Kent Academic Repository

Full text document (pdf)

Citation for published version

Zedler, Julie A.Z. and Mullineaux, Conrad W. and Robinson, Colin (2016) Efficient targeting of recombinant proteins to the thylakoid lumen in Chlamydomonas reinhardtii using a bacterial Tat signal peptide. Algal Research, 19 . pp. 57-62. ISSN 2211-9264.

DOI

https://doi.org/10.1016/j.algal.2016.07.007

Link to record in KAR

http://kar.kent.ac.uk/58522/

Document Version

Author's Accepted Manuscript

Copyright & reuse
Content in the Kent Academic Repository is made available for research purposes. Unless otherwise stated all content is protected by copyright and in the absence of an open licence (eg Creative Commons), permissions for further reuse of content should be sought from the publisher, author or other copyright holder.

Versions of research
The version in the Kent Academic Repository may differ from the final published version.
Users are advised to check **http://kar.kent.ac.uk** for the status of the paper. **Users should always cite the published version of record.**

Enquiries
For any further enquiries regarding the licence status of this document, please contact:
researchsupport@kent.ac.uk

If you believe this document infringes copyright then please contact the KAR admin team with the take-down information provided at **http://kar.kent.ac.uk/contact.html**

KAR Kent Academic Repository

University of Kent

Short Communication

Efficient targeting of recombinant proteins to the thylakoid lumen in *Chlamydomonas reinhardtii* using a bacterial Tat signal peptide

Julie A. Z. Zedler[a], Conrad W. Mullineaux[b], Colin Robinson[a]*

[a] Centre for Molecular Processing, School of Biosciences, University of Kent, Canterbury CT2 7NJ, UK

[b] School of Biological and Chemical Sciences, Queen Mary University of London, London E1 4NS, UK

*Corresponding author: Address correspondence to Colin Robinson. Telephone: +44 1227 823443; email c.robinson-504@kent.ac.uk

© 2016. This manuscript version is made available under the CC-BY-NC-ND 4.0 license http://creativecommons.org/licenses/by-nc-nd/4.0/

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.

34

35 **Keywords**

36 *Chlamydomonas*, Thylakoid lumen, Protein Targeting, TorA signal peptide,
37 Fluorescent Sensor, Antibody Fragment

38

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

fluorescent reporter protein, pHRed, and a biopharmaceutical (scFv antibody fragment) into the thylakoid lumen of *C. reinhardtii*.

2. Material and methods

2.1. Plasmid construction

The sequence for the pHRed fluorescent protein was obtained from the plasmid GW1-pHRed (ORF3, addgene plasmid 31473) [10]. The sequence for scFvIL1B (scFv) was obtained from the plasmid pYU49 [11]. An HA-tag (amino acid sequence (AA) YPYDVPDYA) was added at the C-terminus of every synthetic gene for detection by western blotting. Two constructs were made for each protein, pHRed and scFv respectively: one stroma control (sequence encoding mature protein only) and one with a bacterial Tat export signal peptide from TMO reductase (AA sequence: NNNDLFQASRRRFLAQLGGLTVAGMLGPSLLTPRRATAAQAA inserted after the methionine start codon and the first amino acid of the mature protein sequence) referred to as "TorA signal peptide" [12]. All genes were codon-optimised for chloroplast expression in *C. reinhardtii* using the software 'Codon Usage Optimizer' (codonusageoptimizer.org/download/). The synthetic genes were custom synthesised by GenScript (USA). All constructs made for this study were based on the vectors pASapI [13] and pSRSapI [14] for chloroplast expression in *C. reinhardtii*. An overview of the constructs made for this study is given in Table 1. Plasmid pJZ19 was assembled with the Gibson assembly method [15]. All other constructs were made by cutting with the restriction enzymes *SapI* and *SphI* (NEB) and subsequent

91 ligation into pASapI/pSRSapI. All constructs were sequenced to confirm the correct
92 nucleotide sequence of the synthetic gene.
93
94 **Table 1**: Overview of constructs described in this study. All constructs were made using the
95 transformation vectors pASapI (atpA 3'UTR) [13] or pSRSapI (psaA 3'UTR) [14] for
96 integration downstream of *psbH* in the chloroplast genome. The predicted location of the
97 protein in the chloroplast (*) is based on the presence or absence of a TorA signal peptide in
98 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 Mature size: 27.8	Thylakoid lumen
pJZ22	*atpA 3'UTR-TorA-scFv1L1b-HA*			
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	

99

100

101 **2.2. Cultivation and chloroplast transformation of *C. reinhardtii***

102 All *C. reinhardtii* strains were cultivated in TAP medium using the recipe as
103 described by Gorman and Levine [17] with modified trace element solutions [18]. For
104 selection of chloroplast transformants and confocal imaging (see section 2.6. for
105 details) HSM medium [19] with the modified trace element solutions [18] was used.
106 The protocol for chloroplast transformation was used as described in Economou et al.
107 [13] using the strain TN72 (cw15, *psbH::aadA,* mt+) as a recipient. Further details of
108 the cell line generation were as previously described in Zedler et al [20]. Other than
109 the cell lines generated in this study by transformation with the plasmids as shown in
110 Table 1, a strain with a restored functional *psbH* gene was made by transforming
111 pSRSapI [14] without any synthetic gene integrated into TN72. This strain was named
112 TN72-RP* and served as a negative control for transformants based on the pSRSapI
113 vector. The strain TN72-RP (TN72 transformed with pASapI) [20] was used as a
114 negative control for pASapI based transformants.

115

116 **2.3. Homoplasmy analysis of transformants by PCR**

117 A Chelex-100 resin (Bio-Rad) was used to extract total genomic DNA from *C.*
118 *reinhardtii* using a protocol described elsewhere [13]. Transformants generated with
119 the pASapI vector were analysed by PCR as described in Zedler et al. [20]. The same
120 protocol was used for transformants generated with pSRSapI-based constructs. The
121 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

cushions was centrifuged in a Beckman TL-100 ultracentrifuge at 95 000 rpm, 4°C for two hours using a TLA100.3 rotor (Beckman). The thylakoid fraction was then resuspended in 1x lysis buffer [22]. Samples were boiled at 50°C for 10 minutes and subjected to SDS-PAGE and Western Blotting as described in Section 2.4. The samples were immunoblotted with the HA-antibody and with a PsbO antibody kindly provided by Saul Purton (University College London, UK) as a control for the fractionation.

2.6. Confocal imaging of *Chlamydomonas* cells

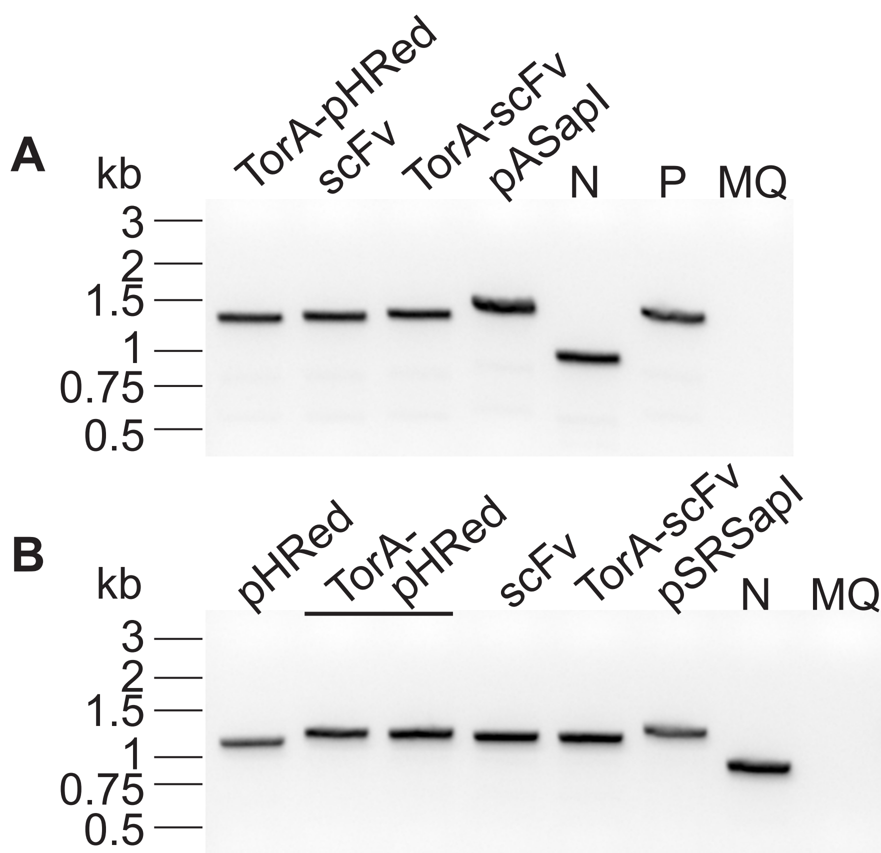
C. reinhardtii cells were taken from liquid cultures in HSM medium, spotted onto glass microscope slides and covered with glass cover-slips. Cells were imaged using a Leica TCS SP5 laser-scanning confocal microscope, using a 63x oil-immersion objective (NA 1.4) and excitation with a 561 nm laser line. Fluorescence emission was detected simultaneously at 600-620 nm for pHRed and 670-720 nm for chlorophyll. The confocal pinhole was set to give a *z*-axis resolution of about 1.5 µm. Images were recorded with scanning at 400 Hz, with each line generated by an average from 6 scans. Quantitative image analysis was with Image J software, with statistics from SigmaPlot 13.0.

3. Results and discussion

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

The aim of this study was to test for targeting of a reporter protein, pHRed, and a biotechnologically-relevant protein (an scFv) to the thylakoid lumen by the Tat

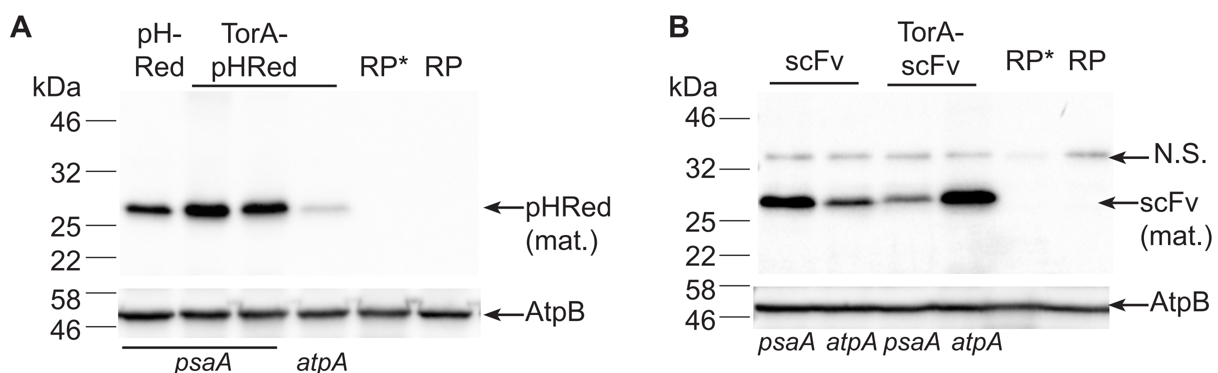
172 pathway. For comparisons of expression levels, and for control purposes, we also
173 expressed the mature-size pHRed protein in the stroma. Screening relied on the
174 restoration of phototrophic growth after transformation, as homologous recombination
175 restores the intactness of the *psbH* gene (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).
183



184

185 **Fig. 1**: Homoplasmy analysis of transformants expressing pHRed and scFv constructs.
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**: Expression of pHRed and scFv with and without TorA signal peptide in the
208 chloroplast of *C. reinhardtii*. **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.

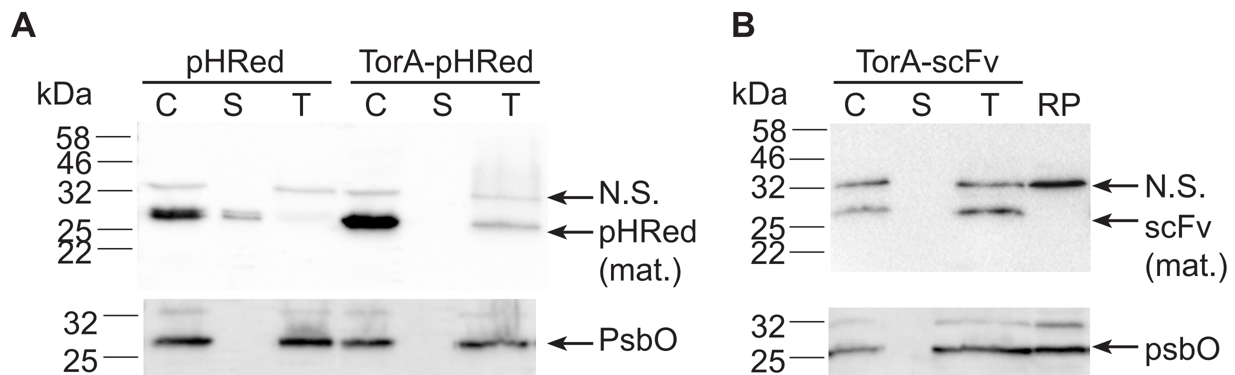
225 In both the A and B panels, the blots were reprobed using antibodies to AtpB
226 as loading controls. It is also notable that in Fig. 2B we detect a band of about 34 kDa
227 which has previously been shown to stem from non-specific reaction of an unknown
228 endogenous protein with the anti-HA antibodies [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

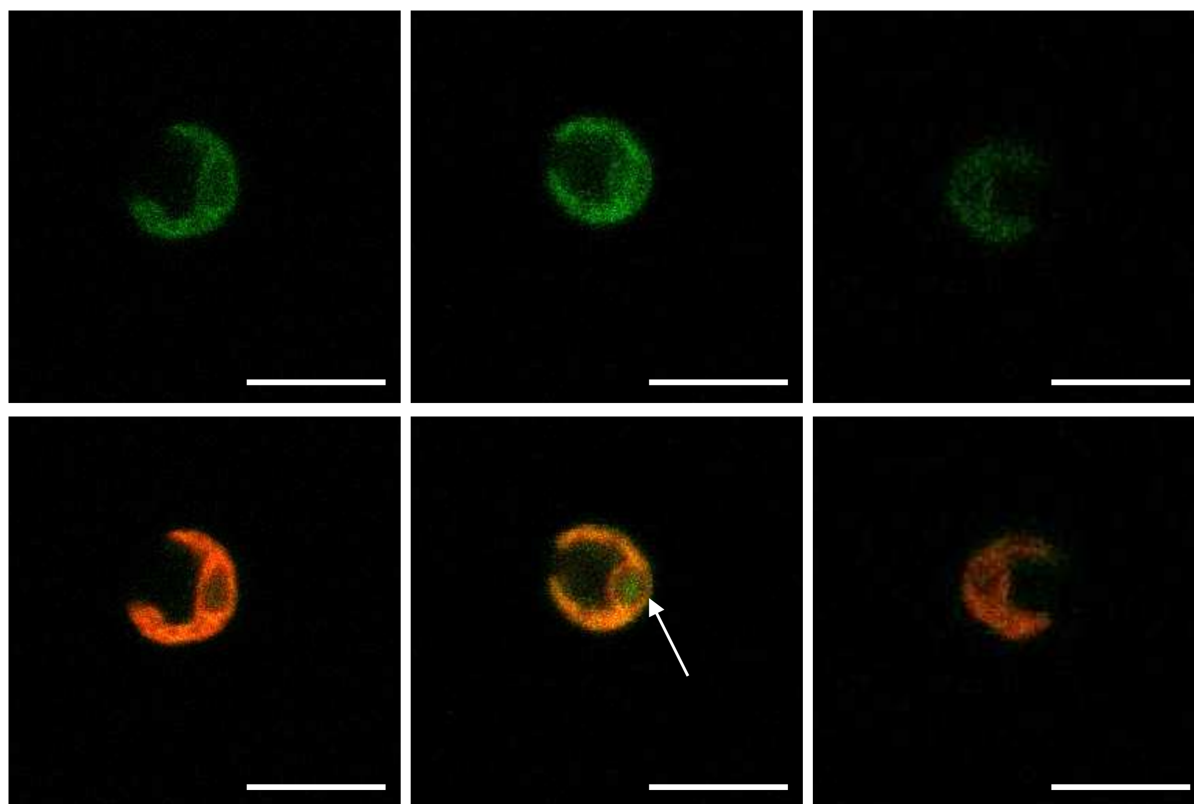
262 antibody, shown in A and B respectively, serves as a control for the fractionation. The
263 PsbO protein is denoted with an arrow.
264

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

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



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

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

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

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

4. Conclusion

We have shown that a bacterial Tat export signal peptide is capable of directing the translocation of model and biotechnologically relevant recombinant proteins into the thylakoid lumen of the *C. reinhardtii* chloroplast. The thylakoid lumen may therefore provide a protective environment for delicate proteins that require tight folding, especially for proteins that are potentially toxic or which are more stable at a lower pH. This process thus represents an addition to the 'algal chloroplast toolkit' with potential for enhancing the competitiveness of microalgae as production platforms.

Research contribution

JAZZ designed the experiments, acquired and analysed the data, drafted and approved the manuscript. CWM acquired the confocal images, analysed the images, wrote and approved the manuscript. CR designed the experiments together with JAZZ, approved and edited the manuscript.

Acknowledgments

The authors thank Tchern Lenn for assistance with the confocal imaging, and we thank Phil Mullineaux (University of Essex) for helpful discussions on the use of pHRed. The research leading to these results has received funding from the People

Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n. 317184. This material reflects only the authors' views and the Union is not liable for any use that may be made of the information contained therein.

References

[1] D. Gangl, J.A.Z. Zedler, P.D. Rajakumar, E.M. Ramos Martinez, A. Riseley, A. Włodarczyk, S. Purton, Y. Sakuragi, C.J. Howe, P.E. Jensen, C. Robinson, Biotechnological exploitation of microalgae, J. Exp. Bot. 66 (2015) 6975–6990.

[2] B.A. Rasala, S.P. Mayfield, Photosynthetic biomanufacturing in green algae; production of recombinant proteins for industrial, nutritional, and medical uses, Photosynthesis Research 123 (2015) 227–239.

[3] A.L. Almaraz-Delgado, J. Flores-Uribe, V.H. Pérez-España, E. Salgado-Manjarrez, J.A. Badillo-Corona, Production of therapeutic proteins in the chloroplast of *Chlamydomonas reinahrdtii*, AMB Express 4 (2014): 57.

[4] G. Potvin, Z. Zhang, Strategies for high-level recombinant protein expression in transgenic microalgae: A review, Biotechnol. Adv. 28 (2010) 910–918.

[5] S.Purton, J.B. Szaub, T. Wannathong, R. Young, C.K. Economou, Genetic Engineering of Algal Chloroplasts: Progress and Prospects, Russ. J. Plant Physiol. 60 (2013) 491–499.

[6] D. Gangl, J.A.Z. Zedler, A. Włodarczyk, P.E. Jensen, S. Purton, C. Robinson, Expression and membrane-targeting of an active plant cytochrome P450 in the chloroplast of the green alga *Chlamydomonas reinhardtii*, Phytochemistry 110 (2015) 22–28.

377 [7] T. Kieselbach, Å. Hagman, B. Andersson, W.P. Schröder, The Thylakoid Lumen
378 of Chloroplasts, J. Biol. Chem. 273 (1998) 6710–6716.
379 [8] A.M. Albiniak, J. Baglieri, C. Robinson, Targeting of lumenal proteins across the
380 thylakoid membrane, J. Exp. Bot. 63 (2012) 1689–1698.
381 [9] K.L. Walker, A.S. Jones, C.Robinson, The Tat pathway as a biotechnological tool
382 for the expression and export of heterologous proteins in Escherichia coli, Pharm.
383 Bioprocess. 3 (2015) 387–396.
384 [10] M. Tantama, Y.P. Hung, G. Yellen, Imaging Intracellular pH in Live Cells with a
385 Genetically-Encoded Red Fluorescent Protein Sensor, J. Am. Chem. Soc. 133 (2011)
386 10034–10037.
387 [11] C.F.R.O. Matos, C. Robinson, H.I. Alanen, P. Prus, Y. Uchida, L.W. Ruddock,
388 R.B. Freedman, E. Keshavarz-Moore, Efficient Export of Prefolded, Disulfide-
389 Bonded Recombinant Proteins to the Periplasm by the Tat Pathway in Escherichia
390 coli CyDisCO Strains, Biotechnol. Prog. 30 (2014) 281–290.
391 [12] V. Méjean, C. Iobbi-Nivol, M. Lepelletier, G. Giordano, M. Chippaux, M.C.
392 Pascal, The anaerobic respiration in *Escherichia coli*: involvement of the *tor* operon,
393 Mol. Microbiol. 11 (1994) 1169–1179.
394 [13] C. Economou, T. Wannathong, J. Szaub, S. Purton, A simple, low cost method
395 for chloroplast trsnsformation of the green alga *Chlamydomonas reinhardtii*, in: P.
396 Maliga (Ed.), Chloroplast Biotechnology, Methods in Molecular Biology 1132
397 (2014): 401-411.
398 [14] R.E.B. Young, S. Purton, Cytosine deaminase as a negative selectable marker for
399 the microalgal chloroplast: a strategy for the isolation of nuclear mutations that affect
400 chloroplast expression, Plant J. 80 (2014) 915–925.

401 [15] D.G. Gibson, L. Young, R.-Y. Chuang, J.C. Venter, C.A. III Hutchinson, O.H.
402 Smith, Enzymatic assembly of DNA molecules up to several hundred kilobases, Nat.
403 Methods 6 (2009) 343–345.
404 [16] I.C. Wilkinson, C.J. Hall, V. Veverka, J.Y. Shi, F.W. Muskett, P.E. Stephens,
405 R.J. Taylor, A.J. Henry, M.D. Carr, High resolution NMR-based model for the
406 structure of a scFv-1L-1b complex, J. Biol. Chem. 284 (2009) 31928–31935.
407 [17] D.S. Gorman, R.P. Levine, Cytochrome *f* and plastocyanin: their sequence in the
408 photosynthetic electron transport chain of Chlamydomonas reinhardi, Proc. Natl.
409 Acad. Sci. U. S. A. 54 (1965) 1665–1669.
410 [18] J. Kropat, A. Hong–Hermesdorf, D.. Casero, P. Ent, M. Castruita, M. Pellegrini,
411 S.S. Merchant, D. Malasarn, A revised mineral nutrient supplement increases biomass
412 and growth rate in *Chlamydomonas reinhardtii*, Plant J. 66 (2011) 770–780.
413 [19] N. Sueoka, MITOTIC REPLICATION OF DEOXYRIBONUCLEIC ACID IN
414 CHLAMYDOMONAD REINAHRDI, Proc. Natl. Acad. Sci. U. S. A. 46 (1960) 83–
415 91.
416 [20] J.A.Z. Zedler, D. Gangl, B. Hamberger, S. Purton, C. Robinson, Stable
417 expression of a bifunctional diterpene synthase in the chloroplast of *Chlamydomonas*
418 *reinhardtii*, J. Appl. Phycol. 27 (2015) 2271–2277.
419 [21] C.B. Mason, T.M. Bricker, J.V. Moroney, A rapid method for chloroplast
420 isolation from the green alga *Chlamydomonas reinhardtii*, Nat. Protoc. 1 (2006)
421 2227–2230.
422 [22] C. Balczun, A. Bunse, C. Schwarz, M. Piotrowski, U. Kück, Chloroplast heat
423 shock protein Cpn60 from *Chlamydomonas reinhardtii* exhibits a novel function as a
424 group II intron-specific RNA-binding protein, FEBS Lett. 580 (2006) 4527–4532.[23]
425 [23]. C. Robinson, C.F.R.O. Matos, D. Beck, C. Ren, J. Lawrence, N. Vasisht, S.

426 Mendel. Transport and proofreading of proteins by the twin-arginine translocation
427 (Tat) system in bacteria. Biochim. Biophys. Acta 1808 (2011), 876-874.
428 [24] J. Bally, E. Paget, M. Droux, C. Job, D. Job, M. Dubald, Both the stroma and
429 thylakoid lumen of tobacco chloroplasts are competent for the formation of disulphide
430 bonds in recombinant proteins, Plant Biotechnol. J. 6 (2008), 46–61.