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5 **Guanosine tetraphosphate (ppGpp) accumulation inhibits chloroplast gene expression and**
6 **promotes super grana formation in the moss *Physcomitrium (Physcomitrella) patens***

7

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22 **Summary**

- 23 • The nucleotides guanosine tetraphosphate and pentaphosphate (or ppGpp) are implicated in
24 the regulation of chloroplast function in plants. ppGpp signalling is best understood in the
25 model vascular plant *Arabidopsis thaliana* where it acts to regulate plastid gene expression to
26 influence photosynthesis, plant development and immunity. However, little is known about
27 the conservation or diversity of ppGpp signaling in other land plants.
- 28
- 29 • We studied the function of ppGpp in the moss *Physcomitrium* (previously *Physcomitrella*)
30 *patens* using an inducible system for triggering ppGpp accumulation. We used this approach
31 to investigate the effects of ppGpp on chloroplast function, photosynthesis and growth.
- 32
- 33 • We demonstrate that ppGpp accumulation causes a dramatic drop in photosynthetic capacity
34 by inhibiting chloroplast gene expression. This was accompanied by the unexpected
35 reorganisation of the thylakoid system into super grana. Surprisingly, these changes did not
36 affect gametophore growth, suggesting that bryophytes and vascular plants may have different
37 tolerances to defects in photosynthesis.
- 38
- 39 • Our findings point to the existence of both highly conserved and more specific targets of
40 ppGpp signalling in the land plants that may reflect different growth strategies.
- 41

42 **Introduction**

43 Guanosine tetraphosphate and pentaphosphate (or ppGpp) are nucleotides that are implicated in stress
44 acclimation in plants and bacteria. Bacterial ppGpp signalling is intensely investigated and
45 mechanistically diverse (Hauryliuk *et al.*, 2015; Steinchen & Bange, 2016; Ronneau & Hallez, 2019).
46 Various stresses and environmental changes can trigger the synthesis of ppGpp from ATP and
47 GDP/GTP by enzymes of the RelA SpoT Homologue (RSH) superfamily. The resulting increase in
48 ppGpp concentration acts to reduce proliferation and activate acclimatory pathways by targeting
49 enzymes involved in transcription, translation, metabolism and replication.

50
51 In plants, ppGpp signalling is less well characterised and its diversity is essentially unexplored. At
52 least three conserved families of chloroplast-targeted RSH enzymes for both ppGpp synthesis and
53 hydrolysis can be found in land plants. These are named RSH1, RSH2/3 and RSH4 (Atkinson *et al.*,
54 2011; Ito *et al.*, 2017; Avilan *et al.*, 2019). In the angiosperm *Arabidopsis thaliana*, where ppGpp
55 signalling is best characterised, ppGpp is known to act as a potent inhibitor of chloroplast gene
56 expression *in vivo* (Maekawa, Mikika *et al.*, 2015; Yamburenko *et al.*, 2015; Sugliani *et al.*, 2016).
57 Increased ppGpp levels lead to major changes in the stoichiometry of photosynthetic complexes such
58 as photosystem II (PSII), and cause a general inhibition of photosynthesis (Maekawa, M. *et al.*, 2015;
59 Sugliani *et al.*, 2016; Honoki *et al.*, 2018). *RSH* mutants with modified ppGpp content are affected in
60 photosynthesis, development and immunity (Sugliani *et al.*, 2016; Abdelkefi *et al.*, 2018). ppGpp is
61 also known to accumulate in response to various different abiotic stresses (Takahashi *et al.*, 2004;
62 Ihara *et al.*, 2015), and when ectopically produced improves the tolerance of plants to nitrogen
63 deprivation (Maekawa, M. *et al.*, 2015; Honoki *et al.*, 2018).

64
65 Relatively little is known about ppGpp signalling in other plants. One of the few studies outside of *A.*
66 *thaliana* is on the function of RSH enzymes in the moss *Physcomitrium patens* (also known as
67 *Physcomitrella patens* and hereafter named *P. patens*) (Sato *et al.*, 2015; Rensing *et al.*, 2020). As a
68 representant of the non-vascular land plants (bryophytes), *P. patens* occupies an interesting
69 phylogenetic position for the study of evolution of plant traits, particularly in comparison with
70 vascular plants (Harrison & Morris, 2018). The *P. patens* genome contains nine *RSH* genes, encoding
71 members of the RSH1, RSH2/3 and RSH4 families, that show different tissue and stress responsive
72 expression patterns (Sato *et al.*, 2015). *PpRSH2a* and *PpRSH2b* appear to play a major role in *P.*
73 *patens* ppGpp biosynthesis because they are highly expressed and encode chloroplast targeted
74 enzymes that show ppGpp synthetase activity *in vitro*. Overexpression of *PpRSH2a* or *PpRSH2b*
75 resulted in a mild slow growth phenotype in *P. patens* grown on media supplemented with glucose
76 (Sato *et al.*, 2015). However, the developmental stage affected was not clear as the moss grown in

77 this condition seemed to be a mix of filamentous protonema and leafy gametophores. Furthermore,
78 comparisons with works on *A. thaliana* are limited because chloroplast phenotypes and
79 photosynthetic capacity were not analysed and we do not know whether ppGpp levels increase in the
80 *P. patens* RSH overexpression lines (Maekawa, M. *et al.*, 2015; Sato *et al.*, 2015; Sugliani *et al.*,
81 2016). To address these problems and to take advantage of the informative evolutionary position of
82 *P. patens* we developed a new strategy to investigate the role of ppGpp. We created a system for the
83 inducible expression of a bacterial ppGpp synthetase that allowed us to increase ppGpp content in the
84 chloroplast independently of endogenous RSH enzymes or their regulatory mechanisms, and in a
85 precisely controllable manner. We focussed our study on *P. patens* shoots, called gametophores,
86 because they are typically the most prominent vegetative developmental stage of moss in the wild.
87 Gametophores also carry leaf-like structures called phyllids that evolved independently to the leaves
88 of vascular plants and are particularly interesting for comparative biology (Fujita *et al.*, 2008; Harrison
89 & Morris, 2018; Glime, 2020; Rensing *et al.*, 2020). In addition, phyllids are a single-cell-layer thick
90 and are therefore highly convenient for microscopic observation (Rensing *et al.*, 2020). Using this
91 approach, we discovered that ppGpp accumulation caused a dramatic drop in photosynthetic capacity
92 by inhibiting chloroplast gene expression that was accompanied by an unexpected reorganisation of
93 the thylakoid system into super grana. Surprisingly, these changes did not appear to significantly
94 affect growth or development. Our results shed new light on the mechanisms and conservation of
95 ppGpp signalling in land plants.

96
97

98 **Results**

99 **An inducible expression system for ppGpp accumulation in *P. patens***

100 To study the role of ppGpp in *P. patens* we created inducible expression lines that upon treatment
101 with estradiol activate the expression of a gene encoding the ppGpp synthase domain of *E. coli* RelA
102 fused to a chloroplast target peptide (SYN) (Fig. 1A). The inducible expression cassette bearing the
103 SYN gene was inserted at the neutral PIG1 locus (*P. patens* InterGenic 1) (Okano *et al.*, 2009) by
104 gene targeting (Fig. S1A). In parallel, control lines (SYN^{D>G}) were created using an inducible cassette
105 encoding a catalytically inactivated version of the SYN gene. SYN and SYN^{D>G} lines with stable
106 single insertions at the target locus were identified by Southern blotting and used for all subsequent
107 experiments (Fig. S1B).

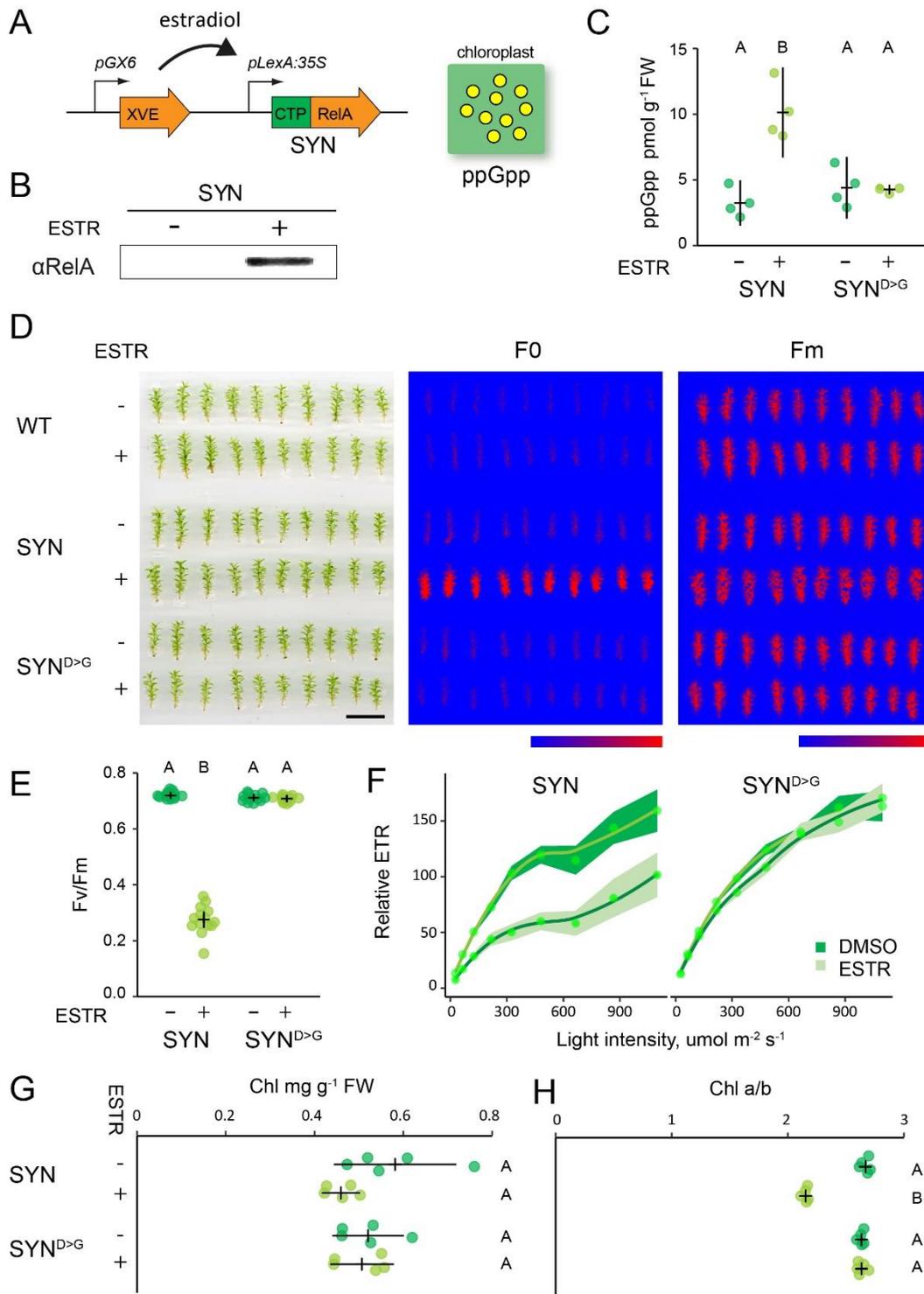
108 We next characterized the properties of the inducible expression system on *P. patens* grown on peat
109 pellets, a condition that favours gametophore development. Induction of SYN gametophores by
110 treatment with estradiol led to the appearance of a protein band that reacted with an antibody raised
111 against RelA, and whose size is consistent with the expected size of the mature SYN protein (Fig.
112 1B). No band was detected in SYN gametophores treated with solvent only, indicating that the
113 estradiol inducible system maintains tight control over the expression of SYN. Expression of the SYN
114 protein was accompanied by an increase in ppGpp to levels three-fold higher than in the non-induced
115 control or the induced SYN^{D>G} control line (Fig. 1C). These data demonstrate that SYN lines are a
116 suitable tool for triggering the *in vivo* production of ppGpp.

117

118 **ppGpp accumulation reduces photosynthetic activity**

119 Studies in *A. thaliana* show that ppGpp accumulation causes a reduction in photosynthetic capacity
120 (Maekawa, M. *et al.*, 2015; Sugliani *et al.*, 2016; Honoki *et al.*, 2018). We therefore analyzed the
121 photosynthetic parameters of induced SYN gametophores to determine whether an increase in ppGpp
122 levels *in vivo* can affect photosynthesis in *P. patens*. We found that the induction of SYN resulted in
123 a large increase in basal chlorophyll fluorescence (F0) that was not observed in the SYN^{D>G} control
124 line or the wild-type, indicating that this change is specific to the accumulation of ppGpp and not
125 other aspects of the inducible expression system (Fig. 1D). Consistent with the increase in F0, induced
126 SYN gametophores also showed a large drop in the maximal efficiency of PSII (Fv/Fm) (Fig 1E).
127 The average Fv/Fm was 0.27 ± 0.013 (mean \pm SE) in induced SYN gametophores and 0.70 ± 0.003
128 (mean \pm SE) in induced SYN^{D>G} gametophores. (Fig. 1E). The decrease in Fv/Fm following SYN
129 induction was detectable 2 days after induction and reached its lowest level after 35 days (Fig. S2).
130 Similar changes in F0 and Fv/Fm were observed in all three independent SYN lines (Fig. S3). The
131 reduction in Fv/Fm in SYN was also accompanied by a strong reduction in photosynthetic operating
132 efficiency at all light intensities, indicating a reduced relative electron transfer rate (ETR) (Fig. 1F).

133 Again, the reduced ETR was observed in all three SYN lines after induction but not in SYN^{D>G} (Fig.
134 S4). While chlorophyll levels did not show clear evidence of a change in induced SYN gametophores
135 (Fig. 1G), we observed a large and significant decrease in the chlorophyll *a/b* ratio, indicating major
136 alterations in the organisation of the photosynthetic machinery (Fig. 1H). Surprisingly, despite the
137 major defects in photosynthesis that we observed in induced SYN lines we did not observe a
138 detectable effect on gametophore growth (Fig. 1D, S2). Altogether, these results indicate that ppGpp
139 accumulation in SYN gametophores reduces photosynthetic capacity without affecting growth.
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Figure 1. Inducible ppGpp production reduces photosynthetic activity. (A) Schematic representation of the estradiol inducible ppGpp overproduction system in SYN lines. (B) Immunoblot on total protein from SYN gametophores after 35 days of induction (DOI) with 0 μM (-) or 5 μM estradiol (+) using an anti-RelA antibody. (C) ppGpp levels in SYN and control SYN^{D>G} lines after 35 DOI (n=4 biological repeats). The photosynthetic status of SYN and SYN^{D>G} gametophores was investigated (D) by imaging basal (F0) and maximal (Fm) chlorophyll fluorescence (scale, 1 cm; false color scale, 20-2000 intensity units), (E) quantifying the maximal efficiency of PSII (Fv/Fm) (n=12-14 gametophores, one-way ANOVA, $P < 0.0001$), and (F) calculating the relative electron transport rate (n=15 gametophores). (G) Chlorophyll concentration and (H) chlorophyll a/b ratios of SYN and SYN^{D>G} gametophores. Experiments performed on gametophores after 35 DOI. Error bars and intervals indicate 95% CI. Significance was tested with one-way ANOVA and post hoc Dunnett test, $P < 0.0001$.

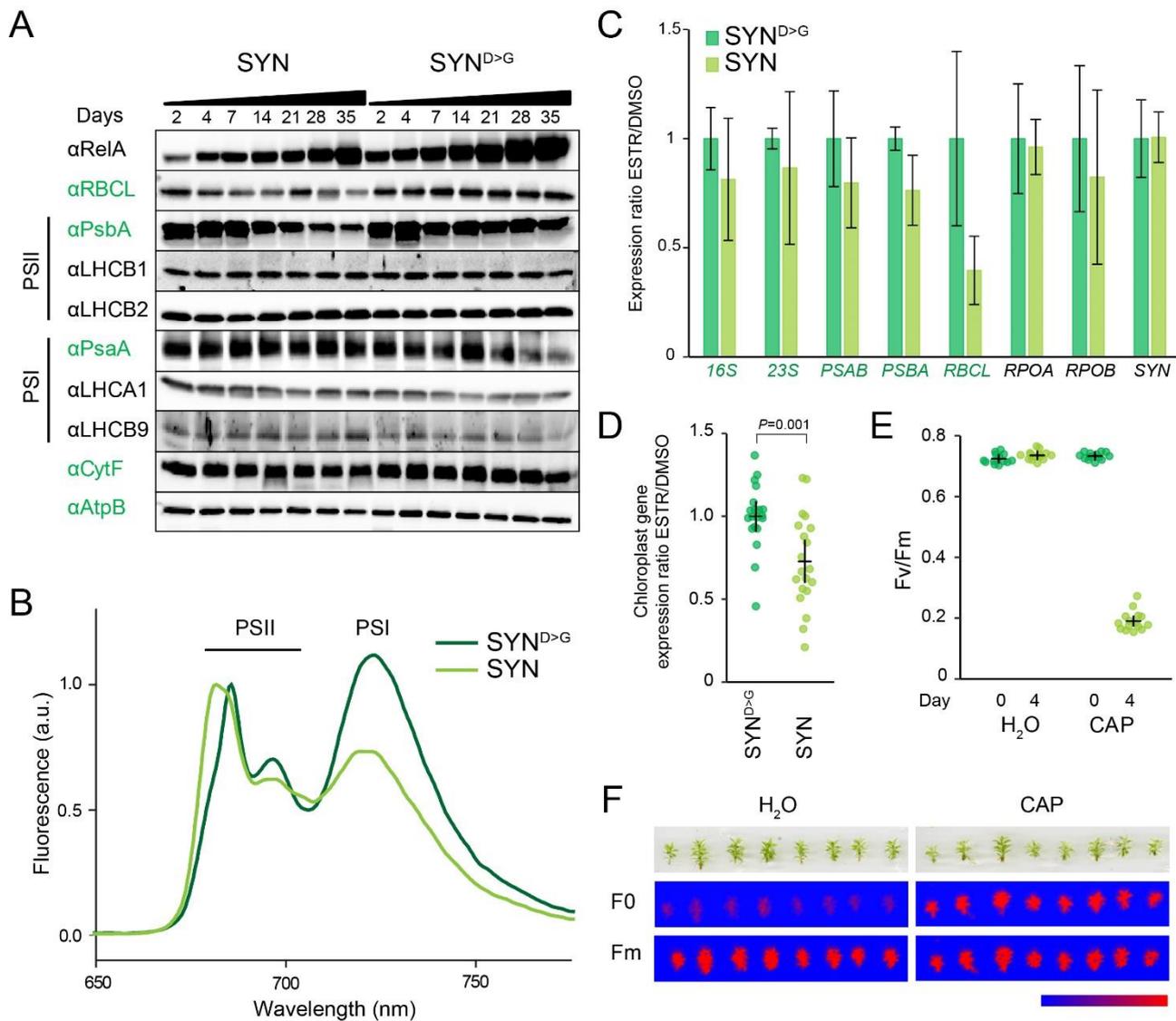
154 **ppGpp accumulation inhibits chloroplast gene expression**

155 The alterations in photosynthetic capacity, chlorophyll fluorescence, and chlorophyll a/b ratio
156 observed in induced SYN gametophores indicate that there is a fundamental remodelling of the
157 photosynthetic machinery in response to ppGpp accumulation. To understand how the photosynthetic
158 machinery is remodelled we analysed the expression of key photosynthetic proteins following SYN
159 induction (Fig. 2A). First, we observed the progressive accumulation of the SYN protein and the
160 SYN^{D>G} protein following induction of the respective lines. This was accompanied by the reduction
161 in the amount of several chloroplast encoded photosynthesis proteins in the SYN line (Fig. 2A and
162 Fig. S5, indicated in green). The most marked reduction was for RBCL, the large subunit of
163 RUBISCO, and PsbA, a subunit of the PSII reaction centre (RC). These were followed by more
164 modest reductions for CytF, a subunit of cytochrome B6f, and AtpB, a subunit of the ATPase. In
165 contrast, we observed either no change or an increase in quantities of the nucleus encoded PSII and
166 PSI light harvesting proteins (LHCB1, LHCB2, LHCA1), and the chloroplast encoded protein PsaA,
167 a subunit of the PSI core. Interestingly, the unusual *P. patens* light harvesting protein LHCB9 also
168 appeared to increase in the SYN line (Fig. 2A). This increase was even more pronounced in another
169 experiment (Fig. S5). Together, these results indicate that ppGpp accumulation inhibits the
170 accumulation of some but not all chloroplast encoded proteins.

171 The increase in the ratio of PSII light harvesting proteins (LHCII) to PSII RC (Fig 2A, Fig. S5)
172 observed in induced SYN gametophores may contribute to the increased chlorophyll fluorescence
173 (F₀) by reducing the efficiency of energy transfer from LHCII into photochemistry. To test this
174 hypothesis, we measured steady state chlorophyll fluorescence at 77K in SYN and SYN^{D>G}
175 gametophores (Fig. 2B). In SYN^{D>G} the expected PSI fluorescence peak at 720 nm and PSII peaks at
176 685 nm and 695 nm were observed. In the SYN line the first PSII peak showed a striking blue-shift
177 to 681 nm and an increase in intensity with respect to PSI. The chlorophylls in isolated LHCII trimers
178 fluoresce at 680 nm, while in intact PSII complexes the fluorescence shifts to 685 nm characteristic
179 of the coupling between LHCII and the low energy chlorophylls of the PSII RC (Lamb *et al.*, 2018).
180 These results indicate that a substantial proportion of the excess LHCII trimers in induced SYN
181 gametophores are energetically uncoupled from the PSII RC, and explains the increase in F₀ and
182 decrease in F_v/F_m in induced SYN gametophores.

183 In *A. thaliana*, ppGpp affects chloroplast gene expression by inhibiting the transcription of chloroplast
184 genes, and in particular by inhibiting the accumulation of chloroplast ribosomal RNA (Yamburenko
185 *et al.*, 2015; Sugliani *et al.*, 2016). We therefore analysed the effects of ppGpp accumulation on
186 chloroplast gene expression by RT-qPCR (Fig. 2C). SYN induction was associated with a small
187 decrease in the abundance of the transcripts for all the chloroplast genes tested, with the most
188 substantial decrease observed for *RBCL*. While the differences were not significant for individual

189 genes, there was overall a clear and significant decrease in the average expression ratio of chloroplast
 190 genes in SYN following induction (Fig. 2D).
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194 **Figure 2. ppGpp accumulation alters chloroplast gene expression and reduces LHCII**
 195 **connectivity to PSII core.** (A) Immunoblots on equal quantities of total protein from SYN and SYN^{D>G}
 196 estradiol induced gametophores using antibodies against signature chloroplast and nuclear encoded
 197 photosynthetic proteins. Chloroplast-encoded proteins are indicated by green text. (B) Low temperature (77K)
 198 fluorescence emission spectra of SYN and SYN^{D>G} gametophores. Spectra are normalized to maximum PSII
 199 emission. (C) RT-qPCR for selected chloroplast-encoded transcripts from SYN and SYN^{D>G} gametophores
 200 after 35 DOI (n= 4 biological replicates). (D) Comparison of the chloroplast gene expression ratio
 201 ESTR/DMSO in SYN and SYN^{D>G} for the chloroplast genes in (B), *P* calculated by two-way Student t-test.
 202 The effects of the chloroplast translation inhibitor chloramphenicol (CAP) on (E) the maximal efficiency of
 203 PSII (Fv/Fm) four days after treatment with CAP and (F) basal (F₀) and maximal (F_m) chlorophyll
 204 fluorescence (false color scale, 20-1000 intensity units). Error bars indicate 95% CI.
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207 We next asked whether the inhibition of chloroplast gene expression alone was sufficient to explain
208 the changes in photosynthesis induced by ppGpp accumulation. We applied chloramphenicol, an
209 antibiotic that inhibits plastid gene expression, to wild-type gametophores and monitored its effect
210 on photosynthetic capacity (Fig. 2E, F). After four days we observed a large rise in F0, and a
211 corresponding drop in Fv/Fm similar to that which occurs following SYN induction (Fig. 1E). This
212 indicates that inhibition of plastid gene expression by ppGpp is sufficient to explain the changes we
213 observe in photosystem organisation and activity.

214

215 **ppGpp accumulation causes major changes in chloroplast structure**

216 In *A. thaliana*, ppGpp accumulation causes a reduction in chloroplast volume that is partially
217 compensated for by an increase in chloroplast number (Sugliani *et al.*, 2016). We therefore visualized
218 chloroplasts in the phyllids of SYN and SYN^{D>G} lines by microscopy. Induced SYN chloroplasts
219 appeared slightly smaller than chloroplasts in SYN^{D>G} (Fig. 3A and Fig. S6) or in the non-induced
220 control, but there was no obvious difference in chloroplast number. However, to our surprise the
221 chloroplasts in the induced SYN line contained dense round structures (8.56 ± 0.40 per chloroplast,
222 $n=75$ chloroplasts; diameter $2.09 \mu\text{m} \pm 0.06$, $n=68$) that were absent from the controls (Fig. 3A, B).
223 The dense round structures were present in the chloroplasts of all the SYN lines (Fig. S6), and started
224 to form three to four days after induction, becoming progressively larger and more well defined with
225 time (Fig. S7).

226 We also found that the inhibition of chloroplast gene expression with chloramphenicol was sufficient
227 to cause the appearance of similar dense round structures (Fig. 3C, D), as we also observed for
228 chlorophyll fluorescence (Fig. 2E, F). Together, these results indicate that ppGpp accumulation drives
229 a major structural change in *P. patens* chloroplasts that is associated with alterations in photosynthetic
230 capacity and which is likely to be the result of the inhibition of chloroplast gene expression.

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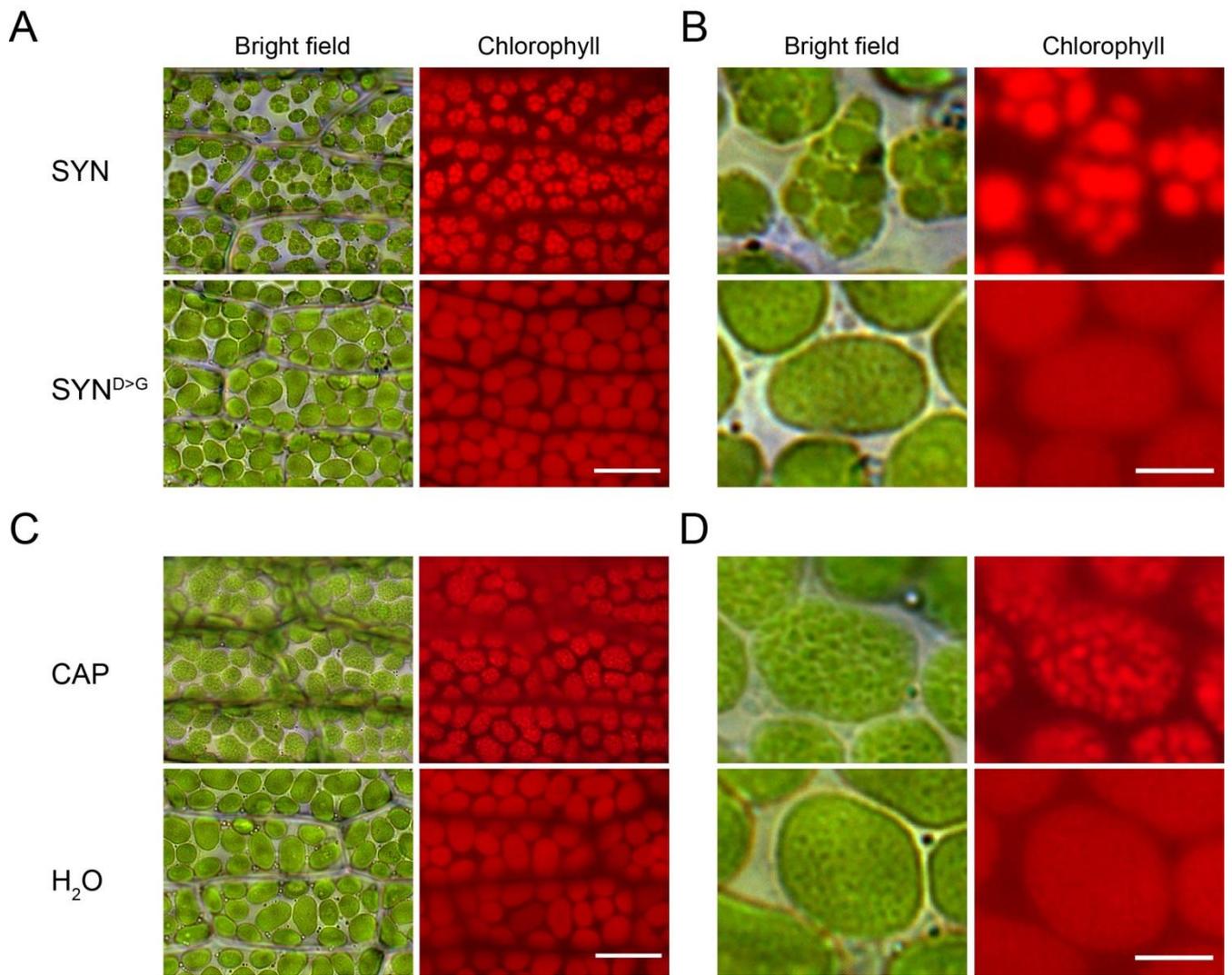
232 **ppGpp accumulation causes the formation of super grana**

233 We used transmission electron microscopy to determine the nature of the structures in the chloroplasts
234 of induced SYN and SYN^{D>G} gametophores (Fig. 4A). SYN^{D>G} chloroplasts contained clear stromal
235 thylakoid membranes with well-defined grana. In contrast SYN chloroplasts contained only two to
236 three very large grana structures (super grana) along the full length of the chloroplast.

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242 **Figure 3. ppGpp accumulation causes a major reorganization of chloroplast structure.** (A) Bright field
 243 and fluorescence microscopy images of phyllids from SYN1 and SYN^{D>G} gametophores at 35 DOI (scale, 20
 244 μm). (B) Close-up images of single chloroplasts in A (scale, 5 μm). (C) Images of phyllids from wild-type
 245 gametophores 4 days after treatment with chloramphenicol (CAP) (scale, 20 μm). (D) Close-up images of
 246 single chloroplasts in C (scale, 5 μm).

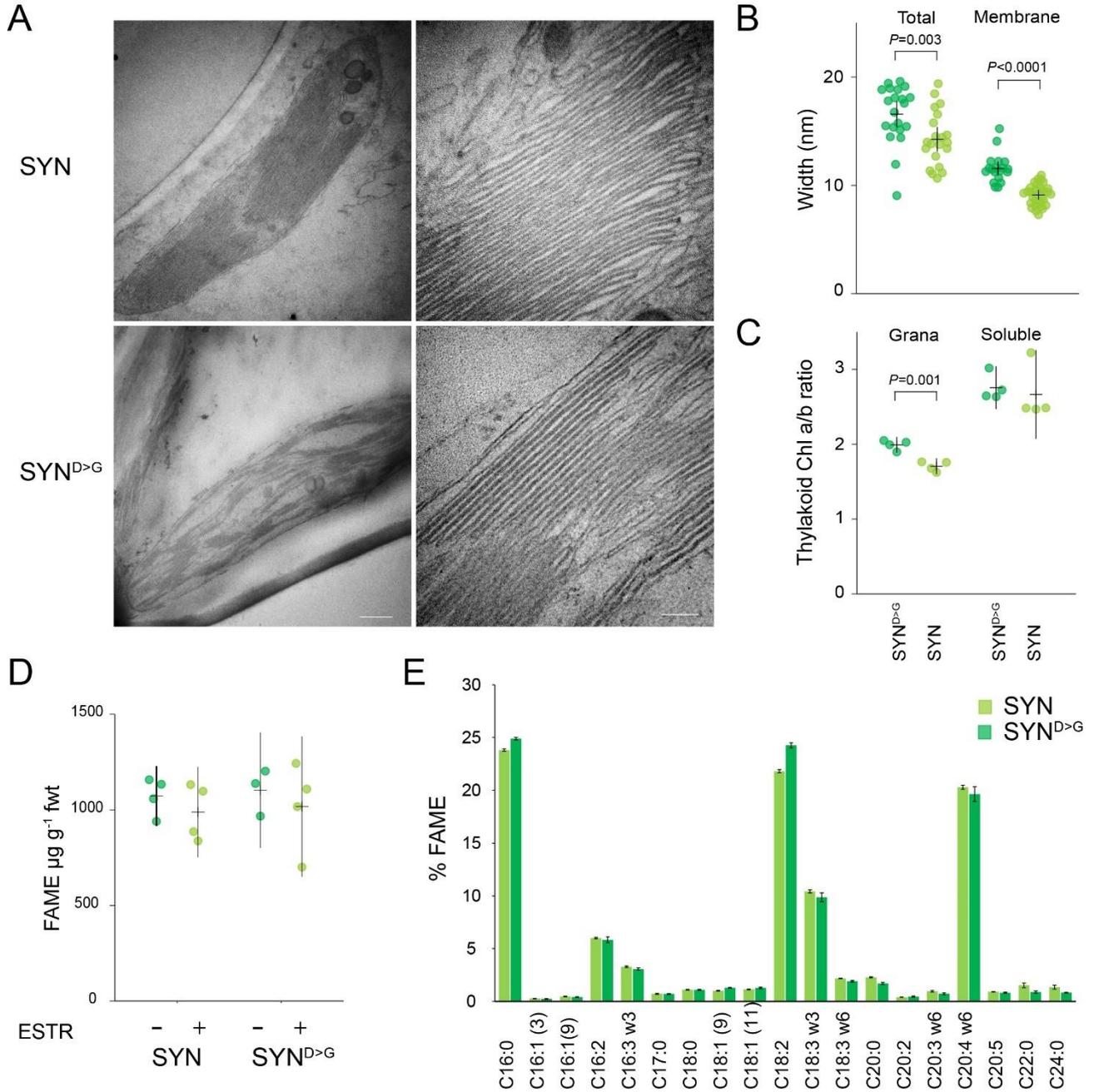
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249 The super grana traversed the complete width of the chloroplast, and were separated by disorganised
250 stromal lamellae. The thylakoid membranes in the SYN super grana were stacked more tightly than
251 in SYN^{D>G}, with reduced membrane and total repeat width (Fig. 4B). The super grana also showed a
252 strikingly low chlorophyll a/b ratio compared to regular grana (Fig. 4C). LHCII trimers have a low
253 chlorophyll a/b ratio of around 1.4 while the PSII RC and PSI have higher chlorophyll a/b ratios
254 (Pinnola *et al.*, 2015). These data indicate that SYN chloroplasts not only have more grana volume
255 than in the control line, but that the grana membranes themselves are also more highly enriched in
256 LHCII trimers.

257 The super grana in induced SYN gametophores represent a major reorganisation of the chloroplast
258 membrane system. We therefore suspected that the super grana could be the result of membrane lipid
259 synthesis or remodeling. However, we found no significant difference in fatty acid content (Fig. 4D)
260 and only minor changes in fatty acid composition (Fig. 4E) indicating that de novo lipid biosynthesis
261 is unlikely to play a major role in super grana formation. Furthermore, analysis of the composition of
262 polar lipid classes also revealed no major changes in the galactolipids monogalactosyldiacylglycerol
263 (MGDG) and digalactosyldiacylglycerol (DGDG) that are the main components of chloroplast
264 membranes (Fig. S8).

265



267

268 **Figure 4. ppGpp accumulation causes the formation of super grana.** (A) Electron microscopy images of
 269 SYN and SYN^{D>G} chloroplasts from gametophores after 35 DOI (scale 500 nm left panels, 100 nm right
 270 panels). (B) Average stack and membrane width in SYN and SYN^{D>G} grana (n=20 membranes, two-way
 271 Student t-test). (C) Chlorophyll a/b ratios for grana and soluble thylakoid fractions (n=3 biological repeats,
 272 two-way Student t-test). Fatty acid methyl ester (FAME) content (D) and composition (E) in SYN and SYN^{D>G}
 273 gametophores after 35 DOI (n=4 biological repeats). Measurements from ESTR induced gametophores are
 274 shown in (E). Error bars indicate 95% CI.
 275

276 **Discussion**

277 In this study we examined the role of ppGpp in the moss *P. patens*. We created an inducible
278 expression system to trigger the accumulation of ppGpp (SYN), with controls that allowed us to
279 separate the specific effects of ppGpp from other effects of the inducible expression system (SYN^{D>G})
280 (Fig.1, Fig. S1). Using these lines we were able to explore the likely role of ppGpp in *P. patens*, and
281 to make comparisons with the role of ppGpp signalling in other photosynthetic organisms.

282

283 The data we present indicate that the effect of ppGpp on photosynthetic function and in particular on
284 PSII is highly conserved across plants and algae. We found that ppGpp accumulation in gametophores
285 caused a major decrease in photosynthetic capacity as shown by a reduction in multiple
286 photosynthetic parameters and the levels of several chloroplast encoded proteins including Rubisco
287 (Fig. 1-2). Similar, although less pronounced, photosynthetic phenotypes were also reported in *A.*
288 *thaliana* plants that overaccumulate ppGpp (Maekawa, M. *et al.*, 2015; Sugliani *et al.*, 2016) as well
289 as more recently in the diatom *P. tricornutum* (Avilan *et al.*, 2021). The conservation of core ppGpp
290 responsive elements is in line with the early origins of the RSH enzyme family for ppGpp homeostasis
291 in the photosynthetic eukaryotes (Atkinson *et al.*, 2011; Ito *et al.*, 2017; Avilan *et al.*, 2019).

292

293 Our results in *P. patens* support the hypothesis that ppGpp acts principally via the inhibition of
294 chloroplast gene expression in plants. In *A. thaliana* ppGpp inhibits chloroplast gene expression via
295 the inhibition of chloroplast transcription, although the molecular mechanism is not yet known
296 (Sugliani *et al.*, 2016; Field, 2018). Here we found that ppGpp accumulation caused a small but
297 significant decrease in the transcript levels of chloroplast encoded genes, consistent with a reduced
298 rate of transcription (Fig. 2). The accumulation of a number of chloroplast encoded proteins, in
299 particular PsbA and RBCL, was also inhibited by ppGpp accumulation (Fig. 2A). However, we note
300 that some chloroplast-encoded proteins such as PsaA appeared to increase in abundance, suggesting
301 that other layers of regulation are also be at play. Nevertheless, in support of a general effect of ppGpp
302 on chloroplast gene expression we also show that treatment with the chloroplast translation inhibitor
303 chloramphenicol is sufficient to cause a similar photosynthetic phenotype to ppGpp accumulation
304 (Fig. 2).

305

306 Certain aspects of the response to ppGpp accumulation in *P. patens* were different to those observed
307 in other organisms. In *A. thaliana* plants ppGpp accumulation causes visible yellowing with a clear
308 reduction in chlorophyll levels (Maekawa, M. *et al.*, 2015; Sugliani *et al.*, 2016). However, here in
309 *P. patens* we observed no detectable effect on gametophore chlorophyll in response to ppGpp
310 accumulation under standard growth conditions (Fig. 1). While we observed a relatively small fold

311 increase in ppGpp levels in response to SYN induction in *P. patens* we also observed much stronger
312 decreases in Fv/Fm and the chlorophyll a/b ratio than previously observed in *A. thaliana* (Sugliani *et*
313 *al.*, 2016), and we also observed the major reorganisation of chloroplast structure (Fig. 3-4).
314 Therefore, we consider it likely that the different response of *P. patens* may reflect differences in the
315 underlying modes of action for ppGpp rather than in the quantity of ppGpp. As such our findings may
316 be evidence of the specialisation of ppGpp signalling in different branches of the photosynthetic
317 eukaryotes.

318

319 We observed the formation of striking super grana structures in *P. patens* chloroplasts in response to
320 ppGpp accumulation (Fig. 3-4). This was surprising because super grana were not previously
321 observed in *A. thaliana* plants that overaccumulate ppGpp (Maekawa, M. *et al.*, 2015; Sugliani *et al.*,
322 2016). Super grana occur naturally in shade-adapted plants like *Alocasia macrorrhiza* (Heitz, 1936;
323 Anderson, 1999), and can also be induced in some plants when exposed to low light levels such as in
324 apple (Skene, 1974). *A. thaliana* does not accumulate super grana under low light. However, growth
325 of *A. thaliana* on the chloroplast translation inhibitor lincomycin provokes the accumulation of super
326 grana in leaves (Belgio *et al.*, 2015). Interestingly, the lincomycin-grown plants have an Fv/Fm of
327 around 0.2, much lower than the Fv/Fm observed in response to ppGpp over accumulation in *A.*
328 *thaliana* and more similar to that observed in *P. patens* (Fig. 1). Interestingly, our data indicate that
329 the reduced Fv/Fm and the accumulation of super grana are likely not due to changes in lipid
330 composition (Fig. 4E, S8), but rather modifications in protein composition. The reduction in Fv/Fm
331 in *P. patens* overaccumulating ppGpp and in lincomycin-grown *A. thaliana* is likely to be due to a
332 substantial energetic uncoupling of PSII antenna from PSII reaction centres (Fig. 2B) (Belgio *et al.*,
333 2015). One way that this can occur is by an increase in the proportion of PSII LHCII trimers to PSII
334 reaction centres. Increased LHCII trimer abundance is also known to promote grana stacking (Pribil
335 *et al.*, 2014; Albanese *et al.*, 2020). However, rather counter intuitively, overaccumulation of ppGpp
336 resulted in an apparently higher LHCII trimer to PSII RC ratio in *A. thaliana* (Sugliani *et al.*, 2016)
337 than we observe here in *P. patens* (Fig. 2A). An explication for this discrepancy is that photosystem
338 composition in *P. patens* is different to that of *A. thaliana* (Alboresi *et al.*, 2008), leading to
339 uncertainty about the quantity and function of each component. For example, *P. patens* possesses
340 many more LHCII isoforms, including the algal-like LHCB9 isoform that is absent from *A. thaliana*.
341 Indeed, we noticed that LHCB9 accumulated in response to ppGpp (Fig. 2 and Fig S5). The increase
342 in the abundance of this isoform, and potentially other isoforms for which we do not have specific
343 antibodies could be responsible for promoting grana stacking and/or causing a larger change in the
344 LHCII trimer / PSII RC ratio than we could observe by immunoblotting (Fig. 2A). This latter
345 possibility is supported by the pronounced blue-shift in the low temperature chlorophyll fluorescence

346 profile for SYN, indicating energetic uncoupling of LHCII trimers from PSII RC (Fig. 2B), as well
347 as the low chlorophyll a/b ratio in gametophores and isolated grana (Fig. 1H, 4C).

348

349 Despite having a dramatic impact on thylakoid organisation and photosynthetic capacity, ppGpp
350 accumulation did not alter gametophore growth (Fig. 1). Our results contrast with the situation in *A.*
351 *thaliana* plants where overexpression of RSH3 or an equivalent SYN protein increased ppGpp levels,
352 reduced photosynthetic capacity and caused a strong reduction in growth (Sugliani *et al.*, 2016).
353 Similarly, the induction of ppGpp synthesis by SYN in the diatom *P. tricornutum* reduced
354 photosynthetic capacity while strongly repressing proliferation (Avilan *et al.*, 2021). These
355 observations strongly suggest that ppGpp has different impacts on growth in divergent photosynthetic
356 eukaryotes. More generally, these results may reflect different responses to a reduction in
357 photosynthetic capacity. Analysis of gas-exchange data shows that bryophytes obtain lower
358 photosynthetic benefits on a per leaf mass area or N content basis than angiosperms (Gago *et al.*,
359 2019). Gago *et al.* (2019) propose that this difference is related to the limitation of CO₂ diffusion by
360 the bryophyte cell wall, differential nutrient investment strategies, and a fundamentally different
361 canopy structure to angiosperms. While it is not clear which of these factors is important, it is likely
362 that they contribute to the low sensitivity of *P. patens* growth to the severe drop in photosynthetic
363 rate and disruption of photosystem organisation caused by ppGpp accumulation. Comparison of the
364 impact of these changes on stress tolerance in both species would need more investigation (Maekawa,
365 M. *et al.*, 2015; Honoki *et al.*, 2018; Gago *et al.*, 2019). It would also be interesting to investigate
366 gametophore growth in *P. patens* mutants affected in photosystem composition to understand whether
367 our observation is specific to ppGpp accumulation or reflects a more general ability of mosses to
368 grow despite defects in photosynthesis (Pinnola *et al.*, 2015; Pinnola *et al.*, 2018; Peng *et al.*, 2019;
369 Storti *et al.*, 2020). Analysis in other model bryophytes, such as the liverwort *Marchantia*
370 *polymorpha*, would also help to determine whether this characteristic can be generalized to all non-
371 vascular plants (Cesarino *et al.*, 2020).

372

373 Altogether, our data indicate that a relatively modest increase in ppGpp can inhibit chloroplast gene
374 expression in *P. patens* and lead to a major downregulation of photosynthetic activity and radical
375 reorganisation of the chloroplast membrane system without a major impact on growth. Our data point
376 to the existence of both highly conserved targets of ppGpp signalling as well as more specialised
377 targets in different organisms. This may be related to the different relationship between
378 photosynthetic capacity and growth observed between bryophytes and vascular plants. Further
379 research is now required to understand the physiological roles of ppGpp during growth and
380 development across land plants, and also the significance and mechanism of super grana formation.

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390 culture methods, and Aurelie Crepin and Stefano Caffarri for assistance with grana isolation and low
391 temperature chlorophyll fluorescence measurements.

392

393 **Contributions**

394 B.M. and B.F. conceived the experiments. S.C. performed the nucleotide quantification; B.L. and
395 Y.L-B. performed and analysed the lipidomics experiments; S.H and A.A performed the electron
396 microscopy experiments; S.H., S.E. and J.V. performed the remaining experiments. S.H., S.E., B.M.
397 and B.F contributed to the interpretation of the results. S.H., B.M. and B.F. wrote the manuscript. All
398 authors provided critical feedback and helped shape the research, analysis and manuscript.

399

400 **Data availability**

401 The data that support the findings of this study are available from the corresponding author upon
402 reasonable request.

403 **Materials and Methods**

404

405 **Plant growth**

406 *P. patens* (Gransden strain) was grown under a 16h light/8h dark photoperiod at 23°C with 50 μmol
407 $\text{m}^{-2} \text{s}^{-1}$ PAR fluorescent lighting. For gametophore generation, fresh protonema cultivated on BCDAT
408 agar media (Roberts *et al.*, 2011) with cellophane disks was collected and dispersed in water with a
409 T10 Basic ULTRA-TURRAX (IKA, Belgium) mixer, 3 ml of the homogenate were inoculated onto
410 a sterile 7.4 mm diameter peat pellet (Jiffy products) and cultured for 25 days unless otherwise stated.
411 To induce the expression of SYN, 25 day old peat pellet-moss cultures containing mainly young
412 gametophores (Fig. S2) were treated with 5 μM of estradiol (from stock dissolved in DMSO) or
413 DMSO control two times a week for 35 days unless stated otherwise. For chloramphenicol treatments,
414 25 days old *P. patens* wild type cultures were sprayed once with 5 mM chloramphenicol (dissolved
415 in water) or water control and then cultured under standard growth conditions.

416

417 **Cloning**

418 A fragment corresponding to amino acids 1 to 386 of RelA was fused by PCR to a genomic sequence
419 coding for the 80-amino acid *P. patens* Rubisco small subunit (Pp3c13_15980V3.1) target peptide.
420 The fused PCR product (SYN) was then introduced into the entry vector pDONR207 (Life
421 Technologies) by Gateway BP cloning. The entry clone was confirmed by sequencing. In parallel an
422 inactive version of RelA with the mutation D275G was used in the same fashion to make
423 SYN^{D275G}. SYN and SYN^{D275G} were then recombined by LR Gateway recombination into the estradiol
424 inducible expression vector pGGW6 (kindly provided by M. Hasebe) (Kubo *et al.*, 2013).

425

426 ***P. patens* transformation**

427 Polyethylene glycol-mediated transformation of the wild-type strain of *P. patens* with linearized
428 plasmid was performed as described previously (Schaefer & Zrýd, 1997) with small modifications
429 (Menand *et al.*, 2007). Selection of stable transformants was performed on BCDAT medium
430 containing 25 $\mu\text{g}/\text{ml}$ hygromycin. Southern blot analysis was performed using DIG labelled probes
431 according to the manufacturer's instructions (Roche). Three SYN and three SYN^{D>G} lines were shown
432 to have one insertion in the correct PIG locus and therefore were selected for the study (Fig. S1). All
433 biochemistry experiments were done with SYN1 and SYN^{D>G}2 lines.

434

435 **Chlorophyll fluorescence measurements**

436 Gametophores were aligned in a petri dish and adapted to the dark for 20 min and then imaged in a
437 Fluorcam FC 800-O imaging fluorometer (Photon System Instruments). F0 and Fm were imaged

438 using the following settings (Shutter=3, Sensitivity=75, Super=50), with F0 acquired over a 4 s
439 period. PSII maximum quantum yield was calculated as $(F_m - F_0)/F_m$. For the relative electron transfer
440 rate (ETR) plants were dark-adapted and exposed to 2 minutes periods of increasing actinic light
441 intensity, with F_v'/F_m' measurements at the end of each period. Relative ETR was calculated as
442 $F_v'/F_m' \times \text{light intensity}$. Steady state 77K chlorophyll fluorescence measurements were obtained
443 from gametophore powder suspended in 85% (w/v) glycerol, 10 mM HEPES, pH 7.5 as described
444 previously (Galka *et al.*, 2012).

445

446 **Immunoblotting**

447 Proteins were extracted and equal quantities of total protein analyzed by immunoblotting as described
448 previously (Sugliani *et al.*, 2016). The following antibodies were used: AtpB (Agrisera;
449 polyclonal, catalog number AS08304), LHCA1 (Agrisera; polyclonal, catalog number AS01 005),
450 LHCB1 (Agrisera; polyclonal, catalog number AS01 004), LHCB9 (Agrisera; polyclonal, catalog
451 number AS15 3088), PetA (Agrisera; polyclonal, catalog number AS08 306), PsaA (Agrisera;
452 polyclonal, catalog number AS04 042), PsbA (Agrisera; polyclonal, catalog number AS05 084, and
453 RelA (1/2000 dilution, raised against *E. coli* RelA and kindly provided by M.Cashel).

454

455 **Gene expression analysis**

456 RNA was extracted from frozen gametophore powder using TriReagent (Sigma-Aldrich), quality was
457 verified by agarose gel electrophoresis, and genomic DNA was removed by treatment with DNase
458 (ThermoFisher). cDNA was synthesized from 500 ng of RNA using Primescript RT Reagent Kit
459 (Takara Bio) with random hexamer primers. qRT-PCR was performed on 1 μ L of 1 in 40 diluted
460 cDNA in 15 μ L reactions using SYBR Premix Ex-Taq II reagent (Takara Bio) in a Bio-Rad CFX96
461 real-time system (see Table S1 for primer pairs). Relative quantification of gene expression adjusted
462 for efficiency was performed using PCR Miner (Zhao & Fernald, 2005). *E2*, *60S* and *APRT* were
463 used as reference transcripts for normalization (Le Bail *et al.*, 2013).

464

465 **Chlorophyll quantification**

466 Chlorophyll was extracted from gametophores with ice-cold 80% acetone saturated with sodium
467 carbonate. The absorbance was measured between 350 and 750 nm in a Varian Cary 300
468 spectrophotometer (Agilent). Chlorophyll concentrations and chlorophyll a/b ratios were calculated
469 using a fitting algorithm as described previously (Croce *et al.*, 2002).

470

471 **Quantification of ppGpp**

472 ppGpp was extracted from gametophores and quantified by LC-MS/MS using a ¹³C-labelled G4P
473 internal standard as described previously (Bartoli *et al.*, 2020).

474

475 **Light and fluorescence microscopy**

476 Gametophores were harvested and fixed in a solution of 2% glutaraldehyde 0.1 M phosphate buffer
477 and stored at 4 °C overnight. Chloroplast structure was visualized in dissected phyllids using an
478 Axioimager M2 microscope and AxioCam HRc Camera (Carl Zeiss Microscopy, Marly le Roi, and
479 France). Chlorophyll auto-fluorescence was visualized with HBO 100 mercury lamp with excitation
480 at BP 560/55, and emission at BP 645/75. Images were captured with AxioVision Rel 4.8 software,
481 and Zerene stacker software version 1.04 was used for focus stacking correction.

482

483 **Electron microscopy**

484 Phyllids were cut from gametophores and fixed in 2.5 % glutaraldehyde. Phyllids were transferred
485 into phosphate buffer pH 7.2 for 5 min, incubated 5 min in agarose at 37°C and left overnight at 4°C.
486 Phyllids were then washed twice 15 min in phosphate buffer 7.2 pH, post-fixed in 1% Osmium
487 phosphate buffer 7.2 pH, 30 min at room temperature, and washed twice in water. The phyllids were
488 then taken through a series of dehydration steps in acetone at 20, 35, 50, 62, 75, 85, & 95 % for 15
489 min each and 3 times 100% for 5 min. The phyllids were kept in acetone and taken through a series
490 of infiltration with SPURR resin (Sigma) (5, 10, 20, 40, and 70 %) 30 min each without accelerator
491 and then 100% with SPURR S2 for 2 hr, and changed to fresh resin and left overnight. The next day,
492 the resin was changed and left for 24 hr. Next, samples were placed in a mould with fresh resin and
493 incubated at 60°C for 48 hr. The blocks were cut into ultra-thin sections of 70 nm using a Cryo-
494 ultramicrotome UCT (Leica). The sections were placed on an EM grid, stained with aqueous uranyl
495 acetate and lead citrate and observed with a FEI Tecnai G2 electron microscope. ImageJ software
496 version 1.45 was used to measure membrane thickness.

497

498 **Analysis of thylakoid composition**

499 Thylakoid membrane preparation and solubilization was performed as described previously, with
500 modifications (Berthold *et al.*, 1981). Approximately 10 g of gametophore was blended in 50 ml of
501 buffer B1 (0.4 M NaCl, 2 mM MgCl₂, 20 mM tricine KOH pH 7.8, 0.2 mM benzamidine, 1 mM
502 hexanoic acid) and passed through a 30 µM filter. The solution was centrifuged 15 min at 1400 g and
503 4°C. Pellets were re-suspended softly with a paint brush in buffer B2 (20 mM tricine KOH pH 7.8,
504 0.15 M NaCl, 5 mM MgCl₂, 0.2 mM benzamidine, 1 mM hexanoic acid) and centrifuged for 10 min
505 at 6000g and 4°C. The pellets representing total thylakoids were then re-suspended in buffer B3 (15
506 mM NaCl, 5 mM MgCl₂, 20 mM Hepes KOH pH 7.5) at a chlorophyll concentration of 2.5 mg ml⁻¹.

507 For solubilisation 100 μ L of total thylakoid was mixed with 18 μ L of Triton buffer (15% (w/v) triton
508 X-100, 15 mM NaCl, 5 mM MgCl₂) and incubated 20 min in the dark on ice with gentle agitation.
509 The mix was centrifuged 3 min at 1500 g and 4°C and then the supernatant was submitted to a final
510 centrifugation for 30min at 29500 rpm at 4°C in an Optima LE-80k ultracentrifuge. The supernatant,
511 enriched for soluble stromal membranes, were separated from the Triton resistant pellets enriched in
512 grana and chlorophyll measured in each fraction.

513

514 **Lipid measurements**

515 Lipids were extracted from gametophores after 35 DOI as described previously (Hara & Radin, 1978)
516 with modifications. Two ml of pre-heated isopropanol (85°C) containing BHT 0.01% (w/v) was added
517 to 200-400 mg frozen gametophore powder in a glass tube with Teflon lined cap. The mixture was
518 vortexed to break the tissue and heated for 5-10 minutes at 85°C in a water bath. After cooling down,
519 internal standards (1 μ g PE17:0/17:0, 1 μ g TAG17:0/17:0/17:0), and 6 ml of MTBE were added to
520 the mixture and vortexed. To allow phase separation, 2 volumes of aqueous sodium chloride NaCl
521 (0.9 w/v) were added and the solutions were vortexed and centrifuged at 3000 g for 2 min. The upper
522 phase containing lipids was collected and transferred to a new glass tube. To maximize extraction, 1
523 ml of MTBE was added to the samples again and vigorously vortexed and centrifuged at 3000 g for
524 2 min. The new upper phase was transferred to the tube containing the first solvent extract. Finally,
525 lipid extracts were stored at -20°C until analysis. Fatty acids were first converted to their methyl esters
526 then analyzed by gas chromatography-mass spectrometry (GC-MS) and lipid molecular species were
527 analyzed by ultra performance liquid chromatography - tandem mass spectrometer (UPLC-MS/MS)
528 as previously described (Legeret *et al.*, 2016)

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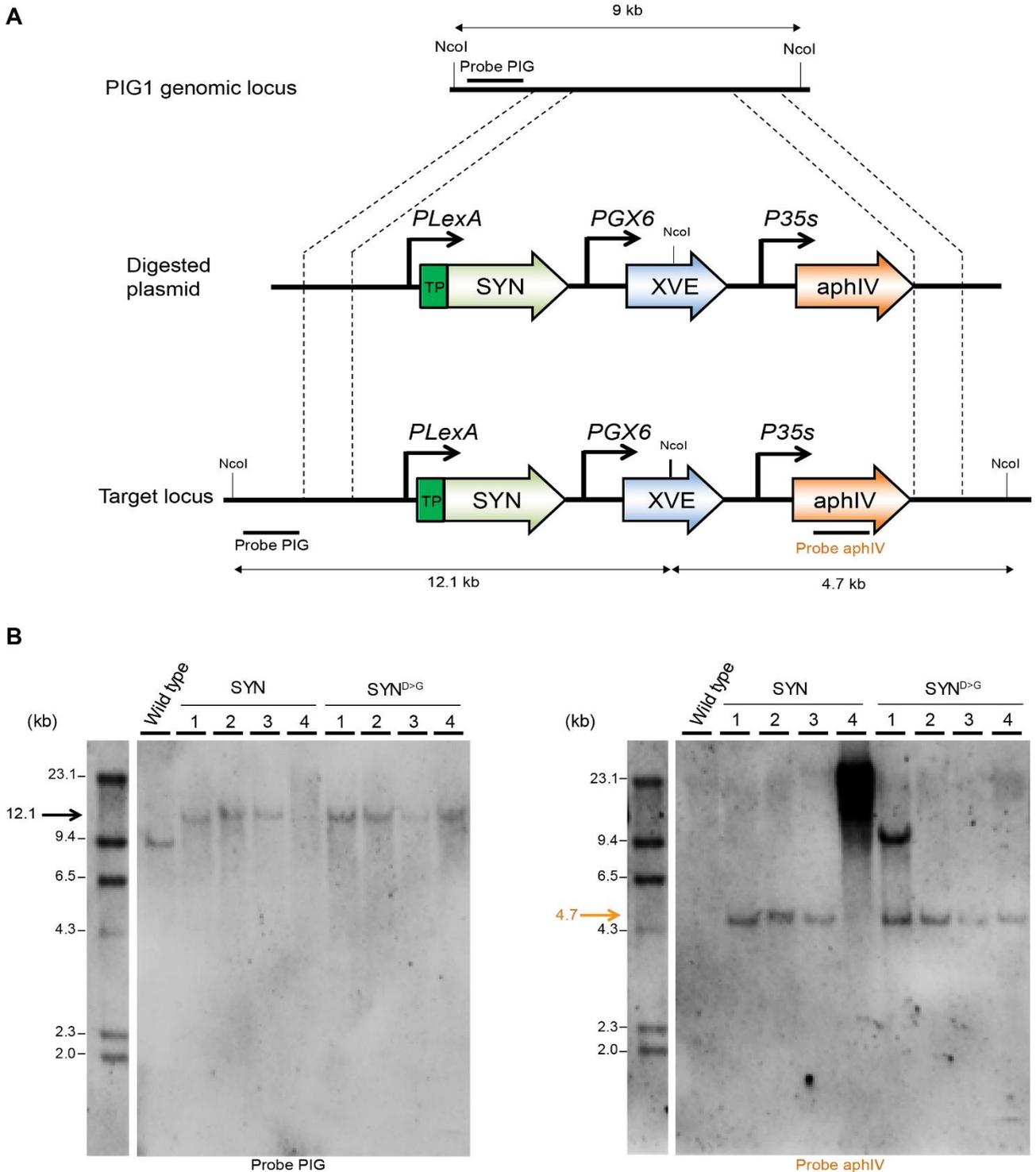
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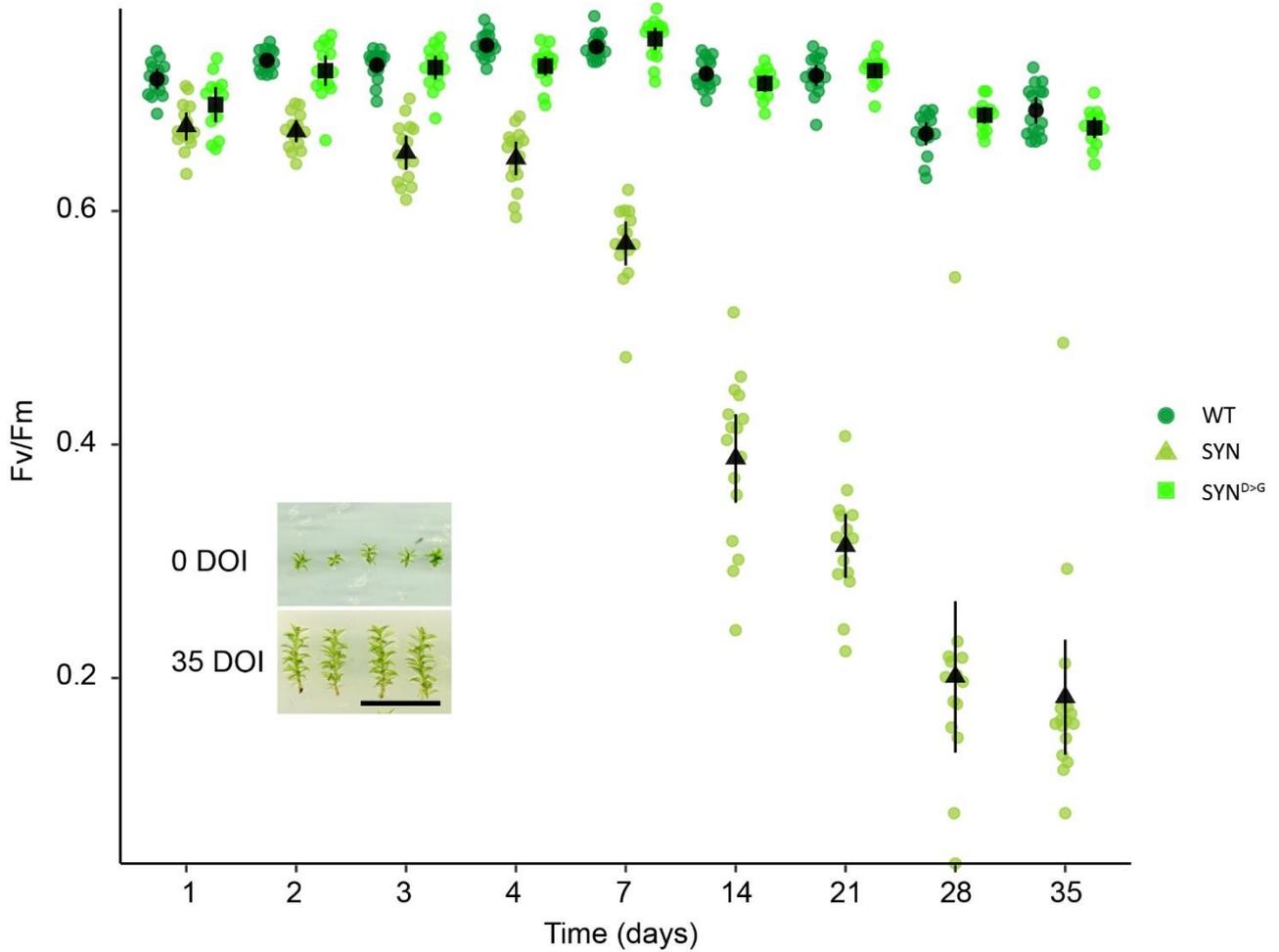
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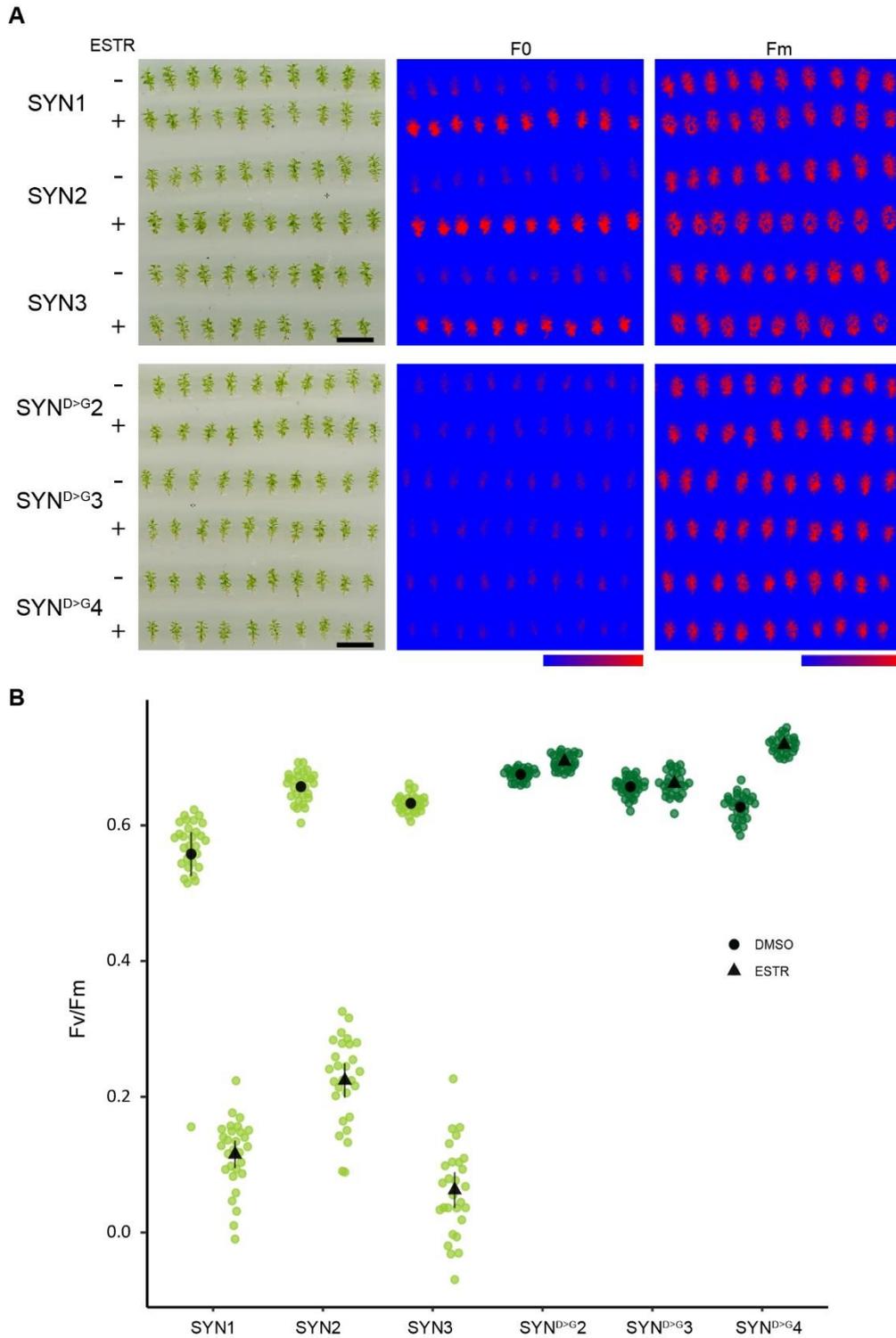


649 **Figure S1. Southern blot analysis of *P. patens* transgenic SYN lines.** (A) Schematic representation of the
 650 PIG locus and the gene targeting construct. Probes for Southern blotting and the expected size of the restriction
 651 fragments they should detect after successful gene targeting are indicated. TP: transfer peptide, SYN: ppGpp
 652 synthetase, XVE: chimeric transcription activator composed of: LexA (X), VP16 (V) and the human estrogen
 653 receptor (E), aphIV: hygromycin phosphotransferase resistant gene, PIG : *P. patens* Intergenic 1. (B) DNA
 654 extracted from different SYN lines was digested with *NcoI* and hybridized using a DIG labelled probe specific
 655 to the PIG locus (left), and then the same membrane was re-probed with a DIG-labelled probe specific to the
 656 antibiotic resistance cassette found in the gene targeting construct (right). The lines SYN 1, 2, 3 and SYN^{D>G}
 657 2, 3, 4 were selected for further study.



659

660 **Figure S2. Evolution of Fv/Fm in different lines in response to estradiol treatment.** Fv/Fm was measured
 661 regularly following the start of estradiol treatment in SYN, SYN^{D>G} and wild-type gametophores (n= 15
 662 gametophores). Inset shows typical gametophore size at the start and end of the experiment (DOI, days of
 663 induction). Scale, 1cm. Error bars indicate 95% CI.
 664

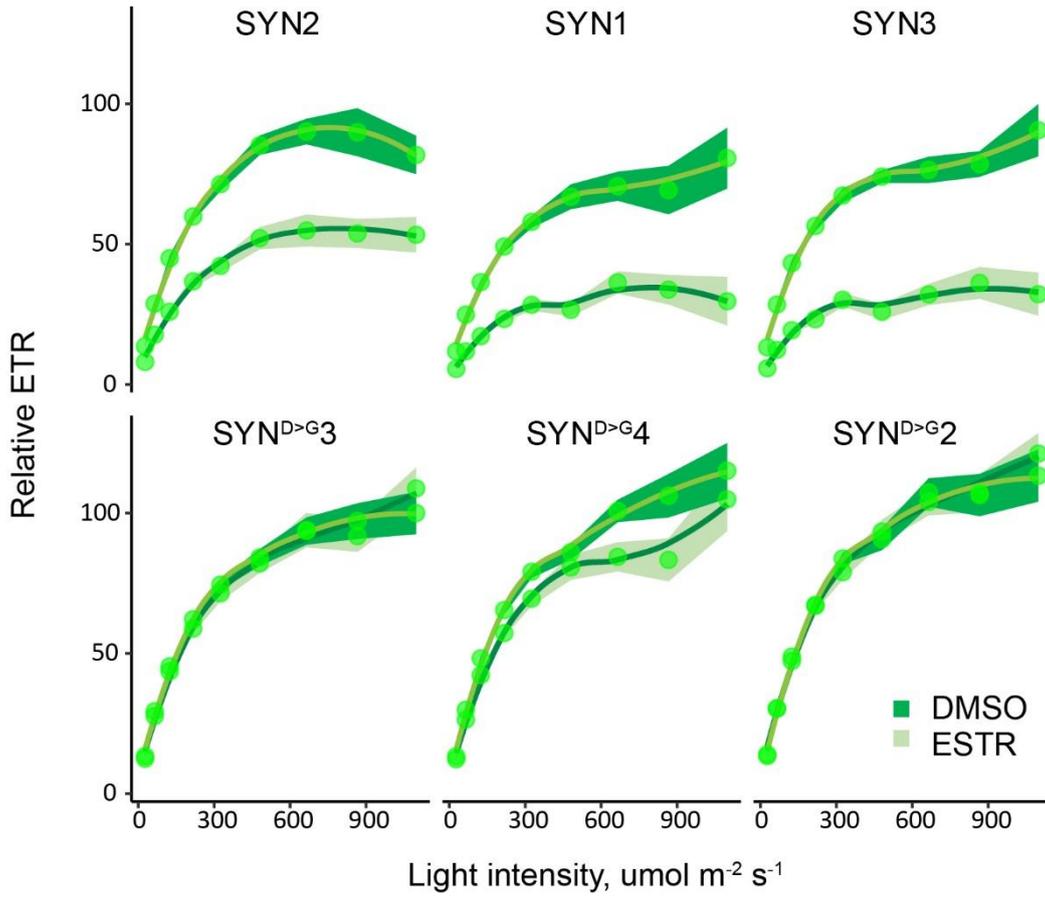


666

667 **Figure S3. Photosynthetic parameters in different SYN lines.** (A) Images of F0 and Fm in different lines
 668 (scale, 1 cm; false color scale, 50-2000 intensity units). (B) Fv/Fm in SYN and SYN^{D>G} lines after 35 DOI
 669 (n=30 gametophores). Error bars indicate 95% CI.

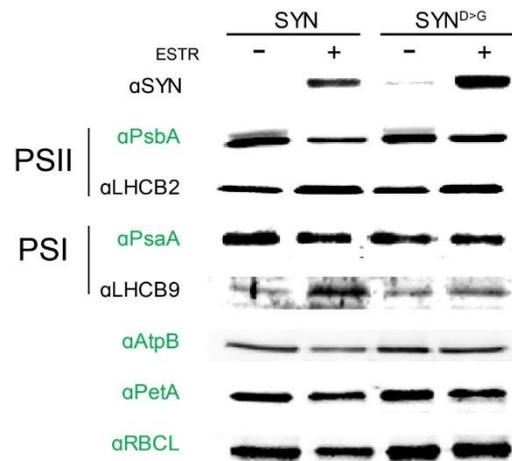
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673 **Figure S4. ETR in different SYN lines.** ETR was determined at different light intensities in SYN and SYN^{D>G}
674 gametophores after 35 DOI (n=30 gametophores). Error ribbon indicates 95% CI.
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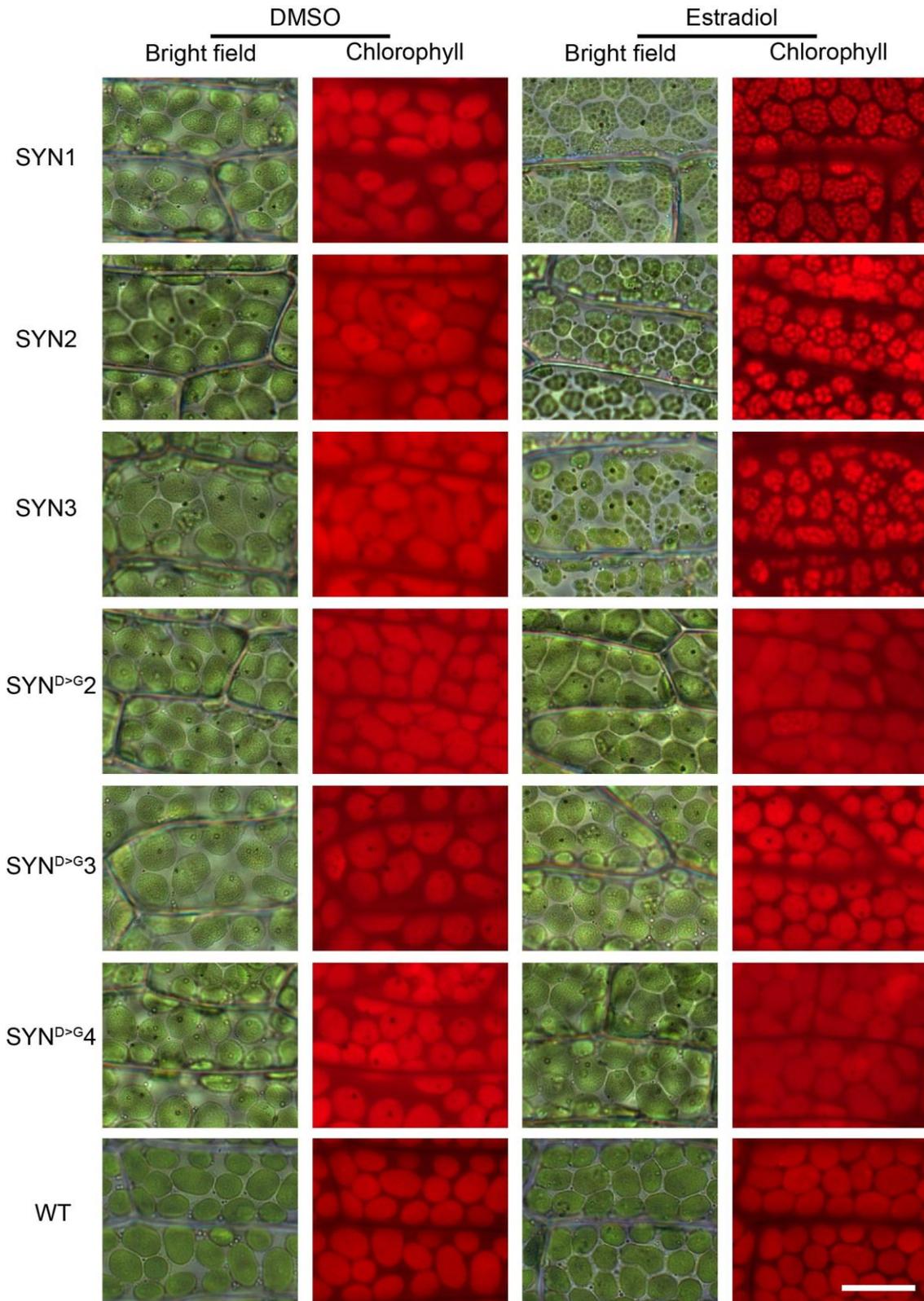
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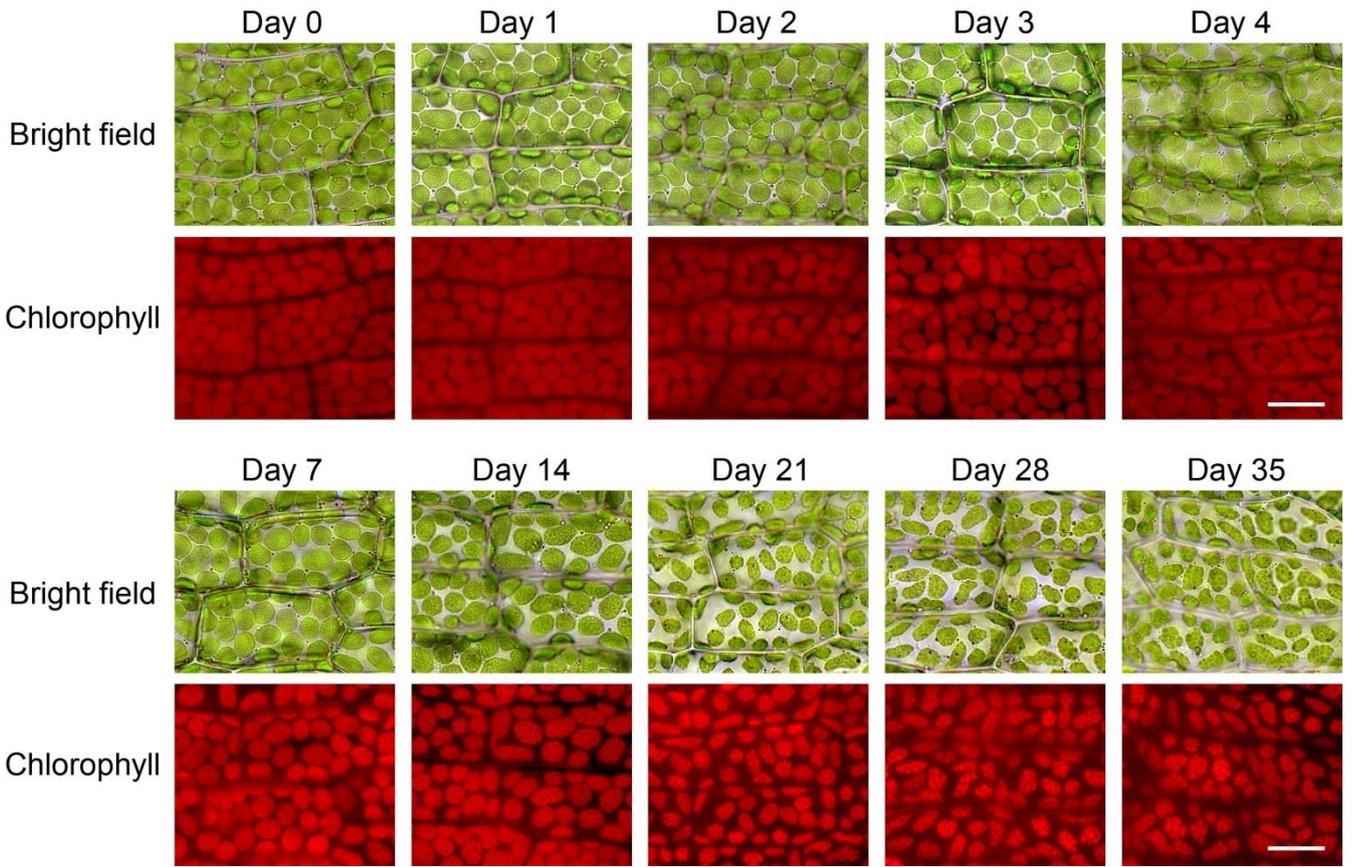
Figure S5. Protein accumulation after 35 DOI. Immunoblots on equal quantities of total protein from SYN and SYN^{D>G} after 35 DOI with 0 μM or 5μM estradiol using antibodies against signature chloroplast and nuclear encoded photosynthetic proteins. Chloroplast-encoded proteins are indicated by green text.



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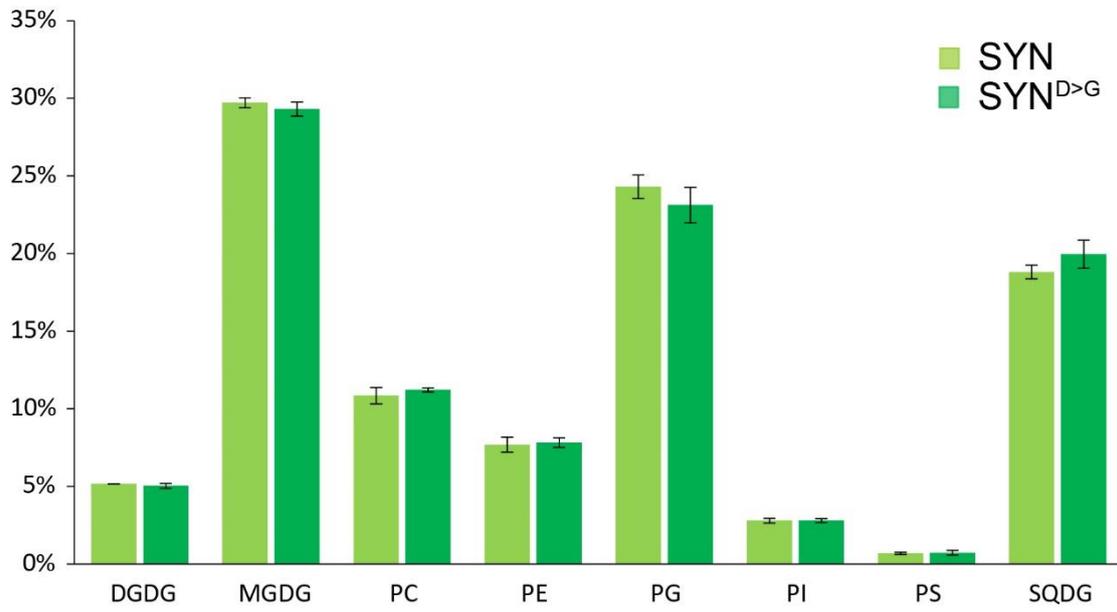
684 **Figure S6. Chloroplast structure in independent SYN lines.** Bright field and fluorescence microscopy
 685 images of phyllids from different SYN lines after 35 DOI (scale, 20 μ M).
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689 **Figure S7. Appearance and evolution of super grana during SYN induction.** Bright field and fluorescence
690 microscopy images of phyllids at multiple timepoints during the induction of SYN (scale, 20 μ M).
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693 **Fig. S8 Polar lipid composition of induced SYN and SYN^{D>G} gametophores.** Polar lipids were
 694 measured in gametophores 35 DOI (n=4 biological repeats). Error bars indicate 95% CI.

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SYN Cloning

SYN-1a (Pp SSU CTP-F)	ATGGCCTCCGCTGTGGCT
SYN-1b (Pp SSU CTP-R)	gtgcacttctaccgcaacGTCATCGCTCAGCGGGGG
SYN-1c (RelA-F)	cccgctgagcgatgacGTTGCGGTAAGAAGTGCAC
SYN-1d (RelA-R)	TTAATGGTGATGGTGATGGTGTCCACCTCCCTCTTCCTGCCACGCAAT

DNA Probes

AphIV-F	GTAGGAGGGCGTGGATATGT
AphIV-R	CGAGTGCTGGGGCGT
PIG-F	CCTTTGGCAGGCTCAGATGT
PIG-R	CAACTGACAGGACCCGACTT

RT-qPCR

PSBA-F	CAACGGTGGTTCCTTACGAGT
PSBA-R	CAGCGATCCAAGGACGCATA
PSAB-F	TCGAAGCATGGGGACAAGAT
PSAB-R	AGCTCCGCCTCGAGTAAATG
16S-F	CGAGGGCAAGCTAACCTCAA
16S-R	TATGGCTGACCGGCGATTAC
23S-F	TGCTTCGGGGAGCTGAAAAT
23S-R	TCAGTTCGCCAGGTTGTCTC
TRNY-F	GGTCGATGCTCGAGTGGTTA
TRNY-R	AAATTGGGCCGAGCTGGATT
RPOA-F	ACGCTCGATTTGCCGTATCT
RPOA-R	ACAAGATGTCCTGCACCGTT
RPOB-F	AGAACCCATTAACGCACGGT
RPOB-R	CCAGCTCGCTATCGACAAGA
60S-F	CAGGAAGCGCAATGATGACG
60S-R	TAACACGGGCGGGGAATAAG
APRT-F	AGTATAGTCTAGAGTATGGTACCG
APRT-R	TAGCAATTTGATGGCAGCTC
E2-F	TACGGACCCTAATCCAGATGAC
E2-R	CAACCCATTGCATACTTCTGAG
SYN-F	TCAAACCTGGCGGAGCGTATT
SYN-R	GTTTCATGCAGCAGTTTGGCA