3’ TO 5’ EXORIBONUCLEASES IN *ARABIDOPSIS THALIANA* PLASTIDS

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3’ TO 5’ EXORIBONUCLEASES IN *ARABIDOPSIS THALIANA* PLASTIDS

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Chloroplast genes and metabolic activities are regulated by the products of nuclear genes, acting mostly at the post-transcriptional level. Polynucleotide phosphorylase (PNP) and Ribonuclease R (RNR1) are the two known nucleus-encoded exoribonucleases and are necessary for the correct maturation and degradation of RNAs. PNP catalyzes both the processive 3’ to 5’ phosphorolysis of chloroplast RNA and its processive polymerization depending on the ratio between inorganic phosphate (Pi) and nucleotide diphosphates (NDPs). RNR1 is a hydrolytically processive 3’ to 5’ exoribonuclease that releases 5’ monophosphate nucleotides.

This study used both null mutants and single amino acid substitutions in the two core catalytic domains of PNP to investigate its role in many aspects of chloroplast RNA metabolism. The phenotypic characterization of null mutants (*pnp1-1* to *1-3*) showed a chlorotic phenotype in young leaves that became less severe as leaves matured, and molecular analysis demonstrated the involvement of PNP in the 3’ maturation, stabilization and/or degradation of many chloroplast RNAs, as well as its importance in the excised intron lariat degradation pathway. Two mutations in the first core domain demonstrated a role for this region in PNP activity, however the residual activity of the PNP mutants permitted the construction of otherwise lethal *pnp/rnr* double mutants.

The resulting *rnr1* mutant plants with reduced PNP activity are chlorotic and display a global reduction in RNA abundance. Such a counterintuitive outcome following

the loss of RNA degradation activity suggests a major importance of RNA maturation as a determinant of RNA stability. The detailed analysis of the double mutant transcriptome revealed that RNR1 completes the maturation of mRNAs 3' termini created by PNPase in a two-step maturation process. In contrast to the double mutant, the *rnr1* single mutant, known to have a substantial decline in rRNA levels, over-accumulated most of the mRNA species examined when compared to the wild-type. Combined with the reduced number of ribosomes, it was not unexpected to find most of the excess mRNAs species present in non-polysomal fractions. Half-life measurements demonstrated a substantial increase in the stability of most mRNA species tested, supporting the hypothesis that RNR1 plays important roles in the maintenance of both chloroplast rRNA and mRNA homeostasis.

Because of the dependence of PNPase activity on the Pi:NDP ratio *in vitro*, we investigated a potential link between PNP and phosphate (P) metabolism of the plant. We found that P-deprived *pnp* mutants develop aborted clusters of lateral roots while a global analysis of metabolites and transcripts supported the hypothesis that the activity of PNP is involved in plant acclimation to P availability.

The most recent results, involving a double mutant lacking both PNP and the endonuclease RNase E, indicate that not only exonucleolytic processing but also endonucleolytic processing is involved in stabilizing chloroplast RNAs. Taken together, these studies deepen our knowledge about the role of RNases in the plastid RNA processing and decay pathways. Indeed, such double mutants reveal the importance of RNA maturation, perhaps as a quality control mechanism.

BIOGRAPHICAL SKETCH

Arnaud Germain was born in Dijon, France, to Christine Germain and Didier Germain. He was raised with his sister Alexia in Dijon where his grand-parents and extended family are located. That is until the age of 10 when his parents relocalized the small family off of the coast of Australia and New-Zealand, in Nouméa, New Caledonia, France. This experience was a blessing for all of them and gave them the travelling bug. It also exposed Arnaud to a huge diversity of flora and fauna, an area that he has always been interested in since he was a small child. After coming back to Dijon, Arnaud attended the University of Burgundy from where he obtained a B.Sc. in Plant Biology in 2002 and a M.Sc. in 2003 after completing an internship in the AFOCEL's Biotechnology Laboratory near Paris, an organization involved in the wood industry of France. There, he acquired his first skills at the laboratory bench and decided to obtain another M.Sc., a joint degree from the Clermont-Ferrand University and the engineering school ENITAC. This included a 6 month internship for which he decided to join David Stern's lab who gave him the great opportunity to stay as a technician after obtaining his degree. Because of his love for Ithaca, Arnaud gladly came back and started establishing a life in Ithaca. A loving partner, great coworkers and awesome friends contributed to an awesome experience. David Stern then suggested that the work he was doing in his lab could earn him a Ph.D., so he followed his advice and was accepted to the Plant Biology Department of Cornell University as a graduate student in 2007. Arnaud enjoyed every moment of his graduate work, both at the bench and in Ithaca and looks forward to keep doing research in Ithaca, on a very closely related topic of chloroplast biology in the laboratory of Maureen Hanson.

DEDICATION

Dedicated to my family, many remarkable mentors and many many incredible friends who, all along my life, have given the support and small building blocks needed to achieve something I would have never thought possible. Thank you!

ACKNOWLEDGMENTS

I would like to wholeheartedly acknowledge my thesis advisor David Stern for his dedication and mentoring efforts, always encouraging me since the beginning and putting me back on the right track by pushing me to try harder. I have been extremely lucky to work on very exciting and fruitful projects in this lab and used a huge variety of techniques that were key to my scientific development. I am also grateful to all the members of the Stern laboratory for the numerous ways they have helped me, both personally when I first arrived in Ithaca, and also scientifically of course. All the scientists I met here, from all around the world, are truly amazing and the atmosphere in the lab has always been supportive and uplifting. The technical support provided by the Boyce Thompson Institute has also been wonderful. Finally, I would like to thank my committee members, Maureen Hanson and Jeffrey Pleiss for their support. It is always wonderful to see where life takes you when you have the courage to do something that seems risky. The rewards have so far been amazing and I look forward to seeing what will happen to me next. In the meantime, I will do as I have been taught by my parents, enjoy the present as much as possible and never have any regrets about the past since the decisions made cannot be changed and were the best decisions with the information at that time. Is that the secret to happiness? Future will tell.

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CHAPTER 1

RNA PROCESSING AND DECAY IN PLASTIDS

ABSTRACT

Plastids were derived through endosymbiosis from a cyanobacterial-like ancestor, whose uptake was followed by massive gene transfer to the nucleus, resulting in the compact size and modest coding capacity of the extant plastid genome. Plastid gene expression is essential for plant development, but depends on nucleus-encoded proteins acquired through endosymbiosis or with non-endosymbiotic origins. The plastid genome is heavily transcribed from numerous promoters, giving post-transcriptional events a critical role in determining the quantity and sizes of accumulating RNA species. The major events reviewed here are RNA editing, which restores protein conservation or creates correct open reading frames by converting C residues to U, RNA splicing, which occurs both in *cis* and *trans*, and RNA cleavage, which relies on a variety of exo- and endoribonucleases. Because the RNases have little sequence specificity, they are collectively able to remove extraneous RNAs whose ends are not protected by RNA secondary structures or sequence-specific RNA-binding proteins. Other plastid RNA-binding proteins, largely members of the helical repeat superfamily, confer specificity to editing and splicing reactions. The enzymes that catalyze RNA processing are also the main actors in RNA decay, implying that these antagonistic roles are optimally balanced. We place the actions of RNA-binding proteins and RNases in the context of

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recent proteomic analyses that compare components of the plastid nucleoid, a protein-DNA complex, with other plastid compartments. These results suggest that a physical separation of proteins could underpin the regulation of RNA maturation and degradation.

INTRODUCTION

Plastids of the eukaryotic supergroup Plantae originated from the single engulfment event of a β-cyanobacterium over one billion years ago (Price *et al.* 2012). Over time, the endosymbiont lost its autonomy as the majority of its genes were integrated into the host cell nucleus. Currently, thousands of nucleus-encoded proteins dominate the plastid proteome, while a little over 100 genes are retained in the plastome, or plastid genome (Timmis *et al.* 2004, Bhattacharya *et al.* 2007). The importance of the remnant plastome is illustrated by the fact that both nucleus- and plastid-encoded subunits form the thylakoid membrane photosynthetic complexes, the CO_2-fixing enzyme Rubisco, the ribosomes and RNA polymerases. In the latter case, transcription is achieved both by a typical prokaryotic plastid-encoded polymerase (PEP) complemented by a family of nucleus-encoded σ-factors, and in vascular plants one (in monocots) or two (in dicots) additional nucleus-encoded polymerases (NEP) of phage-like origin (for review see Liere *et al.* 2011). Similarly, plastid ribosomes are composed of prokaryotic rRNAs and many prokaryotic proteins, but also contain several novel nucleus-encoded proteins that may have acquired specialized functions (Sharma *et al.* 2007). As discussed below, much of RNA metabolism relies on a combination of

bacterial-like enzymes and a variety of proteins with no prokaryotic counterparts. Presumably this chimerism offers evolutionary advantages, perhaps related to the ability of sessile plants to tolerate a wide variety of biotic and abiotic stresses.

The importance of post-transcriptional alterations

Post-transcriptional processes entail modification of newly-synthesized RNA molecules, as well as their ultimate degradation for functional inactivation and nucleotide recycling. Plastid RNA processing includes the generation of 5' and 3' termini, and intergenic cleavage of polycistronic transcripts, the latter phenomenon being largely absent from its cyanobacterial progenitor. Furthermore, although introns are rare within bacterial genomes, over 20 group I and II introns are removed by *cis*- or *trans*-splicing in plastids. Additionally, 20-50 specific editing events, depending on the species, alter the genome-encoded amino acid by generally converting C residues to U. Together, these post-transcriptional alterations can functionally activate an mRNA, affect its translatability, or alter its stability.

Understanding the roles of nucleus-encoded proteins in chloroplast RNA metabolism is indispensable to achieving crop improvement through organelle modification. Numerous examples have demonstrated the potential of chloroplast transgenes to enable the production of therapeutics, bioenergy precursors, or other molecules (Maliga and Bock 2011). Here, we build on recent findings relevant to RNA processing and decay, after briefly summarizing developments in plastid RNA editing and splicing. We conclude by speculating how recent proteomic results may give insight into a potential spatial regulation of chloroplast RNA metabolism.

THE QUEST FOR THE PLASTID EDITOSOME

The study of RNA editing is devoted to understanding why, how and when information encoded by newly-synthesized RNA molecules is altered. In plants the most frequent editing events are C-to-U changes, but U-to-C editing has been described in a few lineages, while no editing has been observed for *Marchantia* (liverworts) or chlorophyte algae such as *Chlamydomonas*. A breakthrough in the elucidation of the editing apparatus was made with the discovery that pentatricopeptide repeat (PPR) proteins play an essential role in editing site recognition (Kotera *et al.* 2005). PPR proteins are the largest of several families of helical repeat proteins that are expanded in land plants (Tillich *et al.* 2010). Based on sequence similarities with nucleotide deaminases and phylogenetic studies, it had been suggested that a particular subclass of PPR proteins (DYW PPRs) could bear the deaminase catalytic activity required for editing (Salone *et al.* 2007). Since then, the list of PPR proteins involved in editing has rapidly grown, but no evidence has emerged that PPR proteins do perform C to U conversion. Instead, they are hypothesized to recruit the deaminase to the target cytosine. Efforts to assign deaminase activity to a specific protein are being actively pursued (for comprehensive reviews see Stern *et al.* 2010, Chateigner-Boutin and Small 2011).

Recent findings suggest that the model of an editosome comprising a specific PPR protein interacting directly with an RNA sequence and a deaminase, or even carrying deaminase activity within its DYW domain, is oversimplified. Two reports describe mutations in a family of ten *Arabidopsis* genes that abolish or lower the editing

efficiency of hundreds of sites in both chloroplasts and mitochondria (Bentolila *et al.* 2012, Takenaka *et al.* 2012). These genes were independently discovered through a screen for mutants that had lost detectable editing at numerous sites, or by pull-down using the PPR editing factor RARE1. The authors concluded that proteins belonging to this family must selectively interact with PPR proteins, hinting at a multifaceted editosome as depicted in Figure 1.1.

Another much-debated question is the “raison d’être” of RNA editing, for which a true regulatory role remains to be established. The three-step model of Covello and Gray (1993) hypothesizes that RNA editing is the result of neutral evolution. In this model, the editing apparatus appeared before organellar mutations and allowed them to become fixed because they could be corrected at the RNA level, meaning there is no selection for the appearance of RNA editing (Lukes *et al.* 2011). An alternative model postulates that RNA editing evolved to correct mutations appearing at the RNA level. This model would only apply where the mutations were not overly detrimental, and a nucleo-cytoplasmic conflict theory has been proposed as a framework (Castandet and Araya 2012).

THE DEPENDENCE OF RNA SPLICING ON NUCLEUS-ENCODED FACTORS

The combined activities of catalytically active intron RNAs (ribozymes) and intron-encoded proteins (IEPs) make introns mobile genetic elements in both bacteria and land plant chloroplasts. Over time, these IEPs have degenerated (only the *matK* gene is conserved in most plastids), rendering these ribozymes immobile, in addition to

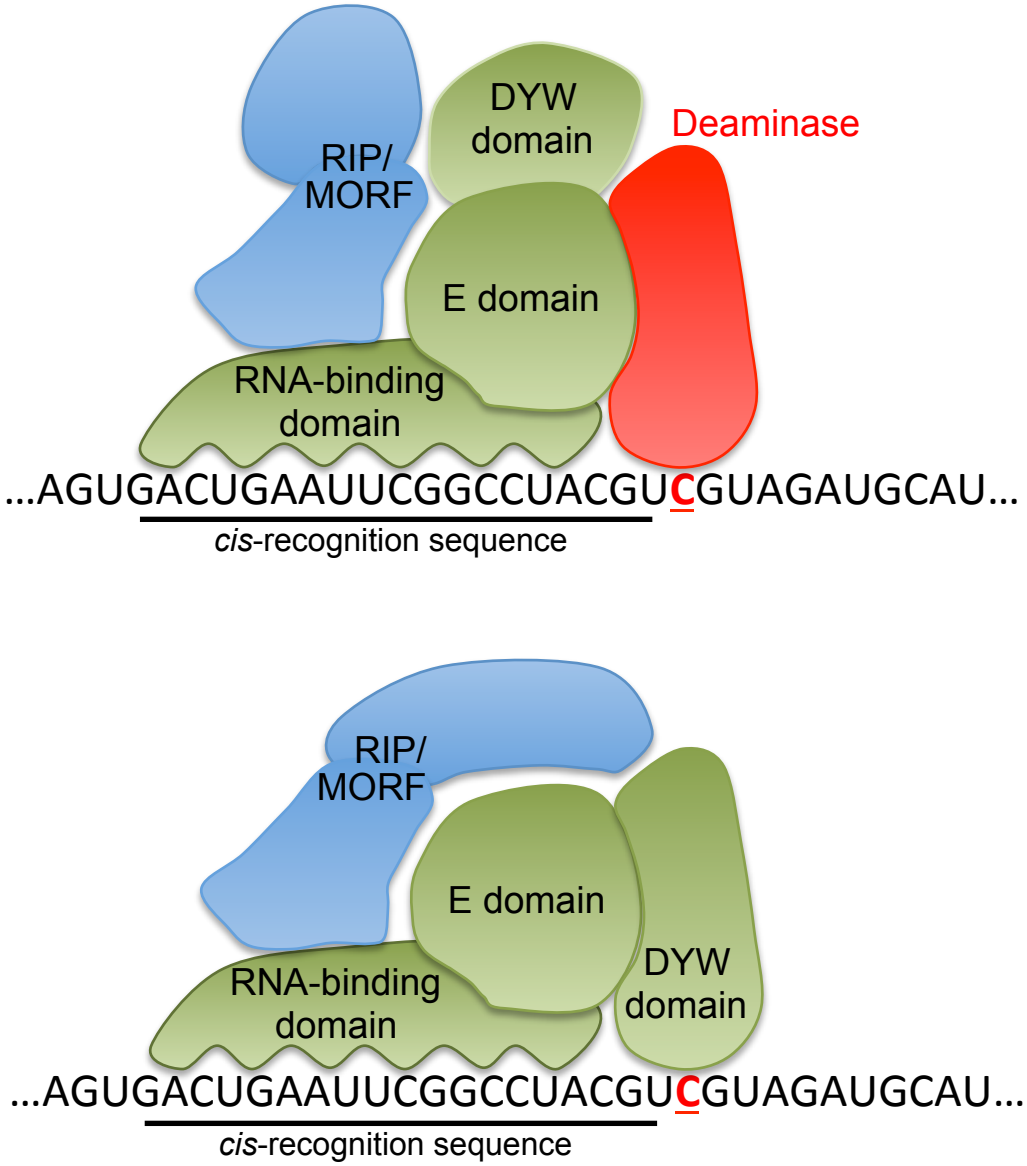

Figure 1.1: Models for plastid RNA editing.
A specific PPR protein, with a conserved E and DYW domain, is directed to an RNA editing site by the *cis* recognition sequence. The RIP/MORF protein interacts with the PPR protein RNA-binding domain and is required for the deamination reaction. In one model (top), this complex recruits an unidentified deaminase to catalyze C-to-U editing. A second hypothesis (bottom) is that the RIP/MORF protein binding causes a shift in the PPR DYW domain that positions it over the target nucleotide. In this case, the DYW domain performs the deamination reaction.

stabilizing their population at a total of about 20 introns. The majority are now group II introns that differ from the group I intron of *trnL* by secondary structure and the form of the excised product, which is linear for group I introns and a lariat for group II introns. Excision of group II introns has become largely if not completely dependent on nucleus-encoded proteins *in vivo*, and they have lost their ability to self-splice *in vitro*. While a large nucleus-located spliceosome is conserved in humans, yeast and plants, organelle intron splicing results from the cooperation of various proteins, unrelated to spliceosome components and often to each other.

A recent model for plastid intron excision in land plants is shown in Figure 1.2. The splicing factors identified to date through mutant or proteomic analysis contain a variety of previously described domains including the CRM, RRM and PPR RNA recognition motifs, the LAGLIDADG motif typically found in group I intron maturases, and conserved domains of unknown function such as PORR or DUF794. RNC1 and WHY1, members of the RNase III and Whirly transcription factor families, respectively, mostly promote subgroup IIA intron splicing. Finally, CRS2, a catalytically-inactive descendant of peptidyl-tRNA hydrolases, plays a role in the splicing of subgroup IIB introns. Plastid splicing factors form protein-dominated complexes typically larger than 500 kDa, which are hypothesized to fold intron sequences into a catalytically active structure.

As for RNA editing, an obligate or regulatory role for plastid introns remains to be demonstrated. Because alternative splicing has never been demonstrated in plastids, any regulatory role would likely be related to splicing efficiency, which can vary according to environmental or developmental context. The prevailing view that plastid

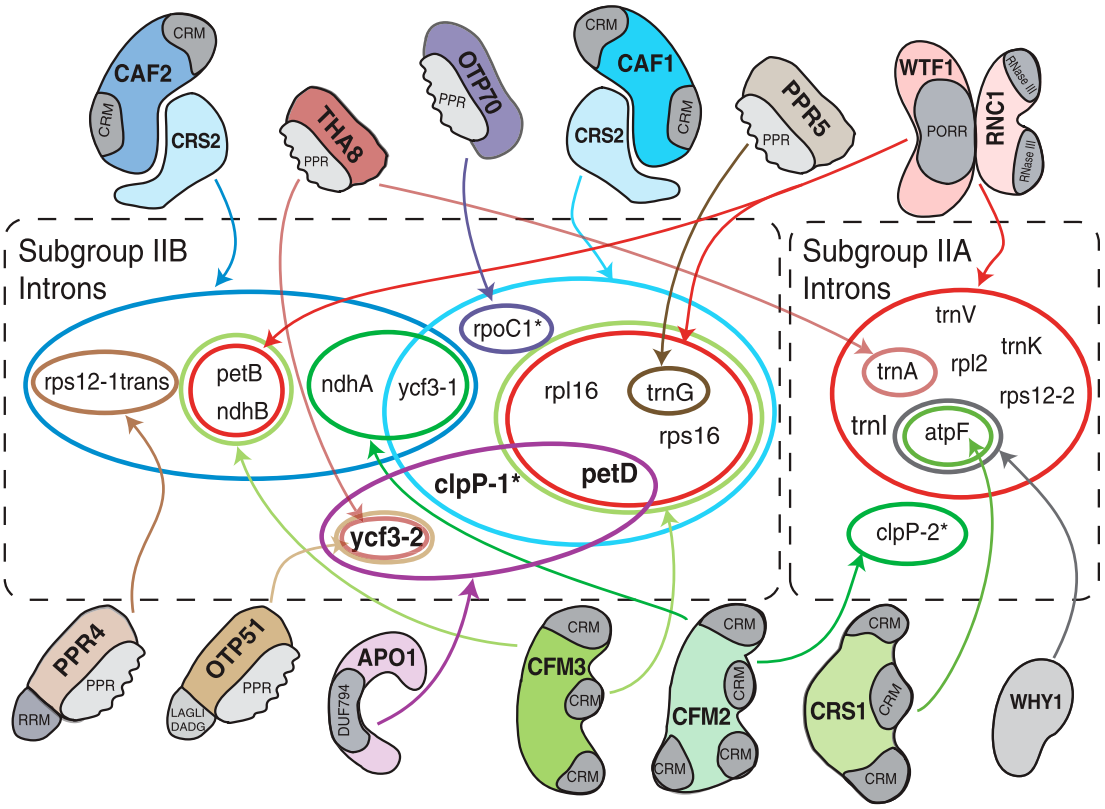

Figure 1.2: Nucleus-encoded factors involved in splicing group II introns in higher plants.
(Modified from Khrouchtchova *et al.* 2012 with permission).

introns are selfish genetic elements is tempered, however, by a recent study demonstrating that the excision of the second *ycf3* intron depends on the presence of its first intron (Petersen *et al.* 2011). This result suggests that selective pressure exists to maintain at least one particular intron. More globally, if introns can affect RNA function far from their insertion sites, this could limit their propagation, as well as select for their preservation.

PROCESSING OF PRIMARY TRANSCRIPTS

Modification of newly-synthesized RNA molecules not only includes editing and splicing, but also substantial cutting and trimming, and large-scale degradation of unwanted segments. The need for massive modification of the RNA population can be ascribed to relaxed transcription in plastids, which arises from a combination of a profusion of transcription initiation sites (Zhelyazkova *et al.* 2012b) and inefficient transcription termination (Figure 1.3, Stern and Gruissem 1987, Rott *et al.* 1996). Compelling evidence for relaxed plastid transcription comes from recent studies utilizing high-throughput strand-specific cDNA sequencing (RNA-Seq), which show that both strands of the entire genome encode accumulating RNAs, albeit in vastly different abundances depending on location (Hotto *et al.* 2011, Zhelyazkova*, et al.* 2012b). This leads to a model where a relatively unselected population of primary transcripts is subjected to competing activities between proteins that bind and protect RNAs, and those that degrade them.

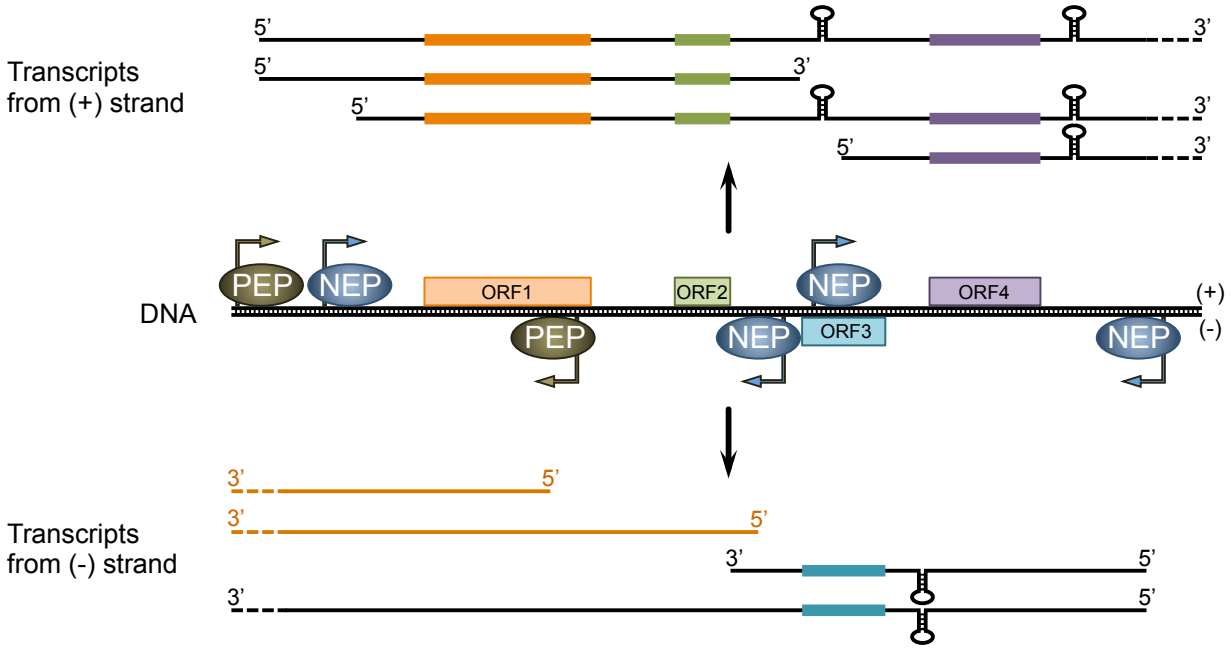

Figure 1.3: Relaxed transcription initiation and inefficient polymerase termination in plastids.
Transcription initiation by NEP (blue ovals) and PEP (brown ovals) occurs at widespread promoters found upstream of known ORFs, within operons and in non-coding regions. Many genes have both NEP and PEP promoters. Inefficient transcription termination creates multiple and poorly-defined 3' ends. The combination of multi-site transcript initiation and imprecise termination creates a diverse pool of plastid RNA precursors, which will vary depending on polymerase concentrations.

Plastids harbor an assortment of nucleus-encoded ribonucleases (RNases, Table 1.1), many of which are homologous to bacterial enzymes. On the other hand, the majority of chloroplast RNA-binding proteins (RBPs) belong to the expanded helical-repeat superfamily, which is almost completely absent in bacteria, with the exception of the OPR family (Rahire *et al.* 2012). Available evidence suggests that these proteins are largely involved in organelle mRNA maturation (reviewed in Schmitz-Linneweber and Small 2008). The RNA population itself can be divided into two distinct classes, mRNAs and non-coding RNAs (ncRNAs). The latter category can be subdivided into rRNAs, tRNAs and other ncRNAs, which includes plastid-localized intergenic and antisense RNAs (asRNAs) collectively referred to as pncRNAs (Hotto *et al.* 2012).

mRNA maturation

Most mRNAs are initially polycistronic

Reflecting their cyanobacterial origin, most chloroplast genes are organized in clusters and transcribed polycistronically, in some cases from both upstream and internal promoters, then cleaved into shorter RNA molecules containing one or more coding regions (Figure 1.4). Recent work suggests that cleavage increases translation efficiency due to a recognition bias by ribosomes for the 5'-most Shine-Dalgarno sequence of polycistronic transcripts (Drechsel and Bock 2011). Other results show that cleavages can alter 5' end secondary structures to facilitate translation (Hammani *et al.* 2012).

While there is an obligate order for certain maturation steps – for example, trimming of internal sequences cannot precede intercistronic cleavage, and proteins that

Protein	Activity	Location[1]	Mutant phenotype	Accession number
PNPase	3' to 5' exonuclease	C	Virescent	At3g03710
RNase II	3' to 5' exonuclease	C/M	Rescued on sucrose	At5g02250
RNase J	5' to 3' exonuclease and endonuclease	C	Embryo lethal	At5g63420
RNase E	Endonuclease	C	Rescued on sucrose	At2g04270
CSP41a/b	Endonuclease; RNA binding	C	Early senescence	At3g63140 / At1g09340
RNase P	Endonuclease	C/M	Embryo lethal	At2g32230
RNase Z	Endonuclease	C	Embryo lethal	At2g04530

[1] C: chloroplast; M: mitochondria

Table 1.1: Characterized nuclear-encoded ribonucleases involved in chloroplast RNA metabolism.

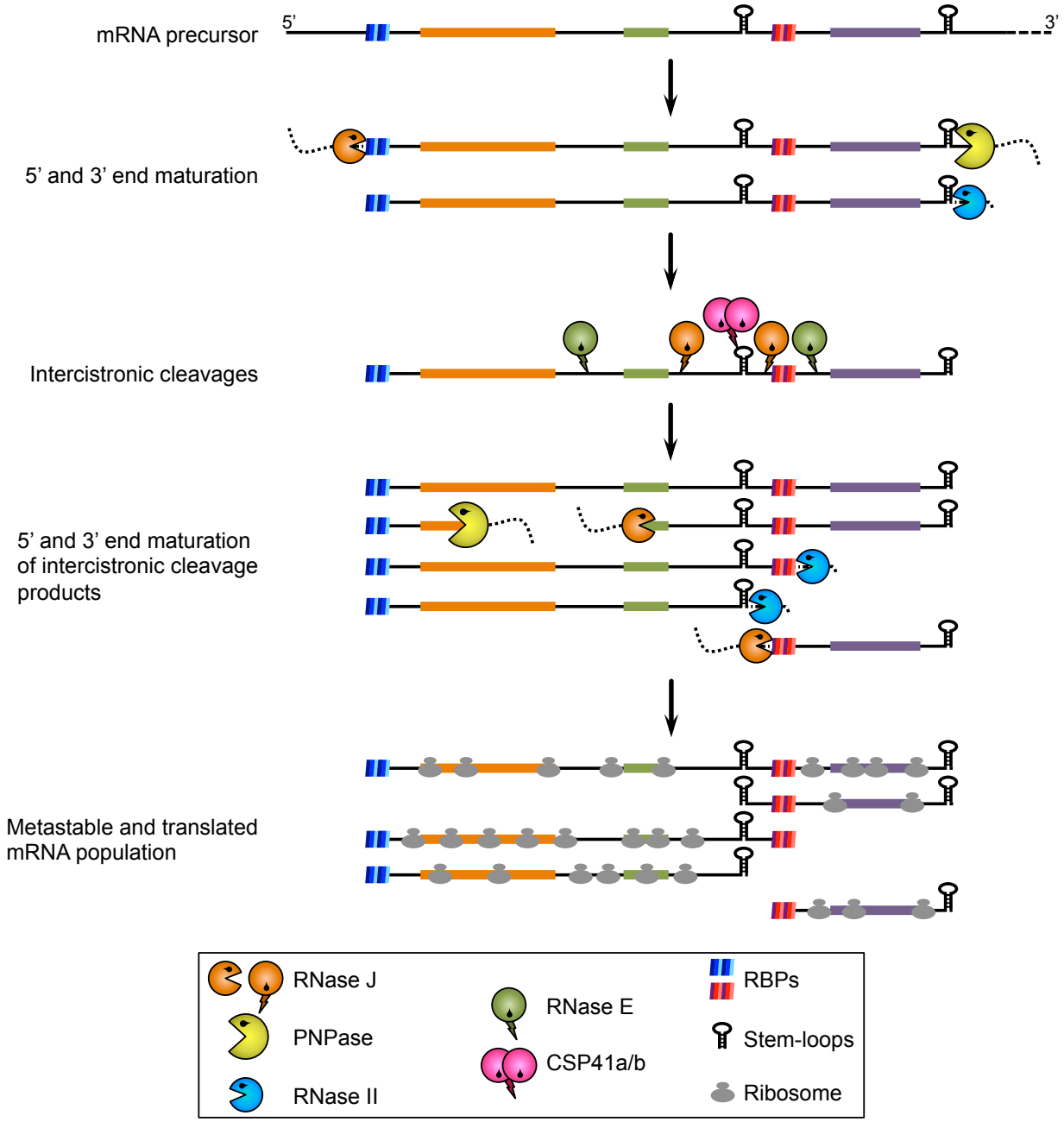

Figure 1.4: Maturation of plastid mRNAs.
A polycistronic mRNA precursor is bound by proteins at specific intergenic sites, and its 5' and 3' ends are matured exonucleolytically. This transcript is also subject to intercistronic cleavage by several RNases whose respective roles remain to be clarified. Specificity could derive from the fact that RNase E prefers AU-rich regions and CSP41a/b preferentially cuts at stem-loops. Intercistronic cleavage produces new 5' and 3' ends, which are again subject to exoribonuclease activity, with RNase II/R generally following PNPase. Once stabilized by secondary structures and RBPs, ribosomes bind to initiate translation, and possibly afford additional protection to the mature transcripts.

protect RNA must bind before that transcript is processed – in general, 5' and 3' end maturation, RNA editing and splicing are considered to occur stochastically. Indeed, the extreme diversity of accumulating RNAs both in terms of the number of ORFs they possess, and the presence of both spliced/unspliced and edited/unedited forms in every conceivable combination, testify to this conclusion.

Roles of exoribonucleases

There are three known chloroplast exoribonucleases. Two have 3'→5' directionality, polynucleotide phosphorylase (PNPase) and RNase II (RNR1), the former being phosphorolytic and the latter hydrolytic. A third, RNase J, degrades RNA in the 5'→3' direction, while also possessing endoribonuclease activity.

Unlike the degradosome-associated bacterial PNPase, chloroplast PNPase is a dimer of trimers (Baginsky *et al.* 2001), whose processive activity can be arrested by secondary structures or RBPs such as maize PPR10 (Yehudai-Resheff *et al.* 2001, Prikryl *et al.* 2011). Depletion of PNPase from *Arabidopsis* or maize chloroplasts leads to 3' extensions in numerous RNA species, revealing its role in trimming (Walter *et al.* 2002, Williams-Carrier *et al.* 2010, Germain *et al.* 2011). In contrast, *Chlamydomonas* strains depleted for PNPase have apparently normal cpRNA length, but their half-lives are increased and the cells are unable to survive phosphorus starvation (Yehudai-Resheff *et al.* 2007). This suggests that 3' end formation in *Chlamydomonas* chloroplasts may rely more heavily on transcription termination or endonucleolytic cleavage, or that PNPase is redundant with RNase II in this species. Another

consideration is that *Chlamydomonas* PNPase was investigated in strains that were depleted by RNAi, which did not fully remove the enzyme.

RNase II was initially described as an RNase II because of its role in mRNA 3' end trimming in *Arabidopsis* mitochondria (Perrin *et al.* 2004b). The enzyme is also targeted to chloroplasts, however, where defects in rRNAs were found (see below). Because of this function, and its better sequence alignment to bacterial RNR1 versus RNase II, in the chloroplast context the enzyme was named RNR1. Still more recently, meticulous analysis of chloroplast RNAs in the *rnr1* mutant showed a failure to remove 3-8 nt mRNA 3' end extensions (Germain *et al.* 2012), corroborating *in vitro* evidence (Prikryl*, et al.* 2011) that RNase II could approach RNA secondary structures or bound RBPs more closely than PNPase. These results indicate that as in mitochondria, RNase II and PNPase cooperate to mature 3' ends.

RNase J appears to be restricted to plastids and while null mutants are embryo lethal, RNase J deficiency has been achieved in both tobacco and *Arabidopsis* using virus-induced gene silencing (VIGS). VIGS-induced RNase J depletion leads to massive accumulation of antisense RNAs (asRNAs), which form duplexes with mRNAs and prevent their translation (Sharwood *et al.* 2011a), likely accounting for embryo lethality. Recombinant *Arabidopsis* RNase J has both endo- and exoribonucleolytic activities, and has been hypothesized to trim heterogeneous 5' termini, leading to the accumulation of uniform species where 5' ends are protected by PPR proteins. This result can be achieved *in vitro* using recombinant PPR proteins and the commercial Terminator 5'→3' exonuclease (Hammani*, et al.* 2012). Chloroplast RNase J does not appear to be sensitive to the 5' status of its substrate, unlike bacterial RNase J (Sharwood*, et al.*

2011a). This suggests that RNase J could be redundant with RNase E in intercistronic RNA processing, even though the global reduction in both precursor and mature RNA species in RNase J knockdowns is not similar to the RNA phenotype displayed in RNase E-depleted tissues (see below). Which catalytic properties of RNase J are most critical *in vivo* remains to be determined.

Roles of endoribonucleases

The four identified endoribonucleases in plastids are CSP41a, CSP41b, RNase E and RNase J, with relatives of RNase III likely to be added to the list (Stoppel and Meurer 2012). CSP41a and CSP41b are diverged products of a presumed gene duplication, which *in vivo* form both homo- and heterodimers, and *in vitro* at least for CSP41a, preferentially bind and cleave stem-loops (Bollenbach and Stern 2003). Mutants lacking only CSP41a have no obvious phenotype, whereas the loss of CSP41b also destabilizes CSP41a, and such plants exhibit a mild chlorosis (Bollenbach *et al.* 2009) that is accentuated under growth stress (Qi *et al.* 2012). The abundant CSP41 proteins have been implicated in transcript synthesis and stability, and have also co-purified with ribosomes. Their precise biological roles remain somewhat enigmatic due to the subtle mutant phenotypes.

Chloroplast RNase E has a catalytic domain with typical bacterial properties, i.e. preferential cleavage of A/U-rich sequences within 5'-monophosphorylated transcripts (Schein *et al.* 2008), as well as a plant-specific domain of unknown function. A recent study shows RNase E functions are supported by the RNA-binding protein RHON1, which is hypothesized to confer sequence specificity (Stoppel *et al.* 2012). Both *rne* and

rhon1 null mutants are chlorotic, and can accumulate very long precursor mRNAs at the expense of processed products, suggesting an important role of RNase E in intercistronic RNA processing (Walter *et al.* 2010). Nevertheless, mature RNA species also accumulate for all transcripts analyzed, indicating that RNase E function is at least partly redundant with other mechanisms or enzymes, such as RNase J.

Roles of RNA-binding proteins

Members of the helical-repeat protein superfamily participate in plastid post-transcriptional processing. First identified was NAC2, a tetratricopeptide repeat (TPR) protein that stabilizes the 5' end of *Chlamydomonas* chloroplast *psbD* mRNA (Boudreau *et al.* 2000). Around the same time, the pentatricopeptide (PPR) family was recognized from *Arabidopsis* genome sequencing (Small and Peeters 2000), and since then the half-a-tetratricopeptide (HAT) and octatricopeptide (OPR) subfamilies have been defined (Auchincloss *et al.* 2002, Hammani*, et al.* 2012). In their mRNA maturation role, helical repeat proteins bind to specific sequences in unprocessed transcripts, and ensure the homogeneity and stability of mature species by preventing the progression of exoribonucleases (Figure 1.4). Sequence specificity seems to arise through variably-sized tandem repeats of loosely conserved amino acids (Barkan 2011, Prikryl*, et al.* 2011, Hammani*, et al.* 2012). The roles of PPR and related proteins in other facets of RNA metabolism have been reviewed elsewhere (Tillich*, et al.* 2010).

Conclusions on mRNA maturation

Parallels between bacterial and plastid mRNA maturation appear to be limited to the initial building block of polycistronic transcripts. The expansion and employment of the helical repeat protein families, in combination with relatively nonspecific nucleus-encoded RNases of bacterial origin, allows the nucleus to shape the accumulating chloroplast transcript population. As such, it is interesting to consider functional mRNAs as metastable degradation intermediates (Barkan 2011).

Processing the primary transcript of the rRNA operon

Chloroplast rRNA genes are co-transcribed with tRNAs in the order 16S-*trnI*-*trnA*-23S-4.5S-5S-*trnR*, and efficient processing of this precursor is essential for maintaining chloroplast translation. Some of the RNases described in the previous section have roles in trimming rRNAs, following endonucleolytic cleavages (Table 1.1). In a number of cases, however, effects on rRNA accumulation and processing have been observed in mutants that are likely to be pleiotropic effects, due to feedback between gene expression dysfunction and ribosome biogenesis. A schematic of rRNA processing is shown in Figure 1.5.

rRNA maturation

RNase P and RNase Z, the endoribonucleases that generate the 5' and 3' ends of tRNAs, respectively, are likely to initiate cleavage of the rRNA operon polycistronic primary transcript. Together with an additional cleavage between the 4.5S and 5S rRNAs, the 16S, 23S-4.5S and 5S rRNA precursors are released. The 16S rRNA

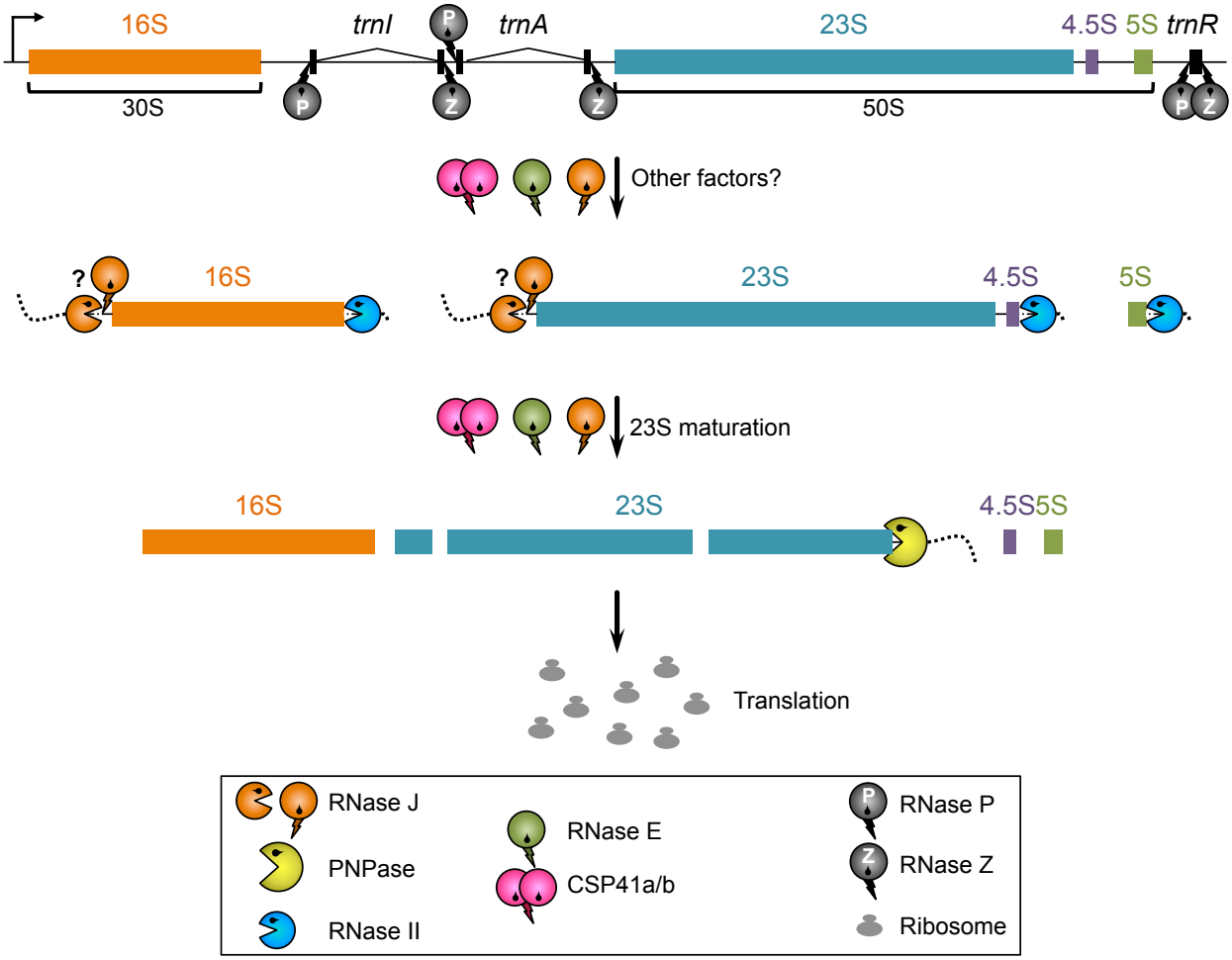

Figure 1.5: Ribosomal RNA maturation.
Top, the rRNA operon is transcribed as a single precursor. In this model, endonucleolytic cleavage by RNase P and RNase Z initiate maturation by releasing the tRNAs. Additional cleavages by other endoribonucleases could also occur at multiple sites, and must occur between the 4.5S and 5S rRNA coding regions. The pool of precursor rRNAs is processed at their 3' ends by RNase II/R and at their 5' ends by RNase J. The 23S and 4.5S rRNAs are initially released as a dicistronic moiety, which is subject to trimming and hidden break processing during or after ribosome assembly. A complete list of factors that directly or indirectly impact rRNA processing are listed in Table 2. While the Figure shows the major enzymes for each step, in their absence other nucleases can also create WT-like ends.

precursor is processed at its 5' end, at least in part, by RNase J, and at its 3' end by RNase II (Kishine *et al.* 2004, Bollenbach *et al.* 2005, Sharwood*, et al.* 2011a). The 23S rRNA 5' end can be matured by RNase J, since RNase J-deficient plants accumulate some 23S transcripts with 5' extensions (Sharwood*, et al.* 2011a). Maturation of the 23S-4.5S rRNA 3' end is completed by RNase II prior to endonucleolytic cleavage between the two regions (Bollenbach*, et al.* 2005), the latter of which occurs following its incorporation into the ribosome (Leal-Klevezas *et al.* 2000, Bellaoui *et al.* 2003, Bollenbach*, et al.* 2005). While the endoribonuclease responsible has not been confirmed, the RHON1 protein mentioned above binds to this region based on immunoprecipitation assays, and may facilitate cleavage by RNase E (Stoppel*, et al.* 2012). The mature 4.5S rRNA 5' end appears to result from this cleavage, while the 23S rRNA 3' end is trimmed by either PNPase or RNase II (Walter*, et al.* 2002, Bollenbach*, et al.* 2005). In dicotyledonous plants, the 23S rRNA is converted into final products of 0.5, 1.3 and 1.1 kb through two internal "hidden breaks" which are hypothesized to occur independently, possibly by different activities. In monocotyledonous plants such as maize, a single cleavage yields mature products of 1.8 and 1.1 kb. Although the underlying mechanism is still unknown, RH39, an RNA helicase, is thought to be involved in processing of the second hidden break, while RHON1 binds to the 23S 5' end (Table 1.2). Additionally, the *Arabidopsis* genome encodes two RNase III-like proteins predicted to be chloroplast targeted. They have high similarity to *Bacillus subtilis* mini-III, which matures the 23S rRNA 5' and 3' ends through cleavage within a bulge in the 23S processing stalk (Redko *et al.* 2008). It is reasonable to speculate that the *Arabidopsis* orthologues may have a similar role.

Protein	Accession number	Protein family / domains	Proposed rRNA function	References
I. Identified in *Arabidopsis*				
CSP41a/b	At3g63140; At1g09340	Rossman Fold; NAD(P) epimerase/dehydrogenase	Processing of 23S 1st hidden break	(Beligni and Mayfield 2008, Bollenbach*, et al.* 2009, Qi*, et al.* 2012)
RHON1	At1g06190	Rho-N	RNase E chaperone binding to 23S 5' end and 23S-4.5S intergenic region	(Stoppel*, et al.* 2012)
RH39	At4g09730	DEAD box helicase	Assist in processing second 23S hidden break	(Nishimura *et al.* 2010)
RH22	At1g59990	DEAD box helicase	50S ribosome assembly	(Chi *et al.* 2012)
RH3	At5g26742	DEAD box helicase	50S ribosome assembly	(Asakura *et al.* 2012)
BPG2	At3g57180	GTP-binding domain; zinc finger motif	Light-regulated pre-rRNA processing	(Komatsu *et al.* 2010, Kim *et al.* 2012)
DAL	At2g33430	DAG family	Ribosome assembly	(Bisanz *et al.* 2003)
SVR1	At2g39140	pseudouridine synthase; RNA binding	rRNA processing	(Yu *et al.* 2008)
SVR2/ClpR1	At1g49970	Clp protease	Regulate rRNA processing factors	(Koussevitzky *et al.* 2007, Yu*, et al.* 2008)
ClpR2	At1g12410	Clp protease	Regulate rRNA processing factors	(Rudella *et al.* 2006, Zybailov *et al.* 2009)
WCO	bt. *nga63* & ATSRP54A on At1	unknown	unknown	(Yamamoto *et al.* 2000)
II. Identified in *Zea mays*				
PPR2	At orthologue At3g06430	RRM and PPR tract	Ribosomal protein mRNA or tRNA processing, or ribosome assembly	(Williams and Barkan 2003)
PPR4	At orthologue At5g04810	RRM and; PPR tract	Splicing of *rps12* intron	(Schmitz-Linneweber *et al.* 2006)

PPR5	At orthologue At4g39620	RRM and PPR tract	Biogenesis of *trnG*	(Cushing *et al.* 2005, Beick *et al.* 2008)
RNC1	At orthologue At4g37510	RNase III	Splicing of tRNAs and ribosomal protein mRNAs	(Watkins *et al.* 2007)
WHY1	At orthologue At1g14410	Whirly Family DNA-binding domain	Processing of 23S or 4.5S	(Prikryl *et al.* 2008)
HCF7	not cloned	unknown	Processing of 23S & 16S, or stabilization of mature species	(Barkan 1993)
CPS1	not cloned	unknown	23S processing or stabilization of mature species	(Barkan 1993)
III. Identified in *Nicotiana benthamiana*				
PRBP	At orthologue At2g37920	loosely resembles an ATP-dependent helicase	4.5S processing	(Park *et al.* 2011)
DCL	At1g45230	DUF 3223	rRNA processing & ribosome assembly	(Bellaoui, *et al.* 2003, Bellaoui and Gruissem 2004)

Table 1.2: Nuclear mutations that affect rRNA maturation.

Although the enzyme acting on the 5S rRNA 5' end remains elusive, it is known that RNase II, at least in part, matures its 3' end. Available evidence suggests that RNase P releases the downstream $tRNA^{Arg}$, and RNase II trims the intergenic region between 5S rRNA and *trnR*. Alternatively and/or additionally, an endonucleolytic cleavage by either RNase E or RNase J can occur in the 5S-*trnR* intergenic region, followed by exonuclease trimming by RNase II or PNPase (Sharwood *et al.* 2011b).

An important observation is that in most cases of perturbed rRNA maturation, some WT-like transcripts still accumulate. This indicates that there is partial redundancy built into rRNA processing: for example, exoribonucleases may partially substitute for endoribonucleases. To further clarify this maturation pathway it will be necessary to investigate plants with combined mutations affecting the aforementioned RNases and other factors, and/or use biochemical approaches. Mutants completely lacking processed rRNAs, however, are likely to exhibit embryo lethality.

Numerous additional mutants exhibit rRNA maturation phenotypes

Analyses of mutants affecting more than 15 proteins, in addition to those mentioned above, have shown defects in rRNA maturation or accumulation (Table 1.2). Most, if not all, of these mutants have pale-green or albino leaf phenotypes, and many have defects in chloroplast development. Of these, seven are defective in proteins of unknown function, three each lack PPR proteins or DEAD-box helicases, and two mutants are deficient for components of the plastid Clp protease. In many cases these proteins were postulated to interact with rRNAs or ribosomal proteins based on immunoprecipitation assays, however, since ribosomal RNAs and proteins are

extraordinarily abundant in the chloroplast, one must be cautious to exclude false positive results. Additionally, primary effects on chloroplast protein import, photosynthetic activity, translation or ribosome assembly are prone to causing pleiotropic effects on rRNA synthesis or processing, that could manifest as the accumulation of precursor transcripts. This would tend to be most pronounced in 23S rRNA processing, since this occurs following ribosome assembly.

The metabolism of tRNAs

The correct processing of tRNA precursors is essential, but achieved by distinctive means depending on the organism considered. This is well illustrated by RNase P, which is a ribonucleoprotein complex in bacteria (Lai *et al.* 2010), but is entirely composed of proteins, and hence referred to as proteinaceous RNase P in human mitochondria and plants (Holzmann *et al.* 2008, Gobert *et al.* 2010). One of the three nucleus-encoded RNase P proteins supports tRNA maturation in both *Arabidopsis* plastids and mitochondria (Gutmann *et al.* 2012).

tRNA 3' trailer sequence removal in bacteria is either mediated by the endoribonuclease RNase Z, or by the successive action of ribonucleases such as RNase E, PNPase, RNase II, RNase PH and/or RNase T (reviewed in Hartmann *et al.* 2009). The second pathway is specific to tRNAs for which the 3' CCA motif is chromosomally encoded, and since eukaryotic tRNAs all require the post-transcriptional addition of the CCA triplet, their 3' maturation is believed to solely depend on RNase Z. *Arabidopsis* encodes four RNase Z proteins, but only deficiency of the chloroplast isoform is embryo lethal, consistent with a required minimum level of chloroplast gene

expression during embryo development (Canino *et al.* 2009, Bryant *et al.* 2011). 5' and 3' matured tRNAs are substrates for tRNA nucleotidyltransferase, which adds the terminal CCA nucleotides (von Braun *et al.* 2007).

Although only RNase P and RNase Z appear to be required to process plastid tRNA precursors, both RNase E and PNPase mutants have abnormal tRNA accumulation. For instance, the accumulation of $tRNA^{fMet}$ and $tRNA^{Pro}$ is proportional to the level of PNPase, with a strong reduction when PNPase is overexpressed (Walter*, et al.* 2002). In an RNase E knockout, while the $tRNA^{Ile}$ level was severely reduced, the opposite was observed for $tRNA^{Arg}$ (Walter*, et al.* 2010). The differing RNase E observations highlight our poor understanding of how plastid tRNA levels are determined.

The metabolism of pncRNAs

Discovery of 60 and 107 pncRNAs in barley and *Arabidopsis*, respectively (Hotto*, et al.* 2011, Zhelyazkova*, et al.* 2012b), raises mechanistic and functional questions (Hotto*, et al.* 2012). Mechanistically, how are these sequences differentiated from other pre-mRNA or antisense sequences that are rapidly degraded? And does this differentiation occur because pncRNAs have maintained or acquired regulatory functions? The biogenesis of so many pncRNAs is made possible by the relaxed transcription described earlier, which leads to the incorporation of eventual pncRNAs into longer precursors. Evidence that pncRNA 3' ends are formed enzymatically comes from the PNPase mutant, where most pncRNAs were shown to be longer and/or more abundant than in the WT, suggesting that pncRNA processing has some parallels with

mRNA and rRNA maturation (Hotto*, et al.* 2011). Initial data were also obtained from plants lacking or deficient for RNase E, RNase J and RNase II. In each mutant background, certain pncRNAs differed in abundance and/or size, hinting at substrate specificity for RNase/pncRNA pairs.

Similar to mRNAs, it is reasonable to assume that RBPs and secondary structures contribute to the accumulation of specific pncRNAs. Indeed, RBPs (e.g. PPR and TPR proteins), which define mRNA termini, have been shown to protect a class of small pncRNAs (18-40 nt) from RNase activity. These pncRNAs commonly map near 5' ends of coding regions or intercistronic regions transcribed in tandem with the flanking genes (Ruwe and Schmitz-Linneweber 2012). Additionally, strong secondary structures such as stem-loops can withstand RNase degradation and accumulate in the chloroplast (Zhelyazkova *et al.* 2012a), much as Repetitive Extragenic Palindromic (REP) sequences stabilize RNAs in bacteria (Stern *et al.* 1984).

RNA DECAY

RNA lifetimes

Structure, sequestration and protein binding can all impact RNA half-life, which must be regulated to achieve quality control and an appropriate rate of protein expression. Of particularly high stability are rRNAs and tRNAs. Both have extensive internal complementarity, which makes them resistant to most RNases, and in addition rRNAs are bound by a coterie of ribosomal proteins. Indeed, rRNA stability is so high that it has not been accurately measured in chloroplasts, and rRNA appears to be

indefinitely stable in bacteria as long as it is part of a correctly assembled ribosome (Deutscher 2009). On the other hand, chloroplast mRNA half-lives are as much as an order of magnitude longer than those in bacteria, being between about 20 minutes and at least 24 hrs (Klaff and Gruissem 1991, Kim *et al.* 1993, Salvador *et al.* 1993, Hwang *et al.* 1996, Germain*, et al.* 2012). The longer lifetimes of chloroplast versus bacterial mRNAs could be a consequence of the types and number or concentrations of RBPs and/or RNases, different catalytic rates for RNases, and/or compartmentalization of RNases relative to their substrates.

Initial degradation of superfluous RNAs

Chloroplast genome probes invariably produce distinct banding patterns on gel blots, corresponding to the accumulation of uniformly-processed RNA species, indicating that intercistronic as well as antisense regions are generally unstable and rapidly eliminated from the RNA pool (Figure 1.3). A good example of the trimming that occurs of the primary transcriptome is revealed by the *atpI* gene cluster transcript pattern observed in the PNPase mutant, where discrete bands are largely replaced by a smear of high molecular transcripts (Germain*, et al.* 2011), which may be the result of imprecise 3' end formation. Another major activity targeting primary transcripts is endonucleolytic cleavage. To date, RNase E mutants have the strongest phenotype in terms of accumulating unusually long transcripts (Walter*, et al.* 2010), although such a phenotype is also observed when PEP is depleted and the more promiscuous NEP becomes responsible for all plastid transcription (Allison *et al.* 1996, Legen *et al.* 2002).

A remarkable phenotype observed when RNase J is depleted, as mentioned above, is the massive accumulation of asRNAs (Sharwood*, et al.* 2011a). The unique property of RNase J is its 5'→3' activity, and since mutants lacking RNase II or PNPase do not accumulate deleterious asRNAs, it seems reasonable to propose that the directionality of RNase J is somehow linked to its role in eliminating these transcripts. This in turn implies that the 5' ends of unwanted asRNA fragments are sensitive to RNase J, whereas their 3' ends may be poor substrates for 3'→5' exoribonucleases. Whether it is because these termini are highly structured, or because they are trapped in dsRNA duplexes, will be an interesting topic for future studies.

The polyadenylation-stimulated RNA degradation pathway is a hallmark of prokaryotes, conserved in plastids (see below and reviewed in Schuster and Stern 2009), and could have a role in the initial degradation of superfluous plastid RNAs. Since polyadenylation accelerates 3'→5' degradation, the pathway should be insensitive to RNase J. It is possible, however, that the heterodisperse transcripts that accumulate in the PNPase mutant represent polyadenylated species. In principle, this could be tested by treatment with oligo(dT) and RNase H, a method used to study the degree of polyadenylation in yeast, for example (Daugeron *et al.* 2001). Use of RNA-Seq could also be considered, as the depth of coverage could give quantitative information on the degree and location of polyadenylation even for low-abundance transcripts.

Inactivation and recycling of transcripts

As in bacteria, plastid RNA degradation is believed to comprise three steps: endonucleolytic cleavage, polyadenylation and exonucleolytic decay (Stern*, et al.* 2010, Barkan 2011). Our knowledge is still limited, however, regarding the relative importance and enzymology of each step.

Is endonucleolytic cleavage the rate-limiting step?

Endoribonucleases are rate limiting in bacterial RNA decay, with the *ams* (RNase E) mutant first described as featuring increased chemical RNA stability (Kuwano *et al.* 1977). An endoribonucleolytic initiation for RNA decay would also make sense for plastids, since such cleavage would strip RNA molecules of their 5' and 3' end protection, rendering them vulnerable to exoribonucleases (Stern*, et al.* 2010). CSP41a was an attractive candidate due to its abundance and specificity for stem-loops *in vitro* (Yang *et al.* 1996, Bollenbach and Stern 2003), and partial depletion of CSP41a increased chloroplast mRNA half-life in a lysed organelle assay (Bollenbach *et al.* 2003). Subsequently, the CSP41a homologue CSP41b was discovered, and null *Arabidopsis* mutants for the two proteins are viable, but have fairly subtle RNA phenotypes (Qi*, et al.* 2012). The most recent hypothesis is that CSP41 protects non-ribosome loaded mRNAs and precursor rRNAs from degradation at night, a role inconsistent with a rate-limiting RNA degradation factor.

Mutant analysis of RNase E and RNase J, described above, also fails to make a strong case for them being rate-limiting endoribonucleases, since neither mutant demonstrably overaccumulates full-length transcripts or degradation intermediates. However, they could be redundant with each other or with CSP41 in this respect. There

could also be an as-yet unidentified endoribonuclease, but there are no major bacterial enzymes that lack known chloroplast counterparts. On the other hand, some chloroplast endoribonucleolytic activities remain to be identified molecularly (Chen and Stern 1991, Liere and Link 1997). In summary, the case is still weak for endonucleolytic cleavage to be the rate-limiting step in chloroplasts.

The role of polyadenylation

It is clear from *in vitro* and *in vivo* approaches that polyadenylation can stimulate RNA decay in chloroplasts, via the enhanced affinity of PNPase for polyadenylated RNAs. Lacking, however, is clear evidence of whether polyadenylation is dispensable in chloroplasts, and how important polyadenylation is in RNA turnover. Indeed, polyadenylation was only rediscovered in *E. coli* through an indirect effect on plasmid replication (Xu *et al.* 1993). An associated complication in *E. coli* (Mohanty and Kushner 2000a), and possibly in plastids, is that PNPase can substitute for poly(A) polymerase in adding tails to RNA fragments. Evidence for a chloroplast PAP is indirect, for example poly(A) tails are still found in the *Arabidopsis* PNPase mutant (Germain*, et al.* 2011), while in contrast a *Chlamydomonas* mutant depleted for PNPase through RNAi has few residual tails (Zimmer *et al.* 2009). Identification of any chloroplast PAP necessitates distinguishing it not only from close relatives that polyadenylate nuclear and possibly mitochondrial transcripts, but also nucleotidyltransferases that add CCA tails to tRNAs during maturation. An initial investigation of this gene family in *Arabidopsis* did not identify a chloroplast PAP (Zimmer*, et al.* 2009).

Tails synthesized by PAP versus PNPase can be distinguished by their composition, with the former consisting of A's, and the latter being heterogeneous

although particularly rich in A and G, probably reflecting available NDP pools. In *Arabidopsis*, for example, tails added to *psbA* fragments include many non-A nucleotides, but *psbA* tails in the PNPase mutant are homopolymeric (Germain*, et al.* 2011). A striking characteristic of many of the long tails found in the wild-type, was that they begin as heterogeneous sequences but end with long A-tracts, hinting at a successive activities of PNPase and PAP.

Ascertaining whether polyadenylation is significant in the chloroplast may depend firstly on the identification of the PAP, and secondly on generating double mutants of PAP and PNPase. Since PNPase mutants also have RNA maturation defects, the analysis would be challenging. It is also important to consider that polyadenylated transcripts are of very low abundance in chloroplasts, with their initial analysis requiring 50 PCR cycles or prior enrichment on an oligo(dT) column (Hayes *et al.* 1996, Kudla *et al.* 1996). They are also seen only sporadically in EST databases generated from polyadenylated RNA pools. It is generally assumed that their low abundance reflects rapid turnover, but it could also reflect a minor role of polyadenylation in chloroplast RNA metabolism.

The final exonucleolytic step

The three known chloroplast exonucleases – PNPase, RNase J, and RNase II - are candidates for the ultimate degradation of RNA transcripts, at least until only short oligoribonucleotides remain, which may be substrates for a chloroplast homologue of oligoribonuclease (Ghosh and Deutscher 1999). Such fragments could be, for example, footprints of PPR protein binding (Ruwe and Schmitz-Linneweber 2012). The role of

PNPase would relate to polyadenylated sequences, possibly in competition with RNase II, which in bacteria can remove short poly(A) tails and thus stabilize transcripts (Marujo *et al.* 2000). A simple model includes successive rounds of polyadenylation and degradation that eventually allow PNPase to breach RNA secondary structures, or new 3' ends would be created within such structures by an endonuclease. The 5'→3' activity of RNase J is well established and is presumably unaffected by structure or sequence at RNA 3' ends, thus being complementary to PNPase. Indirect evidence that RNase J degrades chloroplast mRNAs comes from the analysis of *Chlamydomonas* strains where endogenous genes or transgenes have been modified to contain poly(G) sequences. In these cases, transcripts with poly(G) at their 5' ends accumulate, consistent with a 5'→3' degradative activity (Drager *et al.* 1998, Drager *et al.* 1999, Vaistij *et al.* 2000). Additional evidence for 5'→3' degradative activity in *Chlamydomonas* chloroplasts comes from the analysis of 3' UTR elements that promote degradation of downstream sequences following endonucleolytic cleavage (Hicks *et al.* 2002, Goldschmidt-Clermont *et al.* 2007). Genetic analysis of RNase J in *Chlamydomonas* is currently lacking, however, and the fate of poly(G)-tagged mRNAs in land plant chloroplasts has not been examined.

A key role for RNase II in regulating chloroplast RNA lifetime has only recently been elucidated (Germain*, et al.* 2012). In addition to its action in the maturation of rRNAs described earlier, a previous preliminary observation that certain mRNA precursors overaccumulate (Kishine*, et al.* 2004) has now been more carefully substantiated. This newer work demonstrated a considerably longer half-life for most chloroplast transcripts in the *rnr1* mutant that are not loaded on ribosomes. Thus,

RNase II may be responsible for limiting the pool of RNAs available for ribosomes, possibly in an antagonistic relationship with the abundant chloroplast RBPs of the RRM class, which seem to bind and at least in some cases, protect this same RNA pool (Nakamura *et al.* 2001, Tillich *et al.* 2009).

CONCLUSION

This review has documented the many recent advances in our understanding of the enzymes and RBPs that impact plastid RNA processing and decay. There are also a number of unanswered questions, including whether there is a quality control mechanism that ensures mRNA maturation prior to translation, how enzymes are directed to maturation vs. decay, how many factors remain undiscovered, and the relative importance of individual players.

One perspective that has been little explored and may be relevant to several of the issues highlighted above, is sublocalization within the plastid. For example, in *Chlamydomonas* the *rbcL* mRNA appears to be targeted to eventual pyrenoidal location of its gene product (Uniacke and Zerges 2009), and long ago plastids were recognized to have distinct stromal and membrane-bound populations of polysomes (Tao and Jagendorf 1973, Chua *et al.* 1976). A recent study (Majeran *et al.* 2012) casts light on the plastid nucleoid, long recognized as a DNA-protein complex with roles in DNA replication, transcription and heredity. When highly enriched maize chloroplast nucleoid fractions were compared with non-nucleoid fractions using proteomics, the results strongly suggested that splicing, editing, mRNA processing, and ribosome assembly all take place in the nucleoid (Figure 1.6).

In terms of specific factors discussed above, PNPase, RNase J, RNase E and CSP41b were highly enriched in nucleoid preparations, whereas RNase II appeared to be primarily stromal. This leads us to speculate that the basis for the unanticipated effect of *rnr1* mutants on RNA stability derives from its stromal location, where most mRNA turnover is likely to occur. The endoribonuclease L-PSP was particularly abundant in stromal fractions. L-PSP has not been analyzed in plants, but can inactivate translation in animal systems (Morishita *et al.* 1999), and could conceivably serve as the key endonuclease that initiates RNA turnover. The fact that PNPase is largely in the nucleoid suggests that its predominant role may be RNA processing rather than decay. Numerous RBPs were identified in the nucleoid, including PPRs, TPRs, HATs, as well as the chloroplast RRM proteins. This also is consistent with a model where RNA maturation and protection occur in the nucleoid, and translation and decay occur either in the stroma or after the migration of stromal RNAs to membrane translation sites.

The above mechanisms are likely to be fine-tuned in response to developmental and environmental cues, which are well-known to affect plastid gene expression. Some plastid gene regulators are also targeted to mitochondria, including one of the NEP isoforms in dicot plants, RNase II, and one of the PEP sigma factors (Beardslee *et al.* 2002). This suggests that nuclear mechanisms to coordinate organellar gene expression may exist both at the transcriptional and post-transcriptional levels.

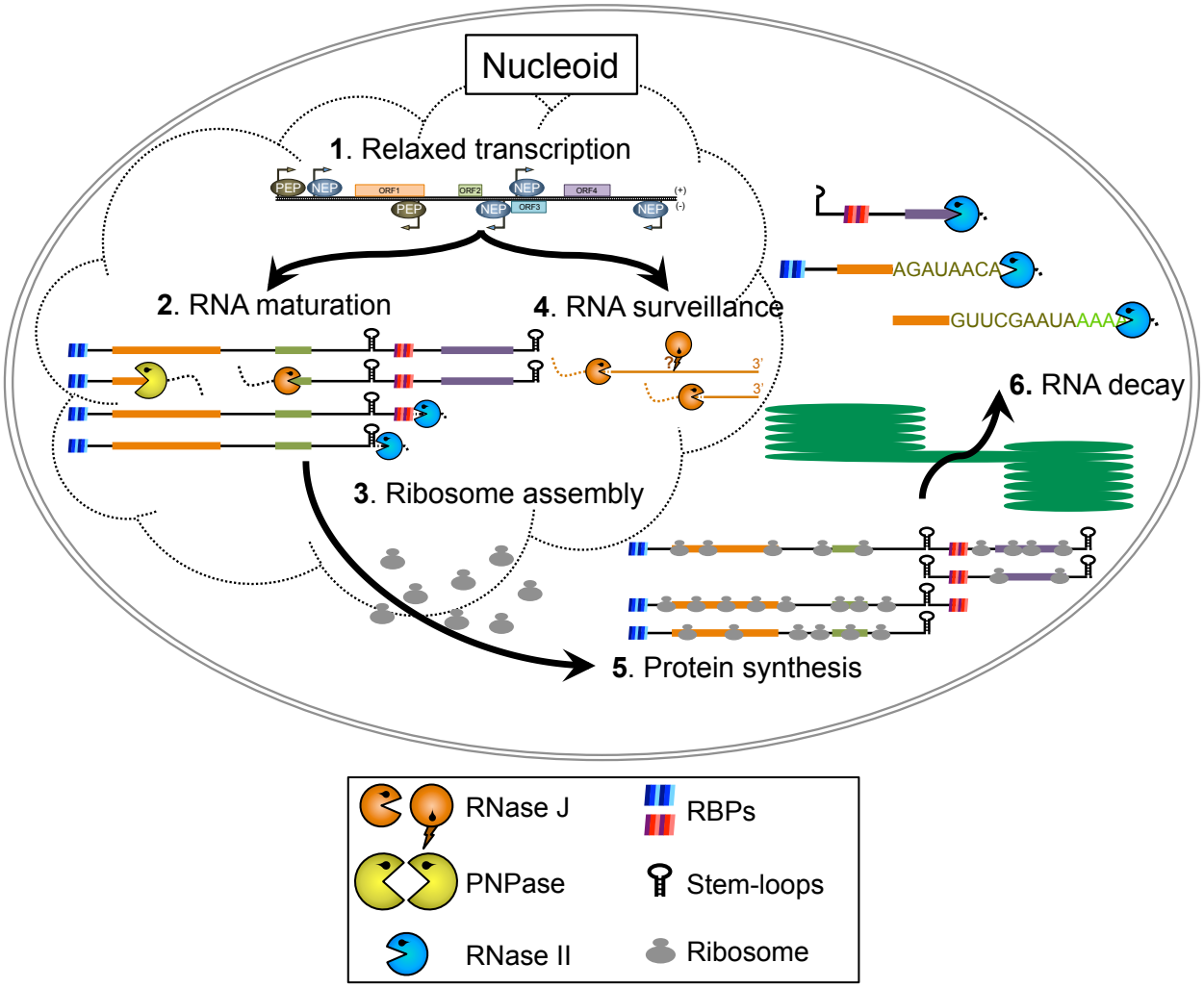

Figure 1.6: Model for the spatial coordination of transcription, RNA maturation and RNA decay.
The nucleoid, shown as a cloud, is home to transcription (1), RNA maturation (2), ribosome assembly (3), and RNA surveillance (4). Mature RNAs and assembled ribosomes exit the nucleoid, where protein synthesis (5) occurs prior to RNA decay (6). RNA decay might be initiated once the protective actions of ribosomes and RBPs discontinue because of dissociation from transcripts. The legend at the bottom denotes the identities of proteins and structures shown in the diagrams.

CHAPTER 2

MUTATIONAL ANALYSIS OF *ARABIDOPSIS* CHLOROPLAST POLYNUCLEOTIDE PHOSPHORYLASE REVEALS ROLES FOR BOTH RNASE PH CORE DOMAINS IN POLYADENYLATION, RNA 3' END MATURATION AND INTRON DEGRADATION*

ABSTRACT

Polynucleotide phosphorylase (PNPase) catalyzes RNA polymerization and 3'→5' phosphorolysis *in vitro*, but its roles in plant organelles are poorly understood. Here, we have used *in vivo* and *in vitro* mutagenesis to study *Arabidopsis* chloroplast PNPase (cpPNPase). In mutants lacking cpPNPase activity, unusual RNA patterns were broadly observed, implicating cpPNPase in rRNA and mRNA 3' end maturation, and RNA degradation. Intron-containing fragments also accumulated in mutants, and cpPNPase appears to be required for a degradation step following endonucleolytic cleavage of the excised lariat. Analysis of poly(A) tails, which destabilize chloroplast RNAs, indicated that PNPase and a poly(A) polymerase share the polymerization role in wild-type plants. We also studied two lines carrying mutations in the first PNPase core domain, which does not harbor the catalytic site. These mutants had gene-dependent and intermediate RNA phenotypes, suggesting that reduced enzyme activity differentially affects chloroplast transcripts. The interpretations of *in vivo* results were confirmed by *in vitro* analysis of recombinant enzymes, and showed that the first core

***** Germain, A., Herlich, S., Larom, S. Kim, S. H., Schuster, G. and Stern, D. B. (2011) Mutational analysis of *Arabidopsis* chloroplast polynucleotide phosphorylase reveals roles for both RNase PH core domains in polyadenylation, RNA 3' end maturation and intron degradation. *Plant J*, **67**, 381-394.

domain affects overall catalytic activity. In summary, cpPNPase has a major role in maturing mRNA and rRNA 3' ends, but also participates in RNA degradation through exonucleolytic digestion and polyadenylation. These functions depend absolutely on the catalytic site within the second duplicated RNAse PH domain, and appear to be modulated by the first RNase PH domain.

INTRODUCTION

Polynucleotide phosphorylase (PNPase) is present in organelles and many bacteria, and was first defined as a reversible polynucleotide polymerase (Grunberg-Manago and Ochoa 1955), a property shared by the spinach chloroplast enzyme (Yehudai-Resheff*, et al.* 2001). The degradative activity of PNPase is processive 3'→5' phosphorolysis, and the chloroplast enzyme is capable of trimming 3' end extensions to near the base of stem-loop structures *in vitro* (Yehudai-Resheff*, et al.* 2001). Similarly, *Arabidopsis* plants depleted for (Walter*, et al.* 2002) or lacking (Marchive *et al.* 2009) chloroplast PNPase (cpPNPase) accumulate several mRNAs as well as 23S rRNA with 3' extensions. Higher plants also encode a mitochondrial PNPase (mtPNPase), which is essential for embryo development. Depletion of mtPNPase revealed its involvement in both mRNA and rRNA maturation and degradation (Perrin *et al.* 2004a, Perrin*, et al.* 2004b). Furthermore, mtPNPase degrades noncoding RNA sequences that result from relaxed transcription (Holec *et al.* 2006).

The polymerization activity of PNPase may have two independent roles in chloroplasts. First, the addition by PNPase of poly(A)-rich tails to RNAs lacking 3'

secondary structures can stimulate 3'→5' exonucleolytic resection and ultimately RNA degradation (reviewed in Schuster and Stern 2009). Indeed, *Chlamydomonas* cells depleted for cpPNPase appear to lack chloroplast polyadenylation (Zimmer*, et al.* 2009). On the other hand, in *Arabidopsis* it was reported that depletion of cpPNPase led to an increase in poly(A) tail amount (Walter*, et al.* 2002). This suggests that higher plant chloroplasts, like bacteria, also utilize a poly(A) polymerase (PAP). Second, poly(A) tail addition to RNAs with strong secondary structures may stimulate mRNA 3' end maturation, as suggested by the cloning of polyadenylated *Chlamydomonas atpB* cDNAs with the tail added at a pre-mRNA processing site (Komine *et al.* 2000).

A number of studies have also revealed intriguing roles for PNPase in cellular functions not directly related to RNA metabolism. For example, *Chlamydomonas* cells depleted for cpPNPase cannot survive phosphorus deprivation (Yehudai-Resheff*, et al.* 2007), and the orthologous *Arabidopsis* mutant fails to elaborate lateral roots under the same growth condition (Marchive*, et al.* 2009). An *Arabidopsis* cpPNPase null mutant was also identified in a screen for mutants resistant to fosmidomycin, an inhibitor of the methylerythritol phosphate pathway (Sauret-Gueto *et al.* 2006). In human mitochondria, PNPase is found in the intermembrane space (Chen *et al.* 2006), separate from the location of mtRNA, yet its depletion somehow alters mtRNA metabolism (Slomovic and Schuster 2008). Furthermore, a recent study implicated mtPNPase in the import of small RNAs into human mitochondria (Wang *et al.* 2010). Clearly, PNPase functions in a diverse array of cellular pathways.

Although PNPase has emerged as a central player in RNA metabolism and other activities, systematic structure-function analysis of cpPNPase has been so far very

limited (Yehudai-Resheff *et al.* 2003). PNPase consists of tandem N-terminal RNase PH-like segments, known as core domains, as well as KH and S1 RNA-binding domains (Figure 2.1a). RNase PH is an exoribonuclease (Deutscher 1993), however PNPase crystal structures from *Streptomyces antibioticus* and *E. coli*, along with a comparison to the archaeal exosome, suggested that only the second core domain would harbor catalytic activity (Symmons *et al.* 2000, Lorentzen *et al.* 2005, Nurmohamed *et al.* 2009). Indeed, the initial observation of catalytic activity for the first core of spinach cpPNPase (Yehudai-Resheff*, et al.* 2003) was not supported by subsequent experiments (G.S., unpublished results). Here, we have taken a mutagenesis approach to study *Arabidopsis* cpPNPase, using both null mutants and point mutants. We find massive disruptions in transcript patterns, and a substantial accumulation of complete or partial intron lariats. We also found two mutations in the first core domain that lead to RNA abnormalities, one of which confers altered PNPase properties *in vitro*, confirming a role for this domain in PNPase catalytic activity.

RESULTS

Phenotypes of *PNPase* T-DNA insertion and single amino acid mutants

To study cpPNPase function, we used both the previously-described T-DNA insertion mutants *pnp1-1* and *pnp1-2* (Marchive*, et al.* 2009), and eleven additional point (TILLING) mutants (Till *et al.* 2003). One of the TILLING mutants was shown to generate a premature stop codon at position 600 (Figure 2.1a), and was designated *pnp1-3*. The other 10 point mutations, five in each core domain, all caused amino acid

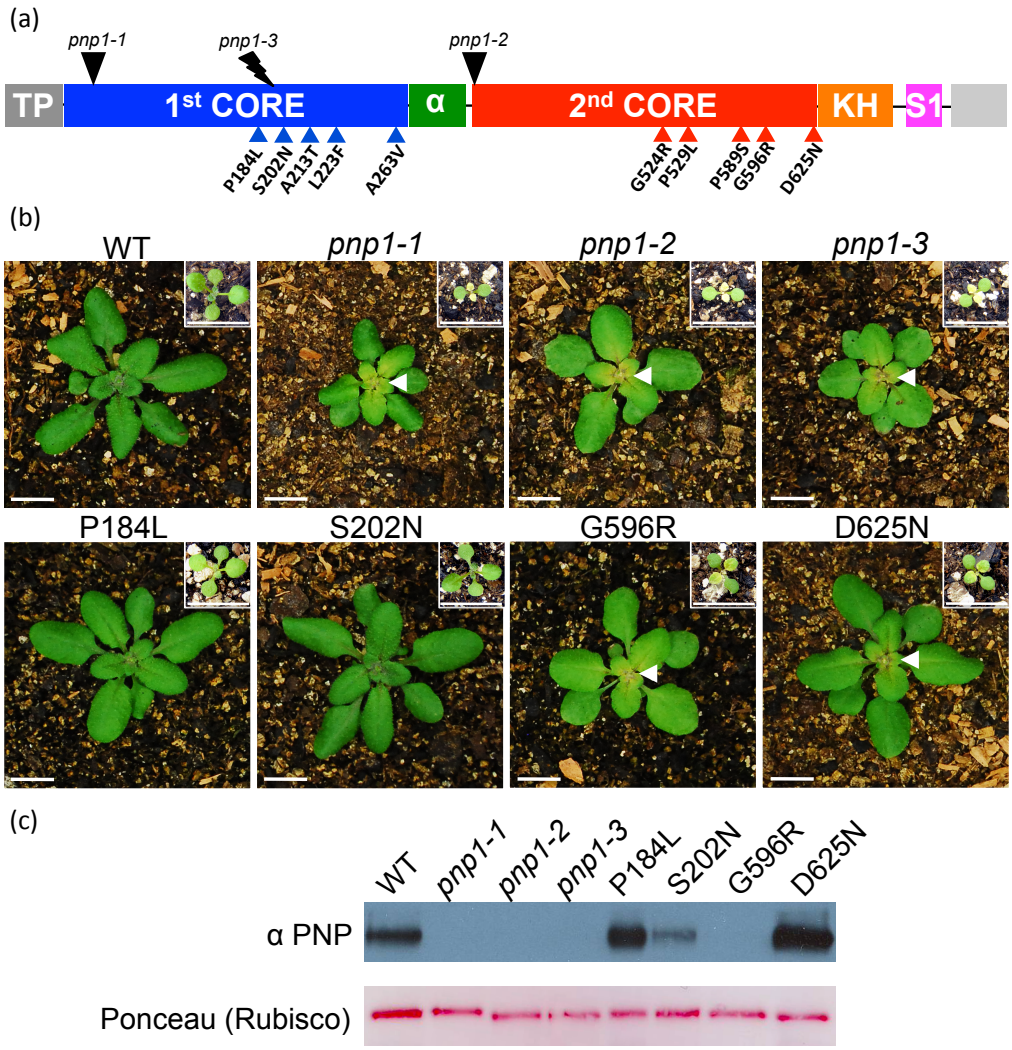

Figure 2.1: Identification of *Arabidopsis pnp* mutants.
(a) Domain structure of *Arabidopsis* cpPNP. TP indicates the chloroplast transit peptide, α represents the α-helical domain and the gray C-terminal domain is an extension unique to cpPNP. Black arrowheads indicate T-DNA insertions (*pnp1-1* and *pnp1-2*), the black lightning bolt indicates a premature translation termination codon caused by a single nucleotide substitution at a splice junction (*pnp1-3*), and the blue and red arrowheads indicate single amino acid substitutions resulting in amino acid changes in the first and the second core domains, respectively.
(b) Phenotypes of a Col-0 wild-type plant (WT) and selected mutants. The white arrowheads point to the pale green emerging leaves. The white bar represents 1 cm.
(c) Immunoblot analysis using a cpPNPase-specific antibody on plastid soluble protein-enriched samples from the WT and mutants. Samples were loaded in a Blue Native gel based on protein concentration, as reflected in Ponceau-S staining of the membrane.

alterations, and were selected based on the conservation of that amino acid amongst chloroplast, mitochondrial and bacterial PNPases (see Figure 7 in Yehudai-Resheff*, et al.* 2003).

Figure 2.1b only shows images of mutants that had growth and/or RNA abnormalities, as is the case in subsequent figures. The null mutants (*pnp1-1 – pnp1-3*) as well as G596R and D625N have similar growth phenotypes, in that their first true leaves and subsequent new leaves emerge pale green (Figure 2.1b), but recover as they mature. Growth of two other mutants that were later found to have milder RNA phenotypes, P184L and S202N, did not differ from the WT.

PNPase accumulation was monitored by Blue Native gel electrophoresis and immunoblot (Figure 2.1c), revealing a signal at 600 kDa, the expected size for the PNPase hexamer (Baginsky*, et al.* 2001), and confirming its absence in the three null mutants. Of the TILLING mutants, D625N appeared to overaccumulate PNPase, P184L was comparable to the WT, S202N slightly lower than the WT, and for G596R, no signal could be detected in either native or denaturing gels. Because qRT-PCR showed that the G596R *PNP* transcript accumulated to about 40% of the WT level (data not shown), we conclude that this substitution compromises PNPase stability.

Both PNPase domains contribute to chloroplast rRNA and mRNA processing

To confirm earlier findings for cpPNPase-depleted plants, and extend them to the point mutants studied here, total RNA was first examined by gel electrophoresis and ethidium bromide staining, revealing a reduced mobility for one of the fragments comprising chloroplast 23S rRNA for the three null mutants, as well as G596R and

D625N (Figure S2.1). This is due to defective exonucleolytic trimming of ~100 nt of the intergenic region between 23S and 4.5S rRNA (Walter*, et al.* 2002). Because G596R lacks cpPNPase (Figure 2.1c), and because D625N resembled *pnp1-1* to *pnp1-3* in this and all subsequent experiments, the five mutants will henceforth be referred to collectively as the null mutants. An intermediate phenotype was observed for P184L, in which a conserved residue located in the phosphorolytically-inactive first core domain is altered. In this case, a mixture of mature and 3'-extended 23S rRNA was observed. Thus, with respect to 23S rRNA maturation, P184L is a weak *pnp* mutant allele; the other mutants appeared as the WT.

We also confirmed (Figure S2.2a) previously published 3' extensions for the monocistronic *rbcL* and *psbA* transcripts (Marchive*, et al.* 2009), which in the case of *rbcL* was verified by gel blot (Figure S2.2b). Again, a mixture of the WT and null mutant pattern was observed for P184L. In addition, we examined the dicistronic *atpB-atpE* gene cluster (Figure S2.2c), whose slightly more complex transcript pattern derives from the presence of two promoters as well as processing to yield *atpE*-specific species (Schweer *et al.* 2006). Again, the pattern was interpretable as 3' end extensions although in this case, P184L appeared as the WT. This suggests that *atpBE* 3' end maturation is less sensitive to the amount of cpPNPase activity.

Analysis of polycistronic gene clusters reveals gene-dependent patterns for both coding and intronic regions

The *Arabidopsis* chloroplast genome encodes several intron-containing polycistronic gene clusters. We analyzed two such clusters, namely those encoding

atpI/*atpH*/*atpF*/*atpA* and *psbB*/*psbT*/*psbN*/*psbH*/*petB*/*petD*. For the *atpI* gene cluster (Figure 2.2), a molecular phenotype common to null mutants for all five probes used is the presence of non-discrete high molecular transcripts, that correlate with the diminution of most of the corresponding discrete bands (Figure 2.2b). These may represent imprecise 3' end formation rather than transcript instability. Indeed, qRT-PCR analysis of the *atpI* coding region showed no difference between the WT and *pnp1-1* (data not shown), although only the band marked "1" remains discrete and unchanged. Even though we did not quantify the *atpA* or *atpF* coding region transcripts, the gel patterns are consistent with a similar conclusion; i.e. that no significant accumulation differences occur in the null mutant backgrounds.

Also of note were four novel bands observed for *atpH* (marked 2-5). Bands 2 and 5 were present only in the null mutants, band 3 was prominent in the null mutants and P184L, and present weakly in another mutant of the first core domain, S202N. Band 4 was present in all mutants, but most strongly in the weak allele P184L. One possible interpretation could be that bands 2-5 represent the products of a succession of endonucleolytic cleavages between *atpH* and *atpF*, that are subsequently trimmed by PNPase. This would be consistent with a recently-proposed model for chloroplast polycistronic RNA processing (Pfalz *et al.* 2009). Their reduced intensities in the weak alleles probably reflect the presence of residual cpPNPase activity in those strains. In the case of band 4, however, the residual activity increases its abundance perhaps, for example, by trimming band 2 or band 3. The precise 3' ends of *atpH* transcripts were compared between the WT and *pnp1-1* using circular RT-PCR (Figure 2.2c). WT transcripts had two main 3' ends. The end nearest the coding region was well

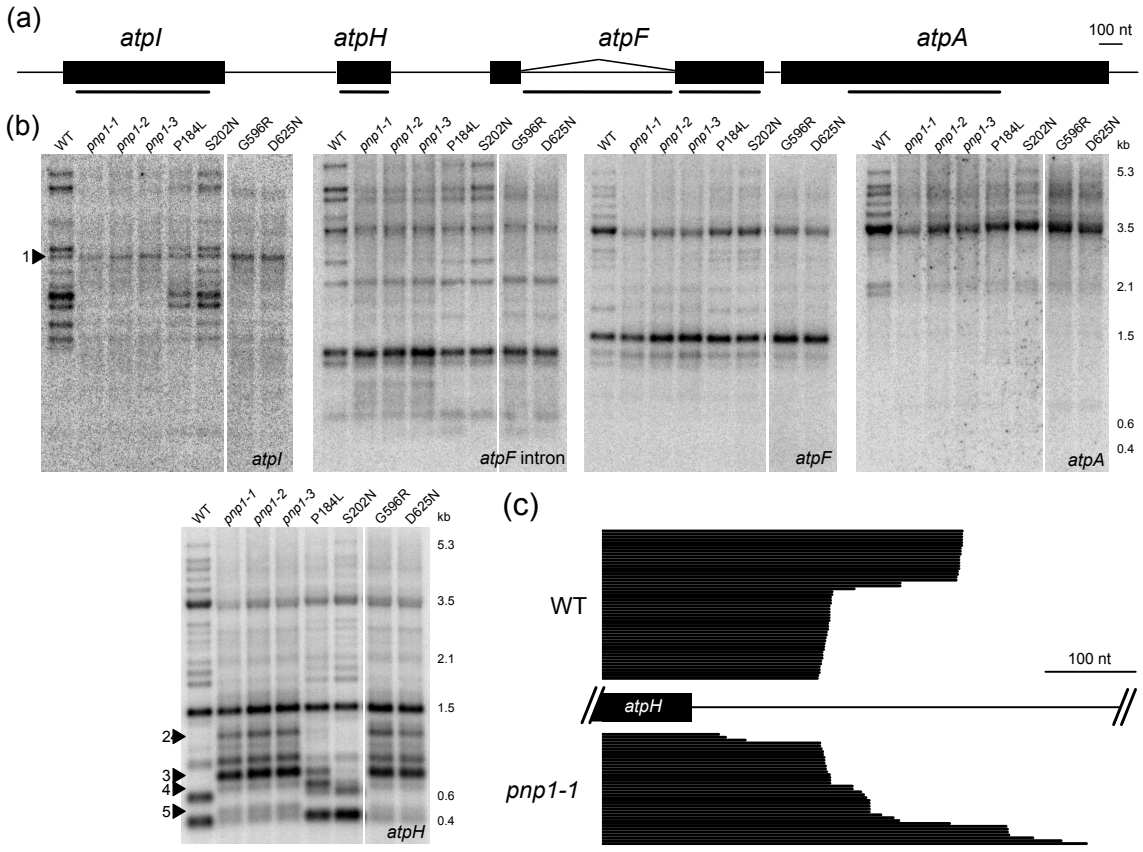

Figure 2.2: RNA gel blot analysis for the *atpI*/*atpH*/*atpF*/*atpA* gene cluster.
(a) Diagram of the *atpI*/*atpH*/*atpF*/*atpA* gene cluster and locations of the probes used for RNA gel blot analysis (black bars).
(b) Results for the five specific probes as indicated at the bottom right of each panel.The numbered arrowheads refer to specific transcripts discussed in the text.
(c) Schematic of cRT-PCR results showing *atpH* 3' ends for WT and *pnp1-1*, with each horizontal line corresponding to a single clone. The number of clones sequenced for WT and *pnp1-1* was 52 and 39, respectively.

represented in *pnp1-1*, suggesting it might be generated by transcription termination. The *pnp1-1* mutant also accumulated a series of extended 3' ends, as suggested by the gel blot, and in keeping with the possibility that they are endonucleolytic cleavage products.difference between the WT and *pnp1-1* (data not shown), although only the band marked "1" remains discrete and unchanged. Even though we did not quantify the *atpA* or *atpF* coding region transcripts, the gel patterns are consistent with a similar conclusion; i.e. that no significant accumulation differences occur in the null mutant backgrounds.

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gel blot, and in keeping with the possibility that they are endonucleolytic cleavage products.

In the *psbB* gene cluster (Figure 2.3), two genes (*petB* and *petD*) contain introns, while *psbN* is expressed from its own promoter on the opposite strand. As shown in Figure 2.3b, the various coding region probes, with the exception of *psbN*, revealed patterns that while more complex, were reminiscent of other genes described above, with the null mutants showing stronger effects, and P184L and S202N intermediate phenotypes. On the other hand, transcripts appeared to be less diffuse than in the case of the *atpI* cluster (Figure 2.2), suggesting that their 3' ends might be more homogeneous. We checked this using *petD* as an example (Figure 2.3c), and found that although it had multiple 3' ends, they were indeed less dispersed than for *atpH* (Figure 2.2c). In the case of *psbN*, the null mutants were nearly depleted for the transcript (Figure 2.3b) which, however, is dispensable for normal PSII function (Zghidi *et al.* 2007). In addition, for *psbB* a band shorter than any WT transcript was observed (band 1), which we speculate is a degradation intermediate. The hybridization patterns for the intron probes are discussed in the next section.

Ultimately, a variety of other transcripts examined in the PNPase mutants showed a range of effects, including apparent 3' end extensions, accumulation of putative degradation intermediates, and instances where no transcript alterations were detected. Also, several other intron probes revealed mutant-specific bands possibly analogous to the one studied in detail below for *petD*. These data are presented in Figure S2.3.

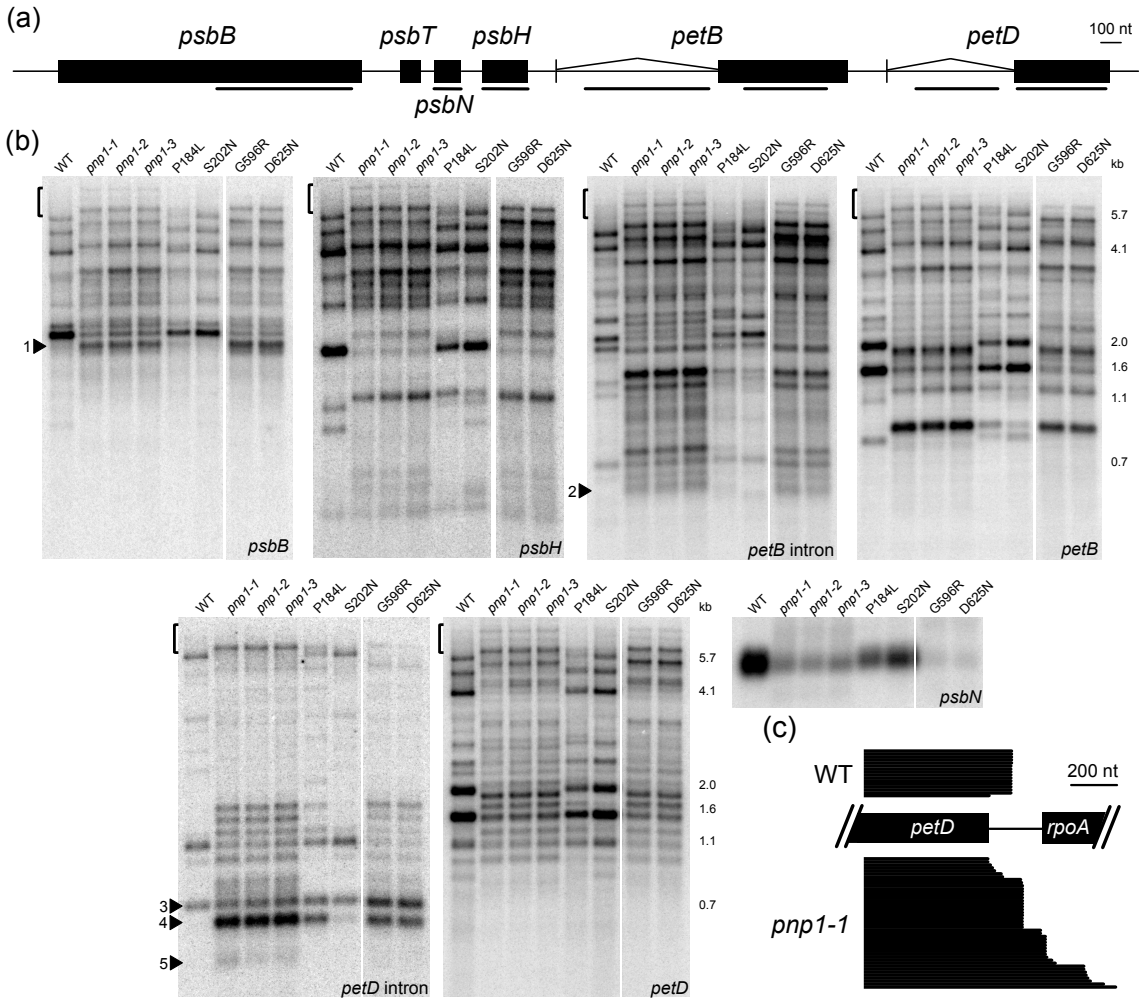


Figure 2.3: RNA gel blot analysis for the *psbB*/*psbT*/*psbN*/*psbH*/*petB*/*petD* gene cluster.

(a) Diagram of the *psbB*/*psbT*/*psbN*/*psbH*/*petB*/*petD* gene cluster and locations of the probes used for RNA gel blot analysis (black bars).

(b) Results for the seven specific probes as indicated at the bottom right of each panel.The numbered arrowheads refer to specific transcripts discussed in the text. Note that *psbN* is on the opposite strand.

(c) Schematic of cRT-PCR results showing *petD* 3' ends for WT and *pnp1-1*, with each horizontal line corresponding to a single clone. The number of clones sequenced for WT and *pnp1-1* was 16 and 44, respectively.

PNPase depletion decreases splicing efficiency and inhibits intron degradation

We were particularly intrigued by the hybridization patterns for the *petB* and *petD* introns (Figure 2.3b), where bands shorter than the complete excised introns (bands 2 and 4) were detected, these being band 3 for the *petB* intron and bands 5 and 6 for the *petD* intron. Given these results, we suspected that *pnp* mutants had a defect in some aspect of intron removal or degradation. To measure splicing efficiency, we used poisoned primer extension, and found that a 1.5 to two-fold change in the ratio of spliced to unspliced RNA occurs in all the null mutants (Figure 2.4a). This relatively small reduction in splicing, however, would not appear to explain the high accumulation of intron-containing fragments.

The introns discussed here belong to Group II, whose splicing mechanism entails lariat formation (reviewed in Stern*, et al.* 2010). The amount of the lariat junction can be measured by qRT-PCR using two primers pointing towards the junction, and Figure 2.4b shows results for the amount of lariat junction-containing *petD* intron fragments in the WT and null mutants. While there was some variability, each mutant accumulates four to eight-fold more lariat junctions than the WT. This suggests that the *pnp*-specific intron-containing bands 5 and 6 in Figure 2.3b include the lariat junction, and because they are shorter than the full-length excised intron (band 4), represent intron degradation intermediates.

We subsequently decided to focus on band 5, the most abundant novel intron-containing band in the null mutants. To map its ends we used circular RT-PCR (cRT-PCR). As shown in the Figure 2.5 inset, samples not treated with T4 RNA ligase gave rise to amplification of the intact intron (open arrowhead), as expected. However, ligated

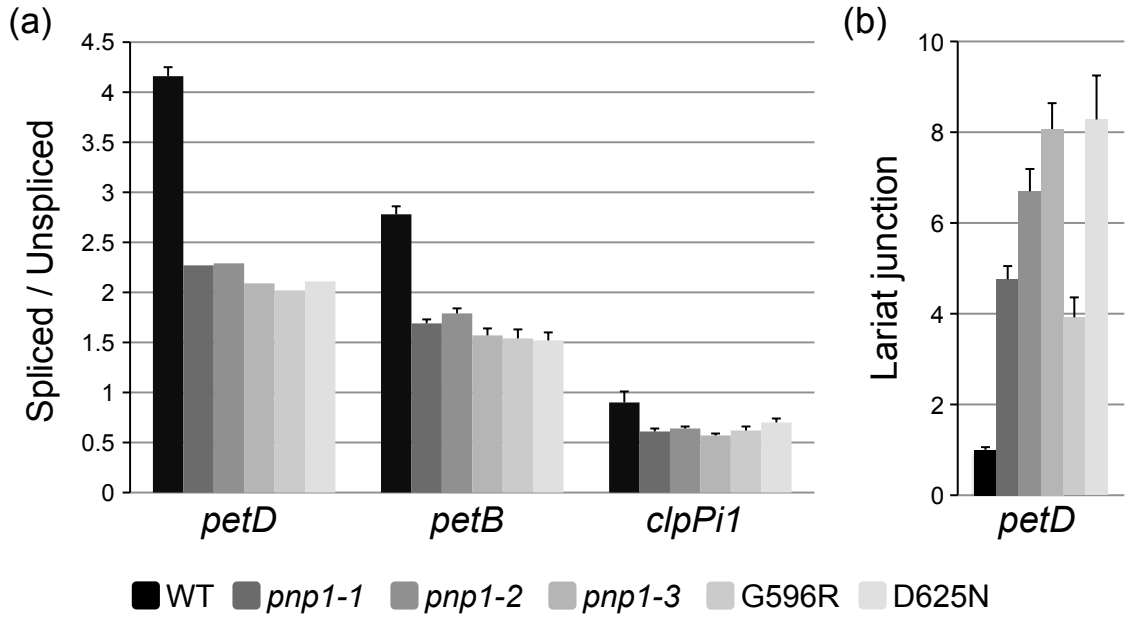


Figure 2.4: Effects of PNP depletion on intron metabolism.
(a) Ratio of spliced to unspliced transcripts for *petD*, *petB* and *clpP* intron 1, as measured by Poisoned Primer Extension. Values shown for *petD* are the average of 2 experiments for the WT, and single samples for each of the null mutants. For *petB* and *clpPi1*, 4 samples for each mutant and 8 WT samples were analyzed. Standard errors are shown.
(b) Relative amounts of *petD* intron fragments containing the lariat junction, measured by qRT-PCR. The results were normalized to actin, and standard error is shown. The data are derived from 5 biological replicates for each genotype.

samples generated additional bands (full arrowheads), with a major *pnp1-1* product absent from the WT sample, suggesting that it might represent the gel band of interest from Figure 2.3b. A faint and smaller band was amplified in the WT. Both products were cloned and sequenced and although their ends were variable (see below), the WT and *pnp1-1* products lacked approximately 240 nt and 200 nt, respectively, in comparison to the 709 nt full-length intron.

Figure 2.5 shows a model of the *petD* intron upon which the 5' and 3' ends of cRT-PCR products are displayed. The cloned 5' ends were similar in the WT and *pnp1-1*, with most being clustered in a 6 nt stretch in the first stem of Domain I, and most of the remaining ones in the loop of Domain II. These 5' ends may represent sites of endonucleolytic cleavage, and would be expected to be impervious to PNPase activity, which has strict 3'→5' polarity. In contrast, the WT and mutant had distinct clusters of 3' ends, both located in Domain I. These were in the exon binding domain 2 (EBS2) for *pnp1-1*, and in a Domain I stem for the WT. These two clusters are separated by approximately 40 nt, which is consistent with the size difference of the cloned PCR products. The simplest interpretation is that PNPase normally completes degradation of the linearized intron, while occasionally stalling at the location marked by 3' ends in the WT. On the other hand, PNPase seems to be required to degrade the intron sequences starting at EBS2.

Only homopolymeric poly(A) tails are detected in the *pnp1-1* mutant

While investigating the 3' and 5' ends of the *petD* intron intermediate degradation products, we found three short non-encoded adenosine tails, as had previously been

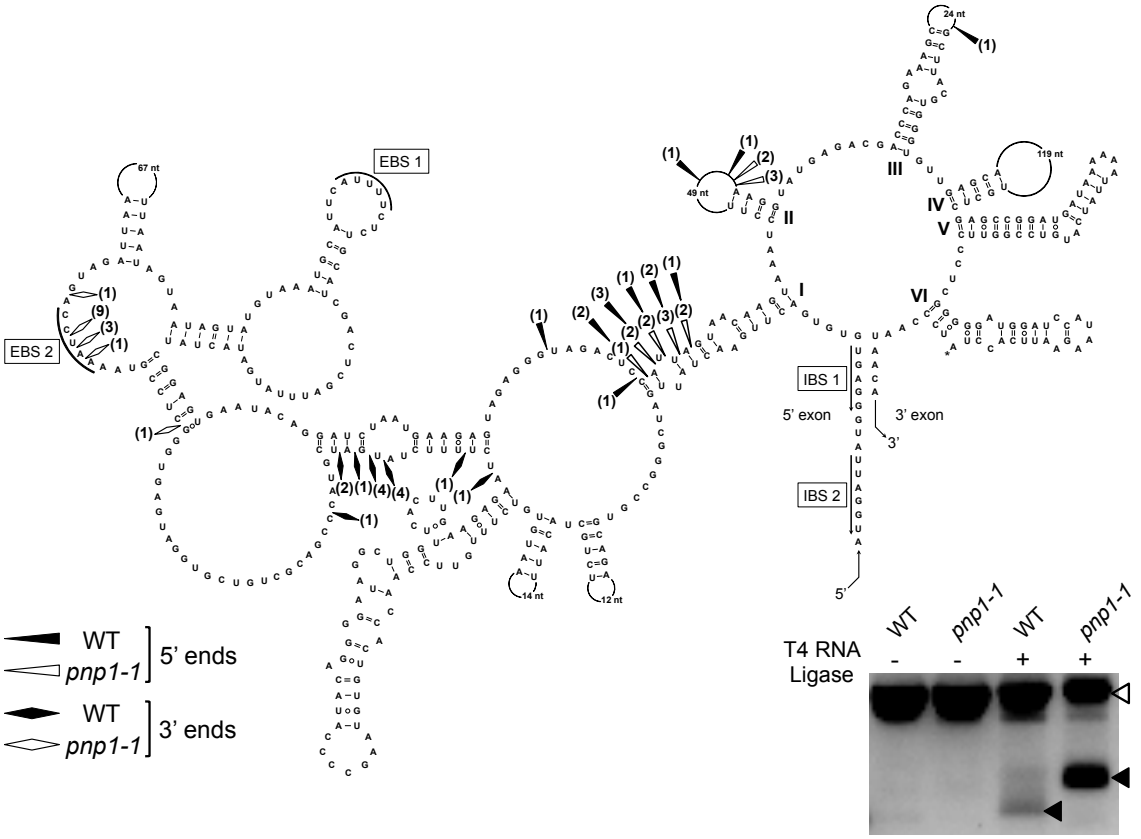


Figure 2.5: Intermediate degradation products of the *petD* intron.
Secondary structure of the *Arabidopsis petD* intron, based on Michel *et al.* (1989) EBS and IBS indicate exon and intron binding sites, respectively, and the Roman numerals I to VI denote particular intron domains. In the schematic, 5' ends are represented by triangles and the 3' ends by diamonds, with the WT and *pnp1-1* ends as filled and open symbols, respectively (see key at lower left). The numbers in parentheses correspond to the numbers of clones representing a particular end, out of 14 for the WT and 15 for *pnp1-1*. The 5' and 3' ends of the intermediates were mapped by sequencing cRT-PCR products (gel at lower right), with filled arrowheads indicating the products that were cloned and sequenced. The open arrowhead marks the intact intron, whose amplification is ligase-independent.

found for a small proportion of intron degradation products from other chloroplast genes (del Campo and Casano 2008). The presence of these tails suggests that the *pnp1-1* mutant retains polyadenylation activity. To extend this analysis, we investigated the composition of poly(A) tails in the WT and *pnp1-1* using oligo(dT)-primed RT-PCR. As shown in Table 2.1, both homo- and heteropolymeric tails were found for the *psbA* mRNA in the WT, while only homopolymeric A-tails were present in *pnp1-1*. The heteropolymeric tails in bacteria and chloroplasts are generated by PNPase, while the homopolymeric tails are produced by poly(A) polymerase (PAP) (Slomovic *et al.* 2008b). Therefore, the difference in composition suggests that in *Arabidopsis* chloroplasts, PNPase significantly contributes to the synthesis of heterogeneous poly(A) tails, whereas in its absence PAP activity creates homogeneous poly(A) tails. Similar features of polyadenylation by PAP and PNPase were initially described in *E. coli* (Mohanty and Kushner 2000a). Also of note was the fact that while the *psbA* 3' end tails were longer in the WT compared to the mutant, 60% of the tails present in WT were heterogeneous at their 5' ends, but terminated in 49-150 nt stretches of adenosines, suggesting that they may have been initiated by PNPase activity and elongated by PAP. The three 23S rRNA tails found from the WT were homogeneous poly(A), as were most of the tails from 16S rRNA. This contrasted with the rather heterogeneous tails of the *psbA* mRNA.

Arabidopsis cpPNPase *in vitro* activity is altered by point mutations

Data presented above for the TILLING mutants suggest that G596R and D625N lack activity, whereas P184L and S202N have diminished activity. The results for G596R were ambiguous, however, because the protein failed to accumulate in mutant

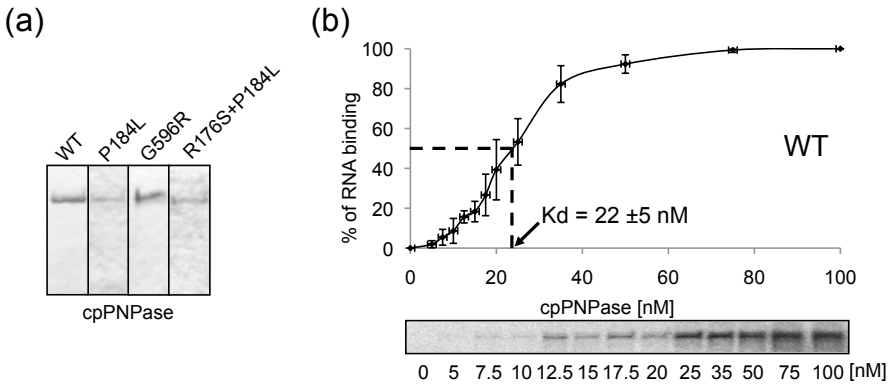
Gene	Genotype	Tail Length	Tail Sequence	Position
psbA	WT	20-22	$\mathbf{A_{20\text{-}22}}$	721
		41	$\mathbf{UA_{31}G_2A_7}$	751
		74	$\mathbf{CAU_2GA_3UGAUCGAU_2GU_2G(UA)_2CUG_2(UC)_2U_3ACAU_2A_2UA_2U_5AC_2U_3AGC_2U_2A_{11}}$	735
		81	$\mathbf{CUCAUC(CAU)_2A_3CA_2CU_2AC_2AGAU_2ACUA_{49}}$	800
		113	$\mathbf{UCAUCU_4CU_2A_{101}}$	753
		114	$\mathbf{A_{39}GA_{74}}$	733
		129	$\mathbf{UGAC_2GUAUG_2U_3AC_2A_2U_2GC_2AGUACA_2UA_{97}}$	729
		152	$\mathbf{GGA_{150}}$	725
	pnp1-1	9-11	$\mathbf{A_{9\text{-}11}}$	724
		11	$\mathbf{A_{11}}$	681, 751, 759
		12-13	$\mathbf{A_{12\text{-}13}}$	721
		13	$\mathbf{A_{13}}$	758
		16-19	$\mathbf{A_{16\text{-}19}}$	732
		19	$\mathbf{A_{19}}$	754
		20-22	$\mathbf{A_{20\text{-}22}}$	721
		22	$\mathbf{A_{22}}$	723
		23-25	$\mathbf{A_{23\text{-}25}}$	721
		28-29	$\mathbf{A_{28\text{-}29}}$	815
23S rRNA	WT	10	$\mathbf{A_{10}}$	2176
		13	$\mathbf{A_{13}}$	1926, 2178
16S rRNA	WT	11	$\mathbf{A_2GA_8}$	1346
		11	$\mathbf{A_{11}}$	1439
		12	$\mathbf{CA_{11}}$	1339
		14	$\mathbf{A_{14}}$	1358
		17	$\mathbf{A_{17}}$	1336
	pnp1-1	12	$\mathbf{A_{12}}$	1325

Table 2.1: Poly(A) and poly(A)-rich sequences from WT and *pnp1-1*.
RNA was used for oligo(dT)-primed RT-PCR for the indicated genes as described in Materials and Methods. The position of tail addition refers to Genbank accession number NC_000932.

tissue (Figure 2.1c). To support and extend these interpretations, we undertook an *in vitro* analysis of the WT enzyme and three point mutants, namely P184L, G596R, and a double mutant R176S-P184L. The double mutant was obtained fortuitously, but allowed us to define an important role for this region of the protein.

To avoid contamination by *E. coli* PNPase, the chloroplast proteins were expressed in bacterial *pnp* mutant cells, and purified to homogeneity (Figure 2.6a). We first tested the RNA-binding properties of these enzymes by UV-crosslinking. As shown in Figure 2.6b, the WT enzyme displayed a dissociation constant (K_d) of 22 nM, in the range of the 11 nM and 16.5 nM values for the *E. coli and* human mitochondrial enzymes (Portnoy *et al.* 2008). Competition with polynucleotides showed that as previously reported for spinach cpPNPase (Yehudai-Resheff*, et al.* 2003), the *Arabidopsis* enzyme has a higher affinity for poly(U) and poly(A) than for poly(G) and poly(C) (data not shown). When the mutant enzymes were tested, P184L did not differ from the WT, R176S-P184L had a K_d value of 60 nM, and the G596R enzyme had no detectable RNA binding activity (Figure 2.6b).

As mentioned previously, cpPNPase is readily reversible *in vitro*; its degradation activity is stimulated by Pi addition, whereas polymerization occurs in the presence of NDPs. Figure 2.7 compares these activities for the WT and mutant enzymes. While the WT enzyme had robust polyadenylation and degradation activities (Figure 2.7a), G596R lacked both, consistent with its failure to detectably bind RNA *in vitro* (Figure 2.7b). P184L had reduced activity in both cases, as evidenced by the requirement for a higher Pi concentration to stimulate degradation, and lower production of poly(A) tails over time (Figure 2.7c). When the R176S-P184L double mutant was checked it had neither



PNPase	Kd [nM]
WT	22 ±5
P184L	22 ±5
G596R	> 100
R176S+P184L	60 ±10
Human	16.5
E. coli	11

Figure 2.6: RNA binding properties of WT and mutant *Arabidopsis* cpPNPase.
(a) SDS-PAGE analysis of purified recombinant WT cpPNPase.
(b) Left, 10 ng WT cpPNPase was analyzed for RNA binding by UV crosslinking using the 3' end [^{32}P]-*psbA* RNA and increasing amounts of the protein as indicated in the figure *inset*. The radioactive signals obtained in three independent experiments were averaged and plotted to determine the observed K_d. The K_d values for the WT and mutants are shown in the table to the right. Those for human and *E. coli* PNPases are taken from Portnoy et al. (2008).

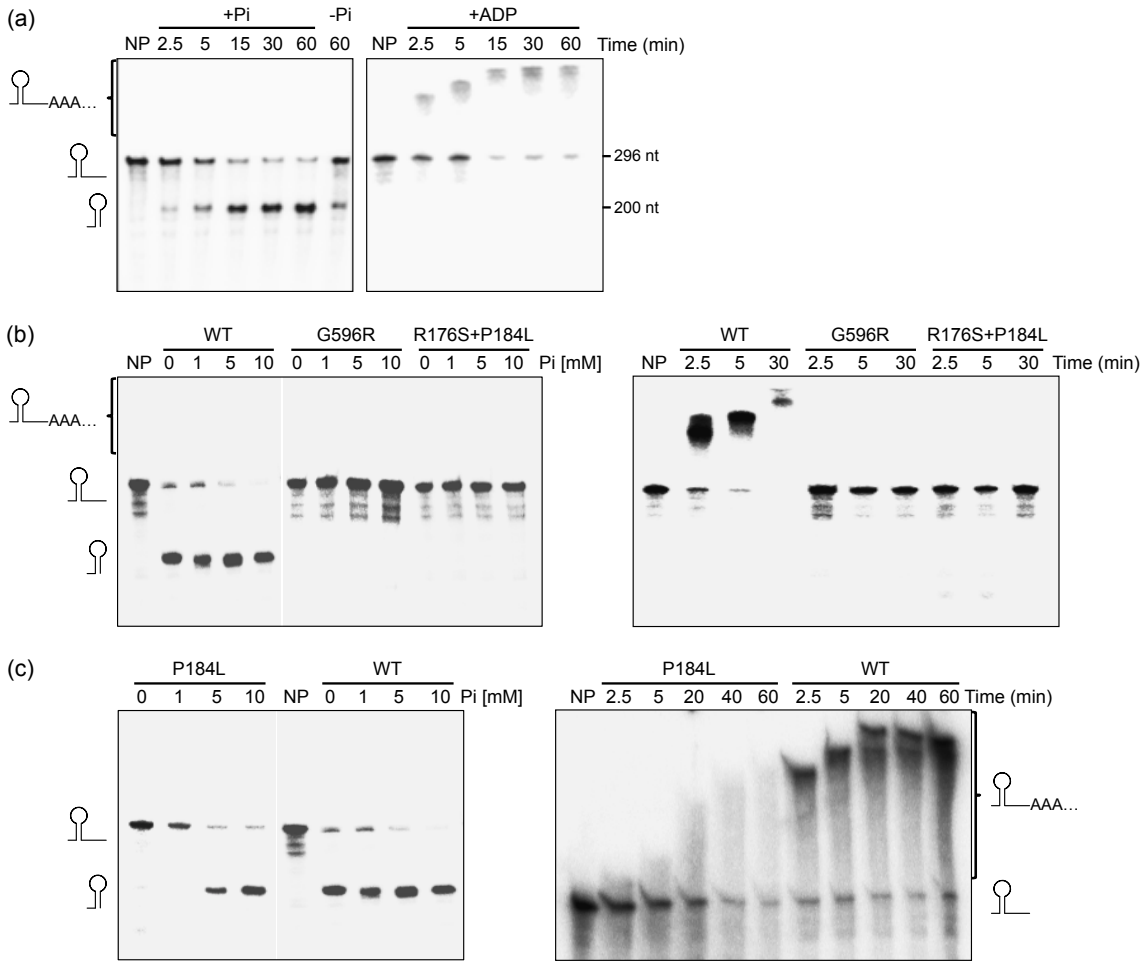


Figure 2.7: Analysis of recombinant cpPNPase for phosphorolytic and polymerization activities.

(a) A [^{32}P]-RNA of 296 nt corresponding to the 3' end of the spinach *psbA* gene was used to analyze the phosphorlysis (left) and polymerization (right) activities of recombinant WT cpPNPase (50 ng for degradation and 100 ng for polymerization), in the presence of 5 mM phosphate or 2 mM ADP, respectively. After incubation, RNA was purified and analyzed by denaturing PAGE and imaging. NP - no protein added to the reaction; -Pi, neither phosphate nor ADP were added and the incubation time was 60 min. The RNA molecules are schematically presented to the left and their lengths in nt are shown at the right.

(b) Analysis as in panel (a), except the WT and two mutant enzymes were compared. Phosphorolysis activity (left) was analyzed by incubating the input RNA for 40 min with increasing Pi concentrations as shown; a time course was used for the polymerization assay (right).

(c) Analysis as in panel (b), except the WT and P184L enzymes were compared.

activity, suggesting that its weak RNA binding ability was insufficient to support catalysis under our experimental conditions (Figure 2.7b).

The significant effects of amino acid substitutions in the first core domain were somewhat unexpected, since it does not harbor the phosphorolytic site. In addition, while the intermediate catalytic activity of P184L was consistent with the RNA phenotypes observed by RNA gel blot analysis, the fact that the enzyme appeared to have normal RNA binding was counterintuitive. To explore this issue, we determined the quaternary structure of the recombinant enzymes. PNPase in *E. coli* is trimeric (Nurmohamed*, et al.* 2009), while spinach cpPNPase is most likely a dimer of trimers (Baginsky*, et al.* 2001). When the WT *Arabidopsis* enzyme was examined by standard or blue native gel electrophoresis, a hexameric form was identified (Figures S2.4a and 2.1c). This was also the case for the P184L and the R176S-P184L mutants, suggesting that their reduced catalytic activities could not be ascribed to altered quaternary structure. When the G596R mutant was analyzed, it migrated as a smear, suggesting a lack of discrete structure. The results for all enzymes were subsequently verified using column chromatography (Figure S2.4b). Therefore, a single amino acid change (G596R) can interfere with the ability to form the correct oligomeric structure, however the two first core domain mutants with altered activities appeared to assemble hexamers normally.

DISCUSSION

The many roles of chloroplast PNPase

Here we have used a variety of null mutants to dissect the roles of cpPNPase in RNA metabolism, supplemented by the analysis of recombinant proteins. Because the recombinant protein analyses are in agreement with the degree of disturbance for the RNA patterns, it is very likely that the observed RNA phenotypes are a direct consequence of reduced or abolished PNPase activity. The null mutants studied here also exhibit chlorosis in young leaves. An initial study of cpPNPase function relied on a cosuppressed line largely depleted for the enzyme, which did not express an obvious growth defect (Walter*, et al.* 2002), suggesting that even small amounts of cpPNPase are sufficient to confer a normal growth phenotype in *Arabidopsis*. Two TILLING mutants in the present study also have normal growth but display multiple RNA abnormalities. This leaves open the question of the proximal cause of young leaf chlorosis in *pnp* null mutants. Given that chloroplast gene expression is generally higher in young vs. mature leaf chloroplasts (Baumgartner *et al.* 1989), we suspect the chlorosis is a threshold effect related to RNA maturation rates or possibly translational efficiency. Indeed, several RNA phenotypes were much more pronounced in young, as compared to mature mutant leaves (Figure S2.5).

Our results reveal a remarkable number of gene expression steps in which PNPase can be implicated (Figure 2.8). These include 3' end maturation (a and b), polynucleotide tail addition (c), tail-stimulated RNA degradation (d), and degradation of specific segments of linearized intron lariats (e and f). While many of these functions

had previously been attributed to PNPase in various organisms, the breadth of its activities in a single context were not previously apparent. Clearly, PNPase is a central player in cpRNA metabolism.

Both mRNAs and rRNAs depend on cpPNPase for correct maturation and degradation

One hallmark of cpPNPase mutant patterns is the accumulation of longer and more heterogeneous transcripts than the WT. Because chloroplast transcription termination is inefficient (Stern and Gruissem 1987), 3' end maturation requires post-transcriptional enzyme activity. cpPNPase was identified as an enzyme capable of catalyzing the 3' to 5' trimming of artificial precursors *in vitro* (Hayes*, et al.* 1996), and appears to partner with the exoribonuclease RNR1 to trim the 3' end of 23S rRNA (Bollenbach*, et al.* 2005). Thus, in the case of monocistronic transcripts, the accumulating species may represent the products of stochastic termination followed by RNR1 trimming, or in some cases such as 23S rRNA, initial endonucleolytic cleavages at downstream tRNA or rRNA processing sites, followed by trimming. RNR1 belongs to the RNase II family whose members, like PNPase, are inhibited by strong RNA secondary structures (Spickler and Mackie 2000). This or an RNA-binding protein (Figure 2.8b) may provide the basis for defining these transcript ends, or some may be generated directly by transcription termination.

A similar rationale cannot readily explain the numerous transcript abnormalities for the more complex gene clusters. A recently proposed model for transcript maturation in these contexts evokes endonucleolytic cleavages between cistrons, followed by

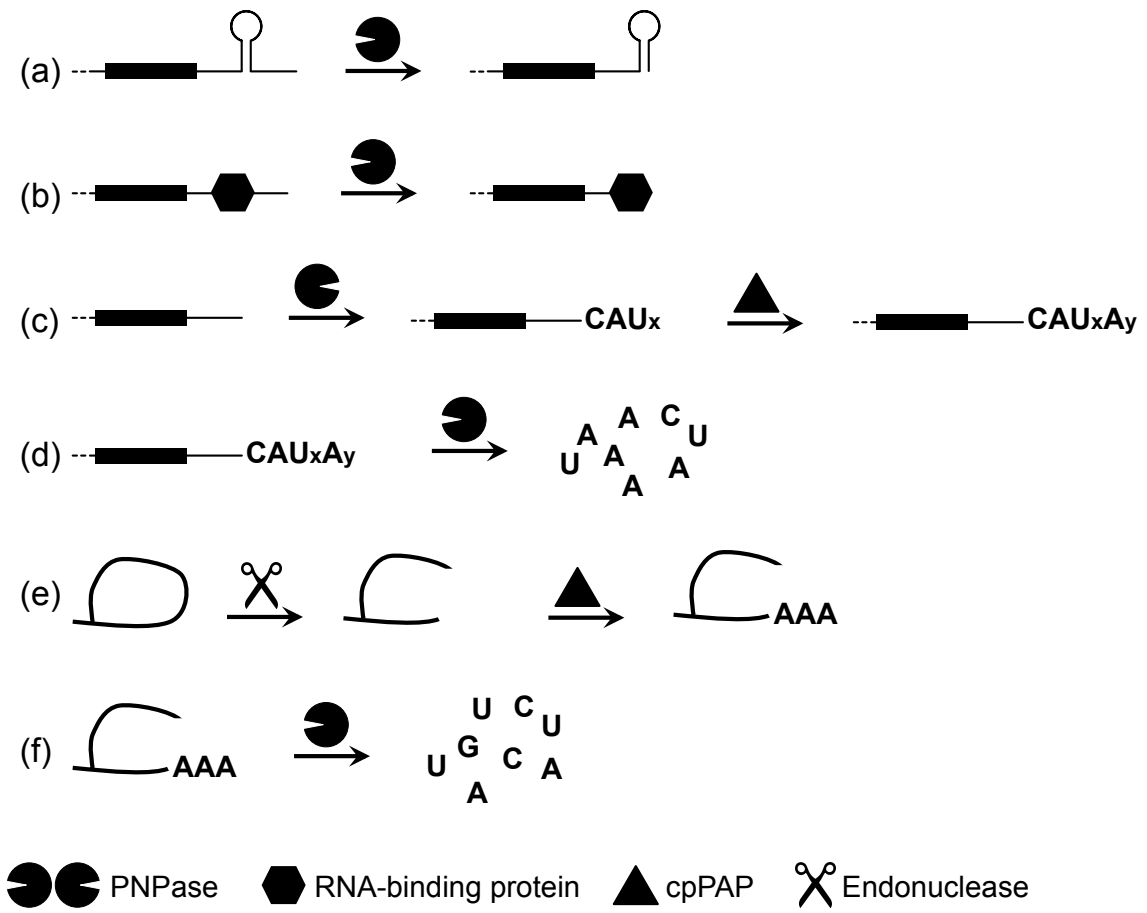


Figure 2.8: The various roles of PNPase in chloroplast RNA metabolism.
(a) PNPase resects the 3' ends of pre-RNAs until it encounters a strong secondary structure.
(b) 3' end maturation can also be defined by RNA-binding proteins such as those of the pentatricopeptide repeat family.
(c) 3' polynucleotide tails that signal degradation may be formed by sequential activities of PNPase and a chloroplast poly(A) polymerase (cpPAP) activity, as shown here for *psbA*. x and y represent arbitrary numbers of heterogeneous nucleotides and adenosines, respectively.
(d) PNPase degrades polyadenylated transcripts in a 3'→5' direction, after the polyadenylation step shown in (c).
(e) Excised intron lariats are hypothesized to be cleaved by an endoribonuclease, and the resulting product may be polyadenylated either by cpPAP as shown here, or by PNPase.
(f) The intermediate generated in (e) is subject to degradation by PNPase; thus, it accumulates to a high level in the *pnp* null mutants.

5’→3’ or 3’→5’ exonucleolytic trimming (Pfalz*, et al.* 2009), with the endpoints of mature transcripts defined by sequence-specific RNA-binding proteins or stem-loop structures (Figures 2.8a and b). Taking the *psbB* gene cluster as an example, we observed 3’ extensions for the three longest full length transcripts (bracketed in Figure 2.3b), which in the WT terminate at the 3’ stem-loop of *petD*, and these would be analogous to what was observed for *psbA* and *rbcL*. However, apart from this, the patterns were highly complex and sometimes quite diffuse (Figures 2.2 and S2.3). One interpretation is that these many transcripts represent endonucleolytic cleavage products by the chloroplast RNase E, which appears to be involved in polycistronic processing (Walter*, et al.* 2010). Because the chloroplast endonucleases CSP41 and RNase J may be partly redundant with RNase E (Pfalz*, et al.* 2009, Stern*, et al.* 2010), the species accumulating in the *pnp* mutants could also be cleavage products of these enzymes. The observed diffuseness could also represent the presence of polyadenylated species. Although it is difficult to measure quantitatively, our observation that it was much easier to obtain poly(A) tails using a *pnp* mutant rather than WT RNA (Table 2.1), hints that this is the case. This observation is also indirect evidence that PNPase has the major role in depleting poly(A)-tailed transcripts in the chloroplast.

PNPase and intron degradation

A surprising result was the accumulation of novel intron-containing fragments, along with a general decrease in splicing efficiency. We suspect that less efficient splicing is a pleiotropic effect of poor growth and/or chlorosis; chloroplast splicing is less pronounced in immature chloroplasts (Barkan 1989) and is reduced when chloroplast

translation is compromised (e.g. Jenkins *et al.* 1997). The accumulation of intron fragments, however, suggests that PNPase plays an essential role in their recycling, and if these fragments sequestered splicing factors, this could also reduce splicing efficiency. We showed that the most abundant *petD* species represents partial degradation of a lariat-containing structure, and it would not be surprising if other intron-containing species seen in *pnp* null mutants, such as for *petB* and *clpP*, had similar structures. Intron degradation has been little studied in chloroplasts, however chloroplasts do not appear to contain the lariat debranching activity that is present in other organisms (Vogel and Borner 2002), suggesting that introns initially undergo an endonucleolytic cleavage (Figure 2.8e).

Importance of both PNPase core domains

Using TILLING, we were able to address roles of the duplicated RNase PH-like core domains of cpPNPase. PNPase crystal structures are available from *Streptomyces antibioticus* and *E. coli*, along with the archaeal exosome, which is very similar to PNPase (Symmons*, et al.* 2000, Lorentzen*, et al.* 2005, Shi *et al.* 2008, Nurmohamed*, et al.* 2009). These studies, as well as others employing site-directed mutagenesis (Jarrige *et al.* 2002), point to the second core domain as the active site. By analogy to the archaeal exosome, the first core domain would be expected to act as an accessory RNA-binding domain and function in trimer formation. In *S. antibioticus*, evidence also points to the first core domain as the site of synthesis of the SOS nucleotide pppGpp (Symmons*, et al.* 2000, Slomovic*, et al.* 2008b), as well as containing a binding site for pppGpp that represses enzyme activity (Gatewood and Jones 2010). Mutagenesis of

the human mitochondrial PNPase revealed the inhibition of activity when a first core amino acid was modified, disrupting the trimeric structure of the enzyme (Portnoy*, et al.* 2008).

In terms of the *Arabidopsis* cpPNPase, we obtained two mutations in or near the catalytic domain, and both plant lines exhibited phenotypes that were nearly indistinguishable from the null mutants. Because the G596R protein did not accumulate *in vivo* this is a trivial observation, although *in vitro* analysis confirmed that this protein is inactive. G596, which is located in the vicinity of the phosphorolytic active site, is conserved in all PNPases (Yehudai-Resheff*, et al.* 2003). We suspect that G596R fails to fold correctly, perhaps as a consequence of its inability to bind phosphate, and is thus marked for degradation. The D625N mutant accumulated at least WT levels of protein, and therefore must be inactive. Indeed, D625 is located in the catalytic site, functions in phosphorolysis, and is conserved in virtually all PNPases from bacteria, organelles as well as the equivalent subunit, Rrp41, of the archaeal exosome. Accordingly, mutagenesis of D625 in other studies also inactivated the enzyme (Jarrige*, et al.* 2002, Lorentzen*, et al.* 2005, Portnoy*, et al.* 2008).

The original TILLING screen generated a large number of mutants, of which those with non-neutral mutations in conserved residues among organellar PNPases were selected for further analysis. In spite of this screen, only two mutations in the first core domain conferred detectable RNA phenotypes. P184L showed a mixture of WT and mutant bands for most RNAs analyzed, whereas S202N only exhibited aberrant patterns for the *psbB* gene cluster, in particular the *petD* intron, as well as the gene cluster containing *psbD-psbC* (Figure S2.3). Because only a subset of chloroplast

genes were analyzed, it is likely that S202N affects other transcripts, presumably those that are particularly dependent on PNPase for their processing or degradation. Although the S202N mutation appeared to result in reduced cpPNPase accumulation (Figure 2.1c), there was no effect on abundant transcripts such as 23S rRNA, suggesting that altered S202N activity rather than its overall abundance, conferred the observed phenotypes. Unfortunately, when expressed in bacteria, this mutant was not obtained as a soluble protein and therefore could not be analyzed *in vitro* for phosphorolysis activity and RNA binding.

The importance of the first core domain for PNPase activity is also evident from the *in vitro* analyses of P184L and R176S-P184L. The double mutant lacks phosphorolysis activity and binds RNA with less affinity compared to the WT protein. These two amino acids are far from the phosphorolytic site, but their substitution is predicted to interrupt the a-helix structure they are part of. It is possible that this disruption in turn causes conformational changes manifested in RNA affinity below the threshold required for activity. As shown by the *in vitro* analysis, the reduced RNA affinity leads to inability of the RNA to enter the phosphorolytic site, and hence a complete inhibition of activity. On the other hand, P184L displays similar RNA affinity to the WT, but intermediate phosphorolytic activity between the WT and the inactive or null mutants. In this case, we hypothesize that the conformational change induced by the interruption of the a-helix still enables RNA binding, and thus reduced but not eliminated enzyme activity. Furthermore, these mutations are located in the vicinity of the *E. coli* PNPase citrate-binding site (Nurmohamed*, et al.* 2009), where modulation of PNPase activity is achieved (Nurmohamed et al., submitted for publication). Indeed, PNPase

catalytic activity appears to involve changes in the quaternary structure (S. Hardwick and B. Luisi, personal communication), which could be relevant to the phenotypes we observed for the first core domain mutants.

We considered that these first core domain mutations might directly impose changes in the oligomeric structure of the enzyme, as shown before for the trimeric human PNPase (Portnoy*, et al.* 2008). As previously suggested (Symmons *et al.* 2002), changes in the oligomeric structure might alter processivity, as indeed can be inferred from the polyadenylation activity of the P184L mutant (Figure 2.7). However, both first core domain mutants still formed hexamers, leaving unclear the reason for the formation of hexameric PNPase in the chloroplast, as compared to the trimers found in bacteria and human mitochondria. Mutants able to form trimers, but not hexamers, would be valuable in this regard.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana experiments used ecotype Col-0. Plants were grown on soil under long days (16 h light/8 h dark) at 22˚C. The *Arabidopsis pnp1-1* and *pnp1-2* T-DNA insertion mutants were previously described (Marchive*, et al.* 2009). The TILLING mutants (Till*, et al.* 2003) were obtained using gene-specific primer pairs (5'-GGAGCGATTCTCAGCTGTAGG-3', 5'-CATATTTCTTTGCTAAAACTT-3'; 5'-TTGGAGGATGAAGACGAG-3', 5'-TCCATTATCTCTAAAGTAATTCCTCCT-3') for the 1st and the 2nd core domains, respectively. Any background mutations caused by the

EMS treatment were removed by four backcrosses to Col-0 WT. Genotyping used the dCAPS Finder 2.0 program (Neff *et al.* 2002).

RNA analysis

Total RNA was isolated and analyzed as described (Marchive*, et al.* 2009). Equal loading was achieved by preparing a 10 µg stock of RNA mixed with loading buffer and visually adjusting the amount of rRNA loaded for each sample. The RNA stock was denatured once at 65ºC, stored at -80ºC and thawed on ice for each gel loading. Poisoned Primer Extension was done according to Asakura and Barkan (2006), quantitative RT-PCR as described by Marchive *et al. (2009)*, using 2 µg of total RNA, and cRT-PCR as described previously by Perrin *et al.* (2004b). Poly(A) tails for the *psbA* transcript were characterized by oligo(dT)-primed RT-PCR according to Slomovic *et al.* (2008a). All primers are listed in Table S2.1.

Protein analysis

Leaves from 4- to 5-week-old plants grown under long day conditions were harvested and plastids were enriched according to van Wijk *et al* (2007) from steps 1 through 6. Once the chloroplasts were pelleted, soluble proteins were extracted in a native soluble protein extraction buffer (100 mM Tris. pH 8.0, 18% sucrose, 40 mM b-mercaptoethanol and 1 mM phenylmethyl sulfonyl fluoride). Proteins were separated in a NativePAGE™ 3-12% Bis-Tris Gel (Invitrogen BN2011BX10) and transferred to an Immun-Blot™ PVDF membrane, according to the protocol of the NativePAGE™ Novex® Bis-Tris Gel System (Invitrogen, XCell SureLock™). The membrane was blocked in TBS-T + 5% milk for 1 hr

at room temperature before adding 1:3,000 PNPase antibody and agitating at 4°C overnight, prior to detection.

Production of recombinant PNPase and its mutated versions

The cDNA encoding the mature protein was amplified from leaf RNA (for primers see Table S2.1). Point mutations were introduced into the WT protein using the QuikChange™ site-directed mutagenesis kit (Stratagene Inc.), and DNA sequences were verified to exclude PCR-induced mutations. In this way, the R176S mutation was discovered. For expression in *E. coli*, PCR products were inserted into Pet20b, which encodes a C-terminal His_6 tag. The proteins were expressed in ENS134-3, which lacks endogenous PNPase (Lopez *et al.* 1999), kindly obtained from Marc Dreyfus (Ecole Normale Supérieure, Paris). The proteins were purified by NTA-agarose affinity followed by MonoQ and Heparin columns (Pharmacia), as previously described (Portnoy*, et al.* 2008), to the stage of a single Coomassie Blue-stained band as visualized by SDS-PAGE (Figure 2.6a). The mutant S202N was not obtained as a soluble protein and therefore was not further analyzed. No contaminating *E. coli* RNase II or RNase E was detected by immunoblot. In order to analyze its oligomeric form, purified PNPase was fractionated by Superdex 200 size exclusion column chromatography (Portnoy*, et al.* 2008). For antibody production, 1.5 mg of recombinant protein was injected into rabbits (Sigma, Inc.)

Polyadenylation and degradation assays; UV-crosslinking

The plasmid used for the *in vitro* synthesis of the RNA corresponding to the 3' end of the spinach chloroplast *psbA* has been described, as well as the procedures for assays of polyadenylation and degradation activities of the recombinant proteins (Yehudai-Resheff*, et al.* 2003). Briefly, [^{32}P]-RNA was incubated with the corresponding proteins (50 ng for degradation; 100 ng for polymerization) in Buffer E with the addition of Pi for degradation assay, or with ADP when polyadenylation activity was to be monitored. Following incubation, the RNA was isolated and analyzed by denaturing PAGE and autoradiography. UV-crosslinking of proteins to radiolabeled RNA was performed as previously described (Portnoy*, et al.* 2008). For the competition assay, the protein was first mixed with the ribohomopolymers and then the radiolabeled RNA was added.

ACKNOWLEDGEMENTS

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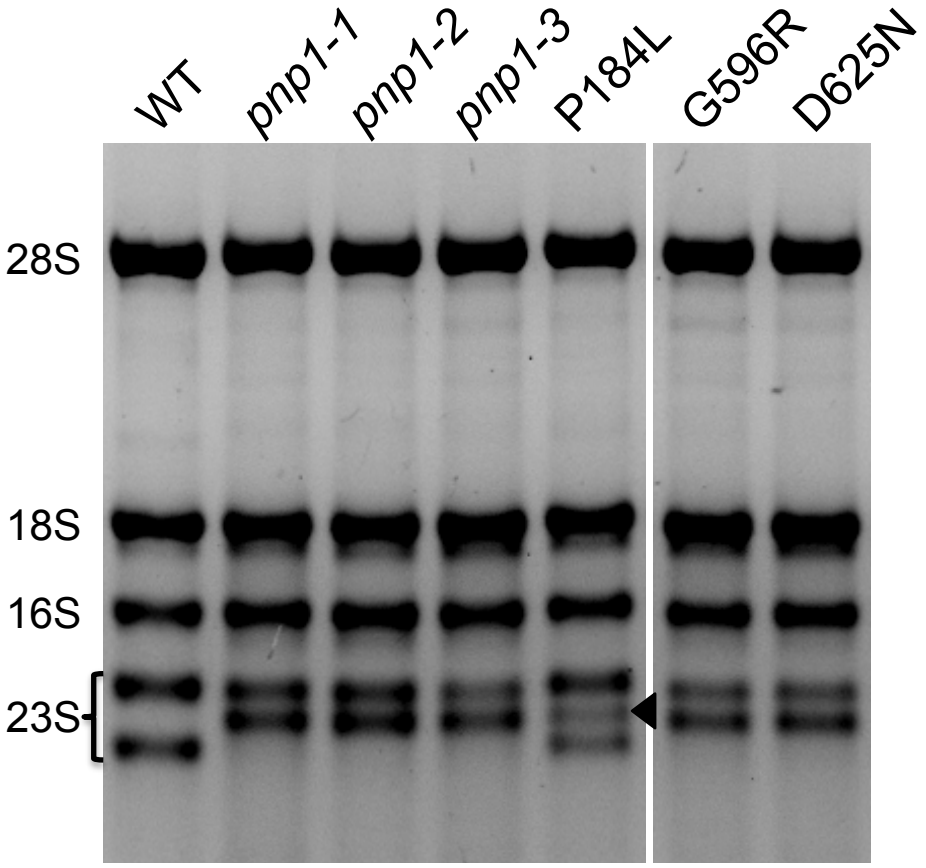


Figure S2.1: Gel electrophoresis to examine ribosomal RNAs.
Total RNA was isolated from green leaves and analyzed by ethidium bromide staining, revealing the reduced mobility of one of the 23S rRNA transcripts, as indicated by the filled arrowhead for P184L.

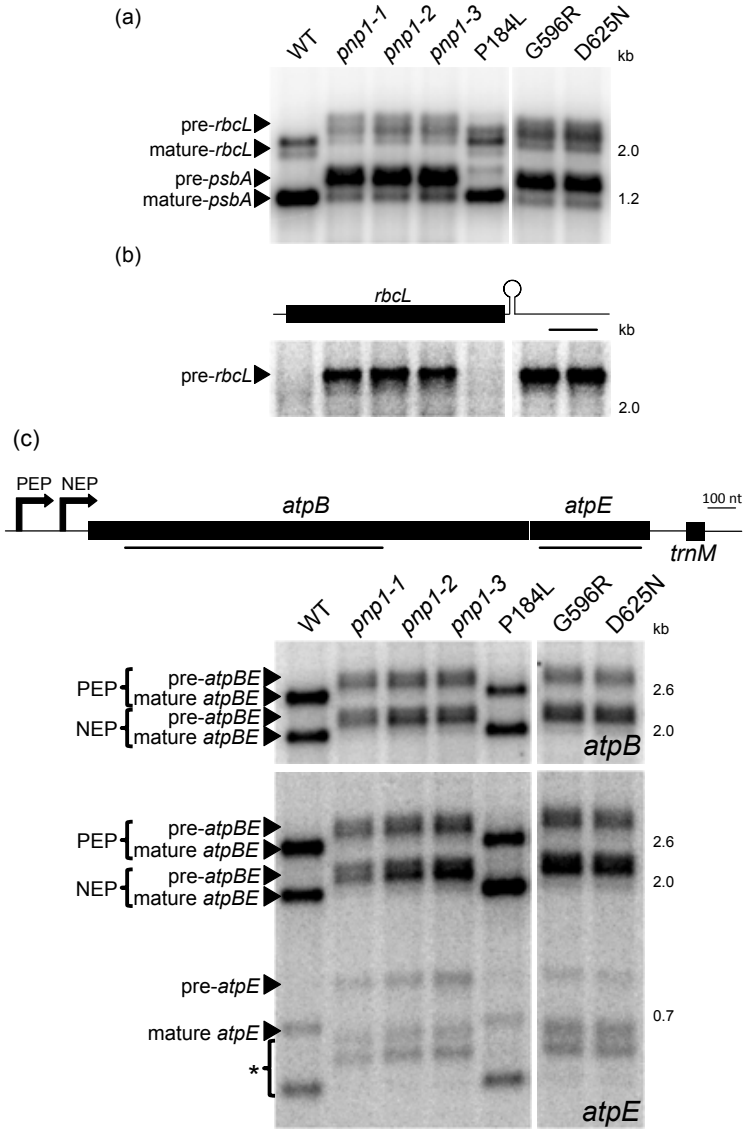


Figure S2.2: RNA gel blot hybridization for two monocistronic mRNAs, *rbcL* and *psbA* and the dicistronic *atpB*/*E* mRNA.
(a) Coding region probes were used simultaneously for *rbcL* and *psbA*. The transcripts labeled pre-*rbcL* and pre-*psbA* include 3' extensions, as discussed in the text.
(b) A 302 bp probe was used, beginning 196 bp downstream of the WT *rbcL 3'* end, as shown in the schematic.
(c) Results for *atpB* (upper blot) and *atpE* (lower blot) are shown. The *atpB*/*E* dicistron is expressed from PEP and NEP promoters as discussed in the text, resulting in four accumulating transcripts in the WT. Precursor RNA species (pre-*atpBE* and pre-*atpE*) are the major RNA species present in the *pnp* mutants. The lower band in the *atpE* blot marked with an asterisk has not been previously described. The schematic above the gels shows promoter locations and extents of the probes used.

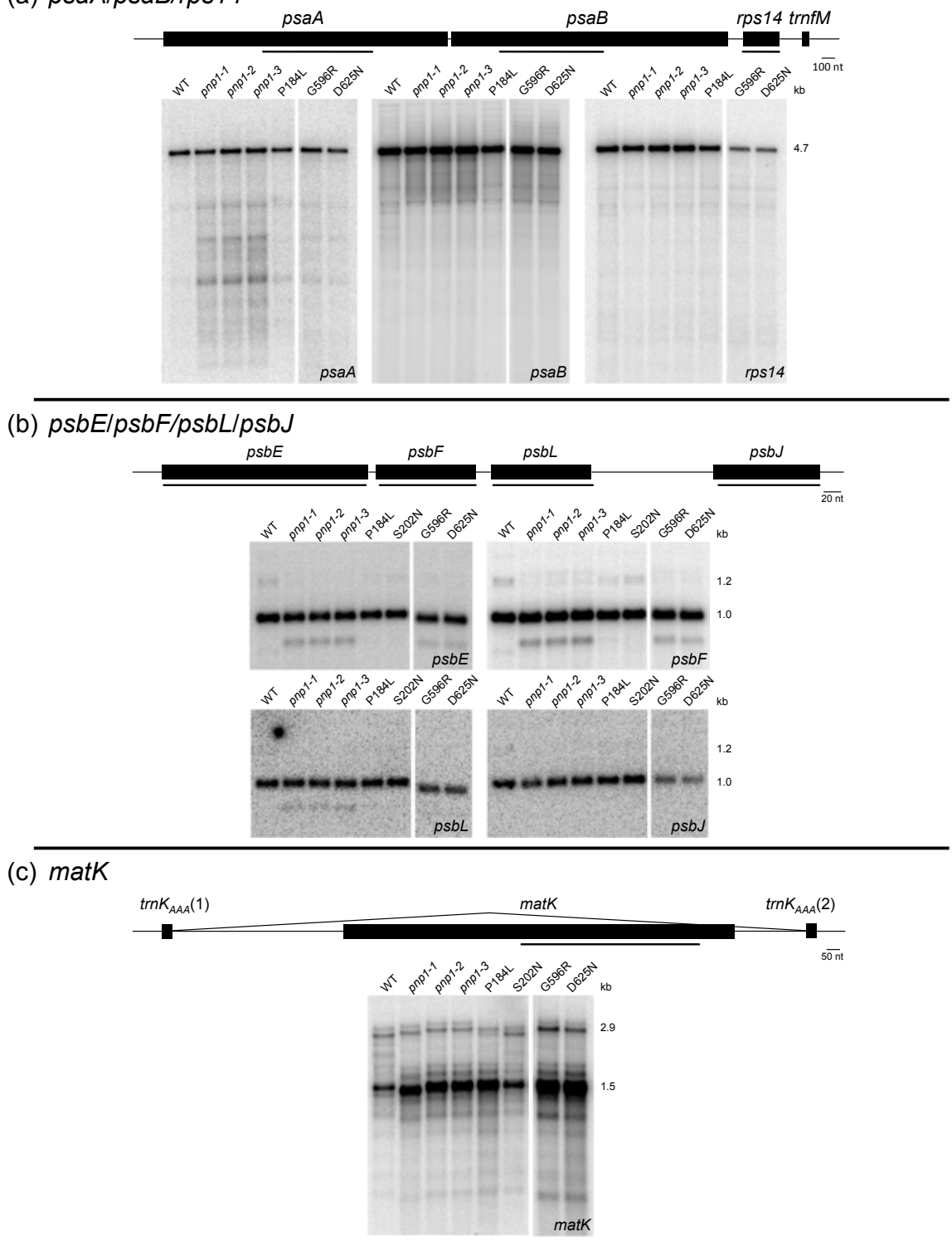


Figure S2.3: RNA phenotypes of PNPase mutants.

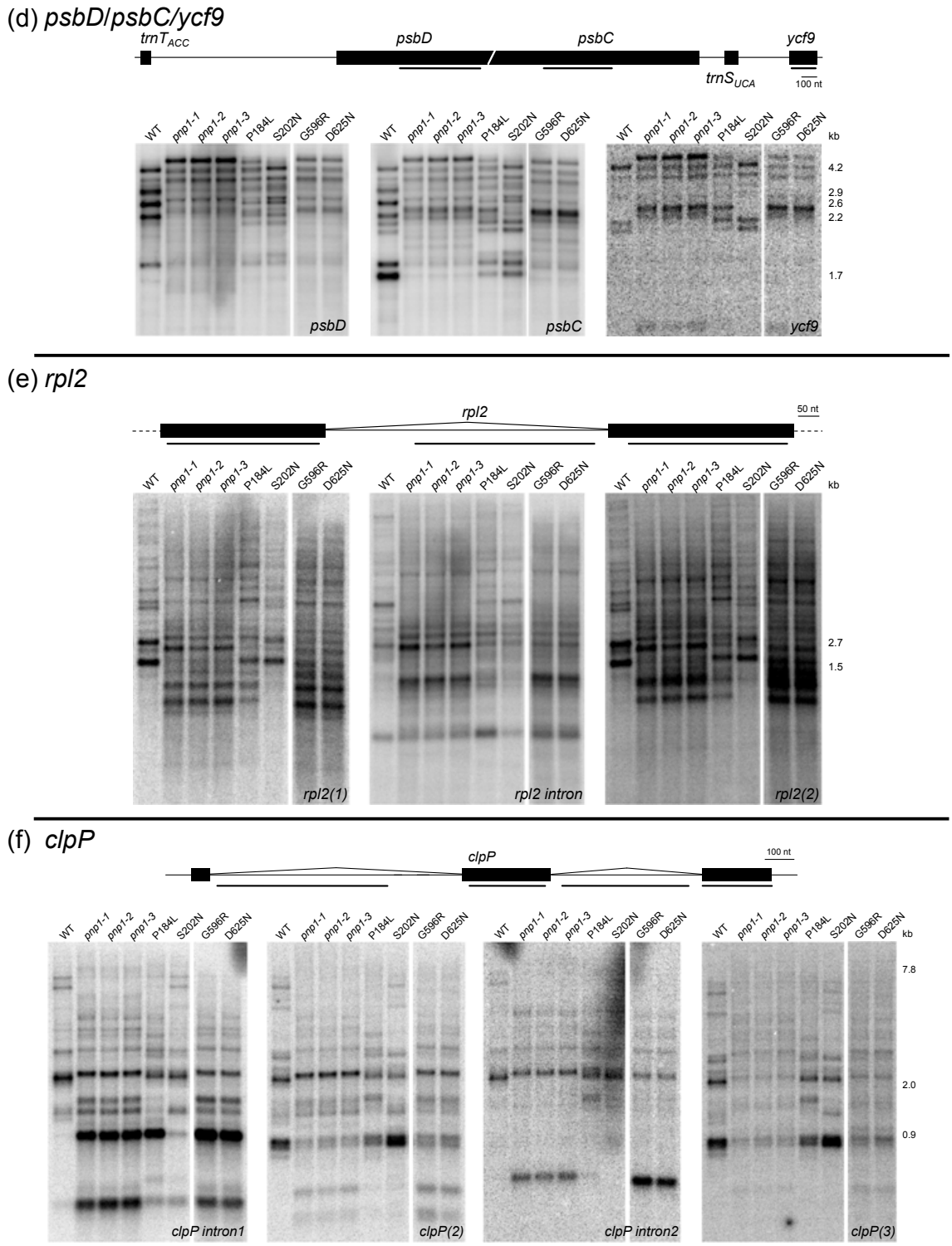
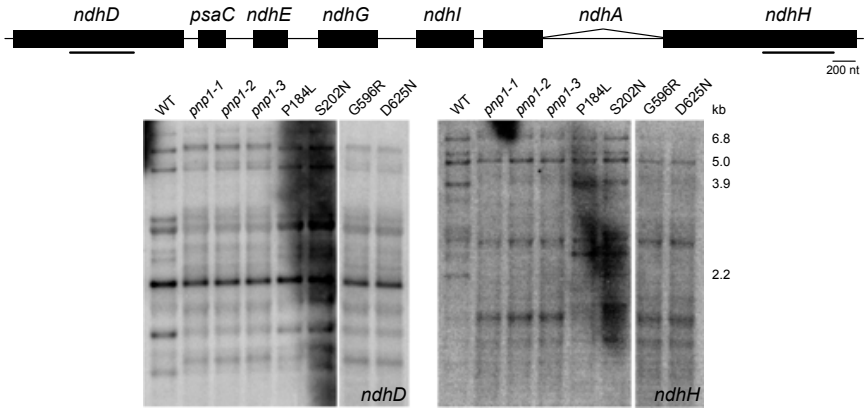
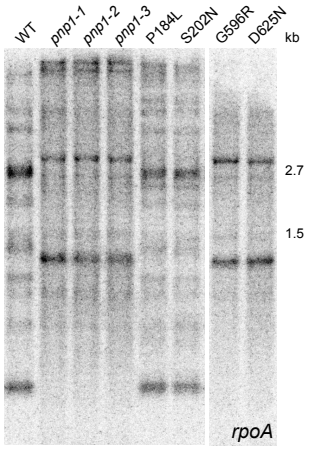


Figure S2.3: RNA phenotypes of PNPase mutants.

(g) *ndhD/psaC/ndhE/ndhG/ndhI/ndhA/ndhH*



(h) *rpoA*



(i) *rpoB/rpoC1/rpoC2*

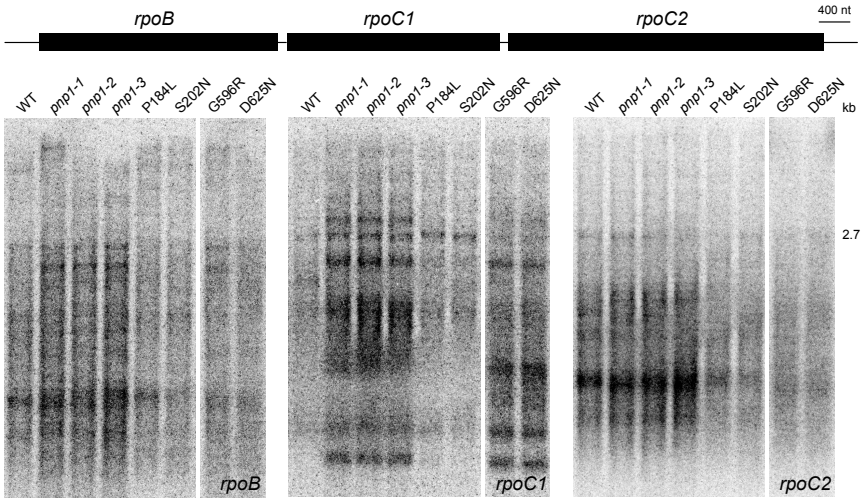


Figure S2.3: RNA phenotypes of PNPase mutants.

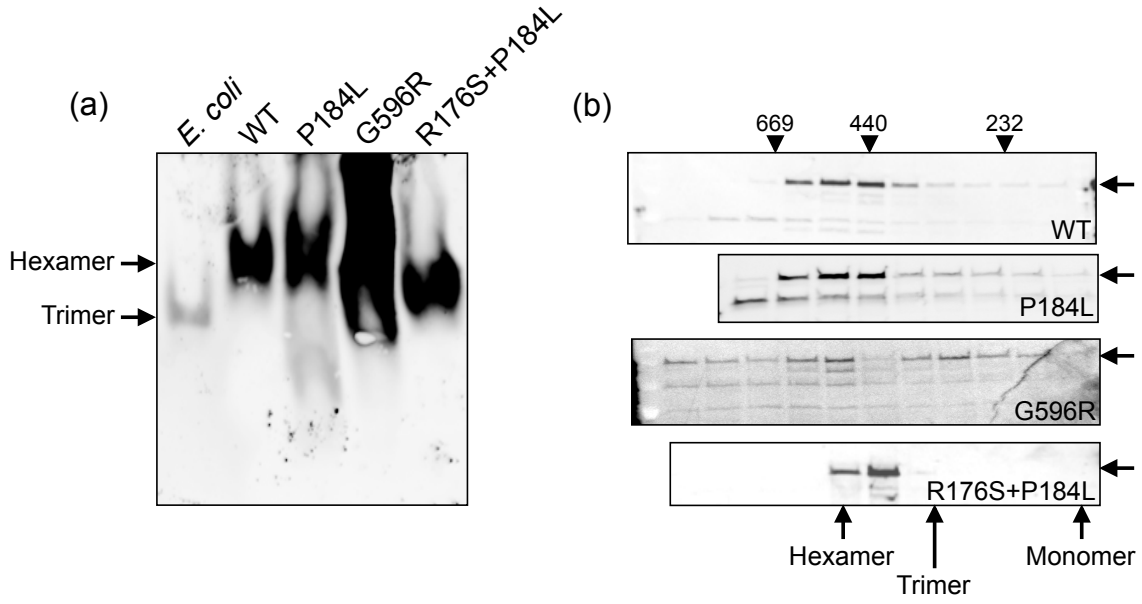


Figure S2.4: Quaternary structures of recombinant PNPases.
(a) The recombinant proteins were fractionated by nondenaturing-PAGE and detected by immunoblot. The *E. coli* PNPase was used as a reference for the trimeric structure and was detected using specific antibodies for that protein. The WT cpPNPase shows the migration of the hexameric structure.
(b) The proteins were fractionated using a Superdex 200 column and the PNPase detected by immunoblotting with specific antibodies. The migration of size markers of known molecular weight are shown across the top, and the inferred oligomeric structures at the bottom.

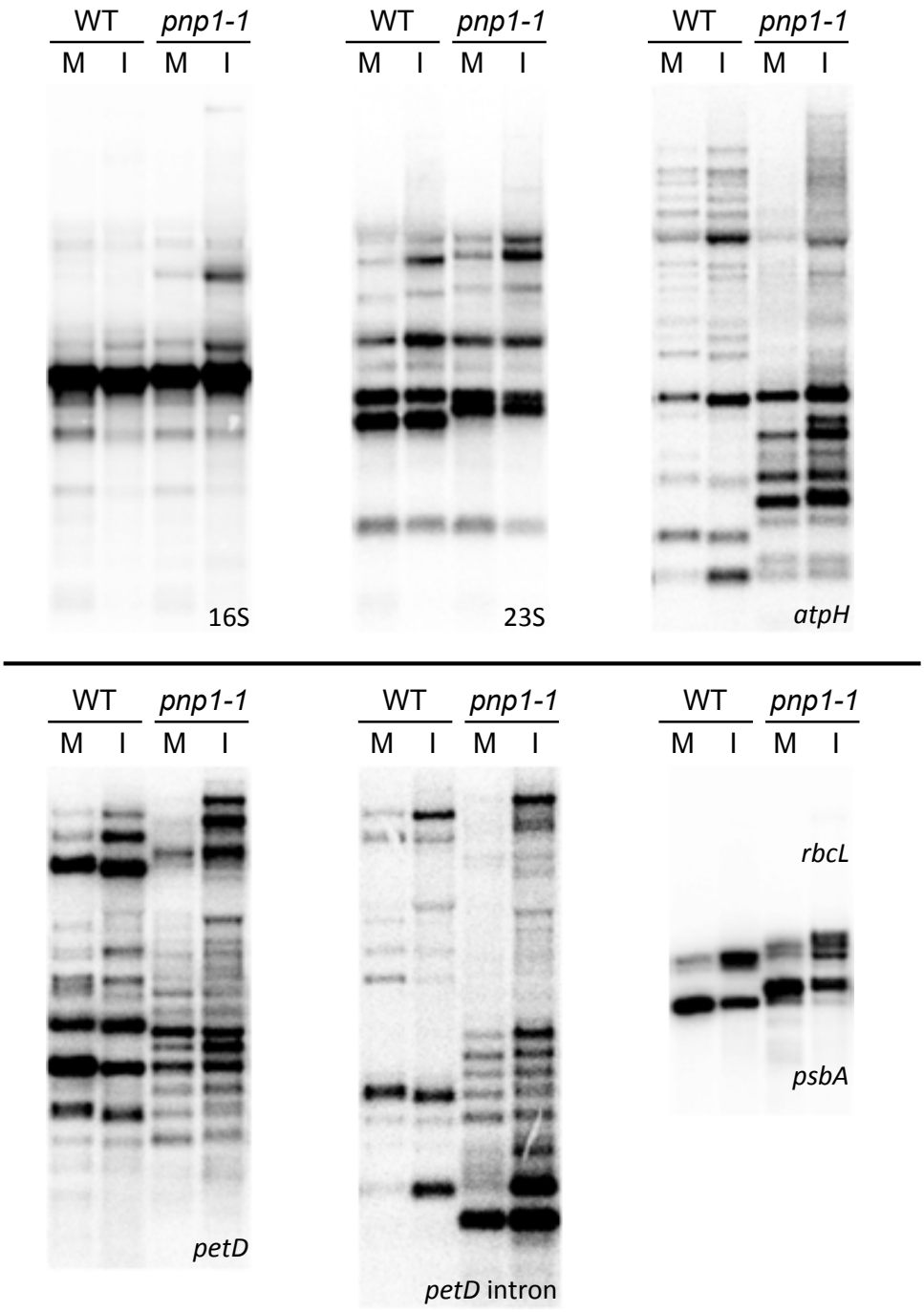


Figure S2.5: RNA gel blot to compare mature and young leaves.
RNA was isolated from mature (M) and immature (I) rosette tissues of the WT and *pnp1-1*. The immature tissues for *pnp1-1* are the ones displaying a chlorotic phenotype in Figure 2.1b. The plants for this experiment were grown under short day conditions to maximize the amount of tissue that could be harvested.

Primer	Sequence (5' to 3')
rbcL-5'	ATGTCACCACAAACAGAG
rbcL-3'	CTACTCTTGGCCATCTAA
psbA-5'	ATGACTGCAATTTTAGAG
psbA-3'	TTATCCATTTGTAGATGGAGCC
rbcL 3'UTR-5'	AACCAAGTATCCCCGCTTCT
rbcL 3'UTR-3'	TAGTTCATTTTCTATTCAGAGTAAGCA
atpB-5'	TCTGGTGGTTAAGGGTCGAG
atpB-3'	CGGGGTCAGTCAAATCATCT
atpE-5'	GACTCCGAATCGAATTGTTTG
atpE-3'	GTGTCCGAGCTCGTCTGAG
atpI-5'	GGTCCATGCCCAAGTTCTTA
atpI-3'	AGCTTGAATCCCGCTTGTAA
atpH-5'	ATCCACTGGTTTCTGCTGCT
atpH-3'	TGCTACAACCAGGCCATAAA
atpF intron-5'	ATCCCGCGAATTACTTCTGA
atpF intron-3'	TTCCCGAACCAAACATGAAT
atpF(2)-5'	TCAGAAGAACTGCGTGAAGG
atpF(2)-3'	CATCGTACCAAACATCCCAAT
atpA-5'	ACGCCTTGGCTAACCCTATT
atpA-3'	AGCAGGTCTGATTCCAGCAT
atpH cRT-PCR1-5'	GGCCTGGTTGTAGCATTAGC
atpH cRT-PCR2-5'	GAGGATGGGGAATTAGCACA
atpH cRT-PCR1-3'	TAACCGAAGCAGCAGAAACC
atpH cRT-PCR2-3'	TTCCTCACACCAAAATAAAGAAA
psbB-5'	CAGCTTTTGTTGTTGCTGGA
psbB-3'	TAGCACCATGCCAAATGTGT
psbN-5'	CTAGTCCCCGTGTTCCTCGA
psbN-3'	ATGGAAACAGCAACCCTAGT
psbH-5'	ATGGCTACACAAACTGTTGA
psbH-3'	CTAATTCACTGAAATTCCAT
petB intron-5'	ACCTCGTAGCCGGACTTCTT
petB intron-3'	ACTCCCCCTCCAAGAACTGT
petB(2)-5'	TATGTTCCTCCGCATGTCAA
petB(2)-3'	TTACCGGAATAGCGTCAGGT
petD intron-5'	AGGGGATAGGCTGGTTCACT
petD intron-3'	CGGCTCGAGCAAGAATTAAC
petD(2)-5'	AAAACCAGATTTGAATGATCCTG
petD(2)-3'	CAATCGGTAATGTTGCTCCA
petD cRT-PCR1-5'	AAATCCATTTCGTCGTCCAG
petD cRT-PCR2-5'	AAATGGGTTCGACTGGAAAA
psbH cRT-PCR-3'	CATTGCAACACCCATCAAAG
psaA-5'	GCAGCCTTAATGCTTTTTGC
psaA-3'	GATCTAATCCGCCACGAAAA
psaB-5'	ATGTCGCTATTCCTGCATCC
psaB-3'	TTGCGCTATGAACGCATAAG
rps14-5'	TGGCAAAGAAAAGTTTGATTTAT
rps14-3'	TTACCAGCTTGATCTTGTTGC
psbE-5'	TGTCTGGAAGCACAGGAGAA
psbE-3'	GCCTTGTCGGCTCTCTGTAA
psbF-5'	GGACCTATCCAATTTTTACAGTGC
psbF-3'	GTTGGATGAACTGCATTGCT
psbL-5'	CAATCAAATCCGAACGAACA
psbL-3'	GAAATAATTCGAAAATAAAACAGCAA
psbJ-5'	CTGGAAGGATTCCTCTTTGG
psbJ-3'	CAGGGATGAACCTAATCCTGA
matK-5'	TTTTTCTACGCAAGCGGTCT
matK-3'	AACCAGAGCCCAACCTTTTT
psbD-5'	CTGGTCCCATTGCTGTTTTT

Table S2.1: Sequences of oligonucleotide primers used in this study.

CHAPTER 3

RNASE II PRESERVES CHLOROPLAST RNA HOMEOSTASIS BY INCREASING MRNA DECAY RATES, AND COOPERATES WITH POLYNUCLEOTIDE PHOSPHORYLASE IN 3' END MATURATION*

ABSTRACT

Ribonuclease R (RNR1) and polynucleotide phosphorylase (cpPNPase) are the two known 3' to 5' exoribonucleases in *Arabidopsis* chloroplasts, and are involved in several aspects of rRNA and mRNA metabolism. In this work, we show that mutants lacking both RNR1 and cpPNPase exhibit embryo lethality, akin to the inviability of the analogous double mutant in *E. coli*. We were successful, however, in combining an *rnr1* null mutation with weak *pnp* mutant alleles, and show that the resulting chlorotic plants display a global reduction in RNA abundance. Such a counterintuitive outcome following the loss of RNA degradation activity suggests a major importance of RNA maturation as a determinant of RNA stability. Detailed analysis of the double mutant demonstrates that the enzymes catalyze a two-step maturation of mRNA 3' ends, with RNR1 polishing 3' termini created by cpPNPase. The bulky quaternary structure of cpPNPase compared to RNR1 could explain this activity split between the two enzymes. In contrast to the double mutants, the *rnr1* single mutant overaccumulates most mRNA species when compared to the wild-type. The excess mRNAs in *rnr1* are present in non-polysomal

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* Germain, A., Kim, S. H., Gutierrez, R. and Stern, D. B. Accepted in *Plant J.*

fractions, and half-life measurements demonstrate a substantial increase in the stability of most mRNA species tested. Together, our data reveal the cooperative activity of two 3' to 5' exoribonucleases in chloroplast mRNA 3' end maturation, and support the hypothesis that RNR1 plays a significant role in the destabilization of mRNAs unprotected by ribosomes.

INTRODUCTION

Substantial regulation of chloroplast gene expression is achieved at the post-transcriptional level by a variety of nucleus-encoded endo- and exoribonucleases, along with a diverse population of RNA-binding proteins (reviewed by Stern*, et al.* 2010, Barkan 2011, Stoppel and Meurer 2012). Inefficient transcription termination (Stern and Gruissem 1987) creates long RNA precursors of varying lengths, which are modified by intercistronic RNA processing (reviewed by Barkan 2011), including endonucleolytic cleavage and 3' and 5' end exonucleolytic trimming. These events, combined with additional post-transcriptional events such as splicing and editing, give rise to the accumulating RNA population. The catalytic and RNA-binding proteins that mediate these activities have a mixture of prokaryotic origins resulting from endosymbiosis, and eukaryotic origins representing acquired functions.

Two enzymes whose importance has been demonstrated in chloroplasts are the prokaryotic-type 3'→5' exoribonucleases ribonuclease R (RNR1) and polynucleotide phosphorylase (PNPase). Both enzymes degrade single-stranded RNA processively, but PNPase releases ribonucleoside-5'-diphosphate by catalyzing a reversible

phosphorolytic reaction, whereas RNR1 hydrolyzes RNA to ribonucleoside-5'-monophosphates (reviewed by Nicholson 1999). Two isoforms of PNPase are encoded by the nucleus of plants and targeted to either the mitochondria (mtPNPase) or the chloroplast (cpPNPase). While only mtPNPase is essential for embryo development, both proteins have been shown to have major roles in mRNA, rRNA and tRNA maturation and/or degradation, as well as in non-coding RNA biogenesis and/or accumulation (Walter*, et al.* 2002, Perrin*, et al.* 2004a, Perrin*, et al.* 2004b, Holec*, et al.* 2006, Marchive*, et al.* 2009, Germain*, et al.* 2011, Hotto*, et al.* 2011). Recombinant cpPNPase is inhibited by RNA secondary structures (Yehudai-Resheff*, et al.* 2001), and cannot progress through regions protected by RNA-binding proteins such as PPR10, a pentatricopeptide repeat protein that defines RNA ends in the *atpI-atpH* intergenic region (Prikryl*, et al.* 2011).

RNR1 was first reported to be a mitochondrial protein, where it was named AtmtRNaseII, because it was shown to trim the 3' end of *atp9* mRNA initially generated by mtPNPase (Perrin*, et al.* 2004b). This enzyme was subsequently found in the chloroplast, and T-DNA-induced null mutants were shown to be chlorotic with severe chloroplast rRNA defects (Kishine*, et al.* 2004, Bollenbach*, et al.* 2005). *In vitro* analysis of the PPR10 protein, however, showed that the native 3' end of *atpI* could not be fully generated by cpPNPase, and it was suggested that RNR1/RNase II might complete the process (Prikryl*, et al.* 2011). Ambiguity over the name of this protein derives from the fact that it is apparently the sole organellar representative of superfamily of exoribonucleases that includes both RNase R and RNase II, which have distinct sensitivities to RNA secondary structure and consequently different biological roles

(Matos *et al.* 2011). To avoid confusion with previous literature, we will use RNR1 here to refer to RNR1/RNase II, although the results presented below suggest that it would more appropriately be named RNase II in future research.

To define the functions of plant RNR1 in detail, and to determine whether it cooperates with PNPase in chloroplast mRNA maturation and/or degradation, we have taken a genetic approach. *E. coli* strains lacking both RNase II and RNase R are essentially normal, whereas strains depleted for either enzyme as well as PNPase are inviable, illustrating the non-redundant and essential roles of the two types of exoribonuclease (Donovan and Kushner 1986, Cheng *et al.* 1998). Here, we demonstrate the embryo lethality of the *pnp1-1/rnr1-3* double mutant in *Arabidopsis*, and circumvent it by taking advantage of weak cpPNPase mutant alleles (Germain*, et al.* 2011). In those double mutants, we find global reduction in chloroplast RNA accumulation, suggesting that correct RNA maturation is required for RNA stability. We also find that as in plant mitochondria, RNR1 completes mRNA 3' maturation initiated by PNPase. Finally, we expand our understanding of RNR1 in chloroplast RNA metabolism, specifically its role in decay of nonpolysomal mRNA. This points to a conflict of the current model of chloroplast RNA metabolism with our data, and we suggest a possible explanation linked to the sublocalization of cpPNPase and RNR1 within the chloroplast.

RESULTS

Analysis of cpPNPase-RNR1 double mutants

To investigate whether cpPNPase and RNR1 had completely distinct roles, we attempted to obtain a *pnp/rnr1* double mutant by crossing the previously-described T-DNA insertion null mutant *pnp1-1* (Germain*, et al.* 2011) to three different *rnr1* alleles (rnr1-1–rnr1-3, Bollenbach*, et al.* 2005). Because DNA analysis did not reveal any doubly homozygous mutants in any of the three original F2 populations, we checked for embryo lethality by analyzing siliques from crosses homozygous for *pnp1-1* and segregating for *rnr1* mutant alleles. As shown in Table 3.1, statistical analysis showed a significantly higher embryo abortion rate where *pnp1-1 rnr1-1* or *pnp1-1 rnr1-3* double mutants were segregating, in comparison to progeny segregating only for *rnr1-1* or *rnr1-3*. Progeny segregating only for *rnr1-2* had an unexpectedly high abortion rate, although lower than the cognate double mutant, for reasons we cannot immediately explain. We can conclude, however, that the complete absence of both cpPNPase and RNR1 leads to a failure to complete embryo development in *Arabidopsis*.

To learn more about the effects on chloroplast gene expression of depleting both major 3'→5' exoribonucleases, we took advantage of two single amino acid cpPNPase mutants with reduced PNPase activity, *P184L* and *S202N*, and a third allele with a WT phenotype, *A263V* (Germain*, et al.* 2011). Most chloroplast transcripts in *P184L* have an intermediate pattern between the null mutant and the wild-type (WT), and the recombinant P184L protein displays a lower catalytic activity. The RNA phenotype of *S202N* is closer to the WT, with a small subset of transcripts showing intermediate phenotypes consistent with reduced cpPNPase activity. Figure 3.1 shows the growth phenotypes of progeny segregating for *rnr1-3* and homozygous for *P184L*, *S202N* or

Parental genotype	Progeny embryo phenotype			P value	
	Aborted	Survived	Total		
PNP1/PNP1 RNR1/rnr1-1	2	621	623	A	0.0004
pnp1-1/pnp1-1 RNR1/rnr1-1	61	473	534	B	
PNP1/PNP1 RNR1/rnr1-2	109	430	539	A	0.3049
pnp1-1/pnp1-1 RNR1/rnr1-2	153	372	525	B	
PNP1/PNP1 RNR1/rnr1-3	13	619	632	A	0.0119
pnp1-1/pnp1-1 RNR1/rnr1-3	26	520	546	B	

Table 3.1: Embryo lethality caused by *pnp1* and *rnr1* mutations.
Plants of the parental genotypes shown were self-pollinated and immature siliques were dissected. Embryos were scored as aborted or survived. Comparison of abortion rates was performed using binary logistic regression analysis to determine if plants A had significantly lower abortion rates than plants B. $P<0.05$ is considered significant.

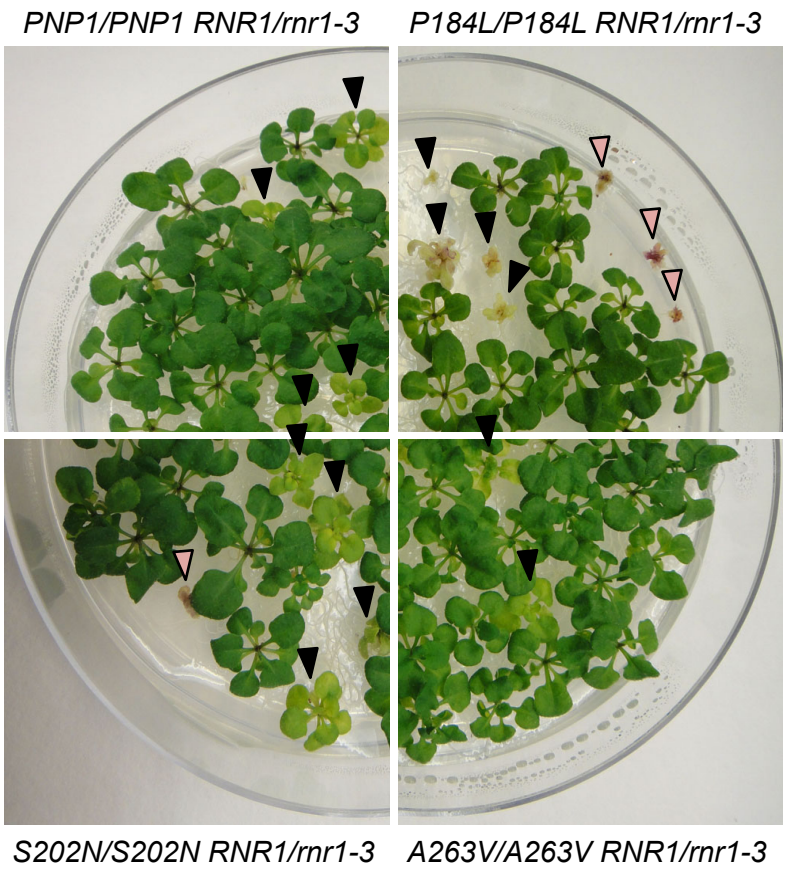


Figure 3.1: Segregating populations with viable *pnp/rnr1* double mutants.

A263V. The progeny giving rise to *P184L*/*rnr1-3* plants display the most severe phenotypes, including the highest percentage of seeds failing to germinate (Table 3.2), numerous plants that do not proceed past early development and accumulate anthocyanins (pink arrowheads in Figure 3.1), as well as the most chlorotic plants as compared to the *rnr1-3* single mutant (black arrowheads in Figure 3.1). The germination failure and anthocyanin accumulation phenotypes were observed to a lesser extent in the progeny segregating *S202N*/*rnr1-3*, while the progeny segregating *A263V*/*rnr1-3* only displayed WT and *rnr1-3* phenotypes, as expected.

Chloroplast rRNA accumulation is severely affected in cpPNPase-RNR1 double mutants

To examine how a combination of cpPNPase and RNR1 deficiency affects chloroplast RNA metabolism, total RNA was examined by ethidium bromide staining, with gel loading based on approximately equal nuclear 28S rRNA amounts (Figure 3.2). This revealed apparently similar chloroplast rRNA patterns in *rnr1-3* and the double mutants, with a general reduction in their accumulation and slightly less distinct banding. The rRNAs, which are transcribed from a polycistronic operon, were then examined in more detail by RNA gel blot, as shown in Figure 3.3a. When probing for 16S rRNA, all *rnr1-3* mutants accumulate lower levels of the mature species (marked 16S in Figure 3.3b), a phenotype previously described for *rnr1-3* by Bollenbach *et al.* (2005), along with the accumulation of the 16S precursor observed in two of the mutants, *rnr1-3* and *A263V/rnr1-3* (pre-16S in Figure 3.3b). The accumulation of pre-16S is, however, similar to WT for *P184L/rnr1-3*, and intermediate in *S202N/rnr1-3*,

Parental genotype	Progeny seedling phenotype			
	No germination	*rnr1-3*	WT	Total
P184L/P184L RNR1/rnr1-3	50 (11.5%)	24 (5.5%)	361 (83%)	435
S202N/S202N RNR1/rnr1-3	17 (5.3%)	56 (17.3%)	250 (77.4%)	323
A263V/A263V RNR1/rnr1-3	1 (0.3%)	92 (21.9%)	327 (77.8%)	420

Table 3.2: Segregation of *pnp*/*rnr1* double mutants.
Plants of the parental genotype were self-pollinated and seeds germinated on MS medium. Seeds were scored as no germination, WT, or *rnr1-3* phenotype if the cotyledons were pink upon germination, according to the phenotype described by Bollenbach *et al.* (2005).

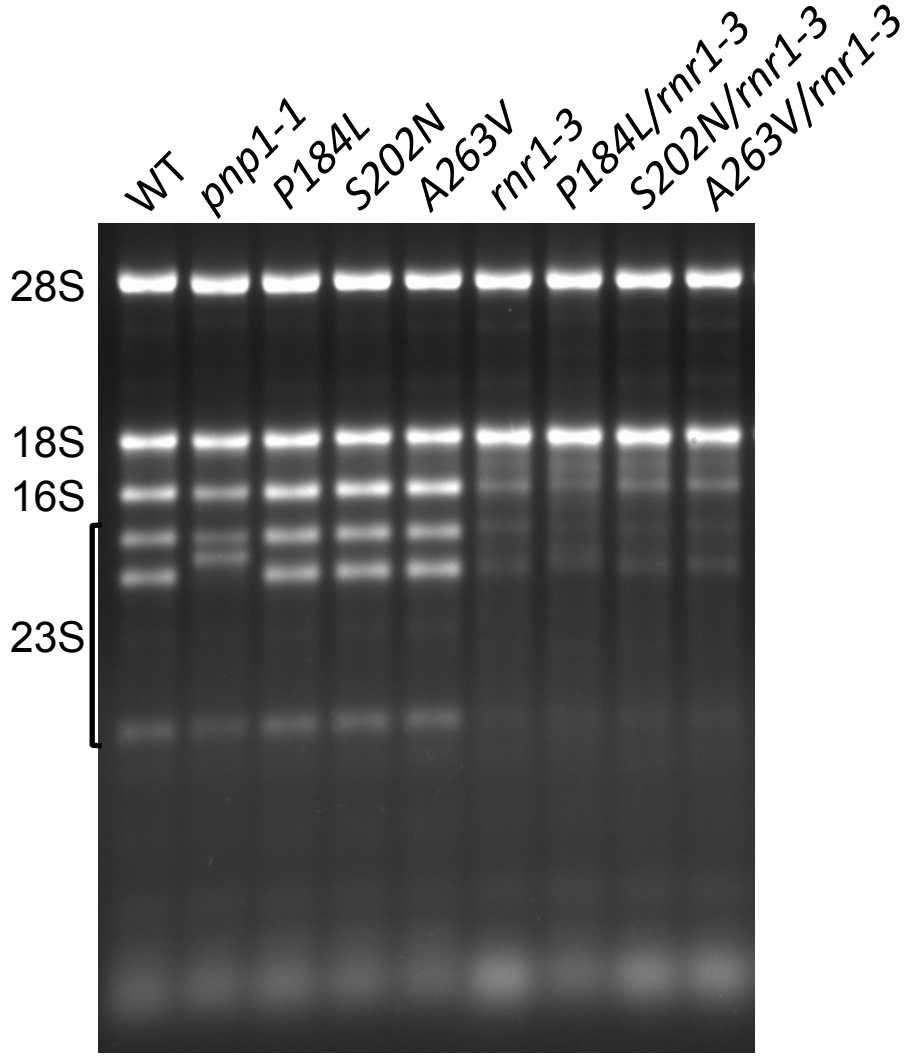


Figure 3.2: Gel electrophoresis to examine ribosomal RNAs.
Total RNA was isolated from whole plants and analyzed by ethidium bromide staining, revealing the reduced accumulation of chloroplast rRNAs for all mutants containing the *rnr1-3* T-DNA insertion.

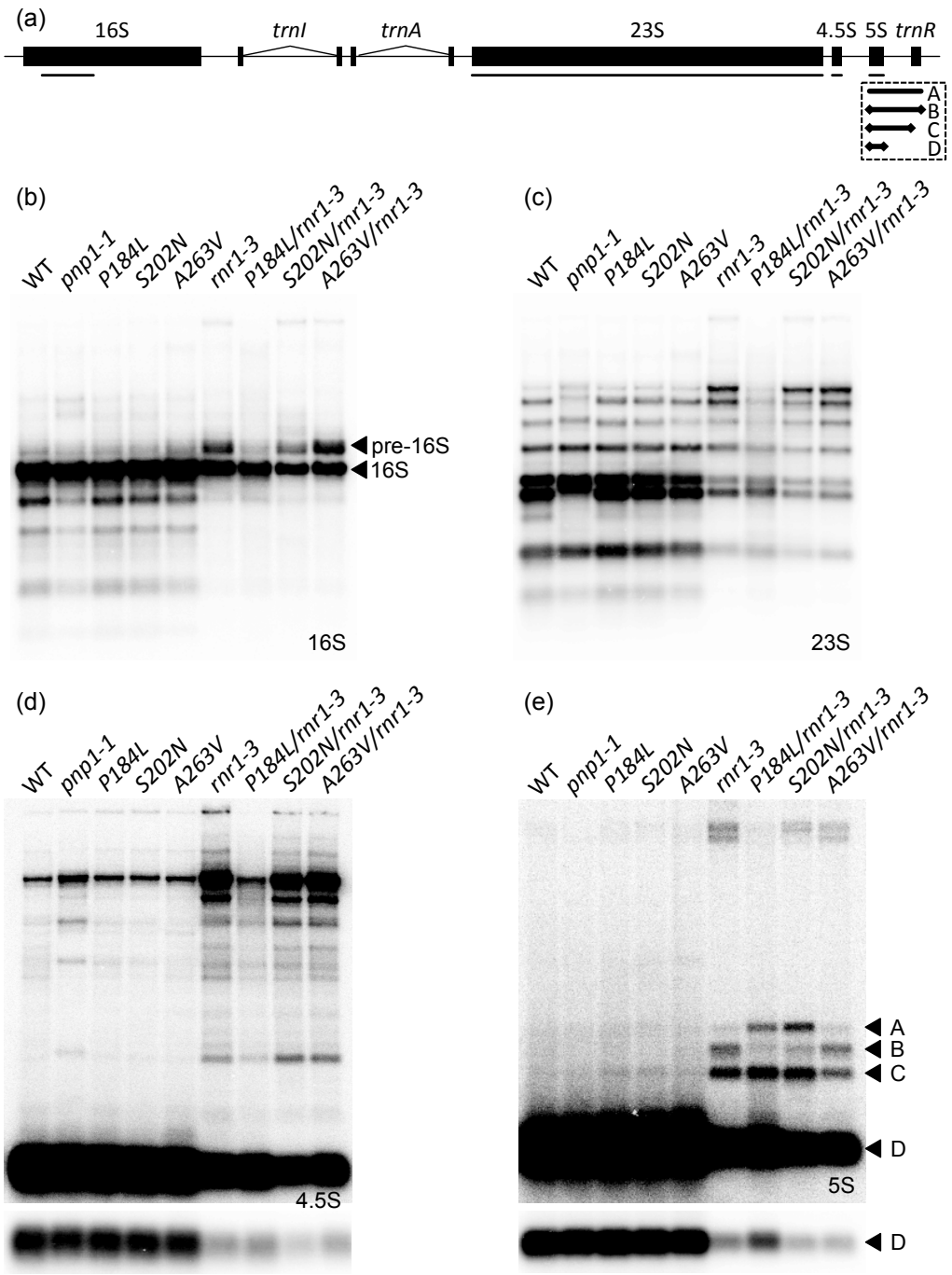


Figure 3.3: Analysis of the *rrn* operon.
(a) Diagram of the *rrn* operon and locations of the probes used for RNA gel blot analysis (black bars). Bars in the dashed box relate to the bands observed in panel e.
(b - e) Results for the four specific probes as indicated at the bottom right of each panel. For 4.5S and 5S rRNAs, an additional shorter exposure is shown to facilitate comparison of mature transcript levels.

indicating a role for cpPNPase in regulating the abundance of pre-16S. Analysis of the 23S and 4.5S rRNAs yielded similar results, with a reduced accumulation of mature rRNAs for the *rnr1-3* mutants coupled with an overaccumulation of precursors except for *P184L/rnr1-3*, in which the precursor levels are similar to those of the WT (Figures 3.3c and d).

We were particularly intrigued by the accumulation pattern of 5S rRNA (Figure 3.3e). In contrast to the reduction in mature 16S, 23S and 4.5S rRNAs for all mutants homozygous for *rnr1-3*, the level of mature 5S transcript (band D in Figure 3.3e) is enhanced in *P184L/rnr1-3* relative to other mutants homozygous for *rnr1-3*, to approximately 20% of the WT level (Figure S3.1). This result implies that cpPNPase plays a role in 5S rRNA degradation. The *rnr1-3* mutants also accumulate some precursors such as band C, band B and band A as described in Sharwood *et al.* (2011b). The accumulation pattern of bands A and B is clearly different between *rnr1-3* and the *P184L/rnr1-3* and *S202N/rnr1-3* double mutants, with an inverted ratio in favor of band A. This result suggests that PNPase has some involvement in the conversion of band A to band B, most likely 3' end trimming of band A until encountering the secondary structure of *trnR* (see right end of panel a).

Overall, the analysis of the rRNA operon frequently reveals an additive effect in the double mutants with a general reduction of rRNA precursors after cpPNPase depletion on top of the already reduced amounts of mature rRNAs carried over from the lack of RNR1. The strongest phenotype was observed for *P184L*/*rnr1-3* and was less noticeable for *S202N*/*rnr1-3*.

pnp/rnr1 double mutants display a global reduction in chloroplast mRNA levels, proportional to the decline in cpPNPase activity

To establish the collective importance of cpPNPase and RNR1 in mRNA maturation and stability, several polycistronic gene clusters as well as the monocistronic *rbcL* and *psbA* transcripts were analyzed by RNA gel blot (Figures 3.4 and S3.2). As previously described in detail (Germain*, et al.* 2011), the *pnp1-1* RNA pattern is very distinct from the WT, with many species migrating more slowly due to 3' extensions as typified by the *psbD*/*psbC*/*ycf9* gene cluster (Figure 3.4a). Another *pnp1-1* RNA phenotype is the barely detectable bands in the higher molecular weight range for the *atpI*/*atpH*/*atpF*/*atpA* gene cluster, while the monocistronic *atpH* bands are still distinct (Figure 3.4b), albeit with 3' extensions. The point mutants *P184L* and *S202N* display an intermediate phenotype between *pnp1-1* and WT, while *A263V* is similar to WT. The *rnr1-3* mutant has been previously described as possessing no obvious changes in mRNA patterns compared to WT, but our present analysis reveals higher accumulation of most mRNA species (e.g. *petD*, *atpI*, *atpH* and *atpA* probes). This observation is discussed further below.

The *P184L*/*rnr1-3* double mutant stood out because of the sharp reduction in the abundance of all mRNA species, with the exception of certain *atpH* and *petD* transcripts (Figures 3.4 and S3.2). This reduction is particularly striking for the *psbD* gene cluster, where no distinct band is detected, especially when compared to the other mutants homozygous for *rnr1-3*. Nonetheless, a comparison of *S202N*/*rnr1-3* to *rnr1-3* shows a similar trend in some cases, for example with the *atpI* and *atpA* probes (Figure 3.4b). To summarize the RNA gel blot data presented so far, lack of cpPNPase increases

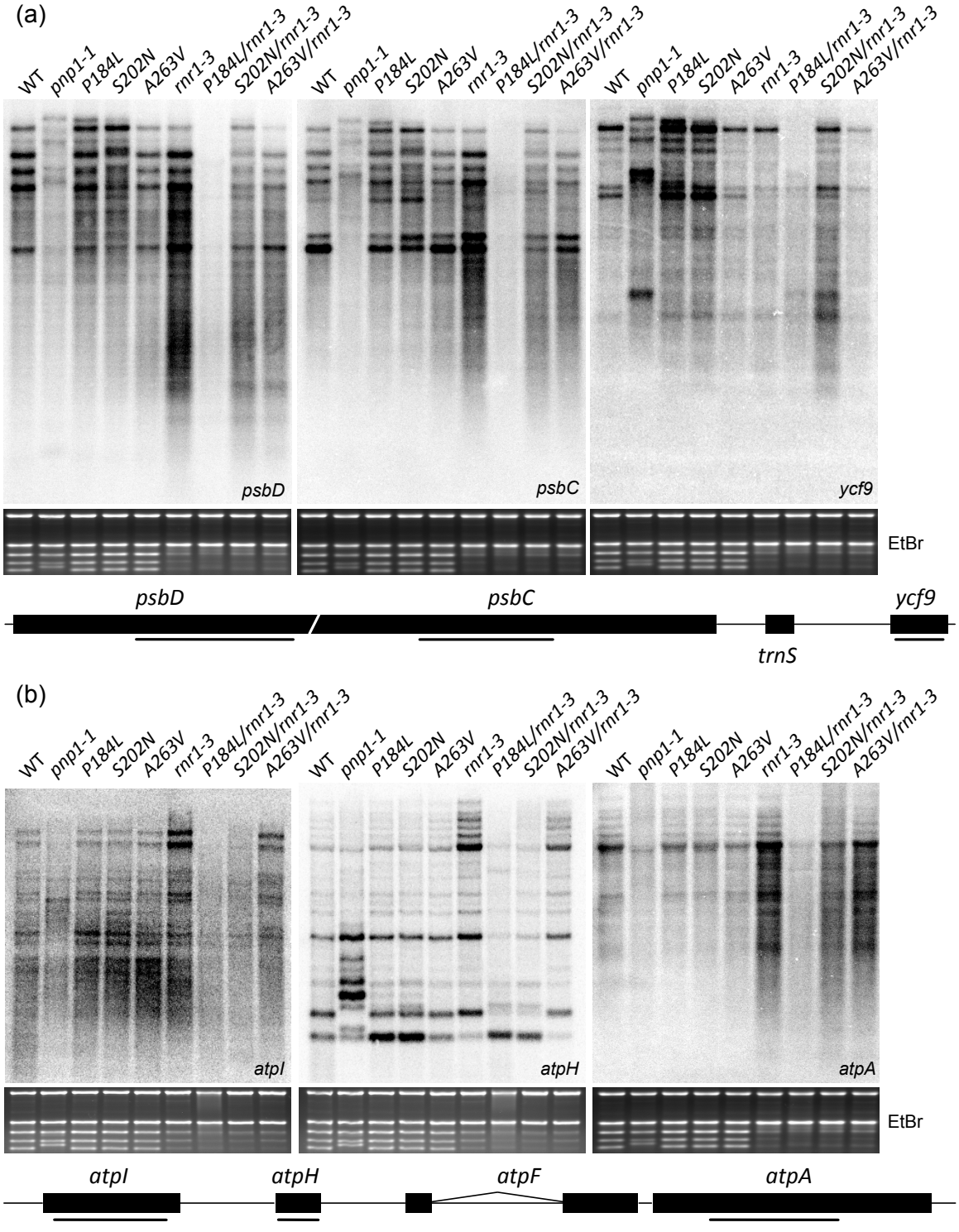


Figure 3.4: Analysis of the *psbD* and *atpI* gene clusters.
(a) Results for the three specific probes as indicated in the bottom diagram (black bars) for the *psbD* gene cluster.
(b) Results for the three specific probes as indicated in the bottom diagram (black bars) for the *atpI* gene cluster.

transcript length and causes 3' end heterogeneity; lack of RNR1 increases RNA abundance, but deficiency of both leads to decreased transcript abundance. We interpret these observations as evidence that the presence of 3'→5' exoribonuclease activity is required for transcript stability, perhaps because at least a minimal level of 3' processing is essential.

To test whether the phenotypes shown in Figure 3.4 held for simpler transcription units, we examined accumulation of the *psbA*, *rbcL* and *matK* monocistronic transcripts (Figures S3.2c-e). These results recapitulate the trend seen for complex gene clusters, i.e. differential accumulation of mRNA species depending on the level of 3'→5' exoribonuclease activity. Exceptions such as the smallest *atpH* band, which is derived from the middle of a gene cluster, indicate that RNA instability rather than lower transcription rates underpin these observations.

Mature RNA 3' ends are generated by the cooperative action of cpPNPase and RNR1

As mentioned in the Introduction, mtPNPase and RNR1 cooperatively generate the 3' end of the *atp9* transcript (Perrin*, et al.* 2004b), and in bacteria the two enzymes are complementary in the sense that PNPase is sensitive to secondary structures while RNase R is not (Arraiano *et al.* 2010). In addition, a double mutant hyperaccumulates small structured RNA (REP) fragments (Cheng and Deutscher 2005). To delineate their respective roles in chloroplasts, we precisely mapped the 3' ends of the *atpI*, *atpH* and *petD* transcripts using circular RT-PCR (Figure 3.5a-c). The *atpI* transcript was chosen because Prikryl *et al.* (2011) had suggested that RNR1 might trim the 3' end of *atpI* to

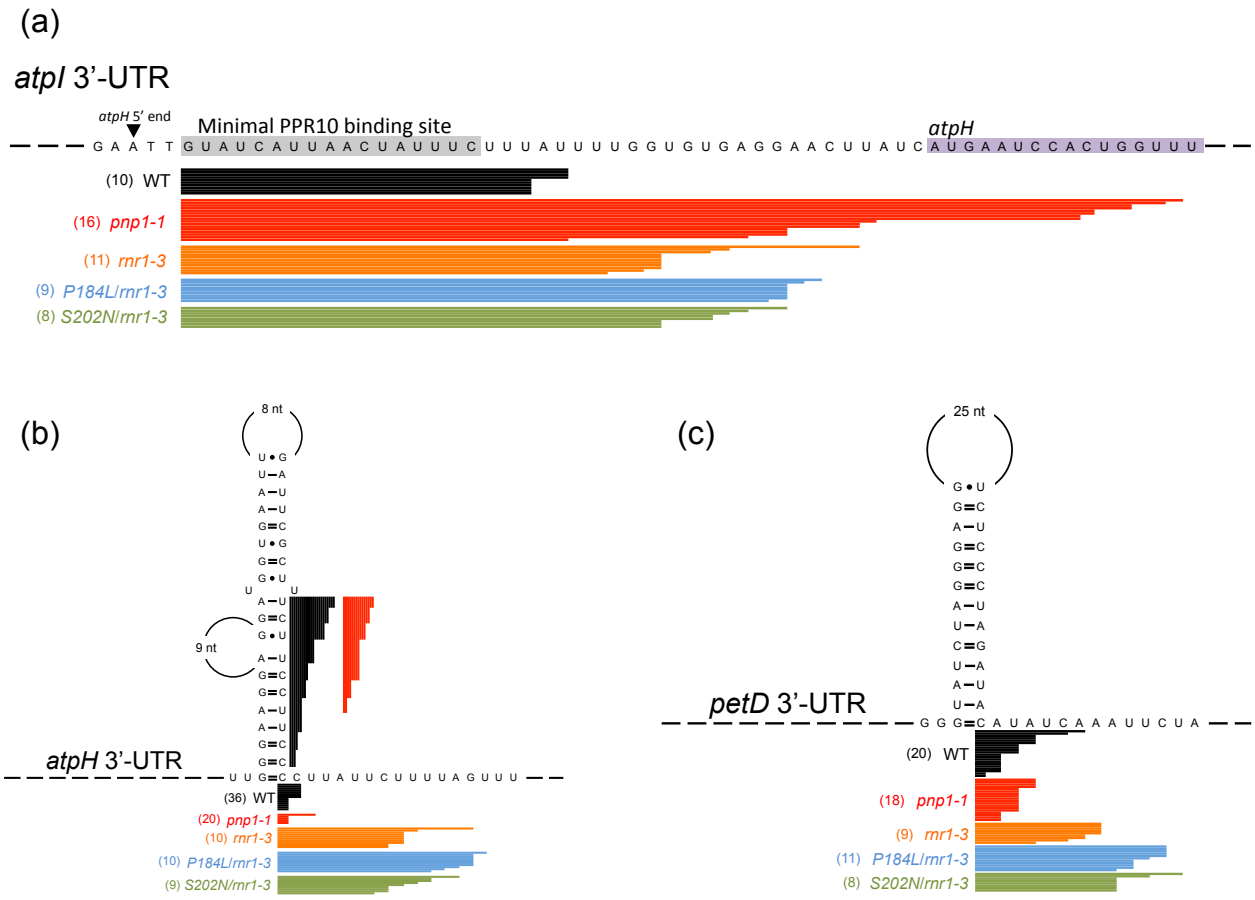


Figure 3.5: Precise 3' end mapping of *atpI*, *atpH* and *petD* transcripts for WT, *pnp1-1*, *rnr1-3*, *S202N/rnr1-3* and *P184L/rnr1-3*.
3' ends are mapped below the sequences with each horizontal line representing a cloned cRT-PCR product. The number in parentheses indicates the total number of clones sequenced. 5' ends are not shown because they were unaffected by genotype. (a) The *atpI-atpH* intergenic region, with the minimal PPR10 binding site shaded in grey. The PPR10-dependent *atpH* 5' end as well as the beginning of the *atpH* ORF, shaded in light purple, are also shown.
(b - c) Putative stem-loops defining the 3' ends of the *atpH* and *petD* transcripts.

abut the PPR10 binding site. As shown in Figure 3.5a, we confirmed that the WT *atpI* 3' end is located 3-5 nt downstream of the minimal PPR10 binding site. In contrast, the 3' ends found in the *pnp1-1* mutant are heterogeneous and extend into the coding region of the neighboring *atpH* gene. This is consistent with the general finding that chloroplast RNAs are 3'-extended in this mutant. When the *rnr1-3* single mutant was analyzed, no WT 3' ends were found. Instead, we found extensions of 2-16 nt, suggesting that cpPNPase cannot fully resect the RNA immediately downstream of bound PPR10. As the activity level of cpPNPase is depleted from the *rnr1-3* mutant, we witnessed an increase in the length of the 3' extensions with those in *S202N*/*rnr1-3* being 5-12 nt, while the majority of the extensions are approximately 12 nt in *P184L*/*rnr1-3*.

For *atpH* and *petD*, stem-loop structures are believed to define 3' ends by blocking further 3'→5' activity. For these genes, analogous results to *atpI* were obtained, with progressively longer 3' extensions as cpPNPase activity was depleted from the *rnr1-3* mutant background. In the case of *pnp1-1*, only the 3' ends that mapped close to the WT are displayed, although a population of transcripts extending much further downstream also exists (Figures 3.2c and 3.3c in Germain *et al.* 2011). In contrast to *atpI* and *petD*, *atpH* 3' ends were heterogeneous for both the WT and *pnp1-1*, including sequences that are part of the predicted stem-loop structure. We suspect that the 9-nt bulge leads to a dynamic secondary structure, allowing RNR1 to process the transcript further than cpPNPase.

Non-polysomal mRNAs over-accumulate in the absence of RNR1

As shown above, examination of the *rnr1-3* single mutant revealed a general overaccumulation of most mRNA species compared to WT (Figures 3.4 and S3.2). Enhanced mRNA accumulation is particularly striking when the fact that rRNAs are much less abundant in *rnr1-3* is taken into account. Because rRNAs are underrepresented in *rnr1-3*, we decided to determine the translational status of some mRNAs by assessing their polysomal association. Figure 3.6 shows that the amount of *psbD*, *psbC*, *atpI* and *atpH* mRNAs assembled with ribosomes (fractions 7-12) is equivalent between WT and *rnr1-3*, whereas mRNA accumulation is much higher in the nonpolysomal (1-6) fractions of *rnr1-3*. Similar polysome loading was observed for *rbcL*, *psaB*, *atpE* and *petD* (Figure S3.3), showing that the phenomenon holds for both mono- and polycistronic transcripts. We conclude that excess mRNA in *rnr1*-3 is not engaged in translation, in agreement with the depleted amount of rRNA and previously documented protein deficiencies (Kishine*, et al.* 2004, Bollenbach*, et al.* 2005).

RNR1 depletion leads to a substantial increase in the stability of most chloroplast mRNA species

The excess accumulation of mRNA in *rnr1-3* nonpolysomal fractions raised the question of whether increased stability was responsible, which would suggest that RNR1 recycles mRNAs that cannot be translated due to limited ribosome capacity. To measure mRNA stability *in vivo*, we treated 2-week-old *Arabidopsis* WT and *rnr1-3* seedlings with actinomycin D (ActD) over a period of 24 hr. ActD is a general inhibitor of RNA polymerases, thus it arrests both NEP and PEP-directed transcription within the

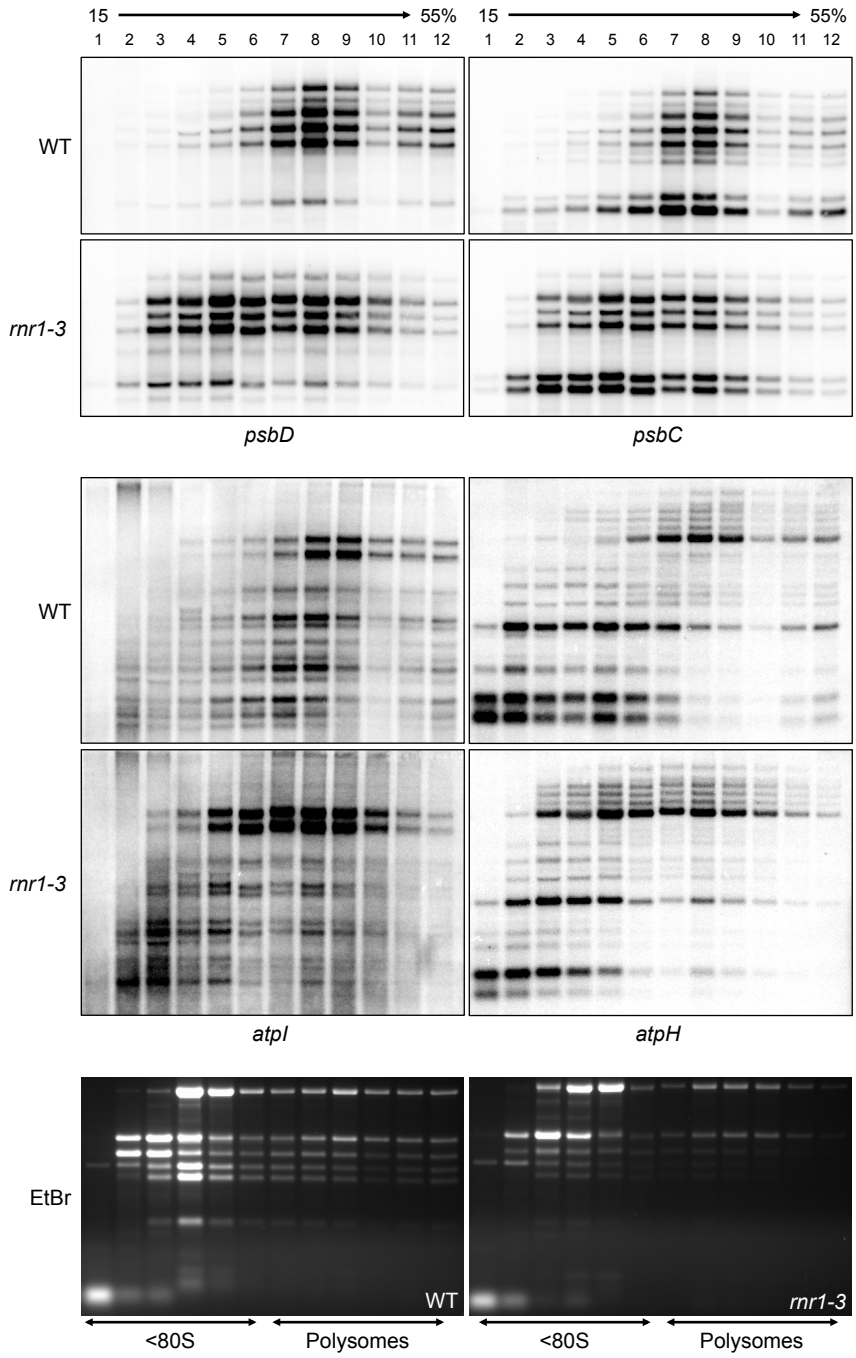


Figure 3.6: Polysomal loading of *psbD*, *psbC*, *atpI* and *atpH* mRNAs for WT and *rnr1-3.*

RNA from 15-55% sucrose density gradient fractions was analyzed by gel blot with the probes indicated at the bottom of each panel. Equal proportions of each fraction were loaded. The bottom panels are ethidium bromide-stained gels to reflect loading. The position of ribosomal (<80S) and polysomal fractions indicated at the bottom, were determined by running puromycin-treated samples in parallel to experimental samples.

chloroplast, and has been used previously for this purpose (Shu and Hong-Hui 2004, Narsai *et al.* 2007). Figure 3.7a shows the profiles of *atpH* mRNAs in ActD-treated WT and *rnr1-3* plants. Of the mRNA species designated 1 to 4, *atpH*-1, *atpH*-2 and *atpH*-4 decay during the WT time course, whereas *atpH*-3 does not appreciably change in abundance. In *rnr1*-3, however, *atpH*-2 and *atpH*-4 appeared to be more stable. To quantify these results, we re-hybridized the blots with a nuclear 28S rRNA probe as an internal control, since this rRNA has been shown to be stable for up to 24 hrs (Narsai*, et al.* 2007). The amount of each *atpH* mRNA species was normalized to the amount of 28S for each time point and plotted on the graphs displayed in Figure 3.7b, which confirmed the initial conclusions drawn from the RNA gel blots. Using linear regression, the half-life of each transcript was calculated within the 24 hr period or extrapolated up to a maximum of 96 hrs (Table 3.3). Under our experimental conditions, only *atpH*-3 had a half-life longer than 24 hrs in WT, whereas all *atpH* mRNA species had half-lives longer than 24 hrs in *rnr1-3*, with changes most pronounced for *atpH*-2 and *atpH*-4. Of note were the higher confidence (R^2 values) for the WT compared to *rnr1-3*, reflecting the difficulty in calculating accurate decay rates for extremely stable species.

The analysis of *psbC*, *psbA* and *rbcL* transcript decay kinetics gave equivalent results with a sometimes substantial increase in the stability of mRNA species in *rnr1-3* as compared to the WT (Table 3.3; Figure S3.4 shows identities of individual mRNA species). Other mRNAs, including the *petD* intron and the intron-encoded *matK* transcript, showed the same trend (Figure S3.5).

As an additional control, we probed for the nuclear *RBCS* transcript and found similar half-lives in WT and *rnr1-3* (Figure 3.8). This result was expected since RNR1 is

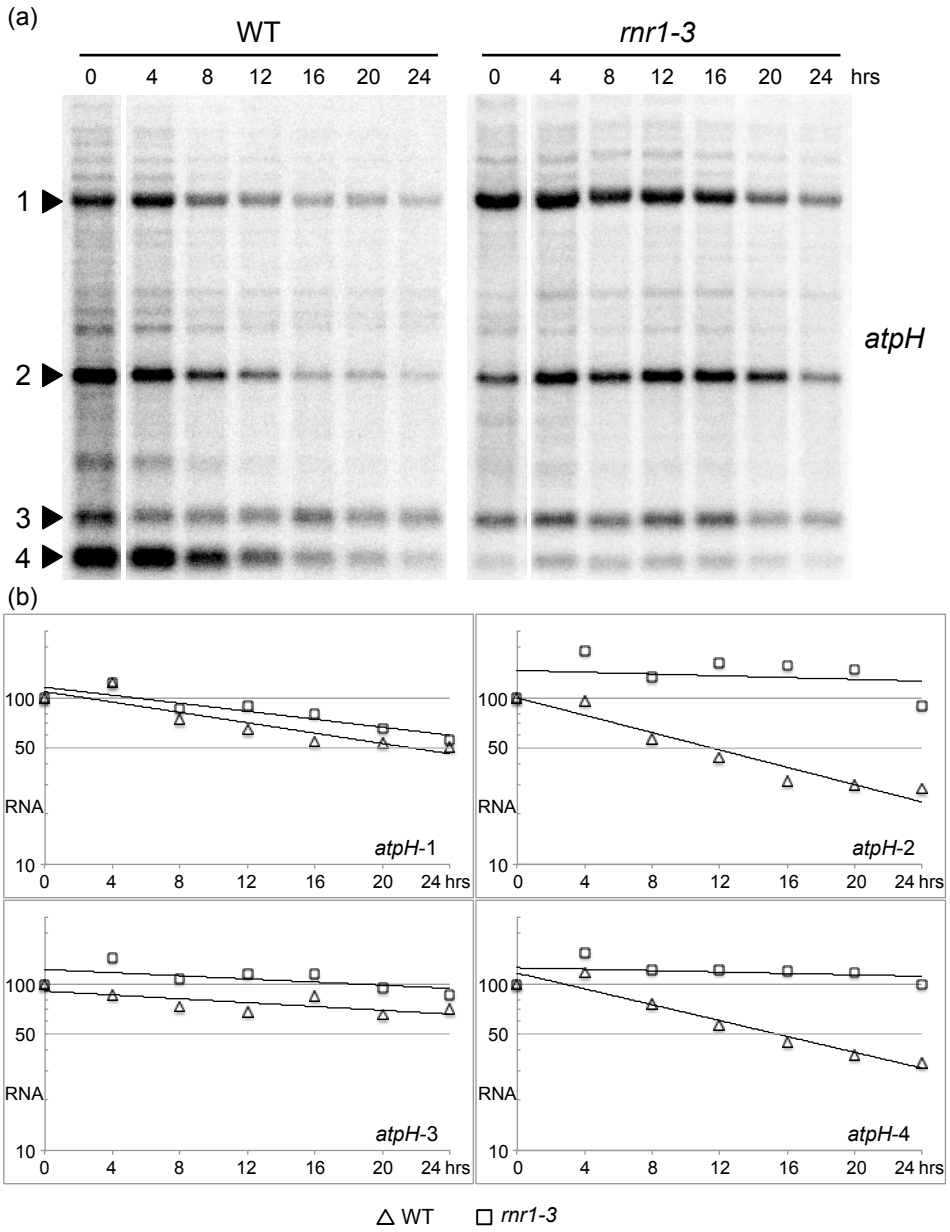


Figure 3.7: Accumulation of *atpH* mRNA in ActD-treated WT and *rnr1-3* plants over a 24 hr time course.
(a) Results using the *atpH* probe indicated in Figure 4b. The numbered arrowheads refer to the transcripts that were quantified and used to derive the graphs in panel (b). (b) Graphs representing linear regression analysis plotted from the gels in panel (a), after normalization to the 28S rRNA signal from the same blot. The regression analysis was used to calculate or extrapolate the mRNA half-lives presented in Table 3.

Band name	WT		*rnr1-3*	
	Half-life (hrs)	R^2	Half-life (hrs)	R^2
atpH-1	22	0.82	30	0.82
atpH-2	11	0.92	>96	0.04
atpH-3	45	0.54	81	0.32
atpH-4	15	0.93	>96	0.09
psbC-1	19	0.91	78	0.27
psbC-2	11	0.92	44	0.47
psbC-3	11	0.98	51	0.38
psbC-4	14	0.94	>96	0.08
psbA	33	0.98	>96	0.004
rbcL-1	14	0.92	29	0.72
rbcL-2	5.2	0.98	16	0.95
RBCS	0.8	0.93	1.1	0.88

Table 3.3: Half-lives of *atpH*, *psbC*, *psbD*, *psbA*, *rbcL* and *RBCS* transcripts in WT and *rnr1-3*.
Half-lives were calculated or extrapolated from the graphs of Figure 7, 8 and S5 with a maximum extrapolation at 96 hrs. R^2 represents the scattering of the data around the linear regression and therefore the confidence of the deduced values for half-lives.

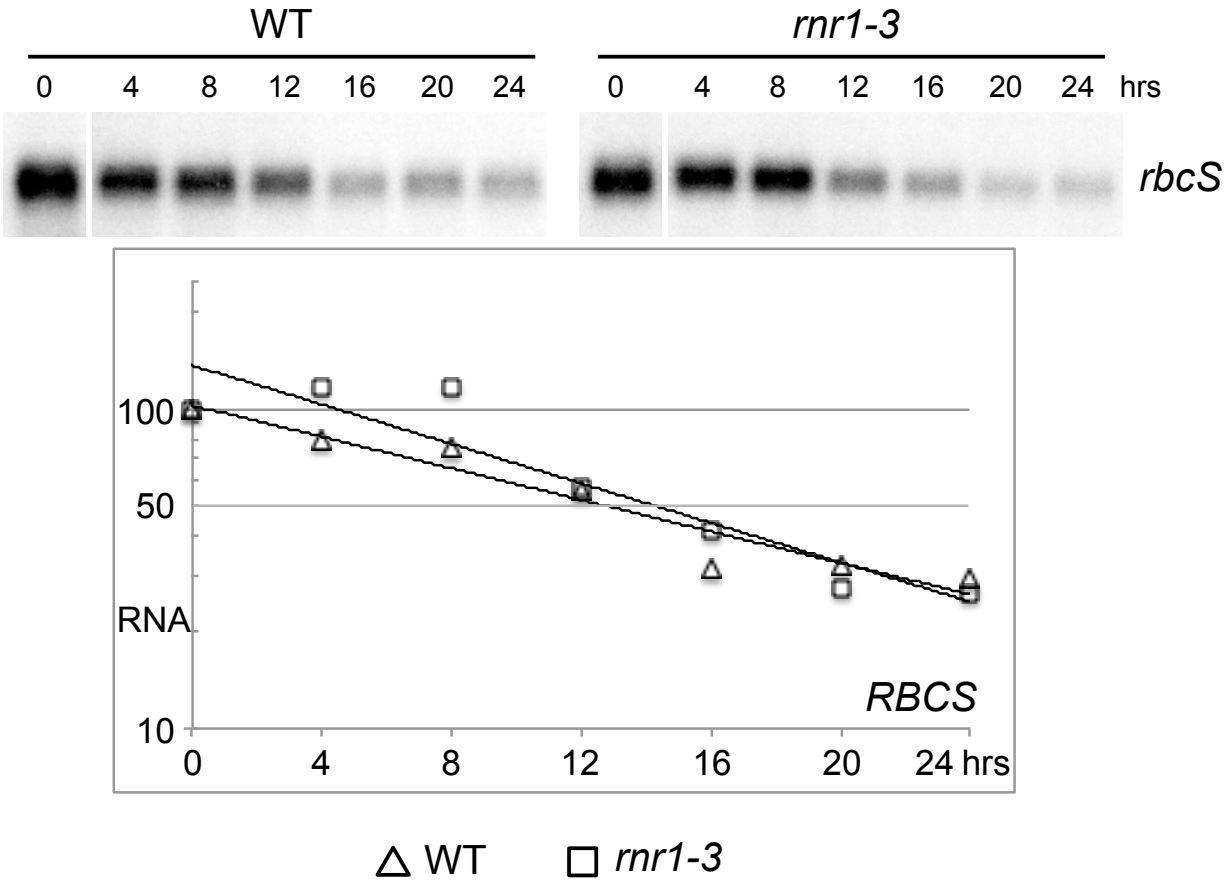


Figure 3.8: Accumulation of *RBCS* mRNA in ActD-treated WT and *rnr1-3*, as described in the legend to Figure 3.7.

organellar and should not influence cytosolic mRNA decay rates, but the experiment largely excludes pleiotropic effects on the results we obtained with *rnr1-3*. Because RNR1 colocalizes to mitochondria, we looked at the influence of RNR1 depletion on the mitochondrial transcripts *cox1*, *atp9* and *rps4* (Figure S3.6). Surprisingly, we observed virtually no decrease in mRNA accumulation following ActD addition, implying that mitochondrial mRNA half-lives in *Arabidopsis* are much greater than those of chloroplast mRNAs. To our knowledge, no mRNA decay kinetics have been published for plant mitochondria, but ActD has been shown to block mitochondrial transcription in animal cells (Ostronoff *et al.* 1995, Piechota *et al.* 2006), with tissue-specific stability of mitochondrial mRNAs varying from half-lives of two hrs to no detectable decline over a period of six hrs (Connor *et al.* 1996). Because we did observe mRNA decay for *RBCS* and many chloroplast mRNAs, our results for mitochondria would appear valid. The results do not, however, allow evaluation of a potential role for RNR1 in mitochondrial mRNA decay.

DISCUSSION

Chloroplast 3' to 5' exonucleolytic activity is essential for embryo development

Even though the depletion of both PNPase and RNase II or RNase R in *E. coli* leads to cell inviability (Donovan and Kushner 1986, Cheng*, et al.* 1998), embryo lethality of the *pnp/rnr1* double mutant was not necessarily expected in *Arabidopsis* since seed formation is supported by the metabolism of the parent plant. There is precedent, however, for impaired chloroplast gene expression resulting in embryo-

defective mutants (e.g. Schmitz-Linneweber*, et al.* 2006, Bryant*, et al.* 2011). In one study, Bryant *et al.* (2011) identified mutations that resulted in embryo defects, and 24 of these were in genes encoding chloroplast RNA synthesis and modification components, including PPR, ribosomal and RNA-binding proteins. In addition, the absence of RNase J, a chloroplast exo- and endoribonuclease (Sharwood*, et al.* 2011a), is known to cause embryo lethality (Tzafrir *et al.* 2004). Possible bases for a plastid gene expression requirement during embryo development are discussed at length in Bryant *et al.* (2011).

RNA accumulation is dependent on 3'→5' exoribonucleolytic activity

Using the weak cpPNPase mutant alleles *P184L* and *S202N*, we were able to reduce cpPNPase activity in the *rnr1-3* mutant background. cpPNPase functions predominantly in mRNA maturation, and no major differences had been observed for overall mRNA accumulation in cpPNPase mutants. On the other hand, previous reports on *rnr1* had focused on the loss of mature rRNA in favor of precursors. Adding *pnp* mutations caused modest changes to rRNA precursor accumulation (Figure 3.3), but much more dramatic was the reduced mRNA accumulation in double mutants (Figures 3.4 and S3.2). This phenotype contrasts with both *E. coli pnp/rnr1* and *pnp*/*rnb* (RNase II) mutants, which accumulate RNA degradation products but not at the expense of mature species (Donovan and Kushner 1986, Arraiano *et al.* 1988). On the other hand, the *E. coli* double mutants were induced by shifting to a non-permissive temperature, as compared to the acclimated state of the *Arabidopsis* mutants. *E. coli* mutants lacking PNPase and another 3'→5' exoribonuclease, RNase PH, are also inviable (Zhou and

Deutscher 1997). In this case, 23S rRNA is highly unstable, more closely resembling our results for mRNAs in the *Arabidopsis* mutant. In contrast, even a quadruple 3'→5' exoribonuclease mutant in *B. subtilis* remains viable (Oussenko *et al.* 2005). These differing results show that the importance of specific ribonucleases can vary depending on which enzymes are present and which activity dominates. In the double mutants described here, we speculate that 3' end maturation by either cpPNPase or RNR1 is required for normal RNA stability, potentially reflecting an RNA quality control mechanism. Incorrect 3' end maturation is linked to degradation of nuclear Pol II transcripts (Schmid and Jensen 2010), and *E. coli* exerts quality control of tRNA through a degradation mechanism targeting abnormal 3' ends (Li *et al.* 2002).

The maturation of mRNA 3' ends is achieved cooperatively

The precise mapping of *atpI*, *atpH* and *petD* 3' ends in the *rnr1-3* mutant corroborates the *atpI*/PPR10 assay using recombinant cpPNPase (Prikryl*, et al.* 2011), where cpPNPase alone left 4-7 nt extensions, that we show here can be removed by RNR1 (Figure 3.5a). HCF107, another chloroplast RNA-binding protein that defines RNA termini, shows similar properties when bound to its RNA substrate *in vitro* and challenged with commercial prokaryotic PNPase and RNase R (Hammani*, et al.* 2012). An analogous result was observed for *petD* and *atpH*, where 3' ends are likely protected by stem-loops rather than bound proteins (Figure 3.5b and c). In all cases, the double mutants accumulate progressively longer 3' ends as cpPNPase activity is depleted in the *rnr1-3* background, demonstrating their cooperative action in mRNA maturation. RNR1 is likely able to progress closer than cpPNPase to the structures or bound

proteins defining transcript 3' ends, because the cpPNPase catalytic site lies within a bulky hexamer (Baginsky*, et al.* 2001), whereas RNase II is a monomer (Shen and Schlessinger 1982) which in *E. coli* has been shown to leave no overhang (Marujo*, et al.* 2000).

Importance of RNR1 in chloroplast RNA homeostasis

A feature of the *rnr1* mutant that was not previously documented is the widespread excess accumulation of mRNA precursors with enhanced stability, and without a concomitant decrease in mature mRNAs. This implicates RNR1 in maintaining a balance between RNA processing and degradation. In *E. coli*, RNase II protects mRNAs by removing the 3' overhang necessary for PNPase to bind, or for PAP1 to bind and add the oligo(A) tail that triggers degradation by either PNPase or RNase II (reviewed by Matos*, et al.* 2011). Moreover, because RNase II preferentially degrades poly(A) tails, it also stabilizes those transcripts by preventing their engagement in the polyadenylated-triggered degradation pathway (Hajnsdorf *et al.* 1994, Marujo*, et al.* 2000, Mohanty and Kushner 2000b). On the other hand, because of its ability to degrade through extensive secondary structures, RNase R is an important participant in mRNA degradation (Cheng and Deutscher 2005). If chloroplast RNR1 were playing mainly a protective role, its absence should lead to increased RNA decay in the presence of PNPase, which is the opposite of what we observed. Analogy to bacterial RNase R also fails, however, because mature mRNA 3' ends, rather than unprocessed precursors, should feature strong secondary structures that would be sensitive to RNR1.

Sublocalization of cpPNPase and RNR1 may lead to their specialization

Competition must exist between the chloroplast RNA processing and degradation pathways, since many actors are shared. Hints as to how these opposing pathways may be balanced comes from a recent proteomic study of chloroplast nucleoids, which are protein-DNA complexes and the site of DNA and RNA synthesis. It was found that cpPNPase is strongly enriched in the nucleoid, being about 20 times less abundant in the stroma (Majeran*, et al.* 2012). On the other hand, RNR1, which is less abundant overall, was found only in the stroma. Taking into account the ready reversibility of cpPNPase, which responds to the nucleoside diphosphate (NDP) to inorganic phosphate (Pi) ratio, a model emerges that would explain why the major role of cpPNPase is in RNA processing, whereas RNR1 plays a key role in RNA decay. A high NDP:Pi ratio favors the polymerase activity of cpPNPase, while a low ratio favors degradation (Yehudai-Resheff*, et al.* 2001). If the local nucleoid NDP:Pi ratio is low, due to the use of (deoxy)nucleoside triphosphates for DNA replication and RNA synthesis, cpPNPase would be mostly degradative in the nucleoid, the activity required for 3' end trimming. On the other hand, if the stromal NDP:Pi ratio is high, due to the degradation of RNA or from other metabolic pathways, stromal cpPNPase would tend to exert polymerase activity. This would be consistent with a role in RNA degradation, where cpPNPase adds heteropolymeric tails to RNA fragments; such tails are absent from the *pnp1-1* mutant (Germain*, et al.* 2011). If these assumptions are correct, RNR1 would be the only 3'→5' exoribonuclease with degradative activity in the stroma, explaining its strong association with the RNA degradation pathway. The implication of this

hypothesis is that long RNA 3' extensions are removed in the nucleoid, then following export or diffusion of RNA from the nucleoid, stromal RNR1 completes 3' end maturation.

Even though mRNA stability in chloroplasts has been shown to be dependent on the developmental stage and on a number of diverse RNA-binding proteins (e.g. Klaff and Gruissem 1991, Nakamura*, et al.* 2001, Pfalz*, et al.* 2009), current models still feature ribosomes to be an important factor in protecting actively translating mRNAs from degradation (Figure 8 in Pfalz*, et al.* 2009). Our results agree with this in the sense that RNR1 appears to have a disproportionate effect on the nonpolysomal RNA pool, perhaps to balance the mRNA population with available ribosomes. On the other hand, our results do not support the concept that ribosomes protect RNAs from endonucleolytic cleavages that would otherwise initiate degradation. Indeed, the chloroplast endoribonuclease mutants analyzed to date (Walter*, et al.* 2010, Qi*, et al.* 2012) do not overaccumulate transcripts to the degree seen in *rnr1*. Finally, nuclear mutants defective in translation of individual mRNAs, do not additionally impact these mRNAs' stability (e.g. Zerges and Rochaix 1994, McCormac and Barkan 1999, Wostrikoff *et al.* 2001).

Arabidopsis RNR1 behaves as an RNase II enzyme

The main difference between *E. coli* RNase II and RNase R is their sensitivity to secondary structures. RNase R is capable of processing through double-stranded RNAs with at least seven nucleotide overhangs, while RNase II creates blunt 3' ends by rapidly degrading extensions, impeding the binding of other exonucleases, including

PNPase, thereby stabilizing RNAs (reviewed by Matos*, et al.* 2011). Our results show that *Arabidopsis* RNR1 is impeded *in vivo* by secondary structures, as well as RNA binding proteins, and shortens the 3' end extensions left by cpPNPase, similar to the characteristics of RNase II. *In vitro* analysis had already shown that RNR1 is arrested by 3' end secondary structures (Bollenbach*, et al.* 2005). Hence, we assert that RNase II is a more appropriate name than RNR1, for this particular gene product.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana Col-0 plants were grown on MS media under long days (16 h light/8 h dark) at 22˚C. The *pnp1-1*, *P184L*, *S202N*, A263V and *rnr1-3* mutants were previously described (Bollenbach*, et al.* 2005, Germain*, et al.* 2011).

Transcription inhibition with Actinomycin D treatment

Two-week old plants grown as described above were transferred to six-well tissue culture plates lined with chromatography paper imbibed with 1 mL of liquid MS containing 200 $\mu g.mL^{-1}$ of Actinomycin D (Sigma-Aldrich). Treatment was started at the beginning of the afternoon and samples were taken every four hrs for 24 hrs under constant illumination.

RNA analysis

Total RNA was isolated and analyzed by RNA gel blots, qRT-PCR and cRT-PCR as described in Germain *et al.* (2011). For polysome analysis, crude cell lysates from six-week old plants grown on MS media were loaded on 15-55% sucrose gradients as described by Barkan (1998). Primers are listed in Table S3.1 except for those used for rRNA gel blot analysis (Bollenbach *et al.*, 2005), mRNA analysis (Germain *et al.*, 2011) and 5S rRNA qRT-PCR (Sharwood *et al.*, 2011).

ACKNOWLEDGEMENTS

This work was supported by Binational Scientific Foundation (BSF) and Binational Agriculture Research and Development Foundation (BARD) awards 2009253 and IS 4152-08, respectively.

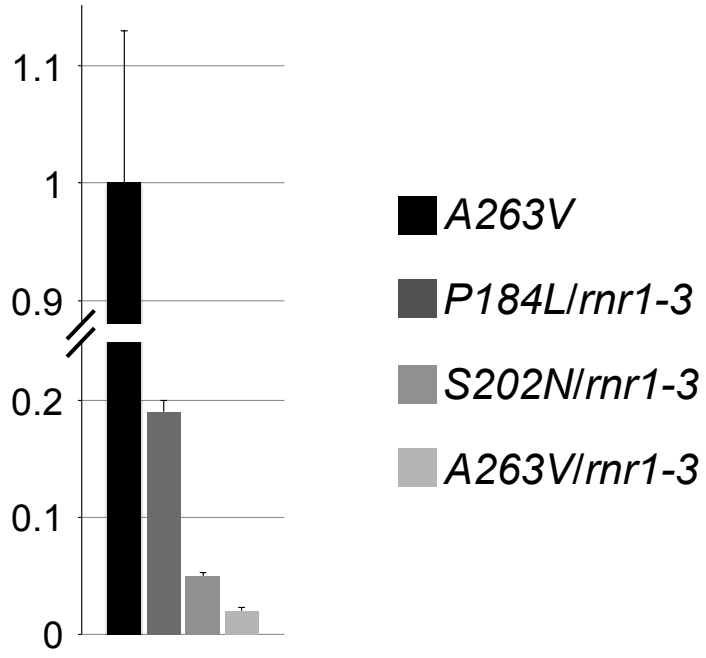


Figure S3.1: Effects on 5S rRNA accumulation of combining *pnp* and *rnr* mutations.
qRT-PCR was used to measure the amount of sequences containing mature 5S rRNA. Reverse transcription was performed using 3' qRT-PCR gene-specific primers as described in Sharwood *et al.* (2011). The results were normalized to actin, and standard error is shown. The amount of 5S rRNA was set to 1 for A263, which acts as a wild-type control. The data are derived from two biological replicates and three technical replicates for each genotype.

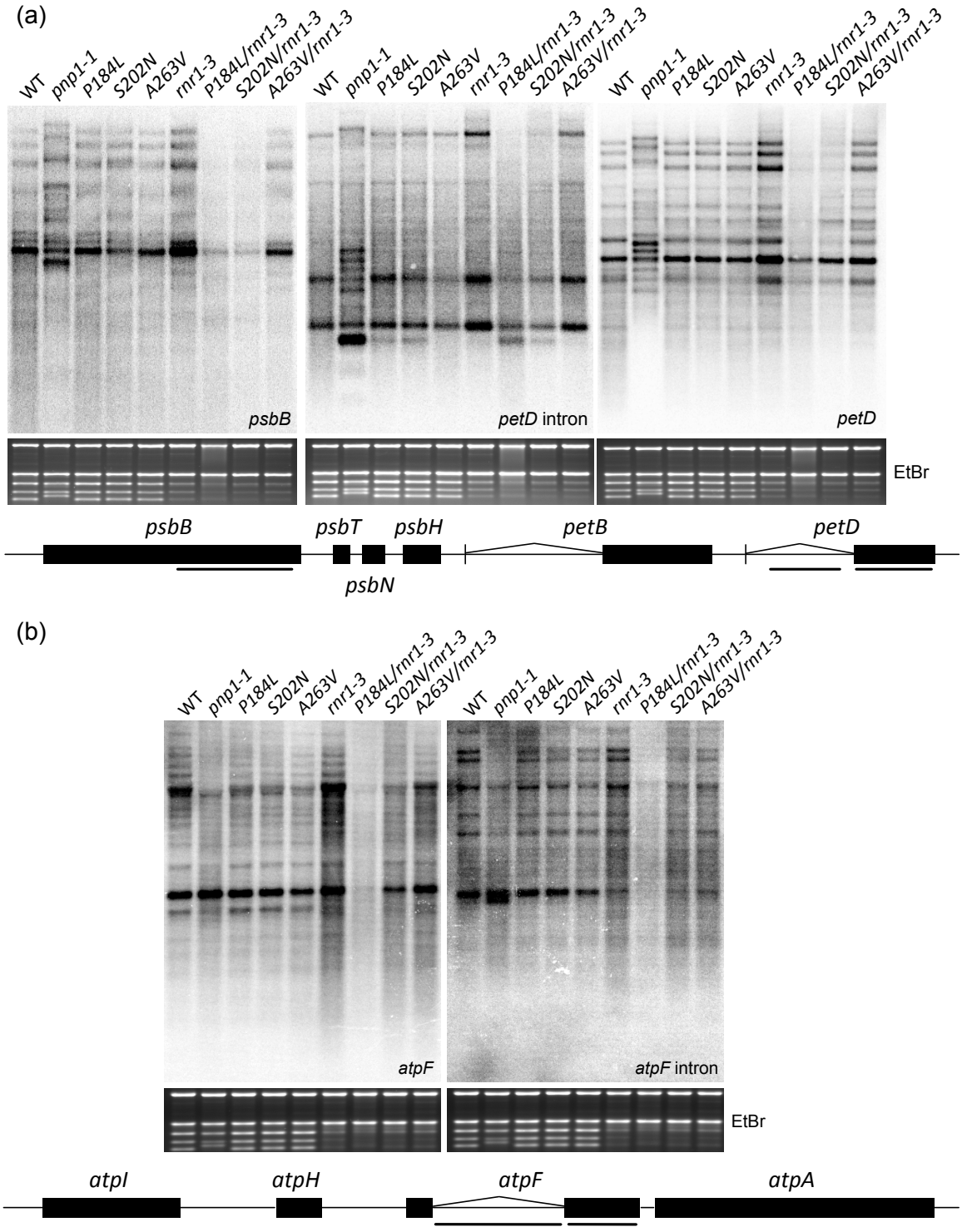
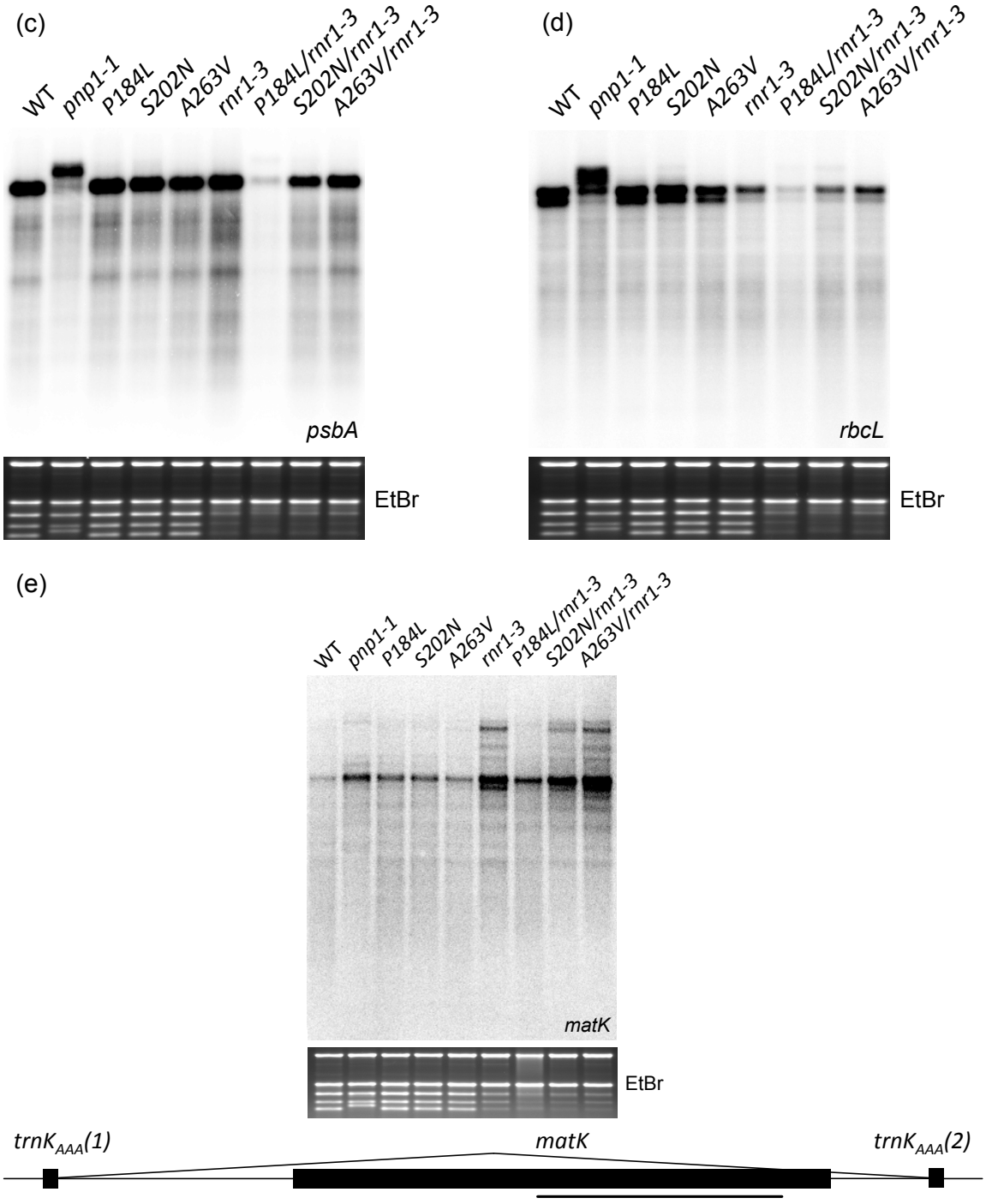


Figure S3.2: RNA phenotypes of single and double mutants.
RNA gel blots were probed with the segments shown in the lower right corner, with their extents indicated by underlining on the schematics.



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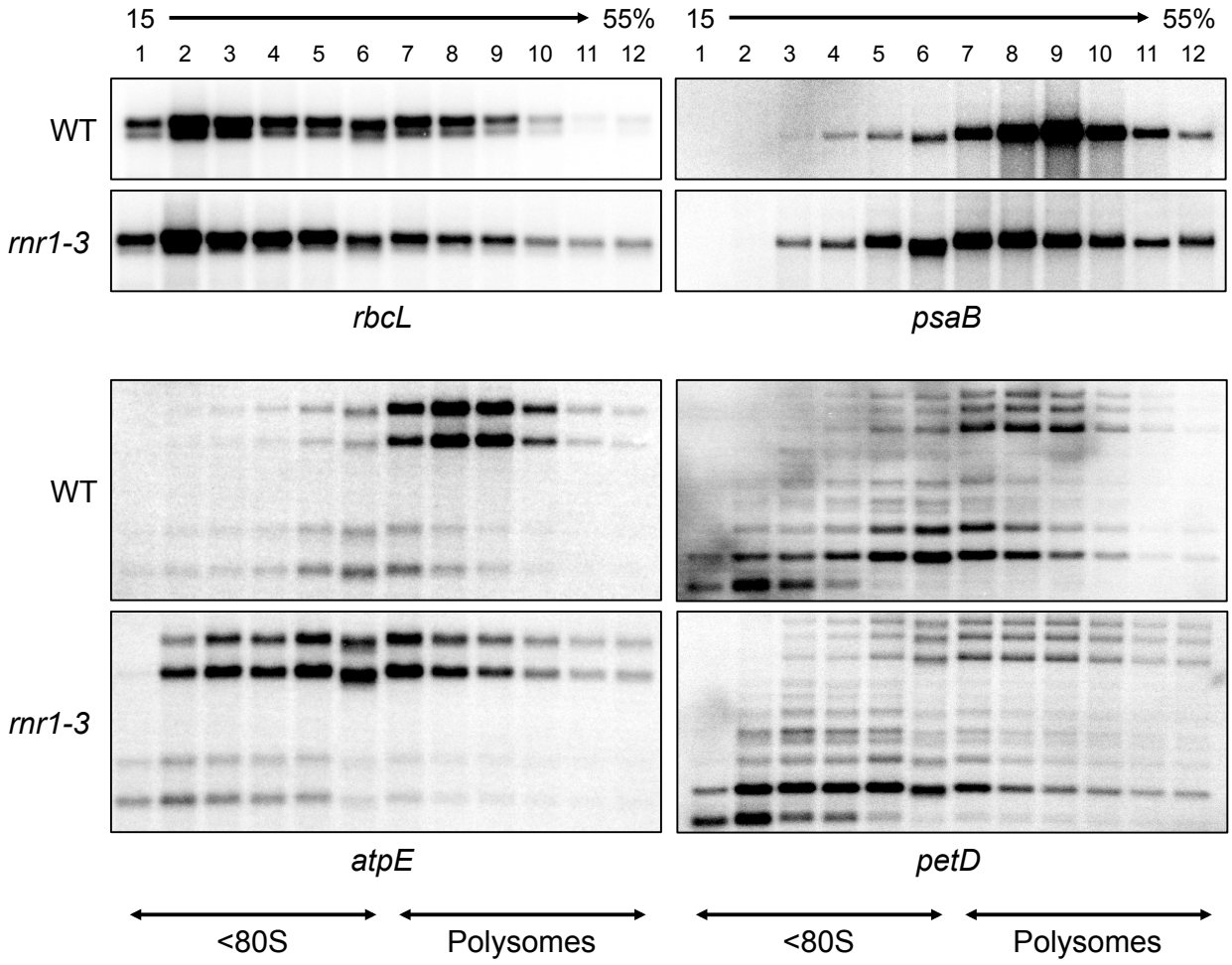

Figure S3.3: Polysomal loading in WT and *rnr1-3.*
Polysomes were separated in sucrose gradients as shown across the top, and RNA from fractions was analyzed by gel blot.

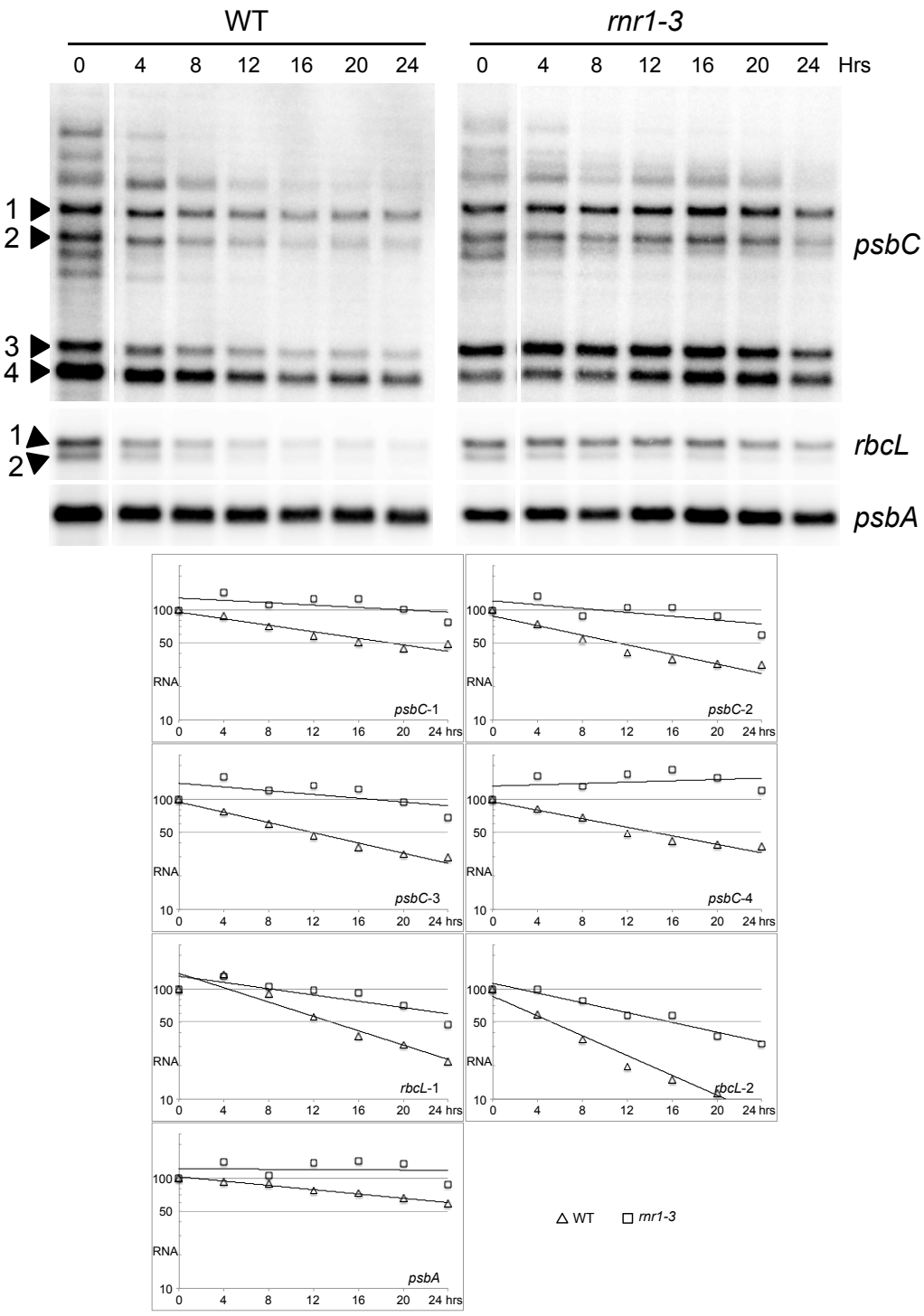

Figure S3.4: Analysis of *psbC*, *rbcL* and *psbA* mRNA in ActD-treated WT and *rnr1-3* plants.

The top part of the figure shows RNA accumulation by gel blot, with numbers at the left corresponding to transcripts whose accumulation is graphed below, as described in the legend to Figure 7.

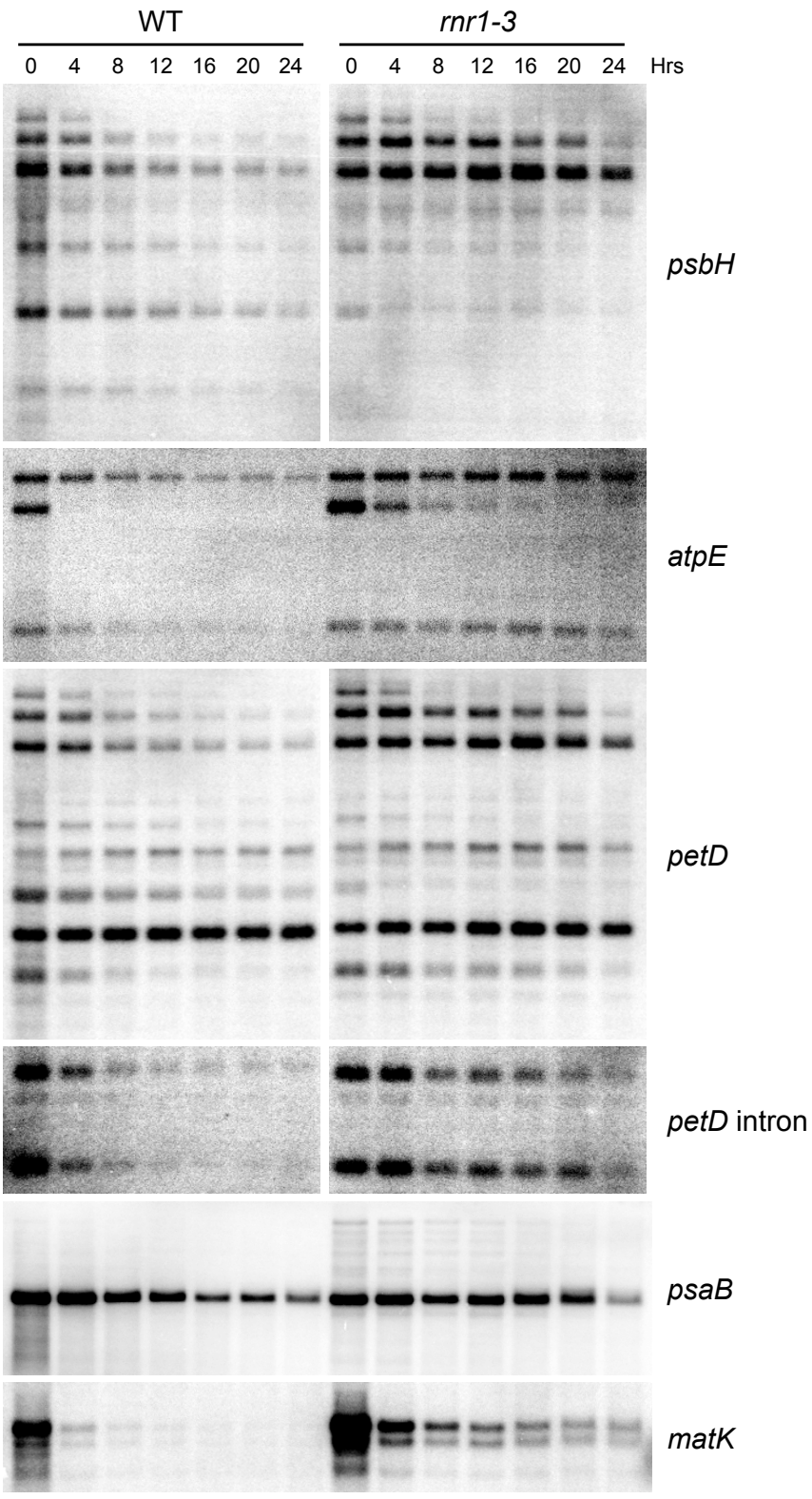

Figure S3.5: Analysis of additional chloroplast transcripts following ActD treatment in WT and *rnr1-3* plants.

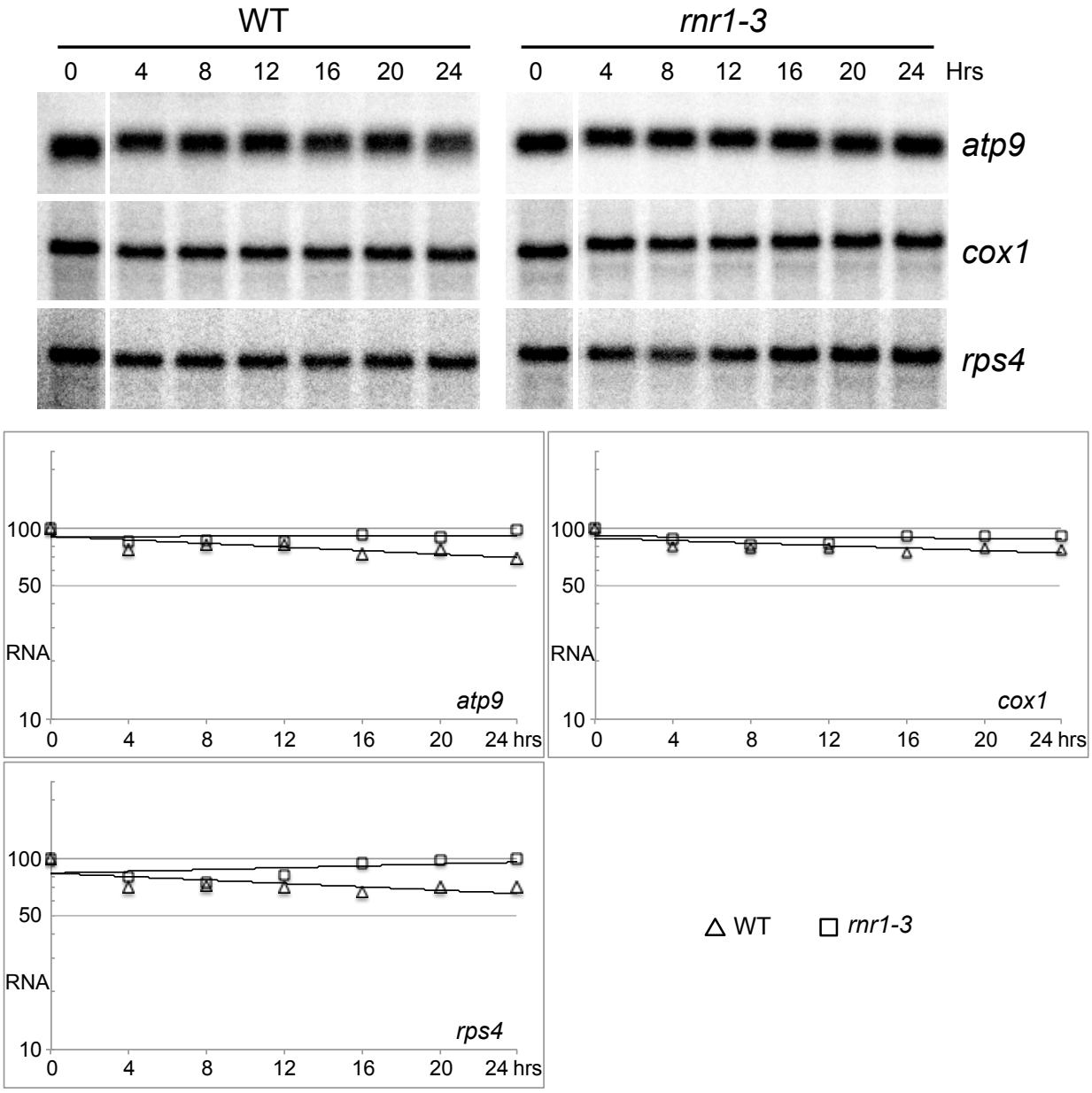

Figure S3.6: Analysis of mitochondrial *cox1*, *atp9* and *rps4* mRNAs in ActD-treated WT and *rnr1-3* plants.

Primer	Sequence (5' to 3')
At atpH cRT-PCR1-5'	GGCCTGGTTGTAGCATTAGC
At atpH cRT-PCR2-5'	GAGGATGGGGAATTAGCACA
At atpH cRT-PCR1-3'	TAACCGAAGCAGCAGAAACC
At atpH cRT-PCR2-3'	TTCCTCACACCAAAATAAAGAAA
At petD cRT-PCR1-5'	AAATCCATTTCGTCGTCCAG
At petD cRT-PCR2-5'	AAATGGGTTCGACTGGAAAA
At petD cRT-PCR1-3'	TTCCCTTCAACTTTTTATGTCAA
At atpI cRT-PCR1-3'	TGATGGAACATGATAAAACATTCA
At atpI cRT-PCR2-3'	ACCTGGAAACCCCCTATTTG
At atpI cRT-PCR1-5'	GGTGAGTCTATGGAAGGTCATCA
At atpI cRT-PCR2-5'	TTTACAAGCGGGATTCAAGC
At atpI cRT-PCR3-5'	ATCCCGCGGATAGGAACTAC
At atpI cRT-PCR4-5'	ACGTCCTAAGTCATTGGATGAT
At rbcS-5'	GGAAAGAAGAAGTTTGAGACTCT
At rbcS-3'	GGCTTGTAGGCAATGAAACTGATG
At atp9-5'	CAACCCGAGATGTTAGAAGG
At atp9-3'	GTGCTTGCTTTATGAGACTG
At rps4-5'	ACCCATCACAGAGATGCACA
At rps4-3'	CGGCATCCCCTCTTAGTTTT
At cox3-5'	CCCAAAAGGGATTGAGGTTT
At cox3-3'	TGATGCTCCTTCGTCAGATG

Table S3.1: Sequences of oligonucleotides primers used in this study.

CHAPTER 4

ABNORMAL PHYSIOLOGICAL AND MOLECULAR MUTANT PHENOTYPES LINK CHLOROPLAST POLYNUCLEOTIDE PHOSPHORYLASE TO THE PHOSPHORUS DEPRIVATION RESPONSE IN *ARABIDOPSIS**

ABSTRACT

The activity of the chloroplast ribonuclease polynucleotide phosphorylase (PNPase) is dependent on phosphate (P) concentrations *in vitro*. Under P starvation conditions, the algae *Chlamydomonas reinhardtii* is able to acclimate and chloroplast RNA levels have been shown to significantly increase. Because this ability was lost in a PNPase deficient strain, we investigated the role of PNPase in *Arabidopsis thaliana* wild-type and *pnp* mutants. Although we found that the viability of the plant was not affected by P starvation, a striking root phenotype was observed, in the form of proteoid-like structures resulting from a decreased auxin responsiveness and cell division. The analysis of chloroplast RNA accumulation did not reveal any significant differences between WT and *pnp* mutants and work from the first two authors of the publication this work was included in supports the hypothesis that the activity of the chloroplast PNPase is involved in plant acclimation to P availability, potentially by ensuring an appropriate balance of metabolites, even under normal growth conditions.

* Marchive, C., Yehudai-Resheff, S., Germain, A. Fei, Z., Jiang, X., Judkins, J. Wu, H., Fernie, A. R., Fait, A. and Stern, D. B. (2009) Abnormal physiological and molecular mutant phenotypes link chloroplast polynucleotide phosphorylase to the phosphorus deprivation response in *Arabidopsis*. *Plant Physiol*, **151**, 905-924.

INTRODUCTION

The role of polynucleotide phosphorylase (PNPase) has been extensively covered in all the previous chapters. I discussed the extensive role of PNPase in chloroplast RNA metabolism in chapter 2, while chapter 3 gives some insight into how PNPase cooperates with RNase II as well as the seemingly crucial role of some amount of 3' processing to occur for plastid RNA stability. Those new data have been integrated along with the latest literature on plastid RNA metabolism in chapter 1 to reflect the current state of the chloroplast RNA field as far as processing and decay is concerned.

This chapter links the regulatory aspect of PNPase with the phosphate (P) metabolism of the plant. Indeed, PNPase has been shown *in vitro* to either consume or release P, depending on the ratio of inorganic P (Pi) to nucleotide diphosphates (NDPs). This results in the liberation of Pi when the Pi:NDPs ratio is low and PNPase is engaged in RNA polymerization, whereas a high ratio leads to RNA degradation by PNPase and the use of Pi (Yehudai-Resheff*, et al.* 2001). Evidently, the latter is not a desired activity in the case of P starvation and it is tempting to speculate about a role of PNPase as a response regulator or as a factor required for the P limitation response.

P is referred to as a macronutrient because it is continually required by every organism and in high amounts, since it is essential both structurally (e.g. nucleic acids) and metabolically. This is especially true in the chloroplast where P is necessary for the metabolisms of ATP, starch but also the regeneration of ribulose-1,6-bisphosphate, the acceptor for CO_2 fixation by RuBisCO. As a consequence, the acquisition and allocation of P is central to plant metabolism, and plants have evolved sophisticated mechanisms

for scavenging P, both from internal and external sources, in order to adjust their metabolism to the limiting conditions that are often encountered. P is in fact considered to be the second most limiting macronutrient after nitrogen (Vance *et al.* 2003).

It is well established that P is a major player in chloroplast metabolism and while the role of PNPase in plastid RNA metabolism is now well-described, "newer evidences have linked PNPase to somewhat unrelated functions. In human cells, for example, a cytosolic fraction of mitochondrial PNPase appears to influence cell differentiation and senescence (Sarkar and Fisher 2006), and in the mitochondrion itself, PNPase is located in the intermembrane space while mitochondrial RNA is in the matrix, suggesting a metabolic rather than RNA catalytic role for PNPase (Chen*, et al.* 2006), through which it can nonetheless influence the accumulation of certain mitochondrial RNAs (Slomovic*, et al.* 2008a). More closely linked to this study, a genetic screen carried out in *Arabidopsis* for resistance to fosmidomycin, which inhibits the plastid methylerythritol phosphate (MEP) pathway identified a chloroplast (cp)PNPase null mutant that was named *rif10* (Sauret-Gueto*, et al.* 2006). This led to the suggestion that the MEP pathway might be regulated by plastid metabolic cues, which in turn could be influenced by PNPase.

This study was stimulated by our finding that in the green alga *Chlamydomonas reinhardtii*, reduced expression of cpPNPase rendered cells unable to acclimate to P deprivation, whereas the same strains had wild-type responses to other nutrient or environmental stresses (Yehudai-Resheff*, et al.* 2007). "The resulting publication compared the response of *Arabidopsis* wild-type (WT) and *pnp* mutant plants under P deprivation with respect to their morphology, metabolite profiles and transcriptomes. My

contribution to this work was focused on analyzing the root morphology as well as investigating the RNA phenotype for both genotypes in either growth conditions.

RESULTS

Lateral root development under P limitation

Amongst many responses to nutrient limitation, drastic modifications in root system architecture are a well-established characteristic that plants use to expand into the rhizosphere. In the case of P starvation, this change translates in the proliferation of lateral roots as well as a significant increase in root hair density is observed (reviewed in Lopez-Bucio *et al.* 2003). Whereas no obvious difference was observed between the WT and two *pnp* mutants (*pnp1-1* and *pnp1-2*) under +P conditions (top panels in Figure 4.1a), the root tip morphology was clearly different under –P conditions (bottom panels in Figure 4.1a). The major difference concerns the presence of proteoid root-like structures that developed in both mutants after four weeks of P starvation. The WT displays normal response to phosphate starvation, with an increased root hair density. We examined the root tip more closely using propidium iodine staining and confocal microscopy (Figure 4.1b). At that level of detail, we can see that many epidermal cells in the *pnp* mutants are swollen and extrude from the surface of the root under –P conditions, a phenotype mostly absent in WT roots under –P conditions. Here again, both WT and the *pnp* mutants display a similar root phenotype under +P growth conditions.

To eliminate the possibility that abnormal chloroplast RNA metabolism was

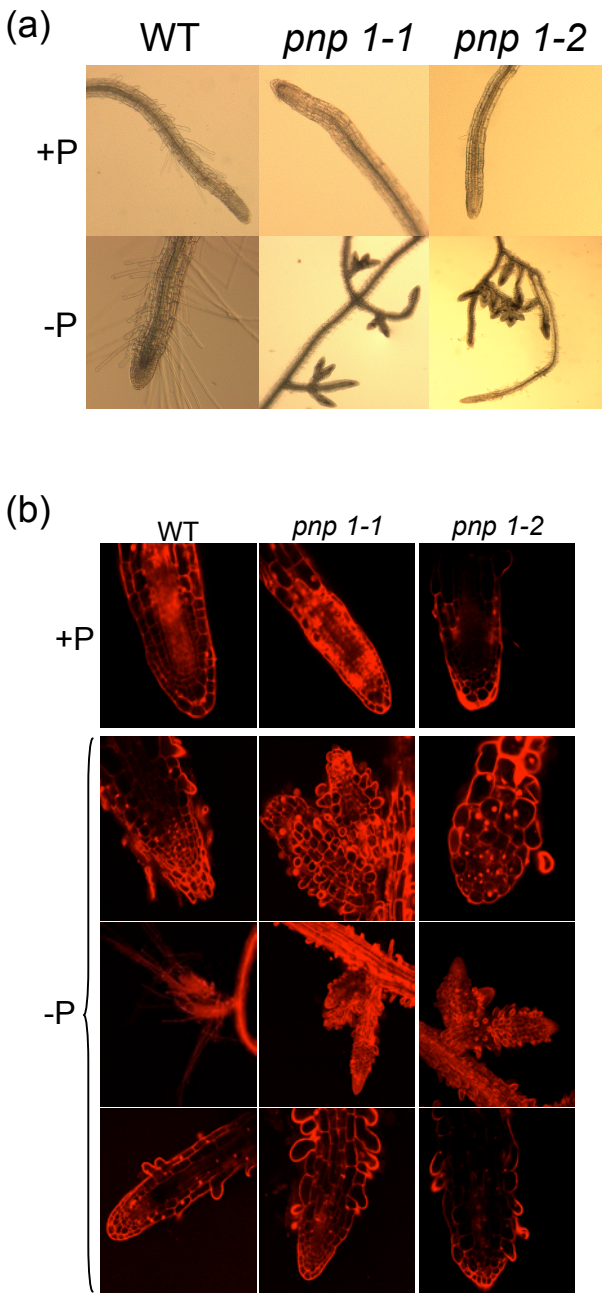

Figure 4.1: Lateral root phenotype under +P or –P conditions, after germination on a full nutrient medium for two weeks, followed by four weeks of either +P or –P medium growth.

WT, Wild type.

(a) Root tips observed using a stereomicroscope (Olympus SZX12).

(b) Root tips observed using a confocal microscope (Leica) after propidium iodine staining.

causing this phenotype by some pleiotropic effect, we incorporated the *csp41b* mutant as a control (Bollenbach*, et al.* 2009). “Because the *csp41b* mutant was indistinguishable from the WT under –P conditions, we concluded that the aberrant root architecture phenotype of *pnp1-1* and *pnp1-2* was not related to altered chloroplast RNA metabolism per se. Instead, we suspected that some function of PNPase was essential to lateral root elaboration”. We therefore decided to examine this phenomenon more closely by backcrossing the *pnp1-1* T-DNA insertion into the two backgrounds expressing the GUS reporter gene under the influence of two distinct promoters commonly used in studies of root physiology under P starvation conditions (Ticconi *et al.* 2004, Sanchez-Calderon *et al.* 2005). The activation of the first one, named DR5, is dependent on the hormone auxin (Ulmasov *et al.* 1997) and the second one is the cyclin B1 promoter (Colon-Carmona *et al.* 1999), which reflects the division status of cells.

Figure 4.2a shows the results after GUS staining for WT and the *pnp1-1* mutant lines, which contain the CYCB1::GUS construct. When grown on +P medium, the staining intensity was comparable between the WT and *pnp1-1*, reflecting a normal rate of cell division in both cases. A slight difference concerns the extend of the dividing zone which doesn’t seem to be as spread along the length of the root of *pnp1-1* (arrows in top panels). This is consistent with the slower overall growth rate observed in the mutant plants. While no significant change is present in the WT root meristematic upon transfer to –P medium, the complete absence of GUS staining from most of the root tips that are part of the proteoid-like cluster is striking in *pnp1-1*. In each case, it looks as if the smallest and most likely newest root primordia is the only one displaying any cell division activity, suggesting the cessation in the majority of lateral root initiates (arrows

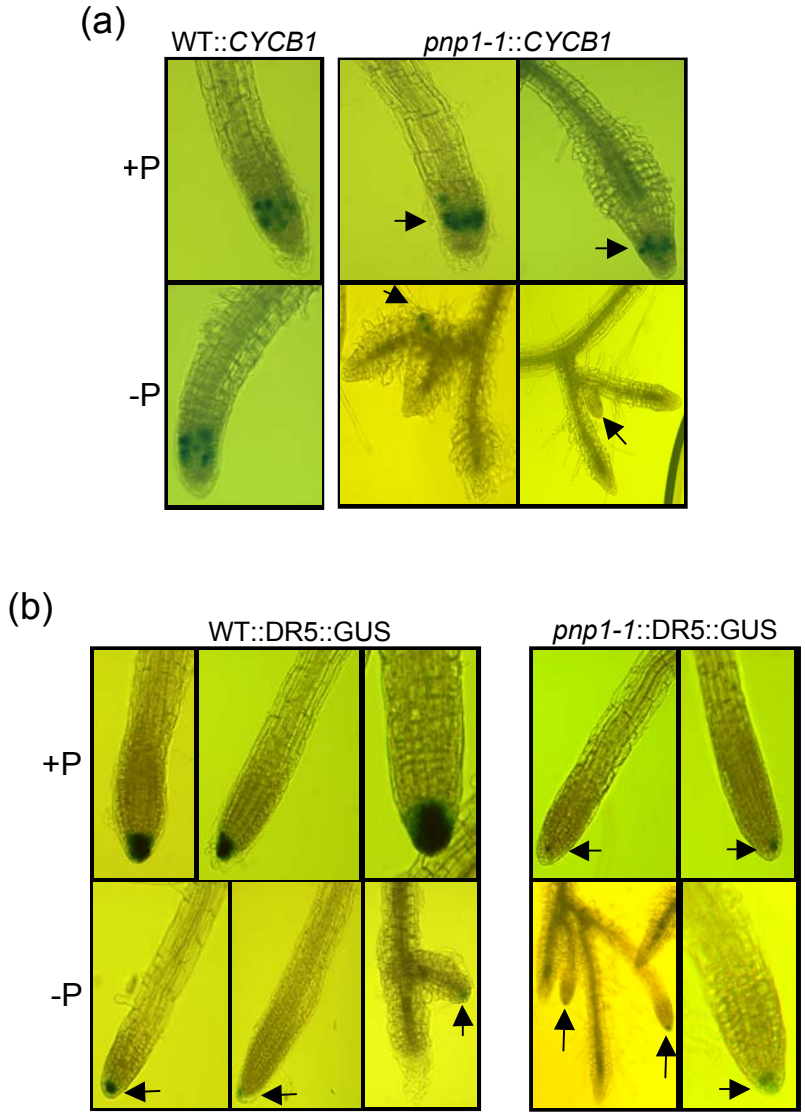

Figure 4.2: Histochemical staining of the GUS reporter enzyme using the technique adapted from Jefferson (1987).
Samples were stained using 2mM 5-bromo-4-chloro-3 indolyl-β-glucuronic acid (Sigma) dissolved in a 100 mM sodium phosphate buffer (pH 7.2) containing 0.2% Triton X-100, 2 mM $K_4Fe(CN)_6$-H_2O, and 2 mM $K_3Fe(CN)_6H_2O$. After washing with distilled water and mounting in 50% glycerol, root tips were viewed using the Olympus SZX12 stereomicroscope.

(a) Reporter gene CYCB1::GUS in the WT and *pnp1-1* background.
(b) Reporter gene DR5::GUS in the WT and *pnp1-1* background.

in bottom panels), a similar phenomenon as reported for the *pdr2* mutant under –P conditions (Ticconi*, et al.* 2004).

Analysis of the DR5::GUS reporter lines gave concordant results, with a strong signal in the WT root tips grown on +P medium, reflecting the high auxin environment necessary for a normally dividing root meristem (top left panels in Figure 4.2b). When grown on –P medium, the WT exhibited reduced staining at the root tip (bottom left panels in Figure 4.2b), a result consistent with another study showing an age-dependent decrease DR5::GUS expression following P starvation as compared with seedlings grown on +P medium (Sanchez-Calderon*, et al.* 2005). This latter signal was the same intensity as in the root tip of *pnp1-1* roots grown on +P medium, once again probably representative of the reduced growth rate observed in the mutant compared to the WT (top right panels in Figure 4.2b). When examining the proteoid-like structures, the same staining pattern as for CYCB1::GUS was detected, suggesting a correlation between auxin level and cell division activity, as expected, but also the auxin-deficient environment of the root tips that did not display any sign of division.

The use of the dye Evans blue, which cannot pass intact membrane barriers, was used to see if cell death had occurred in those root tips that were lacking both GUS signals in Figure 4.2a and b. The majority of those cells stained with this dye and were therefore shown to be dead, indicating that the proteoid-like structures are the result of abortive lateral root initiation under –P conditions, where the aborted roots cease division and undergo cell death.

Lateral root development under P limitation in the single amino acid mutants

We decided to briefly take a look at this intriguing root phenotype in the PNPase single amino acid mutants, for which the location of the substituted amino acid is displayed in Figure 2.1a. Whereas all the mutants had a WT-like rosette phenotype, only *G596R* and *D625N* were like the *pnp* mutants, with new yellow leaves that matured green. The root phenotype did not, however, correlate with the rosette phenotype (Figure 4.3). Indeed, apart from *P529L* and *D625N*, all the mutants had aborted lateral root initiation, with what seems to be an intermediate phenotype for *A213T, A263V* and *P589S*. Of note is the *D625N* mutant, which we show accumulates an inactive PNPase protein but displays a wavy root phenotype, not observed in any of the other mutants.

Chloroplast RNA does not accumulate in response to P limitation

As described in the introduction, the catalytic activity of PNPase is reversible, depending on the Pi:NDPs ratio, with high ratios favoring degradation and low ratio favoring polymerization (Yehudai-Resheff*, et al.* 2001). The hypothesis that the level of RNA would vary according to the P status of the plant comes from the observation by Yehudai-Resheff *et al.* (2007) in the algae *Chlamydomonas*, where the accumulation of the *petD* and *tufA* chloroplast transcripts increased substantially after 24 h of P limitation. We germinated *Arabidopsis* seeds on +P media and grew the plants for 2 weeks before transferring half of them to a +P or –P medium and run the experiment over the course of four weeks, taking samples every week. At that point, we transferred back all the plants on a fresh +P medium and took a final sample two weeks later.

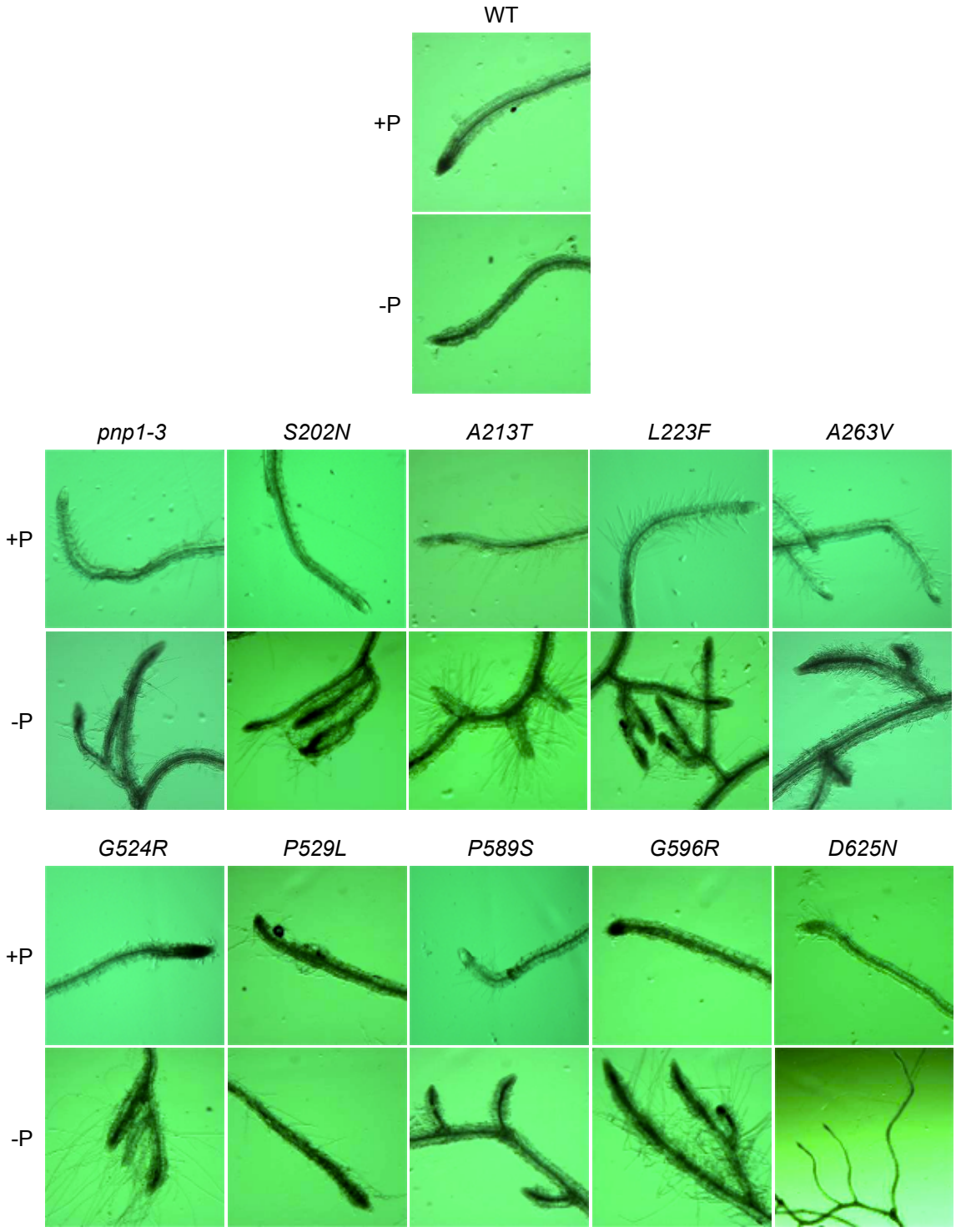

Figure 4.3: Lateral root phenotype under +P or –P conditions, after germination on a full nutrient medium for two weeks, followed by four weeks of either +P or –P medium growth.
WT, Wild type. All the mutants lines presented are introduced in chapter 2.

After RNA extraction, we probed RNA blots for *rbcL* and *psbA*, but no significant increase was observed in WT, under either growth condition (Figure 4.4). When analyzing the two null mutants *pnp1-1* and *pnp1-2*, some variation was detected under –P conditions, with some overaccumulation of the transcripts. Such result was not seen, however, in the single amino acid mutants *G596R* or *D625N*, which do not accumulate any PNPase protein or have an inactive version of it, respectively. Finally, when analyzing two other mutants from either the first or the second core domain of the PNPase protein, namely *A263V* and *P529L*, the same pattern as WT was detected. Overall, no significant or consistent difference was detected for any of the lines analyzed.

DISCUSSION

PNPase deficiency affects root adaptation to –P stress

The inability of the *pnp* null mutant plants to elaborate lateral roots (Figures 4.1 and 4.2) was very similar to the *pdr2* mutant phenotype as a response to phosphate deficiency (Ticconi 2005). The *pdr2* mutation results from a point mutation in At5g23630, which codes for a P-type ATPase and is therefore directly to the P metabolism. PDR2 has been detected in the endoplasmic reticulum (ER), where it interacts with the multicopper oxidase LPR1 (Ticconi *et al.* 2009). Both PDR2 and LPR1 are required for proper expression of scarecrow (SCR), a key regulator of root patterning. All together, it is proposed that this pathway adjusts the root meristem activity to external P status. Because the *pnp* root phenotype was not observed the

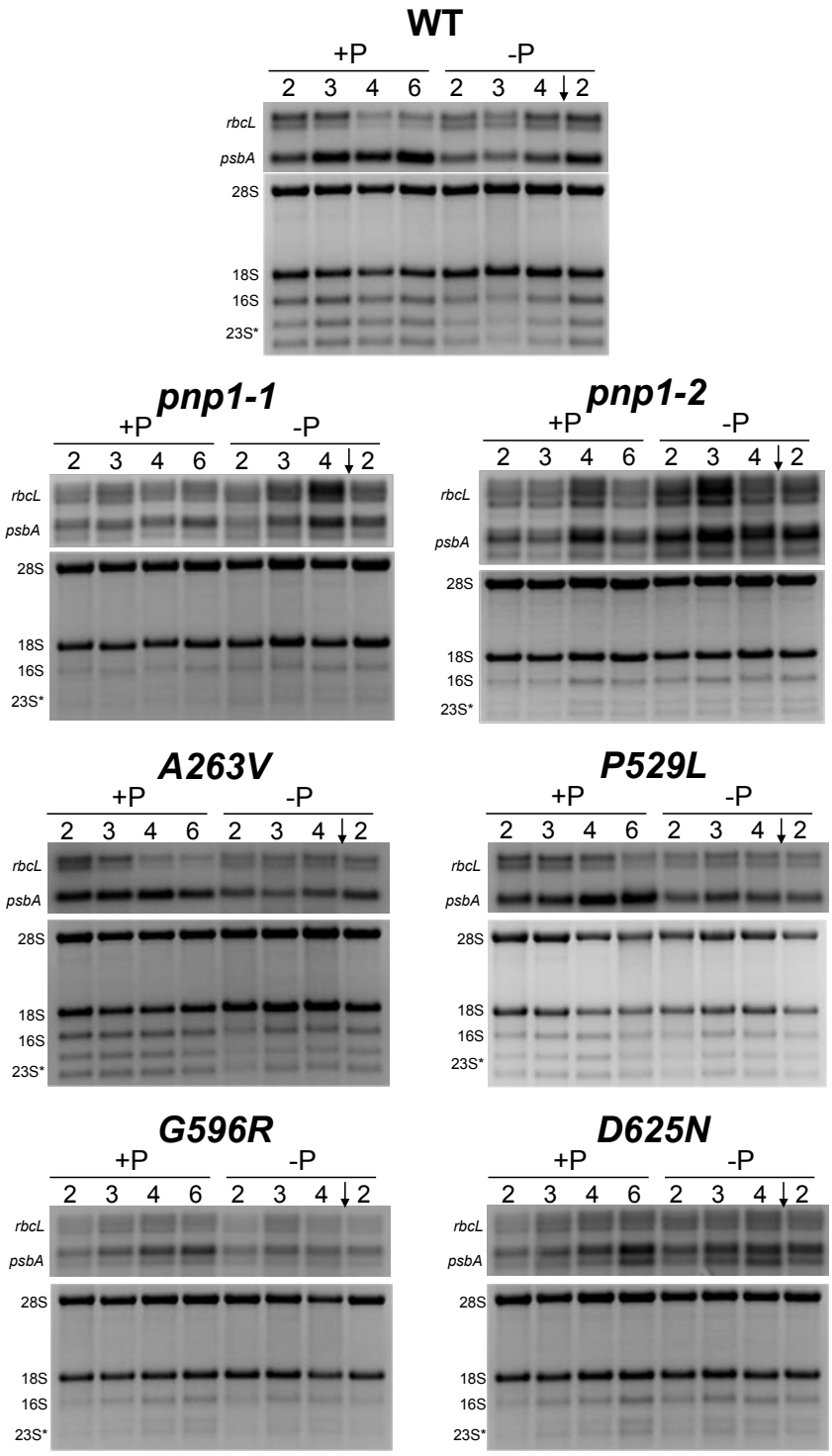

Figure 4.4: Total RNA was isolated and analyzed by RNA gel blots as described in chapter 2.

chloroplast ribonuclease mutant *csp41b*, it does not seem that there is a link between the chloroplast RNA metabolism and root development. Instead, the link might be related to the P metabolism within the chloroplast, which in turn affects the sensory system of the ER pathway, affecting the expression or potential localization of SCR. It might be relevant to test this hypothesis by looking at the expression level of *SCR* in the *pnp* mutant.

The results obtained in the single amino acid mutants are more puzzling, since they seem to disconnect the P metabolism from PNPase, and instead link PNPase directly to the root-patterning pathway. Indeed, it seems that a number of substitution of conserved amino acid leads to proteoid-like root structures (Figure 4.3). The fact that those amino acids are conserved between the first and the second core domain and amongst different species points to their importance even though they did not seem to directly affect the RNA metabolism as mentioned in chapter 2. A thorough analysis of the P metabolism status in those mutants might reveal to be instructive in order to further investigate the unexpected relationship between a chloroplast ribonuclease and the maintenance of the root meristem identity.

The use of the two reporter genes DR5::GUS and CYCB1::GUS gave some additional insight into the *pnp1-1* root phenotype (Figure 4.2). Cell division seems to be restricted to the youngest root primordia of the proteoid-like structures under –P conditions. The involvement of the hormone auxin, was expected since it is known that P starvation enhances auxin sensitivity in *Arabidopsis* roots and helps lead to higher lateral root density and inhibition of primary root elongation (Lopez-Bucio *et al.* 2002). Although these observations are consistent with the fact that auxin is involved, at least

in part, in the failure to elaborate lateral roots in *pnp1-1* under low P conditions, it is once again difficult to imagine the link between PNPase and the auxin metabolism. Moreover, because auxin metabolism is multifaceted, pinpointing a potential direct effect might reveal to be a tough task to achieve.

RNA metabolism under P limitation

Whereas *Chlamydomonas* WT strains have increased chloroplast RNA levels under P starvation conditions, this was not the case in *Arabidopsis* (Figure 4.4). Evidently, it is risky to try and compare a multicellular organism such as *Arabidopsis*, to the unicellular algae *Chlamydomonas*. Indeed, it is well known that multicellular organisms have the ability to remobilize their pools of phosphate form mature leaves to growing tissues, alleviating a potential lack of a major macronutrient (Rouached *et al.* 2010). As a result, it is not necessarily surprising to find divergent strategies in adapting to a particular stress such as P starvation.

Some of those data were published alongside an extensive analysis of the P starvation response and the plant P status for both WT and the *pnp1-1* mutant. Overall, the study concludes that PNPase and more generally the chloroplast homeostasis maintained by PNPase play a major role in the responses required for *Arabidopsis* to adapt to this stress. Indeed, an interesting observation concerns the microarray data where it was concluded that the *pnp* mutant is always in a general state of response to P starvation, even when grown on P medium.

CHAPTER 5

FUTURE PERSPECTIVES

In this study, I used a reverse genetic approach to demonstrate the broad role of PNPase on chloroplast RNA metabolism, presented in chapter 2. As a part of this extensive analysis I was able to isolate single amino acid mutants with weak PNPase alleles. Those mutants were key in bypassing the embryo lethality of a *pnp/rnr* double mutant described in chapter 3. Data presented in chapter 3 also include a deepened investigation of the single *rnr* mutant. As explained in the discussion, the mechanisms behind the strong influence of RNR1 in mRNA half-life is still puzzling and will definitely require further experiments to understand how different from bacteria is the chloroplast RNA decay pathway. Is it just a question of enzyme location (RNR1 seems to be preferentially located in the stroma of maize chloroplasts, Majeran*, et al.* 2012) or some influence of RNR1 on an intermediate step of the decay pathway, such as the ability of transcripts to be polyadenylated or their initial endonucleolytic cleavage?

A similar project that I started should bring some useful information. Indeed, in our lab, we have mutants for all the known chloroplast nucleases and I started crosses between all of them. The enzymes of this list are all described in Table 1 of chapter 1:

- *pnp1-1*
- *P184L*, the weak PNPase allele described in chapter 2
- *rnr1-3*

- *csp41b*, which happens to also be depleted of *csp41a* (Bollenbach*, et al.* 2009)
- *rne*, an RNase E null mutant
- *rnj*, an RNAi inducible *Arabidopsis* line made by an undergraduate Ryan Guttierez, under the supervision of a previous postdoc Tom Bollenbach. An undergraduate intern, David Kim, started the characterization work of this line under my supervision last summer.

The results I very recently obtained for a limited set of double and triple mutants, involving *csp41b*, *pnp1-1*, *P184L* and *rne*, are presented in Figure 5.1 and 5.2. First of all, it is very interesting to note that the double mutants *pnp1-1/csp41b*, *P184L/rne* and *pnp1-1/rne* as well as the triple mutant *pnp1-1/csp41b/rne* are all viable, at least when germinated on sucrose supplemented medium.

Figure 5.1a shows the phenotype of the double mutant *pnp1-1/rne*, which is chlorotic when compared to the WT. The growth rate seems, however, to be very similar under those conditions. After RNA extraction, the single, double and triple mutants were run on an RNA gel (Figure 5.1b). As shown in chapter 2 and as discussed by Walter *et al.* (2002), the mutants containing the *pnp1-1* mutation displays a typical rRNA phenotype, with one of the 23S band migrating slower due to a 3' extension. This RNA gel was loaded according to the amount of nuclear 28S, revealing the slightly reduced amount of chloroplast rRNA in *pnp1-1/rne* and *pnp1-1/csp41b/rne*. This phenotype is, however, not as drastic as observed for the mutants containing the *rnr1-3* mutation, discussed in chapter 3.

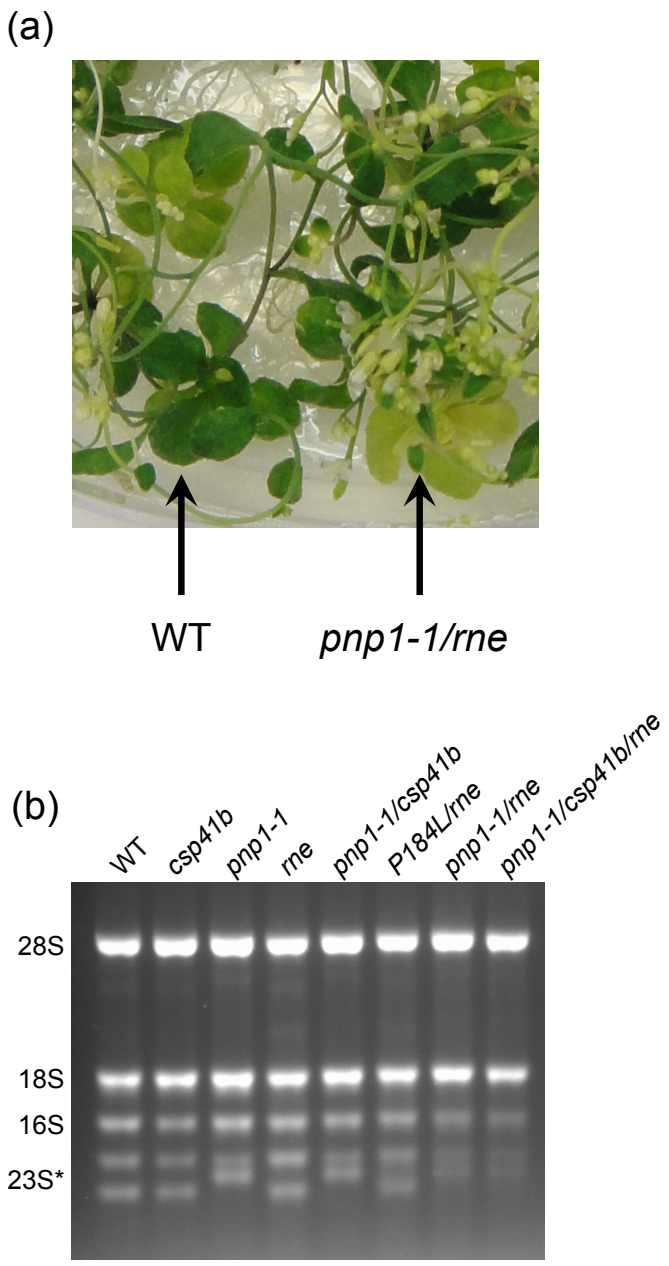

Figure 5.1: *csp41b, pnp1-1* and *rne d*ouble and triple mutant analysis.
(a) Plant phenotype of WT and *pnp1-1/rne.*
(b) RNA gel of the single, double and triple mutants.

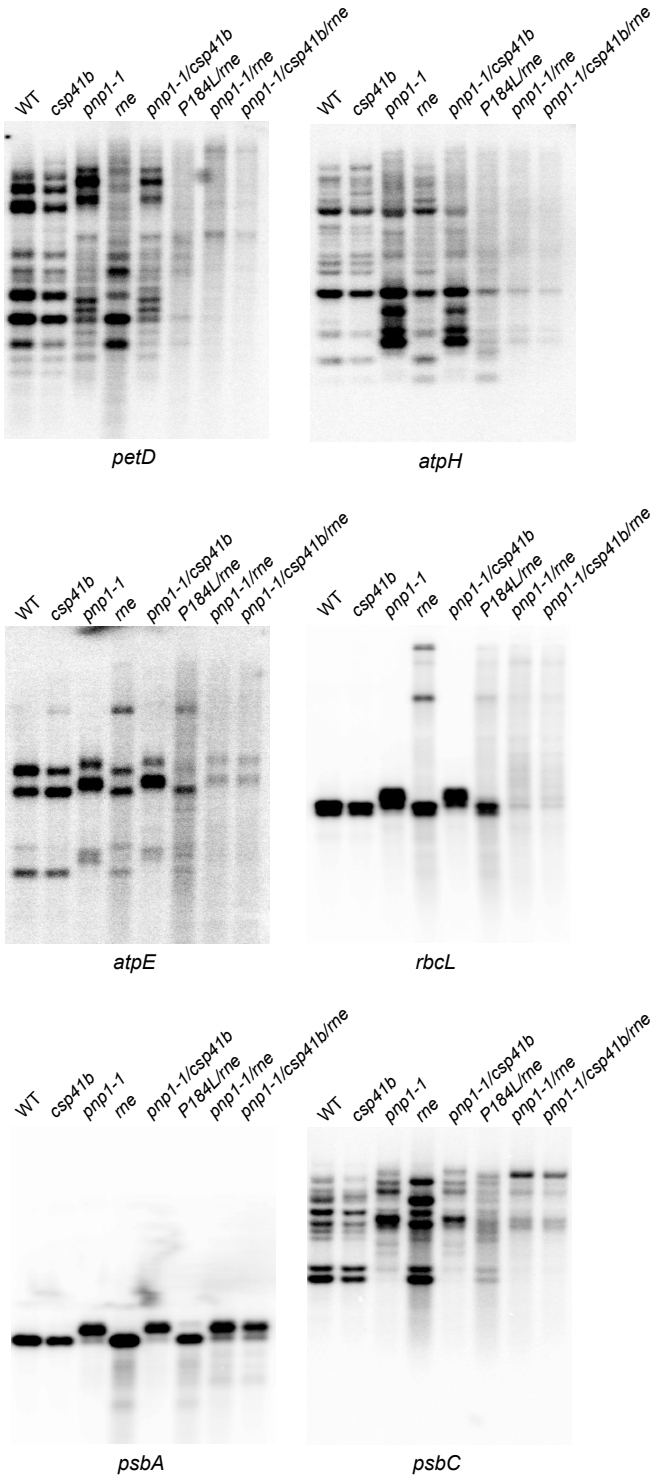

Figure 5.2: Results for the six specific probes *petD, atpH, atpE, rbcL, psbA* and *psbC*.
Those probes are the same as the ones used in chapter 2.

Figure 5.2 illustrates a few different RNA blots resulting from several RNA gels loaded similarly to Figure 5.1b. The RNA phenotype of the *csp41b*, *pnp1-1* and *rne* single mutant lanes confirms published results from Bollenbach *et al.* (2009), Germain *et al.* (2011) and Walter *et al.* (2010), respectively. The *pnp1-1/csp41b* double mutant does not seem to be much different from the *pnp1-1* single mutant, apart maybe from a general but slight decrease in RNA levels for all the probes of Figure 5.2. When looking at the double mutants *P184L/rne* and *pnp1-1-rne*, a general and substantial decrease in RNA level is observed. As in chapter 3, it is interesting to have the weak *pnp* allele *P184L*, which shows an intermediate phenotype in RNA levels between the *rne* single mutant and the *pnp1-1/rne* double mutant. This is most visible for the *atpH, atpE, rbcL* and *psbC* probes. When looking at the last two lanes of the RNA blots, namely the double mutant *pnp1-1/rne* and the triple mutant *pnp1-1/csp41b/rne*, the six probes shown in Figure 5.2 can be divided in three categories; (i) includes *petD* and *atpH*, with a general and substantial decrease in RNA levels into a smear; (ii) comprises *atpE* and *rbcL*, for which the main mature bands are still visible but are much reduced in intensity. The *rbcL* result should be, however, taken with caution since the regulation of the *rbcL* transcript has many other levels of regulation, especially in chlorotic tissues; (iii) includes *psbA* and *psbC* because well-defined bands are detected. In the case of *psbA*, it is interesting to notice that the transcript levels stay somewhat constant in all mutants lines analyzed. This strongly contrasts with the result obtained for the *P184L/rnr1-3* double mutant shown in Figure S3.2 where almost no *psbA* transcript is detected. Finally, the *psbC* probe reveals the disappearance of the mature bands in favor of a

very high molecular weight RNA species, most likely the precursor with a 3' end extension due to the absence of PNPase.

Taken together, those results and the results from chapter 3 show the importance of nucleases for the accumulation of RNA species to WT levels. It is still contradictory to observe that depleting enzymes whose role is to degrade RNAs leads to a severe decrease in the levels of many RNA species. The analysis of this collection of mutant along with the other many possible combinations that need to be germinated will undoubtedly give some insight into the specific role of each enzyme within the chloroplast.

Finally, three other projects will need some attention and should produce interesting results:

- **Suppressor screen of *pnp1-1***. This was started by Chloe Marchive, a previous postdoc in the lab and while it was my plan to finish the screen of the EMS mutant collection as well as using RNA-Seq to map the mutants, I only just finished the screening and have homozygous lines. Those lines need to be crossed to the parental *pnp1-1* line and the F_2 progeny with a WT-like phenotype, pooled and sequenced to map the Single Nucleotide Polymorphism (SNP) responsible for the rescue of the virescent rosette phenotype of *pnp1-1*. Depending on the genes found, further characterization of these mutants should be undertaken.
- **PNPase and RNR1 swapping**. Indeed, those two proteins have the particularity to be present in both chloroplast in mitochondria. While RNR1 is dual targeted,

there is an isoform of PNPase sent into the mitochondria (mtPNPase). My intern, David Kim, from the University of Waterloo, Canada, started the cloning work for this project that is more evolutionary oriented. Concerning PNPase, we are planning on sending the mitochondrial isoform to the chloroplast and vice-versa, in order to see if those two proteins, which have a very high similarity (Yehudai-Resheff*, et al.* 2003), can complement the embryo lethality of the mitochondrial mutant (Perrin*, et al.* 2004b), or the virescent phenotype of the chloroplast mutant. If complementation is not successful, it would be interesting to perform some internal domain swapping between the two proteins, to test their influence and specificity within different organelle, assessing at the same time the effects of evolution of seemingly duplicated genes. As far as RNR1 is concerned, the goal is to target RNR1 to a single organelle at a time, by switching the native transit peptide conferring dual targeting with transit peptides specific to the chloroplast or the mitochondria (Nelson *et al.* 2007). This genetic hemicomplementation has already been achieved with success for *MSH1* (Xu *et al.* 2011), and should give us some answers as to why the *rnr1* mutants require sucrose when germinating.

- Lastly, while Stoppel *et al*. (2012) list the known chloroplast RNases in their tables 1 and 2, as I did in Table 1 of chapter 1, they also have a list of other RNases predicted to be localized to the chloroplast but still uncharacterized. The RNase L-PSP (At3g20390) strongly caught my attention because it was also shown to be one of the most abundant RNase in the stroma (Majeran*, et al.* 2012). I ordered several of the mutants from this table and while the

homozygotes plants did not display any rosette phenotype, homozygous seeds are ready to be germinated for further characterization of their RNA phenotype.

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