CHARACTERIZATION OF THE FOUR GENES ENCODING CYTOPLASMIC RIBOSOMAL PROTEIN S15a IN *ARABIDOPSIS THALIANA*

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By

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ABSTRACT

Eukaryotic cytosolic ribosomes are composed of two distinct subunits consisting of four individual ribosomal RNAs and, in *Arabidopsis thaliana*, 81 ribosomal proteins. Functional subunit assembly is dependent on the equimolar production of each ribosomal component. *Arabidopsis thaliana* r-protein genes exist in multi-gene families ranging in size from two to seven transcriptionally active members. The cytosolic *RPS15a* gene family consists of four members (*RPS15aA*, *-C*, *-D* and *-F*) that, at the amino acid level, share 87-100% identity. Using semi-quantitative RT-PCR I have shown that *RPS15aC* is not expressed and that transcript abundance differs both spatially and temporally among the remaining *RPS15a* genes in non-treated Arabidopsis tissues and in seedlings following a variety of abiotic stresses. A comprehensive analysis of the *RPS15a* 5' regulatory regions (RRs) using a series of deletion constructs was used to determine the minimal region required for gene expression and identify putative *cis*-regulatory elements. Transcription start site mapping using 5' RACE indicated multiple sites of initiation for *RPS15aA* and *-F* and only a single site for *RPS15aD* while all three genes contain a leader intron upstream of the start codon. Analysis of reporter gene activity in transgenic Arabidopsis containing a series of *5′ RR deletion*::*GUS* fusions showed that, similar to previous RT-PCR results, there was a trend for mitotically active tissues to stain for GUS activity. Putative *cis*-elements including the *TELO* box, *PCNA* Site II motif and pollen specific elements were identified. However, there was not always a clear correlation between the presence of a putative element and *RPS15a* transcript abundance or GUS activity. Although variation in transcriptional activity of each *RPS15a* gene has been observed, subcellular localization of both *RPS15aA* and *-D* in the nucleolus has been confirmed *in planta* by confocal microscopy. The results of this thesis research suggest while all three active *RPS15a* genes are transcriptionally regulated, additional post-transcriptional and/or translational regulation may be responsible for final RPS15a levels while differential isoform incorporation into ribosomal subunits may be the final point of r-protein regulation.

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LIST OF ABBREVIATIONS

A-site	aminoacyl site
ABA	(±)cis, trans-abscisic acid
BAP	6-benzylaminopurine
bp	base pair
cDNA	complementary DNA
DFC	dense fibrillar component
E-site	exit site
EF	elongation factor
ER	endoplasmic reticulum
ETS	external transcribed spacer
FC	fibrillar center
GA_3	gibberellic acid
GC	granular component
GUS	β-glucuronidase
IAA	indole-3-acetic acid
ICE	internal control element
IF	initiation factor
ITS	internal transcribed spacer
LSU	large subunit
mRNA	messenger RNA
NAC	nascent-chain associated complex
NOR	nucleolar organizing region
nt	nucleotide
ORF	open reading frame
pre-RNA	precursor RNA
P-site	peptidyl site
pI	isoelectric point
RACE	rapid amplification of cDNA ends
RACK	receptor of activated C-kinase
RNP	ribonucleoprotein
r-protein/RP	ribosomal protein
RR	regulatory region
rRNA	ribosomal RNA
RT-PCR	reverse-transcription polymerase chain reaction
snoRNA	small nucleolar RNA
SRP	signal recognition particle
SSU	small subunit
TOP	terminal oligopyrimidine tract
TOR	target of rapamycin
tRNA	transfer RNA
TSS	transcription start site
UAS	upstream activation sequence
UTR	untranslated region
wt	wild type

CHAPTER 1. LITERATURE REVIEW

1.1. Introduction

Ribosomes are ubiquitous cellular ribozymes responsible for the synthesis of polypeptides from a messenger RNA (mRNA) template. The prokaryotic ribosome has been well characterized, but considerably less is known about eukaryotic, particularly plant cytosolic, ribosomes. Biogenesis of the plant ribosome requires the synthesis of four ribosomal RNAs (rRNAs) and more than 75 ribosomal proteins (r-protein, RP). Plant r-protein genes are organized in multiple gene families in which one or more members are expressed. In the cellular environment, rRNA and r-protein synthesis must be capable of adapting to the dynamic requirements of ribosome biogenesis. The uncoupling of transcription and translation in eukaryotes necessitates export of r-protein transcripts from the nucleus to the cytoplasm for translation, import of the resultant polypeptide into the nucleolus and export of assembled ribosomal subunits to the cytoplasm where they associate with mRNAs to initiate protein synthesis. Due to the complexity of composition and production of the plant ribosome, questions regarding r-protein expression remain to be answered.

The following thesis research is an investigation into the regulation of the four genes encoding cytosolic r-protein S15a (RPS15a) in *Arabidopsis thaliana*. The prokaryotic ortholog of RPS15a, RPS8, has been identified as a primary binder, able to bind specifically and independently to the central domain of the 16S (Svedberg/sedimentation coefficient) rRNA, a required step in the assembly of the 30S subunit platform. This thesis research has compared transcript abundance from the four *RPS15a* genes in wild-type, untreated tissues and in response to a variety of abiotic stresses using reverse transcription PCR (RT-PCR; Chapter 2); mapped the transcription start sites and identified some putative *cis*-elements regulating individual *RPS15a* gene expression through the generation of transgenic plants carrying a series of 5′ regulatory region (RR)-deletion constructs (Chapter 3); and established a strategy to investigate r-

protein heterogeneity within the ribosome using fluorescent protein tags to visualize RPS15a subcellular localization *in planta* (Chapter 4).

1.2. Historical Overview

One of the first references to the ribosome was made late in the 19th century by Garnier who described filamentous structures, located in the cytoplasm, that displayed a high affinity for basic dyes (reviewed in Bielka, 1982). Garnier referred to these structures as ergastoplasm, a highly active form of cytoplasm (reviewed in Palade, 1954). During the 1940's the basophilic nature of ergastoplasm was investigated using biochemical and cytochemical methods (reviewed in Bielka, 1982). It was determined that the cytoplasm contained a high proportion of RNA and moreover, that there was a direct correlation between the amount of RNA present in a cell and the rate of protein synthesis. These results led researchers to conclude that it was the basophilic, RNA containing structures in the cytoplasm that were responsible for protein biosynthesis (reviewed in Bielka, 1982). Concurrently, differential centrifugation was being used by Claude (Claude, 1940; Claude, 1946) to isolate "small granules" (later called microsomes) from normal tissue and tumor extracts. Analysis of the microsome fraction indicated a conserved chemical composition including both phospholipid and ribonucleoprotein components.

Using electron microscopy in the mid-1950s, Palade described small, spherical, RNA rich bodies 10-15 nm in diameter which, depending on the cell type examined, were found either in close association with the outer membrane of the endoplasmic reticulum (ER) or dispersed throughout the cytoplasm (Palade, 1954). These results suggested that the phospholipid component previously identified in isolated microsome fractions of cell homogenates represented the ER membrane and that the ribonucleoprotein particles were themselves cellular organelles (Palade, 1954). It was during this same time period that ultracentrifugation was being employed to isolate and analyze ribonucleoprotein particles from mammals (Petermann et al., 1952; Petermann and Hamilton, 1956), plants (*Pisum sativum*, Ts'O et al., 1956) and yeast (*Saccharomyces cerevisiae*, Chao and Schachman, 1956). The ultracentrifuge patterns obtained following fractionation of cytoplasmic extract from cells of each species

showed multiple, discrete ribonucleoprotein peaks with sedimentation coefficients of approximately 40S, 60S and 80S (Petermann and Hamilton, 1956; Ts'O et al., 1956; Chao and Schachman, 1956; Chao, 1956). The ribonucleoproteins were composed of ~40% RNA and, in pea and yeast, 55% and 58% protein, respectively (Ts'O et al., 1956; Chao and Schachman, 1956).

While advances were being made in the characterization of eukaryotic ribonucleoproteins, several groups were using ultracentrifugation and electron microscopy to analyze particles isolated from various bacterial species (reviewed in Bielka, 1982). In 1958, Tissières and Watson reported that the monomeric, 70S ribonucleoprotein particles from *Escherichia coli* were composed of 30S and 50S subunits and contained 60-65% RNA and 35-40% protein. Based on sedimentation and diffusion data, the molecular weight of the 70S particle was estimated to be approximately 2.8×10^6 daltons. A comparison of prokaryotic and eukaryotic ribonucleoproteins, the latter with an estimated molecular weight of 4×10^6 daltons, led Tissières and Watson (1958) to suggest that while the amount of RNA in the particles remained constant, it was variations in the amount of protein that contributed to the difference in mass. It was during a meeting on ribonucleoprotein particles and protein synthesis in 1958 that the term "ribosome" was first introduced by Roberts to refer to the particles described by Claude and Palade (Bielka, 1982).

In addition to isolation and structural characterization, numerous groups during the 1950's were focusing on the ribosome as the site of protein synthesis (reviewed in Bielka, 1982). Both *in vitro* and *in vivo* experiments demonstrated that the rate of radioactive amino acid incorporation into proteins was highest in the microsomal fraction from liver and pancreas (Borsook et al., 1950; Allfrey et al., 1953; Keller et al., 1954) and furthermore, that the ribosome was the site of protein synthesis in both eukaryotes (Littlefield et al., 1955) and prokaryotes (McQuillen et al., 1959; Tissières et al., 1960). Using cell-free radioactive amino acid incorporation experiments, Zamecnik et al. (1958) were able to show that ribosomes, enzymes from the 105 000 X g supernatant [subsequently shown to be aminoacyl-transfer RNA (tRNA) synthetases; Berg and Ofengand, 1958], "soluble RNA" (tRNA), GTP and ATP were components necessary for protein synthesis. These results led researchers to propose an adaptor

hypothesis suggesting base-pairing between the aminoacyl-tRNA and template RNA based on a triplet code (Hoagland et al., 1957; Crick et al., 1961). The triplet nucleotide nature of the genetic code was later experimentally verified by Nirenberg et al. (1965) and Morgan et al. (1966).

As ribosomes, not DNA, had been shown to be the site of protein synthesis, it was suggested that there must exist an intermediate information carrier. A long held view generally assumed that this intermediate was the RNA component of the ribosome itself (rRNA) and that each gene regulated the production of a specific ribosome that, in turn, was responsible for the synthesis of the corresponding protein (Brenner et al., 1961). However, *in vitro* studies of protein synthesis in phage-infected *E. coli* suggested that the template was instead a metabolically unstable RNA molecule (Volkin and Astrachan, 1956) and that ribosomes were non-specialized, passive translators of genetic information. This template was given the name "messenger RNA" (mRNA; Jacob and Monod, 1961) and was shown to be synthesized from, and complementary to, a single-stranded DNA template (Hall and Spiegelman, 1961). Further work in *E. coli* demonstrated that only active ribosomes, those attached to mRNA, were able to synthesize protein (Brenner et al., 1961; Risebrough et al., 1962). Subsequently, ultracentrifugation and electron microscopic analyses showed that active ribosomes, from both bacterial (Barondes and Nirenberg, 1962; Spyrides and Lipmann, 1962) and animal cells (Warner et al., 1962; Goodman and Rich, 1963), formed aggregates, or polysomes, on a single mRNA molecule. In 1961, Brenner et al. suggested that the process of protein synthesis consisted of a series of successive events which, through the work of numerous research groups, has since been divided into three main events: initiation, elongation and termination (reviewed in Moldave, 1965).

Studies of the structural components of both prokaryotic and eukaryotic ribosomes began in the late 1950s (reviewed in Bielka, 1982). In *E. coli*, these studies culminated in the identification of one 16S rRNA molecule in the small, 30S subunit and single 23S and 5.8S rRNA molecules in the large, 50S subunit (Kurland, 1960). In eukaryotes, the large subunit (LSU) was found to contain a unique 5S rRNA (Brown and Weber, 1968) in addition to the 28S and 5.8S molecules (Hall and Doty, 1959; Forget and Weissman, 1967) while the small subunit (SSU) contained a single 18S

rRNA (Hall and Doty, 1959). The diversity of the r-protein constituent of the *E. coli* ribosome was first described by Waller and Harris (1961) who suggested that the proteins functioned to maintain rRNA in the correct configuration for protein synthesis. Two-dimensional gel electrophoresis was later employed by Kaltschmidt and Wittmann (1970) to determine that the *E. coli* ribosome was composed of 55 r-proteins and, later by Welfle and Bielka (1972) and Sherton and Wool (1972), who estimated the number of r-proteins in rat liver ribosomes to be between 68 and 72. R-protein stoichiometry was investigated by Hardy (1975) who determined that, with the exception of L7/L12, *E. coli* ribosomes contained only a single copy of each individual r-protein.

Structural studies of the ribosome continued into the 1970s and resulted in several major advancements, including the sequencing of r-proteins (reviewed in Wittmann, 1982) and determination of the complete nucleotide sequences of the 5S, 16S and 23S rRNAs from *E. coli* (Brownlee et al., 1967; Brosius et al., 1978; Brosius et al., 1980). Using these components, functionally active small and large ribosomal subunits from *E. coli* were reconstituted *in vitro* (Traub and Nomura, 1968; Nierhaus and Dohme, 1974) and subunit assembly was shown to be a stepwise, cooperative process (Mizushima and Nomura, 1970). Subsequent studies utilized primary structural data to identify individual r-protein binding sites on rRNA using partial nuclease digestion (Zimmermann et al. 1972), electron microscopy (Cole et al., 1978), and chemical and enzymatic probing and primer extension (Stern et al., 1986).

Although it was known that the ribosome was the site of protein synthesis, it was not until the late 1960s that peptide bond formation, catalyzed by a peptidyl transferase (Maden et al., 1968), was identified as an inherent function of the ribosome itself (Monro, 1967). Furthermore, under the conditions of the "fragment reaction", a peptidyl transferase assay, Monro (1967) was able to show that peptide bond formation was dependent on the 50S ribosomal subunit. As all enzymes characterized to this point were protein in nature, subsequent studies focused on identifying the r-protein(s) possessing enzymatic activity. Using protein-depleted ribosome cores and the split r-protein fraction generated by LiCl treatment in reconstitution experiments, it was determined that RPL11, RPL16 or a group of r-proteins including RPL2, RPL3, RPL4, RPL15, RPL16 and RPL18 as well as the 23S rRNA were essential to the restoration of

peptidyl transferase activity (Nierhaus and Montejo, 1973; Moore et al., 1975; Hampl et al., 1981). In addition to the essential proteins, Hampl et al. (1981) also identified "helper proteins" such as RPL20 and RPL24 which, although not directly responsible for enzymatic activity, were involved in early subunit assembly. The importance of RPL16 with respect to the restitution of peptidyl transferase activity to core 50S particles was shown to be due to an RPL16-dependent, rRNA conformational change induced during subunit assembly (Teraoka and Nierhaus, 1978).

The possibility that rRNA was involved in the enzymatic function of the ribosome was first suggested in the 1970s following experiments that showed efficient cross-linking between 23S rRNA and tRNA (reviewed in Noller, 1993). However, it was not until the discovery of RNA enzymes in the early 1980s that this hypothesis was truly entertained. In 1982, Kruger et al. reported that the initial 26S rRNA transcript of *Tetrahymena thermophila* contained an intervening sequence that was spliced out through a series of self-catalyzed, rRNA reactions. In addition, it was shown that the RNA component of ribonuclease P purified from both *E. coli* and *Bacillus subtilus* was the source of catalytic activity (Guerrier-Takada et al., 1983). Using chemically or photochemically labeled tRNAs in affinity binding studies, the peptidyl transferase region was found to include the highly conserved, central loop of domain V of the 23S rRNA (Barta et al., 1984; Steiner et al., 1988). Moreover, chloramphenicol and carbomycin, two antibiotics known to inhibit peptidyl transferase activity, protected specific bases within domain V from chemical probes (Moazed and Noller, 1987). Further, indirect evidence of catalytic 23S rRNA activity, was presented by Noller et al. (1992) who showed that *Thermus aquaticus* 50S subunits retained peptidyl transferase activity following treatment with proteinase K or sodium dodecylsulfate yet were highly sensitive to RNase T1. Definitive proof that the ribosome was indeed a ribozyme however, would not be achieved until 2000 when the atomic structure of the large subunit of *Haloarcula marismortui* complexed with two substrate analogs was reported (Nissen et al., 2000).

The first three-dimensional crystals of the 50S subunit from *Bacillus stearothermophilus* were produced by Yonath et al. (1980). However, it was not until the late 1990s, with improvements in synchrotron light sources and crystallographic

software as well as the development of cryo-crystallography and turnable synchrotron sources that significant advances were made in the determination of ribosome structure (reviewed in Ramakrishnan and Moore, 2001). In 1999, 5 Å and 5.5 Å resolution electron density maps of the 50S subunit from *H. marismortui* (Ban et al.) and the 30S subunit from *T. thermophilus* (Clemons et al.) were generated, respectively. In addition, the crystal structure of the intact, 70S ribosome from *T. thermophilus* complexed with both mRNA and tRNA was resolved at 7.8 Å and provided new insight into the mechanism of translation (Cate et al., 1999). More recently, higher resolution structures have been produced; the atomic structure of the *H. marismortui* 50S subunit has now been resolved to 2.4 Å (Ban et al., 2000) while the 30S subunit from *T. thermophilus* has been solved to 3.0 Å (Wimberley et al., 2000). Ribosome crystal structures contain a wealth of information and have been used to determine the structural basis of antibiotic inhibition of peptide synthesis (reviewed in Steitz, 2004) and the structural and functional roles of individual r-proteins (Hoang et al., 2004; Sharma et al., 2007).

Study of the 80S eukaryotic ribosome has lagged behind that of its prokaryotic counterpart in large part due its greater mass, ~4 MDa compared to ~2.8 MDa, and structural complexity (Verschoor et al., 1996). However, using cryo-electron microscopy, the 3D structure of the 80S ribosome from rabbit reticulocytes (Morgan et al., 2000), wheat germ (Verschoor et al., 1996), *Saccharomyces cerevisiae* (Verschoor et al., 1998; Gomez-Lorenzo et al., 2000; Morgan et al., 2000) and *Chlamydomonas reinhardtii* (Manuell et al., 2005) have been reconstructed. Comparisons between eukaryotic species have shown that the overall structure of the ribosome has been strongly conserved and bears a high degree of resemblance to that of bacterial ribosomes (Verschoor et al., 1996, Verschoor et al., 1998; Gomez-Lorenzo et al., 2000; Morgan et al., 2000, Manuell et al., 2005). Cryo-EM has also been used to visualize ribosome elongation factor-G (EF-G) complexes in *E. coli* (Agrawal et al., 1998; Agrawal; et al., 1999) and the protein-conducting channel complexes of yeast ER (Ménétreat et al., 2000; Beckmann et al., 2001), elucidating details on the processes of translocation and co-translational translocation, respectively.

While rRNA and elucidation of the nature of the peptidyl transferase have been the main focus of ribosome research for nearly forty years, the field currently

encompasses a broad range of topics including rRNA synthesis (Arabi et al., 2005; Grewal et al., 2005), processing (reviewed in Kaczanowska and Rydén-Aulin, 2007) and modification (McCloskey and Rozenski, 2005; Piekna-Przybylska et al., 2008) as well as ribosome assembly (Stagg et al., 2003; Ferreira-Cerca et al., 2007) and transport (reviewed in Johnson et al., 2002). R-proteins are also enjoying a renaissance with ongoing investigations into their roles in disease (Amsterdam et al., 2004; Gregory et al., 2007), mechanisms of their co-ordinate regulation (Perry, 2005) and contributions to ribosome heterogeneity (Chang et al., 2005; Komili et al., 2007).

1.3. Basic Ribosome Structure

As previously discussed, during translation the ribosome is composed of two subunits, a large and a small, each consisting of one or more rRNA and 30-40 (small subunit) or 40-60 (large subunit) associated r-proteins (Figures 1.1 and 1.2). Although the composition of the subunits differs between prokaryotic and eukaryotic cells, the overall function remains the same; the large subunit acts as a static platform (Mears et al., 2002) responsible for catalyzing the peptidyl transferase reaction while the small subunit is dynamic, binding and moving along the mRNA during translation initiation and elongation in addition to interacting with tRNAs (Bailey-Serres, 1998).

The prokaryotic ribosome, best characterized in *E. coli*, has a sedimentaion coefficient of 70S. The small (30S) subunit has a molecular mass of 0.9 X 10^6 daltons and consists of the 16S rRNA and 21 proteins. The large (50S) subunit, consisting of the 23S and 5S rRNAs and 34 proteins, has a molecular mass of 1.6 X 10^6 daltons. The eukaryotic ribosome shares structural similarity with its prokaryotic counterpart but has a sedimentation coefficient of 80S. The mass of the large (60S) subunit varies among eukaryotes from 2.45 - 2.5 X 10^6 daltons in higher plants to 3.0 X 10^6 daltons in mammals (Bielka, 1982). In all eukaryotes, the large subunit contains the 25S (plants) or 28S (mammals) rRNA molecule as well as the 5S and 5.8S rRNA which are analogous to the 5S and 5' end of the 23S rRNA of prokaryotes (Bailey-Serres, 1998).

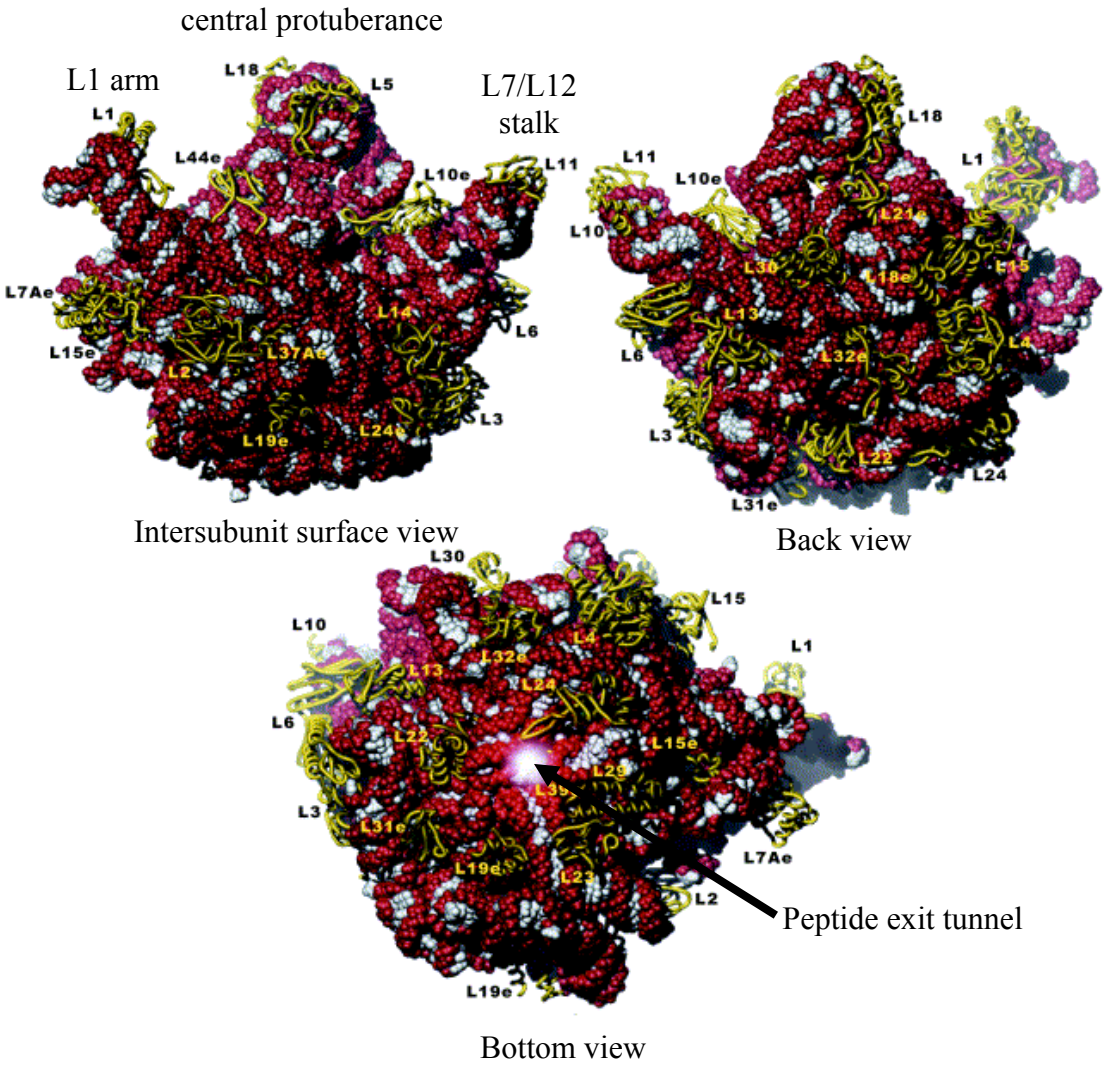
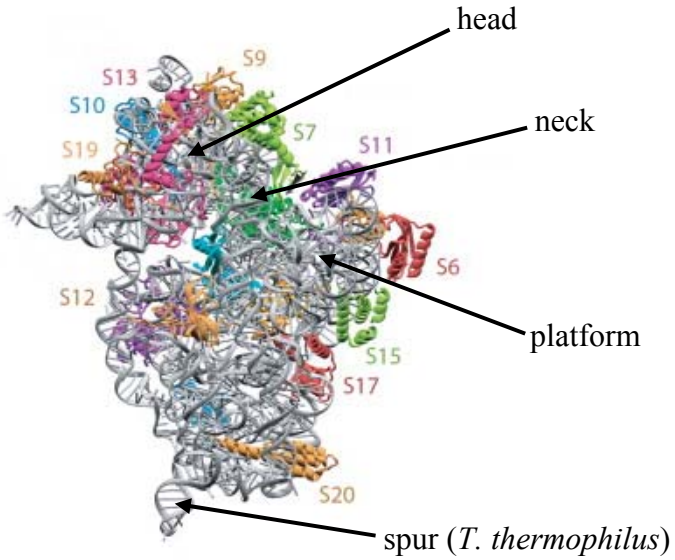
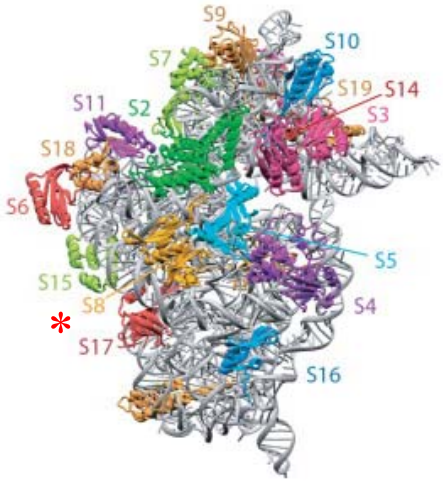


Figure 1.1. Structure of the *H. marismortui* large (50S) ribosomal subunit showing the intersubunit surface, back and bottom views. Sugar-phosphate backbone; bases; ribosomal proteins. Modified from Klein et al. (2004).



Intersubunit surface view



Back view

Figure 1.2. Structure of the *T. thermophilus* small (30S) ribosomal subunit showing the intersubunit surface and back views. 16S rRNA; proteins shown in various colors; (*), indicates the location of RPS8. Modified from Broderson et al. (2004).

The number of r-proteins associated with the large subunit is fairly constant, with the discrepancy in mass among species being attributed to variation of the size of the large rRNA molecule which ranges from 1.2-1.7 X 10^6 daltons (Bielka, 1982). Due to minimal variation of the 18S rRNA and number of associated r-proteins, the mass of the small (40S) subunit is also relatively constant at approximately 1.5 X 10^6 daltons (Bielka, 1982). The increase in the number of eukaryotic ribosomal components is credited to the need for increased translational fidelity and regulation (Verschoor et al., 1996).

1.3.1. rRNA

1.3.1.1. rRNA genes (rDNA)

The genes encoding rRNA are present in two unique sets within the eukaryotic genome. The first set contains genes for the 25-26S (e.g. yeast, Arabidopsis) or 28S (e.g. rat, mouse) rRNA and the 18S and 5.8S rRNAs. These genes are organized in tandem repeats found at distinct chromosomal loci. The second set contains the 5S rRNA gene organized in tandem repeats but located at separate loci (reviewed in Hadjiolov, 1985). The number of rRNA genes per haploid genome is variable among eukaryotes, differing among closely related species, individuals of the same species or among different cell types of a single individual (reviewed in Hadjiolov, 1985). Copy numbers range from 100-140 in yeast, 150-170 in rat (*Rattus norvegicus*) to 13,400 in larch (*Larix decidua*). On average, higher plants contain rRNA copy numbers that are 5-10 fold higher than most eukaryotes (reviewed in Hadjiolov, 1985). Copy numbers in angiosperms range from 630 in orange (*Citrus sinensis*) to 8,500 in maize (*Zea maize*) (reviewed in Hadjiolov, 1985).

The arrangement of rRNA genes in tandem repeats may be responsible for their presence in eukaryotic genomes in numbers that exceed the requirements of ribosome biogenesis (Hadjilov, 1985; Bailey-Serres, 1998). Unequal crossing over of rRNA genes on sister chromatids resulting in duplications and deletions have been shown to occur in *S. cerevisiae* during both meiosis (Petes, 1980) and mitosis (Szostak and Wu, 1980). Therefore, gene amplification of tandem repeats may result in copy number variation within a population (Szostak and Wu, 1980; reviewed in Hadjiolov, 1985).

In most eukaryotes, the 5S rRNA genes are present in multiple copies, arranged in tandem repeats, yet their copy number differs from that of the other rRNA genes (Hadjiolov, 1985). Copy numbers ranging from 150 in yeast (*S. cerevisiae*) to 300,000 in the American red-spotted newt [*Notophtalmus* (*Triturus*) *viridescens*] have been documented (reviewed in Hadjiolov, 1985). Drift of the 5S rRNA genes resulting in their separation from the 18S-5.8S-25/26/28S transcription unit has affected their number, organization and structure (Hadjiolov, 1985). In yeast (reviewed in Planta, 1997), and other relatively simple eukaryotes including *Torulopsis utilis* (torula yeast), *Mucor racemosus* (a filamentous fungus) and *Dictyostelium discoideum* (cellular slime mold) (reviewed in Hadjilov, 1985), the 5S rRNA gene is part of the rRNA transcription unit. When organized in this manner, transcription of the 5S rRNA gene proceeds in the opposite orientation to the other rRNA genes (Planta, 1997) and is carried out by a different RNA polymerase (Hadjiolov, 1985).

The arrangement of the 18S-5.8S-25/26/28S rRNA genes in eukaryotes, similar to the organization found in *E. coli*, has undergone three major changes throughout evolution: increased size of the 16S and 23S rRNA genes, drift of the 5S rRNA gene away from the larger transcription unit and the formation of a new 5.8S rRNA gene in the first internal transcribed spacer (reviewed in Hadjiolov, 1985). Thus, each rRNA transcription unit is comprised of: 5′ regulatory region-external transcribed spacer (ETS)-18S rRNA-internal transcribed spacer 1 (ITS1)-5.8S rRNA- internal transcribed spacer 2 (ITS2)-25/26/28S rRNA 3′. Each transcription unit is separated by a non-transcribed spacer which, when accompanied by its adjacent transcription unit, constitutes one repeating unit.

In plants, as in most eukaryotes, high concentrations of the 18S-5.8S-25S rRNA transcription units are localized at specific chromosomal loci described as nucleolar organizing regions (NORs) (Hadjiolov, 1985). Formation of a nucleolus is dependent upon transcription of the rRNA units by RNA polymerase I and it is the site of several steps of ribosomal subunit biogenesis including processing and base modification of precursor-rRNA (pre-rRNA) transcripts and assembly of the two ribosomal subunits (Maxwell and Fournier, 1995). Eukaryotic nucleoli consist of three distinct regions: the dense fibrillar component (DFC), fibrillar center (FC) and the granular component

(reviewed in Shaw et al., 1996). Production of mature rRNAs begins with transcription of a high molecular weight pre-rRNA (reviewed in Bailey-Serres, 1998) which occurs at the border of the DFC and FC (Shaw et al., 1996). This molecule, ubiquitous to eukaryotic nucleoli (Bielka, 1982), varies in size from 35S in yeast to 45S in vertebrates. The pre-rRNA in plants is referred to as the 45S transcript but varies among species due to inconsistencies in the size of the ETS and ITS regions (reviewed in Bailey-Serres, 1998). The 5S rRNA genes, generally found in clusters within a genome, (Hadjiolov, 1985), are transcribed separately by RNA polymerase III and the transcript imported into the nucleolus.

Following transcription, pre-rRNA processing is performed by small nucleolar RNAs (snoRNAs) that, when complexed with nucleolar proteins, form ribonucleoprotein particles (snoRNPs) (Maxwell and Fournier, 1995). snoRNPs are believed to combine with pre-rRNA, r-proteins and non-r-proteins (methylases, RNases and endonucleases) to form complexes involved in rRNA maturation and subunit assembly. Sequential exo- and endonucleolytic cleavage events remove the ETS and ITS regions from the pre-rRNA (reviewed in Bailey-Serres, 1998) producing the 25S, 18S and 5.8S rRNAs in plants. Processing of pre-rRNA molecules also includes modification of residues to pseudouridine or pseudoserine (Bailey-Serres, 1998) and methylation of the 2'-OH group of the ribose sugar (Bielka, 1982). The specific function of these modifications is unknown (Bailey-Serres. 1998), but conservation of methylated sequences throughout evolution suggests roles in ensuring proper pre-rRNA processing and assembly (Bielka, 1982). Although plant rRNAs are among the most highly methylated among eukaryotes little is known about pre-rRNA modification, including identification of methylation and pseudouridylation patterns (Brown and Shaw, 1998), in comparison to other eukaryotes such as yeast and vertebrates (Barneche et al., 2001).

Pre-ribosome formation proceeds in the nucleolus with rRNA secondary structure formation and the addition of r-proteins which have been imported from the cytoplasm (reviewed in Bailey-Serres, 1998). Addition of r-proteins occurs at two stages during nucleolar ribosome assembly; those added during or immediately following transcription facilitate cleavage and degradation of single stranded rRNA

while those added later may assist in forming the large and small subunits before their release from the nucleolus (Hadjiolov, 1985). Once assembled, the pre-large and pre-small subunits are transported through the nuclear pore complex to the cytoplasm for the final steps of maturation including the addition of remaining r-proteins and release of *trans*-acting factors (reviewed in Zemp and Kutay, 2007).

1.3.2. Ribosomal proteins

Of the eukaryotic organisms studied, 80 r-proteins have been identified in rat (Wool et al., 1995), 79 in yeast (Planta and Mager, 1998; Link et al., 1999) and 75-92 in various plant species (reviewed in Bailey-Serres, 1998). The increase in protein composition of the ribosome from ~54 in eubacteria to ~80 in eukaryotes has not decisively been explained but may be due to the increase in rRNA size (Wool et al., 1995), need for increased translational fidelity and regulation (reviewed in Verschoor et al., 1996), or the addition of functions due to a complicated assembly process (Wool et al., 1995). Although the number of r-proteins is variable, most eukaryotic r-proteins were derived from the same set of ancestral genes (Wool et al., 1995). Complete amino acid sequence comparison between yeast and rat r-proteins revealed that they share, on average, 60% identity, ranging from 40-88% (Wool et al., 1995). A similar range (43-96%) was discovered when the amino acid sequences of rat and plant r-proteins were compared (Bailey-Serres, 1998).

1.3.2.1 R-protein structure

The biochemical characteristics of plant r-proteins, (Bailey-Serres, 1998), are predicted to be similar to those of rat due to their amino acid identity (reviewed by Wool et al., 1995). The majority of r-proteins are basic, the average isoelectric point (pI) being 11.05, and are typified by high proportions of arginine and lysine and low proportions of aspartate and glutamate. Basic or acidic amino acids are organized in clusters found near the carboxy and amino termini of the proteins. Short amino acid repeats found throughout rat r-proteins are speculated to have functional significance and may be involved in nuclear localization or interaction with various species of RNA (Wool et al., 1995). In addition, several proteins were found to contain leucine zipper

(i.e. RPL7, RPL13a and RPS2) or C_2-C_2 zinc finger motifs (i.e. RPL37 and RPL37a) that could potentially mediate rRNA - protein or protein - protein interactions (reviewed in Wool et al., 1995). Unlike other r-proteins, RPS27a, RPS30 and RPL40 from rat are processed by cleavage from an N-terminal ubiquitin, or ubiquitin-like, fusion which acts as a molecular chaperone and assists in ribosome biogenesis (Finley et al., 1989; Catic et al., 2007). Phosphorylated proteins in both rat and plants include P0, P1 and P2, the acidic r-proteins, and RPS6 (Scharf and Nover, 1982; Bailey-Serres and Freeling, 1990; Wool et al., 1995).

1.3.2.2. Intra-ribosomal functions

Since the 1960s ribosome research has largely centered on identification of the peptidyl transferase center and subsequently rRNA while r-proteins were thought to play merely a structural role (Broderson and Nissen, 2005). However, determination of the atomic structure of bacterial 70S ribosomal subunits in 1999-2000 provided a wealth of information on rRNA-protein and protein-protein interactions and suggested that r-proteins have many functions, including direct participation in protein synthesis (Ban et al., 1999; Clemons et al., 1999; Cate et al., 1999; Ban et al., 2000; Wimberly et al., 2000).

1.3.2.2.1. mRNA recognition

In prokaryotes, RPS1, RPS7 and RPS11 are among the proteins responsible for tethering mRNA to the ribosome (Broderson and Nissen, 2005). RPS1 is the largest eubacterial r-protein and is located near RPS7 and RPS11 between the head and the platform of the 30S subunit (Agalarov et al., 2006). RPS1 has been shown to be essential for the translation of almost all *E. coli* mRNAs *in vivo* (reviewed in Gualerzi et al., 2000) and facilitates translation initiation by binding mRNA in a non-sequence specific manner (Broderson and Nissen, 2005). Receptor for activated C-kinase (RACK1) has recently been identified as a eukaryotic r-protein located on the head of the small subunit near the peptide exit tunnel (Sengupta et al., 2004). RACK1 is a scaffold protein that interacts with signaling molecules such as protein kinase C (PKC), Src kinase, Scp160p and integrin-β (reviewed in Nilsson et al., 2004). These

associations suggest that RACK1 mediates specific mRNA binding and translation initiation. In addition, RACK1 may recruit ribosomes to areas requiring local translation, such as focal adhesions, via interactions with integrin-β receptors (reviewed in Nilsson et al., 2004).

1.3.2.2.2. tRNA interactions

Ribosomes contain three tRNA binding sites: the A-site, where the aminoacyl tRNA that correctly matches the mRNA codon is selected; the P-site, where the peptidyl transfer reaction takes place and, the E (exit) site which receives newly deacylated tRNAs. While the A and P-sites are composed mainly of rRNA (Carter et al., 2000), X-ray crystallographic studies of *T. thermophilus* ribosomes have shown that RPS12 is located near the A-site codon-anti-codon helix (Carter et al., 2000; Yusupov et al., 2001) and is involved in maintaining translational fidelity (reviewed in Rodina et al., 2002). The carboxy-terminal tails of RPS9 and RPS13 lie near the anticodon stem-loop of the P-site (Carter et al., 2000). However, recent experimental evidence suggests that the tails are not essential as *E. coli* containing RPS9 and RPS13 double tail deletions were viable (Hoang et al., 2004). Unlike the A and P-sites, the E-site is largely composed of protein (Carter et al., 2000). RPS7 and RPS11 bind to the E-site tRNA anticodon stem-loop and the β-hairpin loop structure of RPS7 may assist in the dissociation of the tRNA from the ribosome (Carter et al., 2000).

1.3.2.2.3. Peptide exit tunnel, signal recognition, secretion and chaperones

Nascent polypeptides must pass through the exit tunnel before emerging from the ribosome. Although most of the tunnel's surface is comprised of the 23S rRNA, RPS4 and RPS22 form a narrow constriction on part of the tunnel wall (Nissen et al., 2000). It has been suggested that this region may act as a monitoring site regarding the functional state of the ribosome and/or the nature of the nascent polypeptide (Broderson and Nissen, 2005), however this hypothesis has yet to be experimentally verified. The area surrounding the tunnel exit site is surrounded by r-proteins including universally conserved RPL22 (RPL17 in eukaryotes), RPL23 (RPL25 in yeast, RPL23a in other eukaryotes), RPL24 (RPL26 in eukaryotes) and RPL29 (RPL35 in eukaryotes) (Nissen

et al., 2000; Harms et al., 2001; Beckmann et al., 2001). The r-proteins in this region of the ribosome serve as binding sites for extra-ribosomal factors that interact with nascent polypeptides (Grallath et al., 2005). For example, in prokaryotes RPL23 is the binding site for signal recognition particle (SRP) (Gu et al., 2003) and Trigger Factor chaperone (Kramer et al., 2002). In yeast, SRP interacts with both RPL25 and RPL35 (Pool et al., 2002) while the nascent-chain associated complex (NAC) binds to RPL25 (Grallath et al., 2005). Cryo-EM reconstruction of the Sec61 complex, the protein-conducting channel embedded in the ER of eukaryotic cells, has shown that in yeast, connections are formed with RPL19, RPL25, RPL26 and RPL35 (Beckmann et al., 2001).

1.3.2.3. Extra-ribosomal functions

In addition to being part of the ribosome, individual r-proteins of *E. coli*, yeast, humans, *Xenopus laevis*, *Drosophila melanogaster*, mouse and rat possess extra-ribosomal functions within the cell including roles in: replication, transcription, RNA processing, DNA repair, autogenous regulation of translation and developmental regulation (Wool, 1996 and references therein). These data support the theory that the primordial ribosome was composed solely of catalytic rRNA and that r-proteins were later recruited from a cellular pool of pre-existing proteins that already possessed designated functions. In addition, proteins most likely to be added to the ribosome were those already possessing an ability to bind nucleic acids, supported by evidence of zinc finger (Chan et al., 1993), bZIP and helix-turn-helix motifs identified in r-proteins that may have once bound DNA (reviewed in Wool, 1996).

1.4. R-protein Gene Expression and Regulation

1.4.1. Prokaryotes

The r-protein genes of *E. coli* are clustered and organized as operons that function as transcriptional units (Mager, 1988). This arrangement, as well as the coupling of transcription and translation, allows r-protein synthesis to be regulated by autogenous feedback mechanisms at the translational level (Mager, 1988). In 1980, Nomura et al. suggested a regulatory mechanism by which some r-proteins recognize and bind to similar sites on mRNA and rRNA. It was further proposed that r-proteins

will preferentially bind rRNA but, if all sites are occupied, will bind to the operator on their own mRNA, preventing translation when produced in excess of rRNA requirements. This mechanism has been illustrated for the L11-L1 operon (reviewed in Guillier et al., 2002) and suggested for the *spc* (reviewed in Guillier et al., 2002) and *IF3* operons which bind RPS8 (RPS15a ortholog) and RPL20 respectively.

1.4.2. Organization of r-protein genes in eukaryotes

Unlike those in *E. coli*, eukaryotic r-protein genes are dispersed throughout the genome and each contains the regulatory elements necessary for its independent transcription (Larson et al., 1991). Eukaryotic r-protein genes may be duplicated and exist in multi-gene families (Mager, 1988). This is the case in yeast where the 79 r-proteins are encoded by 138 genes due to 59 gene duplications (reviewed in Planta, 1997; Link et al., 1999). When occurring in duplicate, both genes are transcribed and encode essentially identical, functional proteins. In mammals, r-protein genes are present in multiple copies, yet in the majority of cases, only one from each family is transcriptionally active (reviewed in Larson et al., 1991). The remainder of the genes lack introns and are present as inactive pseudogenes.

In *A. thaliana*, r-protein genes exist in multi-gene families composed of two to seven members (Barakat et al., 2001). All gene copies in a family may be transcriptionally active although levels of expression may differ (Mager, 1988). It has been proposed that in addition to a constitutively expressed r-protein gene, one or more additional copies under developmental-specific transcriptional regulation could be expressed during periods of increased translation when extra ribosomes are required (Van Lijsebettens et al., 1994). Expression analysis of the *RPL16* gene family from *A. thaliana* shows differential transcription between two members (Williams and Sussex, 1995) of the four gene family (RPL11: Barakat et al., 2001). *AtRPL16A* is regulated in a tissue specific manner and associated with cell elongation and division in roots while *AtRPL16B* expression is correlated with non-tissue specific cell division (Williams and Sussex, 1995). Arabidopsis RPS18 is encoded by three genes of which expression of *RPS18A* is tissue specific and restricted to mitotically active tissues including

meristems, leaf primordia, heart stage embryos and wound sites (Van Lijsebettens et al., 1994).

1.4.3. Regulation of eukaryotic r-protein genes

Production of functional ribosomal subunits is dependent on the coordinate synthesis of both rRNA and r-protein components (Mager, 1988). Studies in eukaryotes have shown that r-protein production may be regulated at the transcriptional, post-transcriptional and/or translational levels in order to maintain ribosomal component balance while allowing for cellular responses to changes in growth conditions or developmental stage (Larson et al., 1991).

1.4.3.1. Transcriptional regulation

1.4.3.1.1. Saccharomyces cerevisiae

Regulation of gene expression has been examined by identifying specific *cis*-acting elements located in the 5' RR of r-protein genes. In yeast, the majority of regulation occurs at the transcriptional level in response to changing growth conditions (reviewed in Planta, 1997). In addition to the core promoter, early studies characterizing the 5' upstream region of yeast r-proteins identified the HOMOL1 and RPG boxes as conserved, duplicated upstream activation sequences (UASs) required for transcription (Leer et al., 1985; Woudt et al., 1986) while a T-rich region, located downstream of the UAS, functioned as a transcriptional enhancer (Rotenberg and Woolford, 1986). The HOMOL1 and RPG boxes are present in many genes encoding proteins involved in translation and bind the transcription factor TUF which was later identified as Rap1 (repressor/activator protein 1; Vignais et al., 1987; Warner, 1989). In addition to a T-rich region, the majority (~90%) of yeast r-proteins contain two Rap1 binding sites however, ~10% of genes contain an alternate, Abf1 (Ars binding factor) site (reviewed in Planta, 1997). Abf1 and Rap1 are organizers of local chromatin structure and, when bound to DNA, form nucleosome boundries while the T-rich region maintains a downstream nucleosome-free region thereby increasing protein accessibility to the exposed DNA (reviewed in Planta, 1997; Lascaris et al., 2000). Rap1 was initially thought to be the factor responsible for the activation and coordination of r-protein

synthesis in yeast, however, Rap1 has many functions including transcriptional activation of genes in a non-r-protein coordinated manner. Recently, genome-wide chromatin immunoprecipitation (ChIP on chip) analysis has shown that in addition to clearing nucleosomes, Rap1 is responsible for recruiting a specific co-factor, Fhl1 (forkhead-like 1), to the UAS of r-protein genes (Wade et al., 2004).

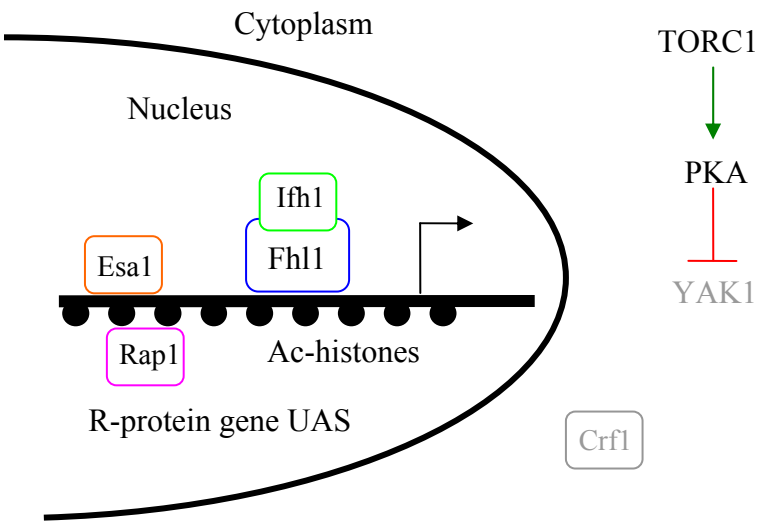
In yeast, the target of rapamycin (TOR) signaling pathway regulates ribosome biogenesis in response to nutrient availability (Powers and Walter, 1999). According to a recent model proposed by Martin et al. (2004), under favorable growth conditions Rap1 binds to the r-protein UAS and recruits Fhl1 (Figure 1.3). In the cytoplasm, the rapamycin sensitive, serine/threonine kinase TORC1 (TOR complex 1), mediates inhibition of the YAK1 kinase via protein kinase A (PKA; Martin et al., 2004). Without active YAK1, Crf1 (co-repressor of Fhl1) remains inactive and sequestered in the cytoplasm allowing the Ifh1 co-activator to bind Fhl1 and activate transcription. During this time Esa1, a histone acetylase, is also recruited to the r-protein UAS in a Rap1 or Abf1 dependent manner (Reid et al., 2000) and is maintained on the UAS by TOR signaling (Rohde and Cardenas, 2003). In unfavorable conditions such as amino acid starvation or heat shock, or following treatment with rapamycin, TORC1 and PKA are inactive and therefore, YAK1 is able to phosphorylate Crf1 (Figure 1.3). Phosphorylated Crf1 accumulates in the nucleus where it competes with Ifh1 for Fhl1 binding sites and represses transcription. In addition, due to the inhibition of the TOR signaling pathway, Esa1 is released from the UAS which is then occupied by the Rpd3-Sin3 histone deacetylase complex (Reid et al., 2000; Martin et al., 2004). This relatively simple model is complicated by the identification of additional factors that may have a role in regulating r-protein transcription such as Hmo1, a high-mobility group protein that binds cooperatively with Rap1 and Fhl1, and may be involved in coordinating r-protein gene and rRNA expression (Hall et al., 2006).

1.4.3.1.2. Mammals

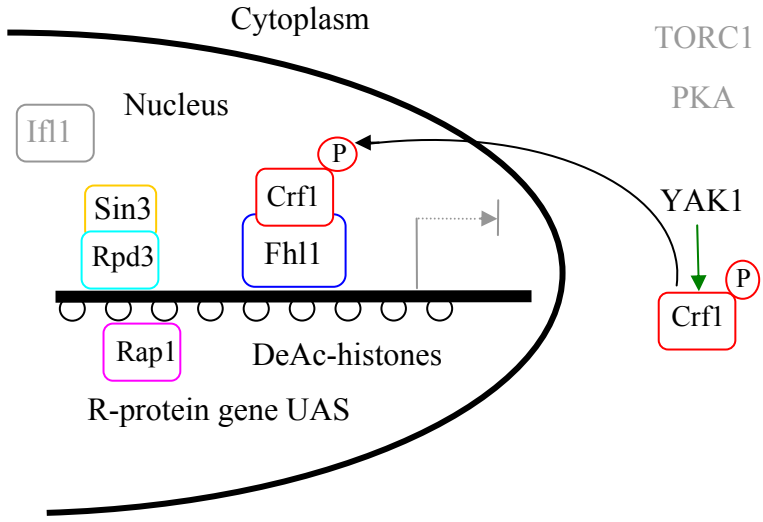
A recent sequence comparison of the 5' regulatory region (RR) of all 79 orthologous human and mouse r-protein genes has identified several evolutionarily

Figure 1.3. Model of TOR-regulated r-protein gene expression in yeast (adapted from Martin et al., 2004). Under favorable growth conditions (TOR active), Rap1 binds to the r-protein UAS displacing nucleosomes and recruiting Fhl1, a transcription factor, and Esa1, a histone acetylase. Dephosphorylated Crf1 remains sequestered in the cytoplasm allowing Ifl1 to bind Fhl1 and transcription to be activated. Under unfavorable conditions (TOR inactive), phosphorylated Crf1 accumulates in the nucleus where it competes for Fhl1 binding sites and represses transcription. Concurrently, Esa1 is released form the UAS and the binding site occupied by the Rpd3-Sin3 histone deacetylase complex. Crf1, co-repressor of Fhl1; Fhl1, forkhead-like 1; Ifl1, interacting with Fhl1; PKA, protein kinase A; Rap1, repressor/activator protein 1; TOR, target of rapamycin; TORC1, TOR complex 1; UAS, upstream activation sequence. See text for additional details.

TOR active



TOR inactive



conserved characteristics including: the separation, by an intron, of sequence involved in transcriptional and translational regulation from sequence with a protein coding function, a polypyrimidine tract with the $(Y)_2C+^1TY(T)_2(Y)_3$ consensus (where Y = any pyrimidine), spanning the transcription start site (TSS) and, binding sites for universal *trans*-acting factors located both upstream (i.e. GABP and Sp1) and downstream (i.e. YY1) of the transcription start site (Perry, 2005). In addition, contrary to previous studies, 35% of r-protein RRs contained a high affinity TATA box at -25 bp (from the TSS) while a further 25% of genes contained A/T rich motifs that could potentially bind TATA binding protein (TBP) with lower affinity (Perry, 2005).

Studies of mouse *RPL7* (Meyuhas and Klein, 1990) and *RPL32* (Chung and Perry, 1989; 1993) have also indicated the presence of regulatory elements located within the transcribed portion of the gene. Individual internal control elements (ICEs) were identified within intron 1 and the first exon-intron junction of *RPL7* as well as within exon 1 and the 5' end of intron 1 of *RPL32*. Chung and Perry (1993) have shown that both ICEs in *RPL32* bind the transcription factor δ, a zinc-finger protein that may function in the regulation of other cellular and viral genes. Sequence similarity and interaction with δ among ICEs identified in *RPL7*, *RPL32* and *RPL30* (reviewed in Meyuhas and Klein, 1990) suggest a role in maintaining the coordinate expression of r-proteins during periods of growth and cellular differentiation during mammalian development.

1.4.3.1.3. Plants

Expression patterns of specific cytosolic r-protein genes from a variety of plant species have shown increased transcriptional activity corresponding to increased mitotic activity. In *Brassica napus*, increased expression of *RPS15a* was observed in mitotically active tissues including young flower buds, leaves and apical meristems while lower levels occurred in mature tissues such as fully expanded leaves (Bonham-Smith et al., 1992). Similar expression patterns have been demonstrated for *RPL2* (Marty and Meyer, 1992), *RPL34* (Dai et al., 1996) and *RPL25* (Gao et al., 1994) in tobacco, *RPS11* and *RPS14* in maize (Lebrun and Freyssinet, 1991; Larkin et al., 1989), *RPL9* in pea (Moran, 2000), *RPL23a* in Arabidopsis (McIntosh and Bonham-Smith, 2005) and

RPS28 in peach (Giannino et al., 2000). Increases in r-protein gene expression have also been observed after wounding and treatment with exogenous plant growth regulators including 2,4-dichlorophenoxyacetic acid (an auxin) and benzyladenine (a cytokinin) (Dai et al., 1996; Gao et al., 1991; Gantt and Key, 1985; McIntosh and Bonham-Smith, 2005). In addition, a 15-20 fold increase in *RPS19* and *RPL7* gene expression has been shown during the early stages of tuberization in potato (*Solanum tuberosum*) (Taylor et al., 1992) while a reduction in *RPS28* and *RPS27A* gene expression has been reported in maize root tips following glucose deprivation (Chevalier et al., 1996). These data suggest that transcription of plant r-protein genes is developmentally and environmentally regulated.

Studies of plant genes encoding components of the translational apparatus have identified several conserved *cis*-elements within the 5' RR. In Arabidopsis, 81% of r-protein genes contain one or more plant *INTERSTITIAL TELOMERE MOTIFS* (*TELO* box, $^{5'}$AAACCCTA$^{3'}$) (Trémousaygue et al., 2003). In cycling cells of Arabidopsis root primordia, the *TELO* box acts in synergy with other *cis*-elements, including the *TEF* box ($^{5'}$ARGGRYANNNNNGT$^{3'}$) or *PROLIFERATING CELLULAR NUCLEAR ANTIGEN* (*PCNA*) Site II motif ($^{5'}$TGGGCC/T$^{3'}$), to regulate gene expression (Regad et al., 1995; Trémousaygue et al., 1999; Manevski et al., 2000). In Arabidopsis, the *TELO* box and Site II motif were found together, in a conserved topological association, in 153 r-protein genes (Trémousaygue et al., 2003). Results from transgene expression analysis suggest that, unlike the *TELO* box, the Site II motif is both necessary and sufficient to direct gene expression and, in plants, may be a conserved element responsible for coordinating the expression of genes up-regulated in mitotically active cells (Trémousaygue et al., 2003).

1.4.3.2. Post-transcriptional regulation

In addition to regulating r-protein synthesis at the level of transcription, plants can potentially exercise post-transcriptional regulation at many levels including: translation, mRNA stability and splicing, 3' - end formation as well as protein stability and modification (Sullivan and Green, 1993). Additional levels of regulation are important for sessile organisms, such as plants, that must adapt to an ever changing

environment and allow for increases in the speed of cellular responses to environmental and physiological cues (Sullivan and Green, 1993). Translational regulation has been demonstrated by r-protein synthesis in α-amanitin treated, germinating axes of maize (Beltran-Pena et al., 1995). Northern analysis confirmed the presence of stored, unspliced transcripts for *RPL3* and *RPL6* as well as mature transcripts for *RPS4* and *RPS6* in dry embryonic axes. These data support the hypothesis that r-protein synthesis at this stage of growth is maintained by stored mRNAs and is regulated at the level of translation.

Translational control has been demonstrated in other eukaryotes under various conditions including: developing *D. discoideum* (Steel and Jacobson, 1987), *X. laevis* (Amaldi et al., 1989) and *D. melanogaster* (Hongo and Jacobs-Lorena, 1991) as well as in glucocorticoid-inhibited mouse P1798 lymphosarcoma cells (Meyuhas et al., 1987) and during mouse myoblast differentiation (Agrawal and Bowman, 1987). Experiments using fertilized *X. laevis* eggs (Mariottini and Amaldi, 1990) and mouse lymphosarcoma cells (Levy et al., 1991) have shown that the 5' untranslated regions (UTRs) of certain r-protein mRNAs were required for translational regulation of fused reporter mRNAs. The 5' termini of characterized r-protein mRNAs in *X. laevis* and mammals contain a polypyrimidine tract (5' TOPs) of variable length, followed by a GC-rich region and an initiation codon in a canonical Kozak context (reviewed in Bailey-Serres, 1998). Phosphorylation of RPS6 has been implicated in the selective translation of TOP mRNAs following mitogenic stimulation (Levy et al., 1991). Previous studies have shown that the mTOR (mammalian target of rapamycin) inhibitor rapamycin blocks S6 kinase (S6K) activity, resulting in a reduction in RPS6 phosphorylation and the translation of TOP mRNAs (Jeffries et al., 1994; Terada et al., 1994; Jeffries et al., 1997). However, recent experimental evidence suggests that translation of TOP mRNAs may be independent of mTOR, S6Ks and RPS6 (Tang et al., 2001; Stolovich et al., 2002; Barth-Baus et al., 2002).

Although there has been no conclusive evidence of 5' TOPs in plant mRNAs, some plant r-protein genes contain regions of five or more polypyrimidines (Bailey-Serres, 1998). In addition, RPS6 phosphorylation was reduced in response to heat-shock in tomato cell cultures (Scharf and Nover, 1982) and hypoxia in maize roots (Bailey-

Serres and Freeling, 1990) but was induced by auxin treatment in maize (Perez et al., 1990; Beltran-Pena et al., 2002). The level of RPS6 phosphorylation corresponded to levels of r-protein synthesis for all three treatments (Scharf and Nover, 1987; Gantt and Key, 1985, Dai et al., 1996). Isolation of two putative *A. thaliana* RPS6 kinases that increase in abundance when exposed to cold or high-salinity stress are believed to induce translation through increased phosphorylation of r-proteins (Mizoguchi et al., 1995). It is hypothesized that phosphorylation of RPS6 may function as a method of translational control by altering ribosome conformation or affinity for specific mRNAs (i.e. mRNAs containing 5' TOPs) (Stewart and Thomas, 1994).

1.5. Ribosome Heterogeneity and the Ribosome Filter Hypothesis

Developmental stage and environmental conditions are often reflected in the cellular transcriptome where mRNA abundance may be controlled by degradation rate (Guhaniyogi and Brewer, 2001), alternative spicing (Modrek and Lee, 2005; Brett et al., 2005) and differential cellular location (Mohr and Richter, 2001). Translation of mRNAs is regulated by a variety of mechanisms while the ribosome has simply been considered a translational machine. The ribosome filter hypothesis, put forth by Mauro and Edelman (2002), suggests that the ribosome is not only responsible for peptide synthesis but can act as a regulatory factor, selectively 'filtering' mRNAs for translation. The filter hypothesis proposes that specific mRNA-rRNA or mRNA-r-protein interactions, primarily at sites on the small ribosomal subunit, play an important role in translational regulation. mRNAs containing different sequences compete for ribosomal binding sites while ribosome heterogeneity further modulates binding interactions by altering affinity for mRNAs at specific ribosomal subunit locations (Mauro and Edelman, 2002).

Ribosome heterogeneity is described as variations in rRNA or r-protein composition, post-translational modifications of ribosomal components, interactions with extra-ribosomal factors and/or ribosome degradation (Mauro and Edelman, 2002; Chang et al., 2005). R-protein heterogeneity within ribosomes has been well documented in the cellular slime mold *D. discoideum* (Ramagopal and Ennis, 1982). A comparison of r-protein content between different developmental stages showed that

RPS5 and RPL18 were present only in the ribosomes of vegetative amoebae while r-proteins A, E and L (no numeric designation) were specific to the ribosomes of germinating spores. In addition, vegetative amoebae ribosomes contained only trace levels of r-protein D and spores only trace levels of RPS10, RPS15 and RPL11. Transcripts encoding all r-proteins were present in germinating spores but mRNAs for RPS20, RPS25, RPS27, RPS28, RPS29, RPS31, RPS33, RPS34, RPL26, RPL31, RPL35, RPL36, RPL38, RPL39, RPL40 or RPL41 could not be detected in dormant spores. Furthermore, a number of r-proteins from both the small and large ribosomal subunits were over-represented in either vegetative or germinating cells, leading the authors to conclude that stoichiometric differences in r-proteins present at unique developmental stages of *D. discoideum* may be a mechanism regulating the translation of distinct protein classes.

During the early stages of *D. discoideum* development, r-protein synthesis is regulated through the binding of mRNAs by a 40S subunit that contains a methylated form of RPS24. If the cells resume growth, methylation is reversed and translation proceeds (Mangiarotti and Giorda, 2002). Conversely, if the developmental stage persists, the methyl group on RPS24 is lost while RPS31 gains a methyl group. R-protein mRNAs bound by 40S subunits containing methylated RPS31 are unstable and are ultimately degraded (Mangiarotti and Giorda, 2002). Studies in rat have shown that RPL40 and RPL29 may be monomethylated at Lys22 and Lys4 respectively; levels of methylated RPL29 varied in liver, brain and thymus tissues (Williamson et al., 1997). Additional post-translational modifications of r-proteins including acetylation, demethionylation, formylation, methylation, hydroxylation, or a combination of the above have also been reported in rat and human although their function has yet to be elucidated (Louie et al., 1996; Odintsova et al., 2003).

Recent studies of *A. thaliana* 80S ribosomes have indicated a high degree of heterogeneity as approximately 26% (Chang et al., 2005) and 45% (Giavalisco et al., 2005) of r-proteins were identified by two or more distinct spots following 2-D polyacrylamide gel electrophoresis. On average, each r-protein was represented by four forms, denoting the expression of multiple members of a single gene family and a degree of post-translational modification (Giavalisco et al., 2005; Carroll et al., 2008).

A group of acidic P-proteins form the tip of the lateral stalk on the 60S ribosomal subunit in all eukaryotic organisms. Although four P-proteins have been identified and characterized in *S. cerevisiae*, they are not absolutely required for protein synthesis (Remacha et al., 1995). Interestingly, the pattern of protein expression differs between wild-type cells and cells deficient of all four P-proteins. Following the addition of exogenous P-proteins to mutant cell extract, the wild-type gene expression profile resumed, confirming the changes were due to acidic r-protein heterogeneity (Remacha et al., 1995). In maize, the acidic r-proteins are designated P0, P1, P2a, P2b and P3, a novel protein found only in higher plants (Bailey-Serres et al., 1997; Szick et al., 1998). Cell type, environment and developmental stage have been found to affect the abundance and phosphorylation of the 12-kDa P-proteins (P1, P2a, P2b and P3), resulting in ribosome heterogeneity (Szick-Miranda and Bailey-Serres, 2001).

In addition to modification of the acidic P-proteins (Szick-Miranda and Bailey-Serres, 2001), phosphorylation of RPS6 has been implicated in the translational regulation of a discrete class of mRNAs (5´TOPs) that includes r-protein and elongation factor transcripts (Jefferies et al., 1994, 1997; Holland et al., 2004). In plants, RPS6 phosphorylation/dephosphorylation is known to regulate mRNA translation and is regulated by environmental conditions such as temperature (Mizoguchi et al., 1995), oxygen availability (Bailey-Serres and Freeling, 1990; Perez et al., 1990) and the presence of phytohormones (Turck et al., 2004; Beltran-Pena et al., 2002).

Ribosome heterogeneity can also be attributed to variations in rRNA due to nucleotide substitutions and/or deletions in the 28S, 18S, 5.8S or 5S ribosomal components (Mauro and Edelman, 2002). Heterogeneity of 28S rRNA genes has been reported in humans while size differences among species are mainly due to contraction or expansion of variable regions (Gonzalez et al., 1985; Hancock and Dover, 1988; Kuo et al., 1996). Although the 18S rRNA is less variable than the 28S rRNA, heterogeneity of 18S rRNA genes has been described in *D. melanogaster* (Tautz et al., 1988). Studies of 5S rRNAs from *Neurospora crassa* have shown the production of many (8-12) 5S rRNAs, with structural heteogeneity occuring when different 5S rRNAs are incorporated into the ribosome (Selker et al., 1985). Three types of 5S rRNA genes have been characterized in *X. laevis*; major oocyte, trace oocyte and somatic (reviewed

in Wolffe and Brown, 1988). The major oocyte and somatic 5S rRNA genes differ in six of 120 nucleotides and, as the nomenclature indicates, are differentially transcribed in oocytes and somatic cells (Wolffe and Brown, 1988).

Interactions with extra-ribosomal factors are thought to promote ribosome heterogeneity by altering subunit conformation, thereby modifying mRNA accessibility to rRNA or r-protein binding sites (Mauro and Edelman, 2002). Fragile X mental retardation protein (FMRP) has been shown to associate with the 60S ribosomal subunit through rRNA binding (Tamanini et al., 1996) while SSB, a cytosolic Hsp70 chaperone expressed in *S. cerevisiae*, is associated with the ribosome nascent-chain complex and exhibits transcript levels similar to those of r-proteins under varying environmental conditions (Lopez et al., 1999). Human laminin binding protein precursor p40 (LBP/p40) tightly binds to RPS21, possibly affecting 40S subunit stability and translation initiation (Sato et al., 1999). Isolation and analysis of cytoplasmic 80S ribosomes from *A. thaliana* identified several associated extra-ribosomal proteins including NAC and RACK1 (Chang et al., 2005; Giavalisco et al., 2005). The RACK1 ortholog in *Schizosaccharomyces pombe*, Cpc2, co-sediments with the 40S ribosomal subunit and affects the translation of specific mRNAs including *RPL25a* (Shor et al., 2003).

1.6. R-protein S15a

RPS15a is the eukaryotic ortholog of bacterial RPS8, a primary binding protein able to bind specifically and independently to the central domain of the 16S rRNA (Ungewickell et al., 1975; Mougel et al., 1993). Binding of RPS8 induces a conformational change in rRNA structure allowing the subsequent addition of RPS6, RPS15, RPS11 and RPS18 which together form the platform of the 30S subunit (Gregory et al., 1984; Svensson et al., 1988; Broderson et al., 2002; Jagannathan and Culver, 2003). In eukaryotes, RPS15a may have a similar role, as a primary binder of the 18S rRNA.

In Arabidopsis, RPS15a is encoded by a six member gene family that can be divided into two evolutionarily distinct clades (Chang et al., 2005). RPS15aA, -C, -D and -F are grouped with RPS15a of rat, Drosophila and yeast RPS22. RPS15aB and -E

are grouped in a separate clade and have been indirectly associated with mitochondrial ribosomes (Adams et al., 2002; Carroll et al., 2008). Of the four genes encoding cytosolic RPS15a, *RPS15aC* is not expressed (Chapter 2; Hulm et al., 2005) while *RPS15aA*, *-D* and *-F* share between 84-90% nucleic acid sequence identity among their open reading frames (ORFs) and 98-100% amino acid identity.

1.7. Objectives

The long-term research objectives of the Bonham-Smith lab are to determine why multiple plant r-protein genes belonging to a single family are expressed and to identify the mechanisms regulating gene expression. To this end, my thesis research has focused on *RPS15a*, a family that contains three expressed cytosolic r-protein genes. The objectives of this research were to 1) determine and compare the expression patterns of *RPS15aA*, *-D* and *-F*, 2) identify and compare *cis*-elements involved in *RPS15a* transcriptional regulation and, 3) visualize RPS15a subcellular location *in planta* as a preliminary investigation of ribosome heterogeneity.

CHAPTER 2: VARIATION IN TRANSCRIPT ABUNDANCE AMONG THE FOUR MEMBERS OF THE *ARABIDOPSIS THALIANA* CYTOSOLIC RIBOSOMAL PROTEIN *S15a* GENE FAMILY

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J.L. Hulm and K.B. McIntosh contributed equally to experimental design and execution. J.L. Hulm performed all RT-PCR reactions, subsequent data analysis and was responsible for preparation of the manuscript.

As a ribonucleoprotein complex, the plant ribosome consists of four ribosomal RNAs (rRNAs) and 75-92 ribosomal proteins (r-proteins), depending on the species. *Arabidopsis thaliana* r-protein genes exist in multi-gene families ranging in size from two to seven transcriptionally active members. The cytosolic *RPS15a* gene family consists of four members that, at the amino acid level, share 87-100% identity. The 5′ regulatory regions of the four genes contain many of the same putative regulatory elements but share only 46-49% nucleotide sequence identity. Semi-quantitative RT-PCR (sqRT-PCR) was used to determine *RPS15a* gene expression patterns and regulatory differences between the four genes. *RPS15aC* expression was not detected in any tissue under any treatment while transcript abundance for *RPS15aA*, *-D* and *-F* was highest in mitotically active tissues e.g. bud and flower. Seedlings showed increased transcript abundance following treatment with the cytokinin 6-benzylaminopurine (BAP) while the auxin indole acetic acid (IAA) induced an increase in *RPS15aF* abundance only. Abscisic acid (ABA) treatment resulted in decreased transcript abundance while gibberellic acid (GA_3) had little effect on all four genes. Similar trends were established for *RPS15aA*, *-D* and *-F* transcript abundance as a result of temperature, mechanical and heavy metal stress. In this chapter we report the differential transcription of the four cytosolic *RPS15a* genes in Arabidopsis and suggest that r-protein *S15a* genes should no longer be considered ‘housekeeping’ genes.

2.1. Introduction

Ribosomes are the ribonucleoprotein particles responsible for peptide synthesis in all living organisms. During translation, the ribosome is composed of two subunits (40S and 60S) consisting of four ribosomal RNA (rRNA) molecules and over 70 associated ribosomal proteins (r-proteins). The number of cytosolic r-proteins differs among eukaryotic species; mammals, including human (*Homo sapiens*; Uechi et al., 2001) and rat (*Rattus norvegicus*; Wool et al., 1996) contain 80 r-proteins while yeast (*Saccharomyces cerevisiae*) ribosomes are composed of 79 r-proteins (Link et al., 1999). The number of identified r-proteins among plant species varies from 75 to 92 (Bailey-Serres, 1998). Studies of *Arabidopsis thaliana* have identified 251 genes encoding 81 r-proteins (33 small-subunit; 48 large-subunit), 79 of which are orthologs of rat r-proteins (Barakat et al., 2001; Chang et al., 2005).

As the enzymatic complex responsible for protein synthesis, the ribosome plays an essential role in cellular growth, differentiation and development. Studies have shown that a reduction in the quantity of individual r-proteins can result in non-lethal, phenotypic abnormalities that may be the result of a decrease in translational efficiency. In *Drosophila melanogaster*, the *Minute* phenotype, distinguished by short, thin bristles, decreased body size and reduced reproductive success, can result from a mutation in one of several different r-protein genes (Lambertsson, 1998; Kongsuwan et al., 1985). Similarly, plants carrying single r-protein gene mutations display abnormal morphology, inhibited growth (*minute* phenotype) and halted embryo development (Van Lijsebettens et al., 1994; Revenkova et al., 1999; Ito et al., 2000; Weijers et al., 2001).

Eukaryotic r-proteins are commonly encoded by more than one gene. In yeast, the 79 r-proteins are encoded by 138 genes resulting from 59 gene duplications (Planta and Mager, 1998; Link et al., 1999). In *A. thaliana*, the r-protein genes are present in multi-gene families composed of two to seven members, with an average copy number of three and are dispersed throughout the genome (Barakat et al., 2001). In rat, the average r-protein multi-gene family contains twelve genes and yet, unlike plants, only one gene from each family is usually transcriptionally active; the remainder of the genes are inactive pseudogenes (Wool et al., 1995). Expression patterns of some cytosolic r-

protein genes from a variety of plant species including rice (*Oryza sativa*; Jain et al., 2004), maize (*Zea maize*; Lebrun and Freyssinet, 1991; Larkin et al., 1989), canola (*Brassica napus*; Bonham-Smith et al., 1992), Arabidopsis (McIntosh and Bonham-Smith, 2005), tobacco (*Nicotiana tabaccum*; Marty and Meyer, 1992; Dai et al., 1996; Gao et al., 1994), pea (*Pisum sativum*; Moran, 2000), peach (*Prunus persica*; Giannino et al., 2000), petunia (*Petunia hybrida*; Lee et al., 1999) and potato (*Solanum tuberosum*; Taylor et al., 1992*)* have shown an increased transcript abundance corresponding to periods of cell growth and development. Increased r-protein gene expression has also been observed following mechanical wounding and treatment with exogenous phytohormones including 2, 4-dichlorophenoxyacetic acid (2, 4-D) and benzyladenine (BAP) (Dai et al., 1996; Gao et al., 1991; Gantt and Key, 1985).

The roles of multiple, functional r-protein isoforms in plants remain unclear although it has been proposed that in addition to a constitutively expressed r-protein gene, additional copies under developmental control could be expressed during periods of increased translation (VanLijsebettens et al., 1994). Within the two gene *RPL16* (*RPL11* in the nomenclature of Barakat et al., 2001) family in *A. thaliana*, *AtRPL16A* is regulated in a tissue specific manner, with expression restricted to lateral root primordia, immature root stele, developing anthers and pollen while *AtRPL16B* expression is correlated with non-tissue specific cell division in apical meristems, cotyledons, vascular tissue and expanding floral organs (Williams and Sussex, 1995).

RPS15a, the eukaryotic ortholog of prokaryotic RPS8, is highly conserved among archea, bacteria and eukaryotes; *Methanococcus jannaschii* RPS8 shares 27-33% amino acid identity with bacterial RPS8 and 45-50% identity with its RPS15a eukaryotic ortholog (Tishchenko et al., 2001). RPS8 is a primary binding protein of the 16S rRNA, located in the central domain of the 30S ribosomal subunit (Brodersen et al., 2002). In addition to its role in 30S subunit assembly in *E. coli*, RPS8 regulates transcription of the *spc* operon containing its own open reading frame (ORF) and those of ten other r-proteins (Yates et al., 1980; Dean et al., 1981). RPS8 binds to a site on the mRNA with structural similarity to its binding site on the 16S rRNA (Gregory et al., 1988; Cerretti et al., 1988).

In this chapter, we report the differential expression of the four Arabidopsis *RPS15a* genes in control tissue and in response to a comprehensive array of treatments. It is important to analyse the expression patterns of all members of a multi-gene family in order to present an accurate view of the overall function of that family. Our analyses allow for both intra- and inter-family comparisons of transcriptional activity among r-protein gene families.

2.2. Materials and Methods

2.2.1. Plant material and seedling cultivation

Arabidopsis thaliana ecotype Columbia-0 plants were used in all experiments. For seedlings grown on culture plates or germinated on filter paper, seed was sterilized overnight (18-20 hours) using a vapor-phase sterilization method (Clough and Bent, 1998). Plate-grown seedlings were grown on ½ Murashige and Skoog medium (MS; Murashige and Skoog, 1962) containing 15 gL^{-1} sucrose and 6 gL^{-1} Phytagar (Gibco Invitrogen, California) on vertically oriented 100 x 15 mm square plates (BD Falcon, New Jersey). All plants were grown at 23°/18°C, 16 h/8 h photoperiod, 50 μmol m^{-2} s^{-1} unless otherwise noted. All tissues were snap frozen in liquid nitrogen following collection. At least three replicate experiments were conducted for each treatment.

2.2.2. Treatments

2.2.2.1. Wild type (non-treated)

Non-treated tissues were collected from five-week-old soil-grown Arabidopsis plants. Tissues included root, leaf, stem, bract, bud, flower, elongating carpels, and green siliques (fully elongated, no floral organs attached).

2.2.2.2. Phytohormones

Seven to ten day-old plate-grown seedlings were treated with 10^{-3} M indole-3-acetic acid (IAA), 6-benzylaminopurine (BAP), (±)-cis,trans-abscisic acid (ABA), or gibberellic acid (GA_3; all phytohormones were from Sigma, Missouri). Treatment of the seedlings was performed essentially as in Williams and Sussex (1995); seedlings were submerged in phytohormone solutions or a water control for 15 minutes, then rinsed

twice with sterile distilled water. Tissue was collected at 0 (immediately following rinsing), 4, and 24 h post-treatment.

2.2.2.3. Temperature stress

Seven to ten day-old plate-grown seedlings were used for all temperature stress experiments. Growth and recovery were carried out at 23°/18°C, 16 h/8 h day/night cycle. Temperature stresses were carried out in a separate growth chamber with a 16 h/8 h day/night cycle. Heat-stressed seedlings were subjected to a 32°C heat stress for 1 h then allowed to recover for up to 24 h. Tissue was collected preceding, during, and following heat stress at 0, 0.5, 1, 1.5, 2, and 24 h. Cold-treated seedlings were subjected to either 5°C or 15°C for 24 h, then allowed to recover for up to 4 h. Tissue was collected at 0, 0.5, 1, 4, 6, 12, 24, 24.25 (15 min recovery), and 28 h (4 h recovery).

2.2.2.4. Wounding

Three week-old soil-grown Arabidopsis plants were subjected to wounding by scoring basal rosette leaves once with a razor blade. Care was taken to score one leaf per plant and to maintain the integrity of the scored leaves. Three or four scored leaves, each from a different plant, were collected at each time point. Leaves were sampled at 0, 5, 10, 15, 30, and 60 min post-wounding.

2.2.2.5. Copper sulfate stress

Surface-sterilized seeds, distributed on damp filter paper, were stratified at 4°C for four days, then allowed to germinate. After 24 h of germination, 2 mL of a water control or 10 μM, 50 μM, 100 μM $CuSO_4$ solution was applied to each plate and seedlings were collected up to 10 h post-treatment. Three plates of germinating seed (approximately 50 mg) were collected per time point (0, 1, 3, 5, 8, and 10 h).

2.2.3. RNA isolation and sqRT-PCR

Total RNA was isolated from 50 - 100 mg of frozen tissue per sample using the RNeasy Plant Mini kit (Qiagen, California) according to the manufacturer's

instructions. RNA was stored in RNase-free water and diluted in 10 mM Tris, pH 7.5 for quantification via UV spectrophotometry (GeneQuant II, Pharmacia Biotech).

Semi-quantitative RT-PCR (sqRT-PCR) was performed using a OneStep RT-PCR kit (Qiagen, California) according to the manufacturer's instructions with the exceptions of primer concentrations (discussed below) and a reduction (1 μL instead of 2 μL) in the amount of Enzyme Mix used in each reaction. All RNA template stocks (4 ng/μL, to a maximum of 1 μg) were treated with 5 U DNaseI (Amersham Biosciences, New Jersey) for 10 min at 37^{o}C to eliminate any possible DNA contamination prior to sqRT-PCR. An RNase-treated control (template RNA treated with 10 ng RNaseA (USB, Ohio) for 10 min at 37^{o}C prior to sqRT-PCR) was included in every set of reactions. RNA template concentration was optimized to produce non-saturated product bands; 64 ng of total RNA was used in all reactions.

All reactions were duplexed with gene specific primers (Table 2.1) for the gene of interest (*RPS15aA*, *RPS15aC*, *RPS15aD*, *RPS15aF*, *COR15A*, or *HSP101)* and a primer/competitive primer (competimer) combination to amplify the 18S internal standard (Sung et al., 2001). The 18S primers and competimers have identical sequence (provided by Fatma Kaplan and Charles Guy, U. Florida, Gainesville) with the competimers terminating with a 3' dideoxynucleotide. Primer to competimer ratio was optimized to a final ratio of 2:8 to give non-saturated product bands. A 30 min reverse transcription step at 50^{o}C was followed by heat-inactivation/HotStarTaq activation at 95^{o}C for 15 min and 30 cycles of PCR at 94^{o}C (1 min for the first cycle, 30 s for subsequent cycles), 52^{o}C (30 s), and 72^{o}C (30 s). All steps were carried out in a PTC-100 thermal cycler (MJ Research). Sequences of amplified DNA were confirmed via automated sequencing (Plant Biotechnology Institute, National Research Council of Canada, Saskatoon).

sqRT-PCR-amplified products were visualized on ethidium-bromide stained gels using the Gel Doc 2000 gel documentation system (Biorad). Gel Doc 2000 Quantity One software was used to calculate average band density measurements, which were recorded and used in graphical analyses. The ratio of target gene product band density to 18S internal control band density was calculated and graphed using

Table 2.1. Oligonucleotide primers used for sqRT-PCR. F, forward primer; R, reverse primer.

Gene Name	Gene Locus	Primer Name	Oligo Sequence	Amplified Fragment Length	Conc. in Reaction
RPS15aA	At1g07770	S15A1F S15A1R	CCGTCACTGAGTACCTGC TCTAGAAGGGAGCAAACGG	365 bp	0.2 μM
RPS15aC	At2g39590	S15A3F S15A3R	CCTCGATATGACCTTGGC CCATGATTCCAGCTGATG	113 bp	0.2 μM
RPS15aD	At3g46040	S15A4F S15A4R	GGTGAGAATCAGTGTGCCTCAAT CCTTCAATCTCCTTAACACC	267 bp	0.2 μM
RPS15aF	At5g59850	S15A2F S15A2R	GTGCGGCTGCCATTTTCG CCATAATACCAGCCGAGG	385 bp	0.2 μM
HSP101	At1g74310	HSP101F HSP101R	AATCGAAGATGAATCCAG TTGATCACTCTTTCAGCA	213 bp	0.2 μM
COR15A	At2g42540	COR15F COR15R	GGCGATGTCTTTCTCCAGGAGC CGGTGACTGTGGATACCATATC	607 bp	0.2 μM
18S	At2g01010	cg359F cg360R (primers)	GGAGCGATTTGTCTGGTT TGATGACTCGCGCTTACT	309 bp	0.01 μM
18S	At2g01010	cg361F cg362R (compet.)	GGAGCGATTTGTCTGGTT TGATGACTCGCGCTTACT	N/A	0.04 μM

Microsoft Excel. Standard error (SE) was determined from three separate biological replicates.

2.2.4. Statistical Analyses

Statistical analyses of sqRT-PCR data were carried out using SAS version 8.2 for Windows (SAS Institute, Inc. Cary, NC, USA). Data from wild type untreated, hormone, and copper sulfate experiments were analyzed within separate mixed models where treatment effects (tissue type, hormone, copper sulfate concentration) and time points were considered fixed and experimental replicates were considered random. Data for different genes (*RPS15aA*, *-D*, *-F*, *HSP101*, and *COR15A*) were combined into a single ANOVA for each of the above models to allow for a quantitative assessment of the interactions of genes with all fixed effects. Wild type tissue, temperature and wounding stress experiments were analyzed within repeated measures mixed models using a compound symmetry covariance structure that was determined to be the most appropriate by SAS model fitting criteria (i.e. Akaike's Information Criterion, AIC and Bayesian Information Criterion, BIC). Orthogonal contrasts (one degree of freedom) were used to compare between levels of fixed effects. The denominator degrees of freedom used to calculate the significance of fixed effects were corrected for small sample size using the Kenward-Roger method (Kenward and Roger, 1997). Differences between fixed effects were considered significant at $p \leq 0.05$.

2.3. Results

2.3.1. RPS15a sequence analysis

The four genes encoding cytosolic RPS15a are located throughout the *A. thaliana* genome on chromosomes I (*RPS15aA*), II (*RPS15aC*), III (*RPS15aD*) and V (*RPS15aF*). Two other genes, originally identified as *RPS15a* family members (Barakat at el., 2001), have been reclassified as mitochondrial *RPS8* genes (Adams et al., 2002). Each of the *RPS15a* genes is comprised of three exons and two variable length introns (Figure 2.1). Although similar in organization and size, sequence comparisons among the *RPS15a* open reading frames (ORFs) using the Needle pairwise alignment program from the European Molecular Biology Laboratory-Bioinformatics Institute

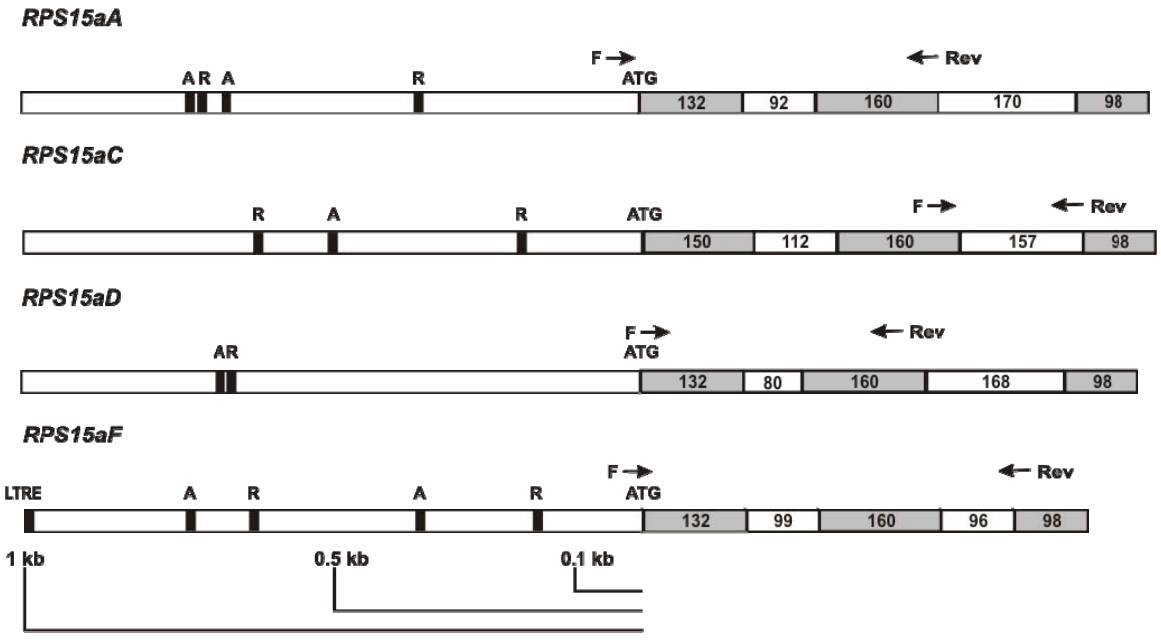


Figure 2.1. Schematic of *RPS15a* genes showing open reading frame and 1 kb of sequence upstream of the ATG start codon. Exons are shown in grey, introns and 5' upstream region are shown in white. Numbers in exons and introns indicate length of segment in base pairs. Black bars indicate putative regulatory elements: **R**, root-specific element (ATATT; Elmayan and Tepfer, 1995); **A**, auxin-responsive element (TGACG; Redman et al., 2002 and ACTTTA; Baumann et al., 1999); **LTRE**, low-temperature-responsive element (CCGAAA; Dunn et al., 1998). Positions of gene-specific primers used for sqRT-PCR amplification of each gene indicated by arrows; **F**, forward primer; **Rev**, reverse primer.

(http://www.ebi.ac.uk/emboss/align/index.html) have shown that *RPS15aA*, *-D* and *-F* share 77%, 76% and 75% sequence identity, respectively, with *RPS15aC* giving putative polypeptides with 88% (*RPS15aA* and *-F*) and 87% (*RPS15aD*) identity with *RPS15aC* (Table 2.2). Sequence identity between the *RPS15aA* ORF and those of *RPS15aD* and *-F* is 84% and 85% while the putative polypeptides are 98% and 100% identical. At 90%, *RPS15aD* and *-F* have the highest degree of ORF sequence identity and the polypeptides have 98% identity at the amino acid level. A comparison of the regulatory regions, 1000 bp upstream of the ATG start codon, shows only 46-49% sequence identity among the four genes. A slight increase in sequence identity (48-51%) occurs among *RPS15aA*, *-D* and *-F* as well as between *RPS15aC* and *-D* when 500 bp upstream of the start codons are compared. While a comparison of the first 100 bp 5′ to the ATG of each gene showed slightly higher sequence identities (52-56%) among *RPS15aA*, *-D* and *-F*, this decreased to between 42-50% when compared to *RPS15aC*.

The Plant Cis-acting Regulatory DNA Elements (PLACE) (http:www.dna.affrc.go.jp/htdocs/PLACE; Higo et al., 1999) database was used to identify putative regulatory motifs in the regions 1000 bp upstream of the ATG start codon of each of the *RPS15a* genes. Although common elements were detected, their arrangement and number varied (Table 2.3). A regulatory element directing root specific gene expression (ATATT; Elmayan and Tepfer, 1995) was present in the 1 kb of upstream sequence of all four genes (Figure 2.1). An ASF-1 binding element (TGACG), found in the promoters of auxin-regulated genes (Redman et al., 2002), is present upstream of the ATG start codon in *RPS15aA*, *-D* and *-F* but not in *RPS15aC* which contains a different auxin-responsive element (ACTTTA; Baumann et al., 1999) -400 bp from the ATG. A low-temperature-responsive element (LTRE) (CCGAAA; Dunn et al., 1998) is only present in the 5′ upstream region of *RPS15aF*.

2.3.2. Optimization of sqRT-PCR

Semi-quantitative RT-PCR was performed to determine the relative transcript abundance from the different *RPS15a* genes compared to an *18S* rRNA internal standard. All reactions were duplexed to amplify both the gene of interest (*RPS15aA*,

Table 2.2. Predicted open reading frame (ORF) and polypeptide sequence identity among the four *RPS15a* genes.

Gene	*RPS15aA*	*RPS15aC*	*RPS15aD*
RPS15aA	ORF / Peptide		
RPS15aC	77% / 88%		
RPS15aD	84% / 98%	76% / 87%	
RPS15aF	85% / 100%	75% / 88%	90% / 98%

Table 2.3. Putative regulatory element position(s) upstream of *RPS15a* start codons (ATG) as determined using the PLACE database.

	Regulatory element position(s) upstream of ATG (bp)		
Gene Name	Root (ATATT)	Auxin (TGACG)	Low Temperature (CCGAAA)
RPS15aA	-708, -363	-716, -666	NP
RPS15aC	-620, -214	-400 (ACTTTA)	NP
RPS15aD	-667	-678	NP
RPS15aF	-628, -190	-364	-1000

*NP – not present

RPS15aC, *RPS15aD*, *RPS15aF*, *HSP101* or *COR15A*) and *18S* rRNA. As rRNA may comprise more than 80% of total cellular RNA, amplification of *18S* rRNA transcript can result in a strong, saturated transcript signal. To reduce this signal, *18S* mRNA was amplified using a combination of primers and competitive primers (competimers) identical to *18S* primer sequence but with a terminal 3′ dideoxynucleotide.

To produce a non-saturated signal over 30 cycles of PCR, following reverse transcription, both template concentration and *18S* primer to competimer ratio were optimized as in Sung et al. (2001). Four, 16, 32, 64 and 256 ng of DNase I-treated total bud RNA were tested in template optimization reactions (data not shown). Bud, a mitotically active tissue, produced strong *RPS15a* and *18S* rRNA transcript signals using 32 ng, 64 ng or 256 ng (data not shown). A concentration of 64 ng was chosen for use in all subsequent sqRT-PCRs, allowing for sufficient signal production from tissues that were relatively transcriptionally inactive, such as leaf and bract. Primer to competimer ratios of 2:2, 2:4, 2:6, 2:8 and 2:10 were tested to determine the optimal ratio for production of non-saturated *18S* rRNA bands; a ratio of 2:8 was chosen for use in all reactions.

2.3.3. RPS15aA, -D and -F transcript abundance in tissues of non-treated plants

Soil-grown, five-week old Arabidopsis Col-0 plants were used to determine the relative transcript abundance of each *RPS15a* gene in an array of mature and developing tissues. *RPS15aC* transcript was not detected in any tissue examined. Transcript abundance was highest in mitotically active tissues; bud > elongating carpel/silique > flower = root = stem > bract > green silique > leaf for *RPS15aA* and *-D*, while *RPS15aF* transcript abundance was highest in bud > flower = elongating carpel/silique = root > green silique > stem > leaf > bract (Figure 2.2). *RPS15aA* and *-D* transcripts were ~1.5 times more abundant in bud than in leaf ($p=0.0053$; $p=0.0407$). The lowest levels of *RPS15aF* transcript occurred in leaf and bract tissues; *RPS15aF* transcript was ~1.9-2.2 times more abundant in bud than in leaf ($p=0.0243$) or bract ($p=0.0271$). Following the maturation of elongating carpel/silique to green silique, *RPS15aF* showed a significant reduction in transcript level ($p=0.0021$). In all tissues, transcript levels of *RPS15aF* were lower than those of *RPS15aA* and *-D*.

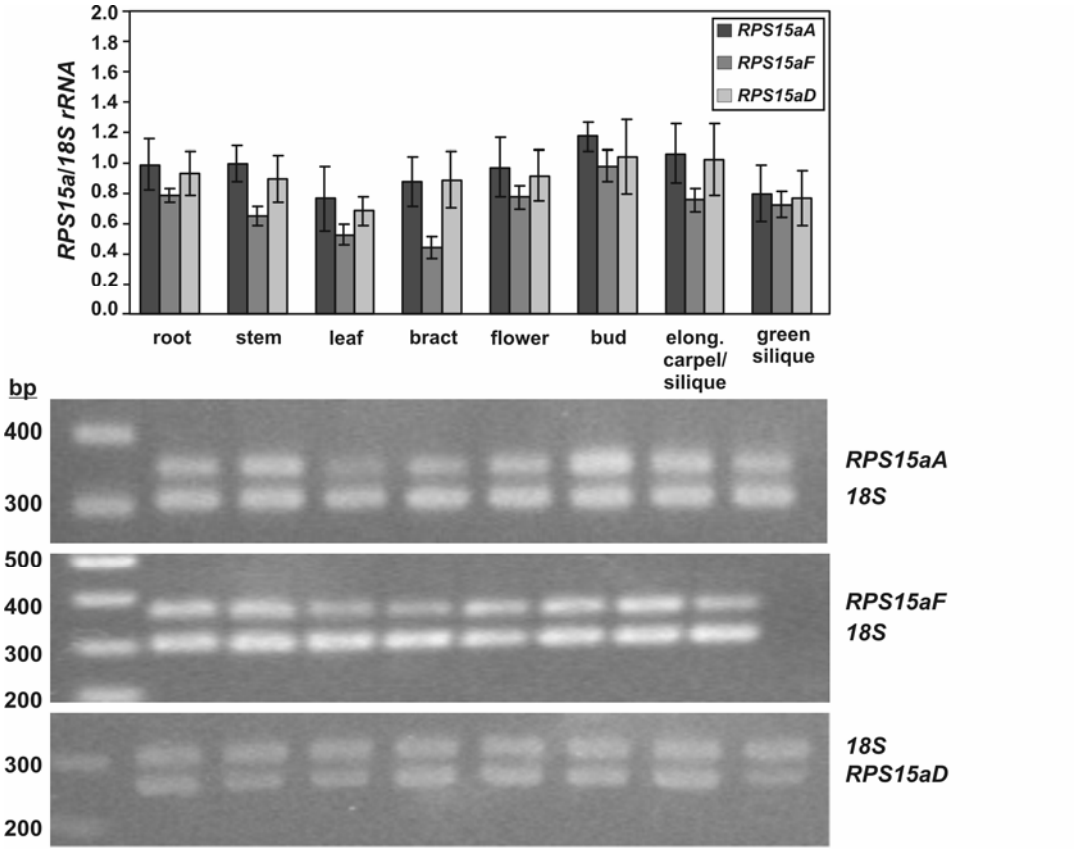


Figure 2.2. *RPS15aA*, *-D* and *-F* transcript levels following sqRT-PCR amplification from a variety of Arabidopsis tissues. Band intensities are relative measurements representing the *RPS15a* to *18S* rRNA average band density ratio. Mean is graphed ± SE (n=3).

2.3.4. Transcript abundance in response to phytohormone treatments

Although PLACE analyses showed the presence of auxin-responsive elements in the 5′ regulatory region of the four *RPS15a* genes (Figure 2.1), only transcript levels of *RPS15aF* increased with auxin treatment; at 24 h *RPS15aF* transcript abundance was ~1.3 times greater than initial levels (p=0.0360; Figure 2.3). At 24 h post-treatment the level of *RPS15aF* transcript was ~1.5 fold greater than that of *RPS15aA* (p=0.1344) and -*D* (p=0.0623), both of which remained constant over the 24 h time period.

BAP treatment resulted in a similar pattern of transcript abundance for all three active genes, *RPS15aA*, -*D* and -*F* (Figure 2.3). Both *RPS15aD* (p=0.0040) and *RPS15aF* (p=0.0089) transcript abundance increased over 24 h although transcript levels were ~1.4 (p=0.0992) to 1.3 (p=0.1389) times lower than those of *RPS15aF*. *RPS15aA* transcript showed a similar level of abundance across the three time points but also the greatest amount of variation among individual replicates.

RPS15aD and -*F* displayed similar patterns of transcript abundance following ABA treatment (Figure 2.3); transcript levels showed an initial decrease at 4 h proceeded by partial recovery at 24 h. *RPS15aF* showed the greatest difference in transcript abundance decreasing ~1.8 fold from 0 h to 4 h (p=0.0092). In the following 20 h *RPS15aF* transcript level increased only slightly. *RPS15aD* transcript, which was present at a lower level than *RPS15aF* at 0 h, also showed an initial decrease in transcript abundance followed by a return to 0 h levels after 24 h. Although initial 0 h transcript levels of *RPS15aF* were ~1.5 fold greater than that of *RPS15aD* (p=0.0563), transcript levels of *RPS15aA*, -*D* and -*F* were all similar at 24 h. Transcript abundance for *RPS15aA*, -*D* and -*F* showed no change following treatment with GA_3 (Figure 2.3). No *RPS15aC* transcript was detected with any of the phytohormone treatments.

2.3.5. Transcript abundance in response to temperature stress

Transcript abundance of *RPS15aA*, -*D* and -*F* were compared to that of *HSP101 (HEAT SHOCK PROTEIN 101),* a known heat inducible gene (Hong, 2001), during a 32°C heat stress and the following recovery period. *RPS15aC* transcript was not detected at any point during the high temperature stress-recovery period. *HSP101*

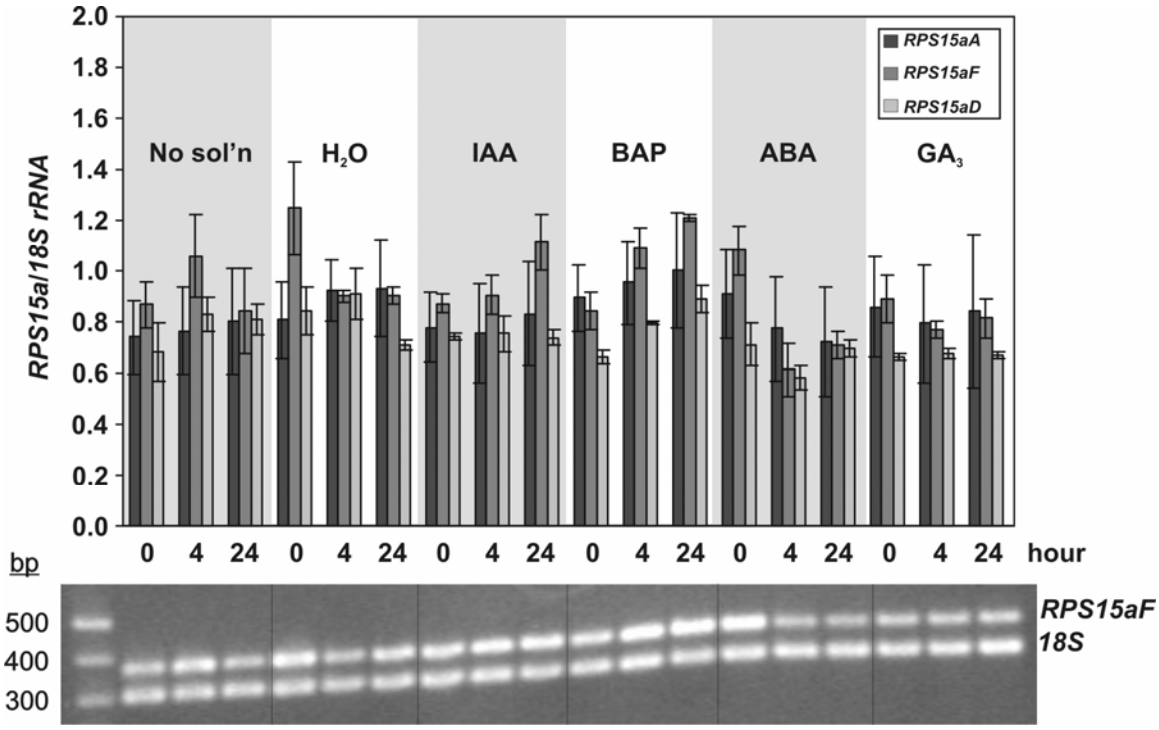


Figure 2.3. *RPS15aA*, *-D* and *-F* transcript levels in response to hormone treatments applied to ~1 week-old seedlings. Seedlings were treated with no solution, water only, or 10^{-3} M IAA, BAP, ABA, or GA_3. Samples were taken at time points 0 (immediately following treatment), 4, and 24 h. Black lines in gel photo were overlaid on top of the gel image for ease of viewing. Band intensities are relative measurements representing the *RPS15a* to *18S* rRNA average band density ratio. Mean is graphed ± SE (n=3).

transcript was detected after 0.5 h of heat stress, remained stable over the 1 h of treatment and progressively decreased to zero after 23 h of recovery (Figure 2.4). The heat stress treatment had no effect on the transcript abundance of *RPS15aA* or *-F* over the time course of the experiment. Although transcript abundance of the three expressed *RPS15a* genes was similar, *RPS15aD* gene expression showed a slight decrease from 0 h to 1 h after heat stress (p=0.0087) relative to *RPS15aA* (p=0.0895) and *-F* (p=0.1328).

During cold (5°C) stress, transcript from a known cold-inducible gene, *COR15A* (*COLD RESPONSIVE*; Lin and Thomashow, 1992), was detected 4 h into the stress, increased over the next 2 h and remained stable until the recovery period, at the end of which (4 h), transcript abundance had decreased to zero (Figure 2.5). *RPS15aA*, *-D* and *-F* showed similar patterns of transcript abundance over the duration of the experiment; transcript levels remained relatively constant except for decreases of *RPS15aA* following transition to the recovery period (p=0.0365) and of *RPS15aF* from 0.25 h to 4 h during recovery (p=0.0054).

During chilling (15°C) treatment *RPS15aA*, *-D* and *-F* showed little change in transcript abundance although there were quantitative differences among the genes (Figure 2.6). Over the time course of the chilling treatment, *RPS15aF* transcript was ~1.4-2.4 times more abundant than *RPS15aA* (p=0.0002-0.0156) and ~1.4-1.7 fold more abundant than *RPS15aD* (p=0.0011-0.0583) transcript, respectively. During the 4 h recovery period transcript levels of *RPS15aF* remained high while those of *RPS15aA* and *-D* showed divergent responses with *RPS15aD* transcript increasing while that of *RPS15aA* decreased. These transcriptional changes resulted in similar levels of *RPS15aD* and *RPS15aF* transcript after 4 h of recovery and a ~2.5 fold difference between *RPS15aF* and *RPS15aA* transcript abundance at the same time point (p=0.0212).

2.3.6. Transcript abundance in response to wounding

Wounding of mature Arabidopsis rosette leaves resulted in similar patterns and levels of transcript throughout the experimental time course for *RPS15aA* and *-D* (Figure 2.7). Transcript levels remained constant and equivalent to those recorded at

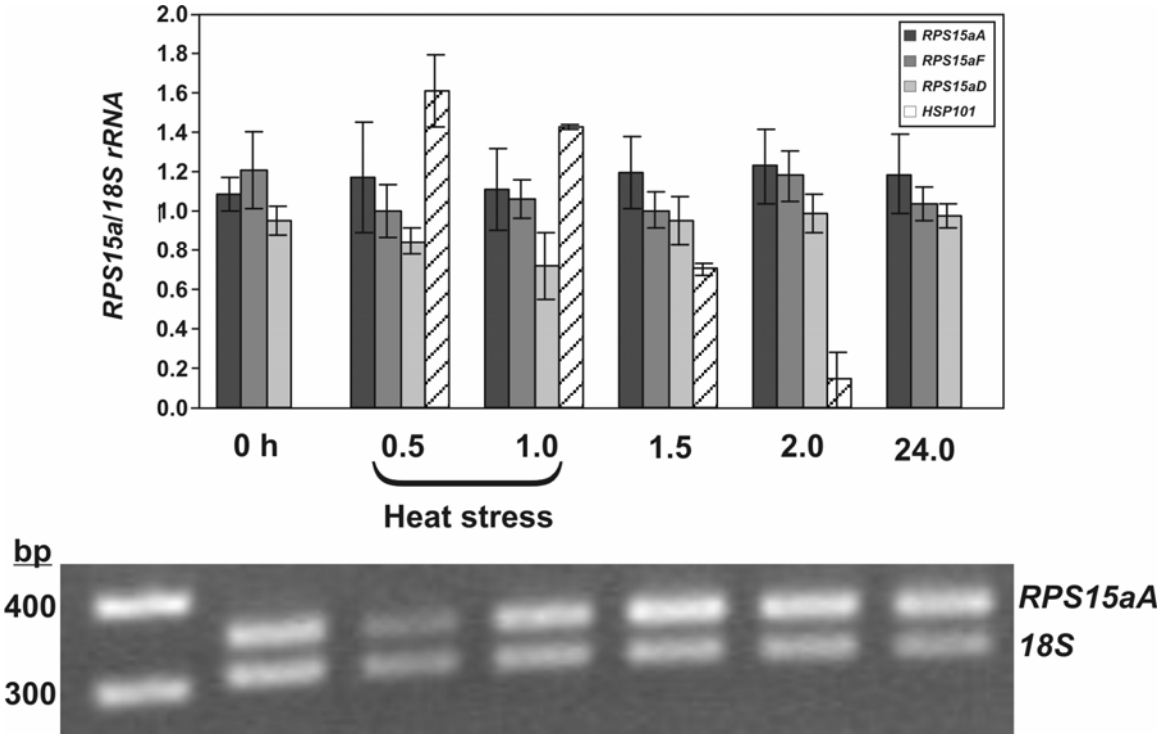


Figure 2.4. *RPS15aA*, *-D* and *-F* transcript levels in response to heat stress applied to ~1 week-old seedlings. *HSP101* transcript levels were amplified as a positive indicator of heat stress. Seedlings were treated at 32^{o}C for 1 h then transferred to normal growth temperature (23^{o}C). Band intensities are relative measurements representing the *RPS15a* to *18S* rRNA average band density ratio. Mean is graphed ± SE (n=3).

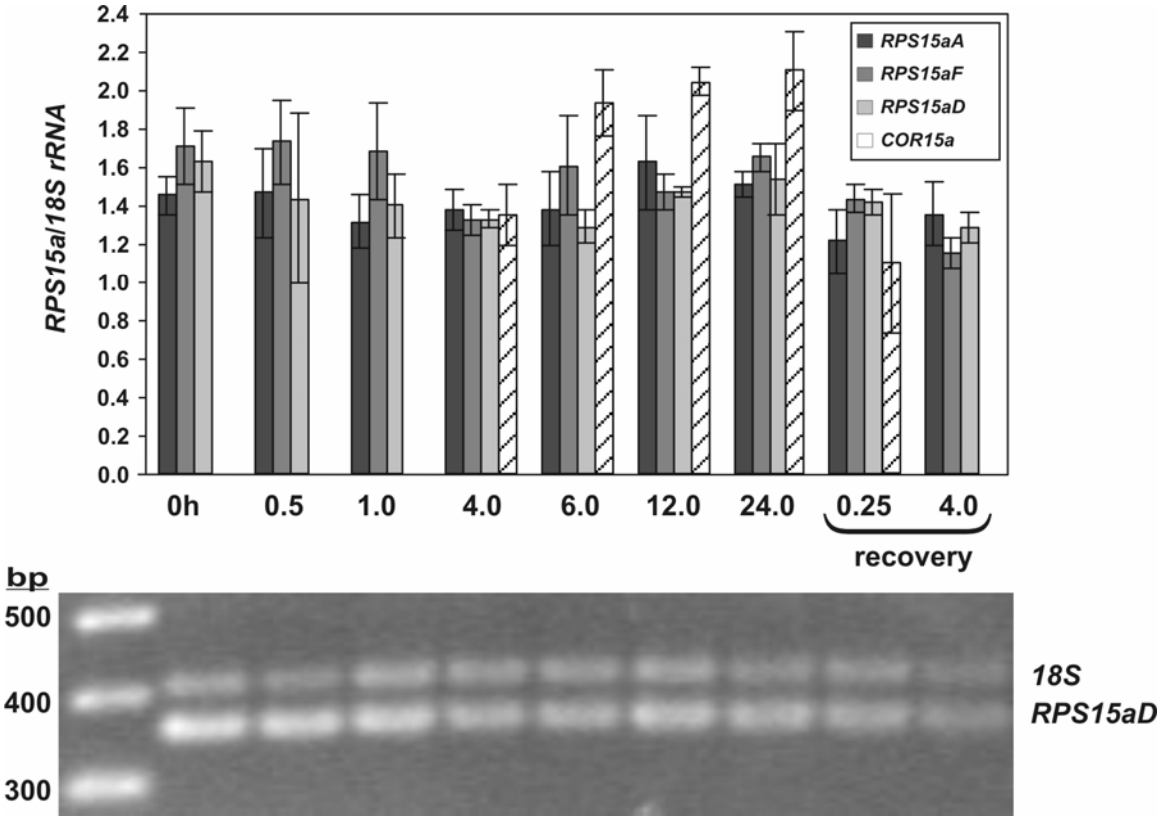


Figure 2.5. *RPS15aA*, *-D* and *-F* transcript levels in response to cold (5°C) stress applied to ~1 week-old seedlings. *COR15A* transcript levels were amplified as a positive indicator of cold stress. Seedlings were incubated at 5°C for 24 h then allowed to recover at normal growth temperature (23°C). Band intensities are relative measurements representing the *RPS15a* to *18S* rRNA average band density ratio. Mean is graphed ± SE (n=3).

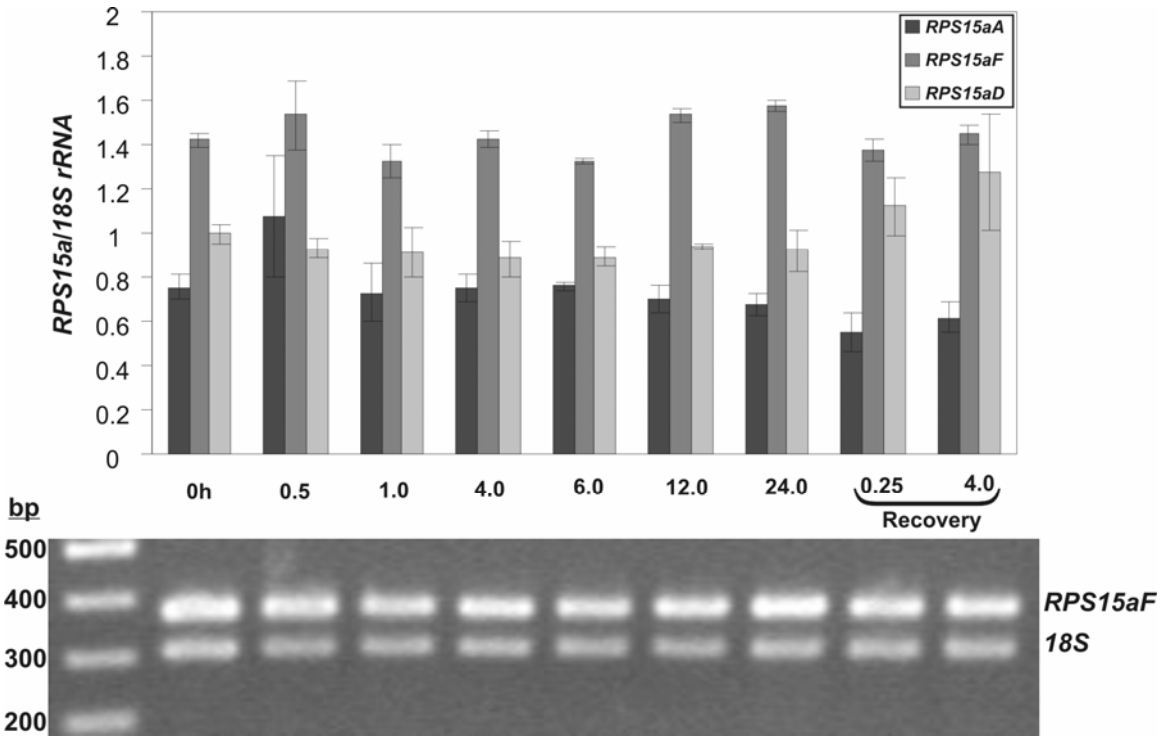


Figure 2.6. *RPS15aA*, *-D* and *-F* transcript levels in response to chilling (15°C) treatment applied to ~1 week-old seedlings. Seedlings were incubated at 15°C for 24 h then allowed to recover at normal growth temperature (23°C). Band intensities are relative measurements representing the *RPS15a* to *18S* rRNA average band density ratio. Mean is graphed ± SE (n=3).

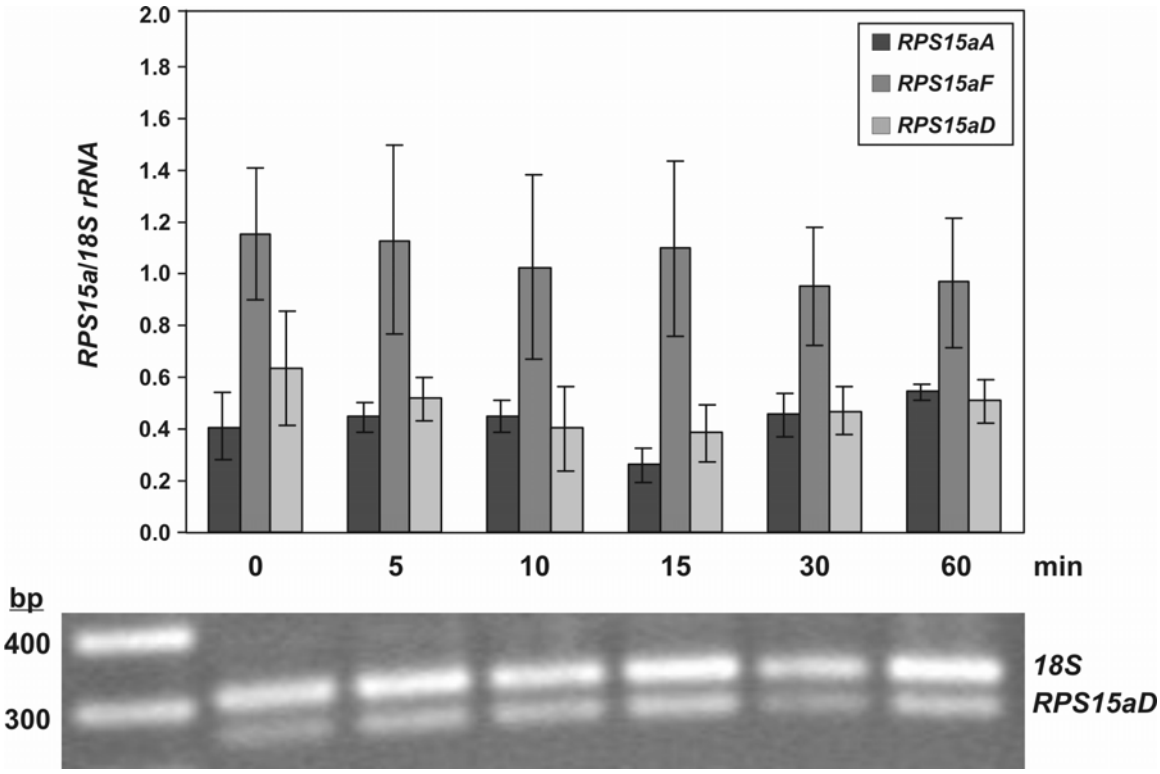


Figure 2.7. *RPS15aA*, *-D* and *-F* transcript levels in response to wounding applied to leaves of 3-week old plants. Leaves were scored on the plants and collected over 1 h following treatment. Band intensities are relative measurements representing the *RPS15a* to *18S* rRNA average band density ratio. Mean is graphed ± SE (n=3).

0 h with the exception of an ~1.7 fold decrease in *RPS15aA* between 10 min and 15 min (p=0.0672). *RPS15aF* transcript was ~1.8-4.2 times more abundant than *RPS15aA* (p=0.1228-0.0317), ~2.5-3.3 times more abundant than *RPS15aD* (p=0.1277-0.0508) and showed a greater degree of variation among replicates. No *RPS15aC* transcript was detected following the wounding treatment.

2.3.7. Expression in response to heavy metal stress

Treatment of germinating Arabidopsis seeds with $CuSO_4$ (10 µM, 50 µM and 100 µM) resulted in the transient decrease of both *RPS15aA* and *-D* transcript levels (Figure 2.8). Although the two genes showed similar patterns of expression *RPS15aD* transcript was ~1.1 (p=0.2498) to 1.5 (p=0.0376) fold greater than that of *RPS15aA*. While down-regulation of *RPS15aA* occurred 3-5 h after treatment with 10 µM $CuSO4_4$ (p=0.0275), *RPS15aD* expression showed a decrease in transcript abundance during the first 5 h post-treatment (p=0.0415); a similar *RPS15aD* transcriptional response was detected following treatment with water (p=0.0400). An increase in $CuSO_4$ concentration to 50 µM resulted in a more pronounced decrease in *RPS15aD* transcript levels as well as an extension of the time period in which transcript levels decreased (p=0.0063) while causing no significant changes in *RPS15aA* expression. A 100 µM $CuSO_4$ treatment resulted in the transient decrease of *RPS15aA* (p=0.0033) and *-D* (p=0.0084) transcript abundance over the entire experimental time course. No *RPS15aC* transcript was detected following $CuSO_4$ stress.

2.4. Discussion

R-proteins genes are often grouped together in expression studies under the umbrella of 'housekeeping genes'. The production of functional ribosomal subunits is considered to be dependent on the coordinate synthesis of r-protein constituents (Gorenstein and Warner, 1976), thus it is thought that r-protein genes would be similarly regulated and share common expression patterns. Yet, in addition to being part of the ribosome, many individual r-proteins perform extra-ribosomal functions

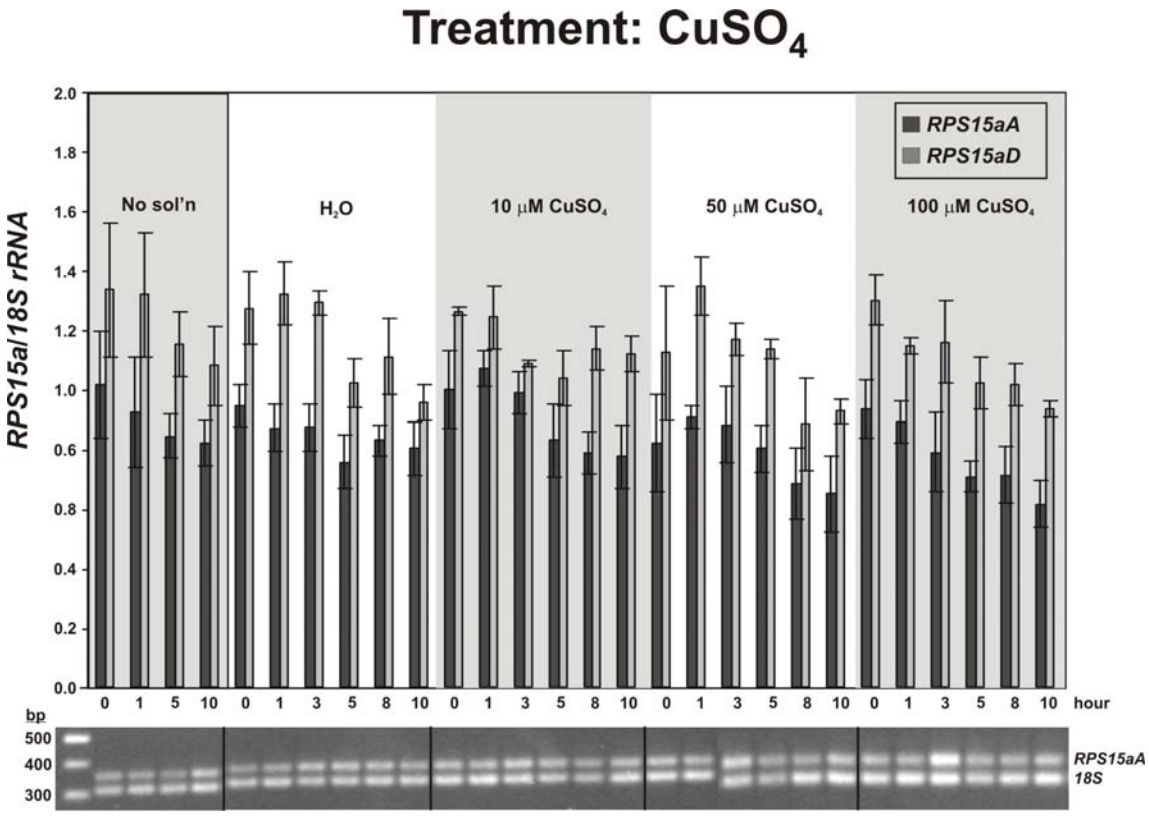


Figure 2.8. *RPS15aA* and *-D* transcript levels in response to metal stress. $CuSO_4$ treatments were applied to germinating seedlings after 24 h of imbibition. Black lines on gel photos were overlaid on top of pictures for ease of viewing. *RPS15aA* gel picture is a composite from two rows run on the same gel due to space constraints. Band intensities are relative measurements representing the *RPS15a* to *18S* rRNA average band density ratio. Mean is graphed ± SE (n=3).

within the cell (Wool, 1996). Revenkova et al. (1999) has shown that although Arabidopsis RPS27A is not necessary for ribosome function, the protein is required for degradation of damaged mRNAs following UV irradiation. In addition, previous studies have shown both spatial and temporal differences in r-protein gene expression; Arbidopsis *RPL16A* (*RPL11* in the nomenclature of Barakat et al. 2001) gene expression is associated with cell elongation and division in roots while *RPL16B* expression is correlated with non-tissue specific cell division (Williams and Sussex, 1995); *RPL25* and *RPL34* gene expression in tobacco is higher in stem and roots than in leaves and flowers (Gao et al., 1994) while *RPS19* and *RPL7* gene expression increases in stolon tips during the early stages of tuberization in potato (Taylor et al., 1992).

RPS15aA, *-D* and *-F* transcripts were detected in all mature tissues examined (Figure 2.2), in germinating, imbibed seed (Figure 2.8), one week-old seedlings (Figures 2.3 to 2.6) and three to five week-old seedlings (Figures 2.2 and 2.7). In addition, developmental expression profiles of *RPS15aA* and *-D* showed the presence of transcript from 20 h to 122 h during seed germination and early seedling development (data not shown). The general expression patterns of *RPS15aA, -D* and *–F* were consistent with those of previous studies (*S14*, Larkin et al., 1989; *S11*, Lebrun and Freyssinet, 1991; *B. napus S15a*, Bonham-Smith et al., 1992; *L2*, Marty and Meyer, 1992; *S19* & *L7*, Taylor et al., 1992; *L25* & *L34*, Gao et al., 1994; *L34*, Dai et al., 1996; *L15* & *L27a*, Lee et al., 1999; *S28*, Giannino et al., 2000; *L9*, Moran, 2000; *L13*, Jain et al., 2004; *L23a*, McIntosh and Bonham-Smith, 2005). *RPS15a* transcript abundance is highest in mitotically active tissues such as flower and bud and lowest in mature leaf and bract (Figure 2.2). Relatively high levels of *RPS15aA*, *-D* and *-F* transcripts were detected in root tissues, agreeing with results showing high levels of *RPL16* transcript in the root cap and during the development of lateral root primordia in Arabidopsis (Williams and Sussex, 1995).

The predominant auxin found in plant cells, IAA, is primarily synthesized in the shoot apical meristem, young leaves and developing fruits while low levels may also be produced in mature leaf and root tip tissue (Taiz and Zeiger, 1998). Endogenous IAA plays an important role in a variety of plant growth and developmental processes while exogenous application of IAA has been shown to rapidly and specifically alter gene

expression (Guilfoyle et al., 1998). Gao et al. (1994) reported a five-fold increase in tobacco *L25* mRNA following the addition of the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) to leaf pieces incubated in MS liquid media, while an approximate eight-fold increase in translatable r-protein mRNAs from 2,4-D treated soybean hypocotyls was described by Gantt and Key (1983). McIntosh and Bonham-Smith (2005) have shown significant increases in *RPL23aA* and *-B* transcript levels following IAA treatment of Arabidopsis seedlings. An increase in rRNA synthesis was shown in artichoke (*Helianthus tuberosus* L.) explants after treatment with 2,4-D due, in part, to increased transcription of precursor rRNA and may have corresponded with increased levels of r-protein mRNA (Melanson and Ingle, 1978). Interestingly, although all three expressed *RPS15a* genes contain putative auxin response elements, only *RPS15aF*, with a putative auxin-responsive element at -364, showed an increase in transcript abundance following exogenous application of IAA (Figure 2.3). Putative auxin-responsive elements found in the upstream regulatory regions of *RPS15aA* and *-D* may be located too far downstream (Table 2.2) of their respective start codons to exert any regulatory effect.

Exogenous application of BAP to Arabidopsis seedlings resulted in an increased transcript level for all three expressed *RPS15a* genes over 24 h (Figure 2.3). The increased level did not match the eight-fold increase in tobacco *L25* transcript following cytokinin treatment reported by Gao et al. (1994). However, this increase followed incubation of wounded leaf tissue in MS media, a treatment that alone resulted in a three-fold increase in *L25* gene expression. Similarly, McIntosh and Bonham-Smith (2005) have described increases in *RPL23aA* and *-B* transcript levels over 24 h following treatment of Arabidopsis seedlings with BAP. Transcript levels of *RPS14* from Arabidopsis rosette leaves and Lupine (*Lupinus luteus*) cotyledons were also found to be increased (Arabidopsis: 6.8 fold greater relative to a 0 h water control; Lupine: 6.4 fold greater relative to a 1.0 h ABA control) reaching a maximum at 5 h; maximum transcript levels were maintained for at least 24 h of a cytokinin treatment (Cherepneva et al., 2003). *RPS16*, *RPL30* and *RPL13A* transcript levels from Lupine cotyledons also showed increases of 4.5, 3.0 and 3.9 fold, respectively, during a cytokinin treatment relative to a 0 h water control (Cherepneva et al., 2003). In a

cytokinin-starved soybean (*Glycine max*) suspension culture, Crowell et al. (1990) demonstrated an increase in transcript abundance of twenty cDNAs after a zeatin treatment. Amino acid sequence similarities indicated that two of the cDNAs, cim9 and cim20, were related to L30 from rat (L32 from yeast) and L44 from yeast and human, respectively. Together, these results suggest a r-protein gene specific induction by cytokinin rather than a global housekeeping r-protein gene induction.

ABA, synthesized in roots and mature leaves, notably during water stress, exerts a number of effects including stomatal closure and the inhibition of shoot growth (Davies, 1995). *RPS14* mRNA accumulation in three-week-old Arabidopsis rosette leaves was suppressed after 5 h of incubation with ABA while *RPS16*, *RPL13A* and *RPL30* transcripts showed a similar decrease in lupine cotyledons (Cherepneva et al., 2003). Transcript abundance of *RPS15aA*, *-D* and *-F* also decreased during the first four hours following ABA treatment although *RPS15aF* and *-D* exhibited a partial recovery to 0 h transcript levels over the remainder of the time course (Figure 2.3). A similar pattern of expression was shown for *RPL23aA* and *-B* from Arabidopsis with transcript levels decreasing in the initial 4 h following ABA treatment and recovery over the next 20 h (McIntosh and Bonham-Smith, 2005).

Gibberellins, a group of phytohormones synthesized in young shoots and developing seeds, are involved in a number of plant developmental processes including the induction of seed germination and bolting in long day plants (Davies, 1995). While the effect of gibberellins on r-protein gene expression has not been extensively studied, McIntosh and Bonham-Smith (2005) showed no effect of GA_3 on *RPL23aA* transcript levels while *RPL23aB* transcript abundance increased over the 24 h time course. None of the four cytosolic *RPS15a* genes contain a gibberellin response element (GARE) within the upstream regulatory region (Figure 2.1) and as such, treatment of Arabidopsis seedlings with GA_3 had no effect on *RPS15aA*, *-D* or *-F* transcript abundance (Figure 2.3).

The expression profiles of *RPS15aA*, *-D* and *-F* showed little change during heat stress, cold acclimation or chilling (Figures 2.4-2.6). Although Volkov et al. (2003) reported a decrease in Arabidopsis *RPL23aA* and tobacco *L25* transcript levels following heat stress, a similar response was not elicited during our experiments;

RPS15aA, *-D* and *-F* transcript levels remained constant (Figure 2.4). These results agree with those of McIntosh and Bonham-Smith (2005) which show unchanged levels of *RPL23aA* and *-B* transcript levels following 1 h of a 32°C treatment. Differences in experimental technique and tissues may account for the contrasting results. Volkov et al. (2003) stressed individual, mature leaves in incubation medium while, in this study, we stressed and harvested intact seedlings thereby reducing any synergistic effects of wounding and anoxia on *RPS15a* transcript abundance.

Low-temperature (5ºC) acclimation is a complex process involving numerous physiological and biochemical alterations resulting from differential gene expression (Thomashow, 1999). Increased transcript levels of *ATPK19* and *ATPK6* in Arabidopsis during cold stress have been reported (Mizoguchi et al., 1995). It was hypothesized that the ATPK19 and ATPK6 proteins, homologous to p70 RPS6 kinases, increased the capacity for protein synthesis following cold stress, through r-protein phosphorylation (Mizoguchi et al., 1995). Furthermore, increased *GmRPS13*, *GmRPS6* and *GmRPL37* r-protein transcript levels have been reported in soybean after three days of cold treatment (Kim et al., 2004) and increased *Brassica napus BnRPL13* transcript (initially identified as *BnC24*; Sáez-Vásquez et al., 2000) in etiolated seedlings, after two days at 4ºC. In contrast, a decrease in *RPS7* transcript abundance in winter rye (*Secale cereale*) following 1, 6 or 24 h at 4ºC has also been reported (Berberich et al., 2000). Transcript levels for *RPS15aA*, *-D* and *-F* in Arabidopsis showed little change during a 5°C cold stress followed by a small decrease in expression during a subsequent recovery period (Figure 2.5). Similar results have been reported by McIntosh and Bonham-Smith (2005) for *RPL23aA* and *-B*. Although the three expressed *RPS15a* genes showed similar levels of expression during treatment, *RPS15aF* is the only gene to contain a putative LTRE in its upstream regulatory region (Figure 2.1). Chilling (15ºC) treatment also had little effect on transcript levels of all three expressed *RPS15a* genes (Figure 2.6). These results are similar to those for *RPL23a* although variation in *RPL23a* transcript abundances were obvious during a recovery period after the chilling treatment (McIntosh and Bonham-Smith, 2005). As temperate crop plants, it is possible that soybean and *B. napus* may have a lower capacity for cold tolerance and acclimation than a hardy plant such as Arabidopsis.

Exposure to physical, chemical and/or mechanical stresses can result in damage to plant tissues and changes in gene expression (Pena-Cortes and Willmitzer, 1995), however, no significant changes in *RPS15a* transcript levels were observed up to 60 min after mechanical wounding of Arabidopsis rosette leaves (Figure 2.7). McIntosh and Bonham-Smith (2005) previously reported no effect of wounding on *RPL23aA* transcript abundance while *RPL23aB* transcript levels were affected. In tobacco leaves, an approximate three-fold increase in *L25* transcript level was observed (Gao et al., 1994) 5 h after wounding. These contrasting results may be explained by the fact that *RPS15a* transcript levels were determined in wounded leaves that remained attached to the plant while, in tobacco, *L25* transcript levels were determined from isolated sections of leaf incubated in liquid medium (Gao et al., 1994).

Previous studies in soybean have demonstrated that a heavy metal stress can elicit transcriptional responses in plants that mimic those detected following wounding and subsequent pathogen attack (Ludwig and Tenhaken, 2001). While little effect was seen on *RPL23aB* transcript levels in $CuSO_4$, increasing concentrations of $CuSO_4$ (10-100 μM) resulted in a more rapid decrease of *RPL23aA* transcript levels (McIntosh and Bonham-Smith, 2005). Treatment of soybean suspension cultures with increasing concentrations of $CuSO_4$ produced a transient decrease in *RPL2* transcript level (Ludwig and Tenhaken, 2001). Arabidopsis seedlings responded to a $CuSO_4$ stress in a similar manner to the soybean cell suspension; *RPS15aA* and *-D* transcript levels decreased over the time course of heavy metal exposure (Figure 2.8). However, transcript levels of Arabidopsis *RPS15aA* and *-D* decreased less rapidly (3-5 h) than that of soybean *RPL2* (1 h). This difference in reaction time was likely a result of slower $CuSO_4$ uptake by Arabidopsis seedlings compared to that of the soybean cell suspension culture. Furthermore, the soybean cell suspension cultures were able to accommodate the $CuSO_4$ stress after 10 h, by which time *RPL2* transcript levels had returned to control levels. No such accommodation was seen in Arabidopsis seedlings where *RPS15aA* and *-D* transcript abundance were still in decline after 10 h in the presence of 100 μM $CuSO_4$, possibly due again to a relatively slow uptake of $CuSO_4$ by Arabidopsis seedlings compared to that of the soybean cell suspension culture.

This study, characterizing the expression patterns of the four cytosolic members of the Arabidopsis *RPS15a* gene family, has identified differences not only in the transcriptional regulation among members of the same gene family but also between r-protein gene families. This work supports the growing body of evidence showing that eukaryotic r-proteins are, in part, differentially regulated at the transcriptional level and that their previously accepted status as 'housekeeping' genes should be re-evaluated.

CHAPTER 3. *RIBOSOMAL PROTEIN S15a*: DISSECTING TRANSCRIPTIONAL REGULATION IN *ARABIDOPSIS THALIANA*

The Arabidopsis cytosolic ribosomal protein (r-protein) *RPS15a* gene family consists of three transcriptionally active members that, at the amino acid level, share 98-100% identity. However, as described in the previous chapter, transcript abundance among the genes differs in untreated, wild type tissues and in response to a variety of abiotic stresses. Therefore, a dissection of the *RPS15a* 5′ regulatory regions (RRs) was performed to determine the minimal region required for gene expression and identify *cis*-regulatory elements. Results of transcription start site mapping indicated multiple initiation sites for *RPS15aA* and *-F* and only a single site for *-D* while all three genes contained a leader intron upstream of the start codon. Analysis of reporter gene activity in transgenic plants containing a series of *RPS15aA*, *-D* or *-F 5′ RR deletion::β-glucuronidase* (*GUS)* fusions showed that although there was a general trend for developing/mitotically active tissues to stain positive for GUS activity, differential regulation was also exhibited. In addition to the prospective regulatory roles of pollen specific elements, *PROLIFERATING CELLULAR NUCLEAR ANTIGEN* (*PCNA*) Site II motifs and inhibitory elements on *RPS15a* expression, the potential for translational regulation is also discussed.

3.1. Introduction

Ribosomes are the ribonucleoprotein complexes that catalyze the peptidyl transferase reaction during the universal, fundamental process of peptide synthesis. Ranging in size from 3-4.5 MDa, the eukaryotic ribosome is the largest enzymatic complex in the cell and, during translation, is composed of two subunits (40S and 60S) consisting of four ribosomal RNA (rRNA) molecules and approximately 80 ribosomal proteins (r-proteins). Although rRNA is the source of enzymatic activity (Nissen et al., 2000), r-proteins are essential to ribosome structure and function. The number of r-

proteins varies among eukaryotic species; human (*Homo sapien*; Uechi et al., 2001) and rat (*Rattus norvegicus*; Wool 1995) ribosomes contain 80 r-proteins while yeast (*Saccharomyces cerevisiae*) contains 79 (Link et al., 1999). Arabidopsis ribosomes have an estimated mass of 3.2 MDa and contain 81 r-proteins including a plant specific acidic phosphoprotein (Bailey-Serres et al., 1997) and an ortholog of the mammalian receptor for activated protein kinase C (RACK1) (Chang et al., 2005).

In eukaryotic cells, r-proteins are generally encoded by more than one gene. In yeast, the 79 r-proteins are encoded by 138 genes, 59 of which are duplicated (Planta and Mager, 1998; Link et al., 1999). Although both members of a gene family are transcriptionally active, their expression levels are often significantly different (Raue and Planta, 1991). In rat, the average r-protein multi-gene family contains 12 genes, yet unlike yeast, only one gene from each family is usually transcribed; the remainder of the genes are present as inactive pseudogenes (Wool et al., 1995). This is especially apparent in the human genome which, according to a recent study, contains over 2000 r-protein pseudogenes (Zhang et al., 2002). In Arabidopsis, r-proteins are encoded by multi-gene families that contain two to seven members, with an average number of three, that are dispersed throughout the genome (Barakat et al., 2001). However, unlike mammals, more than one member of a gene family is transcriptionally active.

R-protein synthesis, which, to ensure the production of functional ribosomes must be coordinated and balanced, has been shown to be regulated at the transcriptional, post-transcriptional, translational and/or post-translational level depending on the species. In yeast, r-protein synthesis is primarily regulated at the transcriptional level, in response to changing growth conditions, to meet the physiological needs of the cell (reviewed in Planta, 1997). In *Escherichia coli*, r-protein genes are not dispersed throughout the genome but are organized into operons. When sufficient amounts of r-proteins have been produced, certain regulatory r-proteins will bind to their own polycistronic mRNA and inhibit translation of the open reading frames (ORFs) present in that operon (Nomura et al., 1980). Although translational regulation of r-protein synthesis has also been shown to occur in vertebrates, it is not through a negative feedback mechanism as r-protein genes are dispersed throughout the genome. Instead, transcripts encoding components of the translational machinery, including r-proteins,

contain a TOP (*t*erminal *o*ligo*py*rimidine) motif within the 5' untranslated region (UTR) that aids in their selective translation (Mariottini and Amaldi, 1990; Levy et al., 1991). In addition, the 5′ regulatory regions (RRs) of mammalian r-protein genes contain evolutionarily conserved features including a TATA box or A/T-rich motif and binding sites for the GABP (GA-Binding Protein) and YY1 (Yin Yang 1) transcription factors that may contribute to coordinated regulation at the transcriptional level (Perry, 2005).

In plants, as in other eukaryotes, the rate of protein synthesis is a determining factor of cellular growth (Ohnish et al., 1990). As components of the translational machinery, increases in cytosolic r-protein gene expression have been shown during periods of growth and development in a variety of plant species, including rice (*Oryza sativa*, Jain et al., 2004), maize (*Zea maize*, Lebrun and Freyssinet, 1991; Larkin et al., 1991), canola (*Brassica napus*, Bonham-Smith et al., 1992), Arabidopsis (McIntosh and Bonham-Smith, 2005; Hulm et al., 2005), tobacco (*Nicotiana tabaccum*, Marty and Meyer, 1992; Dai et al., 1996; Gao et al., 1994), pea (*Pisum sativum*, Moran, 2000), peach (*Prunus persica*, Giannino et al., 2000), petunia (*Petunia hybrida*, Lee et al., 1999) and potato (*Solanum tuberosum*, Taylor et al., 1992). Increased transcript abundance has also been reported following mechanical wounding and treatment with plant growth regulators (Gantt and Key, 1983; Crowell et al., 1990; Gao et al., 1994; Cherepneva et al., 2003; Hulm et al., 2005; McIntosh and Bonham-Smith, 2005). Conversely, mutations in several different Arabidopsis r-protein genes result in altered phenotypes, inhibition of growth and delayed development (Van Lijsebettens et al., 1994; Ito et al., 2000; Weijers et al., 2001).

Studies of plant genes encoding components of the translational apparatus have identified several conserved regulatory *cis*-elements within the 5′ RR. First identified in the Arabidopsis *eukaryotic elongation factor 1A* (*eEF1A*) gene, 174 of 216 Arabidopsis r-protein genes have subsequently been found to contain one or more plant *INTERSTITIAL TELOMERE MOTIFS* (*TELO* box, $^{5'}$AAACCCTA$^{3'}$) (Trémousaygue et al., 2003). However, the *TELO* box must act in synergy with other *cis*-elements, such as the *TEF* box (*TRANSLATION ELONGATION FACTOR 1 BOX*; $^{5'}$ARGGRYANNNNNGT$^{3'}$) or *PROLIFERATING CELLULAR NUCLEAR ANTIGEN* (*PCNA*) Site II motif ($^{5'}$TGGGCC/T$^{3'}$), to regulate gene expression in the cycling cells

of Arabidopsis root primordia (Regad et al., 1995; Trémousaygue et al., 1999; Manevski et al., 2000). In Arabidopsis, 153 r-protein genes contain both a *TELO* box and a Site II motif which occur in a conserved topological association (Trémousaygue et al., 2003). However, unlike *TELO* boxes, Site II motifs were found to be both necessary and sufficient to activate reporter gene expression in root primordia and young leaves (Trémousaygue et al., 2003). Also identified in rice r-protein genes, Site II motifs may be conserved elements that act to coordinate the expression of genes up-regulated in cycling cells (Trémousaygue et al., 2003).

We have previously reported differences in transcript abundance among the cytosolic *RPS15a* gene family of Arabidopsis (Chapter 2; Hulm et al., 2005) and, in this chapter, present a comprehensive analysis of the *RPS15aA*, *-D* and *-F 5′* RRs. In addition to *cis*-regulatory elements, the importance of gene organization including multiple sites of transcription initiation and the presence of leader introns are also discussed.

3.2. Materials and methods

3.2.1. Plant material and cultivation

Arabidopsis thaliana ecotype Columbia-0 was used in all experiments. Seed to be grown on culture plates was vapor-phase sterilized overnight (18-20 h) (Clough and Bent, 1998). Seedlings used for 5' RACE (Rapid Amplification of cDNA Ends) and transgenic selection were grown on ½ Murashige and Skoog (MS) medium (Murashige and Skoog, 1965; *Phyto*Technology Laboratories, Shawnee Mission, KS) containing 15 g L^{-1} sucrose and 6 g L^{-1} Phytagar (Invitrogen, Carlsbad, CA). Five week old plants used for wild type tissue collection were grown in soil. All plants were grown at 23°/18°C, 16 h/8 h photoperiod, 50 µmol photons m^{-2} s^{-1}. Tissues used for RNA extraction were snap frozen in liquid nitrogen immediately following collection.

3.2.2. RNA isolation and 5' RACE

Total RNA was isolated from buds and leaves of five week old plants (50-100 mg frozen tissue per sample) using the RNeasy Plant Mini kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Poly$(A)^+$ RNA was isolated from total RNA

samples using the PolyAT Tract mRNA Isolation System (Promega, Madison, WI) according to manufacturer's instructions; 60-100 μg of total RNA was incubated with 50 U DNaseI (Amersham Biosciences, Piscataway, NJ) for 10 min at 37°C prior to poly(A)$^+$ RNA isolation. Both total and poly(A)$^+$ RNA were used for transcription start site mapping.

Mapping of *RPS15aA*, *-D* and *-F* transcription start sites was performed using a 5' RACE kit (Invitrogen, Carlsbad, CA) according to manufacturer's instructions with the exception of using *Pfu* polymerase (Fermentas, Hanover, MD) in the final nested PCR. Both the PCR of dC-tailed cDNA and final nested amplification were performed as follows: 30 cycles of PCR at 94°C (2 min for the first cycle, 30 s for subsequent cycles), 52°C (30 s), 72°C (45 s) and a final 10 min extension at 72°C. All steps were carried out in a PTC-100 thermal cycler (MJ Research, Miami, FL). Nested amplification products were blunt-ligated into pBluscript KS$^+$ (Stratagene, La Jolla, CA) at *Eco*RV using T4 DNA ligase (Fermentas). Sequences of amplified DNA were confirmed via automated sequencing (Plant Biotechnology Institute, National Research Council of Canada, Saskatoon). Primers used for cDNA synthesis and amplification are listed in Table 3.1.

3.2.3. 5' RR deletion constructs and molecular cloning

Constructs were generated containing each of the *RPS15aA, -D* or *-F* 5′ RRs (defined as the sequence ~1 Kb immediately upstream of the longest mapped transcription start site) or one of a series of consecutive 5′ RR deletion fragments ligated upstream of the *uidA* (*β-glucuronidase*, *GUS*) reporter gene. All PCRs used *Pfu* polymerase (Fermentas), all ligations used T4 DNA ligase (Fermentas), and all restriction endonucleases were obtained from Invitrogen or Fermentas.

RPS15aA (At1g07770) 5′ RR fragments were PCR amplified from BAC F24B9 (Arabidopsis Biological Resource Centre, ABRC, Ohio; GenBank accession no. AC007583); *RPS15aD* (At3g46040) 5′ RR fragments were amplified from genomic DNA isolated from Arabidopsis using the E.Z.N.A. Plant DNA Miniprep kit (Omega Bio-Tek, Doraville, GA) according to manufacturer's instructions; *RPS15aF* (At5g59850) 5′ RR fragments were amplified from BAC MMN10 (ABRC; GenBank

Table 3.1. Oligonucleotide primers used for 5' RACE. AAP, Invitrogen 5' RACE Abridged Anchor Primer (forward primer); AUAP, Invitrogen 5' RACE Abridged Universal Amplification Primer (forward primer). All GSPs (Gene-Specific Primers) are reverse primers. GSP1, used for first strand cDNA synthesis; GSP2, used to amplify dC-tailed cDNA with AAP; GSP3, used for nested amplification with AUAP. I, inosine.

Gene name	Primer name	Oligo sequence (5' - 3')
RPS15aA, *-D* and *–F*	AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG
	AUAP	GGCCACGCGTCGACTAGTAC
RPS15aA	AGSP1	GAAGCCGAGAACCTTGCC
	AGSP2	GCCAGCAGATGTAGTCAGC
	AGSP3	GGCCTGATCATGACCTGCC
RPS15aD	DGSP1	CCAAGAACTTTGCCACCAAC
	DGSP2	CCATGATTCCCGCTGAGGTCG
	DGSP3	GGCCTGATGATAACCTGCC
RPS15aF	FGSP1	CGGAATCCTCTTCTCACC
	FGSP2	CCATAATACCAGCCGAGG
	FGSP3	GGCCTGATCATGACTTGCC

accession no. AB015475). Primers used for 5′ RR fragment amplifications are listed in Table 3.2. Amplified 5′ RR fragments were digested with *Eco*RI and *Bam*HI, cleaned using the QIAquick PCR purification kit (Qiagen) according to manufacturer's instructions and ligated into pCAMBIA1381Z (Cambia, Canberra, AUS) at $^{5'}$*Eco*RI-*Bam*HI$^{3'}$. 5′ RR fragment sequences were confirmed via automated sequencing (PBI, NRC). *Argobacterium tumefaciens* strain LBA4404 carrying the pAL4404 *vir*-containing plasmid (Hoekema et al., 1983) was used as the host for all constructs.

3.2.4. Plant infiltration and transgenic selection

Transgenic Arabidopsis plants were generated using a modified floral dip method (Clough and Bent, 1998). Plants were grown in pots covered with cheesecloth to reduce soil spillage during infiltration. Plants were infiltrated at approximately five weeks post-germination following the production of secondary bolts. Infiltration media was prepared by resuspending *A. tumefaciens* to an OD_{600} of 0.8-1.0 in a 5% sucrose, 0.01% Silwet L-77 solution. Pots of plants were inverted, submerged in infiltration medium and subjected to a 70-100 kPa (~25 mmHg) vacuum for 2 min. Following infiltration, plants were covered by a clear, vented bag for 3-4 days after which the tops of the bags were cut. After 3-4 days the bags were removed and plants allowed to set seed and dry down.

T_1 seed was collected from dried down T_0 (infiltrated) plants. Vapor-phase sterilized T_1 seed was plated on ½ MS medium containing 25 μg ml^{-1} hygromycin (InvivoGen, San Diego, CA) for selection of seedlings containing the T-DNA insert and 200 μg ml^{-1} cefotaxime (Aventis, Quebec, Canada) for suppression of residual bacterial (*Agrobacterium*) growth. Untransformed seedlings turned brown and died at the cotyledon stage while those positive for the T-DNA insert remained green and were transferred to soil at approximately the four leaf stage. Mature T_1 plants were allowed to set seed and dry down.

Table 3.2. Oligonucleotide primers used for amplification of 5' RR fragments. ΔR primers were used as reverse primers for all fragments. NP, not produced.

Gene name	Primer name	Construct	Oligo sequence (5' - 3')	Length of amplified fragment (bp)
RPS15aA	S15aAΔR	All	GCGGGATCCAAGGTGAGCTAGGG	N/A
	S15aAΔ0	Δ0	GCGGAATTCCAATTCAAGTTGCCTTCC	1011
	S15aAΔ1	Δ1	GCGGAATTCCGAAAAGGCTTTACACC	511
	S15aAΔ2	Δ2	GCGGAATTCGAGTTTTGGATCTGCC	401
	S15aAΔ3	Δ3	GCGGAATTCCTCGAGGCTTTTAG	308
	S15aAΔ4	Δ4	GCGGAATTCGCTCAAATTAGATCTGACG	201
	S15aAΔ5	Δ5	GCGGAATTCGGCCTGAAATGAAGC	100
RPS15aD	S15aDΔR	All	GCGGGATCCGACGGAACTATTTTTAGG	N/A
	S15aDΔ0	Δ0	GCGGAATTCGTCAACAACAACCATC	NP
	S15aDΔ1	Δ1	GCGGAATTCCTTCACACGAAAAAAG	514
	S15aDΔ2	Δ2	GCGGAATTCGACCATGAATTAGATAC	404
	S15aDΔ3	Δ3	GCGGAATTCCGTTGGCAGTGAACGGC	310
	S15aDΔ4	Δ4	GCGGAATTCGGGCTTAATACCTAAAT	211
	S15aDΔ5	Δ5	GCGGAATTCGTATCAATAATGGGCC	102
RPS15aF	S15aFΔR	All	GCGGGATCCAAGATGCGAATTAGGG	N/A
	S15aFΔ0	Δ0	GCGGAATTCGAATTCTCAAGG	979
	S15aFΔ1	Δ1	GCGGAATTCCCTTCCCATTCAAAGC	541
	S15aFΔ2	Δ2	GCGGAATTCGCTATAAGACAGTAGC	434
	S15aFΔ3	Δ3	GCGGAATTCGCCTCAGTGATTGATC	NP
	S15aFΔ4	Δ4	GCGGAATTCGCATACTGCTGACG	222
	S15aFΔ5	Δ5	GCGGAATTCCGAGATATTAAGAAAGGC	111

3.2.5. Histochemical GUS *staining*

Histochemical GUS assays, as modified from Sieburth and Meyerowitz (1997), were performed on 11-14 day old seedlings and a variety of mature plant tissues [root, stem, leaf, bud (no visable petals), flower and silique]. Tissues were placed in microcentrifuge tubes, fixed in 90% acetone on ice for 15-20 min and rinsed in a solution of 50 mM $NaPO_4$ (pH 7.2), 0.5 mM $K_3Fe(CN)_6$ and 0.5 mM $K_4Fe(CN)_6$ for 10 min. X-Gluc (5-bromo-4-chloro-3-indoyl β-D-glucuronide; Rose Scientific, Alberta, Canada) staining solution [50 mM $NaPO_4$ (pH 7.2), 0.5 mM $K_3Fe(CN)_6$ and 0.5 mM $K_4Fe(CN)_6$, 2 mM X-Gluc] was added to the samples after rinsing. Tissues were vacuum infiltrated for 2 min at 70-100 kPa and incubated in the dark at 37ºC overnight. Chlorophyll was removed from the tissues by processing through an ethanol series [30%, 50% (50% ethanol, 5% acetic acid, 3.7% formaldehyde), 70%, 85%, 95% and 100%] for a minimum of 1 h at each stage. GUS activity was scored on the basis of X-Gluc staining visualized under a stereomicroscope (Wild MZ3, Wild Heerbrugg).

3.3. Results

3.3.1. RPS15aA, -D and -F transcription start sites and leader intron splicing

Comparisons between genomic sequence and that of the 5' ends of the *RPS15a* cDNAs indicated that the *RPS15aA*, *-D* and *-F* genes each contain an intron upstream of their respective open reading frame (Figures 3.1, 3.2 and 3.3; Table 3.3). The majority (7/10) of *RPS15aA* clones contained a 390 bp leader intron located 23 bp upstream of the ATG start codon and flanked by canonical $^{5'}$GT-AG$^{3'}$ splice sites. However, an alternate $^{3'}$AG splice site was identified in clones derived only from 5' RACE of bud tissue, increasing the size of the leader intron to 393 bp. Comparison of a *RPS15aA* cDNA from Genbank (accession no. AF360284) with the *RPS15aA* genomic sequence showed leader intron splicing at the $^{5'}$GT-AG$^{3'}$ sites found in the majority of 5' RACE clones. *RPS15aD* has a 232 bp leader intron located 18 bp upstream of the ORF flanked by canonical $^{5'}$GT-AG$^{3'}$ splice sites while in *RPS15aF* a 421 bp leader intron is situated 17 bp from the ATG start codon and is flanked by non-canonical $^{5'}$GT-CA$^{3'}$ splice sites. When compared to their respective genomic sequences, the leader intron splice sites of

A) *RPS15aA*

..tataaaaagcgataagaaaaccctagctcaccttctcttcctctaattgcttttctccgtcactgagta

ccttgcctctcgagctcccaatcttaaggtacaattgatcttcactatagtcgttcagaatctgatctgcttat

agaaatattcagaagtgtgagatagctgctaattggtctttactttgtttttttctcttgcttcacatcagaaa

atttaggatcttagagcttttcatagtgaacttaagaacgcttttcgaattagtcagtcttagctctattttgt

ctgtgtgtgtattttctggacacaagacctttgtttcaatttctataagtgatttcggtttacattttaaaagc

ttgatttattcccattgaaataaatattcatgatgagtaaatctgtgcatttgtatcaaatttttagtgctttg

cagattttattttaacgcaagctcttgtttgttgtttgacttgtttagtagaggattgagcttaaggcaag**ATG**

GTAAGAATCAGTGTTCTTAACGATGCTCTCAAGAGCATGTACAATGCTGAGAAACGAGGCAA....

B)

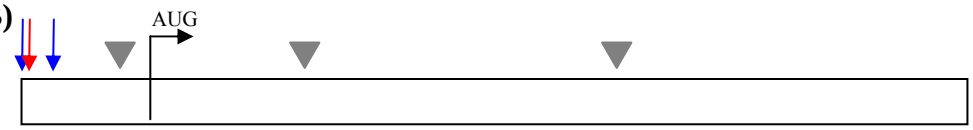


Figure 3.1. A) *RPS15aA* transcription initiation and leader intron splice sites mapped to genomic sequence. **UPPERCASE** - ORF; grey shading - leader intron; **red** arrow - transcription start site determined via 5' RACE in bud and mature leaf tissue; **blue** arrows - transcription start sites determined via 5' RACE in bud tissue; **black** diamonds - $^{5'}$GT-AG$^{3'}$ splice sites identified in leaf and bud; **purple** diamond - alternative splice site identified in bud; **orange** - putative TATA box. B) Schematic representation of *RPS15aA* transcripts. ORF is indicted by the region proceeding the right-angle arrow; **grey** triangles - spliced introns; **red** and **blue** arrows as in A).

A) *RPS15aD*

...aagcgataaaagcgaaaaccctaaaaatagttccgtcttctgtttaatctcttttcaaggccaagcgagc

aagagacgtcggagcggcggctactacttaggtttcaggttagtaatcgccggaatctccatctctcctctaat

ccgatgaactgctttattgttttgttggaacaccggcttagttaatctccccaatttgatgtttctccagagaa

tctagattccaatgtcattttcagagtgtgtaatttttagggaatcgaattcgttccctctgtagatgttttta

tgtacatgtcaataagattcgtgaagctgttgatttatgtttttgcagttttgaaattttgaagaATGGTGAG

AATCAGTGTGCTCAATGATGGTCTTAAGAGTATGTACAATGCAGAGAAGAGAGGAAAGAGGCA..........

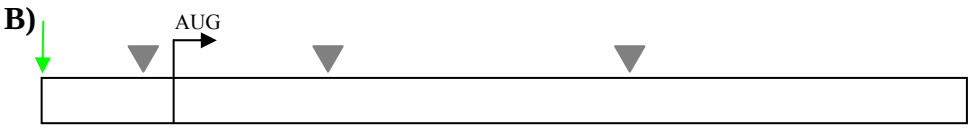


Figure 3.2. A) *RPS15aD* transcription initiation and leader intron splice sites mapped to genomic sequence. **UPPERCASE** - ORF; grey shading - leader intron; **green** arrow - transcription start site determined via 5' RACE in bud and mature leaf tissue; **black** diamonds - $^{5'}$GT-AG$^{3'}$ splice sites identified in leaf and bud; **orange** - putative TATA box. B) Schematic representation of *RPS15aD* transcripts. ORF is indicted by the region proceeding the right-angle arrow; **grey** triangles - spliced introns; **green** arrow as in A).

A) *RPS15aF*

…aataaaaagcaaaaaccctaattcgcatcttgtatttagttctcttgttaaactttgcctcttcgtcttttgc

tccagagtttttgtgcggctgccattttcgatttccaaaggttagagagatctccacagctacgagctttattc

ctgtttccttttctaggatctgctttcatttctgctcaatcatgacgtgaaatctgcttttaaaattctctgaaa

attctcgtagtttcatagtttgagctcgatgctatcgattaacattagtttcttggaccagaatacaattagat

ctatcgaaagtttgcttatagttctatgtttcgttgattgccatttggttattgttgtattcatttcaatatta

gaatcaagttcgttgattgtcgatttagctgccactatagttgtgcatttgaagcatgaatcctcttttaaca

caaaagagactaaatttgattagtcaatgtttattggtgttagcattagtacgtttggtatcggctcagatttg

ttgttaatttggtatcagttttgagctttgaaaa**ATGGTGAGAATCAGTGTGCTTAACGATGCTCTTAAGAGTA**

TGTACAATGCTGAGAAGAGAGGGAAGAGGCA…

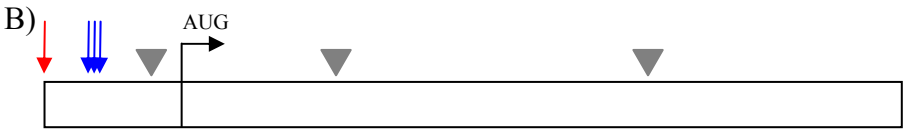


Figure 3.3. A) *RPS15aF* transcription initiation and leader intron splice sites mapped to genomic sequence. **UPPERCASE** - ORF; grey shading - leader intron; **red** arrow - transcription start site determined via 5' RACE in mature leaf tissue; **blue** arrows - transcription start sites determined via 5' RACE in bud tissue; **pink** diamonds - $^{5'}$GT-CA$^{3'}$ splice sites identified in leaf and bud; **orange** - putative TATA box. B) Schematic representation of *RPS15aF* transcripts. ORF is indicted by the region proceeding the right-angle arrow; **grey** triangles - spliced introns; **red** and **blue** arrows as in A).

Table 3.3. *RPS15aA*, *-D* and *-F* gene organization as determined via 5′ RACE.

Gene	Number of identified transcription start sites	Length of leader intron (bp)	Leader intron splice sites	Length of 1st exon (bp)	Length of transcribed fragment 5′ to ATG & 3′of leader introns (bp)	Total length of 5′ UTR (bp)
RPS15aA	Leaf - 1 Bud - 3	390-393	5′ GT - AG 3′	53-69	20-23	76-89
RPS15aD	Leaf & Bud - 1	232	5′ GT - AG 3′	73	18	91
RPS15aF	Leaf - 1 Bud - 3	421	5′ GT - CA 3′	42-82	17	59-99

RPS15aD and *-F* cDNAs from Genbank (accession nos. AY045837 and AY035157) were identical to those identified in their respective 5' RACE fragments.

A single transcription start site, initiating at a cytosine located 66 bp upstream of the leader intron, was identified both in leaf and bud for *RPS15aA* (Figure 3.1; Table 3.3). This site was present in the majority (8/10) of 5' RACE fragments and corresponds with that of the Genbank clone (AF360284). Two alternative start sites, located 53 bp and 69 bp upstream of the leader intron and initiating at a cytosine and an adenine respectively, were also identified in bud. Due to multiple sites of transcription initiation and the presence of an alternate 3' leader intron splice site, the 5' UTR present in processed *RPS15aA* transcripts ranges from 76 bp to 89 bp in length. A single transcription start site (identified in 4/4 fragments), initiating at a cytosine 73 bp upstream of the leader intron and conserved between leaf and bud, was identified for *RPS15aD* (Figure 3.2; Table 3.3). The *RPS15aD* 5' UTR is 91 bp in length. Transcription initiation of *RPS15aF* in leaf occurs at a guanine residue (1/5 fragments) located 82 bp upstream of the leader intron (Figure 3.3; Table 3.3) and corresponds with a Genbank cDNA clone (AY035157). In bud, *RPS15aF* transcription start sites were identified 42 bp (1/5 fragments), 45 bp (1/5 fragments) and 49 bp (2/5 fragments) upstream of the leader intron initiating at guanine (42 bp and 49 bp) and thymine (45 bp) residues. As a result of multiple transcription start sites the *RPS15aF* 5' UTR ranges from 59 bp to 99 bp in length. No transcript is produced from *RPS15aC* therefore no transcription start site could be mapped.

3.3.2. Sequence analysis of the RPS15aA, -D and -F 5´ regulatory regions

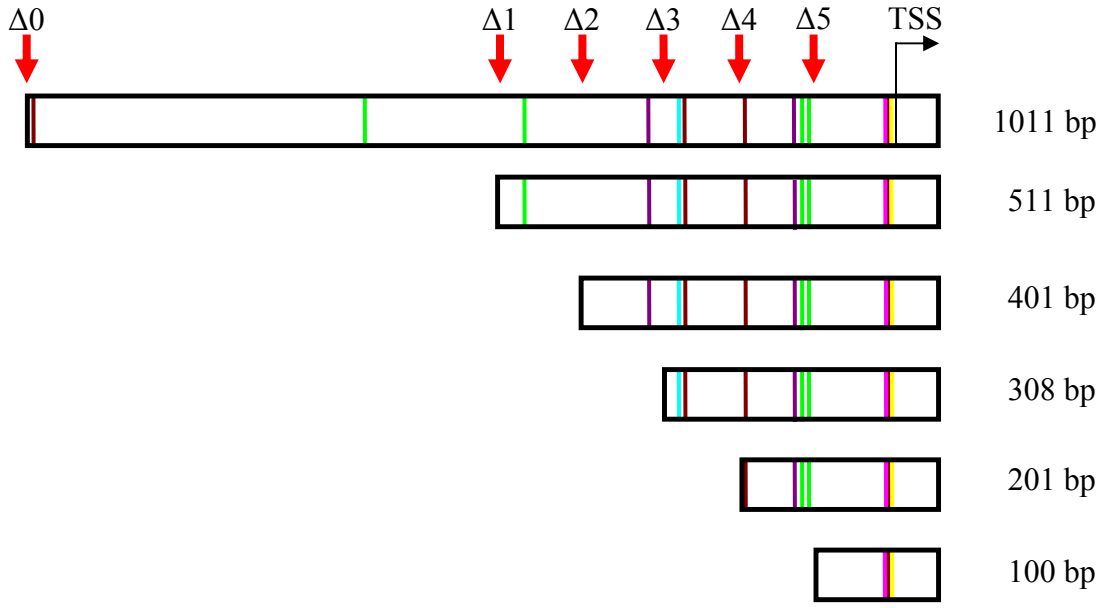
The plant *cis*-acting regulatory DNA elements (PLACE) (http:www.dna.affrc.go.jp/htdocs/PLACE; Higo et al., 1999) database and the FUZZNUC nucleic acid pattern search program from the European Molecular Biology Laboratory-Bioinformatics Institute (Rice et al., 2000) were used to identify putative regulatory motifs in the region 1011, 1022 and 979 bp upstream of the transcription start sites, determined in leaf, for *RPS15aA*, *-D* and *-F* respectively (Figures 3.4, 3.5 and 3.6). Putative TATA boxes are located 34, 30 and 31 bp upstream of the *RPS15aA*, *-D*

Figure 3.4. *RPS15aA* deletion series. A) *RPS15aA* 5' RR upstream of the transcription start site. **Bold, underlined text** - deletion series primers, primer names are listed in the in the right hand margin (Δ0 - Δ5, forward primers; ΔR, reverse primer); *TELO* box; TATA box; *TEF* box; *PCNA* Site II motifs; Late pollen gene g10-related element; Pollen-specific activation element; **black** diamond - transcription start site. B) Schematic of *RPS15aA* 5′ RR deletion series constructs. **Red** arrows - 5' end of each fragment; right angle arrow - transcription start site; *TELO* box; TATA box; *TEF* box; *PCNA* Site II motifs; Late pollen gene g10-related element; Pollen-specific activation element; fragment sizes (bp) are listed in the right hand margin.

A) *RPS15aA*

caattcaagttgccttccgtcagaagcagaaatgggtggctcttagctgggggaatcaatccaac Δ0
aaatgtttcagaagctctttctatccttcaacctgatggaattgatgttagtagcggtatttgcg
gtacagacggtatccagaaggataagtctaagataagctcctttataactgcagttcgctctgta
cactactaatggcaagcaatataaaccacggtaatttatcttgtaactatagttttgagcatcaa
cctgttgtttccaatccatgtcattaagttttgaattgtaactggctgataataataataatgcc
ctttttttcatcagtagtagatttaaattaaaagcgttgggttgtcaccgtaataatgtcgttg
ggctctcaagaacaagatccttgtatgaaatgtattctactgatcatatattatttcatttgatc
gagagttttaccacaggtttctacaaattaatgctttttaatct**cgaaaaggctttacacc**aaa Δ1
acaaaaagaacataatgaatgggccaagatgaatttatgtaacttgtctgcctggttcactgcag
tatgtcctcatgttctttgtccatt**gagttttggatctgcc**tcatagtacacgacatctcctgta Δ2
tcgcttacgaaatggtcttgtttcgcattgcttaaatggtgataataggttta**ctcgaggctttt** Δ3
agattttatagagaaggaacaaacgtattctattggatagaaatcttcacgaggatcattgacgt
gtatatttctcattcgttaaatttatcaat**gctcaaattagatctgacg**gctgagagaaacatca Δ4
ttcatttttacaggttcaagttatagctttgataggcttcaatggtcgtgacaaagaagcccatt
gggcctgaaatgaagcttgagacgcagcgcataacacatgtcaattggtttctttgattttccct Δ5
agtataaaaagcgataagaaaa**ccctagctcaccttc**tcttcttcctctattgcttttctcc... ΔR

B)



A) *RPS15aD*

tcttctctgccggcttcttctctgcctttggtgccatttt**cttcacacgaaaaaag**tttcaaaga Δ1

gattctgaattttagaagatggagtcgtgagatcaagttttgtgtttgttgtttagacgattggt

agctcatgtatatatat**gaccatgaattagatac**agattggttaattctcgtttcacgaggttga Δ2

tgacgtggaatatattacgtaccgttagattagaataaat**ccgtcgttggcagtgaacggca**agc Δ3

atctctcacctttttaaatgggccattaagcagcgaatcaaaattaatttcgagttttacttta

acgggctaaaat**gggcttaatacctaaat**aataatctgttagtcctagacgtgaatcattaagag Δ4

aatgccatcaatgttgatacaaagtccatgtttggttacaaatgggcctaattat**gttcaataat** Δ5

gggcctagttatgtatttataatgggcttagttatacatgaatctgtcccgtgaaagcgataaaa

gcgaaaac**ctaaaaatagttccgt**cttctgtttaatctcttttcaaggccaagcgagc... ΔR

B)

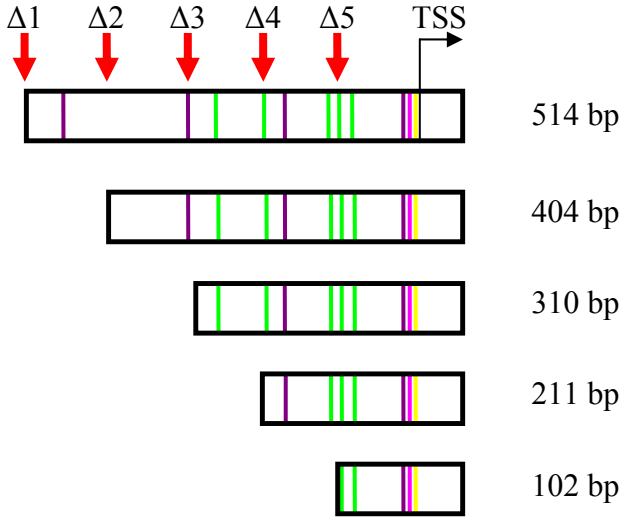


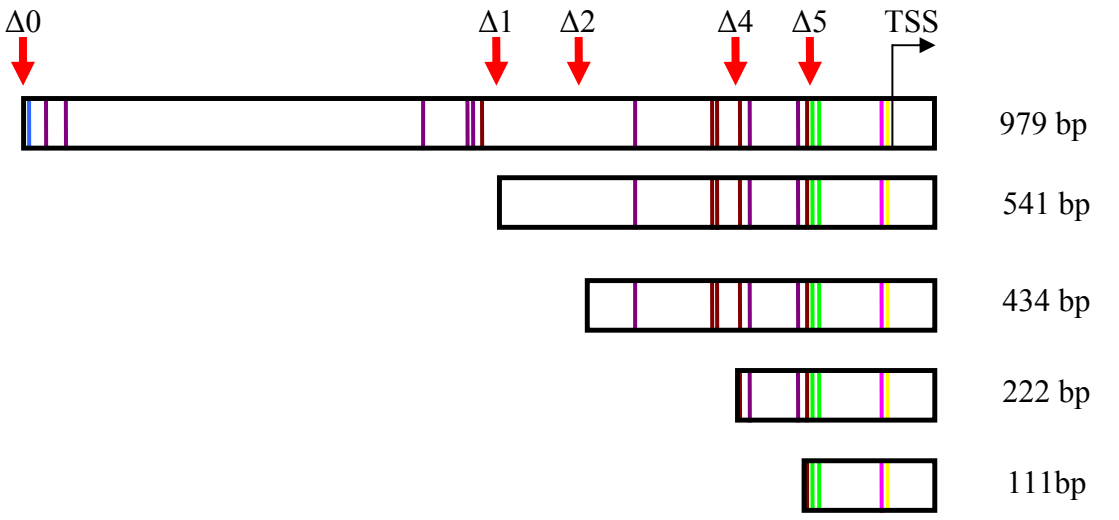
Figure 3.5. *RPS15aD* deletion series. A) *RPS15aD* 5' RR upstream of the transcription start site. **Bold, underlined text** – deletion series primers, primer names are listed in the in the right hand margin (Δ1 - Δ5, forward primers; ΔR, reverse primer); *TELO* box; TATA box; *PCNA* Site II motifs; Pollen-specific activation element; **black** diamond - transcription start site. B) Schematic of *RPS15aD* 5′ RR deletion series constructs. **Red** arrows - 5' end of each fragment; right angle arrow indicates the transcription start site; *TELO* box; TATA box; *PCNA* Site II motifs; Pollen-specific activation element; fragment sizes (bp) are listed in the right hand margin.

Figure 3.6. *RPS15aF* deletion series. A) *RPS15aF* 5' RR upstream of the transcription start site. **Bold, underlined text** - deletion series primers, primer names are listed in the in the right hand margin (Δ0 - Δ2, Δ4 and Δ5 forward primers; ΔR, reverse primer); *TELO* box; TATA box; *TEF* box; *PCNA* Site II motifs; Late pollen gene g10-related element; Pollen-specific activation element; **black** diamond - TSS. B) Schematic of *RPS15aF 5´* RR deletion series constructs. **Red** arrows - 5' end of each fragment; right angle arrow indicates the transcription start site; *TELO* box; TATA box; *TEF* box; *PCNA* Site II motifs; Late pollen gene g10-related element; Pollen-specific activation element; fragment sizes (bp) are listed in the right hand margin.

A) *RPS15aF*

tttataccagaatccgaaatgattcttctgattctc**gaattctcaagg**taattggtacttcagat Δ0
ctctcaggaaatgcatttacttttagagttgtgacagtgtagaactctagaatgatttggtgtgaa
gagtctttagttgttgtgtaaggacagtgtgtgtgccctcttcaatccttgttacttttcagact
tgttgttggtgcttctacttcctttgcagtttccacaaatattagtctgccattaacaatctgaa
aacaaacaaccaaatgcaaagctcagtataatttccaaaagagctcaattaattacacaccaatg
ctgaatattttccttacttttttccgtgaatcgattacgattatgccaaacaagcgattttctgat
ttaagattcattttgtttcaatcaaagccaaagatacatactacatttcaagagtgagtgaagat
tgaagaaaatggatcttca**ccttcccattcaaagc**tttcaaagcctttttgtgcatcatcctcagac Δ1
tcaaaagttatgaatccgaaacctttaggtctctgcgtttgctggtccttaataagacga**gctat** Δ2
aagacagtagcatacaaacgacacaattagttcagacacataatcatagccgtaaaacattcgat
tctgtaatctgaccaaaaggcgcaaacagttgcctcagtgattgatctgtggtgtaagcagagag
tcctgcaagagaacagtttgagaataaaaattcaaaaactgagaaaagcagaaaaactggagatg
tgcaaagttgta**gcatactgctgacg**aaagaaagaaacctgaatttgaaacacaagtgagagtct Δ4
tacctccaattaaacgacggcgcttggatatagacgatctaggctgtgataatgttaa**cgagata** Δ5
ttagaaa**ggc**ccaaaataaagcccattataatgatccattaaccagtctagtaaagtttcataa
ggcgcccc**aataaaaa**gcaaaaacc**ctaattcgcatctt**gtatttagttctcttgttaaact... ΔR

B)



and -*F* transcription start sites respectively. Based on TATA box quality criteria established by Perry (2005) the *RPS15aA* (TATAaAAA) and -*F* (aATAaAAA) TATA boxes comprise high affinity TATA-box binding protein (TBP) sites while that of -*D* (TAaAagcg) is a low affinity site. The TATA box position (-30 to -34 bp) is consistent with the average mean distance of -32 ± 7 bp in plants (Joshi, 1987). The *RPS15a* 5' RRs contain several regulatory motifs present in genes that encode components of the translational apparatus. These include the *TELO* box (Trémousaygue et al., 2003), *PCNA* Site II motif (Manevski et al., 2000; Trémousaygue et al., 1999) and the *TEF* box (Regad et al., 1995). In addition, the *RPS15a* 5' RRs also contain a late pollen gene g10-related element ($^{5'}$GTGA$^{3'}$; Rogers et al., 2001) and/or a pollen-specific activation element ($^{5'}$AGAAA$^{3'}$; Bate and Twell, 1998).

3.3.3. GUS *activity of RPS15a deletion series constructs*

3.3.3.1. Serial deletion constructs and transgenic plants

A series of consecutive 5' deletions of the full length regulatory regions (defined as ~1 Kb upstream of the transcription start site mapped for each *RPS15a* gene in leaf) were generated (Figures 3.4, 3.5 and 3.6). The *RPS15aA* series consisted of six constructs, *AΔ0* through *AΔ5* containing fragments of 1011, 511, 401, 308, 201 and 100 bp respectively. The *RPS15aD* series consisted of five constructs, *DΔ1* through *DΔ5,* containing fragments of 514, 404, 310, 211 and 102 bp respectively. The *RPS15aF* series also consisted of five constructs, *FΔ0*, *FΔ1, FΔ2*, *FΔ4* and *FΔ5,* containing fragments of 979, 541, 434, 222 and 111 bp respectively.

3.3.3.2. GUS expression patterns in Arabidopsis seedlings

To determine the region(s) of the 5' RR required for *RPS15a* gene expression, GUS activity was initially characterized in 11-14 day old Arabidopsis seedlings containing one of the *RPS15aA*, *-D* or *-F* deletion series constructs. Two to ten independent T_2 lines were analyzed per construct and were scored for the presence or absence of GUS activity (Figure 3.7). All untransformed wild type seedlings and those transformed with empty pCAMBIA1381Z were negative for GUS activity. Strong staining for GUS activity was detected in *RPS15aAΔ0* through *AΔ4* seedlings however

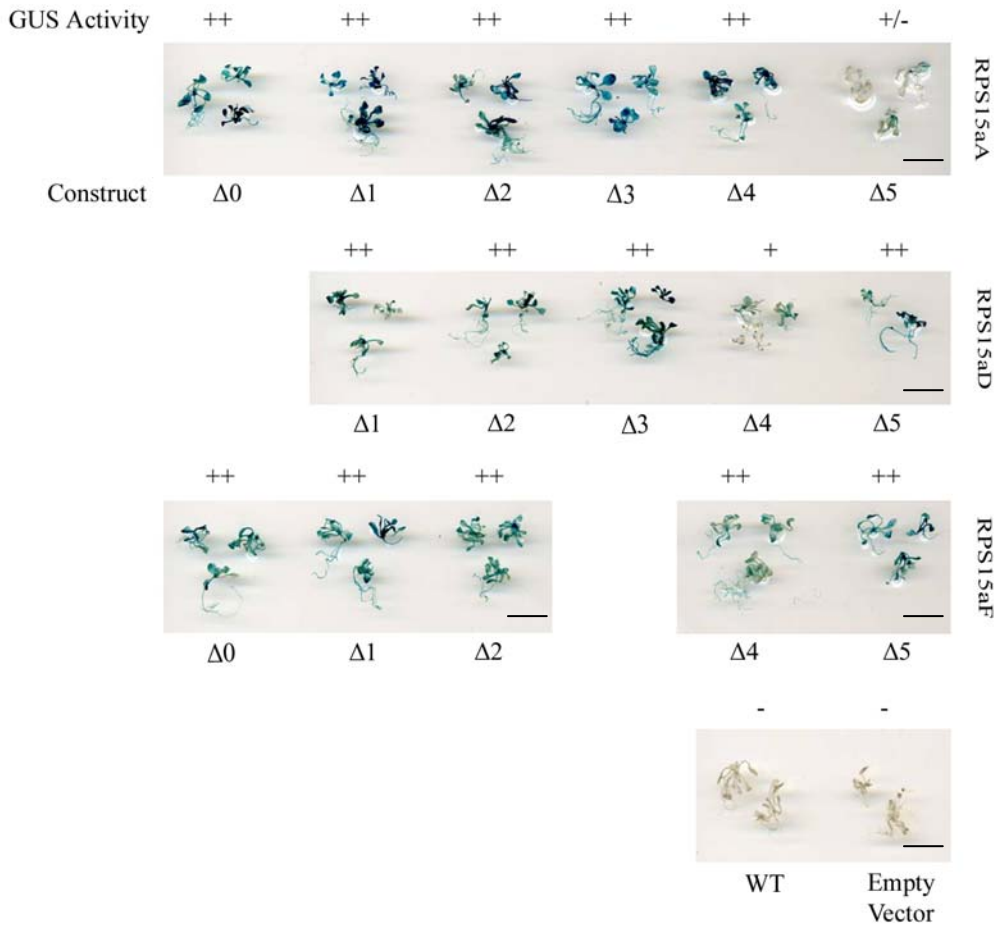


Figure 3.7. GUS activity in 11-14 day old wild type (WT), empty vector, *RPS15aA*, *-D* and *-F* 5' RR deletion series Arabidopsis T_2 seedlings. Seedlings shown are representative of staining patterns of all independent lines sampled for each construct. ++, positive for GUS activity, most or all of the seedling is darkly stained; +, weak positive for GUS activity, only part of the seedling is stained/stain is light; -, negative for GUS activity. Scale bars = 0.5 cm.

staining was reduced or absent in the *AΔ5* seedlings (containing a fragment 100 bp upstream of the transcription start site). Weak staining was observed in seedlings containing *RPS15aDΔ4* fragment (211 bp upstream of the TSS) while *DΔ1, DΔ2, DΔ3* and *DΔ5* transgenics showed strong staining. Seedlings of the entire *RPS15aF* deletion series (*FΔ0, FΔ1, FΔ2*, *FΔ4* and *FΔ5*) stained positive for GUS activity.

3.3.3.3. GUS expression in mature plant tissues

In order to determine specific *RPS15a* expression patterns and, identify the *cis*-elements involved in regulating *RPS15a* transcription, individual, mature Arabidopsis tissues were scored for GUS activity by the presence or absence of staining. For the majority of constructs, three 11-14 day old seedlings from two to five independent T_2 lines that showed consistent GUS staining patterns, were grown to maturity (~six weeks) in soil. Rosette leaves, stem, closed/immature buds, open flowers, root, elongating carpels/siliques <6 mm, siliques 6-10 mm, siliques >10 mm and mature/yellow silques were collected and analyzed for GUS activity. Tissues collected from untransformed wild type plants or transgenics containing the empty pCAMBIA1381Z vector were all negative for GUS activity.

GUS staining patterns in vegetative and floral tissues for each of the *RPS15aA*, *-D* and *-F* deletion series constructs are summarized in Tables 3.4 and 3.5. Although each construct of a particular deletion series did not necessarily direct GUS expression, some general trends did appear. Developing/mitotically active tissues such as lateral root primordia, root tips (Figure 3.8) and elongating carpel/silique (Figure 3.9) were positive for GUS activity. GUS staining was observed in tissues of the stamen including the anther, filament and pollen as well as in the style and ovary of the pistil (Figure 3.10). The vasculature of a variety of tissues including leaves, roots, sepals, petals and filaments (Figures 3.8, 3.10 and 3.11) stained positive for GUS activity as did cut or inadvertent wounding sites (Figure 3.8).

Although the general GUS staining patterns among *RPS15aA*, *-D* and *-F* were similar, there were some interesting differences. While vegetative tissues (leaf, stem and root) containing the *AΔ5* fragment (100 bp upstream of the TSS) were positive for GUS activity, all floral tissues of the same construct, with the exception of weak staining in

Table 3.4. GUS activity in vegetative tissues of wild type, empty vector, *RPS15aA*, *-D* and *-F* 5' RR T_2 plants. ++, positive for GUS activity, most or all tissues in the sample are stained/stain is dark; +, weak positive for GUS activity, only some of the tissues in the sample are stained/stain is light; -, negative for GUS activity. Superscripts indicate specific regions of staining: c, cut sites/ends of stem; e, ends/margins of leaves; v, venation.

Construct	Leaf	Stem	Root
RPS15aA Δ0	$++^{c,v}$	$++^{c,e}$	+
Δ1	$++^{v}$	$+^{c}$	++
Δ2	$+^{c,v}$	$++^{c}$	++
Δ3	$++^{c,v}$	$++^{c,e}$	++
Δ4	$+^{c,e,v}$	$++^{c}$	++
Δ5	+/++	$+^{c}$	++
RPS15aD Δ1	-	-	-
Δ2	$+^{c,e,v}$	-	+
Δ3	$+^{c,e,v}$	$+^{c}/++^{c}$	++
Δ4	-	-	-
Δ5	$+^{c,e}$	$+^{c}$	+
RPS15aF Δ0	$+^{e,v}$	-	+
Δ1	$+^{c,v}$	$+^{c}$	+
Δ2	$+^{e,v}$	$++^{c,e}$	++
Δ4	$+^{v}/++^{v}$	$++^{c,e}$	++
Δ5	$+^{c,e,v}$	$+^{c}$	++
Wild type	-	-	-
Empty vector	-	-	-

Table 3.5. GUS activity in floral tissues of wild type, empty vector, *RPS15aA*, *-D* and *-F* 5' RR T_2 plants. ++, positive for GUS activity, most or all tissues in the sample are stained/stain is dark; +, weak positive for GUS activity, only some of the tissues in the sample are stained/stain is light; -, negative for GUS activity. WT, wild type; EV, empty vector; Sep, sepal; Pet, petal; Stig, stigma; Sty, style; Ov, ovary. Superscripts indicate specific regions of staining: e, end of ovary/silique; p, pollen; r, receptacle; s, seed; sp, stigmatic papillae; st, style; t, tapetum; v, venation.

Construct	Unopened Buds							Open Flowers							Siliques			
	Stamen					Pistil		Stamen					Pistil					
	Sep.	Pet.	Anth.	Fil't	Stig.	Sty.	Ov.	Sep.	Pet.	Anth	Fil't	Stig.	Sty.	Ov.	<6mm	6-10mm	>10mm	Mat.
WT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EV	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AΔ0	+^{p}	-	-	-	+	++	+^{r}	++^{p}	+^{p}	+^{p}/ ++^{p}	++^{p}	-/+	++	++ ^{e,r}	++^{e}	++^{e}	+^{e}	++st
AΔ1	+^{p}	-	-	-	-	++	+^{r}	++^{p}	+^{p}	+^{p}	++^{p}	-	++	+^{r}	+^{e}	+^{e}/ ++^{e}	+^{e,s}	+st
AΔ2	-	-	-	-	-	++	-	+^{p}	-	+^{p}/ ++^{p}	+^{e,p}	-	++	+^{e,r}	+^{e,s}	+^{e}	+^{e,s}	+^{e,sp,st}
AΔ3	+^{p}+^{p}	+^{p}	-	+^{p}	++	++	++^{e,r}	++^{p}	+^{p}	++^{p}	++^{p}	++	++	+^{e,r}	++^{e}	++^{e}	+^{e}	+^{e,st}
AΔ4	-/+^{p}	-	+^{p}	-	++	++	+^{r}	++^{p}	+^{p}	++^{p}	++^{p}	-	++	+^{r}	+^{e,s}	+^{e}	-/+^{e}	-/+^{e,st}
AΔ5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+^{e,st}
DΔ1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DΔ2	-	-	-	-	-	-	-	-	-	+^{p}	-	-	++	+^{e,r,s}	-	-	-	+^{e,sp,st}
DΔ3	+^{p}	-	+^{t}	-	++	++	++^{e,r}	+^{p}	-	+^{p}	+^{e,p}	-	++	+^{e,r,s}	++^{e}	+^{e}	+^{e,sp}	+^{e,sp,st}
DΔ4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DΔ5	+^{p}/+ +^{p}	-	+^{t}	-	-	++	+^{r}	+^{p}	-	+^{p}	+^{e,p}	-	+	+^{r}	+^{e,st}	+^{e}	+^{e}	+^{e,st}
FΔ0	-	-	-	-	-	-	-	+^{p}	-	-	-	-	-	-	-	-	-	-
FΔ1	-	-	-	-	-	-	-	+^{p}	-	+^{p}	+^{e,p}	-	+	+^{r}	+^{e}	-	-	+^{sp,st}
FΔ2	+^{p}	-	+^{t,p}	-	-	+	-/++^{r}	+^{p}	+^{p}	++^{p}	++^{e,p}	-	++	+^{e,r}/ ++^{e,r}	+^{e}	+^{e}	-	+^{e,sp,st}
FΔ4	+^{p}	-	+^{t,p}		++	++	++^{r}	+^{p}	+^{p}	+^{p}	++^{p}	-	++	++^{e,r}	+^{e}	+^{e}	-	+^{sp,st}
FΔ5	+^{p}	-	+^{t}	-	-	-	+^{r}	+^{p}	-	+^{p}	+^{p}	-	++	+^{e,r}	+^{e,s}	+^{e}	-	+^{sp,st}

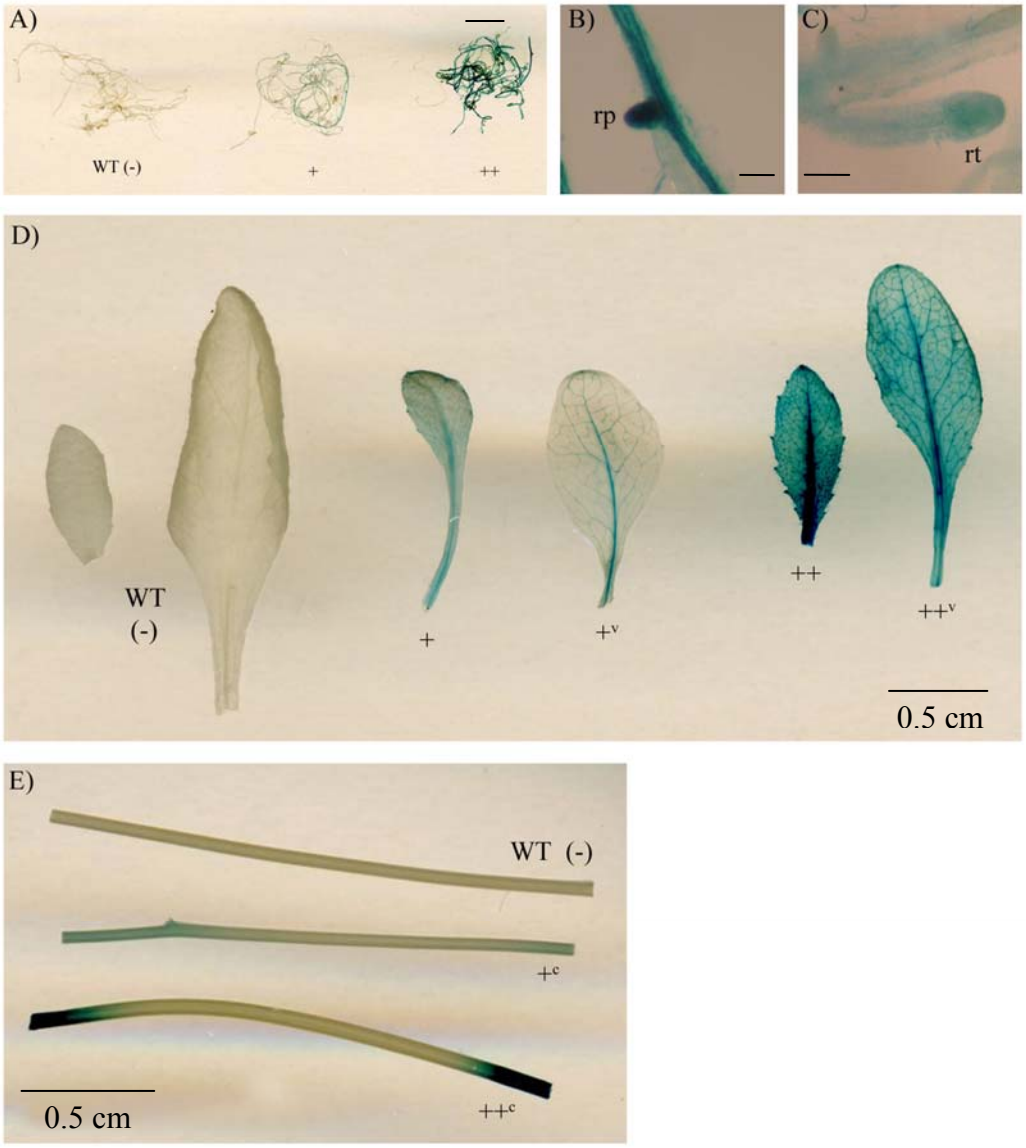


Figure 3.8. GUS activity in wild type (WT) and representative samples of *RPS15aA*, *-D* and *-F* vegetative tissues from T_2 plants. ++, positive for GUS activity; +, weak positive for GUS activity; -, negative for GUS activity. Superscripts indicate region of concentrated staining where applicable: c, cut sites/ends of stem; v, venation. A) Portions of WT and *RPS15a* T_2 roots. Scale bar = 0.5 cm. B) Portion of root showing lateral root primordium (rp). Scale bar = 10 µm. C) Portion of root showing the root tip (rt). Scale bar = 10 µm. D) Selection of WT and *RPS15a* T_2 leaves. E) Stem sections from WT and *RPS15a* T_2 plants.

Figure 3.9. GUS activity in wild type (WT) and representative samples of *RPS15aA*, *-D* and *-F* T_2 siliques. ++, positive for GUS activity, most or all of the silique is stained/stain is dark; +, weak positive for GUS activity, only part of the silique is stained/stain is weak; -, negative for GUS activity. Superscripts indicate regions of concentrated staining where applicable: e, end(s) of the silique; st, stigma.

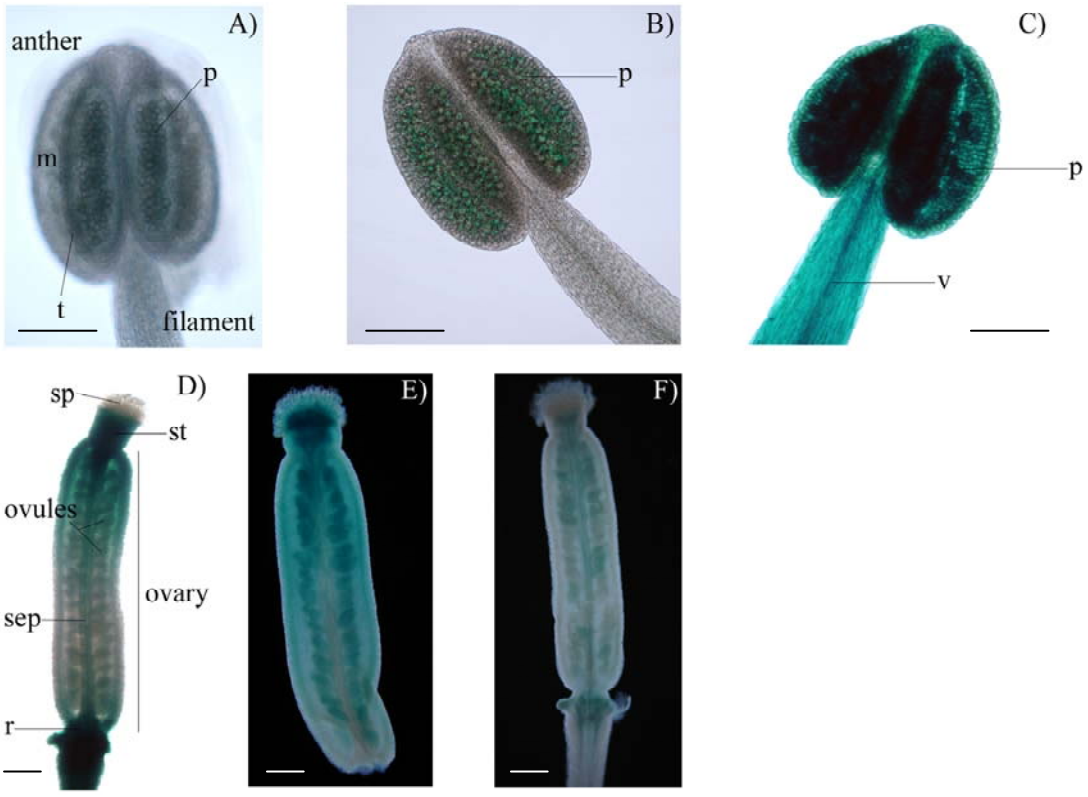


Figure 3.10. GUS activity in representative *RPS15aA*, *-D* and *-F* 5' RR T_2 plants. A-C) Stamens; m, microsporangium; p, pollen; t, tapetum; v, vascular strand. Scale bars = 0.1 mm. D-F) Carpels with all other floral organs removed; r, receptacle; sep, septum; sp, stigmatic papillae; st, style. Scale bars = 0.2 mm.

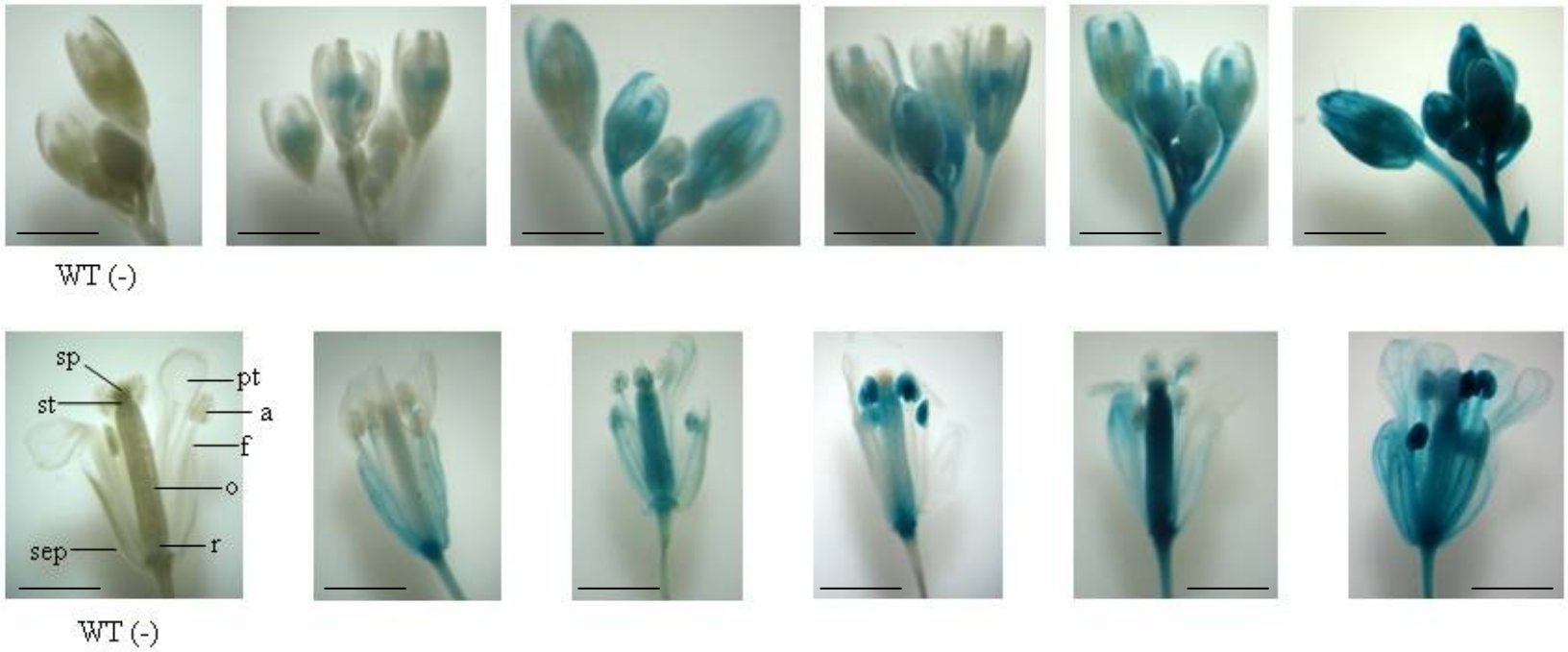


Figure 3.11. GUS activity in wild type (WT) and representative samples of *RPS15aA*, *-D* and *-F* unopened buds and flowers from T_2 plants. Some organs have been removed from flowers to aid viewing; a, anther; f, filament; o, ovary; pt, petal; r, receptacle; sep, sepal; sp, stigmatic papillae; st, style. Scale bars = 0.1 cm.

mature siliques and the ovary of open flowers, were negative. However, both *DΔ5* and *FΔ5* (102 and 111 bp respectively) were sufficient for GUS expression in all vegetative and the majority of floral tissues. It should be noted that GUS staining in dry, mature siliques may be an artifact due to shattering during collection and processing. Staining was also detected in the vegetative tissues of *AΔ1* and *FΔ1* plants (511 and 541 bp respectively) but not in the *DΔ1* (514 bp) transgenics. The majority of floral tissues and all developmental stages of silique containing *AΔ0* (1011 bp), *AΔ1, AΔ2* (401 bp), *AΔ3* (308 bp), or *AΔ4* (201 bp) were positive for GUS activity while those containing *DΔ1* or *DΔ4* (211 bp) were all negative. In *DΔ2* (404 bp) plants, staining was limited to mature siliques and the style, ovary and pollen of open flowers. Similarly, only weak GUS activity was observed in the sepals of *FΔ0* (979 bp) open flowers and was completely absent in *FΔ1* buds.

3.3.3.3.1. GUS *activity within the RPS15aA deletion series*

Comparisons of GUS activity within each individual *RPS15a* deletion series were made to determine the minimal 5′ RR and *cis*-regulatory elements required for expression. Leaf, stem and root tissue of the entire *RPS15aA* deletion series were positive for GUS activity and showed only minor differences in staining intensity (Table 3.4). *AΔ0* and *AΔ1* plants showed similar staining patterns in bud, flower and all stages of developing silique including the reduction/absence of GUS activity in bud compared to flower (Table 3.5). GUS activity was lost in the sepal and ovary of *AΔ2* buds and flower petals but was recovered in those of *AΔ3* plants. All *AΔ3* flower organs, siliques and bud tissues, except for the anther, were positive for GUS activity. Within the *RPS15aA* deletion series, staining of the filament and pollen of the unopened bud was only observed in *AΔ3* plants. The staining pattern of *AΔ4* reproductive and floral organs was similar to that of *AΔ3* however GUS activity was lost in the petal and filament of the bud but observed in pollen. All *AΔ5* siliques, bud and flower organs were negative for GUS activity.

3.3.3.3.2. GUS *activity within the RPS15aD deletion series*

In the *RPS15aD* deletion series the longest, *DΔ1* 5′ RR fragment did not direct GUS expression in any tissue, vegetative or reproductive, sampled (Tables 3.4 and 3.5). Although limited staining was observed in the leaves, roots, flower and mature silique of *DΔ2* plants, the majority of tissues were GUS negative. The *DΔ3* deletion fragment was able to confer GUS activity in leaf, stem, root, all stages of silique and the majority of bud and floral organs however staining was lost in all *DΔ4* plant tissues. GUS activity was recovered in *DΔ5* transgenics and the staining patterns were similar to those of the *DΔ3* plants.

3.3.3.3.3. GUS *activity within the RPS15aF deletion series*

Unlike *RPS15aAΔ0,* all samples containing the *RPS15aFΔ0* deletion fragment were negative for GUS activity with the exception of weak staining in leaf, root and the vasculature of floral sepals (Tables 3.4 and 3.5). While no GUS staining was observed in *FΔ1* buds, staining was observed in the stem, parts of the floral stamen and pistil as well as <6 mm and mature siliques. An increase in staining intensity was seen in the stem and root of *FΔ2* plants as well as the anther, filament, style and ovary of the flower. Parts of the *FΔ2* bud, including sepals, anther, pollen, style and ovary, were also positive for GUS activity. *FΔ4* and *FΔ5* samples showed staining patterns that were similar to those of *FΔ2*, however, GUS activity was only present in pistil tissues of *FΔ4* buds. Interestingly, GUS activity was absent from the petals and filaments of bud, the stigma of the flower, and siliques > 10 mm for all constructs of the deletion series.

3.4. Discussion

In order to conduct an investigation into the mechanisms regulating transcription of the cytosolic *RPS15a* genes from Arabidopsis it was necessary to first map the transcription start sites using 5' RACE. Results showed a single start site for *RPS15aA* and *-F* in leaf while in bud, multiple sites of initiation were mapped for each gene (Figures 3.1 and 3.3). In contrast, a single start site, conserved between leaf and bud was identified for *RPS15aD* (Figure 3.2). Unlike transcription of mammalian r-protein genes, which is usually initiated at a C residue located within a polypyrimidine tract

(Yoshihama et al., 2002), transcription of *RPS15aA* begins at a C or A nucleotide, *-D* at C and *-F* at G or T. Although *RPS15aA* and *-F* transcripts may initiate at more than one nucleotide, there appears to be a preference for the most common sites beginning with C (*RPS15aA*) or G (*RPS15aF*). These results differ from those of McIntosh and Bonham-Smith (2005) which showed that although transcription of *RPL23aA* and *RPL23aB* could be initiated at A, T, C or G residues, *-A* transcripts most commonly began with A and those of *-B* with T. It is interesting to note that *RPS15aA* and *-F* transcripts isolated from bud showed differences in their 5' ends, suggesting that start site selection varies among tissues.

R-protein genes often contain an intron located near the 5' end of the coding region or within the leader sequence (Spingola et al., 1999; Grossman, 2005; Perry, 2005). Similarly, *RPS15aA*, *-D* and *-F* all contain a leader intron upstream of the ATG start codon (Figures 3.1, 3.2 and 3.3). The majority of *RPS15aA* clones contained a 390 bp leader intron, flanked by canonical $^{5'}$GT-AG$^{3'}$ splice sites, 23 bp upstream of the ATG start codon. However, two clones, isolated from bud tissue, contained an alternate $^{3'}$AG splice site that increased the length on the leader intron to 393 bp. This alternate, uncommon splice site may be used during tissue specific processing of *RPS15aA* transcripts or results from mis-splicing given the close proximity to the more common site. However, because the leader intron separates two portions of the 5' UTR in mature transcripts, the reading frame is not affected by alternate splice site utilization. *RPS15aD* contains a 232 bp leader intron flanked by canonical $^{5'}$GT-AG$^{3'}$ splice sites located 18 bp upstream of the ORF. The largest, 421 bp intron, is located 17 bp upstream of the *RPS15aF* start codon. Unlike *RPS15aA* and *-D*, the *-F* leader intron is flanked by a non-canonical $^{5'}$GT-CA$^{3'}$ splice site. Only 0.7% of all splice sites in Arabidopsis genes are non-canonical (Alexandrov et al., 2006) and the effect of a $^{3'}$CA site on *RPS15aF* transcript processing remains to be determined.

A survey of Arabidopsis r-protein genes has indicated that ~21%, dispersed among 25 different gene families, contain a leader intron. The average length of the leader intron is 278 bp, however leader introns in SSU genes average 389 bp in length while those of the LSU are 213 bp. A survey of rice r-protein genes with known

homology to those of Arabidopsis has shown that ~17%, dispersed among 17 gene families, contain a leader intron. Rice locus Os02g27760 is orthologous to *RPS15aA*, *-D* and *-F* and contains a leader intron of 1203 bp. The average length of the leader intron is ~450 bp which is consistent with data indicating that rice introns are ~1.5 times longer than those of Arabidopsis (Alexandrov et al., 2006). However, among human r-protein genes, in which only one member of a gene family is transcriptionally active, ~43% of genes, dispersed among 35 families, contain a leader intron. In addition, unlike the majority of Arabidopsis or rice r-protein genes, human leader intron length may vary due to the use of alternate 5' and/or 3' splice sites. This is the situation with *RPS15a* which contains a leader intron of 1191 or 1125 bp due to the presence of alternate splice sites. The importance of a leader intron on the spatial and temporal expression of potato *Sucrose Synthase* (Fu et al., 1995a; Fu et al., 1995b), maize *Shrunken-1* (Maas et a., 1991; Clancy et al., 1994) and carnation *S-adenosylmethionine decarboxylase* (Kim et al., 2004) gene expression has been shown. However, further experimentation is required to determine the effect, if any, of the leader intron on *RPS15a* expression in Arabidopsis.

Plant 5' UTRs are typically <100 nt in length (Bailey-Serres, 1999). The 5' UTRs of *RPS15a* transcripts vary in size from 91 bp in *RPS15aD* to, due to multiple sites of transcription initiation and/or use of alternate splice sites, 76-89 bp in *RPS15aA* and 59-99 bp in *RPS15aF* (Figures 3.1, 3.2 and 3.3). Alexandrov et al. (2006) has suggested that 5' UTR length is important as, in Arabidopsis, average transcript levels decrease when 5' UTRs are <60 nucleotides long. 5' UTRs are also known to play a role in the translational regulation of r-proteins. Although plant r-protein mRNAs do not contain the pyrimidine tract responsible for translational regulation of vertebrate r-protein transcripts (Mariottini and Amaldi, 1990; Levy et al., 1991), recognition of this sequence by the plant translational apparatus has been shown *in vitro* (Shama and Meyuhas, 1996). While translational regulation of gene expression may require elements in the coding region, 5' and/or 3' UTRs, the 5' UTR is sufficient for translational enhancement of maize *Hsp70* in heat-shocked protoplasts (Pitto et al., 1992) and tomato *lat52*, which is preferentially transcribed during pollen maturation and encodes an essential cysteine-rich protein (Bate et al., 1996). Further

experimentation is required to determine if the 5' UTRs of Arabidopsis r-protein mRNAs, including those of *RPS15aA, -D and -F*, are involved in the translational regulation of their expression.

Following identification of the *RPS15a* transcription start sites, the region(s) of the 5' RR and some of the *cis*-elements required for gene expression were determined by characterizing GUS activity in Arabidopsis seedlings and a variety of individual, mature plant tissues (Tables 3.4 and 3.5). We have previously shown that in Arabidopsis, expression patterns of *RPS15aA*, *-D* and *-F* are similar, with the highest levels of transcript abundance detected in mitotically active tissues such as flower and bud (Hulm et al., 2005). Although differences in reporter gene expression were observed both within each individual *RPS15a* deletion series and among the three genes, results of the present study also indicate a general trend for developing/mitotically active tissues, such as floral reproductive organs, to stain for GUS activity. These results concur with those of previous studies of r-protein gene expression (*S14*, Larkin et al., 1989; *S11*, Lebrun and Freyssinet, 1991; *B. napus S15a*, Bonham-Smith et al., 1992; *L2*, Marty and Meyer, 1992; *S19* & *L7*, Taylor et al., 1992; *L25* & *L34*, Gao et al., 1994; *L34*, Dai et al., 1996; *L15* & *L27a*, Lee et al., 1999; *S28*, Giannino et al., 2000; *L9*, Moran, 2000; *L13*, Jain et al., 2004 and *L23a*, McIntosh and Bonham-Smith, 2005). High GUS activity observed in lateral root primordia and root tips (Figure 3.8A and B) corresponds to relatively high levels of *RPS15a* transcript abundance detected in Arabidopsis roots (Chapter 2; Hulm et al., 2005). In addition, the pattern of *RPS15a* expression is similar to that of *RPL16A* (*RPL11C* in the nomenclature of Barakat et al., 2001) which is associated with cell division and elongation in roots (Williams and Sussex, 1995).

Although it was previously reported that mechanical wounding of Arabidopsis rosette leaves caused no significant changes in *RPS15aA, -D or -F* transcript abundance (Hulm et al., 2005), in the present study wound sites, particularly leaf and stem cut sites (Figure 3.8D and E), were positive for GUS activity. Similar results have been reported by McIntosh and Bonham-Smith (2005) for *RPL23aA* and *-B*. These results also agree with an approximate three-fold increase in *RPL25* transcript levels in tobacco leaves 5 h after wounding (Gao et al., 1994). The restriction of GUS staining to the cut ends of the

stem represents an immediate increase in *RPS15a* expression during tissue collection before samples were fixed in acetone and may account for the relatively high levels of *RPS15aA* and *-D* transcript abundance (Hulm et al., 2005).

Among the *RPS15a* deletion series constructs, GUS staining was observed in the anther, filament and pollen during flower development. In closed buds, staining, when present, was restricted to developing pollen and the tapetal layer of the anther (Figure 3.10). The tapetum is involved in the nutrition of developing pollen grains and is responsible for the synthesis of all major classes of compounds including proteins (reviewed in Mascarenhas et al., 1990). Following anthesis, increased GUS activity was observed in the filament and all anther tissues; an increase in staining was also observed in developing pollen where, at this time, ribosomes accumulate in preparation for pollen tube growth following germination (Figure 3.10) (reviewed in Mascarenhas et al., 1990). A similar pattern of expression was described by Williams and Sussex (1991) for *RPL16A*. Sequence analysis of the *RPS15aA, -D and -F* 5' RRs has identified several previously described pollen specific *cis*-elements which may be responsible for the spatial and temporal regulation of *RPS15a* gene expression in the anther. Analysis of reporter gene expression following deletion or mutation of the various pollen specific elements would further delineate their regulatory roles.

While the *RPS15aA*Δ*5* 5' RR fragment was capable of directing reporter gene expression in vegetative tissues and mature siliques, GUS activity was reduced or eliminated in seedlings, buds and open flowers (Figure 3.7; Table 3.5). The *A*Δ*5* fragment contains a putative, high affinity TATA box ($^{5'}$TATAaAAA$^{3'}$) at -34 bp from the TSS and a *TELO* box ($^{5'}$AAACCCTA$^{3'}$) at -17 bp. However, two *PCNA* Site II motifs ($^{5'}$TGGGCT$^{3'}$, -109 bp; $^{5'}$TGGGCC$^{3'}$, -102 bp from the TSS) present in the *A*Δ*4* fragment are eliminated in the *A*Δ*5* fragment (Figure 3.4). Although it is unclear why GUS activity is retained in *A*Δ*5* roots, leaves and stems (Table 3.4), the lack of GUS activity in the majority of tissues suggests that the Site II elements are necessary for optimal gene expression. Supporting this hypothesis are results indicating that the *D*Δ*5* and *F*Δ*5* fragments, which each contain a *TELO* box and two Site II elements, are sufficient to direct reporter gene expression (Figures 3.5 and 3.6).

RPS15a 5' RRs contain many putative *cis*-regulatory elements that activate gene expression in response to developmental or environmental stimuli yet equally important are elements that inhibit expression. Within the *RPS15aA* series of deletion constructs, bud tissues containing the *AΔ0*, *AΔ1* or *AΔ2* fragments showed reduced GUS staining compared to open flowers (Table 3.5). However, recovery of GUS activity in the majority of *AΔ3* and *AΔ4* bud tissues suggests that there may be one or more *cis*-elements, located between -308 and -401 bp, inhibiting *RPS15aA* expression in buds. Unlike the GUS staining patterns observed in tissues containing the *AΔ1* or *FΔ1* deletion fragments, GUS activity was absent in *DΔ1* mature plant tissues (Tables 3.4 and 3.5). While weak expression was recovered in *DΔ2* leaves, data from mature siliques and mitotically active tissues including pollen, style and ovary, again suggest the presence of inhibitory elements located between -514 and -310 bp from the TSS. Increased staining was observed in *DΔ3* plants, however GUS activity was lost in all *DΔ4* tissues. Interestingly, the *DΔ3* and *DΔ5* staining patterns were similar.

Unlike the strong staining patterns observed in *RPS15aAΔ0* and *AΔ1* plants, GUS staining of *RPS15aFΔ0* and *FΔ1* tissues was weak and present only in vegetative tissues and those of the open flower (Tables 3.4 and 3.5). Although staining in individual tissues was reduced or absent, GUS activity in seedlings containing the *FΔ0, FΔ1, DΔ1* or *DΔ2* 5′ RR fragments was relatively strong (Figure 3.7). *RPS15aFΔ2, FΔ4* and *FΔ5* plants showed GUS activity in a wider range of tissues then the *FΔ0* or *FΔ1* plants. These results may reflect a need for increased numbers of ribosomes in the mitotically active seedling and the presence of negative, tissue specific regulatory elements between -979 and -434 bp. However, sequence analyses of the *RPS15a* 5' RRs using the PLACE and Plant Cis-Acting Regulatory Element (PlantCARE) (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/; Lescot et al., 2002) databases has not identified any known negative regulatory elements.

In this chapter we have demonstrated the importance of transcriptional regulation with respect to the expression of *RPS15aA*, *-D* and *-F* in Arabidopsis. However, this work has also highlighted the complexity of regulating, and coordinating r-protein synthesis and the potential for additional post-transcriptional and translational levels of regulation.

CHAPTER 4: NUCLEOLAR LOCALIZATION OF RPS15aA AND RPS15aD IN TOBACCO LEAF EPIDERMAL CELLS

The Arabidopsis *RPS15a* gene family consists of six members that are divided into two evolutionarily distinct clades. RPS15aA, -C, -D and -F are Type I proteins that are components of the cytosolic ribosome while RPS15aB and -E, Type II proteins, have been putatively associated with mitochondrial ribosomes (Adams et al., 2002; Chang et al., 2005; Carroll et al., 2008). We have previously shown that while *RPS15aA*, *-D* and *-F* are transcriptionally active, transcript abundance among genes differs in wild type, untreated tissues and following various abiotic stresses (Chapter 2; Hulm et al., 2005). As functional plant ribosomes contain only one copy of each ribosomal protein (r-protein; with the exception of 12 kDa, acidic P-proteins), we speculated that individual RPS15a isoforms may be preferentially incorporated into the ribosomes of different tissues, during different developmental stages or under varying environmental conditions. Therefore, a strategy utilizing fluorescent protein tags to visualize RPS15a subcellular location *in planta* was developed. Live cell imaging using a confocal laser scanning microscope (CLSM) showed that transiently expressed RPS15aA and RPS15aD were predominantly localized to the nucleolus and, to a lesser extent, the nucleus of tobacco leaf epidermal cells. Future work employing this technique to characterize the RPS15a nuclear localization signal (NLS) and investigate ribosome heterogeneity is discussed.

4.1. Introduction

Ribosomes are the ribonucleoprotein particles responsible for polypeptide synthesis in all living organisms. Ranging in size from 3-4.5 MDa, the ribosome is the largest enzymatic complex in the cell and is composed of two unequal subunits which associate during translation. Unlike other eukaryotes, plants contain three different types of ribosomes: 70S mitochondrial and 70S chloroplast ribosomes which resemble

those of prokaryotes and the larger, 80S cytosolic ribosome (Bogorad, 1975). The Arabidopsis cytosolic ribosome contains four ribosomal RNA (rRNA) molecules [26S, 5.8S and 5S-large subunit (LSU); 18S-small subunit (SSU)] and 81 ribosomal proteins (r-proteins; 48 LSU, 33 SSU; Barakat et al., 2001; Chang et al., 2005). Arabidopsis r-proteins are encoded by multi-gene families consisting of two to seven members of which more than one gene is transcriptionally active (Barakat et al., 2001). However, with the exception of the acidic P1, P2 and P3 proteins, functional plant ribosomes contain only a single copy of each r-protein, suggesting that multiple r-protein isoforms may be functionally significant.

Variations in rRNA or r-protein composition, post-translational modifications of r-proteins, interactions with extra-ribosomal factors and/or ribosome degradation all contribute to ribosome heterogeneity (Mauro and Edelman, 2002; Chang et al., 2005). Studies in rat have shown that the level of methylated RPL29 varies in liver, brain and thymus tissues (Williamson et al., 1997). Additional post-translational modifications of r-proteins including acetylation, demethionylation, formylation, hydroxylation, or a combination of the above have also been reported in rat and human although their function has yet to be elucidated (Louie et al., 1996; Odintsova et al., 2003). R-protein heterogeneity within ribosomes has been well documented in the cellular slime mold *Dictyostelium discoideum* in which a comparison of ribosomes between vegetative amoebae and germinating spores showed developmentally distinct r-protein composition (Ramagopal and Ennis, 1982). In plants, differences in r-protein composition of ribosomes has been shown in etiolated barley seedlings in which the levels of two r-proteins decreased following illumination while the levels of six r-proteins increased during greening (Koyama et al., 1996). Developmental, environmental and tissue specific heterogeneity of the two RPP2 isoforms has also been described in maize ribosomes (Szick-Miranda and Bailey-Serres, 2001).

Recent studies in Arabidopsis have indicated a high degree of heterogeneity as approximately 26% (Chang et al., 2005) and 45% (Giavalisco et al., 2005) of r-proteins were represented by two or more distinct spots following 2-D polyacrylamide gel electrophoresis. On average, each protein was represented by four forms, suggesting expression of multiple members of a single gene family and/or a degree of post-

translational modification (Giavalisco et al., 2005; Carroll et al., 2008). Post-translational modifications, including phosphorylation of the acidic P proteins (Szick-Miranda and Bailey-Serres, 2001) and RPS6 (Gressner and Wool, 1974; Chang et al., 2005) have been previously identified. The ribosome filter hypothesis, proposed by Mauro and Edelman (2002), suggests that ribosome heterogeneity may modulate mRNA binding interactions by altering affinity for transcripts at specific ribosomal subunit locations.

Ribosome biogenesis is a complex process requiring the synthesis of all rRNA and r-protein components. The uncoupling of transcription and translation in eukaryotes necessitates export of r-protein transcripts from the nucleus to the cytoplasm for translation, import of the resultant polypeptide into the nucleolus, and export of assembled ribosomal subunits to the cytoplasm where they associate with mRNAs to initiate protein synthesis. Subunit assembly is a sequential process as certain r-proteins are required to bind to rRNA earlier than others (Brodersen and Nissen, 2005). Bacterial RPS8, the prokaryotic ortholog of RPS15a, has been shown to be a primary binding protein, able to bind specifically and independently to the central domain of the 16S rRNA (Ungewickell et al., 1975; Mougel et al., 1993). Binding of RPS8 induces conformational changes in rRNA structure and, with the subsequent addition of RPS6, RPS15, RPS11 and RPS18, form the platform of the 30S subunit (Gregory et al., 1984; Svensson et al., 1988; Broderson et al., 2002; Jagannathan and Culver, 2003). In eukaryotes, RPS15a may have a similar role, as a primary binder of the 18S rRNA.

In Arabidopsis, RPS15a is encoded by a six member gene family that, based on a phylogenetic analysis that included 13 eukaryotic S15a, two plastid S8 and two prokaryotic S8 r-proteins, can be divided into two evolutionarily distinct clades (Chang et al., 2005). Type I proteins, RPS15aA, -C, -D and -F, are grouped with RPS15a of rat (*Rattus norvigicus*), *Drosophila melanogaster* and yeast (*Saccharomyces cerevisiae*) RPS22. RPS15aB and -E, Type II proteins, are grouped in a separate clade and have been indirectly associated with mitochondrial ribosomes (Adams et al., 2002; Carroll et al., 2008). Of the four genes encoding cytosolic RPS15a, we have previously shown that *RPS15aC* is not transcriptionally active and that transcript abundance of *RPS15aA*,

-*D* and -*F* differs in wild type, untreated tissues and in response to a variety of abiotic stresses (Chapter 2; Hulm et al., 2005).

In this chapter I assess the feasibility of using fluorescent protein tags to visualize RPS15aA and -D subcellular localization *in planta*. Use of this method as a preliminary indicator of ribosome heterogeneity is also discussed.

4.2. Materials and methods

4.2.1. Fluorescent protein constructs

The fluorescent proteins used in this study were enhanced green fluorescent protein (EGFP; ClonTech, Palo Alto, CA) and monomeric red fluorescent protein (mRFP; Campbell et al., 2002). The spectral properties of EGFP allow for spectral separation from mRFP when covisualized. Standard techniques were used for all molecular cloning (Sambrook et al., 1989). The sequences of cloned products were confirmed via automated sequencing (Plant Biotechnology Institute, National Research Council of Canada, Saskatoon). All gene specific primers (GSPs) used for DNA synthesis and amplification are listed in Table 4.1. All PCR amplifications used *Pfu* polymerase (Fermentas, Hanover, MD), all ligations used T4 DNA ligase (Fermentas) and all restriction endonucleases were obtained from Invitrogen (Carlsbad, CA) or Fermentas.

Clones containing the *RPS15aA* (At1g07770; GenBank accession no. AY081472) or *RPS15aD* (At3g46040; GenBank accession no. AY091373) cDNA were obtained from the Arabidopsis Biological Resource Centre (ABRC, Ohio). *RPS15aA* and -*D* cDNAs were amplified and cloned into pBSKS^{+} at unique $^{5'}$*Eco*RI-*Bam*HI$^{3'}$ restriction sites generating pBSKS^{+}-RPS15aA/-D. The *glutathione S-transferase* (*GST*) linker was PCR amplified from pGEX-4T-3 (GE Healthcare, Piscataway, NJ) and cloned, in frame, with RPS15aA/-D, at $^{5'}$*Bam*HI-*Hind*III$^{3'}$ generating pBSKS^{+}-RPS15aA/-D-GST. mRFP was amplified from pVKH18En6-ST-mRFP (Saint-Jore et al., 2002; Runions et al., 2006) using gene specific primers containing a 3' stop codon and cloned into the $^{5'}$*Hind*III-*Spe*I$^{3'}$ sites of pBSKS^{+} in frame with RPS15aA/-D-GST creating pBSKS^{+}-RPS15aA/-D-GST-mRFP. A tandem repeat of the *Cauliflower Mosaic Virus* 35S promoter (*CaMV* 35S) was PCR amplified from pCAMBIA1381Z

Table 4.1. Oligonucleotide primers used for the amplification of fragments used in molecular cloning.

Gene name	Primer name	Oligo sequence (5' - 3')
RPS15aA (At1g07770)	S15aA-F	GCGGAATTCATGGTAAGAATCAGTGTTC
	S15aA-R	GCGGGATCCATAGAAGAAGCCGAGAACC
RPS15aD (At3g46040)	S15aD-F	GCGGAATTCATGGTGAGAATCAGTGTGC
	S15aD-R	GCGGGATTCGTAAAAGAACCCAAGAAC
GST (U13855)	GST-F	GCGGGATCCATGTCCCCTATACTAGG
	GST-R	GCGAAGCTTACGCGGAACCAGATCCG
mRFP (AF506027)	mRFP-F	GCGAAGCTTATGGCCTCCTCCGAGGACG
	mRFP-R	GCGACTAGTTTAGGCGCCGGTGGAGTGGC
CaMV 35S (AF234306)	35S-F	GCGGGGCCCCCCCAACATGGTGGAGCACG
	35S-R	GCGGAATTCAGAGATAGAATTTGTAGAGAG
Nos terminator	Nos-F	GCGACTAGTCGTTCAAACATTTGGC
	Nos-R	GCGGCGGCCGCCCCGATCTAGTAAC

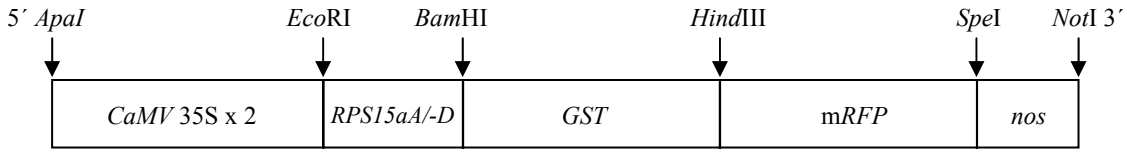
(CAMBIA, Canberra, Australia) and ligated upstream of RPS15aA/-D-GST-mRFP at $^{5'}$*Apa*I-*Eco*RI$^{3'}$. The *nopaline synthase* (*nos)* terminator was PCR amplified from pCAMBIA1381Z and cloned into pGreen0029 at $^{5'}$*Spe*I-*Not*I$^{3'}$. Lastly, the 35S-RPS15aA/-D-GST-mRFP cassette was excised from pBSKS^{+} and cloned into pGreen0029 upstream of the *nos* terminator at $^{5'}$*Apa*I-*Spe*I$^{3'}$, generating the final RPS15aA/-D-GST-mRFP fusion protein cassette under the control of the tandem *CaMV* 35S promoter in pGreen0029 (Figure 4.1A).

To make the nucleolar localized FIBRILLARIN2 (FIB2) control, the FIB2-EGFP fragment from ppk100-FIB2-EGFP (Barneche et al., 2000) was subcloned into pCAMBIA1380 (CAMBIA) at $^{5'}$*Eco*RI-*SpeI*$^{3'}$. A tandem repeat of the *CaMV* 35S promoter was PCR amplified from pCAMBIA1381Z (CAMBIA) and ligated upstream of FIB2-EGFP at $^{5'}$*Apa*I-*Eco*RI$^{3'}$, generating the FIB2-EGFP fusion protein cassette under the control of the tandem *CaMV* 35S promoter in pCAMBIA1380 (Figure 4.1B; R. Degenhardt personal communication).

4.2.2. Transient expression in tobacco

Tobacco (*Nicotiana tabacum* cv Petit Havana) plants used for transient expression were grown in soil in a growth chamber at 23°/18°C with a 16 h light/8 h dark regime and a light intensity of ~170 μmol photons m^{-2} s^{-1}. Electrocompetent *Argobacterium tumefaciens* strain LBA4404 (Hoekema et al., 1983) was transformed with pGreen0029 or pCambia1380 binary vectors containing the 35S::RPS15aA/-D ORF::GST::mRFP and 35S::FIBRILLARIN2::EGFP cassettes respectively (1.5 kV; BTX ECM399 Electroporator, BTX, Holliston, MA). All pGreen constructs were coelectroporated with pSOUP which provides the replication functions, *in trans*, for pGreen (Hellens et al., 2000). Infiltration of tobacco leaves was essentially performed as described by Sparkes et al. (2006). Briefly, *A. tumefaciens* transformed with a fusion protein binary vector was grown overnight at 28°C in LB medium supplemented with 25 μg ml^{-1} kanamycin and 100 μg ml^{-1} streptomycin (pCAMBIA1380) or 50 μg ml^{-1} kanamycin and 150 μg ml^{-1} streptomycin (pGreen0029). Bacterial cultures (1.5 ml) were pelleted by centrifugation at 8000 rpm for 5 min at room temperature, washed once in infiltration medium (IM; 50 mM MES, pH 5.7, 2 mM Na_3PO_4,

A)



B)

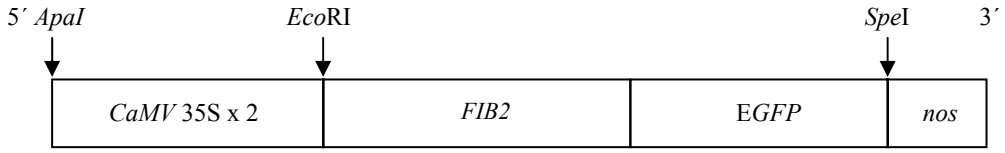


Figure 4.1. A) Fusion protein cassette in the binary vector pGreen0029. *CaMV* 35S x 2, tandem repeat of the *Cauliflower Mosaic Virus* 35S promoter; *RPS15aA/-D*, *RPS15aA* or *-D* cDNA; *GST*, *glutathione S-transferase*; m*RFP*, monomeric *red fluorescent protein*; *nos*, *nopaline synthase* terminator. B) Fusion protein cassette in pCAMBIA1380. *FIB2*, *FIBRILLARIN2* cDNA; E*GFP*, enhanced *green fluorescent protein*.

0.5% glucose [w/v], 100 μM acetosyringone) and resuspended in 1 ml of IM. Cultures were diluted to an OD_{600} of 0.2-0.4 in IM and injected into the lower leaf epidermis of three to six week old tobacco plants using a syringe without a needle. Following infiltration, plants were incubated under normal growth conditions for 48-72 h prior to analysis.

4.2.3. Confocal microscopy

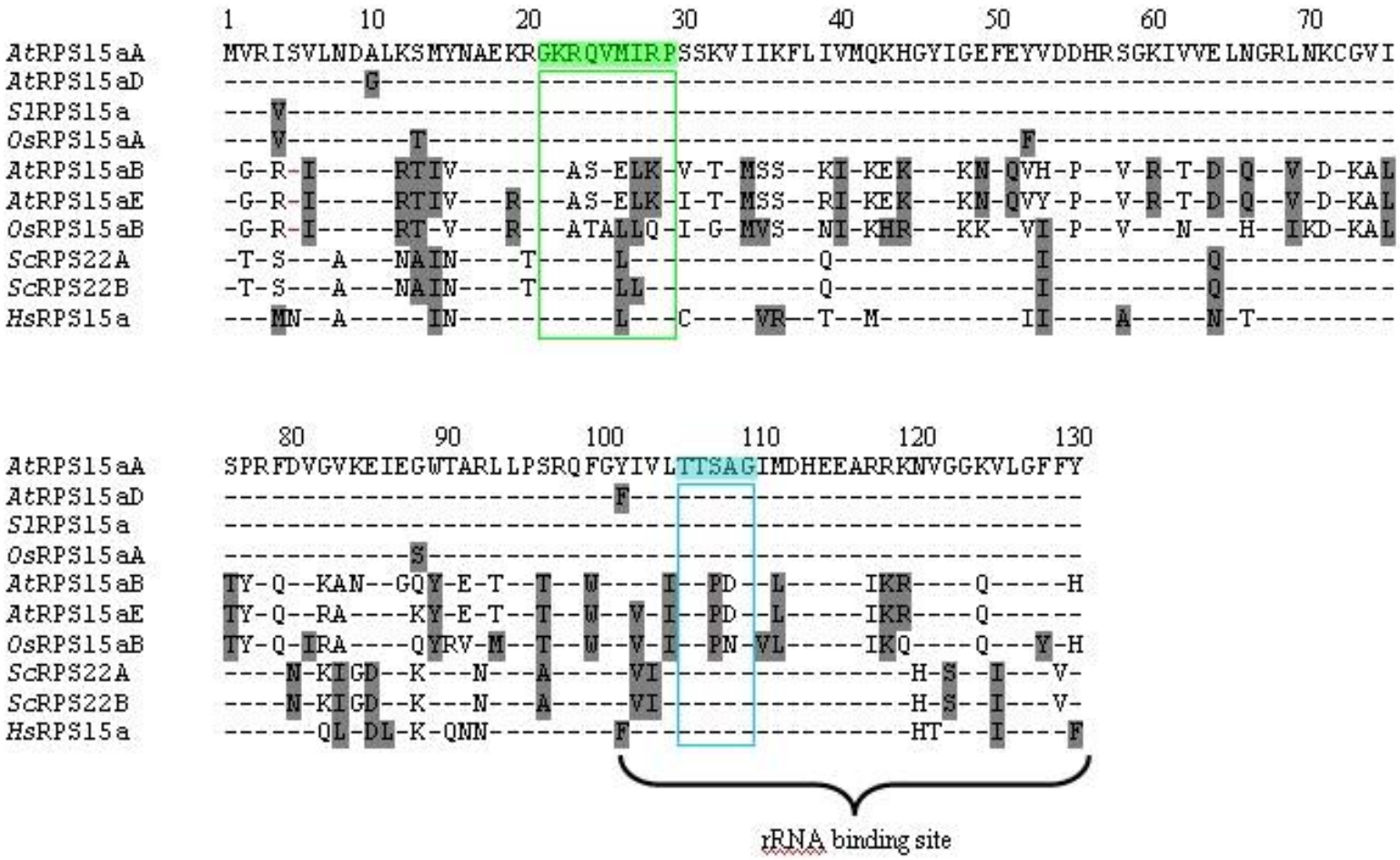
Imaging of transient fluorescence in tobacco leaves was performed using an inverted Zeiss LSM 510 Meta confocal microscope (Jena, Germany). Small, ~1 cm^2, segments of leaf tissue were excised from the infiltrated area, mounted in water on a glass slide and viewed with a 63x water immersion objective. Images of EGFP and mRFP coexpression were acquired using the line switching multi-track option of the microscope to alternate between the 488 and 543 nm excitation lines of the argon ion and helium neon ion lasers, respectively. EGFP fluorescence was detected with a 515 nm dichromatic beam splitter and a 505-530 nm bandpass filter while mRFP fluorescence was detected with a 515 nm dichromatic beam splitter and a 585-615 nm bandpass filter. These settings prevented any cross-talk or bleed-through of fluorescence. Images were initially processed with Zeiss LSM Image Browser software and exported to Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA) for final figure preparation.

4.3. Results

4.3.1. RPS15a *sequence analysis*

The cytosolic Arabidopsis *RPS15a* gene family is comprised of four members: *RPS15aA*, *-C*, *-D* and *-F*. *RPS15aC* is not transcriptionally active and *RPS15aA* and *-F* share 100% amino acid identity. Therefore, *RPS15aA* and *-D*, which share 84.1% sequence identity between their open reading frames (ORFs) and 98% identity at the amino acid level, were chosen for this study. RPS15aA and -D share a high degree of sequence identity with RPS15a orthologs in mono- and dicotyledonous plants (95-97% with *Os*RPS15aA; 98-99% with *Sl*RPS15a), yeast (78-80% with *Sc*RPS22A and -B) and vertebrates (78% with *Hs*RPS15a) (Figure 4.2). RPS15aB and -E share 92% amino acid

Figure 4.2. Clustal W alignment of Arabidopsis Type I (RPS15aA and -D) and Type II (RPS15aB and -E) RPS15a isoforms with tomato (*Solanum lycopersicum*) RPS15a (AK246529), a rice (*Oryza sativa*) RPS15a ortholog (Os2g478600), yeast (*Saccharomyces cereviseae*) RPS22 (NP_013471), and human (*Homo sapien*) RPS15a (NP_001019). Amino acids that differ from AtRPS15aA are indicated. Gaps are indicated by a red dash (-); putative nuclear localization signal is highlighted in green; (S/T)-T-(S/T/P)-X-G motif is highlighted in blue; conservative amino acid substitutions (I/L/M/V; F/W/Y; H/K/R; E/D/N/Q; A/G/P/S/T) are shaded grey.


1 10 20 30 40 50 60 70
AtRPS15aA MVRISVLNDALKSMYNAEKRGKRQVMIRPSSKVIIKFLIVMQKHGYIGEFEYVDDHRSGKIVVELNGRLNKCGVI
AtRPS15aD
SlRPS15a
OsRPS15aA
AtRPS15aB
AtRPS15aE
OsRPS15aB
ScRPS22A
ScRPS22B
HsRPS15a
80 90 100 110 120 130
AtRPS15aA SPRFDVGVKEIEGWTARLLPSRQFGYIVLTTSAGIMDHEEARRKNVGGKVLGFFY
AtRPS15aD
SlRPS15a
OsRPS15aA
AtRPS15aB
AtRPS15aE
OsRPS15aB
ScRPS22A
ScRPS22B
HsRPS15a
rRNA binding site

identity while sharing 75-78% identity with *Os*RPS15aB and only 50-52% identity with *At*RPS15aA and -D, respectively. The putative 18S rRNA binding domain is located in the C-terminus of the peptide and contains the (S/T)-T-(S/T/P)-X-G motif (Figure 4.2; Tishchenko et al., 2001). However, *At*RPS15aB and -E both contain S→P[107] and A→D[108] substitutions within this motif while rice RPS15aB also contains a S→P[107] substitution and an A→N[108] substitution. To date, a NLS has not been identified in any of the Arabidopsis RPS15a isoforms however, the sequence GKRQVMIRP, a NLS recognized in yeast RPS22 (Timmers et al., 1999), is present in both plant and human RPS15a orthologs (Figure 4.2). RPS15aB and -E contain several substitutions within the putative NLS including non-conservative R→A[23], Q→S[24], M→E[26] substitutions as well as conservative I→L[27] and R→K[28] substitutions. A similar array of substitutions is present in *Os*RPS15aB.

4.3.2. RPS15aA *and* -D *are localized to the nucleolus*

To determine the subcellular localization of RPS15aA and -D *in planta,* fluorescent RPS15aA/-D-GST-mRFP fusion proteins were transiently produced in tobacco leaf epidermal cells following *A. tumefaciens* mediated transformation (Sparkes et al., 2006). RPS8, the prokaryotic ortholog of RPS15a, functions as a primary binding protein of the 16S rRNA during assembly of the small ribosomal subunit. Thus, it was hypothesized that in plants, RPS15aA and -D would accumulate to the greatest extent in the nucleolus, the site of cytosolic ribosomal subunit biogenesis in eukaryotes. In order to positively identify nucleolar localization of RPS15aA and -D using CLSM, tobacco leaves were co-infiltrated with a nucleolar localized FIB2-EGFP fusion. Transient expression of RPS15aA/-D-GST-mRFP and FIB2-EGFP fusions were co-visualized using CLSM 72 h following tobacco infiltration. RPS15aA/-D-GST-mRFP and FIB2-EGFP all displayed distinct nucleolar accumulation (Figure 4.3A-D). While the majority of cells contained only one nucleolus, Figure 4.3C clearly shows co-localization of RPS15aD-GST-mRFP and FIB2-EGFP in two separate nucleoli. RPS15aA/-D-GST-mRFP and FIB2-EGFP were also detected in cajal bodies, dynamic structures that are structurally and functionally associated with the nucleolus

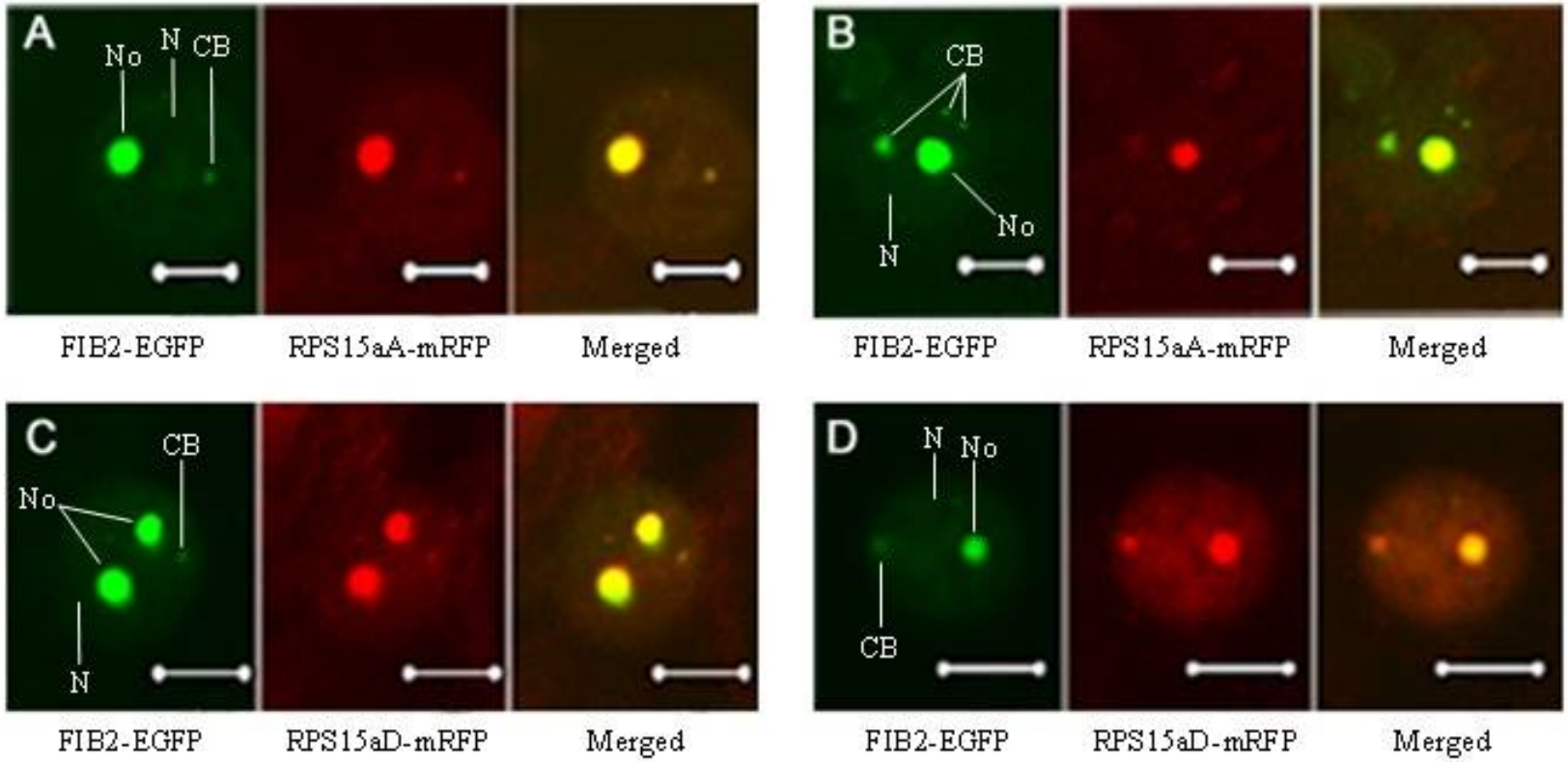


Figure 4.3. Nucleolar localization of RPS15aA and RPS15aD. CLSM images of tobacco leaf epidermal cells transiently co-expressing FIBRILLARIN2 (FIB2)-EGFP and RPS15aA-mRFP (A-B), or FIB2-EGFP and RPS15aD-mRFP (C-D). CB, cajal body; N, nucleus; No, nucleolus. Scale bars = 5 μM.

(Figure 4.3A-D; Kim et al., 2006). Weaker signals from both RPS15aA/-D -GST-mRFP were also detected throughout the nucleoplasm (Figure 4.3A-D).

4.4. Discussion

Consistent with a putative role as a primary 18S rRNA binder, C-terminally tagged RPS15aA/-D fluorescent fusion proteins accumulated predominantly in the nucleoli of tobacco leaf epidermal cells (Figure 4.3A-D). While numerous studies have investigated the nuclear import and nucleolar localization of various r-proteins in animals (Michael and Dreyfuss, 1996; Russo et al., 1997; Annilo et al., 1998; Shu-Nu et al., 2000; Da Costa et al., 2003) and yeast (Moreland et al., 1985; Schaap et al., 1991; Rout et al., 1997; Schlenstedt et al., 1997; Timmers et al., 1999; Lipsius et al., 2005) relatively little work has been done in plants. However, nucleolar localization of C-terminally tagged Arabidopsis RPL23a isoforms has recently been shown in tobacco (R. Degenhardt, personal communication). These results suggest that the use of fluorescent protein tags as a means of visualizing the subcellular localization of r-proteins is feasible however, protein incorporation into functional ribosomal subunits must still be confirmed.

Nuclear import is dependent on the presence of a NLS that, when recognized by a receptor, targets the protein to the nuclear pore complex. Various types of NLSs, including the classical mono- and bipartite sequences, have been identified in many r-proteins (Moreland et al., 1985; Schaap et al., 1991; Russo et al., 1997; Annilo et al., 1998; Rosorius et al., 2000). RPS22, the yeast ortholog of RPS15a, contains a monopartite NLS, GKRQVLIRP, within the N-terminus of the polypeptide (Timmers et al., 1999). This sequence appears to be conserved in both plants and animals and is retained, with only a single amino acid substitution, in Arabidopsis RPS15aA and -D (Figure 4.2). However, the putative NLS of RPS15aB and -E contains several non-conservative substitutions including an $R \rightarrow A^{23}$, $Q \rightarrow S^{24}$, $L \rightarrow E^{26}$. These alterations may affect polypeptide localization or, the NLS may not be required as it has recently been shown, using CLSM, that both RPS15aB and -E are localized to mitochondria (H. Wakely, personal communication). Future experiments using site-directed mutagenesis

to alter the NLSs of RPS15aA/-D and -B/-E could be performed and used to determine the effects of specific residues on RPS15a subcellular localization.

To date, a conserved nucleolar targeting sequence has not been identified however, it has been suggested that interactions with nucleolar proteins and/or rRNA are responsible for protein accumulation within the nucleolus (Russo et al, 1997; Michael and Dreyfuss, 1996; Bouvet et al., 1998; Schmidt-Zachmann and Nigg, 1993; Rosorius et al., 2000). One of the most abundant nucleolar proteins, nucleolin, shuttles continually between the nucleolus and cytoplasm (Orrick et al., 1973; Borer et al., 1989) and may act as an r-protein chaperone and/or facilitate r-protein binding to rRNA (Bouvet et al., 1998). Nucleolin has been shown to bind a subset of rat and human r-proteins through its RGG domain (Bouvet et al., 1998) however, this relationship has yet to be demonstrated in plants. Sequence analysis of RPS15a from plants and animals reveals a highly conserved C-terminal domain that corresponds to the bacterial 16S rRNA binding site (Figure 4.2; Mougel et al., 1993; Wower et al., 1992; Davies et al., 1996; Nevskaya et al., 1998). Included in this domain are several highly conserved amino acid residues and the (S/T)-T-(S/T/P)-X-G motif (Chang et al., 2005; Tishchenko et al., 2001) that, through rRNA interaction, may serve as a nucleolar targeting domain. Further experimentation is required to determine the precise mechanism(s) directing RPS15a nucleolar localization in Arabidopsis.

We have previously shown that *RPS15aA*, *-D* and *-F* are expressed in Arabidopsis (Chapter 2; Hulm et al., 2005) and that the level of transcript abundance of each gene may differ. Therefore, we hypothesized that specific RPS15a isoforms may be preferentially incorporated into ribosomes in a tissue, developmental or environmentally specific manner. In this chapter we have demonstrated that RPS15a subcellular location can be determined using live cell imaging of RPS15a::fluorescent fusion proteins transiently expressed in tobacco leaf epidermal cells. To further evaluate RPS15a heterogeneity, future applications of this technique will include analysis of the subcellular localization of differentially tagged RPS15aA and -D isoforms transiently co-expressed in tobacco and the subsequent generation of stably transformed Arabidopsis plants expressing two or more *RPS15a::fluorescent tag* cassettes. Accumulation of the different RPS15a isoforms could then be visualized *in planta*

following various abiotic treatments and protein incorporation verified using polysome immunopurification (Zanetti et al., 2005).

CHAPTER 5. GENERAL DISCUSSION

As I reflect on the results of my thesis research, I am struck by the apparent complexity of r-protein gene regulation in Arabidopsis and, even more so by the complexity of the ribosome itself. Often regarded as a passive translator of genetic information, the ribosome is now emerging as a putative regulatory factor with the ability to alter mRNA binding interactions through rRNA and/or r-protein heterogeneity (Mauro and Edelman, 2002). The potential implications of this theory become especially interesting in Arabidopsis as recent studies have demonstrated a high degree of ribosome heterogeneity with respect to the r-proteins (Chang et al., 2005; Giavalisco et al., 2005). Certainly, the results of my own work with *RPS15a* have led to the conclusion that the ultimate level of regulation for r-protein synthesis may be at the point of subunit incorporation, a hypothesis I had only begun to investigate with the *in planta* visualization of *RPS15aA* and *-D* nucleolar localization (Chapter 4).

Although eukaryotic r-proteins are commonly encoded by multi-gene families, often only one gene is transcriptionally active or duplicate genes have significantly different expression patterns. In Arabidopsis however, it is common for more than one gene of a r-protein family to be expressed (Barakat et al., 2001) even, as is the case with *RPS15a*, if the encoded proteins are nearly identical. Therefore, I began my research with the initial hypothesis that individual *RPS15a* genes may be preferentially transcribed in a developmental, environmental and/or tissue specific manner. However, although the sequence identity shared among the *RPS15aA, -D* and *-F* full length 5' RRs was relatively low and varied in the number and arrangement of putative *cis*-elements, expression patterns were similar and the differences in transcript abundance minimal (Chapter 2). Furthermore, there was not always a clear correlation between the presence of putative *cis*-elements and transcript abundance or GUS activity (Chapter 3). From these results I concluded that while *RPS15aA*, *-D* and *-F* were, to some degree,

transcriptionally regulated, additional regulation may be occurring at the post-trancriptional and/or translational level.

The production of functional ribosomal subunits depends on the coordinated synthesis of all components and how the cell coordinates expression of such a large set of genes in response to multiple external and internal signals is one of the most interesting questions facing researchers. Although I found that transcription may not be the primary level of r-protein regulation in Arabidopsis, it may represent the first step in coordinated gene expression. Similar to yeast and mammals, conserved *cis*-elements (i.e. *PCNA* Site II motif and *TELO* box) have been identified in the 5' RRs of plant r-protein genes and those of genes encoding other components of the translational apparatus. While the *TELO* box must act synergistically with other elements to regulate gene expression in cycling cells, the Site II motif has been shown to be necessary and sufficient to activate reporter gene expression in both root primordia and young leaves (Regad et al., 1995; Trémousaygue et al., 1999; Manevski et al., 2000; Trémousaygue et al., 2003). In plants, the Site II motif may be responsible for coordinating the expression of genes up-regulated in mitotically active cells (Trémousaygue et al., 2003). The importance of the Site II motif with respect to *RPS15a* gene expression was demonstrated by the reduction or absence of GUS activity in the majority of tissues carrying the *RPS15aA*Δ*5* fragment (Chapter 3). Future experiments, including further 5' deletions of the *RPS15aD*Δ5 and *F*Δ*5* constructs could be used to determine if removal of Site II motifs results in a similar reduction in GUS activity and what combinations of motifs are required for expression.

While my thesis research has focused on transcriptional regulation, I have found that there are features present both in the *RPS15a* genes and processed transcripts that could potentially mediate regulation. Introns appear to be a conserved feature of r-protein genes and are often located near the 5' end of the coding region or within the leader sequence (Spingola et al., 1999; Grossman, 2005; Perry, 2005). Introns in these positions have been shown to enhance gene expression and mRNA accumulation as well as promote translation (Callis et al., 1987; Rose, 2004; Nott et al., 2004). Therefore, the *RPS15aA*, *-D* and *-F* leader introns may be effecting gene expression in a 5' RR-independent manner. Using a series of *RPS15a* leader intron deletion::*GUS*

reporter gene constructs expressed in Arabidopsis, the effect of the leader intron on gene expression could be investigated. Although plant r-protein 5' UTRs lack the polypyrimidine tract responsible for promoting the translation of vertebrate r-protein transcripts, they may contain elements that regulate translation either by acting alone, or in concert with additional elements in the coding region and/or 3' UTR. A series of *RPS15a* 5' UTR::reporter gene constructs, that also contain portions of the 5' RR, coding region and 3' UTR, could be used to determine the effect of the 5' UTR on translation.

In many ways, my investigation of the three cytosolic *RPS15a* genes from Arabidopsis has left me with more questions than answers. The role of multiple expressed plant r-protein isoforms, the biological significance of ribosome heterogeneity, and the coordination and primary regulation of r-protein gene expression are all questions that are just beginning to be answered and ensure an exciting future for the field of plant ribosome research.

APPENDIX 1. ELSEVIER LIMITED LICENSE

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