

1 Genetic autonomy and low singlet oxygen yield support 2 kleptoplast functionality in photosynthetic sea slugs

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25 **Highlight**

26 Isolated *Vaucheria litorea* plastids exhibit upregulation of *tufA* and *ftsH*, key plastid
27 maintenance genes, and produce only little singlet oxygen. These factors likely contribute to
28 plastid longevity in kleptoplastic slugs.

29 **Abstract**

30 *Elysia chlorotica* is a kleptoplastic sea slug that preys on *Vaucheria litorea*, stealing its plastids
31 which then continue to photosynthesize for months inside the animal cells. We investigated the
32 native properties of *V. litorea* plastids to understand how they withstand the rigors of
33 photosynthesis in isolation. Transcription of specific genes in laboratory-isolated *V. litorea*
34 plastids was monitored up to seven days. The involvement of plastid-encoded FtsH, a key
35 plastid maintenance protease, in recovery from photoinhibition in *V. litorea* was estimated in
36 cycloheximide-treated cells. *In vitro* comparison of *V. litorea* and spinach thylakoids was
37 applied to investigate ROS formation in *V. litorea*. Isolating *V. litorea* plastids triggered
38 upregulation of *ftsH* and translation elongation factor EF-Tu (*tufA*). Upregulation of FtsH was
39 also evident in cycloheximide-treated cells during recovery from photoinhibition. Charge
40 recombination in PSII of *V. litorea* was found to be fine-tuned to produce only small quantities
41 of singlet oxygen ($^1\text{O}_2$). Our results support the view that the genetic characteristics of the
42 plastids themselves are crucial in creating a photosynthetic sea slug. The plastid's autonomous
43 repair machinery is likely enhanced by low $^1\text{O}_2$ production and by upregulation of FtsH in the
44 plastids.

45 **Keywords**

46 Kleptoplasty, photoinhibition, photosynthetic sea slugs, PSII repair cycle, reactive oxygen
47 species, singlet oxygen, *Vaucheria litorea*

48 **Abbreviations**

49	$^1\text{O}_2$	singlet oxygen
50	CHI	cycloheximide
51	DCBQ	2,6-dichloro-1,4-benzoquinone;
52	DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
53	DCPIP	2,6-dichlorophenolindophenol
54	F _v /F _M	maximum quantum yield of PSII photochemistry

55	k_{PI}	rate constant of PSII photoinhibition
56	MDA	malondialdehyde
57	OEC	oxygen evolving complex of PSII
58	P_{680}	reaction center Chl of PSII
59	P_{700}	reaction center Chl of PSI
60	P_M	maximum oxidation of P_{700}
61	PPFD	photosynthetic photon flux density
62	PSI	Photosystem I
63	PSII	Photosystem II
64	ROS	reactive oxygen species
65	TEM	transmission electron microscope
66	$TyrD^+$	oxidized tyrosine-D residue of PSII

67 Introduction

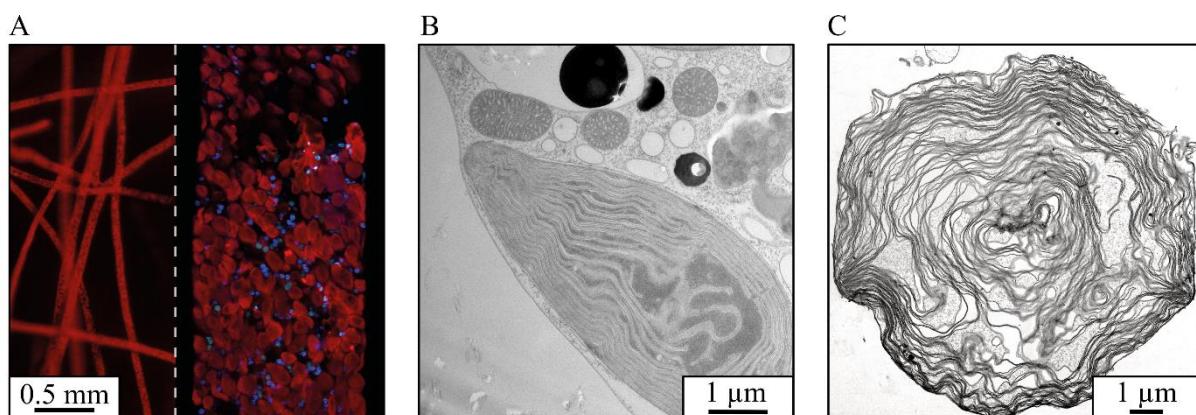
68 Functional kleptoplasty in photosynthetic sea slugs depends on two major components: the first
69 is a slug capable of stealing plastids and retaining them functional within its cells, the second
70 a plastid with a specific genetic repertoire (de Vries *et al.*, 2015). All species that are able to
71 do this belong to the Sacoglossan clade (Rumpho *et al.*, 2011; de Vries *et al.*, 2014). These
72 slugs are categorized based on their plastid retention times, i.e. no retention, short-term
73 retention (hours to ~10 days) and long-term retention species (≥ 10 days to several months)
74 (Händeler *et al.*, 2009). The record holding slug *Elysia chlorotica* can retain plastids for
75 roughly a year (Green *et al.*, 2000). The mechanisms utilized by the slugs to selectively
76 sequester plastids from their prey algae remain uncertain, although recent studies have shown
77 that in *E. chlorotica* it is an active process reminiscent of that observed for symbiotic algae and
78 corals (Chan *et al.*, 2018). The slugs possibly rely on scavenger receptors and thrombospondin-
79 type-1 repeat proteins for plastid recognition (Clavijo *et al.*, 2020).

80 The sacoglossan's ability to sequester plastids tends to distract attention from the unique
81 features of the sequestered organelle, forming the second component of a photosynthetic slug
82 system. Long-term retention sea slugs are only able to maintain functional plastids from a
83 restricted list of siphonaceous algae and usually from only one species. Some sacoglossa have
84 a wide selection of prey algae, but long-term retention of plastids is still limited to specific
85 algal sources (Christa *et al.*, 2013; de Vries *et al.*, 2013). The native robustness of some plastid
86 types was noticed decades ago, and early on suggested to contribute to their functionality inside

87 animals (Giles and Sarafis, 1972; Trench *et al.*, 1973 *a, b*). Studies focusing on the specific
88 properties of the algal plastids, however, are scarce. Reduction of the plastid genome
89 (plastome) during evolution has stripped the organelle of many genes required for self-
90 maintenance (Martin, 2003), but genomic analysis of algal plastomes suggests that three genes
91 (*tufA*, *ftsH* and *psbA*) could be among those critical for plastid maintenance inside a slug cell
92 (de Vries *et al.*, 2013). Out of the three, *psbA* remains in all plastomes, including those of higher
93 plants, whereas *tufA* and *ftsH* are encoded by most algal plastid genomes (Baldauf and Palmer,
94 1990; Oudot-Le Secq *et al.*, 2007; de Vries *et al.*, 2013). It has been suggested that the plastid-
95 encoded translation elongation factor EF-Tu (*tufA*) helps maintain translation, specifically of
96 the thylakoid maintenance protease FtsH (*ftsH*) involved in the repair cycle of Photosystem II
97 (PSII) (de Vries *et al.*, 2013). FtsH degrades the D1 protein (*psbA*) of damaged PSII before the
98 insertion of *de novo* synthesized D1 into PSII (Mulo *et al.*, 2012; Järvi *et al.*, 2015). Without
99 continuous replacement of the D1 protein, light-induced damage to PSII would rapidly curtail
100 photosynthesis (Tyystjärvi and Aro, 1996).

101 Unlike all other known plastid sources of long-term retention slugs, *Vaucheria litorea* (Fig. 1),
102 the sole prey of *E. chlorotica*, is not a green but a yellow-green alga, with plastids derived from
103 red algal lineage through secondary endosymbiosis (Cruz *et al.*, 2013) (Fig. 1B). The plastome
104 of *V. litorea* possesses the three important genes (de Vries *et al.*, 2013). Furthermore, the
105 plastid-encoded FtsH of *V. litorea* has been shown to carry the critical metalloprotease domain
106 that is not present in *ftsH* of other prey algae of long-term retention slugs (Christa *et al.*, 2018).
107 Here, we show that isolated plastids of *V. litorea* (Fig. 1C) maintain highly specific
108 transcription of their genes, and exhibit adequate genetic autonomy in their capability to
109 recover from light induced damage of PSII, i.e. photoinhibition. We also estimated reactive
110 oxygen species (ROS) production in the thylakoid membranes of *V. litorea*. While our results
111 highlight the importance of terminal electron acceptors downstream of Photosystem I (PSI) in
112 limiting ROS production, we show that PSII of *V. litorea* is fine-tuned to decrease the yield of
113 the highly reactive singlet oxygen (${}^1\text{O}_2$). The consequences of our findings to light-induced
114 damage and longevity of the plastids inside photosynthetic sea slugs are discussed in detail.

115



116

117 **Figure 1. Microscope images of *V. litorea*, the main source of plastids for the**
118 **photosynthetic sea slug *E. chlorotica*.** (A) Chlorophyll autofluorescence (red) and nucleus
119 specific dye fluorescence (blue) from *V. litorea* filaments, with a detail of a single filament on
120 the right. (B) Transmission electron micrograph (TEM) showing a plastid *in vivo* in a *V. litorea*
121 cell and in close proximity to several mitochondria, and in (C) an isolated single plastid.

122

123 Materials and Methods

124 Organisms and culture conditions

125 Spinach, *Spinacia oleracea* L. Matador (Nelson Garden, Tingsryd, Sweden), and *V. litorea* C.
126 Agardh 1823 (SCCAP K-0379) were grown in SGC 120 growth chambers (Weiss Technik UK,
127 Loughborough, United Kingdom) in 8/12 h and 12/12 h light/dark cycles, respectively. Growth
128 light (Master TL-D 36W/840; Philips, Amsterdam, The Netherlands) photosynthetic photon
129 flux density (PPFD) was set to 40 $\mu\text{mol m}^{-2}\text{s}^{-1}$ for both species. Temperature was maintained
130 at 22 °C for spinach and 17 °C for *V. litorea*. Spinach plants used in the experiments were
131 approximately 2 months old. *V. litorea* was grown in 500 ml flasks in f/2 culture medium
132 (modified from Guillard and Ryther, 1962) made in 1% (m/v) artificial sea water (Sea Salt
133 Classic, Tropic Marin, Wartenberg, Germany). *V. litorea* cultures were routinely refreshed by
134 separating 1-4 g of inoculate into new flasks, and cultures used in the experiments were 1-2
135 weeks old. Nuclei of *V. litorea* were stained for microscopy with Hoechst 33342 (Thermo
136 Scientific, Waltham, MA, USA) using standard protocols. *In vivo* transmission electron
137 microscope (TEM) images were taken after freeze-etch fixation. The sea slug *Elysia timida*
138 and its prey alga *Acetabularia acetabulum* were routinely maintained as described earlier
139 (Schmitt *et al.*, 2014; Havurinne and Tyystjärvi, 2020).

140 **Gene expression of isolated *V. litorea* plastids**

141 Plastid isolation from *V. litorea* was performed based on Green *et al.* (2005). Briefly, filaments
142 were cut to small pieces, resuspended in 40 ml of isolation buffer (see Table 1) and
143 homogenized with ULTRA-TURRAX® (IKA, Staufen, Germany) using four short bursts at
144 8000 rpm. The homogenate was filtered twice through a layer of Miracloth (Calbiochem,
145 Darmstadt, Germany), centrifuged (1900 x g, 5 min) and the pellet was resuspended in 1 ml of
146 isolation buffer. Percoll solution containing 0.25 M sucrose was diluted to a 75 and 30%
147 solution with 1xTE buffer containing 0.25 M sucrose. The sample was layered between the two
148 dilutions and the assemblage was centrifuged (3500 x g, 20 min) in a swing-out rotor with no
149 deceleration. Intact plastids were collected from the interphase and washed twice by
150 centrifugation (2200 x g, 3 min) with isolation buffer lacking BSA. All steps were carried out
151 at 4 °C in the dark. TEM imaging of the plastids was done after fixing the samples using
152 glutaraldehyde and cryo-fixation followed by freeze substitution.

153 Plastids were kept in isolation buffer for seven days in routine culturing conditions. RNA was
154 isolated at different time points using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St.
155 Louis, MO, USA). Aliquots with 50 ng RNA were subjected to DNase treatment (Thermo
156 Scientific), and treated aliquots amounting to 10 ng RNA were used for cDNA synthesis
157 (iScript™ cDNA Synthesis Kit, BioRad, Hercules, CA, USA). Quantitative real-time PCR was
158 carried out using a StepOnePlus (Applied Biosystems, Foster City, CA, USA) and reagents
159 from BioRad. The primers used in the qPCR were designed using Primer3
160 (<http://frodo.wi.mit.edu/primer3>); the primer sequences are listed in Supplementary Table S1
161 at JXB online. Every reaction was done with technical triplicates and results were analyzed
162 using the $\Delta\Delta Ct$ method (Pfaffl, 2001), in which the qPCR data were double normalized to *rbcL*
163 and time point 0 (immediately after plastid isolation).

164

165 **Supplementary table S1. The list of primers designed for quantitative real-time PCR**
166 **analysis of transcription in isolated *V. litorea* plastids.**

Symbol	Description	Primer (5'→3')
<i>ftsH</i>	FtsH protease subunit	for- TGATGTTGTTTGATGATGTTGC rev- ACTCCTTTGGTATTTAGCACCT

<i>psaA</i>	PSI protein PsaA	for-TGGACTGCTATTGGTGGTT rev-CCATTCAAGTTAGGTGCTGCT
<i>psbA</i>	PSII protein D1	for-ATTCCCACTCACGACCCATA rev- AAACAACATCATTCTGGTGCT
<i>psbB</i>	PSII protein CP47	for-ATGGGCTGGTTCAATGGCTT rev-GCTACACCCCTCAAAACTCCA
<i>psbC</i>	PSII protein CP43	for-TGGTCTGGAAATGCTCGTCTT rev-CAACGCCCATCCTAAAGTA
<i>psbD</i>	PSII protein D2	for-TGGACAAAATCAAGAACGAGGT rev-ACCAACCAATAATACGAAGCGA
<i>psbH</i>	PSII protein PsbH	for-AAAAGTTGCTCCTGGTTGGG rev-ATATTTGCCAATCAACATCTACA
<i>rbcL</i>	RuBisCo large subunit	for-CGCTCTCTCCAACGCATAA rev-GGACTTCGTGGTGGTTAGATT
<i>tufA</i>	Translation elongation factor EF-Tu	for-TATCTACCCATTCAATTATCCCCTTT rev-ATTCCATTGCCAGGTTCAAG

167

168 *In vivo* photoinhibition

169 The capacity to recover from photoinhibition was tested in spinach leaves and *V. litorea* cells
170 in the presence of cycloheximide (CHI), a cytosolic translation inhibitor. Spinach leaf petioles
171 were submerged in water containing 1 mM CHI and incubated for 24 h in the dark. The
172 incubation was identical for *V. litorea* cells, except that the cells were fully submerged in f/2
173 medium supplemented with 1 mM CHI. Control samples were treated identically without CHI.
174 The samples were then exposed to white light (PPFD 2000 $\mu\text{mol m}^{-2} \text{ s}^{-1}$) for 60 min and
175 subsequently put to dark and thereafter low light (PPFD 10 $\mu\text{mol m}^{-2} \text{ s}^{-1}$) to recover for 250
176 min. Temperature was maintained at growth temperatures of both species using a combination
177 of a thermostated surface and fans. The petioles of spinach leaves were submerged in water (-
178 /+ CHI) during the experiments. Cell clusters of *V. litorea* were placed on top of the

179 thermostated surface on a paper towel moistened thoroughly with f/2 medium (-/+ CHI). PSII
180 activity was estimated by measuring the ratio of variable to maximum fluorescence (F_v/F_m)
181 (Genty *et al.*, 1989) with PAM-2000 (Walz, Effeltrich, Germany) fluorometer. During the light
182 treatments, F_v/F_m was measured from samples that were dark acclimated for <5 min, except
183 for the final time point, where the samples were dark acclimated for 20 min. The light source
184 used for all high-light treatments discussed in this study was an Artificial Sunlight Module
185 (SLHolland, Breda, The Netherlands).

186 Membrane proteins were isolated at timepoints indicated in the figures. The same area where
187 F_v/F_m was measured (approximately 1 cm²) was cut out of the leaves/algae clusters and placed
188 in a 1 ml Dounce tissue grinder (DWK Life Sciences, Millville, NJ, USA) filled with 0.5 ml of
189 osmotic shock buffer (Table 1) and ground thoroughly. The homogenate was filtered through
190 one layer of Miracloth and centrifuged (5000 x g, 5 min). The pellet containing the membrane
191 protein fraction was resuspended in 50 µl of thylakoid storage buffer. The samples were stored
192 at -80 °C until use. Membrane protein samples containing 1 µg total Chl were solubilized and
193 separated by electrophoresis on a 10 % SDS-polyacrylamide gel using Next Gel solutions and
194 buffers (VWR, Radnor, PA, USA). Proteins were transferred to Immobilon-P PVDF
195 membranes (MilliporeSigma, Burlington, MA, USA). FtsH was immunodetected using
196 antibodies raised against *Arabidopsis thaliana* FtsH5, reactive with highly homologous
197 proteins FtsH1 and FtsH5, or FtsH2, reactive with FtsH2 and FtsH8 (Agrisera, Vännäs,
198 Sweden). Western blots were imaged using goat anti-rabbit IgG (H+L) alkaline phosphatase
199 conjugate (Life Technologies, Carlsbad, CA, USA) and CDP-star Chemiluminescence Reagent
200 (Perkin-Elmer, Waltham, MA, USA). Protein bands were quantified with Fiji (Schindelin *et*
201 *al.*, 2012).

202 Experiments with *E. timida* were performed on freshly fed individuals. Slugs were kept in the
203 dark overnight both in the absence and presence of 10 mg/ml lincomycin in 3.7 % artificial sea
204 water and then exposed to high light (PPFD 2000 µmol m⁻² s⁻¹) in wells of a 24 well-plate filled
205 with artificial sea water for 40 min. Temperature was maintained at 23 °C throughout the
206 treatment. The slugs were then put to recover overnight in low light (PPFD <20 µmol m⁻² s⁻¹)
207 in their growth conditions. F_v/F_m was measured with PAM-2000 after a minimum 20 min dark
208 period as described earlier (Havurinne and Tyystjärvi, 2020).

209 **Isolation of functional thylakoids for *in vitro* experiments**

210 Functional thylakoids were isolated as described earlier (Hakala *et al.*, 2005) after 24h dark
211 incubation. One spinach leaf per isolation was ground in a mortar in thylakoid isolation buffer
212 (Table 1). The homogenate was filtered through a layer of Miracloth and pelleted by
213 centrifugation (5000 x g, 5 min). The pellet was resuspended in osmotic shock buffer,
214 centrifuged (5000 x g, 5 min) and the resulting pellet was resuspended in thylakoid storage
215 buffer. Chl concentration was determined spectrophotometrically in 90 % acetone using
216 extinction coefficients for Chls *a* and *b* (Jeffrey and Humphrey, 1975). Thylakoid isolation
217 from *V. litorea* was performed using the same procedure, by grinding 2-5 g of fresh cell mass
218 per isolation. The cell mass was briefly dried between paper towels before grinding. Chl
219 concentration from *V. litorea* thylakoids was determined in 90% acetone using coefficients for
220 Chls *a* and *c1 + c2* (Jeffrey and Humphrey, 1975). Protein concentrations of the thylakoid
221 suspensions were determined with DCTTM Protein Assay (Bio-Rad, Hercules, CA, USA).
222 Thylakoids used in functional experiments were kept on ice in the dark and always used within
223 a few hours of isolation.

224

225 **Table 1. Buffer solutions used in sample preparation and measurements.**

Identifier	Composition	Used in
Plastid isolation buffer	0.2 % BSA, 1 mM EDTA, 50mM Hepes-KOH pH 7.6, 1 mM MgCl ₂ , 330 mM sorbitol	Plastid isolation, <i>in organello</i> gene expression
Thylakoid isolation buffer	1 % BSA, 1 mM EDTA, 1 mM glycine betaine, 40 mM HEPES-KOH pH 7.4, 10 mM MgCl ₂ , 0.3 M sorbitol	Thylakoid isolation
Osmotic shock buffer	10 mM HEPES-KOH pH 7.4, 10 mM MgCl ₂ , 5 mM sorbitol	Thylakoid isolation
Storage buffer	10 mM HEPES-KOH pH 7.4, 10 mM MgCl ₂ , 5 mM NaCl, 500 mM sorbitol	Thylakoid isolation, EPR

Photosystem measuring buffer	1 M glycine betaine, 40 mM HEPES-KOH pH 7.4, 1 mM KH ₂ PO ₄ , 5 mM MgCl ₂ , 5 mM NaCl, 5 mM NH ₄ Cl, 330 mM sorbitol,	Thermoluminescence, flash oxygen evolution, fluorescence decay kinetics, ¹ O ₂ production
Photoinhibition buffer	1 M glycine betaine, 40 mM HEPES-KOH pH 7.4, 5 mM MgCl ₂ , 5 mM NaCl, 330 mM sorbitol	<i>In vitro</i> photoinhibition treatments, <i>in vitro</i> P ₇₀₀ ⁺ measurements
PSI measuring buffer	Photosystem measuring buffer + 0.3 mM 2,6-dichlorophenolindophenol (DCPIP), 0.01 mM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 0.12 mM methyl viologen, 32 mM Na-ascorbate, 0.6 mM NaN ₃	Polarographic PSI activity measurements (oxygen consumption)
PSII measuring buffer	Photosystem measuring buffer + 0.5 mM 2,6-dichloro-1,4-benzoquinone (DCBQ), 0.5 mM hexacyanoferrate(III)	Polarographic PSII activity measurements (oxygen evolution)

226

227 **Photosystem stoichiometry**

228 Photosystem stoichiometry was measured from thylakoid membranes with an EPR
229 spectroscope Miniscope MS5000 (Magnettech GmbH, Berlin, Germany) as described earlier
230 (Tiwari *et al.*, 2016; Nikkanen *et al.*, 2019). EPR spectra originating from oxidized tyrosine-D
231 residue of PSII (Tyr_D⁺) and reaction center Chl of PSI (P₇₀₀⁺) of concentrated thylakoid samples
232 (2000 µg Chl ml⁻¹ in storage buffer) were measured in a magnetic field ranging from 328.96 to
233 343.96 mT during illumination (PPFD 4000 µmol m⁻² s⁻¹) (Lightningcure LC8; Hamamatsu
234 Photonics, Hamamatsu City, Japan) and after a subsequent 5 min dark period in the absence
235 and presence of 50 µM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). The dark stable
236 Tyr_D⁺ EPR signal (PSII signal), measured after the post illumination period in the absence of
237 DCMU, and the P₇₀₀⁺ (PSI signal), measured during illumination in the presence of DCMU,
238 were double integrated to determine photosystem stoichiometry.

239 *In vitro* photoinhibition

240 For *in vitro* photoinhibition experiments, thylakoids were diluted to a total Chl concentration
241 of 100 $\mu\text{g ml}^{-1}$ in photoinhibition buffer (Table 1), and 1 ml sample was loaded into a glass
242 beaker submerged in a water bath kept at 22 °C. The samples were exposed to white light
243 (PPFD 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and mixed with a magnet during the 60 min treatments. Aliquots
244 were taken at set intervals to determine PSI or PSII activities using a Clark-type oxygen
245 electrode (Hansatech Instruments, King's Lynn, England). The sample concentration in the
246 activity measurements was 20 μg total Chl ml^{-1} in 0.5 ml of PSI or PSII measuring buffer
247 (Table 1). PSI activity was measured as oxygen consumption, whereas PSII activity was
248 measured as oxygen evolution. Both activities were measured at 22 °C in strong light (PPFD
249 3200 $\mu\text{mol m}^{-2} \text{s}^{-1}$) from a slide projector. The rate constant of PSII photoinhibition (k_{PI}) was
250 obtained by fitting the loss of oxygen evolution to a first-order reaction equation with
251 SigmaPlot 13.0 (Systat Software, San Jose, CA, USA), followed by dark correction, i.e.
252 subtraction of the dark inactivation rate constant from the initial k_{PI} .

253 Lipid peroxidation was measured by detecting malondialdehyde (MDA) formation (Heath and
254 Packer, 1968). A thylakoid suspension aliquot of 0.4 ml was mixed with 1 ml of 20 %
255 trichloroacetic acid containing 0.5 % thiobarbituric acid, incubated at 80 °C for 30 min and
256 cooled down on ice for 5 min. Excess precipitate was pelleted by centrifugation (13500 x g, 5
257 min), and the difference in absorbance between 532 and 600 nm ($\text{Abs}_{532-600}$) was measured as
258 an indicator of the relative amount of MDA in the samples. Protein oxidation was determined
259 by detecting protein carbonylation with Oxyblot™ Protein Oxidation Detection Kit
260 (MilliporeSigma, Burlington, MA, USA). Thylakoid aliquot amounting to a protein content of
261 45 μg was taken at set time points and 10 mM dithiothreitol was used to prevent further protein
262 carbonylation. The samples were prepared according to the manufacturer's instructions and
263 proteins were separated in 10 % Next Gel SDS-PAGE (VWR). Carbonylated proteins were
264 detected with Immobilon Western Chemiluminescent HRP Substrate (MilliporeSigma).

265 The maximum oxidation of P_{700} (P_M) was estimated in an additional experiment. Thylakoids
266 equivalent to 25 μg Chl in 50 μl of photoinhibition buffer were pipetted on a Whatman filter
267 paper (grade 597; Cytiva, Marlborough, MA, USA). The filter was placed inside the lid of a
268 plastic Petri dish, and the bottom of the Petri dish was placed on top of the lid. Photoinhibition
269 buffer was added to the sample from the small openings on the sides of the assemblage. The
270 thylakoids were then illuminated with high light (PPFD 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and the temperature
271 was maintained at 22 °C using a thermostated surface. $\text{F}_\text{v}/\text{F}_\text{M}$ and P_M were measured using a

272 700 ms high-light pulse (PPFD 10000 $\mu\text{mol m}^{-2} \text{s}^{-1}$) with Dual-PAM 100 (Walz) (Schreiber,
273 1986; Schreiber and Klughammer, 2008) at set intervals. The high-light treated samples were
274 dark acclimated for <5 min prior to the measurements.

275 **$^1\text{O}_2$ measurements**

276 $^1\text{O}_2$ was measured from thylakoids diluted to 100 μg total Chl ml^{-1} in 0.3 ml of photosystem
277 measuring buffer, using the histidine method described earlier (Telfer *et al.*, 1994; Rehman *et*
278 *al.*, 2013). Continuously stirred thylakoid samples were exposed to high light (PPFD 3200
279 $\mu\text{mol m}^{-2} \text{s}^{-1}$) from a slide projector at 22 °C in the presence and absence of 20 mM histidine.
280 Oxygen consumption was measured for 60 s using an oxygen electrode (Hansatech), and the
281 difference in the oxygen consumption rates in the presence and absence of histidine was taken
282 as an indicator of $^1\text{O}_2$ production. PSII electron transfer activity (H_2O to DCBQ) in the same
283 conditions was 124.7 (SE±15.4) and 128.4 (SE±10.7) $\mu\text{mol O}_2 \text{ mg Chl}^{-1} \text{ h}^{-1}$ in spinach and *V.*
284 *litorea* samples, respectively, containing 20 $\mu\text{g Chl ml}^{-1}$.

285 **PSII charge recombination measurements**

286 Flash-induced oxygen evolution was recorded at room temperature using a Joliot-type bare
287 platinum oxygen electrode (PSI, Brno, Czech Republic) (Joliot and Joliot, 1968) from
288 thylakoids diluted in photosystem measuring buffer to 50 $\mu\text{g Chl ml}^{-1}$ and supplemented with
289 50 mM KCl, essentially as described in Antal *et al.* (2009). 200 μl of sample was pipetted on
290 the electrode and kept in the dark for 10 min before the measurements. The samples were then
291 exposed to a flash train consisting of 15 single-turnover flashes (4 ns/pulse) at one second
292 intervals, provided by a 532 nm Nd:YAG laser (Minilite, Continuum, San Jose, CA, USA).
293 Charge recombination within PSII was probed by exposing the samples to a preflash and
294 different dark times between the preflash and the flash train used for recording the oxygen
295 traces.

296 The decay of Chl *a* fluorescence yield after a 30 μs single turnover flash (maximum PPFD
297 100 000 $\mu\text{mol m}^{-2} \text{s}^{-1}$) were measured at room temperature from 1 ml samples of thylakoids
298 using FL200/PS fluorometer (PSI). Measurement length was 120 s and 8 datapoints/decade
299 were recorded (2 in the presence of DCMU). The first datapoint was recorded 150 μs after the
300 flash. Single turnover flash and measuring beam voltages were set to 100 % and 60 % of the
301 maximum, respectively. The samples were diluted in photosystem measuring buffer to a total
302 Chl concentration of 20 $\mu\text{g ml}^{-1}$. A set of samples was poisoned with 20 μM DCMU to block
303 electron transfer at the reducing side of PSII.

304 Thermoluminescence was measured from thylakoids using a custom setup (Tyystjärvi *et al.*,
305 2009). Thylakoids were diluted to a total Chl concentration of 100 µg ml⁻¹ in photosystem
306 measuring buffer (Table 1) in the presence and absence of 20 µM DCMU, and a volume of 100
307 µl was pipetted on a filter paper disk that was placed inside the cuvette of the measuring
308 apparatus. The samples were dark acclimated for 5 min before the onset of cooling to -20 °C
309 by a Peltier element (TB-127-1,0-0,8; Kryotherm, Carson City, NV, USA). The samples were
310 then exposed to a flash (E = 1 J) from a FX-200 Xenon lamp (EGandG, Gaithersburg, MD,
311 USA) and heated at a rate of 0.47 °C s⁻¹ up to 60 °C while simultaneously recording
312 luminescence emission.

313 *In vivo* P₇₀₀ redox kinetics

314 Redox kinetics of P₇₀₀ were measured as described by Shimakava *et al.*, (2019) using Dual-
315 PAM 100 (Walz). Spinach plants and *V. litorea* cells were kept in darkness for at least 2 h
316 before the measurements. Anaerobic conditions were obtained using a custom cuvette
317 described in Havurinne and Tyystjärvi (2020). For spinach leaf cutouts, the cuvette was flushed
318 with nitrogen. A combination of glucose oxidase (8 units/ml), glucose (6 mM) and catalase
319 (800 units/ml) in f/2 culture medium was used to create anaerobic conditions for *V. litorea*
320 cells. All samples were treated with 15 s of far red light (PFD 120 µmol m⁻² s⁻¹) and a
321 subsequent darkness lasting 25 s prior to firing a high-light pulse (780 ms, PPFD 10 000 µmol
322 m⁻² s⁻¹).

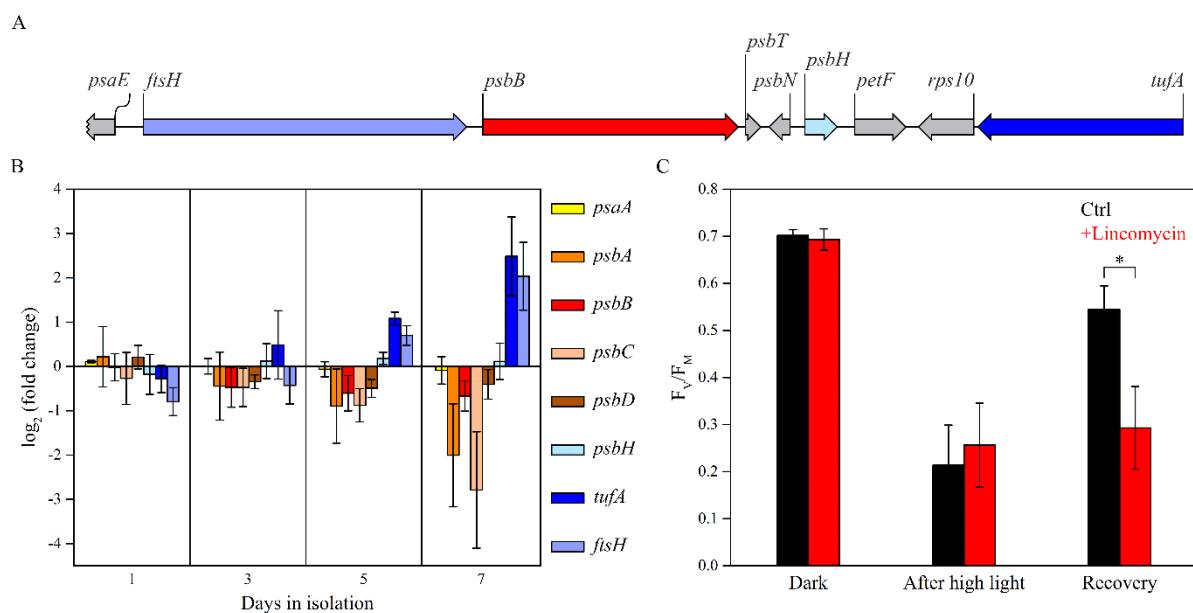
323 Results

324 Isolated *V. litorea* plastids maintain regulated gene expression

325 Laboratory-isolated *V. litorea* plastids exhibited differentially regulated gene expression even
326 after seven days in isolation (Fig. 2). The orientations of selected genes in *V. litorea* plastome
327 are shown in Fig. 2A. PSII core subunit genes *psbA*, *psbB*, *psbC* and *psbD* were downregulated
328 after day 3 of the isolation period, while *psbB* and *psbD*, encoding CP47 and D2 proteins of
329 PSII, reached a stationary level after five days, and the transcription of the genes encoding PSII
330 proteins CP43 (*psbC*) and D1 (*psbA*) were among those downregulated most significantly (Fig.
331 2B). The main protein of PSII targeted for degradation after photoinhibition is D1, whereas
332 release of CP43 from the PSII core has been suggested to precede D1 degradation in higher
333 plants (Aro *et al.*, 2005). One gene, *psbH*, encoding a small PSII subunit involved in proper
334 PSII assembly in cyanobacteria (Komenda *et al.*, 2005), exhibited stationary transcript levels

335 throughout the isolation period, similar to the gene encoding PSI reaction center subunit PsaA.
336 Transcription of *ftsH* and *tufA*, encoding the maintenance protease FtsH and the translation
337 elongation factor EF-Tu, followed an upward trajectory throughout the experiment (Fig. 2B).
338 We also tested the genetic autonomy of plastids sequestered by *E. timida* that feeds on *A.*
339 *acetabulum*. Subjecting the slugs to high light for 40 min resulted in a drastic decrease in PSII
340 photochemistry (F_v/F_M), but the kleptoplasts inside the slugs were capable of restoring PSII
341 activity back to 78 % of the initial level during a 20 h recovery period. Subjecting the slugs to
342 lincomycin, a plastid specific translation inhibitor (Mulo *et al.*, 2003), however, almost
343 completely prevented the recovery (Fig. 2C).

344



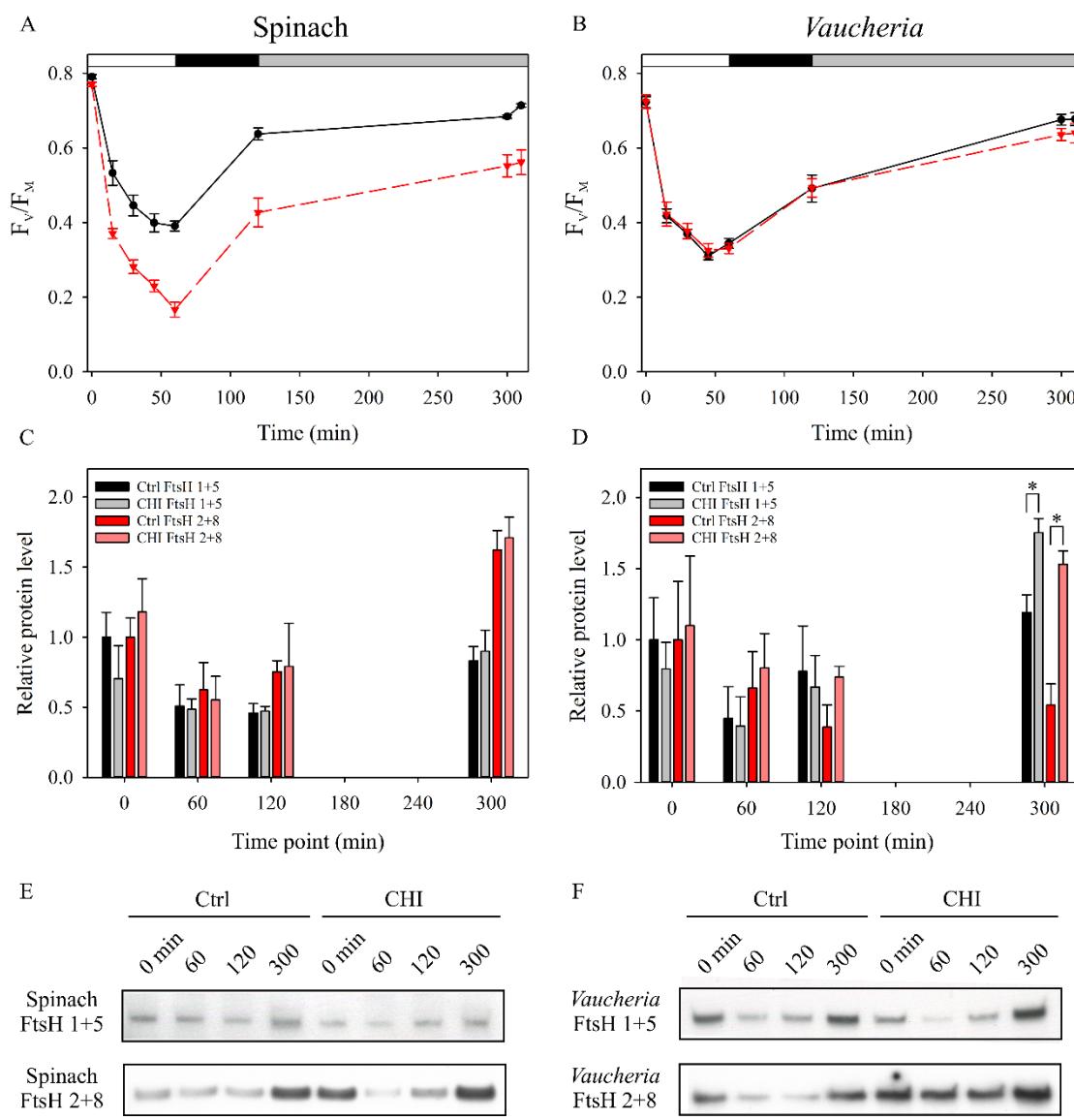
345

346 **Figure 2. Transcription of plastid encoded genes in isolated *V. litorea* plastids and the**
347 **autonomy of kleptoplasts inside the sea slug *E. timida*.** (A) Orientation of specific genes
348 inspected in (B) in *V. litorea* plastid genome. (B) Amounts of transcripts of selected genes
349 during a period of seven days in isolation buffer; each transcript has been compared to the
350 amount measured immediately after plastid isolation. (C) Maximum quantum yield of PSII
351 photochemistry (F_v/F_M) measured at different timepoints of the photoinhibition treatment (40
352 min, PPFD 2000 $\mu\text{mol m}^{-2}\text{s}^{-1}$) and after overnight recovery (PPFD < 20 $\mu\text{mol m}^{-2}\text{s}^{-1}$) in *E.*
353 *timida* slug individuals in the absence and presence of lincomycin. The data in panels (B) and
354 (C) are averages from three and four biological replicates, respectively. Error bars indicate
355 standard deviation. An asterisk indicates a statistically significant difference between the two
356 groups (Welch's t-test, $P < 0.005$).

357

358 **FtsH translation is enhanced in functionally isolated plastids of *V. litorea* during**
359 **recovery from photoinhibition**

360 Treating spinach leaves with CHI, a cytosolic translation inhibitor, resulted in faster loss of
361 PSII activity in high light (Fig. 3A). Also PSII repair was impaired by CHI in spinach. *V. litorea*
362 showed almost no effect of CHI during the same photoinhibition and recovery treatment (Fig.
363 3B). Using two different FtsH antibodies (FtsH 1+5 and FtsH 2+8), we tested the possible
364 involvement of plastid-encoded FtsH of *V. litorea* in the unaffected PSII photochemistry in
365 CHI treated samples. There were no differences in the relative protein levels of FtsH between
366 control and CHI treated spinach during the experiment (Fig. 3C). Genes for FtsH reside in the
367 nucleus in spinach, and our results suggest that the CHI treatment did not inhibit cytosolic
368 translation in the leaves entirely, although *de novo* synthesis of proteins could not be tested by
369 radiolabeling experiments. In *V. litorea*, CHI treatment increased FtsH levels towards the end
370 of the experiment (Fig. 3D). This suggests that not only is expression of plastome genes active
371 in functionally isolated plastids of *V. litorea*, but the translation of specific genes such as *ftsH*
372 can be upregulated when the plastids are deprived from normal cytosolic governance.



374 **Figure 3. *V. litorea* recovers from photoinhibition of PSII in the presence of**
375 **cycloheximide, a cytosolic translation inhibitor, and exhibits upregulation of FtsH.**
376 Quantum yield of PSII photochemistry (F_v/F_m) during photoinhibition treatment and
377 subsequent recovery of (A) spinach and (B) *V. litorea* in the absence (ctrl; black) and presence
378 of CHI (red). 0 min timepoint was measured before the onset of high-light treatment, 60 min
379 timepoint after the high-light treatment (PPFD 2000 $\mu\text{mol m}^{-2}\text{s}^{-1}$), 120 min timepoint after
380 subsequent dark recovery, and 300 min timepoint after recovery in dim light (10 $\mu\text{mol m}^{-2}\text{s}^{-1}$).
381 The final timepoint at 310 min was measured after additional 10 min dark acclimation. The
382 white, black and gray bars on top indicate the high-light treatment, dark and dim light periods,
383 respectively. (C) Relative levels of FtsH in spinach and (D) *V. litorea* during the experiment,
384 as probed by antibodies raised against *A. thaliana* FtsH 5 (FtsH 1+5; black and grey bars for
385 ctrl and CHI treatments, respectively) and FtsH 2 (FtsH 2+8; red and light red bars for ctrl and

386 CHI treatments). The light treatment regime up to 300 min was the same as in (A) and (B).
387 Significant differences between treatments are indicated by an asterisk (Welch's t-test, $P < 0.05$,
388 $n=3$). (E, F) Representative FtsH Western blots from spinach and *V. litorea*, respectively, used
389 for protein quantification in panels (C) and (D). All data in (A) to (D) represent averages from
390 at least three independent biological replicates and the error bars represent SE.

391 **Thylakoids of *V. litorea* exhibit moderate photoinhibition of PSII and elevated
392 ROS damage, but produce little $^1\text{O}_2$**

393 Basic photosynthetic parameters of isolated thylakoids from spinach and *V. litorea* are shown
394 in Table 2. Photoinhibition of PSII during a 60 min high-light treatment of isolated thylakoids
395 proceeded according to first-order reaction kinetics (Tyystjärvi and Aro, 1996) in both species
396 (Fig. 4A). However, spinach thylakoids were more susceptible to damage, as indicated by the
397 larger rate constant of dark-corrected PSII photoinhibition (k_{PI}) (Table 2). General oxidative
398 stress assays of lipids and proteins of the thylakoid membranes exposed to high light showed
399 more ROS damage in *V. litorea* than in spinach thylakoids during the treatment (Fig. 4B,C).
400 Measurements of $^1\text{O}_2$ production, the main ROS produced by PSII (Krieger-Liszakay, 2005;
401 Pospíšil, 2012), from isolated thylakoids showed that the rate of $^1\text{O}_2$ production in *V. litorea* is
402 only half of that witnessed for spinach (Fig. 5A). This suggests that the main ROS, causing the
403 *in vitro* oxidative damage to lipids and proteins (Fig. 4B,C) in *V. litorea*, are partially reduced
404 oxygen species produced by PSI.

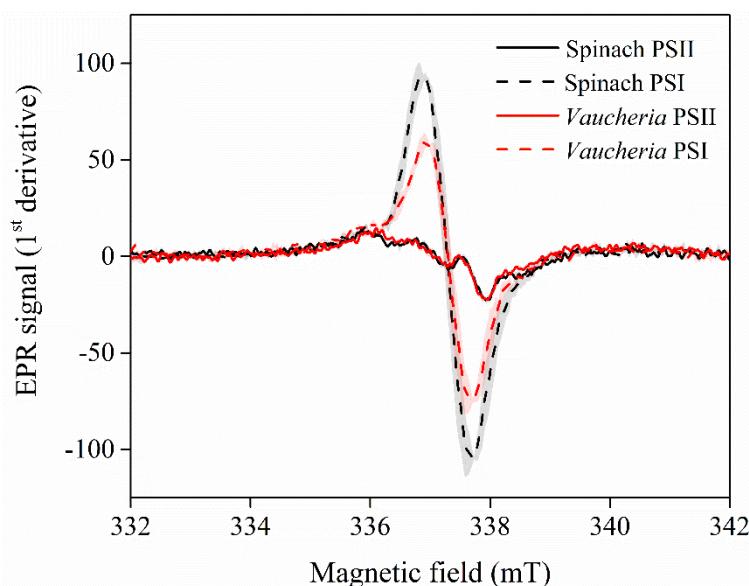
405

406 **Table 2. Photosynthesis-related parameters of isolated spinach and *V. litorea* thylakoid
407 membranes.** The EPR spectra used for estimating the PSI/PSII ratio are shown in
408 Supplementary Fig. S1. The indicated PSII and PSI activities are averages from all initial
409 activity measurements of untreated control samples discussed in this publication. The k_{PI} value
410 was determined from first-order reaction fits of the photoinhibition data in Fig. 4A, and
411 corrected by subtracting the first-order rate constant of PSII inhibition in the dark
412 (Supplementary Fig. S2). All values are averages from a minimum of three biological replicates
413 and SE is indicated in parentheses.

Organism	PSI/PSII	PSII activity (H_2O to	PSI activity (DCPIP to methyl)	k_{PI} (min^{-1})

		DCBQ; μmol O ₂ evolved mg Chl ⁻¹ h ⁻¹)	viologen; μmol O ₂ consumed mg Chl ⁻¹ h ⁻¹)	
Spinach	2.438 (±0.100)	200.12 (±11.53)	758.22 (±77.14)	0.0289 (±0.002)
<i>V. litorea</i>	2.343 (±0.090)	244.54 (±15.71)	797.36 (±93.73)	0.0148 (±0.001)

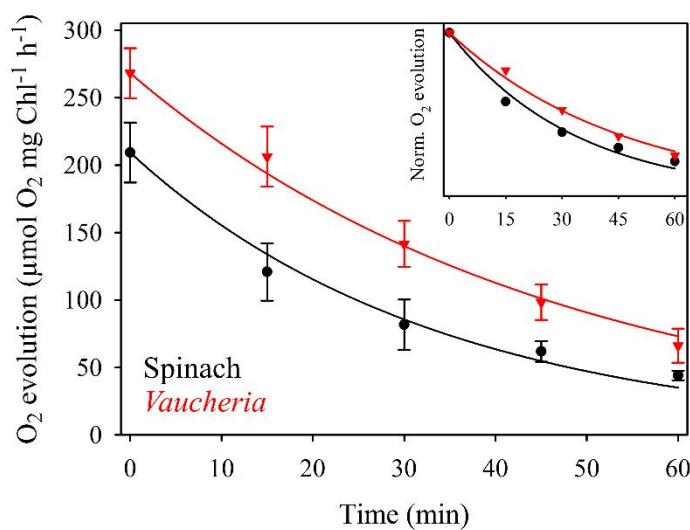
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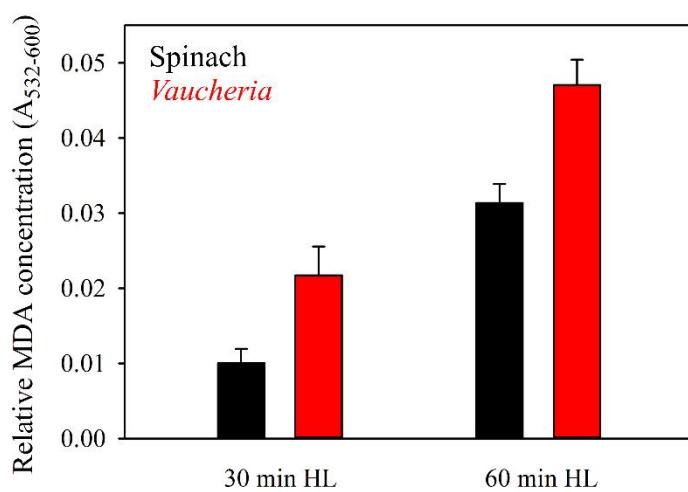
415

416 **Supplementary Figure S1. EPR spectra of PSII (TyrD⁺) and PSI (P₇₀₀⁺) in spinach and *V.***
417 ***litorea* thylakoids.** All spectra were measured from isolated thylakoid samples containing 2000
418 μg total Chl ml⁻¹. Each curve represents an average of three independent biological replicates
419 and the shaded areas around the curves represent SE.

A



B

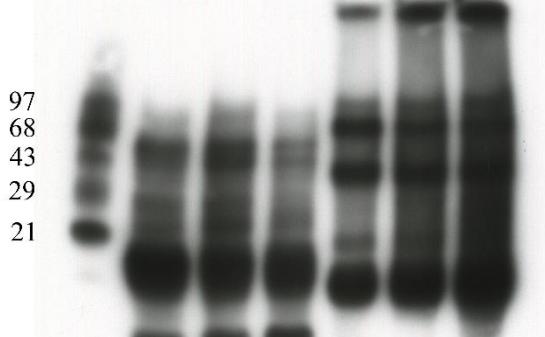


C

Spinach *Vaucheria*

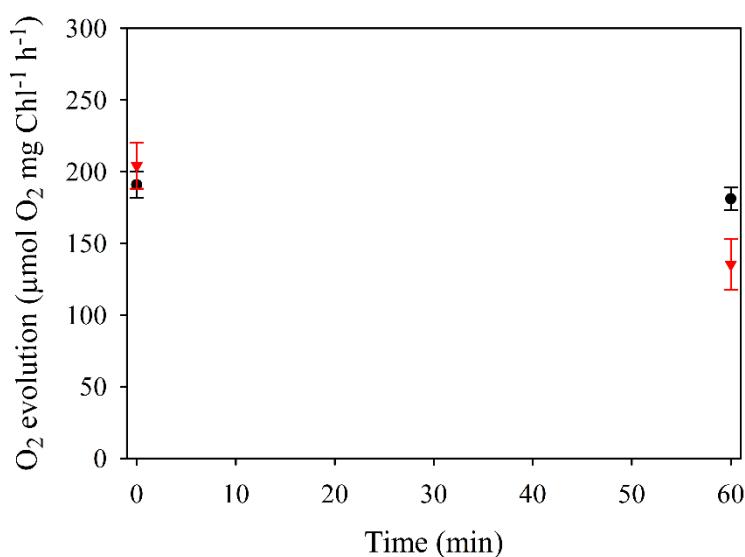
		0 min	30	60	0 min	30	60
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kDa MW 0 min 30 60 0 min 30 60



421 **Figure 4. *In vitro* photoinhibition of PSII and ROS production in spinach (black) and *V.*
422 *litorea* (red) thylakoids in high light.** (A) Photoinhibition of PSII in high light (PPFD 1000
423 $\mu\text{mol m}^{-2}\text{s}^{-1}$), as estimated by oxygen evolution. The curves show the best fit to a first order
424 reaction in spinach and *V. litorea*. Data normalized to the initial oxygen evolution rates are
425 shown in the inset to facilitate comparison. Dark control experiments, shown in supplementary
426 Fig. S2, indicated a 4.9 % (SE \pm 3.6, n=3) and 27.5 % (SE \pm 6.7, n=3) loss of PSII activity after
427 60 min in the dark for spinach and *V. litorea*, respectively. (B) Lipid peroxidation after 30 and
428 60 min of high-light treatment in spinach and *V. litorea*, as indicated by MDA formation. MDA
429 formed during dark control treatments were subtracted from the high-light treatment data. (C)
430 A representative OxyblotTM assay of protein carbonylation during the high-light treatment.
431 Each data point in panels (A, B) represents an average from a minimum of three biological
432 replicates and the error bars indicate SE.

433



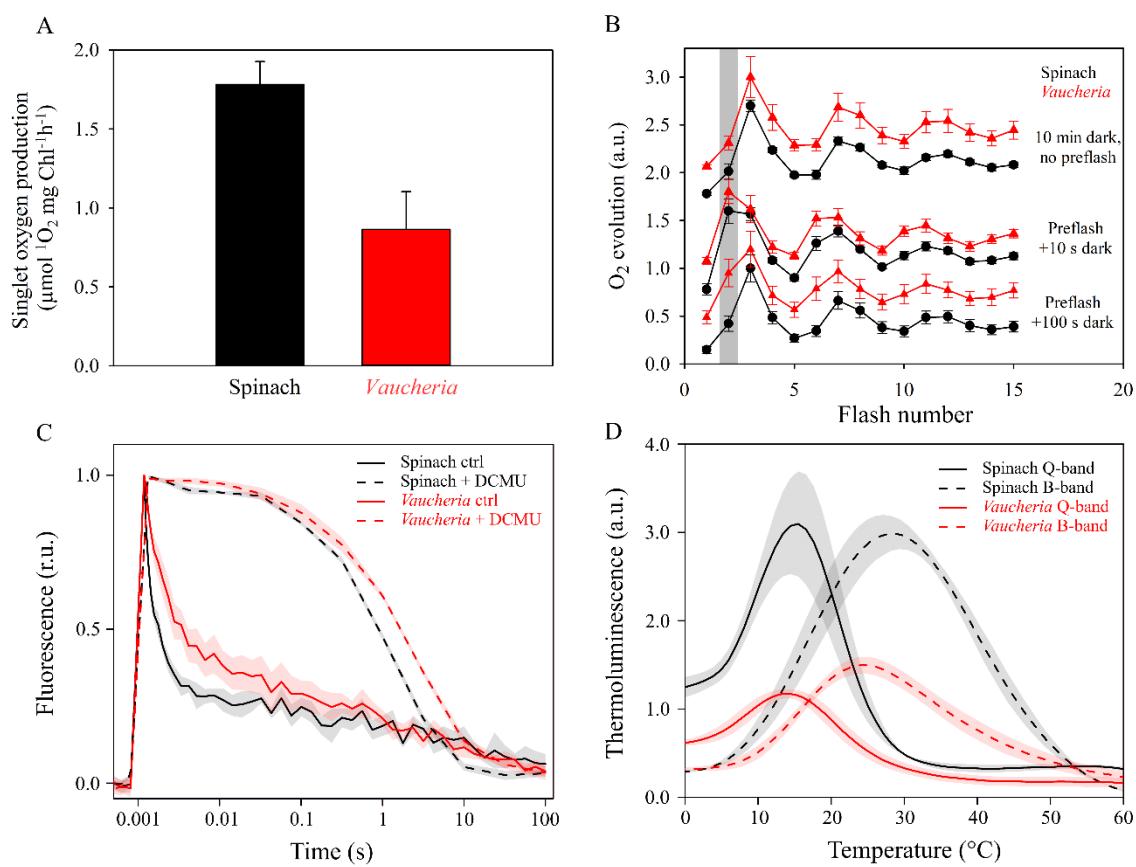
434

435 **Supplementary Figure S2. Dark control treatments of the *in vitro* photoinhibition**
436 **experiments shown in Fig. 4A of the main text.** PSII activities of spinach (black) and *V.*
437 *litorea* (red) at the onset and after a 60 min dark treatment at 22 °C in photoinhibition buffer.
438 Oxygen evolution was measured in the presence of 0.5 mM DCBQ and hexacyanoferrate(III)
439 from samples containing 20 μg total Chl ml^{-1} . Rate constant of PSII dark inactivation was 0.001
440 min^{-1} for spinach and 0.007 min^{-1} for *V. litorea*. Each data point represents an average of three
441 biological replicates and the error bars indicate SE.

442

443 *V. litorea* produces only little $^1\text{O}_2$, likely due to slow PSII charge recombination

444 We probed charge recombination reactions within PSII using three different methods to
445 investigate the role of PSII in the low $^1\text{O}_2$ yield in *V. litorea* thylakoids (Fig. 5A). First, we
446 measured flash-induced oxygen evolution from isolated thylakoids of spinach and *V. litorea*.
447 After 10 min dark acclimation, thylakoids from both species exhibited a typical pattern of
448 oxygen evolution, i.e. the third flash caused the highest oxygen yield due to the predominance
449 of the dark-stable S_1 state of the oxygen evolving complex (OEC), after which the oxygen yield
450 oscillated with a period of four until dampening due to misses and charge recombination
451 reactions (Fig. 5B, top curves). A single turnover pre-flash treatment makes S_2 the predominant
452 state. A 10 s dark period after the pre-flash treatment was not long enough to cause noticeable
453 changes in the S-state distribution in either species, as can be seen from the middle curves of
454 Fig. 5B, where the second flash of the flash train causes the highest yield of oxygen. In spinach,
455 100 s darkness after the pre-flash treatment resulted in nearly complete restoration of the
456 original S-states, whereas in *V. litorea* the second flash still yielded a considerable amount of
457 oxygen (Fig. 5B, bottom curves). This is likely due to slow charge recombination between Q_B^-
458 and the S_2 state of the OEC in *V. litorea* (Pham *et al.*, 2019). The modeled percentage S-state
459 distributions of OEC from spinach and *V. litorea* after different dark times between the pre-
460 flash and the flash train are shown in Supplementary table S2.



461

462 **Figure 5. *V. litorea* thylakoids produce little $^1\text{O}_2$ and exhibit slow charge recombination**
463 **of PSII.** (A) $^1\text{O}_2$ production in spinach (black) and *V. litorea* (red) thylakoid membranes. (B)
464 Flash oxygen evolution after different preflash treatments in spinach and *V. litorea* thylakoids.
465 The grey bar highlights the oxygen yield instigated by the second flash, an indicator of charge
466 recombination reactions taking place during the dark period between a preflash and the
467 measuring flash sequence. Oxygen traces were double normalized to the first (zero level) and
468 third flash and shifted in Y-axis direction for clarity. (C) Chl fluorescence decay kinetics after
469 a single turnover light pulse in untreated (solid lines) and DCMU poisoned (dashed lines)
470 thylakoids, double normalized to zero level before the onset of the pulse and maximum
471 fluorescence measured 150 μs after the pulse. (D) Q (solid lines) and B band (dashed lines) of
472 thermoluminescence, measured in the presence and absence of DCMU, respectively. All data
473 in (A-C) represent averages from at least three biological replicates. Thermoluminescence data
474 in (D) are from three replicates obtained from pooled thylakoid batches isolated from three
475 plants/algae flasks. Error bars and shaded areas around the curves show SE.

476

477 **Supplementary table S2. Percentage distribution of the S-states of the OEC in isolated**
478 **thylakoids from spinach and *V. litorea* after different preflash treatments prior to**
479 **measuring flash induced oxygen evolution.** The flash oxygen data in Figure 5B was modeled
480 essentially as described in Antal *et al.*, (2009) to estimate the S-state distribution.

	S ₀ % (±SE)	S ₁ % (±SE)	S ₂ % (±SE)	S ₃ % (±SE)
Spinach 10 min dark	0.01 (±0.01)	71.01 (±2.78)	24.54 (±1.95)	4.44 % (±1.15)
Spinach preflash + 10 s dark	0	32.30 (±9.36)	57.28 (±5.83)	10.42 (±3.63)
Spinach preflash + 100 s dark	0	65.30 (±0.17)	26.32 (±0.07)	8.39 (±0.24)
Vaucheria 10 min dark	6.84 (±3.20)	63.44 (±3.66)	24.65 (±3.70)	5.08 (±3.20)
Vaucheria preflash + 10 s dark	0.02 (±0.01)	24.69 (±1.19)	60.62 (±0.59)	14.66(±0.83)
Vaucheria preflash + 100 s dark	0.01 (±0.01)	45.21 (±2.03)	39.18 (±0.92)	15.61 (±2.53)

481

482 Next, we measured the decay of Chl *a* fluorescence yield after a single turnover flash from
483 thylakoids in the absence and presence of the PSII electron transfer inhibitor DCMU.
484 Fluorescence decay in the absence of DCMU reflects Q_A⁻ reoxidation mainly by electron
485 donation to Q_B and Q_B⁻. In the presence of DCMU, fluorescence decay is indicative of Q_A⁻
486 reoxidation through various charge recombination reactions (Mamedov *et al.*, 2000), some of
487 which generate the harmful triplet P₆₈₀ Chl through the intermediate P₆₈₀⁺Pheo⁻ radical pair
488 (Sane *et al.*, 2012). The decay of fluorescence yield was slower in *V. litorea* thylakoids than in
489 spinach both in the absence and presence of DCMU (Fig. 5C). In the absence of DCMU, the
490 slower kinetics in *V. litorea* shows that electron transfer from Q_A⁻ to Q_B is not as favorable as
491 in spinach. The slow decay of fluorescence in the presence of DCMU indicates slow S₂Q_A⁻
492 charge recombination.

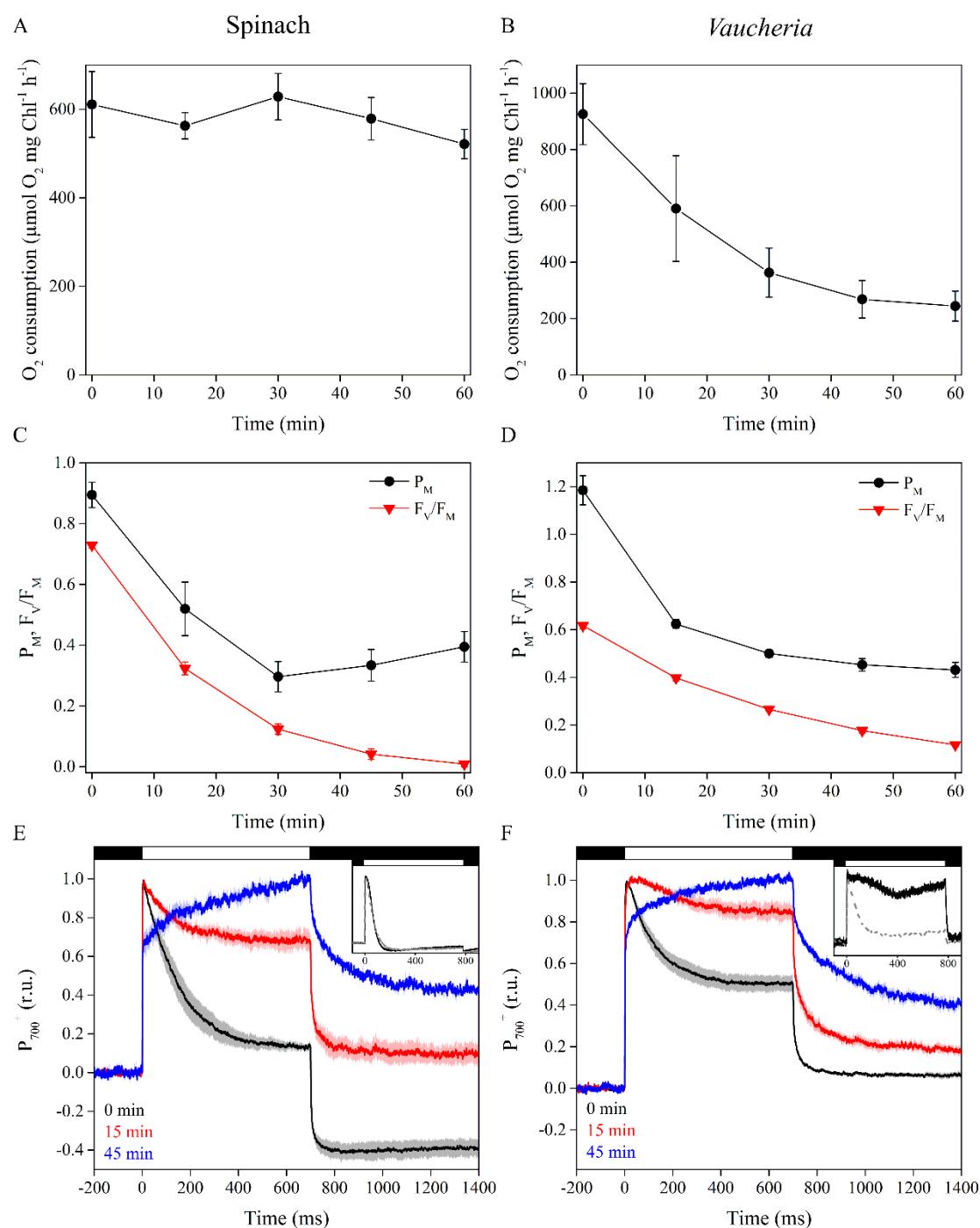
493 Thermoluminescence Q and B bands from thylakoids in the presence and absence of DCMU,
494 respectively, were also measured. For a description on the interpretation of
495 thermoluminescence data, see Tyystjärvi and Vass, (2004) and Sane *et al.*, (2012). Briefly, the
496 thylakoid samples were dark acclimated for 5 min, cooled down to -20 °C, flashed with a single
497 turnover Xenon flash and then heated with a constant rate. The luminescence emitted by the
498 samples at different temperatures is proportional to the rate of the luminescence-producing
499 charge recombination reactions between the S-states of the OEC and downstream electron
500 acceptors, more specifically S_2/Q_A^- (Q band) and $S_{2,3}/Q_B^-$ (B band). The Q and B band emission
501 peaks in spinach were at 15 and 28 °C, whereas in *V. litorea* they were at 14 and 24 °C (Fig.
502 5D). The lower peak temperatures in *V. litorea* would actually suggest that both Q_A^- and Q_B^-
503 are less stable at room temperature in *V. litorea* than in spinach. However, the multiple
504 pathways of recombination (Rappaport and Lavergne, 2009) obviously allow the
505 luminescence-producing minor pathway to suggest destabilization of Q_A^- in *V. litorea* (Fig.
506 5D) even if the total recombination reaction is slower in *V. litorea* than in spinach (Fig. 5B,C
507 and Supplementary table S2). The thermoluminescence signal intensity was lower in *V. litorea*
508 than in spinach, suggesting that the luminescence-producing reaction has a low yield in *V.*
509 *litorea*. The narrow energy gap between Q_A and Q_B in *V. litorea* favors the probability of an
510 electron residing with Q_A . Furthermore, a small Q_A - Q_B energy gap also increases the
511 probability that $S_3Q_B^-$ or $S_2Q_B^-$ recombine directly and non-radiatively without producing
512 triplet P_{680} and subsequently 1O_2 (Ivanov *et al.*, 2003; Sane *et al.*, 2003; Ivanov *et al.*, 2008;
513 Sane *et al.*, 2012).

514 *In vitro* high-light treatment lowers electron donation to methyl viologen and
515 maximal oxidation of P_{700} in *V. litorea*

516 When PSI activity was estimated as electron transfer from DCPIP to methyl viologen (oxygen
517 consumption), spinach PSI remained undamaged during *in vitro* high-light treatment, while *V.*
518 *litorea* seemed highly susceptible to photoinhibition of PSI (Fig. 6A,B). We repeated the
519 photoinhibition experiment, but this time PSII and PSI activities were monitored with Chl
520 fluorescence and P_{700} absorption changes. Again, thylakoid membranes of spinach were more
521 sensitive to photoinhibition of PSII during the high-light treatment than *V. litorea* (Fig. 6C,D).
522 However, this time PSI functionality of both species decreased similarly when estimated as the
523 maximum oxidation of P_{700} (P_M). The decrease in P_M was strong during the first 15 (*V. litorea*)
524 or 30 min (spinach) of the light treatment, whereafter P_M remained at a somewhat stationary
525 level (Fig. 6C,D). The decrease in P_M depended on electron transfer from PSII, as P_M did not

526 decrease in high light in spinach thylakoids in the presence of DCMU (Supplementary Fig.
527 S4).

528 In both spinach and *V. litorea*, redox kinetics of P₇₀₀, measured in aerobic conditions from
529 thylakoids (Fig. 6E,F) were similar as their respective *in vivo* kinetics (Fig. 6E,F insets), i.e.
530 P₇₀₀ in *V. litorea* remained more oxidized during a light pulse than in spinach. Isolating
531 thylakoids from *V. litorea* did, however, cause a decrease in P₇₀₀ oxidation capacity. Unlike in
532 spinach, P₇₀₀ remains oxidized during a high-light pulse in intact *V. litorea* cells if oxygen is
533 present, indicating that alternative electron sinks, such as flavodiiron proteins, function as
534 efficient PSI electron acceptors *V. litorea* (Fig. 6E,F insets), probably protecting PSI against
535 formation of ROS (Allahverdiyeva *et al.*, 2015; Ilík *et al.*, 2017; Shimakawa *et al.*, 2019). In
536 both species, P₇₀₀ redox kinetics changed in the same way during the course of the high-light
537 treatment of isolated thylakoids. The tendency of both species to maintain P₇₀₀ oxidized
538 throughout the high-light pulse in measurements done after 15 min treatment in high light is
539 possibly due to decreasing electron donation caused by photoinhibition of PSII. At 45 min
540 timepoint the damage to PSI is more severe, as indicated by a clear slowing down of P₇₀₀
541 oxidation, which could be associated with problems in electron donation to downstream
542 electron acceptors of PSI, such as ferredoxin (Fig. 6E,F).

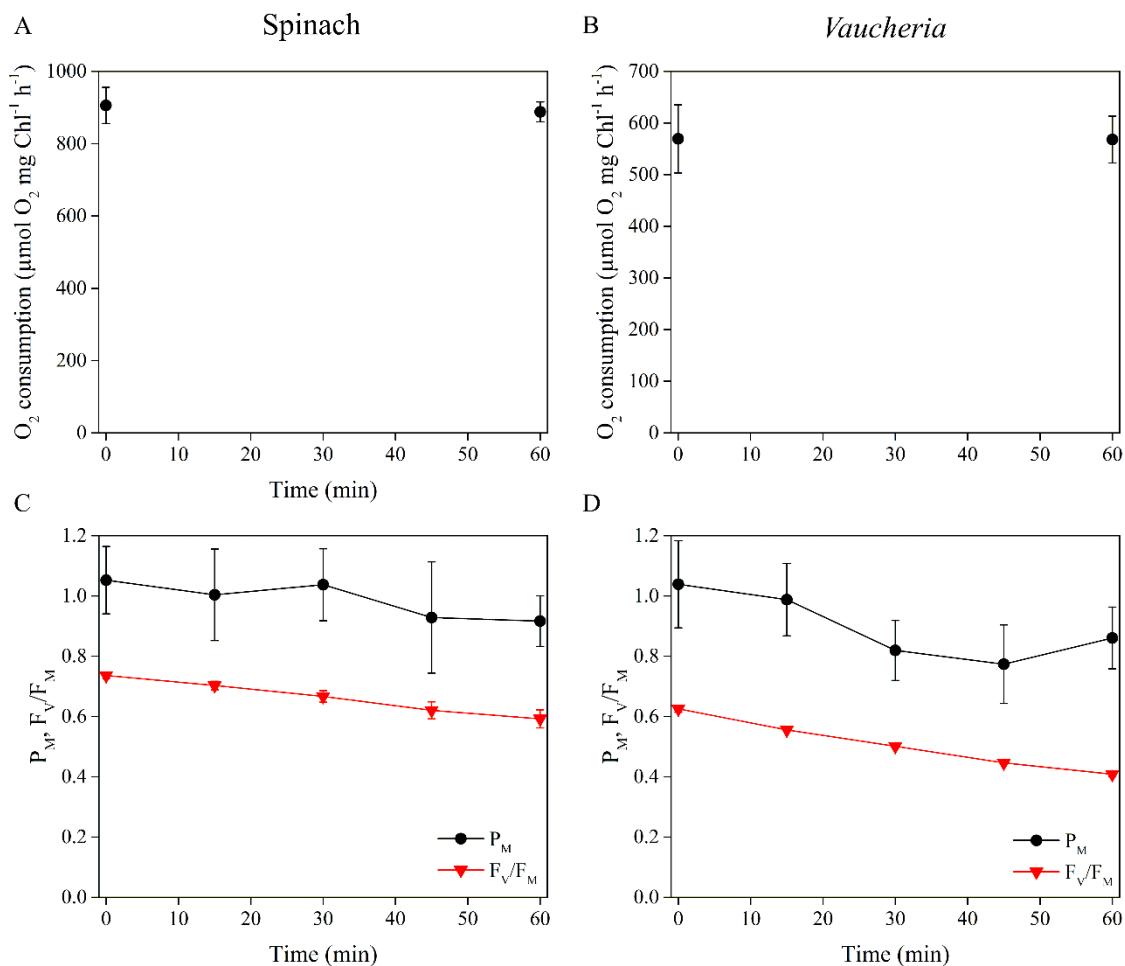


543

544 **Figure 6. Photoinhibition of PSI in isolated thylakoids of spinach and *V. litorea* during**
 545 **high-light treatment, estimated with oxygen measurements or absorption based methods.**
 546 (A, B) Photoinhibition of PSI in spinach and *V. litorea*, respectively, in the same experimental
 547 setup as in Fig. 5. (C, D) Decrease in maximal oxidation of P_{700} reaction center Chl (P_M ; black)
 548 and PSII photochemistry (F_v/F_m ; red) during high-light treatment (PPFD 1000 $\mu\text{mol m}^{-2}\text{s}^{-1}$) in
 549 isolated spinach and *V. litorea* thylakoids. (E) P_{700} redox kinetics of spinach thylakoids during
 550 the high-light pulses used for P_M determination after 0, 15 and 45 min in photoinhibition
 551 treatment (black, red and blue, respectively). Black and white bars on top indicate darkness and

552 illumination by the high-light pulse, respectively. (F) The same measurements as in (E) in *V.*
553 *litorea* thylakoids. The insets of (E, F) show P_{700} redox kinetics from intact spinach leaves and
554 *V. litorea* cells in aerobic (black, solid line) and anaerobic conditions (grey, dashed line). Dark
555 control experiments are shown in Supplementary figure S3. The P_{700} kinetics in (E) and (F)
556 have been normalized to stress the form of the curve. All data are averages from at least three
557 biological replicates and error bars and the shaded areas around the curves indicate SE.

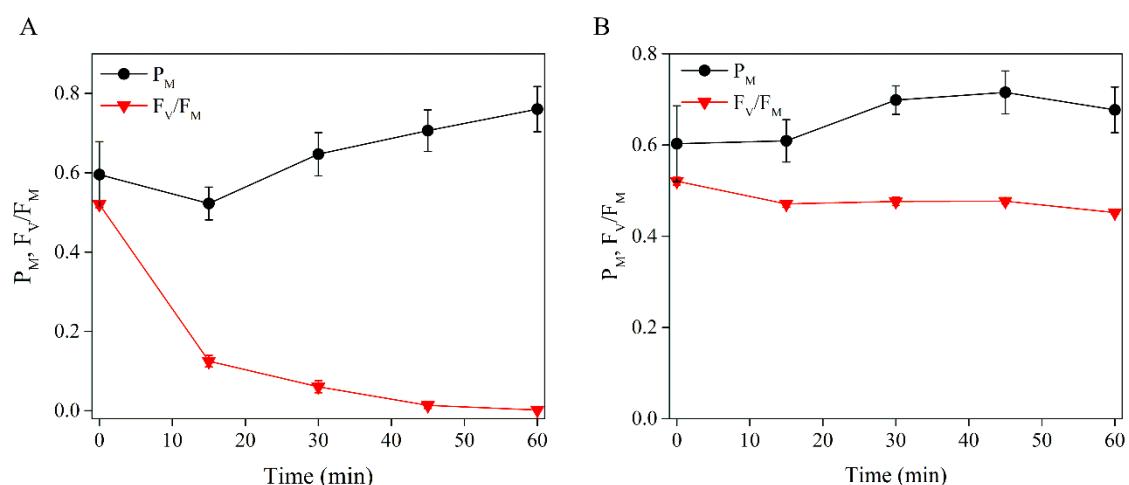
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559

560 **Supplementary figure S3. Dark control treatments of the *in vitro* photoinhibition**
561 **experiments shown in Fig. 6 of the main text.** (A, B) PSI activities of spinach and *V. litorea*
562 during a 60 min dark incubation period in photoinhibition buffer, measured as oxygen
563 consumption. (C, D) PSI and PSII activities in isolated spinach and *V. litorea* thylakoids during
564 a 60 min dark treatment, as estimated by maximal oxidation of P_{700} (P_M ; black) and F_v/F_M
565 (red), respectively. All data are averages from a minimum of three biological replicates and
566 error bars indicate SE.

567



568

569 **Supplementary figure S4. DCMU prevents photoinhibition of PSI in isolated spinach**
570 **thylakoids.** (A) Maximal oxidation of P_{700} (P_M ; black) and maximum quantum yield of PSII
571 (F_v/F_M ; red) were measured during a 60 min high-light treatment (PPFD 1000 $\mu\text{mol m}^{-2}\text{s}^{-1}$) in
572 DCMU treated spinach thylakoids. (B) Dark control experiments using the same setup as in
573 (A). All data are averages from four biological replicates and the error bars indicate SE.

574

575 Discussion

576 Uregulation of FtsH at the center of *V. litorea* plastid longevity

577 Previous studies have shown that the kleptoplasts stemming from *V. litorea* carry out *de novo*
578 protein translation and are generally quite robust inside *E. chlorotica* (Green *et al.*, 2000;
579 Rumpho *et al.*, 2001; Green *et al.*, 2005). Our transcriptomic analysis of *V. litorea* plastids
580 demonstrates active and regulated transcription of the plastome throughout the seven days of
581 isolation we tested (Fig. 2), deepening our knowledge about the factors underpinning their
582 native robustness. Considering gene orientation of the up- and downregulated genes suggests
583 that e.g. *ftsH* and *psbB*, neighboring genes sharing the same orientation, do not constitute an
584 operon (Fig. 2A).

585 Our results highlight the upregulation of *ftsH* and *tufA* during a period of several days after
586 isolation of *V. litorea* plastids. Active transcription of these genes also occurs in the plastids of
587 *E. timida* after a month of starvation (de Vries *et al.*, 2013). FtsH protease is critical for the
588 PSII repair cycle, where it is responsible for degradation of the D1 protein after pulling it out
589 of the PSII reaction center. Recent findings in cyanobacteria, green algae and higher plants
590 imply that FtsH is also important for quality control of a multitude of thylakoid membrane

591 proteins and thylakoid membrane biogenesis (reviewed by Kato and Sakamoto, 2018). These
592 findings may suggest that already the removal of the D1 protein from damaged PSII serves to
593 protect from further photodamage and the production of ROS. The results of our
594 photoinhibition experiments on the long-term retention slug *E. timida* may serve as a model of
595 photoinhibition in other slugs, as they indicate that the kleptoplasts of *E. timida* possess a
596 genetic toolkit capable of maintaining a PSII repair cycle (Fig. 2C).

597 We showed that the capacity of *V. litorea* plastids to recover from photoinhibition of PSII in
598 the presence of CHI is nearly unaffected (Fig. 3B). While our CHI experiments on spinach
599 need further exploration in terms of CHI effects, studies on the green alga *Chlamydomonas*
600 *reinhardtii* (that also lacks *ftsH* in its plastome) have shown severe defects in PSII repair both
601 during high-light and subsequent recovery when exposed to CHI (Fig. 3A, Wang *et al.*, 2017).
602 *C. reinhardtii* mutant lines have also been used to show that abundant FtsH offers protection
603 from photoinhibition of PSII and enhances the recovery process (Wang *et al.*, 2017). In *C.*
604 *reinhardtii*, the FtsH hetero-oligomers responsible for D1 degradation are comprised of FtsH1
605 (A-type) and FtsH2 (B-type) (Malnoë *et al.*, 2014). We probed the relative FtsH protein levels
606 of *V. litorea* during the photoinhibition experiment using antibodies raised against *A. thaliana*
607 A- (FtsH 1+5) and B-type FtsH (FtsH 2+8) in the absence and presence of CHI (Fig. 3D). At
608 the end of the recovery period the CHI treated cells showed elevated levels of FtsH according
609 to both tested antibodies. The elevated FtsH abundance did not enhance the recovery from
610 photoinhibition of PSII in our experimental setup (Fig. 3B), but our results do point to a
611 tendency of both, truly isolated (Fig. 2) and functionally isolated (Fig. 3) *V. litorea* plastids, to
612 upregulate FtsH.

613 **Low $^1\text{O}_2$ yield does not prevent photoinhibition of PSII, but can help maintain
614 efficient repair processes in *V. litorea***

615 A green alga that is nearly immune to photoinhibition of PSII, *Chlorella ohadii*, has been
616 isolated from the desert crusts of Israel (Treves *et al.*, 2013; 2016). Its resilience against
617 photoinhibition of PSII has largely been attributed to very narrow energetic gap between Q_A
618 and Q_B , favoring non-radiative charge recombination pathways within PSII that do not lead to
619 $^1\text{O}_2$ production (Treves *et al.*, 2016). While *V. litorea* does not have as small energetic gap
620 between Q_A and Q_B as *C. ohadii* (temperature difference of *V. litorea* Q- and B-band
621 thermoluminescence peaks was 10 °C, whereas in *C. ohadii* it is only 2-4 °C), PSII charge
622 recombination reactions of *V. litorea* appear to be very slow compared to those of spinach (Fig.

623 5B-D). Furthermore, the low ${}^1\text{O}_2$ yield in *V. litorea* (Fig. 5A) suggests that the charge
624 recombination reactions favor the direct non-radiative pathway. The low ${}^1\text{O}_2$ yield in *V. litorea*
625 likely factors into the lower dark-corrected rate constant of PSII photoinhibition in comparison
626 to that of spinach thylakoids (Table 2) (Vass, 2011). All of our experiments, however, show
627 that *V. litorea* does experience quite regular levels of PSII photoinhibition. This could indicate
628 that the most important effect of the low ${}^1\text{O}_2$ yield is protection of the autonomous maintenance
629 machinery of the plastids, as ${}^1\text{O}_2$ has been shown to be specifically harmful for the PSII repair
630 cycle (Nishiyama *et al.*, 2004).

631 ***V. litorea* thylakoids are highly vulnerable to ROS in the absence of regular
632 stromal electron sinks**

633 Despite the lower rate constant of PSII photoinhibition (Table 2) and ${}^1\text{O}_2$ yield (Fig. 5A), *V.*
634 *litorea* thylakoids exhibited drastic oxidative damage to lipids and proteins under high light
635 (Fig. 4C,D). Isolated thylakoids are stripped of the main electron sink of PSI, the Calvin-
636 Benson-Bassham cycle, and comparing P_{700} redox kinetics of *V. litorea* cells and isolated
637 thylakoids (Fig. 6F and inset) reveals that they are also, at least partially, devoid of a Mehler-
638 like reaction that safely reduces oxygen to water (Allahverdiyeva *et al.*, 2013). This suggests
639 that catalysts of oxygen reduction in *V. litorea* are likely soluble and therefore lost during the
640 isolation procedure. Angiosperm plants like spinach do not rely on a Mehler-like reaction and
641 are susceptible to photoinhibition of PSI in fluctuating light (Shimakawa *et al.*, 2019). The PSI
642 photoprotection by Mehler-like reaction has been assigned to enhanced electron sink capacity
643 that lowers the probability of one-electron reduction of oxygen to superoxide by PSI. In
644 comparison to spinach, this would make intact plastids of *V. litorea* less reliant on other ROS
645 detoxification components that detoxify superoxide and hydrogen peroxide in the water-water
646 cycle (Asada, 1999). Conversely, loss of the Mehler-like reaction during thylakoid isolation
647 would leave the thylakoids highly conducive for ROS production by PSI and very susceptible
648 to oxidative damage of the entire photosynthetic machinery. This is likely behind the finding
649 that *V. litorea* thylakoids lose the ability to reduce methyl viologen in a high-light treatment
650 that does not affect spinach thylakoids (Fig. 6A,B). When damage to PSI was estimated as a
651 decrease in P_M , spinach and *V. litorea* thylakoids showed very similar responses to high light,
652 with both species exhibiting a decrease in PSI activity until electron donation from PSII was
653 diminished due to photoinhibition of PSII (Fig. 6C,D), as suggested earlier (Sonoike, 1995;
654 1996). This, in addition to the highly similar changes in the redox kinetics of P_{700} during the
655 photoinhibition treatment (Fig. 6E,F) between the two species, would suggest that the decrease

656 in oxygen consumption in *V. litorea* thylakoids is caused by a further, more severe damage to
657 PSI than the process causing the decrease in P_M . The nature of this reaction is not known but it
658 may be caused by production of ROS due to continuing electron flow through PSI in thylakoids
659 of *V. litorea* exhibiting a low rate constant of PSII photoinhibition (Table 2) and normally
660 relying on stromal electron acceptors for protection of PSI.

661 PSI of *V. litorea* is not particularly prone to photoinhibition, but our results do confirm that the
662 electron sinks of photosynthesis must be functional in order to avoid large scale oxidative
663 damage. This is especially relevant for animals that host a foreign organelle where uncontrolled
664 ROS production is detrimental (de Vries *et al.* 2015). Our recent results on the LTR slug *E.*
665 *timida* show that oxygen functions as an alternative electron sink in the slug plastids
666 (Havurinne and Tyystjärvi, 2020), but whether the record-holding *E. chlorotica* utilizes the
667 oxygen dependent electron sinks provided by *V. litorea* (Fig. 6F inset) remains to be tested. As
668 for the main electron sink of photosynthesis, the carbon fixation rates of the plastids inside *E.*
669 *chlorotica* are comparable to the rates measured from *V. litorea* cells after incorporation
670 (Rumpho *et al.*, 2001), suggesting that carbon fixation is not a problem in *E. chlorotica*.

671 Conclusion

672 Plastids of *V. litorea* are genetically more autonomous than those of embryophytes, containing
673 genes that help to maintain plastid functionality. Isolating the plastids triggers upregulation of
674 the translation elongation factor EF-Tu and the central maintenance protease FtsH – a
675 phenomenon that may be important for plastid longevity in the foreign cytosol of a sea slug.
676 Low $^1\text{O}_2$ yield protects the functionality of the plastid-encoded maintenance machinery and
677 may slow down photoinhibition of PSII. Interruption of oxygen dependent alternative electron
678 sinks upstream of PSI leads to large scale oxidative damage in *V. litorea*, suggesting that carbon
679 fixation, the main electron sink of photosynthesis, needs to remain in near perfect working
680 order to avoid destruction of the plastids. Our results support decades old data (Trench *et al.*,
681 1973 *a, b*) suggesting that the native stability and associated peculiar functionality of the
682 plastids themselves hold the key to long-term kleptoplast longevity in sacoglossans. Nature has
683 evolved an elaborate suite of photoprotective mechanisms and the unique animal-kleptoplast
684 association allows to explore them and even identify new ones.

685 Supplementary data

686 Supplementary data are available at *JXB* online.

687 *Table S1.* List of primers used in qPCR experiment.

688 *Table S2.* Modeled S-state distribution of the OEC in spinach and *V. litorea*.

689 *Fig. S1.* EPR spectra from spinach and *V. litorea* thylakoids.

690 *Fig. S2.* Dark control treatments of *in vitro* PSII photoinhibition in spinach and *V. litorea*.

691 *Fig. S3.* Dark control treatments of *in vitro* PSI and PSII photoinhibition in spinach and *V.*

692 *litorea*.

693 *Fig. S4.* *In vitro* PSI and PSII photoinhibition in DCMU treated spinach thylakoids.

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701 Author Contributions

702 VH, SBG and ET planned the experiments. VH did all photosynthesis and $^1\text{O}_2$ measurements
703 and wrote the paper with comments from all authors; MH, supervised by SBG, did the gene
704 expression measurements and TEM imaging; MA measured lipid peroxidation, protein
705 oxidation and EPR spectra; SK developed the cuvette system for P_{700}^+ measurements; ET
706 supervised the work.

707 Data Availability

708 The data that support the findings of this study are openly available in Mendeley Data at
709 <http://doi.org/10.17632/535dcxjt2d.1>.

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