

1 **High Light and High Temperature Reduce Photosynthesis**
2 **via Different Mechanisms in the C₄ Model *Setaria viridis***

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27 **Frequently Used Abbreviations:** HL, high light; HT, high temperature; ctrl, control; M,
28 mesophyll; BS, bundle sheath; Rubisco, ribulose-1,5-bisphosphate
29 carboxylase/oxygenase; PSII, photosystem II; PSI, photosystem I; A_{Net}, net CO₂
30 assimilation rates; DEGs, differentially expressed genes; NPQ, non-photochemical

31 quenching; CEF, cyclic electron flow around PSI; RCA, Rubisco activase; ABA, abscisic
32 acid; PG, plastoglobuli; HSP, heat shock protein; HSF, heat shock transcription factor.

33

34 **Abstract**

35 C₄ plants frequently experience damaging high light (HL) and high temperature (HT)
36 conditions in native environments, which reduce growth and yield. However, the
37 mechanisms underlying these stress responses in C₄ plants have been under-explored,
38 especially the coordination between mesophyll (M) and bundle sheath (BS) cells. We
39 investigated how the C₄ model plant *Setaria viridis* responded to a four-hour HL or HT
40 treatment at the photosynthetic, transcriptomic, and ultrastructural levels. Although we
41 observed a comparable reduction of photosynthetic efficiency in HL- or HT-treated leaves,
42 detailed analysis of multi-level responses revealed important differences in key pathways
43 and M/BS specificity responding to HL and HT. We provide a systematic analysis of HL
44 and HT responses in *S. viridis*, reveal different acclimation strategies to these two
45 stresses in C₄ plants, discover unique light/temperature responses in C₄ plants in
46 comparison to C₃ plants, and identify potential targets to improve abiotic stress tolerance
47 in C₄ crops.

48

49 **Introduction**

50 Several of the world's most economically important staple crops utilize C₄ photosynthesis,
51 including *Zea mays* and *Sorghum bicolor*. C₄ photosynthesis concentrates CO₂ around
52 Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) by employing biochemical
53 reactions within mesophyll (M) and bundle sheath (BS) cells^{1,2}. The high local
54 concentration of CO₂ near Rubisco favors carbon fixation over photorespiration, which is
55 initiated by the oxygenase activity of Rubisco^{1,3}. C₄ photosynthesis is hypothesized to
56 have been selected by low CO₂, high light (HL), and high temperature (HT) conditions^{4,5}.
57 C₄ plants typically exhibit higher photosynthetic and water-use efficiencies than their C₃
58 counterparts under HL or HT⁶. However, C₄ crops experience more frequent damaging
59 HL or HT stresses in their natural environments than C₃ crops, with reduced C₄ crop yield
60 regularly occurring in warmer regions⁷. As mean global temperatures continue to

61 increase, maize yields are estimated to decrease between 4 and 12% for each
62 temperature increase in degree Celsius⁷. Photosynthesis in maize leaves is inhibited at
63 leaf temperature above 38°C. Recent data from 408 sorghum cultivars shows that
64 breeding efforts over the last few decades have developed high-yielding sorghum
65 cultivars with considerable variability in heat resilience and even the most heat tolerant
66 sorghum cultivars did not offer much resilience to warming temperatures, with a
67 temperature threshold of 33°C, beyond which sorghum yields start to decline⁸. Under
68 natural conditions, especially at the tops of canopies, direct sunlight can be very intense
69 and thus oversaturate the photosynthetic mechanism in C₄ plants. Sorghum leaves had
70 reduced stomatal conductance and net CO₂ assimilation rates after 4 h exposure to HL
71 mimicking nature sunlight⁹. To improve C₄ crop yields, it is crucial to holistically approach
72 how C₄ plants respond to HL or HT, two of the most influential environmental factors that
73 can compromise C₄ photosynthesis.

74

75 HL responses have been studied extensively in C₃ plants^{10–15}. To cope with reactive
76 oxygen species (ROS) production and photooxidative stress resulting from HL, C₃ plants
77 have evolved many protective mechanisms which act on different timescales^{10,14}. Non-
78 photochemical quenching (NPQ), especially its predominant component, energy-
79 dependent quenching (qE), acts within seconds to dissipate excess light energy as
80 heat^{10,16}. The formation of qE depends on the thylakoid lumen pH, the photosystem II
81 (PSII) polypeptide PsbS, and the accumulation of the xanthophyll pigment zeaxanthin^{17–}
82 ¹⁹. In C₃ plants, under HL, violaxanthin is converted to the intermediate pigment
83 antheraxanthin which is then converted to zeaxanthin by the enzyme violaxanthin de-
84 epoxidase²⁰. Accumulation of zeaxanthin is also necessary for induction of a slower-
85 relaxing component of NPQ, zeaxanthin-dependent quenching (qZ)²¹. State transitions,
86 which restructure the light harvesting complexes (LHCs) around PSII and PSI, occur on
87 the order of minutes^{10,16}. When photoprotective processes are insufficient, HL can result
88 in photoinhibition (qI), which takes hours to recover¹⁰. Following HL exposure, expansion
89 of the thylakoid lumen, swelling of the grana margin, and de-stacking of the thylakoid
90 grana facilitate PSII repair by promoting accessibility and repair of PSII machinery^{15,22–24}.

91 HL stress also results in dynamic transcriptional regulation of photosynthetic genes and
92 induces the abscisic acid (ABA) pathway in Arabidopsis¹¹.

93

94 HT is known to affect many cellular processes in C₃ plants, including various aspects of
95 photosynthesis^{25–29}. C₃ plants under HT have shown decreases in photosynthetic rates,
96 inactivation of Rubisco, reduction of plastoquinone (PQ), and increase in cyclic electron
97 flow (CEF) around photosystem I (PSI)³⁰. Arabidopsis leaves treated with HT of 40°C has
98 swollen M chloroplasts and increased plastoglobuli (PG) formation³¹. PG are thylakoid-
99 associated plastid lipoprotein particles whose size, shape, and counts respond to abiotic
100 stresses³². Additionally, HT induces the expression of heat shock transcription factors
101 (HSFs), many of which have been implicated in transcriptional responses to numerous
102 abiotic stresses, including HL and HT³³. The induced HSFs increase the expression of
103 heat shock proteins (HSPs), which are chaperone proteins involved in proper protein
104 folding in response to HT and other abiotic stresses³⁴.

105

106 Unlike C₃ plants, studies on how C₄ plants respond to HL or HT are largely limited,
107 especially the underlying coordination between M and BS cells and the multi-level effects
108 of HL and HT on photosynthesis, transcriptomes, and ultrastructure of C₄ plants. A recent
109 study examined the effects of HL stress in the C₄ grass *Setaria viridis* over four days, with
110 sampling points for photosynthetic parameters, sugar quantification, and transcriptome
111 analyses every 24 hours³⁵. They reported relatively minor transcriptional changes but a
112 large accumulation of sugars without repression of photosynthesis in HL-treated
113 samples³⁵. These results suggest that prolonged HL-treated leaves undergo adaptive
114 acclimation and transcriptional homeostasis in a few days. However, the short-term
115 transcriptional responses of C₄ plants to HL remain largely unknown. In sorghum leaves,
116 HL induced the avoidance response in M chloroplasts and the swelling of BS chloroplasts
117 (by cross section light microscope images), but the underlying mechanisms are unclear⁹.
118 Research about how C₄ photosynthesis responds to HT is mainly limited to biochemical
119 and gas exchange analyses which suggest that HT results in Rubisco activation³⁶, affects
120 the activities of C₄ carbon fixation enzymes³⁷, decreases the BS conductance while
121 increases CO₂ leakiness^{38,39}. Two transcriptome analyses in maize under HT have been

122 reported^{40,41}, but thorough analysis of C₄ transcriptome with multi-level effects under HT
123 is rare. Additionally, ultrastructural analysis in C₄ plants under HL or HT can help us
124 understand how HL or HT limits C₄ photosynthesis and affects the coordination between
125 M and BS cells, but currently such information is lacking.

126

127 To gain deeper insights into the molecular and physiological responses of C₄ plants to HL
128 or HT, we used the green foxtail *Setaria viridis* as a model. *S. viridis* is an excellent model
129 to study C₄ photosynthesis because of its expanding genetics and genomics toolkit,
130 common growth condition, relatively quick generation time (8~10 weeks, seed to seed),
131 and similarity to important agronomic C₄ crops, e.g. maize and sorghum^{2,42,43}. We
132 hypothesized that HL or HT affected C₄ plants at different levels and linking multi-level
133 changes could improve our understanding of HL or HT tolerance in C₄ plants. We
134 investigated the response of *S. viridis* to moderately HL or HT over a four-hour time-
135 course at photosynthetic, ultrastructural, and transcriptomic levels (Fig. 1a). We
136 monitored the dynamic changes of transcriptomes, pigments, and ABA levels over 4 h
137 time points during the different treatments. We also measured photosynthetic parameters
138 and ultrastructural changes after 4 h treatments, which revealed cumulative changes
139 associated with the different treatments.

140

141 Although we observed a comparable reduction of photosynthetic efficiency in HL- or HT-
142 treated leaves, detailed analysis at multiple levels revealed different acclimation
143 strategies to these two stresses in *S. viridis*. The transcriptional changes under HT were
144 much less but more dynamic than under HL. The HL-treated leaves had over-
145 accumulated starch in both M and BS chloroplasts, which may increase chloroplast
146 crowdedness and inhibit PSII repair. While both HL and HT induced PG formation in
147 chloroplasts, HT-treated leaves also had swollen chloroplasts and grana in M cells.
148 Additionally, we observed different responses of M and BS cells under HT or HL and
149 demonstrated the crosstalk between HL response and ABA signaling in C₄ plants. Our
150 research provides a systematic analysis of HL and HT responses in *S. viridis* and
151 identifies potential targets to improve stress tolerance in C₄ crops.

152

153 **Results**

154 **HL or HT caused comparable reduction of photosynthesis and HL also resulted in**
155 **photoinhibition**

156 *S. viridis* leaves treated with 4 h HL (HL_4h) exhibited significantly reduced maximum
157 efficiencies of PSII (F_v/F_m) as compared to that in 4 h control leaves (ctrl_4h) (Fig. 1b),
158 suggesting HL-induced photoinhibition. Net CO₂ assimilation rates (A_{Net}) were
159 significantly reduced in HL or HT-treated leaves in response to changes in light or CO₂
160 (Fig. 1c,d). Pre-treatment control leaves (ctrl_0h) also had lower A_{Net} as compared to
161 ctrl_4h leaves, suggesting circadian regulation of photosynthesis over the course of the
162 day. The comparisons between different treatments at the 4 h time point should exclude
163 the effects of circadian regulation. Stomatal conductance and transpiration rates in
164 response to light were reduced in HL_4h leaves, especially at the beginning of the light
165 response curve (Supplementary Fig. 3a,c). Stomatal conductance and transpiration rate
166 in response to CO₂ were lower in HL_4h or HT_4h leaves than ctrl_4h leaves
167 (Supplementary Fig. 3b,d). PSII efficiency and electron transport rates in light-adapted
168 leaves were reduced in HL_4h leaves as compared to ctrl_4h leaves in response to light
169 (Supplementary Fig. 3e, g).

170
171 To estimate and model a variety of photosynthetic parameters, we assessed various
172 aspects of leaf-level gas exchange measurements based on the light response curves
173 and CO₂ response curves (Supplementary Fig. 4). HL or HT compromised photosynthetic
174 capacities and reduced several photosynthetic parameters in HL_4h and HT_4h leaves
175 compared to ctrl_4h leaves, including gross maximum CO₂ assimilation rates (A_{max}),
176 maximum carboxylation rates (V_{cmax}), and quantum yields of CO₂ assimilation
177 (Supplementary Fig. 4a,b,c). HL_4h leaves had reduced stomatal conductance (g_s) but
178 increased light compensation point as compared to ctrl_4h leaves (Supplementary Fig.
179 4e, g). HT_4h leaves had reduced light saturation point as compared to ctrl_4h leaves
180 (Supplementary Fig. 4h).

181

182 **Transcriptomics revealed important differences in the key pathways responding to**
183 **HL or HT**

184 To investigate the transcriptional patterns that may underlie the photosynthesis
185 phenomena observed above, we performed RNA-seq analysis (Fig. 1a). Principal
186 component analysis (PCA) of transcripts per million (TPM) (Supplementary Data 1)
187 normalized read counts from ctrl, HL, and HT treatments showed the experimental
188 conditions dominated the variance in the dataset (Fig. 2a).

189

190 Next, we compared differentially expressed genes (DEGs) between HL and HT
191 treatments. Genes that were either up- or down-regulated in at least one time point were
192 included in the lists of DEGs for each condition. Utilizing this method, we were able to
193 broadly compare the trends between the HL and HT transcriptomes. There were more
194 DEGs identified in the HL dataset than the HT dataset (Fig. 2b, Supplementary Fig. 5 and
195 Data 2). Significantly more genes were up-regulated in both HL and HT-treated
196 transcriptomes than would be expected by random chance (Fig. 2b, Supplementary Data
197 4). Additionally, significantly more genes were regulated in opposite directions between
198 HL and HT transcriptomes than would be expected by random chance. To visualize how
199 DEGs were conserved between time points within treatments, we plotted the overlaps
200 between up- and down-regulated genes at each time point. In HL-treated samples, 742
201 genes were up-regulated at 1, 2, 4 h time points, representing the largest subset of
202 uniquely overlapping genes and the core HL-induced genes (Fig. 2c, Supplementary Data
203 3). Similarly, 674 genes were down-regulated at all three time points of HL treatment,
204 representing the core HL-reduced genes. Conversely, in the HT-treated samples, the
205 expression pattern was dominated by genes differentially expressed at a single time point
206 (Fig. 2d), indicating the transcriptional response to HT was more transient and dynamic
207 than that to HL. In HT-treated samples, 102 and 72 genes were up- and down-regulated
208 at all three time points, representing the core HT induced and reduced genes,
209 respectively.

210

211 To reveal transcriptional changes that may explain the reduced photosynthesis under HL
212 or HT, we grouped DEGs into several key pathways. Investigation of genes related to the
213 light reaction of photosynthesis revealed that many genes involved in PSII
214 assembly/repair and photoprotection (e.g., *PsbS*), were up-regulated in HL, while many

215 genes relating to LHCII and the core complexes of PSII/PSI were down regulated in HL
216 (Fig. 3a,b). Although HT treatment did not result in the same extent of differential
217 regulation of light reaction related genes as HL, STN7, a kinase involved in state 1 to
218 state 2 transitions⁴⁴ was induced, while TAP38, a phosphatase involved in state 2 to state
219 1 transitions⁴⁵, was repressed in HT-treated leaves (Supplementary Fig. 6a). This
220 suggests a possible heat induced state transition to move the mobile LHCII from PSII
221 (state 1) to PSI (state 2). Additionally, several genes related to the chloroplast NDH
222 (NADPH dehydrogenase) complex were up-regulated in the HT treatment (Fig. 3b).
223 Furthermore, when investigating genes involved in cyclic electron flow (CEF)
224 (Supplementary Fig. 6), we found that key components of CEF, *PGR5* (*proton gradient*
225 *regulation 5*)⁴⁶ and two copies of *PGRL1* (*PGR5-like photosynthetic phenotype 1*)⁴⁷, were
226 induced under HT, suggesting heat-induced CEF around PSI.

227

228 Under HL treatment, the transcriptional changes of genes involved in the Calvin-Benson
229 cycle were less than those involved in the light reactions of photosynthesis (Fig. 3c).
230 Rubisco activase (RCA) is essential for CO₂ fixation by maintaining the active status of
231 Rubisco^{48,49}. The *S. viridis* genome has two adjacent genes encoding RCAs
232 (Supplementary Fig. 7). Protein sequence alignment of the two *S. viridis* RCAs with
233 Arabidopsis RCAs revealed one SvRCA- α which retains the two conserved redox-
234 sensitive cysteine residues as in AtRCA- α , and one SvRCA- β which has higher basal
235 expression (approximately 700-fold higher) than SvRCA- α and possibly the major RCA
236 in *S. viridis*. SvRCA- α was highly induced during the entire 4 h HT (Fig. 3c).

237

238 Key genes involved in photorespiration, e.g. *GOX1* (glycolate oxidase)^{50,51} and *AGT1*
239 (Serine:glyoxylate aminotransferase)⁵² were down-regulated under HL (Fig. 3c). *GOX1*
240 and several other genes involved in photorespiration, *PGLP1* (2-phosphoglycolate
241 phosphatase)⁵³, *HPR1* (hydroxypyruvate reductase)⁵⁴, and *PLGG1* (plastidic
242 glycolate/glycerate transporter)⁵⁵ were induced under HT, suggesting heat-induced
243 photorespiration.

244

245 Some genes important for C₄ carbon metabolism were up-regulated under HL (Fig. 3c),
246 e.g. *PEPC_B* (phosphoenolpyruvate carboxylase) and *NADP-MDH* (NAD-dependent
247 malate dehydrogenase)¹. Carbonic anhydrase⁵⁶ (*CA_A*) was induced under both HL and
248 HT.

249
250 By investigating pathways associated with photosynthesis, we found HL increased the
251 expression of starch biosynthesis/degradation genes and genes encoding PG-localized
252 proteins (Fig. 4a), but down-regulated several genes in the sugar-sensing pathway (Fig.
253 4b), and differentially regulated several sugar transporter genes (Supplementary Fig. 6b).
254 These transcriptional changes were much less pronounced under HT.

255
256 Several HSFs had highly induced expression under either HL or HT, but interestingly,
257 different HSFs were up-regulated in HL vs HT (Fig. 4c). *HSFA6B* was a notable exception,
258 which was induced in both HL and HT. A set of shared HSPs were induced under both
259 HT and HL, but the induction was quicker and stronger under HT than HL, especially the
260 small HSPs, suggesting shared but also temporally distinct transcriptional responses of
261 HSPs under HL and HT.

262
263 We also investigated genes associated with ROS pathways. Specialized ROS
264 scavenging pathways have evolved in plants⁵⁷. We identified genes encoding antioxidant
265 enzymes in *S. viridis* and investigated their expression patterns under HL or HT
266 (Supplementary Fig. 6c). Three gene families of antioxidant enzymes have many
267 members with strong differential expression in HL-treated leaves: TRX (thioredoxin), POX
268 (peroxidases), and GST (glutathione S-transferase). Interestingly, within each of the three
269 antioxidant pathways, some genes were up-regulated while others were down-regulated
270 in HL-treated leaves. A similar pattern was shown in HT-treated leaves, although with
271 fewer differentially regulated genes.

272
273 The reduced stomatal conductance in HL_4h leaves (Supplementary Fig. 3a) suggested
274 there may be changes in ABA pathways and leaf ABA levels. Our RNA-seq analysis
275 showed that several genes in the ABA pathways were up-regulated in response to HL

276 (Fig. 5a). Additional, ABA levels were increased 3-fold in HL_1h leaves followed by a
277 return to baseline by HL_4h (Fig. 5b).

278

279 To distinguish M and BS specific transcriptomic responses and gain more information
280 about how these two specialized cell types function together in HL or HT responses, we
281 investigated the cell type specificity of our pathways of interest (Supplementary Fig. 8,
282 Supplementary data 6) by using previously published M and BS specific transcriptomes
283 under control conditions⁵⁸. We observed several cell-type specific transcriptional
284 responses to HL or HT, e.g. pathways related to ROS-scavenging, sugar transport, and
285 HSPs.

286

287 **HL treatment induced NPQ in *S. viridis***

288 The increased photoinhibition in HL_4h leaves and the increased *PsbS* transcription
289 under HL prompted us to quantify NPQ and xanthophyll pigments. NPQ was significantly
290 higher in HL_4h leaves than ctrl_4h leaves in response to light and CO₂ (Fig. 6a,b). The
291 HL-induced NPQ measured by LI_6800 was confirmed using MultispeQ with the
292 estimated NPQ, NPQ_(T), based on a method that estimates NPQ in light-adapted leaves⁵⁹
293 (Supplementary Fig. 9c). The increased NPQ was also supported by the observed 4-fold
294 increase of zeaxanthin (Fig 6c) during HL. Additionally, HL treatment doubled the
295 intermediate antheraxanthin level (Fig. 6d) and tripled the overall de-epoxidation state of
296 the xanthophyll cycle (Fig. 6e). In Arabidopsis, lutein also has a role in NPQ or qE and
297 can substitute for zeaxanthin in qE formation⁶⁰. Lutein as well as total carotenoids were
298 significantly induced in HL_4h leaves (Supplementary Fig. 10c,d). These results indicate
299 the occurrence of photoprotection in HL-treated leaves. Ctrl and HT treatments had little
300 effect on leaf pigments.

301

302 **HL or HT altered chloroplast ultra-structures**

303 The reduced photosynthesis (Fig. 1c,d) in HL_4h and HT_4h leaves, and the HL-induced
304 photoinhibition (Fig. 1b) and transcripts related to the starch as well as PG pathways (Fig.
305 4a) led us to investigate the ultrastructural changes of the M and BS chloroplasts in
306 ctrl_4h, HL_4h, and HT_4h leaves by using transmission electron microscopy (TEM).

307 TEM images showed HL_4h leaves had increased relative starch volume fraction and
308 chloroplast area in both M and BS chloroplasts, but decreased relative volume fractions
309 of stroma plus stroma lamellae (unstacked thylakoid membranes) in M chloroplasts as
310 compared to ctrl_4h leaves (Fig. 7, Supplementary Fig. 15), suggesting increased starch
311 accumulation and chloroplast crowdedness under HL. Starch quantification using
312 biochemical assays confirmed 3x starch levels in HL_4h leaves as compared to ctrl_4h
313 leaves (Fig. 7m). In HT_4h leaves, M chloroplasts had reduced relative starch volume
314 fraction but increased chloroplast area (Fig. 7, Supplementary Fig. 15). HT did not affect
315 the relative volume of stroma or stroma lamellae in either M or BS chloroplasts.

316

317 Like in other C_4 plants, grana in *S. viridis* are dominantly present in the M chloroplasts.
318 BS chloroplasts also have some grana, which are absent from the central area but present
319 in the peripheral region (Fig. 7d-f). HL reduced grana width in M chloroplasts and the
320 relative volume, height, and area of grana in BS chloroplasts as compared to the ctrl
321 condition (Fig. 7j, Supplementary Fig. 14,15). The HT effects on grana structure were
322 quite different from HL. M chloroplasts at HT had increased relative volume, height, area,
323 and mean layer thickness of grana, indicating heat-induced grana swelling. However, in
324 BS chloroplasts, HT decreased the relative volume, width, and area of grana, suggesting
325 that HT affected grana structure differently in M and BS chloroplasts.

326

327 HL increased PG count and the total PG area per chloroplast, while it decreased the mean
328 individual PG size in M chloroplasts, indicating smaller but more numerous PGs in M
329 chloroplasts (Fig. 7k, l, Supplementary Fig. 13). Furthermore, HL increased individual PG
330 size and total PG area per chloroplast in BS chloroplasts (Supplementary Fig. 13). HT
331 increased individual PG size and total PG area, suggesting heat-induced PG formation in
332 both M and BS chloroplasts.

333

334 **HL- and HT-treated Leaves had reduced photosynthetic capacity**

335 The over-accumulated starch in HL_4h leaves (Fig. 7) and the increased leaf ABA levels
336 (Fig. 5) led us to investigate photosynthesis under the simulated stress conditions and
337 immediately after different treatments without dark-adaptation in the LI_6800 leaf

338 chamber (Fig. 8). Under the same temperature and light intensity in the LI-6800 leaf
339 chamber, most photosynthetic parameters with or without dark-adaptation were similar
340 (groups 1 vs. 2) (Fig. 8). Under the simulated treatment condition in the LI-6800 leaf
341 chamber (group 3), HL_4h leaves had higher net CO₂ assimilation rates (A_{Net}) and
342 stomatal conductance under 600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ light than ctrl_4h leaves under 200
343 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ light, but both parameters in HL_4h leaves were lower than ctrl_4h
344 leaves under the same light intensity (group 3, 4) (Fig. 8a). This suggests that HL_4h
345 leaves had reduced capacities for A_{Net} and stomatal conductance as compared ctrl_4h
346 leaves under the same condition. Under the simulated treatment condition (group 3),
347 HL_4h leaves under 600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ light had reduced PSII operating efficiency
348 (Fig. 8c), increased electron transport rates (Fig. 8d), and increased NPQ (Fig. 8f) as
349 compared to the ctrl_4h leaves under 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ light, consistent with light
350 induced electron transport and NPQ.

351
352 Without dark-adaptation, HT_4h leaves had similar A_{Net} as ctrl_4h leaves (Fig. 8a, group
353 2). This may be due to the transient recovery of photosynthesis after switching the HT_4h
354 leaves from 40°C in the growth chamber to 25°C in the LI_6800 leaf chamber for
355 measurements. Under the same light intensity, HT_4h leaves had significantly lower A_{Net}
356 (Fig. 8a) and more reduced plastoquinone (Fig. 8e) than ctrl_4h leaves. Under the
357 simulated treatment condition in LI-6800 leaf chamber (group 3), HT_4h leaves had
358 increased stomatal conductance (Fig. 8b) but reduced A_{Net} as compared to ctrl_4h leaves
359 (Fig. 8a), consistent with transpiration cooling of leaf temperature (Supplementary Fig. 1)
360 and reduced photosynthetic capacity in HT-treated leaves (Supplementary Fig. 4a-c).

361

362 **The activity of ATP synthase was inhibited in HL-treated leaves**

363 Based on the HL-induced starch accumulation, we hypothesized that starch may inhibit
364 photosynthesis through feedback regulation. We measured electrochromic shift (ECS)
365 and chlorophyll fluorescence using MultispeQ⁶¹ to evaluate proton fluxes and the
366 transthylakoid proton motive force (pmf) *in vivo*⁶²⁻⁶⁴. Different treatments did not
367 significantly change pmf (Fig. 9a). HL_4h leaves had significantly reduced proton
368 conductivity and lower proton flux rates as compared to ctrl_4h leaves (Fig. 9b,c),

369 indicating reduced ATP synthase activity in HL-treated leaves. The MultispeQ NPQ_(T) data
370 showed that the HL-induced NPQ was more sensitive to *pmf* than ctrl_4h leaves, with
371 higher NPQ produced at a given level of proton motive force in HL_4h leaves than ctrl_4h
372 leaves (Fig. 9d).

373

374 **Discussion**

375 We investigated how the C₄ model plant *S. viridis* responds to HL or HT stresses at
376 multiple levels by employing diverse approaches (Fig. 1a). Our data provide a thorough
377 analysis of HL and HT responses in *S. viridis* at photosynthetic, transcriptomic, and
378 ultrastructural levels and reveal limitations of photosynthesis under HL or HT. The HL
379 (900 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) and HT (40°C) treatments we chose were both moderate
380 stresses within the physiological range for *S. viridis*. Although the impact of moderate
381 stresses can be difficult to analyze due to mild phenotypes, moderate stresses are highly
382 relevant and occur frequently in the field⁶⁵. Understanding the impacts of moderate
383 stresses on C₄ plants is imperative for agricultural research. The moderately HL and HT
384 we used reduced net CO₂ assimilation rates at comparable levels in *S. viridis* leaves (Fig.
385 1c), but via different mechanisms (Fig. 10).

386

387 **Starch over-accumulation may contribute to photoinhibition in HL-treated leaves**

388 In response to HL, *S. viridis* induced NPQ to dissipate excess light energy via increased
389 *PsbS* transcription and zeaxanthin accumulation (Fig. 3a, 6c). At the transcriptional level,
390 HL-treated plants up-regulated transcripts involved in PSII assembly/repair and
391 photoprotection before down-regulating transcripts involved in LHCII, PSII core complex,
392 and PSI complex (Fig. 3), suggesting a strategy to dissipate light and repair damaged
393 PSII before the remodeling of photosystems. With the rapid induction of photoprotective
394 pathways, it was initially surprising to see the significant amount of photoinhibition in HL-
395 treated leaves of *S. viridis* (Fig. 1b), but the HL-induced starch accumulation may provide
396 some insight.

397

398 Our TEM data showed that the mean relative starch volume fraction was increased
399 significantly in both M and BS chloroplasts in HL_4h leaves as compared to ctrl_4h leaves

400 (Fig. 7, Supplementary Fig. 15). The increased starch accumulation likely resulted from
401 increased CO₂ fixation rates (Fig. 8a) but imbalance of starch synthesis/ degradation and
402 sugar transport from downstream pathways under HL. In C₃ plants, starch is mostly
403 present in M chloroplasts where photosynthesis occurs^{66,67}. In C₄ plants, starch is present
404 in both BS and M chloroplasts (Fig. 7a-f), although Rubisco dominantly localizes in the
405 BS chloroplasts⁶⁷. The over-accumulated starch increased the crowdedness of the
406 chloroplasts (Fig. 7, Supplementary Fig. 15), which may hinder PSII repair, especially in
407 M chloroplasts where PSII is enriched. PSII complexes are concentrated in the stacked
408 grana regions; during PSII repair, damaged PSII subunits migrate from the stacked grana
409 region to the grana margin and the unstacked grana region (stroma lamellae) where the
410 proteins involved in PSII repair are localized (e.g., FtsH, Deg proteases that degrade
411 damaged PSII subunits)^{15,68}. In Arabidopsis under HL, the grana lumen and margin swell
412 to facilitate protein diffusion and PSII repair^{23,69}, however, we did not see these changes
413 in HL-treated *S. viridis* leaves (Supplementary Fig. 12d, e, i). Starch overaccumulation
414 and increased chloroplast crowdedness may slow down the migration of damaged PSII
415 subunits and inhibit PSII repair, contributing to the HL-induced photoinhibition (Fig. 1b,
416 10). Additionally, ATP synthase activity was significantly reduced in HL_4h leaves as
417 compared to ctrl_4h leaves (Fig. 9b,c), which may be associated with the starch
418 accumulation and sugar feedback inhibition of photosynthesis. HL-treated Arabidopsis
419 plants had reduced starch in chloroplasts⁷⁰, which may reflect the differences in
420 experimental conditions or the stronger capability to use HL for carbon fixation in C₄ plants
421 than C₃ plants.

422

423 **HL differentially regulated genes involved in sugar-sensing pathways**

424 Sugar signaling integrates sugar production with environmental cues to regulate
425 photosynthesis^{35,71,72}. In C₃ plants, some of the sugar-sensing pathways include: (1)
426 SnRK1 pathway (sucrose-non-fermenting 1 related protein kinase 1, starvation sensor,
427 active under stressful and sugar deprivation conditions to suppress growth and promote
428 survival)⁷³⁻⁷⁵; (2) Trehalose pathway (trehalose is a signal metabolite in plants under
429 abiotic stresses and helps plants survive stresses)^{65,76}. Sugar sensing pathways under
430 abiotic stresses are underexplored in C₄ plants³⁵. Our RNA-seq data showed that two

431 subunits of SnRK1 ($\beta 2$, $\gamma 4$) were highly down-regulated under HL (Fig. 4b), suggesting
432 possible inhibition of the SnRK1 pathway. In the trehalose pathway, trehalose-6-
433 phosphate synthase (TPS) produces trehalose-6-phosphate (T6P); the T6P phosphatase
434 (TPP) dephosphorylates T6P to generate trehalose⁶⁵. A copy of the potential catalytically
435 active *TPS* (*TPSI*) in *S. viridis* was induced and two copies of *TTP* were down-regulated
436 during HL (Fig. 4b), suggesting possible increased level of T6P. T6P is a signal of sucrose
437 availability, inhibits SnRK1 pathway, promotes plant growth and development^{77,78}. Based
438 on the expression pattern of genes involved in sugar-sensing pathways and the over-
439 accumulated starch under HL, we postulated that HL-treated *S. viridis* leaves had
440 increased sugar levels, and possibly up-regulated T6P sugar-sensing pathway to down-
441 regulate the SnRK1 pathway and promote plant growth^{76,79}, which may alleviate the
442 stress of starch over-accumulation and photosynthesis inhibition under HL.

443

444 **Potential links between HL response and ABA pathway exist in *S. viridis***

445 The links between HL responses and ABA have been reported in C₃ plants^{11,12,80,81}.
446 Arabidopsis ABA biosynthesis mutants (e.g., *nced3*) were more sensitive to HL than
447 WT^{11,12}. HL-treated *S. viridis* leaves had reduced capacity for stomatal conductance (Fig.
448 8b), which can most likely be attributed to an acute increase of ABA levels in HL-treated
449 leaves (Fig. 5b). Although ABA levels were only significantly increased at HL_1h and then
450 gradually decreased, the ABA-induced stomatal closure may be prolonged. Consistent
451 with this, RNA-seq data showed increased expression of genes involved in ABA
452 responses and signaling during the 4-h HL treatment (Fig. 5a). Stomatal conductance
453 increases with light to increase CO₂ uptake, which also increases water loss. To reduce
454 water loss and improve water use efficiency, a relatively lower stomatal conductance
455 under HL may be an adaptive response. Our results in *S. viridis* provide insight into the
456 reduced stomatal conductance and photosynthesis in sorghum leaves under HL⁹.

457

458 ABA homeostasis is maintained by the balance of its biosynthesis, catabolism,
459 reversible glycosylation, and transport pathways¹⁹. Several ABA biosynthesis genes were
460 up-regulated during HL (Fig. 5a), including *NCED1* (9-cis epoxy-carotenoid
461 dioxygenase)^{19,82,83} and *ABA1/ZEP1*, suggesting that local, *de novo* ABA biosynthesis

462 may be one source of the rapid and large induction of ABA at HL_1h. The up-regulation
463 of *CYP707As*, which are responsible for ABA degradation⁸⁴, may contribute to the gradual
464 reduction of ABA levels after 1 h HL. Furthermore, the *S. viridis* homolog of Arabidopsis
465 BG1 (glucosidases, hydrolyze inactive ABA-GE to active ABA in endoplasmic reticulum)⁸⁵
466 was induced at HL_2h and HL_4h. Dehydration rapidly induces polymerization of AtBG1
467 and a 4-fold increase in its enzymatic activity⁸⁵. It is possible that the hydrolysis of ABA-
468 GE to ABA by polymerized BG1 may precede the induction of the BG1 transcript,
469 contributing to the transiently increased ABA levels. Several putative ABA transporters
470 were not differentially expressed (Supplementary Data 6), but a *S. viridis* homolog of the
471 Arabidopsis ABA importer *ABCG40* was down-regulated in HL (Fig. 5a), suggesting ABA
472 import from other parts of the plant to leaves may be less likely. Thus, the HL increased
473 ABA level may be due to ABA *de novo* biosynthesis and/or reversible glycosylation from
474 ABA-GE to ABA.

475

476 **HT responses had distinct features in comparison to HL**

477 Compared to HL, HT_4h leaves showed much less change in starch accumulation, little
478 change in chloroplast crowdedness (Fig. 7), and no photoinhibition (Fig. 1). Under HT, M
479 chloroplasts had reduced relative starch volume fraction but increased chloroplast area
480 as compared to ctrl (Supplementary Fig. 13), suggesting heat-induced chloroplast
481 swelling that is independent of starch accumulation. Grana dimension increased in HT-
482 treated M chloroplasts (Supplementary Fig. 13), suggesting heat-induced grana swelling.
483 In contrast, BS chloroplasts have slightly increased starch, no change of chloroplast area,
484 but decreased grana dimension under HT, suggesting cell-type specific heat responses.
485 PG formation was highly induced in both M and BS chloroplasts under HT, which may be
486 associated with heat-increased thylakoid membrane leakiness, consistent with previous
487 reports^{26,86,87}. Induced chloroplast/grana swelling and PG formation may reflect heat-
488 induced damage to chloroplast ultrastructure, which may contribute to the reduced
489 photosynthetic rates under HT.

490

491 The transcriptome changes under HT were less extensive but more dynamic than under
492 HL (Fig. 2-5). HT induced more PG formation than HL (Supplementary Fig. 13), however,

493 surprisingly there were few transcriptional changes of genes encoding proteins that
494 localize to PG under HT (Fig. 4a). These results suggest the heat-induced PG formation
495 may be a direct and physical response of thylakoid membranes to moderately HT and not
496 regulated at the transcriptional level.

497

498 Response to HT also showed some unique transcriptional changes that were absent or
499 minimal under HL. First, HT resulted in high and sustained induction of Rubisco activase
500 (*RCA_α*) (Fig. 3c). RCA removes inhibitors from Rubisco, maintains Rubisco activation,
501 and is important for carbon fixation^{48,49}. Rubisco is thermostable but RCAs are heat labile,
502 resulting in reduced Rubisco activation and CO₂ fixation under HT³⁶. Plants grown in
503 warm environments usually have RCAs that are more thermotolerant⁸⁸⁻⁹⁰. In *S. viridis*,
504 maize, and sorghum, HT induces the protein level of *RCA_α* and the rate of *RCA_α*
505 induction is associated with the recovery rate of Rubisco activation and photosynthesis⁹¹,
506 suggesting the heat-induced *RCA_α* may be the thermotolerant isoform. Understanding
507 the function and regulation of RCAs may help improve thermotolerance of photosynthesis
508 in C₄ plants. Additionally, HT upregulated small *HSPs* much quicker than HL.

509

510 Key genes involved in photorespiration (Fig. 3c) and cyclic electron flow (CEF) around
511 PSI (Supplementary Fig. 6a) were up-regulated under HT, suggesting HT-induced
512 photorespiration and CEF. C₄ plants employ carbon-concentrating mechanisms (CCM) to
513 concentrate CO₂ around Rubisco and reduce photorespiration in the BS chloroplasts.
514 However, *S. viridis* BS chloroplasts have a small number of grana (Fig. 7d-f), where PSII
515 is present and can be a source of O₂ production. Photorespiration increases with
516 temperature faster than photosynthesis^{30,92} and HT may also increase the CO₂ leakiness
517 of BS chloroplasts^{38,39}, promoting photorespiration and reducing photosynthesis. CEF
518 generates only ATP without NADPH, balances ATP/NADPH ratio, generates
519 transthylakoid proton motive force (*pmf*), and protects both PSI and PSII from photo-
520 oxidative damage in C₃ plants^{93,94}. Increased CEF activity has been frequently reported
521 under stressful conditions in C₃ plants^{26,95,96}, indicating its important role in stress
522 protection. To compensate for the extra ATP needed for CCM, C₄ plants are proposed to
523 have high CEF in BS chloroplasts^{3,97}. CEF is reported to increase in *S. viridis* under salt

524 stress⁹⁸. The heat-induced CEF could protect photosynthesis under HT by maintaining
525 transthylakoid *pmf* and generating extra ATP.

526

527 Frey et al. identified 39 heat-tolerance genes in maize that were significantly associated
528 with heat-tolerance and up-regulated in most of the 8 maize inbred lines⁴¹. Five *S. viridis*
529 homologs of the maize heat-tolerance genes were also up-regulated in our RNA-seq data
530 under HT, providing potential engineering targets to improve heat tolerance in C₄ plants
531 (Supplementary Data 5).

532

533 Although HL and HT responses had their own unique features, their transcriptional
534 responses had significant overlaps (Fig. 2b). We identified 42 highly induced genes (FC
535 ≥ 5) and 13 highly repressed genes (FC ≤ -5) in both conditions (Supplementary Fig. 5,
536 Supplementary Data 5). The 42 highly induced genes provide potential targets for
537 improving resistance to HL and HT in C₄ crops, including several putative transcription
538 factors, HSP20/70/90 family proteins, β -amylase, and a putative aquaporin transporter for
539 promoting CO₂ conductivity in C₄ plants^{3,99,100}. Additionally, *HSFA6B* was induced ($2 \leq$ FC
540 ≤ 5) at both HL and HT. It is reported that *HSFA6B* operates as a downstream regulator
541 of the ABA-mediated stress response and is involved in thermotolerance in Arabidopsis,
542 wheat, and barley^{101,102}. This gene may be involved in regulation of genes that are
543 common to both the HL and HT responses and it would be interesting for further study to
544 generate HL and HT tolerant C₄ crops.

545

546 In comparison to the C₃ model plant Arabidopsis, the C₄ model plant *S. viridis* has shared
547 and unique responses under HL and HT. The shared responses include induced NPQ,
548 *PsbS* transcription, zeaxanthin accumulation, PG formation, and ABA levels under HL,
549 and the induced PG formation as well as swollen M chloroplasts under HT. The unique
550 responses in *S. viridis* to HL include the over-accumulated starch in both M and BS
551 chloroplasts and increased chloroplast crowdedness. In HT, the unique responses in *S.*
552 *viridis* include dynamic transcriptome regulation and different heat sensitivities of M and
553 BS chloroplasts. The reduced photosynthetic capacity under HL or HT also demonstrated
554 the need to increase the tolerance to these two stresses in C₄ plants.

555 The different responses in M and BS chloroplasts in *S. viridis* are particularly interesting
556 and warrant further study. We sorted HL or HT induced DEGs into M and BS specific
557 pathways based on previously published M/BS transcriptomes⁵⁸ (Supplementary Fig. 8).
558 Although we cannot rule out some transcripts may have altered cell type specificity under
559 stressful conditions, due to the function specificity of the M and BS cells, a significant
560 fraction of the M and BS specific transcripts likely keep similar cell type specificity under
561 our HL and HT conditions as compared to the published control condition. Our analysis
562 revealed M- and BS-specific transcriptional regulation in response to HL or HT in *S. viridis*
563 (Supplementary Fig. 8). Under HL, the majority of M-specific DEGs related to ROS-
564 scavenging and HSPs were up-regulated while the majority of BS-specific DEGs related
565 to these two pathways were down-regulated, suggesting M cells may require more ROS
566 scavenging and HSPs than BS cells in response to HL, probably due to more ROS
567 production and higher need for maintaining protein homeostasis in M cells than BS cells
568 under HL. In contrast, under HT, many ROS-scavenging DEGs were up-regulated in BS
569 cells but down-regulated in M cells (possibly due to heat-induced photorespiration) while
570 DEGs related to HSPs were up-regulated in both cell types. It is intriguing that HL up-
571 regulated M-specific sugar transporters but down-regulated BS-specific sugar
572 transporters. In Arabidopsis, SWEET16/17 plays a key role in facilitating bidirectional
573 sugar transport along sugar gradient across the tonoplast of vacuoles^{103,104}. The
574 homologous copy of SWEET16/17 in *S. viridis* is M-cell specific and was up-regulated in
575 HL (Supplementary Fig. 6b), suggesting SvSWEET16/17 may mediate sugar uptake into
576 vacuoles in response to a high concentration of cytosolic sugar level in M cells. The down-
577 regulation of BS-specific SWEETs under HL may indicate feedback inhibition of sugar
578 phloem loading due to unmatched sugar usage in downstream processes¹⁰⁵.

579

580 In summary, we elucidated how the C₄ model plant *S. viridis* responds to moderately HL
581 or HT at the photosynthetic, transcriptomic, and ultrastructural levels (Supplementary Fig.
582 14). Our research furthers understanding of how C₄ plants respond to HL and HT by
583 linking the data from multiple levels, reveals different acclimation strategies to these two
584 stresses in C₄ plants, discovers unique HL/HT responses in C₄ plants in comparison to
585 C₃, demonstrates M/BS cell type specificity under HL or HT, distinguishes adaptive from

586 maladaptive responses, and identifies potential targets to improve abiotic stress tolerance
587 in C₄ crops.

588

589 **Methods**

590 **Plant growth conditions and treatments**

591 *S. viridis* ME034 (also known as ME034v) plants were grown in a controlled
592 environmental chamber under constant 31°C, 50% humidity, ambient CO₂ conditions, 12
593 h photoperiod, and leaf level light intensity of 250 μmol photons m⁻² s⁻¹. Similar level of
594 growth light has been used in literatures for *S. viridis* under control conditions^{58,98,106}.
595 Seeds were germinated on Jolly Gardener C/V Growing Mix (BGF Supply Company,
596 Oldcastle, OCL50050041) and fertilized with Jack's 15-5-15 (BGF Supply Company, J.R.
597 Peters Inc., JRP77940) with an Electrical Conductivity (EC) of 1.4. At seven days after
598 sowing (DAS), seedlings were transplanted to 3.14" x 3.18" x 3.27" pots. At 13-DAS, 4 h
599 after light was on in the growth chamber, plants with fourth fully expanded true leaves
600 were selected for 4 h HL (leaf level light intensity of 900 μmol photons m⁻² s⁻¹ and chamber
601 temperature of 31°C) or 4 h HT (chamber temperature of 40°C and leaf level light intensity
602 of 250 μmol photons m⁻² s⁻¹) treatments carried out in separate controlled environmental
603 chambers under 50% humidity and ambient CO₂ conditions. A separate set of plants
604 remained in the control chamber set to growth conditions. Leaf temperature was stable
605 at 31°C under control and HL treatments while it increased gradually from 31°C to 37°C
606 by the end of 4 h treatment of 40°C (Supplementary Fig. 1).

607

608 **Gas-exchange and chlorophyll fluorescence measurements**

609 Leaf-level gas exchange and pulsed amplitude modulated (PAM) chlorophyll *a*
610 fluorescence was measured using a portable gas-exchange system LI-6800 coupled with
611 a Fluorometer head 6800-01 A (LI-COR Biosciences, Lincoln, NE). Fourth fully expanded
612 true leaves of *S. viridis* plants from different treatments were first dark-adapted for 20 min
613 in the LI-6800 chamber to measure maximum PSII efficiency (F_v/F_m) under constant CO₂
614 partial pressure of 400 ppm in the sample cell, leaf temperature 25°C, leaf VPD 1.5 kPa,
615 fan speed 10,000 RPM, and flow rate 500 μmol s⁻¹. We then performed the light response
616 curves followed by CO₂ response curves (A/C_i curve) as described (Supplementary Fig.

617 2). Red-blue actinic light (90%/10%) and 3-6 biological replicates for each treatment were
618 used for all measurements. We used leaf temperature of 25°C for light and CO₂ response
619 curves as described in previous publications for *S. viridis* regardless of growth
620 temperatures^{56,98,107–109}. During all measurements, the instrument parameters were
621 consistent and stable. For CO₂ response curves, all net CO₂ assimilation rates were
622 corrected with the empty chamber data to count for inevitable and minor LI-6800 leaf
623 chamber leakiness during the CO₂ response curves following the established
624 protocols¹¹⁰.

625

626 Photosynthetic parameters were calculated as described⁶² (see formulas, Supplementary
627 Table 1). To estimate the true NPQ, F_m used in the NPQ formula ($F_m / F_m' - 1$) needs to
628 be the maximum chlorophyll fluorescence in fully relaxed, dark-adapted leaves in which
629 there is no quenching^{62,111}. F_m and F_m' are the maximum chlorophyll fluorescence yields
630 in dark-adapted and light-adapted leaves, respectively^{62,111,112}. In ctrl leaves, F_m could be
631 reached with 20 min dark-adaptation without further change after that, but HL-treated
632 leaves needed a much longer recovery period to relax the quenching processes due to
633 the light-induced photoinhibition (Supplementary Fig. 9a). Because the values of F_m in
634 dark-adapted ctrl_4h leaves were highly consistent among different biological replicates
635 and reflected the reference level of F_m (*i.e.*, without stress treatments), we used the mean
636 F_m of ctrl_4h leaves as a baseline to calculate NPQ in leaves with different treatments.

637

638 To investigate photosynthetic performance in plants immediately following 4 h of different
639 treatments (ctrl, HL or HT), we also performed short LI-6800 measurements for 5 min on
640 each plant immediately after 4 h treatments without dark-adaptation at 400 ppm CO₂ with
641 indicated leaf temperatures and light intensities (Fig. 8). To estimate photosynthetic
642 parameters under different treatments as in the growth chambers, the LI-6800 leaf
643 chamber was set to simulate the condition of different treatments: ctrl (31°C, 200 μmol
644 photons $\text{m}^{-2} \text{s}^{-1}$ light), HL (31°C, 600 μmol photons $\text{m}^{-2} \text{s}^{-1}$ light,) or HT (40°C, 200 μmol
645 photons $\text{m}^{-2} \text{s}^{-1}$ light). The temperature and light refer to the conditions in the LI-6800 leaf
646 chamber. The light in LI-6800 leaf chamber (90% red and 10% blue) was different from
647 the white light in the growth chamber, therefore we selected two lights in the LI-6800 leaf

648 chamber that were close to the white lights in growth chambers based on the light
649 quantification in the red (580-670 nm) and blue (440-540 nm) spectrum range. LI-6800
650 light intensities of 200 and 600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ were also two of the conditions used
651 in the light response curves with dark-adaptation (Fig. 1c, 8, group 1), allowing for direct
652 comparison. Individual plants were used for each measurement and replicate.

653

654 The high abundance of PSI in BS chloroplasts of C_4 leaves can affect chlorophyll
655 fluorescence measurement (up to 50%) and underestimate the PSII efficiency (F_v/F_m) and
656 electron transport rates^{113,114}. Thus, our chlorophyll fluorescence data were corrected with
657 $0.5 F_o$, which is the mean minimal chlorophyll fluorescence in dark-adapted leaves under
658 the control condition (ctrl_4h). The PSII operating efficiency calculated from the corrected
659 and uncorrected chlorophyll fluorescence data correlated with each other but the
660 corrected data yielded higher PSII efficiency, with the maximum PSII efficiency in ctrl_4h
661 leaves closer to the theoretical values of 0.86¹¹⁵ (Fig. 1b).

662

663 **Modeling of photosynthetic parameters using leaf-level gas exchange information**

664 To model photosynthetic parameters, we used gas exchange data from light response
665 curves and CO_2 response curves (A/c_i curves). The model parameterization and analyses
666 were conducted in R 3.4.3 Project software® (R Development Core Team 2016). First,
667 light response curves were fitted as previously described¹¹⁶. We fit a non-linear least
668 squares regression (non-rectangular hyperbola) to estimate photosynthetic parameters
669 (Supplementary Fig. 4). A/c_i curves were fitted as previously described¹¹⁷ to estimate the
670 V_{cmax} (the maximum rate of carboxylation). Feng et al. (2013) followed the C_4
671 photosynthesis model using a Bayesian analysis approach¹¹⁸. The normality of the data
672 was verified with the Shapiro-Wilk test. Statistical analysis was performed using Student's
673 two-tailed t-test with unequal variance by comparing ctrl_4h with all other conditions.

674

675 **RNA isolation**

676 To isolate RNA from leaves, four biological replicates containing two 2-cm mid-leaf
677 segments from two plants for each time point and treatment were collected from fourth
678 fully expanded true leaves into screw cap tubes (USA Scientific, 1420-9700) with a

679 grinding bead (Advanced Materials, 4039GM-S050) and immediately frozen in liquid
680 nitrogen and stored at -80°C. Frozen samples were homogenized using a paint shaker.
681 RNA was extracted using a Trizol method with all centrifugation at 4°C and 11,000 RCF.
682 One mL of Trizol Reagent (Invitrogen, 15596018) was added to homogenized leaf tissue
683 and resuspended, then 200 µL of Chloroform:Isoamyl alcohol (25:1) was added and
684 vortexed. Tubes were centrifuged for 15 min. 600 µL from the aqueous layer was
685 transferred to a clean tube with equal volume Chloroform:Isoamyl alcohol, vortexed, and
686 centrifuged for 5 min. Next, 450 µL of aqueous layer was transferred to 0.7x volume 100%
687 Isopropanol, mixed well, and chilled for 30 min in -20°C freezer. Samples were centrifuged
688 for 15 min to pellet RNA. Supernatant was decanted, and RNA pellet was rinsed twice
689 with ice-cold 75% ethanol with a 2-min centrifugation following each rinse. RNA was dried
690 in a laminar flow hood until residual ethanol evaporated and was resuspended in 50 µL
691 of nuclease free H₂O. RNA was quantified using a NanoDrop and Qubit RNA Broad
692 Range (BR) Assay Kit (Thermo Fisher Scientific Inc., Q10210) with the Qubit 3.0 machine.
693 RNA integrity was verified using a Bioanalyzer Nano Assay (Genome Technology Access
694 Center, Washington University in St. Louis).

695

696 **RNA-seq library construction and sequencing**

697 RNA samples were diluted to 200 ng/µL in nuclease free H₂O for a total of 1 µg RNA.
698 Libraries were generated with the Quantseq 3' mRNA-seq library prep kit FWD for Illumina
699 (Lexogen, 015.96). Libraries were generated according to manufacturer's instructions.
700 Cycle count for library amplification for 1 µg mRNA was tested using the PCR add-on kit
701 for Illumina (Lexogen, 020.96). qPCR was performed and a cycle count of 13 was
702 determined for the amplification of all libraries. For library amplification, the Lexogen i5 6
703 nt Dual Indexing Add-on Kit (5001-5004) (Lexogen, 047.4x96) was used in addition to the
704 standard kit to allow all libraries to have a unique combination of i5 and i7 indices. All
705 libraries were quantified using Qubit dsDNA High Sensitivity (HS) Assay Kit (Thermo
706 Fisher Scientific Inc., Q32854) with the Qubit 3.0 machine. Prepared libraries were pooled
707 to equimolar concentrations based on Qubit assay reads. Pooled libraries were submitted
708 to Novogene to be sequenced on the HiSeq4000 platform (Illumina) with paired end, 150
709 bp reads.

710

711 **Mapping and transcript quantification**

712 Single-end reads were trimmed and quality-checked using Trim Galore (version 0.6.2).
713 Trimmed reads from each library were mapped and processed for transcript quantification
714 using Salmon (version 1.1.0) in quasi-mapping mode with a transcriptome index built from
715 the *S. viridis* transcript and genome files (Sviridis_311_v2; Phytozome v12.1)⁴². Salmon
716 outputs were imported into R using the Bioconductor package tximport (1.16.0) to extract
717 gene-level expression values represented by transcript per million (TPM) for each gene
718 across every time point, tissue, and treatment group sampled. Principal component
719 analysis was performed with TPM normalized read counts of all genes using the R
720 package FactoMineR¹¹⁹.

721

722 **Differential expression analysis**

723 Genes that met minimum read count cutoffs of at least 10 raw reads in at least 10% of
724 samples (14,302 genes) were included in differential expression analysis using DeSeq2,
725 FDR < 0.05¹²⁰. HL or HT treatment time points were compared to the control condition
726 from the same time point. Differentially expressed genes between different time points in
727 either HL or HT were visualized in UpSetR¹²¹. To identify genes in key pathways of
728 interest in *S. viridis*, we used the MapMan annotations for the closely related *S. italica*
729 ([RRID:SCR_003543](https://www.ebi.ac.uk/ENSRNOG/003543)). From the *S. italica* MapMan annotations, we identified the best hit
730 in *S. viridis* for genes in pathways of interest. We then manually curated these lists based
731 on relevant literature to obtain genes in pathways of interest (Supplementary Data 6), as
732 well as to provide further annotation information for genes identified using the MapMan
733 annotations. We sorted the differentially expressed genes in pathways of interest into fold
734 change (FC) bins based on their DeSeq2 fold change values and presented their
735 expression patterns. FC bins were defined as follows: highly induced: FC ≥ 5; moderately
736 induced: 5 > FC ≥ 2; slightly induced: 2 > FC > 0; not differentially expressed: FC = 0;
737 slightly repressed: 0 > FC > -2; moderately repressed: -2 ≥ FC > -5; highly repressed: FC
738 ≤ -5. Heatmaps of pathways of interest were generated using the R package pheatmap
739 (version 1.0.12. <https://CRAN.R-project.org/package=pheatmap>).

740

741 **ABA quantification**

742 Leaf samples of three biological replicates were harvested at 0, 1, 2 and 4 hours of ctrl,
743 HL, or HT treatment. The fresh leaf weight was immediately measured after harvesting.
744 The samples were quickly placed in liquid nitrogen and then stored in -80°C freezer until
745 further processing. Frozen leaf tissue was homogenized and 15 ng of [²H₆]-abscisic acid
746 was added as an internal standard. Samples were dried to completeness under vacuum.
747 ABA was resuspended in 200µl of 2% acetic acid in water (v/v) and then centrifuged; an
748 aliquot was then taken for quantification. Foliar ABA levels were quantified by liquid
749 chromatography tandem mass spectrometry with an added internal standard using an
750 Agilent 6400 Series Triple Quadrupole liquid chromatograph associated with a tandem
751 mass spectrometer according to the previously described methods¹²².

752

753 **Pigment analysis**

754 Three biological replicates of one 2-cm middle leaf segment were collected from fourth
755 fully expanded true leaves into screw cap tubes (USA Scientific, 1420-9700) with a
756 grinding bead (Advanced Materials, 4039GM-S050), immediately frozen in liquid
757 nitrogen, and stored at -80°C. During pigment extraction, 600 µl ice-cold acetone were
758 added to the samples before they were homogenized in a FastPrep-24 5G (MP
759 Biomedicals) at 6.5 m/s for 30 s at room temperature. Cell debris were removed by
760 centrifugation at 21,000 x g for 1 min. The supernatant was filtered through a 4 mm nylon
761 glass syringe prefilter with 0.45 µm pore size (Thermo Scientific) and analyzed by HPLC.
762 HPLC analyses were performed on an Agilent 1100 separation module equipped with a
763 G1315B diode array and a G1231A fluorescence detector; data were collected and
764 analyzed using Agilent LC Open Lab ChemStation software. Pigment extracts were
765 separated on a ProntoSIL 200-5 C30, 5.0 µm, 250 mm by 4.6 mm column equipped with
766 a ProntoSIL 200-5-C30, 5.0 µm, 20 mm by 4.0 mm guard column (Bischoff
767 Analysentechnik) and gradient conditions as previously described¹²³. Assuming
768 interconversion of the intermediate antheraxanthin between both zeaxanthin and
769 violaxanthin, the de-epoxidation level can be calculated by (zeaxanthin + 0.5
770 antheraxanthin) / (violaxanthin + antheraxanthin + zeaxanthin)¹²⁴.

771

772 **Transmission electron microscopy (TEM)**

773 *S. viridis* leaves were collected after 4 h of different treatments and prepared for TEM.
774 Four-millimeter biopsy punches were taken from the middle leaf segments of the fourth
775 fully expanded leaves and fixed for 2 h in 2% paraformaldehyde and 2% glutaraldehyde
776 (EM Science, Hatfield, PA, USA) plus 0.1% Tween20 in 0.1 M sodium cacodylate at pH
777 7.4 at room temp and then at 4°C overnight. Samples were then rinsed 3x in buffer and
778 fixed in 2% osmium tetroxide (EM Science, Hatfield, PA, USA) in ELGA water for 2 h,
779 rinsed 3x in ELGA water and placed in 1% uranyl acetate in ELGA water at 4°C overnight
780 and then at 50°C for 2 h. Next, samples were rinsed 5x in water, dehydrated in a graded
781 acetone series and embedded in Epon-Araldite (Embed 812, EM Science, Hatfield, PA,
782 USA). Embedments were trimmed and mounted in the vise-chuck of a Leica Ultracut UCT
783 ultramicrotome (Leica, Buffalo Grove, IL, USA). Ultrathin sections (~60 to 70 nm) were
784 cut using a diamond knife (type ultra 35°C; Diatome), mounted on copper grids
785 (FCFT300-CU-50, VWR, Radnor, PA, USA), and counterstained with lead citrate for 8
786 min¹²⁵. Samples were imaged with a LEO 912 AB Energy Filter Transmission Electron
787 Microscope (Zeiss, Oberkochen, Germany). Micrographs were acquired with ITEM
788 software (ver. 5.2) (Olympus Soft Imaging Solutions GmbH, Germany) with a TRS 2048
789 x 2048k slow-scan charge-coupled device (CCD) camera (TRÖNDLE
790 Restlichtverstärkersysteme, Germany). Ninety electron micrographs were quantified for
791 each experimental treatment using image analysis (FIJI software, National Institutes of
792 Health) and stereology (Stereology Analyzer version 4.3.3, ADCIS, France). Each TEM
793 image was acquired at 8,000X magnification and 1.37 nm pixel resolution with arrays of
794 up to 5X5 tiles using automated Multiple Image Alignment software module (settings:
795 correlation =1, FFT algorithm, overlap area = linear weighted, movement = emphasize,
796 and equalize). TEM images were analyzed with Stereology Analyzer software version
797 4.3.3 to quantify relative volume of various cell parameters including stroma, stroma
798 lamellae, starch granules, and grana within individual chloroplasts (Supplementary Fig.
799 11b). Grid type was set as “point” with a sampling step of 500x500 pixels and pattern size
800 of 15x15 pixels. The percent of relative volume for each parameter was collected after
801 identifying all grid points within one chloroplast and further analyzed in excel. TEM images
802 with a magnification of 8K were used in the Fiji (ImageJ) analysis. The images were

803 scaled to 0.7299 pixel/nm in ImageJ before analyzing the chloroplast area, plastoglobuli
804 area, and grana dimensions. The height of grana margin (positions 1 and 3) and grana
805 core (position 2) were quantified as described previously²³ (Supplementary Fig. 12d, e).
806 The “polygon selections” tool was used to quantify the chloroplast and plastoglobuli area
807 by outlining the target structure. The individual plastoglobuli (PG) size was measured
808 using ImageJ. All PG in a chloroplast were quantified to get the total PG area per
809 chloroplast. The “straight” tool was used to quantify grana height and width. The grana
810 number and PG number were counted manually. Choosing the correct statistical test to
811 reflect the quantified data is essential in making conclusions. Three different statistical
812 tests were used to find the significance of p-values. The negative binomial test was used
813 for counting data that followed a negative binomial distribution. The Kolmogorov-Smirnov
814 test was used for relative volume data since it is commonly used to find significance
815 between data in a form of ratios. A two-tailed t-test with unequal variance was used for
816 all other data that followed a normal distribution. All three statistical tests compared the
817 treatment conditions to the ctrl conditions of the same cell type. Each treatment had three
818 biological replicates and a total of 90~120 images of each treatment were analyzed.

819

820 **Starch quantification**

821 To isolate starch from leaves, three biological replicates of 2-cm mid-leaf segments were
822 collected from fourth fully expanded true leaves into screw cap tubes (USA Scientific,
823 1420-9700) with a grinding bead (Advanced Materials, 4039GM-S050) and immediately
824 frozen in liquid nitrogen and stored at -80°C. Frozen samples were homogenized using a
825 paint shaker. For starch quantification, leaves decolorized by 80% ethanol and starch
826 concentration was subsequently measured using a starch assay kit (Megazyme, K-TSTA-
827 100A).

828

829 **MultispeQ measurement**

830 A MultispeQ⁶¹ v2.0 was used to measure chlorophyll fluorescence parameters and
831 electrochromic shift (ECS) in *S. viridis* leaves at the start or after 4 h treatments of ctrl,
832 HL, or HT. ECS results from light-dark-transition induced electric field effects on
833 carotenoid absorbance bands^{62,126} and is a useful tool to monitor proton fluxes and the

834 transthylakoid proton motive force (*pmf*) *in vivo*^{63,64}. Light drives photosynthetic electron
835 transport along the thylakoid membrane and proton fluxes across the thylakoid
836 membrane. Protons flux into the thylakoid through H₂O oxidation at PSII and plastoquinol
837 oxidation at cytochrome *b₆f* complex; protons flux out of the thylakoid mainly through ATP
838 synthase to make ATP, which is driven by the transthylakoid *pmf*^{63,64}. The total amplitude
839 of ECS signal during the light-dark-transition, ECS_t, represents the transthylakoid *pmf*.
840 The decay time constant of light-dark-transition induced ECS signal, τ_{ECS}, is inversely
841 proportional to proton conductivity ($g_{H^+} = 1 / \tau_{ECS}$), which is proportional to the aggregate
842 conductivity (or permeability) of the thylakoid membrane to protons and largely dependent
843 on the activity of ATP synthase⁶². The proton flux rates, v_{H+}, calculated by ECS_t / τ_{ECS}, is
844 the initial decay rate of the ECS signal during the light-dark-transition and reflects the rate
845 of proton translocation by the entire electron transfer chain, usually predominantly through
846 the ATP synthase⁶². ECS was measured using MultispeQ and the dark interval relaxation
847 kinetics with a modified Photosynthesis RIDES protocol at light intensities of 250, 500,
848 and 1000 μmol photons m⁻² s⁻¹. The MultispeQ v2.0 was modified with a light guide mask
849 to improve measurements on smaller leaves. Parameters at the different light intensities
850 were measured sequentially on the middle segment of a fourth fully expanded true leaf at
851 room temperature with no dark adaptation prior to measurements. The estimated NPQ,
852 NPQ_(T), was measured by MultispeQ based on a method that does not require a dark-
853 adapted state of the leaf for determination of F_m⁵⁹. NPQ_(T) uses the minimal fluorescence
854 (F_o') and maximal fluorescence (F_m') in light-adapted leaves to estimate NPQ. Statistical
855 significance was assigned with a two-tailed t-test assuming unequal variance.

856

857 **Statistics and reproducibility**

858 All data presented had at least 3 biological replicates. Detailed information about statistics
859 analysis were described for each method above.

860

861 **Data availability**

862 The datasets analyzed in this paper are included in this published article and
863 supplementary information files. Other information is available from the corresponding
864 author on request.

865

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886

887 **AUTHOR CONTRIBUTIONS**

888 R.Z. supervised the whole project. R.Z. and C.M.A. designed and planned all the
889 experiments. C.M.A. led the project, performed and analyzed all LI-6800 data, extracted
890 RNA and prepared the RNA-seq library, and led sample harvest for pigment analysis.
891 C.M.A. and E.B. grew all plants needed for the project. T.J.A. provided insight to optimize
892 gas exchange and chlorophyll fluorescence measurements in *S. viridis* using the LI-6800.
893 T.T. and R.V. performed modeling of the leaf-level gas exchange data. N.Z., E.B. and
894 K.J.C. performed TEM analysis. N.Z. quantified starch using assay kits. W.M. helped

895 harvest leaf tissues and performed MultispeQ measurements. E.B. harvested leaves for
896 ABA measurements and S.A.M.M. performed ABA analyses. D.P., O.D., and K.K.N.
897 performed leaf pigment analysis by HPLC. M.B. and A.L.E. provided insight for RNA-seq
898 library preparation. J.Y. and A.L.E. preprocessed RNA-seq data. E.M. led RNA-seq data
899 analysis and generated all the heatmaps. S.P. and R.Z. identified ABA-related genes in
900 *S. viridis*. E.M. identified all other genes used for the heatmaps. J.B. provided suggestions
901 for statistical analysis. M.W. and D.A.N. helped plan, coordinate, and discuss the RNA-
902 seq experiments. R.Z., C.M.A., and E.M. led the writing of the manuscript with the
903 contribution of all other co-authors. All the authors discussed the results, contributed to
904 data interpretation, and helped revise the manuscript.

905

906 **Competing interests**

907 The authors declare no competing interests.

908

909 **Supplementary information**

910 **Supplementary Table 1:** Formulas to calculate photosynthetic parameters.

911

912 **Supplementary Data Files**

913 **Supplementary Data 1:** Normalized read counts in Transcripts Per Million (TPM) for all
914 genes in all time points and biological replicates. Annotation information includes the *S.*
915 *viridis* provisional defline, *A. thaliana* and *O. sativa* best hits and deflines from the Joint
916 Genome Institute bulk annotation information.

917

918 **Supplementary Data 2:** Differential expression data for all genes that were significantly
919 differentially expressed in at least one time point and condition (DeSeq2, FDR < 0.05).
920 Annotation information includes the *S. viridis* provisional defline, *A. thaliana* and *O. sativa*
921 best hits and deflines from the Joint Genome Institute bulk annotation information.

922

923 **Supplementary Data 3:** Genes up-regulated or down-regulated at all time points in high
924 light or high temperature conditions. Annotation information includes the *S. viridis*

925 provisional define, *A. thaliana* and *O. sativa* best hits and defines from the Joint Genome
926 Institute bulk annotation information.

927

928 **Supplementary Data 4:** Overlapping differentially expressed genes between high light
929 and high temperature conditions. Genes differentially expressed in at least one time point
930 were included in lists of up- and down-regulated genes in each condition. Annotation
931 information includes the *S. viridis* provisional define, *A. thaliana* and *O. sativa* best hits
932 and defines from the Joint Genome Institute bulk annotation information.

933

934 **Supplementary Data 5:** Genes highly induced or highly repressed ($FC \geq 5$, or ≤ -5) in
935 both the high light and high temperature treatments during at least one time point.
936 Annotation information includes the *S. viridis* provisional define, *A. thaliana* and *O. sativa*
937 best hits and defines from the Joint Genome Institute bulk annotation information.
938 Additionally, heat tolerance genes identified in maize with homologs in *S. viridis*.

939

940 **Supplementary Data 6:** *S. viridis* v2.1 gene information used to generate heatmaps of
941 pathways of interest.

942

943 **Supplementary figure 1-14**

944

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High Light and High Temperature Reduce Photosynthesis via Different Mechanisms in the C₄ Model *Setaria viridis*

Anderson et al. Main figures

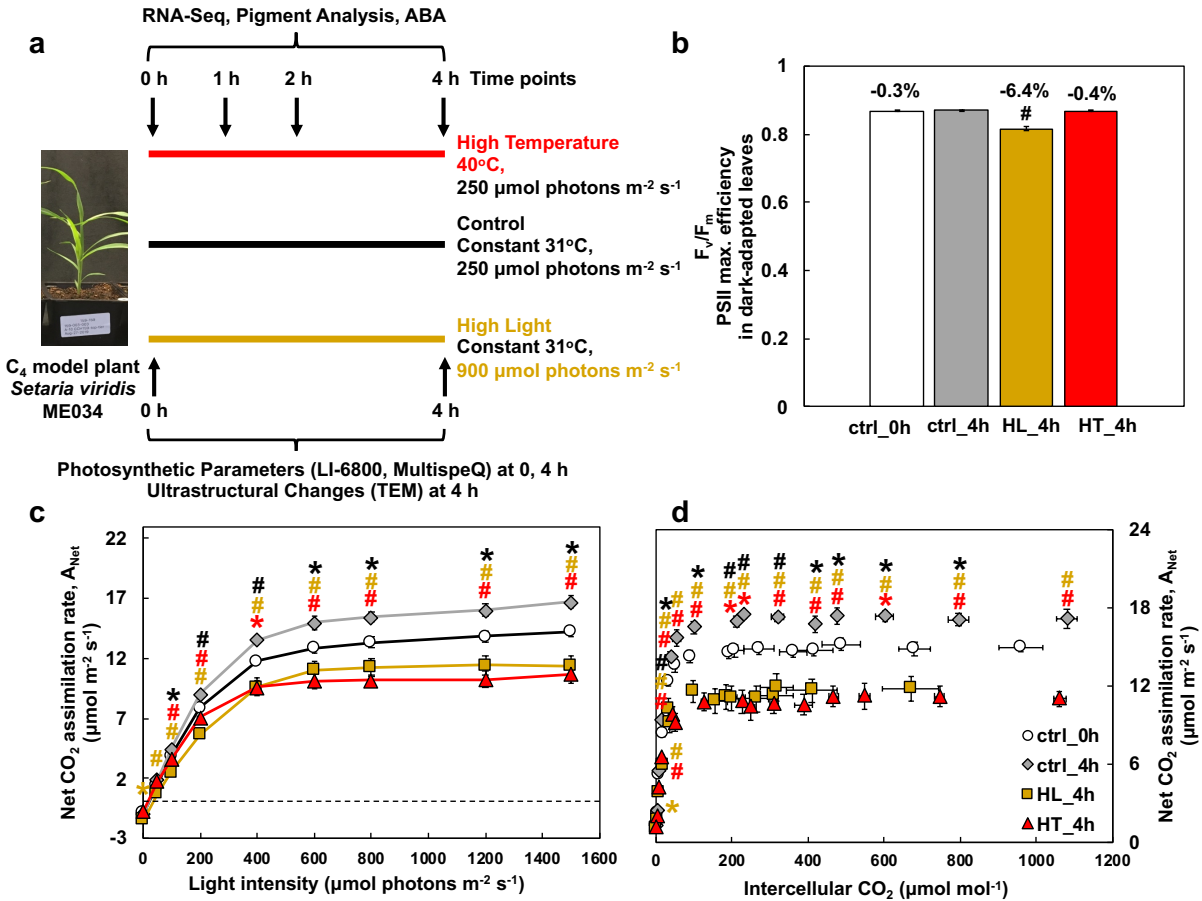


Figure 1: High light (HL) and high temperature (HT) resulted in comparable reduction of net CO₂ assimilation rates and HL also caused significant photoinhibition in *S. viridis* leaves. (a) Experimental overview. We investigated how the C₄ model plant *S. viridis* ME034 responded to HL or HT at different levels. Plants were grown under the control condition (31°C and 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light) for 13 days, then treated with control growth condition or HL (31°C, 900 $\mu\text{mol m}^{-2} \text{s}^{-1}$) or HT (40°C, 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light) in different growth chambers for 4 h. The fourth fully expanded true leaves were utilized for all analyses. Leaf tissues from different treatments were harvested at 0, 1, 2, and 4 h time points for the analysis of RNA-seq, pigments, and leaf

ABA levels. Photosynthetic parameters were measured using intact leaves at 0 and 4 h time points, including gas exchange and chlorophyll fluorescence using LI-6800 and spectroscopic measurements using MultispeQ. Transmission Electron Microscopy (TEM) analysis was performed to investigate chloroplast ultrastructure changes in leaves after 4 h treatments. **(b)** HL-treated leaves had reduced PSII maximum efficiency (F_v/F_m). F_v/F_m was measured by chlorophyll fluorescence in LI-6800 with 20 min dark-adapted leaves. Pound symbols indicate statistically significant differences of ctrl_0h (at the start of treatments), HL_4h (after 4 h HL), and HT_4h (after 4 h HT) compared to ctrl_4h (after 4 h control treatment) using Student's two-tailed t-test with unequal variance (# $p < 0.01$). Percentages indicate reduction in F_v/F_m compared to ctrl_4h. **(c, d)** Net CO₂ assimilation rates during light response and CO₂ response, respectively. Asterisk and pound symbols indicate statistically significant differences of ctrl_0h, HL_4h, and HT_4h compared to ctrl_4h using Student's two-tailed t-test with unequal variance. P-values were corrected for multiple comparisons using FDR (* $0.01 < p < 0.05$, # $p < 0.01$, the colors of * and # match the significance of the indicated conditions, black for ctrl_0h, yellow for HL_4h, red for HT_4h). Mean \pm SE, $n = 3-6$ biological replicates.

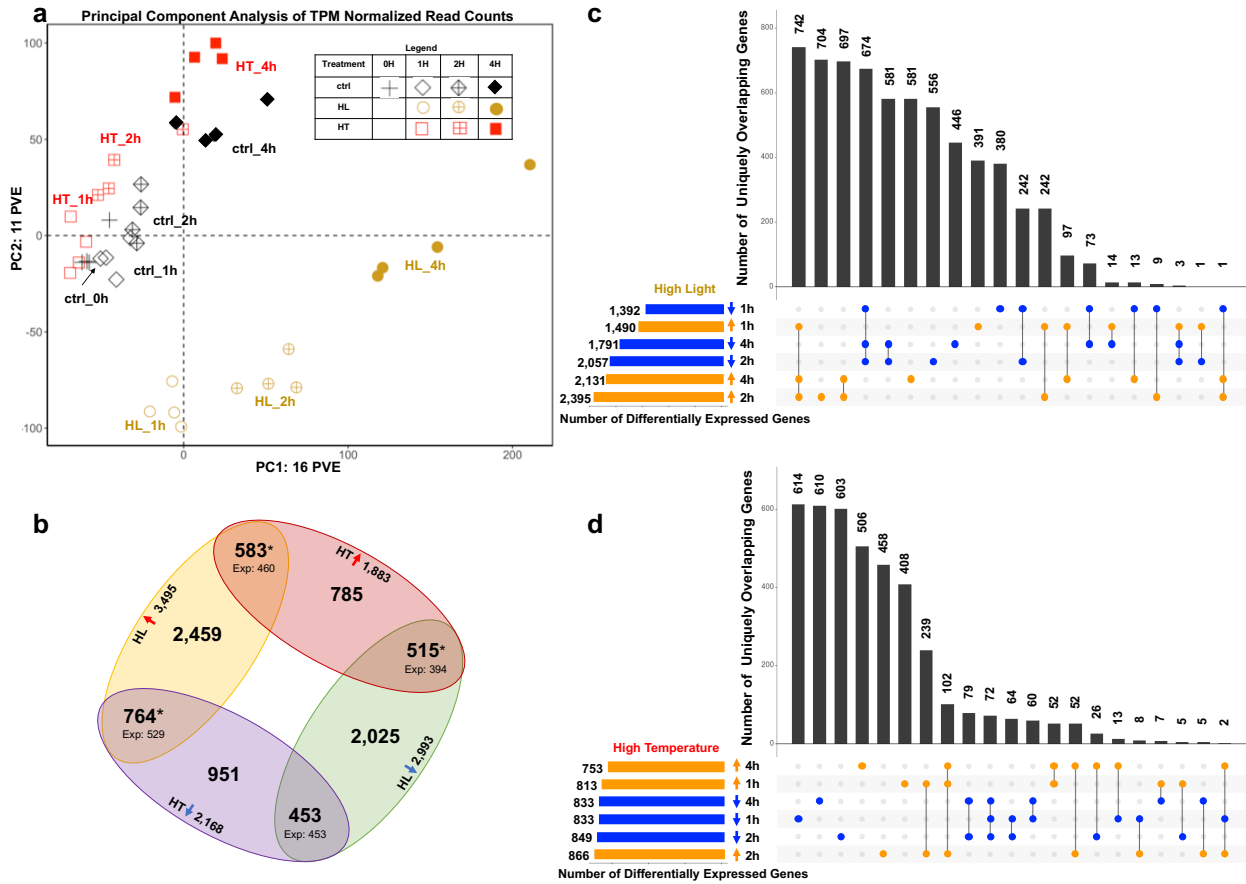


Figure 2: Time course transcriptome data reveal dynamic responses to high light or high temperature stresses in *S. viridis*. (a) Principal Component Analysis of TPM (transcripts per million) normalized read counts in control (ctrl), high light (HL), and high temperature (HT) treated samples. The first two principal components representing the highest percent variance explained are displayed. PC1 explains 16% of the variance in the dataset and mainly separates the samples based on time. PC2 explains 11% of the variance in the dataset and mainly separates the HL samples from the ctrl and HT samples. Black diamonds indicate ctrl samples, yellow circles indicate HL samples, and red squares indicate HT samples. Different fillings for these symbols indicate different time points of each treatment. Each treatment and time point have four biological replicates, represented by symbols with the same shape and color. (b) HL and HT treatments had more overlapping differentially expressed genes than expected by random chance. Gene sets represent the number of genes differentially regulated in at least one time point in the given condition. Red upward arrows denote up-regulation and

blue downward arrows denote down-regulation. Yellow oval denotes HL up-regulated genes, green oval denotes HL down-regulated genes, red oval denotes HT up-regulated genes, purple oval denotes HT down-regulated genes. Expected values (Exp) are the number of the overlapping genes expected by random chance based on size of the gene lists and background of all genes tested via DeSeq2 (14,302). Numbers above expected values are the actual number of overlapped genes between two conditions. * $p < 0.0001$, Fisher's Exact Test. **(c, d)** HT transcriptional responses are more transient than HL. UpSetR plots show number of uniquely overlapping genes between up and down regulated gene sets at each time point in HL and HT, respectively. Horizontal bars indicate the number of genes up or down regulated at each time point. Filled circles indicate the gene sets included in the overlap shown. Vertical bars indicate the number of genes represented in the overlap shown. Overlapping gene sets are arranged in descending order by number of genes. Genes may only belong to a single overlapping gene set and are sorted into the overlapping set with the highest number of interactions.

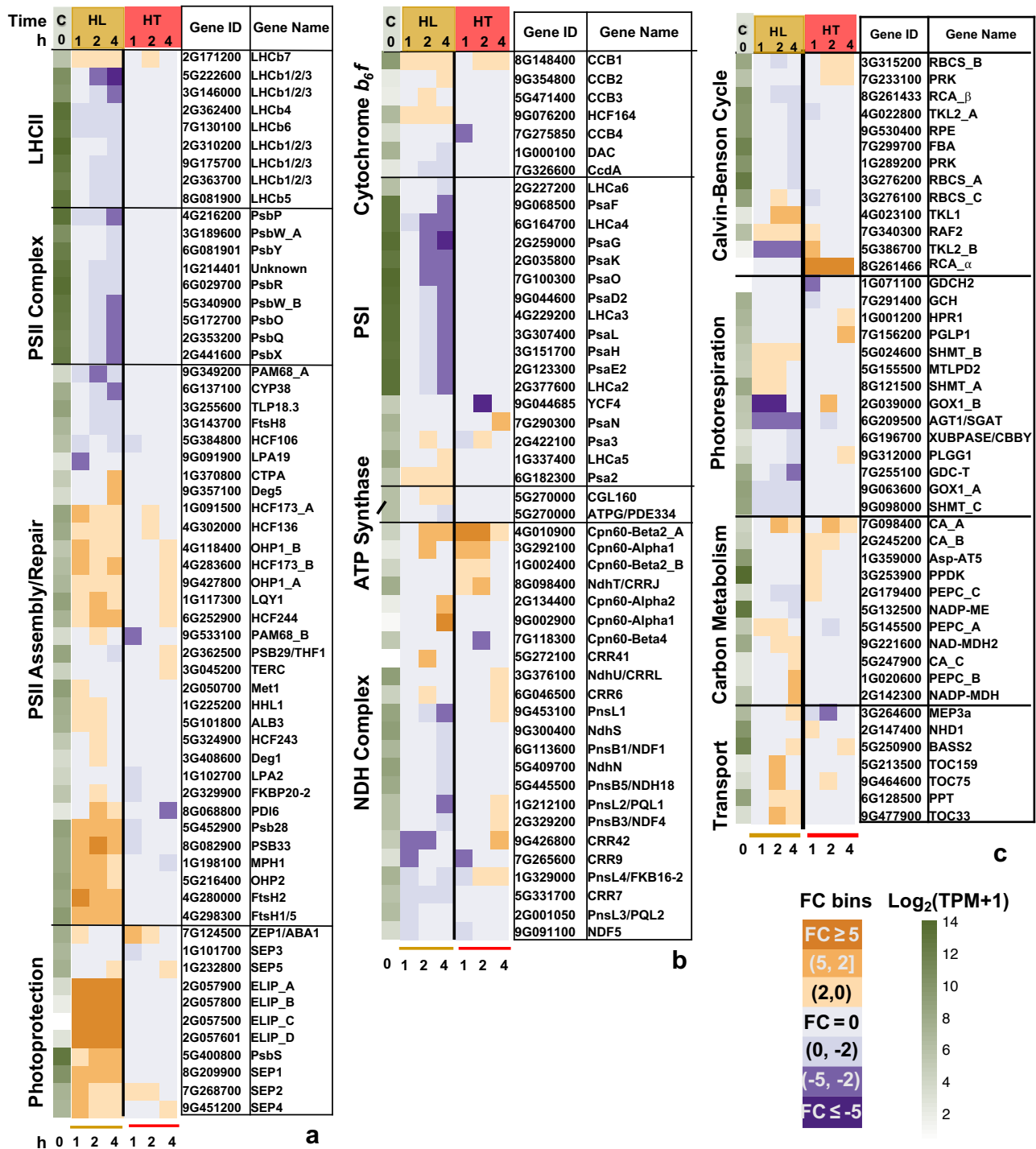


Figure 3: High light (HL) differentially regulated genes involved in photosynthesis more than high temperature (HT). (a, b) Genes related to light reaction of photosynthesis and photoprotection. (c) Genes related to carbon metabolism and chloroplast transport. The first green column displays log₂(mean TPM + 1) at ctrl_0h (at the start of treatments, C). TPM, transcripts per million, normalized read counts. Heatmap

displays the fold change (FC) bin of DeSeq2 model output values at 1, 2, 4 h of HL or HT versus control at the same timepoint ($q < 0.05$). FC bins: highly induced: $FC \geq 5$; moderately induced: $5 > FC \geq 2$; slightly induced: $2 > FC > 0$; not differentially expressed: $FC = 0$; slightly repressed: $0 > FC > -2$; moderately repressed: $-2 \geq FC > -5$; highly repressed: $FC \leq -5$. Gene ID: *S. viridis* v2.1 gene ID, excluding "Sevir.". All genes presented in the heatmaps were significantly differentially regulated in at least one time point.

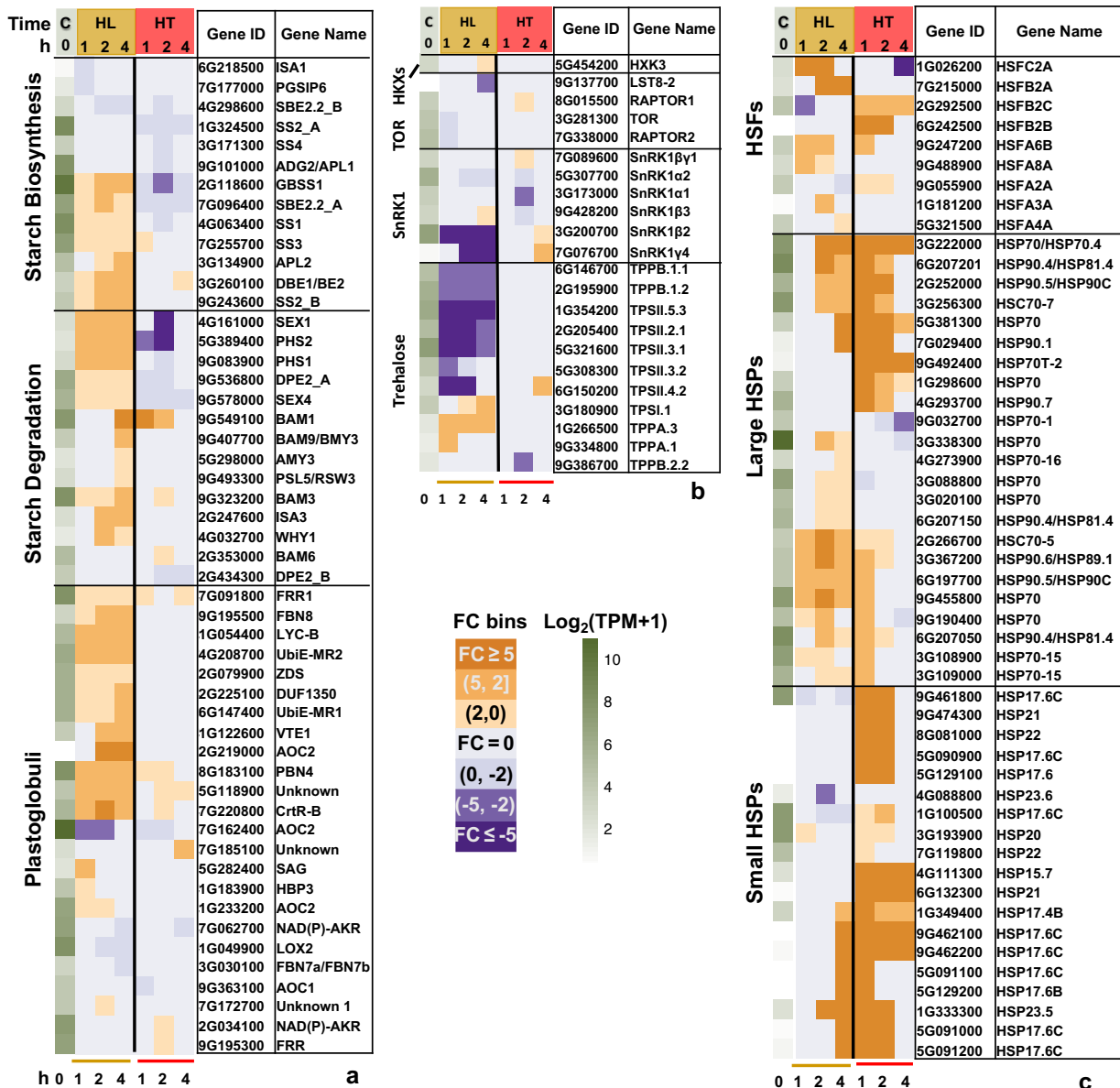


Figure 4: High light (HL) and high temperature (HT) differentially regulated genes involved in several key pathways. (a, b) HL induced genes involved in starch biosynthesis/degradation and genes encoding plastoglobuli-localized proteins; (b) HL down-regulated many genes of the sugar-sensing pathways; (c) Both HL and HT induced genes encoding shock transcription factors (HSFs) and heat shock proteins (HSPs) but the induction was much quicker under HT than HL. The first green column displays $\log_2(\text{mean TPM} + 1)$ at ctrl_0h (at the start of treatments, C). TPM, transcripts per million, normalized read counts. Heatmap displays the fold change (FC) bin of DeSeq2 model output values at 1, 2, 4 h of HL or HT versus control at the same timepoint ($q < 0.05$). FC

bins: highly induced: $FC \geq 5$; moderately induced: $5 > FC \geq 2$; slightly induced: $2 > FC > 0$; not differentially expressed: $FC = 0$; slightly repressed: $0 > FC > -2$; moderately repressed: $-2 \geq FC > -5$; highly repressed: $FC \leq -5$. Gene ID: *S. viridis* v2.1 gene ID, excluding "Sevir.". All genes presented in the heatmaps were significantly differentially regulated in at least one time point.

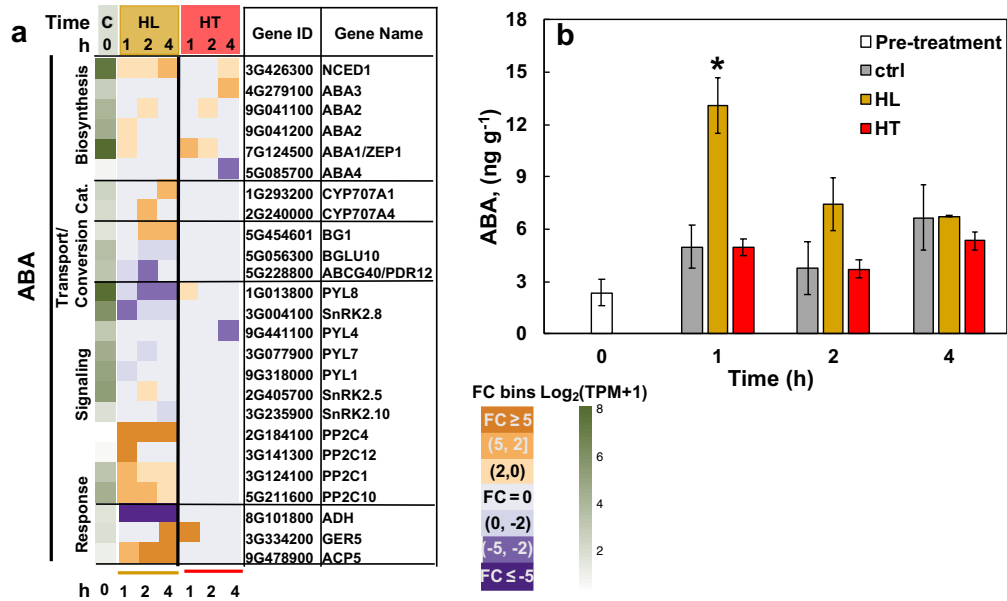


Figure 5: High light (HL) up-regulated genes involved in the abscisic acid (ABA) pathway and transiently increased leaf ABA levels. (a) Heatmap of differentially regulated genes involved in the ABA pathway. Cat: catabolism. The first green column displays $\log_2(\text{mean TPM} + 1)$ at ctrl_0h (at the start of treatments, C). TPM, transcripts per million, normalized read counts. Heatmap displays the fold change (FC) bin of DeSeq2 model output values at 1, 2, 4 h of HL or HT versus control at the same timepoint ($q < 0.05$). FC bins: highly induced: $FC \geq 5$; moderately induced: $5 > FC \geq 2$; slightly induced: $2 > FC > 0$; not differentially expressed: $FC = 0$; slightly repressed: $0 > FC > -2$; moderately repressed: $-2 \geq FC > -5$; highly repressed: $FC \leq -5$. Gene ID: *S. viridis* v2.1 gene ID, excluding “Sevir.”. All genes presented in the heatmaps were significantly differentially regulated in at least one time point. (b) Concentrations of leaf ABA. Mean \pm SE, $n = 3$ biological replicates. Asterisk symbol indicates statistically significant differences as compared to the control condition at the same time point. (Student’s two-tailed t-test with unequal variance, $*0.01 < p < 0.05$).

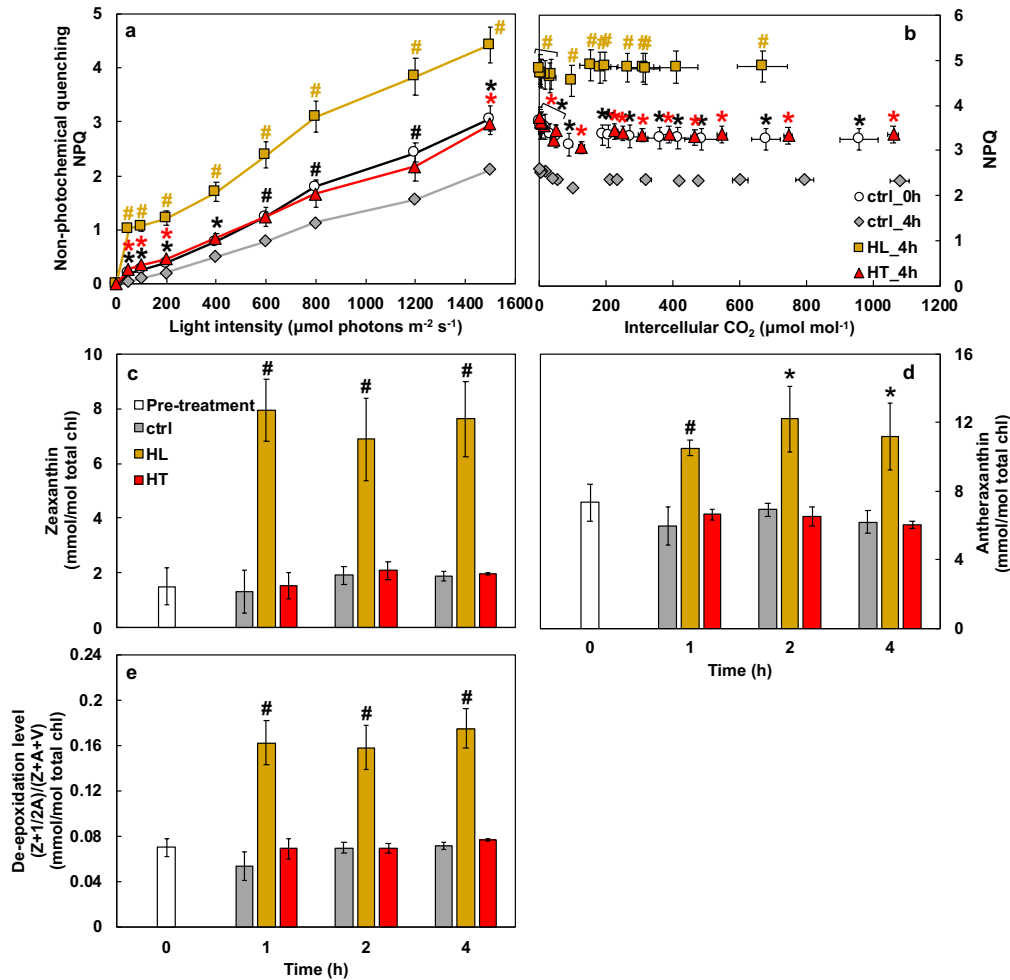


Figure 6: High light (HL) induced non-photochemical quenching (NPQ) and increased zeaxanthin as well as de-epoxidation levels. (a) Light and **(b)** CO_2 response of NPQ. Mean \pm SE, $n = 3-6$ biological replicates. Asterisk and pound symbols indicate statistically significant differences of ctrl_0h (at the start of treatments), HL_4h (after 4 h high light), and HT_4h (after 4 h high temperature) compared to ctrl_4h (after 4 h control treatment) using Student's two-tailed t-test with unequal variance. P-values were corrected for multiple comparisons using FDR ($*0.01 < p < 0.05$, $\#p < 0.01$, the colors of * and # match the significance of the indicated conditions, black for ctrl_0h, yellow for HL_4h, red for HT_4h). **(c, d, e)** Concentrations of zeaxanthin, antheraxanthin, and xanthophyll cycle de-epoxidation. Mean \pm SE, $n = 3$ biological replicates. Asterisk and pound symbols indicate statistically significant differences of high light (HL) or high temperature (HT) treatments compared to the control (ctrl) condition at the same time points using Student's two-tailed t-test with unequal variance ($*0.01 < p < 0.05$, $\#p < 0.01$).

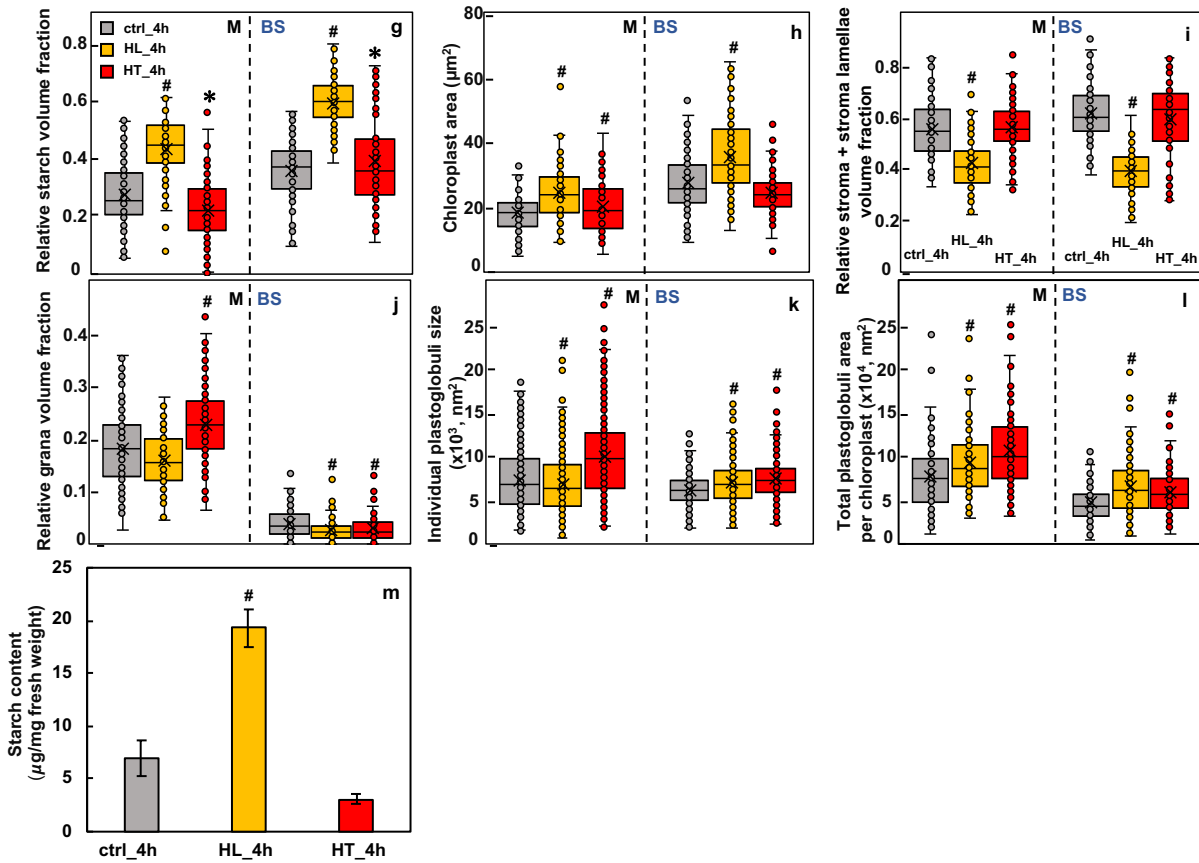
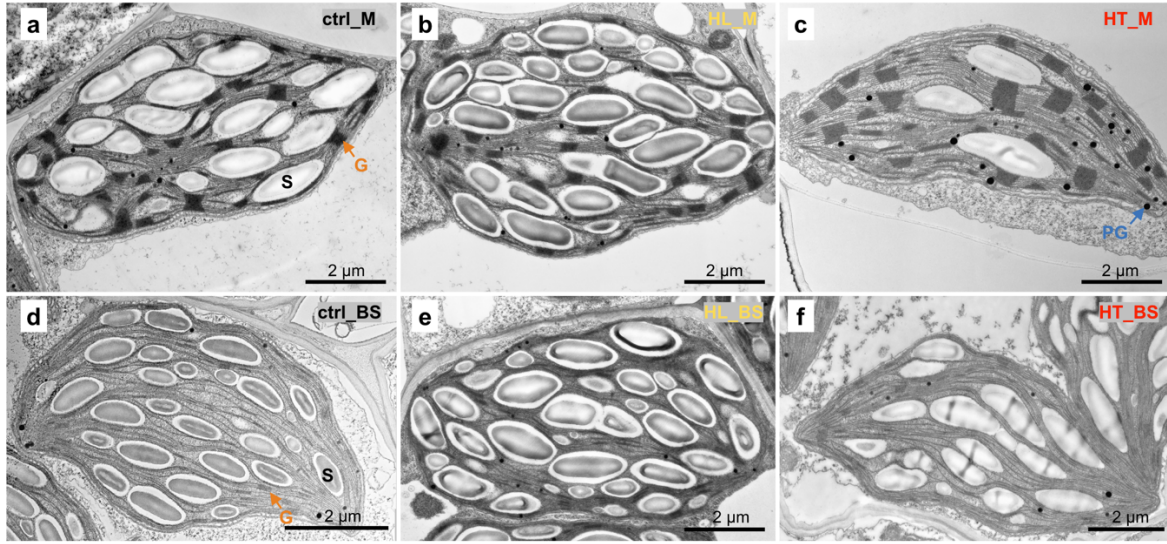


Figure 7: High light (HL) increased starch accumulation and both HL and high temperature (HT) treatments induced chloroplast plastoglobuli formation in *S. viridis* leaves. (a-f) Representative transmission electron microscopy (TEM) images of mesophyll (M) and bundle sheath (BS) chloroplasts in leaves of *S. viridis* after 4 h

treatments of control (ctrl_4h, 31°C, 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light) or high light (HL_4h, 31°C, 900 $\mu\text{mol m}^{-2} \text{s}^{-1}$) or high temperature (HT_4h, 40°C, 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light). TEM images of M (**a, b, c**) and BS (**d,e,f**) chloroplasts. S labels the starch granule; G labels grana, the orange arrows indicate grana in M and BS chloroplasts; PG labels plastoglobuli. (**g, i, j**) Relative volume fraction of indicated parameters were quantified using Stereo Analyzer with Kolmogorov–Smirnov test for statistical analysis compared to the same cell type of the control condition. (**h, k, l**) area and size of indicated parameters were quantified using ImageJ with two-tailed t-test with unequal variance compared to the same cell type of the control condition. Each treatment had three biological replicates, total 90-120 images per treatment. *0.05<p<0.01; #p<0.01. (**m**) Starch quantification using starch assay kits. HL_4h leaves accumulated 4x starch as compared to ctrl_4h leaves. Values are mean \pm SE, $n = 3$ biological replicates. Pound symbols indicate statistically significant differences as compared to ctrl_4h using Student's two-tailed t-test with unequal variance (#p< 0.01).

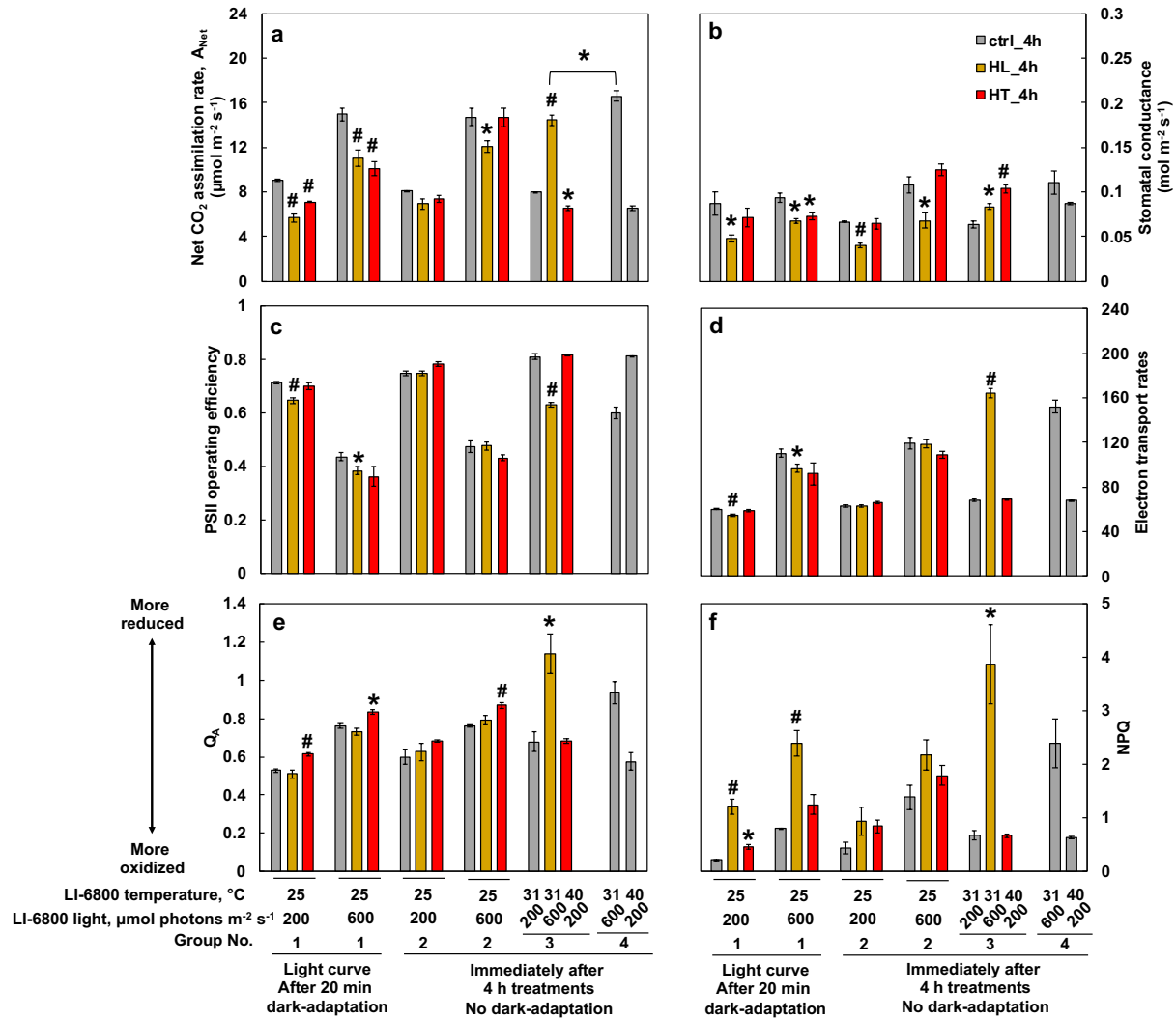


Figure 8: High light or high temperature treated leaves had lower photosynthetic capacities than leaves treated with the control condition. *S. viridis* plants were treated with 4 h of control growth condition (ctrl_4h, 31°C, 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light) or high light (HL_4h, 31°C, 900 $\mu\text{mol m}^{-2} \text{s}^{-1}$) or high temperature (HT_4h, 40°C, 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light) in different growth chambers. After the treatments, an intact fourth fully expanded true leaf from each treated plant was clamped in the LI-6800 leaf chamber to measure various photosynthetic parameters. Group 1 are select data from the light response curves after 20 min dark-adaptation in the LI-6800 leaf chamber with indicated light and temperature. Groups 2,3,4 were measured immediately after 4 h of ctrl, HL, HT treatments without dark-adaptation and under the indicated temperature and light condition in the LI-6800 leaf chamber. Individual plants were used for each replicate. (a) Net CO₂ assimilation

rates. **(b)** Stomatal conductance. **(c)** PSII operating efficiency. **(d)** Electron transport rate. **(e)** Plastoquinone redox status (Q_A). **(f)** NPQ, Non-photochemical quenching. Asterisk and pound symbols indicate statistically significant differences of HL_4h and HT_4h leaves compared to ctrl_4h leaves in the same group or under the same LI-6800 leaf chamber condition using Student's two-tailed t-test with unequal variance (* $0.01 < p < 0.05$, # $p < 0.01$).

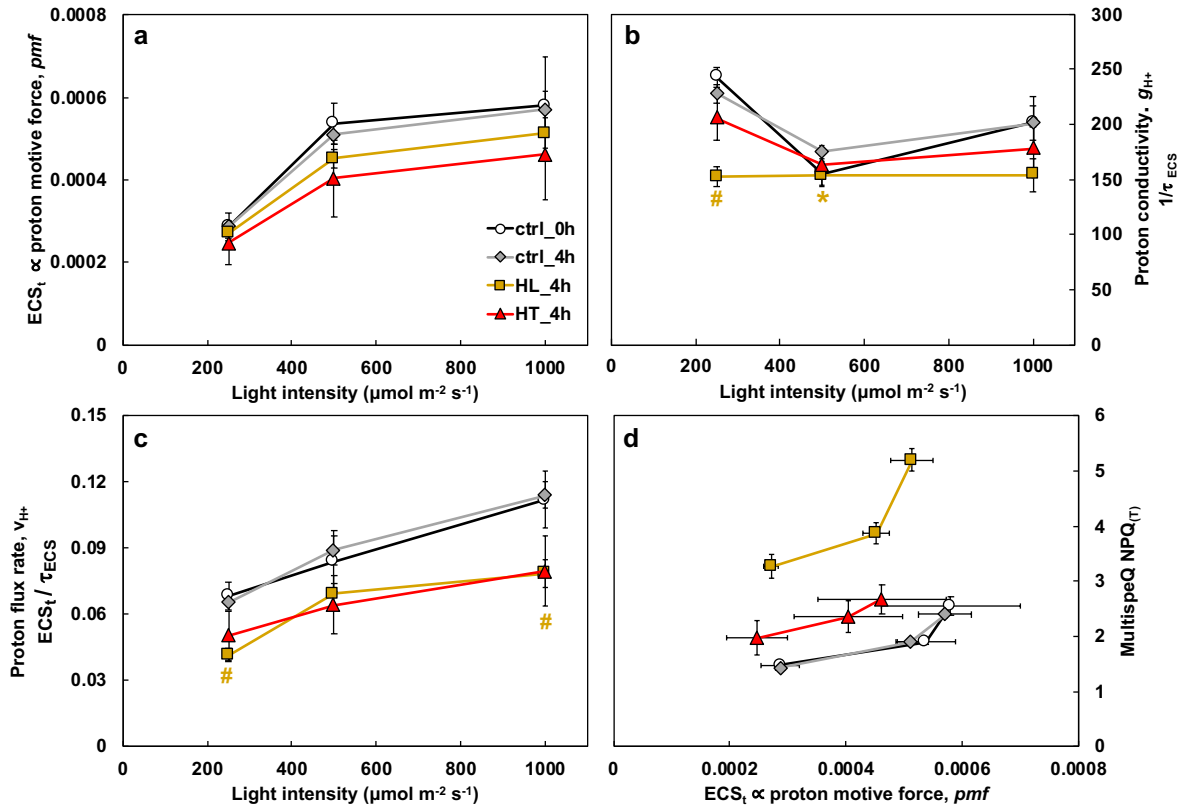


Figure 9: High light treatment inhibited ATP synthase activity. *S. viridis* plants were treated with 4 h of control growth condition (ctrl_4h, 31°C, 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light) or high light (HL_4h, 31°C, 900 $\mu\text{mol m}^{-2} \text{s}^{-1}$) or high temperature (HT_4h, 40°C, 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light) in different growth chambers. After the treatments, photosynthetic parameters in treated leaves were monitored using the MultispeQ instrument. **(a)** ECS_t , measured by electrochromic shift (ECS), representing the transthylakoid proton motive force, *pmf*. **(b)** Proton conductivity ($g_{\text{H}^+} = 1/\tau_{\text{ECS}}$), proton permeability of the thylakoid membrane and largely dependent on the activity of ATP synthase, inversely proportional to the decay time constant of light-dark transition induced ECS signal (τ_{ECS}). **(c)** Proton flux rates, v_{H^+} , calculated by $\text{ECS}_t / \tau_{\text{ECS}}$, the initial decay rate of the ECS signal during the light-dark transition and proportional to proton efflux through ATP synthase to make ATP. **(d)** Non-photochemical quenching (NPQ) measured by MultispeQ. Mean \pm SE, $n = 3$ biological replicates. Asterisk and pound symbols indicate statistically significant differences of ctrl_0h, HL_4h, and HT_4h compared to ctrl_4h using Student's two-tailed t-test with unequal variance. (* $0.01 < p < 0.05$, # $p < 0.01$, the colors of * and # match the significance of the indicated conditions, yellow for HL_4h).

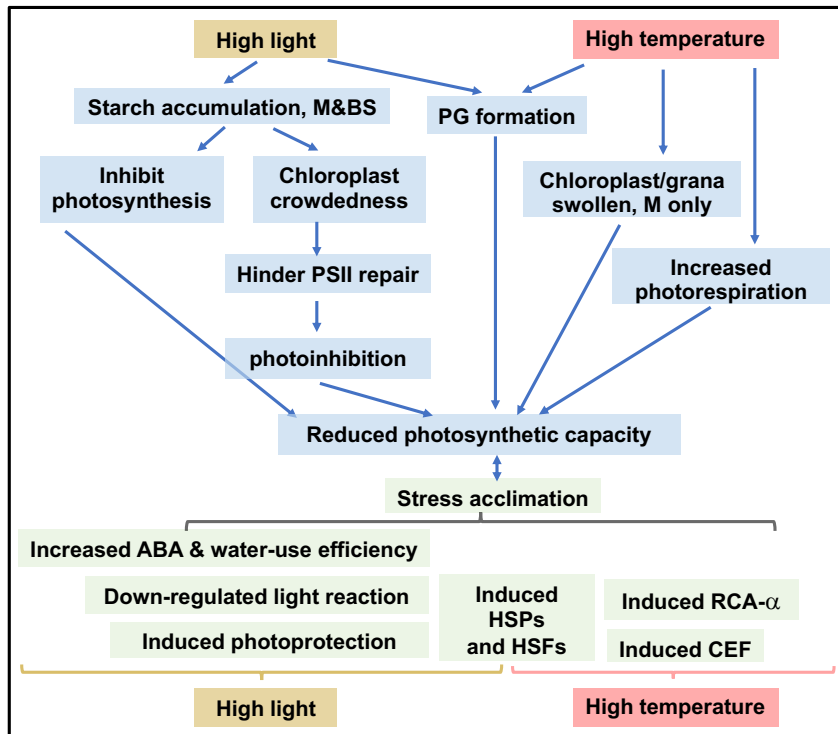
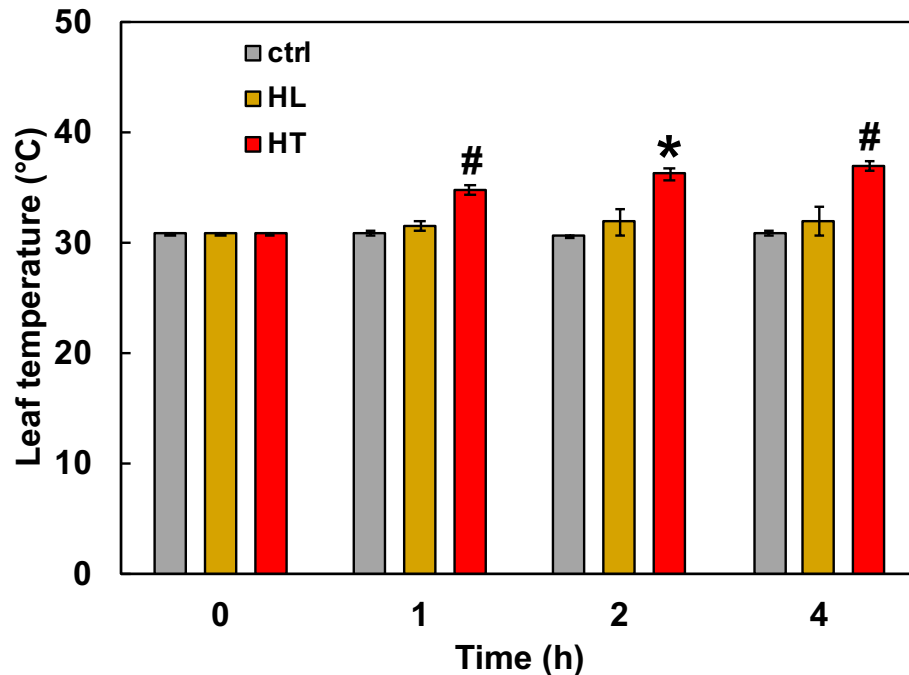


Figure 10: Summary of how *S. viridis* responds to high light (HL) or high temperature (HT). Light blue boxes denote changes that may lead to the reduced photosynthetic capacities; light green boxes denote changes that may be adaptive for stress acclimation. M: mesophyll chloroplasts; BS, bundle sheath chloroplasts. HL-treated leaves had over-accumulated starch and increased chloroplast crowdedness, which may hinder PSII repair and result in photoinhibition. Starch accumulation may also inhibit photosynthesis through feedback regulation. Increased plastoglobuli (PG) formation in HL-treated leaves may affect thylakoid composition and function. Under HT, M chloroplasts had swollen chloroplasts/grana and seem more heat-sensitive than BS chloroplasts. Heat-induced photorespiration and PG formation could further reduce photosynthesis. Meanwhile, HL and HT also induce adaptive responses for acclimation. Under HL, the induced photoprotection, down-regulated light reaction, and increased water-use efficiency through abscisic acid (ABA) can help *S. viridis* acclimate to excess light. Under HT, the induced cyclic electron flow (CEF) and Rubisco activase (RCA- α) can protect photosynthesis from heat stress. The induced heat shock transcription factors (HSFs) and heat shock proteins (HSPs) are adaptive responses to both HL and HT although the induction was much quicker under HT.

High Light and High Temperature Reduce Photosynthesis via Different Mechanisms in the C₄ Model *Setaria viridis*

Anderson et al. Supplementary Figures



Supplementary Figure 1. Leaf temperatures of *S. viridis* stayed constant during the control and high light treatments while increased during high temperature treatment. Leaf temperatures of *S. viridis* measured over the 4 h time course of control or high light or high temperature treatments. *S. viridis* plants were treated with control growth condition (ctrl, 31°C and 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light) or high light (HL, 31°C and 900 $\mu\text{mol m}^{-2} \text{s}^{-1}$) or high temperature (HT, 40°C and 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light) in different growth chambers for 4 h. Leaf temperature was measured at 0, 1, 2, and 4 h for each treatment. Mean \pm SE, $n = 3$ biological replicates. Asterisk and pound symbols indicate statistically significant differences of HL and HT compared to ctrl in a given time point using Student's two-tailed t-test with unequal variance (*0.01< p <0.05, # p <0.01). No significant changes of leaf temperatures during the ctrl and HL condition.

a Stage 1

Dark adaptation:			
Light intensity, $\mu\text{mol photons m}^{-2} \text{s}^{-1}$	Time, min	Logs	Measurement frequency, min
0	20	1	
Total time: 20 min, CO_2 : $400 \mu\text{mol mol}^{-1}$			

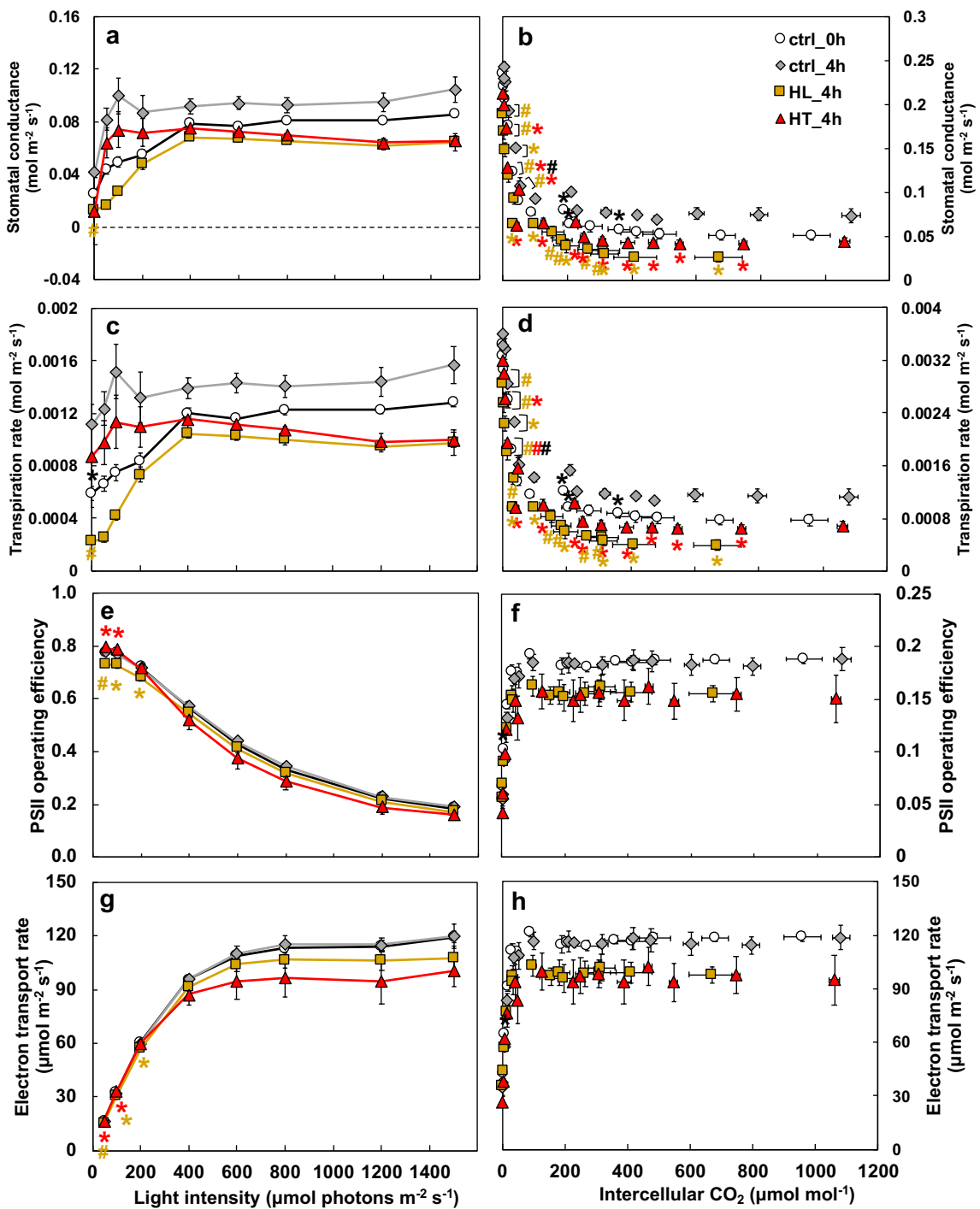
b Stage 2

Light response experiment:			
CO_2 : $400 \mu\text{mol mol}^{-1}$			
Light intensity, $\mu\text{mol photons m}^{-2} \text{s}^{-1}$	Time, min	Logs	Measurement frequency, min
50	5	2	2.5
100	5	2	2.5
200	5	2	2.5
400	5	2	2.5
600	5	2	2.5
800	5	2	2.5
1200	5	2	2.5
1500	10	4	2.5
Total time: 65 min			

c Stage 3

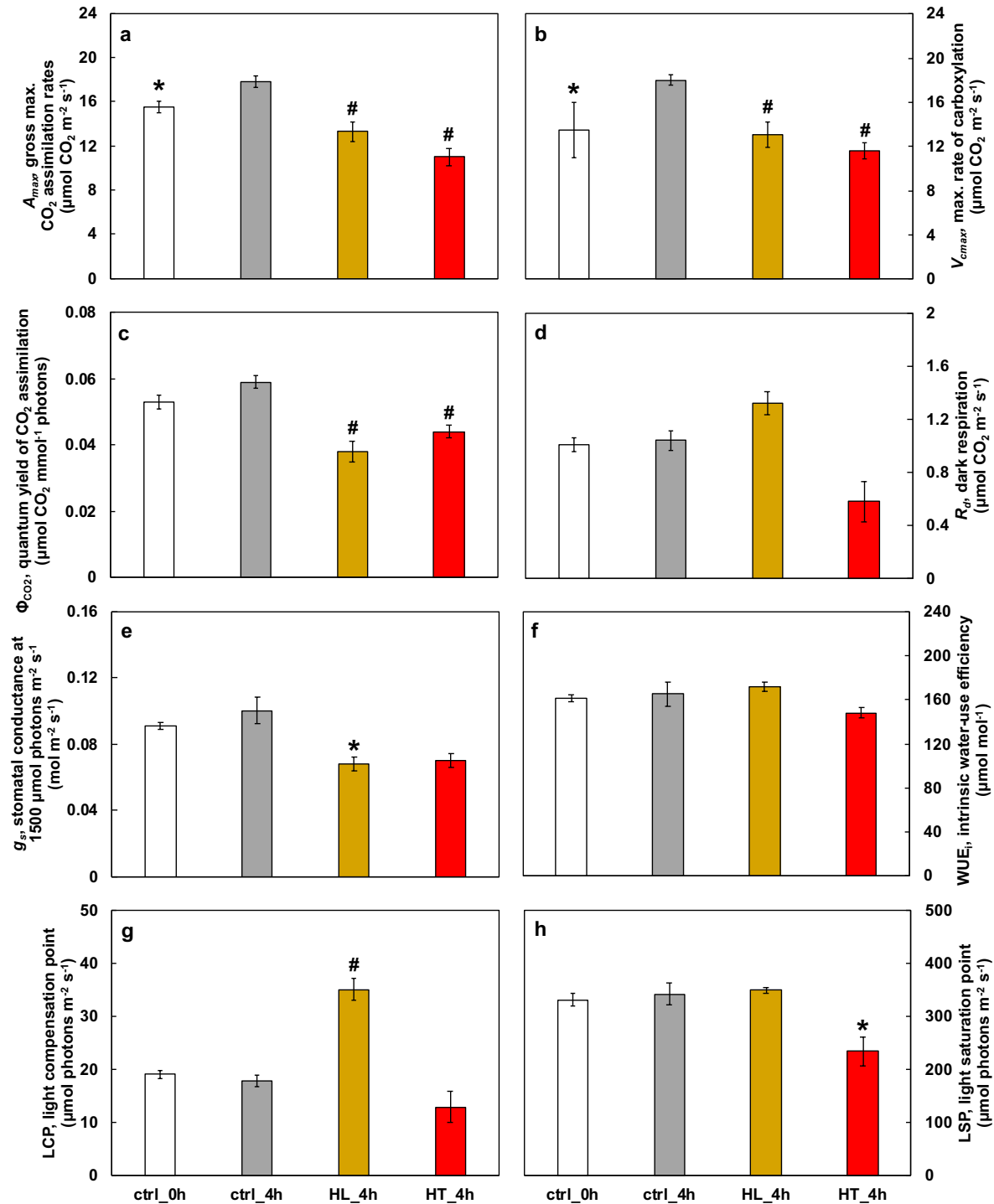
CO₂ response experiment:
Light: $1500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$
[CO ₂] reference
400
300
200
100
50
20
10
400 x 8
500
600
700
800
900
1000
1200
1500
400
Total time: ~45 min

Supplementary Figure 2. LI-6800 protocol for characterizing photosynthetic parameters. Before or after 4 h different treatments, intact *S. viridis* leaves were dark-adapted in LI-6800 leaf chamber for 20 min **(a)** to measure the maximum PSII efficiency (F_v/F_m), followed by **(b)** light response experiment from 50 – 1500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ light and then **(c)** CO₂ response experiment at 1500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ light.



Supplementary Figure 3: High light or high temperature treatments affected photosynthetic parameters measured by gas exchange and chlorophyll fluorescence. Photosynthetic parameters measured during light (a, c, e, g) and CO₂ (b,

d, f, h) response. Mean \pm SE, $n = 3-6$ biological replicates. Asterisk and pound symbols indicate statistically significant differences of ctrl_0h (at the start of treatments), HL_4h (after 4 h high light), and HT_4h (after 4 h temperature) compared to ctrl_4h (after 4 h control treatment) using Student's two-tailed t-test with unequal variance. P-values were corrected for multiple comparisons using FDR (* $0.01 < p < 0.05$, # $p < 0.01$, the colors of * and # match the significance of the indicated conditions, black for ctrl_0h, yellow for HL_4h, red for HT_4h).



Supplementary Figure 4: High light or high temperature treated leaves had reduced photosynthetic efficiency. Photosynthetic parameters were derived from light and CO_2 response curves (mean \pm SE; $n = 3-6$). (a) the maximum gross CO_2 assimilation rates, A_{max} ; (b) the maximum rate of carboxylation, V_{cmax} ; (c) the quantum yield of CO_2

assimilation, Φ_{CO_2} , which is the ratio of the moles of CO_2 fixed in photosynthesis per mole of quanta (photons of light) absorbed, and is a measure of the efficiency in which light is converted into fixed carbon; **(d)** the day-time dark respiration rate, R_d , equal to A_n when light intensity is zero; **(e)** stomatal conductance, g_s ; **(f)** water use efficiency, WUE; **(g)** light compensation point, LCP, the threshold of low light intensity at which photosynthesis is equal to leaf respiration and, therefore A_n is zero; **(h)** light saturation point, LSP, the estimated light intensity where 75 % of A_{max} was reached. Asterisk and pound symbols indicate statistically significant differences of ctrl_0h (at the start of treatments), HL_4h (after 4 h high light), and HT_4h (after 4 h temperature) compared to ctrl_4h (after 4 h control treatment) using Student's two-tailed t-test with unequal variance (* $0.01 < p < 0.05$, # $p < 0.01$).

a

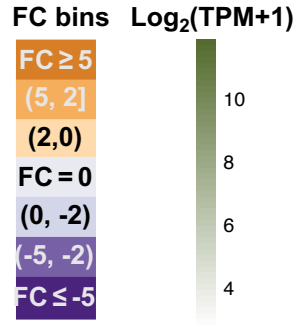
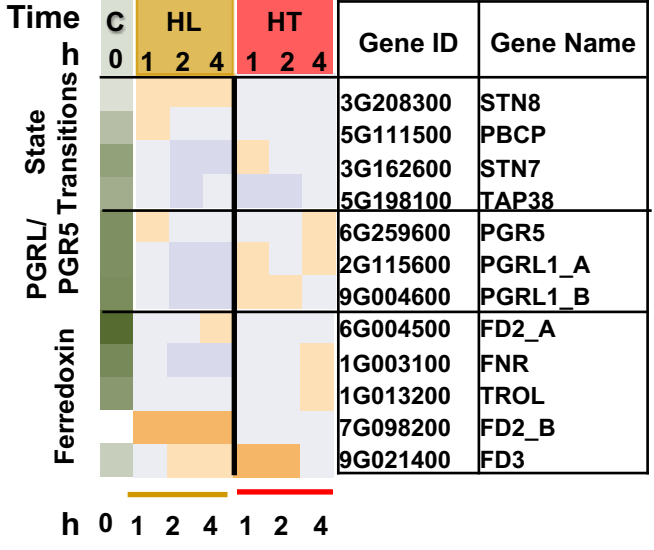
All differentially expressed genes (DEGs) sorted into fold change (FC) bins	HL_1h	HL_2h	HL_4h	HT_1h	HT_2h	HT_4h
Highly induced, $FC \geq 5$	176	292	276	87	71	65
Moderately induced, $2 \leq FC < 5$	738	1075	949	334	278	316
Slightly induced, $0 < FC < 2$	576	1028	906	382	517	372
No change, $FC = 0$	5301	3731	4261	6537	6468	6597
Slightly repressed, $-2 < FC < 0$	534	890	894	341	416	499
Moderately repressed, $-5 < FC \leq -2$	616	894	717	398	340	309
Highly repressed, $FC \leq -5$	242	273	180	94	93	25

b

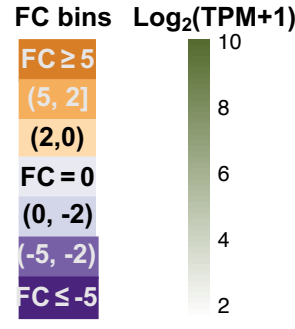
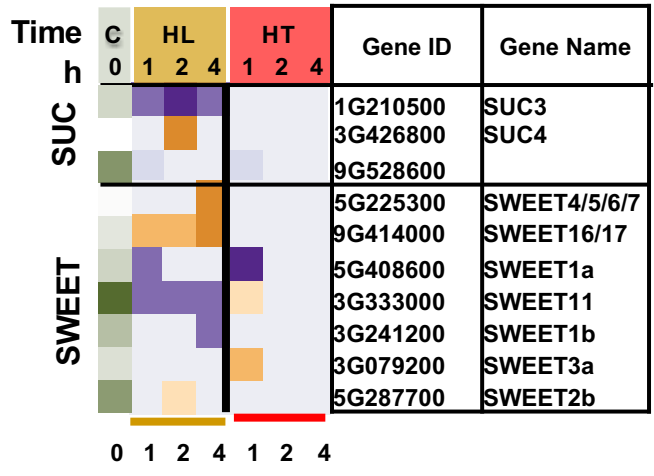
Highly induced or repressed genes in at least 1 time point	Highly Induced $FC \geq 5$	Highly Repressed $FC \leq -5$
High Light	496	424
High Temperature	167	210
High light & high temperature overlapped	42	13

Supplementary Figure 5. High light had more differentially regulated genes (DEGs) than high temperature while both also had overlapping DEGs. (a) Table of DEGs sorted into bins based on their DeSeq2 fold change values at each time point of high light (HL) or high temperature (HT) treatment. All genes that are differentially expressed in at least one timepoint are shown, those that are not differentially expressed at a given time point are represented in the “No change, $FC = 0$ ” category. **(b)** Number of genes that are highly induced ($FC \geq 5$) or highly repressed ($FC \leq -5$) in at least one time point in either HL or HT treatment. 42 genes are highly induced in at least one timepoint in both HL and HT and 13 genes are highly repressed in both HL and HT.

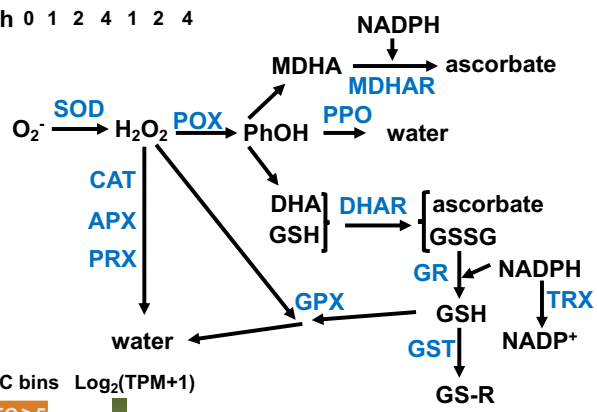
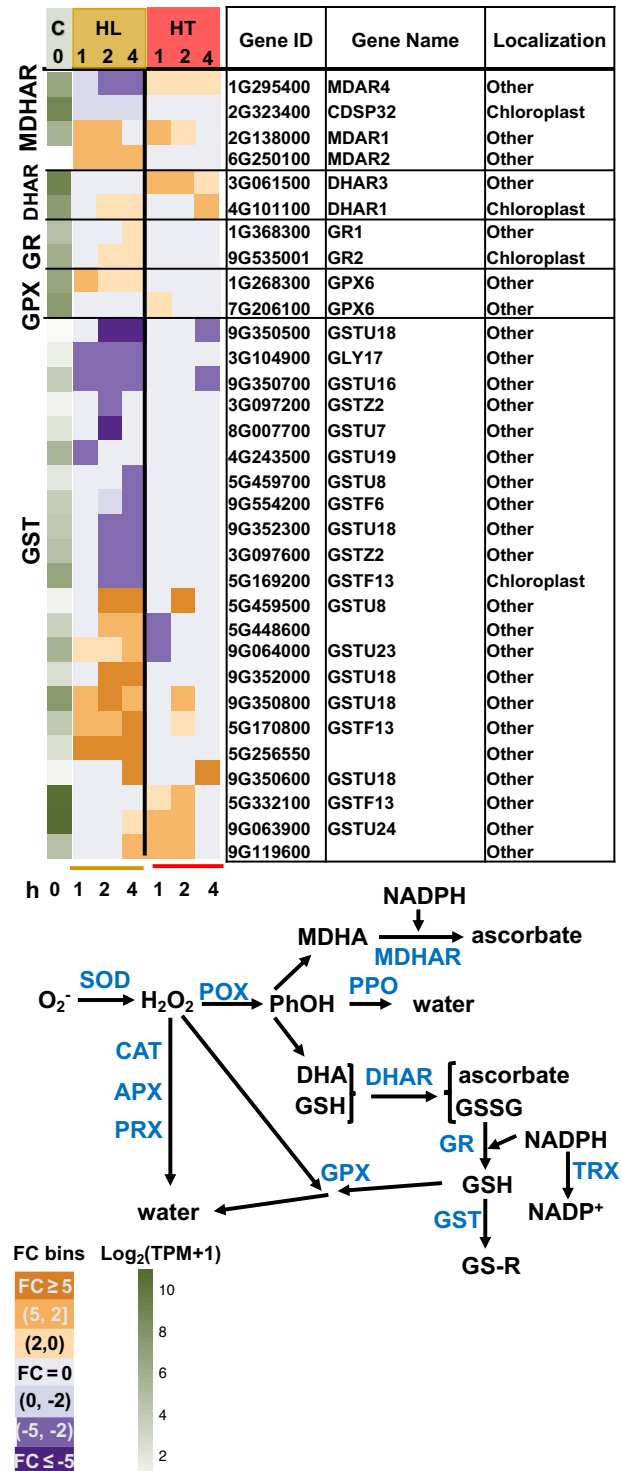
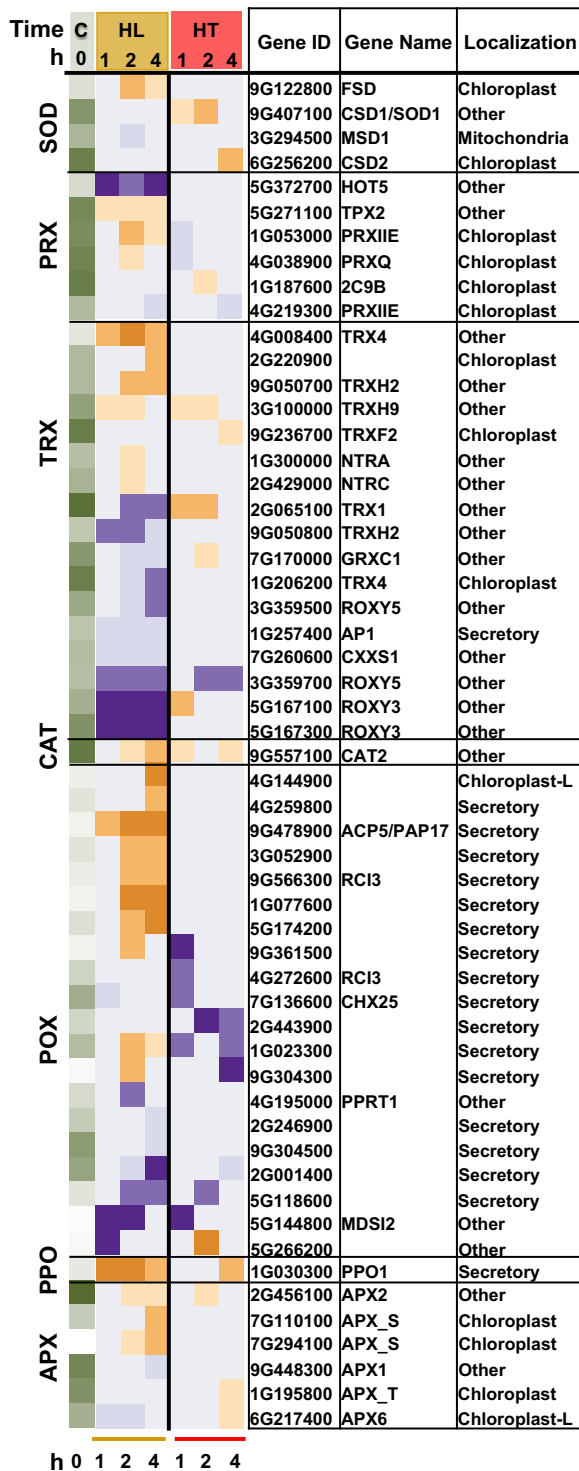
a



b

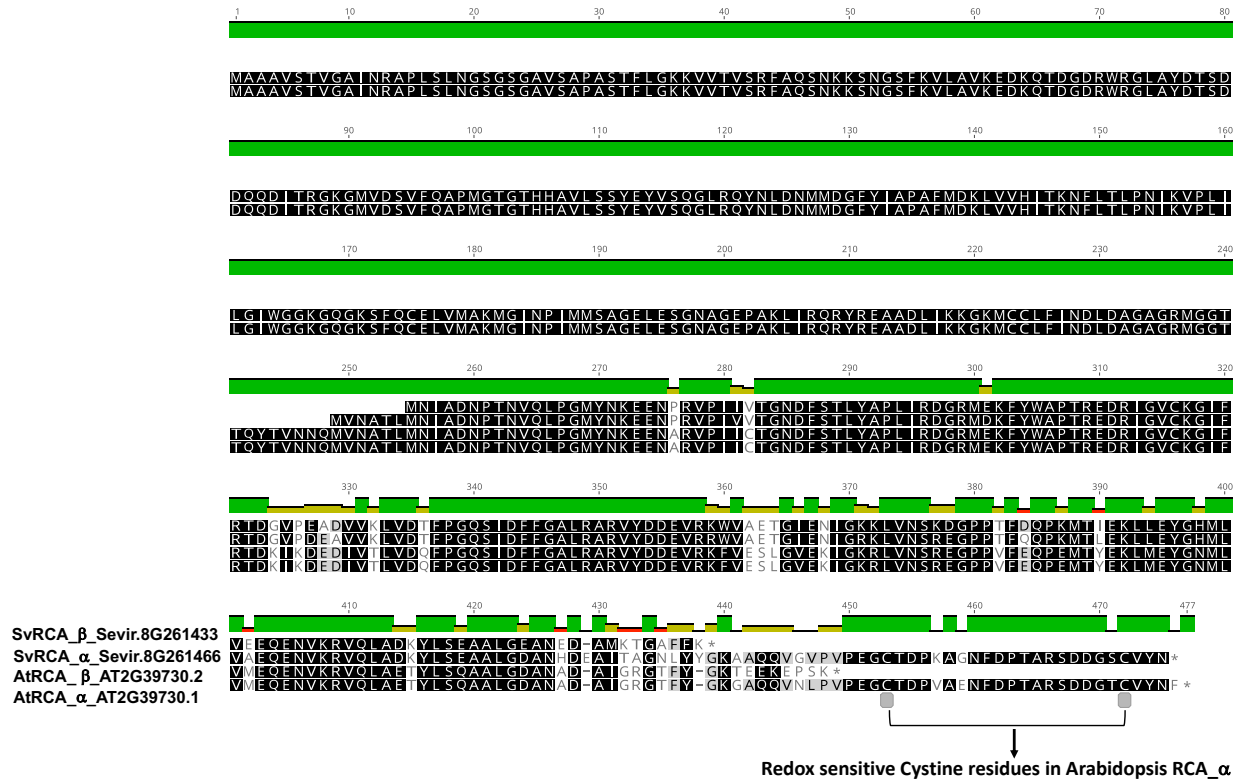


C

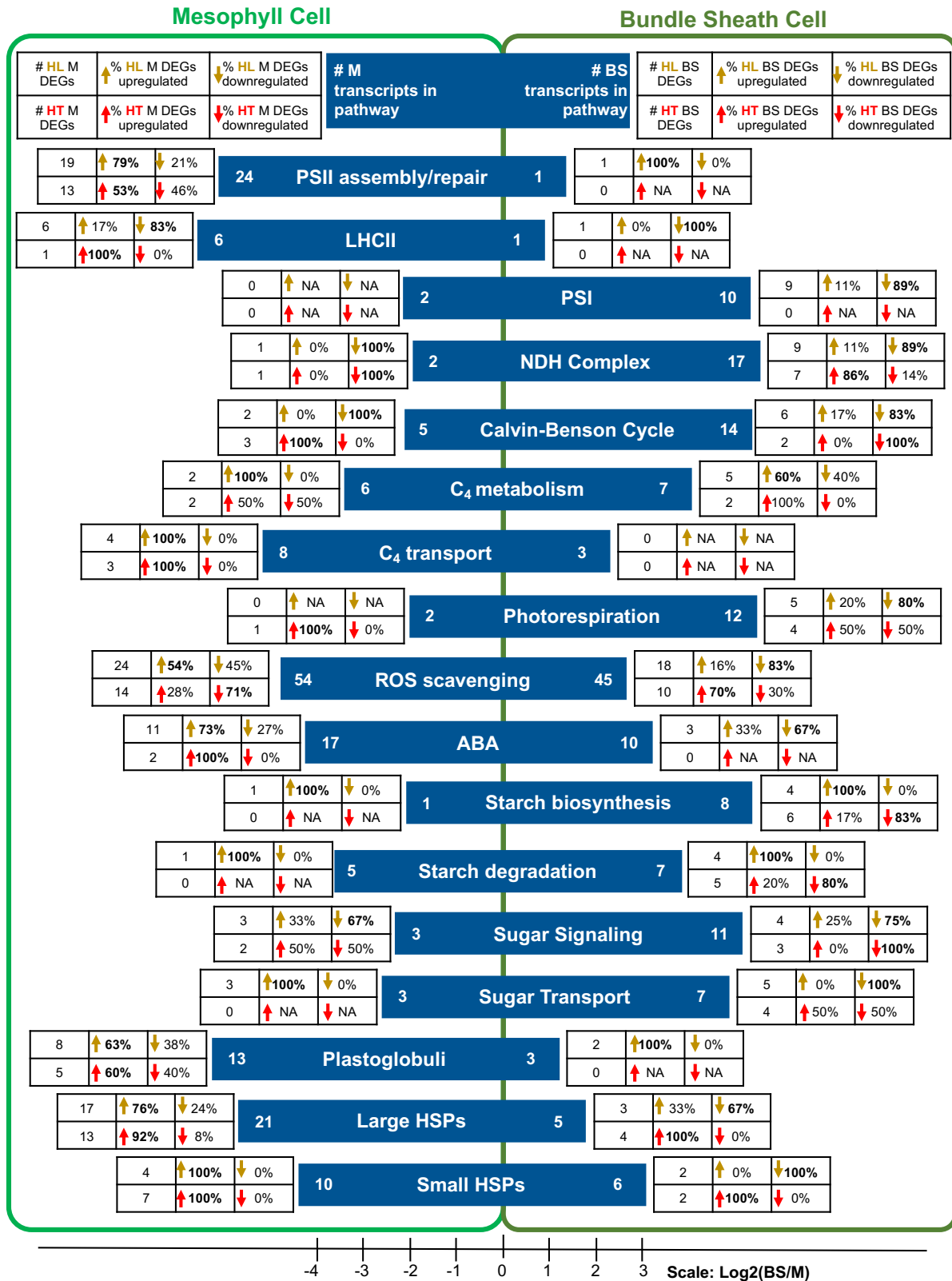


Supplementary Figure 6. High light (HL) or high temperature (HT) differentially regulated genes involved in various pathways associated with photosynthesis. (a) Alternative light reactions of photosynthesis. *PGR5* (proton gradient regulation 5) and

PGRL1 (*PGR5-like photosynthetic phenotype 1*) are genes involved in cyclic electron transport around photosystem I. **(b)** Genes encoding sugar transporters. *SUC* (*Sucrose-proton symporters*) and *SWEET* (*Sugar Will Eventually Exported Transporters*) encode sucrose transporters. **(c)** Genes involved in antioxidant defense pathways. SOD: superoxide dismutase; PRX: peroxiredoxins; TRX: thioredoxin; CAT: catalase. POX: peroxidases. PPO: polyphenol oxidase; APX: ascorbate peroxidase; MDHAR: monodehydroascorbate reductase; DHAR: dehydroascorbate reductase; GR: glutathione reductase; GPX: glutathione peroxidase; GST: glutathione S-transferase. These antioxidant enzymes are colored in blue in the antioxidant defense pathways based on Hasanuzzaman et al⁵⁷. SOD leads the frontline defense in the antioxidant defense system by converting superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2) which is further detoxified to water (H_2O) by one of these enzymes: POX, CAT, APX, PRX, or GPX. MDHA, monodehydroascorbate; PhOH, phenolic compounds; DHA, dehydroascorbate; GSH, reduced Glutathione; GSSG, oxidized glutathione; R, aliphatic, aromatic, or heterocyclic group; NADPH, nicotinamide adenine dinucleotide phosphate. Most antioxidant enzymes have multiple gene family members in *S. viridis*. The first green column displays $\log_2(\text{mean TPM} + 1)$ at ctrl_0h (at the start of treatments, C). TPM, transcripts per million, normalized read counts. Heatmap displays the fold change (FC) bin of DeSeq2 model output values at 1, 2, 4 h of HL or HT versus control at the same timepoint ($q < 0.05$). FC bins: highly induced: $FC \geq 5$; moderately induced: $5 > FC \geq 2$; slightly induced: $2 > FC > 0$; not differentially expressed: $FC = 0$; slightly repressed: $0 > FC > -2$; moderately repressed: $-2 \geq FC > -5$; highly repressed: $FC \leq -5$. Gene ID: *S. viridis* v2.1 gene ID, excluding "Sevir.". All genes presented in the heatmaps were significantly differentially regulated in at least one time point.

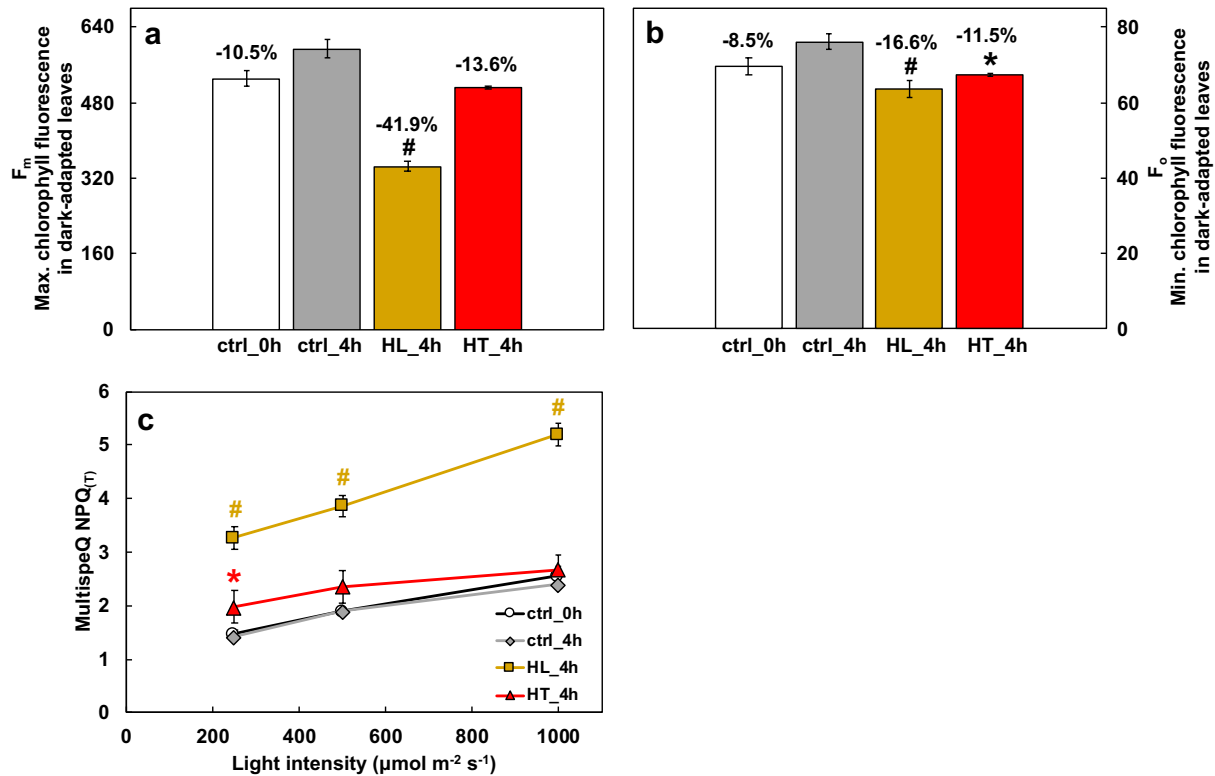


Supplementary Figure 7. Peptide sequence alignment of two *S. viridis* Rubisco Activases (RCAs) with *A. thaliana* RCAs reveals α and β copies of RCA in *S. viridis*. *A. thaliana* RCA_α has two redox sensitive cysteine residues, which are retained in the *S. viridis* RCA_α copy.

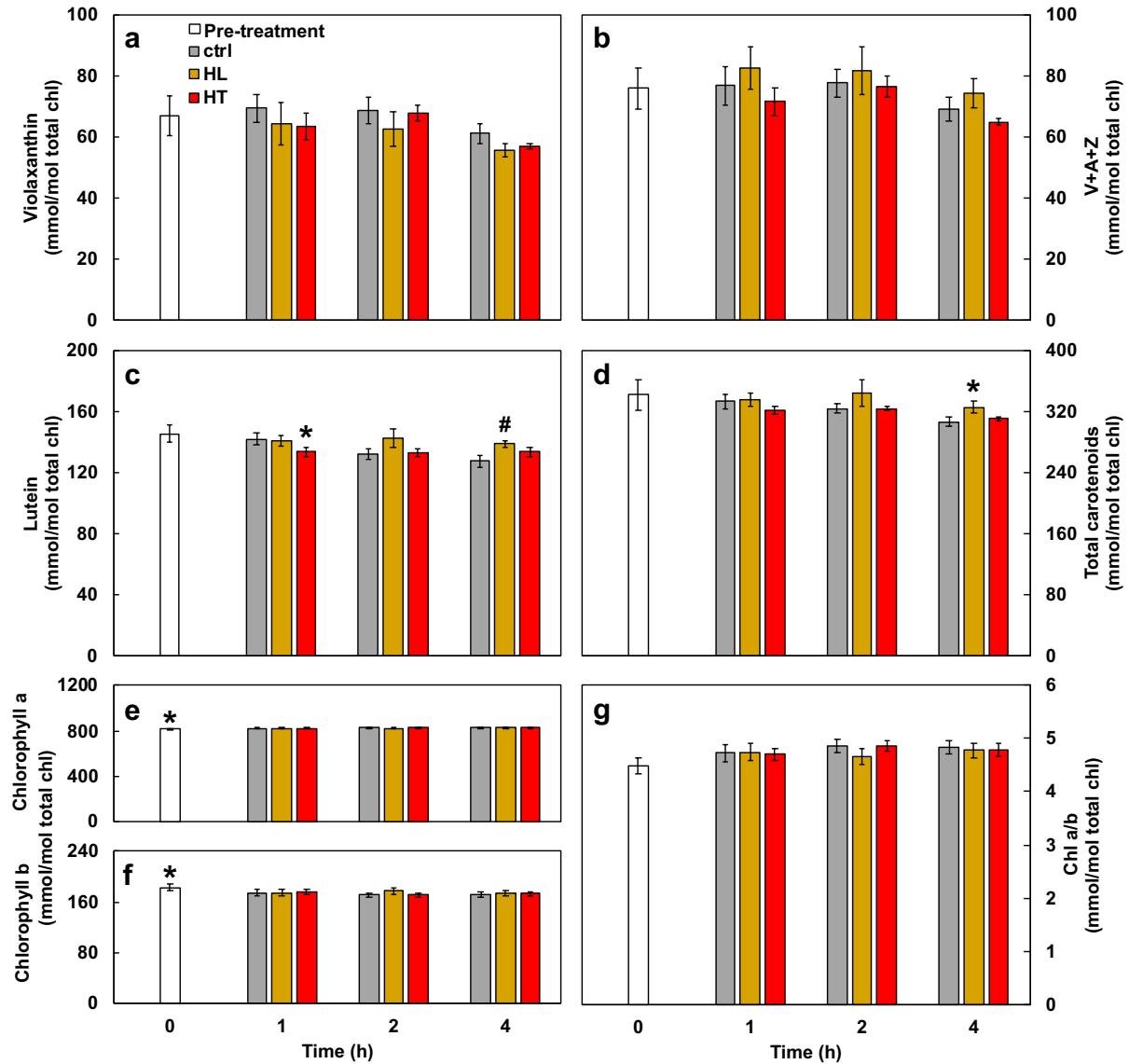


Supplementary Figure 8. Mesophyll (M) and bundle sheath (BS) specificity of

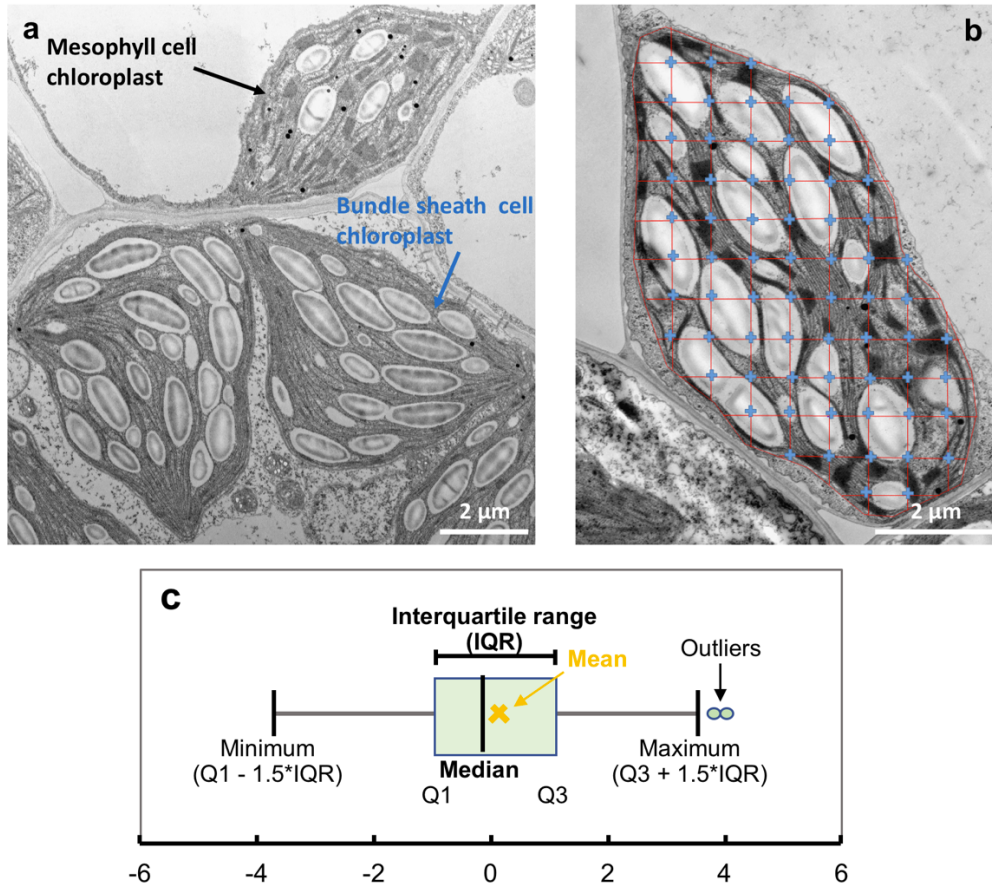
differentially expressed genes reveals cell type specific responses to high light (HL) or high temperature (HT). Light and dark green box denotes M and BS cells, respectively. The blue horizontal bars denote pathways of interest we investigated. Position of blue horizontal bars indicates cell type specificity of pathways, and represents the $\text{Log}_2(\text{number of BS specific transcripts}/\text{number of M specific transcripts associated with a pathway})$ according to the published M and BS specific transcriptome data in *S. viridis* under the control condition⁵⁸. The numbers at the left and right end of each blue horizontal bar represent the numbers of M or BS specific transcripts associated with this pathway. Pathways that are preferentially expressed in M cells (more M than BS specific transcripts, $\text{log}_2(\text{BS}/\text{M}) < 0$) under control conditions include PSII assembly, LHCII, C₄ transport, ROS scavenging, ABA, PG, and HSPs. Pathways that are preferentially expressed in BS cells (more BS than M specific transcripts, $\text{log}_2(\text{BS}/\text{M}) > 0$) under control conditions include PSI, NDH complex, Calvin-Benson cycle, C₄ metabolism, photorespiration, starch biosynthesis/degradation, sugar signaling, and sugar transport. Legends for M and BS specific transcript data in responses to HL or HT are on the top part of the light and dark green box, respectively. Each pathway has a table of data for each cell type. For each table, the first column indicates the number of M or BS specific transcripts related to a pathway that were differentially expressed in HL (top) or HT (bottom) in at least one time point. The rest two columns of the table represent the fraction of up- (2nd Col) or down-regulated (3rd Col) transcripts out of the total number of cell-type specific differentially expressed genes (DEGs) related to a pathway in HL (top) or HT (bottom). Bolded percentage indicates the larger portion (either up- or down-regulated) in HL or HT in each cell type. In HL, 83% of the BS-specific ROS-scavenging DEGs were down-regulated, whereas 54% of M-specific ROS-scavenging DEGs were up-regulated. In contrast, in HT, the majority of BS-specific ROS-scavenging DEGs were up-regulated while M-specific ROS-scavenging DEGs were down-regulated, which may be related to heat-induced photorespiration in BS chloroplasts. In HL, all differentially expressed sugar transports were up-regulated in M cells but down-regulated in BS cells. For HSPs, the majority of M-specific DEGs were up-regulated while the majority of BS-specific DEGs were down-regulated in HL. However, in HT, the majority DEGs of HSPs in both M and BS cells were upregulated.



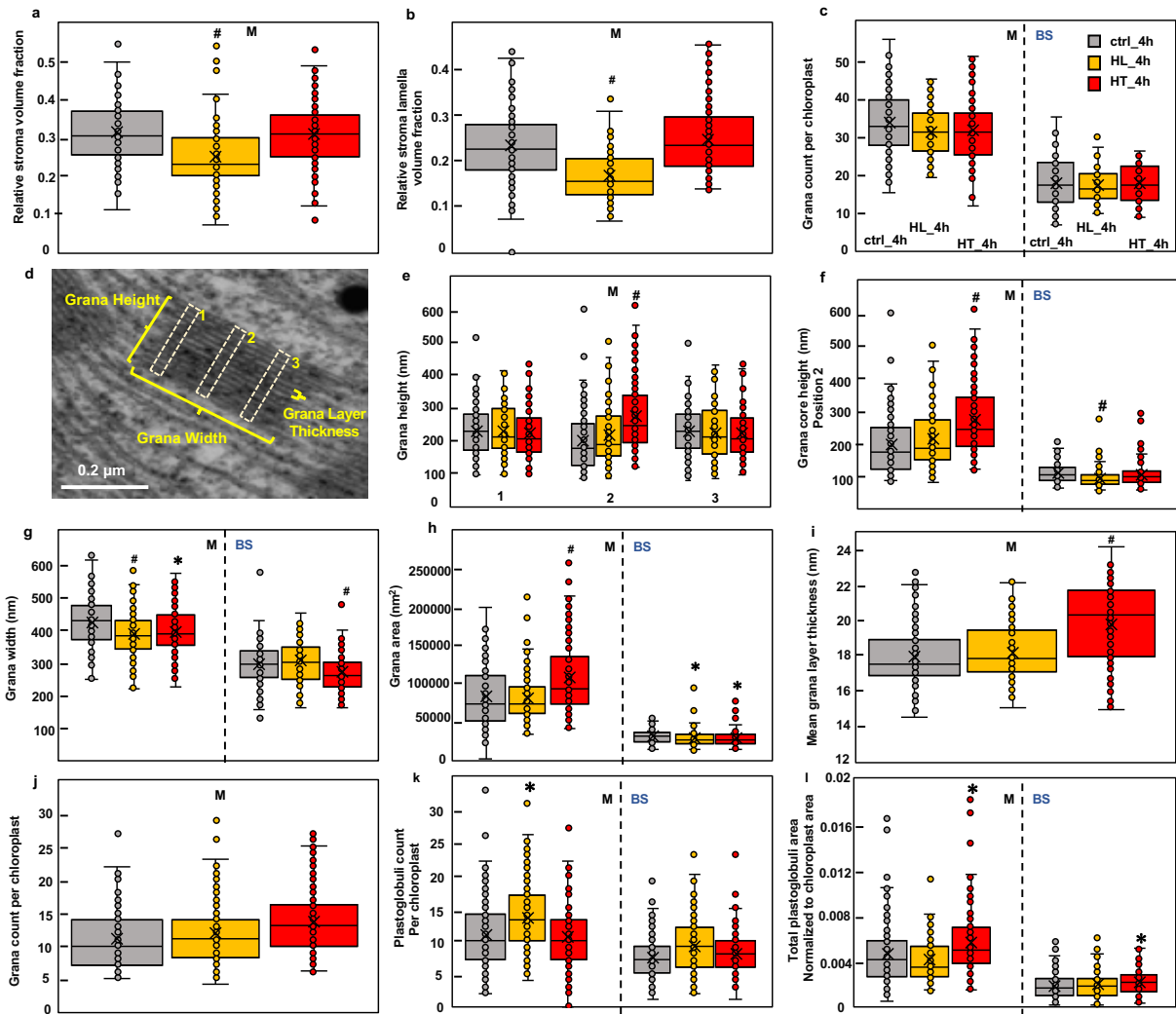
Supplementary Figure 9. High light (HL) resulted in significant reduction of maximum chlorophyll fluorescence (F_m) and the HL-induced NPQ was confirmed by using MultispeQ. (a) HL treatments resulted in significantly reduced maximal chlorophyll fluorescence in 20 min dark-adapted leaves (F_m), however, F_m in ctrl_4h leaves were consistent among replicates. **(b)** HL and HT treatments resulted in reduced minimum chlorophyll fluorescence in dark-adapted leaves (F_0) but F_0 in ctrl_4h leaves were consistent among replicates. Percentages indicate reduction in F_m or F_0 compared to ctrl_4h. **(c)** Estimated Non-photochemical quenching, $NPQ_{(T)}$, calculated by F_0' and F_m' obtained during light response using MultispeQ. F_0' and F_m' are minimum and maximum chlorophyll fluorescence in light-adapted leaves. Mean \pm SE, $n = 3-6$ biological replicates. Asterisk and pound symbols indicate statistically significant differences of ctrl_0h (at the start of treatments), HL_4h (after 4 h HL), and HT_4h (after 4 h HT) compared to ctrl_4h (after 4 h control treatment) using Student's two-tailed t-test with unequal variance (* $0.01 < p < 0.05$, # $p < 0.01$). For panel c, the colors of * and # match the significance of the indicated conditions, yellow for HL_4h, red for HT_4h).



Supplementary Figure 10. High light treatment increased lutein and carotenoids formation. Leaves of *S. viridis* were harvested for high-performance liquid chromatography (HPLC) analysis before treatment or after 1, 2, 4 h treatments of control growth condition (ctrl, 31°C and 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$), or high light (HL, 31°C and 900 $\mu\text{mol m}^{-2} \text{s}^{-1}$) or high temperature (HT, 40°C and 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$). **(a)** Violaxanthin. **(b)** Total xanthophyll pool (violaxanthin + antheraxanthin + zeaxanthin, V+A+Z). **(c)** Lutein. **(d)** Total carotenoids. **(e,f)** Chlorophyll a and b. **(g)** Chlorophyll a/b ratio. Mean \pm SE, $n = 3$ biological replicates. * $0.01 < p < 0.05$, # $p < 0.01$, compared to control leaves at the same time points. Students' two-tailed t-test with unequal variance.



Supplementary Figure 11. Representative transmission electron microscopy (TEM) images illustrate two cell types in *S. viridis* leaves, Stereo Analyzer analysis to quantify chloroplast structures, and boxplot of TEM data. (a) TEM image of chloroplasts of the two cell types in *S. viridis*: mesophyll cells and bundle sheath cells. **(b)** Illustration of Stereo Analyzer analysis for TEM images, which was used to calculate the relative volume of a cellular structures, e.g. starch granules. The Stereo Analyzer outlines a chloroplast with equally spaced uniform grid within the outlined area. The blue crossings of the grid inside the chloroplast are identified as either starch granule, stroma, stroma lamellae, or grana when they overlap with these structures. When all crossings have been identified, the software provides the % of relative volume for each structure of interest. **(c)** Illustration of TEM boxplots based on Tukey-style whiskers. Q1, first quartile; Q3, third quartile; IQR, interquartile range. The median value is represented by the vertical black line between Q1 and Q3. The mean value is represented by the yellow X sign.



Supplementary Figure 12. High light or high temperature altered various chloroplast structures in *S. viridis* leaves. Chloroplast structure changes after 4 h different treatments of control growth condition (ctrl_4h, 31°C and 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$), or high light (HL_4h, 31°C and 900 $\mu\text{mol m}^{-2} \text{s}^{-1}$) or high temperature (HT_4h, 40°C and 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$). M, mesophyll chloroplast; BS, bundle sheath chloroplast. **(a, b)** Relative volume fractions were quantified using Stereo Analyzer with Kolmogorov–Smirnov test for statistical analysis compared to the same cell type of the control condition. **(e, f, g, l)** Parameters related to size and area were quantified using ImageJ with two-tailed t-test with unequal variance compared to the same cell type of the control condition. **(c, j, k)** The counting data was quantified using ImageJ followed by the negative binomial test for significance compared to the same cell type of the control condition. **(d, e)** Position 1 and

3 on grana are to measure the height of grana margin and position 2 is to measure the height of grana core. **(h)** Assuming grana are rectangular, grana area was estimated as grana core height multiplied by grana width. **(i)** The mean grana layer thickness was calculated as grana core height divided by the number of grana layers. Each treatment had three biological replicates with 90-120 images.

Table of Parameters Compared to Control Condition					
		Treatment Condition:			
		HL_M	HL_BS	HT_M	HT_BS
		↑ 58%	↑ 67%	↓ 19%	↑ 10%
Relative Starch Volume		↑ 33%	↑ 29%	↑ 14%	N.S.
Chloroplast Area		↓ 24%	↓ 36%	N.S.	N.S.
Relative Stroma + Stroma Lamella Volume		↓ 20%	N/A	N.S.	N/A
Relative Stroma Volume		↓ 29%	N/A	N.S.	N/A
Relative Stroma Lamella Volume		N.S.	↓ 37%	↑ 24%	↓ 26%
Relative Grana Volume		N.S.	↓ 16%	↑ 36%	N.S.
Grana Core Height, position 2		N.S.	N/A	N.S.	N/A
Grana Margin Height, position 1, 3		↓ 9%	N.S.	↓ 6%	↓ 8%
Grana Width		N.S.	↓ 11%	↑ 29%	↓ 12%
Grana Area		N.S.	N/A	↑ 10%	N/A
Mean Grana Layer Thickness		N.S.	N.S.	N.S.	N.S.
Grana Count per Chloroplast Area		↓ 30%	↓ 24%	↓ 16%	N.S.
Grana Count Normalized to Chloroplast Area		N.S.	N/A	N.S.	N/A
Grana Layer Count		↑ 27%	N.S.	N.S.	N.S.
PG Count per Chloroplast		↓ 7%	↑ 11%	↑ 38%	↑ 19%
Individual PG Size		↑ 20%	↑ 41%	↑ 39%	↑ 26%
Total PG Area per Chloroplast		N.S.	N.S.	↑ 21%	↑ 7%
PG Area Normalized to Chloroplast Area					

Key	
Increase	↑
Decrease	↓
Not Available	N/A
Not Significant	N.S.

Supplementary Figure 13. Summary of chloroplast structure changes by using transmission electron microscopy (TEM) images in leaves after 4 h treatments of high light (HL, 31°C and 900 $\mu\text{mol m}^{-2} \text{s}^{-1}$) or high temperature (HT, 40°C and 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$) as compared to the control growth condition (ctrl, 31°C and 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$). BS, bundle sheath chloroplast; M, mesophyll chloroplast. PG, plastoglobuli. Mean value of each parameter was used for comparison. Yellow highlighted cells and upward arrows denote increased percentages as compared to the control condition. Blue highlighted cells and downward arrows denote decreased percentage as compared to the control condition. Grey highlighted cells and N/A mean data unavailable due to the difficulties to quantify some structures in the BS chloroplasts. White cells and N.S. mean no significant differences between HL or HT as compared to the control treatment.

High light	Parameters	High temperature
↓	net CO ₂ assimilation, after 4 h stress	↓
↓	Stomatal conductance, during stress	↑
↑	Leaf ABA level	No change
Many ↑	ABA pathway transcripts	Little change
↑ M & BS	PG formation in chloroplasts	↑ M & BS
Many ↑	Genes encoding PG localized proteins	Little change
↑ M & BS	Starch accumulation	↑ in BS but ↓ in M
Many ↑	starch biosynthesis/degradation transcripts	Little change
↑	Chloroplast crowdedness	Little change
↓	ATP synthase activity	Little change
↓	Transcripts involved in light reaction	Little change
Many ↑	Transcripts involved in photoprotection	Little change
↑	Zeaxanthin	Little change
Significant ↑	NPQ	Slightly ↑
↑	Photoinhibition	Little change
↑ slowly	Transcripts of HSPs	↑ quickly
↑	HSFs, different genes ↑ under HL or HT	↑
Little change	Transcripts of RCA- α	↑
Slightly ↓	Transcripts involved in CEF	Slightly ↑
Stable	Overall transcriptional changes	Transient

Supplementary Figure 14. Summarized multi-level changes of *S. viridis* in response to 4 h high light or high temperature treatments as compared to the control treatment. Upward arrows denote increase or induction; downward arrows denote decrease or repression. HL, high light; HT, high temperature; ABA, abscisic acid; M, mesophyll chloroplast; BS, bundle sheath chloroplast; PG, plastoglobuli; NPQ, non-photochemical quenching; HSP, heat shock protein; HSF, heat shock transcription factor; RCA, Rubisco activase; CEF, cyclic electron flow around PSI.