CHARACTERIZATION OF REGULATION OF EXPRESSION AND NUCLEAR/NUCLEOLAR LOCALIZATION OF ARABIDOPSIS RIBSOMAL PROTEINS

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ABSTRACT

Ribosomal proteins (RPs), synthesized in the cytoplasm, need to be transported from the cytoplasm to the nucleolus (a nuclear compartment), where a single molecule of each RP assembles with rRNAs to form the large and small ribosomal subunits. The objectives of this research were to identify nuclear/nucleolar localization signals (NLSs/NoLSs; generally basic motifs) that mediate the transport of Arabidopsis RPL23aA, RPL15A and RPS8A into the nucleus and nucleolus, and to study transcriptional regulation and subcellular localization of RPs. While all previous research has shown that nucleolar localization of proteins is mediated by specific basic motifs, in this study, I showed that a specific number of basic motifs mediated nucleolar localization of RPL23aA, rather than any specific motifs. In this protein, single mutations of any of its eight putative NLSs (pNLSs) had no effect on nucleolar localization, however, the simultaneous mutation of all eight completely disrupted nucleolar localization, but had no effect on nuclear localization. Furthermore, mutation of any four of these pNLSs had no effect on localization, while mutation of more than four increasingly disrupted nucleolar localization, suggesting that any combination of four of the eight pNLSs is able to mediate nucleolar localization. These results support a charge-based system for the nucleolar localization of RPL23aA. While none of the eight pNLSs of RPL23aA were required for nuclear localization, in RPS8A and RPL15A, of the 10 pNLSs in each, the N-terminal two and three NLSs, respectively, were absolutely required for nuclear/nucleolar localization.

Considering the presence of only a single molecule of each RP in any given ribosome, which obligates the presence of each RP in the nucleolus in equal quantities, I studied transcriptional regulation of Arabidopsis RP genes and the subcellular localization of five RP families to determine the extent of coordinated regulation of these processes. Variation of up to 300-fold was observed in the expression levels of RP genes. However, this variation was drastically reduced when the expression level was considered at the RP gene family level, indicating that coordinate regulation of expression of RP genes, coding for individual RP isoforms, is more stringent at the family level. Subcellular localization also showed differential targeting of RPs to the cytoplasm, nucleus and nucleolus, together with a significant difference in the nucleolar import rates of RPS8A and RPL15A. Although one could expect coordinated regulation of the processes preceding ribosomal subunit assembly in the nucleolus, my results suggest differential regulation of these processes.

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LIST OF ABBREVIATIONS

A site	aminoacyl site
ABA	abscisic acid
ABRC	Arabidopsis Biological Resource Center
cDNA	complementary DNA
CLSM	confocal laser scanning microscope
DFC	dense fibrillar component
EGFP	enhance green fluorescent protein
E site	exit site
ETS	external transcribed spacer
FC	fibrillar Center
GC	granular component
GUS	β-glucuronidase
IF	translation initiation factor
LSU	large subunit
mRFP	monomeric red fluorescent protein
mRNA	messenger RNA
NLS	nuclear localization signal
NoLS	nucleolar localization signal
NPC	nuclear pore complex
NOR	nucleolar organizing region
ORF	open reading frame
pNLS	putative nuclear localization signal
pNoLS	putative nucleolar localization signal
PTC	peptidyl transfer center
P site	peptidyl site
pI	isoelectric point
pfl	*pointed first leaf*
RNAi	RNA interference
RNP	ribonucleoprotein
RP	ribosomal protein

rRNA	ribosomal RNA
RT-PCR	reverse transcription polymerase chain reaction
snoRNP	small nucleolar RNP
snRNP	small nuclear RNP
SRP	signal recognition particle
SSU	small subunit
TCP	TEOSINTE BRANCHED1, CYCLOIDEA, PCF
TF	transcription factor
TOP	terminal oligopyrimidine tract
tRNA	transfer RNA
UTR	untranslated region

CHAPTER I. LITERATURE REVIEW

1.1. Introduction

Ribosomes, large enzymatic complexes responsible for protein synthesis in all living organisms, are composed of ribosomal RNA (rRNA) and ribosomal proteins (RPs), organized into a large subunit (LSU) and a small subunit (SSU). In the cytoplasm, the two subunits exist as independent units until they are assembled on mRNA to become a translationally active unit (Tate and Poole 2004). Prokaryotes have ribosomes that sediment at 70S, whereas eukaryotes have larger 80S cytoplasmic ribosomes as well as 70S mitochondrial ribosomes and in plants, the additional 70S plastid ribosomes. The LSU with the peptidyl transferase center (PTC) catalyzes peptide bond formation between incoming amino acids and the nascent polypeptide chain, while the SSU mediates the correct interactions between mRNA codons and tRNA anticodons. Both central functions of the ribosome – peptidyl transferase activity and decoding of mRNA – are performed by rRNA, making the ribosome a ribozyme (Nissen et al. 2000; Wimberly et al. 2000).

Although rRNA is central to the catalytic activity of the ribosome, RPs are important in rRNA processing, stabilization of the rRNA structure, ribosomal subunit assembly and transport, and translocation of the nascent polypeptide. Trafficking of RPs from the cytoplasm, the site of their synthesis, to the nucleolus, where they assemble with rRNAs to form ribosomal subunits, is integral to ribosome biogenesis. To arrive at the nucleolus, RPs have to first cross the nuclear membrane. Nuclear localization of proteins is mediated by nuclear localization signals (NLSs) that facilitate protein interactions with nuclear transporters (Chelsky et al. 1989; Kalderon et al. 1984). Intra-nuclear movement of RPs to the nucleolus is believed to be achieved by passive diffusion and/or molecular association with other proteins that shuttle between the nucleolus and the nucleoplasm (Boden and Teasdale 2008).

Prokaryotic ribosomes (70S) contain ~55 RPs while the 80S eukaryotic ribosomes contain ~79 RPs. Only a single copy of each of these proteins, with the exception of L7/L12 (70S) and acidic P0, and P1/P2 (80S), is present in the final ribosome (Ban et al. 2000; Wimberly et al. 2000). Hence, it would be expected that the regulation of RP gene expression and RP localization to the nucleus/nucleolus should be tightly and coordinately regulated in response to growth and environmental stimuli. In eukaryotes, the wide distribution of RP genes throughout the genome and the existence of multigene families of RP genes, with more than one actively

transcribed member, makes any coordinated regulation of RP gene expression a highly complex process (Barakat et al. 2001; Marygold et al. 2007; Planta and Mager 1998). In the model flowering plant *Arabidopsis thaliana* (Arabidopsis), there are 81 RPs encoded by 254 RP genes throughout the genome, in multigene families of 2 to 7 members with two or more members being transcriptionally active (Barakat et al., 2001; Chang et al., 2005).

In this study, I defined the signal requirements for nucleolar localization of Arabidopsis RPL23aA. Localization signals of RPS8A and RPL15 were also identified and compared to those of RPL23aA. I analyzed the extent of coordinate regulation of Arabidopsis RP gene expression using the microarray data analysis tool Genevestigator and identified differences in the nuclear and nucleolar localization patterns for the five two-member RP families RPS3a, RPS8, RPL7a, RPL15 and RPL23a. Nucleolar import rates for RPS8A and RPL15A were also determined.

1.2. The ribosome, a two subunit ribozyme complex

In the 70S prokaryotic ribosome, the ~0.9 MDa 30S SSU is made up of a 16S rRNA and 22 RPs while the ~1.6 MDa 50S LSU is made up of 23S and 5S rRNAs and 34 RPs (Schmeing and Ramakrishnan 2009). Atomic resolution crystal structures of the individual LSU, SSU, intact ribosome (21-25 nm) and the ribosome in various translational states from many different prokaryotic species, have been resolved. The crystal structure of the LSU of *Haloarcula marismortui* at 2.4 Å resolution (Ban et al. 2000), the SSU of *Thermus thermophilus* at 3 Å resolution (Wimberly et al. 2000), the intact ribosome of *Escherichia coli* at 3 Å resolution (Schuwirth et al. 2005), the SSU of *T. thermophilus* complexed with the A-site inhibitor tetracycline, the initiation inhibitor edeine and the C-terminal domain of the translation initiation factor IF3 at 3.2 Å (Pioletti et al. 2001) and the intact ribosome of *E. coli* in intermediate states of ratcheting (rotation of the SSU relative to the LSU required for the positioning of tRNAs in the ribosome) at 3.5 to 4 Å (Zhang et al. 2009), are now available.

At an atomic resolution of ~40 Å, the LSU appears hemispherical with a diameter of ~250 Å. It has three projections radiating outwards from a flat face: a central protuberance (CP) composed of 5S rRNA, with two other projections (L1 arm to the left and L7/12 stalk to the right) positioned on either side at a distance of approximately 60 Å from the CP (Wilson and Nierhaus 2003). The major component of the L1 arm is RPL1, while the L7/L12 stalk is comprised of RPL12 and its acetylated form RPL7. At higher resolution, the presence of rRNA-

rich polypeptide exit tunnel immediately is evident below the peptidyl transferase center, that exits through the bottom of the LSU at an opening surrounded by RPs such as RPL23 (Ban et al. 2000). Many structural details of the SSU such as a large head with a laterally projecting beak, separated from the body by shoulder, platform and thin neck regions have been elucidated at 23 Å resolution (Stark et al. 1995).

Although the tertiary structure, largely determined by rRNA, and basic function of the ribosome are conserved, eukaryotic ribosomes are larger than their prokaryotic counterparts and possess more rRNAs and RPs. The 80S cytoplasmic ribosome (20-30 nm) of eukaryotes is made up of a 40S SSU (1.2 - 1.5 MDa) and 60S LSU (2.0 – 3.0 MDa). The 40S subunit is comprised of 18S rRNA and ~33 evolutionary conserved RPs, whereas the 60S subunit is composed of 5S, 5.8S and 23S-like (25-28S) rRNAs and ~47 evolutionary conserved RPs (Chandramouli et al. 2008; Lecompte et al. 2002; Sengupta et al. 2004).

Three-dimensional structures of the ribosomes from eukaryotic species such as yeast (Verschoor et al. 1998), wheat (Verschoor et al. 1996), rabbit (Srivastava et al. 1995) and human (Spahn et al. 2004) have been derived. Like the prokaryotic LSU, the ellipsoidal LSU of eukaryotes also has three projections; the CP, P-protein-stalk made up of the acidic RPs P1/P2 and P0 (analogous to prokaryotic L7/L12 stalk) and L1-stalk. Structural features of the SSU are also highly conserved (Verschoor et al. 1996; Verschoor et al. 1998). Chandramouli et al. (2008) have resolved the structure of the canine 80S ribosome containing an E site tRNA, at 8.7 Å resolution. This work identified a eukaryote-specific intersubunit bridge separating the LSU and SSU that is thought to aid in resetting the conformation of the ribosome for a new cycle of chain elongation.

1.2.1. rRNAs and their functions

Different rRNAs – 16S, 23S and 5S in prokaryotes and 18S, 23S-like, and 5.8S in eukaryotes – are encoded by a single transcription unit tandemly repeated to form multiple rRNA gene clusters (Srivastava and Schlessinger 1990). In eukaryotes, these clusters of transcription units are present as head-to-tail repeats separated by an intergenic spacer at chromosomal loci called nucleolar organizer regions (NORs). Tandem arrays of 5S rRNA gene repeats are also present in the genome but are located outside of the NORs (Srivastava and Schlessinger 1990). The transcription unit for 18S, 5.8S and 23S-like rRNAs is transcribed by RNA polymerase I (RNA pol I) into a 35S polycistronic pre-rRNA transcript that is subsequently processed into the

individual rRNAs by various small nucleolar ribonucleoprotein (snoRNP) complexes in association with numerous ribosomal and non ribosomal proteins. The 5S rRNA genes are transcribed by RNA pol III (Warner 1989). RNA pol I transcribed rRNA genes are the most expressed genes within the genome, with their transcription accounting for up to 80% of total transcription in rapidly growing cells (Li et al. 1999).

The small subunit 18S rRNA is highly conserved between eukaryotic species although mammalian 18S rRNA sequences are ~10% larger than those of yeast and plants (Van de Peer et al. 2000). However, the size of the 23S-like rRNA varies greatly between mammals and yeast or plants, owing to the insertion of expansion sequences in variable loop regions that increase the overall size in mammals (Schnare et al. 1996).

The catalytic activity of the ribosome is conferred entirely by rRNA. In the atomic crystal structure of the LSU from *H. marismortui*, complexed with two substrate analogs (aminoacyl-tRNA and peptidyl-tRNA), there are no protein side-chain atoms closer than ~18 Å to the peptide bond being synthesized, indicating that the 23S rRNA is solely responsible for peptidyl transferase activity (Ban et al. 2000).

The proofreading capability (monitoring of accurate base-pairing between tRNA anticodon and mRNA codon) of the SSU is also conferred entirely by the SSU rRNA (Wimberly et al. 2000). Furthermore, rRNA is the major constituent of the polypeptide exit tunnel although, the tunnel does contain the RPs L4, L22, and L39e, and the tunnel exit is surrounded by L19, L22, L23, L24, L29, and L31e (Nissen et al. 2000).

1.2.2. Ribosomal proteins (RPs)

1.2.2.1. Features of RPs

RPs are an integral part of the structure and function of the ribosome, as well as being involved in a wide variety of other cellular functions. As integral components of ribosome structure, most RPs are inherently RNA-binding proteins. The highly basic nature of most RPs (pI >10) render them ideal candidates for recruitment to processes involving interactions with nucleic acids or acidic proteins [e.g., NF-κB (nuclear factor of kappa light polypeptide gene enhancer in B-cells 1; Wan et al. 2007)]. As an extension of this rRNA-binding ability, RPs should be able to bind to mRNA sequences that mimic stretches of rRNA sequences. Genomic surveys suggest that many eukaryotic mRNAs contain sequences that mimic those of various

stretches of rRNA (Matveeva and Shabalina 1993; Mauro and Edelman 1997). Binding of RPs to these sequences could play a role in the regulation of processing, cellular localization, translation, and degradation of mRNA (Komili et al. 2007; Warner and McIntosh 2009). Further, an evolution of their RNA-binding properties, with little modification, would allow RPs to acquire DNA binding capacity. In fact, many DNA binding motifs like the K Homology (KH) domain, zinc finger, bZIP and helix-turn-helix motifs are present in various RPs (Chan et al. 1994; Chan et al. 1993; Rice and Steitz 1989; Wan et al. 2007). Furthermore, RPs can undergo a variety of post translation modifications, e.g., phosphorylation (Carroll et al. 2008; Mazumder et al. 2003; Volarevic and Thomas 2001; Yadavilli et al. 2007), methylation (Bachand and Silver 2004; Carroll et al. 2008; Lee et al. 2002; Mangiarotti 2002; Odintsova et al. 2003; Yu et al. 2005), acetylation (Arnold et al. 1999; Carroll et al. 2008; Lee et al. 2002; Odintsova et al. 2003; Yu et al. 2005), hydroxylation (Odintsova et al. 2003), and the removal of initiator methionine (Carroll et al. 2008; Lee et al. 2002; Odintsova et al. 2003; Yu et al. 2005). While phosphorylation of mammalian RPs like RPS6 and RPL13a and its significance in translational regulation of a subset of mRNAs is well characterized (Volarevic and Thomas 2001; Mukhopadhyay et al. 2009), the significance of the most other post-translation modifications of RPs has yet to be elucidated.

1.2.2.2. Conservation of RPs

As suggested by genomic sequence comparisons, RPs across the three domains of life – Archaea, Bacteria, and Eukarya - are highly conserved. Conserved domains in RPs fulfill critical basic roles in ribosome assembly and function (Lecompte et al. 2002; Mears et al. 2002). Comparative analysis of RPs from 66 different species across the three domains revealed that of the known 102 RP families (68 RP families have been identified in Archaea, 57 in Bacteria, and 78 in Eukarya), 34 RP families are conserved in all three domains, 23 families are specific to Bacteria, 33 families are specific to Archaea and Eukarya, 11 families are specific to Eukarya and one family is represented only in Archaea (Lecompte et al., 2002). Within the 34 RP families conserved in all three domains, are the RPs required for; i) early assembly events [S4, S7, S8 (S15a in eukaryotes), S15, S17, L2, L3, L4, L5, L15, L18, L23], ii) the formation of RP–RP or RP–rRNA bridges between the LSU and SSU (S15, S13, S19, L2, L5, L14), iii) surrounding the polypeptide exit tunnel [L22, L23 (L23a in eukaryotes), L24, L29], and iv) interactions with

tRNA (S7, S9, S12, S15, L1, L5) (El-Baradi et al. 1984; Held et al. 1974; Mizushima and Nomura 1970; Rohl and Nierhaus 1982; Yusupov et al. 2001).

1.2.2.3. Functions of RPs

Individual RPs are responsible for a wide range of cellular functions (within and away from the ribosome) that can be classified into the following three categories.

1.2.2.3.1. Ribosome biogenesis and translation

Although rRNAs perform the two key functions of ribosomes, RPs play vital roles in both ribosome biogenesis and translation. RPs are required for rRNA processing, correct tertiary folding of the rRNA into an optimized catalytically active conformation, ribosomal subunit assembly, transport of the precursors of ribosomal subunits, stabilization of the LSU and SSU structures, and interactions of the ribosome with various translation factors (Ban et al. 2000; Brodersen et al. 2002; Brodersen and Nissen 2005; Klein et al. 2004; Wimberly et al. 2000). In addition to these structural roles, various RPs contribute to ribosome function in specific ways. The prokaryotic SSU RPS12, located at the interface between the two subunits and close to the ribosomal A site (where aminoacyl tRNA initially binds and is accepted if the anticodon base-pairs with the mRNA codon), has an important role in decoding of these tRNAs (Ogle et al. 2001). While several RPs such as RPS1, RPS7 and RPS11 are important for the tethering of mRNAs or the binding of tRNAs to the ribosome during translation (Brodersen and Nissen 2005), other RPs, positioned at different strategic regions in the polypeptide exit tunnel, are involved in translation-associated functions, e.g., cotranslational folding, translocation, and protein secretion (Ferbitz et al. 2004; Gu et al. 2003; Woolhead et al. 2004). For instance, in *E. coli*, RPL23 facilitates an interaction between the ribosome and trigger factor that cotranslationally chaperones the folding of nascent polypeptides (Kramer et al. 2002). In eukaryotes, RPL23a and RPL35 have similar roles in the interaction between the ribosome and signal recognition particle (SRP). On interaction with its receptor (SR), SRP targets the ribosome, with the associated nascent chain, to the endoplasmic reticulum (ER), where the nascent polypeptide is processed for further targeting (Pool et al. 2002).

Other RPs, such as RPS3 and RPS4, can facilitate the mRNA helicase activity of the ribosome that is required to denature secondary structures of mRNA, prior to successful translation (Takyar et al. 2005). RPs like L7/L12 help in the recruitment of the GTPase

translation factors involved in translation initiation, elongation and subsequent release of nascent polypeptide chains (Helgstrand et al. 2007; Kavran and Steitz 2007). In *T. thermophilus*, the close proximity of the C-terminal tails of RPS9 and RPS13 to the P site tRNA in the small subunit (Wimberly et al. 2000), and in *H. marismortui,* the proximity of RPL2 and RPL3 to the peptidyl transferase center in the large subunit (Ban et al. 2000) suggests the possibility that RPs may directly influence the catalytic activity of the rRNA in an allosteric way, however, definitive evidence is currently lacking (Brodersen and Nissen 2005).

1.2.2.3.2. Selective translation of mRNAs

The ribosome filter hypothesis states that ribosomes can act as discriminatory structures such that heterogeneous ribosomal subunits can/will differentially translate different mRNAs (Mauro and Edelman 2002). Most RPs are located on the surface of each subunit and thereby are likely to play important roles in the interactions of the ribosome with mRNAs (Ban et al. 2000; Wimberly et al. 2000). Hence, it is reasonable to propose that RPs play critical roles in the differential interactions of ribosomes with mRNAs. This translational regulatory capability of ribosomes is thought to have arisen through ribosomal heterogeneity, a major source of which is RP composition (Carroll et al. 2008; Garcia-Marcos et al. 2008; Giavalisco et al. 2005; Ramagopal 1992; Sugihara et al. 2010; Szick-Miranda and Bailey-Serres 2001). In yeast and plants, most RPs have two or more isoforms and the presence (or absence) of a particular isoform(s) in the ribosome may enhance (or suppress) the ability of a ribosome to translate a particular subset of mRNAs (Etter et al. 1994; Komili et al. 2007). In mammals, where there is predominantly only one isoform of each RP, various posttranslational modifications would generate the heterogeneity required for differential translation (Bachand and Silver 2004; Volarevic and Thomas 2001; Yu et al. 2005). In addition to RP composition, variations in rRNA sequence (Gonzalez et al. 1985; Kuo et al. 1996; Leffers and Andersen 1993; Selker et al. 1985) coupled with post transcriptional modifications of rRNAs (Chow et al. 2007; Esguerra et al. 2008) as well as differential regulation of expression of rRNA genes (Tseng et al. 2008) can also lead to ribosome heterogeneity. However, even rRNA-based ribosome heterogeneity will require RPs able to recognize these variations and to assemble appropriately during ribosome biogenesis.

How does ribosomal heterogeneity, generated from a variety of RPs and/or rRNAs explain differential translation of mRNAs? Possibly by differential binding of the resulting heterogeneous ribosomal subunits to mRNAs (via RP-mRNA or rRNA-mRNA interactions),

and/or by a differential binding to transcript-specific trans factors associated with *cis*-acting elements of mRNAs (Mauro and Edelman 2002).

Evidence supporting the ribosome filter hypothesis comes from the very fact that ribosomes are heterogeneous; considering the cost associated with producing and maintaining a large pool of heterogeneous ribosomes, it would be expected that there would be considerable benefits resulting from such effort. One such benefit would be the generation of a regulatory capacity by these ribosomes. Localized translation of *Saccharomyces cerevisiae Ash1* mRNA in the bud tip, requires a specific combination of isoforms from the 14 duplicated RPs in *S. cerevisiae* implicated in the process (Komili et al. 2007). Furthermore, the study of phenotypic data for various yeast RP mutants suggests that none of the duplicated RP genes have paralogs that share all phenotypic characteristics, suggesting a functional specificity of isoforms (Komili et al. 2007).

It should be noted that apart from RPs possibly facilitating selective translation of mRNAs, the mRNA itself can influence selective translation. The sequence and structure of mRNA, various post transcriptional modifications, and *cis* elements present in mRNA and associated trans factors can also influence how efficiently ribosomal subunits (or other factors required for translation) can access, bind and translate mRNA (Mauro and Edelman 2002).

1.2.2.3.3. Extraribosomal functions

As mentioned above, RPs work coordinately to achieve the tasks related to ribosome biogenesis and function and selective translation. In addition, individual RPs can function independently of the ribosome in extraribosomal processes such as transcription, translation, mRNA processing, DNA repair, apoptosis and tumorigenesis (Lindstrom 2009; Naora 1999; Warner and McIntosh 2009).

1.2.2.3.3.1. Regulation of gene expression

RPs can regulate their own expression (see section 1.2.2.4.2) as well as that of other genes either at the level of transcription or translation. RP control of transcription of genes primarily occurs through associations with, and subsequent activation/deactivation of the transcriptional regulators of these genes. For example, mammalian NF-κB is a transcription factor (TF) that as a dimer binds to, and regulates expression of, various genes involved in immunity, inflammation, and apoptosis (Lenardo and Baltimore 1989; Sen 2006). RPS3 binds to NF-κB and

synergistically enhances its DNA-binding affinity. A loss of RPS3 impacts the ability of NF-κB to transactivate many of its target genes (Wan et al. 2007).

Mammalian RPL11 binds to the oncoprotein transcription factor c-Myc, and in doing so inhibits its ability to mediate transcription of genes involved in cell cycle progression (Dai et al. 2007b). RPL7 is a co-regulator of the heterodimeric vitamin D receptor [VDR] and retinoid X receptor [RXR] TF complex (Berghofer-Hochheimer et al. 1998), while RPL23 negatively regulates the TF Miz1, itself a negative regulator of cell proliferation, by sequestering nucleophosmin (coactivator of Miz1) in the nucleolus (Wanzel et al. 2008).

There is evidence suggesting that RPs can/might directly act as TFs. *S. pombe* RPL32-2, when fused to the GAL4 DNA-binding domain or the GAL4 transactivation domain, in both cases was able to activate transcription of reporter genes driven by the GAL4 promoter (Wang et al. 2006). It was further shown that RPL32-2 could directly bind to the DNA sequence $^{5'}$GGTGTT$^{3'}$. Together, these results suggest that RPL32-2 may be or has the potential to become a TF (Wang et al. 2006). Likewise, yeast acidic RP YP1α has also been shown to have transactivation potential (Tchorzewski et al. 1999).

RPs can also control the expression of mRNAs. A remarkable example is the regulation of translation of *Ceruloplasmin (Cp)* mRNA by human RPL13a. Ceruloplasmin is a copper-carrying protein in the blood that also plays an important role in plasma iron homeostasis. *Cp* mRNA translation is silenced by interferon-gamma; in response to interferon-gamma, RPL13a is phosphorylated and released from the LSU. Free phosphorylated RPL13a, together with three other proteins, forms the IFN-γ-activated inhibitor of translation (GAIT) complex, that binds to GAIT elements in the 3'UTR of *Cp* mRNA to inhibit its translation (Mazumder et al. 2003; Mukhopadhyay et al. 2009). Conversely, RPs can also enhance translation of other mRNAs. In response to DNA damage, human RPL26 binds to the 5' UTR of *p53* mRNA, enhancing its affinity to polysomes, thereby enhancing translation efficiency of this mRNA (Takagi et al. 2005).

1.2.2.3.3.2. Enzymes

RPs do not have any enzymatic activity in the ribosome. However, some RPs do have enzymatic capacity. Mammalian RPS3, with its endonuclease activity, is involved in DNA damage repair (Kim et al. 1995). In response to DNA damage RPS3, phosphorylated at T^{42} by ERK1/2 (Extracellular signal-Regulated Kinase), is translocated to the nucleus, where it is

involved in base excision repair of damaged DNA (Yadavilli et al. 2007). The *Drosophila* orthologue of RPS3 has 8-oxoguanine and apurinic/apyrimidinic (AP) lyase activity (Yacoub et al. 1996).

1.2.2.3.3.3. Regulation of cell proliferation, apoptosis and tumorigenesis

Cell proliferation and differentiation significantly raise the demand for protein synthesis, and as such ribosome biogenesis must be tightly and coordinately regulated with these processes. Deregulation of ribosome biogenesis and consequent perturbation in protein synthesis can lead to either tumorigenesis or cell cycle arrest and apoptosis. Therefore, it is not surprising that ribosome biogenesis is a primary target of many tumor suppressors and oncoproteins. The tumor suppressor proteins p53, retinoblastoma protein (RB), ARF, and phosphatase and tensin homolog (PTEN) all inhibit ribosome biogenesis by reducing the synthesis of RPs and rRNAs (Felton-Edkins et al. 2003; Morton et al. 2007; Zhang et al. 2005), while the oncoprotein c-Myc enhances ribosomal biogenesis (Adhikary and Eilers 2005; Dai et al. 2007b; Felton-Edkins et al. 2003). While oncoproteins and tumor suppressors modulate ribosome biogenesis, they, in turn, are regulated by individual RPs (as described previously), creating elegant feedback surveillance mechanisms and networks for the regulation of cell division. As discussed earlier, RPL26 can directly bind and enhance translation of p53 mRNA in response to DNA damage (Takagi et al. 2005). Whereas, the oncoprotein MDM2 is an E3 ligase, that mediates p53 ubiquitination, thereby targeting it for proteasome degradation. RPL5, RPL11, RPL23, and RPS7 all enhance p53 level in response to various stresses by binding to and inhibiting MDM2 activity towards p53 (Chen et al. 2007; Dai and Lu 2004; Dai et al. 2006; Dai et al. 2004). Like tumor suppressors, oncoproteins e.g., c-Myc can also be regulated by RPs (Dai et al. 2007a; Dai et al. 2007b). Many other RPs also have roles in apoptosis and tumorigenesis. It is interesting that forced expression of some RPs, e.g., RPS29 (Khanna et al. 2003), RPL13a (Chen and Ioannou 1999), and RPS27L (He and Sun 2007) induces apoptosis, while others, e.g., RPS9 (Kim et al. 2003), RPS13, RPL13 (Shi et al. 2004) and RPL35a (Lopez et al. 2002) inhibits apoptosis under differing cellular conditions and in different tissues.

1.2.2.3.3.4. Plant growth, development, biotic and abiotic stress

Many Arabidopsis RP gene mutants have been characterized and a common phenotype among these mutants is embryo lethality (Byrne 2009). Conceivably, lethality is due to the

synthesis of aberrant ribosomes, as a result of the absence/reduced level of a single RP and the consequent perturbation in protein synthetic activity. Some RP gene mutants are viable, but impair specific processes such as determination of organ identity and hormone homeostasis. For example, single mutations in *RPL5A, RPL5B, RPL24B* or *RPL28A* all disrupt the establishment of leaf abaxial-adaxial polarity (Yao et al. 2008). In all of these mutants, the abaxial mesophyll arrangement of loosely packed cells with obvious intercellular spaces, is produced in the adaxial mesophyll domain, where cells are normally tightly arranged (Yao et al. 2008). Leaf patterning is also disrupted in the *piggyback* mutants *pgy1* (*rpl10ab*), *pgy2* (*rpl9c*) and *pgy3* (*rpl5a*) (Pinon et al. 2008). A MYB domain transcription factor ASYMMETRIC LEAVES1 (AS1) is a major determinant of leaf patterning in many plant species (Tattersall et al. 2005). However, in Arabidopsis, *as1* plants show only minor leaf patterning defects (leaves with marginal lobes) (Byrne et al. 2000). However, double mutations of *pgy:as1* enhance the *as1* phenotype resulting in leaf lamina outgrowths on the adaxial side of the leaf, indicating a role for the *PGY* genes (*RPL10aB, RPL9C, RPL5A*) in leaf patterning (Pinon et al. 2008).

Many RP gene mutations have been associated with impaired auxin perception and distribution. The *rps18a* (*pointed first leaf 1* [*pfl1*]), *rps13b* (*pfl2*), and *rps5a* (*Arabidopsis minute-like 1* [*aml1*]) mutants all lead to auxin-related developmental defects; reduced cell division, growth retardation, pointed first leaves, and defects in cotyledon vasculature (Ito et al. 2000; Van Lijsebettens et al. 1994; Weijers et al. 2001), while silencing of *RPL4A* or *RPL23aA* also results in similar phenotypes (Degenhardt and Bonham-Smith 2008; Rosado et al. 2010). In addition, vacuolar trafficking is disrupted in *rpl4a* plants, where fluorescent proteins, carrying sorting signals that would normally target them to vacuoles, are partially secreted to the apoplast (Rosado et al. 2010).

When exposed to high levels of UV light, Arabidopsis plants respond by rapidly degrading the overall cellular mRNA population (Revenkova et al. 1999). This extreme turnover of mRNA may serve two purposes; i) degradation of UV-damaged mRNAs and ii) release of resources to facilitate an up-regulation of transcription of stress-response genes (Revenkova et al. 1999). Arabidopsis *rps27a* plants (one of the paralogs of the three member *RPS27* gene family), are unable to rapidly degrade mRNA after UV treatment (Revenkova et al. 1999). Furthermore, when grown in the presence of methyl methane sulfate (MMS; a genotoxic agent), *rps27a* plants produced tumor-like structures instead of auxiliary roots, whereas under optimal growing

conditions, the *RPS27A* knock out had no effect on plant growth and development (Revenkova et al. 1999). Together, these observations suggest that RPL27A has a role in the degradation of mRNAs in response to genotoxic stress, but is dispensable for protein synthesis under optimal growing conditions (Revenkova et al. 1999).

RPL30E has been identified as a candidate gene for salinity stress tolerance in pea (Joshi et al. 2009), while the loss of *RPL10A* function in Arabidopsis was found to increase susceptibility to geminivirus infection (Carvalho et al. 2008).

1.2.2.4. RP Gene expression

Expression of RP genes is modulated in response to growth stimuli and environmental stress, ensuring sufficient ribosome number and overall protein synthetic capacity required under these differing physiological conditions (Wade et al. 2004). Regulation of RP gene expression occurs both transcriptionally and post-transcriptionally.

1.2.2.4.1. Transcription

RP genes in prokaryotes are arranged in species-specific operons (e.g., *E. coli L11, RIF, str, spc, S10* and *α*) in which a single promoter controls the expression of multiple RP genes, facilitating the expression of different RP genes in equal quantities (Nomura et al. 1984). As in prokaryotes, transcription also appears to be the major control mechanism governing RP levels in yeast. TFs such as High mobility group protein 1 [Hmo1; (Hall et al. 2006)], Repressor/activator site-binding protein1 [Rap1; (Lieb et al. 2001)], Fork head-like transcription factor 1 (Fhl1) and Interacting with fork head 1 [Ifh1; (Wade et al. 2004)] play major roles in transcription regulation of RP genes.

In plants, RP levels can be controlled at the level of transcription as indicated by the variation in abundance of transcripts of some RP genes under various growing conditions. Overall, expression of the majority of RP genes is highest in actively dividing cells and lowest in mitotically-inactive tissue (Hulm et al. 2005; McIntosh and Bonham-Smith 2005; Williams and Sussex 1995). Many RP genes are upregulated under growth stimulating conditions such as treatment with the phytohormones auxins and cytokinins, while they are downregulated under growth inhibiting conditions, such as treatment with abscisic acid or sugar starvation (Contento et al. 2004; Gao et al. 1994; Hulm et al. 2005; Li et al. 2006; McIntosh and Bonham-Smith 2005).

1.2.2.4.2. Post-transcription and translation

An advantage of regulating gene expression at the level of translation is that it enables cells to rapidly repress the synthesis of RPs during a shortage of amino acids or growth arrest and to quickly resume synthesis of RPs when amino acids are replenished or there is a mitogenic stimulation (Meyuhas 2000). Autogenous feedback regulation of translation of RP mRNAs is common in prokaryotes, wherein RPs control translation of their own polycistronic mRNAs by directly preventing translation, inhibiting mRNA splicing or decreasing mRNA half-life (Nomura et al. 1984; Zengel and Lindahl 1992). This extraribosomal role for some RPs, as a translational regulator, has evolved by adaption of an intrinsic rRNA-binding ability. Some eukaryotic RPs (e.g., yeast RPL30, and human RPL7) are also able to autoregulate translation of their own mRNA. Yeast RPL30, RPS14, and human RPS13 autoregulate by inhibiting splicing of their own mRNAs (Fewell and Woolford 1999; Malygin et al. 2007; Neumann et al. 1995; Vilardell and Warner 1994). To autoregulate, yeast RPS28B has evolved a distinctive mechanism whereby it binds to a conserved hairpin structure in the 3'UTR of its own mRNA and mediates decay by recruiting proteins required for decapping (an essential step in mRNA decay) of the mRNA, rapidly followed by 5′ to 3′ exonucleolytic trimming of the transcript (Badis et al. 2004).

A common feature of mammalian RP mRNAs, the 5' terminal oligopyrimidine (TOP) sequence, contains *cis*-regulatory elements required for regulation of translation (Meyuhas 2000). Stimulated by an appropriate mitogenic or growth signal, phosphoinositide-3 (PI-3) kinase turns on the signaling cascade that displaces a repressor protein bound to the TOP sequence in RP mRNAs, permitting the translational machinery to bind and translation to proceed (Meyuhas 2000; Stolovich et al. 2002; Cantrell 2001).

Translation of RP mRNAs can also be controlled by modulation of polysome loading; the recruitment of multiple ribosomes to a single mRNA. In actively dividing cells, or in cells responding to a growth signal, RP mRNAs are found in polysomes, while in cells in resting phase or in cells responding to growth arresting signals, RP mRNAs are shifted to the subpolysomal fraction (Meyuhas 2000). In Arabidopsis, the RP mRNAs found in polysomes significantly decreases, without significant decrease in transcript level, in response to stresses such as sucrose starvation, dehydration or hypoxia (Branco-Price et al. 2005; Kawaguchi et al. 2004; Nicolai et al. 2006).

1.2.2.5. Coordinated regulation of RP gene expression

The ribosome contains only a single molecule of each RP except the acidic RPs, presumably necessitating equimolar availability of different RPs in the nucleolus (Ban et al. 2000; Schuwirth et al. 2005; Spahn et al. 2004; Szick-Miranda and Bailey-Serres 2001; Wimberly et al. 2000). Ribosome biogenesis is an energy-intensive process; a large proportion of a cell's energy is expended in ribosome biogenesis, e.g. in a rapidly growing yeast cell, rDNA transcription accounts for 60% of total transcription, and RP-mRNA splicing accounts for 90% of total mRNA splicing; (Warner 1989). Consequently, RP synthesis for ribosome biogenesis needs to be tightly and coordinately regulated at various levels of gene expression to ensure that no RP is produced in excess or less than their partners.

In contrast to prokaryotes, where coordinated regulation of clustered, operon-arranged RP genes is relatively simple, in eukaryotes, owing to the wide distribution of RP genes across the genome and the absence of any operon arrangements, any coordinated regulation of expression of RP genes is a highly complex process (Perry 2007). For instance, in humans, 75 RP genes are distributed over all 23 chromosomes, with a bias towards chromosome 19 (Kenmochi et al. 1998), while in Arabidopsis, 254 RP genes are scattered across the five chromosomes (Barakat et al. 2001). In plants and yeast, the existence of multigene families of RP genes with more than one transcriptionally active member further complicates coordinated regulation of these genes (Barakat et al. 2001; Warner 1989). In yeast, 59 of the 79 RPs are encoded from two-member gene families with each member being transcriptionally active (Lee et al. 2002; Planta and Mager 1998). In Arabidopsis, where 81 RPs are encoded by 254 genes, there are multigene families of two to seven expressed members (Barakat et al. 2001; Chang et al. 2005). A recent EST data analysis in *Brassica napus* suggests that there are at least 996 genes encoding 79 RPs in this tetraploid species, with some RPs being encoded by as many as 38 genes, but on average with 20.8 genes per family (Whittle and Krochko 2009).

Although the coordinated regulation of RP gene expression in eukaryotes has not been well characterized, considerable efforts have been made to dissect this complex process and identify common *cis*-regulatory elements and associated TFs. In yeast, chromatin immunoprecipitation coupled with microarray analysis suggests that TF Hmo1 binds strongly to the promoters of most RP and rRNA genes, indicating a possible role in the coordinated regulation of these genes (Hall et al. 2006). TF Rap1 can also bind to most yeast RP gene promoters, again suggesting an

important role for this protein in coordinated regulation (Lieb et al. 2001). However, Rap1 is not solely an activator of RP gene transcription; it can also function as a repressor of RP genes. Many Rap1-activated proteins are not coordinately regulated with RPs, therefore, Rap1 alone cannot control coordinated regulation of RP gene expression in yeast. The TF Fhl1, together with its coactivator Ifh1, appears to specifically regulate RP genes in a Rap-1-dependent manner (Wade et al. 2004). Rap1 facilitated nucleosome-displacement and chromatin reorganization aids in the recruitment of Fhl1 to RP gene promoters, which in turn recruits Ifh1; the amount of bound Ifh1 determines the level of transcription, suggesting that Ifh1 association with promoters is a key regulatory step in the coordination of yeast RP gene expression (Wade et al. 2004; Yu and Morse 1999). Environmental stress, inducing a reduction in RP gene expression, also causes a significant reduction in Ifh1 associated with RP gene promoters, but not Fhl1 or Rap1, further corroborating the importance of Ifh1 in coordinated regulation of RP expression (Wade et al. 2004).

A comparative analysis of upstream regulatory regions of 73 human RP genes did not identify any known regulatory motifs common to all RP genes (Yoshihama et al. 2002). A recent analysis of upstream regulatory sequences of 79 pairs of human and mouse RP genes further confirmed the absence of any common motifs among RP genes (Perry, 2005). However, in a computational analysis of the promoters of RP genes in 13 different species (2 plants, 4 yeasts, 2 worms, 2 insects, and 3 mammals), at least one motif common to promoters of all RP genes was found in 11 of the 13 species, the exception being the two worm species (Li et al. 2005). Furthermore, in Arabidopsis, common *cis*-elements have been identified in regulatory regions in many RP genes; synergistically acting *cis*-regulatory elements, *telo*-box ($^{5'}$AAACCCTA$^{3'}$), to which the TF AtPurα binds and the site II motif ($^{5'}$TGGGCY$^{3'}$), to which TF TCP20 (TEOSINTE BRANCHED1,CYCLOIDEA, PCF domain) binds, were identified in the 5' regulatory regions of 153 RP genes (Tremousaygue et al. 2003; Tremousaygue et al. 1999)

Although a coordinated regulation of expression of RP genes to produce equimolar quantities of all RPs appears to be critical for optimal usage of energy in ribosome biogenesis, gene expression profiling under numerous experimental paradigms has suggested differential regulation of expression of individual (or cohorts of) RP genes. Gene expression patterns for 89 RP genes in six adult human tissues identified large variations in the expression of each of these genes within each of the considered tissues. Furthermore, 13 of these genes showed differential

expression across the six studied tissues (Bortoluzzi et al. 2001). In three malignant human nasopharyngeal epithelium derived cell lines, 17 RP genes were identified as being differentially expressed; all 17 genes were downregulated in all three cell lines (TWO1, HONE1 and SUNE1), with the exception of an upregulation of five genes in SUNE1 (Sim et al. 2010). Differential translation of RP mRNAs has also been documented. During the maturation of human monocyte-derived dendritic cells, the mRNAs for 12 LSU RPs were found to be disengaged from polysomes, indicating translational down regulation of these mRNAs (Ceppi et al. 2009).

1.3. The nucleolus – the site of ribosomal subunit biogenesis

In eukaryotes, the assembly of cytoplasmic ribosomal subunits - a multi-step process requiring synthesis, processing and modification of pre-rRNAs, and the assembly of rRNAs with RPs, involving transient interactions with numerous non-ribosomal factors - is coordinated in the nucleolus, a non-membrane bound structure within the nucleus. The association of some RPs and non-RPs with unprocessed 35S pre-rRNA generates a 90S preribosomal particle. Subsequent cleavage of the 35S rRNA to remove flanking and internal spacer regions results in the formation of precursors of the 40S and 60S ribosomal subunits, which are eventually exported through the nuclear pore complex (NPC) into the cytoplasm. Final processing of rRNAs, trimming of the 3'end of the LSU 5.8S rRNA and dimethylation and cleavage of the 20S pre-rRNA to yield the mature SSU 18S rRNA, as well as assembly of remaining RPs occurs in the cytoplasm to form mature ribosomal subunits (Panse and Johnson 2010). Assembly of most RPs on rRNAs takes place in the nucleolus, with only a few late additions of RPs occurring in the cytoplasm (Fromont-Racine et al. 2003; Grandi et al. 2002; Tschochner and Hurt 2003).

The multifunctional nucleolus is a highly dynamic structure formed around tandemly repeated rDNA genes coding for pre-rRNA (Andersen et al. 2005). Nucleoli are assembled during late telophase, persist throughout interphase, and disassemble as a cell enters mitosis (Lam et al. 2005). Nucleoli have three morphologically distinct regions (Figure 1.1) – fibrillar centers (FCs), dense fibrillar components (DFCs), and agglomeration of circular granular components (GCs), each involved in different steps of ribosome biogenesis

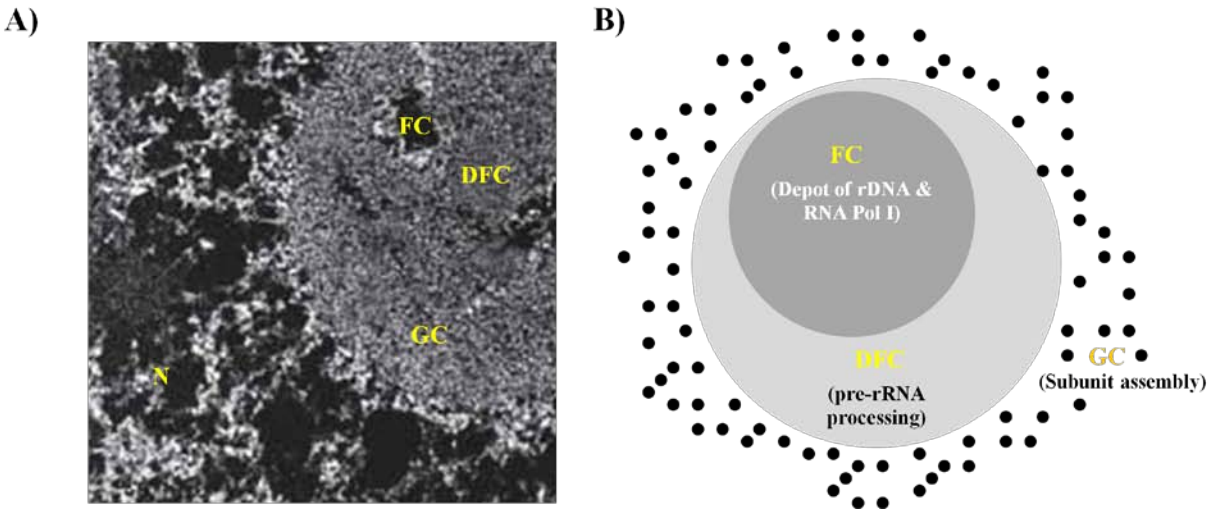


Figure 1.1. The structure of the nucleolus. (A) Electron microscopic image of the ultrastructure of the nucleolus from HeLa cells. (**B)** Outline of the three morphologically distinct regions of the nucleolus and their functions in ribosome subunit assembly. FC, fibrillar centre; DFC, dense fibrillar center; GC, granular center; N, nucleus (Boisvert et al. 2007).

(Shaw and Jordan 1995). Transcription of rDNA to produce the 35S pre-rRNAs takes place at the FC-DFC border. FCs contain RNA pol I, but very little rRNA, while DFCs that do not contain RNA pol I, are rich in rRNA and extensions of nascent pre-rRNA transcripts (Thiry and Lafontaine 2005). The initial steps of 35S pre-rRNA processing, including cleavage and base modifications such as methylation and pseudouridylation take place in the DFCs (Raska et al. 2006). In GCs, the later stages of pre-rRNA processing, as well as the assembly of processed 23S-like and 5.8S rRNA with 5S rRNA and LSU RPs to produce LSUs and 18S rRNA with SSU RPs to produce SSUs takes place (Carmo-Fonseca et al. 2000; Raska et al. 2006). A multitude of snoRNPs and ~150 non ribosomal nucleolar proteins are required in the processing and modification of pre-rRNA transcripts and the subsequent assembly of ribosomal subunits (Carmo-Fonseca et al. 2000; Ferreira-Cerca et al. 2007; Fromont-Racine et al. 2003; Raska et al. 2006).

1.4. Nuclear localization of proteins

Barring a few small proteins (<60 kDa), that can diffuse through NPCs (Breeuwer and Goldfarb 1990; Degenhardt and Bonham-Smith 2008; Fahrenkrog and Aebi 2003; Grebenok et al. 1997; Lim et al. 2008a), most proteins localize to the membrane-bound nucleus through energy-dependent active transport processes via the NPCs (~200 NPCs are present per nucleus in yeast and ~5000 in human). NPCs are ~40-125 MDa multiprotein assemblies made up of ~30 nucleoporin proteins (Cronshaw et al. 2002; Lim et al. 2008b). NPCs are octagonal symmetric structures, with a nuclear envelope embedded scaffold surrounding the central aqueous channel (~30 nm), sandwiched between two rings of nucleoporins, of which one is located on the cytoplasmic side of the channel and the other on the nucleoplasmic side (Figure 1.2; D'Angelo and Hetzer 2008). A third of the nucleoporins are enriched in phenylalanine-glycine repeats (FG repeats), which generally assume an unfolded conformation, creating a meshwork of filaments filling the central channel of NPCs to gate cargos larger than ~60 kDa (Patel et al. 2007).

Targeting of proteins through NPCs is usually mediated by nuclear localization signals (NLSs) comprised of one or more stretches of positively charged (basic) amino acids that interact with negatively charged (acidic) cytosolic nuclear transport receptors belonging to the importin/karyopherin family of proteins (Mosammaparast and Pemberton 2004). Importin α/β heterodimers 'carry' cargo proteins into the nucleus while they shuttle between the cytoplasm

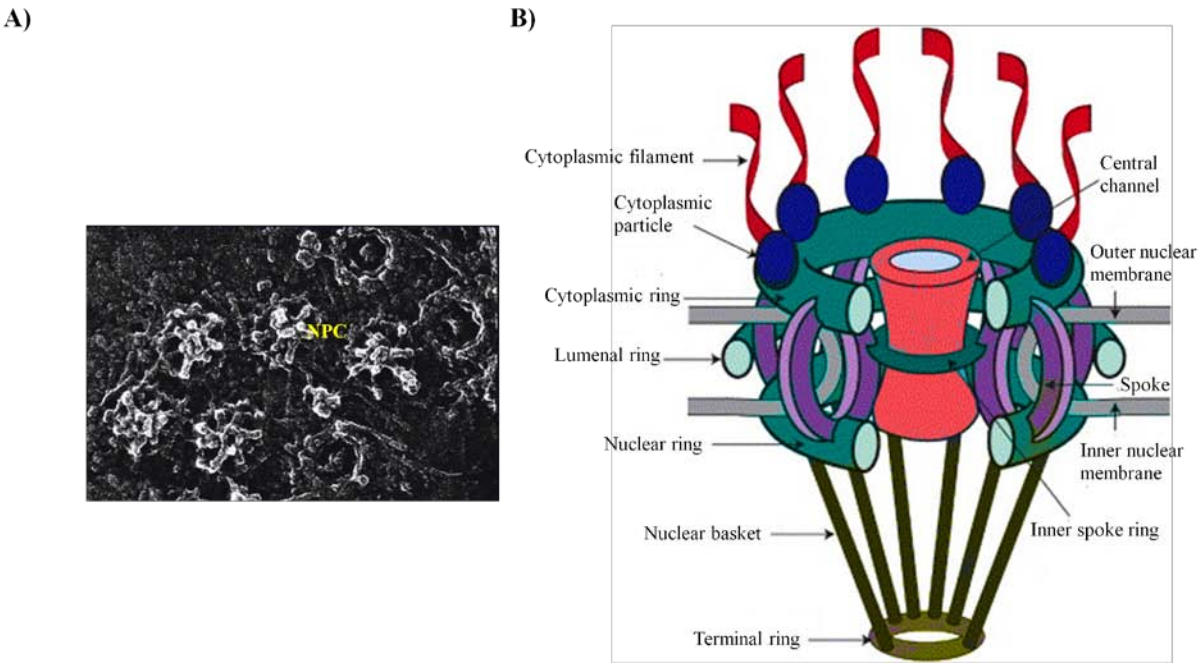


Figure 1.2. The structure of a nuclear pore complex (NPC). (A) Electron micrograph of the nuclear membrane of Dictyostelium with many NPCs. **(B)** Schematic diagram of NPC structure (modified from Suntharalingam and Wente 2003).

and the nucleus (Chook and Blobel 2001; Pemberton and Paschal 2005). Importin α, the adaptor molecule between the cargo and importin β, has two major domains; one recognizes and interacts with the NLS of the cargo, while the importin β-binding (IBB) domain interacts with importin β (Jakel and Gorlich 1998). Importin β, via its interaction with the FG repeat domains of nucleoporins, docks the trimeric (cargo-importin α-importin β) complex to the NPC (Moroianu et al. 1995). From here the complex is translocated into the nucleus by locally disrupting the nucleoporin mesh in the central channel (Frey et al. 2006; Ribbeck and Gorlich 2001; Weis 2003). The FG motifs of the nucleoporins on the nuclear side of the central channel of the NPC have progressively higher affinity for importin β compared to those of the nucleoporins on the cytoplasmic side, possibly facilitating importin β-cargo complex movement in the nuclear direction (Ben-Efraim and Gerace 2001). Like other GTPases, Ran exists in two different nucleotide-bound states – RanGTP and RanGDP. In the nucleus, Ran is predominantly GTP-bound, while in the cytoplasm predominantly GDP-bound. In the nucleus, Ran guanine exchange factor (RanGEF) converts RanGDP to RanGTP, while in the cytoplasm Ran GTPase-activating protein (RanGAP) and Ran binding protein1 (RanBP1) hydrolyze RanGTP to RanGDP, creating a Ran gradient between the cytoplasm and the nucleus; this gradient is a key element in establishing the direction of nucleocytoplasmic transport (Lange et al. 2007). In the nucleus, binding of RanGTP to importin β results in a change in the conformation of importin β, disrupting the interaction between it and the IBB domain of importin α, resulting in its displacement from the trimeric complex (Gilchrist et al. 2002; McLane and Corbett 2009). Within the IBB domain, there is an autoinhibitory region consisting of a KRR motif that mimics a classical NLS (Harreman et al. 2003). Dissociation of importin β from the IBB domain exposes the autoinhibitory region, which now folds over and competes with the NLS of the cargo for binding to the NLS-binding domain of the importin α, resulting in the release of the cargo (Harreman et al. 2003; Lange et al. 2007). The presence of exportin CAS in the nucleus (transport receptor that mediates export of importin α into the cytoplasm) bound to RanGTP accelerates the dissociation of the cargo protein from importin α (Gilchrist et al. 2002; Kutay et al. 1997). Both the free importin α and the importin β-RanGTP complex are now exported to the cytoplasm via the exportin pathway (Izaurralde et al. 1997; Kutay et al. 1997). Importin β can also directly bind to and import some proteins into the nucleus independent of importin α (Sorokin et al. 2007). For instance, human importin β2, also known as transportin, can directly

bind and import hnRNP A1 (Siomi and Dreyfuss 1995; Siomi et al. 1997). Similarly, nuclear import of replication protein A (RPA) via importin β is importin-α-independent. However, the importin β-RPA interaction does require the adaptor protein XRP1α (Jullien et al. 1999).

Some RPs have been reported to use different importins for their nuclear localization. Importin β and importin β-like receptors transportin, RanBP5 and RanBP7 all can directly bind and import human RPS7, RPL23a and RPL5 to the nucleus (Jakel and Gorlich 1998). The nuclear localization of mouse RPL12 is mediated by importin 11, which belongs to a subgroup of karyopherin that includes exportin CAS (Plafker and Macara 2000; 2002). Importin β3 is required for the nuclear import of human RPL7, however, it is not clear whether importin α plays any role in this process (Chou et al. 2010).

1.5. Arabidopsis ribosomal protein gene family *RPL23a*

The Arabidopsis LSU protein gene family *RPL23a* consists of two expressed members – *RPL23aA* (AT2G39460) and *RPL23aB* (AT3G55280) and belongs to the *L23/L25* family that is conserved in all three domains of life. Some members of the L23/L25 family have been shown to be primary rRNA binders and bind directly to domain III, an evolutionary conserved site on the 23S or 23S-like rRNA (El-Baradi et al. 1987; El-Baradi et al. 1984; El-Baradi et al. 1985). L23/L25 is positioned at the exit of the polypeptide tunnel and mediates the interaction between the ribosome with trigger factor and SRP (Kramer et al. 2002; Pool et al. 2002).

Arabidopsis RPL23aA has been shown to complement yeast *l25*, establishing it as a functional homologue of RPL25 (McIntosh and Bonham-Smith 2001). Gene silencing of *RPL23aA* via RNA interference (RNAi) resulted in a pleiotropic phenotype resembling impaired auxin perception and distribution; defects included growth retardation, irregular leaf and root morphology, abnormal phyllotaxy and vasculature and loss of apical dominance. By contrast, a T-DNA knock out of *RPL23aB* had no obvious effect on phenotype (Degenhardt and Bonham-Smith 2008).

RPL23aA and RPL23aB are 94% identical at the amino acid level and both isoforms localize to the nucleolus (Degenhardt and Bonham-Smith 2008). Expression profiling indicated that both *RPL23aA* and *RPL23aB* have highest expression in mitotically-active tissues such as bud, flower, elongating carpel, as well as root and stem, while the lowest expression was detected in mitotically inert mature leaf and bract. However, *RPL23aA* expression was more abundant in all tissues studied compared to *RPL23aB*. IAA and BAP treatment up regulated

expression of both genes while ABA treatment repressed expression of each. Despite the two proteins being highly similar at the amino acid level, the two genes, *RPL23aA* and *RPL23aB* only share ~40–50% primary sequence identity within their 5' regulatory regions and respond differentially to cold-, wounding- and copper-stress (McIntosh and Bonham-Smith 2005).

In yeast L25, amino acid residues 11-17 (**KK**AVV**K**G) and 18-28 (TNG**KKALK**VRT) have been shown to have NLS activity (Schaap et al. 1991). Therefore, we hypothesized that in RPL23aA, the N-terminal basic motif ^{10}**KK**ADPKA**K**AL**K**^{20} is required for nuclear localization. Previously, it has been shown that while RPL23aA efficiently localizes to the nucleolus, RPL23aB often localizes to the periphery of the nucleolus (13.6% of cells) or is excluded from the nucleolus (19.7% of cells) (Degenhardt and Bonham-Smith 2008). The disruption of a putative nucleolin binding site in RPL23aB, which has ^{33}**K**PA**K**^{36} in place of ^{33}**KK**D**K**^{36}, could be a reason for inefficient nucleolar localization of RPL23aB. This suggests that ^{33}**K**PA**K**^{36} could possibility act as a nucleolar retention signal in RPL23aA (Degenhardt and Bonham-Smith 2008).

1.6. Objectives

In the work described in this thesis, I have defined nuclear/nucleolar localization signals of RPL23aA, RPL15 and RPS8, and analyzed the regulation of expression and subcellular localization of Arabidopsis RPs. The objectives are as follows:

1) To characterize nuclear/nucleolar localization signals of Arabidopsis RPL23aA.
2) To identify and compare nuclear/nucleolar localization signals of RPL23aA with those of RPL15 and RPS8.
3) To analyze regulation of expression of Arabidopsis RP genes.
4) To compare subcellular localization of five two-member Arabidopsis RP families.

CHAPTER 2. CHARACTERIZATION OF NUCLEAR/NUCLEOLAR LOCALIZATION OF ARABIDOPSIS RIBOSOMAL PROTEINS RPL23aA, RPL15A AND RPS8A

Ribosomal subunit assembly in the nucleolus is dependent on efficient targeting of ribosomal proteins (RPs) from the cytoplasm into the nucleus and nucleolus. Nuclear/nucleolar localization of a protein is generally mediated by one or more specific stretches of basic amino acids – Nuclear/Nucleolar Localization Signals (NLSs/NoLSs). In this study, I show that nucleolar localization of Arabidopsis RPL23aA is mediated by a specific number of basic motifs, rather than any single or specific combination of motifs. RPL23aA has eight putative NLSs (pNLSs). Site-directed mutagenesis of any single pNLS had no effect on nuclear or nucleolar localization. Mutation of all pNLSs (50% reduction in total basic charge of the protein) completely disrupted nucleolar localization, but had no effect on nuclear localization, confirming that these pNLSs are not required for nuclear localization, but are required for nucleolar localization (putative NoLSs). Subsequent combinatorial mutations showed that simultaneous mutation of any four pNoLSs (25% reduction in basic charge) did not affect nucleolar localization. However, serial mutations of the remaining pNoLSs disrupted nucleolar localization to varying degrees, with mutation of all eight pNoLSs resulting in 100% disruption. Specific NoLSs are not required for nucleolar localization of RPL23aA, however, combinations of pNoLSs resulting in an optimal overall basic charge and/or structure, are required. By contrast, in RPS8A and RPL15A, each with 10 pNLSs, mutation of just two and three N-terminal pNLSs, respectively, disrupted both nuclear and nucleolar localization. The differential signal requirements for nuclear and nucleolar localization, as demonstrated for RPL23aA, RPS8A and RPL15A suggest that different transport mechanisms probably govern the nuclear/nucleolar localization of these three RPs.

2.1. Introduction

Ribosomes are two-subunit macromolecular enzymatic complexes comprised of rRNAs and ribosomal proteins (RPs) that are responsible for protein synthesis in all organisms. RPs, synthesized in the cytoplasm, must be transported into the nucleus and nucleolus, a sub-nuclear compartment where they assemble with rRNAs to form the large and small (LSU and SSU) subunits of the ribosome. Hence nuclear and nucleolar targeting of RPs constitute an important step in ribosome biogenesis.

Most proteins localize to the nucleus through energy-dependent active transport processes via nuclear pore complexes (NPCs) (Breeuwer and Goldfarb 1990; Fahrenkrog and Aebi 2003; Lim et al. 2008a; Mosammaparast and Pemberton 2004; Poon and Jans 2005). Transport of protein cargos through NPCs is usually mediated by stretches of positively charged (basic) amino acids, primarily lysine (K) and arginine (R), forming nuclear localization signals (NLSs) in the cargo; these signals interact with negatively charged cytosolic nuclear transport receptors belonging to the importin/karyopherin family of proteins (Mosammaparast and Pemberton 2004). Importin α/β heterodimers 'carry' cargo proteins into the nucleus while they shuttle between the cytoplasm and the nucleus (Chook and Blobel 2001; Pemberton and Paschal 2005). Importin β docks the trimeric complex (cargo-importin α-importin β) to the NPC (Moroianu et al. 1995), from where the cargo-importins complex is translocated into the nucleus through the interaction of importin β with nucleoporins (Weis 2003).

There are two major classes of NLSs. The classical/canonical monopartite NLSs, primarily comprised of a single cluster of the basic amino acids lysine (K) and/or arginine (R) (Chelsky et al. 1989) and the bipartite NLSs comprised of two clusters of basic amino acids separated by a spacer (Dingwall et al. 1988). A classical monopartite NLS is comprised of at least four consecutive basic amino acids [e.g., SV40 large T antigen NLS - P**KKRK**V (Kalderon et al. 1984)], while a modification of this basic structure, resulting in the consensus sequence **K**-**K/R**-X-**K/R**, where X represents any amino acid, is also recognized [e.g., c-Myc NLS - PAA**KR**V**K**LD (Dang and Lee 1988)]. The consensus bipartite NLS is **K/R**-**K/R**-$X_{10\text{-}12}$-$\mathbf{K/R}_{3/5}$, where $\mathbf{K/R}_{3/5}$ represents at least three K or R out of five consecutive amino acids [e.g., NLS of nucleoplasmin (Dingwall et al. 1988)]. The NLS-binding domain of importin α has two binding pockets; the major binding pocket binds the monopartite NLS or the larger stretch of basic residues in the bipartite NLS, while the minor binding pocket binds the smaller stretch of basic residues in the bipartite NLS (Stewart and Rhodes 1999). From a screening of random peptide libraries to select peptides that bind to importin α, three other consensus NLSs have been identified; **KR**X(W/F/Y)XXAF, LG**KR**(**K/R**)(W/F/Y) and (**R**/P)XX**KR**(**K/R**)(^DE), where (^DE) represents any amino acid except D or E (Kosugi et al. 2009). The consensus sequence of NLSs that are bound directly by importin β2 has also been derived. This NLS called PY-NLS, which is structurally disordered, i.e. lacks secondary structure in its native, unbound state, has an overall basic composition with a central hydrophobic or basic motif followed by a C-terminal

consensus R/H/KX(2-5)PY motif (Lee et al. 2006). Despite the considerable effort to define consensus NLSs, nuclear localization of many proteins has been shown to occur in the absence of any consensus sequences (Nguyen Ba et al. 2009).

NLSs of some RPs have been defined. In yeast L25, the ortholog of Arabidopsis RPL23aA, deletion analysis suggested that the N-terminal 41 amino acids were required for nuclear localization, as a L25 protein lacking this region could not direct nuclear import of fused β-galactosidase (Rutgers et al. 1990). Within this region, amino acid residues 11-17 (**KK**AVV**K**G) and 18-28 (TNG**KKALK**VRT) have been shown to have NLS activity when linked to β-galactosidase. While amino acid residues 1-10 (MAPSAKATAA) did not act as an NLS by itself, it enhanced the nuclear localization mediated by the 11-17 fragment (Schaap et al. 1991). In yeast RPL3, the N-terminal 21 amino acids were identified as sufficient to localize β-galactosidase to the nucleus (Moreland et al. 1985). Yeast RPL29 contains two independent NLSs ^{6}**K**T**RK**H**R**G^{13} and ^{23}**K**H**RK**HPG^{29}, both of which independently can direct β-galactosidase to the nucleus. Human ribosomal protein RPL7 has three basic stretches of amino acids at the N-terminus; ^{18}L**KKKRR**NFAE^{27}, ^{28}L**K**I**KR**L**RKK**FAQ^{39} and ^{40}**K**ML**RK**A**RRK**LIY^{51}, all of which can target EGFP to the nucleus, although the latter had the stronger NLS activity (Chou et al. 2010; Ko et al. 2006). In addition, RPL7 has a bipartite NLS between residues 156 to 167 (**KR**GYG KIN**KKR**I), which was also identified as a required signal for nuclear localization (Chou et al. 2010; Ko et al. 2006). Xenopus RPL5 has two NLSs, NLS-1 (aa 1–25) and NLS-3 (aa 261–285), that resemble the classical NLS of nucleoplasmin (Claussen et al. 1999). Although both NLS-1 and -3 are capable of promoting nuclear transport of a heterologous protein, NLS-1 appears to play the major role in this process as it can bind strongly to different import receptors importin α, importin β, transportin and RanBP7 (Claussen et al. 1999).

Ribosomal subunit assembly occurs in the nucleolus, however, intra-nuclear trafficking of RPs to the nucleolus may not require specific mechanisms. The nucleolus is not a membrane-bound structure and as such proteins may simply diffuse into it from the nucleus. Diffusion would lead to equal distribution of proteins between the nucleus and the nucleolus, however, if these proteins have no function in the nucleus, their accumulation would be a waste of energy and resources. Hence, diffusion between the nucleus and nucleolus alone cannot explain the preferential nucleolar accumulation of proteins like RPs. It has been suggested that when

proteins diffuse into the nucleolus, nucleolar components such as rDNA, rRNA or other nucleolar proteins sequester them in the nucleolus, thereby removing them from the diffusion pool and facilitating further diffusion (Carmo-Fonseca et al. 2000). It has been proposed that nucleolar proteins, such as nucleolin and fibrilarin, concentrate around rDNA and act as hub proteins to which other nucleolar proteins bind and are retained in the nucleolus (Emmott and Hiscox 2009). Alternatively, proteins may localize to the nucleolus by interacting with other nucleus-nucleolus shuttling proteins (Boden and Teasdale 2008; Carmo-Fonseca et al. 2000). For example in mammals, protein phosphatase I (PP1) localizes to the nucleolus by interacting with and being carried by NOM1 (nucleolar protein with MIF4G domain 1) (Gunawardena et al. 2008).

Analyses of the nucleolar proteome have not identified any targeting motifs shared by all nucleolar proteins, suggesting that like nuclear localization, nucleolar localization is also regulated by diverse signals (Andersen et al. 2002; Scherl et al. 2002). Nucleolar localization signals (NoLSs) identified to date, range in size from seven to 30 amino acids and like NLSs, are enriched with K and R residues (Emmott and Hiscox 2009). The nucleolar localization signal (NoLS) of a protein could be part of its NLS or a protein may have distinct NLSs and NoLSs. Human FGF2 (fibroblast growth factor-2) contains a C-terminal 17 amino acid non-canonical bipartite NLS (^{114}TY**RSRK**YTSWYVAL**KR**T^{130}), a portion of which also acts as its NoLS; while K^{119} and R^{129} play a key role in both nuclear and nucleolar localization, K^{128} contributes only to nucleolar localization (Sheng et al. 2004). In contrast, in human parafibromin, distinct signals mediate nuclear and nucleolar localization. Parafibromin contains a bipartite NLS and three distinct NoLSs; ^{76}**RR**AATENIPVV**RR**PD**RK**^{92}, ^{192}**KKR**^{194} and ^{393}**KK**QGCQ**R**ENETLIQ**RRK**^{409} (Hahn and Marsh 2007). In both cases, precise identification of NoLSs is a difficult task as disruption of nuclear localization is always associated with disruption of nucleolar localization. A sequence alignment of the NoLSs of 17 human nucleolar proteins, including nucleolin, identified R/K-R/K-X-R/K as a common motif in these NoLSs, which is also the consensus sequence for a monopartite NLS (Horke et al. 2004). The NoLS of Rev, a nucleolar protein of human immunodeficiency virus type I (HIV-I) consists of clusters of Rs (^{35}**R**QA**RR**N**RRRR**W**R**E**R**Q**R**^{50}), that also encompasses the NLS (Kubota et al. 1989). The NoLS of *Toxoplasma gondii*, GRA10, the causative pathogen of toxoplasmosis, is comprised of repeats of Ks and Rs, ^{199}**RKKRRR**SG**KKKR**G**KR**^{213} (Ahn et al. 2007), while the much smaller

NoLS, ^{29}IM**RRR**GL^{35}, of the polypeptide ligand angiogenin, a potent inducer of angiogenesis, is sufficient to target green fluorescent protein (GFP) to the nucleolus of rat hepatoma cells (Lixin et al. 2001).

While the NLS/NoLSs of many viral, yeast and mammalian proteins have been identified and defined (Emmott and Hiscox 2009; Freitas and Cunha 2009; McLane and Corbett 2009; Nguyen Ba et al. 2009), little is known about the NLS/NoLSs of plant proteins, particularly RPs. In this work, I demonstrate for the first time that the nucleolar localization of a protein can be mediated by the overall basic composition of the protein rather than by any one or combination of specific motif(s). In Arabidopsis RPL23aA, mutating any combination of eight basic putative NLSs/NoLSs, which disrupts more than 25% of the normal positive charge of the RP, affects nucleolar localization in proportion to the percentage charge disruption. Mutation of all eight pNLSs/pNoLSs, a 50% reduction in basic charge, resulted in a total loss of nucleolar localization, with no effect on nuclear localization. In contrast, mutation of just two of 10 basic motifs in RPS8A and three of 10 basic motifs in RPL15A disrupted both nuclear and nucleolar localization of each RP.

2.2. Materials and methods

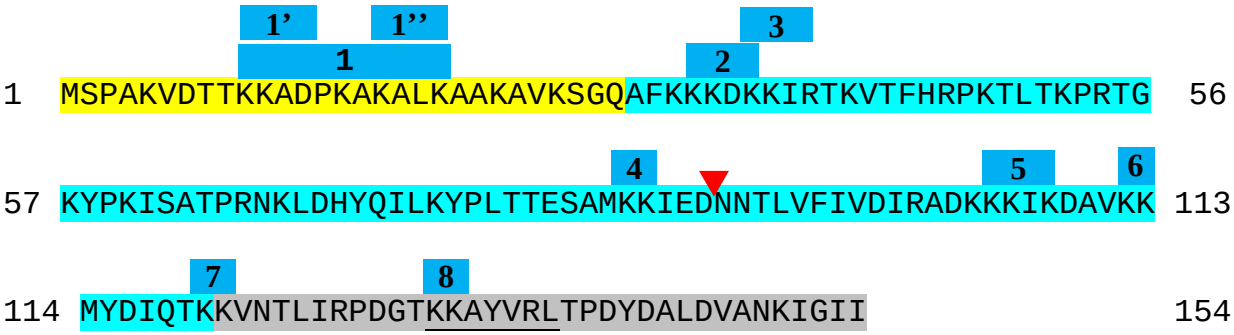
2.2.1. Plant material

Tobacco (*Nicotiana tabacum*) cultivar Petit Havana was used for all transient expression experiments and was grown in a growth chamber with a 23^{o}/18^{o}C temperature regime and a 16 h/8 h photoperiod of ~170 µmol photons m^{-2} sec^{-1}. Six-week-old plants were used for agroinfiltration.

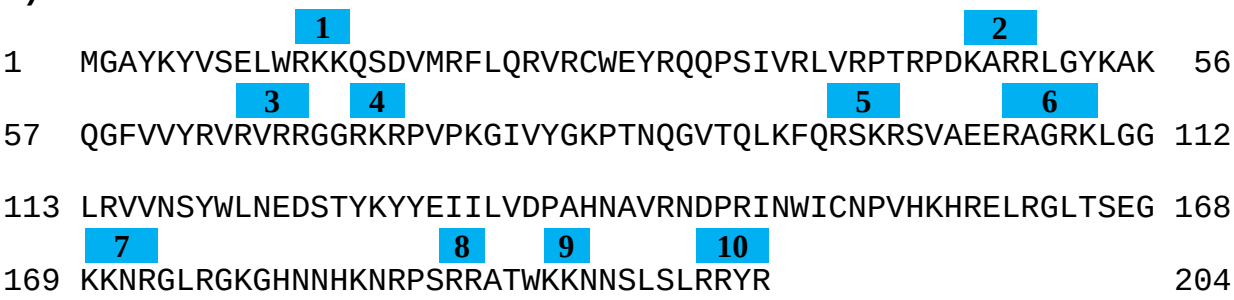
2.2.2. Site-directed mutagenesis

The ORF of *RPL23aA* minus the stop codon was amplified by RT-PCR from total RNA (Degenhardt and Bonham-Smith 2008), while the ORFs of *RPS8A*, and *RPL15A* minus the respective stop codons were amplified (see Appendix A for primers) from cDNA clones obtained from the Arabidopsis Biological Resource Center (ABRC). All ORFs were cloned into the unique *Eco*RI/*Bam*HI sites of pBluescript (pBSKS+). Primers for site-directed mutagenesis (SDM) of putative NLSs (pNLSs) of RPS8A, RPL15A and RPL23aA (Figure 2.1) were designed with mismatches in codons to replace basic lysines (K) and arginines (R) with neutral alanines;

A) RPL23aA



B) RPL15A



C) RPS8A

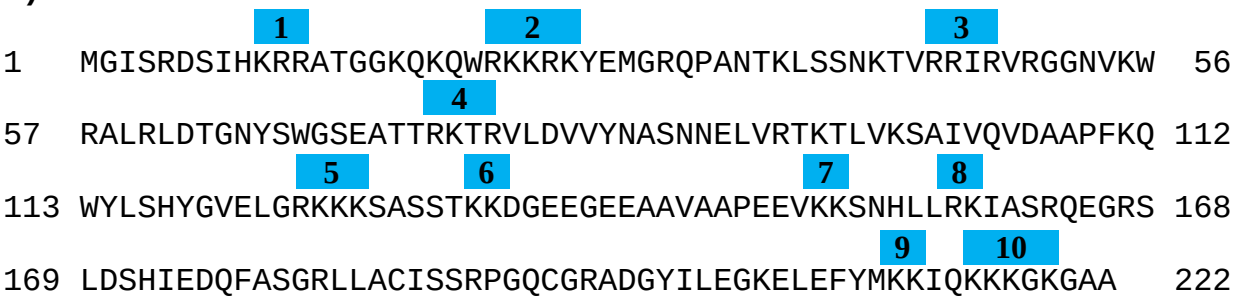


Figure 2.1. pNLSs of A) RPL23aA, B) RPL15A and C) RPS8A. Sequence motifs of two or more of the basic amino acids lysine (K) or arginine (R), as indicated by numbered blue boxes, are considered pNLSs. In RPL23aA sequence, Yellow, blue and grey shading indicates the N-terminal 29, mid-91, and C-terminal 34 amino acids, respectively. Red triangle indicates the start of the C-terminal 64 amino acids. The motif identified as 26S rRNA binding site in yeast L25 is underlined.

in RPL23aA, pNLS1 ^{10}**KK**ADP**K**A**K**AL**K**^{20} was also mutated to ^{10}TTDAP**K**ATDGT^{20}. pBSKS+ plasmids carrying *RPS8A*, *RPL15A* and *RPL23aA* ORFs were amplified; cycle 1 – 95^{o}C for 30 sec, cycle 2 to 16 - 95^{o}C for 30 sec, 55^{o}C for 1 min, 68^{o}C for 3 min, 30 sec using *Pfu* DNA polymerase. The resulting amplicons were treated with *DpnI* and cloned into *E.coli* DH5α. The sequences of all cloned SDM products were confirmed by automated sequencing [National Research Council – Plant Biotechnology Institute (NRC/PBI), Saskatoon, SK, Canada].

Percentage reduction in total basic charge of mutant proteins was calculated as number of basic amino acids mutated/total number of basic amino acids x 100. pI (isoelectric point) of wild type and mutant proteins were calculated using ExPASy pI calculator (http://expasy.org/tools/pi_tool.html).

2.2.3. Fluorescent protein fusion constructs

The binary vector pGREENI0029 (Hellens et al. 2000), modified by the addition of a tandem repeat of the CaMV 35S promoter (35S) in the unique *Apa*I/*Eco*RI restriction sites, a GST linker in the *Bam*HI/*Hind*III sites, a monomeric red fluorescent protein (mRFP) sequence (Campbell et al. 2002) in the *Hind*III/*Spe*I sites, and a nopaline synthase (nos) poly(A) signal (terminator) in the *Spe*I/*Not*I sites (Degenhardt and Bonham-Smith 2008) was used for all fusion protein constructs. The *RPL23aA* ORF and its pNLS1-mutated forms were subcloned from pBSKS+ into the unique *Eco*RI/*Bam*HI sites of pGREENI0029, resulting in pGREENI0029-*35S-L23aA/*Δ*pNLS1-GST-mRFP-nos*. The addition of the GST linker increased the mass of the fusion protein beyond the exclusion limit of the nuclear pore complex (>60 kDa) (Degenhardt and Bonham-Smith 2008), preventing diffusion of the resulting fusion proteins into the nucleus. Two other sets of constructs were made where mRFP was replaced with mCherry (an RFP variant that matures faster and is more photostable (Shaner et al. 2004)) or enhanced green fluorescent protein (EGFP; ClonTech, Palo Alto, CA). The latter form of pGREENI0029 was used to clone pNLS2 to pNLS8-mutated RPL23aA, various regions of the *RPL23aA* ORF (deletion analysis), wild type RPS8A, RPL15A and their pNLS mutants.

AtFIBRILLARIN2, with a C-terminus EGFP tag, under the control of the 35S promoter in the binary vector pCAMBIA1380 was used as a nucleolar marker (Degenhardt and Bonham-Smith 2008). A second nucleolar marker construct pGREENI0029-*35S-AtFIBRILLARIN2-GST-mRFP-nos* was also used.

2.2.4. Transient expression in tobacco and confocal microscopy

The *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al. 1983) was cotransformed with pGREEN constructs and pSOUP by electroporation. pSOUP provides *in trans* replication function for pGREEN (Hellens et al. 2000). Cultures of transformed *A. tumefaciens* (OD_{600} - 0.2) were infiltrated into tobacco leaf epidermal cells following a previously described protocol (Sparkes et al. 2006). For co-expression with a nucleolar marker, a culture of *A. tumefaciens* carrying the *AtFIBRILLARIN2* (*FIB2*) construct was mixed with cultures of *A. tumefaciens* carrying different fluorescent fusions (final OD_{600} 0.1 and 0.2, respectively) and infiltrated into tobacco leaf epidermal cells. Post-infiltration (72 h), live cell imaging was carried out using an inverted Zeiss LSM 510 META CLSM (Jena, Germany). Tissue was DAPI stained 10 h before live cell imaging. Small segments (~0.5 cm^2) of infiltrated leaves were dipped in DAPI solution (2 µg/ml), and vacuum infiltrated for two h followed by an eight h incubation at room temperature. For imaging of EGFP-tagged fusion proteins, an Argon laser (488 nm) was used with a 505-530 nm bandpass filter, whereas for mRFP/mCherry tagged fusion proteins, a HeNe1 laser (543 nm) was used with a 585-615 nm bandpass filter. For imaging of DAPI staining of the nucleus a 405 nm diode was used with a 420-460 nm bandpass filter. Images were processed with the Zeiss LSM Image Browser and Adobe Photoshop software (San Jose, CA, USA) and the cytoplasmic, nuclear and nucleolar intensity of EGFP fusions were measured using McMaster Biophotonics "ImageJ for Microscopy," a collection of imageJ plugins (http://www.macbiophotonics.ca/imagej/). Statistical analysis (student's t-test and ANOVA with n=30 transformed cells, r = 3) was done using Analysis ToolPak of Microsoft Office 2007.

2.2.5. Yeast two hybrid assay

Importin α1 to 6 cDNA clones were obtained from the ABRC and importin α9 from Riken (Japan). The importin α9 clone contained an in-frame stop codon, therefore, the importin α9 ORF was first amplified from this clone, sub cloned into unique *Eco*RI/*Bam*HI sites of pBSKS+ and the stop codon was changed to glutamic acid^{364} by site-directed mutagenesis. Importin α 1-6 and 9 ORFs were cloned into the unique *Sal*I/*Not*I sites of the GAL4 DB (DNA binding domain) vector pDBLeu and the RPL23aA ORF was cloned into the unique *Sal*I/*Not*I sites of the GAL4 AD (activation domain) vector pPC86. These two vectors were coexpressed in

yeast strain MaV203 and transformants were subjected to two hybrid selection on supplemented synthetic dextrose medium lacking leucine, tryptophan and histidine but containing 15 mM 3-amino-1,2,4-triazole. Arabidopsis cyclin-dependent protein kinase inhibitor (ICK1) in pPC86 and Cyclin D3;1 (CYCD3;1) in pDBLeu were used as a positive control (Wang et al. 1998).

2.3. Results

2.3.1. Mutation of pNLS ^{10}KKADPKAKALK^{20} in RPL23aA did not affect nuclear or nucleolar localization

In yeast L25, the orthologue of Arabidopsis RPL23aA, the N-terminal 28 amino acids are required for nuclear localization (Schaap et al. 1991). In RPL23aA, ^{10}**KK**ADP**K**A**K**AL**K**^{20} is the only stretch of basic amino acids in the N-terminal 30 amino acids and hence it was considered as a pNLS. This sequence was mutated to ^{10}TTDAP**K**ATDGT^{20} by SDM (Appendix A) to disrupt the positive charge. Confocal imaging of the localization of wild type RPL23aA (Figure 2.2A-1) and ΔpNLS1-RPL23aA-mRFP (Figure 2.2A-2) showed no difference; both proteins localized to the nucleus and nucleolus, indicating that the ^{10}**KK**ADP**K**A**K**AL**K**^{20} motif is not an absolute requirement for nuclear or nucleolar localization of Arabidopsis RPL23aA. The mCherry- or EGFP- tagged ΔpNLS1-RPL23aA (Figures 2.2A-3, 2.2B-2) also showed wild-type RPL23aA nuclear and nucleolar localization pattern. In addition to mutating Ks to Ts in this pNLS, which may result in phosphorylation of the mutant RPL23aA, Ks were replaced with As, resulting in ^{10}AAADPAAAALA^{20}. These mutations also did not affect nuclear or nucleolar localization of the resulting mutant RPL23aA (Figure 2.2B-3).

2.3.2. Individual pNLS mutations had no effect on localization, while simultaneous mutations did affect nucleolar localization of RPL23aA

Apart from ^{10}**KK**ADP**K**A**K**AL**K**^{20} (pNLS1), RPL23aA has three other monopartite pNLSs; ^{33}**KK**D**K**^{36} (pNLS2), ^{36}**KK**I**R**^{39} (pNLS3), ^{105}**KK**I**K**^{108} (pNLS5) and one bipartite pNLS ^{105}**KKIK**DAV**KK**^{113} (pNLS6). In addition, there are three small stretches of basic amino acids ^{86}**KK**^{87} (pNLS4), ^{120}**KK**^{121} (pNLS7), ^{132}**KK**^{133} (pNLS8) that could possibly be pNLSs (Figure 2.1; Nguyen Ba et al. 2009). These pNLSs are highly conserved between the two Arabidopsis RPL23a isoforms, RPL23aA and RPL23aB that are 94.8% identical, with the exception that pNLS2 in RPL23aB has ^{33}**K**PA**K**^{36} in place of ^{33}**KK**D**K**^{36}. Mutation of each pNLS of RPL23aA individually resulted in no effect on nuclear or nucleolar localization of RPL23aA (Figure 2.3A),

Figure 2.2. Role of pNLS1 in RPL23aA nuclear/ nucleolar localization. Subcellular localization of fluorescent-tagged RPL23aA in tobacco leaf epidermal cells transiently coexpressing the nucleolar marker FIB2-EGFP [left panels in **(A)]** or FIB2-mRFP [left panels in **(B)]** and wild type/L23aA-ΔpNLS1-mRFP/mCherry/EGFP (mid panels). Images in the right panels are a merge of left and mid panel images to show signal overlap. White arrow indicates the nucleus, white arrowhead the nucleolus, and transparent white arrowhead cajal bodies [nuclear structures involved in the formation of small nuclear ribonucleoprotein particles (snRNPs) and snoRNPs (Beven et al., 1995; Kim et al., 2007)]. ΔpNLS1(a) refers to the mutation of pNLS1 to ^{10}TTDAPKATDGT^{20} and ΔpNLS1(b) to ^{10}AAADPAAAALA^{20}. Scale bar = 10 μm.

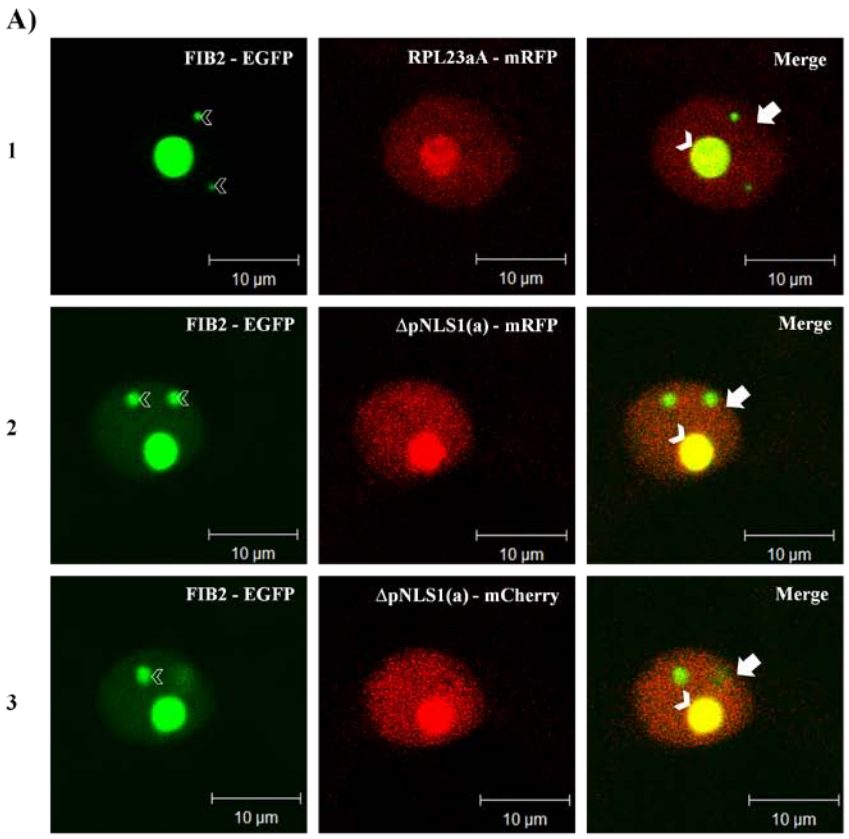
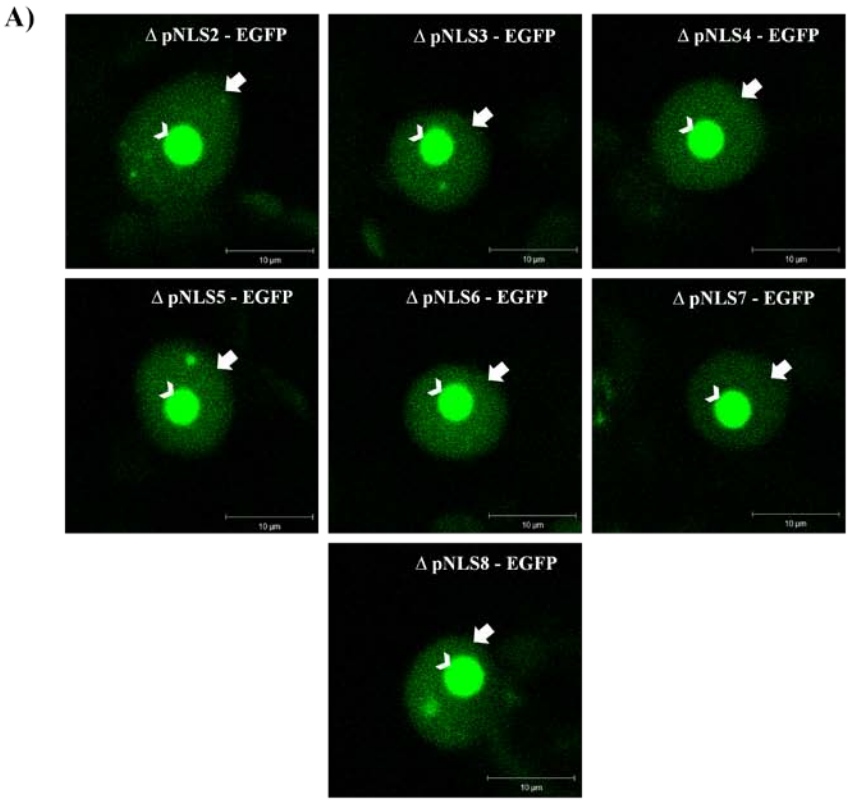
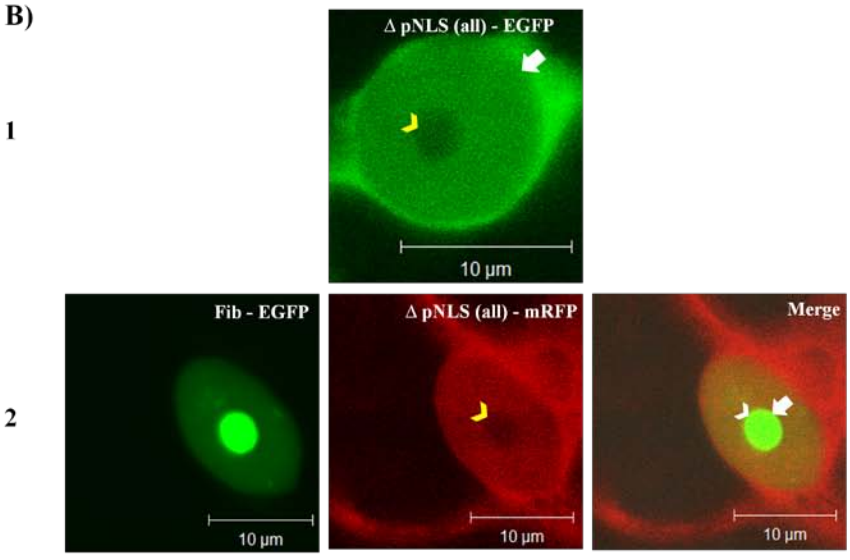
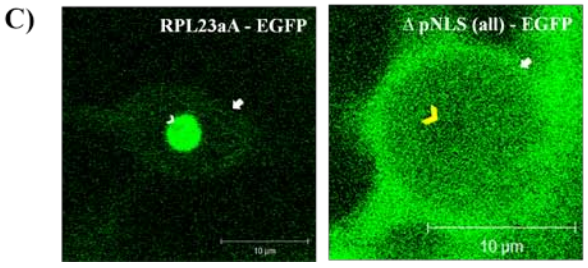

A)
1
FIB2 - EGFP
RPL23aA - mRFP
Merge
10 µm
2
FIB2 - EGFP
ΔpNLS1(a) - mRFP
Merge
10 µm
3
FIB2 - EGFP
ΔpNLS1(a) - mCherry
Merge
10 µm
B)
1
FIB2 - mRFP
RPL23aA - EGFP
Merge
10 µm
2
FIB2 - mRFP
ΔpNLS1(a) - EGFP
Merge
10 µm
3
FIB2 - mRFP
ΔpNLS1(b) - EGFP
Merge
10 µm

Figure 2.3. Individual mutations versus simultaneous mutations of pNLSs of RPL23aA. CLSM images of tobacco leaf epidermal cells transiently expressing **(A)** ΔRPL23aA-EGFP, with various individual pNLSs mutated and **(B)** 1. ΔRPL23aA-EGFP, with all pNLSs [ΔpNLS (all)] mutated 2. FIB2–EGFP (left panel), ΔpNLS (all)-mRFP (mid panel), merge (right panel). **(C)** RPL23aA-EGFP (left panel) and ΔpNLS (all)-EGFP (right panel) driven by the RPL23aA native promoter. Nucleolar exclusion is indicated by yellow arrowhead. White arrow indicates the nucleus and white arrowhead the nucleolus. Scale bar = 10 μm.


A)
Δ pNLS2 - EGFP
Δ pNLS3 - EGFP
Δ pNLS4 - EGFP
Δ pNLS5 - EGFP
Δ pNLS6 - EGFP
Δ pNLS7 - EGFP
Δ pNLS8 - EGFP


B)
1
Δ pNLS (all) - EGFP
10 µm
2
Fib - EGFP
Δ pNLS (all) - mRFP
Merge
10 µm
10 µm
10 µm


C)
RPL23aA - EGFP
Δ pNLS (all) - EGFP
10 µm

indicating possible redundancy of NLSs. It should be noted that although mutation of pNLS2 and pNLS6 disrupted nucleolar localization in ~13 and ~12% cells, respectively, simultaneous mutation of both signals disrupted nucleolar localization in only ~4% cells, similar to a wild type disruption of ~3% cells (Table 2.1). However, mutating all eight pNLSs simultaneously completely disrupted nucleolar localization (Figure 2.3B1, 2), whereas nuclear localization was not affected. Hence, hereafter I refer to these basic motifs as putative nucleolar localization signals (pNoLSs). I also expressed RPL23aA-EGFP and RPL23aA-ΔpNoLS (all)-EGFP from the native RPL23aA promoter. Although expression of these constructs was weaker compared to 35S-driven constructs, the nuclear and nucleolar localization patterns were the same as previously observed for 35S-RPL23aA and 35S-RPL23aA-ΔpNoLS (all) (Figure 2.3C).

2.3.3. Simultaneous mutation of pNoLS2, 5, 6 and 3 did not affect nucleolar localization, but serial mutation of the remaining pNoLSs increasingly disrupted nucleolar localization

Simultaneous mutation of all pNoLSs resulted in complete disruption of nucleolar localization, therefore different combinations of pNoLSs were mutated to determine combinatorial effects. Simultaneous mutation of pNoLS2, 3, 5, and 6 did not affect nuclear or nucleolar localization (Figure 2.4A). However, when the remaining pNoLSs were additionally and serially mutated in the order pNoLS4, 7, 1 and 8, nucleolar localization was increasingly disrupted (Table 2.1), indicating that these four pNoLSs have an accumulative effect on nucleolar localization of RPL23aA. Disruption of nucleolar localization resulted in three patterns of RPL23aA distribution (Figure 2.4B); (a) peripheral nucleolar ring of fusion protein, (b) diffused nuclear-nucleolar pattern that may be a result of weak nucleolar retention of fusion protein (c) nucleolar exclusion of fusion protein. As the number of mutated pNoLSs increased, so did the number of cells showing disrupted nucleolar localization (Table 2.1, Figure 2.4C), eventually leading to nucleolar exclusion in 100% of cells when all eight pNoLSs were mutated.

2.3.4. Nucleolar localization of RPL23aA requires a combined number of pNoLSs, rather than any specific pNoLSs

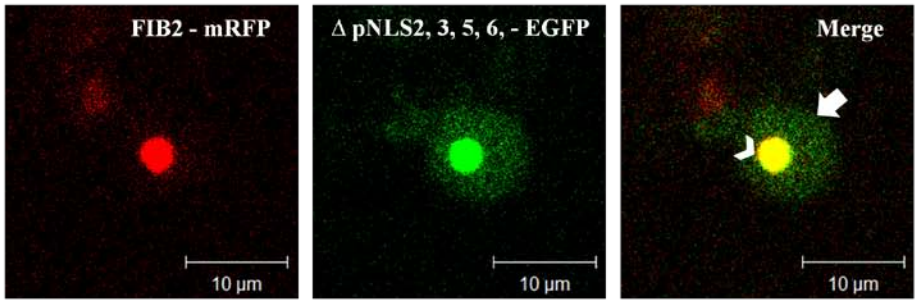
To confirm the cumulative requirement of pNoLS8, 7, 4 and 1 for nucleolar localization, only these four pNoLSs were serially mutated (Table 2.2). None of the resulting mutants showed any disruption of nucleolar localization similar to that of RPL23aA-ΔpNoLS (all)-EGFP (Table 2.2). In fact, more than 90% of transformed cells showed wild type nucleolar localization

Table 2.1. Percentage of cells showing the three different patterns of nucleolar localization in various pNoLS mutants of RPL23aA [n=30 transformed cells, r =3 (scoring of localization patterns in transformed epidermal cells of three different plants)].

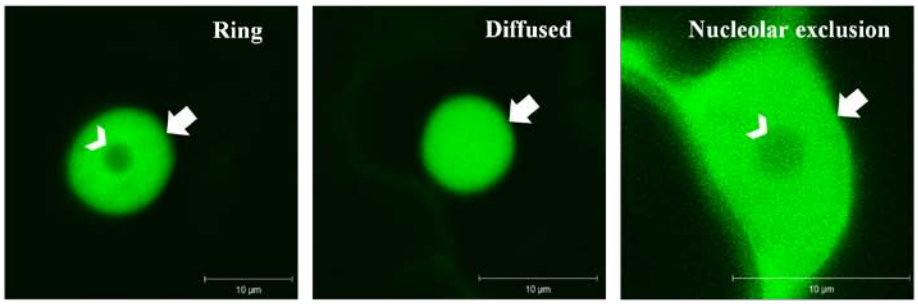
Details of mutations	% Reduction in total basic charge	pI	Wild type localization	Disrupted nucleolar localization			
				Diffused	Ring	Nucleolar exclusion	Total
Wild type RPL23aA	0	10.20	97	0	3	0	3
ΔpNLS1	10	10.12	99	0	1	0	1
ΔpNLS2	7.5	10.14	87	1	11	1	13
ΔpNLS3	7.5	10.11	92	1	7	0	8
ΔpNLS5	7.5	10.14	97	1	0	2	3
ΔpNLS6	12.5	10.10	88	0	6	7	12
ΔpNLS4	5	10.16	99	0	0	1	1
ΔpNLS7	5	10.16	98	0	2	0	2
ΔpNLS8	5	10.16	90	0	10	0	10
ΔpNLS2 + 5 + 6	20	10.02	96	0	2	2	4
ΔpNLS2 + 5 + 6 + 3	25	9.94	100	0	0	0	0
ΔpNLS2 + 5 + 6 + 3 + 4	30	9.87	54	20	19	7	46
ΔpNLS2 + 5 + 6 + 3 + 4 + 7	35	9.79	29	58	11	2	71
ΔpNLS2 + 5 + 6 + 3 + 4 + 7 + 1’	40	9.70	0	96	1	3	100
ΔpNLS2 + 5 + 6 + 3 + 4 + 7 + 1’ + 1’’	45	9.57	0	12	0	88	100
ΔpNLS2 + 5 + 6 + 3 + 4 + 7 + 1’ + 1’’ + 8	50	9.40	0	0	0	100	100

Figure 2.4. Simultaneous mutation of pNoLS2, 3, 5 and 6 of RPL23aA had no effect on nucleolar localization, but serial mutation of the remaining pNoLSs increasingly disrupted nucleolar localization. (A) CLSM images of tobacco leaf epidermal cells transiently coexpressing nucleolar marker FIB2-mRFP (left panel) and RPL23aA-ΔpNoLS 2, 3, 5, 6-EGFP (mid panel). **(B)** Serial mutations of the remaining pNoLSs (4, 7, 1, 8) resulted in the three disrupted patterns of nucleolar localization; (1) ring structure (accumulation of fusion protein at the periphery of the nucleolus), (2) diffused pattern (no distinct nucleolar to nuclear signal), (3) nucleolar exclusion (no nucleolar retention of fusion protein). White arrow indicates the nucleus and white arrowhead the nucleolus. Scale bar = 10 μm. **(C)** Percentage (+SE) of transformed leaf epidermal cells showing the different patterns of nucleolar localization in wild type and ΔpNoLS mutants of RPL23aA (n=30, r=3).

A)


FIB2 - mRFP
Δ pNLS2, 3, 5, 6, - EGFP
Merge
10 µm
10 µm
10 µm

B)


Ring
Diffused
Nucleolar exclusion

C)

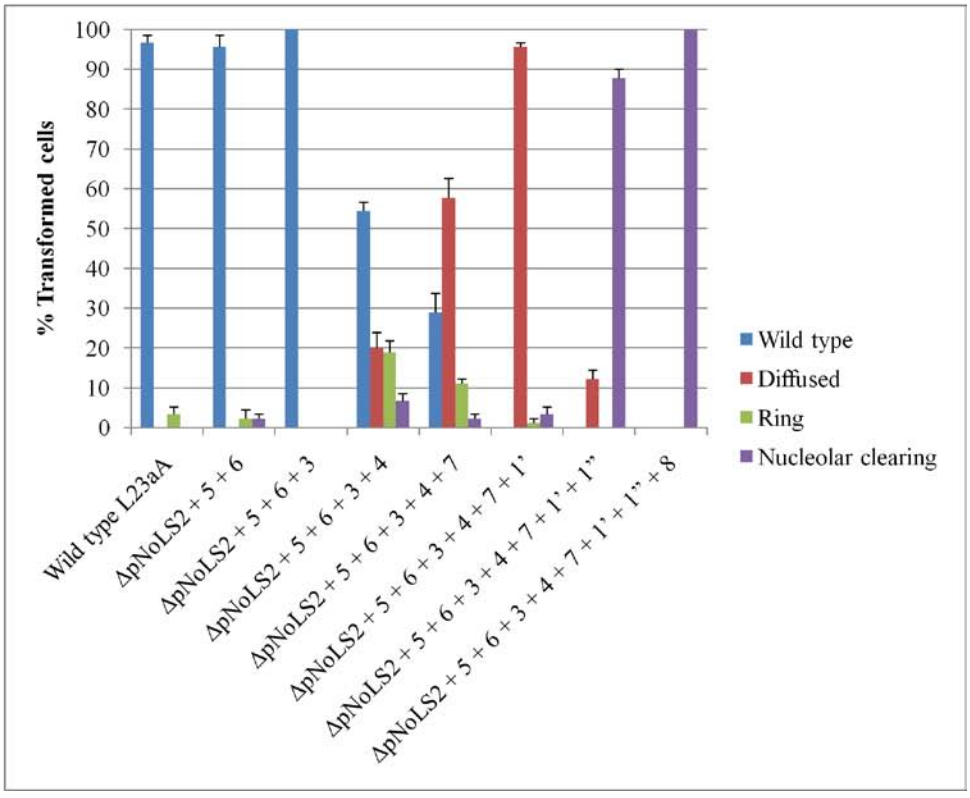

% Transformed cells
100
90
80
70
60
50
40
30
20
10
0
Wild type L23aA
ΔpNoLS2 + 5 + 6
ΔpNoLS2 + 5 + 6 + 3
ΔpNoLS2 + 5 + 6 + 3 + 4
ΔpNoLS2 + 5 + 6 + 3 + 4 + 7
ΔpNoLS2 + 5 + 6 + 3 + 4 + 7 + 1'
ΔpNoLS2 + 5 + 6 + 3 + 4 + 7 + 1' + 1''
ΔpNoLS2 + 5 + 6 + 3 + 4 + 7 + 1' + 1'' + 8
Wild type
Diffused
Ring
Nucleolar clearing

Table 2.2. Percentage of cells showing the three different patterns of nucleolar localization when only pNoLS 8, 7, 4, and 1 of RPL23aA, were serially mutated (n=30 transformed cells, r=3).

Details of mutations	% Reduction in total basic charge	pI	Wild type localization	Disrupted nucleolar localization			
				Diffused	Ring	Nucleolar exclusion	Total
ΔpNLS8	5	10.16	90	0	10	0	0
ΔpNLS8 + 7	10	10.12	94	3	2	0	6
ΔpNLS8 + 7 + 4	15	10.07	99	1	0	0	1
ΔpNLS8 + 7 + 4 + 1’	20	10.02	93	0	7	0	7
ΔpNLS8 + 7 + 4 + 1’ + 1’’	25	9.97	94	4	1	0	6

patterning, with no cells showing nucleolar exclusion (Table 2.2). In light of this unexpected result, the remaining four pNoLSs were serially mutated, in addition to ΔpNoLS8, 7, 4 and 1. As indicated previously, serial mutations of pNoLS2, 3, 5 and 6, in addition to ΔpNoLS8, 7, 4 and 1, increasingly disrupted nucleolar localization, with 100% cells showing nucleolar exclusion when all eight pNoLSs were mutated (Table 2.3). These results suggest that nucleolar localization of RPL23aA is independent of specific pNoLSs and that nucleolar localization results from a combined number of pNoLSs and the total basic charge of the protein. A reduction of more than 25% of the basic charge of RPL23aA disrupted nucleolar localization in proportion to the extent of the reduced charge; a 50% reduction resulted in complete disruption of nucleolar localization (100% nucleolar exclusion).

2.3.5. The N-terminus is dispensable, while the C-terminus is required for nucleolar localization of RPL23aA

Considering the N-terminus requirement for nuclear localization of yeast RPL25, both N- and C-terminal deletions of RPL23aA were tested for nucleolar localization. In contrast to RPL25, deletion of the N-terminal 29 amino acids (containing pNoLS1) of RPL23aA had no effect on nuclear or nucleolar localization of RPL23aA (Table 2.4). However, deletion of the C-terminal 34 amino acids resulted in a disruption of nucleolar retention in more than 95% of transformed cells although this region contains only one pNoLS (Table 2.4). This C-terminal 34 amino acids contains the conserved motif ^{132}**KK**AYV**R**L^{138}, which has been identified as the 26S rRNA binding motif in yeast L25 (Kooi et al. 1994). Mutating the two lysines (pNoLS8) in this motif did not disrupt nucleolar localization (Figure 2.3A), however, mutating the entire motif to ^{132}AAAAAAA^{138} resulted in a diffused nuclear/nucleolar pattern in 89% of transformed cells and a peripheral nucleolar ring pattern in 5% of transformed cells. In the remaining 6% of transformed cells showing wild type localization pattern, the distinction between nuclear and nucleolar signal was significantly reduced (ratio of nucleolar to nuclear signal intensity; wild type = 2.02, Δ**KK**AYV**R**L = 1.21, P = 8.32945E-10, Student's t-test).

Table 2.3. Percentage of cells showing the three different patterns of nucleolar localization when pNoLSs 2, 3, 5, and 6 of RPL23aA were serially mutated, in addition to pNoLS 8, 7, 4, and 1 (n=30 transformed cells, r=3).

Details of mutation	% Reduction in total basic charge	pI	Wild type localization	Disrupted nucleolar localization			
				Diffused	Ring	Nucleolar exclusion	Total
ΔpNLS8 + 7 + 4 + 1’ + 1’’ + 2	32.5	9.86	0	71	11	18	100
ΔpNLS8 + 7 + 4 + 1’ + 1’’ + 2 + 3	37.5	9.75	0	92	0	8	100
ΔpNLS8 + 7 + 4 + 1’ + 1’’ + 2 + 3 + 5	45	9.57	0	0	0	100	100
ΔpNLS8 + 7 + 4 + 1’ + 1’’ + 2 + 3 + 5 + 6	50	9.40	0	0	0	100	100

Table 2.4. Percentage of cells showing the three different patterns of nucleolar localization when different segments of RPL23aA were deleted (n=30 transformed cells, r=3). NΔ29 = deletion of N-terminal 29 amino acids. Mid 91 = fragment spanning amino acid residues 30 to 120. CΔ34 = deletion of C-terminal 34 amino acids.

Details of mutation	# of pNoLSs deleted	pI	Wild type localization	Disrupted nucleolar localization			
				Diffused	Ring	Nucleolar exclusion	Total
NΔ29	1	10.14	100	0	0	0	0
Mid 91	2	10.24	0	9	87	4	100
CΔ34	1	10.30	4	24	70	1	96
CΔ64	4	10.40	2	62	36	0	98

2.3.6. Mutation of pNoLSs increased nuclear and cytoplasmic intensity of RPL23aA-EGFP fusions

Mutating all eight pNoLSs in RPL23aA reduced the overall positive charge by 50% and left no stretches of two or more basic amino acids. As such, it was expected that this mutant *rpl23aA* protein would be excluded from the nucleus as importin-based nuclear localization relies on interactions with stretches of positively charged amino acids in the cargo protein (Wagstaff and Jans 2009). However, RPL23aA-ΔpNoLS (all) not only localized to the nucleus, but with an increased intensity relative to wild type (Figure 2.5A-1 and B; nuclear intensity was ~2.1-fold higher, P = 0.001). Cytoplasmic intensity was similarly increased (Figure 2.5A-2 and B; cytoplasmic intensity was ~6.3-fold higher, P = 1.528E-06, student's t-test).

2.3.7. RPL23aA did not interact with any of the importin αs

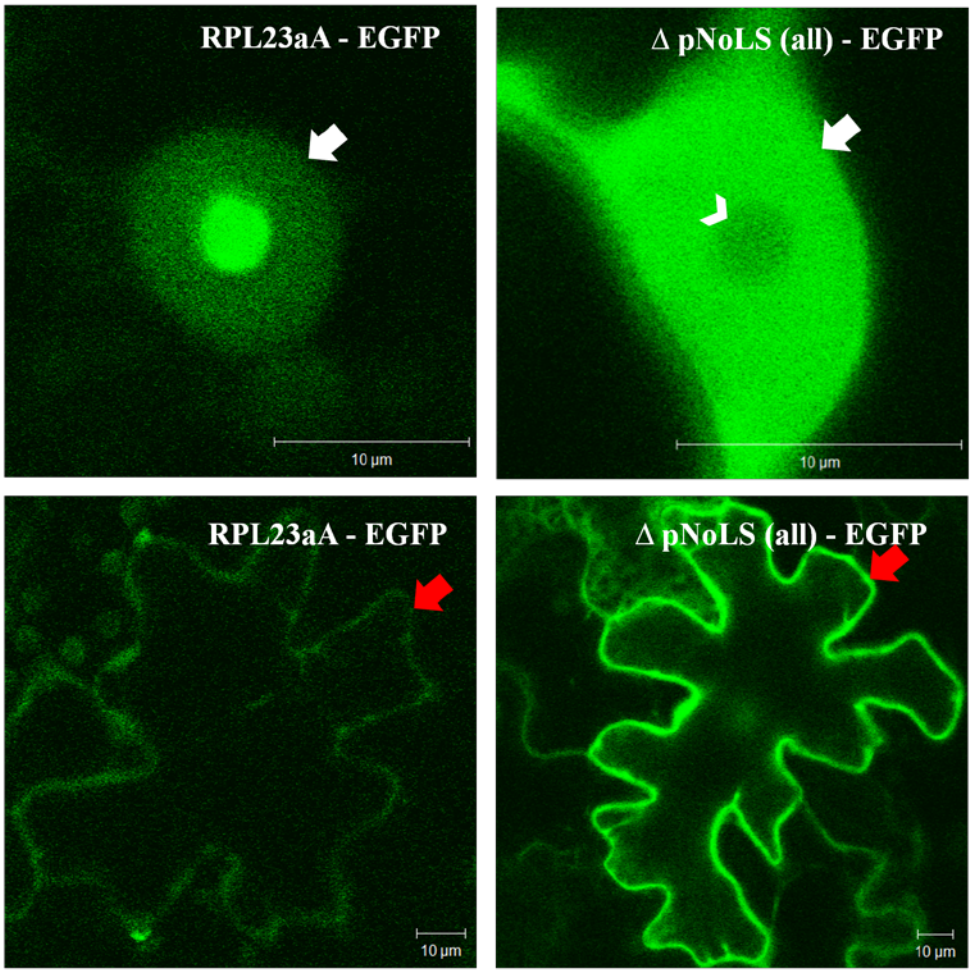
Importin α-mediated nuclear localization generally relies on interactions with stretches of positively charged amino acids in the cargo proteins. The observation that mutations of all 8 NoLSs did not affect nuclear localization raises the question of whether nuclear localization of RPL23aA is independent of importin αs. In Arabidopsis, there are nine importin αs (importin α1 to 9), of which importins α7 and α8 are expressed only during flowering and hence may not be general candidates for mediators of nuclear import of r-proteins. None of the remaining seven importin αs interacted with RPL23aA in a yeast two hybrid assay (Figure 2.6).

2.3.8. Signal requirements for nuclear/nucleolar localization of RPL15A and RPS8A differ to those required for RPL23aA

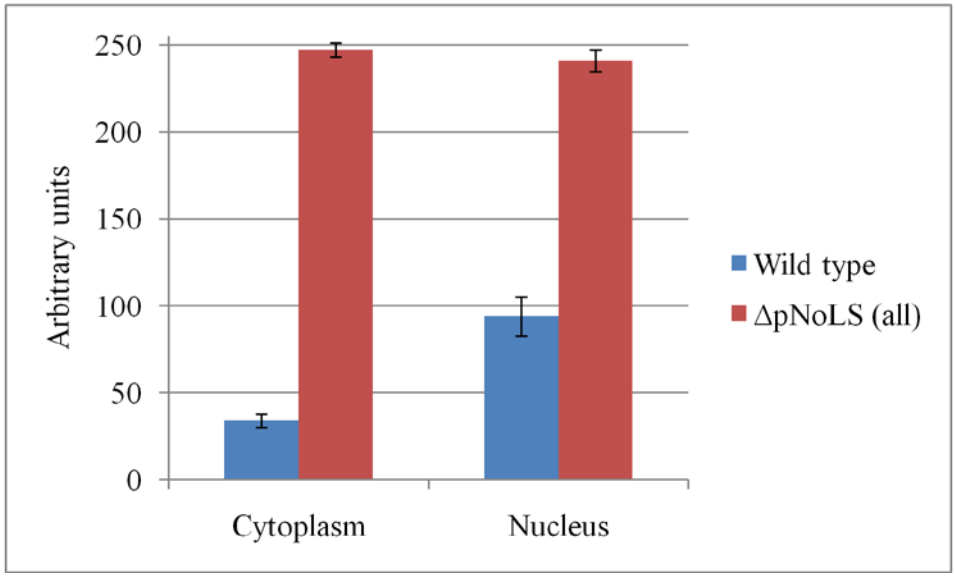
Both RPL15A and RPS8A have 10 pNLSs (Figure 2.1B and C). The amino acid sequences of RPL15A and RPL15B share 99% identity and all 10 pNLSs are conserved between these two isoforms. The RPS8 isoforms, A and B share 81% identity and nine of the 10 pNLSs (pNLS6 is not present in RPS8B) are conserved between these two isoforms.

Figure 2.5. Mutation of pNoLSs increased nuclear and cytoplasmic intensity of RPL23aA-EGFP fusions. (A) CLSM images of tobacco leaf epidermal cells transiently expressing wild type RPL23aA-EGFP or RPL23aA-ΔpNoLS (all)-EGFP. 1. Increased nuclear intensity (white arrow) of ΔpNoLS (all)-EGFP (right panel) compared to wild type (left panel). 2. Increased cytoplasmic intensity (red arrow) of ΔpNoLS (all)-EGFP (right panel) compared to wild type (left panel). Scale bar = 10 μm. **(B)** Mean (+SE) cytoplasmic and nuclear intensities of RPL23aA-EGFP and RPL23aA-ΔpNoLS (all)-EGFP (n=10 transformed cells, r=3).

A)


RPL23aA - EGFP
Δ pNoLS (all) - EGFP
1
10 µm
10 µm
RPL23aA - EGFP
Δ pNoLS (all) - EGFP
2
10 µm
10 µm

B)


250
200
150
100
50
0
Arbitrary units
Cytoplasm
Nucleus
Wild type
ΔpNoLS (all)

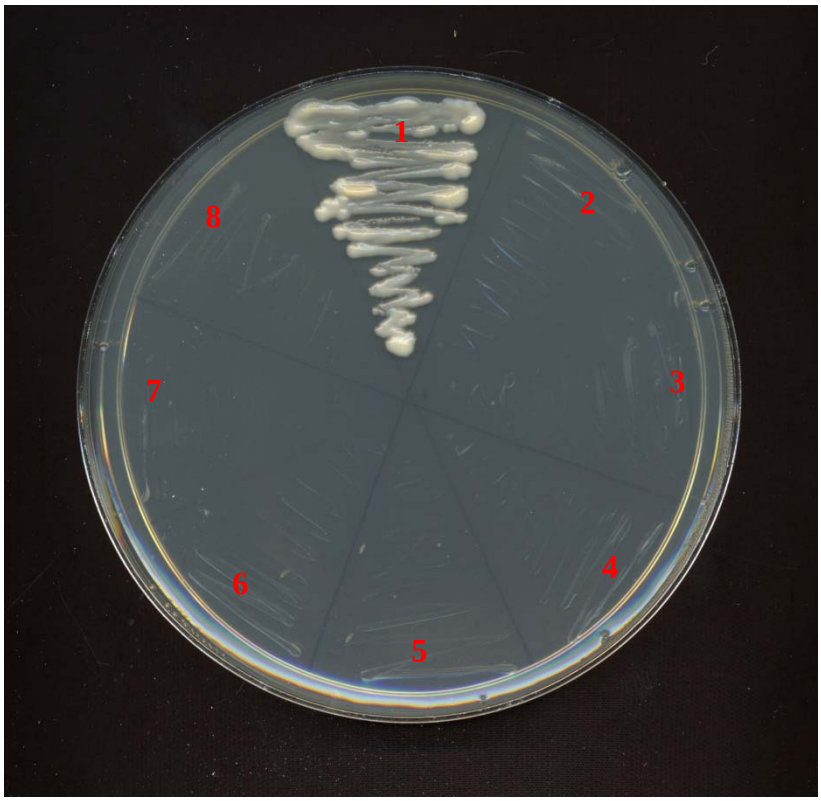


Figure 2.6: Yeast-two-hybrid assay indicated no interaction between RPL23aA and importin αs. 1. positive control (cotransformation of yeast strain MaV203 with pDBLeu - CYCD3;1 and pPC86 - ICK1). 2. negative control (cotransformation of yeast strain MaV203 with empty vectors pDBLeu and pPC86). 3 to 8. interaction between RPL23aA and importin α1 to 6, (cotransformation of yeast strain MaV203 with pDBLeu – importin αs and pPC86 – L23aA. Growth in these cases did not exceed the negative control). Experiment repeated 3 times.

Nuclear and nucleolar localization patterning for both RPS8A and RPL15A are different to that of RPL23aA. RPL15A accumulated in the nucleus and nucleolus to higher levels than RPL23aA (Figure 2.7A; nuclear intensity of RPL15A is ~1.5-fold higher than RPL23aA, P = 0.01, while nucleolar intensity of RPL15A was ~3.6-fold higher than RPL23aA, P = 8.46E-14) whereas, significantly less RPS8A accumulated in the nucleus and the nucleolus compared to RPL23aA (nuclear intensity of RPS8A was 3.1-fold lower than RPL23aA, P = 0.01, while nucleolar intensity was 1.3-fold lower, P = 0.07). RPS8A was primarily localized to the nucleolus or the periphery of the nucleus (Figure 2.7B).

Mutating just pNLS1 (5.6% reduction in basic charge) of RPL15A significantly reduced both nuclear (to background level) and nucleolar localization (Figure 2.8B-1). Nuclear intensity of RPL15AΔpNLS1 was 4.2-fold lower than that of RPL15A (P = 2.67E-06), while nucleolar intensity was 1.5-fold lower (P = 4.32E-03). A double mutation of pNLS1 and 2 (11.3% reduction in basic charge) further reduced nucleolar localization (Figure 2.8B-2). Nucleolar intensity of RPL15AΔpNLS1, 2 was 1.6-fold lower than that of RPL15AΔpNLS1 (P = 0.016), while a triple mutation of pNLS1, 2 and 3 (16.98% reduction in basic charge) resulted in no distinct nuclear or nucleolar signal (Figure 2.8B-3), indicating that these three NLSs have a cumulative effect on nuclear and nucleolar localization of RPL15A. Nuclear intensity of RPL15AΔpNLS3 was 1.2-fold lower than that of RPL15A (P = 0.003), while nucleolar intensity was 2.6-fold lower (P = 2.5E-09). To test if mutating any other set of pNLSs, had a similar effect on nuclear localization of RPL15A, I simultaneously mutated pNLS 5, 6 and 7 (16.98% reduction in basic charge), or pNLS10, 9, 7 and 8 (18.87% reduction in basic charge). No effect on nuclear or nucleolar localization was observed (Figure 2.8C), indicating that while the three NLSs at the N-terminus are absolutely required for nuclear localization of RPL15A, the rest of the pNLSs do not appear to play a role. The localization pattern of RPL15AΔpNLS (all) was the same as that of RPL15AΔpNLS1, 2, 3 (Figure 2.8B-4).

Mutating pNLS1 (6% reduction in basic charge) in RPS8A did not affect nuclear or nucleolar localization (Figure 2.9B-1). However a double mutation of pNLS1 and 2 (16% reduction in basic charge) disrupted both nuclear and nucleolar localization; no detectable nuclear or nucleolar signal was observed (Figure 2.9B-2). Mutation of pNLS2 alone disrupted nuclear or nucleolar localization in 80% of the transformed cells. In the remaining 20% of cells

Figure 2.7. Nuclear and nucleolar localization of RPL15A and RPS8A (A) 1. CLSM images of tobacco leaf epidermal cells transiently expressing wild type RPL15A-EGFP 2. Comparison of nuclear and nucleolar intensity of RPL23aA–EGFP (left panel) and RPL15A–EGFP (right panel) imaged using the same confocal parameters (5% argon laser). **(B)** 1. CLSM images of tobacco leaf epidermal cells transiently expressing wild type RPS8A-EGFP 2. Comparison of nuclear and nucleolar intensity of RPL23aA–EGFP (left panel) and RPS8A–EGFP (right panel) imaged using the same confocal parameters (20% argon laser).The nucleus is indicated by DAPI staining (left panels – A1 and B1). Images in the right panels are merged images of the left and mid panels to show overlap of signal. White arrow indicates the nucleus and white arrowhead the nucleolus. Scale bar = 10 μm.

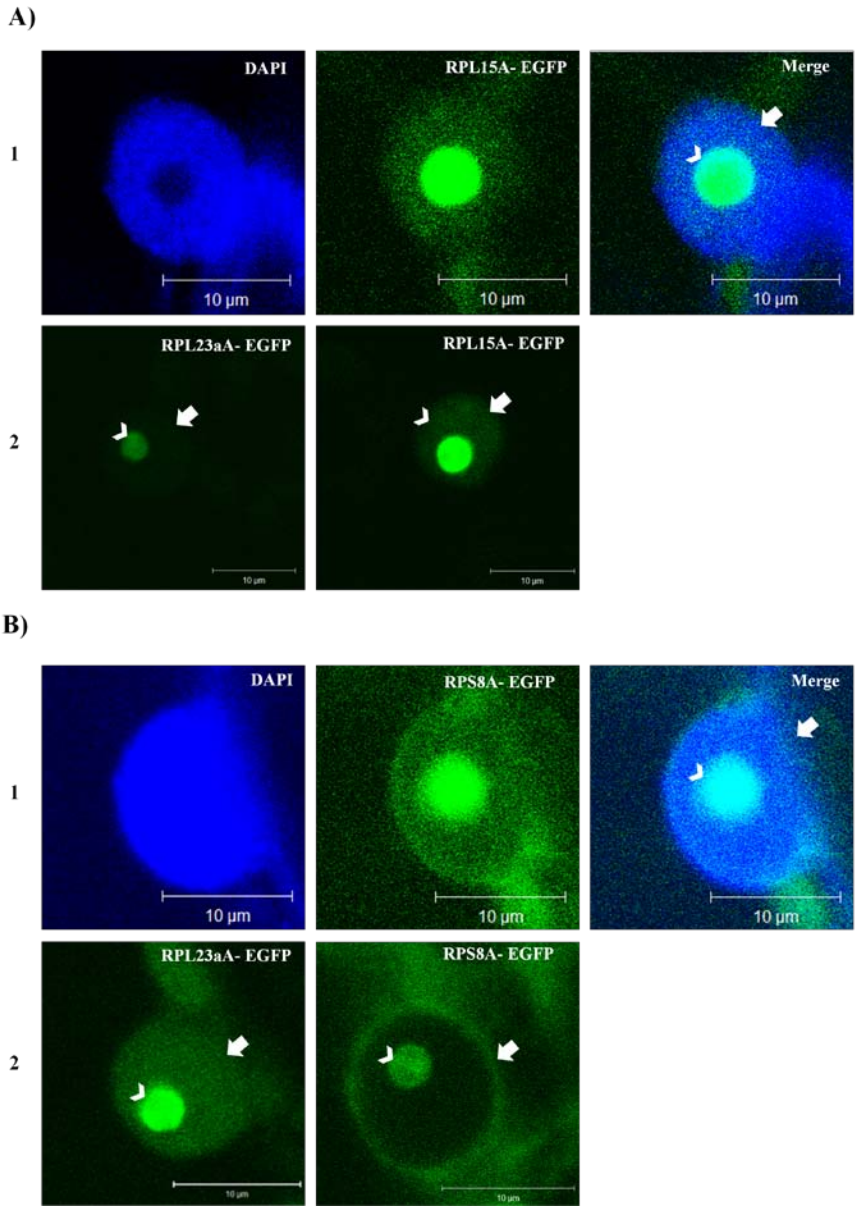

A)
1
DAPI
RPL15A- EGFP
Merge
10 µm
10 µm
10 µm
2
RPL23aA- EGFP
RPL15A- EGFP
B)
1
DAPI
RPS8A- EGFP
Merge
10 µm
10 µm
10 µm
2
RPL23aA- EGFP
RPS8A- EGFP

Figure 2.8. Effect of mutation of pNLSs on localization of RPL15A. CLSM images of tobacco leaf epidermal cells transiently expressing **(A)** wild type RPL15A-EGFP **(B)** RPL15AΔpNLS1-EGFP (1), RPL15AΔpNLS1, 2-EGFP (2), RPL15AΔpNLS1, 2, 3–EGFP (3) or RPL15AΔpNLS(all)-EGFP (4), (mid panels). The nucleus is indicated by DAPI staining (left panels). Images in the right panels are merged images of the left and mid panels to show overlap of signal. Inset images represent the cytoplasmic signal (red arrow) from the cell in the main image to show the absence of nuclear signal is not due to the absence of fusion protein expression. Green spots in the background (white transparent arrowhead) are confirmed (using a 650 nm long pass filter) chloroplast autofluorescence. **(C)** CLSM images of tobacco leaf epidermal cells transiently expressing RPL15AΔpNLS5, 6, 7-EGFP (left panel) and RPL15AΔpNLS10, 9, 7, 8-EGFP (right panel).White arrow indicates the nucleus and white arrowhead the nucleolus. Scale bar = 10 μm.

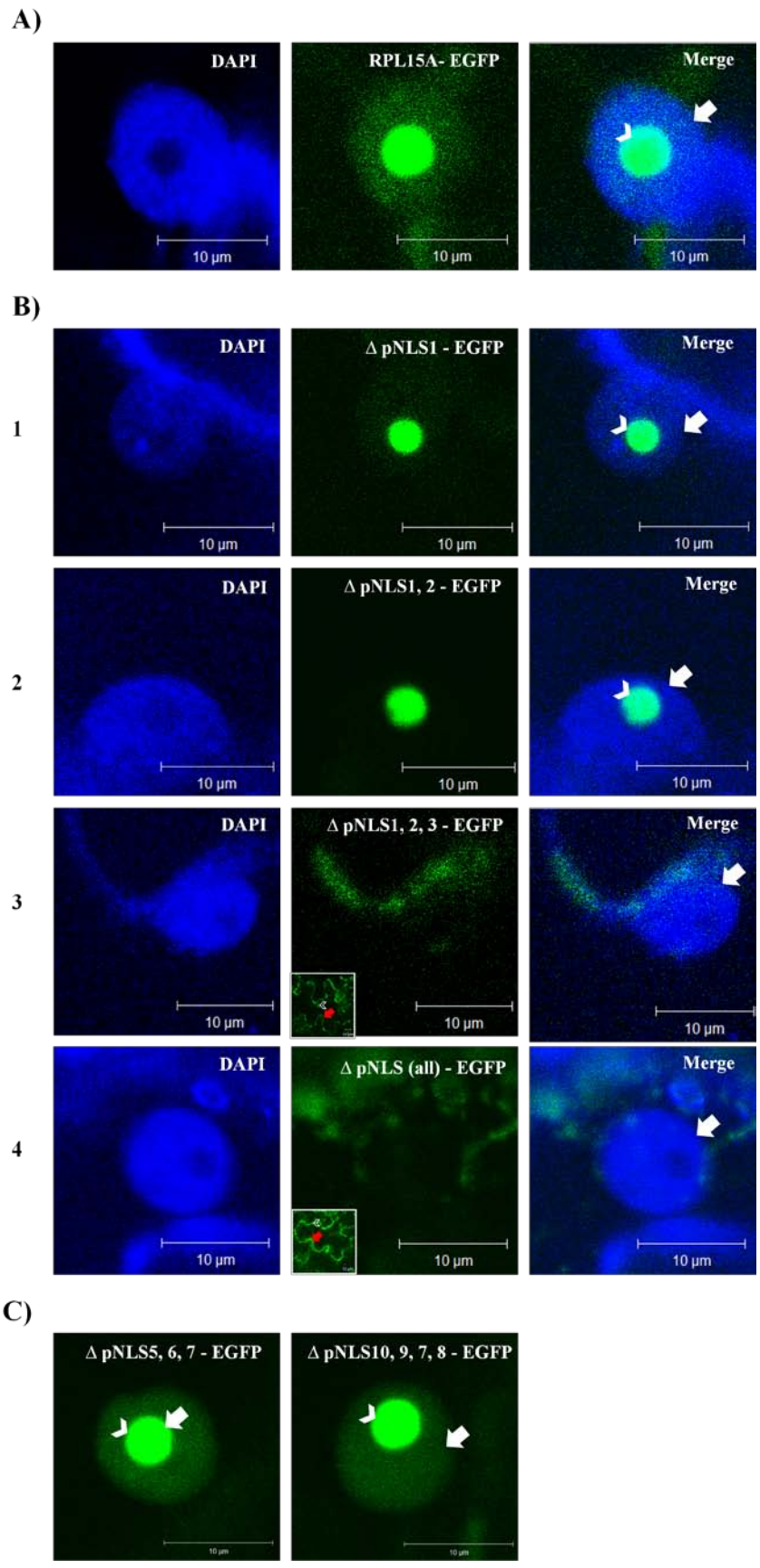
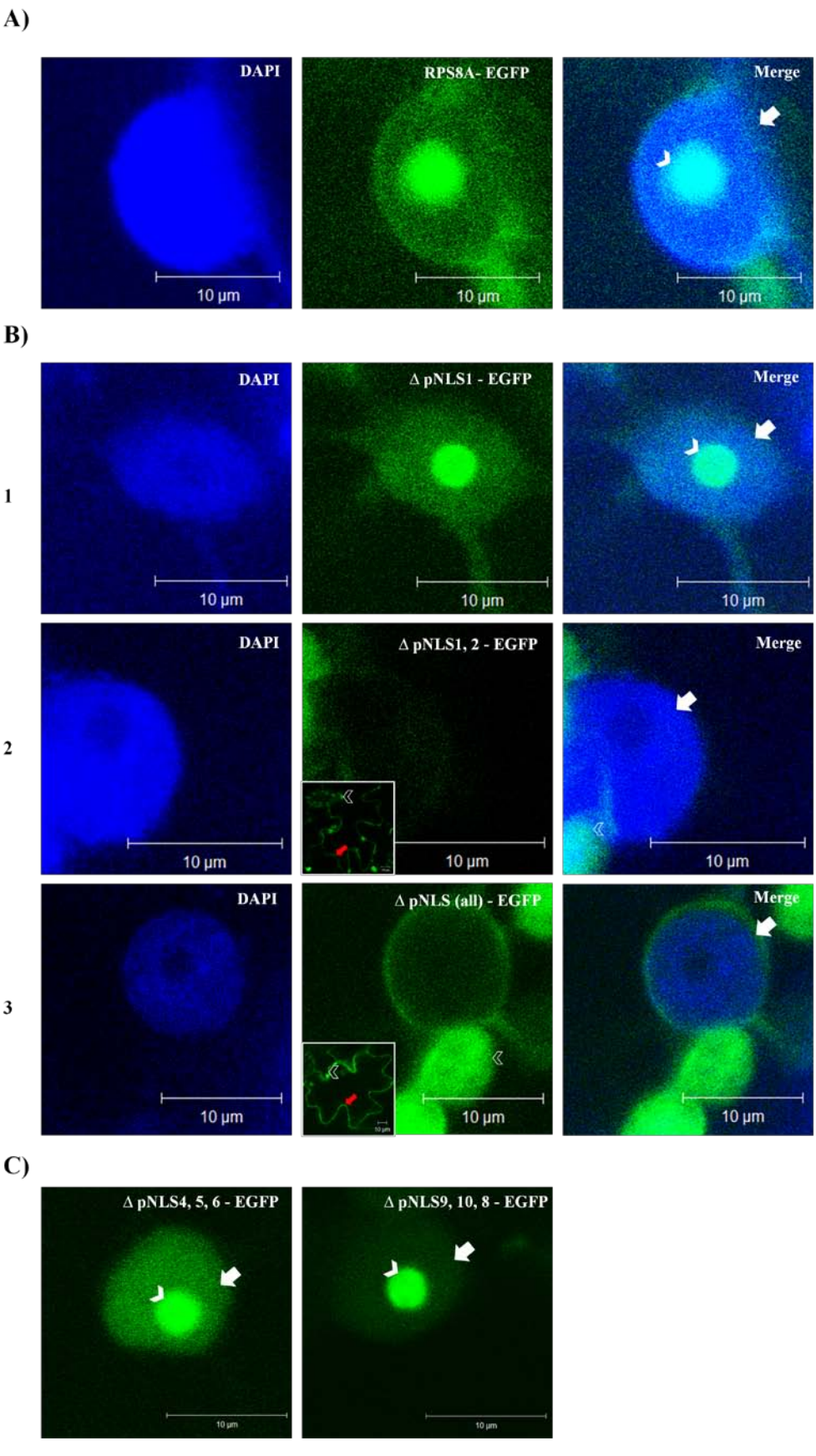

A)
DAPI
RPL15A- EGFP
Merge
10 µm
B)
1
DAPI
Δ pNLS1 - EGFP
Merge
2
DAPI
Δ pNLS1, 2 - EGFP
Merge
3
DAPI
Δ pNLS1, 2, 3 - EGFP
Merge
4
DAPI
Δ pNLS (all) - EGFP
Merge
C)
Δ pNLS5, 6, 7 - EGFP
Δ pNLS10, 9, 7, 8 - EGFP

Figure 2.9. Effect of mutation of pNLSs on localization of RPS8A. CLSM images of tobacco leaf epidermal cells transiently expressing **(A)** wild type RPS8A-EGFP **(B)** RPS8AΔpNLS1-EGFP (1), RPS8AΔpNLS1, 2-EGFP (2) or RPS8AΔpNLS(all)-EGFP (3), (mid panels). The nucleus is indicated by DAPI staining (left panels). Images in the right panels are merged images of the left and mid panels to show overlap of signal. Inset images represent the cytoplasmic signal (red arrow) from the cell in the main image to show the absence of nuclear signal is not due to the absence of fusion protein expression. Green spots in the background (white transparent arrowhead) are confirmed (using a 650 nm long pass filter) chloroplast autofluorescence. **(C)** CLSM images of tobacco leaf epidermal cells transiently expressing RPS8AΔpNLS4, 5, 6-EGFP (left panel) and RPS8AΔpNLS9, 10, 8-EGFP (right panel). White arrow indicates the nucleus and white arrowhead the nucleolus. Scale bar = 10 μm.


A)
DAPI
RPS8A- EGFP
Merge
10 µm
B)
1
DAPI
Δ pNLS1 - EGFP
Merge
2
DAPI
Δ pNLS1, 2 - EGFP
Merge
3
DAPI
Δ pNLS (all) - EGFP
Merge
C)
Δ pNLS4, 5, 6 - EGFP
Δ pNLS9, 10, 8 - EGFP

very faint nuclear and nucleolar signals were observed, wherein nucleolar intensity was 2.1-fold lower (P = 4.6E-05) than that of RPS8A. Nuclear intensity did not change significantly; however, considering that nuclear intensity of wild type RPS8A itself is very low and a decrease in nucleolar localization of this mutant is not associated with the concomitant increase in nuclear intensity as observed with RPL23aA, I believe that the pNLS2 mutation did reduce nuclear localization. These results indicate that pNLS2 plays a major role in nuclear and nucleolar localization of RPS8A. A triple mutation of pNLS4, 5 and 6 (18% reduction in basic charge) or pNLS9, 10 and 8 (16% reduction in basic charge) had no effect on nuclear or nucleolar localization (Figure 2.9C), indicating that while the two NLSs at the N-terminus are required for nuclear localization of RPS8A, the remaining pNLSs do not appear to play a major role. The localization pattern of RPS8AΔpNLS (all) was the same as that of RPS8AΔpNLS1, 2 (Figure 2.9B-3).

In contrast to the marked 6.3-fold increase in cytoplasmic intensity of RPL23aA-ΔpNoLS (all) compared to RPL23aA, there was only a slight increase in cytoplasmic intensity of RPS8A-ΔpNLS (all) ~1.3-fold (P = 0.006), while cytoplasmic intensity of RPL15A-ΔpNLS (all) decreased ~2-fold (P = 1.04E-05), compared to their respective wild types (Table 2.5).

2.4. Discussion

While early nucleolar studies served to highlight the significant structural organisation of this highly conserved sub-nuclear organelle (Jordan and McGovern 1981; Shaw and Jordan 1995), from recent analyses of the human (Andersen et al. 2005) and Arabidopsis (Pendle et al. 2005) nucleolar proteomes, the functional significance and complexity of this organisation is only now being recognized.

I have shown that nucleolar localization of the Arabidopsis ribosomal protein, RPL23aA is mediated by a specific number of basic NoLS motifs, rather than any one or combination of explicit motif(s). This is in contrast to the requirements for nuclear/nucleolar localization of RPL15A and RPS8A and contradicts previous studies that have suggested nucleolar localization of proteins in general is mediated by one or more defined motifs (Emmott and Hiscox 2009). I have demonstrated that, out of eight NoLSs in RPL23aA, any combination of four is sufficient for nucleolar localization and none of the eight are required for nuclear localization. It was also shown that unlike yeast RPL25, where the N-terminal 28 amino acids are required for

Table 2.5. A comparison of localization signals and nuclear/nucleolar localization of RPL23aA, RPL15A and RPS8A.

Details	RPL23aA	RPL15A	RPS8A
Nuclear localization	None of the eight NoLSs have a role	Three N-terminal NLSs	Two N-terminal NLSs
Nucleolar localization	Mediated by any combination of four or more of the eight NoLSs **	Probably mediated by the three N-terminal NLSs *	Probably mediated by the two N-terminal NLSs *
Mutation of all pNLS/NoLSs on nuclear localization	Marked increase	Complete disruption	Complete disruption
Mutation of all pNLS/NoLSs on cytoplasmic localization	Marked increase	Significant decrease	Slight increase

* I cannot rule out the possibility that the disruption of nucleolar localization is solely due to a disruption of nuclear localization and nucleolar localization is mediated entirely by a different set of motifs.

** Although I did not test all combinations of four NoLSs, both the combinations of mutations tested led to a gradual increase in the disruption of nucleolar localization when more than four NoLSs are mutated, suggesting that any four of the eight NoLSs can mediate nucleolar localization.

nuclear localization (Schaap et al. 1991), in Arabidopsis RPL23aA, the N-terminal 29 amino acids are not required for nuclear or nucleolar localization.

Most previously identified NLSs involved in nuclear transport of proteins via the importin pathway, are comprised of one or more stretches of basic amino acids (Freitas and Cunha 2009; McLane and Corbett 2009; Nguyen Ba et al. 2009). These basic motifs in the cargo proteins are essential for protein interactions with the acidic importin α transport proteins. Mutation of all of the pNoLSs in RPL23aA disrupted every stretch of two or more basic amino acids. That mutation of all of these basic motifs in RPL23aA did not affect nuclear localization suggests that nuclear import of RPL23aA is an importin α-independent pathway. Furthermore, none of the Arabidopsis importin α proteins (importin α1 – 9, excluding importin α7 and 8 that are expressed only during flowering) interacted with RPL23aA in yeast two hybrid assays. While it cannot be ruled out that RPL23aA may interact with some of the seven importin α proteins in the presence of other plant-specific proteins, my results do support the probability of an importin α-independent pathway for nuclear localization. In yeast, only 53% of an identified 1515 nuclear proteins contain classical NLSs; the remaining 47% may use importin α-independent mechanisms for nuclear localization (Lange et al. 2007). It has been shown that human RPL23a can directly interact with importin β or importin β-related receptors like transportin, RanBP5 and RanBP7, such that its nuclear localization is not dependent on importin α (Jakel and Gorlich 1998). However, interaction with importin β also often requires stretches of basic amino acids (Chou et al. 2010) and as such may not explain my results with Arabidopsis RPL23aA.

There is evidence for the existence of importin-independent pathways for nuclear transport. The calcium binding protein calmodulin can mediate nuclear import of architectural TFs such as SOX9 (Argentaro et al. 2003), SRY (Sim et al. 2005) and Nhp6Ap (Hanover et al. 2007) by an as yet to be identified mechanism. Calmodulin-mediated nuclear transport, regulated by intracellular calcium levels (Hanover et al. 2009), appears to occur independently of importins, GTP and Ran (Hanover et al. 2007; Sweitzer and Hanover 1996). Although calmodulin-mediated nuclear transport is unlikely to be responsible for RPL23aA import, the existence of such a pathway demonstrates a diversity of nuclear transport mechanisms for different classes of cargo proteins. Some proteins, e.g. β-Catenin, localize to the nucleus, independently of importins and Ran, by directly binding to nucleoporins (Wagstaff and Jans 2009). Similarly, Human T lymphotrophic virus type 1 (HTLV-1) Tax protein localizes to the

nucleus by directly binding to nucleoporin Nup62 (Tsuji et al. 2007), while tumor suppressor proteins SMAD3 and SMAD4 localize to the nucleus by binding to Nup214 (Xu et al. 2003). Furthermore, not only do β-Catenin and Tax localize to the nucleus independently of carrier proteins, but they also act as carriers of other proteins. β-Catenin, piggybacks TF LEF-1, for which β-Catenin acts as a coactivator (Asally and Yoneda 2005), whereas Tax piggybacks NF-κB subunit, p65 (Tsuji et al. 2007). Microtubules have also been implicated in facilitating the nuclear import of some proteins, e.g., tumor suppressor protein p53, and Retinoblastoma (Rb) protein (Giannakakou et al. 2000; Roth et al. 2007). Microtubule-facilitated movement towards the nucleus appears to accelerate the nuclear import of some proteins (Wagstaff and Jans 2009). Likewise, actin filament networks have also been implicated in the nuclear import of NF-κB (Fazal et al. 2007). For most of the above-mentioned cargo proteins, nuclear localization is governed by more than one mechanism to ensure timely and efficient nuclear localization (Wagstaff and Jans 2009). While my results suggest that RPL23aA localizes to the nucleus in an importin-independent mechanism, I cannot completely rule out the possibility of nuclear localization by an importin-dependent mechanism in the absence of basic amino acid-rich motifs. Both human hnRNP A1 (Siomi and Dreyfuss 1995) and SREBP2 (Nagoshi and Yoneda 2001) localize to the nucleus through the importin β-related receptor, transportin, and importin β, respectively, via NLSs that are not enriched in basic residues. Also, as suggested by recent findings, it is possible that NLSs do not necessarily exist as simple linear sequences. The cytomegalovirus UL40, a protein involved in viral DNA replication, contains two clusters of basic amino acids, but neither of these clusters is sufficient for nuclear localization of UL40. It has been proposed that UL40 folds to form a 3-dimensional domain that can bind to importin α (Lischka et al. 2003; McLane and Corbett 2009). TF STAT-1 monomers do not have a classical NLS, however, when they form a homodimer, arginine/lysine residues that are scattered in the linear protein sequence, come together in the three-dimensional structure to form a functional NLS (McLane and Corbett 2009; Melen et al. 2001). In a similar way, basic amino acids of RPL23aA that are not together in the linear sequence may come together when the protein folds to form a NLS that can subsequently interact with importin α in the presence of a plant specific protein(s).

The nucleolus is not membrane bound. The surface and internal organization of the nucleolus has been studied by both SEM and TEM, to show compartmentalization into fibrillar

centres (FCs), surrounded by dense fibrillar components (DFCs), all embedded in the granular component (GC) (reviewed in Saez-Vasquez and Medina, 2008). Ribosome subunit biogenesis occurs as a wave from the FC-DFC complexes through to the GC regions prior to being exported to the cytoplasm. The periphery of the nucleolus appears to be delineated by the density of these structures within the nucleolus. For RPL23aA, I found that some highly mutated ΔpNoLS–RPL23aA proteins accumulated in a fluorescent ring-like arrangement at the periphery of the nucleolus, suggesting a selective permeability at the periphery. A decrease in overall basic charge of the mutated RPL23aA proteins may prevent them from penetrating this nucleus-nucleolus boundary. Furthermore, the three different nucleolar localization patterns observed in this study; ring, diffused and nucleolar exclusion, suggest that nucleolar localization of a protein may actually be the result of three independent steps; penetrating the nucleolar periphery, entering the nucleolus and being retained within the nucleolus. The latter is supported by my observations that removing the C-terminal 30 amino acids, containing the recognized rRNA binding site (AYVRL) for RPL23a (Kooi et al. 1994) resulted in disrupted nucleolar localization of the mutated protein.

K and R are not the only amino acids that are important for nucleolar localization. Nucleolar localization of US11, a viral protein from HSV-1, is mediated by a proline rich NoLS (Catez et al. 2002), while in the NoLS of nucleophosmin (NPM), tryptophan residues play an important role (Nishimura et al. 2002). In this study, I also found that, in addition to basic amino acid-rich NoLSs, the C-terminal 30 amino acids of L23aA, particularly the motif AYVRL, was required for normal nucleolar retention.

Ribosomes contain only a single molecule of each RP (Perry 2007). Hence, some coordinated production and nucleolar localization of RPs is vital. If a RP is synthesized and/or localized to the nucleolus in excess of other RPs, it constitutes a waste of energy and cellular resources. By contrast, if a RP is not localized to the nucleolus in sufficient quantity, the cell cannot produce the required number of ribosomes for normal cell function. It would be therefore reasonable to assume that nuclear/nucleolar localization of individual RPs would be governed by similar/common mechanisms and hence mediated by similar signals. However, my results demonstrate that nuclear and nucleolar localization of RPL23aA, RPS8A and RPL15A are governed by different type of signals (Table 2.5). Differential signal requirements for localization of these three RPs suggest that different mechanisms are likely to govern their

localization. In fact in mammals, nuclear localization of different RPs have been shown to be mediated by different pathways; while nuclear localization of human RPL23a is mediated by importin β or importin β-related receptors like transportin, RanBP5 and RanBP7 (Jakel and Gorlich 1998), nuclear localization of human RPL7 and mouse RPL12 is mediated by importin β3 (Chou et al. 2010) and importin 11 (Plafker and Macara 2002), respectively. Large quantities of RPs are required in the nucleolus to meet the cellular demand for ribosomes in actively dividing cells. Nucleolar localization of such quantities may be ensured by the utilization of different pathways for different RPs, thereby reducing the competition for individual components associated with nucleolar transport.

To study RP localisation I used a heterologous system (tobacco) for transient expression. Considering the high identity between the Arabidopsis and tobacco RPL23aA orthologs, (85% overall identity, 90% identity in amino acids constituting NoLSs and 100% identity in the 26S rRNA binding site), I believe that a conserved mechanism will govern the localization of these RPs and hence, data obtained in tobacco will be applicable to Arabidopsis. Furthermore, the observed effect on nucleolar localization might in fact be due to misfolding of mutant proteins as a result of the large number of basic amino acid mutations, and not simply due to the disruption of basic nucleolar localization signals. Although I cannot rule out this possibility completely, the observed trend (no effect of mutations of four NoLSs and gradual increase of disruption of nucleolar localization with mutation of more than four NoLSs) do support my charge-based conclusions.

Generally, RPs that are not assembled into ribosomes are subject to 26S proteasome-mediated degradation (Lam et al. 2007; Pierandrei-Amaldi et al. 1985). Proteins are marked for proteasome degradation by ubiquitination of lysine residues (Pickart and Cohen 2004). Since L23aA-ΔNoLS (all) accumulation in the nucleus and cytoplasm markedly increases, I assume that mutations of lysines in some of the NoLSs also disrupted ubiquitination sites so that mutant protein is not ubiquitinated and hence survived degradation by 26S proteasome. Although mutation of only ^{132}**KK**AYV**R**L^{138} lead to disruption of nucleolar retention, it did not result in concomitant accumulation in the nucleus or cytoplasm to an extent similar to L23aA-Δ NoLS(all), corroborating the above assumption.

Although coordinate regulation of nuclear and nucleolar localization is presumably a prerequisite for ribosome biogenesis, my results suggest that probably different mechanisms govern the nuclear and nucleolar localization of RPs in Arabidopsis.

CHAPTER 3. DIFFERENTIAL REGULATION OF EXPRESSION AND SUBCELLULAR LOCALIZATION OF ARABIDOPSIS RIBOSOMAL PROTEINS

In Arabidopsis, cytoplasmic ribosomes are comprised of four rRNAs and 81 ribosomal proteins (RPs). Only a single molecule of each RP is incorporated into any given ribosome. Hence, adequate availability of different RPs in the nucleolus would appear to be a prerequisite for efficient use of energy in ribosome biogenesis. I studied transcriptional regulation of 195 of the 254 Arabidopsis RP genes (using Genevestigator, a microarray data analysis tool) and subcellular localization of each of five two-member RP families, to determine to what extent these two processes are coordinated to ensure adequate availability of RPs. Different RP genes are transcribed at different levels representing up to a 300-fold difference. However, when I consider the RP gene families, this difference is drastically reduced (to ~7.5-fold), indicating coordinate regulation of expression of RP genes is more stringent at the family level. Subcellular localization studies showed differential targeting of RPs to the cytoplasm, nucleus and nucleolus, with differential nucleolar import rates for RPS8A and RPL15A. The variation in gene regulation and localization of RPs may be the result of different extra-ribosomal functions, differential acquisition of localization signals during the evolution of eukaryotic RPs or differential ubiquitination and degradation of RPs that are not assembled into ribosomes.

3.1. Introduction

Arabidopsis ribosomes are comprised of four rRNAs (26S, 5.8S, 5S and 18S) and 81 ribosomal proteins (RPs). In ribosomes, although rRNAs perform the key peptidyl transferase reaction and mRNA decoding functions, (Nissen et al. 2000; Wimberly et al. 2000), RPs play critical roles in ribosome biogenesis and function. RPs are involved in rRNA processing and folding, ribosomal subunit assembly and transport, stabilization of the subunit structure, interactions of the ribosome with various translation factors, folding and targeting of nascent polypeptides (Ban et al. 2000; Brodersen et al. 2002; Brodersen and Nissen 2005; Klein et al. 2004; Wimberly et al. 2000).

Regardless of the quantity of each RP produced, only a single molecule of each (except acidic proteins P0, P1, P2 and P3) can be incorporated into any given ribosome (Ban et al. 2000; Schuwirth et al. 2005; Wimberly et al. 2000), presumably necessitating an equimolar availability of the different RPs in the nucleolus to ensure an optimal level of ribosome biogenesis.

Equimolar availability can be ensured by coordinated regulation of RP gene expression at the transcriptional and post-transcriptional (mRNA turn over and translation) levels. Production of a RP in excess or less than its partners could both be deleterious. Haploinsufficiencies of some RPs can lead to growth retardation and other developmental abnormalities in yeast (Deutschbauer et al. 2005), *Drosophila* (Saeboe-Larssen et al. 1998) and mammals (Gazda and Sieff 2006; Oliver et al. 2004) as well as in plants (Ito et al. 2000; Van Lijsebettens et al. 1994; Weijers et al. 2001). It should be noted that equimolar expression of different RP genes would be expected if RPs are involved only in ribosome function. However, recent reports suggest that many individual RPs have wide-ranging extraribosomal roles in processes such as transcription, translation, mRNA processing, DNA repair, apoptosis and tumorigenesis (Lindstrom 2009; Naora 1999; Warner and McIntosh 2009). The variety of cellular functions performed by different RPs is exemplified by their wide distribution in the cell; RPs can localize to the nucleolus (Degenhardt and Bonham-Smith 2008; Kruger et al. 2007; Lam et al. 2007), and to the mitochondria (Adams et al. 2002), and in plants to the plastids (Ma and Dooner 2004) for cytoplasmic ribosomal subunit assembly with rRNAs. RPs can localize to the cell surface (Sibille et al. 1990) or be secreted out of the cell (Dai et al. 2010), and can also accumulate in the cytoplasm and nucleoplasm (Dai et al. 2010; Kruger et al. 2007; Mazumder et al. 2003; Yadavilli et al. 2007). Because of extraribosomal functions some RP genes may be expressed at higher levels than others.

In yeast, where 59 of the 79 RPs are encoded by two-member gene families, coordinated regulation occurs primarily at the level of transcription (Planta and Mager 1998; Sengupta et al. 2004). However, such coordinated regulation result only in similar, but not identical, amounts of each RP mRNA being produced. In exponentially growing cultures, a difference in transcript levels of up to five-fold has been reported between various RP mRNAs, although most are within a two-fold range (Holstege et al. 1998).

In mammals, as a result of expression from only a single gene copy for most RPs, variations in RP mRNA abundance are kept within a fairly narrow range, with a few exceptions that appear to be cell type-specific (Angelastro et al. 2002; Ishii et al. 2006; Perry 2007). Regulation of expression of RPs can also occur post-transcriptionally through modulated recruitment of RP transcripts to polysomes, largely mediated by 5' TOP sequences in these transcripts (Meyuhas 2000; Volarevic and Thomas 2001; Wool et al. 1995). Also, in both yeast

and mammals, RPs produced in excess of biological demand, are subjected to 26S proteasome-mediated degradation (Maicas et al. 1988; Pierandrei-Amaldi et al. 1985; Tsay et al. 1988)

Coordinated regulation of expression of RPs in plants, where RPs are encoded by multigene families, many of which are comprised of more than two active members, is much more complex. Members of many plant RP families exhibit differential expression in different tissues, developmental stages or in response to stress (Hulm et al. 2005; McIntosh and Bonham-Smith 2005; Williams and Sussex 1995). For instance, Arabidopsis *RPS5A* is strongly expressed in dividing cells, while its paralog *RPS5B* is predominantly expressed in differentiating cells (Weijers et al. 2001). *RPL11B* is highly active in proliferating tissues such as shoot and root apical meristems, whereas its paralog *RPL11A* is active in the root stele and in anthers (Williams and Sussex 1995). In response to UV-B treatment, expression of Arabidopsis *RPL10C* is upregulated, while expression of *RPL10B* is downregulated (Falcone Ferreyra et al. 2010). Differential expression of RP isoforms, within a single tissue, has also been reported in *B. napus* (Whittle and Krochko 2009). While there has been some effort to study the expression patterns of members within a number of RP families in Arabidopsis, there is little information pertaining to coordinate regulation across RP families.

A coordinated regulation at various levels of RP gene expression cannot ensure an equimolar availability of RPs in the nucleolus for ribosomal subunit assembly, if there are differences in localization patterns and rates of localization. Recruitment of individual RPs for functions outside of the ribosome (Lindstrom 2009; Warner and McIntosh 2009) and in different compartments of the cell adds further complexity to the ‘coordinated’ availability of RPs for ribosome biogenesis. However, there are little data available to compare subcellular localization patterns and nucleolar import rates of RPs and their effect on ribosome biogenesis.

Using Genevestigator (Hruz et al. 2008) to analyze Arabidopsis 22k microarray data, I report an analysis of RP gene expression at both the individual RP gene and the RP gene family levels. I have also compared the subcellular localization patterns of five two-member RP families; RPS3a, RPS8, RPL7a, RPL15 and RPL23a and of these families, I have investigated differences in the nucleolar import rate of RPS8A and RPL15A.

3.2. Materials and methods

3.2.1. Plant material

Six-week-old plants of tobacco (*Nicotiana tabacum*), cultivar Petit Havana, grown in a growth chamber under a $23^{o}/18^{o}$C temperature regime and a 16 h/8 h photoperiod of ~170 µmol photons m^{-2} sec^{-1} were used for all transient expression analyses.

3.2.2. RP gene expression analysis

To analyze the expression of RP genes in different developmental stages, GENEVESTIGATOR (https://www.genevestigator.com), a database and data mining interface for microarray data (Affymetrix GeneChip data), was used (Hruz et al. 2008). Values of RP gene expression during different developmental stages from ATH1: 22k high quality arrays in wild type Columbia-0 genetic background were used.

3.2.3. Fluorescent fusion protein constructs

The ORFs of *RPL23aA* and *B* minus the stop codons were amplified by RT-PCR from total RNA (Degenhardt and Bonham-Smith 2008), while the ORFs of *RPS3aA* and *-B, RPS8A* and *-B, RPL7aA* and *-B*, and *RPL15A* and *-B* minus the respective stop codons were amplified (see Appendix A for primers) from cDNA clones obtained from the Arabidopsis Biological Resource Center (ABRC). Fluorescent fusion protein constructs were prepared as described in section 2.2.3.

3.2.4. Transient expression in tobacco and confocal microscopy

Fluorescent fusion protein constructs were transiently expressed in tobacco leaf epidermal cells and subsequently imaged as described in section 2.2.4. Statistical analysis (student's t-test, ANOVA and correlation coefficient) was carried out using the Analysis ToolPak of Microsoft office 2007.

3.2.5. Fluorescence Recovery after Photobleaching (FRAP)

Due to the movement of nuclei/nucleoli within tobacco leaf epidermal cells, that could not be overcome by treatment with the actin depolymerization agent latruncilin B, FRAP assays were carried out manually. Prior to photobleaching, five images of EGFP fluorescence in the nucleolus were acquired. The complete area of the nucleolus was photobleached for three

minutes using 100% argon laser power. Recovery of fluorescence was monitored for 90 minutes. During this period, single section images were collected with <10% laser power at ~2 minute intervals for 40 min and at ~10 min interval for the remaining time period. To generate FRAP recovery curves, nucleolar fluorescence intensities of pre and post bleaching images were measured using McMaster Biophotonics "ImageJ for Microscopy." Fluorescence recovery curves were generated using the Analysis ToolPak of Microsoft office 2007.

3.3. Results

3.3.1. Differential level of expression of individual RP genes

On the Affymetrix 22k array, 195 of the 254 Arabidopsis RP genes are represented by a probe set that is highly specific to each single gene. The analysis of RP gene expression during various developmental stages suggests that there is a wide variation in the level of expression of different RP genes. For instance, in germinated seeds 14 genes have a signal intensity of less than 100, while nine genes have a signal intensity of more than 30000, representing an ~300-fold difference in expression level, while the expression level of most of the rest of the genes (129) is in the range of 5001-25000 (Figure 3.1A). Similarly, in the seedling stage, 16 genes have a signal intensity of less than 100 (including 14 of the same genes in the germination stage, although their order of ranking varies), whereas 11 genes (including nine of the same genes with a signal intensity of more than 30000 in the germination stage) have a signal intensity of more than 20000, representing a 200-fold difference in expression level (the expression levels of 126 genes are in the range of 5001-25000; Figure 3.1B). A similar trend was observed in other developmental stages such as young rosette, developed rosette, bolting, young flower, developed flower and mature siliques (Table 3.1).

Among the 195 RP genes on the array, *RPL23C*, followed by *RPS8A*, showed the highest level of expression in all stages of development. Furthermore, *RPP0B*, *RPL37aC*, *RPL10A*, and *RPL3A* all appeared in the list of the ten highest expressed genes in each developmental stage (Table 3.2). Interestingly, *RPL26A* expression was one of the top 10 in all tissues except mature siliques. *RPS9A* showed least expression in seven developmental stages and second least expression in the remaining two stages (bolting and young flower stage), while *RPS15B*, *RPL4B*, *RPL37aB*, *RPS15aC*(3), *RPS25C* and *RPS15E* all appeared in the list of the ten least expressed genes (signal intensity of less than 100) across all developmental stages (Table 3.3).

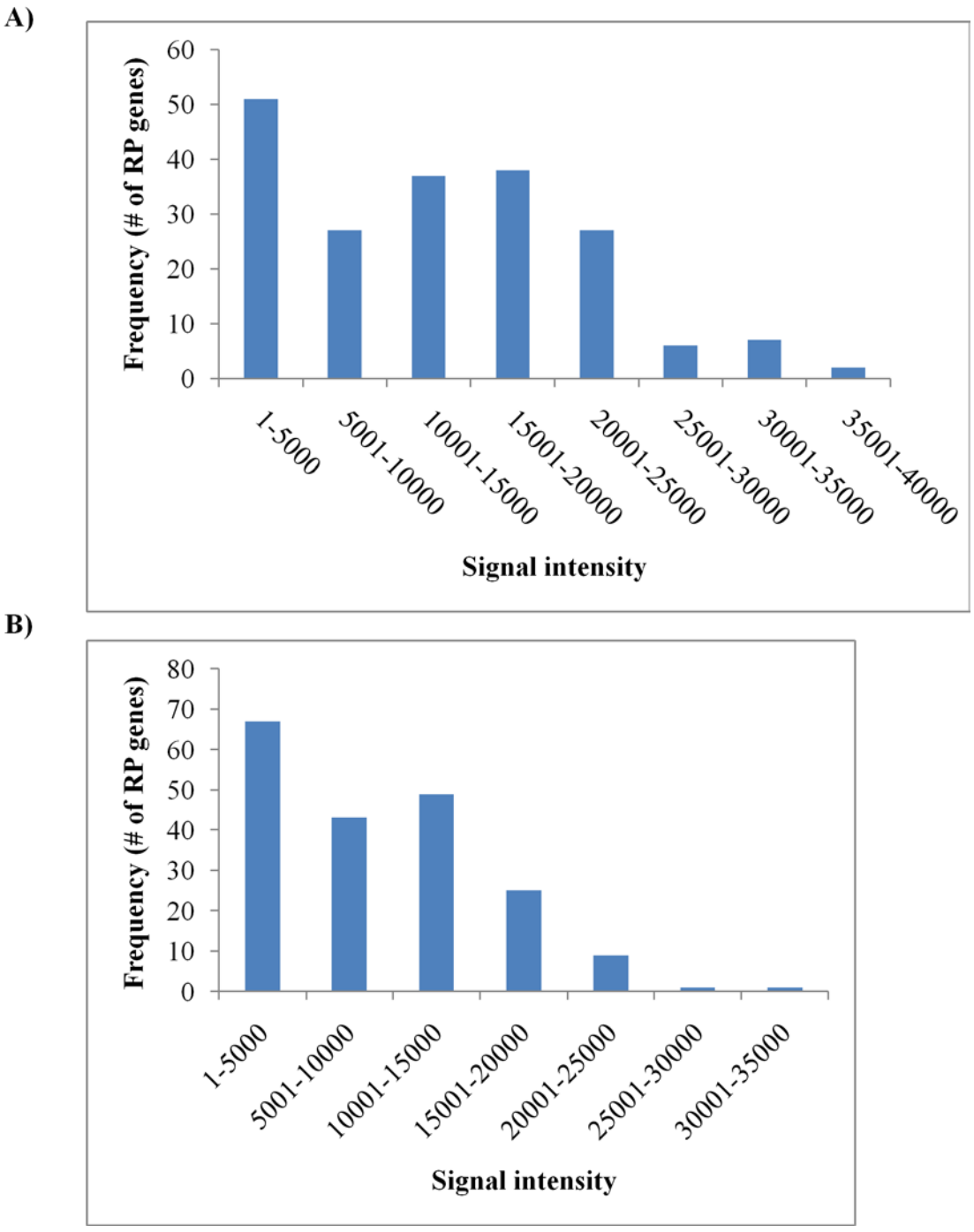


Figure 3.1. Differential expression of individual RP genes. Histogram showing frequency of RP genes with different levels of expression during germination **(A)** and seedling **(B)** stages.

Table 3.1. Frequency of RP genes showing different expression levels in the different developmental stages of Arabidopsis (22k microarray data from GENEVESTIGATOR).

Signal intensity (range)	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers & siliques	Mature siliques
4-100	14	16	14	15	15	11	14	11	15
101-1000	14	17	17	17	18	24	20	22	19
1001-5000	23	34	36	36	32	57	35	33	55
5001-10000	27	43	43	48	37	63	49	51	53
10001-15000	37	49	53	49	50	29	47	43	31
15001-20000	38	25	22	19	27	9	20	21	14
20001-25000	27	9	6	9	11	1	8	9	5
25001-30000	6	1	2	1	3	1	1	3	2
30001-35000	7	1	2	1	1	0	1	1	1
35001-37734	2	0	0	0	1	0	0	1	0

Grey shading = Expression levels of the highest number of RP genes in each developmental stage.

Table 3.2. List of the ten RP genes showing the highest expression levels in the different developmental stages of Arabidopsis.

	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
1	RPL23C	RPL23C	RPL23C	RPL23C	RPL23C	RPL23C	RPL23C	RPL23C	RPL23C
2	RPS8A	RPS8A	RPS8A	RPS8A	RPS8A	RPS8A	RPS8A	RPS8A	RPS8A
3	RPP0B	RPP0B	RPL3A	RPL26A	RPP0B	RPL3A	RPL10A	RPL26A	RPL10A
4	RPL37aC	RPL10A	RPL37aC	RPL37aC	RPL26A	RPL10A	RPL26A	RPL37aC	RPL3A
5	RPL10A	RPL26A	RPL26A	RPL10A	RPL3A	RPP0B	RPL30C	RPL3A	RPP0B
6	RPS9B	RPL3A	RPL37B	RPP0B	RPL10A	RPS9B	RPL37aC	RPL10A	RPL37aC
7	RPL3A	RPS9B	RPL10A	RPL3A	RPL37aC	RPL26A	RPL3A	RPS9B	RPL30C
8	RPL19A	RPL37aC	RPS9B	RPL30C	RPS9B	RPL30C	RPP0B	RPL37B	RPSaA
9	RPL26A	RPL37B	RPP0B	RPS29C	RPL37B	RPL37aC	RPS29C	RPL19A	RPL19A
10	RPSaA	RPL19A	RPL19A	RPL37B	RPL19A	RPS29C	RPS9B	RPP0B	RPL18aB

Light blue = RPL23C	Purple = RPL37aC	Yellow = RPL26A
Light red = RPS8A	Aqua = RPL10A	
Olive green = RPP0B	Orange = RPL3A	

Table 3.3. List of the ten RP genes showing the lowest expression level in the different developmental stages of Arabidopsis.

	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
1	RPS9A	RPS9A	RPS9A	RPS9A	RPL13A	RPL13A	RPS9A	RPS9A	RPS9A
2	RPS15B	RPL13A	RPL13A	RPL13A	RPS9A	RPS9A	RPL13A	RPL37aB	RPL13A
3	RPL4B	RPS15B	RPL37aB	RPL37aB	RPS15B	RPS15B	RPL37aB	RPL13A	RPL37aB
4	RPL37aB	RPL4B	RPL4B	RPL4B	RPL37aB	RPL37aB	RPS15B	RPL4B	RPL4B
5	RPS15aC	RPL37aB	RPS15B	RPS15B	RPS25C	RPL4B	RPL4B	RPS15E	RPS15E
6	RPS25C	RPS25C	RPP2E	RPS25C	RPL4B	RPS25C	RPS25C	RPS25C	RPS15aC
7	RPP2E	RPS15E	RPS15E	RPP2E	RPS15E	RPS15E	RPS15E	RPS15B	RPS25C
8	RPS15E	RPP2E	RPS25C	RPS15E	RPS15aC	RPL5C	RPS15aC	RPS15aC	RPS15B
9	RPL5C	RPS15aC	RPS15aC	RPS15aC	RPL5C	RPS15aC	RPP2E	RPL5C	RPS21A
10	RPL21F	RPS15F	RPS21A	RPL5C	RPS15C	RPS21A	RPL5C	RPL21F	RPL35aA

Light blue = RPS9A	Purple = RPL37aB	Grey = RPS15E
Light red = RPS15B	Aqua = RPS15aC	Yellow = RPL13A
Olive green = RPL4B	Orange = RPS25C	

Interestingly, *RPL13A* was one of the least 10 expressed genes in all tissues except germinated seeds.

3.3.2. Regulation of expression of RP genes at the family level

Although each of the 81 Arabidopsis RPs has two or more isoforms, only one isoform of each RP can assemble into a given ribosome. So RP gene expression may be coordinately regulated at the RP gene family level rather than at the individual RP gene level. To verify this possibility, I analyzed combined RP gene expression at the level of the gene family. Of the 81 RP gene families, expression data is available in Genevestigator for all members of 55 families. In germinated seed, as opposed to up to a ~300-fold difference in the expression levels of individual RPs, expression of different RP gene families (sum of expression of individual family members) varies only up to 4.3-fold. Furthermore, as many as 41 families have signal intensities in 30001-50000 range, representing only an ~1.6-fold difference in expression level between all of these families (Figure 3.2A). In the seedling stage, a difference in the expression level of up to ~6.5-fold was observed, although most of the families (44) are in the range of a 2-fold (20001-40000) difference (Figure 3.2B). A similar trend was observed for all other stages (Table 3.4). The above data indicate that regulation of expression of RP genes is more coordinated at the level of the gene families than at the level of individual RP genes. However, it should be noted that up to ~7.5-fold difference (in mature siliques) still exists at the family level.

3.3.3. Grouping of RP families based on the gene expression levels of family members.

RP families in Arabidopsis can be classified into two groups based on the levels of expression of individual members. Type I; RP families with members having similar levels of gene expression i.e. no significant difference among the members in average expression across the nine developmental stages ($\alpha = 0.01$, student's t-test) and gene expression levels of the members across different developmental stages correlates with a coefficient of >0.85 (Pearson's correlation coefficient; Figure 3.3A and B). Type II; RP families of members with differing levels of gene expression (Figure 3.3C and D). Out of 55 RP families, eight (*RPS3a*, *RPS6*, *RPS7*, *RPS13*, *RPL7a*, *RPL15*, *RPL36a*, *RPL38*) are comprised of members with similar levels of gene expression (Table 3.5). Of these eight families seven are two-member families, while *RPS7* is a three-member family. The remaining 47 families are comprised of members with differential levels of gene expression (Table 3.5).

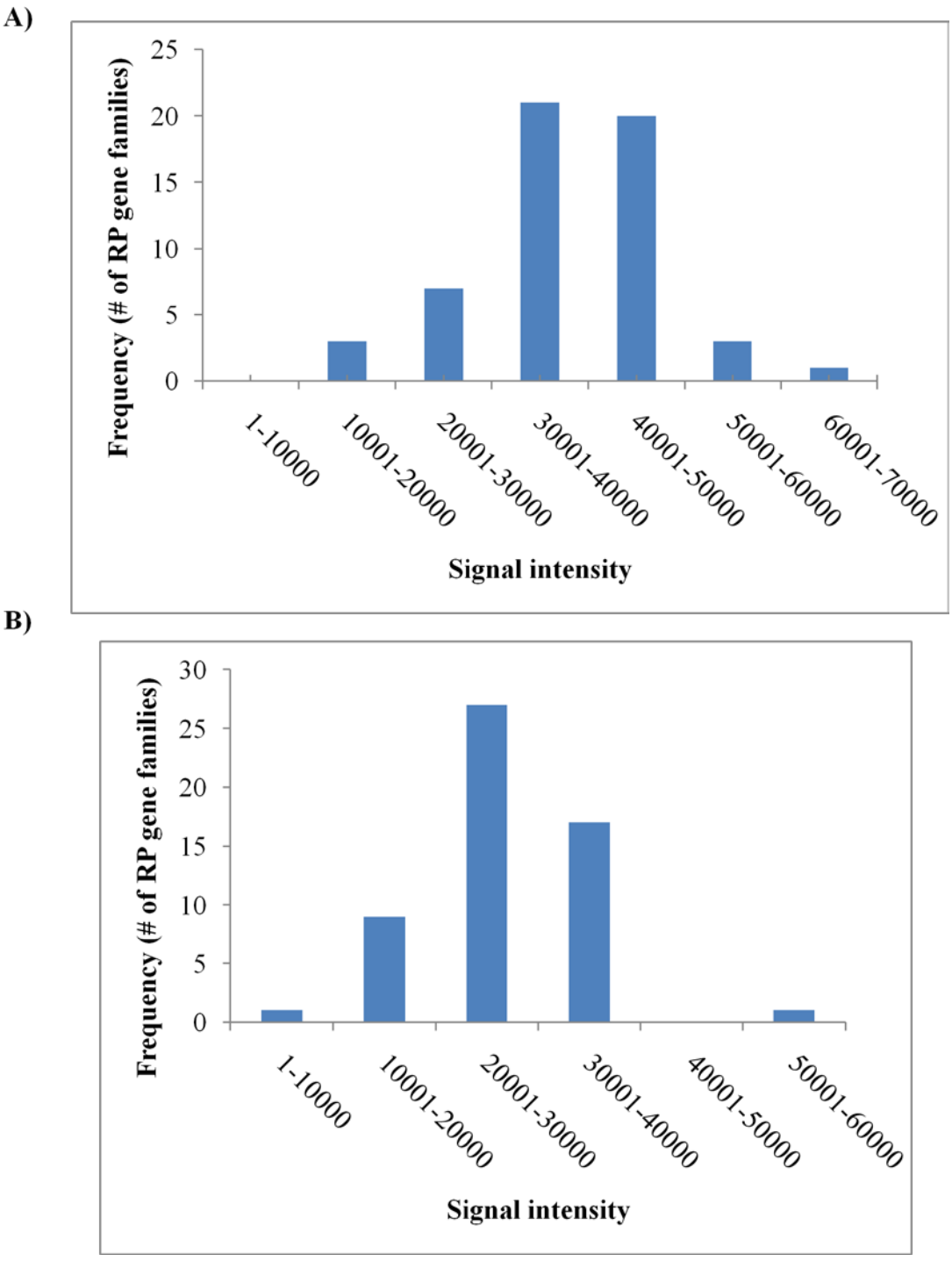


Figure 3.2. Expression levels of RP gene families. Histogram showing frequency of RP gene families with different levels of expression during germination **(A)** and seedling **(B)** stages.

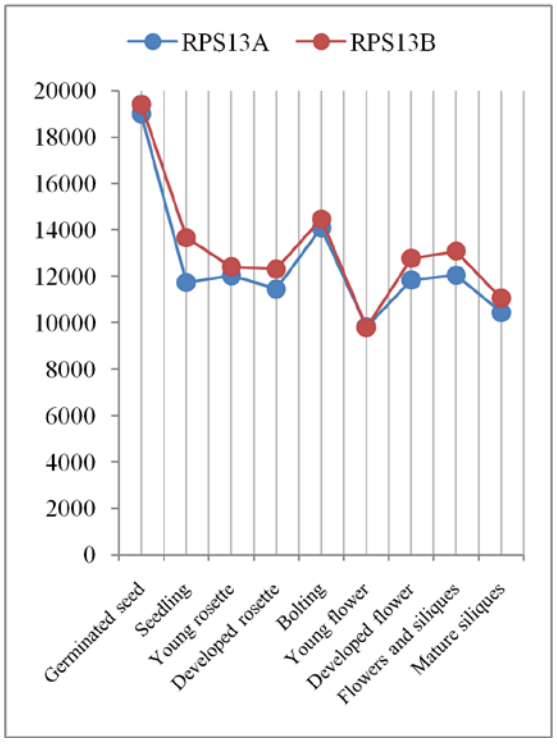
Table 3.4. Frequency of RP gene families with different expression levels in the different developmental stages of Arabidopsis.

Signal intensity (range)	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers & siliques	Mature siliques
5312-10000	0	1	1	2	1	5	2	2	3
10001-20000	3	9	8	10	7	30	10	8	20
20001-30000	7	27	30	27	24	18	30	26	27
30001-40000	21	17	14	15	19	2	12	17	3
40001-50000	20	0	1	0	3	0	0	1	2
50001-60000	3	1	1	1	1	0	1	1	0
60001-65319	1	0	0	0	0	0	0	0	0

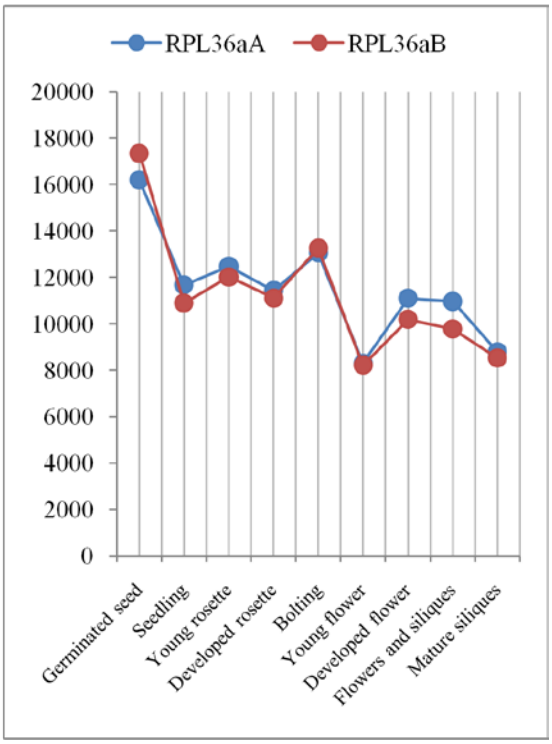
Grey shading = Range of expression levels that highest number of RP gene families have in each developmental stage.

Figure 3.3. Groupings of RP families. Representative type I RP families with members showing similar level of expression (*RPS13*; **A**) and (*RPL36a*; **B**), and type II RP families with members showing differential level of expression (*RPS8*; **C**) and (*RPL10*; **D**). n = 80 (germinated seed), 692 (seedling), 382 (young rosette), 121 (developed rosette), 55 (bolting), 233 (young flower), 375 (developed flower), 89 (flowers and siliques), and 42 arrays (mature siliques). Error bars = SE.

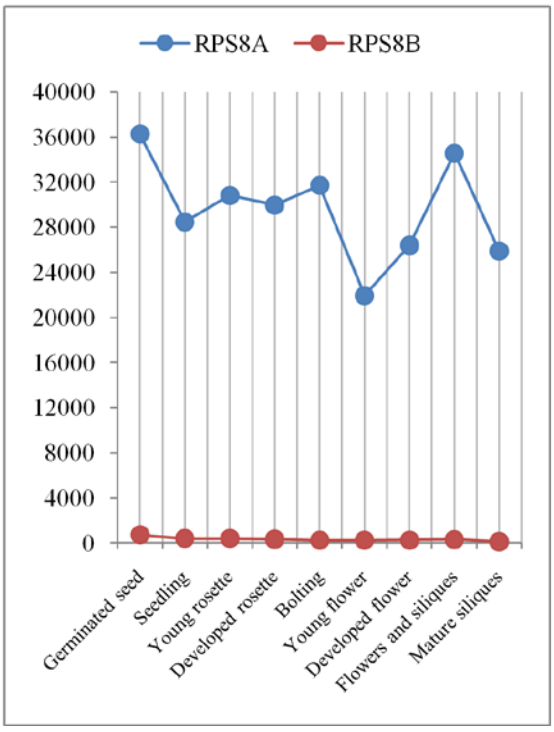
A)


RPS13A
RPS13B
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Germinated seed
Seedling
Young rosette
Developed rosette
Bolting
Young flower
Developed flower
Flowers and siliques
Mature siliques

B)


RPL36aA
RPL36aB
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Germinated seed
Seedling
Young rosette
Developed rosette
Bolting
Young flower
Developed flower
Flowers and siliques
Mature siliques

C)


RPS8A
RPS8B
40000
36000
32000
28000
24000
20000
16000
12000
8000
4000
0
Germinated seed
Seedling
Young rosette
Developed rosette
Bolting
Young flower
Developed flower
Flowers and siliques
Mature siliques

D)

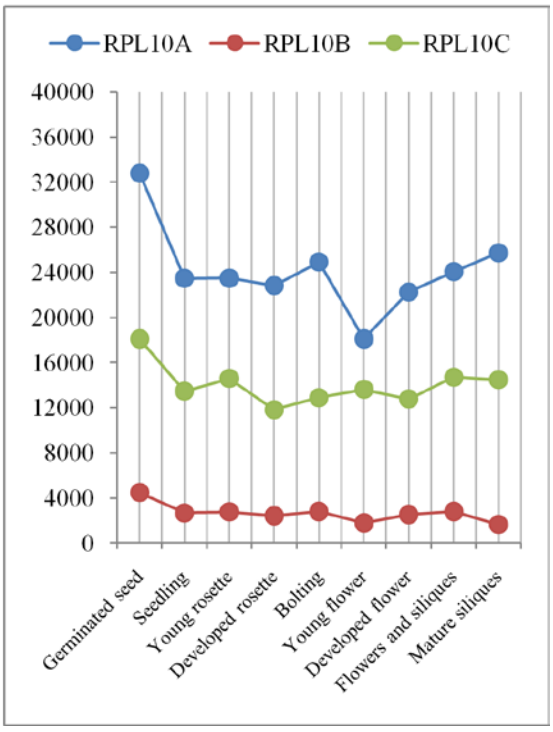

RPL10A
RPL10B
RPL10C
40000
36000
32000
28000
24000
20000
16000
12000
8000
4000
0
Germinated seed
Seedling
Young rosette
Developed rosette
Bolting
Young flower
Developed flower
Flowers and siliques
Mature siliques

Table 3.5. Grouping of RP families based on the expression levels of family members

RP gene family	Family members	Average expression across developmental stages	Type
RPS3a	RPS3aA; RPS3aB	14946; 14092	I
RPS6	RPS6A; RPS6B	11980; 13234	I
RPS7	RPS7A; RPS7B; RPS7C	10322; 11311; 11815	I
RPS13	RPS13A; RPS13B	12499; 13219	I
RPL7a	RPL7aA; RPL7aB	11953; 15175	I
RPL15	RPL15A; RPL15B	16166; 14005	I
RPL36a	RPL36aA; RPL36aB	11556; 11263	I
RPL38	RPL38A; RPL38B	8843; 9393	I
RPSa	RPSaA; RPSaB	19851; 1304	II
RPS8	RPS8A; RPS8B	29558; 340	II
RPS9	RPS9A; RPS9B; RPS9C	7; 22206; 1674	II
RPS10	RPS10A; RPS10B; RPS10C	5550; 12847; 12067	II
RPS11	RPS11A; RPS11B; RPS11C	9630; 1394; 9797	II
RPS12	RPS12A; RPS12C	16129; 7610	II
RPS14	RPS14A; RPS14B; RPS14C	11762; 14343; 6198	II
RPS15	RPS15A; RPS15B; RPS15C; RPS15D; RPS15E; RPS15F	18139; 29; 91; 3044; 44; 106	II
RPS15a	RPS15aA(1); RPS15aB(2); RPS15aC(3); RPS15aD(4); RPS15aE(5); RPS15aF(6)	16148; 520; 55; 9670; 457; 10033	II
RPS18	RPS18A; RPS18B; RPS18C	10592; 5847; 10770	II

RP gene family	Family members	Average expression across developmental stages	Type
RPS19	RPS19A; RPS19B; RPS19C	13492; 1641; 10314	II
RPS21	RPS21A; RPS21B; RPS21C	84; 10513; 16414	II
RPS24	RPS24A (S19); RPS24B	6195; 6047	II
RPS25	RPS25A; RPS25B; RPS25C; RPS25E	261; 3671; 37; 9963	II
RPS28	RPS28A; RPS28B; RPS28C	3817; 11762; 6247	II
RPS30	RPS30A; RPS30B; RPS30C	4089; 11745; 10105	II
RPL5	RPL5A; RPL5B; RPL5C	11274; 9720; 73	II
RPL7	RPL7A; RPL7B; RPL7C; RPL7D	2039; 16734; 10591; 3904	II
RPL10	RPL10A; RPL10B; RPL10C	24186; 2652; 14047	II
RPL10a	RPL10aA; RPL10aB; RPL10aC	10467; 11381; 5502	II
RPL12	RPL12A; RPL12B; RPL12C	12379; 16106; 6899	II
RPL13	RPL13A; RPL13B; RPL13C; RPL13D	23; 17313; 126; 6979	II
RPL13a	RPL13aA; RPL13aB; RPL13aC; RPL13aD	13249; 16380; 4486; 3511	II
RPL14	RPL14A; RPL14B	3480; 18701	II
RPL17	RPL17A; RPL17B	7949; 18434	II
RPL18	RPL18A; RPL18B; RPL18C	134; 13147; 13971	II
RPL18a	RPL18aA; RPL18aB; RPL18aC	3447; 19388; 8486	II
RPL19	RPL19A; RPL19B; RPL19C	21174; 3743; 7244	II
RPL22	RPL22A; RPL22B; RPL22C	98; 13574; 8046	II
RPL23	RPL23A; RPL23B; RPL23C	9401; 9687; 32994	II
RPL23a	RPL23aA; RPL23aB	11317; 6122	II

RP gene family	Family members	Average expression across developmental stages	Type
RPL24	RPL24A; RPL24B	9979; 14168	II
RPL26	RPL26A; RPL26B	23442; 6512	II
RPL27	RPL27A; RPL27B; RPL27C	830; 6046; 8698	II
RPL29	RPL29A; RPL29B	6090; 4157	II
RPL30	RPL30A; RPL30B; RPL30C	175; 14196; 20817	II
RPL31	RPL31A; RPL31B; RPL31C	3437; 9201; 11433	II
RPL34	RPL34A; RPL34B; RPL34C	9012; 17932; 5939	II
RPL35	RPL35A; RPL35B; RPL35C; RPL35D	11516; 3359; 903; 4215	II
RPL36	RPL36A; RPL36B; RPL36C	2000; 9524; 7544	II
RPL37	RPL37A; RPL37B; RPL37C	1034; 21098; 10092	II
RPL39	RPL39A; RPL39B; RPL39C	5471; 2207; 6865	II
RPL40	RPL40A; RPL40B	6952; 19214	II
RPPO	RPPOA; RPPOB; RPPOC	615; 23415; 1739	II
RPP1	RPP1A; RPP1B; RPP1C	17642; 7079; 10079	II
RPP2	RPP2A; RPP2B; RPP2C; RPP2D; RPP2E	12744; 13974; 1795; 5780; 81	II
RPP3	RPP3A; RPP3B	2908; 5798	II

The above classification of Arabidopsis RP families using Genevestigator data is consistent with experimentally determined expression levels of some of these RP gene families e.g., *RPS15a* (Hulm et al. 2005), *RPS18* (Van Lijsebettens et al. 1994), *RPL10* (Falcone Ferreyra et al. 2010) and *RPL23a* (McIntosh and Bonham-Smith 2005), all are comprised of members with differential levels of expression, while the *RPS6* family is comprised of two members with similar levels of expression (Creff et al. 2010).

Although individual members of 47 type II families differ significantly in their level of expression, members of most of these families follow a similar expression pattern (fold change in expression over different developmental stages) across the developmental stages. A pair-wise comparison of expression pattern between 129 member pairs within each of these families shows that ~72% of pairs have correlation coefficients of >0.8. Family members with expression levels lower than 300 were not included in this analysis. Expression level in these instances was so low that, more sensitive assays may be required to accurately determine fold change across the different developmental stages.

3.3.4. Differential localization of RPs

As indicated by the previous data, regulation of RP gene expression at the level of transcription may not completely ensure the availability of equimolar quantities of RPs required for ribosome biogenesis. Therefore, mechanisms may exist at the post-transcriptional level to ensure that all RPs are available in the nucleolus in equimolar quantities for ribosome biogenesis. To investigate RP localization to the nucleolus, I studied the subcellular localization of five two-member RP families. As indicated by the fluorescence intensity of these RP-EGFP fusions in different cellular compartments, there were significant differences (ANOVA, n=10 transformed cells and r=3) in RP localization to the cytoplasm (P = 2.3E-07), the nucleus (P = 7.7E-11) and the nucleolus (P = 1.9E-10) (Figure 3.4 and 3.5A). The highest cytoplasmic accumulation was observed for both RPL7aA and B, with RPL7aB being the highest, while cytoplasmic accumulation of RPL15B and RPL23aA was the lowest. RPL23aB exhibited the strongest nuclear localization, while very little RPS8A accumulated in the nucleus. RPL15A/B showed the strongest nucleolar localization, while RPS3aB and RPS8B showed the weakest nucleolar localization (Figure 3.4 and 3.5A). There was a strong inverse correlation between cytoplasmic

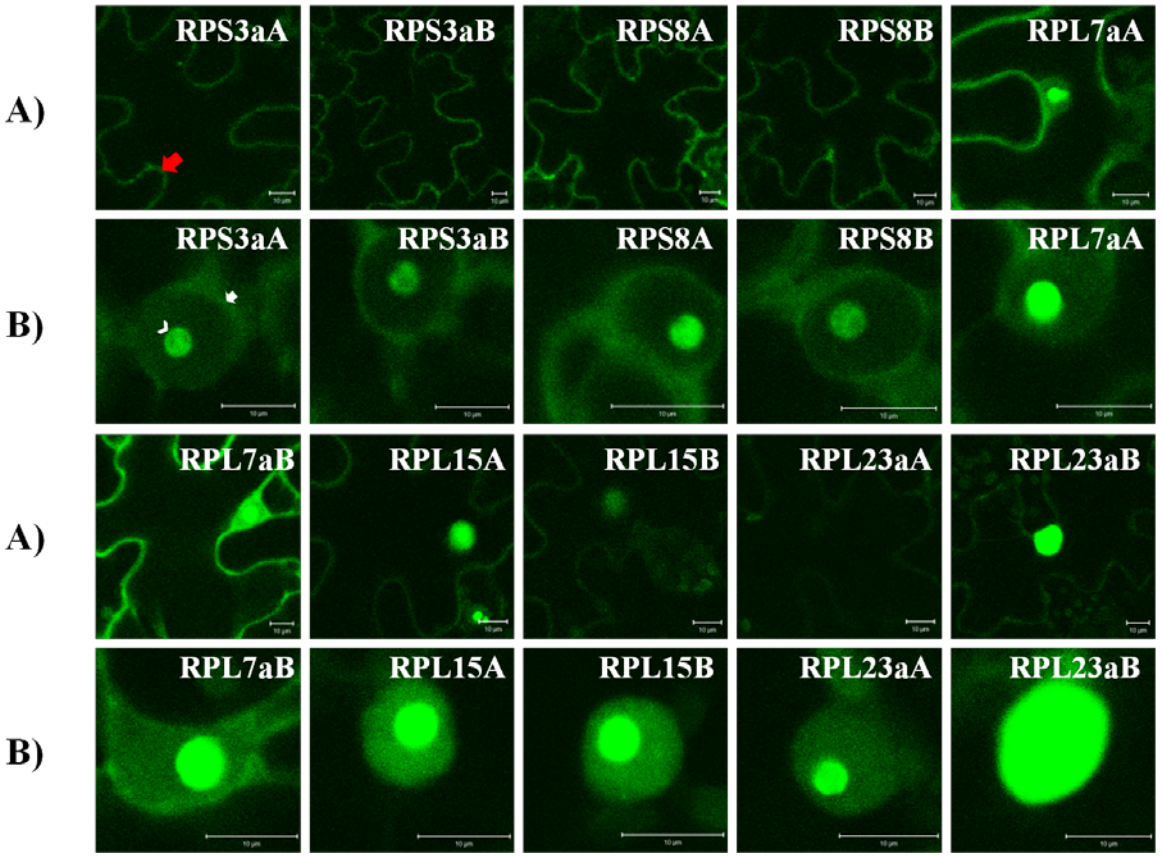


Figure 3.4. Subcellular localization of RPS3aA/B, RPS8A/B, RPL7aA/B, RPL15A/B and RPL23aA/B. CLSM images of tobacco leaf epidermal cells transiently expressing different RPs tagged to EGFP. Cytoplasmic **(A)**, nuclear and nucleolar **(B)** localization of different RPs tagged with EGFP. White arrowhead indicates the nucleolus, white arrow the nucleus, and red arrow the cytoplasm. Scale bar = 10 µm.

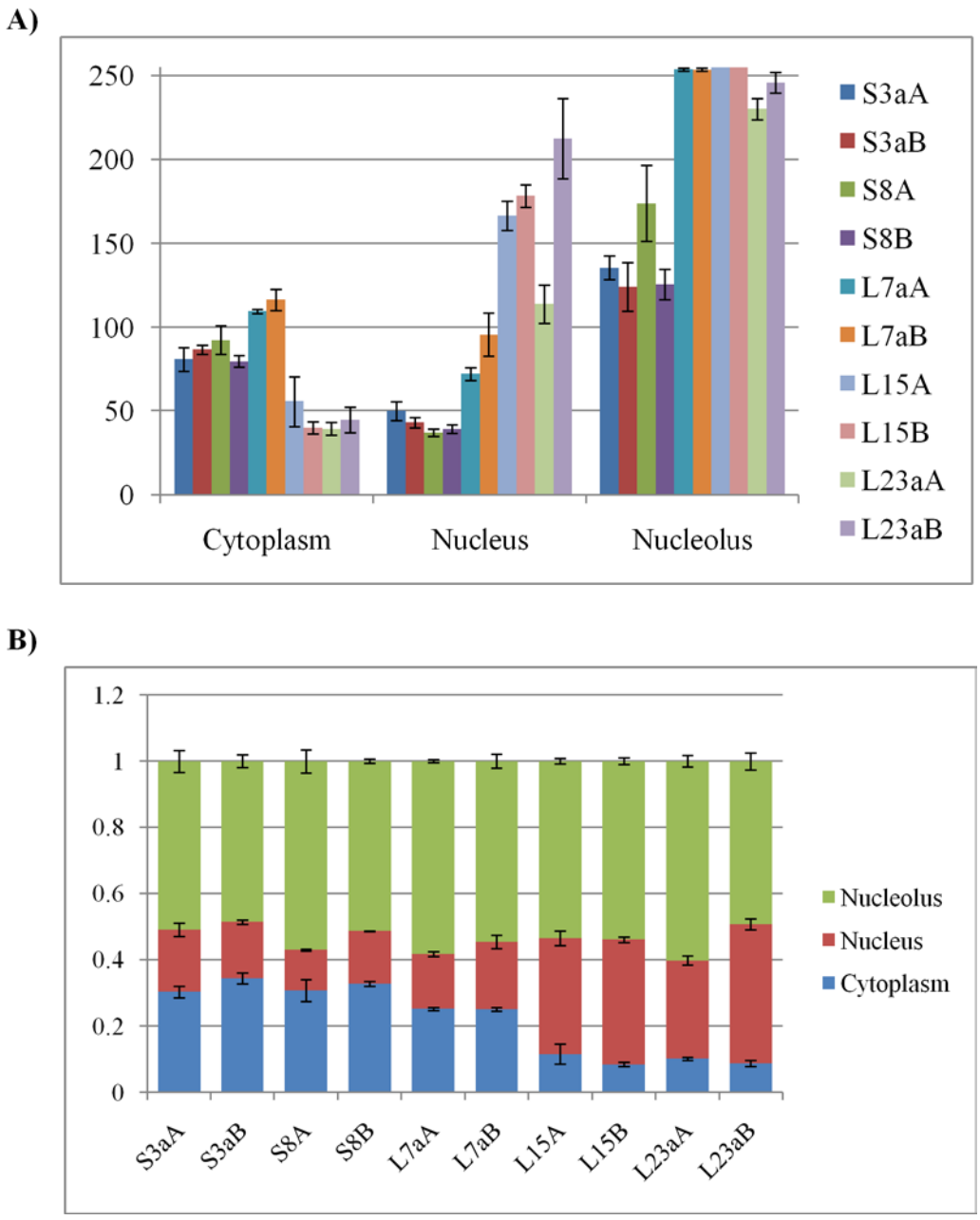


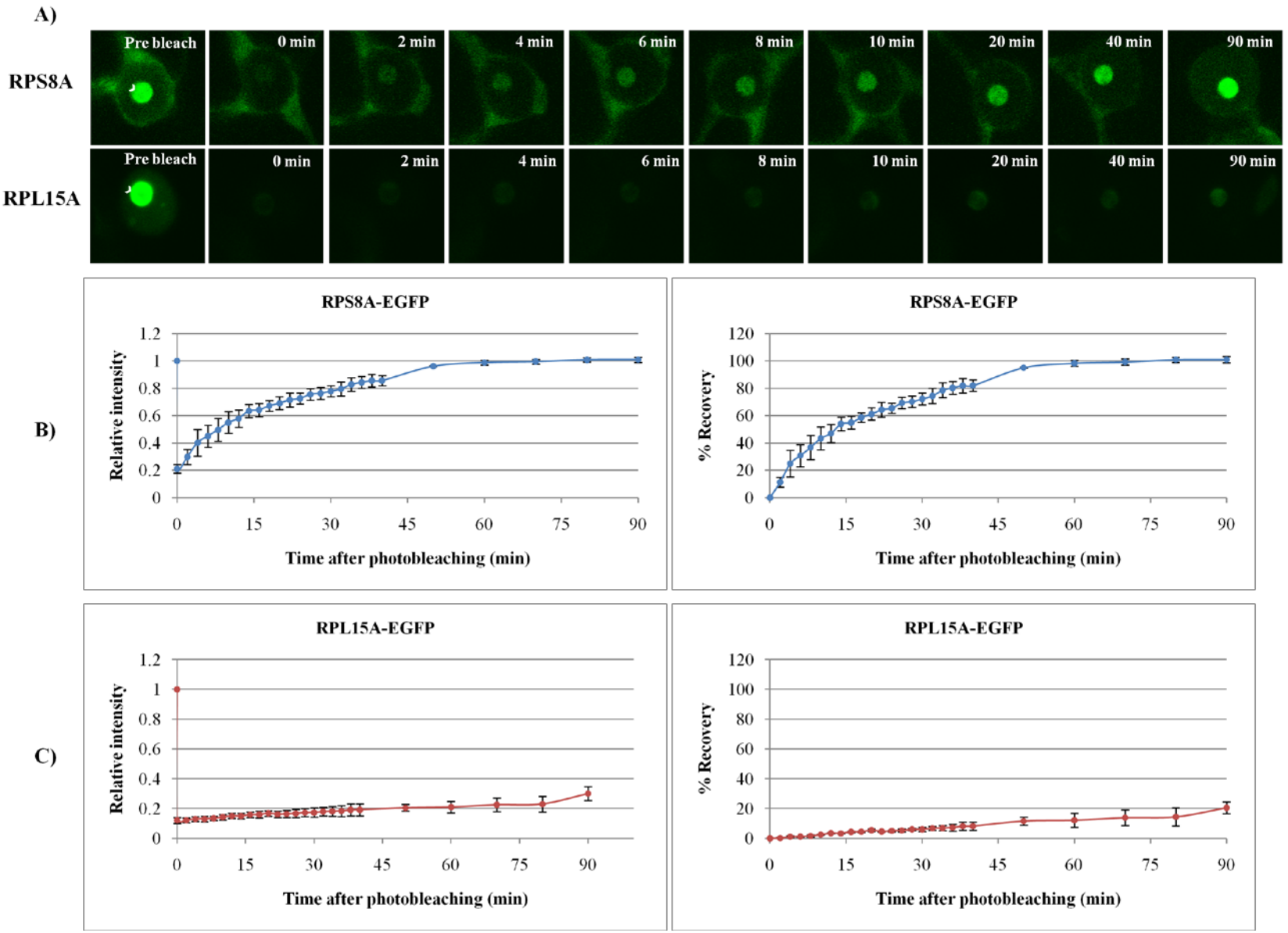
Figure 3.5. Quantification of differential localization of RPs. (A) The cytoplasmic, nuclear and nucleolar intensities (arbitrary units, 0-255) of EGFP tagged RPs. (**B)** Relative fluorescence intensities (proportion) of different RPs within the cytoplasm, nucleus and nucleolus.

and nucleolar localization of RPs (R = -0.87). Higher cytoplasmic accumulation of RPS3aA/B and RPS8A/B is associated with a relatively lower nucleolar localization, whereas RPL15A/B and RPL23aA/B showed the opposite trend. A third trend was seen for RPL7Aa/B where a higher cytoplasmic localization, compared to other RPs, is not associated with lower nucleolar localization, but does correlate with lower nuclear localization. There was a significant difference in relative fluorescence intensity (proportion of cytoplasmic, nuclear and nucleolar intensity of a RP) of RPs across the cytoplasm (P = 5E-11), the nucleus (P = 2.19E-12) and the nucleolus (P = 0.01) (Figure 3.5B), indicating that differential localization of RPs to a specific cellular compartment is not due to a difference in protein level itself, but due to differential localization. However, it should be noted that the relative fluorescence intensities of RPL15A and RPL15B may not be accurate as fluorescence intensities of these two RPs are saturated (Figure 3.5A). Nonetheless, there is a significant difference in relative fluorescence intensities among the remaining eight RPs (P < 0.01). Another notable observation was a frequent accumulation of some RPs at the periphery of the nucleus (Figure 3.4B – RPS3aA, RPS3aB, RPS8A, RPS8B, RPL7aA and RPL7aB). Percentage of transformed cells (n = 30) showing peripheral nuclear localization; RPL7aA = 40%, RPS3aA = 43%, RPL7aB = 50%, RPS3aB = 63%, RPS8B = 70%, and RPS8A = 77%, however, RPL15A/B and RPL23aA/B never accumulated at the periphery of the nucleus. A significant differential localization within the family was observed for RPL23a, with a significantly lower level of RPL23aA accumulated in the nucleus compared to RPL23aB (Figure 3.4A). Previously, it was observed that RPL23aB accumulated at the periphery of the nucleolus in a significantly higher number of cells compared to RPL23aA (Degenhardt and Bonham-Smith 2008). A relatively inefficient nucleolar localization leading to reduced assembly into ribosomes could be a reason for a buildup of RPL23aB in the nucleus, leading to increased fluorescence intensity.

3.3.5. RPS8A is imported into the nucleolus faster than RPL15A

To determine if the level of RP localized to the nucleolus was dependent on import rate, I carried out FRAP analyses to determine the kinetics of nucleolar influx of two proteins RPS8A (molecular weight = 24.99 kDa, pI = 10.32) and RPL15A (molecular weight = 24.24 kDa, pI = 11.44), that have a significant difference in nucleolar localization. Considering that RPL15A had a much higher nucleolar accumulation than RPS8A (Figure 3.5A), I expected that RPL15A would have a higher rate of nucleolar import. However, FRAP results (Figure 3.6A and B)

Figure 3.6. RPS8A had higher nucleolar import rate than RPL15A. Dynamics of nucleolar import observed in FRAP assays of nucleoli of tobacco leaf epidermal cells transiently expressing RPS8A-EGFP and RPL15A-EGFP. **(A)** Pre-bleach and post-bleach images of nucleolar RPS8A-EGFP and RPL15A-EGFP taken at the indicated times (min) after photobleaching during a time course of 90 min. White arrowhead indicates the nucleolus. Kinetics of nucleolar recovery of RPS8A-EGFP **(B)** and RPL15A-EGFP **(C)** after photobleaching. Relative intensity = nucleolar fluorescence intensity at an indicated time point after photobleaching normalized to prebleach fluorescence intensity. % recovery = nucleolar fluorescence recovered at an indicated time normalized to nucleolar fluorescence lost during bleaching expressed as a percentage [(fluorescence intensity at an indicated time point – intensity immediately after the bleaching)/prebleach intensity - intensity immediately after bleaching) x 100]. The experiment was repeated three times. Error bars = SE.


A)
RPS8A
RPL15A
Pre bleach
0 min
2 min
4 min
6 min
8 min
10 min
20 min
40 min
90 min
B)
RPS8A-EGFP
Relative intensity
% Recovery
Time after photobleaching (min)
C)
RPL15A-EGFP

showed that RPS8A had a much higher import rate than RPL15A. After photobleaching, 50% of RPS8A-EGFP fluorescence was recovered in 13 (SE = ± 2.79) min, whereas during the same period RPL15A-EGFP showed only a 3% (SE = ± 0.22) recovery (Figure 3.6A and C). By 50 min the RPS8A-EGFP nucleolar intensity had recovered to 95% (SE = ± 0.28) level, while during this period there was only an 12% (SE = ± 2.74) recovery of RPL15A-EGFP fluorescence, which showed just 21% (SE = ± 3.96) recovery even after 90 minutes. These results indicate that higher nucleolar localization of RPL15A was not due to a higher rate of import. Nucleolar import rate of RPL23aA [50% recovery in 9 (SE = ± 1.37) minutes, and in 50 minutes 90% (SE = ± 1.33) recovery] was similar to that of RPS8A.

3.4. Discussion

Owing to the presence of only a single molecule of each of the 81 RPs in a given Arabidopsis ribosome, presumably all RPs need to be available in the nucleolus in equimolar quantities. Although coordinated regulation to ensure equal levels of expression of the different RP genes would be the most efficient way to achieve equimolar availability, considering the existence of multigene families for RPs in plants and the various extraribosomal functions of some RPs, I hypothesized that all RP genes might not be transcribed at the same level. Consistent with this hypothesis, a Genevestigator analysis of the Arabidopsis Affymetrix 22k chip for RP gene expression over various developmental stages showed up to a 300-fold difference in transcript levels, indicating that a general coordinated regulation of RP gene expression is not evident at the level of individual RP genes. At the level of the gene family, the difference in expression between families is reduced to ~7.5-fold, suggesting that some coordination between families does occur. This is a reasonable outcome, as although each RP has more than one isoform, only a single isoform can assemble into any given ribosome. In many RP families, a low expression of one member is compensated for by a higher expression of another member of the family.

In spite of a more stringent coordinated regulation of expression at the RP gene family level, different families are not expressed at identical levels – differences of up to ~7.5-fold still exist. For instance in the germination stage, the RPL23 family has a signal intensity of 65319, whereas the RPS24 family has a much lower signal intensity of 16770. It is not clear whether RPs with lower expression, like RPS24, are limiting factors in ribosome biogenesis or whether

there is a mechanism to accommodate the lower transcript level by higher translational efficiency of these transcripts. Alternatively, RP families with higher expression like RPL23 may be required for extraribosomal functions (Table 3.6) in addition to their conventional role in ribosome biogenesis and function. Some high expression RP families are comprised of members, all of which show expression higher than the lowest expressed family. For instance, signal intensities of the *RPL15* family members *RPL15A* is 23590 and *RPL15B* is 22404 during germination stage. Both of these signal intensities are individually higher than the combined signal intensity of the RPS24 family (16770), suggesting that one of the members of *RL15A* is sufficient to meet the demand for this RP in ribosome biogenesis given that RPS24 could be a limiting factor. It is possible that while one member of *RPL15* is recruited to ribosomal function, the other member(s) is recruited to extraribosomal function.

Families of RP genes, originating from gene or genome duplication events during evolution, may contain genes that have become; I) nonfunctionalized, where one copy is simply silenced by degenerative mutations (Lynch and Conery 2000; Veitia et al. 2008). *RPS15aC*, one of the six members of the Arabidopsis *RPS15a* family, is transcriptionally inactive in all tissues and at all developmental stages that have been studied (Hulm et al. 2005). Across all developmental stages studied, the signal intensity of *RPS15aC*,as reported by Genevestigator, is ~40-69. The same range of signal intensity is seen for *RPS9A*, *RPS15B*, *RPL4B*, *RPL37aB*, *RPS25C* and *RPS15E*, suggesting that these genes have become nonfunctionalized or are on an evolutionary path to nonfunctionalization. However, we cannot rule out the possibility that these proteins may perform functions that require minor amounts of protein or function in tissues or developmental stages yet to be analyzed. II) Subfunctionalized, where duplicated genes share ancestral gene function (Lynch and Conery 2000). RP families *RPS3a*, *RPS6*, *RPS7*, *RPS13*, *RPL7a*, *RPL15*, *RPL36a*, and *RPL38* all have members with similar expression levels and pattern of expression, suggesting that these members may share ancestral protein function within or away from the ribosome. Also, considering their similar expression level and pattern of expression, the members of these families are likely to share the same transcriptional machinery, and hence one can expect transcriptional compensation within the family; if expression of one member is knocked out/down as a result of mutation in its regulatory regions, the expression of the other member is upregulated owing to the reduced competition for the components of the transcriptional machinery. III) Neofunctionalized, where one copy has acquired a novel,

Table 3.6. Extraribosomal functions of some RPs

RP	Organism	Extra ribosomal functions	References	% Identity with Arabidopsis orthologs
RPS3	Human	Co-regulator of transcription factor NF-kB, DNA endonuclease	Lenardo and Baltimore 1989; Sen 2006 Kim et al. 1995	RPS3A - 82.0 RPS3B - 81.9 RPS3C - 76.3
RPL7	Human	Co-regulator of transcription factor complex VDR/RXR	Berghofer-Hochheimer et al. 1998	RPL7A – 40.0 RPL7B – 60.0 RPL7C – 67.0 RPL7D – 61.0
RPL13a	Human	Inhibition of translation of *ceruloplasmin* mRNA	Mazumder et al. 2003; Mukhopadhyay et al. 2009	RPL13aA - 61.6 RPL13aB - 61.1 RPL13aC - 61.6 RPL13aD - 60.5
RPL26	Human	Promotion of translation of *p53* mRNA	Takagi et al. 2005	RPL26A - 73.4 RPL26B - 67.6
RPL11	Human	Inhibition of c-Myc-mediated transcription	Dai et al. 2007a; Dai et al. 2007b	RPL11A -72.0 RPL11B -72.0 RPL11C -72.0 RPL11D -72.0
RPL23	Human	Sequesters co-activator of Miz1, nucleophosmin in the nucleolus	Wanzel et al. 2008	RPL23A - 84.9 RPL23B - 84.9 RPL23C - 84.9
RPS13	Human	Autogenous inhibition of splicing of own mRNA	Malygin et al. 2007	RPS13A - 78.1 RPS13B - 78.8
RPS14	*S. cerevisiae*	Autogenous inhibition of splicing of own mRNA	Fewell and Woolford 1999	RPS14A – 80.8 RPS14B – 80.8 RPS14C – 80.0

RP	Organism	Extra ribosomal functions	References	% Identity with Arabidopsis orthologs
RPL30	*S. cerevisiae*	Autogenous inhibition of splicing of own mRNA	Vilardell and Warner 1994	RPL30A - 61.0 RPL30B - 61.0 RPL30C - 63.0
RPS28B	*S. cerevisiae*	Mediates decay of own mRNA	Badis et al. 2004	RPS28A - 70.0 RPS28B - 70.0 RPS28C - 69.0
RPL30E	*Pisum sativum*	Salt tolerance	Joshi et al. 2009	RPL30A - 89.0 RPL30B - 88.0 RPL30C – 87.0
RPL10A	Arabidopsis	Resistance to geminivirus infection	Carvalho et al. 2008	NA
RPS27A	Arabidopsis	UV-stress response	Revenkova et al. 1999	NA
RPL5A/B RPL9 RPL10a RPL24B RPL28A	Arabidopsis	Determination of leaf abaxial-adaxial polarity	Yao et al. 2008; Pinon et al. 2008	NA
RPS18A RPS13B RPS5A RPL4A RPL23aA	Arabidopsis	Auxin homeostasis	Ito et al. 2000; Van Lijsebettens et al. 1994; Weijers et al. 2001; Degenhardt and Bonham-Smith 2008; Rosado et al. 2010	NA
RPL4A	Arabidopsis	Vacuolar trafficking	Rosado et al. 2010	NA

beneficial function, while the other copy(ies) retain(s) the original ancestral function(s) (Lynch and Conery 2000). For instance, while two of the three member Arabidopsis *RPS27* family have retained the original ancestral function associated with the ribosome, *RPS27A* has acquired a new role in the plant response to genotoxic (DNA-damaging) stress; *rps27a* mutants are unable to rapidly degrade mRNA after UV treatment, which is an essential step in the UV-stress response, but under optimal growing conditions, the *RPS27A* knock out has no effect on plant growth and development (Revenkova et al. 1999).

Considering that divergence of duplicated genes and the subsequent acquisition of new adaptive functions is often associated with differential expression, there may be many more RP genes that have been neofunctionalized. Out of 55 families, 47 families (type II) have members with different levels of expression.

As mentioned above, coordinated regulation of expression of RP gene families at the level of transcription ensures only similar, but not identical amounts of transcript of each RP family, necessitating some regulation at the post-transcriptional and translational levels. There is considerable evidence that in plants, signals such as hypoxia, sucrose starvation and dehydration stress, leading to growth arrest, result in a coordinated decrease in the translation of RP mRNAs (Branco-Price et al. 2005; Kawaguchi et al. 2004; Nicolai et al. 2006). Removal of these signals leads to a coordinated reversal of translational repression, indicating the presence of mechanisms able to coordinately regulate the translation of RP mRNAs (Branco-Price et al. 2005; Kawaguchi et al. 2004; Nicolai et al. 2006). However, when there are different transcript levels in the first place, coordinated regulation of translation of these transcripts will produce unequal amounts of RPs, unless there is a reciprocal difference in the translation efficiency of these transcripts. At least in yeast, such a reciprocal difference does not appear to exist; RP mRNAs with four to five-fold differences in abundance have similar rates of translation (Maicas et al. 1988; Perry 2007). Therefore there may be some regulation mechanisms operating at the level of subcellular localization of RPs to ensure their equimolar availability in the nucleolus. My subcellular localization studies of members of five RP families, however, suggest that RPs do not localize to the nucleolus in equal quantities or rates. While RPs like RPL15A showed strong nucleolar localization, RPs like RPS3a had weak nucleolar localization. There was also a significant variation in accumulation of RPs in the cytoplasm and nucleus. In agrobacterium-mediated plant cell transformation, the number of T-DNA insertions may vary between cells, leading to

differential expression levels. Although this might be a reason for differential localization of RPs, the consistency of fluorescence intensities between cells and replicates (Figure 3.5) largely rule out such possibilities.

Why do RPs exhibit differential localization patterns and accumulate to different levels in different cellular compartments? Some individual RPs are known to perform various extraribosomal functions in different cellular compartments (Table 3.6). For instance, mammalian RPS3 with an endonuclease activity, is involved in DNA damage repair (Kim et al. 1995). RPS3 generally accumulates in the cytoplasm, but in response to DNA damage is phosphorylated at T^{42} by ERK1/2 (Extracellular signal-Regulated Kinase) and translocates to the nucleus, where it is involved in base excision repair of the damaged DNA (Yadavilli et al. 2007). Similarly, Arabidopsis RPL10A, which normally accumulates in the cytoplasm, is phosphorylated and translocates to the nucleus in response to viral infection (Carvalho et al. 2008). RPs also function in the nucleus in association with TFs to regulate transcription of target genes; mammalian RPL11 in association with TF c-Myc and mammalian RPL7 in association with the heterodimeric TF complex, vitamin D receptor [VDR] and retinoid X receptor [RXR], control the transcription of the target genes of these TFs (Berghofer-Hochheimer et al. 1998; Dai et al. 2007b). In the nucleolus itself, RPs can perform functions unrelated to ribosome biogenesis. For instance, RPL23 in the nucleolus can sequester nucleophosmin, the coactivator of TF Miz1, thereby negatively regulating transcription of Miz1 target genes in the nucleus (Wanzel et al. 2008). It is possible that in Arabidopsis also some of the RPs we studied may perform extraribosomal functions in the nucleus, cytoplasm and nucleolus, and hence some RPs as I observed show stronger localization to these compartments than others.

Too much of a RP in the nucleus/nucleolus is probably not a good thing therefore, in mammals, degradation of excess RPs, that are not assembled into ribosomes, occurs via the 26S-proteasome pathway in the nucleus (Lam et al. 2007). A similar mechanism may also exist to degrade excess RPs in the cytoplasm (Hirsch and Ploegh 2000; Shirangi et al. 2002). Differential ubiquitination and degradation of unassembled RPs may also contribute to the differential accumulation of RPs in the nucleus (e.g., very little nuclear accumulation of RPS8 versus strong nuclear accumulation of RPL23aB) and cytoplasm (e.g., strong cytoplasmic accumulation of RPL7a versus weak cytoplasmic accumulation of RPL23a). A 26S proteasome degradation

pathway for RPs has recently been confirmed in Arabidopsis (Degenhardt and Bonham-Smith, submitted 2011).

Differential localization of RPs could also be the result of a differential acquisition of localization signals over evolutionary time. While targeting of RPs to the nucleolus is an essential step in eukaryotic ribosome biogenesis, in prokaryotes, no such targeting process is required as the cell is non-compartmentalized. Indeed as documented in chapter II differences in signal requirements for the localization of RPL23aA, RPL15A and RPS8A are apparent.

I also observed differences in the nucleolar import rates of RPs; RPL15A has a much lower nucleolar import rate compared to RPS8A. However, it is surprising that although RPL15A-EGFP has much stronger nucleolar localization compared to RPS8A-EGFP, it has a lower nucleolar import rate. It is possible that to make up for its slower import rate some RPL15A is always stored in the nucleolus to ensure that its import rate is not a limiting factor when demand for ribosome biogenesis increases. Alternatively, following its import into the nucleolus, the rate at which RPL15A is incorporated into ribosomal subunits may be slower than RPS8A.

Although I could not determine the relationship between transcript level of a RP and its localization efficiency (in my subcellular localization study all RP genes were driven by the 35S promoter and the level at which corresponding endogenous RPs were present was unknown), my results do suggest that there is an inherent difference in localization pattern and nucleolar localization efficiency of RPs.

CHAPTER 4. SUMMARY, CONCLUSIONS AND FUTURE WORK

In this thesis, I investigated the regulation of expression of Arabidopsis RP genes, subcellular localization of five RP families, and signal requirements for the nuclear/nucleolar localization of RPL23aA, RPL15A and RPS8A.

As a result of whole and localized duplication events, there are two or more genes for each of the 81 Arabidopsis cytoplasmic RPs (Barakat et al. 2001). Although some of the duplicated genes might have been lost altogether from the evolving genome, or a few of them such as *RPS15aC* may have lost their function over the course of evolution (Hulm et al. 2005), all Arabidopsis RP gene families now have two or more transcriptionally active members (Barakat et al. 2001). However, only a single molecule of each RP can assemble into a given ribosome, hence different RPs need to be present in equimolar quantities in the nucleolus to ensure efficient use of energy in ribosomal subunit assembly. Although equimolar availability could be efficiently achieved by coordinated regulation of expression of RP genes, considering the presence of multiple isoforms of RPs in plants and the various extraribosomal functions that individual RPs may perform, I assumed that different RP genes would have different rates of transcription. To verify this assumption, I analyzed Arabidopsis RP gene expression during various developmental stages, using the microarray data analysis tool Genevestigator. This data analysis suggests that, consistent with the above assumption, there is up to a 300-fold difference in the amount of transcripts of different individual RP genes, indicating that RP gene expression is not generally coordinated at the individual RP gene level. When transcript abundance is analyzed at the level of the RP gene family, there is only a difference of up to ~7.5-fold, indicating that there is a higher level of coordinated regulation of RP gene expression at the family level. The lower expression of one (some) family member(s) is compensated for by a higher expression of other family member(s). However, it is notable that, despite the higher level of coordinated regulation at the RP gene family level, different families are not expressed at an identical level. Therefore, mechanisms may exist at the post-transcriptional level to compensate for the difference in transcript abundance, by means of a reciprocal difference in mRNA half-life, efficiency of translation or nucleolar targeting, to attain an equimolar availability of different RPs in the nucleolus.

Different RPs do not localize to the nucleolus in equal quantities, nor do they localize to the nucleus or accumulate in the cytoplasm in equal quantities. There is also a difference in the

nucleolar import rate of RPs. Interestingly, RPL15A, with a stronger nucleolar localization, compared to RPS8A, has a slower nucleolar import rate. Some of the possible explanations for differential transcription and localization of RPs are; i) some RPs might have extraribosomal roles. (Warner and McIntosh 2009; Wool 1996), ii) differential ubiquitination and degradation of unassembled RPs, and iii) differential acquisition of localization signals during the evolution of eukaryotic RPs, as a result of which some RPs localize to the nucleolus more efficiently than others, making those with the lower nucleolar accrual the limiting factor for ribosome subunit assembly.

Differential localization of RPs raises the question of whether localization of different RPs is mediated by different signals. To address this question, I studied the signal requirements for nuclear and nucleolar localization of three RPs that show significant difference in their localization pattern – RPL23aA, RPL15A and RPS8A. The results of this study support the suggestion that differences in signals mediate different rates and location of RP accumulation.

In RPL23aA, mutation of all eight basic pNLS motifs did not affect nuclear localization, but completely disrupted nucleolar localization (pNoLSs). Mutation of any four of the eight pNoLSs did not affect nuclear or nucleolar localization, while the serial mutations of the remaining pNoLSs increasingly disrupted nucleolar localization, leading to 100% disruption when all eight NoLSs were mutated. These data support the notion that nucleolar localization of RPL23aA is mediated by a charge-based mechanism and not specified by a single NoLS. It should be noted that although the total basic charge is a major determinant of nucleolar localization, the positions of the NoLSs conferring this basic charge might also be equally important as the positions of these NoLSs could significantly impact the protein folding. In contrast, mutations of just three N-terminal basic motifs in RPL15A disrupted both nuclear and nucleolar localization. The mutation of the first N-terminal basic motif in RPS8A did not affect localization, while the additional mutation of the second basic N-terminal motif disrupted both nuclear and nucleolar localization. Therefore unlike RPL23aA, nuclear/nucleolar localization of both RPL15A and RPS8A required specific N-terminal NLSs. The mutation of other combinations of basic motifs within RPL15A and RPS8A had no effect on nuclear or nucleolar localization of these RPs. Differential signal requirements for the localization of RPL23aA, RPL15A and RPS8A suggest that different mechanisms likely govern the localization of these RPs. Interestingly, the NLSs of RPL15A and RPS8A, identified in this study, match classical

monopartite NLS, while such signals, although present in RPL23aA, do not have a role in nuclear localization of this RP. In humans, nuclear localization of different RPs has been shown to be mediated via different pathways (Chou et al. 2010; Jakel and Gorlich 1998; Plafker and Macara 2002).

The availability in the nucleolus of sufficient quantities of RPs to meet the cellular demand for ribosome biogenesis, may be ensured by employing different pathways for the localization of RPs. As demonstrated by the results in this work, in the eukaryotic cell, the transport of different RPs to the nucleolus, subsequent to their synthesis in the cytoplasm, is one of the complex processes of the cell and probably involves many different pathways.

Future work

Future work is required to: i) study by yeast two hybrid and bimolecular fluorescence complementation (BiFC) assays, the interaction of RPL23aA with other proteins, especially, importin βs, to elucidate the mechanism of nuclear import of RPL23aA, ii) determine if RPL23aA interacts with 26S rRNA and if so, the sequence motifs required for this interaction, to elucidate the mechanism of retention of RPL23aA in the nucleolus. This type of study may also provide insights into the role of RPL23aA in pre-rRNA processing; the effects of mutations of identified RPL23aA motifs involved in rRNA interactions, on pre-rRNA processing could be identified.

It is necessary to study the ability of various NoLS mutants of RPL23aA to assemble into ribosomes and to study the effect of these mutations on growth. This can be done by testing the ability of these mutant proteins to complement the yeast *l25* strain YCR61 (McIntosh and Bonham-Smith 2001).

To identify common requirements for coordinate regulation of RP gene expression, it will be necessary to identify common TF binding sites in the regulatory regions of RP genes. It has been observed that, while most RP genes have almost similar expression levels, some have very high or low levels of expression. Such expression levels may be a result of unique TF binding sites present in the regulatory regions of these genes, which need to be identified. Furthermore, is the observed difference in transcript levels of RP genes compensated for by a reciprocal difference in the translation efficiency of their transcripts? To address this question, it will be

necessary to determine translation rates of different RP transcripts by the polysome profiling and DNA microarray combined with subsequent western blot analysis.

Considering my observation that the five RP families I studied have differential localization patterns, it would be necessary to study subcellular localization patterns of all RPs, which may identify the extent of general, coordinated regulation of nucleolar localization of RPs. These studies would also aid in the identification of extraribosomal functions of RPs, as localization of a RP to a particular cell location would be an indication that the RP has a function in that locale.

5. APPENDIX A. LIST OF OLIGONUCLEOTIDE PRIMERS

Table A.1. Primers used for cloning (restriction sites are in bold and underlined)

ORF	Primer	Primer sequence	Restriction site
RPS3aA	Forward	**GAATTC**ATGGCCGTCGGGAAAAAC	*Eco*RI
	Reverse	**GGATCC**AGCTCCGATGATTTCAG	*Bam*HI
RPS3aB	Forward	**GAATTC**ATGGCTGTCGGGAAGAAC	*Eco*RI
	Reverse	**GGATCC**AGCTCCGATGATTTCTG	*Bam*HI
RPS8A	Forward	**GAATTC**ATGGGTATTTCTCGTGAC	*Eco*RI
	Reverse	**GGATCC**AGCAGCACCCTTGCCC	*Bam*HI
RPS8B	Forward	**GTCGACAATTG**ATGGGTATCTCTCGTG	*Sal*I, *Mfe*I
	Reverse	**TCTAGATCT**TGCAGCACCAGCATTC	*Xba*I, *Bgl*II
RPL7aA	Forward	GCG **GAATTC**ATGGCCCCGAAGAAAGG	*Eco*RI
	Reverse	GCG **TCTAGATCT**ATTCATCCTTTGGGCAG	*Xba*I, *Bgl*II
RPL7aB	Forward	GCG **GAATTC**ATGGCACCGAAGAAGGG	*Eco*RI
	Reverse	GCG **TCTAGATCT**GTTCATTCTCTGGGCAG	*Xba*I, *Bgl*II
RPL15A	Forward	**GAATTC**ATGGGTGCGTACAAGTATG	*Eco*RI
	Reverse	**GGATCC**ACGGTAACGACGAAGGCTG	*Bam*HI
RPL15B	Forward	**GAATTC**ATGGGTGCGTACAAATAC	*Eco*RI

ORF	Primer	Primer sequence	Restriction site
	Reverse	**GGATCC**CCGGTAACGACGGTGTG	*Bam*HI
RPL23aA	Forward	GCG**GAATTC**ATGTCTCCGGCTAAAG	*Eco*RI
	Reverse	GCG**GGATCC**GATGATGCCGATCTTGTTAG	*Bam*HI
RPL23aB	Forward	GCG**GAATTC**ATGTCTCCAGCTAAAG	*Eco*RI
	Reverse	GCG**GGATCC**GATGATCCCGATTTTGTTAG	*Bam*HI
mCherry	Forward	GCG**AAGCTT**ATGGTGAGCAAGGGC	*Hind*III
	Reverse	GCG**ACTAGT**TTACTTGTACAGCTCGTC	*Spe*I
EGFP	Forward	GCG**AAGCTT**ATGGTGAGCAAGGGCG	*Hind*III
	Reverse	GCG**ACTAGT**TTACTTGTACAGCTCGTCC	*Spe*I
AtFIBRILARIN2	Forward	GCG**GAATTC**ATGAGACCCCCAGTTACAG	*Eco*RI
	Reverse	GCG**GGATCC**TGAGGCTGGGGTCTTTTG	*Bam*HI
RPL23aA-NΔ29	Forward	**GAATTC**ATGGCCTTCAAGAAGAAGGAC	*Eco*RI
	Reverse	GCG**GGATCC**GATGATGCCGATCTTGTTAG	*Bam*HI
RPL23aA-mid 91	Forward	**GAATTC**ATGGCCTTCAAGAAGAAGGAC	*Eco*RI
	Reverse	GCG**GGATCC**CTTGGTCTGGATGTC	*Bam*HI
RPL23aA-CΔ34	Forward	GCG**GAATTC**ATGTCTCCGGCTAAAG	*Eco*RI
	Reverse	GCG**GGATCC**CTTGGTCTGGATGTC	*Bam*HI

ORF	Primer	Primer sequence	Restriction site
RPL23aA-CΔ64	Forward	GCG**GAATTC**ATGTCTCCGGCTAAAG	*Eco*RI
	Reverse	GCG**GGATCC**GTCTTCAATCTTCTTC	*Bam*HI
RPL23aA-5'RR (native promoter)	Forward	**GGGCCC**TTTTCCGGCGGCGGAGAG AGACTTTG	*Apa*I
	Reverse	GCG**GAATTC**GGCTTGAAATGATTCTTC	*Eco*RI

Table A.2. Primers used for site-directed mutagenesis (nucleotides corresponding to amino acid substitutions are underlined)

ORF	Primer ID	Mutation details	Sequence
RPL23aA	pNLS1'(a)-F	^{10}KKAD^{13} to ^{10}TTDA^{13}	CT AAA GTT GAT ACT ACC ACG ACG GAT GCT CCT AAG GCC AAG G
	pNLS1'(a)-R		C CTT GGC CTT AGG AGC ATC CGT CGT GGT AGT ATC AAC TTT AG
	pNLS1''(a)-F	^{17}KALK^{20} to ^{17}TDGT^{20}	GAT CCT AAG GCC ACG GAC GGG ACG GCG GCA AAG GCT GTG
	pNLS1''(a)-R		CAC AGC CTT TGC CGC CGT CCC GTC CGT GGC CTT AGG ATC
	pNLS1(a)-F	^{10}KKADPKAKALK^{20} to ^{10}TTDAPKATDGT^{20} (RPL23aA ORF with ^{10}KKAD^{13} to ^{10}TTDA^{13} mutation was used as template)	GCT CCT AAG GCC ACG GAC GGG ACG GCG GCA AAG GCT GTG
	pNLS1(a)-R		CCC AGC CTT TGC CGC CGT CCC GTC CGT GGC CTT AGG AGC
	pNLS1'(b)-F	^{10}KKAD^{13} to ^{10}AAAD^{13}	GAT ACT ACC GCG GCG GCT GAT CCT AAG
	pNLS1'(b)-R		CTT AGG ATC AGC CGC CGC GGT AGT ATC
	pNLS1''(b)-F	^{17}KALK^{20} to ^{17}AALA^{20}	GCT GAT CCT GCG GCC GCG GCC TTG GCG GCG GCA AAG
	pNLS1''(b)-R		CTT TGC CGC CGC CAA GGC CGC GGC CGC AGG ATC AGC
	pNLS2-F	^{33}KKDK^{36} to ^{33}LADA^{36}	GGT CAA GCC TTC AAG CTG GCG GAC GCA AAG ATT AGG ACC AAG G
	pNLS2-R		C CTT GGT CCT AAT CTT TGC GTC CGC CAG CTT GAA GGC TTG ACC
	pNLS3-F	^{36}KKIR^{39} to ^{36}AAIR^{39}	C AAG AAG AAG GAC GCA GCG ATT GCG ACC AAG GTC ACC TTC C
	pNLS3-R		G GAA GGT GAC CTT GGT CGC AAT CGC TGC GTC CTT CTT CTT G

ORF	Primer ID	Mutation details	Sequence
	pNLS2, 3-F	^{33}KKDKKIR^{36} to ^{33}LADAAIR^{36} (RPL23aA ORF with ^{33}KKDK^{36} to ^{33}LADA^{36} mutation was used as template)	C AAG CTG GCG GAC GCA GCG ATT GCG ACC AAG GTC ACC
	pNLS2, 3-R		GGT GAC CTT GGT CGC AAT CGC TGC GTC CGC CAG CTT G
	pNLS4-F	^{86}KK^{87} to ^{86}AA^{87}	GAA TCT GCG ATG GCG GCG ATT GAA GAC AAC
	pNLS4-R		GTT GTC TTC AAT CGC CGC CAT CGC AGA TTC
	pNLS5-F	^{105}KKIK^{108} to ^{105}AAIK^{108}	GCT GAC AAG GCG GCG ATT GCG GAT GCT GTT AAG
	pNLS5-R		CTT AAC AGC ATC CGC AAT CGC CGC CTT GTC AGC
	pNLS6-F	^{105}KKIKDAVKK^{113} to ^{105}AAIKDAVAA^{113} (RPL23aA ORF with ^{105}KKIK^{108} to ^{105}AAIA^{108} mutation was used as template)	G GAT GCT GTT GCG GCG ATG TAT GAC ATC
	pNLS6-R		GAT GTC ATA CAT CGC CGC AAC AGC ATC C
	pNLS7-F	^{120}KK^{121} to ^{120}AA^{121}	GAC ATC CAG ACC GCG GCA GTG AAC ACA CTC
	pNLS7-R		GAG TGT GTT CAC TGC CGC GGT CTG GAT GTC
	pNLS8-F	^{132}KK^{133} to ^{132}AA^{133}	CCT GAT GGA ACC GCG GCG GCT TAC GTG AGG
	pNLS8-R		CCT CAC GTA AGC CGC CGC GGT TCC ATC AGG
	pRBS-F		ACC GCG GCG GCT GCC GCG GCG GCT ACA CCA GAC

ORF	Primer ID	Mutation details	Sequence
	pRBS-R	^{132}KKAYVRL^{138} to ^{132}AAAAAAA^{138} (RPL23aA ORF with ^{133}KK^{134} to ^{133}AA^{134} mutation was used as template)	GTC TGG TGT AGC CGC CGC GGC AGC CGC CGC GGT
RPL15A	pNLS1-F	^{11}RKK^{13} to ^{11}AAA^{13}	CT GAG CTA TGG GCG GCG GCA CAG TCC GAT GTG ATG
	pNLS1-R		CAT CAC ATC GGA CTG TGC CGC CGC CCA TAG CTC AG
	pNLS2-F	^{47}KARR^{50} to ^{47}AAAA^{50}	CT ACT CGT CCT GAT GCG GCT GCT GCT TTG GGT TAC AAG G
	pNLS2-R		C CTT GTA ACC CAA AGC AGC AGC CGC ATC AGG ACG AGT AG
	pNLS3-F	^{65}RVRR^{68} to ^{65}AVAA^{68}	GTG TAC CGT GTA GCT GTG GCA GCT GGT GGA CGC AAG
	pNLS3-R		CTT GCG TCC ACC AGC TGC CAC AGC TAC ACG GTA CAC
	pNLS3, 4-F	^{65}RVRRGGRKR^{73} to ^{65}AVAAGGAAA^{73} (RPL15A ORF with ^{65}RVRR^{68} to ^{65}AVAA^{68} mutation was used as template)	GTG GCA GCT GGT GGA GCC GCG GCG CCA GTG CCT AAG
	pNLS3, 4-R		CTT AGG CAC TGG CGC CGC GGC TCC ACC AGC TGC CAC
	pNLS5-F	^{96}RSKR^{98} to ^{96}ASAA^{98}	CAA CTC AAG TTC CAG GCT AGC GCG GCT TCT GTT GCT GAG GAG
	pNLS5-R		CTC CTC AGC AAC AGA AGC CGC GCT AGC CTG GAA CTT GAG TTG
	pNLS6-F	^{105}RAGRK^{109} to ^{105}AAGAA^{109}	GCT GAG GAG GCT GCT GGC GCG GCA TTG GGT GGT C
	pNLS6-R		G ACC ACC CAA TGC CGC GCC AGC AGC CTC CTC AGC
	pNLS7-F	^{169}KKNR^{172} to ^{169}AANA^{172}	CC TCA GAG GGA GCG GCG AAC GCA GGT CTC CGC

ORF	Primer ID	Mutation details	Sequence
	pNLS7-R		GCG GAG ACC TGC GTT CGC CGC TCC CTC TGA GG
	pNLS8-F	^{188}RR^{189} to ^{188}AA^{189}	G AAC CGT CCA TCT GCC GCG GCT ACA TGG
	pNLS8-R		CCA TGT AGC CGC GGC AGA TGG ACG GTT C
	pNLS9-F	^{193}KK^{194} to ^{193}AA^{194}	GCT ACA TGG GCG GCA AAC AAC TCT CTC
	pNLS9-R		GAG AGA GTT GTT TGC CGC CCA TGT AGC
	pNLS10-F	^{201}RRYR^{204} to ^{201}AAYA^{204} (nucleotides in bold are part of the plasmid pBSKS+)	TCT CTC AGC CTT GCT GCT TAC GCT **GGA TCC TTA TCG**
	pNLS10-R		**CGA TAA GGA TCC** AGC GTA AGC AGC AAG GCT GAG AGA
RPS8A	pNLS1-F	^{10}KRR^{12} to ^{10}AAA^{12}	GAC TCT ATC CAC GCG GCG GCT GCC ACT GGA GGC AAG
	pNLS1-R		CTT GCC TCC AGT GGC AGC CGC CGC GTG GAT AGA GTC
	pNLS2-F	^{22}RKKRK^{26} to ^{22}AAAAA^{26}	G CAG AAG CAA TGG GCG GCG GCG GCA GCG TAT GAG ATG GGA AGG C
	pNLS2-R		G CCT TCC CAT CTC ATA CGC TGC CGC CGC CGC CCA TTG CT T CTG C
	pNLS3-F	^{45}RRIR^{48} to ^{45}AAIA^{48}	GC AAC AAG ACG GTC GCA GCA ATA GCA GTT CGT GGT GG
	pNLS3-R		CC ACC ACG AAC TGC TAT TGC TGC GAC CGT CTT GTT GC
	pNLS4-F	^{75}RKTR^{78} to ^{75}AATA^{78}	GAA GCA ACT ACC GCC GCG ACC GCA GTC CTT GAT GTG G
	pNLS4-R		C CAC ATC AAG GAC TGC GGT CGC GGC GGT AGT TGC TTC
	pNLS5-F	^{124}RKKK^{127} to ^{124}AAAA^{127}	GGT GTT GAG CTT GGG GCC GCG GCG GCG AGT GCT TCT TCC
	pNLS5-R		GGA AGA AGC ACT CGC CGC CGC GGC CCC AAG CTC AAC ACC
	pNLS6-F	^{133}KK^{134} to ^{133}AA^{134}	GCT TCT TCC ACC GCG GCG GAC GGA GAG

ORF	Primer ID	Mutation details	Sequence
	pNLS6-R		CTC TCC GTC CGC CGC GGT GGA AGA AGC
	pNLS7-F	^{151}KK^{152} to ^{151}AA^{152}	CCT GAG GAG GTC GCG GCG AGC AAC CAC
	pNLS7-R		GTG GTT GCT CGC CGC GAC CTC CTC AGG
	pNLS8-F	^{158}RK^{159} to ^{158}AA^{159}	CAC CTC CTG GCA GCG ATT GCA AGC CGT C
	pNLS8-R		G ACG GCT TGC AAT CGC TGC CAG GAG GTG
	pNLS9-F	^{211}KK^{212} to ^{211}AA^{212}	GAG TTC TAC ATG GCG GCG ATC CAG AAG AAG
	pNLS9-R		CTT CTT CTG GAT CGC CGC CAT GTA GAA CTC
	pNLS9, 10-F	^{211}KKIQKKKGK^{219} to ^{211}AAIQAAAGA^{219} (RPS8A ORF with ^{211}KK^{212} to ^{211}AA^{212} mutation was used as template)	GCG GCG ATC CAG GCG GCG GCG GGC GCG GGT GCT GC
	pNLS9, 10-R		GC AGC ACC CGC GCC CGC CGC CGC CTG GAT CGC CGC
Importin α9	Forward	To mutate in frame stop codon to correct (^{364}glutamic acid) codon	CGTGTTTTAAAGAAGGAAGCTGCTTGGGTGC
	Reverse		GCACCCAAGCAGCTTCCTTCTTTAAAACACG

Table A.3. Primers used for yeast two hybrid assays (restriction sites are in bold and underlined)

ORF	Primer	Primer sequence	Restriction site
RPL23aA	Forward	GCG**GTCGACC**ATGTCTCCGGCTAAAG	*Sal*I
	Reverse	GCG**GCGGCCGC**GATGATGCCGATCTTGTTAG	*Not*I
Importin α1	Forward	GCG**GTCGACC**ATGTCACTGAGACCCAAC	*Sal*I
	Reverse	GCG**GCGGCCGC**GCTGAAGTTGAATCC	*Not*I
Importin α2	Forward	GCG**GTCGACC**ATGTCTTTGAGACCTAAC	*Sal*I
	Reverse	GCG**GCGGCCGC**CTGGAAGTTGAATCC	*Not*I
Importin α3	Forward	GCG**GTCGACC**ATGTCTCTCAGACCTAGC	*Sal*I
	Reverse	GCG**GCGGCCGC**AATAAAGTTGAATTG	*Not*I
Importin α4	Forward	GCG**GTCGACC**ATGTCGCTGAGGCCGAGC	*Sal*I
	Reverse	GCG**GCGGCCGC**GGCAAATTTGAATCC	*Not*I
Importin α5	Forward	GCG**GTCGACC**ATGTCCTTGCGACCGAGC	*Sal*I
	Reverse	GCG**GCGGCCGC**ACGAGAAAAATCAAAC	*Not*I
Importin α6	Forward	GCG**GTCGACC**ATGTCTTACAAACCAAGC	*Sal*I
	Reverse	GCG**GCGGCCGC**ACCAAAGTTGAATCC	*Not*I
Importin α9	Forward	GCG**GTCGACC**ATGGCGGATGATGGCTC	*Sal*I
	Reverse	GCG**GCGGCCGC**TCATTCATCGATTCC	*Not*I

ORF	Primer	Primer sequence	Restriction site
Importin α9	Forward	GCG**GAATTC**ATGGCGGATGATGGCTC	*Eco*RI
	Reverse	GCG**GGATCC**TCATTCATCGATTCC	*Bam*HI
ICK1	Forward	GCG**CTCGAGC**ATGGTGAGAA AATATAG	*Xho*I
	Reverse	GCG**GCGGCCGC**CTCTAACTTTACCCATTC	*Not*I
CYCD3;1	Forward	GCG**GTCGACC**ATGGCGATTC GGAAGG	*Sal*I
	Reverse	GCG**GCGGCCGC**TGGAGTGGCTACGATTGC	*Not*I

6. APPENDIX B. EXPRESSION VALUES OF ARABIDOPSIS RP GENES

Table B.1. Signal intensities of RP genes on the Affymetrix 22k array across different developmental stages extracted by Genevestigator. Values in parenthesis are SE.

RP gene	AGI	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
RPSaA	AT1G72370	29902 (1610)	19299 (252)	18972 (267)	17636 (577)	21180 (880)	14154 (368)	17800 (381)	19469 (956)	20247 (1095)
RPSaB	AT3G04770	2497 (219)	1241 (22)	1272 (24)	1138 (47)	1215 (84)	993 (26)	1352 (50)	1242 (58)	785 (56)
RPS2C	AT2G41840	24425 (1218)	17593 (219)	17738 (224)	16037 (500)	17734 (756)	12152 (300)	16386 (411)	18210 (790)	15506 (949)
RPS2D	AT3G57490	4607 (465)	1772 (46)	1598 (32)	1631 (88)	1866 (126)	1749 (60)	2137 (85)	1561 (102)	1319 (113)
RPS3C	AT5G35530	21277 (1066)	13924 (176)	14570 (188)	12424 (413)	14958 (654)	10001 (240)	12641 (281)	13982 (537)	12601 (696)
RPS3aA	AT3G04840	21555 (1156)	16066 (242)	14765 (227)	14394 (438)	17643 (800)	11209 (297)	14594 (385)	14155 (752)	10129 (857)

RP gene	AGI	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
RPS3aB	AT4G34670	20570 (1259)	14089 (178)	14539 (234)	12975 (450)	14901 (770)	9778 (206)	13389 (318)	15417 (685)	11167 (681)
RPS4A	AT2G17360	9336 (571)	6391 (86)	7263 (111)	6339 (240)	7024 (368)	4945 (113)	6744 (188)	6313 (292)	5934 (368)
RPS4B	AT5G07090	9679 (682)	6215 (100)	8074 (130)	6041 (232)	7820 (388)	4870 (156)	6519 (203)	6123 (345)	4729 (377)
RPS4D	AT5G58420	11515 (770)	6747 (126)	7425 (146)	5724 (242)	6982 (444)	4156 (137)	6282 (221)	6152 (298)	3717 (308)
RPS6A	AT4G31700	19432 (1046)	14259 (251)	11182 (192)	10891 (377)	12784 (721)	7887 (217)	10979 (304)	12049 (693)	8358 (606)
RPS6B	AT5G10360	18911 (912)	12958 (176)	13787 (176)	12084 (378)	13646 (634)	9504 (232)	12691 (306)	13609 (581)	11919 (763)
RPS7A	AT1G48830	14630 (627)	10557 (143)	10029 (132)	9627 (235)	11165 (424)	7464 (176)	9682 (199)	9753 (416)	9989 (573)
RPS7B	AT3G02560	16456 (935)	11670 (155)	11402 (157)	11139 (351)	12633 (659)	7986 (223)	11140 (290)	11868 (538)	7506 (481)

RP gene	AGI	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
RPS7C	AT5G16130	17795 (954)	12600 (179)	12335 (196)	11140 (354)	13730 (681)	8901 (248)	11353 (279)	11073 (567)	7410 (645)
RPS8A	AT5G20290	36271 (1871)	28475 (398)	30829 (285)	29962 (678)	31704 (976)	21936 (512)	26395 (393)	34574 (1596)	25877 (1536)
RPS8B	AT5G59240	721 (112)	407 (23)	401 (14)	334 (30)	252 (18)	238 (10)	256 (8)	328 (27)	121 (17)
RPS9A	AT4G12160	7 (1)	8 (0.4)	4 (0.1)	6 (1)	10 (2)	8 (1)	7 (1)	7 (1)	6 (1)
RPS9B	AT5G15200	31764 (1548)	22187 (276)	23178 (209)	20586 (524)	23814 (742)	17179 (378)	20033 (350)	22949 (944)	18165 (1031)
RPS9C	AT5G39850	2783 (318)	1259 (29)	1230 (27)	1319 (82)	994 (71)	1018 (34)	1233 (47)	1817 (115)	3416 (380)
RPS10A	AT4G25740	8310 (547)	5783 (92)	5366 (80)	5898 (198)	6381 (345)	3795 (105)	5675 (158)	5246 (250)	3493 (341)
RPS10B	AT5G41520	17725 (991)	12631 (146)	13920 (170)	13544 (339)	13458 (581)	9590 (158)	12831 (229)	13510 (517)	8415 (577)

RP gene	AGI	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
RPS10C	AT5G52650	16647 (930)	12519 (207)	9874 (154)	12017 (369)	14162 (769)	8923 (242)	12387 (339)	11724 (636)	10354 (808)
RPS11A	AT3G48930	14612 (931)	9851 (152)	9496 (162)	9107 (323)	10111 (552)	6555 (193)	9165 (256)	9931 (510)	7844 (594)
RPS11B	AT4G30800	2789 (315)	1258 (40)	1455 (38)	1193 (69)	1551 (137)	1014 (44)	1255 (51)	1362 (104)	664 (72)
RPS11C	AT5G23740	14650 (851)	10050 (154)	9597 (150)	10267 (335)	11313 (536)	6418 (226)	10057 (257)	9929 (461)	5896 (588)
RPS12A	AT1G15930	24282 (1457)	15916 (275)	17172 (276)	15799 (476)	17720 (832)	11054 (328)	15433 (370)	16951 (747)	10833 (840)
RPS12C	AT2G32060	13512 (1028)	7710 (184)	7050 (146)	7007 (303)	8774 (658)	4452 (159)	7373 (243)	7419 (465)	5197 (469)
RPS13A	AT3G60770	19002 (1188)	11736 (181)	12037 (146)	11451 (322)	14090 (579)	9838 (238)	11839 (277)	12052 (557)	10445 (629)
RPS13B	AT4G00100	19407 (1073)	13664 (157)	12412 (166)	12312 (383)	14471 (575)	9795 (222)	12771 (288)	13082 (570)	11058 (753)

RP gene	AGI	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
RPS14A	AT2G36160	16912 (981)	10951 (141)	12337 (167)	11318 (396)	12474 (601)	8269 (203)	11576 (291)	12687 (523)	9336 (531)
RPS14B	AT3G11510	19425 (1094)	14243 (176)	13360 (180)	14275 (473)	16537 (682)	11331 (291)	14791 (431)	14527 (671)	10601 (973)
RPS14C	AT3G52580	10716 (762)	5996 (109)	6407 (139)	5288 (231)	7056 (531)	3661 (145)	5179 (162)	6385 (358)	5096 (358)
RPS15A	AT1G04270	28688 (1793)	18712 (260)	15637 (248)	18384 (593)	20242 (894)	13422 (373)	18617 (477)	18735 (898)	10811 (893)
RPS15B	AT5G09490	25 (3)	24 (1)	19 (1)	24 (3)	19 (2)	20 (2)	21 (1)	52 (11)	54 (12)
RPS15C	AT5G09500	87 (7)	96 (3)	80 (2)	71 (4)	72 (7)	99 (17)	81 (5)	143 (18)	92 (10)
RPS15D	AT5G09510	5384 (460)	2862 (50)	2718 (46)	2668 (91)	3301 (148)	2558 (77)	3166 (97)	2767 (153)	1973 (141)
RPS15E	AT5G43640	62 (4)	48 (2)	35 (1)	35 (3)	48 (4)	40 (2)	44 (2)	43 (4)	38 (4)

RP gene	AGI	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
RPS15F	AT5G63070	82 (6)	77 (2)	97 (2)	88 (4)	84 (6)	100 (4)	100 (3)	138 (11)	186 (26)
RPS15aA(1)	AT1G07770	17883 (799)	15670 (157)	16675 (164)	17789 (348)	17256 (631)	11824 (222)	16946 (552)	16845 (570)	14447 (988)
RPS15aB(2)	AT2G19720	659 (52)	529 (10)	495 (9)	521 (20)	651 (38)	370 (12)	488 (12)	558 (36)	411 (34)
RPS15aC(3)	AT2G39590	42 (4)	59 (2)	40 (1)	51 (3)	50 (4)	68 (3)	65 (2)	69 (5)	47 (7)
RPS15aD(4)	AT3G46040	12518 (757)	8754 (118)	10341 (122)	9226 (248)	10831 (578)	6996 (164)	9298 (205)	9705 (378)	9357 (557)
RPS15aE(5)	AT4G29430	745 (73)	410 (8)	516 (11)	432 (19)	404 (21)	358 (8)	436 (12)	456 (24)	357 (24)
RPS15aF(6)	AT5G59850	13845 (878)	9824 (133)	10442 (169)	10049 (361)	10621 (551)	7733 (174)	10520 (304)	9661 (490)	7602 (600)
RPS16B	AT3G04230	1550 (100)	1083 (23)	1426 (41)	1196 (66)	943 (58)	833 (22)	882 (21)	1558 (97)	712 (67)

RP gene	AGI	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
RPS17C	AT3G10610	6533 (438)	3762 (79)	3575 (82)	3083 (137)	3936 (262)	2212 (97)	3368 (123)	3491 (231)	1929 (148)
RPS18A	AT1G22780	15120 (1004)	10573 (147)	11420 (178)	10146 (347)	11875 (617)	8014 (229)	10191 (268)	10333 (482)	7659 (639)
RPS18B	AT1G34030	9222 (695)	5858 (93)	5803 (92)	5529 (194)	5996 (286)	3944 (113)	5649 (151)	5800 (293)	4823 (359)
RPS18C	AT4G09800	14972 (726)	10810 (145)	9899 (151)	10186 (292)	11453 (434)	7765 (186)	10595 (231)	12079 (448)	9167 (554)
RPS19A	AT3G02080	20652 (1142)	13366 (195)	12922 (214)	12744 (415)	14618 (650)	9309 (297)	12450 (287)	14535 (663)	10828 (814)
RPS19B	AT5G15520	2455 (183)	1651 (35)	1665 (38)	1634 (70)	1747 (104)	1059 (34)	1674 (55)	1782 (85)	1098 (98)
RPS19C	AT5G61170	17527 (1046)	10840 (175)	10461 (175)	9567 (341)	11865 (604)	7203 (224)	9777 (289)	9548 (572)	6033 (491)
RPS20B	AT3G47370	12011 (889)	6846 (129)	5717 (111)	5668 (241)	7137 (420)	4599 (137)	5818 (158)	6162 (359)	4511 (287)

RP gene	AGI	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
RPS21A	AT3G27450	92 (7)	91 (2)	71 (2)	88 (5)	79 (5)	86 (3)	93 (4)	103 (8)	56 (7)
RPS21B	AT3G53890	15114 (963)	11017 (173)	9916 (163)	10354 (417)	12363 (595)	7849 (217)	10528 (321)	10423 (527)	7051 (645)
RPS21C	AT5G27700	22495 (1163)	16362 (228)	14798 (259)	17454 (573)	18135 (859)	11614 (276)	18013 (1145)	17580 (677)	11277 (832)
RPS24A (S19)	AT3G04920	8576 (613)	6142 (85)	5658 (99)	6421 (224)	6736 (347)	4130 (107)	6051 (167)	6645 (343)	5401 (482)
RPS24B	AT5G28060	8194 (627)	5762 (93)	7293 (127)	6188 (219)	6650 (386)	4466 (131)	6312 (174)	7047 (379)	2508 (303)
RPS25A	AT2G16360	233 (20)	245 (5)	243 (4)	265 (13)	220 (15)	256 (7)	295 (8)	303 (12)	288 (18)
RPS25B	AT2G21580	6948 (534)	3514 (63)	3329 (58)	3252 (139)	3976 (257)	2213 (80)	3272 (106)	3606 (198)	2929 (205)
RPS25C	AT3G30740	44 (4)	32 (1)	36 (1)	29 (2)	29 (3)	28 (2)	37 (2)	46 (5)	51 (6)

RP gene	AGI	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
RPS25E	AT4G39200	15207 (1146)	10334 (266)	13619 (524)	8619 (516)	9686 (715)	5696 (244)	8846 (353)	10910 (772)	6753 (429)
RPS26C	AT3G56340	18347 (1181)	10725 (179)	11267 (169)	10364 (347)	12708 (597)	8307 (226)	10778 (291)	10741 (481)	7149 (593)
RPS27A	AT2G45710	4884 (380)	2673 (38)	2498 (43)	2628 (107)	3126 (162)	2374 (63)	3179 (100)	2821 (136)	3601 (306)
RPS27B	AT3G61110	20885 (1214)	15157 (220)	14528 (186)	15327 (539)	18459 (800)	11635 (337)	16423 (688)	15749 (657)	16221 (1539)
RPS27D	AT5G47930	15523 (996)	10998 (147)	12204 (165)	10935 (345)	11683 (525)	7987 (200)	11324 (275)	11090 (440)	7844 (545)
RPS27aA	AT1G23410	1117 (114)	713 (24)	1238 (53)	665 (66)	621 (74)	268 (14)	492 (28)	606 (53)	241 (46)
RPS28A	AT3G10090	5785 (458)	3761 (70)	4083 (87)	4123 (157)	4296 (260)	2555 (68)	3726 (83)	3755 (161)	2265 (176)
RPS28B	AT5G03850	15746 (925)	11763 (155)	10956 (147)	12242 (442)	12458 (605)	7969 (178)	12045 (425)	12308 (548)	10369 (734)

RP gene	AGI	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
RPS28C	AT5G64140	10173 (695)	6291 (122)	5361 (97)	5675 (217)	6939 (361)	4456 (121)	5996 (158)	5942 (302)	5393 (396)
RPS29C	AT4G33865	23614 (1302)	18829 (208)	19640 (253)	21069 (504)	20644 (836)	15128 (264)	20251 (542)	20373 (625)	14578 (956)
RPS30A	AT2G19750	6541 (558)	3645 (63)	4411 (73)	3645 (130)	4442 (218)	3020 (84)	4114 (112)	4045 (239)	2940 (254)
RPS30B	AT4G29390	19009 (1037)	11483 (186)	11533 (252)	10217 (419)	11345 (681)	6873 (198)	9728 (230)	11558 (549)	13963 (834)
RPS30C	AT5G56670	14424 (933)	9764 (183)	10325 (165)	10280 (351)	11821 (721)	6407 (224)	9525 (241)	10822 (652)	7574 (662)
RPL3A	AT1G43170	31135 (1445)	22448 (287)	26478 (245)	21445 (589)	25179 (948)	18456 (406)	21212 (416)	25066 (1174)	22602 (1269)
RPL3B	AT1G61580	2335 (280)	1492 (45)	1217 (40)	1011 (55)	1219 (83)	1013 (39)	847 (24)	972 (62)	522 (60)
RPL4A(L1)	AT3G09630	20590 (1014)	15103 (192)	17436 (219)	14123 (399)	15567 (781)	10556 (241)	13627 (325)	15206 (599)	12565 (588)

RP gene	AGI	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
RPL4B	AT1G35200	29 (4)	26 (1)	18 (1)	18 (2)	30 (3)	26 (2)	29 (2)	36 (5)	30 (5)
RPL4D	AT5G02870	21974 (1169)	16595 (232)	17831 (220)	14999 (438)	17358 (856)	12974 (315)	14967 (361)	15054 (685)	11535 (837)
RPL5A	AT3G25520	16713 (807)	12051 (161)	12339 (187)	10532 (344)	12345 (572)	8263 (198)	10605 (275)	10830 (551)	7785 (619)
RPL5B	AT5G39740	13514 (886)	9339 (148)	12105 (205)	9335 (335)	10855 (562)	7194 (215)	8966 (251)	9335 (478)	6841 (541)
RPL5C	AT5G40130	76 (9)	80 (3)	87 (2)	68 (5)	65 (6)	63 (3)	69 (2)	72 (7)	77 (13)
RPL6A	AT1G18540	15802 (927)	11258 (178)	9812 (177)	10005 (319)	12278 (611)	7427 (192)	9699 (239)	10777 (612)	8642 (497)
RPL7A	AT1G80750	3834 (413)	1914 (39)	1948 (36)	1739 (82)	2395 (214)	1452 (52)	1877 (65)	1832 (110)	1356 (89)
RPL7B	AT2G01250	22748 (1209)	16818 (235)	18734 (261)	16271 (449)	18720 (787)	11602 (297)	15539 (362)	16197 (778)	13976 (999)

RP gene	AGI	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
RPL7C	AT2G44120	16056 (1030)	11133 (168)	10306 (200)	9972 (419)	12134 (723)	7537 (198)	10070 (283)	9838 (544)	8272 (710)
RPL7D	AT3G13580	5764 (430)	3634 (64)	4634 (96)	3846 (152)	4061 (304)	2714 (83)	3506 (95)	4112 (188)	2869 (221)
RPL7aA	AT2G47610	19417 (1104)	12520 (175)	12698 (239)	10835 (427)	12663 (750)	8164 (231)	10553 (289)	11732 (644)	9001 (630)
RPL7aB	AT3G62870	22048 (1348)	15762 (214)	15734 (220)	14269 (408)	16680 (683)	11670 (247)	14286 (290)	15059 (644)	11063 (707)
RPL8B	AT3G51190	155 (11)	185 (4)	190 (4)	215 (8)	174 (16)	217 (6)	239 (6)	215 (8)	216 (14)
RPL9D	AT4G10450	6810 (522)	4257 (76)	3806 (90)	4257 (216)	4835 (311)	3182 (107)	4471 (149)	3858 (256)	1958 (199)
RPL10A	AT1G14320	32808 (1725)	23464 (288)	23502 (276)	22834 (499)	24906 (748)	18099 (323)	22259 (342)	24054 (885)	25744 (1041)
RPL10B	AT1G26910	4465 (330)	2684 (48)	2769 (59)	2419 (120)	2796 (203)	1780 (54)	2531 (88)	2795 (158)	1628 (134)

RP gene	AGI	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
RPL10C	AT1G66580	18103 (883)	13466 (149)	14576 (178)	11817 (316)	12888 (513)	13620 (367)	12776 (242)	14708 (867)	14470 (1218)
RPL10aA	AT1G08360	15162 (872)	10384 (154)	11243 (169)	9862 (278)	11555 (519)	7565 (188)	9565 (209)	10751 (437)	8120 (524)
RPL10aB	AT2G27530	16682 (897)	11132 (142)	10849 (179)	10652 (337)	12585 (525)	8257 (185)	10810 (249)	12049 (582)	9415 (570)
RPL10aC	AT5G22440	10041 (708)	5846 (115)	5769 (155)	5258 (268)	6477 (494)	3001 (121)	4938 (184)	5558 (369)	2626 (260)
RPL11A	AT2G42740	3250 (316)	1703 (40)	2112 (58)	1652 (98)	2105 (198)	967 (41)	1672 (67)	1952 (121)	1243 (93)
RPL11B	AT3G58700	6677 (469)	3964 (77)	3993 (86)	3686 (150)	4270 (237)	2742 (78)	3768 (93)	3859 (172)	2937 (171)
RPL12A	AT2G37190	18576 (1093)	13835 (221)	12187 (228)	11980 (447)	13801 (774)	8482 (293)	11316 (327)	13031 (669)	8204 (801)
RPL12B	AT3G53430	23056 (1176)	16457 (212)	15456 (214)	15400 (414)	18429 (715)	12982 (273)	15494 (301)	15861 (721)	11820 (920)

RP gene	AGI	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
RPL12C	AT5G60670	9297 (522)	7343 (112)	7685 (145)	7258 (257)	7920 (437)	4884 (147)	6848 (190)	6475 (355)	4381 (522)
RPL13A	AT3G48130	127 (23)	12 (1)	8 (1)	7 (1)	5 (0.4)	8 (1)	9 (1)	23 (12)	12 (2)
RPL13B	AT3G49010	24906 (1427)	17822 (256)	17906 (226)	15894 (435)	19130 (751)	13003 (350)	16144 (330)	16942 (742)	14071 (903)
RPL13C	AT3G48960	124 (9)	135 (3)	134 (3)	128 (6)	113 (7)	114 (4)	140 (5)	133 (8)	110 (11)
RPL13D	AT5G23900	11221 (632)	7238 (101)	7636 (114)	6760 (220)	7291 (329)	4847 (108)	6487 (165)	6990 (314)	4338 (302)
RPL13aA	AT3G07110	22336 (1316)	13758 (240)	11853 (207)	12192 (432)	15358 (749)	9401 (289)	12241 (329)	12853 (680)	9251 (627)
RPL13aB	AT3G24830	20552 (1010)	15958 (207)	17856 (200)	15747 (406)	17588 (719)	11854 (246)	15624 (290)	16826 (657)	15413 (930)
RPL13aC	AT4G13170	8140 (547)	5175 (100)	3807 (69)	4449 (167)	5378 (271)	3000 (90)	4245 (109)	3870 (221)	2313 (189)

RP gene	AGI	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
RPL13aD	AT5G48760	5520 (377)	3387 (54)	3021 (54)	3236 (152)	4585 (337)	2537 (82)	3497 (101)	3441 (221)	2377 (173)
RPL14A	AT2G20450	7607 (608)	3348 (78)	3310 (75)	3248 (160)	3822 (275)	1908 (74)	3137 (102)	3199 (222)	1738 (116)
RPL14B	AT4G27090	22753 (1174)	17990 (206)	19134 (256)	18696 (463)	18801 (736)	13375 (292)	17734 (305)	22118 (951)	17711 (1083)
RPL15A	AT4G16720	23590 (1254)	16261 (240)	15631 (194)	15733 (407)	17614 (691)	11640 (306)	15147 (312)	16428 (678)	13452 (826)
RPL15B	AT4G17390	22404 (1229)	15813 (238)	12691 (221)	13912 (474)	15853 (910)	9009 (276)	12559 (327)	14612 (800)	9194 (733)
RPL17A	AT1G27400	14206 (966)	8304 (137)	8562 (160)	7103 (293)	8927 (544)	5302 (177)	7255 (230)	7857 (413)	4028 (391)
RPL17B	AT1G67430	27100 (1407)	19105 (284)	17599 (251)	16297 (549)	19753 (906)	13098 (328)	16148 (348)	18597 (916)	18210 (1194)
RPL18A	AT2G47570	130 (9)	117 (3)	109 (3)	112 (7)	104 (10)	120 (5)	159 (5)	166 (10)	187 (14)

RP gene	AGI	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
RPL18B	AT3G05590	19308 (1008)	14256 (199)	12585 (190)	12861 (395)	15273 (717)	9729 (239)	12082 (288)	13140 (678)	9091 (492)
RPL18C	AT5G27850	20580 (1282)	14664 (212)	14156 (244)	13082 (416)	15186 (731)	9357 (251)	13031 (319)	13921 (623)	11759 (697)
RPL18aA	AT1G29970	3079 (252)	3300 (92)	2439 (52)	3254 (122)	2268 (131)	3500 (95)	3755 (261)	4326 (268)	5104 (950)
RPL18aB	AT2G34480	27979 (1308)	20449 (282)	17105 (231)	18000 (528)	21070 (816)	14080 (346)	17579 (348)	18993 (889)	19236 (1462)
RPL18aC	AT3G14600	12348 (667)	8651 (148)	7099 (140)	8109 (330)	9633 (680)	5378 (143)	7738 (233)	9330 (472)	8090 (474)
RPL19A	AT1G02780	30977 (1644)	20548 (280)	22666 (279)	19302 (489)	22171 (922)	14603 (389)	18394 (353)	22206 (1002)	19697 (1021)
RPL19B	AT3G16780	8277 (741)	3728 (113)	4102 (106)	3199 (171)	3863 (296)	1939 (78)	2884 (103)	3392 (235)	2306 (163)
RPL19C	AT4G02230	10105 (504)	6457 (92)	7974 (125)	7185 (222)	7552 (423)	5442 (126)	6454 (140)	8738 (371)	5288 (361)

RP gene	AGI	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
RPL21F	AT3G57820	81 (5)	83 (2)	72 (1)	79 (3)	74 (5)	104 (3)	92 (2)	82 (5)	90 (6)
RPL22A	AT1G02830	88 (7)	93 (2)	101 (2)	93 (4)	95 (7)	102 (3)	120 (3)	91 (6)	100 (10)
RPL22B	AT3G05560	20497 (1150)	13782 (227)	14159 (214)	13081 (397)	15593 (761)	9555 (269)	12624 (292)	13359 (668)	9518 (746)
RPL22C	AT5G27770	10028 (743)	7683 (109)	8930 (120)	8222 (245)	9196 (416)	6565 (151)	8490 (206)	8163 (322)	5141 (371)
RPL23A	AT1G04480	14985 (1044)	10065 (171)	9081 (139)	9093 (304)	10831 (561)	6823 (173)	9300 (240)	8919 (386)	5515 (373)
RPL23B	AT2G33370	12600 (761)	10532 (135)	10461 (147)	9917 (276)	11209 (553)	7016 (204)	9825 (238)	9294 (410)	6327 (541)
RPL23C	AT3G04400	37733 (1941)	30949 (385)	32171 (295)	34461 (898)	35118 (1262)	25456 (501)	33113 (834)	35148 (1293)	32796 (2167)
RPL23aA	AT2G39460	17616 (1254)	11883 (181)	10041 (160)	10937 (352)	12688 (582)	7887 (206)	10771 (245)	11790 (658)	8242 (677)

RP gene	AGI	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
RPL23aB	AT3G55280	10414 (763)	6075 (108)	5879 (117)	5696 (233)	6336 (339)	3880 (136)	5878 (183)	6202 (352)	4735 (413)
RPL24A	AT2G36620	16155 (1078)	10386 (193)	10323 (207)	9046 (363)	11800 (668)	7361 (221)	9391 (274)	9243 (608)	6102 (589)
RPL24B	AT3G53020	19337 (1212)	14411 (192)	15130 (206)	13704 (393)	15562 (680)	9894 (245)	13328 (290)	14307 (604)	11840 (792)
RPL26A	AT3G49910	30765 (1863)	23332 (318)	24484 (294)	24105 (638)	25370 (1025)	17147 (538)	22070 (449)	25855 (1153)	17849 (1567)
RPL26B	AT5G67510	11123 (707)	6561 (125)	6047 (117)	6101 (229)	7383 (470)	4941 (137)	6509 (187)	6630 (390)	3313 (259)
RPL27A	AT2G32220	1344 (115)	758 (16)	707 (16)	718 (41)	672 (36)	609 (22)	837 (28)	768 (48)	1057 (119)
RPL27B	AT3G22230	9246 (849)	5669 (105)	6679 (127)	5821 (208)	6663 (351)	4398 (140)	6119 (175)	5662 (291)	4154 (342)
RPL27C	AT4G15000	13770 (837)	8980 (145)	9846 (162)	7928 (273)	9499 (492)	5977 (199)	8039 (224)	8249 (447)	5994 (505)

RP gene	AGI	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
RPL27aA	AT1G12960	107 (9)	88 (2)	141 (3)	110 (4)	94 (8)	112 (4)	123 (4)	108 (7)	103 (9)
RPL28A	AT2G19730	24140 (1559)	16217 (281)	14860 (248)	14917 (499)	17969 (863)	11006 (345)	13925 (332)	15777 (753)	12509 (992)
RPL28C	AT4G29410	12160 (1029)	6797 (132)	6588 (126)	5855 (233)	7503 (471)	4044 (136)	5949 (183)	6631 (369)	3889 (299)
RPL29A	AT3G06700	11436 (954)	6129 (153)	5862 (132)	5308 (253)	6479 (471)	4102 (135)	5106 (141)	5954 (350)	4434 (395)
RPL29B	AT3G06680	7002 (497)	4403 (107)	4744 (100)	3874 (208)	5099 (359)	2497 (132)	4238 (161)	3557 (277)	1999 (248)
RPL30A	AT1G36240	141 (12)	157 (3)	166 (3)	175 (7)	160 (10)	193 (6)	222 (8)	182 (8)	179 (17)
RPL30B	AT1G77940	20111 (1092)	13869 (178)	13244 (225)	13183 (398)	14742 (647)	10472 (259)	13917 (333)	12608 (636)	15622 (991)
RPL30C	AT3G18740	25773 (1572)	19894 (243)	19256 (249)	21142 (691)	22102 (647)	16673 (325)	21763 (670)	20208 (827)	20540 (1241)

RP gene	AGI	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
RPL31A	AT2G19740	4935 (410)	3112 (43)	3300 (51)	3438 (106)	3687 (152)	3077 (55)	3616 (76)	3867 (139)	1898 (166)
RPL31B	AT4G26230	13597 (963)	9029 (160)	10264 (172)	9513 (343)	10302 (487)	6833 (186)	9408 (268)	9110 (460)	4756 (468)
RPL31C	AT5G56710	17419 (1185)	11512 (219)	12078 (198)	10182 (342)	12593 (705)	7662 (228)	10207 (264)	10716 (572)	10531 (904)
RPL34A	AT1G26880	14140 (884)	9311 (162)	8978 (151)	8782 (317)	10345 (520)	6564 (193)	8690 (215)	8837 (468)	5461 (472)
RPL34B	AT1G69620	23045 (1215)	17788 (232)	18301 (232)	17485 (396)	19405 (861)	13094 (271)	16869 (337)	18987 (832)	16417 (1281)
RPL34C	AT3G28900	9909 (787)	5759 (94)	5269 (102)	5622 (240)	7199 (539)	3693 (143)	5799 (203)	5269 (338)	4935 (464)
RPL35A	AT3G09500	14990 (831)	11041 (168)	12486 (161)	11214 (321)	12414 (536)	8184 (214)	11274 (263)	11465 (480)	10575 (739)
RPL35B	AT2G39390	5739 (492)	3723 (77)	3636 (71)	3082 (135)	3280 (202)	2505 (82)	3284 (97)	2971 (140)	2006 (123)

RP gene	AGI	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
RPL35C	AT3G55170	1474 (144)	793 (18)	1142 (29)	942 (43)	818 (54)	580 (20)	966 (34)	981 (62)	428 (41)
RPL35D	AT5G02610	6518 (493)	4141 (65)	3788 (59)	4279 (144)	4818 (227)	3321 (75)	4384 (110)	3998 (193)	2687 (208)
RPL35aA	AT1G06980	276 (40)	357 (19)	149 (5)	152 (11)	197 (20)	184 (10)	219 (14)	123 (11)	77 (12)
RPL35aC	AT1G74270	6400 (376)	4032 (60)	3574 (66)	4158 (135)	4046 (182)	2927 (75)	4083 (123)	4177 (182)	3667 (273)
RPL36A	AT2G37600	2717 (239)	1737 (31)	1772 (34)	1970 (87)	2311 (140)	1384 (43)	2232 (79)	2100 (131)	1773 (208)
RPL36B	AT3G53740	14158 (1044)	8331 (164)	11438 (208)	8937 (291)	9987 (629)	5697 (231)	8421 (213)	10392 (466)	8356 (694)
RPL36C	AT5G02450	10079 (679)	7407 (135)	9575 (157)	7690 (300)	8059 (519)	5218 (166)	7375 (220)	7498 (323)	4994 (401)
RPL36aA	AT3G23390	16200 (1183)	11676 (180)	12474 (201)	11443 (382)	13051 (647)	8304 (241)	11100 (268)	10964 (545)	8792 (839)

RP gene	AGI	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
RPL36aB	AT4G14320	17359 (1387)	10891 (216)	12023 (194)	11116 (443)	13262 (701)	8219 (290)	10195 (241)	9775 (570)	8529 (825)
RPL37A	AT1G15250	2149 (231)	840 (21)	750 (16)	884 (47)	1057 (75)	602 (25)	971 (33)	985 (57)	1066 (113)
RPL37B	AT1G52300	29623 (1654)	20564 (345)	23716 (356)	20734 (563)	22413 (1155)	14188 (433)	18968 (403)	22312 (986)	17361 (1006)
RPL37C	AT3G16080	15577 (1211)	9787 (203)	10756 (195)	10137 (356)	11635 (625)	7067 (215)	9704 (260)	9426 (502)	6736 (611)
RPL37aB	AT3G10950	29 (4)	30 (2)	16 (1)	16 (2)	21 (3)	22 (1)	21 (1)	21 (3)	23 (5)
RPL37aC	AT3G60245	33009 (1949)	22072 (347)	26303 (320)	23979 (615)	24871 (1151)	16401 (445)	21559 (435)	25735 (1126)	20803 (1304)
RPL38A	AT2G43460	12200 (774)	9026 (127)	8621 (131)	9494 (315)	10037 (532)	6657 (186)	9312 (294)	8928 (420)	5314 (477)
RPL38B	AT3G59540	13365 (1013)	8285 (145)	9751 (175)	9616 (322)	9655 (525)	6480 (210)	9427 (302)	9846 (423)	8112 (753)

RP gene	AGI	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
RPL39A	AT2G25210	10841 (907)	4739 (112)	6394 (167)	4657 (251)	5686 (465)	2966 (133)	4861 (176)	5273 (286)	3823 (353)
RPL39B	AT3G02190	3216 (269)	1874 (42)	1537 (40)	1870 (96)	1778 (128)	1730 (49)	1876 (53)	2606 (125)	3377 (306)
RPL39C	AT4G31985	11045 (894)	5935 (133)	6946 (142)	6259 (294)	7339 (448)	4911 (177)	6472 (216)	7544 (392)	5334 (412)
RPL40A	AT2G36170	9522 (632)	6902 (101)	7399 (124)	7008 (244)	7799 (433)	4817 (132)	7242 (197)	7666 (361)	4209 (439)
RPL40B	AT3G52590	22766 (1187)	17899 (222)	19181 (211)	19355 (539)	21055 (753)	15091 (301)	18973 (368)	20134 (827)	18470 (1196)
RPPOA	AT2G40010	1060 (83)	763 (15)	553 (11)	533 (25)	690 (51)	397 (20)	592 (26)	547 (35)	401 (38)
RPPOB	AT3G09200	33239 (1688)	23820 (308)	22828 (300)	21796 (625)	25920 (1037)	17712 (381)	21193 (452)	22185 (977)	22042 (1116)
RPPOC	AT3G11250	3176 (268)	1724 (39)	1527 (27)	1387 (54)	1909 (124)	1264 (42)	1712 (55)	1653 (108)	1298 (68)

RP gene	AGI	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
RPP1A	AT1G01100	22443 (1280)	17049 (235)	17675 (231)	17125 (543)	19442 (903)	13207 (363)	17745 (577)	18524 (743)	15572 (1075)
RPP1B	AT4G00810	9456 (585)	7138 (95)	6864 (99)	7942 (246)	8402 (345)	5870 (139)	7667 (189)	6834 (309)	3534 (326)
RPP1C	AT5G47700	15395 (954)	10696 (166)	9353 (155)	9717 (360)	11526 (641)	7483 (229)	10676 (457)	8986 (475)	6883 (576)
RPP2A	AT2G27720	17248 (1080)	12277 (160)	12102 (192)	12964 (417)	13779 (734)	8809 (221)	13034 (372)	13331 (576)	11153 (802)
RPP2B	AT2G27710	19834 (1089)	14848 (199)	11976 (219)	14112 (508)	15461 (701)	10910 (268)	13701 (322)	13343 (735)	11585 (1001)
RPP2C	AT3G28500	224 (15)	269 (8)	606 (22)	1123 (225)	2657 (739)	1261 (226)	2205 (224)	5057 (679)	2752 (412)
RPP2D	AT3G44590	11274 (842)	5604 (117)	6401 (150)	5393 (267)	6590 (530)	3309 (130)	5383 (202)	5316 (378)	2750 (212)
RPP2E	AT5G40040	47 (5)	50 (2)	32 (2)	35 (6)	102 (21)	120 (35)	68 (8)	185 (32)	91 (12)

RP gene	AGI	Germinated seed	Seedling	Young rosette	Developed rosette	Bolting	Young flower	Developed flower	Flowers and siliques	Mature siliques
RPP3A	AT4G25890	4755 (381)	2845 (57)	2990 (69)	3034 (140)	3091 (202)	1878 (64)	3008 (125)	3371 (163)	1196 (133)
RPP3B	AT5G57290	10322 (702)	5146 (97)	5981 (127)	5227 (243)	5719 (354)	3435 (105)	5654 (267)	5945 (337)	4757 (307)

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