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1   Short Communication

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3   Efficient targeting of recombinant proteins to the thylakoid lumen in

4   *Chlamydomonas reinhardtii* using a bacterial Tat signal peptide

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16

19   **Abstract**

20   Interest in the exploitation of microalgae for biotechnological applications has

21   increased over the last decade, and microalgae are now viewed as offering a

22   sustainable alternative to traditionally used host chassis. A number of recombinant

23   proteins have been expressed in genetically modified algal strains, with the green alga

24   *Chlamydomonas reinhardtii* being a particularly popular host strain. While nuclear

25   transformation is possible with this organism, chloroplast transformation offers more

26   reliable expression, and several proteins have been expressed in the stroma. Here we

27   present the first utilisation of the thylakoid lumen for recombinant protein production

28   in microalgae. A bacterial export signal peptide was used to efficiently translocate

29   two recombinant proteins, a fluorescent reporter protein (pHRed) and a

30   biopharmaceutical model substrate (scFv) into the thylakoid lumen. This approach

31   expands the algal chloroplast genetic toolkit and offers a means of expressing proteins

32   that are difficult to express in the stroma for reasons of toxicity, stability or a

33   requirement for disulphide bonding.

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39   **1. Introduction**

40   The green alga *Chlamydomonas reinhardtii* has been used as a host for the

41   expression of a variety of recombinant proteins, and its biotechnological potential has

42   been explored in many studies over the last decade. A number of heterologous

43   proteins have been expressed, including vaccines, antibody fragments and terpene

44   synthesis enzymes [for recent reviews see 1-3]. Tools for the genetic engineering of

45   this green microalga have advanced remarkably, and it is now possible to transform

46   both the nuclear and chloroplast genomes with reasonable efficiency. Chloroplast

47   transformation offers the advantage that gene integration occurs by homologous

48   recombination at specific sites, whereas nuclear transformation is essentially random

49   with frequent gene silencing [e.g. 4, 5]. However, all of the chloroplast transformants

50   reported to date have involved expression of the target protein in the stroma, with the

51   exception of a study in which the target protein, a cytochrome P450, was targeted into

52   a membrane (probably the thylakoid membrane) [6].

53   In this study we present a novel approach to expand the genetic tool kit of the

54   algal chloroplast involving targeting to the thylakoid lumen. The thylakoid lumen is

55   an important compartment playing a key role in photosynthesis and energy generation

56   in chloroplasts. However, it has a relatively small proteome [7] and it offers a very

57   different environment compared to the stroma: for example, the pH is lower and the

58   lumen is an oxidising environment that is conducive to disulphide bonding. This

59   could have advantages for the expression of some proteins and enrich the potential of

60   the algal chloroplast as a production platform. In chloroplasts, proteins are naturally

61   targeted across the thylakoid membrane by the Sec or Tat pathways, and attachment

62   of a Sec or Tat signal peptide to a heterologous protein often results in correct

63   targeting and maturation (reviewed in [8]). Here, we used the TorA Tat signal peptide

64   from *Escherichia coli* which has been used to direct the export of biotechnologically

65   relevant proteins to the periplasm in *E. coli* (reviewed in [9]) as a targeting peptide to

66   translocate recombinant proteins into the thylakoid lumen of the *C. reinhardtii*

67   chloroplast. We show that the Tat signal peptide can target and translocate both a

68   fluorescent reporter protein, pHRed, and a biopharmaceutical (scFv antibody

69   fragment) into the thylakoid lumen of *C. reinhardtii*.

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2. Material and methods

2.1. Plasmid construction

74   The sequence for the pHRed fluorescent protein was obtained from the

75   plasmid GW1-pHRed (ORF3, addgene plasmid 31473) [10]. The sequence for

76   scFvIL1B (scFv) was obtained from the plasmid pYU49 [11]. An HA-tag (amino acid

77   sequence (AA) YPYDVPDYA) was added at the C-terminus of every synthetic gene

78   for detection by western blotting. Two constructs were made for each protein, pHRed

79   and scFv respectively: one stroma control (sequence encoding mature protein only)

80   and one with a bacterial Tat export signal peptide from TMO reductase (AA

81   sequence: NNNDLFQASRRRFLAQLGGLTVAGMLGPSLLTPRRATAAQAA

82   inserted after the methionine start codon and the first amino acid of the mature protein

83   sequence) referred to as "TorA signal peptide" [12]. All genes were codon-optimised

84   for chloroplast expression in *C. reinhardtii* using the software 'Codon Usage

85   Optimizer' (codonusageoptimizer.org/download/). The synthetic genes were custom

86   synthesised by GenScript (USA). All constructs made for this study were based on the

87   vectors pASapI [13] and pSRSapI [14] for chloroplast expression in *C. reinhardtii*.

88   An overview of the constructs made for this study is given in Table 1. Plasmid pJZ19

89   was assembled with the Gibson assembly method [15]. All other constructs were

90   made by cutting with the restriction enzymes *SapI* and *SphI* (NEB) and subsequent

4

ligation into pASapI/pSRSapI. All constructs were sequenced to confirm the correct

nucleotide sequence of the synthetic gene.

Table 1: Overview of constructs described in this study. All constructs were made using the

transformation vectors pASapI (atpA 3'UTR) [13] or pSRSapI (psaA 3'UTR) [14] for

integration downstream of *psbH* in the chloroplast genome. The predicted location of the

protein in the chloroplast (*) is based on the presence or absence of a TorA signal peptide in

the presequence of the protein.

Plasmid	Synthetic gene	Encoded protein	Expected MW (kDa)	Predicted location*
pJZ21	*psaA 3'UTR-scFv1L1b-HA*	scFv-HA Single-chain Fv (recombinant antibody fragment, C-terminal HA-tag) against interleukin 1β [11,16]	27.8	Stroma
pJZ20	*atpA 3'UTR-scFv1L1b-HA*			
pJZ23	*psaA 3'UTR-TorAsp-scFv1L1b-HA*	TorA-scFv-HA (scFv-HA with N-terminal TorA leader peptide)	Pre sequence: 32.3	Thylakoid lumen
pJZ22	*atpA 3'UTR-TorA-scFv1L1b-HA*		Mature size: 27.8	
pJZ25	*psaA 3'UTR-pHRed-HA*	pHRed-HA [10]	27.3	Stroma
pJZ26	*psaA 3'UTR-torA-pHRed-HA*	TorA-pHRed-HA (pHRed-HA with N-	Pre sequence: 31.7	Thylakoid lumen

| pJZ19 | atpA 3'UTR-torA-pHRed-HA | terminal TorA leader peptide) | Mature size: 27.3 | |

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2.2. Cultivation and chloroplast transformation of *C. reinhardtii*

All *C. reinhardtii* strains were cultivated in TAP medium using the recipe as described by Gorman and Levine [17] with modified trace element solutions [18]. For selection of chloroplast transformants and confocal imaging (see section 2.6. for details) HSM medium [19] with the modified trace element solutions [18] was used. The protocol for chloroplast transformation was used as described in Economou et al. [13] using the strain TN72 (cw15, *psbH::aadA,* mt+) as a recipient. Further details of the cell line generation were as previously described in Zedler et al [20]. Other than the cell lines generated in this study by transformation with the plasmids as shown in Table 1, a strain with a restored functional *psbH* gene was made by transforming pSRSapI [14] without any synthetic gene integrated into TN72. This strain was named TN72-RP* and served as a negative control for transformants based on the pSRSapI vector. The strain TN72-RP (TN72 transformed with pASapI) [20] was used as a negative control for pASapI based transformants.

2.3. Homoplasmy analysis of transformants by PCR

A Chelex-100 resin (Bio-Rad) was used to extract total genomic DNA from *C. reinhardtii* using a protocol described elsewhere [13]. Transformants generated with the pASapI vector were analysed by PCR as described in Zedler et al. [20]. The same protocol was used for transformants generated with pSRSapI-based constructs. The primers FLANK1, rbcL.F (both previously described [20]) were used in conjunction

122   with the primer psaA.R (5'-GGATTTCTCCTTATAATAAC-3') in a standard PCR

123   protocol with an annealing temperature of 54°C. Sequences for the primer design

124   were kindly provided by Saul Purton (University College London, UK).

125

126   **2.4. Cell lysis, SDS-PAGE and western blotting**

127   Crude cell lysates for protein expression analysis were prepared from *C.*

128   *reinhardtii* cells that were grown in 6 well plates in TAP medium at 25°C, 110 rpm

129   shaking and approx. 50 μE. A volume of cells equivalent to 0.5 mL of a culture with

130   an optical density of $OD_{750}=1$ measured on a DU 730 UV/Vis Spectrophotometer

131   (Beckman Coulter) were harvested from each sample and resuspended in 0.1 ml 10

132   mM Tris-HCl (pH 8.0). 0.025 ml 5x SDS protein gel loading buffer (containing β-

133   mercaptoethanol as a reducing agent) were added to the samples and then boiled at

134   95°C for 5 minutes. The crude lysates were separated and analysed by SDS-PAGE on

135   a 15% sodium dodecyl sulphate-polyacrylamide gel and immunoblotted. An HA-

136   antibody (Sigma-Aldrich) was used to detect the target protein and an AtpB-antibody

137   (Agrisera, Sweden) as a loading control.

138

139   **2.5. Chloroplast isolation and fractionation**

140   Chloroplasts were isolated from 1 L liquid cultures that were grown in TAP

141   medium to mid log phase at 25°C, 120 rpm shaking and constantly illuminated with

142   approx. 50μE. The protocol described by Mason et al. [21] was used for chloroplast

143   isolation. After washing the isolated chloroplasts in 0.1% BSA isolation buffer, the

144   chloroplasts were directly resuspended in hypotonic lysis buffer for fractionation into

145   stroma and thylakoids (a membrane fraction also including the chloroplast envelope

146   membranes) as described in Balczun et al [22]. The lysate loaded on 1 M Sucrose

147   cushions was centrifuged in a Beckman TL-100 ultracentrifuge at 95 000 rpm, 4°C

148   for two hours using a TLA100.3 rotor (Beckman). The thylakoid fraction was then

149   resuspended in 1x lysis buffer [22]. Samples were boiled at 50°C for 10 minutes and

150   subjected to SDS-PAGE and Western Blotting as described in Section 2.4. The

151   samples were immunoblotted with the HA-antibody and with a PsbO antibody kindly

152   provided by Saul Purton (University College London, UK) as a control for the

153   fractionation.

154

155   **2.6. Confocal imaging of *Chlamydomonas* cells**

156   *C. reinhardtii* cells were taken from liquid cultures in HSM medium, spotted

157   onto glass microscope slides and covered with glass cover-slips. Cells were imaged

158   using a Leica TCS SP5 laser-scanning confocal microscope, using a 63x oil-

159   immersion objective (NA 1.4) and excitation with a 561 nm laser line. Fluorescence

160   emission was detected simultaneously at 600-620 nm for pHRed and 670-720 nm for

161   chlorophyll. The confocal pinhole was set to give a *z*-axis resolution of about 1.5 µm.

162   Images were recorded with scanning at 400 Hz, with each line generated by an

163   average from 6 scans. Quantitative image analysis was with Image J software, with

164   statistics from SigmaPlot 13.0.

165
166
167   **3. Results and discussion**

168

169   **3.1. pHRed and an scFv are robustly expressed in the chloroplast**

170   The aim of this study was to test for targeting of a reporter protein, pHRed,

171   and a biotechnologically-relevant protein (an scFv) to the thylakoid lumen by the Tat

172   pathway. For comparisons of expression levels, and for control purposes, we also

173   expressed the mature-size pHRed protein in the stroma. Screening relied on the

174   restoration of phototrophic growth after transformation, as homologous recombination

175   restores the intactness of the *psbH* gene (see [13] for details). One series of constructs

176   was cloned into the plasmid pASapI, which uses the *atpA* promoter, and a second

177   series of transformations was carried out using constructs based on the plasmid

178   pSRSapI, which uses the *psaA* promoter. *C. reinhardtii* chloroplast transformants

179   expressing the constructs detailed in Table 1 were successfully generated using the

180   recipient strain TN72 as detailed in Economou et al. [13]. Homoplasmy analysis by

181   PCR confirmed that all strains were homoplasmic ensuring stable integration of the

182   gene into the chloroplast genome (Fig. 1).

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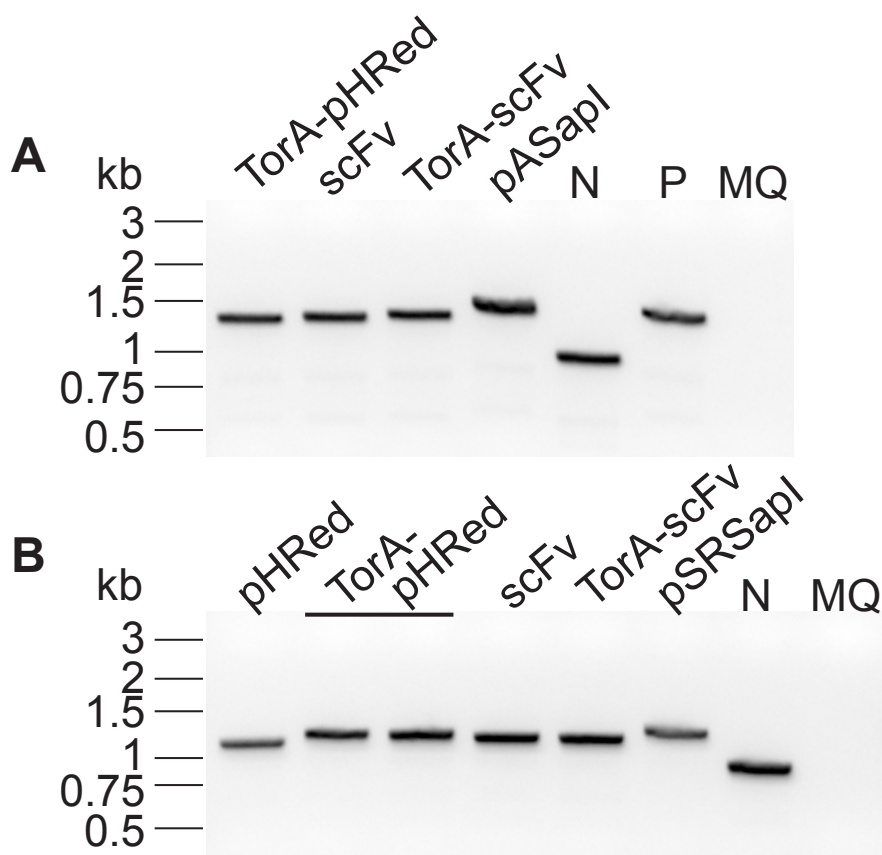
184

185 **Fig. 1**: <u>Homoplasmy analysis of transformants expressing pHRed and scFv constructs</u>.
186 PCR for homoplasmy analysis was carried out on the TN72 strains transformed with
187 constructs based on pASapI (Fig. 1A) and pSRSapI (Fig. 1B). All lanes indicate the
188 construct that was used for transformation (details shown in Table 1) to generate the
189 respective strain. pJZ26a/26b represent two separate transformants expressing TorA-
190 pHRed. N denotes the strain TN72, used as a negative control for the PCR reaction
191 (i.e. with no gene integrated), P denotes a positive control (i.e. gene has been
192 integrated). In the lanes 'MQ' water was used instead of a DNA template as a
193 negative control for the PCR reaction.
194

195   Further analysis of the transformants by SDS-PAGE and western blotting of

196 crude cell lysates showed that the cells were expressing the respective protein, i.e.

197 scFv or pHRed, in the chloroplast at stable levels (Fig. 2). Fig. 2A shows (from left to

198 right) blots of stromal pHRed and TorA-pHRed (in 2 different transformants),

199 expressed from the same *psaA* promoter. The protein is clearly detected as a band of

200 ca. 27 kDa and the levels of the stromal and lumen-targeted versions are reasonably

201 similar. The next lane shows that lower TorA-pHRed levels were obtained when

202 expressed from the *atpA* promoter, and the band is absent from the control strains

203 transformed with empty pSRSapI or pASapI vector (RP*, RP).

204

205
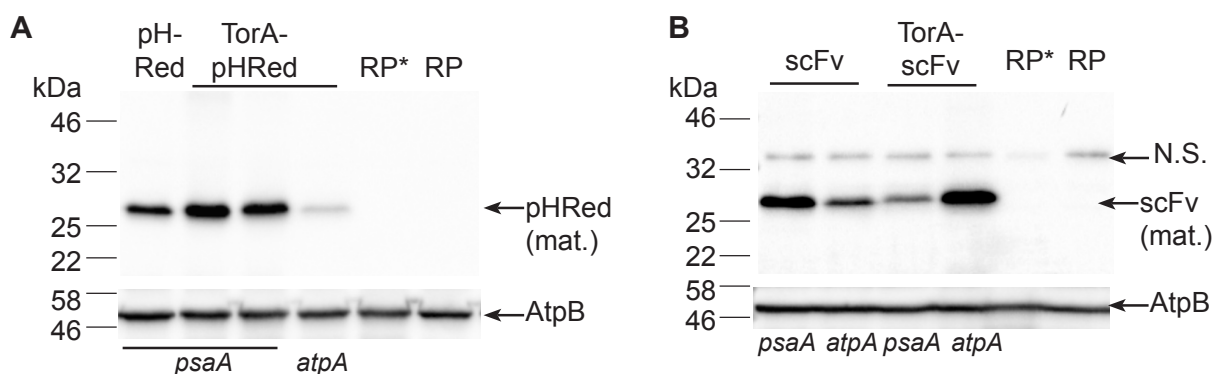
206
207 **Fig. 2**: <u>Expression of pHRed and scFv with and without TorA signal peptide in the</u>
208 <u>chloroplast of *C. reinhardtii*.</u> **A** shows an immunoblot of cell lysates from strain TN72
209 transformed with the following constructs from left to right: pJZ25 (pHRed); JZ26a,

210 pJZ26b, pJZ19 (TorA-pHRed); pSRSapI (RP*); pASapI (RP). The blot was probed
211 with antibodies to the HA tag on the C-termini of the target proteins, and the arrow
212 indicates the mature protein size of pHRed. **B** shows a blot of cell lysates from TorA-
213 scFv and mature-size scFv constructs. The TN72 transformant strains from left to
214 right are: pJZ21, pJZ20 (scFv); pJZ23, pJZ22 (TorA-scFv); pSRSapI (RP*), pASapI
215 (RP). The mature-size scFv protein is marked with an arrow. N.S. indicates a non-
216 specifically reacting band that has been previously observed [20]. In both A and B the
217 promoter used to drive expression is shown below the blot. The blots below the anti-
218 HA blot were probed with an anti-AtpB antibody showing approximately equal
219 loading of lysates in all lanes; the AtpB protein is marked with an arrow.
220

221   Fig. 2B shows blots of transformants expressing the 28 kDa scFv in the stroma

222 from the *psaA* or *atpA* promoter, with slightly higher levels detected in the former.

223 Slightly surprisingly, expression of TorA-scFv yields somewhat different results, with

224 protein levels higher when expressed from the *atpA* promoter.

225   In both the A and B panels, the blots were reprobed using antibodies to AtpB

226 as loading controls. It is also notable that in Fig. 2B we detect a band of about 34 kDa

227 which has previously been shown to stem from non-specific reaction of an unknown

228 endogenous protein with the anti-HA antibodies [20].

229

230 **3.2. The precursor proteins TorA–pHRed and TorA–scFv are processed**
231 **efficiently**

232   Interestingly, in the strains expressing TorA-pHRed and TorA-scFv (Fig. 2),

233 only the mature protein sizes 27/28 kDa were detected, with the same molecular

234 weights as the stromal versions. No precursor protein, which would be expected to be

235 around 32 kDa for both proteins, was observed in our experiments. Lumen-targeted

236 precursor proteins are processed to the mature size after translocation by a lumen-

237 facing processing peptidase [8], so this provides preliminary evidence that both

238 proteins may be targeted to the thylakoid lumen and processed to the mature size.

239

240 **3.3. The TorA export signal peptide enables protein translocation to the**

241 **thylakoid lumen in the algal chloroplast**

242   Although the absence of the precursor protein is indirect evidence of targeting

243 to the lumen, fractionation studies were deemed essential to confirm this point, and

244 chloroplasts were therefore isolated and fractionated into stroma and thylakoids by

245 hypotonic lysis. The results of the fractionation are shown in Fig. 3, with the target

246 proteins again detected by immunoblotting with antibodies to their C-terminal HA

247 tags. Both TorA-pHRed (Fig. 3A) and TorA-scFv (Fig. 3B) were translocated into the

248 thylakoids, with the mature-size proteins ('mat') clearly detected in the thylakoid

249 fraction (T) but not the stroma (S). The stromal pHRed is detected in the stroma as

250 expected (Fig. 3A) as was the stromal scFv (data not shown). The 34 kDa band from

251 non-specific binding of the anti-HA antibody is also apparent.

252
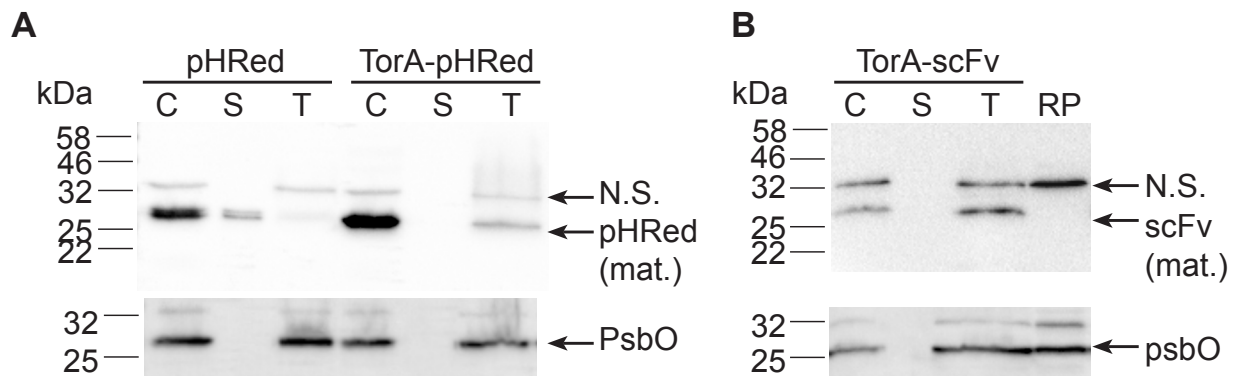
253

254

255 **Fig. 3**: Fractionation of chloroplasts into stroma and thylakoids (including envelope
256 membranes). Whole chloroplast lysates (C), the stroma fraction (S) and the thylakoid
257 fraction (T) are shown. **A** shows an Anti-HA immunoblot of chloroplast fractions
258 from strain TN72 transformed with pJZ25 (pHRed) and pJZ26 (TorA-pHred). **B**
259 shows the fractions of TN72 transformed with pJZ23 (TorA-scFv). RP denotes the
260 negative control showing that the band indicated with N.S. is a non-specific reacting
261 band present in the negative control. A second immunoblot probed with an PsbO

262 antibody, shown in A and B respectively, serves as a control for the fractionation. The
263 PsbO protein is denoted with an arrow.
264

265   The fractions were also probed with a PsbO antibody as a control for the

266 fractionation; PsbO is a well-known lumenal protein that forms part of the

267 photosystem II oxygen-evolving complex. This control confirms that the targeted

268 proteins are indeed in the thylakoid fraction. From these results, it is apparent that a

269 bacterial Tat export signal peptide is suitable for translocation of proteins to the

270 thylakoid lumen in *C. reinhardtii*.

271

272 **3.4. TorA–pHRed is specifically targeted to the thylakoids**

273   Representative confocal images of the strains expressing the fluorescent

274 reporter gene *pHRed* are shown in Fig. 4. All images were recorded with 561 nm

275 excitation and emission at 600-620 nm, a combination which proved to give the most

276 selective visualisation of pHRed relative to the background fluorescence from the

277 photosynthetic pigments. However, even at these wavelengths there was significant

278 non-pHRed fluorescence from the thylakoid membranes (see right-hand panels of Fig.

279 4, showing the control strain), which complicates analysis of the distribution of

280 pHRed. For quantitative comparison of fluorescence yields we manually selected

281 either the whole cell or the pyrenoid region in the chloroplast (see Fig. 4) and

282 measured the mean fluorescence in these regions, a procedure which automatically

283 corrects for differences in cell size. For cells expressing stromal pHRed, mean

284 fluorescence at 600-620 nm was 36% higher than in the control strain ($n = 20$, $p = $

285 0.00013 from a Student's *t*-test). The difference was even more pronounced when

286 fluorescence was measured only from the pyrenoid region, an area of the chloroplast

287 stroma where there is an optically-resolvable gap between the thylakoid membranes

13

288   so that the background fluorescence from the photosynthetic pigments is lower in this

289   region (see Fig. 4). In the pyrenoid region, 600-620 nm mean fluorescence from cells

290   with stromal pHRed was 65% higher than in the control strain ($n = 20$, $p = 0.000087$),

291   and most cells with stromal pHRed showed an obvious fluorescence signal from the

292   pyrenoid that was absent from the other strains (Fig. 4).

293
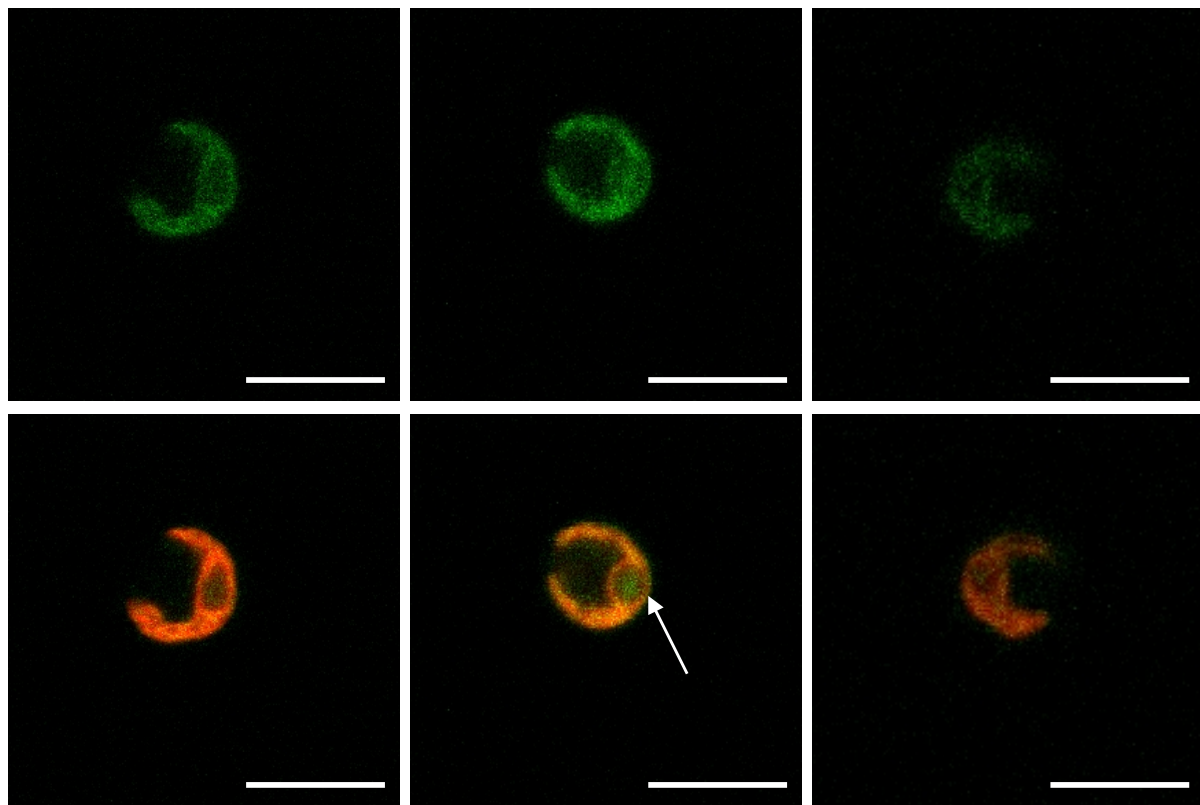
TorA-pHRed (lumenal)   pHRed (stromal)   control (RP*)

294

295   **Fig. 4** Representative confocal fluorescence images of *C. reinhardtii* cells expressing
296   pHRed and TorA-pHRed, with cells having the empty transformation plasmid
297   integrated (RP*) as a control. Top: images of fluorescence in the pHRed region 600-
298   620 nm, shown in green. Bottom: the same images merged with chlorophyll
299   fluorescence at 670-720 nm, shown in red. The white arrow highlights stromal pHRed
300   fluorescence from the pyrenoid region. Scale bar: 10 μm.
301

302   Cells expressing TorA-pHRed) showed mean cell fluorescence only

303   marginally (6%) higher than the control strain, without compelling statistical

14

304 significance for a difference ($n = 20$, $p = 0.24$). Western blots indicate that TorA-

305 pHRed protein is present at similar levels to stromal pHRed (Fig. 2), so it appears that

306 fluorescence from lumenal pHRed must be somewhat quenched relative to stromal

307 pHRed. This quenching cannot be a simple consequence of pH difference, since

308 pHRed should show enhanced fluorescence with excitation at 561 nm at the lower pH

309 expected in the thylakoid lumen [10]. Our fluorescence images confirm different

310 distributions of stromal and lumenal pHRed demonstrated by the fractionation

311 experiments (Fig. 3A). Lumenal pHRed appeared largely absent from the pyrenoid

312 region of the stroma, since fluorescence in this region was 28% lower than in the

313 strain expressing stromal pHRed ($n = 20$, $p = 0.00038$) and only marginally higher

314 than in the control strain (18% higher, $n = 20$, $p = 0.026$).

315

316 **3.5. Potential of lumen targeting in microalgae for biotechnology**

317   The Tat machinery is specialised for the translocation of fully folded proteins

318 and it has previously been shown that the bacterial Tat system has quality control

319 (proofreading) capabilities, such that *correctly*-folded proteins are preferentially

320 transported (reviewed in [23]). Correct protein folding is highly advantageous for

321 recombinant protein production, and if the thylakoid Tat system has similar

322 properties, the lumen may therefore offer certain advantages over the stroma; with the

323 transported proteins exhibiting high folding fidelity. The lumen may also represent a

324 beneficial environment for the production of disulphide-bonded proteins. In tobacco

325 chloroplasts, disulphide bond formation tested with a recombinant protein (alkaline

326 phosphatase) was reported to be more efficient in the thylakoid lumen than in the

327 stroma [24]. This supports the idea of the thylakoid lumen as a novel compartment for

328 recombinant protein production. Finally, a number of potential target proteins may be

329 toxic in the stroma, or may catalyse unwanted metabolic processes, and the lumen
330 may offer a 'safe haven' for such proteins.

331

332 **4. Conclusion**

333 We have shown that a bacterial Tat export signal peptide is capable of

334 directing the translocation of model and biotechnologically relevant recombinant

335 proteins into the thylakoid lumen of the *C. reinhardtii* chloroplast. The thylakoid

336 lumen may therefore provide a protective environment for delicate proteins that

337 require tight folding, especially for proteins that are potentially toxic or which are

338 more stable at a lower pH. This process thus represents an addition to the 'algal

339 chloroplast toolkit' with potential for enhancing the competitiveness of microalgae as

340 production platforms.

341

342

343 **Research contribution**

344 JAZZ designed the experiments, acquired and analysed the data, drafted and

345 approved the manuscript. CWM acquired the confocal images, analysed the images,

346 wrote and approved the manuscript. CR designed the experiments together with

347 JAZZ, approved and edited the manuscript.

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