome footprints. Deep-sequencing offers greater sensitivity than the microarray approach and is especially well suited for detecting changes in ribosome distribution within an ORF at codon resolution. We used the null mutant *Zm-chlH-3* for this experiment to ensure that the trace amounts of chlorophyll present in the *Zm-chlH-1/2* mutants used for the microarray experiment did not mask any effects that chlorophyll might have on ribosome behavior. The normalized abundance of ribosome footprints mapping to each chloroplast gene is plotted in Figure 3. Translational efficiencies were calculated by normalizing ribosome footprint abundance to RNA abundance (Figure 3C). Unlike the *Zm-chlH-1/2* mutant, four genes (*cemA*, *ndhE*, *ndhI*, and *rpoC1*) showed more than three-fold decrease of translational efficiency in the *Zm-chlH-3* mutant compared to wild-type. However, as observed by microarray analysis of the *Zm-chlH-1/2* mutant, no substantial differences in translational efficiency of mRNAs encoding chlorophyll apoproteins were detected between wild-type and the *Zm-chlH-3* mutant.

The dynamics of ribosome movement along an ORF are reflected by the relative abundance of ribosomes at each codon, with longer ribosome dwell times resulting in a higher abundance of ribosome footprints (Ingolia, 2014). To determine whether chlorophyll impacts ribosome pausing, we analyzed the distribution of ribosomes along plastid mRNAs encoding chlorophyll-binding apoproteins (Figure 4). The profiles of peaks and valleys in these ribosome coverage plots are very similar between the mutant and wild-type, suggesting that the deficiency of chlorophyll does not substantially alter pausing at specific sites or the relative rates of initiation and elongation. Minor differences in ribosome distribution were



FIGURE 2 | Microarray-based plastome-wide analysis of ribosome footprint and transcript abundances in the wild-type and the *Zm-chlH-1/-2* mutant. Plots are based on data that are provided in **Supplementary Dataset S1**. Genome positions refer to the reference maize chloroplast genome (Maier et al., 1995). **(A)** Gene map indicating protein-coding genes of the maize chloroplast genome created with OGDRAW (Lohse et al., 2013). The circular map of the chloroplast genome was linearized and shows only the first of the two large inverted repeat regions. Asterisks mark genes with defects in gene expression based on the microarray data in B-F (wild-type to mutant signal ratio > 3). Dashed lines connect these genes on the map with peaks in the plots below. **(B)** Normalized ratios of ribosome footprint signals (Ribo footprints) in wild-type versus mutant are plotted as a function of genome position. Peaks designate regions with more ribosome footprints in the wild-type compared to the mutant. **(C)** Normalized ratios of total RNA signals in wild-type versus mutant are plotted as a function of genome position. Peaks represent regions with higher RNA accumulation in the wild-type compared to the mutant. **(D)** Translational efficiencies were calculated as the ratios of ribosome footprint ratios (shown in **B**) to total RNA ratios (shown in **C**). **(E)** Normalized ribosome footprint signal intensities obtained from wild-type (red) and mutant (green). **(F)** Normalized total RNA signal intensities obtained from wild-type (red) and mutant (green). **(G)** Median ribosome footprint signals for chlorophyll apoprotein-coding ORFs (signals plotted in \log_{10} -scale). **(H)** Median total RNA signals for chlorophyll apoprotein-coding ORFs (signals plotted in \log_{10} -scale). **(I)** Median translational efficiency values for chlorophyll apoprotein-coding ORFs.

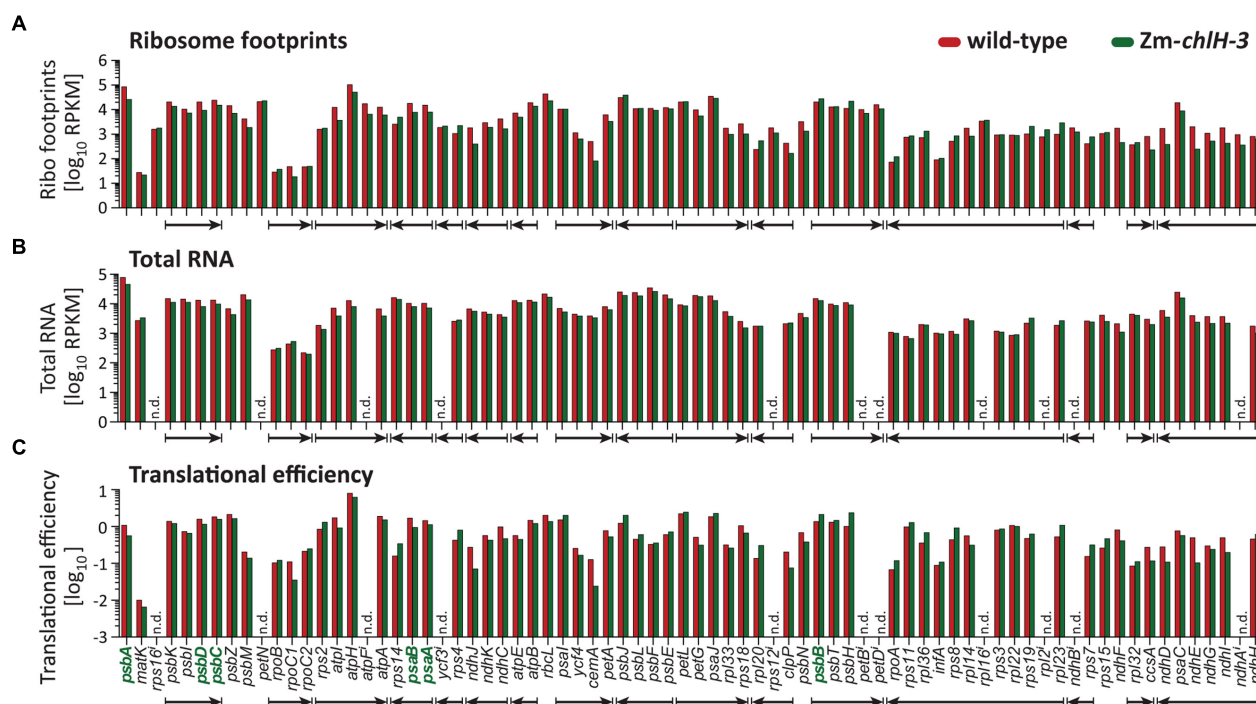


FIGURE 3 | Summary of deep-sequencing analysis of plastid ribosome footprint and transcript abundances in wild-type and *Zm-chlH-3* mutant leaf tissue. Genes encoding chlorophyll-binding proteins are shown in bold green font. The data are displayed as the number of reads per ORF after normalizing to ORF length (kilobase) per million reads mapping to nuclear genome coding sequences (RPKM; values are shown in **Supplementary Dataset S2**). Translational efficiencies are calculated as the ratios of ribosome footprint to transcript reads. Co-transcribed genes are marked with arrows according to the direction of transcription. **(A–C)** Ribosome footprint abundance, transcript levels, and the derived translational efficiencies are displayed according to native gene order on the maize chloroplast genome. RNA levels and translational efficiencies of *petV* and intron containing ORFs (marked with i) were not determined (n.d.) due to technical limitations that preclude accurate quantification of the mRNAs. The ribosome footprint values provided for intron-containing ORFs come only from the last exon or, in the case of *rps12*, from exon 2.

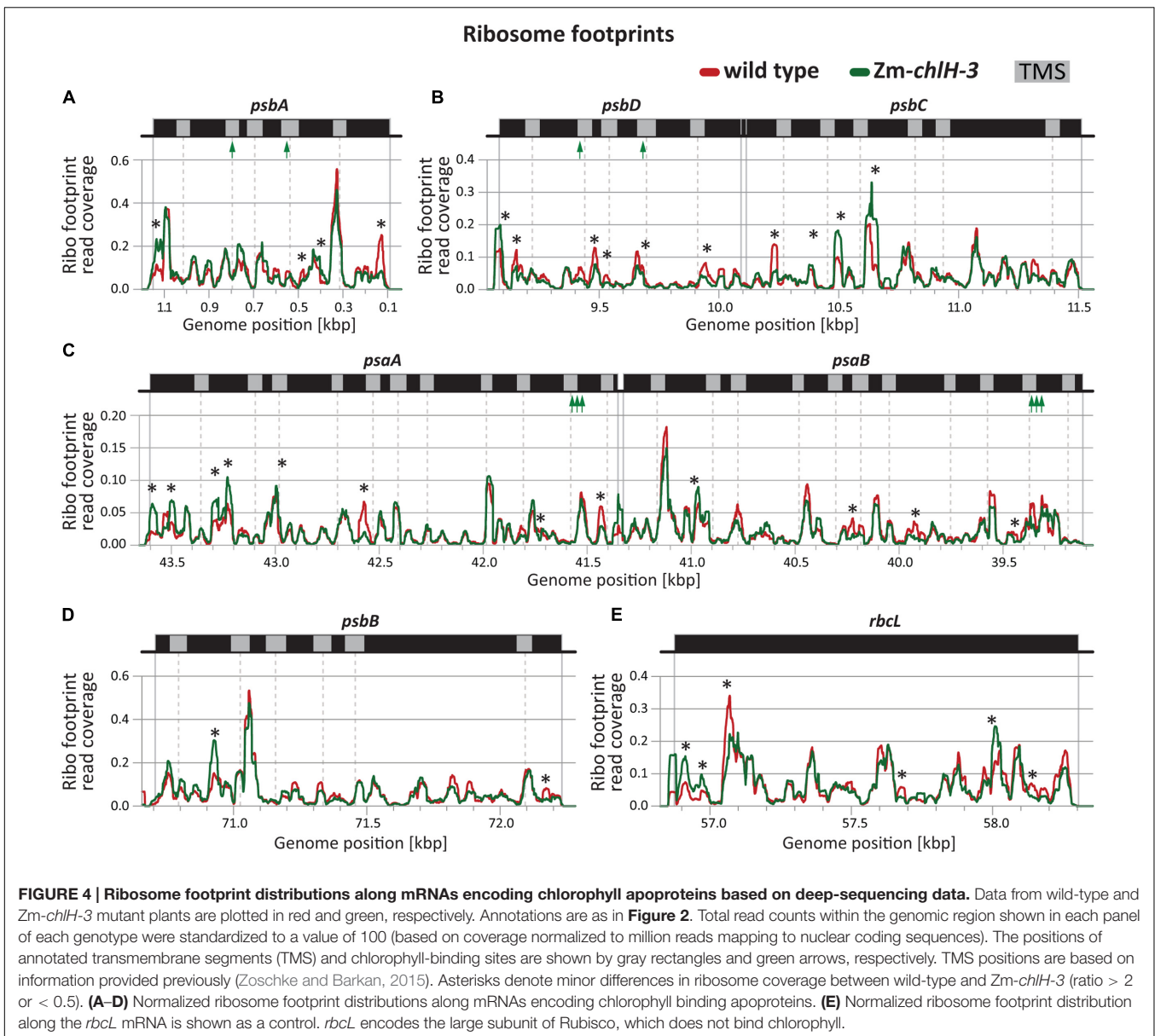
detected between wild-type and the *Zm-chlH-3* mutant at several positions and may indicate chlorophyll-dependent changes in apoprotein translation behavior (more than two-fold diminished or increased ribosome occupancy is marked by asterisks in **Figures 4A–D**). However, similar features were found for many other reading frames that do not code for chlorophyll-binding proteins (**Figure 4E** shows *rbcl* as an example). Consequently, these differences are not likely to be a direct consequence of the presence or absence of chlorophyll on ribosome dynamics.

In sum, our results show that the distribution of ribosomes among and within ORFs encoding chlorophyll apoproteins is not markedly altered in *Zm-chlH* mutants. This provides

strong evidence that chlorophyll does not act as a specific regulator of the synthesis of plastid-encoded chlorophyll apoproteins.

Co-translational Membrane Engagement of Nascent Chlorophyll-binding Apoproteins Is Not Altered in a *Zm-chlH* Mutant

To address whether chlorophyll availability impacts the co-translational engagement of chlorophyll-binding apoproteins with the thylakoid membrane, we used a previously described



approach that reports the partitioning of ribosome footprints between the membrane and soluble fractions (Supplementary Figure S3); this method reveals the point in nascent peptide synthesis at which co-translational membrane engagement occurs (Zoschke and Barkan, 2015). We isolated ribosome footprints from separated membrane and soluble fractions of the *Zm-chlH-1/2* mutant and examined them by competitive hybridization to our maize chloroplast microarrays (Figure 5). The results did not reveal any substantial difference in co-translational membrane engagement of nascent thylakoid proteins in the *Zm-chlH* mutant compared to wild-type plants (Figures 5B,C). We observed the same set of proteins to be co-translationally membrane-engaged (including the chlorophyll apoproteins; shown as green shaded regions in Figures 5B,C), and the relative signal intensities of

ribosome footprints recovered from membrane and soluble fractions are similar in *Zm-chlH* mutant and wild-type plants (Figures 5B–E).

High-resolution views of the same data (Figure 6) showed that the point at which membrane engagement of nascent chlorophyll apoproteins takes place is very similar between wild-type and *Zm-chlH* mutant plants (Figures 6A–D). Furthermore, the topographies of the mutant plots closely resemble those of the wild-type plots, providing further evidence that chlorophyll has little if any effect on the dynamics of ribosome movement through these ORFs. Minor isolated differences were detected for several probes: e.g., peaks were observed for membrane-attached ribosome footprints in the *psaB* and *psbB* coding regions in the *Zm-chlH* mutant that were absent in the wild-type (marked by asterisks in Figures 6C,D bottom panels).

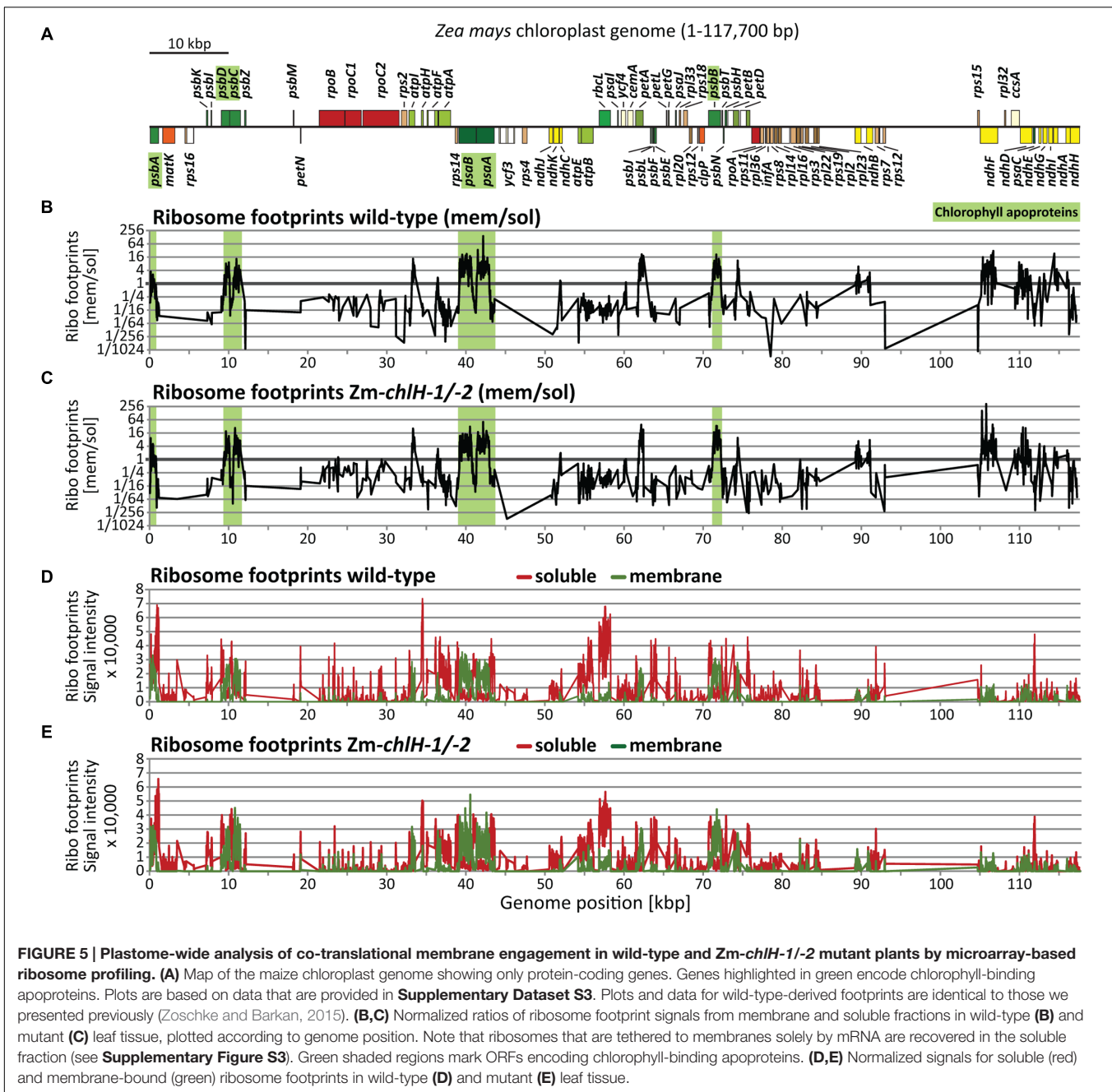


FIGURE 5 | Plastome-wide analysis of co-translational membrane engagement in wild-type and Zm-*chlH*-1/-2 mutant plants by microarray-based ribosome profiling. **(A)** Map of the maize chloroplast genome showing only protein-coding genes. Genes highlighted in green encode chlorophyll-binding apoproteins. Plots are based on data that are provided in **Supplementary Dataset S3**. Plots and data for wild-type-derived footprints are identical to those we presented previously (Zoschke and Barkan, 2015). **(B,C)** Normalized ratios of ribosome footprint signals from membrane and soluble fractions in wild-type **(B)** and mutant **(C)** leaf tissue, plotted according to genome position. Note that ribosomes that are tethered to membranes solely by mRNA are recovered in the soluble fraction (see **Supplementary Figure S3**). Green shaded regions mark ORFs encoding chlorophyll-binding apoproteins. **(D,E)** Normalized signals for soluble (red) and membrane-bound (green) ribosome footprints in wild-type **(D)** and mutant **(E)** leaf tissue.

This might reflect ribosome pauses that differ between wild-type and mutant. However, we favor the view that these differences result from technical variations because we did not detect analogous changes in ribosome distribution when profiling unfractionated chloroplast lysates (**Figures 2B,E, 4C,D**).

Altogether, our results demonstrate that chlorophyll availability does not impact the initial co-translational engagement of plastid-encoded chlorophyll apoproteins with the thylakoid membrane. However, our assay does not address any possible effects of chlorophyll on the integration of downstream transmembrane segments.

DISCUSSION

Chlorophyll-independent Translation of Plastid-encoded Chlorophyll Apoproteins

Compared to the sophisticated knowledge about the structure of the photosystems and the location of chlorophylls therein (Umena et al., 2011; Croce, 2012; Mazor et al., 2015), little is known about the coordination of apoprotein synthesis with chlorophyll availability. Although it is well established that chlorophyll binding proteins do not accumulate in the absence of chlorophyll (e.g., Klein et al., 1988a; Herrin et al.,

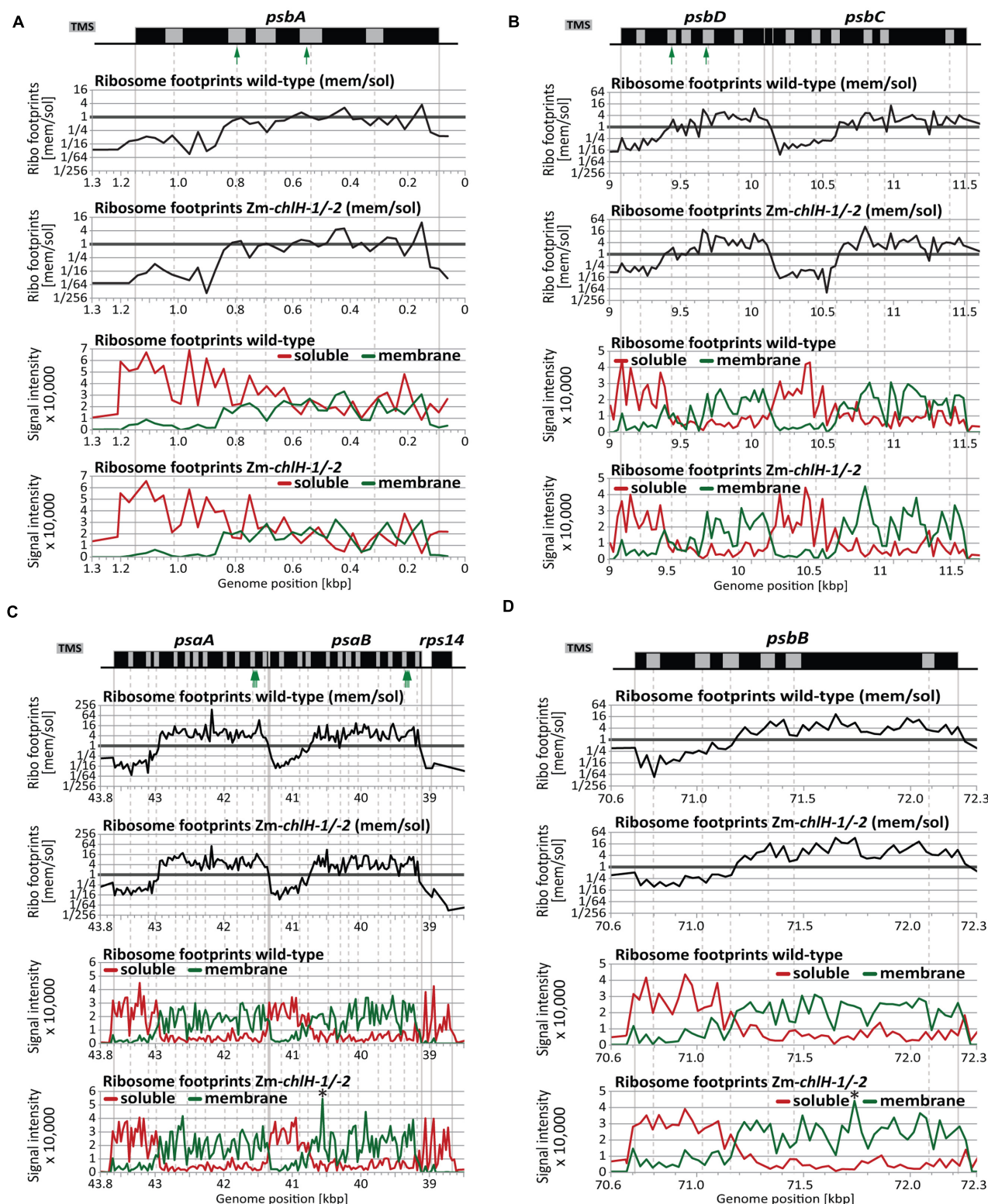


FIGURE 6 | Continued

FIGURE 6 | Zoom-in views of the co-translational membrane engagement of chlorophyll-binding apoproteins in wild-type and *Zm-chlH-1/-2* mutant plants. Gene maps are drawn to scale above the plots. The positions of annotated transmembrane segments (TMS) and chlorophyll-binding sites are shown by gray rectangles and green arrows, respectively. TMS positions are based on information provided previously (Zoschke and Barkan, 2015). The plots of wild-type data were taken from Zoschke and Barkan (2015). The upper two plots in each panel show normalized ratios of ribosome footprint signals from membrane and soluble fractions of plants of the indicated genotype. The lower two plots in each panel show the signals for membrane (green) and soluble (red) ribosome footprints in plants of the indicated genotype. Asterisks denote minor differences in ribosome coverage between wild-type and *Zm-chlH-1/-2* (see Results). **(A)** Co-translational membrane engagement of the nascent chlorophyll apoprotein PsbA (D1). **(B)** Co-translational membrane engagement of the nascent chlorophyll apoproteins PsbD (D2) and PsbC (CP43) encoded by the overlapping *psbD* and *psbC* reading frames. **(C)** Co-translational membrane engagement of the nascent chlorophyll apoproteins PsbA and PsbB encoded by the adjacent *psaA* and *psaB* genes. Data from the *rps14* gene, which is co-transcribed with *psaA* and *psaB*, is included to illustrate the origin of the soluble ribosome footprints derived from the *psaB* 3'-UTR. **(D)** Co-translational membrane engagement of the nascent chlorophyll apoprotein PsbB (CP47).

1992; Eichacker et al., 1996), the relative contributions of increased protein instability and reduced protein synthesis remain unclear. In this study, we used three different ribosome profiling approaches (each employing independent mutant tissue) to comprehensively analyze the translation of chloroplast mRNAs in chlorophyll-deficient *chlH* maize mutants. The results consistently showed no substantial effect of chlorophyll deficiency on the abundance or distribution of ribosomes on plastid mRNAs encoding chlorophyll apoproteins. These findings strongly argue against a chlorophyll-dependent regulation of the synthesis of plastid-encoded chlorophyll apoproteins in plants.

This interpretation of our data relies on the assumption that ribosome footprint abundance is a valid proxy for rates of protein synthesis, an assumption that is widely made when interpreting ribosome profiling data (Ingolia, 2014). This view is based on a considerable body of evidence that gene-specific differences in translation rate under any particular condition generally result from differences in the rate of translation initiation (e.g., Shah et al., 2013; Hersch et al., 2014). The global rate of translation elongation can be modulated by environmental inputs (Shalgi et al., 2013), but examples of gene-specific differences in translation elongation rates on mRNAs that are native to the host organism are rare. An example of particular relevance to the question we address here involves a nascent peptide that can modulate ribosome stalling at a specific site in response to the small molecule arginine (Fang et al., 2004). It has been suggested that ribosome pause sites may facilitate the co-translational binding of chlorophyll (Kim et al., 1991), but our results do not address that possibility. However, the fact that ribosome distributions along ORFs encoding chlorophyll-binding apoproteins are unaltered in the *Zm-chlH* mutants provides strong evidence against site-specific effects of chlorophyll on ribosome stalling. Our results are consistent with previous findings from experiments with isolated barley chloroplasts, which showed that the chlorophyll apoproteins PsbA, PsbD, and PsbC indeed can be synthesized independent from chlorophyll (Kim et al., 1994a).

The fact that the abundance of Rubisco and ATP synthase subunits are reduced in the *Zm-chlH-3* mutant suggests a global decrease in translation rate in the mutant chloroplasts. Our data are consistent with the possibility that the mutants experience a global reduction in the rates of translation initiation and elongation in the chloroplast such that the distribution of ribosomes within and among genes shows only minor variations. Validation of this possibility and investigation of the underlying

mechanism are potential subjects of future investigation. That said, our data do provide strong evidence against any selective effect of chlorophyll on the translation of open reading frames encoding chlorophyll apoproteins.

Altogether, our data strongly support the idea that, in plants, the adjustment of apoprotein accumulation to chlorophyll levels is mainly achieved by co- or post-translational proteolysis of apoproteins when they are not bound by their chlorophyll cofactors. Indeed, it has been suggested that chlorophyll-deficient apoproteins may incorrectly fold or assemble into complexes and thereby trigger their rapid proteolytic turnover (e.g., Kim et al., 1994a; Eichacker et al., 1996). In line with that, binding of chlorophyll can induce folding and assembly of LHC chlorophyll *a/b*-binding proteins *in vitro* (Paulsen et al., 2010). Several thylakoid membrane proteases have been assigned to chlorophyll apoprotein processing and homeostasis and are candidates for a proteolytic adjustment of apoprotein levels to chlorophyll availability (van Wijk, 2015; Nishimura et al., 2016). It is important to note that different synthesis and assembly mechanisms apply for PsbA during biogenesis and repair (Jarvi et al., 2015). Since we studied translation in seedlings containing primarily “biogenic” tissue, we cannot rule out that chlorophyll may regulate *psbA* translation during the D1 repair cycle.

Chlorophyll Is Not Required for the Co-translational Membrane Engagement of Nascent Chlorophyll-binding Apoproteins

The chlorophyll apoproteins PsbA/B and PsbA/B/C/D engage the thylakoid membrane co-translationally (e.g., Kim et al., 1994a; Zoschke and Barkan, 2015; see also Figures 5, 6). Assuming co-translational binding of chlorophyll to nascent apoproteins and a coupling to apoprotein folding and membrane integration, it can be speculated that chlorophyll availability may influence the co-translational integration of nascent apoproteins. However, our analysis of ribosome footprints in separated membrane and soluble fractions showed that the position at which the nascent chlorophyll apoproteins engage the thylakoid membrane is not influenced by chlorophyll deficiency. This is perhaps unsurprising, given that none of the chlorophyll interaction sites is located upstream of the first transmembrane segment (UniProt annotations: PsbA (P04966), PsbB (P04967), PsbC (P48183), PsbD (P48184); and Croce, 2012), which comprises the signal that initially engages the membrane (Zoschke and Barkan, 2015). In

line with that, the terminal chlorophyll synthesis enzymes and carrier were found to be associated with the thylakoid membrane, which would enable chlorophyll attachment to apoproteins only after membrane engagement of the nascent apoproteins (Wang and Grimm, 2015). It remains possible that the integration of downstream located transmembrane segments that occurs subsequent to chlorophyll attachment is, in fact, influenced by chlorophyll availability in a way that does not change ribosome progression, a possibility that cannot be addressed by ribosome profiling technologies.

AUTHOR CONTRIBUTIONS

RZ and AB designed the research; RZ and PC performed the research; RZ, PC, and AB analyzed the data; RZ and AB wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2017.00385/full#supplementary-material>

FIGURE S1 | Characteristics of ribo-seq data demonstrate that the reads derive from bona fide ribosome footprints. (A) Distributions of sequencing read sizes within the coding sequences of chloroplasts, mitochondria and cytosol

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are similar to previously published distributions of ribosome footprint sizes (Chotewutmontri and Barkan, 2016). **(B)** Metagenome analysis of cytosolic reads around start and stop codons of all nuclear genes indicates specific occupancy of the reads to the coding region. These reads exhibit 3-nucleotide periodicity depicting the characteristic codon movement of ribosomes. Number of reads were normalized per million reads mapped to nuclear coding sequences. **(C)** Comparison of RNA-seq and ribo-seq read coverages for a representative chloroplast transcription unit (coding for *atpH/-H/-F/-A*). RNA-seq reads map to the entire transcription unit whereas ribo-seq reads map specifically to the coding sequences as expected for ribosome footprints.

FIGURE S2 | Ribosome footprint distribution along the *Zm-chlH* mRNA.

Ribosome footprint reads aligning to the *Zm-chlH* gene were normalized to the reads mapping to nuclear coding sequences and are displayed per million for wild-type and *Zm-chlH-3* mutant plants (note the different y-axis scales of the diagrams). A dashed vertical line indicates the *Mu* transposon insertion site in *Zm-chlH-3* to illustrate the absence of translation downstream of the transposon. The about ten-fold reduced ribosome footprint coverage upstream of the transposon insertion is caused by a reduced accumulation of the *Zm-chlH* transcript in the mutant (as detected in our transcript dataset).

FIGURE S3 | Model for the co-translational membrane engagement of nascent chlorophyll-binding apoproteins and its resulting spatially resolved ribosome profiling data (modified from Zoschke and Barkan, 2015).

(A) Ribosomes translating chlorophyll-binding apoproteins become attached to the membrane in a nuclease-resistant fashion by co-translational thylakoid membrane engagement of the nascent peptide. This occurs shortly after the co-translational exposure of a transmembrane segment from the ribosome (see Zoschke and Barkan, 2015). The supposed co-translational binding of chlorophyll (Chl) is indicated. Ribonuclease pre-treatment releases translating ribosomes to the stroma if they are tethered to the membrane in an mRNA-mediated manner (scissors represent RNase-facilitated ribosome release). A hypothetical membrane channel is shown for illustration only and is not intended to imply any particular membrane insertion mechanism. **(B)** Results of the spatially resolved ribosome profiling analysis of stromal and thylakoid membrane-bound ribosomes for the co-translational membrane engagement shown in **A**. Top panel: the signals of soluble (red line) and membrane-bound (green line) ribosome footprints predominate the 5'- and 3'-regions of the reading frame, respectively. Bottom panel: The ratio of membrane to soluble ribosome footprint signals reverses between the 5'- and 3'-end of the reading frame.

DATASET S1 | Data set of microarray-based ribosome profiling and total RNA control experiments illustrated in Figure 2. Data were processed and analyzed as previously described (Zoschke et al., 2013).

DATASET S2 | Data set of deep-sequencing-based ribosome profiling and total RNA control experiments displayed in Figures 3, 4. Data were processed and analyzed as previously described (Chotewutmontri and Barkan, 2016).

DATASET S3 | Data sets of spatially resolved microarray-based ribosome profiling experiments shown in Figures 5, 6. Data were processed and analyzed as previously described (Zoschke and Barkan, 2015). The wild-type data were taken from Zoschke and Barkan (2015).

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Non-canonical Translation in Plant RNA Viruses

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Viral protein synthesis is completely dependent upon the host cell's translational machinery. Canonical translation of host mRNAs depends on structural elements such as the 5' cap structure and/or the 3' poly(A) tail of the mRNAs. Although many viral mRNAs are devoid of one or both of these structures, they can still translate efficiently using non-canonical mechanisms. Here, we review the tools utilized by positive-sense single-stranded (+ss) RNA plant viruses to initiate non-canonical translation, focusing on *cis*-acting sequences present in viral mRNAs. We highlight how these elements may interact with host translation factors and speculate on their contribution for achieving translational control. We also describe other translation strategies used by plant viruses to optimize the usage of the coding capacity of their very compact genomes, including leaky scanning initiation, ribosomal frameshifting and stop-codon readthrough. Finally, future research perspectives on the unusual translational strategies of +ssRNA viruses are discussed, including parallelisms between viral and host mRNAs mechanisms of translation, particularly for host mRNAs which are translated under stress conditions.

Keywords: non-canonical translation, RNA structure and function, translation enhancers, translational recoding, protein synthesis, IRES, 3'-CITE

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INTRODUCTION

Viruses usurp the metabolism of the host cell in their own benefit. Viral mRNA translation is a paradigmatic illustration of this, as the hallmark of viruses is that their genomes do not code for a protein synthesis apparatus. Thus, viruses have evolved many subtle ways to use and control the translational machinery of their hosts (Jiang and Laliberté, 2011; Echevarría-Zomeño et al., 2013; Walsh et al., 2013), and in fact the host range of a given virus may be determined by its ability to efficiently translate viral mRNAs using host translation factors, as we have shown recently for a plant virus (Truniger et al., 2008; Nieto et al., 2011; Miras et al., 2016). From a strategic point of view, understanding how viruses translate their own proteins may significantly contribute to the identification of therapeutic (Robert et al., 2006; Cencic et al., 2011) or breeding targets (Nicaise et al., 2003; Gao et al., 2004; Ruffel et al., 2005; Stein et al., 2005; Nieto et al., 2006; Naderpour et al., 2010). Also, understanding the peculiarities of viral mRNA translation can provide important biotechnological tools for protein overexpression (Sainsbury and Lomonosoff, 2014; Lomonosoff and D'Aoust, 2016), given the very efficient translation of some viral mRNAs in diverse conditions. From a fundamental point of view, viral mRNAs constitute powerful probes to uncover the varied and fascinating mechanisms of protein translation and their control. In this review, we describe current knowledge on the mechanisms used by positive-sense single-stranded (+ss) RNA plant viruses to initiate translation, focusing on *cis*-acting sequences present in viral mRNAs. We also

describe other protein translation strategies used by plant viruses to optimize the usage of the coding capacity of their very compact genomes, including leaky scanning initiation, ribosomal frameshifting and stop-codon readthrough.

CANONICAL TRANSLATION OF EUKARYOTIC mRNAs

To understand the mechanisms of non-canonical translation of viral mRNAs, we first review briefly how canonical eukaryotic mRNA translation proceeds. Most eukaryotic mRNAs are appended at the 5' end with a m⁷G(5')ppp(5')N cap structure, and a poly(A) tail at the 3' end, which are critical *cis*-acting elements during canonical translation. Traditionally, translation is divided into four distinct steps: initiation, elongation, termination and ribosomal recycling. Translation initiation is the rate limiting and most highly regulated step (reviewed in Aitken and Lorsch, 2012) and begins with the formation of the 43S preinitiation complex (PIC). PIC is composed of the ternary complex (TC) eIF2-Met-tRNA-GTP bound to the 40S ribosome subunit through the P-site and the eukaryotic initiation factors (eIFs) eIF3, eIF5, eIF1A, and eIF1 (Sonenberg and Hinnebusch, 2009). EIF3, which is a large thirteen-subunit complex (Sun et al., 2011; Browning and Bailey-Serres, 2015; Smith et al., 2016), interacts with eIF2 via its subunit eIF3a and indirectly via eIF5 bridging these two factors (Valášek et al., 2002; Jivotovskaya et al., 2006). Interestingly, the eIF3d subunit can act as a cap-binding protein and is required for specialized cap-dependent translation (Lee et al., 2016).

In parallel to PIC formation, recognition of the mRNA is facilitated through binding of the cap-binding protein eIF4E to the 5' cap and the poly(A)-binding protein (PABP) to the 3' poly(A) tail (Pestova et al., 2001). EIF4G interacts with eIF4E through its highly conserved canonical binding domain and forms, together with the helicase eIF4A, the eIF4F complex. Very recently, a second eIF4E-binding domain has been described in eIF4G, suggesting a bipartite eIF4E-eIF4G binding mode for higher eukaryotes (Grüner et al., 2016). EIF4G can also recruit other factors, including eIF3 and PABPs through direct protein-protein interactions. It is thought that the eIF4G-PABP interaction promotes the circularization of the message enhancing translation efficiency (Gray et al., 2000; Paek et al., 2015). This model is supported by biochemical data and by atomic force microscopy studies that confirm the interactions and the circularization of the mRNA (Wells et al., 1998; Kahvejian et al., 2001). However, there is increasing evidence that circularization may vary in importance for stimulation of translation among different organisms (i.e., yeast) and cells types. For example, the eIF4G-PABP interaction is not required for wild-type cell growth in yeast and mammals (Hinton et al., 2007; Park et al., 2011). Similarly, it was observed by cryo-EM that the formation of circular polyribosomes was independent of the cap structure and poly(A) tail (Madin et al., 2004; Afonina et al., 2014). These results suggest alternative mechanisms for mRNA circularization that may mimic the strategies used by +ssRNA viruses detailed in this review.

Once the mRNA is circularized, the 43S PIC in its open conformation is able to bind to the mRNA near its 5' end. The exact mechanistic details are unknown, but eIF3 and eIF4G appear to facilitate this step (Aitken and Lorsch, 2012). The 43S PIC searches for the mRNA start codon, scanning downstream of the leader sequence resulting in the entry of the 5' proximal start codon into the 40S subunit P-site (Kozak, 2002). Start codon selection requires cooperation between the scanning ribosome and eIF1, eIF2, and eIF5, forming the 48S preinitiation complex (Pestova and Kolupaeva, 2002). Once the start codon enters the P-site, the 60S subunit joins, with the release of eIF2, eIF1, and eIF5 and the association with eIF5B-GTP (Pestova et al., 2001). With the formation of the resulting 80S complex, the GTP molecule associated with eIF5B is hydrolyzed and released (Pestova et al., 2001).

Translation continues with the elongation phase, where the polypeptide is formed. In the elongation stage, entering amino acyl-tRNAs (aa-tRNA) bind to the A-site through the second codon of the mRNA (Lewin, 2008). After the aa-tRNA is located at the A-site, the peptidyl-tRNA is relocated from the P-site to the A-site. Once the peptide bond is formed, the translocation step occurs when the ribosome moves in a 3' direction along the mRNA, placing a new codon at an empty A-site while the new peptidyl-tRNA is moved to the P-site and the deacylated tRNA in the E-site is ready to exit the ribosome (Julián et al., 2008; Rodnina and Wintermeyer, 2009). After the nascent polypeptide has been released, ribosomes remain bound to the mRNA and tRNA. It is only during the ribosomal recycling phase when the ribosome subunit dissociation occurs leaving them free to bind new mRNAs (Pisareva et al., 2011; Dever and Green, 2012).

NON-CANONICAL TRANSLATION INITIATION OF VIRAL mRNAs

Mechanisms of non-canonical translation initiation include those that function independently of a 5' cap or/and a poly(A) tail. These can be mediated by stimulators present in *cis* in the 5'-UTR, for example internal ribosome entry sites (IRESes) or genome-linked viral proteins (VPgs), in the 3'-UTR, for example cap-independent translation elements (3'-CITE) or tRNA-like structures (TLS), and also in intergenic regions, for example intergenic IRESes (Table 1).

Enhancers Located in the 5'-UTR: Internal Ribosome Entry Sites and VPgs in *Potyviridae*

The family *Potyviridae* is the largest among plant viruses with RNA genomes. The potyviral genome acts as mRNA and codes for a single polyprotein which is cleaved by viral proteases rendering 10 final functional proteins (Revers and García, 2015). Potyviral RNAs resemble those of the animal-infecting picornaviruses: they possess a small viral protein covalently bound to their 5' ends (VPg), instead of a 5' cap structure, and they are polyadenylated at their 3' ends (Adams et al., 2005). However, VPgs in different virus families differ greatly in size

TABLE 1 | Translation enhancers known in RNA plant viruses.

Type	Virus	Family	5'/3' structures	Genome localization	Type of 3'-CITE	RNA secondary structure	eIFs/Other	18S RNA complementarity	References
IRES	TEV	Potviridae	VPg/pol(A)	5'-UTR		Two pseudoknots ^a	eIF4G	✓	Zeenko and Gallie, 2005; Ray et al., 2006
	PVY	Potviridae	VPg/pol(A)			Two stem-loops ^b			Yang et al., 1997
	TuMV	Potviridae	VPg/pol(A)			Stem-loop ^b	RPS6		Basso et al., 1994; Yang et al., 2009
	TrMV	Potviridae	VPg/pol(A)			Two stem-loops ^a	eIF4G/eIFiso4G		Roberts et al., 2015, 2017
	BRV	Secoviridae	VPg/pol(A)			Stem-loop ^b			Karetnikov and Lehto, 2007
IRES	PRLV	Luteoviridae	5'pppN/-OH	Intergenic region		Bulge stem-loop ^b			Jaag et al., 2003
	HCRSV	Tombusviridae	5'pppN/-OH			Bulge stem-loop ^b			Koh et al., 2003
	PFBV	Tombusviridae	5'pppN/-OH			Bulge stem-loop ^b			Fernández-Miragall and Hernández, 2011
	TCV	Tombusviridae	5'pppN/-OH			Bulge stem-loop ^b			May et al., 2017
	σTMV	Virgaviridae	m ⁷ GpppN/TLS			None			Dorokhov et al., 2002
TLS	TYMV	Tymoviridae	m ⁷ GpppN/TLS	3'-UTR		tRNA-like ^a	eEF1a/ 40S		Matsuda et al., 2004; Colussi et al., 2015
	BMV	Bromoviridae	m ⁷ GpppN/TLS						Barends et al., 2004
CPB	AMV	Bromoviridae	m ⁷ GpppN/-OH	3'-UTR		Six stem-loops ^b	CP/eIF4G/iso4G		Neelaman et al., 2004; Krab et al., 2005
3'CITE	STNV	Tombusviridae	5'pppN/-OH	3'-UTR	TED	Long stem-loop ^a	eIF4F/iso4F		Meulewaeter et al., 1998; Gazo et al., 2004
	PLPV	Tombusviridae	5'pppN/-OH		TED	Long stem-loop ^a			Blanco-Pérez et al., 2016
	MNSV	Tombusviridae	5'pppN/-OH		ISS	Stem-loop ^a	eIF4F		Truniger et al., 2008; Miras et al., 2016
	MNeSV	Tombusviridae	5'pppN/-OH		ISS	Stem-loop ^a	eIF4F		Nicholson et al., 2010
	MNSV-N	Tombusviridae	5'pppN/-OH		CXTE	Two helices protruding central hub ^a	eIF4E independent		Miras et al., 2014
	BYDV	Luteoviridae	5'pppN/-OH		BTE	Basal helix plus 3 helices ^a	eIF4G/40S	✓	Treder et al., 2008; Sharma et al., 2015
	RSDaV	Luteoviridae	5'pppN/-OH		BTE	Complex BTE structure ^a	eIF4G	✓	Wang et al., 2010
	RCNMV	Tombusviridae	5'pppN/-OH		BTE	Basal helix plus 5 helices ^a	eIF4G	✓	Wang et al., 2010
	TINV-D	Tombusviridae	5'pppN/-OH		BTE	Basal helix plus 2 helices	eIF4G	✓	Shen and Miller, 2004
	TBTV	Tombusviridae	5'pppN/-OH		BTE	Basal helix plus 2 helices ^a	eIF4G	✓	Wang et al., 2010
	TBSV	Tombusviridae	5'pppN/-OH		YSS	Three helices ^a		✓	Fabian and White, 2004
	CIRV	Tombusviridae	5'pppN/-OH		YSS	Three helices ^a	eIF4F/eIFiso4F		Nicholson et al., 2013
	PEMV2	Tombusviridae	5'pppN/-OH		PTE	Pseudoknot ^a	eIF4E		Wang et al., 2009b, 2011
	PMV	Tombusviridae	5'pppN/-OH		PTE	Pseudoknot ^a			Batten et al., 2006
	SCV	Tombusviridae	5'pppN/-OH		PTE	Pseudoknot ^a			Chattopadhyay et al., 2011
	TCV	Tombusviridae	5'pppN/-OH		TSS	tRNA-like ^a	60S		Stupina et al., 2008; Zuo et al., 2010
	PEMV2	Tombusviridae	5'pppN/-OH		TSS	tRNA-like ^a	40S/60S		Gao et al., 2013, 2014
	BRV	Secoviridae	VPg/pol(A)		-	Pseudoknot ^b		✓	Karetnikov et al., 2006

^aSolution probed RNA secondary structure.^bPredicted RNA secondary structure.

and function. The well-characterized VPg of *Poliovirus* (genus *Enterovirus*, family *Picornaviridae*) is only 22 amino acids (aa) long, while that of potyviruses consists of around 192 aa.

Early studies using the model potyvirus *Tobacco etch virus* (TEV, genus *Potyvirus*, family *Potyviridae*) showed that its 5'-UTR contains a sequence that was able to enhance translation 8- to 21-fold in tobacco protoplasts (Carrington and Freed, 1990). Deletion studies identified two regions in the TEV 5'-UTR including nucleotides 26-85 and 66-118 which were able to stimulate translation 10-fold with respect to a capped RNA control (Zeenko and Gallie, 2005); these regions were consequently named cap-independent regulatory elements (CIRE) 1 and 2 (Zeenko and Gallie, 2005). The TEV CIREs promoted translation of a second ORF when placed in a dicistronic reporter construct, suggesting that they were able to promote internal initiation like IRESes (Niepel and Gallie, 1999). However, the addition of a stem loop structure upstream of CIRE-1 and CIRE-2 in its natural 5' end context reduced translation 30 and 70%, respectively, suggesting that the TEV leader might require an accessible 5' end for ribosomal scanning (Niepel and Gallie, 1999). The TEV CIRE-1 folds into an AU-rich pseudoknot structure (PK1, nucleotides 38-75) which is essential for cap-independent translation. Interestingly, one loop of PK1 is complementary to a conserved region of the 18S rRNA and mutations in the 7 nt-complementary sequence (61-UACUUCU-67) were responsible for an approximately 80% decrease in translation compared to wild type (Zeenko and Gallie, 2005). This type of complementarity also occurs between the 18S rRNA and the sequence 4836-GAUCCU-4841 that belongs to the translation enhancer located in the 3'-UTR of *Barley yellow dwarf virus* (BYDV; genus *Luteovirus*, family *Luteoviridae*) (see Section on CITEs) and the polypyrimidine-rich tracts located in both IRES elements found in *Blackcurrant reversion virus* (BRV; genus *Nepovirus*, family *Comoviridae*) (Karetnikov and Lehto, 2007; Sharma et al., 2015), suggesting that these translation elements could recruit the 40S ribosomal subunit before loading to the 5' end of the mRNA to start the scanning.

Early experiments using partially eIF4F depleted wheat germ extract showed that the TEV 5'-UTR conferred a competitive advantage over non-viral mRNAs which seemed to be lost when eIF4F was added back to wheat germ extract (Gallie and Browning, 2001). These results suggest that the TEV genome recruits eIF4F more efficiently than plant mRNAs when the concentration of this factor is limiting. Further analysis showed that, like for *Picornaviridae* IRESes, TEV translation is eIF4F-dependent and that eIF4G binds directly to both, the TEV 5' leader and PK1 having a large entropic contribution (Ray et al., 2006). Moreover, the poly(A) tail functions synergistically with the TEV IRES to increase translation (Gallie et al., 1995), as also shown for animal-infecting picornaviral IRES-mediated translation (de Quinto et al., 2002; Thoma et al., 2004).

Like that of TEV, the 5' leaders of *Potato virus Y* (PVY; genus *Potyvirus*, family *Potyviridae*), *Turnip mosaic virus* (TuMV, genus *Potyvirus*, family *Potyviridae*), and *Triticum mosaic virus* (TriMV; genus *Poacevirus*, family *Potyviridae*) (Table 1) have been shown to stimulate cap-independent translation. The 5'-UTR of PVY also contains an IRES that directs efficient

translation of an ORF in a dicistronic vector (Levis and Astier-Manifacier, 1993), and IRES mapping showed that a 55 nt 3' terminal region was fundamental for translation enhancement in tobacco protoplasts (Yang et al., 1997). The 131-nt long 5' leader of TuMV conferred translational activity when placed upstream of a GUS reporter gene flanked at its 5' end by a 33 nt vector-sequence (Basso et al., 1994); this RNA was able to promote translation *in vitro* to a similar level as capped mRNAs inhibiting cap-dependent translation when added *trans* (Basso et al., 1994). The study from Yang et al. (2009) demonstrated that the TuMV RNA requires the ribosomal protein RPS6 for accumulation in *Nicotiana benthamiana*, and RPS6 is up-regulated under TuMV infection in *Arabidopsis thaliana*. The silencing of RPS6 abolished TuMV infection and also that of the non-related *Tomato bushy stunt virus* (TBSV; genus *Tombusvirus*, family *Tombusviridae*) (Yang et al., 2009). The TBSV viral RNA is uncapped and not polyadenylated, having no VPg. The RPS6 protein is related to other ribosomal proteins implicated in picornaviral and alphaviral infection and indispensable for *Hepatitis C virus* (HCV, genus *Hepacivirus*, family *Flaviviridae*) replication (Cherry et al., 2005; Montgomery et al., 2006; Huang et al., 2012).

It should be noted that the above reported IRESes of potyviruses may not be as strong as the IRESes of picornaviruses or HCV, for example. The 5'-UTRs of potyviruses are much shorter than the IRESes of the *Picornaviridae*, and lack strong structure or conserved sequence, and AUG triplets (Niepel and Gallie, 1999; Zeenko and Gallie, 2005). As mentioned above, an upstream stem-loop inhibited downstream translation mediated by the IRES, which lends doubt on whether it truly facilitates internal ribosome entry. Moreover, translation directed by the TEV 5'-UTR sequence from the internal position was orders of magnitude less efficient than when located at the natural 5' end (Niepel and Gallie, 1999). Also, capped potyviral transcripts containing the 5'-UTR (including the IRES), linked to a reporter gene, translated more efficiently than uncapped transcripts (Carrington and Freed, 1990; Khan et al., 2008). These observations support the notion that conventional ribosome scanning from the 5' end is important for efficient translation of potyviral RNAs.

One singular potyviral 5'-UTR that resembles a true animal virus-like IRES, is that of *Triticum mosaic virus* (TriMV) (genus *Tritimovirus*, *Potyviridae*). The exceptionally long (739 nt) 5'-UTR is much longer than that of other potyvirids and translation initiates at the 13th AUG triplet (Roberts et al., 2015). The minimal region of the TriMV leader for cap-independent translation resides in a 300-nt long sequence forming a secondary structure consisting of two long stem-loop-containing bulges. A hairpin structure at nucleotide positions 469-490 is required for cap-independent translation and internal translation initiation, and plays a role in its ability to compete with capped RNAs (Roberts et al., 2015). A unique feature of the TriMV IRES compared to those of other potyviruses is that it can mediate translation when a stem-loop structure is added upstream of the 5' leader, thus its translation is 5' end independent. The TriMV 5'-UTR interacts with eIF4G or eIFiso4G *in vitro*, and requires eIF4A helicase activity to mediate translation initiation

(Roberts et al., 2017). These properties are true hallmarks of an IRES.

The VPg covalently attached to the 5' end of potyviral RNAs may contribute directly to translational efficiency by interacting with translation initiation factors (Khan et al., 2008; Miyoshi et al., 2008). The addition of the TEV VPg together with eIF4F to a depleted wheat germ extract enhanced translation of an uncapped TEV RNA reporter (Khan et al., 2008). This enhancement correlated with an increase in the eIF4F-TEV RNA affinity in the presence of the VPg mediated through a direct interaction of the VPg with eIF4E. The disruption of VPg-eIF4E binding abolished stimulation of IRES-mediated translation *in vitro* (Khan et al., 2008). In contrast, TuMV VPg binds the isoform of eIF4E, eIFiso4E *in vitro* and *in vivo* (Leonard et al., 2004; Khan et al., 2008). PABP increases the binding affinity and stabilization of VPg with eIF4F or eIFiso4F in both viruses (Khan et al., 2009; Khan and Goss, 2012). Similarly to the TEV and the TuMV VPg, *Potato virus A* (PVA, family *Potyviridae*) VPg binds eIF4E and eIFiso4E and enhances viral translation in plants (Eskelin et al., 2011). Silencing of those host factors abolished PVA VPg-mediated stimulation of translation. Ribosomal protein P0 enhanced translation synergistically together with VPg and eIFiso4E and its stimulation depended on the PVA 5'-UTR (Hafren et al., 2013). Further on, Hafren et al. (2015) showed that viral HC-Pro and the host RNA binding protein varicose, both components of potyviral RNA granules, stimulated VPg-promoted translation of PVA.

All of the above mechanisms involve the VPg stimulating RNA translation *in trans*, leaving open the question of how the VPg specifically recognizes only the viral RNA. It is unknown whether the VPg acts *in cis* when it is covalently attached to the 5' end, to simply replace the 5' cap function in recruiting eIF4E and stimulating translation. The much smaller VPg of picornaviruses does not participate in translation, as polysome-associated picornaviral RNA lacks the VPg (Nomoto et al., 1977). Instead it primes picornavirus RNA synthesis (Paul et al., 1998). It is likely that the VPgs of all viruses also have this latter role, but to our knowledge, priming of RNA synthesis has not been demonstrated for the VPg of any plant virus.

The potyvirus VPg may functionally resemble the 13–15 kDa VPg of calici- and noroviruses (*Caliciviridae*) (Goodfellow, 2011). Like the potyvirus VPg, calicivirus VPg binds eIF4E (Goodfellow et al., 2005). This interaction is required for translation of *Feline calicivirus* (FCV, genus *Vesivirus*, family *Caliciviridae*) RNA, so the VPg acts as a functional analog of the cap (Goodfellow et al., 2005; Hosmillo et al., 2014; Zhu et al., 2015). In contrast, the VPg on norovirus RNA binds and requires eIF4G for translation initiation (Chung et al., 2014). This difference in factor binding may be associated with the different structures of their VPgs. While FCV and *Porcine sapovirus* (PSaV, genus *Sapovirus*, family *Caliciviridae*) VPgs adopt a compact three-helical bundle structure, *Murine norovirus* (MNV, genus *Norovirus*, family *Caliciviridae*) VPg has only two helices (Leen et al., 2013; Hwang et al., 2015). The MNV VPg-eIF4G interaction was mapped to the HEAT-1 domain in eIF4G and to the 20 C-terminal residues in VPg

(Leen et al., 2016), with this latter domain differing from the eIF4E-interacting domains in FCV and PSaV VPgs. VPgs vary widely in sequence, even within a genus, so it would be difficult to extrapolate this structural information to potyvirus VPgs. Instead, to experimentally determine whether the potyvirus VPg plays the role of replacing the 5' cap in translation, it would be valuable to determine whether translating potyvirus RNA on polysomes contains a VPg, and the effect of removing this VPg on potyvirus RNA translation.

Viruses in the family *Secoviridae* and in the genus *Sobemovirus* also have VPgs linked to their genomic RNA. The VPg of the sobemovirus *Rice yellow mottle virus* has been shown to interact with eIFiso4G and this interaction is required for viral multiplication, but a role in translation has not been published for this interaction (Hébrard et al., 2010). The role in translation of secovirids VPgs is poorly understood (Léonard et al., 2002).

Intergenic Region Enhancers

IRESes have also been found in internal genomic positions within certain viral genomes (Table 1). For example, the crucifer strain of *Tobacco mosaic virus* (crTMV; genus *Tobamovirus*, family *Virgaviridae*) harbors two IRESes that stimulate the synthesis of the CP and movement protein (MP), 75 and 148-nucleotides long, respectively (Dorokhov et al., 2002, 2006). The CP IRES contains a bulged stem-loop structure that is flanked by two purine-rich repeats that are crucial for IRES activity. To find the minimal purine-rich sequence the authors reported that 16 consecutive GAAA repeats were sufficient to provide high IRES activity in plants and human cells (Dorokhov et al., 2002). However, apparently this observation has not been repeated in other labs (e.g., Fan et al., 2012). A low level of CP translation from genomic RNA of carmoviruses *Hibiscus chlorotic ringspot virus* (HCRSV) (Koh et al., 2003; Fernández-Miragall and Hernández, 2011), *Pelargonium flower break virus* (PFBV) (Fernández-Miragall and Hernández, 2011), and *Turnip crinkle virus* (TCV) (May et al., 2017) has also been reported to be IRES-mediated. Like the crTMV IRES, the TCV IRES seems to require only to be A-rich and lack of structure and its activity is inversely correlated with the size of the RNA.

Another virus that shares the crTMV polypurine tract in its IRES sequence is *Potato leafroll virus* (PLRV; genus *Polerovirus*, family *Luteoviridae*). This IRES, which is in a highly unexpected location, 22 nt downstream of the start codon and within a region of the PLRV RNA genome that is characterized by non-canonical translation mechanisms such as −1 ribosomal frameshifting, leads to translation of replication-associated protein (Rap1) (Jaag et al., 2003). The PLRV IRES element, in conjunction with the 22 nt spacer sequence, are sufficient to mediate cap-independent translation *in vitro* but not *in vivo* (Jaag et al., 2003), which sheds doubt on its biological relevance. Furthermore, this reported IRES function and the resulting translated ORF are not conserved in related poleroviruses.

Given the unstructured and sequence non-specific nature of the IRES RNA in the examples above, which is unlike the much longer, highly structured and powerful mammalian viral and dicistrovirus IRESes, we think these observations should be interpreted with caution. It may be possible that, due to lack of

structure, the RNA is sensitive to nuclease cleavage providing a 5' end, which, being unstructured, may be a very efficient leader to allow detectable translation of CP (or Rep1) ORF from undetectable amounts of degraded RNA. This alternative mechanism of expression may still be biologically relevant, or simply an artifact of the assays, but would not result from an IRES.

Enhancers Located in the 3'-UTR

tRNA-Like Structures

Viruses from the family *Bromoviridae* and the genera *Tobamovirus* and *Tymovirus* possess a 5' cap structure but lack a 3' poly(A) tail. In contrast, they contain tRNA-like structures (TLSs) at their 3' termini that perform many viral processes, such as (i) serving as a telomere by interacting with CTP:ATP nucleotidyl transferase which adds CCA in a non-templated fashion to the 3' end (Rao et al., 1989), (ii) regulation of negative strand synthesis (Dreher, 2009), (iii) translation enhancement (Gallie and Walbot, 1990; Choi et al., 2002; Matsuda and Dreher, 2004), and (iv) packaging of the viral RNA in the virion (Annamalai and Rao, 2007). Three basic types of 3' terminal TLS have been described in the genomes of *Turnip yellow mosaic virus* (TYMV; genus *Tymovirus*, family *Tymoviridae*), TMV and *Brome mosaic virus* (BMV; genus *Bromovirus*, family *Bromoviridae*). Because of their multiple functions, it has been difficult to tease out the mechanisms of each role, but the translational enhancement structures and mechanisms have been well characterized for TMV and TYMV (Table 1).

The TYMV TLS requires aminoacylation of the 3'-CCA terminus for maximal translational efficiency and the 5' cap synergistically promotes this activity (Matsuda et al., 2004). Translational enhancement maps principally to the TLS, although the upstream adjacent pseudoknot is important for optimal translation, possibly serving as a sequence spacer (Matsuda and Dreher, 2004). The aminoacylated TLS binds to eukaryotic elongation factor 1A (eEF1A) and is a substrate for tRNA-modifying enzymes (Dreher and Goodwin, 1998; Matsuda et al., 2004) mimicking tRNA activity. The 5'-proximal AUG in the TYMV genome serves as start codon for a 69 kDa ORF (p69), and the second AUG is the start codon for the main polyprotein ORF (p206) with which ORF p69 overlaps. Based on only *in vitro* translation assays, Barends et al. (2004) proposed a "Trojan Horse" model of translation initiation in which the aminoacylated TLS delivers its amino acid to the start codon of the polyprotein ORF. However, the Dreher lab provided *in vitro* and *in vivo* evidence that a more likely mechanism is classical leaky scanning, except that the efficiency of initiation at the second AUG correlated with its proximity to the first AUG (Matsuda and Dreher, 2006). In addition, the translation efficiency of the polyprotein ORF depended on a 5' cap, and not the 3' TLS. This and additional data support an "initiation coupling" model in which the close proximity (7 nt) of the two AUG codons is necessary for maximum translation of the polyprotein ORF (Matsuda and Dreher, 2007).

How the TLS interacts with the 5' end to stimulate translation in the scanning-dependent manner is suggested by the crystal

structure of the TYMV TLS. The TLS has a tRNA-like shape, but it uses a very different set of intramolecular interactions (Colussi et al., 2015). These interactions allow the TLS to switch conformations and to interact with the ribosome, docking within it to regulate the folding and unfolding state to permit dual functionality in viral translation and replication. This leads us to hypothesize that TLS recruits the ribosome, which is delivered to the 5'-UTR by communication with the 5' end through the cap-eIF4E-eIF4G-eIF3-40S chain of interactions.

A different function for tRNA mimicry occurs in the only IRES that occurs naturally between ORFs: the intergenic region (IGR) IRES of dicistroviruses (Wilson et al., 2000; Khong et al., 2016). In the IGR IRES, a pseudoknot mimics the structure of the anticodon loop of a tRNA basepaired to a codon in mRNA, facilitating instant elongation as the ribosome joins the viral RNA with no initiation steps (Costantino et al., 2008).

In the case of BMV RNA, its 3'-UTR has been shown to provide translation enhancement, and the disruption of its TLS reduced translation *in vitro* (Barends et al., 2004). On the other hand, the TMV TLS is structurally similar to the TYMV TLS and functions as minus-strand promoter (Chapman and Kao, 1999), but it does not mediate translation enhancement. However, the 3'-UTR of TMV contains an upstream pseudoknot domain that stimulates translation in a way that is replaceable by a poly(A) tail (Gallie et al., 1991; Leathers et al., 1993). Additionally, TMV RNA also harbors in its 5'-UTR the 68-nt omega (Ω) sequence which highly stimulates cap-dependent translation (Gallie and Kado, 1989). Ω is recognized by the heat shock protein 101 (HSP101), mediating translational activity (Wells et al., 1998) and interacts with eIF4F via eIF4G (Gallie, 2002, 2016). Similarly, the Brassicaceae-specific eIFiso4G2 isoform also contributes in Ω -mediated translation, unlike eIFiso4G which did not affect Ω -dependent translation (Gallie, 2016). These results suggest that eIFiso4G2 exhibits more functional similarity with eIF4G than eIFiso4G. Regarding translational activity, Ω is one of the most efficient mRNA leaders *in vitro* and *in vivo* and it was used for biotechnological applications such as transgene expression (Gallie et al., 1987; Fan et al., 2012).

3'-UTR Mediated Translation of the *Alfalfa mosaic virus* Genome

The non-polyadenylated *Alfalfa mosaic virus* (AMV, genus *Alfavirus*, family *Bromoviridae*) RNA requires the viral CP for efficient translation and infection. The 3'-UTR of AMV also plays a role in translation due to its ability to bind the CP, adopting the CP-binding (CPB) conformation. This binding avoids the minus-strand promoter activity and enhances translation, possibly acting as a mimic of the poly(A) tail (Olsthoorn et al., 1999). The CPB structure folds into a series of stem-loops separated by an AUGC motif and mutations in this motif led to the loss of binding to CP, correlating with reduction of translation in protoplasts (Reusken and Bol, 1996; Neeleman et al., 2004). The crystal structure of CP-bound RNA revealed a novel RNA fold in which RNA forms two hairpins separated by the linker AUGC motif and oriented in right angles (Guogas et al., 2004). The presence of the CP promotes the base pairing between linker motifs, leading a compact structure. Moreover, pulldown assays revealed that the

CP interacts with eIF4G/eIFiso4G subunits (Krab et al., 2005). This interaction may stimulate mRNA circularization in a similar fashion as found for rotaviruses (Groft and Burley, 2002). In addition to the CPB form, AMV RNAs 3' termini also fold into a pseudoknot structure that resembles a TLS conformation. The 3'-UTR can be recognized by a tRNA-specific enzyme and by the viral replicase and this recognition is inhibited by the addition of CP (Olsthorn et al., 1999; Chen and Olsthorn, 2010). Thus, it suggests that TLS conformation acts as a minus-strand promoter and the CP interaction and pseudoknot stability may regulate a conformational switch between translation and replication (Chen and Olsthorn, 2010).

Cap-Independent Translation Elements

Members of the *Tombusviridae* and *Luteoviridae* plant virus families lack both 5' cap and 3' poly(A) elements, but contain in their 3' ends structured RNA elements capable of enhancing translation in the absence of cap (cap-independent translation elements, CITEs). Most 3'-CITEs have in common their ability to bind translation initiation factors of the eIF4E or eIF4G families, as well as the presence of small sequence stretches within or near the 3'-CITE capable of base-pairing to sequences in the 5'-UTR of the mRNA to establish long-distance RNA:RNA interactions (Table 1). By definition, 3'-CITEs functionally substitute for the 5' cap with high efficiency. They recruit translation initiation factors leading to ribosome entry at or near the 5' terminus followed by ribosome scanning to the initiation codon (Fabian and White, 2004; Rakotondrafara and Miller, 2008; Nicholson and White, 2011); therefore, in contrast to IRESes, 3'-CITEs do not promote internal ribosome entry. To date, seven different classes of 3'-CITEs have been described (Simon and Miller, 2013; Miras et al., 2014) which share little secondary structure and sequence similarity. Due to space limitations and a previous comprehensive review on 3'-CITEs (Simon and Miller, 2013), we will describe only briefly each 3'-CITE and recent updates.

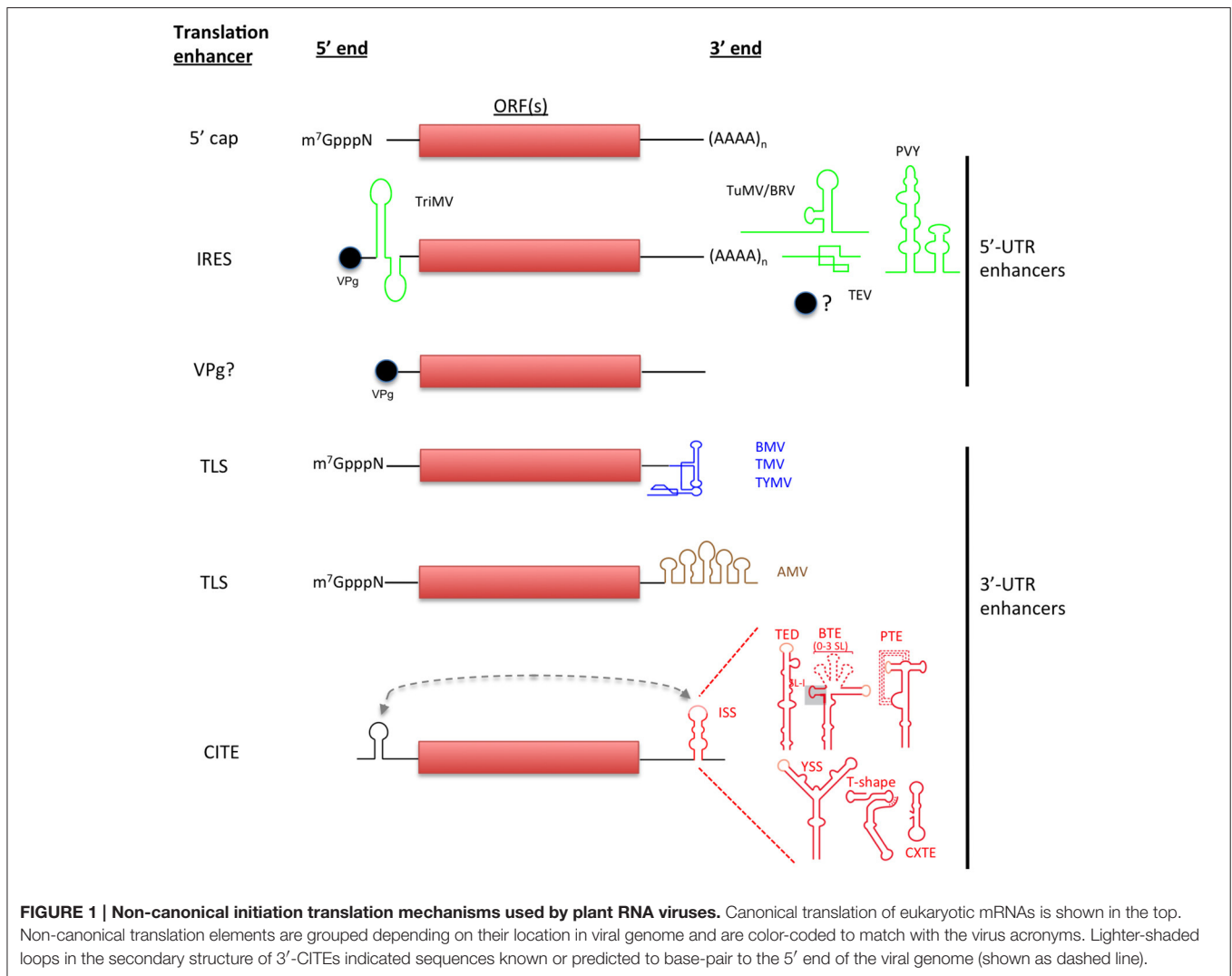
The first 3'-CITE was discovered in *Satellite tobacco necrosis virus* (STNV) and is located in a 120-nt sequence termed translation enhancer domain (TED) (Danthinne et al., 1993; Timmer et al., 1993; Meulewaeter et al., 1998). The TED is predicted to form a long stem-loop with several internal bulges (Van Lipzig et al., 2002). This element was shown to be functional in enhancing translation *in vitro* and *in vivo*. TED binds eIF4F or eIFiso4F (Gazo et al., 2004), and is proposed to interact with the 5'-UTR via a predicted RNA:RNA long-distance interaction with the apical loop of the 5' end. However, mutations that disrupted this potential long-distance base-pairing reduced translation only slightly, and covarying mutations designed to restore base pairing did not restore translation to wild type levels (Meulewaeter et al., 1998). The STNV 3'-CITE confers cap-independent translation *in vitro* when it is moved to the 5'-UTR of an uncapped reporter (Meulewaeter et al., 1998). Another member of the *Tombusviridae* family, *Pelargonium line pattern virus* (PLPV, genus *Carmovirus*) was recently shown to harbor a 3'-CITE in the TED class (Blanco-Pérez et al., 2016). In this case, PLPV TED was shown to require a long-range RNA:RNA kissing stem-loop interaction with a hairpin in the coding sequence of the PLPV

p27 ORF for efficient translational activity (Blanco-Pérez et al., 2016).

The shortest CITEs are the I-shaped structures (ISS) present in the 3'-UTRs of *Maize necrotic spot virus* (MNeSV, *Tombusvirus*, family *Tombusviridae*) and *Melon necrotic spot virus* (MNSV, genus *Carmovirus*, family *Tombusviridae*) (Truniger et al., 2008; Nicholson et al., 2010; Miras et al., 2016), and are apparently similar in secondary structure to the TED. MNeSV ISS has been shown to preferentially interact with the eIF4E subunit of eIF4F. As for TED and most other CITEs, base pairing between the 3'-CITE and the 5'-UTR is predicted to deliver the translation factor to the 5' end, facilitating recruitment of the 43S preinitiation complex (Nicholson et al., 2010). In support of this model, it has been shown that the interacting 5'-UTR:I-shaped 3'-CITE of MNeSV together with eIF4F form a complex *in vitro*. In addition, ribosome toe printing demonstrated that while bound to eIF4F, the I-shaped CITE can simultaneously base pair with the 5'-UTR and recruit ribosomes to the 5' end of the viral fragment (Nicholson et al., 2010).

In the case of MNSV ISS, genetic evidence for interaction of the ISS with eIF4E has been shown in melon. A single amino acid change in melon eIF4E strongly reduces translation efficiency controlled by MNSV ISS and makes melon resistant to MNSV infection (Nieto et al., 2006; Truniger et al., 2008). The minimal 3'-CITE sequence, named Ma5TE (MNSValpha5-like translation enhancer), was mapped to a 45 nt region. *In vitro* binding assays revealed that Ma5TE forms a complex with eIF4F and this interaction was mapped to a conserved guanosine residue located in a Ma5TE internal loop (Miras et al., 2016). Additionally, mutational analyses in eIF4E residues involved in its interaction with eIF4G showed that eIF4F complex formation is necessary for efficient cap-independent translation driven by Ma5TE (Miras et al., 2016). Identification of a new resistant-breaking isolate of MNSV revealed a new class of 3'-CITE, the CXTE, which was acquired from *Cucurbit aphid-borne yellows virus* (CABYV, genus *Polerovirus*, family *Luteoviridae*) Xinjiang by interfamilial recombination, conferring to the recipient MNSV isolate the advantage to translate efficiently and infect resistant melon varieties (Miras et al., 2014). Thus, the 3'-UTR of this MNSV isolate harbors two 3'-CITEs, Ma5TE, and CXTE, with CXTE secondary RNA structure folding into two helices protruding from a central hub. Both 3'-CITEs are active in susceptible melon, while only the CXTE functions in resistant melon and in the absence of eIF4E (Miras et al., 2014).

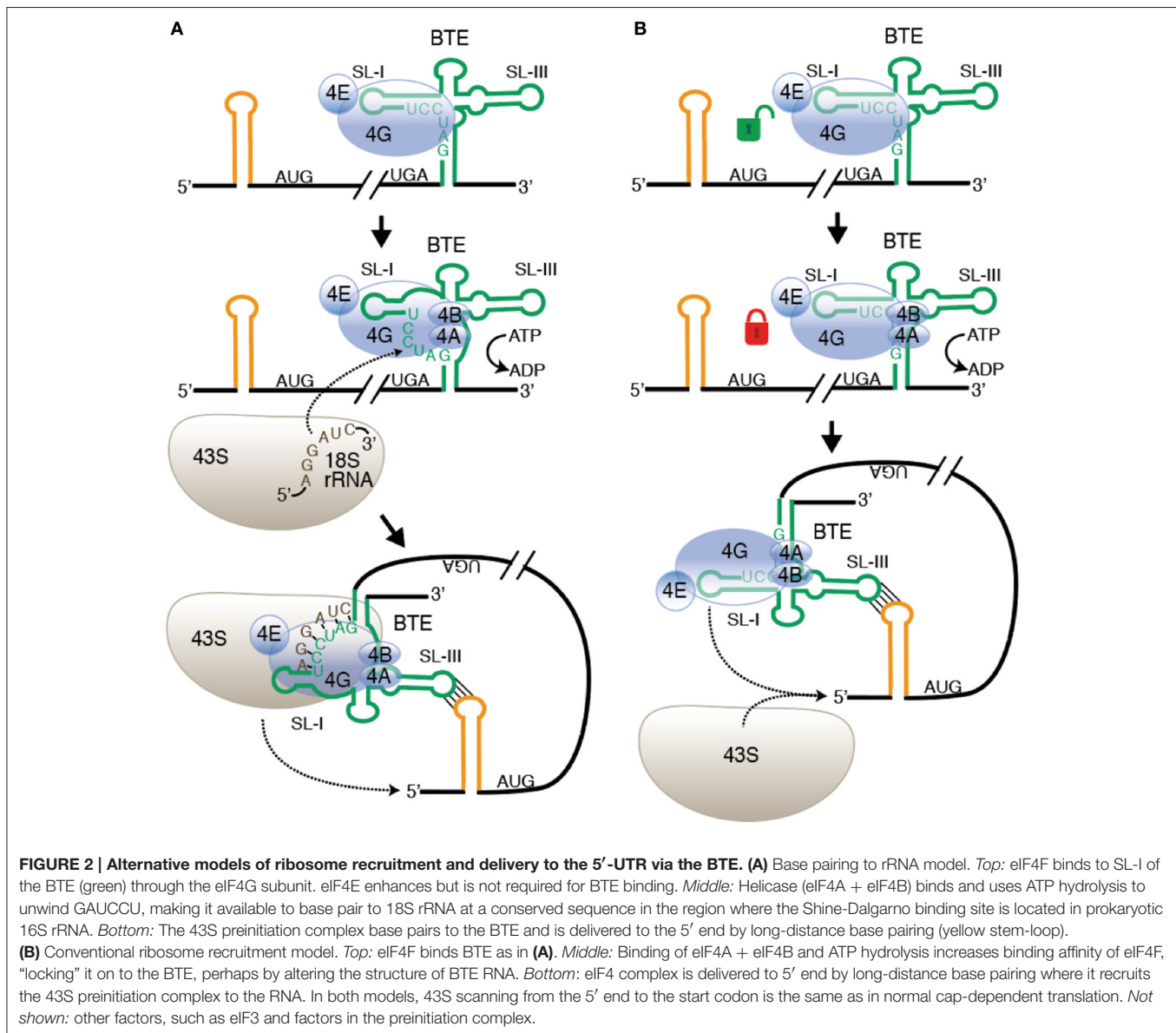
The *Barley yellow dwarf virus*-like translation element (BTE) is one of the best-characterized 3'-CITEs and is found in all members of the *Luteovirus*, *Dianthovirus*, *Alphanecrovirus*, *Betanecrovirus*, and *Umbravirus* genera (Wang et al., 2010; Simon and Miller, 2013). All BTEs share a long basal helix from which two to five additional helices radiate (Figure 1). BTEs contain a highly conserved 17-nucleotide sequence GGAUCCUGGGAAACAGG that includes SL-I (formed by pairing of underline bases). The BTE binds preferentially to the eIF4G subunit of the eIF4F heterodimer (Treder et al., 2008). The eIF4G-binding site in the BTE was revealed by SHAPE footprinting, which showed that eIF4G protects SL-I and nearby bases around base of the hub from which all



helices protrude (Kraft et al., 2013). Addition of eIF4E enhanced the level of protection and stimulated translation by about 25%. Deletion analysis of eIF4G revealed that only the core domain (including eIF4A and eIF3 binding sites, but lacking the eIF4E and PABP binding sites) and an adjacent upstream RNA binding domain are necessary for binding to the BTE and to stimulate translation (Kraft et al., 2013; Zhao et al., 2017).

A long-distance kissing stem-loop interaction between a loop in the BTE and the 5'-UTR is required for BTE-mediated translation (Guo et al., 2001). This long-distance RNA:RNA interaction can be replaced by complementary non-viral sequences outside the BTE (Rakotondrafara et al., 2006). This interaction is conserved among all BTEs except the BTE of *Red clover necrotic mosaic virus* (RCNMV, genus *Dianthovirus*, family *Tombusviridae*), in which mutations in potential complementary loops had no effect on translation and possess the longest BTE and 3'-CITE (Sarawaneeyaruk et al., 2009).

After eIF4F binds the BTE, it appears that the eIF4A helicase, eIF4B plus ATP bind in order to recruit the 40S subunit directly to the BTE. The long-distance base pairing would then deliver the 40S complex to the 5' end for scanning to the first AUG (Sharma et al., 2015; **Figure 2A**). This differs from a previous model in which it was proposed that the long-distance base pairing places the factors near the 5' end, at which point the 40S complex is recruited (Rakotondrafara et al., 2006). However, the dependence on helicase activity may support an older model in which a six base tract in the 17 nt conserved sequence (GAUCCU) base pairs directly to 18S rRNA at the position where the Shine-Dalgarno sequence is located in prokaryotic ribosomal RNA (Wang et al., 1997). Because much of this tract is base paired internally in both the BTE and in 18S rRNA, the helicase activity may be required to disrupt this base pairing, freeing the complementary tracts in the BTE and 18S rRNA to base pair to each other. This base pairing would recruit the 40S subunit directly to the BTE (**Figure 2A**). However, recently the presence of eIF4A, eIF4B and ATP was also found to enhance the binding affinity of the BTE to eIF4G



in the absence of the ribosome (Zhao et al., 2017). This enhanced binding affinity may be the consequence of helicase activity of eIF4A/eIF4G/ATP altering BTE structure. This greater affinity of eIF4G to the BTE may facilitate efficient recruitment of the 40S subunit by conventional factor interactions without need for base pairing to ribosomal RNA (Figure 2B). Future experiments are necessary to determine which model is correct. On the other hand, RCNMV possesses an A-rich sequence (ARS) with strong affinity to PABP in addition to its BTE in its 3'-UTR. Both sequences, ARS and 3'-CITE, have been shown to coordinately recruit eIF4F/ eIFiso4F and the 40S ribosomal subunit to the viral RNA (Iwakawa et al., 2012).

Tomato bushy stunt virus (TBSV, genus *Tombusvirus*, family *Tombusviridae*) and other viruses belonging to the genus *Tombusvirus*, contain 3'-CITEs resembling Y-shaped structure

(YSS), formed by three helical regions. The efficiency of translation controlled by the YSS of TBSV depends on a long-distance interaction with the 5'-UTR of the genome. Mutational analysis of TBSV YSS showed that alterations in junction residues between helices and in a large asymmetric bulge in the major supporting stem disrupted translation (Fabian and White, 2004, 2006). Moreover, the YSS of *Carnation Italian ringspot virus* (CIRV, genus *Dianthovirus*, family *Tombusviridae*) requires addition of the eIF4F or eIFiso4F complex to a factor-depleted wheat germ extract to promote efficient translation (Nicholson et al., 2013). Translation assays showed the ability of the CIRV YSS to function efficiently *in vitro* and *in vivo*, whereas TBSV YSS was detectable only in *in vivo*, suggesting that this difference is due to a misfolding in the TBSV RNA and the lack of eIFs required in translation (Fabian and White, 2004).

The *Panicum mosaic virus*-like Translation Enhancer (PTE) was first identified in *Panicum mosaic virus* (PMV, genus *Panicovirus*, family *Tombusviridae*) (Batten et al., 2006) and later in *Pea enation mosaic virus 2* (PEMV2, genus *Umbravirus*, family *Tombusviridae*) (Wang et al., 2009b). The PEMV2 PTE consists of a three-way branched helix with a large G-rich bulge in the main stem (Wang et al., 2009b). The formation of a magnesium-dependent pseudoknot between the G-rich bulge and a C-rich sequence at the three-helix junction of the PTE is critical for translation and eIF4E recruitment by the PTE (Wang et al., 2011). Unlike most other CITEs, the PEMV2 PTE may not participate in a long-distance RNA:RNA interaction with the 5'-UTR. Instead, upstream of the PTE, there is an element, the kl-TSS, that participates in a long range RNA:RNA interaction with a 5' proximal hairpin located in the p33 ORF (Gao et al., 2012).

Most other PTEs contain a loop predicted to base pair to the 5'-UTR. Indeed, *Saguaro cactus virus* (SCV, genus *Carmovirus*, family *Tombusviridae*), harbors a PTE which participates in a long-distance RNA:RNA interaction with a hairpin located in the p26 ORF (Chattopadhyay et al., 2011). Interestingly, the sequence involved in the interaction has the same conserved motif found in carmovirus TED-like elements and I-shaped structures (Simon and Miller, 2013).

The 3'-UTR of another member of the *Tombusviridae* family, *Turnip crinkle virus* (TCV, genus *Carmovirus*, family *Tombusviridae*), contains an internal T-shaped structure (TSS) that consists of three hairpins, two pseudoknots and multiple unpaired single stranded linker regions (Zuo et al., 2010). Interestingly, the TSS resembles a three-dimensional tRNA-like structure (Zuo et al., 2010). The TCV TSS recruits and binds the 60S subunit of the 80S ribosome (Stupina et al., 2008). For this element, no base pairing between 3'-CITE and 5'-UTR has been identified. It was proposed that the ribosomal subunits form a protein bridge with the UTRs, where the 40S subunit binds the 5'-UTR and the 60S subunit binds the TSS (Stupina et al., 2008). Two additional TSSs were found in the PEMV2 3'-UTR, one upstream of the PTE and another near to the 3' terminus (Gao et al., 2013). Interestingly, both TSSs can also bind the 60S ribosomal subunit and although they are essential for virus accumulation *in vivo*, mutations that disrupted the downstream TSS had no effect in translation (Gao et al., 2013, 2014). However, when this TSS element was positioned proximal to the reporter ORF enhanced translational activity. This report points out the importance of the reporter constructs in the identification of 3'-CITE that participate in translation. A recent report showed that TCV RdRp binds to A-rich sequence upstream of the TSS and using optical tweezers and steered molecular dynamic simulations showed that elements of TSS unfold when it is interacting with RdRp which may promote the conformational switch between translation and replication (Le et al., 2017).

More classes of 3'-CITE await discovery, as the 3' UTRs of several members of the *Tombusviridae* contain no structure that obviously resembles a known 3'-CITE (Simon and Miller, 2013). Thus, viruses have evolved a plethora of structures to achieve the same goal: recruitment of eIF4F and ultimately the ribosome to their RNAs.

5'- and 3'-UTR Dependent Translation of Nepovirus Genomic RNAs

As mentioned above, nepovirus (family *Secoviridae*, order *Picornavirales*) genomes contain a VPg linked to their 5' end, thus are uncapped but polyadenylated requiring also cap-independent translation mechanisms. The two genomic RNAs (gRNA) of *Blackcurrant reversion virus* (BRV; genus *Nepovirus*, subfamily *Comoviridae*), have translation enhancing sequences in their 5'- and 3'-UTRs. The 5' leader sequences of the two gRNAs of BRV contain IRES elements that facilitate translation when placed either at the 5'-end of a non-capped reporter RNA or internally between two reporter genes (Karetnikov and Lehto, 2007, 2008). The BRV IRESes contain little secondary structure, harboring only one predicted single stem-loop structure at the 5' end. Also, the 5'-UTRs of both gRNAs have at least six AU-rich tracts of 8–10 nt predicted to base pair to 18S rRNA. Deletion of these sequences reduced cap-independent translation activity, suggesting a disruption of the required complementarity or other 5'-UTR functional features (Karetnikov and Lehto, 2007, 2008).

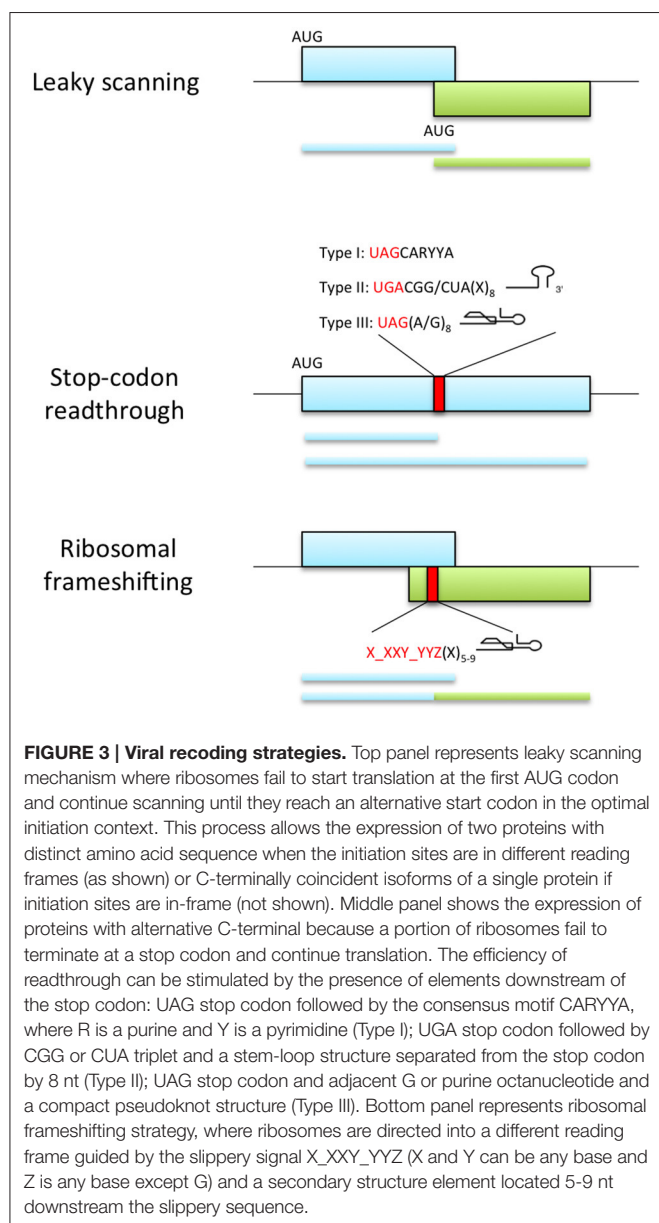
In addition to these IRESes, CITE activity was mapped to the 3'-UTRs of BRV RNA1 and RNA2 (Karetnikov et al., 2006; Karetnikov and Lehto, 2008). This activity depended on the presence of a predicted stem-loop structure located immediately downstream of the last ORF. Moreover, translation efficiency was shown to be dependent on a long-distance RNA interaction with a stem-loop structure present in the 5'-UTR (Karetnikov and Lehto, 2008). Secondary structures of the 3'-CITE and 5'-UTR have not been determined, although they are predicted to fold as a pseudoknot and a stem-loop, respectively. Presence of a poly(A) tail (which is naturally present in the BRV RNA, unlike in other 3'-CITE-containing viruses) in reporter mRNAs stimulated translation several fold, thus playing a major role in CITE-mediated translation. Many of the key elements identified in BRV RNAs, including 5'-3'-UTR RNA interactions and sequence complementarity with the 18S rRNA in the 5'-UTR, are predicted to be conserved in the RNAs of other nepoviruses (Karetnikov and Lehto, 2008), although their precise biological functions remain unknown.

OPTIMIZATION OF CODING CAPACITY

RNA viruses often contain overlapping genes, which allows a very efficient use of the sequence to maximize the coding capacity. Expression of these overlapping genes is achieved by (i) initiation of translation at multiple start codons in different reading frames, by leaky scanning of ribosomes, (ii) frameshifting by a portion of the ribosomes during the elongation phase of translation, or (iii) generating subgenomic mRNAs that allow translation of each ORF from a separate mRNA. The latter will not be discussed, as it is not a translational control mechanism.

Leaky Scanning

Leaky scanning occurs when a proportion of ribosomes fail to initiate translation at the first AUG codon and continue downstream until they reach an AUG codon in the optimal caA(A/C)aAUGGCg initiation context (Figure 3; Joshi et al.,



1997; Kozak, 2002). If the two AUG codons are in the same reading frame, the protein derived by initiation at the second AUG is an N-terminally truncated version of that made by initiation at the first AUG. If the two AUGs are in different frames, then the two proteins have entirely different amino acid sequences. Examples of the latter are long overlaps of replication genes and the triple gene block (TGB) that encodes the movement proteins of several viruses: For instance, the TGB3 of *Potato virus X* (PVX, family *Flexiviridae*, genus *Potexvirus*) and *Barley stripe mosaic virus* (BSMV, family *Virgaviridae*, genus *Hordeivirus*) and the TGB2 of *Peanut clump virus* (PCV, family *Virgaviridae*, genus *Pecluvirus*) are expressed by leaky scanning (Herzog et al., 1995; Zhou and Jackson, 1996; Verchot et al., 1998). In addition, leaky scanning may also

be facilitated by the use of non-AUG initiation codons, which require a strong initiation context (Kozak, 1989). In this respect, *Shallot virus X* (ShVX; family *Flexiviridae*, genus *Allexivirus*) contains a non-canonical ORF for its TGB3 protein (Kanyuka et al., 1992; Lezzhov et al., 2015). ShVX TGB3 translation initiates in a CUG triplet, which has been shown previously to be the most efficient non-AUG initiator (Firth and Brierley, 2012). This triplet and flanking sequences give an optimal context for translation initiation and are conserved in all allexviruses (Lezzhov et al., 2015). Similarly, translation of the second movement protein from *Pelargonium line pattern virus* (PLPV, family *Tombusviridae*, genus *Pelarspovirus*) and *Maize chlorotic mottle virus* (MCMV, family *Tombusviridae*, genus *Machlomovirus*) were suggested to be initiated in a GUG or CUG start codons, accomplished by leaky scanning (Scheets, 2000; Hernández, 2009).

In the main subgenomic RNA of poleroviruses and luteoviruses all three reading frames are used. The tiny, 45 codon first ORF, which encodes a long-distance movement protein, always starts with a non-AUG codon, such as GUG, CUG or AUU. Thus, most scanning 40S ribosomes skip this codon (Smirnova et al., 2015). The second ORF, which encodes the coat protein, starts with AUG in a poor context, while the third ORF, a movement protein gene, starts with AUG in a strong context. The secondary structure encompassing these two AUGs also affects initiation preference (Dinesh-Kumar and Miller, 1993). Other examples of leaky scanning in replicase ORFs have been described. In tymoviruses, the first AUG initiates an ORF encoding a 69 kDa protein that overlaps with the main replicase-encoding ORF initiated by the second AUG. While Kozak context plays a role, unlike “conventional” leaky scanning, the second AUG must be in close proximity (e.g., 7 nt) of the first to efficiently initiate translation (Matsuda and Dreher, 2006). Recently, a small ORF in the sobemoviruses, ORFx, was discovered that overlaps ORF2a and is essential for *Turnip rosette virus* (TRoV, genus *Sobemovirus*) to establish systemic infection (Ling et al., 2013).

Translational Recoding: Frameshift and Readthrough

Recoding consists of the redefinition of individual codons in response to signals in an mRNA. Such signals could be RNA secondary structures, complementary interactions with ribosomal RNA or alteration of the ribosomal state (Atkins and Baranov, 2010). In ribosomal frameshifting a proportion of translating ribosomes are guided into a different reading frame by induced slippage of the ribosome by the mRNA structure (exhaustively reviewed by Miller and Giedroc, 2010; Atkins et al., 2016), while in readthrough mechanisms, a portion of ribosomes fail to terminate at a stop codon and continue translation (Figure 3). This generates proteins with alternative C-termini. Viruses use often these processes to express the RNA-dependent RNA polymerase domain of the replicase.

Ribosomal Frameshifting

Many plant viruses utilize programmed ribosomal frameshifting (PRS) to translate overlapping ORFs. This recoding event can

occur in the + or – direction relative to the normal 0 frame of mRNA translation by shifting the ribosome in one or two nucleotides forward or backward. Productive frameshifting normally competes poorly with standard decoding, so the efficiency of frameshifting in viruses varies from 1% in BYDV to 82% in cardioviruses (Barry and Miller, 2002; Finch et al., 2015). Thus far, most frameshifting by plant viruses is in the -1 direction. These include members of the *Sobemovirus*, *Umbravirus*, and *Dianthovirus* genera and the *Luteoviridae* family (Brault and Miller, 1992; Demler et al., 1993; Kujawa et al., 1993; Mäkinen et al., 1995; Kim et al., 1999; Lucchesi et al., 2000; Barry and Miller, 2002; Tamm et al., 2009). Members of the non-related family *Closteroviridae* (genus *Closterovirus*, *Crinivirus* and *Ampelovirus*) are predicted to use a +1 frameshift to synthesize their viral replicases (Agranovsky et al., 1994; Karasev et al., 1995; Melzer et al., 2008).

The -1 PRS usually requires two signals in the mRNA, a slippery sequence of the type X_XXY_YYZ, where X normally represents any nucleotide, Y represents A or U and Z represents A, C or U (gaps delimit codons in the original 0 frame); and a downstream secondary structure element separated from the slippery sequence by a spacer region of 5-9 nt (Dinman, 2012). In plant viruses these structural elements, acting as stimulators of frameshifting, fall into three structural classes: an apical loop with a bulge, a compact hairpin-type pseudoknot or a stem-loop (Figure 3) (reviewed by Miller and Giedroc, 2010).

The -1 PRS stimulatory elements of BYDV, PEMV-RNA2 and RCNMV fold into a stem-loop with an internal bulge in a similar manner (Kim et al., 1999; Paul et al., 2001; Barry and Miller, 2002; Gao and Simon, 2015). For BYDV, this element participates in a long-distance interaction with the apical loop of a stem-loop located in the 3'-UTR (about 4 kb downstream of the frameshift site). This interaction is required for the low expression levels of RdRp and thus replication (Barry and Miller, 2002). Similar long-range base pairing interactions were shown in RNAs of RCNMV and PEMV2 (Tajima et al., 2011; Gao and Simon, 2015). For PEMV2 RNA, this interaction modifies the lower stem of the structure, possibly due to a rise of its stability or the approximation of other sequence near the 3' end. Curiously, the distant -1 PRS element of PEMV2 RNA appeared to inhibit, rather than stimulate frameshifting, because in its absence, the frameshift rate increased 72% with respect to the wild type viral genome (Gao and Simon, 2015).

On the other hand, the frameshift stimulatory elements from poleroviruses *Beet western yellows virus*, *Potato leaf roll virus* and *Sugarcane yellow leaf virus* (BWYV, PLRV, and ScYLV, family *Luteoviridae*, genus *Polerovirus*) and PEMV1 (family *Luteoviridae*, genus *Enamovirus*) form h-type pseudoknots (Egli et al., 2002; Cornish et al., 2005; Pallan et al., 2005; Giedroc and Cornish, 2009). The frameshift regulatory element of BWYV was the first to be determined at atomic resolution showing a compact pseudoknot with a triple-stranded region (Egli et al., 2002). It was suggested that pseudoknots provide a kinetic barrier to the ribosome and that the unfolding of this element correlates with frameshifting stimulation (Giedroc and Cornish, 2009).

Stop-Codon Readthrough

Stop-codon readthrough is a common strategy found in plant viruses to encode protein variants with an extended C-terminus from the same RNA. During readthrough, some ribosomes do not stop at the stop codon but continue until the next termination codon. Members of the *Tombusviridae*, *Luteoviridae* and *Virgaviridae* families employ readthrough of UGA and UAG stop codons in their replicase and coat protein genes. Flanking nucleotides as well as long-range RNA-RNA interactions influence stop-codon readthrough (Figure 3; Firth and Brierley, 2012; Nicholson and White, 2014). Depending on the sequence motifs and the stop codon, three types of readthrough can be described: The type I motif employs a UAG codon in the replicase gene and is followed by the consensus motif CARYYA (where R is a purine and Y is a pyrimidine) (Skuzeski et al., 1991); this type is used by tobamoviruses, benyviruses and pomoviruses (Pelham, 1978; Firth and Brierley, 2012). The type II motif is used by tobamoviruses, pecluviruses, furoviruses and pomoviruses to generate their viral RdRp and by furoviruses to express the coat protein (Skuzeski et al., 1991; Zeffass and Beier, 1992). It involves a UGA stop codon followed by a CGG or CUA triplet and a stem-loop structure about 8 nts downstream of the stop codon (Firth et al., 2011). The type III class comprises an UAG stop codon, a downstream G or purine-rich octanucleotide and a 3' RNA structure (Firth and Brierley, 2012) and appears in carmovirus and tombusvirus genomes. For example, the tombusvirus CIRV uses stop-codon readthrough to generate its viral RdRp and requires a long-distance interaction between an RNA structure located downstream of the readthrough site and also a sequence in the 3'-UTR (Cimino et al., 2011). *Tobacco necrosis virus-D* (TNV-D, genus *Betanecrovirus*, family *Tombusviridae*) employs a complex series of downstream interactions. A stable bulged readthrough stem-loop (RTSL) immediately downstream of the leaky stop codon contains a G-rich bulge which must base pair to a distant readthrough element (DRTE) located 3 kb downstream in the structure required for replication initiation (Newburn et al., 2014). A pseudoknot immediately 3' to the RTSL, and a stem-loop adjacent 5' to the DRTE in the 3'-UTR are also necessary for optimal readthrough (Newburn and White, 2017). The long-distance interactions within the viral genome required for frameshifting and readthrough may play a regulatory role as switch between translation and replication (Cimino et al., 2011), by allowing replicase entering at the 3' end of the genome to stop its own translation 3–4 kb upstream, as it disrupts this essential long-distance interaction (Miller and White, 2006).

Readthrough of the CP stop codon of viruses from the *Luteoviridae* family appears to use a fourth class of *cis*-acting signals (Brown et al., 1996). The stop codon is usually UAG, but can be UGA or UAA. Instead, readthrough requires a tract of 8–16 repeats of CCXXXX beginning about 8 nt downstream of stop codon and requires additional sequence about 700–750 nt downstream in the coding region of the readthrough ORF, in the example of BYDV (Brown et al., 1996). Although the resulting CP-readthrough protein fusion is not essential for virus particle assembly or infectivity it is assembled into the virion and

is required for persistent, circulative aphid transmission (Brault et al., 1995; Chay et al., 1996).

PERSPECTIVES

This review provides an outlook of the vast diversity of non-canonical mechanisms that RNA viruses use to translate their RNAs. With some significant exceptions, knowledge is still superficial for a large number of cases. It would be highly desirable to obtain additional and deeper information on specific cases and mechanisms. For example, secondary structure data is available for only a few translation initiation elements (Wang et al., 2009a; Zuo et al., 2010; Nicholson and White, 2011; Kraft et al., 2013; Miras et al., 2014), and high-resolution three-dimensional structures are known only for the small H-type pseudoknot frameshift structures of the polero- and enamoviruses (Miller and Giedroc, 2010), and for plant translation factor eIF4E (Monzingo et al., 2007; Ashby et al., 2011). There is no structural data on bipartite or multipartite virus-host complexes. This represents a significant methodological challenge, but the current advancement of techniques like cryo-electron microscopy may significantly contribute to tackle it. Structural data would provide additional mechanistic insight and could contribute to uncover interacting regions with regulatory roles, providing molecular targets for intercepting productive host-virus interactions.

It is important to note that mutations in translation initiation factors that disrupt interactions with viral proteins or RNA might not only prevent infection in resistant varieties of susceptible host species but also contribute to non-host resistance. Mutations in viral factors conferring compatibility with translation initiation factors of otherwise non-host plants can contribute to broaden the host range of potyviruses (Calvo et al., 2014; Estevan et al., 2014; Svanella-Dumas et al., 2014) and a carmovirus (Nieto et al., 2011). Also, the development of techniques to monitor the translational dynamics based on fluorescent and optical methods could provide more complete pictures of how and where translation occurs.

The diversity of mechanisms is particularly striking when identified in a single viral RNA. BRV provides an example of this, with gRNAs carrying VPg, poly(A), IRES, and CITE (Karetnikov et al., 2006; Karetnikov and Lehto, 2008), and there are other viral RNAs for which multiplicity of *cis*-acting elements has been recognized, including MNSV (Miras et al., 2014) and PEMV2 (Gao et al., 2013, 2014). This multiplicity may exist for different reasons, including the use of different mechanisms during different steps of the infection cycle or to infect different hosts, or the overlapping of templates for transcription of mRNAs which, again, may be translated during different steps of the infection cycle and/or in different cellular environments. This brings us to various additional methodological aspects that may require attention for further development of this research field: On the one hand, the dissection of the infection cycle is still a difficult task for plant virologists, as there is a lack of experimental systems in which synchronous infections can be established. On the other hand, experimental systems appropriate for performing arrays

of experiments covering biochemistry, genetics and cellular biology are also missing. For instance, wheat germ extract has been and still is very useful for biochemistry experiments, but genetics or cellular biology experiments are difficult using wheat as a host, because it is hexaploid and difficult to transform. In another example, *N. benthamiana* is an excellent host to perform cellular biology experiments, but its genetic tractability is rather poor, and *N. benthamiana* is not particularly advantageous for biochemistry experiments. In this regard, the preparation of translationally active extracts from evacuated protoplasts (Murota et al., 2011) from different plant species may contribute to solve this problem, particularly if prepared from genetically tractable and microscopy amenable hosts such as *Arabidopsis*.

From the point of view of the cellular translational machinery and how viruses use it, the described diversity of translation mechanisms points toward the different ways that viruses use and control the basic translation machinery of the cell, but it also seems to point toward the existence of a diversity of associations of RNA and protein translation factors used for the uninfected cell to synthesize proteins from different mRNA populations under different micro-environmental conditions and/or subcellular locations. It is tempting to speculate that during evolution plant viruses may have adopted cellular preexisting mechanisms to translate their proteins; quite likely, there is a significant overlap between translation mechanisms of viral RNAs and translation of cellular mRNAs in uninfected cells under abiotic stress conditions (Spriggs et al., 2010), and viruses might be viewed as useful probes to uncover the cellular mechanisms of translation. In this regard, it has been shown that active plant virus replication associates with host gene shutoff (Wang and Maule, 1995; Aranda and Maule, 1998), but even during active replication there are host mRNAs which are over-expressed, suggesting common mechanisms for host and viral mRNA expression, including translation; structural data may provide important information on how these transcripts can recruit the host machinery efficiently during cellular shut-off. Interestingly, host mRNAs over-expressed during virus replication include stress response transcripts (Aranda et al., 1996) and, in fact, at least a maize HSP101 and ADH1 transcripts have been shown to contain IRES-like elements (Dinkova et al., 2005; Mardanova et al., 2008). Large screenings of the human genome revealed widespread identification of cap-independent translation elements located in the 5'-UTR and 3'-UTR of human transcripts, but their mode of regulation remains unknown (Weingarten-Gabbay et al., 2016). In plants, there are few reports on ribosome profiling under abiotic stresses such as drought, varying external light conditions or in response to reactive oxygen species (Liu et al., 2013; Benina et al., 2015; Lei et al., 2015), but not under viral infection conditions, limiting the identification of potential parallelisms.

Last but not least, the diversity of *cis*-acting translation elements identified in plant viruses may contribute to the design of tools for synthetic biology (Ogawa et al., 2017), and in vectors for the overexpression of proteins in biofactory cell-free systems, cell cultures, or whole plants (Fan et al., 2012), or, perhaps, in

other organisms used for industrial overexpression of proteins, if mechanisms employed by plant viruses are universal or at least conserved in the species of interest.

AUTHOR CONTRIBUTIONS

MM wrote Sections Intergenic Region Enhancers to Optimization of Viral mRNA Coding Capacity, WM, VT, and MA edited and added specific information to all sections,

MA supervised MM writing and wrote Sections Introduction and Perspectives.

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Emerging Roles and Landscape of Translating mRNAs in Plants

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Plants use a wide range of mechanisms to adapt to different environmental stresses. One of the earliest responses displayed under stress is rapid alterations in stress responsive gene expression that has been extensively analyzed through expression profiling such as microarrays and RNA-sequencing. Recently, expression profiling has been complemented with proteome analyses to establish a link between transcriptional and the corresponding translational changes. However, proteome profiling approaches have their own technical limitations. More recently, ribosome-associated mRNA profiling has emerged as an alternative and a robust way of identifying translating mRNAs, which are a set of mRNAs associated with ribosomes and more likely to contribute to proteome abundance. In this article, we briefly review recent studies that examined the processes affecting the abundance of translating mRNAs, their regulation during plant development and tolerance to stress conditions and plant factors affecting the selection of translating mRNA pools. This review also highlights recent findings revealing differential roles of alternatively spliced mRNAs and their translational control during stress adaptation. Overall, better understanding of processes involved in the regulation of translating mRNAs has obvious implications for improvement of stress tolerance in plants.

Keywords: mRNA, translational regulation, ribosomal associations, stress, development

INTRODUCTION

Plant ‘omics’ research is currently focusing on at least two important fronts that can have major implications for crop breeding: (1) sequencing and re-sequencing of plant genomes with phylogenetic or agronomic importance and (2) comparative and functional genomic approaches for identifying genes with important roles in plant development and stress adaptation. Such genes can be edited using the editing technology such as CRISPR-Cas9 to develop crops with high value traits (Chang et al., 2016; Zhou et al., 2016). Aforementioned approaches are also components of ‘systems biology,’ which broadly refers to a common framework to understand the functional component of plant genomes and their subsequent adaptation to changing climates (Cramer et al., 2011). Recently, high throughput transcriptome profiling approaches such as RNA-seq have revolutionized the discovery and functional characterization of genes associated with agronomically important traits. In addition, several traits of agronomic importance and recently various forms of quantitative trait loci (QTL) such as expression QTL (e-QTL), *cis*- and *trans*-QTL have been mapped for functional crop improvement (Druka et al., 2010; Wang et al., 2014). These approaches have been widely applied across a variety of stress conditions

(Hirayama and Shinozaki, 2010; Debnath et al., 2011) to understand the regulatory role of stress responsive genes and associated transcription factors (Fu et al., 2016).

In addition, recently emerging co-expression analyses (Serin et al., 2016) and network modeling approaches have been widely used to identify key networks or modules and certain transcription factors that modulate these networks in those regulatory modules (Serin et al., 2016). Although transcriptome profiling methods have been informative, they do not necessarily provide a thorough understanding of whether transcriptional changes observed under a condition actually mirror the abundance of mRNAs (translating mRNAs) associated with ribosomes. Indeed, ribosome profiling approaches, which profiles mRNAs fragments associated with ribosomes provides a direct estimate of mRNAs to be translated into proteins. A thorough understanding of this process will enable us to focus only on those mRNAs bound to ribosomes for functional analyses of gene function. Emerging evidence from recent studies have suggested the discordance between transcriptome and proteome can even be greater during stress responses. Indeed, translating mRNAs are reduced by ~50% under heat stress as only those mRNAs encoding proteins mainly involved in translation and stress responses, are enriched for binding to ribosomes, suggesting that they are selectively translated and demonstrates a level of selective enrichment of certain mRNAs during stress (Yángüez et al., 2013). Similarly, 77% decrease in the pool of translating mRNAs was observed under hypoxia stress indicating that the selective enrichment of hypoxia specific genes in the translating mRNA pool (Branco-Price et al., 2005). Taking into account, the collective information, we can infer the swathing information about the translational control of plant under stress and development conditions using the translating mRNAs as an index of measure. In this review, we highlight the role of ribosome profiling in identifying translating mRNAs involved in stress responses and plant development.

PROFILING APPROACHES FOR TRANSLATING mRNAs

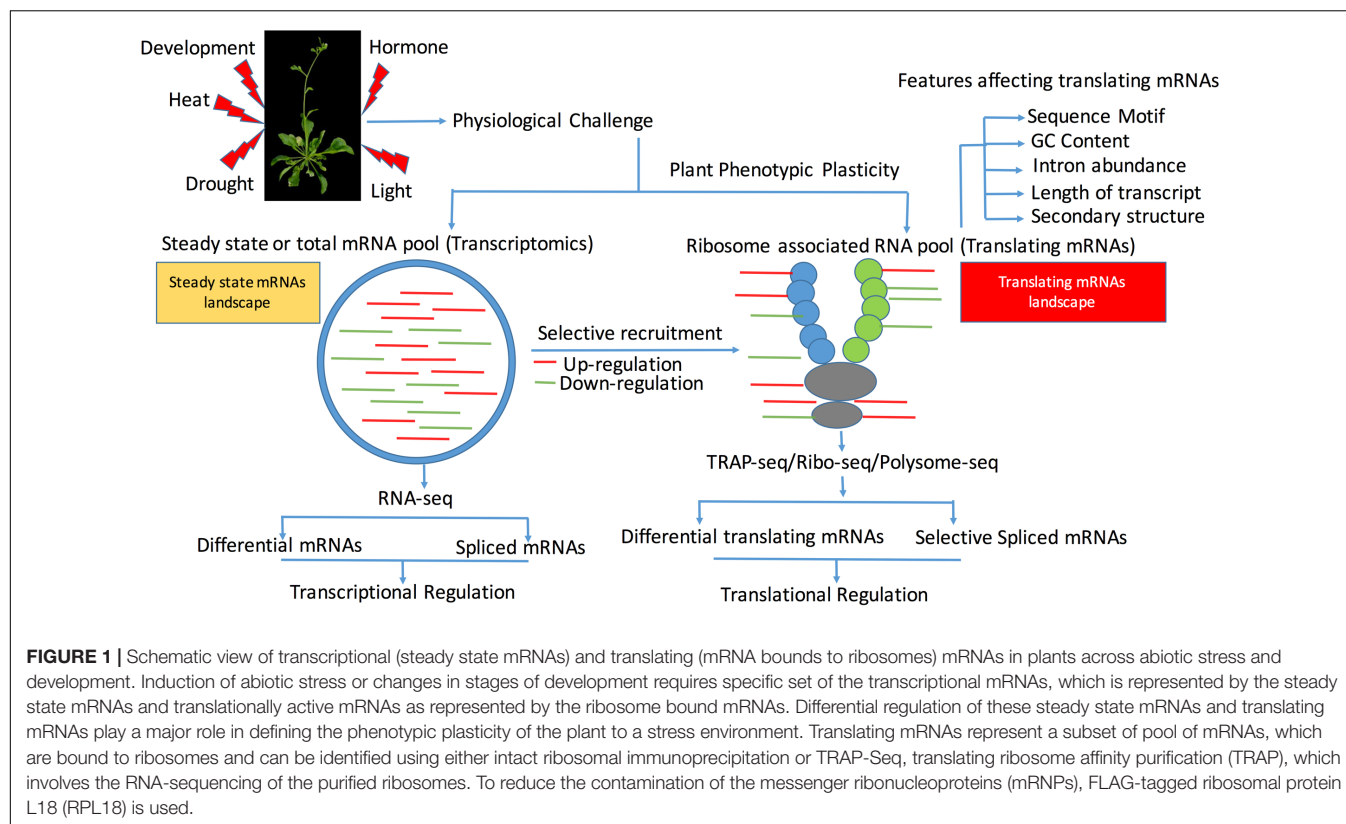
Proteomic-based approaches have been used to establish a correlation between observed fluctuations in transcript expression and the actual peptide abundance during plant development (Galland et al., 2014). However, proteomic approaches are laborious and expensive and preparation of samples and quantification of proteome using techniques such as 1-dimensional (1-D) or 2-dimensional (2-D) or 2D-coupled with iso-electric focusing (2D-IEF) followed by peptide sequencing also requires specialized technical expertise and provide limited resolution of spatio-temporal resolution of translated mRNAs. Another limitation of proteome-based approaches is the identification of an algorithm of sequenced peptides, which mainly relies on BLAST searches against the proteome of the corresponding species or against previously annotated or un-annotated proteins. Recently, ribosomal foot-printing (Ingolia et al., 2009, 2012, 2014), which sequences ribosome-protected

mRNA fragments, has been applied to identify mRNAs bound to ribosomes. Additionally, the genome-wide profiling of ribosome-protected mRNA fragments has highlighted the role of 5' and 3'-regulatory regions and the presence of sequence motifs which could accelerate the initiation of translating mRNAs (Bai et al., 2016).

To isolate translating mRNAs, either intact ribosomes or immunoprecipitated ribosomes enriched for polysome-associated mRNAs have been used (Zanetti et al., 2005; Reynoso et al., 2015; Zhao et al., 2017). Translational abundance is then analyzed using ribosome-profiling methods such as translating ribosome affinity purification (TRAP) followed by RNA sequencing (TRAP-seq) (Zanetti et al., 2005). The use of FLAG-tagged ribosomal protein L18 (RPL18) in this method gives an intricate view of functional ribosomes by reducing the contamination of messenger ribonucleoproteins (mRNPs) (Juntawong et al., 2013; Zhao et al., 2017). **Figure 1** represents a summary view of applications of these approaches to study stress conditions and developmental patterns. These approaches have been undertaken in both model and non-model species with model species benefiting from the availability of genome-based mapping methods, which can reveal the translational efficiency as a measure of the ribosomal scores (Guttman et al., 2013). Recently, ribosomal scores have been used to measure the translational efficiency of sense and antisense transcripts in maize (*Zea mays*) under drought stress (Xu et al., 2017), which demonstrated that ribosomal scores can be used as a measure to estimate the translational efficiency. **Table 1** shows examples from the application of these approaches into model plants. As for proteomics, several factors also contribute to the sequence diversity among the pool of translating mRNAs such as the association of RNA binding proteins to translating mRNAs and translational elongation (Tebaldi et al., 2012; Browning and Bailey-Serres, 2015). Additionally, sequence features such as the length and the GC content of transcripts (Zhao et al., 2017) and the presence or absence of smallORFs, can affect the rate by which mRNAs can be translated (Zhao et al., 2017).

TRANSLATING mRNAs AND THEIR ROLES IN STRESS FUNCTIONAL GENOMICS

Transcriptional profiling has played a vital role in understanding the regulation of genes during stress. Also revealed are a wide array of genes involved in stress responses (Kreps et al., 2002; Buitink et al., 2006; Kianianmomeni, 2014; Valliyodan et al., 2014). However, the translating dynamic landscape of these transcriptionally active mRNAs was mostly lacking with only few reports addressing the role of translating mRNAs in stress (Mustroph et al., 2009; Juntawong et al., 2014). Given that the advent of the ribosome- profiling approaches made the identification of translating mRNAs possible we are now in a much better position to determine how transcriptional changes occurring during stress correlate with those of translating mRNAs (Zhao et al., 2017). In the following section, a



few specific examples from recent studies will be briefly discussed.

Light plays a key role in the adaptation of plant species to any environment and controls the rate of photosynthesis, which is a source point for energy required for growth. Several stress studies have used light measures and photosynthetic efficiency

as a first point to understand plant responses to stress (Dunaeva and Adamska, 2001; Soitamo et al., 2008). Previously, wide arrays of genes have been profiled in response to light stress in model and non-model species and attempts made to reveal the light-based regulation and evolution of C3–C4 biosynthetic cycle (Perduns et al., 2015). Light-regulated responses occur diurnally with alternative light- and dark- phases. Different sets of the transcriptionally active genes have been identified during diurnal shifts (Violet-Chabrand et al., 2017). Ribosome profiling approaches revealed mRNAs encoding ribulose biphosphate carboxylase (*RBCS*) and ribulose biphosphate carboxylase small chain 1A (*RBCS1A*) as major translating mRNAs (Liu et al., 2013; Juntawong et al., 2014). *RBCS* plays an important role in carbon fixation and regulatory role of this enzyme at the level of translation can help understand the minimum cost of translation and translation efficiency for carbon fixation. These observations can be linked to the energy cost of translation, which is defined as the minimum energy required to translate particular mRNAs (Branco-Price et al., 2008) and have also been recently shown in light regulated translating mRNAs (Juntawong and Bailey-Serres, 2012). Missra et al. (2015) observed phase shifts and state transitions of the translating mRNAs with ribosomal proteins and mitochondrial respiration associated translating mRNAs showing peak translation states during night cycles. Notably, central clock mRNAs revealed a wide variation in the abundance of translating mRNAs, with *CCA1*, a clock-associated mRNA, showing phase shifts and light-dark phase regulated ribosomal associations (Missra

TABLE 1 | Summary of the translating mRNAs studies.

Plant species	Condition	Reference
<i>A. thaliana</i>	Hypoxia	Branco-Price et al., 2005; Mustroph et al., 2009; Niedojadlo et al., 2016
<i>Zea mays</i>	Drought stress	Lei et al., 2015
<i>A. thaliana</i>	Circadian cycle	Missra et al., 2015
<i>A. thaliana</i>	Gibberellin signaling	Ribeiro et al., 2012
<i>A. thaliana</i>	Heat stress	Yángüez et al., 2013
<i>A. thaliana</i>	Lipid metabolism	Li et al., 2015
<i>A. thaliana</i>	Developing flowers	Jiao and Meyerowitz, 2010
<i>A. thaliana</i>	Root development	Rajasundaram et al., 2014
<i>A. thaliana</i>	Multiple stresses	Palusa and Reddy, 2015
<i>A. thaliana</i>	Seed germination	Galland et al., 2014; Bai et al., 2016
<i>A. thaliana</i>	Thermal stress	Lukoszek et al., 2016
<i>Oryza sativa</i>	Tissue and stress	Zhao et al., 2017
<i>A. thaliana</i>	Brassinosteroid signaling	Vragović et al., 2015
<i>A. thaliana</i>	Pollen tubes	Lin et al., 2014
<i>A. thaliana</i>	Light stress	Gamm et al., 2014

et al., 2015). These associations reveal the diurnal transition of translating mRNAs involved in the circadian clock (Missra et al., 2015).

Translating mRNAs also showed alterations under various stress conditions (Table 1). For instance, under hypoxia (low oxygen stress) only 70% of the cytosolic mRNAs were found to be associated with ribosomes to conserve cellular kinetic energy (Branco-Price et al., 2008; Juntawong and Bailey-Serres, 2012). Among the favored translating mRNAs found under hypoxia were those that either promote the conservation of ATP as a source for cellular energy or facilitate the shift toward anaerobic metabolism (Juntawong and Bailey-Serres, 2012). This observation is consistent with the increase observed in translating mRNAs encoding for anaerobic enzymes (Mustroph et al., 2009), thus providing support for the selective enrichment of mRNAs during physiological changes. Previous observations were further supported by whole genome ribosome foot-printing in hypoxia (Juntawong et al., 2014). Previous reports indicate fewer number of ribosomes associated with up-regulated transcripts under hypoxia thus illustrating the lack of translational initiation as compared to elongation (Juntawong et al., 2014). However, recent studies established the correlation between the transcriptional and translational coordination under drought stress in maize (Lei et al., 2015).

Recent studies highlighting the differences in the pool of translating cytoplasmic and nuclear mRNAs under hypoxia have presented new insights into the selection of translating mRNAs (Niedojadło et al., 2016). For example, no preferential enrichment of translating *ADH1* (*Alcohol Dehydrogenase 1*) mRNAs, a core hypoxia-induced gene, was observed in nucleus among the selectively retained translating mRNAs (Niedojadło et al., 2016). Interestingly, post-aeration (a mechanism to restore the plant from hypoxia-induced stress) revealed selective increase in cytoplasmic mRNAs (Niedojadło et al., 2016). This indicated that cytoplasmic mRNAs come as a first point of contact to the translational machinery during stress recovery while mRNAs, which are not involved in stress responses, are stored pre-dominantly in the nucleus (Niedojadło et al., 2016). Similar patterns of condition-associated selective enrichment of translating mRNAs have been observed by Tiruneh et al. (2013). Juntawong et al. (2013) showed a positive association between cold shock protein (CSP) abundance and the translation of ribosomal mRNAs in *Arabidopsis thaliana*, demonstrating the selective abilities of CSPs as molecular chaperones to selectively load mRNAs.

To understand the processes involved in the regulation of translating mRNAs, genes involved in this process have been identified and functionally characterized using mutant lines. Since translating mRNAs represent the pool of polyA mRNAs associated with ribosomes, it is imperative to highlight the role of PAB1, which is a poly-A binding protein that exerts a level of translational control by bringing the 5' cap and 3' poly-A tail together (Tiruneh et al., 2013). However, this process is compromised in the poly-(A) binding protein mutant, *pab2 pab8*, as well as in a mutant of a large ribosomal subunit protein, *rpl24b/shortvalve1* (Tiruneh et al., 2013).

Comparative assessment of translating mRNAs across these mutants revealed that only one-fifth of the mRNAs showed a highly plastic translational control and the lack of poly-A binding protein mutations has only affected proteins involved in late embryogenesis. However, no significant effect of the *rpl24b* mutation on translating mRNAs was found, suggesting that the pool of translating mRNAs is independent of the *RPL24b* gene.

TRANSLATING mRNA POOLS ACROSS DEVELOPMENTAL LANDSCAPE

The APETALA2 (AP2) gene family plays an important role during plant development (Zhao et al., 2007; Wu et al., 2009). Jiao and Meyerowitz (2010) laid the founding work by demonstrating the role of translating mRNAs in flower development by incorporating FLAG-tagged RPL18 (large subunit ribosomal protein L18) protein under the control of either APETALA1 (AP1), APETALA3 (AP3) or AGAMOUS (AG) promoter. Notably, the enrichment of petal and stamen development was seen as enriched in the AP3 domain as compared to the AP1 and AP2 domain. Interestingly, they observed the enrichment of the GO terms specific to chloroplast functions in the AG domains and the abundance and the enrichment of these terms were found to be positively correlated to flower development, suggesting that the chloroplast translating mRNAs play an important role in flower development. Another example highlighting the role of cell specific translating mRNA comes from the *Arabidopsis thaliana* transcriptome cell-specific mRNA atlas (Mustroph and Bailey-Serres, 2010). Mining of the *Arabidopsis* transcriptome cell-specific mRNA atlas revealed mRNAs encoding suberin and cutin biosynthesis proteins showing cell-type specific regulation at the translational level, further suggesting a role for translational regulation in cell determination and differentiation (Mustroph and Bailey-Serres, 2010). Augmenting these previous observations, selective enrichment and distinctiveness of translating mRNAs vary not only across different cell types but also across different tissues. Tissue specific enrichment of translating mRNAs is also supported by recent findings in *Oryza sativa*, revealing a distinct profile of the GC rich translating mRNAs across tissues (Zhao et al., 2017).

The enrichment of the translating mRNAs also showed variations during developmental phases and selective enrichment of the sub-set of steady state mRNAs during plant development (Yamasaki et al., 2015). For example, using 2-D proteomics with a radiolabeled amino acid precursor, namely [³⁵S]-methionine, a higher proportion of translating mRNAs was seen from phase I to phase II, both of which are defined as germination *sensu stricto* as compared to phase II to phase III (resumption of water uptake) transitions during *Arabidopsis thaliana* seed germination (Galland et al., 2014). Galland et al. (2014) specifically highlighted the role of the nuclear cap-binding complex, which plays an important role in the selective export of nuclear mRNAs. This finding is also in line with those from recent studies suggesting that the nucleus serves as a host for retention of mRNAs and depending on the nature of the stress response,

actively selects translated mRNAs and recruits them to the cytoplasmic pool (Niedojadło et al., 2016). Galland et al. (2014) demonstrated the selective mRNA translation during the seed germination using proteomics based approaches, which recently has been re-visited in *Arabidopsis thaliana* illustrating the role of translating mRNAs mainly to two temporal shifts: seed hydration and germination (Bai et al., 2016). Interestingly, they found a significant overlap (25%) with hypoxia regulated translating mRNAs, which might be attributed to low oxygen during the seed germination (Bai et al., 2016). Basbouss-Serhal et al. (2015) demonstrated the involvement of translating mRNAs in seed germination by comparatively analyzing dormant and non-dormant seeds. A correlation could not be established between the transcriptional and translational landscape, except for few genes such as *ACO1*, *GASA6*, and *HSP70*, leading to the conclusion that seed germination is mainly translationally controlled. A closer look at the functional categories using GO analysis demonstrated specific enrichment of GO categories in non-dormant and dormant seeds specifically highlights the role of redox and lipid metabolism associated genes in dormancy maintenance (Basbouss-Serhal et al., 2015).

Translational regulation through the regulation of translating mRNAs also plays an important role in sexual reproduction, specifically within pollen tube growth. Transcriptomics based approaches have highlighted the role of *POP2*, which plays an important role in pollen tube growth (Palanivelu et al., 2003). The role of LURE peptides, which are defensin-like peptides secreted from synergids has been widely elucidated as signaling components (Kanaoka and Higashiyama, 2015). However, the detection of translating mRNAs has been lacking until the studies of Lin et al. (2014), which used LAT52: HF-RPL18 transgenic *Arabidopsis* expressing the ribosomal protein L18 (RPL18) tagged with a His6-FLAG and driven by pollen specific promoter (ProLAT52) (Twell et al., 1989). A comparative analysis of the *in vivo* and *in vitro* pollen tubes showed 41 specific transcripts that were enriched in *in vivo* pollen tubes, including *IV6* (xyloglucan endotransglucosylase/hydrolase), *IV4* (putative glutathione transferase) and *IV2* (putative methyltransferase), which are involved in micropylar guidance. Lin et al. (2014) also highlighted the difference in the pool of translating mRNAs as compared to the previously transcribed mRNAs suggesting the difference in the steady state population of mRNAs and ribosome associated mRNAs (Lin et al., 2014).

Hormonal regulation plays an important part in plant growth (Fridman et al., 2014). Plant patterning and architecture is a widely studied developmental process with most studies focusing on spatio-temporal regulation of shoot apical meristem (Gendron et al., 2012). Hormonal regulation of translating mRNAs dates back to the first study by Jiao and Meyerowitz (2010). These authors, using AP1, AP2, and AP3 domain specific pool of translating mRNAs, highlighted the role of several hormones such as ethylene, jasmonic acid, brassinosteroids, cytokinins, and gibberellins in the regulation of translating mRNAs. Specifically, they observed that genes with in AP3 domains showed pattern of up- and down-regulation at specific stages of flower development in response to gibberellins and jasmonic acid. Notably, they observed these phytohormones regulate the flower development

by down-regulating the specific genes in the AP3 domain (Jiao and Meyerowitz, 2010). Ethylene plays a central role in plant development and most importantly its perception to the stomatal opening and activating the stress perception in plants. Among the most widely characterized ethylene pathways, *EIN2* (ETHYLENE INSENSITIVE 2), which plays a key role in the perception and signaling of the response from the endoplasmic reticulum to nucleus (Zheng and Zhu, 2016) has been shown to be under the translational control previously using the ribosome profiling methods. Interestingly, in parallel to the *EIN2*, non-sense mediated decay proteins UPFs also act synergistically to control the translational control of *EIN2* (Merchante et al., 2015). It has been further demonstrated that the translational control of the *EBF3*, which is a critical component of the master ethylene pathway is under the translational control of the *EIN2*, UBFs and 3' long UTRs of EBFs (Merchante et al., 2015).

Ribeiro et al. (2012), using the FLAG-epitope tagged ribosomal protein L18 (FLAG-RPL18), demonstrated the role of gibberellins (GAs) in modulating the pool of translating mRNAs in *Arabidopsis thaliana* shoots. Translating mRNA profiling revealed the feedback regulation of GA biosynthetic genes, demonstrating the correlation between the carbon availability and growth. The role of brassinosteroids has been widely elucidated in regulating root and shoot development (Fridman et al., 2014). Recently *BZR1*, a brassinosteroid specific transcription factor, has been shown to regulate the expression of transcripts involved in development (Jaillais and Vert, 2016). A recent investigation highlights the role of *BZR1* in suppressing the cup-shaped cotyledon (*CUC*) gene, which regulates the morphogenesis processes taking place in the shoot apical meristem (Gendron et al., 2012). Translating mRNA-profiling approaches revealed tissue specific regulation of BR (Vragović et al., 2015). Interestingly, contrasting patterns of gene expression were observed, with epidermal cells inducing the cell division as a signal from BR by stimulating auxin gene expression and stele suppresses the epidermal induction (Vragović et al., 2015) resulting in coordinated growth and meristem size determination. Perception and involvement of auxin in TOR signaling pathway has been first elucidated by uncovering the translational control of the up-stream open reading frame (uORF), which depends on the translational elongation factor eIF3h (Schepetilnikov et al., 2013). To delineate the functional association TOR inhibitor Torin-1 was used, which in case of non-inhibition activity recruits the SK6K1 to polysomes for phosphorylation, whereas in the presence of the Torin-1 auxin promotes the SK61 dissociation and functional association of the TOR to polysomes thus functionally elucidating the TOR pathway, which plays a key role in response to hormones and nutrients (Schepetilnikov et al., 2013).

FEATURES AFFECTING SELECTIVE RECRUITMENT OF TRANSLATING mRNAs

Translating mRNA pools have been widely studied to understand sequence features that allow for the selective association of

translating mRNAs with ribosomes under specified conditions (Zhao et al., 2017). Among the features that have been widely correlated with translating mRNA are the GC content, minimal free energy and uORFs, which act as a check point for translating mRNAs (Lei et al., 2015). For example, a recent study by Zhao et al. (2017) indicated the selective enrichment of GC rich and short coding sequences with translating mRNAs across tissues. Similar features have been observed during stress and development, suggesting that plant translating mRNAs represent the minimum energy cost budget defined as the minimal energy required for subsequent elongation and termination of translating mRNA (Juntawong et al., 2013; Basbous-Serhal et al., 2015). It is interesting to see that minimal free energy is one of the factors that also controls the population of translating mRNAs and their subsequent association with ribosomes (Lei et al., 2015). Minimal free energy affects RNA folding and has been previously widely linked to the ribosomal rates, which is defined as the rate of the association of the ribosome to the corresponding mRNAs. Recently, this has been addressed using the ribosome drafting technique, which typically links the accelerated rate of ribosome binding to mRNAs as compared to the canonical rate of mRNA folding (Borujeni and Salis, 2016). However, whether ribosomes drafting occurs for transcripts that accelerate to populate with the ribosomes under defined abiotic or biotic stress conditions in plants has not yet been established.

In addition to sequence features, recent investigations by Lukoszek et al. (2016) have demonstrated the role of mRNA secondary structures which influence the association of translating mRNAs with ribosomes. Secondary mRNA structures can have a direct influence on their folding energy as well. Lukoszek et al. (2016) estimated the folding energy of profiled mRNA and found that up-regulated translating mRNAs have relatively higher folding energy up-stream of the start codon. Interestingly, this scenario has not been observed in the case of down-regulated translating mRNAs, suggesting that the up-stream enhancement of the folding energy is a feature associated with rapidly translating mRNAs to increase the ribosomal occupancy at a given time point. Previous studies have shown that mRNAs with stable structures encode proteins that are more compact and mRNA length acts as a determinant of the folding energy (Faure et al., 2016). Several studies have indicated toward the selective recruitment of shorter transcripts with high GC content to ribosomes (Liu et al., 2013; Zhao et al., 2017). However, the correlation of GC richness with codon usage, a measure that represents the usage of codons in synonymous sites, needs to be also taken into account as the biased and preferential usage of codons varies from one species to another. Interestingly, Bai et al. (2016) observed a positive correlation between translating mRNAs and GC content at the third synonymous sites in codons, providing the basis of selective enrichment of the GC rich transcripts for frequent association to ribosomes. In addition to the aforementioned sequence features, translating mRNAs such as the ones pertaining to seed germination have been recently shown to have enriched motifs (Motif3c) (Bai et al., 2016). It is likely that

the presence of this motif allows enhanced translational initiation (Bai et al., 2016).

TRANSLATING mRNAs AND SPLICING DIVERSITY

Protein diversity and the evolution of protein diversity through the mechanism termed alternative splicing (AS) has been widely studied in plants (for a review see Reddy et al., 2013). Plants show a high proportion of splicing diversity with as much as 60% of the *Arabidopsis thaliana* proteome is resulted from alternatively spliced transcripts (Reddy et al., 2013). Juntawong et al. (2014) provided the first evidence of translating spliced mRNAs, revealing a link between alternative splicing and the ribosomal association of spliced transcripts. The preferential ribosomal association of the most abundantly spliced gene family (serine/arginine-rich (SR) proteins) has recently been shown (Palusa and Reddy, 2015). The non-small nuclear ribonucleoprotein (snRNP) spliceosomal protein family shows a differential recruitment of splice variants during development and in response to heat and cold stress (Palusa and Reddy, 2015). There are 100 distinct splice variants from 14 SR genes in this family (Reddy et al., 2013). However, ribosomal association seems to be affecting only three SR genes (*SR30*, *SR34a*, and *SR34b*) and their splice variants (Palusa and Reddy, 2015). Intron retention, a dominant form of splicing variation in plants (Mastrangelo et al., 2012; Min et al., 2015) has been shown to regulate the preferential recruitment of these splice variants as translating mRNAs (Palusa and Reddy, 2015). Interestingly in *Oryza sativa*, features of translating mRNAs revealed fewer association of transcripts with retained introns to ribosomes (Zhao et al., 2017), which is in line with the translating mRNAs observed in seed germination (Bai et al., 2016). Together, these findings suggest that exploring the association between intron splicing and translating mRNAs may be able to establish the role of splicing machinery at translational level. This in turn will help unravel how splicing machinery may alter the translational output in species-specific alternative splicing and may reveal differential pools of spliced translating mRNAs associated with development and abiotic stress in plants.

TRANSLATING mRNAs AND POLYPLOIDY

It is now widely accepted that the majority of extant plants are currently polyploid (neopolyploid) or were in a state of polyploidy at some point in their evolutionary history (paleopolyploid) (Wood et al., 2009). A subsequent effect of whole genome duplication or ploidy-induced chromosome doubling is the abundance of in-paralogs with respect to orthologous genes. Following polyploidization, global transcriptional patterns shift dramatically relative to patterns observed in progenitor species. Following this initial state of flux, polyploids move toward functional diploidization. During this evolutionary process, redundant genes are silenced or lost (Schnable et al., 2011; Sehrish et al., 2014) and the patterns

of homoeolog expression bias and expression dominance are established (Feldman and Levy, 2009). Therefore, polyploidy-associated phenomena should be taken into consideration when studying translating mRNAs within polyploid crop species.

Previous studies by Tiruneh et al. (2013) revealed differences in translating paralogous mRNAs encoding ribosomal proteins; however, whether the correlation of this observed translational state to the *rpl24b* mutation is yet to be established. Interestingly, a difference in the association of paralogous translating mRNAs with ribosomes was seen in *Glycine dolichocarpa* (~100 MYA allotetraploid), revealing wide variations in one-quarter of the translating mRNAs, with categories mostly involved in photosynthesis (Coate et al., 2014). Specifically, Coate et al. (2014) indicated that translational shifts might be possible in polyploid genomes, and can cause expression shifts in whole chromosome homoeologs. Transcriptional response in hexaploid wheat genome indicates a sub-genome bias toward the transcriptional response to biotic stresses indicating a preferential expression of defense related genes from B and D sub-genomes (Powell et al., 2017). However, it is yet to be ascertained whether the homoeolog expression divergence observed occurred at the translating mRNA level. However, translating mRNA regulations represent association with homoeologs retained with after paleopolyploid event and provide a proof of concept for further exploration of links between the role of homoeologs and their subsequent association and divergence with ribosomes.

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CONCLUSION AND FUTURE DIRECTIONS

Recent profiling of the transcriptional landscape in crop and model plants has produced numerous insights that can be used to potentially enhance crop productivity by aiding selection of genotypes resilient to stresses. However, a thorough understanding of post-transcriptional changes and in particular translating changes is still lacking. Recently developed techniques such as ribosomal profiling has started revealing potential roles of translating mRNAs in stress responses (Browning and Bailey-Serres, 2015), which will be the next major leap forward to accelerate the improvement of stress tolerance in diverse crop species.

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