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1 **Title:**

2 Disentangling chloroplast ATP synthase regulation by proton motive force and thiol modulation in  
3 Arabidopsis leaves

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17

18 **Abstract:**

19 The chloroplast ATP synthase (CF<sub>1</sub>F<sub>o</sub>) contains a specific feature to the green lineage: a  $\gamma$ -subunit  
20 redox domain that contains a cysteine couple which interacts with the torque-transmitting  
21  $\beta$ DELSEED-loop. This thiol modulation equips CF<sub>1</sub>F<sub>o</sub> with an important environmental fine-tuning  
22 mechanism. *In vitro*, disulfide formation in the  $\gamma$ -redox domain slows down the activity of the CF<sub>1</sub>F<sub>o</sub> at  
23 low transmembrane electrochemical proton gradient ( $\Delta\tilde{\mu}_{H^+}$ ), which agrees with its proposed role as  
24 chock based on recently solved structure. The  $\gamma$ -dithiol formation at the onset of light is crucial to  
25 maximize photosynthetic efficiency since it lowers the  $\Delta\tilde{\mu}_{H^+}$  activation level for ATP synthesis *in*  
26 *vitro*. Here, we validate these findings *in vivo* by utilizing absorption spectroscopy in *Arabidopsis*  
27 *thaliana*. To do so, we monitored the  $\Delta\tilde{\mu}_{H^+}$  present in darkness and identified its mitochondrial  
28 sources. By following the fate and components of light-induced extra  $\Delta\tilde{\mu}_{H^+}$ , we estimated the ATP  
29 lifetime that lasted up to tens of minutes after long illuminations. Based on the relationship between  
30  $\Delta\tilde{\mu}_{H^+}$  and CF<sub>1</sub>F<sub>o</sub> activity, we conclude that the dithiol configuration *in vivo* facilitates photosynthesis  
31 by driving the same ATP synthesis rate at a significative lower  $\Delta\tilde{\mu}_{H^+}$  than in the  $\gamma$ -disulfide state. The  
32 presented *in vivo* findings are an additional proof of the importance of CF<sub>1</sub>F<sub>o</sub> thiol modulation,  
33 reconciling biochemical *in vitro* studies and structural insights.

34

35 Keywords: chloroplast ATP synthase, electrochromic shift, redox regulation, photosynthesis,  
36 Arabidopsis, electrochemical proton gradient

## 37 Introduction

38 During photosynthesis, the electron transfer is coupled to a movement of protons across the  
39 thylakoid membrane, generating an electrochemical proton gradient ( $\Delta\tilde{\mu}_{H^+}$ ) which consists of an  
40 osmotic component (the concentration gradient of hydrogen ions,  $\Delta pH$ ) and a membrane potential  
41 ( $\Delta\Psi$ ). For efficient photochemistry, the  $\Delta\tilde{\mu}_{H^+}$  partitioning must be dynamically regulated via  
42 thylakoid ion channels and antiporters [1, 2], as well as the chloroplast ATP synthase ( $CF_1F_o$ ). The  
43 latter catalyzes the conversion of the  $\Delta\tilde{\mu}_{H^+}$  into the synthesis of ATP, subsequently used in the  
44 Calvin-Benson-Bassham cycle. The enzyme consists of two portions [reviewed in 3, 4]: a membrane-  
45 spanning  $F_o$  (subunits  $IV_1I_1II_1III_{14}$ , or  $a_1b_1b'_{1c_{14}}$ ) and a membrane-attached  $F_1$  (subunits  $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$ ). In  
46 the chloroplast enzyme of vascular plants, during a 360° rotation of subunits  $\gamma\epsilon_{c_{14}}$  against the static  
47 subunits  $\alpha_3\beta_3\delta abb'$ , 14  $H^+$  are translocated along the electrochemical gradient while 3 molecules of  
48 ATP are synthesized [5]. Perfect coupling is observed in isolated chloroplasts, i.e.,  $H^+$  slip processes do  
49 practically not occur *in vitro* [6, 7, for a different view see Ref. 8]. Being reversible,  $CF_1F_o$  catalyzes  
50 both the synthesis and hydrolysis of ATP and the reaction direction is determined by the extent of  
51 the  $\Delta\tilde{\mu}_{H^+}$  and the  $[ATP]/([ADP][P])$  ratio [9].

52 The activity of the  $CF_1F_o$  plays a central role in photosynthesis; it fuels the chemical phase of  
53 photosynthesis with ATP but can also, via its conductivity for  $H^+$ , participate in the regulation of the  
54 photochemical phase by modulating the osmotic component of the  $\Delta\tilde{\mu}_{H^+}$  [10]. Indeed, the luminal  
55 pH is involved in the regulation of the photosynthetic electron transfer at two levels. It regulates the  
56 photoprotective mechanism in photosystem II (PSII) [11, 12] and the turnover of the cytochrome  $b_6f$   
57 through the so-called photosynthetic control [13]. Being at the crossroads of the dark and light  
58 phases of photosynthesis, the activity of  $CF_1F_o$  needs to be fine-tuned and, although other  
59 regulations exist [10, 14, 15], two main regulatory mechanisms can modulate the rates of the  $CF_1F_o$ .  
60 The first one is relevant for F-ATP synthase in general which is a regulation by its energy source, the  
61  $\Delta\tilde{\mu}_{H^+}$ . Above a threshold value of  $\Delta\tilde{\mu}_{H^+}$ , the transition of the inactive  $CF_1F_o$  to a fully active form was  
62 observed in isolated chloroplasts [16, 17]. Since the structural  $H^+/ATP$  ratio (subunits  $c/\beta$ ) differs  
63 slightly from the thermodynamic one, it is possible that a small  $\Delta\tilde{\mu}_{H^+}$  fraction is lost for ATP synthesis  
64 during the  $CF_1F_o$  activation process when the membrane is energized from an equilibrated state [8].  
65 Although the  $\Delta\tilde{\mu}_{H^+}$  must contain a minimal  $\Delta\Psi$  to allow for ATP synthesis at thermodynamic  
66 equilibrium [18], several studies on chloroplasts and leaves demonstrated that each of the two  
67 components of the  $\Delta\tilde{\mu}_{H^+}$  can activate the enzyme if they allow the  $\Delta\tilde{\mu}_{H^+}$  to reach this critical level  
68 [19-23]. We call this level  $\Delta\tilde{\mu}_{H^+}^{activation}$  hereafter and it can be reached by high  $[ATP]/([ADP][P])$  ratios  
69 *in vivo*, alternatively to illumination. Specific to  $CF_1F_o$  in the green lineage is a second mechanism,  
70 “thiol modulation” or “redox regulation”, which involves an insertion in the  $\gamma$ -subunit. The additional  
71 domain harbors a redox-active Cys couple which forms a disulfide in the dark. Formation of the  $\gamma$ -  
72 disulfide is, in part, mediated by a thioredoxin-like2/2-Cys peroxiredoxin redox cascade in  
73 Arabidopsis where electrons are transferred from the dithiol to  $H_2O_2$  [24]. Dithiol formation is  
74 catalyzed by thioredoxin at moderate light intensities [25] and by NADPH thioredoxin reductase C in  
75 dim light [26, 27]. However, it is not fully understood why the redox switch evolved. An Arabidopsis  
76 mutant, termed *gamera*, remains in the  $\gamma$ -dithiol state in the dark by expressing a redox-insensitive  $\gamma$ -  
77 subunit isoform [28] and displays a stay green phenotype after several days in the dark [29]. It was  
78 suggested that altered pH-dependent protein import might partially contribute to the phenotype.  
79 The hypothesis that the slowdown of ATP hydrolysis preserves ATP in the dark [30], when the  $\Delta\tilde{\mu}_{H^+}$  is  
80 low and the disulfide is present, could not explain all the phenotypes upon extended dark adaptation

81 [29]. Moreover, point mutants of the redox-active Cys in *Arabidopsis thaliana* are viable [31]. This  
82 suggests that the impact of thiol modulation may be more versatile than originally proposed. New  
83 discoveries regarding developmental regulation of chloroplast ATP recruitment from the cytosol [32]  
84 and photosynthetic  $\gamma$ -disulfide formation [24] support this view.

85 From a structural perspective, the  $\gamma$ -redox domain in the disulfide form was found to act as an ATP  
86 hydrolysis chock that is vicinal to the torque-transmitting  $\beta$ DELSEED-loop [33, 34], supporting earlier  
87 findings on critical  $\gamma$ -/ $\beta$ -subunit interactions during thiol modulation [35]. The redox switch also  
88 affects ATP synthesis which was first demonstrated *in vitro*. The authors showed that the  
89 dependency between the redox and  $\Delta\tilde{\mu}_{H^+}$  regulations of  $CF_1F_0$  stems from a lower  $\Delta\tilde{\mu}_{H^+}^{activation}$   
90 threshold in presence of the  $\gamma$ -dithiol [36], yielding half-maximal rates  $\sim 0.7$   $\Delta$ pH units apart. A recent  
91  $CF_1F_0$  structure of the  $\gamma$ -dithiol conformation suggests diminished torsional constraints upon  
92 reduction of the disulfide [37]. Another level of steric adjustments may be established by the  $\Delta\tilde{\mu}_{H^+}$   
93 which facilitates chemical labeling of various structurally buried residues in the  $\gamma$ -/ $\beta$ -subunit  
94 interface, including the dithiol [reviewed in 38]. Taken together, this supports the idea that  $CF_1F_0$   
95 experiences intertwined  $\Delta\tilde{\mu}_{H^+}$ - and redox-dependent structural changes.

96 The main aim of this study is to confirm *in vitro* findings by Junesch and Gräber [36], thus stimulating  
97 further  $CF_1F_0$  thiol modulation investigations *in vivo*. It is of utmost interest to fully understand this  
98 evolutionary strategy of photosynthesis in the green lineage since the beneficial effect of thiol  
99 modulation is still under discussion while new structural insights emerge [29, 34, 37]. We  
100 differentiated between distinct redox states by using the WT and the dithiol-locked *gamera* mutant  
101 [28], in addition to an intermediate redox situation in the WT upon infiltration with the thiol  
102 reductant TCEP. Then, we obtained a  $\Delta\tilde{\mu}_{H^+}$ -dependent  $CF_1F_0$  activity profile in the redox states by  
103 introducing a spectroscopic technique that can also be utilized for  $CF_1F_0$  mutant characterization  
104 beyond thiol modulation.

105 Because the  $CF_1F_0$  activity is both  $\Delta\tilde{\mu}_{H^+}$  and redox regulated, the study of its redox regulation  
106 requires the *in vivo* measurement of two parameters. First, the  $CF_1F_0$  activity which can be routinely  
107 measured since photosynthetic membranes are spectroscopic voltmeters. Accordingly, the  $\Delta\Psi$  can  
108 be probed linearly by ECS, the electrochromic shift of photosynthetic pigments [39, 40, reviewed in  
109 41]. Following a light-induced membrane energization in dark-adapted samples, the  $\Delta\Psi$  (and ECS)  
110 decay is tightly linked to  $H^+$  translocation via  $CF_1F_0$ . ECS decay kinetics following a single-turnover  
111 laser flash are therefore commonly used to probe  $CF_1F_0$  activity *in vitro* [e.g., 17] as well as *in vivo*  
112 [e.g., 28, 42]. The second parameter is the measurement of the  $\Delta\tilde{\mu}_{H^+}$  extent. This is a methodological  
113 bottleneck which can be circumvented on the basis of a previous protocol [23], best explained  
114 through a simple analogy: To determine the unknown water volume in an opaque bottle one could  
115 add more water until reaching the *maximal carrying capacity*. Thus, even if the absolute value of the  
116 spillage point remains unknown, the added water volume can be quantified and expressed in relation  
117 to this reference/leak value. Regarding the measurement of the  $\Delta\tilde{\mu}_{H^+}$  in the dark, Joliot and Joliot  
118 [23] have shown that a threshold of  $\Delta\tilde{\mu}_{H^+}$  exists, which is a constant in a given photosynthetic  
119 material and corresponds to the leak of protons through the thylakoid membrane *in vivo* ( $\Delta\tilde{\mu}_{H^+}^{leak}$ ,  
120 representing the *maximal carrying capacity*). In brief, the ECS-based protocol to measure the extent  
121 of the  $\Delta\tilde{\mu}_{H^+}$  in the dark ( $\Delta\tilde{\mu}_{H^+}^{dark}$  hereafter) consists in energizing the membrane with a very strong  
122 light pulse and probing the ECS increase until the  $\Delta\tilde{\mu}_{H^+}^{leak}$  is reached (i.e., measuring the *remaining*  
123 *empty volume*).

124 Since this work is founded on a previously introduced method [23], we tested two implicit  
125 hypotheses *before* investigating the thiol modulation mechanism: (i) The method remains valid in

126 dark-adapted or illuminated leaves whatever the redox state of the  $\gamma$ -subunit domain, and (ii) there is  
127 no significant  $\Delta\text{pH}$  generation during the short saturating pulse so that the increase of ECS (which  
128 probes  $\Delta\Psi$ ) reflects the increase of  $\Delta\tilde{\mu}_{\text{H}^+}$ . After presenting the deconvolution basics first, we  
129 continue to demonstrate that those hypotheses are respected. We measure  $\Delta\tilde{\mu}_{\text{H}^+}$  partitioning as a  
130 function of pulse duration and the ATP lifetime upon illumination before finally confirming that  $\text{CF}_1\text{F}_o$   
131 is mostly in the oxidized state in dark-adapted leaves (in contrast with a previous report [23]).  
132 Validating *in vivo* the model of Junesch and Gräber, our results show that the  $\Delta\tilde{\mu}_{\text{H}^+}$  activation of a  
133 disulfide-containing  $\text{CF}_1\text{F}_o$  was lowered (by  $\Delta\Psi = 2.6$  charge separations per photosystem I) and this  
134 was confirmed in leaves infiltrated with TCEP, where the efficiency of the chemical reduction of the  
135  $\gamma$ -subunit was a little less than 50%.

136

137

## 138 **Material and Methods**

139

140 *Plant material and growth conditions.* The Columbia ecotype of *Arabidopsis thaliana* served as wild  
141 type in this study. The *gamera* mutant and related rescued control lines were previously published  
142 [28], and seeds were a kind gift of Dr. Jörg Meurer (Ludwig Maximilian University of Munich). Plants  
143 were grown on soil in a growth chamber under a 16-h photoperiod at 22°C (18°C at night) for about  
144 three weeks. The light intensity was set to 100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ .

145

146 *Absorption changes.* The experimental setup was as described previously [23]. Absorption changes  
147 were measured using a JTS spectrophotometer (Biologic). Pulses of saturating light are provided by  
148 LEDs peaking at 630 nm ( $\sim 2500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ), unless otherwise stated. This light irradiance  
149 corresponds to  $\sim 2000$  photons absorbed per second per photosystem I (PSI) or per PSII, based on the  
150 measurement of changes in the membrane potential ( $\Delta\Psi$ ) according to the method described  
151 previously [23].

152

153 *Correction and normalization of ECS signals.* The light-induced membrane potential changes ( $\Delta\Delta\Psi$ )  
154 were measured through the absorption changes at 520 nm (reflecting mostly ECS) for experiments  
155 with short pulses (less than 20 ms), and 520 nm–546 nm for longer pulses (Fig 2). Indeed, subtracting  
156 546 nm allows to correct for the contribution of cytochromes, P700 and scattering. However, the  
157 comparison between corrected data (520–546) and uncorrected data (520) shows that the  
158 correction was not necessary for short pulses (Fig S1). To express the measured ECS signals in charge  
159 separations per PSI, we calibrated the ECS signal by the ECS increase corresponding to one charge  
160 separation per PSI. For that, we divided all measured ECS signals in this work by  $\frac{1}{2}$  the ECS increase  
161 following a single-turnover flash, provided by a dye laser at 690 nm pumped with a Nd:Yag laser.  
162 Since all photosystems will generate one charge separation upon such a flash, the ECS measured 250  
163  $\mu\text{s}$  after the flash is proportional to the number of active PSI + PSII. Assuming equal concentrations of  
164 both photosystem reaction centers, half this signal is equal to the sole contribution of PSI.

165

166 *Reproducibility.* The kinetics of ECS rise and decay were reproducible between different leaves of  
167 different batches, but it was not the case of the extent of the ECS. This is because its amplitude  
168 depends on the density of reaction centers and ECS probes per leaf area, which slightly varied in

169 different cultures. For this reason, for each experiment where the amplitude of ECS was probed, we  
170 compared samples from the same batch of culture.

171

172 *Chemicals and Inhibitors.* We used chemical compounds by vacuum infiltration since the method did  
173 not interfere with the  $\Delta\Psi$  increase and decay kinetics as compared to non-infiltrated samples (see  
174 Fig S2). All chemicals were purchased from Sigma-Aldrich. Tris(2-carboxyethyl)phosphine  
175 hydrochloride (TCEP) was dissolved in water at 0.3 M and aliquots, adjusted to pH 7.0, were frozen  
176 until further use. Solutions of antimycin-A (AA, 40 mM), nonactin (40 mM), nigericin (Nig, 10 mM)  
177 and Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, 1 mM) were dissolved in ethanol  
178 and usually served as 1000x stocks that were stored at  $-20^{\circ}\text{C}$ . All vacuum-infiltrated leaves were  
179 incubated in the dark for at least 15 min until measurements.

180

181

## 182 **Results**

183

### 184 *1. Following membrane potential developments during and after saturating light pulses.*

185

186 In order to study  $\text{CF}_1\text{F}_o$  in  $\gamma$ -disulfide- and  $\gamma$ -dithiol-promoting conditions, we will first elaborate on an  
187 improved protocol which is based on a previous study [23] and allows to measure the  $\Delta\tilde{\mu}_{\text{H}^+}$  already  
188 present in dark-adapted leaves ( $\Delta\tilde{\mu}_{\text{H}^+}^{\text{dark}}$ ). Compared to the baseline  $\Delta\Psi$  before light perturbation,  
189 the method measures  $\Delta\Psi$  changes ( $\Delta\Delta\Psi$ ) induced by a short saturating light pulse, and the return to  
190 baseline level (Fig 1). In this section, we will present the deconvolution routine of raw kinetics (ECS  
191 amplitudes and decay phases) which will be revisited below in sections 3 and 4. We make the  
192 reasonable hypothesis that short pulses ( $< 20$  ms) induce exclusively  $\Delta\Delta\Psi$  and that changes in the  
193  $\Delta\text{pH}$  can be neglected [23] due to the high buffering capacity inside the lumen [43]. According to this  
194 hypothesis (tested in section 2), ECS changes which are strictly speaking proportional to  $\Delta\Delta\Psi$ ,  
195 equally reflect changes in the electrochemical proton gradient ( $\Delta\Delta\tilde{\mu}_{\text{H}^+}$ ).

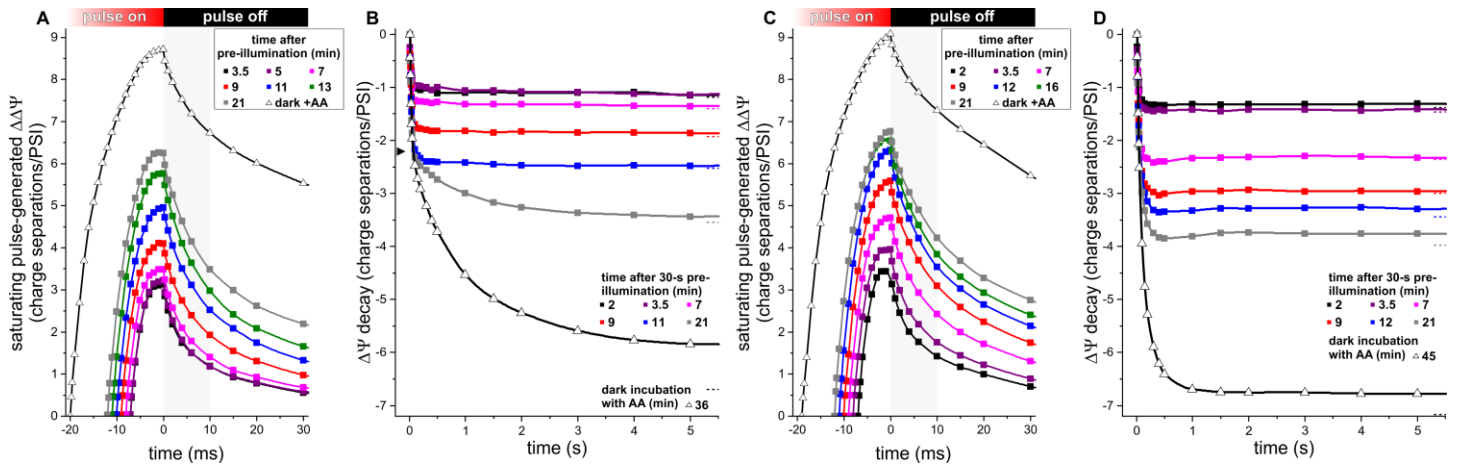
196 Fig 1A illustrates how the baseline  $\Delta\tilde{\mu}_{\text{H}^+}^{\text{dark}}$  prior to the pulse influences the amplitude and kinetics of  
197 the pulse-induced ECS (or  $\Delta\Delta\tilde{\mu}_{\text{H}^+}$ ). To do so, we analyzed in water-infiltrated leaves at high and low  
198  $[\text{ATP}]/([\text{ADP}][\text{P}])$  ratios, respectively. Low  $[\text{ATP}]/([\text{ADP}][\text{P}])$  ratios were obtained when respiratory  
199 ATP production was hampered in dark-adapted leaves by application of the cytochrome  $bc_1$  inhibitor  
200 antimycin-A (AA, open symbols in Fig 1). AA addition exploits the genetically regulated nucleotide  
201 import machinery which metabolically couples chloroplast ATP levels to mitochondrial respiration  
202 [23, 32, 44-47]. On the contrary, high  $[\text{ATP}]/([\text{ADP}][\text{P}])$  ratios were reached after building up ATP  
203 following a 30-s illumination (closed symbols). In all curves of Fig 1A, the ECS reached a plateau  
204 during the pulse which was followed by a fast decay phase in darkness. Both amplitudes of the  
205 plateau level and the fast decay phase depend on the light intensity of the applied pulse (Fig S3, not  
206 varied in the main text). The fast phase likely involves  $\text{H}^+$  leaks above a critical  $\Delta\tilde{\mu}_{\text{H}^+}$  ( $\Delta\tilde{\mu}_{\text{H}^+}^{\text{leak}}$ ), which  
207 is an *absolute*  $\Delta\tilde{\mu}_{\text{H}^+}$  reference in a given material *in vivo* [23]. This fast phase finishes after 10 ms  
208 when the ECS decay kinetics becomes independent of the pulse light intensity (Fig S3), reflecting ATP  
209 synthesis [23]. The 10-ms ECS signal in darkness ( $\Delta\Psi_{10\text{ms}}$ ) is therefore independent of pulse intensity,

210 and also independent of the physiological conditions preceding the pulse. Thus, the  $\Delta\Psi_{10\text{ms}}$  will be  
211 used hereafter as the *experimental* reference of  $\Delta\tilde{\mu}_{\text{H}^+}$ . In turn, the ECS data in the following will be  
212 expressed as negative values ( $\Delta\Psi - \Delta\Psi_{10\text{ms}}$ ; Fig 1B) which corresponds to the “remaining empty  
213 volume” of the opaque bottle analogy. Considering the outlined method, the larger pulse-induced  
214 ECS amplitude in the presence of AA than after the pre-illumination is related to a lower basal  
215  $\Delta\tilde{\mu}_{\text{H}^+\text{dark}}$  before the pulse in AA samples (Fig 1A). This was revealed after rescaling to our  
216 *experimental* reference  $\Delta\Psi_{10\text{ms}}$  (-6.7 vs. >-3.5 charge separations/PSI, Fig 1B). After the 30-s  
217 illumination and the following re-adaptation to darkness, the increase of the pulse amplitude with  
218 the time in darkness (Fig 1A), again, indicated the continuous decrease of the  $\Delta\tilde{\mu}_{\text{H}^+\text{dark}}$  (Fig 1B) and  
219 reflected the consumption of the extra ATP produced during the 30-s illumination.

220 According to Fig 1B and a previous report [23], the ECS decay kinetics after the pulse displayed three  
221 phases. The first phase, completed in 10 ms and discussed before, was systematically discarded. A  
222 second phase was completed in ~100 ms (phase 2) and was linked to ATP synthesis. Then, a  
223 multiphasic slow decay (phase 3, completed in ~1 min) was observed only once the  $\Delta\tilde{\mu}_{\text{H}^+}$  fell below a  
224 threshold (at the end of phase 2, ~100 ms). This threshold corresponds to the  $\Delta\tilde{\mu}_{\text{H}^+\text{activation}}$  described  
225 before [17, 19-23] and represented a second reference level, about 2 charge separations/PSI below  
226  $\Delta\Psi_{10\text{ms}}$  (arrowhead in Fig 1B). Phase 3 indicated a slowly active  $\text{CF}_1\text{F}_o$ , or, alternatively, a small  $\text{CF}_1\text{F}_o$   
227 fraction staying fully active.

228 We first decided to check whether this quantitative measurement of the  $\Delta\tilde{\mu}_{\text{H}^+\text{dark}}$  remained valid  
229 regardless of the redox regulation mechanism. Figs 1C and 1D show similar experiments on WT  
230 leaves infiltrated with the thiol reductant TCEP to cleave the  $\gamma$ -disulfide. Furthermore, the samples  
231 were in the same physiological conditions as in Figs 1A and 1B (AA-treated or pre-illuminated  
232 samples). In all cases, the ECS generation and the decay in the first tens of ms after the end of the  
233 pulse resembled the untreated sample (Fig 1C). As in water-infiltrated leaves, AA-treated leaves in  
234 the presence of TCEP showed a lower  $\Delta\tilde{\mu}_{\text{H}^+\text{dark}}$  and illumination increased this pedestal (dashed lines  
235 in Fig 1D), reflecting ATP accumulation during pre-illumination and consumption during re-  
236 adaptation to darkness. The  $\Delta\tilde{\mu}_{\text{H}^+}$  decay kinetics always seemed monophasic; we did not observe the  
237 slow phase of ECS decay (absence of phase 3 in Fig 1D). This was true even in conditions where  
238  $\Delta\tilde{\mu}_{\text{H}^+\text{dark}}$  was below the  $\Delta\tilde{\mu}_{\text{H}^+\text{activation}}$  of Fig 1B, i.e., when dark re-adaptation after the 30-s  
239 illumination was longer than 9 min or in AA-treated samples. This observation at low  $\Delta\tilde{\mu}_{\text{H}^+}$  was in  
240 agreement with the higher  $\text{CF}_1\text{F}_o$  activity measured *in vitro* upon cleaving the  $\gamma$ -disulfide by chemical  
241 thiol reductants [36]. A quantitative analysis will be given in section 4, demonstrating how the effect  
242 of the  $\gamma$ -redox state influences the  $\Delta\tilde{\mu}_{\text{H}^+}$  dependence of  $\text{CF}_1\text{F}_o$  activity. The comparison of the control  
243 and TCEP-treated leaves validate our first hypothesis (mentioned in the introduction): The proposed  
244 protocol to measure the  $\Delta\tilde{\mu}_{\text{H}^+\text{dark}}$  works in both the reduced and oxidized state, provided that no  
245  $\Delta\text{pH}$  is generated during the pulse. This absence of pulse-induced  $\Delta\text{pH}$  corresponds to the second  
246 hypothesis, which will be addressed in the next section.

247



248

249 Fig 1: Pulse-induced ECS kinetics are shown in water-infiltrated leaves in the absence (A-B) or presence (C-D) of  
 250 the mild reductant TCEP at 50 mM. Dark-adapted leaves were illuminated for 30 s (green-orange LED, 1000  
 251  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ), followed by a dark re-adaptation for several minutes (indicated in the legend). +AA:  
 252 infiltrated with 40  $\mu\text{M}$  antimycin-A without illumination. All the measurements were made on different leaves  
 253 from the same plant. In panels A and C, the dark-adapted (baseline) ECS level was arbitrarily set to 0 and only  
 254 the ECS kinetics during the pulse and in the initial relaxation phases are shown. In panels B and D, the  $\Delta\Psi_{10\text{ms}}$   
 255 values of all curves were set to 0 and only the ECS relaxation in the dark is shown (the value reached after 1  
 256 min relaxation is indicated with three dots on the right). The rapid transition between the fast and slow  
 257 relaxation phases ( $\Delta\tilde{\mu}_{\text{H}^+ \text{ activation}}$ ) is indicated in panel B by an arrowhead. The homothetic ECS curves in TCEP  
 258 samples of panel D decayed without an apparent  $\Delta\tilde{\mu}_{\text{H}^+ \text{ activation}}$ , and the major decay finished within  $\sim 1$ -s.

259

## 260 2. Obtaining the relative extent of $\Delta\Psi$ and $\Delta\text{pH}$ induced by short light pulses.

261 For the interpretation of the previous results regarding the evolution of  $\Delta\tilde{\mu}_{\text{H}^+}$ , we followed the  
 262 opaque bottle analogy and have made the hypothesis that the  $\Delta\Psi_{10\text{ms}}$  reference served as our  $\Delta\tilde{\mu}_{\text{H}^+}$   
 263 reference. This implied that the  $\Delta\text{pH}$  generated until reaching  $\Delta\Psi_{10\text{ms}}$  was insignificant, and only  $\Delta\Psi$   
 264 increased during the short saturating light pulses. The demonstration of a negligible  $\Delta\text{pH}$   
 265 contribution is crucial for this ECS-based method before introducing  $\Delta\tilde{\mu}_{\text{H}^+}$ -dependent  $\text{CF}_1\text{F}_0$  activity  
 266 profiles in section 4, focusing on thiol modulation.

267 To test our hypothesis, we applied saturating light pulses of variable duration to dark-adapted leaves  
 268 and probed the extra  $\Delta\Psi$  ( $\Delta\Delta\Psi$ ), extra  $\Delta\text{pH}$  ( $\Delta\Delta\text{pH}$ ) and their sum ( $\Delta\Delta\tilde{\mu}_{\text{H}^+}$ ) remaining after a  
 269 relatively short (15 s) dark recovery. To obtain those values, we applied a short 12-ms probing pulse  
 270 15 s after the first one and compared to a similar 12-ms probing pulse applied on a non-perturbed  
 271 sample (dark line and symbols, Fig 2A). The  $\Delta\Psi$  was monitored throughout the measurement and  
 272  $\Delta\Delta\Psi$  was calculated as the difference between the dark-adapted ECS signal before the first light  
 273 perturbation and the ECS signal 15 s later (Fig 2A). The  $\Delta\Delta\text{pH}$  after 15 s was derived from the 12-ms  
 274 probing pulse, i.e., from the difference of its  $\Delta\Psi_{10\text{ms}}$  value compared to fully dark adapted leaves (Fig  
 275 2A). A  $\Delta\Delta\text{pH}$  usually appeared after prolonged light pulses but this was not the case in Fig 2A.  
 276 Therein, the ionophore nigericin (Nig) was used to exchange  $\text{H}^+$  with  $\text{K}^+$  as a proof of principle to favor  
 277  $\Delta\Delta\Psi$  at the expense of  $\Delta\Delta\text{pH}$ . It is important to note that Nig also collapsed the proton motive force  
 278 for mitochondrial ATP synthesis and thus interfered with metabolic coupling between the organelles.  
 279 Accordingly, Nig infiltration resulted in a collapse of  $\Delta\tilde{\mu}_{\text{H}^+ \text{ dark}}$  in the chloroplast (within  $\sim 15$ -30 min

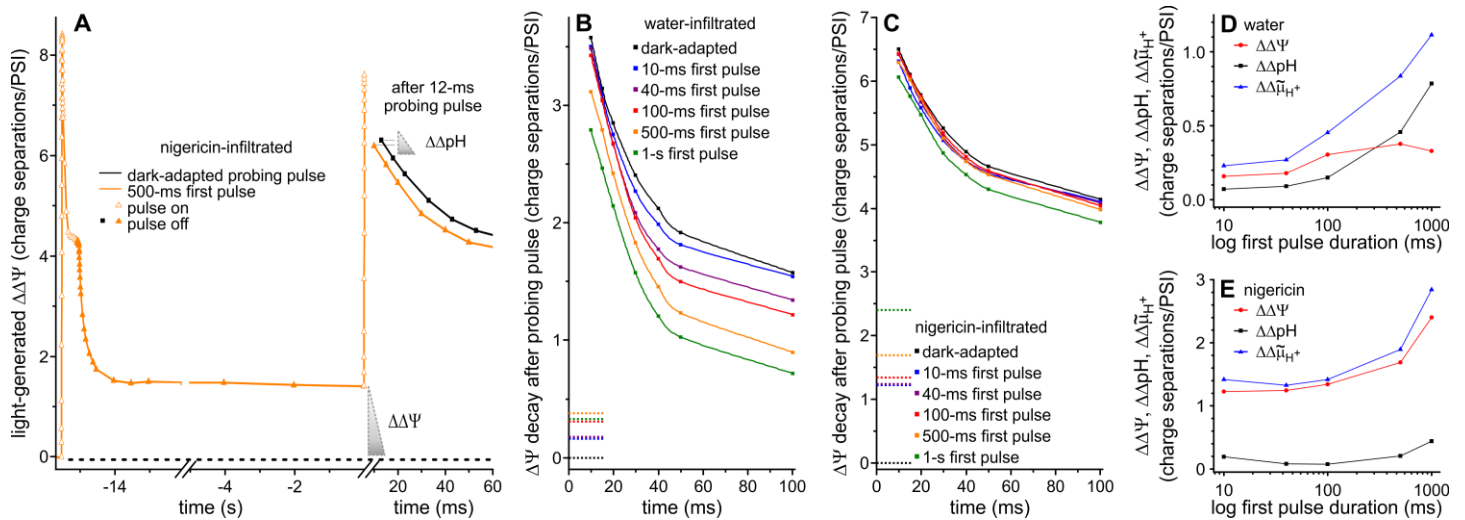


280 incubation, see Fig S4 and Supplementary discussion). A duration of 15 s dark was necessary in this  
281 experiment to allow a partial relaxation of the electron carriers toward the dark-adapted state.  
282 Indeed, at the end of the first light pulse, the photochemistry of both photosystems was low due to  
283 the absence of acceptors (oxidized plastoquinones or oxidized ferredoxins) or donors (reduced P700).  
284 If the second light pulse was applied too early, photochemistry was insufficient to reach the  $\Delta\tilde{\mu}_{H^+}^{leak}$   
285 which made measurement of the  $\Delta\tilde{\mu}_{H^+}$  impossible.

286 We followed those parameters in water-infiltrated (Fig 2B) and in Nig-treated leaves (Fig 2C) for  
287 different durations of the first pulse (10 ms to 1 s light perturbation). Regardless of the first pulse  
288 duration, in both treatments the  $\Delta\Psi_{10ms}$  upon the second pulse was smaller than in the  
289 corresponding dark-adapted sample. This indicated that a significant amount of extra ATP, generated  
290 during and after the first illumination, was still present after 15 s dark and increased the  $\Delta pH$  ( $\Delta\Delta pH$ )  
291 compared to the dark-adapted level. The ionophore Nig attenuated  $\Delta\Delta pH$  (Fig 2C) but in water-  
292 infiltrated samples (Fig 2B), the  $\Delta\Delta pH$  increment was significant only between the 100-ms and 500-  
293 ms light perturbations. These samples also sustained a significant  $\Delta\Delta\Psi$ , up to 0.4 charge  
294 separation/PSI (dashed line in Fig 2B), although a stagnation for pulses longer than 100-ms was  
295 observed. The  $\Delta\Delta\Psi$  after 15 s was always higher than 1 charge separation/PSI when Nig was present,  
296 even for the shortest durations of the light pulse (dashed line in Fig 2C). The lowered  $\Delta\tilde{\mu}_{H^+}^{dark}$  by the  
297 drug also influenced the  $\Delta\Delta\tilde{\mu}_{H^+}$  15 s after a 10-ms pulse, increasing it from 0.2 in controls (Fig 2D) to  
298 1.4 charge separations/PSI in Nig samples (Fig 2E). For a 1-s pulse, those values reached 1.1 and 2.8  
299 charge separations/PSI for control and Nig samples, respectively.

300 The fact that short pulses (below 100 ms, Fig 2D) generated only small changes in  $\Delta pH$  (smaller than  
301 0.1 charge separation/PSI) validated our initial hypothesis. This indicated that, if probing light pulses  
302 remain short, the ECS pulse method allowed the quantitative measurement of  $\Delta\tilde{\mu}_{H^+}$  with a potential  
303 error of 0.1 charge separation/PSI only. The higher  $\Delta\tilde{\mu}_{H^+}$  storage after 15 s dark in the Nig samples  
304 was simply due to the slower relaxation of the pulse-induced  $\Delta\tilde{\mu}_{H^+}$  when the  $\Delta\tilde{\mu}_{H^+}^{dark}$  was far below  
305 the  $\Delta\tilde{\mu}_{H^+}^{activation}$ , as we described before in the case of the AA treated sample (Fig 1B). The  
306 uncoupling by Nig was more efficient in collapsing  $\Delta\tilde{\mu}_{H^+}^{dark}$  compared to AA but, like other  
307 uncouplers, Nig modified the different phases of ECS decay kinetics (Fig S4, see also Supplementary  
308 discussion). Therefore, membrane integrity-preserving AA will be preferred for unbiased  $CF_1F_0$   
309 analysis at low  $\Delta\tilde{\mu}_{H^+}^{dark}$  (section 4).

310 In the first two sections, we have shown that our methodology is valid regardless of the redox state  
311 of  $CF_1F_0$ . Moreover, it allows quantitative measurements of the  $\Delta\tilde{\mu}_{H^+}^{dark}$  (relative to the  $\Delta\tilde{\mu}_{H^+}$   
312 reference given by  $\Delta\Psi_{10ms}$ ) provided that the saturating light pulse is (i) strong enough to reach the  
313  $\Delta\tilde{\mu}_{H^+}^{leak}$  (which is valid for the 2000 photons/PSI/s light irradiance used here), and (ii) short enough  
314 to avoid the generation of  $\Delta pH$  (which is valid for pulses shorter than 100 ms). This allows to follow  
315 the kinetics of the  $\Delta\tilde{\mu}_{H^+}^{dark}$  after a light perturbation in the next part.



316

317 Fig 2: The estimation of  $\Delta\Delta\Psi$ ,  $\Delta\Delta pH$  and  $\Delta\Delta\tilde{\mu}_{H^+}$  generated by an illumination is shown. The parameters were  
 318 measured 15 s after a saturating pulse of various duration. (A) Exemplary ECS measurement in the presence of  
 319 nigericin (open symbols light on, closed symbols darkness) measured as the difference between the absorption  
 320 changes at 520 nm and the one at 546 nm (see Methods). The leaf was illuminated with a first pulse of 500-ms  
 321 ( $t \sim -15$  s), then ECS was followed for 15 s in the dark and a 12-ms probing pulse was used to determine the  
 322 new  $\Delta\Psi_{10ms}$  reference (the probing pulse ended at  $t = 0$  s). For the sake of visualization, the dark-adapted ECS  
 323 decay was shifted on the x-axis by 3 ms. (B) and (C) The initial phase of the  $\Delta\Psi$  decay after the 12-ms pulse is  
 324 shown for leaves infiltrated with (B) water and (C) nigericin. The  $\Delta\Delta\Psi$  at the end of the 15-s dark period is  
 325 indicated by horizontal dashed lines. The  $\Delta\Delta pH$  corresponds to the difference between the  $\Delta\Psi_{10ms}$  after the  
 326 saturating pulse and the one of a dark-adapted leaf (black closed squares). (D) and (E) The relationship of  $\Delta\Delta\Psi$ ,  
 327  $\Delta\Delta pH$  and  $\Delta\Delta\tilde{\mu}_{H^+}$  with the duration of the first light pulse are shown for (D) water and (E) nigericin infiltrated  
 328 leaves. All the measurements in this figure were performed with leaves from the same plant, and are the  
 329 representative outcome of two independent experiments.

330

331 3. Showing that the sustained  $\Delta\tilde{\mu}_{H^+}^{dark}$  elevation is a function of extra ATP after an illumination.

332

333 In this section, we use the method to follow the fate of the  $\Delta\tilde{\mu}_{H^+}$  after an illumination by supposing  
334 that the thermodynamic equilibrium is met during a dark period before the ECS measurement, and  
335 that following  $\Delta\tilde{\mu}_{H^+}$  is equivalent to following the [ATP]/([ADP][P]) ratio.

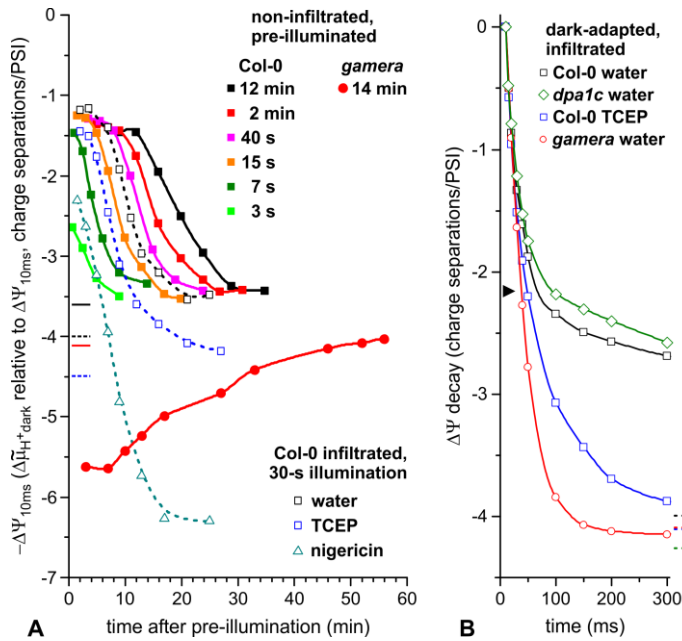
336 After the 30-s illumination in water-infiltrated leaves (Fig 1B and additional measurements from the  
337 same experiment), we re-plotted the  $\Delta\tilde{\mu}_{H^+}^{dark}$  values as a function of the time in darkness (Fig 3A,  
338 black open squares). The  $\Delta\tilde{\mu}_{H^+}^{dark}$  after the 30-s illumination was  $\sim -1.2$  charge separations/PSI  
339 relative to the  $\Delta\tilde{\mu}_{H^+}$  reference ( $\Delta\Psi_{10ms}$ ) in the water-infiltrated leaf (black open squares in Fig 3A);  
340 being significantly higher than in the dark-adapted sample ( $\sim -4$  charge separations/PSI, black dashed  
341 line). After ATP formation in the light, the elevated  $\Delta\tilde{\mu}_{H^+}^{dark}$  remained constant for 5 min and, due to  
342 slow ATP consumption [23], relaxed towards the dark-adapted value in  $\sim 30$  min (Fig 3A). When  
343 leaves were infiltrated with Nig before the 30 s illumination (open triangles in Fig 3A), the  $\Delta\tilde{\mu}_{H^+}^{dark}$   
344 reached a lower level ( $-2.3$  charge separations/PSI after 2 min dark) and relaxed to an even lower  
345 value within  $\sim 15$  min ( $-6.3$  charge separations/PSI). As discussed, Nig may interfere with  
346 mitochondrial ATP production in darkness, resulting in a lower  $\Delta\tilde{\mu}_{H^+}^{dark}$  and more rapid depletion of  
347 extra ATP upon illumination.

348 The closed symbols in Fig 3A also show the lifetime of  $\Delta\tilde{\mu}_{H^+}^{dark}$  and ATP in non-infiltrated WT leaves  
349 after illuminations of different durations (between 3-s and 12-min). Interestingly, the elevated  
350  $\Delta\tilde{\mu}_{H^+}^{dark}$  became independent on the illumination duration beyond 7 s (between  $\sim -1.3$  to  $-1.5$  charge  
351 separations/PSI). Moreover, a lag was observed in the relaxation, which increased with the time of  
352 illumination. Once ATP levels decreased slowly over  $\sim 20$  min, the  $\Delta\tilde{\mu}_{H^+}^{dark}$  reached a stable dark level  
353 which corresponded to the dark-adapted value ( $\sim -3.5$  charge separations/PSI, black solid line). The  
354 water infiltration treatment had no effect, at least for illuminations not longer than 30 s, since the  
355 curve for infiltrated leaves illuminated for 30 s was in between the 15-s and 40-s illumination curves  
356 of the non-infiltrated ones (Fig 3A). To conclude, the  $\Delta\tilde{\mu}_{H^+}^{dark}$  is dependent on the physiological  
357 conditions. Nucleotide exchange between cell compartments is strictly controlled and, compared to  
358 the reversible  $CF_1F_o$ , passive ion movements across the membrane are slow [23, 32, 44-47].

359 We also analyzed the light-induced changes of  $\Delta\tilde{\mu}_{H^+}^{dark}$  in the reduced and oxidized  $CF_1F_o$  forms  
360 before establishing a specific  $\Delta\tilde{\mu}_{H^+}$  dependency of  $CF_1F_o$  activity (next section). Besides infiltrating  
361 leaves with the thiol reductant TCEP, we also looked at the behavior of the *gamera* mutant, in which  
362 the  $CF_1F_o$  is in its reduced isoform ( $\gamma$ -dithiol) in the dark [28]. The  $\Delta\tilde{\mu}_{H^+}$  threshold ( $\Delta\tilde{\mu}_{H^+}^{activation}$ ) at  
363  $\sim 100$  ms in darkness is characteristic for water-infiltrated samples with the  $\gamma$ -disulfide (arrowhead in  
364 Fig 3B, see also Fig 1B). It disappeared in presence of TCEP due to accelerated  $\Delta\tilde{\mu}_{H^+}$  decay kinetics at  
365 the end of phase 2. The  $\Delta\tilde{\mu}_{H^+}$  decay kinetics was even more accelerated in the *gamera* mutant. This  
366 phenotype, obtained in the *dpa1* genetic background [28], could be unambiguously attributed to the  
367 dithiol since the behavior of the *ATPC1*-complemented *dpa1* mutant, *dpa1c*, was similar to the WT  
368 (Fig 3B; *ATPC1* is the thioredoxin-sensitive  $\gamma$ -subunit isoform and *ATPC2* is the isoform present in  
369 *gamera*). Based on the pronounced slowdown of the  $\Delta\tilde{\mu}_{H^+}$  decay after phase 2, we conclude that  
370  $CF_1F_o$  was present mainly in its oxidized state in dark-adapted WT leaves, in contradiction with a  
371 previous report [23]. Incomplete  $\gamma$ -disulfide reduction by TCEP infiltration resulted in a mixture of  
372 active and inactive  $CF_1F_o$  (distributed homogeneously along the thylakoid membrane) and a  
373 continuous decay kinetics of the  $\Delta\tilde{\mu}_{H^+}$ .

374 In TCEP-treated leaves, the  $\Delta\tilde{\mu}_{H^+}^{dark}$  attained after the 30-s illumination was similar to the one in  
375 water-infiltrated leaves ( $\sim -1.5$  charge separations/PSI, blue open squares in Fig 3A). The capacity to

376 sustain an elevated  $\Delta\tilde{\mu}_{\text{H}^+_{\text{dark}}}$  after illumination was slightly less pronounced in TCEP samples. We  
377 cannot rule out that TCEP infiltration also influenced the metabolic ATP sink capacity in the dark or  
378 produced mild uncoupling of the membranes. We tested whether the effect of TCEP on thiol  
379 modulation developed further during the illumination and following relaxation in the dark, and  
380 concluded that this effect is stable throughout the experiment (Fig S6). It is noteworthy that, at  
381 variance with the effect of commonly used dithiothreitol (DTT), the kinetics of the fluorescence  
382 increase in a dark-adapted leaf was not altered upon TCEP infiltration, eliminating a potential side  
383 effect on the plastoquinone pool redox state (not shown). In a similar experiment, we analyzed the  
384 effect of a 14-min pre-illumination on a non-infiltrated *gamera* mutant leaf (closed circle in Fig 3A).  
385 At variance with the results obtained in the WT, we observed that illumination of the *gamera* mutant  
386 induced a decrease of the  $\Delta\tilde{\mu}_{\text{H}^+_{\text{dark}}}$  that returned to the dark-adapted level very slowly in  $\sim 45$  min  
387 (see red horizontal line). We conclude that ATPC2 produced a perturbation of  $\text{CF}_1\text{F}_o$  that prevented  
388 long term ATP accumulation during illumination. A lower *gamera* proton conductivity in steady-state  
389 light was reported [28] which might reflect this  $\text{CF}_1\text{F}_o$  perturbation. The latter could be related to the  
390 large slowdown of Calvin-Benson cycle activation (about a factor 4) and the larger transient NPQ  
391 formation observed on the mutant with respect to the WT (see Fig S5). This section demonstrates  
392 that the ECS-based  $\Delta\tilde{\mu}_{\text{H}^+_{\text{dark}}}$  estimations provide thermodynamic snapshots of the  $[\text{ATP}]/([\text{ADP}][\text{P}])$   
393 ratio in the chloroplast.  
394



395

396 Fig 3: Light-induced ATP lifetime measurements and membrane potential ( $\Delta\Psi$ ) decay kinetics in various  
 397 Arabidopsis genotypes are shown. (A) The effect of the  $\gamma$ -disulfide bond reduction on ATP accumulation and  
 398 relaxation after an illumination of different durations is displayed. The  $\Delta\tilde{\mu}_{H^+}^{dark}$  values are relative to the  
 399  $\Delta\Psi_{10ms}$  and horizontal lines refer to fully dark-adapted, non-infiltrated (solid) and infiltrated (dashed) samples.  
 400 The water-/TCEP-infiltrated WT samples were from the same plant, and the varied pre-illumination  
 401 experiments were performed on another plant. (B) Initial  $\Delta\Psi$  decay kinetics of the WT are shown (infiltrated in  
 402 the dark with water or 50 mM TCEP), as well as the *gamera* mutant and the *ATPC1*-complemented *dpa1c*  
 403 mutant, termed *dpa1c*. The slowdown at  $\sim 100$  ms is shown with an arrowhead. Dashed lines indicate the  $\Delta\Psi$   
 404 decay value 1 min after the pulse.

405

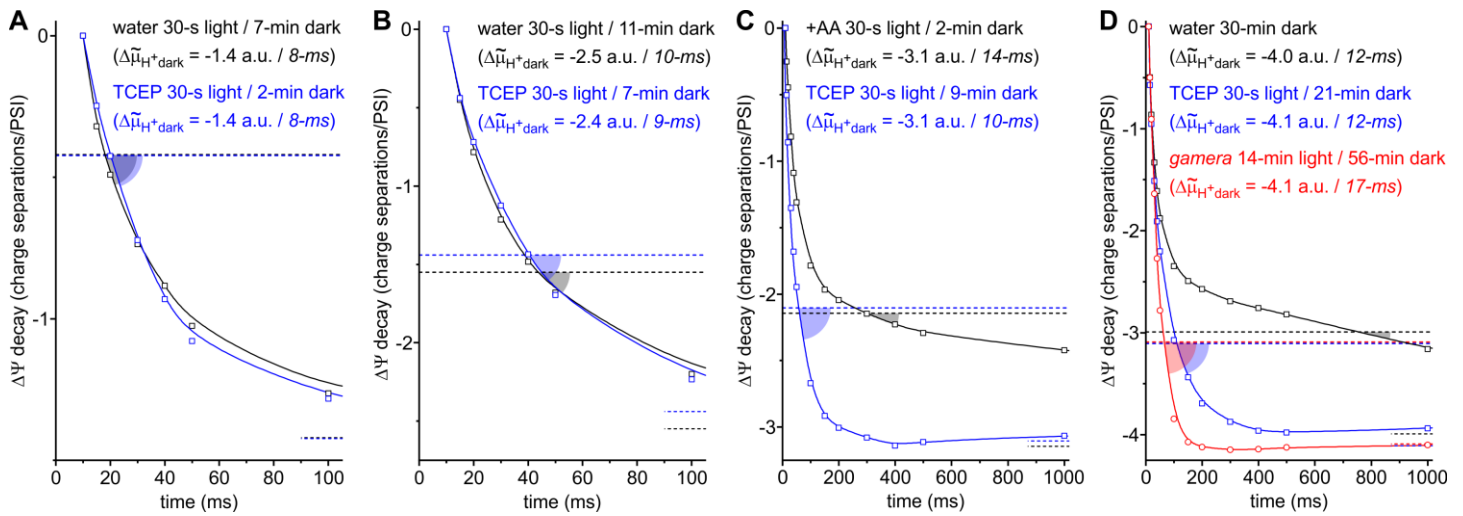
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#### 407 4. Obtaining $\Delta\tilde{\mu}_{H^+}$ -dependent $CF_1F_o$ activity profiles in the two $\gamma$ -subunit redox states.

408

409 The final aim of this work is to determine how the reduction/oxidation of the  $\gamma$ -disulfide modifies the  
 410  $\Delta\tilde{\mu}_{H^+}$  regulation of  $CF_1F_o$  activity. As outlined above, the pulse-induced ECS method to measure  $\Delta\tilde{\mu}_{H^+}$   
 411 has been established and validated in both redox states. For  $CF_1F_o$  activity determination, the ECS  
 412 decay rate after a single turnover laser flash is routinely obtained from exponential decay functions  
 413 [e.g., Ref. 42]. However, there is a bias due to the “*b* phase”: a  $\sim 10$ -ms ECS rise after the flash due to  
 414 electrogenic contribution of the cytochrome *b<sub>6</sub>f* activity which superimposes on the  $CF_1F_o$ -related ECS  
 415 decay. Moreover, we observed another poorly understood but precedingly documented [16, 17]  
 416 source of variation when using flashes. In short, the ECS decay rate after a flash not only depends on  
 417 the  $\Delta\tilde{\mu}_{H^+}^{dark}$  but also on whether the  $\Delta\tilde{\mu}_{H^+}^{activation}$  was surpassed during the light perturbation. We  
 418 tried to circumvent these sources of error and mitigate their contributions to the ECS decay by  
 419 surpassing  $\Delta\tilde{\mu}_{H^+}^{activation}$  in all of our measurements, thus producing  $\Delta\tilde{\mu}_{H^+}$ -activated  $CF_1F_o$  (by 8-ms to  
 420 17-ms saturating pulses depending on the  $\Delta\tilde{\mu}_{H^+}^{dark}$ ; see Fig 4). We then measured the rate constant  
 421 of  $CF_1F_o$  as the slope of the ECS decay when ECS signals reached the level expected after a saturating  
 422 laser flash (i.e.,  $\Delta\tilde{\mu}_{H^+}^{dark} + 1$  charge separation/PSI; see Fig 4). This procedure offered another  
 423 advantage: At the time of the slope measurement, i.e., few tens or hundreds of ms after the end of

424 the pulse, the contribution of the *b* phase was negligible compared to CF<sub>1</sub>F<sub>0</sub> activity, although *b<sub>f</sub>*-  
 425 catalyzed electron transfer between P700<sup>+</sup> and PQH<sub>2</sub> lasted longer in our measurements.



426  
 427

428 Fig 4: Calculation of the CF<sub>1</sub>F<sub>0</sub> rate from the ECS decay kinetics is shown in water- and TCEP-infiltrated WT, as  
 429 as well as *gamera* (only in panel D since the mutant could not accumulate ATP in the light and therefore sustained  
 430 a lower  $\Delta\tilde{\mu}_{H^+}^{dark}$ ). The data is part of Fig 5A and the rationale was to calculate the CF<sub>1</sub>F<sub>0</sub> rate when the  
 431 electrochemical proton gradient reached  $\Delta\tilde{\mu}_{H^+}^{dark} + 1$ , in relation to the classical single turnover flash ECS decay  
 432 protocol. Each panel compares samples with similar  $\Delta\tilde{\mu}_{H^+}^{dark}$  (-1.4, -2.5, -3.1 and -4 charge separations/PSI)  
 433 from Figs 1B, 1D and 3B. The dashed lines on the bottom right indicate the  $\Delta\tilde{\mu}_{H^+}^{dark}$  and the upper dashed lines  
 434 represent  $\Delta\tilde{\mu}_{H^+}^{dark} + 1$  charge separation/PSI. The semi-transparent angles visualize the ECS decay rate at this  
 435 point, expressed as *R* in Fig 5A. The  $\Delta\tilde{\mu}_{H^+}^{dark}$  was estimated by a ms-pulse and its duration is given in italics. In  
 436 panels A and B, the  $\Delta\tilde{\mu}_{H^+}^{dark}$  remained above the  $\Delta\tilde{\mu}_{H^+}^{activation}$  and only a fast phase of ECS decay was seen in  
 437 both  $\gamma$ -redox states. In panels C and D, the  $\Delta\tilde{\mu}_{H^+}^{dark}$  was below the  $\Delta\tilde{\mu}_{H^+}^{activation}$  of water-infiltrated WT leaves  
 438 and the ECS decay kinetics became biphasic in these samples. In the latter, the break in the  $\Delta\tilde{\mu}_{H^+}$  decay kinetics  
 439 at the end of phase 2 revealed a CF<sub>1</sub>F<sub>0</sub> transition from an active toward an inactive form.

440

441 Fig 5A sums up all the CF<sub>1</sub>F<sub>0</sub> rate constants (obtained at  $\Delta\tilde{\mu}_{H^+}^{dark} + 1$  charge separation/PSI) as a  
 442 function of the corresponding  $\Delta\tilde{\mu}_{H^+}$  values. The data presented in this manuscript so far lays the  
 443 foundations for Fig 5A: WT leaves (dark-adapted or during relaxation following a 30-s illumination),  
 444 TCEP-treated WT leaves in the same conditions, as well as *gamera* leaves (dark-adapted or following  
 445 a 14-min illumination). Fig 5A also contains additional measurements obtained in comparable  
 446 experiments, such as dark-adapted *gamera* leaves in the presence of AA. The CF<sub>1</sub>F<sub>0</sub> activity vs.  $\Delta\tilde{\mu}_{H^+}$   
 447 curves of the reduced (*gamera* isoform) and oxidized (water-infiltrated WT) enzyme resembled the  
 448 ones obtained *in vitro* [36]. The TCEP-infiltrated WT was intermediate, in agreement with a mixed  
 449 CF<sub>1</sub>F<sub>0</sub> redox population. Fig 5A shows that below  $\Delta\tilde{\mu}_{H^+}^{activation}$  of the oxidized form (~-2.2 charge  
 450 separations/PSI, see arrowhead in Fig 1B, Fig 3B), the CF<sub>1</sub>F<sub>0</sub> was always faster in the (partially)  
 451 reduced form. The rate constant of CF<sub>1</sub>F<sub>0</sub> increased exponentially with  $\Delta\tilde{\mu}_{H^+}$  in the reduced form, as  
 452 soon as  $\Delta\tilde{\mu}_{H^+}$  was above ~-4.7 charge separations/PSI (Fig 5A).

453 Both  $\Delta\tilde{\mu}_{H^+}$ -dependent activities of (chemically unmodified) CF<sub>1</sub>F<sub>0</sub> in WT and *gamera* have been fitted  
 454 with a sigmoid function, similar to the *in vitro* study in spinach using thiol agents [36]. In fact, the  
 455 plotted CF<sub>1</sub>F<sub>0</sub> rate constants of both genotypes were processed in one calculation by using a sum of

456 two sigmoid functions (Fig 5A). In Equation 1, we forced via the constant  $c$  the first summand to be  
457 zero in the *gamera* plot, and the second summand to be zero in the WT plot:

458

$$459 \quad y = c \times \left( \frac{A1}{1 + 10^{(x0.ox-x) \times p}} \right) + (1 - c) \times \left( \frac{A1}{1 + 10^{(x0.red-x) \times p}} \right) \quad (\text{Eq.1}).$$

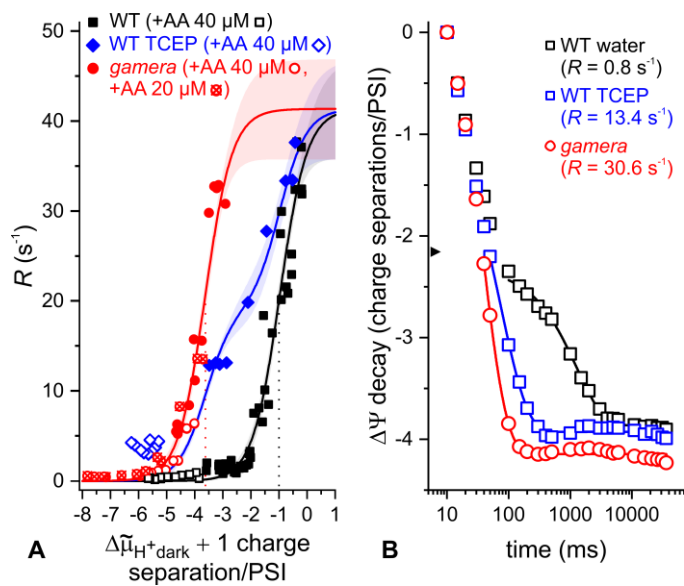
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461  $A1$  defines shared maximal rate constants of  $41.4 \pm 2.8 \text{ s}^{-1}$  ( $\pm$  standard error). The slope parameter of  
462 the function is expressed as  $p$  ( $0.9 \pm 0.1$ ). The  $\Delta\tilde{\mu}_{H^+}$  where the half-maximal  $CF_1F_o$  activities were  
463 obtained for WT and *gamera* are defined as  $x0.ox$  ( $-1.0 \pm 0.1$  charge separations/PSI) and  $x0.red$  ( $-3.6$   
464  $\pm 0.1$  charge separations/PSI), respectively. The TCEP sigmoid fitting curve in Fig 5A was obtained  
465 with  $c = 0.54 \pm 0.04$ , suggesting about 54% of  $CF_1F_o$  in the oxidized  $\gamma$ -disulfide conformation.  
466 Incomplete  $\gamma$ -disulfide cleavage could be related to the limited efficiency of TCEP [48]. This efficiency  
467 value was close to the fraction of reduced  $CF_1F_o$  in the presence of TCEP in Fig 5B, estimated from the  
468 ECS decay kinetics in *gamera* and WT (already presented in Fig 3B). In Fig 5B, the relaxation of ECS  
469 from the  $\Delta\tilde{\mu}_{H^+ \text{ activation}}$  (arrowhead) to  $\Delta\tilde{\mu}_{H^+ \text{ dark}}$  ( $\sim -4$  charge separation/PSI for the 3 curves) was fitted  
470 with a mono-exponential decay. Rate constants of  $0.8 \text{ s}^{-1}$  in water-infiltrated WT were obtained in  
471 the presence of  $\sim 100\%$   $\gamma$ -disulfide, and  $30.6 \text{ s}^{-1}$  were measured with 100%  $\gamma$ -dithiol in *gamera*.  
472 Accordingly, Fig 5B suggests that TCEP infiltration produced  $\sim 58\%$   $\gamma$ -disulfide cleavage in WT  $CF_1F_o$   
473 since the rate constant was  $13.4 \text{ s}^{-1}$ . Unexpectedly, the partially reduced TCEP samples in Fig 5A  
474 showed a faster ECS decay at the low  $\Delta\tilde{\mu}_{H^+ \text{ dark}} + 1$  charge separation/PSI when AA was present (an  
475 ECS decay kinetics comparison with fully reduced *gamera*  $CF_1F_o$  is shown in Fig S7).

476 Supporting previous reports [36, 49], we did not observe an effect of the redox regulation on the  
477 maximal  $CF_1F_o$  activity at high  $\Delta\tilde{\mu}_{H^+}$  in WT. However, we did not manage to sustain high enough  
478  $\Delta\tilde{\mu}_{H^+ \text{ dark}}$  to fully describe the plateau of maximal  $CF_1F_o$  activities (see 95% confidence intervals in Fig  
479 5A). This was due to experimental constraints: First, in the oxidized form, it was not possible to reach  
480 higher values of  $\Delta\tilde{\mu}_{H^+}$  because a sufficient dark period was needed after the 30 s illumination to  
481 reoxidize PSI and PSII acceptors. Second, illumination decreased the  $\Delta\tilde{\mu}_{H^+}$  in the reduced *gamera*  
482  $CF_1F_o$  (Fig 3A). Still, the observation that half-reduced TCEP samples and oxidized WT leaves produced  
483 similar rate constants at  $\Delta\tilde{\mu}_{H^+} \sim -0.5$  charge separation/PSI suggests that the  $CF_1F_o$  activities in both  
484 redox states were similar in this range of  $\Delta\tilde{\mu}_{H^+}$  (Fig 4A, Fig 5A).

485 Taken together, this section demonstrates that the  $CF_1F_o$  redox state has a significant impact on its  
486 enzymatic activity *in vivo* and confirms previous *in vitro* studies: The reduced form of  $CF_1F_o$  (in the  
487 *gamera* mutant, but also partially in the TCEP-treated WT) exhibited higher activity at low membrane  
488 energization.

489



490

491 Fig 5: The regulation levels of  $\Delta\tilde{\mu}_{H^+}$  and thiol modulation on ATP synthesis are shown. (A) The  $\Delta\tilde{\mu}_{H^+}$ -  
 492 dependence of the  $\text{CF}_1\text{F}_0$  activity in the oxidized (black) and reduced (red) forms are shown, as well as in the  
 493 intermediate situation of TCEP-infiltrated WT leaves (blue). Closed symbols: untreated leaves, open symbols:  
 494 leaves were additionally infiltrated with 40  $\mu\text{M}$  and 20  $\mu\text{M}$  antimycin-A (+AA). The  $\Delta\tilde{\mu}_{H^+}$  in each color-coded  
 495 group was modulated in independent samples ( $n$  in closed/open black 3/2, blue 1/2, red 1/3). Decay rate  
 496 constants,  $R$ , were obtained according to Fig 4. Fitted curves were calculated with Equation 1 and the 95%  
 497 confidence intervals are transparent. The water-infiltrated WT showed a  $\Delta\tilde{\mu}_{H^+ \text{ activation}}$  of  $\sim -2.2$  charge  
 498 separations/PSI, and half-maximal  $R$  at  $\sim -1.0$  charge separations/PSI (dotted black line). The  $\gamma$ -dithiol-containing  
 499 *gamera* showed a half-maximal  $R$  at  $\sim -3.6$  charge separations/PSI (dotted red line) and a  $\Delta\tilde{\mu}_{H^+ \text{ activation}}$  of  $\sim -4.7$   
 500 charge separations/PSI. (B) Different kinetics of the ECS decay are shown in the control and TCEP-treated WT,  
 501 as well as the *gamera* mutant at similar  $\Delta\tilde{\mu}_{H^+ \text{ dark}}$ . Indicated by fitted ECS relaxation curves, a mono-exponential  
 502 decay function starting below  $\Delta\tilde{\mu}_{H^+ \text{ activation}}$  of the WT water sample (arrowhead) was used to calculate  $R$  of the  
 503  $\text{CF}_1\text{F}_0$ .

504



505 **Discussion**

506 Before finally visualizing the changing  $CF_1F_o$  activity *in vivo* (Results section 4), various methodical  
507 requirement were met to refine the ECS protocol [23] that estimates the  $\Delta\tilde{\mu}_{H^+}^{dark}$ . To do so, we  
508 presented in section 2 that short saturating pulses do not generate a  $\Delta pH$  and that pulse-induced ECS  
509 changes reflect changes in  $\Delta\tilde{\mu}_{H^+}$ . In section 3, we showed that the measured  $\Delta\tilde{\mu}_{H^+}^{dark}$  is a metabolic  
510 feature, defined by the  $[ATP]/([ADP][Pi])$  ratio at thermodynamic equilibrium, and that leak processes  
511 are negligible. The final  $CF_1F_o$  characterization let us conclude that the WT enzyme is in an oxidized  
512 state in the dark, contrary to a previous report [23]. The rate constants we measured in the low  $\Delta\tilde{\mu}_{H^+}$   
513 range (Fig 5A) were not zero although it was previously proposed that the  $CF_1F_o$  was fully inactive  
514 below the  $\Delta\tilde{\mu}_{H^+}^{activation}$  [16, 17]. If so, the activities below the apparent  $\Delta\tilde{\mu}_{H^+}^{activation}$  in Fig 5B ( $0.8\text{ s}^{-1}$  in  
515 WT vs.  $30.6\text{ s}^{-1}$  in *gamera*) would mean that 2-3% of the  $CF_1F_o$  was in the reduced form in the dark-  
516 adapted state *in vivo*, matching *in vitro* findings [49].

517 In the following four sections, we will discuss (i) the caveats of the presented  $CF_1F_o$  data for strict  
518 comparisons with the *in vitro* results, (ii) the sustained impact of an illumination period on  $\Delta\tilde{\mu}_{H^+}^{dark}$ ,  
519 (iii) possible side effects of chemical infiltration with TCEP, and (iv) structure-function considerations.

520

521 (i) *Precautions for strict comparisons of the presented  $CF_1F_o$  data with in vitro results.*

522 Overall, the obtained  $CF_1F_o$  rate versus  $\Delta\tilde{\mu}_{H^+}$  curves *in vivo* resembled earlier *in vitro* results in  
523 spinach chloroplasts where maximal ATP synthesis activity was independent from the redox state,  
524 while half-maximal rates of reduced  $CF_1F_o$  occurred at a lower  $\Delta pH$  of  $\sim 0.7$  units [36]. This  
525 corresponds to a difference of  $\sim 42$  mV when expressed in  $\Delta\Psi$  equivalent. In our results, half-  
526 saturation of  $CF_1F_o$  was 2.6 charge separations/PSI higher in the oxidized WT form compared to the  
527 *gamera* mutant. The *in vivo* and *in vitro* results are therefore roughly compatible by accepting a value  
528 of  $\sim 20$  mV per PSI charge separation, which is the most accepted estimate [40, 50, 51].

529 However, our results are not suited for strictly comparing the absolute values of the electrochemical  
530 gradient needed to reach half-saturation. The  $\Delta\tilde{\mu}_{H^+}$  values in our work are expressed in relative  
531 values (by comparison to  $\Delta\Psi_{10ms}$ ), whereas absolute values are reported in the *in vitro* work of  
532 Junesch and Gräber. For a proper *in vitro* and *in vivo* comparison, our results should be expressed in  
533 absolute values, too, which requires to obtain a situation where  $\Delta\tilde{\mu}_{H^+}^{dark}$  is zero. Several studies [23,  
534 47] worked with appropriate inhibitors to completely collapse the  $\Delta\tilde{\mu}_{H^+}^{dark}$ , including ours. The  
535  $\Delta\tilde{\mu}_{H^+}^{dark}$  is built at the expense of the ATP present in the plastid in dark-adapted leaves, which  
536 originates from mitochondrial ATP production as has been shown previously for algae [52], diatoms  
537 [47], and vascular plants [23]. Although antimycin-A (AA) inhibits the cytochrome *bc\_1* complex in the  
538 mitochondrial respiratory chain, it is known that the treatment does not eliminate this cellular ATP  
539 source completely [47]. In that regard, we identified more effective treatments in WT (Fig S4) but  
540 lowering  $\Delta\tilde{\mu}_{H^+}^{dark}$  by AA is preferred in our  $CF_1F_o$  characterization study despite individual differences  
541 between samples (Supplementary discussion). Most importantly, the  $\Delta\tilde{\mu}_{H^+}^{dark}$ -collapsing efficiency of  
542 the chemical does not involve direct modification of the membrane and its electric permeability.

543 With this in mind, it is possible to discuss our results in the light of the 2.7 and 3.4  $\Delta pH$  units for half-  
544 saturation of the reduced and oxidized ATP synthesis rate *in vitro* (see reference 36 and Fig S8A). Let  
545 us focus on oxidized samples where half-maximal rates *in vitro* were measured at a  $\Delta\tilde{\mu}_{H^+}$  equivalent  
546 to  $\sim 201$  mV [36]. This value yields  $\sim 10$  charge separations/PSI with an estimated 20 mV per reaction  
547 center turnover [40, 50, 51]. By referring to  $\Delta\Psi_{10ms}$ , our relative  $\Delta\tilde{\mu}_{H^+}$  values indicated half-  
548 saturation of oxidized  $CF_1F_o$  at -1.0 charge separation/PSI. We obtained the lowest values of  $\Delta\tilde{\mu}_{H^+}^{dark}$

549 for AA-infiltrated *gamera* leaves in our ECS data set which equilibrated down to  $\sim$ -8.8 charge  
550 separations/PSI (note that x-axis in Fig 5A is  $\Delta\tilde{\mu}_{H^+_{dark}} + 1$ ). We expect a residual  $\Delta\tilde{\mu}_{H^+_{dark}}$  when solely  
551 relying on the respiratory inhibitor AA (Supplementary discussion). When matching half-saturation in  
552 both systems, the remaining  $\Delta\tilde{\mu}_{H^+_{dark}}$  in our oxidized  $CF_1F_0$  data set should be  $\sim$ 2.2 charge  
553 separations/PSI ( $10 - 8.8 + 1.0$ ), corresponding to  $\sim$ 44 mV or  $\sim$ 0.7  $\Delta$ pH units. Thus, we do  
554 acknowledge a significant discrepancy between our results and the ones obtained *in vitro* [36]. A  
555 tentative conversion of our relative oxidized  $CF_1F_0$  half-saturation values to an absolute  $\Delta\tilde{\mu}_{H^+}$   
556 expression yields  $156 \pm 22$  mV ( $(8.8 - 1.0) \times 20$  mV; for reduced samples see also Fig S8B). We also  
557 consider the possibility that the  $\Delta\tilde{\mu}_{H^+}$  at ATP synthesis half-saturation of the fully reduced ATPC1  $\gamma$ -  
558 dithiol in WT leaves does not resemble  $CF_1F_0$  in *gamera* (see sections iii and iv).

559

560 (ii) *What does the lifetime of ATP depend on?*

561 In this work, we could measure the ATP lifetime after an illumination and showed that the  
562 accumulated ATP was saturated after 7 s of light. Prolonging the light duration only modified the lag  
563 before the ATP pool relaxed. We favor the hypotheses that the increased ATP lifetime, as a function  
564 of the pre-illumination period (closed squares in Fig 3A), was associated with the accumulation of  
565 high energy bonds in different plastid metabolites and/or that free ATP levels were buffered by the  
566 stromal adenylate kinase activity as proposed recently [53, 54]. The view that metabolic ATP  
567 consumption occurs within the chloroplast at higher rates than leaking towards other compartments  
568 is supported in a nucleotide transporter study. Therein, the ATP exchange rate between chloroplast  
569 and cytosol was rather slow, and free ATP diminished rapidly in the dark after illumination [32].  
570 According to the fluorescent ATP sensor data, free MgATP levels varied in the stroma from 0.2 mM  
571 (darkness) to 0.5 mM (light), while cytosolic MgATP was about 2 mM [32, 55]. Thus, a delayed post-  
572 illumination decline of the  $\Delta\tilde{\mu}_{H^+_{dark}}$  in Fig 3A could be related to slow metabolite conversions that  
573 consume plastidic ATP. For instance, high levels of ribulose-1,5-bisphosphate and 1,3-  
574 bisphosphoglycerate in the Calvin-Benson cycle could be part of a feedback inhibition that prevents  
575 their own ATP-consuming synthesis. Furthermore, the deactivation of the Calvin-Benson cycle in the  
576 dark [23] was in the same time range as the decay of  $\Delta\tilde{\mu}_{H^+_{dark}}$ . On the other hand, the thioredoxin  
577 targets fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase may have become  
578 partially reduced when infiltrating leaves with TCEP (midpoint redox potential of  $-290$  mV [56]), thus  
579 favoring their activation. Additionally, it is not known whether TCEP influences the phosphate group  
580 transfer between ATP and AMP (yielding 2 ADP) through cysteine redox chemistry in chloroplast  
581 adenylate kinase 5 [53]. In turn, this could explain a slightly earlier onset of the consumption of ATP  
582 after 30-s illumination in TCEP-infiltrated samples (cf. open squares in Fig 3A).

583

584 (iii) *Possible side effects of TCEP infiltration*

585 In Fig 5A, the fit of the TCEP-infiltrated experimental data by the sum of two sigmoids failed at low  
586  $\Delta\tilde{\mu}_{H^+}$ : significantly elevated rates were observed in the presence of TCEP + AA (open blue diamonds,  
587 see also Fig S7 for kinetics comparison with *gamera* + AA). We provide three possibilities for our  
588 observations, none of which we can endorse at this point: First, it is possible that  $\gamma$ -disulfide cleavage  
589 by TCEP was further advanced ( $>$ 50%) when the WT  $CF_1F_0$  population was under the influence of an  
590 increased cellular redox poise due to respiration inhibition. To further substantiate this hypothesis,  
591 the measurement of approximative  $R < 1$  s $^{-1}$  would have required the  $\Delta\tilde{\mu}_{H^+_{dark}}$  to collapse even below  
592 the values obtained in Fig 5A for TCEP + AA. The associated difficulties of achieving such situations  
593 are outlined in the Supplementary discussion. This hypothesis also implies that ATP synthesis onset in

594 reduced WT CF<sub>1</sub>F<sub>o</sub> is below that shown in Fig 5A for the *gamera* CF<sub>1</sub>F<sub>o</sub> isoform. Secondly, we cannot  
595 rule out that TCEP infiltration had weak uncoupling effects or, thirdly, modified ion  
596 channel/antiporter activity. Possible TCEP targets could be an activation of  $\Delta\Psi$ -consuming anion  
597 channels such as VCCN1 [2], or a deactivation of  $\Delta\Psi$ -generating H<sup>+</sup>/cation antiporters such as KEA3  
598 [1]. However, there is currently no evidence of VCCN1/KEA3 thiol regulation, nor were their activities  
599 analyzed at low  $\Delta\tilde{\mu}_{H^+}^{dark}$ . It has been reported that reduction of chloroform-extracted CF<sub>1</sub> by DTT  
600 resulted in the reversible dissociation of the  $\epsilon$ -subunit which would produce membrane uncoupling  
601 [57-59], if this was the case with TCEP *in vivo*. Accordingly, the slightly earlier onset of  $\Delta\tilde{\mu}_{H^+}^{dark}$   
602 decrease upon illuminating TCEP-infiltrated samples might be due to a slow leak process (Fig 3A).  
603 Considering the remaining uncertainties on the TCEP + AA data, further studies are necessary to  
604 clarify whether CF<sub>1</sub>F<sub>o</sub>-independent processes produce slightly elevated ECS decay rate constants.

605

#### 606 (iv) *Combining function, structure, and physiology*

607 In the following, we underpin our thiol modulation study in the light of structural findings since  
608 contribution of  $\Delta\tilde{\mu}_{H^+}$  is poorly understood. The  $\gamma$ -redox domain as such forms an L-shaped double  
609 hairpin (Fig 6A, light green in magnified area). The second hairpin interacts with the  $\beta$ -subunit  
610 DELSEED motif and supposedly forms an ATPase chock during rotation in the oxidized state [34].  
611 Alternatively, the  $\gamma$ -disulfide stabilizes the redox domain intrinsically rather than establishing the  
612 chock element [37]. Based on a proportion shift of the three rotary states, lower energetic barriers  
613 may occur during rotary transitioning due to an increased flexibility and lower torsional constraints in  
614 the reduced enzyme [37].

615 Critical structural alterations between the interactors ( $\gamma$ -redox domain and  $\beta$ -DELSEED loop) can be  
616 expected as a function of  $\Delta\tilde{\mu}_{H^+}$ . For instance, various buried residues (e.g.,  $\gamma$ K222 and  $\beta$ K399 in Fig  
617 6A) have been chemically modified in illuminated thylakoids only [reviewed in 38, 60, 61]. Although  
618 details of these CF<sub>1</sub>F<sub>o</sub> rearrangements are not clear yet, the  $\Delta\tilde{\mu}_{H^+}$  may transiently modify the sum of  
619 interactions with the  $\beta$ -DELSEED loops by uncovering parts of the  $\gamma$ -redox domain in a reversible  
620 fashion. In turn, the  $\gamma$ -redox domain exposure may enhance the chemical-to-mechanical energy  
621 transduction efficiency and could be a feature of the CF<sub>1</sub>F<sub>o</sub> activation. The influence of  $\Delta\tilde{\mu}_{H^+}$  quantity  
622 [8] and quality [18] on CF<sub>1</sub>F<sub>o</sub> activation has been investigated by various groups.

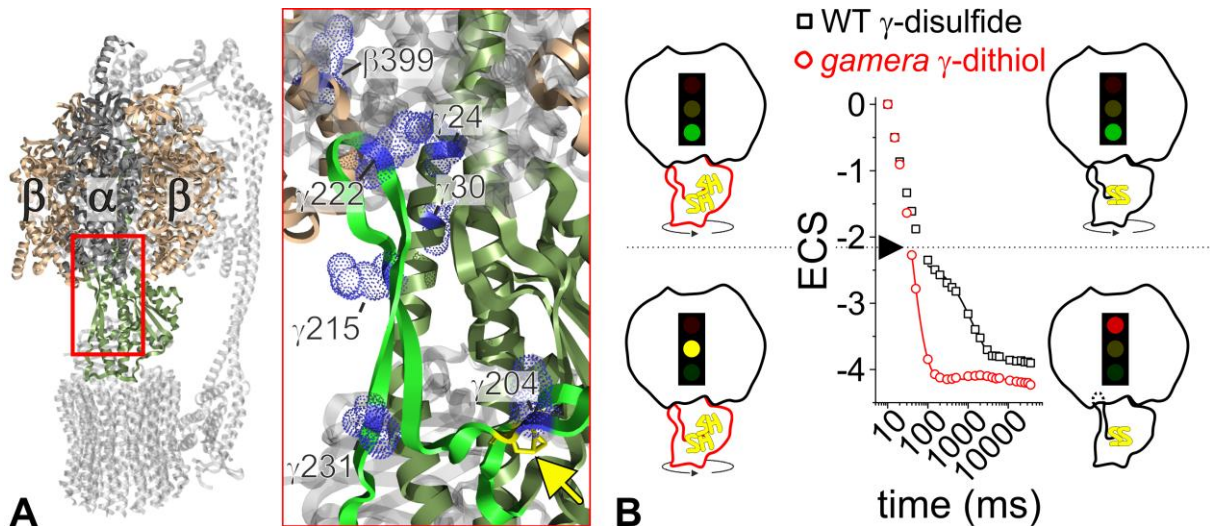
623 Upon reaching  $\Delta\tilde{\mu}_{H^+}^{leak}$  in our experiments at the end of the ms-pulses, the  $\Delta\tilde{\mu}_{H^+}$ -activated CF<sub>1</sub>F<sub>o</sub> was  
624 obtained in both redox states (Fig 6B above dashed line). The surplus of  $\Delta\tilde{\mu}_{H^+}$  was consumed at  
625 maximal rates during this ~100-ms phase in the dark until reaching a turning point, i.e.,  $\Delta\tilde{\mu}_{H^+}^{activation}$   
626 in WT (arrowhead in Fig 6B, the corresponding threshold in *gamera* was below the  $\Delta\tilde{\mu}_{H^+}^{dark}$ ).  
627 Stabilized by the disulfide, we hypothesize that the  $\gamma$ -redox domain is buried again which slows down  
628 the WT by altering the sum of interactions with the  $\beta$ -DELSEED loops (Fig 6B, below dashed line). It  
629 was shown that the redox domain experiences the  $\Delta\tilde{\mu}_{H^+}$ , becoming more solvent-accessible [25-27,  
630 49, 62, 63]. The intrinsic  $\Delta\tilde{\mu}_{H^+}^{activation}$  in WT may represent the critical driving force level for ATP  
631 synthesis where transient resting times in between rotary states are long enough to allow for  
632 molecular relaxation during dwells. A different dwelling without the rigidity-enhancing  $\gamma$ -disulfide  
633 might account for a lower  $\Delta\tilde{\mu}_{H^+}^{activation}$  in *gamera*. Note that the amino acid sequences between  
634 ATPC1 (WT) and ATPC2 (*gamera*) are significantly different in the redox domain with profound  
635 effects on steady-state rates [64], which may relate to isoform-specific contacts with the  $\beta$ -DELSEED  
636 loop. Considering the effects and limits of chemical reduction (section iii), it is likely that disrupting  
637 the disulfide in ATPC1 by mutation will yield a different activity profile as shown in Fig 5A for *gamera*.

638

639 Conclusion

640 The intertwining of  $CF_1F_0$  and  $\Delta\tilde{\mu}_{H^+}$  includes various regulatory loops such as thiol modulation which  
641 has physiological consequences. By increasing light utilization efficiency, a highly conductive  $CF_1F_0$  at  
642 low  $\Delta\tilde{\mu}_{H^+}$  maximizes the photosynthetic output for  $CO_2$  fixation. Here, we introduced and discussed a  
643 versatile spectroscopic *in vivo* assay to disentangle the molecular consequences of thiol modulation  
644 on the  $\Delta\tilde{\mu}_{H^+}$  to ATP conversion efficiency. Moreover, the ECS protocol may be transferred to other  
645  $CF_1F_0$  mutants in the future, for instance of the  $\epsilon$ -subunit. Its C-terminus was found retracted in the  
646 spinach structure [34, 37] which dismissed its previously established role as intrinsic ATPase inhibitor  
647 [65-67]. Our assays may help to consolidate those views since some  $\epsilon$ -subunit features were also  
648 entangled with  $\Delta\tilde{\mu}_{H^+}$  [65, 68]. By following the  $[ATP]/([ADP][Pi])$  ratio at thermodynamic equilibrium,  
649 we revealed a hampered ATP storage capacity in the *gamera* mutant in extension to its other  
650 phenotypes [28, 29]. Likewise, additional independent insights on chloroplast nucleotide transporter  
651 regulation may be revealed, which currently rely on fluorescent ATP sensors [32]. Finally, time-  
652 resolved characterization of  $\Delta\tilde{\mu}_{H^+}$  partitioning may become accessible by describing the  $\Delta\tilde{\mu}_{H^+}$  after  
653 short illumination periods with our method, as well as thylakoid ion channel/antiporter effects during  
654 the photosynthetic induction phase.

655



656 **A**  
 657 Fig 6: A selection of  $\Delta\tilde{\mu}_{H^+}$ -dependent structural rearrangements in spinach  $CF_1F_o$  and a tentative model for  
 658 different ATP synthesis rates as a function of  $\Delta\tilde{\mu}_{H^+}$  are shown. (A) The structure of spinach  $CF_1F_o$  (PDB ID: 6FKH)  
 659 magnifies the redox loop on the right (light green, arrow pointing to disulfide in yellow sticks). The frontal  $\alpha$ -  
 660 subunit is magnified half-transparent for the sake of visibility of the  $\gamma$ -subunit (dark green). The ATPase choc  
 661 structure is formed by the  $\gamma$ -hairpin loop around position 222 which is stabilized by the disulfide [34, 37]. A  
 662 variety of structural rearrangements are depending on the  $\Delta\tilde{\mu}_{H^+}$ , including the  $\gamma$ -hairpin. Various residues (blue  
 663 spheres) become available for chemical labeling or trypsin digestion exclusively in illuminated thylakoids  
 664 [reviewed in 38]. (B) The exposed position of the choc structure in  $\Delta\tilde{\mu}_{H^+}$ -activated  $CF_1F_o$  allows for efficient  
 665 rotational catalysis in both  $\gamma$ -redox states (top cartoons of subunits  $\alpha_3\beta_3\gamma$ ). Below the WT activation threshold  
 666 (arrowhead and horizontal line), the disulfide-stabilized choc structure prevents efficient ATP synthesis. The  
 667 *gamera* sustains an active conformation at this  $\Delta\tilde{\mu}_{H^+}$  since the rotating choc structure is less efficiently  
 668 stabilized inside the  $\alpha_3\beta_3$  cavity, owing to the absence of the  $\gamma$ -disulfide.  
 669  
 670  
 671

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676

677 **Competing interests:**

678 All authors declare no competing interests.

679

- 681 [1] U. Armbruster, L.R. Carrillo, K. Venema, L. Pavlovic, E. Schmidtman, A. Kornfeld, P. Jahns, J.A.  
682 Berry, D.M. Kramer, M.C. Jonikas, Ion antiport accelerates photosynthetic acclimation in fluctuating  
683 light environments, *Nat Commun*, 5 (2014) 5439.
- 684 [2] A. Herdean, E. Teardo, A.K. Nilsson, B.E. Pfeil, O.N. Johansson, R. Unnep, G. Nagy, O. Zsiros, S.  
685 Dana, K. Solymosi, G. Garab, I. Szabo, C. Spetea, B. Lundin, A voltage-dependent chloride channel  
686 fine-tunes photosynthesis in plants, *Nat Commun*, 7 (2016) 11654.
- 687 [3] W. Junge, N. Nelson, ATP synthase, *Annu. Rev. Biochem.*, 84 (2015) 631-657.
- 688 [4] T. Hisabori, E.I. Sunamura, Y. Kim, H. Konno, The chloroplast ATP synthase features the  
689 characteristic redox regulation machinery, *Antioxidants & Redox Signaling*, 19 (2013) 1846-U1216.
- 690 [5] H. Seelert, A. Poetsch, N.A. Dencher, A. Engel, H. Stahlberg, D.J. Muller, Structural biology. Proton-  
691 powered turbine of a plant motor, *Nature*, 405 (2000) 418-419.
- 692 [6] R.E. McCarty, J.S. Fuhrman, Y. Tsuchiya, Effects of adenine nucleotides on hydrogen-ion transport  
693 in chloroplasts, *Proc Natl Acad Sci U S A*, 68 (1971) 2522-2526.
- 694 [7] G. Groth, W. Junge, Proton slip of the chloroplast ATPase: its nucleotide dependence, energetic  
695 threshold, and relation to an alternating site mechanism of catalysis, *Biochemistry*, 32 (1993) 8103-  
696 8111.
- 697 [8] P. Turina, J. Petersen, P. Gräber, Thermodynamics of proton transport coupled ATP synthesis,  
698 *Biochim. Biophys. Acta*, 1857 (2016) 653-664.
- 699 [9] P. Mitchell, Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic  
700 type of mechanism, *Nature*, 191 (1961) 144-148.
- 701 [10] A. Kanazawa, D.M. Kramer, *In vivo* modulation of nonphotochemical exciton quenching (NPQ) by  
702 regulation of the chloroplast ATP synthase, *Proc. Natl. Acad. Sci. USA*, 99 (2002) 12789-12794.
- 703 [11] P. Horton, A.V. Ruban, D. Rees, A.A. Pascal, G. Noctor, A.J. Young, Control of the light-harvesting  
704 function of chloroplast membranes by aggregation of the LHClI chlorophyll-protein complex, *FEBS*  
705 *Lett.*, 292 (1991) 1-4.
- 706 [12] A.V. Ruban, Light harvesting control in plants, *FEBS Lett.*, 592 (2018) 3030-3039.
- 707 [13] J.N. Nishio, J. Whitmarsh, Dissipation of the Proton Electrochemical Potential in Intact  
708 Chloroplasts (II. The pH Gradient Monitored by Cytochrome f Reduction Kinetics), *Plant Physiol.*, 101  
709 (1993) 89-96.
- 710 [14] K. Takizawa, A. Kanazawa, D.M. Kramer, Depletion of stromal Pi induces high 'energy-dependent'  
711 antenna exciton quenching ( $q_E$ ) by decreasing proton conductivity at CFo-CF1 ATP synthase, *Plant*  
712 *Cell Environ*, 31 (2008) 235-243.
- 713 [15] F. Buchert, B. Bailleul, T. Hisabori, A  $\gamma$ -subunit point mutation in *Chlamydomonas reinhardtii*  
714 chloroplast  $F_1F_0$ -ATP synthase confers tolerance to reactive oxygen species, *Biochim. Biophys. Acta*,  
715 1858 (2017) 966-974.
- 716 [16] W. Junge, B. Rumberg, H. Schroder, The necessity of an electric potential difference and its use  
717 for photophosphorylation in short flash groups, *Eur. J. Biochem.*, 14 (1970) 575-581.
- 718 [17] W. Junge, The critical electric potential difference for photophosphorylation. Its relation to the  
719 chemiosmotic hypothesis and to the triggering requirements of the ATPase system, *Eur. J. Biochem.*,  
720 14 (1970) 582-592.
- 721 [18] G. Kaim, P. Dimroth, ATP synthesis by F-type ATP synthase is obligatorily dependent on the  
722 transmembrane voltage, *EMBO J.*, 18 (1999) 4118-4127.
- 723 [19] J.H. Kaplan, E. Uribe, A.T. Jagendorf, ATP hydrolysis caused by acid-base transition of spinach  
724 chloroplasts, *Arch Biochem Biophys*, 120 (1967) 365-370.
- 725 [20] P. Gräber, H.T. Witt, Relations between the electrical potential, pH gradient, proton flux and  
726 phosphorylation in the photosynthetic membrane, *Biochim. Biophys. Acta*, 423 (1976) 141-163.
- 727 [21] D.A. Harris, A.R. Crofts, The initial stages of photophosphorylation. Studies using excitation by  
728 saturating, short flashes of light, *Biochim. Biophys. Acta*, 502 (1978) 87-102.
- 729 [22] D.M. Kramer, A.R. Crofts, Activation of the chloroplast ATPase measured by the electrochromic  
730 change in leaves of intact plants, *Biochimica et Biophysica Acta*, 976 (1989) 28-41.

731 [23] P. Joliot, A. Joliot, Quantification of the electrochemical proton gradient and activation of ATP  
732 synthase in leaves, *Biochim. Biophys. Acta*, 1777 (2008) 676-683.

733 [24] K. Yoshida, A. Hara, K. Sugiura, Y. Fukaya, T. Hisabori, Thioredoxin-like2/2-Cys peroxiredoxin  
734 redox cascade supports oxidative thiol modulation in chloroplasts, *Proc. Natl. Acad. Sci. USA*, 115  
735 (2018) E8296-E8304.

736 [25] J.D. Mills, P. Mitchell, P. Schurmann, Modulation of coupling factor ATPase activity in intact  
737 chloroplasts - The role of the thioredoxin system, *FEBS Lett.*, 112 (1980) 173-177.

738 [26] B. Naranjo, C. Mignee, A. Krieger-Liszka, D. Hornero-Mendez, L. Gallardo-Guerrero, F.J. Cejudo,  
739 M. Lindahl, The chloroplast NADPH thioredoxin reductase C, NTRC, controls non-photochemical  
740 quenching of light energy and photosynthetic electron transport in Arabidopsis, *Plant Cell Environ*, 39  
741 (2016) 804-822.

742 [27] L.R. Carrillo, J.E. Froehlich, J.A. Cruz, L.J. Savage, D.M. Kramer, Multi-level regulation of the  
743 chloroplast ATP synthase: the chloroplast NADPH thioredoxin reductase C (NTRC) is required for  
744 redox modulation specifically under low irradiance, *Plant J.*, 87 (2016) 654-663.

745 [28] K. Kohzuma, C. Dal Bosco, A. Kanazawa, A. Dhingra, W. Nitschke, J. Meurer, D.M. Kramer,  
746 Thioredoxin-insensitive plastid ATP synthase that performs moonlighting functions, *Proc. Natl. Acad.  
747 Sci. USA*, 109 (2012) 3293-3298.

748 [29] K. Kohzuma, J.E. Froehlich, G.A. Davis, J.A. Temple, D. Minhas, A. Dhingra, J.A. Cruz, D.M.  
749 Kramer, The Role of Light-Dark Regulation of the Chloroplast ATP Synthase, *Frontiers in Plant  
750 Science*, 8 (2017) 1248.

751 [30] D.R. Ort, K. Oxborough, *In situ* regulation of chloroplast coupling factor activity, *Annu. Rev. Plant  
752 Physiol. Plant Mol. Biol.*, 43 (1992) 269-291.

753 [31] G. Wu, D.R. Ort, Mutation in the cysteine bridge domain of the  $\gamma$  subunit affects light regulation  
754 of the ATP synthase but not photosynthesis or growth in Arabidopsis, *Photosynthesis Res.*, 97 (2008)  
755 185-193.

756 [32] C.P. Voon, X. Guan, Y. Sun, A. Sahu, M.N. Chan, P. Gardestrom, S. Wagner, P. Fuchs, T. Nietzel,  
757 W.K. Versaw, M. Schwarzlander, B.L. Lim, ATP compartmentation in plastids and cytosol of  
758 Arabidopsis thaliana revealed by fluorescent protein sensing, *Proc. Natl. Acad. Sci. USA*, 115 (2018)  
759 E10778-E10787.

760 [33] J.Z. Pu, M. Karplus, How subunit coupling produces the  $\gamma$  subunit rotary motion in  $F_1$ -ATPase,  
761 *Proc. Natl. Acad. Sci. USA*, 105 (2008) 1192-1197.

762 [34] A. Hahn, J. Vonck, D.J. Mills, T. Meier, W. Kuhlbrandt, Structure, mechanism, and regulation of  
763 the chloroplast ATP synthase, *Science*, 360 (2018).

764 [35] F. Buchert, H. Konno, T. Hisabori, Redox regulation of  $CF_1$ -ATPase involves interplay between the  
765  $\gamma$ -subunit neck region and the turn region of the  $\beta$ DELSEED-loop, *Biochim. Biophys. Acta*, 1847 (2015)  
766 441-450.

767 [36] U. Junesch, P. Gräber, Influence of the redox state and the activation of the chloroplast ATP  
768 synthase on proton-transport-coupled ATP synthesis hydrolysis, *Biochim. Biophys. Acta*, 893 (1987)  
769 275-288.

770 [37] J.H. Yang, D. Williams, E. Kandiah, P. Fromme, P.L. Chiu, Structural basis of redox modulation on  
771 chloroplast ATP synthase, *Communications biology*, 3 (2020) 482.

772 [38] M.L. Richter, R. Hein, B. Huchzermeyer, Important subunit interactions in the chloroplast ATP  
773 synthase, *Biochim. Biophys. Acta*, 1458 (2000) 326-342.

774 [39] W. Junge, H.T. Witt, On the ion transport system of photosynthesis--investigations on a  
775 molecular level, *Z Naturforsch B*, 23 (1968) 244-254.

776 [40] H.T. Witt, Energy conversion in the functional membrane of photosynthesis. Analysis by light  
777 pulse and electric pulse methods. The central role of the electric field, *Biochim. Biophys. Acta*, 505  
778 (1979) 355-427.

779 [41] B. Bailleul, P. Cardol, C. Breyton, G. Finazzi, Electrochromism: a useful probe to study algal  
780 photosynthesis, *Photosynthesis Res.*, 106 (2010) 179-189.

781 [42] G. Wu, G. Ortiz-Flores, A. Ortiz-Lopez, D.R. Ort, A point mutation in *atpC1* raises the redox  
782 potential of the Arabidopsis chloroplast ATP synthase  $\gamma$  subunit regulatory disulfide above the range  
783 of thioredoxin modulation, *J. Biol. Chem.*, 282 (2007) 36782-36789.

784 [43] W. Junge, W. Auslander, A.J. McGeer, T. Runge, The buffering capacity of the internal phase of  
785 thylakoids and the magnitude of the pH changes inside under flashing light, *Biochim. Biophys. Acta*,  
786 546 (1979) 121-141.

787 [44] C. Lemaire, F.A. Wollman, P. Bennoun, Restoration of phototrophic growth in a mutant of  
788 *Chlamydomonas reinhardtii* in which the chloroplast *atpB* gene of the ATP synthase has a deletion:  
789 an example of mitochondria-dependent photosynthesis, *Proc. Natl. Acad. Sci. USA*, 85 (1988) 1344-  
790 1348.

791 [45] J. Reiser, N. Linka, L. Lemke, W. Jeblick, H.E. Neuhaus, Molecular physiological analysis of the  
792 two plastidic ATP/ADP transporters from Arabidopsis, *Plant Physiol.*, 136 (2004) 3524-3536.

793 [46] O. Trentmann, T. Mühlhaus, D. Zimmer, F. Sommer, M. Schroda, I. Haferkamp, I. Keller, B.  
794 Pommerrenig, H.E. Neuhaus, Identification of Chloroplast Envelope Proteins with Critical Importance  
795 for Cold Acclimation, *Plant Physiol.*, 182 (2020) 1239-1255.

796 [47] B. Bailleul, N. Berne, O. Murik, D. Petroustos, J. Pihoda, A. Tanaka, V. Villanova, R. Bligny, S.  
797 Flori, D. Falconet, A. Krieger-Liszkay, S. Santabarbara, F. Rappaport, P. Joliot, L. Tirichine, P.G.  
798 Falkowski, P. Cardol, C. Bowler, G. Finazzi, Energetic coupling between plastids and mitochondria  
799 drives CO<sub>2</sub> assimilation in diatoms, *Nature*, 524 (2015) 366-369.

800 [48] D.J. Cline, S.E. Redding, S.G. Brohawn, J.N. Psathas, J.P. Schneider, C. Thorpe, New water-soluble  
801 phosphines as reductants of peptide and protein disulfide bonds: reactivity and membrane  
802 permeability, *Biochemistry*, 43 (2004) 15195-15203.

803 [49] H. Konno, T. Nakane, M. Yoshida, H. Ueoka-Nakanishi, S. Hara, T. Hisabori, Thiol modulation of  
804 the chloroplast ATP synthase is dependent on the energization of thylakoid membranes, *Plant Cell*  
805 *Physiol.*, 53 (2012) 626-634.

806 [50] W. Schliephake, W. Junge, H.T. Witt, Correlation between field formation, proton translocation,  
807 and the light reactions in photosynthesis, *Z Naturforsch B*, 23 (1968) 1571-1578.

808 [51] A.A. Bulychev, W.J. Vredenberg, Effect of ionophores A23187 and nigericin on the light-induced  
809 redistribution of Mg<sup>2+</sup>, K<sup>+</sup> and H<sup>+</sup> across the thylakoid membrane, *Biochim. Biophys. Acta*, 449 (1976)  
810 48-58.

811 [52] P. Bennoun, Chlororespiration revisited: Mitochondrial-plastid interactions in *Chlamydomonas*,  
812 *Biochim. Biophys. Acta*, 1186 (1994) 59-66.

813 [53] P.R. Lange, C. Geserick, G. Tischendorf, R. Zrenner, Functions of chloroplastic adenylate kinases  
814 in Arabidopsis, *Plant Physiol.*, 146 (2008) 492-504.

815 [54] A.U. Igamberdiev, L.A. Kleczkowski, Optimization of ATP synthase function in mitochondria and  
816 chloroplasts via the adenylate kinase equilibrium, *Front Plant Sci*, 6 (2015) 10.

817 [55] V. De Col, P. Fuchs, T. Nietzel, M. Elsässer, C.P. Voon, A. Candeo, I. Seeliger, M.D. Fricker, C.  
818 Grefen, I.M. Møller, A. Bassi, B.L. Lim, M. Zancani, A.J. Meyer, A. Costa, S. Wagner, M. Schwarzländer,  
819 ATP sensing in living plant cells reveals tissue gradients and stress dynamics of energy physiology,  
820 *Elife*, 6 (2017) e26770.

821 [56] P.K. Pallela, T. Chiku, M.J. Carvan, 3rd, D.S. Sem, Fluorescence-based detection of thiols in vitro  
822 and in vivo using dithiol probes, *Anal. Biochem.*, 352 (2006) 265-273.

823 [57] R.J. Duhe, B.R. Selman, The dithiothreitol-stimulated dissociation of the chloroplast coupling  
824 factor-1  $\epsilon$  subunit is reversible, *Biochim. Biophys. Acta*, 1017 (1990) 70-78.

825 [58] R.J. Duhe, B.R. Selman, Studies on the heterogeneity of the soluble chloroplast coupling factor 1:  
826 The formation of  $\epsilon$ -deficient isozymes, *Biochim. Biophys. Acta*, 974 (1989) 294-302.

827 [59] M.L. Richter, W.J. Patrie, R.E. McCarty, Preparation of the  $\epsilon$  subunit and  $\epsilon$  subunit-deficient  
828 chloroplast coupling factor-1 in reconstitutively active forms, *J. Biol. Chem.*, 259 (1984) 7371-7373.

829 [60] M. Komatsu-Takaki, Energizing effects of illumination on the reactivities of lysine residues of the  
830  $\gamma$  subunit of chloroplast ATP synthase, *Eur. J. Biochem.*, 236 (1996) 470-475.



831 [61] M. Komatsu-Takaki, Effects of Energization and Substrates on the Reactivities of Lysine Residues  
832 of the Chloroplast ATP Synthase  $\beta$  Subunit, *Eur. J. Biochem.*, 228 (1995) 265-270.  
833 [62] S.R. Ketcham, J.W. Davenport, K. Warncke, R.E. McCarty, Role of the  $\gamma$  subunit of chloroplast  
834 coupling factor-1 in the light-dependent activation of photophosphorylation and ATPase activity by  
835 dithiothreitol, *J. Biol. Chem.*, 259 (1984) 7286-7293.  
836 [63] K. Yoshida, Y. Matsuoka, S. Hara, H. Konno, T. Hisabori, Distinct redox behaviors of chloroplast  
837 thiol enzymes and their relationships with photosynthetic electron transport in *Arabidopsis thaliana*,  
838 *Plant Cell Physiol.*, 55 (2014) 1415-1425.  
839 [64] G.A. Davis, A. Kanazawa, M.A. Schottler, K. Kohzuma, J.E. Froehlich, A.W. Rutherford, M. Satoh-  
840 Cruz, D. Minhas, S. Tietz, A. Dhingra, D.M. Kramer, Limitations to photosynthesis by proton motive  
841 force-induced photosystem II photodamage, *Elife*, 5 (2016).  
842 [65] E.A. Johnson, R.E. McCarty, The carboxyl terminus of the  $\epsilon$  subunit of the chloroplast ATP  
843 synthase is exposed during illumination, *Biochemistry*, 41 (2002) 2446-2451.  
844 [66] E.A. Johnson, Altered expression of the chloroplast ATP synthase through site-directed  
845 mutagenesis in *Chlamydomonas reinhardtii*, *Photosynthesis Res.*, 96 (2008) 153-162.  
846 [67] J.A. Cruz, B. Harfe, C.A. Radkowski, M.S. Dann, R.E. McCarty, Molecular dissection of the  $\epsilon$   
847 subunit of the chloroplast ATP synthase of spinach, *Plant Physiol.*, 109 (1995) 1379-1388.  
848 [68] M. Komatsu-Takaki, Energy-dependent conformational changes in the  $\epsilon$  subunit of the  
849 chloroplast ATP synthase ( $CF_0CF_1$ ), *J. Biol. Chem.*, 264 (1989) 17750-17753.

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