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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

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Abstract

Interest in the exploitation of microalgae for biotechnological applications has increased over the last decade, and microalgae are now viewed as offering a sustainable alternative to traditionally used host chassis. A number of recombinant proteins have been expressed in genetically modified algal strains, with the green alga *Chlamydomonas reinhardtii* being a particularly popular host strain. While nuclear transformation is possible with this organism, chloroplast transformation offers more reliable expression, and several proteins have been expressed in the stroma. Here we present the first utilisation of the thylakoid lumen for recombinant protein production in microalgae. A bacterial export signal peptide was used to efficiently translocate two recombinant proteins, a fluorescent reporter protein (pHRed) and a biopharmaceutical model substrate (scFv) into the thylakoid lumen. This approach expands the algal chloroplast genetic toolkit and offers a means of expressing proteins that are difficult to express in the stroma for reasons of toxicity, stability or a requirement for disulphide bonding.

Keywords

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

1. Introduction

The green alga *Chlamydomonas reinhardtii* has been used as a host for the expression of a variety of recombinant proteins, and its biotechnological potential has been explored in many studies over the last decade. A number of heterologous

proteins have been expressed, including vaccines, antibody fragments and terpene synthesis enzymes [for recent reviews see 1-3]. Tools for the genetic engineering of this green microalga have advanced remarkably, and it is now possible to transform both the nuclear and chloroplast genomes with reasonable efficiency. Chloroplast transformation offers the advantage that gene integration occurs by homologous recombination at specific sites, whereas nuclear transformation is essentially random with frequent gene silencing [e.g. 4, 5]. However, all of the chloroplast transformants reported to date have involved expression of the target protein in the stroma, with the exception of a study in which the target protein, a cytochrome P450, was targeted into a membrane (probably the thylakoid membrane) [6].

In this study we present a novel approach to expand the genetic tool kit of the algal chloroplast involving targeting to the thylakoid lumen. The thylakoid lumen is an important compartment playing a key role in photosynthesis and energy generation in chloroplasts. However, it has a relatively small proteome [7] and it offers a very different environment compared to the stroma: for example, the pH is lower and the lumen is an oxidising environment that is conducive to disulphide bonding. This could have advantages for the expression of some proteins and enrich the potential of the algal chloroplast as a production platform. In chloroplasts, proteins are naturally targeted across the thylakoid membrane by the Sec or Tat pathways, and attachment of a Sec or Tat signal peptide to a heterologous protein often results in correct targeting and maturation (reviewed in [8]). Here, we used the TorA Tat signal peptide from *Escherichia coli* which has been used to direct the export of biotechnologically relevant proteins to the periplasm in *E. coli* (reviewed in [9]) as a targeting peptide to translocate recombinant proteins into the thylakoid lumen of the *C. reinhardtii* 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

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 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

with the primer psaA.R (5'-GGATTTCTCCTTATAATAAC-3') in a standard PCR protocol with an annealing temperature of 54°C. Sequences for the primer design were kindly provided by Saul Purton (University College London, UK).

2.4. Cell lysis, SDS-PAGE and western blotting

Crude cell lysates for protein expression analysis were prepared from *C. reinhardtii* cells that were grown in 6 well plates in TAP medium at 25°C, 110 rpm shaking and approx. 50 µE. A volume of cells equivalent to 0.5 mL of a culture with an optical density of OD_{750}=1 measured on a DU 730 UV/Vis Spectrophotometer (Beckman Coulter) were harvested from each sample and resuspended in 0.1 ml 10 mM Tris-HCl (pH 8.0). 0.025 ml 5x SDS protein gel loading buffer (containing β-mercaptoethanol as a reducing agent) were added to the samples and then boiled at 95°C for 5 minutes. The crude lysates were separated and analysed by SDS-PAGE on a 15% sodium dodecyl sulphate-polyacrylamide gel and immunoblotted. An HA-antibody (Sigma-Aldrich) was used to detect the target protein and an AtpB-antibody (Agrisera, Sweden) as a loading control.

2.5. Chloroplast isolation and fractionation

Chloroplasts were isolated from 1 L liquid cultures that were grown in TAP medium to mid log phase at 25°C, 120 rpm shaking and constantly illuminated with approx. 50µE. The protocol described by Mason et al. [21] was used for chloroplast isolation. After washing the isolated chloroplasts in 0.1% BSA isolation buffer, the chloroplasts were directly resuspended in hypotonic lysis buffer for fractionation into stroma and thylakoids (a membrane fraction also including the chloroplast envelope 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

pathway. For comparisons of expression levels, and for control purposes, we also expressed the mature-size pHRed protein in the stroma. Screening relied on the restoration of phototrophic growth after transformation, as homologous recombination restores the intactness of the *psbH* gene (see [13] for details). One series of constructs was cloned into the plasmid pASapI, which uses the *atpA* promoter, and a second series of transformations was carried out using constructs based on the plasmid pSRSapI, which uses the *psaA* promoter. *C. reinhardtii* chloroplast transformants expressing the constructs detailed in Table 1 were successfully generated using the recipient strain TN72 as detailed in Economou et al. [13]. Homoplasmy analysis by PCR confirmed that all strains were homoplasmic ensuring stable integration of the gene into the chloroplast genome (Fig. 1).

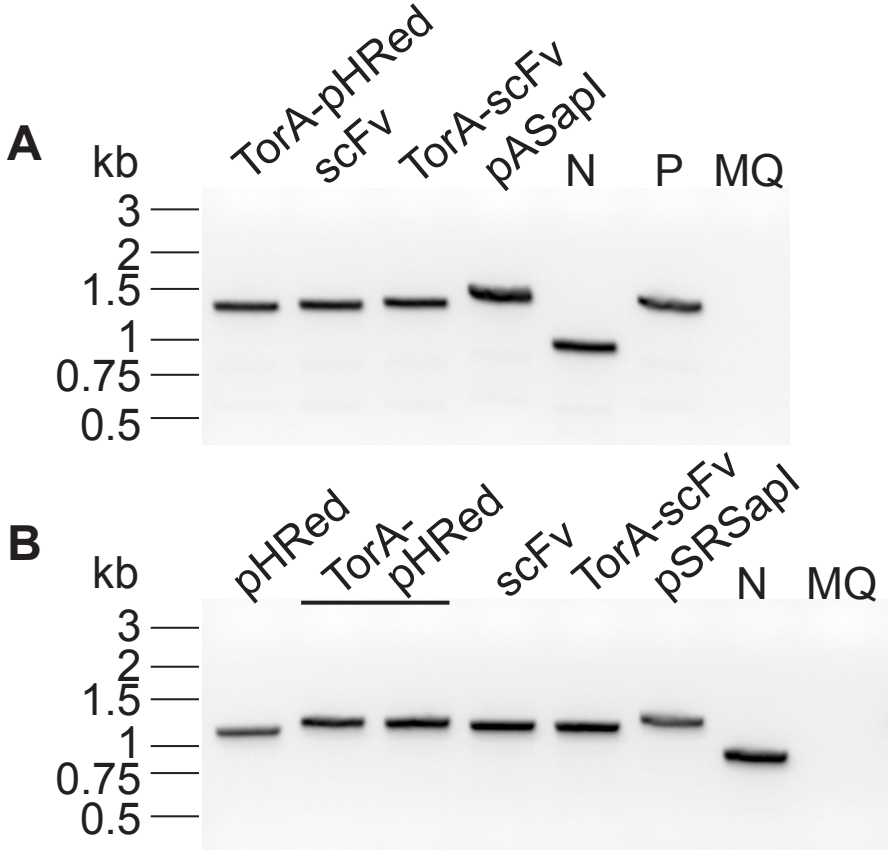


Fig. 1: Homoplasmy analysis of transformants expressing pHRed and scFv constructs. PCR for homoplasmy analysis was carried out on the TN72 strains transformed with constructs based on pASapI (Fig. 1A) and pSRSapI (Fig. 1B). All lanes indicate the construct that was used for transformation (details shown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

Further analysis of the transformants by SDS-PAGE and western blotting of crude cell lysates showed that the cells were expressing the respective protein, i.e. scFv or pHRed, in the chloroplast at stable levels (Fig. 2). Fig. 2A shows (from left to right) blots of stromal pHRed and TorA-pHRed (in 2 different transformants), expressed from the same *psaA* promoter. The protein is clearly detected as a band of ca. 27 kDa and the levels of the stromal and lumen-targeted versions are reasonably similar. The next lane shows that lower TorA-pHRed levels were obtained when expressed from the *atpA* promoter, and the band is absent from the control strains transformed with empty pSRSapI or pASapI vector (RP*, RP).

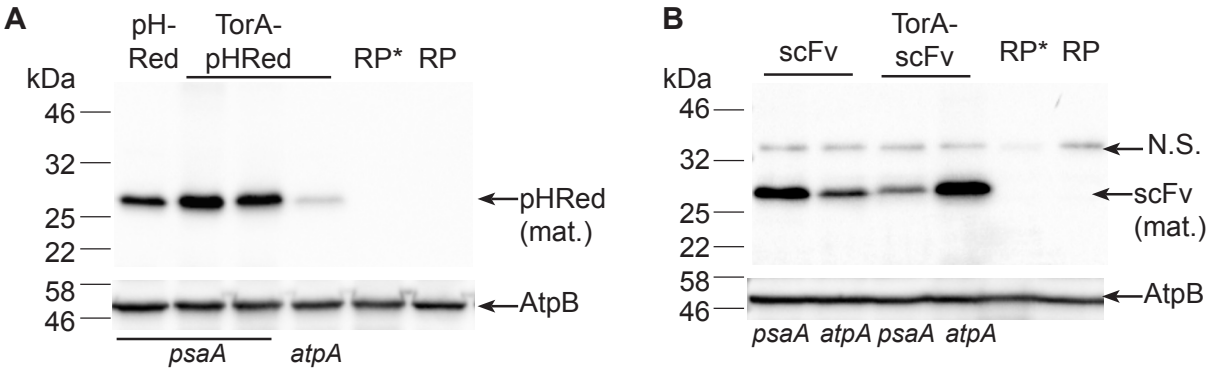


Fig. 2: Expression of pHRed and scFv with and without TorA signal peptide in the chloroplast of *C. reinhardtii*. **A** shows an immunoblot of cell lysates from strain TN72 transformed with the following constructs from left to right: pJZ25 (pHRed); JZ26a,

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

Fig. 2B shows blots of transformants expressing the 28 kDa scFv in the stroma from the *psaA* or *atpA* promoter, with slightly higher levels detected in the former. Slightly surprisingly, expression of TorA-scFv yields somewhat different results, with protein levels higher when expressed from the *atpA* promoter.

In both the A and B panels, the blots were reprobed using antibodies to AtpB as loading controls. It is also notable that in Fig. 2B we detect a band of about 34 kDa which has previously been shown to stem from non-specific reaction of an unknown endogenous protein with the anti-HA antibodies [20].

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

Interestingly, in the strains expressing TorA-pHRed and TorA-scFv (Fig. 2), only the mature protein sizes 27/28 kDa were detected, with the same molecular weights as the stromal versions. No precursor protein, which would be expected to be around 32 kDa for both proteins, was observed in our experiments. Lumen-targeted precursor proteins are processed to the mature size after translocation by a lumen-facing processing peptidase [8], so this provides preliminary evidence that both proteins may be targeted to the thylakoid lumen and processed to the mature size.

3.3. The TorA export signal peptide enables protein translocation to the thylakoid lumen in the algal chloroplast

Although the absence of the precursor protein is indirect evidence of targeting to the lumen, fractionation studies were deemed essential to confirm this point, and chloroplasts were therefore isolated and fractionated into stroma and thylakoids by hypotonic lysis. The results of the fractionation are shown in Fig. 3, with the target proteins again detected by immunoblotting with antibodies to their C-terminal HA tags. Both TorA-pHRed (Fig. 3A) and TorA-scFv (Fig. 3B) were translocated into the thylakoids, with the mature-size proteins ('mat') clearly detected in the thylakoid fraction (T) but not the stroma (S). The stromal pHRed is detected in the stroma as expected (Fig. 3A) as was the stromal scFv (data not shown). The 34 kDa band from non-specific binding of the anti-HA antibody is also apparent.

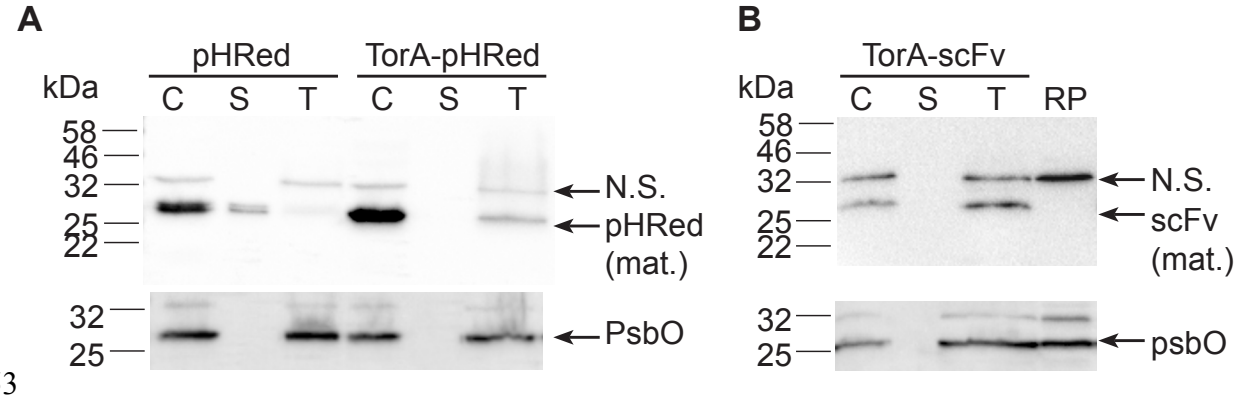


Fig. 3: Fractionation of chloroplasts into stroma and thylakoids (including envelope membranes). Whole chloroplast lysates (C), the stroma fraction (S) and the thylakoid fraction (T) are shown. **A** shows an Anti-HA immunoblot of chloroplast fractions from strain TN72 transformed with pJZ25 (pHRed)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

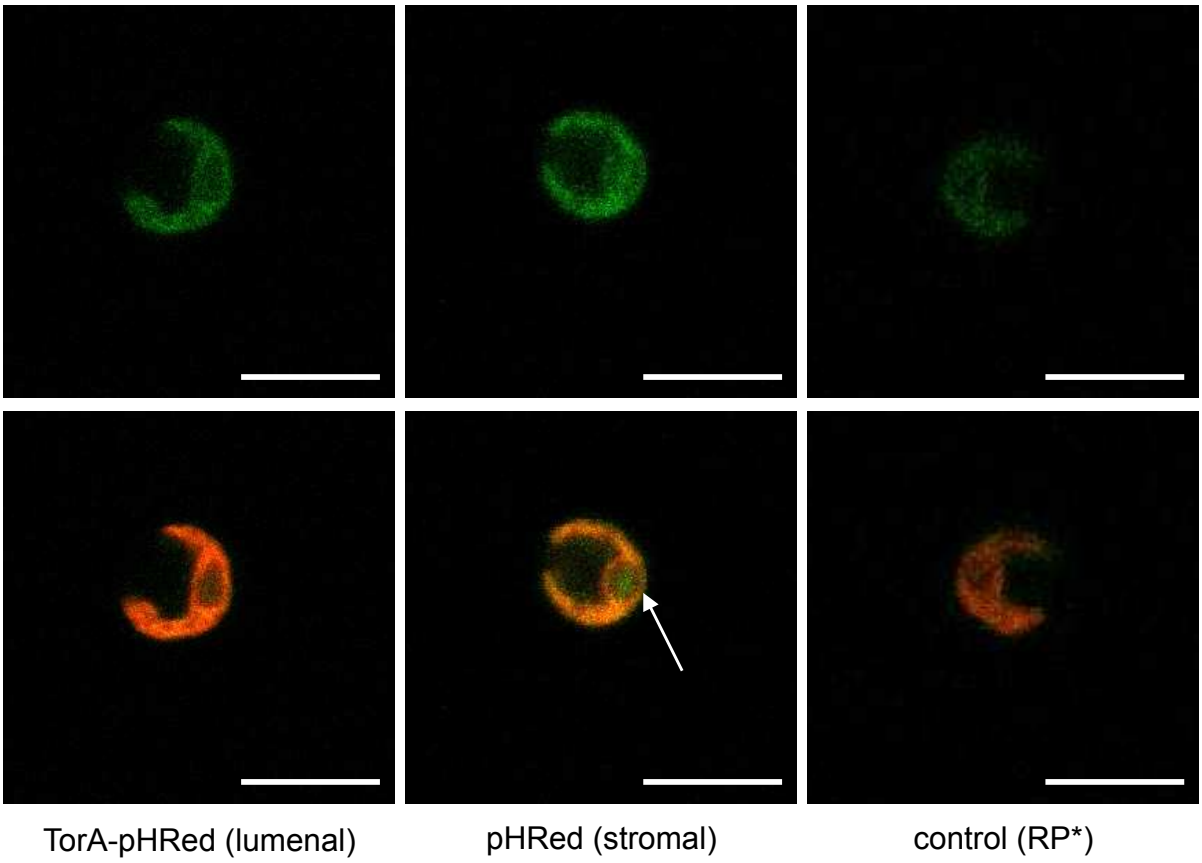
antibody, shown in A and B respectively, serves as a control for the fractionation. The PsbO protein is denoted with an arrow.

The fractions were also probed with a PsbO antibody as a control for the fractionation; PsbO is a well-known lumenal protein that forms part of the photosystem II oxygen-evolving complex. This control confirms that the targeted proteins are indeed in the thylakoid fraction. From these results, it is apparent that a bacterial Tat export signal peptide is suitable for translocation of proteins to the thylakoid lumen in *C. reinhardtii*.

3.4. TorA–pHRed is specifically targeted to the thylakoids

Representative confocal images of the strains expressing the fluorescent reporter gene *pHRed* are shown in Fig. 4. All images were recorded with 561 nm excitation and emission at 600-620 nm, a combination which proved to give the most selective visualisation of pHRed relative to the background fluorescence from the photosynthetic pigments. However, even at these wavelengths there was significant non-pHRed fluorescence from the thylakoid membranes (see right-hand panels of Fig. 4, showing the control strain), which complicates analysis of the distribution of pHRed. For quantitative comparison of fluorescence yields we manually selected either the whole cell or the pyrenoid region in the chloroplast (see Fig. 4) and measured the mean fluorescence in these regions, a procedure which automatically corrects for differences in cell size. For cells expressing stromal pHRed, mean fluorescence at 600-620 nm was 36% higher than in the control strain (n = 20, p = 0.00013 from a Student's t-test). The difference was even more pronounced when fluorescence was measured only from the pyrenoid region, an area of the chloroplast 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

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.

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