

# mRNA localisation, reaction centre biogenesis and thylakoid membrane targeting in cyanobacteria

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## Abstract

The thylakoid membranes of cyanobacteria form a complex intracellular membrane system with a distinctive proteome. The sites of biogenesis of thylakoid proteins remain uncertain, as do the signals that direct thylakoid membrane-integral proteins to the thylakoids rather than to the plasma membrane. Here, we address these questions by using Fluorescent *in situ* Hybridisation to probe the subcellular location of mRNA molecules encoding core subunits of the photosystems in two cyanobacterial species. These mRNAs cluster at thylakoid surfaces mainly adjacent to the central cytoplasm and the nucleoid, in contrast to mRNAs encoding proteins with other locations. Ribosome association influences the distribution of the photosynthetic mRNAs on the thylakoid surface, but thylakoid affinity is retained in the absence of ribosome association. However, thylakoid association is disrupted in a mutant lacking two mRNA-binding proteins, which likely play roles in targeting photosynthetic proteins to the thylakoid membrane.

27 Cyanobacterial thylakoid membranes form a complex intracellular membrane system that has been inherited, with  
28 modifications, in chloroplasts. Cyanobacterial thylakoids are the sole site of photosynthetic electron transport and  
29 the major site of respiration, and they have a proteome distinct from that of the plasma membrane<sup>1-3</sup>. Membrane  
30 architecture varies between species, but the thylakoids usually form a series of flattened sacs located between the  
31 plasma membrane and the central cytoplasm<sup>4-8</sup>. Electron tomography indicates that the thylakoids and the plasma  
32 membrane are not contiguous<sup>5,7</sup>, although in some cyanobacteria, such as *Synechocystis* sp. PCC 6803 (hereafter  
33 *Synechocystis*), the sacs converge on a membrane tube which closely approaches the plasma membrane at some  
34 sites<sup>7</sup>.

35  
36 Thylakoid membranes have a distinctive proteome that includes all the complexes involved in photosynthetic  
37 electron transport, and most of the respiratory complexes<sup>1,3</sup>, organised in a dynamic and variable protein landscape<sup>9</sup>.  
38 An early study suggested that the initial steps of photosystem biogenesis occurred in the plasma membrane<sup>10</sup>, but an  
39 improved cell fractionation procedure showed that Photosystem II (PSII) biogenesis takes place in the thylakoids<sup>11</sup>.  
40 Biochemical and mutagenesis studies have revealed an intricate, co-ordinated sequence of events by which the  
41 photosynthetic reaction centre apoproteins, along with their chlorophylls and other co-factors, are synthesised and  
42 assembled into mature reaction centres<sup>1,12-14</sup>, but the exact locations of these processes remain uncertain. In  
43 *Synechocystis*, a specialised biogenic region was proposed at the convergence membrane adjacent to the plasma  
44 membrane. PrtA, a periplasmic protein implicated in the delivery of manganese ions to the water-oxidising complex  
45 of PSII, is concentrated near the convergence zones, suggesting that these are a major site of PSII biogenesis<sup>12,15,16</sup>.  
46 Cryo-electron tomography shows some ribosomes associated with the convergence membranes, although the vast  
47 majority of thylakoid-associated ribosomes are found at the innermost thylakoid surface, facing the central  
48 cytoplasm<sup>7</sup>. An earlier electron tomographic study highlighted a high density of ribosomes associated with  
49 protrusions of the thylakoid system into the central cytoplasm<sup>6</sup>.

50  
51 Mechanisms for specific targeting of proteins to the thylakoid or plasma membrane remain uncertain. Membrane-  
52 targeted proteins commonly carry N-terminal leader sequences that should be specific for the Sec or Tat  
53 translocons<sup>17</sup>. However, *Synechocystis* has only a single set of genes for each translocon, and it is likely that the same  
54 sets of Sec and Tat components are present in both thylakoid and plasma membranes<sup>17</sup>. Intensive studies of the

55 leader sequences have not revealed any differences that could lead to predictable targeting to a specific  
56 membrane<sup>17</sup>. It was accordingly suggested that protein sorting might occur post-translationally, based on differences  
57 in the electrostatic properties of the C-terminal portions of the polypeptides<sup>1</sup>. This would require connections (at  
58 least transiently) between the thylakoid and plasma membranes, to allow sorting by lateral diffusion after  
59 translation. Such connections are rarely observed<sup>7</sup>.

60  
61 Here, we use Fluorescent *in situ* Hybridisation (FISH) to probe the subcellular location of mRNA molecules encoding  
62 core subunits of the photosystems in two species of cyanobacteria. The results give clues to the sub-cellular location  
63 of the first stage of photosystem assembly, and the mechanism that targets photosynthetic proteins to the  
64 thylakoids.

### 66 **Rationale for RNA-FISH experiments**

67 To better understand the targeting and biogenesis of cyanobacterial thylakoid membrane proteins, we probed the  
68 subcellular location of specific mRNAs encoding photosynthetic proteins. The expectation is that cyanobacterial  
69 membrane-integral proteins should be inserted co-translationally via the Signal Recognition Particle (SRP)-  
70 dependent pathway<sup>17,18</sup>. Essentially all *psbA* mRNA in *Synechocystis* and in *Synechococcus elongatus* PCC 7942  
71 (hereafter *Synechococcus*) is ribosome-associated: translational control is achieved through pausing at distinct  
72 sites<sup>19,20</sup>. Therefore, the location of the mRNA should reveal the site of membrane integration of the protein. We  
73 used a single-molecule RNA-FISH protocol which uses a set of 40-48 short single-stranded DNA probes, each about  
74 20 bases long and with a fluorophore attached to the 3' end<sup>21</sup>. The high sensitivity and specificity of the technique  
75 come from the large number of fluorophores that can be associated with each mRNA molecule, and the need for  
76 multiple probes to hybridise in the same place to produce a fluorescent focus<sup>21</sup>.

77  
78 We selected two unicellular cyanobacterial model species. *Synechocystis* is the most widely-used cyanobacterial  
79 model for photosynthesis research, and its membrane architecture has been intensively studied<sup>6,7</sup>. Except where  
80 otherwise stated, we used the PCC-M variant<sup>22</sup>, which has larger cells than some of the sub-strains. *Synechococcus*  
81 has a different cell architecture, with rod-shaped cells and thylakoid membranes that lack obvious convergence  
82 zones or central extensions<sup>23,24</sup>. The thylakoids approximate to a set of nested concentric cylinders aligned along the

83 long axis of the cell, and their regular conformation is conducive to quantitative microscopy<sup>23,25,26</sup>. Both organisms  
84 have chlorophyll *a* and phycocyanin as their major photosynthetic pigments. We therefore employed oligonucleotide  
85 probes linked to TAMRA (5-Carboxytetramethylrhodamine) whose absorption and emission maxima (respectively  
86 552 nm and 576 nm) are distinct from the photosynthetic pigments.

### 88 **Practical aspects of RNA-FISH in cyanobacteria**

89 RNA-FISH experiments require cell fixation followed by permeabilisation with 70% ethanol before probing<sup>21</sup>. This  
90 treatment removes much of the chlorophyll, but the phycobilins are retained. Phycobilin fluorescence allows  
91 visualisation of the thylakoids in the treated cells, and cell structure as probed by confocal fluorescence microscopy  
92 shows little difference from live cells (Extended Data Fig. 1a-e). The fixed cells retained high background  
93 fluorescence across the spectrum, mostly originating from the thylakoids (Extended Data Fig. 1f,g). Therefore, we  
94 could only detect reliable FISH signals from abundant mRNA species, and imaging required extreme care since  
95 exposure to the excitation light easily photobleached the FISH signal whilst enhancing the background fluorescence.  
96 This issue precluded repeated imaging to obtain z-stacks or to image multiple probes simultaneously. FISH imaging in  
97 cyanobacteria proved less straightforward than in e.g. *Escherichia coli*, where background fluorescence is low  
98 compared to the FISH signal<sup>21</sup>. However, we found that the problems in cyanobacteria could be mitigated because  
99 the background signal in the TAMRA channel is reliably predictable from the thylakoid image in the red channel. This  
100 allows subtraction of the background from images of probed cells to give a cleaner image of mRNA location  
101 (Extended Data Fig. 2).

### 103 **Probing for *psbA* mRNA**

104 We first probed for the highly expressed *psbA* mRNAs encoding the D1 subunit of PSII (Fig. 1,2). We designed probes  
105 against the most highly expressed *psbA* genes (*psbA2* in *Synechocystis*<sup>27</sup> and *psbA1* in *Synechococcus*<sup>28</sup>), but, in both  
106 species, the strong nucleotide sequence conservation within the *psbA* gene families means that the FISH probes  
107 (Supplementary Table 1) must recognise a mixture of *psbA* mRNAs. A *Synechocystis psbA2* null mutant still showed a  
108 *psbA* FISH signal (likely from *psbA3* mRNA<sup>27</sup>), albeit at reduced intensity (Extended Data Fig. 3). We checked for  
109 specificity of the probes for the *psbA* species using a *Synechocystis* triple knockout mutant lacking all three *psbA*  
110 genes<sup>29</sup>. Unlike the wild type (Extended Data Fig. 1-3), this mutant shows occasional concentrations of fluorescence

111 in the TAMRA channel even in unprobed cells, likely reflecting accumulation of pigment precursors or breakdown  
112 products due to perturbed reaction centre biogenesis. However, the signal was not significantly different in probed vs  
113 unprobed cells (Extended Data Fig. 3).

114

115 *psbA* FISH signals were decreased, although not completely abolished, by treatment with the RNA polymerase  
116 inhibitor rifampicin for 1 hour (Extended Data Fig. 4). The residual signal likely reflects slow degradation of some  
117 mRNA following rifampicin treatment. *psbA* mRNAs have relatively long half-lives in *Synechococcus* (18-25 min for  
118 *psbA1*)<sup>28</sup>. The FISH signal increased in high light-treated cells, where *psbA* expression is expected to increase<sup>27,28</sup> (Fig.  
119 2a,b). In some cells, the *psbA* FISH signals were clearly distinct from the location of the nucleoid, as probed with 4',6-  
120 diamidino-2-phenylindole (DAPI) staining (Extended Data Fig. 5). This suggests that the FISH probe does not hybridise  
121 with DNA sequences, as expected since the RNA-FISH protocol does not include a denaturation step to separate  
122 double-stranded DNA<sup>21</sup>.

123

#### 124 **Location of *psbA* mRNA**

125 Cells probed with the *psbA* probe-sets showed concentrations of TAMRA fluorescence with distributions distinct  
126 from the background (Fig. 1,2). Given the low overlap between TAMRA emission and cell absorption, and the  
127 generally low level of light absorption at the single-cell level<sup>30</sup>, distortion of the patterns by fluorescence re-  
128 absorption is not a significant concern. In *Synechocystis*, *psbA* FISH signals were concentrated at the inner surface of  
129 the thylakoid system and around protrusions of the thylakoid system into the central cytoplasm (Fig. 1a). TAMRA  
130 fluorescence in the central cytoplasm was almost invariably associated with such protrusions, which were  
131 identifiable by fluorescence in the red channel from photosynthetic pigments. Fig. 1a shows representative examples  
132 of these fluorescence distributions as observed by confocal microscopy, with line profiles to demonstrate the close  
133 coincidence between concentrations of TAMRA fluorescence and thylakoid membrane signals.

134

135 To investigate the relationship between *psbA* mRNA and thylakoid membrane structures at higher spatial resolution,  
136 we used fluorescence microscopy with super-resolution radial fluctuations (SRRF)-Stream technology<sup>31</sup> (Fig. 1b).  
137 These images have higher spatial resolution than the confocal images, but lower spectral resolution (see Methods),  
138 and we were not able to use background subtraction to remove the background autofluorescence as with the

139 confocal images. Therefore, these images show background autofluorescence in addition to the RNA-FISH signal.  
140 However, the SRRF images confirm the presence in many cells of very fine thylakoid membrane protrusions into the  
141 central cytoplasm, and suggest that these protrusions are frequently associated with concentrations of *psbA* mRNA  
142 (Fig. 1b). We did not observe similar thylakoid protrusions in the thinner and rod-shaped *Synechococcus* cells, where  
143 all the photosynthetic pigment fluorescence comes from regular tubes of thylakoid membrane layers surrounding  
144 the thinner central cytoplasm (Fig. 2). In *Synechococcus* cells grown under our standard conditions, *psbA* FISH signals  
145 were concentrated in localised patches at the thylakoids (Fig. 2 a,d). The regular conformation of the cells and the  
146 thylakoids in *Synechococcus* enables quantitation of distributions and merging data from multiple cells. Fig. 2d shows  
147 line profiles drawn along the long and short axes, merged from 50 cells. The merged long-axis profile reveals a high  
148 frequency of *psbA* mRNA spots towards the inner edge of the thylakoid system near the poles of the cell, although  
149 spots can also be found in other locations (Fig. 2d). The merged short-axis profile shows that *psbA* mRNA is  
150 concentrated near the inner edge of the thylakoid system, with a distinct dip in signal in the central cytoplasm and  
151 no detectable signal at the outer edge of the thylakoids adjacent to the plasma membrane (Fig. 2d). *Synechocystis*  
152 cells also show very little *psbA* FISH signal adjacent to the plasma membrane: the signal is overwhelmingly  
153 concentrated at the inner surfaces of the thylakoid system (Fig. 1).

#### 154 155 ***psbA* mRNA in PSII biogenesis vs. repair**

156 Cyanobacterial *psbA* expression is often connected with PSII repair after photodamage, rather than *de novo*  
157 biogenesis<sup>13</sup>. To get a clearer picture of mRNA location associated with the two processes, we took advantage of the  
158 regular conformation of *Synechococcus* cells to quantify *psbA* mRNA distribution under different conditions. We  
159 induced photodamage by exposure of low light-grown cells to high light (HL) for 1 h. Such treatments are known to  
160 induce high levels of *psbA* mRNA<sup>28</sup>, and as expected, we found that HL-treatment caused a strong increase in the  
161 mean cellular *psbA* signal (Fig. 2b), although expression levels differ markedly in different individual cells (Fig. 2a,b).  
162 Although the *psbA* FISH signal in HL cells was still clearly thylakoid-associated, it appeared more evenly distributed  
163 along the thylakoid surface than in low light cells. To quantify this effect, we used a method previously used to  
164 quantify the patchiness in the distribution of a GFP-tagged thylakoid protein<sup>25</sup>: this involves tracing a line around the  
165 thylakoids in each cell image and plotting fluorescence as a function of distance along the line. Evenly-distributed  
166 fluorescence fluctuates little along the line profile and therefore has a low standard deviation, whereas patchy

167 fluorescence has a high standard deviation<sup>25</sup>. The standard deviation (normalised to the total fluorescence) therefore  
168 provides a quantitative measure of the patchiness of the signal. This analysis demonstrates that HL treatment makes  
169 the *psbA* FISH signal significantly less patchy (Fig. 2c).

### 171 **Influence of ribosomes on *psbA* mRNA location**

172 To explore the role of ribosomes in determining *psbA* mRNA localisation, we carried out FISH measurements on cells  
173 preincubated with lincomycin, which blocks translation elongation<sup>20</sup>, and puromycin, which blocks translation  
174 elongation and additionally releases the mRNA from the ribosome<sup>32</sup>. We confirmed that inhibitor treatments did not  
175 increase the background fluorescence in unprobed cells, and that puromycin at 50  $\mu\text{g ml}^{-1}$  was sufficient to kill all  
176 cells in the culture (Extended Data Fig. 6). In our FISH experiments we used puromycin at 500  $\mu\text{g ml}^{-1}$ . Both inhibitors  
177 induced substantial increases in FISH signal in a subset of the cells (Fig. 1a,c; Fig. 2 d,e). Blocking translation has  
178 previously been shown to increase the stability of *psbA* transcripts<sup>33,34</sup>, and Northern blot analysis confirmed that the  
179 inhibitor treatments increase *psbA2* transcript levels in *Synechocystis* (Extended Data Fig. 7). Both ribosome  
180 inhibitors significantly changed the mRNA distribution, which became less patchy in the treated cells (Fig. 1a,d; Fig  
181 2d,f). In puromycin-treated *Synechocystis*, *psbA* mRNA often appeared to coat the inner edge of the thylakoid  
182 system, whereas lincomycin treatment led to accumulation of the transcript in the central cytoplasm away from the  
183 thylakoids (Fig. 1a). Similar effects were quantifiable in *Synechococcus* (Fig. 2 d,g,h) where both inhibitors caused a  
184 significant increase in the mean distance between the *psbA* FISH signals and the thylakoids (Fig. 2h). However, the  
185 effect of lincomycin was greater (Fig. 2h), and, in lincomycin-treated cells, the short axis line-profiles suggest very  
186 little thylakoid association (Fig. 2g). By contrast, *psbA* mRNA in puromycin-treated cells retains a distinct bias in its  
187 distribution towards the thylakoids (Fig. 2g). The significant effects of the inhibitors indicate that *psbA* mRNA  
188 location is influenced by association with ribosomes, consistent with previous findings that *psbA* mRNA is all  
189 ribosome-associated in cyanobacteria<sup>19</sup>. Nevertheless, a clear association of *psbA* mRNA with the thylakoid inner  
190 surface and protrusions was retained after treatment with a high concentration of puromycin (Fig. 1a; Fig 2d,g,h).

### 192 **Location of *psaA* mRNA**

193 To probe the location of Photosystem I (PSI) biogenesis, we carried out similar FISH experiments with probes  
194 hybridising to the *psaA* part of the *psaAB* transcript encoding the PSI core subunits (Fig. 3; Extended Data Figs 8,9).

195 The *psaA* FISH signals show sharply punctate distributions. As with *psbA* (Figs 1,2), there are concentrations in  
196 discrete patches at the thylakoid inner surface in *Synechococcus* (Fig. 3b), and around the thylakoid extensions in  
197 *Synechocystis* (Fig. 3a). In contrast to their effects on *psbA* transcript levels (Fig. 1,2; Extended Data Fig. 7), both  
198 puromycin and lincomycin strongly decreased the *psaA* FISH signal, suggesting destabilisation of the *psaAB* mRNA  
199 (Fig. 3). Northern blot analysis confirmed this effect (Extended Data Fig. 7). Inhibited cells showed residual FISH  
200 signals in patches at the thylakoid inner surfaces (Fig. 3). Although the low residual signal precluded plotting of  
201 distance distributions as for *psbA* (Fig. 2h), results are consistent with a thylakoid affinity that does not depend on  
202 active translation or ribosome association.

203

#### 204 **Location of other mRNAs**

205 To test whether location at the inner surface of the TM was specific to mRNAs encoding membrane-integral  
206 thylakoid components, we probed the locations of two abundant mRNA species encoding proteins with other  
207 locations: *cpcAB* in *Synechocystis* and *rbcl* in *Synechococcus* (Fig. 4). *cpcAB* encodes the  $\alpha$ - and  $\beta$ -subunits of  
208 phycocyanin, the major protein component of the phycobilisome light-harvesting complexes. These proteins are  
209 water-soluble and associated with the cytoplasmic surface of the thylakoid membrane<sup>35</sup>. *rbcl* encodes the large  
210 subunit of ribulose-1,5-bisphosphate carboxylase, a cytoplasmic enzyme that is mainly packaged into carboxysomes:  
211 icosahedral organelles with a protein shell that are located in the central cytoplasm<sup>36–39</sup>. *cpcAB* mRNA showed a  
212 patchy distribution mainly in the central cytoplasm of *Synechocystis* (Fig. 4a). Comparison of *cpcAB* distribution with  
213 *psbA* and *psaA* confirms closer association of both *psbA* and *psaA* mRNAs with the membrane in *Synechocystis* (Fig.  
214 4b). *psbA* and *psaA* mRNAs showed similar distance distributions, but *cpcAB* mRNA was significantly further from the  
215 thylakoids (Fig. 4b). We probed *rbcl* mRNA in a *Synechococcus* mutant expressing GFP-tagged RbcL<sup>39,40</sup>. The GFP  
216 signal is concentrated in semi-regularly spaced spots in the central cytoplasm which clearly correspond to the  
217 carboxysomes<sup>36</sup> (Fig. 4c). The *rbcl* FISH signal is also found in spots in the central cytoplasm (Fig. 4c). These spots  
218 sometimes coincide with the carboxysomes, but they are much less numerous (Fig. 4c). It is possible that *rbcl* mRNA  
219 co-localises with a sub-population of nascent carboxysomes, but this point needs further investigation. Short-axis  
220 line profiles show that the distribution of *rbcl* mRNA is centred in the middle of the central cytoplasm, similarly to  
221 the RbcL-GFP signal (Fig. 4d) and in contrast to *psbA* (Fig. 2g). The distances of spots of *rbcl* mRNA from the nearest



222 thylakoid (Fig. 4e) show a different distribution from *psbA* and *psaA* (Fig 3e). *psbA* and *psaA* mRNAs are quantifiably  
223 closer to the thylakoids than *rbcl* ( $p = 10^{-6}$  for *psbA* and  $10^{-5}$  for *psaA*).

224

### 225 **Involvement of mRNA-binding proteins**

226 Results in Figs 1-4 suggest that proximity to thylakoid surfaces may be specific to mRNAs encoding membrane-  
227 integral thylakoid proteins, and that thylakoid affinity of these mRNAs can be independent of ribosome association.  
228 This raises the possibility of RNA-binding proteins (RBPs) that might bind specifically to this set of mRNAs and help to  
229 anchor them at the thylakoid membrane. Accordingly, we investigated two putative *Synechocystis* RBPs: Rbp2  
230 (*ssr1480*) and Rbp3 (*slr0193*). Mutants were constructed in the glucose-tolerant *Synechocystis* GT-I background<sup>41</sup> in  
231 which each of these proteins was FLAG-tagged, and pull-downs of the FLAG-tagged protein were probed for *psbA2*  
232 and *psaA* mRNAs. Rbp2 bound *psbA2* mRNA (Fig. 5a) and Rbp3 bound both *psbA2* and *psaA* mRNA, including an  
233 untranslated upstream region resulting from a second transcriptional start-site (Fig. 5b). Biochemical fractionation  
234 showed that Rbp3 is strongly membrane-associated (Extended Data Fig. 10). We then investigated a mutant lacking  
235 Rbp2 and Rbp3 (GT-I:: $\Delta$ *rbp2/3*). This mutant showed some growth perturbation, with a prolonged lag phase after  
236 transfer to higher light intensity and slightly slower growth than wild type in the presence of glucose (Extended Data  
237 Fig. 10). It had perturbed pigment content, with lower pigment per cell and a lower content of PSI relative to PSII  
238 (Extended Data Fig. 10). FISH measurements showed perturbed location of both *psbA* and *psaA* mRNAs in GT-  
239 I:: $\Delta$ *rbp2/3*, with concentrations in the central cytoplasm that appeared detached from the thylakoids (Fig. 5c,f).  
240 Analysis of the distance between the mRNA concentration and the nearest thylakoid confirmed that the location of  
241 both mRNAs was significantly perturbed, but with a stronger effect on *psaA* (Fig. 5i). In addition, cellular levels of  
242 *psaA* mRNA were significantly reduced (Fig. 5g) whereas there was a slight increase in *psbA* mRNA (Fig. 5d).

243

### 244 **Discussion**

245 By probing the subcellular locations of specific mRNA molecules in two species of cyanobacteria, we have shown  
246 that the mRNAs encoding two core membrane-integral photosynthetic proteins (PsbA and PsaA) are concentrated at  
247 the inner surfaces of the thylakoid system adjacent to the central cytoplasm (Fig. 1-3). Biochemical fractionation  
248 indicates that about half of *psbA* mRNA is membrane-associated in *Synechococcus*<sup>42</sup>. From our images we cannot  
249 precisely quantify membrane-associated vs. cytosolic mRNA, but our results indicate that the membrane-associated

250 fraction must be overwhelmingly at the inner surfaces of the thylakoids. Two other mRNA species that encode  
251 proteins with other locations, the mRNAs encoding RbL and phycocyanin subunits, are both found concentrated in  
252 spots in the central cytoplasm and are quantifiably less closely thylakoid-associated than *psbA* and *psaA* mRNAs (Fig.  
253 4).

254

255 The locations of *psbA* and *psaA* mRNAs could in principle reflect either sites of translation or storage locations, or  
256 both. However, it is known that *psbA* mRNA in *Synechococcus* and *Synechocystis* is strongly ribosome-associated<sup>19,20</sup>  
257 and we found that treatment with ribosome inhibitors quantifiably changes the distribution of *psbA* and *psaA*  
258 mRNAs (Fig. 1,2). Lincomycin, which blocks translation elongation but does not detach the mRNA from the  
259 ribosome, induces dissociation of the mRNAs from the thylakoids (Figs 1,2) which is consistent with previous  
260 biochemical studies<sup>20</sup>. The major change in distribution of the mRNAs induced by lincomycin implies that the mRNA  
261 locations we observe reflect sites of active translation and therefore membrane insertion via the SRP-dependent  
262 pathway<sup>17,18</sup>.

263

264 In *Synechococcus*, we were able to quantify differences in *psbA* mRNA location induced by high light treatment (Fig  
265 2). The mRNA was always concentrated at the inner edge of the thylakoid system, but distributions varied in the  
266 extent to which the mRNA was concentrated into localised foci. In cells in standard low light conditions, *psbA* mRNA  
267 was strongly concentrated into a small number of sharp foci (Fig. 2) similarly to *psaA* mRNA (Fig. 3). In cells that had  
268 been subjected to photodamage by a short high light-treatment, *psbA* mRNA levels were higher on average, and the  
269 mRNA was much more evenly distributed over the inner edge of thylakoid (Fig. 2a,c). Under these conditions, PsbA  
270 production is predominantly implicated in PSII repair rather than *de novo* biogenesis<sup>13</sup>, so the result suggests that *de*  
271 *novo* biogenesis is more localised than PSII repair. Overall, our data suggest that the first steps in photosystem  
272 biogenesis take place around the inner surfaces of the thylakoid system, adjacent to the central cytoplasm and the  
273 nucleoid. In *Synechocystis*, our results highlight the importance for biogenesis of thin extensions of the thylakoid  
274 system into the central cytoplasm. *psaA* mRNA is particularly concentrated at these extensions (Fig. 3) but they are  
275 also a major location for *psbA* mRNA (Fig. 1). Similar thylakoid extensions were not apparent in *Synechococcus*,  
276 where translation and insertion occur in discrete foci in the smooth innermost layers of the thylakoid system, with a  
277 preponderance near the cell poles (Figs 2,3). Technical constraints meant that we could not simultaneously probe

278 *psaA* and *psbA* mRNAs, and therefore it remains to be determined whether the same zones are involved in insertion  
279 of components of both photosystems.  
280  
281 The photosynthetic translation zones indicated by our FISH experiments match closely to the locations of thylakoid-  
282 associated ribosomes in *Synechocystis* as observed by electron tomography<sup>6,7</sup>. In a recent cryo-electron tomographic  
283 study, the vast majority of thylakoid-associated ribosomes were found on the inner surfaces of the thylakoid system  
284 adjacent to the central cytoplasm<sup>7</sup>, and an earlier electron tomographic study also highlighted ribosome-covered  
285 extensions of the thylakoid system into the central cytoplasm<sup>6</sup>. Our results do not provide support for suggestions  
286 that photosystem biogenesis takes place initially in the plasma membrane<sup>1,10</sup>, or in thylakoid zones immediately  
287 adjacent to the plasma membrane<sup>7,11,15,16</sup>. However, photosystem assembly is a complex multi-step process<sup>12-14</sup>. Our  
288 data only give information on sites of translation and not on the next steps of photosystem assembly, and we cannot  
289 exclude the possibility that newly-translated proteins could migrate to sites closer to the plasma membrane, where  
290 Mn integration into the PSII water-oxidising complex has been suggested to occur<sup>15,16</sup>.  
291  
292 Our FISH images show that both ribosome inhibitors (Figs 1,2) and HL (Fig. 2, Extended Data Fig. 4) induce markedly  
293 different levels of *psbA* mRNA in different individual cells. The reasons remain to be determined, but it highlights  
294 variations that do not appear in bulk measurements of transcript levels. There are precedents for strong  
295 transcriptional variation among individual bacterial cells<sup>43</sup>.  
296  
297 Strikingly, we found that *psbA* and *psaA* mRNAs remain concentrated near the thylakoid membrane in the presence  
298 of high concentrations of puromycin, which decouples mRNAs from the ribosomes<sup>32</sup> (Figs 1-3). This indicates that  
299 ribosome-uncoupled thylakoid mRNAs can be targeted to the thylakoid surface. There are precedents for such  
300 ribosome-independent mRNA targeting to membranes. In *Escherichia coli*, membrane protein mRNAs show  
301 translation-independent targeting to the plasma membrane<sup>44</sup>. In the chloroplast of the green alga *Chlamydomonas*  
302 *reinhardtii*, several mRNA species, including *psbA*, are localised at specialised translation zones around the pyrenoid  
303 by mechanisms that are partially independent of translation<sup>44</sup>. Translation-independent mRNA localisation in the  
304 eukaryotic cytoplasm influences the eventual destination of proteins<sup>45</sup>. The destination of thylakoid proteins in  
305 cyanobacteria could be strongly influenced by initial targeting of the mRNA to the thylakoid surface, prior to

306 ribosome association (Fig. 6). This may be the key to the unresolved membrane protein sorting problem in  
307 cyanobacteria<sup>17</sup>.  
308  
309 The mechanism of membrane targeting likely involves dedicated RNA-binding proteins that recognise specific  
310 features of the mRNA molecules (Fig. 6). Indeed, we identified two *Synechocystis* RBPs that bind photosynthetic  
311 mRNAs (Fig. 5) and found that a deletion mutant lacking both these RBPs has a significant loss of thylakoid  
312 association of both *psbA* and *psaA* mRNAs (Fig. 5), accompanied by perturbations to the photosynthetic apparatus  
313 and slower growth under some conditions (Extended Data Fig. 10). Our mRNA-FISH data do not clearly show  
314 whether the RBPs simply bind the free mRNA and localise it at the membrane (as illustrated in Fig. 6) or whether  
315 they bind ribosomes and mRNA together. However, a database of protein and mRNA association in fractionated  
316 *Synechocystis* cells<sup>46</sup> suggests that the RBPs that we identified do not associate with the ribosome fraction, favouring  
317 the model shown in Fig. 6. Both these *Synechocystis* RBPs are partial homologs of a *Chlamydomonas* chloroplast RBP  
318 implicated in *psbA* mRNA binding and localisation at the translation zones<sup>47</sup>. This suggests that elements of a  
319 cyanobacterial membrane targeting system mediated by RBPs have been retained during the evolutionary transition  
320 from cyanobacteria to chloroplasts. However, much remains to be determined about the roles of different RBPs in  
321 membrane targeting, the membrane binding partners of the RBPs, and the features of specific mRNAs that they  
322 recognise.

## 323 324 **METHODS**

### 325 **Strains and growth conditions**

326 *Synechocystis* sp PCC 6803 (the motile PCC-M variant<sup>22</sup> or the glucose-tolerant GT-I<sup>41</sup> where specified) and  
327 *Synechococcus elongatus* PCC 7942 cells were grown in BG11 media<sup>48</sup> supplemented with  
328 tris(hydroxymethyl)methylamino propane sulfonic acid (TAPS), pH 8.2 at 30°C under constant low white light (~5  
329  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ ). Liquid cultures were maintained in tissue culture flasks (Sarstedt) with continuous shaking (130  
330 rpm). Cultures were also maintained on BG11 plates containing 1.5% (w/v) Bacto-agar (VWR, UK) supplemented with  
331 0.3% (w/v)  $\text{Na}_2\text{S}_2\text{O}_3$ . For the *Synechocystis*  $\Delta\text{psbA2}$  mutant, media were supplemented with 25  $\mu\text{g ml}^{-1}$   
332 chloramphenicol (Sigma-Aldrich, UK), and for the *Synechocystis* triple *psbA* knockout media were supplemented with  
333 5 mM glucose, 30  $\mu\text{g ml}^{-1}$  chloramphenicol and 5  $\mu\text{g ml}^{-1}$  gentamycin (Sigma-Aldrich, UK), For the *Synechocystis*

334  $\Delta rbp2/3$  mutant, media were supplemented with 10  $\mu\text{g ml}^{-1}$  chloramphenicol and 50  $\mu\text{g ml}^{-1}$  kanamycin (Carl Roth,  
335 Germany). The media were additionally supplemented with 2.5  $\mu\text{g ml}^{-1}$  gentamycin (Carl Roth, Germany) if used for  
336 the mutant strains complemented with genes encoding proteins with a C-terminal triple FLAG-tag under control of  
337 the copper inducible *petE* promoter<sup>49</sup>. The *Synechococcus rbcL-gfp* mutant<sup>39,40</sup> was grown in medium supplemented  
338 with 50  $\mu\text{g ml}^{-1}$  apramycin and prior to FISH measurements it was HL-treated (260  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$  for 30-40 min  
339 at 22 °C) to enhance *rbcL* expression<sup>39,50</sup>. For the affinity pull-downs and RT-qPCR, the mutants complemented with  
340 FLAG-tagged versions of Rbp2 and Rbp3 were cultivated in freshwater media (50 mM  $\text{NaNO}_3$ , 15 mM  $\text{KNO}_3$ , 10 mM  
341  $\text{NaHCO}_3$ , 2.4 mM  $\text{K}_2\text{HPO}_4$ , 2 mM  $\text{MgSO}_4$ , 1.6 mM  $\text{KH}_2\text{PO}_4$ , 0.5 mM  $\text{CaCl}_2$ , 0.15 mM  $\text{FeCl}_3$ , 0.15 mM  $\text{Na}_2\text{-EDTA}$ , 10  $\mu\text{M}$   
342  $\text{MnCl}_2$ , 1  $\mu\text{M}$   $\text{ZnSO}_4$ , 0.1  $\mu\text{M}$   $\text{Na}_2\text{MoO}_4$ , 30 nM  $\text{CoCl}_2$ , 25 nM  $\text{H}_3\text{BO}_3$ ) in a CellDeg system (CellDEG, Germany). The  
343 cultures were supplied with 15 ml  $\text{min}^{-1}$  10 %  $\text{CO}_2$  and shaken at 30 °C. For the first 48h the illumination intensity was  
344 set at 130  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , then increased to 250-300  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  for the next 24h and then set to a  
345 final illumination intensity of 700  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . The cultures were inoculated to an  $\text{OD}_{750}$  of 0.5 and protein  
346 expression was induced at an  $\text{OD}_{750}$  of 8-10 by addition of 2  $\mu\text{M}$   $\text{CuSO}_4$  for 24h.

347

## 348 **Construction of mutants**

349 The  $\Delta psbA2$  mutant was generated in the *Synechocystis* wild type (PCC-M)<sup>22</sup> background by replacing the entire  
350 *slr1311* (*psbA2*) gene sequence with the *cmIA* gene conferring chloramphenicol resistance ( $\text{Cm}^R$ ).  $\text{Cm}^R$  was  
351 introduced into the genome using a vector (pGEM-T Easy) carrying ~500 bp of *slr1311* flanking sequences either side  
352 of  $\text{Cm}^R$  to assist double homologous recombination. The plasmid vector was generated by Gibson assembly<sup>51</sup> using  
353 the NEBuilder HiFi DNA assembly master mix (NEB, UK). Primers used to amplify the DNA fragments are listed in  
354 Supplementary Table 2. NEB5-alpha competent *E. coli* cells (NEB, UK) were used to clone the plasmid. *Synechocystis*  
355 transformation was done following the protocol described by Clerico *et al*<sup>52</sup>. Successful transformation and full  
356 segregation were confirmed by colony PCR (Supplementary Fig. 3). Since the  $\text{Cm}^R$  gene sequence (763 bp) is smaller  
357 than *psbA2* (1083 bp), a second PCR was carried out to confirm the full segregation status of the mutant. For this  
358 PCR, a primer was designed to bind within the *psbA2* sequence. Along with the reverse primer of the *psbA2*  
359 downstream sequence, this *psbA2* sequence-specific primer generated an amplicon from the wild type, which was  
360 absent in the mutant after full segregation (Supplementary Fig. 3).

361 The *Δrbp2* disruptant was generated in the *Synechocystis* GT-I background<sup>41</sup> by replacing the entire target gene  
362 sequence with the drug resistance marker genes. The up- and downstream regions of the gene (*rbp2: ssr1480*) and  
363 the chloramphenicol resistance marker gene were amplified by PCR with respective primers (Supplementary Table  
364 3). Amplified fragments were mixed and fused by recombinant PCR. For the construction of the *rbp3* (*slr0193*)  
365 disruptant, the DNA fragment containing the flanking regions of *rbp3* and a kanamycin-resistant gene was amplified  
366 from the genomic DNA of *rbp3* mutant (provided by Prof. Masahiko Ikeuchi). The resulting PCR products were used  
367 for the transformation of *Synechocystis* GT-I. Complete segregation was confirmed by PCR using the appropriate  
368 primers (Supplementary Table 3).

369 *Δrbp* mutant strains complemented with C-terminal triple FLAG-tagged protein versions under control of the copper  
370 inducible promoter *petE* were generated by PCR amplification of the *rbp* genomic regions including their full 5' and  
371 3'UTRs, flanked by 5' *HindIII* and 3' *XhoI* restriction sites and followed by blunt end ligation into pJet1.2 (CloneJET PCR  
372 cloning kit, Thermo Fisher Scientific). The constructed vector was subjected to multiple steps of inverse PCR to  
373 introduce the bacteriophage lambda *oop* transcription terminator downstream of the 3'UTR of the respective gene  
374 and the triple FLAG tag upstream of the stop codon, followed by *DpnI* digestion (Thermo Fisher Scientific), 5'  
375 phosphorylation (T4 polynucleotide kinase, Thermo Fisher Scientific), self-ligation (T4 DNA ligase, Thermo Fisher  
376 Scientific) and heat-shock transformation into chemically competent *E. coli* DH5 $\alpha$  cells. Hereafter, the native  
377 promoter was replaced with the *petE* promoter via AQUA-cloning<sup>53</sup> and the final constructs were ligated into the  
378 multi-host vector pVZ322 using the *HindIII* and *XhoI* restriction sites and verified by Sanger-sequencing (GATC,  
379 Eurofins, Germany). Aliquots of 60  $\mu$ L electrocompetent *Synechocystis* cells in 1 mM HEPES buffer pH 7.5 were  
380 transformed with 1  $\mu$ g pVZ322 plasmid DNA by electroporation with 2.5 kV for 4 ms<sup>54</sup> (MicroPulser, Bio-Rad, 2 mm  
381 electrode distance), resuspended in 50 ml BG11, incubated at 30°C for 24 h, harvested and plated on BG11 agar  
382 plates, supplemented with 1  $\mu$ g ml<sup>-1</sup> gentamycin (Carl Roth, Germany) (Primer: Table S4).

383

#### 384 **mRNA-FISH (mRNA-Fluorescent *in situ* hybridisation)**

385 mRNA probing was done based on the mRNA-FISH protocol described by Skinner *et al*<sup>21</sup> with some modifications.  
386 For each of the target mRNAs, a set of 40-48 oligonucleotide probes (each 20 nucleotides long) was designed using  
387 free online software: the Stellaris RNA FISH Probe Designer program (<https://www.biosearchtech.com/stellaris->

388 designer). The probes were generated against the target transcript sequence, having a minimum of 2 bp inter-probe  
389 separation and a GC content of around 50%. The probe set was purchased from LGC Biosearch Technologies  
390 (California, USA and Risskov, Denmark), pre-labelled with a TAMRA (5-Carboxytetramethylrhodamine) fluorophore  
391 at the 3' end of each oligonucleotide. The probe sets used for this study are listed in Supplementary Table 1.

392 For the optimal detection of the photosynthetic transcripts, cultures were grown in liquid BG11 media with a starting  
393 OD (optical density) at 750 nm of 0.2. The OD of the culture was routinely measured with a UV-1800  
394 spectrophotometer (Shimadzu). Cells were collected for mRNA-FISH when the OD<sub>750</sub> reached between 0.4 and 0.6.  
395 For each sample roughly  $3 \times 10^8$  cells were used, as calculated using a conversion factor obtained by counting cells in  
396 a haemocytometer. Cells were harvested by centrifugation (3,000xg, 6 minutes) and fixed immediately by treating  
397 with 1 ml PBS (Phosphate-Buffered Saline) containing 3.7% (vol/vol) formaldehyde at room temperature for 30 min.  
398 Cells were washed twice with 1 ml PBS and collected by centrifugation at 6,000xg for 1.5 min. The fixed cells were  
399 permeabilized with 70% ethanol for 1 h at room temperature<sup>21</sup>. Ethanol washed away most of the chlorophyll from  
400 the cell, which helps to reduce the background signal in the TAMRA detection channel. To achieve an even  
401 background signal from all the cells studied, an additional 2 h of 70% ethanol incubation was performed at 4 °C in  
402 dark. Then, cells were washed with 1 ml of 40% (w/vol) formamide, 2× SSC (Saline Sodium Citrate). The washed cell  
403 pellet was collected by centrifugation at 6,000xg for 1.5 min and resuspended in 25 µl hybridization buffer  
404 containing the probe set at 5 µM. The final concentration of the hybridisation buffer was adjusted to 40% (w/vol)  
405 formamide, 2× SSC, 2.5 mg dextran sulphate sodium salt, 25µg *E. coli* tRNA and 10 nM Ribonucleoside Vanadyl  
406 Complex. Hybridisation reactions were carried out overnight at 30 °C in the dark. Cells were then washed three times  
407 using 200 µl of 40% (w/v) formamide, 2× SSC at 30°C with 30 minutes incubation in between each wash. The cell  
408 pellets were finally re-suspended in 50-200 µl of imaging buffer (2X SSC). Cell suspensions were spotted onto a 1.5%  
409 agarose plate (Low melt agarose, dissolved in 1X PBS). Small blocks of agar with the dried spots were cut out,  
410 mounted on a glass coverslip and placed in a custom-built sample holder for confocal imaging.

#### 411 **Pre-treatments before mRNA-FISH**

412 When required, cells were treated with the inhibitors puromycin, lincomycin or rifampicin before fixation. After  
413 collecting the desired quantity of cells for mRNA-FISH, the cell pellet was resuspended in 1 ml of fresh BG11 medium  
414 containing either puromycin (500 µg ml<sup>-1</sup>), lincomycin (400 µM) or rifampicin (500 µg ml<sup>-1</sup>) and incubated for 1 h at

415 room temperature in ambient light ( $\sim 5 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ ). For high-light (HL) treatment, the cell pellet was  
416 resuspended in 1 ml BG11 medium, transferred to a 1.5 ml microfuge tube and incubated at  $600 \mu\text{mol photons m}^{-2}\text{s}^{-1}$   
417 white light for 1h at  $30^\circ\text{C}$ . Cells were fixed immediately after the pre-treatment, as described in the mRNA-FISH  
418 section. Inhibitor experiments shown are representative of 2-3 full biological replicates.

#### 419 **Affinity pull-downs and RT-qPCR**

420 The *Synechocystis* cultures were crosslinked with 0.1 % (v/v) formaldehyde for 15 min, The reaction was stopped by  
421 addition of 125 mM glycine and incubation for 5 min. Afterwards, the cultures were washed twice with ice cold TBS  
422 (150 mM NaCl, 50 mM Tris-HCl, pH 7.5) and the pellet was resuspended in 800  $\mu\text{l}$  of FLAG buffer (50 mM  
423 HEPES/NaOH pH 7, 5 mM  $\text{MgCl}_2$ , 25 mM  $\text{CaCl}_2$ , 150 mM NaCl, 10% [w / v] glycerol, 0.1 % Tween20) containing  
424 protease inhibitor (cOmplete, Roche) and RNase inhibitor (RiboLock, Thermo Fisher Scientific). The cells were  
425 mechanically disrupted with glass beads using an MM 400 (Retsch, Germany) cell disruptor. The lysate was cleared  
426 from glass beads and unbroken cell debris by centrifugation at  $4^\circ\text{C}$  and the cleared supernatant was subjected to  
427 affinity purification using 50  $\mu\text{l}$  packed gel volume of FLAG M2 magnetic beads (Sigma Aldrich, Germany) and  
428 incubated for 1 h at  $4^\circ\text{C}$  on a rotation device. The flow-through was separated from the beads using a DynaMag-2  
429 device (Thermo Fisher Scientific). Afterwards, the beads were washed six times with 20 fold packed gel volume FLAG  
430 buffer + protease inhibitor and proteins were eluted by incubating twice with a 5 fold packed gel volume of  $100 \mu\text{g}$   
431  $\mu\text{l}^{-1}$  3xFLAG Peptide (Sigma Aldrich) for 30 min with low shaking. Both elution fractions were concentrated using  
432 Amicon<sup>®</sup> Ultra 0.5 10K (Merck) concentrator columns. For subsequent RNA purification, eluates were digested by  
433 proteinase K (5 % SDS, 50 mM Tris / HCl pH 7.5 and 2.5 mg/ml proteinase K) for 30 min at  $30^\circ\text{C}$ , followed by addition  
434 of PGTX buffer<sup>55</sup> and incubation at  $65^\circ\text{C}$  for 15 min. Phase separation was performed by addition of  
435 chloroform/isoamyl alcohol (24:1). Afterwards, the aqueous phase was mixed 1:1 with 100 % ethanol and further  
436 RNA purification and DNase treatment was performed using the RNA-Clean and concentrator-5 kit (Zymo Research,  
437 Germany) according to the manufacturer protocol. For the reverse transcription reaction, the QuantiTect Reverse  
438 Transcription kit (Qiagen, Germany) was used. Reaction mixtures without addition of reverse transcriptase (no RT)  
439 served as negative control. The qPCR was performed in a 7500 Fast Real-Time PCR system (Applied Biosystems) using  
440 the Power SYBR Green PCR master mix (Applied Biosystems). All reactions were performed in triplicate for each of  
441 two biological replicates. All samples were tested for the presence of residual DNA during quantitative real-time PCR  
442 with an RT-minus control. The RT-qPCR data were analyzed using the 7500 software version 2.3. As endogenous



443 standard the RNase P RNA (*rnpB* gene) was used. *Synechocystis* cultures expressing FLAG-tagged sGFP were used as  
444 references samples for the relative quantity and therefore the relative quantity of all GFP samples were set to 1  
445 (primers: Table S5).

446

#### 447 **Chlorophyll/OD<sub>750</sub> ratio measurement, spectroscopy and growth curves**

448 Chl concentration from whole cells was determined by diluting samples by a factor of 10 in 100% methanol,  
449 incubating at 68 °C for 15 minutes and pelleting insoluble material by centrifugation. Chl concentration was then  
450 calculated from the absorbance at 665 nm (UV-1800 spectrophotometer, Shimadzu) using an extinction coefficient  
451 of 12.7 mM<sup>-1</sup>cm<sup>-1</sup><sup>56</sup>. The Chl/OD ratio was obtained by dividing Chl concentration by OD<sub>750</sub> measured for the culture  
452 in the same spectrophotometer. Cell absorption spectra were measured for suspensions at 3-4 μM Chl *a* with a  
453 modernised Aminco DW-2000 UV/Vis spectrophotometer (Olis, USA). Spectra were normalised to the  
454 reading at 750 nm. Fluorescence emission spectra were recorded at 77K with a Perkin-Elmer LS55  
455 luminescence spectrometer equipped with a liquid nitrogen housing. Cells were harvested from  
456 exponential-stage cultures and resuspended to 5 μM Chl in BG11 medium. Cells were then loaded into  
457 silica capillary tubes and dark-adapted for 5 min before freezing by plunging into liquid nitrogen. Spectra  
458 were recorded for the frozen samples with excitation at 435 nm or 600 nm and emission at 620-750 nm.  
459 Excitation and emission slit-widths were 5 nm. Emission spectra were corrected for the instrument spectral  
460 response and normalised to the PSI or phycobilin fluorescence peaks after subtracting the background  
461 signal. For growth curves, technical triplicates of the *Synechocystis* cultures were grown as in "Strains and  
462 growth conditions" above except that light intensities were 50 μmol photons m<sup>-2</sup>s<sup>-1</sup> (low light) and 80 μmol  
463 photons m<sup>-2</sup>s<sup>-1</sup> (high light), in the presence of 10 mM glucose where specified. Optical density at 750nm  
464 was measured using a Genequant 1300 (Biochrom).

#### 465 **Confocal microscopy and image processing**

466 Images were recorded with a Leica TCS-SP5 laser scanning confocal microscope equipped with a 63X oil-immersion  
467 objective (numerical aperture 1.4). The confocal pinhole was set to give a section thickness in the z-direction of  
468 ~0.72 μm and all images were recorded as a single slice at the same z-position. Images were recorded in 12-bit, 1024

469 x 1024 pixel format and acquired with 16x line averaging at 400 Hz line scan speed. Each pixel was 24 x 24 nm. For  
470 RNA-FISH samples, excitation was with a 561 nm laser source. Photosynthetic pigments were detected with an  
471 emission window of 660-700 nm, and TAMRA was detected at 565-580 nm. Live cells were imaged with excitation at  
472 488 nm and emission 670-720 nm. Microscope control and image acquisition used Leica LAS-AF software. In all  
473 measurements, we were careful to avoid saturation of the fluorescence signal in any pixels.

474 Image processing was with the Fiji ImageJ package<sup>57</sup>. Image analysis was done after smoothing the images (below  
475 optical resolution) by blurring over a 2x2 pixel window and correcting the TAMRA channel for background  
476 autofluorescence. This was done by measuring the fluorescence in the 565-580 nm detection window relative to the  
477 signal in the 660-700 nm window for unprobed cells. The 660-700 nm image for probed cells was then multiplied by  
478 this ratio to predict the background autofluorescence image at 565-580 nm. This background image was subtracted  
479 from the observed image for probed cells to eliminate the background fluorescence. Cellular RNA-FISH signal  
480 intensities were measured using cell boundaries determined by thresholding the thylakoid (red) channel. Cells not  
481 completely in the field of view were excluded, and cells that were in contact or undergoing division were handled  
482 manually. To analyse the patchiness of the RNA-FISH signal in *Synechocystis*, cells were segmented into thylakoid and  
483 cytoplasmic regions using a level-sets plugin of Fiji ImageJ. For *Synechococcus*, patchiness was analysed by extracting  
484 a fluorescence profile from a line (8 pixels wide) drawn around the boundary between the thylakoid zone and the  
485 cytosol. In both cases, the standard deviation of the signal was measured and normalised to mean fluorescence  
486 intensity. Standard deviation and positional analyses included all cells found completely within the field of view that  
487 had a detectable FISH signal. Intensity analyses included all cells. For image presentation, the brightness of the  
488 images was adjusted at the same level for all parallel samples. Statistical significance was assessed from *p*-values  
489 obtained from two-tailed Student's *t*-tests, carried out with Microsoft Excel software.

#### 490 **Super-resolution imaging**

491 Super-resolution spinning disc confocal fluorescence imaging was performed on a Dragonfly microscope (Andor)  
492 using super-resolution radial fluctuations (SRRF)-Stream technology<sup>31</sup>. A 63x oil-immersion objective (numerical  
493 aperture 1.46) and excitation at 561 nm were used. Applied settings for SRRF were: SRRF frame count=100, SRRF  
494 radiality magnification=4, SRRF ring radius=1.00 px, SRRF temporal analysis=mean, symmetrical binning 1x1. The  
495 confocal pinhole was set to 40  $\mu$ m. TAMRA fluorescence was detected using a 600 nm filter (bandwidth 50 nm) and

496 native pigment fluorescence was detected using a 700 nm filter (bandwidth 75 nm). Images were processed with the  
497 FIJI image processing package<sup>57</sup>.

#### 498 **Northern Blot analysis**

499 RNA was isolated according to Pinto *et al.*<sup>55</sup>, separated on a denaturing 1.3% agarose gel and blotted onto Roti-Nylon  
500 plus membrane (Carl Roth, Germany). *In vitro* transcription of PCR fragments with the Ambion T7 polymerase  
501 maxiscript kit (Thermo Fisher Scientific, Germany) and [ $\alpha$ -<sup>32</sup>P]-UTP (Hartmann Analytics, Germany) was used to  
502 generate radioactively-labeled RNA probes for the 5' UTR of *psbA2* mRNA and the control RNA RnpB. A PCR fragment  
503 covering the *psaA* sequence was labeled using the Rediprime II DNA labeling system (GE Healthcare Life Sciences).  
504 Hybridization signals were detected on a Typhoon FLA4500 imaging system (GE Healthcare) and quantified using  
505 Quantity One software (Bio-Rad Laboratories, Germany). Primers used to amplify PCR products for labeling  
506 reactions are given in Supplementary Table 6.

#### 507 **Antiserum against *Synechocystis* Rbp3 protein.**

508 For the expression of 6×His-tagged Rbp3 protein, the PCR-amplified *rbp3* gene was cloned into pETNH vector<sup>58</sup> using  
509 In-Fusion Cloning kit (TaKaRa, Shiga, Japan). After verification of the sequences, the plasmids were introduced into *E.*  
510 *coli* Rosetta competent cells (TaKaRa) and used for the purification of recombinant Rbp3 proteins as follows. The  
511 resulting *E. coli* strain was grown in 100 ml of LB medium at 37°C. When the culture reached an OD<sub>600</sub> of 0.5, IPTG  
512 was added to medium at a final concentration of 1 mM and the growing temperature was shifted to 20°C. After 24 h,  
513 cells were harvested by centrifugation, washed with a purification buffer (20 mM Tris-HCl (pH 8.0 at 4°C) and 250  
514 mM NaCl), and stored at -80°C until use. For protein purification, frozen cells were suspended in 5 ml of purification  
515 buffer containing 1 mM PMSF. The cells were disrupted by sonication and centrifugated at 15,000 × g for 30 min at  
516 4°C, the resulting supernatant was mixed with 1 ml of Ni<sup>2+</sup>-NTA agarose resin (QIAGEN) equilibrated with purification  
517 buffer and loaded onto a column. After washing by 10 ml of washing buffer 1 [20 mM Tris-HCl (pH 8.0), 250 mM  
518 NaCl, and 5 mM imidazole] and 50 ml of washing buffer 2 [20 mM Tris-HCl (pH 8.0 at 4°C), 250 mM NaCl, and 20 mM  
519 imidazole], proteins were then eluted with 10 ml of an elution buffer [20 mM Tris-HCl (pH 8.0 at 4°C), 250 mM NaCl,  
520 and 200 mM imidazole], and dialyzed against the dialysis buffer [20 mM HEPES (pH 7.5), 5 mM MgSO<sub>4</sub>, 1 mM EDTA,  
521 0.0001% (w/v) BSA, 0.05% Tween 20, 150 mM NaCl, and 30% Glycerol]. The purity of the protein was confirmed by

522 SDS–PAGE, and a polyclonal antibody to the protein was generated by immunising a rabbit (Eurofins, Ebersberg,  
523 Germany).

#### 524 **Cell fractionation and Western blotting.**

525 Exponential phase cells of *Synechocystis* PCC-M strain were harvested and subjected to fractionation according to  
526 the procedure described previously<sup>20</sup> except for the polysome isolation. After the fractionation, crude extracts were  
527 prepared using 10% trichloroacetic acid as described previously<sup>59</sup>. 25 µg and 15 µg of samples were analysed by  
528 Western blotting using anti-Rbp3 and anti-Rbcl (Agrisera) antibodies as the primary antibody (dilution 2000x), and  
529 HRP-conjugated anti-rabbit IgG antibody (GE Healthcare) as the secondary antibody (dilution 10000x).

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657 **DATA AVAILABILITY**

658 The datasets analysed during the current study are available from the corresponding author on reasonable request.

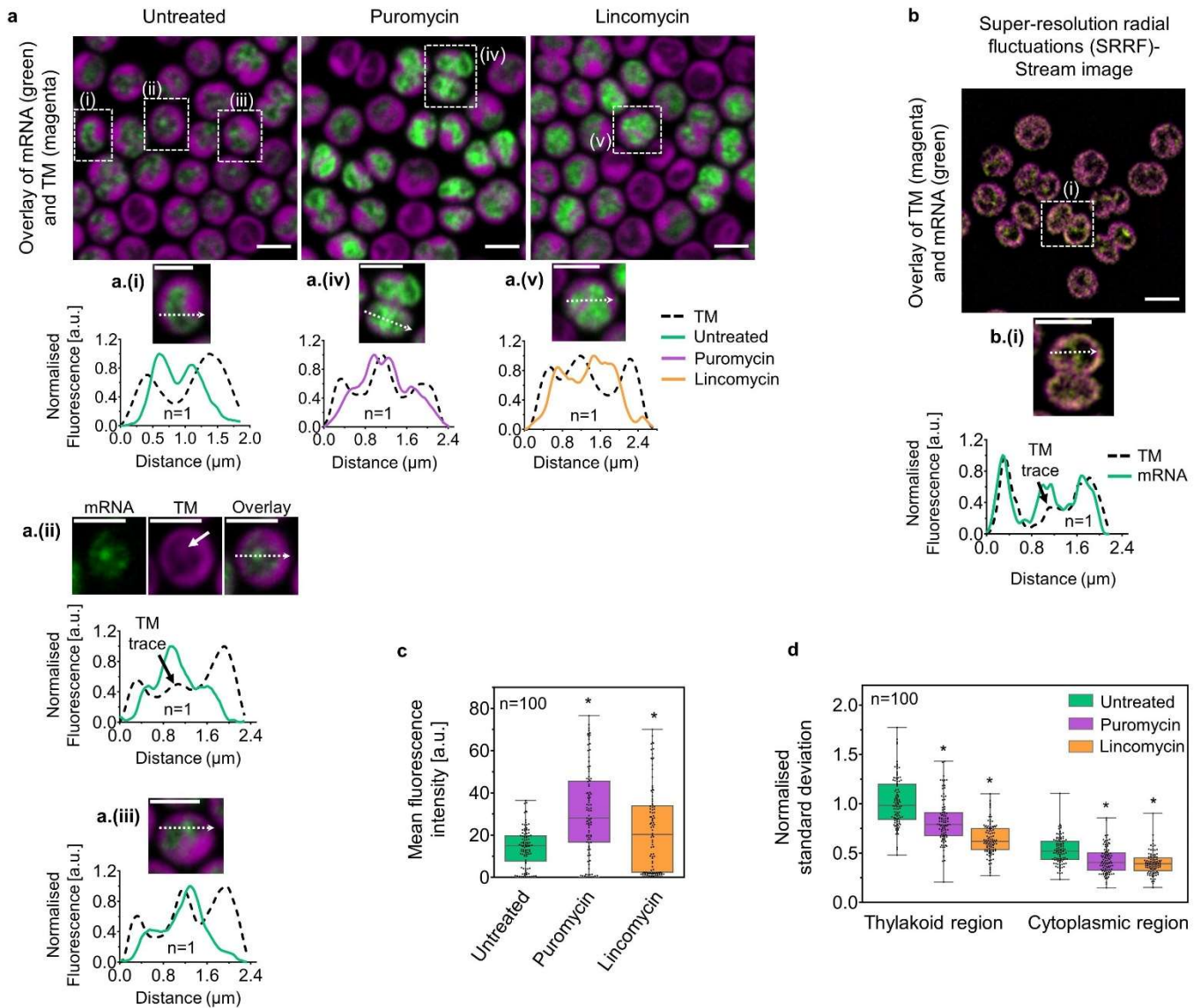
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668  
669 **AUTHOR CONTRIBUTIONS**

670 MM carried out the bulk of the experimental work and data analysis. LH, MR, SW and WRH generated and  
671 characterised RBP mutants and analysed RBP-mRNA association. YY and RK carried out *cpcAB* and *rbcl* FISH  
672 measurements. HC assisted with data analysis. CE helped to establish the RNA-FISH technique in the lab and  
673 discussed data. TH and L-NL performed super-resolution microscopy and data analysis and provided the *rbcl-gfp*  
674 mutant. AW designed and analysed Northern Blot hybridisation experiments and discussed data. CWM and MM  
675 devised the study and wrote the paper, with input from all authors.





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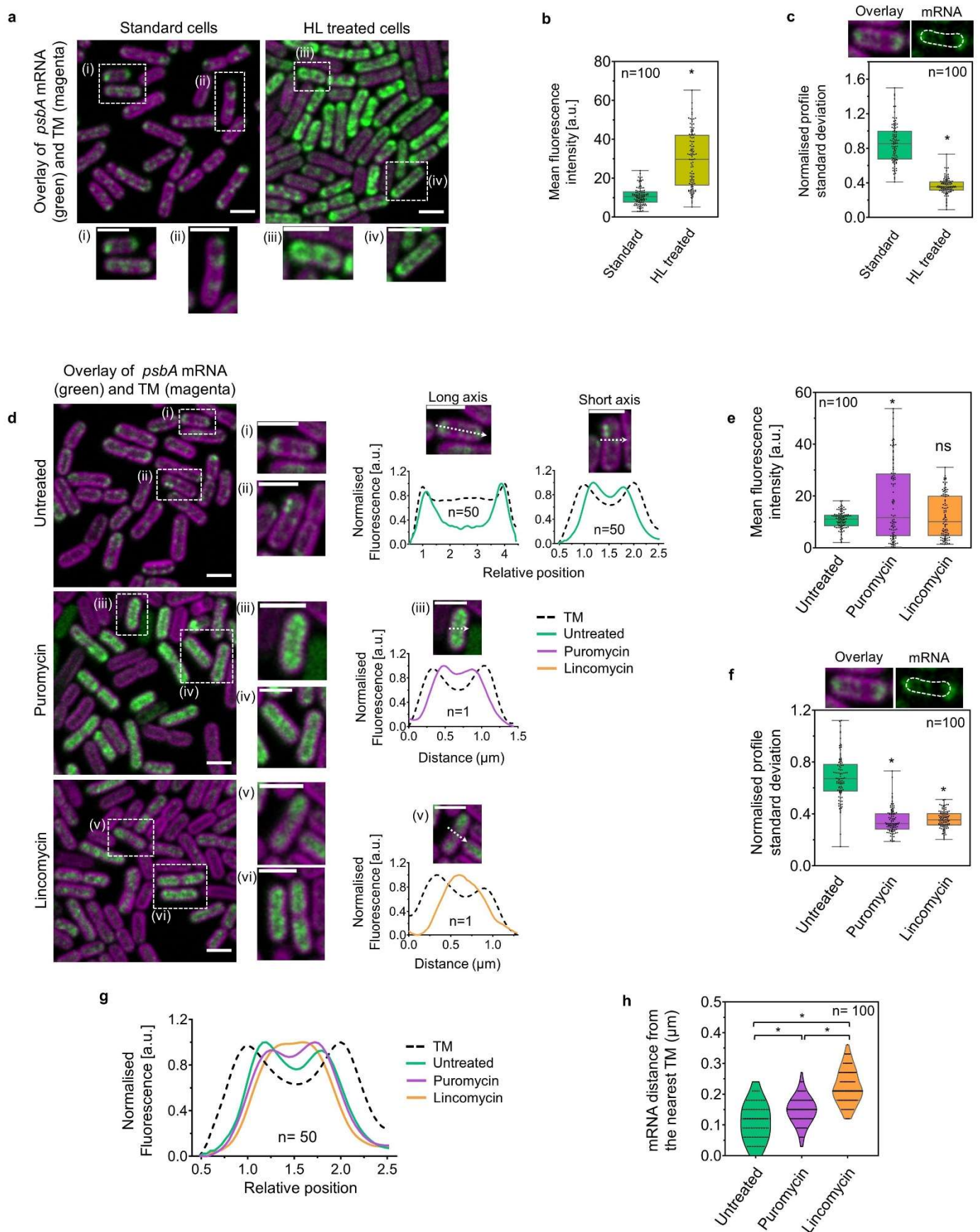
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**Figure 1: Location of mRNAs encoding PsbA proteins in *Synechocystis*, with effects of ribosome inhibitors. a,** *Synechocystis psbA* mRNA (green) relative to thylakoid membrane (magenta) with and without pre-exposure to puromycin and lincomycin. Representative cells (**a, i-v**) are shown in enlarged form below the corresponding micrographs. Line profiles from the representative cells (**a, i-v**) show location of transcript signal relative to thylakoid membrane (TM). **b,** Super-resolution fluorescence image showing the localisation of *psbA* mRNA relative to TM. Line profile of a representative cell (**b, i**) shows a TM projection (arrow) coinciding with the mRNA peak in the middle of the cell. **c,d,** Quantitation of inhibitor effects on *psbA* mRNAs, showing relative FISH signal per cell (**c**:  $p=2 \times 10^{-11}$  for puromycin vs untreated,  $3 \times 10^{-4}$  for lincomycin vs untreated) and the patchiness of its subcellular distribution, assessed by the normalised standard deviation in the FISH signal (**d**: in thylakoids  $p=2 \times 10^{-8}$  for puromycin vs. untreated,  $4 \times 10^{-27}$  for lincomycin vs untreated; in cytoplasm  $p=5 \times 10^{-7}$  for puromycin vs. untreated,  $4 \times 10^{-12}$  for lincomycin vs untreated). Error bars in the box plots indicate the range of values recorded, the centre line shows the

688 median and the box spans the interquartile range. n: number of cells measured, \*: significant difference from the  
689 untreated cells, at  $p < 0.001$ , measured by unpaired two-tailed Student's t-test. All scale bars:  $2\mu\text{m}$ .  
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**Figure 2: Distribution of *psbA* mRNA in *Synechococcus* under different conditions. a-c, Abundance and**

distribution of *psbA* mRNA, comparing standard conditions with high light-treated cells (HL,  $600 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ ,

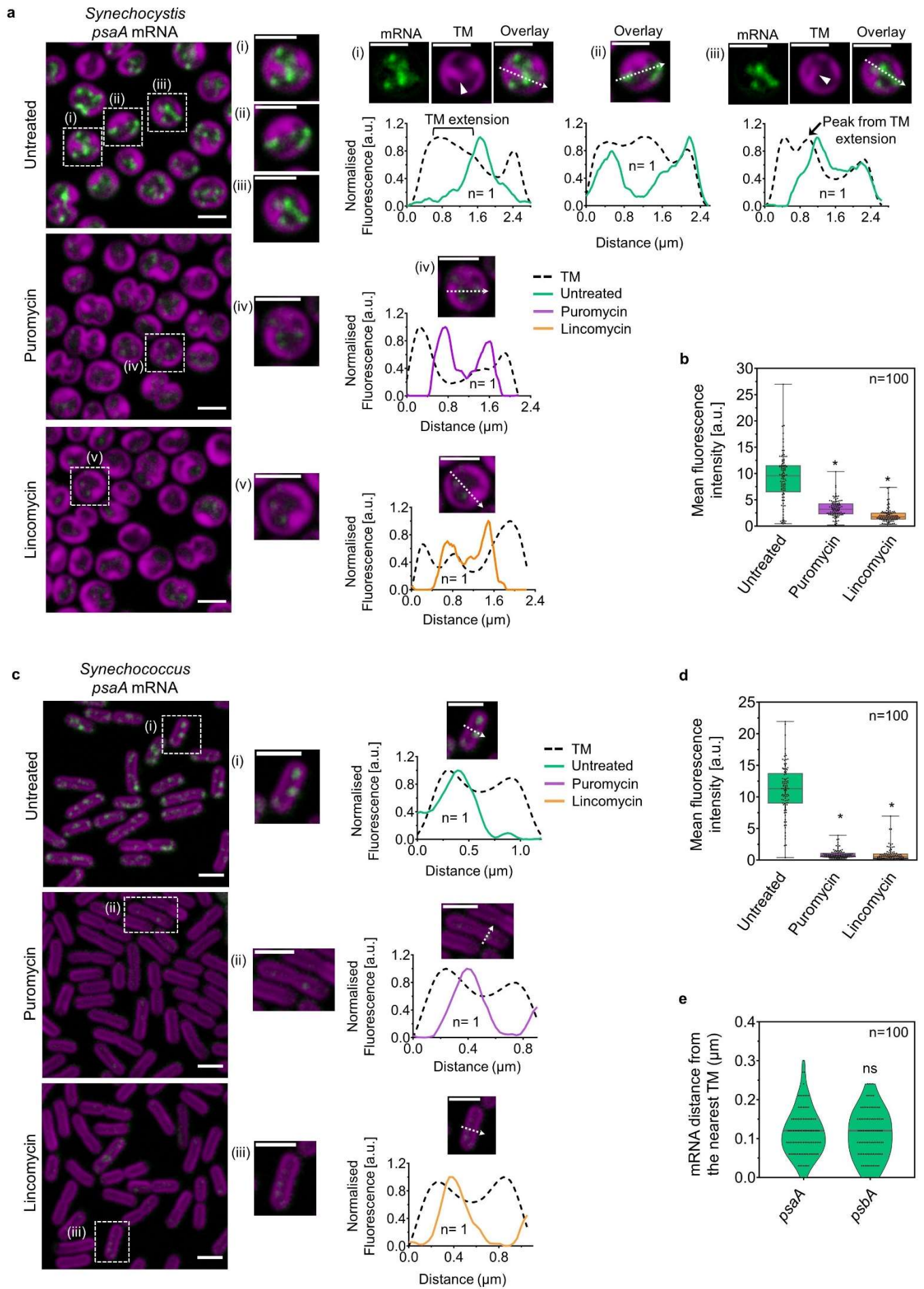
1h); **a**, Micrographs of the cells showing mRNA in green and thylakoid membrane (TM) in magenta; **b**, relative mRNA

signals per cell ( $p=8 \times 10^{-15}$ ); **c**, Patchiness of mRNA distribution, assessed by measuring the standard deviation in the

696 FISH signal along a profile drawn around the TM as shown ( $p=2\times 10^{-40}$ ); **d-h**, Effects of ribosome inhibitors; **d**,  
697 Fluorescence micrographs: representative cells are shown enlarged at the right of corresponding micrograph. Line  
698 profiles from each of the samples are presented next to the enlarged micrographs, showing mRNA signal relative to  
699 TM. For untreated cells, long-axis and short-axis line profiles are averaged from 50 cells; for puromycin and  
700 lincomycin, line profiles next to the enlarged micrographs are drawn from a single representative cell; **e,f**,  
701 Quantitation of inhibitor effects showing relative FISH signal intensity ( $p=10^{-5}$  for untreated vs puromycin, 0.18 for  
702 untreated vs lincomycin) (**e**) and the patchiness of the mRNA distribution ( $p=2\times 10^{-29}$  for untreated vs puromycin,  
703  $2\times 10^{-28}$  for untreated vs lincomycin) (**f**). **g**, averaged line profiles across the short axis of the cells showing effects of  
704 inhibitors on the subcellular distribution of mRNA; **h**, Distance of mRNA from the nearest TM. Peak-to-peak distances  
705 measured from short-axis line profiles ( $p=3\times 10^{-6}$  for untreated vs puromycin,  $8\times 10^{-30}$  for untreated vs lincomycin,  
706  $7\times 10^{-21}$  for puromycin vs lincomycin). The thick line at the middle of each violin plot shows the median. Error bars in  
707 the box plots indicate the range of values recorded, the centre line shows the median and the box spans the  
708 interquartile range. n: number of cells measured for b, c, e, f and number of mRNA peaks measured for h, \*:  
709 significant difference from the condition shown at the left of the plot, at  $p<0.001$ , measured by unpaired two-tailed  
710 Student's t-test; ns:  $p$ -value non-significant. All scale bars: 2  $\mu\text{m}$

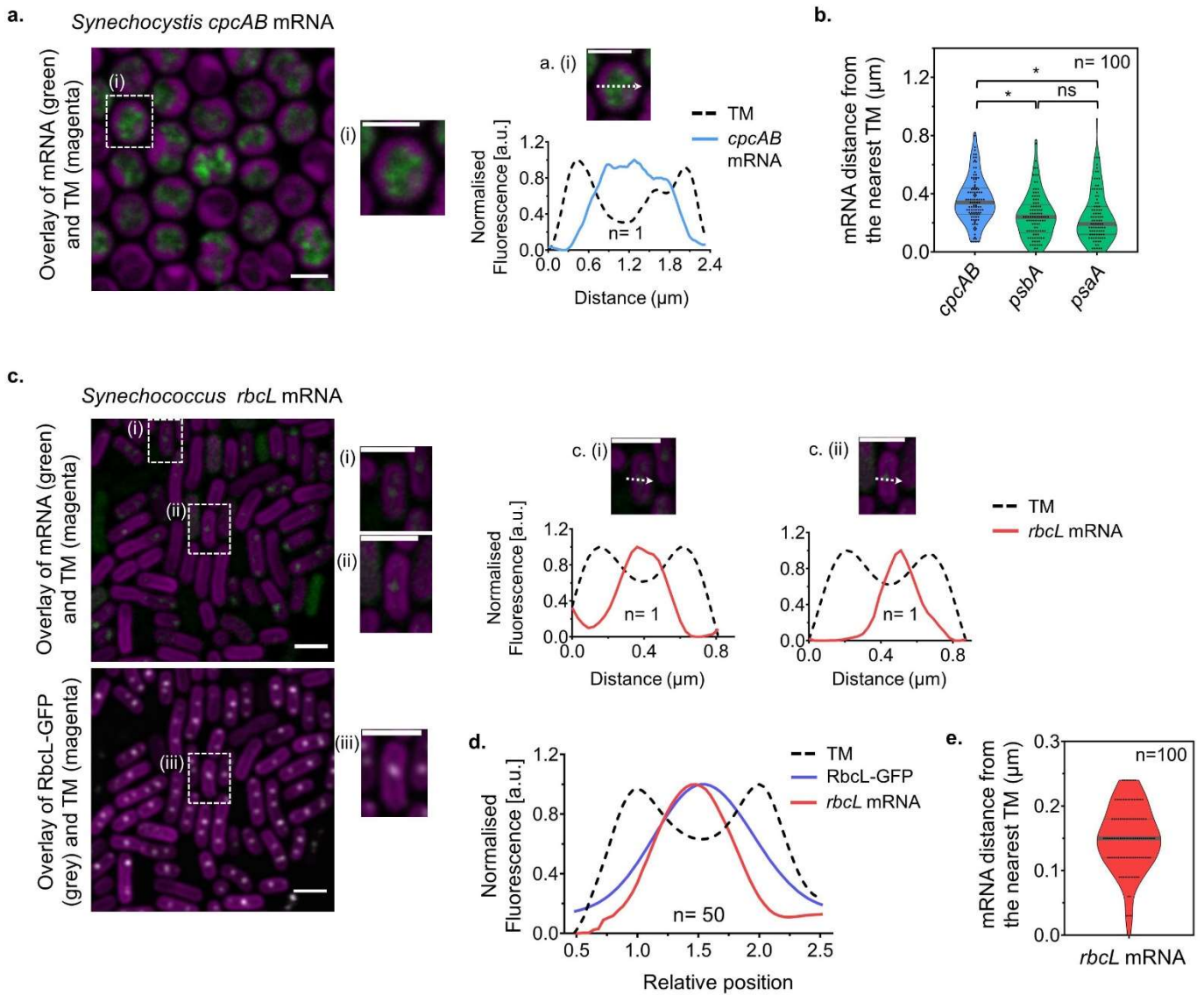
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713 **Figure 3: Location of mRNAs encoding PsaA proteins in *Synechocystis* and *Synechococcus*, with effects of ribosome**  
714 **inhibitors. a,** *psaA* mRNA FISH signal (green) in *Synechocystis* relative to thylakoid membrane (magenta) ± inhibitor  
715 pre-treatment. Representative cells (a, i-v) are shown in enlarged form at the right of the corresponding  
716 micrographs. Line profiles from the representative cells (a, i-v) show location of transcript signal relative to thylakoid  
717 membrane (TM). Thylakoid extension towards the cytoplasm is marked with arrowheads **b,** Quantitation of effects of  
718 inhibitors on the mean FISH signal per cell in *Synechocystis* ( $p=4\times 10^{-21}$  for puromycin vs untreated,  $2\times 10^{-27}$  for  
719 lincomycin vs untreated). **c,** *psaA* mRNA FISH signal (green) in *Synechococcus* relative to thylakoid membrane  
720 (magenta) ± inhibitor pre-treatment: enlarged examples shown below with line profiles. **d,** Quantitation of effects of  
721 inhibitors on the mean FISH signal per cell in *Synechococcus* ( $p=2\times 10^{-47}$  for puromycin vs untreated,  $5\times 10^{-49}$  for  
722 lincomycin vs untreated). **e,** Comparison of the distance of mRNA FISH signals from the closest TM for *psbA* and *psaA*  
723 in *Synechococcus* ( $p=0.5$ ). Detail of the *psbA* mRNA of *Synechococcus* is shown in Fig. 2. The thick line at the middle  
724 of each violin plot represents the median). Error bars in the box plots indicate the range of values recorded, the  
725 centre line shows the median and the box spans the interquartile range. n: number of cells measured, \*: significant  
726 difference from the untreated cells, at  $p < 0.001$ , measured by unpaired two-tailed Student's t-test; ns:  $p$ -value non-  
727 significant; all scale bars:  $2\mu\text{m}$ .

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**Figure 4: Location of mRNAs that do not encode TM-integral proteins: *cpcAB* in *Synechocystis* and *rbcL* in**

***Synechococcus*. a, *cpcAB* mRNA FISH signal (green) in *Synechocystis* relative to thylakoid membrane (magenta).**

Representative cell (a, i) is shown in enlarged form at the right of the micrograph. Line profile from the

representative cell show location of transcript signal relative to thylakoid membrane (TM). **b, Comparison of the**

distance of mRNA FISH signals from the closest TM for three mRNA species: *cpcAB*, *psbA* and *psaA* in *Synechocystis*

( $p=7 \times 10^{-7}$  for *cpcAB* vs *psbA*,  $9 \times 10^{-7}$  for *cpcAB* vs *psaA*, 0.72 for *psbA* vs *psaA*). The thick line at the middle of each

violin plot represents the median. n: number of cells measured. \*: significant difference ( $p < 0.001$ ), measured by

unpaired two-tailed Student's t-test; ns:  $p$ -value non-significant. **c, *rbcL* mRNA FISH signal (green: top) and RbcL-GFP**

signal (grey: bottom) in *Synechococcus* relative to thylakoid membrane (magenta). Representative cells (c, i-iii) are

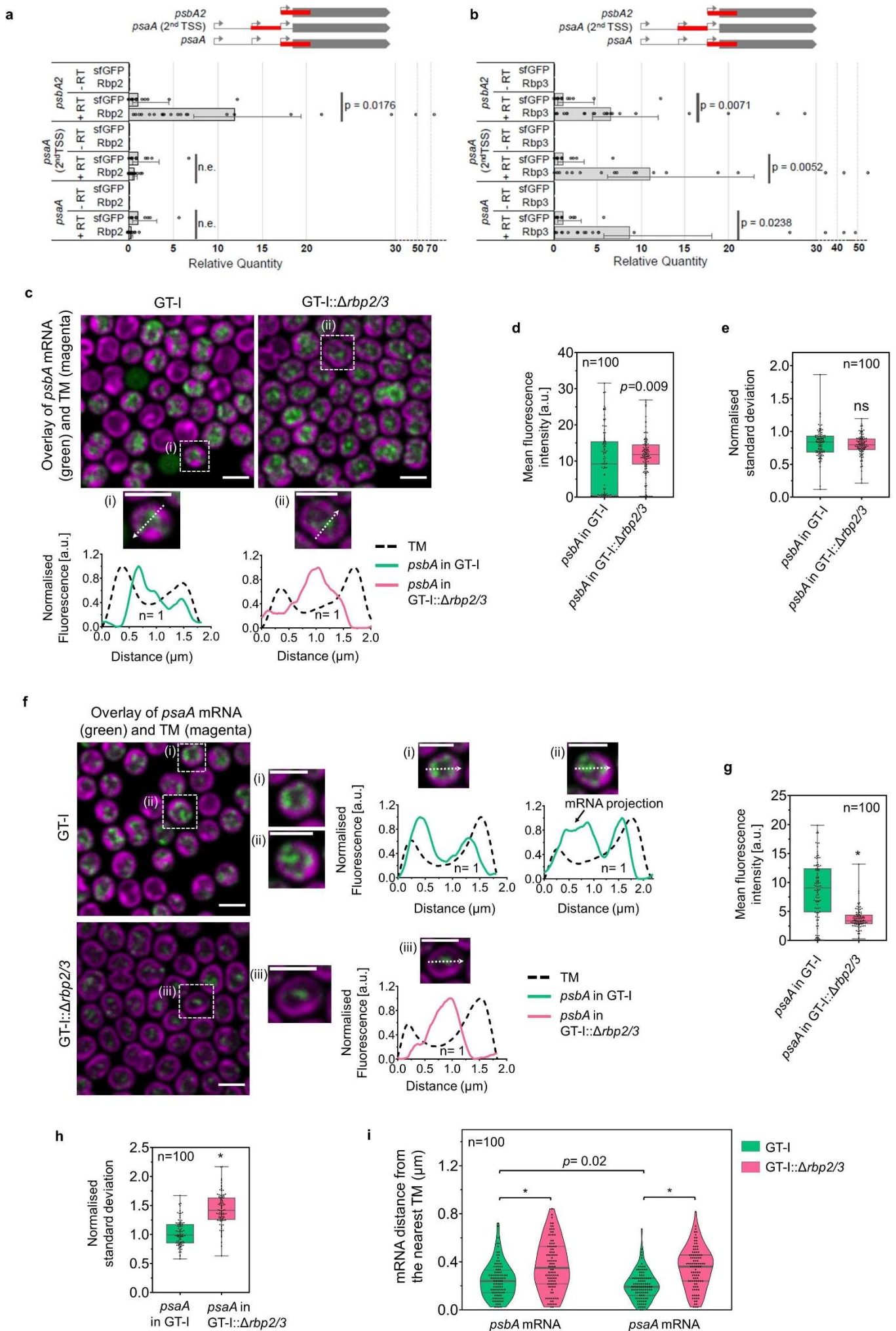
shown in enlarged form at the right of the micrograph. Short-axis line profiles from representative cells (c, i-ii) show

location of transcript signal relative to TM. **d, Averaged short-axis line profiles showing the distributions of *rbcL***

mRNA and RbcL-GFP relative to TM in *Synechococcus*. **e, Violin plot showing distance from *rbcL* mRNA**

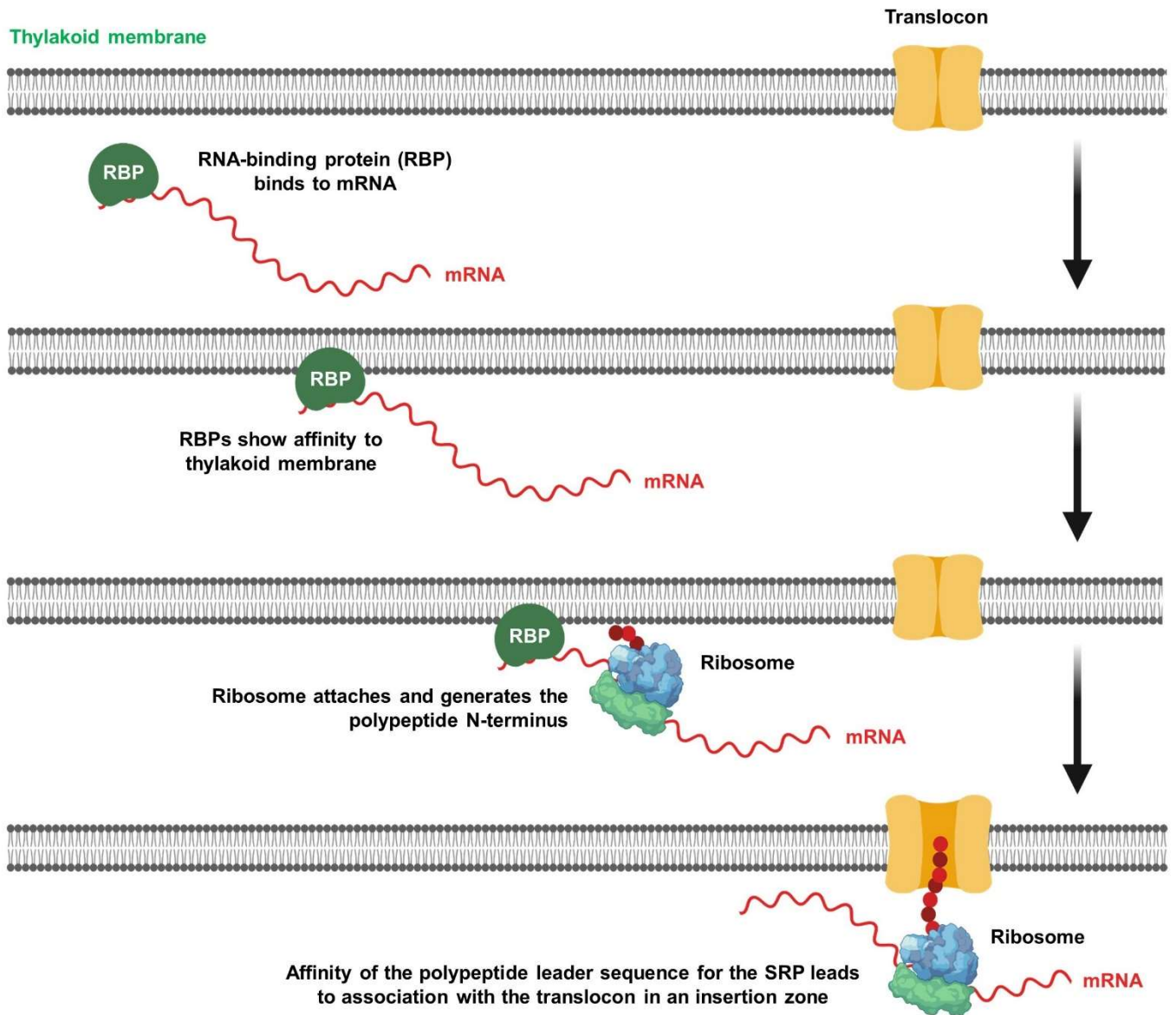
742 concentrations to the nearest TM.  $n = 100$  cells. *rbcL* mRNA is significantly further from the TM than *psbA* mRNA and  
743 *psaA* mRNA (Fig. 3e:  $p$  from two-tailed Student's  $t$ -tests =  $10^{-6}$  for *psbA* and  $10^{-5}$  for *psaA*). All scale-bars:  $2\mu\text{m}$ .  
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746 **Figure 5: RNA-binding proteins in *Synechocystis* GT-I and their effects on mRNA localisation. a,b**, RT-qPCR analysis  
747 of *psbA2*, *psaA* and *psaA* proximal 5' UTR (2<sup>nd</sup> TSS) mRNA binding to FLAG-tagged RBP2 (a) and RBP3 (b), with FLAG-  
748 tagged GFP as negative control and *rnpB* as endogenous control for normalisation. *n* = 6 for all data (triplicate  
749 reactions from 2 biological replicates). Error bars in in RT-qPCR represent the mean of the minimum and maximum  
750 relative quantities from all measurements. Asterisks label significant difference from the sfGFP control, at *p* < 0.05,  
751 measured by unpaired two-tailed Student's t-test. n.e.: not enriched. **c**, Micrographs showing *psbA* mRNA (green)  
752 location relative TM (magenta) in GT-I and GT-I::Δ*rbp2/3*. Representative cells (**c. i and ii**) are shown in enlarged form  
753 below the corresponding micrographs. Line profiles from the representative cells show location of transcript signal  
754 relative to thylakoid membrane (TM). **d**, Relative *psbA* mRNA signal intensity in GT-I and ΔGT-I::Δ*rbp2/3* cells. **e**,  
755 patchiness of the *psbA* mRNA distribution patterns assessed from normalised standard deviation in the signal (*p*=  
756 0.28). **f**, Micrographs showing *psaA* mRNA (green) location relative to TM (magenta) in GT-I and ΔGT-I::Δ*rbp2/3*.  
757 Representative cells (**f. i-iii**) are shown in enlarged form at the right of the corresponding micrographs. Line profiles  
758 from the representative cells show location of transcript signal relative to TM. **g**, Relative *psaA* mRNA signal intensity  
759 in GT-I and ΔGT-I::Δ*rbp2/3* cells (*p*=4x10<sup>-16</sup>). **h**, patchiness of *psaA* mRNA distribution assessed from the standard  
760 deviation in the FISH image, (*p*=6x10<sup>-21</sup> for GT-I vs. ΔGT-I::Δ*rbp2/3*). **i**, Violin plot comparing the distance of *psbA* and  
761 *psaA* mRNA spots from the closest thylakoid membrane in GT-I vs. GT-I::Δ*rbp2/3* (*p*=7x10<sup>-6</sup> for *psbA* mRNA; 2x10<sup>-10</sup>  
762 for *psaA* mRNA). The thick grey line in each violin plot represents the median distance to TM. Error bars in the box  
763 plots indicate the range of values recorded, the centre line shows the median and the box spans the interquartile  
764 range. *n*: number of cells measured, \*: significant difference from the untreated cells, at *p* < 0.001, measured by  
765 unpaired two-tailed Student's t-test; ns: *p*-value non-significant; scale bars: 2μm.

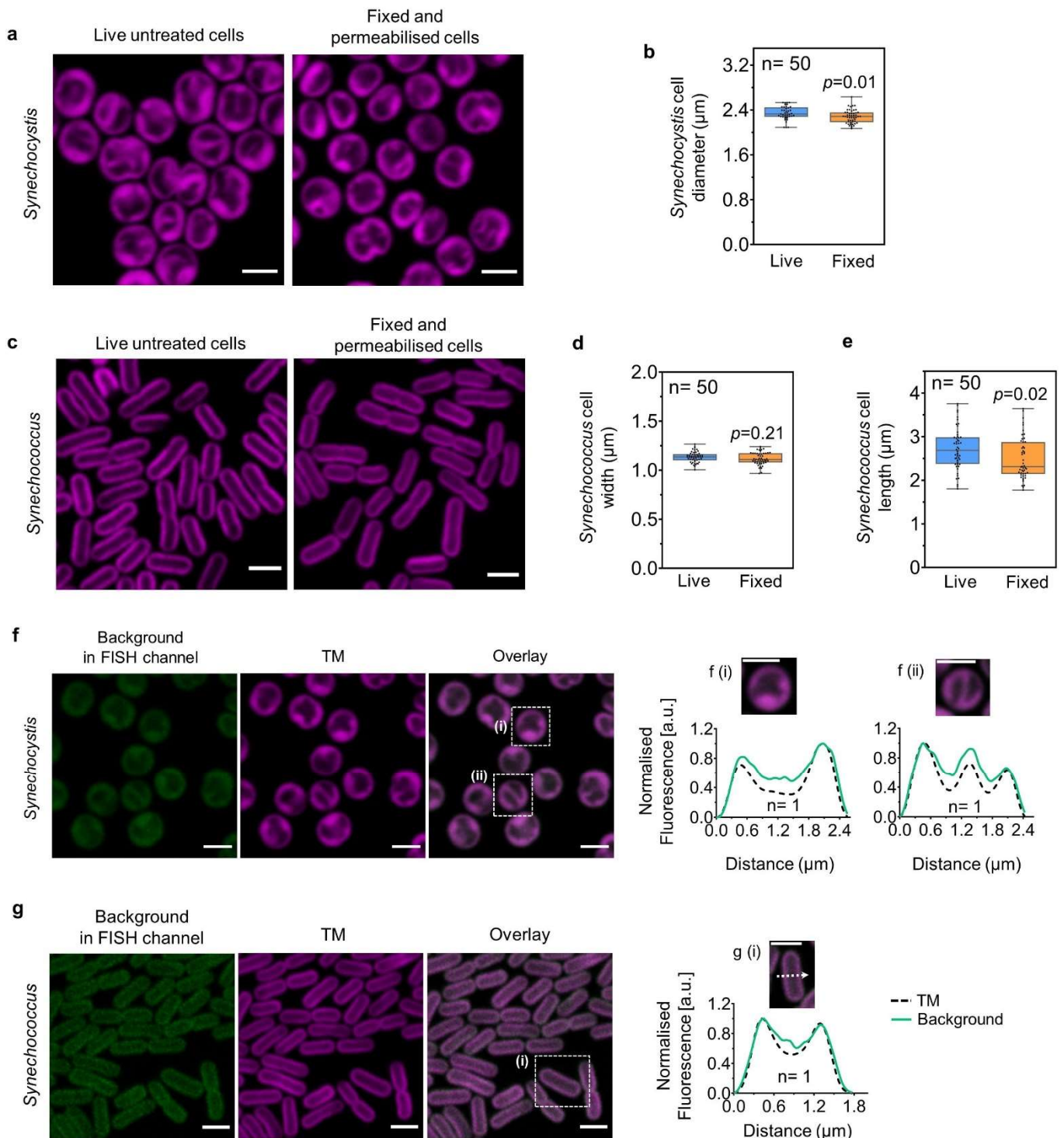
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768 **Figure 6: Model for thylakoid membrane targeting based on recognition of thylakoid-mRNA molecules by specific**  
 769 **RNA-binding proteins with affinity for the thylakoid membrane surface.** Following association with the thylakoid  
 770 membrane surface, ribosomes bind and initiate translation. Affinity of the N-terminal leader sequence of the nascent  
 771 polypeptide for the Signal Recognition Particle (SRP) then promotes association with the translocon, which is  
 772 concentrated in specific zones at the inner surface of the thylakoid system (adjacent to the central cytoplasm) or on  
 773 thylakoid protrusions into the central cytoplasm in *Synechocystis*. In *Synechococcus*, the initial association with the  
 774 thylakoid is quite delocalised over the membrane surface (as observed in the presence of puromycin) but active  
 775 translation leads to concentration at insertion zones. Ribosome binding likely displaces RBPs from the mRNA, leading  
 776 to a loss of thylakoid association in the presence of lincomycin.

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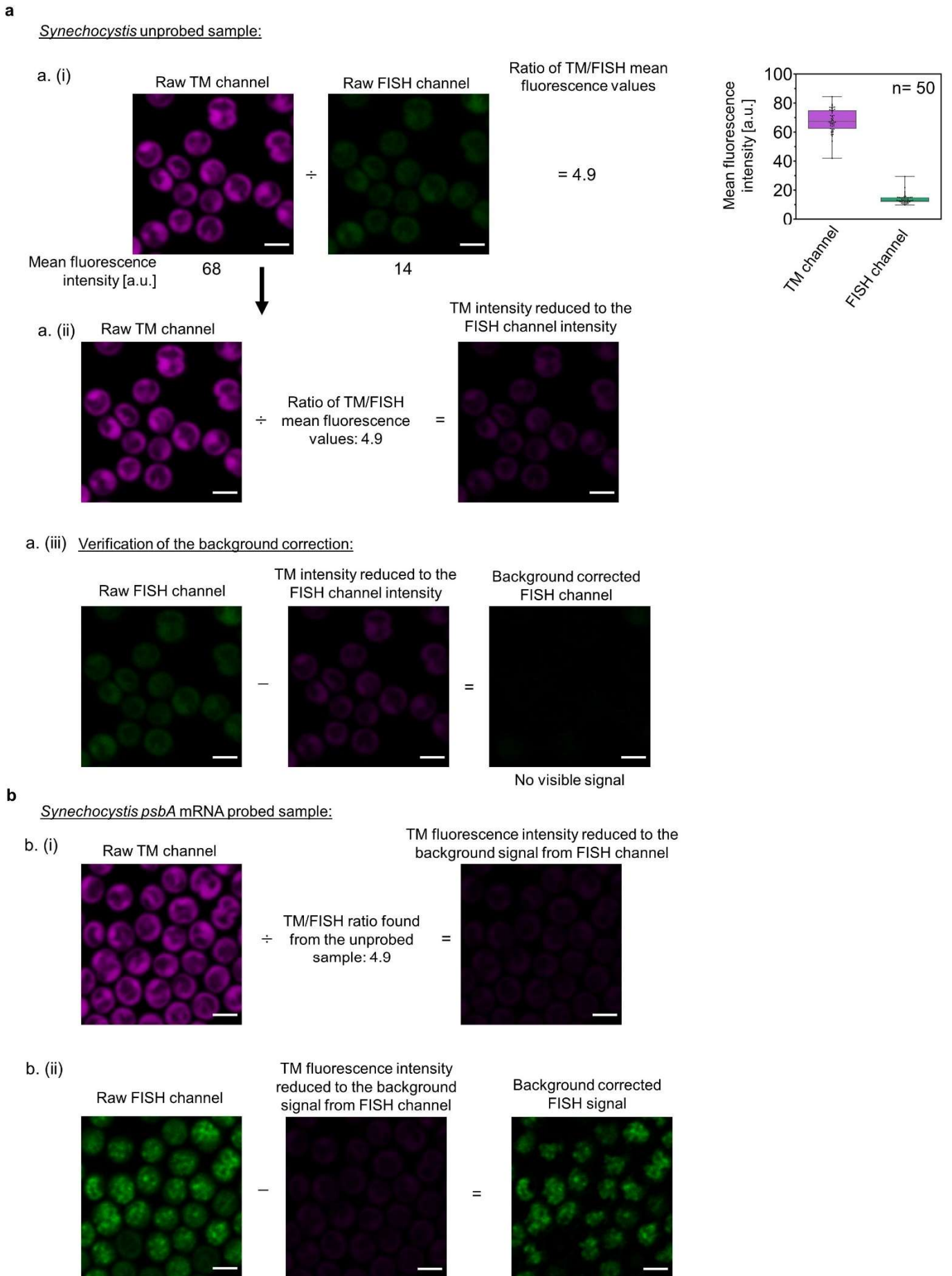
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**Extended Data Figure 1: Background fluorescence in fixed cells of *Synechocystis* and *Synechococcus*.** **a**, Confocal fluorescence images of photosynthetic pigments in *Synechocystis* cells showing thylakoid membrane organisation without and with the fixation and permeabilisation used for mRNA-FISH probing. **b**, statistics for cell diameter in fixed vs live cells ( $n = 50$  cells: no adjustments for multiple comparisons). **c**, Confocal fluorescence images of photosynthetic pigments in *Synechococcus* cells showing thylakoid membrane organisation in live vs. fixed cells. **d,e**, statistics for cell width and length in fixed vs live cells ( $n = 50$  cells: no adjustments for multiple comparisons). **f,g**, Confocal micrographs of *Synechocystis* (**f**) and *Synechococcus* (**g**) cells (fixed and permeabilised but not probed)



786 showing the background signal (green) in the TAMRA detection channel in comparison to fluorescence from the  
787 photosynthetic pigments (TM, in red). Line profiles drawn from representative cells of both *Synechocystis* (**f, i,ii**) and  
788 *Synechococcus* (**g, i**) confirm that the background signal (green line) colocalises with the TM (black dashed line).  
789 TAMRA channel shown without background correction. Images are representative of at least 2 independent  
790 experiments. Error bars in the box plots indicate the range of values recorded, the centre line shows the median and  
791 the box spans the interquartile range. *p* values are from unpaired two-tailed Student's *t*-tests. Scale bars: 2  $\mu\text{m}$ .  
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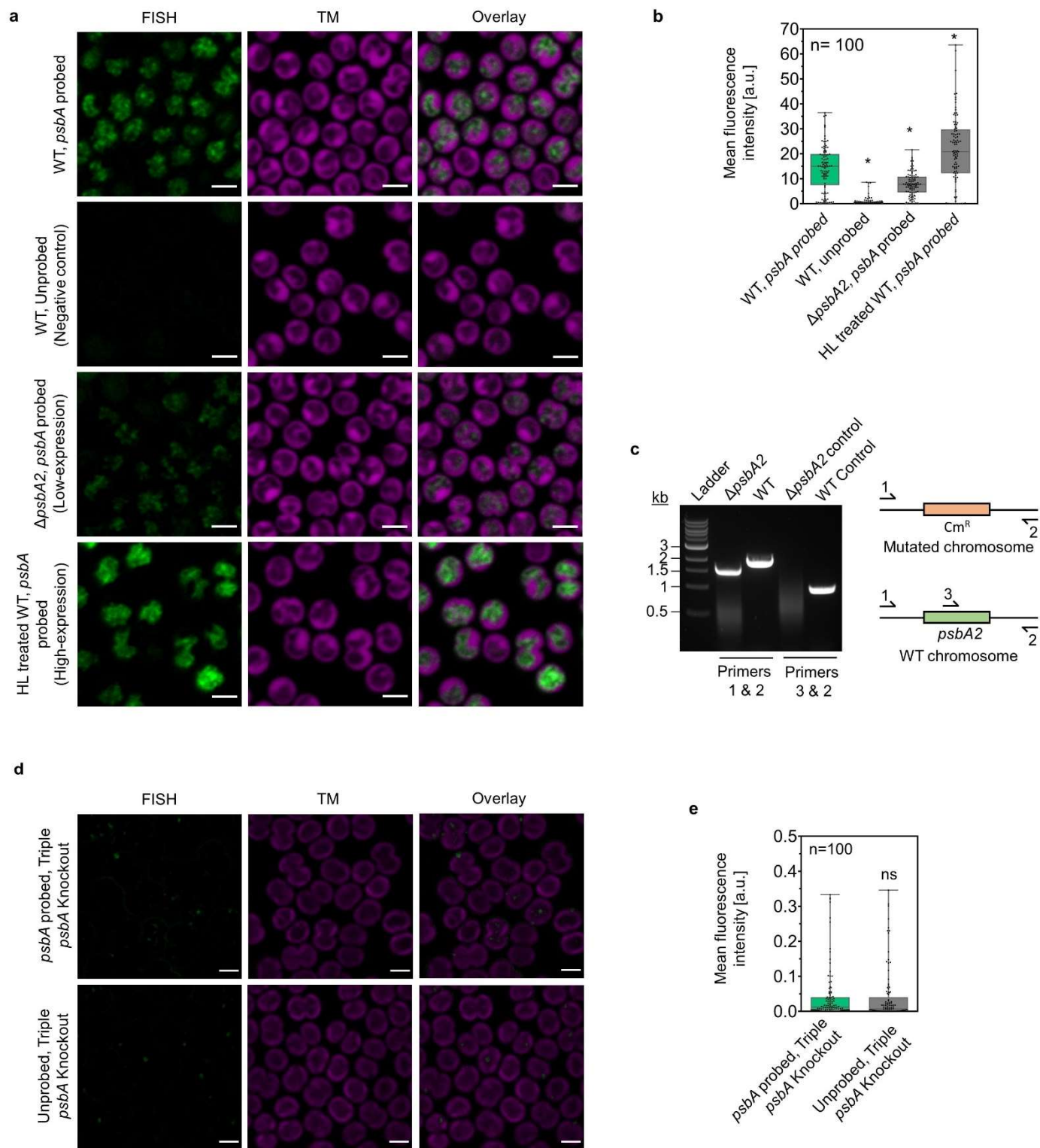
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**Extended Data Figure 2: Correcting for background fluorescence in RNA-FISH micrographs.** Step-by-step illustration of the procedure used to subtract background fluorescence from the FISH images, illustrated with *psbA* mRNA in

797 *Synechocystis* as an example. **a**, demonstration that the signal in the TAMRA channel is a predictable fraction of the  
798 signal in the TM channel in unprobed cells ( $n = 50$  cells); **b**, use of this principle to remove background signal from  
799 FISH images in probed cells. Images are representative of at least 2 independent experiments.

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**Extended Data Figure 3: Controls for specificity of *psbA* mRNA-labelling in *Synechocystis*.** **a**, Variation in *psbA*

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mRNA signal intensity between WT grown under standard conditions (first row) and three control samples:

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unprobed,  $\Delta psbA2$  mutant (probed with *psbA2* mRNA probes) and high light (HL: 600  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ , 1 hour) -

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treated cells similarly probed. Micrographs showing the FISH channel (green) vs. TM (magenta). **b**, Mean intensity of

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the mRNA signal in the different samples. Analysis was done after smoothing the images (below optical resolution)

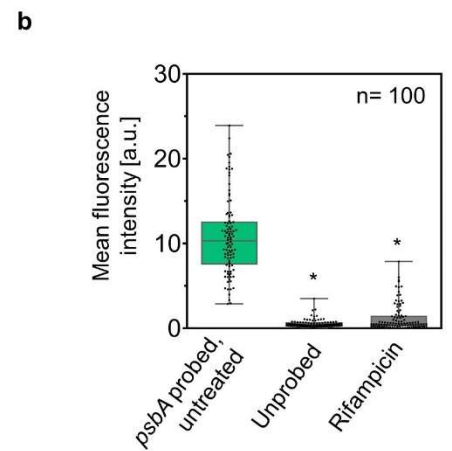
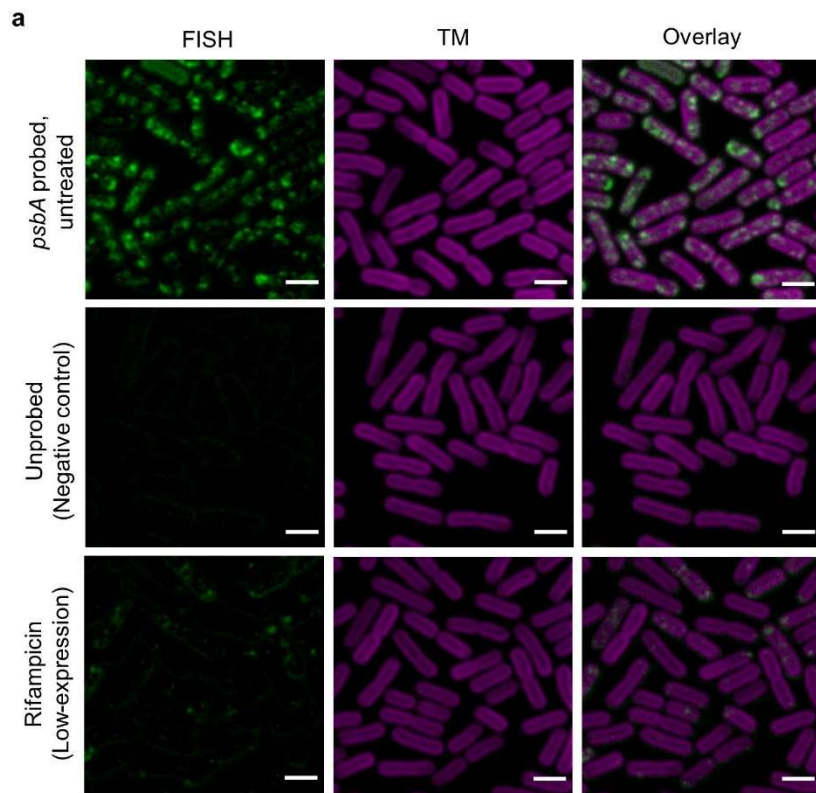
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and correcting the background signal in the FISH detection channel (detail in Methods):  $n = 100$  cells;  $p = 4 \times 10^{-27}$  for



808 WT probed vs unprobed,  $5 \times 10^{-9}$  for  $\Delta psbA2$  vs WT,  $4 \times 10^{-5}$  for HL vs normal growth. **c**, Confirmation of full segregation  
809 status of  $\Delta psbA2$  mutant by PCR amplification using the primer pair illustrated at the right hand side of the gel image  
810 (detail in Methods); **d**, Micrographs showing the triple *psbA* knockout<sup>29</sup>  $\pm$  *psbA* probe; **e**, Quantification of FISH signal  
811 from the triple *psbA* knockout  $\pm$  *psbA* probe ( $n = 100$  cells;  $p = 0.9$ ). Analysis was done after smoothing the images  
812 (below optical resolution) and correcting the background signal in the FISH channel (detail in Methods). Images are  
813 representative of at least 2 independent experiments. Error bars in the box plots indicate the range of values  
814 recorded, the centre line shows the median and the box spans the interquartile range,  $n$ : number of cells measured,  
815 \*: significant difference from the untreated cells, at  $p < 0.001$ , measured by unpaired two-tailed Student's t-test; ns=  
816  $p$ -value non-significant; scale bars:  $2 \mu\text{m}$ .

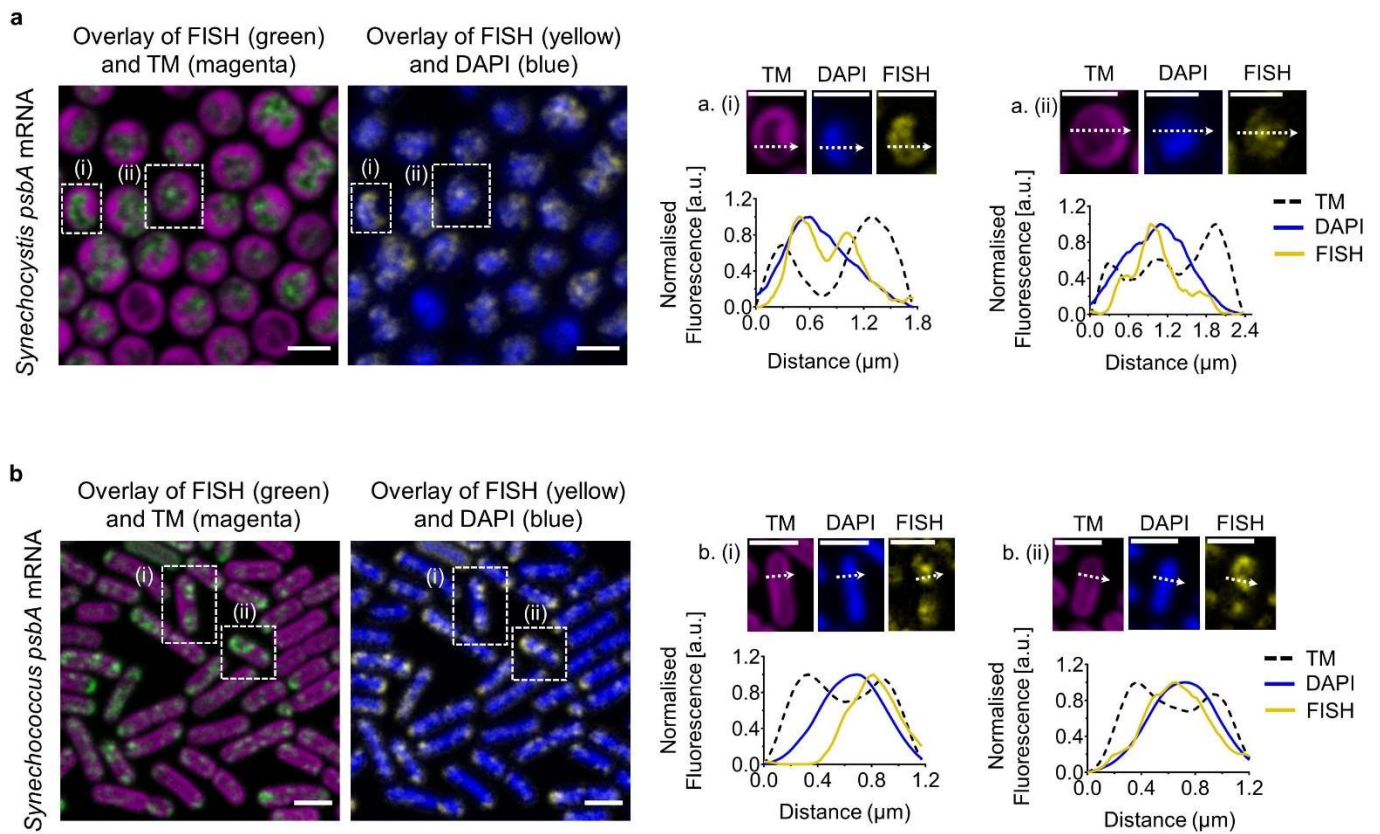
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819 **Extended Data Figure 4: Controls for specificity of *psbA* mRNA-labelling in *Synechococcus*.** **a**, Comparison of *psbA*  
820 mRNA signals in cells grown under standard conditions (first row) and unprobed and rifampicin-treated cells.  
821 Micrographs of the mRNA FISH signal (green) and TM (magenta). **b**, Mean fluorescence intensity per cell of the  
822 mRNA signal in cells from the different samples:  $p=6 \times 10^{-41}$  for probed vs unprobed,  $5 \times 10^{-40}$  for rifampicin vs  
823 untreated. Analysis was done after smoothing the images (below optical resolution) and correcting the background  
824 signal in the FISH channel (detail in Methods). Error bars in the box plots indicate the range of values recorded, the  
825 centre line shows the median and the box spans the interquartile range. Images are representative of at least 2  
826 independent experiments. n: number of cells measured, \*: significant difference, at  $p < 0.001$ , measured by unpaired  
827 two-tailed Student's t-test; scale bars:  $2 \mu\text{m}$ .

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**Extended Data Figure 5: Localisation of *psbA* FISH signals relative to thylakoid membrane (TM) and nucleoids**

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**(DAPI-stained) in *Synechocystis* and *Synechococcus*. a, *psbA* mRNA in *Synechocystis* relative to TM and DAPI. Line**

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**profiles (a, i-ii) across representative cells show the distribution of TM, FISH and DAPI signals in *Synechocystis*. b,**

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***psbA* mRNA in *Synechococcus* relative to TM and DAPI. Line profiles (b, i-ii) across the short axis of representative**

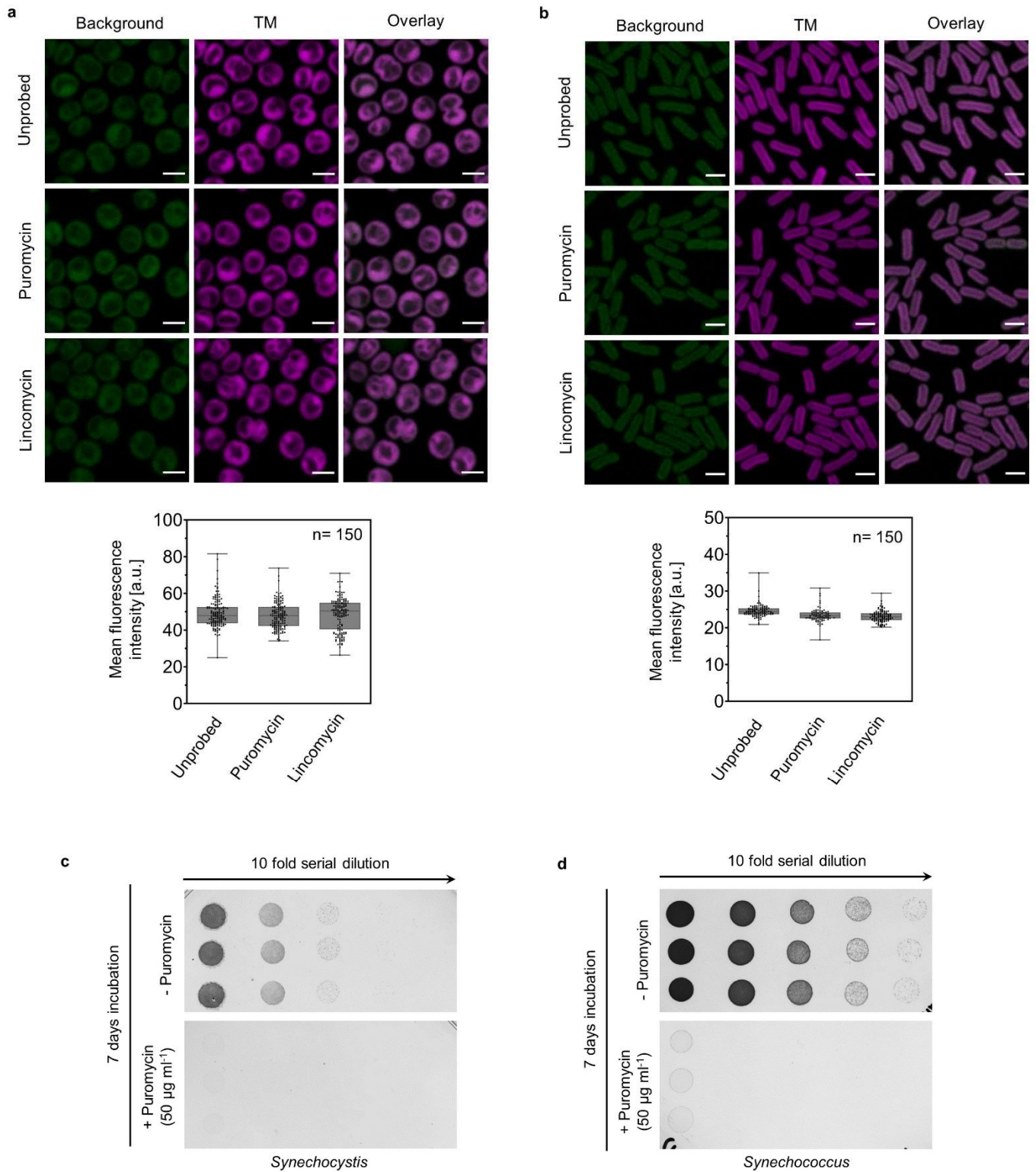
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**cells show the distribution of TM, FISH and DAPI signals in *Synechocystis*. Images are representative of at least 2**

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**independent experiments. Scale bars: 2µm.**

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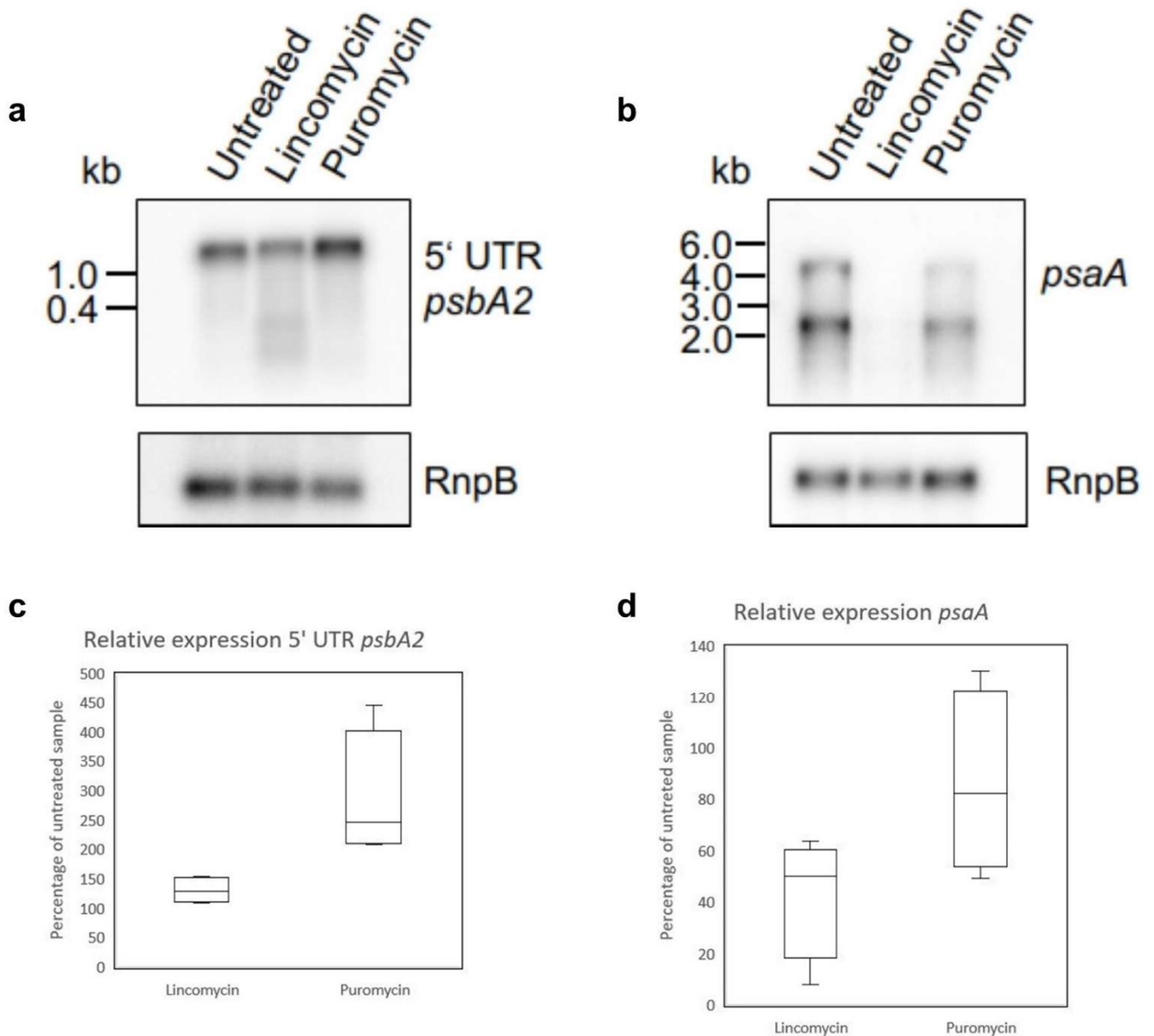
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**Extended Data Figure 6: Effects of puromycin and lincomycin treatments.** **a,b**, Controls showing that puromycin and lincomycin treatments do not increase background autofluorescence. Confocal fluorescence micrographs and mean fluorescence intensity per cell of the background signal in the TAMRA channel for unprobed cells of (a) *Synechocystis* and (b) *Synechococcus*, showing similar intensity of the background autofluorescence with and without inhibitor treatment. Error bars in the box plots indicate the range of values recorded, the centre line shows the median and the box spans the first to third quartiles; none of the differences are significant. n: number of cells

844 measured; Scale bars: 2  $\mu\text{m}$ . **c,d**, Effects of puromycin on growth of *Synechococcus* and *Synechocystis* cells. Ten-fold  
845 serial dilutions of three independent cultures were spotted on BG11 plates  $\pm$  puromycin (50  $\mu\text{g}/\text{ml}$ ). Plates were  
846 photographed after seven days of growth. Images are representative of 2 independent experiments.

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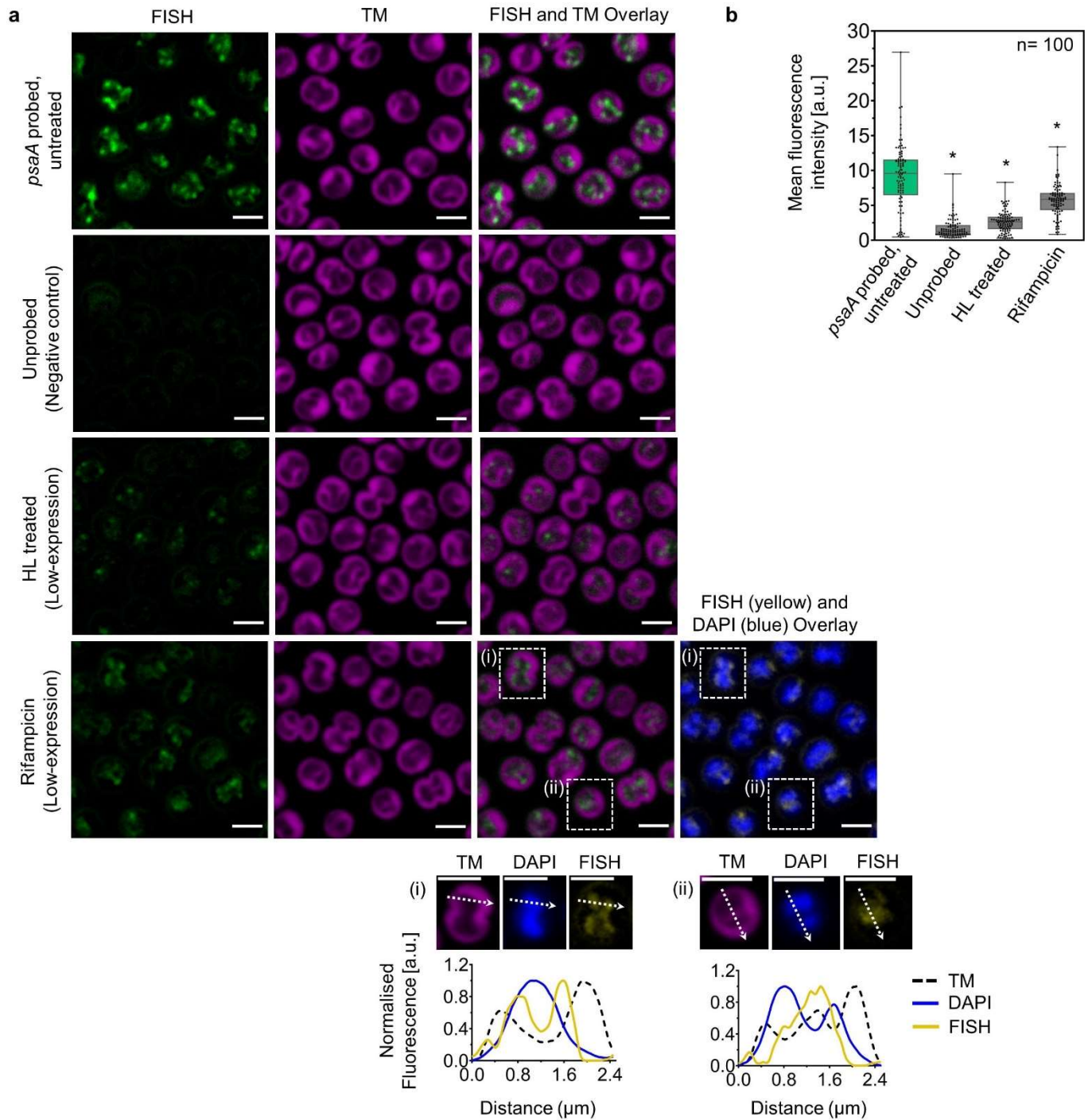
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**Extended Data Figure 7: Effects of ribosome inhibitors on *psbA2* and *psaA* transcript levels.** RNA isolated from *Synechocystis* cells treated with lincomycin or puromycin was separated by electrophoresis, blotted onto a nitrocellulose membrane and hybridized with radioactively labeled probes against the 5' UTR of *psbA2* mRNA and *psaA*. A probe against the RNA subunit of the ribonuclease P (RnpB) was used as a control. **a,b**, Representative gel images. See Source Data for full-length gel images. Results shown are representative of 2 biological replicates, each with 2 technical replicates. **c,d**, Plots showing *psbA2* (c) and *psaA* (d) mRNA levels (normalised to the RnpB signal) in inhibitor-treated cells relative to untreated cells, combining data from all 4 replicates. Error bars in the box plots indicate the range of values recorded, the centre line shows the median and the box spans the first to third quartiles. The puromycin-treated sample hybridized with *psaA* is not significantly different from the untreated sample ( $p = 0.245$ ). Other differences are all significant at  $p < 0.05$  ( $p = 0.036$  for *psbA2*/lincomycin;  $0.021$  for *psbA2*/puromycin;  $0.009$  for *psaA*/lincomycin), measured by unpaired two-tailed Student's t-tests.



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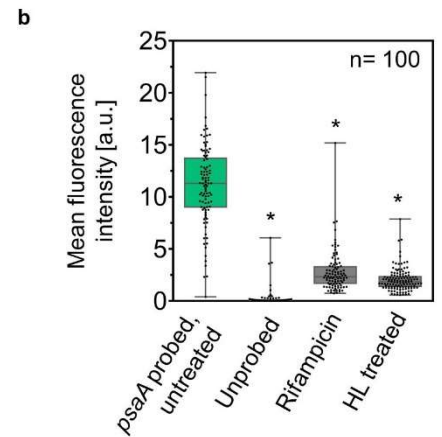
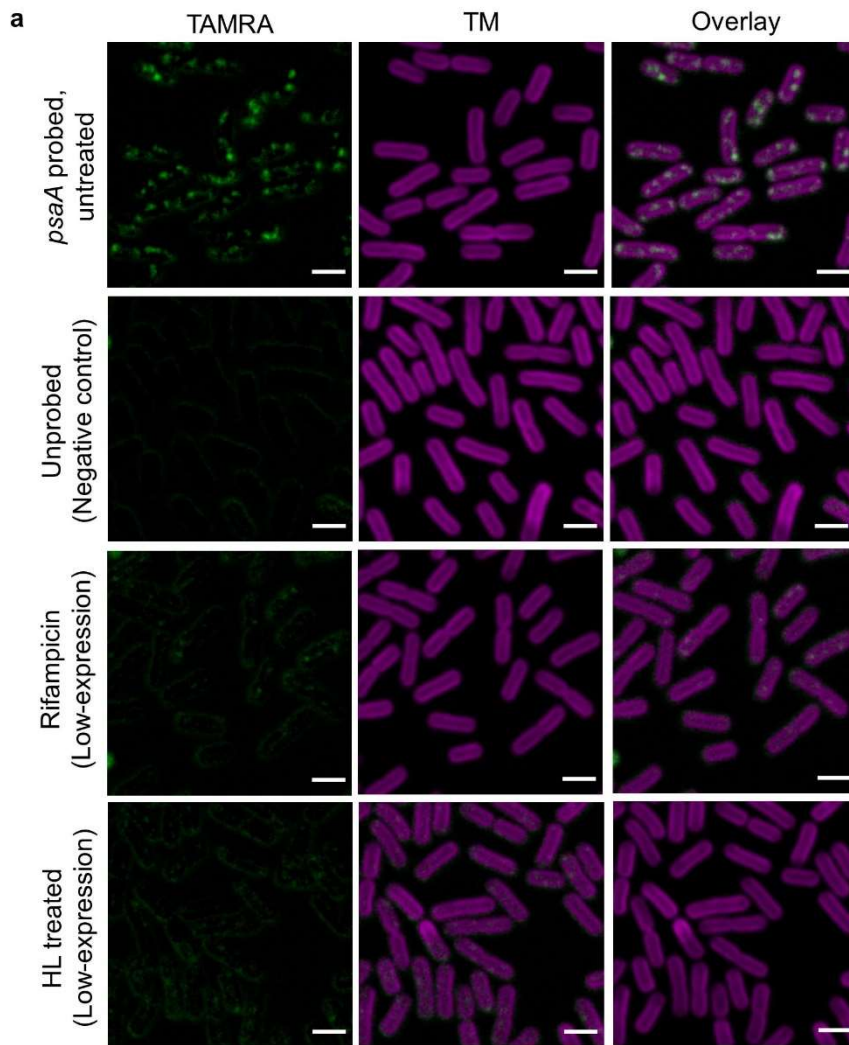
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**Extended Data Figure 8: Controls for specificity of *psaA* mRNA labelling in *Synechocystis*.** **a**, Variation in *psaA* mRNA signal intensity between cells grown under standard conditions (first row) and 3 control samples: unprobed, high light (HL)-treated cells ( $600 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ , 1 hour) and Rifampicin-treated ( $400 \mu\text{g ml}^{-1}$ , 1 hour) cells. Confocal fluorescence micrographs showing FISH signal in green and TM in magenta. An overlay between the DAPI-stained nucleoid region (blue) and FISH signal (yellow) is shown for rifampicin-treated cells. Line profiles from representative cells are shown below the corresponding micrographs. **b**, comparison of mRNA FISH signals per cell in the different conditions ( $p=3 \times 10^{-29}$  for probed vs unprobed,  $8 \times 10^{-25}$  for HL vs normal growth and  $2 \times 10^{-9}$  for rifampicin vs untreated). Images smoothed (below optical resolution) and corrected for the background signal in the FISH channel.

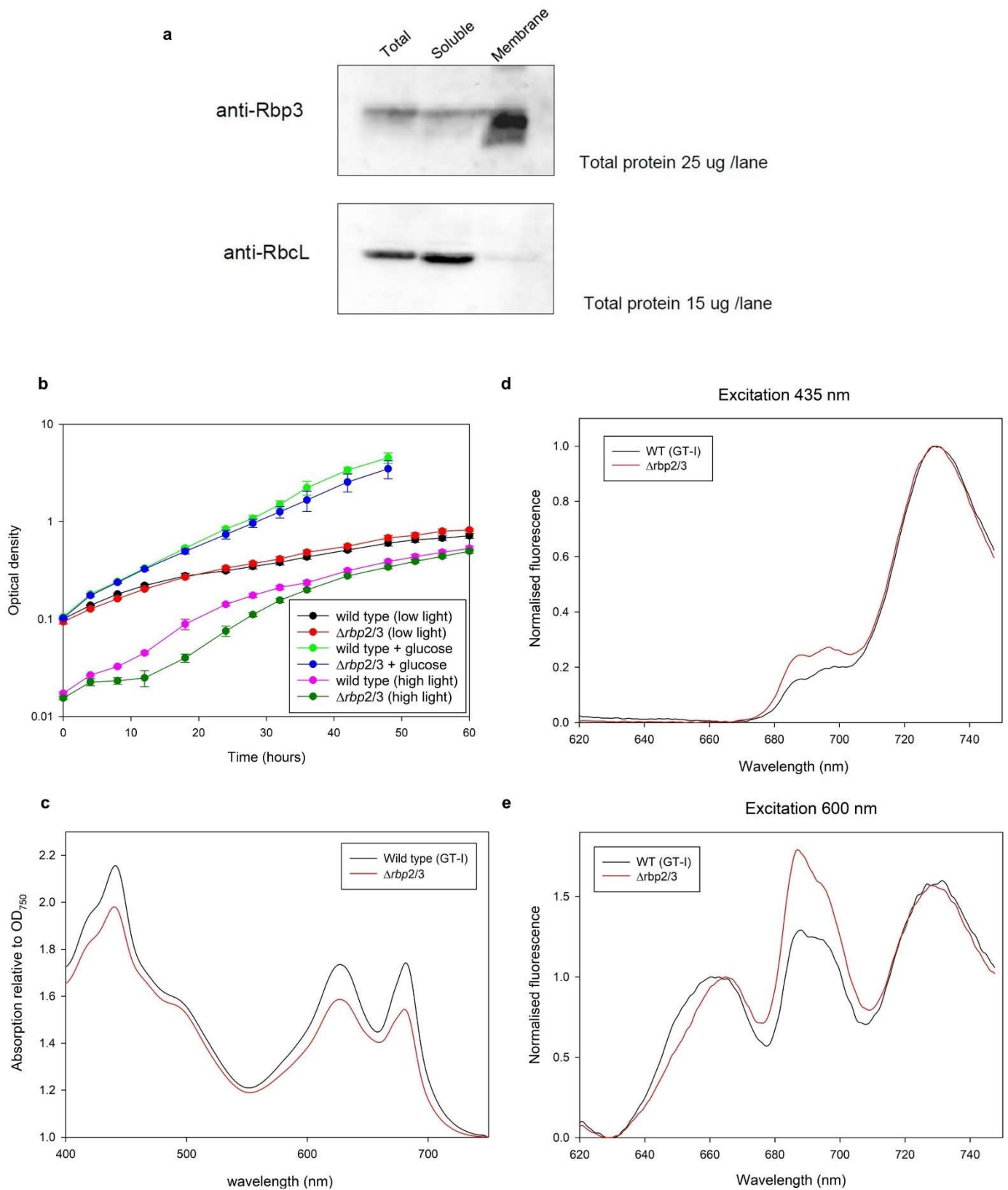
869 Error bars in the box plots indicate the range of values recorded, the centre line shows the median and the box spans  
870 the first to third quartiles. n: number of cells measured, \*: significant difference from the untreated cells, at  $p <$   
871 0.001, measured by unpaired two-tailed Student's t-test; scale bars: 2 $\mu$ m.

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 874 **Extended data Figure 9: Controls to demonstrate specificity of *psaA* mRNA labelling in *Synechococcus*.** **a**, Variation  
 875 in *psaA* mRNA signal intensity between cells grown under standard conditions (first row) and the three control  
 876 samples: unprobed, Rifampicin-treated ( $400 \mu\text{g ml}^{-1}$ , 1 hour) and high light (HL)-treated cells ( $600 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ , 1 hour). Confocal fluorescence micrographs showing FISH signal in green and thylakoid membrane region in  
 877 magenta. **b**, Comparison of mRNA signal in the control samples compared with the experimental sample. Images  
 878 smoothed (below optical resolution) and corrected for the background signal in the FISH channel ( $p=2 \times 10^{-50}$  for  
 879 probed vs unprobed;  $8 \times 10^{-42}$  for rifampicin vs untreated;  $8 \times 10^{-44}$  for HL vs normal growth). Error bars in the box plots  
 880 indicate the range of values recorded, the centre line shows the median and the box spans the interquartile range.  
 881 Images are representative of at least 2 independent experiments. n: number of cells measured, \*: significant  
 882 difference from the untreated cells, at  $p < 0.001$ , measured by unpaired two-tailed Student's t-test; scale bars:  $2 \mu\text{m}$ .  
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**Extended data Figure 10: *Synechocystis* Rbp3 location and photosynthetic phenotype of the  $\Delta rbp2/3$  mutant. a,**

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Western blotting showing that Rbp3 is associated with the membrane fraction. See Source Data for full-length gel

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images with molecular weight markers. Data shown are representative of 2 biological replicates with similar results.

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**b,** growth curves for  $\Delta rbp2/3$  vs. the wild-type (GT-I) background in different conditions: 50  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$

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(low light), 80  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$  (high light), or low light in the presence of 10 mM glucose. Error bars

891 indicate standard deviations from 3 replicate cultures. **c**, absorption spectra of cell suspensions, normalised to  
892 turbidity at 750 nm. *Δrbp2/3* has lower pigment per OD<sub>750</sub>. **d**, florescence emission spectra at 77K with chlorophyll  
893 excitation (435 nm) normalised to PSI emission at 725 nm. **e**, florescence emission spectra at 77K with phycocyanin  
894 excitation (600 nm) normalised to phycobilin emission at 660 nm. *Δrbp2/3* has lower PSI emission (725 nm) relative  
895 to PSII (peaks at 685 and 695 nm). All spectra are representative of similar results from 3 independent cultures.

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**Supplementary Tables**

**Supplementary Table 1: mRNA-FISH probe sets**

**a, Oligonucleotide probes designed against *Synechocystis psbA2* (slr1311) gene sequence.**

Probe number	Sequence (5' to 3')	Probe number	Sequence (5' to 3')
1	TGTTGGAGAGTCGTTGTCAT	23	TGTGCTCAGCTTGGAACACG
2	AGGTCACCCACTGACAAAAC	24	TGGAAGGGGTGCATCAGGAT
3	ACATAAATCCGGTTGTTGGT	25	TACACCAGCCACACCTAACA
4	GATCATCAAGGTACCGAACC	26	CGGAGAACAAGCTACCACCG
5	AAGTGGTGGCAGTTAAGAGG	27	GTTACCAAGGAACCGTGCAT
6	CGGCGATGAAGGCAATGATG	28	TGGTTTCACGCACCAAGGAG
7	GATACCGTCGATGTCAACGG	29	CGTAGTTCTGGGATTCAACT
8	AGCAAAGAACCAGCAACGGG	30	TTCTTCTTGACCGAATTTGT
9	CCAGAGATGATGTTGTTACC	31	CGGCAACGATGTTGTAGGTT
10	CGATAGCGTTGGAAGAAGGT	32	GATCAACCGACCAAAGTAGC
11	CAGATGGGGTAGAAGTGCAA	33	CGGCTGTTGTTGAAAAGAAGC
12	CTCATCTAAGGAAGCGGCTT	34	AAGCACCCAAGAAGAAGTGC
13	TAAGGACCACCGTTGTACAA	35	GAACCAGATGCCGATTACAG
14	GCCGATGAGGAAGTGAATA	36	CATGGTGCTTACACCCATAG
15	GACGACCCATGTAGCAGAAA	37	GTTGAAACCGTTCAGGTTGA
16	CTAAGCGGTAGGAAAGTTCC	38	CTATCCAAGATGGACTGGTT
17	ACACAAATCCAAGGACGCAT	39	TTGGCTCGGTTCAATACATC
18	GGATACGGGGCAGAGTAAG	40	ATTGCGTTCGTGCATTACTT
19	GATCAAGAATACGGCGGTGG	41	CTAAGTCGAGGGGGAAGTTG
20	AGAAGGAGCCTTGACCAATG	42	TCAAAGCCACAGGAGCTTGC
21	ATACCCAAGGGCATAACCATC	43	TAACCGTTGACAGCAGGAGC
22	CATGAAGTTGAAGGTACCAG		

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**b, Oligonucleotide probes designed against *Synechococcus psbA1* (Synpcc7942\_0424) gene sequence.**

Probe number	Sequence (5' to 3')	Probe number	Sequence (5' to 3')
1	TCGCGAAGAATGCTGGTCAT	21	ACATGAAGTTGAAGGTGCCG
2	GATCCCAAACGTTATCGCGG	22	TTGTGCTCTGCTTGGAACAC
3	CTGGTTACCCACTCACAAA	23	TGTGGAAGGGGTGCATCAA
4	CGAACCAACCCACGTAGATG	24	AGAACAGCGAACCCACCGAAC
5	AGAGTGGGGATCATCAGCAC	25	CACCAACGAACCGTGCATTG
6	ATGAAGCAGATGGTGGCGGT	26	TAGTTTTGGCTCTCGGTCTC
7	AGGGGCTGCAATGAACGCAA	27	CTCTTGACCAAATTTGTAGC
8	ATACATGAGAGAGCCGGCAA	28	CCACGATGTTGTAGGTCTCT
9	CGCCGGAAATGATGTTGTTG	29	ATCAAGCGACCGAAGTAACC
10	GCGTTGCTGGAAGGAACAAC	30	GTTGAACGATGCGTATTGGA
11	GCTTCCCAAATCGGATAGAA	31	GGAAGAAGTGCAGCGAACGG
12	TTGTACAGCCACTCGTCGAG	32	CATGGAGGTAAACCAGATGC
13	CCACTAATTGGTAAGGACCA	33	TGAACGCCATGGTGCTGATG
14	ATACCCAGCAAGAAGTGGAA	34	ACTGGTTGAAGTTGAAACCA
15	ATTGACGACCCATGTAGCAG	35	TTGCCTTGGCTATCCAAAAC
16	ATACCGAGGCGGTACGACAG	36	ATCTGCCCAAGTGTGATCA

17	ATGCAACACAGATCCAAGGG	37	CCAAGTTGGCACGGTTCAAC
18	AAAAGCAGCCGAGAGTGGAG	38	ATTACGCTCGTGCATCACTT
19	CCGATCGGGTAGATCAGAAA	39	AAGTCGAGCGGGAAGTTGTG
20	CATGCCGTCCGAGAACGAAC	40	ATTGAAGGCGCAGTCAAAGC

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**c, Oligonucleotide probes designed against *Synechocystis psaA* (slr1834) gene sequence.**

Probe number	Sequence (5' to 3')	Probe number	Sequence (5' to 3')
1	TCGGGTGGACTAATTGTCAT	25	ATTGAGCGTGCCAAGAAGTA
2	TTATCAACCGAGACTTTGGC	26	AAGAACCTAACAGGGCGAGG
3	TCTCGAAGGAAGTTGGTACC	27	TGTTGTGCAACGATGATCGT
4	CTAAAGTCCGGTCCGAAGTGA	28	GGTGGGTGAACAGGGATAAC
5	ATTCCAAATCCAAGTGGTGG	29	ATCACGCACCATGAAGATGG
6	TCTAAATCGCTGGTCTGACT	30	TTATTAACGTTCTTGGCGGG
7	CCCAAAGTGAGCACTGAAGA	31	ACCCAGTTGAGATGGGAAAT
8	AAAATTTGCGCGCCGTGGAAG	32	AGCTATGGAAGCCGAGGAAA
9	CACTGGGTTTAATGTGGGTA	33	GTCGTTGTGGATGTAGAGAC
10	AAGATGCCTTGACCGACAAT	34	AAGATCGGTTGCAGTTGGAT
11	AACAGGCCAGACGTAATCTG	35	GGTAAAAGCGTGGATGTGGT
12	TGATAGCTGTCGGTGAAACC	36	ATATAGAACCCCTTTGAGGA
13	CATAACCAAACCGCCAATGG	37	TATCAGGGACAAGGCGAGAG
14	CAATTTGGGAGCTTTGACGT	38	CACAGGGGAAACGAAACCG
15	CATCATCGACTCCACATTTT	39	AGACCGAGGAAAACGTGGTC
16	CCCAGCATCCAAAAGTTTAT	40	AAGGGAGTTGTACATCCAGA
17	AAGGGGAATGTCCTTAGGAG	41	CATCGGATTGCATTTTCCAA
18	ATCTTGCTCGGTTCCAAAAT	42	TAGGAGTTGATGACGTTGGC
19	TCAAACCTTGGGCAAAGCTG	43	GGCAAAGACAAAGTGCCGG
20	AGGAAGTCTGAGTAGACTCC	44	CGTCCACTGAACAGGAACAT
21	GATTCAATCCCCCTTTAAAG	45	CCAGACAATGGACTCGATCA
22	AAATGGTGGTGGAGCGGTATC	46	CCACATTGAGTTTGTGTTGA
23	TGATGAACAGGACGGCGATC	47	ACGACCTTGAATGATGCTCA
24	GAGGATCTCTTCATGCTAT	48	ACAATACCTCCGAGGAGATA

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**d, Oligonucleotide probes designed against *Synechococcus psaA* (Synpcc7942\_2049) gene sequence.**

Probe number	Sequence (5' to 3')	Probe number	Sequence (5' to 3')
1	GTCGGAACCGGATTTTTATC	25	CTAGGTTGATCGACAGTTGG
2	CTTACCCCACTTCTCAAAG	26	ACGATGATGCTGATCGAACC
3	TCCAAATCCAAGTTGTGGTT	27	AAGTACGGATACGGAGGCAT
4	AAATCGTGAGCGTTAGCGTG	28	CAATCCAGATGTGGTGGAGTA
5	TCAAGGTCACTGGTATGACT	29	TGAAAATGGCAGCGTGAGCA
6	ACCAAAGTGAGCGCTGAAGA	30	AGGAGGTTGTCGACATTCTT
7	ACCAGATAAAGATCACCGCA	31	GTGTCGTTGTGGATGTAGAG
8	CAGCCGCTGAAATTGGAGAA	32	CGAGAACATGTCTTGGGGAC
9	GAAGATCGGCCAAACGACTT	33	AAGATGGGCTGCAGTTGAAT
10	GATCTGAATGCCATGGAAGC	34	GTGCAAGGGCATGAATGTTT
11	CCGTGACGTAGAGCTGAAAC	35	CAAACACTTGGCTGACCGAA
12	CTTTGTGGTAGTGGAACCAG	36	GAATCGTGAAGGCGTGGATG

13	TCGACGTTTTGGAACCATTC	37	GAACTCCGAGCGTAGAGAAC
14	CAAGTGGTGGTTCAACATCG	38	AAGGTTGGCTTTGTCAGGAA
15	CGACACGTGAATCTGGTGAC	39	GAACAAGCCTAGGAACACGT
16	TTTTGCCGTTGAGAACCAAC	40	TCGACAGGGAGTTGTACATC
17	CGGAATATCAGCCGCAGAAG	41	CCAAACATCGGACTGCATTT
18	TCAGGCTGACATCCAAGAAC	42	TTGAGCAAAGTTGCCATTCG
19	AGCGTGAAGAAGGCTTTCAC	43	GACAAAGTGAGCACCCAAGA
20	ACCTTTGAAGGTCAGGAAGT	44	GCCACTGAACAGGAACATCA
21	GTGACCCGCAACAATGAAGA	45	CAGACGATGGACTCGATCAG
22	CCAAGATTTCTTTGAGGCTG	46	CACTTTGAGCTTGTTGTGAG
23	GTGAAAGGACCTTTGTGAGC	47	AGACCATGTGGTCACAATTC
24	TCTCATAGAGACCTTTGTGG	48	AACCAACTGCAATGATGCGG

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**e, Oligonucleotide probes designed against *Synechocystis cpcAB* (sll1577 and sll1578) gene sequence.**

Probe number	Sequence (5' to 3')	Probe number	Sequence (5' to 3')
1	TGTGATAGTTTCTCTGGTGG	25	CTTCTAGAACGGAAGCGTCTG
2	TGTGGTTTGTCTGTGATCTA	26	CAACGTAGGTTTCACGGAGA
3	TTCATCAGGAGCCATAAACC	27	CATTTTTTGAACGCCAGCAG
4	TAACGAAGTCGGGATCGTCT	28	CCATTGGGATCGTTAACGAT
5	TCTCTACAGGTGGGTATAGA	29	GCAACGATAGCACTGCAATC
6	ATCCCACCATTTTGATATTC	30	GTCGAAGTAACCAGCGATTT
7	CTACTTTCCTTTTTGAGCTT	31	ATAACCAGACTAGGCTACGG
8	AAGACTGTTGCCTAGGGAAC	32	CGGGTATGGAACCTGAGTAA
9	GTGCGAACTTTTCTGTTTT	33	GTCAGCTTTAAGCTGGATTT
10	GTTTCGTTACGCTTATGTTT	34	TATCTCCCCTTAGAATGTGA
11	TAATGTTGCAGGGGATTCTC	35	GCTTCAGTTAAAGGGTTTT
12	GTGATGGGACTTATGTCTGT	36	TTGAGAGTCAGCGGTGGAAA
13	TAGGCTTGAATGCTTTTGCA	37	AATTCGGTGCTGCTCAGAAA
14	ATCCAGGGAGAAACACGGAG	38	TGCAAACCAGCATTAGCTTG
15	TTAACTACCCGGGGATTAT	39	ATTGTCGGTCAGAGCTTTAG
16	GGATAAACTGAGGGAGTCCA	40	GTTATAAACGGCTTGGGCAG
17	ACAACCCGAGTGAATACGTC	41	CAGTAGGTAACGATGCGGAG
18	GAGAACCAGAGAGGTAICTG	42	GCGATCAAGTACTCATCCAA
19	GTAGCGCTCAAAGCATCTAA	43	AAGGTGCGGTTGATTTATC
20	CGGTGATGCGGTTAACAGAA	44	GAGGTAGGAATTAGCTTCGT
21	CAGCGTTGAAACGATAGCG	45	TGACTAGCTCAGAGCATTGA
22	TGGATTAATTGGGGCTGTTC	46	CCAGGCCAGCTAGAATTAAA
23	ACGCAAACAAGCAGCCATAC	47	AGTTGTTAACACTTTCCAC
24	GTAGGTAACATAGCGGAGGA	48	GTTCTCCTAGATAAGTGTC

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**f, Oligonucleotide probes designed against *Synechococcus rbcL* (Synpcc7942\_1426) gene sequence.**

Probe number	Sequence (5' to 3')	Probe number	Sequence (5' to 3')
1	TTATAGCCTGCGGCAGATTG	25	TGCGATTTGTGGATTGCATC
2	TGAGTTTGTAGTCTTCACC	26	TTTGATTTACCGGTTTCTG
3	GTGTAATCGGGGGTGAATA	27	CGGTCACGTTACGGTAGTGA
4	CAGCAGGTCAGTGTCTTTGG	28	TTTCATCATTTCTTCGCAGG
5	CTGAGGGCTGAAGCGGAAAG	29	AGTTCTTTAGCGAACTCAGC

6	GGTACCGGTCGAAGATTCAG	30	CATGCATGATGATCGGCATG
7	GATCCATGTCTGGTCAGCAAG	31	GTGAAACCAGCCGTCAAGAA
8	GCTCGATGTGGTAGCACTTG	32	ATTTTGCCAAGGTGGTGTTG
9	TAGGAGTTCTCTTCGCCTTG	33	ATTGCACGGTGGATGTGCAG
10	CGGGTAAGCGATGAACGCAA	34	TTACGCTGACGGTCGATCAC
11	ACCCTTCTCAAACAGGTCTG	35	AAGACACGGAAGTGAATCCC
12	GAGGTCAGGATGTTGGTGAC	36	GGACAGACGCAAACACTTGG
13	AAGCCAAACACGTTACCGAC	37	CAAGGTCGAAGCTTTGTCTGC
14	ACGCAGCGAACGGATAGCTT	38	TTCGCGCATCAAGTCAACAA
15	ACGGGGAAGCGGATGTCTTC	39	CCCAATCTGGGTGAAGAAG
16	CCTTGAAGGTTTTGACCAA	40	TGGATACCACCGGAAGCAAC
17	GACTTGGATACCGTGGGGAG	41	ATCACCGAAGATTTCCACCA
18	CGTACTTGTTTCAGCAGGTCTG	42	CACCGAACTGGAGAACGGAG
19	GTTTTGGTTTGATCGTGCAA	43	CAAGCTTCCAAGGCAACACG
20	GTAGTTTTTCGCCGACAGAC	44	TTCACGGTAGAGGTCTCGGAC
21	CAGACATTCGTAGACGGCAC	45	CAGCTTACGAAGGATGTCTG
22	GTCTTTGGTGAAGTCCAGAC	46	ACTTGATCTCTTCCAGAGG
23	GCTGCGAGTTGATGTTTTCTG	47	CTTGTCCATCGTTTCGAATT
24	CCACAAACAGGAAGCGATCG		

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**Supplementary Table 2: Oligonucleotide primers used to generate  $\Delta psbA2$  mutant of *Synechocystis***

Purpose		Sequence (5' to 3')
Amplification of <i>psbA2</i> (slr1311) upstream sequence	Forward primer:	TAGCGTTCCAGTGGATATTTGCTGG
	Reverse primer:	GGGCGTAACGATGTTTCAGATTGGAAGTCTG
Amplification of <i>psbA2</i> (slr1311) downstream sequence	Forward primer:	ACGTGCCGATCATTCTTGGTGTAATGCCAAC
	Reverse primer:	ATTCAATCGCTCTAGGCGATCG
Amplification of CmR	Forward primer:	CAGTTCCAATCTGAACATCGTTACGCCCCGCCCTGCCAC
	Reverse primer:	TGGCATTACACCAAGGAATGATCGGCACGTAAGAGGTTTC
Amplification of pGEM-T Easy	Forward primer:	CGATCGCTAGAGCGATTGAATAGCTTGAATATTCTATAGTGTC
	Reverse primer:	CCAGCAAATATCCACTGGAACGCTAAATTCGCCCTATAGTGAGTCTG
<i>psbA2</i> (slr1311) specific sequence to check segregation status of the mutant	Forward primer:	TGTTCCAAGCTGAGCACAAAC

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916**Supplementary Table 3: Oligonucleotide primers used to construct the  $\Delta rbp2/3$  mutant and *rbp3* expression vector.**

Purpose		Sequence (5' to 3')	PCR template
Amplification of <i>rbp2</i> (ssr1480) upstream sequence	Forward primer:	CCGGCCACCCCGATTAATGTG	Genomic DNA of <i>Synechocystis</i> GT-I
	Reverse primer:	GATTTATTTATTCTAAATTAGCTCCAAAAC CAGAGAA	
Amplification of <i>rbp2</i> (ssr1480) downstream sequence	Forward primer:	GGGCGGGGCGTAAGTTTTGCCTAATTAC CTGAATTTAAG	Genomic DNA of <i>Synechocystis</i> GT-I
	Reverse primer:	TGGTGGCTCCTAATTCCCGCAGTT	
Amplification of CmR	Forward primer:	TTTTGGAGCTAATTTAGAATAAATAAATCC TGGTGTG	Chloramphenicol resistant gene in pUC303 <sup>58</sup>
	Reverse primer:	AATTAGGCAAAAACCTTACGCCCCGCCCTG CCACTC	
Fusion of DNA fragments ( <i>rbp2</i> KO) and check segregation status of the mutant	Forward primer:	CCGGCCACCCCGATTAATGTG	Mixture of three DNA fragments or genomic DNA of <i>rbp2</i> mutant
	Reverse primer:	TGGTGGCTCCTAATTCCCGCAGTT	
Amplification of <i>rbp3</i> (slr0193) KO DNA fragment and check segregation status of the mutant	Forward primer:	ACCTCACTGCGTATGACTTCC	Genomic DNA of <i>rbp3</i> mutant provided from Prof. Masahiko Ikeuchi
	Reverse primer:	CACCAATTTGCCACTGTCTACC	
Construction of <i>rbp3</i> expression vector	Forward primer:	CATCATCATGAATTCATGTCCATTCGTCTC TACG	Genomic DNA of <i>Synechocystis</i> GT-I
	Reverse primer:	GTGGTGGTGTCTCGAGCTACTGGGCCGCT GTCAGTT	

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**Supplementary Table 4: Primers used for construction of inducible FLAG-tagged *rbp2* and *rbp3* mutants**

Oligonucleotide name	Sequence (3' → 5')	Description
HindIII-ssr1480_fwd	AAGCTTTAGGGTCAGTTGACCGG	Amplification of <i>ssr1480</i> ( <i>rbp2</i> ) and introducing Hind III and XhoI restriction sites
XhoI-ssr1480_rev	CTCGAGTCCCCAGTCTATCAGC	
HindIII-slr0193_fwd	AAGCTTATGCTTATTCCCGTTTGATTG	Amplification of <i>slr0193</i> ( <i>rbp3</i> ) and introducing Hind III and XhoI sites
XhoI-slr0193_rev	CTCGAGGTTTTTATTAAGCTCTAAACAGGACAAAG	
pJet-sll0517-Toop_fwd	CGCCGGGCGTTTTTATTCTCGAGATCTTTCTAGAAGATCTCTAC AATATTC	Introduction of the ooP-Terminator into the pJet1.2::sll0517 constructs by inverse PCR
pJet-sll0517-Toop_rev	GCAACCGAGCGAACAGGATTTAGCCCGATTTCCCACCAGAAATA CGGGTAG	
pJet-sll0517-3xFLAG_fwd	TATTGATTATAAAGATGATGATGATAAATAGGGCTTAGTTTTGTTCG TCGGTTAGTGAAACTTTTTTG	Introduction of a 3x FLAG-Tag into the pJet1.2::sll0517-Toop construct by inverse PCR
pJet-sll0517-3xFLAG_rev	TCATGATCTTTATAATCGCCATCATGATCTTTATAATCCATGTAGCGG CTACCACCATAGCTTTTAC	
pJet-ssr1480-Toop_fwd	CGGGCGTTTTTATTCTCGAGATCTTGCTGAAAACTCGAGCC	Introduction of the oop terminator into the pJet1.2::ssr1480 construct by inverse PCR
pJet-ssr1480-Toop_rev	GCGGCAACCGAGCGAATCCCCAGTCTATCAGGCAAGCCTGCC TAGCAAA	
pJet-ssr1480-3xFLAG_fwd	TGATATTGATTATAAAGATGATGATGATAAATAAGTTTTTGGCCTAAT TACCTGAATTTAAGATTTTCATTC	Introduction of a 3x FLAG tag into the pJet1.2::ssr1480-Toop construct by inverse PCR
pJet-ssr1480-3xFLAG_rev	TGATCTTTATAATCGCCATCATGATCTTTATAATCCATACGAGGGGTT CTCGGTCTTGC	
pJet-slr0193-Toop_fwd	CGGGCGTTTTTATTCTCGAGATCTTTCTAGAAGATCTCC TACAATATT	Introduction of the ooP terminator into the pJet1.2::slr0193 construct by inverse PCR
pJet-slr0193-Toop_rev	GCGGCAACCGAGCGAAGTTTTTATTAAGCTCTAAACAGGACAAAG	
pJet-slr0193-3xFLAG_fwd	ATATTGATTATAAAGATGATGATGATAAATAGGTCCACAGTTTTTCCC TGAACCGGAACTGTTC	Introduction of a 3x FLAG tag into the pJet1.2::slr0193-Toop construct by inverse PCR
pJet-slr0193-3xFLAG_rev	CATGATCTTTATAATCGCCATCATGATCTTTATAATCCATCTGGGCCG CTGTCAGTTTTTCTTTTAC	
PpetE-pjet-sll0517 fwd	ATGTCAATTTATGTAGGCAACCTGTC	Amplification of the pJet1.2::sll0517 construct for inserting the PpetE via Aqua-cloning
PpetE-pjet-sll0517 rev	AAGCTTATCTTGCTGAAAACTCGAG	
pjet-sll0517-PpetE fwd	TCGAGTTTTTCAGCAAGATAAGCTTCTGGGCTACTGGGCTATTC	Amplification of the <i>petE</i> promoter and introducing sequences homologous to pJet1.2::sll0517 constructs up- and downstream
pjet-sll0517-PpetE rev	TTGCCTACATAAATTGACATACTTCTTGCGATTGTATCTATAGG	
PpetE-pjet-ssr1480 fwd	ATGTCCATTTATGTCGGG	Amplification of the pJet1.2::ssr1480 constructs for inserting PpetE via Aqua-cloning
PpetE-pjet-ssr1480 rev	AAGCTTATCTTTCTAGAAGATCTC	
pjet-ssr1480-PpetE fwd	CGTATCACGAGGCCAAGCTTCTGGGCTACTGGGCTATTC	Amplification of the <i>petE</i> promoter and introducing sequences homologous to pJet1.2::ssr1480
pjet-ssr1480-PpetE rev	TTCCCGACATAAATTGACATACTTCTTGCGATTGTATCTATAGG	

		constructs up- and downstream.
PpetE-pjet-slr0193 fwd	ATGTCCATTCGTCTCTACGTCCGGTAACC	Amplification of the plet1.2:: <i>slr0193</i> construct for inserting PpetE via Aqua-cloning
PpetE-pjet-slr0193 rev	AAGCTTATCTTGCTGAAAACTCGAGCCATC	
pjet-slr0193-PpetE fwd	TCGAGTTTTTCAGCAAGATAAGCTTCTGGGCCTACTGGGCTATTC	Amplification of the <i>petE</i> promoter and introducing sequences homologous to plet1.2:: <i>slr0193</i> up- and downstream
pjet-slr0193-PpetE rev	ACGTAGAGACGAATGGACATACTTCTTGCGATTGTATCTATAGG	
pVZ322-hindIII-seq_fwd	TACAACCTATTAATTTCCCTC	Colony PCR and sequencing of the pVZ322 plasmid and its derivatives
pVZ322-xhoI-seq_rev	ATGAACAATAAACTGTCTGCT	

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923 **Supplementary Table 5: Primers used for RT-qPCR**

Oligonucleotide name	Sequence (3' → 5')	Description
psbA2_qPCR fw	GTTCCAATCTGAACATCGACAAATAC	Primer for <i>psbA2</i> amplification for RT/qPCR from fished RNA
psbA2_qPCR rev	CACTGACAAAACCTGTTCCAC	
RnpB_qPCR fw	GCACCAATTTCCAAGACTAC	Primer for <i>RnpB</i> amplification for RT/qPCR from fished RNA
RnpB_qPCR rev	TCTCTTTTCTAGTGCCATTG	
psaA_2TSS_qPCR fw	GTGATGTTTGCTGAAAACGCC	Primer for the amplification of the second TSS of the <i>psaA</i> mRNA for RT/qPCR from fished RNA
psaA_2TSS_qPCR rev	GCAGAATAGTGTAATAGAGGAAG	
psaA_qPCR fw	ATAGGAAACCTTAATAGTTCATTG	Primer for <i>psaA</i> amplification for RT/qPCR from fished RNA
psaA_qPCR rev	GTTGTTATCAACCGAGACTTTG	

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**Supplementary Table 6: Primers used for Northern Blot analysis**

Name	Sequence (5' to 3')
<i>psbA2_T7</i>	TAATACGACTCACTATAGGGGAGCGCGCTGTTGGAGAGTCGTTGTC
<i>psbA2_R</i>	AGTCAGTTCCAATCTGAACATCGAC
<i>psaA_F</i>	TGGTTCCACTACCACGTCAA
<i>psaA_R</i>	ACCATGAAGTCGGCAGTACC
<i>rnpB_F</i>	GAGTTAGGGAGGGAGTTGCGG
<i>rnpB_T7</i>	TAATACGACTCACTATAGGGGCACTGTCCTCACGCTCGC

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