New perspectives on the molecular mechanisms of stress signalling by the nucleotide guanosine tetraphosphate (ppGpp), an emerging regulator of photosynthesis in plants and algae

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Tansley review

New perspectives on the molecular mechanisms of stress signalling by the nucleotide guanosine tetraphosphate (ppGpp), an emerging regulator of photosynthesis in plants and algae.

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Contents

Summary

The nucleotides guanosine tetraphosphate and guanosine pentaphosphate (together (p)ppGpp) are found in a wide range of prokaryotic and eukaryotic organisms where they are associated with stress signalling. In this review we will discuss recent research highlighting the role of (p)ppGpp signalling as a conserved regulator of photosynthetic activity in the chloroplasts of plants and algae, and the latest discoveries that open up new perspectives on the emerging roles of (p)ppGpp in acclimation to environmental stress. We explore how rapid advances in the study of (p)ppGpp signalling in prokaryotes are now revealing large gaps in our understanding of the molecular mechanisms of signalling by (p)ppGpp and related nucleotides in plants and algae. Filling in these gaps is likely to lead to the discovery of conserved as well as new plant and algal specific (p)ppGpp signalling mechanisms that will offer new insights into the taming of the chloroplast and the regulation of stress tolerance.

Keywords

ppGpp, guanosine tetraphosphate, chloroplast, nitrogen, algae, RSH, photosynthesis

I. Introduction

Thanks to the domestication of the chloroplast plants and algae are among the most successful and important organisms on the planet. A pair of purine nucleotides called guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), collectively (p)ppGpp, may have helped tame the chloroplast and at the same time allow efficient acclimation to environmental fluctuations. These signalling molecules (also known as alarmones), which are synthesized from GTP/GDP and ATP by RelA SpoT Homologue (RSH) enzymes (Fig. 1A), were discovered more than 50 years ago in bacteria where they play a major role in growth control and in acclimation to environmental change by targeting a wide range of effector enzymes to slow proliferation and promote resilience (Bange et al., 2021; Irving et al., 2020). (p)ppGpp signalling (also referred to as the stringent response) was discovered more recently in plants and algae (Takahashi et al., 2004; van der Biezen et al., 2000), where it takes place in the chloroplast (Boniecka et al., 2017; Field, 2018). The chloroplast is the site of photosynthesis, which fuels plant growth and nearly all life on earth by converting sunlight into chemical energy, and is also a hub for stress perception and regulation (Kleine et al., 2021). (p)ppGpp signalling is therefore well placed to play important roles in regulating both the nutrition and stress acclimation of photosynthetic eukaryotes. Indeed, as we will discuss, recent studies highlight (p)ppGpp as a conserved regulator of photosynthetic activity and open new perspectives on the emerging roles of (p)ppGpp in acclimation to environmental stress. We will then look at how rapid advances in the study of prokaryotic (p)ppGpp signalling are now revealing gaps in our understanding of the molecular mechanisms of (p)ppGpp signalling in plants and algae. Filling in these gaps is likely to lead to the discovery of highly conserved mechanisms as well as new plant and algal specific mechanisms that will offer fresh insights into the remarkable success of the cohabitation between the chloroplast and the eukaryotic cell, and a greater understanding of stress acclimation in these organisms.

II. Specialisation of RSH enzymes for (p)ppGpp metabolism in plants and algae

RSH superfamily enzymes carry out the synthesis and hydrolysis of (p)ppGpp (Fig. 1A). A synthetase domain catalyses the Mg^{2+}-dependent transfer of a pyrophosphate group from ATP to the ribose 3'-OH of GDP (or GTP) to form (p)ppGpp while a hydrolase domain catalyses the Mn^{2+}-dependent removal of the 3'-diphosphate from (p)ppGpp to produce GDP (or GTP) and pyrophosphate. Multi-domain long RSH enzymes that possess both (p)ppGpp synthetase and hydrolase domains, as well as the related single-

domain small alarmone synthetases (SAS) and hydrolases (SAH), have been identified in almost all bacterial groups studied, as well as in photosynthetic eukaryotes, and some members of the Archaea (Atkinson et al., 2011; Avilan et al., 2019; Ito et al., 2017). An interesting recent development is the identification of the small alarmone synthetase called Metazoan SpoT homologue 1 (MESH1) in animals, along with the presence of (p)ppGpp (Ito et al., 2022; Sun et al., 2010; Young et al., 2021). However, the absence of an obvious enzyme responsible for (p)ppGpp synthetase activity, and the dual activities of MESH1 as an efficient hydrolase of both (p)ppGpp and NADPH means that the physiological role played by (p)ppGpp in animals is not yet fully resolved (Mestre et al., 2022). Plants and algae have at least three conserved families of long RSH enzymes, RSH1, RSH2/3, RSH4 (or Ca^{2+}-activated RSH, CRSH) (Atkinson et al., 2011; Avilan et al., 2019; Ito et al., 2017) (Fig. 1B). These families originated at an early stage in the evolution of the Archaeplastida because representatives can be found in the three major lineages- green plants and algae (Viridiplantae), red algae (Rhodophyta), and blue-green algae (Glaucophyta). Evolutionary inference in multiple studies places the Archaeplastida RSH families far from the cyanobacteria, the likely ancestors of the chloroplast, pointing to a complex evolutionary history that may not be possible to explain by simple vertical descent. Indeed, the RSH1 family groups with RSH from the Deinococcus-Thermus bacteria (Atkinson et al., 2011; Avilan et al., 2019; Ito et al., 2017), and there are signs of the more recent emergence of clades of diatom RSH that may have involved lateral gene transfer from bacteria (Avilan et al., 2019).

Plant and algal RSH enzymes show important differences in domain structure from bacterial RSH, as well as a higher diversity and functional specialisation (Fig. 1C). The majority of plant and algal RSH so far tested are nuclear-encoded and possess a predicted or experimentally verified chloroplast transit peptide (CTP). Except for the CTP and an N-terminal extension, members of the RSH1 family show a strong resemblance to bacterial long RSH enzymes (Fig. 1C), with both (p)ppGpp hydrolase and synthetase domains, and a bacteria-like C-terminal regulatory region (CTR) with the threonyl-tRNA synthetase-GTPase-SpoT (TGS) and helical domains. While Arabidopsis lacks a clearly identifiable aspartate kinase–chorismate mutase–TyrA (prephenate dehydrogenase) (ACT) domain in the CTR, this domain is found in the CTR of many other plant and algal RSH1 (Avilan et al., 2019). In bacteria the CTR controls the switch between hydrolase and synthetase activities by interacting with partners such as stalled ribosomes (Fig. 1D)(Arenz et al., 2016; Brown et al., 2016; Loveland et al., 2016) and regulatory proteins (Battesti and Bouveret, 2006; Krüger et al., 2021; Ronneau et al., 2018). It is not yet known whether RSH1 family enzymes are also regulated by interactions at the CTR in a similar way. However, the report of an evolutionary conserved interaction

between Arabidopsis RSH1 and the chloroplastic ribosome-associated GTPase spo0B-associated GTP-binding protein (ObgC) by yeast-two-hybrid suggests that such interactions are a real possibility (Bang et al., 2012). In the land plant-clade of RSH1 enzymes, despite an early report showing ppGpp synthetase activity in *E. coli* complementation assays (van der Biezen et al., 2000), it is now generally accepted that the synthetase domain is not catalytically functional (Avilan et al., 2019; Mizusawa et al., 2008; Sugliani et al., 2016), and the enzyme functions as the main (p)ppGpp hydrolase limiting (p)ppGpp levels *in planta* (Li et al., 2022; Sugliani et al., 2016)(Fig. 2).

RSH2/RSH3 family enzymes show bifunctional (p)ppGpp synthetase / hydrolase activity and in Arabidopsis act as the major ppGpp synthetases during the day (Maekawa et al., 2015; Sugliani et al., 2016) and are required for constraining (p)ppGpp levels at night (Ono et al., 2020). In addition to the catalytic region plant RSH2/RSH3 enzymes have significant N-terminal region (NTR) and CTR extensions with high sequence conservation, but which bear little or no homology to their bacterial counterparts (Fig. 1C). The RSH2/RSH3 NTR and CTR may therefore be involved in novel, plant-specific regulatory processes.

The RSH4 / CRSH family enzymes identified so far all contain a non-functional (p)ppGpp hydrolase domain, and except for the CTP they lack an extension in the NTR. In plants and some green algae the CTR contains EF-hand domains which permit the Ca^{2+}-mediated activation of (p)ppGpp synthetase activity (Avilan et al., 2019; Masuda et al., 2008a; Tozawa et al., 2007)(Fig. 1C). Interestingly, the acquisition of novel domains is frequent among algal members of the RSH4 family (Avilan et al., 2019; Ito et al., 2017). This suggests that the regulation of synthetase domain activity observed in bacterial RSH can readily be repurposed to permit new regulatory connections.

III. The physiological roles of (p)ppGpp in photosynthetic eukaryotes

Although the (p)ppGpp pathway was discovered some time ago in plants and algae, it is only recently that significant progress has been made in understanding its physiological roles. Progress has come principally from manipulating ppGpp levels *in vivo* (pppGpp is not usually detected in plants) via the use of different *RSH* mutants or the expression of ppGpp synthetases and hydrolases initially in Arabidopsis (Abdelkefi et al., 2018; Goto et al., 2022; Honoki et al., 2018; Maekawa et al., 2015; Ono et al., 2020; Romand et al., 2022; Sugliani et al., 2016; Yamburenko et al., 2015), and more recently using similar approaches in rice, moss and algae (Avilan et al., 2021; Harchouni et al., 2022; Imamura et al., 2018; Ito et al., 2022; Li et al., 2022). These studies have highlighted the role of (p)ppGpp signalling in regulating

chloroplast function (and in particular photosynthesis) during growth and development, acclimation to nitrogen starvation, the onset of night and immune responses (Fig. 2).

ppGpp is a conserved regulator of photosynthesis in plants and algae

A common theme emerging from multiple studies on plants and algae is that manipulation of ppGpp levels alters photosynthetic activity. Specifically, ppGpp accumulation causes a decrease in photosynthesis-reducing maximal quantum efficiency (Fv/Fm) and operating efficiency (or quantum yield) and electron transport rate (ETR) (Avilan et al., 2021; Harchouni et al., 2022; Honoki et al., 2018; Ito et al., 2022; Li et al., 2022; Romand et al., 2022; Sugliani et al., 2016). In Arabidopsis, moss and diatoms these changes are associated with modifications in the architecture of the photosynthetic electron transport chain (PETC). Notably at photosystem II (PSII) there is a decrease in the quantity of PSII reaction centres compared to the peripheral light-harvesting antenna (Avilan et al., 2021; Harchouni et al., 2022; Maekawa et al., 2015; Sugliani et al., 2016). Photosystem I (PSI) would appear to be less affected, although more work is required to determine the relative impact of ppGpp on PSI and PSII. Rubisco levels also drop markedly in response to ppGpp accumulation, and *RSH* mutants deficient in (p)ppGpp metabolism show defects in nitrogen remobilisation from Rubisco during stress-induced senescence (Harchouni et al., 2022; Honoki et al., 2018; Li et al., 2022; Maekawa et al., 2015; Romand et al., 2022; Sugliani et al., 2016). Interestingly, while the decrease in photosynthetic activity in response to ppGpp accumulation is conserved, certain features vary. For example, Rubisco is not sensitive to even very high levels of ppGpp in the diatom *Phaeodactlyum tricornutum* (Avilan et al., 2021). This suggests that (p)ppGpp is able to trigger specific responses in different photosynthetic organisms.

The effects of ppGpp on photosynthesis were first established via the artificial overaccumulation of ppGpp. The relevance of these effects was also demonstrated at physiological levels of ppGpp in wild type organisms, as well as in the absence of stress (Romand et al., 2022; Sugliani et al., 2016). *RSH* mutants deficient for (p)ppGpp biosynthesis or hydrolysis show small defects in photosynthetic parameters under standard growth conditions (Honoki et al., 2018; Sugliani et al., 2016). More recently very large ppGpp-dependent effects on photosynthesis were observed during nitrogen starvation in Arabidopsis (Romand et al., 2022), and also in field-grown rice plants carrying a mutation in the (p)ppGpp hydrolase gene *RSH1* (also known as *ABC1 REPRESSOR2*, *ARE2*)(Li et al., 2022). These points are discussed in more detail below.

(p)ppGpp signalling influences growth and development

Several studies have reported that the perturbation of (p)ppGpp levels has effects on growth and development. Such effects might be expected given the role of (p)ppGpp in the regulation of photosynthetic activity as discussed above. However, the situation is complex because (p)ppGpp over-accumulation in plants including Arabidopsis, rice and moss has variously been reported to increase (Maekawa et al., 2015), decrease (Li et al., 2022; Sugliani et al., 2016), or have no detectable effect on plant size (Harchouni et al., 2022; Ito et al., 2022). These conflicting results may be related to differing levels of (p)ppGpp, species specific effects, differences in light intensity / quality, and nutrient levels. Indeed, there is recent support for the idea that variation in nutrient levels, which can vary considerably during the course of plant culture and in different growth substrates, might explain the different effects reported for ppGpp on plants size in Arabidopsis. Goto and colleagues showed that plants with high ppGpp levels can grow to a greater size than wild type plants under moderately low nitrogen conditions (Goto et al., 2022). Defects in (p)ppGpp signalling also affect senescence. In Arabidopsis dark-induced and natural senescence are accelerated in mutants of the (p)ppGpp hydrolase RSH1 that have higher (p)ppGpp levels, and delayed in mutants unable to accumulate wild type levels of (p)ppGpp (Li et al., 2022; Sugliani et al., 2016).

A pivotal role in acclimation to nitrogen deprivation

(p)ppGpp signalling was recently shown to play a major role in acclimation to nitrogen deprivation (Romand et al., 2022). The artificial accumulation of ppGpp in an *RSH3* over-expression line was found to protect Arabidopsis plants against nitrogen limitation (Goto et al., 2022; Honoki et al., 2018; Maekawa et al., 2015) suggesting that (p)ppGpp might be involved in acclimation. Using mutants defective in ppGpp accumulation Romand et al. (2022) demonstrated that ppGpp accumulation is required for the acclimation of Arabidopsis plants to nitrogen limitation under physiological conditions that may be encountered in nature. Interestingly, another study performed in parallel further supports these findings by showing that the increase in ppGpp levels caused by a mutation in the rice (p)ppGpp hydrolase gene *RSH1* can suppress and overcome the constitutive nitrogen-starved phenotype of the *ABNORMAL* CYTOKININ *RESPONSE1* mutant (Li et al., 2022). During nitrogen starvation (p)ppGpp signalling is required for the safe downregulation of the photosynthetic machinery, whose products are no longer necessary due to a general growth arrest. ppGpp-mediated downregulation of the photosynthetic

machinery is associated with downregulation of chloroplast transcript levels, a reduction in the GTP pool, and remodelling of PSII (Romand et al., 2022). These changes are very similar to those observed in ppGpp-overaccumulating lines (Maekawa et al., 2015; Sugliani et al., 2016). Limiting ppGpp biosynthesis during nitrogen starvation delays the downregulation of photosynthesis, and results in increased reactive oxygen species (ROS) accumulation, tissue damage, and a major disruption of the co-ordination between chloroplast and nuclear gene expression (Romand et al., 2022). Surprisingly, ppGpp levels do not increase to very high levels during nitrogen starvation, suggesting that (p)ppGpp signalling is somehow potentiated during stress by other factors. For example, potentiation of (p)ppGpp signalling could be related to the increase in the ppGpp/GTP ratio that occurs under nitrogen starvation, which would enhance the inhibition of enzymes where ppGpp is a competitive inhibitor. In any case, these findings show that the strong connection between (p)ppGpp signalling and photosynthesis is physiologically relevant and demonstrate a clear role for (p)ppGpp in abiotic stress acclimation. Downregulation of the photosynthetic machinery reduces carbon assimilation and at the same time liberates significant quantities of nitrogen as the machinery accounts for over half of leaf nitrogen in C3 plants (Evans and Clarke, 2019). Therefore, while (p)ppGpp can reduce the risk of ROS accumulation from excessive photosynthetic activity during nitrogen limitation, it is likely that the nitrogen liberated from the photosynthetic machinery also serves a major role in supporting other cellular processes. Indeed, the photosynthetic and growth phenotypes of (p)ppGpp mutants under normal growth conditions (Honoki et al., 2018; Li et al., 2022; Sugliani et al., 2016) suggest that (p)ppGpp signalling continually fine-tunes the cellular carbon / nitrogen equilibrium.

(p)ppGpp for quiet nights?

(p)ppGpp signalling is likely to be involved in regulating chloroplast gene expression at night in plants. In Arabidopsis ppGpp levels increase at the onset of night (Ihara et al., 2015) in a CRSH dependent manner (Ono et al., 2020), although the final concentration requires the participation of RSH1, RSH2 and RSH3. The onset of night also triggers a transient Ca^{2+} flux into the chloroplast stroma (Johnson et al., 1995; Sai and Johnson, 2002) which may be responsible for directly activating CRSH via the EF-hand domains in the CTR. While a *CRSH* mutant did not show any obvious growth phenotype, the authors observed a probable defect in the night-triggered downregulation of transcript levels for certain chloroplast-encoded genes (Ono et al., 2020). This may therefore be one of the processes by which dark-induced stromal Ca^{2+} transients can influence chloroplast function (Rocha et al., 2014). Cyanobacteria and algae also accumulate (p)ppGpp in the dark but do not have RSH enzymes with EF hand domains for Ca^{2+} binding (Hood et al.,

2016; Jin et al., 2022; Puszynska and O'Shea, 2017). This suggests that dark-induced (p)ppGpp signalling is widespread in photosynthetic organisms, although the activation of RSH enzymes must occur via distinct mechanisms.

(p)ppGpp signalling influences plant immunity

The chloroplast, and in particular chloroplast-generated ROS, plays a key role in plant immunity (Littlejohn et al., 2021). Therefore, it is perhaps not surprising that (p)ppGpp signalling, with its ability to downregulate photosynthesis where ROS are generated, can influence plant immunity against pathogens. The expression of plant *RSH2/3* genes is upregulated by plant pathogens, wounding, pathogen-associated molecules and defence-related hormones (Abdelkefi et al., 2018; Givens et al., 2004; Kim et al., 2009; Petrova et al., 2021; Takahashi et al., 2004). However, *RSH2/3* upregulation is associated with pathogen susceptibility suggesting that under at least some cases (p)ppGpp accumulation can favour the pathogen (Petrova et al., 2021). Consistent with this, over-accumulation of ppGpp in Arabidopsis leads to strong reductions in the levels of transcripts for defence-related genes (Abdelkefi et al., 2018). Furthermore, high ppGpp levels lead to greater susceptibility to Turnip Mosaic virus while lower levels are associated with increased resistance, accumulation of the defence hormone salicylic acid, and precocious expression of the defence-related protein PATHOGENESIS-RELATED 1 (Abdelkefi et al., 2018). Pathogen-associated molecular pattern (PAMP) triggered immunity (PTI) provokes stromal Ca^{2+} fluxes in a similar way to darkness, suggesting that CRSH might be activated during PTI. However, treatment of a *CRSH* mutant with the PAMP flagellin22 induced defence-related genes in a similar fashion to the wild-type control (Ono et al., 2020). Therefore, the links between (p)ppGpp and Ca^{2+} signalling during immunity remain uncertain. Altogether, more work is required for understanding the full role that (p)ppGpp signalling has on plant immunity, and in particular during interactions with biotrophic and necrotrophic pathogens as well as herbivores. Furthermore, comparing the pathogenicity of wild-type pathogens with mutants unable to deliver their effector machinery may allow the identification of pathogens that are able to subvert (p)ppGpp signalling to overcome host defence.

IV. Molecular mechanisms of (p)ppGpp signalling in plants and algae

Despite recent advances in understanding the physiological roles of (p)ppGpp as discussed above, few if any chloroplastic effectors of (p)ppGpp have been firmly identified. This contrasts with the situation in bacteria where (p)ppGpp and related nucleotides are known to interact directly with specific effector enzymes to regulate growth rate and promote stress acclimation and survival (Bange et al., 2021; Irving et al., 2020). Over recent years the development of systematic approaches has led to a considerable expansion in the number of known (p)ppGpp binding proteins and effectors in bacteria. These advances were driven by techniques such as the differential radial capillary action of ligand assay (DRaCALA), a rapid and quantitative method that can be used for testing candidate protein-(p)ppGpp interactions in crude extracts and without the need for protein purification (Corrigan et al., 2016; Roelofs et al., 2011; Zhang et al., 2018), as well as by the use of ppGpp analogues to directly capture and identify ppGpp-binding proteins in cellular extracts (Haas et al., 2022; Wang et al., 2019). Such approaches may also have considerable potential for identifying effectors in plants and algae. At the same time, the growing list of (p)ppGpp targets in bacteria also provides insights into the possible molecular mechanisms of (p)ppGpp signalling in plants. Indeed, bacterial ppGpp effectors are known today in transcription, nucleotide metabolism, translation, ribosome assembly, fatty acid biosynthesis and amino acid metabolism (Bange et al., 2021; Irving et al., 2020; Kanjee et al., 2012; Steinchen et al., 2020). Many of these processes are conserved in the chloroplasts of plants and algae and should therefore be considered potential targets of (p)ppGpp signalling (Table 1).

Does (p)ppGpp directly inhibit chloroplast transcription?

Multiple studies show that (p)ppGpp accumulation *in vivo*, either artificially or during stress, results in the downregulation of chloroplast transcript levels in plants (Harchouni et al., 2022; Maekawa et al., 2015; Romand et al., 2022; Sugliani et al., 2016). Direct analysis of transcription by chloroplast run-on or labelling of nascent transcripts in Arabidopsis indicates that the reduction in chloroplast transcript abundance caused by (p)ppGpp is due to the inhibition of transcription (Sugliani et al., 2016; Yamburenko et al., 2015). Chloroplast transcription is carried out by a bacterial-like plastid-encoded polymerase (PEP) and a phage-like nucleus-encoded polymerase (NEP). Some studies have observed a preferential effect of (p)ppGpp on the levels of PEP transcripts (Sato et al., 2009; Sugliani et al., 2016), while others have not observed a clear separation between NEP and PEP transcripts (Romand et al.,

2022). The role of (p)ppGpp in regulating chloroplast transcription is therefore established, however, it is not yet clear exactly how (p)ppGpp is able to mediate this effect.

In *E. coli*, (p)ppGpp directly modulates the activity of the RNA polymerase (RNAP) to downregulate the expression of ribosomal RNAs (rRNA) and upregulate the expression of genes involved in stress acclimation. The bacterial RNAP core is a complex composed of two α subunits, a β subunit, a β' subunit, a ω subunit and a σ subunit ($\alpha_2\beta\beta'\omega\sigma$)(Fig. 3A, Table 1). There are two allosteric (p)ppGpp binding sites on RNAP that are conserved in *E. coli* and other proteobacteria. Site 1 is located at the interface between the β' and ω subunits (Ross et al., 2013), and site 2 at the interface between the β' subunit and the transcription factor DksA (Ross et al., 2016). The major transcriptional effects of (p)ppGpp accumulation in *E. coli* can be explained by (p)ppGpp binding at these two sites, although it does not explain all the effects of (p)ppGpp on growth (Wang et al., 2019). In plants, PEP has a bacterial-like core complex consisting of two α subunits, a β subunit, β' and β'' subunits, and a σ subunit ($\alpha_2\beta\beta'\beta''\sigma$)(Borner et al., 2015; Igloi and Kössel, 1992; Suzuki et al., 2003)(Fig. 3A, Table 1). The β' and β'' subunits correspond to the N-terminal and C-terminal portions of the bacterial β' subunit, and the same split is also present in the RNAP of cyanobacteria which is presumably ancestral. The plant PEP has also acquired additional co-purifying accessory factors called PEP-associated proteins (PAPs) that are not present in bacteria or even green algae (Pfannschmidt et al., 2000; Steiner et al., 2011; Suzuki et al., 2004). Strikingly, with regard to the action of (p)ppGpp, PEP completely lacks site 1 and site 2: there is no ω subunit, the conserved β' K615 residue required for (p)ppGpp binding (Myers et al., 2020) is lacking from the corresponding Arabidopsis β'' subunit, and there is no orthologue of the DksA transcription factor in plant or algal genomes (Fig. 3A, Table 1). From an evolutionary perspective the lack of *E. coli* like (p)ppGpp binding sites is not surprising because the *E. coli* mechanism is a relatively recent evolutionary innovation that is restricted to the proteobacterial (Ross et al., 2016), and the RNAP of other bacterial groups is insensitive to (p)ppGpp.

Despite the clear absence of proteobacteria-equivalent ppGpp binding sites for the control of PEP, several studies nevertheless indicate that PEP may be directly targeted by (p)ppGpp. Takahashi and colleagues first showed that exogenous application of ppGpp or pppGpp can inhibit transcription in chloroplast extracts (Takahashi et al., 2004). A follow-on study then showed that ppGpp is able to specifically inhibit transcription in extracts enriched for PEP and not in extracts enriched for the alternative chloroplast RNA polymerase NEP (Sato et al., 2009). Furthermore, radiolabelled 6-thio-ppGpp

was found to bind to the PEP β' subunit (Sato et al., 2009). It is therefore reasonable to suppose that a novel ppGpp binding site on the β' subunit is necessary for PEP inhibition, although the exact residues involved remain to be identified and tested. ppGpp was also found to inhibit transcription of the chloroplast 16S ribosomal RNA in crude extracts from the unicellular red alga, *Cyanidioschyzon merolae* (Imamura et al., 2018). In both plants and algae, the concentration of ppGpp required for the *in vitro* inhibition of chloroplast transcription is at the high end of ppGpp sensitivities observed for bacterial enzymes (Steinchen et al., 2020), and is higher than the levels estimated to naturally occur within the chloroplast under non-stress conditions (~1-3 μM) (Ihara et al., 2015; Ito et al., 2022; Sugliani et al., 2016). The authors of both the plant and algal PEP studies therefore propose that, *in vivo,* other unidentified factors may potentiate the action of ppGpp on PEP in a similar manner to DksA (Imamura et al., 2018; Sato et al., 2009). The possibility of very local peaks in ppGpp concentration has also been suggested, and these findings could also point to the existence of strong and weak targets of (p)ppGpp in the chloroplast to allow for a graded response to (p)ppGpp levels as observed in bacteria (Steinchen et al., 2020).

Purine nucleotide metabolism, a universal target of (p)ppGpp signalling?

Purine biosynthesis has emerged as a major target of (p)ppGpp signalling in diverse bacteria (Bange et al., 2021; Irving et al., 2020). A large part of the purine biosynthetic pathway takes place in the chloroplast of plants and algae and involves orthologues of the bacterial enzymes (Fig. 3B, Table 1)(Kusumi and Iba, 2014; Smith and Atkins, 2002; Witte and Herde, 2020). These enzymes include orthologues of the bacterial (p)ppGpp targets adenylosuccinate synthetase (PurA or ADSS)(Stayton and Fromm, 1979; Wang et al., 2019; Yang et al., 2020a), amidophosphoribosyltransferase (PurF or ASE)(Wang et al., 2019), inosine-5'-monophosphate dehydrogenase (GuaB or IMDH)(Gallant et al., 1971; A. Kriel et al., 2012) guanylate kinase (GmK)(A. Kriel et al., 2012) and the RSH enzymes themselves (Steinchen et al., 2015; Yang et al., 2020b; Zhang et al., 2018). Currently there is evidence that the chloroplast guanylate kinase of plants is inhibited at physiological ppGpp levels *in vitro* (Nomura et al., 2014). Furthermore, there is evidence that (p)ppGpp signalling can affect plant purine metabolism *in vivo* with the recent demonstration that (p)ppGpp accumulation is required to promote a decrease in total GTP levels during nitrogen starvation stress in Arabidopsis (Romand et al., 2022). In addition, overexpression of *RSH3* in the conditional GmK mutant *virescent-2* in rice strongly enhanced the mutant phenotype, suggesting an interaction between increased ppGpp levels and reduced GmK function (Ito et

al., 2022). However, the situation may be more complex than it appears because artificially increasing (p)ppGpp levels does not always affect the total GTP pool (Avilan et al., 2021; Bartoli et al., 2020).

The inhibition of bacterial purine nucleotide metabolism by (p)ppGpp may occur for several reasons. These include the conservation of metabolic precursors to allow a rapid return to growth, meeting the reduced demands of bulk RNA biosynthesis which itself is also a target of inhibition by (p)ppGpp, and reducing GTP levels to downregulate growth and potentiate the competitive inhibition of GTP-dependent enzymes targeted by (p)ppGpp (Wang et al., 2020). Indeed, a (p)ppGpp-mediated decrease in GTP is required for downregulation of transcription in the Firmicute, Actinobacteria and Deinococcus–Thermus groups of bacteria where RNAP is (p)ppGpp insensitive (Krasny and Gourse, 2004; Liu et al., 2015). In the model Firmicute *B. subtilis*, (p)ppGpp accumulation causes a drop in GTP levels via the inhibition of guanylate kinase. This reduction in GTP levels inhibits transcription from genes where GTP is the initiating NTP, which notably includes the rRNA genes. A similar mechanism may explain the observed downregulation of chloroplast transcription by ppGpp in plants (Sugliani et al., 2016; Yamburenko et al., 2015). As discussed above, the chloroplastic GmK is specifically inhibited by ppGpp (Nomura et al., 2014), and GTP levels drop in a ppGpp-dependent fashion under physiological stress conditions (Romand et al., 2022). GTP is also the initiating NTP for the chloroplast operon containing the 23S and 16S rRNAs in at least several plant species (Sugliani et al., 2016). Therefore, multiple lines of evidence point to the existence of a firmicute-like mechanism for regulating transcription in chloroplasts. However, more detailed direct investigations into the role of GmK in plant (p)ppGpp signalling are required for demonstrating a direct causal link between ppGpp-mediated inhibition of GmK and the inhibition of chloroplast transcription.

A role for ppGpp in regulating chloroplast translation?

In bacteria (p)ppGpp signalling downregulates global translation by targeting a wide range of GTP-binding enzymes involved in translation as well as in ribosome biogenesis and ribosome hibernation / recycling (Table 1)(Bange et al., 2021; Irving et al., 2020; Zegarra et al., 2022). (p)ppGpp is also likely to directly regulate translation in the chloroplast: many features of the prokaryotic translation mechanism are retained in the chloroplast (Zoschke and Bock, 2018) and, extending on previous observations (Masuda, 2012; Masuda et al., 2008b), we can identify chloroplast orthologs of all the major bacterial (p)ppGpp-targeted enzymes involved in translation regulation (Fig. 3C, Table 1).

Despite the promising theoretical situation there is still relatively little experimental evidence on the effects of (p)ppGpp on chloroplast translation. Using *an in vitro* chloroplast translation system from pea (*Pisum sativum),* ppGpp was found to inhibit the peptide elongation cycle of chloroplast translation by about 50% at 400 µM (Nomura et al., 2012). This is consistent with the presence of LepA (Ji et al., 2012) and SNOWY COTYLEDON1 (SCO1)(Albrecht et al., 2006) in the chloroplast, orthologues of EF4 and EF-G which participate in polypeptide elongation in bacteria and are well known targets of inhibition by (p)ppGpp (Fig. 3C, Table 1)(Bange et al., 2021). Artificial accumulation of ppGpp *in vivo* was also found to have a major effect on chloroplast translation, as measured by the incorporation of the antibiotic puromycin (a structural analogue of aminoacyl-tRNA) into nascent peptide chains (Sugliani et al., 2016). However, the observed inhibition was difficult to separate from the transcriptional downregulation of rRNA and tRNA that is also caused by ppGpp.

Recently, the role of (p)ppGpp in promoting the stress induced hibernation of bacterial ribosomes has received particular attention (Bange et al., 2021; Irving et al., 2020; Prossliner et al., 2018; Trösch and Willmund, 2019). Under stress conditions bacterial 70S ribosomes are inactivated as monomers or dimers that are also known as 100S ribosomes. Inactivation contributes to the downregulation of translation, and also allows rapid re-activation of translation upon return to favourable conditions. Notably, (p)ppGpp accumulation promotes the transcriptional upregulation of hibernation factors such as ribosome-associated inhibitor A (RaiA), ribosome modulation factor (RMF) and hibernation promoting factor (HPF) that trigger ribosome inactivation. The chloroplasts of plants and algae possess an HPF orthologue named plastid-specific ribosomal protein 1 (PSRP1, Fig. 3C) that can trigger the formation of inactive 70S monomers (Sharma et al., 2010). However, the physiological function of PSRP1 is not yet elucidated (Swift et al., 2020), and it is unlikely to be transcriptionally regulated by (p)ppGpp as it is encoded on the nuclear genome. The ribosome-associated GTPase high frequency of lysogeny X (HflX) is also implicated in ribosome inactivation in bacteria. In *Staphylococcus aureus* HflX is able to dissociate the hibernating 100S complex and this activity is inhibited by (p)ppGpp binding (Basu and Yap, 2017). Hflx, along with other GTPases involved in ribosome biogenesis and assembly (RsgA, RbgA, Era, Obg) are all inhibited by (p)ppGpp to reduce subunit maturation or prevent 70S assembly in the translation cycle (Bennison et al., 2019). Notably, and as discussed above, Obg and its chloroplast orthologue ObgC share conserved interactions with RSH enzymes (Bang et al., 2012; Chen et al., 2014; Wout et al., 2004)

indicating that there is a profound link between (p)ppGpp signalling and ribosome biogenesis that appears to have been maintained over a vast expanse of evolutionary time.

Are there chloroplast specific targets of (p)ppGpp signalling?

Since the original acquisition of the chloroplast there has been ample time for the evolution of new (p)ppGpp signalling mechanisms. Furthermore, the cohabitation of the chloroplast and the eukaryotic cell, the development of multicellularity and the colonisation of new niches including the land would have provided powerful selection pressures to drive the emergence of novel mechanisms. Chloroplastic GTPases are prime candidates as ppGpp targets simply because ppGpp has a tendency to target GTPases in bacteria (Fig. 3C, Table 1). Outside translation, only a handful of chloroplast GTPases are known, and these play roles in ribosome assembly, photosynthesis, chloroplast division, vesicle trafficking, and membrane remodelling. The circularly permuted GTPases SUPPRESSOR OF VARIEGATION 10 (SVR10) and BRZ INSENSITIVE PALE GREEN2 (BPG2) are implicated in chloroplast ribosome assembly (Qi et al., 2016); the GTPase PsbO is a subunit of the oxygen evolving complex involved in the turnover of the PSII reaction centre (Lundin et al., 2007; Spetea et al., 2004); chloroplast FtsZ tubulin-like GTPases ensure the formation of a contractile ring within the stroma during chloroplast division (Osteryoung and Vierling, 1995; Yoshida et al., 2016), the chloroplast-localised Rab family small GTPases are implicated in chloroplast vesicle trafficking (Alezzawi, 2014; Alezzawi et al., 2014; Ebine et al., 2011; Karim et al., 2014; Karim and Aronsson, 2014); and finally the GTPase vesicle-inducing protein in plastids 1 (VIPP1), is essential for the biogenesis and maintenance of thylakoid membranes (Gupta et al., 2021; Ohnishi et al., 2018). Interestingly, accumulation of ppGpp was shown to cause hyper-stacking of the thylakoid membranes in the moss *Physcomitrium patens* (Harchouni et al., 2022). While this might simply be explained by an increase in the quantity of PSII antenna subunits, it also raises the possibility that ppGpp can promote membrane remodelling by acting on proteins like VIPP1. Beyond the GTPases there may be evidence that other classes of protein are targeted by ppGpp. For example, we previously speculated that the higher sensitivity of some chloroplast proteins to ppGpp, and Rubisco in particular, could involve the ppGpp-mediated regulation of chloroplast protein turnover (Romand et al., 2022). However, it is clear that more intensive studies aimed specifically at identifying chloroplast (p)ppGpp targets using both candidate-based and more open-ended approaches will be required to move beyond speculation.

A larger family of related signalling nucleotides in plants?

In this review we have dealt exclusively with (p)ppGpp. However, these are just two of a larger family of related nucleotides that also include pGpp and (p)ppApp. Discovered in the 1970s (Nishino et al., 1979; Oki et al., 1976), pGpp and (p)ppApp can be synthesised by RSH and SAS enzymes, and their functions are stimulating renewed interest among microbiologists. pGpp, ppGpp and pppGpp act in similar ways, though have preferences for different target enzymes (Gaca et al., 2015; Yang et al., 2020b). (p)ppApp, on the other hand, can bind RNAP on a different site to (p)ppGpp and is able to activate transcription (Bruhn-Olszewska et al., 2018; Travers, 1978). To our knowledge, there are no reports of the detection of pGpp or (p)ppApp in plants or algae. Furthermore, while ppGpp is now readily detected (Bartoli et al., 2020; Ihara et al., 2015; Jin et al., 2018), pppGpp has only been reported once (Takahashi et al., 2004) suggesting that it is unstable or present only under certain circumstances. In the future it will be interesting to determine if other members of the extended (p)ppGpp family of nucleotides are present in plants and algae, and what role they play.

V. Concluding remarks

(p)ppGpp was originally discovered more than fifty years ago (Cashel and Gallant, 1969). Since that time work on bacteria, algae, plants and more recently animals have revealed the extraordinary diversity and reach of (p)ppGpp signalling. Over recent years our understanding of (p)ppGpp signalling in plants and algae has advanced considerably thanks to studies of its physiological roles *in vivo*, notably revealing the conserved action of (p)ppGpp on photosynthesis and its likely role in regulating cellular carbon / nitrogen status. To understand how (p)ppGpp acts at a molecular level it will be necessary to build on the early *in vitro* experiments, and adopt new approaches including those so successfully employed in bacteria for identifying the physiologically relevant targets of (p)ppGpp and related nucleotides. Likewise, the functional diversification of plant and algal members of the RSH superfamily promises to reveal exciting new features of (p)ppGpp signalling.

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Table 1. Bacterial (p)ppGpp effectors and their likely chloroplastic orthologues in *Arabidopsis thaliana*.

Bacterial (p)ppGpp targets	Chloroplast orthologue(s)	*E. coli*	*B. subtilis*	Chloroplast	References
Transcription					
Core RNAP complex	PEP complex	*	-	*	Imamura et al., 2018, Sato et al., 2009, Takahashi et al., 2004
alpha1 (RpoA)	RpoA/AtCg00740				
beta (RpoB)	RpoB/AtCg00190				
beta' (RpoC)	RpoC1/AtCg00180	*			Ross et al., 2013, Sato et al., 2009
	RpoC2 /AtCg00170				
omega (RpoZ)	no orthologue	*			Ross et al., 2013
sigma	Sig1/At1g64860, Sig2/At1g08540, Sig3/At3g53920, Sig4/At5g13730, Sig5/At5g24120, Sig6/At2g36990				
Transcription factor, DksA	no orthologue	*			Ross et al., 2016
Ribosome and translation associated GTPases					
Translation initiation factor 2 (IF2)	At1g17220/FUG1	*			Legault et al., 1972; Milon et al., 2006
Elongation factor TU (EF-TU)	At4g20360/SVR11	*			Legault et al., 1972; Rojas et al., 1984
Elongation factor G (EF-G)	At1g62750/SCO1, , At1g45332, At2g45030	*			Mitkevich et al., 2010; Rojas et al., 1984
Elongation factor 4 (EF4/LepA)	At5g08650/LepA	*			Zhang et al., 2018
GTPase Der (Der/EngA)	At3g12080/Der	*			Bharat et al., 2014
GTPase Era (Era)	At5g66470/Era1	*			Corrigan et al., 2016; Zhang et al., 2018
GTPase Obg (ObgE/CgtA)	At5g18570/ObgC	*	*		Buglino et al., 2002; Persky et al., 2009 ; Zhang et al., 2018
GTPase HflX (Hflx)	At5g57960/HflX	*	*		Zhang et al., 2018 ; Corrigan et al., 2016
GTPase BipA (BipA/TypA)	At5g13650/SVR3	*			Fan et al., 2015; Kumar et al. 2015
GTPase RsgA (RsgA)	At1g67440/RsgA	*			Zhang et al., 2018
GTPase RbgA (RbgA)	At4g02790/RbgA		*		Corrigan et al., 2016
Translation release factor RF3	no orthologue	*			Kihira et al., 2012; Zhang et al., 2018
Purine metabolism					
Adenylosuccinate synthetase (PurA)	At3G57610/ADSS	*			Stayton and Fromm, 1979
Amidophosphoribosyltransferase (PurF)	At2g16570/ASE1, At4g34740/ASE2, At4g38880/ASE3	*	-		Wang et al., 2019
Inosine-5'-monophosphate dehydrogenase (GuaB)	At1g16350	(*)	*		Kriel et al., 2012; Pao et al., 1981; Wang et al., 2019
Guanylate kinase (GmK)	At3g06200/GMK3/GKpm	-	*		Kriel et al., 2012; Liu et al., 2015, Nomura et al., 2019

Hypoxanthine phosphoribosyltransferase (Hpt)	no chloroplast orthologue	*	*		Anderson et al., 2019; Hochstadt-Ozer et al.,1972; Kriel et al., 2012; Zhang et al., 2018
Xanthine phosphoribosyltransferase (XpT)	no orthologue		*		Anderson et al., 2020
Adenine phosphoribosyltransferase (Apt)	no chloroplast orthologue	*	*		Haas et al., 2022; Hochstadt-Ozer et al.,1972
Nucleotide 5'-monophosphate nucleosidase (YgdH/PpnN)	no orthologue	*			Zhang et al., 2018
Others					
DNA primase (DnaG)	no orthologue	*			Maciag et al., 2010; Wang et al., 2007
Lysine decarboxylase (LdcI)	no orthologue	*			Kanjee et al., 2011a
Lysine decarboxylase (Ldcc)	no orthologue	*			Kanjee et al., 2011b
Ornithine decarboxylase (SpeF)	no orthologue	*			Kanjee et al., 2011b
Ornithine decarboxylase (SpeC)	no orthologue	*			Kanjee et al., 2011b
pppGpp pyrophosphatase (GppA)	no chloroplast orthologue	*			Keasling et al., 1993
(p)ppGpp synthetase (RelA)	At4g02260/RSH1, At3g14050/RSH2, At1g54130/RSH3, At3g17470/CRSH	*			Zhang et al., 2018; Shyp et al., 2012
Hydrogenase maturation factor (HypB)	no orthologue	*			Zhang et al., 2018
3-hydroxydecanoyl-[acyl-carrier-protein] dehydratase (FabA)	no orthologue				Stein et al., 1976
3-hydroxyacyl-[acyl-carrier-protein] dehydratase (FabZ)	At2g22230, At5g10160				Stein et al., 1976
acetyl coenzyme A carboxylase (ACC)					Polakis et al., 1973
alpha subunit (AccA)	CAC3/At2g38040				
beta subunit (AccD)	ACCD/AtCg00500				

A green square indicates the presence of a gene encoding the enzyme in the host genome. Lighter green indicates subunits of the same enzymatic complex. In the case of Arabidopsis only chloroplast targeted (predicted or demonstrated) enzymes are shown. A white asterisk indicates experimental evidence for (p)ppGpp binding, white dashes indicate experimental evidence showing a lack of (p)ppGpp binding. Brackets indicate conflicting evidence. FabA, FabZ, ACC and Arabidopsis guanylate kinase are inhibited by (p)ppGpp but binding has not been directly shown. References for studies demonstrating inhibition, activation or binding of the indicated enzymes by (p)ppGpp are listed to the right. (Anderson et al., 2020, 2019; Bharat and Brown, 2014; Buglino et al., 2002; Corrigan et al., 2016; Fan et al., 2015; Haas et al., 2022; Hochstadt-Ozer and Cashel, 1972; Kanjee et al., 2011a, 2011b; Keasling et al., 1993; Allison Kriel et al., 2012; Kumar et al., 2015; Legault et al., 1972; Liu et al., 2015; Maciag et al., 2010; Milon et al., 2006; Mitkevich et al., 2010; Pao and Dyes, 1981; Persky et al., 2009; Polakis et al., 1973; Rojas et al., 1984; Ross et al., 2016, 2013; Shyp et al., 2012; Stayton and Fromm, 1979; Stein and Bloch, 1976; Wang et al., 2019, 2007; Zhang et al., 2018)

Figure 1. RelA SpoT Homologue (RSH) enzymes are involved in guanosine tetraphosphate (ppGpp) and pentaphosphate (pppGpp) biosynthesis in plants, algae and bacteria. (A) Outline of the synthesis and hydrolysis of (p)ppGpp by RSH superfamily enzymes. (B) A schematic outline showing the evolutionary relationship of RSH enzymes based on the phylogenetic analysis of Avilan et al. (2019). The three main plant and algal families are shown (RSH1, RSH2/3 and RSH4/CRSH). (C) The domain structure of long RSH in bacteria and Arabidopsis. For bacteria we show the structure of a typical member of the Rel subgroup which is thought to represent the original bacterial long RSH (Atkinson et al., 2011). (D) The structure of the *E. coli* RelA, a bacterial long RSH, when bound to the ribosome. Inset shows the position of RelA on the ribosome, and the uncharged tRNA (grey) that interacts with RelA in the AH domain of the regulatory C-terminal region. Protein Data Bank identifier, 5L3P. CTP, chloroplast transit peptide; hydrolase, (p)ppGpp hydrolase domain; synthetase, (p)ppGpp synthetase domain; TGS, ThrRS, GTPase, and SpoT; AH, alpha-helical domain; RIS, ribosome-intersubunit domain; ACT, aspartate kinase–chorismate mutase–tyrA (prephenate dehydrogenase); EF *X*2, two EF-hand domains. Crossed-out text indicates the presence of a domain that is not catalytically active.

Figure 2. The physiological roles of (p)ppGpp in *Arabidopsis thaliana*. A simplified outline of the known physiological roles of (p)ppGpp signalling in *Arabidopsis thaliana*. Dashed lines indicate potential interactions where there is no direct evidence. ROS, reactive oxygen species.

Figure 3. Known and potential targets of (p)ppGpp in the chloroplast. (A) (p)ppGpp (purple circles) may be able to modulate chloroplast transcription through an interaction with the β' subunit of the plastid encoded polymerase (PEP) (right). This interaction site is distinct to those found in *E. coli* RNA polymerase (RNAP) (left). PAPs, PEP associated proteins (B) Chloroplastic (p)ppGpp targets in purine metabolism. GmK is directly inhibited by (p)ppGpp in vitro, and other enzymes of purine metabolism are potential targets based on their predicted chloroplastic localization and targeting by (p)ppGpp in bacteria. PRPP, phosphoribosylpyrophosphate; IMP, inosine monophosphate; AdS, adenylosuccinate. (C) Chloroplastic enzymes implicated in ribosome biogenesis, translation and ribosome hibernation / recycling that may be inhibited by (p)ppGpp. PSRP1, plastid-specific ribosomal protein 1 an orthologue of bacterial hibernation promoting factor (HPF). IC, initiation complex. See Table 1 for list of bacterial (p)ppGpp targets and their chloroplastic orthologues in plants.

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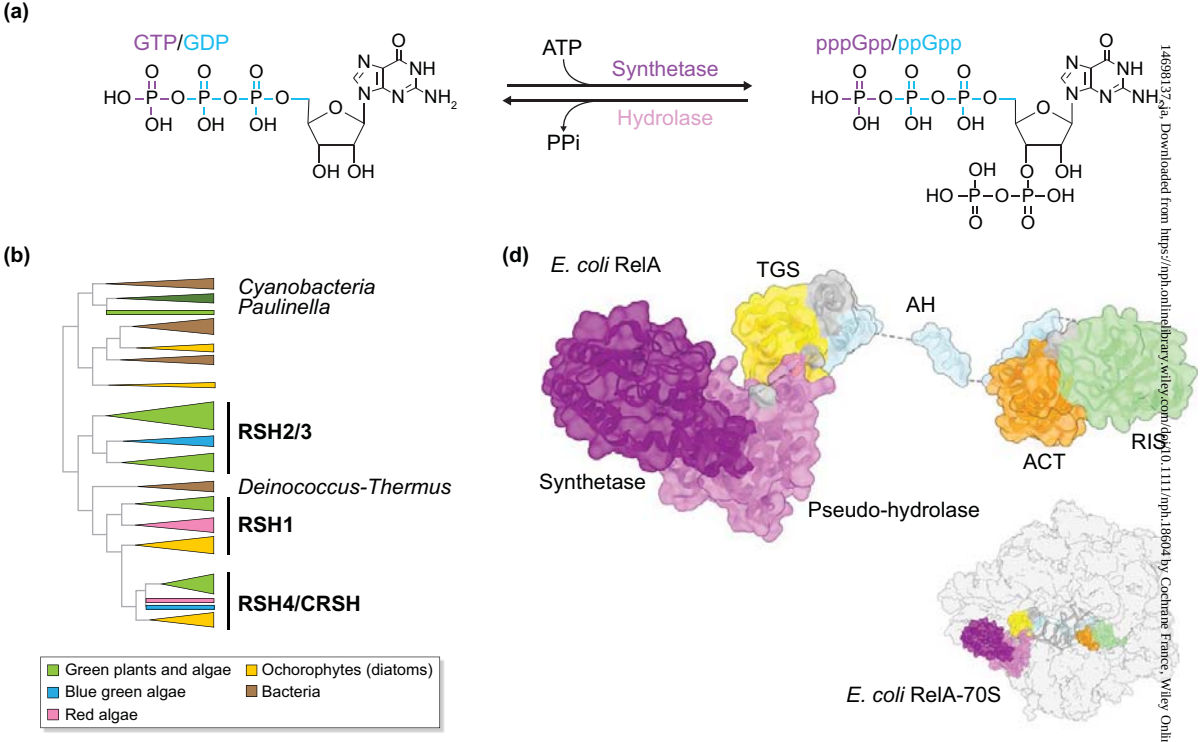

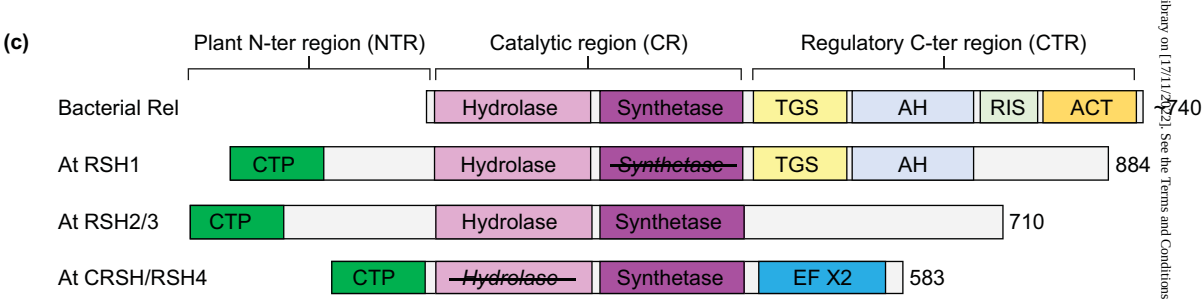

Figure 1

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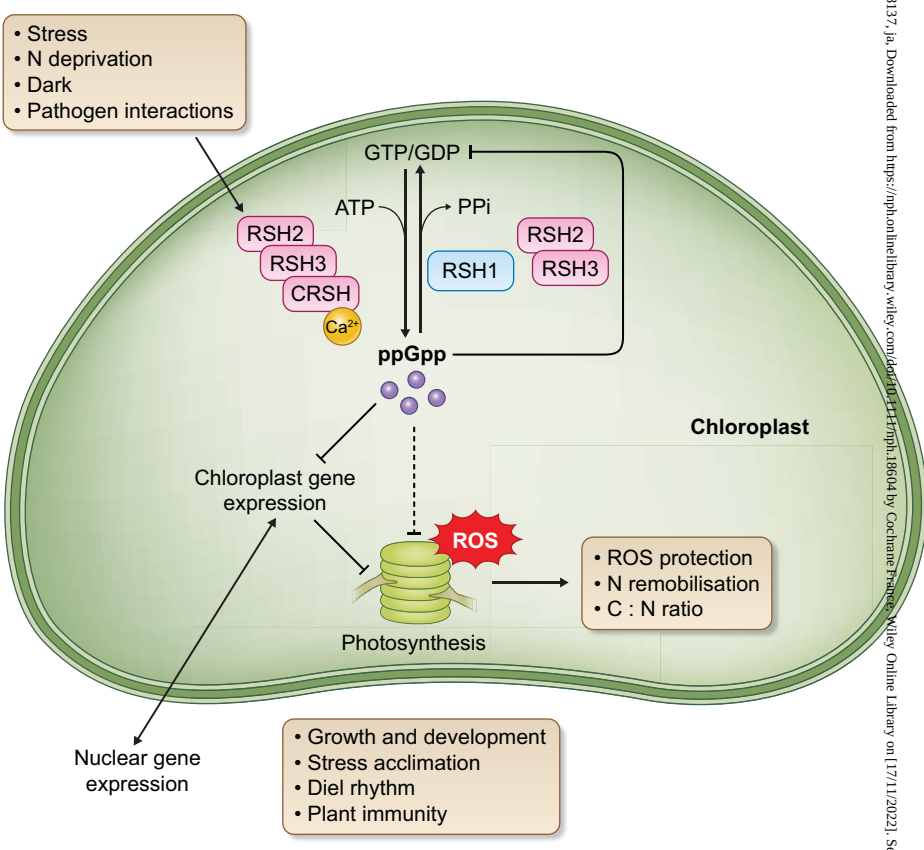

Figure 2

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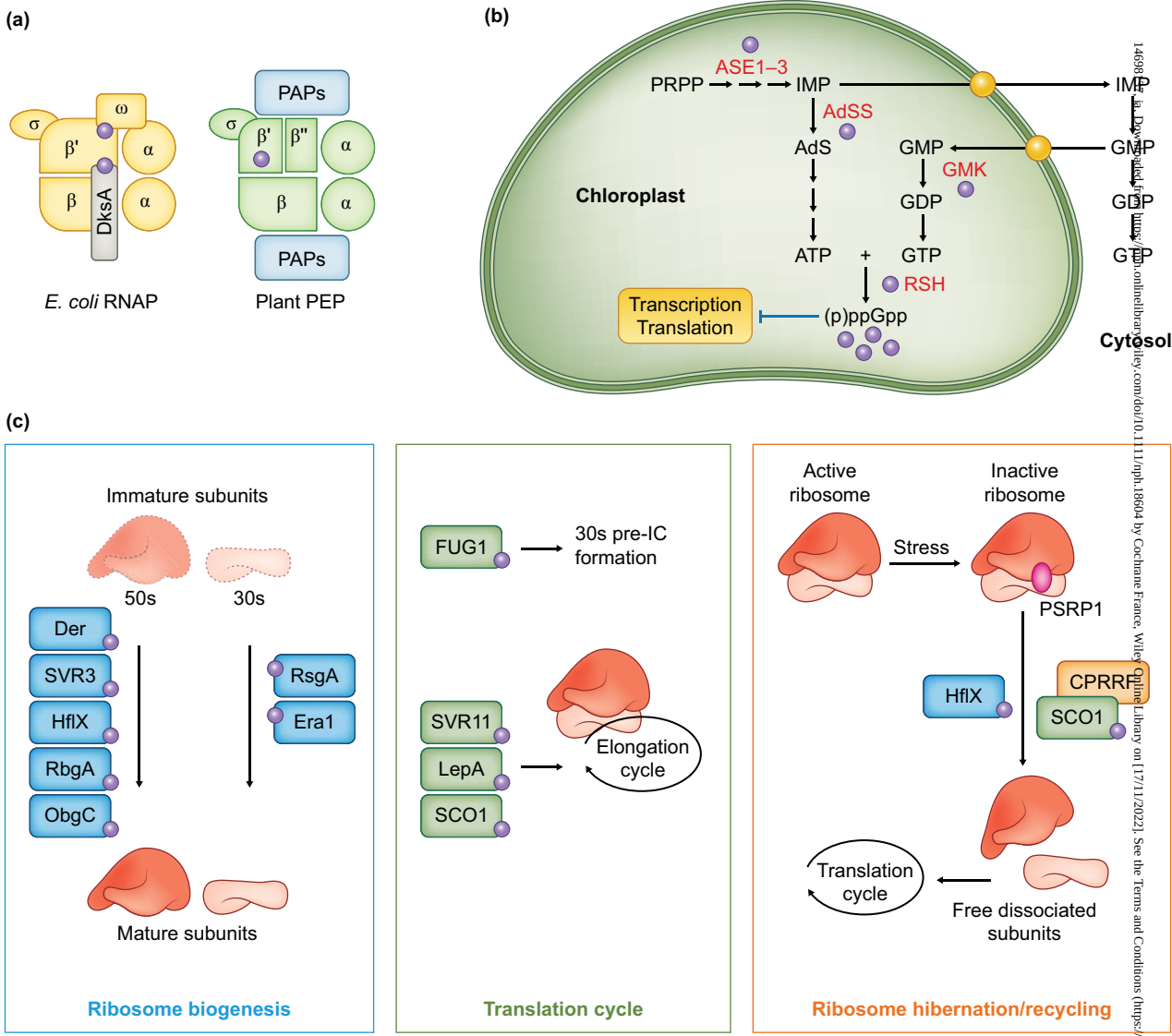

Figure 3

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