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Role of RNA Binding Proteins and Post-Transcriptional Regulation in Response to Environmental Changes in *Arabidopsis thaliana*

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ABSTRACT OF THE DISSERTATION

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Light and temperature are two of the most important factors that regulate plant growth and development. The adaptation to alterations in light and temperature involves programed changes in gene expression that are necessary for physiological and morphological adaptations. Transcript abundance is frequently used to monitor changes in gene expression in response to sub-optimal growth conditions. However, transcript accumulation may not accurately mirror gene expression due to extensive post-transcriptional and post-translational regulation. In this dissertation, the significance in post-transcriptional regulation in response to unanticipated alterations in light availability was evaluated in seedlings of *Arabidopsis thaliana*. Early darkness resulted in translational inhibition and sequestration of a subset of cellular mRNAs. The translationally regulated mRNAs were enriched in transcripts encoding chloroplastic and protein synthesis machinery. The reduced engagement of the majority of these transcripts with ribosomes was rapidly reversed upon re-illumination. These results suggest that

regulation of the translational status of chloroplastic and protein synthesis mRNAs may aid in energy conservation during unanticipated darkness. Towards the elucidation of RNA binding proteins that control selective mRNA translation, the complexes of *Arabidopsis* Cold Shock Proteins (CSPs 1-4) were characterized. Transgenic lines over-expressing epitope-tagged CSPs were established and used for cellular fractionation and mass spectrometric protein identification of immunopurified CSP complexes. Fluorescently-tagged CSPs and associated proteins were localized in transiently transformed cells by confocal microscopy. Together, the results of this survey indicate *Arabidopsis* CSPs are involved in multiple processes of post-transcriptional regulation, including pre-mRNA/rRNA processing and mRNA translation. Of the four CSPs, CSP1 co-fractionated with ribosome. A mild RNase A treatment of ribosome complexes combined with sucrose gradient fractionation confirmed that CSP1 is a polysome-associated RNA binding protein. CSP1 accumulated under normal growth condition and was induced by low-temperature. A polyclonal antibody prepared to specifically recognize CSP1 protein was used to co-immunopurify native CSP1 complexes. DNA microarray hybridization was used to compare total (transcriptome), polysomal (translatome), and CSP1-associated mRNAs from plants grown under normal or low-temperature conditions. The results demonstrate that CSP1 preferentially associated with mRNAs involved in RNA processing and protein synthesis. Many of the CSP1-associated mRNA also have high 5'-UTR with a high G+C content. The transcriptome and translatome adjustments during low-temperature stress were highly correlated, in contrast to the findings with early darkness. This work provides new perspectives on post-

transcriptional gene regulation in response to environmental cues in *Arabidopsis*, as well as a foundation for future in-depth characterization of mRNA-RNP control networks in plants.

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Chapter 1

RNA binding proteins and their roles in post-transcriptional regulation

1.1 Introduction

Gene expression is a continuum of processes leading from the gene via its mRNA to the functional protein. This involves regulation at the levels of chromatin structure, DNA methylation, initiation of transcription, pre-mRNA capping, splicing, and polyadenylation, followed by a quality-control round of mRNA translation at the nuclear pore, which triggers non-sense mediated decay (NMD) or export to the cytoplasm where localization, translation, storage, and ultimately mRNA degradation occur. Many of these processes involve RNA binding proteins (RBPs) that are often components of mRNA-ribonucleoprotein (mRNP) complexes. The recognition of differentially expressed genes, based on modulation of abundance of individual mRNAs, is a keystone of reverse genetic analyses in plants. But, unfortunately, the isolation of the mRNA from homogenized organs typically in under condition that dissociates all RNPs, so transcripts that are stored or targeted for degradation may be indistinguishable from those that are undergoing processing and translation. Consequently, steady-state transcript abundance merely provides a snapshot that reflects the balance between mRNA production and turnover. A more precise view of gene regulation requires the quantitative evaluation of mRNAs associated with specific RBPs or mRNP complexes.

Plant genomes encode hundreds of RBPs that possess one or more RNA-binding motif (Lorković & Barta, 2002) such as RNA recognition motif (RRM) (Cléry *et al.*, 2008), K homology domain (KH) (Valverde *et al.*, 2008), Pumilio or Puf domain (PUM) (Lu *et al.*, 2009), and pentatricopeptide repeat (PPR) and tetratricopeptide repeat (TPR) (Schmitz-Linneweber & Small, 2008) (Table 1.1). The majority of the ~1200 *Arabidopsis thaliana* (*Arabidopsis*) proteins with putative RNA binding or modification activity are uncharacterized; many appear to be unique to plants and therefore might participate in plant-specific processes (Lorković & Barta, 2002). In the nucleus, RBPs function in the addition of the 5'-m^7GpppN-cap to the pre-mRNA, the co- and post-transcriptional removal of introns (Lorković & Barta, 2002; Reddy, 2007; Lorković, 2009), cleavage and 3' polyadenylation (Hunt *et al.*, 2008) mRNA export (Chekanova *et al.*, 2007), NMD (Pendle *et al.*, 2005), and *siRNA* and *miRNA* biogenesis (Voinnet, 2009) (Figure 1.1). Some nuclear RBPs participate in pre-mRNA processing and ribosome biogenesis within the nucleolus (Pendle *et al.*, 2005), chromatin modification (Pontes & Pikaard, 2008), and telomere maintenance (Kannan *et al.*, 2008). Genetic screens have uncovered a number of nuclear RBPs that are critical to development and stress responses (Chinnusamy *et al.*, 2007; Lorković, 2009). Importantly, some RBPs associate with transcripts in the nucleus and remain bound after export to the cytoplasm. The history of nuclear processing events these RBPs report provides a continuum between nuclear and cytoplasmic processes. In the cytoplasm, RBPs are constituents of a number of mRNPs including the mRNA-40S ribosome pre-initiation complex, ribosome, signal recognition particle (SRP), processing bodies (PBs), stress granules (SG), and mRNA

localization machinery (Figure 1.1). Importantly, some cytosolic RBPs bind to cohorts of transcripts via conserved motifs and thereby facilitate the coordinated localization and activity of cadres of mRNAs within the cytoplasm (Okita & Choi, 2002). Keene (2007) proposed a post-transcriptional RNA regulon model, in which RBPs co-ordinate the expression of sub-population mRNAs that encode proteins for specific biological processes. This RNA regulon concept has not been explored in plants. Through the study of mRNPs and the constituents of RNP complexes, networks of gene regulation operating at the post-transcriptional level may be elucidated.

1.2 Nuclear processing of pre-mRNA

The association of RBPs with pre-mRNA occurs co-transcriptionally. The pre-mRNAs associate with RBPs and their interacting proteins in complexes that facilitate a series of pre-mRNA processes including 5' capping, intron splicing, 3' cleavage and polyadenylation (Figure 1.1). The primarily nuclear RBPs, hnRNPs (heterogeneous nuclear RNPs) immediately bind to the newly transcribed mRNAs. To date, *ca.* 20 hnRNP protein families have been identified and proposed to have distinct functions in animals (Han *et al.*, 2010). hnRNPs have some degree of RNA sequence-specificity (Dreyfuss *et al.*, 1993; Singh & Valcárcel, 2005), suggesting that each mRNA is associated with a specific combination of hnRNP proteins. These proteins are important for nucleocytoplasmic transportation of mRNAs and presumably influence mRNA fate in the cytoplasm (Dreyfuss *et al.*, 2002).

Following transcription of the first 20 nucleotides, capping of the 5'-end of the pre-mRNA with a 7-methyl-guanosine (m^7G) is the first pre-mRNA processing event (Neugebauer, 2002). In eukaryotes, the mRNA-capping enzymes are responsible for the triphosphatase, guanylyltransferase and methyltransferase activities. The 5' mRNA capping event augments subsequent pre-mRNA biogenesis processes including splicing by spliceosome complexes (Izaurralde *et al.*, 1994), cleavage and polyadenylation by stabilizing the interaction of the polyadenylation machinery (Cooke & Alwine, 1996), nuclear export by interacting with RNA export factors (Hamm & Mattaj, 1990; Izaurralde *et al.*, 1992, 1995), and mRNA stability by protection of the transcript from 5'→3' exonuclease activity (Balatsos *et al.*, 2006).

Based on sequence similarity, *Arabidopsis* contains two orthologs of guanine-N^7 cap methyltransferase (Bujnicki *et al.*, 2001) and three different bifunctional triphosphatase-guanylyltransferase enzymes with distinct expression patterns (Yamada *et al.*, 2003). The 5'-cap is important for the binding of the nuclear cap-binding complex (CBC) that is critical in the first or "pioneering" round of the translation and transport to the cytoplasm (Merkle, 2010). *Arabidopsis* encodes two orthologs of the small and large CBC proteins, CBC20 and CBC80, respectively (Kmieciak *et al.*, 2002). Mutation of AtCBP80 disturbed the ABA signaling pathway (Hugouvieux *et al.*, 2002), suggesting that 5'-capping and association with CBC80 contributes to ABA signal transduction. Recently, it was shown that mutation of *Arabidopsis* CBC (*cbc20*, *cbc80*, and *cbc20/cbc80* double mutants) resulted in significant changes in alternative splicing profiles of many transcripts in *Arabidopsis* rosette leaves (Raczynska *et al.*, 2010).

Newly transcribed and 5'-capped pre-mRNA contains both coding sequences (exons) and non-coding sequences (introns). The intron must be removed by precise splicing to generate the mature mRNAs. In mammals, the spliceosome, a macromolecular RNP complex required for pre-mRNA splicing, consists of five small nuclear ribonucleoprotein particles (snRNPs), U1, U2, U4, U5 and U6 snRNPs, and several splicing factors (Krämer, 1996). At completion, the splicing machinery marks the exon junction with a protein complex called the exon junction complex (EJC). In mammals, the EJC binding site is about 20-24 nucleotides upstream of the exon/exon junction point (Le Hir *et al.*, 2001) and functions as a binding platform for RBPs involved in mRNA export and NMD (Le Hir *et al.*, 2001; Lykke-Andersen *et al.*, 2001).

Two families of RBPs, hnRNPs and Serine/Arginine-rich proteins (SRPs) participate in splicing regulation. Typically, SRPs contain N-terminal RRM and C-terminal Arginine/Serine rich domain. In mammals, SRPs interact with the spliceosome, facilitating recruitment of U1 and U2 snRNPs to pre-mRNAs, whereas, hnRNPs inhibit early steps of spliceosome assembly (Singh & Valcárcel, 2005).

In plants, the splicing process and intron/exon organization are similar to that of other eukaryotes, with a few exceptions. These include a plant-specific branch point sequence, generally shorter intron length, and higher UA-rich intronic elements (Lorković *et al.*, 2000b). In *Arabidopsis* protoplasts, the over-expression of the hnRNP-like UBP1 protein, a member of U-rich binding protein family (UBPs), was shown to

enhance splicing of poorly spliced introns suggesting that UBPs may function in pre-mRNA splicing (Lambermon *et al.*, 2000).

A study of *Arabidopsis* nucleolar proteomes identified several putative pre-mRNA splicing proteins with human and yeast orthologs, suggesting that plants operate similar mechanisms of transcript processing (Bae *et al.*, 2003; Brown *et al.*, 2005; Pendle *et al.*, 2005). However, many SRPs appear to be plant specific (Lorković *et al.*, 2000b), and are proposed to regulate alternative splicing processes (Barta *et al.*, 2008). It has been shown that environmental stresses regulate the alternative splicing pattern of some SR proteins and the changes of SRP concentration and isoforms may also affect the alternative splicing of downstream target genes (Reddy, 2007). Recently, Filichkin *et al.* (2010) utilized high-throughput RNA sequencing technology to study genome-wide alternative splicing in *Arabidopsis* and showed that alternative splicing enhances transcriptome plasticity under high-light, heat, cold and high-salinity conditions. In addition, they showed that the *AtSRP30* transcripts undergoes alternative splicing, resulting in a shift of AtSRP30 protein isoforms under different stress conditions. However, the detailed mechanisms orchestrating this network of alternative splicing remains to be illuminated.

The formation of a mature mRNA also requires the cleavage and polyadenylation of the 3'-end of the transcript, which provides protection from degradation and aids translation (Gray *et al.*, 2000). In mammals, the first step of polyadenylation is the cleavage of the pre-mRNA at a site located downstream of a

canonical AAUAAA sequence where a Cleavage and Polyadenylation Specificity Factor (CPSF) binds (Neugebauer, 2002). The second step is binding of a downstream U+G rich region by a Cleavage Stimulatory Factor (CStF) (Neugebauer, 2002). The 3'-end cleavage is performed by Cleavage Factors I and II (CFI and CFII), and polyadenylation is performed by Poly (A) Polymerase (PAP) bound to CPSF and the Nuclear Poly (A)-Binding Protein (PABN) which controls the processivity of PAP (Neugebauer, 2002). PABN remains associated with the poly(A) tail of the mRNA until the nucleocytoplasmic translocation of the mRNP is completed. Finally, Cytoplasmic Poly(A) binding protein (PABC) binds to the poly(A) tail to promote translation by recruiting translational initiation factors and 40S ribosome subunit (Gray *et al.*, 2000)

In plants, the conserved AAUAAA sequence can be present but is not required for polyadenylation (Li & Hunt, 1997; Hunt, 2008). Plant polyadenylation signals consist of a tripartite combination of *cis*-elements: a Far Upstream Element (FUE), one or more Near Upstream Element (NUE), and the polyadenylation Cleavage Site (CS). Moreover, a downstream U+G rich region is not conserved in plants (Li & Hunt, 1997). Many plant RBPs have been proposed to regulate pre-mRNA cleavage and polyadenylation, although, the composition of the complexes that carry this out remain poorly characterized (Belostotsky & Rose, 2005; Hunt *et al.*, 2008).

Post-transcriptional control may be regulated by alternative polyadenylation site selection. Alternative polyadenylation usually affects mRNA translation or mRNA stability, moreover, it can result in change of the carboxyl terminal amino acid sequence

and protein function (Hunt, 2008). Two lines of evidence indicate that alternative polyadenylation impacts gene expression in plants. First, the *Arabidopsis* polyadenylation factor, FY regulates alternative polyadenylation of *FLOWERING TIME CONTROL* (*FCA*) mRNA (Quesada *et al.* 2003; Simpson *et al.* 2003). Second, there is pollination-induced mRNA poly(A) tail-shortening of tobacco (*Nicotiana tabacum*) transmitting tissue-specific mRNAs (Wang *et al.*, 1996). Future studies should consider variation in poly(A) sites and tail lengths at the 3'-ends of individual mRNAs and their effects on gene regulation. This information can be delivered by long-read high-throughput RNA sequencing technology.

1.3 The ribosome - a dynamic cytoplasmic mRNP contributing to a gene regulation

The most well characterized catalytically active RNP of the plant cytoplasm is the 80S ribosome, comprised of a large (60S; 2.01 MDa) and small (40S; 1.6 MDa) subunit (Chang *et al.*, 2005). The plant ribosome resembles those of other eukaryotes in its structural architecture (Verschoor *et al.*, 1996) and components (Chang *et al.*, 2005; Giavalisco *et al.*, 2005; Manuell *et al.*, 2005; Carroll *et al.*, 2008). The 60S subunit consists of 25-26S, 5.8S and 5S rRNAs and an estimated 42 ribosomal proteins (RPs), whereas the 40S subunit consists of the 18S rRNA and 32 RPs (Table 1.1). RPs are generally basic proteins that assemble into pre-ribosomes in the nucleolus. After ribosomes are transported to the cytoplasm the ~12 kDa acidic P-proteins (RPPs), designated P1, P2, and P3, bind P0 of the 60S subunit to form a motile stalk (Szick-Miranda & Bailey-Serres, 2001), which enhances the translocation step of protein

synthesis (Gonzalo & Reboud, 2003). A recent study by (Li *et al.*, 2010) shows that the motile stalk attracts eEF2 and protects the ribosome from depurination by ricin chain A, a ribosome inactivating protein.

The receptor of activated kinase (RACK1) also joins the 40S subunit in the cytoplasm (Chang *et al.*, 2005). This WD-domain protein is a scaffold for translational regulators in eukaryotes (Sengupta *et al.*, 2004). Despite the remarkable conservation in their structure and function, plant ribosomes are heterogeneous due to an array of posttranslational modifications of RPs, variable presence of the 12 kDa RPPs and RACK1, and the co-expression of gene paralogs that encode non-identical RPs (Chang *et al.*, 2005). The functional significance of this heterogeneity in translational regulation needs to be explored.

1.3.1 The role of the ribosome in different phases of translation

In plants, the ribosomal subunits participate in the three phases of translation: initiation, elongation and termination (Bailey-Serres, 1999; Kawaguchi & Bailey-Serres, 2002; Gallie, 2004). As the secondary structure at the 5'-untranslated region is relaxed, the 43S complex (40S subunit and the eIF2α-GTP-tRNA^{met} ternary complex) scans from the 5'-end of the mRNA until an AUG codon is found in the correct context (Kawaguchi & Bailey-Serres, 2002). The 60S subunit joins the 40S subunit at the selected AUG codon to begin peptidyl transferase and translocation activities required for polypeptide synthesis (Figure 1.2b). A polyribosome (polysome) of two or more ribosomes threaded on a single mRNA is generated by sequential recruitment of ribosomes. This large mRNP

can be stabilized with cycloheximide and separated from free-ribosomal subunits and 80S monoribosomes by differential centrifugation of cell extracts through a sucrose gradient (Figure 1.2a). This method is routinely used to monitor the translation state of individual mRNAs, which is independent of message abundance. If the elongation or termination is impaired, then the number of ribosomes per mRNA can increase due to ribosome pausing and stacking within the coding sequence. Although there are examples of selective inhibition of elongation and/or termination in plants (Bailey-Serres, 1999; Kawaguchi & Bailey-Serres, 2002), including the recent demonstration of a nascent polypeptide that impairs elongation in a metabolite dependent manner (Onouchi *et al.*, 2005, 2008), most cases of translation control involve regulation during the initiation phase. Examples include adjustment of translation of individual mRNAs during development and in response to cues (see Section 1.3B). If the initiation or re-initiation rate of ribosomes on a transcript is reduced, the mRNA will shift into smaller polysomes or non-polysomal mRNP complexes. The rate of translation of individual mRNAs has not been measured *in planta* but might be assessed as accomplished for yeast (*Saccharomyces cerevisiae*) (Ingolia *et al.*, 2009). In that report, the position and spacing of ribosomes on individual mRNAs was determined by digesting polysomes with nuclease, followed by high-throughput sequencing and mapping of the circa 30 nt ribosome-protected fragments along individual gene transcripts.

1.3.2 Changes in the association of mRNAs with ribosomes in response to environmental stimuli

Change in regulation of mRNA translation occurs in response to environmental stimuli and growth-regulatory substances as well as during development (Bailey-Serres, 1999). Light, the most important parameter that controls growth and development of plants and other photosynthetic organisms, has been shown to involve several aspects of gene regulation, including post-transcriptional control. Early studies showed that in golden amaranth (*Amaranthus hypochondriacus*) the expression of *ribulose 1,5-bisphosphate carboxylase* small and large subunit genes were regulated at the translational level in response to a change in illumination (Berry *et al.*, 1986, 1988). In addition, light and circadian cycles also affect mRNA stability, as shown by time-course microarray analysis of transcripts from *Arabidopsis* seedlings treated with the transcription inhibitor cordycepin (Gutiérrez *et al.*, 2002). In transgenic tobacco (*Nicotiana tabacum*), heterologous pea *Ferredoxin-1* (*Fed-1*) mRNAs were stabilized and actively translated in the light, whereas, darkness induced polysome transcript dissociation and *Fed-1* mRNA degradation (Petracek *et al.*, 1997). Dickey *et al.* (1998) demonstrated that the change in pea *Fed-1* mRNA abundance and ribosome association in response to light availability required an element in the 5'-untranslated region of the message. Following this work, Tang *et al.* (2003) showed that darkness induced the dissociation of several nuclear-encoded chloroplast mRNAs from the ribosomes in tobacco leaves, including *Light Harvesting Complex A4* (*LHCA4*), *Photosystem A Subunit K* (*PSAK*), and *Chloroplast Protein 12* (*CP12*). Together, these findings provide

strong evidence that light availability modulates expression of some nuclear-encoded chloroplast genes by regulation of mRNA stability and translation. However, to date, there has no genome-level study of mRNA translation in response to changes in light availability, such as an unanticipated shift to darkness followed by re-illumination.

Recently the engagement of individual mRNAs with polysomes was successfully quantified at the genomic level in *Arabidopsis* (Kawaguchi *et al.*, 2004; Kim *et al.*, 2004; Branco-Price *et al.*, 2005, 2008; Nicolai *et al.*, 2006; Mustroph *et al.*, 2009; Matsuura *et al.*, 2010) (Table 1.2). The pool of mRNAs associated with ribosomes was analyzed by using DNA oligonucleotide microarrays to quantify mRNAs in the non-polysomal and polysomal fractions or total and polysomal fractions. These studies provided information on the translation state of individual mRNAs, more specifically quantitation of the relative amount of a message associated with polysomes. Several of these studies considered the effect of growth conditions on translation status. The profiling of mRNAs in polysomal mRNPs under suboptimal growth conditions including dehydration, hypoxia, sucrose starvation, and salinity confirmed that the translation of individual mRNAs is highly regulated in *Arabidopsis* (Kawaguchi *et al.*, 2004; Branco-Price *et al.*, 2005, 2008; Nicolai *et al.*, 2006; Mustroph *et al.*, 2009; Matsuura *et al.*, 2010). An important finding was that gene expression could be severely limited through translational control. It should be noted that the effect of cold temperature on genome-wide translational control in higher plants has not yet been examined.

The repression of translation of a sub-set of cellular mRNAs was particularly evident in the response of seedlings to brief or prolonged hypoxia, which reduced levels of ~70% of cellular mRNAs in large polysomes or epitope-tagged ribosomes (Branco-Price *et al.*, 2005, 2008; Mustroph *et al.*, 2009) (Figure 1.4). A similar suppression of translation was confirmed when the ratio of polysomal versus non-polysomal mRNA was evaluated in rosettes following mild dehydration (Kawaguchi *et al.*, 2004; Kawaguchi & Bailey-Serres, 2005). Remarkably, only half of the mRNAs that increased at the level of steady-state abundance in response to either stress concomitantly increased in polysomes. Thus, a rise in transcript abundance in response to a cue does not guarantee increased synthesis of the encoded protein. In the case of hypoxia, a number of mRNAs induced at the steady-state level were not recruited onto polysomes until re-oxygenation, indicating that cells are primed for recovery from stress (Branco-Price *et al.*, 2008).

1.3.3 Ribosome activity and energy status

The differential translation of cellular mRNAs leads to discordance between the steady-state abundance of mRNAs and the proteins they encode in yeast (MacKay *et al.*, 2004) and mammalian cells (Conrads *et al.*, 2005; Bianchini *et al.*, 2008). It can be hypothesized that differential mRNA translation is responsible for some of the disparity observed between levels of mRNAs, active enzymes and metabolites of central carbon and nitrogen metabolism in leaves of *Arabidopsis* rosettes over the diurnal cycle (Gibon *et al.*, 2006). Levels of protein synthesis reflect cellular energy status, since polysome levels parallel ATP content in *Arabidopsis* seedlings exposed to hypoxia and re-

oxygenation (Branco-Price *et al.*, 2008). The repression of translation during hypoxia, dehydration and sucrose starvation is not universal; it affects mRNAs encoding proteins of processes that are pertinent to stress acclimation, such as the abundant mRNAs of the protein synthesis machinery (*i.e.* RPs and eukaryotic elongation factors (eEFs)) and cell wall components (Kawaguchi *et al.*, 2004; Branco-Price *et al.*, 2005; Kawaguchi & Bailey-Serres, 2005; Nicolai *et al.*, 2006). In the case of hypoxia, most of the poorly translated mRNAs were stable and returned to polysomes upon re-oxygenation. Recently, Piques *et al.* (2009) showed that light availability and the circadian clock affect the steady-state accumulation and ribosome-association of mRNAs encoding enzymes for central metabolism, suggesting that the light-mediated energy balance contributes to post-transcriptional regulation of gene expression. Thus, discontinuity in mRNA accumulation and translation can result from an economization of energy through the selective repression of translation of mRNAs encoding proteins destined for energy demanding cellular processes.

The dramatic increase in monosomes during these energy-limited stresses indicates that the repression largely occurs at the initiation phase of translation. Translation is regulated during an energy crisis in animal cells by signal transduction from AMP kinase via Target of Rapamycin (TOR) to p70 S6 kinase (S6k), which modifies phosphorylation of eukaryotic initiation factor (eIF) 4E-binding protein, eIF4B, eEF2, and ribosomal protein small subunit 6, RPS6, culminating in differential mRNA translation (Proud, 2007). Plants possess TOR and S6k orthologs; and abiotic stresses affect the phosphorylation of translation factors and RPS6 (Bailey-Serres, 1999;

Kawaguchi & Bailey-Serres, 2002; Williams *et al.*, 2003). The recognition of plant SnRK1 (KIN10/11) kinases as the functional complement of AMP kinases in the sensing of cellular energy status (Baena-Gonzalez *et al.*, 2007; Baena-González, 2010; Reinbothe *et al.*, 2010) has raised the possibility that they participate in regulation of differential translation of certain mRNAs through a TOR and S6k-mediated pathway (Branco-Price *et al.*, 2008; Hummel *et al.*, 2009) (Figure 1.1).

1.4 Involvement of RBPs in initiation of translation

With few exceptions, plant mRNAs are monocistronic and possess a 5'-m^7GpppN-cap and a 3'-polyadenylated (poly(A)) tail that are juxtaposed through interactions between proteins that bind each terminus (Bailey-Serres, 1999; Kawaguchi & Bailey-Serres, 2002; Williams *et al.*, 2003) (Figure 1.2c). Plants have two distinct cytosolic cap-binding complexes (*i.e.* eIF4F and eIF(iso)4F), comprised of the related cap-binding proteins (eIF4E or eIF(iso)4E) and two highly divergent scaffold proteins (eIF4G or eIF(iso)4G). Both cap-binding complexes stimulate the *in vitro* translation of unstructured mRNAs, but eIF4F more effectively enhances the translation of mRNAs with a structured 5'-terminus or no 5'-cap (Gallie & Browning, 2001). The cap-binding complex associates with the RNA helicase eIF4A and the RRM-containing protein eIF4B, which together remove secondary structures in the 5'-leader to promote scanning of the pre-initiation complex (Cheng & Gallie, 2006; Cheng *et al.*, 2008).

The poly(A) binding protein (PABP) is an RRM-containing RBP that binds in multiple copies to the 3'-tail of the transcript and forms a bridge to the 5'-end through

binding to either eIF4G or eIF(iso)4G, as well as to eIF4B (Cheng & Gallie, 2007). This multifaceted interaction between proteins and mRNA termini results in a circular conformation that stimulates the primary initiation and subsequent re-initiation events (Bailey-Serres, 1999; Le *et al.*, 2000; Kawaguchi & Bailey-Serres, 2002; Belostotsky, 2003; Williams *et al.*, 2003). Plant PABPs have diversified relative to those of other model eukaryotes, leading to the suggestion that temporal and spatial distinctions in PABP expression contribute to the regulation of mRNA biogenesis, export and translation (Bravo *et al.*, 2005). For example, some PABPs possess a C-terminal motif (PABC) that binds PABP-interacting motif 2 (PAM2) proteins (Wang & Grumet, 2004; Siddiqui *et al.*, 2007). It is not known if a PAM2-PABP interaction *in planta* disrupts the circularization of the mRNA, however the conserved stress-induced PAM2 protein, Enhanced Dehydration Response 15 (ERD15) of cucumber (*Cucumis sativus*) inhibits translation of poly(A)-tailed mRNAs *in vitro* if the PAM2 motif has not been mutated (Rangan *et al.*, 2008). Plants possess several conserved PAM2 proteins with RRM or LSM domains of RBPs that deserve further study (Wang & Grumet, 2004). This diversity in cytosolic cap-binding, PABP and PAM2 proteins raises questions about their roles in differential mRNA translation.

1.5 Processing bodies and stress granules

Once nuclear post-transcriptional processing is completed the mRNA is transported to the cytoplasm where it is translated. In mammalian cells, cytoplasmic mRNAs are usually found in three different mRNP complexes; polysomes, Processing

Bodies (P-bodies: PBs) and Stress Granules (SGs) (Balagopal & Parker, 2009). PBs and SGs are dynamic points of cytoplasmic mRNA sorting, storage, and degradation (Kedersha *et al.*, 2005; Decker *et al.*, 2007) (Figure 1.1 and Figure 1.2b). PBs usually contain translationally repressed mRNAs and associate with the cytoplasmic decapping machinery including 5'-m^7GpppN decapping enzymes (DCPs), 5'→3' exonuclease (XRN1), and other regulators of mRNA decay (Table 1.1). The interaction between *Arabidopsis* DCP1 and DCP2 is accompanied by a scaffold protein VARICOSE (VCS) that is essential for regulation of post-embryonic development (Xu *et al.*, 2006; Goeres *et al.*, 2007). In addition, Goeres *et al.* (2007) demonstrated the physical interaction between DCP2 and VCS and confirmed an interaction between *Arabidopsis* XRN4, the ortholog of human XRN1, and both DCP2 and DCP1 by bimolecular fluorescence complementation in protoplasts of *Nicotiana plumbaginifolia*. Weber *et al.* (2008) showed the accumulation of fluorescently-tagged DCP1 was induced by heat shock and hypoxia suggesting the formation of PBs is dynamic and involved in the translational down-regulation of mRNAs. Moreover, the same group also reported that in the *xrn4-5* mutant that exhibits reduced cytosolic 5'→3' exonuclease activity, the formation of cytoplasmic foci was disrupted, indicating PBs are important for mRNA degradation (Weber *et al.*, 2008). Zhang *et al.* (2010) recently identified *SUPPRESSOR OF VARICOSE* (*SOV*) containing an RNase II domain and demonstrated that SOV is involved in selective cytoplasmic mRNA decay. The interactions between DCP1, DCP2, VCS, SOV and their relationships in mRNA degradation need to be further explored.

In mammals, SGs function in mRNA sequestration (Kedersha *et al.*, 2005). SG components include TIA1, TIAR, eIF4E, eIF4G, eIF4A, eIF3, PABP, G3BP1, and the small ribosomal subunit (Kedersha *et al.*, 2005). Animal TIA1 is important for SG formation and also functions as a translation silencer, in alternative splicing and as mRNA decay regulator (Del Gatto-Konczak *et al.*, 2000), and nucleocytoplasmic transport (Eisinger-Mathason *et al.*, 2008). The *Arabidopsis* orthologs of TIA1 protein, UBP1 (A-C), RBP45 (A-D) and RBP47 (A-C, C') (Table 1.1) were proposed to regulate pre-mRNA processing and stability (Lambermon *et al.*, 2000, 2002; Lorković *et al.*, 2000a). UBP1 stimulates pre-mRNA splicing of low efficiency introns (Lambermon *et al.*, 2000), can translocate to the cytoplasm and is incorporated in SGs under heat stress (Weber *et al.*, 2008). The dynamic formation of SGs and PBs in plant cells emphasizes that both the storage as well as the turnover of transcripts is highly regulated, particularly in response to sub-optimal growth conditions.

1.6 Cytosolic RBPs that function in post-transcriptional regulation in response to changes in light availability and cold temperatures

A number of RBPs play a role in regulation of individual or cohorts of mRNAs in response to environmental variables, including the alteration of light and cold stress. Plants overcome disparity in production and consumption of energy in response to rapid fluctuations in light by quickly adjusting light harvesting capability (Allen, 2003). The selective translation of mRNAs encoding photosynthetic machinery in the cytoplasm and chloroplast is important for this photo-acclimation (Pogson *et al.*, 2008). Early

studies revealed that light controls the abundance, stability and translation of a number of nuclear-encoded transcripts (reviewed by Bailey-Serres, 1999; Kawaguchi & Bailey-Serres, 2002). In the algae *Chlamydomonas reinhardtii*, the RBP NAB1 was found to transiently sequester *Light Harvesting Complex* (*LHCBM*) mRNAs under high light conditions (Mussgnug *et al.*, 2005). NAB1 contains an RRM and an evolutionarily conserved Cold Shock domain (CSD), characteristic of bacterial cold responsive proteins (CSPs) that bind nucleic acid and chaperone RNAs. Although a CSP similar to NAB1 has not been found in higher plants, *Arabidopsis thaliana* encodes four CSPs that additionally contain Glycine-rich (GR) and zinc finger domains and purportedly function as RNA chaperones during cold acclimation (Lorković & Barta, 2002; Karlson *et al.*, 2002; Kim *et al.*, 2005; Nakaminami *et al.*, 2006).

1.6.1 *Arabidopsis* Cold Shock Proteins (CSPs)

In *Arabidopsis*, Cold Shock Proteins (CSPs 1-4 encoded by At4g36020, At4g38680, At2g17870, and At2g21060, respectively) contain a highly conserved N-terminal CSD. These proteins also have GR regions and two (CSP2 and CSP4) or seven (CSP1 and CSP3) $Cys(X)_2Cys(X)_4His(X)_4Cys$ (CCHC)-zinc finger domains (Figure 1.3).

The CSD belongs to the oligosaccharide/oligonucleotide (OB) fold superfamily, a single stranded DNA/RNA binding domain widely recognized in eukaryotic and prokaryotic species (Murzin, 1993). OB-fold proteins are involved in diverse cellular processes of transcriptional and post-transcriptional regulation (Sommerville, 1999). Although the function of the CSD in nucleic acid binding is well

characterized, the functional roles of GR and CCHC-zinc finger motifs have not been well established. Study of the *Drosophila melanogaster* Nanos protein, a CCHC zinc finger containing RBPs, revealed that the CCHC zinc-finger domain binds RNA *in vitro* with high affinity, but with little sequence specificity (Curtis *et al.*, 1997). In *Trypanosoma cruzi*, the poly-zinc finger protein PZFP1 contains seven CCHC-zinc finger motifs, interacts with DNA/RNA oligonucleotides, localizes in both the cytoplasm and nucleus, and interacts with specific SR proteins in a yeast two-hybrid assay (Espinosa *et al.*, 2003). These findings indicated that CCHC-zinc finger motif is important for nucleic acid binding.

In addition to the CSD family, a related-group of RBPs that possesses an S1 RNA binding domain (S1-D as contained in bacterial ribosomal protein S1) has been included as members of the OB-fold superfamily (Sommerville, 1999). In *Arabidopsis*, the S1 domain containing proteins are found in exosome complexes and participate in RNA degradation (Zhang *et al.*, 2010)

Bacterial CSPs are reported to function as RNA chaperones. Their function is to convert double stranded RNA to single stranded RNA with low sequence specificity (Jiang *et al.*, 1997), thereby stimulating growth and accommodating cold acclimation. Recently, *Bacillus subtilis* CSPs were found to function along with cold-inducible DEAD box RNA helicases (CshA and CshB) to unwind mis-folded mRNA molecules and control translation initiation (Hunger *et al.*, 2006). Similar to bacterial CSPs, eukaryotic CSD/S1 containing proteins (for example, YB-1, Lin-28, UNR, RRP40, RRP44, eIF1A,

eIF2α, eIF5A, and plant CSPs) have been shown to be important in post-transcriptional regulation including mRNA processing, degradation and translation (Mihailovich *et al.*, 2010).

The accumulation of *AtCSP* transcripts is induced by low temperature (Karlson *et al.*, 2002) leading to the suggestion that CSPs may function in adaptation to the cold. Heterologous expression of *AtCSP1* and *AtCSP3* in the cold sensitive *Escherichia coli* (*E. coli*) BX04 mutants that lacks four CSDs, rescues the cold sensitive phenotype (Kim *et al.*, 2007b, 2009). This has lead to the suggestion that CSP1 and CSP3 functionally complement bacterial CSPs. However, expression of *AtCSP2* in the BX04 mutant did not enhance cold tolerance (Kim *et al.*, 2007b). In *Arabidopsis*, the over-expression of *AtCSP1* or *AtCSP2* delayed or accelerated seed germination under high-salinity or drought conditions (Park *et al.* 2009). Moreover, it can rescue a cold-sensitive *atgrp7-1* mutant from freezing damage (Park *et al.*, 2009). In addition, an *atcsp3-2* mutant was sensitive to freezing as compared to wild-type plants under cold acclimated and non-acclimated conditions, whereas *AtCSP3* over-expression conferred a freezing resistant phenotype (Kim *et al.*, 2009).

In addition to their apparent roles in the response to cold stress, *AtCSP2* and *AtCSP4* transcripts accumulate in developing embryos, shoot apices and during floral induction (Fusaro *et al.*, 2007; Sasaki *et al.*, 2007; Nakaminami *et al.*, 2009), suggesting they function during growth and development. Together, these findings suggest CSPs act

under both stress and non-stress conditions, although the precise roles they play remain obscure.

1.6.2 Plants Glycine Rich-RNA Binding Protein (GR-RBPs)

A group of proteins related to plant CSPs, the GR-RBPs, lack the CSD but possesses GR and RRM motifs. The *Arabidopsis* genome encodes eight GR-RBPs (GR-RBPs 1-8, At2g16260, At4g13850, At5g61030, At3g23830, At1g74230, At1g18630, At2g21660, At4g39260, respectively) (Lorković & Barta, 2002; Wang & Brendel, 2004) and three additional related GR-RBP proteins, AtRZ-1a (At3g26420), AtRZ-1b (At1g60650), and AtRZ-1c (At5g042800) (Figure 1.3). The AtRZ-1 family is related to mammalian hnRNP-G proteins (Wang & Brendel, 2004), in which function in pre-mRNA processing. The roles of plant GR-RBPs and AtRZ-1s have been linked to regulation of stress responses, including low temperature stress (Lorković & Barta, 2002; Chinnusamy *et al.*, 2007; Zhu *et al.*, 2007; Kim *et al.*, 2010), although their role in regulating specific cohorts of transcripts is unknown.

1.7 The relationship of *miRNA* roles to translation or storage of their target mRNAs in plants

Another group of RBPs that function in post-transcriptional gene regulation within the cytoplasm are those involved in *miRNA*-mediated gene silencing. *miRNA*s integrate into the mRNP called RISC (RNA-Induced Silencing Complex) that contains the catalytic Argonaute (AGO) and RNase H, and other proteins (Table 1.1). It is well established that *miRNA*-mediated gene silencing in plants involves slicing and

degradation of the target mRNA (Voinnet, 2009). However, there are examples of *miRNA*s that do not destabilize their mRNA target(s) but limit production of the gene product (Figure 1.1). An example is *Arabidopsis miR172*, which reduces accumulation of AP2 protein but not its mRNA (Aukerman & Sakai, 2003; Chen, 2004). It should be noted that recent report has shown that mir172 can target other AP2-like mRNAs such as *SCHLAFMU¨ TZE (SMZ)* or *SCHNARCHZAPFEN (SNZ)* mRNAs to cleavage and degradation (Mathieu *et al.*, 2009). Repression of gene expression downstream of mRNA accumulation has also surfaced for *Arabidopsis miR156/157* and *miR854* (Arteaga-Vázquez *et al.*, 2006; Gandikota *et al.*, 2007). In animals, *miRNA*s typically bind the 3'-UTR of their target in imperfect duplexes present in one or more copies and inhibit or in unusual cases stimulate translation (Vasudevan *et al.*, 2007). Translational inhibition usually requires a 5'-m^{7G}pppN-cap on the target mRNA and can be facilitated by a 3'-poly(A) tail; the inhibition is mediated by competition between AGO and eIF4E for binding to the 5'-cap (Meister, 2007; Filipowicz *et al.*, 2008).

Four lines of evidence indicate that silencing can occur at the level of mRNA activity in plants. First, *miR172*, *miR156/157* and *miR854* targets remain at high levels, despite limited protein production. These mRNAs are weakly uncapped compared to other *miRNA* targets in inflorescence tissue (Jiao *et al.*, 2008), although the significance of this is unknown. Second, the *miRNA-action deficient* (*mad*) mutants, *mad5* and *mad6*, are functional in *miRNA*-mediated mRNA slicing but are defective in attenuating production of the protein encoded by the targets of *miR156* and other *miRNAs* (Brodersen *et al.*, 2008). Third, similar to animals the decapping complex scaffold protein VCS is

required for slicing-independent *miRNA*-mediated silencing (Brodersen *et al.*, 2008). Finally, Jiao and Meyerowitz (2010) utilize immunopurified polysome RNP complexes and deep-sequencing to demonstrate that most miRNA targets, including AP2 transcripts, are weakly translated. These observations support the conclusion that plant *miRNAs* can act via a mechanism other than mRNA slicing and degradation, but it remains to be clarified whether this involves a direct inhibition of translation and/or sequestration of the target mRNA. This intricate and dynamic relationship deserves further scrutiny since some animal *miRNAs* are associated with RBPs found in PBs, such as GW182, but their targets are not necessarily degraded (Filipowicz *et al.*, 2008).

1.8 Role of RBPs in intra- and intercellular trafficking of mRNAs

The intracellular cytoskeleton can transport polysomes and RNP complexes to subcellular domains, for example a particular region of ER (Figure 1.1) (Muench & Park, 2006). The study of rice RBP, OsTudor-SN, proved the association of this RBP with *Prolamine* and *Glutamine* mRNAs by specific sequence motifs (Wang *et al.*, 2008). OsTudor-SN targeted prolamine mRNA along actin filaments and release the mRNA to inclusions in the lumen of the ER (Choi *et al.*, 2000). A Second example is the *Arabidopsis* RBP phragmoplastin-interacting protein, PHIP1, which can bind *Ran2* mRNA, interact with Rop1 and Ran2, and may involve in the cell plate formation during cytokinesis (Ma *et al.*, 2008). Another group of *Arabidopsis* RBPs contained the Pumillo Homology domain (PUM) (AtPUMs 1-26) has been proposed to participate in cytoplasmic and mRNA localization (Tam *et al.*, 2010), based on the localization of

fluorescent-tagged AtPUMs (AtPUM7 (At1g78160), AtPUM8 (At1g22240), AtPUM9 (At1g35730), AtPUM10 (At1g35750), AtPUM12 (At5g56510), AtPUM14 (At5g43110), AtPUM18 (At5g60110), AtPUM23 (At1g73210), and AtPUM24 (At3g16810)). In addition, genomic-level studies in yeast demonstrated that PUMs control cytoplasmic transport of specific mRNAs (Hogan *et al.*, 2008).

It has been shown that plasmodesmata and phloem sap can facilitate cell-to-cell and symplastic mRNAs trafficking. The systemic transport pathways of specific mRNAs and *si*/*miRNA*s co-ordinate physiological process, including regulation of development and stress responses (Kehr & Buhtz, 2008). The first example is the movement of viral RNAs by plant viral movement proteins through plasmodesmata (Lucas, 2006). The second example is the *Cucurbita maxima* phloem RNA binding protein (RBP50), a poly-pyrimidine tract binding protein that transports several mRNAs symplastically within the pumpkin phloem translocation stream (Ham *et al.*, 2009). In addition to mRNAs, phloem sap contains several non-coding RNAs including *miRNA*, siRNA, tRNA , and rRNA (Yoo *et al.*, 2004; Zhang *et al.*, 2009). Future studies are anticipated to unveil additional RBPs and mRNAs that participate in intercellular trafficking of RNAs and to reveal the mechanisms of RNA translocation.

1.9 Conclusions

The plant cell contains a diverse repertoire of mRNPs and RBPs that orchestrate a network of post-transcriptional processes that begins in the nucleus and continues in the cytoplasm. Despite the recognition of hundreds of RBPs encoded by

plant genomes, only a small proportion are recognized components of known RNPs or have assigned biological function. Plant RBPs can be studied by use of routine methods for cellular localization and mutant analysis. The demonstration that cytoplasmic polysome can be immunopurified and the proteins and mRNAs of these complexes have been characterized, raised the prospect of targeted isolation of mRNA-RBP complexes by immunopurification (Figure 1.4)

1.10 Dissertation objectives

The objective of this dissertation research was to further explore dynamics in mRNA translation in response to environmental stimuli in *Arabidopsis thaliana*. The specific goals were:

(1) To determine if selective translation of individual transcripts occurs in response to changes in light availability. Chapter 2 presents a global transcriptome and translatome analysis in seedlings in response to early darkness and re-illumination. The project utilized the method developed for immunopurification of polysome complexes from transgenic *Arabidopsis*. The results provide evidence of selective dampening of translation of mRNAs encoding for chloroplast and protein synthetic machinery in response to early darkness. The translation repression is rapidly reversed by re-illumination. The study included bioinformatics analyses of mRNA sequence features and motif characteristic of light-regulated mRNAs.

(2) To characterize the Arabidopsis Cold Shock Protein complexes. Based on the role of the CSD protein, NAB1, in *Chlamydomonas* and the finding of light-

modulated translation in *Arabidopsis* (Chapter 2), it was hypothesized that CSPs may function in light-regulated mRNA translation in higher plants. In Chapter 3, the cellular fractionation and immunoprecipitation of the transgenic *Arabidopsis* expressing epitope-tagged-CSPs 1-4 were carried out to identify CSP associated proteins in order to begin assigning function to these proteins. Selected protein associations were confirmed by fluorescent co-localization in transgenic transformed *Nicotiana benthamiana* leaf pavement cells. The results suggest that CSPs are involved in diverse post-transcriptional activities including nuclear pre-RNA processing and mRNA translation. Of the four CSPs, CSP1 was the only CSP found in association with polysomes.

(3) To determine the functional role of Arabidopsis CSP1 in post-transcriptional regulation under low temperature stress. In Chapter 4, biochemical and genetic studies showed that CSP1 is a cold-induced polysome-associated RBP that may be involve in cold stress adaptation. A method to isolate CSP1-mRNP complexes was developed that entailed immunopurification with a polyclonal antibody raised against CSP1-specific peptide. The global transcriptome, translatome, and CSP1-regulated mRNAs were quantitatively evaluated in response to cold and non-stress by use of oligo-based DNA microarrays. The results provide molecular evidence that cold-inducible CSP1 preferentially binds to a subpopulation of cellular mRNAs with high G+C 5' in their UTR. The study provides a clear demonstration of an RNA regulon in a plant.

Figure 1.1 RBPs mediate post-transcriptional gene regulation. Transcript biogenesis is tightly coupled with pre-mRNA processing events including 5'-capping, intron splicing and 3'-polyadenylation. RBPs bind to the transcript to form an mRNP. The exon junction (EJC) complex marks the sites of intron removal, whereas other RBPs bind the 5'-cap, 3'-poly(A) tail, and along the mRNA in sequence specific or non-specific manner. Nuclear mRNPs are exported to the cytoplasm and must pass a quality control checkpoint or be subjected to non-sense mediated decay (NMD). The cytoplasmic routing and activity of mRNAs is determined by sequence features, binding of specific RBPs, or characteristics of the nascent peptide, such as a signal peptide. mRNAs and polysomes may be targeted within the cell along microtubules (MT) or actin filaments (AF), or to neighboring cells by passage through plasmodesmata. The initiation of translation is a regulated process that results in the formation of a polysome. Regulation of initiation can shift the balance between actively translating polysomes and large mRNP aggregates, such as stress granules and processing bodies, which are associated with mRNA sequestration or degradation, respectively. Signaling initiated by reduced energy status is hypothesized to inhibit the target of Rapamycin (TOR) and S6 kinase (S6K) pathway and repress translation. Micro RNA (*miRNA*) processing and export involves participation of multiple RNPs. Plant *miRNA* functions in mRNA slicing and degradation, and might also contribute to translational repression. Abbreviations: Ago1, Argonaute1; XRN, exoribonuclease; eIF4E, Eukaryotic initiation factor 4E; ISE2, Increased size exclusion limit 2; nCBPs, Nuclear cap-binding proteins; UBP1, Oligouridylate binding protein 1; PABP, Poly(A) binding protein; RBP47, RNA binding protein 47; RH12, RNA helicase12; VCS, Varicose. (modified from Bailey-Serres *et al.* 2009)

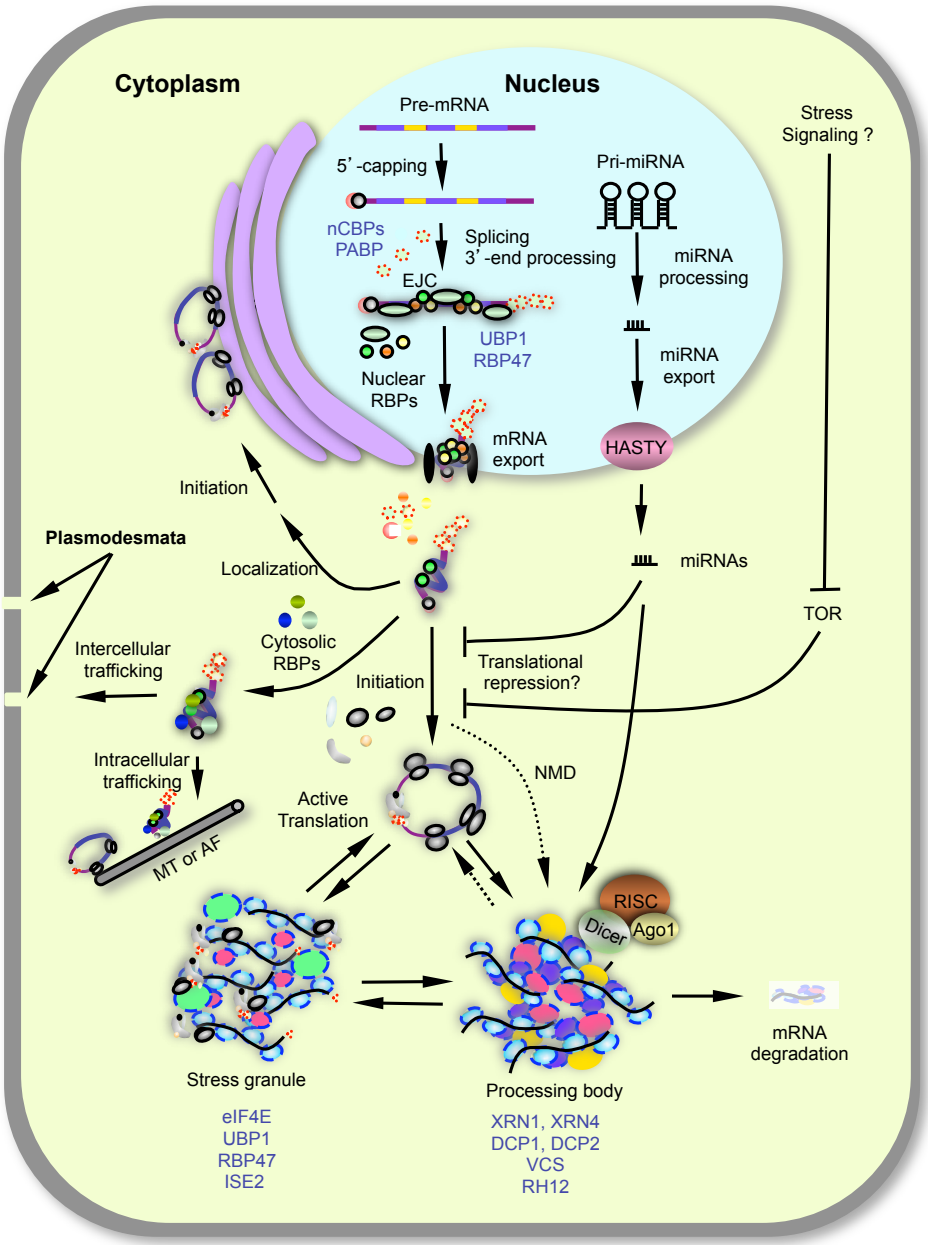
Cytoplasm
Nucleus
Pre-mRNA
5' -capping
nCBPs
PABP
Splicing
3' -end processing
EJC
UBP1
RBP47
Nuclear
RBPs
mRNA
export
Pri-miRNA
miRNA
processing
miRNA
export
HASTY
miRNAs
Stress
Signaling ?
TOR
Initiation
Plasmodesmata
Localization
Cytosolic
RBPs
Intercellular
trafficking
Translational
repression?
Initiation
Intracellular
trafficking
MT or AF
Active
Translation
NMD
RISC
Dicer
Ago1
mRNA
degradation
Stress granule
eIF4E
UBP1
RBP47
ISE2
Processing body
XRN1, XRN4
DCP1, DCP2
VCS
RH12

Figure 1.2 Cytoplasmic mRNPs associated with translation, sequestration and degradation (a) UV absorbance profile of extracts from *Arabidopsis* rosette leaves after centrifugation over a 20-60% sucrose gradient. Non-polysomal region of the gradient (left side) includes mRNPs (mRNA-RBP complexes), 43S pre-initiation, 80S monosome and 80S monosome-mRNA complexes. Polysomal region of the gradient (right side) contains mRNAs associated with multiple ribosomes. (b) States of translation, storage and degradation. Interactions between the 5'-cap/3'-poly(A) tail of the mRNA mediate efficient initiation and reinitiation (recycling) of ribosomes. Dotted arrows propose alterations in translation status due to reduced primary initiation and reinitiation or elongation. Disruption of the 5'-cap/3'-tail interaction leads to fewer ribosomes per mRNA. Impaired elongation, as a result of ribosome pausing, alters the number and spacing of ribosomes per mRNA without increasing polypeptide synthesis. mRNAs can be sequestered in stress granules (SGs) or directed to processing bodies (PBs) where degradation can occur. Proteins found in PBs and SGs are partially overlapping, with 40S subunits found in SGs. *Micro RNAs* (*miRNA*s) might interrupt translation at some stage of initiation or reinitiation and promote sequestration. (c) The translation pre-initiation complex has a circular conformation established by interactions between the proteins at the 5'-cap and 3'-tail of the mRNA. eIF4E (4E) or eIFiso4E binds to eIF4G (4G) or eIFiso4G, and the 5′-7mGpppN-cap of the mRNA. eIF4B binds eIF4A, eIF4G and eIF4E. Poly(A) binding protein (PABP) binds the poly(A) tail in multiple copies, with each molecule protecting approximately 25 nt of RNA. PABP independently binds either eIF4G or eIF4B. The eIF3 complex, ribosomal 40S subunit, and eIF2-GTP-met-tRNA ternary complex are also shown. Abbreviations: 2, eIF2; 3, eIF3; 3h, eIF3h; 4A, eIF4A; 4B, eIF4B. Complexes, proteins and mRNAs are not drawn to scale. (modified from Bailey-Serres *et al.* 2009)

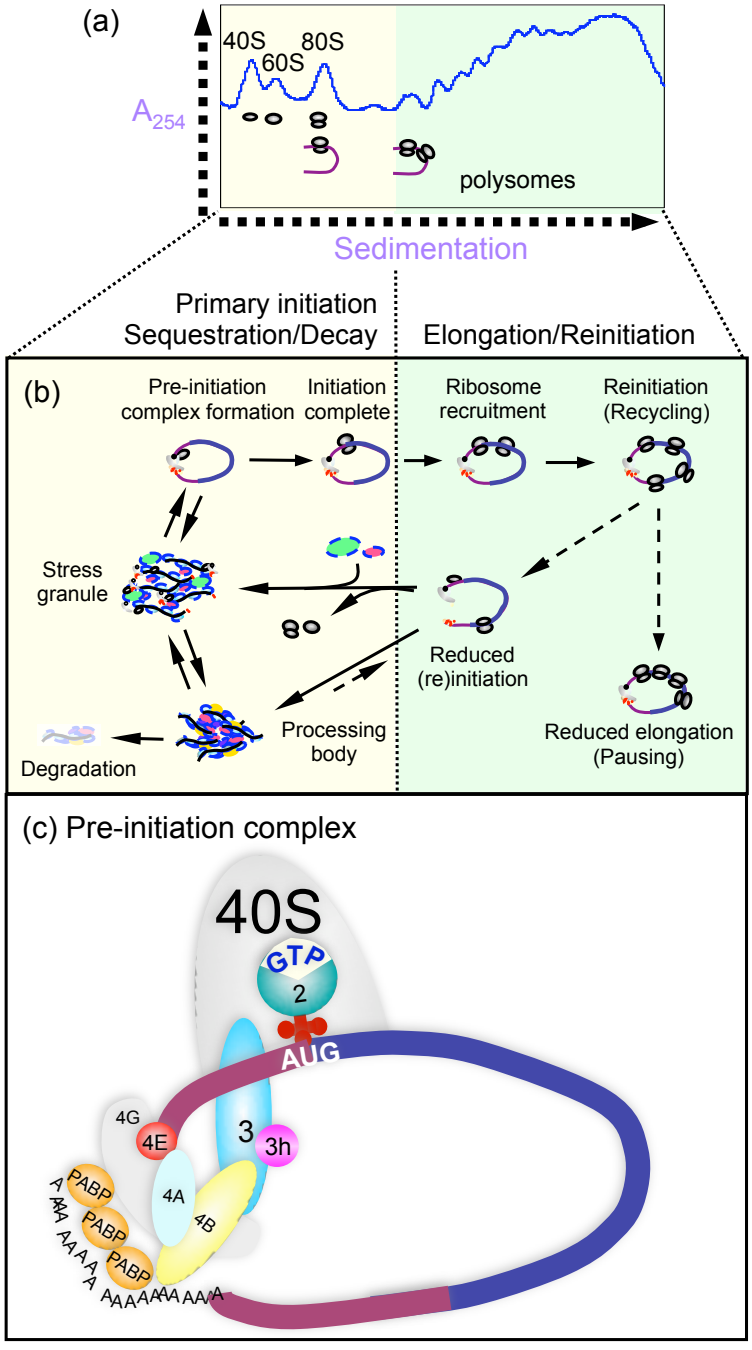
(a)
40S
60S
80S
A_{254}
polysomes
Sedimentation
Primary initiation
Sequestration/Decay
Elongation/Reinitiation
(b)
Pre-initiation
complex formation
Initiation
complete
Ribosome
recruitment
Reinitiation
(Recycling)
Stress
granule
Reduced
(re)initiation
Reduced elongation
(Pausing)
Processing
body
Degradation
(c) Pre-initiation complex
40S
GTP
2
AUG
4G
4E
3
3h
4A
4B
PABP
PABP
PABP

Figure 1.3 The domain structures of *Arabidopsis* CSPs and GR-RBPs. AtCSPs 1-4; At4g36020, At4g38680, At2g17870, and At2g21060. GR-RBPs 1-8; At2g16260, At4g13850, At5g61030, At3g23830, At1g74230, At1g18630, At2g21660, At4g39260. AtRZ-1a; At3g26420, AtRZ-1b; At1g60650, and AtRZ-1c; At5g042800. Domains are not drawn to scale.

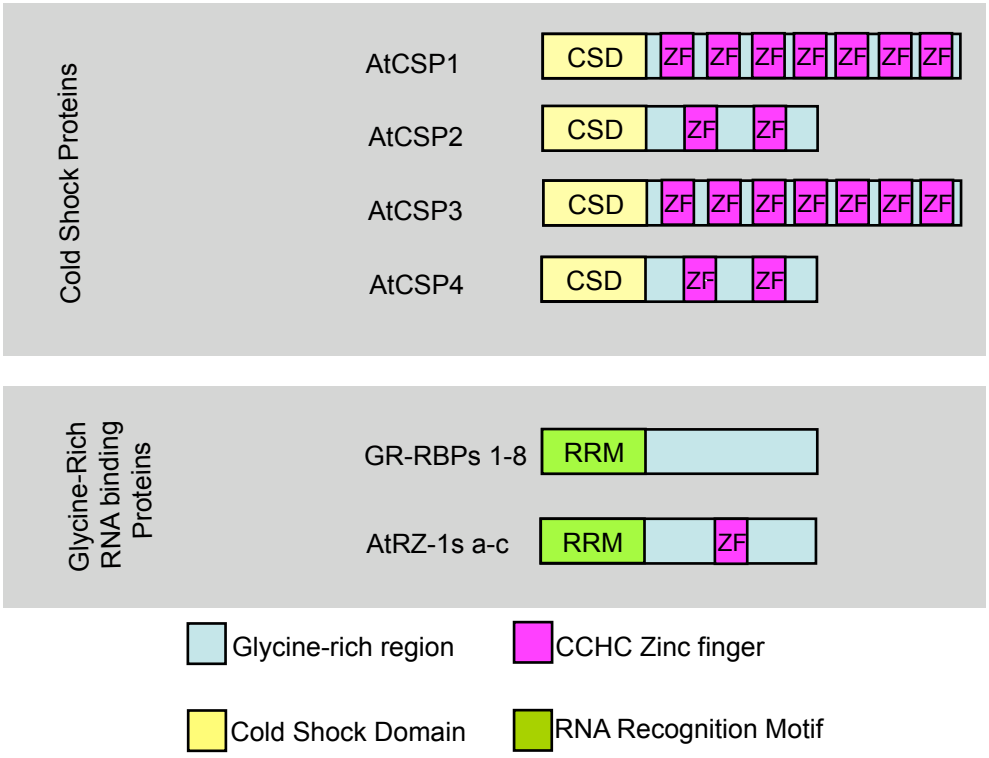

Figure 1.4 Immunopurification of ribosomes and other RNPs for transcript and proteome analyses. Conventional fractionation of ribosome and mRNP complexes involves tissue homogenization in buffer containing detergents to dissociate ribosomes from the ER and cytoskeleton, cycloheximide to inhibit ribosome release, and magnesium to stabilize subunit interactions, followed by centrifugation of clarified cell extracts over a sucrose density gradient (See Figure 1.1). A simplified ribosome purification method was achieved by engineering a 24 amino acid (2 kDa) epitope tag (FLAG) on the amino terminus of *Arabidopsis* RPL18, a protein located on the periphery of the 60S subunit distant from the subunit interaction site, polypeptide and mRNA tunnels. Tissue of transgenic seedlings expressing the FLAG-epitope tagged RPL18 can be used for immunoprecipitation of tagged ribosomes of similar mass and mRNA complexity as those isolated by conventional procedures (Zanetti *et al.*, 2005). The proteome of these complexes include RPs, translation factors, and several RBPs. The purification of mRNAs by this method is highly reproducible (Hogan *et al*., 2008). The use of developmentally regulated promoters for FLAG-RPL18 expression facilitates isolation of mRNA-ribosome complexes from specific types of cells (Mustroph *et al.*, 2009; Jiao & Meyerowitz, 2010). The use of ribosome immunopurification for translatome analyses has also been reported for mice (*Mus musculus*) (Doyle *et al.*, 2008; Heiman *et al.*, 2008) and yeast (Halbeisen & Gerber, 2009) The method of immunopurification of RNPs can be extended to decipher roles of uncharacterized RBPs, as accomplished for a diverse group of RBPs of yeast . Alternatively, antisera against a specific RBP can be used to immunopurify mRNA-protein complexes, as demonstrated for maize (*Zea mays*) plastid RBPs (Schmitz-Linneweber *et al.*, 2005). Future characterization of plant RBPs and their passenger mRNAs can be accomplished through use of similar approaches to immunopurify specific mRNP complexes. In some cases, mRNA-RBP interaction might need to be stabilized by UV or chemical cross-linking prior to immunoprecipitation. (modified from Bailey-Serres *et al.*, 2009)

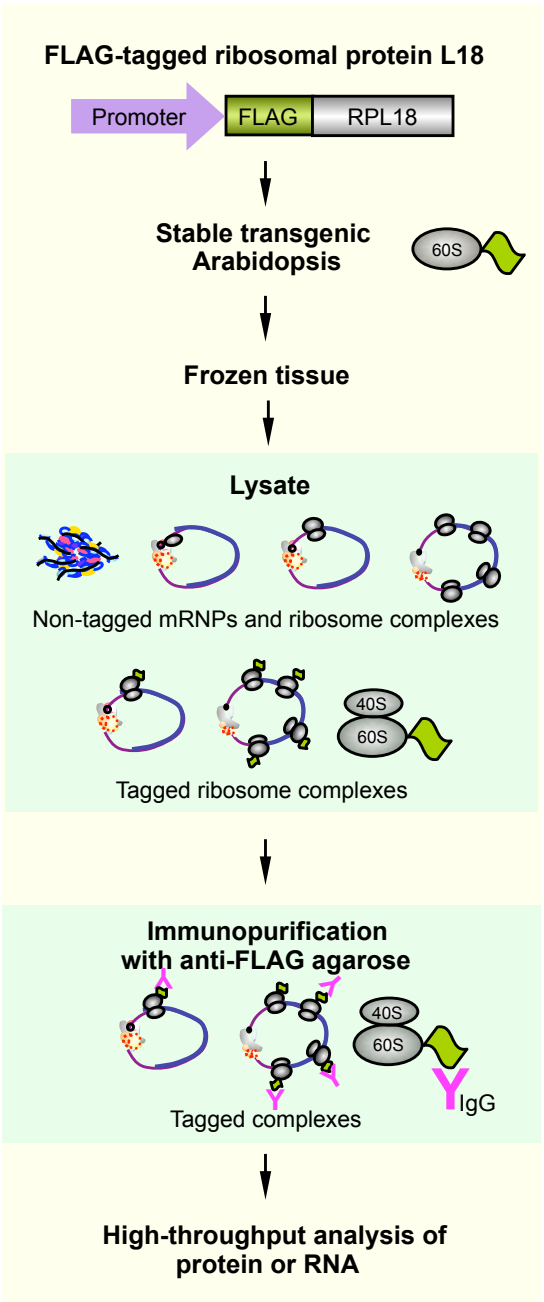
FLAG-tagged ribosomal protein L18
Promoter
FLAG
RPL18
Stable transgenic Arabidopsis
60S
Frozen tissue
Lysate
Non-tagged mRNPs and ribosome complexes
40S
60S
Tagged ribosome complexes
Immunopurification with anti-FLAG agarose
40S
60S
IgG
Tagged complexes
High-throughput analysis of protein or RNA

Table 1.1. Overview of mRNP proteins and predicted RNA Binding Proteins of *Arabidopsis*

Protein Group **(Gene no. in *A. thaliana*)**	**Proteins (no. in *A. thaliana* and specific names[1])**	**References**
	Cytoplasmic Ribosome RNP	
RPL(127)	Large (60S) ribosomal subunit proteins (48 families)	Chang *et al.*, 2005; Giavalisco *et al.*, 2005; Manuell *et al.*, 2005; Nishimura *et al.*, 2005
RPS(102)	Small (40S) ribosomal subunit proteins (32 families); RACK1(3)	Chang *et al.*, 2005; Giavalisco *et al.*, 2005
RPP(15)	60S ribosomal P proteins (6 families)	Chang *et al.*, 2005
	Cytoplasmic mRNA management	
Processing Bodies (PB)[2] (putative, 8)	AtCAR1, AtDCP2, AtDCP1, AtRH12, AtRH6, AtRH8, VARICOSE, AtXRN4, SOV	Xu *et al.*, 2006; Goeres *et al.*, 2007; Weber *et al.*, 2008; Zhang *et al.*, 2010
Stress Granules (SG)[2] (putative, 51)	AtNUC-L1, NTF-RRM(9), RBP45(4), RBP47(4), RHAU-like(9), PUM(26), UBP1(3)	Lambermon *et al.*, 2000; Lorković *et al.*, 2000a; Weber *et al.*, 2008; Lu *et al.*, 2009
mi/siRNA processes(28)	AGO(10), DCL1(4), DDL, DRB(5), SDE(5), SE, SGS3, RDR(6)	Fang & Spector, 2007; Voinnet, 2009
Tudor/Agenet(47)	subcellular trafficking of mRNAs (*i.e.* similar to OsTudor-SN) TSN (2)	Wang *et al.*, 2008; dit Frey *et al.*, 2010
	Cytoplasmic translation factors	
eIF(67)	eukaryotic initiation factors (eIF4E(3), eIF(iso)4E(1), eIF4A(3) and eIF4B(3), eIF5A(3), PABP(9),eIF4G(1), eIF(iso)4G(3)	Browning, 1996; Bailey-Serres, 1999; Kawaguchi & Bailey-Serres, 2002; Kim *et al.*, 2007a; Feng *et al.*, 2007; Weber *et al.*, 2008; Liu *et al.*, 2008; Ma *et al.*, 2010
eEF(12); eRF(4)	eukaryotic elongation factors; eukaryotic release factors	Browning, 1996

Table 1.1. Continued

Protein Group (Gene no. in *A. thaliana*)	Proteins (no. in *A. thaliana* and specific names[1])	References
	RNPs involved in nuclear processes	
Pre-mRNA processing (putative, 76)	CBP20, CBP80, CC1-like(3), AtRBPA/B(6), AtRBPH/F(2), AtRBPI/P(3), AtRSP31, RSp41, RSZp22, RSZ33, SCL30a, AtSF1/BBP, AtSRp30, SR45, AtSRp34b, SR-RRM, U1-70K, U1A, AtU2AF35a, AtU2AF65a, AtU2AF65b, U2B", U2SF3b53b, hnRNP (19), Sm core proteins (23)	Kuhn & Schroeder, 2003; Wang & Brendel, 2004; Reddy, 2007; Moore & Proudfoot, 2009
Exon Junction Complex(8)	AtALY-4, AteIF4A-III, AtRNPS1, AtUAP56-2, UPF1,UPF2, UPF3, AtY14	Yoine *et al.*, 2006; Arciga-Reyes *et al.*, 2006; Koroleva *et al.*, 2009; Simpson *et al.*, 2010
	Other RBPs	
Glycine-rich-RBP(17)	RNA chaperones involved in stress acclimation, precise functions unknown: AtRZ-1(3), GR-RBP(8), ATE1, AtCSP(4)	Kim *et al.*, 2005, 2010; Nakaminami *et al.*, 2006; Lee *et al.*, 2008; Streitner *et al.*, 2008
Other RRMs(133)	Diverse nuclear and cytoplasmic processes or function unknown: AtAML(5), AtCSTF-64, AtCUG-BP(2), AtCYP59, FCA(2), FLK, FPA, AtHB54, AtKINESIN13A, AtLA1, MCT2, nucleolin-like, PHIP1, PTB(3), AtRANGAP1, RBP37(6), AtREF(4), S-RBP(16), TAF15(2), TBP-BP, TEL(2), 30K-RRM(8), RRM+KH+ZnFinger[CCHC](1), RRM+ZnFinger(16), RRM+Lupis La(4), RRM+other(7), RRM+enz. domain(20), RRM[only recognized domain](26),	Lorković & Barta, 2002; Ma *et al.*, 2008; Wang & Okamoto, 2009
K-homology (KH)(25)	Diverse processes or functions unknown: FLOWERING LOCUS K, HEN4(2), PEPPER, KH+Zn Finger[CCCH](2), KH+other(4), KH[only recognized domain](15)	Lorković & Barta, 2002

Table 1.1. Continued

Protein Group (Gene no. in *A. thaliana*)	Proteins (no. in *A. thaliana* and specific names[1])	References
DEAD-Box RNA Helicase(73) excludes 36 proteins with chromatin remodeling domains	Diverse processes or function unknown: BIRH1, DRH1, HEN2(3), LOS4, PRH75, RecQ-like(2), STRS1, STRS2, AtSUV3-like, RH+Zn finger(5), RH+DUF1605(16), RH+Q-motif(36), DEAD2(4)	Xu *et al.*, 2006; Kobayashi *et al.*, 2007; Kant *et al.*, 2007
Pentatricopeptide repeat proteins (PPR)(485) and Tetratricopeptide repeat protein (TPR)	Plastid, mitochondrial, and nuclear RNA processing, splicing, editing, translation, degradation AtPRP39 (TPR domain), PPR5	Wang *et al.*, 2007; Beick *et al.*, 2008; Schmitz-Linneweber & Small, 2008

Abbreviations: At, *Arabidopsis thaliana*; Os, *Oryza sativa*. [1]*Arabidopsis* gene names based on The *Arabidopsis* Information Resource. [2]Association with these complexes might be transient.

Table 1. 2. Profiling of plant mRNAs associated with polysomes

Stimulus or Mutation	*Arabidopsis*	Analysis	Major Finding	Ref.
Hypoxia	7 d-old-seedling	Translation state: Polysomal (≥ 5 ribosomes per mRNA) *versus* total mRNA; Affymetrix ATH1 array	Average polysome loading of mRNA declined from 51 to 32% following 12 h stress; mRNAs that have long or GC rich 5'-UTRs are more poorly translated	Branco-Price *et al.*, 2005
Hypoxia and reoxygenation	7 d-old-seedling grown on medium	Translation state: Immunopurified polysomal versus total mRNA; Affymetrix ATH1 array	Translational repression mirrors ATP content and is rapidly reversible; some induced mRNAs are not translated until reoxygenation	Branco-Price *et al.*, 2008
Hypoxia	7-d-old-seedling grown on medium	Cell-type specific translation state: Immunopurified polysomal versus total mRNA; Affymetrix ATH1 array	Cell-specific profiling of translating mRNAs in seedlings and cell-type specific selective translation in response to hypoxia and normal growth-condition	Mustroph *et al.*, 2009
Mild dehydration	Mature rosette tissue	Translation state: Polysomal (≥ 2 ribosomes) versus non-polysomal mRNA; Affymetrix 8.2K and ATH1 arrays	Average ribosome loading of mRNA declined from 82 to 72%; mRNAs with 5'-UTRs that are long, GC rich, form potential secondary structure and/or contain uORFs are poorly translated under control conditions; under stress GC content further decreases translatability	Kawaguchi *et al.*, 2004; Kawaguchi & Bailey-Serres, 2005

Table 1.2 Continued

Stimulus or Mutation	*Arabidopsis*	Analysis	Major Finding	Ref.
eIF3h-1 mutant	10 to 12 d-old-seedlings grown on medium	Translation state: Polysomal (≥ 2 ribosomes) *versus* non-polysomal mRNA; Affymetrix ATH1 array	eIF3h facilitates translation of mRNAs with 5'-UTRs that are long or have uORFs; polysome loading of ribosomal and photosynthetic protein mRNAs increased in a *eif3h-1* mutant	Hayden & Jorgensen, 2007
Sugar starvation	Cultured cells	Translation state: Polysomal (≥ 2 ribosomes) versus total mRNA; CATMA array	183 of 224 mRNAs that significantly changed in the polysomal but not in the total population showed inhibition in translational initiation	Nicolai *et al.*, 2006
Standard growth conditions	2 wk-old-seedlings grown on soil	Ribosome subpopulation: mRNAs of membrane-bound *versus* soluble polysomes; CATMA array	Improved prediction of genes encoding membrane-associated and secreted proteins	de Jong *et al.*, 2006
High temperature and salinity	Cultured cells	Translation state: polysomal (≥ 2 ribosomes) *versus* non polysomal mRNA; Agilent *Arabidopsis* 3 array	Decrease in polysomal mRNA loading; 90% and 46% following high temperature and salinity stress, respectively	Matsuura *et al.*, 2010
Standard growth conditions	Inflorescence from plant grown on soil	Cell-type specific translation state: Immunopurified polysomal versus total mRNA; Translating Ribosome Affinity Capture and deep Sequencing (TRAP-seq)	Cell-specific profiling of translating mRNAs in developing flowers, post-transcriptional regulation at intron splicing and translational stages, and polysome associated non-coding RNA	Jiao & Meyerowitz, 2010

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Chapter 2

Comparative analysis of transcriptome and translatome dynamics in response to light availability in *Arabidopsis thaliana*

2.1 Abstract

Light, a dynamic environmental parameter, is an essential regulator of plant growth and development. Light-regulated transcriptional networks are well documented, whereas light-regulated post-transcriptional regulation has received only limited attention. In this study, dynamics in translation of cytosolic mRNAs were evaluated at the genome-level in *Arabidopsis thaliana* seedlings grown under a typical light / dark regime, shifted to darkness at midday and briefly re-illuminated. The shift to early darkness reduced levels of polyribosomes (polysomes) by *circa* 17% relative to illumination. Quantitative comparison of the total cellular population of transcripts (the transcriptome) to those associated with ≤ 80S ribosome complexes (the translatome) identified over 1600 mRNAs that are differentially translated in response to light availability. To confirm translational regulation of selected mRNAs, ribosome complexes were fractionated by differential centrifugation through sucrose gradients and mRNAs were monitored in each of twelve fractions. The results indicate that early darkness causes the sequestration of a subset of mRNAs to limit synthesis of proteins involved in photosynthesis and the energy demanding processes of ribosome biogenesis. The reduced

engagement of the majority of these transcripts with ribosomes is rapidly reversed upon re-illumination. We propose that regulation of translational status of chloroplast and protein synthesis mRNAs serves as an energy conservation mechanism during darkness.

2.2 Introduction

Light is an essential and variable environmental factor that greatly impacts the morphogenesis and development of photosynthetic organisms. On a daily basis, fluctuations in the quantity and quality of light alter energy and carbon resources in plants. In daylight, photosynthesis results in carbon fixation and starch accumulation that supports nighttime metabolic and growth activities. The alteration between light and darkness, circadian regulation, and the resultant fluctuation of sugars has been shown to induce changes in the accumulation and stability of mRNA transcripts in model plants including *Arabidopsis thaliana* (Lidder *et al.*, 2005; Bläsing *et al.*, 2005; Usadel *et al.*, 2008; Graf *et al.*, 2010). Light-regulated mRNAs encode proteins involved in diverse cellular processes, ranging from the components of the photosynthetic machinery to enzymes involved in carbon sequestration.

Plants can acclimatize to changes in light quantity and quality. Rapid light-fluctuations stimulate short-term responses (*e.g.* chlorophyll energy quenching) that are reversible within minutes (Külheim *et al.*, 2002; Allen, 2003), whereas progressive changes in the light environment induce long-term responses such as the modification of the number and size of photosynthetic complexes within the thylakoid membrane through changes in gene expression (North *et al.*, 2005; Bräutigam *et al.*, 2009). Dietzel and

Pfannschmidt (2008) have shown that the long-term response to light quality, for example changes in photosynthetic complex stoichiometry and light harvesting complex antenna size, is an important acclimation strategy for improving plant survival under changing light quality conditions.

Both nuclear and chloroplast genomes encode the components of chloroplast light harvesting and photosynthetic complexes. This necessitates coordinated regulation of gene transcription and protein production within the nucleus, cytoplasm and the chloroplast. Chloroplast gene expression is modulated by the availability of light (Pogson *et al.*, 2008) through regulation at levels including transcription, mRNA processing, stability and translation (Nickelsen *et al.*, 1994; Pfannschmidt *et al.*, 1999; Tullberg *et al.*, 2000; Rochaix, 2001; Pfalz *et al.*, 2009; Valkov *et al.*, 2009; Pokorska *et al.*, 2009; Johnson *et al.*, 2010; Stern *et al.*, 2010). It has been shown that the stability and translation of chloroplast mRNA is orchestrated by RNA binding proteins (RBPs) that complex with 5' or 3' untranslated regions (UTRs) (Bruick & Mayfield, 1999).

The production of nuclear-encoded proteins of the photosynthetic machinery is also highly regulated by light fluency and quantity. The control includes the modulation of chromatin organization as well as the activity and stability of transcription factors (Hiratsuka & Chua, 1997; Ma *et al.*, 2001; Rutitzky *et al.*, 2009). Despite detailed mechanistic knowledge of signal transduction pathways mediated by light that manifest transcriptional control, there is only limited knowledge of the extent of post-transcriptional control of gene regulation in response to light availability. Several studies

confirmed that light and circadian cycles impact the stability and translation of specific gene transcripts (Berry *et al.*, 1986, 1988; Sullivan & Green, 1993; Petracek *et al.*, 1997, 1998; Dickey *et al.*, 1998; Gutiérrez *et al.*, 2002; Tang *et al.*, 2003). More recently, Piques *et al.* (2010) reported that light availability and the circadian clock affects the steady-state accumulation and ribosome-association of mRNAs encoding 35 enzymes of central metabolism, suggesting that the light-mediated energy balance contributes to post-transcriptional regulation of gene expression. To date, there has not been a genome-level study of mRNA translation in response to changes in light availability, such as an unanticipated shift to darkness followed by re-illumination.

The translation state of individual mRNAs can be measured by comparison of steady-state abundance and polysome association. In *Arabidopsis*, this has been accomplished at the genomic-level by isolation of polysomes through differential centrifugation of detergent-treated cell extracts (Kawaguchi *et al.*, 2004; Kim *et al.*, 2004, 2007; Kawaguchi & Bailey-Serres, 2005; Branco-Price *et al.*, 2005; Nicolaï *et al.*, 2006; Matsuura *et al.*, 2010) or by the immunoprecipitation of polysomes from transgenic plants expressing a FLAG-tagged ribosomal protein integrated into functional 60S ribosomal subunits (Zanetti *et al.*, 2005; Branco-Price *et al.*, 2008; Mustroph *et al.*, 2009). These studies have shown that mRNA translation is perturbed by sub-optimal environmental conditions, including water deficit, hypoxia, sucrose starvation, and high temperature, as well as mutation of genes encoding non-essential components of the translational machinery. Each of these environmental stresses causes a re-prioritization of mRNAs for translation.

In this study, we examined global dynamics in mRNA translation status in *Arabidopsis* seedlings subjected to rapid unanticipated changes in light availability. The quantitative evaluation of the total steady-state and polysome populations isolated from 2-week old seedlings confirmed that unanticipated darkness transiently limits the translation of a sub-population of nuclear-encoded mRNAs without a concomitant effect on transcript abundance.

2.3 Material and Methods

2.3.1 Plant material, growth conditions and treatments

Transgenic *Arabidopsis 35S:HF-RPL18* (Col-0 ecotype), that expresses an amino-terminally His_6-FLAG (HF)-tagged copy of ribosomal protein L18B (RPL18) under a control of the near-constitutive Cauliflower Mosaic Virus (CaMV) 35S promoter, was used to enable the immunoprecipitation of polysomes (Zanetti *et al.*, 2005). Seeds were surfaced sterilized in a 15 mL Falcon tube with 10 mL 95% (v/v) ethanol for 5 min, 10 mL 20 % (v/v) household bleach plus 0.1 % (v/v) Tween-20 for 5 min, rinsed 3 times with 10 mL sterile water, and stratified in 10 mL sterile water at 4 °C for 48 h in darkness. Seeds were plated on sterile solid Murashige and Skoog (MS) medium containing 0.43 % (w/v) MS salts (Caisson Laboratories, North Logan, UT), 1 % (w/v) Sucrose, 1 % (w/v) Agar [pH 5.7] and vertically orientated in a growth chamber (model CU-36L5C8, Percival Scientific, Inc.) under a 16 h light (~60 μE sec^{-1} m^{-2}) and 8 h dark cycle at 23 °C.

At the middle of the day (Zeitgeber time 8, ZT8), 14-day-old seedlings were subjected to changes in light availability. For the early darkness treatment, plates were placed vertically in a plastic box that was completely wrapped in foil and kept in a growth chamber for 1 h. After 1 h of darkness, the foil was removed and plants were re-illuminated in the growth chamber for 10 min. For the light control, plates from the growth chamber were harvested at ZT9. Dark treated seedlings were collected in the dark using a dim-green light (Corning glass filter No 4010, Corning Life Science, Union City, CA). Seedlings were quickly frozen in liquid nitrogen, ground to fine powder, and stored at -80 °C until use.

2.3.2 Quantitative measurement of polysomes from concentrated ribosome pellets

Polysomes were isolated and quantified by differential centrifugation as described previously (Mustroph *et al.,* 2009a). Briefly, 5 mL of packed frozen tissue was thawed in 10 mL polysome extraction buffer (PEB: 200 mM Tris-HCl [pH 9.0], 200 mM KCl, 36 mM $MgCl_2$, 25 mM EGTA, 5 mM DTT, 1 mM PMSF, 1% (v/v) Triton X-100, 1% (v/v) Brij-35, 1% (v/v) Tween 20, 1% (v/v) NP-40, 50 µg/mL cycloheximide and 50 µg/mL chloramphenicol), transferred to a glass mortar, and homogenized with a glass teflon pestle. The crude cell extract was clarified by centrifugation at 16,000*g* for 20 min at 4 °C. The supernatant was filtered though sterile Miracloth (Calbiochem, La Jolla, CA), layered on top of an 8 mL 1.75 M sucrose cushion (400 mM Tris-HCl [pH 9.0], 200 mM KCl, 30 mM $MgCl_2$, 1.75 M sucrose, 5 mM DTT, 50 µg/mL chloramphenicol, 50 µg/mL cycloheximide), and centrifuged at 135,000*g* for 18 h at 4 °C (70Ti rotor,

Beckman, Brea, CA) to obtain a crude ribosome pellet. The pellet was resuspended in 250 µL polysome buffer (PB: 200 mM Tris-HCl [pH 9.0], 200 mM KCl, 36 mM $MgCl_2$, 25 mM EGTA, 5 mM DTT, 50 µg/mL cycloheximide and 50 µg/mL chloramphenicol and 20 U/mL RNaseOUT (Invitrogen, Carlsbad, CA)). Approximately 2000 units (OD_{260}) were loaded on top of a 20-60 % (w/v) sucrose gradient (Kawaguchi *et al.*, 2004) and centrifuged at 275,000*g* for 1.5 h at 4 °C (SW55 Ti rotor, Beckman), then passed through a UA-5 detector and 185 gradient fractionator (ISCO, Lincoln, NE). Data was analyzed using the Icruncher 2.2 program (Williams *et al.*, 2003). Quantitative polysome gradient fractionation was performed using three biological replicate samples. The mean and standard deviation (in percent) of RNA in polysomes was calculated.

2.3.3 Quantitative measurement of polysome and RNA extraction from crude cell lysates

Polysomes were extracted from 0.5 mL packed frozen tissue that was thawed in 1 mL PEB, homogenized, clarified, and filtered as described in section 2.3.2. The clarified supernatant (500 µl) was loaded on top of a 20-60 % (w/v) sucrose gradient (Kawaguchi *et al.*, 2005), centrifuged, and passed through a UA-5 detector and 185 gradient fractionator (ISCO, Lincoln, NE) as described in section 2.3.2. Twelve fractions of equal volume were collected and RNA extraction was performed by use of the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

2.3.4 Polysome immunoprecipitation and RNA extraction

Ribosome complexes were isolated by immunoprecipitation as described previously (Zanetti *et al.*, 2005; Mustroph, *et al.*, 2009b). Briefly, 7.5 mL of frozen packed tissue was thawed in 15 mL of PEB, homogenized, and centrifuged at 16,000*g* for 20 min at 4 °C. Total extract was centrifuged at 16,000*g* for 20 min at 4 °C. The clarified supernatant of the extract was passed through Miracloth and 13 mL was combined with 400 µl EZ view Red Anti-FLAG affinity beads (Sigma, St. Louis, MO) that had been pre-washed twice with 6 mL PB. The mixture was incubated at 4 °C for 2 h with gentle shaking. The unbound fraction was removed by centrifugation in clinical centrifuge at 700*g* for 2 min and beads were washed 4 times with 6 mL PB for 5 min at 4 °C with gentle shaking. The elution was performed by incubation of beads with 600 µL PB containing 200 ng/µL 3x FLAG peptide (Sigma) for 30 min at 4 °C.

Total RNA was extracted from 1 mL of the clarified extract. Both the total cell extract and immunoprecipitation eluate were subjected the TRIzol RNA extraction (Invitrogen, Carlsbad, CA). RNA was further purified by use of the RNaeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA concentration and quality was determined by absorbance 260 and 280 nm by use of a spectrophotometer (ND-1000, Nanodrop, Wilmington, DE). The RNA integrity was examined with an Agilent Bioanalyzer 2100 on RNA 6000 nano chip (Agilent Teachnologies, Santa Clara, CA)

2.3.5 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Transcript levels were evaluated by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). For total RNA, polysomal RNA, and sucrose gradient fractioned RNA the complementary DNA (cDNA) synthesis was performed using 400 ng RNA in 20 µl reaction buffer (2.5 µM oligo-dT (Promega, Madison, WI), 5X reaction buffer (250 mM Tris-HCl, [pH 8.3], 250 mM KCl, 50 mM $MgCl_2$, 2.5 mM spermidine, 50 mM DTT), 0.2 mM dNTP mix, 10 mM DTT, 40 U RNaseOUT (Invitrogen), 200 U Superscript II reverse-transcriptase (Invitrogen)) at 65 °C for 5 min for oligo-dT annealing, 42 °C for 50 min for reverse transcription and then at 70 °C for 15 min for termination. PCR was performed in 20 µl reaction buffer (25 mM Tris-HCl, [pH 8.3], 25 mM KCl, 2 mM $MgCl_2$, 2.5 mM each oligonucleotide primer and 0.5 U Taq polymerase (Qiagen)). PCR Amplification was carried out with denaturation at 95 °C for 1 min, annealing at 50 to 55 °C for 45 sec and elongation at 72 °C for 60 sec for 20 to 30 cycles in a thermocycler (PTC-0200 DNA Engine, MJ research Inc., Waltham, MA). See Table 2.1 for primers, annealing temperature and cycle of PCR amplification. An equal volume (20 µl) of each RT-PCR sample was mixed with 5 µl of 5x DNA gel loading dye (125 µl 2% (v/v) bromophenol blue in ddH_2O, 125 µl of 2% (v/v) xylene cyanol in ddH_2O, 25 % (v/v) glycerol, 5 % (v/v) 0.5 M EDTA) was analyzed on a 1.2 % agarose gel containing 0.01 % (w/v) ethidium bromide with Tris-Acetate-EDTA buffer (40 mM Tris-HCL [pH8.4], 20 mM acetic acid, 1 mM EDTA) and photograph under UV light.

2.3.6 Microarray hybridization and data analysis

Total mRNA and immunopurified polysomal mRNA was analyzed by hybridization to ATH1 (GeneChip System, Affymetrix, Santa Clara, CA) GeneChips at the Genomic Core Facility, Institute for Integrative Genome Biology, University of California, Riverside, CA. cRNA synthesis and hybridization was conducted with 1.5 µg RNA using One-Cycle cDNA synthesis Kit (Affymetrix, Santa Clara, CA). Biotin-labeled cRNA was synthesized by use of the GeneChip IVT Labeling Kit (Affymetrix). Hybridizations were performed at 45 °C for 16 h, in a rotating platform with 10 µg of biotin-labeled cRNA. Microarray hybridization was performed by use of two independent biological replicate samples. Raw expression data obtained in .cel files was extracted by use of the Bioconductor package of the R statistical software. Low-level normalization followed using Affymetrix the MAS 5.0 (MAS5) platform, and included probe-specific and multichip background corrections. The microarray datasets were clustered by use of a bootstrapped hierarchical cluster analysis (nboot = 1000; p-value < 0.05) with the normalized signal values. Present, Marginal or Absent (P/M/A) calls, describing signals for probe pair sets that were above or below the background default threshold, were obtained with the MAS5 platform.

To accurately quantify the amount of individual mRNAs in polysomes, the normalized MAS5 data values from the polysomal RNA hybridizations were further adjusted based on the relative polysome content, as described previously (Kawaguchi *et al.*, 2004, Branco-Price *et al.*, 2005; 2008). Briefly, the normalization factor was

generated from quantitative measurements of polysome content in tissue from each treatment sample by use of sucrose density gradient absorbance profiles of three biological replicates. In each case, the relative proportion of RNA in polysomes was determined from the integrated area of the polysome peak region divided by that of the complete gradient profile (total RNA content) (A_{254} units in polysomes / A_{254} = relative polysome level). Normalized expression values for polysomal RNA were obtained by multiplication of the MAS5 value by the relative polysome level (light = 0.729, early darkness = 0.603 and re-illumination = 0.738). The normalized data were used to calculate signal $\log_2$ ratio (SLR) values for total and polysomal mRNA samples using these comparisons: Dark/Light (L→D), Re-illumination/Dark (D→R), and Re-illumination/Light (R vs. L). False discovery rates (FDR) for significant differences between mRNA in the samples compared were generated using P-value distributions (Smyth, 2004). These values were used to identify differentially expressed genes (DEGs). Normalized SLR data of probe pair sets (genes) detected without an "absent" call in both bioreplicates and with a FDR < 0.05 were used as the data set for the subsequent cluster and gene category analyses (n = 2,508, 1,224 of which had a |SLR| of ≥ 1 in at least one comparison).

2.3.7 Identification of co-regulated genes by clustering and their ontology

The data were sorted into co-regulated gene by use of Fuzzy *k*-means clustering with Euclidean correlation for the distance measure, a membership exponent of 1.1, maximal number of iterations of 5,000 and ten clusters (Mustroph *et al.*, 2009b). The

mean SLR value for each cluster was determined for summary visualization. Clusters membership was analyzed for gene ontology (GO) category enrichment analysis by use of the GOHyperGall function according to Horan *et al.* (Horan *et al.*, 2008). GO annotations were obtained from http://geneontology.org/ (TAIR, 08/03/2010 release). The PAGEMAN tool (Usadel *et al.*, 2005, 2006) was used for an independent interpretation of biological significance of differentially expressed genes, through the overview of significant data sets in diagrams of known pathways. The PAGEMAN annotation map utilized TAIR version 9 (TAIR 9: January, 2010 release). Enrichment was decided by Wilcoxon rank sum statistical analysis with Benjamini-Hochberg FDR, where a z score of 1.96 represents a FDR < 0.05. The annotation of chloroplast and ribosomal proteins categories were obtained from TAIR (ATH_GO_GOSLIM, date 09/14/2010).

2.3.8 Sequence motif analyses

5'-UTR and 3'-UTR sequences were extracted from http://www.arabidopsis.org (TAIR 9: 06/19/2009 release). Over-represented motifs were identified by use of the MEME suite (Multiple EM for Motif Elicitation, http://meme.nbcr.net/) (Bailey *et al.*, 2009) using the Two-Component Mixture (TCM) model with minimum and maximum widths set at 6 and 50 bases, respectively. The motif consensuses derived from MEME were represented graphically using the WebLogo application (Crooks *et al.*, 2004). The over-represented motifs derived from MEME were used to search against sequence databases for motif occurrences by FIMO (Find

Individual Motif Occurrences) with significant p-value < 1E-04 (Bailey *et al.*, 2009). Motif discovery rates (MDR) were calculated by use of equations:

$$(1) \qquad MDR = \frac{number\quad of\quad motif\quad found}{number\quad of\quad gene}$$

$$(2)\ relative\_MDR = \frac{MDR(Focused\quad sequence\quad collection)}{MDR(Arabidopsis\quad gene\quad set)}.$$

2.4 Results

2.4.1 Light availability dynamically and reversibly modulates seedling polysome levels

The experimental strategy used to evaluate modulation of mRNA translation in response to dynamic changes in light availability by *Arabidopsis* seedlings is shown in Figure 2.1. To evaluate whether light availability affects global levels of translation, seedlings at midday of a typical light/dark diurnal cycle (ZT8, Light), shifted to early darkness for 1 h (ZT8 to ZT9 in dark, Dark), or re-illuminated for 10 min after 1 h of darkness (Re-illumination) were harvested and immediately cryopreserved. This tissue was subsequently used for the isolation, fractionation by differential ultracentrifugation and quantitation of polysomes. In the dark-shifted seedlings, a decrease in polysome levels was accompanied by an increase in 80S monosomes and ribosomal subunits (Figure 2.2a), characteristic of a reduction in the initiation of translation. After 10 min of re-illumination, polysome levels were restored and monosomes decreased. Quantitative

analysis confirmed that the amount of RNA in polysome complexes of light, dark-shifted and re-illuminated seedlings was 72.88 ± 0.55 %, 60.31 ± 1.02 % and 73.80 ± 0.90 %, respectively (n = 3 independent experiments). We confirmed a *circa* 17 % (17.25 ± 1.67 %) decline in polysome complexes in seedlings transferred to early darkness that was rapidly restored by 10 min of re-illumination (Figure 2.2b). Together, these findings suggest that the shift to early darkness transiently reduces polysome levels through modulation of the initiation of translation.

2.4.2 Light-mediated changes in translation status

To simultaneously evaluate the effect of changes in light availability on the accumulation and translation of individual mRNAs, we quantitatively profiled the total and polysomal mRNA populations from light, dark-shifted and re-illuminated seedlings using the Affymetrix ATH1 DNA microarray platform. To obtain polysomal mRNAs, we utilized the *35S:HF-RPL18* line characterized and implemented previously to immunopurify ribosome complexes (60S subunits, 80S monosomes, and polysomes) (Zanetti *et al.*, 2005; Branco-Price *et al.*, 2008; Mustroph *et al.*, 2009a). Of the 14,362 probe sets with signal levels above the limit of detection in at least one of the samples, 2,508 genes (probe sets) displayed a significant change in transcript abundance in one or more of six SLR comparisons (FDR < 0.05 in one or more of the SLR comparisons as indicated in the "Materials and Methods" (Supplementary Table 2.1)). Of the 2,508 differentially expressed genes (DEGs), 1,224 mRNAs also showed a two-fold increase or decrease in abundance in one or more mRNA populations ($|SLR| \geq 1$). The hybridization

signal values were highly correlated between biological replicate samples (R^2 value range from 0.96-0.98), confirming the reproducibility of the biological response and polysomal mRNA immunopurification method.

To obtain a global perspective of the dynamics in the transcriptomes and translatomes monitored in this study, we compared the SLR of the 2,508 DEGs by comparison of total versus polysomal mRNA (Figure 2.3). In this analysis, the genes that plot above or below the solid diagonal line are positively or negatively regulated at the level of translation, respectively. In the dark shifted seedlings (Light→Dark), early darkness affected the abundance of mRNAs in both the total and polysomal mRNA populations, but not to the same degree (R^2=0.66) (Figure 2.3a). Many mRNAs with limited change in steady-state level were reduced in polysome complexes.

We also enumerated the genes that were differentially regulated in this analysis based on both fold-change and FDR (|SLR| > 1, FDR < 0.05) (Figure 2.4). In the case of the shift to darkness 61 mRNAs were reduced in the total mRNA population, whereas ten-fold more (n = 647) were significantly reduced in polysome-association (Figure 2.4, Light→Dark panel). This observation indicates that expression of a cohort of mRNAs was down-regulated by the shift to darkness through restriction in translational initiation, as evidenced by the decline in association with polysomes. On the other hand, most of the mRNAs that were increased at the steady-state level by darkness were increased to a similar extent in the polysomal mRNA population.

Consistent with the down-regulation of translation of a cohort of mRNAs in darkness, there was limited correlation between adjustments in the transcriptome *versus* the translatome for the DEGs in response to re-illumination ($R^2 = 0.03$) (Figure 2.3b; Dark→Re-illumination). The large number of genes plotting above the diagonal line reflects an increase in translation of many DEGs upon re-illumination relative to darkness. In fact, 10 min re-exposure to light did not result in any changes in steady-state abundance of mRNAs of ≥ 2-fold (FDR < 0.05) (Figure 2.3), but significantly elevated the translation of 380 mRNAs, of which 179 were reduced in translation in the Dark→Light comparison. The comparison between the total and polysomal mRNA populations of the Light and Re-illuminated seedlings samples confirmed these populations were highly concordant ($R^2 = 0.85$) (Figure 2.3a), indicating that transcript accumulation and translation status of individual mRNAs was similar prior the shift to darkness and after brief re-illumination. Together, these results indicate that translation of over 2,500 gene transcripts is affected by the shift to early darkness and re-illumination.

2.4.3 Light regulates the accumulation and translation of individual mRNAs

To further evaluate the response to light availability, we considered the biological role of DEGs that were co-regulated at the level of transcript accumulation and translation in response to early darkness and re-illumination. Co-regulated genes were recognized by use of fuzzy k-means clustering, sorting the DEGs into ten groups (gene clusters) ($k = 10$; n = 2,508 DEGs) (Figure 2.5; Supplemental Table 2.1). The display of the median $\log_2$ SLR of each cluster in a heat map revealed that the DEGs fall into three groups of genes

comprising two to five clusters. Group 1 mRNAs were up-regulated at the transcriptional and translational level by early darkness (n = 492 mRNAs; Clusters 1-3). The abundance and translation of these mRNAs was not dramatically affected by brief re-illumination. Group 2 mRNAs showed a proportional decline in abundance and translation in response to early darkness (n = 403 mRNAs; Clusters 4-5). Of these, Cluster 4 mRNAs were slightly more elevated at the level of polysome association in response to re-illumination than Cluster 5 mRNAs. The third group of mRNAs was those maintained at similar steady-state levels in the light, following the shift to darkness, and upon re-illumination. Notably, these mRNAs were translationally repressed upon the shift to darkness and re-recruited back to polysomes upon re-illumination, with the exception of Cluster 10 mRNAs (n = 1,613 mRNAs; Clusters 6-10). These results suggest that light availability influences both mRNA abundance and translation status.

To determine biological function of the co-regulated mRNAs, we performed Gene Ontology (GO) analysis with the genes from the fuzzy *k*-means clusters (Supplementary Table 2.2). The dark-induced and well-translated mRNAs mainly consisted of genes involved in metabolic processes (*i.e.* Cluster 1, very-long-chain fatty acid metabolic process (3.8E-02); Cluster 2, glutamate dehydrogenase (2.40E-03); Cluster 3, xyloglucan transferase activity (1.41E-03); glycosyl hydrolase activity (1.97E-03); pyruvate phosphate dikinase activity (4.84E-02); cellular amino acid catabolism (2.13E-03)). In addition, this cohort of mRNAs was enriched for transcription factor activity (Cluster 2, 8.50E-05; Cluster 3, 8.91E-03), response to stimuli (Cluster 1, 1.07E-09; Cluster 2, 5.37E-16), and response to the absence of light (Cluster 3, 1.47E-04). The

transcripts that were dark unstable / re-illumination-induced and poorly translated in darkness until re-illumination were enriched for transcriptional factor activity (Cluster 4, 3.44E-06), as well as carbohydrate anabolism (*i.e.* glycoside biosynthesis process (Cluster 5, 2.78E-03), UDP-glucosyl transferase activity (Cluster 4, 8.63E-03)), regulation of heterochronic development (Cluster 4, 8.78E-06), chlorophyll biosynthesis (Cluster 5, 1.91E-02), and the chloroplast (Cluster 6, 1.78E-19). The clusters of mRNAs that remained stable in the dark but were translationally repressed encode proteins involved in energy-consuming processes such as ribosome biogenesis (Cluster 6, 9.22E-03; Cluster 7, 2.65E-25; Cluster 9, 4.40E-27), photosynthesis (Cluster 6, 5.71E-08), generation of precursor metabolites and energy (Cluster 7, 1.64E-02), reductive pentose-phosphate cycle (Cluster 8, 4.12E-02), guanylate kinase activity (Cluster 10, 5.44E-03), and nitrogen compound metabolic process (Cluster 10, 2.54E-02).

The PAGEMAN tool was used to further evaluate a possible connection between molecular function and regulation of steady-state and polysomal mRNA of the DEGs (Usadel *et al.*, 2005, 2006). This platform utilizes gene categorization similar to GO and plots groups of genes with significantly different SLR values in one or more of the six comparisons (Wilcoxon sign rank test / Benjamini-Hochberg FDR < 0.05) (Figure 2.6). This independent method revealed further distinctions in the dynamics of mRNAs in the total and polysomal mRNA populations. For example, genes associated with photosynthetic light reactions displayed significant regulation at steady-state and polysomal mRNA levels. These transcripts were translationally repressed by early darkness but increased in abundance upon re-illumination and were proportionally

recruited to polysomes. As shown in the GO analysis, PAGEMAN recognized mRNAs encoding cytosolic ribosomal proteins as targets of translational repression upon the shift to darkness. The analysis also highlighted genes associated with sucrose transport, trehalose metabolism, WRKY transcription factors, a sub-class of receptor kinases and ethylene and calcium signal transduction that were translationally enhanced by early darkness. Whereas, proteins associated with cell wall modifications and secondary metabolism had reduced translation status. Together, the fuzzy *k*-means clustering and PAGEMAN analysis confirm that light availability dynamically coordinates both accumulation and translation of individual mRNAs.

2.4.4 Validation of differential accumulation and polysomal mRNA association during light acclimation

To verify the results from microarray analysis, semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) was used to survey the association of three mRNAs under the three treatment conditions. The selected genes represent mRNAs with a decline in polysomal mRNA abundance in response to early darkness. The ribosomal pellet of detergent-solubilized cell-extracts was centrifuged through a 20-60 % (w/v) sucrose gradient and collected in 12 gradient fractions. These mRNA in these fractions were then analyzed by RT-PCR with gene specific primer for three genes (Figure 2.2c). To confirm the reliability of the polysome immunoprecipitation technique, we also performed semi-quantitative RT-PCR with immunoprecipitated polysomal and total cellular mRNAs from the same tissue samples (Figure 2.2d). The results confirmed

a greater association of *LIGHT HARVESTING COMPLEX A4* (*LHCA4*) and *RIBOSOMAL PROTEIN LARGE SUBUNIT 12A* (*RPL12A*) mRNAs with large polysomes (≥ five ribosomes; fractions 9-12) in the Light and Re-illuminated seedling samples. Whereas, in the seedlings shifted to early darkness, these mRNAs accumulated to a greater extent in the free mRNA, monosome and small polysome fractions (fractions 1-5). By contrast, the total abundance of these transcripts was not affected by light availability, suggesting that a portion of these mRNAs were sequestered during darkness but rapidly remobilized onto polysomes within 10 min re-illumination. These data suggest that the production of the proteins encoded by these mRNAs is regulated in part by light-regulated translational activity, consistent with the microarray data. By contrast, the mitochondrial *ATP SYNTHASE ß-SUBUNIT (ß-ATP)* transcript remained stable and was generally well-translated under the three treatment conditions. Together, these data provided strong evidence of differential translation of individual mRNAs following a change in light availability.

2.4.5 Regulation of chloroplast and ribosomal protein mRNA stability and translation as an energy conversing mechanism during light acclimation

The mRNAs that were stable but poorly translated during the darkness and became quickly associated with polysome upon brief re-illumination (Group 3, Clusters 6-10) were highly enriched for chloroplast, photosynthesis and ribosomal proteins (Supplementary Table 2.2). Since translation is an energetically demanding process the down-regulation of these abundant mRNAs in the dark may limit the consumption of

cellular energy. Through evaluation of the hybridization signal values for individual gene transcripts *versus* the hybridization signal value of all mRNAs present in the light, we determined that Cluster 6–10 mRNAs comprised 80 % of all DEG transcripts and 19 % of all mRNAs, based on the total cellular mRNA content in light-grown seedlings (Figure 2.7 a and b). The Group 1 and 2 mRNAs comprised 11 % and 9 % of the DEG mRNA signal value, respectively, but did not represent a sizable portion of the total mRNA in the light (Figure 2.7 b). The finding that early darkness down-regulated translation of 19 % of all gene transcripts is highly consistent with the finding that polysome levels declined by 17 % in response to early darkness (Figure 2.2 b).

Since Group 3 mRNAs (reduced in translation status but not abundance by early darkness) were enriched in chloroplast proteins and structural components of the ribosome, we determined the percent of cellular mRNA in light-grown seedlings that encode chloroplast, chloroplast ribosomal proteins, and other ribosomal proteins. We found that the total hybridization signal of Group 3 mRNAs corresponded to 51 % of the DEG mRNA signal value (Figure 2.7c). The gene transcripts categorized by GO as chloroplast, chloroplast ribosomal or other ribosomal proteins represent 36 % of all mRNAs present in light grown seedlings (Figure 2.7d). Although only about half of the genes in this category were significantly translationally regulated, those that were accounted for half of the 19% reduction in global translation observed in response to early darkness.

2.4.6 Over-represented 5' UTR motifs that correlated with light regulation

We attempted to find cis-regulatory elements that are important for light-regulated translation of chloroplast and ribosomal protein mRNAs by identification of over-represented motifs in 5'- and 3'-UTRs. By examination of the chloroplast targeted mRNAs that were translationally repressed by darkness from Cluster 6 and the ribosomal proteins and translation factor mRNAs from Clusters 6, 7, 8 and 9 with the unsupervised multiple expectation maximization for motif elicitation (MEME) tool, we identified an over-represented A-rich motif (consensus: [AU][AGC]AA[GA]AA[AU][GAC]A[GA][AC][CAU][UG][ACG]) (Figure 2.8 a and c) present in the 3'-UTRs of chloroplast mRNAs (26 of 44 Cluster 6 mRNAs; E-value = 5.30E-03) and an over-represented AC-rich motif (consensus: [AG][AG]AAACCUA[AG]) (Figure 2.8 b and d) present in the 5'-UTR of translationally repressed ribosomal protein or translation factor mRNAs (41 of 120 Cluster 6-9 mRNAs; E-value = 1.2E-035). Despite the significant enrichment of these motifs in chloroplast and ribosomal protein mRNAs, respectively, these motifs were not limited to the translationally regulated mRNAs (Figure 2.8e and f).

2.5 Discussion

2.5.1 Dynamic and reversible regulation of individual transcripts in response to light availability

The data presented here demonstrate that light availability dynamically regulates gene expression at the level of transcription and translation in *Arabidopsis*

seedlings. Most mRNA expression profiling studies have focused on the total cellular mRNA population, which is readily isolated from tissue samples. Here, we utilized an efficient method of polysome immunopurification to isolate ribosome-associated mRNAs following brief periods of environmental perturbation. The comparative quantitative analysis of total mRNA (transcriptome) and ribosome-associated mRNA (translatome) populations uncovered rapid and reversible adjustments in the mRNA translation status in response to light availability. We found that the shift to early darkness followed by re-illumination dynamically regulated the abundance of a subset of cellular mRNAs in either the transcriptome or translatome (n= 2,508). These included 497 dark-induced mRNAs, 403 dark-destabilized mRNAs, and 1,613 dark translationally-impaired mRNAs. The latter class of mRNAs shifted out of polysome complexes, most likely due to inhibition of translational initiation following the darkness-shift.

In general, photosynthetic organisms utilize light energy for photosynthesis and ATP production. Branco-Price *et al.* (2008) reported that oxygen deprivation causes a deficiency in cellular ATP content that is strongly correlated with global levels of polysome levels in *Arabidopsis* seedlings. Although ATP levels were not monitored in this study, we found that the 17 % reduction in global polysome levels of seedlings after 1 h of early darkness was rapidly reversed by 10 min of re-illumination, concomitant with similar adjustments in mRNAs in polysome complexes (Figure 2.2 a and b; Figure 2.7b).

Our results demonstrated that darkness induced the accumulation and translation of mRNAs involving in carbohydrate catabolism, confirming the idea that dark-shifted seedlings utilized available substrates for cellular respiration and ATP production. The induction of trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) under darkness (Figure 2.6) has been linked with increased expression of stress-related genes such as Sucrose-non-fermenting-Related Kinase 1 (*SnRK1*) and S6 kinase 2 (*S6K2*) and the regulation of sugar metabolism by controlling the rate of starch synthesis (Baena-González, 2010). Although, the manipulation of light in this study did not alter *SnRK1* transcript accumulation or translational status, we discovered differentially regulated mRNAs encoding *SnRK* protein family members (SnRK2.1, SnRK2.2, SnRK2.3, SnRK3.4, SnRK3.10, SnRK3.12, SnRK3.14, SnRK3.17, SnRK3.21, and SnRK3.22) (Supplementary Table 2.1) that are proposed to be involved in ABA, stress responses, and sugar signaling (Halford & Hey, 2009).

Our study revealed that the shift to early darkness inhibited translation of 368 mRNAs associated with chloroplasts and 134 mRNAs associated with the protein synthesis machinery, including ribosomal proteins and translation factors. Following a short period of re-illumination, these mRNAs quickly re-associated with polysomes. Interestingly, these chloroplast and ribosomal protein mRNAs represent a large proportion (38 %) of all mRNAs present in light grown seedlings (Figure 2.7c). mRNA translation and protein synthesis is an energetically-costly step in the gene expression pathway due to ATP and GTP requirements during 43S-pre initiation complex formation and scanning of the 5'-UTR, the completion of the initiation processing, charging of

tRNAs, and ribosome translocation. The reduced translation of chloroplast and translation machinery mRNAs is likely to conserve carbon and ATP energy. However, the alteration between light and darkness is a recurring phenomenon. Thus, the conservation of ATP energy could be improved by sequestration of these mRNAs in translationally inactive complexes reducing the need to generate new mRNAs. Once light becomes available, the rapid reversal of mRNA sequestration would allow rapid recovery of photosynthesis and protein production. Based on the recovery of polysome complexes and return of mRNAs to 80S ribosome complexes within 10 min of re-illumination, the reversal of this process is extremely rapid.

2.5.2 Translational regulation in response to changes in light availability

Our findings revealed that the shift to darkness repressed translation of ribosomal protein mRNAs similar to what was found in response to mild dehydration of rosette-stage *Arabidopsis* plants (Kawaguchi & Bailey-Serres, 2005) and low-oxygen stressed seedlings (Branco-Price *et al.*, 2005, 2008). In mammals, the regulation of ribosomal protein mRNA translation is modulated by nutrient avialability and cellular stresses through the energy-sensing mammalian target of rapamycin (mTOR), TOR regulatory protein (RAPTOR) and S6 kinase (Lie *et al.*, 2006), which modifies phosphorylation of eukaryotic initiation factor (eIF) 4E-binding protein, eIF4B, eEF2, and RPS6 (Proud, 2007). *Arabidopsis* possesses functional orthologs of mTOR pathway components and multiple stresses affect the phosphorylation of translation factors and RPS6 (Turck *et al.*, 1998, 2004; Kawaguchi & Bailey-Serres, 2002; Williams *et al.*,

2003; Mahfouz *et al.*, 2006; Arsham & Neufeld, 2006). In our study, *Arabidopsis* orthologs of *S6K1* (*At3g08730*) and *S6K2* (*At3g08720*) were differentially regulated. Early darkness induced accumulation and translation of *S6K2* mRNA (Cluster 1, Supplementary Table 2.1), whereas light maintained and induced translation of *S6K1* mRNA (Cluster 6). Mahfouz *et al.* (2006) proposed distinct roles for the two *Arabidopsis* S6Ks, in which S6K1 functions in the plant TOR pathway and S6K2 regulates the phosphorylation of RPS6 in the nucleolus.

Variability in light causes several cellular adjustments, for example, changes in the redox balance of the plastoquinone pool and accumulation of reactive oxygen species (ROS). Mühlenbock *et al.* (2008) discovered that Lesion Simulating Disease (LSD1) is important for light acclimation processes by regulating ROS levels and the redox state of plastoqiuinone. Interestingly, re-illumination increased the polysome-association of *LSD1* transcripts (Cluster 10). In addition, elevation of singlet oxygen, a major ROS involved in photooxidative damage, has been shown to down-regulate translation of photosynthetic mRNAs including Rubisco large and small subunits and LHCB2 chlorophyll binding protein, correlating with a decline in the phosphorylation state of RPS6 (Khandal *et al.*, 2009). Reinbothe *et al.* (2010) hypothesized that singlet oxygen may interfere the S6K signaling network. We speculate that changes in cellular redox state or ROS production upon the shift to darkness and upon re-illumination are relevant to translational regulation of nuclear-encoded chloroplast mRNAs.

Differential translational regulation in plants is likely to be regulated by *cis*-acting sequences within the 5'- or 3'-UTR of the mRNA. Studies have shown that 5'-UTR length, G+C content and limited potential for secondary structure contributes to differential translation of mRNAs under dehydration and low-oxygen stress (Kim *et al.*, 2004; Kawaguchi & Bailey-Serres, 2005; Branco-Price *et al.*, 2005). Additional mRNA feature such as the 5'-UTR upstream open reading frame (uORF) has been shown to regulate translation initiation and polysome loading of some mRNAs (Kim *et al.*, 2004; Kawaguchi & Bailey-Serres, 2005). In the case of light-enhanced selective translation, a CAUU repeat in the 5'-UTR of the pea ferredoxin mRNAs is required for light-regulated mRNA accumulation and polysome association in tobacco (Petracek *et al.*, 1997, 1998; Dickey *et al.*, 1998). This regulatory sequence most likely functions as a *cis*-binding site for an RBP that binds to the mRNA and increases its stability or polysome association. We attempted to find cis-regulatory elements that are important for light-regulated translation of chloroplast and ribosomal protein mRNAs by identification of the over-represented motif at the 5'- and 3'-UTR. Although we found two sequences that were significantly enriched in the translationally regulated chloroplast and ribosomal protein mRNAs, the sequences were not limited to the regulated mRNAs.

In *Chlamydomonas*, the RBP *NAB1* has been shown to inhibit translation of *LHCBm* mRNAs by sequestration under highlight conditions (Mussgnug *et al.*, 2005). A recent report from Wobbe *et al.* (2009) confirmed that modification of NAB1 cysteine-226 by the cellular redox state regulated the binding of NAB1 to *LHCBm* mRNAs. These

findings, together, emphasize the role of RBPs and cellular redox state in post-transcriptional regulation of nuclear-encoded chloroplast mRNAs.

mRNA sequestration in eukaryotes involves at least two types of cytoplasmic ribonucleoprotein (mRNP) complexes: stress-granules (SGs) and processing bodies (PBs). SGs usually contain the 40S ribosomal subunit and translation initiation components (eIF4E, eIF4G, eIF4A, eIF4B, poly(A)-binding protein (Pabp), eIF3, and eIF2) and specific RBPs involved in mRNA storage (Buchan & Parker, 2009). PBs contain mRNA decay machinery and function in turnover of transcripts (Balagopal & Parker, 2009). Formation of SGs and PBs is dynamic and highly regulated, especially in response to environmental changes. In *Saccharomyces cerevisiae*, PBs are involved in ribosomal protein mRNA sequestration under glucose starvation in a manner dependent on phosphorylation of Decapping Complex Protein 2 (DCP2) (Yoon *et al.*, 2010). *Arabidopsis* possesses putative orthologs of mammalian PB and SG proteins (Bailey-Serres *et al.*, 2009). The functions of these mRNPs in storage and turnover are emphasized by the dynamic formation of SGs, PBs and other mRNPs with translationally inactive mRNAs, particularly in response to sub-optimal growth conditions (Bailey-Serres *et al.*, 2009).

The analysis of light-regulated adjustments in the seedling transcriptome and translatome confirmed that translation is selectively modulated by light availability. Future studies should consider the RBPs and mRNPs complexes that contribute to this translational regulation. It would be interesting to examine if the phosphorylation state of

translation factors, PB or SG components contributes to the selective translation of chloroplast and ribosomal protein mRNAs in response to change in light availability. In addition, the mutation of the putative regulatory proteins, for example, SG and PB components, combined with a genomic technology such as RNP-immunopurification followed by high throughput sequencing using Illumina technology (RIP-RNA-seq) could be used to identify subset of mRNAs regulated by specific RBPs.

2.6 Acknowledgements

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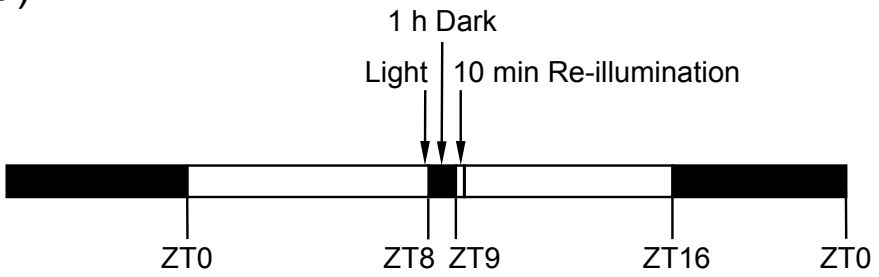
Figure 2.1. Quantitative analysis of molecular response to the shift to early darkness and re-illumination in *Arabidopsis* (Col-0) seedlings (a) Experimental scheme of the light cycle used for seedling growth for 14 d on MS medium supplemented with 1% sucrose. Arrows indicate time of day relative to the beginning of the light period (ZT0). For one hour light-control, seedlings were harvested in the light at ZT9. For one hour darkness and re-illumination, seedlings were transferred to early darkness at ZT8 for 1 h and subsequently re-illuminated for 10 min before harvesting. (b) Flow diagram of experimental strategy for quantitative assessment of gene transcript abundance of the total mRNA and immunopurified polysomal mRNA populations.

(a)


1 h Dark
Light
10 min Re-illumination
ZT0
ZT8
ZT9
ZT16
ZT0

(b)

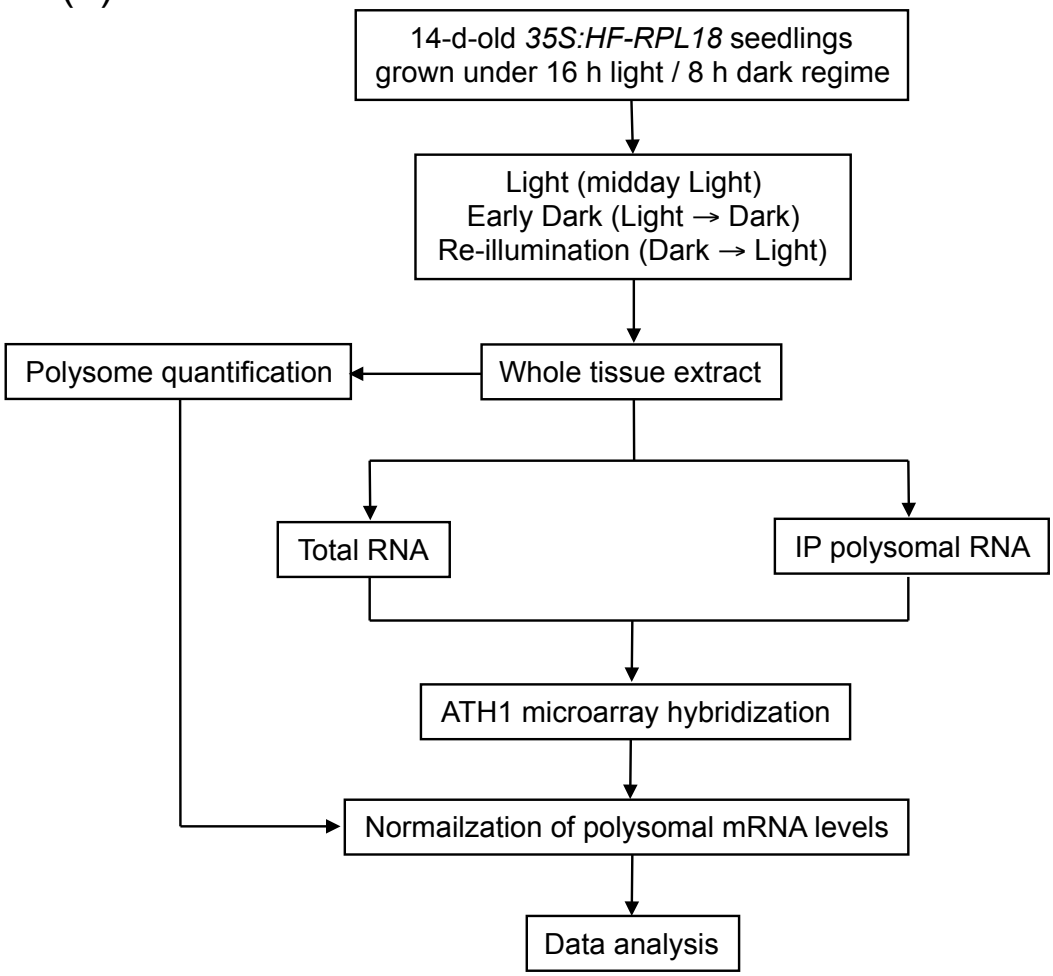

14-d-old 35S:HF-RPL18 seedlings grown under 16 h light / 8 h dark regime
Light (midday Light)
Early Dark (Light → Dark)
Re-illumination (Dark → Light)
Polysome quantification
Whole tissue extract
Total RNA
IP polysomal RNA
ATH1 microarray hybridization
Normailzation of polysomal mRNA levels
Data analysis

Figure 2.2. Reversible global response in protein synthesis to altered light availability and confirmation of microarray data for selected mRNAs (a) Quantitative sucrose density gradient analysis of ribosomal subunit and polysome complex levels. Whole seedlings from three biological replicate samples were pulverized under liquid nitrogen, hydrated in a membrane-disrupting and ribosome-stabilizing buffer, and centrifuged to obtain a ribosome pellet. The ribonucleoprotein complexes were re-suspended and separated by centrifugation through 20-60% sucrose density gradients. Absorbance profiles at 254 nm were recorded. The ribosomal subunit and polysome peak areas were quantified after adjustment of the absorbance profiles to equivalent total optical density units per gradient. Line traces show representative absorbance profiles of fractionated ribosomes of seedlings shifted to darkness for 1 h (D, red), re-illuminated for 10 min (R, grey) and maintained in the light (L, black). (b) Mean (in percent) cellular RNA content in polysome complexes, calculated from three replicate experiments. Error bars represent standard deviation and an asterisk indicates a significant difference from the seedlings maintained in the light, as determined by Student's *t*-test, $P < 0.05$. (c) mRNA obtained from sucrose density gradient fractionated polysomes evaluated by semi-quantitative reverse-transcriptase PCR with selected specific primer pairs using tissue from light (L), dark (D) and re-illuminated (R) seedling samples. (d) Monitoring of total and immunopurified polysomal mRNA levels by semi-quantitative reverse-transcriptase PCR of light (L), dark (D) and re-illuminated (R) seedlings. *LHCA4, LIGHT HARVESTING COMPLEX A4; RPL12, RIBOSOMAL PROTEIN 12A; ß-ATP, (MITO-CHONDRIAL ATP SYNTHASE ß-SUBUNIT.*

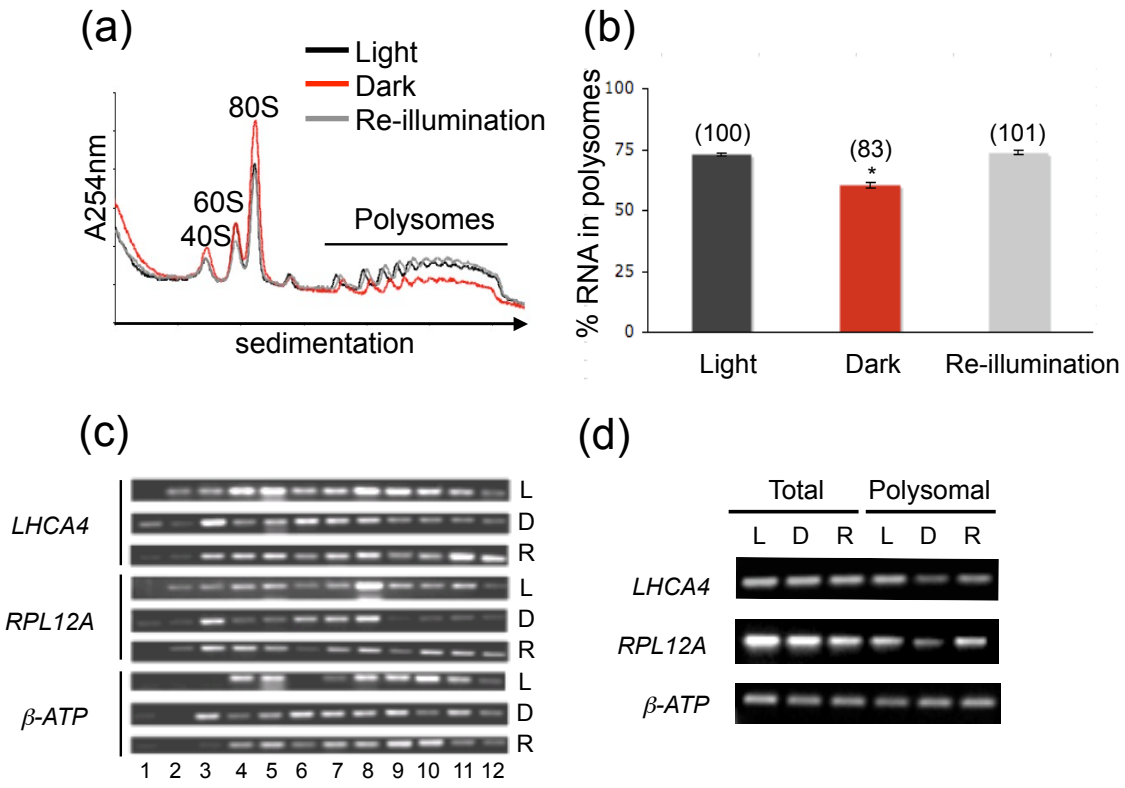

(a)
Light
Dark
Re-illumination
80S
60S
40S
Polysomes
A254nm
sedimentation
(b)
% RNA in polysomes
100
75
50
25
0
(100)
(83)
*
(101)
Light
Dark
Re-illumination
(c)
LHCA4
RPL12A
β-ATP
L
D
R
1 2 3 4 5 6 7 8 9 10 11 12
(d)
Total
Polysomal
L D R L D R
LHCA4
RPL12A
β-ATP

Figure 2.3. Comparison of abundance of total and polysomal mRNA in seedlings in the light, following transfer to early darkness, and after brief re-illumination. The treatment and harvest times for the seedling material was as indicated in Figure 2.1 a. Change in total steady-state mRNA abundance (*x*-axis, transcriptome) *versus* change in immunopurified polysomal mRNA (*y*-axis, translatome) A: (a) Comparison of transcriptome and translatome of seedlings in light to those shifted to darkness (Light→Dark) (b) Comparison of transcriptome and translatome of re-illuminated seedlings to those shifted to early darkness (Dark→Re-illumination) (c) Comparison of transcriptome and translatome in re-illuminated seedlings *versus* those in light. Each point on the graph represents the mean $\log_2$-transformed signal $\log_2$ ratio (SLR) value for an individual gene by comparison of two different treatments (*e.g*. SLR = $\log_2$ signal Treatment A gene i/$\log_2$ signal Treatment B gene i), using genes without an "absent" call in the bio-replicates (FDR < 0.05; n = 2,508 probe pair sets).

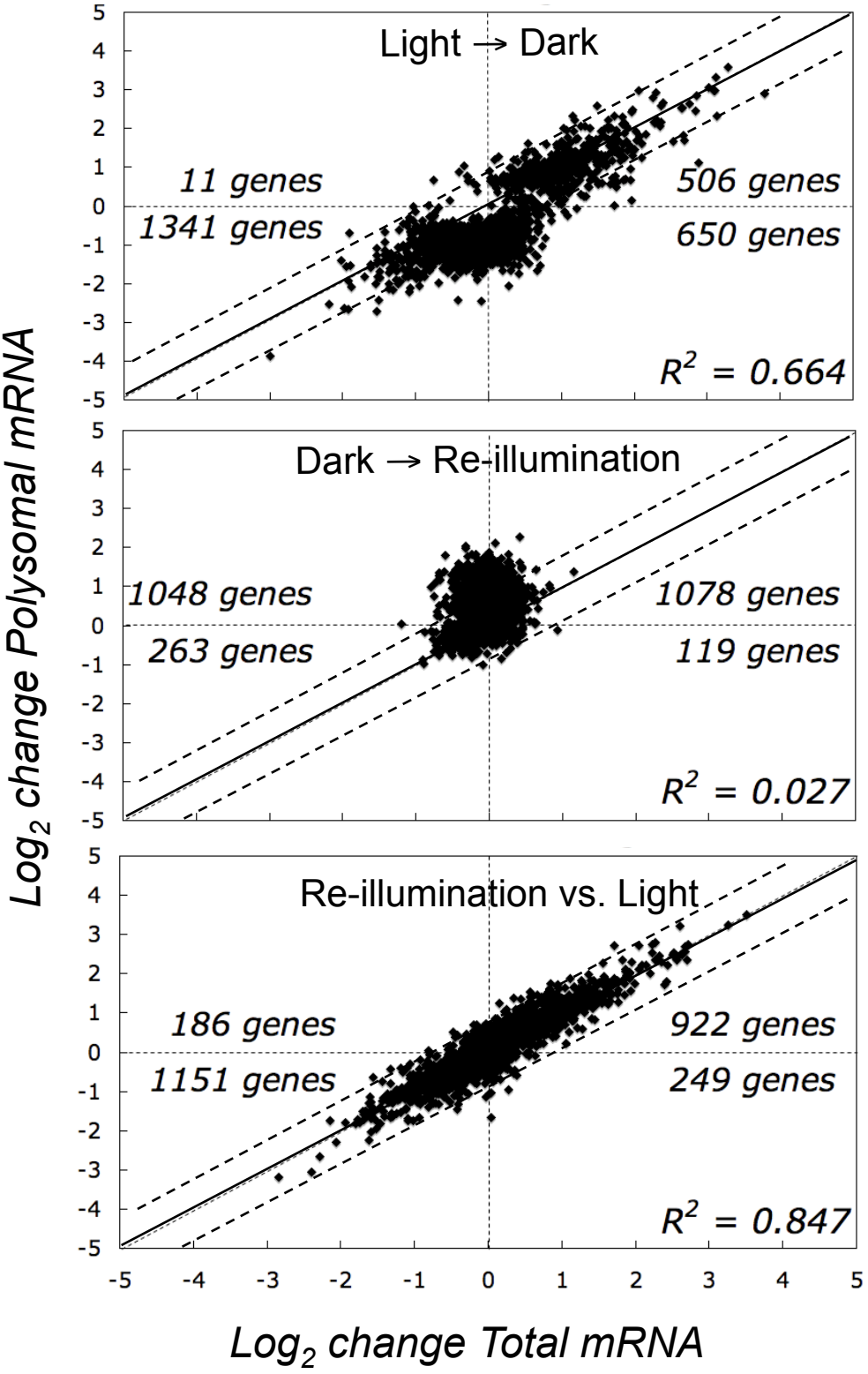

Light → Dark
11 genes
506 genes
1341 genes
650 genes
R^2 = 0.664
Dark → Re-illumination
1048 genes
1078 genes
263 genes
119 genes
R^2 = 0.027
Re-illumination vs. Light
186 genes
922 genes
1151 genes
249 genes
R^2 = 0.847
Log_2 change Polysomal mRNA
Log_2 change Total mRNA

Figure 2.4. Comparison of changes in mRNA abundance in the transcriptome and translatome. Venn diagrams indicate the number of genes in the total and immunopurified polysomal mRNAs that displayed a significant increase or decrease in abundance following the shift to early darkness or re-illumination. mRNAs analyzed included those without any "absent" calls in the bio-replicates and with a significant increase or decrease in abundance in total or polysomal mRNA (|SLR|>1; FDR < 0.05; n = 1,224 probe pair sets).

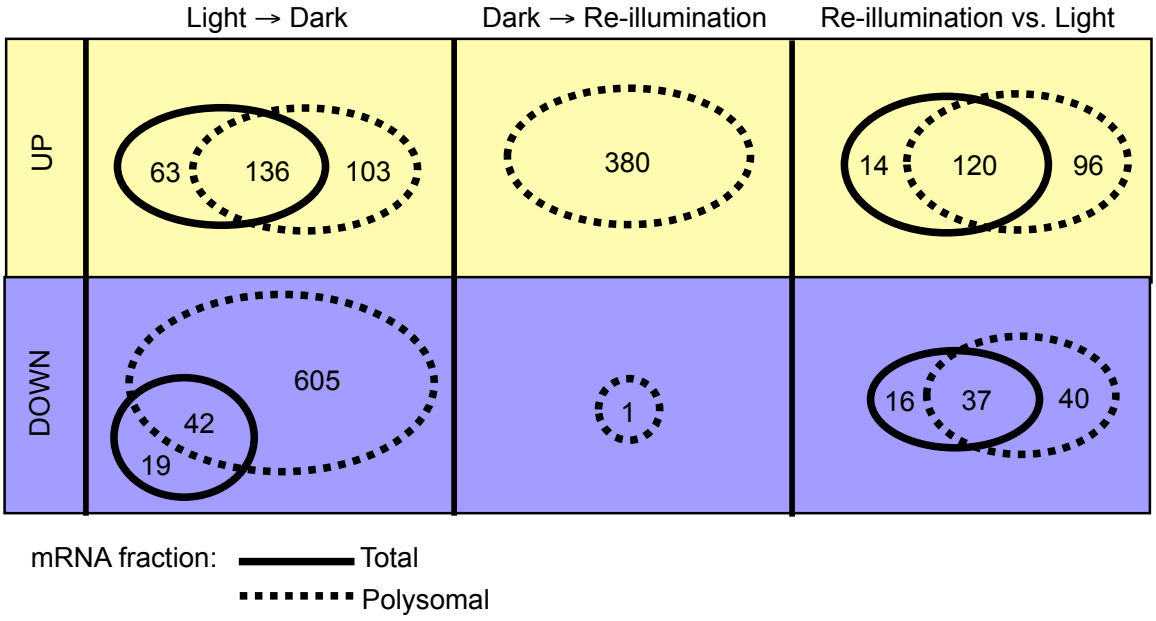


Figure 2.5. Light availability alters both transcript accumulation and translation status. Fuzzy *k*-mean clustering analysis performed on six mean signal log ratio (SLR) comparisons of total and immunopurified polysomal mRNA transcript abundance. Genes included in the analysis were those without an "absent" and significantly different in abundance (FDR < 0.05; n = 2,508 probe pair sets). The comparisons and genes evaluated are the same as those represented in Figure 2.3 (Supplemental Table 2.1). The heatmap shows median SLR values for each of ten groups (Clusters) of mRNAs that displayed similar responses to light availability based on the total and polysomal mRNA populations; color indicates increase (yellow), decrease (blue) or no change (black) in mRNA abundance. Columns indicate cluster ID, number of genes (No. genes)

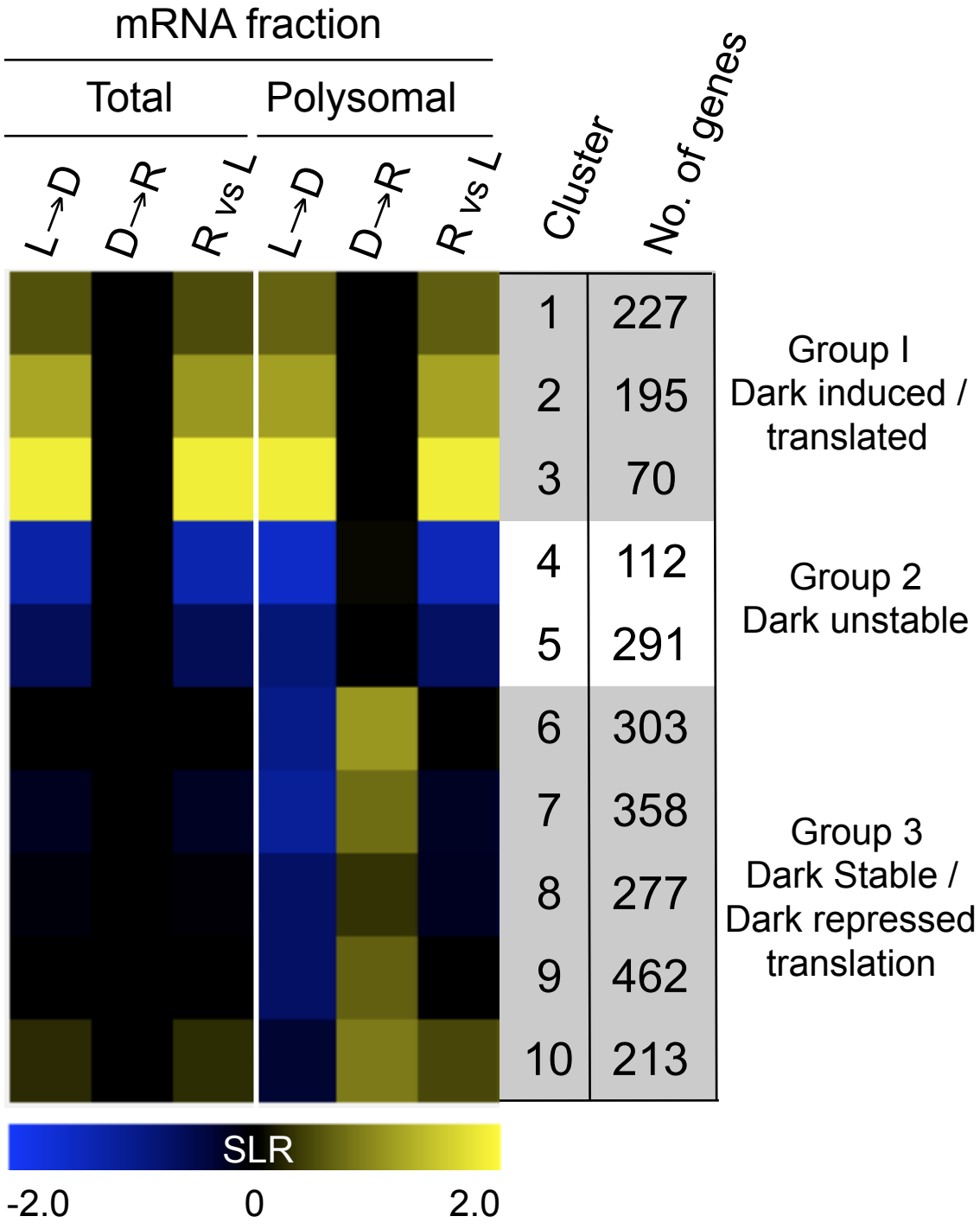

mRNA fraction
Total
Polysomal
L→D
D→R
R vs L
L→D
D→R
R vs L
Cluster
No. of genes
1
227
2
195
3
70
4
112
5
291
6
303
7
358
8
277
9
462
10
213
Group I
Dark induced /
translated
Group 2
Dark unstable
Group 3
Dark Stable /
Dark repressed
translation
SLR
-2.0
0
2.0

Figure 2.6. Light availability influences co-regulation of genes encoding proteins with similar molecular function. Analysis of genes differentially expressed at the level of transcript abundance or translational status by use of the PAGEMAN tool. The genes are the same as those represented in Figures 2.3 and 2.5. Significant co-regulation was determined by the Wilcoxon rank sum test with the Benjamini-Hochberg FDR control (Usadel *et al.*, 2005, 2006) Significant categories were collapsed for display. Statistical differences are represented by a false color heatmap; color indicates increase (yellow), decrease (blue) or no change (white) in mRNA abundance, where a z-score of 1.96 represents a FDR < 0.05.

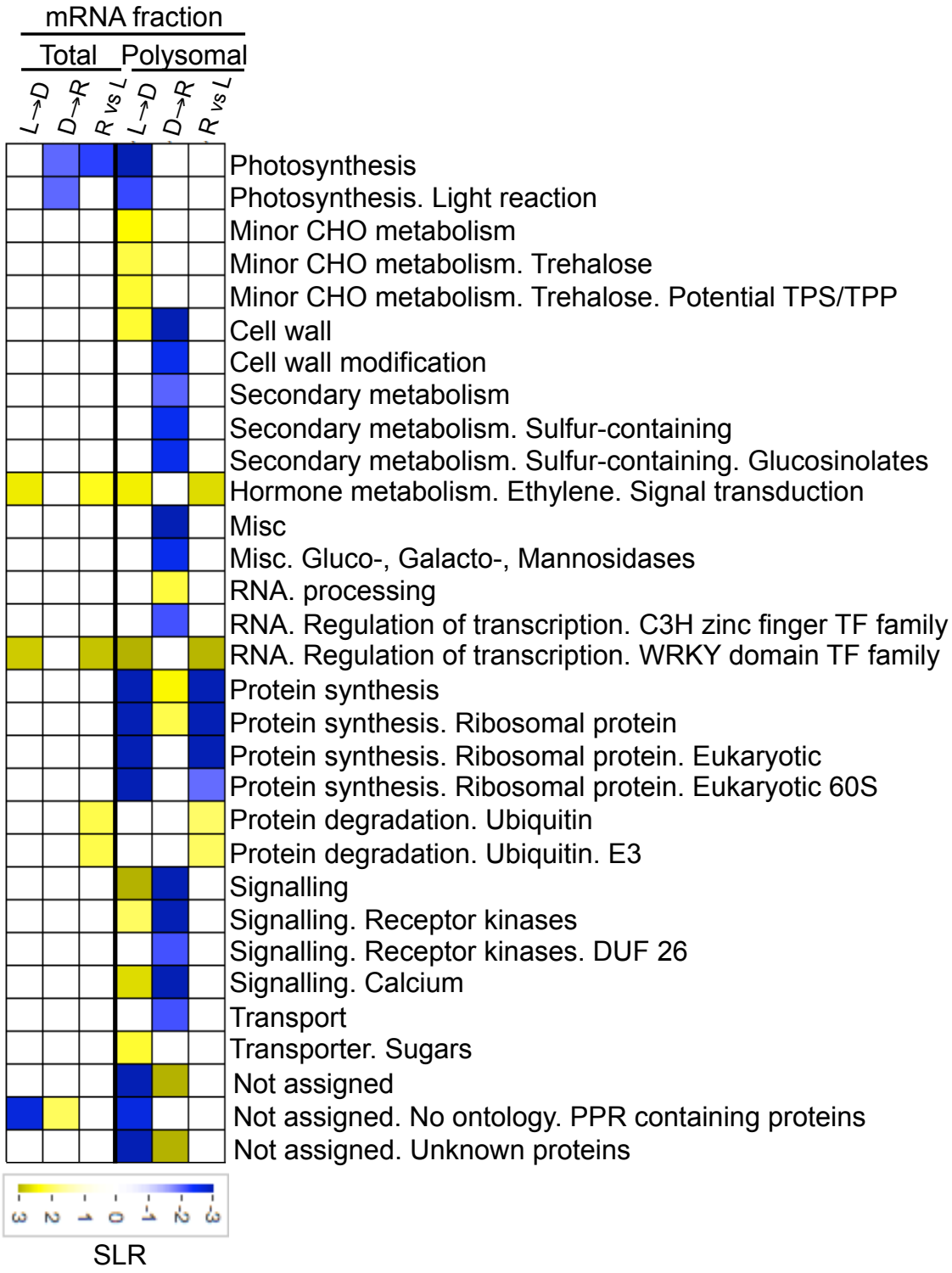

mRNA fraction
Total
Polysomal
L→D
D→R
R vs L
L→D
D→R
R vs L
Photosynthesis
Photosynthesis. Light reaction
Minor CHO metabolism
Minor CHO metabolism. Trehalose
Minor CHO metabolism. Trehalose. Potential TPS/TPP
Cell wall
Cell wall modification
Secondary metabolism
Secondary metabolism. Sulfur-containing
Secondary metabolism. Sulfur-containing. Glucosinolates
Hormone metabolism. Ethylene. Signal transduction
Misc
Misc. Gluco-, Galacto-, Mannosidases
RNA. processing
RNA. Regulation of transcription. C3H zinc finger TF family
RNA. Regulation of transcription. WRKY domain TF family
Protein synthesis
Protein synthesis. Ribosomal protein
Protein synthesis. Ribosomal protein. Eukaryotic
Protein synthesis. Ribosomal protein. Eukaryotic 60S
Protein degradation. Ubiquitin
Protein degradation. Ubiquitin. E3
Signalling
Signalling. Receptor kinases
Signalling. Receptor kinases. DUF 26
Signalling. Calcium
Transport
Transporter. Sugars
Not assigned
Not assigned. No ontology. PPR containing proteins
Not assigned. Unknown proteins
SLR

Figure 2.7. Regulation of mRNA stability and translation status contributes to energy conservation upon transfer to early darkness. Pie charts in (a) and (b) represent the percentage of total cellular mRNAs in seedlings in the light, as determined from average signal values. (a) The proportion of total cellular mRNA of contrastingly regulated gene clusters; differential expressed gene (DEG) mRNAs in Clusters 1-3 and 4-5 shown in Figure 2.5. (b) Proportion of total cellular mRNA of the three groups of DEGs represented in Figure 2.5. (c) Pie chart represents the percentage of cellular mRNA content in Clusters 6-10 (19% of the total cellular mRNA in the light) that encodes genes annotated as chloroplast localized, chloroplast ribosomal protein (RP), cytosolic or mitochondrial ribosomal protein (Other RP) or some other category of molecular function or location. (c) Calculated from Clusters 6-10 mRNAs. (d) Proportion of total cellular mRNA of the four groups of genes evaluated in (c).

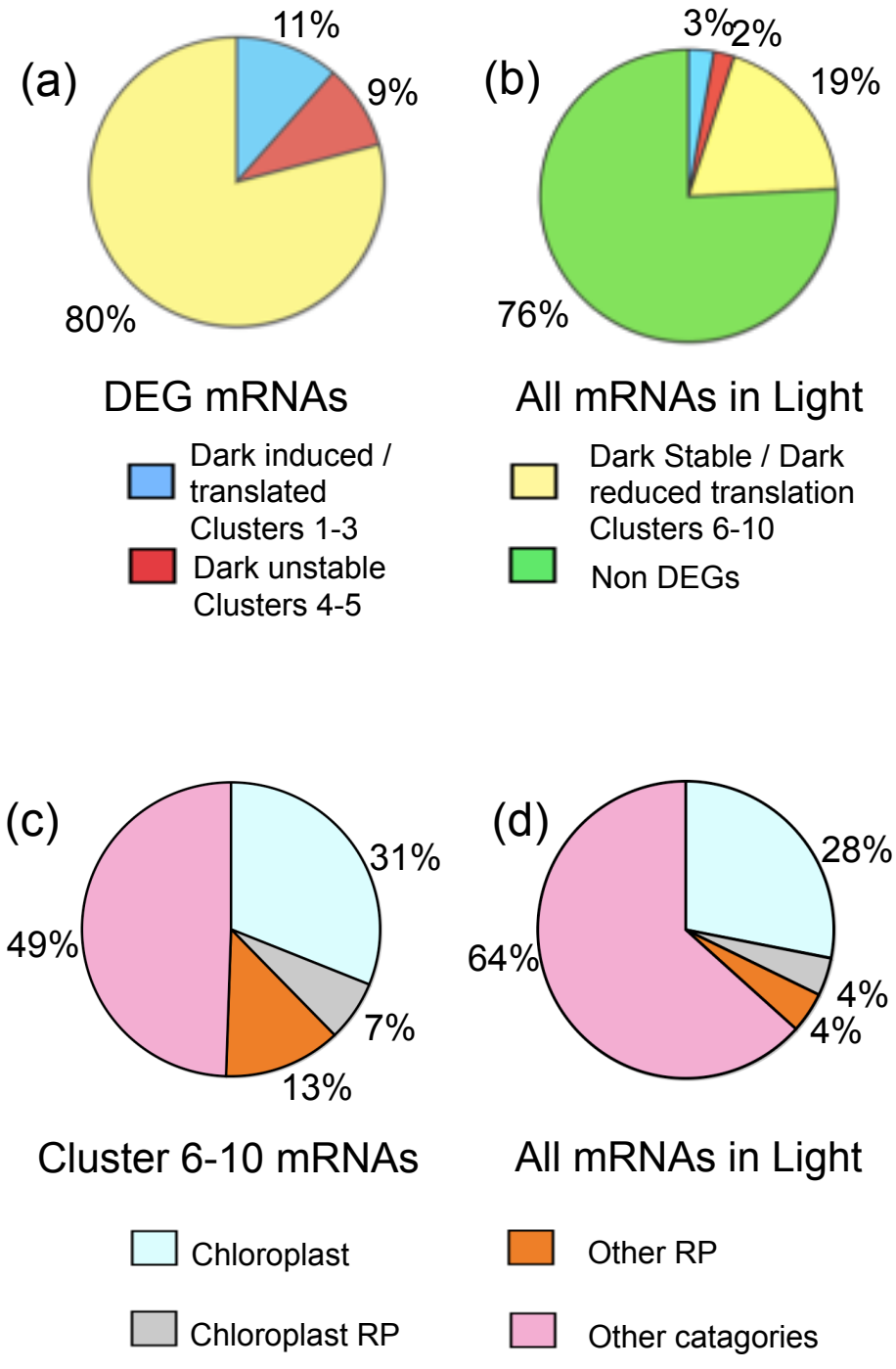

(a)
11%
9%
80%
DEG mRNAs
Dark induced / translated Clusters 1-3
Dark unstable Clusters 4-5
(b)
3%
2%
19%
76%
All mRNAs in Light
Dark Stable / Dark reduced translation Clusters 6-10
Non DEGs
(c)
31%
49%
7%
13%
Cluster 6-10 mRNAs
Chloroplast
Chloroplast RP
(d)
28%
64%
4%
4%
All mRNAs in Light
Other RP
Other catagories

Figure 2.8. 5'- and 3'-UTR over-represented motifs of dark stabilized and translationally repressed chloroplast and protein synthesis machinery (PS) mRNAs as analyzed by MEME suite. Forty-four chloroplast mRNAs from Cluster 6 and 120 ribosomal protein and translation factor mRNAs from Clusters 6, 7, 8 and 9 were independently analyzed for over-represented sequences using MEME. This identified a consensus sequence present in the 3'-UTR of dark-regulated and translationally-repressed mRNA encoding proteins targeted to the chloroplast (a) and a consensus present in the 5'-UTR of ribosomal protein and translation factor mRNAs (b). Relative motif occurrence rate identified by FIMO in the whole transcriptome (all genes), 2,508 light regulated mRNAs (DEGs) and 44 chloroplast mRNAs in Cluster 6 (c) and protein synthesis machinery mRNAs in Clusters 6-9 (PS Cluster 6-9). Number in brackets above each category indicated the q-value for motif-enriched discovery. The numbers (n) of 5' or 3' UTR used for each analysis were varied based the availability of the 5' or 3' UTR sequences for each mRNA. (d). Distribution by Cluster of DEGs with the 3'-UTR chloroplast mRNA motif or (e) the 5'-UTR protein synthesis machinery mRNA motif (f).

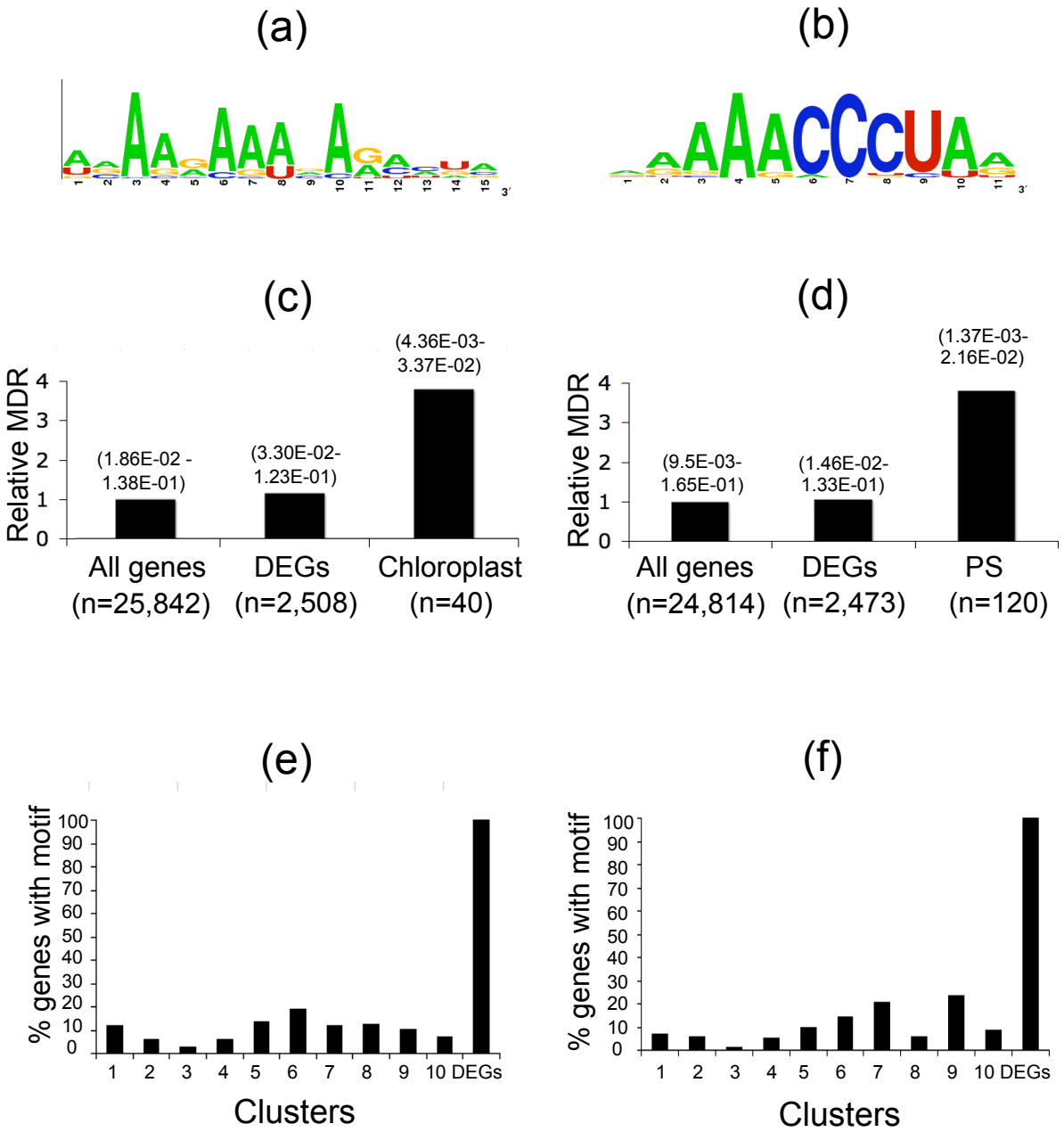

(a)
(b)
(c)
Relative MDR
(1.86E-02 - 1.38E-01)
(3.30E-02- 1.23E-01)
(4.36E-03- 3.37E-02)
All genes (n=25,842)
DEGs (n=2,508)
Chloroplast (n=40)
(d)
Relative MDR
(9.5E-03- 1.65E-01)
(1.46E-02- 1.33E-01)
(1.37E-03- 2.16E-02)
All genes (n=24,814)
DEGs (n=2,473)
PS (n=120)
(e)
% genes with motif
Clusters
(f)
% genes with motif
Clusters

Table 2.1. List of primers and RT-PCR condition used for validation of microarray analysis

Name	***AGI***	***Primer sequence***	***PCR size (bp)***	***Annealing temperature/ cycle***
LHCA4	At3g47470	Forward 5'-*ATG GCT ACT GTC ACT ACT CAT GCC TC*-3' Reverse 5'-*GAT TCC GAT CTT GGT GAA AAC TTC CG*-3'	350	54 °C/ 23 cycles
RPL12A	At2g37190	Forward 5'-*GTT TTC GGA ACT TCG ATC GTA* G-3' Reverse 5'-*CCA AAA CTG ACT TGT CGT TG*-3'	456	52 °C / 24 cycles
ATP SYNTHASE ß-SUBUNIT	At5g08670	Forward 5'-*GAT CAT GAC ATC TCT CGA GG*-3' Reverse 5'-*TGG TAA GGA GCA AGG AGA TC*-3'	348	54 °C / 27 cycles

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Chapter 3

Characterization of *Arabidopsis* Cold Shock Protein Complexes

3.1 Abstract

The four *Arabidopsis thaliana* Cold Shock Proteins (CSPs) contain a highly conserved cold-shock DNA/RNA binding domain (CSD) found in bacterial and eukaryotic species. Recent studies associate the CSD-containing protein of non-plant species with the regulation of translation of individual mRNAs. To survey the proteins found in association with the CSPs, we generated transgenic *Arabidopsis thaliana* (Col-0) lines that over-express His_6-FLAG-tagged versions of the four CSPs. Fractionation of cellular complexes by differential centrifugation identified CSP1 as the only one of the four that co-purified with ribosomes. CSP1, CSP2, CSP3, and CSP4 were immunopurified by use of an anti-FLAG agarose resin. To identify CSP-associated proteins, these immunoprecipitates were subjected to on-bead trypsin digestion followed by Liquid Chromatography (LC), Electron Spray Ionization (ESI) Quadrupole Time-of-Flight (Q-TOF) mass spectrometry analysis. To validate the proteomic data, fluorescent protein-tagged CSP1, CSP2, CSP3 and putative CSP-associated proteins were transiently expressed in *Nicotiana benthamiana* leaves to evaluate sub-cellular protein localization and co-localization. The results indicate CSPs reside in the nucleus and cytoplasm in association with RNA binding proteins involved in post-transcriptional processes

including pre-mRNA splicing, polyadenylation, and translation. The co-immunopurification of CSPs, glycine-rich RNA binding proteins (GR-RBP) and tetratricopeptide repeat proteins (TPRs) indicates a network involving these three classes of RBPs.

3.2 Introduction

Gene expression is a multifaceted process involving the regulation of chromatin structure, DNA methylation, initiation of transcription, and complex post-transcriptional processes. The modulation of pre-mRNA capping, splicing and polyadenylation, followed by mRNA quality control, transport, circularization and translation or storage, and finally degradation can each subtly and significantly impact production of the gene protein. A cadre RNA binding proteins (RBPs) play roles in the regulation of these processes by forming mRNA-ribonucleoprotein (mRNP) complexes of diverse function either in the nucleus or cytoplasm. RBPs usually contain one or more RNA binding domains, for example, the RNA Recognition Motif (RRM, RBD or RNP domain), K Homology (KH) domain, Zinc-finger, RGG box, DEAD/DEAH box, Pumilio (PUF) domain, or pentatricopeptide repeat (PPR) (Bailey-Serres *et al.*, 2009). In many cases RBPs contain one or more of the same or different RNA binding elements that make up a specialized domain, such as the Cold Shock Domain (CSD).

The Cold Shock Proteins (CSPs) were initially characterized as cold-shock-inducible proteins in *Escherichia coli* (Newkirk *et al.*, 1994; Schindelin *et al.*, 1994; Yamanaka *et al.*, 1998). Bacterial CSPs contain a highly conserved 70-amino-acid

DNA/RNA binding domain called the CSD that forms a beta-barrel structure with five anti-parallel beta-strands (Graumann & Marahiel, 1996; Yamanaka *et al.*, 1998). The CSD contains two consensus RNA-binding structures, RNP1 and RNP2 that facilitated nucleic acid binding (Newkirk *et al.*, 1994; Schindelin *et al.*, 1994; Schröder *et al.*, 1995). Low-temperature causes the stabilization of secondary structures in DNA and RNA, resulting in reduced efficiency of transcription and translation. In bacteria, these secondary structures are relaxed by cold induced CSPs (Phadtare *et al.*, 1999). Additional studies have shown that bacterial CSPs can function as RNA chaperones that unwind RNA secondary structure (Jiang *et al.*, 1997) and as transcription anti-terminators (Bae *et al.*, 2000).

Eukaryotic CSD-containing proteins typically contain an N-terminal CSD and C-terminal auxiliary domains. For example, mammalian Y-box binding protein-1 (YB-1) contains a single CSD and four basal/aromatic repeats. Studies show that YB-1 is a component of nucleocytoplasmic mRNPs involved in pre-mRNA splicing, and regulation of mRNA stability and translation (Matsumoto & Wolffe, 1998; Evdokimova & Ovchinnikov, 1999; Sommerville, 1999; Kohno *et al.*, 2003). In *Xenopus laevis*, the YB-1 ortholog, FRGY2 is a component of maternally stored mRNPs in oocytes (Murray *et al.*, 1992) that is required for translational repression and stabilization of maternally produced mRNAs (Matsumoto *et al.* 1996; 2003). Another CSD protein, *Caenorhabditis elegans* Lin-28, is a cytoplasmic RBP consisting of one CSD and two CCHC-zinc-finger motifs (Balzer & Moss, 2007). Lin-28 contributes to the regulation of microRNA (miRNA) metabolism by binding to the *let-7* pre-miRNA and blocking production of its

mature miRNA (Viswanathan *et al.*, 2008). In addition, Lin-28 co-fractionates with polysomes and binds to *IGF2* mRNA, which promotes the synthesis of the IGF2 (Polesskaya *et al.*, 2007). Together these observations implicate CSPs in several post-transcriptional activities.

The *Arabidopsis thaliana* genome encodes four CSD-containing proteins, Cold Shock Protein 1 to 4 (encoded by CSPs 1-4; At4g36020, At4g38680, At2g17870, At2g21060, respectively), each of which contains a CSD as well as C-terminal glycine-rich and zinc-finger domains (Chapter 1, Figure 1.3) (Karlson & Imai, 2003). Levels of *CSP* transcripts were significantly elevated by low temperatures (Karlson & Imai, 2003; Park *et al.*, 2009; Kim *et al.*, 2009), leading to the suggestion that CSPs function as RNA chaperones to aid adaptation during cold acclimation (Nakaminami *et al.* 2006; Kim *et al.* 2009). In support of this, heterologous expression of *CSP1* and *CSP3* rescues the cold sensitive phenotype of *E coli* strain BX04 that lacks four native *CSPs* (Kim *et al.* 2007; Kim *et al.* 2009) and over-expression of *CSP1* and *CSP2* in *Arabidopsis* rescued a cold-sensitive *atgrp7-1* (At2g21660) mutant from freezing damage (Park *et al.*, 2009). Moreover, the *atcsp3-2* knockout mutant is more sensitive to freezing than Col-0 (wild-type) under cold acclimated and non-acclimated conditions whereas the over-expression of *CSP3* conferred freezing resistance (Kim *et al.*, 2009). It has also been reported that *AtCSP2* and *At*CSP*4* transcripts accumulate in developing embryos, shoot apices and during floral induction (Fusaro *et al.*, 2007; Nakaminami *et al.*, 2009; Park *et al.*, 2009), suggesting they also function in a developmental context. Indeed, *AtCSP1* and *AtCSP2* overexpression lines showed delayed or accelerated seed germination under various

stress conditions (Park *et al.*, 2009). Other than their ability to unwind RNA and DNA helices, the functional roles of CSPs and the mRNAs they regulate remain largely unknown.

We hypothesized that CSPs are multi-functional and involved in post-transcriptional regulation under both stress and non-stress conditions. In *Chlamydomonas reinhartdii*, a CSD-containing protein, NAB1, differentially binds to *LIGHT HARVESTING COMPLEX BM (LHCBM)* mRNAs, sequestering it from the translational machinery under high light conditions (Mussgnug *et al.*, 2005). Based on the role of NAB1 in *Chlamydomonas* and the finding of light-modulated translation in *Arabidopsis* (Chapter 2), it was hypothesized that CSPs may function in light-regulated mRNA translation in higher plants. To investigate this possibility, I sought to determine if any of the CSPs co-fractionate with ribosomes, making them candidates for regulators of translation of individual mRNAs. The goal of this chapter was to produce transgenic *Arabidopsis* plants that over-express epitope-tagged CSPs, characterize the fractionation of the CSP complexes relative to ribosomal subunits and polysomes, and to determine the proteins that co-immunopurify with CSPs. The results suggest that CSPs are involved in diverse post-transcriptional activities, based on their association with RBPs and proteins that govern splicing, 3'-end cleavage and polyadenylation, and ribosomal RNA processing. Of the four CSPs, only CSP1 co-fractionated primarily with ribosomes. Some of the cellular CSP1 and CSP2 could be co-purified and co-localized, suggesting these CSP domains are connected within the post-transcriptional regulatory network.

3.3 Material and Methods

3.3.1 Plant material and growth conditions

The *Arabidopsis thaliana* ecotype Col-0 and *Nicotiana benthamiana* plants were used. Lines were bulked by growth of seeds in soil (Sunshine Mix LC1, JM McConkey, Sumner, WA) plus 150 g Osmocote 14-14-14 fertilizer (Scotts catalog #90036) and 75 g Marathon pesticide (Crop production services, Riverside, CA) per 3.8 ft^3 of Sunshine Mix LC1) in a controlled environmental growth room (16 h at ~100 μE light: 8 h dark at 23 °C).

Seeds were surface sterilized in a 15 mL Falcon tube with 10 mL 95% (v/v) ethanol for 5 min, 10 mL 20 % (v/v) household bleach plus 0.1 % (v/v) Tween-20 for 5 min, rinsed three times with 10 mL sterile water, and stratified in 10 mL sterile water at 4 °C for 48 h under darkness. Seeds were plated in 100 x 15 mm Petri dishes on sterile solid Murashige and Skoog (MS) medium containing 0.43 % (w/v) MS salts (Caisson Laboratories, North Logan, UT), 1 % (w/v) Sucrose, 1 % (w/v) Agar [pH 5.7]. The plates were oriented vertically in a growth chamber (model CU-36L5C8, Percival Scientific Inc.) under 16 h at ~50 μE light: 8 h dark at 23 °C.

3.3.2 Generation of *35S:AtCSP1-FH, 35S:HF-AtCSP2, 35S:HF-AtCSP3* and *35S:HF-AtCSP4* transgenic lines

CSP1 (*At4g36020*), *CSP2* (*At4g38680*), *CSP3* (*At2g17870*), and *CSP4* (*At2g21060*) full-length open reading frames were generated by RT-PCR from

Arabidopsis ecotype Col-0 seedlings by use of gene specific primers: CSP1 forward 5'-*CAC CAT GGC TTC AGA GGA TC*-3' and CSP1 reverse 5'-*TTA AGC TAC AGA AGA ACA TTC CCT TG*-3'; CSP2 forward 5'-*CAC CAT GAG CGG AGA CAA CG*-3' and CSP2 reverse 5'-*TTA ACG TCC ACC GCT GG*-3'; ATCSP3 forward 5'-*CAC CAT GGC GAT GGA AGA TCA ATC*-3' and ATCSP3 reverse 5'-*TTA AGC AAC CGA AGT ACA TTC C*-3'; CSP4 forward *5'-CAC CAT GAG CGG AGG AGG AG-3'* and CSP4 reverse *5'-TCA ACG AGC ACC ACC G-3'*. Total RNA was extracted from 10-d-old seedlings (see "Materials and Methods", Chapter 2). cDNA was synthesized from 400 ng total RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) using oligo d(T). PCR amplification was performed with gene specific primers by use of PrimeSTAR HS DNA polymerase (Takara-Bio Inc., Madison, WI) according to the manufacturer's protocol. PCR products were separated on a 1 % (w/v) agarose Tris-acetate gel. DNA bands were excised and gel purified with QIAquick gel extraction kit (Qiagen, Valencia, CA). The PCR products were cloned into pENTR/D-TOPO vector (Invitrogen), transformed into One Shot TOP10 Chemically Competent *E. coli* (Invitrogen) and selected on LB agar plates containing 50 μg/mL kanamycin. Verified clones were recombined into the p35S:GATA-FH or p35S:HF-GATA, Gateway compatible vectors (Mustroph *et al.*, 2010), transformed to *E coli* strain *DH5α* and selected on LB agar plates containing 50 μg/mL chloramphenicol. The vectors provided a N-terminal His-FLAG (HF) tag ($M(H)_6(G_3DYKDDDDK(G)_7$) or the C-terminal FLAG-His (FH) tag ($(G)_7DYKDDDDK(G)_3(H)_6$), respectively. Four constructs were generated: C-terminal HF-tagged CSP1 (p35S:AtCSP1-FH), N-terminal HF-tagged CSP2 (p35S:HF-AtCSP2),

N-terminal HF-tagged CSP3 (p35S:HF-AtCSP3) and N-terminal HF-tagged CSP4 (p35S:HF-AtCSP4) in a Ti binary plasmid with a neomycin phosphotransferase II (NPTII) gene providing kanamycin resistance. The CaMV 35S promoter, the TMV Ω 5'-untranslated leader, and coding sequence of each CSP were confirmed by sequencing both strands of the construct (Institute of Integrative Genome Biology, University of California, Riverside). For plant transformation, plasmid DNA was electroporated into *Agrobacterium tumefaciens* strain *GV3101* and selected on LB agar plates containing 50 µg/mL chloramphenicol. *Arabidopsis* Col-0 ecotype transformation was performed by the floral dip method (Clough & Bent, 1998). Briefly, *A. tumefaciens* cultures were grown overnight at 28 °C and pelleted by centrifugation at 5,000 *g* for 20 min at room temperature. The cell pellet was resuspended in 5 % (w/v) sucrose and 0.02 % (v/v) Silwet L-77 to a final concentration of *circa* 0.8 OD600. Six-week old plants were floral-dipped in the *Agrobacterium* medium for 10 sec, laid horizontally and left in the dark for overnight before returning to the growth room. Seeds were collected and selected on MS agar plates with 1% (w/v) sucrose plus 50 µg/mL of kanamycin. Kanamycin resistant seedlings were transferred to soil for propagation. T2 and T3 seedlings were used for biochemical and proteomic analysis.

3.3.3 Cellular fractionation by differential ultracentrifugation

Extraction of polysome and other mRNP complexes was based on the procedures of Zanetti *et al.* (2005) and Mustroph *et al.* (2009). Three mL pulverized frozen tissue was thawed in 7 mL polysome extraction buffer (200 mM Tris-HCl [pH

9.0], 200 mM KCl, 36 mM $MgCl_2$, 25 mM EGTA, 5 mM DTT, 1 mM PMSF, 50 ng/mL cycloheximide, 50 ng/mL chloramphenical, 1% (v/v) Triton X-100, 1% (v/v) Brij-35, 1% (v/v) Tween-40, 1% (v/v) NP-40, 1% (v/v) PTE). After removal of cell debris by centrifugation at 16,000 *g* for 20 min at 4 °C, the supernatant (Crude cell extract supernatant; S-16) was filtered through sterilized Miracloth (Calbiochem, La Jolla, CA). The pass-through supernatant was layered on top of an 8 mL 1.75 M sucrose cushion (400 mM Tris-HCl [pH 9.0], 200 mM KCl, 30 mM $MgCl_2$, 1.75 M sucrose, 5 mM DTT, 50 ng/mL chloramphenicol, 50 ng/mL cycloheximide) and centrifuged at 135,000 *g* for 18 h at 4 °C (70Ti rotor, Beckman, Brea, CA). This yielded the ribosome pellet (P-170) and the ribosome-depleted supernatant (S-170).

3.3.4 Protein separation and immunodetection

Proteins were separated in 12.5 % (w/v) Sodium Dodecyl Sulfate-polyacrylamide gels by electrophoresis (SDS-PAGE), transferred to Hybond ECL membranes (Amersham, Picastaway, NJ) and subjected to immunodetection with anti-FLAG horseradish-peroxidase (HRP) conjugated monoclonal antibody (1:1000; Sigma, St. Louis, MO) or a polyclonal antibody against maize (*Zea mays* L.) ribosomal protein S6 (RPS6) (1:10,000) (Williams *et al.*, 2003). Horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000; Biorad, Hercules, CA) was used as a secondary antibody. Visualization was performed with the ECL Plus chemiluminescence system according to the manufacturer's protocol (Amersham).

3.3.5 Immunoprecipitation of CSP complexes

The protocol developed for immunoprecipitation (IP) of ribosomes (Mustroph *et al.*, 2009) was modified to adopt a less disruptive extraction buffer. Approximately 0.5 mL frozen 10-d-old whole seedling tissue was thawed in 1 mL IP buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EGTA, 2 mM $MgCl_2$ and 0.2 % (v/v) NP-40 and 10 µL/mL Protease inhibitor cocktail for plants (Sigma)). The total extract was centrifuged at 16,000 *g* for 10 min at 4 °C. The supernatant was passed through Miracloth and incubated with 30 µL EZ-view anti-FLAG M2 agarose gel (Sigma) that was pre-washed twice with 1 mL IP buffer. The mixture was incubated at 4 °C for 2 h with gentle shaking. The unbound fraction was removed by centrifugation at 8,200 *g* and the beads were washed 4 times with 1 mL IP buffer for 5 min at 4 °C. The elution of protein complexes from the agarose-conjugated IgGs was achieved by incubation with 200 µL IP buffer that additionally contained 200 ng/µL 3x FLAG peptide (Sigma) for 30 min at 4 °C.

3.3.6 Mass Spectrometry and Proteomic analysis

Following immunoprecipitation, the beads were washed two additional times with 1 mL 50 mM NH_4HCO_3 [pH 7.8] and subjected to on-bead trypsin digestion. Briefly, 200 µL of 50 mM NH_4HCO_3 [pH 7.8] was added, followed by 24 µL of trypsin (100 ng/µL in 1 mM HCl; Promega, Madison, WI) and incubated overnight at 37 °C with gentle shaking. Mass Spectrometry (MS) was performed by use of Liquid Chromatography (LC), Electron Spray Ionization (ESI) Quadrupole–Time Of Flight (Q-

TOF) mass spectrometer (Waters, Milford, MA) in the W.M. Keck Proteomics Facility, Institute for Integrative Genome Biology, University of California, Riverside. Protein identification was performed using the National Center for Biotechnology Information (NCBI) non-redundant database for *Arabidopsis thaliana* by use of the Mascot MS/MS Ion Search algorithm (http://matrixscience.com (Perkins *et al.*, 1999)). The peptides identified in the immunopurified complexes of a mock IP sample were subtracted from those transgenic lines identified in and proteomics analysis performed with Col-0 tissue. All retained peptides had masses within 100 ppm of the calculated mass and a Mascot score of >30 or no non-plant peptides of the same mass were found in the NCBI database.

3.3.7 CSP tagging with fluorescent proteins, sub-cellular localization and co-localization in transiently transformed *Nicotiana benthamiana*

Ti binary plasmid expression constructs were prepared as follows: full-length open reading frames were generated by RT-PCR from *Arabidopsis* Col-0 seedling cDNA with gene specific primers: COR47 (At1g20440) forward 5'-*CAC CAT GGC TGA GGA GTA CAA GAA C*-3' and COR47 reverse 5'-*ATC ATC AGA CTC TTT TTC TTT CTT CAC*-3'; CSP2 (At4g38680) forward 5'-*CAC CAT GAG CGG AGA CAA CG*-3' and CSP2 reverse 5'-*ACG TCC ACC GCT GGT GCA ATC*-3'; AtRZ-1c (At5g04280) forward 5'-*CAC CAT GGC TGC AAA AGA AGG TAG*-3' and AtRZ-1c reverse 5'-*ATA ACG GTC AAA AGT GGA CG*-3'; CSIF2 (At4g25550) forward 5'-*CAC CAT GGC TAT GTC TCA GG*-3' and CSIF2 reverse 5'-*CGA ACT AAT CAT GTT GAA ATG GAA TC*-3'.

The PCR products were cloned into the pENTR/D-TOPO vector (Invitrogen) and transformed into TOP10 *E. coli* cells as described above. Verified clones were re-combined into the Gateway-compatible expression vector pGWB554 (Nakagawa *et al.*, 2007a, b) to generate C-terminal tagged monomeric Red Fluorescent Protein (mRFP) Ti binary plasmids carrying a hygromycin phosphotransferase (HPT) gene for hygromycin resistance. A pENTR/D-TOPO construct for CSP1 was recombined into the Gateway-compatible expression vector pEarleyGate101 (Earley *et al.*, 2006) to generate a C-terminal tagged Yellow Fluorescent Protein (YFP) Ti binary plasmid with a bialaphos resistance (BAR) gene for glufosinate (Basta) resistance. A pENTR/D-TOPO construct for CSP3 was recombined into the Gateway-compatible expression vector pEarleyGate104 (Earley *et al.*, 2006) to generate a N-terminal tagged YFP Ti binary plasmid with a BAR gene for Basta resistance.

The promoter, untranslated leader and coding sequence of each gene construct were confirmed by sequencing both strands. The verified clones were electroporated into *Agrobacterium* stain *GV3101* and selected on plates supplemented with 100 µg/mL spectinomycin for the mRFP constructs or 50 µg/mL kanamycin for the YFP expression constructs.

For *N. benthamiana* leaf infiltration, *Agrobacterium* stain *GV3101* cultures, each containing a Ti binary expression construct, were grown overnight at 28 °C in LB medium supplemented with the appropriate antibiotic and 20 µM acetosyringone. Cells were pelleted by centrifugation at 7,000 *g* for 15 min and resuspended in 10 mM MES

[pH 5.7], 10 mM $MgCl_2$ and 150 μM acetosyingone to a density of 1 OD600 unit and incubated at room temperature for 3 h. Four to 6-week-old leaves of *N benthamiana* plants were used for leaf infiltration by injection of the *Agrobacterium* culture into the adaxial leaf surface with a 3-mL syringe tip (without a needle). For co-localization of proteins, equal amounts of *Agrobacterium* cultures containing the desired expression constructs were mixed immediately before leaf infiltration and plants were returned to the growth room. Forty eight to 72 h later, injected leaves were vacuum infiltrated with 10 μg/mL 4', 6-diamidino-2-phenylindole (DAPI) solution for 20 min. Cells were visualized by a confocal laser scanning microscope (Leica SP2, Bannockburn, IL) at the Microscopy Core Facility, Institute for Integrative Genome Biology, University of California, Riverside. mRFP was visualized by excitation at 543 nm and emission at 590–630 nm. YFP was visualized by excitation at 514 nm and emission at 520-540 nm. DAPI stained nuclei were visualized with a UV laser by excitation at 350 nm and emission at 400-500 nm.

3.4 Results:

3.4.1 CSP1 co-fractionates with ribosomes

To investigate the role of CSPs, His-FLAG tagged versions of CSP1, CSP2, CSP3 and CSP4 were constructed under the control of the near-constitutive CaMV 35S promoter and introduced into the Col-0 ecotype of *Arabidopsis* (Figure 3.1). Stable transgenics were used to determine whether the ectopically expressed epitope-tagged CSPs fractionate with ribosomes or in the post-ribosome supernatant of detergent-

solubilized cell extracts from seedlings. This was accomplished by a series of differential centrifugation steps, yielding a crude cell extract supernatant (S-16), a ribosome pellet (P-170), and a ribosome-depleted supernatant (S-170) (Figure 3.2). The proteins of these fractions were separated by SDS-PAGE and immunodetection was performed with an anti-FLAG antibody to monitor the presence of the tagged CSP1. This analysis confirmed that epitope-tagged CSPs accumulated to detectable levels in the crude cell supernatant fraction. A greater proportion of CSP1-FLAG was present in the ribosome pellet fraction (P-170), whereas FLAG-CSP2 and FLAG-CSP3 fractionated in the post-ribosomal supernatant fraction (S-170) (Figure 3.3). The fractionation of FLAG-CSP4 was unclear, although this protein was later immunopurified from the crude cell supernatant (Figure 3.4). The same immunoblot was cross-reacted with an antiserum that recognizes ribosomal protein S6 (RPS6) to confirm the efficient depletion of ribosomes in S-170 fraction.

3.4.2 Proteomic analysis of CSP complexes identified RNA binding proteins and pre-mRNA processing factors

To explore the functional roles of CSPs, we preformed co-immunoprecipitations using *Arabidopsis* transgenic lines stably expressing FLAG-tagged CSPs. The immunopreciptations were performed using anti-FLAG agarose and cell extracts from seedlings in a manner similar to the method used to capture FLAG-tagged ribosome complex and associated mRNAs (Zanetti *et al.*, 2005). The immupurification procedure was also carried out with tissue from Col-0 seedlings as control for non-

specific binding of protein to the affinity matrix. Following the purification and SDS-PAGE separation of proteins, silver-staining of proteins and FLAG detection confirmed that each epitope-tagged CSP was present in the total cell extract and immunoprecipitated (IP) fractions, but not in the supernatant fraction that remained after immunoprecipitation (Unbound) (Figure 3.4). This result demonstrates that FLAG-CSPs can be efficiently affinity-purified. The silver-stained proteins of the CSP1 and Col-0 control were similar, despite the confirmation of the immunopurification of the epitope-tagged proteins. This indicates that there is some non-specific binding of proteins to the anti-FLAG agarose affinity matrix.

To investigate whether other proteins co-immunopurify with CSP1-FH, HF-CSP2, HF-CSP3, and HF-CSP4, the immunoprecipated proteins attached to the affinity matrix were digested with trypsin and the resultant peptides were separated by liquid chromatography (LC) and monitored by electro-spray ionization Quadruple-Time Of Flight MS (Q-TOF MS). Because of the non-specific binding of proteins to the anti-FLAG agarose, an immunopreipitation was performed with Col-0 as a control. The peptides obtained from the MS analysis of the tagged-CSP samples were compared to those of *Col-0* and any common peptides or proteins were disregarded as CSP-associated proteins. After this background subtraction, three to ten proteins were identified in the FLAG-CSP1, CSP2, CSP3, and CSP4 immunoprecipitates, in addition to the target protein. Based on the peptide analyses, the co-purifying proteins were primarily RBPs or proteins with known roles in post-transcriptional processes.

The proteins that co-immunopurified with CSP1 included Glycine-Rich Protein 3 short isoform (GRP3S; At2g05380), Cold-regulated 47 (COR47; At1g20440) and a putative pre-mRNA splicing factor (PRP8; At4g38780, At1g80070) (Table 3.1). The proteins that co-immunopurified with CSP2 included several putative RNA binding proteins, snRNP-G (At2g23930), PRP19-like protein (At2g33340), Fibrillarin 1 (FIB1, At5g52470), U2A' (At1g09760), RBP45B (At1g11650), and CSP1 (Table 3.2). The proteins that co-immunopurified with CSP3 included FIB1, as well as an unknown nucleic acid binding protein (At1g29250), COR47, a Glycine-rich RNA binding protein (AtRZ-1c, At5g04280), and Cleavage and polyadenylation factor 2 (CSIF2; At4g25550) (Table 3.3). The proteins that co-immunopurified with CSP4 included an uncharacterized Glycine-Rich Protein (At1g27090) and eukaryotic translation initiation factor 5A-2 (eIF5A-2; At1g01320) (Table 3.4). The predominance of RBPs in the co-purifying proteins strongly suggests the immunopurification method yielded biologically relevant associations with CSPs.

3.4.3 Sub-cellular localization of CSPs and confirmation of co-association of selected proteins

To investigate the sub-cellular localization of CSP1, a C-terminal YFP-tagged CSP1 (CSP1-YFP) was transiently expressed in *N. benthamiana* leaves by use of *Agrobacterium* infiltration. Confocal microscopy imaging confirmed that CSP1 was present in nuclei and granular cytoplasmic complexes in the leaf pavement cells (Figures 3.5 and 3.6). Within the nuclei, CSP1 accumulation was most prevalent within the

nucleolus. The cytoplasmic distribution of CSP1 included a general diffuse distribution as well as aggregates resembling the association of cytoplasmic RNPs in stress granules (SG) and/or processing bodies (PB) (Weber *et al.*, 2008). Since cellular hypoxia stress promotes formation of SG-like complexes (Weber *et al.*, 2008), we tested whether the restriction of oxygen availability caused by placing a seedling in water and between a glass slide and coverslip was sufficient to promoted movement of CSP1 into granular foci. Monitored immediately after mounting, cytoplasmic CSP1 distribution included few granular foci (Figure 3.6). However after 20 and 40 min the foci brightened and the diffuse distribution was reduced. This dynamic localization suggests that hypoxic stress induces the formation of cytoplasmic granules that contain CSP1.

To provide secondary validation of association between CSPs and other proteins, selected proteomic-identified interactors of CSP1 were tagged with mRFP (Protein-mRFP) and co-infiltrated with CSP1-YFP. COR47-mRFP accumulated in the cytoplasm but did not form granules that co-localized with YFP-CSP1 (Figure 3.7). Whereas both CSP2-mRFP and YFP-CSP1 displayed a unique pattern of cellular accumulation, both were found in the nucleus and cytoplasm (Figure 3.7 b-f), as confirmed by DAPI staining. High-resolution micrography revealed that CSP1 and CSP2 co-localize and accumulate in granules that form around the nucleus and in the cytoplasm (Figure 3.7 d and f).

We applied the same strategy to confirm co-association with CSP3. A transiently expressed CSP3-YFP-tagged fusion protein accumulated in both nuclei and

the cytoplasm (Figure 3.8 a-d). By contrast, mRFP-tagged AtRZ-1c and CSIF2 accumulated in nuclei (Figure 3.8 a-d), as confirmed by DAPI staining. The nuclear localization of CSP3 with these proteins was very similar, except that CSP3 staining extended beyond the nuclear periphery with both a diffuse and granular pattern of accumulation.

3.5 Discussion

3.5.1 Identification of putative *Arabidopsis* Cold Shock Protein Complexes

RBPs play important roles in the post-transcriptional processes of gene expression. The CSP-RBPs have been linked to mRNA chaperone and secondary structure removal activities in response to cold stress, during flowering and seed development (Nakaminami *et al.* 2006; Sasaki *et al.* 2007; Kim *et al.* 2009; Nakaminami *et al.* 2009; Park *et al.* 2009). However, the complexes they form and the processes they participate in have not been elucidated. In this study we generated transgenic *Arabidopsis* that ectopically over-express and accumulate His_6-FLAG-tagged-CSPs (Figures 3.1, 3.3-3.4). Cell fractionation under conditions that stabilize mRNP and polysome complexes followed by differential centrifugation led to the determination that CSP1 co-fractionated with ribosomes, whereas CSP2-4 fractionated in the post-ribosomal supernatant (Figure 3.3). By use of co-immunoprecipitation and proteomic analysis we identified CSP-associated proteins (Figure 3.4, Tables 3.2-3.3). Evaluation of sub-cellular distribution and confirmation of selected CSP / protein interactions was accomplished through the transient expression of fluorescently-tagged proteins and confocal

microscopy in tobacco (Figures 3.5 – 3.8). We found that CSPs accumulate in nucleoplasm, nucleolus, and cytoplasm in diffuse and focused distributions. Based on co-immunopurification, CSPs associate with RBPs that function in pre-mRNA and pre-rRNA processing, mRNA polyadenylation, and mRNA translation.

3.5.2 CSP1

CSP1 contains an N-terminal CSD and seven zinc-finger and glycine-rich motifs. Previously, CSP1 was proposed to act in chaperoning of mRNA and removal of mRNA secondary structure under low temperature stress (Karlson & Imai 2003; Kim *et al.* 2005; Park *et al.* 2009). In this study we showed that CSP1 co-fractionates with ribosomes and polysomes (Figure 3.3), is distributed in the nucleoplasm, nucleolus and cytoplasm, and becomes concentrated in cytoplasmic foci in response to hypoxia. Three associated proteins were identified by immunopurification of CSP1 from seedlings grown under control conditions, including two RBPs and a protein associated with cold and dehydration stress.

Our proteomic analysis of CSP1 immunoprecipitate identified the glycine-rich protein GRP3S, the dehydrin COR47 (SK_2 repeat type), and pre-mRNA processing factor 8 (PRP8). Although, there has been no report on GRP3S RNA-binding ability, with the association of this protein with Wall Associated Kinase 3 (WAK3) is linked to abiotic and biotic stress responses (Hou *et al.*, 2005). The function of dehydrins is not fully understood, although, several studies proposed that these dehydration- and cold-induced proteins function as molecular chaperones of cold-sensitive enzymes and protect lipid

membranes from peroxidation (Rorat, 2006). Hara *et al.* (2009) reported that a *Citrus unshiu* cold-regulated dehydrin (CuCOR15) can interact non-specifically with DNA and RNA, suggesting that it may function in stabilization of the nucleic acids during cold stress. Since the immunopurification study was performed on non-stressed seedling tissue, COR47 was present in limited abundance. It therefore seems reasonable to suggest that the co-purification of CSP1 with COR47 may indicate a function of a dehydrin in post-transcriptional regulation. We also identified the putative splicing factor PRP8 in association with CSP1. The PRP8 ortholog of *Saccharomyces cerevisiae* is a core component of the spliceosome that binds directly to RNA (Beggs *et al.*, 1995). Altogether, the analysis of CSP1 fractionation, sub-cellular localization, and associated proteins suggest that this member of the CSP family functions in multiple post-transcriptional processes, including pre-mRNA splicing and translation. This is reminiscent of the multi-functional mammalian CSD-protein YB-1, which participates in pre-mRNA splicing, regulation of mRNA stability, and translational control (Evdokimova *et al.*, 1995, 1998, 2001; Evdokimova & Ovchinnikov, 1999).

3.5.3 CSP2

CSP2 contains an N-terminal CSD and two zinc-finger glycine-rich motifs. Previously, CSP2 was proposed to act in chaperoning the mRNA under cold stress (Sasaki *et al.*, 2007) and in the regulation of flower and seed development (Fusaro *et al.*, 2007). In this study we found that CSP2 is not ribosome-associated and accumulates in a diffuse pattern in the nucleus and cytoplasm (Figure 3.3; Figure 3.7). The distribution of

CSP1 and CSP2 is similar, however CSP2 accumulation is more pronounced in the nucleoplasm, and less pronounced in the nucleolus, cytoplasm and the cytoplasmic granules. The immunopurification of CSP2 identified several associated RBPs, including four pre-mRNA processing factors (snRNP-G, PRP19, U2A', and RBP45B), a pre-rRNA processing factor (Fibrillarin 1), and CSP1.

The CSP2-associated proteins are all known to reside in the plant nucleus, consistent with the primarily detection of CSP2-mRFP within the nucleus (Figure 3.7). The CSP2-associated proteins included snRNP-G, a member of small nuclear RNP (snRNP) family that contains an RNA binding Sm and Sm-like domain that is important for spliceosome formation in yeast (Camasses *et al.*, 1998) and animals (Heinrichs *et al.*, 1992). Lorkovic *et al.* (2004) reported that *Arabidopsis* snRNP-G and other snRNPs localize to the nucleus, suggesting their functions are conserved in plants. The CSP2-interactor PRP19, is a member of the U-box family of E3 ubiquitin ligases. In *S. cerevisiae*, the ortholog of *Arabidopsis* PRP19 is required for pre-mRNA splicing processes (Chan & Cheng, 2005). Monaghan *et al.* (2009) showed that the *Arabidopsis* orthologs of PRP19, MAC3A and MAC3B, localize to the nucleus, interacts with putative spliceosomal protein MOS4. Intriguingly, MAC3 proteins serve some function in basal and R-mediated defense and regulate plant immune response (Monaghan *et al.*, 2009). CSP2 was also found associated with the U2-snRNP specific protein U2A'. This protein interacts with U2B" and plays an essential role in spliceosome assembly (Caspary & Séraphin, 1998). Lorković *et al.* (2004) demonstrated that *Arabidopsis* U2A' and U2B" reside in the nucleus of *Arabidopsis* mesophyll protoplasts. RBP45B is an oligo-

uridylate RBP. In *Nicotiana plumbaginifolia*, RBP45 was localized to the nucleus and shown to interact with nuclear poly(A)+ RNA (Lorković *et al.*, 2000). CSP2 also co-purified with an unknown RBP (At4g03110). A BLAST search of this protein against NCBI non-redundant protein database revealed a 39% identity to CUG-BP (CUG-binding protein), a regulator of cell-specific alternative splicing in human cardiac troponin T (cTNT) exon 5 (Ladd *et al.*, 2001). It is possible that the association of CSP2 with the product of At4g03110 is related to alternative-mRNA splicing. The predominate localization of CSP2 in the nucleus together with its association with several RBPs associated with pre-mRNA processing, provides strong support that CSP2 participates in the nuclear mRNA processing pathway.

However, the function of CSP2 may also extend to processes in the nucleolus and cytoplasm. Our confocal imaging indicated this protein is also present within the nucleolus and cytoplasm, although at levels lower than the nucleoplasm. Moreover, CSP2 was found associated with the pre-rRNA processing protein, Fibrillarin 1. The *Arabidopsis* genome encodes two *FIBRILLARIN* genes (Fibrillarin 1-2, At5g52470 and At5g52490, respectively) Fibrillarin 1 and 2 display functions similar to *S. cerevisiae* fibrillarin protein (Nop1p) in the binding of small nucleolar RNA (snoRNA) (Barneche *et al.*, 2000). Finally, CSP2 co-localized and co-purified with CSP1. The association of these proteins was confirmed by transient co-expression of FP-tagged proteins. Future studies are needed to investigate the molecular basis and significance of the association of CSP1 and CSP2.

3.5.4 CSP3

CSP3 contains an N-terminal CSD and C-terminal seven zinc-fingers and glycine-rich motifs. This structure is similar to CSP1, which shares 61% amino acid sequence identity. As for CSP1, previous studies predicted that CSP3 plays a role in the chaperoning of mRNA necessary for freezing tolerance (Kim *et al.*, 2009). Similar to CSP1, we found that CSP3 is distributed in the nucleoplasm, nucleolus and cytoplasm (Figure 3.8), however unlike CSP1, CSP3 did not co-purify with ribosome complexes (Figure 3.8). We also succeeded in identifying proteins that co-immunopurify with CSP3, including several putative RBPs. Notably, the dehydrin COR47 was identified as a CSP1- and CSP3-associated protein in the co-immunoprecipitates of seedlings grown under control growth conditions. The other CSP3-associated proteins were not identified in the analysis of CSP1. These included the Cleavage and polyadenylation factor 2 (CSIF2), the ortholog of mammalian CFim25 (Cleavage Factor Im 25 kDa) (Hunt *et al.*, 2008), that is involved in poly(A) site selection (Kubo *et al.*, 2006). *Arabidopsis* CSIF1, a paralog of CSIF2, interacts with AtFip1, a core component of the polyadenylation machinery (Forbes *et al.*, 2006). The sub-cellular co-localization of CSP3 and CSIF2 was confirmed by transient expression in *N. benthamiana* (Figure 3.8 c-d). Both proteins predominantly localized to the nucleus and co-localized with CSP3. Another RBP that co-purified with CSP3 is a putative DNA/RNA binding protein of unknown function (At1g29250). This RBP contains Alba DNA/RNA binding motif (Pfam domain: PF01918), originally described as feature of a DNA binding protein common to archaea taxa and later found in a protein that interact with the pre-rRNA processing enzyme RNaseP (Pop7/Rpp20) and

a pre-tRNA processing enzyme (MRP2) of eukaryotes (Aravind *et al.*, 2003). Plants possess putative orthologs of Rpp20 and MRP2; At1g29250 is one of three related proteins in Rpp20 family based protein sequence but not functional data. Interestingly, CSP3 associated with a cold-inducible GR-RBP (AtRZ-1c, At5g04280) that is related to mammalian hnRNP-G. In a large survey of nucleolar-enriched proteins this GR-RBP was shown to strongly accumulate in the nucleoplasm and weakly in the nucleolus of *Arabidopsis thaliana* (Brown *et al.*, 2005). Recent studies by Kim *et al.* (2010) demonstrated AtRZ-1c transcript accumulation induced by cold stress and the nuclear localization pattern of AtRZ-1c in *Arabidopsis* seedlings. In addition, mammalian hnRNP-G is a component of spliceosome and regulates alternative splicing by binding to the CC(A/C) rich region in pre-mRNAs (Heinrich *et al.*, 2009). We determined a stronger nucleoplasmic and weaker nucleolar localization of this protein, as well as validated its co-localization with CSP3 (Figure 3.8, a-b). Unlike CSP3, the cytoplasmic accumulation of AtRZ-1c was extremely limited. Taken together, these data indicate that the CSP1-like CSD protein CSP3 is predominantly nuclear localized and found in association with proteins involved in varied steps in mRNA maturation.

3.5.5 CSP4

CSP4 contains an N-terminal CSD and C-terminal two zinc-finger glycine-rich motifs. Nakaminami *et al.* (2009) reported that *CSP4* transcripts accumulated in developing embryos and shoot apices suggesting its function in a developmental context. This CSD is most closely related to CSP2 (77% amino acid identity). Similar to CSP2,

CSP4 was not found in association with ribosomes. This sub-cellular location of this protein has not yet been explored.

The immunopurification of CSP4 from transgenic *Arabidopsis* seedlings over-expressing CSP4 identified three associated proteins. These included a glycine-rich protein (GRP, At1g27090) and a eukaryotic translation initiation factor 5A-2 (eIF5A-2) (Table 3.4). Little has been reported about the function of CSP4-associated GRP, except for the observation that this protein was among the proteins co-purified from *Arabidopsis* cell extracts with m7GTP-Sepharose, which binds the cap-binding complex (Bush *et al.*, 2009). eIF5A, on the other hand is not a *bone fide* translation factor, but appears to function as a nucleocytoplasmic shuttle protein by translocating specific mRNA from the nucleus to the cytosol (Thompson *et al.*, 2004). The *Arabidopsis* eIF5A-2 mutant, *fumonisin B-resistant 12 (fbr12)*, displayed developmental defects in floral organs and defective sporogenesis, both associated with abnormal cell division and cell growth (Feng *et al.*, 2007). More recently, eIF5A has been associated with xylem development (Liu *et al.*, 2008) and osmotic stress response (Ma *et al.*, 2010). The association of eIF5A-2 with CSP4 could hint at roles in post-transcriptional key processes to development and environmental responses. Future studies on CSP4 could begin with sub-cellular localization studies and investigation of direct interaction with the proteins identified by co-immunopurification.

3.5.6 Interaction of CSPs with pentatricopeptide and tetratricopeptide repeat proteins

The immunopurification of CSP2, CSP3 and CSP4 yielded a number of pentatricopeptide repeat (PPR) and tetratricopeptide repeat (TPR) proteins as putative associated proteins. The PPR family consists of ca. 450 members, of these, 75% predicted to be targeted to the chloroplast or mitochondria (Saha *et al.*, 2007; Schmitz-Linneweber & Small, 2008). The function of PPR proteins and the related TPR proteins, is linked to post-transcriptional processes within organelles, for example, pre-mRNA splicing, mRNA editing, and translational regulation (Schmitz-Linneweber & Small, 2008). These proteins bind RNA, most likely in a sequence specific manner as confirmed by different experimental methods including, gel shift (Kobayashi *et al.*, 2007), UV cross-linking (Nakamura *et al.*, 2003) and RNP-complex immunopurification and hybridization (Schmitz-Linneweber *et al.*, 2005, 2006). Roles of PPR and TPR proteins in the plant cell nucleus or cytoplasm have not yet been described. Notably, we found that the large (est 198.65 kDa) TRP encoded by At1g01320 was associated with CSP2, CSP3 and CSP4. Since both PPR and TPR proteins co-purified with CSP complexes, these proteins may be involved in post-transcriptional regulation of gene expression in the plant nucleus.

3.5.7 Networking among CSPs and GR-RBPs

Our co-immunopurification and proteomic analysis identified several RBPs that share a similar functional context. The co-immunopufication and co-association

between CSP1 and CSP2 was confirmed by proteomic identification and co-localization *in planta*. In the case of CSP3, the cold-inducible GR-RBP (AtRZ-1c) was found to co-purify and co-localize with CSP3. Since, CSP and many GR-RBP transcripts are induced by low temperature stress, we propose an mRNA/protein network that includes both CSPs, GR-RBPs, PPRs and potentially the dehydrin COR47 that exists within plant cells. Although the analysis presented here did not include evaluation of protein associations in cold stressed seedlings, an increase in abundance of these proteins in the cold and other conditions is proposed to further enhances the formation of complexes including these proteins. The specific mRNA/protein interactions, individual complexes, and their molecular function within an integrated RBP-mRNA network remains to be explored.

In conclusion, this study of the four CSPs provides evidence that these CSD, zinc finger and glycine-repeat proteins participate in post-transcriptional gene regulation in the nucleus and cytoplasm of plant cells. As a next step towards discovering their biological function it may be feasible to combine phenotypic characterization of *CSP* gene mutants with genomic technologies such as RNP-immunopurification followed by microarray hybridization (RIP-CHIP) or high throughput sequencing (RIP-seq) to identify the mRNAs targeted by individual CSPs.

3.6 Acknowledgements

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Figure 3.1. Diagrammatic representation of *CSP* transgenes introduced into *Arabidopsis thaliana* by stable transformation with a binary T-DNA vector. Each *CSP* was epitope-tagged with FLAG at the N- or C-terminus. Gene elements are not drawn to scale; details of the tag and vector are provided in the Materials and Methods.

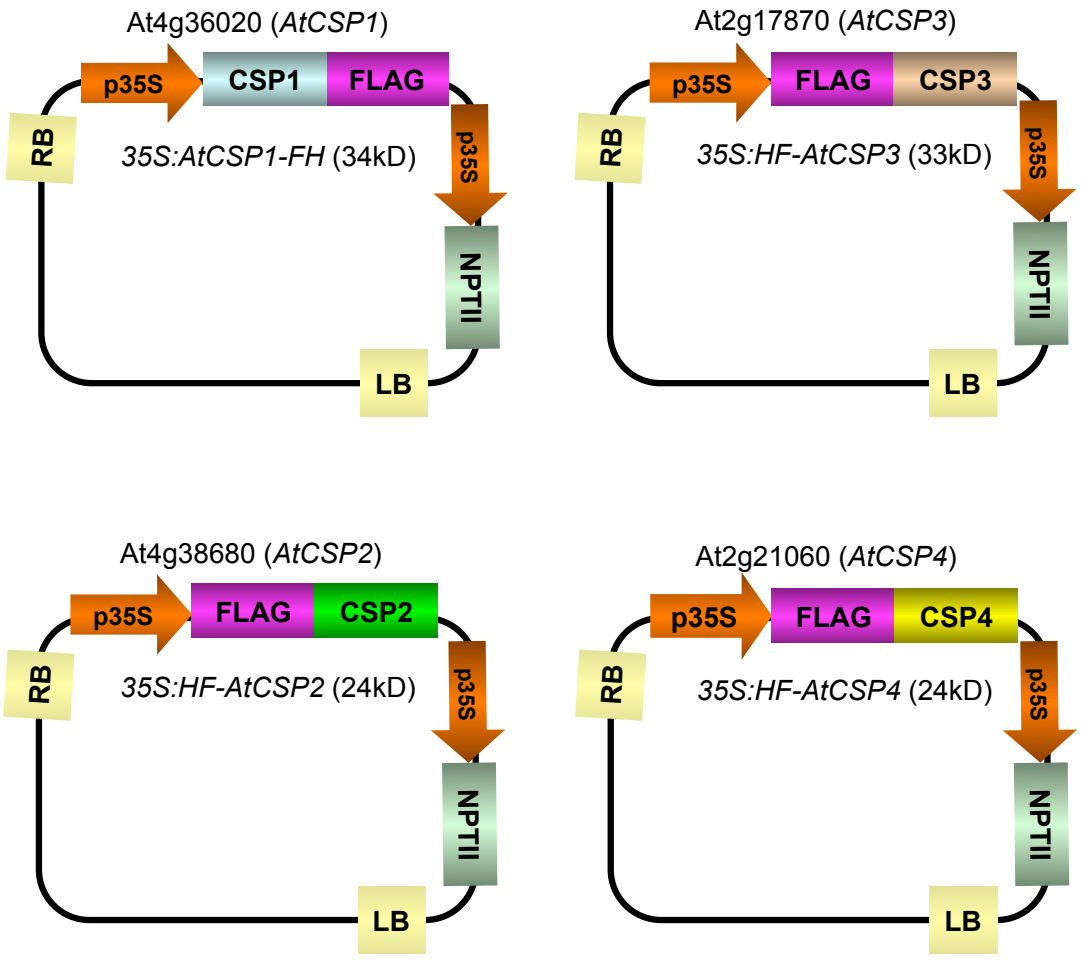

Figure 3.2. Flow diagram of cellular fractionation of mRNP complexes from cryo-preserved seedling tissue of *Arabidopsis thaliana*.

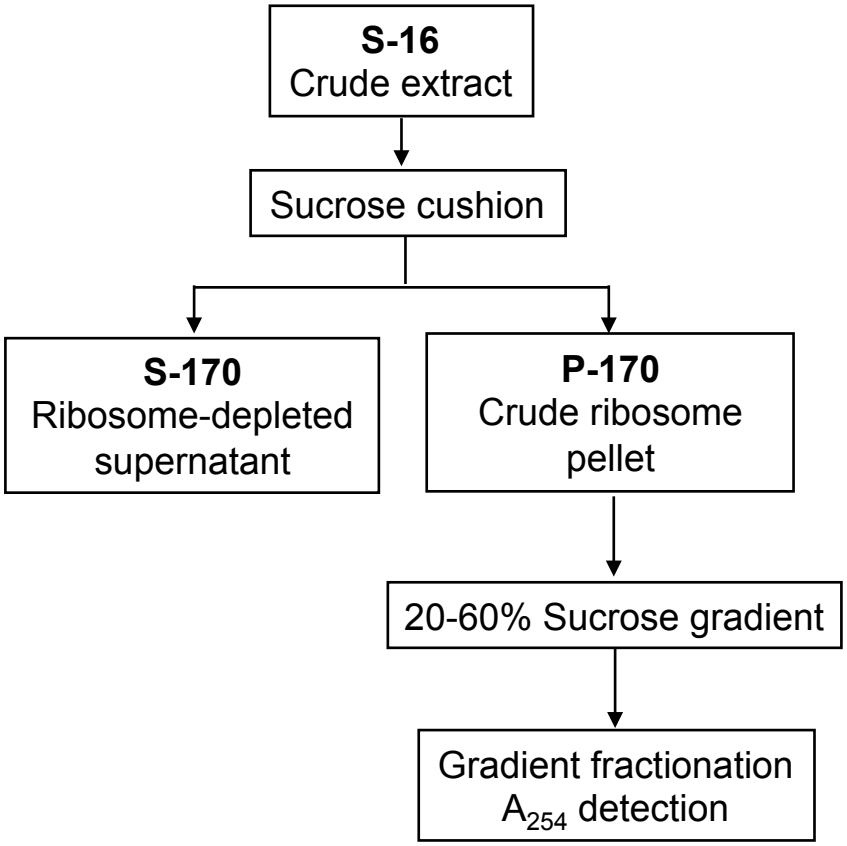

Figure 3.3. Fractionation of FLAG-tagged CSPs. A crude detergent-treated cell extract supernatant (S-16) from 10-d-old seedlings was fractionated by ultracentrifugation through a sucrose cushion to obtain ribosome pellet (P-170) and a ribosome depleted supernatant (S-170). Cellular fractions from *35S:AtCSP1-FH*, *35S:HF-AtCSP2*, *35S:HF-AtCSP3*, and *35S:HF-AtCSP4* transgenic plants were separated by 12.5% SDS-PAGE, transferred to a nitrocellulose membrane and immunodetected with a monoclonal anti-FLAG antibody and polyclonal anti-RPS6 antiserum. The same quantity of protein was analyzed in each S-16, S-170 and P-170 sample.

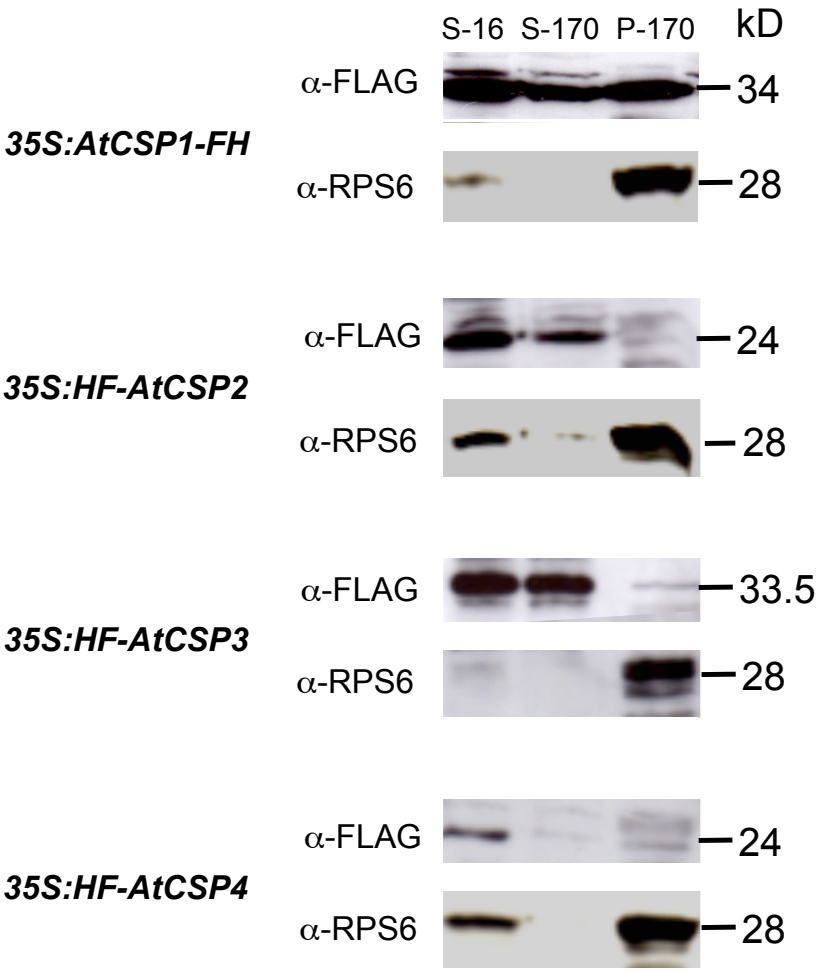

Figure 3.4. Immunopurification of FLAG-tagged CSPs using anti-FLAG agarose. Crude extracts from 10-d-old seedlings were used for immunopurification of proteins with anti-FLAG M2 agarose. Immunopurification was performed with Col-0 seedling tissue as a control for non-specific protein binding to the anti-FLAG agarose. Total, unbound, and immunoprecipitate (IP) fractions were separated by 12.5% SDS-PAGE and silver-stained or transferred to nitrocellulose membrane and immunodetected with an anti-FLAG antibody. The affinity matrix (M2) was also evaluated as a control.

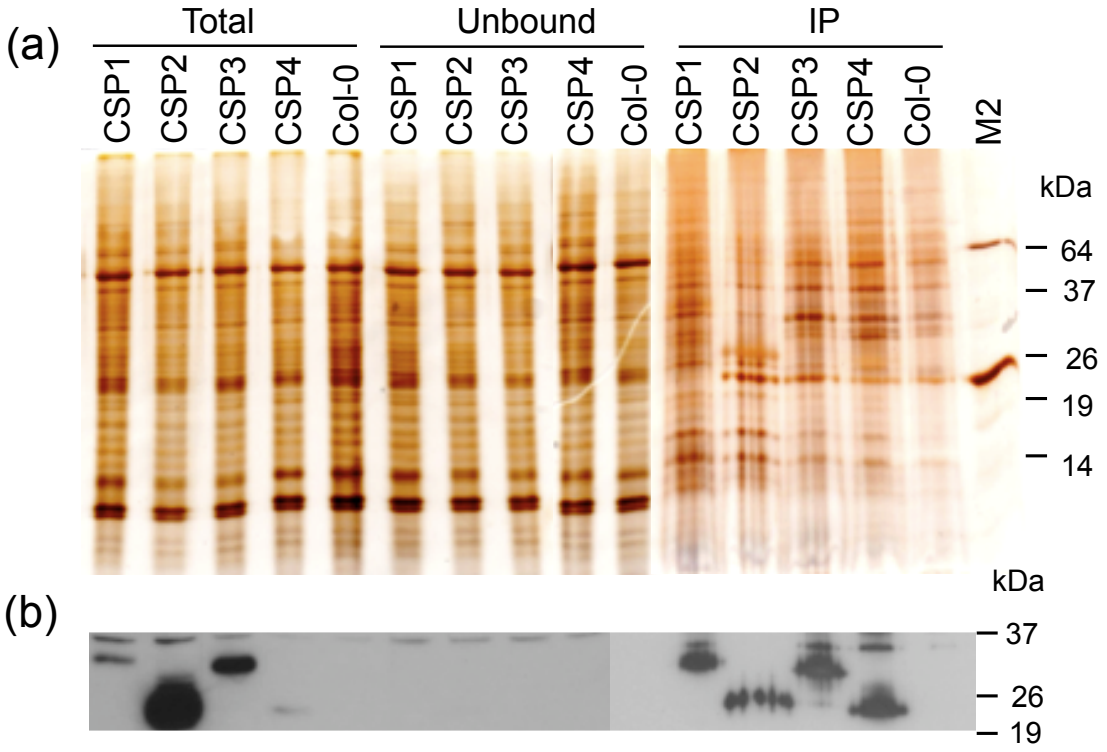

Figure 3.5. Sub-cellular localization of CSP1. Confocal microscopic images of *Nicotiana benthamiana* leaf pavement cells transiently expressing CSP1-YFP fusion protein and stained with DAPI. Micrographs show a representative cell. (a) YFP detection, (b) DAPI detection, and (c) bright-field image.

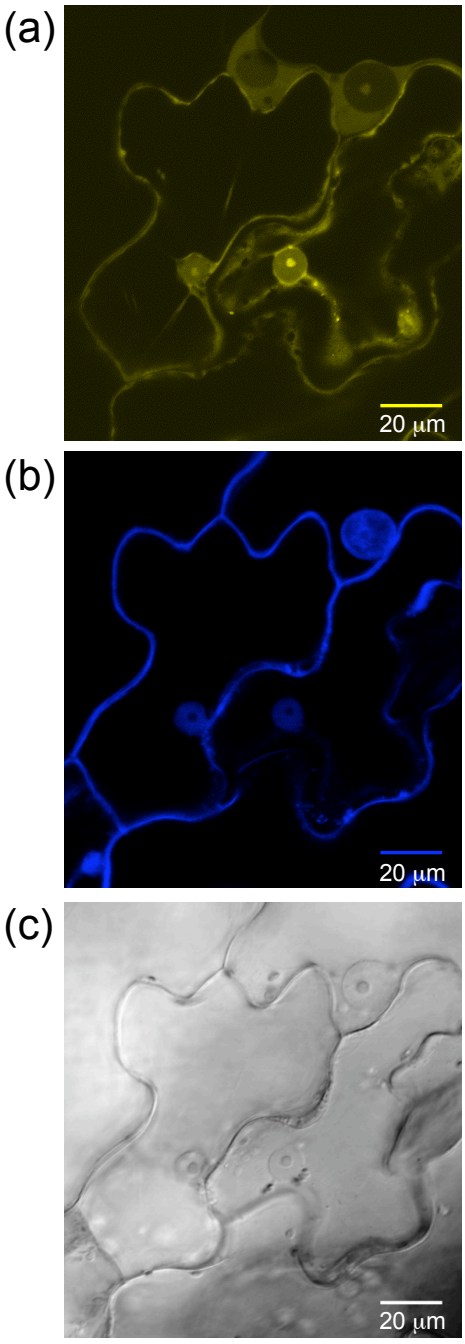

Figure 3.6. Coverslip-induced CSP1 cytoplasmic granule formation. Representative confocal microscopic image of *Nicotiana benthamiana* leaf pavement cells transiently expressing CSP1-YFP fusion protein. A series of Z-stack photographs were taken within 2 min of sealing the leaf pieces between a glass slide and coverslip, and then 20 and 40 min later.

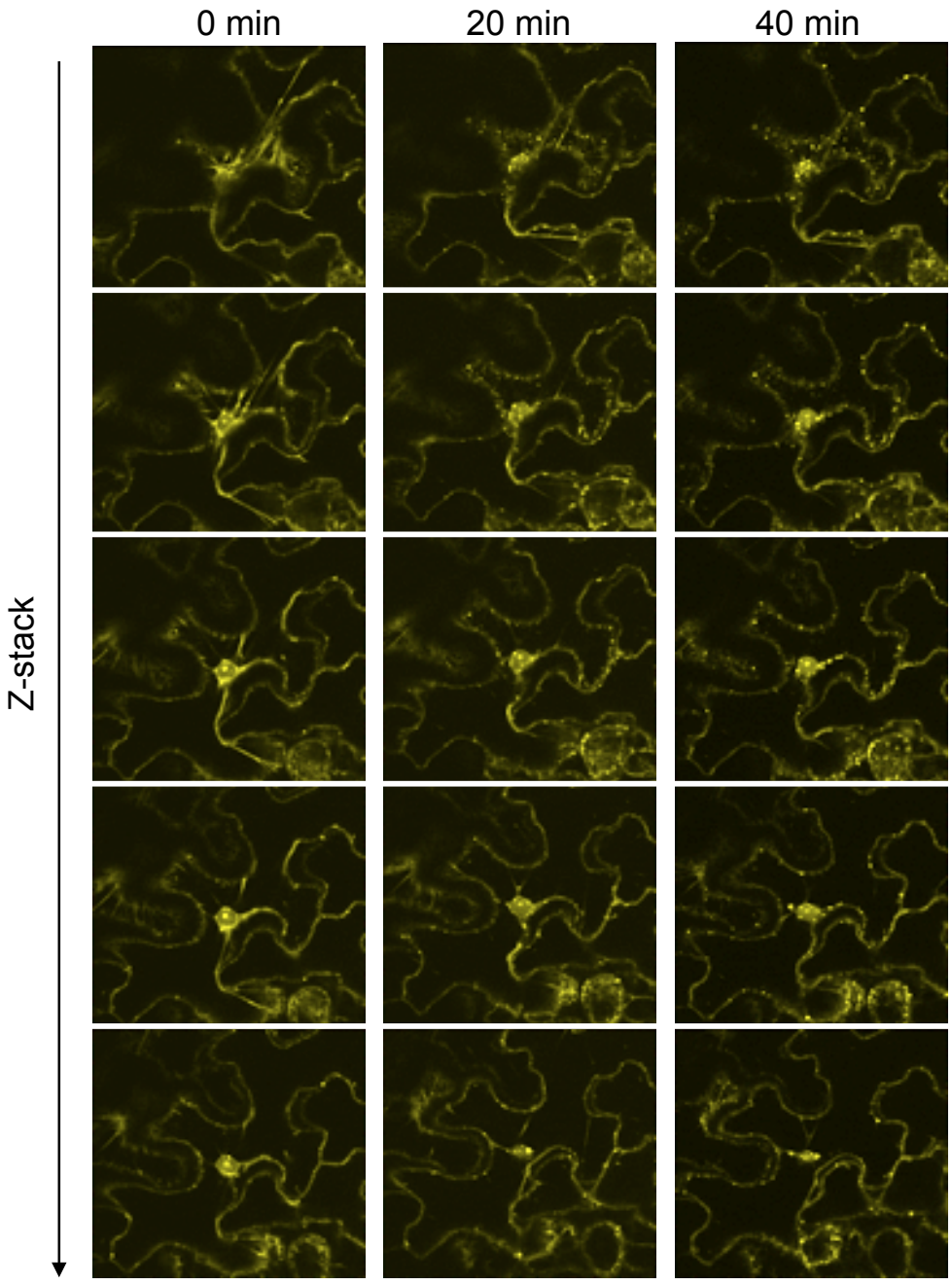

Figure 3.7. Co-localization of CSP1 and CSP2. Representative confocal microscopic images of *Nicotiana benthamiana* leaf pavement cells transiently producing CSP1-YFP and COR47-mRFP (a), and CSP1-YFP and CSP2-mRFP (b-f) fusion proteins and stained with DAPI. (d). Magnification of the nuclear region of (c) and (e) in (d) and (f), respectively

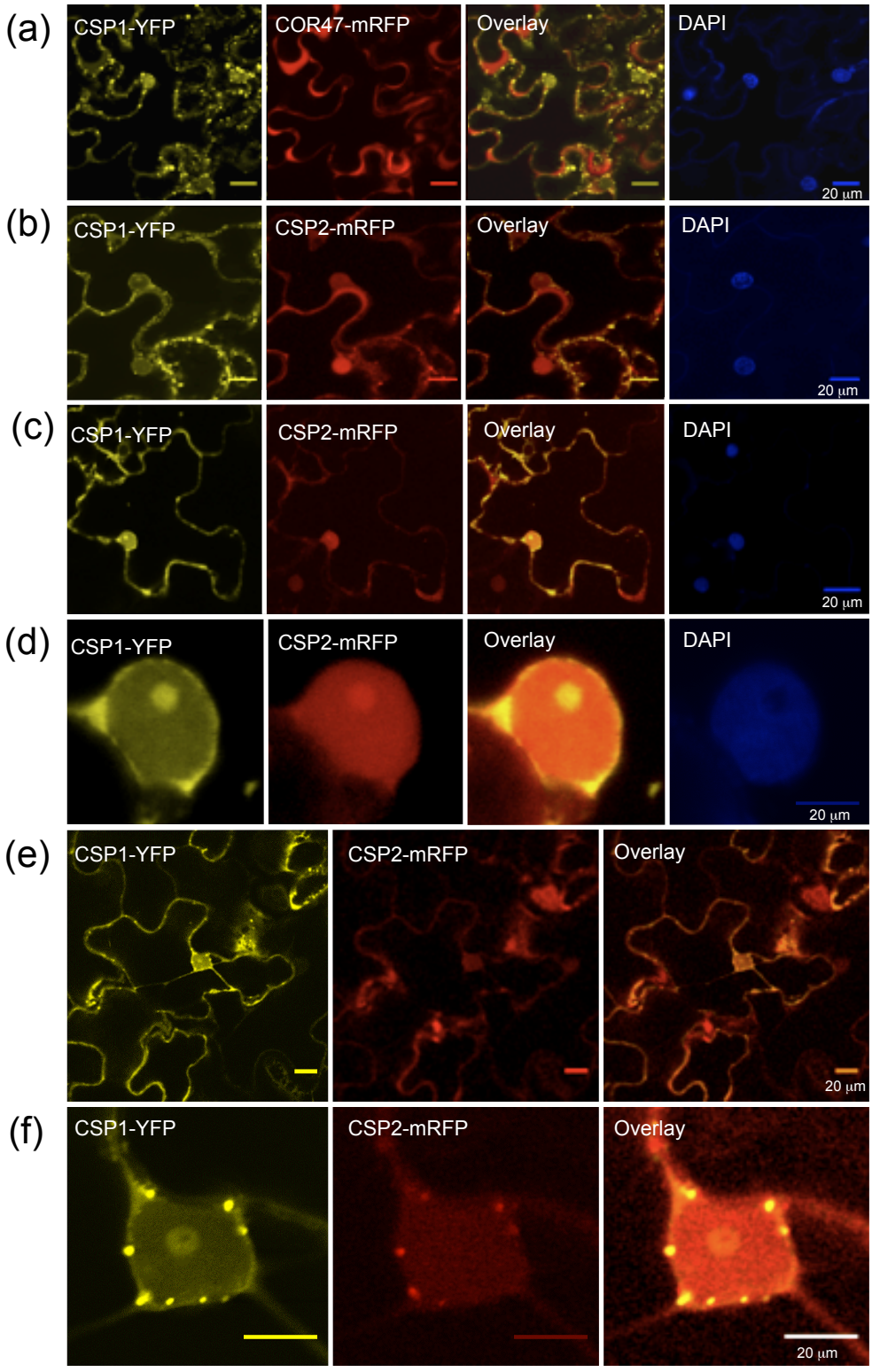
(a)
CSP1-YFP
COR47-mRFP
Overlay
DAPI
20 μm
(b)
CSP1-YFP
CSP2-mRFP
Overlay
DAPI
20 μm
(c)
CSP1-YFP
CSP2-mRFP
Overlay
DAPI
20 μm
(d)
CSP1-YFP
CSP2-mRFP
Overlay
DAPI
20 μm
(e)
CSP1-YFP
CSP2-mRFP
Overlay
20 μm
(f)
CSP1-YFP
CSP2-mRFP
Overlay
20 μm

Figure 3.8. Co-localization of CSP3, AtRZ-1c, and CSIF2. Representative confocal microscopic images of *Nicotiana benthamiana* leaf pavement cells transiently expressing CSP3-YFP and AtRZ-1c-mRFP (a), and CSP3-YFP and CSIF2-mRFP (b) fusion proteins and stained with DAPI. Magnification of the nuclear region of (a) and (c) in (b) and (d), respectively.

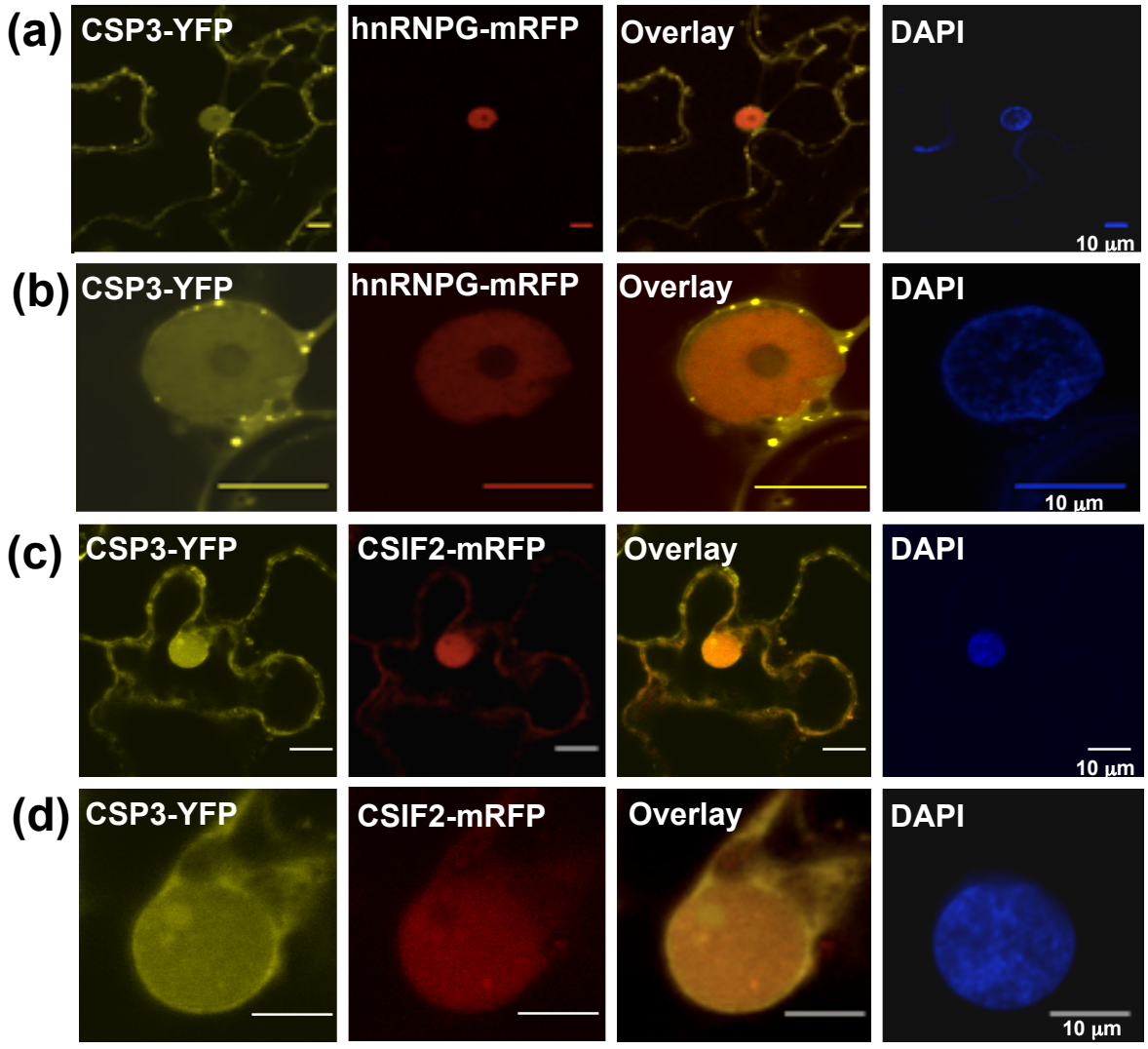

Table 3.1. Identification of proteins that co-immunopurify with CSP1. Ten-d-old seedlings of the *35S:AtCSP1-HF* transgenic line were used to immunoprecipitate CSP1-FH and associated proteins. The immunopurified proteins were digested with trypsin *in situ* on the anti-FLAG agarose matrix and qualitatively analyzed by ESI Q-TOF MS. Protein identification was performed by use of the Mascot algorithm and *Arabidopsis* NCBInr2009 protein database. The maximum number of missed cleavage sites allowed was two and mass tolerance was set at <100 ppm. The peptides identified were compared to those of a mock immunoprecipitate performed in parallel with Col-0 seedling tissue. All peptides or proteins identified in the mock immunoprecipitated sample were subtracted from the *35S:AtCSP1-FH* sample.

Protein Identity	Mass/ Charge (D)	Peptide mass (D)	Amino acid sequence	ppm	Mascot Score	Protein Molecular mass (kDa)
CSP1	585.84	584.83	K.AVNVTAPGGGSLK.K	21.40	42	30.07
Arabidopsis Cold Shock Protein 1	649.89	648.89	T.KAVNVTAPGGGSLK.K	28.70	33	
(At4g36020)	649.90	648.90	K.AVNVTAPGGGSLKK.E	43.80	38	
	948.00	946.99	R.SLTVGDAVEFAITQGSDGK.T	29.60	83	
	987.17	986.16	K.GYGFITPDDGSVELFVHQSSIVSEGYR.S	24.10	140	
GRP3S	610.61	609.61	R.YQGGGGHGGHGGGGYQGGGGR.Y	26.30	74	11.58
Glycine-rich protein 3 short isoform						
(At2g05380)						
COR47	1076.06	1075.06	K.AQISEPELAAEHEEVKENK.I	28.90	58	29.88
Cold-regulated 47	1163.25	1162.24	M.AEEYKNNVPEHETPTVATEESPATTTEVTDR.G	35.10	43	
(At1g20440)						
PRP8	714.09	713.08	R.TDVIQALGGVEGILEHTLFK.G	35.50	64	275.25
Pre-mRNA processing factor 8						
(At4g38780, At1g80070)						

Table 3.2. Identification of proteins that co-immunopurify with CSP2. Immunopurification and protein identification as described in Table 3.1.

Protein Identity	Mass/ Charge (D)	Peptide mass (D)	Amino acid sequence	ppm	Mascot Score	Protein Molecular mass (kDa)
CSP2	1049.95	1048.94	K.AIDVSGPDGAPVQGNSGGGSSGGR.G	39.05	136	19.87
Arabidopsis Cold Shock Protein 2	792.01	791.00	R.SLAAEEAVEFEVEIDNNNRPK.A	57.44	142	
(At4g38680)	1190.52	1189.51	K.GFGFITPDDGGDDLFVHQSSIR.S	36.46	175	
snRNP-G	770.90	769.89	R.GNSIVTVEALEPVGR.S	31.32	73	8.83
Probable small nuclear ribonucleoprotein G						
(At2g23930)						
PRP19	607.77	606.76	R.AAVDEELGPDAK.K	50.74	70	58.90
Pre-mRNA splicing factor 19						
(At2g33340)						
RNA-binding protein (unknown)	719.33	718.32	K.NVSEAEVQSLFSK.Y	51.69	53	48.17
(At4g03110)						
CSP1	585.81	584.80	K.AVNVTAPGGGSLK.K	36.42	43	30.07
Arabidopsis Cold Shock Protein 1						
(At4g36020)						
TPR containing protein	1145.13	1144.12	K.VLPVIVDVIVNLPDETEAILK.G	33.90	39	198.65
Tetratricopeptide repeat containing protein						
(At1g01320)						
Fibrillarin1	480.74	479.74	K.GKEDALVTK.N	56.59	38	32.81
At5g52470	620.83	619.82	R.TNVIPIIEDAR.H	36.81	34	
	666.81	665.80	K.NLVPGEAVYNEK.R	51.11	43	

Table 3.2 continued.

Protein Identity	Mass/ Charge (D)	Peptide mass (D)	Amino acid sequence	ppm	Mascot Score	Protein Molecular mass (kDa)
U2A' U2 small nuclear ribonucleoprotein A (At1g09760)	764.89	763.88	K.AAIINSQTIEEIAR.L	42.00	37	28.07
PPR repeat-containing protein Pentatricopeptide repeat containing protein (At2g48000)	1088.50	1087.49	K.MSRSMFHGLMIMYGSQK.R	54.10	30	55.00
RBP45B RNA-binding protein 45B (At1g11650)	555.73	554.72	R.FSDESEQIR.A	54.31	29	43.95
PPR repeat-containing protein Pentatricopeptide repeat containing protein (At5g16640)	637.27	636.26	R.VSEAEEFYEEMIRR.S	8.26	25	57.17

Table 3.3. Identification of proteins that co-immunopurify with CSP3.
Immunopurification and protein identification as described in Table 3.1.

Protein Identity	Mass/ Charge (D)	Peptide mass (D)	Amino acid sequence	ppm	Mascot Score	Protein Molecular mass (kDa)
CSP3	629.37	628.36	I.EVTAPGGGSLNKK.E	35.60	68	29.55
Arabidopsis Cold Shock Protein 3	657.37	656.37	K.AIEVTAPGGGSLNK.K	26.50	35	
(At2g17870)	481.29	480.28	K.AIEVTAPGGGSLNKK.E	29.90	53	
	977.16	976.15	K.GYGFITPDDGGEELFVHQSSIVSDGFR.S	31.90	164	
Fibrillarin 1	819.44	818.43	R.ISVQNEDGTKVEYR.V	31.60	79	32.81
(At5g52470)	636.41	635.40	K.LAAAILGGVDNIWIKPGAK.V	51.20	130	
TPR containing protein	837.99	836.98	K.LVADFGSLELSPVDGR.T	55.90	61	198.65
Tetratricopeptide repeat containing protein	1145.20	1144.20	K.VLPVIVDVIVNLPDETEAILK.G	28.80	41	
(At1g01320)						
ATCSIF2	895.53	894.52	K.LLAVPLFELYDNVQR.Y	40.00	84	22.82
Cleavage and poly adenylation factor 2						
(At4g25550)						
COR47	1076.08	1075.07	K.AQISEPELAAEHEEVKENK.I	44.50	59	29.88
Cold-regulated 47						
(At1g20440)						
Nucleic acid binding protein	1169.60	1168.60	MEEITEGVNNMNLAVDTQKK.N	47.30	46	14.56
(At1g29250)						
Glycine-rich RNA-binding protein	635.05	634.04	R.IFVGGLSPEVTDRDLER.A	68.00	35	33.47
AtRZ-1c, hnRNPG						
(At5g04280)						

Table 3.4. Identification of proteins that co-immunopurify with CSP4.
Immunopurification and protein identification as described in Table 3.1.

Protein Identity	Mass/ Charge (D)	Peptide mass (D)	Amino acid sequence	ppm	Mascot Score	Protein Molecular mass (kDa)
CSP4	1139.59	1138.59	R.SLAAEESVEFDVEVDNSGRPK.A	42.20	144	19.065
Arabidopsis Cold Shock Protein 4	784.70	783.70	K.GFGFITPSDGGDDLFVHQSSIR.S	13.23	129	
(At4g21060)						
Glycine-rich protein	777.47	776.47	R.SKPAVVILIDELEK.I	17.20	77	45.99
(At1g27090)	608.36	607.35	R.SKPAVVILIDELEKIR.A	16.17	30	
eIF5A	592.28	591.27	M.SDDEHHFEASESGASK.T	54.80	56	17.13
eukaryotic translation initiation factor 5A						
(At1g26630)						
TPR containing protein	1145.15	1144.14	K.VLPVIVDVIVNLPDETEAILK.G	16.45	44	198.65
Tetratricopeptide repeat containing protein						
(At1g01320)						

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Chapter 4

Post-transcriptional role of *Arabidopsis* Cold Shock Protein 1 in the response to low-temperature stress

4.1 Abstract

The *Arabidopsis thaliana* Cold Shock Protein 1 (CSP1) contains an evolutionary conserved Cold Shock Domain (CSD) characteristic of bacterial cold responsive proteins (CSPs) that bind to nucleic acids and chaperone RNAs. The CSD has been extensively studied in both prokaryotes and eukaryotes, including humans. However, the functional roles of plant CSPs are unknown. Previous studies showed that *AtCSP1* transcript accumulation is induced by low temperature suggesting CSP1 acts in response to this environmental change. Stable transgenic lines over-expressing a C-terminal His_6-FLAG (HF)-tagged CSP1 were generated. Cellular fractionation using differential ultracentrifugation identified CSP1 in association with polyribosome complexes. RNase A treatment of polyribosomes disrupted this association, indicating binding is not directly with the ribosome. Low temperature time-course treatment demonstrated that CSP1 protein accumulates under cold stress in wildtype and transgenic *35S:AtCSP1-FH* plants. *atcsp1-1* knockdown plants displayed decreased freezing tolerance. To characterize the mRNAs that selectively associate with CSP1, we performed immunopurification using a polyclonal antibody prepared against a CSP1-specific peptide. By use of the ATH1 DNA microarray platform, the effect of 12 h of cold treatment (4 ^{o}C) on the total, polysomal,

and CSP1-associated mRNA populations were quantitatively evaluated. This revealed that AtCSP1 associates with a subpopulation of cellular mRNAs, with preferential binding to transcripts with (G+C) rich 5'-untranslated regions. However, the manipulation of CSP1 abundance had little impact on steady-state or polysomal mRNA levels under non-stress and cold conditions. This suggests that low levels of CSP1 are sufficient for its RNA chaperone activity.

4.2 Introduction

As sessile organisms, higher plants are continuously exposed to environmental changes. The regulation of gene expression via transcriptional and post-transcriptional control is required for the adaptive responses to the changes in temperature. Low-temperature poses a major limitation on growth and development. Some plants can acclimate to cold or freezing temperatures, however, this usually requires reprogramming of gene expression in order to accumulate freezing protective proteins and metabolites (Zhu *et al.*, 2007). Transcriptome adjustment in response to cold stress has been well characterized in model and major crop plants (Gulick *et al.*, 2005; Lee *et al.*, 2005; Rensink *et al.*, 2005; Hewezi *et al.*, 2006; Wong *et al.*, 2006; Winfield *et al.*, 2010). However, post-transcriptional mechanisms that contribute to adaptation to low-temperatures are not well understood.

Post-transcriptional regulation involves multiple processes from processing of pre-mRNA, nucleo-cytoplasmic transport, regulation of mRNA decay, mRNA localization, and translation (reviewed by Bailey-Serres *et al.*, 2009). Post-transcriptional

control is regulated by RNA-binding proteins (RBPs), which directly or indirectly interact with individual mRNAs (reviewed in Chapter 1). Keene (2007) proposed an RNA regulon model in which multiple mRNAs encoding functionally related proteins are coordinately regulated by one or more RBPs in response to growth and developmental clues in mammalian cells and yeast. For example, the Pufs, which are a family of structurally related cytoplasmic RNA binding proteins, have been shown to regulate mRNA translation and stability through binding to the 3'-UTR of their target mRNAs. In yeast (*Saccharomyces cerevisiae*), Puf1p and Puf2p bind mostly mRNAs encoding membrane-associated proteins, Puf3p exclusively targets messages for nuclear-encoded mitochondrial proteins, and Puf4p and Puf5p associate primarily with transcripts encoding proteins bound for the nucleus (Moore, 2005). To date, there has been no report of a plant RNA regulon.

RBPs usually contain one or more RNA binding domains (Bailey-Serres *et al.*, 2009; Lorković, 2009). Cold Shock Proteins (CSPs) contain a specialized DNA/RNA binding domain called a Cold Shock Domain (CSD). CSPs are an ancient protein found in eubacteria, archaebacteria and eukaryotes (Graumann & Marahiel, 1998). Bacterial CSPs accumulate under cold stress and function as RNA chaperones to remove nucleic acid secondary structure (Sommerville, 1999). CSPs are recognized in plants, including *Arabidopsis* (Karlson & Imai, 2003), wheat (*Triticum aestivum*) (Karlson *et al.*, 2002), and rice (*Oryza sativa*) (Chaikam & Karlson, 2008). Previous studies showed that the four *Arabidopsis CSP* (*AtCSPs 1-4*) gene transcripts accumulate in response to cold treatment (Karlson & Imai, 2003), suggesting they function in cold adaptation. Kim *et al.*

(2007, 2009) reported that *Arabidopsis* CSP1 and CSP3 function as RNA chaperones in response to cold stress. However, there is no information regarding the mRNAs that might be targeted by *Arabidopsis* CSPs or their functional role in post-transcriptional regulation under cold stress.

Selective and coordinated regulation of mRNAs translation occurs in response to environmental cues (reviewed in Chapter 1). In *Arabidopsis*, dehydration, hypoxia, heat, high salinity, and sugar starvation resulted in selective translation of mRNAs (Kawaguchi *et al.*, 2004; Branco-Price *et al.*, 2005, 2008; Nicolai *et al.*, 2006; Mustroph *et al.*, 2009b; Matsuura *et al.*, 2010). A previous report found that transfer of maize (*Zea mays* L.) seedlings to 8 ^{o}C for resulted in a reproducible increase in the size of polyribosome (polysome) complexes, although the elevation was not statistically significant (Williams, 2001). In contrast to anoxia stress, cold stress did not affect the synthesis of housekeeping proteins (Guy, 1990). This information suggested that translational regulation during cold-adaptation could be different than the other stresses. However, the influence of sub-ambient growing temperatures on translational has not been evaluated at a global scale.

The results presented here indicate that *Arabidopsis thaliana* CSP1 is a polysome-associated RBP. The accumulation and the polysomal-association of CSP1 were induced by cold stress. CSP1 bound preferential to a subset of cellular mRNAs under cold and non-stress conditions, confirming it is a selective RNA chaperone.

4.3 Materials and Methods

4.3.1 Plant material and growth conditions

The *Arabidopsis thaliana* genotypes *Col-0, 35S:HF-RPL18* (Zanetti *et al.*, 2005), *atcsp1-1* (ABRC, SALK_048960: T-DNA insertion into promoter of *At4g36020*) and *35S:AtCSP1-FH-11-2* (Section 3.3.2) were used (Figure 4.1). Seed sterilization and growth conditions were as described in Section 3.3.1.

4.3.2 Establishment of the *atcsp1-1* homozygous line

A homozygous *atcsp1-1* T-DNA insertion mutant was identified from a segregating SALK line by Polymerase Chain Reaction (PCR) amplification of genomic DNA with primers: *AtCSP1*-gen-forward 5'-*GTG TAT TGA CAA TGA GCG ATC ACT G*-3'; *AtCSP1*-reverse 5'-*TTA AGC TAC AGA AGA ACA TTC CCT TG*-3'; and T-DNA left border primer LBa1 5'-*TGG TTC ACG TAG TGG GCC ATC G*-3'. To evaluate transcript accumulation, total RNA extraction was performed with 100 mg of pulverized tissue from 10-d-old whole seedlings with TRIzol (Invitrogen, Carlsbad, CA). Approximately 400 ng of total RNA was used for complimentary DNA (cDNA) synthesis with oligo d(T) primer (Promega, Madison, WI) and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) was performed using gene specific primers: *AtCSP1*-forward 5'-*CAC CAT GGC TTC AGA GGA TC*-3' and *AtCSP1*-reverse 5'-*TTA AGC TAC AGA AGA ACA TTC CCT TG*-3'; *ACTIN2*-forward 5'-*ATG ATG CCC CGA GAG CAG*-3' and *ACTIN2*-reverse 5'-*GGA TGC AAG GAT TGT CCT C*-3'. RT-

PCR reaction was performed under 55 °C annealing temperature and 28 cycles for both *AtCSP1* and *ACTIN2*-specific primers.

4.3.4 Multiple sequence analyses

AtCSP 1-4 (At4g36020, At4g38680, At2g17870, and At2g21060, repectively) protein sequences were derived from TAIR. *Escherichia coli* CSP_A (AP_004238.1) and *Homo sapiens* YB-1 (NP_004550.2) protein sequences were obtained from NCBI. Multiple sequence alignment was performed by CLUSTAL_W (Thompson *et al.*, 1994).

4.3.5 Freezing tolerance and electrolyte leakage test

Three-week-old *atcsp1-1*, *35S:HF-RLP18* and *35S:AtCSP1-HF* plants grown in soil were used for freezing tolerance and electrolyte leakage tests. For cold acclimation treatment, plants were placed at 4 °C for 4 d before leaves were sampled for the freezing tolerance assay. For each treatment, fully expanded leaf (leaf number 5 to 8) was excised at the base of the petiole and, placed in a glass test tube (12 x 75 mm) containing 100 µl of ddH_2O, and which placed in a refrigerated water bath (model 1187; VWR Scientific, San Francisco, CA) with the temperature set to 0 °C. The temperature of the water bath was programmed to decrease to -10 °C, with a decrement of 1 °C every 30 min. When the temperature reached -1 °C, tiny pieces of ice were added to allow ice nucleation. The tubes were removed from the water bath at specific temperatures and placed on ice to allow gradual thawing overnight. Leaf was transferred to a new tube (16 x 150 mm) containing 15 mL of ddH_2O, which was shaken at 25 °C (~120 rpm) overnight before the conductivity of the solution was measured with an electro conductivity meter (model

1054; VWR Scientific). The tubes with leaves were capped and autoclaved (121 °C, 15 psi, 15 min), cooled to room temperature, and the conductivity of the solution was measured again. The percentage of electrolyte leakage was calculated as the percentage of electrolyte conductivity before and after autoclaving. Four biological replicates were performed for each sample.

4.3.6 Cold treatment

Twenty-five-d-old plants grown in 5.08 cm square pots were placed in a 4 ºC chamber (16 h, ~ 50 μE sec^{-1} m^{-2} light: 8 h dark at 4 °C) for 0 to 48 h or in a 23 ºC growth chamber (16 h, ~ 100 μE sec^{-1} m^{-2} light: 8 h dark at 4 °C). Cold treatment began 2 h following the initiation of light period (ZT2). Cold-treated or control rosette leaves were collected at specific time points, flash frozen in liquid nitrogen, pulverized, and stored at -80 °C.

For RNA immunopurification, rosette leaves of 25-d-old plants were collected and cross-linked with formaldehyde solution. Briefly, leaves were washed with ice-cold ddH_2O and cross-linked by bathing in 1% (v/v) formaldehyde (Molecular biology grade, Fisher, Fremont, CA) for 15 min by vacuum infiltration. Cross-linking was stopped by addition of 2 M glycine [pH 7.0] to a final concentration of 125 mM and vacuum-infiltrated for 5 min. Leaves were washed twice with ice-cold ddH_2O, ground in liquid nitrogen and stored at -80 °C.

4.3.7 Total and polysomal RNA preparation and protein extraction

The extraction procedure was based on that of Zanetti *et al.* (2005) and Mustroph *et al.* (2009a). Briefly, 3 mL of pulverized frozen tissue was thawed in 7 mL Polysome Extraction Buffer (200 mM Tris-HCl [pH 9.0], 200 mM KCl, 36 mM $MgCl_2$, 25 mM EGTA, 5 mM DTT, 1 mM phenylmethanesulfonylfluoride (PMSF), 50 μg/mL cycloheximide, 50 μg/mL chloramphenicol, 1% (v/v) Triton X-100, 1% (v/v) polyoxyethylene (23) lauryl ether (Brij-35), 1% (v/v) Tween-40, 1% (v/v) Nonidet P-40 (NP-40), 1% (v/v) Polyoxyethylene 10 tridecyl ether (PTE)). After removal of cell debris by centrifugation at 16,000g for 20 min at 4 °C, the crude cell supernatant (S-16) was filtered through sterilized Miracloth (Calbiochem, La Jolla, CA) and 300 μL was saved for extraction of total RNA. The pass-through supernatant was layered on top of an 8 mL 1.75 M sucrose cushion (400 mM Tris-HCl [pH 9.0], 200 mM KCl, 30 mM $MgCl_2$, 1.75 M sucrose, 5 mM DTT, 50 μg/mL chloramphenicol, 50 μg/mL cycloheximide) and centrifuged at 170,000 *g* for 3 h at 4 °C (70Ti rotor, Beckman, Brea, CA). The ribosome pellet (P-170) was resuspended in 250 μL Polysome Resuspension Buffer (200 mM Tris-HCl [pH 9.0], 200 mM KCl, 36 mM $MgCl_2$, 25 mM EGTA, 5 mM DTT, 50 μg/mL cycloheximide, 50 μg/mL chloramphenicol) and 20 U/mL RNAseout (Invitrogen)). Approximately 2000 unit of ribosomes (OD_{260}) was loaded on top of a 20-60 % (w/v) sucrose gradient (Kawaguchi *et al.*, 2005). For RNase A treatment, 2000 unit of ribosomes (OD_{260}) were incubated with 2.5 μg RNase A for 10 min at room temperature before loading to the sucrose gradient. Gradients were centrifuged at 275,000 *g* for 1.5 h

at 4 °C (SW55Ti rotor, Beckman), passed through a UA-5 detector to obtain a UV absorbance profiles, and a 185 gradient fractionator (ISCO, Lincoln, NE) to fractionate the gradient into 12 equal (~ 0.45 mL) fractions in microcentrifuge tubes. The polysome profile data were analyzed using Icruncher 2.2 (Williams *et al.*, 2003).

Proteins from each fraction were precipitated by addition of two volumes of 99 % (v/v) ethanol, incubation on ice overnight and centrifugation 16,000g at 4 °C for 15 min. Each pellet was washed with 70 % (v/v) ethanol, speed-vacuum dried and resuspended in SDS loading buffer (10 mM Tris-HCl [pH 6.8], 1 % (v/v) beta-mercaptoethanol, 2.5 % (v/v) glycerol, 4 % SDS, 1 % (w/v) bromophenol blue).

Total RNA and polysomal RNA (typically gradient fraction #7 to #13) were extracted by use of TRIzol solution (Invitrogen) according to the manufacturer's protocol. Total RNAs were subjected to DNAse treatment by use of RNase-Free DNase Set (Qiagen, Chatsworth, CA) according to the manufacturer's protocol. Total and polysomal RNA were cleaned by use of the RNAeasy Plant mini kit (Qiagen).

4.3.8 Immunoprecipitation of CSP1 complexes

A peptide (C_{151}AGNGDQRGATKGGN_{163}) specific to *Arabidopsis thaliana* CSP1 (Figure 4.4) was synthesized and injected into a rabbit to generate a polyclonal antiserum against CSP1, which was subsequently affinity-purified using the CSP1 peptide (anti-CSP1, Genscript, Piscataway, NJ).

EZview Red™ Protein A Affinity Gel (200 μL) (Sigma, St. Louis, MO) were coated with anti-CSP1 antiserum (ratio: 0.1 μg antibody to 1 μl suspended beads) in Polysome Extraction Buffer for 16 h at 4 °C with gentle shaking. The beads were washed 3 times with 5 mL extraction buffer and stored in 200 μL Polysome Extraction Buffer at 4 °C until use. One mL pulverized frozen plant tissue was thawed in 2 mL extraction buffer, mixed and centrifuged at 16,000 *g* at 4 °C for 15 min to obtain a supernatant and passed through Miracloth. The clarified extracts were added to the antibody-coated Protein A beads and incubated at 4 °C for 2 h with gentle shaking. The supernatant (Unbound fraction) was removed and the beads were washed 4 times by resuspension in 6 mL wash buffer (200 mM Tris-HCl [pH 9.0], 200 mM KCl, 36 mM $MgCl_2$, 25 mM EGTA, 5 mM DTT, 1 mM PMSF, 50 μg/mL cycloheximide, 50 μg/mL chloramphenicol, 1.125 % (v/v) Triton X-100, 0.125 % (v/v) Brij-35, 0.125 % (v/v) Tween-40, 0.125 % (v/v) NP-40) and centrifuged to discard the wash solution. To elute the bound CSP1 and any associated molecules, 150 μL elution buffer (100 mM Tris-HCL [pH 8.0], 25 mM EDTA, 1 % (w/v) SDS) was added to the beads, which were incubated 10 min at room temperature with shaking, and centrifuged to obtain the eluate fraction. Elution were repeated by addition of 150 μL elution buffer following by incubation at 65 °C for 10 min. The eluate was combined and 2 μL proteinase K (20 μg/mL) was added, and incubated at 65 °C for 1 h to reverse the cross-linking. Immunopurified RNAs were extracted with TRIzol (Invitrogen) according to the manufacturer's protocol and further purified by use of the RNAeasy plant mini kit (Qiagen).

4.3.9 Protein separation and immunodetection

Proteins were separated by 12.5 % (w/v) Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Laemmli, 1970), transferred to Hybond ECL membranes (Amersham, Piscataway, NJ) and subjected to immunodetection with anti-FLAG horseradish-peroxidase (HRP) conjugated monoclonal antibody (1:1000; Sigma), the affinity-purified polyclonal antibody against a AtCSP1 peptide, or a polyclonal antibody against maize (*Zea mays* L.) ribosomal protein S6 (RPS6) (1:10,000; Williams *et al.*, 2003). HRP-conjugated goat anti-rabbit IgG (1:10,000; Bio-Rad, Hercules, CA) was used as a secondary antibody. Visualization was performed with the ECL Plus Chemiluminescence system according to the manufacturer's protocol (Amersham).

4.3.10 Microarray hybridization and data analyses

The integrity of RNA used for microarray hybridization was examined with an Agilent Bioanalyzer 2100 on RNA 6000 nano chip or RNA 6000 pico chip (Agilent Technologies, Santa Clara, CA). The total, polysomal, and immunoprecipitated RNA samples were analyzed by hybridization to ATH1 (Affymetrix, Santa Clara, CA) GeneChips at the Genomic Core Facility, Institute for Integrative Genome Biology, University of California, Riverside, CA. Amplified RNA (aRNA) synthesis and biotin-labeling was conducted with 150 total or polysomal RNA, or 3-6 ng immunoprecipitated (IP) RNA using GeneChip® 3' IVT expression (Affymetrix). Hybridizations were performed at 45 °C for 16 h, in a rotating platform with 10 µg of biotin-labeled aRNA for

total and polysome RNAs and 7-10 µg of biotin-labeled aRNA for IP RNA. Microarray hybridization was performed by use of three (for samples that derived from *35S:HF-RPL18* (WT)) or two (for samples that derived from *35S:AtCSP1-FH* (OE) and *atcsp1-1* (KD)) independent biological replicate samples. Expression data obtained in .cel files was extracted by use of the Bioconductor package of the R statistical software. Low-level normalization followed using Robust Multichip Average (RMA), and included probe-specific and multichip background corrections. Present, Marginal or Absent (P/M/A) calls, describing signals for probe pair sets that were above or below the background default threshold, were obtained with the Affymetrix MAS 5.0 platform.

The normalized data were used to calculate signal $\log_2$ ratio (SLR) values for total and polysomal and immunoprecipitated mRNA samples. False discovery rates (FDR) for significant differences between mRNA in the samples compared were generated using P-value distributions (Smyth, 2004). These values were used to identify differentially expressed genes (DEGs). Normalized SLR data of probe pair sets (genes) detected with 'present' call across all comparisons, at least one comparison with an FDR < 0.05, and with at least one comparison with $|SLR| \geq 1$ were used as the DEGs for the subsequent cluster and gene category analyses (DEG set 1, n=1,876 probe pair sets; DEG set 2, n=1,345 probe pair sets).

4.3.11 Identification of co-regulated genes by clustering and their ontology

The data were sorted into co-regulated gene by use of Fuzzy *k*-means clustering with Euclidean correlation for the distance measure, a membership exponent of

1.1, maximal number of iterations of 5000 and twenty clusters (Mustroph *et al.*, 2009b). The mean SLR value for each cluster was determined for summary visualization. Clusters membership was analyzed for gene ontology (GO) category enrichment analysis by use of the GOHyperGall function according to Horan *et al.* (2008). GO annotations were obtained from http://geneontology.org/ (TAIR, 08/03/2010 release). The PAGEMAN tool (Usadel *et al.*, 2005, 2006) was used for an independent interpretation of biological significance of differentially expressed genes, through the overview of significant data sets in diagrams of known pathways. The PAGEMAN annotation map utilized TAIR version 9 (TAIR 9: January, 2010 release). Enrichment was decided by Wilcoxon statistical analysis with Benjamini-Hochberg FDR where a *z*-score of 1.96 represents a FDR < 0.05.

4.3.12 Analysis of 5'-untranslated regions and transcript length

5'-UTR and transcript sequences were extracted from http://www.*Arabidopsis*.org (TAIR 9: 06/19/2009 release) based on sequence availability.

4.4 Results

4.4.1 CSP1 associates with polysomes via mRNAs

To study the functional role of CSP1, we began by generating a carboxy-terminal epitope tagged CSP1 (CSP1-FH) over-expressed ectopically in the Col-0 ecotype background driven by the Cauliflower Mosaic Virus (CaMV) 35S promoter

(see Materials and Methods, Chapter 2). To further evaluate the co-fractionation of CSP1 (Figure 3.2) of 7-d-old seedlings, the ribosome pellet (P-170K fraction) was obtained by differential centrifugation and further fractionated by centrifugation through a 20 to 60 % sucrose gradient to separate complexes by density and visualize relative quantities of ribosomal subunits, monosomes and polysomes. Figure 4.2 shows the UV absorbance profiles obtained for *35S:AtCSP1-FH* and Col-0 seedlings, which accumulate similar levels of polysomes relative to 80S monosomes. Although a slightly more pronounced peak in large polysomes was observed in the *35S:AtCSP1-FH* line, this was not a reproducible difference. The immunoblot evaluation of gradient fractions showed that FLAG-tagged CSP1 co-fractionates with polysomes similar to RPS6 (Figure 4.2). CSP1-FLAG accumulated primarily in the polysome-containing fractions and in limited amount in the 80S monosome, 60S, and 40S ribosome subunit-containing fractions. By comparison, the 40S ribosomal subunit protein, RPS6 fractionated with polysomes as well as in the monosome and subunit-containing fractions of the gradient.

To determine whether the co-fractionation of CSP1 with polysomes was via association with mRNA, the polysome pellet fraction (P-170) from *35S:AtCSP1-FH* and *35S:HF-RPL18* (Zanetti *et al.*, 2005) were subjected to RNase A treatment before centrifugation through sucrose gradients. In this experiment, the *35S:HF-RPL18* line was used instead of Col-0 so that the large ribosomal subunit could be monitored with an anti-FLAG antiserum. The purpose of the RNaseA treatment was to digest accessible mRNAs region. For both genotypes, RNaseA treatment resulted in the appearance of ribosome subunits and monosomes, concomintant with the loss of polysomes (Figure 4.3).

Immunoblot of the independent gradient fractions determined that the majority of the CSP1-FLAG shifted to the less dense fractions (Figure 4.3b). The change in CSP1-FLAG sedimentation was distinct from RPS6, which co-fractionated with ribosome subunits and monosomes after the nuclease treatment. By comparison, in the *35S:HF-RPL18* seedlings, RNase treatment resulted in a similar shift in FLAG-tagged RPL18 and RPS6 to the fractions enriched in monosomes and ribosome subunits. These data demonstrate that the fractionation of CSP1 with polysomes is not directly via a ribosomal subunit but is via mRNA or interaction with another protein that binds the mRNA during translation.

4.4.2 Response of CSP1 to cold stress

Previous studies have shown that *AtCSP1* mRNA is induced by cold stress (Karlson & Imai, 2003). To study the effect of cold stress on CSP1 protein accumulation, 25-d-old *Arabidopsis* plants were transferred to 4 °C for 0 to 48 h, the entire rosette was harvested and used to evaluate the effect on endogenous CSP1 levels by immunoblot hybridization with anti-CSP1 antiserum (Figure 4.4). As a control, CSP1 accumulation was simultaneously monitored in plants maintained at control 23 °C under similar illumination. CSP1 was present in rosette leaves grown at 23 °C. The low temperature time course analysis (Figure 4.5) showed that CSP1 levels increased in response to cold stress relative to FLAG-tagged RPL18. CSP1 accumulation peaked between 9 to 24 h after the transfer to the cold. These data indicate that CSP1 is constitutively present and increases in response to cold stress.

4.4.3 Cold increases CSP1 association with polysomes

To determine if the role of polysome-association of CSP1 is altered in response to low temperature stress, we compared the level of CSP1 in ribosomes in three genotypes: *atcsp1-1* knockdown mutant (Figure 4.1), *35S:HF-RLP18,* and *35S:AtCSP1-HF* seedlings grown under standard conditions for 14 d, and subjected to cold stress at 4 °C for 9 h. The seedling tissue was used to obtain a polysome pellet (P-170) that was fractionated, the UV absorbance profile recorded, and immunoblotting performed on the gradient fractions to compare the abundance and sedimentation of CSP1 relative to RPS6. CSP1 was detectable in the ribosomal subunit, monosome, and polysome fractions of all three genotypes under both conditions (Figure 4.6 a-c). Immunoblotting of polysome gradient fractions from *atcsp1-1* and *35S:HF-RLP18* lines detected two bands of endogenous CSP1 (~30 kDa) (Figure 4.6 a and b). In addition, immunodetection of polysome-associated CSP1 from *35S:AtCSP1-FH* identified endogenous CSP1 and CSP1-FLAG (~34 kDa) as a protein doublet (Figure 4.6c). These results suggest that both the polysome-associated endogenous and CSP1-FLAG may be subjected to post-translational modification. In mammals, the CSP ortholog YB-1 can be phosphorylated by the serine/threonine kinase Akt at Ser-102 inside the CSD region (Figure 4.4), which stimulates translation of its targeted mRNAs (Evdokimova *et al.*, 2006). It should be noted that the putative Ser-102 phosphorylation site is conserved in CSP1 (Figure 4.4).

By comparison of level of polysome-associated CSP1 in *atcsp1-1* to *35S:HF-RPL18* seedlings, using RPS6 as a control, a slight reduction in polysome-associated

CSP1 was evident in *atcsp1-1*. Cold stress elevated the amount of polysome-associated CSP1 in all genotypes. The accumulation of CSP1 in polysomes suggests a functional role in translational control that may be further promoted under conditions of low temperature.

4.4.4 *CSP1* may involve in cold-acclimation

Because earlier studies showed that *atcsp3-2* mutants are affected in survival of freezing stress (Kim *et al.*, 2009), we decided to quantitate CSP1 levels in rosette leaves from *atcsp1-1*, *35S:HF-RPL18*, and *35S:AtCSP1-FH* lines in plants grown under non-stress and cold temperatures. Immunoblotting of crude cell extracts (S-16) was performed to evaluate CSP1 composition and abundance using a peptide antiserum prepared against CSP1. The accumulation of CSP1 in *atcsp1-1* rosette tissue was lower than in *35S:HF-RPL18* and *35S:AtCSP1-FH* (Figure 4.7). In the *35S:HF-RPL18* line, endogenous CSP1 was detected as a 30 kDa protein at higher level. The *35S:AtCSP1-FH* over-expression line accumulated two forms of CSP1, the endogenous 30-kDa and the FLAG-tagged 34 kDa form. After 4 d of cold acclimation at 4 ^{o}C, we observed increases in CSP1 in all three genotypes.

To investigate whether over-expression and 9 of *AtCSP1* has phenotypic consequences on cold-treated plants, the three genotypes *atcsp1-1*, *35S:HF-RPL18*, and *35S:AtCSP1-FH* were grown at 4 ^{o}C for up to 2 weeks. However, there was no evident phenotypic distinction between the lines (data not shown). To further consider an effect of low temperature on these lines, we examined the freezing tolerance of non-stressed (23

^{o}C) or cold-acclimated (4 ^{o}C) leaves from *atcsp1-1*, *35S:HF-RPL18*, and *35S:AtCSP1-FH*. This was accomplished by exposing excised leaves to freezing temperature ranged from -2 to -10 ºC. This analysis confirmed that *atcsp1-1* rosette leaves that were cold-acclimated or non-acclimated displayed significant higher percent of electrolyte leakage than *35S:HF-RPL18* leaves at -6 to -10 ºC (Figure 4.7). However, *35S:AtCSP1-FH* and *35S:HF-RPL18* leaf electrolyte leakage was indistinguishable in non-acclimated and cold-acclimated plants. These data suggest that the rosette leaves of the knock-down *atcsp1-1* mutant are freezing hypersensitive.

4.4.5 Native CSP1 complexes can be immunopurified

To validate whether the anti-CSP1 antibody could be used to purify endogenous and epitope-tagged CSP1 protein, we performed immunopurification on the crude cell supernatant (S-16) fraction from rosette leaves. Immunoblot analysis on the total (S-16), post-immunoprecipitation supernatant (unbound) and immunoprecipitate (IP) fractions confirmed that CSP1 can be successfully purified using the anti-CSP1 peptide antibody coupled to Protein A agarose beads (Figure 4.8). This analysis also re-confirmed the distinctions in accumulation of CSP1 in rosette leaves of the *atcsp1-1*, *35S:HF-RLP18*, and *35S:AtCSP1-FH* genotypes.

The immunopurification of CSP1 did not co-purify RPS6, consistent with the immunopurification results obtained with CSP1-FLAG (Chapter 3, Table 3.1). This supports the conclusion that CSP1 association with ribosomes is via the mRNA or another non-ribosomal interaction. However, because some CSP1 remains in the

unbound fraction of the immunoprecipitate, we cannot rule out the possibility that immunoprecipitated that CSP1 associated with mRNAs engaged in translation cannot be immunoprecipitated with the anti-CSP1 antiserum by use of CSP1-FLAG.

Since the formation of mRNP complexes is dynamic and often temporally and spatially regulated, *in vivo* formaldehyde cross-linking can be performed to insure more precise characterization of RNA-protein interactions by preventing dissociation of the RBP from its target and the re-assortment of protein and RNA components during cell lysis (Niranjanakumari *et al.*, 2002). The results in Figure 4.9 demonstrate that vacuum infiltration of rosette leaves with 1 % (v/v) formaldehyde to cross-link protein and protein-RNA complexes did not affect the efficiency of CSP1 immunopurification. In leaves of both non-stressed and cold-stressed plants, immunopurification of CSP1 again failed to yield RPS6, even after cross-linking. Hence, reversible formaldehyde cross-linking can be used to identify CSP1-mRNA complexes, although not the complexes associated with translating ribosomes.

4.4.6 A distinct set of cellular mRNAs is associated with CSP1

To evaluate the binding of CSP1 to mRNAs and the effect of low-temperature on the accumulation and translation of individual mRNAs, we quantitatively profiled the total, polysomal, and CSP1-immunoprecipitated mRNA populations from cold treated and non-treated rosette leaves using the Affymetrix ATH1 DNA microarray platform (Figure 4.11). In this experiment we utilized differential centrifugation to isolate the polysomal RNA fraction (≥ two ribosomes) from three genotypes *35S:HF-RPL18* (used

as WT), *35S:AtCSP1-FH* and *atcsp1-1* and the anti-CSP antibody to purifiy CSP-associciated mRNAs from *35S:HF-RPL18* (used as WT) and *35S:AtCSP1-FH*. The RNAs immunoprecipitated with the anti-CSP1 antibody did not contain ribosomal-RNAs (rRNAs) (Figure 4.11).

Of the 2,420 probe sets with signal levels above the limit of detection across all GeneChips, 2,287 genes (probe pair sets) were differentially regulated and displayed a significant change in transcript abundance in one or more of eighteen SLR comparisons (FDR < 0.05 in one or more of the SLR comparisons as indicated in the "Materials and Methods" (Supplementary Table 4.1)). Of these differentially expressed genes, 1,876 mRNAs (DEGs set 1) also showed at least a two-fold increase or decrease in abundance in one or more mRNA populations ($|SLR| \geq 1$). The hybridization signal values were highly correlated between biological replicate samples (R^2 values ranged from 0.95-0.98 for total and polysomal mRNAs and from 0.81-0.95 for the CSP1-immunoprecipitated samples), confirming the reproducibility of the biological response. A higher variation of R^2 value from the CSP1 immunoprecipitated samples of cold-stress seedlings suggests that CSP1-mRNA complex formation is highly dynamic.

To determine whether CSP1-enriched mRNAs were biased towards highly abundant transcripts, we compared mRNA abundance in the total RNA and CSP-enriched RNAs of DEGs set 1. This confirmed that CSP1-enriched mRNAs not only included highly abundant mRNA, but also included low and moderately abundant mRNAs (Figure 4.12). These findings indicate that CSP1-enriched mRNAs comprise a

subpopulation of cellular mRNAs, confirming selectivity in binding. To further evaluate the response to low-temperature and the binding of CSP1 to mRNAs, we considered the biological role of the DEG set 1 that were co-regulated at the level of transcript accumulation, translation, and CSP1-association. Co-regulated genes were recognized by use of fuzzy *k*-means clustering, which was used to sort the DEGs into 20 co-regulated group of genes (gene clusters) (k = 20; n = 1,876 DEGs) (Figure 4.13; Supplemental Table 4.1). The display of the median $\log_2$ SLR of each cluster in a heat map revealed that the DEGs fall into four groups of genes comprising three to seven clusters (Figure 4.13). Group 1 mRNAs were not dramatically affected by low-temperature at the transcriptional and translational level. However, these mRNAs were associated with CSP1 under both cold and non-stress conditions (n = 627 mRNAs; clusters 1-6). Of these, cluster 1 mRNAs were slightly more elevated at the level of CSP1-association in both conditions than cluster 2-6 mRNAs. Group 2 mRNAs were induced in abundance and translation in response low temperature stress (n = 126 mRNAs; clusters 7-9). Group 3 mRNAs was those maintained at similar steady-state levels in both cold and non-stress conditions except for clusters 10, 13, and 15. These mRNAs were not associated with CSP1 (n = 859 mRNAs; clusters 10-16). This group was more enriched in the immunopurified mRNAs from the CSP1 over-expression line than that of the wild type under cold stress. Group 4 mRNAs were unstable under cold stress (n = 264; cluster 17-20). Notably, there were no clear-differences between the transcriptome, translatome and genotypic effects in response to low-temperature stress for DEGs set 1. Consistent with

this, we observed no change in the overall levels of polysomes from these genotypes in response to cold stress (data not shown).

To evaluate the biological function of the co-regulated mRNAs, we performed Gene Ontology (GO) analysis with the gene clusters (Supplementary Table 4.2). The cold-stabilized and CSP1-associated mRNAs (Group 1) mainly consisted of genes involved in energy consuming processes for example mitochondria respiratory chain, RNP complex, and ribosome biogenesis (*i.e.* cluster 1, ATP hydrolysis coupled proton transport (2.84E-02); cluster 2, structural constituent of ribosome (2.15E-10); ribonucleoprotein complex biogenesis (5.52E-05); cluster 3, respiratory chain complex I (1.69E-03); cluster 4; respiratory chain complex III (4.17E-06); cluster 5; DNA-directed RNA polymerase activity (2.50E-05); structural constituent of ribosome (2.40E-24); DNA-directed RNA polymerase activity (2.50E-05); ribonucleoprotein complex (5.86E-24); cluster 6; structural constituent of ribosome (9.81E-40)). The transcripts that were induced and translated in response to cold-stress (Group 2) were enriched for abiotic stress, cold, water deprivation, and reactive oxygen species (cluster 7, 3.25E-10; cluster 8, 4.95E-07, 4.89E-03; 1.29E-02, respectively). The clusters of mRNAs that remained stable under cold stress but were not associated with AtCSP1 (Group 3) encode for chloroplast proteins (cluster 15, 1.40E-10; cluster 16, 8.16E-66). Notably, this group was also enriched in ribosomal protein mRNAs (cluster 13, 5.56E-30). The transcripts that were unstable and poorly translated under cold (Group 4) encode proteins involving in secondary metabolite synthesis including glucosinolate biosynthesis (cluster 18, 3.44E-04; cluster 20, 7.75E-06).

We utilized similar strategy described in section 2.4.3 to evaluate the connection between molecular function and regulation of transcriptome, translatome, and CSP1-associated mRNAs from different genotypes. The PAGEMAN tool (Wilcoxon sign rank test / Benjamini-Hochberg FDR < 0.05) identified distinctions in the CSP1-associated mRNAs. The mRNAs encoded proteins for photosynthesis, central metabolism, and transport display no changes in the steady-state accumulation and polysomal mRNA levels following the cold-treatment. This group was not enriched in CSP1 complexes (Figure 4.14). The mRNAs involves in secondary metabolism, including glucosinolate synthesis were unstable and not well translated under cold-stress. The group of mRNAs that associated with CSP1 encoded proteins for RNA processing and protein synthesis. This group of mRNAs was also induced and translated under cold conditions. The results suggested no differences in transcriptome and translatome, comparing between genotypes. Taken together, the fuzzy *k*-means clustering and PAGEMAN analysis confirm that CSP1 regulates mRNAs involved in the post-transcriptional processes and ribosome biogenesis.

4.4.7 Specific mRNA features characteristic for CSP1-bound mRNAs

We made an effort to identify *cis*-regulatory features that are important for the binding of CSP1. We found that there were significant differences in the G+C content of the 5'-UTR of CSP1-associated mRNAs (Figure 4.15a). The mRNAs from clusters 1-2 and 5-6 displayed significant higher G+C content at the 5'-UTR comparing with those from the DEGs set 1 (p-value < 0.005). Notably, cluster 1 (strongest enrichment in CSP1

complexes) displayed the highest 5'-UTR G+C percentage. By contrast, the clusters that were not enriched in CSP1 complexes displayed lower 5'-UTR G+C content, with clusters 8 and 20 showing significant differences (p-value < 0.005). We also tested for G+A and C+U biases in the 5'-UTRs of each cluster, however, there were no differences between the CSP1-enriched and CSP1 non-enriched mRNAs (data not shown).

The comparison of the length of the transcript from each cluster revealed significant differences between CSP1-enriched and CSP1 non-enriched mRNAs (Figure 4.15b). The transcript length from clusters 1-6 was shorter than that of the DEGs set 1, except for cluster 4. There were significant differences in transcript length of clusters 1-4 and 6 (p-value < 0.005). By contrast, the length of transcripts from CSP1-unbound mRNAs was longer than that of the DEGs set 1, except for clusters 8, 17 and 19. There were also significant differences in the length of clusters 10, 11,14,17, and 19 (p-value < 0.005).

4.4.8 Cold-regulated transcriptome and translatome are highly correlated

To further evaluate the effect of low-temperature on the accumulation and translation of individual mRNAs, we compared changes in the total and polysomal, mRNA populations from cold treated and non-treated rosettes (Figure 4.16). Of the 8,597 probe sets with signal levels above the limit of detection in the total and polysomal RNA populations from the in all three genotypes and the two conditions evaluated, 1,792 genes (probe pair sets) displayed a significant change in transcript abundance in one or more of six SLR comparisons (FDR < 0.05 in one or more of the SLR, Supplementary Table 4.3).

Of the 1,792 genes, 1,345 mRNAs (DEG set 2) showed at least a two-fold increase or decrease in abundance in one or more mRNA population ($|SLR| \geq 1$). The Venn diagrams in Figure 4.16 show that the expression of subsets of the DEG set 2 mRNAs was up-regulated or down-regulated under cold stress in all three genotypes. The regulation of total and polysomal mRNA abundance was highly overlapping, indicating a correlation between the transcriptome and translatome adjustment in response to cold-stress (Figure 4.14). Gene ontology analyses of cold-induced or translated mRNAs identified mRNAs that involve is response to stimulus (5.87E-21) (Supplementary Table 4.4). The cold-destabilized or non-translated mRNAs included mRNAs that involves in glucosicolate metabolic process (1.18E-17), response to stress (2.52E-09), and starch metabolic process (1.29E-08). These findings confirmed that gene regulation in response to cold stress was mainly regulated by changes in transcript abundance rather than through stress-regulated mRNA translation.

4.5 Discussion

4.5.1 CSP1 is a polysome-associated RNA chaperone

In this study we determined that the cold-induced RNA binding protein CSP1 co-purifies with other RBPs and polysomes and binds a subset of cellular transcripts. Previous studies have confirmed that CSP1 has double- and single-stranded RNA and DNA binding activity, leading to the suggestion that it acts as a mRNA chaperone under cold stress (Kim *et al.*, 2007; Park *et al.*, 2009). However, the molecular mechanism underlying this process and the mRNA species that CSP1 regulates were unknown. Our

study utilized the transgenic *Arabidopsis* that ectopically over-expresses and accumulates His_6-FLAG-tagged-CSP1 (see Section 3.3.2) to evaluate CSP1 fractionation from cell extracts by differential centrifugation (Figure 4.2). This determined that CSP1 co-fractionated with polysomes in a manner that was disrupted by mild RNase A digestion of accessible single-stranded RNA (Figure 4.3). By comparison of the effect of RNase A digestion of polysome complexes with the sedimentation of CSP1 and the core ribosomal proteins RPS6 and RPL18, we confirmed that binding of CSP1 to polysomes is via mRNA. This is likely to be via direct binding to the mRNA since CSP1 has RNA binding activity, however indirect association via interaction with a protein such as a translation factor cannot be ruled out.

Our study showed that CSP1 accumulation (Figure 4.5) and its association with polysomes (Figure 4.5) was enhanced after 6 h or more of cold treatment. Moreover, we found that the *atcsp1-1* knock-down mutant is significantly sensitive to freezing, based on an electrolyte leakage test. *atcsp1-1* rosettes accumulated less CSP1 under non-stress and cold-stress conditions (Figure 4.5) in the crude extract and polysome complexes (Figure 4.6). Despite the hypersensitivity to freezing, we did not detect any dysfunction in tolerance of prolonged cold tolerance in the *atcsp1-1* mutant as compared to the wildtype and a CSP1 over-expression line, based on growth and levels of polysomes (data not shown). This suggests that the level of CSP1 in the *atcsp1-1* mutant is sufficient to maintain protein synthesis and growth. In support of this, we observed limited differences in the transcriptome and translatome adjustments to 12 h of cold stress treatment in wildtype, *35S:AtCSP1-FH* and *atcsp1-1* rosettes (Figure 4.15).

To gain new insight into the functional role of CSP1, we developed a peptide antiserum that specifically recognized the protein and was effective in immunopurification of CSP1 (Figures 4.8 and 4.9) and non-ribosomal RNAs (Figure 4.10). To identify and quantitatively assess the mRNAs that bind to CSP1, hybridizations were performed to the ATH1 GeneChip platform. The DNA microarray results revealed that CSP1 preferentially binds to a subpopulation of mRNAs, primarily encoding proteins involved in energy-demanding process, including RNP complex formation and ribosome biogenesis (Figures 4.12-4.14). These indicate a CSP1-RNA regulon exists that coordinates or augments the expression of a sub-population mRNAs. CSP1 showed limited association with abundant mRNAs with roles in photosynthesis and central carbon metabolism. Interestingly, many of the CSP1-associated mRNAs had a high G+C content in their 5'-UTR and shorter than average total transcript length (Figure 4.15). Since a high G+C content correlates with more thermodynamically stable secondary structures (Kawaguchi & Bailey-Serres, 2005),

CSP1 may be important for removal of secondary structure to facilitate access of the translation machinery and the scanning of the 5'-UTR by the 43S pre-initiation complex. Under low temperatures, the ability to maintain translation of mRNAs encoding ribosomal proteins could be necessary to sustain growth. *Arabidopsis thaliana* plants can cold-acclimate, grow, and reach maturity under low-temperature condition (4-6°C) (Miquel *et al.*, 1993; Schneider *et al.*, 1995). Similar to *Arabidopsis*, wheat is tolerant to cold-stress (Crespi *et al.*, 1991; Houde *et al.*, 1992). Interestingly, both *Arabidopsis* and wheat possess CSPs that accumulate under cold-stress condition (Figure

4.5) (Karlson *et al.*, 2002; Sasaki *et al.*, 2007). On the other hand, in rice, a cold-sensitive species, CSP accumulation was constant following low-temperature treatment (Chaikam & Karlson, 2008). These findings indicate that the accumulation of CSPs may be important for cold-adaptation. It would be interesting to know whether or not cold-stress has a greater impact on global protein synthesis in rice than in Arabidopsis.

4.5.2 Possible functional redundancy among RNA chaperones

Low temperatures promote formation of nucleic acid secondary structures. RNA chaperones are ubiquitous and abundant proteins that function in resolving these structures and facilitate the interaction between the mRNA and other regulatory factors (Jiang *et al.*, 1997). Bacterial RNA chaperones are important for regulation of transcription, translation, and ribosome assembly (Rajkowitsch *et al.*, 2007). In *Arabidopsis*, several RBPs were proposed to function as RNA chaperones under cold-stress, including GRPs 1-8, RZ-1 a-c, and CSPs 1-4 (Kim *et al.*, 2005, 2007, 2008, 2009, 2010; Kwak *et al.*, 2005; Nakaminami *et al.*, 2006, 2009; Sasaki *et al.*, 2007; Lee *et al.*, 2008). Heterologous expression of *AtCSP1* and *AtGRP7* (At2g21660) has been shown to complement the cold sensitive phenotype of *E coli* strain BX04 that lacks four native *CSPs* (Kim *et al.*, 2007), suggesting they function similar to bacterial CSPs by chaperoning mRNAs under low temperature conditions. More recently, evaluation mRNA location at a subcellular level by poly(A) mRNA *in situ* hybridization provided evidence that an *atgrp7* mutant was defective in transport of mRNA from the nucleus to the cytoplasm at cold temperatures (Kim *et al.*, 2010). The over-expression of *AtCSP1* in

Arabidopsis rescued an *atgrp7-1* mutant from freezing damage (Kim *et al.*, 2007), indicating that these RBPs may function in a redundant manner. It will be of interest to determine whether or not *Arabidopsis* CSP1 functions during transport in addition to translation. The co-immunopreciptation of CSP1 with another glycine-rich protein (GRP3S) and a pre-mRNA splicing factor (PRP8) and the accumulation of CSP1-YFP in both the cytoplasm and the nucleus is consistent with a role of this protein within the nuclear domain (Chapter 3, Figures 3.5-3.7).

In our study, we did not observe any marked differences between the transcriptomes and translatomes of three genotypes that accumulate different levels and CSP1 in non-stressed and cold-stressed rosette tissue. This may be due to the functional redundancy among cold-inducible RNA chaperones including other GR and CSPs (*i.e*. the closely related CSP3) or the residual amount of CSP1 produced by *atcsp1-1* mutants. The redundancy or residual CSP1 may be sufficient to maintain translation at 4 ^{o}C but insufficient to protect against cell damage at freezing temperatures. To further investigate these possibilities, *atcsp1-1, atcsp3-2,* and *atgrp7-1* double and triple mutants might be produced and studied. Alternatively, an inducible microRNA system targeting *AtCSP1* expression more severely limit production of CSP1 (Ossowski *et al.*, 2008).

4.5.3 Translational regulation in response to low-temperature stress

In contrast to the translational regulation in response to dehydration (Kawaguchi *et al.*, 2004; Kawaguchi & Bailey-Serres, 2005), hypoxia (Branco-Price *et al.*, 2005, 2008), and light/dark transition (Chapter 2), we did not observe marked

differences between transcriptome and translatome adjustments in response to cold-stress in rosette tissue. Moreover, we found that polysome levels following 12 h of cold-stress were indistinguishable from normal growth conditions. Early investigation of the influence of low temperatures on levels of large and small polysome complexes showed that cold treatment did not affect the distribution of the different classes of polysomes, but did increase overall levels of, polysomes in wheat seedlings (Fehling & Weidner, 1986; Perras & Sarhan, 1990) and rye (*Secale cereale*) seedlings (Laroche & Hopkins, 1987), both cold-tolerant plants. Antikainen and Pihakaski (1993) proposed that the increase in polysome content reflected the adjustment of plant metabolism to aid cold adaptation. Castiglioni *et al.* (2008) showed that by overexpressing bacterial CSPs A and B, cold-tolerance in *Arabidopsis* seedlings was improved (grown at 8 °C for 6 weeks). Moreover, the same group showed that over-expression of bacterial CSPs improved cold and heat-tolerance in rice seedlings, and drought-tolerance in rice seedlings and in several developmental stages of maize (Castiglioni *et al.*, 2008). It would be interesting to examine whether or not the stress-tolerance in these plants is correlated with polysome accumulation and translational regulation.

In *Arabidopsis*, the transcript level of protein synthesis regulators, ribosomal protein S6 kinase 1 and 2 (*AtS6K1* (At3g08730); *AtS6K2* (At3g08720)) accumulated rapidly under cold-treatment (Mizoguchi *et al.*, 1995), suggesting S6Ks may function in the adaptation of plants to low-temperature stress. Our microarray data indicated that both *AtS6K1* and *AtS6K2* transcripts accumulate under cold treatment in all three genotypes (Supplementary Table 4.1), although accumulation in the transcriptome and

translatome populations was not statistically different. Intriguingly, the poly-phosphorylation of the carboxy terminus of RPS6, a target of S6k, is enhanced by cold stress of maize seedlings (Williams *et al.*, 2003). By contrast, in *Arabidopsis* seedlings exposed to hypoxia, *AtS6K2* mRNA was induced but not recruited to polysomes until reoxygenation (Branco-Price *et al.*, 2008). In maize, hypoxia reduced the overall phosphorylation of RPS6 (Williams *et al.*, 2003). Together, these data suggest that overall polysome levels and the translation of ribosomal protein mRNAs is maintained during adaptation to low temperatures, in contrast to the decline in protein synthesis and particularly in ribosomal protein mRNA translation observed in response to other abiotic stimuli.

4.6 Conclusion

In conclusion, this study provides the first molecular evidence that the cold-inducible RBP, CSP1, of the higher plant *Arabidopsis thaliana* associates with polysomes in an RNA-dependent manner and preferentially binds a sub-population of cellular mRNA species. The study also provides the first genome-level evaluation of adjustments in steady-state abundance and polysome-association of transcripts in response to cold temperatures. Finally, the study is the first to successfully capture a sub-population of nuclear-encoded gene transcripts of plant cells, beyond those associated with polysomes.

Future studies can more fully elucidate the molecular mechanisms of CSP1 function. Important unanswered questions include: (1) Is there specificity in CSP1

binding at the sequence or structural level? (2) Does CSP1 binding initiate in the nucleus during mRNA maturation? (3) Is CSP1 binding relevant to transport from the nucleus to the cytoplasm and/or the pioneer translation event? (4) Does CSP1 binding reduce secondary structure and act synergistically with RNA helicase activity associated with the cap-binding complex (eIF4) and the scanning 43S pre-initiation complex? Toward this end, CSP1-mRNA binding sequences may be identified through *in vivo* cross-linking, nuclease digestion of unprotected mRNA and CSP1-immunopurification followed by high throughput sequencing (CLIP-seq) (Ule *et al.*, 2003, 2005). Finally, strategic production of *AtCSP* and *AtGRP* mutants or identification of chemical compounds that specifically bind to CSPs might be used to provide further resolution of the biological activities of the RNA chaperone CSP1.

4.7 Acknowledgements

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Figure 4.1. Confirmation of the *atcsp1-1* mutant (a) Diagram showing the position of the T-DNA insertion and primers (arrows, 1:AtCSP1-gen-forward, 2: LBa1, 3: AtCSP1 reverse) used for genotyping. (b) Total RNA from Col-0 and *atcsp1-1* homozygotes was extracted from 10-d-old seedlings and RT-PCR was performed and separated on agarose gels. *ACTIN2* expression was used as a loading control.

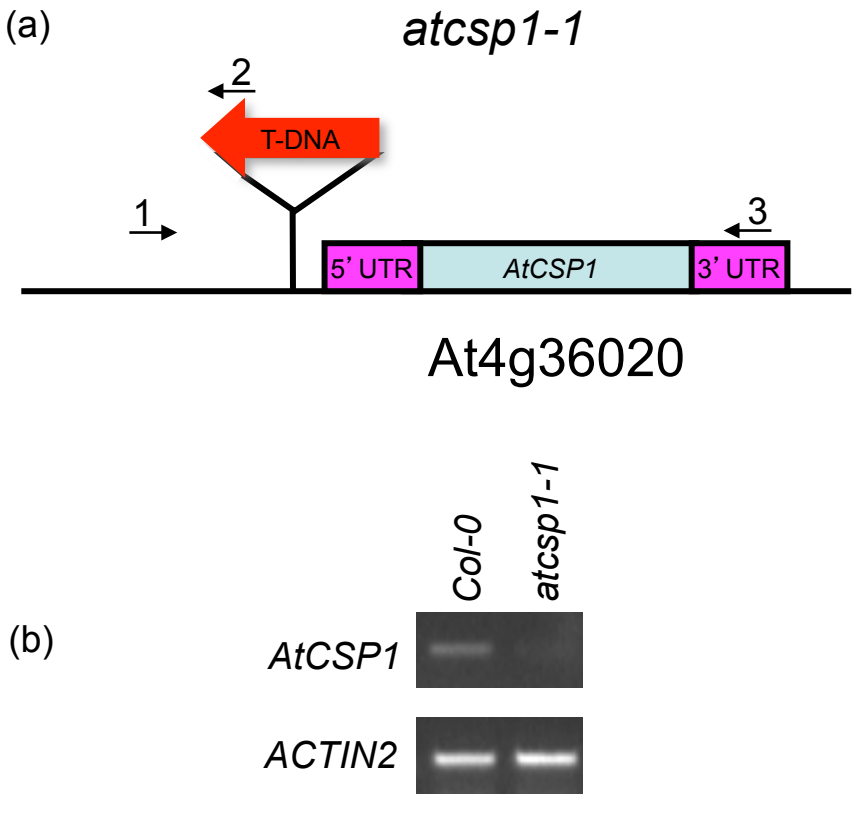


Figure 4.2. CSP1 co-migrates with polysome complexes. Polysome profiles were obtained from whole Col-0 and *35S:AtCSP1-FH* seedlings grown for 7 d on solid MS agar containing 1 % sucrose. Proteins from each fraction were concentrated by precipitation, separated by 12.5 % SDS-PAGE, and subjected to immunoblot analysis with anti-FLAG-HRP or anti-RPS6 antisera. The apparent molecular mass of CSP1-FLAG is 34 kDa and that of RPS6 is 28 kDa.

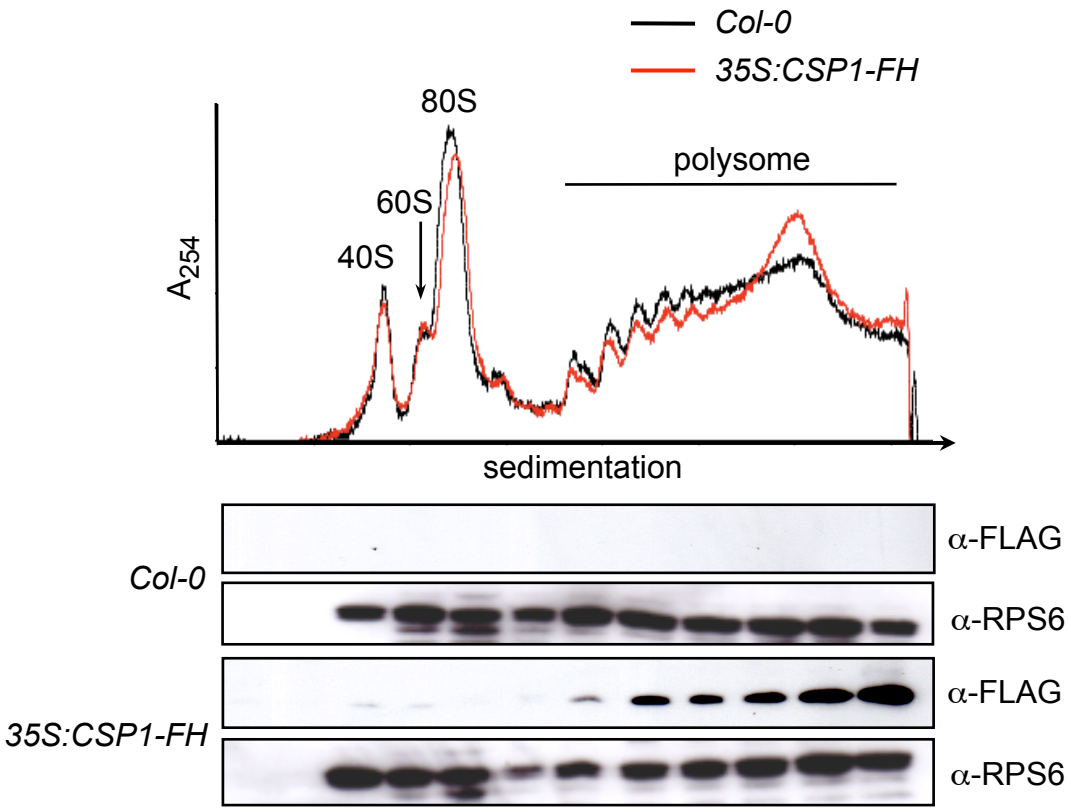


Figure 4.3. CSP1 association with polysomes via RNA. Polysome profiles from 10-d-old *35S:HF-RPL18* (a) and *35S:AtCSP1-HF* (b) whole seedlings, control (non-treated) and treated with RNase A. Proteins from each fraction were precipitated, fractionated on 20-60% sucrose gradients, separated by 12.5 % SDS-PAGE, and subjected to immunoblot analysis with anti-FLAG-HRP (for HF-RPL18 and CSP1-FLAG detection) or anti-RPS6 antisera (for RPS6 detection). The FLAG-tagged protein in the *35S:HF-RPL18* line is RPL18 (22 kDa apparent molecular mass); the FLAG-tagged protein in the *35S:AtCSP1-HF* line is CSP1 (34 kDa apparent molecular mass). The apparent molecular mass of RPS6 is 28 kDa.

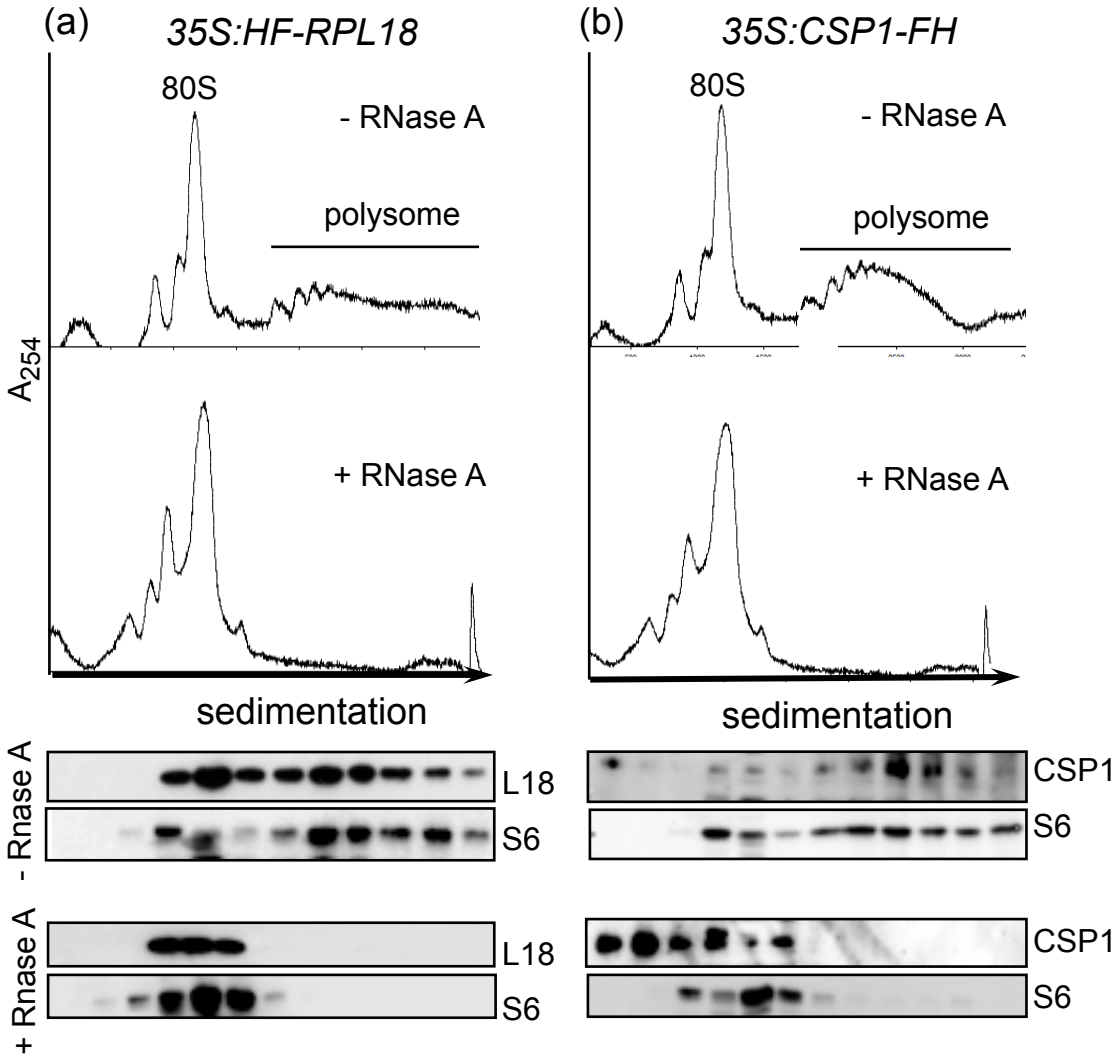


Figure 4.4. The alignment of *Arabidopsis* CSPs amino acid sequences. Four *Arabidopsis* CSPs (*AtCSPs* 1-4, At4g36020, At4g38680, At2g17870, At2g21060, respectively), *Escherichia coli* CSP A (AP_004238.1) and *Homo sapiens* YB-1 (NP_004550.2) were included in the analysis. Identical conserved consensus amino acids are indicated by asterisks; conserved substitutions are indicated by colons and periods. Sequences were aligned using CLUSTAL_W (Thompson *et al.*, 1994). Gaps were introduced to ensure maximum homology. Yellow highlight indicates the *Arabidopsis* CSP1 region used to synthesis a CSP1-specific peptide for polyclonal antibody production. Red highlight indicates a conserved Serine phosphorylation site found in YB-1 (Ser-102) (Evdokimova *et al.*, 2006). Black underlines indicate CSD domain.

```
AtCSP1 ------------------------------------------MAS----EDQSAARST 12
AtCSP3 ------------------------------------------MAM----EDQSAARSI 12
AtCSP2 ------------------------------------------MSG----DNGGGERRK 12
AtCSP4 ------------------------------------------MSGGGDVNMSGGDRRK 16
CSP_A  ----------------------------------------------------SGKMT 5
YB-1   MSSEAETQQPPAAPPAAPALSAADTKPGTTGSGAGSGGPGGLTSAAPAGGDKKVIATKVL 60
                                                           . :

AtCSP1 GKVNWFNASKGYGFITPDDGSVELFVHQSSIVSEG----YRSLTVGDAVEFAITQGSDGK 68
AtCSP3 GKVSWFSDGKGYGFITPDDGGEELFVHQSSIVSDG----FRSLTLGESVEYEIALGSDGK 68
AtCSP2 GSVKWFDTQKGFGFITPDDGGDDLFVHQSSIRSEG----FRSLAAEEAVEFEVEIDNNNR 68
AtCSP4 GTVKWFDTQKGFGFITPSDGGDDLFVHQSSIRSEG----FRSLAAEESVEFDVEVDNSGR 72
CSP_A  GIVKWFNADKGFGFITPDDGSKDVFVHFSAIQNDG----YKSLDEGQKVSFTIESGAKG- 60
YB-1   GTVKWFNVRNGYGFINRNDTKEDVFVHQTAIKKNNPRKYLRSVGDGETVEFDVVEGEKG- 119
       * *.**.  :*:***. .*   ::*** ::* .:.     :*:   : *.: :  . ..

AtCSP1 TKAVNVTAPGGGSLKKENNSRGN----GARRGGGGSGCYNCGELGHISKDCGIGGGG--- 121
AtCSP3 TKAIEVTAPGGGSLNKKENSS---------RGSGGN-CFNCGEVGHMAKDCDGGSGGKSF 118
AtCSP2 PKAIDVSGPDGAPVQG--------------NSGGG---------------SSGGRGG--F 97
AtCSP4 PKAIEVSGPDGAPVQG--------------NSGGGG--------------SSGGRGG--F 102
CSP_A  PAAGNVTSL-------------------------------------------------- 69
YB-1   AEAANVTGPGGVPVQGSKYAADRNHYRRYPRRRGPPRNYQQNYQNSESGEKNEGSESAPE 179
       . * :*:.

AtCSP1 GGGERRSRGGEG-CYNCGDTGHFARDCTSAGNGDQRGATKGGNDGCYTCGDVGHVARDCT 180
AtCSP3 GGGGGRRSGGEGECYMCGDVGHFARDCRQSGGGNSGGGGGGGRP-CYSCGEVGHLAKDCR 177
AtCSP2 GGG--RGGGRGS-------GGGYGG--GGGGYGGRGGGGRGGSD-CYKCGEPGHMARDCS 145
AtCSP4 GGGGGRGGGRG--------GGSYGGGYGGRGSGGRGGGG-GDNS-CFKCGEPGHMARECS 152
CSP_A  ---------------------------------------------------------- 
YB-1   GQAQQRRPYRRRRFPPYYMRRPYGRRPQYSNPPVQGEVMEGADN--QGAGEQGRPVRQNM 237

AtCSP1 QKSVGNGDQRGAVKG-GNDGCYTCGDVGHFARDCTQKVAAGNVRSGGGGSGTCYSCGGVG 239
AtCSP3 GGSGGNRYGGGGGRGSGGDGCYMCGGVGHFARDCRQN-GGGNV--GGGGS-TCYTCGGVG 233
AtCSP2 EGGGG---YGGGGGGYGGGGGYGGGGGGY----------GGGGRGGGGGGGSCYSCGESG 192
AtCSP4 QGGGG---YSGG----GGGGRYGSGGGG----------------GGGGGGLSCYSCGESG 189
CSP_A  ---------------------------------------------------------- 
YB-1   YRGYRPRFRRGPPRQRQPREDGNEEDKEN----------QGDETQGQQPPQRRYRRNFNY 287

AtCSP1 HIARDCATKRQPS-----RGCYQCGGSGHLARDCDQRGSG---GGGNDNACYKCGKEGHF 291
AtCSP3 HIAKVCTSKIPSGGGGGGRACYECGGTGHLARDCDRRGSGSSGGGGGSNKCFICGKEGHF 293
AtCSP2 HFARDCTS----------------GGR---------------------------------- 203
AtCSP4 HFARDCTS----------------GGAR--------------------------------- 201
CSP_A  ---------------------------------------------------------- 
YB-1   RRRRPENPKPQ-------------DGKETKAADPPAENSSAPEAEQGGAE---------- 324

AtCSP1 ARECSSVA 299
AtCSP3 ARECTSVA 301
AtCSP2 --------
AtCSP4 --------
CSP_A  --------
YB-1   --------
```

Figure 4.5. Immunoblot analysis of CSP1 protein accumulation under cold stress. *35S:HF-RPL18* 25 d-old plants were exposed to 4 ºC for indicated time points (0-48 h). Total protein was extracted from rosette leaves, separated by 12.5 % SDS-PAGE, and subjected to immunoblot analysis with anti-CSP1 or anti-FLAG antisera. The FLAG-tagged protein is RPL18 (22 kDa apparent molecular mass). The apparent molecular mass of CSP1 is 30 kDa.

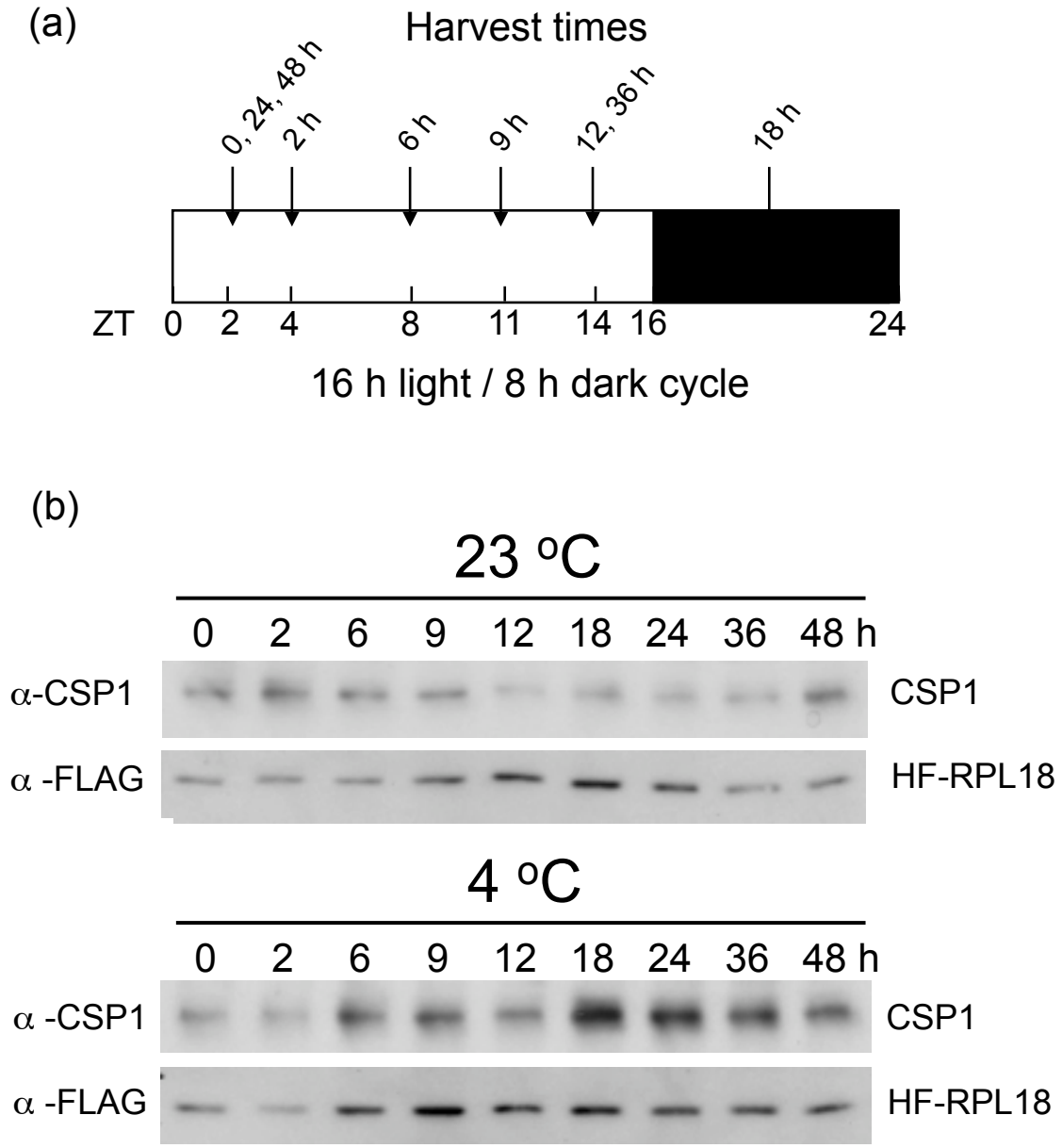


Figure 4.6. The effect of low temperature on accumulation of CSP1 in complexes. Polysome profiles from 14-d-old *atcsp1-1* (a), *35S:HF-RPL18* (b) and *35S:AtCSP1-FH* (c) whole seedlings were subjected to non-stress treatment (NS: black line) or exposed to 4 ^{o}C for 9 h (Cold: red line). Proteins from each fraction were precipitated, fractionated by 12.5 % SDS-PAGE, and subjected to immunoblot analysis with anti-CSP1 (CSP1) or anti-RPS6 (S6) antisera. The anti-CSP1 antiserum detects both the endogenous CSP1 and the FLAG-tagged version of this protein in *35S:AtCSP1-FH* seedlings. The apparent molecular mass of CSP1, CSP1-FLAG and RPS6 are 30, 34 and 28 kDa respectively.

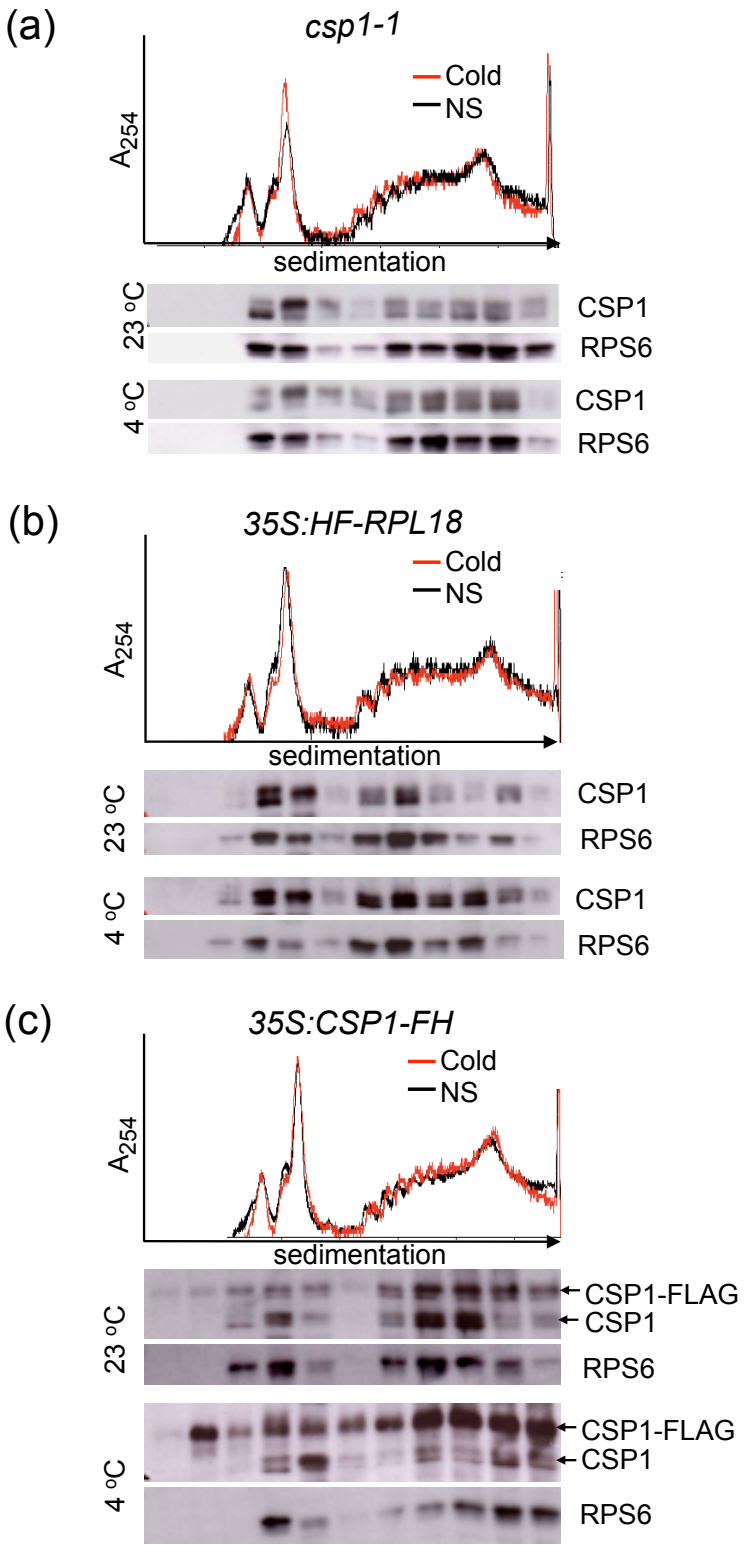

(a)
csp1-1
Cold
NS
A254
sedimentation
23 °C
CSP1
RPS6
4 °C
CSP1
RPS6
(b)
35S:HF-RPL18
Cold
NS
A254
sedimentation
23 °C
CSP1
RPS6
4 °C
CSP1
RPS6
(c)
35S:CSP1-FH
Cold
NS
A254
sedimentation
23 °C
CSP1-FLAG
CSP1
RPS6
4 °C
CSP1-FLAG
CSP1
RPS6

Figure 4.7. Freezing sensitivity of *atcsp1-1* (*csp1-1*), *35S:HF-RPL18* (L18) and *35S:AtCSP1-HF* (CSP1). Three-week-old plants grown for 4 d at 23 ºC or 4 ºC. Leaves were cut at the base of petiole and used for electrolyte leakage measurement (a) or detection of CSP1 in crude cell extracts following fractionation by 12.5% SDS-PAGE and detection with anti-CSP1 antiserum (b). Error bars represent standard deviation (n = 4). The asterisks represent (a) significant differences between the means of percent electrolyte leakage in leaves of *csp1-1* and *L18* (Student's t-test, p-value < 0.05) and (b) non-specific cross-reactive proteins.

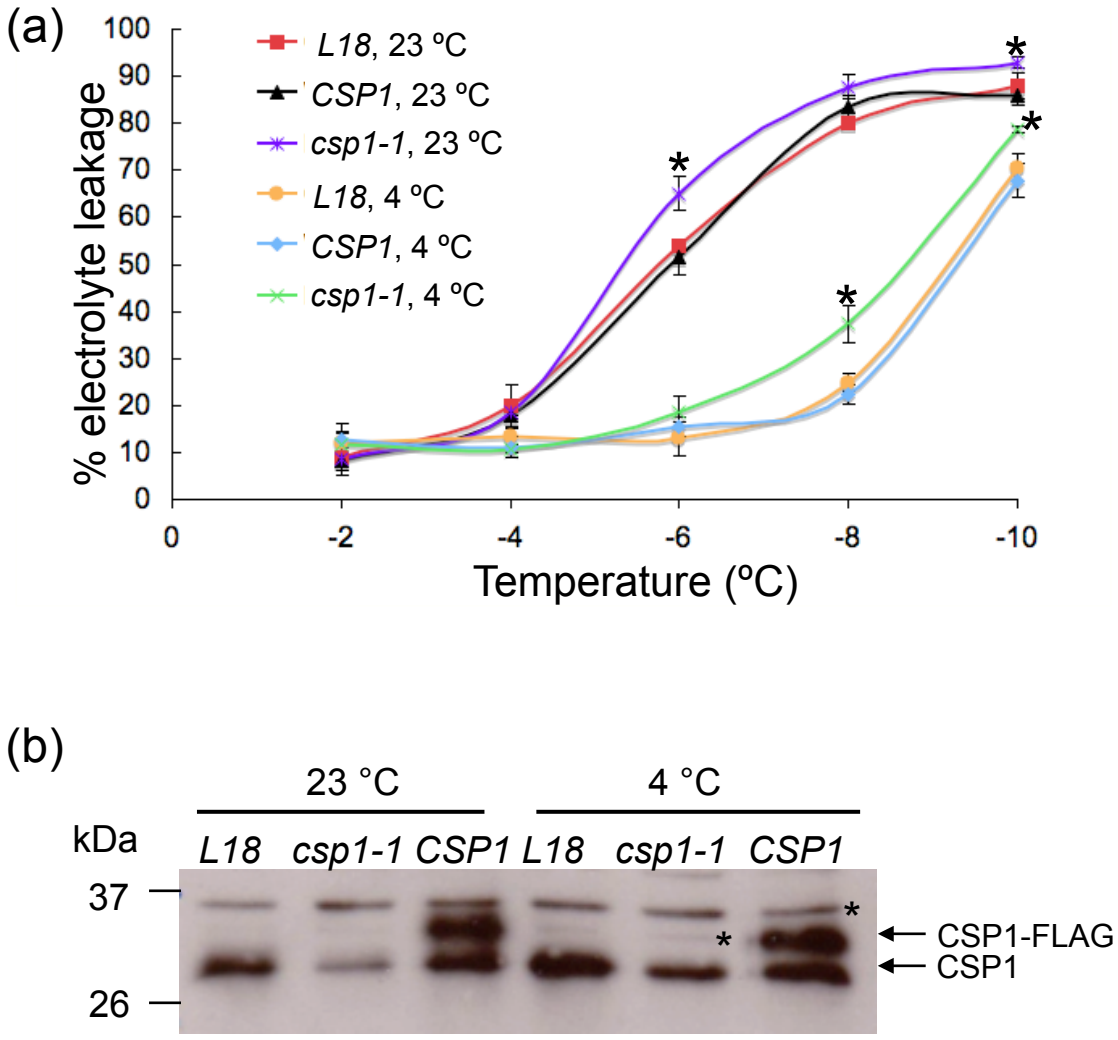


Figure 4.8. Demonstration of the specificity of the anti-CSP1 antiserum. Crude cell extracts from 14-d-old seedlings were used for immunoprecipitation of CSP1 with the antiserum prepared against a CSP1-specific peptide. The crude sample (total), supernatant of the immunoprecipitation (Unbound) and immunoprecipitate (IP) were evaluated. Following 12.5 % SDS-PAGE, immunoblot blot detection was performed with anti-CSP1 or anti-RPS6 antisera. Asterisks represent non-specific cross-reactive proteins. Arrows indicate the IgGs present in the IP fraction, CSP1-FLAG, endogenous CSP1, and RPS6.

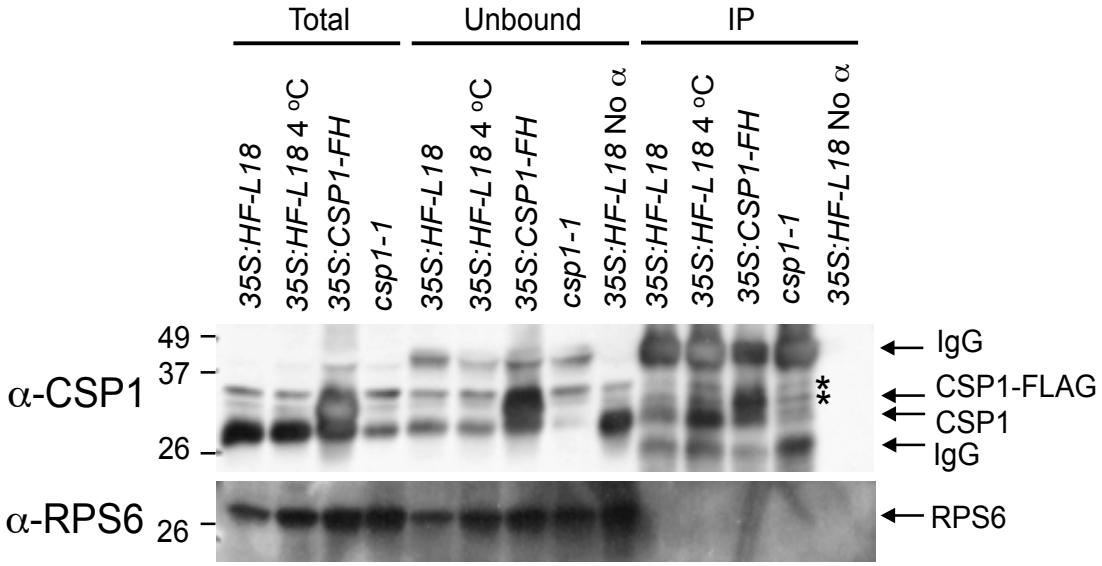


Figure 4.9. Formaldehyde cross-linking did not further improve immunopurification of CSP1. Immunoblot results from CSP1-immunopurifications using leaves of 25 d old plants (*35S:HF-RPL18*) subjected to formaldehyde (FA) cross-linking (+) or without formaldehyde cross-linking (-). Total, unbound and immunoprecipitated (IP) samples were evaluated as described in Figure 4.7. Results from non-stressed (a) and cold-stressed plants (b).

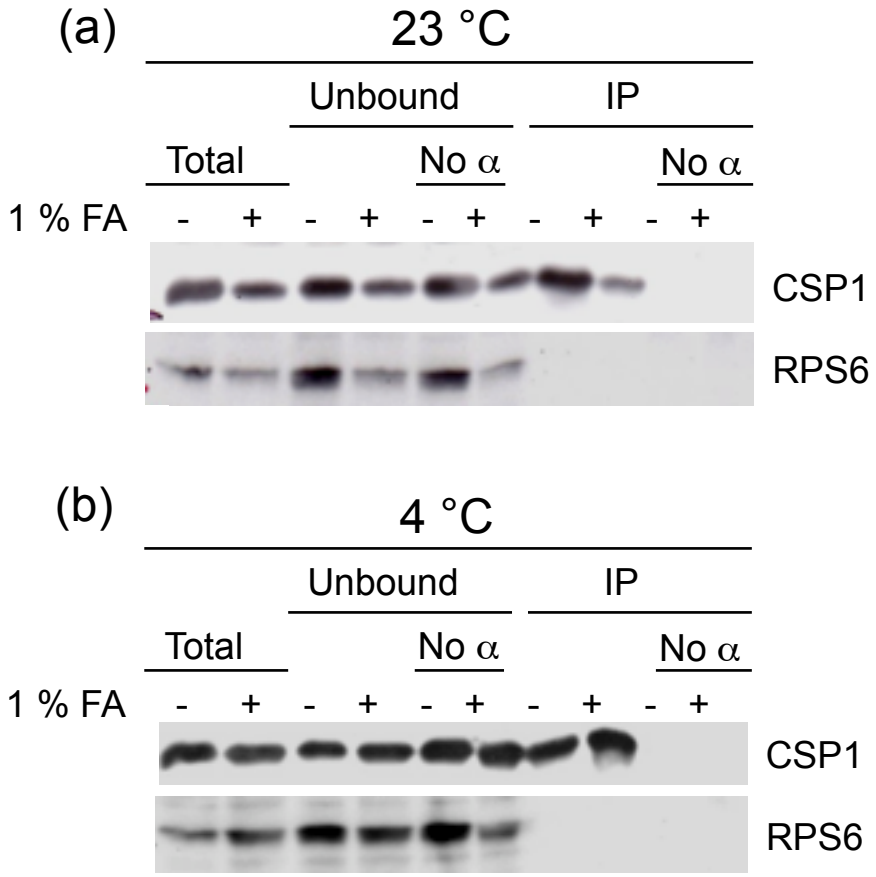


Figure 4.10. RNA bioanalyzer gel of total, polysomal, and CSP1-immunoprecipitated (IP) RNAs from rosette leaves of *35S:HF-RPL18* plants grown at 23 °C. Nuclear rRNAs (25S, 18S, 5.8 and 5s) were labeled.

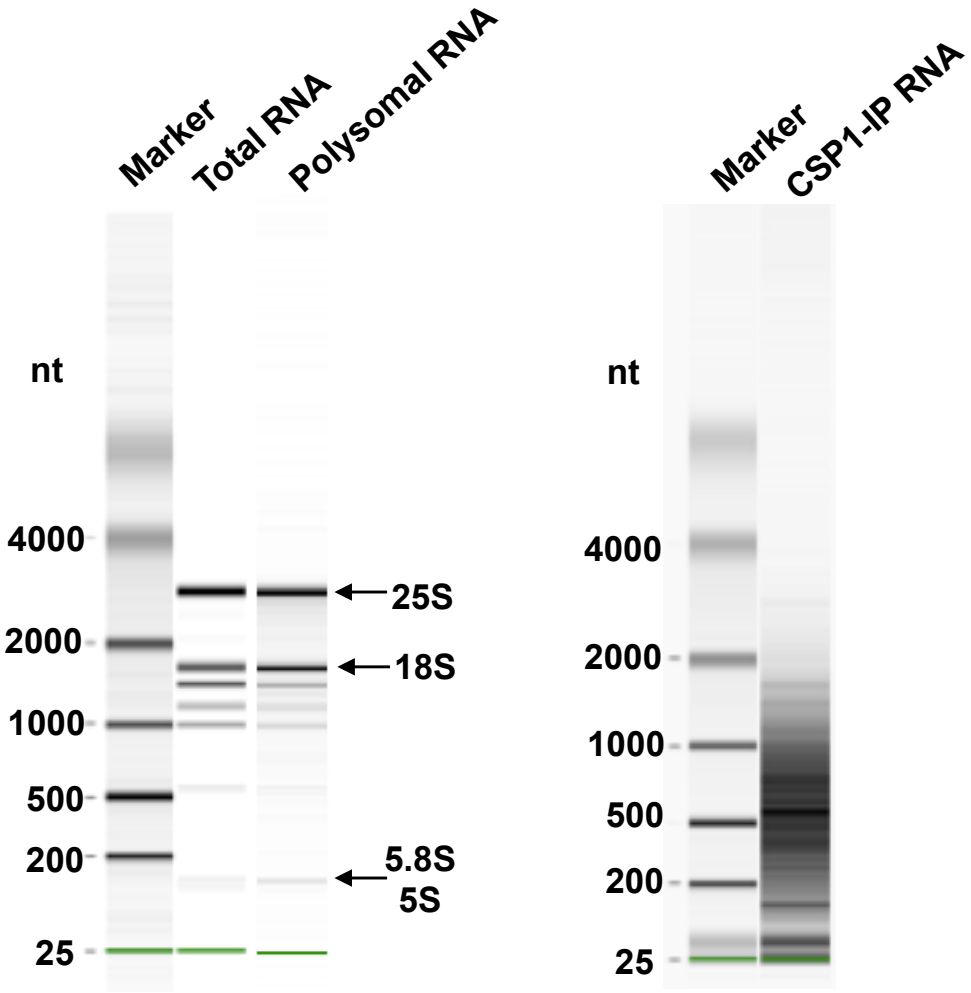
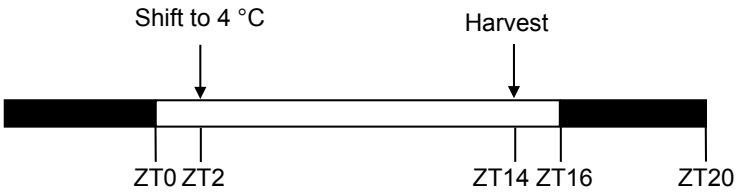


Figure 4.11. Quantitative analysis of low temperature-induced changes in the total, polysomal, and CSP1-associated mRNA populations of rosette leaves. (a) Experimental scheme of the light cycle and treatment used for 25-d-old plants grown in soil. Arrows indicate time of day relative to the beginning of the light period (ZT0). For the non-stressed control samples, leaves from plants grown at 23 °C were harvested in the light at ZT14. For the 12 h cold-treatment, plants were transferred to 4 °C at ZT2 for 12 h before harvesting. (b) Flow diagram of experimental strategy for quantitative assessment of gene transcript abundance of the total, polysomal, and CSP1-associated mRNA populations.

(a)



(b)

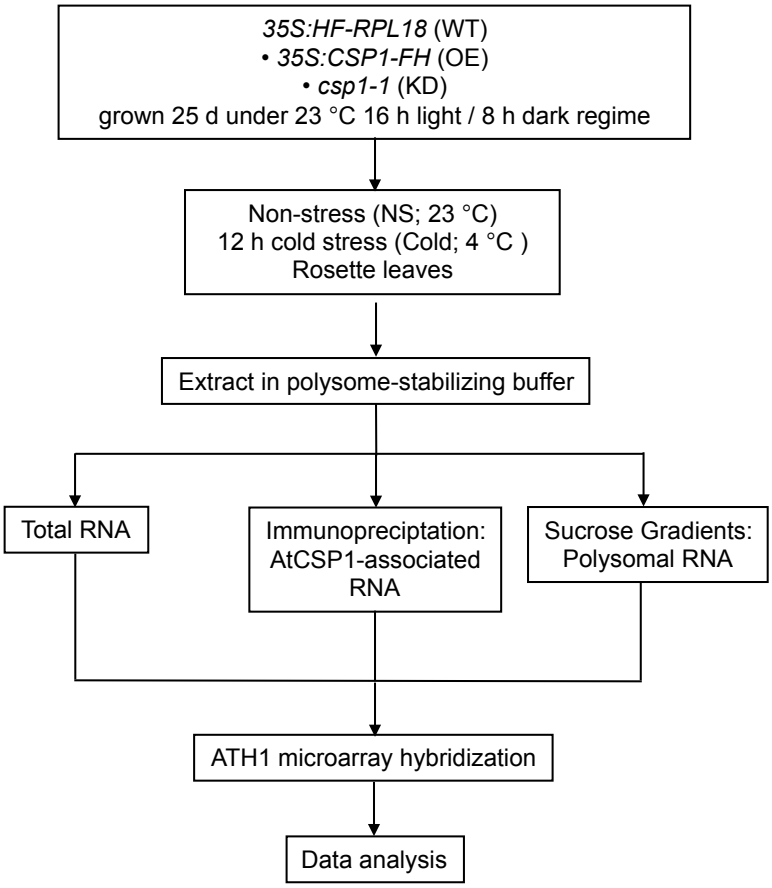


Figure 4.12. Immunopurification of CSP1 identified mRNAs that varied in the abundance. Graphs represent a comparison between mRNA abundance (RMA raw values) of the total RNA of differential expressed genes 1 (DEG set 1, n = 1,876), CSP1-enriched mRNAs (DEG set 1, clusters 1-6), and DEG set 1, cluster 1. (a) Comparison under non-stress condition. (b) Comparison under cold-stress condition.

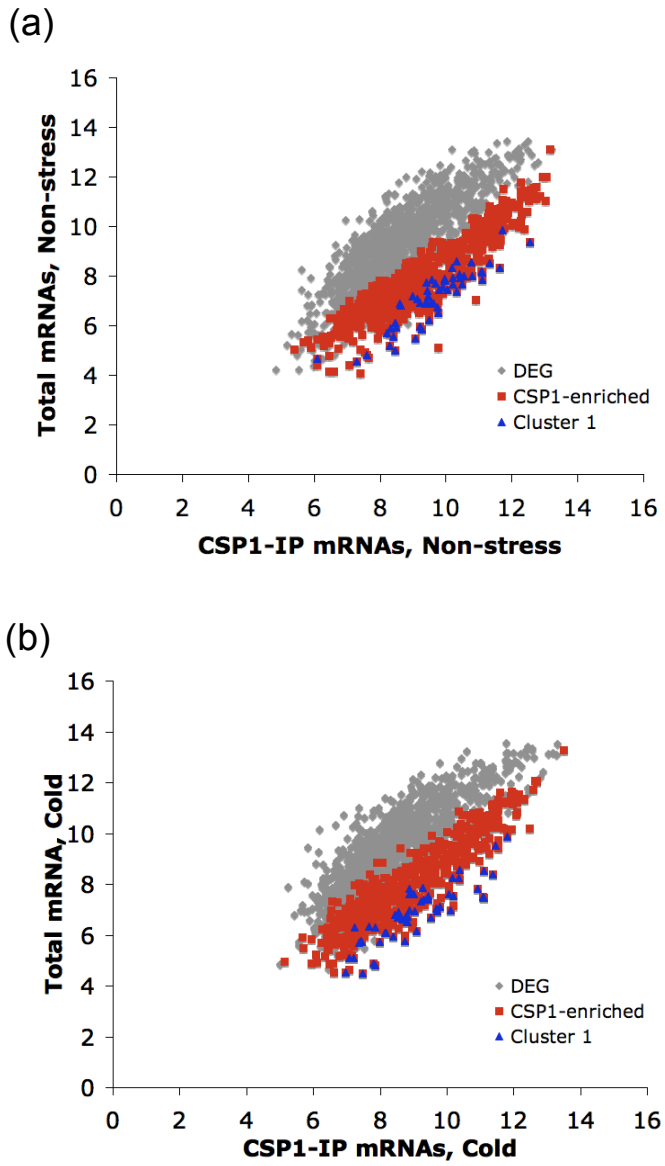


Figure 4.13. Identification of mRNAs associated with CSP and their steady-state abundance and translation state. Fuzzy *k*-means clustering analysis was performed on 11 mean signal log ratio (SLR) comparisons of total, polysomal and CSP1-immunopurified mRNA transcript abundance. Comparisons included: Log signal in Cold sample – Log signal in non-stress sample (C/NS) in the total (T) and polysomal (P) mRNAs of the three genotypes (*35S:HF-RPL18* (WT), *35S:AtCSP1-FH* (CSP1-OE), *atcsp1-1* (*csp1-1*); Log signal in the CSP-1 IP – Log signal in Total samples (IP/T) and Log signal in the CSP-1 IP – Log signal in Polysomal samples (IP/P) for WT and CSP1-OE; Log signal in the *35S:AtCSP1-FH* (OE) IP sample - Log signal in the *35S:FH-RPL18* (WT) IP sample (OE/WT) under cold (C) and non-stress (NS) conditions. Genes included in the analysis were those with a "present" call across all chips and significantly different in abundance in at least comparison ($|SLR| \geq 1$; FDR < 0.05, n = 1,876 probe pair sets (Supplementary Table 4.1)). The heatmap shows median SLR values for each of twenty groups (clusters) of mRNAs that displayed similar responses to cold treatment based on the total, polysomal or CSP1-immunopurified mRNA populations; color indicates increase (yellow), decrease (blue) or no change (black) in SLR value. Columns indicate cluster ID, number of genes (No. genes).

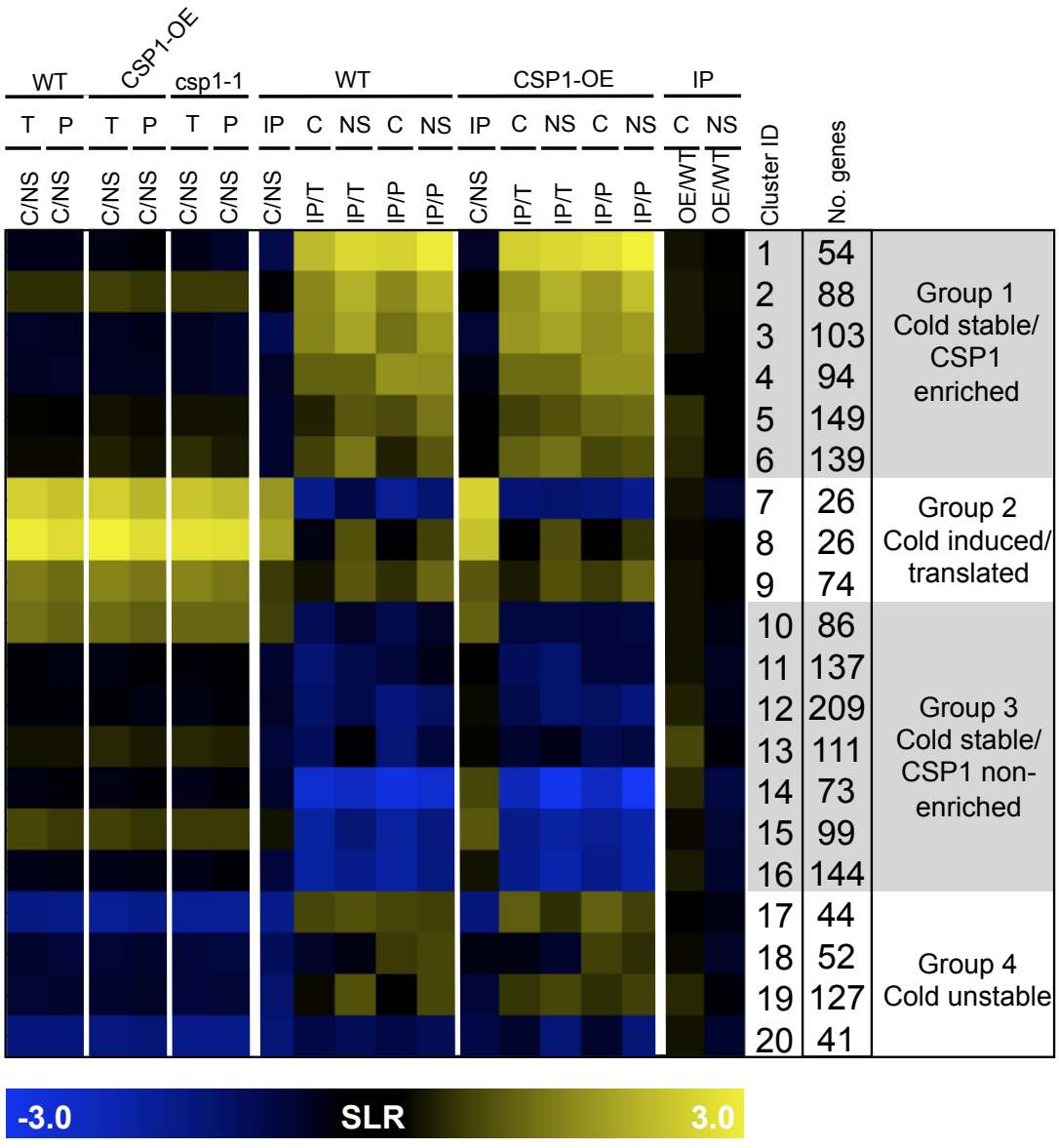

WT
CSP1-OE
csp1-1
WT
CSP1-OE
IP
T
P
T
P
T
P
IP
C
NS
C
NS
IP
C
NS
C
NS
C
NS
C/NS
C/NS
C/NS
C/NS
C/NS
C/NS
C/NS
IP/T
IP/T
IP/P
IP/P
C/NS
IP/T
IP/T
IP/P
IP/P
OE/WT
OE/WT
Cluster ID
No. genes
1 54
2 88
3 103
4 94
5 149
6 139
Group 1 Cold stable/ CSP1 enriched
7 26
8 26
9 74
Group 2 Cold induced/ translated
10 86
11 137
12 209
13 111
14 73
15 99
16 144
Group 3 Cold stable/ CSP1 non-enriched
17 44
18 52
19 127
20 41
Group 4 Cold unstable
-3.0
SLR
3.0

Figure 4.14. CSP1 co-regulated genes encode proteins with similar molecular function. Analysis of genes differentially expressed at the level of steady-state transcript abundance, translational status, and association with ASP1 by use of the PAGEMAN tool. The 11 comparisons and 1,876 differentially expressed genes are the same as those represented in Figure 4.13. Significant co-regulation was determined by the Wilcoxon rank sum test with the Benjamini-Hochberg FDR control (Usadel *et al.*, 2005, 2006). Significant categories were collapsed for display. Statistical differences are represented by a false color heatmap; color indicates increase (yellow), decrease (blue) or no change (white) in SLR value, where a *z*-score of 1.96 represents a FDR < 0.05.

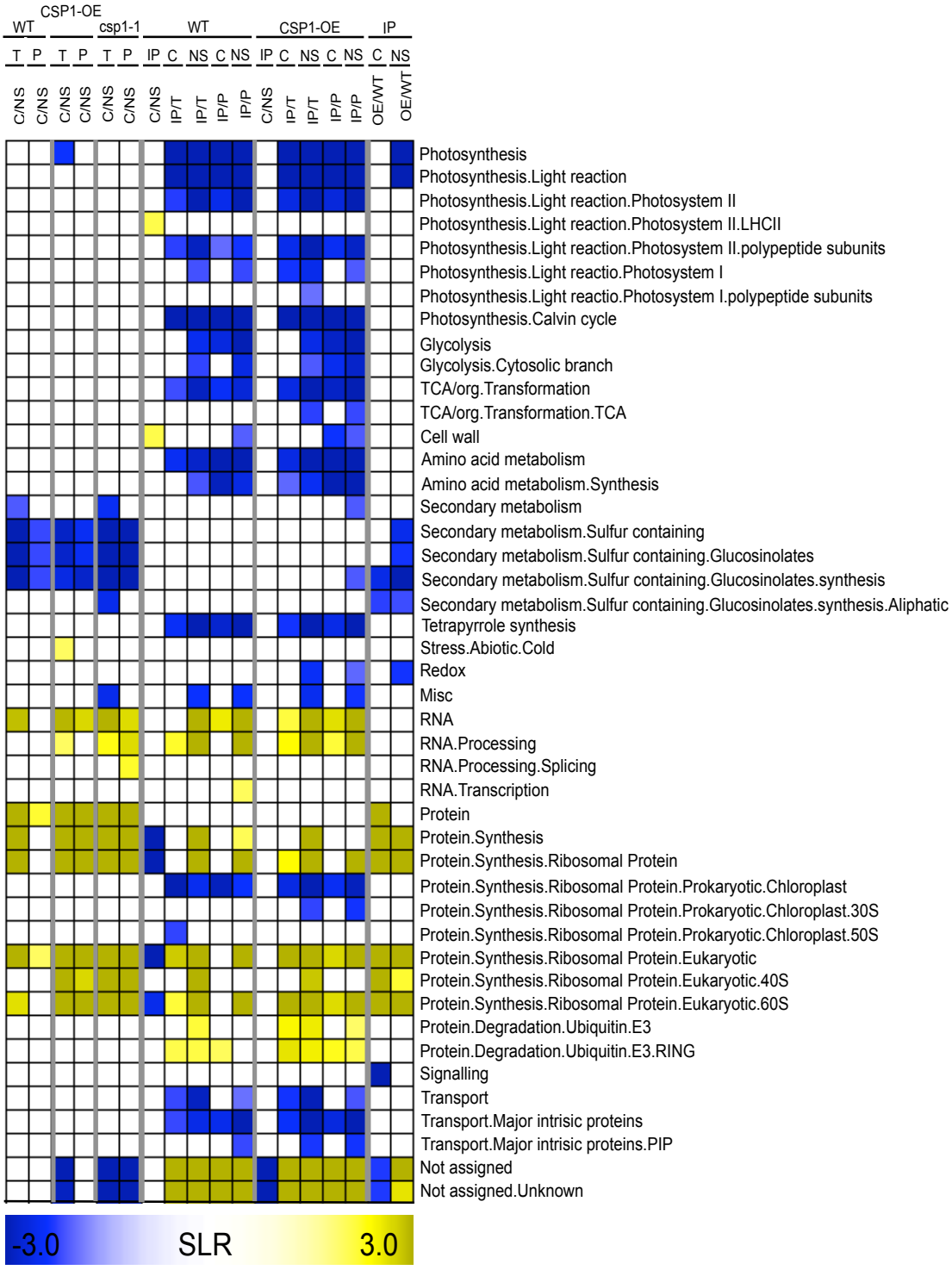
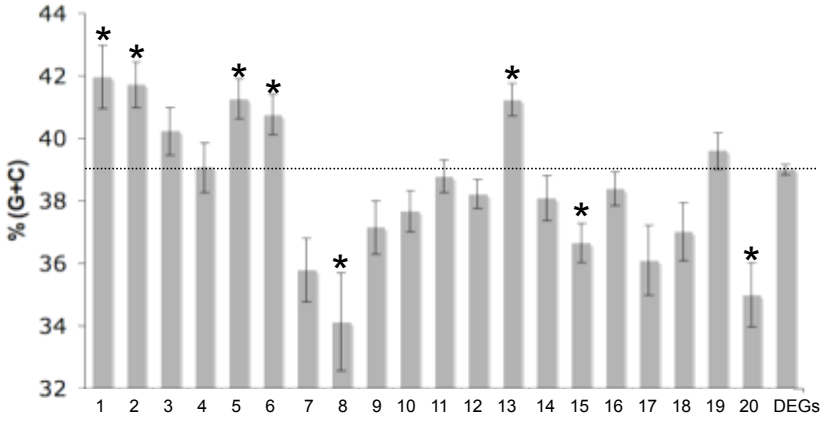

CSP1-OE
WT
csp1-1
WT
CSP1-OE
IP
T P T P T P IP C NS C NS IP C NS C NS C NS
C/NS C/NS C/NS C/NS C/NS C/NS C/NS IP/T IP/T IP/P IP/P C/NS IP/T IP/T IP/P IP/P OE/WT OE/WT
Photosynthesis
Photosynthesis.Light reaction
Photosynthesis.Light reaction.Photosystem II
Photosynthesis.Light reaction.Photosystem II.LHCII
Photosynthesis.Light reaction.Photosystem II.polypeptide subunits
Photosynthesis.Light reactio.Photosystem I
Photosynthesis.Light reactio.Photosystem I.polypeptide subunits
Photosynthesis.Calvin cycle
Glycolysis
Glycolysis.Cytosolic branch
TCA/org.Transformation
TCA/org.Transformation.TCA
Cell wall
Amino acid metabolism
Amino acid metabolism.Synthesis
Secondary metabolism
Secondary metabolism.Sulfur containing
Secondary metabolism.Sulfur containing.Glucosinolates
Secondary metabolism.Sulfur containing.Glucosinolates.synthesis
Secondary metabolism.Sulfur containing.Glucosinolates.synthesis.Aliphatic
Tetrapyrrole synthesis
Stress.Abiotic.Cold
Redox
Misc
RNA
RNA.Processing
RNA.Processing.Splicing
RNA.Transcription
Protein
Protein.Synthesis
Protein.Synthesis.Ribosomal Protein
Protein.Synthesis.Ribosomal Protein.Prokaryotic.Chloroplast
Protein.Synthesis.Ribosomal Protein.Prokaryotic.Chloroplast.30S
Protein.Synthesis.Ribosomal Protein.Prokaryotic.Chloroplast.50S
Protein.Synthesis.Ribosomal Protein.Eukaryotic
Protein.Synthesis.Ribosomal Protein.Eukaryotic.40S
Protein.Synthesis.Ribosomal Protein.Eukaryotic.60S
Protein.Degradation.Ubiquitin.E3
Protein.Degradation.Ubiquitin.E3.RING
Signalling
Transport
Transport.Major intrisic proteins
Transport.Major intrisic proteins.PIP
Not assigned
Not assigned.Unknown
-3.0
SLR
3.0

Figure 4.15. Identification of mRNA sequence features characteristic of CSP1-immunopurified mRNAs. (a) The average percent G+C nucleotide content of the 5'-UTRs of the mRNAs from each gene cluster defined in Figure 4.13. (b) The average of transcript length (nt) of the mRNAs from each gene cluster. Error bars represent standard error of the mean. Asterisks indicate a significant difference between the average 5'-UTR percent G+C or transcript length from each gene cluster to those of all 1,876 differentially expressed genes evaluated (Student's *t*-test; p-value < 0.005). The dashed lines represent in (a) the average 5'-UTR G+C content (39%) from *Arabidopsis* 5'-UTR genomic data, in (b) the average transcript length (+UTRs,- introns) (1640.7 nt). These data were obtained from Kawaguchi & Bailey-Serres (2005).

(a) 5' UTR G+C content



(b) Transcript length

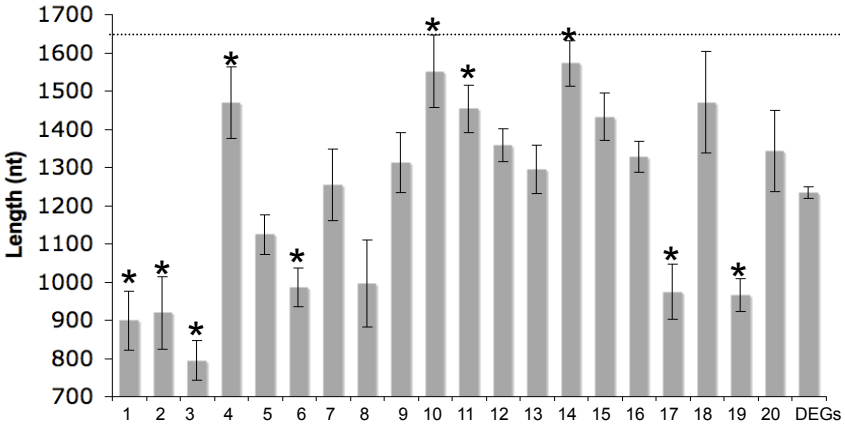
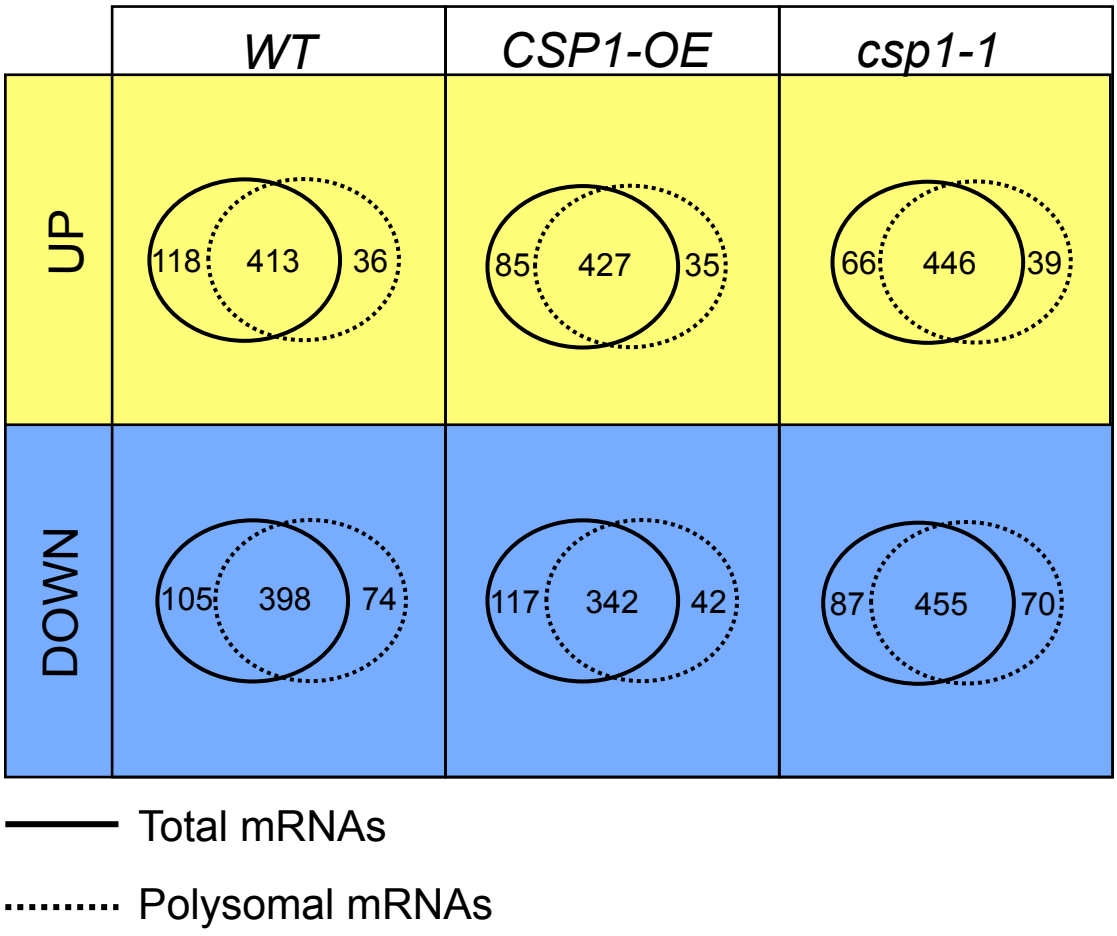


Figure 4.16 Comparison of changes in mRNA abundance in the transcriptome and translatome in response to low-temperature. (a) Venn diagrams indicate the number of genes in the total and polysomal mRNAs that displayed a significant increase or decrease in abundance following 12 h cold-treatment. mRNAs analyzed included those with "present" calls in all twelve hybridization and with a significant increase or decrease in abundance in total or polysomal mRNA (|SLR|>1; FDR < 0.05; DEGs set 2, n = 1,345 probe pair sets).



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Chapter 5

General conclusions

Change in the regulation of gene expression in response to environmental stimuli is frequently measured by monitoring dynamics in the transcriptome, the total mRNA pool with a cell, tissue or organ. However, gene expression is a multifaceted process involving transcriptional modulation, post-transcriptional regulation, and post-translational modification (Bailey-Serres *et al.*, 2009). RNA Binding Proteins (RBPs) become associated directly or indirectly with mRNAs as they are transcribed and processed in the nucleus and ultimately regulate the nuclear to cytoplasmic export, localization, sequestration, translation, targeting and turnover of transcripts in the cytoplasm. The eukaryotic RNA regulon model posits that an essential role of RBPs is in coordinating the regulation of gene cohorts, much like a gene operon coordinates transcriptional and translational regulation within a bacterium (Keene, 2007). However, the standard procedures for mRNA extraction from cells dissociate the distinct populations of mRNA-RBP (mRNP) complexes within the cell. To overcome the loss of information provided by the proteins and complexes associated with an individual transcript, mRNP complexes can be isolated by immunopurification, and subsequently characterized by RNA extraction or protein purification. Utilizing RBP-based immunopurification, dynamics in distinct subpopulations of mRNAs can be characterized. This strategy was developed for the model plant *Arabidopsis thaliana* by epitope tagging a single protein of the large ribosomal subunit, which made feasible the

isolation of polysome complexes from whole organs or distinct cell-types, through the use of specific promoters to drive the tagged protein in transgenic plants (Zanetti *et al.*, 2005; Mustroph *et al.*, 2009). The combination of polysome immunopurification with high-throughput gene profiling, using microarrays or RNA sequencing, has provided quantitative measurement of translatome in the context of development and environmental responses (Branco-Price *et al.*, 2008; Mustroph *et al.*, 2009; Jiao & Meyerowitz, 2010).

The environmental condition, for example temperature, light, nutrient availability, water status, presence of pests or pathogens, salt or other ions, as well as oxygen and carbon dioxide levels, affect plant growth and development. Plants experience daily changes in light quality and quantity, from sudden shading, to the diurnal cycle, to seasonal changes under different temperature condition. In this dissertation, my aim was to utilize genetic resources, genomic technologies and bioinformatics methods to elucidate post-transcriptional mechanisms that regulate plant adaptation to changes in light availability and low temperature.

In Chapter 2, I demonstrated that an unanticipated shift of *Arabidopsis* seedlings to darkness caused a rapid and reversible decrease in protein synthesis. Transcriptome and translatome analysis reveal that early darkness induces translational inhibition of many chloroplast and protein synthesis mRNAs. Since photosynthesis requires light, the shift to darkness rapidly limits ATP production. I propose that the dark-induced inhibition of synthesis of components of the photosynthetic apparatus and the

ribosome serves as a mechanism of energy conservation. In *Chlamydomonas reinhardtii,* the RBP NAB1 post-transcriptionally regulates *Light Harvesting Complex BM* mRNAs in by their sequestration from the translational machinery under high light conditions (Mussgnug *et al.*, 2005). In *Saccharomyces cerevisiae,* Processing bodies (P-bodies) are involved in the sequestration of ribosomal protein mRNAs during glucose starvation (Yoon *et al.*, 2010). These findings suggest a role of RBPs in post-transcriptional control of light regulation. Because *C. reinhardtii* NAB1 contains an evolutionally conserved Cold Shock DNA/RNA binding domain (CSD), I sought the function of the four partially characterized CSD containing proteins (CSPs) of *Arabidopsis*. These proteins had been shown to bind single and double stranded nucleic acids (Karlson *et al.*, 2002; Kim *et al.*, 2007, 2009) and a mutation of *AtCSP3* had been shown to impair cold acclimation and freezing tolerance (Kim *et al.*, 2009).

To probe the function of the four *Arabidopsis* CSPs (AtCSPs 1-4), I developed stable *Arabidopsis* transgenics that overexpress epitope-tagged CSPs. In Chapter 3, I utilized differential centrifugation, co-immunopurification, mass spectrometric protein identification, and confocal microscopy to characterize the CSP complexes. From the cellular fractionation, proteomic examination, and sub-cellular localization of CSP complexes, I conclude that CSPs are involved in post-transcriptional regulation, including the processing of RNAs in the nucleus and regulation of translation in the cytoplasm. Of the four CSPs, CSP1 was the only one that co-fractionated with ribosome complexes. This finding motivated me to investigate whether AtCSP1 functions in the regulation of translation of a sub-population of cellular mRNAs.

In Chapter 4, biochemical examination of *Arabidopsis* CSP1 revealed that it is a polysome-associated RBP. CSP1 accumulates under non-stress growth conditions but abundance and polysome association is increased by low-temperature stress. To examine the functional role of CSP1, I developed a method for co-immunopurification of native CSP1 complexes. This strategy, combined with gene expression microarray analyses, led to the identification of *Arabidopsis* mRNAs that interact with CSP1. I also examined the effect of low-temperature stress on transcriptome and translatome adjustments using *Arabidopsis* lines that differ in CSP1 accumulation. The results revealed that CSP1 preferentially binds to a subpopulation of mRNAs that are involved in energy-demanding processes including RNP complex formation and ribosome biogenesis. These results provide unambiguous support for a CSP1-mRNA regulon.

The importance of the CSP1-mRNA regulon may be to coordinate or enhance the expression of mRNAs required for cell growth, particularly in plants acclimated to cold temperatures. By contrast, CSP1 showed limited association with abundant mRNAs with roles in photosynthesis and central carbon metabolism. Although the role of CSP1 in light to dark transitions should be studied, these finding suggests that CSP1 is unlikely to be involved in light/dark regulation of synthesis of chloroplast-targeted proteins. Many of the CSP1-associated mRNAs have a high G+C content in their 5'-UTR which is correlated with the potential for secondary structure formation (Kawaguchi & Bailey-Serres, 2005). Therefore, I propose that CSP1 binding is likely to assist in the removal of secondary structures, particularly when stabilized in the cold, to aid mRNA translation.

The manipulation of CSP1 level had little effect on genome-wide transcript abundance and polysome engagement in my studies. This may reflect functional redundancy among cold-inducible RNA chaperones (CSPs or related Glycine-Rich RBPs [GR-RBPs]) or indicate that a small amount of CSP1 is sufficient for the cold acclimation conditions tested. To gain further insight into this question, mutants of multiple *CSPs* and *GR-RBPs* or transgenic lines with inducible microRNA targeting *AtCSP1* can be obtained and the translational regulation of CSP1-targeted mRNAs examined.

Future studies should also aim to identify the CSP1/mRNA interaction site. This could be accomplished by a method called "CLIP-Seq" (Ule *et al.*, 2003, 2005), which combines *in vivo* cross-linking, RNP immunopurification, RNA fragmentation and digestion, and finally a high-throughput sequencing to identify RBP-protected RNA sequences. The CLIP-Seq approach might also provide information on CSP1 bound to polysome-associated mRNAs. Finally, biochemical and genetic analysis can be used to further examine roles of CSP1 in post-transcriptional regulation during development.

The analysis of cold-regulated transcriptome and translatome adjustments revealed that cold stress has little impact on translational regulation in *Arabidopsis*. This finding contrasts with the selective translation observed in response to dehydration (Kawaguchi *et al.*, 2004; Kawaguchi & Bailey-Serres, 2005), hypoxia (Branco-Price *et al.*, 2005, 2008; Mustroph *et al.*, 2009), sucrose starvation (Nicolaï *et al.*, 2006), and light/dark transitions (Chapter 2). Each of these environmental perturbations dampened the translation of ribosomal protein mRNAs, in addition to more stress-specific regulation

of other mRNAs. The finding of a CSP1-regulon enriched in ribosomal protein mRNAs suggests that this RBP may contributed to the circumvention of translational repression in the cold, in contrast to other stresses. Future experimentation might consider whether the putative translational regulators, ribosomal protein S6 kinases, are less active under cold-stress than these other abiotic stress conditions. It would also be worthwhile to test if the over-expression of CSP1 alters the translational inhibition of ribosomal protein mRNAs during light/dark transitions or other stresses.

This dissertation provides new perspectives on post-transcriptional gene regulation from the global to gene-specific level in response to environmental cues. In addition to the CSP1-mRNA regulation, other RBPs are likely to direct the translational activity of groups of mRNAs, such as photosynthetic gene transcripts in response to early darkness. To elucidate this layer of the web of gene regulation, the functional role of stress granule proteins, P-body components, and CSPs 2-4 might be explored. This could be performed by the approach developed in Chapter 4 for quantitative assessment of mRNA/RNP association. This will require high-throughput genomic analysis, such as DNA microarray and deep-sequencing technologies. Here, we used DNA microarray analysis due to the extensive coverage of the transcriptome and well-established bioinformatic pipelines. However, other approaches for evaluation of the CSP1-associated mRNAs have advantages. For example *in vivo* cross-linking and immunoprecipitation following by massively parallel sequencing of the associated mRNAs may yield additional information on the transcript isoforms (*i.e.* incompletely or alternatively spliced mRNAs) that are missed by use of oligo-based microarray platforms.

In conclusion, this dissertation provides a foundation for future in-depth characterization of mRNA-RNP control networks in plants.

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