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1 **A guanosine tetraphosphate (ppGpp) mediated brake on photosynthesis is required for**
2 **acclimation to nitrogen limitation in Arabidopsis.**

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27

28 **Abstract**

29 Guanosine pentaphosphate and tetraphosphate (together referred to as ppGpp) are
30 hyperphosphorylated nucleotides found in bacteria and the chloroplasts of plants and algae. In
31 plants and algae artificial ppGpp accumulation can inhibit chloroplast gene expression, and
32 influence photosynthesis, nutrient remobilisation, growth, and immunity. However, it is so far
33 unknown whether ppGpp is required for abiotic stress acclimation in plants. Here, we
34 demonstrate that ppGpp biosynthesis is necessary for acclimation to nitrogen starvation in
35 *Arabidopsis*. We show that ppGpp is required for remodeling the photosynthetic electron
36 transport chain to downregulate photosynthetic activity and for protection against oxidative
37 stress. Furthermore, we demonstrate that ppGpp is required for coupling chloroplastic and
38 nuclear gene expression during nitrogen starvation. Altogether, our work indicates that ppGpp
39 is a pivotal regulator of chloroplast activity for stress acclimation in plants.

40

41 **Introduction**

42 Plants cannot easily escape harsh environmental fluctuations, and so their survival hinges on
43 facing each threat. To this end plants have developed intricate stress perception and response
44 mechanisms (Devireddy et al., 2021), where the chloroplast is recognized as both a major
45 signaling hub and a target for acclimation (Kleine et al., 2021). Likely candidates for
46 regulating chloroplast stress signaling are the hyperphosphorylated nucleotides guanosine
47 pentaphosphate and tetraphosphate (together referred to as ppGpp) that are synthesized from
48 ATP and GDP/GTP by chloroplast localized enzymes of the RelA SpoT Homologue (RSH)
49 family (Boniecka et al., 2017; Field, 2018). In bacteria, where ppGpp was originally
50 discovered, a considerable body of work indicates that ppGpp and related nucleotides interact
51 directly with specific effector enzymes to regulate growth rate and promote stress acclimation
52 (Ronneau and Hallez, 2019; Bange et al., 2021; Anderson et al., 2021). In plants, ppGpp
53 signaling is less well understood both at the physiological and mechanistic levels. ppGpp
54 levels increase transiently in diverse plants in response to treatment with a range of abiotic
55 stresses and stress related hormones (abscisic acid, jasmonate and ethylene)(Takahashi et al.,
56 2004; Ihara et al., 2015). The accumulation of ppGpp is controlled by the ppGpp synthesis
57 and hydrolysis activity of three families of chloroplast targeted RSH enzyme, RSH1, RSH2/3
58 and RSH4/CRSH (Atkinson et al., 2011; Ito et al., 2017; Avilan et al., 2019). In the
59 angiosperm *Arabidopsis thaliana* (Arabidopsis), where ppGpp signaling is best characterized,
60 RSH1 lacks ppGpp synthase activity and acts as the main ppGpp hydrolase (Sugliani et al.,
61 2016), the closely related RSH2 and RSH3 act as the major ppGpp synthases during the day
62 (Mizusawa et al., 2008; Maekawa et al., 2015; Sugliani et al., 2016), and the calcium
63 activated RSH (CRSH) is responsible for ppGpp synthesis at night and in response to
64 darkness (Ihara et al., 2015; Ono et al., 2020). RSH2 and RSH3 are bifunctional ppGpp
65 synthase / hydrolase enzymes, and their hydrolase activity was recently shown to be necessary
66 for constraining CRSH-mediated ppGpp production at night (Ono et al., 2020). The artificial
67 accumulation of ppGpp itself has been shown to repress the expression of certain chloroplast
68 genes *in vivo* via the inhibition of chloroplast transcription (Yamburenko et al., 2015;
69 Maekawa et al., 2015; Sugliani et al., 2016; Ono et al., 2020). The chloroplastic effectors of
70 ppGpp signaling in plants have not yet been identified. Nevertheless, ppGpp is able to
71 specifically repress activity of the chloroplast guanylate kinase, suggesting the existence of a
72 ppGpp effector mechanism that is found in many firmicute bacteria (Nomura et al., 2014;
73 Field, 2018; Bange et al., 2021). According to the firmicute model, guanylate kinase
74 inhibition causes a drop in GTP concentration, which in turn results in reduced transcription

75 from rRNA-coding genes where GTP is usually the initiating nucleotide. Over accumulation
76 of ppGpp in the chloroplast by the over-expression of RSH3 or bacterial ppGpp synthase
77 domains has also shown that ppGpp may act as a conserved repressor of photosynthesis in
78 both land plants (Maekawa et al., 2015; Sugliani et al., 2016; Honoki et al., 2018; Harchouni
79 et al., 2021) and algae (Avilan et al., 2021). Interestingly, the artificial accumulation of ppGpp
80 also appears to protect plants against nitrogen deprivation (Maekawa et al., 2015; Honoki et
81 al., 2018). In addition, a few studies have now demonstrated that ppGpp is required for
82 different physiological processes; these include the regulation of plant growth and
83 development (Sugliani et al., 2016; Ono et al., 2020), plant immunity (Abdelkefi et al., 2018),
84 and photosynthesis under standard growth conditions (Sugliani et al., 2016). However, there
85 are so far no demonstrations that ppGpp is required for abiotic stress acclimation in plants.

86

87 Here, we looked at the role of ppGpp in acclimation to nitrogen deprivation. ppGpp was
88 discovered in bacteria during research into the acclimation of *Escherichia coli* to amino acid
89 limitation (Cashel and Gallant, 1969). We reasoned that a similar role was likely to be
90 maintained in plants due to a shared and fundamental requirement for amino acids and the
91 nitrogen to synthesize them. In particular, chloroplasts, and the photosynthetic machinery
92 within, represent a major nitrogen store that must be remobilized during periods of nitrogen
93 limitation and during senescence. We found that ppGpp biosynthesis by RSH2 and RSH3 is
94 necessary for acclimation to nitrogen starvation in Arabidopsis. During nitrogen starvation
95 ppGpp acts by remodeling the photosynthetic electron transport chain to downregulate
96 photosynthetic activity, and by protecting against oxidative stress and tissue damage.
97 Furthermore, we show that ppGpp couples chloroplastic and nuclear gene expression during
98 nitrogen starvation. Overall, our work indicates that ppGpp is a pivotal regulator of
99 chloroplast activity with a photoprotective role and that ppGpp signaling is required for the
100 acclimation of plants to harsh environmental conditions.

101

102 **Results**

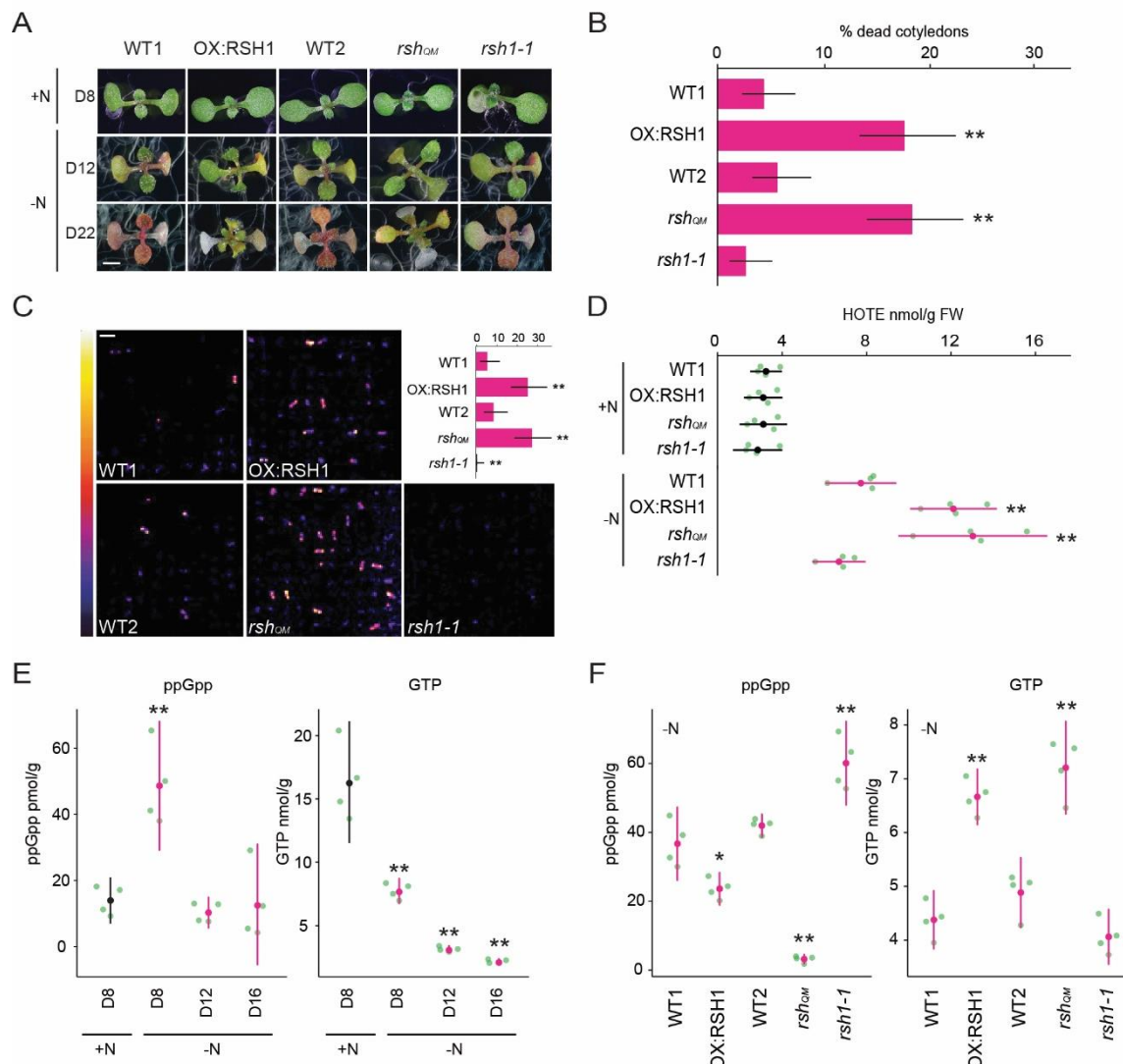
103 **ppGpp is required for acclimation to nitrogen deprivation**

104 To determine whether ppGpp plays a significant physiological role during nitrogen starvation
105 we grew a series of *RSH* lines on a nitrogen limiting medium that imposes a progressive
106 nitrogen starvation (Fig. 1A). The *RSH* lines were previously shown to accumulate lower
107 (OX:RSH1, *RSH* quadruple mutant [*rsh_{QM}*]) or higher (*rsh1-1*) amounts of ppGpp under
108 standard growth conditions (Sugliani et al., 2016). Growth arrest occurred for all lines 9-10
109 days after sowing on nitrogen limiting media and was followed by the production of
110 anthocyanins and loss of chlorophyll (Fig. 1A, Fig. 1- figure supplement 1). Strikingly, we
111 observed that the low ppGpp lines OX:RSH1 and *rsh_{QM}* showed significantly higher rates of
112 cotyledon death than in the corresponding wild-type plants (Fig. 1A, B). Interestingly,
113 between 16 and 22 days we also saw a greater rate of new leaf initiation in the ppGpp
114 deficient lines (Fig. 1A).

115

116 We reasoned that the increased cotyledon death in the low ppGpp lines could be due to
117 overproduction of reactive-oxygen species (ROS). We therefore imaged the autoluminescence
118 of lipid peroxides, a signature of ROS accumulation (Fig. 1C). Under nitrogen-deprivation
119 conditions strong autoluminescence was observed only in the OX:RSH1 and *rsh_{QM}* low
120 ppGpp lines, while high ppGpp *rsh1-1* plants showed almost no autoluminescence (Fig. 1C).
121 Quantification of lipid peroxidation products (hydroxy-octadecatrienoic acids [HOTEs], the
122 oxidation products of linolenic acid, the major fatty acid in Arabidopsis leaves) by HPLC
123 further supported these findings: HOTEs increased in the wild-type in response to nitrogen
124 deprivation, and this increase was significantly larger in the ppGpp deficient lines (Fig. 1D).
125 These results indicate that nitrogen deprivation promotes ROS accumulation, and that ppGpp
126 is required to prevent overaccumulation of ROS, oxidative stress and death of cotyledons
127 under these conditions.

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Figure 1. ppGpp is required for acclimation to nitrogen deprivation. (A) Images of seedlings grown on nitrogen replete (+N) or nitrogen limiting (-N) medium for 8 (D8), 12 (D12) and 22 (D22) days. Scale, 3 mm. WT1 (Col-0) and WT2 (*qrt1-2*) are the wild type for OX:RSH1 and *rsh_{QM}* / *rsh1-1* respectively. See Figure 1–figure supplement 1 for additional images. (B) Percentage of plants with dead cotyledons (completely white with collapsed tissue) for different genotypes grown on -N medium for 22 days. Three pooled experimental replicates, median +/- 95% CI, n = 285 - 298 seedlings per genotype. (C) Bioluminescence emission from lipid peroxides in seedlings grown on -N medium for 16 days. Inset graph shows median number of luminescent seedlings +/- 95% CI, n = 100 seedlings. Scale, 1.1 cm. (D) Quantification of HOTEs from seedlings grown in -N for 12 days or +N for 8 days where the developmental stage is similar. Mean +/- 95% CI, n = 4 experimental replicates. Concentrations of ppGpp and GTP in wild-type plants were determined (E) at the indicated time points during growth on +N and -N medium and (F) in different genotypes after 12 days of growth in -N medium (equivalent to 10 days in the experiment in panel E). The ppGpp/GTP ratio is shown in Figure 1–figure supplement 2. Mean +/- 95% CI, n = 4 experimental replicates. Statistical tests shown against respective wild-type controls, * $P < 0.05$, ** $P < 0.01$. Source data and statistical test reports are shown in Figure 1- source data 1.

147 **ppGpp levels increase at an early stage of nitrogen deprivation**

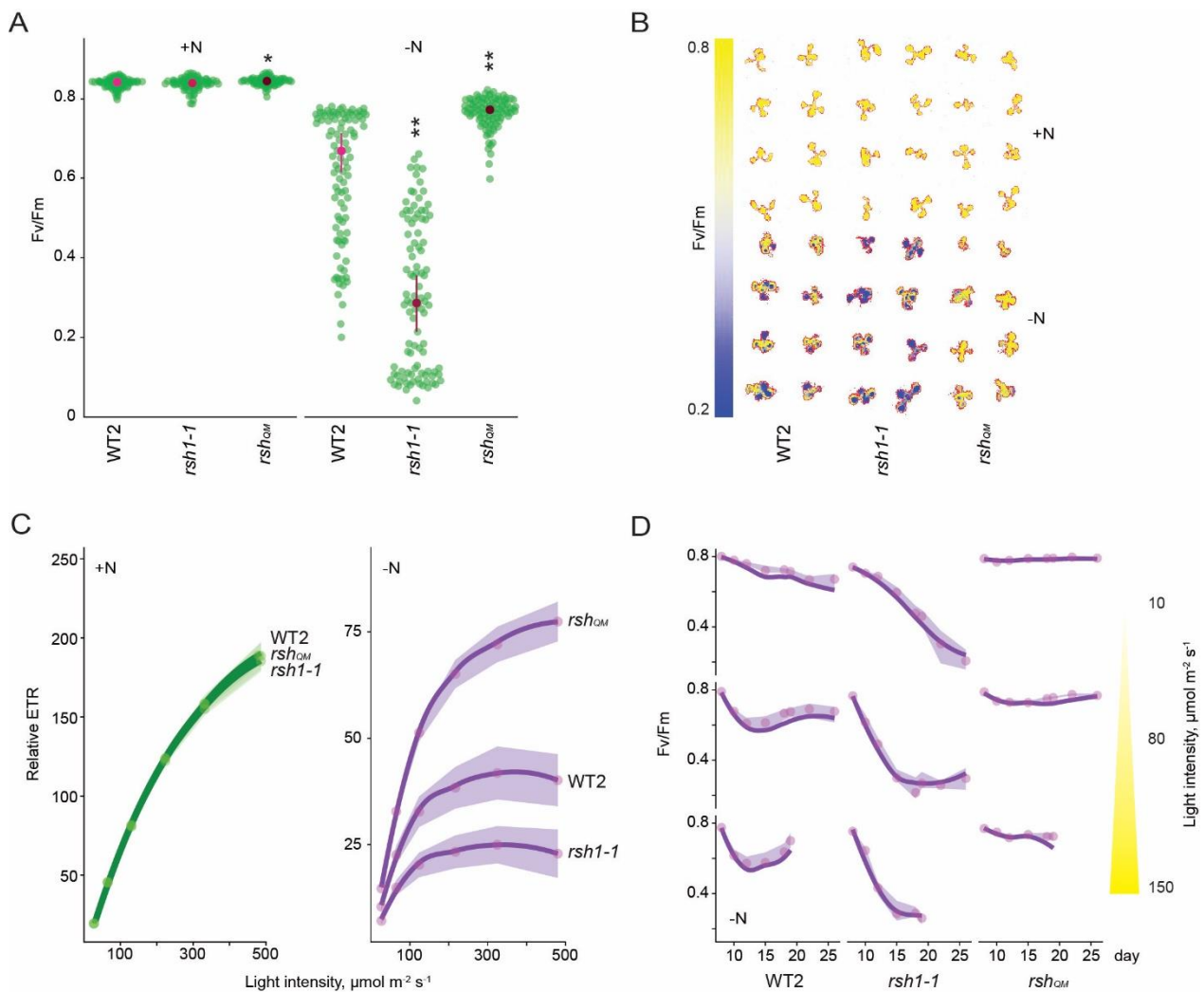
148 In bacteria and in plants ppGpp is known to peak in response to stress before stabilizing at
149 lower levels (Varik et al., 2017; Takahashi et al., 2004a; Ihara et al., 2015). To determine the
150 kinetics of changes in ppGpp concentration during nitrogen deprivation we quantified ppGpp
151 levels in wild-type plants at different timepoints during growth on nitrogen limiting media.
152 We observed a peak in ppGpp levels after 8 days on nitrogen limiting medium that was not
153 observed in plants of the same age grown on nitrogen replete medium (Fig. 1E). The ppGpp
154 concentration decreased and stabilised after 12 days on nitrogen limiting medium. In contrast,
155 the level of GTP decreased throughout the timecourse. We next determined how (p)ppGpp
156 levels were affected in each of the *RSH* lines during growth on nitrogen limiting medium (Fig.
157 1F). As anticipated we found that ppGpp levels in OX:RSH1 and *rsh_{QM}* lines were lower than
158 in wild-type plants, while they were higher in *rsh1-1* plants. Strikingly, we found that the
159 lines with low ppGpp levels (OX:RSH1 and *rsh_{QM}*) maintained higher levels of GTP than in
160 the wild-type controls or *rsh1-1* (Fig. 1F), resulting in dramatic differences in the ppGpp/GTP
161 ratio (Fig. 1- figure supplement 2). Therefore, nitrogen limitation leads to an early increase in
162 ppGpp levels and is followed by a progressive diminution of the GTP pool that is dependent
163 on ppGpp accumulation. These results suggest that ppGpp inhibits GTP biosynthesis, perhaps
164 through inhibition of guanylate kinase (Nomura et al., 2014), and supports the hypothesis that
165 plants have a ppGpp effector mechanism that operates through purine metabolism as found in
166 many bacteria.

167

168 **Nitrogen deprivation promotes a ppGpp-dependent drop in photosynthetic activity**

169 Under standard growth conditions artificial production of ppGpp has been shown to
170 downregulate photosynthetic activity in Arabidopsis (Sugliani et al., 2016; Maekawa et al.,
171 2015). Defective downregulation of photosynthesis during nitrogen starvation could cause the
172 over-accumulation of ROS and lipid peroxides observed in the low ppGpp lines due to
173 oversaturation of photosynthesis in the presence of diminished metabolic sinks. We therefore
174 measured different photosynthetic parameters in the wild type and *RSH* lines during growth
175 on nitrogen limiting medium. A decrease in the maximum quantum yield (Fv/Fm) of
176 photosystem II (PSII) caused by an increase in basal fluorescence (Fo, see Materials and
177 Methods for formula) was observed in the cotyledons and first set of true leaves of wild-type
178 seedlings (Fig. 2A-B, Fig. 2- figure supplement 1, Fig. 2- source data 1). Remarkably, this
179 decrease was almost completely suppressed in the low ppGpp *rsh_{QM}* and OX:RSH1 lines, and
180 was enhanced in the high ppGpp *rsh1-1* mutant (Fig. 2A, 2B, Fig. 2- figure supplement 2A).

181 Analysis of *rsh2-1* and *rsh3-1* single mutants, as well as the complementation of an *rsh2-1*
 182 *rsh3-1* double mutant with the genomic version of *RSH3*, indicates that the RSH3 enzyme is
 183 the major ppGpp synthase responsible for driving the Fv/Fm decrease under nitrogen
 184 starvation conditions (Fig. 2- figure supplement 2B). Likewise, the enhanced Fv/Fm decrease
 185 in the high ppGpp *rsh1-1* mutant and complementation of the *rsh1-1* phenotype by expression
 186 of the genomic *RSH1* indicate that the ppGpp hydrolase RSH1 acts antagonistically to RSH3,
 187 probably by constraining ppGpp accumulation during nitrogen deprivation (Fig. 1F. 2A, 2B,
 188 Fig. 2- figure supplement 2C).



189 **Figure 2. Nitrogen deprivation promotes a ppGpp-dependent drop in photosynthetic capacity.**
 190 Seedlings were grown 8 days on nitrogen replete media (+N) or 12 days on nitrogen limiting (-N)
 191 media and (A) the maximal yield of PSII (Fv/Fm) measured by fluorescence imaging individual
 192 seedlings. Median +/- 95% CI, n = 95-100 seedlings. (B) Fv/Fm images of whole seedlings grown on
 193 +N and -N media for 12 days. (C) Relative ETR measurements in different lines grown 8 days on +N
 194 media or 12 days on -N media. Median +/- 95% CI, n = 95-100 seedlings. (D) Fv/Fm time-courses
 195 from seedlings grown on -N media and transferred to three different light intensities (photosynthetic
 196 photon flux density, 10, 80 and 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$) after 6 days. Median +/- 95% CI, n = 95-100
 197 seedlings. Tests shown against respective wild-type controls, * $P < 0.05$, ** $P < 0.01$. Additional
 198 supporting data is presented in Figure 2- figure supplements 2-4. Source data and statistical test
 199 reports are shown in Figure 2- source data 1.

200

201 While unlikely, it is possible that RSH enzymes could influence Fv/Fm via mechanisms that
202 do not require ppGpp synthesis or hydrolysis. To exclude this possibility we conditionally
203 overexpressed a heterologous chloroplast targeted ppGpp hydrolase from *Drosophila*
204 *melanogaster* (Metazoan SpoT homolog, MESH)(Sugliani et al., 2016) (Fig. 2- figure
205 supplement 2D). We observed that ppGpp depletion by MESH suppresses the Fv/Fm decrease
206 in response to nitrogen limitation, confirming a specific role for ppGpp in this process.

207

208 Our experiments were carried out on plate-grown seedlings and in the presence of exogenous
209 sugar in the media. To test the robustness of our observations to different experimental set-
210 ups we exposed mature plants grown in quartz sand to nitrogen starvation conditions (Fig. 2-
211 figure supplement 2E). We observed responses from the wild type, *rsh_{QM}* and *rshI-1* mutant
212 that were consistent with those observed for plate grown seedlings, confirming the robustness
213 of the ppGpp-dependent decrease in Fv/Fm in response to nitrogen limitation.

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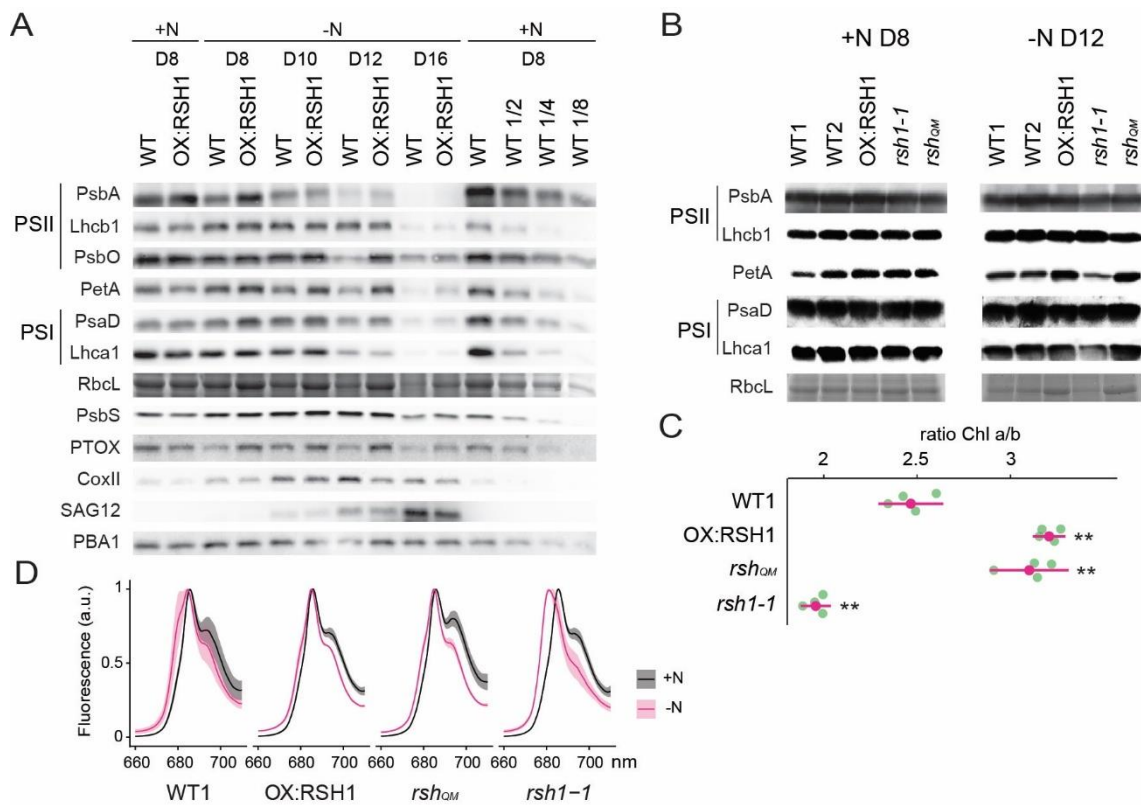
215 Fv/Fm measurements provide information principally on PSII. To understand the state of the
216 entire photosynthetic electron transport chain we next measured the relative rate of electron
217 transport (ETR). We found that nitrogen deprivation led to a large decrease in ETR in wild-
218 type plants (Fig. 2C, Fig. 2- figure supplement 4A). The *RSH* lines showed similar ETRs to
219 the wild type under nitrogen replete conditions. However, nitrogen deprivation led to large
220 differences in the *RSH* lines compared to the wild type: a low ETR was observed in the high
221 ppGpp *rshI-1* mutant, and a substantially higher ETR in the low ppGpp *rsh_{QM}* and OX:RSH1
222 lines. Induction of the MESH ppGpp hydrolase also prevented much of the decrease in ETR
223 observed under nitrogen deprivation, again indicating that this phenomenon is dependent on
224 the activity of RSH enzymes and ppGpp accumulation (Fig. 2- figure supplement 4B).

225

226 **The ppGpp-dependent decline in photosynthesis occurs even at low light fluences**

227 The ppGpp-dependent drop in Fv/Fm during nitrogen deprivation is reminiscent of
228 photoinhibition, a process whose rate is proportional to light intensity (Tyystjarvi and Aro,
229 1996). We therefore asked whether the ppGpp-dependent decrease in Fv/Fm is similarly
230 dependent on the excitation pressure on the photosystems. Strikingly, under nitrogen
231 deprivation the Fv/Fm of the wild type decreased to a similar minimum regardless of the light
232 intensity (photosynthetic photon flux density; low light, 10 $\mu\text{mol m}^{-2}\text{s}^{-1}$; growth light, 80
233 $\mu\text{mol m}^{-2}\text{s}^{-1}$; and higher light, 150 $\mu\text{mol m}^{-2}\text{s}^{-1}$)(Fig. 2D). However, the rate of the Fv/Fm

234 decrease was proportional to light intensity, showing a slower decrease to the minimum under
 235 low light than under normal light or high light. A similar phenomenon was observed in the
 236 high ppGpp *rsh1-1* line, except that the Fv/Fm dropped at a faster rate and to a considerably
 237 lower level. In contrast, in the low ppGpp *rsh_{QM}* mutant we observed almost no decrease in
 238 Fv/Fm, regardless of the light intensity tested. The insensitivity of the *rsh_{QM}* mutant indicates
 239 that the process is completely dependent on ppGpp biosynthesis. Furthermore, the response of
 240 the wild-type and even stronger response of the *rsh1-1* mutant at all light fluences indicate
 241 that ppGpp-initiated regulation of Fv/Fm is triggered in response to nitrogen deprivation
 242 rather than to changes in the excitation status of the photosynthetic electron transport chain
 243 (Fig. 2D).



244 **Figure 3. ppGpp-dependent alterations in the photosynthetic machinery during nitrogen**
 245 **deficiency. (A)** Immunoblots showing evolution in abundance of the indicated proteins in seedlings
 246 grown in nitrogen replete (+N) or nitrogen limiting (-N) media for the indicated number of days. RbcL
 247 was revealed by Coomassie Brilliant Blue. Equal quantities of total proteins were loaded and PBA1, a
 248 subunit of the proteasome, was used as a protein normalization control **(B)** Immunoblots showing the
 249 abundance of the indicated proteins in purified thylakoid membranes from seedlings grown in +N for 8
 250 days or -N for 12 days. RbcL was revealed by Coomassie Brilliant Blue staining. Equal quantities of
 251 total chlorophyll were loaded. Immunoblots after ppGpp-depletion by induction of chloroplastic MESH
 252 are shown in Figure 3- supplement 1. **(C)** Chlorophyll a/b ratios in extracts from seedlings subjected to
 253 -N for 12 days. Means +/- 95% CI, data from four experimental replicates. **(D)** Emission spectrum of
 254 chlorophyll fluorescence at 77°K between 660 and 720 nm, normalized to the PSII peak at 685 nm.
 255 The full spectra are shown in Figure 2- supplement 2. Measurements were made on seedlings grown
 256 in +N for 8 days or -N for 12 days. Means ± 95% CI; data from four experimental replicates. Statistical
 257 tests, ** $P < 0.01$. Uncropped immunoblots are available in Figure 3- source data 1 for 3A and source
 258 data 2 for 3B. Numeric data and statistical test reports are shown in Figure 3- source data 3.

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ppGpp is required for the timely degradation of photosynthetic proteins

In order to better understand the changes in the photosynthetic machinery that underlie the reduction of photosynthetic capacity during nitrogen starvation we analyzed the abundance of representative photosynthetic proteins in the wild type and the low ppGpp line OX:RSH1 (Fig. 3A). In the wild type, we observed a decrease in proteins representative of nearly all the major photosynthetic complexes analysed (PsbA, Lhcb1, PsbO, PetA, PsaD, Lhca1, RBCL) and PTOX analyzed between 8 and 16 days of growth under nitrogen deprivation. The decrease in photosynthetic proteins was defective in OX:RSH1 plants, which showed a marked delay for the decrease in abundance of PsbA, PsbO, PetA, PTOX, PsaD and RBCL. This delay was especially visible after 12 days on nitrogen limiting medium. On the contrary, changes in Lhcb1 and Lhca1, the nuclear encoded light harvesting proteins of PSII and PSI, were indistinguishable between wild-type and OX:RSH1 plants.

In contrast to the downregulation of many components of the photosynthetic chain during nitrogen deprivation, mitochondrial activity appeared to increase. The mitochondrial marker protein COXII, a subunit of the mitochondrial complex IV, increased to reach a maximum on day 12 of nitrogen deprivation. Interestingly, the COXII maximum was higher in the wild type than in OX:RSH1 plants suggesting increased ppGpp levels could also positively influence mitochondrial activity. PsbS, a key component of non-photochemical quenching, and SAG12, a marker of senescence, showed similar patterns of accumulation in WT and OX:RSH1 plants.

We next extended our analysis from OX:RSH1 to the other RSH lines to confirm that ppGpp is responsible for the observed changes in photosynthetic proteins during nitrogen deprivation (Fig. 3B). In this experiment we normalized to total chlorophyll to exclude possible artefacts linked to normalization on total protein, yet we obtained broadly similar results. PetA and RBCL, the proteins with the greatest differences in OX:RSH1 during the timecourse, were more abundant in the low ppGpp lines OX:RSH1 and *rsh_{QM}* at day 12 under nitrogen deprivation. The same proteins were also less abundant than the wild type in the high ppGpp line *rsh1-1*. Interestingly, PsbA accumulation did not appear different between the lines, contrasting with results from the OX:RSH1 time course. However, this difference is likely linked to the chlorophyll normalization because Lhcb1 levels were higher in the wild type and *rsh1-1* mutant, indicating a similar direction of change for the ratio of PSII core to antenna.

293 Finally, to unequivocally link these changes to ppGpp we analyzed representative protein
294 levels in induced MESH ppGpp hydrolase lines, which again showed the ppGpp is required
295 for diminution of PetA and RBCL levels during nitrogen deprivation (Fig. 3- figure
296 supplement 1). Altogether our immunoblotting experiments indicate the ppGpp is required for
297 the timely reduction in abundance of key photosynthetic proteins during nitrogen starvation,
298 and that proteins such as PsbO (PSII core), PetA (Cyt b₆f) and RBCL (Rubisco) appear to be
299 preferentially affected.

300

301 **Nitrogen starvation promotes ppGpp-dependent energetic uncoupling of PSII antenna**

302 The ppGpp dependent drop in Fv/Fm and drop in the PsbA / Lhcb1 ratio suggest that ppGpp
303 specifically remodels the structure of the PSII complex during nitrogen deprivation.

304 Supporting this idea, we also found a ppGpp dependent drop in the Chl a/b ratio (Fig. 3C),
305 indicating a substantial decrease in PSII RC, which lacks Chl b, to Chl b rich PSII antenna.

306 An increase in relative PSII antenna abundance might be accompanied by the energetic
307 uncoupling from the PSII RC. Low temperature chlorophyll fluorescence spectra confirmed
308 this hypothesis. During nitrogen deprivation there was a shift of the PSII antenna emission
309 peak towards lower wavelengths in the wild type, indicating an energetic decoupling from
310 PSII RC (Crepin et al., 2016)(Fig. 3D, Fig. 3- figure supplement 2). This shift is mainly
311 ppGpp dependent because it was smaller in the low ppGpp OX:RSH1 and *rsh_{QM}* lines, and
312 much stronger in the high ppGpp *rsh1-1* line. Therefore, multiple approaches show that
313 ppGpp is necessary for PSII remodeling and decreasing the excitation pressure on the
314 photosystems during nitrogen starvation.

315

316 **ppGpp plays a major role during acclimation to nitrogen deprivation**

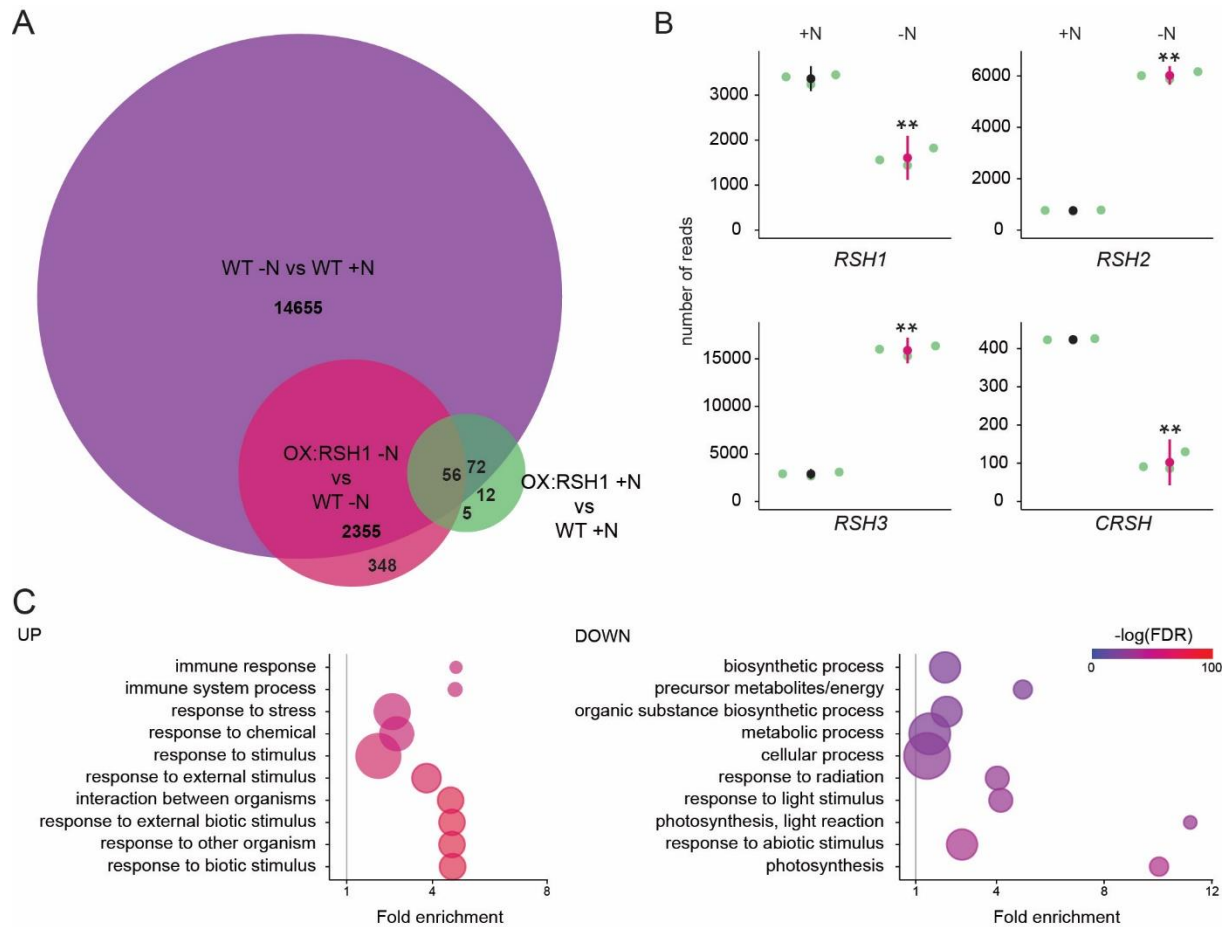
317 Our results show that defects in the capacity of plants to accumulate ppGpp lead to aberrant
318 photosynthesis and stress phenotypes under nitrogen deprivation. To determine the extent and
319 specificity of the impact of ppGpp on cellular processes we analyzed global nuclear and
320 organellar transcript abundance in wild-type and OX:RSH1 plants.

321

322 Nitrogen deprivation caused a massive alteration in transcript levels in both the wild type and
323 OX:RSH1, significantly affecting the accumulation of about 15,000 transcripts (Fig. 4A, Fig.
324 4- source data 1). For the wild type there was substantial overlap (80%) with differentially
325 accumulating transcripts recently identified in plants grown under chronic nitrogen limitation
326 for 60 days (Luo et al., 2020). Strikingly, we observed large increases in *RSH2* and *RSH3*

327 transcript levels in response to nitrogen deprivation (Fig 4B). These were accompanied by a
328 decrease in *RSH1* transcript levels, suggesting a coordinated upregulation of ppGpp
329 biosynthetic capacity. The expression profile of OX:RSH1 plants showed clear differences to
330 the wild type, especially under nitrogen deprivation. Under control conditions OX:RSH1
331 plants had a very similar gene expression profile to the wild type, with the differential
332 accumulation of only 150 transcripts (Fig. 4A, Fig. 4- source data 1). However, under
333 nitrogen deprivation we observed the differential accumulation of 2700 transcripts in
334 OX:RSH1. The greater deregulation of the OX:RSH1 transcript profile under nitrogen
335 deprivation indicates that ppGpp plays a major and specific role in the acclimation response.
336

337 Analysis of enrichment for gene ontology terms corroborated our findings that low ppGpp
338 plants are unable to properly acclimate to nitrogen deprivation. OX:RSH1 plants accumulated
339 more transcripts associated with stress, including oxidative stress than the wild type (Fig. 4C,
340 Fig. 4- source data 2). We also observed a significant decrease in the abundance of transcripts
341 for genes involved in photosynthesis and chloroplast activity. Notably, these downregulated
342 genes were all nucleus encoded (Fig. 4C).
343



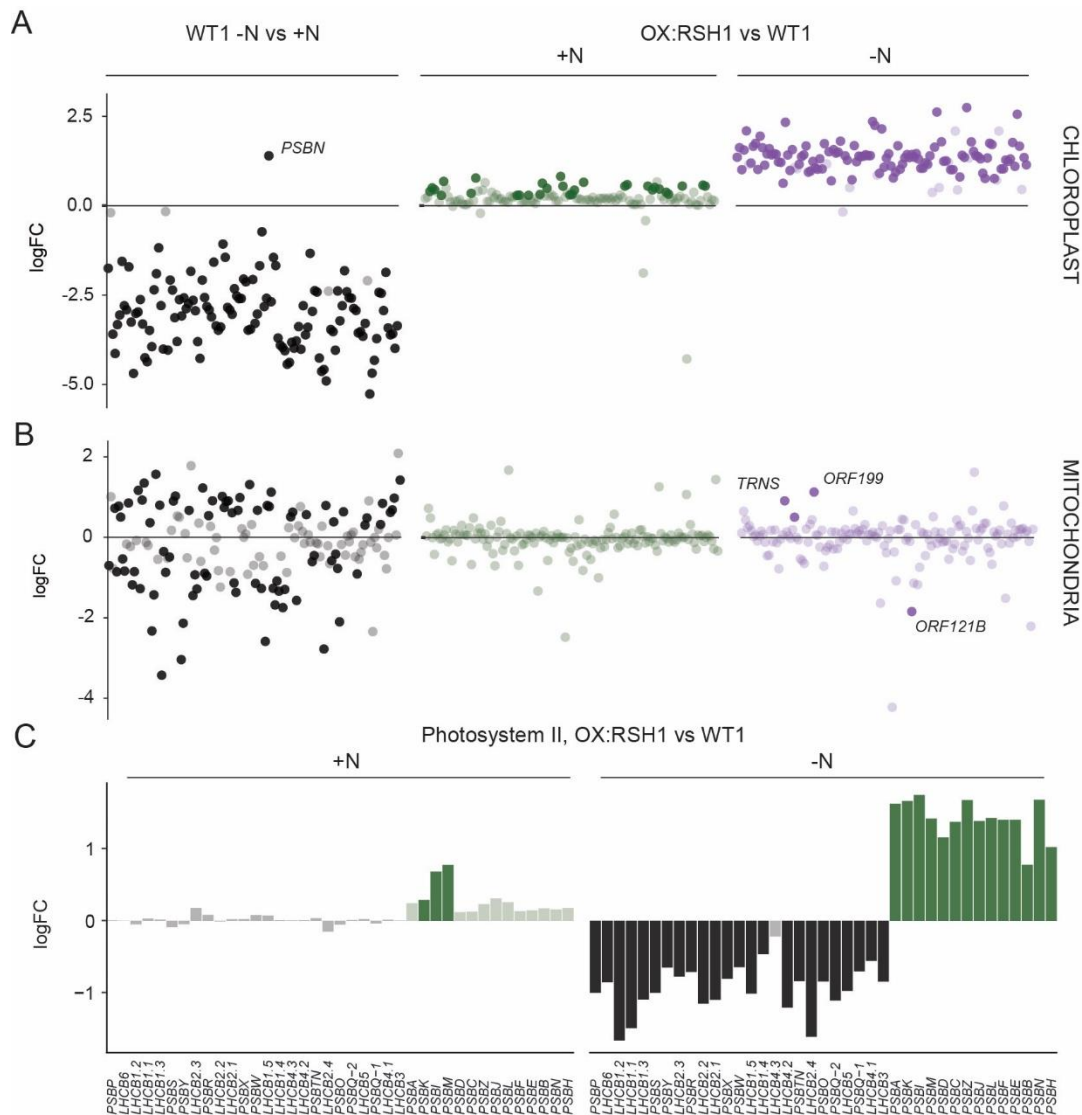
345
346 **Figure 4. ppGpp plays a major role during acclimation to nitrogen deprivation.** RNA-seq
347 experiments were performed on WT and OX:RSH1 seedlings grown 8 days on nitrogen replete media
348 (+N) or 12 days on nitrogen limiting (-N) media, n=3 experimental replicates. **(A)** Venn diagram for
349 transcripts showing differential accumulation for each of three comparisons. All differentially
350 accumulating transcripts are listed in Figure 4- source data 1. **(B)** RNA-seq transcript levels for the
351 four *RSH* genes in the WT, +N vs -N. Mean +/- 95% CI, ** $P < 0.01$. **(C)** Enriched gene ontology terms
352 among significantly up- and down-regulated transcripts in OX:RSH1 vs WT under -N. The ten most
353 significant terms are shown, point size is proportional to gene number. FDR, false discovery rate. The
354 full GO analysis is presented in Figure 4- source data 2. Source data and statistical test reports are
355 shown in Figure 4- source data 3.

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361 **ppGpp is required for global downregulation of chloroplast gene expression**

362 We next turned to the effects of nitrogen deprivation on organellar gene expression. Nitrogen
363 deprivation leads to a strong decrease in the levels of all chloroplast transcripts except *PsbN*
364 in the wild type (Fig. 5A, Fig. 5- source data 1). The OX:RSH1 mutant showed minor
365 differences to the wild type under normal growth conditions. However, under nitrogen
366 starvation the vast majority of chloroplast transcripts (120 of 133 transcripts analyzed) were

367 present at significantly higher levels in OX:RSH1 than in the wild type, indicating that ppGpp
368 is required for globally reducing chloroplast gene expression. In contrast to the
369 monodirectional response of the chloroplast to nitrogen deprivation, we found that
370 mitochondrial transcripts showed diverse responses in the wild type (Fig. 5B, Fig. 5- source
371 data 2). Significantly, mitochondrial transcript levels in the OX:RSH1 line were highly
372 similar to the wild type under control and nitrogen deprivation conditions. Taken together,
373 these results indicate that ppGpp is required for the downregulation of chloroplast gene
374 expression during nitrogen starvation and demonstrate that ppGpp acts specifically within the
375 chloroplast.
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Figure 5. ppGpp is required for the downregulation of chloroplast gene expression during nitrogen deficiency. (A) The differential expression of chloroplast transcripts ordered along the chloroplast genome (Figure 5- source data 1). (B) The differential expression of mitochondrial transcripts ordered along the mitochondrial genome (Figure 5- source data 2). (C) Relative transcript levels in OX:RSH1 versus the wild-type control for nuclear (black) and chloroplast (green) genes encoding subunits of the photosystem II complex. Solid colors indicate significantly different changes in expression ($P < 0.05$), transparent colors indicate non-significant changes. Source data for 5C is shown in Figure 5- source data 3.

389 ppGpp is required for coordinating chloroplast and nuclear gene expression

390 Our gene expression analysis showed the ppGpp depletion during nitrogen starvation
391 appeared to have opposite effects on transcript abundance for nuclear encoded chloroplast
392 genes and chloroplast encoded genes. Many chloroplast protein complexes contain subunits
393 encoded by both the nuclear and chloroplast genomes. Notably, in the wild-type chloroplast
394 protein complexes showed coordinate downregulation of both nuclear and chloroplast

395 transcripts in response to nitrogen deprivation (Fig. 5- figure supplement 1). However, ppGpp
396 depletion caused widespread mis-regulation of transcript abundance for complex subunits
397 (Fig 5C- source data 3). For the PSII complex there was little difference between OX:RSH1
398 and the wild type under nitrogen replete conditions. However, in OX:RSH1 nitrogen
399 deprivation caused the large scale uncoupling of the coordination of gene expression between
400 the nuclear and chloroplast genomes: all but one of the transcripts for nuclear-encoded
401 subunits of PSII were downregulated, while all the transcripts for chloroplast-encoded
402 subunits of PSII were upregulated (Fig. 5C). Similar genome uncoupling was observed in
403 OX:RSH1 for transcripts encoding subunits of the chloroplastic Cyt b_6f , PSI, ATP synthase,
404 NDH, TIC, transcription and translation complexes (Fig. 5- source data 3). These results
405 indicate first that nitrogen deprivation promotes the coordinated downregulation of genes
406 encoding chloroplastic proteins in the nuclear and chloroplastic genomes, and second that
407 ppGpp plays a pivotal role in coupling gene expression between nuclear and chloroplast
408 genomes under nitrogen limitation.
409

410 **Discussion**

411 Nitrogen deprivation has long been known to cause a drop in photosynthetic capacity in plants
412 (Terashima and Evans, 1988; Nunes et al., 1993; Verhoeven et al., 1997; Lu and Zhang, 2000;
413 Garai and Tripathy, 2018). Here we show that ppGpp signaling plays a major role in this
414 process. We demonstrate that nitrogen starvation leads to an early and transient increase in
415 ppGpp levels (Fig. 1E), and that the capacity to synthesize and accumulate ppGpp is then
416 required for protecting plants against excess ROS accumulation, tissue damage and stress
417 (Fig. 1A-D, 4C). We show that ppGpp is likely to mediate the acclimation response by
418 affecting the ppGpp/GTP ratio (Fig. 1- figure supplement 2), promoting the downregulation
419 of photosynthetic capacity (Fig. 2A-D, 4C, 5A) and remodeling the photosynthetic machinery
420 (Fig. 3A-D, 5C). Finally, we show that ppGpp may function by specifically downregulating
421 chloroplast transcript abundance during nitrogen deprivation to maintain an equilibrium
422 between chloroplast and nuclear gene expression (Fig. 5A-C, Fig. 5- source data 1). ppGpp
423 was initially discovered thanks to the identification of bacterial mutants that were unable to
424 downregulate rRNA transcription during amino acid starvation (Stent and Brenner, 1961;
425 Cashel and Gallant, 1969). Our results here therefore indicate that the fundamental
426 involvement of ppGpp signaling in acclimation to nitrogen deprivation is likely to have been
427 maintained despite the large evolutionary distance separating bacteria from plants that
428 includes major shifts including the domestication of the cyanobacterial ancestor of the
429 chloroplast, and the different regulatory logic and signaling networks of photosynthetic
430 eukaryotes.

431

432 ***The physiological relevance of ppGpp signaling in plants***

433 Depletion or removal of ppGpp is usually necessary for directly establishing the implication
434 of ppGpp in a physiological process. Up to now a handful of studies in plants have directly
435 implicated ppGpp physiological processes by showing a requirement for ppGpp synthesis
436 (Sugliani et al., 2016; Abdelkefi et al., 2018; Honoki et al., 2018). We now add to these
437 studies by using multiple approaches, including three different ppGpp depletion methods
438 (OX:RSH1, *rsh_{QM}* and MESH), to demonstrate that ppGpp is required for acclimation to
439 nitrogen deprivation. Previous work hinted that ppGpp may play such a role by showing that
440 plants ectopically over-accumulating ppGpp appear able to better withstand transfer to media
441 lacking nitrogen (Maekawa et al., 2015; Honoki et al., 2018). Notably, Honoki et al. (2018)
442 also used a similar *rsh2 rsh3* double mutant in their experiments though, apart from a delay in
443 Rubisco degradation, did not observe clear-cut differences from the wild type in terms of

444 photosynthetic activity after transfer to media lacking nitrogen. The reasons for this are not
445 clear, but it could be related to differences in experimental set up such as the timepoints for
446 analysis, how nitrogen starvation was imposed, or the different RSH3 mutant allele used.

447

448 Our findings further reinforce the evidence that RSH enzymes function in the chloroplast.
449 GFP fusion experiments have shown that RSH enzymes are localised to the chloroplasts
450 (Maekawa et al., 2015), and the pleiotropic phenotypes of RSH3 overexpressing plants can be
451 suppressed by chloroplast expression of a ppGpp hydrolase (Sugliani et al., 2016). However,
452 some proteins, such as ATP synthase subunit beta-3, show low levels of dual targeting that
453 are not detectable using full length fluorescent protein fusions (Sharma et al., 2019). Coverage
454 of the full mitochondrial transcriptome here shows that overexpression of RSH1 has almost
455 no effect on the large-scale changes in mitochondrial transcript abundance that occur in
456 response to nitrogen deprivation (Fig. 5B). These results therefore confirm that RSH enzymes
457 operate within the chloroplast.

458

459 *New insights into ppGpp signaling in planta*

460 The kinetics of changes in ppGpp concentration during nitrogen deprivation provide new
461 information on how ppGpp acts *in planta*. We observed a transient peak in ppGpp levels after
462 eight days of growth on nitrogen limiting medium (Fig. 1E). Similar transient peaks of ppGpp
463 accumulation were also observed in plants subjected to different stresses (Takahashi et al.,
464 2004; Ihara et al., 2015; Ono et al., 2020), and are observed in bacterial cells (Varik et al.,
465 2017). Notably, the peak in ppGpp that we observed in response to nitrogen deprivation
466 occurs prior to major changes in Fv/Fm (Fig. 2D), protein levels (Fig. 3A), or anthocyanin
467 accumulation (Fig. 1- figure supplement 1). These factors, along with the progressive nature
468 of the nitrogen starvation suggest that a signaling mechanism activates ppGpp biosynthesis
469 relatively soon after perception of nitrogen supply limitation. The upregulation of *RSH2* and
470 *RSH3* expression (Fig. 4B) indicates that there is at least a transcriptional component to this
471 response. However, additional layers of regulation are likely, including for example the
472 allosteric activation of RSH enzymes by interaction with other proteins or small molecules as
473 found for bacterial RSH (Ronneau and Hallez, 2019; Irving and Corrigan, 2018). After the
474 initial peak the ppGpp concentration then drops to levels not very different to those observed
475 in plants grown in nitrogen replete conditions (Fig. 1E). Furthermore, OX:RSH1 plants only
476 show slightly lower levels of ppGpp than the wild type despite showing very similar
477 phenotypes to the *rsh_{QM}* mutant (Fig. 1F). Together these observations indicate that ppGpp

478 continues to exert an effect after peaking, suggesting the activation of irreversible processes
479 or increased sensitivity to the presence of ppGpp. The second of these possibilities is
480 supported by the phenotype of the high ppGpp *rsh1-1* mutant which displays much stronger
481 photosynthesis phenotypes than wild-type plants despite a relatively modest increase in
482 ppGpp levels (Fig. 1F, 2A-D, 3A-D).

483
484 GTP is a key nucleotide in ppGpp signaling because it is both the substrate for ppGpp
485 biosynthesis and enzymes in its biosynthetic pathway such as guanylate kinase are potential
486 ppGpp effectors (Field, 2018). We observed a striking decrease in GTP levels during nitrogen
487 deprivation (Fig. 1E). Remarkably GTP levels were closely and inversely correlated to ppGpp
488 levels in the wild type and the *RSH* lines (Fig. 1E, F). GTP is present at levels almost three
489 orders of magnitude higher than ppGpp, so the relationship between ppGpp and GTP cannot
490 be simply explained by the consumption of GTP for ppGpp biosynthesis. Therefore, ppGpp
491 accumulation drives the depletion of the cellular GTP pools by other means, which could for
492 example include the inhibition of enzymes involved in chloroplastic GTP biosynthesis such as
493 guanylate kinase (Nomura et al., 2014). Even more remarkable is that the ppGpp-driven GTP
494 depletion occurs specifically under nitrogen deprivation because accumulation of ppGpp to
495 high levels in OX:RSH3 plants does not affect the GTP pool under normal growth conditions
496 (Bartoli et al., 2020). The reduction in GTP levels may contribute to ppGpp signaling in two
497 ways. First, reduced GTP levels are likely to directly contribute to the observed ppGpp-
498 dependent reduction in chloroplast transcription in a similar fashion to ppGpp-signaling in
499 firmicute bacteria (Bange et al., 2021). Second, the increase in the ppGpp / GTP ratio will
500 significantly augment the action of ppGpp on effectors where ppGpp competes with GTP for
501 binding. GTP-binding effectors are common in bacteria, and many are conserved in
502 chloroplasts, particularly those involved in translation and ribosome biogenesis (Pausch et al.,
503 2018; Field, 2018). We propose that the increase in the ppGpp / GTP ratio during nitrogen
504 limitation is may also explain how ppGpp maintains its influence despite decreasing in
505 absolute amounts.

506

507 ***The impact of ppGpp on photosynthesis under nitrogen deprivation***

508 Our work also reveals the wide-ranging physiological effects of ppGpp on photosynthetic
509 activity during nitrogen deprivation. These effects are reminiscent of the photosynthetic
510 changes that occur during senescence (Krieger-Liszkay et al., 2019), and are remarkably
511 similar to those found using the ectopic over-accumulation of ppGpp in Arabidopsis, moss

512 and algae (Maekawa et al., 2015; Sugliani et al., 2016; Imamura et al., 2018; Honoki et al.,
513 2018; Avilan et al., 2021; Harchouni et al., 2021). However, compared to these studies our
514 more detailed analysis here highlights first the global effect of ppGpp on chloroplast
515 transcript abundance (Fig. 5A), and second the strong effect of ppGpp on certain
516 photosynthetic proteins such as PsbO from PSII, PetA from Cyt b₆f, and RbcL (Fig. 3A, B).
517 ppGpp controls the abundance of these proteins via the downregulation of chloroplast
518 transcript abundance (Fig. 5A), reducing the quantity of transcripts available for translation as
519 well as translation capacity via a likely reduction in rRNA transcription. The downregulation
520 of transcript abundance may involve a GTP concentration dependent inhibition of
521 transcription as discussed above. However, the strong reduction in subunits from complexes
522 that are usually stable like Cyt b₆f suggests that more active processes could also be involved.
523 This idea is supported by previous work showing the high stability of Cyt b₆f in tobacco
524 (Hojka et al., 2014), the selective loss of Cyt b₆f during natural senescence (Krieger-Liszky
525 et al., 2019; Roberts et al., 1987), and the protease-mediated degradation mechanism for Cyt
526 b₆f and Rubisco that occurs under nitrogen limitation in *Chlamydomonas* (Majeran et al.,
527 2000; Wei et al., 2014). Another argument supporting the idea that ppGpp may act on protein
528 stability is the contrast between reduced transcript and stable protein levels for the nuclear
529 encoded photosynthetic genes Lhcb1 and Lhca1 in low ppGpp plants (Fig. 3A-B, Fig. 5C,
530 Fig. 5- source data 3). This contrast suggests that ppGpp could promote the degradation of
531 these proteins during nitrogen limitation.

532

533 PSII architecture was also modified in a ppGpp-dependent manner during nitrogen limitation
534 with an increase in the ratio of PSII antenna to RC (Fig. 3A-D), causing an increase in basal
535 fluorescence and contributing to the decrease in PSII maximal efficiency (Fv/Fm) (Fig. 2A,
536 D). While this phenomenon has similarities to photoinhibition, our data suggest that ppGpp
537 does not increase PSII RC photoinactivation, but instead inhibits the resynthesis of PSII RC
538 via the inhibition of chloroplast transcription. Due to the rapid degradation of
539 photoinactivated PSII RC this leads to an increase in the ratio of PSII antenna to RC.

540

541 ***ppGpp is implicated in photoprotection during nitrogen limitation***

542 During nitrogen deficiency plants must remobilize nitrogen to drive acclimation processes. At
543 the same time, growth arrest leads to a strong decrease in demand for the products of
544 photosynthesis which has the potential to lead to overexcitation and ROS production.
545 Therefore, in addition to remobilizing nitrogen, the plant must also downsize the

546 photosynthetic machinery in a controlled fashion to avoid producing damaging levels of ROS.
547 Here, we show that ppGpp promotes a reduction in the photosynthetic machinery, a major
548 nitrogen store, and at the same time plays a role in protecting against excess ROS production.
549 We show that ROS increases in the wild type in response to nitrogen deprivation, and that
550 defects in ppGpp biosynthesis lead to greater increases in ROS and cell death (Fig. 1B, D).
551 The limited ROS increase in the wild type is in agreement with a recent study also showing
552 ROS accumulation in the leaves of nitrogen starved plants (Safi et al., 2021). Chloroplasts are
553 the major source of ROS in photosynthetic plant leaves (Rogers and Munné-Bosch, 2016;
554 Domínguez and Cejudo, 2021). The role for ppGpp signaling in the prevention of ROS over-
555 accumulation and tissue death during nitrogen limitation (Fig. 1A-D) is therefore very likely
556 related to its role in downregulating photosynthetic activity (Fig. 2, Fig. 2- figure supplement
557 2, Fig. 2- figure supplement 4). As part of this process, ppGpp signaling alters the
558 stoichiometry of PSII components, reducing excitation pressure on PSII and thus electron
559 flow in the photosynthetic chain. Note that the reduced Rubisco levels we observe under
560 nitrogen limitation (Fig. 3A, 3B) suggest that PSI may be acceptor side limited due to reduced
561 activity of the Calvin-Benson cycle, which could lead to increased superoxide formation by
562 reduction of molecular oxygen at PSI. Indeed, others have proposed that the reduction in
563 levels of active PSII RC during photoinhibition can protect PSI against overexcitation damage
564 (Tikkanen et al., 2014). Interestingly, the ppGpp-mediated alteration of PSII architecture
565 appears to be independent of the global reduction in photosynthetic proteins during nitrogen
566 limitation: photosynthetic proteins are downregulated in low ppGpp plants without major
567 alterations in PSII activity (Fig. 3A-B, Fig. 2C-D). On the basis of these results, we suggest
568 that one of the functions of ppGpp signaling may therefore be to optimize nitrogen
569 remobilization, notably via the degradation of the most abundant leaf protein Rubisco,
570 modulating at the same time the photosynthetic machinery to prevent overexcitation and
571 photodamage. The most straightforward explanation based on the evidence presented here is
572 that ppGpp acts through a general and coordinated down-regulation of chloroplast gene
573 expression, and further evidence would be needed to determine whether ppGpp is also able to
574 play a more specific role in remodeling photosynthesis.

575

576 We demonstrate a new physiological role for ppGpp signaling and offer new insights into the
577 molecular mechanisms underlying its action. The wide range of stresses known to induce
578 ppGpp accumulation (Takahashi et al., 2004) and the association between stress and
579 overexcitation of the photosynthetic electron transport chain (Bechtold and Field, 2018),

580 together suggest that ppGpp signaling may allow plants to anticipate and acclimate to harsh
581 environmental conditions that would otherwise lead to overexcitation of the photosynthetic
582 machinery and oxidative stress. At the same time, our work also highlights important
583 questions about ppGpp function that are not yet fully answered: how does nitrogen limitation
584 activate ppGpp signaling? does ppGpp target a single process (transcription) or several? and
585 does ppGpp signaling interact with known photoprotective pathways (Pinnola and Bassi,
586 2018; Malnoë, 2018)?
587

Key Resources Table				
Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Genetic reagent (<i>Arabidopsis thaliana</i>)	Col-0	Nottingham Arabidopsis Stock Centre (NASC)	WT1, Columbia, N1093 (NASC)	
Genetic reagent (<i>Arabidopsis thaliana</i>)	<i>qrt1-2</i>	Nottingham Arabidopsis Stock Centre (NASC), Coppenhaver et al. (2000)	WT2, N8846 (NASC)	Col-3 ecotype
Genetic reagent (<i>Arabidopsis thaliana</i>)	<i>qrt1-2/rsh1-1</i>	Nottingham Arabidopsis Stock Centre (NASC), Sugliani et al. (2016), Sessions et al. (2002)	TDNA insertion SAIL_391_E11, N818025 (NASC)	<i>RSH</i> mutant
Genetic reagent (<i>Arabidopsis thaliana</i>)	<i>qrt1-2/rsh2-1</i>	Nottingham Arabidopsis Stock Centre (NASC), Sugliani et al. (2016), Sessions et al. (2002)	TDNA insertion SAIL_305_B12, N814119 (NASC)	<i>RSH</i> mutant
Genetic reagent (<i>Arabidopsis thaliana</i>)	<i>qrt1-2/rsh3-1</i>	Nottingham Arabidopsis Stock Centre (NASC), Sugliani et al. (2016), Sessions et al. (2002)	TDNA insertion SAIL_99_G05, N862398 (NASC)	<i>RSH</i> mutant
Genetic reagent (<i>Arabidopsis thaliana</i>)	<i>qrt1-2/rsh2-1 rsh3-1</i>	Sugliani et al. (2016)	DM-23	<i>RSH</i> mutant
Genetic reagent (<i>Arabidopsis thaliana</i>)	<i>qrt1-2/rsh_{QM}</i>	Sugliani et al. (2016)	<i>rsh1-1, rsh2-1, rsh3-1, crsh-ami / Qmaii</i>	<i>RSH</i> mutant
Genetic reagent (<i>Arabidopsis thaliana</i>)	Col-0/ OX:RSH1	Sugliani et al. (2016)	OX:RSH1-GFP (10.4)	overexpression line
Genetic reagent	<i>qrt1-2/rsh2-1 rsh3-1</i>	Sugliani et al.	C11	complementation

<i>(Arabidopsis thaliana)</i>	pRSH3:RSH3	(2016)		line
Genetic reagent <i>(Arabidopsis thaliana)</i>	<i>qrt1-2/</i> <i>rsh2-1 rsh3-1</i> pRSH3:RSH3	Sugliani et al. (2016)	C43	complementation line
Genetic reagent <i>(Arabidopsis thaliana)</i>	<i>qrt1-2/</i> <i>rsh2-1 rsh3-1</i> pRSH3:RSH3	Sugliani et al. (2016)	C131	complementation line
Genetic reagent <i>(Arabidopsis thaliana)</i>	<i>qrt1-2/</i> <i>rsh2-1 rsh3-1</i> pRSH3:RSH3	Sugliani et al. (2016)	CX3	complementation line
Genetic reagent <i>(Arabidopsis thaliana)</i>	Col-0/ pOP:MESH	Sugliani et al.(2016)	MESH	DEX inducible MESH
Genetic reagent <i>(Arabidopsis thaliana)</i>	<i>qrt1-2/</i> <i>rsh1-1</i> pRSH1:RSH1	This study.	C110	Materials and methods: Creation of <i>rsh1-1</i> complementation lines
antibody	anti-COXII (rabbit polyclonal)	Agrisera	Ref. AS04 053A	dilution (1:2000)
antibody	anti-LHCA1 (rabbit polyclonal)	Agrisera	Ref. AS01 005	dilution (1:2000)
antibody	anti-LHCB1 (rabbit polyclonal)	Agrisera	Ref. AS01 004	dilution (1:2000)
antibody	anti-PBA1 (rabbit polyclonal)	Abcam	Ref. ab98861	dilution (1:2000)
antibody	anti-PetA (rabbit polyclonal)	Agrisera	Ref. AS08 306	dilution (1:2000)
antibody	anti-PsaD (rabbit polyclonal)	Agrisera	Ref. AS04 046	dilution (1:2000)
antibody	anti-PsbA (rabbit polyclonal)	Agrisera	Ref. AS05 084	dilution (1:2000)
antibody	anti-PsbO (rabbit polyclonal)	Agrisera	Ref. AS05 092	dilution (1:2000)
antibody	anti-PsbS (rabbit polyclonal)	Agrisera	Ref. AS09 533	dilution (1:1000)
antibody	anti-PTOX (rabbit	Uniplastomic	Kindly provided by	dilution (1:2000)

	polyclonal)		X.Johnson	
antibody	anti-SAG12 (rabbit polyclonal)	Agrisera	Ref. AS14 2771	dilution (1:2000)
chemical compound, drug	13C-ppGpp	Kindly provided by J.Bartoli and E.Bouveret		internal standard for ppGpp quantification
chemical compound, drug	13C-GTP	Sigma-Aldrich	710687	internal standard for GTP quantification
chemical compound, drug	15-HEDE	Cayman Chemical	Item No. 37700	internal standard for HOTE quantification
commercial assay or kit	Oasis WAX 1 cc Vac Cartridge	Waters	Ref. 186002491	Nucleotide quantification
chemical compound, drug	Nucleozol	Macherey Nagel	Ref. 740404.200	RNA extraction
chemical compound, drug	4-bromoanisole	Sigma-Aldrich	B56501	RNA extraction
commercial assay or kit	Clean & Concentrator™-25 kit	Zymo Research	Cat. No. : R1017	RNA extraction
commercial assay or kit	Ribo-Zero rRNA Removal Kit (Plant)	Illumina	Ref. MRZPL116	RNA treatment
other	Open FluorCam	Photon System Instruments	FC 800-O/2020-GFP	Chlorophyll fluorescence
software, algorithm	R	R Core Team (2020)		Data analysis
software, algorithm	ggplot2 package	Wickham (2009)		Data analysis
software, algorithm	Rmisc package	Hope (2013)		Data analysis
software, algorithm	boot package	Canty and Ripley, (2021); Davison and Hinkley, (1997)		Data analysis
software, algorithm	rstatix package	Kassambara (2021)		Data analysis
software, algorithm	Rcompanion package	Salvatore (2021)		Data analysis
software, algorithm	prepare_gene_ontology.pl script	This study		Data analysis, available at: https://github.com/c

				ecile- lecampion/gene- ontology-analysis- and-graph
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590

591 **Plant materials and growth conditions**

592 *Arabidopsis thaliana* mutant lines, overexpressors and complementation lines and respective
593 wild-type controls are listed in the Key Resources table. In each experiment the seeds for each
594 line were derived from the same batch of plants grown together. For growth on plates, seeds
595 were surface sterilized with 75% ethanol, rinsed with 100% ethanol, dried and placed in a ten
596 by ten grid pattern on square culture plates containing 45 ml of nitrogen replete (+N) (0.5X
597 Murashige and Skoog salts [Caisson Labs], 1% sucrose, 0.5 g L⁻¹ MES and 0.4% Phytigel
598 [Merck Sigma-Aldrich], adjusted to pH 5.7 with KOH) or nitrogen limiting (-N) (+N medium
599 diluted 1/25 in 0.5X Murashige and Skoog medium without nitrogen [Caisson Labs], 0.5 g/L
600 MES and 0.4% Phytigel [Merck Sigma-Aldrich], adjusted to pH 5.7 with KOH) growth
601 medium (for detailed medium composition see Supplementary File 1). Plates were placed at
602 4°C for 2 days in the darkness, and then transferred to a 16 h light (at 22°C) / 8 h darkness (at
603 19.5°C) photoperiod with 80 μmol photons m⁻² s⁻¹ lighting. For growth in quartz sand, seeds
604 were germinated in soil, and then transferred into pots containing quartz sand at 7 days after
605 germination. The plants were then grown under a 8 h light (at 22°C)/16 h dark (at 19.5°C)
606 photoperiod at 18°C / 22°C with 110 μmol photons m⁻² s⁻¹ lighting. Plants were treated
607 weekly with a complete nutrient solution. After 50 days the pots were rinsed, and then treated
608 weekly with 0.5X Murashige and Skoog medium with or without nitrogen for three weeks.

609

610 **Creation of *rsh1-1* complementation lines**

611 The 9.1 kb genomic *RSH1* sequence including the 3' untranslated region, 5' untranslated
612 region, and 3 kb of upstream sequence containing the promoter was amplified from
613 *Arabidopsis* genomic DNA using Phusion polymerase (New England Biolabs) using flanking
614 primers and then the primers RSH1-F (5'-TCCGTCTTGTCTGAATCAGCT-3') and RSH1-R
615 (5'-TTTCTAGATTTACTTTGGTTTTGTCCA-3') with attB1 / attB2 adapters. The resulting
616 PCR product was then introduced by Invitrogen BP Gateway recombination (Thermofisher)
617 into pDONR207. The entry clone was confirmed by sequencing and recombined by
618 Invitrogen LR Gateway recombination into the binary vector pGWB410 (Nakagawa et al.,
619 2007) which carries a kanamycin resistance cassette for selection in plants. The resulting
620 construct was transferred into *Agrobacterium tumefaciens* (strain GV3101) and used to
621 transform *rsh1-1* plants by floral dipping.

622

623 **Autoluminescence imaging and quantification of HOTEs**

624 Peroxidated lipids were visualized by autoluminescence imaging as described previously
625 (Birtic et al., 2011) and similar results were observed in four biological replicates. The
626 intensity of the leaf autoluminescence signal is proportional to the amount of lipid peroxides
627 present in the sample, the slow spontaneous decomposition of which produces luminescent
628 species. For quantification of HOTEs, lipids were extracted from about 300 mg of seedlings
629 in methanol / chloroform and analyzed by HPLC-UV as detailed elsewhere (Montillet et al.,
630 2004; Shumbe et al., 2017).

631

632 **Nucleotide triphosphate quantification**

633 Nucleotides were extracted from about 150 mg of seedlings, and quantified by HPLC-MS/MS
634 using stable isotope labelled ppGpp and GTP standards as described previously (Bartoli et al.,
635 2020).

636

637 **Chlorophyll fluorescence measurements**

638 Plants were dark adapted for 20 min and chlorophyll fluorescence was measured in a
639 Fluorcam FC 800-O imaging fluorometer (Photon System Instruments). PSII maximum
640 quantum yield (F_v/F_m) was calculated as $(F_m - F_o)/F_m$. For relative electron transfer rate
641 (ETR) measurements plants were exposed to 2 min periods of increasing actinic light
642 intensity, with PSII quantum yield measurements at the end of each period. Relative ETR was
643 calculated as quantum yield X light intensity (photosynthetic photon flux density).
644 Photosynthetic photon flux density was measured using a Universal Light Meter 500 (ULM-
645 500, Walz). All experiments on photosynthetic parameters were repeated two to five times
646 with similar results. Steady state 77K chlorophyll fluorescence measurements were obtained
647 from frozen seedling powder suspended in 85% (w/v) glycerol, 10 mM HEPES, pH 7.5 as
648 described previously (Galka et al., 2012).

649

650 **Protein extraction and immunoblotting**

651 Protein extraction and immunoblotting were performed on 100 bulked seedlings as described
652 previously (Sugliani et al., 2016), with the addition of a protein precipitation step with 20%
653 TCA after protein extraction. The precipitation step was necessary to remove compounds in
654 nitrogen-limited seedlings that interfered with the bicinchoninic acid assay for determining
655 protein concentration. The following primary antibodies were used against CoxII (Agriseria;
656 polyclonal; ref. AS04 053A), Lhca1 (Agriseria; polyclonal, ref. AS01 005), Lhcb1 (Agriseria;
657 polyclonal, ref. AS01 004), Pba1 (Abcam; polyclonal; ref. ab98861), PetA (Agriseria;

658 polyclonal, ref. AS08 306), PsaD (Agrisera; polyclonal; ref. AS04 046), PsbA (Agrisera;
659 polyclonal, ref. AS05 084), PsbO (Agrisera; polyclonal; ref. AS05 092), PsbS (Agrisera;
660 polyclonal, ref. AS09 533), PTOX (Uniplastomic, Biviers, France; kindly provided by Xenie
661 Johnson), SAG12 (Agrisera; polyclonal; ref. AS14 2771).

662

663 For the analysis of thylakoidal proteins, 100 bulked seedlings were harvested and then ground
664 in liquid nitrogen. Proteins were extracted in an extraction buffer adapted from Pesaresi et al.
665 (2011) (0.4 M sucrose, 10 mM NaCl, 5 mM MgCl₂, 10 mM tricine KOH pH 7.5, 100 mM
666 ascorbate, 0.2 mM PMSF, 5 mM aminocaproic acid). Samples were centrifuged at 1000g for
667 5 min at 4°C, then the pellets were resuspended in extraction buffer. The samples were
668 centrifuged again at 1000g for 5 min at 4°C, and the resulting chloroplast-enriched pellets
669 suspended in lysis buffer as previously described (Chen and Schröder, 2016) (10 mM Tricine
670 -NaOH pH 7.8) and incubated for 30 min. The samples were centrifuged at 5000g for 5 min at
671 4°C, and the pellets containing the membrane fraction were re-suspended in lysis buffer.
672 Samples were centrifuged at 1000g for 5 min at 4°C, then the pellets were resuspended in
673 extraction buffer (100 mM Tris pH 6.8, 20% glycerol, 10% SDS). The samples were heated to
674 40°C for 5 min and then centrifuged at 1300 g for 10 min at room temperature. The
675 supernatant was recovered, and the samples were normalized on the amount of chlorophyll.

676

677 **Chlorophyll quantification**

678 Pigments were extracted in 80% acetone then separated and quantified by HPLC-UV as
679 described previously (Campoli et al., 2009).

680

681 **RNA sequencing**

682 RNA sequencing was performed with three biological replicates on 100 bulked seedlings per
683 line grown on +N medium for 8 days or -N medium for 12 days. RNA was extracted from
684 frozen seedling powder with Nucleozol (Macherey-Nagel) with 4-bromoanisole to reduce
685 DNA and anthocyanin contamination. Total RNA was cleaned and concentrated using RNA
686 Clean & ConcentratorTM-25 (Zymo Research) according to the manufacturer's instructions.
687 Genomic DNA was removed by treatment with DNase. RNA-seq libraries were constructed
688 by the POPS platform (IPS2) using the TruSeq Stranded mRNA library prep kit (Illumina)
689 with RiboZero plant (Illumina). Libraries were sequenced in single-end (SE) with a read
690 length of 75 bases for each read on a NextSeq500 (Illumina). Approximately 30 million reads
691 by sample were generated. Adapter sequences and bases with a Q-Score below 20 were

692 trimmed out from reads using Trimmomatic (v0.36) (Bolger et al., 2014) and reads shorter
693 than 30 bases after trimming were discarded. Reads corresponding to rRNA sequences were
694 removed using sortMeRNA (v2.1) (Kopylova et al., 2012) against the silva-bac-16s-id90,
695 silva-bac-23s-id98, silva-euk-18s-id95 and silva-euk-28s-id98 databases. Read quality checks
696 were performed using FastQC (Version 0.11.5)(Andrews, 2010). The raw data (fastq) was
697 then aligned against the Arabidopsis transcriptome
698 (Araport11_cdna_20160703_representative_gene_model.fa) concatenated with non-coding
699 RNA (TAIR10.ncrna.fa) using Bowtie2 (version 2.2.9)(Langmead and Salzberg, 2012).
700 Default parameters were used. Reads were counted using a modified version of a command
701 line previously described (Van Verk et al., 2013). Differential expression analysis was
702 performed with SARTools (version 1.7.3) (Varet et al., 2016) using edgeR. Study details and
703 Fastq files were deposited at the European Nucleotide Archive (<https://www.ebi.ac.uk/ena>)
704 under accession number PRJEB46181. Workflow and analysis reports for RNAseq data
705 analysis are provided in Supplementary File 2.

706

707 **Data analysis**

708 The majority of analysis was conducted in R (R Core Team, 2020) and custom annotated R
709 markdown scripts are provided in Supplementary File 3. Graphs were produced using the
710 package ggplot2 (Wickham, 2009) with confidence intervals determined for normally
711 distributed data using the Rmisc package (Hope, 2013), and non-parametric data with
712 bootstrap confidence interval calculation using the boot package (Canty and Ripley, 2021;
713 Davison and Hinkley, 1997). Individual plants were counted as biological replicates, and
714 experiments were repeated at least two times with similar results. For the timecourse in Fig.
715 3A and the nitrogen deprivation performed on mature plants in Fig. 2- figure supplement 2E
716 single experimental replicates were performed. Statistical analyses were performed on
717 normally distributed data using ANOVA with posthoc Tukey test and non-parametric data
718 using the Kruskal-Wallis test with posthoc Dunn test in the rstatix package (Kassambara,
719 2021). For categorical data (Fig. 1B,C) significance was calculated using a proportion test and
720 posthoc Fisher test using the Rstatix and Rcompanion packages (Kassambara, 2021;
721 Salvatore, 2021). All statistical tests included adjustments for multiple comparisons.
722 GO enrichment analysis for the RNAseq data was performed using a custom
723 [prepare_gene_ontology.pl](https://github.com/cecile-lecampion/gene-ontology-analysis-and-graph) script ([https://github.com/cecile-lecampion/gene-ontology-](https://github.com/cecile-lecampion/gene-ontology-analysis-and-graph)
724 [analysis-and-graph](https://github.com/cecile-lecampion/gene-ontology-analysis-and-graph), commit c170f8e90323f3061d44a87fcb76d6b0e8b00f63) which
725 automatically uses PANTHER and REVIGO for the identification and simplification of

726 enriched GO terms according to the procedure proposed by Bonnot et al. (2019). Results were
727 plotted using ggplot2.

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735

736 **Competing interests**

737 The authors declare no competing interests.

738

739 **References**

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- 949

950 **List of figures, figure supplements, source data and supplementary information**

951 Figure 1. ppGpp is required for acclimation to nitrogen deprivation.

952 Figure 1- figure supplement 1. Extended timecourse of nitrogen deprivation.

953 Figure 1- figure supplement 2. GTP/ppGpp ratios in RSH lines.

954 Figure 1- source data 1. Source data and statistical test reports for Figure 1 and supplements.

955

956 Figure 2. Nitrogen deprivation promotes a ppGpp-dependent drop in photosynthetic capacity.

957 Figure 2- figure supplement 1. Changes in additional photosynthetic parameters (Fo, Fm and

958 Fo / Fm) for panel 2A.

959 Figure 2- figure supplement 2. Role of ppGpp in the nitrogen deprivation induced decrease in

960 PSII maximal yield.

961 Figure 2- figure supplement 3. Changes in additional photosynthetic parameters (Fo, Fm and

962 Fo / Fm) for Figure 2- Figure supplement 2.

963 Figure 2- figure supplement 4. ppGpp is required for reducing ETR during nitrogen

964 deprivation.

965 Figure 2- source data 1. Source data and statistical test reports for Figure 2 and supplements.

966

967 Figure 3. ppGpp-dependent alterations in the photosynthetic machinery during nitrogen

968 deficiency.

969 Figure 3- figure supplement 1. ppGpp depletion by MESH also affects abundance of

970 chloroplast proteins.

971 Figure 3- figure supplement 2. Full 77K chlorophyll fluorescence spectra under nitrogen

972 deprivation.

973 Figure 3- source data 1. Uncropped immunoblots for 3A.

974 Figure 3- source data 2. Uncropped immunoblots for 3B.

975 Figure 3- source data 3. Source data and statistical test reports for Figure 3 and supplements.

976

977 Figure 4. ppGpp plays a major role during acclimation to nitrogen deprivation.

978 Figure 4- source data 1. Differentially accumulating transcripts.

979 Figure 4- source data 2. GO enrichment analysis.

980 Figure 4- source data 3. Source data and statistical test reports for Figure 4B.

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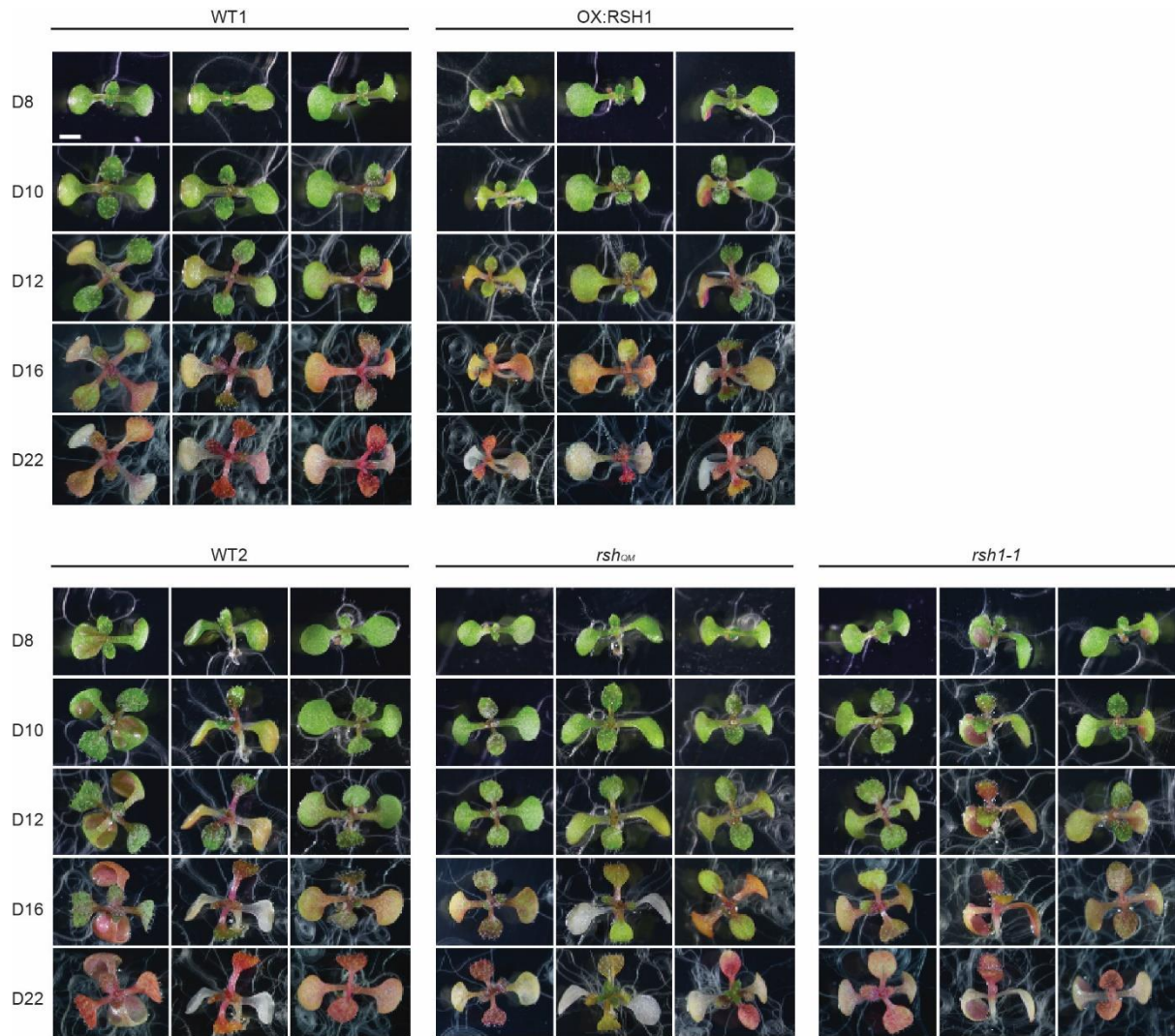
982 Figure 5. ppGpp is required for the downregulation of chloroplast gene expression during

983 nitrogen deficiency.

984 Figure 5- source data 1. Chloroplast gene expression.
985 Figure 5- source data 2. Mitochondrial gene expression.
986 Figure 5- source data 3. Source data for Figure 5C including extended figures for multiple
987 photosynthetic complexes.
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989 Supplementary file 1. Media composition
990 Supplementary file 2. RNA-seq analysis reports. Related to Figure 4.
991 Supplementary file 3. R markdown scripts. Related to all figures.
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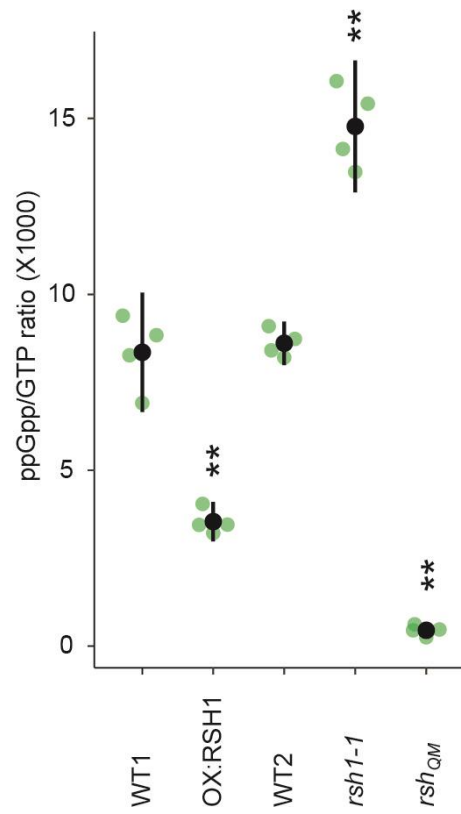
996 **Figure 1- figure supplement 1. Extended timecourse of nitrogen deprivation.**

997 Images of seedlings grown on nitrogen limiting medium for the indicated number of days. Scale, 3

998 mm.

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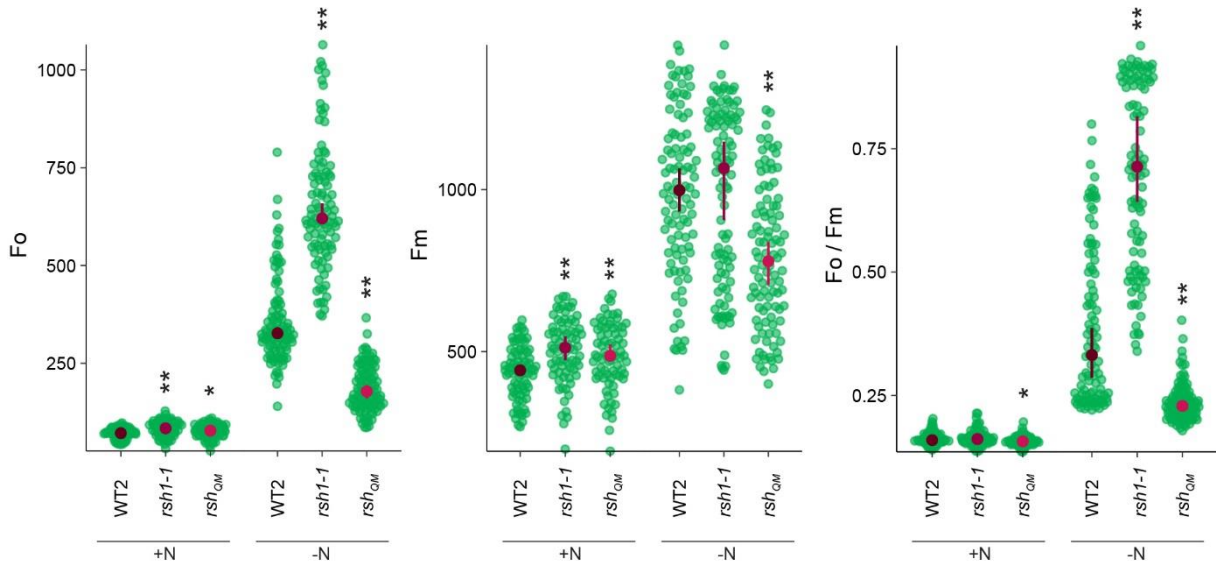
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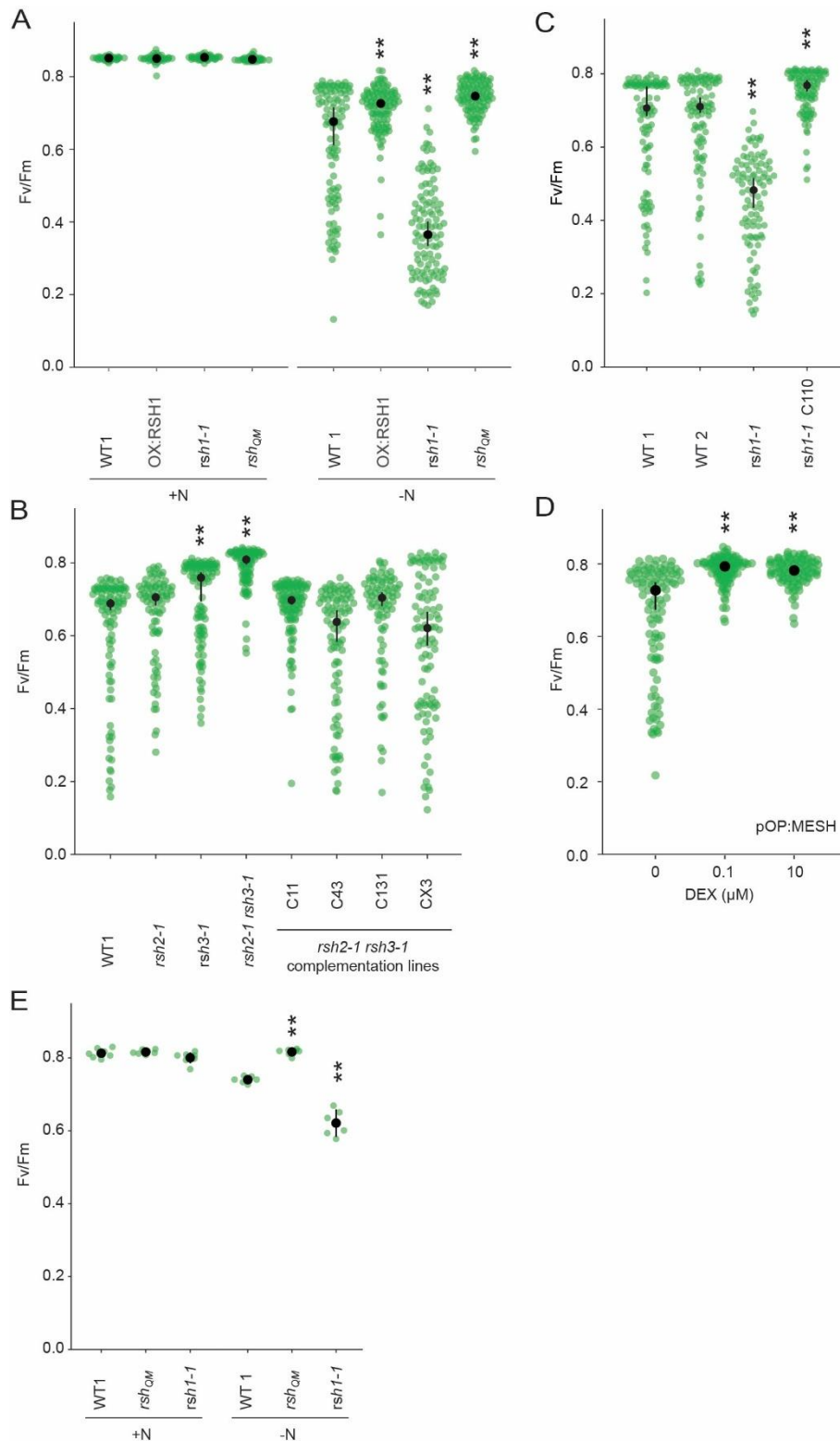
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Figure 1- figure supplement 2. GTP/ppGpp ratios in RSH lines. The ratio of ppGpp to GTP concentration in seedlings grown for ten days on nitrogen limiting media. Mean +/- 95% CI, n = 4 experimental replicates. Statistical tests shown against respective wild-type controls, * $P < 0.05$, ** $P < 0.01$.

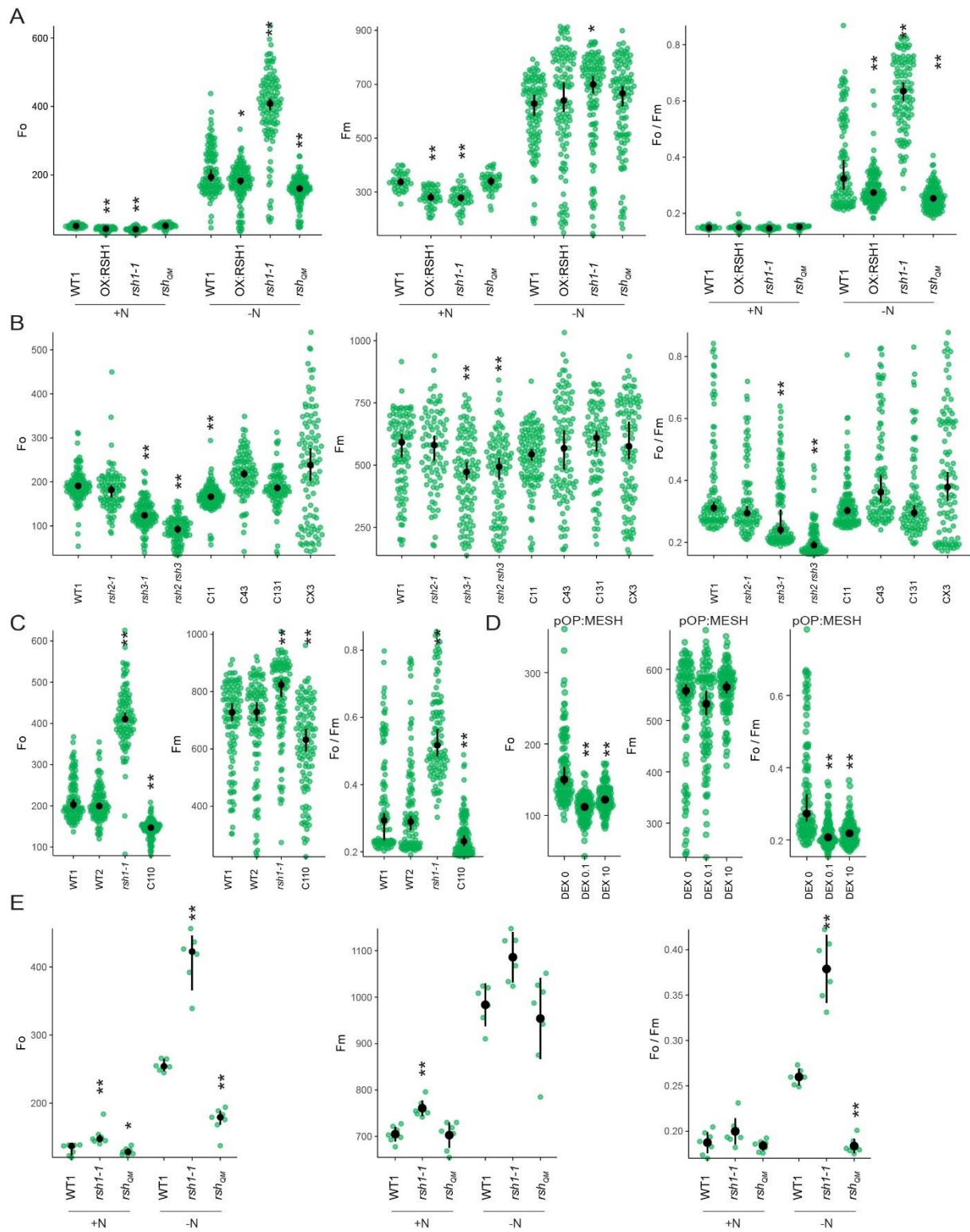


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Figure 2- figure supplement 1. Changes in additional photosynthetic parameters (Fo, Fm and Fo / Fm) for lines shown in panel 2A. Seedlings were grown 8 days on nitrogen replete media (+N) or 12 days on nitrogen limiting (-N) media. Median +/- 95% CI, n = 95-100 seedlings. Statistical tests shown against respective wild-type controls, * $P < 0.05$, ** $P < 0.01$, Kruskal-Wallis test with post-hoc Dunn tests.

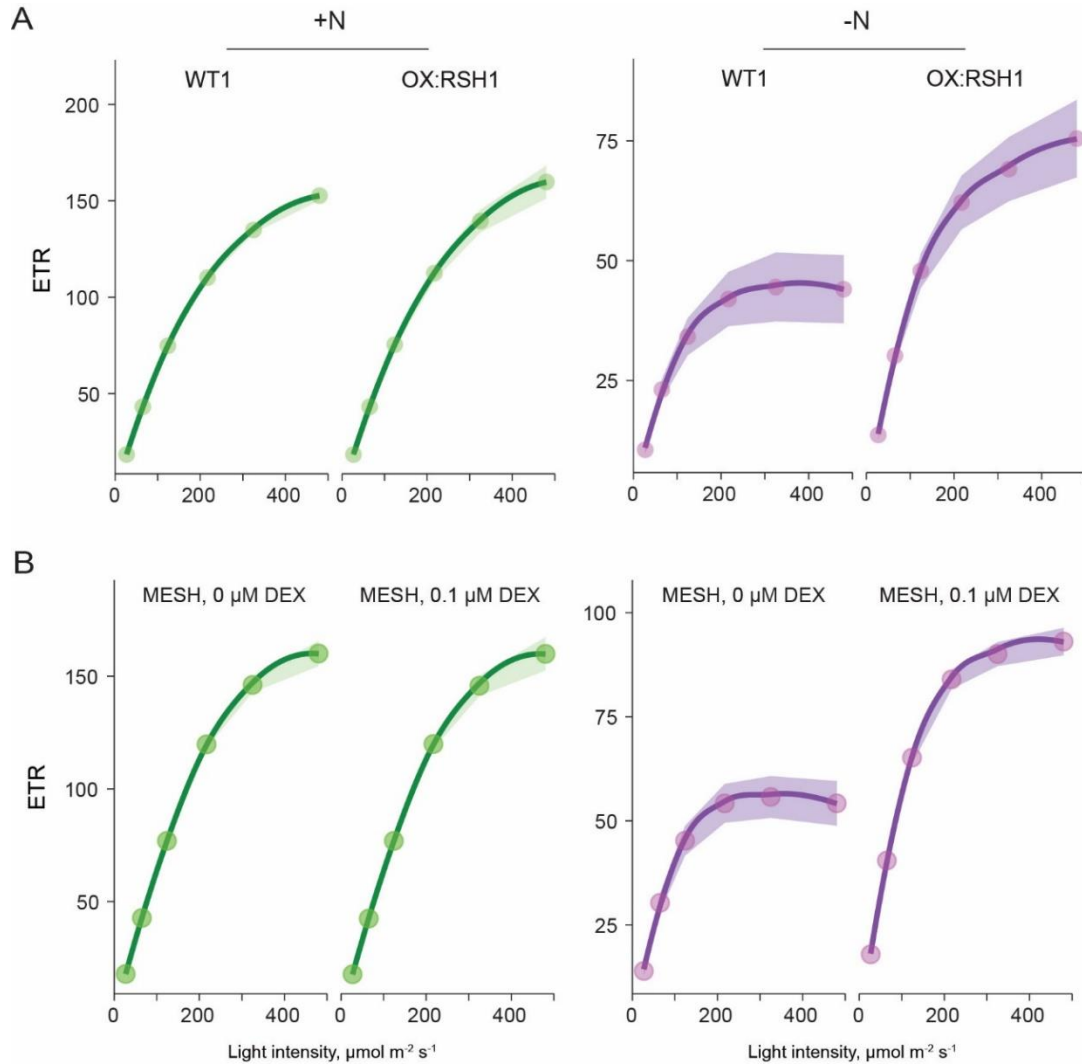


1016 **Figure 2- figure supplement 2. Role of ppGpp in the nitrogen deprivation induced decrease in**
 1017 **PSII maximal yield. (A)** Fv/Fm in seedlings of indicated lines grown 8 days on nitrogen replete media
 1018 (+N) or 12 days on nitrogen limiting (-N) media. **(B)(C)(D)** Fv/Fm in seedlings of indicated lines grown
 1019 12 days on -N. *rsh1-1* C110 is complementation line complemented with the full length *RSH1*
 1020 (*pRSH1:RSH1*). pOP:MESH is a dexamethasone (DEX) inducible line that expresses a chloroplast
 1021 targeted ppGpp hydrolase MESH. **(E)** Fv/Fm in mature plants grown on quartz sand supplemented
 1022 with nitrogen replete media (+N) or nitrogen free (-N) media. Median +/- 95% CI, n = 95-100 seedlings
 1023 (A-D), 6 plants (E). Statistical tests shown against respective wild-type controls, * $P < 0.05$, ** $P < 0.01$.
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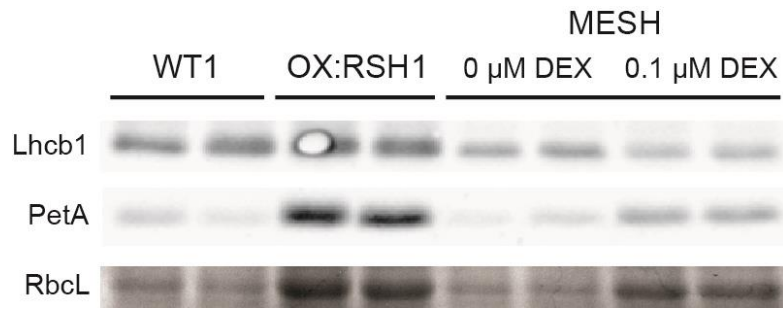
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 1026 **Figure 2- figure supplement 3. Changes in additional photosynthetic parameters (Fo, Fm and**
 1027 **Fo / Fm) for Figure 2- Figure supplement 2. (A)** In seedlings of indicated lines grown 8 days on
 1028 nitrogen replete media (+N) or 12 days on nitrogen limiting (-N) media. **(B)(C)(D)** In seedlings of
 1029 indicated lines grown 12 days on -N. C110 is an *rsh1-1* complementation line complemented with the
 1030 full length *RSH1* (*pRSH1:RSH1*). pOP:MESH is a dexamethasone (DEX) inducible line that expresses
 1031 a chloroplast targeted ppGpp hydrolase MESH. **(E)** In mature plants grown on quartz sand
 1032 supplemented with nitrogen replete media (+N) or nitrogen free (-N) media. Median +/- 95% CI, n =
 1033 95-100 seedlings (A-D), 6 plants (E). Statistical tests shown against respective wild-type controls, *
 1034 $P < 0.05$, ** $P < 0.01$. Kruskal-Wallis test with post-hoc Dunn tests (A-D), one-way Anova with post-hoc
 1035 Tukey test (E).

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Figure 2- figure supplement 4. ppGpp is required for reducing ETR during nitrogen deprivation. Relative ETR measurements in different lines grown 8 days on +N media (left) or 12 days on -N media (right). (A) A comparison between OX:RSH1 and the wild-type control. (B) ETR in the dexamethasone (DEX) inducible pOP:MESH line grown on non-inducing medium (0 μM DEX), or inducing medium (10 μM DEX). MESH is a chloroplast targeted ppGpp hydrolase. Median +/- 95% CI, n = 95-100 seedlings.



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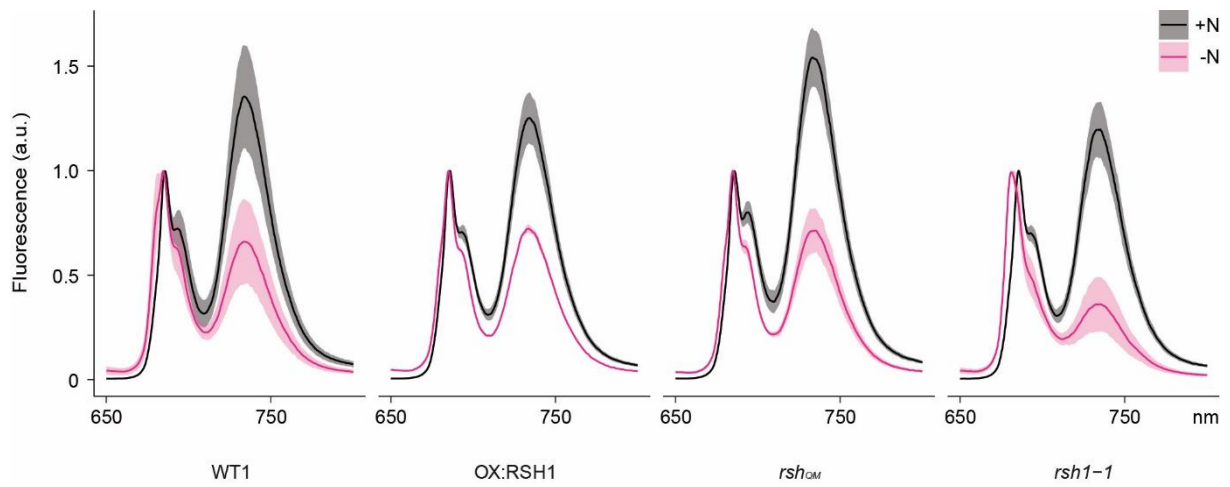
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Figure 3- figure supplement 1. ppGpp depletion by MESH also affects abundance of chloroplast proteins. Immunoblots showing the abundance of the indicated proteins in extracts from 100 bulked seedlings grown in -N for 12 days from two experimental replicates. MESH seedlings were induced by inclusion of dexamethasone (DEX) or not in the growth medium. RbcL was revealed by Coomassie Brilliant Blue. Equal quantities of total protein were loaded. Uncropped immunoblots are shown in Figure 3- source data 3.

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Figure 3- figure supplement 2. Full 77K chlorophyll fluorescence spectra under nitrogen deprivation. Emission spectrum of chlorophyll fluorescence at 77°K normalized to the PSII peak at 685 nm. Measurements made on 8 or 12 day-old seedlings grown under +N or -N. Means \pm 95% CI; data from four experimental replicates.